HERBICIDES – PROPERTIES, SYNTHESIS AND CONTROL OF WEEDS

Edited by **Mohammed Naguib**
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Preface

Considerable advances in the understanding of herbicides in soils and/or in plants have been made over the past decade. Elucidation of the synthetic pathways of herbicides is continuing apace and, while their function is still open to controversy, it is now widely acknowledged that these compounds are not, in general, biomaterials but are artificially synthesized.

Exciting discoveries are continually being made in the field of herbicides. Since the completion of this book, it has been reported that the mode of action of herbicides is still in need of further investigation in order to cope with weed control.

The overall purpose of this book is to show that plants do not haphazardly produce a large number of chemical compounds, but that each compound is synthesized for a definite purpose and that the majority of herbicides and pesticides are artificially synthesized in the labs; investigating synthetic pathways and properties, and that all products produced to overcome weeds and insects which causes low production of crops.

The format of the book is based on two main sections: Synthesis and Properties of Herbicides and Control of Weeds. This book contains 25 review articles on a wide range of important topics. Several chapters review herbicide progress in specific crops. Other chapters deal with the synthesis and properties of herbicides and weed control. I hope therefore that readers who are interested in a synthesis and properties and control of weeds, for example in winter wheat of early growth stages, effects of herbicides on fresh water zooplankton, and herbicide tolerant food legume crops, will benefit from the wider range of topics also discussed within this book.

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Part 1

Synthesis and Properties

1

Preparation and Characterization of Polymeric Microparticles Used for Controlled Release of Ametryn Herbicide

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1. Introduction

There is increasing pressure to improve agricultural productivity, due to rapid population growth, increased consumption and global demand for high quality products. As a result, agricultural chemicals have become essential for the control of weeds, pests and diseases in a wide range of crops. Ametryn (2-ethylamino-4-isopropylamino-6-methylthio-s-2,4,6-triazine) is a selective herbicide belonging to the s-triazine family, whose activity is the result of inhibition of photosynthesis by blocking of electron transport. The ametryn molecule (Figure 1) contains a symmetrical hexameric aromatic ring in its chemical structure, consisting of three carbon atoms and three nitrogen atoms in alternate positions. The herbicide is classified as a methylthiotriazine, due to the presence of the SCH_3 group (Tennant et al., 2001).

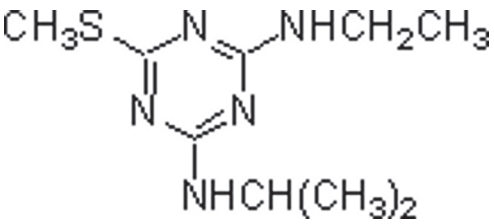

Fig. 1. Structural formula of ametryn.

Ametryn is used for the control of graminaceous and broad-leaved weeds in plantations of annual crops (Tennant et al., 2001). Once in the soil the herbicide may be taken up by plants, absorbed by the soil and plant residues, biodegraded, or undergo chemical transformations that increase its volatilization and photocatalytic decomposition. Studies have shown that prolonged human exposure to triazine herbicides can lead to serious health problems including contact dermatitis, intoxication, hormonal dysfunction and cancers (Friedmann et

al., 2002). It is therefore desirable to develop techniques whereby the physico-chemical properties of these chemicals can be altered and their usage made safer. The goal is to enable the use of soil management strategies that can produce foods at the current high levels of demand, without significant human or environmental risk.

Micro- and nanostructured polymeric materials can be used as transport systems for active chemicals. Advantages of these materials include good physical, chemical and biological stability, simple and reproducible preparation procedures, and applicability to a wide range of chemicals. In use, the active principle is released slowly and continuously, enabling the use of smaller quantities with greater efficiency, which reduces the risk of adverse environmental impacts (Sinha et al., 2004; Sopena et al., 2009).

Controlled release systems have been extensively used in the food and pharmaceutical industries for active substances including nutrients, drugs and aromas (El Bahri & Taverdet, 2007; Grillo et al., 2008; Mello et al., 2008; Moraes et al., 2010), and there has been a recent increase in their application in medicine (Natarajan et al., 2011; Parajo et al., 2010; Vicente et al., 2010).

Amongst the new controlled-release system technologies under development, the use of polymeric micro- and nanoparticles is of special interest in agribusiness. Several studies have investigated controlled-release systems for bioactive compounds in agricultural applications (Ahmadi & Ahmadi, 2007; Bin Hussein et al., 2010; El Bahri & Taverdet, 2005, 2007; Grillo et al., 2010; Hirech et al., 2003; Li et al., 2010; Lobo et al., 2011; Silva et al., 2010; Singh et al., 2008, 2010). Materials that have been used include silica, bentonite and sepiolite clays, and polymeric substances such as alginate, lignin and synthetic polymers. The latter include the poly(hydroxyalkanoates) (PHAs) (Salehizadeh & Loosdrecht, 2004), of which poly(3-hydroxybutyrate) (PHB) and its hydroxyvalerate copolymer (PHBV) have been most widely used (Amass & Tighe, 1998). The advantages of using polymers such as PHB and PHBV are that they are fully biodegradable, inexpensive and easily prepared by bacterial fermentation (Pouton & Akhtarb 1996; Reis et al., 2008). These polymers are isotactic and highly crystalline (55-80 %), so that their degradation rates are relatively slow compared to those of lactate (PLA) and glycolate (PGA) copolymers (Sudesh et al., 2000).

The objective of this work was to develop a novel release system for ametryn, employing microparticles prepared using two different polymers, PHB and PHBV (either individually or as mixtures). It was envisaged that the encapsulation of the herbicide in these microparticles would improve its chemical stability and enable the use of smaller quantities of the chemical, hence reducing the risk of environmental contamination.

2. Experimental

2.1 Materials

Polyvinyl alcohol (PVA), poly(3-hydroxybutyrate) (PHB, MW = 312,000 g mol^{-1}), poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV, MW = 238,000 g mol^{-1}) and ametryn (Pestanal®) were purchased from Sigma Chem. Co. The solvents employed in the chromatographic analyses were acetonitrile, HPLC grade methanol (JT Baker) and Milli-Q water. The solutions were filtered using 0.22 μm nylon membranes (Millipore, Belford, USA).

2.2 Methodology

2.2.1 Determination of ametryn

The HPLC analyses were performed using a Varian ProStar instrument fitted with a PS 210 pump, a UV-VIS detector (PS 325), a Metatherm oven and an automatic injector (PS 410). The chromatograms were acquired and processed using Galaxy Workstation software. The eluent used was acetonitrile/water (70:30, v/v), at a flow rate of 1.4 mL min^{-1}, and separation was achieved using a Phenomenex Gemini C_{18} reversed phase column (5 µm, 110 Å, 150 mm x 4.60 mm i.d.). Ametryn was detected at a wavelength of 260 nm. The injection volume was 100 µL, and all samples were previously filtered through 0.22 µm nylon membranes.

2.2.2 Preparation of the polymeric microparticles containing ametryn

Microparticles were prepared with the PHB and PHBV polymers, used either individually or as a mixture, by formation of oil in water emulsions using the emulsification-solvent evaporation technique (Coimbra et al., 2008; Conti et al., 1995; Lionzo et al., 2007; Lobo et al., 2011). 200 mg of polymer (PHB, PHBV or a mixture of the two polymers, as described in Table 1) and 10 mg of herbicide were dissolved in 10 mL of chloroform to form the organic phase. The aqueous phase (200 mL) was prepared using 0.5 % (w/v) polyvinyl alcohol, at 50 °C. The organic phase was transferred to the aqueous phase (at 50 °C) with magnetic stirring (1000 rpm for 15 min). The chloroform was then evaporated from the emulsion. The suspension of microparticles formed was stored in an amber flask (to avoid any photodegradation of the herbicide). The final concentration of ametryn was 50 mg L^{-1}.

Formulation	PHBV (mg)	PHBV %	PHB (mg)	PHB %
A	200	*100*	0	*0*
B	150	*75*	50	*25*
C	100	*50*	100	*50*
D	50	*25*	150	*75*
E	0	*0*	200	*100*

Table 1. Proportions of polymers used to prepare the different formulations.

2.2.3 Measurements of encapsulation efficiency

Portions (10 mg) of the different microparticles containing herbicide were dissolved in 50 mL of acetonitrile, and the association rate of the herbicide with the microparticles was determined by the technique described previously, which involves ultrafiltration/ centrifugation and analysis using HPLC (Kilic et al., 2005; Schaffazick et al., 2003). The samples were centrifuged in regenerated cellulose ultrafiltration filters that had a molecular size-exclusion pore size of 30 KDa (Microcon, Millipore), and the filtrate was analyzed using HPLC. The ametryn concentration was obtained from an analytical curve. The association

rate of ametryn was calculated from the difference between the concentration measured in the filtrate and the total concentration (100 %) in the microparticle suspension. The total concentration was measured after diluting the suspension with acetonitrile, which dissolved the polymer and ensured complete release of the herbicide. The measurements were performed in triplicate for each formulation. The encapsulation efficiency (EE, %) was expressed as the ratio:

$$EE(\%) = \frac{W_S}{W_{TOTAL}} x\ 100\% \quad (1)$$

Where, W_s is the quantity of ametryn in the microparticles and W_{total} is the amount of ametryn used in the formulation.

2.2.4 Scanning electron microscopy (SEM)

A scanning electron microscope (Model JSM-6700F, JEOL, Japan) was used to investigate the size distribution and surface morphology of the microparticles. Suspensions of microparticles containing the herbicide were filtered and the particles were then washed with 150 mL of distilled water. The solid residues were dried overnight over Na_2SO_4 in a desiccator. The samples were then attached to metallic supports (stubs) with double-sided tape, and metalized by deposition of a gold layer at a current of 25 mA for 150 s. Images (electron micrographs) of the samples were then generated using the microscope. Particle sizes were measured using the ImageJ 1.42 program, and the size distributions of the different microparticles were obtained using OriginPro 7.0. At least 1000 individual particles of each sample were used for these measurements.

2.2.5 Release of ametryn from the microparticles

The release profiles of ametryn, either free or associated with the microparticles, were investigated using a two-compartment experimental system. A cellulose membrane (Spectrapore, with a molecular exclusion pore size of 1000 Da) separated the donor compartment, containing 4 mL of solution (or suspension) of the herbicide, from the acceptor compartment, which contained 50 mL of deionized water maintained under gentle agitation at ambient temperature (Paavola et al., 1995). The pore size of the membrane only allowed passage of the free herbicide, while the herbicide associated with the microparticles was retained in the donor compartment until the equilibrium was shifted so as to release the ametryn present within the particles. The size of the microparticles prevented their passage through the pores of the membrane. These experiments were conducted under *dilution sink* conditions, whereby the volume of the dissolution medium was sufficiently large that the herbicide concentration never exceeded 10 % of the value of its saturation concentration (Aulton et al., 2002).

Samples were retrieved from the acceptor compartment as a function of time, and analyzed by HPLC at a detector wavelength of 260 nm. During the first hour, samples were collected every 15 min, during the second hour every 30 min, and subsequently at hourly intervals until the peak area stabilized. The peak area values were then converted into the percentage of herbicide released as a function of time (De Araújo et al., 2004).

2.2.5.1 Mathematical modeling of ametryn release

Mathematical modeling is increasingly used to investigate the release profiles of bioactive compounds in polymeric systems, since it can provide important information concerning the release mechanism. Analysis of the mechanism of release of ametryn from the microparticles employed the zero order, first order, Higuchi and Korsmeyer-Peppas models (Colombo et al., 1995, 2005; Costa & Lobo, 2001; Ferrero et al., 2000; Hariharam et al., 1994; Ritger & Peppas, 1987a, 1987b).

3. Results and discussion

The encapsulation efficiency values obtained for the different microparticles are listed in Table 2. Formulation A (100 % PHBV) showed the highest encapsulation efficiency (76.5 %). The efficiency decreased as the proportion of PHBV decreased, and formulation E (100 % PHB) provided the lowest encapsulation efficiency (26.2 %). The values obtained for formulations A and B were fairly high, relative to values that have been reported in the literature for other active principles (Bazzo et al., 2009; Grillo et al., 2010; Lobo et al., 2011; Sendil et al., 1999). Grillo and colleagues (2010) showed that the encapsulation efficiency of the herbicide atrazine in PHBV microparticles was in excess of 30 %. Lobo et al. (2011), using an experimental design optimization procedure, obtained an encapsulation efficiency of 24 % for atrazine in PHBV microparticles.

Formulation	PHBV (%)	PHB (%)	EE (%)
A	100	0	76.5
B	75	25	54.7
C	50	50	40.5
D	25	75	29.3
E	0	100	26.2

Table 2. Encapsulation efficiencies (EE, %) of the different microparticles.

The relationship between the percentage of PHBV and the encapsulation efficiency is illustrated in Figure 2. There was a polynomial relationship between the encapsulation efficiency and the PHBV concentration, which was positive for PHBV and negative for PHB. This can probably be explained by the structural differences between the microparticles, due to the different polymer ratios used in their preparation (Table 1).

The morphological characteristics of the microparticles, as well as the influence of the encapsulation of ametryn, were analyzed using the SEM procedure. Electron micrographs of the microparticles containing ametryn are illustrated in Figure 3. All types of microparticle were spherical, although the surface structures were different. Most of the PHB microparticles possessed smooth surfaces with few pores, while most of the PHBV microparticles were rough-surfaced with many cavities and pores, some of which were quite large, as can be clearly seen for formulation A (Figure 3, a1 and a2). Grillo et al. (2010) also found that PHBV microparticles, prepared using the same methodology as that

described here, were rough-surfaced with pores, while PHB microparticles had smooth surfaces and fewer pores.

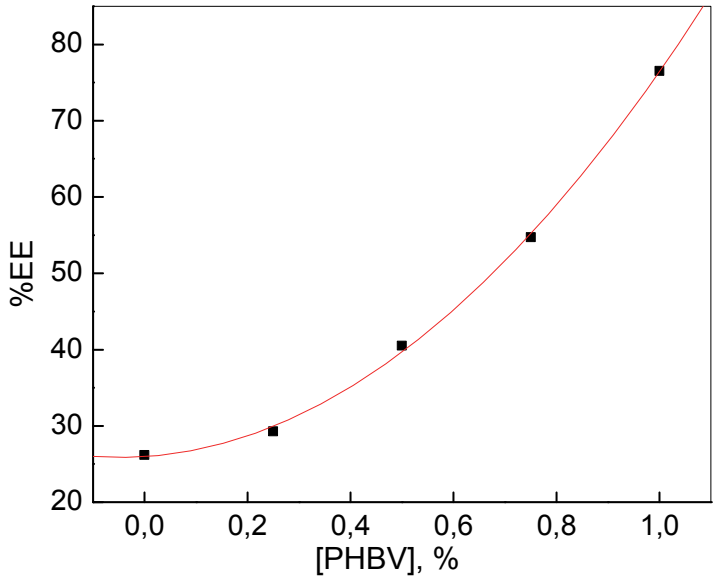

Fig. 2. Encapsulation efficiency according to PHBV content of the microparticles.

A higher encapsulation efficiency of ametryn was therefore related to a greater number of pores in the microparticles, probably due to greater contact (and/or affinity) of the herbicide with the microparticles during the formulation preparation procedure. Ametryn is likely to have greater affinity for the PHBV polymer, since both of these molecules possess alkyl branches, with interaction being further enhanced by the porosity of the PHBV microparticles.

The size distribution profiles (Figure 4) differed between microparticle formulations (it was not possible to measure the size distribution of the formulation D microparticles due to focusing problems). The average size of the microparticles (Table 3) increased as the PHBV concentration decreased and the PHB concentration increased, and was greatest for the PHB microparticles (formulation E). These size differences could be related to the incorporation of the herbicide as well as to associations between the molecules (as discussed above). At higher encapsulation rates, the amount of ametryn present within the microparticle increased, and the potential for reactions and interactions with the polymer therefore also increased. Ametryn is likely to have a higher affinity for PHBV, and as a result of this affinity (and/or reaction) the polymer contracts due to the formation of linkages between the polymer chains. As the proportion of PHBV decreases, the affinity of ametryn for the polymer mixture also diminishes (due to the lower affinity of ametryn for PHB), so that there is less shrinkage.

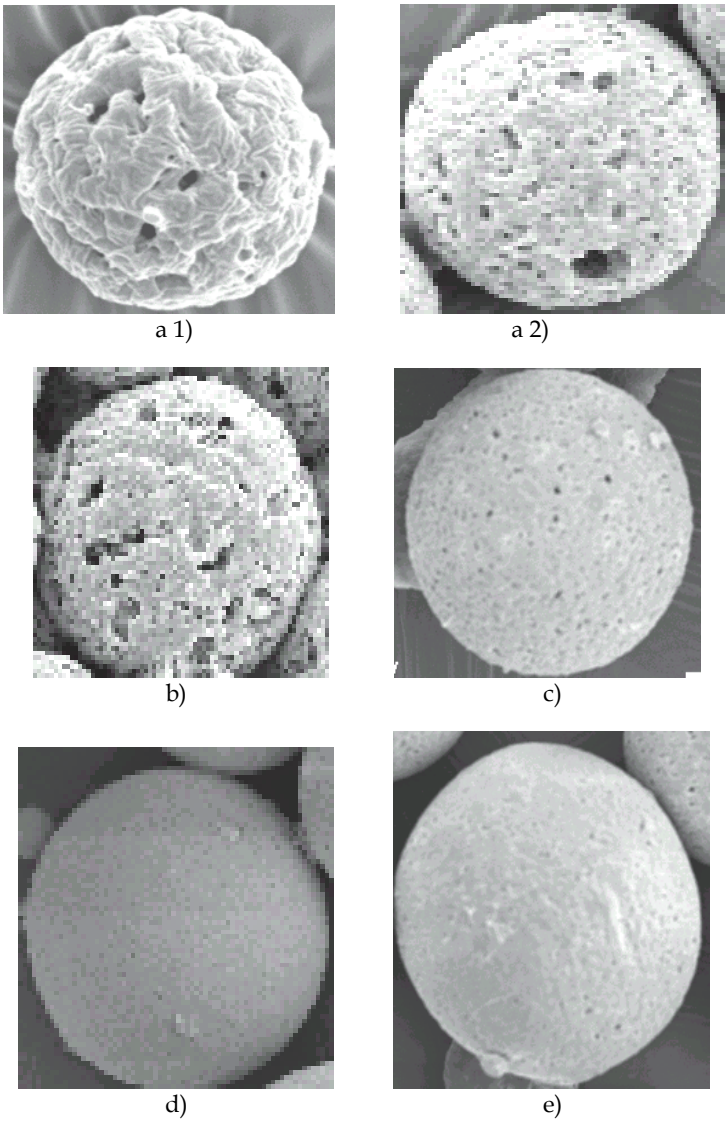

Fig. 3. SEM images of the polymeric microparticles: a) Formulation A; b) Formulation B; c) Formulation C; d) Formulation D; e) Formulation E.

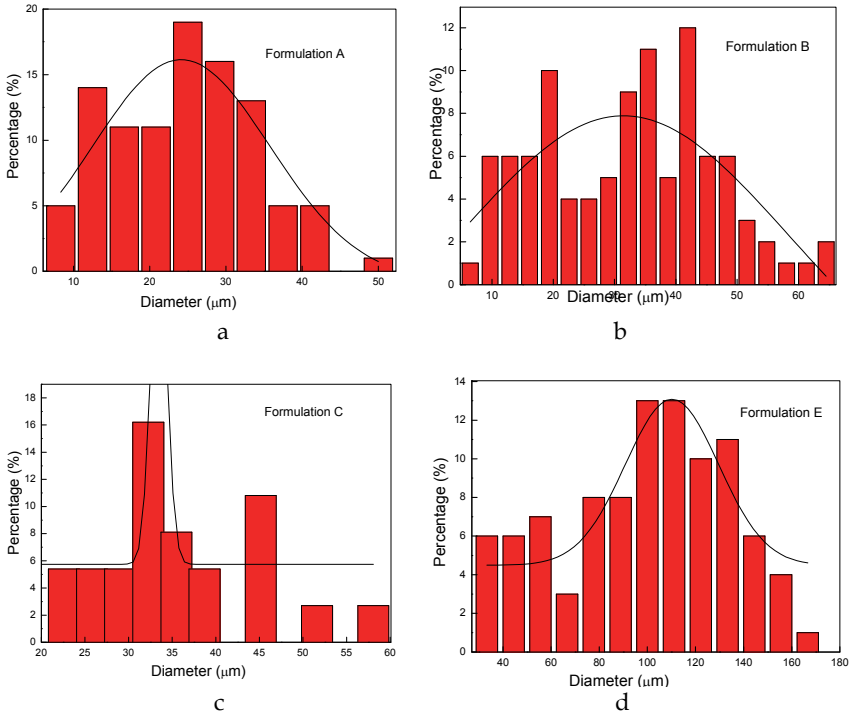

Fig. 4. Size distributions of the polymeric microparticles: a) Formulation A; b) Formulation B; c) Formulation C; d) Formulation E.

Formulation	PHBV (%)	PHB (%)	Average size (μm)
A	100	0	24.14 ± 1.606
B	75	25	31.45 ± 2.797
C	50	50	33.5 ± 3.22
D	25	75	*
E	0	100	110.2 ± 3.881

* Not determined.

Table 3. Average sizes (± SD) of the different microparticles.

The release profiles of free ametryn (as the reference) and ametryn encapsulated in the microparticles are illustrated in Figure 5, as a function of time (up to approximately 360 min). In these experiments the herbicide could traverse the pores of the membrane, while the microparticles were retained, so that it was possible to measure the influence of the association of ametryn with the polymeric matrix of the microparticles on its release rate. The release kinetics of free ametryn was faster than that of the encapsulated herbicide, with

almost total release after 360 min. Association with the microparticles resulted in retarded release, with around 70 % (formulations A and B), 30 % (formulation C), 20 % (formulation D) and 40 % (formulation E) being released after 360 min.

The release of other bioactive compounds from systems composed of microstructured polymers has been described in the literature, but usually for only one type of polymer (Grillo et al., 2010; Maqueda et al., 2009; Sendil et al., 1999; Singh et al., 2010; Wang et al., 2007). However, interpretation of release profiles relies to a large extent on knowledge of the composition and structural characteristics of the microparticles concerned, and in this respect studies that use more than one type of microparticle are advantageous. In the present work, the release of ametryn increased in line with the content of PHBV for formulations A-D, indicating that increased porosity aided the exit of ametryn molecules due to increased contact with the solvent. However formulation E was an exception to the rule, since it was composed of PHB alone and showed the fastest release of ametryn. There are two possible explanations for this observation. Firstly, the encapsulation efficiency of this formulation was lower than those achieved using the other formulations, which could have resulted in higher concentrations of ametryn crystals in the solution, and consequently higher release rates. Secondly, it is possible that lengthy refrigerated storage of this sample could have resulted in solubilization of the herbicide, due to increased contact time with the solvent.

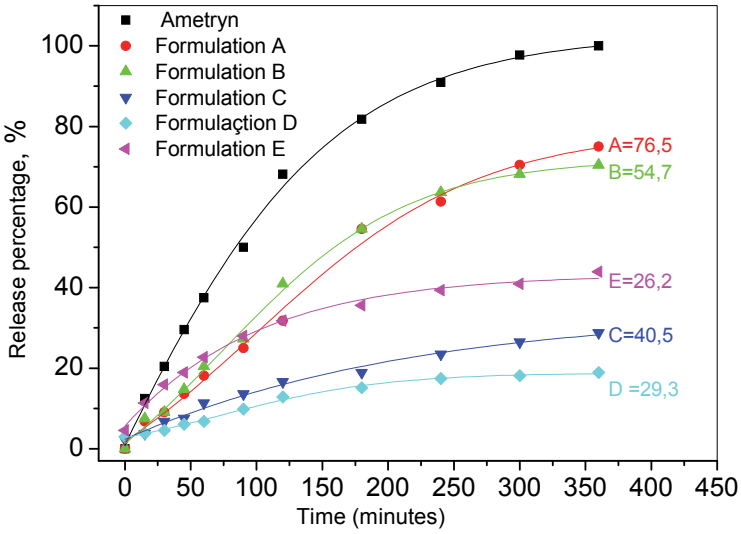

Fig. 5. Results of the release experiments, comparing the kinetic profiles of free ametryn and ametryn associated with the different microparticles (PHB, PHBV and PHBV+PHB), at ambient temperature (n = 3).

Analysis of release curves can provide important information concerning the mechanisms involved in the release of compounds from microparticles (Polakovic et al., 1999). Possible mechanisms include desorption from the surface of the polymeric matrix, diffusion through the pores or wall of the matrix, disintegration of the microparticle with subsequent release

of the active principle, and dissolution and erosion of the matrix or the polymeric wall (Polakovic et al., 1999; Schaffazick et al., 2003).

A number of mathematical models have been extensively used to analyze the characteristics of the release of substances from polymeric systems (Costa & Lobo 2001). Here, the results of the release experiments (Figure 5) were analyzed using the zero order, first order, Higuchi and Korsmeyer-Peppas models (Table 4). For the formulations investigated, the Korsmeyer-Peppas model provided the best explanation of the ametryn release mechanism, according to the correlation coefficient obtained. The curves obtained for each formulation using this model are illustrated in Figure 6.

	Zero order	First order	Higuchi	Korsmeyer-Peppas
Formulation A				
				n = 0,82641
Release constant (k)	4.59184 min^{-1}	0.00667 min^{-1}	4.49925 $min^{-1/2}$	0.00628min^{-n}
Correlation coefficient (r)	0.92307	0.98452	0.97721	0.99364
Formulation B				
				n = 0.79373
Release constant (k)	0.20767 min^{-1}	0.00624 min^{-1}	4.35701 $min^{-1/2}$	0.0072min^{-n}
Correlation coefficient (r)	0.89455	0.96782	0.98115	0.9879
Formulation C				
				n = 0.62532
Release constant (k)	0.07283 min^{-1}	0.00581 min^{-1}	1.52624 $min^{-1/2}$	0.0162 min^{-n}
Correlation coefficient (r)	0.86545	0.97701	0.9893	0.9929
Formulation D				
				n = 0.5671
Release constant (k)	0.048 min^{-1}	0.00495 min^{-1}	1.00913 $min^{-1/2}$	0.0194min^{-n}
Correlation coefficient (r)	0.90337	0.96059	0.97587	0.98839
Formulation E				
				n = 0.42726
Release constant (k)	0.0983 min^{-1}	0.00441 min^{-1}	2.17047 $min^{-1/2}$	0.0429min^{-n}
Correlation coefficient (r)	0.79093	0.92828	0.99035	0.9927

Table 4. Results of the application of four mathematical models to the release curves of ametryn associated with different microparticles.

The Korsmeyer-Peppas model is based on a semi-empirical equation (Korsmeyer & Peppas, 1991; Korsmeyer et al., 1983) that is widely used when the release mechanism is unknown. When the release exponent (n) is equal to 0.43 the mechanism involved is diffusion. When the value of the exponent is greater than 0.43 but smaller than 0.85, the release occurs due to anomalous transport that does not obey Fick's Law. Values less than 0.43 are indicative of porous systems in which transport occurs by a combination of diffusion through the polymeric matrix and diffusion through the pores. The values obtained (Table 4) differed

according to formulation, as expected considering the different structural characteristics of the microparticles, so that the release mechanisms were not identical. Nonetheless, the values obtained for all formulations were in the range $0.43 < n < 0.85$, indicating that in all cases the release occurred as a result of anomalous transport, involving diffusion and relaxation of the polymeric chains. This information concerning the release mechanism is of vital importance in order to be able to adjust and optimize the release of the active principle according to circumstances.

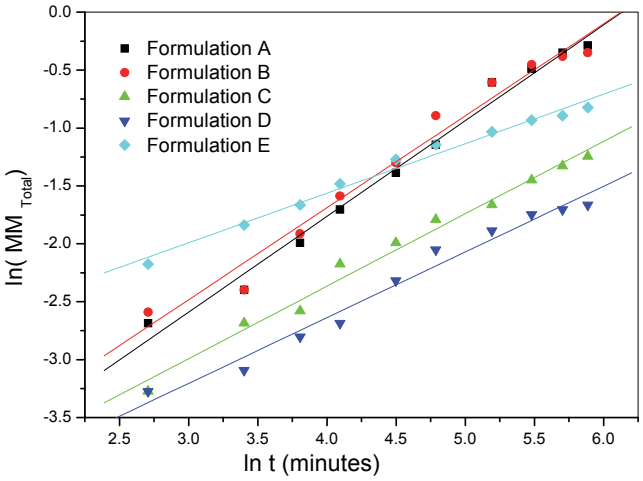

Fig. 6. Results obtained using the Korsmeyer-Peppas model applied to formulations A-E.

4. Conclusions

Ametryn herbicide was efficiently encapsulated in microparticles composed of PHB, PHBV and mixtures of the two polymers. The highest encapsulation efficiencies were achieved when higher proportions of PHBV were used. SEM analysis showed that the microparticles were spherical, although with different surface features (either smooth or rough with pores). The release profile of ametryn was modified when it was encapsulated, with slower and more sustained release compared to the free herbicide. This finding suggests that the use of encapsulated ametryn could help to mitigate adverse impacts on ecosystems and human health. This is particularly important given the increasingly widespread and intensive use of agents such as ametryn in modern agriculture.

5. Acknowledgments

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2

Benzoxazolinone Detoxification and Degradation – A Molecule´s Journey

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1. Introduction

Benzoxazinoids are important secondary products of maize, several other Poaceae and a few dicotyledonous species belonging to the Acanthaceae, Lamiaceae, Scrophulariaceae and Ranuculaceae. The synthesis which was investigated in maize by the group of Gierl and Frey starts with the conversion of indole-3-glycerol phosphate to indole. The following steps involve four cytochrome P450 dependent monooxygenases (*BX2-BX5*) that convert indole to benzoxazinone by incorporation of oxygen. Glucosylation at the 2-position of DIBOA results in DIBOA-glucoside, an intermediate of the final product DIMBOA-glucoside (Frey et al., 1997; Glawischnig et al., 1999; von Rad et al., 2001; Jonczyk et al., 2008; Schuhlehner et al., 2008). Whereas the benzoxazinoid acetal glucosides are stable under neutral conditions, the aglycones with the 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one skeleton underlay a degradation by ring contraction and release of formic acid which yields the benzoxazolinones BOA or MBOA (Sicker et al., 2000; Sicker & Schulz, 2002). These derivatives are more stable and can be detected in the soil of rye or wheat fields over a period of several weeks until they are absorbed by other plants or they are converted by microorganisms. The release of benzoxazinoids into the environment and their final degradation are cornerstones within the lifetime of these molecules. In between, a complex set of (re)-modulations and conversions take place due to the activities of a variety of organisms, such as higher plants, fungi and bacteria. Our contribution will give an impression of shuttles between those organisms that end up in the degradation of phenoxazinone(s) as the final conversion products with a limited life time but will also present several reactions of maize to the treatment with benzoxazolinone BOA.

Investigations of weed specific and of benzoxazinoid producing crops specific reactions, reactions of microorganisms, effects on the biodiversity of soil organisms and the elucidation of degradation processes are unequivocally necessary before bioherbicides can be used.

2. Functions of benzoxazinoids

Benzoxazinone glucosides are stored in the vacuole until the tissue is damaged, for example by herbivores, and hydrolysis of the sugar moiety by ß-glucosidases takes place. The highly bioactive aglycones can be released into the environment also by root exudation or by plant residue degradation (Barnes & Putnam, 1987). The mutagenic benzoxazinoids are electrophilic compounds that interact with proteins, intercalate with nucleic acids and are deleterious for many cellular structures and activities (Frey et al., 2009; Sicker & Schulz, 2002). In maize, DIMBOA may have an additional endogenous function. Recently Frebortova et al. (2010) discussed a possible role in cytokinin degradation. Oxidative cleavage of DIMBOA led to coniferron, an electron acceptor of cytokinin dehydrogenase. However, benzoxazinoids have first of all an outstanding role as chemical weapons against other organisms (Niemeyer 2009). Aside of their insecticidal, fungicidal and bactericidal properties, benzoxazinoids are phytotoxic to susceptible plants. Often observed reactions are an inhibited germination but particularly the reduction of seedlings growth. Therefore, the compounds could play an important role in sustainable agricultural systems for natural weed and pest control in innovative agricultural systems.

3. Factors that influence benzoxazinone accumulation

The amount of benzoxazinoids varies highly with plant age, organ and cultivar. Investigated rye cultivars differ in the total benzoxazinoid amounts from 250 to 1800 µg g^{-1} dry tissue in young plants to about 100 µg g^{-1} or less in old plants (Reberg-Horton et al., 2005; Rice et al., 2005; Zasada et al., 2007). In rye cultivars used as mulches by Tabaglio et al. (2008), the content ranges from 177 to 545 µg g^{-1}. High differences in the concentrations among rye cultivars are also reported by Burgos et al. (1999). Water stress conditions and high temperatures increase the content of DIMBOA and DIBOA (Gianoli &Niemeyer 1997; Richardson & Bacon 1993). Nitrogen fertilization has a significant influence on the benzoxazinoid content (Gavazzi et al., 2010). In maize, we found a 3-4 fold higher benzoxazinone accumulation under sulfur deficiency conditions compared to the control plants which were cultivated under optimal nutrient supply (Fig. 1).

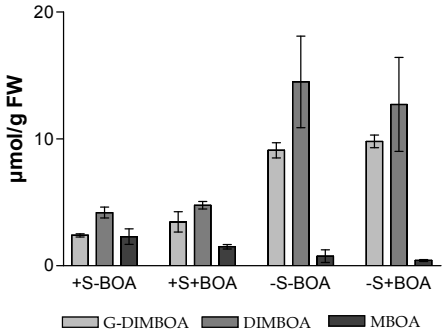

Fig. 1. Influence of sulfur on the benzoxazinoid accumulation in maize roots. Three week old plants were used for the incubation with 0.5 mM BOA (40 ml=20 µmol/g FW). Plant cultivation, BOA incubation, extraction and analyses were performed as described in Knop et al. (2007) and Sicker et al. (2001). *N* =5.

The reason for the stress induced benzoxazinoid accumulation is unclear since the biosynthesis of the compounds is developmentally regulated (Frey et al., 2009). Recently Ahmad et al. (2011) found an increased apoplastic accumulation of DIMBOA-glucoside, DIMBOA and HDMBOA-glucoside in maize leaves during defined stages of infestation with *Rhopalosiphum padi* and *Setosphaeria turtica*. Thus, the translocation of benzoxazinoids out of the cell may be an important step of a process which can lead to an increased stress tolerance and biocidal defense.

4. Effects of benzoxazolinone in maize – increase of glutathione transferase actvity and glutathione levels

Several groups investigated the mode of action of benzoxazolinones (Baerson et al., 2005; Batish et al., 2006; Sanchez-Moreiras et al., 2010, 2011; Singh et al., 2005) on *Lactuca sativa*, *Arabidopsis thaliana* or *Phaseolus aureus*. In plants BOA induces oxidative stress, membrane damage and lipid peroxidation. A prolonged exposure to high BOA concentrations (45 µmol, *Arabidopsis thaliana*) up to 8 days led to a decline in photosynthetic efficiency, induced senescence and death. At sub lethal concentrations, *A. thaliana* reacts with a strong alteration of the gene expression pattern, which comprises about 1% of the total genome. Burgos et al. (2004) found reduced densities of ribosomes, dictyosomes and mitochondria together with a lower amount of starch granules in roots of cucumber seedlings after treatment with BOA or DIBOA. These authors assume that BOA and DIMBOA induces changes in cellular ultrastructure, reduces root growth by disrupting lipid metabolism, by a decreased protein synthesis, and by a reduced transport or reduction of secretory capabilities.

Although maize roots are relatively resistant to BOA (Knop et al., 2007), their physiology is affected when exposed for 24 hours to levels considered to be non-toxic (500 µM and lower). As indicated by the marker compound malondialdehyde (MDA) lipid peroxidation is one of the earliest effects in roots of 6 to 7 days old maize seedlings. An increase is already observed after 1 min and a maximum between 5 to 40 min (Fig. 2). Subsequently, the MDA amount drops below the control value. This indicates the fast activation of mechanisms that counteract cellular damage, also an important action to avoid autotoxicity. During the next hours the level of GST activity is slightly increased (17-30% compared to +S -BOA conditions) in BOA incubated root tips of plants cultivated under optimal sulfur supply. At the same time the major detoxification product glucoside carbamate starts to accumulate (see below). Root tips from -S-plants have only about 40% of the GST activity found in +S-plants. The activity increases up to 50% during the course of incubation, but the presence of BOA has no influence (Fig. 3). Thus, -S-plants have deficits in providing GSTs that have a function in stress reactions.

The soluble plant glutathione transferases are categorized in defined classes: *Θ* (GSTF), *T* (GSTU), *Φ* (GSTT), *Z* (GSTZ), *Λ* (GSTL), dehydroascorbate reductase and tetrachlorohydroquinone dehalogenase like enzymes. Phi and tau class enzymes have a well known function in herbicide detoxification. GSTs respond to many processes that induce ROS production. Up regulating of GST gene expression can be triggered by herbicide safeners (Riechers et al., 2010). GSTs could have as well a function in the detoxification of endogenous substrates (Dixon et al., 2010). Because of the lack of *in vivo* accumulating natural glutathionylated products, Dixon et al. (2010) postulate unstable reaction products, which may decay or which are immediately transformed to other products by metabolic

channeling. GSTs may have also non-catalytic functions as transporters of unstable GS-conjugates, which can be generated spontaneously via radical formation of an acceptor molecule in presence of glutathione. In *Arabidopsis*, BOA induces the up regulation of several GST genes (Baerson et al., 2005). If these GSTs are involved in BOA detoxification pathways is unclear since glutathione conjugates have not yet been found in *Arabidopsis,* maize or other plants. However, it cannot be excluded that GSTs have a role in the transport of unstable intermediates of BOA detoxification products. This question is currently under investigation.

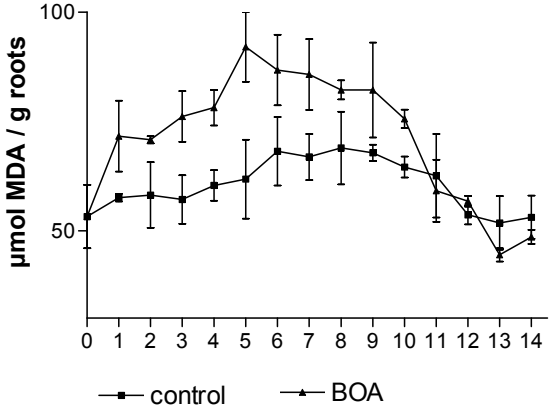

Fig. 2. Course of malondialdehyde (MDA) production in maize roots of 6-d-old seedlings. 0: control; 1-5: intervals of measurements 1 min; 6-10: intervals 15 min; 11: 90 min, 12: 3h, 13: 3h, 14: 4h after start of the incubation. Seedlings were grown and incubated as described in Schulz & Wieland (1999). Samples were prepared and MDA was determined according the method of Wong et al. (2001). *N* = 5.

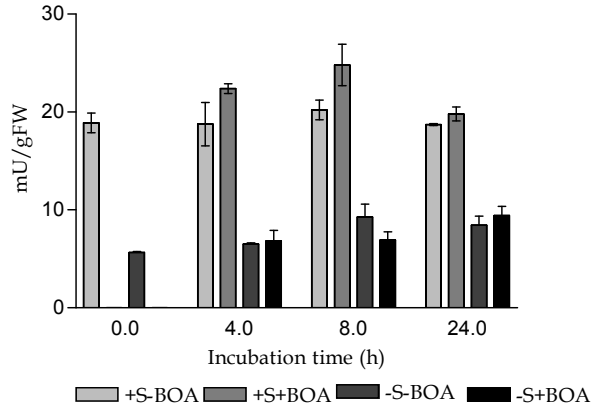

Fig. 3. GST activity in root tips of aeroponically cultured maize plants (greenhouse condition) according the method of Habig & Jacoby (1981). Sulfur deficiency was induced as described in Knop et al. (2007). *N* = 3.

The tripeptide glutathione (GSH) is the major thiol inter alia in plants and a substrate for the GSTs. The multiple functions of GSH in organisms include important roles in redox-homeostatic buffering, in cellular signaling, root development, sulfur assimilation, in defense and stress reactions and in the detoxification of xenobiotics (see review article of Noctor et al., 2011).

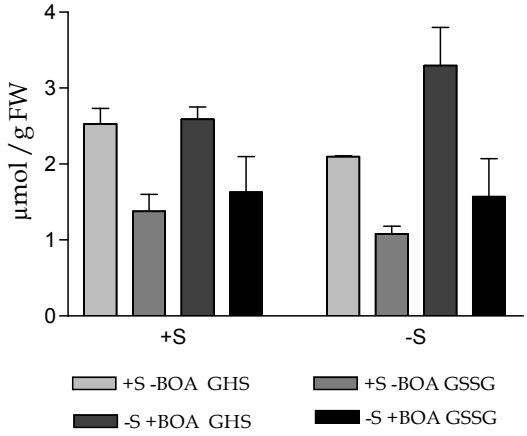

Fig. 4. Reduced and oxidized glutathione in root tips of aeroponically cultured maize plants (greenhouse). The plant material was extracted as described by Gamczarka (2005); measurement of glutathione was done according to Teare et al. (1993). *N* = 3.

The glutathione concentration measured in +S-root tips is similar to the values published by Kocsy et al. (2000). In -S-root tips, the total GHS content is decreased. However, we found slightly increased content of the total GHS in the root tips of –S-maize plants after 24 h exposure to BOA (Fig. 4). The content of GSSH in all samples indicates the induction of some oxidative stress due to the incubation conditions (see also Fig. 2), but there is a tendency of higher GSSG contents in BOA incubated plant material which indicates oxidative stress. Our results are conform to the findings that herbicides can increase the content of glutathione and the activity of associated enzymes such as glutathione reductase in herbicide tolerant plants (Kocsy et al., 2000). The response of –S-maize glutathione content to exogenously applied BOA is obviously very similar to that observed with certain herbicides. Glutathione synthetase gene induction under stress conditions is known (Hruz et al., 2008). An induction of glutathione synthesis even under -S-conditions would involve a change in the priority of the sulfur use in sulfur deficient plants and the mobilization of sulfur from sulfur containing molecules in the root tip. The exact role of glutathione in BOA detoxification is still under investigation. At present it is already unambiguous that the sulfur availability is important in the plant´s coping with BOA (see below).

5. Actin cytoskeleton, cytoarchitecture, and auxin transport

Although maize root growth is not inhibited significantly by BOA, we have scored subtle but relevant effects on the actin cytoskeleton and root apex cytoarchitecture which are

stronger exhibited in –S-plants. In cells of the meristem and transition zone, nuclei are affected in their typical central position and shifted laterally and/or axially (Fig. 5, 6). Similar effects were reported in maize root cells having affected their actin cytoskeleton due to impacts of the actin polymerization inhibitors or in mutant of maize *lilliputian* having aberrant actin cytoskeleton, irregular cell files and root anatomy (Baluška et al., 2001a, 2001b). Importantly, the actin cytoskeleton under the plasma membrane (Fig. 8), especially at the synaptic cell-cell adhesion domains is affected (Baluška et al., 2005). These domains are depleted in their abundant F-actin (Baluška et al., 1997) whereas there are over-polymerized F-actin foci assembled around nuclei of BOA-exposed root cells, shifted out of cellular centres (Fig. 6-8). Similar impacts on the cytoarchitecture were reported in maize root cells exposed to vesicular secretion inhibitor refeldin A (Baluška & Hlavacka 2005), as well as to mastoparan which is affecting phosphoinositide signalling (Baluška et al., 2001c), and to auxin transport inhibitors (Schlicht et al., 2006). Unique BOA-induced effect on the actin cytoskeleton of the transition zone cells is the prominent local assembly of F-actin patches at corners of cross-walls (plant synapses) which resemble published data of the *brk1* mutant line of *Arabidopsis* (Fig. 7C, D) in this chapter and Figure 8 in Dyachok et al., 2008). Interestingly, the BRK1 protein localizes to the cross-wall corner sites of *Arabidopsis* root apices showing aberrant actin organization both in the root cells of the *brk1* mutant and in the BOA-exposed root cells (Fig. 4). BRK1 is a component of the evolutionary conserved SCARE complex that acts as F-actin nucleator and BOA might directly target the SCARE complex. This would be a very attractive scenario and it should be tested in future. BOA is also known to inhibit activity of the PM H^+-ATPase of root cells (Friebe et al., 1997) and it is of interest to note that the actin cytoskeleton is controlling permeability of the plant plasma membrane (Hohenberger et al., 2011). Interestingly, maize mutants *lrt1* and *rum1*, and especially the *lrt1/rum1* double mutant, which are affected in the polar auxin transport, showed similar F-actin depletion at the synaptic cell-cell adhesion domains and shifted nuclei (Schlicht et al., 2006). In general, all polar auxin inhibitors resemble BOA in affecting the actin cytoskeleton especially at the transition zone of the root apex which is the most active zone with respect of F-actin rearrangements (Baluška et al., 1997, 2001a, 2001b, 2001d), the polar auxin transport (Baluška et al., 2010; Mancuso et al., 2005, 2007).

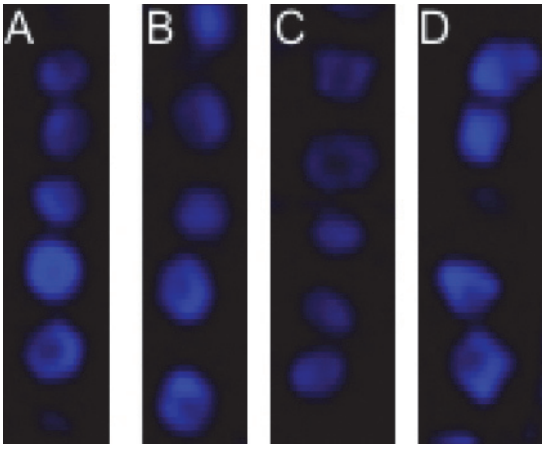

Fig. 5. Shifted nuclei (visualized with DAPI) in root apex cell files. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA.

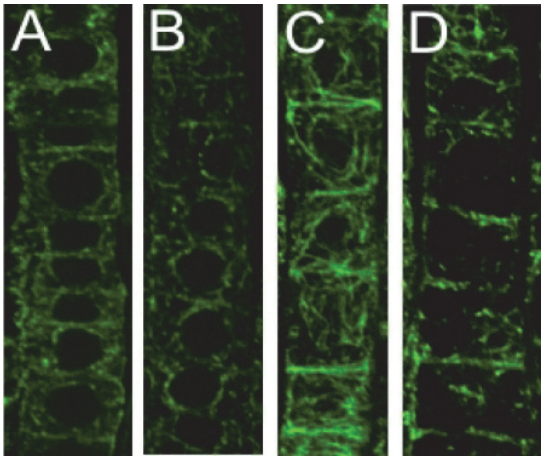

Fig. 6. Overview of the actin cytoskeleton in maize root apex. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA.

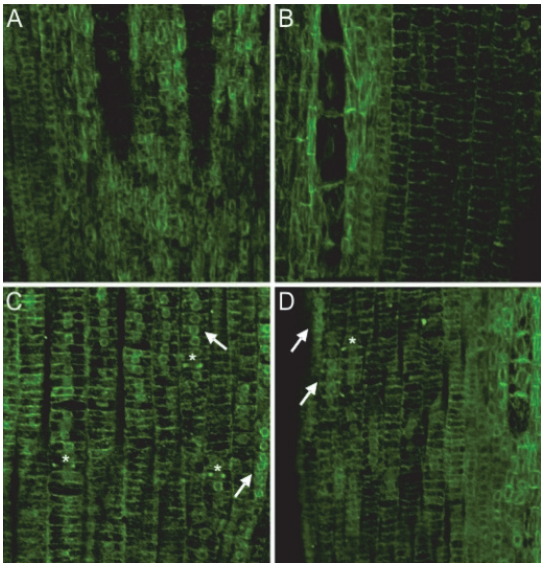

Fig. 7. Details of the actin cytoskeleton in cortical cells of the transition zone. Note the aberrant over-polymerization of F-actin in cortex cells of the BOA-exposed roots. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA

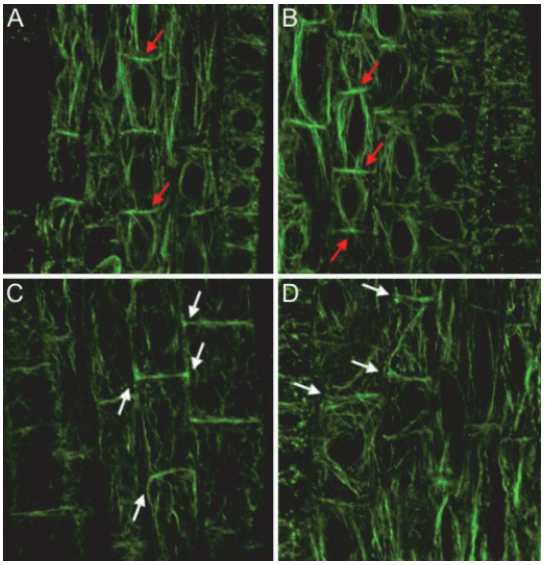

Fig. 8. Details of the actin cytoskeleton in cells of the transition zone. Red arrows indicate abundant F-actin at the cross walls (plant synapses) of pericycle and endodermis cells. White arrows indicate depleted F-actin at the cross walls (plant synapses) of pericycle/ endodermis cells of the BOA-exposed roots. Note the over-polymerization of F-actin in the cell corners. **A**: +S-BOA, **B**: -S-BOA, **C**: +S+BOA, **D**: -S+BOA.

For the experiments, apical root segments (~7mm) encompassing the major growth zones were excised, fixed with 3.7% formaldehyde, and embedded in the Steedman's wax. Ribbons of 7-mm sections were dewaxed and incubated with a mouse anti-actin monoclonal antibody, clone C4 from ICN Pharmaceuticals (Costa Mesa, CA, USA) diluted 1:200 and a rabbit maize polyclonal anti-actin (gift of Chris Staiger, Purdue University, USA) diluted 1:100 in PBS buffer. After rinsing with PBS buffer and incubation with secondary antibodies, sections were mounted under a coverslip and examined in the confocal microscope Olympus Fluoview 1000.

Similarly to *Arabidopsis* roots, the actin cytoskeleton is affected by auxin transport inhibitors in similar manner as we have reported here for the maize root cells (Rahman et al., 2007; Dhonukshe et al., 2008). Importantly in this respect, BOA is known to act as anti-auxin and to block lateral root formation (Anai et al., 1996; Baerson et al., 2005; Burgos et al., 2004; Hoshi-Sakoda et al., 1994) a process well-known to be based on auxin transport in both monocots and dicots roots (Hochholdinger & Zimmermann, 2008; Peret et al., 2009). Finally, the root apex transition zone emerges as specific target of allelochemicals, particularly this unique zone of the root apex (Baluška et al., 2010). Future studies should focus on these effects of BOA on the polar auxin transport and on other processes and activities characteristic for the root apex transition zone (Baluška et al., 2010).

6. Detoxification of benzoxazolinones in higher plants

Plants react to BOA in a species- and dosage dependent manner. Generally, members of the Poaceae were found to be less sensitive to the compounds than dicotyledoneous species,

although there are exceptions. Moreover, ecotypes (for instance, *Chenopodium album* ecotypes or *Portulaca oleracea* garden forms) and varieties can differ in their accumulation and detoxification activities (Schulz unpublished). One important reason for the different sensitivity is the better developed ability of most Poaceae to reduce the toxicity benzoxazolinone(s), in comparison to dicots. Deleterious effects on the biochemistry, physiology and cell biology are therefore limited in good detoxifiers (see effects on maize described above). Interestingly, Macias et al. (2005) found an almost 100% inhibition of *Allium cepa* and *Lycopersicon esculentum* root growth whereas *Triticum aestivum* root growth was inhibited to 50 %, when low concentrations of DIMBOA-glucoside (5 µmol = 5 ml of a 1mM solution used as the highest concentration) were applied to 10 or 25 seeds in Petri dishes. It is generally known that the glucosides of benzoxazinones are much less toxic than the aglycons, but they obtained similar results with DIBOA. The growth of *Lepidium sativum* was stimulated to about 20%.

Almost all investigated higher plant species detoxify benzoxazolinone (BOA) via 6-hydroxylation and subsequent O-glucosylation (Tab. 1). *Portulaca oleracea* and a few other related species produce BOA-5-O-glucoside as a byproduct (Hofmann et al., 2006). Monocots perform, mainly or at least to a considerable portion, glucoside carbamate (Schulz et al., 2006; Schulz & Wieland, 1999; Sicker et al., 2000, 2004; Wieland et al., 1998). In contrast to BOA-6-OH and its glucoside, glucoside carbamate is not toxic up to concentrations of 1 mM and is therefore a most suitable detoxification product. First found in maize, glucoside carbamate is subsequently modified by malonylation or by addition of a second glucose molecule yielding gentiobioside carbamate (Hofmann et al., 2006). BOA-6-O-glucoside is, however, the major detoxification product when maize or other seedlings are incubated with MBOA. Glucoside methoxycarbamate occurred only in maize as a minor compound when the incubation was extended to more than 48 h. Identified stable detoxification products are illustrated in figure 9. The accumulation of BOA-6-OH is a good marker for a high sensitivity to BOA (for example *Vicia faba*). This hydroxylation product is twice as toxic as BOA and causes necrosis in the root tips within 24 h.

The BOA-detoxification process in maize roots starts with the production of BOA-6-O-glucoside. However, after 3 to 6 h glucoside carbamate accumulation is initiated. About 10 h after incubation start, this compound becomes the major detoxification product, whereas BOA-6-O-glucoside does not further accumulate although the glucosyltransferase activity responsible for glucosylation of BOA-6-OH is still abundant (Schulz et al., 2008). The increasing accumulation of gentiobioside carbamate and malonyl-glucoside carbamate 18 to 20 h after start of the incubation is a late event in the detoxification process. Avoidance of BOA uptake can be another strategy to escape the harmful effects of BOA.

In a recent study, we found a significant reduction of redroot pigweed (*Amaranthus retroflexus* L.) and common purslane (*Portulaca oleracea* L.), whereas common lambsquarters (*Chenopodium album* L.) and velvetleaf (*Abutilon theophrasti* Medicus) were moderately or not suppressed, respectively (Gavazzi et al., 2010; Tabaglio et al., 2008).

One possibility to explain the different reactions of the four weeds could be differences in the detoxification activities or accumulation characteristics that minimize the harmful effects of rye allelochemicals (BOA and related compounds). This affects a direct correlation between the benzoxazinoid content of rye mulch used in the study and weed suppression. The four warm season weeds exhibit remarkable differences in their detoxification behavior

with a high correlation to the sensitivities of the weeds previously observed in experiments with rye mulch under greenhouse conditions. These studies demonstrate for the first time that detoxification processes are important for the survival of adapted weeds in environments enriched with benzoxazinoids, such as maize, wheat or rye fields (Schulz et al., submitted). Moreover, nutrients together with stress conditions have an influence on the detoxification processes. For instance, sulfur deficiency in combination with herbicide treatment can lead to a breakdown of the BOA detoxification process in maize (Knop et al., 2007). Optimal sulfur supply seems to be an emerging factor to guarantee well functioning of detoxification pathways. This is particularly important since sulfur deficiency is increasing in many areas of the word (Scherer, 2001, 2009).

Family	**Species**	**A**	**B**	**C**	**D**	**E**	**F**
Poaceae	*Avena sativa*	xx	xx				
	Avena fatua	xxx	xx				
	Digitaria sanguinalis	xxx	x				
	Lolium perenne	xx	xxx				
	Hordeum vulgare	xx	xxx				
	Triticum aestivum	x	xxx				
	Secale cereale	x	xxx				
	Zea mays	(x)	xxx		xx	xxx	
Portulacaceae	*Portulaca oleracea* cv Gelber	xx	xx		x	x	xx
Chenopodiaceae	*Chenopodium album (ecotypeI)*	xx	x	x			
Brassicaceae	*Arabidopsis thaliana*	xxxx	x				
	Rhaphanus sativus	xxx	x				
	Diplotaxis tenuifolia	xx		x			
Amaranthaceae	*Amaranthus albus*	xxx					
	Amaranthus retroflexus	xx	xx		x	x	
Ranunculaceae	*Consolida orientalis*	xxx	x				
	Consolida regalis	xxx	x				
Apiaceae	*Coriandrum sativum*	xxx	xx				
	Daucus carota	xxx	x	x			
Asteraceae	*Galinsoga ciliate*	xxx		x			
	Helianthus annuus	xxx					
Fabaceae	*Vicia faba*	xx		xx			

Table 1. Some plant species (6-10 days old seedlings) and their major BOA detoxification products after 24 h of incubation with 0.5 mM BOA (40 ml / g FW). Maize: compounds present after 48 h are considered. Major compound: xxx; xx: minor compound: x; traces (x). **A**: BOA-6-O-glucoside; **B**: glucoside carbamate; **C**: BOA-6-OH; **D**: gentiobioside carbamate; E: malonylglucoside carbamate; F: BOA-5-O-glucoside.

There are also some hints that the ecobiochemical potential of species to detoxify benzoxazolinone drives the membership to certain plant associations (Schulz & Wieland, 1999).

A portion of the detoxification products are released again by root exudation (Sicker et al., 2002). When BOA incubated maize plants are transferred to tap water, BOA-6-O-glucoside and glucoside carbamate can be identified in the water. After several days, the compounds

cannot be detected anymore in the soluble fraction prepared from the plants. A similar result is obtained with *Galinsoga ciliata* and *Coriandrum sativum*, indicating that exudation of soluble detoxification products is a more general phenomenon. The exuded products can get in contact with endophyts and microorganisms of the rhizosphere.

7. Microbial degradation products and fate of exuded plant degradation products

Many fungi are known to be sensitive to benzoxazinones and benzoxazolinones. However, some are able to detoxify the compounds (Fig. 10). Species of *Fusarium* have been investigated for their growth in presences of benzoxazinone (Friebe et al. 1998; Glenn et al. 2001). Eleven of 29 *Fusarium* species had some tolerance to BOA, the most tolerant species was *F. verticillioides* with only one sensitive strain of the 56 ones tested (Glenn et al., 2001). The first step in the degradation of benzoxazolinone-2(*3H*)-one (BOA) is a hydrolysis yielding 2-aminophenol. This step is performed by bacteria as well, also by seed born ones (Bacon et al. 2007; Burdziak et al., 2001). 2-Aminophenol is not stable but is spontaneously dimerized to 2-amino-3*H*-phenoxazin-3-one (APO) or it can be captured by several fungi which convert the compound to N-(2-hydroxyphenyl)malonamic acid (oHPMA) and 2-acetamidophenol (AAP) (Carter et al., 1999; Friebe et al., 1998; Glenn et al., 2001, 2002, 2003). Several endophytic fungi (*Plectosporium tabacinum, Gliocladium cibotii, Chaetosphaeria sp., Fusarium sambucinum*) from *Aphelandra tetragona* are described to produce 2-amino-(*3H*)-phenoxazinone derivatives when incubated with benzoxazinones (Baumeler et al., 2000; Zikmundova et al., 2002a, 2002b). *Fusarium verticillioides*, an endophytic fungus of maize, did not convert benzoxazolinone to any known microbial degradation product when sterile grown maize seedlings were inoculated with the fungus whereas the seedlings produced their known detoxification products since gentiobioside carbamate and glucoside carbamate could be detected in the medium. APO, AAP and oHPMA can have effects on plant growth. Absorbed traces of AAP and oHPMA stimulated maize radicle growth; traces of AAP stimulated that of cress. Phenoxazinone inhibited the growth of cress radicles at concentrations higher than 500 µM, whereas maize radicles were hardly affected (Knop et al., 2007).

In another study (Schulz et al., unpublished), the growth of some representative fungi was monitored over a period of 10 days in presence of BOA and APO. Generally, BOA was always less toxic than APO. The ability to grow in presence of BOA is influenced by the availability of nutrients. Several species changed the sensitivity to BOA, when BOA had to be used as N-source.

Once released into the soil, the plant and microbial detoxification products can be degraded by fungi. All compounds are finally converted to phenoxazinone(s): The degradation work of *Botrytris cinerea* (B.cin), *Drechslera tuberose* (D.tub), *Fusarium heterosporum* (F.het), *F. verticillioides* , *F. oxysporum* (F.oxy), *F. culmorum* (F.cul) , *F. solanum (*F.sol*), Trichoderma viride* (T.vir) is presented in Fig. 11.

In the media of *Fusarium verticillioides* and *Drechslera tuberosa*, some benzoxazolinone-2(*3H*)-one (BOA) is present after the incubation with glucoside carbamate, the medium of the other fungi contained only traces of BOA. This indicates an opening of the carbamate heterocycle followed by the release of glucose. *Botryis cinerea* has only a rather limited ability to degrade

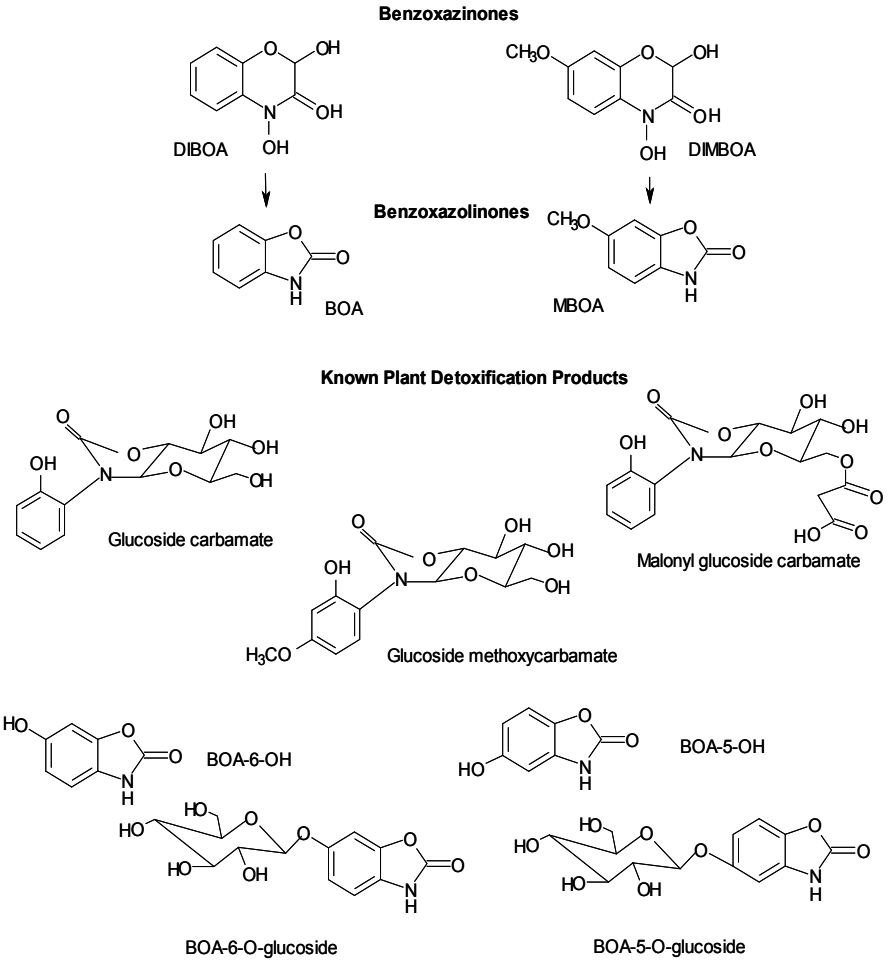

Fig. 9. Benzoxazolinone detoxification compounds produced by plants.

the compound, while *Paecilomyces farinosus* is unable to convert it. The behavior is, however, highly dependent on the different strains of a given species.

In the media of all of the species able to degrade glucoside carbamate a new, hitherto unknown intermediate occurred. The new compound was isolated for structural analysis. The ^{1}H spectrum showed signals for an *ortho*-substituted phenyl ring and well resolved signals in the sugar region with all couplings, too. The complete assignment was made by use of H,H-COSY, HMQC and HMBC. The latter technique was decided to prove that the hydrolytic ring opening of the oxazolinone precursor 1-(2-hydroxyphenylamino)-1-deoxy-ß-glucoside 1,2-carbamate (glucoside carbamate) led to a carbamic acid structure instead of a regioisomeric carbonate with 2-OH from the sugar moiety. Accordingly, H-1 of the glucose unit appears at 5.84 ppm and shows in the HMBC two cross peaks with C-3 of glucose at 74.0 ppm and the COOH group (158.3 ppm), each by coupling via three bonds.

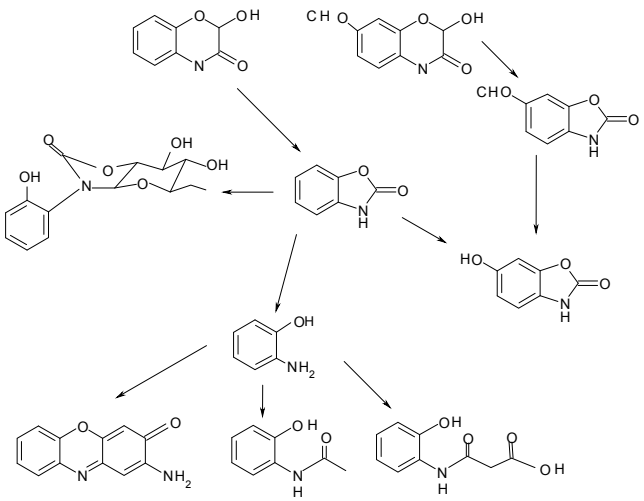

Fig. 10. Microbial degradation products derived from BOA.

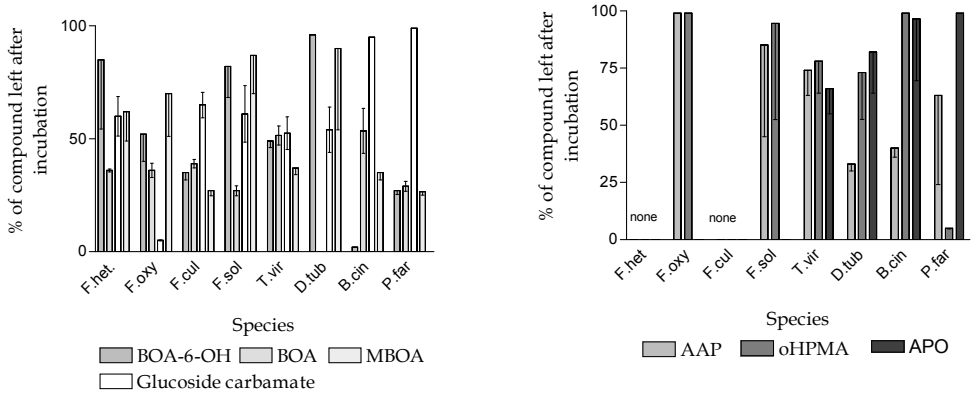

Fig. 11. Mycelial plugs from agar plates (discs 0.5-1 cm in diameter) were transferred into 250 ml flasks with 100 ml sterilized Czapek medium. When mycelia were well developed, 0.5 mg were transferred to 100 ml flasks containing sterile medium without sucrose (controls) and with addition of 10 µmol BOA, BOA-6-OH, MBOA, glucoside carbamate, AAP, oHPMA, or APO (1 µmol). Cultures were grown at 25 ^{0}C in the dark without shaking. Species which did not grow without sucrose were incubated with the different compound in presence of sucrose. BOA and 2-acetamidophenol were from Aldrich, MBOA was synthesized (Sicker 1989) as well as BOA-6-OH (Wieland et al., 1999) and oHPMA (Friebe et al., 1998). Glucoside carbamate) was prepared as described (Wieland et al., 1998; Sicker et al., 2001). The cultures were harvested after 14 days of cultivation. Mycelia were separated by filtration through 100µm nylon nets, dried between paper sheets and weighted. The medium was extracted with ethyl acetate. The organic and aqueous phases were evaporated to dryness, the residues dissolved in 70 % methanol and analyzed by HPLC. N = 5.

The following data could be obtained by MS-analysis: In the positive ion mode (with addition of formic acid for a better ionization), several peaks appear: a protonated monomer ion at 298.09216 da (exact theoretical mass 298.09213 da) besides two sodium-adducts of appropriate mono- and dimer ions at 320.07465 da (exact theoretical mass 320.07407 da) and 617.15936 da (exact theoretical mass 617.15892 da), respectively. By addition of sodium formate to the sample solution, the two last-mentioned signals increase to the most intensive peaks in the spectrum, accompanied by a further dimer peak at 639.14197 da ([2M-H,+2Na]$^+$, exact theoretical mass 639.14087 da).

In the negative ion mode, applied to the initial methanolic solution without buffer, a corresponding weak signal at 296.07845 da (exact theoretical mass 296.07758 da) appears. With ammonia as buffer this monomer signal at 296.07806 da increases and is still accompanied by a weak dimer signal. By use of a stronger base like triethylamine, the above mentioned mono- and dimers appear again, however, now accompanied by an additional ion pair of low intensity at 314.08320 da and 629.13868 da. The latter ion was already detected in the ammonia-spectra. This at first glance odd behavior can be easily understood as follows: Object of investigation is compound **1** from a well separated peak of the HPLC chromatogram. The retention time of **1** is distinctively different from that of the precursor glucoside carbamate. Hence, our findings from the mass spectra lead to the conclusion, that under the ESI conditions the carbamic acid **1** reacts almost completely back to the glucoside carbamate by dehydration. Only under strongly basic conditions signals for the intrinsic carbamic acid with the formula $C_{13}H_{17}NO_8$ appear. Thus, by means of the MS and NMR data analysis the new compound was identified as N-ß-D-glucopyranosyl-N-(2'-hydroxyphenyl) carbamic acid (**1**), (N-glucosylated carbamic acid, Fig. 12).

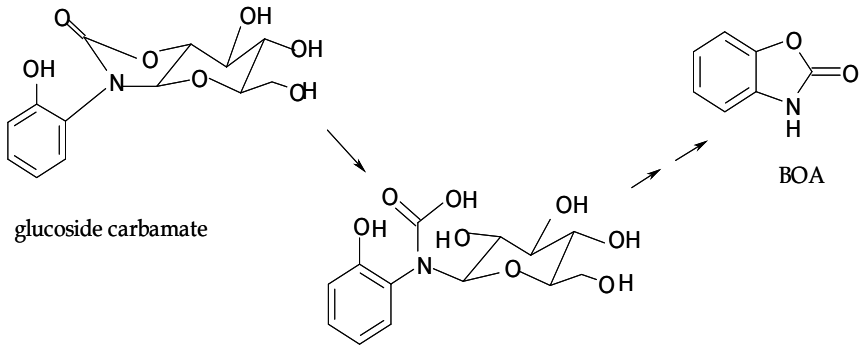

Fig. 12. Glucoside carbamate is hydrolysed to N-glucosylated carbamic acid, than deglucosylated and rearranged back to BOA. N-glucosylated carbamic acid was isolated the medium of the species mentioned in the text and purified by HPLC.

The carbamic acid feature is a rare one among natural products. Hitherto, only five representatives are known. Pallidin, a N-carboxyindole alkaloid, has been isolated from the sponge *Rhaphisia pallid* (Su et al., 1996)). Echinosulfone A, isolated from a Southern Australian marine sponge *Echinodictyum*, is a related derivative of the N-carboxyindole moiety (Ovenden et al., 1999). 1,2-Pyrrolidinedicarboxylic acid was identified as constituent of propolis balsam (Greenaway et al., 1991). N-1'-carboxybiotin has been studied in respect

of the formation of enzyme N1′-carboxybiotin complexes during biochemical transformations (Jockel et al., 2000; Legge et al., 1996). However, the feature of a glycosyl carbamic acid as found in **1** has not at all been described for natural products. The most similar compound reported is synthetic ß-D-glucopyranosyl carbamic acid (Ulsperger et al., 1958). The identification of compound 1 was possible with the help of Diana Hofmann (Universität Leipzig, Institut für Analytische Chemie) and Lothar Hennig (Universität Leipzig, Institut für Organische Chemie).

8. Degradation of 2-amino-3H-phenoxazin-3-one (APO)

Fungi differ considerably in their sensibility to APO (Fig. 13). A low sensitivity is correlated with the ability to decompose the compound. All tested strains of the *Fusarium* species, *Drechslera tuberosa* and *Trichoderma viride* are able to degrade APO. With the *Fusarium* species the compound disappeared completely. The medium of *T. viride* contains traces of several phenoxazinones, indicating that some APO is modified by substitutions. *Botrytis cinerea*, which was found to be highly sensitive to APO in the growth tests, has only a low activity to degrade the compound as well as the most sensitive species *Paecilomyces farinosus*.

Since it is rather likely that APO degradation is started by oxidation, we performed experiments to elucidate how fungi can initiate oxidation processes that result in APO destruction. When 200 nmol APO was incubated with H_2O_2 in methanolic solution at room temperature, no decrease or precipitation of the compound was observed over a period of 24 h. The same result is obtained when Czapek medium (contains 10 mg $FeSO_4$/l) is used without addition of H_2O_2 (Tab. 2). Czapek medium with H_2O_2, however, led to an almost complete destruction of APO within 24 h via several intermediates. Thus, in combination with H_2O_2 and Fe^{2+} (Tab. 2), APO is easily destroyed via Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$, in which the mechanism of radical production is still a matter of debate.

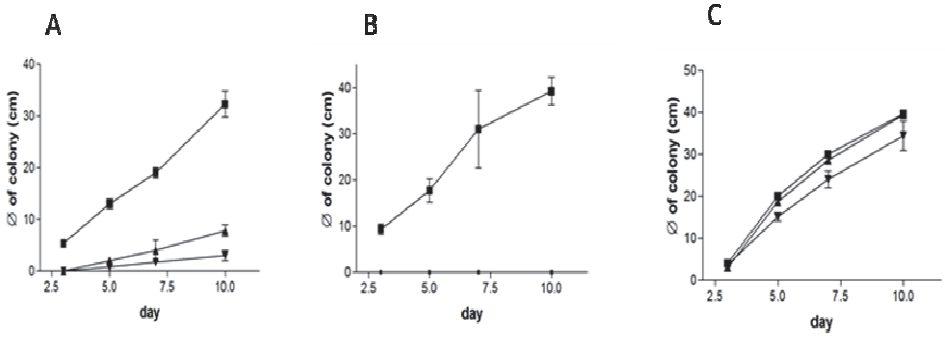

Fig. 13. Examples for the differences in the sensitivity to APO. **A**: *Botrytis cinerea* F-00646; **B**: *Paecilomyces farinosus* F-01073 and **C**: *Fusarium avenaceum* F-00475. ■: control; ▲1 µmol APO;▼2µmol APO in Czapek medium. Growing fungal mycelium was placed on the center of each Petri dish (20 cm i.d.) and incubated in the dark at 25°C. Fungal growth was measured at the 3rd ,5th ,7th and 10th day of growth. Each experiment was repeated three times. *P. farinosus* was completely inhibited by APO, *B. cinerea* strongly inhibited by both APO concentrations. *F. avenaceum* showed no inhibition.

Hyde & Wood (1997) reported on the presence of a Fe(II) oxalate complex in aerobic solution that can lead to hydroxyl radicals without any other source. Therefore we examined the excretion of oxalate by the two fungi: *Fusarium heterosporum* F-00195 as a strain able to degrade APO rather fast and *Paecilomyces farinosus* F-01073 as a strain with a low degradation activity. Oxalate determination was done with the Trinity biotech oxalate kit No. 591.The mediums of the fungi were analyzed for secreted oxalate during the first 8 h of incubation in presence of APO, the one of *F. heterosporum* was also tested for the presence of H_2O_2 (National Diagnostics hydrogen peroxide assay kit Cl-204). During the incubation period, the pH of the media was lowered from pH 7.0 to 5.5 (*F. heterosporum*) and 5.7 (*P. farinosus*). *F. heterosporum* started to secrete oxalate already 30 min after start of the incubation (Fig. 14-16). The amount strongly increased during the next hour, but drops later on. Oxalate secretion by *P. farinosus* was measurable after 4 h of incubation, but reached only about 10% of the highest *F. heterosporum* value after 8 h. The oxalate excretion profile of *F. heterosporum* corresponds to the disappearance of APO already 1 h after starting the incubation. Contrarily, *P. farinosus* started some APO degradation after 8 h. In the *F. heterosporum* medium, H_2O_2 was measurable immediately after start of the incubation and again 8 h later. Hydrogen peroxide was extremely low during the major phase of oxalate release and APO degradation. It is assumed that oxalate excretion and the release of H_2O_2 are causative for the APO degradation of all other APO insensitive *Fusarium* strains tested.

Incubation time	APO (200 nmol)	APO + H_2O_2 (35 mmol)	APO + H2O2 + Fe^{2+} (100 nmol)	APO + Czapek medium	APO + Czapek medium + H_2O_2	APO + 5 µmol oxalate + 5 µmol Fe^{2+}	Assay volume: 400 µl
Start (t 0)	1.02 nmol	1.15	0.96	1.20	1.24	1.20	Volume
End (t 24h)	1.04 nmol	1.16	0.30	1.25	0.16	0.70	analyzed by HPLC: 2 µl

Table 2. Destruction of APO in presence of H_2O_2 or oxalate and Fe^{2+} and controls. Average values of 5 independent experiments.

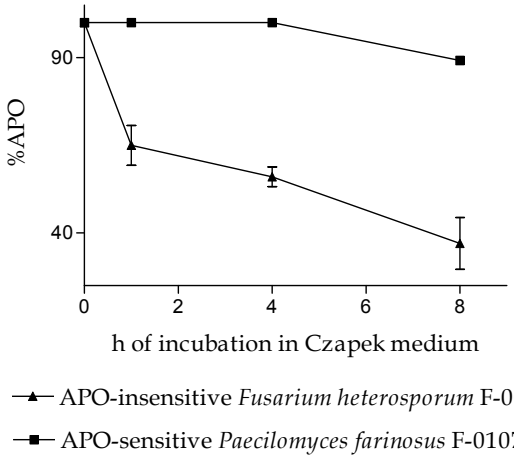

Fig. 14. Decrease of APO in the medium of *F. heterosporum* and *P. farinosus*. *N* = 3.

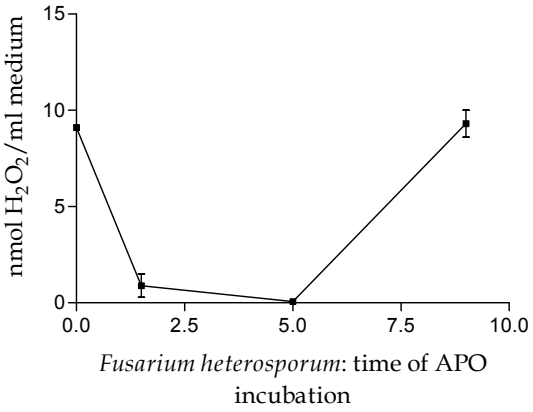

Fig. 15. During the major degradation phase, H_2O_2 is poorly present in the medium. *N* = 3.

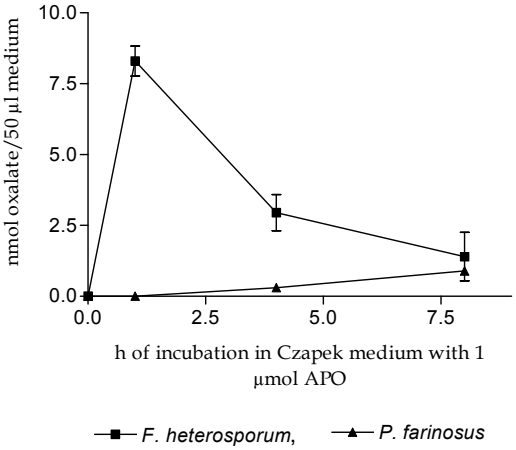

Fig. 16. High excretion of oxalate by *F. heterosporum* during the major phase of APO degradation. *N* = 3.

Many fungi are known to secrete oxalate, some in mmolar concentrations under certain conditions (Cessna et al., 2000; Dutton & Evans, 1996). According to Varela & Tien (2003) oxalate facilitates hydroxyl radical formation at low concentration. Oxalate sequestering of ferric ions are also discussed as a protection of the fungi. Moreover, certain fungi are known to release H_2O_2. According to our study, oxalate must have a function in radical formation because oxalate can replace H_2O_2 (Tab. 2). Figure 15 illustrates that the depletion of H_2O_2 during the major phase of *F. heterosporum* APO degradation is accompanied by a strong excretion of oxalate. Thus, existing H_2O_2 is used for APO degradation and excreted oxalate lead to new hydroxyl radicals.

The oxidation of BOA, BOA-6-OH, glucoside carbamate, AAP and oHPMA was also tested (100 µmol each) for degradation via Fenton reaction. None of these compounds was

destroyed in Czapek medium supplemented with H_2O_2. We assume the participation of exuded fungal enzymes that modify these compounds prior to APO production and the subsequent oxidative destruction of phenoxazinones by the Fenton reaction. It is concluded that the complete biodegradation of BOA detoxification products and phenoxazinones is a concerted action of various fungi with different metabolic properties which is probably supported by bacteria. At least the sugar moiety of the plant detoxification products can be metabolized by the microorganisms. Interestingly, Chen et al. (2010) found an increase in soil fungi after DIMBOA and MBOA application. The authors assume affects on the soil microbial community structure with a change in fungi populations in the wheat rhizosphere. Saunders and Kohn (2009) described a significant influence of benzoxazinoids on fungal endophyte communities.

Phenoxazinones are not only compounds originated from benzoxazinone degradation. They are synthesized by a variety of different organisms, for instance by members of the genus *Pycnoporus* (Sullivan & Henry 1971; Temp & Eggert 1998) or by *Streptomyces* species (Suzuki et al., 2006). Clearly, these compounds are relatively wide-spread in nature. Macias et al. (2005) reported on a life time of APO of more than 80 days in some soils. Unfortunately, neither the source of the soil nor its quality is mentioned. Also Bacon et al. (2007) take APO for a stable compound. In contrast, Krogh et al. (2006) determined $1.4x10^{-11}$ M APO as the highest concentration in sandy loam soil after incorporation of one rye seedling 300 mg^{1-} soil. The APO concentration decreased rapidly during 10 days to about 30%.

For APO degradation in nature, several soil properties such as a high diversity of microorganisms including ones that excrete chelator agents (e.g. oxalate), generation of H_2O_2, the presence of iron, and a pH lower than 6 are certainly prerequisites for starting APO degradation via Fenton reaction. Even if the life time is variable depending on the soil conditions, APO is fortunately not a stable compound but an allelochemical which can be completely degraded over time. The Fenton reaction is the key reaction in the oxidation of membrane lipids, oxidation of amino acids and biologic reactions where biological reduction agents are present. The reaction is common in chemical, biological, and environmental systems (Barbusinski, 2009; Prousek, 2007). The importance of the Fenton reaction in natural environments and in waste treatments for degradation of phenolic and other compounds has been recognized during the last two decades (Pignatello et al., 2006; Vlyssides et al., 2011).

9. Conclusion

The bio-accumulation of conventional herbicides/pesticides and their often highly toxic degradation products is a well-known problem. Another problem is their persistence in soil and ground water. Some of these compounds are not only genotoxic, carcinogenic, neurotoxic and immunotoxic but have also negative effects on the fertility of vertebrates and are toxic to bees. Moreover, plants have developed new strategies in the resistance against common herbicides (Gainesa et al., 2010; Powles &Yu, 2010). Yuan et al. (2006) summarize in their article the dramatic increase in herbicide-resistant weed biotypes which became obvious since the late 80ies of the last century. This demands innovative and environmental-friendly strategies based on sustainable resources using natural, plant–own compounds for weed control which are acceptable by consumers. Breeding crops suitable for natural product applications has to be aimed. Allelopathic concepts are more and more attractive in

agriculture. However a number of prerequisites are of importance. Among others, the selectivity and the relatively fast and complete biodegradation of the compounds is of outstanding importance to avoid environmental damage and the destruction of biodiversity. The same priority has the development of new agricultural systems. Applications of large quantities of a natural compound, perhaps booted by artificial substitutions, instead of conventional herbicides, cannot be a solution. The same problems as obtained with common herbicides will occur soon. Therefore, the reactions of plant, including detoxification strategies, of microorganisms, of plant and microbial genotypes, soil properties, fertilizer management, occurrence and degradation of the mother substances and their derivatives have to be investigated carefully prior to the use of any natural compound as a bioherbicide. This knowledge will help to design cultural systems promoting natural compounds to agricultural crops with a beneficial impact on environmental safety as well as consumers' health. Benzoxazinoids could be a group of allelochemicals that seem to fulfill several of the prerequisites. The "journey" of benzoxazolinone and its final degradation demonstrates at least some of the advantages of the use of suitable natural bioactive compounds in agriculture: highly selective efficiency and a short existence (Fig. 17). It gives also an impression of the complexity of the ecological net involved in the transformation and degradation of an allelochemical, although only a few aspects could be directed in this article.

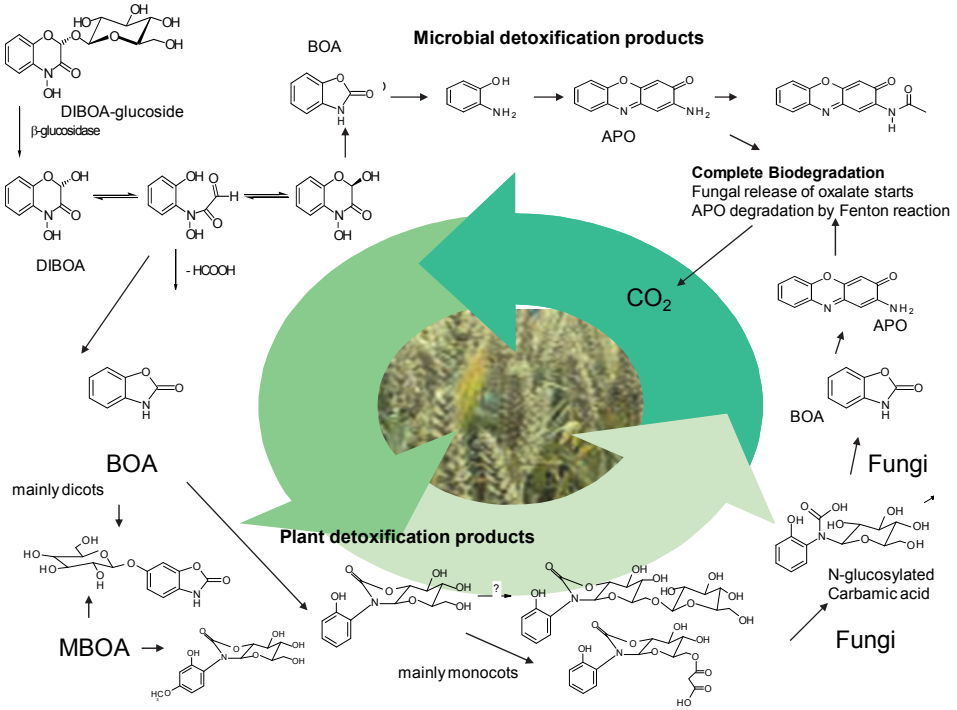

Fig. 17. Illustration of the journey. BOA is released from DIBOA degradation, can be absorbed by other plants, for example, weeds or individuals of the same species (rye, wheat, maize). Plant and microbial detoxification products can be exuded and are converted by defined microorganisms. Phenoxazinone(s) can be completely degraded via Fenton reaction.

10. References

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3

Fate and Determination of Triazine Herbicides in Soil

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1. Introduction

Triazine herbicides belong to the group of the most widely used herbicides worldwide. In this review paper, encompassing mostly the relevant research and publications done in the last decade, the fate of triazine herbicides after their introduction in the environment will be discussed. They are transformed in a variety of transformation products after their application, and some of these products are at least as important for the assessment of the overall fate of triazine herbicides and their impact on the environment. Both parent compounds and transformation products will be discussed with particular emphasis on their behaviour in the soil. Analytical methods for the determination of their residues and transformation products in the soil will be reviewed along with the consideration of the impact of the current analytical approaches on our knowledge about the fate of triazines.

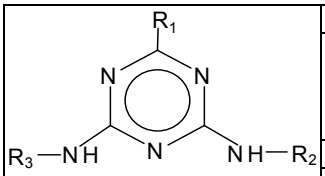
2. Physico-chemical properties of triazines

Chemically, triazine herbicides are comprised of asymmetrical triazines (triazinones, triazidinones) and symmetrical or 1,3,5-triazines (*s*-triazines): chlorotriazines, methoxytriazines, methylthiotriazines. Structures of the more important triazines and their transformation products (TPs) are shown in Fig. 1.

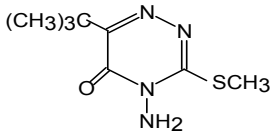
Physico-chemical properties of compounds relevant for their behaviour in the environment are their polarity (expressed as *n*-octanol-water partitioning coefficient K_{ow}), linked to water solubility, moreover their acido-basic properties (expressed as dissociation constant K_a) and volatility (usually expressed as vapour pressure). These are listed in Table 1 for the more environmentally important triazines.

3. Toxicity and environmental effects of triazines

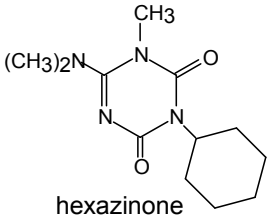
Triazine herbicides are generally of low acute toxicity for birds and mammals, although certain species show unexpected vulnerability for some of them, e.g. for sheep the fatal dose of simazine has been reported as 500-1400 mg/kg, while LD_{50} for rats is >5000 mg/kg (Stevens & Sumner, 1991). Acute toxicity data for some compounds are shown in Table 2.



General s-triazine structure (see table on the right)



metribuzin (triazinone)



hexazinone (triazidinone)

	R_1	R_2	R_3
Chlorotriazines			
atrazine	Cl	CH_2CH_3	$CH(CH_3)_2$
simazine	Cl	CH_2CH_3	CH_2CH_3
propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$
cyanazine	Cl	CH_2CH_3	$C(CH_3)_2CN$
terbutylazine	Cl	CH_2CH_3	$C(CH_3)_3$
Methoxytriazines			
atratone	OCH_3	CH_2CH_3	$CH(CH_3)_2$
prometon	OCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$
terbumeton	OCH_3	CH_2CH_3	$C(CH_3)_3$
Methylthiotriazines			
ametryn	SCH_3	CH_2CH_3	$CH(CH_3)_2$
simetryn	SCH_3	CH_2CH_3	CH_2CH_3
prometryn	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$
terbutryn	SCH_3	CH_2CH_3	$C(CH_3)_3$
Degradation products			
desethylatrazine	Cl	H	$CH(CH_3)_2$
desisopropylatrazine	Cl	CH_2CH_3	H
desethyldesisopropylatrazine	Cl	H	H
hydroxyatrazine	OH	CH_2CH_3	$CH(CH_3)_2$

Fig. 1. Structures of some widely used triazines and more important transformation products.

Name	M / g/mol	Water sol. / mg/L	logK_{ow}	pK_a	p / Pa
Atrazine	215.7	33 (20 °C)	2.2-2.7	1.7	$4.0 \cdot 10^{-5}$ (20 °C)
Simazine	201.7	5 (20-22 °C)	2.2-2.3	1.65	$8.1 \cdot 10^{-7}$ (20 °C)
Cyanazine	240.7	171 (25 °C)	1.8-2.0	1.85	$2.1 \cdot 10^{-7}$ (25 °C)
Terbutylazine	229.8	8.5 (20 °C)	2.6-3.0	2.0	$1.5 \cdot 10^{-4}$ (25 °C)
Atraton	211.3	1800 (20-22 °C)	2.3-2.7	4.2	NA
Terbumeton	225.3	130 (20 °C)	2.7-3.1	4.7	$2.5 \cdot 10^{-5}$ (25 °C)
Ametryn	227.1	185 (20 °C)	2.7-3.1	4.0	$1.1 \cdot 10^{-4}$ (20 °C)
Prometryn	241.4	33-48 (20 °C)	3.3	4.1	$1.3 \square 10^{-4}$ (20 °C)
Terbutryn	241.4	25 (20 °C)	3.1-3.7	4.3	$2.2 \cdot 10^{-4}$ (25 °C)
Desethylatrazine	187.7	3200	1.5	1.65	$1.2 \cdot 10^{-2}$ (25 °C)
Desisopropylatrazine	173.6	670	1.1-1.2	1.58	NA
Hydroxyatrazine	197.3	5.9	1.4	5.2	$1.1 \cdot 10^{-3}$ (25 °C)

Table 1. Some relevant physico-chemical parameters for the environmentally important triazines and their transformation products (Kaune et al., 1998; Noble, 1993; Shiu et al., 1990; Tomlin, 1994). NA - not available.

Name	oral LD_{50} /mg/kg (rats)	oral LD_{50} /mg/kg (other species)
Atrazine	1900-3000	750 (rabbits)
Simazine	>5000	500-1400 (sheep)
Cyanazine	180-380	NA
Terbutylazine	1000-1590	NA
Atraton	1465-2400	NA
Terbumeton	>650	NA
Ametryn	110-1750	NA
Prometryn	3150-5235	NA
Terbutryn	2000-2980	3880 (mice)

Table 2. Acute toxicity data for some triazines (IUPAC Agrochemical Information, 2011; Stevens & Sumner, 1991). NA - not available.

However, the situation is less plausible when assessing the chronic toxicity of triazines. Significant scientific and public controversy has been increasing in the last decade especially regarding the effects of environmentally relevant concentrations of atrazine and its main transformation products desethylatrazine, desisopropylatrazine and hydroxyatrazine, resulting in the 2003 ban of atrazine products in European Union (Sass & Colangelo, 2006). Initial studies reported some carcinogenic, mutagenic and teratogenic effects of triazines only at the dose exceeding the maximal tolerable dose (Stevens & Sumner, 1991). However, environmentally relevant low concentrations of atrazine were later shown to adversely affect the normal male development in amphibians (Tavera-Mendoza et al., 2002), although the evidence is still not conclusive (Solomon et al., 2008). Adverse effects of atrazine were shown also for rats, both on male reproductive tract (Kniewald et al., 2000) and on oestrus in females (Eldridge et al., 1999). The latter is presumably due to the effect on hypothalamic-pituitary-gonadal axis and not on intrinsic estrogenic effect of atrazine (Eldridge et al., 1999; Taketa et al., 2011). Similar effects have been observed for the main atrazine transformation products (Stanko et al., 2010). Besides these endocrine-disrupting properties, atrazine has been shown to affect immune function in mice and the effects persist some time after the exposure (Filipov et al., 2005). Other triazine herbicides are not that extensively covered regarding their chronic toxicity, presumably because they are less widely applied. However, USA Environmental Protection Agency (EPA) concludes that triazines and TPs with chlorine attached to the ring (see Fig. 1) have the same common mechanism of toxicity regarding their endocrine-related developmental, reproductive and carcinogenic effects (Environmental Protection Agency [EPA], 2011).

4. Distribution of triazines in the environmental compartments

After the introduction in the environment, triazines are distributed between the three main environmental compartments, namely gaseous (air), aqueous (ground and surface waters) and solid (soil, sediments). The fourth important compartment interacting with the environment is biota: uptake of triazines into microorganisms and plants, which will be considered separately. Distribution is governed by the physicochemical properties of the compounds (Table 1) and is an ongoing process. There is a dynamic interchange of temporary equilibrium states and re-distribution, influenced by weather conditions, input of materials and various pollutants into the environment etc.

Volatilization of triazines and their long-range atmospheric transport is a poorly researched process. It is supposed that, similar to other semivolatiles, triazines are transported by air masses absorbed on the particulate matter and deposit in cold atmospheric conditions (high mountains, higher geographical latitudes) mainly by wet deposition. Snow is an effective scavenger of particulate matter and associated pollutants from the atmosphere. Triazines have been detected both in snow and rainwater (Polkowska et al., 2000; Usenko et al., 2005).

Triazines are distributed mainly between aqueous and solids compartments. The main processes are partitioning and sorption on solid components, as well as solubilisation in the aqueous compartment followed by leaching into lower solid layers and eventually into groundwater. Living organisms present in both compartments contribute to the transport by uptaking the compounds and returning them mainly as transformation products. The majority of research has been done on atrazine in the last decade of 20th century and is encompassed in a recent review paper (Mudhoo & Garg, 2011). However, atrazine residues in soil have proven to be more persistent than previously expected (Jablonowski et al., 2011) and thus there is an ongoing need for further research on the soil behaviour of this compound (Barton & Karathanasis, 2003; Jablonowski et al., 2011; Kovaios et al., 2006; Ling et al., 2006). Atrazine is expected to be in its non-ionized form at the environmentally relevant pH values (see Table 1) and for uncharged compounds, it is generally accepted that they are sorbed on organic carbon fraction of the soils/sediments (Mudhoo & Garg, 2011). The main mechanism in operation is partitioning between aqueous and organic carbon phase, predominantly humic substances. Both overall partition coefficient K_d and partition coefficient for organic carbon K_{oc} are used to quantitatively express the extent of interaction. The reported values for the latter differ considerably from 25 to 600 L/kg OC (Mudhoo & Garg, 2011), which may reflect the differences in organic matter structure. Humic substances (HS) are heterogeneous and still poorly characterized macromolecules or supramolecular associations (Schaumann, 2006). A number of mechanisms have been proposed for the interaction of atrazine and HS: partitioning resulting from hydrophobic interactions (Lima et al., 2010; Prosen & Zupančič-Kralj, 2000), hydrogen bonding (Prosen & Zupančič-Kralj, 2000), electron transfer, charge transfer (Mudhoo & Garg, 2011). While atrazine is sorbed primarily onto soil organic matter (SOM), presence or addition of dissolved organic matter (DOM) may enhance the sorption at lower DOM concentration, but decrease it at higher DOM concentration (Ling et al., 2006; Mudhoo & Garg, 2011), which is a consequence of increased solubilisation of atrazine in the aqueous fraction with DOM.

Atrazine is sorbed on some mineral components of soils/sediments as well: aluminium-saturated smectite (Mudhoo & Garg, 2011), silicagel (Kovaios et al., 2006) and Florisil (SiO_2+MgO) (Prosen et al., 2007), but not calcite or alumina (Kovaios et al., 2006; Prosen et al., 2007). The proposed mechanism is electrostatic or electron-transfer interaction of atrazine with silanol groups (Kovaios et al., 2006; Prosen et al., 2007). Besides soil organic matter (SOM) content and presence of adsorbing minerals, other parameters govern the extent of atrazine sorption to environmental solids: pH, ionic strength, surface area, particle and pore size, presence of other compounds, especially surfactants (J.F. Lee et al., 2004), temperature (Mudhoo & Garg, 2011). Contact time is another important factor. Desorption hysteresis has been observed for longer contact times (Drori et al., 2005; Prosen & Zupančič-Kralj, 2000). The currently accepted model explaining the effect of contact time, nonlinear sorption kinetics, desorption hysteresis and conditioning effect of sorbate on sorbent affinity is the dual-mode sorption process of sorbate in the interchangeable rubbery and glassy state of polymerous SOM material (Schaumann, 2006).

Leaching of atrazine into lower layers of the soil and eventually groundwater is generally affected by the same parameters as sorption. The mobility of compound in soil/sediment is expressed by retardation factor R_f as determined by column lysimeters (Weber et al., 2007). For atrazine, R_f has been shown to be inversely proportional to SOM content and related to pH and soil leaching potential (Weber et al., 2007). Presence of more polar SOM with higher ratio of polar functional groups, e.g. from the manure, has been postulated to result in stronger hydrogen bonding of atrazine and reduced desorption and mobility (Lima et al., 2010), although completely opposite results, i.e. stronger bonding to more hydrophobic humic matter, were reported elsewhere (Celano et al., 2008). Desorption and leaching is enhanced by the presence of surfactants, especially anionic (J.F. Lee et al., 2004; Ying et al., 2005), as well as dissolved organic matter (DOM) (Ling et al., 2006). However, great caution is needed when extrapolating results from these studies to predict the dissipation behaviour of atrazine, as gross underestimations have been observed (Jablonowski et al., 2011).

Considerably less information about sorption and mobility in soil and sediments is available for other triazines or transformation products. Chlorotriazines are generally assumed to behave similarly to atrazine and this has been confirmed in some experiments for simazine (Mudhoo & Garg, 2011; Ying et al., 2005) or terbutylazine for humic organic matter (Celano et al., 2008). The latter is a less polar compared to atrazine and has been shown to exhibit greater extent of sorption on HS (Erny et al., 2011; Prosen & Zupančič-Kralj, 2000). In comparison of methylthio-, methoxy- and chlorotriazine sorption on sediments and mineral soil components, sorption intensity was related to the basicity (pK_a) and water solubility of compounds, but not their $\log K_{ow}$ (Prosen et al., 2007; Stipičević et al., 2009) - Fig. 2. Dealkylated triazine transformation products are weakly sorbed on humic substances compared to parent compounds (Erny et al., 2011), while hydroxyatrazine, a dechlorinated atrazine TP, is extensively sorbed on mineral components of the soil/sediment (Stipičević et al., 2009).

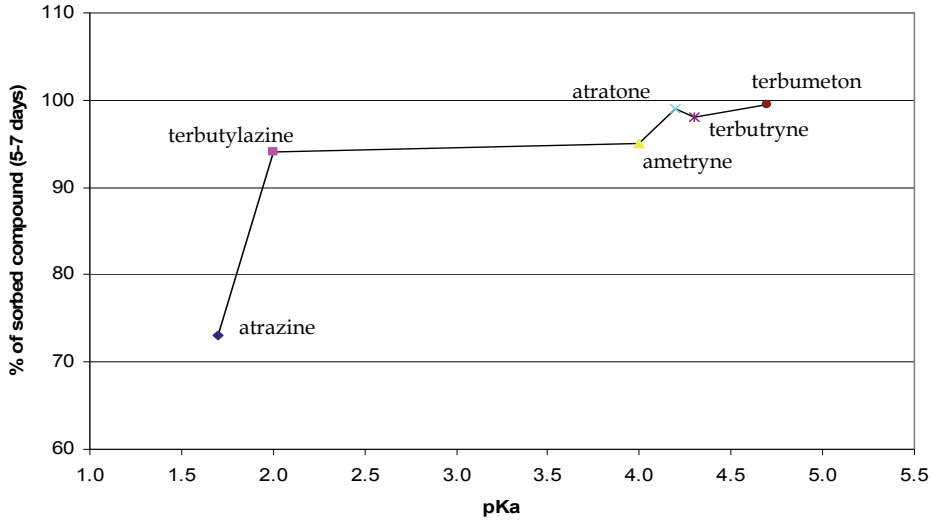


Fig. 2. Relation between pK_a and % of sorbed compounds after 5-7 days of batch equilibrium experiment on Florisil (SiO_2, MgO). Adapted after Prosen et al. (2007).

Knowledge of sorption/desorption behaviour of triazines is frequently applied in bioremediation either to enhance their leaching or to stabilise the residues in the contaminated sites (Delgado-Moreno et al., 2010; Jones at al., 2011; J.F. Lee et al., 2004; Lima et al., 2010; Mudhoo & Garg, 2011; Ying et al., 2005).

5. Triazine degradation and uptake in the soil

The sorption behaviour of triazines in soil directly influences their bioavailability to soil microorganisms and plants (Mudhoo & Garg, 2011), leading to their uptake and biodegradation. Numerous studies are available for atrazine as the most widely applied and apparently also persistent triazine in the soil (Jablonowski et al., 2011). Plant uptake of triazines from the contaminated soils is extensively studied as a means for bioremediation. The C4-metabolism plants show the greatest resistance to triazines and detoxify them by hydrolysis. Examples of plants shown to be useful in degrading atrazine in their rhizosphere are *Polygonum lapathifolium*, *Panicum dichotomiflorum* (Mudhoo & Garg, 2011), *Pennisetum clandestinum* (Popov & Cornish, 2006; Singh et al., 2004).

Ongoing research in the soil microorganisms capable of utilizing triazines as their energy source has resulted in an extensive array of isolated strains: *Acinetobacter* sp., *Cytophaga* sp., *Pseudomonas* sp., *Ralstonia* sp., *Agrobacterium* sp. (Mudhoo & Garg, 2011), *Klebsiella* sp. and *Comamonas* sp. (Yang et al., 2010), *Nocardioides* sp. and *Arthrobacter* sp. (Vibber et al., 2007). Most of them are capable of extensive mineralization of triazines (Mudhoo & Garg, 2011; Yang et al., 2010) and have a limited access even to aged herbicide residues in the soil (Jablonowski et al., 2008; Mudhoo & Garg, 2011). The species most often used for triazine degradation is *Pseudomonas* sp., its efficacy has been shown to be influenced by citrate addition (Jablonowski et al., 2008), soil humidity (Ngigi et al., 2011) and microorganism adsorption on simulated soil particle aggregates (Alekseeva et al., 2011). Green algae and diatoms (Mudhoo & Garg, 2011), as well as cyanobacteria (Gonzalez-Barreiro et al., 2006) are also capable of atrazine uptake and are thus a valuable option for the bioremediation of the contaminated waters. Certain fungal species able to grow on atrazine-contaminated soils and capable of its uptake have been identified as well (Mudhoo & Garg, 2011).

Compared to biotic degradation by microorganisms and higher plants, abiotic degradation of triazines in soils is a minor dissipation route. Humic substances at low pH catalyse the hydrolysis of atrazine and its chlorinated transformation products to their hydroxy analogues (Prosen & Zupančič-Kralj, 2005). Photolysis of atrazine under solar irradiation and in the presence of humic substances was found to be negligible (Prosen & Zupančič-Kralj, 2005); however, simazine and terbutylazine were found to dissipate faster under solar irradiation of the soil (Navarro et al., 2009). Photolytic transformation and eventual mineralization is enhanced by using a suitable photocatalytic agent, e.g. TiO_2, which holds a potential for clean-up of contaminated sites (Konstantinou & Albanis, 2003).

6. Analytical approaches and cautions for triazine determination in soil

Triazine determination data for soil and other solid environmental samples are used to estimate the extent of the site pollution and potential toxicity (Jablonowski et al., 2011). However, determination of triazines and their TPs in solid samples is prone to many problems, as schematically depicted in Fig. 3.

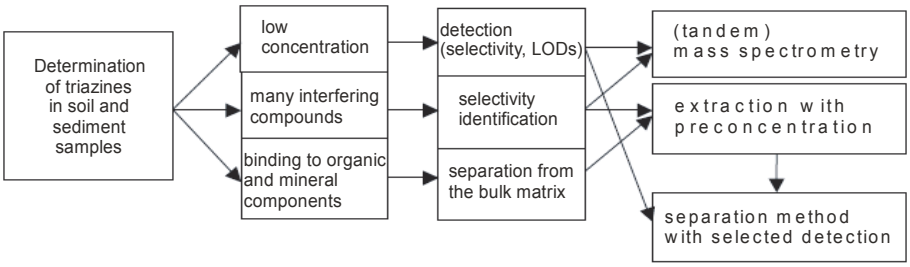


Fig. 3. Schematic representation of the problems and solutions for triazine determination in solid environmental samples.

Technique	Principle	Advantages	Disadvantages
Soxhlet Extraction (SE)	continuous percolation of organic solvent	- recoveries not dependent on sample type - cheap	- time-consuming - high consumption of organic solvents - extracts have to be concentrated
Ultrasonication Extraction (USE)	mixing, desorption of analytes from sample components	- recoveries not dependent on sample type - cheap	- moderately time-consuming - high consumption of organic solvents - work-intensive
Supercritical Fluid Extraction (SFE)	supercritical fluid of low viscosity better penetrates the sample	- fast method - solvent CO_2 non-toxic, environmentally acceptable	- limited sample amount - recoveries depend on sample type - high initial cost
Microwave-Assisted Solvent Extr. (MASE)	microwave- assisted desorption of analytes and sample components	- fast method - low consumption of organic solvents - additional regulation parameters	- polar solvents only - non-selective - extensive extract clean-up needed - high initial cost
Pressurised Liquid Extraction (PLE) / Accelerated Solvent Extr. (ASE)	enhanced extraction efficiency of analytes due to solvents at high temperature and pressure (liquids above boiling point)	- fast method - low consumption of organic solvents	- non-selective - extensive extract clean-up needed - high initial cost

Table 3. Common extraction techniques for triazines from the solid environmental samples (Andreu & Pico, 2004; Camel, 2000; Lesueur et al., 2008; Lopez-Avila, 1999).

The analytical procedure usually comprises of a suitable extraction technique (Table 3), preferably enabling preconcentration as well, possibly a clean-up step, and an appropriate determination technique (Andreu & Pico, 2004; Camel, 2000; Lesueur et al., 2008; Lopez-Avila, 1999). The first dilemma encountered is whether to use an exhaustive extraction technique or a more mild one. Extraction techniques regarded as exhaustive under most conditions are Soxhlet's, MASE and PLE (Camel, 2000). There is a high probability that even triazines bound to soil components would be extracted, although this may depend on the type of compound (Kovačić et al., 2004). However, most of the unwanted organic compounds from the sample would be transferred to extract as well, and these interferences have to be selectively removed prior to analysis by an appropriate clean-up technique. The key word in this case is selectivity, as the clean-up may otherwise lead to significant loss of analytes as well. A selection of frequently applied clean-up techniques is listed in Table 4. In the second case, i.e. by applying a mild extraction technique (0.01 M $CaCl_2$ solution or aqueous methanol), the obtained extract would better reflect the actual fraction of the triazines and TPs available to plants and microorganisms (Regitano et al., 2006) and could thus be more useful for the actual assessment of the residual toxicity of triazines (Jablonowski et al., 2008; Jablonowski et al., 2011).

Technique	Principle	Advantages	Disadvantages	Variants and improvements
Liquid-Liquid Extraction (LLE)	partitioning between two immiscible solvents	- high recoveries - broad choice of solvents	- time-consuming - automatisation difficult - environmentally problematic	supported liquid membrane extr. (SLME) liquid-phase microextr. (LPME) / single-drop microextraction
Solid Phase Extraction (SPE)	adsorption / partitioning between aqueous and solid phase, followed by desorption with organic solvents	- high recoveries - low solvent consumption - automatisation possible (on-line)	- lower selectivity - narrower choice of sorbents	restricted access material (RAM) molecularly imprinted polymer (MIP) immunosorbents multi-walled nanotubes
Solid Phase Micro-extraction (SPME)	partitioning between aqueous and non-polar phase on fibre, follow by thermal or solvent desorption	- fast - solventless - automatisation possible	- mainly for volatile compounds - poor repeatability - non-exhaustive (low recoveries)	in-tube SPME

Table 4. Common clean-up techniques for triazines in soil/sediment extracts (Andreu & Pico, 2004; Hylton & Mitra, 2007; Jonsson & Mathiasson, 2000; Masque et al., 2001; Min et al., 2008; Psillakis & Kalogerakis, 2002; Stalikas et al., 2002).

Determination of triazines in the extracts after extraction and clean-up is usually accomplished using either gas (GC) or liquid chromatography (HPLC) (Andreu & Pico, 2004). Both techniques can be coupled with mass spectrometry, enabling simultaneous confirmation of compound identity (Andreu & Pico, 2004; Lesueur et al., 2008; Min et al., 2008; Tsang et al., 2009; Usenko et al., 2005). Other detectors frequently used in triazine analysis are spectrophotometric, preferably diode-array detector for HPLC (Andreu & Pico, 2004; Kovačić et al., 2004; Prosen et al., 2004), and nitrogen-phosphorous detector for GC (Andreu & Pico, 2004; Stalikas et al., 2002).

Besides chromatography, other analytical techniques are seldom applied to triazine determination, although they may offer some significant advantages: electromigration techniques, e.g. micellar electrokinetic chromatography (Lima et al., 2009; Prosen et al., 2004); voltammetry (De Souza et al., 2007). Biosensors and bioassays are used for preliminary screening of samples or sample extracts, but because of their cross-reactivity the samples with analyte content above the cut-off value should be re-analysed by a more specific analytical technique. The most widely applied is antibody-based ELISA, but some innovative approaches have been developed, e.g. sensors based on photosystem-II inhibition from plant photosynthetic membranes (Bengtson Nash et al., 2005; Varsamis et al., 2008).

Analytical determination of triazines in solid samples, although often seen as a routine procedure, is prone to many errors. Starting with sampling, the sample taken for analysis should be representative of that part of environment for which the information about pollutant concentration should be obtained. To achieve this goal, an appropriate number of samples, as well as time and site of sampling should be considered. Preservation of samples during the transport and storage is important as well and should be carefully selected (Kebbekus & Mitra, 1998). An example is the need to completely dechlorinate drinking water to prevent rapid degradation of triazines (Smith et al., 2008). Next step, namely extraction with clean-up, is again critical due to the possibility of significant analyte losses because of improper sample preparation conditions. These should be optimised and tested for every analyte. The choice between exhaustive and milder extraction techniques has already been mentioned, but mild conditions are also needed to avoid thermal degradation. Most triazines and their TPs are thermally stable, but not all (Tsang et al., 2009). Another caveat with extraction is the significant difference in analyte binding and thus extraction recoveries between freshly-spiked blank samples and real-life samples containing the so-called »aged residues«. Various authors have proposed to reproduce aging under environmental conditions by leaving spiked blank samples at room temperature for anything between 3 days and 2 years (Andreu & Pico, 2004). However, simulation may not necessarily yield equivalent results to field conditions (Louchart & Voltz, 2007). Finally, determination technique is important in terms of selectivity, limits of detection and reliable quantification. To achieve the latter, standard solutions for the calibration should always match the actual matrix as close as possible to avoid the significant matrix effects seen with some types of detectors (Kovačić et al., 2004), especially with electrospray interface for LC-MS.

7. Elucidation of triazine fate in the soil as influenced by analytical determination

As already explored in subchapter 4 of this review, we are mainly concerned with triazine sorption, desorption, leaching and plant/microorganism uptake when dealing with triazine

fate in the soil. Sorption in its broadest sense (i.e. partitioning, non-covalent and covalent binding) is usually evaluated by sorption isotherms conforming to various theoretical models: Freundlich, Langmuir, Polanyi-Dubinin-Manes, etc. (Aboul-Kasim & Simoneit, 2001; Kleineidam et al., 2002). The most frequently used method to obtain the experimental data for isotherm construction remains the batch equilibrium method (Celano et al., 2008; Kleineidam et al., 2002; Konda et al., 2002; Kovaios et al., 2006; Lima et al., 2009; Ling et al., 2006; Stipičević et al., 2009). Other approaches are by chromatographic estimation (Bermudez-Saldana et al., 2006) or indirectly by structural descriptors (Schüürmann et al., 2006). In batch equilibrium method, several variables may influence the process of sorption and have to be carefully optimised: organic solvent content, ionic strength and pH, solid/solution ratio, sorption time (Celano et al., 2008; Kleineidam et al., 2002; Kovaios et al., 2006; Prosen & Zupančič-Kralj, 2000; Prosen et al., 2007). After the equilibrium is reached, the solution has to be separated from the sorbent either by centrifugation or filtration (Kleineidam et al., 2002). By using the latter, another potential source of error is introduced as more hydrophobic compounds may bind to certain types of filters.

The equilibrium concentration of the pollutant in the solution after the separation is determined by any of the analytical methods mentioned in subchapter 6. Preferably, it should be performed without previous extraction as this introduces another equilibrium and another possible source of error. Thus, direct HPLC (Celano et al., 2008; Prosen & Zupančič-Kralj, 2000) or electromigration techniques (Erny et al., 2011; Lima et al., 2009) are the methods of choice. If radiolabelled compounds are used, their equilibrium concentration can be measured by radioactivity measurement (Jablonowski et al., 2008). A different approach is to determine the free concentration directly in a multi-phase system by a non-exhaustive solid-phase microextraction and subsequent GC analysis (Heringa & Hermens, 2003; S. Lee et al., 2003; Prosen et al., 2007). The depletion of the compounds from the solution is considered to be negligible, thus giving the opportunity to measure the true equilibrium concentration in the solution (Heringa & Hermens, 2003). Distribution coefficients K_d obtained by SPME-GC determination of equilibrium concentrations after the sorption experiment have been reported to be significantly different compared to those obtained by other determination methods (S. Lee et al., 2003).

As well as for sorption/desorption, the understanding of the leaching behaviour of triazines is significantly influenced by the determination method. The usual approach is to evaluate the mobility of the compound in soil columns by lysimeters (Jablonowski et al., 2011; Weber et al., 2007), but experiments should be conducted under the appropriate time-scale to avoid gross underestimations (Jablonowski et al., 2011). A different approach is the use of ceramic suction cups, but these are also prone to errors due to ageing effects (Domange et al., 2004).

8. Conclusions

This review attempts to cover a vast subject of triazine behaviour in the environment, especially soil, as well as their analytical determination in the same. Special attention was given to the various problems encountered in both. However, the broadness of the subject prevents its detailed evaluation; the interested reader can find more information in other excellent reviews that focus more on triazine behaviour in solid environmental compartment (Jablonowski et al., 2011; Mudhoo & Garg, 2011), their degradation and elimination (Konstantinou & Albanis, 2003) or the applied analytical methods (Andreu &

Pico, 2004; Camel, 2000; Hylton & Mitra, 2007; Jonsson & Mathiasson, 2000; Lopez-Avila, 1999; Masque et al., 2001; Psillakis & Kalogerakis, 2002).

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4

The Influence of Biochar Production on Herbicide Sorption Characteristics

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1. Introduction

Biochar is the by-product of a thermal process conducted under low oxygen or oxygen-free conditions (pyrolysis) to convert vegetative biomass to biofuel (Jha et al., 2010). There are a wide variety of end-products that can be manufactured depending on processing parameters and initial feedstocks (Bridgewater, 2003). The pyrolytic process parameters such as temperature, heating rate, and pressure can change the recovery amounts of each end-product, energy values of the bio-oils, and the physico-chemical properties of biochar (Yaman, 2004).

Biochars are recalcitrant forms of carbon and, depending on properties, can remain in the soil for greater than 1000 years (Skjemstad et al., 2002). The long-term persistence of this carbon form is due to slow microbial degradation and chemical oxidation rates (Sanchez et al., 2009). In addition, biochar interacts with soil materials such as ions, organic matter, and clays that generally increase the persistence of biochar within the soil. However, biochars, unlike commercial fertilizers, are not precisely defined materials and vary widely in properties depending on organic material source and manufacturing process (Karaosmanoglu et al., 2000; McHenry, 2009; Sohi et al., 2010). Increasing pyrolytic temperature decreases biochar recovery but increases C concentration of the char compared with char recovered at lower temperatures (Daud et al., 2001; Katyal et al., 2003). For example, as temperature increased from 300^0 to 800^0 C, biochar C content increased from 56 to 93% whereas biochar yield decreased from 67 to 26% (Okimori et al., 2003). Other pyrolytic parameters, such as sweep gas flow, can influence biochar particle size with higher flows reducing the particle size but increasing heating values (Katyal et al., 2003; Demirbas, 2004). Biochar also can be influenced by reactor design and other reaction parameters including heating rate, residence time, pressure, and catalyst used. Feedstock type, quality, and initial physical characteristics of the material (e.g. particle size, shape, and structure) can impact the bio-oil yield and properties, as well as the type and amounts of biochar formed (Bridgewater et al., 1999).

Landspreading biochar for a soil amendment is suggested to improve crop production efficiency because regardless of the initial manufacturing process, biochars have a high charge density and surface area. The use of biochar as a soil amendment is not a new concept. Dark earths (terra preta) discovered in the Amazon Basin were found to have

received deliberate land applications of charred materials and residues of biomass burning by Amer-indian populations before European arrival (Erickson, 2003; Sombroek et al. 2003). Pyrogenic C in terra preta is very resistant to microbial decay over centuries due to its complex aromatic structure and acts as a significant C sink (Glaser et al., 2001).

The benefits of biochar application have been hypothesized to include: increasing plant available soil water; building soil organic matter; enhancing nutrient cycling; lowering soil bulk density; acting as a liming agent if high in pH; and reducing transfer of pesticides and nutrients to surface and ground water (Laird, 2008) thereby improving water quality. The application of biochar to soil has been reported to have a positive impact on physical properties such as soil water retention and aggregation (Piccolo et al., 1996) and may decrease erosion potential. Glaser et al. (2002) observed an increase in field water holding capacity by 18% in charcoal enriched Anthrosol due to an increase in surface area. Biochar application has been shown to improve other soil physical, chemical, and biological properties (Glaser et al., 2002; Lehmann and Rondon, 2006) leading to positive impacts on plant growth and development. For example, Chidumayo (1994) observed enhanced seed germination (30%), shoot height (24%), and biomass production (13%) of seven indigenous woody crops with the application of charcoal compared with the crops on undisturbed Zambian Alfisols and Ultisols. Kishimoto and Sugiura (1985) also found increases in height (26 to 35%) and biomass (2.3 X greater) production of sugi trees (*Cryptomeria japonica L.*). Similar enhancement was observed in yields of annual crops such as maize (*Zea mays L.*) on Nigerian Alfisols and Inceptisols with the application of charcoal (Mbagwu and Piccolo, 1997) due to an increase of soil pH that resulted in greater micro-nutrient availability and decreased deficiencies. However, biochars also have been shown to have an extreme affinity for essential plant nutrients (Sanchez et al., 2009) that can provide a slow release mechanism.

Some biochars that have high pH (e.g. >9.5) can provide liming capacity and increase the soil pH (Sanchez et al., 1983; Mbagwu and Piccolo, 1997). For example, application of coal ash at the rate of 110 Mg ha^{-1} increased the pH of an eroded Palouse soil from 6.0 to 6.8 (Cox et al., 2001). Exchangeable bases also were observed to increase in sandy and loamy soils with the additions of hardwood and conifer charcoals (Tryon, 1948). Application of charcoal to highly weathered soils with low-ion retention capacities increased the cation exchange capacity (CEC) by 50% compared to unamended soil (Mbagwu and Piccolo, 1997). Oguntunde et al. (2004) reported a significant increase in soil pH, base saturation, electrical conductivity (EC), exchangeable Ca, Mg, K, Na, and available P in charcoal kiln sites and reported an increase in grain and biomass yield of maize of 91% and 44% respectively, with a coal char application. Leaching of NH_4^+ from an unfertilized Ferralsol was reduced with the application of charcoal due to its high C content, although the retention properties of chars may differ for other ionic species (e.g. K, Ca, Mg) if the char already contains high concentrations of the ion of interest (Lehmann et al., 2002). Because of biochar's diverse properties and potential for high reactivity in soils, a 'one-recommendation-fits-all situations' mentality for the use as of biochar as a soil amendment needs to be avoided. To date, the greatest positive impacts of biochar have been primarily observed on degraded soils and those with low fertility whereas applications on highly productive soils have been reported to have low or minimal impacts (Woolf et al., 2010).

Agrichemicals such as pesticides, growth regulating chemicals, and nutrients are applied to crops to control pests and increase yield potential. Depending on the type and amount of

biochar applied, the changes in soil properties associated with the application (e.g. soil pH, EC) as well as the physio-chemical properties of the char itself, may impact the use, rates, efficacious properties, and fates of agrichemicals used in agronomic management. The environmental fate (e.g. leachability, rate of decomposition, etc.) and efficacy of soil applied pesticides are influenced strongly by their reaction and retention with soil particles and organic matter (Brown et al., 1995). Agrichemical molecules can be removed from soil solution through attraction or attachment to the surfaces of organic materials and soil particles (adsorption) or movement into the matrix (like water into a sponge) (absorption). Often, experiments cannot distinguish between these processes so that the general term sorption is used.

Sorption is controlled by properties of the chemical of interest including the water solubility, pH, dissociation constant (pKa), octanol/water partition coefficient, and other factors (Weber, 1995) and can be used to help describe the fate of an herbicide in the environment (Wauchope et al., 2002). The sorption of the chemical also is affected by soil properties including water, organic matter, clay, sand, and oxide contents, and soil pH (Koskinen and Clay, 1997; Laird and Koskinen, 2008). Soils high in sand generally sorb much less chemical than loamy or clay type soils. Agricultural practices that involve modifying soil organic matter content often increase chemical retention. Indeed, studies have shown that adding biochar to soil can result in greater sorption of pesticides (Cao et al., 2009; Spokas et al., 2009; Yu et al., 2009). The distribution of chemical between a solution and solid phase gives an indication of the amount of chemical available in solution and is defined using a sorption coefficient (K_d) where:

$$K_d = \frac{\text{mass of herbicide sorbed per g of solid}}{\text{amount of chemical remaining in solution at equilibrium}} \quad (1)$$

Large K_d values (typically over 100) indicate that a high amount of the chemical originally in solution is sorbed to the solid interface, with low amounts of chemical remaining in solution. Sorption of a chemical from the liquid phase of soil may result in the chemical being: 1) less available to plants, so there may be less uptake; 2) less available to soil organisms, thereby increasing the chemical's residence time and slowing degradation; and 3) less available to leach with water percolating through the soil, which could result in improved groundwater quality.

The biochar source-processing combination provides a rich diversity of biochars to evaluate for soil amendment use (Lehmann et al., 2009). The potential of a specific biochar for a specific use will depend on the physical and chemical properties of the biochar, as well as soil characteristics. The challenge of amending soil with biochar is to identify the benefits that biochar can provide (e.g. fertility, increased water holding capacity) (Lehmann, 2007) and balance these against any negative effects that the char may have. Site-specific application recommendations of specific biochars require an examination of the products of different production and processing scenarios. Much of the biochar research has been based on slow pyrolysis with a goal to optimize biochar properties for a specific goal such as improved soil fertility, greenhouse gas mitigation, or heating value. Little work has been done with biochar produced from fast pyrolysis processes and even less with biochar produced from microwave pyrolysis reactors.

Feedstock is a key factor governing the status of physio-chemical properties of biochar. All types of materials including, but not limited to, palm shells, rapeseed (*Brassica rapa*) stems, sunflower (*Helianthus annuus*), and wood have been used or are being proposed as potential feedstock sources for use in the biofuel industry. In the Midwestern U.S., maize stover and switchgrass (*Panicum virgatum*) biomass are feedstocks that bioenergy companies are exploring for use.

2. Biochar influence on herbicide sorption to soil

This study examined atrazine and 2,4-D sorption to several biochars that were the result of microwave pyrolysis using varying temperatures and processing times of maize and switchgrass biomass. In addition, sorption characteristics of these two chemicals to soil amended with these biochars at two application rates were determined.

2.1 Materials and methods

2.1.1 Biochar and soil

Biochar was produced from maize stover (stalks and other residues remaining after maize grain harvest) and switchgrass biomass collected from fields near Brookings, South Dakota, USA (44.31, -96.67). Briefly, the material was dried at room temperature and pulverized mechanically using a Thomas-Wiley laboratory mill (Model No. 3375-E15, Thomas Scientific, USA) to pass through a 4 mm screen. The ground materials were processed by microwave pyrolysis using the SDSU Ag and Biosystem Eng. Dept. microwave system (specific processing methods reported in Lei et al., 2009). Processing temperatures ranged from 530^0 to 670^0 C and microwave residence times ranged from 8 to 24 minutes with seven maize and nine switchgrass biochars produced (Table 1 and Figures 1 and 2). The energy output, product types, particle size distribution, and elemental analysis of the biochar recovered from maize stover using these processing conditions are reported in Lei et al. (2009).

For this study, the maize biochars were used alone or mixed with the A horizon soil of a Brandt silty clay loam (Fine-silty, mixed, superactive, frigid Calcic Hapludoll, [Soil Survey Staff, 2011]) soil at 1 or 10% (w/w) to examine their effect on solution pH, EC, and atrazine and 2,4-D K_ds (sorption coefficients) for each biochar and biochar/soil combination. For switchgrass biochars, the 1 or 10% amendments to soil were used for pH and EC measurements, however, for herbicide sorption studies only biochar alone or soil mixed with 10% biochar were used, due to limited biochar supply. To maximize homogeneity, each soil/biochar combination was individually mixed by adding air-dry soil and biochar to each individual tube.

2.1.2 Solution characteristics

Biochars, soil, and soil with biochar amendments were analyzed for pH using a 0.01 *M* $CaCl_2$ slurry (1:1 w/v) and a standardized pH electrode. The solution pH was recorded after the reading had stabilized. Electrical conductivity (EC) was determined on a slurry that was mixed 1:1 (v/w) with 0.01 *M* $CaCl_2$ and biochar, soil, or soil amended with biochar. The slurry was shaken for 0.5 hr and EC measured using a commercially available EC electrode.

2.1.3 Herbicide sorption

Atrazine solution was diluted to a final concentration of 13 µM in 0.01 $CaCl_2$ using technical grade atrazine. This solution was spiked with about 0.4 kBq of uniformly-ring-labeled [^{14}C] atrazine (specific activity of 1000 MBq mmol^{-1} with > 99% purity; Sigma Chemical Co., St. Louis, MO). The 2,4-D solution was made in a similar manner, with technical grade 2,4-D added to 0.01 M $CaCl_2$ to have a final concentration of 13 µM. This solution was spiked with uniformly-ring-labeled [^{14}C]-2,4-D (specific activity of 1000 MBq mmol^{-1} with > 99% purity; Sigma Chemical Co., St. Louis, MO).

A 4-mL aliquot of herbicide solution was added to 2 g soil or soil amended with 1 or 10% biochar (final slurry solution 2:1 v/w) in glass centrifuge tubes sealed with a Teflon-lined cap. A 5-mL aliquot of herbicide solution was added to 0.5 g biochar when biochar was used as the sorbent, with the final solution/biochar ratio of was 10:1 v/w, due to the highly sorbent characteristics of the biochar.

After solution addition, the mixtures were shaken or vortexed to form a slurry. Tubes containing the slurries were shaken for 24 hr, centrifuged, and a 250-µL aliquot of supernatant removed. The amount of ^{14}C remaining in the supernatant solution was determined by liquid scintillation (Packard Model 1600TR) counting after the addition of scintillation cocktail. The amount of radioactivity sorbed was determined by comparing the counts in the supernatant samples with counts recorded from the original soil-free blank solution samples. The sorption coefficients (Kd) of the samples were then calculated as L kg^{-1}, correcting for the differences in volume added g^{-1} of material.

2.1.4 Statistical analysis

Experimental treatments were run in triplicate and studies were repeated in time. Results were combined for the studies due to similarity of means and homogeneity of variance between studies. Means presented were averaged over all treatment replicates and statistically separated by least significant difference calculation at $P \leq 0.05$.

2.2 Results

2.2.1 Biochar pH and EC values

The biochars produced in this study ranged in pH from acidic (4.06) to alkaline (9.88), and were dependent on feedstock, pyrolysis temperatures, and processing times (Table 1). Differences were observed among maize and switchgrass feedstocks. For maize stover, three of the microwave pyrolysis reactions at high temperatures ($\geq$650^{o}C), regardless of processing time, resulted in biochars that were very alkaline (pH>9). Two processes at lower temperatures (530^{o}C and a processing time of 16 min or 550^{o}C with a processing time of 10 min) resulted in biochars with pH <5. The 22 min processing time at 550^{o}C resulted in a biochar with a more neutral (7.6) pH. For switchgrass, four processes resulted in biochars that were acidic (pH < 4.6) and the biochars were more acidic than biochars from maize at the same time and temperature. The acidic biochars were formed from processes that had low temperatures (<600^{o}C) or shorter times at 600^{o}C (8 min), or 10 min at 650^{0}C. The most alkaline switchgrass biochar was the result of processing at 670^{o}C for 16 min. This biochar had a pH of ~9.1, which was lower than the alkaline maize biochars that ranged in pH from

Pyrolysis parameters		Maize (*Zea mays*)					
		pH			EC		
Temp	time	Biochar	soil + 1% biochar	soil + 10% biochar	Biochar	soil + 1% biochar	soil + 10% biochar
°C	min					mS cm^{-1}	
530	16	4.59	6.39	5.85^{-}	0.3	2.4	1.4
550	10	4.77	6.38	6.04^{-}	2.3	1.8	2.2
	22	7.60	6.47	6.61^{+}	1.9	1.8	1.8
600	8	5.68	6.44	6.44	2.1	1.8	2.0
650	10	9.88	6.46	6.75^{+}	2.0	1.9	1.9
	22	9.43	6.43	6.76^{+}	2.0	1.8	1.9
670	16	9.65	6.43	6.73^{+}	1.1	1.9	1.9
		Switchgrass (*Panicum virgatum*)					
530	16	5.32	6.17	6.70	0.3	1.80	1.67
550	10	4.12	6.49	5.67^{-}	2.1	2.13	1.97
	22	4.06	6.49	5.71^{-}	1.5	1.87	2.13
600	8	4.15	6.60	5.90^{-}	1.9	1.80	1.83
	16	6.47	6.45	6.76^{+}	1.7	1.30	1.33
	24	5.60	6.61	6.44	1.8	1.67	1.87
650	10	4.57	6.44	6.11^{-}	2.0	2.07	2.20
	22	8.28	6.48	6.80^{+}	2.9	1.97	2.37^{+}
670	16	9.10	6.48	6.85^{+}	2.5	1.67	1.90

Table 1. The influence of seven maize stover and nine switchgrass biochars produced with microwave pyrolosis with different processing times and temperature conditions on 100% biochar and soils amended with 1% or 10% (w/w) biochar. The soil used for this study was the A horizon of a Brandt silty clay loam (Fine-silty, mixed, superactive, frigid Calcic Hapludoll, [Soil Survey Staff, 2011]) from Aurora, SD (44.31, -96.67) with an unamended pH in a 1:1 solution of 0.01 *M* $CaCl_2$ of about 6.40 and an EC value of 1.63 mS cm^{-1}. A '-' sign indicates significantly lower value and a '+' sign indicates significantly higher value compared with unamended soil.

~9.4 to 9.9. The pH of these biochars can be compared with other biochar data. A wood ash/biochar that was the by-product of a commercial ethanol plant (Chippewa Valley Ethanol Company, Benson, MN) was obtained and used for comparison purposes. The wood ash had a pH of over 11. In comparison, broiler litter biochar obtained from pyrolysis reactions at either 350 or 700°C was found to have a fairly uniform acidic pH (5.5) (Uchimiya et al., 2010). These data indicate that the pH of different types of biochar are dependent on processing time, temperature, and initial feedstock material.

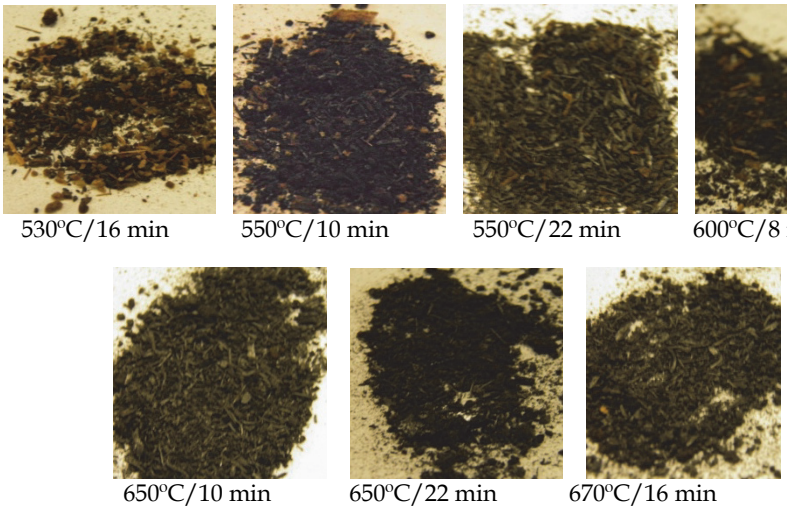

Fig. 1. Examples of biochars formed after exposure of maize (*Zea mays*) stover feedstocks to microwave pyrolosis at varying temperatures and times (see Lei et al., 2009).

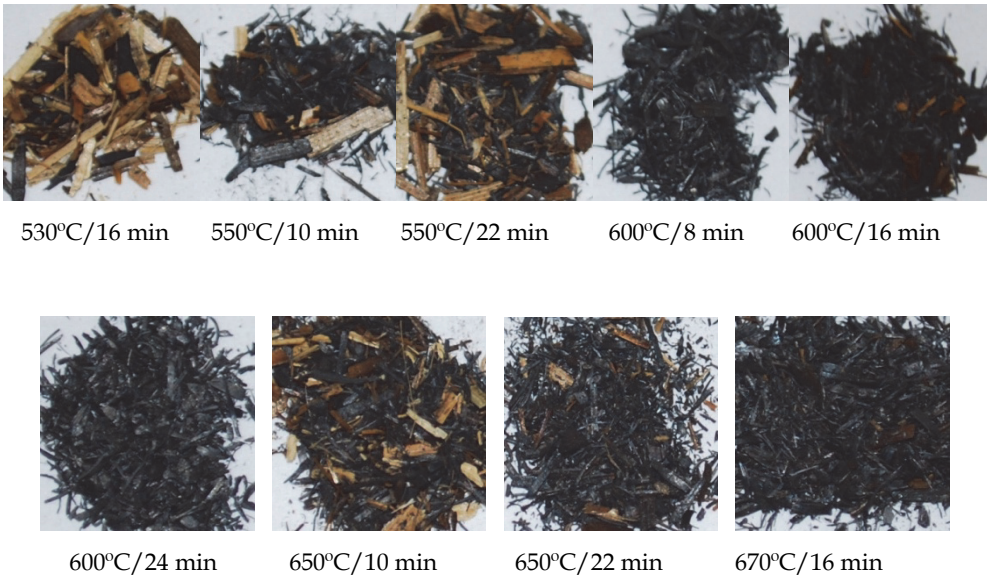

Fig. 2. Examples of biochars formed after exposure of switchgrass (*Panicum virgatum)* feedstocks to microwave pyrolosis at varying temperatures and times.

Electrical conductivity provides an indication of the amount of neutral soluble salts in the material or its salinity. High soil salinity often impedes the growth of most agricultural plants. Adding amendments that increase soil salinity, even though other beneficial

properties such as water holding capacity would increase, would be counterproductive. Saline soils are recognized worldwide (Food and Agriculture Organization, FAO) as soils with an EC reading of >4 mS cm^{-1} (Richards, 1954; Abrol et al., 1988). In the U.S., the Soil Science Society of America (SSSA) uses a value of >2 mS cm^{-1} boundary for the saline classification. Woodchip biochar had an EC value of 3.6 mS cm^{-1}. Biochar produced from maize stover had EC values ranging from 1.1 to 2.3 mS cm^{-1} with five out of the seven >1.9 mS cm^{-1}. The switchgrass biochars had EC values ranging from 1.5 to 2.9 mS cm^{-1} with the highest EC when materials were processed at 650° C for 22 min.

2.2.2 Influence on biochar amendment on soil pH and EC properties

The Brandt soil chosen for this study was a silty clay loam with a pH of 6.4. Due to the inherent soil properties and buffering capacity of this soil, it was expected that even high applications of the most acidic or alkaline biochar would have minimal impact on soil pH. When 1% maize or switchgrass biochars were added to soil, pH changes were minimal (generally <3%) (Table 1). When soils were amended with 10% biochar, pH was influenced to a greater extent. The slurry pH decreased from 4 to 8% when low pH biochars were added and increased a maximum of 9% when high pH biochars were added.

Soil EC was 1.63 mS cm^{-1}, well below the salinity values for saline soil. Adding either maize or switchgrass biochar to soil at 1% increased soil salinity, but with the exception of one switchgrass sample, did not increase the salinity to >2 mS cm^{-1}. Amending soil with 10% with the maize biochar that had the greatest EC value (2.3 mS cm^{-1}) was the only maize biochar that increased soil salinity above 2 mS cm^{-1}. Adding switchgrass biochar at 10% had greater impact than maize stover biochar and increased EC values an average of 11% when compared with ECs of unamended soil. Three switchgrass biochars increased EC values from 23 to 36% (Table 1) with final soil slurry EC values above 2 mS cm^{-1}, the SSSA value for saline soil classification. However, even with a 10% amendment, all final EC values were well below the FAO saline soil value of 4 mS cm^{-1}. If significant amounts of these biochars were applied frequently to the same field, managers must be cognizant of the potential for changes to EC values. Saline soil remediation can be expensive and often requires long-term management interventions, rather than short-term programs.

2.2.3 Atrazine sorption to biochar and soils amended with biochar

Atrazine is a chemical in the triazine family and has a slightly positive charge in soil solutions (Laird and Koskinen, 2008). The positive charge on the molecule, when in solutions above its pK_a, causes the molecule to be sorbed to materials that have a negative charge. Atrazine sorption to soil is considered moderate with K_d values ranging from 1 to 5 (Koskinen and Clay, 1997). The value is dependent on many soil properties including pH, organic matter, and clay content (Koskinen and Clay, 1997). In this study, atrazine sorption to biochar ranged from 7 to 92 L kg^{-1} (Figure 3). The sorption was dependent on feedstock type and processing method. These values ranged from 200 to 2300% greater than sorption to soil.

In general, the biochars from maize had much more variability in K_d values than switchgrass biochar (Figure 3). Three of the seven maize biochars had K_ds less than 20 L kg^{-1} whereas the other four had values of 55 L kg^{-1} or greater. In general, the switchgrass biochars had lower

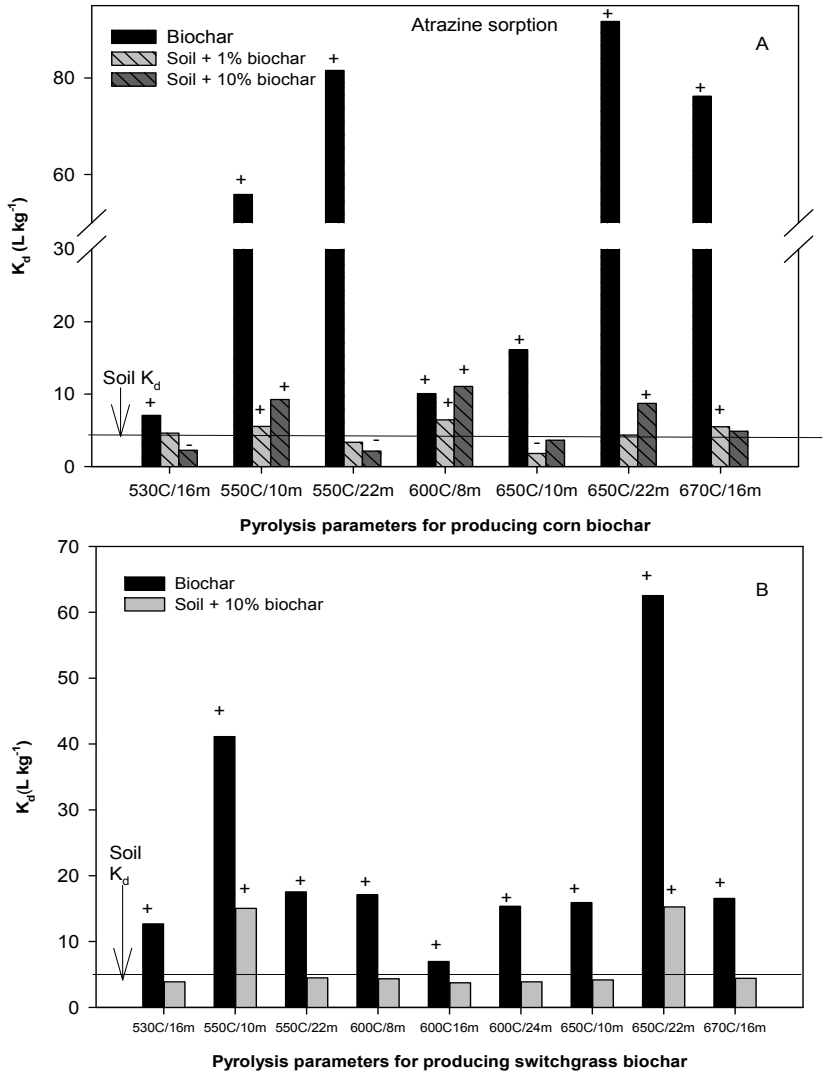

Fig. 3 A and B. Atrazine sorption (K_d) values to biochar from maize (*Zea mays*) stover (A) and switchgrass (*Panicum virgatum*) (B) produced by microwave pyrolysis at various processing times and temperatures. K_d values of sorption for the A horizon of a Brandt silty clay loam (Fine-silty, fmixed, superactive, frigid Calcic Hapludoll, [Soil Survey Staff, 2011]) soil when amended with 1 or 10% maize biochar or 1% switchgrass biochar. K_d sorption value of atrazine to unamended soil averaged about 3.86 L kg^{-1}. A "–" sign indicates lower sorption at $P \leq 0.05$ and a "+" sign indicates greater sorption at $P \leq 0.05$ than unamended soil.

K_d values for atrazine than maize, with only two of the nine samples having sorption values >18 L kg^{-1}. Correlation analysis was conducted to examine pH of biochar vs K_d but these parameters were poorly to moderately correlated for maize (r = 0.4) and not correlated for switchgrass.

Amending soil with maize biochar at 1% increased the K_d with three biochars and decreased the K_d for one biochar. The maximum increase was 66% more sorbed than unamended soil. The 10% additions decreased the amount sorbed by soil in two samples by about 43%. This was surprising as one of the biochars alone had double the K_d of soil (K_d = 7 L kg^{-1}) and a pH of 4.5 and the other had very high sorption (K_d = 82 L kg^{-1}) value and pH of 7.6. It is unclear what properties of this biochar would result in lower atrazine sorption. The soil amended with three maize biochars used at 10% amendment had nearly 3 times as much atrazine sorbed (K_ds ranging from 8.7 to 11.0 L kg^{-1}) when compared with soil alone. Two switchgrass biochars with the highest atrazine sorption also increased atrazine sorption when added as a 10% soil amendment, and raised the K_ds nearly 4-fold, with a K_d of about 15 L kg^{-1}. Other switchgrass biochars had no or only a slight influence on atrazine sorption.

2.2.4 2,4-D sorption to biochar and soils amended with biochar

Unlike atrazine which has a positive charge in most soils, 2,4-D with a pKa of 2.8 is a weak acid in most soil solutions (Wauchope et al., 1992). This chemical was chosen as a model compound to explore the effect of biochar on these types of compounds. The negative charge on the 2,4-D, as well as other chemicals in this auxin-type chemistry, often results in low or no sorption to soil (Clay et al., 1988). If these types of chemicals have a long residence time in soil (e.g. picloram), there is a high potential for leaching, although, because 2,4-D often is reported to have a ½ life of 10 d or less, leaching of this chemical is not usually considered a problem.

The K_d sorption value of 2,4-D to unamended Brandt soil was about 1 L $kg^{-1,}$ a four-fold lower sorption than atrazine to this soil. All biochar samples had much greater sorption coefficients than soil alone (Figure 4), with switchgrass biochars generally sorbing more 2,4-D than maize biochars. The K_d values for all biochars, regardless of feedstock type ranged from about 3 to >80 L kg^{-1} and was much greater than soil. K_d values for soil amended with 1% maize biochars were similar to K_d of unamended soil (Figure 4). Amending soil with 10% biochar (either maize or switchgrass) resulted in a few treatment combinations that had increased sorption compared to soil. Maize biochar resulting from processing stover at 600°C for 8 min increased 2,4-D sorption 3.3 times over unamended soils, whereas maize biochar formed from processing at 650°C for 22 min increased 2,4-D sorption by 4.5 times. Switchgrass biochar added at 10% to soil had little impact on 2,4-D sorption with two exceptions. The first was the biochar formed when processed at 550°C for 10 min where a 9.4- fold sorption increase was measured and the second when switchgrass was processed at 650°C for 22 min where a 15-fold sorption increase was measured. These two switchgrass biochars also dramatically increased atrazine sorption. The char produced at the higher temperature did influence soil EC values at 10% addition (Table 1), however, it is not known what the exact properties of these biochars or their interactions with soil/solution resulted in these increased sorption amounts.

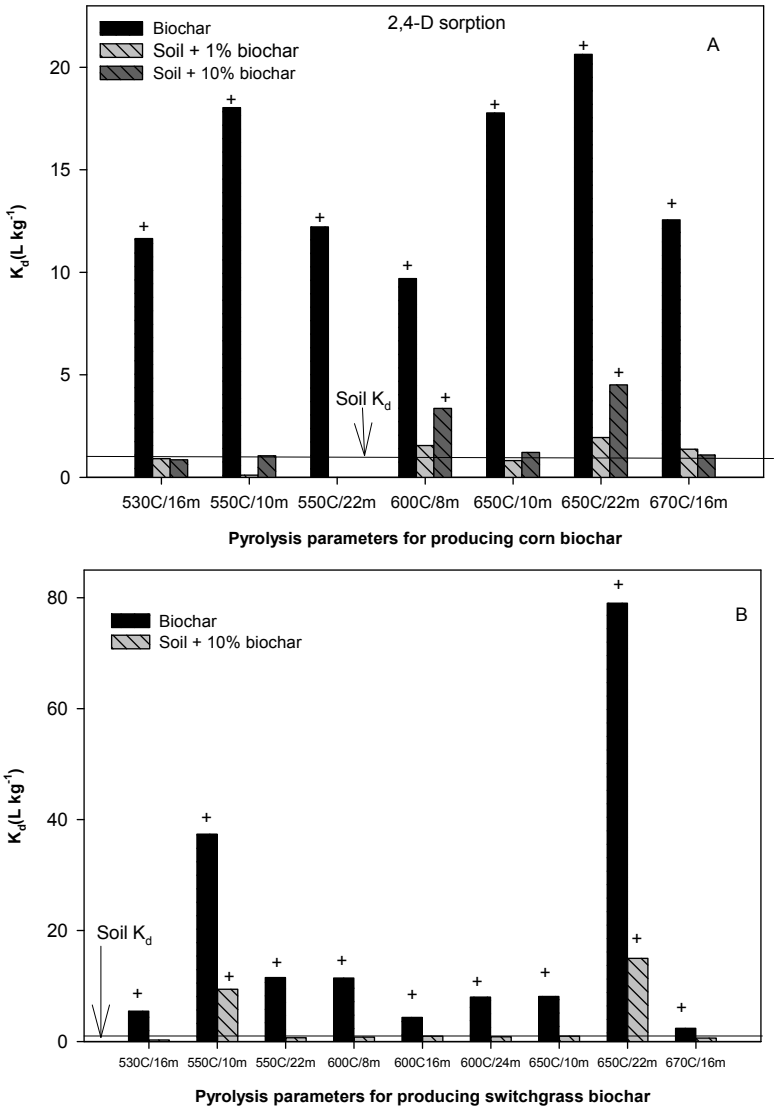

Fig. 4 A and B. 2,4-D sorption (K_d) values to biochar from maize (*Zea mays)*stover and switchgrass (*Panicum virgatum*) produced by microwave pyrolysis at various processing times and temperatures; K_d values of sorption for the A horizon of a Brandt silty clay loam (Fine-silty, mixed, superactive, frigid Calcic Hapludoll, [Soil Survey Staff, 2011]) soil when amended with 1 or 10% maize biochar or 10% switchgrass biochar. K_d sorption value of unamended soil averaged about 1.0 L kg^{-1}. A "+" sign indicates greater sorption at $P \leq 0.05$ than unamended soil.

3. Conclusion

Biochars, the by-products of pyrolitic conversion processes of vegetative biomass to gas, bio-oil, or other fuels, are proposed soil amendments for many diverse purposes. Biomass feedstocks and production processes vary depending on the desired end-products. This study measured the influence of several microwave pyrolitic conversion processes, which varied temperature and residence time, on pH and EC characteristics of the resulting biochars produced from maize stover and switchgrass. These biochars were used to amend a silty clay loam soil and examined the solution pH, EC, and sorption properties of a weakly cationic herbicide, atrazine, and an anionic herbicide, 2,4-D.

The microwave pyrolysis parameters of processing time and temperature of maize stover and switchgrass produced biochars that had a range of characteristics, with enough variation that they should not be thought of as a single entity with uniform properties. Short processing times (<10 min) of either feedstock at high (650^{o}C) or low (550^{o}C) temperature resulted in biochar with a pH < 4.5. Biochars produced with processing times >15 min at high temperature resulted in materials with pHs >8. Processing at intermediate temperatures and times resulted in char pHs ranging from 5.6 to 6.5. Adding 1% char to soil did not impact soil pH (6.4) whereas adding 10% biochar decreased soil pH a maximum of 12% when low pH biochars were used or increased soil pH up to 7% when high pH biochars were applied. "Native" soil EC was 1.63 mS/cm. Soils amended with 1% or 10% biochar ranged from -20% lower up to 39% higher EC values depending on biochar type and amount added. The biochars used in this study would be considered 'fresh', and not aged or post-process treated. Aging biochar or treating with steam or oxygen has been reported to dramatically change pH and other properties. Studies on these materials would need to be conducted to determine if results are similar to those reported for this study.

In a 2010 literature review, Kookana (2010) stated that there were limited published studies on the effect of biochars on pesticide efficacy and fate in soil, although in the few studies where sorption is reported, the sorption coefficients could be as high as >2000 times those of soil. Results from our study confirmed that when biochars were used as a single sorption material very high sorption amounts could be observed for both a cationic and an anionic compound. Herbicide sorption K_d to all biochars alone was very high compared with soil but varied among biochar types. Soil amended with 1% maize stover biochar had herbicide sorption values similar to unamended soil. However, adding 10% biochar amendment increased both atrazine and 2,4-D sorption coefficients by many-fold. A neutral herbicide, alachlor, has also been shown to have increased sorption in soils amended with woodchip biochar addition (Spokas et al., 2009). If biochars are applied to production fields, biochars may reduce atrazine preemergence weed control due to decreased availability to emerging seedlings. Kookana (2010) also discussed the possibility of longer residence time of pesticides due to reduced bioavailability, which may influence further the impact of a pesticide on ecotoxicology and potential accumulation. Indeed, Jones et al. (2011) reported biochar addition suppressed simazine biodegradation due to limiting availability to soil microbes through increased sorption, although leaching potential was reduced simultaneously.

The results of this study along with other reports have implications on best use of biochar in agricultural fields. If biochar has no or little effect on pesticide sorption, efficacy, or EC values, then the material may be suitable for general application in agricultural fields and

highly desirable if it can be used to increase water holding capacity or as a nutrient source. Biochars, if high in sorption capacity, may be applied strategically and could accomplish important roles in ecosystem health and environmental quality. Biochar, added in filter strips and waterways, eroded landscapes, or other areas where increased sorption is desired, may aid in cleaning water running off fields by sorbing undesirable contaminants. Increased sorption may also slow or stop herbicides from leaching, so highly sorbent biochar types may be desired over shallow aquifers or in areas low in native organic matter (Wang et al., 2010). Herbicide bioavailability in some cases may be reduced, protecting sensitive plants.

Conversely, the effect of spreading biochars across entire fields may have negative results and be undesirable. One consequence may be that the materials increase soil EC values to saline levels. In addition, if the biochar reduces the efficacy of soil-applied herbicides or other pesticides this may have negative impacts. Reduced pesticide efficacy would require higher herbicide application rates to be as effective as lower rates. This would have monetary implications for growers and field managers by increasing management costs. Increased sorption, in some cases, also may increase the recalcitrance of pesticides leading to longer residence times in the environment. The occurrence of greater recalcitrance may be desirable if bioactivity was still acceptable and longer activity of the pesticide was desired to control the pest of interest. However, longer residence time may lead to other long-term environmental problems, such as greater leaching potential or carry-over problems into the following season.

Prior to any regular field applications of any biochar, the biochar properties must be examined to determine the suitability of the material for the long-term management of a particular site. The reasons for the application should be defined clearly and the outcomes closely monitored to determine if expectations and results are synonymous.

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5

Chemical Behaviour and Herbicidal Activity of Cyclohexanedione Oxime Herbicides

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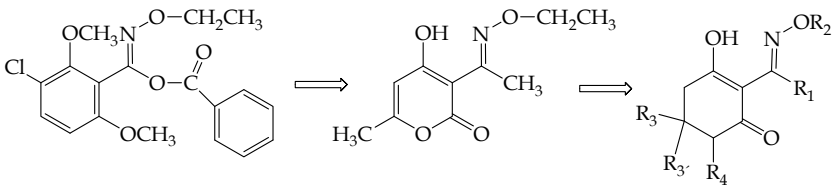
1. Introduction

Benzoximate (I; Fig. 1) is an acaricide developed by Nippon Soda in 1971 (Iwataki, 1992). However, scientifics of the company observed that some benzohydroxamates showed weak herbicidal activity. After much synthetic developments, a new lead compound, an ethoxyimino dehydroacetic acid derivative (II; Fig. 1), showed a strong pre-emergence herbicidal activity against annual grass weed without any effects towards broadleaf plants. Further developmental research was performed on the cyclohexanedione skeleton to develop a new post-emergence herbicide. It was observed that the ethoximine group between the two keto groups was essential for the herbicidal activity (III; Fig. 1). Besides, when hetero atoms were introduced in the ring, the compounds showed high pre-emergence activity. When the ring was formed by carbons, so-called cyclohexane derivatives, the compounds showed high pre- and post-emergence activities. Therefore, the synthetic research was focused towards the substituents on the cyclohexanodione skeleton. The activity was higher when side chain substituents R_1 and R_2 (Fig. 1) were alkyl groups. As for the ring substituents, mono i-Pr and germinal dimethyl at the R_3 and $R_{3'}$ position and ciano and methoxycarbonyl groups at the R_4 position provided the maximum activity. This way, alloxydim–sodium was discovered and introduced in the market in 1978.



I Benzoximate II III

Fig. 1. Pathway of lead compounds towards the discovery of cyclohexanedione oxime herbicides.

However, though alloxydim-sodium showed a potent activity against annual grass weeds, it did not against perennial grass weeds. Therefore, the synthetic research was focused towards the introduction of different substituents on the cyclohexane ring, since the structure-activity pattern of the skeletal had been already identified. It was disclosed that

substituents having a hetero atom such as chlorine, oxygen or sulfur increased the herbicidal activity. Some sulphur-containing cyclohexanodione derivatives showed very high activity against annual and perennial grass weed with post-emergence treatment. Thus, alkyl- and aryl- thioalkyl groups were introduced in position R_3 (III; Fig. 1) resulting in the discovery of other cyclohexanedione herbicides such as, sethoxydim, clethodim or cycloxydim (Fig. 2).

Thus sethoxydim, introduced in 1982, showed excellent herbicidal activity against various weed species in particular *Sorghum halepense*. Afterwards, clethodim, discovered by Chevron Chemical (Tomlin, 2006), presented almost the same herbicidal spectrum to sethoxydim but the application rates seems to be lower (Iwataki, 1992). Cycloxydim, discovered by BASF, showed a very broad spectrum with application rates similar to sethoxydim. Other herbicides like tralkoxydim have an herbicidal spectrum narrower than sethoxydim and clethodim. It has been used only for the control of annual winter grass weeds in wheat fields (Roberts, 1998). The last cyclohexanedione oxime herbicide was profoxydim (Fig. 2) developed by BASF, and first registered in 1998 for the control of grass weeds in rice.

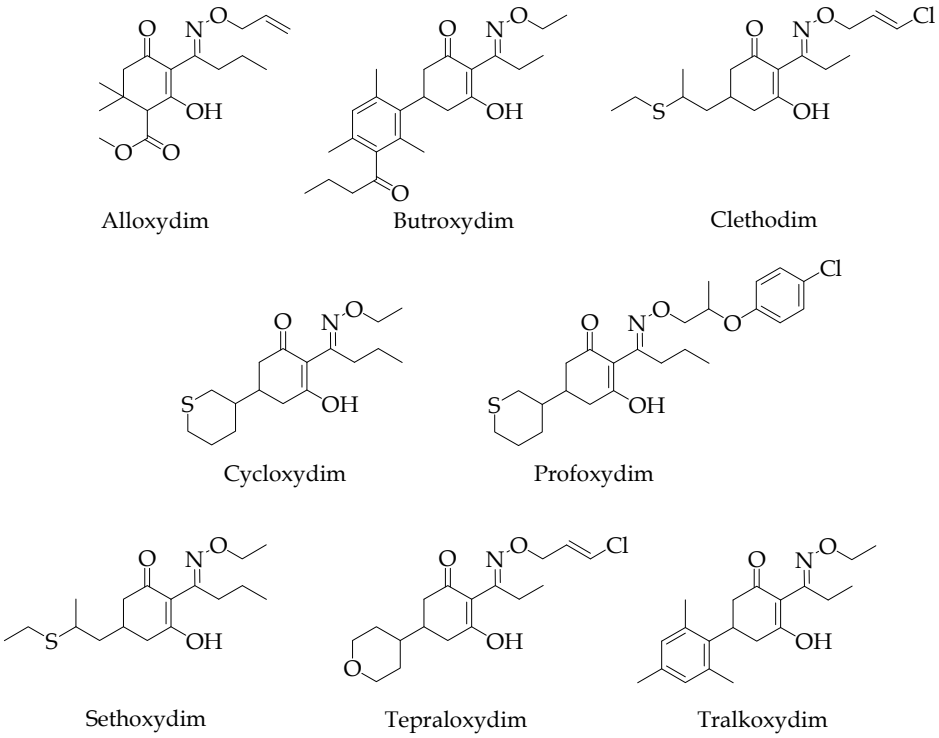
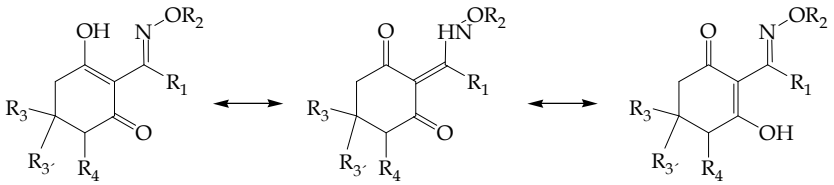


Fig. 2. Chemical structures of cyclohexanedione oxime herbicides.

The basic structure of the cyclohexanedione oxime herbicides is shown in Fig. 3. These compounds show a keto-enol tautomerism, where the enolic forms (IV and VI) is generally predominant to the keto form (V) (Iwataki, 1992; Iwataki & Hirono, 1978). Thus, general formulas for this class of compounds are expressed as the enolic form. The term "dione" is used, however, as the general term for these compounds because of its simplicity.



IV V VI

Fig. 3. Keto-enolic tautomerism of cyclohexanedione oxime herbicides.

As can be observed in Fig. 3, these herbicides present two isomers *E* and *Z* relating to the chloroallyloxy side chain, though the *E* form is more active and more stable than the Z form (McInnes et al., 1992; Sandín-España et al., 2003) and they are commercialized in this isomeric form.

Herbicides of this family are weak acids with pKa below 5. Thus, they can readily ionize, and if the pH increases above the pKa value of the herbicide, the ionized form will predominate. The protonated form of a herbicide will penetrate the plant cuticle more rapidly than the ionized form (Bukovac et al., 1971).

Their solubilities and partition properties are highly dependent on pH. They are easily decomposed by pH variations and by sunlight radiation (Roberts, 1998). These properties are highly relevant to their environmental fate.

Degradation is so rapid in aqueous media that in some cases it is questioned if herbicidal activity is maybe due to some degradation product (Iwataki, 1992). Due to the rapid degradation, most degradation products are common to most systems and in some cases it is not clear if a by-product is produced by chemical or biochemical degradation.

Therefore, it is of utmost importance to study the degradation routes of these herbicides to estimate the persistence of the residues of these compounds and to identify the factors that influence their behaviour in the environment.

2. Abiotic transformations, degradation pathways and degradation products

When an herbicide is introduced into the environment, it is subjected to different biotic and abiotic processes. Abiotic transformations may include chemical (mainly hydrolysis and thermolysis) and photochemical reactions. Complete mineralization of herbicides into inorganic constituents such as carbon dioxide, ammonia, water, mineral salts, and humic substances often occurs slowly in the environment. Different compounds, however, are formed before herbicides can be completely degraded. The organic compounds formed by the different transformation processes are referred to by several names such as degradates, by-products, etc. The most widely used are "transformation product" and "degradation product". "Metabolites" is a term usually inappropriately employed as it should be use only when the transformation product is a result of biological transformation. Therefore, in this chapter mainly dedicated to abiotic processes, we will refer to these compounds as degradation products, by-products or photoproducts if they are formed by transformations induce by sunlight.

In general, one or two transformations in the molecular structure of some herbicides are enough to modify its properties. In fact, the biological activity and/or environmental contamination attributed to the parent compound can be due to the degradation products.

The understanding of the total consequences for herbicide use is limited to the fact that most studies have focused on the parent compound and generally did not consider their transformation products (Somasundaram & Coats, 1991).

Historically, some of the most serious concerns about the safety of herbicides have raised from its transformation products than can cause detrimental side effects. Different studies confirm that many degradation products are more mobile and some others are more persistent than their respective parent compound (Boxall et al., 2004; Green & Young, 2006; Kolpin et al., 2004; Tixier et al., 2000).

Information about these degradation routes is necessary to estimate the persistence of these compounds and to identify the factors that influence their behaviour in the environment.

Nowadays, some herbicide transformation products are considered as "emerging pollutants" (Richardson, 2009; Rodríguez-Mozaz et al., 2007) as most of them have been presented in the environment for a long time, but their significant and presence are only now been elucidated and, therefore, they are generally no included in the legislation. Besides, there is still a lack of knowledge about long-term risks that the presence of these emerging pollutants may pose for organisms as well as for human health. Consequently, transformation products have become a new environmental problem and have awakened great concern among scientists in the last years (Richardson, 2006; Richardson, 2007).

Besides, herbicide degradates can either be less toxic or have similar or greater toxicity than their parent compounds (Belfroid et al., 1998; Tuxhorn et al., 1986). Thus, obtaining data on parent compounds and their primary degradation products is critical for understanding the fate of herbicides in the environment.

As mentioned before, the different abiotic/biotic processes that take place in the environment modified the physicochemical properties of the parent molecule.

Most of the oxidative reactions (hydroxylation, sulfoxidation, dealkylation…) and hydrolysis impart also some degree of increase polarity and hence water solubility to the molecule. Therefore, the new xenobiotics are more mobile in soil. Reduction reactions are characterized in environments with low oxygen concentrations, low pH and anaerobic microorganism. These reactions are less commonly observed and generally give rise to products with lower polarity.

Recent studies indicate that about one-third of the degradation products derived from a range of pesticides types have an organic carbon absorption coefficient (K_{oc}) of at least one order of magnitude lower than that of the corresponding parent compound. Thus, these transformation products may be more likely to be transported to surface and groundwater.

Boxall and co-workers (Boxall et al., 2004) showed that among different classes of pesticides and its transformation products, 41% of the transformation products were less toxic than parent compound and 39% had a similar toxicity to their parents, but 20% were above 3 times more toxic and 9% were above ten times more toxic than their parent compounds.

Oxidation reactions occur frequently in some soil and are an extremely important transformation pathway. S-containing herbicides, like some cyclohexanediones, are often rapidly oxidized to sulfoxide and afterwards more slowly to sulfones (Roberts, 1998). Sulfoxidation of herbicides can occur in soil and water mediated by chemical or biological reactions. This oxidation is so rapid and complete that sulfoxides are often the compounds found in soil shortly after application of the parent sulfide compound. Furthermore, in some cases, sulfoxides and sulfones are suspected to have pesticidal activity (Ankumah et al., 1995; Campbell & Penner, 1985; Tuxhorn et al., 1986).

2.1 Chemical degradation

Chemical degradation of organic compounds includes mainly hydrolysis and thermolysis reactions. With regard to hydrolysis, pH of water is responsible for the transformation of some pesticides in solution, especially in conjunction with extreme pH (García-Repetto et al., 1994).

Furthermore, organic compounds sensitive to pH give rise to its rapid degradation even with a slight variance of pH (Dannenberg & Pehkonen, 1998; Santos et al., 1998; Sanz-Asencio et al., 1997) and hence they have a low environmental persistence.

Alloxydim-sodium is a sodium salt of an acid, alloxydim (Fig. 4), having a pKa of 3.7, which is present as the monoanion and/or the acid in aqueous solution, and the possibility of many tautomeric forms should be considered to understand the transformations in the molecule when degradative reactions occur.

Alloxydim-sodium is neutralized to the sodium free compound (alloxydim) by the action of carbon dioxide in air or components in plants or in soil.

Studies of thermal degradation of alloxydim gave two oxazole derivatives (VII and VIII; Fig. 4) at 120 ºC. The ratio was found to be 3 to 2 (VII:VIII) (Iwataki & Hirono, 1978). The mechanism of the formation of these oxazoles seems to take place when in certain tautomeric isomers, the cyclohexane ring of alloxydim is coplanar with the six membered ring which is formed by hydrogen bonding and the allyloxy group should be in the anti position against the cyclohexane group on the C=N bond. Therefore, the Beckmann rearrangement reaction occurs, which coincides with the intramolecular cyclization to form the oxazoles.

In the same way, when alloxydim is heated at 30, 40 and 50 ºC in a dark incubator for 20 days gives the mixture of VII and VIII (Fig. 4). These three degradation products also appeared when alloxydim-sodium is applied in the leaves of plants (Hashimoto et al., 1979a; Koskinen et al., 1993).

The acid hydrolysis of alloxydim gives rise mainly to the butyrylamido derivatives and the imine salt. The alkaline hydrolysis forms the demethoxycarbonylated butyrylamido derivative (Iwataki & Hirono, 1978).

Chemical degradation of herbicides can also take place when the herbicide gets in contact with water that possesses substances that promote its degradation. In this sense, it is known that the presence of substances employed for the disinfection of water such as hypochlorite and chloramines degrade the herbicide to compounds more or less toxic than the active substance (Lykins et al., 1986; Magara et al., 1994; Reckhow & Singer, 1990).

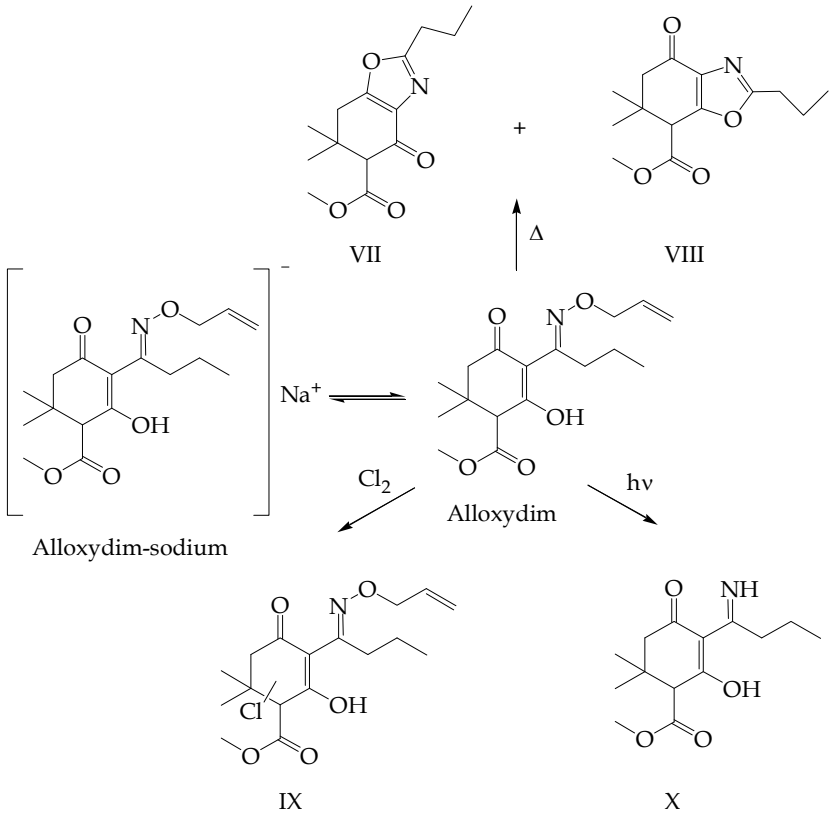


Fig. 4. Main degradation pathways of alloxydim-sodium.

Studies of alloxydim degradation in chlorinated water showed that the herbicide degrades very fast with half-lives less than one minute. As a result of the reaction one degradation product is formed that is isolated by solid phase extraction and identified by means of mass spectrometry as a chlorinated cyclohexanedione oxime compound IX (Fig. 4) (Sandín-España et al., 2005b).

Other cyclohexanedione oxime herbicide, clethodim is also an labile acid. Degradation rates significantly decreases from pH 7 to pH 5. After 20 hours, clethodim loss was 37% at pH 5 , 8% at pH 6 and 0% at pH 7 (Falb et al., 1990). Nine by-products were separated by liquid chromatography, though none of them was identified.

Falb et al., 1991 (Falb et al., 1991) also observed an increased in the degradation of clethodim when pH decreased. 19 peaks of degradation products more polar than parent clethodim molecule were separated by liquid chromatography.

As mentioned before, cyclohexanedione herbicides are marketed as the *E*-isomer at the oxime ether double bond (Fig. 5). It has been stated that some of them, like clethodim, may equilibrate with the *Z*-isomer in a polar medium such as water (Falb et al., 1990; Sandín-España et al., 2003). 4% of isomerization of clethodim to *Z*-isomer was observed when

preparation of aqueous solution and 40% after two months (Sandín-España et al., 2005a). This isomerization has been also observed in herbicide tepraloxydim where equilibrium between both isomers was slowly attained in aqueous solution. It took about 7 days to reach equilibrium with a final ratio between isomers of 2:1 (*Z:E*) (Sandín-España et al., 2003). Other studies show that isomer Z also appears in acidic water samples of *E*-tepraloxydim after 72 h of storage at 4 ^{o}C (Sandín-España et al., 2002).

Degradation of clethodim in chlorinated water either with sodium hypochlorite or chloramines was very rapid with half-lives below 10 minutes (Sandín-España et al., 2005a). The main degradation processes was the oxidation to clethodim sulfoxide (Fig. 5). Experiments continued to follows degradation of the sulfoxide molecule. Its half-life was 4.4 seconds with hypochlorite and 9.3 hours with chloramines. Subsequent oxidative reaction of the sulfoxide generates the formation of clethodim sulfones (Fig. 5) and other minor products. The fast degradation of clethodim in chlorinated water (either with hypochlorite or chloramines) practically precludes any possible exposure of consumers to this compound when tap water is subjected to a chlorinated treatment. However, it is not possible to ensure complete destruction of the reaction product clethodim sulfoxide before the distribution point when chloramines are used for disinfection due to the slower degradation rate. Besides, whereas some minor degradation products remain unidentified, none of the major degradation products of clethodim contain more chlorine atoms than the parent compound which represents a positive aspect of this compound with respect to consumers´ safety.

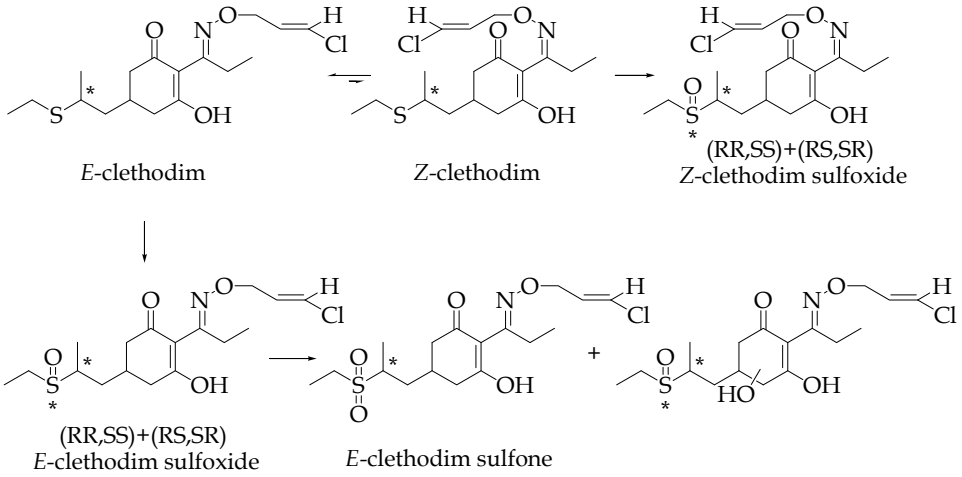


Fig. 5. Proposed degradation pathway of clethodim in chlorinated water.

Sethoxydim dissolves in water is found to be unstable at room temperature or when kept at -20^{o} C; only 6 and 24% or the parent sethoxydim remained after 72 hours (Campbell & Penner, 1985). This herbicide also undergoes chemical decomposition at acid pH (Shoaf & Carlson, 1992; Smith & Hsiao, 1983) and in alkaline solution (Shoaf & Carlson, 1986).

Others cyclohexanedione herbicides such as profoxydim or tralkoxydim have also been reported to undergoes hydrolysis depending on the pH (Walter, 2001) of the solution and being easily degraded in aqueous solution (Sevilla-Morán et al., 2011; Srivastava & Gupta,

1994) and in chlorinated water (Sandín-España, 2004). However, scarce data exists in the open literature about degradation pathways or degradation products.

2.2 Photochemical degradation

Photochemical reactions are one of the major transformation processes affecting the fate of pesticides in the environment, especially in the aquatic compartment (Dimou et al., 2004; Neilson & Allard, 2008). Two ways of photodegradation reactions occur in sunlit natural water. In direct photolysis, organic compounds absorb light and as a consequence of that light absorption, undergo transformation. For this to occur in the water, the emission of the sun (290-800 nm) needs to fit the adsorption spectrum of the pesticide. In indirect photochemical reactions organic chemical are transformed by energy transfer from another excited species (e.g., components of natural organic matter) or by reaction with very reactive, short-lived species formed in the presence of light (e.g., hydroxyl radicals, single oxygen, ozone, peroxy radicals, etc.). Absorption of actinic radiation by nitrate and dissolved organic matter (DOM) leads to the formation of most of these species. Therefore, the composition of the aquatic media plays an important role on the phototransformation of pesticides in this compartment.

The hydroxyl radical, $OH^{\bullet}$, is one of the most reactive of the aforementioned reactive intermediates due to its non-selective and highly electrophilic nature.

Ideally solar radiation should be used in studies of environmental photochemistry, however, meteorological conditions in most countries and a slow degradation rate do not permit reproducible experimentation. In a first approach to study the photochemical behavior of organic compounds in different matrices, it is common to conduct the degradation under controlled conditions. Generally, the use of xenon arc lamp, with light above 290 nm (provided by a filter), is preferred as its spectral emission distribution are very close to the solar radiation spectrum (Marcheterre et al., 1988). It has been demonstrated that the use of different light sources under identical aqueous conditions can produce similar degradation products, with the only difference being in their kinetic of formation (Barceló et al., 1996). As for the experimental equipment, quartz glass is preferred instead of other glass material since it permits a greater transmission of radiation (Peñuela et al., 2000).

The composition of aquatic media also plays an important role in the phototransformation of pesticides. Various authors point out that particulate matter, such as sediment particles, and dissolved substances present in natural waters could be responsible for the different photolysis rates observed between natural and distilled water (Dimou et al., 2004; Schwarzenbach Rene et al., 2002; Tchaikovskaya et al., 2007). The most important light-absorbing species that may induce indirect photolytic transformation of organic pollutants in natural waters are the chromophores present in dissolved organic matter (DOM) where humic acids are important absorbing constituents of it and in a lesser extent, fulvic acids.

Diverse studies are available from literature where humic acids act enhancing (Sakkas et al., 2002a, 2002b; Santoro et al., 2000; Vialaton & Richard, 2002) or inhibiting (Bachman & Patterson, 1999; Dimou et al., 2004; Dimou et al., 2005; Elazzouzi et al., 1999; Sevilla-Morán et al., 2010a; Sevilla-Morán et al., 2008) the degradation of pesticides. In the first case, humic acids behave as a "sensitizer" where the excited states of humic acids can participate in a charge-transfer interaction with pesticides, or generate reactive intermediates, such as

hydroxyl radicals, singlet oxygen, solvated electrons or hydrogen peroxide. In the second case, humic acids act as photon trap (optical filter effect), decreasing the photodegradation rate of pesticide.

Consequently, information about photodegradation of pesticides is necessary to estimate the persistence and to identify the factors that influence their behavior in the environment. Furthermore, it is important to investigate what these compounds degrade into, its persistence of by-products relative to the parent compounds, and whether the degradation products retain the activity of the active substance to cause a toxicological effect on non-target organisms in aqueous systems.

Studies show that alloxydim is degraded on the leaf surface by photochemical reactions. After two days, the deallyloxylated compound X (Fig. 4) is the main degradation product identified. This by-product is formed by photoreduction. Other minor degradation products identified, that accounted less than 0.5% of the applied radioactivity, were two isomeric oxazoles and a demetoxycarbonylated compound (Hashimoto et al., 1979b; Soeda et al., 1979).

When photodegradation of alloxydim was studied in sterilized soil (Ono et al., 1984), only 22% of the ^{14}C-alloxydim applied was remained. The main by-products identified by thin-layer chromatography and mass spectrometry were again the deallyoxylated compound (X) and the two oxazole isomers (VII, VIII) (Fig. 4). The demethoxycarbonylated compound was formed in a minor extent (Ono et al., 1984).

Photochemical transformation of a methanol solution of ^{14}C-alloxydim on silica gel plate, irradiated with UV light also gives by-products VII, VIII and X (Soeda et al., 1979).

Sevilla-Morán et al., studied the photodegradation of alloxydim-sodium under simulated solar irradiation using a xenon arc lamp (Sevilla-Morán et al., 2008). This light source fits the solar radiation spectrum best over the whole range of spectral emission (Marcheterre et al., 1988). Indirect photolysis under the presence of various concentrations of humic acids, nitrate and iron ions was also investigated. Results show that degradation rate in direct photolysis was higher as the radiation intensities increased. Irradiation of aqueous solutions of alloxydim containing different concentrations of humic acids (1-20 mg L^{-1}) show that increasing concentrations of humic acids, decrease photolysis rate of this herbicide, indicating that absorbed most of the photons emitted, thereby slowing down direct photochemical reaction of alloxydim. The presence of nitrate ions had no effect on the degradation rate. On the contrary, iron ions accelerate the rate of photolysis of alloxydim possibly due to the formation of a complex and later undergo a direct photolysis (Boule, 1999; Park & Choi, 2003). Simultaneously to the irradiation experiments, control experiments in absence of radiation were performed in order to discard other type of dark reactions (hydrolysis, thermolysis, ...). Quantitative recoveries of clethodim during the entire exposure period to simulated solar irradiation enable to ignore other transformation processes that are not initiated by radiation.

As for the identification of transformation products, HPLC-ESI-QTOF-MS technique was employed. QTOF provides elevated resolution and sensitivity, high mass accuracy for both parent and fragment ions in combination with the possibility of performing MS/MS acquisitions obtaining more structural information (Aguera et al., 2005; Ibáñez et al., 2004). In this way, QTOF allows the assignments of a highly probable empirical formula for

unknown compounds. Two main degradation pathways were identified. The main reaction was the photoreduction of the N-O bond dissociation of allyloxyamino moiety to give the imine (X; Fig. 4). The second reaction was the isomerization of the oxime moiety to give the *Z*-isomer (Sevilla-Morán et al., 2008).

In the same way, under UV light technical clethodim was greatly accelerated as compared to dark conditions. The degradation half-lives were 2.5, 2.6 and 3.2 hours at pH 5, 6 and 7 (Falb et al., 1990). The HPLC system separated 13 photoproducts though none of them were identified. The addition of adjuvants to clethodim increased the rate of photodegradation with UV radiation by 2 to 7 fold. The rate of degradation under sunlight was increased with the addition of adjuvant by 7 to 27 fold over the control (Falb et al., 1990).

Falb and co-workers, (Falb et al., 1991) also observed that clethodim undergoes degradation when exposed to UV light and developed a method for the separation of 31 degradation products, but none of them were characterized.

It is recommended that spraying at late evening or night may improve cyclohexanedione oxime herbicides efficacy due to a reduction in the amount of UV light present (McMullan, 1996). Applying cyclohexanedione herbicides at times when the UV light is lowest, such as late evening or at night, will maximize weed control with these herbicides.

Cyclohexanedione herbicide efficacy was affected by spray solution pH. This was probably due to the predomination of the ionized form of the cyclohexanedione molecule at high spray solution pH and the possible formation of cyclohexanedione sodium salts (Nalewaja et al., 1994).

Experiments on the efficacy of clethodim and tralkoxydim (McMullan, 1996) showed that filtering UV light 4 hours after treatment improved the efficacy between 13 and 55%. These results are in agreement with results published by McInnes et al. with sethoxydim (McInnes et al., 1992).

Indirect photodegradation of clethodim in the presence of humic acid, nitrate ions and iron ions in aqueous solution has been also studied (Sevilla-Morán et al., 2010a). The presence of humic acid increased the half-life of herbicide photolysis compared to direct photolysis [$t_{1/2([HA]=1\ mg/L])}$ = 44,2 min *vs.* $t_{1/2ultrapure\ water}$ = 28,9 min]. This retarding effect, as in alloxydim, indicates that these substances can act as an "optical filter" absorbing most of the photons emitted and thereby slowing the direct photochemical reaction of clethodim. Nitrate ions had no effect on the photodegradation of clethodim and the presence of iron ions increase the rate of photolysis up to 6 times when 20 mg L^{-1} of Fe(III) ions were present in the solution. In these experiments up to nine different by-products were observed all of them more polar than clethodim (Sevilla-Morán et al., 2010a). Identification was performed by using a QTOF mass spectrometer. A detailed study based on the exact mass measurements and fragmentation pattern makes possible for the first time to elucidate the structures of the nine photodegradation by-products. Figure 6 shows the proposed photodegradation pathway of clethodim in aqueous solution. The by-products identified were the following; *Z*-isomer of clethodim at the oxime ether double bond. The *Z*-isomer is much more polar that clethodim (26 *vs.* 41 minutes). This could be due to an internal hydrogen bond formed between the oxime oxygen and the hydroxyl group of the cyclohexane ring. Several authors stated that some *E*-isomer of cyclohexanedione oxime herbicides may equilibrate with the *Z*-isomer in polar solvents (Falb et al., 1990; Sandín-

España et al., 2002) or in chlorinated water (Sandín-España et al., 2005a). Besides, it has been reported that isomerization can be induced by light and temperature (Curtin et al., 1966; Sevilla-Morán et al., 2008).

The oxidation of the sulphur atom of the molecule generates two pairs of enantiomers (RR+SS and RS+SR) and diastereomers are chromatographically separated in two peaks, containing a pair of enantiomers each. In the same way, *Z*-isomer of clethodim gave rise to the corresponding pairs of enantiomers of *Z*-clethodim sulfoxide. As in alloxydim, photoreductive reaction of *E*- and *Z*-clethodim forms the corresponding imine. Oxidation of imine gave rise to two pair of enantiomers of clethodim imine sulfoxides. The oxidative cleavage of the C-S bond of clethodim imine gave rise to clethodim imine ketone.

Various authors have also studied the photodegradation of sethoxydim (Campbell & Penner, 1985; Sevilla-Morán, 2010; Sevilla-Morán et al., 2010b; Shoaf & Carlson, 1986; Shoaf & Carlson, 1992). The herbicide was completely lost within seconds in aqueous media either in incandescent or UV light at pH 3.3 and 6.0 and methanolic solutions of the herbicide were transformed more than 50 % after 10 min of exposition to UV light (Shoaf & Carlson, 1992) (Shoaf & Carlson, 1992). Upon 1 hour of UV irradiation sethoxydim sulfone was identified as one of the degradation products formed during the experiment. Campbell and Penner suggested the rapid degradation of sethoxydim in water and organic solvents (Campbell & Penner, 1985). These authors exposed aqueous solutions of sethoxydim to artificial light and observed that only 2 % remained after 3 h. In the same way, they also observed a rapid photodegradation on glass disks of sethoxydim dissolved in n-hexane (81 % of herbicide was transformed after 1 h). In both systems, 6 major products were detected. Five of these products were transitory and only one appeared to be the single end product. One of these compounds was isolated and identified by mass spectrometry as desethoxy-sethoxydim.

Evidence of rapid transformation of sethoxydim expose to light suggested that herbicidal activity resulted from the more stable transformation products. Five of these products were applied to barnyardgrass to determine phytotoxicity. It was found that two degradation products had significant activity as did sethoxydim and three of them showed no herbicidal activity. Though none of these isolated by-products were quantified as relative herbicidal potencies could not be determined, this is relevant information about phytotoxicity of degradation products.

Under field conditions, were sethoxydim would be applicated by spraying water-oil emulsion during daylight, it is likely that sethoxydim transformation would be rapid and levels of degradation products would be present. These findings suggest that some of the degradation products are actually the herbicidal agent (Campbell & Penner, 1985; Shoaf & Carlson, 1992).

In order to obtain results close to real field conditions different experiments have been carried out to study the photodegradation of sethoxydim in natural waters (mineral, well and river) and under natural sunlight. The degradation rates in natural waters were lower than in ultrapure water and degradation under natural sunlight also decrease the degradation rates. These results indicate that under real environmental conditions photodegradation of sethoxydim is retarded compare to laboratory studies. Besides, significant differences among types of water suggest that degradation of sethoxydim has a strong dependence on the composition of water sample. Figure 7 shows the photo-

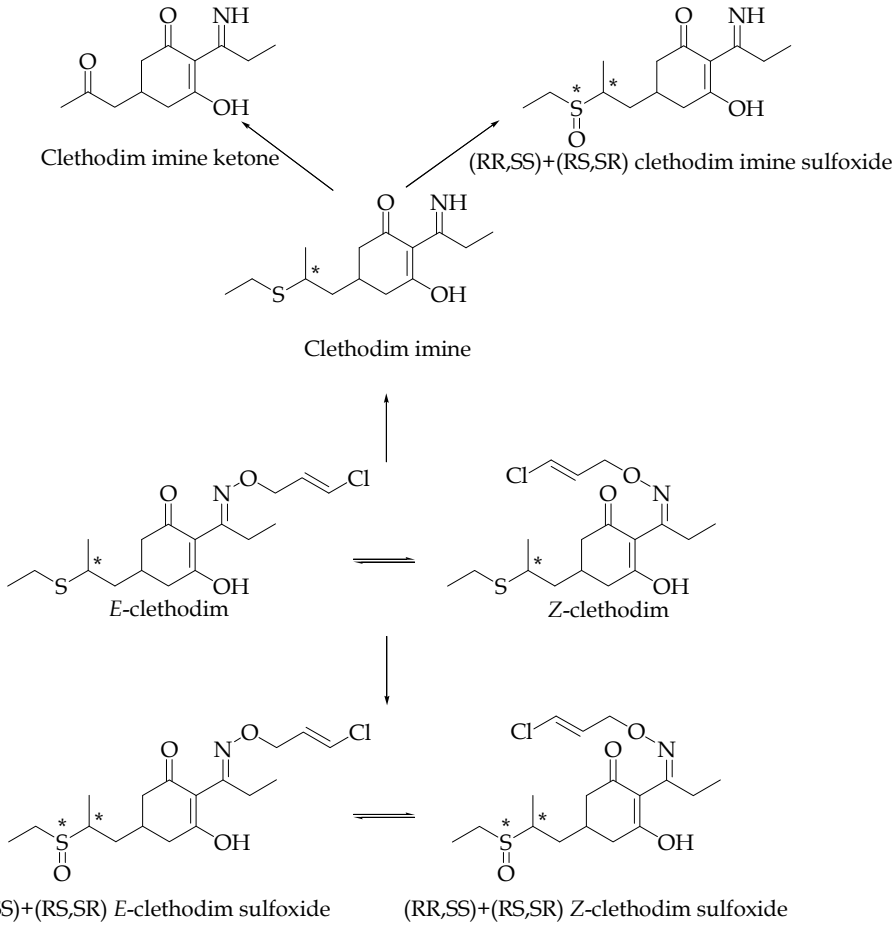


Fig. 6. Proposed photodegradation pathway of clethodim in aqueous solution.

degradation curves of sethoxydim in natural waters under solar irradiation. The photolysis rate decreases in the following order, river<well≈mineral<ultrapure water. Photodegradation of sethoxydim-lithium in natural water was approximately 5 times slower than in ultrapure water showing a half-live of 436.9 ± 0.8 min for river water and 82.1 ± 0.7 min for ultrapure water. These significant differences observed in the three water samples, under both types of irradiation, indicated that the degradation of sethoxydim-lithium depends to a large extent on the composition of aqueous matrix. Natural water contains some substance/s that induces a retardant effect of the photodegradation of sethoxydim-lithium. Thus, this difference observed (2-5 folds) could be attributed to the presence of increasing concentrations of TOC (Total Organic Carbon) in the natural waters, where river water has the highest concentration of TOC (2.865 mg L^{-1}) and ultrapure water has the lowest (0.005 mg L^{-1}).

The photodegradation of sethoxydim-lithium in all water types under simulated light was quicker than under natural sunlight (e.g. $t_{1/2(\text{river water-natural sunlight})}$=436.9 ± 0.8 min *vs.* $t_{1/2}$ (river

$_{water)}$ 135.5 ± 0.3), which is well correlated with the lower intensity of natural sunlight (460 W m^{-2} at the middle of the day *vs.* 750 W m^{-2} of xenon lamp). These results show a great influence of the intensity radiation on the degradation of sethoxydim-lithium and they are in accordance with other authors where the degradation of active substance is dependent on the irradiation energy (Dimou et al., 2005; Sakkas et al., 2002a).

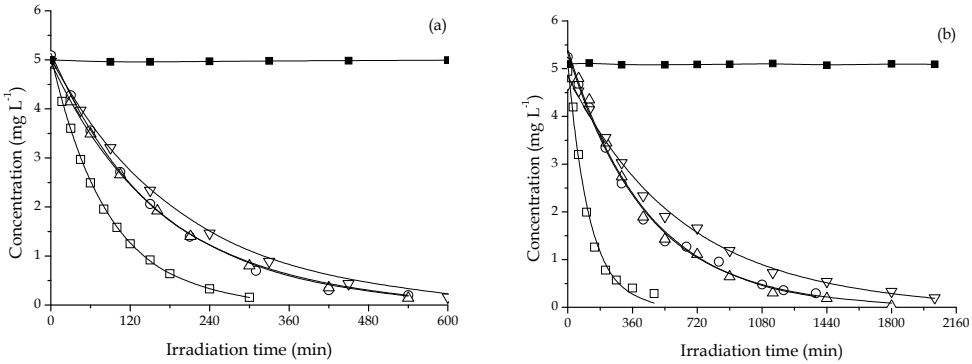


Fig. 7. Photodegradation of sethoxydim-lithium in various types of water under simulated light (a) and natural sunlight (b): (□) ultrapure water, (○) mineral water, (Δ) well water, (∇) river water, (■) dark experiment.

3. Bioassay methods to detect herbicide phytotoxicity

Weeds are a continuous problem in agriculture. The success of modern agricultural practices is due in part to the discovery and adoption of chemicals for weed control. The introduction of selective herbicides has greatly facilitated farmers' work by suppressing the need for manual weeding. Indeed, the tremendous increase in crop yields associated with the "green" revolution would not have been achieved without the contribution of these synthetic compounds. The abundance of high quality food in developed nations has eliminated concerns about access to food in these countries. However, concerns over the potential impact of pesticides on the environment have now arisen and as consequence, more pressing and more stringent pesticide registration procedures have been introduced.

The basis for much of the work done in weed control is the testing of the response to the herbicides. Bioassay methods have been developed to determine the residue level of many herbicides in soil and water (Fig. 8). There are different types of bioassays, depending on the species, the type of herbicide used, its mode of action, substrate and other environmental conditions, as well as the measured parameter. Their use offer several advantages such as the detection of very low phytotoxic residues and the detection of its bioavailability. Therefore, bioassays can be used to complement the analytical chemical methods and are useful tools to screen herbicide phytotoxicity and provide information about the phytotoxicity of herbicide residue in the soil at sowing time. The sensitivity, low cost and reproducibility of bioassays fulfill the criteria for a good technique.

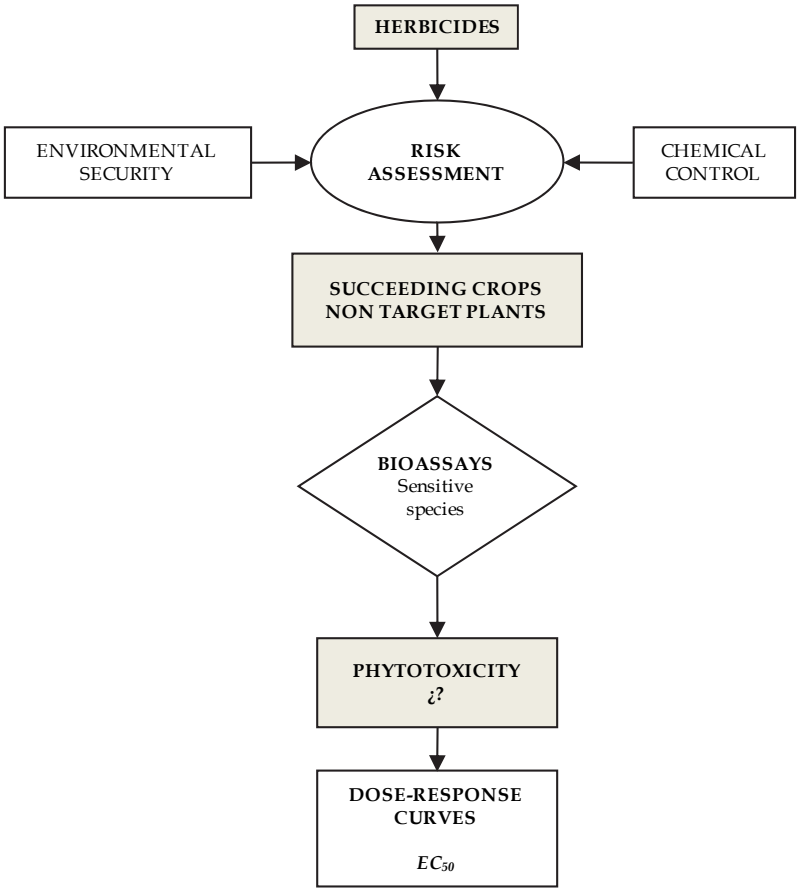


Fig. 8. Process of conducting bioassays in testing response of herbicides.

The classical bioassay, often used to quantify the amount of herbicide in soil, employs a single "standard" dose-response curve. This standard curve shows the plant response to different herbicide concentrations and report information of different concepts related to herbicide efficacy, such as selectivity, tolerance and resistance. These methods are of the utmost importance in studies of crop selectivity, herbicide resistance development and herbicide resistant weeds detection.

A typical dose-response curve is sigmoid in shape. One example of such a curve is the log-logistic curve (Seefeldt et al., 1994). The mathematical expression relating the response Y to de dose X is the following;

$$Y=C+\frac{D-C}{1+\exp\left\{b\cdot\left[\ln(X+1)-\ln(EC_{50}+1)\right]\right\}}$$

where C is the lower limit, D is the upper limit, b is the slope, and EC_{50} is the dose giving 50% response. The log-logistic is the most common model used in bioassays to describe

dose-response relations. Other relevant sigmoid curves might be the Gompertz (Ritz et al., 2006), are used sometimes, for instance, in cases where a log-logistic model did not fit well to the data. Also, the toxicity factor used in the risk assessment is usually the EC_{50} (concentration required to give 50% reduction of the plant growth with respect to the control).

The consequences of herbicide introduction into the environment could not be limited to active ingredient. It is very important to know whether any phytotoxic effects detected due to active substance applied or to some of its metabolites or degradation products. In this case, it is necessary to know the route and rate of degradation not only for the active substance but also for the products of degradation (Berenzen et al., 2005).

Besides, the herbicide formulations contain many other compounds called adjuvants. Adjuvants are defined as ''an ingredient in the pesticide prescription, which aids or modifies the action of the principal ingredient'' (Foy, 1993). These products are added to increase the effectiveness (bioavailability) of the formulation by enhancing the solubility, or the compatibility of the active ingredients. Other functions can be to improve adsorption, penetration and translocation of the active ingredients into the target, increase rain fastness, and alter selectivity of the active ingredient toward different plants (Foy, 1993; Foy & Witt, 1992; Li & Foy, 1999). Furthermore, many papers concerning effect studies of pesticides have compared effectiveness of different adjuvants (Foy, 1993). Only very few papers discuss the environmental toxicity and risk of adjuvants; Chow and co-workers have previously noted this lack of information on the effects of adjuvants on the environment (Chow & MacGregor, 1983). Especially, information on both simple toxicological properties of adjuvants and their influence in complex ecosystems is required. Actually, the ecotoxicological aspects of the use of surfactants in cleaning products mostly for household have been published (Junghans et al., 2006; Krogh et al., 2003).

The potential problems that residues of these products may present, as the effect on non-target species or in successive crops, do not provide information needed to relate these effects with the chemical nature of the residue. Therefore, it is also necessary to study the nature of this residue by conventional analytical methods to identify potential causes of environmental problems.

3.1 Herbicidal activity of cyclohexanedione oxime herbicides

The graminicides are a group of commercially important selective herbicides (Harwood, 1999). One of major chemical classes of post emergence herbicides belongs to the cyclohexanedione class. This herbicides´ group is effective against a wide range of annual and perennial grasses (monocotyledonous) in a large variety of broad-leaved crop plants (dicotyledonous). Their biochemical target site is the enzyme acetyl coenzyme-A carboxylase (ACCase) (Burton et al., 1991; Rendina et al., 1990; Secor & Cseke, 1988), which catalyzes the first step in fatty acid biosynthesis. They are also known as group A herbicides or group 1 herbicides (Park & Mallory-Smith, 2004; Price et al., 2003). Since their introduction in the late 1970s, the ACCase-inhibiting herbicides have been widely used worldwide to control a number of grass weed species (Devine & Shimabukuro, 1994). Ciclohexanodione herbicides are also a family used at low-dose rate as they are biologically active at very low concentration (0.2-0.5 kg a.i. ha^{-1}). It is currently being registered in

Europe, for weed control in broad-leaved crops, mainly in sugarbeet (*Beta vulgaris* L.), soya bean (*Glycine max* L. Merr.), and pea (*Pisum sativum* L.) crops, although its use is recommended in other broad-leaved crops such as rape (*Brassica napus* L.), potato (*Solanum tuberosum* L.) and beans (*Phaseolus vulgaris* L. and *Vicia faba* L.) (Sandín-España et al., 2003).

Cyclohexanedione oxime herbicides cause a rapid cessation of growth followed by destruction of shoot meristems in susceptible species. Studies on the uptake, translocation, and metabolic fate in tolerant and susceptible plants have shown that these herbicides inhibited *de novo* fatty acid biosynthesis in isolated chloroplasts, cell cultures, or leaves of susceptible grasses such as corn, wheat, and wild oats but not in tolerant broad-leaved plants such as soybean, spinach, and sugar beet (Burgstahler & Lichtenthaler, 1984). Injury symptoms tend to develop rather slowly in sensitive plants treated with cyclohexanedione herbicides. Growth (leaf elongation) stops within 24-48 h after herbicide application. Chlorosis is first observed on the youngest tissue, usually the emerging leaves. This reflects the fact that the initial phytotoxicity occurs primarily at the apical meristem, the major site of cell division and *de novo* fatty acid synthesis in these plants. In fact, 48-72 h after treatment the youngest emerged leaf can be quite easily separated from the rest of the plant by gently pulling it upwards; again, this reflects the tissue damage at the meristem. Chlorosis then spreads slowly through the rest of the plant, although it may take 7-10 days for the entire plant to be affected. Phloem translocation of these herbicides through the plant is limited, resulting in relatively small amounts reaching the roots. For this reason, these herbicides provide excellent control of perennial grass weeds. However, uncertain conditions some control of perennials can be achieved. No injury symptoms appear on dicotyledonous crops or weeds treated at typical use rates. Physiological injury can occur in cereal crops under certain conditions (e.g., low temperature at time of application), presumably due to reduced rates of herbicides detoxification. However, most plants recover from this temporary injury within 7-10 days (Walker et al., 1988).

The cyclohexanedione oxime class has been developed at low doses to reduce the adverse effects of the use of some herbicides and fulfill environmental requirements, water and soil pollution set by international legislation. However, due to high phytotoxicity, small amounts of residual herbicide in soil may affect sensitive succeeding crops. Also their polar character makes them easily leach to groundwater and potentially contaminate at levels above 0.1 μg L^{-1} (Sandín-España et al., 2003). In this context, studies about mobility, degradation and persistence in soil and water were performed with a variety of analytical techniques like gas and liquid chromatography, mass spectroscopy, photodegradation studies, studies with ^{14}C, immunoassays, etc. Most studies have been made in water and soil; occasionally there are some bioassays in microalgae (Santín-Montanya et al., 2007). The last results obtained confirm that could be susceptible specie capable to detect the presence of some herbicides (Table 1 & Fig. 9).

Herbicides	Log-logistic curve doses-response	R^2 (%)
Alloxydim	y= 0,19+((0,63-0,19)/(1+exp(1,74*(Ln(Dose+1)-Ln(285,22+1)))))	78,03
Sethoxydim	y=-0,0022+((0,66+0,0022)/(1+exp(0,62*(Ln(Dose+1)-Ln(110,33+1)))))	83,76
Metamitron	y=0,11+((1,08-0,11)/(1+exp(0,95*(Ln(Dose+1)-Ln(0,128+1)))))	98,14
Clopyralid	Not adjusted to regression equation	

Table 1. Regression equations by Seefeldt model that describe the relationships between increased doses of herbicides and growth of *Dunaliella primolecta* (unpublished data).

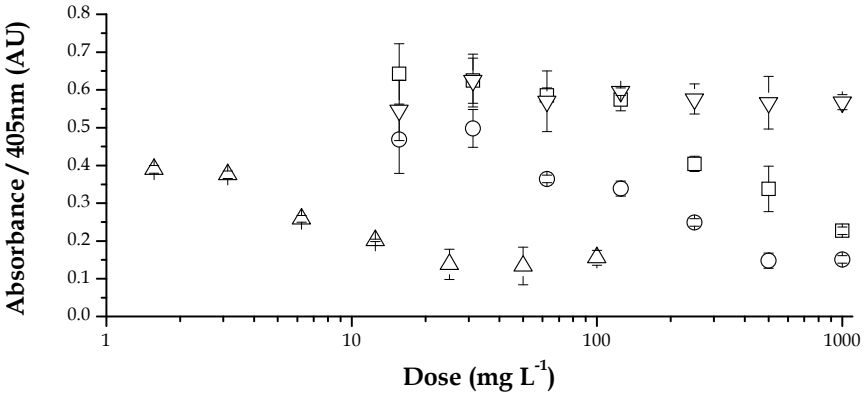


Fig. 9. Response of microalgae *Dunaliella primolecta* growth in presence of different concentrations of alloxydim (□), sethoxydim (○), metamitron (△) and clopyralid (▽) (unpublished data).

Other bioassays have been developed to detect phytotoxic residues of herbicide sethoxydim (Hsiao & Smith, 1983). In our group, initial attempts to obtain a practical hydroponic bioassay that allowed us to quantify tepraloxydim were frustrated due to the lack of repeatability and random results. Therefore, an investigation was carried out to determine the fate of tepraloxydim under bioassay conditions in order to clarify the reason for poor bioassay repeatability. The presence of residual chlorine in water was identified as the key factor on the repeatability of the bioassay.

Finally, an extensive research was conducted to develop and optimize a bioassay based on the high sensitivity of wheat (*Triticum aestivum* L.) to tepraloxydim in hydroponic culture using chlorine free mineral water (Sandín-España et al., 2003). Afterwards, similar studies were carried out with tralkoxydim (Table 2&3).

Dose (μg L^{-1})	Tepraloxydim		Tralkoxydim	
	Bioassay 1	Bioassay 2	Bioassay 1	Bioassay 2
0	11.9 a	12.1 a	9.5 a	11.9 a
2	10.2 b	10.7 b	8.2 b	11.9 a
4	6.7 c	5.8 c	7.7 b	9.3 b
8	3.5 d	3.2 d	3.9 c	3.9 c
16	1.5 e	1.6 e	1.4 d	1.2 d
32	0.7 e	0.7 e	0.8 d	0.6 d

Table 2. Mean values of root growth wheat treated with herbicides 7 days after treatment. Different letters after value, for each column, indicate differences at $p<0.05$.

	Tepraloxydim		Tralkoxydim	
	Bioassay 1	**Bioassay 2**	**Bioassay 1**	**Bioassay 2**
EC_{50} (μg L^{-1})	4,6	3,8	6,8	7,0
R^2 (%)	99,8	97,2	98,4	99,6

Table 3. EC_{50} parameter of by Seefeldt model to two ciclohexanodione oxime herbicides in hydroponic culture of wheat.

It has been demonstrated that water chlorination with disinfection purposes degrades completely any possible residue of herbicide clethodim (Sandín-España et al., 2005a). This degradation is very rapid, giving rise to different degradation products. In this sense, we have studied the phytotoxicity of alloxydim and its main metabolite with hydroponic bioassays on wheat (Sandín-España et al., 2005b).

A chlorinated degradation product (IX; Fig. 4) of alloxydim was the main product obtained in its degradation with chlorine, one of the most common disinfectant agents employed in water treatment. Results showed that after seven days of treatment the most sensitive biological parameter for alloxydim was root length, causing in the root growth of plants a 40% of significative reduction at the dose of 0.3 mg L^{-1} and 94% of reduction at the highest dose. However, the effect of metabolite on root growth only occurred at the highest metabolite dose (10 mg L^{-1}), causing a 32% of reduction in root growth. Root system control presented normal growth (main tap root plus secondary roots), while those from injured plants were increasingly deformed (main tap root twisted and lack of secondary roots). Root growth was increasingly affected with doses from 0.1 mg L^{-1} to the highest dose (Table 4).

The foregoing results suggested that the use of low dose herbicides can produce damage on succeeding crops, neighbouring crops and on non-target plants. Overall, there is no one species or endpoint that is consistently the most sensitive for all species or all chemicals in all soils, and differences in bioavailability among compounds may confound comparison of test results (Clark et al., 1993). Therefore, bioassays can provide additional information, with acceptable reproducibility (Ritz et al., 2006) on herbicide uptake and translocation (Horowitz, 1980; Reineke et al., 2002).

Dose (mg L^{-1})	**RG with Alloxydim (cm)**		**RG with Metabolite (cm)**	
0	15.20 a		12.60 a	
0.1	13.67 a		13.15 a	
0.2	11.65 a		13.07 a	
0.3	9.04 b	↓ **40** %	13.59 a	
0.4	4.93 c		13.30 a	
0.5	2.64 d		13.35 a	
0.7	1.58 e		12.94 a	
1	0.90 e	↓ **94** %	12.23 a	
5			12.60 a	
10			8.55 b	↓ **32** %

Table 4. Response of root growth (RG) of wheat plants to different doses of alloxydim and its metabolite 7 days after treatment (means values of root growth). Different letters after value, for each column, indicate differences at $p<0.05$.

3.2 Occurrence of weed resistance to cyclohexanedione oxime herbicides

The reliance on herbicides for weed control has resulted in shifts in the weed flora and, more importantly, in the selection of herbicide-resistant weed populations. This is particularly true for herbicides with a single target, such as herbicides inhibiting acetyl coenzyme-A carboxylase (Devine & Shimabukuro, 1994).

Since their introduction to world agriculture in the 1980s, cyclohexanedione oxime herbicides have been widely used to control a variety of grass weeds. As a consequence, they rapidly selected, and are still selecting, resistant plants within grass weed species.

The resistance to acetyl coenzyme-A carboxylase-inhibiting herbicides was reviewed in detail (Devine & Shimabukuro, 1994), the number of grass weed species in which resistant plants have been reported increased from 9 to 34 (Delye et al., 2005) and resistance to ACCase-inhibiting herbicides has been reported in 26 countries. The estimates of cultivated land surfaces concerned by this resistance vary between 3 to 4.6 million hectares (Delye et al., 2005).

One of the best-studied weeds is black-grass (*Alopecurus myosuroides* Huds.), a major grass weed in winter crops in Europe. Similar findings were obtained in annual ryegrass (*Lolium rigidum* Gaud.) (Delye et al., 2003a), green foxtail (*Setaria viridis* L. Beauv.), wild oat (*Avena fatua* L.) and winter wild oat (*Avena sterilis* ssp. *Ludoviciana* Malzew.) (Christoffers & Kandikonda, 2006; Delye et al., 2003b; Delye et al., 2005; Shukla et al., 2004; Zagnitko et al., 2001), slender foxtail (*Eleusine indica* L. Gaertn.), barnyardgrass (*Echinochloa colona* L. Link) and little canarygrass (*Phalaris minor* Retz.).

Species	Location	Chemical class*
Avena fatua	Canada, USA, Australia, UK	AOPP, CHD
Avena sterilis	Australia, UK	AOPP
Alopecurus myosuroides	UK, Spain, Germany, France	AOPP
Digitaria ischaemum	USA	AOPP
Digitaria sanguinalis	USA	AOPP
Echinochloa colona	Costa Rica	AOPP
Eleusine indica	Malaysa	AOPP, CHD
Festuca rubra	USA	CHD
Lolium rigidum	Australia, Spain	AOPP
Lolium multiflorum	UK, USA	AOPP
Phalaris minor	Israel	AOPP
Setria faberi	USA	AOPP, CHD
Setaria viridis	Canada	CHD
Sorghum halepense	USA	AOPP

Table 5. Weed species exhibiting resistance to ACCase-inhibiting herbicides. Resistance has been conferred to one or more biotypes or accessions of the above weed species by reduced ACCase sensitivity. * AOPP: Aryloxyphenoxypropionate, CHD: Cyclohexanedione.

The occurrence of resistance in grass weeds, *Setaria faberi* Herrm. and *Digitaria sanguinalis* L.) Scop., to cyclohexanedione herbicides has been confirmed also in the United States (Stoltenberg & Wiederholt, 1995; Wiederholt & Stoltenberg, 1995). Sethoxydim had been the

only ACCase-inhibiting herbicides applied to fields in which resistant plants were identified. In whole-plant dose–response experiments, resistant *S. faberi* was 134-fold resistant to sethoxydim (Stoltenberg & Wiederholt, 1995), but showed low levels of resistance to the cyclohexanedione herbicide clethodim. Similarly, Table 5 summarizes the weed species that are known to have developed resistance to ACCase-inhibiting herbicides around the world (Delye et al., 2005; Hatzios, 2001).

The development of resistance to ACCase-inhibiting herbicides in several grass weeds is an increasing problem in several parts of the world. Resistance to these herbicides can arise easily following selection pressure with cyclohexanedione herbicides for six to ten years. The judicious use of ACCase-inhibiting herbicides in combination with herbicides from other classes and methods of non-chemical weed control will be important for prolonging the usefulness of the cyclohexanedione herbicides.

4. Conclusion

The continuous use of plant protection products has led to the contamination of different environmental compartments, such as water, soil and air, being water contamination of great concern due to the risks for human consumption.

The synthetic research of herbicides in the last decades has shifted from long-life compounds to less persistent and more polar compounds, in order to avoid their accumulation in the environment. However, the low persistence of these compounds does not imply their completely mineralization but they are going to degrade to smaller molecules with different physicochemical properties than the active substances. In fact, it has been demonstrated that some of their transformation products are more mobile, persistent and/or more toxic than the parent molecule. Therefore, the knowledge of the fate of herbicides in the environment is underestimated if we do not take into account their transformation products whose behaviour and agro-environmental fate is in many cases unknown.

Besides, the lack of data on the phytotoxic effects of herbicide residues has been highlighted. In this sense, it is necessary to study and develop simple methods for evaluating the environmental impact of these products based on hard scientific data. Furthermore, it is also important not only study the phytotoxicity of the herbicide by means of bioassays, but also of their degradation by-products. From these studies should be able to derive recommendations for agricultural practices for the use of these products are environmentally friendly in general and in particular the agricultural environment capable of guaranteeing the future productivity of farms in the context of sustainable agriculture.

Cyclohexanedione oxime herbicides have been developed for the post-emergence control of grasses in dicotyledonous agricultural crops. These herbicides are unstable in aqueous solution and are very sensitive to pH and sunlight. These properties are highly relevant to their environmental fate. Degradation is so rapid that degradation products apparently could contribute to the activity of the parent molecule. Until now, some of these degradation products and the degradation pathways have been identified. However, the fate, significance and phytotoxicity of their degradation products is not fully known and future research is still need to attain a complete understanding of the fate of cyclohexanedione herbicides in the environment.

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6

1(Heterocyclyl),2,4,5-Tetrasubstituted Benzenes as Protoporphyrinogen-IX Oxidase Inhibiting Herbicides

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1. Introduction

It is well known that agrochemicals have played a important role in agricultural production that provided for about 700 million people during the past 50 years. At the same time, the increasing world population seems to be a major driving force for the need to enhance the output of food production. The agrochemical industry has been very successful in developing new herbicides and other agrochemicals. Herbicides are used widely in the world in protecting crops from undue competition from weeds (Price & Kelton, 2011).

The first commercial inhibitor of protoporphyrinogen oxidase (Protox) is the nitrofen that belongs to diphenyl ether (DPE), which was introduced in 1963 by Rohm & Hass (Now Dow AgroSciences) (Matsunaka, 1976). Some years later, the oxadiazon as the fisrt compound of the 1(heterocyclyl), 2, 4, 5-tetrasubstituted benzene (HTSB) family was introduced in 1968 by Rhone-Poulenc (Metivier et al., 1968). Nitrofen and oxadiazon represent the earliest examples of Protox inhibiting herbicides (Fig. 1). Although their chemical structures are completely different from each other, they share a common mode of action, inhibition of the protoporphyrinogen oxidase enzyme, though this was not known until the late 1980s.

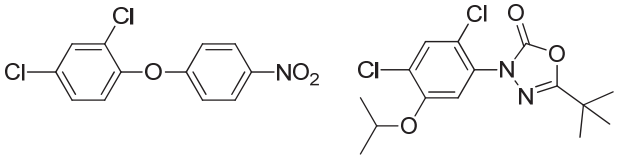


Fig. 1. Chemical structures of two early examples of Protox inhibitors.

Several early inventions of HTSB herbicide in 1960s had a significant impact on our understanding of the structure–activity of this kind herbicides. Rhone-Poulenc first introduced 3-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2(3H)-one in 1965 (Boesch et al., 1965). Further lead optimization at the phenyl ring soon led to the discovery in 1968 of the 2,4-dichloro-5-isopropoxyphenyl substitution pattern of the herbicide oxadiazon

(Boesch et al.,1968). Oxadiazon was the first compound of the cyclic imide family introduced into the markert for the control of annual grasses and broadleaf weeds in pre-emergence or early post-emergence treatment by Rhone-Poulenc in 1969. The second cyclic imide herbicide, chlorophthalim, was introduced by Matsui in 1972. The 2, 4-dihalo-5-substituted pattern at the aromatic ring would become the basis for much of the 2,4,5-trisubstituted phenyl tetrahydrophthalimide research that followed in this area of chemistry.

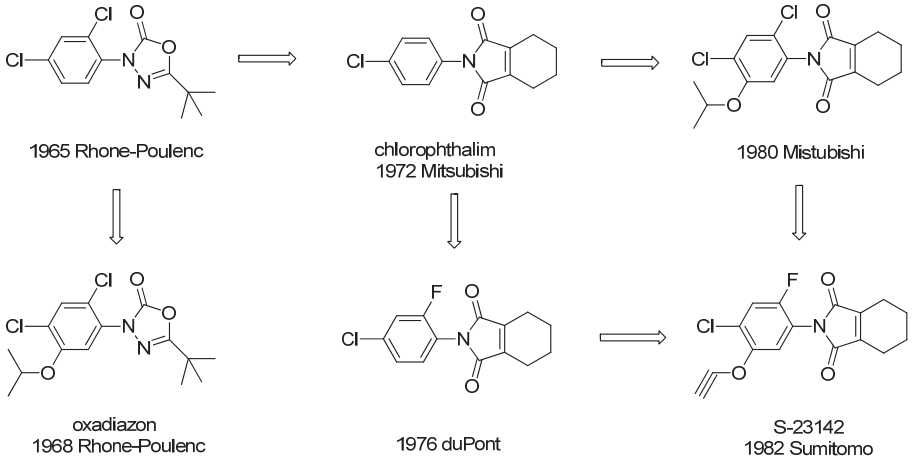


Fig. 2. Incorporation of the 2,4-dihalo-5-alkoxy aromatic pattern of oxadiazon into new phenyl tetrahydrophthalimide ring systems.

A breakthrough discovery was the increasing biological activity caused by the replacement of chlorine by fluorine at the 2-phenyl position. In 1976, DuPont introduced the first example of a 2-fluoro-4-chlorophenyl tetrahydrophthalimide Protox inhibitor (Goddard, 1976) (Fig. 2). The dramatic increase in biological activity caused by the fluorine in the 2 position of the phenyl ring would, in the next decade, the 1980s, influence the lead optimization work in the HTSB area, such as the discovery of the 4-chloro-2-fluorophenyltetrahydrophthalimide herbicide S-23142 (Nagano et al., 1982). Since then, various HTSB derivatives with high herbicidal activity (10-50 g/ha) have been discovered by many companies.

2. Commercialized protoporphyrinogen-IX oxidase inhibiting herbicides

A number of Protox inhibiting compounds have been already commerciallized. Their structures and biological activities are introduced as follows.

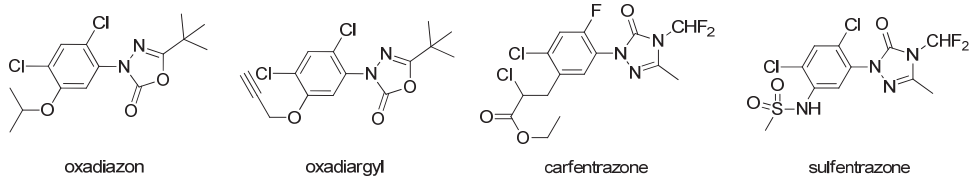


Fig. 3. Chemical structure of oxadiazon, oxadiargyl, carfentrazone, sulfentrazone.

Both oxadiazon and oxdiargyl are developed by Rhone-Poulenc (Fig. 3). Oxadiargyl is a pre-emergence and early post-emergence herbicide in sugarcane and sunflower fields or orchards to control both annual broadleaf and grass weeds. It application rate is rather high at 500-2000 g a. i. /ha. It is also developed as an herbicide for rice and turf.(Oe et al., 1995). FMC developed carfentrazone-ethyl and sulfentrazone. Carfentrazone-ethyl is a post-emergence herbicide in rice, cereals and maize fields. It shows excellent activity against annual broadleaf weeds such as *Gallium*, *Lamium*, and *Veronica* in wheat at 20-35 g a. i./ha. It also controls *Euphorbia*, *Polygogum*, *Abutilon*, *Ipomea*, *Kochia*, *Salsola*, etc. in foliar application (Vansaun et al., 1993; Mize, 1995). Sulfentrazone is a pre-emergence herbicide in soybean and sugarcane fields. It controls *Ipomea*, *Amaranthus*, *Chenopodium*, *Abutilon*, *Polygonum*, *Datura*, etc. at 350-420 g a. i. /ha. (Oliver et al., 1996; Vidrine et al., 1996)

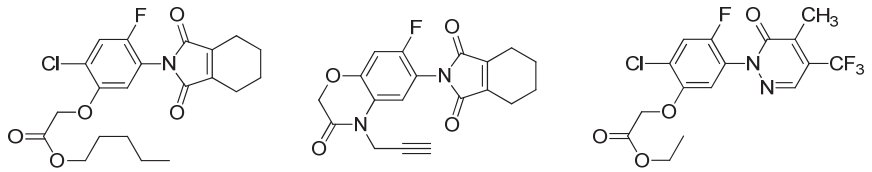


Fig. 4. Chemical structure of flumiclorac pentyl, flumionazin, flufenpr-ethyl.

The above three herbicides in figure 4 are all developed by Sumitomo. Flumiclorac pentyl was commercialized as the first cyclic imide herbicide in Europe as early as 1993. It controls annual broadleaf weeds such as *Abutilon*, *Euphorbia*, *Chenopodium*, *Datura*, *Ambrosia* and *Xanthium* at 30-60 g a. i. /ha in post- emergence treatment in soybean and maize fields, especilly, it shows excellent activity against *Abutilon at progressed leaf stage at 60* g a. i. /ha (Kurtz & Pawlak, 1992, 1993; Satio et al., 1993). Flumioxazin controls annual broadleaf weeds such as *Abutilon*, *Euphorbia*, *Chenopodium*, *Ipomea* and *Sida* at 50-100 g a. i. /ha in pre-emergence treatment in soybean and peanut fields. But it is less active against annual grass weeds at the same dosage rate.(Yoshida et al., 1991). Flufenpyr-ethyl is commercillized recently as an herbicide in soybean, cotton, corn and sugarcane. It is supposed that it is more selective.

Pentoxazone was discovered by Sagami Chemical Research Center and Kaken Pharmaceutical as a pre-emergence and early post-emergence herbicide in rice (Fig. 5.). It shows excellent activity against both annual and perennial weeds such as *Echinochloa*, *Eleochairs*, *Sagittaria* and *Cyperus* at 145-150 g a. i. /ha (Yoshimura et al., 1992; Hirai et al., 1995). Pyraflufen-ethyl was developed by Nichino, It shows excellent selectivity for winter cereals at the extremely low rates of 6 to 12 g /ha, and also long-term residual activity brought out by chemical and biological stabilities , as a post-emergent contact herbicide active on broadleaf weeds such as cleavers, henbit, clickweeds and wild chamomile; especially provided effective control of 5- to 6-leaf stage of cleavers at the low rate. Azafenidin is developed by Du Pont as a non-selective pre- and post-emergence herbicide for non-cropland and orchard. It has wide herbicidal spectra and controls both annual and perennial weeds at 560 g a. i. /ha (Netzer et al., 1996). Saflufenacil was developed by BASF as an herbicide which is used alone or in mixtures with glyphosate for burn down weed

control, with foliar and residual activity against more than 70 broadleaf weeds, introduced in Nicaragua, Chile, and Argentina as Heat in 2009.

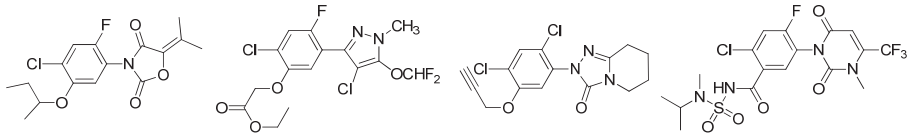


Fig. 5. Chemical structure of pentoxazone, pyraflufen-ethyl, azafenidin, saflufenacil.

3. Mode of action of 1(heterocyclyl),2,4,5-tetrasubstituted benzenes protoporphydrinogen-IX oxidase inhibiting herbicides

The mode of action of 1(heterocyclyl),2,4,5-tetrasubstituted benzenes (HTSB) protoporphyrinogen oxidase (Protox) inhibiting herbicides has been extensively reviewed (Duke et al., 1990). HTSB herbicides inhibit the enzyme Protox in the chlorophyll biosynthesis pathwy (Matringe & Scalla, 1988; Witkowski & Halling, 1988; Lydon & Duke, 1988). The Protox enzyme catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX by molecular oxygen. Inhibiting the Protox enzyme, which is located in the chloroplate envelop, results in an accumulation of the enzyme product protoporphyrinogen IX, but not the substrate, via a complex process that has not been entirely elucidated. Enzymatic oxidation of protoporphyrin IX in the cytoplasm yeilds a significant accumulation of protoporphyrin IX from the location of the chlorophll biosynthesis, sequence in choroplasts. In the presence of light, protoporphyrin IX generates large amounts of singlet oxygen ($^1O_2$), which results in the peroxidation of the unsaturated bonds of fatty acids found in cell membranes (Fig. 6). The end result of this peroxidation process is the loss of membrane integrity and leakage, pigment breakdown, and necrosis of the leaf that results in the death of the plant. This is a relatively fast process, with leaf symptoms such as a flaccid wet appearance observed within hours of plant exposure to the Protox herbicides under sunlight.

4. Structure-activity relationships of 1(heterocyclyl),2,4,5-tetrasubstituted benzenes protoporphydrinogen-IX oxidase inhibiting herbicides

4.1 Overviw of structure-activity relationships of 1(heterocyclyl),2,4,5-tetrasubstituted benzenes protoporphydrinogen-IX oxidase inhibiting herbicides

It is very important to analyze structure-activity data accumulated during past trials when formulating ratinonal structure-activity relationships (SARs). The relationships could be utilized as possible guiding principles for further structure transformation leading to novel peroxided herbicides. The information about (sub)molecular mechanisms of biological action may be extracted from the relationship. The structure-activity of Protox herbicides has been extensively reviewed (Fujita & Nakayama, 1999). Figure 6 shows the SARs of 2-fluoro-4-chloro-5-substituted-phenyl heterocycles (Theodoridis, 1997). SAR studies of 2,4,5-trisubstituted-phenyl heterocycles have shown that position 2 of the phenyl ring required a halogen group for optimum biological activity, with fluorine generating the highest overall activity. Introducing a substituent in position 3 of phenyl ring resulted in dramatic decrease

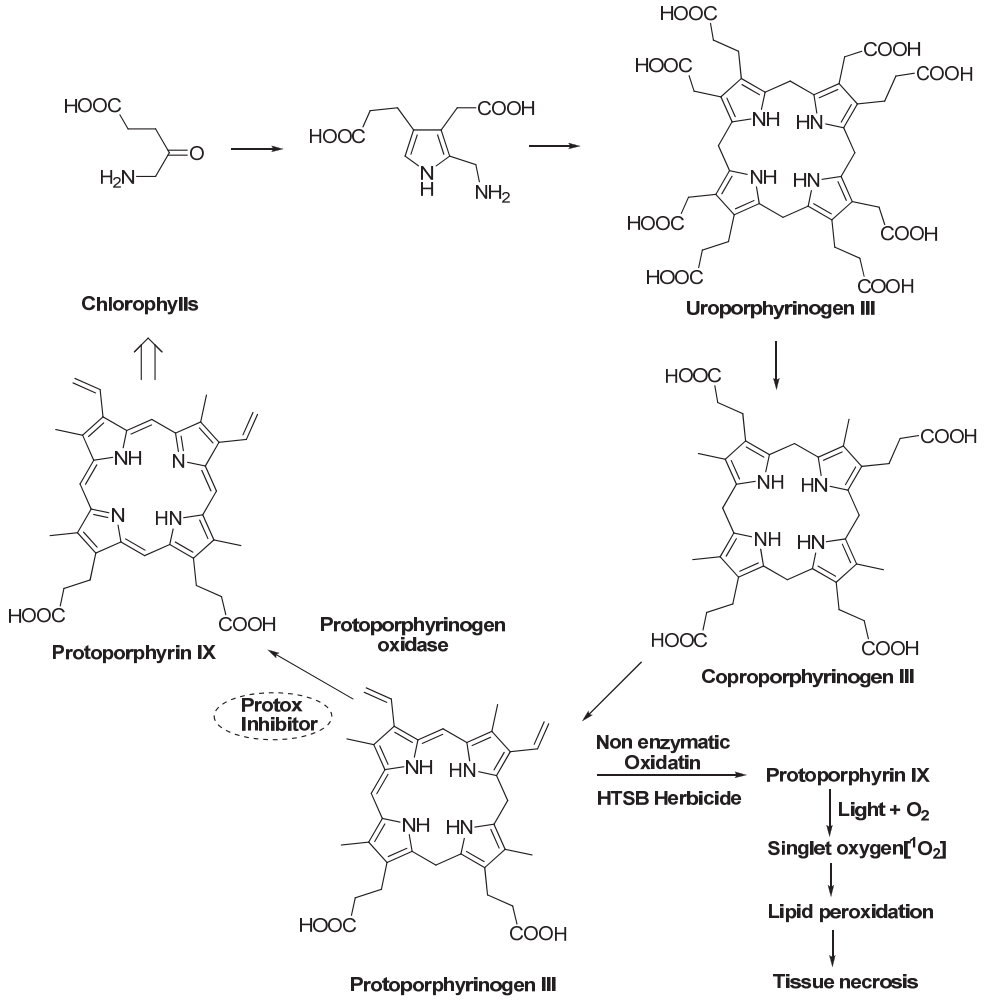


Fig. 6. Chlorophyll biosynthetic pathway.

of herbicidal activity. Position 4 of the phenyl ring required a hydrophobic, electronegative group such as halogen for optimum activity, with chlorine resulting in the best activity. Electron-donating groups such as methoxy resulted in significant loss of biological activity. As shown in Figure 7, the substituent R have a great effect on the bioactivity. Considering herbicidal activity and limited crop selectivity, OCH_2CCH is more favorable than other sunstituents. Considering weed spectrum and multicrop selectivity, R is $NHSO_2Et$ generating the highest overall activity. Many heterocyclic systems, usually attached to aromatic rings via a nitrogen atom, have been introduced in the past fifteen years. Oxadiazolinone (Metivier et al., 1968), oxazolidinedione (Hirai et al., 1989), tetrahydrophthalimide (Matsui et al., 1972), tetrazolinone (Theodoridis et al., 1990), triazolinone (Theodoridis, 1989), pyrimidinedione ring (Wenger, et al., 1988) showed relative higher herbicidal activity than other heterocyclic systems.

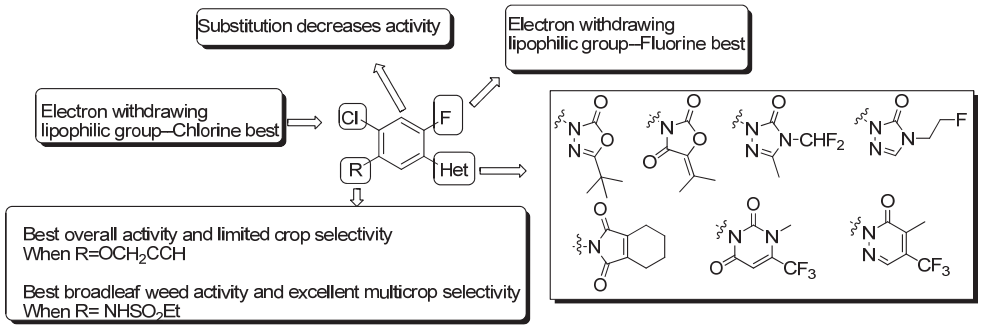


Fig. 7. Structure-activity relationships of the two aromatic rings of 2,4,5-trisubstituted-phenyl heterocyclic systems.

Linking the 4 and 5 positions of phenyl ring to give a new benzoheterocyclic ring, such as benzoxazinone, quinolin-2-one, benzimidazole, resulted in two new classed of Protox herbicides, both increased biological efficacy and new SARs (Fig. 8) (Lyga et al., 1999; , Grawford et al., 1997). As privious studies, position 2 of the phenyl ring required a halogen group for optimum biological activity, with fluorine generating the highest overall activity. The substituent R has a great effect on the herbicidal activity. Introducing propargyl resulted in dramatic increase in the bioactivity.

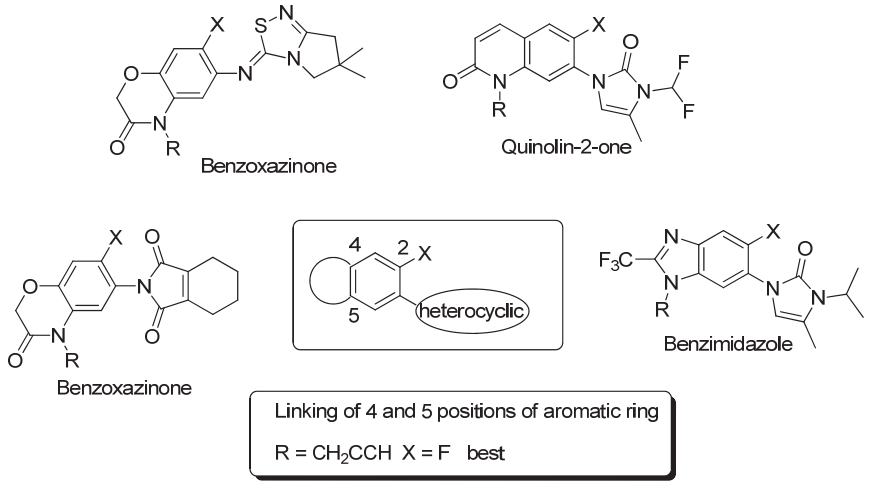


Fig. 8. Structure-activity relationships of benzoheterocycles resulting from linking aromatic positions 4 and 5 of phenyl heterocyclic systerms.

The second class of benzoheteroaryl Protox herbicides are obtained when aromatic positon 5 and 6 are linked togther to form various benzoherocyclic rings, which can be attached to a wide range of heterocycles (Fig. 9). The 6-trifluoromethyl group (R_1=CF_3) in the uracil ring is essential for bioactivity, replacing it with methyl results in complete loss of activity (Thecodoridis et al., 2000 and 1994). Increaseing the size and length of R_2 group resulted in a significant reduction in bioactivity. Substituents X had a dramatic effect on the weed

spectrum and crop selectivity. Compounds with fluoroine and hydrogen resulted in broad-spectrum control of weeds and high herbicidal activity (Lyga et al., 1999).

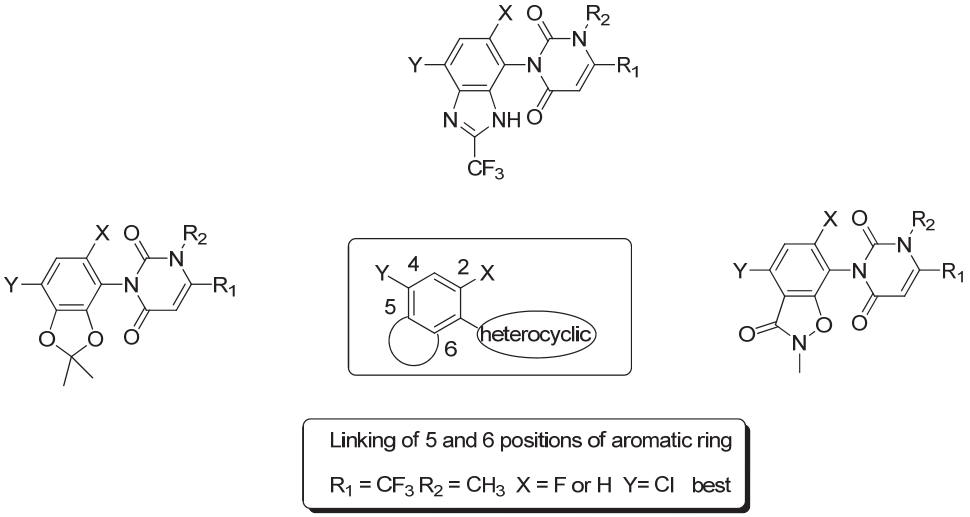


Fig. 9. Structure-activity relationships of benzoheterocycles resulting from linking aromatic positions 5 and 6 of phenyl heterocyclic systerms.

4.2 Pharmacophore analysis

Many molecular modeling studies of ligand binding require some knowledge of the pharmacophore as a starting point. The pharmacophore mode could show the significant structural similarities and identifies the active conformation. The model itself identifies the list of feature classes required and the distances between them. Eight HTSB compounds in table 1 was selected as the testing compounds for the pharmacophore study. A DISCO model of the pharmacophore was developed based on information from X-ray crystal structures of compound I-1 and Sybyl using the Tripos force field. Key pharmacophore elements are a polarizable functionality separated by a fixed distance from two H-bond accepting elements. The compound I-1 was choosed as a reference compound. The crystal data was listed in table 2 and the structure was shown in figure 10. The 3D structures of all the compounds in table 1 were built by SYBYL 6.9/ Sketch, and then optimized using MMFF94 force field, by powell method with energy termination of 0.005 kcal/mol, and a maximum of 1000 iterations. Then, the Gasteiger–Hückel charges were added. Compounds I-1 was selected as the training set and the test set.

As shown in figure 11, the pharmacophore model contains two hydrophobic centers and two acceptor atoms. One hydrophobic center was closed to the 1-heterocyclic ring, which may be interacted with phenylalanine 392 in Protox. Another hydrophobic center, which was in the center of phenyl group, may be interacted with leucine 356 and 372 in Protox. The acceptor atom was oxygen atom located in the carbonyl group and the 5-positon of phenyl group, respectively. The acceptor atom may have interaction with hydrogen atom in target enzyme.

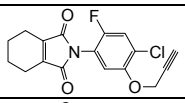
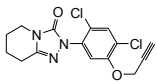
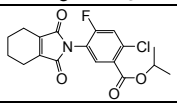
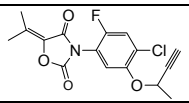
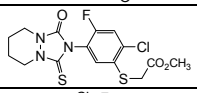
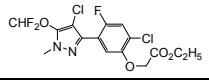
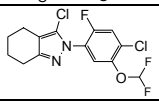
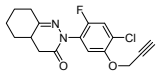
Compound	Structure	PPO pI_{50}	Compound	Structure	PPO pI_{50}
I-1		8.97	I-5		8.49
I-2		8.86	I-6		8.57
I-3		8.92	I-7		8.49
I-4		8.55	I-8		8.96

Table 1. The HTSB compounds for pharmacophore study.

Formula	C17 H13 Cl F N O3
Formula weight	333.73
Color/shape	colorless/prism
Crystal system	Triclinic
Space group	P-1
Unit cell dimensions	a = 9.313(5) Å alpha = 99.663(9) deg. b = 9.380(5) Å beta = 104.263(9) deg. c = 10.485(6) Å gamma = 112.778(8)
Volume	deg.
Z	782.1(8) Å3
Calculated density	2
Absorption coefficient	1.417 g.cm-3
F(000)	0.269 mm-1
Crystal size/mm	344
Temp. /K	0.36 x 0.24 x 0.22
θ ranges/°	293(2)
Limiting indices	2.46 to 25.00 deg.
Reflections collected / unique	-11<=h<=9, -9<=k<=11, -10<=l<=12
Completeness to theta = 25.00	4071 / 2747 [R(int) = 0.0174]
Absorption correction	99.4 %
Max. and min. transmission	Semi-empirical
Refinement method	1.000000 and 0.689808
Data / restraints / parameters	Full-matrix least-squares on F2
Goodness-of-fit on F2	2747 / 1 / 208
Final R indices [I>2sigma(I)]	1.022
R indices (all data)	R1 = 0.0428, wR2 = 0.1095
Largest diff. peak and hole	R1 = 0.0632, wR2 = 0.1228 0.440 and -0.291 e.A-3

Table 2. Crystal data and structure refinement of compound I-1.

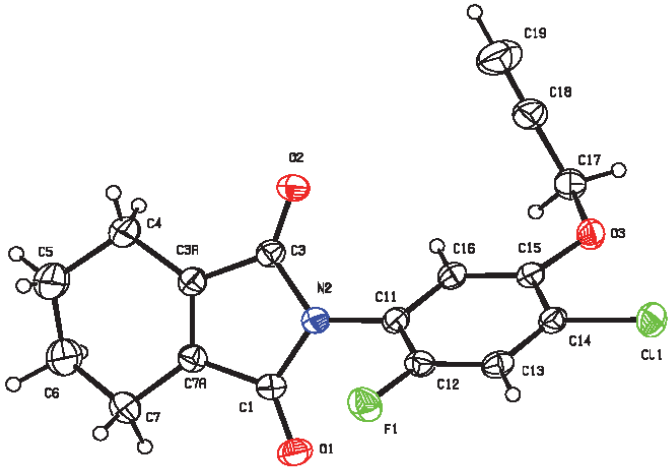
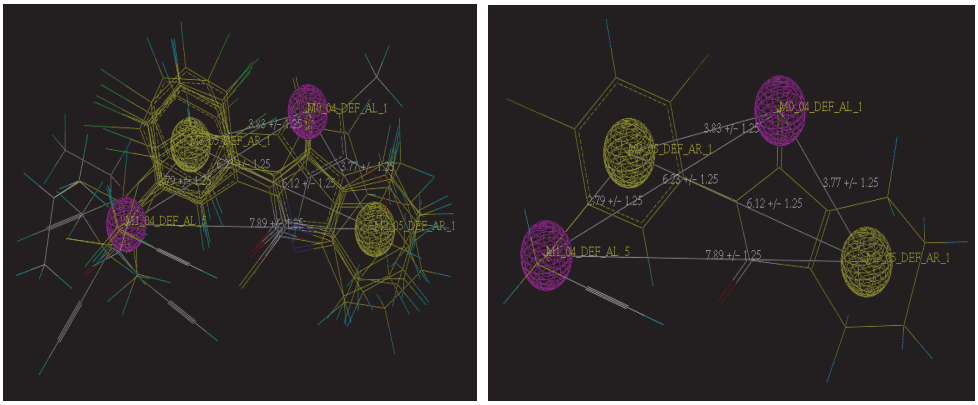


Fig. 10. Molecular structure of crystal compound I-1.



A B

Fig. 11. Pharmacophore A: overlap of all eight compounds; B: model with refererce compound I-1.

4.3 CoMFA analysis

QSAR (quantitative structure-activity relationships) have been reported about the effects on the substituents in phenyl group (Fujita & Nakayama, 1999; Jiang et al., 2010), but little literature about comparative molecular field analysis (CoMFA) on the effect of heterocyclic rings (Zhang et al., 2011). In order to understand the heterocyclic rings and substituents in

phenyl group effects on the PPO inhibition of a series of cyclic imide compounds, the method of CoMFA was applied to understand the quantitative structure–activity relationships. Using the information help us to increase the efficiency of syntheses of new candidate compounds.

37 HTSB compounds in table 3 was selected for the CoMFA study (Fujita & Nakayama, 1999). The 3D structures of all the compounds were built by SYBYL 6.9/ Sketch, and then optimized using MMFF94 force field, by Powell method with energy termination of 0.005 kcal/mol, and a maximum of 1000 iterations. Pharmacophore-based molecule alignment method was applied to superimpose all the compounds by using GALAHAD in SYBYL 6.9. The steric and electrostatic field energies for CoMFA were calculated using the SYBYL default parameters: 2.0 grid points spacing, a sp^3 carbon probe atom with +1 charge and a van der Waals radius of 1.52, and column filtering of 2.0 kcal/mol. The CoMFA descriptors were used as independent variables, and pI_{50} values were used as dependent variables in partial leastsquares (PLS) regression analyses to derive 3D-QSAR models. Leave-one-out (LOO) cross-validated PLS analyses were performed to determine the optimal number of components to be used in the final QSAR models and to check the predictive ability of the models. To visualize the 3D-QSAR results in term of field contributions, isocontour maps were generated using the field type 'stdev * coeff' and the contour levels were set to default values. In CoMFA, compounds II-1 was selected as the training set and the test set.

The alignment of HTSB compounds was shown in figure 12. The molecular modeling studies found good overlap between the 37 HTSB compounds. As listed in Table 3, a predictive CoMFA model was established with the conventional correlation coefficient R^2 = 0.908, the standard error s = 0.319, and F-test value F = 49.5. The contribution of steric and electrostatic fields are 49.5% and 50.5%, respectively. The observed and calculated activity values for all the compounds are given in Table 3, and the plots of the caculated versus the actual activity values for all the compounds are shown in Figure 13.

In Figure 14, the isocontour diagrams of the steric and electrostatic field contributions (''stdev*coeff'') obtained from the CoMFA analysis are illustrated together with exemplary ligands. The steric field contour map is plotted in Figure 14A. The green region highlights positions where a bulky group would be favorable for higher PPO inhibition activity. In contrast, yellow indicates positions where a decrease in the bulk of the desired compounds is favored. As shown in Figure 14A, the CoMFA steric contour plots indicated that a big yellow region is located around the group of phenyl in 4, 5-position, while a big green region surrounded the heterocycle group. This map means that the substituents of phenyl in 4, 5-position should be bulky. This steric map explained clearly why compound II-20 and II-21 displayed lower activity than other compounds. The electrostatic contour plot is shown in Figure 14B. The blue contour defines a region where an increase in the positive charge will result in an increase in the activity, whereas the red contour defines a region of space where increasing electron density is favorable. As shown in Figure 14B, the electrostatic contour plot showed that a blue region is around the Z group in the position of 5 of phenyl ring, whereas a red region is around the carbonyl group. The electrostatic contour plot indicated the target compounds bearing an electron-withdrawing group at the position of 5 of phenyl will display higher activity. This contour map indicated that the more electronegative of the oxygen atom of the carbonyl, the higher the activity of inhibitors. This means the carbon atom of one of the carbonyl group played an important role in

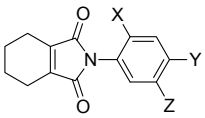
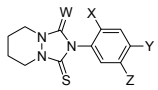
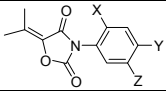
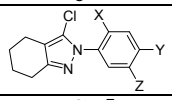
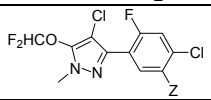
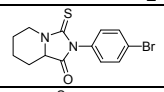
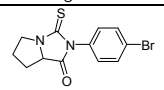
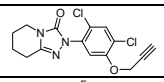
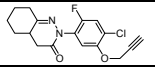
Com-pound	Structure	X, Y, Z	PPO-pI_{50} Obs.	PPO-pI_{50} Cal.	Deviation
II-1		2-F, 4-Cl, 5-OCH(Me)CCH	9	8.69	0.31
II-2		2-F, 4-Cl, 5-OCH_2CCH	8.97	9.04	-0.07
II-3		4-Cl, 5-COO-i-Pr	8.9	8.69	0.21
II-4		2-F, 4-Cl, 5-COO-i-Pr	8.86	8.82	0.04
II-5		2-F, 4-Br	8.6	8.51	0.09
II-6		2-F, 4-Cl, 5-OMe	8.52	8.76	-0.24
II-7		2-F, 4-Cl	8.43	8.54	-0.11
II-8		4-Cl, 5-COOEt	8.43	8.17	0.13
II-9		4-Cl, 5-$COOCH_2COOMe$	8.3	8.36	-0.06
II-10		4-Cl, 5-COOMe	8.05	8.12	-0.07
II-11		2-F, 4-Cl, 5-$OCHF_2$	7.96	7.95	0.01
II-12		4-Cl, 5-COO-t-Bu	7.85	8.03	-0.18
II-13		4-Br	7.67	6.80	0.87
II-14		4-Cl	7.6	6.83	0.77
II-15		4-OMe	7	7.11	-0.11
II-16		4-CF_3	6.55	6.57	-0.02
II-17		4-NO_2	6.4	6.62	-0.22
II-18		4-F	6.37	6.97	-0.60
II-19		4-Me	6.08	6.05	0.03
II-20		H	5.8	6.55	-0.75
II-21		3-Cl	5.6	5.76	-0.16
II-22		W=S; 2-F, 4-Cl, 5-OCH2C*CH	9.05	9.09	-0.04
II-23		W=S; 2-F, 4-Cl, 5-O-i-Pr	8.92	8.88	0.04
II-24		W=S; 4-Cl	8.17	8.32	-0.15
II-25		W=S; 2-F, 4-Cl	8.14	8.12	0.02
II-25		W=S; 4-Br	8.1	8.12	-0.02
II-27		W=O; 2-F, 4-Cl, SCH_2COOMe	8.08	8.08	0.0041
II-28		2-F, 4-Cl, 5-OCH(Me)C*CH	8.57	9.02	-0.45
II-29		4-Cl	7.66	7.32	0.34
II-30		2-F, 4-Cl, 5-OCH_2C*CH	9.14	9.24	-0.10
II-31		2-F, 4-Cl, 5-$OCHF_2$	8.55	8.46	0.09
II-32		OCH_2COOEt	8.49	8.40	0.09
II-33		H	8.49	8.51	-0.02
II-34			8.29	8.17	0.12
II-35			7.77	7.81	-0.04
II-36			8.49	8.42	0.07
II-37			8.96	8.79	0.17

Table 3. CoMFA study on the HTSB compounds.

determining the activity of PPO inhibitors. The stronger the ability of the carbonyl group to accept electrons from receptor, the higher the activity of PPO inhibitor.

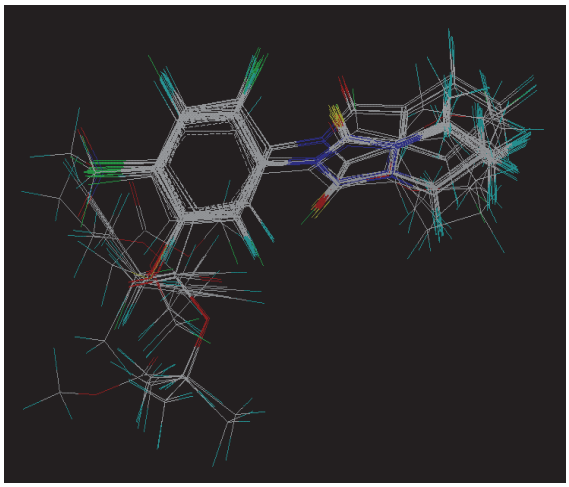


Fig. 12. Alignment of 37 HTSB compounds.

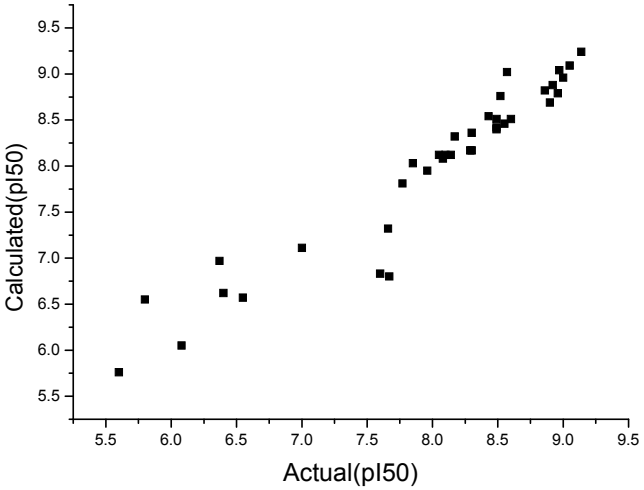
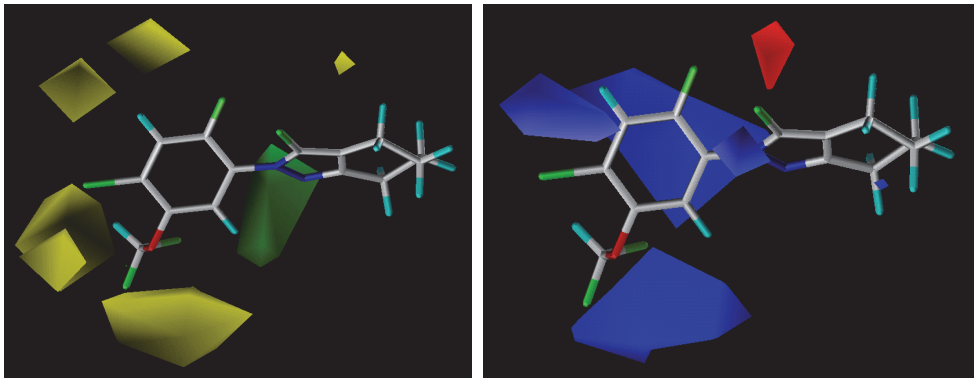


Fig. 13. Calculated pI_{50} (Y-axis) are versus actual pI_{50} (X-axis) values. The dots represent training compounds.



A B

Fig. 14. CoMFA contour maps with compound II-1 as the reference structure. (A) Steric contours. Scattered green areas are regions where bulky substituents are favorable, yellow areas are unfavorable. (B) Electrostatic contours. The red areas are the regions where negative potential is favorable for the activity, blue areas are unfavorable.

5. Conclusions

HTSB herbicides are characterised with their high herbicidal activities, fast acting, and environmentally benign. However, most of them cause short-term damage to the crops applied, which makes them no significant market share in the last 30 years (Qasem, 2011).

In recent years, the development of Protox inhibitor-resistant crops (Li and Nicholl, 2005; Vencill, 2011) began a new era for the use of Protox herbicides. Furthermore, weed shifts observed in genetically modified crops, caused by the development of weed resistance to the widely used glyphosate herbicide, will offer market opportunities for herbicides with other modes of action, such as Protox inhibiting herbicides. So, Protox inhibiting herbicides will continue to be an important area of interest to agrochemical companies, with most efforts focused on fine tuning the 5 position of the phenyl ring. The application of CoMFA approach will seed up the discovery processes.

6. Acknowlegments

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7

Persistence of Herbicide Sulfentrazone in Soil Cultivated with Sugarcane and Soy and Effect on Crop Rotation

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1. Introduction

In the deployment of an agricultural area through a cultivation system, there are serious and significant changes in geomorphic subsystems, edaphic and biological, making them simpler (agroecosystem), compared with the ecosystem, this, a more complex system. This change results in drastic reduction of the self-regulatory system, making it more unstable and susceptible to energy inputs. One major consequence of this transformation is the excessive increase of some species of insects, microorganisms and nematodes populations, and wild plants in such a way as to significantly impair the production, making uneconomical the productive units, so, they are named as agricultural pests.

When the wild plants interfere with cultivated plants, specifically, they become weeds, which unlike others agricultural pests, they are by nature always present in agroecosystems, they are difficult to control and are directly (competition, allelopathy, etc.) or indirectly (reservoir of pathogens, insects and nematodes) responsible for the drastic decrease in economic production of crops.

For a long time, agricultural researches have demonstrated that the control of weeds in various agroecosystems is a key factor for the success of crop production. All the technological development in crop science, nutrition, or breeding, can be compromised if the weeds were not controlled. The weed control is done by combining several methods, such as preventive, cultural, and chemical weeding, this, by the use of herbicides.

Herbicides are chemicals used to eliminate plants. They are applied in suitable doses directly on the vegetation for foliar absorption (post-emergence treatment), or on the soil for absorption by the plant tissues formed after the seed germination, before the plant emergence from the soil surface (pre-emergence treatment). They are generally used to control of weeds in different agro-ecosystems, or in any other favorable ecological niches of these organisms: wasteland, margins of highways, railroad beds, parking lots, and aquatic environments.

To selection of which herbicide will by used to weed control, you should always have an ecological focus using this agronomic technique aiming the maximum production. This

duality, choice, besides the type, dose, number and mode of application, should always seek the dichotomy of maximum efficiency and minimum environmental impact, thus maximizing the benefits of their use and minimizing their environmental and toxicological risks

Even so, the use of these chemicals is not without risks, with possible presence of residues in agroecosystems that can cause toxicity to susceptible plants used in rotation with the crops originally treated with the herbicides.

Brazil is a country where agricultural production is has world importance, notably in the growing of sugarcane and soybean, which since the end of the last century, with the implementation of the program using ethanol to replace gasoline, and the cultivation of soybeans in the Brazilian Cerrado (savanna), their productions and productivities have increased significantly each year.

These two crops are cultivated in extensive areas, because of their ease of use and performance; and the use of herbicides in these crops is intensive and in many cases, especially in sugarcane, in that most herbicides have long residual power, persisting in soil for a very long time. Thus, these two crops, sugar cane and soybeans, must be those with the greatest potential risk of occurrence of problems related to the persistence of herbicide molecules in the soil for a longer period than it is desirable, with the possibility of causing environmental contamination and phytotoxicity to sensitive crops used in rotation, especially with soybean that has a shorter cycle.

These themes are linked with the herbicide ecotoxicology, and only in 1960 began the interest in studies on the ecological effects of chemicals, when then the society begins to worry about their effects on environmental contamination due to, primarily, the world press reports on the effects of insecticides for agricultural use on the wildlife. A classic example occurred in the 60's in Mississippi and Atchafalaya rivers, United States, resulting in the deaths of ten millions fishes from water polluted with the insecticide endrin (Madhun & Freed, 1990). In 1962, there was a great repercussion around the world, with the release of the book "Silent Spring", written by Rachel Carson (1964), which projected an obscure future for planet Earth, if the man did not stop using pesticides in an indiscriminate way.

Soon after this season, in 1975, it was started the development of Ecotoxicology, a branch of science created by Rene Thruhaut in Paris (Astolfi et al, 1984), studying the mechanisms of environmental contamination by natural and artificial chemicals (xenobiotics) as well as the action such substances and their effects on living beings that inhabit the biosphere. Ecotoxicology is a natural extension of Toxicology.

One test that has contributed to understanding the behavior of pesticides are the ecotoxicological field tests to verify and monitor the persistence, accumulation, degradation and leaching of these products in soil.

2. Behaviour of herbicides in soils, especially sulfentrazone

The agricultural soil is the final destination of a large number of herbicides, either when they are applied directly to the soil or on the shoots of plants (Walker, 1987). When the herbicides, reach the ground, interacting with the environment, their fate is governed by three general types of processes: physical (sorption-desorption, volatilization, leaching by

water erosion and transportation along the ground by wind and water); chemicals (photodecomposition, sorption, chemical reactions with the soil constituents) and biological (represented by the microbial decomposition of the molecule and removal of soil by plants), Sheets, 1970, Blanco, 1979.

All these processes are described in the opinion of Briggs (1969, 1976, 1981), Blanco (1979), Walker (1983), Walker & Allen (1984) and Velini (1992), dependent on the chemical and physical soil nature and climatic conditions, particularly temperature and soil moisture, and soil characteristics (texture, structure, content and nature of colloid, pH, temperature, humidity, and others). The chemical nature of each herbicide, in turn, is a function of its molecular structure, molecule ionization, water solubility, lipid solubility, polarity and volatilization of the molecule. On the other hand, several external factors may play an important role in herbicide-soil interactions, such as dose and application mode, the herbicide formulation and soil microbial community

It is understood by adsorption to the accession of a molecule, ion or particle of the surface in any other particle, resulting from the interaction of a force field emanating from the adsorbent surface (clay and organic matter) and the surface of the adsorbate (in this case a herbicide). Herbicide Particles can also be absorbed by soil colloids. In 1994, Harper pondered the difficulty of distinguishing between the phenomena of absorption and adsorption, suggesting the use of the term sorption, which refers both cases.

Briggs (1969, 1976) reported that the extent and intensity of the processes involved in the phenomenon of sorption / desorption depend largely on molecular properties (physical and chemical) of the herbicide and the temperature, humidity, and soil pH and colloids. Herbicides can be molecular, weak acids or bases with their ionization depending on the soil pH, when the herbicide is the value of its ionization constant (pK), near the soil pH, the predominant form (molecular or ionic) , can vary greatly with a slight change in soil pH, Figure 1 illustrates it for sulfentrazone (pk = 6.56), characterized with weak acid.

Figure 1 shows the effect of soil pH rate on the percentage of the herbicide shape, ionized (anion) or molecular, and helps to understand the article of Grey et al. (1997), who analyzed the sorption of sulfentrazone when applied in multiple doses, varying the soil pH index, note that this characteristic has very significantly influence on the phenomenon of herbicide sorption to soil colloids, that decreases in response to a increasing pH, especially when this increase occurs above the pK of the herbicide (6.56), that because of the increased concentration of ionized form (anion) and a decrease in molecular form, in reverse, there is increased sorption to colloids when the index decreases, especially when this value below the pK of sulfentrazone.

It should be noted that this proportionality has a logarithmic behavior, so that a small variation in pH values leads to a major shift in the predominant form of the herbicide (molecular or ion).

This is a reality for Brazilian soils that have a pH range that include the pK of sulfentrazone (6.56), theoretically in two soils in the same climatic region with soil texture and organic matter equal but with the soil pH ranging from 5.5 to 7.2, the percentage of ionization would vary from 8.01 and 81.32% respectively, and may thus influence significantly the sorption of herbicides to soil colloids and in consequence, there are different persistence just for this variation in soil pH.

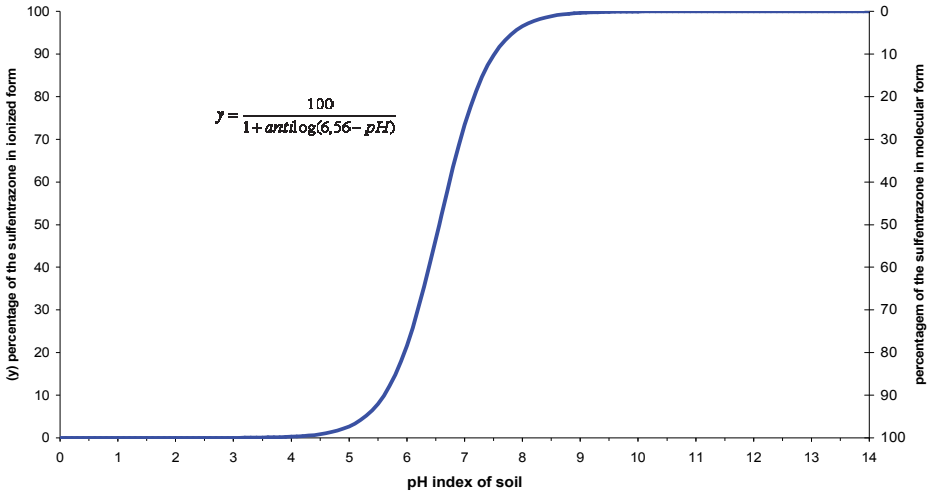

Fig. 1. Logistic model showing the ionization variation and molecular percentage of sulfentrazone (pk = 6.56), depending the content of the soil pH.

It should be noted that at low pH, even with a higher proportion of ionized form in the proton hydrogen (H^+) in soil solution competes with the ionized form of the herbicide by the site of sorption to soil colloids, so in many cases there is a greater sorption when soil pH is equal to the pK of the acid herbicide.

Reddy & Locke (1998), studying the sulfentrazone interaction on soil microorganisms using the labeled carbon method in the radical of the phenol molecule, found that after 77 incubation days, only 2.1% of 14 C-sulfentrazone were degraded by respiration edaphic to 14 CO_2, concluding that the native population of microorganisms edaphic had a small adaptation to the herbicide, could not dispel and degrade it, suggesting that this pathway is not the most efficient for their removal of the environment.

Chemical dissipation of the herbicide sulfentrazone is not yet fully understood, we know that does not show hydrolysis, photolysis are stable when applied to the soil, but extremely sensitive to this process in the water (EPA, 2011, Reddy & Locke 1998). All of these processes and factors influencing the sulfentrazone soil persistence.

3. Herbicides persistence in soils

Seeds of agricultural crops, when thrown to the ground, germinate at once and have slow initial growth in relation to the weeds. Herbicides applied directly on the ground need to persist in action on the ground with multiple streams of weed emergence (residual power) until the final limit of the critical competition period that for some crops is relatively long in Brazil.

For example, for the soybean crop in Brazilian conditions, this period is 30 to 50 days after emergence, while the sugarcane is planted in southeastern Brazil, at different periods during the year, the critical competition periods of varies according to these periods, a shorter period for spring and summer plantings, 15 to 60 days, and a longer, 60 to 90 days after emergence of seedlings, for autumn / winter planting because of drought that halts the growth of the plants until the next rain season (Blanco et. al 1978, 1979, 1981), so the herbicides used in this condition will persist for a long time with residual action to weeds in order to control their first flows allowing the plant to grow and expanding their leaves from parallel planting lines, shading the spacing of planting, controlling these plants through of light competition.

By definition, the persistence of herbicides in soil can be of three types, according to the method and objective of its biological, agronomic and chemical persistence determination. The biological persistence is determined by biological methods (bioassays) the time of the residue effect on living beings (bioactivity), in which case the agronomic persistence is determined using plant test, a biological persistence is individualized, therefore, measurements of the time that the herbicide residue with activity remains in soil can affect plants grown in a system of succession or rotation of crops. The chemical persistence concerns at time of the residue remains in soil that can be detected by chemical or radiometric methods.

The chemical methods (residues analysis) quantify the level of the herbicide while the biological (bioassay) qualify the presence of this, and in many cases this method is more sensitive, as all methodologies, advantages and limitations, and their use depend on the purpose of each test. Figure 2, show inferences about the persistence of herbicides in soil and their action.

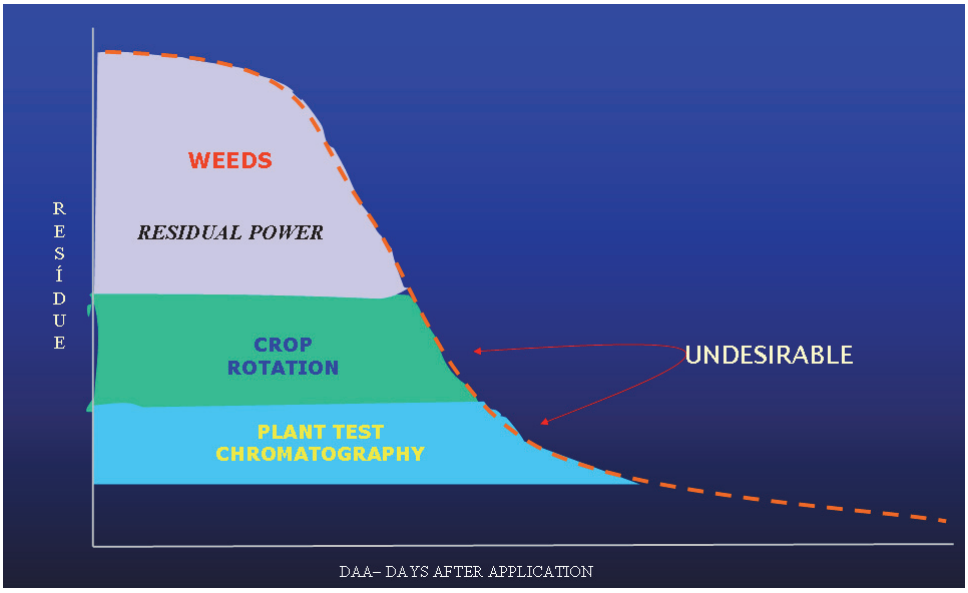

Fig. 2. Show a theoretical model by a logistic function, emphasis areas for action or method capable of detecting the herbicide, thus illustrating the decreasing variation of the residual herbicide in soil in time function (persistence).

We observe that the highest concentrations of the herbicide residues in soil correspond to the desirable effect of weed control (residual power). Over time, the herbicide concentration in soil decreases to levels that no weed control, but its concentration can affect crops in succession to that in which the herbicide was applied originally; their presence can be detected by symptoms of phytotoxicity expressed in these crops when they are sensitive. From this time, the residual level of the herbicide is very low, being detected only by plants with extremely susceptibility to the herbicide (plant test) or by analytical methods (eg chromatography). From this point, the concentration of herbicide in the soil is so low that current technology can not detect its presence on the ground.

Thus, the ideal herbicide would be that for which there is a coincidence of the final period of their persistence with the final period of the critical competition period the crop, a situation that unfortunately does not occur for current herbicides.

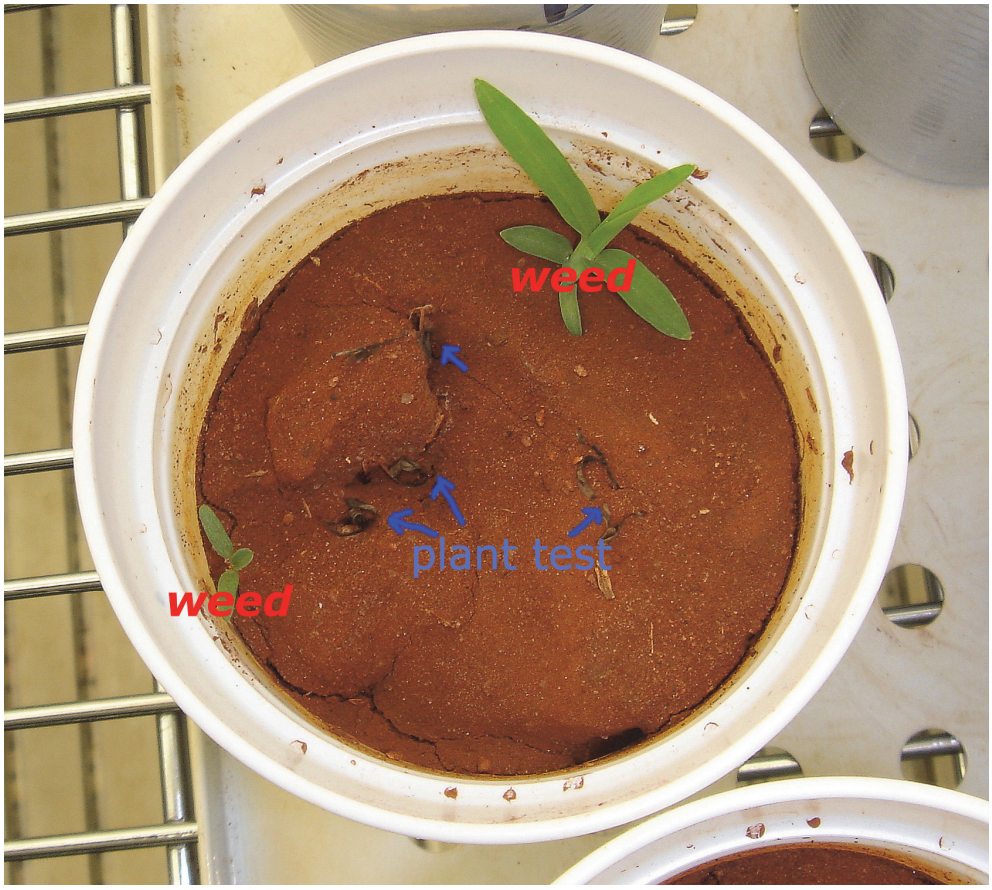

Fig. 3. Bioassay showing different action of the herbicide. Photo: Flávio M. G. Blanco.

Figure 3 explains the difference between persistence and residual power of the herbicide. Although the herbicide is present (persistent) in the soil, because it affects the plant test, does not affect the weeds, in this case the herbicide persists in the soil, but without power or residual effect on weeds.

Aiming aspects of selectivity for crops in succession to determine the persistence using the methodology to plant test is more efficient because the residual level in the soil did not affect the plant test, the more sensitive and also not cause injury to succession crops.

It should be emphasized that the use of results from ecotoxicological research, with aim to study the persistence of herbicides in soils in other countries (not Brazil) should be used with reservation, since different soil and climatic conditions often change the action of herbicides in soil, moreover, even when considering the Brazilian territory, it is difficult to extrapolate, for example, the results obtained in the Southeast to the Northeast Region.

In Brazil, the research works of herbicides in soils in agroecosystems are still few, especially when obtained under natural conditions of cultivation, such as the determination of persistence in the soil sulfentrazone, as well as its effects on cultures in a system of succession

3.1 Soil persistence of sulfentrazone in Brazilian conditions

3.1.1 Persistence determination by bioassays

Sulfentrazone herbicide is registered in Brazil for soybean, sugarcane, coffee and citrus. Belongs to the aryl-triazolinonas herbicide group, solubility of 780 mg L^{-1} (pH_7), vapor pressure 1.10^{-9} mmHg (25 C^o), dissociation constant (pK) 6.56 and partition coefficient ($Kow_{(pH7)}$ 9.8), belongs to group aryl triazolinonas chemical, mode of action is to the destruction of cell membranes by inhibiting the enzyme Protox is the accumulation of protoporphyrin IX causing peroxidation of O_2 and consequently the destruction of cell membranes.

Rossi et al. (2003) using bioassays, evaluated the leaching of sulfentrazone (0.86 kg ha^{-1}), in two Brazilian soils (Red Nitosol and Neosoil Quartzarênico), in PVC columns; 0.10 and 0.50 m (diameter and length) using sorghum (*Sorghum bicolor*) under different rainfall regimes for 15 days, determined that over rainfall of 90 mm, the herbicide was detected by 7.5 and 12.5 respectively in Red Nitosol and Neosoil Quartzarênico soil.

These data are consistent with Vivian et al. (2006), also in brazilian conditions, investigating the actions of sulfentrazone, up to 20 cm deep, applied at the dosage of 0.9 kg ha^{-1} in sugarcane crop, determined with bioassays using sorghum as plant test, the leaching of herbicide was significant only on 0-10 cm layer of soil and persisted up to 467 days. However, when reapplying the herbicide in sugarcane crop, the order of persistence could not be determined, because until the last evaluation (640 days) the herbicide still persisted in the soil. In the same work, it was given the relationship of sorption (RA) in 3.6, indicating that this herbicide, when sorbed onto colloids, has a tendency for slow release into soil solution.

For new herbicides the difficulty is to determine which plant test should be used, especially when the group is composed of a few chemical elements. To determine the sensitivity, several mathematical models can be tested, to quantify and explain the phenomenon:

exponential, reciprocal, logistic, quadratic, cubic, Gompertz, exponential, quadratic and cubic, and others. The important aspect is not to analyze only the mathematical aspect of the model, such as the coefficient of determination that reflects the goodness of fit, but also the logic of biological phenomena, thus the choice of model will have a higher probability of success.

For Streibig et al. (1988, 1995), when compared with other methods for the detection of residues in agroecosystems, such as chromatography, the mathematical modeling studies using bioassay methods, is not fully standardized yet. There are several types of models, in the literature, to estimate the phenomenon, however, the authors indicate the logistic or log-logistic model as the most appropriate to explain the dependence of plant development with herbicide dose variation, and especially for calculating the RG 50 (herbicide dose that reduces growth by 50% of the plant). The same opinion is shared by Seefeldt et al. (1995).

Thus, Blanco (2002), using the bioassay method, tested two plant species, candidates as test plant for sulfentrazone herbicide, evaluating the biological responses of sorghum (*Sorghum bicolor*), cv AG 2002 and sugar beet (*Beta vulgaris*), cv. Early Wonder, exposed to a series of herbicide dilutions, growing for 14 days in 300 ml plastic cups, without percolation, with 250 g of soil with medium texture, kept at 80% of field capacity by daily watering in a fitotron Conviron® model PVG386, at 20° C, 70-80% relative humidity and photoperiod of 16 hours, light intensity of 35,400 lumens m^{-2}, to determine the dose that reduces 50% of their fresh weight of the epigeal part (RG 50%), using logistic function as the mathematical model (Fig. 4).

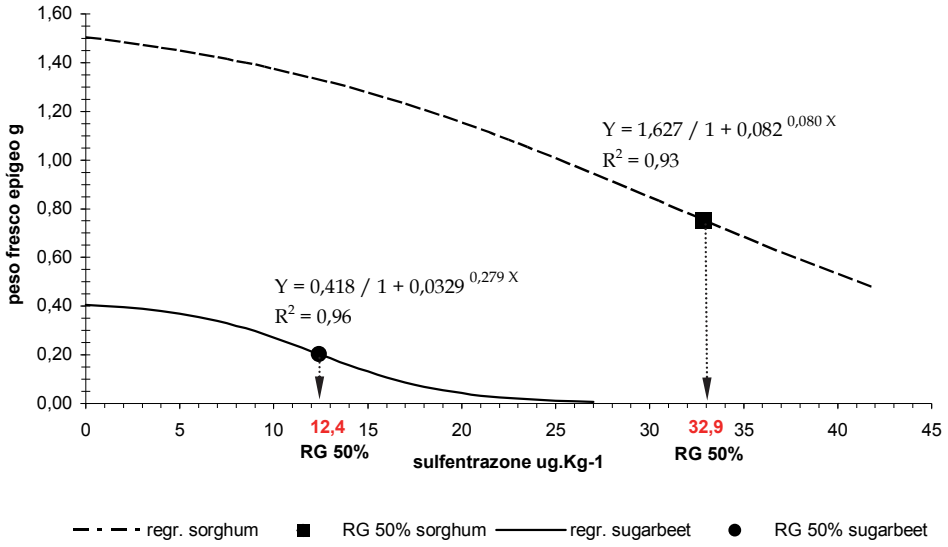

Fig. 4. Effect of increasing doses of sulfentrazone on epigeal fresh weight of sugar beet and sorghum and determination of the RG (50%) of each plant by logistic regression models, mean values of 10 replicates. (Blanco, 2002).

The analysis of the sensitivity of the plant test, using the methodology to evaluating the "fresh epigeal mass" instead of "epigeal dry mass," is due to feature of the herbicide, which belongs to the family of aryl triazolynonas, and is a desiccant, so, the methodology of evaluating the "epigeal dry mass," would tend to remove all differences between treatments, making all of them similar to the control, considering that a turgid leaf, when dried, can present the same symptoms (chlorosis, necrosis and desiccation) as a leaf treated with herbicide. Drying the plant material, it decreases the variability between treatments and, consequently, increasing the difficulty in finding significant differences between them, either by treatments analysis of variance (F test) or by an independent medium test

As observed in Figure 4, the logistic model adapts well to explaining the phenomenon, either by their biological logic - gradual reduction in mass with the increase of doses tested, as well as mathematical logic - high coefficients of determination, 0.93 and 0.96, showing excellent fit of the data to the chosen model. It should be noticed that to obtain the mathematical models, we used an appropriate number of observations, depending upon the extent of the analyzed data, fifteen and ten grain sorghum and sugar beet, respectively with a spacing between them of 3 µg of sulfentrazone, contributing to good accuracy of the obtained models

It is emphasized that the high light intensity, 35,400 lumens m^{-2}, and the photoperiod of 16 hours, for the bioassay, increased the sensitivity of the test plant, because sulfentrazone has its mode of action activated by the light intensity.

In figure 4, it is shown that both plants were very sensitive to the herbicide, notably beets with the highest sensitivity with RG (50%) equal to 12.4 $\mu g.kg^{-1}$, less than the half of the value observed to sorghum. Thus, the beet was used as the test plant for biological assays to determine the persistence of sulfentrazone in sugarcane and soybean crops in the proceedings of Blanco & Velini, 2005 and Blanco et al. 2010, described below.

3.1.2 Soil persistence of sulfentrazone applied in sugarcane crops.

Blanco et al. 2010, studying the sulfentrazone (0.6 and 1.2 kg ha^{-1}) in Brazilian conditions, soil pH 6.4 and organic matter, 11 g dm-3, using the bioassay method evaluating herbicide persistence under field conditions, the sugarcane crop until 704 days after treatment application, obtained the following biological responses for the plant test, described in the Figure 5.

During the test period, the soil was sampled in 23 seasons, immediately after application (0), up to 704 DAT, where the soil was sampled to determine the persistence by bioassay method, using sugar beet as test plant, growing for 14 days in a phytotron set at 20 ° C, 70-80% relative humidity and photoperiod of 16 hours, with light intensity of 35,400 lumens $m^{-2.}$

The climatic condition during the test is represented by figure 6.

The doses evaluated had different behavior dose of 0.6 kg ha^{-1} increased gradually from fresh epigeal not deferred test $t_{(p<5\%)}$ from the control 601 DAT by the end of sampling at 704 DAT. The dose 1.2 kg ha^{-1}, the averages of fresh epigeal also increased and differed significantly from the control, until the end of sampling.

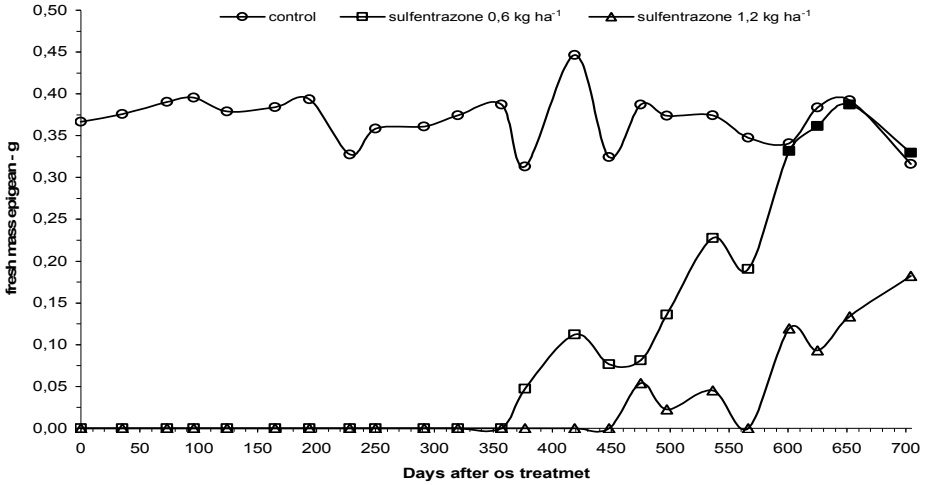

Fig. 5. Temporal variation of fresh epigeal a function of days after treatment, ■ represents no significant difference (p> 0.005), compared with the control. Each symbol represents the mean value of 5 replicates (Blanco et al. 2010).

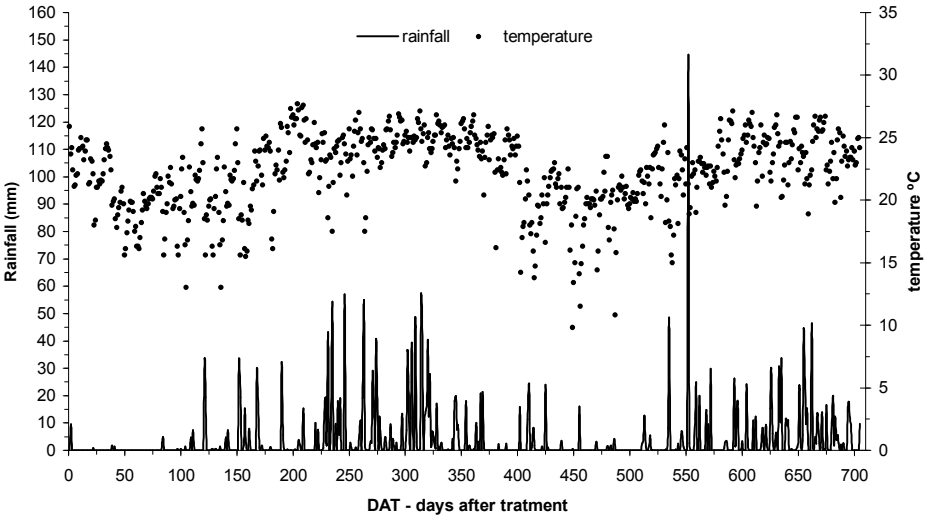

Fig. 6. Daily rainfall and average air temperature in the period from 30/03/2000 to 04/03/2002. (Blanco et al. 2010).

Through these values, we can determine the final limit of the persistence of the herbicide at 0.6 kg ha^{-1}, 601 DAT, the highest dose was found this persistence has lasted over 103 days, signaling that the sulfentrazone persistence when applied at 1.2 kg ha^{-1}, may exceed 704 DAT.

The timing of herbicide in early fall was characterized by a gradual decrease in rainfall and water content in soil, which benefited the herbicide sorption to soil colloids and discouraging the development of the microbial community, participatory processes dissipation herbicides (Alexander 1965; Weber 1970), it favored the retention of the herbicide in the soil, which would explain the lack of plant development in the first test ratings, which occurred only after 150 DAT, with the onset of rains and the increase in temperature, remaining until 450 DAT.

This condition favored the development of the microbial community and the desorption of the herbicide to the colloids, thus making it available to the dissipative processes (Blanco, 1979, Walker and Allen, 1984; Reddy and Locke, 1998), reducing its concentration in the soil and thus the beginning of the first plant test germination, the DAT 377 and 475 for treatments 0.6 and 1.2 kg ha^{-1}, respectively (Figure 1).

However, the increase in temperature and precipitation was only sufficient to that the herbicide was dissipated partly because after the dry season (448-556 DAT) was necessary to even more a rainy season (550 until 704 DAT), so that there were further dissipation of the herbicide and progressive reduction of their concentration in the soil, notably for the dose of 0.6 kg ha^{-1}, where it was possible to determine the end of the persistence of sulfentrazone, the highest dose was also detected by this plant test until the end of the evaluations, at 704 DAT.

3.1.3 Soil persistence of sulfentrazone applied in soybean and its effect on crops rotation

Blanco & Velini, 2005 studying the persistence of sulfentrazone applied in soybean cv. Embrapa 48, at the same doses of the test previously described , 0.6 and 1.2 kg ha^{-1}, pH 5.8 and soil organic matter content of 43 g dm-3, using the same bioassay method, also evaluated the effect of this herbicide on five crops in succession: millet cv. Italian and oats cv. White, wheat cv. IAC 24, sunflower cv. Uruguay and kidney bean cv. Carioca, under field conditions for 539 days after the treatments. The biological response of the beet test plant for persistence of the herbicide is described in Figure 7.

The Figure 7 shows the results of the action of sulfentrazone on the beet plant test; it is observed that when the test was carried out with the soybean crop, there were six samples and from these, the growth of beet plants was observed only in the control treatment. After the soybean harvest, it was initiated the preparation of the area for planting crops in rotation, at 159 DAT. Despite all the procedures for preparing the soil for planting, it was found that the management was not sufficient to dissipate the herbicide. The results of the bioassays from samples collected from each culture, at the harvest 278 DAT (oats, beans and millet), 286 DAT (wheat) and 305 DAT (sunflower), demonstrated that the persistence of the herbicide was not influenced by crop type neither by specific cultivation practices that were undertaken for each crop.

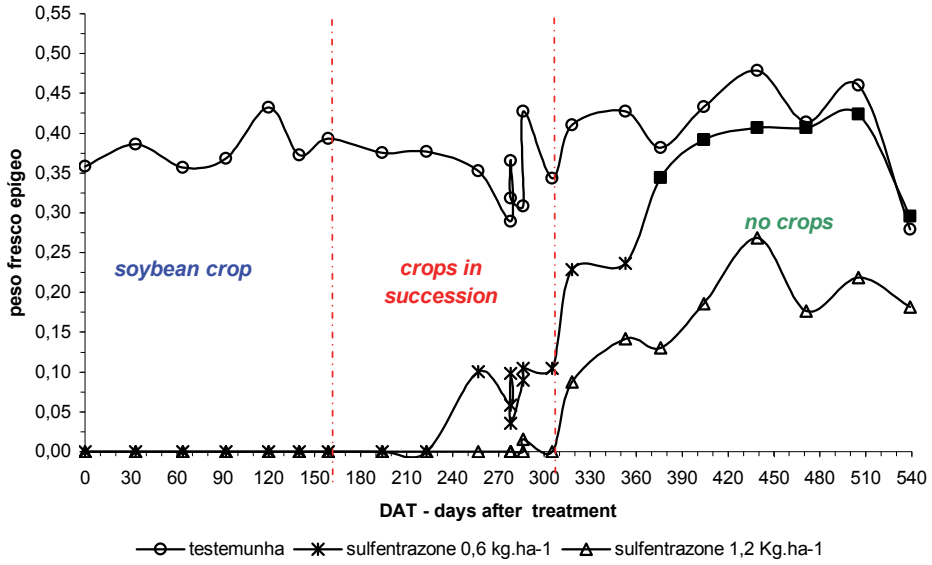

Fig. 7. Temporal variation of fresh epigeal a function of days after treatment, ■ represents no significant difference (p> 0.005), compared to control. Each symbol represents the mean of 5 repetitions (Blanco & Velini, 2005).

In all samplings, tests of means (*t* test at 5% probability) were carried out contrasting the control treatment with herbicide doses in each season. The results indicated that only for the lowest dose, from 376 DAT, there was no significant difference to the control until the end of the test (539 DAT). For this reason, 376 DAT can be defined as the final limit of sulfentrazone persistence at the dose of 0.6 kg ha^{-1}. At the highest dose used, the fresh weight of the plant test was significantly lower than that of the control, in all evaluated periods, indicating that, for the dose of 1.2 kg ha^{-1}, the final limit of persistence was not achieved, thereby; the persistence of sulfentrazone, in soil in this case, was longer than 539 days after herbicide application.

Figure 8 shows the rainfall and average temperature during the research work.

The soil where the assay was carried out showed a high content of organic matter (43 g dm3) and clay (46.3%), this fact combined with the predominant molecular form (85.2%) of the be herbicide, favored the sorption of the herbicide to soil colloids (Weber 1970, Grey et al., 1997). However, as the herbicide was sprayed in the summer, characterized by frequent rainfall and high temperatures (Figure 6), the conditions were not favorable for the herbicide sorption to the soil colloids, but tending to stay in the solution available for dissipative processes and leaching (Briggs, 1976; 1984; Weber, 1970; Walker & Allen, 1984).

This condition was maintained until 80 DAT, the period when due to the dry season, there was the most favorable condition for the herbicide sorption to the colloids, until 200 DAT, when a new rainy condition favored the persistence of the herbicide in the soil solution and subjected to the dissipative processes and leaching. This argument is strengthened by, the

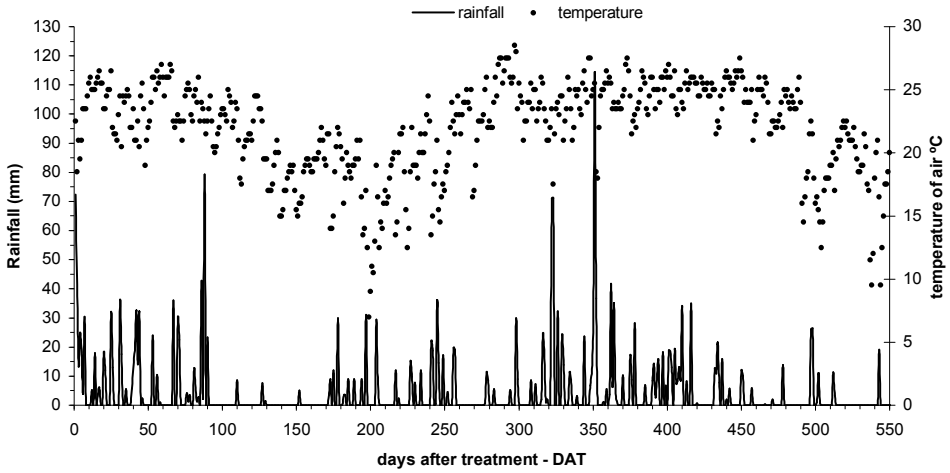

Fig. 8. Daily rainfall and average air temperature in the period from 13/01/2000 to 04/07/2001 (Blanco & Velini, 2005).

first occurrences of test plants in the bioassays at the initial phase of this period, indicating the beginning of the herbicide dissipation.

This condition of high intensity and abundance of rainfall and high temperatures was maintained until almost the end of the assay, at 500 DAT, was favorable to the dissipation of the herbicide, and it was possible to define the limit of the persistence of sulfentrazone in its lowest doses, at 376 DAT. After this period, the weather conditions changed to dry, with cool temperatures, characteristic of the end of the experiment (fall/winter). This phase was favorable to the herbicide sorption to the soil colloids, becoming not available to the processes of dissipation and thus the herbicide residue level in the soil was able to affect significantly the test plant,not being able to detect the final limit of the persistence of sulfentrazone at the highest dose, 1.2 kg ha^{-1}.

For the persistence data obtained in this experiment, beyond the Ecotoxicological aspects, it is important to notice that after the harvest of soybean crop, there were still residues of the herbicide in a reasonable concentration in the soil, resulting in two immediate risks for the crops in succession to soybean: injuries to sensitive crops and when the crop is tolerant to the herbicide, if the same chemical is used for weed control, as it was already present in the soil, there may be an initial concentration higher than the tolerance threshold level and cause damage to the crop that would theoretically be tolerant to the herbicide. To evaluate the selectivity of sulfentrazone on soybean crops in succession, several development parameters of these crops were measured at different periods: visual symptoms of phytotoxicity, stand, height, leaf area, leaf number, fresh and dry weight and also production.

Analysis of the results showed that sulfentrazone, independent of the dose, affected the millet and oat development. On the other hand, for sunflower and bean crops, the variance

analysis showed that the treatments were not significant, thus demonstrating the selectivity of this herbicide for these crops. For wheat crop, the selectivity of the herbicide was variable according to the dose tested. It was selective for the lowest dose and not selective for the highest dose, 0.6 and 1.2 kg ha^{-1}, respectively

4. Conclusion

The methodology of bioassays for the detection of residues of sulfentrazone in soil is adequate.

The herbicide when applied in the cultivation of sugarcane cv. SP8018160 and soybean cv. Embrapa 48 shows long persistence in the soil and affects significantly the development of millet cv. Italiano and oats cv. White and wheat cv. IAC 24 (at 1.2 kg ha^{-1}) in soybean rotation, however, for sunflower cv.Uruguay and kidney bean cv . IAC Carioca and wheat cv. IAC 24 (dose 0.6 kg ha^{-1}), sulfentrazone does not affect the development of these plants.

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8

Paraquat: An Oxidative Stress Inducer

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1. Introduction

Paraquat (1,1_-dimethyl-4,4_-bipyridinium dichloride), is a foliar-applied and non selective bipyridinium herbicides, and it is one of the most widely used herbicides in the world, controlling weeds in a huge variety of crops like corn, rice, soybean, wheat, potatoes; major fruits: apples, oranges, bananas; beverages: coffee, tea, cocoa; and processed crops: cotton, oil palm, sugarcane and rubber.

For a foliar absorbed herbicide to completely kill a plant, it must be capable of accessing the whole plant, as growing leaves and newly emerging roots. This often means that the herbicide not only needs to damage at the point of its absorption, but must also be translocated to parts of the plant not contacted by the herbicide during application.

Paraquat is a cation formed by two pyridine rings, each having a quaternary amine and thus charged 2+. Although the majority of herbicides are passively transported as noionic molecules, paraquat cation movement by diffusion across membrane lipid bilayer is unlikely. Transporter studies to explain paraquat compartment were made using several systems. ABC transporters, large membrane proteins which use ATP for the active transport of several compounds including paraquat have been described. Other groups of transporters are small antiporter proteins which exchange protons for some other molecules using the proton electrochemical potential gradient (Morymio et al., 1992, Yerushalmi, et al., 1995). In animal tissues it has been shown that paraquat transport occurs by carriers that also function as carriers of other molecules such as polyamines (Rannels et al., 1989, Jóri et al., 2007). Hart et al. (1992a 1992b) demonstrated that paraquat movement across plasma membrane root epidermal and cortical maize cells has a concentration-dependent kinetic and that the herbicide binds to cell wall, and its transport is facilitated by a carrier that normally functions in the movement of molecules that has a similar chemical structure or similar charge distribution such us diamines like putrescine and cadaverine. Using maize protoplast Hart et al. (1993) showed that paraquat uptake has similar concentration-kinetic to that observed in intact cells and the accumulation inside cells increase in a time-dependent manner and is saturated after 10 min, although 50% of uptake occurs during the first 10 s. The saturable K_m for paraquat uptake in maize cells and protoplasts was determined at 90 μM and 132 μM respectively, similarly the K_m in rat lung was 70 μM

suggesting in both animal and vegetal tissues a carrier-mediated process (Rannels et al., 1985).

In order to investigate paraquat uptake, compartmentation and translocation, maize plantlets with their root immersed in paraquat solution for several loading periods were used (Hart et al., 1993). The lack of chloroplasts in roots provides a system to minimize the short-term phototoxic effect. The paraquat accumulation in the root vacuole was linear over a 24 h loading period. The vacuolar paraquat content, with respect to the total accumulated increased from 15% to 42% after 2 h and 24 h loading period, respectively. In contrast to the vacuole, total cytoplasmic paraquat content appeared to approach saturation whereas paraquat associated with the cell wall fraction remained relatively constant, suggesting that this phase is rapidly saturated. Even though paraquat is considered to be relatively immobile, linear paraquat (PQ) translocation occurred from roots to shoots and was estimated that approximately 50% of the paraquat effluxing from roots started translocation to shoots 5 h after the beginning of loading period (Hart et al., 1993b).

Paraquat acts as a redox cycler with a great negative reduction potential ($E_{0'} = -0.446$ V). This feature restricts its interaction with strong reductant compounds. When dication of paraquat (PQ^{2+}) accepts an electron from a reductant form the paraquat monocation radical (PQ^{+}), which then rapidly reacts with oxygen (O_2 $E_{o'} = 0.16$ V) to initially produce superoxide radical ($O_2^{\bullet -}$) (k 7.7 x 108 M^{-1} s^{-1}) and subsequently the other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH).

In plants, paraquat is principally reduced within chloroplasts, where it acts as an alternative electron acceptor taking electron from Fe-S proteins of photosystem I; inhibiting the ferredoxin reduction, the NADPH generation, and also the regeneration of ascorbic acid. In consequence, paraquat is a potent oxidative stress inducer, because it greatly increases the ROS production and inhibits the regeneration of reducing equivalents and compounds necessary for the activity of the antioxidant system.

Paraquat also induces the increase of superoxide radical production in mitochondria, where complexes I and III are the major electron donors. For this reason paraquat has been widely used to induce mitochondrial oxidative stress in many experimental systems such as isolated mitochondria, cultured cells, and whole organisms including plants, *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* and rodents (Cocheme & Murphy, 2008).

2. Generation and role of ROS

Superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are highly reactive compounds that induce protein and pigment degradation, lipid peroxidation, nucleic acid damage, affecting key components of plant cell metabolism that can finally lead to cell death. These deleterious reactions triggered by ROS are known as oxidative stress phenomenon (Casano et al., 1994, 1997; Lascano et al., 1998, 1999).

Even though all ROS are highly reactive compounds their effects and plant responses depend on the ROS in question as well as on its concentration, site of production, interaction with other stress molecules and on the developmental stage and plant cell previous history.

In green tissues under light, chloroplasts are the main intracellular source of ROS (Asada, 1999) and peroxisomes, through photorespiration, are other important ROS producers (del Río et al., 2006). While mitochondria, are the principal source of ROS in darkness and non green tissues. On the other hand, the NADPH oxidase complex, peroxidases and amino oxidases are major sources of apoplastic ROS (Sagi & Fluhr, 2006).

Primarily, the chloroplasts mainly produce $O_2^{\cdot -}$ at photosystem I (PSI) and $^1O_2$ at photosystem II (PSII), and the mitochondria produce $O_2^{\cdot -}$ at complexes I and III (Asada, 1999). The peroxisomes produce H_2O_2 as byproduct of photorespiratory glycolate oxidase reaction, fatty acid β-oxidation and reaction of flavin oxidase, and $O_2^{\cdot -}$ is generated by xanthine oxidase and by electron transport chains in the peroxisomal membrane (del Río et al., 2006).

Various interconverting reactions occur among different ROS. Superoxide is spontaneously or enzymatically converted to H_2O_2 by disproportion mechanism and H_2O_2 and $O_2^{\cdot -}$ can interact to produce $^{\cdot}OH$ through the Fenton reaction catalyzed by free transition metal ions (Fridovich, 1986).

Different ROS have different features. Hydrogen peroxide is a non radical, apolar molecule and, in consequence, it is a relatively stable compound with half-life around 1 ms. In plant tissues, its concentration could be in the micro to millimolar range. The half-lives of the other ROS are very short, ranging from nano to micro second, and then they are present at very low concentrations (Asada, 1999).

Reactive oxygen species also have different reactivities. Hydrogen peroxide (Eo 1.77 V), not a highly reactive ROS *per se*, mainly oxidizes thiol groups, in presence of transition metal ions it catalyzes $^{\cdot}OH$ generation by Fenton reaction. Superoxide radical (Eo -0.33V) oxidizes ascorbate and NADPH, reduces metal ions and cytochrome C and reacts with protein Fe-S centers. Singlet oxygen is particularly reactive with conjugated double bonds of polyunsaturated fatty acids. Whereas $^{\cdot}OH$ (Eo 2 V), the most oxidant ROS, reacts with all types of macromolecular cellular components. The differential ROS reactivity means that they leave different footprints in the cell in the form of different oxidatively modified components (Moller et al., 2007).

Cellular membranes are the principal targets of ROS. Peroxidation of polyunsaturated fatty acids (PUFAs) is a common oxidative stress effect. Linoleic acid (18:2) and linolenic acid (18:3) are major fatty acid present in galactolips of thylakois and phospholipids of all membranes. PUFAs peroxidation generates mixtures of lipid hydroperoxides several aldehydes, e.g., 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), hydroxyl and keto fatty acids and oxidative modification in membrane protein. The consequences over the membrane function are the fluidity and selectivity decreases (Halliwell et al.,1999; Halliwell, 2006). Some of the PUFA peroxidation products act directly or after enzymatic modification as secondary messengers either, e.g. oxylipins (Muller et al., 2004).

ROS induce mainly irreversible covalent modification on proteins. The reversible modifications on sulfur containing amino acid are very important in the redox or oxidative signaling. Cystein thiol groups are initially oxidized to disulfide and in further oxidation to sulfenic and sulfinic acid. The highest level of cysteine oxidation, cysteic acid seems to be irreversible and damaging (Ghezzi & Bonetto; 2003). Nitrosylation and glutathionylation are

other cystein thiol modification mediated by nitric oxide, reactive nitrogen species (RNS) and glutathione. RNS are generated by the interaction between nitric oxide and ROS. (Costa et al., 2003; Halliwell, 2006). Carbonylation, a common oxidative protein modification affecting particularly Arg, His, Lys, Pro, Thr, and Trp; and conjugation with peroxidation PUFA products, mainly with HNE, are other oxidative protein modifications (Shacter, 2000; Winger et al., 2005).

The generation of 8-Hydroxyguanine is the most common DNA modification induced by ROS. The nucleotide bases are attacked by. OH and $^1O_2$ while H_2O_2 and $O_2^{\cdot -}$ do not react at all (Wiseman & Halliwell, 1996). Chloroplastic and mitochondrial DNAs are into the two major source of ROS where potentially high rates of modification might occur (Thorslund et al., 2002). Another indirect oxidative modification to DNA is the conjugation of MDA with guanine (Jeong, 2005). The DNA oxidative modification could induce changes in cytosines methylation patterns, and then in the regulation of gene expressions. ROS-induced DNA modification seems to be a not completely random process (Halliwell, 2006).

Carbohydrates can be oxidatively modified by $^{\cdot}OH$, being the formic acid the main breakdown product of sugar oxidation (Isbell et al., 1973).

In spite of its toxic effects, increasing evidence indicates that ROS are signaling molecules that participate in many processes, such as cell cycle, cell elongation, cell death, plant growth and development, senescence, hormone signaling, responses to biotic and abiotic stress and in symbiotic interaction with microorganisms (Bustos et al., 2008; Mittler et al., 2004; Muñoz et al. 2011, submitted; Rodriguez et al., 2010). The H_2O_2 molecular properties make it a good second messenger that could cross membrane by diffusion or aquaporins. However, all ROS can act as signaling molecules directly or by oxidized product. NADPH oxidase complex, the main source of apoplastic ROS, has a key role in oxidative signaling (Sagi & Fhlur, 2004).

The dual role of ROS, as toxic or signaling molecules, depends on the ratio and subcellular location of its generation, thus the tight regulation of the steady-state level of ROS in different subcellular compartments has both signaling and oxidative damage protection purposes. The function of ROS as signaling molecules is intrinsically related to the interaction with non-enzymatic antioxidants, such as ascorbate and glutathione, which are redox buffers and also signal molecules *per se* (Foyer & Noctor 2005 a, 2005b).

The relationship among ROS, antioxidants, reducing equivalents, sugars, the redox state of chloroplastic and mitochondria electron transport chains are major determinants of the cellular redox state, which has a critical function in the environmental perception and modulation of defense, acclimation and tolerance responses (Foyer & Noctor, 2005 a; 2005b; Lascano et al., 2003; Melchiorre et al., 2009; Robert et al., 2009).

3. Antioxidant system in plants

Plants have evolved a complex antioxidant system composed by both non-enzymatic and enzymatic components, to prevent the harmful effects of ROS.

Low-molecular-mass metabolites soluble in both aqueous and lipid phases lipid with high ROS reactivity such as ascorbate, glutathione tocopheroles, flavonoids, alkaloids, carotenoids, proline and amines, form non-enzymatic part of the antioxidant system (Apel & Hirt, 2004; Sharma and Dietz, 2006).

Superoxide dismutase (SOD) (E.C.: 1.15.1.1), ascorbate peroxidase (APX) (E.C.: 1.11.1.11), catalase (CAT) (EC 1.11.1.6), and glutathione reductase (GR) (E.C.: 1.6.4.2) are key antioxidant enzymes that modulate the concentration of two of the Haber/Weiss and Fenton reaction substrates, $O_2^{\cdot -}$ and H_2O_2, preventing the formation of the highly toxic $^{\cdot}OH$ radical (Asada, 1999). Approximately, 80% of SOD, GR, and APX activity is located in the chloroplast (Asada, 1999). CAT activity is located in peroxisomes and mitochondria (Scandalios, 1994). SOD catalyses the disproportionation of $O_2^{\cdot -}$ to H_2O_2, and is present in multiple isoforms: copper/zinc (CuZn-SOD), iron (Fe-SOD) and manganese (Mn-SOD) (Bowler et al, 1992). In most plants, CuZn-SOD and Fe-SOD are present in the chloroplasts, CuZn-SOD in the cytosol and Mn-SOD in mitochondria (Casano et al., 1997; Scandalios, 1993). Degradation of H_2O_2, in the chloroplasts and in the cytosol is carried out by the ascorbate-glutathione cycle, which involves APX and GR activities (Lascano et al., 1999, 2003). APX has chloroplastic and cytosolic isoforms, and catalyses the conversion of H_2O_2 to water using ascorbate as electron donor (Asada, 1999).

Reduced glutathione (GSH) and ascorbic acid are the most important soluble non-enzymatic antioxidants and in chloroplasts they are present at millimolar concentrations (Noctor & Foyer, 1998). Ascorbate acts as a ROS quencher and it is involved in the regenerations of tocopherol and violoxanthine depoxidase activity of xanthophylls cycle (Noctor & Foyer, 1998). Reduced glutathione is a tripeptide γ-glutamylcysteinyl glycine (γ-Glu-Cys-Gly) involved in: direct reaction with ROS, the regeneration of the ascorbate pool and as electron donor of glutaredoxins which are linked to type II peroxiredoxin activity. Likewise, GSH participates in the glutathionylation, a post-transcriptional modification of protein thiols groups that regulates the function of proteins like glyceraldehyde-3-phosphate dehydrogenease and thioredoxin activities (Michelet et al., 2005; Zaffagnini et al., 2007). The reduction of oxidized glutathione is NADPH-dependent and carried out by GR, a ubiquitous flavoenzyme with many isoforms, located in chloroplasts, cytosol, and mitochondria (Lascano et al., 2001; Tanaka et al., 1994).

Other more recently identified components of enzymatic antioxidant system are peroxiredoxins and glutathione peroxidase, non-heme-containing peroxidase which activity depend on cystein residues (Bryk et al., 2000; König et al., 2003).

4. The use of paraquat in stress response studies

Plants as sessile organism are permanently exposed to changing environment that become stressful conditions affecting their growth, development and productivity. Tolerance to environmental stress is a major selection criterion in plant breeding. The cellular and molecular tolerance mechanisms of plants to different stresses have been intensively studied.

Reactive oxygen species are produced as byproduct of normal aerobic metabolism and the life under aerobic conditions is strictly dependent on the presence of antioxidant system. Nowadays, it is widely accepted that the generation of ROS is enhanced under abiotic and biotic stress conditions. Depending on stress intensity and its associated-ROS levels the plant responses range from tolerance to death.

Likewise, the positive response of the antioxidant system correlates, in part, with the tolerance to many different environmental stress conditions. ROS and antioxidant system

are central components of the cross-tolerance phenomenon which states that a tolerant genotype to one stress condition could be also tolerant to other kinds of stress.

Paraquat treatments have been frequently used, as a potent oxidative stress inducer, in many different basic studies like: oxidative stress tolerance and cross tolerance responses associated with the antioxidant system responses (Lascano et al., 1998, 2001, 2003), forward and reverse genetic approaches to study the function of different antioxidant system components (Melchiorre et al., 2009 and references therein,) ROS signaling (Robert et al., 2009), ROS and NO-induced cell death (Tarantino et al., 2005), and to mimic the drought effect on carbon and nitrogen metabolism of nodules (Marino et al., 2006).

Several attempts made to enhance tolerance photooxidative stress conditions have been tested with paraquat treatments. These have involved the overexpression of enzymes associated with the Asada-Halliwell pathway including SOD (Arisi et al., 1998; Bowler et al, 1991; McKersie et al., 1999; Melchiorre et al., 2009; Perl et al., 1993; Pitcher et al, 1991; Sen Gupta et al., 1993;Tepperman et al, 1990; Van Camp et al.,1996), and GR (Aono et al., 1991, 1993; Creissen et al., 1995; Foyer et al., 1991, 1995; Melchiorre et al., 2009). The tolerance to different oxidative stress conditions was dependent on the copy numbers and overexpression levels; the isoform overexpressed; the subcellular location where the overexpressions were targeted; and the induction of other antioxidant enzymes. The results of chloroplasts-targeted Mn-SOD or GR overexpression in wheat chloroplasts, suggest that antioxidant enzyme overexpression effects on tolerance response not only depend on their antioxidant capacities but also on their effects on the cellular redox state, which modulates the responses to photooxidative stress in a pathway where apoplastic superoxide generation could be involved (Melchiorre et al., 2009). The photooxidative activations of NADPH oxidase complex, the main source of apoplastic ROS, can be mimicked by paraquat treatment (Robert et al., 2009).

Paraquat has also been used as an efficient inducer of cell death in both animal and plant cells (Dodge, 1971; Suntres, 2002). The cell death processes in plants are major regulatory mechanism of growth, development, and responses to biotic and abiotic stresses (Lam et al., 2001; Pennel & Lamb, 1997). Environmental or developmental conditions where cellular redox balance is disturbed and significant ROS accumulation occurred, could lead to the induction of cell death processes (Dat et al., 2000). In this context, two type of ROS-associated stress intensity-dependent death can be defined: Ordered or Programmed Cell Death (PCD) when the cell maintains the membrane and energy generation systems, and Disordered or Necrosis, when these systems are overwhelmed by the oxidative burst. Continuous or transient light-dependent H_2O_2 accumulation, provoke necrosis or PCD, respectively indicating the existence of a ROS levels threshold below which PCD is triggered and above which necrotic cell death prevail (Montillet et al., 2005).

Programmed Cell Death in plant cells shares some similarities with that of animal cells, like organelle degeneration, nuclear condensation, nuclear DNA fragmentation and eventually cell shrinkage. Interestingly, animal anti-apoptotic protein (Bcl-2, Bcl-xL, and CED-9) expressed in plant, prevented apoptosis-like death mediated by chloroplasts photooxidadative stress induced by paraquat (Chen & Dickman, 2004; Mitsuhara et al., 1999).

The *in vivo* relationship between ROS-associated to environmental stress condition like drought and biological nitrogen fixation (BNF) inhibition in the legume–Rhizobium

symbiosis were studied using different dose of paraquat to induce oxidative stress in nodules. Paraquat produced cellular redox imbalance leading to an inhibition of biological nitrogen fixation (BNF). The low paraquat dose provoked BNF decline, preceded by a decrease in sucrose synthase gene expression protein content and activity, while high paraquat induced a faster and more pronounced BNF inhibition, coinciding with a decline in sucrose synthase and also with a reduction in leghaemoglobin content. These results support the occurrence of two regulation pathways for BNF under oxidative stress, one of these involving carbon shortages and the other involving leghaemoglobin /oxygen flux (Marino et al., 2006, 2008).

4.1 Paraquat resistant mutants

To date, several mutants, ecotypes, and biotypes with paraquat resistance have been characterized in a few plant species. Paraquat-resistant mutants have been shown to be cross tolerant to other oxidative stress conditions and have been used to study the tolerance to other photooxidative stress condition (Tsugane et al., 1999).

There are several paraquat-resistant *Arabidopsis* mutants. Photoautotrophic salt tolerate 1 (*pst1*), an *Arabidopsis* mutant that can grow under high salt concentrations, is nearly 10 times more tolerant to paraquat than wild-type seedlings. This mutant, which is also tolerant to high light intensities exhibits higher SOD and APX activities under paraquat, salt, and high light intensities treatments (Tsugane et al., 1999).

The paraquat-resistant *Arabidopsis thaliana* mutant, allelic to the ozone sensitive mutant *rcd1-1*(radical-induced cell death1-1) (Overmyer et al., 2000), called *rcd1-2*, is also tolerant to UV-B and freezing. The tolerance in this mutant is also related to higher levels of the ROS-scavenging enzymes, particularly chloroplastic CuZn-SOD and APX, and also with an increased accumulation of flavonoids (Fujibe et al, 2004). *Arabidopsis* Cvi ecotype also shows a higher resistance to paraquat, which seems to be determined by a new allele of plastidic CuZnSOD (Abarca et al., 2001).

Gigantea, a late-flowering *Arabidopsis* mutant, is resistant to paraquat (Kurepa et al., 1998), however, the resistance mechanism remains unknown (Huq et al., 2000). In the broadleaf weed *Archoteca calendula* (L) paraquat tolerance has been associated with increases in antioxidant defense. This species also exhibit cross tolerance to other stress conditions (Soar et al., 2003).

Arabidopsis paraquat resistant2-1 (par2-1) mutant show an anti-cell death phenotype. Paraquat treatment induce similar superoxide production in *par2-1* and wild-type plants, suggesting that PAR2 acts downstream of superoxide to regulate cell death. *par2-1* encode a S-nitrosoglutathione reductase (GSNOR) that catalyze a major biologically active nitric oxide species, S-nitrosoglutathione. Compared to wild type, *par2-1* mutant showed higher nitric oxide level, suggesting that nitric oxide level and nitrosylation protein modification regulates cell death in plant cells (Chen et al., 2009).

Other paraquat-resistant genotypes have also been reported; like the grass weed *Hordeum glaucum* (Lasat et al., 1997) and *Conyza bonariensis* (Fuerst et al., 1985; Norman et al., 1994). The resistance mechanism seems to be related to a higher herbicide compartmentalization in root vacuoles of the resistant biotype than in the susceptible one. On the contrary, the amount of paraquat accumulated in the cytoplasm of the susceptible biotype was double that found in the resistant biotype.

Additionally, paraquat tolerance has been associated with the expression of transporters able to carry molecules with similar chemical structure or charge distribution to paraquat, like polyamines (Tachihara et al., 2005). Pharmacological treatments with blockers of proton pump ATPases, such us nitrate, carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) and N4N1- dicyclohexylcarbodiimide (DCCD) were used in order to study their effects on paraquat moving into inactive compartments in *C. canadiensis* (Jóri et al., 2007). Recovery after paraquat treatment in tolerant biotypes was strongly inhibited by nitrate, as nitrate selectively blocks ATPases in the vacuoles -responsible for energy supplies to vacuolar membranes- the results suggested that paraquat sequestration uses energy from the proton gradient (Jóri et al., 2007).

Regarding the relationship between paraquat tolerance and leaf age, some studies have shown that young leaves are more tolerant than mature ones (Kuk et al., 2006; Ohe et al., 2005), it is worth nothing that responses are closely related with detoxify mechanism and antioxidative responses as well as with morphological leaf characteristics such as epicuticular wax content and leaf cuticule development which is the first and most significant barrier for foliar-applied chemicals. Although damage originated by paraquat treatment in *Cucurbita spp* varied among cultivars, the injury provoked by herbicide application was lower in younger leaves than in older ones as it was observed by lesser conductivity values and malondialdehide production which indicate membrane damage with cellular leakage and membrane lipid peroxydation respectively. These responses correlated also with higher antioxidant activity and increases in ascorbate content as well as with higher epicuticular wax in young leaves (Yeol Yoon , 2011).

5. Conclusion

Paraquat is potent oxidative stress inducer, which beyond the widely use as desiccant herbicide, it has been a very useful tool in plant biology basic research. Many aspect of oxidative stress in plants, the toxic and signaling roles of ROS, the native and transgenic plant tolerance/susceptibility responses to many environmental stress conditions, the cross tolerance phenomenon and different cell death processes have been studied using paraquat treatments.

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9

Herbicides and the Aquatic Environment

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1. Introduction

The quality of water resources is perhaps currently the most discussed topic when it comes to environmental preservation, since aquatic ecosystems have been suffering changes worldwide in most cases irreversible. Such changes are often associated with human activities such as deforestation, release of industrial and domestic effluents, and even the use of pesticides in agricultural fields, which is one of sources that most contributes to the fall of quality of water resources.

Pesticides are important to the agricultural system. However, it is crucial that they be used with responsibility in order to preserve the quality of the final product and the natural resources that support the production, especially soil and water (Oliveira Junior & Regitano, 2009).

Pesticides are products whose function is to eliminate organisms causing damage to agricultural crops thus ensuring high productivity. Their classification is made according to target species (insecticides, herbicides, fungicides, acaricides, nematicides, etc..) (Alves-Silva & Oliveira, 2003, Sanches et al., 2003), patter of use (defoliants, repellents, and others) (Alves-Silva & Oliveira, 2003; Laws, 1993; Sanches et al., 2003), mechamisns of action (acetylcholinesterase inhibitor, anticoagulants, etc) (Alves-Silva & Oliveira, 2003) or chemical structure (pyretroids, organophosphates, carbamates, etc) (Alves-Silva & Oliveira, 2003; Laws, 1993).

Although these molecules, when applied, have target organisms as their final destination, according to Macedo (2002) 99% of applied pesticides go into the air, water and soil, ie, only 1% reaches its target. This finding is quite disturbing as the world population grows; it means that the use of pesticides will increase (thus increasing food productivity) and natural resources will remain under intense threat from these molecules.

2. Pesticides market in Brazil

Pesticides started to become popular in the middle of the Second World War, when the world discovered the DDT. The ease of accesses of this product and its low cost made it to

be extremely used before the discovery of its negative effects. The great successes of this compound in pest control made new products being produced strengthening the agrochemical industry today (Bull & Hathaway, 1986).

Currently, according to the data from National Health Surveillance Agency Anvisa (2010), Brazil is the largest consumer of pesticides in the world and has the largest market for these products with 107 companies authorized to register this compounds, responding for 16% of the world market. According to the sales in Brasil, only in 2010, the industry negotied 342,590 tons of active ingredients and its clear that this number is increasing in recent years (Figure 1).

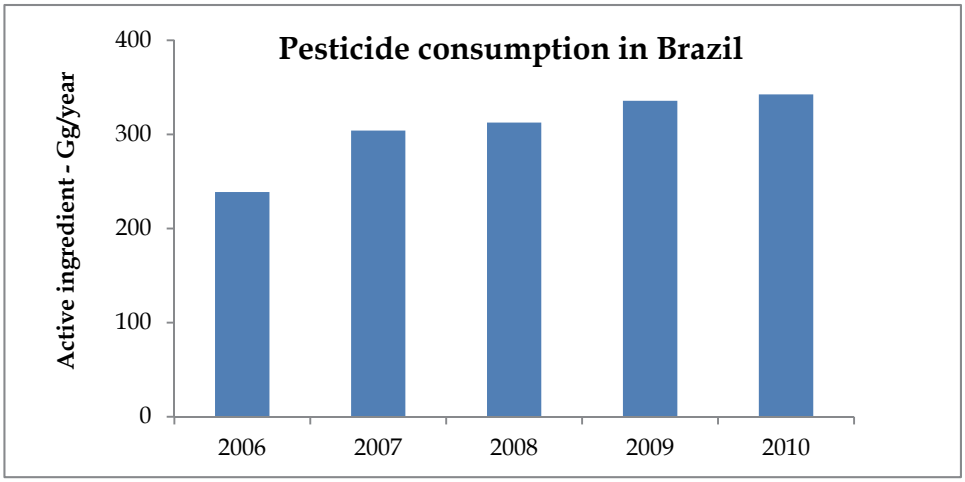

Fig. 1. Pesticide consumption in Brazil, in gig grams of active ingredient, in the period of 2006 to 2010.

Among the classes of pesticides, herbicides are those that make up most marketed worldwide (Moura et al., 2008). These molecules are chemical substances that act by killing or suppressing the development of weeds that impair the productivity of crops of commercial interest (Roman et al., 2007). According to the National Association of Products Industry for Agricultural Defense, only Brazil, one of the leading countries in agriculture with the use of pesticides, 725 000 tons of formulated products were sold in 2009 and herbicides are the main class with 59% (429,693 tons), followed by insecticides and acaricides with 21% (150,189 tons), fungicides 12% (89,889 tons) and others 8% (55,806 tons) (Figure 2) (Sindag, 2010). The problem is that many of these substances are likely to contaminate water resources due to characteristics such as high shift-potential in the soil profile (leaching), high persistence in soil, low to moderate water solubility and moderate adsorption to organic matter present in soil colloids (Almeida et al., 2006). Once present in aquatic environments, these molecules can be absorbed by organisms, and since they live in continual interaction with each other in a complex system of food chains, contamination can result in a drastic imbalance in the ecosystem.

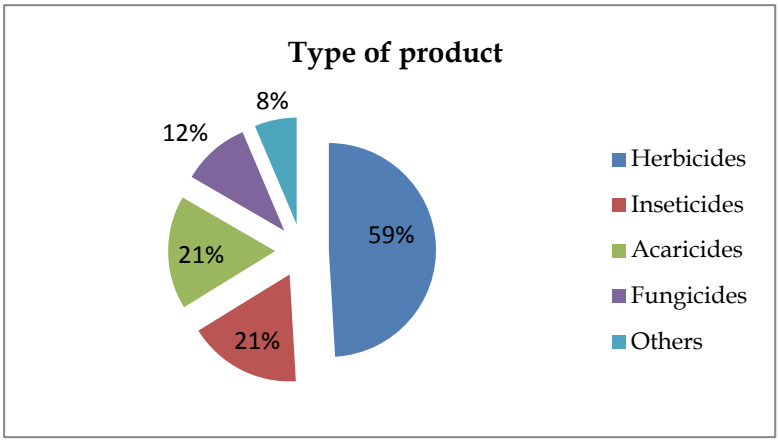

Fig. 2. Pesticide consumption in Brazil by type of product in gig grams of active ingredient, in 2009.

3. Herbicides: leaching and residual effects

Pesticides have an important role in modern agriculture, with new formulations being introduced regularly. Among these, the chlorinated acid-phenoxy herbicides such as 2,4-D and MCPA are commonly used to control weeds in wheat, rice, corn, sugar cane and pasture. The massive use of pesticides has resulted in their presence in the environment in the form of sub-lethal pollution, and problems such as contamination of surface and groundwater have been observed (Legrouri et al., 2005). The concern of environmental protection agencies with the presence of these molecules in soils, water and air has increased greatly in recent times, particularly as it relates to protecting the quality of drinking water (Lagaly, 2001). Due to the commercial importance of agriculture in world and pesticides industry, probably the extensive use of these substances will last for a long period. Therefore, the most feasible would be the rational use of these products through a strict control of its use and handling, aiming, mainly, avoid over dosing, application in undue places and improper washing of packaging and application equipment that many times are held on the banks of rivers (Trovo et al., 2005). Thus, contamination of soils and water due to the extensive use of pesticides over large areas in modern agriculture is a problem that requires research to its remediation (Ignatius et al., 2001).

Considering the transport processes in the environment with which herbicides are related after applied to agricultural areas, leaching and runoff deserve some attention. Surface runoff favors surface water contamination, since the molecule is carried and adsorbed to eroded soil particles or in solution. On the other hand, leaching results in contamination of groundwater, and in this case, chemical substances are carried in solution with the water that feeds the ground water (Spadotto, 2002). Only a low percentage of herbicides in soil are used bioactivity, ie, the remainder is distributed in the environment. This loss of product requires a high amount application, increasing the damage to the environment and consequently to health (Dich et al., 1997).

The knowledge of sorption-desorption processes is of great importance, once determining the amount of product present in the soil is possible to control other processes that can affect the dynamics of these molecules in the soil. If the degree of sorption of a pesticide increases, this compound concentration in water and air decreases. Consequently, the speed of concentration-dependent processes such as volatilization, bioavailability, and vertical movement of pesticides through the soil profile also decrease, thus reducing the risk of contamination of surface and groundwater (Cox et al., 1999).

The aquatic environment has become extremely vulnerable to contamination, Herbicides with high leaching potential, ie, those with low capacity to be retained in the soil are potentially more damaging in this environment by being subject to loading by the underground water flow and deposit with final residual effect on aquatic community. The water pollution is still of concern since often agricultural fields are near lakes, streams and rivers potentiating this environment exposure (Moore et al., 2001) to soluble herbicides. Depend on the physical and chemical characteristics, the residue in water, can bind to the material in suspension, accumulate in the sediment or can be absorbed by aquatic organisms. They can be trasported through the aquatic system by diffusion of water in streans or in the bodies of organisms. Some products may also return to the atmosphere through volatilization. Thus, it is evident that there is a continuous interaction between pesticides, sediment and water, affected by water movement, turbulence and temperature (Nimmo, 1995). This interaction can result in a longer exposure of aquatic organisms to toxic compounds.

Solubility in water is defined as the maximum amount of pure molecule that can be dissolved in water (Lavorenti et al., 2003), being considered the most important physical property related to the transport and fate of organic molecules in aquatic systems, such as herbicides, and also one of determinants of soil sorption coefficient. Thus, herbicides with high solubility have a tendency to be less sorbed to soil colloids (Lavorenti et al., 2003). Therefore, sorption to soil and water solubility becomes important parameters to predict the herbicide trend to move horizontally or vertically in the ground (Extoxnet, 1998).

Another measure of leaching potential for a herbicide is the n-octanol-water partition coefficient (Kow), which measures the hydrophobic or hydrophilic character of a molecule. The Kow is defined as the ratio of the solubility of a compound in octanol (a non-polar solvent) to its solubility in water (a polar solvent). The higher to Kow, the more non-polar the compound (U.S.E.P.A, 2009). In environmental studies, this parameter is also correlated with water solubility, soil sorption coefficient and sediments and bioconcentration in aquatic organisms (Lyman et al., 1990; Sablji et al., 1995; Ran et al., 2002). Herbicides with high log Kow values (> 4.0) or lipophilic tend to accumulate in lipid material, for example, soil organic matter and, consequently, present low mobility (Lavorenti et al., 2003). On the other hand, hydrophilic herbicides (log Kow <1.0) are considered more soluble in water and consequently will present low sorption (Lavorenti et al., 2003) and greater potential for damage to the aquatic community.

Several studies on environmental contamination by pesticides are reported in the literature (Jacomini et al., 2006; Henares et al., 2008). Herbicides of the triazine group, which includes ametryne prometryne, atrazine, simazine, among others, are used worldwide and often detected in samples of soil and water, having high mobility in the environment, lasting

years in the ground, water and organisms (Costa Queiroz et al., 1999; Kolpin et al., 2002; Jacomini et al., 2006).

The herbicide atrazine is one of the most widely used herbicides in Brazil, and its use is registred for sorghum, corn, sugarcane and other crops (Rodrigues & Almeida, 2005). Due to its wide use, high persistence and moderate mobility in soil, this herbicide has been detected in several compartments of the environment, especially in surface waters (Buser, 1990) and groundwater (Dörfler et al., 1997). Highest losses of atrazine have been correlated with the first rain or irrigation after its application (Belamie & Gouy, 1992; Patty et al., 1997; Correia et al., 2007). The shorter the time between herbicide application and irrigation or rainfall, the higher the herbicide transport by leaching.

Several authors highlight the problem of contamination of surface and subsurface waters by atrazine (Buser, 1990; Pick et al., 1992; Dörfler et al., 1997; Yassir et al., 1999) so that its use was banned in European Union in 2003 (Sass & Colangelo, 2006). Jablonowski et al. (2009), conducted studies on the persistence of atrazine for more than 20 years after application. Concentrations were detected on average four times higher in the subsurface compared to surface in soil, indicating high risk of contamination of groundwater, even past the experimental period. Armas et al. (2007) found concentrations of atrazine in surface waters of Corumbataí river (São Paulo, Brazil) above the level permitted by Brazilian law. In the United States, atrazine was found at high incidence in surface water and groundwater; research included 178 streams and over 2700 wells (Kolpin et al., 2002). In Australia, atrazine and its metabolites were also detected in low concentrations in groundwater/surface water in several states (Ahmad et al., 2001).

The herbicide clomazone also has high water solubility and persistence in soil and can reach, under aerobic conditions, more than 270 days (California, 2003; Senseman, 2007). After its application on the soil surface, the product may leach into the deeper layers, presenting a potential risk of groundwater contamination and, consequently, watercourses contamination as well (Santos et al., 2008). The clomazone fate and behavior is influenced by organic matter and texture (Loux & Slife, 1989) with edaphic half-life ranging between 5 and 117 days depending on the type of soil and environmental conditions (Curran et al., 1992; Mervosh et al., 1995; Kirksey et al., 1996). Senseman (2007) reports that clomazone persistence is lower in sandy soils than in clay soils.

Monitoring conducted for three years (2000-2003) in two Brazilian rivers (Vacacaí and Vacacaí-Mirim) in Rio Grande do Sul (Brazilian state) detected the presence of the herbicides clomazone, in higher concentration, quinclorac and propanil (Marchesan et al., 2007).

Santos et al. (2008) in a study conducted in shallow waters around the rice-growing areas in Rio Grande do Sul showed that 90% of samples contained clomazone residues. Bortoluzzi et al. (2006) found the presence of this product in surface water adjacent to tobacco crops. The presence of clomazone in water and soil samples have been reported in the literature not only in Brazil but also in other countries such as Spain (Nevado et al., 2007), Italy (Palmisano & Zambonin, 2000), China (Li et al., 2010), Uruguay (Carlomagno et al., 2010) and United States (Gunasekara et al., 2009).

Another herbicide that has concerned researchers is ametryne, whose half-life is between 50 and 120 days in soil and about 200 days in natural water with pH 7.0 and temperature ranging from 5 to 29 °C. This period has been reported as dangerous to the environment by

the power of contamination of soils and surface water/groundwater by this product. (Cumming et al., 2002; Laabs et al., 2002; Armas, 2006). Ametryne also has potential to contaminate aquatic environments, once in addition to being transported by runoff, this molecule can undergo leaching. Ametryne residues have also been found in surface waters of Brazil (Armas et al., 2007) although Brazilian law has not yet set a permissible limit in surface waters.

Within the broad class of herbicides, there is no doubt that the most commercialized worldwide is glyphosate. Its occurrence in groundwater was cited only once, in Texas, USA, reported by Hallberg (1989) - under review presented by Amarante Junior et al. (2002) - but the concentration measured was not specified. The direct application as herbicide in surface water to eliminate aquatic plants may be responsible for the presence of glyphosate in surface water.

Due to the rapid adsorption to soil, glyphosate is not readily leached, being unlikely the groundwater contamination. On rare occasions, this herbicide has been detected in water samples, but in general, this occurs due to the difficulty of separating the compounds and also by not being considered a serious water contaminant.

In the case of water pollution, glyphosate can be adsorbed by sediments being carried by them. This interaction is normally fast and occurs within 14 days resulting in much slower natural decay process. The Environmental Protection Agency of the United States (USEPA) sets limits of 700 μg/L glyphosate in drinking water as a "health advisory limit". However, across Europe is established the limit of 0.1 mg/L as "maximum allowable concentration" for pesticides in drinking water as individual substances, since total concentration does not exceed 0.5 mg/L (IAEAC, 1994). Due to its broad-spectrum herbicide properties, ie being non-selective, systemic and low toxicity to animals, as discussed, it became one of the most used in the world, increasing the need for implementation of monitoring programs.

Various processes for water treatment have been investigated regarding their efficiency in removal of certain herbicides present in fresh water samples. Among them, the anaerobic degradation, electrochemical destruction by photo-Fenton reactions, adsorption on activated carbon, adsorption on clays saturated with inorganic or organic cations and the sorption of anionic molecules in lamellar double hydroxides (HDLs) through the processes of anion exchange or merge, among others might be cited.

Atrazine degradation by anaerobic microorganisms was studied by Ghosh & Philip (2004). Authors demonstrated that the degradation of this molecule is dependent on the amount of product in the effluent and the high organic content in the effluent reduces its rate of degradation.

Based on the properties of clays and clay minerals, several authors studied the removal of herbicides present in water, such as phenoxyacetic acid (Yurdakoc & Akcay, 2000), 2,4-D (Hermosín & Cornejo, 1992), prometrine (Socias-Viciana et al., 1998), dicamba (Carrizosa et al., 2001), linuron, atrazine, acephate, diazinon (Villa et al., 1999).

4. Ecotoxicology

The pesticide toxicity is quite complex and overall the goal is to determine what concentration in a particular product is toxic to an organism. The manifestation of a toxic effect resulting from a chemical substance may occur at a point distant from where was

found the entry in the medium, since when it reaches a water environment for example, the pollutant can be transported by droplets or particles in suspension thorugh long distances (Pedrozo & Chasin, 2003).

Pollutant toxicity can be expressed by the effective dose or effective concentration (EC_{50} or ED_{50}) which is the amount of a substance affecting half of one group of organisms. Exposure effects to organisms vary according to the product's physico-chemical properties (solubility, chemical reactivity, stability, particle size, etc.) route of exposure (oral, inhalation, dermal), duration and frequency of exposure , species tested (there are differences in susceptibility among species and the type of effect on each one, differences in the effects on individuals of different sex and age, young and elderly are more sensitive than adults), among others (Chasin & Azevedo, 2003). Considering the possibility of contamination in aquatic environments and the need for using herbicides in order to increase agricultural productivity, scientists around the world are working to learn, alert and minimize the effect of these substances in organisms living in these environments. This concern led to the creation of Ecotoxicology, which according to the french toxicologist René Truhaut is the science that studies the effects of natural or synthetic substances on living organisms, populations and communities, animals or plants, terrestrial or aquatic, that make up the biosphere, thus including the interaction of substances with the environment in which organisms live in an integrated context (Plaa, 1982; Niederlehner & Cairns, 1995).

Toxicity tests are used to know the effects of substances in organisms. These represent an important tool in ecotoxicology enabling to determine the toxic effect or not in a particular substance. In the 80's, environmental agencies around the world especially in the United States and Europe began to develop standardized protocols for toxicity test using aquatic organisms (Usepa, 1996; Oecd, 1984-2004). In 1984, the Usepa established the use of organisms for monitoring water quality (USEPA, 1984). Concomitantly, the Organization for Economic Cooperation and Development (OECD) launched a series of test protocols for toxicity to aquatic organisms, including algae, fish and microcrustaceans in Europe. In Brazil, the first initiative to do a focused approach to the subject was in 1975. After this year, other methodologies using groups of organisms have emerged, highlighting algae (Abnt, 1992; Cetesb, 1994), microcrustaceans (Abnt, 1993, 2005; Cetesb, 1994) and fish (Cetesb, 1990).

These tests are used as mechanisms for understanding the effects of anthropogenic impacts on living organisms which act as representative organisms (Campagna, 2005). Toxicity tests allow assessing the environmental contamination by various pollution sources such as agricultural, industrial and domestic waste, chemical products and medicines in general (Marschner, 1999; Lombardi, 2004) and even also detecting the ability of a toxic agent or a mixture to produce deleterious effects showing the extent to which substances are harmful, how and where effects are manifested (Magalhaes & Filho, 2008). They even provide information about the potential danger of a toxic substance to aquatic organisms such as mortality, carcinogenesis, mutagenesis, teratogenesis, behavioral disorders, cumulative physiological, antagonistic and synergistic effects (Baudo, 1987).

The toxicity depends on the susceptibility of the organisms to a particular chemical compound. Different species have different sensitivities according to their feeding habits, behavior, development, physiology and others (Silva & Santos, 2007). Young individuals are usually more susceptible to chemicals than adults, probably due to the diference in degree

of development or detoxification mechanisms (Silva & Santos, 2007). Stressed organisms due to of previous exposure to other toxicants may be more sensitive (Rand & Petrocelli, 1985), a commom scenario in the environment .

Toxicity tests are divided into acute and chronic. The acute test aims to assess the effects on organisms to a short period of exposure, whose goal is determining the concentration of a test substance that produces deleterious effects under controlled conditions. For fish, the observed effect is lethality, from which is determined the toxic agent concentration that causes 50% mortality (LC_{50}). For microcrustaceans there is no mobility from which is calculated the average estimate concentration (EC_{50}) that causes 50% immobility (Rand & Petrocelli, 1985). There are also chronic toxicity tests whose organisms are continually exposed to toxic substances for a significant period of time of their life cycle that can vary from half to two thirds of the cycle (Rand & Petrocelli, 1985). These tests assess sublethal effects such as changes in growth and reproduction, changes in behavior (difficulty in movement, increased frequency of opening of the operculum), physiology, biochemistry and tissue changes (Laws, 1993; Adams, 1995). Chronic toxicity tests directly depend on the results of acute toxicity tests, since sublethal concentrations are calculated from the LC_{50} and EC_{50}. For the choice of test organism are often use the following selection criteria: abundance and availability; significant ecological representation within biocenoses; species cosmopolitanism, knowledge of its biology, physiology and dietary habits; genetic stability and uniformity of its populations; low seasonality index, constant and accurate sensitivity; commercial importance; ease of cultivation in the laboratory and, if possible, species should be native for better representation of ecosystems (Rand & Petrocelli, 1995).

Since Ecotoxicology was created several studies have been made always aiming to evaluate the toxicity of a substance for a particular test organism. For example, Botelho et al. (2009) studied the toxicity of various herbicides for tilapia (*Oreochromis niloticus*) including atrazine, paraquat and some mixtures as alaclhor + atrazine, diuron + MSMA and 2,4D + picloram. The LC_{50} (96 hours) was 5.02 $mg.L^{-1}$ for atrazine. These authors also reported weight loss of organisms at 2.5 and 5.0 $mg.L^{-1}$. In relation to the other products, after 48 hours of exposure, the misture alaclhor + atrazine was the only one that caused 100% of mortality to the organisms. Other studies involving atrazine showed the following LC_{50} values (96 hours): 18.8 $mg.L^{-1}$ for the fish *Cyprinus carpio* (Neskovic et al., 1993), 10.2 mgL^{-1} for *Rhamdia quelen* (Kreutz et al., 2008) and 42.38 mgL^{-1} to *Channa punctatus* (Nwani et al., 2010). In toxicity studies, sensitivity of organisms can vary even if used the same product, as shown in the aforementioned studies with atrazine.

Several other studies involving atrazine has been performed, highlighting Hayes et al. (2002a), which showed that low atrazine concentration (0.1 ppb) stopped the gonadal development of male frogs, confirming the reports of Parshley (2000). In a laboratory study, Hayes et al. (2002b, 2002c) also reported that this herbicide was related to the feminization of *Rana pipiens*. Palma et al. (2009) found that atrazine concentrations affected the reproduction of *Daphnia magna*.

The herbicide atrazine is classified as a toxic agent, carcinogen and hormone disrupter (Friedmann, 2002) which includes potentially carcinogenic compounds to humans (Biradar & Rayburn, 1995). The presence of this product in the environment presents a risk to wildlife and the ecosystem in general, interfering with hormonal activity in animals and human in

low doses. Studies have shown that atrazine can also effect the human reproductive system, decreasing the amount of sperms and increasing the infertility (Pan, 2011).

A research of the University of California analyzed the development of 40 males African frogs since the tadpoles stage to adult phase in water with concentration of atrazine within the limits considered safe by the Environmental Protection Agency (EPA). This group of frogs was compared with another without exposure to contaminated water. Among the frogs developed in the water with the herbicide, 10% became functional females. The others 90% despite having characteristics of males, had low testosterone levels and fertility (Hayes, 2010). Strandberg and Scott-Fordsmand (2002) considering organisms exposed to the herbicide simazine, reported ecological effects, including, bioaccumulation in aquatic organisms.

5. Final remarks

There is a growing choice of weed's chemical management by farmers in many agricultural regions of Brazil and the worldwide. The use of herbicides within technical recommendations offers low risk of contamination of non-target sites; however, when applied intensively and without liability, negative environmental impacts may occur.

The application of leachable products such as atrazine and clomazone concerns researchers. It is necessary to achieve sustainable alternatives to the use of these products: the replacement of non-leachable and less toxic products to the environment or follow the banishment example already performed in some countries.

The adoption of bioremediation techniques to areas already contaminated and investment in pesticide application technology as preventive alternative are some of the possibilities to reduce the waste of these molecules in surface water and groundwater.

The lack of supervision by the authorities in small and large agricultural areas coupled with the lack of knowledge of peoples who apply these products and the facility of acquisition contribute to an intensive use and without responsibility.

If professionals and research groups involved in agribusiness can hardly do for the reduction of environmental impacts from domestic and industrial origin, on the other hand, they have fundamental role in the use and dissemination of Good Agricultural Practice to producers, which will be essential for maintaining the activity in a sustainable way at long term.

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10

Comparative Assessment of the Photocatalytic Efficiency of TiO_2 Wackherr in the Removal of Clopyralid from Various Types of Water

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1. Introduction

Many pyridine derivatives have found widespread application as herbicides. Because of their frequent use, chemical stability and resistance to biodegradation, they are encountered in waste waters, and, due to their hazardous effects on ecosystems and human health, their removal is imperative (Stapleton et al., 2006). With this in mind, we have recently paid significant attention to the study of the model compounds (Abramović et al., 2003; Abramović et al., 2004a, 2004b) and pyridine containing pesticides (Abramović et al., 2007; Šojić et al., 2009; Abramović & Šojić, 2010; Abramović et al., 2010; Guzsvány et al., 2010; Šojić et al., 2010a, 2010b; Banić et al., 2011).

Clopyralid (3,6-dichloro-2-pyridinecarboxylic acid, CAS No. 1702-17-6, $C_6H_3Cl_2NO_2$, M = 192.00 g mol^{-1}) (CLP) is a systemic herbicide from the chemical class of pyridine compounds, i.e., pesticides of picolinic acid. It has been used effectively for controlling annual and perennial broadleaf weeds in certain crops and turf. It also provides effective control of certain brush species on rangeland and pastures. The acidic form of CLP and three CLP salts (triethylamine, triisopropylamine, and monoethanolamine), which are very soluble in water, are commonly used in commercial herbicide products. Its chemical stability along with its mobility allows this herbicide to penetrate through the soil, causing long-term contamination of the ground water, as well as surface water supplies (Cox, 1998; Huang et al., 2004; Donald et al., 2007; Sakaliene et al., 2009). Due to these properties, CLP has recently been reported to occur in drinking water at concentrations above the Permitted Concentration Value of 0.1 μg L^{-1} for an individual pesticide (EU directive 98/83/EC). Although the occurrence of CLP in surface, ground and drinking waters has been widely reported, there are only a few studies concerning with its photocatalytic removal from water (Šojić et al., 2009; Šojić et al., 2010a, 2010b; Tizaoui et al., 2011). These studies showed that the degradation of this herbicide takes place most effectively in the presence of Degussa P25 as photocatalyst. However, several recent studies of photocatalytic activity reported that some cosmetic pigments (TiO_2, Wackherr's

''Oxyde de titane standard'') are even more efficient than TiO_2 Degussa P25 in the photodegradation of phenol (Rossatto et al., 2003; Vione et al., 2005) and herbicides with a pyridine ring (Abramović et al., 2011).

The aim of this work was to study the effect of water type (double distilled (DDW), tap and river water) on the efficiency of TiO_2 Wackherr toward photocatalytic degradation of CLP. First of all, the study is concerned with the transformation kinetics and efficiency of photocatalytic degradation of CLP in DDW. The study encompasses the effects of a variety of experimental conditions such as the effect of the type of irradiation, catalyst loading, the initial concentration of CLP, temperature, pH, presence of electron acceptors, and hydroxyl radical ($^{\bullet}OH$) scavenger on the photodegradation kinetics in DDW. The results were compared to the most often used TiO_2 Degussa P25. An attempt has also been made to identify the reaction intermediates formed during the photo-oxidation process of CLP, using the LC–ESI–MS/MS method. The cell growth activity of CLP alone or in the mixture with its photocatalytic degradation intermediates was evaluated *in vitro* in rat hepatoma and human fetal lung cell line, using colorimetric Sulphorhodamine B assay. Finally, the matrix effect of river and tap water on photocatalytic removal of CLP was also studied.

2. Experimental

2.1 Water samples, chemicals and solutions

All chemicals were of reagent grade and were used without further purification. CLP, 99.4%, pestanal quality, was manufactured by Riedel-de Haën; 85% H_3PO_4 was obtained from Lachema (Neratovice, Czech Republic) and NaOH from ZorkaPharm (Šabac, Serbia). The other chemicals used, such as 30% H_2O_2, *cc* acetic acid and 96% ethanol, were obtained from Centrohem (Stara Pazova, Serbia), $KBrO_3$, $(NH_4)_2S_2O_8$ and 60% $HClO_4$, from Merck, while 99.8% acetonitrile (ACN) and HPLC gradient grade methanol (MeOH) were products of J. T. Baker and humic acids (HUM) technical, of Fluka. All solutions were made using DDW. The pH of the reaction mixture was adjusted using a dilute aqueous solution of $HClO_4$ or NaOH. Aspirin® was purchased from Bayer, doxorubicin (Doxorubicin-Teva®) from Pharmachemie B.V. (Haarlem, Netherlands) and gemcitabine (Gemzar®) from Lilly France S.A. (Fegersheim, France), fetal calf serum (FCS) from PAA Laboratories GmbH (Pashing, Austria), penicillin and streptomycin from Galenika (Belgrade, Serbia), trypsin from Serva (Heidelberg, Germany), and EDTA, trichloroacetic acid (TCA), mercury(II) chloride from Laphoma (Skopje), and tris(hydroxymethyl)amino methane (TRIS) from Sigma Aldrich.

Wackherr's ''Oxyde de titane standard'' (100% anatase form, surface area 8.5±1.0 $m^2\ g^{-1}$, crystallite size 300 nm (Vione et al., 2005) hereafter ''TiO_2 Wackherr'', and TiO_2 Degussa P25 (75% anatase and 25% rutile form, 50 $m^2\ g^{-1}$, about 20 nm, non-porous) were used as photocatalysts.

The tap water sample was taken from the local water supply network (Novi Sad, Serbia). River water, collected from the Danube (Novi Sad, Serbia) in May 2010, was filtered through Whatman filter paper 42 (diameter: 125 mm, pore size: 0.1 μm, ashless) before use. The physicochemical characteristics of the water samples, along with that of DDW are given in Table 1.

Parameter	Water type		
	DDW	Tap water	Danube river
pH	6.5	7.3	7.8
El. conductivity at 25 ^{0}C (μS mL^{-1})	2.9	516	365
TOC (mg L^{-1})	1.04	1.80	5.60
Carbonate hardness (^{o}dH)	0.37	13.06	8.36
HCO_3^- (mg L^{-1})		285	182

Table 1. The physicochemical characteristics of the analysed water types.

2.2 Photodegradation procedures

The photocatalytic degradation was carried out in a cell made of Pyrex glass (total volume of ca. 40 mL, liquid layer thickness 35 mm), with a plain window on which the light beam was focused. The cell was equipped with a magnetic stirring bar and a water circulating jacket. A 125 W high-pressure mercury lamp (Philips, HPL-N, emission bands in the UV region at 304, 314, 335 and 366 nm, with maximum emission at 366 nm), together with an appropriate concave mirror, was used as the radiation source. Irradiation in the visible spectral range was performed using a 50 W halogen lamp (Philips) and a 400 nm cut-off filter. The outputs for the mercury and halogen lamps were calculated to be ca. 8.8×10^{-9} Einstein mL^{-1} min^{-1} and 1.7×10^{-9} Einstein mL^{-1} min^{-1} (potassium ferrioxalate actinometry), respectively. In a typical experiment, and unless otherwise stated, the initial CLP concentrations were 1.0 mM, and the TiO_2 Wackherr loading was 2.0 mg mL^{-1}. The total suspension volume was 20 mL. The aqueous suspension of TiO_2 Wackherr was sonicated (50 Hz) in the dark for 15 min before illumination, to uniformly disperse the photocatalyst particles and attain adsorption equilibrium. The suspension thus obtained was thermostated and then irradiated at a constant stream of O_2 (3.0 mL min^{-1}). During the irradiation, the mixture was stirred at a constant speed. All experiments were performed at the natural pH (~ 3.5), except when studying the influence of the pH on the photocatalytic degradation of the substrate. In the investigation of the influence of electron acceptors, apart from constant streaming of O_2, H_2O_2, $KBrO_3$ or $(NH_4)_2S_2O_8$ was added to the CLP solution to make a 3 mM concentration. Where applicable, ethanol (400 μL) was added as a hydroxyl radical scavenger.

2.3 Analytical procedures

2.3.1 Kinetic studies

For the LC–DAD kinetic studies of the CLP photodegradation, samples of 0.50 mL of the reaction mixture were taken at the beginning of the experiment and at regular time intervals. Aliquot sampling caused a maximum volume variation of ca. 10% in the reaction mixture. Each aliquot was diluted to 10.00 mL with DDW. The obtained suspensions were filtered through a Millipore (Millex-GV, 0.22 μm) membrane filter. The absence of the CLP adsorption on the filters was preliminarily checked. After that, a 20 μL sample was injected and analysed on an Agilent Technologies 1100 Series liquid chromatograph, equipped with a UV/vis DAD set at 225 nm (absorption maximum for CLP), and a Zorbax Eclipse XDB-

C18 (150 mm × 4.6 mm i.d., particle size 5 μm, 25 °C) column. The mobile phase (flow rate 1 mL min^{-1}, pH 2.56) was a mixture of ACN and water (3:7, v/v), the water being acidified with 0.1% H_3PO_4. Reproducibility of repeated runs was around 5–10%.

The total organic carbon (TOC) analysis was performed on an Elementar Liqui TOC II according to Standard US EPA Method 9060A. In studying the influence of the initial pH on the photocatalytic degradation use was made of a combined glass electrode (pH-Electrode SenTix 20, WTW) connected to a pH-meter (pH/Cond 340i, WTW).

2.3.2 Identification of the reaction intermediates

For the LC–ESI–MS/MS evaluation of intermediates, a 1.0 mM of CLP solution was prepared. Aliquots were taken at the beginning of the experiment and at regular time intervals during the irradiation. Then, a 20 μL sample was injected and analysed on an Agilent Technologies 1200 series liquid chromatograph with Agilent Technologies 6410A series electrospray ionisation triple-quadrupole MS/MS. The mobile phase (flow rate 1.0 mL min^{-1}) consisted of 0.05% aqueous formic acid and MeOH (gradient: 0 min 30% MeOH, 10 min 100% MeOH, 12 min 100% MeOH, post time 3 min). Components were separated on an Agilent Technologies XDB-C18 column (50 mm × 4.6 mm i.d., particle size 1.8 μm) held at 50 °C; UV/vis signal of the eluate was monitored at 210 nm, 225 nm and 260 nm (bandwidth 16 nm for each); continuous spectrum in the range from 200 to 400 nm (2 nm step) was also recorded. The eluate was forwarded to the MS/MS instrument without flow splitting. Analytes were ionised using the electrospray ion source, with nitrogen as drying gas (temperature 350 °C, flow 10 L min^{-1}) and nebuliser gas (45 psi), and a capillary voltage of 4.0 kV. High-purity nitrogen was used as the collision gas. Full-scan mode (*m/z* range 100–800, scan time 100 ms, fragmentor voltage 100 V), using positive or negative polarity (depending on compound), was used to select precursor ion for CLP and each degradation product, as well as to examine isotopic peaks distribution (Table 2). Then, product ion scan MS^2 mode (fragmentor voltage 100 V, scan time 100 ms, collision energy 0–40 V in 10 V increments) was used for structure elucidation of each degradation product.

2.4 Cell growth activity

Cell lines. For the estimation of cell growth effects, the cell lines H-4-II-E (rat hepatoma) and MRC-5 (human fetal lung) were grown in RPMI 1640 (H-4-II-E) and DMEM medium (MRC-5) with 4.5% glucose, supplemented with 10% heat inactivated FCS, 100 IU mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin. Investigated cell lines grew attached to the surface. They were cultured in 25 mL flasks (Corning, New York, USA) at 37 °C in atmosphere of 5% CO_2 and 100% humidity, sub-cultured twice a week and a single cell suspension was obtained using 0.1% trypsin with 0.04% EDTA.

Samples and controls used in cell growth experiments. For the analysis of cell growth effects, serial dilutions in distilled water were used. Samples were filtered through a 0.22 μm micro filters (Sartorius) to obtain sterility. The final concentrations of CLP before beginning the irradiation as well as in the CLP solution that was not irradiated were in the range from 6.25 to 100 μM, i.e. the dilution was from 10 to 160. Solution of CLP, filtered suspension of TiO_2 Wackherr catalyst and Aspirin® were used as negative controls, while cytotoxic drugs doxorubicin and gemcitabine, as well as $HgCl_2$ were used as positive controls.

	Compound	t_R (min)	M_{MI} (g mol-1)	UV max. (nm)	Mode	Precursor ion *m/z*	V_{col} (V)	Product ions *m/z*, rel. abundance
1	CLP	1.16	191	222, 281	NI	190	0	190 (82), 146 (100)
							10	146 (100)
							20	146 (100)
2	3,6-Dichloro-4,5-dihydroxypyridine-2-carboxylic acid	0.81	223	216, 278	NI	222	0	222 (51), 178 (100)
							10	178 (100), 142 (48), 106 (11)
							20	178 (71), 142 (100), 106 (40)
							30	142 (29), 106 (100), 78 (61), 66 (50)
3	3,6-Dichloro-pyridin-2-ol*	2.31	163	235, 315	PI	164	0	164 (100)
							10	164 (100), 146 (5), 124 (31)
							20	164 (43), 146 (86), 128 (100), 110 (37), 100 (26), 73 (78)
							30	146 (37), 128 (10), 110 (93), 75 (9), 73 (100), 62 (6)
							40	110 (79), 75 (34), 73 (100), 62 (11)
					NI	162	0	162 (100)
							10	162 (100)
4	3,6-Dichloro hydroxypyridine-2-carboxylic acid*	1.39	207	204, 248, 295	PI	208	0	208 (3), 190 (100)
							10	190 (100), 162 (42)
							20	190 (14), 162 (100), 107 (25)
							30	162 (45), 134 (9), 107 (100), 98 (15)
							40	107 (100), 98 (15)
					NI	206	0	206 (100), 162 (36)
							10	206 (12), 162 (100), 90 (11)
							20	162 (100), 126 (12), 90 (95), 62 (11)
							30	162 (7), 90 (100), 62 (94)
5	6-Chloro-3-hydroxypyridine-2-carboxylic acid or 3-Chloro-6-hydroxypyridine-2-carboxylic acid	1.57	173	233, 307	NI	172	0	172 (100), 128 (9)
							10	172 (54), 128 (100)
							20	128 (100)

Table 2. MS/MS fragmentation data of CLP photodegradation intermediates (Part I).

	Compound	t_R (min)	M_{MI} (g mol^{-1})	UV max. (nm)	Mode	Precursor ion *m/z*	V_{col} (V)	Product ions *m/z*, rel. abundance
6	3,6-Dichloro pyridinediol	1.65	179	~227sh, 310, ~350sh	PI	180	0	180 (100)
							10	180 (100), 162 (6), 144 (11), 88 (12)
							20	180 (69), 162 (52), 144 (29), 107 (50), 98 (7), 89 (12), 88 (100)
							30	162 (9), 107 (100), 89 (9), 88 (53)
							40	107 (100), 98 (10), 89 (15), 88 (39), 72 (7), 53 (7), 52 (13)
					NI	178	0	178 (100), 142 (16)
							10	178 (100), 142 (51), 78 (21)
							20	78 (100)
7	3,6-Dichloro hydroxypyridine-2-carboxylic acid*	0.85	207	220, 265, 312–320**	PI	208	0	208 (100), 190 (24)
							10	190 (100), 162 (57)
							20	190 (12), 162 (100), 134 (11), 107 (59)
							30	162 (18), 107 (100)
							40	134 (10), 107 (100), 98 (7), 83 (8)
					NI	206	0	206 (92), 162 (100)
							10	162 (100), 126 (23), 90 (7)
							20	162 (45), 126 (76), 98 (52), 90 (100)
							30	126 (5), 98 (75), 90 (100), 66 (8), 62 (49)

* previously identified using TiO_2 Degussa P25
** wide spectral band
M_{MI} – monoisotopic weight

Table 2. MS/MS fragmentation data of CLP photodegradation intermediates (Part II).

Sulphorhodamine B (SRB) assay. Cell lines were harvested and plated into 96–well microtiter plates (Sarstedt, Newton, USA) at a seeding density of 4 x 10^3 cells per well (Četojevic-Simin et al., 2011), in a volume of 180 µL, and preincubated in complete medium supplemented with 5% FCS, at 37 °C for 24 h. Serial dilutions and solvent were added (20 µL/well) to achieve the required final concentrations and control. Microplates were then incubated at 37 °C for additional 48 h. Cell growth was evaluated by the colorimetric SRB assay according to Skehan et al. (1990). Cells were fixed with 50% TCA (1 h, +4 °C), washed with distilled water (Wellwash 4, Labsystems; Helsinki, Finland) and stained with 0.4% SRB (30 min, room temperature). The plates were then washed with 1% acetic acid to remove unbound dye. Protein-bound dye was extracted with 10 mM TRIS base. Absorbance was measured on a microplate reader (Multiscan Ascent, Labsystems) at 540/620 nm. The effect on cell growth was expressed as a percent of the control, and calculated as: % Control = (At/Ac) x 100 (%), where At is the absorbance of the test sample and Ac is the absorbance of the control.

Statistical analysis. The results of cell growth activity were expressed as mean ± SD of two independent experiments, each performed in quadruplicate (n = 8). Differences between control and treated groups were evaluated using one-way analysis of variance at the significance level of $p < 0.05$ (Microsoft Office Excel 2003 software). IC_{50} values were calculated using Calcusyn for Windows (Version 1.1.0.0.; Biosoft).

3. Results and discussion

3.1 Effects of the type of TiO_2

The photocatalytic activity of TiO_2 Wackherr was compared to that of the most often used Degussa P25 under UV and visible irradiation. As can be seen from Figure 1, practically no degradation was observed under the visible light irradiation, either in the presence or absence of TiO_2. The lack of CLP disappearance in the presence of TiO_2 under these conditions also allows the exclusion of a significant adsorption of CLP on the catalyst surface during the course of the irradiation. In contrast, significant CLP removal could be observed under UV, and the process involving TiO_2 Wackherr was slightly faster compared to that observed in the presence of Degussa P25. This insignificant acceleration of the degradation of CLP in the presence of TiO_2 Wackherr is noteworthy, considering that this TiO_2 specimen has much larger particles (average radii in solution are 3–4 times larger) than Degussa P25 and a surface area that is almost six times lower (Vione et al., 2005).

The direct photolysis of CLP was also checked under the adopted irradiation conditions, in the absence of a catalyst (Figure 1). It appears that CLP can be degraded by direct photolysis in the near UV region, but at a significantly lower rate compared to the photocatalytic process.

Under the relevant experimental conditions, the reaction followed a pseudo-first order kinetics. On the basis of the kinetic curves ln$c$ (substrate concentration) vs. t, the values of the pseudo-first order rate constant k' were calculated. The degradation rate of CLP was calculated for all the investigated as the product $k' c_0$, where c_0 is the initial concentration of CLP.

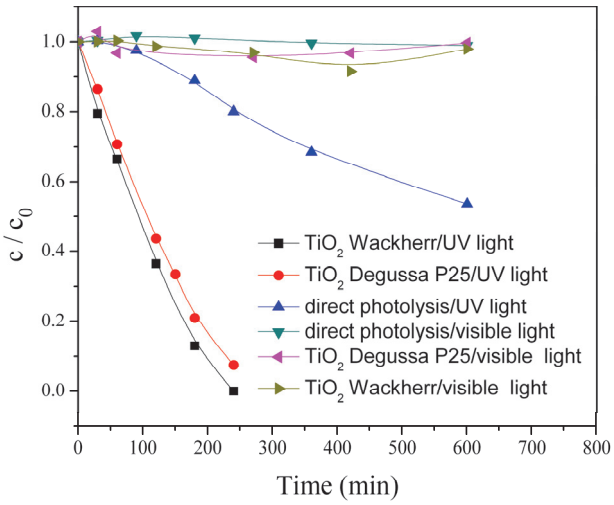

Fig. 1. Kinetics of the photolytic and photocatalytic degradation of CLP (1.0 mM). When present, the TiO_2 loading was 2.0 mg mL^{-1}. Operation conditions: t = 40 °C at pH ~ 3.5.

A comparison of the mineralisation capacities of TiO_2 Wackherr and Degussa P25, presented in Figure 2, shows that the mineralisation efficiency in the presence of TiO_2 Wackherr is significantly higher — 90% of CLP was mineralised during 240 min, whereas in the case of Degussa P25 only about 60%. Also, the ratio of the removal rate of the parent compound and total mineralisation in the case of TiO_2 Wackherr is about 1.20, whereas in the case of P25 this ratio is significantly higher, amounting to even 2.80. If these results are compared with those of the photocatalytic degradation of pyridine pesticides such as picloram and triclopyr (Abramović et al., 2011) it can be seen that the ratios of the mineralisation rate to the rate of the parent compound degradation are different and that they depend on the type of the substituent in the pyridine ring. Namely, in the photocatalytic degradation of triclopyr, TiO_2 Degussa P25 showed a higher efficiency both in the process of mineralisation and degradation of the parent compound. In the case of picloram, TiO_2 Wackherr showed higher photocatalytic efficiency than Degussa P25. However, the ratio of the rates of removal of the parent compound and total mineralisation in the presence of TiO_2 Wackherr is much higher in the case of picloram than of CLP, and it amounts to about 9, whereas in the presence of Degussa P25 this ratio is about 3, which is similar to the value obtained also for CLP.

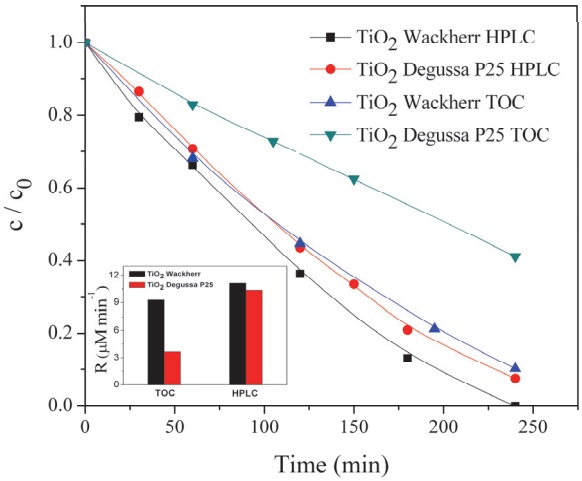

Fig. 2. Comparison of the photocatalytic removal parent compound and mineralisation of CLP. The inset shows the degradation rate (R) calculated over 240 min of irradiation. Operation conditions: $c(CLP)_0$ = 1.0 mM, TiO_2 = 2.0 mg mL^{-1}, t = 40 °C, at pH ~ 3.5.

3.2 Effect of catalyst loading

Due to the inherent nature of heterogeneous photocatalytic systems, there is always an optimum catalyst concentration at which the removal rate is at its maximum. In this study, the optimum was determined by changing the concentration of TiO_2 Wackherr over the loading range from 0.2 to 2.0 mg mL^{-1}, as shown in Figure 3. As can be seen from the figure inset, the increase in TiO_2 loading up to 1.0 mg mL^{-1} was accompanied by an increase in the degradation rate, but a further increase caused an opposite effect. Theoretically, the increase in the catalyst loading above an optimum value has no effect on the photodegradation rate since all the light available is already utilized. However, higher loading of TiO_2 led to the aggregation of its particles and thus to a decrease in the contact surface between the reactant and photocatalyst particles, which caused a decrease in the number of active sites, resulting in a lower rate of photodegradation. Also, when TiO_2 is overdosed, the intensity of the incident UV light is attenuated because of the decreased light penetration and increased scattering, which attenuates the positive effect coming from the dosage increment, and therefore the overall performance decreases (Wong & Chu, 2003). Optimum catalyst concentration is a complex function of a number of parameters including catalyst agglomeration, the suspension opacity, light scattering, mixing, reactor type, and the pollutant type (Toor et al., 2006; Mendez-Arriaga et al., 2008); hence it is not constant for all photocatalytic systems. Indeed, optimum catalyst concentrations have been reported to vary between as low as 0.1 mg mL^{-1} to as high as around 10 mg mL^{-1} (Mendez-Arriaga et al., 2008; Alhakimi et al., 2008; Chu et al., 2009a). An optimum catalyst concentration of around 1 mg mL^{-1} has been generally reported in many studies (Chen & Ray, 1998; Lu et al., 1999; Mendez-Arriaga et al., 2008; Rajeswari & Kanmani, 2009; Tizaoui et al., 2011). However, by comparing these results with our previously finding that the optimal catalyst loading of

TiO_2 Degussa P25 was 4.0 mg mL^{-1} (Šojić et al., 2009), it can be concluded that the effect of catalyst loading on the efficiency of the photocatalytic removal of CLP is influenced by the type of TiO_2 used. If we compare the efficiencies of the two catalysts at their optimal loadings (i.e. 1.0 mg mL^{-1} for TiO_2 Wackherr and 4.0 mg mL^{-1} for Degussa P25), it comes out that TiO_2 Wackherr, although being present in a lower amount, is more efficient in the removal of CLP.

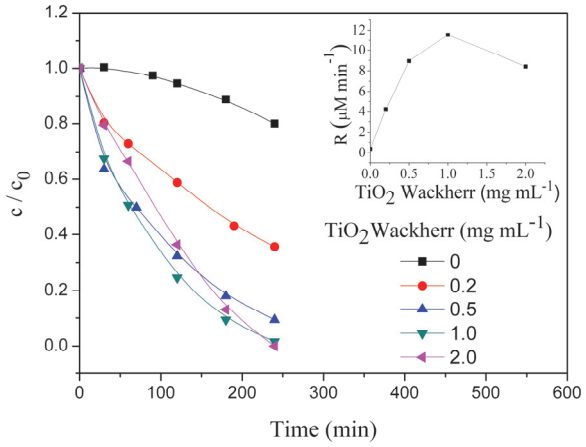

Fig. 3. Effect of the TiO_2 Wackherr catalyst loading on the kinetics of CLP photodegradation. The inset shows the effect of TiO_2 Wackherr loading on the degradation rate (R) determined after 120 min of irradiation. Operation conditions: $c(CLP)_0$ = 1.0 mM, t = 40 °C at pH ~ 3.5.

3.3 Effect of the initial CLP concentration

Many studies have shown that the initial pollutant concentration has a significant effect on the rate of its photocatalytic removal. In this work, the effect of the initial concentration of CLP on the photodegradation rate was studied under UV light using TiO_2 Wackherr in the loading range from 0.25 to 1.0 mM (Figure 4). As can been seen from the inset of Figure 4, the degradation rate decreased with increase in the CLP concentration above to 0.5 mM. Such behaviour may be explained by the fact that at an increased concentration of CLP more of its molecules can be adsorbed on the photocatalyst surface, needing thus a larger catalyst area for their degradation. However, as the intensity of light, irradiation time and amount of catalyst are constant, the relative amounts of O_2^- and $^{\bullet}OH$ radicals on the catalyst surface do not increase (Atiqur Rahman & Muneer, 2005; Qamar et al., 2006).

An alternative explanation for the effect of the substrate concentration is the competition for reactive species between the substrate and the transformation intermediates, the concentration of which increase with increasing substrate concentration, or the poisoning of the photocatalyst surface by the intermediates themselves (Abramović et al., 2011).

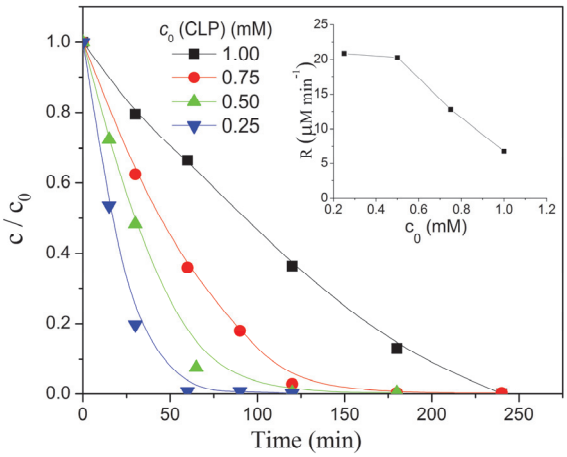

Fig. 4. Effect of the initial CLP concentration on the kinetics of photodegradation. The inset shows the effect of initial CLP concentration on the CLP degradation rate (R) calculated for 60 min of irradiation. Operation conditions: TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 40 °C at pH ~ 3.5.

3.4 Effect of temperature

The photocatalytic degradation of CLP was studied in a temperatures range from 25 to 40 °C (Figure 5) and the rate constant k' was determined from the pseudo-first order plots. In general, the rate constant is expected to increase at higher temperatures, but it appeared that CLP more easily degraded at lower temperatures in the TiO_2 Wackherr suspension. Thus, the decrease in the rate constant observed in this temperature range may be attributed to the physisorption between the TiO_2 surface and the CLP molecules (Ishiki et al., 2005). Namely, the temperature of fastest CLP removal was, surprisingly, 25 °C, and hence all the further measurements were carried out at this temperature. The energy of activation, E_a, was calculated from the Arrhenius plot of ln$k$ versus $1/T$ (K^{-1}), and it amounted to 37.9 kJ mol^{-1}. Obviously, this value is somewhat higher than that obtained for the photocatalytic degradation of CLP in the presence TiO_2 Degussa P25 (Šojić et al., 2009), but it is acceptable since for TiO_2 photocatalyst, irradiation is the primary source of the electron-hole pair generation at ambient temperature, as the band gap energy is too high to be overcome by thermal activation (Topalov et al., 2004).

3.5 Effect of the initial pH

The effect of pH is very important in the heterogeneous photocatalytic removal of organic molecules since it influences both the surface charge of TiO_2 and the ionic form of the reactant, influencing thus the electrostatic interactions between the reactant species and the catalyst surface. Moreover, the pH influences the sizes of TiO_2 aggregates, interaction of the

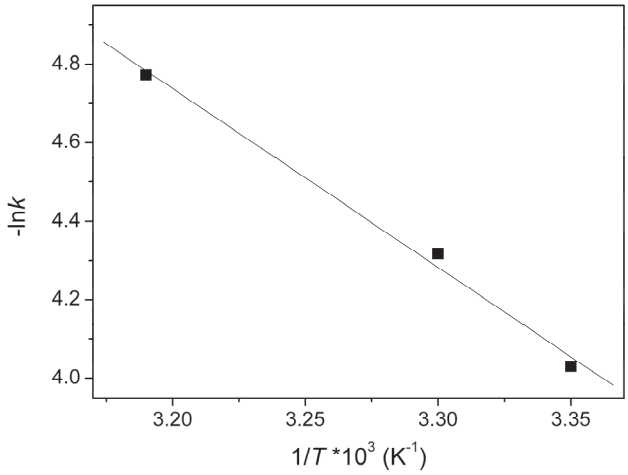

Fig. 5. Arrhenius plot of ln*k* versus 1/*T* for the photocatalytic degradation of CLP for the first 120 min of irradiation. Operation conditions: $c(\mathrm{CLP})_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1} at pH ~3.5.

solvent molecules with the catalyst and the type of radicals or intermediates formed during the photocatalytic reaction (Muneer & Bahnemann, 2002; Šojić at al., 2009; Tizaoui et al., 2011). All of these factors have a significant effect on the adsorption of solutes on TiO_2 surfaces and, as a result, on the observed photodegradation rates. Because the real effluent stuff of pesticide can be discharged at a different pH, the pH effect on the photocatalytic rate degradation of CLP was studied in the pH range from 2.4 to 9.8 (Figure 6). The point of zero charge (pH_{pzc}) of anatase is 5.8 (Karunakaran & Dhanalakshmi, 2009). Thus, the TiO_2 surface will be positively charged ($TiOH_2^+$) in acidic media (pH < pH_{pzc}) and negatively charged (TiO^-) in alkaline media (pH > pH_{pzc}). On the other hand, the pK_a values for CLP are 1.4±0.1 and 4.4±0.1 (Corredor et al., 2006), so that at pH < 1.4 the herbicide is mainly present in its protonated form, and at pH > 4.4 in the anionic form. In the pH interval from 2.4 to 3.5, one can expect a great increase in the photodegradation rate, arising as a consequence of the dissociation of the carboxylic group and deprotonation of the pyridine nitrogen (to a significantly smaller extent). In this way, favourable electrical forces are generated that are manifested as the attraction between the positively charged surface of the catalyst and CLP anion. As can be seen in Figure 6 (inset), in the pH interval from 3.5 to 4.8, a distinct decrease of the photodegradation rate is observed, arising probably as a consequence of the decrease in the number of positive sites on the catalyst surface. A further increase in the pH up to 9.8 caused a decrease in the photodegradation rate, which was probably a consequence of the influence of several factors. Namely, at pH > pH_{pzc}, the TiO_2 surface is negatively charged, causing the repulsion of the CLP anion. Besides, unfavourable electrical forces are generated, i.e., the repulsion between the negatively charged surface of the catalyst and OH^-.

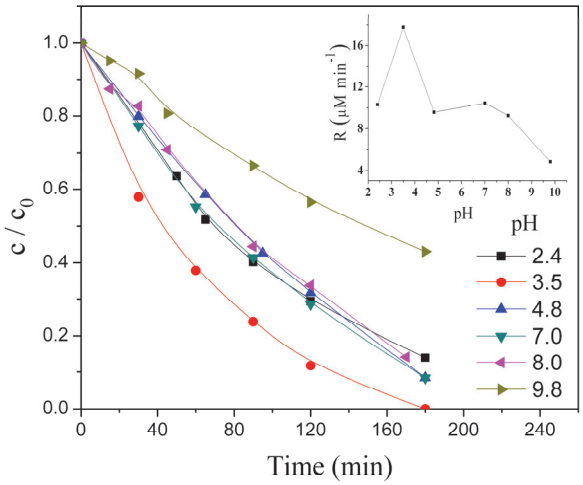

Fig. 6. Effect of the pH on the kinetics of CLP photocatalytic degradation. The inset shows the effect of pH on the degradation rate (R) calculated for 120 min of irradiation. Operation conditions: c $(CLP)_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 25 °C.

3.6 Effect of electron acceptors

A practical problem arising in the use of TiO_2 as a photocatalyst is the undesired e^-–h^+ pair recombination. One strategy to inhibit e^-–h^+ pair recombination is to add other (irreversible) electron acceptors to the reaction mixture. They may have several different effects such as (1) to increase the number of trapped electrons and, consequently, avoid recombination, (2) to generate more radicals and other oxidising species, (3) to increase the oxidation rate of intermediate compounds, and (4) to avoid problems caused by low oxygen concentration. In highly toxic wastewater, where the degradation of organic pollutants is the major concern, the addition of electron acceptors to enhance the degradation rate may often be justified (Singh et al., 2007). The rates of photocatalytic degradation and mineralisation of CLP in the presence of various electron acceptors such as $KBrO_3$, H_2O_2, and $(NH_4)_2S_2O_8$ in addition to the molecular oxygen are shown in Figure 7.

As can be seen, the mentioned electron acceptors showed different effects. Namely, only the addition of $KBrO_3$ enhanced the rate of photocatalytic degradation of the parent compound (by a factor of 1.4), indicating that this compound is a more effective electron acceptor compared with other oxidants employed in this study. A possible explanation might be the change in the reaction mechanism of the photocatalytic degradation, since the reduction of BrO_3^- by electrons does not lead directly to the formation of $^{\bullet}OH$, but rather to the formation of other reactive radicals or oxidising reagents e.g. BrO_2^- and HOBr. Furthermore, BrO_3^- by themselves can act as oxidising agents (Singh et al., 2007). However, the mineralisation rate is slightly lower (by a factor of 1.1).

However, the presence of H_2O_2 caused a decrease in both the rate of removal of CLP (by a factor of 1.7) and its mineralisation (by a factor of 1.3). Such a negative effect of H_2O_2 is probably a consequence of the fact that it can also act as an $^{\bullet}OH$ scavenger, generating much less reactive hydroperoxyl radicals ($HO_2^{\bullet}$). The $HO_2^{\bullet}$ can further react with the remaining strong $^{\bullet}OH$ to form ineffective oxygen and water. Besides, at a higher dose, H_2O_2 might absorb and thus attenuate the incident UV light available for the photocatalysis process (Chu & Wong, 2004; Muruganandham & Swaminathan, 2006).

The presence of $S_2O_8^{2-}$ had an insignificant effect on the rate of degradation of CLP, but it decreased the rate of mineralisation more than $KBrO_3$ and H_2O_2, which can be explained by an increase in the concentration of SO_4^{2-} adsorbed on the TiO_2 surface, reducing thus the catalytic activity. The excess of adsorbed SO_4^{2-} also reacts with the photogenerated holes and with the $^{\bullet}OH$ (San et al., 2001; Muruganandham & Swaminathan, 2006).

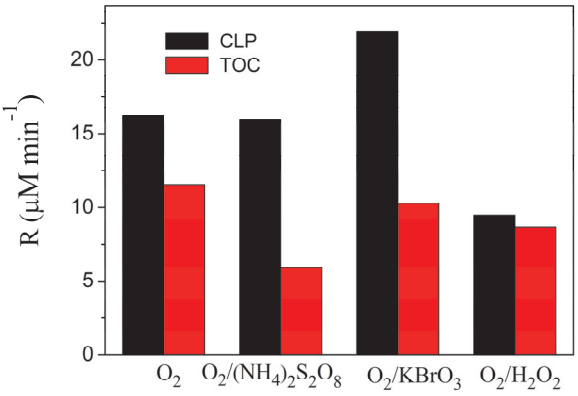

Fig. 7. Comparison of the degradation rate (R) of CLP removal and mineralisation in the presence of different electron acceptors (3 mM) calculated for 60 min of irradiation. Operation conditions: $c(CLP)_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 25 °C, pH ~3.5.

3.7 Effect of $^{\bullet}OH$ scavenger

In order to investigate whether the heterogeneous photocatalysis takes place via $^{\bullet}OH$, ethanol was added to the reaction mixture. Namely, it is known that alcohols, e.g. ethanol, act as $^{\bullet}OH$ scavengers (Daneshvar et al., 2004). The results obtained (data not shown) indicate that the degradation rate was significantly slower (by about 100 times) compared to that observed in the absence of ethanol, which proves that the reaction of photocatalytic degradation proceeded via $^{\bullet}OH$.

3.8 Intermediates and the mechanism of photodegradation

The LC-MS analysis of the irradiated CLP solutions indicated the formation of six intermediates (labelled 1–7, Table 2), whose kinetic curves are shown in Figure 8. Three of

them, 3,6-dichloro-pyridin-2-ol (compound **3**) and isomeric 3,6-dichloro hydroxypyridine-2-carboxylic acids (compounds **4** and **7**) were previously identified (Šojić et al., 2009) in the presence of TiO_2 Degussa P25. Using the positive and negative ionization MS^2 spectra, it was possible to identify the remaining compounds and propose a photocatalytic degradation scheme (Figure 9).

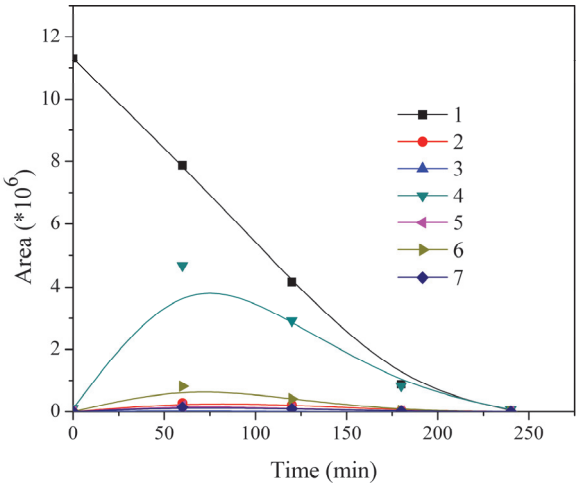

Fig. 8. Kinetics of the appearance/disappearance of CLP and intermediates in the photocatalytic degradation of CLP monitored by LC-ESI-MS/MS. Operation conditions: $c(CLP)_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 25 °C, pH ~3.5.

Compound **2**, eluting at 0.81 min, had M_{MI} 223, two chlorine atoms (on the basis of the A+2 isotopic peak intensity, as well as two consecutive losses of HCl in the MS^2 spectra: 178→142 and 142→106) and an odd number of nitrogen atoms (odd molecular weight), and was visible only in negative mode. In both the first-order and second-order MS spectra, the loss of CO_2 was observed (222→178), pointing out to the presence of carboxylic group. On the basis of the molecular weight (32 units higher than that of CLP) and spectral data, it was concluded that the compound is 3,6-dichloro-4,5-dihydroxypyridine-2-carboxylic acid.

Compound **5** eluted at 1.57 min. On the basis of the A+2 isotopic peak intensity and molecular weight, it could be concluded that it contains one chlorine atom and an odd number of nitrogen atoms. The only fragmentation observable in the NI MS^1 and MS^2 spectra was the loss of the carboxylic group as CO_2 (172→128). The monoisotopic weight of 173 mass units could be explained by the loss of one chlorine atom (which is in agreement with the isotopic profile) from the CLP molecule and introduction of one hydroxyl. Thus, the compound was identified as either 6-chloro-3-hydroxypyridine-2-carboxylic acid or 3-chloro-6-hydroxypyridine-2-carboxylic acid.

Finally, compound **6** was characterized by the odd monoisotopic weight of 179 units (pointing out to the odd number of nitrogen atoms), presence of two chlorine atoms, and the absence of carboxylic group loss both in positive mode (no sequential loss of H_2O and CO)

and in negative mode (no loss of CO_2 or •COOH). Based on the molecular weight (12 units lower than that of CLP, which corresponds to the loss of COO and the introduction of two oxygen atoms), the compound was identified as 3,6-dichloro pyridinediol (the exact positions of hydroxyls could not be determined).

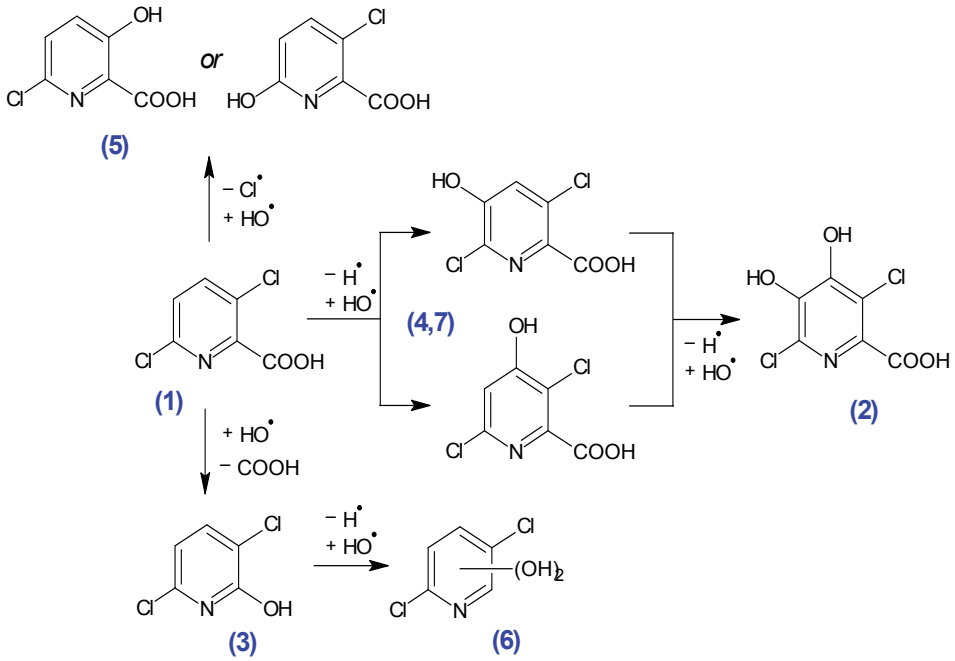

Fig. 9. Tentative pathways for photocatalytic degradation of CLP.

3.9 Cell growth activity

The cell growth activity of CLP, as well as of the mixture of CLP and its photocatalytic degradation intermediates was evaluated *in vitro* in a panel of two cell lines: H-4-II-E (rat hepatoma) and MRC-5 (human fetal lung) at 160, 80, 40, 20 and 10-fold dilutions that correspond to 6.25, 12.5, 25.0, 50.0 and 100 μM concentrations of CLP at the beginning of experiment (before the irradiation process). The toxicity was evaluated using the SRB assay (Skehan et al., 1990), which determines both specific rate of protein synthesis and cell growth rate.

Cell growth inhibition of CLP reached 5 to 7% in the MRC-5 and H-4-II-E cell line, respectively (Figure 10). The reaction mixture obtained after different irradiation times showed a higher toxicity toward the MRC-5 cell line compared to the parent compound after 120 min of irradiation at 20-fold dilution and after 240 min in the whole concentration range (Figure 10b). A comparison of the evolution of toxicity and degradation kinetics indicates that the toxicity toward the MRC-5 cell line was mildly increased after 120 min of irradiation at higher concentrations, i.e. at 20-fold dilution, and after 240 min in the whole concentration range. This implies that irradiation longer than 120 min contributed to the

concentration of toxic degradation intermediates and to the toxicity of the mixture that is no longer dominated by the parent compound.

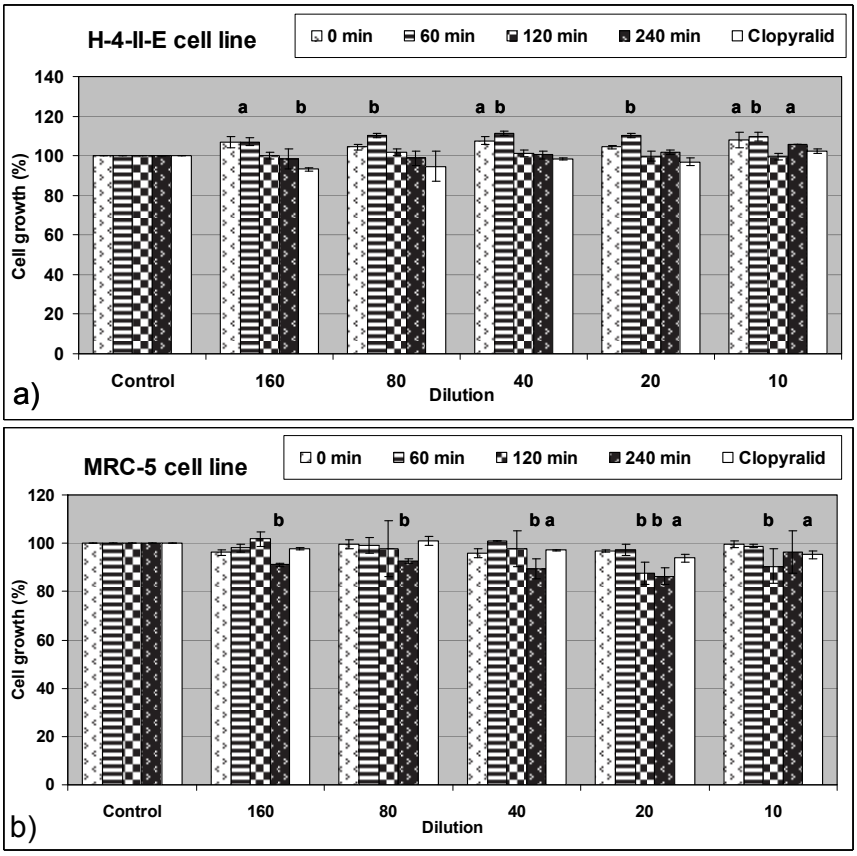

Fig. 10. Cell growth activity of the serial dilutions of CLP and its photocatalytic degradation intermediates obtained after different irradiation times in a) H-4-II-E and b) MRC-5 cell line. (One-way analysis of variance, compared to the control; **a**: $p < 0.05$, **b**: $p < 0.01$). Results are expressed as mean ± SD of two independent experiments, each performed in quadruplicate ($n = 8$).

On the other hand, in the H-4-II-E cell line, the reaction mixture obtained after 60 minutes of irradiation produced significant ($p < 0.01$) stimulation of cell growth compared to control (Figure 10a). The effects that are not concentration-dependent may be explained by the well known concept of hormesis (low dose stimulation and high dose inhibition). Since hormetic effects have been reported in a highly diverse array of biological models, for numerous organs and endpoints and chemical/physical stressors, it is evident that no single mechanism can account for these phenomena. In pharmacology, such dose responses have been studied with the aid of synthetic agonists and antagonists of receptors which mediate hormetic biphasic effects (Calabrese & Baldwin, 2001). A single agonist with differential

binding (i.e. high and low receptor affinities) that affects two opposite acting receptors will induce hormetic-like biphasic dose responses in numerous biological systems, as has been shown for dozens of receptor systems. Pollutants, for example, may initiate significant changes in complex receptor systems, and affect biphasic dose responses by inducing changes in the concentrations of endogenous agonists. When such changes occur over a broad dose range, biphasic dose responses typically become manifested (Calabrese, 2005).

The IC_{50} values of Aspirin®, two well known cytotoxic drugs (Doxorubicin® and Gemcitabine®) and $HgCl_2$ (Table 3), as well as cell growth inhibition of TiO_2 Wackherr (Figure 11) were obtained in the same panel of cell lines.

Cell line	IC_{50} (μM)			
	ASP[a]	DOX[b]	GEM[c]	$HgCl_2$
H-4-II-E	>5551	0.272	0.004	3.189
MRC-5	>5551	0.408	0.384	69.578

[a]Aspirin® ; [b]Doxorubicin®; [c]Gemcitabine®

Table 3. IC_{50} values (μM) of Aspirin®, Doxorubicin®, Gemcitabine® and $HgCl_2$ in selected cell lines.

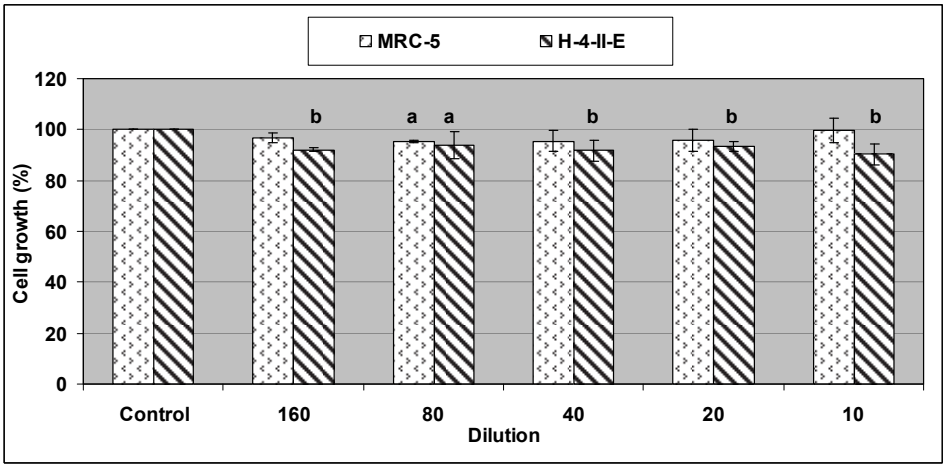

Fig. 11. Cell growth activity of different concentrations of TiO_2 Wackherr catalyst in the MRC-5 and H-4-II-E cell lines. (One-way analysis of variance, compared to the control; **a**: $p<0.05$, **b**: $p < 0.01$). Results are expressed as mean ± SD of two independent experiments, each performed in quadruplicate ($n = 8$).

In order to check whether the cell growth activity presented in Figure 10 is a consequence of the presence of CLP and its degradation intermediates only, it was necessary to run a blank test. To this end, aqueous suspension of TiO_2 Wackherr (2 mg cm^{-1} without CPL) was sonicated in the dark for 15 min, as in the case of photodegradation of CLP, filtered through Millipore membrane filter, to apply then the same dilutions from 10 to 160. There were no significant effects ($p < 0.01$) on the growth of MRC-5 cell line. On the other hand, the

H-4-II-E cell line appeared to be more sensitive to the presence of catalyst compared to the MRC-5 cell line (Figure 11). In the H-4-II-E cell line, the inhibition of cell growth influenced by TiO_2 Wackherr was significantly different compared to control ($p < 0.01$), even at the 160 dilution (Figure 11), but in all cases was below 10%. Solvent (DDW) was also tested after different irradiation times and it was shown to be nontoxic, i.e. all values were at the level of control that was treated with DDW (data not shown).

The effects of examined samples on the growth of selected cell lines were dependent on the type of cell line, concentration and time of irradiation. It can be concluded that CLP and reaction mixture of CLP and its photocatalytic degradation intermediates effected mildly the cell growth of both cell lines. In the examined concentration range, none of the treatments produced cell growth inhibition higher than 50%, i.e. the IC_{50} values were not reached, either by the parent compound nor by samples obtained after different irradiation times. All examined samples exhibited lower toxicity in selected cell lines compared to the cytotoxicity of controls and $HgCl_2$.

A low level of free oxygen species is necessary for the promotion of cell proliferation (Burdon & Gill, 1993; Wei & Lee, 2002). The redox alterations play a significant role in a signal transduction pathway important for cell growth regulation. It is reasonable to propose that the examined samples obtained using UV irradiation in the presence of O_2 and TiO_2 Wackherr catalyst might influence the cell redox state, altering the cell proliferation.

Multi-endpoint bioassays that are based on whole cell response in human cell lines are a powerful indicator of metabolic, biochemical and genetic alterations that arise under the influence of evaluated compounds. This study presents an example of a systematic and simple first tier method to assess the toxicity of degradation products.

3.10 Effect of water type

Since natural aquatic systems contain dissolved organic matter (DOM) and different ionic species, it can be expected that they may complicate the photodegradation process. It has been reported that higher contents of inorganic and organic matter in tap and river water affect the efficiency of removal by UV/TiO_2 process (Buxton et al., 1988). To functionalise a TiO_2 water treatment process, the basic understanding of the effect of these inorganic ions on the photocatalytic performance is essential (Crittenden et al., 1996). Due to the zwitter ionic nature of the TiO_2 particles, it is also possible that the pH might have a profound effect on the selective inhibition of inorganic ions on the surface of the TiO_2 particles (Guillard et al., 2003). In this study, the effect of the matrix on the photocatalytic degradation of CLP was studied on the example of drinking and Danube water. As can be seen from Table 4, the rate of CLP removal from the samples of tap and river water was by about two/three times slower than that from DDW. The observed decrease in the degradation rate can be a consequence of the presence of HCO_3^- and HUM in the examined water samples (Neppolian et al., 2002). Namely, the addition of HCO_3^- and HUM to DDW in the amounts present in Danube water (Table 1), caused a decrease in the rate of photocatalytic degradation compared to that observed in DDW. As can been seen in Table 4, the concentration of HCO_3^- in the examined tap water was somewhat higher, whereas the river water contained more HUM.

	HCO_3^- (mg L^{-1})	HUM (mg L^{-1})	R (μM min^{-1})
DDW			10.44
DDW	285	5	4.98
Tap water			5.41
DDW	182	15	4.51
Danube water			3.82

Table 4. The influence of water type on the degradation rate (R) of CLP determined after 120 min of irradiation. Operation conditions: $c(CLP)_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 25 °C, pH ~7.0.

In the literature, the inhibition of photocatalytic properties in the presence of ions is often explained by the scavenging of •OH radical by ions. Of ionic species, HCO_3^- can especially, inhibit the degradation rate due to the high rate constant k' of its reaction with •OH (8.5×10^6 M^{-1} s^{-1}) (Buxton et al., 1988). Because of that we focused our attention on the influence of different concentrations of this ion on the photocatalytic degradation (Figure 12). Expectedly, an inhibition of CLP degradation was observed after adding HCO_3^- to DDW up to about 285 mg L^{-1}.

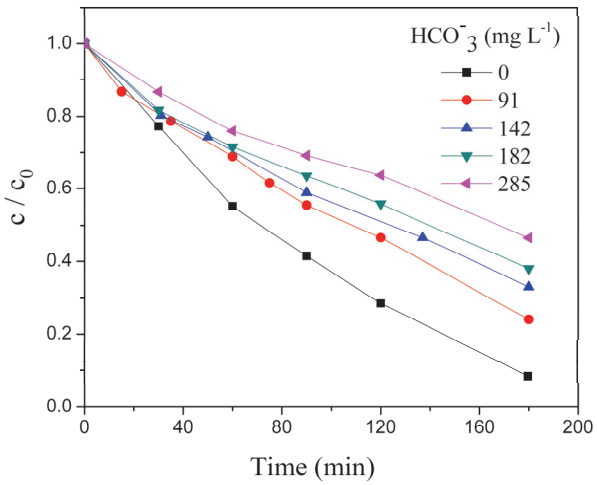

Fig. 12. Effect of the concentration of HCO_3^- on photodegradation of CLP in DDW. Operation conditions: $c(CLP)_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 25 °C, pH~7.0.

The effect of HUM can be explained by the reaction with •OH, which lowers the availability of the latter for the reaction with CLP. Moreover, the actually available UV radiation reduces because some organic matters (especially aromatic compounds) absorb strongly UV

irradiation (Chu et al., 2009b). Expectedly, the degradation rate decreased after the addition of HUM up to 20 mg L^{-1} (Figure 13). The behaviour observed in the presence if HUM suggests a predominant effect of the •OH radical inhibition, due to the complex structure of HUM and their high reactivity with •OH radicals (Basfar et al., 2005; Prados-Joya et al., 2011).

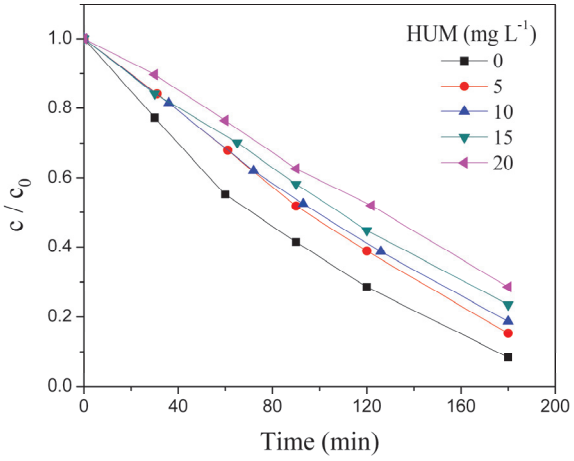

Fig. 13. Effect of the concentration of HUM on photodegradation of CLP in DDW. Operation conditions: $c(CLP)_0$ = 1.0 mM, TiO_2 Wackherr = 2.0 mg mL^{-1}, t = 25 °C, pH~7.0.

4. Conclusion

The results of this study clearly indicate that under the UV irradiation TiO_2 Wackherr was more efficient than Degussa P25 in both the process of removal of CLP from water and its mineralisation. The reaction followed the pseudo-first order kinetics. The optimum loading of TiO_2 Wackherr was 1.0 mg mL^{-1} at pH 3.5. The photodegradation rate was dependent on the temperature, and the apparent activation energy was 37.9 kJ mol^{-1}. Along with molecular oxygen, $KBrO_3$ was the most efficient electron acceptor when concerning the degradation of the parent compound, whereas its mineralisation was most efficient in the presence of O_2 only. It was found that the presence of ethanol as a scavenger of •OH inhibited the CLP photodecomposition, suggesting that the reaction mechanism mainly involved free •OH. The LC–DAD, and LC–ESI–MS/MS monitoring of the process showed that six intermediates were formed. The analysis of the intermediate product formed during the photocatalytic degradation could be a useful source of information about the degradation pathways. The rate of photodegradation of CLP in DDW was about two/three times higher than in tap and river waters. The photodegradation rate was dominantly influenced by the pH of the medium and the presence of HCO_3^- and DOM. Our work validates the presented screening methodology of ecotoxicological risk assessment for transformation products, and can be used as a first step in toxicity assessment of degradation products and for prioritisation and planning of more detailed investigations.

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11

Row Crop Herbicide Drift Effects on Water Bodies and Aquaculture

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1. Introduction

Aquatic ecosystems produce substantial amounts of aquatic products; including all new sources of seafood, from aquaculture. Level land with clay soils and the availability of water supplies makes riverine alluvial plains favorable areas for row crops and aquaculture. Aquaculture ponds are susceptible to impacts from row crop production through drift of herbicides. To assess these impacts we have conducted field research in replicated mesocosms filled with water and associated naturally-occurring communities from various pond ecosystems and subjected to expected levels of drift from all major aerially-applied herbicides currently in use. Rather than an organismal approach and LC_{50}'s, data indicates community-level approaches better approximate ecosystem impacts. Herbicide drift that affects phytoplankton adversely or in a stimulatory manner will similarly impact the ecosystem, as phytoplankton produce oxygen, take up ammonia and nitrite and provide food for zooplankton. Drift levels are below toxic levels to most other aquatic organisms, including fish (Spradley, 1991). Drift amounts reaching water bodies and ponds, including fish ponds, depend on many factors, but the cumulative range is most affected by the size of the water body. Thus, other than in direct overflight, larger catfish ponds (6-8 ha) have a drift range of 1-10% and smaller more recent designs of 4 ha, 5-20%. Even smaller ponds, used for fingerling production and baitfish production (0.8-2 ha), may receive drift amounts of up to 30% of the field rate.Herbicide drift may be expected to impact small water bodies through death or reduction in the photosynthetic rats of phytoplankton, which could reduce the supply of dissolved oxygen, inhibit removal of toxic nitrogenous wastes, and reduce production of zooplankton by reducing their food supply. These conditions could also result in death, disease, or lower growth rates of managed or cultured fishes. Triazine herbicides (atrazine and simazine), as well as amides (propanil), phenylureas (diuron), triazones, uraciles and phenolics, act through inhibition of photosystem II (PSII) of photosynthesis (Cobb, 1992). They are widely used in agriculture, since they provide a low-cost basal weed control (Jay et al., 1997). Using mesocosms and naturally-occurring plankton communities in a multi-day study provides better extrapolations to real environments than laboratory studies on a single species (Juettner et al., 1995), and possibly prevent overestimate of impacts (Macinnis-Ng and Ralph, 2002). The major drift source is aerial application, with an

estimated 20 X higher drift deposition compared to application by ground spray booms (Hill et al., 1994).

2. Evaluation of 40 aerially-applied row crop herbicide effects on water bodies

Recent studies at the University of Arkansas at Pine Bluff (UAPB) have assessed the effects of herbicide drift from 40 herbicides used on adjacent soybean, rice, cotton, corn and winter wheat row crops to plankton and water quality in adjacent flood plain ponds (Perschbacher et al., 1997, 2002, 2008; Perschbacher and Ludwig, 2004). Herbicide drift may be expected to impact ponds through death or a reduction in the photosynthetic rate of phytoplankton, which could reduce the supply of dissolved oxygen, inhibit removal of toxic nitrogenous wastes, and reduce production of zooplankton by reducing the food supply (Waiser and Robarts, 1997). Aerial application has drift deposition 20 times higher compared to application by ground spray booms (Hill et al., 1994).%). The mode of action of herbicides impacting phytoplankton, is reversable inhibition of photosynthesis at photosystem II (PSII) and should not be species specific (Cobb, 1992; Solomon et al., 1996). Photosystem II inhibitors are widely used in agriculture, since they provide a low-cost basal weed control (Solomon et al., 1997; Jay et al., 1997).

2.1 Methods and materials

These studies were conducted to determine if aerially-applied herbicides would cause measurable plankton and water quality changes in outdoor pool mesocosms filled with water from a fish pond. Rates used encompassed the estimated range of drift and a field rate (full) equivalent to direct application. The studies were conducted at the UAPB Aquaculture Research Station at the approximate time of the year when the respective herbicides are applied. The experimental plankton mesocosms used were above ground, circular 500-L fiberglass tanks arranged in four rows on a cement pad. When filled, water depth of tanks was 0.7 m (slightly less than the average depth of most fish ponds) and there was no mud substrate. Water surface area of each tank was 0.78 m^2 and diameter was 1.0 m, similar to those used in a prior study of atrazine effects on plankton and water quality (Juettner et al., 1995). Tanks were filled immediately prior to herbicide application with water pumped from an adjacent 0.1-ha pond.

Herbicides were applied over the tank surfaces at one of three levels: field rates (equal to overspray) and high and low drift rates of 1/10 and 1/100th of this level, respectively (Perschbacher et al., 1997). A control, without herbicide addition, was the fourth treatment. Each herbicide was tested at the recommended application rate (Baldwin et al., 2000). Commercial formulations were used without addition of adjuvants or wetting agents. Approximately 30 ml of distilled water was used to dissolve the herbicide. Each treatment was replicated three times in randomly- assigned tanks. Tanks were flushed and air-dried between trials.

Each herbicide was added to the tanks at approximately 0900. A set of measurements was taken immediately prior to application and again 24, 48 and 72 hours after application. If effects were noted, sampling was continued approximately weekly until morning oxygen (DO) levels of drift treatments did not significantly different from the control (ie. recovery). Dissolved oxygen is the water quality parameter most sensitive to herbicide effects (Juettner

et al., 1995) and most critical to fish culture. Water temperature, dissolved oxygen and pH were measured with a multiprobe meter (OI Analytical, College Station, TX). Total ammonia nitrogen (TAN) and nitrite-nitrogen were measured by Nessler and diazotization methods (Hach Co., Loveland, CO), respectively. Unionized ammonia (UIA) levels were calculated from measured temperature, TAN and pH. Chlorophyll *a,* corrected for pheophytin *a,* and a 2-h light and dark bottle estimation of phytoplankton net primary productivity (NPP) by the oxygen method followed APHA (2005), except for use of ethanol as the solvent for chlorophyll (Nusch, 1980). Major zooplankton group concentrations were also determined in each replicate in the following manner. Three, 1-l samples were obtained with a tube sampler that encompassed the entire water column. The samples were concentrated by being strained through a 70-um Wisconsin plankton net and then preserved in 70% isopropyl alcohol. Samples were identified and quantified by using a Sedgwick-Rafter cell and a microscope (Ludwig, 1993). Statistical analysis was by SAS statistical software package. ANOVA (after pretesting for normality) and LSD were used to test for significant differences ($P \leq 0.05$) among treatments for each day during each trial.

2.2 Results and discussion

Atrazine lowered NPP on d 2; and effects on ecosystems from field studies have been summarized as short-lived, with quick recovery at concentrations less than 50 ug/l (Solomon et al., 1996). Solomon et al. (1996) observed stimulation of chlorophyll *a* on d 7 post-application of 50 ug/L atrazine. This was also found by us at d 7 with propanil (Perschbacher et al. 1997, 2002). Edziyie (2004) noted drift from propanil affected fry ponds with ≤10 ug/l chlorophyll *a* less than culture ponds with levels of 50-85 ug/l of chlorophyll *a*, such as were present in this study. This may explain the reduced effects of atrazine, but is in need of further study. Carfentrazone drift rates resulted in significantly lower rotifer and nauplii numbers compared to control levels the day after application, but not on the second day. Reductions ranged from 5-30% of control numbers and could not be explained from chlorophyll *a* data or net primary productivity. Zooplankton from diuron and atrazine were noted greatly impacted (Table 2). Propanil at 1 and 10% drift rates did not result in significant effects, although full field rates did (Perschbacher et al., 2002). Further evaluation of propanil is considered in the section 3.

These studies indicate that drift effects from 40 common aerially-applied herbicide applications on plankton and water quality were limited to atrazine, diuron and carfentrazone (Table 1). Of the 40 herbicides, diuron presents the greater risk for reduced water quality and for a longer time period, of at least 4 weeks (Table 2).

3. Evaluation of drift levels to small alluvial plain water bodies: atrazine, propanil and diuron

Small water bodies, equal to or less than 1.2 ha, in alluvial plains may be subjected to greater drift concentrations from adjacent row crops, due to reduced surface areas and volumes (Perschbacher and Ludwig 2007). These small ponds may be used for growing early and vulnerable stages of commercial aquaculture crops, and for fish consumption by farm pond owners. The three herbicides causing appreciable impacts, atrazine, propanil and diuron, were further tested at maximum drift rates expected of 30% of field rates.

Common Name	Trade Name	Date Applied	A.I (kg/ha)	Chl. α (ug/l)
Soybean				
Bentazon	Basagran	8/23	0.57	200
Imazaquin	Image	8/2	0.14	240
Fomesafen	Flexstar	8/16	0.43	250
Aciflourfen	Blazer	8/9	0.43	270
Fluzifop	Fusilade	8/16	0.10	240
Clethodim	Prism	7/26	0.07	300
Chlorimuron	Canopy	8/9	0.004	125
Glyphosphate	Roundup	8/2	0.43	500
Flumiclorac	Resource	6/8	0.045	135
Sethoxydim	Vantage	6/1	0.45	239
Carfentrazone*	Aim	3/2	0.03	400
Rice				
Clomazone	Command	5/23	0.60	280
Thiobencarb	Bolero	5/29	3.40	400
Pendamethalin	Prowl	6/5	1.10	250
Propanil	Stam	6/12	4.50	160
Quinclorac	Facet	6/20	0.60	450
Halosulfuron	Permit	6/27	0.07	475
Bensulfuron methyl	Londax	7/5	0.07	240
2.4-D-amine	2,4-D	7/5	1.70	45
Molinate	Ordram	7/25	5.60	450
Triclopyr	Grandstand	7/11	0.40	115
Fenoxyprop-ethyl	Acclaim	6/15	0.13	114
Cyhalofop	Clincher	7/5	0.30	65
Bispyribac-sodium	Regiment	7/12	0.036	114
Cotton				
Diuron (burndown)*	Direx	3/5	1.40	390
Diuron (defoliant)	Direx	9/23	0.165	850
Paraquat	Gramaxone	4/10	0.83	160
Quizalofop	Assure	6/18	0.05	300
Dimethipin	Harvade	9/16	0.15	750
Tribufos	Def	10/7	1.00	1075
Ethephon	Finish	10/14	1.76	1000
Sodium chlorate	Defol	10/21	5.30	520
Glufosinate	Liberty	3/13	0.55	344
Flumioxazin	Valor	4/6	0.03	334
Corn				
Mesotrione	Callisto	5/30	1.80	150
Metolachlor	Dual	3/8	0.10	350
Atrazine*	AAtrex	5/6	0.90	30
Rimsulfuron	TranXit	5/3	0.90	40
Nicosulfuron	Steadfast	4/29	0.90	105
Winter Wheat				
Thifensulfuron + Tribenuron	Harmony Extra	3/25	0.028	189
* significant effects noted				

Table 1. Summary of mesocosm tests of drift from aerially-applied herbicides by major crop, common name, trade name, date applied, recommended active ingredient (A.I.) field rate and approximate levels of pond plankton.

Days Post-Application	Diuron 1/100	Diuron 1/10	Atrazine 1/100	Atrazine 1/10
DO				
1	92*	92*	80	102
2	93*	81*	100	104
7	83*	71*	102	111*
Recovery (days)	21	>28	0	0
NPP				
1	49*	21*	124	105
2	37*	25*	82*	79*
7	41*	22*	84	82
Chlorophyll *a*				
1	97	95	110	102
2	99	96	89	93
7	83*	58*	96	115
pH				
1	96*	95*	100	100
2	98*	91*	100	100
7	89*	87*	100	100
TAN				
1	194*	122*	94	101
2	120	80	92	85
7	243*	356*	133	94
UIA				
1	100	50*	100	100
2	84	12*	80	87
7	35*	37*	150*	125
Nitrite-N				
1	100	100	112	93
2	120	20	141	151*
7	100	133	25	150
Rotifers				
1	67	78	200	150
2	126	226	128	57
7	115	96	22*	67
Copepod nauplii				
1	238	163	74	76
2	66	92	104	95
7	100	100	199	300
Copepod adults				
1	100	160	173	110
2	75	150	110	113
7	64	21*	120	94
Cladocerans*				
1	NA	NA	120	94
2	NA	NA	73	127
7	NA	NA	100	82
*no cladocerans observed in diuron trials				

DO = 0900 Dissolved Oxygen; Recovery = return of morning DO to control levels;
NPP = Net Primary Productivity; TAN = Total Ammonia Nitrogen; UIA = Unionized Ammonia

Table 2. Comparison of mean low (1/100 direct application rate) and high (1/10 direct application rate) drift effects of diuron and atrazine, expressed as percentage of control levels. Means significantly different ($P \leq 0.05$) from control means, indicated by *.

3.1 Methods and materials

The study was conducted at the University of Arkansas at Pine Bluff (UAPB) Aquaculture Research Station The experimental mesocosms were 500-l, above ground, circular fiberglass tanks arranged in four rows on a cement pad. When filled, water depth of the tanks was 0.7 m (slightly less than the average depth of most fish ponds) and there was no soil substrate. Surface area of each tank was 0.78 m^2 and diameter was 1.0 m, similar to those used by Juettner et al.(1995). Tanks were filled immediately prior to herbicide application with water pumped from an adjacent 0.1-ha Aquaculture Research Station experimental pond. Total dissolved solids were 290 mg/l, hardness 185 mg/l and alkalinity 197 mg/l as calcium carbonate.

Commercial formulations, without adjuvants or wetting agents, were applied over the tank surfaces at 30% of field rates (Baldwin et al., 2000) in four randomly selected pools each. Four additional pools received no herbicide and served as controls. The level used was equivalent to highest potential cumulative drift concentrations based on graphs in Hill et al. (1994) to water bodies of 1.2 ha surface area. The experimental dose was added to 30 ml of distilled water for more uniform application over the tank surface.

Immediately following filling, the first set of measurements were taken. The suite of measurements was subsequently taken 24, 48 and 72 h after application. If impacts were noted, sampling was continued approximately weekly until morning oxygen levels of drift treatments did not significantly differ from the control. Dissolved oxygen is the water quality parameter most sensitive to herbicide effects (Juettner et al., 1995) and most critical to aquatic life. Water temperature, dissolved oxygen, total dissolved solids (TDS), and pH were measured with a multiprobe meter (YSI, Yellow Springs, OH). Total ammonia nitrogen and nitrite-nitrogen were measured by Nessler and diazotization methods (Hach Co., Loveland, CO), respectively. Unionized ammonia levels were obtained from water temperature, TDS, TAN and pH. Chlorophyll *a,* corrected for pheophytin *a* and using ethanol as a solvent (Nusch, 1980), and a 2-h light and dark bottle estimation of net phytoplankton primary productivity by the oxygen method followed Standard Methods (APHA, 2005). Concentrations of the major zooplankton groups (rotifers, copepod nauplii, adult copepods and cladocerans) were also determined in each replicate in the following manner. Six, 1-L samples were obtained with a tube sampler that encompassed the entire water column. The samples were concentrated by being strained through a 70-um Wisconsin plankton net and then preserved in 70% isopropyl alcohol. Samples were identified and quantified by using a Sedgwick-Rafter cell and a microscope. Phytoplankton were enumerated and identified to genus (Prescott, 1962) in Sedgwick-Rafter cells with Whipple grid at 150X (APHA, 2005) from 20 ml unconcentrated samples obtained with a 0.9-m polyvinyl chloride (PVC) column sampler and preserved with 1 ml of formalin. Cyanobacteria were further identified to species using Cocke (1967). A randomized block design was used. Means from each sample date were tested for significant differences ($P \leq 0.05$) with controls by paired, single tail Student's t-tests.

3.2 Results

Propanil levels were 58 ug/l and atrazine levels were 19.5 ug/l. Significant changes from control treatment values were found for several parameters in all three herbicide treatments (Perschbacher and Ludwig, 2007). Following application on 20 June, net primary

productivity was significantly depressed on d 1 in the propanil treatments, but increased on d 2 and 3. Morning dissolved oxygen was lower on d 1-3, but not to critical levels. Also, in the presence of propanil, pH and consequently UIA were lower from d 1-3. Atrazine reduced morning DO on d 2 and 3, but not net primary productivity. Nitrite-N, however, was significantly higher on d 1. Phytoplankton total numbers, and the cyanobacterium *Chroococcus* sp. which dominated, were reduced by propanil on d 1-3; similarly affected by atrazine on d 2 and 3. Numbers of green algae, *Scenedesmus* sp. and *Coelastrum* sp., and diatoms were however stimulated by propanil and diatoms by atrazine. Zooplankton were little affected by either herbicide.

Due to the greater impacts of diuron (at levels equivalent to 30 ug/l), response of important environmental metrics to diuron drift are presented in Tables 3-5. No significant differences in pre-application sampling were found. Following application of diuron, net primary productivity was reduced by 97%, and recovered on d 7 (Table 1). Morning oxygen concentration also declined on day 1 by 32%, and was at stressful levels from d 2-3. Recovery was attained on d 14. Chlorophyll *a* and pheophytin *a* levels were significantly higher on d 2-14. Levels of pH were reduced by diuron addition from d 1-14. With lower pH values, unionized ammonia was significantly less from d 2-14. Plankton were also significantly impacted. Cyanobacteria, with the exception of *Chroococcus* spp., were reduced from d 1 and green algae, especially *Scenedesmus* spp. were stimulated (Table 4, 5). The other major group of phytoplankton, pinnate diatoms, were unchanged with the exception of a decline on d 7. In terms of percentage composition of the phytoplankton community, in diuron-treated mesocosms cyanobacteria declined from 24 to 20%, while green algae increased from 45 to 72%. Diatoms also declined from 26 to 8% (Table 5).

Zooplankton groups with significantly reduced mean abundances included: nauplii-616/l compared to control level of 1750/l on d 7, and cladocerans-0/l treatment level compared to 33/l on d 2. Copepod numbers however increased: from 1483/l control level to treatment level of 2133/l on d 3, and from 1150/l control level to the 2133/l treatment level on d 4. Rotifers were not impacted, in contrast to the findings of Zimba et al. (2002) who found an increase in rotifers.

3.3 Discussion

Diuron is a urea herbicide, that is 4-6 times more potent in photosynthesis inhibition than simazine herbicide (Ashton and Crafts, 1981). The concentration used in this study and representing the highest drift level of diuron (Direx) was 30 ug/l. Cyanobacteria were most susceptible to diuron, found previously with diuron (Zimba et al. 2002) and propanil and atrazine (Voronova and Pushkar 1985, Leboulanger et al. 2001, Perschbacher and Ludwig 2007). An increase in chlorophyll *a* was also noted by Ricart et al. (2009) in biofilms exposed to 0.07-9.0 ug/L diuron. This was attributed to a so-called "shade-adaptation" response to reduced photosynthetic efficiency from diuron. Zimba et al. (2002) observed no decrease in phytoplankton biomass, as measured by chlorophyll *a*, during 9 weekly treatments of 10 ug/l diuron each, but found the phytoplankton composition was altered. Numbers of filamentous cyanobacteria decreased, while ultraplankton coccoid cyanobacteria, diatoms and chlorophytes increased and chlorophyll *b* indicative of chlorophytes was significantly higher on one sample date.

Although drift levels of diuron in the present study were 3 times higher than in the Perschbacher and Ludwig (2004) study, which evaluated maximum drift effects to water bodies over 7 ha, inhibition of photosynthesis was longer lasting in the 2004 study. The dominance of cyanobacteria which formed surface scums and were thus unstable in the former study may have been responsible for the greater impacts, as found for propanil (Edziyie, 2004).

The present study found that in small eutrophic ponds, typical in agricultural environments and with relatively high chlorophyll *a* levels, short-term negative impacts would be expected on morning DO from atrazine, propanil and diuron. However, they may also benefit water quality by reducing pH, a major concern in eutrophic ponds utilized for recreational fish production and commercial fish culture (Barkoh et al., 2005; Ludwig et al., 2007) and which in turn resulted in lowered unionized ammonia levels.

Parameters	Treatment			Time	(d)		
		0	1	2	3	7	14
DO (mg/l)	C	16.13	14.83*	11.23*	8.63*	6.73*	8.73
	D	16.07	11.02	3.10	2.50	4.63	7.87
NPP (mg O_2/l/h)	C	1.28	0.63*	0.51*	ND	0.13	0.35
	D	1.47	0.05	0.07	ND	0.22	0.32
Chlorophyll *a* (ug/l)	C	202.4	113.0	81.0*	70.8*	37.1*	ND
	D	209.2	131.6	126.5	108.0	118.1	ND
Pheophytin *a* (ug/l)	C	31.4*	41.7	19.4*	23.6	4.2*	ND
	D	45.9	47.9	38.7	33.7	21.2	ND
pH	C	8.57	8.73*	8.63*	8.42*	8.20*	8.47*
	D	8.60	8.60	8.07	7.73	7.75	8.17
UIA (mg/l)	C	0.01	0.02	0.02*	0.01*	0.01*	0.10*
	D	0.02	0.02	0.01	0.00	0.00	0.00

DO = 0900 Dissolved Oxygen; NPP = Net Primary Productivity; UIA = Unionized Ammonia; ND = No Data

Table 3. Mean (SE) water quality differences in diuron (D) and control (C) treatments. Column means significantly different have different letters ($P \leq 0.05$).

Species/Genera	Treatment		Time	(d)	
		0	2	7	14
Scenedesmus spp.	C	30.6	19.3	10.0	0.3*
	D	19.0	32.7	20.9	12.7
Ankistrodesmus spp.	C	1.4	2.3	1.5	0.5*
	D	2.1	2.5	1.5	2.5
Coelastrum spp.	C	1.1	0.8	0.1*	0.0*
	D	1.2	1.1	1.1	0.3
Anabaena levanderi	C	0.3	0.3	1.3*	12.3*
	D	0.2	0.1	0.0	0.0
Anabaena circinalis	C	0.1	0.2	0.5*	3.5*
	D	0.1	0.1	0.0	0.1
Oscillatoria angustissma	C	7.1	11.3*	27.3*	27.9*
	D	3.9	5.6	18.7	0.1
Chroococcus dispersus	C	3.3	2.7	6.7	0.2
	D	8.0	1.3	3.9	4.5
Pinnate diatoms	C	10.7	8.8	16.5*	1.0
	D	10.4	6.8	4.7	1.8

Table 4. Mean phytoplankton (10^3 cells/ml) in diuron (D) and control (C) treatments. Column means significantly different have * ($P \leq 0.05$).

Groups	Treatment		Time	(d)	
		0	2	7	14
Cyanobacteria	C	23.1	31.8*	55.7*	95.6*
	D	24.1	12.5	14.3	20.0
Green	C	51.6	47.4*	18.8*	2.0*
	D	45.4	71.8	68.1	72.0
Diatom	C	22.5	20.6	25.5	20.0*
	D	25.7	15.7	17.5	7.8

Table 5. Mean % composition of major phytoplankton groups by natural units, with and without diuron addition, over time. Column means significantly different are * ($P \leq 0.05$).

4. Modifiying factors due to algal state from *in situ* mesocosm testing

Aquaculture ponds often have surface floating scums predominately composed of cyanobacteria. These scum-forming algae are common in eutrophic ponds, including aquaculture ponds, especially during the growing season with warm temperatures and high nutrient loadings. Cyanobacteria in a suface scum state are unstable and prone to sudden die-offs (Boyd et al., 1975). The objective of this study was to test the effect of propanil on a pond with algal scums.

4.1 Methods and materials

The experiment was conducted at the University of Arkansas at Pine Bluff mesocosm facility. A completely randomized design was used, with three replicates for each treatment in 12 mesocosms and with water of approximately 400 ug/l chlorophyll *a* from a goldfish pond. The treatments used were: a control with no propanil, 1%, 10% and 100% of the recommended field rates (0.45 kg/ha). Variables measured included: morning dissolved oxygen, pH, nitrite-nitrogen, total ammonia nitrogen, unionized ammonia , net primary productivity, chlorophyll *a* and phytoplankton composition. Methods followed Standard Methods (APHA 2005). Samples were taken before and after treatments were added.

4.2 Results and discussion

Microsystis and *Anabaena* dominated the phytoplankton and formed the surface scum. Significantly lower DO and net primary productivity resulted after application in the 10% and full treatment. However, recovery was noted after 48 h. Also lower was pH following application. Tan and UIA were higher on d 2.

In the earlier trials (Perschbacher et al., 1997, 2002) without surface scum algae, propanil at 10% drift resulted only in elevated chlorophyll *a*, but no significant differences were noted in chlorophyll *a* and phytoplankton composition in the present study. Thus, the significant negative impacts found in the present study were not expressed in previous studies, and the difference is attributed to the algal state.

Effects of propanil drift depended on the level of chlorophyll *a* found in the systems, in the study by Edziyie (2005). The greatest impact was on water quality in ponds with chlorophyll *a* levels 50-200 ug/l and lesser impacts below 20 and above 300 ug/l. Phytoplankton at high levels have been proposed to modify pesticide effects by sorption to the algae (Day and Kaushik, 1987; Waiser and Robarts, 1997; Stampfli et al., 2011).

5. Conclusions

5.1 Large pond (7 ha and larger) simulations

Of the 40 herbicide applications tested, significant effects from drift levels of 1 and 10% (the range possible in ponds equal to and larger than 7 ha) were noted for diuron (used for burndown) and atrazine. Diuron presents the greater risk for reduced water quality and for

a longer time period (in excess of 4 wks). Atrazine effects are short-lived . Carfentrazone resulted in brief zooplankton reductions.

5.2 Surface scum algal populations simulations

Algal populations forming scums appear more susceptible to these drift levels. Propanil levels which did not result in reductions in water quality in mixed water column populations, resulted in adverse reactions equal to the direct overspray. The concentration of algae at the surface and the propensity for algae in this stage to be unstable (crash) are judged responsible.

5.3 Differing chlorophyll *a* level simulations

Effects of propanil and atrazine drift, and perhaps of other herbicides, depend on the level of chlorophyll *a* found in the systems. The greatest impact of propanil was on water quality in ponds with chlorophyll *a* levels 50-200 ug/l and lesser impacts below 20 and above 300 ug/l. Absorption by algae, and other factors, may be responsible.

5.4 Small pond (1.2 ha and smaller) simulations

Simulations in small ponds, equal to or less than 1.2 ha, used drift rates up to 30%. Although atrazine and propanil did not cause concern, diuron caused DO drops that were below 3 mg/l for several days and recovery was not noted until 14 days.

5.5 Beneficial aspects of herbicide drift

Beneficial effects of atrazine, propanil and diuron included reduction or elimination of cyanobacteria, and reduced pH (Ludwig et al., 2007) and thus reduced UIA. Clorophyll *a* levels were stimulated by propanil and atrazine.

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Part 2

Control of Weeds

12

Evaluation of the Contamination by Herbicides in Olive Groves

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1. Introduction

The application of phytosanitary products on the ground is one of the main employed procedures to solve the problems of plagues and diseases in olive groves. As a result, there is an increasing unease related to the possible presence of residues from these products in the olive-derived foods. Recent studies have shown that these residues can be found in olives and olive oil (mainly insecticides and fungicides) due to the lipophillic nature of the used plaguicides. This is also the effect that has been observed with the introduction of herbicides in this cultive. However, the detected levels of all these phytosanitary products are usually below the maximum residue limits (MRLs) established.

Herbicides are used by olive farmers to weed olive plantations with two essential goals: (1) eliminate the competition for water resources between olive trees and weeds, especially in particular moments of the productive cycle; and (2) keep the soil clean around the olive tree (in that way, the harvest of the olive fruits is easier if they fall off the tree after maturation is over). The main herbicides commonly used in olive groves are triazines (simazine, atrazine, trietazine, terbuthylazine and terbutryn), phenylurea diuron and phenylether oxifluorphen. All of them are directly applied on the ground of the plantation and nowadays widely used in intensified-traditional and modern-intensive olive groves. Herbicide residues remain highly concentrated in the top 5-15 cm of soil, even after several months. This fact presents two main consequences. First, herbicide residues are washed into streams, rivers and reservoirs with the soil that is eroded in heavy rains, polluting surface waters. Second, when harvesting is done using various devices to shake the olives off the trees without extending nets under the tree, olives come in contact with the herbicides present in the soil. This constitutes a risk of these herbicides being incorporated in olives and, consequently, olive oil.

Olive oil is a very important commodity in the Mediterranean basin. This product has a great importance in the sustainable economy of important regions from the main olive oil producers in the world: Spain, Italy and Greece. Due to the facts that virgin olive oil production has increased in recent years and that it is being exported to different countries, exhaustive quality controls are required. Different regulations regarding MRLs in olives and olive oil have been established by the European Union and the Codex Alimentarius of the Food and Agriculture Organization of the United Nations. In addition, new regulations will be established in following years with MRLs of 10 μg/kg.

There are alternative methods available for the analysis of herbicides in different kinds of samples. However, the common methods of analysis for their determination are Gas Chromatography (GC) and Liquid Chromatography (LC). GC is usually the chosen technique due to the high separation efficiency and compatibility with a wide range of detection techniques. Our research group has performed thorough investigation over the analysis of herbicides in soils, olive fruits and virgin olive oils. In particular, we have developed different GC analytical methods with mass spectrometric detection for the analysis of all the main herbicides used in olive groves. In this way, sensitive and reliable procedures can be implemented for the quality control of olives and olive oils in the industry. These methodologies, together with the obtained results over real samples from Spain, will be commented in this chapter.

2. Composition and properties of olive oil. Classification

Olive oil is composed mainly of triglycerides (98-99% of the olive oil) and contains small quantities of free fatty acids (FFA), being the proportion of FFA variable and related to the degree of hydrolysis of the triglycerides. The composition of fatty acids in the oil depends on the variety of the olive tree, climatic conditions and geographical localization of the grove. Both the International Olive Council (IOC) and the Codex Alimentarius Comission have established maximum and minimum percentages for each fatty acid in the composition of the olive and pomace-olive oils. Olive oil is basically composed of mono-insaturated fatty acids. Regarding the degree of insaturation, fatty acids composition is as follows: 72% mono-insaturated, 14% poli-insaturated and 14% saturated.

There are also minor components in olive oil, which are specific markers of its physico-chemical authenticity and they also add unique sensory and biological characteristics:

- Squalene is the major olive oil terpenoid hydrocarbon (300-700 mg L^{-1}), whereas β-carotene, biological precursor of vitamin A, is found in small quantities (mg L^{-1}).
- Triterpenoids alcohols (24-methylenecycloartanol, cycloartenol, α-amirine and β-amirine) are especially important from the biological point of view. Eritrodiol is also important from the analytical perspective, because it can be used to detect the presence of pomace-olive oil.
- Sterols can be used to construct a fingerprint in order to authenticate the olive oil, specifically using the β-sitosterol content, which represents around 93% of the total content of sterols.
- Tocopherols, especially α-tocopherol or vitamin E (150-300 mg L^{-1}) are important antioxidants.
- Phenolic compounds, some of them contribute to the characteristic flavor of olive oil, increase the antioxidant properties of the oil.
- Approximately one hundred aromatic compounds are also present, being the exact chemical composition dependent on the variety, climatic conditions and quality of the oil.

Virgin olive oil is the most digestible of the edible fats and it helps to assimilate vitamins A, D and K. It contains essential acids that cannot be produced by our own bodies and slows down the aging process. It also helps bile, liver and intestinal functions. It is noteworthy that olive oil has a beneficial effect in the dietary treatment of diabetes. In addition, it helps to control blood

pressure and increases the bone mass. Moreover, olive oil has a favorable effect on the development of the central nervous and vascular systems, in brain development as well as normal child development (Cicerale et al., 2010; El & Karakaya, 2009).

The human body easily absorbs olive oil. This means that the body absorbs the good ingredients such as vitamin E and phenols, which have anti-oxidizing properties and prevent the oxidization of fatty tissue, therefore helping to delay the aging process. In addition, it is not only easy to digest but it also helps the digestion of other fatty substances because it helps the secretions of the peptic system and stimulates the pancreatic enzyme lipase. On the other hand, olive oil consumption has a very positive effect on blood cholesterol (limits the oxidizing of bad cholesterol because it is rich in anti-oxidizing agents, as it was indicated before).

Olive oil, as any fatty substance, deteriorates during the frying process, especially if it is used over and over and if the frying temperature is very high. High temperature destroys the good ingredients of any oil while it creates harmful agents for the liver, the arteries and the heart. However, it is important to take into consideration that these harmful agents are less likely to be created in olive oil than in all other known vegetable oils because of the different composition. Olive oil contains a high percentage of oleic acid, which is much more resistant to oxidization than polyunsaturated acids (found in large amounts in seed oils). As a result, olive oil is the most stable fat and it stands up well to high frying temperatures.

Taking into account all the procedure in order to obtain the oil, edible olive oil is marketed in accordance with the following designations and definitions (International Olive Council, 2010):

1. Virgin olive oil: it is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration. The virgin olive oils that are fit for consumption are extra virgin olive oil and virgin olive oil, presenting a free acidity, expressed as oleic acid, of not more than 0.8 and 2 grams per 100 grams, respectively. They also have to fulfil different quality criteria that, according to the IOC, include good organoleptic characteristics (taste and aroma) and low peroxide value.
2. Olive oil: it is the oil consisting of a blend (approximately 80:20 v:v) of refined olive oil and virgin olive oils fit for consumption as they are. It has a free acidity, expressed as oleic acid, of not more than 1 gram per 100 grams and its other characteristics correspond to those fixed for this category in this standard.
3. Olive pomace oil: it is the oil obtained by treating olive pomace with solvents or other physical treatments, to the exclusion of oils obtained by re-esterification processes and of any mixture with oils of other kinds. The marketed olive pomace oil is the oil comprising the blend (approximately 80:20 v:v) of refined olive-pomace oil and virgin olive oils fit for consumption as they are. It has a free acidity of not more than 1 gram per 100 grams.

Chemical processing may improve high acidity olive oil and make it edible; however, it takes away some extremely valuable ingredients such as vitamins and phenols. As a result, processed olive oil (refined) lacks the desirable properties and characteristics that can be found in abundance in (extra) virgin olive oil.

3. Processing and elaboration of olives and olive oil

In this section, the steps required for obtaining high-quality olive oil (extra virgin) and table olives will be described. It is important to know the details of the processing in order to understand in which step can the contamination by herbicides take place.

3.1 Olive oil

3.1.1 Harvest of olives

The harvest of the olives could be understood as an independent activity from the elaboration of the oil. However, the characteristics of the oil are highly influenced by the harvest time and method employed.

Olives must be picked at the moment of optimum ripeness, where the fruit presents the maximum content of oil and the best characteristics. Olives reach their ripeness in autumn and picking starts at the end of November, lasting up to February or March. The methods to harvest the olives have not changed much from the ancient time. The methods used should not damage the fruit and should avoid breaking of boughs or shoots. The high-quality olive oil is obtained by "milking" the olives into a sack tied around the harvester's waist (using ladders for the highest boughs) and extending canvases at the foot of the trees, where the olives will fall when the tree is beaten (using flexible poles). Recent methods make use of harvester machines (shakers) that generate the vibration on the tree for the falling of the fruit. This vibration machines can be small vibrators operated by the farmer in the required boughs, of full-equipped tractors, with vibration units for the whole trunk of the tree and an umbrella to pick the olives.

Harvest by hand is impossible in olive trees of 4-5 meters high, even with the use of ladders. In general, the trunks are to wide to allow the use of vibration machines and the olives are picked directly from the ground after they fall down when they reach ripeness. Hence, harvest period lasts longer, even until spring when it is a high-production year. When the olives are picked from the ground, weeds have to be completely eliminated from the zone in order to make it easier the picking. The quality of the olive oil obtained from this olives is poor due to organoleptics flaws such as soil flavor.

3.1.2 Washing

Traditionally, agricultural workers have cleaned the fruit in the fields by means of sieves. But this cleaning is not complete and the fruit goes into the olive-oil mill with a great quantity of impurities, leaves, branches, mud, etc, which are necessary to eliminate. Therefore, for this purpose, cleaners that use air current or shaking sieves are used to eliminate leaves, branches and other impurities lighter than the fruits. In addition, washing devices are employed to eliminate heavier impurities such as stones or dust. In next figures, both cleaning steps, the one with the air for light impurities (Figure 1) and the other one by washing for heavy impurities (Figure 2), are shown.

Once the fruit has been cleaned and weighed, it is stored in hoppers until it can be crushed. The storage period must be as short as possible in order to avoid alterations, which will produce oils with a higher degree of acidity, lower stability and worse flavor.

Fig. 1. Cleaning steps for olive fruit.

Fig. 2. Washing step for olive fruit.

3.1.3 Preparation

The process of releasing the oil from the plant tissue begins by milling the olives to tear the flesh cells in order to let the oil run out of the vacuoles. This is followed by stirring the olive paste to permit the formation of large drops of oil and to break up the oil–water emulsion. In so-called "dual-phase decanters" the oil is then separated by direct continuous centrifugation from the pomace, which consists of vegetable matter and water. The yield of oil varies from 80 to 90% of the total oil content of the olives, because the oil in the olive paste is only partially free to escape and part of it remains in the unbroken cells or is trapped in the tissues of the cytoplasm, or is emulsified in the aqueous phase.

After the extraction of virgin olive oil from the olives, the remaining paste is called pomace and still contains a small quantity (2-6%) of oil that can only be extracted with chemical solvents. This is done in specialized chemical plants, not in the oil mills and the obtained oil is called pomace oil.

The FFA limit in olive oil for direct consumption is 2%. Virgin olive oils with higher content are called "lampantes" and must be refined prior to consumption. The components to be removed are all those ones that are detrimental to the flavor, color and stability of the oil, mainly FFA, phosphoacylglycerols, pigments, volatiles and contaminants. The standard processes used are chemical and physical refining. The main difference between both processes is that chemical refining procedure includes caustic soda treatment to neutralize the oil while, following physical refining, FFA are eliminated by distillation during deodorization. Physical refining reduces the loss of neutral oil, minimizes pollution and enables recovery of high quality FFA. Nevertheless, not all oils can be physically refined.

3.2 Table olives

"Table olives" means the product: a) prepared from the sound fruit of varieties of the cultivated olive tree (*Olea europaea L.*) that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh taste, firmness and ease of detachment from the stone make them particularly suitable for processing; b) treated to remove its bitterness and preserved by natural fermentation or by heat treatment, with or without the addition of preservatives; c) packed with or without covering liquid (International Olive Oil Council, 2004).

Table olives are classified in one of the following types according to the degree of ripeness of the fresh fruits:

- Green olives: Fruits harvested during the ripening period, prior to colouring and when they have reached normal size.
- Olives turning color: Fruits harvested before the stage of complete ripeness is attained, at colour change.
- Black olives: Fruits harvested when fully ripe or slightly before full ripeness is reached.

To prevent olive damage, fruits destined for table olives production are picked by hand and carefully placed in special padded basket that are hung by their neck. Olive transportation is carried out in perforated plastic containers, which have plastic netting as walls (supported by an iron structure). The perforated walls permit the aeration of the fruits and the reduced weight also contributes to minimizing the damage of the fruits. Sometimes, olives are also transported in bulk, although this transportation system is not recommended due to the increased risk of damaging the olives.

Once the olives have been transported, their processing takes place. In general, any processing method aims to remove the natural bitterness of the fruit, caused by the glucoside oleuropein. The bitterness may be removed by alkaline treatment, by immersion in a liquid to dilute the bitter compound, or by biological processes. The product so obtained may be preserved in brine according to its specific characteristics, in dry salt, in a modified atmosphere, by heat treatment, my preservatives, or by acidifying agents. The most common trade preparations are (Sánchez et al., 2006):

- Treated olives: Olives that have undergone alkaline treatment, then packed in brine in which they undergo complete or partial fermentation, and preserved or not by the addition of acidifying agents. The most common preparation is "treated green olives in brine".

- Natural olives: Olives placed directly in brine in which they undergo complete or partial fermentation, preserved or not by the addition of acidifying agents. The most common preparation is "natural black olives".
- Olives darkened by oxidation: Olives preserved in brine, fermented or not, darkened by oxidation in an alkaline medium and preserved in hermetically sealed containers subjected to heat sterilisation; they shall be a uniform black color. They are also known as "black olives".

Other trade preparations include dehydrated and/or shrivelling olives and specialities prepared in different forms.

4. Pesticides: definition and classification

The denomination pesticides (or plaguicides) include a wide variety of products that are very different in their chemical composition and characteristics. A plaguicide can be described as a substance (or formulation containing at least one of them) that presents any of the following uses:

- Fight agents that can be harmful for the crops or prevent the potential effects of these agents.
- Control or regulate the vegetable production.
- Protect the vegetable production, including woods.
- Destruct weeds.
- Destruct part of the vegetables or prevent undesirable growths.
- Destruct or prevent the action of potentially harmful organisms different to the ones that attack plants.

Taking into account the specific action of the plaguicides, different classifications can be made, being the decimal classification one of the most frequently used:

- Insecticides are used against insects and they include ovicides and larvicides (against eggs and larvae of insects, respectively). Nearly all insecticides have the potential to significantly alter ecosystems, being many of them toxic to humans.
- Acaricides kill members of the Acari group, which includes ticks and mites.
- Fungicides are chemical compounds or biological organisms used to kill or inhibit fungi or fungal spores.
- Nematocides, disinfectants and fumigants in general, used to kill parasitic nematodes.
- Herbicides are used to kill unwanted plants while leaving the desired crop relatively unharmed.
- Phytoregulators and similar products can be used to improve the potential of the trees.
- Molusquicides and rodenticides are used to control molluscs (slugs and snails) and rodents pests.
- Post-harvest pesticides and seeds.
- Protectors of woods, fibers and derivatives.
- Other specific plaguicides.

Laboratory studies show that pesticides can cause health problems, such as birth defects, nerve damage, cancer, and other effects that might occur over a long period of time. However, these effects depend on how toxic the pesticide is and how much of it is

consumed. Some pesticides also pose unique health risks to children. For these reasons, the governments carefully regulate pesticides to ensure that their use does not pose unreasonable risks to human health or the environment.

The mechanisms of action of the plaguicides over the organism are very different depending on the chemical composition. These mechanisms are well-known for some pesticides, even at the molecular level. However, they are completely unknown for some others. Even among pesticides from the same family, some of them can be classified as scarcely toxic while others are very toxic. As a result, it is difficult to establish general rules when dealing with the toxicity of plaguicides.

Herbicides are required to control the growth of weeds. The absence of weeds in the soil around the olive tree presents two major benefits: the weeds do not waste water resources for the olive trees and the harvest of olives from the soil is easier when they naturally fall down from the tree.

Depending on the period of application, herbicides can be classified as:

- Pre-emergents, applied at the starting of autumn.
- Early post-emergents, applied by the middle of autumn, after the early rainings.
- Post-emergents, applied in spring against perennial weeds.

In Table 1, the herbicides that are usually employed in the Spanish olive groves are shown. Simazine, which is forbidden, is also present because it is very persistent and has to be analyzed in order to ensure the absence of residues. The herbicides comprise triazines (simazine, atrazine, trietazine, terbuthylazine and terbutrine), phenylurea diuron and phenylether oxyfluorphen.

The water solubility (W.S.) at 25 ^{o}C and the $K_{O/W}$ value are also presented in Table 1. The $K_{O/W}$ value is the partition coefficient between octanol and water, and its logarithm is an indication of fat solubility of the pesticide. A high value of the coefficient $K_{O/W}$ for a particular plaguicide indicates that its solubility in water is low; hence, this plaguicide would be fat-soluble and there would be risks of bio-accumulation in fatty tissues. It has been demonstrated that fat-soluble pesticides tend to concentrate in olive oil during its production and extraction from the olives. For this reason, higher concentration levels of fat-soluble plaguicides are expected in the oil than in the olives from which the oil was produced. On the other hand, polar pesticides do not tend to preconcentrate in olive oil and their concentration is lower.

Monitoring herbicide residues in olive oil and table olives is of great interest to ensure food safety related to their use. The development of multi-residue methods is required in this case in order to determine all the herbicides in the same analysis. These methods need to present a high sensitivity in order to be able to analyze the samples at the legislated MRLs.

The analysis of herbicides in these samples is very challenging, because of the inherent complexity of the matrix. As a result, it is necessary to extract the pesticide fraction from the whole matrix to isolate the compounds that will be analyzed. Taking into account that some herbicides are fat-soluble, it is difficult to completely separate them from the matrix. Hence, a clean-up step is required after the extraction procedure.

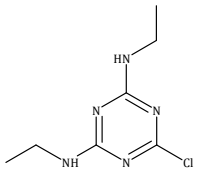
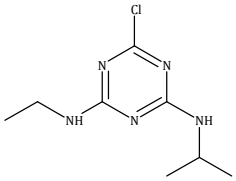
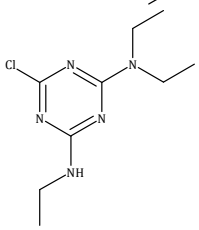
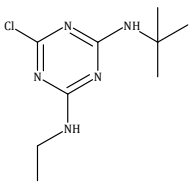
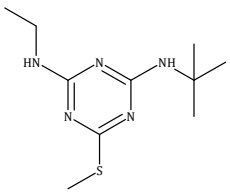
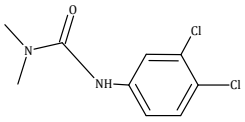
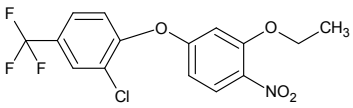
Plaguicide	Structure	W.S. (mg L^{-1})	$K_{O/W}$ 25°C
Simazine		1.3	2.10
Atrazine		33	2.50
Trietazine		20	3.34
Terbuthylazine		8.5	3.21
Terbutrine		22	3.65
Diuron		36.4	2.85
Oxyfluorfen		0.12	4.86

Table 1. Herbicides in olive groves.

5. Sample preparation

The first step required in the analysis of herbicides is to separate the analytes from the matrix and other interfering compounds, therefore isolating the herbicides from the rest of the sample. This separation includes extraction, pre-concentration and clean-up steps, although sometimes they can be performed at the same time (Gilbert-López et al., 2009; Mukherjee & Gopal, 1996).

This part of the analysis is critical, because the final results will be completely related to the success of the separation process. The sample has to come into contact with an extractant (solid, liquid or supercritical fluid) in optimized conditions in order to weaken the analyte-matrix interactions and increase the analyte-extractant interactions.

There is a continuous development of sample-treatment procedures for the isolation of herbicides in samples with relatively high fat content, such as olive oil and olives. The preparation of oil samples for their determination by chromatographic techniques requires the removal of the fatty components from the sample. The main problem associated when working with this kind of samples is that dirty extracts may harm the instruments employed. For this reason, proper extraction and clean-up steps are required. In addition, the different nature and physicochemical properties of the classes of herbicides to be analyzed makes it more complex to select the appropriate extraction methodology.

There are numerous extraction and clean-up procedures. However, the most common ones are solid-liquid or liquid-liquid extraction, gel permeation-chromatography (GPC) and solid-phase extraction (SPE) (Gilbert-López et al., 2009; Mukherjee & Gopal, 1996; Walters, 1990).

5.1 Solid-liquid extraction

In this case, the sample comes to contact with an appropriate solvent. After that, different procedures can be applied to homogenize both phases: ultrasonic agitation and microwave extraction.

Ultrasonic agitation: the solvent interacts with the sample simply by a shaking process. Ultrasonic radiation (frequency of 25-40 Hz) causes the vibration of the molecules, increasing the collision between them. Hence, the contact between sample and solvent is enhanced. After the analyte is dissolved in the extractant, a filtration step and the evaporation of the solvent are required.

Microwave extraction: samples are enclosed in high quality Teflon vessels together with the solvent and heated to a controlled temperature with microwave power, being the extract filtered when the process finishes. Electromagnetic irradiation is used to heat the solvent that acts as extractant, which is in most cases a mixture between hexane and acetone.

5.2 Liquid-liquid extraction

Usually, it has been the chosen method for the extraction, preconcentration and clean-up of liquid samples. It is based on the relative solubility of the analyte in two different immiscible liquids; therefore, it is an extraction of a substance from one liquid phase into another liquid phase. The liquid sample comes to contact with an appropriate solvent (immiscible with the

sample) and, after a mixing time, both phases (sample and extractant) are separated. During the mixing period, the analyte is distributed between both phases until it reaches the equilibrium.

The $K_{O/W}$ value is critical to decide the appropriate extractant. If this value is high, it indicates that the analyte tends to dissolve in the organic phase instead of in water. As a general rule, the organic solvents selected are volatile substances that present high affinity for the analytes and are immiscible with the sample.

5.3 Gel-permeation chromatography

It is probably the most extensively used technique for the analysis of pesticide residues in olive oil, usually after a liquid-liquid extraction. In this technique, an aliquot of an olive oil extract (obtained from the extraction step) is injected into the GPC system. The selected fraction is collected and, after a solvent-exchange step, the sample is analyzed by GC. A GPC system is composed of a chromatographic pump, a fraction collector and a detector. The columns are made from polymeric porous microspheres that enable the separation of compounds according to their molecular weights (which are related to the size of the compound). Using this principle, the herbicide fraction is separated from the triglyceride fraction, which presents higher molecular weight. In GPC, the compounds are eluted from higher to lower molecular weight.

5.4 Solid-phase extraction

It is a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. SPE is used to concentrate and purify samples for analysis and to isolate the analytes of interest from a wide variety of matrices.

SPE uses the affinity of the analytes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase. The portion that passes through the stationary phase is collected or discarded, depending on whether it contains the desired analytes or undesired impurities. If the portion retained on the stationary phase includes the desired analytes, they can then be removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed with an appropriate eluent.

The stationary phase comes in the form of a packed syringe-shaped cartridge that can be mounted on its specific type of extraction manifold. The manifold allows multiple samples to be processed by holding several SPE media in place and allowing for an equal number of samples to pass through them simultaneously. A typical cartridge SPE manifold can accommodate up to 24 cartridges and is equipped with a vacuum port. Application of vacuum speeds up the extraction process by pulling the liquid sample through the stationary phase. The analytes are collected in sample tubes inside or below the manifold after they pass through the stationary phase.

Solid phase extraction cartridges are available with a variety of stationary phases, each of which can separate analytes according to different chemical properties. Most stationary phases are based on silica that has been bonded to a specific functional group.

6. Detection techniques for the analysis of pesticides. Gas chromatography

Pesticides (herbicides, fungicides or insecticides) are the most abundant environmental pollutants found in soil, water, atmosphere and agricultural products, and may exist in harmful levels and pose an environmental threat. Even low levels of these contaminants can cause adverse effects on humans, plants, animals and ecosystems. As it has been commented in the Introduction, there are several alternatives for the determination of pesticides in different kinds of samples. In this sense, automated systems of analysis with spectroscopic or electrochemical detection have been developed (Llorent-Martínez et al., 2011). In particular, the development of sensors have been specially useful. Electrochemical sensors present the advantages of miniaturization, simplicity, possibility of in-situ measurements and low-cost. In general, the main contribution of these sensors to pesticide analysis has consisted of the development of methods of analysis for determining a whole family of compounds (Du et al., 2009; Halámek et al., 2005; Liu & Lin, 2006). In spectroscopic methods, fluorescence (Calatayud et al., 2006; Mbaye et al., 2011) or chemiluminescence (Catalá-Icardo et al., 2011; López-Paz & Catalá-Icardo, 2011) detections have been usually employed due to their intrinsic sensitivity and selectivity. Among the spectroscopic methods of analysis, the design of flow-through optosensors has also been paid particular attention (Llorent-Martínez et al., 2011). These sensors present enhanced sensitivity and selectivity and have been applied to the determination of a small number of pesticides in different kind of samples (Llorent-Martínez et al., 2005; Llorent-Martínez et al., 2007; López Flores et al., 2007).

In general, electrochemical sensors can provide a useful tool when a whole family of compounds is targeted, while optosensors provide an interesting approach in order to quantify a small number of analytes in a particular sample. However, chromatographic techniques are still the chosen ones when a multi-residue analysis is required, being GC the most common one for the analysis of pesticides.

GC is an analytical technique that is used for separating and analysing compounds that can be vaporized without decomposition (Harris, 2007; Skoog et al., 1996). Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture. In GC, the mobile phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called column. A gas chromatograph uses a flow-through narrow tube known as the column, through which different chemical constituents of a sample pass in a gas stream (carrier gas, mobile phase) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the stationary phase. As the chemicals exit the end of the column, they are detected and electronically identified. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, column length and the temperature.

In a GC analysis, a known volume of gaseous or liquid sample is injected into the head of the column using a micro syringe. As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of the analyte molecules either onto the column walls or onto packing materials in the column. The rate at which the molecules progress along the column depends on the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different times. A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined.

Different detectors can be used in GC. The most common ones until recent years were the flame ionization detector (FID), the thermal conductivity detector (TCD) and the electron capture detector (ECD). However, nowadays most of the developed analytical methods use GC coupled to mass spectrometry (MS) detectors. In the analytical methods that will be described later, only ECD, thermoionic specific detector (TSD) and MS have been used in our research.

ECD is used for detecting electron-absorbing compounds. It uses a radioactive beta particle (electron) emitter. The electrons are formed by collision with a nitrogen molecule because it exhibits low excitation energy. The electron is then attracted to a positively charged anode, generating a steady current. Therefore, there is always a background signal present in the chromatogram. As the sample is carried into the detector by the carrier gas, analyte molecules absorb the electrons and reduce the current between the collector anode and a cathode. The analyte concentration is thus proportional to the degree of electron capture. ECD is particularly sensitive to halogens, organometallic compounds, nitriles, or nitro compounds.

TSD is a very sensitive but specific detector that responds almost exclusively to nitrogen and phosphorous compounds. It contains a rubidium or cesium silicate (glass) bead situated in a heater coil, at little distance from the hydrogen flame. The heated bead emits electrons by thermionic emission. These electrons are collected under a potential of few volts by an appropriately placed anode, and provides a background current. When a solute containing nitrogen or phosphorous is eluted from the column, the partially combusted nitrogen and phosphorous materials are adsorbed on the surface of the bead. The adsorbed material reduces the work function of the surface and, as consequence, the emission of electrons is increased, raising the current collected at the electrode.

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. In MS detection: 1) the analytes undergo vaporization; 2) they are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions); 3) the ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields; 4) the ions are detected, usually by a quantitative method; and 5) the ion signal is processed into mass spectra.

7. Analysis of herbicides

Our research has focused mainly on the determination of herbicides in olives destined for production of olive oil and olive oil. However, at this moment, we are continuing with this research, analyzing herbicide residues in table olives. On average, 5 kg of olives are required for the production of 1 L of oil. Taking into account that most pesticides (including herbicides) are lipophilic, a concentration effect could occur when obtaining the olive oil. Thus, MRL for herbicides have been set by the European Union in both olives and olive oil. For this reason, our research group has developed analytical methods that allow the analysis of the selected herbicides in olive oil and olives. In this section we will describe the analytical procedures that have been employed for this purpose as well as the results obtained.

For olive oil samples (Guardia-Rubio et al., 2006b), the preparation procedure included a liquid-liquid extraction followed by GPC clean-up step: 1) Two grams of the olive oil sample were dissolved in n-hexane saturated in acetonitrile. The solution was transferred to a separation funnel where it was extracted three times with acetonitrile saturated in n-hexane. The extracts were combined in a round-bottomed flask and were concentrated to dryness in a rotary evaporator. 2) The residue was dissolved in the GPC mobile phase (ethyl acetate-cyclohexane, 1:1 (v:v)) and injected into the GPC column. The collected eluate fraction was transferred to a round-bottomed flask and concentrated to dryness in a rotary evaporator. 3) The residue was redissolved in cyclohexane and analyzed by GC-MS.

For olive samples (Guardia-Rubio et al., 2007c), approximately 130 g of olives (including the seeds) were first crushed by means of a hammer mill. Afterwards, a 100 g portion was weighed in a glass tube and 50 g of anhydrous sodium sulphate were added. The sample was then extracted twice with light petroleum by homogenization with Ultra-Turrax (a high flow mixing tool) and the extracts evaporated using a vacuum rotary evaporator. The solid residue was dissolved in n-hexane saturated in acetonitrile and the same liquid-liquid extraction and GPC clean-up procedure employed for olive oil were performed before the GC-MS analysis.

The following Table shows the retention times (t_R) and analytical parameters obtained for each selected herbicide, including the detection limit (DL). The procedures previously detailed were applied to the determination of the cited herbicides in olives and olive oil samples.

As it was commented in Section 3, there are different olive harvesting methods. Depending on this, the olive fruits can be grouped into three categories: a) fruits picked directly from the tree without any contact with the soil (flight olives); b) fruits picked from the ground (soil olives); and c) fruits that are not separated before the elaboration process (non-separated olives). The separation of flight and soil olive fruits before olive oil elaboration is critical in order to obtain appropriate results (high-quality virgin olive oil). If both fruits are not separated, the quality of the oil and the percentage of extra virgin olive oil obtained decrease. In addition, the harvesting method may be very important for the presence of herbicide residues in olives, because they remain concentrated in the top 5-15 cm of soil, even after several months since their application. 94 and 33 samples of olives and olive oil, respectively, were analyzed. Diuron and terbuthylazine were found in many of these samples, 79 of olives (specially soil olives) and 31 of olive oil. In four of the soil olives,

diuron levels were higher than the MRL established by the European Union (0.2 mg kg^{-1}); however, none of the olive oil samples presented levels higher than the corresponding MRL (0.8 mg kg^{-1}). Terbuthylazine was also quantified in many samples with values over the MRL in some of them, being the established MRLs 0.05 and 0.2 mg kg^{-1} for olives and olive oil, respectively. Although other herbicides were also detected, in most cases the levels were below the quantification limit of the system. In general, the levels of herbicides found in soil olives have been significantly higher than those ones found in flight olives, which have not been in contact with the soil. These results suggest that herbicide residues are mainly caused by the contamination of the olives when they come to contact with the soil after falling down (Guardia-Rubio et al., 2006b; Guardia-Rubio et al., 2006c).

Herbicide	t_R (min)	Olives		Olive oil	
		Linear Range (μg kg^{-1})	DL (μg kg^{-1})	Linear Range (μg kg^{-1})	DL (μg kg^{-1})
Simazine	11.971	0.75-125	0.25	3-2000	1
Atrazine	12.095	1.25-250	0.50	5-1000	2
Trietazine	12.621	2.50-200	1.25	10-800	5
Terbuthylazine	12.666	0.25-250	0.12	1-1000	0.5
Terbutrine	18.125	5.00-250	1.25	20-1000	5
Diuron	6.908	1.25-250	0.12	5-1000	0.5
Oxyfluorfen	25.918	2.50-250	1.25	10-1000	5

Table 2. Analytical parameters for olives and olive oil determination.

The fruit goes into the olive-oil mill with a great quantity of impurities, leaves, branches, mud, etc, which are necessary to eliminate. Therefore, for this purpose, cleaners and washing devices are employed to eliminate impurities (Section 3), especially present in soil olives. It would be interesting to evaluate what fraction of the herbicides could be eliminated from the olives after the washing process in the olive mill, previous selection of the herbicides that frequently appear in these samples. The selected plaguicides were diuron and terbuthylazine. Our research group presented the first exhaustive study of the influence of the olives washing in the mills over the concentration of these herbicides (Guardia Rubio et al., 2006a; Guardia Rubio et al., 2007a; Guardia-Rubio et al., 2007b; Guardia-Rubio et al., 2008). Olive samples were collected before and after the washing process in the mill and were analyzed by GC-MS.

The most outstanding conclusion from the obtained results was the drastic reduction in the levels of herbicides in soil olives after the washing process when compared to the same olive samples before the washing step. The washing process significantly diminished the levels of residues of herbicides in soil olives, while the influence of the washing step was not clearly appreciated in flight olives. In the case of non-separated olives, it is interesting to remark that some of the washed olives presented higher levels of herbicides than the non-washed ones. This can be due to the contamination of herbicides-free olives during the washing process. The washing machines for the olives are cleaned and filled with fresh water at the starting of the day. With this water, up to 160000 kg of olives can be washed before the water is changed next day. The water can be contaminated after the washing of soil olives, therefore contaminating following herbicides-free olives in the washing process. Therefore, the water and mud from the olive washing devices were analyzed to confirm this theory. The procedures employed for both type of analyses follow:

a. In the case of washing water samples, they were first filtered and then slowly passed through a SPE cartridge packed with C_{18} using a 12-port SPE vacuum manifold. The retained herbicides were then eluted from the solid phase with dichloromethane. The eluate, filtered and dried with anhydrous Na_2SO_4, was evaporated to dryness and the residue was dissolved in cyclohexane for GC-MS analysis.
b. In the case of mud samples, a solid-liquid extraction was carried out with a mixture of cyclohexane/acetone (3:1) in an ultrasonic water bath. The extracts were then filtered to eliminate particulate material, dried with anhydrous Na_2SO_4 and evaporated to dryness by means of a rotary evaporator. After that, an additional clean-up step was necessary in order to remove any remaining fat in the extract after the extraction procedure. This step involved the use of a chromatographic column that was packed with activated alumina suspended in cyclohexane. Once the extract was applied to the column, a mixture of cyclohexane/acetone (3:1, v:v) followed by dichloromethane was used to elute the herbicides. Once the eluate was evaporated and redissolved in cyclohexane, the sample could be analyzed by GC with ECD or TSD detection.

From the analyses carried out over mud and washing water samples, the following results were obtained: a) regarding water analysis, the waters from an olive mill were collected at different times during the same day, and it was observed that the concentration of herbicides increased continuously along the day. An increase in the amount of washed

olives meant an increase in herbicide residues. These results confirmed that the waters were being continuously contaminated with herbicides and a decontamination process would be required in the middle of the day (Guardia-Rubio et al., 2008); b) with respect to mud samples, 18 samples were analyzed. Diuron and terbuthylazine were found in nearly all the analyzed samples. Diuron appeared in all samples at concentration levels that ranged between 2.8 and 401.3 ng g^{-1}. Terbuthylazine was detected in 16 samples at concentration levels between 7 and 1031.4 ng g^{-1}. Simazine, prohibited in olive farming in the European Community but a very persistent pollutant, was detected in four samples, although in three of them the concentration was below the quantification limit (Guardia Rubio et al., 2006a). In general, the analysis of mud and waters from the washing device showed that, although the washing process eliminate a high percentage of the herbicide residues, a control over the re-used washing water needs to be performed.

8. Conclusions

The production of virgin olive oil has increased in recent years and it is being exported to different countries, representing an important part of the economy in some Mediterranean countries. As we described in this chapter, it poses a lot of beneficial properties for the human health. However, it is important to carry out exhaustive quality controls in order to maintain its high standards. One of the most important ones is the analysis of residues of pesticides (including herbicides), which can be very dangerous for the human body, presenting different adverse effects over the organism depending on their toxicity. Here, we have described the methods developed in our research group for the analysis of herbicides in olives and olive oil. It has been shown that diuron and terbuthylazine are commonly found in these samples, although usually at levels below the established MRLs. In addition, the separation of flight and soil olives, together with the washing process in the olive mills, is a critical step to control the levels of herbicides. However, the efficiency of the washing step decreases over the time and the washing waters need to be replaced in order to keep the process being useful along the whole working day. Although the studies here presented have focused on the analysis of olive oil and olives destined to oil production, further research is currently being performed for the analysis of processed table olives.

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13

Prediction of Herbicides Concentration in Streams

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1. Introduction

Natural and anthropogenic variables of stream drainage basins such as hydrogeologic parameters (permeability, porosity etc.), amount of agricultural chemicals applied, or percentage of land planted affect agricultural chemical concentration and mass transport in streams. The use of herbicides, pesticides, and other chemicals in agricultural fields increase the concentration of chemicals in streams which severely affects the health of human and environment. The transport of chemical pollutants into river or streams is not straight forward but complex function of applied chemicals and land use patterns in a given river or stream basin. The factors responsible for transport of chemicals may be considered as inputs and chemical concentration measurements in streams as outputs. Each of these inputs and outputs may contain measurement errors. Present work exploited characteristics of fuzzy sets to address uncertainties in inputs by incorporating overlapping membership functions for each of inputs even for limited data availability situations. Soft computing methods such as the fuzzy rule based and ANN (Artificial Neural Networks) is used for characterization of herbicides concentration in streams. The fuzzy c-means (FCM) algorithm is used for the optimization of membership functions of fuzzy rule based models for the estimation of diffuse pollution concentration in streams. The general methodology based on fuzzy, ANN and FCM for estimation of diffuse pollution in streams is presented. The application of the proposed methodology is illustrated with real data to estimate the diffuse pollution concentration in a stream system due to application of a typical herbicide, atrazine, in corn fields with limited data availability. Solution results establish that developed fuzzy rule base model with FCM outperform fuzzy or ANN and capable for the estimation of diffuse pollution concentration values in water matrices with sparse data situations.

Application of pesticides, insecticides and herbicides, cause diffuse pollution, commonly referred to as non-point source pollution in river or streams. Diffuse pollution from agricultural activities is a major cause of concern for the health of human and environment. Diffuse (non-dot, dispersed) pollution generally arises from land-use activities (urban and rural) that are dispersed across a catchment or subcatchment, where as point sources of pollution arise as a process industrial effluent, municipal sewage effluent, deep mine or farm effluent discharge (Novotny 2003, based on CIWEM (D'Arcy et al., 2000)). Potential point sources of pollution is characterised by its location, magnitude and duration of activity; and the sources of pollution is characterized when these parameters are identified

(Mahar and Dattta 2000; Singh and Datta, 2004, and Singh and Datta, 2006a and 2006b). In diffuse sources of pollution or non-point sources of pollution, sources of pollution is moving with polluting media thus making it more difficult and complex problem to solve.

Often diffuse pollution is individually minor but collectively constitutes significant sources at basin scale. Although nonpoint or diffuse sources may contribute many of the same kinds of pollutants, these pollutants are generated in different volumes, combinations, and concentrations (Jha et al., 2005). Thus, diffuse pollution comprises true non-point source pollution together with inputs from a multiplicity of minor point sources. The important characteristics of diffuse pollution are, therefore, not whether anyone can identify the source or sources, but the collective impact of diffuse pollutants and the mechanisms through which they move through the environment. The concept of diffuse pollution is useful because it explains features of pollution in receiving water bodies that differ from the point sources of pollution that are typically well characterized, monitored, and quantified. Some of the characteristics of diffuse pollutants are that the concentrations of some pollutants actually may increase with flow rather than it has diluted, pollution peaks are variable and difficult to predict, and impacts are often slow to develop and become evident years later (e.g. contamination of groundwater). For diffuse pollution, it is the proportion of the land use from which the pollution is derived, is more important.

Agricultural activities such as application of herbicides result in the contamination of surface water with agricultural chemicals. Numerous recent investigations (Goolsby and Battaglin, 1993 and 1995; Schottler et al., 1994; Baker and Richards, 1990) indicate that significant quantities of some herbicides are flushed from cropland to streams each spring and summer during rainfall events following the applications. Peak concentration of several herbicides can exceed 10 µg/l during these events (Coupe et al., 1995; Scribner et al., 1994). Pareira (1990), Crawfard (1995, 2001), Capel and Larson (2001), and Smith and Wheater (2004) in their studies on pesticides/herbicides, identified the major factors that control the pollutant transport. Herbicides and pesticides concentrations in surface waters are affected by natural and human factors. For example, concentrations of atrazine, a herbicide widely used on corn fields, tended to be higher in an agricultural basin with permeable, well drained soils, than in an agricultural basin with less permeable, more poorly drained soils (Crawfard, 1995). Capel et al. (2001) estimated the annual pollutant transport as percent of use (load as percent of use - LAPU). Larson and Gilliom (2001) developed a regression model for the estimation of pollutants.

Water resources professionals, managers and government authorities involved in surface water management are increasingly pressed to make appropriate decisions on land use and development policies such that these decisions will not adversely affect the health and environment. At the same time, they are constrained by inadequate budgets, limited resources, and incomplete information, which compel them to rely on models to evaluate or to estimate the pollution characteristics in the water bodies, and the implications of their decisions based on those evaluations. In this regard, the role of complex stream quality simulation models e.g. SWAT (Arnold et al. 1983), AGNPS (Young et al., 1989) etc. in evaluating runoff pollution conditions under various agricultural chemicals and land use patterns is also limited. These models incorporate rainfall, catchments, and pollutant characteristics, requiring extensive calibration and verification. However, their results are not without large uncertainties. These uncertainties arise both in the representation of the

physical, chemical, and biological processes as well as in the data acquisition and parameters for model algorithms. Consequently, the complexities of these models and their resource-intensive nature are significant obstacles to their application (Charbeneau and Barrett 1998).

There is a need for the development of simpler methods of agricultural stream quality predictions that provide the required information to the analyst and water managers with minimal effort and limited data requirements as compared to complex process models. As an alternative or supplement to complex runoff quality simulation models, fuzzy rule based model with FCM is proposed to estimate pollutant concentration due to applications of agricultural chemical, herbicide, atrazine, in the streams.

The herbicide atrazine (2-chloro-4-[ethylamino]-6-[isopropylamino]-1,3,5-triazine), a chlorinated herbicide, has been one of the most heavily used herbicides in the world. Atrazine is toxic to many living organism. The maximum contaminant level (MCL) of atrazine is restricted to 3 μg/l for drinking water (USEPA, 2001). Because atrazine is water soluble, it has the potential to leach into ground water and run off to surface water. Atrazine is associated with developmental effects (USEPA, 2002), such as birth defects, structural anomalies, and adverse hormone changes. Thus, its accurate estimation in water matrices is imperative.

In this study, a fuzzy rule based model optimized by fuzzy c-Means, is developed to obtain the estimate of atrazine concentrations from agricultural run-off using limited available information. The work discusses the methodology to develop the fuzzy rule base model using annual average use of herbicide atrazine per unit area, extent of herbicide atrazine applied area and herbicide atrazine application season as inputs to fuzzy rule based model and observed herbicide concentration at the basin outlet as the output for the fuzzy model. The data of White River Basin, a part of the Mississippi River system, USA, is used for developing the fuzzy rule base model.

2. Agricultural diffuse pollution concentration simulation in streams

Natural and anthropogenic variables of stream drainage basins such as hydrogeologic parameters (permeability, porosity etc.), amount of agricultural chemicals applied, or percentage of land planted affect agricultural chemical concentration and mass transport in streams. The general form of model that simulates the concentration measurement in a watershed can be represented by (Tesfamichael et al., 2005)

$$C = f(\mathbf{W}, \mathbf{H}, \mathbf{A}) \tag{1}$$

where C is the stream agricultural diffuse pollution observed concentration measurement values; **W** is a vector of watershed characteristics; and **H** is a vector of hydrological variables such as precipitation, runoff, etc., and **A** is a vector of relevant agricultural practices including actual chemical application rate in the field in lb/acre.

For a particular watershed, watershed characteristic, **W**, may be assumed to be constant. Also, for a particular hydrological unit, **H** may be assumed to be of similar characteristics. Then, Equation (1), though simplified, may be represented by

$$C = f(\mathbf{A}) \tag{2}$$

The **A** may be further represented by

$$\mathbf{A} = f(\mathbf{A_C}, \mathbf{A_L}) \quad (3)$$

where $\mathbf{A_C}$ represent the vector of applied agricultural chemical characteristics such as type of agricultural chemical (insecticide, herbicides etc.), application rate, application season etc., and $\mathbf{A_L}$ is the land use patterns such as type of crop grown, percentage of cropped area, etc.

Here, agricultural chemical considered is herbicide, atrazine, and crop considered is corn. In this study fuzzy rule based model with FCM simulates the stream system behavior from inputs of agricultural practices and corresponding observed concentration measurement values. In fact the model tries to emulate the mechanism that produced the data set. In this way, the mathematical description of the physical system is learned by the model, and therefore utilized as a tool for stream system simulation. The cluster centers of inputs and outputs obtained using FCM model, in essence, represents a typical characteristics of the system behaviour, and hence utilized in the formation of rule base of the fuzzy model.

3. Methodology

Statistical methodologies have been traditional being utilized for diffuse pollutants predictions in streams. However, transport of herbicides is complex and uncertain phenomena and traditional methods like regression are not able to incorporate uncertainty in model predictions. Present work will discuss methodologies based on recent soft computing techniques like fuzzy, artificial neural network (ANN) and their hybrids. The application of the proposed methodology is illustrated with real data to estimate the diffuse pollution concentration in a stream system due to application of a typical herbicide, atrazine, in corn fields with limited data availability.

3.1 Modeling approach

The models based on fuzzy logic and ANN, also known as intelligent or soft computing models, are potentially capable of fitting a nonlinear function or relationships. Identification of model architecture is decisive factor in the simulation and comparison. The identification of model architecture is crucial in ANN model building process. While the input and output of the ANN model is problem dependent, there is no direct precise way to determine the optimal number of hidden nodes (Nayak et al., 2005).The model architecture is selected through a trial and error procedure (Singh et al.. 2004). The fuzzy model, on the other hand, may be considered as a mapping of input space into output space by partitions in the multidimensional feature space in inputs and outputs. Each partition represents a fuzzy set with a membership function.

3.2 Fuzzy rule based system

Fuzzy logic emerged as a more general form of logic that can handle the concept of partial truth. The pioneering work of Zadeh (1965) on fuzzy logic has been used as foundation for fuzzy modeling methodology that allows easier transition between humans and computers for decision making and a better way to handle imprecise and uncertain information. Human being think verbally, not numerically. As the fuzzy logic systems involves verbal

statements and, therefore, the fuzzy logic is more in line with human perception (Zadeh, 2000). Fuzzy logic has an advantage over many statistical methods in that the performance of a fuzzy expert system is not dependent on the volume of historical data available. Since these expert systems produce a result based on logical linguistic rules, extreme data points in a small data set do not unduly influence these models. Because of these characteristics, fuzzy logic may be a more suitable method for diffuse pollution forecasting than the usual regression modeling techniques used by many researchers (e,g. Goolsby and Battaglin (1993); Larson and Gilliom (2001); and Tesfamichael et al. (2005) etc.) for estimation of diffuse pollution concentration in streams or other water bodies.

3.2.1 Fuzzy rule based system architecture

The most common way to represent human knowledge is to form it into natural language expression of the type,

IF premise (antecedent), THEN conclusions (consequent) (4)

The form in expression (4) is commonly referred to as the IF-THEN rule based form (Ross, 1997). It typically expresses an inference such that if a fact (premise, hypothesis, antecedent) is known, then another fact called a conclusion (consequent) can be inferred or derived. Fuzzy logic systems are rule base systems that implements a nonlinear mapping (Dadone and VanLandingham, 2000) between stresses (represented by consequents) and state variables (represented by antecedents). Creating a fuzzy rule based system may be summarized in four basic steps (Ross 1997; Mahabir et al. 2003; Singh and Singh 2005):

a. For each variable, whether an input variable or a result variable, a set of membership functions must be defined. A membership function defines the degree to which the value of a variable belongs to the group and is usually a linguistic term, such as high or low.
b. Statements, or rules, are defined that relate the membership functions of each variable to the result, normally through a series of IF–THEN statements.
c. The rules are mathematically evaluated and the results are combined. Each rule is evaluated through a process called implication, and the results of all of the rules are combined in a process called aggregation.
d. The resulting function is evaluated as a crisp number through a process called defuzzification.

Subjective decisions are frequently required in fuzzy logic modeling, particularly in defining the membership functions for variables. In cases such as in this study, where large data sets are not available to define every potential occurrence scenario for the fuzzification of model, expert opinion is used to create logic in the rule base system.

3.2.2 Membership functions

Membership functions used to describe linguistic knowledge are the enormously subjective and context dependent part of fuzzy logic modeling (Vadiee, 1993). Each variable must have membership functions, usually represented by linguistic terms, defined for the entire range of possible values. The key idea in fuzzy logic, in fact, is the allowance of partial belongings of any object to different subsets of universal set instead of belonging to a single set

completely. Partial belonging to a set can be described numerically by a membership function which assumes values between 0 and 1 inclusive. Intuition, inference, rank ordering, angular fuzzy sets, neural networks, genetic algorithms, and inductive reasoning can be, among many, ways to assign membership values or functions to fuzzy variables (Ross, 1997). Fuzzy membership functions may take on many forms, but in practical applications simple linear functions, such as triangular ones are preferable due to their computational efficiency (Khrisnapuram, R¸1998). In this study, triangular shapes are utilized to represent the membership functions.

3.3 Fuzzy c-means partitioning

Fuzzy rule based models represent the system behaviour by means of if then fuzzy rules. The basic requirement of fuzzy rule based model is to fuzzify or partition the inputs and outputs representation of a physical system. Assigning the number, shape, overlaps etc. of membership functions is most complex part of the fuzzy rule based model building. In most of the cases the optimality of the membership assigned to different fuzzy variables are not guaranteed. FCM is one of the methods to determine the fuzzy partitions of the available data sets into a predetermined number of groups. The data points are divided into group of points that are close to each other. Each data point belongs to a group or cluster with a membership function. Closeness between data points is defined by a metric distance or data center, and each metric yields a different portioning. This cluster centers are utilized in assigning overlaps of triangular shape membership function in this study.

Fuzzy c-means (FCM) is a method of clustering which allows one piece of data to belong to two or more clusters. The FCM method (developed by Dunn (1973) and improved by Bezdek (1981)) is frequently used in pattern recognition. It is based on minimization of the following objective function:

$$J_m = \sum_{i=1}^{N}\sum_{j=1}^{C_N} u^m{}_{ij} \left\| x_i - c_j \right\|^2, 1 \le m < \infty \tag{5}$$

where m is any real number greater than 1, uij is the degree of membership of xi in the cluster j, xi is the ith of d-dimensional measured data, cj is the d-dimension center of the cluster, and ||*|| is any norm expressing the similarity between any measured data and the center. The N represents total number of data points, and CN represents the total number of fuzzy centers. Fuzzy partitioning is carried out through an iterative optimization of the objective function shown above, with the update of membership *uij* and the cluster centers *cj* by:

$$u_{ij} = \frac{1}{\sum_{k=1}^{C_N} \left(\frac{\left\| x_i - c_j \right\|}{\left\| x_{i-c_k} \right\|} \right)^{\frac{2}{m-1}}} \tag{6}$$

$$c_j = \frac{\sum_{i=1}^{N} u^m{}_{ij} \cdot x_i}{\sum_{i=1}^{N} u^m{}_{ij}} \tag{7}$$

This iteration will stop when maxij{$|$uijk+1-uijk $|$}< ε , where ε is a termination criterion between 0 and 1, where as *k* are the iteration steps. This study used FCM algorithm (Matlab version 6.5), and ε is equal to 0.1 - 10^{-5} to obtain the pre-specified fuzzy centers.

This study implements FCM algorithm (Matlab version 6.5), m=2, and ε equal to 10^{-5} to obtain the pre-specified fuzzy centers.

3.4 Fuzzy rule based system with FCM for estimation of diffuse pollution concentration in streams

The watershed of the streams plays a vital role in influencing the diffuse pollution concentration in the streams. Basic Steps 1 through Steps 4 as discussed earlier in section Rule Based System are implemented by partitioning the input and output spaces into fuzzy regions with FCM, generation of fuzzy rules from available data pairs, assigning a degree to each rule, construction of a combined fuzzy rule base, and mapping from the input space to the output space using the rule base and a defuzzification (Wang and Mendel, 1992).

The vector AC and AL as represented by equation (2) are characterized for the specified watershed of the streams. As explained earlier, AC represents the vector of applied agricultural chemical characteristics such as type of agricultural chemical (insecticide, herbicides etc.), application rate, application season etc. The AL is the land use patterns such as type of crop grown, percentage of cropped area, etc. and C is the stream agricultural diffuse pollution observed concentration measurement values. Patterns were generated using a known set of input-output data pairs. The input data pairs AC and AL values and corresponding output values of C for a particular year constitutes a pattern. While AC and AL are constant for a particular year, the C is temporally and spatially varying at each of the monitoring station sites.

Fuzzy rules are building-blocks of fuzzy rule base systems. Partitioning the fuzzy variables into linguistic variables is necessary step towards designing the rule base system. Fuzzy partitions for the input and output variables are defined or generated according to the type of data as discussed in the membership section (Singh, 2008). In this work, FCM model is utilized to supply optimum number data centers to partition the input and output fuzzy variables.

It is absolutely possible to obtain the redundant and inconsistent rules from the data patterns having same antecedent parts. As mentioned, each rule is assigned a degree or weight by multiplying the membership functions of inputs and outputs for that rule. In the standard approach the rule having largest degree is adopted (Wang and Mendel, 1992). As an improvement, the degree of each rule is multiplied by a redundancy index to obtain the effective degree for that rule. The redundancy index may be defined as:

$$\text{Redundancy Index (R.I.)} = \frac{\mathrm{r_i}}{T_r} \tag{8}$$

where, ri represents the redundant rule with same i antecedents; and Tr represents the sum of all the redundant rules. Final fuzzy rule base includes the rules having the highest effective degree.

The fuzzy inference mechanism uses the fuzzified inputs and rules stored in the rule base for processing the incoming inputs data and produces an output. The fuzzy rules are processed by fuzzy sets operations as discussed in rule based section as basic steps for fuzzy rule base system. The fuzzy rule based design is accepted to be satisfactorily completed when its performance during training and testing satisfies the stopping criteria based on some statistical parameters.

3.5 ANN based methodology for estimation of diffuse pollution concentration in streams

The ANN learns to solve a problem by developing a memory capable of associating a large number of example input patterns, with a resulting set of outputs or effects. ANN is discussed in ASCE Task Committee (2000), etc. An overview of artificial neural networks and neural computing, including details of basics and origins of ANN, biological neuron model etc. can be found in Hassoun (1999), Schalkoff (1997), and Zurada (1997). The details of ANN model building process and selection of best performing ANN model for a given problem is available in (Singh et al., 2004).

As illustrated in the fuzzy model building for estimation of diffuse pollution concentrations in streams, the AC and AL values for a particular year in a watershed are inputs, and corresponding C values in the stream is out put for the ANN model. The values of AC, AL and C for a particular year constitute a data pattern. A standard back propagation algorithm (Rumelhart et al., 1986) with single hidden layer is employed to capture the dynamic and complex relationship between the inputs and outputs utilizing the available patterns. The ANN architecture that perform better than other evaluated architectures based on certain performance evaluation criteria, both in training and testing, was selected as the final architecture.

3.6 Performance evaluation criteria

The performance of the developed models are evaluated based on some performance indices in both training and testing set. Varieties of performance evaluation criteria are available (e.g. Nash and Sutcliffe 1970; WMO 1975; ASCE Task Committee on Definition of Criteria for Evaluation of Watershed Models1993 etc.) which could be used for evaluation and inter comparison of different models. Following performance indices are selected in this study based on relevance to the evaluation process. There can be other criteria for evaluation of performance.

3.6.1 Correlation coefficient (R)

The correlation coefficient measures the statistical correlation between the predicted and actual values. It is computed as:

$$R = \frac{\sum_{i=1}^{n}(Xai - \bar{X}ai)(Xpi - \bar{X}pi)}{\sqrt{\sum_{i=1}^{n}(Xai - \bar{X}ai)^2 \sum_{i=1}^{n}(Xpi - \bar{X}pi)^2}} \tag{9}$$

where Xai and Xpi are measured and computed values of diffuse pollution concentration values in streams; $\overline{X}ai$ and $\overline{X}pi$ are average values of Xai and Xpi values respectively; i represents index number and n is the total number of concentration observations.

The correlation coefficient measures the statistical correlation between the predicted and actual values. A higher value of R means a better model, with a 1 meaning perfect statistical correlation and a 0 meaning there is no correlation at all.

3.6.2 Root sean square error (RMSE)

Mean-squared error is the most commonly used measure of success of numeric prediction, and root mean-squared error is the square root of mean-squared-error, take to give it the same dimensions as the predicted values themselves. This method exaggerates the prediction error - the difference between prediction value and actual value of a test case. The root mean squared error (RMSE) is computed as:

$$RMSE = \sqrt{\frac{1}{n}(\sum_{i=1}^{n}(Xai - Xpi)^2)} \tag{10}$$

For a perfect fit, Xai = Xpi and RMSE = 0. So, the RMSE index ranges from 0 to infinity, with 0 corresponding to the ideal.

3.6.3 Standard error of estimates (SEE)

The standard error of estimate (SEE) is an estimate of the mean deviation of the regression from observed data. It is defined as (Allen, 1986):

$$SEE = \sqrt{\frac{\sum_{i=1}^{n}(Xai - Xpi)}{(n-2)}} \tag{11}$$

3.6.4 Model efficiency (Nash–Sutcliffe coefficient)

The model efficiency (MENash), an evaluation criterion proposed by Nash and Sutcliffe (1970), is employed to evaluate the performance of each of the developed model. It is defined as:

$$\mathrm{ME}_{\mathrm{Nash}} = 1.0 - \frac{\sum_{i=1}^{n}(Xa_i - X_{pi})^2}{\sum_{i=1}^{n}(X_{ai} - \overline{X}ai)^2} \tag{12}$$

A value of 90% and above indicates very satisfactory performance, a value in the range of 80–90% indicates fairly good performance, and a value below 80% indicates an unsatisfactory fit.

4. Data synthesis and architecture identification of models

In this work, the diffuse pollution concentration in stream is considered due to herbicide atrazine application in corn fields of the watershed. Concentration measurements data were obtained from the National Water Quality Assessment (NAWQA) program of the U S Geological Survey (USGS) (http://water.usgs.gov/nawqa/naqamap.html) for the period 1992 to 2002. The stream considered is White River, and monitoring site for the atrazine concentration measurement, is Hazeltone (Crawford, C.G, 1995), the outlet site of the watershed of White River Basin in Indiana State. At Hazeltone site, Latitude is 38°29'23", and Longitude is 87°33'00" and Drainage area 11,305.00 square miles. The White River basin is a part of the Mississippi River system where the application of atrazine accounts for 24 percent of all agricultural herbicides. The major agricultural chemical characteristics, AC, which contribute to the atrazine concentration at the watershed outlet are identified as its application rate (lb/Acre) and application time. The major land use patterns, AL, is the extent of cropped area (percentage of cultivated area (Pareira, 1990; Crawfard, 2001; and Capel and Larson, 2001).

Time series of data (average monthly values) from 1992-2001 are utilized for model building and validation. The major agricultural chemical characteristics, AC, which contribute to the atrazine concentration at the watershed outlet are identified as its application rate (lb/acre) and application time. The major land use pattern, AL, is the extent of cropped area (percentage of cultivated area (Crawford, 2001, 1995).These data are utilized for identification of fuzzy and ANN based models architectures by applications of the methodologies discussed in previous sections. The performance evaluations criteria are utilized to judge the predictive capability of the best performing fuzzy and ANN models. The procedure of developing fuzzy logic rule based model is implemented using the data of atrazine application rate as first input, atrazine application season as second input, and the percentage area applied with atrazine as third input. The atrazine concentration measurement values observed at the monitoring site is the output for the fuzzy rule based model. The weighted average of herbicide application rates and percentage of area applied of the corn and soybean cropped area are given in Table 1. The seven years data (1992-1998) are utilized for training and the three years data (1999-2001) (Table 1) are utilized for testing models.

Year	Weighted Percentage Area	Application Rate (lb/Acre)
1992	79	1.35
1993	91	1.31
1994	87	1.35
1995	87	1.31
1996	91	1.31
1997	84	1.33
1998	89	1.36
1999	91	1.26
2000	80	1.41
2001	94	1.35

Table 1. Agricultural Herbicide Atrazine Application Rate and Percentage Area Applied for the Corn Crop.

4.1 Evaluation of fuzzy c-means centers

The FCM model represented by equation (5) is used to partition the input data into fuzzy partitions. The FCM algorithm is implemented using MATLAB version 6.5 for ε equal to 10^{-5} to obtain the pre-specified fuzzy centers. The 3, 4, and 5 fuzzy centers for the inputs application rate and weighted percentage area obtained using the FCM model is shown in Table 2. Instead of iterating for the optimal number of fuzzy centers, a prior knowledge about the fuzzy partitioning for the fuzzy rule based models were utilized in implementing fuzzy c-means algorithm.

Fuzzy Partition centers by FCM Model		
Fuzzy Partitions	Input Application Rate (lb/Acre)	Application Rate (lb/Acre)
3-Fuzzy Centers	1.26	80.38
	1.31	86.68
	1.37	90.75
4-Fuzzy Centers	1.26	79.50
	1.31	84.02
	1.33	87.21
	1.36	90.88
5-Fuzzy Centers	1.26	80.00
	1.31	86.67
	1.33	87.00
	1.35	89.17
	1.41	91.0

Table 2. Different Fuzzy Partition Centers Using FCM Model

4.2 Training and testing the fuzzy rule based model with FCM

The seven years data (1992-1998) are utilized for training and the three years data (1999-2001) are utilized for testing the fuzzy rule based model with FCM. The model is assumed to be performing satisfactory when model efficiency coefficient (MENash) as given by equation (12) is greater than 90 percent, and other performance indices are also improved. Although arbitrary, it may be used as stopping criteria to limit the processing of large number of rules with increase in linguistic fuzzy variables for the inputs.

Performance of fuzzification of inputs application rate and weighted percentage area were studied by assigning 3, 5, and 7 fuzzy variables without using FCM (Singh, 2008). Though performance of fuzzifiction with 7 variables worked better than fuzzification with 3 and 5 variables; fuzzification by 5 fuzzy variables are comparable to fuzzification with 7 variables as shown in Table 3. Fuzzy rule based models with 3, 5 and 7 fuzzy variables are represented by Fuzzy_3M, Fuzzy_5M, and Fuzzy_7M models respectively in the Table 3. As 3 partitions are not adequate, four fuzzy partitions were specified for the use of fuzzy rule based system with FCM model. The four centers as shown in Table 2, obtained using FCM are partitioned into four linguistic fuzzy variables as low, medium, high, and very high. A

sample schematic representation of membership function is shown for the input atrazine application rate in Figure 1.

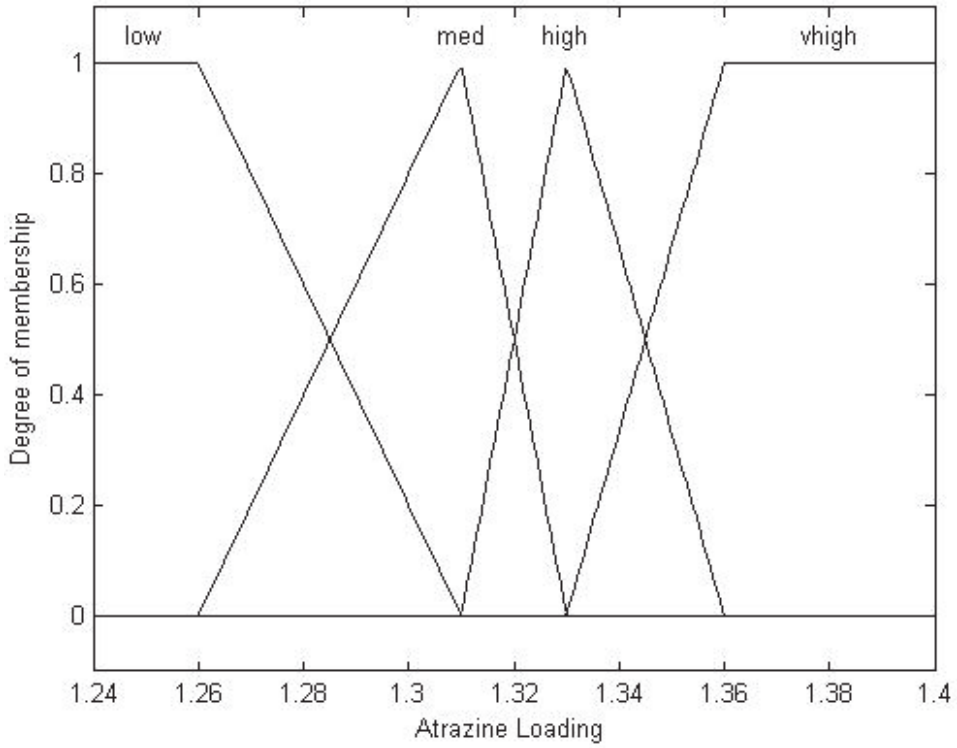

Fig. 1. A sample representation of linguistic variables membership function for first input.

The input application season is assigned 12 fuzzy variables, S1-S12 corresponding to each month of a year. The output concentration measurement values of atrazine is represented by 25 fuzzy centers by FCM model and represented by fuzzy variables, C1-C25, so that all the ranges of atrazine concentration measurement values in the data set for the period 1992-2001, is adequately represented. All the fuzzy variables in inputs and outputs are represented by triangular shape, except at the domain edges, where they are semi trapezoidal. This representation has been selected based on literature due to their computational efficiency (Khrisnapuram R 1998; Guillaume and Charnomordic, 2004). A sample representation of the membership functions is shown in Figure 1 for the first input. Of course, other divisions of the inputs and output domain regions and other shapes of membership functions are possible. The total number of rules in case of 4 linguistic variables for inputs application rate and weighted percentage area, and 12 fuzzy variables for seasons are 192. The total number of rules was much high i.e. 588 when 7 fuzzy variables were used for inputs application rate and weighted percentage area. The model building process is completed by creating combined fuzzy rule base using inputs-output pair values of training set data. Finally, the defuzzification converts fuzzy output produced by the fuzzy rule base model as crisp output corresponding to any new inputs.

5. Concentration measurement estimation results

The performance of the FCM based fuzzy rule based model is evaluated based on performance indices as described in performance evaluation criteria. These include root mean square error (RMSE), correlation coefficient (R) between the actual and estimated monthly average concentration measurement values of atrazine herbicides, standard error of estimate (SEE) and MENash. The performance evaluation results of the fuzzy rule based model with four fuzzy variables obtained using FCM, represented as Fuzzy_4_FCM, is also compared with that of the fuzzy rule based models with 3, 5, 7 linguistic variables for both of the input 1 and input 3. The performance of the Fuzzy_4_FCM model is also compared with solution results of an artificial neural network (ANN) based model using back propagation algorithm (Rumelhart et al. 1986) as represented by ANN_M in Table 3.

Models	Training Error (1992-198)				Testing Error (1999-2001)			
	RMSE	**R**	**SSE**	**ME_{Nash}**	**RMSE**	**R**	**SSE**	**ME_{Nash}**
Fuzzy_3M	1.318	0.891	1.377	0.550	0.703	0.886	0.771	0.623
Fuzzy_5M	0.836	0.969	0.837	0.894	0.455	0.952	0.498	0.855
Fuzzy_7M	0.706	0.970	0.775	0.915	0.342	0.975	0.375	0.914
ANN_M	1.153	0.918	1.264	0.752	0.906	0.759	0.993	0.446
Fuzzy_4M_FCM	0.492	0.998	0.539	0.967	0.725	0.968	0.416	0.901

Table 3. Comparison of training and testing errors for different models.

It can be noted from the Table 3 that the error statistics are better for Fuzzy_4M_FCM model than those of Fuzzy_3M, Fuzzy_5M and ANN_M model in both the training and testing in prediction in atrazine concentration measurement values. Its performance is even better than Fuzzy_7M model in training. Model efficiency (MENash) in training is 94.3 percent whereas it is 91.5 percent for Fuzzy_7M model. Similarly, RMSE, R, and SSE values are also comparable. In testing, results are also comparable though error statistics for Fuzzy_7M model is slightly better than Fuzzy_4_FCM. Thus, the FCM optimized fuzzy membership functions partitions in Fuzzy_4_FCM model are performing comparable to almost double the fuzzy partitions without FCM in Fuzzy_7M model. Figure 2 shows better RMSE value by Fuzzy_4_FCM model in comparison to other models.

It can also be noted from Table 3 that performances of fuzzy rule based model is better than those obtained using an ANN model with 2 inputs (atrazine application rate and weighted percentage area), 12 outputs (average monthly concentration measurements), and 11 hidden nodes (selected on the basis of experimentation) represented by ANN_M model. The poor performance by ANN_M model may be due to inadequate training patterns for experimentation, as the total number of free parameters become more than the number of training patterns even for 1 hidden node in hidden layer.

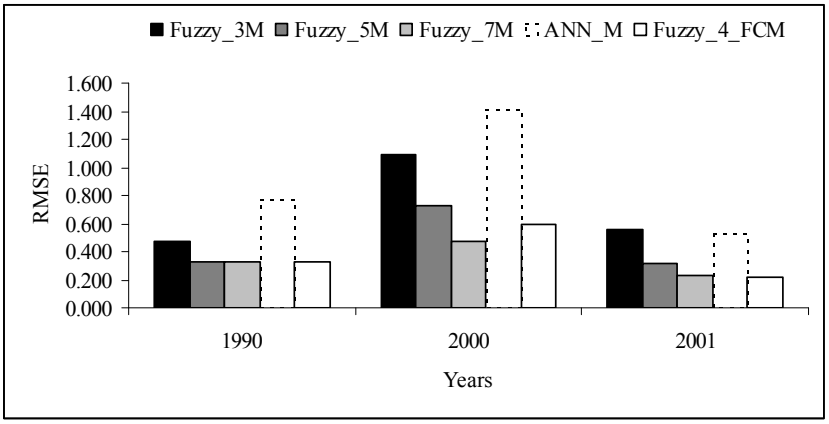

Fig. 2. Performance comparison of models.

Scatter plots of average monthly observed and predicted atrazine concentration measurement in the stream for model Fuzzy_4_FCM are plotted for the testing period 1999, 2000, and 2001. Comparison of actual and model estimated values are also presented for average monthly variations of atrazine concentration in the stream during the testing period, 1999-2001. Figure 3 represents scatter plot, and Figure 4 represents comparison of actual and Fuzzy_4_Model estimated values for the period 1999. Scatter plots between the observed and Fuzzy_4_FCM predicted average atrazine concentration measurement values in stream followed a 1:1 line except for a few cases of high magnitudes. The high values of coefficient of determination, R^2 (0.933), indicate that there is a good match between the observed and model predicted atrazine concentration. Figure 4 shows a comparison of observed and, Fuzzy_4_FCM model predicted average monthly atrazine concentration measurement values in the stream. The observed and Fuzzy_4_FCM predicted values match well except for the occurrence of peak value.

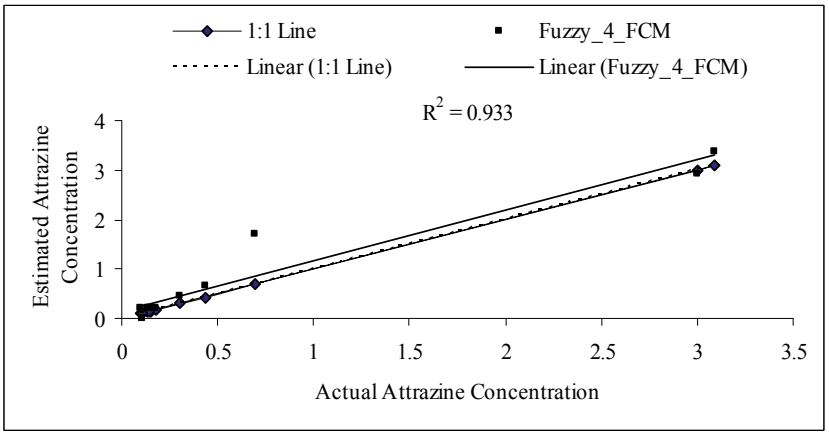

Fig. 3. Scatter plot of observed and Fuzzy_4_FCM Model predicted average monthly atrazine concentration for the testing period year 1999.

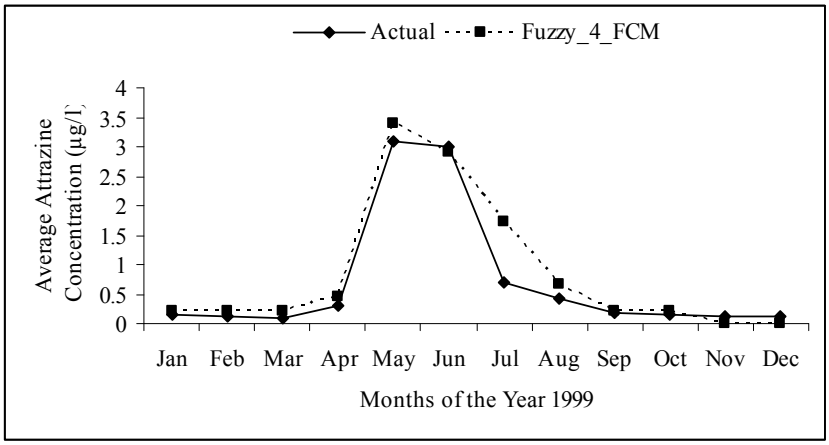

Fig. 4. Comparison of observed and Fuzzy_4_FCM predicted average monthly atrazine concentration for the testing period year 1999.

Figure 5 represents scatter plot of observed and Fuzzy_4_FCM predicted values, and Figure 6 represents comparison of observed and Fuzzy_4_FCM predicted atrazine concentration values for the period 2000. Scatter plots between the observed and Fuzzy_4_FCM predicted average atrazine concentration measurement values in stream followed a 1:1 line with R^2 value of 0.95. In this case though initial and final months values matches well, intermediate months values including peak value does not mach well as shown in Figure 6.

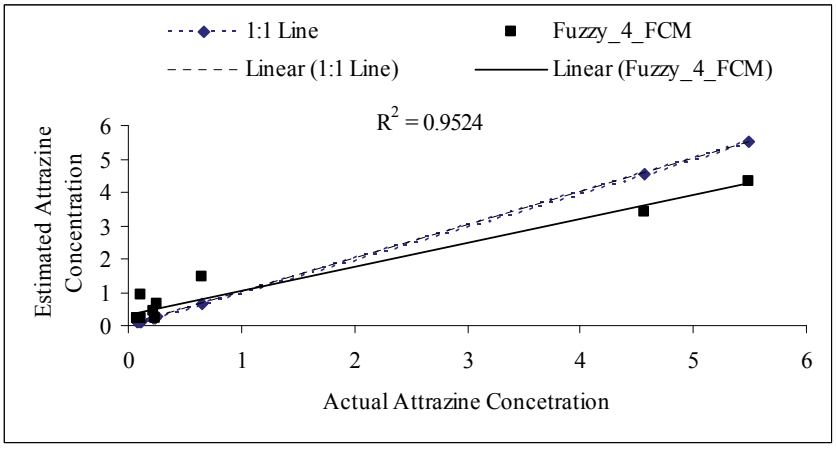

Fig. 5. Scatter plot of observed and Fuzzy_4_FCM Model predicted average monthly atrazine concentration for the testing period year 2000.

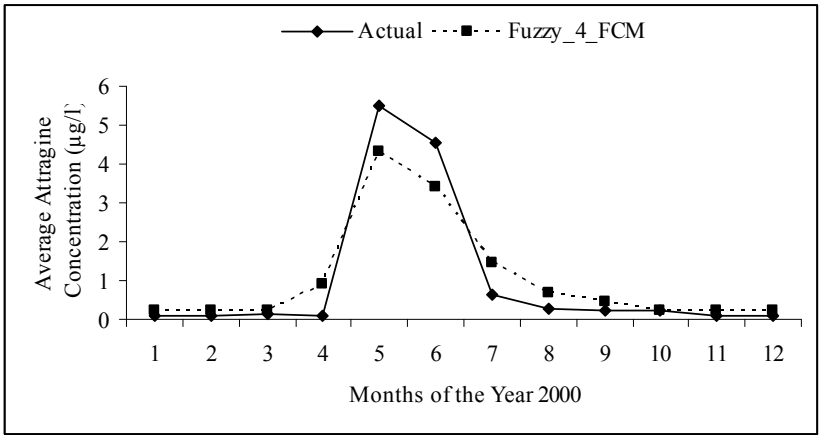

Fig. 6. Comparison of observed and Fuzzy_4_FCM predicted average monthly atrazine concentration for the testing period year 2000.

Figure 7 represents scatter plot of observed and Fuzzy_4_FCM predicted values, and Figure 8 represents comparison of observed and Fuzzy_4_FCM model predicted atrazine concentration values for the period 2001. Scatter plots between the observed and Fuzzy_4_FCM predicted average atrazine concentration measurement values in stream followed a 1:1 line with high value R^2 (0.93).

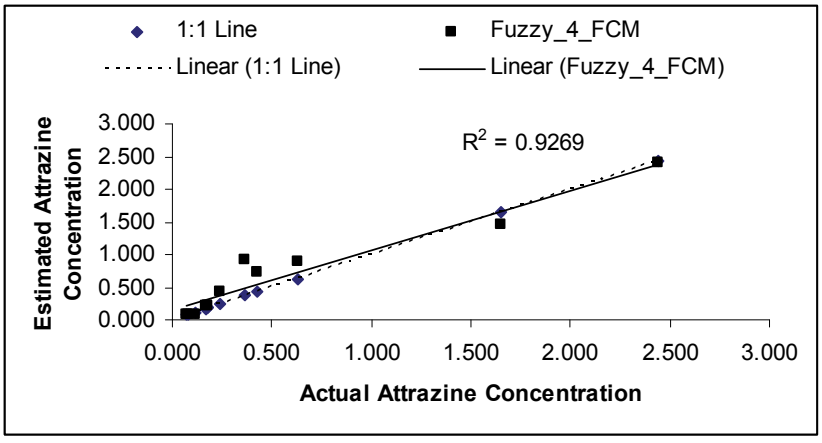

Fig. 7. Scatter plot of observed and Fuzzy_4_FCM Model predicted average monthly atrazine concentration for the testing period year 2001.

6. Discussion of results

The performance evaluation results presented in this study establish the potential applicability of the developed methodology in estimation of monthly atrazine concentration measurement values using fuzzy rule based models with FCM. However, the comparative

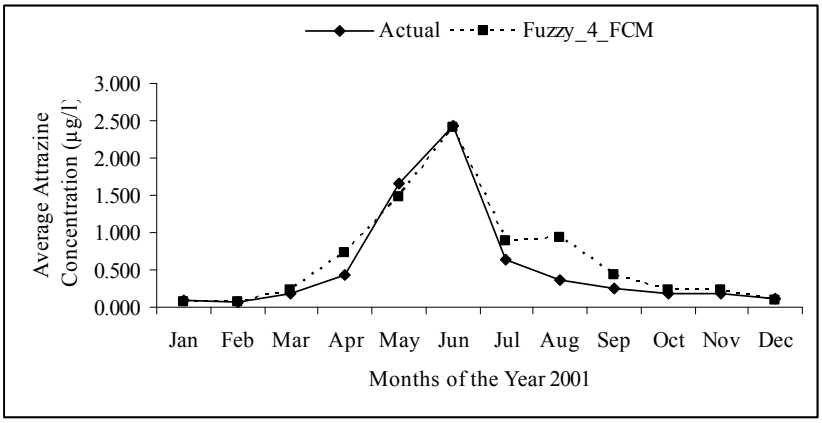

Fig. 8. Comparison of observed and Fuzzy_4_FCM model predicted average monthly atrazine concentration for the testing period year 2001.

performance of the methodology in different evaluation periods, under or over prediction of peak values, fuzzy rule based model control parameters (shape, total number of fuzzy centers, overlaps etc. of membership functions; fuzzy set operations i.e, defuzzification methods etc.) needs to be investigated further.

The performance of fuzzy rule based model with FCM is better than those without FCM model with even more number of fuzzy partitions. This is inferred by comparison of performances of Fuzzy_4_FCM model with Fuzzy_3M, Fuzzy_5M, and Fuzzy_7M models. In all the evaluation results obtained by Fuzzy_4_FCM model for the period 1999-2001, the R^2 values from scatter plots, and MENash values obtained from observed and model predicted values are high (around 0.9). This implies good match between the observed and model predicted values. The fuzzy rule with FCM model also performed better than the ANN based model. It establishes that the developed fuzzy rule based model with FCM is potentially suitable for estimation of concentration measurement values with limited data availability. The performances of the developed models are better in comparison to performance of regression models developed for the Mississippi River Systems (Battaglin and Goolsby, 1997). Their study show that multiple linear regression models estimate the concentration of selected agricultural chemicals with maximum R-squared value is 0.514, and in the case of atrazine, R-squared value is 0.312. In this study, almost all the developed models have R-squared value greater than 0.55. However, this comparison is limited as the White River basin considered in this study is only a part of (one of 10 basins) of Mississippi River Systems considered by them (Battaglin and Goolsby, 1997).

The estimation results obtained using fuzzy rule based models are encouraging but not conclusive. In almost all the evaluations, though initial months and final months concentration measurement values matches well, the intermediate values including the peak values are either over predicted or under predicted except for the year 2001 where peak predicted value matched well with the observed value. As the intermediate months, from April to July observes most of the changes in atrazine observed concentration measurement values, the same dynamics are exactly not reflected in model predictions. Thus, though the FCM model works better than ANN model in case of limited data availability, its

performance is also affected due to limited data sets. In the present study, the inputs were assigned with triangular shape. Further improvement in the performance of the methodology may be possible with more extensive evaluations of membership functions shape, number of data centers for membership functions for each variables, and overlap between two membership functions. Present methodology utilized centroid method for defuzzification. Performance of other defuzzification method also need to be investigated. The error in prediction of peak values shows the limitation of the methodology. However, these results show potential applicability of the proposed methodology. The main advantage of the developed methodology is incorporate some prior knowledge into the model frame work, and its ability to perform in case of limited availability of data than other methods such as ANN.

7. Conclusions

The present study describes the framework for evaluating average monthly concentration of agricultural non point source pollution due to herbicide atrazine in streams by fuzzy rule based model with FCM utilizing limited amount of data. The values of statistical performance evaluation criteria indicate the model is able to simulate the behaviour of diffuse pollution sources from agricultural fields like attrazin in streams. The fuzzy rule based model with FCM performs comparatively better than the fuzzy rule based model without FCM and even with more fuzzy partitions. The proposed methodology also performs better than the ANN model when applied to the same problem. However, the model predicts with lesser accuracy for the intermediate months concentration measurement values including peak values. An extensive evaluation of the effect of more number of FCM based fuzzy centers and shapes of membership functions may fully establish the applicability of the methodology.

However, the proposed fuzzy rule based approach with FCM uses least amount of information in terms of number of inputs required, incorporate prior knowledge about fuzzy partitions, and also uses linguistic variables which make it relatively easy to interpret the rules. Prior knowledge about the physical system in the form of rule base can also be directly incorporated in the suggested approach. This preliminary study shows that the developed fuzzy rule based approach with FCM is potential suited to estimation of diffuse pollution concentration like atrazine in streams.

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14

Forty Years with Glyphosate

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1. Introduction

If one were to pick the most notified pesticide of the turn of the millennium, the choice would most likely be glyphosate. Although DDT remains to be the all-time star in the Hall of Fame of pesticides, the second most admitted pesticide active ingredient must be the phosphonomethylglycine type compound of Monsanto Company, glyphosate.

Indeed, the two boasted pesticides show certain similarities in their history of discovery and fate. Both were synthesised first several decades prior to the discovery of their pesticide action. DDT and glyphosate were first described as chemical compounds 65 and 21 years before their discovery as pesticides, respectively. Both fulfilled extensive market need, therefore, both burst into mass application right after the discovery of their insecticide/herbicide activity. They both were, to some extent, connected to wars: a great part of the use of DDT was (and remains to be) hygienic, particularly after Word War II, but also the Vietnam War; while glyphosate plays an eminent role in the "drug war" (Plan Colombia) as a defoliant of marijuana fields in Mexico and South America. And last, not least, ecologically unfavourable characteristics of both was applauded as advantageous: the persistence of DDT had been seen initially as a benefit of long lasting activity, and the zwitterionic structure and consequent outstanding water solubility of glyphosate, unusual among pesticides, also used to be praised, before the environmental or ecotoxicological disadvantages of these characteristics were understood.

Yet there are marked differences as well between these two prominent pesticide active ingredients. Meanwhile the career of DDT lasted a little over three decades until becoming banned (mostly) worldwide, the history of glyphosate has gone beyond that by now, since the discovery of its herbicidal action (Baird et al., 1971). And while DDT is the only Nobel prize laureate pesticide, glyphosate was the "first billion dollar product" of the pesticide industry (Franz et al., 1997). Moreover, meanwhile the course of DDT was rather simple: rapid rise into mass utlilisation, discovery of environmental persistence, development of pest resistance, loss of efficacy, and subsequent ban; the history of glyphosate is far more diverse: its business success progressed uncumbered, receiving two major boosts. First, the patent protection of glyphosate preparations was renewed in the US in 1991 for another decade on the basis of application advantages due to formulation novelties, and second, its sales were further strengthened outside Europe with the spread of glyphosate-tolerant (GT) genetically modified (GM) crops. This market success has been limited significantly neither

by the recognition of the water-polluting feature of the parent compound, nor by the emerging weed resistance worldwide.

It is not a simple task to predict whether glyphosate continues to rise in the near future, or its application will be abating. To facilitate better assessment of these two possibilities, the present work attempts to provide a summary of the utility and the environmental health problems of glyphosate applications.

2. Glyphosate and its biochemistry

2.1 The discovery of glyphosate

The molecule *N*-(phosphonomethyl)glycine was first synthesised in 1950 by a researcher of the small Swiss pharmaceutical firm Cilag, Henri Martin (Franz et al. 1997). Yet, showing no pharmaceutical perspective, the compound has not been investigated any further. A decade later through the acquisition of the company, it was transferred to the distributor of laboratory research chemicals, Aldrich Chemical Co., along with research samples of Cilag. This is how it came to the attention of Monsanto Company (St. Louis, MO) in the course of its research to develop phosphonic acid type water-softening agents, through testing over 100 chemical substances related to aminomethylphosphonic acid (AMPA). Monsanto later extended the study of these compounds to herbicide activity testing, and observed their potential against perennial weeds (Dill et al., 2010). *N*-(phosphonomethyl)glycine (later termed glyphosate) was first re-synthesised and tested by Monsanto in 1970. Its herbicidal effect was described by Baird and co-workers in 1971, the subsequent patent (US 3799758), followed by numerous others, was claimed and obtained by Monsanto, and was introduced as a herbicide product Roundup® (formulation of the isopropylamine salt of glyphosate with a surfactant). Upon its introduction in the mid seventies, glyphosate jumped to a leading position on the pesticide market, became the most marketed herbicide active ingredient by the nineties, and more or less holds that position ever since. A great change came about, when the original patent protection expired in many parts of the world outside the United States in 1991. As a result, an almost immediate price decline occurred (by 30% in one year, 40% in two years and about 50% in two decades (Cox, 1998). Upon the expiration of the patent protection also in the United States in 2000, sales of generic preparations intensively expanded (main international producers include Dow, Syngenta, NuFarm, etc.), but the leading preparation producer remained Monsanto (Duke & Powles, 2008).

The current situation of the international active ingredient producers shows a rather different picture. Recently, Chinese chemical factories (e.g., Zhejiang Wynca Chemical Co., Zeijang Jinfanda Biochemical Co. and Hubei Xingta Chemical Group., Nantong Jiangshan Agrochemical and Chemical Co., Sichuan Fuhua Agricultural Investment Group, Jiangsu Yangnong Chemical Group, Jiangshu Good Harvest-Welen, etc.) gained leading parts of this business. At present, the global glyphosate production capacity is 1.1 million tonnes, while the global demand is only 0.5 million tonnes. The overall glyphosate production capacity of Chinese companies rose from 323,400 tonnes in 2007 to 835,900 tonnes in 2010, by a compounded annual growth rate of 37 percent (Yin, 2011). China has enough glyphosate capacity to satisfy the global demand even if all other glyphosate manufacturers cease production. The domestic demand of China is only 30-40 thousand tonnes, about 0.3 million tonnes of glyphosate is produced for export. Presently Chinese glyphosate production

facilities have been suspended being limited by the market demand. Extended use of GT plants in the Word would help on this problem, even if Europe is hesitant to allow commercial cultivation of this kind of GM plants. The overall situation has led to continously decreasing glyphosate prices on the Word market, and has significant effects on dispread of GT plants.

2.2 Mode of action

Glyphosate is a phosphonomethyl derivative of the amino acid glycine. It is an amphoteric chemical substance containing a basic secondary amino function in the middle of the molecule and monobasic (carboxylic) and dibasic (phosphonic) acidic sites at both ends (Fig. 1). Containing both hydrogen cation (H^+) donor (acidic) and acceptor (basic) functional groups, it can form cationic and anionic sites within the small molecule, the dissociation constants (pK_a) of these three functional groups are 10.9, 5.9 and 2.3, and therefore, similarly to amino acids, glyphosate can form a zwitterionic structure (Knuuttila & Knuuttila, 1979). This is reflected in excellent water solubility (11.6 g/l at 25 ^oC). Consequently, its lipophilicity is very low (logP < -3.2 at 20 ^oC, pH 2-5), and is insoluble in organic solvents e.g., ethanol, acetone or xylene (Tomlin, 2000). To further increase its already good water solubility it is often formulated in form of its ammonium, isopropylammonium, potassium, sodium or trimethylsulphonium (trimesium) salts. The order of water solubility is glyphosate << ammonium salt < sodium salt < potassium salt < isopropylammonium salt < trimesium salt, the solubility of the trimesium salt being two orders of magnitude higher than that of glyphosate.

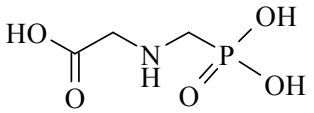

Fig. 1. The chemical structure of *N*-(phosphonomethyl)glycine, glyphosate, containing a basic function (amine) in the middle of the molecule and two acidic moieties (carboxylic and phosphonic acids) at both ends.

It has been known since the early seventies that glyphosate acts by inhibiting aromatic amino acid biosynthesis in plants (Jaworski, 1972; Amrhein et al., 1980), and elaborate research has revealed that the responsible mechanism is blocking a key step in the so-called shikimate pathway (Herman & Weaver, 1999), responsible for the synthesis of aromatic amino acids and critical plant metabolites. Glyphosate exerts this effect by inhibiting the activity of the enzyme 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) catalyzing the transformation of phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P) (Amrhein et al., 1980). This metabolic pathway exists in plants, fungi, and bacteria, but not in animals (Kishore & Shah 1988). Although higher order living organisms lack this metabolic route, therefore, are not expected to be directly affected by this herbicide, the environmental consequences of the widespread use of glyphosate have been reported (Cox, 2000; Santillo et al., 1989).

Being an amino acid (glycine) derivative itself, glyphosate inhibits the formation of the main intermediate, by binding as an analogue of the substrate PEP to its catalytic site on the enzyme. The inhibition of this catabolic pathway blocks the synthesis of triptophan, phenylalanine and tyrosine, and in consequence, the synthesis of proteins. The lack of the

synthesis of these essential amino acids and the proteins that contain them leads to rapid necrosis of the plant. Because this metabolic pathway is present in all higher order plants, and because the amino acid sequence of the active site of the EPSPS is a very conservative region in higher plants, the herbicidal effect is global among plant species.

Moreover, through its excellent solubility features glyphosate is a systemically active herbicide ingredient. As it is capable to be transported in the plant from the leaves towards the roots, it belongs to the relatively uncommon group of basipetally translocated herbicides (Ashton & Crafts, 1981). Its uptake and translocation is relatively rapid in diverse species (Sprankle et al., 1975).

2.3 Transition state analogue theory of enzyme inhibition

A unique feature of the mechanism of the inhibition of EPSPS by glyphosate is that glyphosate is reported to show close similarity in its structure to the tetrahedral phosphoenolpyruvoyl oxonium ion derivative of PEP, formed during its catalytic conversion to S3P, and the adduct formation with EPSPS has been verified by nuclear magnetic resonance spectroscopy (Christensen & Schaefer, 1993). Therefore, it has been proposed that glyphosate exerts its inhibitory activity as transition-state analogue (TSA) of the putative phosphoenolpyruvoyl oxonium ion derivative of PEP from plants (Anton et al., 1983; Steinrücken & Amrhein, 1984; Kishore & Shah, 1988) and bacteria (Du et al., 2000; Arcuri et al., 2004).

The so-called transition state theory has been advanced by Pauling (1948) to explain the mechanism of enzymatic reactions. Enyzmes are catalysts therefore they accelerate a reaction without influencing its equilibrium constant. One way to achieve that is to diminish the energy barrier of the reaction by lowering the energy of the transition state, transient, unstable intermediate of the reaction. This may be accomplished through stabilizing the transition state by binding to it as soon as it has occurred, and thus facilitating its formation. This results in the enzymatic effect that lowers the activation energy of the catalyzed reaction. Based on this idea, extremely potent inhibitors can be developed for a given enzymatic reaction if one can synthesize "transition state analogues" or "transition state mimics": stable chemical compounds resembling the transition state (Wolfenden, 1969). The TSA theory has therefore been successfully applied to the development of various biologically active substances, including insect control agents (Hammock et al., 1988), sulfonylurea microherbicides (Schloss & Aulabaugh, 1990) or compounds relatd to glyphosate (Marzabadi et al., 1992; Anderson et al., 1995).

The TSA hypothesis as it applies to the mechanism of the inhibition of EPSPS by glyphosate, became widely accepted as it has been evidenced in numerous studies that glyphosate forms a tight ternary complex with EPSPS (Herman & Weaver, 1999). It is easy to understand, however, that a classical TSA inhibitor would cause irreversible inhibition of the enzyme, competeable (although possibly with a low affinity) by the natural substrate of the enzyme. In later studies, it has been evidenced by biochemist researchers of Monsanto that glyphosate was an inhibitor of EPSPS uncompetitive with EPSP, and therefore, the TSA hypothesis has been reconsidered (Sammons et al., 1995; Schönbrunn et al., 2001; Alibhai & Stallings, 2001; Funke et al., 2006). The effects of glyphosate on aromatic amino acid synthesis in *Escherychia coli* have been attributed to chelation of Co^{2+} and Mg^{2+} (Roisch & Lingens, 1980), cofactors for enzymes in this pathway. Moreover, it is interesting, that glyphosate does not inhibit the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase

(Samland et al., 1999), structurally and mechanistically closely related EPSPS, and playing a key role in the biosynthesis of UDP-muramic acid.

2.4 Other biochemical effects of glyphosate

Various biochemical interactions of glyphosate, besides its identified mode of action, in plants and microorganisms were summarised by Hoagland and Duke (1982). The authors refer to numerous secondary or more complex indirect effects of glyphosate, and point out that a compound with such a powerful growth retardant effect or strong phytotoxicity will ultimately affect virtually all biochemical processes in the affected cells.

The effects of glyphosate in the plant possibly include influences on the regulation of hormonal processes. Methionine levels are greatly reduced by glyphosate (Duke et al., 1979), which suggests that this herbicide may alter ethylene biosynthesis. Results of Baur (1979) suggest that glyphosate may inhibit auxin transport by increasing ethylene biosynthesis. Glyphosate may also affect the biosynthesis of non-aromatic amino acids. Nilsson (1977) suggested that the build-up of glutamate and glutamine in glyphosate-treated tissue might be due to blocked transamination reactions.

It has been hypothesised that glyphosate lower phenylalanine and tyrosine pools not only by its primary mode of action, but possibly also by induction of phenylalanine ammonia-lyase (PAL) activity. Indeed, pronounced PAL activity has been detected in glyphosate-treated maize and soy (Duke et al., 1979; Cole et al., 1980), yet not by direct effect according to *in vitro* tests. Therefore, although glyphosate has been evidenced to cause profound effects on extractable PAL, substrate(s) and end products, increased PAL activity has been evaluated as a secondary effect (Hoagland & Duke, 1982).

Glyphosate did not appear to cause direct effects on photosynthesis, but its possible effect on chlorophyll biosynthesis has been considered, and its strong inhibitory effect on chlorophyll accumulation has been shown (Kitchen et al., 1981). Experimental result indicated that the effect of glyphosate on chlorophyll may be indirect through photobleaching and/or peroxidation of chlorophyll.

Glyphosate has been shown to significantly affect the membrane transport of cellular contents only at very high concentrations (Brecke & Duke, 1980; Fletcher et al., 1980). Phosphorous uptake was retarded (Brecke & Duke, 1980), but loss of membrane integrity, decrease in energy supply or external ion chelation were excluded as causes. Moreover, uptake of amino acids, nucleotides and glucose were also found to be retarded by glyphosate in isolated cells (Brecke & Duke, 1980). Other studies (Cole et al., 1980; Duke & Hoagland, 1981) found inhibition of amino acid uptake by glyphosate not severe. Glyphosate has been reported to uncouple oxidative phosphorylation in plant (Olorunsogo et al., 1979) and mammalian (Olorunsogo & Bababunmi, 1980) mitochondria, the latter is likely to be due to altered membrane transport processes, as glyphosate was found to enhance proton permeability of mitochondrial membranes in a concentration-dependent manner (Olorunsogo, 1990).

3. Pre-emergent application technology of glyphosate

Glyphosate, exerting global herbicidal action, has originally been intended to pre-emergent weed control treatments of field vegetation and weed control of orchards and ruderal areas.

Post-emergent applications are impossible solely with glyphosate-based herbicide formulations due to the phytotoxicity of the compound to the crop as well.

Common first visible phytotoxicity effects of glyphosate include rapid (within 2-10 days upon application) chlorosis, usually followed by necrosis (Suwannamek & Parker, 1975; Putnam, 1976; Campbell et al., 1976; Fernandez & Bayer, 1977; Marriage & Khan, 1978; Segura et al., 1978; Abu-Irmaileh & Jordan, 1978), possibly accompanied with morphological leaf deformities (Marriage & Khan, 1978), root and rhizome damage (Suwannamek & Parker, 1975; Fernandez & Bayer, 1977). Glyphosate accumulation has been reported in the meristems (Haderlie et al., 1978). It is rather surprising that although glyphosate inhibits seedling growth as well, it did not exert significant effect on the germination of various species (Haderlie et al., 1978; Egley & Williams, 1978).

3.1 Formulated glyphosate-based herbicides

Glyphosate-based formulations such as Roundup®, Accord® and Touchdown® represent the most common types used for agricultural purposes (Franz et al., 1997). These formulated herbicides can be used for weed control in agricultural practice, including in no-till agriculture to prepare fields before planting, during crop development and after crop harvest; as well as in silvicultural, urban and, lately, aquatic environments. The main herbicide products currently distributed are listed in Table 1. These preparations contain glyphosate as formulated in form of its ammonium (AMM), dimethylammonium (DMA), isopropylammonium (IPA), potassium (K) or trimesium (TRI) salts. The very first formulations containing IPA, sodium and ammonium salts were patented by Monsanto in 1974. A unique form is the trimesium salt of outstanding water solubility, patented by ICI Agrochemicals (later Zeneca Agricultural Products Inc, then Novartis CP, and after 2000 Syngenta) in 1989 (Tomlin, 2000).

As the actual active ingredients of the formulations are salts, differing from each other in the cation(s) and consequently the molecular mass of the salts, active ingredient concentrations are specified as glyphosate equivalent, in other term acid equivalent (a.i.) referring to the free acid form of glyphosate. This provides instant comparability among various formulations. Moreover, the use of a.i. units is common practice in residue analysis of glyphosate as well.

3.2 Formulating agents

Formulated glyphosate-based herbicides contain various non-ionic surfactants to facilitate their uptake by the plants (Riechers et al., 1995). These components, as all other pesticide additives and diluents, are assumed to be inert, which as it turns out, is not the case for several such ingredients. The most common surfactant applied in combination with glyphosate is polyethyloxylated tallowamine (POEA), which itself has been found to exert ecotoxicity, also in synergy with glyphosate, causing the formulated herbicide (e.g., Roundup) more toxic than its technical grade active ingredient (Folmar et al., 1979; Atkinson, 1985; Wan et al., 1989; Powell et al., 1991; Giesy et al., 2000; Tsui & Chu, 2003; Marc et al., 2005; Benachour et al., 2007; Benachour & Séralini, 2009).

The apparent synergistic toxic effects of the assumedly inert ingredients with glyphosate triggered a legal case between Monsanto and the New York Attorney General's Office in

Manufacturer	a.i. salt [a]	Product [b]
AAKO B.V.	IPA	Akosate
Agriliance LLC	IPA	Cornerstone
Agro-Chemie Ltd.	IPA	Fozát
Albaugh Inc./Agri Star	IPA	Aqua Star, Gly Star Original
Astrachem Ltd.	IPA	Tiller
Barclay Chem. Mfg. Ltd.	IPA	Gallup
Calliope S.A.	IPA	Kapazin
Chemical Products Technologies LLC	IPA	ClearOut; ClearOut Plus
Cheminova	IPA	Glyfos; Glyfos X-tra
Control Solutions Inc.	IPA	Spitfire
Crystal Chem. Inter-America	IPA	Glifonox
Dow AgroSciences	IPA	Dominator; Durango; Glyphomax; Glyphomax Plus; GlyPro; Panzer; Ripper; Rodeo; Vantage
	DMA	Durango DMA; Duramax
Drexel Chem. Co.	IPA	Imitator
	K	DupliKator
FarmerSaver.com LLC	IPA	Glyphosate 4
Griffin LLC	IPA	Glyphosate Original
Growmark Inc.	IPA	FS Glyxphosate Plus
Helena Chemical Co.	IPA	Rattler
	IPA + AMM	Showdown
Helm Agro US Inc.	IPA	Glyphosate 41%; Helosate Plus
Loveland Products Inc.	IPA	Mad Dog; Mirage
Makhteshim-Agan	IPA	Eraser, Gladiator; Glyphogan; Hardflex; Herbolex; Taifun
Micro Flo	IPA	Gly-Flo
Monsanto Co.	IPA	Accord; Aquamaster; Azural; Clinic; Glialka; Honcho; Ranger Pro, Roundup Bioforce / Classic / Original / UltraMAX
	K	Roundup Forte / Mega / PowerMAX / WeatherMAX; VisionMAX
Nufarm	IPA	Amega; Credit; Credit Extra
	IPA + MA	Credit Duo
Oxon Italia S.p.A.	AMM	Buggy
Pinus TKI d.d.	IPA	Boom Efekt
Sinon Corporation	IPA	Glyfozat; Total
Syngenta AG	AMM	Medallon Premium
	DMA	Touchdown IQ
	K	Refuge; Touchdown HiTech / Total; Traxion
	TRI	Coloso; Ouragan
Tenkoz Inc.	IPA	Buccaneer
UAP	IPA	Makaze
Universal Crop Protection Alliance LLC	IPA	Gly-4
Winfield Solutions LLC	IPA	Cornerstone

[a] AMM = ammonium; DMA = dimethylamine; IPA = isopropylamine; K = potassium; TRI = trimesium
[b] Formulations containing only glyphosate salts as active ingredient are listed, herbicide combinations are not included

Table 1. Formulated herbicide preparations containing glyphosate as active ingredient.

1996 (Attorney General of the State of New York, 1996). The toxicological basis of the legal claim was that Monsanto inaccurately implied toxicity data of the active ingredient glyphosate on the formulated product Roundup. As a result of the lawsuit, Monsanto was fined, and agreed to drop description of being "environmentally friendly" and "biodegradable" from the advertisements of the herbicide.

Concerns about application safety, triggered by the above studies and findings on teratogenic effects (see 6.3 Teratogenic activity of glyphosate), have brought re-registration of glyphosate and its formulated products in focus in the European Union, as part of the regular pesticide revision process due to take place in 2012. Nonetheless, the EU Commission dismissed these findings, based on a rebuttal by the EU "rapporteur" member state for glyphosate, Germany, provided by the German Federal Office for Consumer Protection and Food Safety (BVL), and postponed the review of glyphosate and 38 other pesticides until 2015 (European Commission, 2010). To protest against such delay in re-evaluation of these 39 pesticides, the Pesticides Action Network Europe and Greenpeace brought a lawsuit against the EU Commission, and the dismissal of the reported teratogenicity data from the official current evaluation has been judged by several researchers as irresponsible act (Antoniou et al., 2011).

4. Post-emergent application technology of glyphosate

A group so far of the highest financial importance within GM crops has been modified to be tolerant to this active ingredient, outstandingly broadening its application possibilities.

4.1 Glyphosate-tolerant crops

Upon pre-emergent applications of the global herbicide glyphosate, the majority of the weeds decays, perishes, and does not get consumed by wild animals. This situation has been changed tremendously by the appearance of GT crops, leading to increasing environmental herbicide loads due to approved post-emergent treatments (2-3 applications in total). Of these crops, the varieties of Monsanto became most publicised, under the trade mark Roundup Ready® (RR), indicating that these plants can be treated with the herbicide preparation of Monsanto, Roundup® containing glyphosate as active ingredient even, after the emergence of the crop seedlings. Similar varieties by Bayer CropScience, Pioneer Hi-Bred and Syngenta AG are termed Gly-Tol™, Optimum® GAT® and Agrisure® GT, respectively. Two strategies have been followed by plant gene technology in the development of GT varieties: either the genes (*cp4 epsps*, *mepsps*, *2mepsps*) of mutant forms of the target enzyme less sensitive to glyphosate or genes (*gat*, *gox*) of enzymes metabolizing glyphosate have been transferred into the GM plant varieties (Table 2). The genetically created tolerance to glyphosate does not alter the mode of action of the compound: the molecular mechanism of glyphosate tolerance has been elucidated (Funke et al., 2006), and the sole mechanism of inhibition remains blocking of the shikimate pathway when applied at very high doses on GT soybean and canola (Nandula et al., 2007).

The first GT crop was RR soybean by Monsanto in 1996, followed by GT cotton, GT maize, GT canola, GT alfalfa and GT sugarbeet (Dill et al., 2008). GT crops allow a new form of technology, post-emergent application of glyphosate. The utilizability of post-emergent applications was systematically tested in 2002 and 2003 in field experiments in the United States (Parker et al., 2005). The extensive study involving GT maize and GT soybean sites at

Variety owner	Crop	Genetical event	Transgene introduced [a]
Bayer CropSience (part of Sanofi-Aventis)	Cotton	*GHB614*	*2mepsps*
Monsanto Co.	Cotton	*MON 1445*	*cp4 epsps*, *nptII*, *aad*
	Cotton	*MON 88913*	*cp4 epsps*
	Maize	*MON 88017*	*cp4 epsps*, *cry3Bb1*
	Maize	*NK603*	*cp4 epsps*
	Rape	*GT 73*	*cp4 epsps*, *gox*
	Soybean	*MON40-3-2*	*cp4 epsps*
	Soybean	*MON 87705*	*cp4 epsps*, *FAD2-1A*, *FATB1-A*
	Soybean	*MON 89788*	*cp4 epsps*
	Sugar-beet [b]	*A5-15*	*cp4 epsps*, *nptII*,
	Sugar-beet [c]	*H7-1*	*cp4 epsps*
Pioneer Hi-Bred (part of DuPont)	Maize	*DP-98140*	*Gat4601*, *als*
	Soy	*DP-356043*	*gat4601*
Syngenta	Maize	*GA21*	*mepsps*

[a] *aad* – gene of *Escherchia coli* origin, encoding resistance against aminoglycoside antibotics (streptomycin and spectinomycin); *als* – gene (*zm-hra*) of maize origin, enhancing tolerance of ALS inhibiting herbicides (e.g., chlorimuron and thifensulfuron); *cry3Bb1* – gene of *Bacillus thuringiensis* origin, encoding Cry3 toxin; *FAD2-1A* – gene of soy origin, encoding fatty acid desaturease enzyme, silencing of which enhances the proportion of monounsaturated fatty acids; *FATB1-A* – gene of soy origin, encoding medium-chain fatty acid thioesterase, silencing of which reduces the proportion of saturated fatty acids; *cp4 epsps* – *epsps* gene of *Agrobacterium* sp.; *mepsps* – *epsps* gene of maize origin; *2mepsps* – double mutated *epsps* gene of Mexican black, sweet maize origin; *gat4601* – gene of *Bacillus lichiformis* origin, encoding glyphosate acetyltransferase enzyme; *gox* – gene of *Ochrobactrum anthropi* origin, encoding glyphosate oxidase enzyme; *nptII* – gene of *Escherichia coli* K12 origin, encoding neomycin phosphostransferase, causing neomycin and kanamycin resistance.

[b] together with Danisco Seeds and DLF Trifolium as variety owners

[c] together with KWS Saat Ag. as variety owners

Table 2. Glyphosate tolerant crop variety groups under registration process in the European Union.

seven locations, as well as regular or directed post-emergent applications of 10 formulated glyphosate preparations (ClearOut 41 Plus™, Gly Star™, Glyfos®, Glyfos® X-tra, Glyphomax™, Roundup Original™, Roundup UltraMAX®, Roundup WeatherMAX™, Touchdown® and Touchdown Total™) containing isopropylamine or potassium salts of glyphosate found no herbicide efficacy or produce quality differences, no phytotoxicity to maize and medium phytotoxicity to cotton at high doses in some instances, and therefore proposed post-emergent glyphosate applications. As a result, the use of glyphosate has expanded almost 20-fold by 2007 in the United States (Pérez et al., 2011).

Another impact of GT crops on agricultural practices is the spread of no-till agriculture. As the crop tolerates the active ingredient, intensive herbicide treatments are possible to be carried out, instead of former tillage practices, to eradicate vegetation in the field. This has greatly increased herbicide use and consequent chemical pressure on the environment. No-till practice is particularly common in GT crop cultivating areas in South America, including Brazil, Argentina, Paraguay and Uruguay (Altieri & Pengue, 2006).

An interesting detail is that in parallel to industrial development of GT crops, illegal genetic modification projects are also being carried out to achieve "crops" that are resistant to

glyphosate e.g, a new marijuana (*Canabis* sp.) hybrid that can be cultivated all year and cannot be controled with herbicides (Anonymous, 2006). The GT marijuana hybrid, first appeared in Mexico in 2004, allows 8-9-times higher yields than "conventional" varieties, and became the plant of choice for drug traffickers in Michoacan.

4.2 The effect of glyphosate-tolerant crops on glyphosate residues

As a result of the combined effect of the expiration of the patent protection of glyphosate (in 2000 in the United States) and the spread of cultivation of GT GM crops (since 1996 in the United States), the use of glyphosate products is again increasing (Woodburn, 2000). Besides GT GM crops, energy crop cultivation is also an and emerging source of glyphosate contamination (Love et al., 2011). Moreover, due to the modified metabolic pool in the GT GM crops, residues of the systemic glyphosate active ingredient are expected to occur in the surviving plants. In case of EPSP-mutant (RR and Agrisure GT) varieties, the residue composition is expected to be similar to those seen at regular glyphosate applications, while in the case of the boosted glyphosate metabolizing (regardless whether *epsps* or *gox* transgene based) varieties, increased amounts of *N*-acetylglyphosate (NAG) (Optimum GAT variety) or aminomethylphosphonic acid (AMPA) (RR and Agrisure GT varieties) are expected in the plants. In turn, residue patterns not yet seen in food and feed are to be expected. Summarizing the results of their studies in Argentina between 1997 and 1999, Arregui and co-workers (2004) reported glyphosate residue levels after 2-3 glyphosate applications as high as 0.3-5.2 mg glyphosate/kg and 0.3-5.7 mg AMPA/kg in the leaves and stem of RR soy during harvest, and 0.1-1.8 mg glyphosate/kg and 0.4-0.9 mg AMPA/kg in the produce. In turn, glyphosate occurred as surface water, soil and sediment contaminant in a GM soybean cultivating area in Argentina (Peruzzo et al., 2008).

5. The environmental fate of glyphosate

5.1 Residue analysis of glyphosate

Present analytical methods developed for the detection of glyphosate are mostly based on separation by liquid chromatography (LC), as previous methods utilizing gas chromatography (GC) have become of much lesser importance than they used to be (Stalikas & Konidari, 2001). The main obstacle in the GC detection of glyphosate and its main metabolite AMPA is the polaric and zwitterionic structure of these compounds, which required laborious sample preparation steps prior to instrumental analysis. The earliest method accredited for authoritative analytical determination of glyphosate (US FDA, 1977) employed aqueous extraction, anion and cation exchange purification, *N*-acetylation derivatisation with trifluoroacetic acid and trifluoroacetic anhydride, and subsequent methylation of both the carboxylic acid and phosphonic acid moieties on the parent compound, followed by GC analysis with phosphorous-specific flame ionisation detection. Recoveries above 70% were achieved by the method in plant samples, the limit of detection (LOD) was 0.05 mg/kg. The basis of the protocol was the GC-MS derivatisation method developed by Monsanto (Rueppel et al., 1976). A later method by Alferness and Iwata (1994) also employs aqueous extraction, followed by washing with dichloromethane/chloroform, purification on cation exchange column, derivatisation to trifluoroacetate and heptafluorobutyl ester, followed by GC analysis with mass spectrometry (MS) detection, and a similar methods have also been developed (Tsunoda, 1993; Natangelo et al., 1993;

Royer et al., 2000; Hudzin et al., 2002). Validated LC methods also resulting in similar analytical parameters (Cowell et al., 1986; Winfield et al., 1990; DFG, 1992) utilise washing with chloroform and hydrochloric acid, purification on ion exchange column, and upon neutralisation and derivatisation with o-phthalic aldehyde and mercaptoethanol, determination by high performance liquid chromatography (HPLC) with fluorescence detection. Yet the LOD of the official method (Method 547) established by the U.S. Environmental Protection Agency is as high as 6 μg/l in reagent water and 9 μg/l in surface water (Winfield et al., 1990). Ninhydrin or 9-methlyfluorenyl chloroformiate have also been applied as derivatising agents (Wigfield & Lanquette, 1991; Sancho et al., 1996; Nedelkoska & Low, 2004, Peruzzo et al., 2008). More recent LC procedures with somewhat simplified sample preparation steps offer rapid and more economic analytical methods than GC procedures always requiring complex, often several step derivatisation. As a result, GC methods remain being used solely due to their analytical parameters, including sensitivity. Nonetheless, LODs of LC and ion chromatographic methods were achieved to be lowered (Mallat & Barceló, 1998; Vreeken, 1998; Bauer et al., 1999; Grey et al., 2001; Patsias et al., 2001; Lee et al., 2002a; Nedelkoska & Low, 2004; Ibáñez et al., 2006; Laitinen et al., 2006; Hanke et al., 2008; Popp et al., 2008) to meet the strictening maximal residue levels (MRLs) in environmental and health regulations. The most recent LC-MS methods using electrospray ionisation (Granby et al., 2003; Martins-Júnior et al., 2011) easily meet the MRL by the EU for given pesticide residues in drinking water, 0.1 μg/l, but the instrumentation demand of these methods is substantial.

Among novel innovative analytical methods for the detection of glyphosate, mostly capillary electrophoresis (CE) and immunoanalytical methods are to be mentioned. Initial drawbacks of the CE methods included relatively high LOD and the need for derivatisation or external fluorescent labeling (Cikalo et al., 1996; You et al., 2003; Kodama et al., 2008), later solved by coupling CE with MS (Goodwin et al., 2003) and microextraction techniques (Hsu and Whang 2009; See et al., 2010). Among various immunoanalytical techniques, enzyme-linked immunosorbent assays (ELISAs) gained the highest utility. While in the early nineties we considered yet that effective antibodies are not produced against glyphosate and similar zwitterioninc compounds due to their low immunogeneity (Hammock et al., 1990), difficulties in immunisation have been overcome within a decade, and sensitive ELISAs, also employing derivatisation, were developed (Clegg et al., 1999; Lee et al., 2002b; Rubio et al., 2003; Selvi et al., 2011), proven to be of great utility in environmental analytical studies for glyphosate (Mörtl et al., 2010; Kantiani et al., 2011). On the basis of the immunoassay principle, sensors using glyphosate-sensitive antibodies (González-Martínez et al., 2005) or molecularly imprinted polymers (MIPs) (Zhao et al., 2011) were also developed.

5.2 Glyphosate and its decomposition products

Decomposition of glyphosate takes place mostly by two processes: decarboxylation or dephosphorylation, and the corresponding intermediate metabolites are AMPA or glycine, respectively. The first pathway is catalyzed by oxidoreductases, the second by C–P lyases cleaving the carbon-phosphorous bond. Both pathways occur in environmental matrices (water, soil) and plants, but the main metabolite in all cases is AMPA (Fig. 2). The environmental fate, behaviour and analysis of both AMPA and glyphosate has received considerable attention (Stalikas & Konidari, 2001).

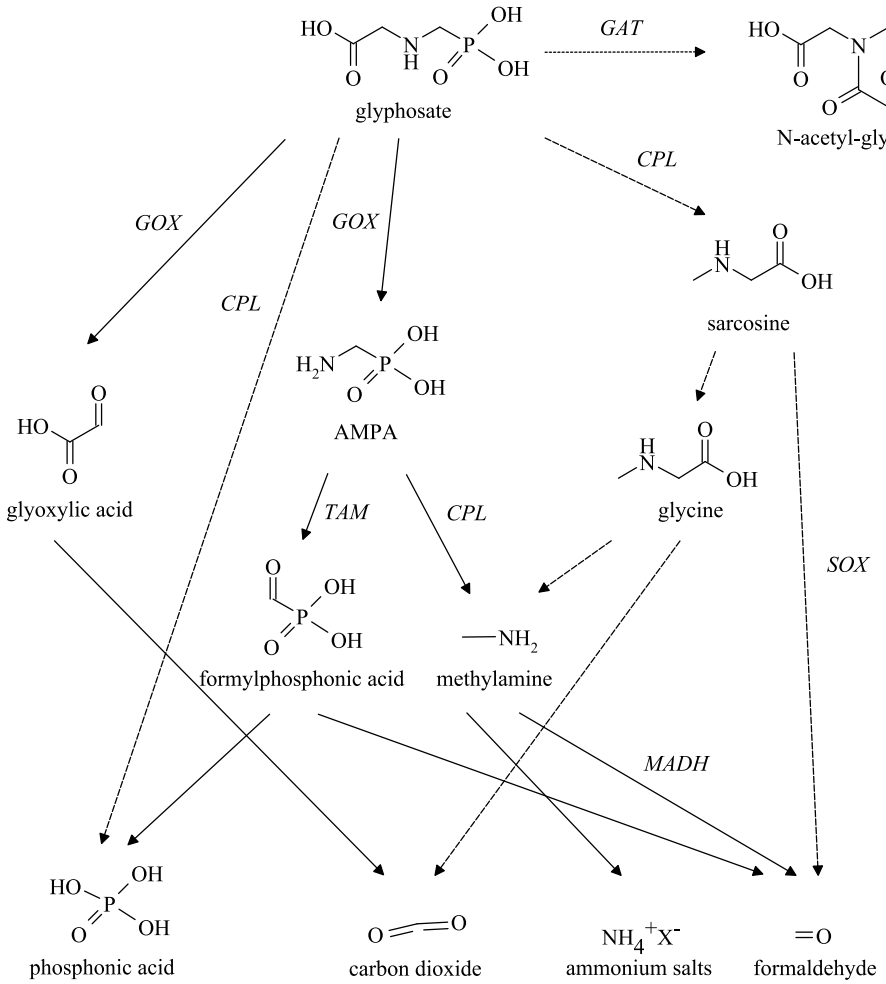

Fig. 2. Possible fate of glyphosate by various metabolizing pathways. Oxidative decomposition (*solid arrows*), non-hydrolytic decomposition (*dashed arrows*), inactivation in plants (*dotted arrow*). Processing enzymes (*Italics letters*) – GOX: glyphosate oxidoreductase, GAT: glyphosate *N*-acetyltransferase, CPL: C–P-liase, SOX: sarcosin oxidase, TAM: transaminase, MADH: methylamine dehydrogenase.

AMPA has been reported to be rapidly formed microbiologically, but not by chemical action, in water and in various loam soils (Drummer silty clay loam, Norfolk sandy loam, Ray silt loam, Lithonia sandy loam) (Rueppel et al., 1977; Aizawa, 1982; Mallat & Barceló, 1998), and was shown to be degraded subsequently completely to carbon dioxide (Sprankle et al., 1975, Rueppel et al., 1977; Moshier & Penner, 1978). Chemical processes of degradation are ineffective because of the presence of a highly stable carbon-phosphorus bond in the compound (Gimsing et al., 2004). Which pathway is predominant in the microbial degradation depends on bacterial species. The first (AMPA) pathway is

commonly seen in mixed soil bacterial cultures (Rueppel et al., 1977) and certain *Flavobacterium* sp. The glycine pathway is characteristic to certain *Pseudomonas* and *Arthrobacter* sp. strains (Jacob et al., 1988). AMPA is further metabolised, providing phosphorus for growth, although the amount eliminated is typically set by the phosphorus requirement of the bacterium in question. Sarcosine and glycine are other possible main degradation products in soils (Rueppel et al., 1977).

As for decomposition in water or soil, the stability of glyphosate depends of a number of parameters. It strongly interacts with soil components by forming tight complexes with numerous metal ions in solution and by being adsorbed on soil particles, including clay minerals. Adsorption is strongly influenced by cations associated with the soil (Carlisle & Trevors, 1988), and it is mainly the phosphonic acid moiety that participates in the process, therefore, phosphate competes with glyphosate in soil adsorption (Gimsing & dos Santos, 2005). As a result of its adsorption on clay particles and organic matter present in the soil, upon application glyphosate remains unchanged in the soil for varying lengths of time (Penaloza-Vazquez et al., 1995). Adsorption of chelating agents by surfaces has been shown to decrease biodegradability. It can be expected that phosphonates with their higher affinity to surfaces are much slower degraded in a heterogeneous compared to a homogeneous system, as seen for glyphosate (Zaranyika & Nyandoro, 1993).

Therefore, differences have been observed between half-lives (DT_{50}) of glyphosate determined in laboratory or field studies. Half-lives were found quite favourable in laboratory, 91 days in water and 47 days in soil. Nonetheless, half-life of the parent compound ranged between a few days to several months or even a year in field studies, depending on soil composition. A reason of such delayed decomposition is partly binding to the soil matrix, through which glyphosate absorbed on soil particles can form complexes with metal (Al, Fe, Mn, Zn) ions (Vereecken, 2005). By the increased solubility of its various alkali metal, ammonium or trimesium salts, the active ingredient can leach into deeper soil layers, in spite of its rapid decomposition and strong complex formation capability under certain conditions (Vereecken, 2005). Its primary metabolite AMPA is more mobile in soil than the parent compound (Duke & Powles, 2008).

Moreover, decomposition dynamics of glyphosate is greatly dependent on the microbial activity of soil, with mostly *Pseudomonas* species as most important microbial components (Borggaard & Gimsing, 2008). If microbial activity is elevated, glyphosate is degraded with reported laboratory and field half-life of < 25 days and 47 days, respectively (Ahrens, 1994). Moreover, glyphosate itself affects the survival of soil microorganisms (Carlisle & Trevors, 1988; Krzysko-Lupicka & Sudol, 2008). Studies of glyphosate degrading bacteria have involved selection for, and isolation of pure bacterial strains with enhanced or novel detoxification capabilities for potential uses in biotechnology industry and biodegradation of polluted soils and water. Microorganisms known for their ability to degrade glyphosate in soil and water include *Pseudomonas* sp. strain LBr (Jacob et al., 1988), *Pseudomonas fluorescens* (Zboinska et al., 1992), *Arthrobacter atrocyaneus* (Pipke et al., 1988) and *Flavobacterium* sp. (Balthazor & Hallas, 1986). Soil microbial activity, however, depends on a number of additional parameters, including soil temperature, abundance of air and water, and a number of not yet defined factors, creating rather variable conditions for the decomposition of glyphosate (Stenrød et al., 2005; 2006). Other studies have also shown that soil sorption and degradation of glyphosate exhibit great variation depending on soil composition and properties (de Jonge et al., 2001; Gimsing et al., 2004a, 2004b; Mamy et al.,

2005; Sørensen et al., 2006; Gimsing et al., 2007). Laitinen and co-workers (2006; 2008) reported that phosphorous content in the soil affects the environmental behaviour of glyphosate e.g., its absorbance on soil particles, and its occurrence in surface waters. Weaver and co-workers (2007) claim that its effects on soil microbial communities are short and transient, and that decomposition characteristics of glyphosate do not change significantly in lower soil layers in Mississippi with various tilling methods (Zablotowicz et al., 2009). Outstandingly different result were obtained in an environmental analytical study carried out in Finland, who detected 19% of the applied glyphosate undecomposed and 48% in form of AMPA 20 months after application in Northern European soils of low phosphorous content (Laitinen et al., 2009). This also sheds a light on the high reported glyphosate contamination levels in Scandinavian surface waters (Ludvigsen & Lode, 2001a; 2001b). The phosphorous content of the soil may also play a key role in the low decomposition rate seen through its effect on microbial communities, as soil phosphorous has been shown to be able to stimulate decomposition of glyphosate (Borggaard & Gimsing, 2008). An interesting interaction observed is that persistence of glyphosate significantly increased in soils treated with Cry toxins of *Bacillus thuringiensis* subsp. *kurstaki*, while a similar effect was not seen when soils were treated with purified Cry1Ac toxin (Accinelli et al., 2004; 2006). Therefore, it is worthwhile reconsidering the fate of glyphosate in soils, including sorption, degradation and leachability.

Due to its strong sorption and relatively fast degradation in soil, glyphosate has been claimed to cause very limited risk of leaching to groundwater (Giesy et al., 2000; Busse et al., 2001; Vereecken, 2005; Cox & Surgan, 2006). Yet, other investigations indicates possible leaching and toxicity problems with its use (Veiga et al., 2001, Strange-Hansen, 2004; Kjær, 2005; Landry et al., 2005; Relyea, 2005b; Torstensson et al., 2005; Siimes et al., 2006) and consequent effects on aquatic microbial communities (Pérez et al., 2007; Pesce et al., 2009; Vera et al., 2010; Villeneuve et al., 2011), except cyanobacteria (Powell et al., 1991). Just like soil bacteria, aqueous microorganisms e.g., microalgae may also utilise glyphosate as source of phosphorous (Wong, 2000). An interesting detail is that glyphosate may be formed during water treatment for purification from organic micropollutants. Glyphosate and AMPA were found to be formed during ozonisation of dilute aqueous solution of the complexing agent ethylenediaminetetra(methylenephosphonic acid) (Klinger et al., 1998; Nowack, 2003). The wide use, and hence ubiquity of glyphosate makes great demands on glyphosate safety, i.e. the absence of any harmful environmental effect except on target organisms (the undesirable weeds).

Glyphosate is very stable in higher plants (Putnam, 1976; Zandstra & Nishimoto, 1977; Chase & Appleby, 1979; Gothrup et al., 1976; Wyrill & Burnside, 1976). Through its metabolism, AMPA has been identified as the main metabolite in plants as well e.g., in montmorency cherry (*Prunus cerasus* L.) leaves, field bindweed (*Convolvulus arvensis* L.), henge bindweed (*Convolvulus sepium* L), Canada thistle (*Cirsium arvense* (L) Scop.), tall morning glory (*Ipomea purpurea* (L.) Roth.) and wild buckwheat (*Polygonum convolvulus* L.) (Sandberg et al., 1980; Aizawa, 1982; Aizawa, 1989).

Besides AMPA, its certain derivatives e.g., N-methyl-AMPA or N,N-dimethyl-AMPA have been also found as metabolites, mostly in plants (FAO/WHO, 2006). Decomposition in GT plants is even more complex, as some of these plants have been designed for enhanced degradation of glyphosate. In such plants, further AMPA derivatives e.g., N-acetyl-AMPA,

N-malonyl-AMPA, N-glyceryl-AMPA and various conjugates of AMPA have also been identified (FAO/WHO, 2006).

5.3 Environmental monitoring of glyphosate

Glyphosate shows unique characteristics in soil as compared to other pesticide active ingredients. With predominantly apolar groups pesticides typically bind to the organic matter in soil (Borggaard & Gimsing, 2008). In contrast, glyphosate is of amphoteric (zwitterionic) character, analytical determination of which is to date a great challenge to analytical chemists. As a result of the unusual chemical behaviour of the parent compound (*N*-phosphonomethylglycine) and its metabolite (AMPA), routine environmental analytical methods do not detect them with sufficient sensitivity. It is also due to the difficult analytical procedure that glyphosate is often not targeted or overlooked in environmental studies, or has been considered of neglectable level. Certain studies, however, report frequent occurrence. In the United States, surface water contamination has been reported due to run-off from agricultural areas (Edwards et al., 1980; Feng et al., 1990) or pesticide drift (Payne et al., 1990; Payne, 1992). Glyphosate has been listed among pesticides of potential concern in surface water contamination in the Mediterranean region of Europe in the mid' nineties (Barceló & Hennion, 1997), and glyphosate and AMPA were found as contaminants in two small tributaries of the river Ruhr in North-Rhine-Westphalia, Germany at up to 590 ng/l concentration (Skark et al., 1998). A monitoring study carried out in Norway found frequent occurrence of glyphosate and its metabolite AMPA in surface water samples. In 54% of the 540 surface water samples collected between 1995 and 1999 glyphosate or AMPA was detected. The maximal concentration was 0.93 μg/l (average 0.13 μg/l) for glyphosate, and 0.2 μg/l (average 0.06 μg/l) for AMPA (Ludvigsen & Lode, 2001a; 2001b). The monitoring study, therefore, indicated broad occurrence of glyphosate and its metabolite at low concentrations. In a study carried out in surface waters of the Midwest in the United States in 2002 glyphosate was detected in 35-40% of the samples (maximal concentration 8.7 μg/l) and AMPA in 53-83% of the samples (maximal concentration 3.6 μg/l) (Battaglin et al., 2005), and both glyphosate and AMPA were detected in vernal snow-flood at concentrations up to 328 and 41 • g/l, respectively, in 2005-2006 in four states of the US (Battaglin et al., 2009). Analysing water samples from 10 wastewater treatment plants in the United States, the U.S. Geological Survey detected AMPA in 67.5% and glyphosate in 17.5% of the samples (Kolpin et al., 2006). The study concluded that urban use of glyphosate contributes to glyphosate and AMPA concentrations in streams in the United States. In a study carried out in Canada in 2004-2005, 21% of the analysed 502 samples contained glyphosate with a maximum concentration of 41 μg/l, and the peak concentration of AMPA was 30 μg/l glyphosate equivalent (Struger et al., 2008). In France, glyphosate and AMPA were detected in 2007 and 2008 due to urban runoff effect (Batta et al., 2009). In fact, Villeneuve et al. (2011) adjudge glyphosate to be one of the herbicides most often found in freshwater ecosystems worldwide, and state that AMPA is the most often detected and glyphosate is the third most frequent pesticide residue in French streams. Elevated glyphosate levels were detected in surface water, soil and sediment samples due to intensive herbicide applications in a GM soybean cultivating area in Argentina (Peruzzo et al., 2008). These studies are warning signs indicating that this herbicide active ingredient of intensive use, that is expected to further expand with the commercial cultivation of GM crops, became an ubiquitous contaminant in surface waters, and therefore, a permanent pollutant factor, which deserves pronounced attention by ecotoxicology.

6. Adverse environmental effects of glyphosate

6.1 Glyphosate and *Fusarium* species

Sanogo and co-workers (2000) observed that crop loss in soy due to infestation by *Fusarium solani* f. sp. *glycines* increased after glyphosate applications. Kremer and co-workers (2005) described a stimulating effect of the root exsudate of GR soy sampled after glyphosate application on the growth of *Fusarium* sp. strains. Treatments caused concentration dependent increase on the mycelium mass of the fungus. Nonetheless, Powel and Swanton (2008) could not confirm these observations in their field study. Kremer and Means (2009) claim that certain fungi utilise glyphosate released from plant roots into the soil as a nutritive, which facilitates their growth. Soil manganese content also affects the above consequence of glyphosate through chelating with the compound and thus, modifying its effects. Considering the fact that numerous plant pathogenic *Fusarium* species produce mycotoxins, an increasing proportion of these species is far not favourable as a side-effect. Johal and Huber (2009) lists numbersome plant pathogens (e.g., *Corynespora cassicola* or *Sclerotinia sclerotiorum* on soy) they claim to grow increasingly after glyphosate treatments, and the list contains several *Fusarium* species (*F. graminearum*, *F. oxysporum*, *F. solani*). They hypothesize that glyphosate causes disturbances in microelement metabolism in plants, and in parallel, deteriorate the defense system of the plants, thereby increasing the virulence of certain plant pathogens. Zobiole and co-workers (2011) confirmed the above effects by their observation that glyphosate treatments facilitate colonisation of *Fusarium* species on the soy roots, but reduces the fluorescent *Pseudomonas* fraction of the rhizosphere, the level of manganese reducing bacteria and of the indoleacetic acid producing rhizobacteria. As a combined result of these effects, root and overall plant biomasses were found to be reduced.

6.2 Toxicity of glyphosate to aquatic ecosystems and amphibians

Substances occurring in surface waters deserve special attention by ecotoxicologists, as they enter a matrix that is the habitat of numerous aqueous organisms and the basis of our drinking water reserves. Drinking water is an irreplaceable essential part of our diet, and is a possible vehicle for chronic exposure (the basis of chronic diseases) in daily contact/consumption.

Glyphosate has been known to cause toxicity to microalgae and other aquatic microorganisms (Goldsborough and Brown 1988; Austin et al., 1991; Anton et al., 1993; Sáenz et al., 1997; DeLorenzo et al., 2001; Ma 2002; Ma et al., 2002; Ma et al., 2003), in fact a green algal toxicity test has been proposed for screening herbicide activity (Ma & Wang, 2002). In contrast, cyanobacteria have been found to show resistance against glyphosate (López-Rodas et al., 2007; Forlani et al., 2008). Tsui and Chu (2003) tested the effect of glyphosate, its most common polyoxyethyleneamine (POEA) type formulating materials, polyethoxylated tallowamines, and the formulated glyphosate preparation (Roundup) on model species from aquatic ecosystems, bacteria (*Vibrio fischeri*), microalgae (*Selenastrum capricornutum, Skeletonema costatum*), protozoas (*Tetrahymena pyriformis*, *Euplotes vannus*) and crustaceans (*Ceriodaphnia dubia*, *Acartia tonsa*). The most surprising result of the study was that the assumedly inert detergent formulating agent, POEA was found to be the most toxic component. In light of this it is far not surprising that Cox and Surgan (2006) and Reuben (2010) propounded the question, why tests only on the active ingredients are necessary to be specified in the documentation required by the Environmental Protection Agency of the

Unites States (US EPA), when several of the used formulating components are known to exert biological activity.

Although acute toxicity and genotoxicity of glyphosate have been evidenced to certain fish (Langiano & Martinez, 2008; Cavalcante et al., 2008), glyphosate shows favourable acute toxicity parameters on most vertebrates, and therefore, has been classified as III toxicity category by US EPA. The European discretion is stricter, listing the compound among substances causing irritation (Xi) and severe ocular damage (R41). It has to be noted, however, that that model species of neither amphibians, not reptilians are represented in the toxicological documentations required nowadays. It may not be surprising, therefore, that after atrazine (Hayes et al., 2002; 2010), glyphosate is the second herbicide active ingredient that is questioned due to its detrimental effects on the animal class, considered the most endangered on Earth, amphibians.

Mann and Bidwell (1999) studied the toxicity of glyphosate on tadpoles of four Australian frogs (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis* and *Litoria moorei*). The toxicity of Roundup and its 48-hour LC_{50} values were found to be 3-12 mg glyphosate equivalent/l. Tolerance of the adult frogs was substantially greater. A glyphosate-based formulated herbicide preparation (VisionMAX) caused no significant effects on the juvenile adults of the green frogs (*Lithobates clamitans*) when applied at field application doses, only marginal differences in statistics of infection rates and liver somatic indices in relation to exposure estimates (Edge et al., 2011). Chen et al. (2004) observed that the toxicity of glyphosate on the frog species *Rana pipiens* was greatly affected by lacking food resources and the pH of the medium as stress factors. Relyea (2005a) reported tadpole (*Bufo americanus*, *Hyla versicolor*, *Rana sylvatica*, *R. pipiens*, *R. clamitans* and *R. catesbeiana*) mortality related to glyphosate applications. The effect, occurred at 2-16 mg glyphosate equivalent/l concentrations, was linked with the stress caused by the predator of the tadpoles, salamander *Notophthalmus viridescens*. Later Relyea and Jones (2009) included further frog species (*Bufo boreas*, *Pseudacris crucifer*, *Rana cascadea*, *R. sylvatica*) into the study, and found LC_{50} values to be 0.8-2 mg glyphosate equivalent/l. Testing four salamander species (*Amblystoma gracile*, *A. laterale*, *A. maculatum* and *N. viridescens*), the corresponding values ranged between 2.7 and 3.2 mg glyphosate equivalent/l. In this case, glyphosate was formulated with detergent POEA. Further studies also shed light on the fact that another stress factor, population density, playing an important part in the competition of the tadpoles increased the toxic effect of glyphosate (Jones et al., 2010). Lajmanovich and co-workers (2010) detected lowered enzymatic activities (e.g., acetylcholine esterase and glutathion-*S*-transferase) in a frog species, *Rhinella arenarum* upon glyphosate treatments.

Sparling and co-workers (2006) detected lowered fecundity of the eggs of the semiaquatic turtle, red-eared slider (*Trachemys scripta elegans*) if treated with glyphosate at high doses.

6.3 Teratogenic activity of glyphosate

The teratogenicity of the pesticide preparations containing glyphosate deserves special attention. The very first examples of observed teratogenicity of glyphosate preparations have also been linked to amphibians. Using the so-called FETAX assay, Perkins and co-workers (2000) observed a formulation dependent teratogenic effect of glyphosate on embryos of the frog species *Xenopus laevis*. The concentrations that triggered the effect were relatively high (the highest dose applied in the study was 2.88 mg glyphosate equivalent/l),

but not irrealisticly high with respect to field doses of glyphosate, indicating, that high allowed agricultural doses cause glyphosate levels close to the safety margin. Lajmanovich and co-workers (2005) studied the effects of a glyphosate preparation (Glyfos) on the tadpoles of *Scinax nasicus*, and found that a 2-4-day exposure to 3 mg/l glyphosate caused malformation in more than half of the test animals. The treatment was carried out nearly at the LC_{50} level of glyphosate. Dallegrave and co-workers (2003) found fetotoxic effects on rats treated with glyphosate at very high, 1000 mg/l concentration on the 6th-15th day after fertilisation. Nearly half of the newborn rat progeny in the experiments were born with skeletal development disorders.

Testing the effects of glyphosate preparations on the embryos of the sea urchin, *Sphaerechinus granularis*, Marc and co-workers (2004a) observed a collapse of cell cycle control. Inhibition affects DNA synthesis in the G2/M phase of the first cell cycle (Marc et al., 2004b). The authors estimate that glyphosate production workers inhale 500-5000-fold level of the effective concentration in these experiments. A marked toxicity of the formulating agent POEA has also been observed on sea urchins (Marc et al., 2005). The very early DNA damage was claimed to be related to tumour formation by Bellé and co-workers (2007), and the authors consider the sea urchin biotest they developed as a possible experimental model for testing this effect. Jayawardena and co-workers (2010) described nearly 60% developmental disorders on the tadpoles of a Sri Lanka frog (*Polpedates cruciger*) upon treatment with 1 ppm glyphosate.

The teratogenicity of herbicides of glyphosate as active ingredient have been tested lately on amphibian (*X. laevis*) and bird (*Gallus domesticus*) embryos. Applied with direct injection at sublethal doses caused modification of the position and pattern of rhobomeres, the area of the neural crest decreased, the anterior-posterior axis shortened and the occurrence of cephalic markers was inhibited at the embryonic development stage of the nervous system. As a result, frog embryos became of characteristic phenotype: the trunk is shortened, head size is reduced, eyes were improperly or not developed (microphthalmia), and additional cranial deformities occurred in later development. Similar teratogenic effects were seen on embryos of Amniotes e.g., chicken. These developmental disorders may be related to damages of the retinoic acid signal pathway, resulting in the inhibition of the expression of certain essential genes (*shh, slug, otx2*). These genes play crucial roles in the neurulation process of embryogenesis (Paganelli et al., 2010). These findings were later debated by several comments. On behalf of the producers, Saltmiras and co-workers (2011) questioned certain conclusions in the work of Paganelli and co-workers (2010), claiming that the standardised pilot teratogenicity tests, carried out under good laboratory practice (GLP) by the manufacturers, have been evaluated by independent experts of several international organisations. They also considered the dosages used by Paganelli and co-workers exceedingly high, and the mode of application (microinjection) irrealistic in nature. Similar criticism has been voiced by Mulet (2011) and Palma (2011). In his answer, Carrasco (2011) emphasised their opinion that the company representatives ignore scientific facts supporting teratogenicity of atrazine, glyphosate and triadimefon through retinoic acid biosynthesis. He also emphasized that of 180 research reports of Monsanto, 150 are not public, or have never been presented to the scientific community. He also included that they obtained similar phenotypes in their studies with microinjection, than by incubation of the preparations. As a follow-up, Antoniou and co-workers (2011) compiled an extensive review of 359 studies and publications on the teratogenicity and birth defects caused by glyphosate,

and heavily criticize the European Union for not banning glyphosate, but rather postponing its re-evaluation until 2015 (European Commission, 2010).

6.4 Genotoxicity of glyphosate

Occupational exposure to pesticides, including glyphosate as active ingredient, may lead to pregnancy problems even through exposure of men (Savitz et al., 1997). Such phenomenon has been first described in epidemiology with Vietnam War veterans exposed to Agent Orange with phenoxyacetic acid type active ingredients contaminated with dibenzodioxins. Although glyphosate has been claimed not to be genotoxic and its formulation Roundup "causing only a week effect" (Rank et al., 1993; Bolognesi et al., 1997), Kale and co-workers (1995) observed mutagenic effects of Roundup in *Drosophila melanogaster* recessive lethal mutation tests. Lioi and co-workers (1998) described increasing sister chromatide exchange in human lymphocytes with increasing glyphosate doses. Walsh and co-workers (2000) detected in murine tumour cells the inhibitory activity of Roundup on the biosynthesis of a protein (StAR) participating in the synthesis of sex steroids. This reduced the operation of the cholesterol – pregnenolon – progesteron transformation pathway to a minimal level. As it often happens in exploring mutagenic effects of chemical substances, additional studies have not found glyphosate mutagenic, and therefore, it is not so listed in the GAP2000 program compiled from US EPA/IARC databases. However, Cox (2004) describes chronic toxicity profile of several substances applied in the formulation of glyphosate.

Studying the activity of dehydrogenase enzymes in the liver, heart and brain of pregnant rats, Daruich and co-workers (2001) concluded that glyphosate causes various disorders both in the parent female and in the progeny. According to results of the study by Benedettia and co-workers (2004), aminotransferase enzyme activity decreased in the liver of rats, impairing lymphocytes, and leading to liver tissue damages. In *in vitro* tests McComb and co-workers (2008) found that glyphosate acts in the mitochondria of the rat liver cells as an oxidative phosphorylation decoupling agent. Mariana and co-workers (2009) observed oxidative stress status decay in the blood, liver and testicles upon injection administration of glyphosate, possibly linked to reproductional toxicity.

Prasad and co-workers (2009) detected cytotoxic effects, as well as chromosomal disorders and micronucleus formation in murine bone-marrow. Poletta and co-workers (2009) described genotoxic effects of Roundup on the erythrocytes in the blood of caimans, correlated with DNA damages.

According to the survey of De Roos and co-workers (2003), the risk of the incidence of non-Hodgkin lymphoma is increased among pesticide users. As the authors found it, this applies to herbicide preparations with glyphosate as active ingredient. Focusing the study solely on glyphosate preparations a year later in the corn belt of the United States, of the majority of malignant diseases, only the incidence of abnormal plasma cell proliferation (*myeloma multiplex*, *plasmocytoma*) showed a slight rise (De Roos et al., 2004). Myeloma represents approximately 10% of the malignant haematological disorders. Although the cause of the disease is not yet known, its risk factors include autoimmune diseases, certain viruses (*HIV* and *Herpes*), and the frequent use of certain solvents as occupational hazard. On the basis of murine skin carcinogenesis, George and co-workers (2010) reported that glyphosate may act as a skin tumour promoter due to the induction of several special proteins.

6.5 Hormone modulant effects of glyphosate and POEA

Studying chronic exposure of tadpoles of *Rana pipiens*, Howe and co-workers (2004) found that in addition to developmental disorders, gonads in 15-20% of the treated animals developed erroneously, and these animals showed intersexual characteristics. Arbuckle and co-workers (2001) registered increased risk of abortion in agricultural farms after glyphosate applications. In addition, excretion of glyphosate has been determined in the urine of agricultural workers and their family members (Acquavella et al., 2004).

Richard and co-workers (2005) evidenced toxicity of glyphosate on the JEG3 cells in the placenta. Formulated Roundup exerted stronger effect than glyphosate itself. Glyphosate inhibited aromatase enzymes of key importance in estrogen biosynthesis. This effect has also been evidenced in *in vitro* tests by binding to the active site of the purified enzyme. The formulating agent in the preparation enhanced the inhibitory effect in the microsomal fraction. Benachour and co-workers (2007) tested the effect of glyphosate and Roundup Bioforce on various cell lines, and also determined the aromatase inhibiting effect of glyphosate and the synergistic effect of the formulating agent. They suppose that the hormone modulant effect of Roundup may affect human reproduction and fetal development. Testing these human cell lines, Benachour and Séralini (2009) found that glyphosate alone induces apoptosis, and POEA and AMPA applied in combination exert synergistic effects, similarly to the synergy seen for Roundup. The synergy was reported to be further acerbated with activated Cry1Ab toxin related to that produced by insect resistant GM plants, raising concern regarding stacked genetic event GM crops exerting both glyphosate tolerance and Cry1Ab based insect resistance (Mesnage et al., 2011). Moreover, the combined effect caused cell necrosis as well. Effect enhancement is likely to be explained by the detergent activity of POEA facilitating the penetration of glyphosate through cell membranes and subsequent accumulation in the cells. The aromatase inhibitory effect of the formulated preparation was four-fold, as compared to the neat active ingredient. The authors consider it proven, that POEA, previously believed to be inert, is far not inactive biologically. As the authorised MRL of glyphosate in forage is as high as 400 mg/kg, Gasnier and co-workers (2009) studied in various *in vitro* tests, what effects this may cause in a human hepatic cell line. All treatments indicated a concentration-dependent effect in the toxicity tests were found genotoxic in the comet assay for DNA damages, moreover, displayed antiestrogenic and antiandrogenic effects.

6.6 Glyphosate resistance of weeds

Frequent applications of glyphosate and the spread of GT crops outside of Europe escalate the occurrence of glyphosate in the environment, exerting severe selection pressure on the weed species. It has been well known that certain weeds have native resistance against glyphosate e.g., the common lambsquarters (*Chenopodium album*), the velvetleaf (*Abutilon theophrasti*) and the common cocklebur (*Xanthium strumarium*).

The first population of GT *Lolium rigidum* was described in 1996 by Pratley and co-workers in Australia. This was followed in 1997 by GT goosegrass (*Eleusine indica*) in Malaysia (Lee & Ngim, 2000), GT horseweed (*Conyza canadensis*) in the United States (VanGessel, 2001), GT Italian ryegrass (*Lolium multiflorum*) in Chile (Perez & Kogan, 2003). Further known GT weed species include *Echinochloa colona* (2007), *Urochloa panicoides* (2008) and *Chloris truncata*

(2010) in Australia; *Conyza bonariensis* (2003) and ribwort plantain (*Plantago lanceolata*, 2003) in South Africa; ragweed (*Ambrosia artemisifolia*, 2004), *Ambrosia trifida* (2004), *Amaranthus palmeri* (2005), *Amaranthus tuberculatus* (2005), summer cypress (*Bassia scoparia*, 2007) and annual meadow grass (*Poa annua*, 2010) in the United States; *Conyza sumatrensis* (2009) in Spain; Johnsongrass (*Sorghum halepense*) (2005), Italian ryegrass (*Lolium perene*, 2008) in Argentina; *Euphorbia heterophyla* (2006) in Brazil; *Parthenium hysterophorus* (2004) in Colombia and *Digitaria insularis* (2006) in Paraguay (Heap, Epubl). GT Johnsongrass was reported in a continuous soybean field in Arkansas, United States (Riar et al., 2011). Price (2011) claims that agricultural conservation tillage is threatened in the United States by the rapid spread of GT Palmer amaranth (*Amaranthus palmeri* [S.] Wats.) due to wide range cultivation of transgenic, GT cultivars and corresponding broad use of glyphosate. GT amaranths were first identified in Georgia, and later reported in nine states, Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Tennessee, and a closely related GT amaranth, common waterhemp (*Amaranthus rudis* Sauer) in four states, Illinois, Iowa, Minnesota, and Missouri. Moreover, GT Italian ryegrass populations collected in Oregon, United States appeared to show cross-resistance to another phosphonic acid type herbicide active ingredient, glufosinate (Avila-Garcia & Mallory-Smith, 2011).

Powles and co-workers (1998) described a *L. rigidum* population resisting 7-11-fold dosage of glyphosate in Australia. Shrestha and Hemree (2007) found GT subpopulations of 5-8 leaf stage *Conyza canadensis* surviving only 2-4-fold glyphosate doses. According to Powles (2008), it is not coincidental that in countries, where GT crops are on the rise (Argentina and Brazil), the occurrence of GT weeds is more frequent. Moreover, he considers this one of the main obstacles of the spread of GT crops in the agricultural practice. Glyphosate tolerance is an inherited property, therefore, accumulation of weeds in the treated areas is to be expected. Genomics studies of the GT populations revealed that mutation of the gene (*epsps*) encoding the target enzyme responsible for tolerance is not infrequent in nature. (The mutant alleles (*mepsps*, *2mepsps*) responsible for tolerance has been found in maize as well, see Table 2.). Reduced or modified uptake or translocation of glyphosate has also been observed, and the metabolic fate of the compound may also become altered in the cell (Shaner, 2009), possibly resulting in GT populations. It is not difficult to predict, that prolonged cultivation of GT crops will necessitate supplemental herbicide administrations with active ingredients other than glyphosate.

7. References

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15

Effects of Herbicide Atrazine in Experimental Animal Models

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1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a widely used herbicide in many countries for the control of broadleaf and grassy weeds in agricultural crops. The State of São Paulo, located in Brazil Southeast, is an important sugarcane, soybean and corn producing area with high use of chemicals in agriculture and potential risk of environmental contamination because of the pesticide dissemination, among them the atrazine (ATZ) leaching to groundwater (Cerdeira et al., 2005).

The prolonged use of ATZ and its persistence involves the risk of its retention in crops and soils; moreover, these compounds may also pass from surface to ground waters (Figure 1). In this way, ever-increasing agriculture has caused contamination of natural water sources (Mundiam et al., 2011). The maximum contaminant levels for the most of triazines in drinking water are 3 parts per billion (μg/L) (Costa Silva et al., 2010).

Some European countries have included ATZ on the list of pesticide residues to be controlled because it is a potential contaminant due to its chemical characteristics, including lipophilicity, slow hydrolysis, moderate to low water solubility, and high solubility in organic solvents with high absorption by organic matter, clay, and fat tissues (Ross et al., 2009). The lack of data about the effects of ATZ metabolites has prompted the U.S. Environmental Protection Agency (U.S. EPA) to state that the toxicity of atrazine's metabolites is equivalent to that of its parent compound and that exposure to these metabolites should be taken into account for risk assessment purposes (Ralston-Hooper et al., 2009).

Mohammad & Itoh (2011) presented the relative risk of various scenarios of exposure and recovery with an aquatic test organism submitted a long-term exposure to herbicides and demonstrated the toxicity of isolated or mixtures of ATZ at different concentrations. However, the patterns of accumulation of xenobiotics vary depending on the organism, characteristics of the chemical compound, quantity of this substance present in the environment, and the balance between assimilation and metabolic rates (Nwani et al., 2011).

It is necessary to study the effects of atrazine exposure in a great variety of experimental animal models in order to understand its action in the organisms and their target organs. In this context, it is very important to verify the effect of high concentration of herbicides in animal tests as positive controls. Saal & Welshons (2006) related the importance of positive controls in toxicological research to determine whether conclusions from experiments that report no significant effects in low-dose of the toxicant are valid or false.

Since in the last decade many efforts have resulted in intensive research about action of ATZ in various organisms, it could be necessary to identify morphological, molecular, biochemical or physiological biomarkers that detect biological effects of this triazine herbicide on the organisms (Campero et al., 2007). So we present in this chapter an extensive bibliographical review about this herbicide in animal tissues, focusing some target-organs, in order to gain insight into its cellular mechanisms, highlighting the results obtained by our research group.

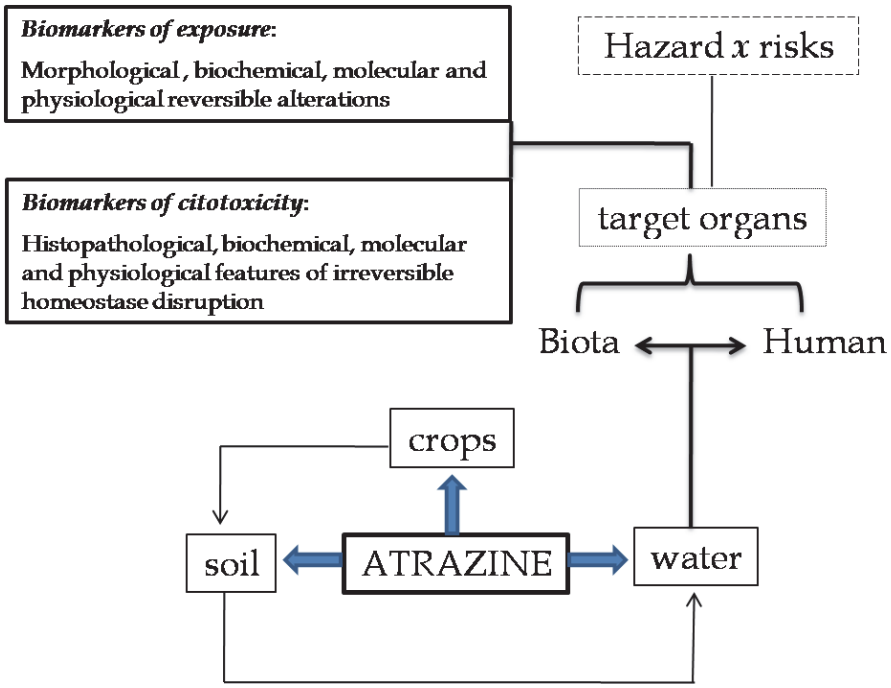

Fig. 1. Environmental contamination way of atrazine. Studies about biota and human health hazard are the basis for risk assessment and, in this context, cellular markers in target organs from organisms exposed to this herbicide could be used in monitoring programs.

2. Morphological and molecular alterations caused by ATZ

2.1 Hepatotoxicity

In liver, organ responsible for detoxification process, our researches with Wistar rats orally exposed to 400 mg/kg body weight (bw) of ATZ for 14 days, showed reduced accumulation

of hepatic glycogen and early symptoms of cytotoxicity. This event is attributed to the hepatotoxic effect of ATZ, which inhibits the activity of key enzymes of glyconeogenesis such as hexokinase, glycogen synthase, and glucokinase (Glusczak et al., 2006) and it can explain decrease in animals' body weight observed in our study. This finding agrees with Curic et al. (1999), who studied fish exposed to low doses of ATZ (2 mg/kg) for two weeks and observed the decrease of glycogen and the increase of lipids in the liver.

On the other hand, no differences in glycogen or lipid storage were noted in livers of *Xenopus laevis* tadpoles exposed to both atrazine concentrations 200 and 400µg/L (Zaya et al., 2011). Livers of ATZ-exposed tadpoles were significantly smaller and those from 400µg/L-exposed tadpoles had higher numbers of activated caspase-3 immuno-positive cells suggesting increased rates of apoptosis. The changes noted in body and organ size at 200 and 400µg/L ATZ indicated that exposure throughout development compromised the tadpoles. Additionally, fat body size decreased significantly after exposure to 200 and 400µg/L of ATZ, although this organ still contained some lipid and lacked any pathology. Zaya et al. (2011) suggested that significant reductions in fat body size could potentially decrease their ability to survive the stresses of metamorphosis or reduce reproductive fitness as frogs rely on lipid storage for these processes.

In male Japanese quail (*Coturnix japonica*), vacuolar degeneration in liver was observed at high doses of ATZ (500 mg/kg bw) ingested orally by 45 days (Hussain et al., 2011). Additionally, biliary hyperplasia and mild renal tubular necrosis were observed in these quails. In our studies with Wistar rats orally exposed to 400 mg/kg body weight (bw) of ATZ for 14 days, similar data were observed in liver and renal tubular necrosis was also observed.

Zebrafish (*Danio rerio*) is other model organism that presented histopathological effects in liver, which were induced by atrazine exposure. Yuanxiang et al. (2011) found seven proteins that were upregulated >2- fold, whereas 6 protein were downregulated >2-fold, after 10 and 1000 µg/l ATZ exposures in zebrafish for 14 days. They suggested that these changes in protein regulation were associated with a variety of cellular biological processes, such as response to oxidative stress, oncogenesis and others.

Another example of cellular biological process that could be changed in response to atrazine exposure is the lipid metabolism and insulin resistance. Study performed in Sprague-Dawley rats treated for 5 months with vehicle or ATZ (30 or 300 µg kg^{-1} day^{-1}), supplied in drinking water, showed prominent accumulation of lipid droplets in the livers of ATZ-treated rats (Lim et al., 2009). By means of transmission electron microscopy, some liver mitochondria from the ATZ-treated group showed partially disrupted cristae. Despite the fact that mitochondrial morphology was altered in liver and, additionally, in muscle, protein expression levels of mitochondrial OXPHOS complex subunits in liver and muscle tissues were not changed significantly by ATZ administration. Since no treatment-related changes in food or water intake or physical activity were observed at any point during the study, Lim et al. (2009) believe that the development of insulin resistance by ATZ might be related to energy metabolism and they suggest that long-term exposure to the herbicide ATZ might contribute to the development of insulin resistance and obesity, particularly where a high-fat diet is prevalent.

2.2 Reproductive toxicity

In review of Sifakis et al. (2011) about pesticide exposure and health, related issues in male and female reproductive system have been presented and they showed that ATZ seems to have estrogenic and anti-androgenic properties.

Our research group evaluated histopathological effects of low and high doses of ATZ in ovary and testicles from exposed Wistar rats and the compilation of data are presented in the Table 1.

Testicular lesions observed in our studies (Table 1) also be detected, associated with reduced germ cell numbers, in teleost fish, amphibians, reptiles, and mammals; and induces partial and/or complete feminization in fish, amphibians, and reptiles (Hayes et al., 2011). Then, ATZ is an endocrine disruptor that demasculinizes and feminizes the gonads of male vertebrates by means of the reduction in androgen levels and the induction of estrogen synthesis - demonstrated in fish, amphibians, and reptiles - that represent plausible and coherent mechanisms to explain these effects, according to Hayes et al. (2011). ATZ reduce testicular testosterone in male rats and it was associated with poor semen quality (Sifakis et al., 2011).

Morphological alterations induced by atrazine oral exposure			
Ovaries		**Testis**	
0,75mg/kg	**400mg/kg**	**0,75mg/kg**	**400mg/kg**
Primordial follicles without alterations	Primordial follicles without alterations	Normal histoarchitecture	Disorganized histoarchitecture
Primary follicles without alterations	Primary follicles without alterations	Absence of degeneration in seminiferous epithelium	Degeneration in some areas of the seminiferous epithelium
Presence of some multioocytic Preantral follicles	Preantral follicles with disorganized granulose layer and/or a degenerating oocyte	Germinative cells keep their typical morphology	Some germinative cells presented apoptotic or necrosis features
Antral follicles without alterations	Presence of some Antral follicles with a degenerating oocyte	Germinative cells were not released to tubular lumen	Releasing of germinative cells to the tubular lumen
Atretic antral follicles with intensification of apoptosis in granulose cells	High intensity of apoptosis in the granulose cells of Atretic antral follicles	Intertubular tissue around seminiferous tubules remained intact	Intertubular tissue around seminiferous tubules remained intact

Table 1. Histopathological analysis of ovaries and testis from Wistar rats submitted to subchronic oral exposure: 0,75mg atrazine/kg/day during 30 days; and subchronic oral exposure: 400mg atrazine/kg/day during 14 days.

A study developed by Hussain et al. (2011) that intended to determine the pathological and genotoxic effects of ATZ in male Japanese quail (*Coturnix japonica*) demonstrated that testis from ATZ treated birds were comparatively smaller in size and seminiferous tubules in group treated with 500 mg/kg bw exhibited decreased number of spermatocytes, necrotic nuclei of spermatids, and lesser number or absence of spermatozoa.

A significant dose dependent induction in the levels of mRNA expression of genes of steroidogenic acute regulatory protein (STAR), cytochrome P450-11A1, 3β-hydroxysteroid dehydrogenase (3β-HSD), and other steroidogenic proteins were observed in cells exposed to ATZ. These data suggest the applicability of these selected marker genes of steroidogenesis as an indicator of short term exposure of ATZ induced rat testicular toxicity in interstitial Leydig cells (ILCs) (Abarikwu et al., 2011).

Pogrmic-Majkic et al. (2010) examined Leydig cells treated for 24 h with the concentrations 0.001, 1, 10, 20, and 50μM of ATZ and they observed increased basal and human chorion gonadotropin-stimulated testosterone production and accumulation of cAMP in the medium of treated cells. The stimulatory action of atrazine on androgen production but not on cAMP accumulation was abolished in cells with inhibited protein kinase A. They observed that Leydig cells obtained from rats treated with 200 mg ATZ/kg body weight, by gavage, during the first 3 days of treatment, stimulated the expression of mRNA transcripts for steroidogenic factor-1, steroidogenic acute regulatory protein, cytochrome P450(CYP)-17A1, and 17b-hydroxysteroid dehydrogenase (HSD), as well as the activity of CYP17A1 and 17bHSD and cAMP accumulation and androgen production. However, this behavior is followed by a decline during further treatment (6 days). These results indicate that ATZ has a transient stimulatory action on cAMP signaling pathway in Leydig cells, leading to facilitated androgenesis.

ATZ exposure (120 or 200 mg/kg body weight ATZ orally for 7 and 16 days) has a dose-dependent adverse effect on the testicular and epididymal sperm numbers, motility, viability, morphology, and daily sperm production in rats (Abarikwu et al., 2009). Although the testis of the ATZ -treated animals appear normal, few tubules had mild degeneration with the presence of defoliated cells, similar to observed in our research group for rat testis (Table 1). Likewise, no perceptible morphological changes were observed in the epididymis. The results suggest that ATZ impairs reproductive function and elicits a depletion of the antioxidant defense system in the testis and epididymis, indicating the induction of oxidative stress. Glutathione (GSH) and glutathione-S-transferase (GST) activities were elevated in the high-dose group, whereas the activity of superoxide-dismutase (SOD), catalase (CAT); ascorbate (AA), and malondialdehyde (MDA) levels and hydrogen peroxide production were unchanged in the testis during the 7-day exposure protocol. When ATZ treatment was increased to 16 days, GSH levels remained unchanged, but lipid peroxidation levels were significantly increased in both the testis and epididymis. This corresponded to the significant diminution in the activities of GST and SOD. CAT activities were unaffected in the testis and then dropped in the epididymis. These experiments performed by Abarikwu et al. (2009) was important to understand the antioxidant defense system in the testis and epididymis; and it is interesting to note that ATZ can also affect mitochondrial electron transport and oxidative stress in the insect *Drosophila melanogaster* (Thornton et al., 2010).

Eggs of alligator *Caiman latirostris*, at stage 20 of embryonic development, were exposed to 0,02ppm of ATZ and incubated at 33ºC resulted in male hatchlings. Tortuous seminiferous tubules with increased perimeter, disrupted distribution of peritubular myoid cells (desmin positive), and emptied tubular lumens characterized the testis of pesticide-exposed *Caiman* (Rey et al., 2009).

ATZ is a known ovarian toxicant which increase progesterone (P4) secretion and induce luteal cell hypertrophy following repeated administration. The aim of Taketa (2011) study group was to define the pathways by which these compounds exerted their effects on the ovary and hypothalamic-pituitary-gonadal (HPG) axis. They demonstrated that 300 mg ATZ/kg were orally given daily from proestrus to diestrus in normal cycling rats resulted in significant increased serum P4 levels, upregulation of the follow steroidogenic factors: scavenger receptor class B type I, steroidogenic acute regulatory protein, P450 cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase (HSD), and so downregulation of luteolytic gene 20α-HSD. ATZ may directly activate new corpora lutea by stimulating steroidogenic factor expressions and the authors suggest that multiple pathways mediate its effects the HPG axis and luteal P4 production in female rats *in vivo*.

One of the molecular events that may be triggered by stressful conditions, like pesticide exposure, is the synthesis of heat shock proteins (HSP). Additionally to histophatological analysis of rat ovaries (Table 1), our studies also emphasized the immunohistochemical labeling of 90 KD heat shock protein (HSP 90) in order to evaluate the role played by this protein in the ovary, under stressed conditions induced by ATZ exposure. Our results indicated that atrazine induced impaired folliculogenesis, increased follicular atresia and HSP90 depletion in female rats submitted to subacute treatment, while the subchronic treatment with the lowest dose of ATZ could compromise the reproductive capacity reflected by the presence of multioocytic follicle and stress-inducible HSP90 (Juliani et al., 2008).

Experiments developed by our research group also showed that low doses of ATZ, which does not affect estrous cyclicity, induced a higher HSP70 expression in cells of the oviduct when compared to the control group, indicating that HSP70 may be acting in the tissue response to stress caused by chronic exposure to the herbicide. In subacute exposure, with the dose that disrupts the estrous cycle, the expression of HSP70 was higher than the control group and the subchronic treatment (Figure 2), probably indicating a major protective function of HSP70 in addition to the estrogen receptor baseline level maturation. In literature, HSP70 is related to the maturation of the estrogen receptor in the oviduct (Mariani et al., 2000). We concluded that the increased expression of HSP70 induced by ATZ is mainly related to the protective effect of these chaperones in response to chemical stress generated by exposure to this herbicide.

2.3 Glandular alterations

Due to the adrenal gland is reported to be the most common endocrine organ associated with chemically induced lesions, our research group also evaluated adrenal glands of adult rats submitted to subacute and subchronic treatment with this herbicide, respectively. The morphological and histochemical analyses were performed and the results indicated that the subacute treatment induced drastic alterations in the cortex of the adrenal glands as well as

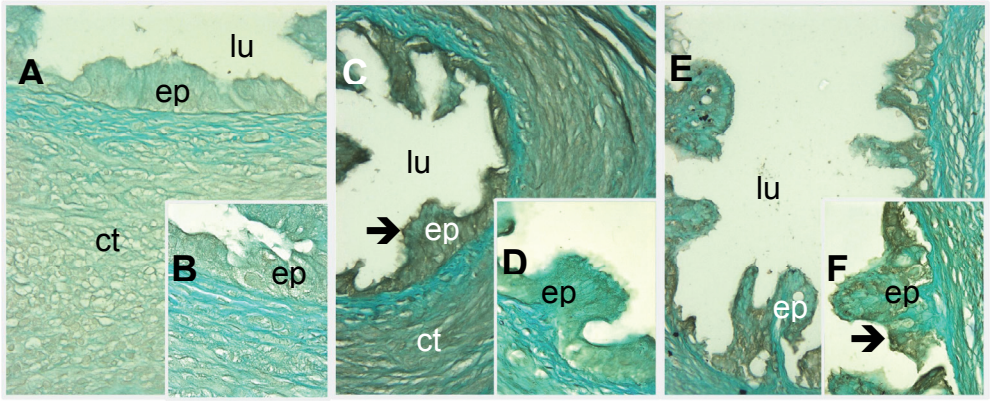

Fig. 2. Immunohistochemical detection of HSP70 (Heat Shoch Protein - 70) in oviduct from Wistar rats. A–B) Control Group; C-D) Experimental group submitted to subchronic oral exposure: 0,75mg atrazine/kg/day during 30 days; E-F) Experimental group submitted to subchronic oral exposure: 400mg atrazine/kg/day during 14 days. Oviducts present different degrees of immunopositive reaction (brown color) in epithelium (ep) and connective tissue (ct). Arrows indicate areas with high imunolabeling of HSP70. A, C, E – Magnification = 200x; B, D, F – Magnification = 400x.

in the medullar region. The subchronic treatment with the low dose of ATZ caused slight morphological alterations in the cortex of adrenal glands, but not in the medullar region. The histochemical analyses showed abnormal accumulations of lipid droplets mainly in the Reticularis Zona of the adrenal cortex suggesting alteration in the steroidogenesis process that occur in this region (Figure 3).

Foradori et al. (2011) demonstrated that high doses of ATZ (200mg/kg), administered for 4 days, suppress luteinizing hormone (LH) release and increase adrenal hormones levels. Considering the known inhibitory effects of adrenal hormones on the hypothalamo-pituitary-gonadal axis, the authors investigated the possible role that the adrenal gland has in mediating ATZ inhibition of LH release and observed that adrenolectomy had no effect on ATZ inhibition of the LH surge but prevented the ATZ disruption of pulsatile LH release. These data indicate that ATZ selectively affects the LH pulse generator through alterations in adrenal hormone secretion. Adrenal activation does not play a role in ATZ's suppression of the LH surge and therefore, ATZ may work centrally to alter the preovulatory LH surge in female rats.

2.4 Genotoxicity

Although the toxic properties of ATZ are well known, there is not a consensus about the genotoxic effects of ATZ. On aquatic organisms they are rather scarce. To evaluate the genotoxic effects of ATZ and an ATZ-based herbicide (Gesaprim®) on a model fish species *Carassius auratus* L., 1758, (Pisces: Cyprinidae) using the micronucleus test and the comet assay in peripheral blood erythrocytes, fish were exposed to 5, 10 and 15 µg/L ATZ and to its commercial formulation for 2, 4 and 6 days (Cavas, 2011). The results revealed significant increases in the frequencies of micronuclei and DNA strand breaks in erythrocytes of *C.*

auratus, following exposure to commercial formulation of ATZ and thus demonstrated the genotoxic potential of this pesticide on fish.

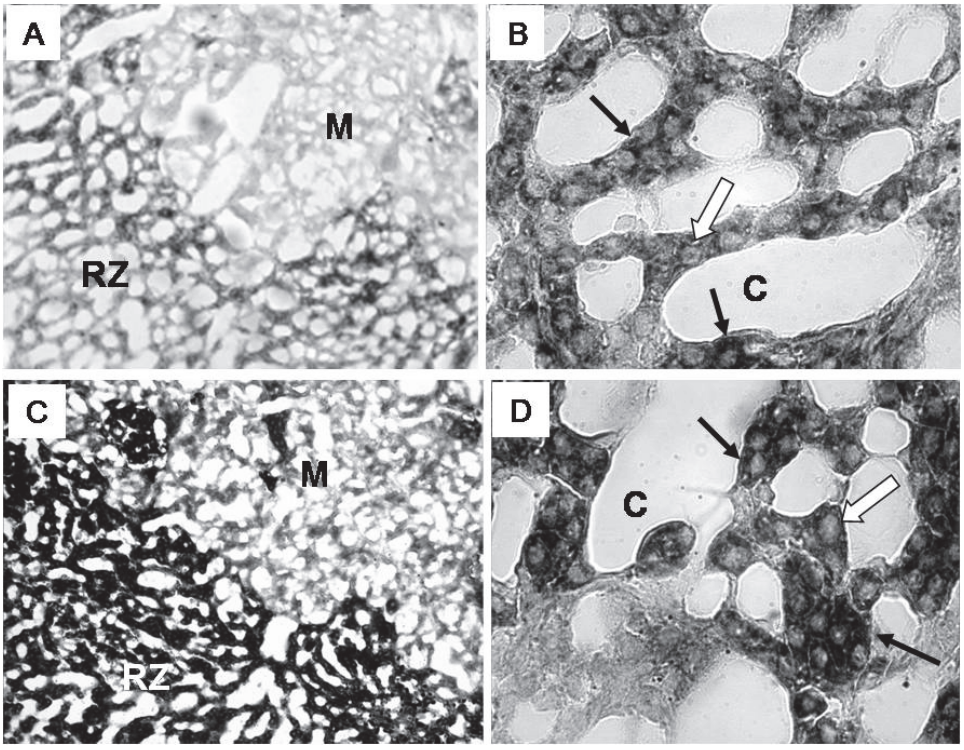

Fig. 3. Cryosections of adrenal gland from Wistar rats, stained with Sudam Black. The dark-brown color indicates the presence of lipids in the Reticularis Zone (RZ) but not in the Medullar region (M). A–B) Control Group; C-D) Experimental group submitted to subchronic oral exposure: 0,75mg atrazine/kg/day during 30 days. In (B) and (D), lipids in cells of Reticularis Zone are indicated with arrows (black arrow= the strongest intensity; white arrow= strong intensity). A, C – Magnification = 200x; B, D – Magnification = 400x

Significantly longer comet tails of DNA damage in leukocytes and isolated hepatocytes of male Japanese quail (*Coturnix japonica*) were recorded with 500 mg/kg bw ATZ (Hussain et al., 2010).

In our results with rats treated with 400mg ATZ/kg bw too have been observed a significant increase of micronucleated polycromatic erythrocytes (data not published), corroborating that authors and suggesting a possible genotoxic potencial of ATZ in mammals, which have to make its use highly controlled.

2.5 Mutagenicity and cancer

Chronic studies of ATZ and simazine and their common metabolites show an elevated incidence of mammary tumors only in female Sprague Dawley (SD) rats. On the basis of the

clear tumor increase in female SD rats, ATZ was proposed to be classified as a likely human carcinogen by US Environmental Protection Agency (EPA) in 1999. With Fischer rats, all strains of mice, and dogs, there was no evidence of increased incidence of ATZ -associated tumors of any type. Evidence related to the pivotal role of hormonal control of the estrus cycle in SD rats appears to indicate that the mechanism for mammary tumor induction is specific to this strain of rats and thus is not relevant to humans. In humans the menstrual cycle is controlled by estrogen released by the ovary rather than depending on the LH surge, as estrus is in SD rats. However, the relevance of the tumors to humans continues to be debated based on endocrine effects of triazines. No strong evidence exists for ATZ mutagenicity, while there is evidence of clastogenicity at elevated concentrations. ATZ does not appear to interact strongly with estrogen receptors α or β but may interact with putative estrogen receptor GPR30 (G-protein-coupled receptor). A large number of epidemiologic studies conducted on manufacturing workers, pesticide applicators, and farming families do not indicate that triazines are carcinogenic in these populations. A rat-specific hormonal mechanism for mammary tumors has now been accepted by US EPA, International Agency for Research on Cancer, and the European Union. Chlorotriazines do influence endocrine responses, but their potential impact on humans appears to be primarily on reproduction and development and is not related to carcinogenesis (for revision, see Jowa & Howd, 2011).

According an extensive review, epidemiology studies do not provide consistent, scientifically convincing evidence of a causal relationship between exposure to ATZ or triazine herbicides and cancer in humans. Based upon the assessment studies, there is no scientific basis for inferring the existence of a causal relationship between triazine exposure and the occurrence of cancer in humans (Sathiakumar et al., 2011).

A study developed by NIEHS (National Institute of Environmental Health Sciences) that extended analysis of cancer risk associated with occupational hazard of ATZ showed that there was no strong or consistent evidence of an association between ATZ and any cancer. There was a non-statistically significant increased risk of ovarian cancer related to occupational hazard for female who reported to use ATZ compared to those who did not; however, this observation was based on a small number of cases among ATZ users. The authors found an elevated risk of thyroid cancer, has not been previously reported, for the highest versus lowest category of intensity weighted ATZ use, but the trend was not monotonic and not statistically significant when lifetime days of use was considered as the exposure metric. In contrast, they observed little evidence for an association between ATZ occupational use and other cancers previously reported in the literature, such as NHL non-Hodgkin lymphoma) and leukemia, or with cancers of the breast or prostate, for which ATZ has been hypothesized to be a risk factor because of its hormonal properties (Freeman et al., 2011).

Although there is conflicting information about relationship between ATZ and cancer some researches have been demonstrated preoccupation with this aspect and they highlighted the importance of many studies to confirm or not this supposition.

3. Conclusion

We concluded that:

- In adult model animals, lower doses of atrazine generally induce accumulation of lipids in hepatocytes, otherwise higher doses induce hepatotoxicity with degree variation

according to animal. Amphibian tadpole's liver presents morphological response pattern, which is different those from the adult model animals.

- In rat and bird testis, atrazine has a dose-dependent adverse effects varying from no perceptible morphological changes to degeneration of seminiferous epithelium because ATZ impairs reproductive function and induces a depletion of the antioxidant defense system, according to the dose and time of exposure. Otherwise, in testis of teleost fish, amphibian and reptile, atrazine has a demascunilization/feminization effect that can be partial or complete what depends on the dose and time of exposure.
- High concentrations of atrazine induce morphological alterations in rat ovarian follicles, but not in oviduct. Induction of HSP70 in oviduct (low and high doses) could be used as exposure cell marker, as well as HSP90 depletion (high dose) or HSP90 increasing (low dose) could indicate the degree of ATZ exposure.
- In adrenal glands of rats, atrazine exposure induced varied degree of morpho-physiological alterations, which is observed in a dose-dependent way due its endocrine disruptor property.
- There is not a consensus about the genotoxic effects of atrazine, and then it is necessary further studies in experimental animal models.
- Although high doses of atrazine induce clastogenicity, there is not consistent evidence that associate mutagenecity with cancer in humans.

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16

A Critical View of the Photoinitiated Degradation of Herbicides

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1. Introduction

The application of herbicides to agricultural soil is a well established and effective practice to control weed growth. Another areas of herbicide application are roads and railways where herbicides are used to mantain the quality of the track and a safe working environment for railway personnel (Torstenson, 2001). Some of total herbicides are used in urban areas, or as algicides in paints and coatings (Lindner et al.,2000). Among the wide range of herbicides available, phenyl-urea and triazine derivatives represent a prominent group, the variety and use of which having increased markedly during the past decades. Many of the compounds in both families are biorecalcitrant, i.e. their microbiological degradation is slow or totally ineffective, they therefore persist in the environment for many weeks or even months after application.

The partial water solubility of triazines and phenylurea herbicides results in their leaching or washing into surface and ground waters from the place of application.

For many important classes of pesticides including phenylurea and triazine herbicides, photoinitiated transformation may be the only relevant elimination process in surface waters. In waste-waters, advanced photochemical oxidation processes (EPA Handbook, 1998) using oxidative agents/UV combination have been under study.

2. Photoinitiated reactions

Each reaction started by an absorption of radiation may be classified as a photochemical or photoinitiated reaction. According to the mechanism of the photoinitiated reaction, photolytic, photosensitized and photocatalytic reactions can be distinguished.

A photolytic reaction is usually understood as a reaction in which the quantum of radiation absorbed has enough energy to cause the breaking of a covalent bond in the substrate compound. Usually highly energetic UV radiation (less than 250 nm) is necessary for this purpose. These reactions cannot proceed on the Earth´s surface since solar radiation reaching the Earth´s surface contains wavelengths greater than 290 nm.

A photosensitized reaction needs a sensitizer molecule. This is a molecule that is able to absorb radiation and to transfer the absorbed excitation energy onto another molecule. The

energy can be transferred either onto an organic molecule, substrate (e.g. herbicide molecule), or onto an oxygen molecule as shown in Eqs. 1 - 5.

$$^1\text{Sens} + h\nu \rightarrow {}^1\text{Sens}^* \quad (1)$$

$$^1\text{Sens}^* + {}^1\text{Substrate} \rightarrow {}^1\text{Substrate}^* + {}^1\text{Sens} \rightarrow \text{Product} + {}^1\text{Sens} \quad (2)$$

$$^1\text{Sens}^* \rightarrow \textit{through ISC} \rightarrow {}^3\text{Sens}^* \quad (3)$$

$$^3\text{Sens}^* + {}^3O_2 \rightarrow {}^1O_2 \quad (4)$$

$$^1O_2 + {}^1\text{Substrate} \rightarrow \text{Oxidized product} \quad (5)$$

Eq.1 represents excitation of the sensitizer from the ground state (which is always a singlet state, i.e. all electrons in the molecule are paired) to the first excited singlet state. Eq. 2 represents energy transfer onto the substrate and its subsequent reaction into a product. Eq. 3 represents the conversion of the sensitizer from the first excited singlet state (all electrons are paired in the molecule in a singlet state) into the first triplet state (where two electrons are unpaired) through so called intersystem crossing (ISC). The sensitizer in the triplet state is able to react with molecular oxygen dissolved in the reaction mixture (Eq.4) because the ground state of molecular oxygen with its two unpaired electrons is a triplet state. If this ISC process did not occur, the reaction would not proceed since a reaction between a singlet and a triplet state molecule is spin-forbidden. The reaction results in the formation of a powerful oxidative species, singlet oxygen that oxidizes organic substrate molecules (Eq. 5).

Photocatalysis may occur as a homogeneous process or as a heterogeneous process. In homogeneous photocatalytic reactions light produces a catalytically active form of a catalyst. E. g. ferric ions may be reduced photochemically in the presence of an electron donor to ferrous ions that exhibit much higher catalytic activity. The subsequent catalytic reaction of a substrate is a ´dark´ reaction, i.e. not photochemical, since the reaction does not need light. Heterogeneous photocatalysis includes photochemical reactions on semiconductors. It proceeds via the formation of an electron-hole pairs under irradiation. These holes and electrons react with the solvent (water) and dissolved oxygen to produce an oxidative species, mainly OH radicals (Eqs. 6 – 11).

$$h^+ + H_2O \rightarrow HO^\bullet + H^+ \quad (6)$$

$$h^+ + OH^- \rightarrow HO^\bullet \quad (7)$$

$$O_2 + e^- \rightarrow O_2^{\bullet-} \quad (8)$$

$$O_2^{\bullet-} + H^+ \rightarrow HO_2^\bullet \quad (9)$$

$$2HO_2^\bullet \rightarrow H_2O_2 + O_2 \quad (10)$$

$$H_2O_2 + O_2^{\bullet-} \rightarrow HO^\bullet + O_2 + OH^- \quad (11)$$

3. Characterisation of s-triazine and phenylurea herbicides

S-triazine herbicides contain an aromatic ring with three N heteroatoms. The formula of a triazine herbicide, atrazine, is shown in Fig. 1., the formula of a phenylurea herbicide, chlorotoluron, in Fig. 2.

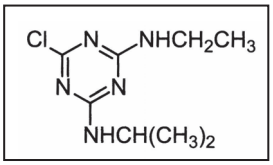

Fig. 1. The structural formula of a triazine herbicide, atrazine.

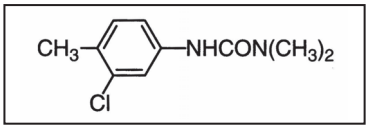

Fig. 2. The structural formula of a phenylurea herbicide, chlorotoluron .

The triazine herbicides were introduced in the 1950s (Gysin & Knüsli, 1957, Gast et al., 1956, both in Tomlin, 2003), phenylurea pesticides a decade later (L´Hermite et al., 1969, in Tomlin 2003).

The solubilities of these herbicides in water are in milligrams or at most tens of milligrams per liter as shown for three trazine and one phenylurea herbicide in Table 1. Table 1 also summarizes the DT_{50} values for the selected herbicides. DT_{50} signifies 50% dissipation time, i.e. the amount of time required for 50% of the initial pesticide concentration to dissipate. Unlike half-life dissipation time does not assume a specific degradation model.

Herbicide	solubility (mg/l)	DT_{50} (days)
Atrazine	33 (22°C)	field: 16 – 77, median 41 natural waters: 10 –105 groundwaters: 105 - >200
Propazine	5.0 (20°C	soil: 80 - 100
Simazine	6.2 (20°C)	soil: 27 - 102
chlorotoluron	74 (25°C)	soil: 30- 40 water: >200

Table 1. Solubilities and DT_{50} values of selected triazine and phenylurea herbicides as given in Tomlin (2003).

All these herbicides are photosynthetic electron transport inhibitors at the photosystem II receptor site. They are all also systemic herbicides. Systemic herbicides (in comparison with contact herbicides) are translocated through the plant, either from foliar application down to the roots or from soil application up to the leaves. They are capable of controlling perennial plants and may be slower in action but ultimitaly more effective than contact herbicides.

4. Biodegradation of selected triazine and phenylurea herbicides

4.1 Biodegradation of triazines

In spite of the fact that triazine and phenylurea herbicides persist in the natural environment for a long time and do not undergo biodegradation easily there are some higher plants and microorganisms capable of metabolizing these compounds.

In tolerant plants triazines as well as phenylurea herbicides are readily metabolized. Plant metabolites include the hydroxy- and dealkylated derivatives of parental compounds. Atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) is metabolized in tolerant plants to hydroxyatrazine and amino acid conjugates, with further decompositon of hydoxyatrazine by degradation of the side-chains. The resulting amino acids on the ring are hydrolyzed and mineralized (i.e. degraded to CO_2). In sensitive plants, unaltered atrazine accumulates, leading to chlorosis (a condition in which leaves produce insufficiant amounts of chlorophylls) and death. The similar degradation or action pathways apply for propazine (6-chloro-N^2,N^4-di-isopropyl-1,3,5-triazine-2,4-diamine) and simazine (6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine). With chlorotoluron (3-(3-chloro-p-tolyl)1,1-dimethylurea), metabolites found in winter wheat include 3-chloro-p-toluidine,3- (3-chloro-4-methylphenyl)-1-methylurea and 1-(3-chloro-4-methylphenyl)urea (Tomlin, 2003).

Behki and Khan studied agricultural soils to which atrazine was applied for a long time. They isolated three bacteria strains (*Pseudomonas* family) capable of utilizing atrazine as the sole source of carbon (Behki & Khan, 1986). Those bacteria use the side-chain carbon, thus N-dealkylation resulting in desisopropylatrazine and desethylatrazine was observed. Two bacterial strains were able to cause the splitting of chlorine from atrazine as well as from the dealkylated metabolites. The same authors proved the capacity to degrade atrazine, propazine, and simazine in the bacteria of *Rhodococcus* species (Behki & Khan, 1994), the degradation rates being however lower than in *Pseudomonas* bacteria.

Not only bacteria but also other organisms such as soil fungal communities have been found to be able to attack and degrade triazines (Kodama et al., 2001).

A *Pseudomonas* bacterial strain was used to degrade atrazine by Wenk (Wenk et al., 1998). The rate of atrazine disappearance was shown to depend on the water content of the soil and on the number of inoculated bacteria; the time necessary for atrazine removal differed ranging from 1 to 25 days. A partial mineralisation of atrazine into CO_2 was also observed.

Such results are in agreement with the findings of Crawford and his coworkers (Crawford et al., 2000), who concluded that the biodegradation rate is affected by the properties of soils and sediments, by agricultural cultivation practices and by the history of triazine application onto the particular soil.

Two genes responsible for s-triazine degradation have been found in four bacterial phyla (Jason Krutz et al., 2010).

4.2 Biodegradation of phenylurea chlorotoluron

Biotransformation of phenylurea herbicides by soil microorganisms (bacterial and fungi) has been reported by several authors (Badawi et al., 2009; Khadrani et al., 1999; Sørensen et al., 2003; Tixier et al., 2002). Bacteria degrade phenylurea herbicides by successive N-

dealkylation to substituted aniline products. Fungal pathways result in successive dealkylated metabolites as well as aniline derivatives, but Badawi (Badawi et al., 2009) reported the detection of a new major metabolite which (according to thin layer chromatography and nuclear magnetic resonance spectrometry) is a non-aromatic diol.

Biodegradation by some bacterial and fungal strains leads to the formation of very toxic substituted anilines which have even higher levels of LD_{50} - the dose required to kill half the members of a tested population after a specified test duration time (Tixier et al., 2000a; Tixier et al, 2009). The same applies to products of photochemical degradation (Tixier et al., 2000b).

5. Photochemical degradation of triazine and phenylurea herbicides

5.1 Possible photoinitiated pathways for herbicide degradation

An organic substrate may undergo the following photoinitiated reactions under natural sunlight or artificial source irradiation:

- direct sunlight photodegradation;
- homogeneous photocatalytic degradation in the presence of dissolved metal ions;
- heterogeneous photocatalytic degradation on particulate metal compounds in natural waters;
- heterogeneous photocatalytic degradation on semiconductors;
- photosensitized reaction - reaction in the presence of sensitizers;
- photolytic degradation by short-wavelength irradiation.

For a pollutant the processes given above are schematically visualized in Fig. 3.

5.2 Direct sunlight photodegradation

Direct sunlight photodegradation can proceed with substrates that are able to absorb the solar action spectrum. Solar radiation reaching the Earth´s surface has wavelengths ranging from about 300 nm upwards. Triazine and phenylurea compounds, which absorb at range well below 300 nm (absorption maxima at 220 – 235 nm) cannot therefore undergo direct sunlight photodegradation.

5.3 Homogeneous photocatalytic degradation in the presence of dissolved metal ions

Homogeneous photocatalytic reactions of triazine herbicides in the presence of dissolved metal ions were studied for ferric, copper, and manganese ions (Klementova & Hamsova, 2000). Cupric and manganese (II) ions exhibited only small activities, and only in high concentrations. Table 2 shows the results for atrazine degradation in aqueous solutions under irradiation at a range of wavelengths from 300 to 350 nm. When no metal ions are added, no reaction occurs.

In the case of atrazine the addition of Cu (II) or Mn(II) ions results in conversion below 15 % or less. Ferric ions in comparable concentration cause the conversion of practically all the atrazine in 90 minutes of irradiation. The degradation of atrazine was shown to be strongly dependent on the ferric ion concentration (Fig. 4). Simazine and propazine did not show such a strong dependence on the added ferric ions.

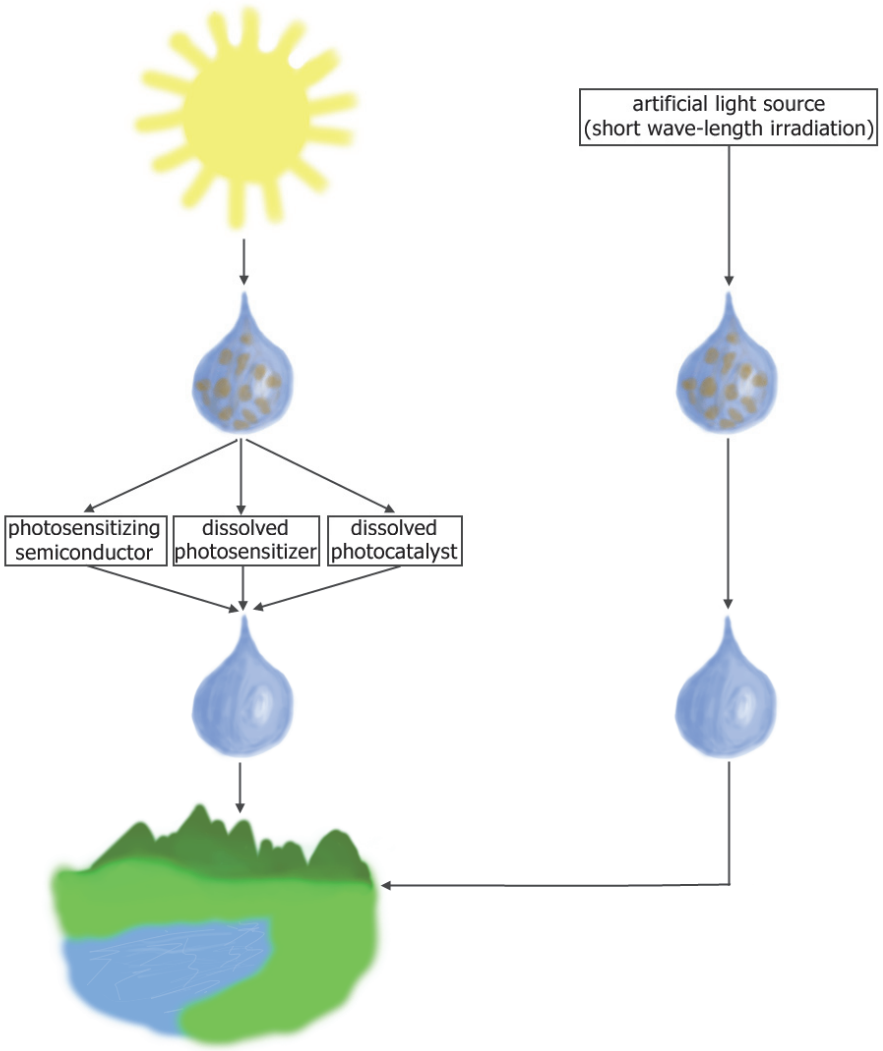

Fig. 3. Scheme of possible degradation pathways of a pollutant non-absorbing solar radiation.

In order to prove the photocatalytic mechanism of the degradation in the triazine solutions, formation of Fe^{2+} ions was measured in the reaction system. The results are set out in Fig. 5.

	atrazine consumption (% of initial concentration)						
time of irrad. (minutes)	no added metal ions	Cu(II) $3.3*10^{-4}$ mol/l	Cu(II) $1.0*10^{-3}$ mol/l	Mn(II) $1.6*10^{-4}$ mol/l	Mn(II) $1.0*10^{-3}$ mol/l	Fe(III) $1.0*10^{-4}$ mol/l	Fe(III) $3.3*10^{-4}$ mol/l
0	0	0	0	0	0	0	0
30	0	6	8	1	7	30	97
60	0	8	12	4	8	64	98
90	0	14	15	6	9	98	99

Table 2. Degradation of atrazine in photoinitiated reaction in air saturated aqueous solution in the presence of metal ions. Initial concentration of atrazine $5.0*10^{-5}$ mol/l. Irradiation: Rayonet photochemical reactor RPR 100, lamps 3000Å, emission up to 290 nm filtered by optical glass. (From Klementová & Hamsová, 2000.)

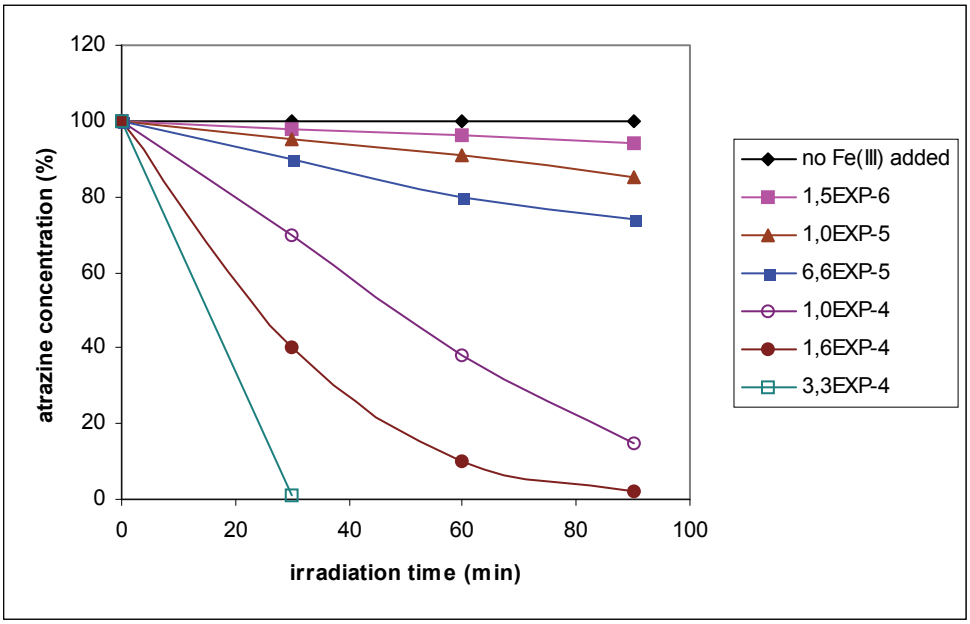

Fig. 4. Effect of ferric ions concentration on atrazine photochemical degradation (conditions of irradiation see Tab.2). Initial concentration of atrazine $5.0*10^{-5}$ mol/l. (From Klementová & Hamsová, 2000).

The photoreduction of ferric to ferrous ions occurs quickly under the irradiation of all three triazines, atrazine, propazine and simazine, though the reaction mixtures were saturated by the air. In the steady state, about 23% of added ferric ions are present in the reduced form in the reaction mixture of atrazine, about 70% in the reaction mixture of propazine, and nearly 90% in the reaction mixture of simazine.

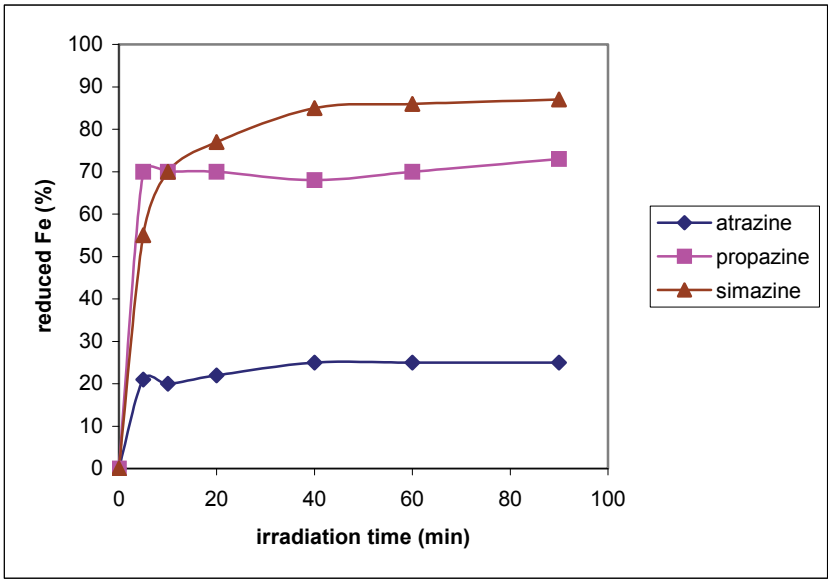

Fig. 5. Photochemical reduction of Fe(III) in the reaction systems with atrazine, propazine and simazine, resp., in the air saturated reaction mixtures. Concentration of substrates $5.0*10^{-5}$ mol/l, concentration of initial Fe^{3+} ions $1.0*10^{-4}$ mol/l. Conditions of irradiation – see Table 2. (From Klementová & Hamsová, 2000).

Homogeneous photocatalytic reactions in the presence of ferric ions may provide a possible pathway for the photochemical degradation of atrazine in water bodies; the problem being that the iron content in natural surface waters is about $1*10^{-5}$ mol/l, a relatively ineffective concentration for atrazine degradation. Other triazine derivatives, propazine and simazine, seem not to be affected by homogeneous photocatalytic degradation in the presence of the ions that are most abundant in natural waters (iron and manganese).

5.4 Heterogeneous photocatalytic degradation

There are no data on the heterogeneous photocatalytic degradation of herbicides with particulate matter in natural waters. Ample studies deal on the other hand with heterogeneous photochemical degradation in relation to semiconductors especially in the context of decontamination option for drinking water and in waste-water treatment.

Semiconductor photocatalysis uses solid catalytic systems where five discrete stages associated with conventional heterogeneous catalysis can be distinguished:

a. transfer of liquid or gaseous phase reactant to the catalytic surface by the diffusion;
b. adsorption of the reactant on the catalyst surface;
c. reaction of the adsorbed molecules;
d. desorption of products;
e. removal of products from the interface region by the diffusion.

The photocatalytic reaction occurs in the stage where the reactants are absorbed on the catalyst surface, the activation of the reaction being photonic activation. The semiconductor is activated by irradiation from a light source of appropriate wavelength depending on the band gap energy of the semiconductor. The activation generates a pair of charge carriers, a hole, h^+, and an electron, e^-; the charge carriers generated photochemically can react with molecules on the surface of the semiconductor (Eqs. 6 – 11 and Fig. 6).

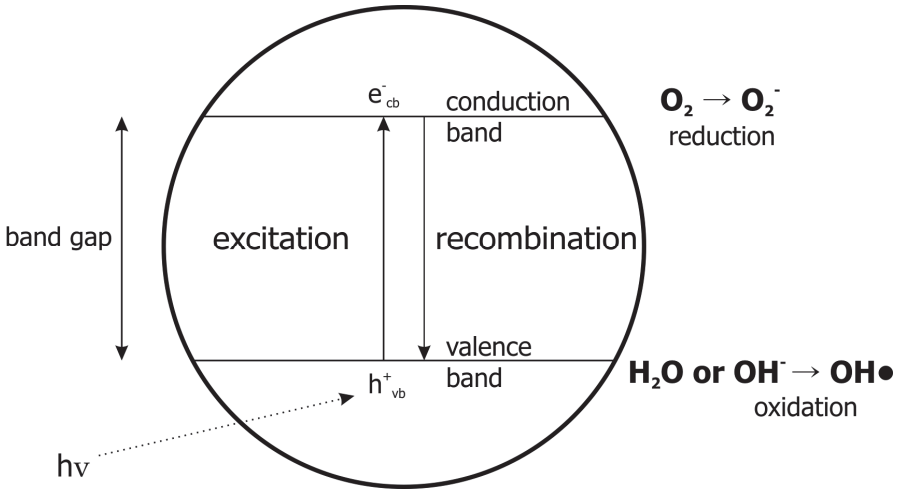

Fig. 6. Scheme of oxidative species production on semiconductors under irradiation.

Various metal oxides, e.g. TiO_2 (Hashimoto et al, 2005; Héquet et al., 2001; Konstantinou et al., 2001a; Linsebigler et al., 1995; Pelizzetti et al, 1990; Penuela& Barceló, 2000) ZnO (Byrappa et al., 2006), CeO_2 (Yongging Zha et al., 2007), ZrO_2 (Bota et al., 1999), WO_3 (Guo et al., 2007) and many other composites of semiconductors or doped semiconductors have been used as catalysts in semiconductor photocatalytic reactions (e.g. Dunliang et al. , 2009).

TiO_2 – the most widely used semiconductor in contaminant photocatalysis – occurs in three distinct polymorphs: anatase, rutile and brookite. Of these three forms only anatase is functional as a photocatalyst. Anatase is a typical n-type semiconductor with a band gap of about 3.2 eV. Photons with a wavelength shorter then 385 nm have enough energy to excite electrons from the valence band to the conduction band of this material. Since the 1970s, anatase has been a popular choice as semiconductor photocatalyst in research efforts because it is non-toxic and mechanically stable, has high photo-activity and low cost, and exhibits a reasonable overlap with the ultra-violet portion of the solar spectrum which makes it attractive for solar applications. Up to now a multitude of compounds have been investigated as target pollutants in photocatalytic oxidation studies on TiO_2. The studies have been performed at bench scale using small reactors operating as batch or flow reactor systems. Besides pollutant degradation successful tests for the treatment of bacteria, viruses, fungi, and tumor cells have been reported. Construction materials coated with TiO_2 exhibit self-cleaning properties (Devilliers, 2006).

Triazine herbicides photocatalytic degradation on TiO_2 has been studied by several authors, e.g. Héquet et al., 2001; Konstantinou et al., 2001;Pelizzetti et al., 1990; Penueala & Barceló,

2000), in some cases with the addition of oxidative species such as hydrogen peroxide or photo-Fenton system, H_2O_2/Fe(III), providing hydroxyl radicals. Atrazine was found to be degraded to desethylatrazine and desisopropylatrazine, i. e. the same compounds that are metabolites of biodegradation. These metabolites are not easily further degraded in the photocatalytic process on TiO_2.

In our group (Klementová, 2011), we compared the degradation of atrazine in the homogeneous photocatalytic reaction in the presence of Fe (III) and the photocatalytic degradation on TiO_2 (batch experiment, glass coated with TiO_2, irradiation by Philips TLD 15 W 08 lamps). The reaction constant of the heterogeneous photocatalytic reaction (0.018 min^{-1}) was comparable with the reaction constant in reaction mixtures with higher concentrations of ferric ions (0.021 min^{-1} for Fe(III) concentration $1.4*10^{-4}$ mol/l).

The degradation of phenylurea herbicides on TiO_2 has been studied e.g. by Amorisco et al.(2006), Haque et al. (2006) and Lhomme et al. (2005). The results of such studies show the importance of operational conditions (adsorption capacity, initial concentrations chlorotoluron, TiO_2 forms – coated or in suspension (Lhomme et al., 2005). The pathway of chlorotoluron degradation contained a substitution of chloride ion by the hydroxyl group on the aromatic ring, the demethylation of N group on the side chain, and in some cases a breaking down of the aromatic ring was observed.

Heterogeneous photocatalysis may represent a feasible pathway for the degradation of herbicides in waste-water treatment or even drinking water treatment, especially under conditions where the aromatic ring structure is broken down.

5.5 Photosensitized reactions

Photosensitized reactions may proceed in natural waters in the presence of natural sensitizers such as humic substances. Humic substances originate from the decay of plant and animal biomass and humification reactions in the decaying material. The molecules of humic substances are of variable structure and size (molecular weight ranging from several hundreds to several hundreds of thousands). Humic substances are classified into three operational classes:

- humic acids, which are non-soluble under low pH values,
- fulvic acids, which are soluble at all pH values,
- humins, which is the insoluble fraction.

Humic acids and fulvic acids have an acidic character due to their substential content of carboxylic and phenolic functional groups (Schnitzer & Khan, 1972); Dojlido & Best, 1993). The basic structural features of humic and fulvic acids are shown in Fig. 7.

Humic and fulvic acids have featureless absorption spectra with increasing absorption from the short-wavelengths of visible light through the ultraviolet radiation range.

Photosensitizing properties resulting in the production of singlet oxygen molecules ($^1O_2$), superoxide anions (O_2^-), hydroxyl radicals ($HO^\bullet$), peroxyradicals ($ROO^\bullet$), and hydrated electrons (e_{aq}^-) have been well established (Cooper et al., 1989; Hoigné et al., 1989; Mill T., 1989; Simmons & Zepp, 1986).

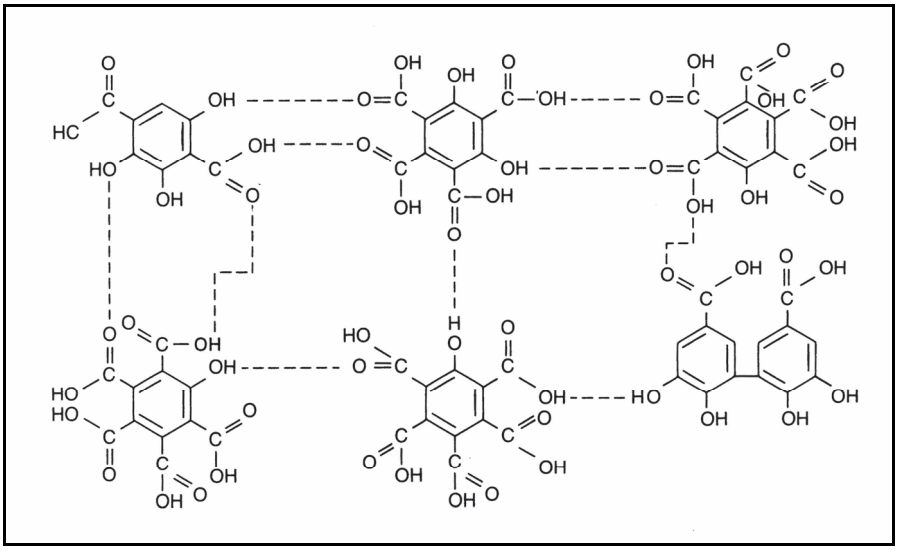

Fig. 7. Structure of fulvic acids. (From Dojlido & Best, 1993).

The photosensitized degradation of triazine and phenylurea herbicide in the presence of humic substances has been studied by several authors, e.g. Amine-Khodja et al. (2006); Comber (1999); Gerecke et al. (2001); Klementova & Piskova (2005); Konstantinou et al. (2001b), Minero et al. (1992) and Schmitt et al. (1995). The results suggest that there is no unambiguous answer about the influence of humic substances. Some authors report better degradation of the substrates, other report decrease in reaction rates in the presence of humic substances. The explanation probably lies in the combination of absorption characteristics of humic samples, their concentrations and the light sources used in the studies. In concentrated humic waters, inner filtration (i. e. the absorption of a significant part of the radiation energy by the photosensitively inactive parts of humic molecules) may play an important role and cause a decrease in the reaction rate of degradation. The heterogeneous chemical character of humic fractions may also be responsible for the variable photosensitizing activities of individual humic samples.

Two groups of artificial sensitizers which provide defined oxidative species were studied in our group for triazine and triazine metabolite degradation: phthalocyanines, i.e. photosensitizers providing singlet oxygen, and anthraquinonesulfonate causing formation of superoxide anions (Klementová & Hamsová, 2000). To our surprise phthalocyanines (aluminium-chloro-phthalocynanine-disulfonate and zinc-phthalocyanine-trisulfonate) showed no observable effect. Anthraquinonesulfonate presence in the aqueous solutions of triazine herbicides (atrazine, propazine, simazine) and the two of atrazine metabolites (desethylatrazine and desisopropylatrazine) resulted in a relatively swift degradation (Fig. 8). Anthraquinonesulfonate was repeatedly added to the reaction mixtures since its molecules are degraded by UV light. This result suggests that triazine herbicides are readily degradable by superoxide species. Nevertheless, the aromatic ring is not broken down so the decomposition is incomplete as it is in other sensitized and catalyzed reactions.

5.6 Photolytic degradation by short-wavelength radiation

Direct photolytic degradation is a decomposition that follows the absorption of a photon (and therefore a rearrangement in the electron density distribution of the molecule in the excited state). The reaction includes only one reactant, i.e. the molecule that undergoes photolysis. The products of a photolytic splitting may undergo another photolytic decompositon if the radiation is of a suitable wavelength. The reaction follows the first order kinetics scheme (Eq. 12).

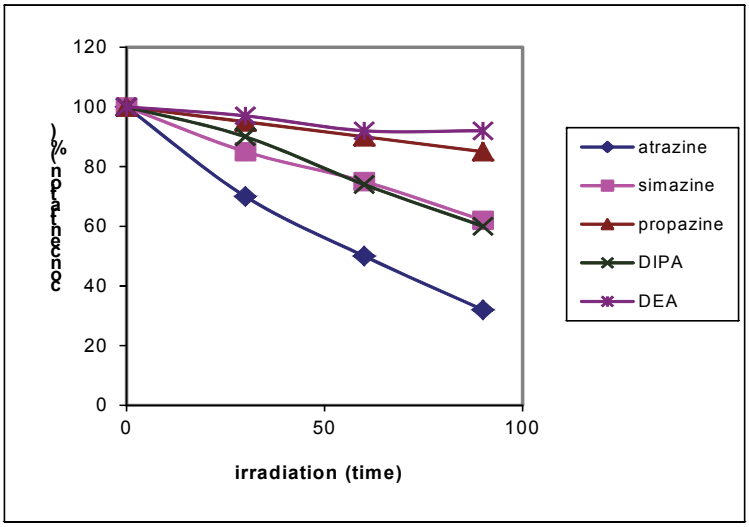

Fig. 8. Photosensitized degradation of triazine herbicides atrazine, simazine and propazine, and atrazine metabolites desethylatrazine (DEA) and desisopropylatrazine (DIPA) with anthraquinone sulfonate as the sensitizer. Initial concentration of individual substrates: $5.0*10^{-5}$ mol/l. Concentration of anthraquinonesulphonate after addition: $1*10^{-4}$ mol/l, addiotions each 30 minutes. Irradiation: Rayonet photochemical reactor RPR 100, lamps 3000Å, emission up to 290 nm filtered by optical glass. (From Klementová & Hamsová, 2000).

$$A \rightarrow B \tag{12}$$

To achieve a photolytic decomposition highly energetic radiation is necessary. Usually a low pressure mercury lamp (emitting most radiation energy at the 254 nm wavelength) is used in these experiments. It is therefore obvious that such processes cannot contribute to herbicide degradation on the Earth´s surface, but have their potential in waste-water and drinking water treatment.

Photolytic degradation of triazine and phenylurea herbicides has been studied by several authors. Frimmel & Hessler (1994) irradiated atrazine, desethyatrazine and simazine by low pressure mercury lamp. The rate constants of individual reaction were identical ($1.9*10^{-4}$ s^{-1}). Palm & Zetzsch (1996) carried out kinetic experiments with atrazine, propazine and simazine irradiated by xenon lamp in quartz vessels. Their kinetic evalutation gave the rate constants similar to those calculated by Frimmel & Hessler (1994); slightly higher rate constants and differing for the individual substrates studied were gained by Klementová & Píšková (2005) who irradiated atrazine, simazine, propazine, desethylatrazine and desisopropylatrezine by RPR 3000Å lamps (wavelength range 250 – 350 nm) – see Table 3.

triazine	atrazine	propazine	simazine	DEA	DIPA
rate constant (s^{-1})	$4.64*10^{-4}$	$4.35*10^{-4}$	$5.45*10^{-4}$	$5.86*10^{-4}$	$6.33*10^{-4}$

Table 3. First-order kinetics rate constant for photolytic UV degradation (lamps RPR 3000Å) of triazine and triazine derivatives. DEA – desethylatrazine; DIPA – desisopropylatrazine.

Phenylurea herbicides UV photolysis has been studied e.g. by Benitez et al. (2006) for chlorotoluron, diuron, isoproturon, and by Klementová & Zemanová (2008) for chlorotoluron. Benitez et al. (2006) reported a dependence of the reaction rate on the pH value of the solution; the results published by Klementova & Zemanová (2008) did not support the reported pH dependence, the degradation was pH independent in the range of pH values from 2 to 11.

Measuring the content of dissolved organic carbon (DOC) by DOC analyzer revealed that photolysis in solutions saturated with air results in the partial mineralization of organic substrates, i.e. decomposition of the organic carbon into CO_2. About 20 % of organic carbon was mineralized in 90 minutes of irradiation.

Photolytic degradation by short-wavelength radiation therefore apparently represents a powerful tool for herbicides degradation in waste-water and drinking water treatment, since it leads to total decomposition of organic matter.

5.7 Photochemical degradation of triazine and phenylurea herbicides – common features

In all cases where photochemical degradation was observed in our experiments, the initial step of the degradation of the triazine and phenylurea herbicides and triazine herbicide metabolites was dechlorination and hydroxyderivative formation. Chlorine was found in the solution as chloride ions, Cl^-, that were detected in the reaction mixtures by ion chromatography. Hydroxyderivatives were detected by high performance liquid chromatography with a mass spectrometer as an analyzer. Fig. 9 shows one example of herbicide (chlorotolurone) degradation, and chloride ions and hydroxyderivative formation. In this case, as well as in the case of other triazine substrates, the plots of the substrate decomposition and the chloride formation are perfectly symmetrical. Hydroxyderivatives are intermediates that decompose further with a reaction rate constant nearly equal to that of the original substrate decomposition.

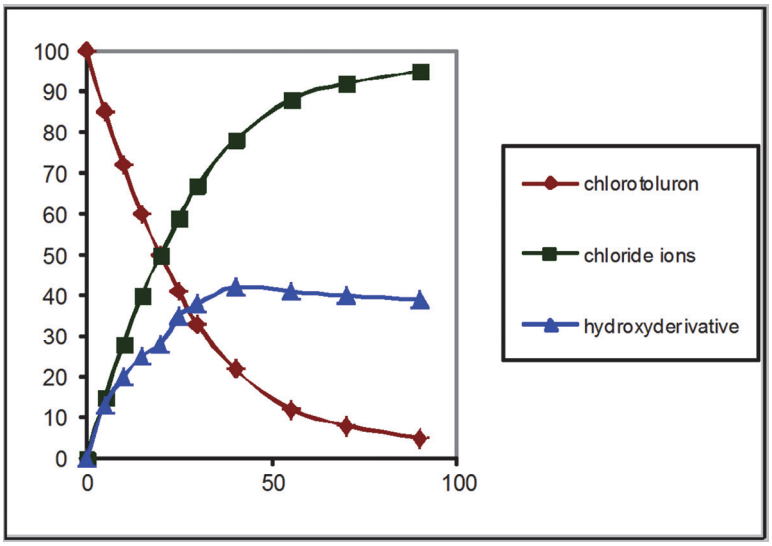

Fig. 9. Chloride ion release and hydroxyderivative formation in chlorotoluron photodecomposition.

6. Conclusions

In order to summarize the findings presented in this chapter on the photoinitiated degradation of triazine and phenylurea herbicides we can conclude:

- Direct sunlight photodegradation cannot proceed in natural surface waters since the substrates absorption maxima do not correspond to the solar action spectrum.
- In most cases natural (humic) sensitizers do not seem to have significant effects on degradation of the substrates. If the concentration of humic sensitizers is low, only a small amount of reactive oxidative species is formed and the degradation is ineffective. If the concentration of humic sensitizers is high, they absorb a lot of radiation themselves, thus radiation is reduced due to inner filtration and cannot reach molecules under the thin surface layer.
- Some artificial sensitizers cause herbicide degradation, but their application in waste-water and drinking water treatment cannot be expected; such sensitizers are expensive for other than small scale laboratory experiments and they themselves together with their degradation products would contaminate the water to which they were applied.
- Homogenous photocatalytic degradation seems to be able to contribute to photodegradation of the substrates in the natural water environment, the typical iron concentrations in natural waters are however not sufficient to bring about a significant conversion of the substrates.
- Heterogeneous photocatalysis with immobilised semiconductors and photolysis remain the only potentially helpful methods forthe removal of the recalcitrant herbicides from waste-waters and perhaps even from contaminated drinking waters. The obstacles connected with the use of these two approaches on a larger scale arise from the three-dimensional nature of water purification: in assuring the delivery of sufficient amounts

of light energy to enable purification of higher columns of solutions. With heterogenous photocatalysis the three-dimensionality has one more aspect: the photocatalytic reaction on a semiconductor is a surface process, thus the reactant must be captured by the photocatalyst surface.

- With all processess demanding artificial irradiation the cost of lamps and energy must be taken into consideration.

Nevertheless, environmental pollution including water and soil pollution with herbicides is an increasingly grave problem, and with herbicides resistent to biodegradation and persisting for a long time in the environment the possibilities of photochemical degradation will not cease to attract attention. The possibilities for further development are open especially in the area of heterogeneous photocatalysis. An important key to success will be the utilisation of nano-sized photocatalyst powders dispersed on substrates with extremely large surface areas. Another approach is the modification of TiO_2 to make it sensitive to visible light. So far the researchers investigating in this field are struggling with the issue of low reproducibility and chemical stability, nonetheless heterogeneous photocatalysis represents a promising prospect for 21 century.

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17

Oxidative Stress as a Possible Mechanism of Toxicity of the Herbicide 2,4-Dichlorophenoxyacetic Acid (2,4-D)

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1. Introduction

Chlorophenoxy herbicides are widely used in agriculture and forestry, for the control of broad-leaved weeds in pastures, cereal crops, as well as along public rights of way. Structurally, these herbicides consist of a simple aliphatic carboxylic acid moiety attached to a chlorine-substituted aromatic ring via an ether linkage. One of the most commonly used herbicides of this type is 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 1). In congruence with the similitude between its molecular structure and that of the plant hormone indole-acetic acid, 2,4-D acts as a plant growth regulator that can interfere with normal hormonal action and plant growth (Munro et al., 1992).

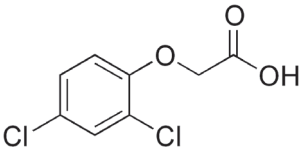

Fig. 1. Structure of the 2,4-Dichlorophenoxyacetic Acid.

2.4-D was synthesized for the first time in 1941 and commercially marketed in the United States (U.S.) in 1944 (IARC, 1986) and worldwide since 1950 (Munro et al. 1992). The widespread use of 2,4-D as a domestic herbicide and as a component of Orange Agent encouraged the study of its toxicity.

Human exposure to chlorophenoxy herbicides may occur through inhalation, skin contact or ingestion. The predominant route for occupational exposure to 2,4-D has been the absorption of spills or aerosol droplets through the skin.

Several studies have shown that doses of 50, 70 or 100 mg/kg body weight (bw)/day of 2,4-D produce a wide range of toxic effects on the embryo and on the reproductive and neural

systems in animal (mostly rat) and human models (Rosso et al., 2000; Barnekow et al., 2001; Charles et al., 2001). Doses of 50 mg/kg bw/day of 2,4-D have been reported to increase ventral prostate weight in rats. Treatment of human prostate cancer cell cultures with 10 nM 2,4-D enhanced the androgenic activity of dihydroxytestosterone (DHT) on cell proliferation and transactivation (Kim et al., 2005). In cultured chinese-hamster ovary cells, 2.0 to 10.0 µg/ml 2,4-D were reported to produce DNA damage and sister chromatid exchange (Gonzalez et al., 2005). Importantly, although the 2,4-D toxicity in low doses is controversial, the U.S. Environmental Protection Agency (U.S. EPA, 2006) established a LD50 of 639 mg/kg based on rat studies.

There could be particular situations in which the susceptibility of a population exposed to environmental pollutants can be dangerously enhanced. This may be the case for many rural populations subjected to some specific nutritional deficiencies, as often observed in developing countries. Such situation may be worthy of attention during the development stage, especially concerning the endocrine and nervous systems.

It has been recently found that 2,4-D administered to lactating rats can pass to suckling pups, an can also inhibit the suckling-induced hormone release in the mother. Thus, gestational and lactational periods –including the neonatal and prepubertal stages– seem to be particularly favorable for the induction of 2,4-D effects in rodents (Stürtz et al., 2000; 2006).

2. Adverse effects on developing nervous system

In human studies, prenatal exposure to 2,4-D was associated with mental retardation of the children (Casey, 1984). Comparable animal experiments in chicken and rats showed that prenatal exposure altered some behavioral patterns of the offspring (Sanders & Rogers, 1981; Sjoden & Soderberg, 1972).

In the rat, one critical period for normal maturation during growth seems to be that corresponding to the perinatal development of the brain—''the brain growth spurt''—spanning the first 3 or 4 weeks of life (Diaz & Samson, 1980). Therefore, exposure of rats to pesticides during the first weeks of life would have adverse effects on growth and behavior, as well as on the locomotor activity, as affected by anatomical changes. Noteworthy, the age at exposure is an important factor (Kolb & Wishaw, 1989).

This selective susceptibility of the developing nervous system may be due to several toxicokinetic factors and a partial lack of a blood–brain barrier (BBB) in the fetus. In humans, the BBB is not fully developed until the middle of the first year of life (Rodier, 1995).

Gupta et al. (1999) have shown that different classes of pesticides are able to change the permeability characteristics of the BBB in rats when administered during some susceptible periods of the BBB development, and that this effect may persist after exposure for variable periods. An altered BBB may render the nervous system more vulnerable to other toxics that would not be able to pass the BBB otherwise.

Therefore, although the developing nervous system has some capacity to adapt to or compensate for early perturbations, many chemical agents have been shown more toxic on the developing than on the adult nervous system (Tilson, 1998).

In the last two decades many different alterations have been reported in neonatal rats exposed to 2,4-D through breast milk, at a dose producing no overt signs of toxicity in dams. Alterations in astroglial cytoarchitecture and neuronal function (Brusco et al., 1997) as well as neuro-behavioral changes were observed in pups and adult rats after an early exposition to the herbicide (Bortolozzi et al., 1999, 2001). Other reported effects in neonate rats were a deficit in myelin lipid deposition (Konjuh et al., 2008) and changes in the ganglioside pattern in some brain regions (Rosso et al., 2000).

2.1 Metals and monoamines levels

Studies in well-fed or undernourished rat offsprings showed that the mechanisms for the induction of the above effects would include some changes in brain monoaminergic system (Ferri et al., 2000) and in iron (Fe), copper (Cu) and zinc (Zn) brain levels (Ferri et al., 2003).

Importantly, the combination of neonatal undernourishment plus mothers' exposure at 2,4-D low dose (70 mg/kg bw) induced a higher modification of the measured parameters than those induced by undernourishment or 2,4-D exposure alone. The data showed a different pup's brain areas susceptibility to the 2,4-D effects and an increased vulnerability to the herbicide, including an increased mortality at a higher dose (100 mg/kg bw), a feature which was not observed in well-nourished animals.

In addition, the results suggest that malnutrition or exposure to 2,4-D exert their effects independently (Tables 1 & 2) (Ferri et al., 2003) and the fact that the alterations observed are very different according to the area involved, reinforces the idea of a selective susceptibility for each brain region.

2.2 Oxidative stress

Different studies suggest some functional relationships between the oxidative status of the Central Nervous System (CNS) and the protecting level of catecholamines (Kumiko et al., 2001) and metals, like Fe and Cu, the major generators of reactive oxygen species –ROS- in Alzheimer's disease (Huang et al., 1999), related with a decreased glutathione (GSH) content (Dringer, 2000) and also involved in Fenton's and Haber Weiss' redox reactions . (Halliwell & Gutteridge, 1998; Milton, 2004). Other data have shown that 2,4-D affects the redox chain, thus altering cell energetic metabolism and redox balance (Palmeira et al., 1994; Sulik et al., 1998; Bukowska et al., 2003; Duchnowicz et al., 2002).

In rat pups, exposure to 2,4-D through breast milk induced a number of changes in different brain areas, such as disparate changes in the activity of some protective enzymes, an increase in reactive oxygen species (ROS) levels, and a depletion of reduced glutathione (GSH) content (Tables 3, 4 & 5, respectively) (Ferri et al., 2007).

Therefore, as long as a high oxygen consumption by the CNS increases its sensitivity to oxidative stress (Emerit et al., 2004), the observed changes in the levels of metal ions and neurotransmitters, particularly catecholamines, as well as the oxidative status imbalance, would point out oxidative stress as one possible mechanism of adverse 2,4-D effects on the CNS.

AREA	Treatment	NE	DA	DOPAC	HVA	TRP	5-HT	5-HIAA
PFc	**DMSO**	0.93 ± 0.04	3.20 ± 0.44	0.97 ± 0.09	0.28 ± 0.03	20.71 ± 0.61	1.07 ± 0.13	1.08 ± 0.07
	2,4-D	1.10 ± 0.06* (↑20%)	2.01 ± 0.30* (↓37%)	0.90 ± 0.17	0.25 ± 0.03	11.26 ± 0.51** (↓46%)	1.48 ± 0.09* (↑38%)	1.03± 0.05
Str	**DMSO**	4.24 ± 0.42	20.79 ± 1.61	9.37 ± 0.48	3.19 ± 0.12	27.46 ± 1.61	2.98 ± 0.31	2.84 ± 0.29
	2,4-D	2.36 ± 0.44* (↓44%)	17.03 ± 3.31	6.96 ± 0.69** (↓26%)	2.05 ± 0.32** (↓36%)	28.06 ± 1.76	1.85 ± 0.25* (↓38%)	2.81 ± 0.42
Hipp	**DMSO**	0.91 ± 0.12	0.70 ± 0.09	0.44 ± 0.07	0.30 ± 0.04	5.55 ± 0.35	0.74 ± 0.09	1.63 ± 0.11
	2,4-D	1.67 ± 0.24* (↑83%)	0.92 ± 0.09* (↑31%%)	0.58 ± 0.06* (↑31%)	0.50 ± 0.05* (↑66%)	3.68 ± 0.20** (↓34%)	1.10 ± 0.13* (↑49%)	1.75 ± 0.10
Hyp	**DMSO**	9.05 ± 1.19	1.58 ± 0.35	1.46 ± 0.21	1.08 ± 0.20	3.81 ± 0.26	1.52 ± 0.13	3.09 ± 0.59
	2,4-D	13.57 ± 1.44* (↑50%)	1.80 ± 0.33	1.03 ± 0.17	1.14 ± 0.18	2.96 ± 0.35	2.08 ± 0.31	2.68 ± 0.26
MB	**DMSO**	3.16 ± 0.57	1.54 ± 0.31	0.65 ± 0.14	0.36 ± 0.06	31.88 ± 1.21	2.79 ± 0.21	3.54 ± 0.40
	2,4-D	3.96 ± 0.17	1.96 ± 0.17	0.78 ± 0.12	0.24 ± 0.02	23.34 ± 0.97** (↓27%)	4.14 ± 0.19** (↑48%)	4.78 ± 0.37* (↑35%)
Cereb	**DMSO**	1.58 ± 0.12	0.17 ± 0.06	0.31 ± 0.01	0.09 ± 0.01	7.39 ± 0.55	0.46 ± 0.03	0.48 ± 0.03
	2,4-D	2.44 ± 0.08** (↑54%)	0.21 ± 0.04	0.29 ± 0.01	0.13 ± 0.01	4.86 ± 0.21** (↓34%)	0.43 ± 0.04	0.43 ± 0.02

Monoamine content is expressed as pMol/mg of tissue. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value.*p < 0.05; **p < 0.01; n= 6/group; 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex), Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), NE (Norepinephrine), DA (Dopamine), DOPAC (3,4-Dihydroxyphenylacetic acid), HVA (Homovanillic Acid), TRP (Tryptophan), 5-HT (Serotonin) and 5-5-HIAA (Hydroxyindoleacetic acid); other abbreviations as indicated in the text.

Table 1. Monoamine levels in different brain areas of 25-day-old, 2,4-D-expossed pups.

Metal	Treatment	PFc	Str	Cereb	Hipp	MB	Hyp
Fe	**DMSO**	19.58 ± 2.36	17.07 ± 0.90	16.43 ± 1.27	14.23 ± 0.73	18.79 ± 2.03	19.48 ± 2.25
	2,4-D 70 mg/kg	24.48 ± 1.83* (↑25.05 %)	16.65 ± 2.65	17.53 ± 0.86	12.86 ± 1.20	15.54 ± 0.55	20.95 ± 1.76
	2,4-D 100 mg/kg	29.48 ± 2.19* (↑50.56 %)	15.75 ± 2.65	20.26 ± 0.68* (↑23.31%)	13.98 ± 1.80	14.95 ± 0.35	21.31 ± 2.68
Zn	**DMSO**	12.51 ± 1.20	34.10 ± 2.40	24.40 ± 1.90	25.40 ± 2.10	32.65 ± 1.10	29.80 ± 3.40
	2,4-D 70 mg/kg	14.64 ± 2.20	28.63 ± 2.40* (↓16.04 %)	21.50 ± 0.85	27.89 ± 1.60* (↑9.80%)	29.55 ± 1.74	27.11 ± 2.56
	2,4-D 100 mg/kg	17.93 ± 2.00* (↑43.32%)	13.10 ± 2.00** (↓61.58%)	24.40 ± 0.70	34.30 ± 3.50* (↑35.04%)	24.35 ± 1.54** (↓25.42%)	26.37 ± 3.20
Cu	**DMSO**	1.85 ± 0.08	2.25 ± 0.11	1.88 ± 0.07	1.84 ± 0.02	2.21 ± 0.19	1.95 ± 0.08
	2,4-D 70 mg/kg	1.97 ± 0.18	2.17 ± 0.13	2.00 ± 0.10	2.01 ± 0.20	2.16 ± 0.21	1.91 ± 0.16
	2,4-D 100 mg/kg	2.31 ± 0.21* (↑ 24.86%)	2.38 ± 0.21	2.20 ± 0.08** (↑17.02%)	2.23 ± 0.17* (↑21.19%)	2.14 ± 0.20	1.91 ± 0.15

Metal contents are expressed as micrograms per gram of wet tissue. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value. *p < 0.05 with reference to DMSO control values. **p <0 .01 with reference to DMSO control values; n= 6/group. 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex, Str (Striatum), Cereb (Cerebellum), Hipp (Hippocampus), MB (Midbarin), Hyp (Hypothalamus), and other abbreviations as in the text.

Table 2. Effects of 2,4-D on iron, zinc and copper levels in different brain areas of well-nourished pups.

Enzime	Treatment	Brain	PFc	Str	Cereb	Hipp	MB	Hyp
Cu,Zn-SOD	DMSO	2330 ± 119	1950 ± 200	2460 ± 150	2730 ± 330	2330 ± 200	1950 ± 120	2320 ± 160
	2,4-D	2400 ± 93	2450 ± 310* (↑ 25.6%)	2540 ± 220	2610 ± 240	2980 ± 320* (↑ 27.9%)	2140 ± 90	2400 ± 120
Mn-SOD	DMSO	250 ± 110	250 ± 80	310 ± 120	320 ± 130	270 ± 90	240 ± 120	370 ± 140
	2,4-D	284 ± 135	150 ± 70	280 ± 100	280 ± 90	390 ± 60	290 ± 110	350 ± 110
CAT	DMSO	2556 ± 150	2950 ± 250	2740 ± 200	2300 ± 130	2580 ± 160	2360 ± 200	2740 ± 150
	2,4-D	1978 ± 133* (↓ 22.5%)	2200 ± 200* (↓ 25.4%)	2250 ± 150* (↓ 17.9%)	2530 ± 170	2690 ± 210	1850 ± 120* (↓ 21.6%)	2810 ± 210
Se-GPx	DMSO	31.52 ± 1.24	30.00 ± 1.43	28.19 ± 2.10	25.62 ± 9.10	29.05 ± 1.14	31.93 ± 1.06	28.89 ± 1.85
	2,4-D	26.76 ± 1.14* (↓ 15.10%)	24.19 ± 1.90* (↓ 19.4%)	22.29 ± 2.86* (↓ 20.9%)	29.52 ± 2.10* (↑ 15.2%)	32.29 ± 1.00* (↑ 11.1%)	27.57 ± 1.03* (↓ 13.6%)	27.97 ± 1.56
noSe-GPx	DMSO	17.71 ± 0.69	20.80 ± 1.08	18.55 ± 0.62	18.18 ± 0.98	20.94 ± 0.69	22.65 ± 0.77	15.81 ± 0.69
	2,4-D	20.32 ± 0.94* (↑ 14.7%)	18.99 ± 0.99	15.65 ± 0.46* (↓ 15.6%)	17.95 ± 1.05	19.90 ± 0.87	19.35 ± 0.82* (↓ 14.6%)	17.01 ± 0.71

Enzyme activities are expresed as miliUnits per miligram of protein. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value. *p < 0.05, n= 6/group. 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex, Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), Cu,Zn-SOD (Copper,Zinc superoxide dismutase), Mn-SOS (Manganese superoxide dismutase), CAT (catalase), Se-GPx (selenium-glutathione peroxidase), noSe-GPx (non selenium-glutathione peroxidase),and other abbreviations as in the text

Table 3. Protective Enzymes Activities in brain areas of 25-old-day pups lactationally exposed to 2,4-D.

	Brain	PFc	Str	Cereb	Hipp	MB	Hyp
DMSO	45.1±2.5	17.8±0.7	22.6±0.8	24.0±0.9	18.0±1.0	20.3± 1.0	22.6±1.2
2,4-D	38.0±1.4* (↓ 15.7%)	20.6±0.5* (↑ 15.7%)	25.1±0.7* (↑ 11.1%)	23.7±1.1	18.1±1.1	23.8±0.7* (↑ 17.2%)	21.1±1.3

ROS levels are expresed as IF per mg of protein. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value.; *p < 0.05, n= 6/group. 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex, Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), other abbreviations as in the text.

Table 4. ROS levels in brain areas of 25-old-day pups lactationally exposed to 2,4-D.

	Brain	PFc	Str	Cereb	Hipp	MB	Hyp
DMSO	1.22±0.40	1.23±0.29	1.31±0.24	0.79±0,19	0.88±0.19	1.06±0.12	1.07±0.41
2,4-D	1.25±0.38	1.29±0.20	0.82±0.18* (↓ 37.4%)	0.80±0.22	0.94±0.24	0.70±0.15* (↓ 34.0%)	1.08±0.30

GSH levels are expresed as microgram per miligram of protein. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value. *p < 0.05, n= 6/group. 100 mg 2,4-D/kg bw of mother. PFc (Pre frontal cortex, Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), other abbreviations as in the text.

Table 5. GSH levels in brain areas of 25-old-day pups lactationally exposed to 2,4-D.

3. Prostate, ovary and breast

The endocrine system of many vertebrate embryos seems to be particularly susceptible to a variety of substances or either natural or anthropogenic origin, including pesticides (Crews et al., 2000). However, there are few studies on developmental toxicology that focus on the 2,4-D's effects on hormone-sensitive organs such as the prostate, ovary and breast.

Free radicals are associated with oxidative stress and are also thought to play some significant roles in reproduction. Induction of oxidative stress by many environmental contaminants—such as pesticides—has also been pointed out during the last decade as a possible mechanism of some toxic effects on the reproductive system (Bagchi et al., 1992; Abdollahi et al., 2004). It is already known that reproductive cells and tissues will remain stable only when antioxidant and oxidant status are in balance (Lee et al., 2010). ROS levels are a double-edged sword, as long as they not only serve as key signal molecules in physiological processes, but also have a role in pathological processes involving the female reproductive tract (Agarwal et al., 2005).

On the other hand, there are diverse environmental chemical contaminants which can be potentially harmful to the mammary gland in association with estrogens. Oxidative catabolism of both estrogen and those compounds, a mechanism mediated by the same enzymes, generates reactive free radicals that can cause oxidative damage. Xenobiotic chemicals may exert their pathological effects through generation of reactive free radicals (Mukherjee et al., 2006).

There is growing evidence that free radicals can exert a wide spectrum of deleterious effects on the reproductive system and asocciated glands (Saradha et al., 2008). Thus, Pochettino et al. (2010) investigated the effect of 2,4-D on oxidative stress and antioxidative system and on some hormone-sensitive organs such as ventral prostate, ovaries and breasts, exposed to the herbicide during the pre- and the postnatal period, as described next (Pochettino et al., 2010).

3.1 Prostate

In rat ventral prostate, 2,4-D caused oxidative stress during the whole development, through a significant increase in lipid peroxides, hydroxyl radical levels and protein oxidation. Morevover, the antioxidant enzyme activity was increased at any age, as shown for Glutathione S-transferase (GST), catalase (CAT) and selenium-glutathione peroxidase (Se-GPx), with the exception of Se-GPx administered at the 90th postnatal day (PND 90). Nevertheless, at PND 90 a reduced activity of Glutathione Reductase (GR) was detected (Table 6).

GST is relevant to detoxification of endogenous compounds and xenobiotic substances such as environmental pollutants, drugs, and natural toxins (Pietsch et al., 2001; Padros et al., 2003; Cazenave et al., 2006). Several studies have demonstrated that enhanced GST activity by ROS in the testis could represent an adaptative response to oxidative stress, probably targeted to achieve a detoxification of peroxide-containing metabolites (Kaur et al., 2006).

As far as the testis is intimately related to the prostate, this interpretation looks coherent with the observed ROS-induced increase in GST activity in the prostate.

		PND 45	PND 60	PND 90
Hydroxyl radical	**Control**	3.25±0.34	2.97±0.39	1.09±0.13
	2,4-D	8.75±0.61* (↑169%)	6.53±0.09* (↑119%)	2.03±0.18* (↑85%)
Carbonyl groups	**Control**	3.54±0.12	10.66±1.07	7.02±0.88
	2,4-D	4.84±0.11* (↑37%)	15.01±1.32* (↑47%)	12.42±1.11* (↑77%)
Total Thiols	**Control**	491±12	642±86	341±24
	2,4-D	520±14	748±14	333±8
MDA	**Control**	29.09±0.32	27.74±3.74	38.27± 2.14
	2,4-D	41.91±3.05* (↑44%)	42.69±3.13* (↑54%)	47.48 ± 2.54* (↑24%)
GST	**Control**	8.93±0.67	10.53±2.53	13.37±2.09
	2,4-D	19.07±2,45* (↑113%)	15.95±1.04* (↑45)	18.14±0.26* (↑36)
CAT	**Control**	10.93±1,20	5.44±0.21	5.99±0.21
	2,4-D	14.74±1.26* (↑35%)	11.44±0.34* (↑110%)	7.77±0.39* (↑28%)
Se-GPx	**Control**	312±18	518±57	562±32
	2,4-D	436±33* (↑36)	880±41* (↑70%)	530±13
GR	**Control**	10.05±0.86	33.01±2.52	31.09±4.36
	2,4-D	10.47±1.78	37.75±3.48	10.57±0.05* (↓72%)

Hydroxy radical are expresed as 2,3 dihydroxybenzoic acid/salicilc acid rario; carbonyl groups and total thiols are expresed as micromol per miligram of protein; MDA is expresed as nanomol per microgram of protein. GST, CAT and GR activities are expresed as Units per miligram of protein; and Se-GPx is expresed as miliUnits per miligram of protein. Each value is the mean ± SEM. Values between brackets are % of increase (↑) or decrease (↓); *$p < 0.05$, n= 6/group. 70 mg 2,4-D/kg cw of mother. Abbreviations as in the text.

Table 6. Oxidative parameters in ventral prostate.

Therefore, the 2,4-D-induced increase in all ROS level, lipid peroxidation and protein oxidation may have caused some critical oxidative stress in ventral prostate. Nevertheless, the increased activity of some antioxidant enzymes in the prostate could have not been strong enough as to counteract the oxidative stress produced by the herbicide at different stages of rat development. Moreover, it is not a general rule that increase in oxidative species stimulates antioxidant activity (Celik & Tuluce, 2007).

3.2 Ovary

The complex ovarian structure varies widely during differentiation. Free radicals play important regulating roles during the ovarian follicular cycle, possibly through inhibition of steroid production (Behrman et al., 2001). There is also a delicate balance between ROS and antioxidant enzymes in the ovarian tissues (Agarwal et al., 2005). Non-physiological effects of free radicals include premature ovarian follicular atresia via cell apoptosis. Many pesticides— e.g. the xenoestrogen pesticide methoxychlor — can induce oxidative stress and apoptosis in the ovary (Gupta et al., 2006). Moreover, clinical studies have reported increased levels of reactive oxygen species associated to a decreased female fertility (Agarwal et al., 2006).

		PND 45	PND 60	PND 90
Hydroxyl radical	**Control**	3.65±0.26	1.89 ± 0.22	1.09 ± 0.13
	2,4-D	8.75±0.89	1.98 ± 0.13	4.35 ± 0.53* (↑93%)
Carbonyl groups	**Control**	14.77±2.75	5.74 ± 0.13	6.22 ± 0.94
	2,4-D	23.71±0.47* (↑60%)	8.80±0.72* (↑55%)	5.64±0.73
Total Thiols	**Control**	1462±162	672±24	519±38
	2,4-D	1360±176	676±39	537±22
MDA	**Control**	87.71±14.02	34.12±2.24	34.68±1.31
	2,4-D	192.5±17.8* (↑119%)	42.49±1.35* (↑24%)	39.39±0.89* (↑14%)
GST	**Control**	38.51±0.41	10.59±0.81	10.99±0.18
	2,4-D	25.15±1.37 (↓34.6%)	7.97±0.54* (↓24.7)	9.91±0.57
CAT	**Control**	42.89±3.14	27.86±1.08	16.08± 0.42
	2,4-D	43.41±0.67	15.34±0.43* (↓44.9%)	16.38±0.71
Se-GPx	**Control**	691±97	411±48	514±29
	2,4-D	1622±117* (↑135)	549±24* (↑33%)	593±23* (↑15%)
GR	**Control**	14.48±3.44	17.15±1.67	28.64±2.31
	2,4-D	12.67±2.61	16.88±1.45	19.62±1.75* (↓31%)

The parameters are expresed as in Table 7. Each value is the mean ± SEM. Values between brackets are % of increase (↑) or decrease (↓); *p < 0.05, n= 6/group. 70 mg 2,4-D/kg cw of mother. Abbreviations as in the text.

Table 7. Oxidative parameters in ovary.

On analyzing the 2,4-D toxic effects on the ovary, Pochettino et al. (2010) found an increase in lipid peroxide (LPO) evidenced by augmented levels of malondialdehyde (MDA) and decrease antioxidant enzyme activity. These effects differed with age, while an increase in Se-GPx activity was exceptionally observed at all ages (Table 7). These effects could reflect the natural diversity of rat ovarian cell types at different ages. Another explanation would be the well-known, protecting effect of estrogens against apoptosis and oxidative stress in a variety of tissues and cells (Spyridopoulos et al., 1997; Tomkinson et al., 1997; Garcia-Segura et al., 1998; Pelzer et al., 2000). Estrogens increase all ovarian weight, follicular growth, and the mitotic index of granulose cells, and also control granulosa cell apoptosis (Richards, et al., 1980; Bendell & Dorrington, 1991) and have exerted varied antioxidant effects (Chatterjee & Chatterjee 2009). Further studies are needed to analyze the time-course of the effects observed.

3.3 Breast

Pocchetino et al. (2010) observed that 2,4-D increased MDA levels at all ages (Table 8). It is known that MDA reflects the extent of oxidant status and is considered a good marker of oxidative stress (Wen et al., 2006). Both, singlet oxygen and hydroyl radicals have a high potential to initiate free-radical chain reactions in lipid peroxidation (Celik & Tuluce, 2007). As the hydroyl radical level was unchanged in that study, 2,4-D could have stimulated LPO by increasing singlet oxygen levels. In addition, 2,4-D inhibited the activity of anti-oxidative enzymes such as CAT, Se-GPx, GR and GST (Table 9).

		PND 45	PND 60	PND 90
Hydroxyl radical	**Control**	2.61±0.11	3.04±0.11	4.31±0.45
	2,4-D	2.65±0.44	3.49±0.52	4.49 ± 0.11
Carbonyl groups	**Control**	19.25±0.82	28.57±3.86	59.38±10.69
	2,4-D	21.34±5.47	23.31±5.49	57.37±14.89
Total Thiols	**Control**	942±5	1072±77	3551±757
	2,4-D	951±25	667±46* (↓62%)	1560±226* (↓56%)
MDA	**Control**	52.62±1.57	71.07±4.68	158.41±2.59
	2,4-D	70.65±7.48* (↑34%)	139.2±17.94* (↑96%)	217.8±18.95* (↑37%)
GST	**Control**	17.18±0.59	19.41±1.51	72.81±7.41
	2,4-D	10.41±1.91* (↓40%)	13.54±0.92*(↓30%)	32.35±5.98* (↓55%)
CAT	**Control**	59.38±3.03	137.62±10.73	358.21±36.31
	2,4-D	62.55±1.57	81.27 ± 2.55* (↓41%)	122.11±17.42* (↓66%)
Se-GPx	**Control**	538±44	1430±31	5596±1015
	2,4-D	198±19* (↓63%)	695±15* (↓51%)	2257±474* (↓60%)
GR	**Control**	15.94±0.91	90.75±5.51	228.81±14.31
	2,4-D	7.39±1.54* (↓53.6%)	60.02±9.05* (↓34%)	76.02±10.95* (↓67%)

The parameters are expresed as in Table 7. Each value is the mean ± SEM. Values between brackets are % of increase (↑) or decrease (↓); *p < 0.05, n= 6/group. 70 mg 2,4-D/kg cw of mother. Abbreviations as in the text.

Table 8. Oxidative parameters in breast

Therefore, the decreased activity of anti-oxidative enzymes may decrease the protection against oxidants (Amstad et al., 1991).

In that regard, Dimitrova et al. (1994) suggested that the superoxide radicals, either by themselves or after transformation to H_2O_2, stimulate cysteine oxidation and inhibit the activity of the enzymes. Furthermore, Regoli & Principato (1995) demonstrated that the flux of superoxide radicals inhibits CAT activity. Consequently, the decreased CAT activity might have reflected a flux of superoxide radicals promoted by 2,4-D. Moreover, GR also plays an important role in cellular antioxidant protection, catalyzing the reduction of glutathione disulfide (GSSG) to GSH (Kim et al., 2010).

Thus, the decrease in thiol groups could reflect GSH depletion in the breast. Therefore, 2,4-D produced oxidative imbalance, mainly during puberty and adulthood, probably because the gland is more sensitive to xenobiotics at these stages of development.

4. *In vitro* studies

It has been observed that 2.4-D concentrations of 1 to 2 mM impaired neurite outgrowth, disrupted the cytoskeleton, and disorganized the Golgi apparatus in cultured cerebellar granule cells (CGC) (Rosso et al., 2000). Futhermore, Kaioumuva et al. (2001b) have demonstrated that the dimethylamonium salt of 2,4-D (DMA 2,4-D) at 0.1 to 5 mM induces apoptosis in a dose- and time-dependent pattern in peripheral blood lymphocytes of healthy individuals and in Jurkat cells. Whereas, Tuschl & Schwab (2003) showed that 4 to 16 mM 2,4-D induces cytotoxic effects and apoptosis in HepG2 cells.

In rat CGC, either 1 or 2 mM 2,4-D induced similar increases of cellular death. The herbicide decreased significantly mean neuronal survival (46.4%) after 48 h, while no affect was observed after 24 h of treatment (Bongiovanni et al., 2007, 2011) (Fig. 2).

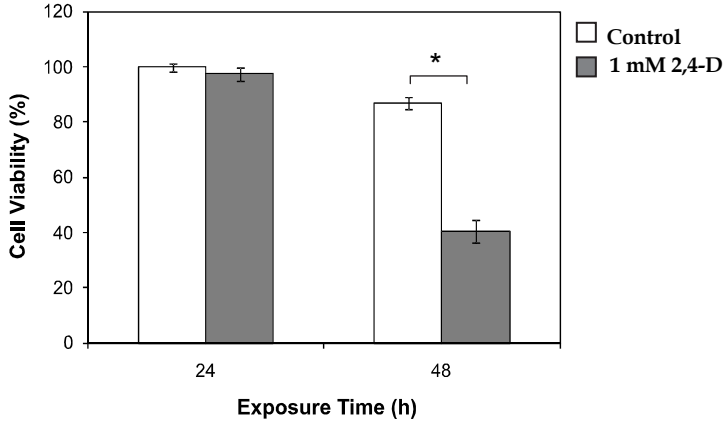

Fig. 2. Effect of 2,4-D on rat cerebellar granule cell viability. Cell cultures were incubated for 24 or 48 h in presence or ausence of 1 mM 2,4-D. Values are means ± SEM; * indicates $p < 0.001$ vs. control group; n= 10/group.

Bongiovanni et al. (2007, 2010) studied oxidative stress as a possible mechanism of toxicity aiming to elucidate the mechanism of death induction by 2,4-D. Oxidative stress parameters were altered: ROS level and Se-GPx activity increased whereas CAT activity decreased at both treatment times (24 and 48 h). GSH content was reduced only after 48 h of 2,4-D treatment. However, neither Mn-SOD nor Cu,Zn-SOD activities nor reactive nitrogen species (RNS) levels were affected (Tables 9 & 10). Interestingly, although the oxidative parameters evaluated were modified at the two time-limits studied, the cell viability only decreased at 48 h of treatment. This finding could be explained by a time dependency of this latter alteration.

Parameters	24 h		48 h	
	Control	1 mM 2,4-D	Control	1 mM 2,4-D
ROS	1.03 ± 0.25	2.30 ± 0,22* (↑ 123%)	2.28 ± 0.35	4.13 ± 0.32* (↑ 81%)
RNS	7.45 ± 1.13	8.23 ± 1.85	4.82 ± 0.27	6.05 ± 0.47
GSH	2.408 ± 0.09	2.225 ± 0.09	1.508 ± 0.061	1.125 ± 0.031* (↓ 25%)

Parameters are expresed as micrograms per miligram of protein. Values between brackets are % of increase (↑) or decrease (↓); *$p < 0.001$, n= 10/group. Abbreviations are indicated in the text.

Table 9. ROS, RNA and GSH levels (means ± SEM) in rat cerebellar granule cell in culture for 24 or 48 h in presence or ausence of 1 mM 2,4-D.

Enzimes	24 h		48 h	
	Control	1 mM 2,4-D	Control	1 mM 2,4-D
CAT	30.97 ± 1.26	15.80 ± 1.23* (↓ 49%)	15.82 ± 1.59	6.52 ± 0.83* (↓ 59%)
(Zn,Cu) SOD	10.43 ± 1.23	10.56 ± 1.45	8,49 ± 1,20	7.05 ± 1.65
(Mn) SOD	4.45 ± 0,88	5.97 ± 1.90	2,86 ± 1,90	1.98 ± 1.00
Se-GPx	9.71 ± 1.20	39.75 ± 2.90* (↑ 309%)	12.73 ± 1.75	33.73 ± 4.31* (↑165%)

Parameters are expresed as Units per miligram of protein. Values between brackets are % of increase (↑) or decrease (↓); *p < 0.001, n= 10/group. Abbreviations are indicated in the text.

Table 10. CAT, SODs and GPx activities (means ± SEM) in rat cerebellar granule cells in culture for 24 or 48 h in presence or ausence of 1 mM 2,4-D.

On using a PC-12 cell model, other authors have been previously shown that a depletion of mitochondrial and cytoplasmatic GSH results in increased ROS levels, disruption of the mitochondial transmembrane potential, rapid loss of mitochondial function, decrease in the ATP concentration, and eventually a higher cell death rate (Nieminen et al., 1995; Wüllner et al., 1999).

Therefore, the alteration in oxidative parameters suggest that the possible mechanisms of chlorophenoxy herbicide toxicity could involve dose-dependent cell membrane damage, uncoupling of oxidative phosphorylation, acetylcoenzyme disruption (Bradberry et al., 2000), and an indirect disruption of mitochondrial transmembrane potential which may lead to caspase inactivation (Kaioumova et al., 2001a). Mitochondrial structural modifications and increased permeability of the pores were also reported in association with a ROS increase (Belizário et al., 2007). In contrast, other studies suggest that 2,4-D cytotoxic effects are exerted by apoptosis induction via a direct effect on mitochondria (Tuschl & Schwab, 2003).

In this regard, Bongiovanni et al. (2011), in agreement with De Moliner et al. (2002), demonstrated that 2,4-D induces apoptosis and necrosis in CGC. While De Moliner et al. (2002) showed that 2,4-D-induced apoptosis is associated with and increase in caspase-3 activity preceded by cytochrome-c release from mitochondria, the quantification of ultrastructural changes showed that 1 mM 2,4-D stimulated neuronal death. As much as 49% of necrotic cells and 20% of apoptotic cells were observed, while only 31% of CGC presented normal growth with respect control group ($p<0.001$; Fig. 3 compared with Fig. 4) (Bongiovanni et al., 2011).

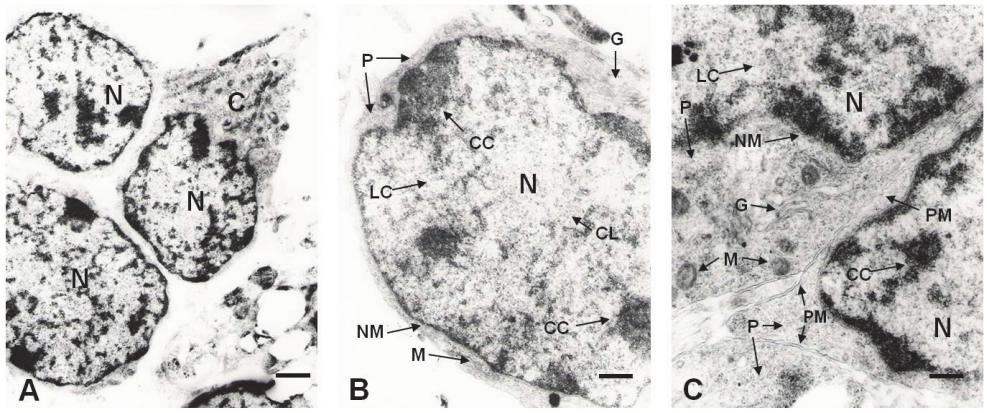

Fig. 3. Electron photomicrographies showing cerebellar granular neurons cultured in a control medium (NaCl 0.9%) for 48 h. a–b. Cell morphology is preserved (nucleus with laxe chromatin, dense chromatin patch close to the nucleus envelope, scarce cytoplasm, and the presence of neurites). Bars correspond to 1 µm in (a) and 160 nm in (b); c. Cells show preserved ultrastructural characteristics (Golgi apparatus, polyribosomes and mitochondrial characteristics of normal granular cerebellar cells). Bars correspond to 320 nm in (c). C cytoplasm, CC dense chromatin, G Golgi apparatus, LC laxe chromatin, M mitochondria, N nucleus, NM nuclear membrane, P polyribosome, PM plasmatic membrane.

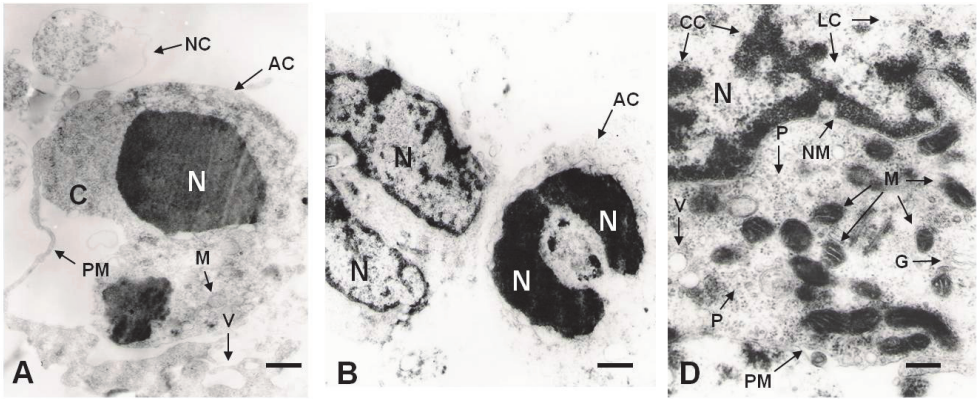

Fig. 4. Electron photomicrographies showing the ultrastructural cytoplasmatic characteristics of cerebellar granular cells after 2,4-D addition to the medium for 48 h. a–b. An apoptotic cell (nuclear fragmentation and very dense chromatinic accumulus), a necrotic cell (cytoplasm very scarce, no nucleus), and cells with scarce cytoplasm and small nucleus are shown, allowing comparison with the control group (Cf Figs. 3a, b). Bars correspond to 1 µm. c. A cell with cytoplasmatic protutions, vacuoles, disorganization of the cytoplasmatic reticulum, distended cisterns of the Golgi apparatus, and mitochondrial swelling. Bars correspond to 400 nm. AC apoptotic cell, NC necrotic cell, V vacuole, and other abreviations in Fig. 3.

In these studies, melatonin and amphetamine were used as phamacological tools aiming to improve the analysis of oxidative stress as a mechanism of toxicity, by assessing whether these compounds could be effective in preventing the toxic effect of 2,4-D in the redox balance of CGC *in vitro* (Bongiovanni et al., 2007, 2011).

A remarkable body of evidence indicates that melatonin exerts antioxidative protection in cell culture and *in vivo* systems (Pandi-Perumal et al., 2006). Regarding to 2,4-D toxicity, the oxidative stress induced by 1 mM 2,4-D was counteracted by the concomitant addition of 0.1 or 0.5 mM melatonin in CGC cultures (Bongiovanni et al., 2007).

On the other hand, amphetamine has constistently been reported to accelerate the recovery of several functions in animals and humans with brain injury (Goldstein, 2000; Martinsson & Eksborg, 2004). Amphetamine was also shown to stimulate both the dendritic growth in the ventral tegmental area (Mueller et al., 2006) and the neurotrophic and neuroplastic responses after brain damage (Moroz et al., 2004; Adkins & Jones 2005). However, few data are available regarding any possible protective effect of amphetamine. In this regard, Bongiovanni et al., (2011) demonstrated that 1 or 10 µM amphetamine reverted the 2,4-D-induced apoptosis and oxidative stress in CGC. Nevertheless, amphetamine alone induced no significant changes with respect to the control culture. Noteworthy, at 1 µM AMPH plus 2,4-D, 39% of the cells were normal; 53% were necrotic, and 8% showed apoptosis. At 10 µM AMPH plus 2,4-D, 57% of the cells were normal, 43% were necrotic, and no apoptotic cells were observed by electron microscopy (Fig. 4 compared with Fig. 5).

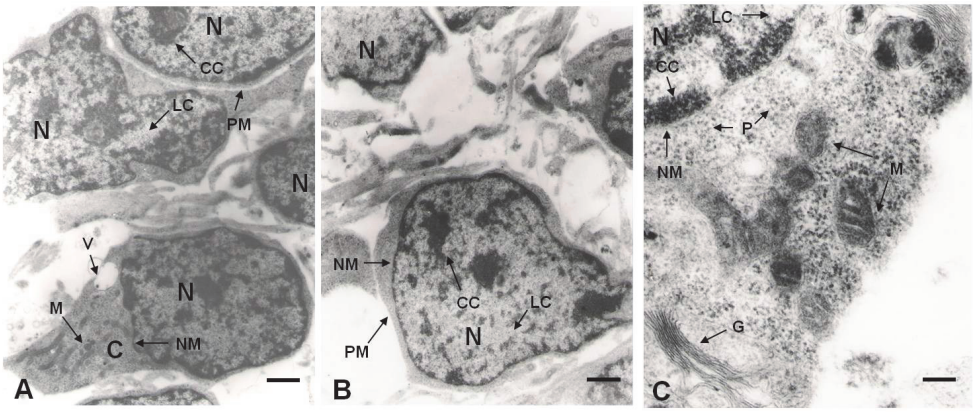

Fig.5. Electron photomicrographies showing the ultrastructural cytoplasmatic characteristics of cerebellar granular cells after 2,4-D and 10 µM AMPH addition to the medium for 48 h. a–b. Cells present more conserved morphology (nucleus and cytoplasm) than those treated with 2,4-D alone (Cf Figs. 4a, b). Bars correspond to 1 µm. c. The cell shows mitochondria and Golgi cisterns more preserved than those of the cells treated with 2,4-D alone (Cf Fig. 4c). Bars correspond to 600 nm. AC apoptotic cell, NC necrotic cell, V vacuole and other abreviations in Fig. 3.

The collected evidence would indicate a protective effect of melatonin and amphetamine against 2,4-D-induced cell death, possibly due to an inhibition of the oxidative mechanisms, as judged by the close relationship between ROS and apotosis induction (Carmody &

Cooter, 2001). While apoptosis and necrosis present some early features that may be common to both, mithocondrial disorders could be irreversibly compromised in necrotic, but not in apoptotic neurons (Nicotera & Leist, 1997). This could explain why amphetamine decrease apoptosis but not necrosis in 2,4-D-treated cells.

In summary, 2,4-D would induce necrosis and apoptosis, the latter being possibly mediated by an oxidative imbalance.

5. Concluding remarks

A great body of evidence suggests that exposure to 2,4-D or to its ester or salt formulations is associated with a wide range of adverse effects in human and different animal species (Berkley & Magee, 1963; Bortolozzi et al, 2001, 2003; Ferri et al., 2003, 2007; Konjuh et al., 2008; Stürtz et al., 2010).

Oxidative stress may affect the cells as a result of imbalance between the (physiological) production of potentially toxic ROS and some (physiological) scavenging activities (Park et al., 1999). Xenobiotics that interact with one or several complexes of the mitochondrial electron transport system, impairing the normal electron flow, may enhance ROS generation, leading to an imbalance between prooxidant species and cellular antioxidants (Jurado et al., 2011).

This review has analyzed the oxidative stress as a possible mechanism of toxicity by the herbicide 2,4-D. The collected evidence confirms that 2,4-D is an environmental pollutant that induces oxidative stress and could determine important deleterious changes in the development of the neural and reproductive systems in the studied models (Ferri et al., 2007; Bongiovanni et al., 2007, 2011; Pocchettino et al., 2010).

While the reported results showed that 2,4-D induces both necrosis and apoptosis, the evidence suggests that apoptosis would be mediated by or associated to an oxidative imbalance (Bongiovanni et al., 2011). Then, the oxidative stress would produce cytochrome-c release from mitochondria and a consequent activation of caspase-3 in the affected cells (De Molliner et al., 2002). However, as mitochondria contribute to both apoptosis and necrosis, intracellular ATP and GSH could determine cell death by one or both of these mechanisms (Leist et al., 1997; Yutaka et al., 1997; Qian et al., 1999; Nieminen, 2003; Bongaerts, 2008). Therefore, the 2,4-D cytotoxic actions may involve some permissive effect on either necrosis or apoptosis induction.

Finally, the experimental evidence reported that 2.4-D can not only affect the nervous system or other hormone-sensitive organs, but also exert a very important, deleterious effect on embryonic and fetal development.

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7. References

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18

Weed Population Dynamics

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1. Introduction

Clearly, the growing infestation of weeds in agricultural systems causes damage to crops, with sharp declines in productivity, either by direct competition for factors of production, whether by allelopathic compounds released into the soil (MARTINS and PITELLI, 1994). There are many factors related to population dynamics of these plants. However this chapter will be referred to those who, according to research, seem to be the most important.

2. Factors influencing the population dynamics of weed

In various cultures was observed the influence of farming system, being of fundamental importance to understand these dynamics through studies on the floristic composition and phytosociological structure of the same. The cultivation of maize intercropped with tropical forages in the system of direct planting can reduce the incidence of weeds due to the high biomass production and allelopathy provided by surface deposition of straw on the soil. The presence of *B. brizantha* intercropping reduced weed density. Therefore, the use of intercropping maize with *B. brizantha* provides control rate of 95% of the weeds in the soil (BORGHI et al., 2008).

A survey of weeds in conventional farming sunflower family Poaceae was the most representative among species (SILVA et al., 2010). In the experiment carried out by MARQUES et al. (2010) the plants originate in poultry farming sprouts in cowpea had the highest rates of importance values. However, they are dependent on the season and the continuity of the system.

Mechanized harvesting of raw cane enables the maintenance of the layer of straw on the surface, so by reducing the movement of soil and alter the dynamics of herbicides. These changes promote changes in microclimatic conditions, which in turn affect the composition of specific weeds. In this culture, the population dynamics of weeds in no-tillage system reduces up to 531% the incidence of weeds compared to the conventional system after treatment with herbicides. This provides 27% reduction in the productivity of cane sugar in conventional tillage soil (DUARTE JR et al., 2009).

The results obtained by VAZ-DE-MELO et al. (2007) showed that the practices adopted in growing organic green corn under no-tillage system, provide the appropriate management of weeds while with adoption of soil cover with oat straw. Among the weed

species, *B. pilosa* was the one that had the highest relative importance, that due to higher efficiency of this species to produce biomass in the absence of competition. In addition, this species shows high capacity for regrowth after mowing adopted in the organic system. The maize cultivars also interfere with the relative importance of *C. rotundus,* indicating the importance of knowledge of the floristic composition and the cultivar of corn to be adopted for the proper management of weeds.

Another important factor in weed control is to define the ability of weed species to compete for water, light and nutrients that are the factors responsible for reduced productivity of the main crop.

According GAZZIERO et al. (2004), features such as growth rate, efficiency of space occupation of the soil, shading, release of toxic chemicals to weeds, crop residues produced different and the specific methods of control used in each culture are also considered important features of competition between the crop and weeds.

2.1 Humic substances in the dynamics and allelopathy and weed control

The herbicides have specific mechanisms of interaction with organic compounds in humic matter in soil. This interaction interferes with the dynamics of molecules of herbicides in soil as well as implementing the recommendations.

The humic substances present in agricultural systems are caused by the biological degradation of plant and animal remains in the soil. The maintenance of straw on the soil surface and the permanence of the root system of crops harvested in the soil increases, the medium and long term, its organic matter content. This enables the maintenance of temperature and soil moisture at adequate levels, favoring the perfect physiological functioning of plants, ensuring the survival of a wide variety of organisms such as fungi and bacteria, which are primary decomposers of crop residues and serve as food for the small animals (MATZENBACHER, 1999).

In addition, the products then formed associate themselves into complex structures more stable, dark colored, high molecular weight, separated on the basis of solubility characteristics. Classified into: humin, humic acids, fulvic and hymatomelâmicos.

The humic substances increase the CTC CTA and soil, protecting and providing the cations and anions for the plants. This property of ion exchange of soil humic substances, when properly managed, enhances the efficiency of pesticides and fertilizers, and reduce the contaminant action (FOLONI e SOUZA, 2010, 2010).

The cultures used as soil cover in general have the ability to recycle nutrients, promote decompression of the soil, increase organic matter content and suppress weeds (THEISEN et al., 2000). The suppression occurs through the production of secondary metabolites, called allelochemicals, which accumulate in various organs of plants and are released with important ecological function. The main forms of release into the environment occur through the processes of volatilization, exudation from roots, leaching and decomposition of waste (DURIGAN and ALMEIDA, 1993).

The allelopathic action, both during vegetative growth and during the decomposition process, interspecific inhibition exerts on other species. The inhibition is linked primarily to reduced availability of light and to allelopathic effects, which have potent phytotoxic and can act as inhibitors of photosystem II (CZARNOTA et al. 2003; KADIOGLU et al., 2005).

The biochemical production of inhibitors, the remains of crops or the soil microorganisms can inhibit the germination and emergence of some species (MATEUS, 2004), as well as reduce the initial growth of plants.

Sorghum and millet are C4 plants, which have fast growth and good ability to cover soil. Furthermore, sorghum has allelopathic compound that is exuded by their roots, sorgoleone, which is able to differentially suppress the growth of various weeds and crops (NIMBAL et al., 1996).

Another factor that may alter the production of allelopathic compounds and modify the intensity of the effects found in the field, are the characteristics of the environment where these allelochemicals are produced. According to MARTINS et al. (1999) in the system of production of sugarcane, if allelopathic compounds are present in the straw, without fire, will be released larger amounts of these substances in the soil and may promote weed control or cause reductions shoots in culture due to autointoxication, similar to that observed in the cultivation of *Brachiaria brizantha* (RODRIGUES and REIS, 1994).

Thus, determining the nature of the effects of straw on the seed germination process and may lead to the adoption of different techniques of behavior control of invasive plants. In addition to the species and numbers of viable weed seeds that are dependent on culture, the presence of humic substances in the soil and in the application solution interferes with the population dynamics of weeds.

2.2 Weed control through trash on the soil

In Brazil, the adoption of production systems where crops are planted on some kind of plant waste / trash has increased in several regions. The layer of straw on the soil is essential to the success of no-tillage system. It creates an extremely favorable environment for the improvement of physical, chemical and biological soil, contributing to weed control, stabilization and recovery of production or maintenance of soil quality. The system of crop rotation and succession must be suitable for the maintenance of a minimum cover the soil with straw.

The weed control by vegetation can occur by physical effect, preventing the incidence of light, which can be maximized by reducing the spacing between rows, favoring rapid soil cover (BARROS et al 2009), and by allelopathic effects (KREMER, 1998; THEISEN and VIDAL, 1999; FÁVERO et al., 2001 and MESCHEDE et al., 2007).

According to RODRIGUES and ALMEIDA (2005), species such as *Galinsoga parviflora* (buttercup) and *Sonchus oleraceus* (milkweed) did not germinate in soil covered, while *Raphanus raphanistrum* (wild radish) sprouts normally. The greater the amount of straw, the greater the physical barrier that will influence the germination of weed seeds and the higher the amount of allelochemicals produced.

The amount of straw depends on the source material, soil and climatic conditions of the region and the management system. The decomposition of the mulch depends directly on the relationship between the levels of carbon and nitrogen in each material. C / N ratio indicates a high content of high cellulose and lignin. In places where climatic conditions favor the rapid decomposition of the mulch should be preferred to species with high C / N ratio, for example, grains.

According to ALVARENGA et al. (2002), can be considered that 6 t ha $^{-1}$ of residue on the soil surface constitutes an adequate amount to no-tillage system, with which it can proper rate of soil cover. However, for this same author, the quantity and quality of straw on the soil surface depends largely on the type of plant cover and management practices is given. Therefore, this amount can vary greatly depending on the ease or difficulty of biomass production or the rate of decomposition. In this case, one must consider the permanence of straw on the soil surface. It is known that the C / N ratio becomes larger as the plant grows, and the C / N ratio around 40 seems satisfactory when the objective is to collect straw.

A plant of adequate coverage is one that maintains or improves soil conditions. Grasses have fasciculate roots, making them useful in reconstructing the structure of the soil, improving water infiltration and controlling erosion. Since legumes are the most efficient in the process of biological nitrogen fixation, rapidly decomposing waste by the lower C / N ratio (PECHE-FILHO et al., 1999). In choosing these plants, is a decisive factor to know their adaptation to the region and its ability to grow in an environment less favorable, since the crops are laid down in auspicious times. Therefore the straw to form the Brazilian Cerrado conditions, can be sown corn, sorghum, millet, pigeon pea, sunflower and crotalaria after cultivation of main crop (second crop).

The cultivation of oats and other species to cover the soil, either alone or in consortium, within a system of crop rotation, promotes significant increases in the yield of subsequent crops, and make them more lucrative by reducing the use of mineral fertilizers (MATZENBACHER, 1999). DERPSCH et al. (1985), reported that oat winter covering produced larger amounts of dry matter (8670 kg ha $^{-1}$) and high levels of total N (147 kg ha $^{-1}$), while reducing the amplitude of variation of temperature and soil humidity. To VIDAL et al. (1998), the mulch originated from oat straw to reduce weed infestations.

Results of experiments conducted in five tillage in Nebraska, USA, indicated that five seven t ha $^{-1}$ of wheat straw residue on the soil reduced the biomass of weeds in 21 and 73%, respectively, compared with soil discovered (WICKS et al., 1994). CRUTCHFIELD et al. (1985) reported that five t ha $^{-1}$ of wheat residue reduced weed density by 65%, contrasted with soils without residue.

Thus, we can say that the presence of plant residue / straw affects the establishment of weeds in different ways. Among the forms of interference cites are: formation of physical barrier to be broken by the plant in emergency temperature control on the soil surface, increased microbial biomass that can reduce the seed bank in the soil, apart from possible allelopathic effects that inhibit germination (FOLONI e SOUZA, 2010).After the biochemical transformation of these residues occur in the presence of soil humic substances, which will interfere with the dynamics of molecules of herbicides in the soil, or even the recommended doses of herbicides.

Herbicides when applied to the soil come, either directly or by incorporation of vegetable crop residues. In this sequence, there is a branch of the most complex and fascinating study of environmental soil chemistry, where little is known.

3. Organic matter and sorption of herbicides in the soil

Organic Matter (OM) present a strong ability to absorb herbicides (STEVENSON, 1972) and this reduces the mobility and biological activity of chemical compounds applied to soil

(SCHEUNERT et al., 1992). The pronounced reactivity of the OM is mainly related to its high specific surface area and presence of various functional groups such as carboxyl, hydroxyl and amine, and aliphatic and aromatic structures (STEVENSON, 1972; STEARMAN et al. 1989; KUCKUK et al. 1997).

CHEFETZ et al. (2004) observed greater adsorption of ametryn in sediment with 1.25 dag kg^{-1} of organic carbon in relation to the other with 1.63 dag $kg^{-1.}$ The authors attributed this behavior to the fact that most of the sediment showed higher adsorption of aromatic compounds in the organic fraction, followed by a smaller number of polysaccharides, which favors its adsorption capacity.

Among the compartments of soil organic matter, humic substances are reported as the main responsible for the sorption of herbicides (PUSINO et al. 1992; CELIS et al., 1997). Most humic substances, especially in tropical regions, is in the form of clay-organic complexes (52 to 98%), according to STEVENSON (1994), whose total binding energy depends on the different forms of interaction promoted by functional groups of components organics. In this condition, neutralize their functional groups with loads of clay minerals, which reduces its sorption capacity of herbicides (PROCOPIO et al., 2001).

3.1 Quality of OM and HS on the sorption of herbicides

The quality of organic matter because of their functional groups determine the sorption of atrazine in soils, since different humic substances show different sorption intensities (PICCOLO et al., 1992). Among the constituents of organic matter, humic acid is responsible for about 70% of the sorption capacity of atrazine (BARRIUSO et al., 1992). Another important physical and chemical sorption of pesticides in soil is pH. In this sense, TRAGETTA et al. (1996) observed maximum sorption of atrazine in humic and fulvic acid near pH 3, while for pH conditions normally found in soils (5-7) sorption is lower.

PROCOPIO et al. (2001) observed that the sorption of atrazine by humic acids isolated was approximately nine times greater than that found sorption to kaolinite, goethite and ferridrita also examined separately. This indicates the high affinity between the herbicide and humic acids (MARTIN-NETO et al., 1994).

According MARTIN-NETO et al. (1999), the high intensity of hydrophobic sites to which the fraction of atrazine coupled (non-ionic behavior) can bind would be the main mechanism involved in sorption of atrazine with humic substances.

FERRI et al. (2005), in his study of sorption of herbicides on different substrates and found that the sorption ability of acetochlor was higher in the soil under no-tillage compared to conventional tillage.This behavior was explained only partially by higher content of C in the treatment of the sample of direct seeding.

When isolated, humin and humic acids showed acetochlor ability to absorb higher than when contained in the soil. Humin had higher ability to absorb acetochlor than the humic acids (FERRI et al., 2005).

Spectroscopic studies performed by PICCOLO et al. (1996) demonstrated that the main mechanism linking glyphosate and humic substances can be hydrogen bonds. The sorption of this herbicide may vary depending on the macromolecular structure and size of humic substances. The less aromatic C, the greater the sorption of the molecule.

3.2 Humic substances on the recommendation of herbicides

The humic acids in general allow a better exchange of chemicals, so that some authors suggest a decrease in those with the maintenance of efficacy was observed that the FOLONI and SOUZA (2010) working with cane sugar, concluding in his work that the use of humic acid in doses of 3.0 and 6.0 L ha $^{-1}$ did not cause phytotoxicity apparent effect on the culture of sugar cane plant. The addition of the use of humic acid with Dual Gold in different combinations, even with dose reduction of 25% allowed an excellent control of the main weeds present, equaling or surpassing traditional treatments until 120 DAT.

In assessing the remobilization of bound residues of 14C-anilazina fulvic acids in two soils of Germany, LAVORENTI et al. (1998) observed a small percentage of this waste in the humic and humin fractions and that the microorganisms were stimulated by applying the treatments corn stover (1.5 g 100 g $^{-1}$ dry soil) and glucose + peptone (0.2 g + 0.2 g 100 g^{-1} dry soil).

4. Allelochemicals release and control weed

4.1 Concept and production of allelopathy

Allelopathy is said to have any effect that plants have on the production of other chemical compounds released into the environment (RICE, 1984). Plants are able to produce chemicals with properties that affect beneficial or harmful in other plant species phenomenon called allelopathy, which is of Greek origin meaning *allelon* (from one to another) and *pathos* (suffering) (MOLISCH, 1937). Currently, this conceptual definition has become broader, expanding into the animal kingdom, since the interaction can occur between them and the plants and between animals and plants (GARDEN OF FLOWERS, 2001).

The chemicals responsible for allelopathy are called allelochemicals, whose function is primarily protective (SORIANO, 2001). The natural compounds with phytotoxic properties may have a high potential to control weeds (SOUZA-FILHO, 2006). According to MORALES et al. (2007), these compounds tend to have low toxicity to non-target organisms of control, as a potential source in the discovery of new molecules of herbicides less harmful to the ecosystem.

Unlike the common occurrence of compounds with allelopathic properties in higher plants, the amount and composition of these may vary depending on the species, age of the organ of the plant, temperature, light intensity, nutrient availability, microbial activity of the rhizosphere and composition of the soil in which they are the roots (PUTNAM, 1985; EINHELLIG and LEATHER, 1988).

Many are organic compounds produced by higher plants or microorganisms that have been identified as allelochemicals, which are: terpenes, steroids, organic acids, soluble in water, aliphatic aldehydes, ketones, long chain fatty acids, polyacetylenes, naphthoquinones, anthraquinones and complex quinones, originate from the mevalonate metabolic pathway of acetate (REZENDE and PINTO, 2003). Already the simple phenols, benzoic acids and derivatives, cinnamic acids and derivatives, coumarins, amino acids, polypeptides and sulfides and glycosides, alkaloids, cianidrina, flavonoids, and purine nucleoside derivatives, quinones and hydrolysable and condensed tannins are derived from the acid metabolic pathway shikimic (REZENDE and PINTO, 2003).

These compounds can be released from allelopathic plants through leaching and volatilization, root exudation and decomposition of plant residues (WEIR et al., 2004). A large number of allelopathic compounds such as oxalic acid, the amygdalin, coumarin and transcinâmico acid, are released into the rhizosphere and can act directly or indirectly in plant-plant interactions and the action of microorganisms.

The allelopathic effects may occur in the forms of auto toxicity and hetero toxicity (MILLER, 1996). The autotoxicidade occurs when the plant produces toxic substances that inhibit seed germination and growth of plants of the same species. Research has shown that alfalfa plants contain water-soluble phytotoxic compounds that are released into the soil environment by means of fresh leaves, stems and crown tissues as well as dry material, decaying roots and seeds (HALL and HENDERLONG, 1989). The phytotoxic hetero toxicity occurs when substances are released by leaching and root exudation and decomposition of waste in any type of plant on seed germination and growth of other plants (NUÑEZ et al., 2006). This second form is more potential to be explored by science, as a subsidy for the control of weeds in organic farming systems, or even as a tool to reduce costs with herbicides in conventional systems management.

4.2 Mode of action of allelochemicals

The action of allelochemicals and modification involves inhibition of growth or development of plants. According to SEIGLER (1996), the allelochemicals can be selective in their actions and plants can be selective in their response, which is why it is difficult to clarify the mode of action of these compounds. Several mechanisms of action of allelochemicals can affect the processes of respiration, photosynthesis, enzyme activity, water relations, stomatal opening, level of hormones, mineral availability, division and cell elongation, structure and permeability of membranes and cell wall (REZENDe et al. 2003). The same authors reported that many of these processes occur as a result of oxidative stress. One of the many effects of allelochemicals in plants is to control the production and accumulation of reactive oxygen species (ROS), which accumulates in cells in response to the allelochemical, thereby being responsible for damaging the cells causing their death (TESTA, 1995). Among them, blocking chain that carries electrons, where electrons are free and easily react with O_2 to form superoxide. Another known mechanism in the formation of ROS is the activity of allelochemicals on the NADPH oxidase, an enzyme that transfers electrons from the NDPH and donates to an acceptor (O_2) forming superoxide (FOREMAN et al., 2003).

Some allelochemicals rapidly depolarize cell membranes, increasing permeability and inducing lipid peroxidation, causing a generalized cell disorder that leads to cell death (YU et al., 2003).

4.3 Weed control by allelopathic effect

Seeds of *Coronilla L varies*. showed reduced germination rate when exposed to aqueous extracts of *Eucalyptus camaldulensis* and *Juglans regia* (ISFAHAN and SHARIAT, 2007). Soils planted with species *E.grandis, E.* and *E. urophylla grandis x urophylla* contains water-soluble phenolic compounds that inhibit the germination and early growth of black beans *(Phaseolus vulgar)* (ESPINOSA-GARCIA et al., 2008). According BURGOS et al. (2004), the

allelochemicals produced by *Secale cereale L.* reduces root growth of *Cucumis sativus L.* causing changes in cellular structures of the roots. Thus, a large part of allelochemicals acts on oxidative stress by producing reactive oxygen species, which act directly or as flags to the processes of cell degradation, thus preventing the germination and early development, as well as physiological processes of plants.

Another effective technique for controlling weeds, mainly due to physical and allelopathic effects is the use of green manure. FONTANÉTTI et al. (2004) observed that species velvetbean *(Stizolobium aterrimum)* and pork-bean *(Canavalia ensiformis)* significantly reduced the number of nutsedge when incorporated into the soil before planting romaine lettuce and cabbage. Already CAVA et al. (2008) found that plants such as *C. juncea, C. spectabilis, M. aterrima* and *M. pruriens* have high competitive capacity by reducing the production of dry mass and number of weeds. SEVERINO and CHRISTOFFOLETI (2001) found that the use of green manures *Arachis pintoi, C. juncea* and *Cajanus cajan* have significantly reduced the seed bank of species *Brachiaria decumbens, Panicum maximum* and *Bidens pilosa.*

Thus, it is clear that the population dynamics of weed species is a function not only of the main crop, soil and planting season, but also because of the tillage system, the quantity and quality of dry matter present in the soil surface. This is due to the fact that each species used in land cover has production of metabolites that interfere with specific control of weeds. The association provides interaction with herbicides and microorganisms in the soil fauna, as well as decreasing the dose of herbicides due to the increased amount of straw on the soil surface.

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19

Ecological Production Technology of Phenoxyacetic Herbicides MCPA and 2,4-D in the Highest World Standard

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„Selectivity is a major goal in modern synthetic chemistry"
Bartman W., Trost B. M.

1. Introduction

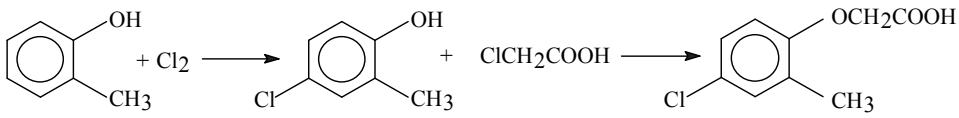
Herbicides MCPA (4-chloro-2-metylphenoxyacetic acid) and 2,4-D (2,4-dichloro-phenoxyacetic acid), which belong to the group of chlorophenoxyacetic acids, have been produced in Poland since the break of the sixties in the scale of many thousands of tons per year, which constitutes 5-7% of the world's production. Acids and their salts are exported to all continents. An advantage of herbicides within this group is their harmlessness for man and environment in doses used in agriculture. The condition is the high content and purity of the active substance in utility preparations. Unfortunately, classic technologies based on the reaction of phenols chlorination or their derivatives used until today all over the world do not ensure high purity, and significant quantities of highly toxical chloroorganic waste compounds originate in the production process. The main cause lies in the low selectivity of the reaction of chlorination of phenols' aromatic ring. Numerous producers enrich the purity of chlorophenols with the method of rectification. As our research has shown, in the process of rectification and while burning post-rectification wastes, dioxines and dibenzofurans can originate.

Below we present research conducted in the Institute of Industrial Organic Chemistry (IPO) in Warsaw with strict cooperation with production plants „Rokita Agro" in Brzeg Dolny and „Organika Sarzyna" in Nowa Sarzyna over the development of technologies of chlorophenoxyacetic herbicides. Commonly used „classical" technologies of production of MCPA and 2,4-D are based on two subsequent reactions – chlorination of phenol or 2-methylphenol and the reaction of the obtained chlorophenol with chloroacetic acid (MCAA), commonly called condensation. The first stage – chlorination reaction – is critical for the nature of the entire reaction, i.e. the number of operations, selection of equipment, purification methods, wastes. Electrophilic reaction of chlorination of aromatic phenol ring is non-selective. Isomers and polychlorinated compounds are always originated. In order to increase the selectivity of reaction and the purity of the the final product, various technologies of chlorination and purification of chlorophenols are used all over the world,

including regioselective catalysts, replacement of chlorine with sulfuryl chloride, rectification of chlorophenol and reversed technologies, i.e. condensation first, and then chlorination of the obtained phenoxyacetic acid. Also in our country technologies of production of MCPA and 2,4-D have been changed many times, achieving higher and higher selectivity of the chlorination reaction. The current production of MCPA (Chwastoks) is based on a unique technology of chlorination of 98% selectivity, while in the 2,4-D technology 96% selectivity has been achieved, and after crystallization it ensures the purity of the product equal to 98%. Technologies minimize or eliminate waste chloroorganic compounds. The final products have the highest quality standard.

2. MCPA

In the years 1959-1961 in IPO-Warszawa an experimental production MCPA in the scale of 5 tons per year of 30% „Chwastoks R-30" preparation was started. It made possible the conduct of vast agricultural research, including the research at 15500 linum plantations. In 1962 in Nowa Sarzyna was started the first technical installation of 1000 tons of Chwastoks R-30 (Moszczyński et al, 1963). The synthesis was based on chlorination of 2-methylphenol with gaseous chlorine. Technical 4-chloro-2-methylphenol without purification was exposed to condensation with monochloroacetic acid (MCAA). The diagram of the reaction is presented below.



2-methylphenol 4-chloro-2-methylphenol 2-methyl-4-chlorophenoxyacetic acid (MCPA)

The reaction of chlorination of melted 2-methylophenol had a very low selectivity – 60 to 70%. Technical 4-chloro-2-methylphenol was a mixture of chlorophenols of the following composition:

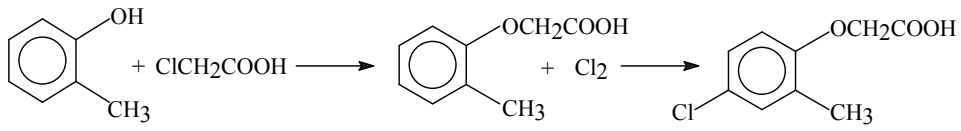
4-chloro-2-methylphenol	60-70%
6-chloro-	12-20%
4,6-dichloro-	3-9%
2-methylphenol-	4-10%

After the condensation of technical 4-chloro-2-methylphenol with chloroacetic acid there was derived a mixture of chloromethylphenoxyacetic acids containing 60 to 70% of MCPA. Below such a product is referred to as „MCPA 70". In the process of condensation the efficacy varied within broad limits and there always remained several per cent of non-reacted chloromethylphenols.

The final product Chwastoks R-30 was separated from the post-condensation mass without purification, the preparation contained up to 6% of free chloromethylphenols. A similar MCPA technology was applied by many producers, e.g. in Leuna-Werke plants, GDR, where the raw post-chlorination mixture was exposed to vacuum rectification. Together

with that 40% of waste chloromethylphenols was originated. The preparation Chwastoks R-30 had a good opinion among its users. It did not freeze in winter in unheated warehouses, it perfectly dissolved in water. It was cheap. In IPO a comparative research of the activity of MCPA-70 with pure MCPA was conducted, and the activity of each chloromethylphenoxyacetic acids separately, and also their mixtures. It appeared that isomeric 6-chloro-2-methylphenoxyacetic acid had a high biological activity, and in the mixtures the synergism of activity was observed. After 10 years of MCPA-70 production in a small installation in 1972 a new production facility of 4500 tons per year capacity was started without substantial changes in technology. In the discussed decade in the European market there appeared MCPA preparations of high purity, without free chloromethylphenols containing 80-90% of the pure active component. In the technologies sulfuryl chloride was used as a more selective chlorinating agent, and sometimes additionally vacuum rectification of technical 4-chloro-2-methylphenol. Chemische Werke Schwarzweide in GDR started the production of MCPA basing on sulfuryl chloride on the license purchased in Great Britain. The technology based on sulfuryl chloride is currently commonly used by chemical companies all over the world.

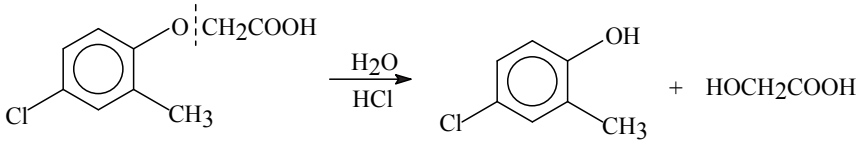
Skeeters was the first to describe in 1956 the MCPA synthesis through chlorination of 2-methylphenoxyacetic acid (MPA) in 1,2-dichloroethane (Skeeters et al., 1956). In the seventies such a method was used in industry. It was a new generation technology, below referred to as the reverse method, and the obtained product – „MCPA 90". The diagram of the reaction is presented below.



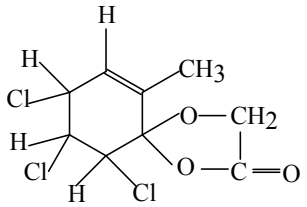
2-methylphenol — 2-methylphenoxyacetic acid (MPA) — MCPA

The aromatic ring of MPA acid is less prone to electrophilic substitution, and the side chain is a steric hindrance for creating the isomer 6-chloro-. In the reaction of MPA, chlorination selectivity of 80-90% is achieved, i.e. higher than in the classical method, where sulfuryl chloride without purification of the product by rectification is used. In the reverse technology a barrier which is difficult to lift is the high melting temperature of MPA (157°C) and the low solubility (7%) in organic solvents. Chlorination of MPA acid without solvents in hot water cannot be performed to the end and there remains 20% of non-chlorinated MPA. In Bratysława, the chlorination of 20-30% of water solution of MPA sodium salt MPA in the temperature of 90-100 °C was used on the industrial scale. The final product, containing 90% of the active substance, contained 5% of isomer 6-chloro- and 3-6% of free MPA (Rapoš et al, 1960). Chimzawod in Ufa, USSR, launched a multi-ton installation of MPA acid chlorination in 7% solution in solvent. The license with the full equipment was purchased from the Fisons company (Great Britain). The MCPA purity was 85-90%, the product contained 1-3% of free MPA, efficiency about 92% (Moszczyński, 1971).

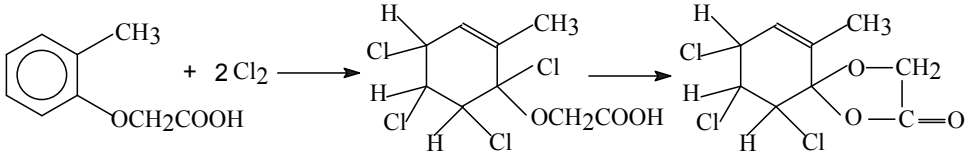
In Poland, in 1976, after the complete redevelopment of MCPA-70 system (Moszczyński et al., 1975), a new generation reverse technology was developed and used in industry. MPA acid heated with tetrachloroethylene (TCE) in post-condensation mass (brine) forms an eutectic of melting temperature of 100°C. It enables the separation of the liquid organic phase MPA/TCE from brine effluent from the post-condensation mass. The ogranic phase of MPA/TCE without drying was chlorinated in the temperature of 90-100%°C. TCE was removed from the post-chlorination mass with the use of destillation with water vapour. The obtained product was of 90% purity, with a mix of 7-9% of isomer 6-chloro-, and up to 3% of free chloromethylphenols. With the evaluation of efficiency in precise balance tests, 3 to 5% of the product was missing, and the quantity of free chloromethylphenols was higher that the quantity derived from MPA. In the technology used in Ufa, MPA before chlorination was effectively dephenoled by the extracting method, and in spite of that, in the final product chloromethylphenols were present. In patent literature the low efficiency of MPA chlorination attracts attention, as well as the lack of material balance of reaction. We have recognized that as a normal known phenomenon of breaking MPA and MCPA ether bonding in the presence of hydrogen chloride according to the following diagram:



The hydrolysis research which we performed did not confirm the presence of such a reaction in the conditions of MCPA synthesis, in the MPA post-chlorination mass there was found and separated several per cent and a compound with spirolactone structure was identified. An identical compound was depicted earlier by H. Lund (Lund, 1958).

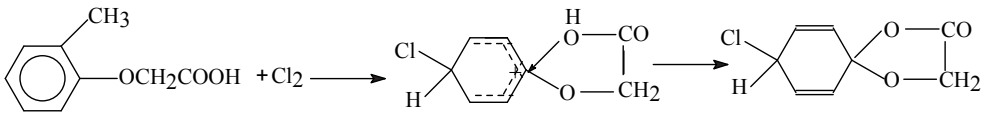


H. Lund suggests the mechanism of spirolactone origination, a derivative of MPA, based on joining 2 molecules of Cl_2 to the aromatic ring before the formation of cyclic ketal (spirolactone) according to the diagram:



He refers to known phenols reactions, which in the process of long-lasting chlorination, after saturation of all the places with chlorinum, form cyclohexanones. It is difficult to agree with

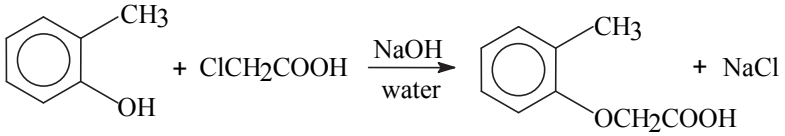
such a mechanism, where in the chlorination of MPA with one chlorinum atom, in mild conditions, with the help of hypochlorite with the known susceptibility of phenol aromatic ring to replacement of *o*- and *p*-, there might occur such a significant addition of chlorinum atoms. The life of cyclohexanone ring should not be conditioned by the presence of lactone side ring. Meanwhile, the reconstruction of the side ring transforms the cyclic structure into the aromatic one. W. Moszczyński suggests a completely different mechanism (Moszczyński, 1998). In the process of MPA chlorination after binding chlorinum atom in the electrophilic reaction there is formed a transitory sigma complex, which is subject to preservation in the intramolecular nucleophile trap according to the following diagram:



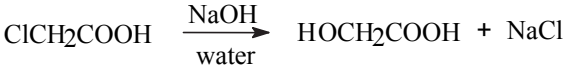
sigma complex sigma complex preserved in the intramolecular nucleophile trap

The preserved sigma complex is easily disintegrated into chloromethylphenol and glycolic acid. The discussed reaction is of a general nature, we repeated it while chlorinating 2,4-dichlorophenoxyacetic acid and chloromethylphenoxypropionic acid, obtaining appropriate preserved sigma complexes. Our discovery that chlorophenoxyalkanecarboxylic acids are traps of sigma complexes in electrophilic reaction of chlorination in the environment of organic solvents explains the causes of appearance of free chloromethylphenols and losses of the final product in the synthesis with the reverse method.

In MCPA production with the reverse method, the first stage was the synthesis of 2-methylphenoxyacetic acid (MPA) in the Williamson's reaction according to the following diagram:



The main reaction is always accompanied by the side reaction of hydrolysis of chloroacetic acid into glycolic acid.



In the domestic industrial production of MPA and 2,4-D, for many years the efficiency of condensation calculated to chloroacetic acid varied within broad limits, achieving on average 85%. Research conducted by W. Moszczyński with the use of regression function explained the causes of changeable efficiency (Moszczyński, 1999). Repetitively the efficiency of MPA synthesis of about 92% was achieved. It was established that the first 15 minutes of reaction are critical for the efficiency of the main reaction. In that time about 90% of MCAA reacts. The nucleophilic reaction of synthesis of MPA with Williamson's method proceeds according to the bimolecular mechanism according to the scheme:

$$V = k[ArO^-] [ClCH_2COO^-]$$

In the temperature <90°C, in the water alkaline environment its speed is low, while in the temperature of 90-110°C full substract reaction occurs after 1 hour (Moszczyński, 1994). pH has very clear influence on this speed, and the concentration of ions of phenoxyl ArO - and hydroxyl OH^- depends on pH. Both mentioned nucleophilic agents compete with each other in the reaction with substract – apart from the main reaction there occurs hydrolysis of chloroacetic acid into glycolic acid. Hydrolysis of substract makes the reaction's kinetics more complicated, and the formula of speed of reaction of synthesis of MPA needs to be corrected.

Hydrolysis of chloroacetic acid has a course which is clearly distinct from the main reaction. According to Berhenke and Britton, it occurs both in acidic environment and in alkaline environment and in the range of pH 3 – 10 it has similar speed. (Berhenke, Britton, 1946). Occurrence of hydrolysis both in the acidic and lightly alkaline environment as well as acceleration of speed of hydrolysis in the final stage of MPA synthesis along with sodium chloride accumulation proved by this research confirm that the reaction proceeds according to the mechanism S_N1. With pH = 11 - 11,5 MCAA hydrolysis is 5,5%, and with pH = 12 - 12,5 it increases to 42%, which suggests that with a greater concentration of OH^- the reaction proceeds according to the bimolecular mechanism type S_N2 with the participation of OH^- ions. This research confirms the findings of Dawson and Pycock (Dawson, Pycock, 1936) that the reaction of MCAA hydrolysis is a combination of mechanisms S_N1 and S_N2.

For research on the optimalization of parametres of MPA synthesis with the use of mathematical model the following parametres were accepted as critical: reagent molar ratio (X_1), reagent molar ratio (X_2), pH (X_3). Optimum values of variables X_1 and X_2 marked by experiment and calculated for the synthesis of MPA are respecively 0,8-1, and 3,3-3,7 mol/dm^3.

pH of reaction environment has a critical influence on the efficiency of MPA synthesis. With pH <9,5 MPA synthesis does not occur, while with pH >11, ion ArO^- loses in the competition with ion OH^-. The optimum value of pH indicated on the mathematical model is 10-10,7.

In the period of MCPA production with the reverse method in „Organika Sarzyna" in the seventies, countries classical technologies of chlorination of 2-methylphenol with sulfuryl chloride used in different were modernized. New regioselective catalysts combined with vacuum rectification of technical 4-chloro-2-methylphenol were commonly used. It meant an improvement of the purity of preparations with over 90 to 96% of active substance.

IPO for the third time has joined the competition in the market, overtaking, in terms of modernity, all technologies based on sulfuryl chloride. MCPA of 98% purity was offered directly from the synthesis, without purification and wastes, with an outstanding simplification of technology.

After several years of research on regioselective catalysts of chlorination of MPA with chlorinum, sodium hypochlorite and *t*-butyl, a laboratory synthesis „MCPA 98" of 98% of purity of active substance was developed. In May 1992 a combined research-implementation team, composed of 25 specialists from IPO was assigned, W. Moszczyński, I. Górska et al., and 19 from „Organika Sarzyna", including. T. Jakubas, J. Peć to conduct technical tests,

supervising redevelopment of construction and to conduct the launch of production. Synthesis of MCPA 98 consisted in one-stage condensation of MCAA with *o*-cresol to MPA and chlorination of MPA in water in room temperature with the use of sodium hypochlorite against amine catalyst (Moszczyński et al., 1992). A simple laboratory technology appeared to be unrepeatable in industrial conditions. For two years of ½-technical and technical tests not a single manufactured unit of product of >94% purity was obtained.

In the laboratory in glass all syntheses without exception had selectivity and efficiency of 98-99%. Meanwhile, with any multiplication of scale in glass and apparatuses from 50 l to 2,5 m^3 after exceeding the stage of about 2/3 pure chlorination *para*- suddenly broke down and a product of 90-94% purity of isomer *para*- was originated. Only the research of W. Moszczyński on the mechanism of reaction showed that we deal wigh a new, unknown in this group of compounds, aromatic free radicals reaction of 99% selectivity (Moszczyński, 1998). Free radicals reactions, as opposed to ion reactions, are highly selective, but sensitive to the conditions of reactions and dozens of external agents, and anytime there may occur a break of chain generation of radicals and return to the ion mechanism of a lower selectivity. Phenol reactions occupy a special place in this group. They are subject to reactions with such free radicals, which in other cases are inactive. There are proofs that with the presence of unpaired electrones delocalized into the aromatic ring, the replacement only occurs into the location *orto*- or *para*- (Dermer & Edmison 1957). The presence of free radicals was proved by EPR method at the University of Wrocław (Jezierski et al., 1999). It allowed to discover and eliminate radicals inhibitors and easily repeat the synthesis on an industrial scale. The installation launch took place in 1995. For the third time a new MCPA production installation was assembled with the full automatic control of the technological process and continuous chlorination and educing of MCPA acid. In the table below a standard of active substance of domestic MCPA was shown, according to the technology from 1976 and the last from 1996 with the purity declared in that time by leading western producers (Moszczyński et al. 2010). MCPA 98 from „Organika Sarzyna" is the best in all parametres.

No.	Parametres	MCPA 90 „Organika Sarzyna" 1976	MCPA AK 20 The Netherlands prospectus 1992	MCPA BASF Germany prospectus 1992	MCPA 98 „Organika Sarzyna" 1995
		[%]			
1	MCPA	85-90	96.0	94.0	97-98
2	6-chloro-2-methylphenoxyacetic acid	8-14	1.0	1.5	0.7
3	2-methylphenoxyacetic acid (MPA)	2	1.0	1.5	0.2
4	4,6-dichloro-2-methylphenoxyacetic acid	2	1.5	2.5	0.2
5	chlorocresols	1-3	0.5	0.5	traces

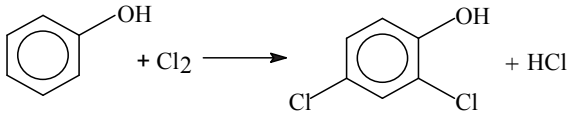
Table 1. Comparison of MCPA quality standards.

MCPA 98 authors have won a series of awards in national competitions for the technical and ecological level of technologies.

3. 2,4-D

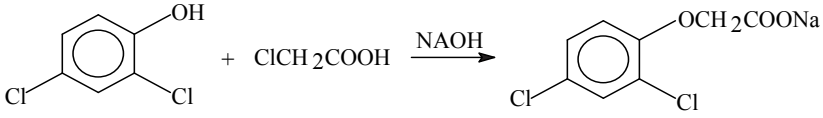
In Poland in a short time after the publication of Hamner and Tukey in Science (Hamner, Tukey, 1944) about the discovery of selective chemical herbicide 2,4-D there was started research on production and agricultural use of 2,4-D. 2,4-dichlorophenoxyacetic acid and its sodium salt are marked with the symbol 2,4-D. The dynamic development of technology and production of 2,4-D and its derivatives in our country is a constant keeping pace with the growing needs of agriculture. Sodium salt 2,4-D („Pielik"), the first modern chemical selective herbicide in the domestic market, quickly became a strategical item in agricultural cultivation. In the years 1952-53 in the Chemical Plant „Rokita", an experimental production of 20-30 tons per year according to the technology developed in the Intitute of Industrial Organic Chemistry was launched (Hirszowski, Moszczyński, 1952).

The technology was based on the reaction of dichlorophenol (2,4-DCP) with chloroacetic acid (MCAA):



2,4-dichlorophenol

88-90% of isomer 2,4-



MCAA

2,4-D 88-90% izomer 2,4-

96% 2,4-D after crystalization from water

In 1960 in the Chemical Plants „Rokita", a production installation of 500 tons per year was launched. One year later ½-technique, and in 1964 a technical installation of esters 2,4-D, so called Pieliki Płynne. In 1968 in IPO there was developed the technology of technical enrichment of 90% 2,4-DCP with the help of vacuum rectification. The process turned out to be highly energy-consuming and costly. Because of similar boiling temperatures of 4-chloro-2,4- and 2,6-dichlorophenols within the limits of 210-220°C, strongly corrosive for the equipment and generating 15% of chlorophenols in the form of cube residue. In that time we did not have the knowledge about the possibility of forming dioxines in the process of chlorophenols rectification.

The turning point in the production and usage of 2,4-D occurred after the appearance and launching production of dimethylamine salt, so called Aminopielik in 1976. Aminopieliks

are liquid preparations soluble in water, easy to use and prepare working liquids. Previously used sodium salt 2,4-D, so called Pielik, in the form of powder was poorly soluble in water, and in hard water residues of magnesium, calcium and iron salt were precipitated. In 1968 a brand new production installation 3000 tons per year was launched. As was shown in the diagram above, the synthesis of 2,4-D is based on two subsequent reactions of chlorination and condensation. This method is used by producers of 2,4-D in all countries, invariably since the first synthesis published in 1941 by J. Pokorny (Pokorny, 1941). Below, this technology is referred to as „classical". This method is based on easily available resources and the standard prodution equipment, which makes it generally accessible. A disadvantage to this method is the low quality of the product, burdened with 10-12% of undesired chlorophenoxyacetic acids, as well as free chlorophenols.

In the process of production as a result of standard purification of the product with the method of crystallization and washing there are originated 15 tons of wastes per 1 ton of 2,4-D, burdened with chloroorganics, chlorophenoxyacetic acids, chlorophenols, glycolic acid and sodium chloride. With multi-thousand scale of production, it means a serious environmental problem. For several thousand tons of annual production for many years there were created many thousand tons of wastes in the solid form and in liquid wastes. In some manufactured units there was confirmed the presence of nanogram quantities of highly toxic dioxines. Technology 2,4-D required research and solutions eliminating dioxines. Thermal processing, including burning of chloroorganic compounds, particularly chlorophenols, conduces originating dioxines. For some period waste chlorophenols were separated from the liquid wastes and chlorinated up to pentachlorophenol PCF, a known fungicide used mainly for the preservation of railway sleepers. In the first period in the installation 500 tons of 2,4-D per year, chlorophenol wastes were oxygenated with sodium hypochlorite on separated uncovered ground fields, a technology of Ostrowska J. IPO. The breakthrough occurred only after condensation was mastered at the beginning of the nineties, thanks to using several metre long reactors with stirrers for the reaction of disintegration of chlorophenols with sodium hypochlorite and additional cleaning of liquid wastes on biological treatment plant.

The border of purity of 2,4-D in the classical technology is determined by 90% of isomer 2,4-DF in the process of phenol chlorination and 96% of the active substance in acid and preparations 2,4-D after crystallization and washing.

Composition of 2,4-D obtained in the classical technology [% GC]	
chlorophenols	0.3
o-chlorophenoxyacetic acid	2.0
p- chlorophenoxyacetic acid	0.4
2,6-di chlorophenoxyacetic acid	0.9
2,4- di chlorophenoxyacetic acid	95.6
2,4,6-tri chlorophenoxyacetic acid	0.3

Table 2. 2,4-D obtained in the classical technology. (Białek, Moszczyński, 2009).

For many years of production of 2,4-D on bigger and bigger installations, apart from the cumulation of wastes, a weak link was the poor efficiency of condensation reaction, maximum 90% of calculated on 2,4-dichlorophenol. In particular manufactured units, it

varied up to several per cent. The reacting mass, initially thin, sometimes as a result of a violent reaction and salting out the product, was getting thick so quickly that it could not be stirred and it was thrown out of the hatchway from the reactor. Never ending experiments with a change of concentration and order of introducing products, molar ratio, temperature and height of pH gave no results. During the synthesis, in the course of reacting of chloroacetic acid pH was changing, which required adding sodium lye. The measurement and adjustment of pH was done by hand and as a rule – delayed in relation to the speed of reaction. As it was mentioned above, in „Organika Sarzyna" was produced since 1962 on IPO technology 4-chloro-2-methylphenoxyacetic acid (MCPA, Chwastoks). In the reaction of condensation of 4-chloro-2-methylphenol with chloroacetic acid there occurred similar difficulties. They were mastered only after partial continuity of the process and using automatic adjustment of pH with the help of pH-metre controlling the dispensing of MCAA and sodium lye.

The causes of difficulties occurring at MCAA and phenols condensation were only explained by the research of Moszczyński over the mechanism of the main reaction and the competitive MCAA hydrolysis reaction depicted before (Moszczyński, 1999). The research concerned the reaction of *o*-krezol with chloroacetic acid, but they are of a general nature and they can be related to 2,4-dichlorophenol (2,4-D) and other alkilo- and chlorophenols. Particular phenols depending on the substituents and solubility of the final chlorophenoxyacetic acid react in a slightly different way. The maximum industrial capacity of condensation in MPA synthesis is 92%, while in 2,4-D synthesis 97% is achieved.

The composition of mass after condensation of raw 2,4-dichlorophenol with MCAA acid was shown below in table 3.

Composition of post-condensation mass	**[%GC]**
o-chlorophenoxyacetic acid	2.5
p- chlorophenoxyacetic acid	0.5
2,6-dichlorophenoxyacetic acid	7.4
2,4- dichlorophenoxyacetic acid	84.6
2,4,6-trichlorophenoxyacetic acid	1.2
o-chlorophenol	0.0
2,4-dichlorophenol	2.2
p-chlorophenol	0.0
2,6-dichlorophenol	1.1
2,4,6-trichlorophenol	0.4
NaCl glycolic acid water	

Table 3. Composition of reaction mass after condensation of raw 89% 2,4-DCP with MCAA.

Purification of acid with crystallization method has a limited efficiency. Isomer 2,6 is easily eliminated, while 2-chlorophenoxyacetic acid is hard to dissolve and remains in 2,4-D. 2,4-D of 96% purity of active substance is obtained. Rectification of about 89% raw 2,4-dichlorophenol is non-economical and threatened with originating dioxines in cube residue. The difficulty in mastering the technology of selective chlorination, purifying raw 89% 2,4-

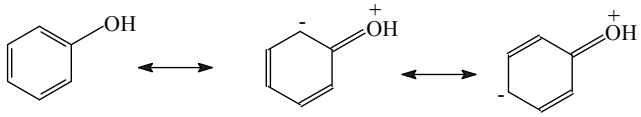
dichlorophenol or technical 2,4-D acid is soundly confirmed by the EU position regarding purity of commercial 2,4-D and its derivatives.

In the countries of the European Union, after the process of verification and re-registration according to the directive 91/414/EWG, only preparations of the highest quality were allowed into the market, broadly tested, man friendly and natural environment friendly. In the nineties, over one half of active substances used in preparations of plant protection was recalled from sales. With allowing a plant protection agent into the market full chemical specification and toxicity tests of all pollutions of the active substance which were found in the quantity of over 0,1% were required.

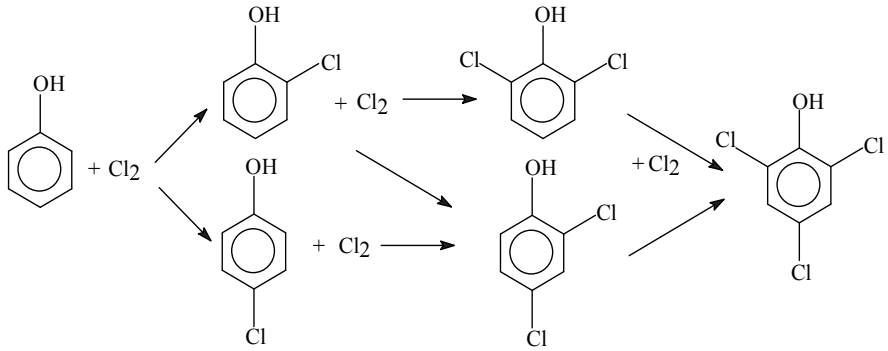
In the meantime, the working team for toxicological and ecotoxicological evaluation appointed under directive 91/414 EEC (over 200 of open and closed sorts of research) 2,4-D, consisting of 12 producers and suppliers of preparation 2,4-D in the European Union, including DowElanco Europe UK, Nufarm-Agrolinz Austria, Rhone-Poulenc France and AH Marks Co Ltd. UK submitted to the European Committee for registration 2,4-D of the quality of 96% of active substance. This standard was approved for UE producers by the directive 2001/103/EC of 28.11.2001. Maintaining the traditional standard 96% of active substance 2,4-D, in spite of a great pressure of environmentalists, shows difficulties in obtaining product of a higher purity.

Moszczyński and Białek have conducted a vast research on selectivity of reaction of phenol chlorination to 2,4-dichlorophenol (Białek, Moszczyński, 2009).

Phenol is a substrate which easily undergoes electrophylic substitution in aromatic ring. Additional pair of electrons from hydroxyl group after delocalization and because of messomerism shifts to moves in aromatic ring which results in higher density of electrons in *ortho*- and *para*- positions.



Substitution in the ring with an electrophilic agent is fast but not regio- and chemoselective. Just after using 1 M of chlorine for 1 M of phenol a mixture of six products is formed (Watson, 1985).



The following conclusion can be drawn from the scheme above: obtaining *p*-chlorophenol as an intermediate product in synthesis of 2,4-dichlorophenol is much more advantageous than obtaining *o*-chlorophenol which is followed by forming 2,4-dichlorophenol and 2,4,6-trichlorophenol or even 2,6- dichlorophenol.

It is commonly known that many factors are included in the selectivity of reaction. Electron density in the substrat molecule – active phenol centres are located in the position *ortho-*, *para-* with preference for *para-* . The dissociation of hydroxyl group in phenol in different conditions of pH influences the orientation of electrophilic substitution. The activity of electrophilic agent is of crucial significance has. Chlorine molecules are a strong electrophilic agent reacting violently with active aromatic substrats. The decisive significance should be ascribed to steric effect and catalysts. Intermolecular hydrogen bondings through hydroxyl group are formed in melted phenol. The association of molecules makes it more difficult to replace in the position *ortho-*. A similar effect is achieved at phenol chlorination in strong acids and polar proton dissolvents. The presence of hydrogen bondings in phenols was confirmed by IR methods and proton NMR method.

Our research mostly concerned catalysts and chlorinating agents, including high-molecular ones.

It is not necessary to use catalyst during the synthesis of 2,4-dichlorophenol from melted phenol and gaseous chlorine. The process of chlorination stops at 2,4,6-trichlorophenol. Substitution of next chlorine atoms is only possible when Friedel-Crafts catalysts are used. It was stated that Lewis acids used at chlorination of less active substrates do not influence the selectivity of substitution of highly reactive phenols. Catalysts from amines group direct substituents into *ortho-* position. Catalysts used by Moszczyński in MPA production appeared to be totally inactive. Sulphur catalysts have a limited directing effect. The combination of Lewis catalysts with sulphur compounds resulted with a high regioselectivity into *para-* position. Probably in the reaction a high-molecular complex is originated, where the catalytic effect interferes with the steric effect.

There were also performed tests of chlorination with the reverse method, i.e. phenoxyacetic acid (PA). Przondo, in cooperation with fellow workers in the installation ½-technical in Chemical Plants „Rokita", did research on chlorination of PA acid with chlorine in alkaline water solution. Satisfactory results were not achieved. The process required significant dilutions of the reaction mass. The technology appeared to be highly waste-generating and the selectivity of chlorination not much better than at phenol chlorination (Dudycz et al. 1985).

Our team IPO and Rokita continued research on the reversed process. Using *tert*-butyl hypochlorite in the place of chlorine (t-BuOCl) of spatially developed molecule. A high selectivity of chlorination was achieved (98%) and purity of 2,4-D up to 99% of active substance. A very high selectivity of chlorination reaction is the result of the interference of two steric effects – side chain and volumetric molecule t-BuOCl (Moszczyński et al. 2008). A negative side to the technology is the necessity of operating flammable t–BuOCl and t-BuOH.

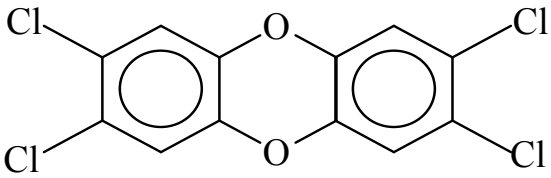
A technology based on sulfuryl chloride was developed and confirmed in the ½ technical scale results presented by Watson (Watson, 1974). The process is easy to implement and control. A negative side is the necessity of SO_2 recycling.

Three new methods of 2,4-D synthesis were developed, each of them of > 95% selectivity. The barrier of 90% selectivity of isomer 2,4-D in phenol chlorination was lifted. Results were shown below in table 4.

Chlorinating agent, catalyst,	Cl_2	Cl_2 catalytic complex, Lewis acids, sulphur compounds	SO_2Cl_2 catalytic complex	*tert*-BuOCl phenoxyacetic acid, reverse method
Selectivity of chlorination [%]	90	96	98	98
Production scale	industrial	industrial (Moszczynski et al., 2002)	experimental (Moszczyński et al., 2009)	laboratory

Table 4. Progress in selectivity of the process of chlorination in technology 2,4-D.

While discussing the technology of production of 2,4-dichlorophenol, the problem of trace pollutions must be mentioned, which includes remains of catalyst, present always in industrial water iron, polychlorinated compounds, cyclohexenones derivatives. There may also appear polychlorodibenzodioxines and polychlorodibenzofurans. Trace pollutions in 2,4-DCP are undesired, because they influence the quality and stability of 2,4-D. Iron compounds may worsen the hue, remains of sulphur catalysts – odour, form during storing liquid forms slimy residues and also, as an effect, decrease the durability of 2,4-D preparations. Used by some 2,4-D producers, the vacuum rectification of technical 2,4-DCP is efficient, but it threatens with dioxines forming. In the tests of vacuum rectification of technical 2,4-dichlorophenol there was formed about 15% of post-destillation tars, containing significant quantities of dioxines, including the most toxic 2,3,7,8-TeCDD and TeCDF, which was shown below in table 5.



2,3,7,8-TCDD

Authors have developed the technology of removing catalysts and trace pollutions (Moszczyński et al. 2005).

2,4-dichlorophenol obtained with the method of phenol chlorination in the presence of catalysts is washed off with mineral acid, it is neutralized to pH >10,5 and dilutes with water to 50%. A suspension of mineral sorbents is introduced to chlorophenolate obtained in this way. Chlorophenolate with sorbents is stirred for 30 minutes and the introduced sorbents are filtered, which are then destroyed thermally.

Congener PCDD / PCDF	Toxicity equivalency factor (TEF)	Congener content in an specimen, m_i [ng/g]	Toxicity TEQ m_i x TEF [ng-TEQ/g]
2,3,7,8-TeCDD	1	2.32	2.3200
1,2,3,7,8-PeCDD	1	51.71	51.7100
1,2,3,4,7,8-HxCDD	0.1	0.28	0.0280
1,2,3,6,7,8-HxCDD	0.1	16.22	1.6220
1,2,3,7,8,9-HxCDD	0.1	6.63	0.6630
1,2,3,4,6,7,8-HpCDD	0.01	0.76	0.0076
OCDD	0.0001	3.11	0.0003
2,3,7,8-TeCDF	0.1	10.69	1.0690
1,2,3,7,8-PeCDF	0.05	1.45	0.0725
2,3,4,7,8-PeCDF	0.5	1.50	0.7500
1,2,3,4,7,8-HxCDF	0.1	0.55	0.0550
1,2,3,6,7,8-HxCDF	0.1	4.58	0.4580
1,2,3,7,8,9-HxCDF	0.1	3.16	0.3160
2,3,4,6,7,8-HxCDF	0.1	0.17	0.0170
1,2,3,4,6,7,8-HpCDF	0.01	5.90	0.0590
1,2,3,4,7,8,9-HpCDF	0.01	0.00	0.0000
OCDF	0.0001	67.75	0.0068
Result in **ng TEQ/g**			**59.15 ± 0,05**

Table 5. Contents of PCDD/F in the cube residue from the destillation of 2,4-DCP (Białek, 2009).

Authors have developed the technology of removing catalysts and trace pollutions (Moszczyński et al. 2005).

2,4-dichlorophenol obtained with the method of phenol chlorination in the presence of catalysts is washed off with mineral acid, it is neutralized to pH >10,5 and dilutes with water to 50%. A suspension of mineral sorbents is introduced to chlorophenolate obtained in this way. Chlorophenolate with sorbents is stirred for 30 minutes and the introduced sorbents are filtered, which are then destroyed thermally.

Trace pollutions in purified 2,4-dichlorophenol are given below in table 6.

Iron contents [ppm]	Derivatives DPS [%]	PCDD/F [TEQ ng/g]
<5	<0.001	0.027

Table 6. Trace pollutions in purified 2,4-dichlorophenol.

The product contains trace pollutions PCDD/F in the amount allowed by technical producers norms.

The world production of 2,4-dichlorophenol without Russia, China and India is estimated for 44 000 tons per year. The current production of 2,4-DCP in Poland is 6 to 8 000 tons per year. It places our country in the strict world lead. After using in 2004 a new technology of selective chlorination of phenol, two barriers were lifted: chlorination selectivity increased from 89 to 96% and the purity of commercial acid 2,4-D from 96 to 98%. Waste chloroorganic compounds were decreased by one half in the production process, also organic pollutions introduced to the soil during agricultural operations were decreased by one half. Recommendations of directive 91/414 EEC on the improvement of ecological conditions in the production and usage of plant protection agents were implemented.

The author team W. Moszczyński, A. Białek, E. Makieła, B. Rippel, Listopadzki E., Okulewicz, Z. Dancewicz G. for the technology and product won prizes in national and world competitions, including:

- Złoty Orbital (Gold Orbital) of the monthly Rynek Chemiczny (Chemical Year), year 2004
- The main prize in the competition „Polish Product of the Future" („Polski Produkt Przyszłości") in category „Technology of the Future" („Technologia Przyszłości") organized by the Polish Agency for Enterprise Development, year 2004
- The Gold Medal with Mentions in the World Inventions, Research and Innovations Exhibition „Brussels Eureka", year 2005.

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20

Vegetative Response to Weed Control in Forest Restoration

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1. Introduction

Longleaf pine (*Pinus palustris* Mill.) stands once occupied an estimated 24 million ha in the southeastern USA (Stout & Marion, 1993). Fire suppression, timber harvest, and land conversion reduced its extent to around one million ha (Outcalt & Sheffield, 1996). In recent times, widespread interest in restoring longleaf pine ecosystems or planting the species for timber production has motivated private landowners, industrial forest owners, and public agencies to establish more longleaf pine forest. Over 33 million longleaf pine seedlings were produced for the 2005-2006 planting season in the southeastern United States (McNabb & Enebak, 2008), and 54 million produced in 2008-2009 (Pohl & Kelly, 2011).

Longleaf pine ecosystems are fire-adapted and support a diverse understory plant community when ground fires are frequent (Peet & Allard, 1993). Longleaf pine seedlings germinate and develop into a grass-like clump, and later transition from this "grass stage" to become woody saplings. Seedlings in the grass stage resist fire, but become vulnerable to fire upon emergence from the grass stage until height growth elevates their terminal bud beyond reach of fire and their bark thickens (Boyer, 1990). Early fire resistance is thought to be an adaptation to frequent fire. During the grass stage, seedlings invest energy in root development in preparation for rapid shoot extension upon emergence. This strategy for re-occupying disturbed sites gives the slower-growing longleaf pine a competitive advantage over less fire-hardy pines and hardwood competitors (Outcalt, 2000). However, in the absence of fire, longleaf pine seedlings are quickly overtopped by competing vegetation. Therefore rapid restoration of longleaf pine forests will necessarily involve some disturbance of competing vegetation. Hardwood regeneration is usually prolific following disturbances such as removal of forest cover. A suite of hardwood species regenerate as stump sprouts and root suckers, developing quickly from established root systems. Grasses and vines also develop quickly after disturbance in the warm humid climate of southeastern USA. Various forms of above- and belowground competition impact on survival and growth of planted longleaf pine seedlings (Harrington et al., 2003; Pecot et al, 2007) and other pine species (e.g., Richardson et al., 1996b; Amishev & Fox, 2006).

Tools available for control of competing vegetation in longleaf pine forest restoration include prescribed fire, mechanical methods, and chemical weed control with herbicides. Prescribed fire most closely mimics the natural disturbance regime in longleaf pine forests,

but it may not carry in areas with insufficient quantity or quality of fuels, and it may not be appropriate or acceptable on some ownerships. Mechanical weed control methods include portable saws and machine-mounted mowers or masticators. These methods are more expensive than prescribed fire treatments, but can have similar effects: competing vegetation is disturbed above ground but not always killed; much of it re-sprouts. Herbicides can provide effective and economical control of competing vegetation, but their use may not be appropriate or acceptable in some areas amid concerns over effects on non-target organisms, movement and drift, and persistence in the environment. Fire or broadcast herbicide treatments can eliminate live vegetation cover, exposing soil to erosive forces and temporarily reducing biodiversity. Applying herbicide in spots as opposed to broadcast applications has the advantage of reducing chemical usage while maintaining some continuity of vegetation cover and preserving biodiversity between treated spots (Richardson et al., 1996a).

Research into longleaf pine forest establishment and weed control has focused on the Coastal Plain region of the southeastern USA. Field research on the Coastal Plain indicated that mechanical weed control treatments were inferior to chemical weed control in terms of enhancing longleaf pine seedling survival and growth (Knapp et al., 2006). Chemical weed control with herbicide has proven effective in several longleaf pine restoration studies on the Coastal Plain (Brockway & Outcalt, 2000; Ramsay et al., 2003; Knapp et al., 2006; Haywood, 2007; Freeman & Shibu, 2009; Shibu et al., 2010). Longleaf pine is native to the Coastal Plain, but also occurs naturally in the mountainous regions further inland, and across the Piedmont Region. The Piedmont is a physiographic region extending from the State of New Jersey down to central Alabama, spanning over 200,000 km^2 of rolling foothills between the Appalachian Mountains and the Coastal Plain (Anon, 2000). Little has been reported on longleaf pine restoration in the Piedmont, but restoration experiments have been established (Berrill & Dagley, 2009).

Data from a replicated field experiment established on degraded Piedmont forest sites are presented here. To our knowledge no other experiment simultaneously addresses questions of repeat herbicide applications versus single treatments each of varying spot sizes, and compares all these weed control treatments to non-herbicide management options. We established non-contiguous single-tree plots in a randomized complete block design with multiple treatment levels nested in a split-plot arrangement within contiguous fixed-area treatment plots. Our objective was to determine the influence of frequency and extent of chemical weed control on planted trees and competing vegetation using commonly-used, widely-available herbicides, and to compare herbicide treatments with mechanical weed control and a no-treatment control. Specifically, we sought to answer the following four questions:

i. How does planted seedling survival and growth differ between various herbicide treatments and two alternative experimental treatments: mechanical weed control, and zero weed control?
ii. Is one herbicide treatment sufficient for control of vegetation competing with tree seedlings planted for restoration? Or, will a second 'repeat application' treatment be required?
iii. How large of an area needs to be treated with herbicides around each planted seedling (when making a single herbicide treatment, and/or when making a repeat application)?

More specifically, what is the trade-off between size of treated area (termed 'spot size') and tree seedling response in terms of both survival and growth?

iv. What is the response of weeds to the various treatments? How quickly did each type of weed (grasses, vines, woody vegetation) develop after treatment?

2. Study sites

The restoration experiment was established at four disturbed sites on the 1,900 ha Hitchiti Experimental Forest (N 33^0 02′ W 83^0 42′) in Jones County, Georgia, USA. Southern pine beetles (*Dendroctonus frontalis* Zimmerman) had killed patches of even-aged conifer plantation throughout the forest in 2007. The kill areas totalled 10% of the forest area. Salvage harvesting in 2007 was followed by broadcast burning that consumed most of the scattered woody debris and residual hardwoods. Fire failed to carry through some areas due to lack of fuels. Containerized 1-0 'mountain variety' longleaf pine seedlings were hand planted in late March 2008 at a spacing of approximately 3.65 x 3.65 m (740 stems/ha).

Vegetation naturally regenerating throughout the study sites consisted primarily of hardwood stump sprouts and root suckers, vines, forbs, and various grasses. Natural regeneration of 22 tree species was recorded, including an abundance of dogwood (*Cornus florida* L.), loblolly pine (*P. taeda* L.), persimmon (*Diospyros virginiana* L.), sweetgum (*Liquidambar styraciflua* L.), and water oak (*Quercus nigra* L.). Five shrub species, 49 forb species, and eight vine species were recorded. The most common forb was American burnweed (*Erechtites hieracifolia* (L.) Raf.). Throughout the four sites selected as experimental replicates for the restoration study, muscadine grapevines (*Vitis rotundifolia* Michx.) were abundant and expanding laterally to occupy the disturbed sites.

Elevation of the four study sites ranged from 120-150 m above sea level. Soils were classified as a mixture of Davidson and Vance soil series with remnants of loamy surface layer over clay subsoil. The rolling hills were incised by a series of narrow, shallow gullies (Brender 1952). Before the beetle attack in 2007, the four study sites were forested with planted stands of loblolly pine 24-100 years old. Site index ranged from 24.4 m to 27.4 m at base age 25 years for loblolly pine (Clutter & Lenhart, 1968).

Climate at the study site is humid and warm in summer months, and cool in winter. Monthly average low temperatures range from -1°C in January to 19°C in July, and monthly highs range from 13°C in January to 32°C in July. Extreme temperatures were the record high of 40°C in July 1986 and the record low of -20°C in January 1985. The average annual rainfall of 1180 mm is distributed throughout the year; March being the wettest month with 140 mm, and October the driest with 70 mm average monthly rainfall (www.weather.com).

2.1 Experimental design

One experimental replicate block was established in each of four beetle-killed areas at different locations across the forest. Within each replicate block (study site), four treatment plots were established. The 25 x 25 m square treatment plots were surrounded by 4 m wide buffers. Treatments applied to each plot were either mechanical weed control, chemical weed control (repeated in two plots), and control (i.e., no weed control). In a split-plot arrangement, each chemical weed control treatment measurement plot (considered the experimental unit for main treatments) was divided into approximately 12 replicates of

three single-tree plots where a single longleaf pine seedling became the experimental unit. Within a split-plot replicate of three adjacent longleaf pine seedlings, each of the three seedlings was randomly assigned a different 'spot size' spray area treatment: a small, medium, or large circular herbicide spot sprayed around the planted seedling.

2.1.1 Weed control treatments

Chemical and mechanical treatments were applied approximately three months after planting, in late June 2008. The objective of the chemical weed control treatment was to reduce above- and belowground competition in the vicinity of longleaf pine seedlings. Glyphosate in the form of isopropylamine salt of N-(phosphonomethyl) glycine was delivered using a backpack sprayer with 2% active ingredient in water at a rate of 6.9 liters active ingredient in 360 liters of solution per ha (D'Anieri et al., 1990). Longleaf pine seedlings were covered with large paper cups prior to spraying. One week after glyphosate application, competing vegetation was mowed close to ground level, and cut stumps of woody species within each randomly-assigned spot treatment area immediately treated with an 8% triclopyr water-based solution of triethylamine salt (5.74% triclopyr acid equivalent). Triclopyr was only used when woody vegetation was present within the treatment spots. Therefore the volume of triclopyr applied differed between small, medium, and large spots, and due to variations in density of woody vegetation within and between study sites. Across all sites, the sum of all spot areas in herbicide plots (0.133 ha) and surrounding buffers (0.060 ha) was 0.193 ha. A total of 0.132 liters of triclopyr active ingredient was applied in these spots, giving an average application rate of 0.69 liters per hectare. These application rates would equate to the volume applied per hectare if the entire area was treated. We applied much less volume to our herbicide treatment plots (total area 0.89 ha at four sites) because it was only applied in spots. The chemical weed control treatment applied in circular spots around each longleaf pine seedling resulted in very different volumes of active ingredient being applied in small, medium, and large spots. We calculated that if, for example, three land managers each prescribed one of the spot size treatments we tested, then the prescription with medium size spots would require approximately four times more active ingredient per hectare than the small spots we tested, and four times less herbicide than if the large spots were prescribed (Table 1). Therefore, even with a second 'repeat' application of herbicide in the same spot size, total chemical usage in small spots sprayed a second time would be half the volume used in a single application in medium size spots, and so forth. Implementing the largest spot size across an area would result in 74% of the ground area being treated if 740 stems/ha were planted (Table 1).

Spot size	Small	Medium	Large
Spot radius (m)	0.455	0.892	1.784
Spot area (m^2)	0.650	2.500	10.000
Treated area (ha)	0.048	0.185	0.740
Glyphosate usage (liters ai/ha)	0.332	1.280	5.110

Table 1. Herbicide spot treatment sizes, and comparison of anticipated chemical usage assuming each spot size treatment was applied to 740 seedlings planted on one hectare i.e., treated area is the combined area of 740 spots, and glyphosate usage is the total volume of active ingredient (ai) needed to implement 740 small, medium, or large herbicide spots.

Mowing in the chemical weed control treatment plots extended beyond the circular spots to cover the entire plot area to uniformly reduce aboveground competition. Vegetation in the mechanical treatment plot was also mowed close to ground level manually using motorized brush saws.

Prior to treatment in late June 2008, the following data were collected in all 25 x 25 m measurement plots: longleaf pine seedling status (live/dead), health and physical condition (brown spot infection, sparseness of live foliage, damaged/covered), and total height (if emerging from grass stage). Within 30 cm of each longleaf seedling (0.3 m^2 sample area), herbaceous ground cover percent was estimated occularly and maximum height of herbaceous cover was measured. Within approximately 50 cm of each longleaf seedling (1 m^2 sample area), vine cover percent and woody vegetation cover percent were recorded, and the maximum height of woody vegetation measured. Survival was also assessed at the end of the first growing season, in October 2008. This did not include assessment of competing vegetation due to seasonal discrepancies in cover caused by loss of leaf area among annual plants and deciduous perennials (Fig. 1).

The vegetation assessments were repeated in early June 2009, 11 months after the first assessment and the first set of weed control treatments were applied. All competing vegetation within 1 m^2 quadrats centred on each longleaf pine seedling was assessed. Immediately after the year-two assessment, chemical weed control was re-applied in one of the two chemical treatment blocks at each study site. This repeat herbicide application treatment was named treatment "H2". No treatments were applied in year two to the other chemical weed control plot at each study site. This 'single application' herbicide treatment was named treatment "H1". The mechanical weed control treatment (named "M") was repeated at each study site in year two, reducing aboveground competition from herbaceous vegetation, vines, and woody perennials in the measurement plot and surrounding buffer. Mowing was also applied in the H2 treatment in year two, completing reduction of above- and belowground competition. No treatments were applied to control plots (named "C"). We returned annually thereafter to monitor the development of planted longleaf pine seedlings and competing vegetation, assessing longleaf pine seedling survival, emergence from the grass stage, height of emerged longleaf pine seedlings, and competing vegetation height and cover percent.

Seedling survival and growth data were subjected to monthly growth adjustment assuming an 8-month growing season from April to November. This procedure gave seasonally-adjusted age estimates for seedlings at each assessment event i.e., data for assessments in the first growing season were assigned age 0.5 years (end of June) and 0.875 years (October), with subsequent assessments at age 1.375 years in June of the second growing season, age 2.25 years in May of the third season, and age 3.5 years in July of the fourth growing season. Seedlings were assigned age 0 years at the time of planting in the winter month of March 2008.

3. Survival of planted seedlings

Survival of longleaf pine seedlings was assessed post-treatment, twice in the first growing season, and annually thereafter. Survival over the year immediately following the first treatment (herbicide and mechanical) was highest following chemical control of competing

Fig. 1. Study sites at Hitchiti Experimental Forest in the first spring after broadcast burning and planting of longleaf pine seedlings (A), close-up of newly-planted seedling (B), middle of first growing season, before treatment (C) and immediately following mechanical treatment (D), herbicide spots at end of first growing season (E), and no-treatment control at end of first growing season. *Photo credit: J-P. Berrill (A-D) & Rex Dagley (E, F).*

vegetation (treatments H1 & H2), intermediate following mowing of competing vegetation (M), and lowest in the no-treatment control (C). The repeat application of herbicide to

competing vegetation in the second growing season (H2) enhanced survival, whereas survival declined in the mechanically-treated areas where competing vegetation was rapidly recovering from mowing (Fig. 2).

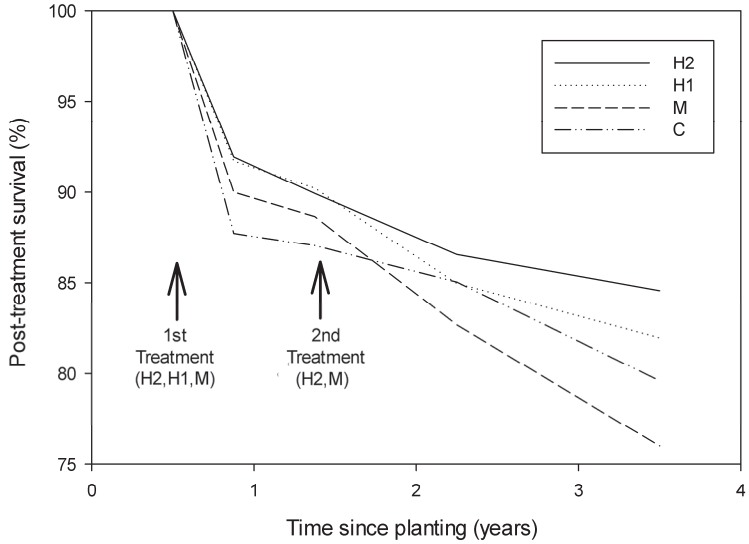

Fig. 2. Survival of planted longleaf pine seedlings from the time of application of the first series of weed control treatments: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Sample size: n=627 (H2: n=157, H1: n=145, M: n=159, C: n=166).

4. Growth of planted seedlings

Rapid restoration of longleaf pine forest requires that seedlings emerge from the grass stage and sustain a higher rate of height growth than adjacent competing vegetation (Fig. 3).

Fig. 3. Longleaf pine seedlings in grass stage (left) and emerged from grass stage (right). *Photo credit: David Combs, USDA Forest Service Southern Research Station, Athens, GA.*

4.1 Emergence from grass stage

The number of seedlings emerging from the grass stage was compared between mechanical and chemical weed control treatments, and between different herbicide spot sizes.

4.1.1 Mechanical vs. chemical weed control

Longleaf pine seedlings treated with herbicide were more likely to emerge from the grass stage sooner than seedlings receiving mechanical weed control or no weed control. Over 60% of seedlings receiving a single herbicide treatment had emerged from the grass stage by the fourth growing season. The repeat application of herbicide in year two resulted in a modest enhancement in emergence with 75% of seedlings emerging by the time of assessment midway through the fourth growing season (Fig. 4). By this time, across the four study sites, the number of emerged seedlings in measurement plots equated to 468, 368, 284, and 236 stems/ha in the H2, H1, M, and C treatments, respectively. The highest frequency of emergence among seedlings occurred sometime between consecutive assessments of the experiment in the months of June in the second growing season and May in the third growing season.

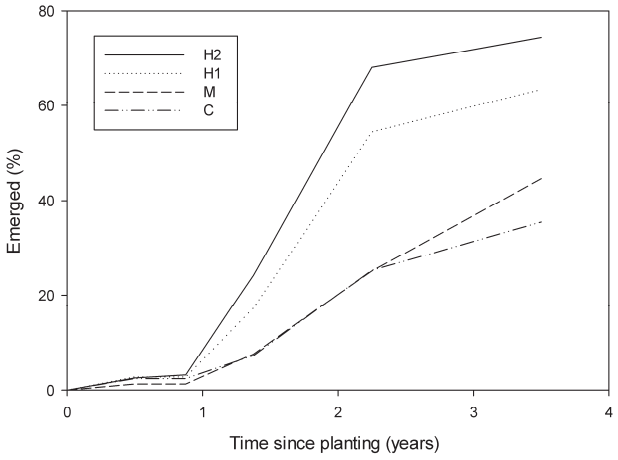

Fig. 4. Proportion of longleaf pine seedlings emerged from grass stage in each weed control treatment: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Sample size: n=627 (H2: n=157, H1: n=145, M: n=159, C: n=166).

4.1.2 Herbicide spot size

The number of seedlings emerging from the grass stage in the year after the initial herbicide treatment ranged from 11-23% and was not significantly affected by size of herbicide spot. The repeat application of herbicide appeared to promote a modest 'wave' of emergence from the grass stage, but without any apparent relation to herbicide spot size (Fig. 5).

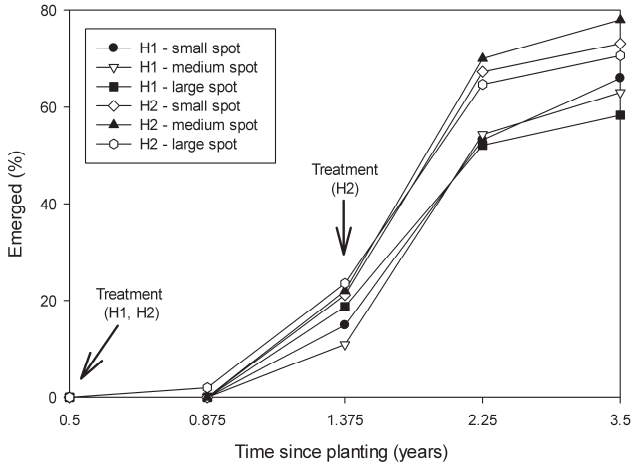

Fig. 5. Proportion of longleaf pine seedlings emerged from grass stage in each herbicide spot size weed control treatment: herbicide applied once in year 1 (H1) and herbicide applied twice (H2), in small (0.65 m^2), medium (2.5 m^2), and large (10 m^2) spots around each planted seedling. Sample size: n=302 (H1-S: n=49, H1-M: n=47, H1-L: n=49, H2-S: n=53, H2-M: n=52, H2-L: n=52).

4.2 Planted seedling height growth

The height development of longleaf pine seedlings that had emerged from the grass stage was compared between mechanical and chemical weed control treatments, and between different herbicide spot sizes.

4.2.1 Mechanical vs. chemical weed control

Height growth of individual seedlings was variable within and between treatments (Fig. 6). Among seedlings that emerged from the grass stage within a year of the first treatments being applied, average height development was most rapid after repeat application of herbicide. Height growth was similar in plots receiving either mechanical treatment or a single herbicide treatment, and slowest in the un-treated control (Fig. 7).

Seedlings emerging at different times caused the average height to rise and fall; the average height of seedlings emerging early increased over time, while later emergence introduced new, shorter seedlings to the calculation of average height. This presented challenges for analysis and testing for differences between treatments. Isolating height data for seedlings that emerged between two consecutive re-measurements somewhat mitigated the problem, and allowed us to test for differences in periodic height increment (rate of growth over a specified period) among seedlings that emerged within the same time period. The periodic average height increment between the third and fourth growing seasons was significantly greater after repeat application of herbicide (78 cm/yr; $p = 0.03$). Periodic height growth was similar in plots receiving either mechanical treatment (64 cm/yr) or a single herbicide treatment (63 cm/yr), and slowest on average in the un-treated control (48 cm/yr).

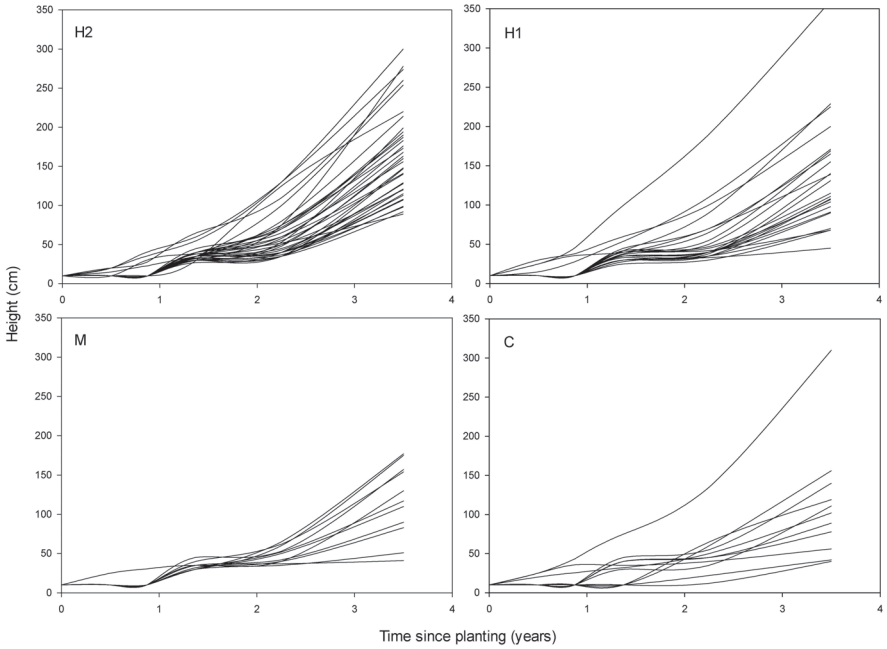

Fig. 6. Height development of individual longleaf pine seedlings that had emerged from grass stage between the time of planting and the middle of the second growing season in each weed control treatment: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Sample size: n=83 (H2: n=37, H1: n=24, M: n=11, C: n=11).

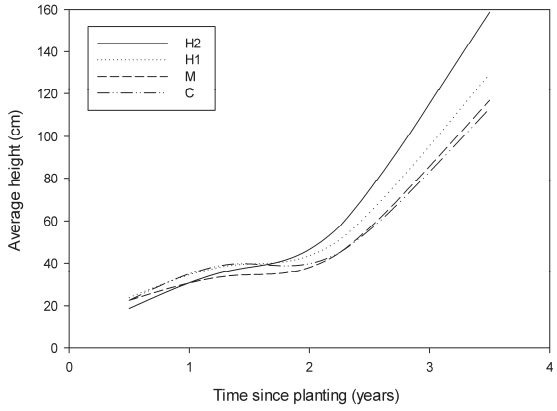

Fig. 7. Average height of longleaf pine seedlings that had emerged from grass stage between the time of planting and the middle of the second growing season in each weed control treatment: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Sample size: n=83 (H2: n=37, H1: n=24, M: n=11, C: n=11).

4.2.2 Herbicide spot size and height growth

Average height development of longleaf pine seedlings that emerged within the year following application of herbicide treatments in year one was enhanced by the repeat application of herbicide. Among spot sizes tested, average height was greatest within large spots and lowest in medium-sized spots (Fig. 8). Part of these differences between treatments was likely caused by a random variable that we were not able to control for: variations in timing of emergence from the grass stage and initiation of height growth. This problem was mitigated by examining the rate of longleaf pine seedling height growth between the third and fourth growing seasons. This 'periodic' height increment was greater on average among seedlings receiving a repeat application of herbicide (Fig. 9). However, differences in height growth between the repeat herbicide applications in small, medium, and large spots were not significant ($p = 0.43$). These repeat treatments resulted in significantly greater seedling height growth than among seedlings treated once with the smallest size of herbicide spot ($p = 0.03$). The statistical significance of differences between spot size treatments was likely understated because: (i) our sample sizes decreased when we restricted the analysis to seedlings emerging within one year of the first herbicide, and (ii) due to variability in periodic height growth data among young longleaf pines in each treatment.

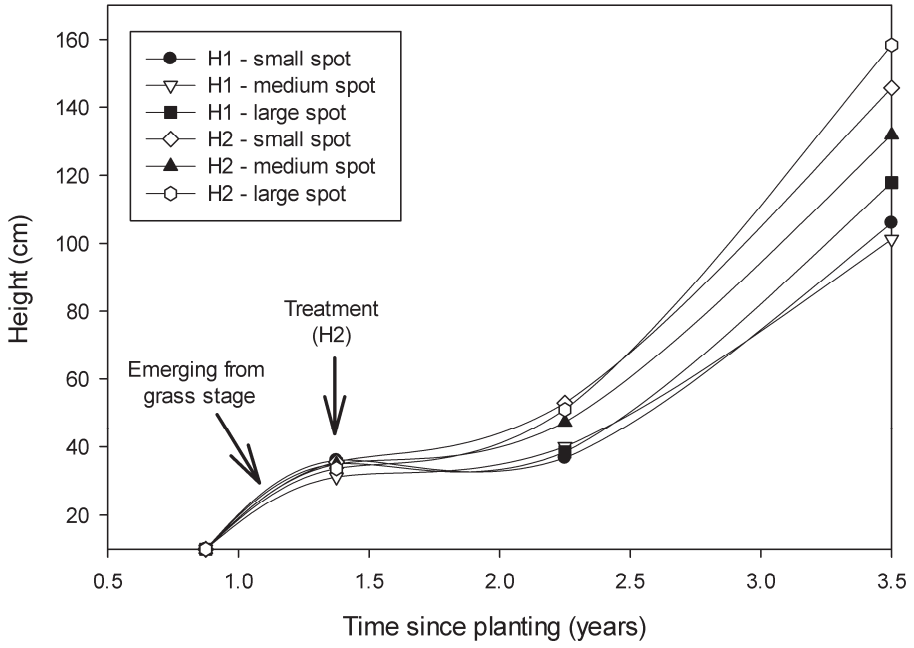

Fig. 8. Average height of longleaf pine seedlings receiving herbicide weed control treatment once (H1) and twice (H2) in small (0.65 m^2), medium (2.5 m^2), and large (10 m^2) spots around each planted seedling. Height data represent average height of seedlings that emerged from grass stage within one year of the first herbicide application. Sample size: n=51 (H1-S: n=7, H1-M: n=4, H1-L: n=8, H2-S: n=11, H2-M: n=10, H2-L: n=11).

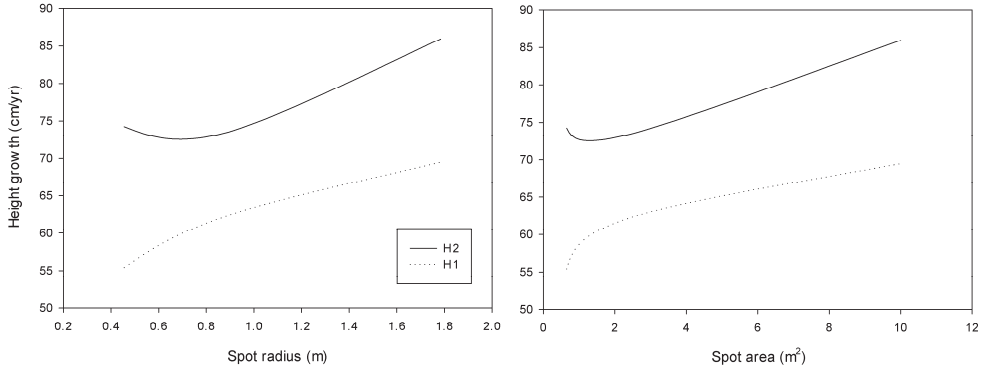

Fig. 9. Relationship between herbicide spot size, number of herbicide applications, and height growth of longleaf pine seedlings emerging from grass stage within one year of the first herbicide application. Height growth calculated as the periodic height increment between the third and fourth growing seasons. Sample size: n=51 (H1-S: n=7, H1-M: n=4, H1-L: n=8, H2-S: n=11, H2-M: n=10, H2-L: n=11).

5. Control of competing vegetation

The extent of competing vegetation cover and its composition were monitored over consecutive growing seasons. Assessment of 1m² quadrats centred on each longleaf pine seedling gave estimates of the percent cover and type of vegetation adjacent to, and presumably competing with, the planted seedlings.

5.1 Weed coverage and composition

Competing vegetation developed quickly in the first growing season. Approximately half of the bare ground around planted seedlings was covered by grasses and forbs, vines, and woody vegetation by the time of the first treatments, three months after planting longleaf pine. The herbicide treatment removed competing vegetation cover in the vicinity of planted seedlings, but only temporarily. Competing vegetation re-occupied herbicide-treated spots at a slower rate than before treatment. Total vegetation cover at the end of the first growing season was only 20% after herbicide treatment, whereas it had attained over 60% cover in the absence of any treatment and following mechanical treatment. In the second growing season, competing vegetation expanded to cover approximately 90% of ground area surrounding planted seedlings in the no-treatment control area and after mechanical treatment. It only covered approximately 50% of ground area in plots receiving a single herbicide treatment by the end of year two, and approximately 25% of ground area in plots receiving a repeat herbicide application in the second growing season. Grasses increased in relative abundance following mechanical treatment. Vine cover increased at the same rate in the control and mechanical treatment areas. Woody vegetation increased in relative abundance, at the expense of grass cover, in the no-treatment control areas. Herbicide treatments had a lasting impact on the development of woody vegetation cover, especially after herbicide was re-applied in the second growing season (Fig. 10).

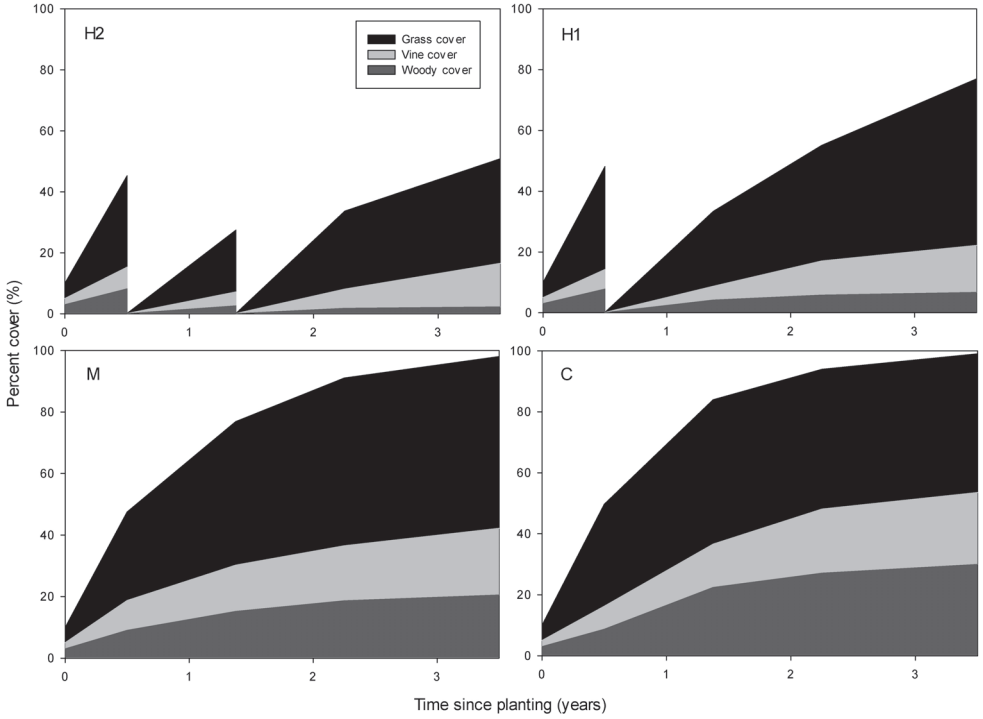

Fig. 10. Weed coverage of ground around planted longleaf pine seedlings. Cover percent is the average cover of each type of competing vegetation in each treatment: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Sample size: n=627 (H2: n=157, H1: n=145, M: n=159, C: n=166).

5.2 Weed height development

Calculating the average of height data for the tallest competing vegetation adjacent to each longleaf pine seedling gave an approximation of the 'top height' or 'dominant height' of the vegetation canopy. The dominant height and percent cover of competing vegetation recovered from each treatment at similar rates, with one exception: mechanical treatment appeared to stimulate height growth of competing vegetation (Fig. 11). Calculating dominant height for different components of the competing vegetation gave separate estimates for woody vegetation and for herbaceous vegetation (grasses and forbs). The height of woody vegetation increased steadily, whereas the height of herbaceous vegetation appeared to attain its maximum within two years of treatment. The time taken for vegetation cover or height to return to pre-treatment levels – referred to as 'treatment persistence' – was shorter (rapid recovery; low treatment persistence) for herbaceous vegetation height than for woody vegetation height or total vegetation cover. The repeat application of herbicide doubled herbicide treatment persistence in terms of vegetation cover, and checked hardwood height development by approximately three years (Fig. 11).

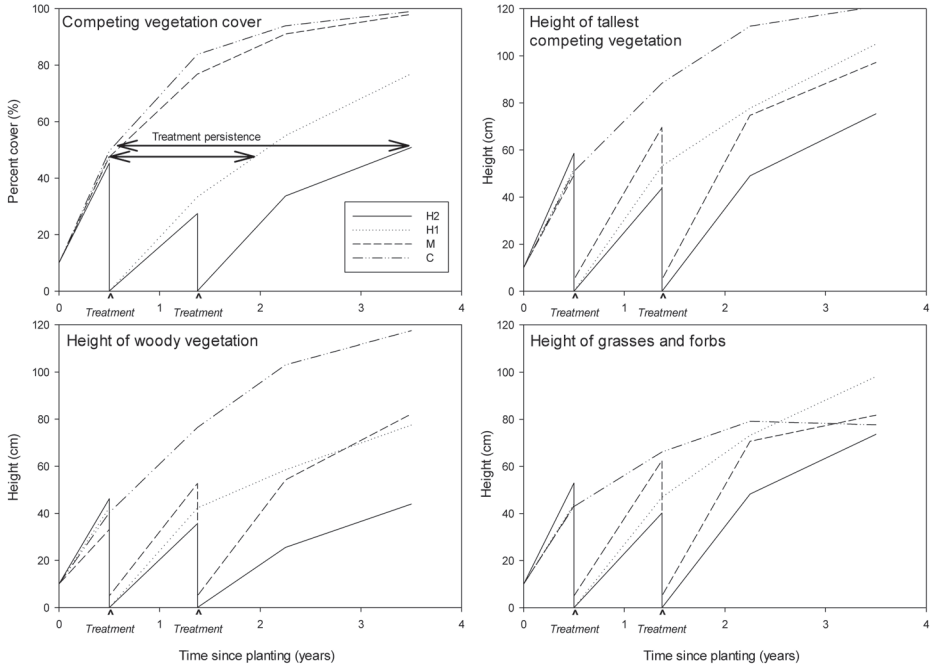

Fig. 11. Development of competing vegetation cover and height in each treatment: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Height of competing vegetation represented by average height of the tallest individual competitor (herbaceous or woody vegetation) in 1 m² quadrat centred on each longleaf pine seedling. Sample size: n=627 (H2: n=157, H1: n=145, M: n=159, C: n=166).

6. Comparing growth of crop trees and woody competitors

The most vigorous individuals in any cohort of planted trees are of notable importance in forest restoration. The expectation is that these trees will dominate and form the main forest canopy. Woody vegetation could represent an ongoing threat to successful restoration of longleaf pine because, unlike herbaceous vegetation, it can sustain height growth and compete with the longleaf pines for light and growing space over the longer term. Longleaf pines that outsize their competitors by several meters should be able to maintain long live crowns, remain vigorous, and retain dominance over competing vegetation. We compared height growth of the tallest longleaf pine seedlings, in terms of average height of the tallest 200 stems/ha, with height growth of their major competitor: naturally-regenerating woody vegetation. The repeat application of herbicide in year two was the only treatment that allowed longleaf pine seedlings to gain a substantial height advantage over adjacent woody vegetation by the fourth growing season. The average height of the tallest 200 stems/ha of longleaf pine in the H2 treatment was 115 cm greater than the average height of competing woody vegetation. By the fourth growing season, the tallest 200 longleaf pine seedlings/ha in no-treatment control plots were an average of 45 cm shorter than the average height of competing woody vegetation in the absence of mechanical or herbicide treatment (Fig. 12).

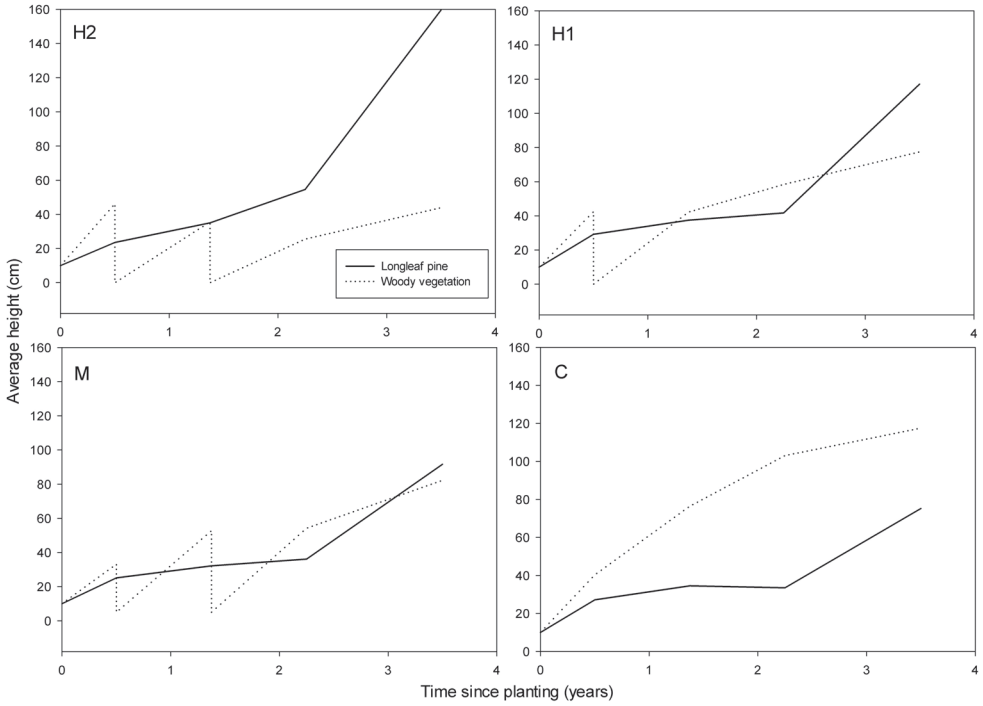

Fig. 12. Height development of the tallest 200 stems/ha longleaf pine seedlings and competing woody vegetation in each treatment: herbicide applied once in year 1 (H1), herbicide applied twice (H2), mechanical weed control (M), and no-treatment control (C). Height of competing vegetation represented by average height of the tallest woody vegetation in 1 m^2 quadrat centred on each longleaf pine seedling. Sample size: n=200 longleaf pine seedlings (n=50 per treatment, representing 200 stems/ha), and n=454 quadrats containing woody vegetation (H2: n=113, H1: n=105, M: n=120, C: n=116).

7. Conclusion

Mechanical control of competing vegetation provided an early enhancement in survival and emergence of longleaf pine seedlings planted in beetle-killed areas, but the beneficial effects were short lived. Herbaceous vegetation exhibited the most aggressive early response to mechanical treatment. The mechanical treatment also appeared to stimulate height development of woody vegetation, resulting in low treatment persistence. Our data suggest that mechanical treatments may need to be repeated regularly if sufficient numbers of longleaf pine are to overtop the competing vegetation. Repeat application of herbicide provided lasting control of competing vegetation, enhanced survival and emergence from the grass stage, and promoted rapid height growth of longleaf pine seedlings planted on the four sites in the central Georgia Piedmont region.

Seedlings emerging from the grass stage began their height growth at different times, providing challenges for summary and analysis of treatment effects on height growth. The

problem was not completely mitigated by examining a subset of data for seedlings that emerged during a single time period between consecutive re-measurements of the experiment; sample size was reduced and differences in timing of emergence still introduced variability in height growth estimates. More frequent re-measurements should overcome this problem by allowing for the study of subsets of seedlings emerging from the grass stage at similar times.

We found no evidence that treating larger areas around planted seedlings with herbicide would promote earlier emergence from the grass stage. Once emerged, the seedlings grew marginally more rapidly, on average, in larger spots. Height growth was significantly more rapid following the repeat application of herbicide in the second growing season than among seedlings receiving only one herbicide treatment in the smallest spot size. Therefore if only one treatment will be applied in future restoration projects, we recommend a larger size of herbicide spot treatment. However, total chemical usage is lower when implementing smaller spots, and more vegetation cover is maintained between the smaller spots. If repeat herbicide treatments are planned, then our results suggest that smaller spot sizes applied twice will provide adequate enhancement of survival, emergence, and growth among planted longleaf pine seedlings.

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Sugar Beet Weeds in Tadla Region (Morocco): Species Encountered, Interference and Chemical Control

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1. Introduction

Sugar beet occupies each year about 65.000 hectares in Morocco which allows a production that approaches or exceeds three million tons of roots, with an average yield of 46 tonnes per ha (54% of national needs sugar consumption). Since its introduction in Morocco in 1962-1963, sugar beet yield increased significantly in quantity and quality. In Morocco, the sugar beet is a very important crop because of its products and by-products, mainly:

- Production of sugar for sugar consuming population.
- Producing leaves, beet tops and pulp wet and dry food that are essential for cattle sheep that is either intended for milk production or to that of meat. It is important to note that major investments such as installation of various agro-industrial units were made.

In Morocco, sugar beet is planted from September through June - July. Yield obtained by farmers, averaging 46T/ha, is significantly below the request potential that would be 90 to 100 T/ha. Many factors contribute to low sugar beet production. Poor stand establishment, inadequate weed control, inadequate insect control and inadequate nitrogen fertilization are the main causes of low tonnage and poor quality sugar beet in Morocco.

The sugar beet is an important strategic crop in the irrigated perimeter of Tadla. During these 5 last years, an annual surface of 12000 ha is emblaved by this crop representing 23% / of the national area. The average yield obtained in the region is approximately 45 to 50 T/ha, which is very low compared to the potential yield.

Sanitary problems particularly weed management is a great constraint to sugar beet production and weeds may cause high yield losses (Rzozi et al., 1990). This paper presents the main results of investigations and experiments conducted in Tadla region to improve the weed management program by identifying mains weed species encountered in sugar beet field, studying the effect of weeds on sugar beet growth and estimating yield losses and determining the critical period of weed control and evaluating herbicide treatments.

2. Sugar beet weeds

2.1 Introduction

The sugar beet is an important strategic crop in the irrigated perimeter of Tadla. During these 5 last years, an annual surface of 12000 ha is emblaved by this crop. The average yield obtained in the region is approximately 45 to 50 T/ha, which is very low compared to the potential yield which would be of 100 T/ha. Several constraints of technical order are at the origin of this low production, among which the weak control of sanitary problems particularly weed management. In order to achieve a good control of weeds, these last must be well identified. Tanji and Boulet (1986) drew up a general floristic and biological inventory of these weeds in Tadla area (All crops included). The objective of this work was to study thoroughly this inventory in sugar beet.

2.2 Material and methods

2.2.1 Presentation of the study area

The plain of Tadla is located at the foot of the Middle Atlas Mountain (Center of Morocco) (Figure 1). This plain has an area of about 360,000 hectares. The altitude varies between 250 m and 500 m and an average of 400 m. According to Emberger climagram , the plain of Tadla has an arid climate with mild winter for the area north of the Oued Oum Er Rabia; winter to charge for the south as well as some of Beni Amir.

In general, natural vegetation is limited to the most degraded soils, the shallower and less suitable for agriculture are sheltered pastures. The average rainfall varies between 556 mm in Beni Mellal as maximum and 327 mm in Dar Ould Zidouh and is averaging 346.6 mm.These datas are decreasing because of climate change. Average monthly temperatures range from 10.2 ° C in January to 28 ° C in August. Minimum monthly temperatures range from 3.23 ° C in January and 18.5 ° C in August and the average maximum temperatures range from 17.8° C in January and 37.5°Cin August.

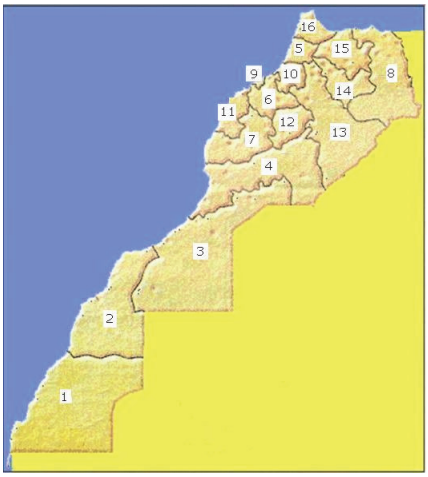

Fig. 1. Localisation of the studied region in Morocco map (12).

2.2.2 Prospecting and sampling

A total of 126 sugar beet fields were explored. Only fields not chemically treated and weedy full kept by farmers were prospected. A stratified sampling according to Gounot (1969) was established taking account of some factors mainly type of soil, rainfall and temperatures. Meanwhile, farmers were questioned about cultural practices and soil samples were taken in order to characterize soil texture and total calcium content.

The method of the "tower field" has been adopted to identify the weed species present (Maillet, 1981), for which an Abundance-Dominance Index (ADI) (+, 1, 2, 3, 4, 5) according to the scale of Montegut (Not dated) modified by Boulet et al. (1989) has been assigned. This index is as follows:

+: Very rare species (1 to 5 feet), virtually no recovery.
1: scarce species, recovery very low, irregular distribution
2: averagely abundant species, low recovery, irregular distribution
3: abundant species, covering less than 50%, regular distribution
4: abundant species, recovery of 50 to 75%, regular distribution
5: very abundant species, recovery from 75 to 100%, regular distribution

The agronomic importance of each species is judged based on its relative frequency and covering. The estimation of the average abundance of species during the reading was conducted assuming equivalences between the ADI and its average covering in percentage (Boulet et al, 1984). The methodology was as follows:

ADI	Covering	Average covering
5	75 – 100	87,5
4	50 – 75	65,5
3	25 - 50	37, 5
2	5 – 25	17, 5
1	1 – 5	5
+	< 1	1

These values allow calculating the average covering R of each species at reading time. The combination of this index, of the absolute frequency of species and their ethological type, allowed attribution of a “Partial Nuisibility Index” (PNI) to species (Bouhache et al, 1984).

PNI = (Sum of coverage/number of reading) x 100. The perennial species are underlined and only species with a frequency higher than 20% are taken in consideration.

Species encountered were identified by using some documents such as Flora Europea (Tutin et al., 1964- 1984), Catalogue des Plantes du Maroc (Jahandiez and Maire, 1931-34) and Mauvaises herbes des regions arides et semi arides du Maroc occidental (Tanji et al., 1988). The ethological type for each species was determined according to classification elaborated by Raunkiaer (1905). The biogeographical origin of weed species was derived from Quezel and Santa (1962-63) and Negre (1961-62) on flora investigations.

2.3 Results and discussion

2.3.1 Systematic aspect

A total of 144 weed species including volunteer wheat belonging to 30 botanical families (Table 1) were inventoried in the 162 sugar beet fields prospected. This number correspond respectively to 43,6% and 17,2% of the total weed flora of Tadla region (Tanji and Boulet, 1986) and of Central West Morocco (Boulet et al., 1989) and is relatively low compared to that observed in the Gharb region (Tanji et al., 1984), more important than that showed in Doukkala region (Bouhache and Ezzahiri, 1993) and similar to that found in Moulouya region (Taleb and Rzozi, 1993).

Dicotyledonous species are prevalent (118 species) and correspond to 81,9% of total encountered. Similar results are shown in other regions where sugar beet is grown. Six families dominated particularly the weed flora (Table 1): Asteraceae, poaceae fabaceae, brassicaceae apiaceae and caryophyllaceae. They provide 51.8% of the total. Representing 81 species , these six families are also dominant in sugar beet in Gharb region (Tanji et al.,1984), in cereals (Taleb and Maillet, 1994) and generally for the national flora (Bouhache and bouleT, 1984 and Ibn Tatou and Fennane, 1989). The most dominant family is the asteraceae, that is represented by 19 species, representing 13.2% of the weed flora found. The Asteraceae is also the richest family in species by about 20.000 species worldwide (Taleb, 1995)

2.3.2 Ethological aspect

According to RAUNKIAER classification, the 144 species surveyed belong to five ethological types (Table 2). The ethological spectrum is dominated by annuals (therophytes) with 119 species or 82,6% of the total. This data is similar to that obtained by the main regional botanical and floristic studies of sugar beet weed flora (Tanji et al., 1984; Bouhache and Ezzahiri, 1993; Taleb and Rzozi, 1993. The Geophytes follow with 17 species (11.8%), bisannuals (hemicryptophytes) with 5 species (3.5%) and the chamaephytes and others with 2 species (2.1%). The geophytes encountered are mainly monocotyledonous species with rhizomes, bulbs and tubers. The most important geophytes species inventoried are Convolvulus arvensis L., Solanum elaeagnifolium Cav. And Cynodon dactylon (L.) Pers. They cause serious problems to the crop.

2.3.3 Biogeographical distribution of species

The Mediterranean weed species (broadly defined) dominate the flora inventoried with 56.2%. This high rate of Mediterranean species confirms those of other authors (Bouhache and Boulet, 1984; Loudyi, 1985; Tanji and Boulet, 1986; Careme, 1990; Taleb, 1995; Wahbi, 1994; Bensellam, 1994) or for the entire Moroccan flora (about 2 / 3 according to Braun-Blanquet and Maire, 1924). European and eurasiatic species represent 5,5 and 4,9 % of the total. Cosmopolitan and sub- cosmopolitan are well represented (8,3%). This seems to be high comparatively to that reported by Bouhache and al., 1993. Concerning endemic species to north west of Africa, they are represented only by *Diplotaxis tenuissiliqua* Del., also reported by Tanji and Boulet (1986).

Famillies	Number of species	Contribution (%)	Ranking
Asteraceae	19	13,2	1
Poaceae	19	13,2	1
Fabaceae	18	12,5	3
Brassicaceae	9	6,2	4
Apiaceae	8	5,5	5
Caryophyllaceae	8	5,5	5
Amaranthaceae	6	4,2	7
Chenopodiaceae	5	3,5	8
Euphorbiaceae	4	2,8	9
Liliaceae	4	2,8	9
Papaveraceae	4	2,8	9
Plantaginaceae	4	2,8	9
Polygonaceae	4	2,8	9
Rubiaceae	4	2,8	9
Convolvulaceae	3	2,1	15
Malvaceae	3	2,1	15
Solanaceae	3	2,1	15
Lamiaceae	3	2,1	15
Boraginaceae	2	1,4	19
Geraniaceae	2	1,4	19
Ranunculaceae	2	1,4	19
Scrophulariaceae	2	1,4	19
Araceae	1	0,7	30
Cyperaceae	1	0,7	30
Iridaceae	1	0,7	30
Portulacaceaa	1	0,7	30
Primulaceae	1	0,7	30
Rhamnaceae	1	0,7	30
Urticaceae	1	0,7	30
Verbenaceae	1	0,7	30

Table 1. Specific contribution of botanical families encountered.

Biological type	%
Therophytes (Annuals)	82.6
Geophytes (Perennials)	11.9
Hemicryptophytes (Bisannuals)	3.4
Chamaephytes and nanophanerophytes	2.1

Table 2. Ethological aspect of sugar beet weed flora in Tadla.

2.3.4 Agronomic aspect

The number of weed species per Sugar beet field varied from 9 to 26 and averaged 17,5. It is relatively low compared to that reported at Doukkala region. The weed survey allowed

Species	**PNI**
Group 1: species with IPN>1000	
Lolium rigidum Gaudin.	1919
Phalaris brachystachys Link.	1530
Triticum aestivum L.	1209
Triticum durum L.	1112
Avena sterilis L.	1059
Convolvulus arvensis L.	1024
Group 2: species with 500<IPN<1000	
Lolium multiflorum Lam.	910
Cichorium endivia L.	787
Anagallis foemina Miller	768
Papaver rhoeas L.	700
Ridolfia segetum L.	672
Medicago polymorpha L.	651
Melilotus sulcata Desf.	642
Phalaris minor Retz.	640
Galium tricornitum Dandy	638
Chenopodium murale L.	635
Chenopodium album L.	590
Sonchus oleraceus L.	572
Lamium ampexicaule L.	570
Sinapis arvensis L.	528
Solanum elaeagnifolium Cav.	521
Malva parviflora L.	501
Fumaria parviflora Lam.	501
Group 3: species with 250<IPN<500	
Emex spinosa (L.) Campd.	401
Rumex pulcher L.	381
Chrysanthemum coronarium L.	325
Bromus rigidus L.	315
Calendula Arvensis L.	301
Vicia sativa L.	270
Chrysanthemum segetum L.	250
Group 4: species with IPN<250	
Polygonum aviculare L.	237
Phalaris paradoxa L.	220
Antirrhinum orontium L.	201
Reseda alba L.	187
Plantago afra L.	150
Scorpiurus vermiculatus L.	132
Vaccaria hispanica Med.	120
Lathyrus ochrus (L.) DG.	104
Cynodon dactylon (L.) Pers.	92

Table 3. Partial Nuisibility Index (PNI) of the most frequent weed species in sugar beet.

identifying 39 major weed species including volunteer wheat that are relatively frequent and cause serious problems and yield loss for the crop (table 3). These species were divided into four groups on the basis of their PNI.

Weeds belonging to group 1 are mainly monocotyledonous species such as *Lolium rigidum* Gaudin., *Phalaris brachystachys* Link., *Avena sterilis* L. And volunteer wheat (*Triticum aestivum* L. And *Triticum durum* L.). This later generally precede sugar beet in the plot. These species competes highly with sugar beet because of their relatively high covering and early emergence in the season. The perennial rhizomatous weed *Convolvulus arvensis* L. is also a dangerous species and it is very difficult to control because of its important vegetative multiplication.

Group 2 contain many species with PNI between 500 and 1000 that also could be noxious for the crop regarding their covering. These weeds are mainly dicotyledonous species such as *Anagallis foemina* Miller, *Papaver rhoeas* L., *Medicago polymorpha* L., *Chenopodium album* L., *Sinapis arvensis* L., *Galium tricornitum* Dandy. *Solanum elaeagnifolium* Cav. is deep rooted weed and a very troublesome species in all Tadla region.

Other species with relatively low covering (Groupe 3 and 4) are often encountered in sugar beet field but they are less competitive compared to those belonging to group 1 and 2: *Rumex pulcher* L., *Chrysanthemum coronarium* L., *Bromus rigidus* L., *Calendula Arvensis* L., *Vicia sativa* L., *Chrysanthemum segetum* L., *Reseda alba* L., *Plantago afra* L., *Scorpiurus vermiculatus* L., *Vaccaria hispanica* Med.

2.4 Conclusion

The sugar beet weed flora in Tadla region is much diversified. Effectively, 144 species belonging to 30 botanical families were encountered in the 126 field prospected. The most represented families are asteraceae, poaceae, fabaceae, bracassicaceae, apiaceae and caryophyllaceae. Therophytes (annuals) and dicotyledonous species dominate with 82,6% and 81,9 respectively. The floristic diversity vary from 9 to 26 species per field and it average 17, 5. The weed survey allowed identifying 39 major weed species including volunteer wheat that are relatively frequent and cause serious problems and significant yield losses for the crop.

3. Weed interference and critical period

3.1 Introduction

Weeds compete with crop plants for water, light nutrients and space and cause considerable yield losses. Integrate weed management (IWM) involves a combination of cultural, mechanical, biological, genetic and chemical methods for effective and economical weed control (Swanton and Weise, 1991). The principles of IWM should provide the foundation for developing optimum weed control systems and efficient use of herbicides. The critical period for weed control (CPWC) is a key component of an IWM program. Weeds are limiting factors in sugar beet production (Cooke and Scott, 1993). Integrated weed control management is necessary for minimizing weeds interference and maximizing the crop yield (Schweizer, 1983; Cooke and Scott, 1993).

The critical period of weed interference refers to the period during which a crop must be kept free of weeds in order to prevent yield loss. It represents the time interval falling between two separate components: (a) the minimum length of time after seeding that a crop must be kept weed-free so that later-emerging weeds do not reduce yield, and (b) the maximum length of time that weeds which emerge with the crop can remain before they become large enough to compete for growth resources (Radosevich and Holt, 1984; Zimdahl, 1988; Weaver *et al*., 1992; Baziramakenga and Leroux, 1994; Ghadiri, 1996).

Sugar beet can tolerate weeds until 2-8 weeks after emergence, depending on the weed species, planting date, the time of weed emergence relative to crop and environmental conditions (Cooke and Scott, 1993). The presence of weeds can decrease sugar beet yield by 90%. For example, a single presence of barnyardgrass *Echinochloa crus-galli* (L.) *Beauv.* plant per 1.5 m2 resulted in yield reduction of 5 to 15 % (Norris, 1996). The earliest date at which weeding could cease in sugar beet without significant yield loss has been shown to be between 4 and 12 weeks, depending on sowing date, rainfall and weed infestation (Link and Koch, 1984; Scott et aI., 1979; Singh et aI., 1996). Studies on the competitive effect of weeds in sugar beet have been numerous under temperate climates (Dawson, 1965; Farahbakhsh and Murphy, 1986; Schweizer and Dexter, 1987; Scott et aI., 1979; Zimdahl and Fertig, 1967). Continuous post-planting hand-weeding for 17 weeks and 15 weeks in 1990, and for 15 weeks and 12.5 weeks in 1991 were required to limit sugar beet root yield loss to 5% and 10%, respectively In Gharb region (Alaoui et al., 2003). Based on 10% loss of yield, the beginning of the critical period of weed control (CPWC) was 25 and 5 days after planting for the first year and the second year, respectively. On this basis, the end of the critical period of weed control was 78 days for the first year and 88 after planting for the second year (Salehi et al., 2006).

This research was conducted to study (i) the effect of weed competition on sugar beet growth parameters and (ii) determine the minimum period sugar beet should be kept weed-free after planting (CPWC) in the Tadla region to limit yield loss from late emerging weeds

3.2 Material and methods

3.2.1 Experimental site localization and characterization

Field experiment was conducted during two growth seasons 2003- 2004 and 2004-2005 at Afourer experimental station of the National Institute of Agricultural Research in Tadla region. The soil characteristic are as follows: 2.72 % organic matter, 11% sand, 37.2% silt, 51.6 % clay, and pH 8.1. Plots were plowed, disked three times and harrowed for seedbed preparation. Sugar beet cv. 'lydia', a mono germ variety, was seeded manually in a 2 cm deep in 70-cm wide rows with a spacing of 10 cm between seeds (population of 83,000 plants/ha) on October 15 in 2003 and November 25 in 2004.

Fertilization, irrigation and diseases and predators control were achieved in experimental plots according to those recommended by the sugar regional comity.

3.2.2 Competition duration

To determine the critical period of weed control in sugar beet, an experiment was conducted and consisting of 16 treatments. Weed free treatments included the removal of weeds at 4, 7,

9, 11, 13, 17 and 21 weeks after emergence (WAE) of sugar beet. In weed infested treatments, weeds were allowed to interfere with sugar beet crop 4, 7, 9, 11, 13, 17 and 21 weeks after emergence sugar beet crop. Two control treatments (full-season control of weeds and full-season interference of weeds) were also included. Individual plots consisted of 10 rows, each 10 m long.

3.2.3 Experimental design and statistical analysis

The experiment was a randomized complete block design with four replicates. Data on weeds and on sugar beet growth parameters and yield components were subjected to an analysis of variance using statistical STATITCF software. The means were compared using Fisher's protected LSD ($\alpha = 0.05$).

3.2.4 Measurements

Weed density is not as reliable as biomass to assess weed interference in a crop (Scott et aI., 1979; Tomer et aI., 1991; Wilson and Peters, 1982), especially for species which have a high capacity to compensate for low densities through tillering and branching. Therefore, the impact of weed-free and weedy duration on weed growth and on crop growth and crop yield was assessed through weed dry weight. Weed dry weight were measured during the entire growing season for all individual plots. Four 0.5 m x 0.5m quadrates per plot were placed randomly over the plot. Weeds within the sampling area were removed by hand, taken to laboratory and dried at 60 C for 48 h to determine total weed dry weight. Sugar beet growth was assessed at the same time as weed sampling. Six sugar beet plants without root were taken randomly in plot but not on central rows that served for estimating yield. The number of leaf per plant, leaf area and dry matter was determined. Because of unavailability of an electronic leaf area meter, a graduated table was used for measuring leaf area. Sucrose percentage and the concentration of impurities (sodium, potassium, amino-N) were measured at the regional sugar factory.

3.3 Results and discussion

3.3.1 Effect of Weed free and weedy periods on weed dry matter

The dominant weeds observed in 2003 were volunteer wheat (ADI = 4), *Phalaris brachystachys* Link.(3), *Avena sterilis* L. (2), *Cichorium endivia* L. (4), *Papaver rhoeas* L. (3), *Ridolfia segetum* L. (3), *Sinapis arvensis* L. (3), and *Galium tricornitum* Dandy (2). With the exception of field bindweed (*Convolvulus arvensis* L.) (3), the same weed species were dominant in 2004. Weed free periods resulted in lower weed dry matter and weedy periods resulted in high weed dry matter (Figure 2). Maximum total weed dry weight generally decreased as weed-free duration was increased. The statistical analysis showed a highly significant difference (Not shown).

These findings are similar to those observed by Salehi et al. (2006), Rzozi (1993) and Alaoui et al. (2003). Weed growth was reduced drastically after a weed free duration greater than 17 WAE in both years. Same results were obtained for all the two years 2003 an 2004. For the later, weed dry matter was relatively lower because the later date of sowing results generally in low weeds density.

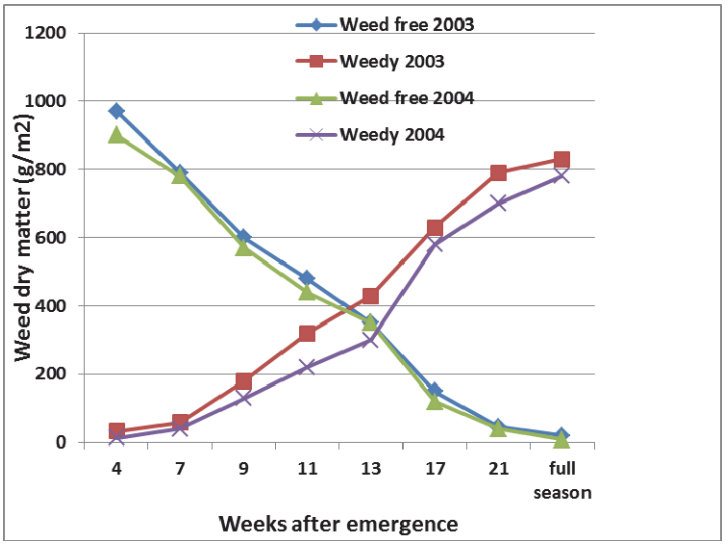

Fig. 2. Effect of competition duration on weed dry matter.

3.3.2 Effect of weed free and weedy periods on sugar beet growth parameters

All sugar beet growth parameters were affected by the presence of weeds. Effectively, the sugar beet leaf number decreased as weedy periods increased and in contrast it increased as weed free periods increased (Figure 3). Also, the leaf area decreased as weedy periods increased. This parameter was highly significantly reduced because of the important competitive effect of weeds. (Figure 4). The crop leaf dry matter was also significantly reduced by the weed competitive effect. The longer the weedy period the lower sugar beet dry matter. The later increased as the weed free period increased (Figure 5). These results confirm those of Alaoui et al. (2003) reporting that the leaf area and the other growth parameters are vigorously decreased by the competitive effect of weeds.

3.3.3 Effect of weed free and weedy periods on sugar beet yield, on sugar yield and sugar content

Weed infestation reduced root yield in all treatments. The presence of weeds during the entire growing season decreased root yield by 97.6 % and 68.9 % in 2003 and 2004, respectively, as compared to full season weed free check. Although sugar content did not show any significant difference between various treatments in both years, weed infestation decreased sugar yield, their corresponding yields decreased considerably in infested treatments. For example, season-long weed infestation decreased sugar yield by 89.8% and 81.1 % in 2003 and 2004, respectively, as compared to weed free check (data not shown). The concentration of sugar beet impurities such as potassium, sodium and amino nitrogen were not affected by weed competition (data not shown).

In most years in Morocco, weeds can cause more than 75% yield reduction (Rzozi, unpublished data; Rzozi et al., 1990). Such reductions indicate complete crop failure because small sugar beet roots produced under severe weed competition cannot be processed. In

other countries, weeds also seriously suppress sugar beet yield (Schweizer and Dexter, 1987).

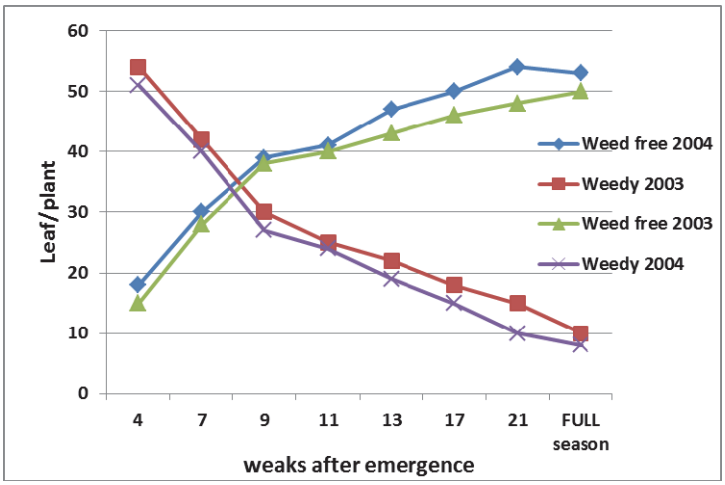

Fig. 3. Effect of weeds on sugar beet leaf number.

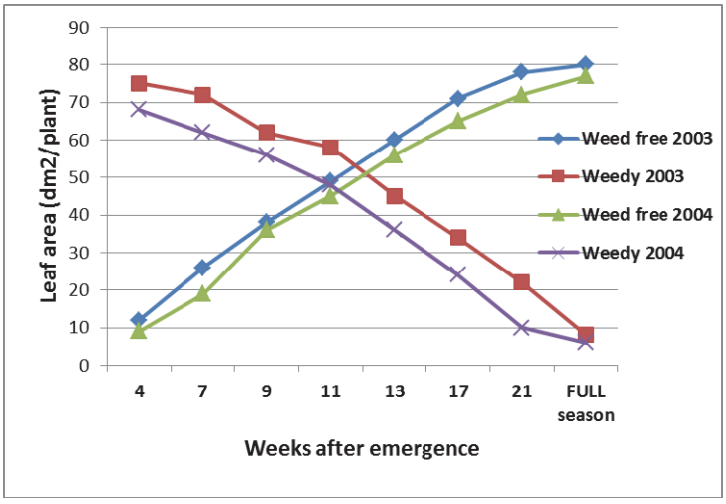

Fig. 4. Effect of weeds on sugar beet leaf surface.

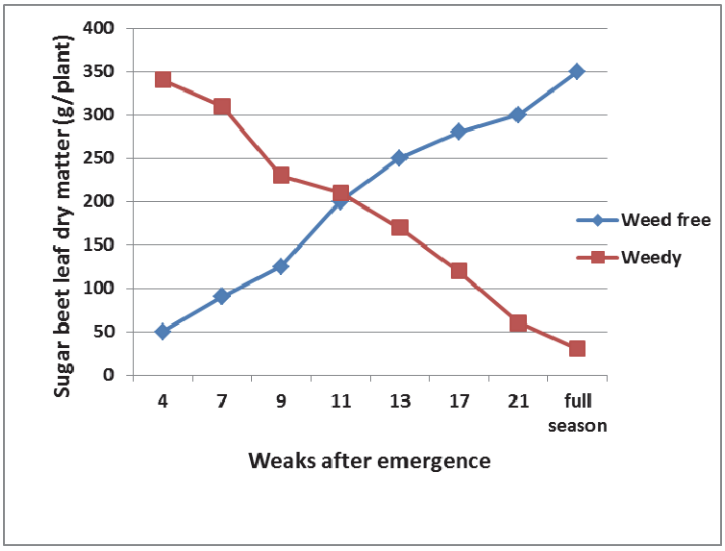

Fig. 5. Effect of weeds on sugar beet leaf dry matter.

3.3.4 Critical period of weed control

Weed interference caused a sharp decline in sugar beet root yield in both years (Figure 6 and 7). Based on 10 % permissible decrease in root yield, weeding should start from 4 WAE and 7 WAE in 2003 and 2004, respectively (Figure 6 and 7). For the given 10% root yield reduction, weed control should be continued until 15 WAE and 12 WAE in 2003 and 2004, respectively (Figure 5 and 6). Weed interference caused a sharp decline in sugar yield (data not shown). Based on 10 % permissible decrease in root yield, weeding should start from 3.5 WAE and 7 WAE and must be continued until 15 WAE and 11 WAE in 2003 and 2004 respectively.

The results show that the critical period begins earlier in 2003 and its duration is longer comparatively to that observed in 2004 which is shorter and begins relatively later. This may be due to date of sowing. Effectively, in 2003, sugar beet was sown October 15 and this allows to many weed species, particularly gramineous including volunteer wheat, to germinate and emerge in great number and vigorously at the same time of the crop germination and emergence. In 2004, sugar beet was sown 25 November. At this time, a great number of weed species (mainly gramineous) has germinated and emerged from soil and destructed during the seedbed preparation.

Emergence time of weeds influences the critical period of weed control (Zimdahl, 1987; Weaver *et al.*, 1992; Mesbah *et al.*, 1994; Ghadiri, 1996). In Shahrekord, sugar beet is planted in May and June; this delay in seedbed preparation and planting may lead to earlier germination of weeds over the sugar beet crop. Therefore, critical period of weed control starts earlier and its duration is longer. At early growth stages, sugar beet has a low competitive ability against weeds; as a result critical period would start sooner. In 2003, presence of weeds for the entire growing season reduced root yield by 97.6% relative to weed free control. In 2004, the reduction was 68.6 %. A similar 71% root yield reduction was

also observed by Shahbazi and Rashed Mohassel (2000). Dawson (1977) showed that annual weeds that germinate during a 2-week period after planting or a 4-week period after two-leaf stage in sugar beet reduce root yield by 26 to 100%. Therefore, effective control of weeds at early stages seems to be more important than that of later developed stages. The closure of crop canopy at later growth stages suppresses the late-emerging weeds. The increased period of weed competition reduces the photosynthesis and crop growth

(Zimdahl, 1987; Ghadiri, 1996). Longer presence of weeds caused more use of environmental resources (light, water, and nutrients) and more accumulation of dry matter in weeds, making the critical period longer and, therefore reducing root and white sugar yield of the sugar beet crop.

3.4 Conclusion

A field experiment was conducted during two growing seasons 2003/2004 and 2004/2005 to assess the effect of weeds on sugar beet growth parameters and sugar beet yield and to determine the critical period of weed control (CPWC). Weed free treatments and weed infested treatments included the removal (or not) of weeds at 4, 7, 9, 11, 13, 17 and 21 weeks after emergence of sugar beet. Dry matter of weed, sugar beet leaves/plant, sugar beet leaf area and sugar beet dry weight was measured during all growing season. Weed free periods resulted in lower weed dry matter and weedy periods resulted in high weed dry matter. Maximum total weed dry weight generally decreased as weed-free duration was increased. The presence of weeds during the entire growing season decreased root yield by 97.6 % and 68.9 % in 2003 and 2004, respectively. All crop growth parameters were significantly reduced by weed infestation.

The critical period of weed control began at 4 and 7 weeks after sugar beet emergence (WAE) and continued until 15 and 12 WAE in 2003/2004 and 2004/2005 respectively depending on sowing period. It was concluded that the CPWC is longer in 2003/2004 than in 2004/2005.

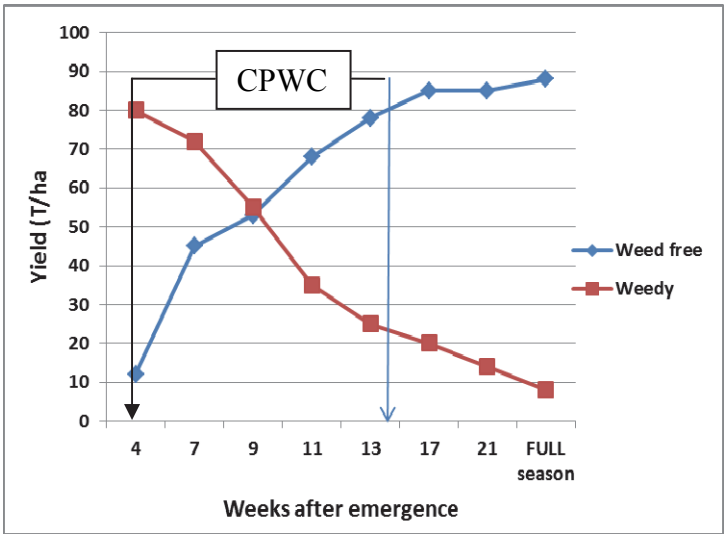

Fig. 6. Critical period of weed control (2003/2004).

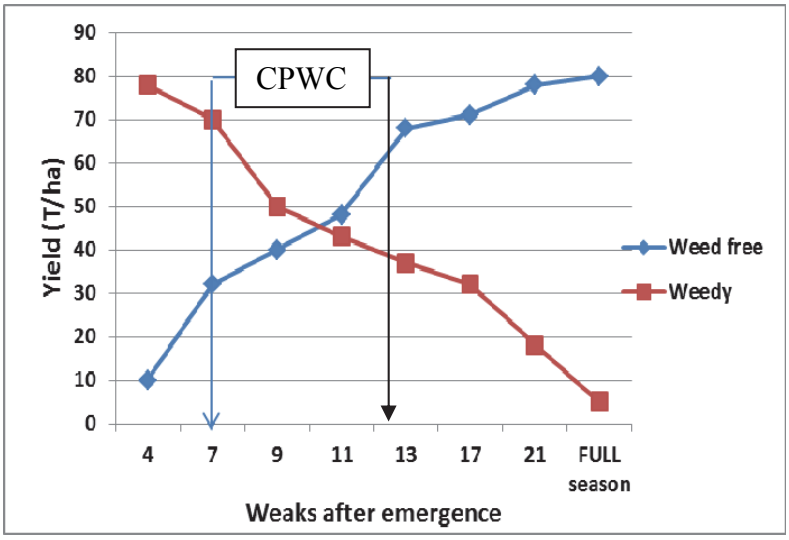

Fig. 7. Critical period of weed control (2004/2005).

4. Chemical control of sugar beet weeds

4.1 Introduction

In the area of Tadla, sugar beet is regarded as an important crop. Weeds constitute a great constraint to crop production improvement and cause important yield losses (Rzozi and al., 1990). The farmers do not use herbicides efficiently. Generally, only one herbicide is applied and the results are not satisfactory (Baye et al, 2004). This work aims to develop a chemical weed control program by evaluating the effectiveness of some herbicide treatments.

4.2 Material and methods

4.2.1 Field experiment localization

A field experiment was conducted during the two sugar beet growing seasons 2003/2004 and 2004/2005 in three location Fqih Ben Salah, Afourer and Deroua to assess the efficacy of some herbicide treatments. These locations were chosen in order to have diversified weed flora and then have maximum information about herbicide activity spectrum.

4.2.2 Herbicides and herbicide treatments studied

The main and important herbicides homologated on sugar beet and registered in Morocco such as ethofumesat, desmedipham, phenmedipham, metamitron, triflusulfuron methyl and lenacil were experimented (Table 4). Thes active ingredients were tested either alone or in mixture (Table 5). A hand weeding taked place for all treatments when it was necessary.

4.2.3 Observations on weeds

The importance of weeds encountered in field experiments was estimated according to the Abundance – Dominance-Index (ADI).

4.2.4 Evaluation of herbicide efficacy

Weed dry weight were measured during at 60 days after treatments (DAT) for all individual plots. Four 0.5 m x 0.5m quadrates per plot were placed randomly over the plot. Weeds within the sampling area were removed by hand, taken to laboratory and dried at 60° C for 48 h to determine total weed dry weight. The efficacy in percentage (%) for each treatment is calculated comparing its dry matter to that of the check.

4.2.5 Observations on the crop

The sugar beet yield was estimated on the two central rows at harvest. Sucrose percentage and the concentration of impurities (sodium, potassium, amino-N) were measured at the regional sugar factory.

4.2.6 Experimental design and statistical analysis

The experiment was a randomized complete block design with four replicates. Individual plots were 4m x 8m size. Data on efficacy (%) were first transformed to Arc Sin% if necessary. Sugar beet yield and efficacy data were subjected to an analysis of variance using statistical STATITCF software. The means were compared using Fisher's protected LSD (α = 0.05).

Commercial product	Active ingredient
Tramat Combi	30 % ethofumesat + 12 % lenacil
Betanal Progress	16 g/l desmedipham + 62 g/l phenmedipham + 128 g/l ethofumesat
Goltix	70 % metamitron
Safari	70 % triflusulfuron methyl
Venzar	80 % lenacil
Fusilad Super	125 g/l Fluazifop p-butyl

Table 4. herbicides tested.

4.3 Results and discussion

4.3.1 Importance of weed flora

In Fqih Ben Salah location, weed flora is dominated by gramineous mainly volunteer wheat. Some dicotyledonous species such as *Malva parviflora, Medicago polymorpha, Emex spinosa* and *fumaria parviflora* are important (Table 6). In Deroua location, infestation by gramineous was low and *cichorium endivia*, *Sinapis arvensis* and *convolvulus arvensis* were dominant in 2003/2004 and *Rumex pulcher*, *Papaver rhoas* and *Ridolfia segetum* dominated the weed flora in 2004/2005. Concerning Afourer, C*ichorium endivia*, *Sinapis arvensis, Polygonum aviculare, Lamium amplexicaule and Ridolfia segetum* were the most important species in both two growing season.

4.3.2 Efficacy of the herbicide treatments

Generally, fluazifop- p- butyl (Fusilade Super) achieved a good gramineous control (data not showed). However, it is important to mention that some ray grass (*Lolium* spp) population had recently developed resistance to this herbicide.

Treatments	Herbicide treatments tested
T1	Tramat Combi (3,5l/ha) in post sowing preemergence
T2	Goltix (5kg/ha) applied in post sowing preemergence
T3	Goltix (5kg/ha) in 2 applications (2,5 + 2,5) kg/ha post emergence (2 true leaves stage)
T4	Safari (60g/ha) in 2 applications (30+30) g/ha) post emergence (2 true leaves stage)
T5	Betanal Progress (5l/ha) in 2 applications (3 + 2) l/ha g/ha) post emergence (2 true leaves stage)
T6	(Safari (30g/ha) + Venzar (200g/ha) applied twice post emergence (2 true leaves stage)
T7	(Betanal Progress(1,25l/ha) + Safari (30g/ha)) applied twice post emergence (2 true leaves stage)
T8	(Betanal Progress (1,25l/ha) + Goltix (1kg/ha)) applied twice post emergence (2 true leaves stage)
T9	(Goltix (1kg/ha) + Safari (30g/ha) applied twice post emergence (2 leaf stage)
T10	(Betanal Progress (1L/ha) + Goltix (300g/ha) + Venzar (100g/ha)) applied twice post emergence (2 true leaves stage)
T11	(Betanal Progress (0,8L/ha) + Safari (30g/ha) + Goltix (300g/ha) + Venzar (100g/ha)) applied twice post emergence (2 true leaves stage)
T12	Hand weeding (Three times in the season)
T13	Check (Not treated)

Table 5. Herbicides treatments experimented.

In order to control gramineous species, all first post emergence application are mixed with Fusilade Super (1l/ha); an oil concentrate adjuvant (Seppic at 1/ha) is adjusted to the two application to obtain satisfactory activity. The second application is made 10 days after the first.

Concerning post sowing preemergence application treatments, Tramat combi (T1) provided good efficacy (90 % and more) and protected then the crop for a long period more than 2 months (Table 7). This allowed to sugar beet to grow vigorously. The treatment controlled both dicotyledonous and monocotyledonous species except *Emex spinosa* that showed some tolerance to this herbicide. The other treatment applied preemergence (T2) showed not satisfactory with efficacy lower than 68 %. This herbicide did not control monocotyledonous (volunteer wheat included) and many other dicotyledonous species such as *Medicago polymorpha and Melilotus sulcata.*

For post emergence applications, it was noted that when treatments were applied alone (not mixed), the efficacy was not satisfactory. Effectively, efficacy was generally below 70 % except T5 in 2004/2005 at Deroua (Table 7). In this case, the percent control is above 80 %.

This difference in efficacy is explained mainly by the herbicide activity of each one. Safari (T4) provides low control against *Papaver rhoeas, Chenopodium album, Anagallis foemina, stellaria media, Cichorium endivia and Fumaria parviflora.* In contrast, it achieves good control against many other important species particularly malvaceae, *Malva parviflora*, apiaceae such

as *Ridolfia segetum* and *Ammi majus* and brassicaceae maily *Sinapis arvensis.* Goltix (T3) did not control apiaceae, malvaceae and other species; however, it provides good control of polygonaceae such as *Rumex pulcher* and *Emex spinosa.* Betanal Progress presented the most large herbicide activity spectrum and controlled great number of species even applied alone in some times. This is the case of Deroua in 2003/2004. The efficacy obtained is 82%.

Generally, treatments achieved good efficacy when applied in tank mixtures than when applied individually alone because of their complementarily in eliminating maximum weed species. So, this must be taken in consideration in a weed chemical management program.

	Fqih Ben Salah		**Afourer**		**Deroua**	
Species	**2003/04**	**2004/05**	**2003/04**	**2004/05**	**2003/04**	**2004/05**
Volunteer wheat	4	3	1	2	2	2
Phalaris brachystachys	1	3	3	3	2	2
Lolium rigidum	3	2	2	1	1	1
Avena sterilis	2	2	1	2	2	2
Bromus rigidus	+	+	+	+	+	1
Malava parviflora	4	3	+	+	+	1
Emex spinosa	3	1	2	+	+	2
Rumex pulcher	+	+	1	1	2	4
Anagallis foemina	1	4	3	3	+	2
Chenopodium album	2	3	3	1	1	2
Fumaria parviflora	3	3	1	1	+	1
Cichorium endivia	+	2	4	4	4	2
Convolvulus arvensis	+	1	2	3	3	3
Sinapis arvensis	1	1	4	4	1	2
Sonchus oleraceus	1	1	2	2	1	2
Polygonum aviculare	1	+	4	3	+	1
Lamium amplexicaule	1	+	4	3	1	2
Medicago polymorpha	4	+	3	2	1	2
Melilotus sulcata	2	1	2	+	1	1
Papaver rhoeas	2	+	3	2	+	4
Ridolfia segetum	+	+	2	4	1	3
Ammi majus	-	+	+	+	+	+
Stellaria media	-	+	+	+	+	3
Veronica polita	-	+	+	1	-	1
Torilis nodosa	-	-	+	+	-	-
Euphorbia exigua	-	-	+	+	-	-
Galium aparine	-	-	1	1	-	1
Capsella bursa-pastoris	-	-	+	+	2	3

Table 6. Weed species encountered in field experiments.

4.3.3 Effect of herbicide treatments on sugar beet yield

Weed presence in sugar beet during all season caused yield losses between 86 and 93% following the nature of weed flora and the location. Herbicide treatments did not affect the sugar content percentage (Data not showed). Sugar beet yield was significantly affected by the herbicide treatments (Table 8). The post sowing preemergence treatment (Tramat Combi) achieved a satisfactory yield averaging 75 T/ha. This is due to its good weed control achievement during a long period. When used in tank mixtures (particularly 3 and 4 products), herbicide treatments provide high yields (Table 8). It is important to mention that weed chemical treatment alone is generally not sufficient to provide good root sugar beet production and it must be followed by other weed control methods such as mechanical, cultivation and hand weeding.

Treatments	Fqih Ben Salah		Afourer		Deroua	
	2003/04	2004/05	2003/04	2004/05	2003/04	2004/05
T1	89.5a	86a	87.3a	86.9a	90.1a	92.6a
T2	62c	60.3c	65c	65.9b	64bc	68.4b
T3	69.3b	60.7c	65.2c	66.8b	69.5b	65.2bc
T4	65.4bc	69b	63.5c	65.2b	61.4c	50.1d
T5	72b	79ab	75b	70b	82a	62.9c
T6	75b	72.2b	65c	69b	72.6b	62c
T7	84a	86.4a	87.8a	85.7a	86.9a	66bc
T8	75.1b	79b	76b	62b	80.1a	74.1b
T9	72b	75.6b	69.3c	67b	65b	72.b
T10	75b	77b	62c	60b	69b	75b
T11	88.2a	86.7a	88.1a	86.3a	88.6a	80.6a
T12	70b	73b	74b	69b	73b	65bc

Means within columns followed by different letters are significantly different at $\alpha = 0.05$.

Table 7. Efficacy of herbicide treatments (%) at 60 DAT.

Treatments	Fqih Ben Salah		Afourer		Deroua	
	2003/04	2004/05	2003/04	2004/05	2003/04	2004/05
T1	74a	72.9a	75a	74.6a	78a	80.2a
T2	52.3c	53.6c	51c	49c	53.3bc	51.6c
T3	54.3bc	51c	50.9c	52.3c	53.4bc	50.3c
T4	52c	53.2b	52.6c	51c	50c	46c
T5	60b	62b	60.3b	59.2b	68a	51c
T6	62.1b	61.4b	54bc	58bc	60.6b	50.6c
T7	69a	70.5a	71.2a	72a	71.9a	73.2a
T8	61.6b	63b	64.5b	65.1b	69a	61b
T9	53c	60b	51c	50c	49c	52.3c
T10	60.8b	62.6b	57bc	55.4c	59b	61b
T11	70.2a	71.3a	72.6a	71.9a	73.3a	72.8a
T12	49.8c	50.9c	51c	52c	50.2c	51.9c
T13	7.2d	5d	3.9d	8d	4.8d	6.3d

Means within columns followed by different letters are significantly different at $\alpha = 0.05$.

Table 8. Effect of herbicide treatments on sugar beet yield.

Many studies relative to sugar beet weed chemical control were achieved in Morocco and other counties. Bensellam et al. (1993) reported that phenmediham + pyrazone achieved good control of weeds in sugar beet. Rzozi et al. (1990) found that nor metamitrone followed by phenmedipham neither chloridazone applied preemergence gave good efficacy. El Antri (2002) reported that triflusulfuron methyl + lenacil + clopyralid achieved good control of weeds in sugar beet. El Ghrasli and Allali (2002) estimated that farmers in Gharb region could use Safari, Goltix, Betanal and Venzar to control weeds in sugar beet. The pre sowing and preemergence herbicides: Tramat Combi and Goltix and the post emergence safari, Goltix, Betanal Progress and Venzar are widely used in France (Anonymous, 1999) and in USA (Stachler, 2011).

4.4 Conclusion

A field experiment was conducted during two growing seasons 2003/2004 and 2004/2005 in three locations in Tadla region to evaluate the effectiveness of some herbicides treatments. The main and important herbicides homologated on sugar beet and registered in Morocco such as ethofumesat, desmedipham, phenmedipham, metamitron, triflusulfuron methyl and lenacil were experimented individualy alone or in tank mixtures.

Tramat combi (Ethofumesate + lenacil) applied post sowing preemergence provided good efficacy (90 % and more) and protected then the crop for a long period more than 2 months.

Generally when applied post emergence, herbicides ethofumesate, metamitron, triflusulfuron methyl, phenmedipham, desmedioham and lenacil achieved good efficacy in tank mixtures than applied individually alone because they are complementarily in eliminating maximum weed species. So, this must be taken in consideration in a weed chemical management program. These herbicide treatments allow to crop to grow without weed competitiveness nearly until the end of the critical period and are often followed by a mechanical cultivation or a hand hoeing.

5. General conclusion

In Morocco, sugar beet is an important strategic crop. It is planted from September through June - July. Yield obtained by farmers, averaging 50 T/ha, is significantly below the request potential that would be 90 to 100 T/ha. Many factors contribute to low sugar beet production. Poor stand establishment, inadequate weed control, inadequate insect control and inadequate nitrogen fertilization are the main causes of low tonnage and poor quality sugar beet in Morocco.

This paper presents the main results of investigations and experiments conducted in Tadla region to improve the weed management program by identifying mains weed species encountered in sugar beet field, studiying the effect of weeds on sugar beet growth and estimating yield losses and determining the critical period of weed control and evaluating herbicide treatments.

One hundred twenty six (126) fields of sugar beet were surveyed by stratified sampling in Tadla region (Center of Morocco). In total, 144 weed species belonging to 30 botanical families were recorded. Six among them asteraceae, poaceae, fabaceae, brassicaceae, apiaceae and caryophyllaceae account 81 species (56,1% of total species). Dicotyledonous (81,9%), annuals (82,6%) and the Mediterranean floristic element (56,2%) were predominant and characterized the weed flora. The agronomic study made it possible to distinguish 24 species and volunteer wheat causing appreciable problems to the crop. Statistical analysis using soil-climatic factors allowed distinguishing four ecologic groups.

To determine the critical period of weed control in sugar beet, an experiment was conducted and consisting of 16 treatments. Weed free treatments included the removal of weeds at 4, 7, 9, 11, 13, 17 and 21 weeks after emergence (WAE) of sugar beet. In weed infested treatments, weeds were allowed to interfere with sugar beet crop 4, 7, 9, 11, 13, 17 and 21 weeks after emergence sugar beet crop. Weed infestation reduced root yield in all treatments. The presence of weeds during the entire growing season decreased root yield by 97.6 % and 68.9 % in 2003 and 2004, respectively. Based on 10 % permissible decrease in root yield, weeding

should start from 4 WAE and 7 WAE in 2003 and 2004, respectively. For the given 10% root yield reduction, weed control should be continued until 15 WAE and 12 WAE in 2003 and 2004. The results show that the critical period begins earlier in 2003 and its duration is longer (77 days) comparatively to that observed in 2004 which is shorter (35 days) and begins relatively later.

A field experiment was conducted during two sugar beet growing seasons 2003/2004 and 2004/2005 in three locations to assess the efficacy of some herbicide treatments. These locations were chosen in order to have diversified weed flora and then have maximum information about herbicide activity spectrum. Concerning post sowing preemergence application treatments, Tramat combi (T1) provided good efficacy (90 % and more) and protected then the crop for a long period more than 2 months. This allowed to sugar beet to grow vigorously. The treatment controlled both dicotyledonous and monocotyledonous species. For post emergence applications, it was noted that when treatments were applied alone (not mixed), the efficacy was not satisfactory. Generally, herbicides (ethofumesate, metamitron, triflusulfuron methyl, phenmedipham, desmedioham and lenacil) achieved good efficacy when applied in tank mixtures than when applied individually alone because they are complementarily in eliminating maximum weed species. So, this must be taken in consideration in a weed chemical management program. These herbicide treatments allow to crop to grow within weed competitiveness nearly until the end of the critical period and are often followed by a mechanical cultivation or a hand hoeing.

6. Acknowledgments

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22

Adverse Effects of Herbicides on Freshwater Zooplankton

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1. Introduction

The use of herbicides to control weeds is a part of agricultural management throughout the world. Unfortunately, the indiscriminate use of these herbicides may have impacts on non-target organisms (Sarma et al., 2001; Nwani et al., 2010). The long persistence of many herbicides in freshwater suggests that they are capable of producing adverse effects on freshwater zooplankton. Dalapon persist in water for 2 to 3 days, paraquat and diquat persist more than dalapon, and 2,4-D amine salt persist for 4 to 6 weeks; chlorthiamid breaks down into dichlobenil that stays for three months in water. On the other hand, terbutryne and diuron persist for more than three months in the water. These periods of time in the water show that most herbicides will cause serious adverse effects in the populations of freshwater zooplankton (Newbold, 1975). The herbicide n-chloridazon (n-CLZ) is degraded to desphenyl-chloridazon (DPC). This transformation product is more toxic than n-CLZ, and can last more than 98 days in surface water. Maximum concentrations of 7.4 µg/L DPC have been found in Germany (Buttiglieri et al., 2009). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is one of the most commonly used herbicides found in the rural environments, easily transported and one of the most detected pesticides in streams, rivers, ponds, reservoirs and ground waters (Battaglin et al., 2003; Battaglin et al., 2008). It has a hydrolysis half-life of 30 days and relatively high water solubility (32 mg/L), which aids in its infiltration into ground water. Atrazine concentrations of 20 to 700 µg/L in runoff surface waters have been reported (Nwani et al., 2010). Table 1 show some physicochemical properties of herbicides which are used to determine the toxic effects on freshwater zooplankton, as well as lethal values of some of these herbicides.

Table 1. Toxicological properties of some herbicides used to determine lethal and sublethal toxicity.

Herbicides	CAS Registry number	Molecular formula	Brekdown in water	Mobility in water	Species	LC 50 mg/l 24h	48 h	96 h	Reference
2,4 –D	94-75-7	$C_8H_6Cl_2O_3$	4 to 6 weeks		*Pteronarcys califórnica* (I)		1.8		Walker (1971)
					Daphnia pulex (C)		3.2		Walker (1971)
					Simochepalus serrulatus (C)		4.9		Walker (1971)
					Daphnia magna(C)		>100		Newbold (1975)
Dalapon	75-99-0	$C_3H_4Cl_2O_2$	2 to 3 days	very mobile	*Pteronarcys califórnica* (I)		100		Sanders and Cope (1968)
					Simocephalus serrulatus (C)		16		Walker (1971)
					Daphnia pulex (C)		11		Walker (1971)
					Daphnia magna (C)		6		Newbold (1975)
Dichlobenil	1194-65-6	$Cl_2C_6H_3CN$	2 to 3 months	low	*Hyalella azeteca* (A)		12.5	8.5	Wilson and Bond (1969)
					Callibaetis sp. (I)		15.2	12	Wilson and Bond (1969)
					Limnephilus sp. (I)		23.3	13	Wilson and Bond (1969)
					Enallagma sp. (I)		24.2	20.7	Wilson and Bond (1969)
					Pteronarcys califórnica (I)		8.4		Cope (1966)
					Daphnia pulex (C)		3.7		Cope (1966)
					Simocephalus serrulatus (C)		5.8		Cope (1966)
					Daphnia magna (C)		3.7		Newbold (1975)
Diquat	2764-72-9	$C_{12}H_{12}N_2$	8 to 11 days	immobile	*Hyalella azeteca* (A)		0.12	0.048	Wilson and Bond (1969)
					Callibaetis sp. (I)		65	33	Wilson and Bond (1969)
					Limnephilus sp. (I)		> 100	> 100	Wilson and Bond (1969)
					Enallagma sp. (I)		> 100	> 100	Wilson and Bond (1969)
					Daphnia magna (C)		7.1		Newbold (1975)
Paraquat	4685-14-7	$C_{12}H_{14}N_2$	7 to 14 days	immobile	*Simocephalus serrulatus* (C)		0.45		Walker (1971)
					Daphnia pulex (C)		0.24		Walker (1971)
					Daphnia magna (C)		3.7		Newbold (1975)
Terbutryn	886-50-0	$C_{10}H_{19}N_5S$			*Daphnia magna* (C)		1.4		Newbold (1975)
Diuron	330-54-1	$C_9H_{10}Cl^2N_2O$	3 months	low	*Daphnia magna* (C)		1.4		Newbold (1975)
Propanil	709-98-8	$C_9H_9Cl_2NO$		low	*Daphnia magna* (C)	43.74	5.01		Villarroel et al. (2003)
					Daphnia magna (C)		0.14		Rohm and Haas (1991)
					Ceriodaphnia dubia (C)		1.65		Moore et al. (1998)
Tebuthiuron	34014-18-1	$C_9H_{16}N_4OS$		very mobile	*Daphnia magna* (C)	44.2			Meyerhoff et al. (1985)

(A) = Amphipoda; (C) = Cladocera; (I) = Insecta.

2. Generalities of the adverse effects of herbicides on freshwater zooplankton

Ecological effects of herbicides in freshwater systems occur direct and indirectly. Indirect effects of herbicides are defined as observed effects on consumer populations in freshwater invertebrates that are not caused by direct toxicity but due to adverse effects on primary producers such as algae and macrophytes (Fairchild, 2011). An herbicide induced death suddenly because cuts off oxygen supply during a period when growth and reproduction by freshwater zooplankton are taking place. Individuals of *Simocephalus vetulus* (Crustacea) may have died in the diquat treated ponds because of lower oxygen supply that benefited *Daphnia longispina* because increased its populations (Brooker & Edwars, 1973).

Fairchild (2011) argues that atrazine did not produce neither direct nor indirect effects on aquatic invertebrates/vertebrates. However a recent review by Rohr and McCoy (2010) concluded that atrazine produces indirect and sublethal effects on fish and amphibians at environmentally relevant concentrations. These effects were observed in reproductive success, sex ratios, gene frequencies, populations, and communities. However, these effects remain uncertain and restricted to few species. Other authors report of many indirect effects of pesticides on freshwater zooplankton obtained through meso- and microcosm experiments (see section 8 of this chapter).

The study of the direct effects of herbicides on freshwater zooplankton results in a complex mixture of data on lethal and sublethal values obtained from standard toxicity tests assessing one species relationship with chemicals of high purity in the lab, to meso- and microcosms experiments, field studies, use of biomarkers, and DNA microarrays. However, aside from environmental health protection agencies reports, the data on the mainstream scientific literature is scarce and restricted to: a) few test species, b) models, and c) small number of herbicides. The result of this diagnosis is a scattered picture with many uncertainties, but also with many opportunities for environmental toxicology research. Perhaps the fact that many authors argue that there are no direct effects of herbicides on freshwater zooplankton at environmental concentrations (Fairchild, 2011) or that herbicides do not represent a threat to aquatic communities (Relyea, 2005; Golombieski et al., 2008) has discourage research in this area. However, these authors failed to consider a series of circumstances that might be consider while analyzing the potential of herbicides for adverse effects:

a. Many herbicides are applied as commercial formulae and the formulae can be more toxic to non-target organism than the active ingredient. That is the case of glyphosate and its different commercial formulae (Domínguez-Cortinas et al., 2008).
b. The safe standards and good application techniques for herbicides are not followed as strictly as they should in developed countries and certainly less so in underdeveloped or poorly developed countries. That means that the theoretical concentrations in which many Quantitative Structure/Activity Relationship (QSAR´s) model for herbicides are based on might not apply in many cases and true environmental concentrations might be underestimated.
c. Relyea & Hoverman (2006) argue that results have shown that some herbicides may interact with a range of different natural stressors and that synergism among herbicides and other pesticides has not been studied at all. Therefore, the interaction between herbicides and the cocktail of toxicants found in many polluted sites throughout the

world has not been analyzed, and therefore, the assumption that some herbicides do not interact with other toxicants at environmentally relevant concentrations to produce direct adverse effects on freshwater zooplankton is just unsustainable (just to put it in ecological terms).

d. Ecotoxicogenomics and the development of new and more sensitive biomarkers that are unveiling effects on freshwater zooplankton (especially on endocrine disruption) at very low environmentally relevant concentrations (see sections 8 and 9 of this chapter) might change the opinion of many researchers on adverse effects of herbicides.
e. The data (at least in the mainstream scientific literature) on potential effects of herbicides on freshwater zooplankton is extremely scarce and restricted to no more than five or six taxonomic groups and less than 30 herbicides.

Herbicides can produce bioaccumulation and biomagnification, but the data is buried in different reports and few scientific articles, that a review is greatly needed. For instance, some herbicides like benfluralin, bensulide, dacthal, ethalfluralin, oxadiazon, pendimethalin, triallate, and trifluralin have the potential to accumulate in sediments and aquatic biota (USGS, 1999).

Lethal effects of a few herbicides have been determined so far in only the following freshwater zooplankton groups: amphipods, cladocerans, copepods, malacostracans, and rotifers. The information on herbicide toxicity on freshwater zooplankton is limited and mainly focused on studies of population dynamics and effects on the biodiversity of the community. Sublethal effects of herbicides on freshwater zooplankton species have focused on demographic parameters (mainly life tables and determination of "r" values), of three groups: amphipods, cladocerans, and rotifers.

Herbicides may affect the population dynamics of freshwater zooplankton by controlling individual survival and reproduction, and by altering the sex ratio. Herbicides might also produce the following effects at the community and ecosystem levels: a) induction of dominance by small species, b) an increase of species richness and diversity, and c) elongation of the food chain and reduction of energy transfer efficiency from primary producers to top predators (Hanazato, 2001).

Biomarkers used so far to study effect of herbicides on freshwater zooplankton correspond to: a) enzyme inhibition, b) mRNA expression levels, c) gen induction, and d) grazing rate inhibition.

3. Mechanism of action of herbicides related to adverse effects on freshwater zooplankton

Herbicides represent a broad variety of chemical classes of compounds, which acts over diverse sites of metabolic functions and energy transfer in plant cells (Duke, 1990). Only a few herbicides classes have a known molecular site of action, moreover, the molecular site of action and the mechanism of several important herbicide classes is still unknown (Duke, 1990). Among known mechanisms of action of herbicides, there are herbicides that inhibit photosynthesis, those that inhibit pigments and those that inhibit seedling growth (Duke, 1990; Prostko & Baughman, 1999; Gunsolus & Curran, 1999). An undesirable side-effect of herbicides is that they may enter freshwater ecosystems by spray drift, leaching, run-off, and/or accidental spills (Cuppen et al., 1997). Surface water contaminations by herbicides

have been reported to have direct toxic effects on phytoplankton, epiphyton, and macrophytes. Furthermore, herbicides have indirect effects over zooplankton and animal populations (Relyea, 2005, 2009; Cuppen et al., 1997), affecting all trophic chains in freshwater reservoirs. Several studies show that herbicides selectively decreased primary producers, leading to a bottom-up reduction in the abundance of consumers due to food limitation (Fleeger et al., 2003). Contaminant-induced changes in behavior, competition and predation/grazing rate can alter species abundances or community composition, and enhance, mask or spuriously indicate direct contaminant effects (Fleeger et al., 2003). Thus, the impacts that herbicides exerts on freshwater communities are one of the main concerns about the use of these chemical compounds. The mechanisms of action of herbicides are classified according to site or specific biochemical process that is affected and are summarized in Table 2; these mechanisms have been described in plants. Below are some examples of the adverse effects of some herbicides according to their mechanism of action in freshwater zooplankton.

3.1 Amino acid synthesis inhibitors

One of the most important herbicides in this category is glyphosate because is extensively used in the aquatic environment. Martin et al. (2003), determined the acute toxicity of technical-grade glyphosate acid, isopropylamine (IPA) salt of glyphosate, Roundup and its surfactant polyoxyethylene amine (POEA) in Microtox® bacterium (*Vibrio fischeri*), microalgae (*Selenastrum capricornutum* and *Skeletonema costatum*), protozoa (*Tetrahymena pyriformis* and *Euplotes vannus*) and crustaceans (*Ceriodaphnia dubia* and *Acartia tonsa*); generally the toxicity order of the chemicals was: POEA > Roundup® > glyphosate acid > IPA salt of glyphosate, while the toxicity of glyphosate acid was mainly due to its high acidity. In *Ceriodaphnia dubia* the LC50 = 147 mg/L to glyphosate acid and for *Acartia tonsa* was LC50 = 35.3 mg/L. Glyphosate produced adverse effects on the embryonic development on time (3 and 8 mg/L), duration of juvenile and reproductive periods, average lifespan, net reproductive rate (8.0 and 10.50 mg/L), and the intrinsic population increasing rate on the freshwater rotifer *Brachionus calyciflorus* (Chu et al., 2005).

Meyerhoff et al. (1985) observed a lower length in *D. magna* exposed to the herbicide tebuthiuron than in blank control animals when the cladocerans were exposed to 44.2 mg/L herbicide. Hanazato (1998) indicated that the neonatal body size determines the size at maturation. The reduced growth rate of neonates due to the chemicals will result in a smaller size at maturation and thus a smaller adult size, leading to smaller clutch sizes.

3.2 Cell-membrane disrupters

The way in which terbutryn exerts its toxicity to rotifers is not clear. The survival curves for all *Brachionus* sp. cultures fed with terbutryn-exposed microalgae showed a drastic mortality showed that population density decreased as terbutryn concentration increased in the microalgal cells. In fact, this species of rotifer did not survive beyond four days when fed with microalgae exposed to 500 nM terbutryn. Percentage of reproductive females in rotifer populations fed with terbutryn-exposed microalgae decreased significantly as herbicide concentration increased (Rioboo et al., 2007). Interestingly the highest concentration of herbicide tested is no toxic to the algae *Chlorella vulgaris* viability, at least after 24 h of exposure (González-Barreiro et al., 2006).

Mechanism of action	Herbicide Family Chemistry	Affected site or biochemical process
Amino Acid Synthesis Inhibitors	Sulfonylureas	Inhibition of Acetolactate synthase enzyme (ALS)
	Imidazolinone	
	Triazolopyrimidine	
	Pyrimimidinylthiobenzoate	
	Sulfonyl amino carbonyl triazolinones	
Cell-Membrane Disrupters	Bipyridliums	Inhibition of protoporphyrinogen oxidase (PPO oxidase)
	Diphenylethers	Electron acceptors, formation of reactive oxygen species (ROS)
Growth Regulators	Triazolinone	Alteration of hormonal balance
	Oxadiazoles	
	Arsenical	
	Phenoxi acids	
	Benzoic acids	
	Pyridine acids	
	Quinilonecarboxylic	
Lipid Synthesis Inhibitors	Aryloxyphenoxyproprionates	Inhibition of Aceytl Coenzyme-A carboxylase (ACCase)
	Cyclohexanediones	

Table 2. Mechanism of action of herbicides (Plimmer et al. 2005).

The herbicide molinate was tested in *Daphnia magna*, and the reproduction was significantly reduced when molinate concentration was increased in the medium, but only this effects was higher in the parental daphnids (F0) than the F1-1st and F1-3rd offspring, seem to be adapted to the herbicide molinate, showing more longevity and reproduction than their parental (Sánchez et al., 2004). Similar result were found by Julli & Krassoi (1995) who observed a significant decreased in total young per female in three broods of *Moina australiensis* when exposed to molinate.

Paraquat was toxic to almost all compartments of the plankton community including zooplankton like: rotifers (*Brachionus calyciflorus*, *Lecane* sp., *Conochiloide* sp., *Asplanchna* sp., and *Hexarthra* sp.), copepods (*Thermocyclops decipiens*, *Mesocyclops* sp.) and cladocerans (*Diaphanosoma excisum*), leading to a reduction in biomass, numbers, and overall trophic functioning, in fact *Thamnocephalus decipiens* exhibited dose-dependent sensitivity to paraquat (Leboulanger et al., 2011). Paraquat may induce peroxidation processes in non-target animal species. Furthermore, paraquat may interfere with the cellular transport of polyamines. Cochón et al. (2007), investigate some aspects related to paraquat-induction of oxidative stress (lipoperoxidation, enzymatic activities of catalase and superoxide dismutase) and also the levels of polyamines (putrescine, spermidine and spermine) in two species of freshwater invertebrates, the oligochaete *Lumbriculus variegatus* and the gastropod *Biomphalaria glabrata*. In *L. variegatus* did not induce membrane lipoperoxidation and only a transient decrease in CAT activity was observed. After 48 h of exposure, an increase of lipoperoxidation and a decrease of SOD activity were registered in the snails. It could be hypothesized that the higher resistance of *L. variegatus oligochaetes* could be due in part to a lower ability to activate the paraquat and also to a protective role of polyamines.

3.3 Growth regulators

Sarma et al. (2001) reported that the herbicide 2,4-Dichlorophenoxy acetic acid had a negative influence on the population growth of *Brachionus patulus* when the rotifers were directly exposed via water and food. Interestingly, Relyea (2005) reported 2,4-Dichlorophenoxy acetic acid had no effect on zooplankton. But exists LC_{50} = 363 and 389 mg/L values (96 h) for the *Daphnia magna* (Johnson & Finley, 1980; Verschueren, 1983, respectively). Boyle (1980) determinate the effects on 2,4-D herbicide applied two concentration 5 and 10 kg/ha, and quantifier the planktonic invertebrates (number per liter of water) rotifers and crustaceans: with a concentration of 5 kg/ha of 2,4-D, found 320 rotifers species and 40 of crustaceans, and found 207 rotifers species and 34 crustaceans with 10.0 kg/ha.

3.4 Lipid synthesis inhibitors

Metazachlor is a frequently used herbicide with high concentrations in surface waters and effects on zooplankton caused by changes in habitat structure in species such as *Keratella quadrata*, *Lecane* spp, *Brachionus calyciflorus*, *Polyathra dolicoptera* and *Bosmia longirostris*. For species such as *K. quadrata*, *Alonella excisa*, *Acropercus harpae*, *Chydorus sphaericus* and some ostracods species with negative weights indicated a decrease in abundance after metazachlor application. In contrast, species like *P. dolichoptera* or *Ceriodaphnia quadrangula* increased in abundance in the treatments as compared to the controls as indicated by the positive weight (Mohr et al., 2008). Direct toxic effects of metazachlor were not expected

since this group is generally unable to synthesize fatty acids and therefore membrane functions will not be disrupted directly. EC_{50} value of 22.3 mg/L (48h) was found for *Daphnia magna* (FAO, 1999).

Another lipid synthesis inhibitors herbicide is norflurazon and is a bleaching, preemergence. Horvat et al. (2005) found that the toxicity of norflurazon caused mortality in *Polycelis feline,* and morphological and histological changes in treated animals compared to corresponding controls. The most prominent histological changes were damage of the outer mucous layer, lack of rhabdites, damage to epidermis and extensive damage to parenchyma cells.

3.5 Pigment inhibitors

Pigments inhibitors affecting plant cell by preventing the formation of photosynthetic pigments (chlorophyll and carotenoids) localized in leaf tissues, trough interfere both the chlorophyll and terpenoid synthesis pathway, inhibiting their synthesis (Duke, 1990; Prostko & Baughman, 1999; Gunsolus & Curran, 1999). This condition cause rapid photobleaching of green tissue of leafs, due the Photosystem I (PS I) reduce a chemical group of the structure of these herbicides to a radical that reduce molecular oxygen to superoxide radical. This reaction repeats continuously to form large amounts of superoxide radical; producing lipids peroxidation and photobleaching (Duke, 1990), giving to affected plants a white or translucent appearance. Because this effect, pigment inhibitors are often called "bleaching herbicides" or "photobleachers" (Prostko & Baughman, 1999). This herbicide class includes isoxazolidinones (i.e. clomazone), pyridazinones (i.e. norflurazon), fluridone, difunone, amitrole and *m*-phenoxybenzamides (Duke, 1990).

This type of herbicides has not direct effects on freshwater zooplankton, but can have indirect negative effects on them. The mechanism of action of these herbicides is targeted to photosynthetic organisms (plants), in the case of freshwater communities, the phytoplankton are the organisms that suffers direct negative effects, which affect them drastically reducing their population. However, the reduction of phytoplankton population may cause indirect negative effects on the zooplankton due a reduction of feed availability for zooplankton, reducing their abundance and/or inducing changes in the taxa composition of zooplankton (Relyea, 2005, 2009).

3.6 Photosynthesis inhibitors

Herbicides that inhibit photosynthesis are the most common type. These herbicides disrupt the vital process of photosynthesis that allows plants to convert the solar light energy into glucose. This type of herbicides binds to the quinone-binding protein (D1 protein) of photosynthetic electron transport, blocking the electron transport. Photosynthesis inhibitors herbicides include triazines (i.e. atrazine), phenylureas (i.e. linuron), uracils, nitriles and benzothidiazoles (Duke, 1990; Gunsolus & Curran, 1999; Prostko & Baughman, 1999). Diuron blocks photosynthetic electron transfer in plants and algae, it might also affect freshwater zooplankton (Leboulanger et al., 2011).

Photosynthesis inhibitors have not direct effects on freshwater zooplankton, but can have indirect effects on them. These herbicides affect mainly to phytoplankton that suffers direct toxic effects, which entails to reducing their population. Thus, the reduction of food supply, modifications of both reproduction and feeding behavior of zooplankton may cause indirect

effects on the zooplankton, resulting in decrease of the abundance of some taxa (indirect negative effect), increase of some taxa (indirect positive effect), both decrease of diversity and changes in species composition of zooplankton (Solomon et al., 1996; Cuppen et al., 1997; Hanazato, 1995; Relyea, 2005, 2009; Chang et al., 2008).

Chang et al. (2008) studied the effects of application of simetryn (20 and 80 μg/L), a methylthiotriazine herbicide, and the fungicide iprobenfos (100 and 600 μg/L), on zooplankton community composed by rotifers and cladocerans. They applied four treatments (low and high concentrations of both pesticides), and their results showed that the herbicide have less apparent direct impact on zooplankton abundance within a short period; however, they observed that the diversity and species composition changed with simetryn application, suggesting that the structure of zooplankton can be altered by the herbicide application (Chang et al., 2008).

The mode of action of atrazine is blocking electron transport in photosystem II leading to chlorophyll destruction and blocking photosynthesis (Nwani et al., 2011). Dodson et al. (1999), found that atrazine have effects on male production of *Daphnia*, changing the sex ratio, which exerts a control of *Daphnia* population dynamics.

Cuppen et al. (1997) studied the effects of a chronic application of linuron (at concentrations of 0.5, 5, 15, 50 and 150 μg/L during 28 days) on freshwater microcosms, which included phytoplankton, zooplankton and macroinvertebrates. They observed that the direct negative effect of linuron on several algae (cryptophytes, diatoms) and the positive effect on green algae *Chlamydomonas* resulted in a decrease of several Rotatoria and an increase in Copepoda, and to a lesser extent, Cladocera.

3.7 Seedling growth inhibitors

This type of herbicides includes dinitroanilines (i.e. trifluralin), acetanilides (i.e. acetochlor) and thiocarbamates (i.e. EPTC). The seedling growth inhibitors are divided into two groups: a) root inhibitors; and b) shoot inhibitors. The first group binding to tubulin protein and disrupt the cell division, which inhibit the root elongation and lateral root generation. About second group, little is known about their mechanism of action, but is believe that disrupt protein synthesis and waken cell wall (Duke, 1990; Prostko & Baughman, 1999).

These herbicides may impact indirectly on freshwater zooplankton, due the direct negative effects on phytoplankton, which may be sensitive to disruption of their cell division process, limiting the growth and multiplication of phytoplankton, reducing the feed availability for zooplankton, decreasing their reproduction rate and their population (Fleeger et al., 2003; Relyea, 2009).

Relyea (2009) examined the effect of acetochlor and metolachlor on zooplankton at low concentrations (6-16 p.p.b.); he encountered that there was no clear indication of any indirect effects from the addition of these herbicides to zooplankton, and in one zooplankton taxon (*Ceriodaphnia*) the mixture of five herbicides (acetochlor, metolachlor, glyphosate, atrazine and 2,4-D) added at concentrations of 6-16 p.p.b. caused an increase in abundance. The few studies about acetochlor and other herbicides (atrazine and 2,4-D) suggest that low concentrations of these herbicides have not effect in cladoceran survival, or may cause an increase of their population due to high reproduction rate in cladocerans (Relyea, 2009).

3.8 Other kind of herbicides whose mechanism is unknown

Only two other molecular sites of action of herbicides are known. One is the herbicide asulam, which inhibits folate synthesis by inhibiting dihydropteroate synthase, although there may also be a second site of herbicide action associated with cell division. In another hand, the herbicide dichlobenil inhibits cellulose synthesis, but its molecular site of action is unknown. Photoaffinity labeling of cotton fiber proteins with a photoaffinity dichlobenil analogue resulted in specific labeling of an uncharacterized 18 kD protein (Duke, 1990). Amoung the seedling growth inhibitors, the group that inhibits plant shoot elongation have a mode of action almost unknown until today, is believe that this inhibitors disrupt protein synthesis and waken cell wall (Duke, 1990; Prostko & Baughman, 1999). In another hand, is too believed that these inhibitors could have multiple sites of action (Gunsolus & Curran, 1999).

4. Lethal effects of herbicides on freshwater zooplankton

The information on herbicide toxicity on freshwater zooplankton is limited and mainly focused on studies of population dynamics and effects on the biodiversity of the community. Some authors claim that herbicides apparently do not pose a threat to the aquatic communities, or have a lesser adverse effect than other pesticides (Golombieski et al., 2008). Relyea (2005) argue that glyphosate and 2,4-D, have no significant adverse effect on zooplankton biodiversity. Perhaps lethal effects are not so evident. However, symetrin can cause shifts in species composition, diversity and dominance of freshwater zooplankton (Hanazato, 2001; Chang et al., 2008). Therefore, it is convenient to consider data on lethal toxicity to determine the most sensitive species which might enable us to predict the direction of indirect effects on a community (Relyea & Hoverman, 2006).

Few if any environmentally relevant concentrations have been shown to have direct effects on zooplankton, fish, or amphibians in the laboratory (Fairchild, 2011). However a recent review by Rohr & McCoy (2010) concluded that atrazine produces indirect and sublethal effects on fish and amphibians at environmentally relevant concentrations. Furthermore, Domínguez-Cortinas et al. (2008) found that both glyphosate and its commercial product Faena® produce lethal toxicity to the freshwater invertebrates *Daphnia magna* and *Lecane quadridentata* at environmental concentrations (the highest concentration of glyphosate in runoff waters, 5.2 mg/L, was found in runoff occurring 1 day after treatment at the highest rate (8.6 Kg/ha of Roundup®)) (Edwards et al., 1980).

Sublethal effects of glyphosate and its formulae could be found at protective values, like the 65 µg/L value published in the Environmental Guide for protecting aquatic life of the Canadian Government (Environment Canada, 1987) for glyphosate. This value is 6.5-fold higher than the esterase inhibition NOEC value for glyphosate and 2-fold higher than the Faena® esterase inhibition NOEC value obtained by Domínguez-Cortinas et al. (2008). On the other hand, the US EPA (1986) has established a value of 700 µg/L of glyphosate for drinking water, which according to Domínguez-Cortinas et al. (2008) esterase inhibition results may represent a risk (LOEC = 62 µg/L, EC50 = 280 µg/L) especially when we consider the ample presence of acetylcholinesterases in the test organisms (Pérez-Legaspi et al., 2011).

Herbicide	Species	Criteria	Endpoint (mg/L)	Reference
Acroleine	*Daphnia magna* (C)	48-h	LC50 = 0.051mg/L	Holcombe et al., 1987
	Pennaeus aztecus (M)	48-h	LC50 = 0.100mg/L	Eisler, 1994
Atrazine	*Daphnia pulex* (C)	3-h	LC50 > 40	Keith et al., 1995
"	"	48-h	EC50 =36 –46.5	"
"	"	48-h	LC50 = 33	"
"	*Daphnia magna* (C)	26-h	LC50 = 3.6	"
"	"	48-h	LC50 = 9.4	"
"	"	48-h	EC50 = 3.6	"
"	"	24h,48h	EC50 > 39	"
"	"	48-h	LC50 = 6.9 MATC = 0.14-0.25	"
"	*Daphnia macrocopa* (C)	3-h	LC50 > 40	"
"	*Ceriodaphnia dubia* (C)	7-d	LC50 = 2.0	"
"	*Daphnia carinata* (C)	48-h	EC50 = 24.6	Phyu et al., 2004
"	*Hyalella azteca* (A)	96-h 21-d	LC50 = 3.0 LC50 = 1.8	Ralston-Hooper et al., 2009
"	*Diporeia* sp (A)	96-h 21-d	LC50 > 3.0 LC50 = 0.24	"
DEA (desethylatrazine)	*Hyalella azteca* (A)	96-h 21-d	LC50 = 5.1 LC50 > 3.0	Ralston-Hooper et al., 2009
"	*Diporeia* sp (A)	96-h 21-d	LC50 > 3.0 LC50 = 0.33	"
DIA (deisopropylatrazine)	*Hyalella azteca* (A)	96-h 21-d	LC50 = 7.2 LC50 > 3.0	Ralston-Hooper et al., 2009
"	*Diporeia* sp (A)	96-h 21-d	LC50 > 3.0 LC50 = 0.3	"
Diuron	*Daphnia pulex* (C)	96-h 7-d	LC50 = 17.9 LC50 = 7.1	Nebeker and Schuytema, 1998
"	*Hyalella azteca* (A)	96-h	LC50 = 19.4	"
"	"	10-d	LC50 = 18.4	"
Glyphosate	*Daphnia magna* (C)	48-h	NOEC = 120 LOEC = 140 LC50 = 146	Domínguez-Cortinas et al., 2008
"	*Lecane quadridentata* (R)	48-h	NOEC = 120 LOEC = 140 LC50 = 150	"
Glyphosate < 74 % (Faena ®)	*Daphnia magna* (C)	48-h	NOEC = 3.3 LOEC = 6.5 LC50 = 7.9	Domínguez-Cortinas et al., 2008
"	*Lecane quadridentata* (R)	48-h	NOEC = 9.8 LOEC = 13.0 LC50 = 13.1	"
Glyphosate (IPA)	*Ceriodaphnia dubia* (C)	48-h	LC50 = 415.0	Tsui and Chu, 2003
Glyphosate (POEA)	*Daphnia pulex* (C)	96-h	EC50 = 2.0	Servizi et al., 1987
Glyphosate 48 % (RON-DO®)	*Daphnia magna* (C)	24-h 48-h	EC50 = 95.96 EC50 = 61.72	Alberdi et al., 1996
"	*Daphnia spinulata* (C)	24-h 48-h	EC50 = 94.87 EC50 = 66.18	"
Glyphosate (Roundup®)	*Phyllodiaptomus annae* (Co)	48-h	LC50 = 1.06	Ashoka Deepananda et al., 2011

Herbicide	Species	Criteria	Endpoint (mg/L)	Reference
"	*Caridina nilotica* (M)	72-h	LC50 = 107.53	Folmar et al., 1979
		96-h	LC50 = 60.97	
	Daphnia magna(C)	48-h	EC50 = 3.0	Folmar et al., 1979
	Ceriodaphnia dubia (C)	48-h	LC50 = 5.7	Tsui and Chu, 2003
	Daphnia pulex (C)	96-h	EC50 = 8.5	Servizi et al., 1987
	Gammarus pseudolimnaeus (A)	48-h	LC50 = 62.0	Folmar et al., 1979
	Hyalella azteca (A)	48-h	LC50 = 1.5	Tsui and Chu, 2004
Glyphosate (Rodeo®)	*Ceriodaphnia dubia* (C)	48-h	LC50 = 415.0	Tsui and Chu, 2004
	Hyalella azteca (A)	48-h	LC50 = 225.0	Tsui and Chu, 2004
Metribuzin (Sencor®)	*Diaptomus mississippiensis* (Co)	24-h 48-h	LC50 =205.0	Syed et al., 1981
	Eucyclops agilis (Co)		LC50 =150	
Molinate	*Brachionus calyciflorus* (R)	24-h	LC50 = 11.37	Ferrando et al., 1999
"	*Daphnia carinata* (C)	48-h	EC50 = 26.5	Phyu et al., 2004
Paraquat	*Diaptomus mississippiensis*(Co)	24-h 48-h	LC50 =10 LC50 = 5.0	Syed et al., 1981
	Eucyclops agilis (Co)			
"	*Diaphanosoma excisum* (C)	24-h	LOEC = 0.057	Leboulanger et al., 2008
"	*Moina micrura* (C)	24-h	LOEC = 0.577	"
Paraquat 27.6 % (OSAQUAT)	*Daphnia magna* (C)	24-h 48-h	EC50 = 16.47 EC50 = 4.55	Alberdi et al,. 1996
"	*Daphnia spinulata* (C)	24-h 48-h	EC50 = 9.91 EC50 = 2.57	"
Paraquat + metribuzin (1:1) 91% + 9%	*Diaptomus mississippiensis* (Co)	24-h 48-h	LC50 = 29 LC50 = 17	Syed et al., 1981
	Eucyclops agilis (Co)			
Pendimethalin 60%	*Daphnia magna* (C)	24-h 48-h	LC50 = 112 LC50 = 53	Kyriakopoulou et al., 2009
S-metolachlor 31.2% + Terbuthilazine 18.8%	*Daphnia magna* (C)	24-h 48-h	LC50 = 20 LC50 = 9.5	Kyriakopoulou et al., 2009
Simazine (Aquazine)	*Daphnia pulex* (C)	48-h	LC50 > 50	Fitzmayer, et al., 1982
Thiobencarb	*Brachionus calyciflorus* (R)	24-h	LC50 = 47.82	Ferrando et al., 1999
2,4-D (2,4-dichlorophenoxyacetic acid)	*Brachionus calyciflorus* (R)	24-h	LC50 = 117	Snell et al., 1991
3,4- DCA (3,4-dichloroaniline)	*Daphnia magna* (C) (adults)	48-h	LC50 = 12	Ferrando and Andreu-Moliner, 1991
"	*Brachionus calyciflorus* (R)	24-h	LC50 = 61.47	"
"	*Daphnia magna,* larva (C)	24-h 48-h 96-h 7-d 14-d 3-w	LC50 = 0.40 LC50 = 0.23 LC50 = 0.16 LC50 = 0.12 LC50 = 0.10 LC50 = 0.10 EC50 = 0.01	Adema and Vink, 1981

Herbicide	Species	Criteria	Endpoint (mg/L)	Reference
"	*Daphnia magna,* adult (C)	48-h	LC50 = 12	"
		96-h	LC50 = 1.0	
		7-d	LC50 < 0.58	
"	*Brachionus calyciflorus* (R)	24-h	LC50 = 62	Snell et al., 1991

Abbreviations. (C) Cladocerans, (R) Rotifers, (Co) Copepods, (A) Amphipod, (M) Malacostracan. LC50 = Median Lethal Concentration, EC50 = Concentration where 50% inhibition occurs, MATC = Maximum Acceptable Toxicant Concentration, LOAEL = Lowest Observed Adverse Effect Level, NOAEL = No Observed Adverse Effect Level, LOEC = Lowest Observed Effect Concentration, NOEC = No observed effect concentration.

Table 3. Lethal toxicity values of herbicides with different species of freshwater zooplankton. Criteria of mortality include different exposure time to herbicide in hours (h), days (d) or weeks (w).

Lethal toxicity tests with freshwater invertebrates are based on standard protocols which are simple, reproducible, and with certain ecological relevance. They are valuable tools to estimate the adverse effect of single chemicals in short periods of exposure (usually 24 and 48 h), with or without food. The most common evaluation parameter is the death or immobility which is represented by the median lethal toxicity (LC50) or the median effect concentration (EC50) (Sarma et al., 2001; Pérez-Legaspi et al., 2011). The cladocerans (*Daphnia* sp., *Ceriodaphnia sp.* and *Moina* sp.) and the rotifer genus *Brachionus*, are among the most used freshwater organisms in toxicity tests (Table 3), mainly due to their great availability, high sensitivity towards many toxicants, ease of handling and culture and high rates of growth and reproduction (Snell & Janssen, 1998; Sancho et al., 2001; Sarma & Nandini, 2006). The amphipod (*Hyalella* sp.) and copepods have also been used (Table 3). Some of these protocols have been recognized by International Standard Organizations (ISO), USEPA, OECD, ASTM, Standard Methods (Snell & Janssen, 1995; Persoone et al., 2009).

Among herbicides, the most studied with freshwater zooplankton are atrazine (Table 3) and glyphosate (Pérez et al., 2011; Table 3). However, the most toxic herbicides are: acroelin (LC50 = 0.051 and 0.100 mg/L), the commercial formula of glyphosate, Faena® for the cladoceran *Daphnia magna* (48h-LC50 = 7.9 mg/L), Roundup® for the copepod *Phyllodiaptomus annae* (48h-LC50 = 1.06 mg/L), and 3,4- DCA (24h-LC50 = 0.40 mg/L) for *D. magna*. On the other hand, glyphosate the active ingredient is less toxic for *D. magna* (48h-LC50 = 146 mg/L) and the freshwater rotifer *Lecane quadridentata* (48h-LC50 = 150 mg/L) than its herbicide formula Roundup®; which suggests that in this particular case the substances present in the commercial formula contribute through synergistic effects to increase the toxicity towards non-target organisms (Domínguez-Cortinas et al., 2008). The 24 and 48 h exposure periods are the most common in the lethal tests, but some tests might last several days. In the case of 3,4-Dichloroaniline (3,4-DCA) the range of *D. magna* LC50 values (0.40 – 0.10 mg/L) decrease as the exposure time increases. Presence of food (microalgae) is a factor that decreases the toxicity of the herbicide as test animals are better fed; they seem to be more resistant (Sarma et al., 2001). In general among freshwater zooplankton the most sensitive model organisms to herbicides are amphipods and crustaceans. However, more toxicity testing with freshwater zooplankton are necessary because data on different species and toxicant are scarce making predictions of herbicide toxicity on zooplankton an

unexplored area, and some herbicides have the potential to alter the dynamics and structure of aquatic communities.

5. Chronic effects of herbicides on freshwater zooplankton

Lethal toxicity data is considered by many environmental health protection agencies in world as reliable and significant, because comes from standard and simplified protocols. However, mortality or immobility is a parameter of lesser sensitivity in estimating adverse effects on freshwater zooplankton. Chronic tests are usually more sensitive because are based on growth, reproduction, physiological, biochemical and genetic characteristics in lower concentrations and longer exposure periods (Table 4). In other words, they assess the first responses (stress, physiological, behavioral and reproductive) to toxicants (Nimmo & McEwen, 1994). Chronic toxicity is usually expressed as the median effective concentration (EC50) or the concentration in which 50% of a specific effect is determined. Many chronic tests rely on life tables that examine demographic parameters (r, Ro, Vx, T and e_o) in freshwater invertebrates. Some chronic tests focus only on growth inhibition arguing that this is an outstanding parameter since involves all steps of a life cycle (embryos, juveniles and adults) during the test period, which makes these tests rapid, sensitive, and relevant ecologically (Snell & Moffat, 1992; Sancho et al., 2001). Besides demographic parameters, tests of chronic effects of herbicides on freshwater zooplankton also involve ingestion rate, enzymatic inhibition and behavioral parameters (Table 4). The most commonly used species belong to cladocerans, rotifers, and one species of amphipod (Table 4). Atrazine is the most studied herbicide regarding chronic effects on freshwater zooplankton; although, studies have been restricted to crustaceans. The most toxic herbicide studied so far is glyphosate, EC50 = 0.28 mg/L, for *in vivo* esterase inhibition in *L. quadridentata*, followed by thiobencarb (EC50 = 0.75 mg/L) for 21 days survival and growth inhibition tests in *D. magna*. The least toxic herbicide is 2,4-D (EC50 = 500 mg/L) for *B. patulus* and EC50 = 128 mg/L, for *B. calyciflorus* (Table 4).

As for lethal tests, the scarcity of data related to chronic effects on freshwater zooplankton becomes a research opportunity to increase the number of taxonomic groups and different herbicides studied, and to diversify the list of chronic parameters as recommended by the American Society for Testing Materials (ASTM) (Sancho et al., 2001). Such an effort would enhance our comprehension of the effects of herbicides in freshwater ecosystems (Hanazato, 2001).

6. Biomarkers assessing adverse effects of herbicides on freshwater zooplankton

The need to rely in parameters more sensitive to estimate adverse effects of toxicants in small concentrations has led to the development of biomarkers. These biomarkers detect small biochemical, cellular, genetic, physiological, morphologic and behavioral variations which can be easily and non-destructively determined in most organisms (Hagger et al., 2006; Walker et al., 2006). These small variations can led to changes in all levels of the biological organization (Hyne & Maher, 2003). These effects are usually more rapid in lower levels of biological organization and can therefore offer more sensitive responses to toxicant exposure inside the populations (Hagger et al., 2006). Therefore, Walker et al. (2006), define a biomarker as any biological response towards an environmental chemical substance

Herbicide	Test organism	Criteria	Endpoint (mg/l)	Reference
Atrazine	*Ceriodaphnia dubia* (C)	4-d	Chronic value = 6.9 NOEC = 5.0 –10 LOEC = 10–20	Keith et al. 1995
"	"	7-d	Chronic value = 3.5 NOEC = 2.5 LOEC = 5.0	"
"	"	7d	NOEC = 5.0	"
"	*Scapholeberis mucronata* (C)	F	1.0	"
"	"	ED 30 – 45-d	1.0	"
Diuron	*Daphnia pulex* (C)	R 7-d	LOAEL = 7.7 NOAEL = 4.0	Nebeker and Schuytema, 1998
"	*Hyalella azteca (A)*	S 10-d	LOAEL = 15.7 NOAEL = 7.9	"
Glyphosate	*Lecane quadridentata* (R)	cFDAam 30-m	NOEC =0.032 LOEC = 0.062 EC50 = 0.28	Domínguez-Cortinas et al. 2008
"	"	PLA2 30-m	NOEC = 5.0 LOEC = 10.0 EC50 = 17.6	"
Glyphosate < 74 % (Faena ®)	*Lecane quadridentata* (R)	cFDAam 30-m	NOEC = 9.8 LOEC =13.0 EC50 = 13.1	Domínguez-Cortinas et al. 2008
"	"	PLA2 30-m	NOEC = 0.4 LOEC = 1.3 EC50 = 4.6	"
Glyphosate (Vision®)	*Simocephalus vetulus*	8-d survivorship and reproduction	0.75 mg/L	Chen et al., 2004
Molinate	*Brachionus calyciflorus* (R)	Ro T r	EC50 = 2.24 EC50 = 5.6 EC50 = 2.7	Ferrando et al. 1999
Paraquat	*Moina micrura* (C)	Population growth rate	not significant effect > 0.022	Leboulanger et al. 2008
Thiobencarb	*Brachionus calyciflorus* (R)	Ro T r	EC50 = 3.4 EC50 = 3.86 EC50 = 3.5 MATC = 3.16 NOEC = 2.0 LOEC = 5	Ferrando et al. 1999
Thiobencarb (S-4-chlorobenzyl diethylthiocarbamate)	*Daphnia magna* (C)	24-h	EC50 = 3.01	Sancho et al. 2001
"	"	R	> 0.30	"
"	"	S, r 21-d	0.75	"
2,4-D (2,4-dichlorophenoxyacetic acid)	*Brachionus calyciflorus* (R)	r 2-d	Chronic value = 70 NOEC= 58 LOEC=83 EC50= 128	Snell and Moffat, 1992

Herbicide	Test organism	Criteria	Endpoint (mg/l)	Reference
"	*Brachionus calyciflorus (R)*	r 2-d	NOEC = 2.5 EC10= 2.38 EC20= 4.91 EC50= 16.8	Radix et al. 1999
2,4-D (technical grade)	*Brachionus patulus* (R)	r	500	Sarma et al., 2001
3,4- DCA (3,4- dichloroaniline)	*Brachionus calyciflorus* (R)	S e_o Ro r Vx T	5.0, 10, 20 > 2.5 ≥ 5.0 > 5.0 2.5 > 5.0	Ferrando et al. 1993

Abbreviations. (C) Cladocerans, (R) Rotifers, (A) Amphipod. LC50 = Median Lethal Concentration, EC50 = Concentration where 50% inhibition occurs, MATC = Maximum Acceptable Toxicant Concentration, LOAEL = Lowest Observed Adverse Effect Level, NOAEL = No Observed Adverse Effect Level, LOEC = Lowest Observed Effect Concentration, NOEC = No observed effect concentration.

Table 4. Chronic toxicity of herbicides assessed to several species of freshwater zooplankton. Criteria consider a decrease or inhibition of the parameter at different exposure time to herbicide in minutes (m), hours (h) or days (d). Parameters: F = Fecundity, ED = Embryonic Development, R = Reproduction, S = Survival, cFDAam = Esterase activity, PLA2 = Phospholipase A2 activity, Ro = Net reproductive rate, T = Generation time, r = Intrinsic rate of population growth, e_o = Life expectancy, and Vx = Reproductive value.

distinct from the normal status of the individual or system health. Biomarkers are classified in three types:

1. Effect biomarkers, which record the exposure of the organism to a toxicant or stressor without being directly related with the specific mechanism of action of the toxicant, and therefore, do not provide information on the level of adverse effect that this change causes (Hagger et al., 2006; Walker et al., 2006).
2. Exposure biomarkers, which provide qualitative and quantitative estimations of exposure to several compounds. These biomarkers are well characterized and associated with the mechanism of action of the toxicant showing the relationship between levels of modification of the biomarker with respect to level of adverse effect (Hagger et al., 2006).
3. Susceptibility biomarker, which provide information of the system´s health and are sensitive to toxicant exposure (Domingues et al., 2010).

There are different types of exposure biomarkers that involve important biological functions and that have been used to assess the adverse effect of many chemical substances. However, use of these biomarkers regarding aquatic invertebrates have been limited due to low availability of biological material, specificity, duration and costs (Hyne & Maher, 2003).

During a risk assessment, it is valuable to consider the range of specificity of the biomarkers. For instance, acethylcholinesterase (AChE) inhibition is consider specific for organophosphate, organochloride, and carbamate pesticides (Walker et al., 2006); and it is necessary to consider enough time to detect the presence of neurotoxic substances in the environment. Besides, AChE inhibition has been assesses in different aquatic invertebrate

species. Therefore, it can be used as a good biomarker for these pesticides. The knowledge of AChE activity and its inhibition by certain herbicides can be used to relate enzymatic activity with the decrease of population densities in the field (Hyne & Maher, 2003). De Coen et al. (2001) demonstrated the relationship between parameters from carbohydrate enzymatic metabolism in *D. magna* and the specific effects of a toxicant suggesting that the activity of the piruvate kinase could potentially be the first warning sign about prolonged effects and to predict quantitative changes in the population.

Records on the use of biomarkers estimating the effect of herbicides on freshwater zooplankton are scarce. Barata et al. (2007) performed *in situ* bioassays with *D. magna*, reporting severe effects on the grazing rate, AchE, catalase, and glutathion S-transferase inhibition associated with the presence of bentazone (487 µg/L), methyl-4-chlorophenoxyacetic acid (8 µg/L), propanil (5 µg/L), molinate (0.8 µg/L), and fenitrothion (0.7 µg/L) in water. Domínguez-Cortinas et al. (2008) found that esterase and phospholipase A2 inhibition are good exposure biomarkers when the freshwater rotifer *L. quadridentata* and the cladoceran *D. magna* are exposed to the herbicide glyphosate and its commercial formula Faena (Table 1 and Table 2).

According to Barata et al. (2007) and Walker et al. (2006), the use of biomarkers is valuable to identify and assess the biological effects whenever toxicants are present in enough concentration to induce a detectable effect. Besides, Hagger et al. (2006), suggest that if the measurement of these effects shows the first responses in lower concentrations than the usual parameters of traditional toxicology, then the sensitivity of biomarker is of great use. It is important to consider that some chronic or sublethal effects can be irreversible and that can take place in ecosystems apparently healthy and where initially they were not detected (Hyne & Maher, 2003). Finally, a biomarker used as an integral parameter has the potential of establishing evidence of adverse effects caused by the presence of chemical substances in a system that can then be related with other levels of biological organization. Therefore, is fundamental to develop more research using biomarkers on freshwater zooplankton that allow to assess the adverse effect of all kind of toxicants (including herbicides), and to use these biomarkers regularly to monitor aquatic ecosystems.

7. Herbicides as endocrine disruptors of freshwater zooplankton species

Although many of the adverse physiological effects of chemicals affecting the neuroendocrine system have been known for over three decades, special attention to this issue only materialized in the early 1990s (Tackas et al., 2002). Given the high volume of use, high level of toxicity to primary producers, and long persistence in the environment, many studies have addressed the capacity of herbicides to disrupt endocrine function at concentrations that commonly occur in surface waters during application periods (Porter et al., 1999). An endocrine disruptor is defined as an exogenous agent that directly interferes with the synthesis, secretion, transport, binding action, or elimination of endogenous hormones and neurohormones, resulting in physiological manifestations of the neuroendocrine, reproductive or immune systems in an intact organism (Tackas et al., 2002).

Aquatic toxicity studies have shown that cladoceran fecundity and survival endpoints are not affected at atrazine concentrations below 100 µg/L (Takacs et al., 2002). However, Dodson et al. (1999) revealed that chronic exposure of *Daphnia pulicaria* to very low

concentrations (0.5 μg/L) of atrazine induced a shift in the population sex ratio due to increased male production, indicating sex ratio is a very sensitive, ecologically-relevant endpoint. Males were produced in stress situations, in response to environmental signals such as shortening day length, reductions in food supply and pheromones produced in crowded populations (Dodson et al., 1999).

Villarroel et al. (2003) compared acute toxicity, reproductive and growth, and feeding activity alterations in *D. magna* exposed to several concentrations of propanil herbicide in a 21-days study. Some parameters analyzed were affected by herbicide: Survivorship did not decrease with increasing concentration of propanil, except with higher concentration (0.55 mg/L); number of neonates born, brood size and number of broods per female as well as the intrinsic rate of growth (r) decreased as the concentrations of propanil increased in the medium. EC50 values indicated that reproductive parameters, like the number of young per female (0.21 mg/L) and brood size (0.26 mg/L) were the most sensitive endpoints in response to propanil exposure. The filtration and ingestion rates were reduced significantly after 5-h exposure to this herbicide; this would be related with lose of coordination and paralysis caused for toxic effects of herbicide on nervous system of *D. magna* (Villarroel et al., 2003).

Other studies have shown that uptake of herbicides can directly affect survival, population growth, reproduction and feeding of rotifers. Riobbo et al. (2007) found that the *Brachionus* sp. population density decreased when females were fed with *Chlorella vulgaris* cells previously exposed to different concentrations of terbutryn, with a maximum survival of 4-days with 500 nM terbutryn in the medium. Terbutryn accumulated in *C. vulgaris* provoked a decrease in the feeding rate of *Brachionus* cultures, and a 66% reduction of the number of eggs per reproductive female compared to controls.

These results suggest that endocrine effects on zooplankton are caused by direct or indirect exposure to herbicides, where population growth rate and sex ratio can be the more sensitive parameters.

8. Field studies, mesocosms, and microcosms, involving herbicides and freshwater zooplankton

Among non-target organisms affected by herbicides in freshwater bodies, plankton and its components (bacterio-, phyto-, and zooplankton) are known to respond on short timescales to low levels of pollutants (Daam et al., 2009), mainly owing to their intrinsic sensitivity and high population turnover (Relyea, 2005). Secondary effects of herbicides on these organisms are difficult to predict since they depend on interactions between species, herbicides and the original structure of the ecosystem (Wendt-Rasch et al., 2003). For aquatic ecosystems, toxicity testing ranges from standard tests under laboratory conditions to field studies, including microcosm and mesocosm experiments (Caquet et al., 2000). These studies in enclosures are valuable tools that can help to understand how herbicides exposure may affect ecosystems as a whole, and be an aid in the assessment of the various risk scenarios resulting from the use of these chemicals (Wendt-Rasch et al., 2003).

Most of the information on the ecotoxicity of herbicides in aquatic communities is related to individual o combined effects of exposure to these chemicals at the ecosystem level (Thompson, 2006). Wendt-Rasch et al. (2003) reported no significant effects on copepod nauplii

and rotifers from exposure during 14 days to metsulfuron methyl (0, 1, 5, 20 μg/L) in 24 enclosures of 80 L (height: 0.65 m, diameter: 0.4 m) in water bodies adjacent to agricultural fields. Metsulfuron methyl is a sulfonylurea herbicide that affects the synthesis of essential amino acids in plants, and hence inhibits cell division. It is highly water-soluble and has a low sorption coefficient (Tomlin, 1997). However, herbicide exposure had a significant effect on the conductivity, pH and total nitrogen in the enclosures (Wendt-Rasch et al., 2003).

Plankton communities from a tropical freshwater reservoir in Mozambique were monitored for 5 days after exposure to nominal concentrations of diuron (2.2 and 11 μg/L) and paraquat (10 and 40.5 μg/L), commonly used in the tropics for agriculture and disease vector control. Diuron blocks photosynthetic electron transfer in plants and algae, and paraquat generates superoxide O_2^- that affects all cellular components (Leboulanger et al., 2011). In general, zooplankton was slightly sensitive to diuron, and very sensitive to paraquat. Nauplii or cyclopidae copepodites and adults did not differ in microcosms inoculated with diuron relative to the controls. However, the adult stages of the copepod *Diaphanosoma excisum* were slightly reduced in high concentration compared with the control. A reduction in rotifer biomass was also noticed with a below significance level ($p = 0.072$). Low concentration of paraquat caused a significant reduction in *Thermocyclops decipiens* copepodite biomass relative to controls, whereas high treatments reduced the carbon biomass in all groups of zooplankton, mainly the cladocera and copepod nauplii (Leboulanger et al., 2011).

In PVC tanks of 150 L with water from the Paraná River, Gagneten (2002) evaluated the effects of paraquat (0.1, 0.2, 0.4 and 0.8 ml/L) on zooplankton community for 35 days of exposure. Contrary to what was observed with the species richness dominated by rotifers (55%), cladocerans (18%), and copepods (15%), paraquat negatively affected the zooplankton density, especially in higher concentrations. The chemical effect of the herbicide was higher on rotifers *Anuraeopsis*, *Lecane*, *Phylodina* and *Conochilus*; on the cladoceran *Ceriodaphnia*; on copepods *Eucyclops* and *Notodiaptomus*, and on thecamoebians *Arcella* and *Cucurbitella*. Dissolved oxygen, pH and water hardness did not vary significantly between controls and treatments during the experimental period. According to Pratt and Barreiro (1998), it is necessary to consider species composition, inter- and intraspecific interactions and environmental factors, such as physicochemical parameters, when analyzing the impact of herbicides on aquatic communities. This interaction between herbicides and biological and environmental factors may reduce or increase the impact of pollution on aquatic ecosystems (Gagneten, 2002).

Interactions of herbicides with others environmental stressors have also been studied. Chen et al. (2004, 2008) examined effects of interactions among pH (5.5 and 7.5), two levels of food concentrations, and the formulated products Vision® (glyphosate: 0.75 and 1.50 mg acid equivalent/L) and Release® (triclopyr) on cladoceran *Simocephalus vetulus.* Herbicide treatments resulted in significant decreases in survival, reproductive rate, and development time for *S. vetulus* at levels 5–10× below predicted worst case environmental concentrations (2.6 mg/L). High pH increased the toxic effects of the herbicide on all response variables even though it improved reproductive rate of *S. vetulus* over pH 5.5 in the absence of herbicide. Stress due to low food also interacted with pH 5.5 to diminish *S. vetulus* survival. These results support the general postulate that multiple stress interactions may exacerbate chemical effects on aquatic biota in natural systems.

Atrazine is a selective herbicide with long residual activity used on crops such as corn, sorghum, sugarcane, conifers, forestry and lawn care applications (Solomon et al., 1996). Degradation rates in water are highly variable. The DT50 in water has been estimated to range from 3-90 d or more and in sediment the range was 15-35 d (Huber, 1993). Several invertebrate community studies have been conducted with atrazine in field situations using mesocosms or whole ponds. The population density of cladocerans in ponds treated at 20 μg/L was lower than that in control ponds even one year after contamination. The most sensitive effect concentration for invertebrates in outdoor enclosures was 0.1 μg/L in which herbivorous zooplankton were reduced in abundance (Tackas et al., 2002).

Indirect effects on zooplankton were reported by Jüttner et al. (1995) during a 6 week mesocosms study. Total numbers of the cladoceran *Daphnia longispina* declined in all 7 enclosures following treatment with atrazine. This was accompanied by reduced egg ratios between day 3 and day 21. In both cases, effect concentration was 318 μg/L. Likewise, effect concentration on reduction in the density of copepod nauplii, *Synchaeta* sp. and *Polyarthra* sp was from 68, 132, and 318 μg/L atrazine, respectively. Van den Brink et al. (1995) detected only slight reductions in primary productivity over 7 weeks in multispecies microcosms exposed to 5 μg/L atrazine, and observed no significant effects on cyclopoid and cladoceran species or on the amphipod *Gammarus* and the rotifer *Keratella*.

Lozano et al. (1992) studied the temporal variation in abundance (% of control) of zooplankton following a single dose of esfenvalerate in 5 different concentrations (0.01, 0.08, 0.2, 1.0, 5.0 μg/L). Mesocosms were shallow (0.5 - 1.1 m depth), had sediment and macrophytes and ranged between 25 – 1100 m^3 in volume. Dose-response curves showed that the initial impact on abundance and the subsequent recovery were dependent on the concentration: decreasing in Cladocera and Copepoda, and increasing in phytoplankton and Rotifera. Perschbacher et al. (2002) and Perschbacher and Ludwig (2004) tested the adverse impacts of common aerially applied herbicides for rice on phytoplankton, zooplankton, and water quality in 12 mesocosms (500 L, 0.7 m depth). Clomazone (0.6 kg active ingredient/ha), thiobencarb (3.4), pendamethalin (1.1), quinclorac (0.6), halosulfuron (0.07), bensulfuron methyl (0.07), triclopyr (0.4), 2,4-D-amine (1.7), and molinate (5.6) produced no measurable effects on plankton or water quality. Propanil (4.5) and diuron (1.4) significantly reduced oxygen production by 75% after their application and stimulated chlorophyll *a*, too. It was assumed to be related to compensatory action by the algae for photosynthesis inhibition. The increase in chlorophyll *a* concentration suggests an increase in food availability for zooplankton and is ultimately believed to have been responsible for the observed increase in numbers of rotifers and copepods, but not cladocerans (Perschbacher et al., 2002).

Marcial and Hagiwara (2008) determined acute toxicity of the mefenacet herbicide on the copepod *Tigriopus japonicus*, the cladoceran *Diaphanosoma celebensis* and the rotifer *Brachionus plicatilis*. Compound exposure was carried out in 6-well polystyrene plates, and mortality was evaluated after 24 h. Although species showed different sensitivities to herbicide, a dose-response relationship was consistent in all cases. *B. plicatilis* was particularly resistant to mefenacet, while *T. japonicus* and *D. celebensis* are comparatively sensitive.

Mohr et al. (2008) monitored for 140 days the effects of metazachlor (5, 20, 80, 200, and 500 μg/L) on stream and pond communities. In this study, metazachlor strongly affected

mesocosms communities at all concentrations. Direct negative effects were most prominent for chlorophytes whereas diatoms and cryptophytes seemed insensitive. The effects on zooplankton were caused by changes in habitat structure due to the strong decline of macrophytes. The slow degradation of metazachlor combined with the absence of recovery in both chlorophytes and macrophytes was likely to cause long-lasting effects on aquatic ecosystems.

Jenkins and Buikema Jr. (2009) studied effects of simazine (0.1, 0.5 and 1.0 mg/L) on zooplankton and physical-chemical parameters in *in situ* microcosms for 21 days. Herbicide induced decreases in dissolved oxygen and pH, but induced increases in nitrate and ammonia levels compared to control microcosms. Rotifers dominated the zooplankton and were differentially affected by simazine. The dominant species, *Kellicottia bostomensis*, exhibited a positive response to simazine, as did *Keratella cochlearis*, due to lesser mortality in higher concentrations of simazine. *Polyarthra vulgaris* was unaffected, but *Synchaeta pectinata* was impaired by simazine at day 21.

These micro- mesocosms studies indicate that decrease in zooplankton density in the treated ponds probably was not caused by direct toxic effects of the herbicides, but to indirect effects resulting from reduced algal productivity, a change in the food source or a change in the competition for a food source.

9. Molecular genetics, DNA and protein microarrays, environmental genomics relating herbicides and freshwater zooplankton

The integration of genomic-based tools and ecotoxicology is a promising approach that may provide a broad view of how living systems respond to a given stressor (Neumann & Galvez, 2002; Robbens et al., 2007; Snape et al., 2004).

Transcription profiling using microarrays is one of the most prominent genome-wide technologies within ecotoxicogenomics since it provides an overview of changes in gene expression linked to chemical exposure (Pereira et al., 2010). Very recently, cDNA microarray-related techniques have been successfully used to address transcriptional responses of *D. magna* to different environmental toxicants, including pharmaceuticals, heavy-metals, pesticides and PAHs (Connon et al., 2008; Heckmann et al., 2008; Soetaert et al., 2006, 2007; Watanabe et al., 2007).

The evaluation of herbicides genotoxicity has been an important research line, to investigate the alterations in the molecular pathway in the organism. The most important organism for this test is *Daphnia magna*. Table 5 shows some alterations and DNA damages caused for some herbicides.

The effects of herbicides on freshwater zooplankton has been studied on molecular pathways and DNA, for example Pereira et al. (2010), to understanding the genomic responses of *D. magna* to chemical challenges, exposed to the herbicide propanil to compare phenotypic effects with changes in mRNA expression level. Propanil highly promoted synthesis of innate immunity response systems (more details in Table 3) and elicited specific up-regulation of gene transcription within neuronal pathways, including dopa decarboxylase and syntaxin 6. Atrazine induced hemoglobin genes (dhb1, dhb2 and dhb3) in *D. magna* through the hormonal pathways. This hypothesis was tested by modeling the

combined effects of atrazine and the terpenoid hormone mimic pyriproxyfen on hemoglobin mRNA levels assuming the same mechanism of action (concentration addition model) and alternatively, assuming different mechanisms of action (response addition model) (Rider & Leblanc, 2006).

Herbicide	DNA alterations	Reference
Terbutryn	Cytogenetic damage Primary DNA damage	Moretti et al., 2000
Atrazine	Mutagenic and genotoxic potencial	Kaya et al., 2000
	DNA damage	Pino et al., 1988 Clements et al., 1997 Tennant et al., 2001
	Expression of haemoglobin genes	Rider and LeBlanc, 2006
Propanil	Promoted transcriptions genes of: Haemoglobin synthesis Neuronal pathways Up-regulated genes specifically related to defense mechanisms	Pereira et al., 2010

Up-regulated genes specifically related to defense mechanisms

Table 5. DNA alterations by herbicides.

10. Conclusions and future research

The study of the adverse effects of herbicides on freshwater zooplankton is an unexplored field. Studies in Quantitative Structure/Activity Relationship (QSAR´s) are scarce or missing (at least from mainstream scientific literature). Ecotoxicogenomics studies are scarce and restricted to few herbicides and one species: *Daphnia magna*. Regarding biomarkers applied to herbicide exposure the small set of data available suggest that the potential of herbicides for producing adverse effects on freshwater zooplankton can be high, and warrants future research. Presently, atrazine and glyphosate are the two herbicides of great regulatory concern because of their widespread use, common detection in water having relatively long persistence in freshwater. Lethal toxicity in amphibians has been demonstrated (Reylea, 2005). Still, some authors pose serious doubts about the results suggesting direct and indirect effects of herbicides on invertebrates, amphibians and fish exposed to environmentally relevant concentrations (Fairchild, 2011). These doubts have to be clarified using well designed experiments that include effects on endocrine and immune function. Mesocosms studies will help identify and characterize the mechanisms that modify the sensitivity of zooplankton by exposure to herbicides. Compared to laboratory experiments, mixtures of herbicides combined with physical and chemical factors at the natural environment, could identify physiological, biochemical and behavioral changes more significant on zooplankton communities, mainly rotifers and copepods for which information reported is scarce. However, this chapter already includes recent data on lethal tests that suggest that at least for brief periods of time, some herbicides at environmentally

relevant concentrations can produce mortality, and other relevant sublethal effects in freshwater zooplankton (for example, reduction in rate population growth).

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23

Herbicide Tolerant Food Legume Crops: Possibilities and Prospects

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1. Introduction

Weeds are one of the major problems in agriculture. Weeds compete with other crops for water and nutrients and, as a result, decrease yields and productivity. Without weed control it is extremely difficult to harvest crops. The advent of mechanization replaced much of the hand labour in the developed world as well as the developing parts of the third world. Mechanical weed control is fraught with high-energy costs, facilitates soil erosion and compaction and has been mostly replaced by chemical weed control using herbicides (Gressel J, 2000). As countries industrialize and develop economically, cheap farm labour becomes unavailable, thus increasing the necessity for cost-effective chemical weed control. In India, weeds cause the highest loss (33%) followed by pathogens (26%), insects (20%), storage pests (7%), rodents (6%) and others (8%). It has been estimated that the potential losses due to weeds in different field crops would be around 180 million tonnes, valued at Rs. 105,0000 millions annually (Anonymous, 2008). Globally, herbicide constitutes 50 percent of the total pesticides sale and in some countries like USA, Germany and Australia; the figure is as high as 60-70 percent. In India, however, the position is different as herbicides form a meager 15 percent of the total pesticide consumption. But still, the consumption has increased rapidly from 4100 metric tons (MT) in 1988-89 to 13,764 MT in 2004 and it is likely to further increase in future (Varshney and Mishra, 2008). Given the harmful economic implications of poor weed management, it is hardly surprising that herbicide production is a main driver of the agrochemical industry. Too often there is no selective chemical that can control a particular weed in a particular crop, as most selectivity between crop and weed are due to catabolic degradation of the herbicide by the crop. Therefore, closely related weeds are to be expected to have similar catabolic pathways as the crop and thus escape the chemical effect. This is one major reason that genetically modified herbicide-resistant crops (GM-HRC) have become so useful, and that biotechnology has been utilized to produce such crops as well as to find new herbicide targets. Selectivity can be enhanced by inserting exogenous resistance genes into the crops or by selecting natural mutations. However, one major concern about transgenic herbicide resistant crops (HRCs) is that the transgene could genetically introgress into related weeds, and make them resistant and therefore, their careful management comes into account.

2. Chemical weed control

The controlling of weeds in the growing crops with weedicides increases their yields and ensures the efficient use of irrigation, fertilizers and plant-protection measures, such as the spraying of insecticides and fungicides. The removal of weeds from the growing crops facilitates easy harvesting and gives a high-quality produce without admixture with weed seeds. Chemical weed control can be adopted quite in time and in situations and under conditions, which make manual or mechanical weeding difficult. A great advantage of this method lies in killing weeds in the crop row or in the immediate vicinity of crop plants. The chemical method is easier, less time-consuming and less costly than weeding by hired laborers. However, there are several disadvantages like environment pollution, human and animal health issues related to its use.

3. Biological weed control

Biological weed control is the action of parasites, predators, or pathogens to maintain another organism's population at a lower average density than would occur in their absence. Biological control is usually thought of as intentional introduction of parasites, predators, or pathogens to achieve control, but it is also a natural phenomenon. Biological control will never be the solution to every weed problem. It is employed as one weed management practice among many. Using tools of biotechnology, it is possible to engineer a more potent parasite, predator or mutant which can be deployed to weed control. The biological weed control can be permanent weed management because once an organism is released, it may be self-perpetuating and control will continue without further human intervention. Besides, there are no chemical environmental residues from biological control other than the organism. Bio control may be the best option for management of invasive species. In ideal cases, initial costs are nonrecurring and usually, once the organism is established, no further inputs are needed. There are some situations where biological control is not appropriate. If a plant is a weed in one place and valued in another place, in the same general geographic region, biological control is inappropriate. Spread of a biological control organism, once introduced, cannot be controlled. Biological control is inherently slow, and results are not guaranteed. Some species are geographically local, minor weeds, and development of a biological control for them would be very expensive and not financially wise because of the small-infested area. Release of a biological control organism can induce competitive suppression or extinction of native biological control organisms and other desirable organisms. Biocontrol, particularly in disturbed cropping situations, will not control as many different weeds as other techniques. It won't eradicate weed problems, but most other techniques won't either.

4. Biochemistry and molecular biology of weed control

The need for developing cost effective chemical weed control systems has led to a vast industrial investment to find and develop selective herbicides and later GM-HRC. Virtually all herbicides marketed are the result of random screening of chemicals. Once success is obtained, further syntheses around the identified chemical are used to find compounds with greater activity and then selectivity. After such compounds have been

found and marketed, they become research tools of the physiologists and biochemists, first to find a site of action and then as 'anti-metabolites' to further understand and modulate metabolic pathways. Thus the advent of 2,4-D assisted in understanding auxin action, atrazine and diuron (DCMU) in understanding photosystem II, paraquat for photosystem I, dinitroanilines in tubulin to microtubule assembly, dichlobenil for cellulose biosynthesis, etc. Herbicides are the anti-metabolites of choice in dealing with key enzymes such as glutamine synthase [glufosinate (phosphinothricin)], acetolactate synthase (ALS) (many herbicides), acetyl-CoA carboxylase (ACCase) (many herbicides), dihydropteroate synthase (asulam), enolpyruvate-shikimate phosphate synthase (EPSP) (glyphosate) and phytoene desaturase (many herbicides). The genes for most of these enzymes have been isolated and used in transgenic programs. Such research transcended plant biochemistry and agriculture. For example, it was discovered through comparative genomics that plant and trypanosome β-tubulins were similar to each other and different from mammalian β-tubulin. The dinitro-aniline herbicides then proved to be excellent trypanocides (Chan *et al*., 1993; Bell, 1998). The repetitive (mis) use of single herbicides in monoculture over many years predictably leads to the evolution of herbicide-resistant weeds (Gressel & Segel, 1978). The advent of triazine resistance was crucial to the understanding of the role of the *psb*A gene product in the photosystem II binding site, leading to innumerable studies of photosynthesis, biophysics and biochemistry correlated with molecular structure of the gene product. The mutant and natural *psb*A gene products were crystallized and analyzed, leading to new insights into 'drug' (ligand) binding and design (Michel & Deisenhofer, 1988; Deisenhofer & Michel, 1989). Information from herbicide resistance provided the theoretical underpinning for designing transient drought resistant plants. Harvey and Harper (1982) first promoted the idea that paraquat resistance can be similar to oxidative stress tolerance. This was later extrapolated to being similar to transient drought tolerance (Malan *et al*., 1990). This has allowed developing quick pre-tests with paraquat to ascertain the level of transient drought tolerance of transgenic plants bearing genes designed to confer oxidative stress resistance. Genes coding for herbicide resistance developed for agriculture became the selectable markers of choice for generating transgenics, supplanting antibiotic resistance, even when there was no plan for registering the herbicide for use in that crop. The huge corporate investment in HRC and *Bacillus thuringiensis* (*Bt*) toxin containing crops due to perceived market size resulted in the gain of much of our knowledge on promoters, organelle-specific and transit peptides, as well as more recently in organelle transformation. This corporate investment in basic plant molecular biology was manifold greater than the public sector effort, and the spill-over was great. It is important to understand that the transgenic research is market driven and the market is for weed control.

5. Recombinant DNA technology used to achieve herbicide resistance

The techniques used to achieve herbicide tolerance have been reviewed by Cole (1994). Crops which have been transformed to become herbicide tolerant include are shown in Table 1.

In general, the herbicide tolerance gene is expressed as a determinant which is integrated at a single nuclear locus. Tobacco has often been used as a model crop to study and optimise alien gene performance; this reflects the ease of transformation in this species.

Herbicide	Novel gene product	Gene Function	Gene Source	Transformed agricultural crops
Sulfonylureas	Acetolactate	mts	Higher plant	Chicory, cotton, flax,lettuce, lucerne, melon, sugarbeet, tomato
Imidazolinones	Acetolactate synthase	mts	Higher plant	Tobacco
Glyphosate	Enolpyruvylshikimic acid phosphate synthase	mts	Soil and enteric bacterium, higher plant	Rape, soybean, tomato
	Glyphosate oxidoreductase	detox	Soil bacterium	Maize, rapeseed, soybean
Atrazine	"DI" protein	mts	Higher plant	Soybean
Glufosinate	N-acetyl transferase	detox	Bacterium	Cotton, lucerne
Bromoxynil	Nitrilase	detox	Soil bacterium	Cotton, potato, rape, tomato
2,4-D	Mono-oxygenase	detox	Soil bacterium	Cotton

Table 1. Transformation of crop species for herbicide tolerance.

6. Mechanisms for conferring herbicide tolerance in crops

Tolerance to herbicides can be achieved by various mechanisms and genes:

a. *bar* gene

Members of the genus *Streptomyces* (Actinobacteria: Actinomycetales) produce hundreds of antibiotics, one of which is bialaphos (also known as bilanafos or PTT). Its chemical structure is given below (Fig 1). Bialaphos is an inhibitor of the key enzyme in the nitrogen assimilation pathway, glutamine synthetase (GS). It becomes active after removal of the alanine residues by intracellullar peptidases. The remaining glufosinate compound inhibits GS and as a result leads to accumulation of toxic levels of ammonia in bacteria and plant cells.

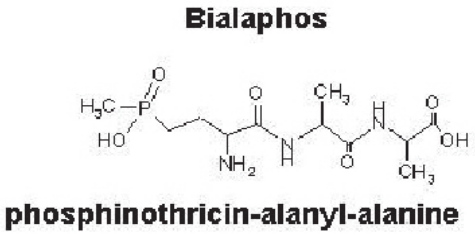

Fig. 1. Structure of bialapos.

Some microorganisms can detoxify glufosinate by producing an enzyme that causes acetylation of the amino group. The gene encoding the acetylating enzyme has been isolated from *Streptomyces hygroscopicus* (Thompson *et al*., 1987) and from *S. viridochromogenes* (Wohlleben *et al*., 1988). It has been referred to as *bar* (for bialaphos resistance) and PAT gene, respectively. The *bar* gene encodes a phosphinothricin acetyl transferase (PAT). In the few countries commercial transgenic crops such sugar beet, canola, soybean, rice and maize carrying the *bar* gene has already been released and cultivated commercially.

b. Detoxifying enzyme coding gene

Continuous search for new herbicides that are highly effective and safe for animals and the environment is the need of the hour. A new class of herbicides that fulfils these needs acts by inhibiting specific amino acid biosynthesis pathways in plants (La Rossa 1984). However, most of these herbicides do not distinguish between weeds and crops. Modifying plants to become resistant to such broad-spectrum herbicides would allow their selective use for crop protection. As a consequence, a major effort has been devoted in several laboratories to engineer herbicide-resistant plants. Two approaches have been followed. In the first, a mutant form of the target enzyme is produced which is still active but less sensitive to the herbicide. In this way, mutant plants producing an altered form of the enzyme acetolactate synthase have been selected which are resistant to the sulfonylurea and imidazolinone herbicides (Shaner and Anderson, 1985). In another example, a mutant form of the bacterial aro A gene was expressed in tobacco and conferred tolerance to the herbicide glyphosate (Comai *et al*., 1985). The second approach involves overproduction of the target enzyme. It has been demonstrated that overexpression of the plant enzyme 5-enol-pyruvylshikimate- 3 phosphate synthase conferred glyphosate tolerance in transgenic petunia plants (Shah *et al*., 1986).

Glyphosate was released by Monsanto Chemical Co. in 1971. Its discovery and release were as revolutionary in weed science as the discovery of 2,4-D. The structure of the amino acid glycine is underlined in following Figure.2 Glyphosate, the N-phosphonomethyl derivative of glycine, is a nonselective, foliar herbicide with limited to no soil activity because of rapid and nearly complete adsorption. It controls perennial grasses and has an advantage over paraquat, because glyphosate translocates. It is the only available herbicide that inhibits EPSP synthase. The enzyme is common in the synthetic pathways leading to the aromatic amino acids phenylalanine, tyrosine, and tryptophan. These amino acids are essential in plants as precursors for cell wall formation, defense against pathogens and insects, and production of hormones (Duke, 1990). The enzyme is not found in animals. Low application volume is more effective than high volume, and small plants are more readily controlled than large ones. In contrast, paraquat, a photosynthetic inhibitor, acts quickly (one or two days) on most plants. Glyphosate activity usually cannot be detected as quickly and may take several days to appear after application. One glyphosate formulation is also used as an aquatic herbicide. Transgenic crops resistant to glyphosate have been developed and marketed. Resistant species include Palmer amaranth, common ragweed, hairy fleabane, goosegrass, Italian ryegrass, rigid ryegrass, and buckhorn plantain. Resistance has been found in Australia, Chile, South Africa, Spain, and in 15 US states.

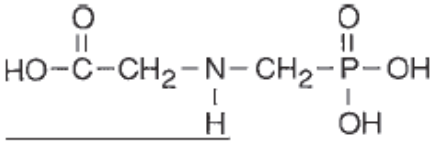

Fig. 2. Structure of glyphosate with glycine underlined.

7. Glutamine synthase

Glutamine synthetase (GS) is essential for assimilation of organic nitrogen as ammonia (Duke, 1990). Glufosinate (phosphinothricin) is the only available herbicide that inhibits GS. It is available in the United States for complete weed control in non crop areas and as a directed spray in field- and container-grown nursery stock. It is rapidly degraded in soil with a half-life of seven days. Even though it is not adsorbed tightly, it does not leach because it is degraded quickly. Glufosinate is nearly nonselective. It has been made selective in corn because a gene coding for phosphinothricin acetyl transferase activity was isolated from the soil bacteria, *Streptomyces hygroscopicus*, and cloned into corn. The acetyl transferase enzyme converts glufosinate to its nonphytotoxic acetylated metabolite, enabling crops to achieve resistance by rapidly metabolizing glufosinate.

8. Uses of molecular data in weed control

Not all applications of molecular biology are commercial. There is a necessity to taxonomically classify weeds, as there are differences in selective control among related weeds, as epitomized with weeds of the genus *Amaranthus* (Mayo *et al.,* 1995). Indeed, the classical taxonomy is so complicated that it was found using molecular techniques that many accessions of wild species in the collections were mis-classified (Martin *et al*., 1997). Molecular taxonomy has been of great assistance and has often provided the decisive data in many cases on whether two similar *Amaranthus* species were actually one, or were separate, or were hybrids, depriving classical taxonomists of their endless battles. Knowing relatedness will be required for assessing the risks of crop gene introgression into weeds, and has been used to trace whether a biotype evolved resistance by introgression, vs. by its own internal mutation (Wetzel *et al*., 1999b). This is very important to know, as the wild species do not introgress freely with cultivated species and the feral form does exist (Morishima, 1984; Ling-Hwa & Morishina, 1997; Mariam *et al.,* 1996; Majumder *et al*., 1997; Cohen *et al*., 1999). Most of the present uses of molecular biology are to find new herbicide targets and to generate HRC.

9. Herbicide tolerance

After using an herbicide continuously to control a weed over time, it may no longer be effective in controlling that weed. In other words, the weed species becomes resistant to the herbicide and is no longer controlled by it. However, the entire population of the weed species may not necessarily behave in the same manner; some members of the same species may still be controlled by the herbicide. There are two basic processes by which herbicide

tolerance develops. Most of the time, it develops as a result of selection pressure. In this case, a very small fraction of the population of a particular weed species may possess a slightly different genetic makeup from the rest of the population -- referred to as a biotype -- that makes it tolerate herbicide "X" the first time it is used. Another process by which resistance develops is through mutations. In this case, one or more members of a weed species undergo a change in genetic makeup due to frequent exposure to the herbicide. The modifications usually occur at the site where the herbicide binds at the target site in order for it to be effective.

The mode of action is the mechanism by which a given herbicide travels to a target site within a plant where it exerts activity by inhibiting a growth process vital to the plant. Certain families of herbicides may inhibit the process of photosynthesis. Others may inhibit the synthesis of chlorophyll or amino acids vital to plant's growth, still other groups may cause leaks to plant cells resulting in plant kill. If a weed species develops resistance to two or more herbicides belonging to the same family, the phenomenon is called Cross Resistance. If it develops resistance to two or more herbicides belonging to different families, it is called Multiple Resistance.

There are three physiological mechanisms for natural or induced tolerance or resistance to an herbicide:

1. Reduced sensitivity at a molecular site of action,
2. Increased metabolic degradation, and
3. Avoidance of uptake or sequestration (hiding) after uptake (Duke *et al.,* 1991).

Each of these has potential use in development of resistance in crops. Most of those modified to be resistant to glyphosate and glufosinate are commercially available and grown. Roundup Ready TM soybeans, corn, cotton, and canola have achieved commercial success in the United States and Canada. Other glyphosate-resistant crops are being developed by Monsanto.

The primary cause of herbicide resistance is selection pressure or repeated use of the same herbicide or other herbicides with the same mode of action. Therefore, the most effective step is to use all possible methods of weed control rather than depending upon a single tactic. This helps to avoid the use of the same or similar herbicide repeatedly. An Integrated Pest Management (IPM) method that encompasses cultural, mechanical, chemical, and biological control methods, rotating with different families of herbicides, tank-mixing herbicides having different modes of action, and occasionally using a non selective herbicide to control all weeds are practical methods to reduce resistance buildup. Resistance is real and widely present, but it can be managed. It is well understood that it results from repeated use of the same herbicide or herbicides with the same mode of action in fields. It is not created by the herbicides; it is selected for. The plants that are susceptible are killed. The resistant population survives and comes to dominate. It is a process of evolution by chemical selection. The time for development of resistance has proven to be short. Several species have evolved cross-resistance to more than one herbicide. Since 1982 the number of resistant weeds has more than tripled, and the land area involved has increased 10 times. Multiple resistances have been observed and occur when resistance to several herbicides results from two or more distinct resistance mechanisms occurring in the same species. In general, but not always, there are enough alternative herbicides and other control measures

(e.g., rotation, tillage) to manage resistant weeds effectively. Resistance to some of these herbicides has developed in as little as three years. It is equally incorrect to assume that the phenomenon of resistance is the death knell for herbicides. Resistant weeds are not super weeds and are often less fit ecologically than their susceptible relatives. It is important to recognize that resistance is possible and to determine the reasons for it. Management of herbicide resistance will require reducing reliance on herbicides as the primary tool for weed management and developing integrated weed management systems that require the substitution of human intellect and skill for chemical technology (Shaner, 1995).

Most Important herbicide resistant crops are given bellow:

1. Rigid grass : *Lolium rigidum*
2. Wild oat : *Avena fatua*
3. Redroot Pigweed : *Amaranthus retroflexus*
4. Common Lambsquarters : *Chenopodium album*
5. Green Foxtail : *Setaria viridis*
6. Bamyardgrass : *Echinochloa crus-galli*
7. Goosegrass *: Eleusine indica*
8. Kochia *: Kochia scoparia*
9. Horseweed *: Conyza canadensis*
10. Smooth Pigweed : *Amaranthus hybridus*

10. Herbicide resistance GM crops

Genetically modified crops are the most rapidly adopted technology in agricultural history due to the social and economic benefits these crops may offer. Crops that are genetically altered to be tolerant to herbicide, followed by crops resistant to insects, were the first agricultural biotechnology inventions successfully commercially exploited worldwide. Until the emergence of genetically modified crops, selective herbicides (herbicides that only kill a specific weed) were the answer. The development of selective herbicides is not an easy task and for this reason only a few common weed species could be targeted. Given that each weed requires a different herbicide, herbicide application was frequent, in large volumes and very costly. The advent of herbicide resistant crops caused a major shake-up in the agro-chemical industry. Demand for selective herbicides fell significantly. In certain countries, for the crops that have herbicide resistance, are widely planted and otherwise non-selective (broad spectrum) herbicides are primarily used for weed management. Provided that the field crops are genetically modified to carry gene(s) for herbicide resistance, these broad-spectrum herbicides will not harm the crop. Broad-spectrum herbicides, such as glufosinate and glyphosate, are comparably biodegradable, display low levels of toxicity, and to date, weeds have shown minimal resistance to repeated applications. Resistance to these broad-spectrum herbicides depends upon the genes that have been inserted into the crop plant.

11. Global scenario

The global area planted with transgenic crops is increasing continuously and according to the recent data available (2010); the total global area of transgenic crops is 148 million hectares, a more than 48 fold increase from 1996. More than eight and a half million farmers

in 28 odd countries have grown transgenic crops. The majority of the growth occurred in the United States (63%), Argentina (20.5%), Canada (6.5%) and Brazil (4.4%). Almost one third (30%) of the global acreage was grown in developing countries. Total area under transgenic crops in India is around 9.4 million hectare. In 2003, herbicide resistant crops made up 73% of the total genetically modified (GM) crop-growing area, while insect resistant crops constituted 18%. GM crops containing genes for both herbicide resistance and insect resistance comprised 99% of the total GM crop growing area. It is expected that the overall global area of transgenic crops and the number of countries growing transgenic crops will increase in near future. Currently, the agricultural GM market is dominated by a single company, Monsanto. Monsanto produces approximately 90% of genetically engineered crops worldwide. This most likely reflects the ownership by Monsanto of patents on the *bar* gene which confers herbicide resistance as well as patent ownership of various *Bt* toxin genes for insect resistance. Another four companies, Syngenta, Bayer Crop Science, Dow and DuPont produce the remaining 10% of transgenic crops. All major herbicide companies have research programs to incorporate herbicide tolerance through genetic engineering in crops. Success has been achieved with several herbicides. The work has focused on major crops: corn, soybean, wheat, rice, cotton, and tobacco. The technology for agricultural crops was introduced as early as the mid-1980s.

12. Metabolically resistant genetically modified – herbicide resistant crops (GM-HRC)

Many crops bearing transgenes coding for highly specific enzymes that metabolically catabolize herbicides have been generated (Cole & Rodgers, 2000). These include for example, bromoxynil resistance crops bearing a nitrilase (Freyssinet *et al*., 1996), glufosinate-resistant crops bearing an acetyl-transferase (Vasil, 1996), 2, 4-D resisting crops bearing a highly specific soluble cytochrome P-450 monooxygenase (Streber & Willmitzer, 1989), phenmidipham resisting crops bearing a bacterial gene and dalapon resisting crops bearing a dehalogenase (Buchanon-Wallaston *et al*., 1992). Of these, only the bromoxynil- and glufosinate resistant crops have reached commercialization. All the herbicide tolerant genes used commercially are of bacterial/ actinomycete origin, despite the fact that plants contain genes for herbicide resistance, which is the basis for most natural metabolic selectivity used for 50 years, yet plant genes conferring metabolic resistance have not been used commercially as yet. There are recent reports using non-prokaryotic genes to confer resistance, but none are yet commercialized, and whether they confer sufficient resistance is not clear. The examples include a rabbit esterase gene conferred resistance to thiazopyr via degradation (Feng *et al*., 1998), the expression of plant and animal P450 transgenes conferred phenyl urea resistance (Inui *et al*., 1999; Siminsky *et al*., 1999). Transgenes encoding maize glutathione transferases increased the level of herbicide resistance (Jepson *et al*., 1997). The crops generated with metabolic resistance seem to be problem-free, with little metabolic load conferred by generating the small amount of enzyme needed. The toxicology is simplified because the transgene product typically initiates a cascade of events whereby the herbicide eventually disappears. There has been an assumption that one cannot use catabolic enzymes to confer resistance to fast acting herbicides. However, inhibitors of protoporphyrinogen IX oxidase (protox), which actually cause the accumulation of the photodynamically- toxic product induce photodynamic death of plants within 4–6 h in bright sunlight. Beans are immune to some members of this group, e.g. acifluorfen, because

they possess a specific homoglutathione transferase and contain enough homoglutathione to stoichiometrically degrade these herbicides before they can damage the crop (Skipsey *et al*., 1997). Similarly, strains of *Conyza bonariensis* contain a complex of enzymes capable of detoxifying the reactive oxygen species generated by the photosystem I blocker paraquat, and keeping the plants alive until the paraquat is dissipated (Ye *et al*., 2000; Ye & Gressel, 2000). As almost all herbicides are either degraded in the soil or in some plant species, one should be able to find more genes for catabolic resistance to those herbicides and then be able to rapidly generate herbicide-resistant crops with metabolic resistance than with target site resistance.

13. The success of genetically modified herbicide resistant crops (GM-HRC)

Millions of hectares are being planted with GM-HRC, with insect resistance in second place, with both traits often 'stacked' in the same seeds to enhance their value. The real values of GM-HRC come from instances where there really are no viable weed control methods (e.g. due to evolved herbicide resistances in weeds), and the impact that such GM-HRC could lead to a more sustainable world food production. The easiest way to obtain selectivity among closely related species is to engineer resistance to a general herbicide into the crop. For example, it has already been shown that rice (*Oryza sativa*) is easily controlled by glufosinate. The transgenic rice (Sankula *et al*., 1997a, b) bearing the *bar* gene confers resistance to this herbicide (Oard *et al*., 1996). The immediate answer to multiple resistance problems in weeds of wheat in major growing areas is to engineer resistances to inexpensive herbicides (Gressel, 1988). Neither the chemical nor the biotechnological industries have shown particular interest in generating GM-HRC in wheat, rice, millets, pulses or oilseed crops. As too little profit is perceived to come from wheat, rice or other seed or even from generic herbicides, it may be necessary to have wheat, rice, millets and pulses engineered by the public sector. Glufosinate resistance has been engineered into wheat, more as a marker gene than for agronomic utility (Weeks *et al*., 1993). GM-HR wheat, rice and food legume crops may be an answer to the major problems of these crops. Inserting a gene into wheat or rice conferring resistance to a broad spectrum herbicide can control weeds that evolved resistance in wheat and even closely-related grasses, including red, weedy, and other wild rices (Gressel, 1999a, b, c). The transgenes will allow problems of resistance that have evolved to be overcome especially, the problems of cross-resistances (where one evolutionary step confers resistance to a variety of chemicals) and multiple resistances (where a sequence of evolutionary steps with different selectors, confers resistance to a variety of chemicals). The use of non-plant transgenes may also allow farmers to overcome the natural resistances in weeds closely related to the crop. The problems of interclass cross-resistances and multiple resistances in wheat have necessitated considering the generation of GM-HR oilseed rape, especially in Australia (Gressel, 1999b). Oilseed rape has become an excellent rotational crop alternating with wheat in many places where wheat is grown. There are many agronomic advantages to rotating a dicot with a monocot, especially vis a vis weeds. It should be far easier to eliminate grass weeds in oilseed rape than in wheat, as there are more selective graminicides available for use in dicot crops. There are far too few concrete molecular and biochemical data published about the properties of these crops and thus there are problems in evaluating their properties to allow suggestions for improvements. Thus, some of what will be said below should be considered as speculative. Two types of gene have been used to generate herbicide-resistant crops: (1) where the gene

product detoxifies the herbicide; (2) where the herbicide target has been modified such that it no longer binds the herbicide. One could envisage other types such as exclusionary mechanisms, sequestration, etc., but they have yet to be found and thus not utilized. The resulting GM-HRC with each type of resistance is rather different.

14. Herbicide tolerant food legume crops

The food legumes like chickpea, pigeonpea, fieldpea, lentils, urdbean and mungbean are very important for food and nutritional security of poor people in India. These crops suffer to a great extent (33%) due to infestation by weeds. At present no post emergence selective weedicide is available which can be effective to control weeds as these crops are highly sensitive to application of herbicides. Hence, mechanical or manual weeding is considered to be only management options for weed control. In general, food legumes are highly sensitive to available post emergence weedicides. It is, therefore, required to develop resistance/ tolerance in these crops against post emergence weedicides. Development of GM-HRC can be one of the potential options. However, in mid eighty when priorities in area of plant biotechnology were decided in country for developing GM crops, herbicide tolerant was kept out of priority because very cheap agricultural labour were available for these operations in the country. However, with increasing industrialization there is acute shortage of farm labours in the country. Therefore, the need of GM-HRC is realized these days. In view of this, genetic transformation has been successfully attempted in chickpea, pigeonpea and fieldpea with bar gene (used as selectable marker) and stable transformants have been recovered which show considerable degree of resistance to phosphinothricin (Singh *et al.,* 2009). In azuki bean, genetic transformation was done by introducing binary plasmid (pZHBG) comprising the bar gene coding the enzyme, phosphinothricin acetyltransferase which directly inactivates the herbicides phosphinothricin and confers resistance to the commercial herbicides, bialaphos (Confaloneirri *et al.*, 2000). In Cowpea, stable gene transformation was obtained by using particle gun method by Ikea *et al.,* 2003. However, the level of expression of introduced genes in cowpea cells is very low and this accounted for the high mortality rate of progenies under Basta spray. Transgenic plants of the model legume Lotus japonicus were regenerated by hypocotyl transformation using a bar gene as a selectable marker (Lohar *et al.,* 2001). The production of PPT herbicide-resistant L. japonicus plants has shown significant commercial applications in crop production. Brar *et al.,* 1994 developed transgenic plants of peanut of cultivars Florunner and Florigiant, two of the most widely cultivated peanut cultivars in the USA, using the ACCELL® gene delivery method. Gus and bar genes exhibited predictable segregation ratios in the R_1 and R_2 generations and were genetically linked. Integration of the bar gene conferred resistance to BASTA™, a wide-spectrum herbicide, applied at 500 ppm of active ingredient. This work has paved the way to develop herbicide tolerant transgenics in these crops. However, there are far too few concrete molecular and biochemical data published about the properties of these crops and thus there are problems in evaluating them for improvements. With the increasing use of herbicide tolerant crops,there comes an increasing use of glyphosate based herbicide sprays. In some areas glyphosate resistant weeds have developed causing farmers to switch to other herbicides. Some studies also link widespread glyphosate usage to iron deficiencies in some crops, which is both a crop production and a nutritional quality concern, with potential economic and health implications.

15. Risks and concerns of GM HRC

It is generally incurred that the development of herbicide tolerant crop will encourage heavy use of herbicides. Hence, concern has been expressed about water or food contamination from increased herbicide use. Additional concern centres on use of herbicides in crops that do not metabolize the herbicide. Therefore, the unaltered herbicide could be consumed by people. As herbicide resistant crops develop, it is important to remember that no technology is ever proved to be perfectly safe. Scientists look for evidence of harm, and if none is found, conclude that there is none or that it must be looked for in a different way. Second, this technology, like all technologies (e.g., herbicides, cell phones, computers), has both its good and bad uses. We must be cautious about demonizing the potential but unknown bad effects of legitimate uses by good people and weigh them carefully against illegitimate uses by bad people.

Environmental concern: Environmental concern is related to herbicide use. It is suggested that transgenic crops have the potential to create a more sustainable agricultural system than present chemically based systems but will fail "in enabling a fully sustainable agriculture." As genetic traits that have a higher potential of enabling truly sustainable agricultural systems have not been developed due to, the lack of EPA and regulatory policies that specifically promote sustainable traits.

An agricultural biotechnology industry is dominated by agricultural chemical companies.

Patent law and industry policies prevent farmers from saving transgenic seed and thus tailoring transgenic crops to their local ecological conditions.

Social concern. Social concern is related to the following:

1. Fear that the technology will favour large farms and lead to loss of more small farms and small-scale farmers.
2. Cost of food production and food cost to the consumer will rise.

Weed control concerns. There are three major concerns related to weed control:

1. **Development of herbicide resistance***:* Herbicide resistance among weeds may become more widespread because of continued use of an herbicide to which a crop is resistant (Sandermann, 2006).
2. **Resistant gene flow to sexually compatible plants**: This is acknowledged as a potential risk of introducing any genetically engineered (transgenic) crop variety. The risk is transfer of desired herbicide resistance from the crop to a weed where undesirable resistance persists by natural selection. It is worth noting that this has happened when genes from herbicide resistant canola moved to a non-weedy relative in the mustard family and then to wild mustard in a short time. The risk may be especially high where the crop and weed are closely related and can interbreed—for example, red rice and rice or Johnson grass and grain sorghum.

Once such gene(s) is transferred within wild populations, it is suggested that a selective advantage could be conferred on the recipients, so altering their biology and influencing their ecological relationship with native genotypes or other species (Lefol *et al,* 1997; Linder, 1998). It is considered that this could constitute a threat to biodiversity. The possibilities for transfer of any trait from crop to weed will depend on the two occurring together, their

synchronous flowering, successful pollen transfer and compatibility of the pollen that would allow successful fertilization and embryo development. Any seed produced would then need to germinate and the trait would need to be exhibited in the resulting plant. To maintain the trait, success as a pollen donor, as a seed producer, or both, would be needed. Where a trait carrier is self fertile or where more than one individual has been produced, the F_2 generation may be produced by hybrid mating, though the more likely scenario is for introgression into the recipient species' genome as a result of backcrossing. Crop plants and some weeds are derived from the same ancestors and retain a number of common characteristics. They may also still grow in close association within the geographical area in which both originated and give rise to crop-weed complexes (van Raamsdonk and van der Maesen, 1996) in which introgression of weed characters into the crops and crop characters into the weeds can occur, and may have done so over an extended period of time. Little Seed Canary Grass (*Phalaris minor*) is a monocot weed in the Poaceae family. In India, this weed first evolved resistance to Group C2/7 herbicides in 1991 and infests wheat. Group C2/7 herbicides are known as Ureas and Amides (Inhibition of photosynthesis at photosystem II). Research has shown that these particular biotypes are resistant to isoproturon and they may be cross-resistant to other Group C2/7 herbicides.

3. **Resistant crop plants becoming hard-to-control volunteer weeds:** The quite legitimate concerns of epistasis and pleiotrophy must also be recognized. Another common critique of herbicide resistant crops is that the technology will promote the use of herbicides, not decrease it, while continuing to develop what many view as an unsustainable, intensive monocultural agriculture. It is also suggested that herbicide-resistant crops will reinforce farmers' dependence on outside, petroleum-based, potentially polluting technology. An associated concern is that there is no technical reason to prevent a company from choosing to develop a crop resistant to a profitable herbicide that has undesirable environmental qualities such as persistence, leachability, harm to nontarget species, and so on. It is undoubtedly true that nature's abhorrence of empty niches will mean that other weeds will move into the niches created by removal of weeds by the herbicide used in the newly resistant crop. In other words, herbicide resistance will solve some but not all weed problems. Weeds that are not susceptible to the herbicide to which the crop is resistant will appear. Development of herbicide-resistant crops is proceeding rapidly, and there are important advantages that provide good reasons for their development. Many argue that the technology will provide lower-cost herbicides and better weed control. These are powerful arguments in favour of the technology because both can lead to lower food costs for the consumer. It is also true that herbicide-resistant crops are providing solutions to intractable weed problems in some crops. Glyphosate resistance has been created in several crops. It is an environmentally favourable herbicide, and therefore, it is better to use it in lieu of other herbicides that are not environmentally favourable. An important argument in favour of the technology is that it has the potential to shift herbicide development away from initial screening for activity and selectivity and later determination of environmental acceptability to the latter occurring first. Resistance to herbicides that are environmentally favourable but lack adequate selectivity in any crops or in a major crop so their development will be profitable could be engineered and the herbicide's usefulness could be expanded greatly. This has important implications for minor crops (e.g., vegetables, fruits) where few

herbicides are available because the market is too small to warrant the cost of development. If resistance to an herbicide already successful in a major crop (e.g., cotton) could be engineered into a minor crop, manufacturers and users would benefit. The public doubts about genetic modification of anything are raised, and it is in this context that these doubts must be addressed. Weed scientists and others involved with GMOs often think if we can just educate the public about our science, the problem will be solved as technology is already widely promoted, accepted and used.

16. Future prospects

Plant genetic engineering and biotechnology is now moving from the initial euphoria to the phase of course correction. Several environmental problems related to plant genetic engineering prevent realization of its full potential. One such common concern is the escape of foreign genes through pollen dispersal from transgenic crop plants engineered for herbicide resistance to their weedy relatives creating "superweeds" or causing gene pollution among other crops. Such dispersal of pollen from transgenic plants to surrounding non-transgenic plants has been well documented. The high rate of such gene flow from crops to wild relatives (as high as 38% in sunflower and 50% strawberries) is certainly a serious environmental concern. Clearly, maternal inheritance of foreign genes is highly desirable in such instances where there is no potential for out-cross (Daniell *et al.,* 1998). Since the transgenic crops have been available for some time, we know what has been done with genetically modified herbicide resistant crops. The technology is so new and changing so rapidly that we do not—perhaps cannot—know what might be done. That is, the direction of research is clear, but the final destination is not. We cannot be sure what new possibilities will be discovered as the technology of herbicide resistance continues to develop rapidly. Adoption of molecular-based methods in weed science research will bring a new dimension to the science and can have "far reaching benefits in agriculture and biotechnology" (Marshall, 2001). One potential benefit of genomics research is the discovery of new targets for herbicide action (Hess *et al.,* 2001). Other benefits may include identification and use of genes that contribute to a crop's competitive ability (e.g., early shoot emergence, rapid early growth, fast canopy closure, production of allelochemicals). Genomics may also discover genes that contribute to weediness, a plant's perennial growth habit, seed dormancy, and allelopathy (Weller *et al.*, 2001).

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24

Influence of Degree Infestation with *Echinochloa crus–galli* Species on Crop Production in Corn

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1. Introduction

Corn continues to be globally one of the main crops, ranking third, after wheat and rice. In Romania it is the main agricultural plant, whose economic importance, especially in the private sector is growing. Given the particularities of this culture, with particular reference to the high sensitivity at infestation with weeds, especially in the early stages of vegetation, corn crop is feasible only if weeds are controlled through various methods. The damage caused by weeds in maize crop are mostly of 30-70% (Sarpe, 1975; Budoi and Penescu, 1996; Oancea, 1998; Bilteanu, 2001; Berca, 2004; Gus et al., 2004; Bogdan et al., 2005; Rusu, 2008) and when the infestation is strong, culture can be fully compromised. The presence in a culture of a small number of weeds is not harmful, but damage caused by weeds grow along with increasing the degree of infestation, depending on the species and age of occurrence of weeds, soil and climate conditions and the moment when weeds are combat (Paunescu, 1996; Bosnic and Swanton, 1997; Perron and Legere, 2000; Bogdan et al., 2001; Fukao et al., 2003; Clay et al., 2005; Rusu et al., 2009). Therefore, specifying the economic threshold of pest is difficult to establish considering the fact that the number of researches in this field until now, is reduced.

Echinochloa crus-galli is one species with a large requirement to water being able to behave as mesophita, mesohygrophita, hygrophita and hygrohelophita (Anghel et al., 1972; Bogdan et al., 2007). It is especially met on the luvosoils, fertile and wet soils, being wide-spread in all the country but in the north sides it has a lower abundance and general dominance than in the south ones. *Echinochloa crus-galli* is met growing on a large variety of soils and grains, from clay sand or sandy clay soils to medium hard soils. The soils with a relative big capacity of water holding and large fertility insure an ideal sublayer.

Echinochloa crus-galli is a weed with a fascicular, powerful root which is hardly drawn by weeding and it easily sprouts after mowing or while weeding. The seeds get to maturity progressively and they can keep the germinal sufficiency till 8-9 years, germinating by installment. They do not support the flooding (Dimancea, 1967).

The success of this weed can be imputable to a very low number of seeds generation, easily dispersed from the plant, owning a latent state of the seeds, a fast development and capacity of blooming in a large range of photoperiods (Păunescu, 1997). The number of seeds made

by a plant varies between 200 and 10,000. Chirilă (1967) establishes as limits of seeds number/plant from 150 to 10,000. The medium mass of 1,000 bobsleighs is 2.48 g. (Anghel et al., 1972; Berca, 1996). The reserve of *Echinochloa crus-galli* seeds that can be found in the soil can reach impressive values, correlative with the production potential of the species and the vegetative conditions specific to the infestation areas. The number of seeds found by Kott (1953) reported to the surface of one hectare gets to 1-2.5 billion *Echinochloa crus-galli* seeds. Berca (1996) referring to the seeds of this species and their germination, shows that germination happens after one year of seeds forming, by instalment both as structure and life. The germination happens all over the year, especially in the spring time, 1-2 cm depth when the temperature is over 10 ^{o}C. The *Echinochloa crus-galli* seeds have a post maturation period that happens into the soil, especially the upper side of the soil. The length of seminal rest depends by a lot of internal and external factors (Berca, 1996).

The period of germination-rising starts for *Echinochloa crus-galli* in April, depending by the temperature provided by the soil, the minimum germination temperature is 8^{o}C, and it ends in September. The maximum germination is between May and June, after this period there comes an attenuation of germination proportion and plants rising, so that in October it is accomplished to a very low level.

The elongation of the *Echinochloa crus-galli* plants is in a strong connection with the temperature. In the spring time when the temperature is low the elongation is a slow one but in the summer time when the temperature is high the plants grow very fast (Păunescu, 1997; Rusu et al., 2010). At the beginning the plants grow slowly, after 2-3 weeks after their rising starts the tillering period after that the plants start a very fast growing if the conditions of light, humidity and nutritive substances are assured (Berca, 1996). After the floral branches cutting of this weed, or after the first fructification they sprout again during the same year and fructify for the second time (Staicu, 1969).

Echinochloa crus-galli belongs to the yearly monocotiledonate weeds class with late spring germination very harmful for the corn cultures. Growing very fast it asphyxiates the corn crop and infamies the crop.

2. Material and methods

Our researches highlight in terms of Transylvania, the influence of *Echinochloa crus-galli* species (L.) Pal. Beauv. and other weeds on corn production, according to the degree of infestation. Researches have been conducted on Experimental Teaching Resort of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. The experiments were located on the northern slope, weak to moderately sloping land, with soil type preluvosol (SRTS, 2003), medium fertile, humus content 3%, texture loam-clay, 42-45% clay. Experience was held between 2004 and 2009 and had more objectives:

2.1 The vegetative cycle and the productivity characters of *Echinochloa crus-galli* depending on the rise period, at Cluj area

The researches were made between 2004 and 2007, respecting the climatic conditions of the soil appropriate to every year. The researches were made outdoors onto 1 m^2 plots, where we sowed 20 caryopsis of *Echinochloa crus-galli* per plot, at the beginning of April, May, June,

July and August. The observations were made every 10 days between rising and maturity. We analyzed the rising period according to the sowing date; the leaves appearance, the sprouting and the stem elongation, the panicle appearance and the flourishing beginning, the plants' maturation, the caryopsis' maturation and the dissemination and the productivity's characters variation.

In each lot the plants were rarefied, the observations regarding the productivity elements being done upon a number of 3 plants on surface unit. We chose this density for the reason of the necessary space protection for the *Echinochloa crus-galli* growing in order to touch the maximum values of the productivity parametres according to the biological potential of the plant.

The years of experiment with climatic specific (May 1 – August 31)
2004: 405.7 mm (excessively wet climate) and 17.6 ^{o}C (normal temperatures conditions);
2005: 349.6 mm (excessively wet climate) and 18.05 ^{o}C (normal temperatures conditions);
2006: 455.4 mm (excessively wet climate) and 18.5 ^{o}C (warm temperatures conditions);
2007: 167.5 mm (excessively dry climate) and 18.8 ^{o}C (warm temperatures conditions).

Year	Specification	The daily average temperature, ^{0}C	Rainfall, mm
2004	Value	14.88	562.4
	Deviation	- 0.75	+ 204.4
2005	Value	16.1	505
	Deviation	+ 0.47	+ 147
2006	Value	16.83	572
	Deviation	+ 1.2	+ 214
2007	Value	16.98	250.6
	Deviation	+ 1.35	- 107.4
The normal values		15.63	358

Table 1. The climate conditions during the 1st of April and the 30th of September in Cluj.

2.2 Productivity elements variation of *Echinochloa crus-galli* in accordance to density

In order to follow the variations of productivity parameters to *Echinochloa crus-galli*, the experiences were fixed on the field, in 4 random repetitions, after blocks method, on 1m^2 lot surface. In the last decade of April there were seeded 200 caryopsis of *Echinochloa crus-galli* on each lot so that the rising of the plants to be assured for the beginning of May.

After the plants rising and the forming of two first leaves, there was done their spacing in order to achieve the density of 50, 20, 10, 5, 3, 2 plants per m^2. The rating of the plants growing parameters and plants productivity was done in the last decade of July – first decade of August – when the plants were mature enough having as goals: plants height, tillering, panicles length, number of seeds (production).

The results interpretation was done by means, percents, statistical elaboration (variance analyzes). In order to analyze the values that were obtained there were used control data, medium values of the parameters obtained in the variant of density 3 plants/m^2.

2.3 Influence of degree infestation with *Echinochloa crus–galli* species on crop production in corn

Experience was held between 2008 and 2009. Biological material was the hybrid Turda 201, recommended for this area of culture. The research was done on two agrofonds: unfertilized and mineral fertilized (MF) with NPK 100 kg/ha.

In the unfertilized maize crop were made four variants (I-IV) with different degrees of infestation with *Echinochloa crus-galli*, from about 40 to 100 plants/m^2 and witness – 2 holings.

In fertilized plots were used the next herbicides for weed control: V_1 - dimetenamid 900 g/l – 2 l/ha applied p.p.i. (pre plant incorporated). V_2 - acetoclor 860 g/l – 2 l/ha applied preemergent. V_3 - isoxaflutol 750 g/l – 0.15 g/ha, applied p.p.i. + (bentazon 320 g/l + dicamba 90 g/l) – 2 l/ha applied postemergent.

Herbicide application was made with the pomp for experience, applying 300 l solution/ha. The experience was organized after randomized blocks method, in four repetitions and area of a plot is 25 m^2. Competition between corn plants and weeds present was studied in natural density infestation, in unfertilized plots and in those fertilized in which the process of herbicides took place. Weed biomass, corn plants and grain production was measured in the ripening stage. Samples of plants and weeds were harvested using metric frame of 50/50 cm.

3. Results and discussion

3.1 The vegetative cycle and the productivity characters of *Echinochloa crus-galli* in accordance to rising period

The biological particularities of weeds make them be superior to the cultivated plants, as they use more effective the vegetation conditions and the afferent inputs of an agricultural area. *Echinochloa crus-galli* is an annual monocotyledonous species, which germinates late in spring. This species is spread onto extensive areas in the world, covering all continents between 50^0 northern latitude and 40^o southern latitude. In Romania there is plenty of it in all regions, prevailing in the south western part of the country (90%) and in the eastern part (75%). In the other areas, the species varies between 9% (Dobrogea) and 57% (Transylvania). It is very harmful for hoed cultures. In the Cluj County, *Echinochloa crus-galli* represents between 36% and 52% of the weedy rate of the hoed cultures. *Echinochloa crus-galli* produces big damages in Romania's agriculture: in maize – over 70%, in rice – 60 – 65%; in sunflower – 30%; in soybean – 15 – 20%, in sugar beet 25%, in wheat – 10%, in flax – 10%.

In autumn or early spring sowed cultures (that cover the soil to a large extent) *Echinochloa crus-galli* hardly forms a small stem, but when the cultures are harvested off the field, the weed heavily sprouts and it produces a large amount of seeds as it has more space, light, nutrition and moisture.

The high ecological plasticity and adaptability of this species, completed by the possibility of flourishing in a wide range of photoperiods are biological particularities of *Echinochloa crus-galli.*

The plants rising takes place monthly in different percentages until September, when the rising is reduced. The rising period is of 8 – 16 days since the sowing depending on the

temperatures. The correlations established between the soil temperature conditions and the *Echinochloa crus-galli* plants rising are very significantly positive. From the specific equations for the experiment years result that the percentage of the plants that are rising is increasing by 5.75 – 6.87 per 1 ^{0}C of the soil temperature - beginning with 8^{0}C, the minimum germination temperature.

The plants' growth varies according to the rising period. So, the plants that rise up during the second half of April pass through each specific vegetative stage for a longer period comparing to the ones that rise up during the next months – when the temperatures increase (Table 2).

The rising period/The vegetative stage	April	May	June	July	August
1-3 leaves phase	8 – 10	6 - 8	6 – 7	6	5 – 6
Tillering beginning	19 – 23	17 - 20	13 – 17	12 – 15	12 – 14
The intensive tillering, the adventitious roots rising	32 – 38	28 – 34	20 – 21	18 – 20	16 – 19
The end of the tillering, the culm elongation	50 – 56	48 – 50	30 – 34	28 – 30	26 – 30
The skin stage*	70 – 80	64 – 68	43 – 46	38 – 43	36 – 41
The panicle apparition, flowering*	83 – 90	74 – 79	58 – 61	49 – 54	48 – 50
The grains filling *	94 – 105	80 – 88	64 – 70	60 – 63	59 – 63
The seeds' maturation; dissemination*	110 - 115	95 – 99	79 - 82	70 - 79	65 - 72

*This information is specific for the main stem. The shoots pass progressively these stages after the main stem.

Table 2. The period in days passed by a *Echinochloa crus-galli* plant from it's rising to each vegetative stage (the average period for the years 2004 – 2007 on Cluj-Napoca conditions).

The daytime influences the flourishing period so that the plants that rise later (July, August) reach the flourishing phase in a much shorter period (48 -54 days), comparing to the plants that rise in April (83 days). The shorter days of late August and early September stimulate precocious flourishing and ageing.

The caryopses are maturating in a 20 – 30 days period, after heading (depending on the rising period).

The first panicle dissemination is taking place during 10 -16 August for the plant that rose in April; 15 – 19 August for those that rose in May (first decade); 28-30 August for those that rose in June; 25 – 28 September for the plants that rose in July and 15 – 18 October for those that rose in August.

At the beginning of September the first plants dry out; they are those that rose in April, while those that rose in August dry out at the end of October.

The vegetative cycle of *Echinochloa crus-galli* plants is taking place in summer (Fig. 1). It begins in April for the plants sowed in April and it ends in August. But the cycle for the plants sowed in August, it ends in October.

The length of the vegetation period for a plant and the productivity characters (the height, the shoots number, the panicles number, the panicles' length, the caryopsis number of a plant, the bio weight) are reduced as the plants' rising is late (Table 3).

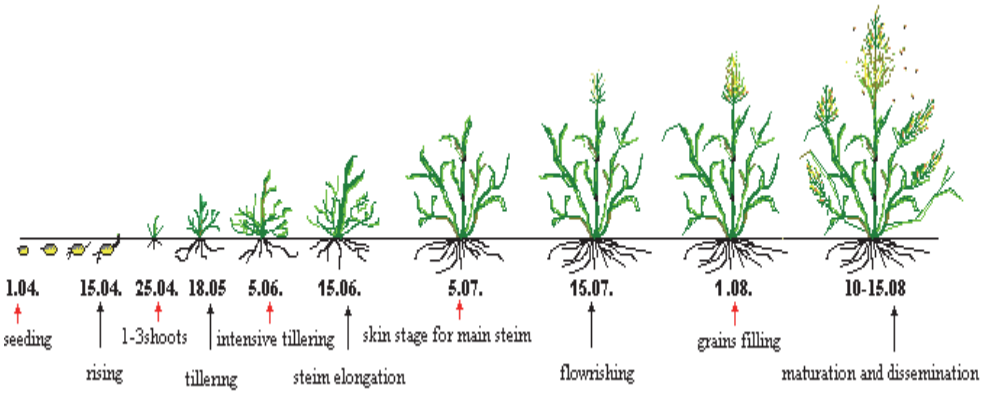

Fig. 1. The growth cycle of *Echinochloa crus-galli* (L.) P.B. on Cluj-Napoca conditions.

The variation of the vegetation period of this species (85 – 140 days) reflects the extraordinary flexibility and the excellent adaptability of *Echinochloa crus-galli* in different environment conditions.

The plants height decreases from 170 cm (the plants that rose in April) to 55 cm (the plants that rose in August). The vegetative growths are reduced as the vegetative period is decreasing and the daily average temperatures are increasing. The plants that rise in spring (April, May) grow and tiller very intensely, they reach considerable heights as a consequence of late flourishing, as the daytime is longer.

The roots of the early plants grow more intensely.

The whole weight reaches impressive values for the long vegetation period plants; it decreases drastically for the plants that rose late. The seeds production decreases as the vegetative periods diminish. It is notable that *Echinochloa crus-galli* seeds production is important (8435 caryopsis/plant) even for the plants that rose in August.

The rising period/The features	April	May	June	July	August
The vegetative period (days)	135-140	125-130	115-120	95-100	85-90
The plants height (cm)	150-170	140-150	120-130	90-105	55,75
Number of shoots	23-25	19-25	16-18	12-14	13-15
Number of panicles	27-31	20-24	16-18	10-16	12-14
The length of the panicles (cm)	12.5-20.4	11.2-18.5	8.5-10.6	7.5-9.4	5.6-7.2
Number of seeds (average/plant)	15794	13406	10898	8762	8435
The length of the roots (cm)	39-45	35-38	30-34	24-27	13-16
Bioweight (gr./plant – herb weight)	895	794	586	338	212

Table 3. The vegetation period and the productivity features of *Echinochloa crus-galli* depending on the rising period on Cluj-Napoca conditions.

There have been variations of the productivity characters among the 4 experimentation years, according to the temperatures and the pluviometric quantities.

The climate of 2006 and 2007 significantly influenced the productivity characters of this species. So, during 2006 – the wealthiest in precipitation, the vegetative growth of the *Echinochloa crus-galli* plants was impressive: the maximum height was 218 cm, the shoots number was 49, and the developed panicles number was 45. During 2007 – when it was drought, there were the lowest values for the vegetative growth, but there were a lot of seeds comparing to the plants height.

3.2 The productivity features variation of *Echinochloa crus-galli* in accordance to density

The productivity features variations of *Echinochloa crus-galli* depend by the plants density to surface unit and climatic conditions specific to experimental years.

The height of the plants is strongly influenced by density, increasing significantly to a low density (2 plants/m² than 3 plants/m²) and a high one (50 plants/m² than 3 plants/m²) due to a strong shading and lack of light (Table 4). This feature (height) did not present a constant variation regarding the climatic conditions specific the experiment years, the single year when the medium difference of height was significantly negative was 2007.

Density / Year	2004	2005	2006	2007	Average
50 plants/m²	172.6***	135.6***	184.5*	65.8ooo	139.6***
10 plants/m²	159.8***	120.1***	158.6ooo	76.3oo	128.7ns
5 plants/m²	142.2oo	101.3oo	180.2o	81.4**	126.3ns
2 plants/m²	152.6***	118.9***	205.6***	84.2***	140.3***
Control 3 pl/m²	145.6	103.5	182.4	78.5	127.5
LSD 5%	1.85	1.19	1. 58	1.41	1.75
1%	2.63	1.70	2.24	2.01	2.48
0.1%	3.80	2.46	3.25	2.91	3.56

Note: ns – not significant, * signification positives, 0 signification negatives

Table 4. The height of *Echinochloa crus-galli* (cm) in accordance to density.

The other characters of productivity are strongly influenced by the plants density on the surface unit. Between 3 and 5 plants /m² it is achieved a close tillering, panicles number, panicles length and seeds number, with no significant differences while the density growing is 5 plants bigger on the surface unit all these conditions are decreased to limits between signification negative to very signification negative. The density attenuation under 3 plants on square metre has as effect the growing of the species productivity potential.

The danger that this plant represents even to a reduced infestation of the cultures comes from the possibility of achievement both a high biomass through the vegetative growing elongation, and a very high production of seeds that will represent the source of weeding for the next cultures.

The influence of the nutritional space size and development is very strong upon this species *Echinochloa crus-galli.* The tillering, the panicles number and panicles length are very significantly reduced to densities bigger than 5 plants per surface unit (Table 5).

The differences that appear among the years represent a consequence of species adaptability for adjustment conditions of the productivity in accordance to climatic conditions. If during the rainy years the tillering is influenced from distinctive significant to very negative significant by plants density growing with 2 samples per surface unit, during the very dry year – 2007 - this condition does not suffer any adjustment having a significant growing by density increasing with 2 plants per surface unit. The explanation of this fact is found into the high capacity of tillering of this species when the height growing is diminuated.

Density/Year	2004	2005	2006	2007	Average
50 plants/m²	3.2^{ooo}	2.9^{ooo}	4.2^{ooo}	2.4^{ooo}	3.2^{ooo}
10 plants/m²	12.9^{ooo}	12.1^{ooo}	14.3^{ooo}	9.3^{ooo}	12.2^{ooo}
5 plants/m²	19.3^{oo}	18.8^{oo}	20.4^{ooo}	16.9*	18.9^{ns}
2 plants/m²	23.4^{ns}	20.1^{ns}	26.2*	18.5***	22.1*
Control 3 pl/m²	22	20	25	16	20.5
LSD 5%	1.59	0.73	0.97	0.88	1.35
1%	2.26	1.04	1.38	1.25	2.13
.1%	3.27	1.5	2.00	1.81	2.97

Table 5. The tillering per plant on *Echinochloa crus-galli* in accordance to density.

The number of panicles per plant (table 6) follows, in general, the same tendency of a very significant decreasing to densities bigger than 5 plants per surface unit and increasing or decreasing from insignificant to very significant in the situation of a density increasing with only 2 plants, in accordance to the climatic conditions of the year. The weed density decreasing under 3 plants /m² has as effect in both character situations (tillering and panicles number) increasing from insignificant to very positive significant. The number of panicles is, especially, the most influenced positively character by the density decreasing.

Density/Year	2004	2005	2006	2007	Average
50 plants/m²	2.4^{ooo}	2.6^{ooo}	3.1^{ooo}	3.9^{ooo}	3.0^{ooo}
10 plants/m²	13.1^{ooo}	14.5^{ooo}	16.6^{ooo}	9.6^{ooo}	13.5^{ooo}
5 plants/m²	20.8^{ooo}	21.2**	26.8^{ns}	18.2^{ns}	21.7^{ns}
2 plants/m²	25.2***	26.1***	32.4***	20.8***	26.1***
Control 3 pl/m²	23	20	27	18	22
LSD 5%	0.96	0.83	0.95	1.22	1.12
1%	1.36	1.19	1.35	1.73	1.49
0.1%	1.97	1.72	1.95	2.50	2.38

Table 6. The number of panicles on *Echinochloa crus-galli* in accordance to density.

The panicles length is very significant reduced to plants densities of *Echinochloa crus-galli* bigger than 10 plants /m² while density of 5 plants /m² does not make significant differentiations (Table 7).

The production of caryopsis per plant is very significant reduced to increasing of plants density per surface unit starting with density of 5 plants /m². On this density, where the other productive features are less influenced compared with witness density (3 plants/m²), the seeds production suffers major decreasing especially during the years that are rich in

precipitations, when the productive potential of the species is directed to vegetative features (Table 8).

Density/Year	2004	2005	2006	2007	Average
50 plants/m²	11.4^{ooo}	11.3^{ooo}	12.2^{ooo}	10.3^{ooo}	11.3^{ooo}
10 plants/m²	14.1^{ooo}	13.4^{o}	14.3^{ooo}	12.1^{ooo}	13.5^{ooo}
5 plants/m²	16.1^{ns}	13.6^{ns}	16.2^{ns}	13.4^{ns}	14.8^{ns}
2 plants/m²	16.4^{ns}	15.1***	17.1*	14.9*	15.9*
Control 3 pl/m²	16.1	13.9	16.4	13.9	14.85
LSD 5%	0.56	0.43	0.63	0.73	0.65
1%	0.79	0.61	0.83	1.04	1.25
0.1%	1.15	0.89	1.29	1.52	1.56

Table 7. The length of panicles on *Echinochloa crus-galli* (cm) in accordance to density.

Density/Year	2004	2005	2006	2007	Average
50 plants/m²	1,289^{ooo}	1,216^{ooo}	1,482^{000}	2,105^{ooo}	1,523^{ooo}
10 plants/m²	9,462^{ooo}	8,324^{ooo}	9,874^{ooo}	7,304^{ooo}	8741^{ooo}
5 plants/m²	13,821^{ooo}	10,918^{ns}	12,956^{ooo}	10,021^{oo}	12,179^{oo}
2 plants/m²	15,659^{ns}	12,164*	16,102^{ns}	11,434*	13,840^{ns}
Control 3 pl/m²	15,208	11,303	16,018	10,795	13,406
LSD 5%	457.05	667.1	669.8	511.3	678.6
1%	649.71	948.3	952.1	731.08	973.4
0.1%	940.74	1,373.1	1,378.7	1,058.5	1,354.8

Table 8. The number of seeds/plant on *Echinochloa crus-galli* in accordance to density.

The productivity features of *Echinochloa crus-galli* suffer changes in accordance to the weed density per unit surface, to high densities the increasing in high are very visible while the tillering, panicles number, panicles length and the number of seeds produced by a plant are reduced very significant. Between 2 and 5 plants of *Echinochloa crus-galli* /m², the productivity parameters vary in more reduced limits, being in the most cases the consequence of the climatic conditions of the experimentation years. The inter specific concurrency is felt even when speaking about the increasing with one plant per surface unit, but this one becomes hypercriticalism in case of density growing with more than 5 plants/m².

3.3 Influence of degree infestation with *Echinochloa crus–galli* species upon the maize crop

Echinochloa crus-galli is known as a weed which germinate in late spring, invades especially weeding crops on wetlands, fattened with manure, grows very quickly, suppress and compromise the culture. Precipitation in April - May 2009 (102 mm in April compared to 47 mm multiannual average and 105 mm in May compared to 76 mm) have delayed corn seeding until the end of the optimal period and promoted the accumulation of moisture in the soil of 30% on average depth from 0 to 50 cm and a reserve of water on the same depth of 977 m³/ha. Under these conditions, sown late, high humidity, fertilization in the last year

with manure, favoured an excessive infestation of the culture, with species that germinate in late spring and especially *Echinocloa crus-galli*. At the same time, shortcomings on internal drainage of the soil aggravate the maintenance of crops in critical periods. Under these circumstances, competition for factors of vegetation was quickly won by *Echinochloa crus-galli* which influenced the subsequent development of maize and other weeds (Fig. 2).

In the unfertilized variant, corn invaded by weeds grows anemic and has a yellowish green color, develops storied, on the upper *Echinochloa crus-galli* dominate, in the middle floor develops *Setaria glauca* (L.) Beauv. and in the lower floor a number of dicotyledonous: *Galinsoga, Convolvulus, Matricaria, Lapsana, Hibiscus, Plantago* etc. (Table 9). The amount of weeds, obviously influenced production levels of maize grain and green mass (Fig. 3). Thus, it is found that on unfertilized agrofond with 22,113 kg/ha weed, maize green mass production is 2,100 kg/ha and with 200 kg/ha weed, maize green mass production is 29,790 kg/ha. The total amount of green mass (weed + maize/ha) varies in very close limits between 24,213 kg/ha to 31,740 kg/ha. On fertilized variant, the competition between weeds and maize, on the one hand and between monocotyledonous and dicotyledonous on the other hand, is more balanced, as dicotyledonous come from 1,700 kg/ha in unfertelized variants (Table 9), to 4,159 kg/ha in untreated, mineral fertilized variant (Table 10). On fertilized agrofond in untreated plot, the whole plant corn production was 27,600 kg/ha, and the grain production was 1,965 kg/ha, while the total mass of monocotyledonous weeds weighed 19,560 kg/ha and dicotyledonous weeds 4,159 kg/ha. In the variant treated with dimethenamid the whole plant corn production increased to 48,500 kg/ha, and the grain at 5,070 kg/ha, while total weed mass was 9,671 kg/ha. Similar results were obtained in the variant treated with acetochlor.

The highest production of whole plant corn 53,600 kg/ha and 7,020 kg/ha grain were obtained in the variant treated with isoxaflutol + (bentazon + dicamba). In this variant, because of the high efficiency of herbicides, the total amount of weeds was the smallest, only 950 kg/ha. In this experience, on fertilized background, in variant treated with herbicides, the amount of corn (27,600 kg/ha) + weeds (23,719 kg/ha) totals 51,393 kg/ha, which is practically equal to the best variants treated with isoxaflutol + (bentazon + dicamba), where were obtained 53,600 kg/ha maize and 950 kg/ha weeds, thus in total 54,550 kg/ha.

The reserve of *Echinochloa crus-galli* seeds in the 0-10 cm soil layer determinates at maize harvest shows the danger constituted by late infestations of maize crops with weeds, in maintaining the cultural hygiene of exploitation. From a valuable point of view this reserve of seeds is about 22,264 seeds/m² (average on the three years) in the variant of no disproof, 3,512 seeds/m² in the variant of a classical disproof, 5,394 seeds/m² in a chemical disproof variant through a pre emergent treatment, 6,042 seeds/m² in a chemical disproof variant through a post emergent treatment and 3,816 seeds/m² in a chemical disproof variant through two treatments (p.p.i. + postem.). We can state that the *Echinochloa crus-galli* seed reserve accumulated in the superficial soil layer is tightly related to the biomass of the weeds present in the culture before maize harvest.

3.4 The influence of climatic and technological factors upon the weed characteristics

The variable characteristics of the clime in the hilly area in the spring time, especially in April-May, completed with the particularities of soils workability from this area build for

many times one impediment to assure the optimal conditions for corn seeding and establish the optimal time for seeding. The repercussions of these deficiencies can be found for the most times in: culture late rising, culture irregularity, a bigger number of weeds, the passing of some phonological phases by corn plants during inappropriate periods, the differentiation of productivity organs during dryness periods, reduced productions.

Field	Group	Species	Plants/ m^2	Mass, kg/ha		
				Species	Group	Total
Plot I	Corn whole plant		4	-	-	2,100
	grains					288
	Mono	*Echinochloa crus – galli*	104	18,052	20,364	
		Setaria glauca	10	2,312		
	Dico	*Galinsoga parviflora*	25	1,516		22,113
		Convolvulus arvensis	4	72	1,749	
		Matricaria, Lapsana, Hibiscus	7	161		
Plot II	Corn whole plant		4	-	-	4,630
	grains					1,116
	Mono	*Echinochloa crus – galli*	95	12,633	12,804	
		Setaria glauca	8	171		
	Dico	*Galinsoga parviflora*	12	1,341		14,587
		Convolvulus arvensis	3	81	1,783	
		Matricaria,Lapsana, Hibiscus	7	361		
Plot III	Corn whole plant		4	-	-	130,000
	grains					2,526
	Mono	*Echinochloa crus – galli*	58	8,323	8,433	
		Setaria glauca	4	110		
	Dico	*Galinsoga parviflora*	22	1,293		10,230
		Convolvulus arvensis	4	102	1,797	
		Plantago,Matricaria, Lapsana	9	402		
Plot IV	Corn whole plant		4	-	-	19,720
	grains					3,866
	Mono	*Echinochloa crus – galli*	47	7,080	7,283	
		Setaria glauca	6	203		
	Dico	*Galinsoga parviflora*	14	1,012		8,464
		Convolvulus arvensis	2	90	1,185	
		Shymphytium, Lapsana	3	83		
Witness (2 holings)	Corn whole plant		4	-	-	29,790
	grains					5,157
	Mono	*Echinochloa crus – galli*	3	990	990	
	Dico	*Convolvulus arvensis*	2	240	960	1,950
		Shymphytium officinalis	2	720		

Mono – Monocotyledonous; Dico – Dicotyledonous.

Table 9. Influence of the density of *Echinochloa crus – galli* species and of other weed species upon the maize crop in the case of unmineral fertilized soil and without any measure of chemical weed control.

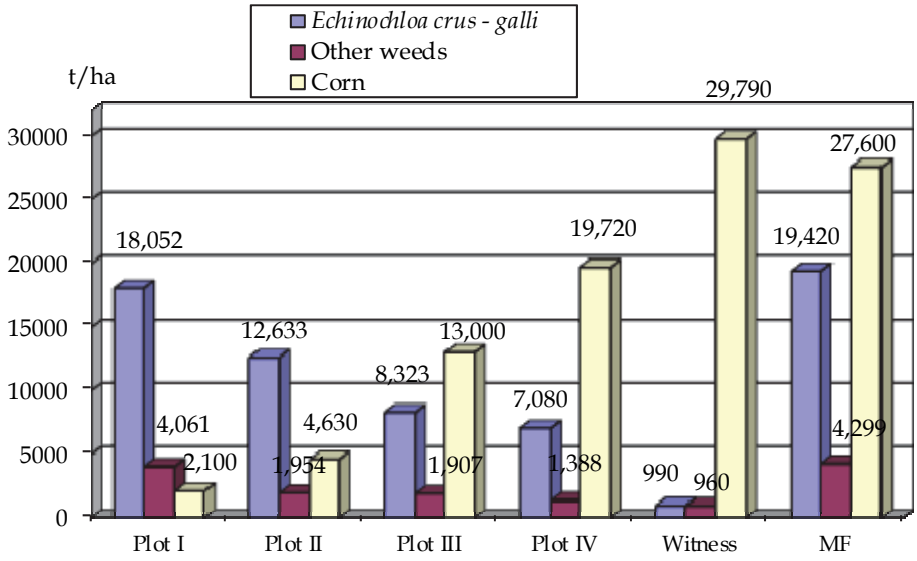

Fig. 2. Influence of *Echinochloa crus – galli* species on the development of other weeds and the green mass corn yield (t/ha).

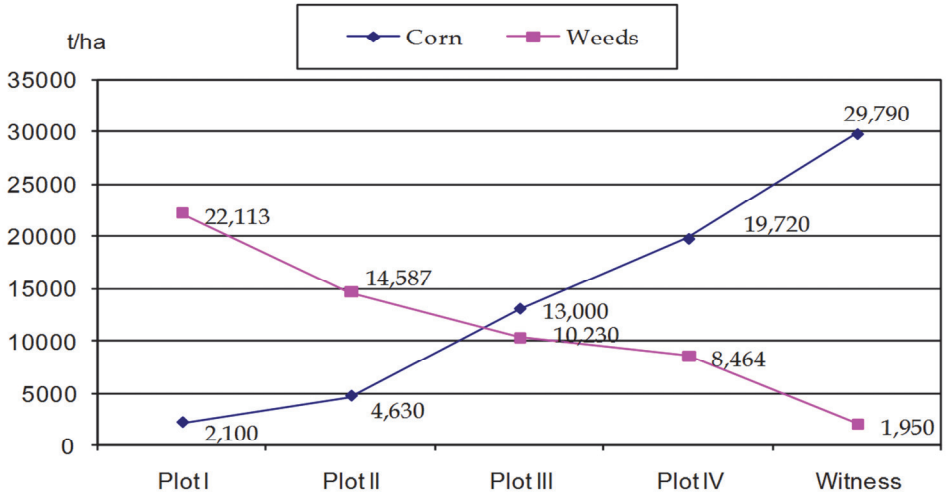

Fig. 3. Correlation between the weeds quantity and the greenery corn (t/ha).

Herbicides kg/ha	Group	Species	Plants /m²	Mass, kg/ha		
				Species	Group	Total
Untrated	Corn whole plant		4	-	-	27,600
	grains					1,965
	Mono	*Echinochloa crus – galli*	34	19,420	19,560	
		Setaria glauca	3	140		
	Dico	*Galinsoga parviflora*	9	891		
		Chenopodium album	8	1,040		23,719
		Polygonum convolvulus	8	1,241	4,159	
		Matricaria, Cirsium				
		Euphorbia helioscopia	14	987		
V_1 - dimetenamid	Corn whole plant		4	-	-	48,500
	grains					5,070
	Mono	*Echinochloa crus – galli*	2	2,060	3,100	
		Setaria glauca	4	1,040		
	Dico	*Amarantus retroflexus*	5	3,230		9,671
		Cirsium arvense	3	940	6,571	
		Chenopodium,Gallinsoga	7	2,401		
V_2 – acetoclor	Corn whole plant		4	-	-	49,120
	grains					5,421
	Mono	*Echinochloa crus – galli*	7	3,880	4,890	
		Setaria glauca	6	1,010		7,092
	Dico	*Amaranthus, Cirsium*	10	2,202	2,202	
V_3 - isoxaflutol + (bentazon + dicamba)	Corn whole plant		4	-	-	53,600
	grains					7,020
	Dico	*Amaranthus retroflexus*	1	950		950

Mono – Monocotyledonous; Dico – Dicotyledonous.

Table 10. Influence of density *Echinochloa crus – galli* species upon the maize crop in the case of a mineral fertilized soil and measure of chemical weed control.

The determined correlations confirmed a very strong connection between the climatic conditions and weed amount inclusively with *Echinochloa crus-galli.* There are also very significant direct relations between the overtaking of the optimal seeding date (April 15) and weed of the culture (Table 11).

One significant correlation exists between the quantity of precipitations and *Echinochloa crus-galli* (r = 0.875), but this species has a lower dependence to humidity, at least in the first periods of growing comparatively to other weeds, fact that explains the big number of exemplaries, even in the years with a low amount of precipitations and soils with a low reserve of humidity. The air temperature has a lower influence upon the weeding (r=0.571) especially during the first period of corn vegetation in conditions in that there were not significant variations of this climatic parameter.

The overtaking of seeding optimal date determinates the increasing range of weed inclusively the amount of *Echinochloa crus-galli* per surface unit. The relation is very significant, the correlation coefficient has values between 0.766 and 0.840 (very significantly)

and the regression equation y=2.5148 x + 288.96 shows that every day of seeding delay conduce to weed increase with more than 2 weeds/square metre.

The explanation of the identified correlations is found in the climatic characteristics of the experimental years. The dry periods influence negatively the corn germination and rising taking in consideration the spent period from seeding rising, culture density and its homogeneity. The weeds are also influenced less as frequency and more as rising and development period. During the years that are rich in precipitations the weeds succeeded in germination, rising and assurance of a high infestation of the culture. The plus of humidity and temperature from May and June favoured the weeding both as frequency and phonological development especially between May 30 and June 30. The weeds concurrence to the corn plants in this period it was an acerbic one.

The existent weeds mass in the corn crop before harvesting reflects on one side the climatic specific of the agrarian year, but mostly the effectiveness of each applied method to combat the weeds and not lastly the capacity of weeds concurrence.

The correlations established between the biomass achieved at harvesting moment of *Ecinochloa crus-galli* and yield (Fig. 4, Fig. 5 and Fig. 6) are – very strong, proving once again the fact that this species is a majoritary one both as frequency in corn crops from Cluj area but also as a corn concurrency potential bringing to significant production reductions. The correlation coefficient (r) is very negative significant having values between 0.861 and 0.952.

Characteristic	1	2	3	4	5	6
1. Weed number/m^2	1	0.65**	0.85**		0.92***	0.84***
2. Covering range, %		1		0.571^o		0.90^{ooo}
3. Number of *Echinochloa crus-galli* /m^2			1		0.578*	0.766***
4. Medium rising temperature – 15 days after seeding (^{0}C)				1		
5. The amount of precipitations rising - 15 days after seeding (mm)					1	0.859***
6. Number of days behind seeding						1

r / p 5% = 0.497; 1% = 0.623; 0.1% = 0.742

Table 11. The existent correlations between the weed characteristics, climatic and technological conditions from the corn crop.

The assessment of each combating method both under efficiency in corn crop weeds control aspect and achieved productions level after weeds combating (Fig. 7) it is compulsory and objective. The combating range of *Echinochloa crus-galli* accomplishes with the production a strong positive relation r = 0.959***. Therefore, in the case of a 10% increasing of combating range, the production rises with 48.65 kg/ha.

4. Conclusion

Significant particularity of *Echinochloa crus-galli* species is its growing plasticity according to the rising period and the respective climate during the vegetation period. The vegetative phases and vegetative parameters are adjusted so that the plant would completely pass the generative phase and would assure the species perpetuation.

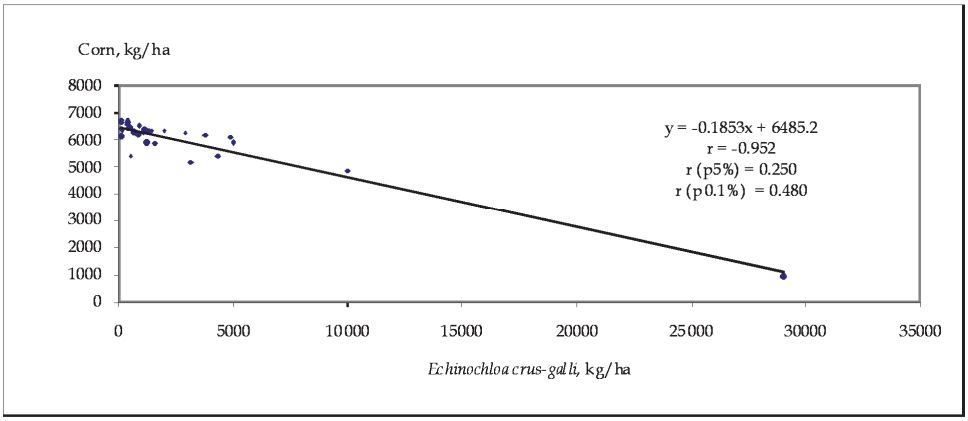

Fig. 4. The relation between biomass of *Echinochloa crus-galli* and corn yield during the dry years.

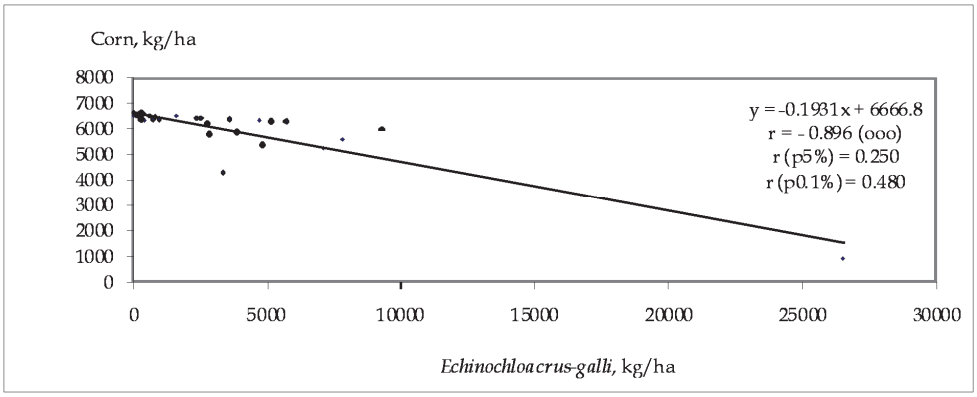

Fig. 5. The relation between biomass of *Echinochloa crus-galli* and corn yield during the rainy years.

The problem of the influence of different species of weeds on the production of agricultural plants has been studies by many researchers. The damage caused by weeds in maize crop is mostly of 30-70%, and when the infestation is strong culture can be fully compromised. Our researches highlight in terms of Transylvania, the influence of *Echinochloa crus-galli* species (L.) Pal. Beauv. and other weeds on corn production, according to the degree of infestation. Researches have been conducted at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.

The researches was done on two agrofunds: unfertilized and mineral fertilized with NPK 100 kg s.a./ha. The unfertilized maize crop has been made in four variants with different degrees of infestation of *Echinochloa crus-galli*, from about 40 to 100 plants/m^2 and witness – 2 holdings. In fertilized plots were used 4 herbicides for weed control (isoxaflutol 750 g/l; acetoclor 860 g/l; dimetenamid 900 g/l; bentazon 320 g/l + dicamba 90 g/l). Weed biomass, corn plant and grain production was measured in the ripening stage.

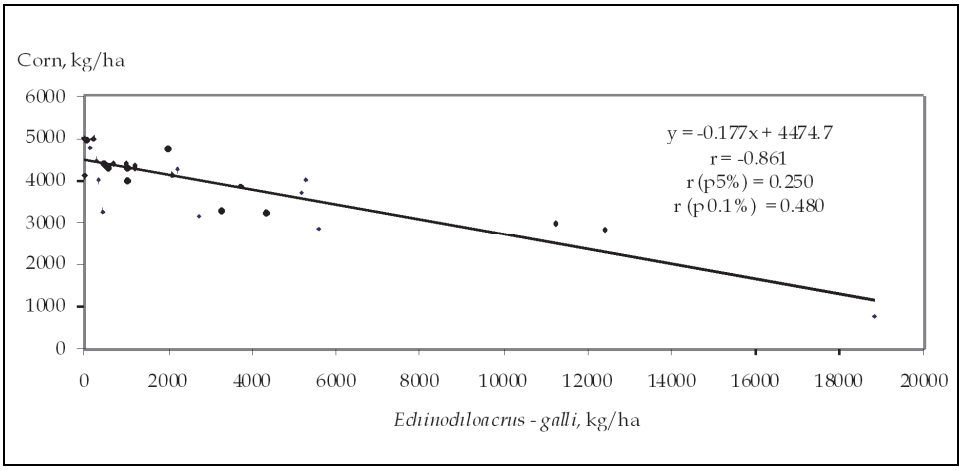

Fig. 6. The relation between biomass of *Echinochloa crus-galli* and corn yield during normal climatic conditions.

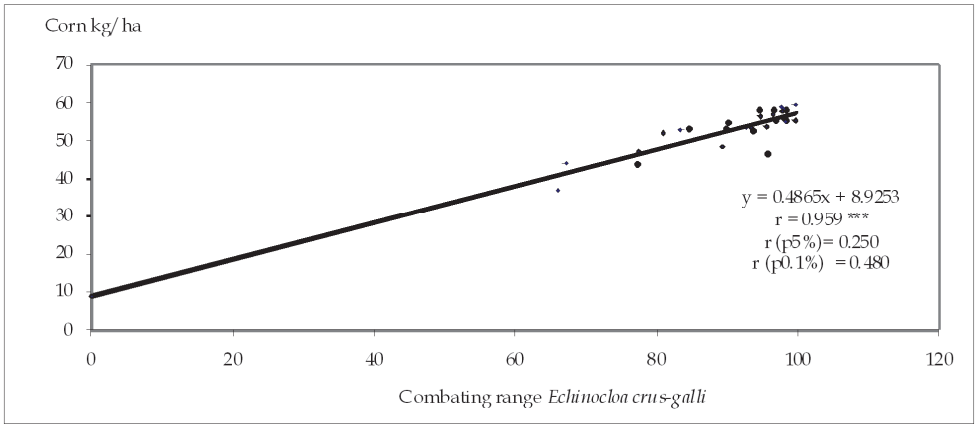

Fig. 7. The relation established between the *Echinochloa crus-galli* combating range assured in the tested variants and production level.

Corn invaded by weeds grows anaemic and has a yellowish green colour, in the unfertilized variant the corn develops storied, on the upper *Echinochloa crus-galli* dominates, in the middle floor develops *Setaria glauca* (L.) Beauv. and in the lower floor a number of dicotyledonous. On fertilized variant, the competition between weeds and maize, on the one hand and between monocotyledonous and dicotyledonous on the other hand, is more balanced, as dicotyledonous come from 1,700 kg/ha in mineral fertilized variants, to 4,100 kg/ha in mineral fertilized variant. The amount of weeds, obviously influenced production levels of maize grain and green mass. *Echinochloa crus - galli* had favorable conditions for maize crop infestation; the losses of production are depending on the degree of weed infestation and can reach 5,000 kg/ha maize grain, compared with those obtained in conditions of weeds control. Production losses in terms of green mass per hectare can be

considered equal to the weight of green weeds. At a density of 104 plants/m² of *Echinochloa crus-galli* with green mass of 18,052 kg/ha corn crop is fully compromised.

The prevention of maize crops infestations with weeds and weed control must be adjusted to topoclimate conditions. Along with agro technical, physiomechanical, biological and control means against weeds to share an equal importance for maize crops. All these must be so established as to succeed in efficiently controlling weeding all through the vegetation period of maize.

In central Transylvania, abundant rainfalls in July, August and even September and high temperatures favours late infestation of maize crops with annual species, very plastic as concerns the springing period and the bio-mass accumulated in the time period, especially *Echinochloa crus-galli.* Thus, when maize is harvested we can observe a high weeding level and the weed seeds reserve accumulating in the soil increases.

The protection of maize crops in the centre of Transylvania against weeds must to be into consideration some factors that are specific for that area. These factors are: large weed seeds reserve in soil, which, every year, provides a high weeding degree of crops; whimsical rainfalls; alternative springing of dominant weed species and their biological specific features, in order to reduce specific maize weeding under the economic deleterious level.

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Herbicides in Winter Wheat of Early Growth Stages Enhance Crop Productivity

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1. Introduction

Herbicides are chemical substances destroying undesirable plants (weeds) or suppressing their growth.

Wheat (*Triticum* spp.) is a cereal that is cultivated worldwide. It is the most important human food grain (Hanee M. Al-Dmoor, 2008). Traditionally, herbicides in winter wheat are applied from two leaf stage till the end of tillering (Triasulphuron - *Logran*) and in spring at tillering (Propoxycarbazone-sodium - *Atribut*, Sulphosulphuron - *Monitor*, Iodosulphuron-methyl-sodium - *Husar*) and from tillering till booting (Florasulam + 2.4-D 2-ethylhexyl ester - *Mustang*). *Atribut, Monitor and Husar* best fit for control of annual monocotyledonous weeds as *Apera spica-venti, Avena fatua, Poa pratensis* etc. and some annual dicotyledonous weeds as *Galium aparine, Tripleurospermum perforatum, Viola spp., Lamium spp*. etc. *Logran* best fits for control of annual dicotyledonous weeds as *Sinapis spp., Capsella bursa-pastoris, Thlaspi arvense* and etc. while *Mustang* is designed for control of dicotyledonous weeds as *Chenopodium album, Centaurea cyanus, Myosotis arvensis, Sonchus arvensis, Cirsium arvense* and others (Rimavičienė, 2005). Appropriate selected and in time applied herbicides destroy spreading weeds in crop or suppress weed growth and new seed production. Crop weediness is considerably reduced when soil is adequately cultivated, herbicides are applied and crop rotations are practiced (Barberi et al., 1997). It was determined that the field crops of cultured plants are plant associations or so called agrophytocenoses, and that the total biomass of a crop stand (crop and weed biomass) is more or less constant and that the crop yield is inversely proportional to the weed biomass (Lazauskas, 1990, 1993). Effectiveness of chemical weed control is determined by three main specifications: selection of an adequate herbicide, its optimal norm and duration of application. In the process of weed control it should be remembered that wet climate, cold spring weather, long autumn are the factors that help them grow and develop. Another important factor is the ratio of weed biological groups. The prevailing weeds in Lithuania are short-lived annual dicotyledons that comprise 70-90% of all the weeds (Pilipavičius, 2005). Many annual weeds successfully survive till spring because of climate warming; however, they were naturally frozen during winter time just 10-15 years ago. Global warming is the increase in the average temperature of the Earth's near-surface air and the oceans since the mid-twentieth century and its projected continuation. Including uncertainties in future greenhouse gas concentrations and climate sensitivity, the IPCC, scientific intergovernmental body set up by the World Meteorological Organization (WMO) and by the

United Nations Environment Programme (UNEP), anticipates a warming of 1.1°C to 6.4°C by the end of the 21st century, relative to 1980–1999 (Summary for policymakers, Climate change, 2007). Conventionally herbicides are used in spring for weed control in winter cereals, therefore, perennial and winter annual weeds have favourable conditions to grow and compete with cereals when vegetation in spring is renewing. Winter wheat *Triticum aestivum* L. is sensitive to weed competition in early stages of growth and development. Therefore, intensive agricultural systems seek to destroy all growing weeds in crops and avoid of weed seed bank replenishment with new matured weed seeds that can survive in soil for decades (Koch & Hurle, 1978; Niemann, 1981). Weed seed bank in the soil changes in two directions: regularly cleans from seeds and is replenished by them. Balance between these processes decides seed bank change dynamics in the soil (Pilipavičius, 2004, 2007b). Many researchers (House, 1989; Faravani & Khaghani, 2004; Sikkemaa et al., 2007; Stasinskis, 2009) have investigated the effect of herbicide application on field weediness of wheat crops. However, there are reported data on conventional standard time of use of pre-emergence herbicides in autumn or post-emergence herbicides in spring. The potential use of herbicides at early stages of development of winter wheat in autumn has not been clearly considered, and published research data are still insufficient. Intensive use of herbicides following the traditional crop growing technologies, however, does not entirely solve the problem of weediness.

The work hypothesis: application of herbicides in autumn will control weeds that survive during winter time and winter wheat will not be damaged and better conditions for crop competition in spring after renewing of vegetation would be created.

The aim of this work was to evaluate various herbicide active substance applications in autumn at early stages of winter wheat *Triticum aestivum* L. development, its influence on crop weediness and productivity.

2. Chemical weed control development in winter wheat

The research was carried out in Kaunas county, Prienai district, Ašminta region, Strielčiai village. Winter wheat fore-crop was black fallow. Experimental field was ploughed in autumn using semi-helical plough Overum 4 to the depth of 24 cm, cultivated using a cultivator with comb harrow KPŠ-15 to the depth of 10 cm and the surface of soil was levelled off with a roller PP-7. The field was fertilized in autumn (10 September, 2005) by amofos 100 kg ha^{-1} and potassium chloride 200 kg ha^{-1}. Winter wheat cv. Ada was sown on 12 September, 2005. The sowing-machine SPU – 6 (inter-beds 12.5 cm) was used. The amount of seeds comprised 240 kg ha^{-1} and they were sown in the depth of 4-5 cm. Winter wheat cv. Ada develops well on all soils growing it by the conventional technologies. Cultivar Ada has high overwintering qualities evaluated by 8-9 points from 9. It is resistant to frost, when at tillering node temperature subsides till minus 14^0C died just 6% of plants. Average productivity 6.36 t ha^{-1}; stem medium high 90-94 cm (Characteristics of wheat varieties ..., 2011; Lithuanian national list of plant varieties, 2011). The investigated active substances of herbicides according to the scheme of research were applied in autumn at BBCH 14-15 of winter wheat (7 October, 2005).

Experimental design:

1. Control treatment, not sprayed with herbicides in autumn*
2. Monitor 75% g. (Sulphosulphuron 750 g kg^{-1}), 26.7 g ha^{-1}

3. Atribut 70% w.s.g. (Propoxycarbazone-sodium 700 g kg^{-1}), 0.120 g ha^{-1}
4. Mustang 458.75 g L^{-1} c.s. (Florasulam + 2.4-D 2-ethylhexyl ester 6.25 + 452.5 g L^{-1}), 0.5 L ha^{-1}
5. Logran 20% w.s.g. (Triasulphuron 200 g kg^{-1}), 0.03 g ha^{-1}
6. Husar 5% w.s.g. (Iodosulphuron-methyl-sodium 50 g kg^{-1}), 0.200 g ha^{-1}.

Note: g – granules; w.s.g. – water-soluble granules; g L^{-1} c.s. – grams in a litre of concentrated suspension.

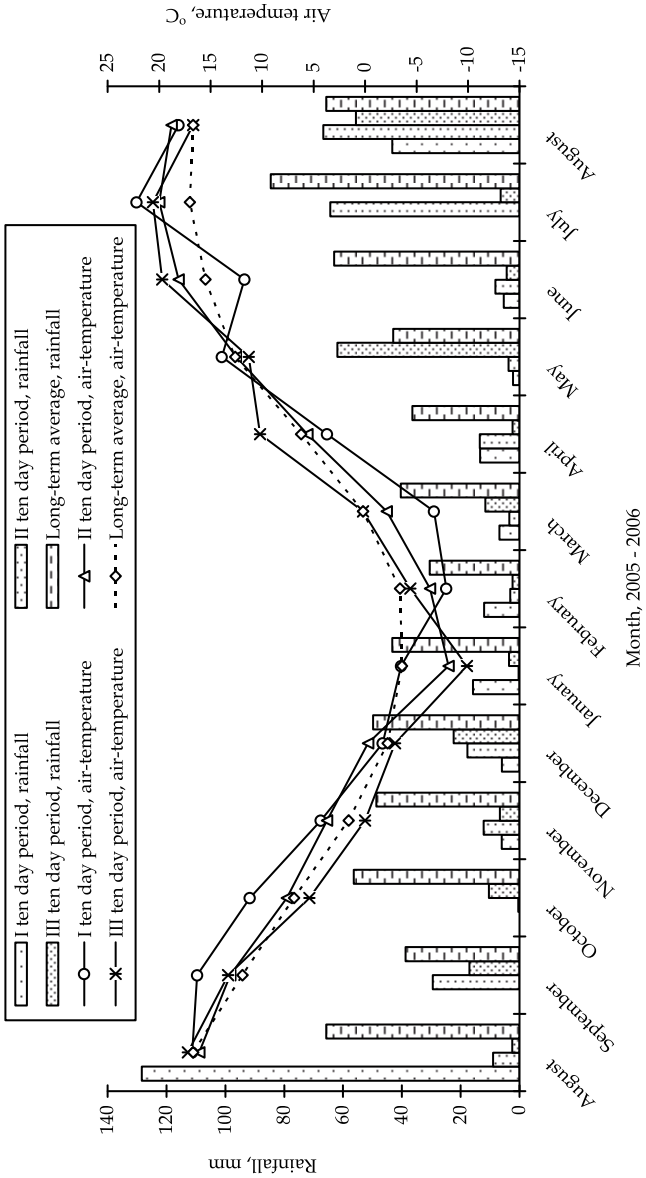
The experiment was carried out in four replications. The experimental data were evaluated using analysis of variance by *Selekcija* (Tarakanovas, 1997) and correlation-regression analysis by *SigmaPlot 8.0* (SPSS Sciences, 2000).

For the first fertilization in spring (12 April, 2006) 250 kg ha^{-1} of ammonium nitrate was used and for the second fertilization (8 May, 2006) 200 kg ha^{-1} of ammonium nitrate was applied. A *composite of herbicides Sekator 300 g ha^{-1} and MCPA 1 L ha^{-1} with a growth regulator Cycocel 1.5 L ha^{-1} was used for conventional spraying at BBCH 22-23 (2 May, 2006, sprayed all experimental field, standard technology). At the beginning of winter wheat stem elongation (25 May, 2006) the composite of insecticide Fastak 100 g ha^{-1}, fungicide Folikur 0.75 L ha^{-1} and complex fertilizer Wuxal 5 L ha^{-1} were sprayed.

2.1 Meteorological conditions

Lithuanian territory occupies intermediate geographical position between west Europe oceanic climate and Eurasian continental climate. Climate of the Lithuania territory forms in different radiation and circulation conditions. Differences in these conditions hardly cross the boundaries of microclimatic differences; therefore, Lithuania belongs to western region of the Atlantic Ocean continental climatic area (Basalykas et al., 1958). During 2005-2006 meteorological conditions were favourable for winter wheat crop establishment. Autumn of 2005 was warm and rainy, i.e. suitable for crop emergence and early growth. The beginning of winter delivered well balanced conditions for wintering, however, during January – March 2006 the temperatures were rather low with insufficient snow cover on the soil. April – June 2006 was cool with high variation of rainfall which principally did not exceed the long-term average. Significant increase of rainfall in August resulted in complicated conditions for winter wheat maturing and harvesting (Pilipavičius et al., 2010b). Meteorological conditions during vegetation of winter wheat crop experiment are summarised in figure 1. September of 2005 was enough warm, average air temperature during the second ten day period was higher by 2^0C comparing with long-term average. However, the first ten day period of September was very rainy with rainfall of 68.3 mm while during the second ten day period it compounded 21.5 mm and the third ten day period pasted without rainfall. The first ten day period of October was warm but already at the second and the third ten day periods average air temperatures dropped to 8^0C which were by 1.1^0C and 1.4 ^{0}C warmer comparing with long-term average, accordingly. Rainfall during the first ten day period in October reached 31.3 mm while during the second and the third ten day periods decreased till 6.0 mm and 10.4 mm, accordingly. Hence, warm and humid first ten day period in October formed favourable conditions for winter wheat tillering. During November average air temperatures decreased till 1.6^0C, 5.6^0C and 6.1^0C and amount of rainfall consisted of 6.1 mm, 12.2 mm and 6.7 mm. Gradual decrease of temperatures and rainfall was adequately fitting biological needs of winter wheat. During December average air temperatures fell down below 0, i.e. till -1.7^0C while long-term average was -2.2^0C. Precipitation consisted 6.0 mm at the first ten day period of December,

17.7 mm at the second and 22.4 mm at the third. It was lower than long-term average and formed proper conditions for winter wheat cv. Ada wintering.



Note. Long-term average of rainfall (600.4 mm) and average air temperature (6.8°C) by ten day periods during 1974-2004, Kaunas Hidrometeorological station

Fig. 1. Meteorological conditions, rainfall and average air temperature by ten day periods during vegetation of winter wheat crop, Kaunas county, Prienai district, Lithuania.

Meteorological conditions during January, February and March were very unfavourable for winter wheat overwintering, because there was few precipitation formed insufficient snow cover with rather low average air temperatures of -7.2^{0}C, -6.3^{0}C and -2.7^{0}C accordingly. During January and February precipitation was by 54% and 42% lower comparing with long-term average. April weather was cool, just at the second ten day period it started to warm till +10.2^{0}C with rainfall of 29.3 mm while long-term average was 36.4 mm. During May average air temperatures reached 12.5^{0}C that was slightly lower than long-term average of 12.6^{0}C. The third ten day period of May was very rainy reaching 61.9 mm that consisted 83% of the whole month standard comparing with long-term norm average. The first ten day period in June was cool 11.7^{0}C comparing with long-term average 15.5^{0}C while during the second and the third ten day periods average air temperatures reached 18.1^{0}C and 19.7^{0}C accordingly. June was very dry with 18 mm rainfall when long-term average is 63 mm. It was by 71% lower than long-term average and it was very inappropriate for winter wheat growth and development (Fig. 1). As a consequence, average winter wheat grain yield 2.46 t ha^{-1} in Lithuania in 2006 was the lowest comparing with 2005-2010 (Statistics Lithuania, 2011). July was warm with average air temperature of 20.9^{0}C and 64.3 mm of rainfall that was by 20.3 mm lower than long-term average. The rainiest month during winter wheat vegetation was August with 165.6 mm rainfall exceeding long-term average by 99.9 mm and created very unfavourable conditions for winter wheat grain maturing and wet soil aggravated grain harvesting.

2.2 Soil weed seed bank

2.2.1 Soil agrochemical characteristics

Soil samples for agrochemical analysis and establishment of weed seed bank, seed varietal composition and quantity, were taken from 0-20 cm soil layer at the end of September from 10 sites of all treatments and their replications, making combined samples. Soil agrochemical characteristics were established in the Centre of Agrochemical Research, Lithuanian Agricultural Institute and weed seed bank composition was established at the Lithuanian University of Agriculture. Experimental field soil was *Gleyic Cambisols CMg*. Topsoil layer was alkaline, average in humus, rich in phosphorus and average rich in potassium (Table 1).

Agrochemical soil properties			
pH	Humus %	P_2O_5 mg kg^{-1}	K_2O mg kg^{-1}
7.1	2.17	152	146

Table 1. Experimental field soil agrochemical characteristics of 0–20 cm soil layer.

2.2.2 Weed seed bank

Weed seeds from soil samples were washed through 0.25 mm sieve and separated by saturated solution of high specific mass of NaCl (Rabotnov, 1958; Warwick, 1984, Pilipavičius, 2004). Seeds of 12 weed species (10 annual and 2 perennial) were identified in the soil weed seed bank. Annual weed seeds dominated with 88.0%-95.7% of weed seed bank, from them winter annual weed seeds (*Viola arvensis*, *Tripleurospermum perforatum*,

Thlaspi arvense etc.) comprised 21%-60% (36%-54% from the whole seed bank). Seeds of the perennial weeds (*Cirsium arvense*, *Rumex crispus*) were in the minority with 4.3%-12.0% from the whole soil weed seed bank (Table 2). In winter wheat crop perennial weeds were in the minority as well (Fig. 3 & 4). From separated weed seed species, the seeds of *Chenopodium album* prevailed in the soil seed bank. They comprised 34%-48% of the whole soil weed seed bank. However, *Chenopodium album* was recessive weed in the crop as it is summer annual weed and consequently is freezing during winter time (see subchapter 2.3). *Viola arvensis* was the other dominant weed in the soil seed bank that covered 16%-30% of seed bank (Table 2). The main change in the number of weed species was influenced by appearance and disappearance of weed seeds that were low in number. It was either actual for the crop.

Weeds	Treatment					
	Control	Monitor	Atribut	Mustang	Logran	Husar
	Weed seeds					
Chenopodium album L.	3.0	2.0	4.25	2.75	4.0	4.75
Cirsium arvense (L.) Scop.	0.0	0.0	0.25	0.0	0.0	0.0
Fallopia convolvulus L.	0.0	0.0	0.0	0.0	0.5	0.0
Galeopsis tetrahit L.	0.0	0.0	0.25	0.0	0.0	0.0
Myosotis arvensis (L.) Hill.	0.0	0.25	0.25	1.0	0.25	1.75
Persicaria lapathifolia L.	0.0	0.0	0.0	0.0	0.0	0.25
Rumex crispus L.	0.75	0.25	1.0	0.75	0.75	1.5
Sinapis arvensis L.	0.25	1.0	0.0	0.25	0.25	0.25
Stellaria media (L.) Vill.	0.75	0.5	1.75	0.0	0.5	1.75
Thlaspi arvense L.	0.0	0.0	0.25	0.5	0.25	0.25
Tripleurospermum perforatum (Merat) M. Lainz	0.50	0.5	0.75	0.0	0.0	0.0
Viola arvensis Murray	1.0	1.25	3.75	2.0	2.5	2.75
All weed seeds	6.25	5.75	12.5*	7.25	9.0	13.25*
LSD_{05}	4.34					

Note. * - essential differences at 95% level of probability, compared to control treatment

Table 2. Weed seed bank in winter wheat crop soil of 0–20 cm layer, weed seeds in 100 g of air-dry soil (Pilipavičius et al., 2010a).

Direct chemical soil weed seed bank control is rather indeterminable, therefore ecological and cultural weed control methods should be applied for weed seed control. One of possibilities to control weed seed bank is harvesting cereal at earlier stage of maturity for the whole plant silage. It is essentially important factor, decreasing the amount of coming new weed seeds to the soil terminating weed seed rain while only a small part of weed seeds pours at milk or milk–dough stages of cereal maturity (Pilipavičius & Lazauskas, 2000, Pilipavičius, 2002, 2006) and delivering more fodder for animals from the same plot area (Pilipavičius, 2007b, 2012). When the amount of new weed seeds getting into the soil decreases, soil is cleaning quicker from them (Pilipavičius, 2004). Another important factor is weed seed position in the soil. The more weed propagation rudiments are decreased in the top soil layer, the less is weediness of the crop – the number and the mass of weeds (Pilipavičius, 2007a, Pilipavičius et al., 2009). Existing weed seed bank and vegetative weed

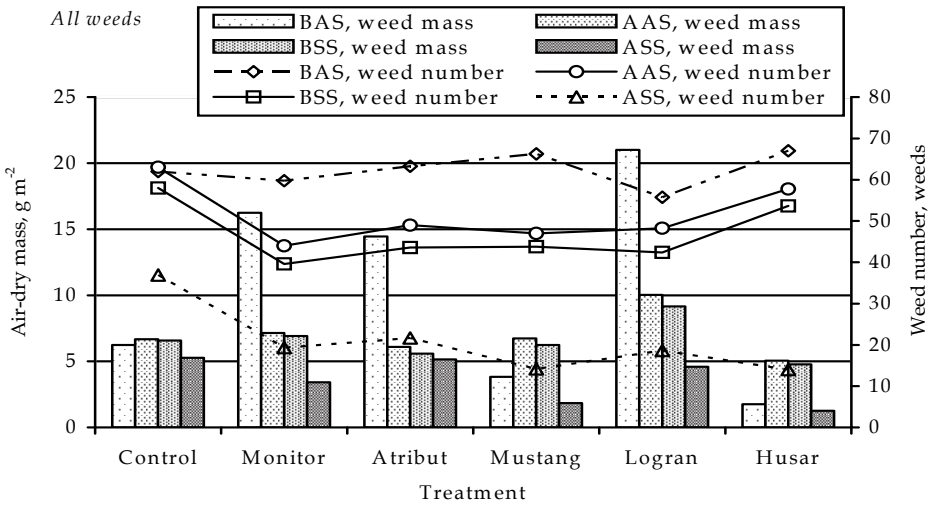
parts in soil can be managed in non-chemical way. According to theoretical preconditions and data of the experiments, it is proved that total turnover of the arable soil layer in organic agriculture is a very important means of weed control decreasing weediness of the crop and increasing harvest. Comparing technological ploughing processes and ploughs, it was concluded that two layer ploughs can help to carry out this process the most effectively in organic agriculture (Lazauskas & Pilipavičius, 2004) and can be successfully applied in conventional agriculture.

2.3 Crop weediness

Weediness of winter wheat crop was established by a quantitative-weight method. Four samples with wire rim 50 x 50 cm (0.25 m^2) were taken from each experimental plot to establish weed density and mass (Pilipavičius, 2005) in autumn and spring during the winter wheat tillering before and after spraying with herbicides. Collected weeds were air-dried and distributed into species. Nomenclature of Latin plant names was based on the Institute of Botany's edition *Vascular plants of Lithuania* (Gudžinskas, 1999).

Twenty weed species in experimental field were established in autumn before spraying with herbicides. Seventeen of them were annual and three perennial ones. After autumn spraying with herbicides number of established weed species increased by one annual and one perennial, however, weed biomass essentially decreased (Fig. 2).

After overwintering the number of weed species in the crop principally did not change while after conventional spring spraying with herbicides it decreased by one annual weed species. The main change in the number of weed species was influenced by appearance and disappearance of weeds as *Chenopodium album* L., *Erysimum cheiranthoides* L., *Sinapis arvensis* L., *Myosotis arvensis* (L.) Hill, *Veronica arvensis* L., *Cerastium arvense* L., *Fumaria officinalis* L., *Viola arvensis* Murray, *Galeopsis tetrahit* L., *Polygonum aviculare* L. and some other weeds that were low in number. It was confirmed that more weed species were established in the crop (Fig. 3 & 4) than in soil weed seed bank (Table 2). Before autumn application of herbicides there were no considerable differences in weediness of winter wheat but after herbicide spraying the number of weeds decreased by 15.4 – 28.4% and their air-dry biomass lessened even up to 56.8% (Fig. 2). Monitor (Sulphosulphuron) was the most effective herbicide in destroying weeds in winter wheat crop in autumn compared to the control treatment with no herbicide application in autumn and other herbicides used. Crop weediness decreased by 18 weeds in m^{-2} and by 9.5 g m^{-2} of air-dry mass; the reduction comprised 28.4% and 56.8% respectively. Assessing the effectiveness of different herbicide active substances, it was established that after autumn spraying the number of weeds decreased from 32.4% to 91.7% compared to not sprayed by herbicides in autumn control. Assessing winter wheat crop in spring, it was determined that autumn herbicide application resulted in reduced weediness also after crop wintering. It was established that herbicide application in autumn at early stages of winter wheat development significantly decreased crop weediness as well in spring vegetation after over-wintering (Fig. 2). Later, Latvian researchers received analogous results while winter wheat crop in plots where herbicides were applied in autumn was more even, denser and better developed than in plots just with spring herbicide application. In the spring-treated plots the crops became thin and in open places weed plants that were not controlled by the herbicides could regrow and develop well during the growing season up to harvest time (Vanaga et al., 2010).

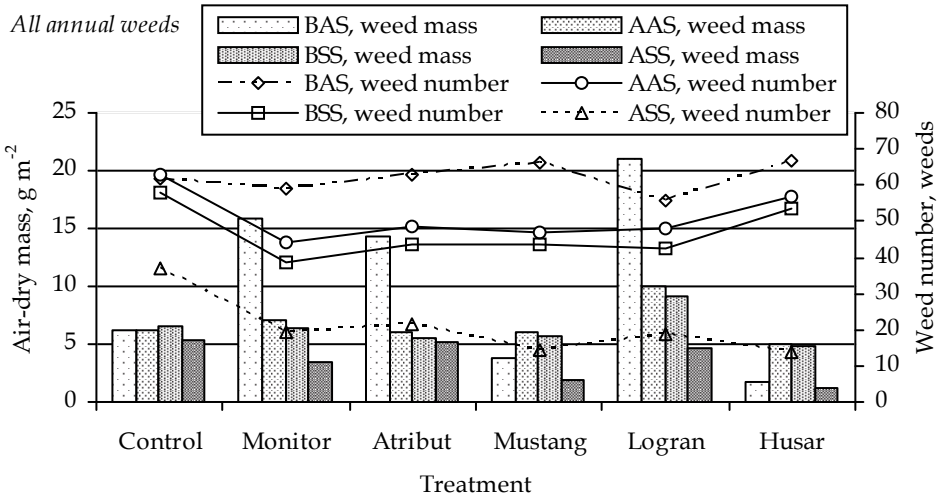


Note. LSD_{05}=24.18 for air-dry weed mass g m^{-2} before autumn application of herbicides; LSD_{05}=7.34 for air-dry weed mass g m^{-2} after autumn application of herbicides; LSD_{05}=22.85 for weed number before autumn application of herbicides; LSD_{05}=23.28 for weed number after autumn application of herbicides; BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 2. Winter wheat crop weediness before and after autumn and spring application of herbicides.

Annual weeds such as *Centaurea cyanus* (Fig. 6), *Raphanus raphanistrum* (Fig. 8), *Thlaspi arvense* (Fig. 11) and *Tripleurospermum perforatum* (Fig. 12) prevailed in winter wheat crop whereas among perennial weeds only of *Sonchus arvensis* and *Plantago major* and a few plants of *Antennaria dioica* and *Poa trivialis* emerged in the crop (Fig. 4). Short-lived annual weeds in the crop of winter wheat in autumn and after application of the intended herbicides comprised 96%-100% and 93%-100% respectively, whereas in spring before and after application of background spring herbicides they comprised 89%-100% and 99%-100% respectively (Pilipavičius et al., 2010a) (Fig. 2 & 3).

This means that perennial weeds are better adapted to wintering than the short-lived ones because the increase of air-dry biomass up to 11% of perennial weeds was established in spring before the application of chemical weed control measures (Fig. 4). Assessing the effectiveness of diverse herbicides, it was established that the number of weeds after spraying in autumn decreased by 32–91% compared to the control treatment plot not sprayed in autumn. Assessing winter wheat crop in spring, it was determined that autumn application of herbicides resulted in lessened crop weediness after its wintering even before spring spraying (Pilipavičius et al., 2010a). The number of weeds decreased by 70–92% compared to the control (Fig. 2) with no autumn herbicide application.

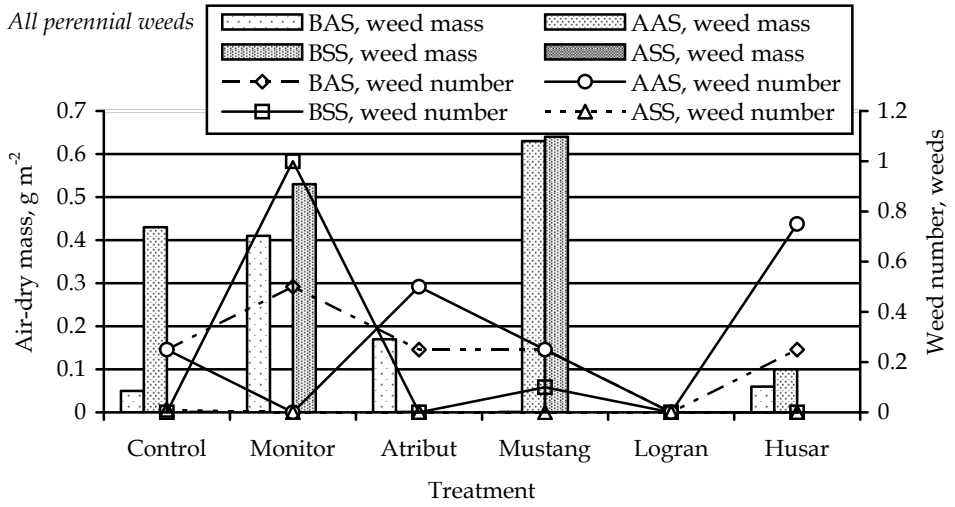


Note: Annual weed species: *Capsella bursa-pastoris* (L.) Medik (Fig. 5), *Centaurea cyanus* L. (Fig. 6), *Galium aparine* L. (Fig. 7), *Raphanus raphanistrum* L. (Fig. 8), *Raphanus sativus* L. (Fig. 9), *Stellaria media* (L.)Vill (Fig. 10), *Thlaspi arvense* L. (Fig. 11), *Tripleurospermum perforatum* (Merat) M.Lainz (Fig. 12), other annual weeds - *Chenopodium album* L., *Erysimum cheiranthoides* L., *Sinapis arvensis* L., *Myosotis arvensis* (L.) Hill, *Veronica arvensis* L., *Cerastium arvense* L., *Fumaria officinalis* L., *Viola arvensis* Murray, *Galeopsis tetrahit* L., *Polygonum aviculare* L.;
BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 3. Annual weeds in winter wheat crop before and after autumn and spring application of herbicides.

Comparing weed over-wintering possibilities as crop weediness change dynamics, is important to pay attention for the development of one average weed plant air-dry mass (g per plant). In our experiment, it was shown that short-lived annual weeds successfully survived winter frosts even increasing its average one weed plant mass (Table 3).

Though, moderated increase of annual weed mass during winter time was in conformity with the research hypothesis that many annual weeds successfully survive winter time as earlier was not usual. Naturally, perennial weeds have the highest tolerance to winter frosts as biologically well adapted to over-wintering. The highest mass of one its over-wintered plant before spring application of herbicides reaches 6.4 gram increasing it from 2.52 gram in autumn. However, perennial weeds were rare in our experimental field (Fig. 4) and even were not present in some experimental plots overall (Table 3). Received analogous cereal crop weediness variations mostly depend on experimental field weediness heterogenity especially in intensive operating fields with low weediness as each weed observation is made randomly (Pilipavičius, 2005). Similar trend of annual and perennial weed populations has been noticed by Geisselbrecht-Taferner et al., 1997; Colbach et al., 2000; Rew et al., 2001 and other researchers. Dominating annual weeds in the winter wheat crop directly influenced all weed average plant mass that remained analogous to average annual weed plant mass (Table 3).



Note. Perennial weed species - *Antennaria dioica* L., *Plantago major* L., *Poa trivialis* L., *Sonchus arvensis* L.; BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying.

Fig. 4. Perennial weeds in winter wheat crop before and after autumn and spring application of herbicides

Spring spraying with composite of herbicides *Sekator* and *MCPA* was low effective as standard technology in control treatment (average weed plant mass increase from 0.10 gram in autumn till 0.14 gram in spring after application). Winter wheat crop spring spraying with herbicides was either low effective as average weed plant mass has tendency to increase comparing weed average mass in autumn after experimental application of herbicides or in spring before application of standard chemical weed control technology with average weed one plant mass after spring application of herbicides (Table 3).

Weed air-dry biomass in the crop before spraying by herbicides in spring regularly depended on left weed air-dry mass in the crop after autumn spraying by herbicides r = 0.608** using chemical weed control (according to the experimental design) in early winter wheat growth and development stages. The reliable linear dependence (1) best described this regularity.

$$y = 4.077 + 0.335\ x;\ P = 0.0016 \quad (1)$$

Weeds left in the crop after autumn spraying by herbicides reliably increased crop weediness in spring before conventional spraying. Weed air-dry biomass of 1 g m^{-2} left in winter wheat crop in autumn increased crop weediness by 0.335 g m^{-2} in spring after renewing of vegetation (Pilipavičius et al., 2010b). Other researchers (Spiridonov et al., 2006) have affirmed high biological and economical efficiency of autumn application of herbicide (*Difezan*) in winter wheat crop in comparison with the conventional spring period of treatment. The successful post emergence control of weeds in the winter cereal crops at the BBCH 11-25 in autumn with herbicide *Atlantis* was reported by Brink and Zollkau 2004.

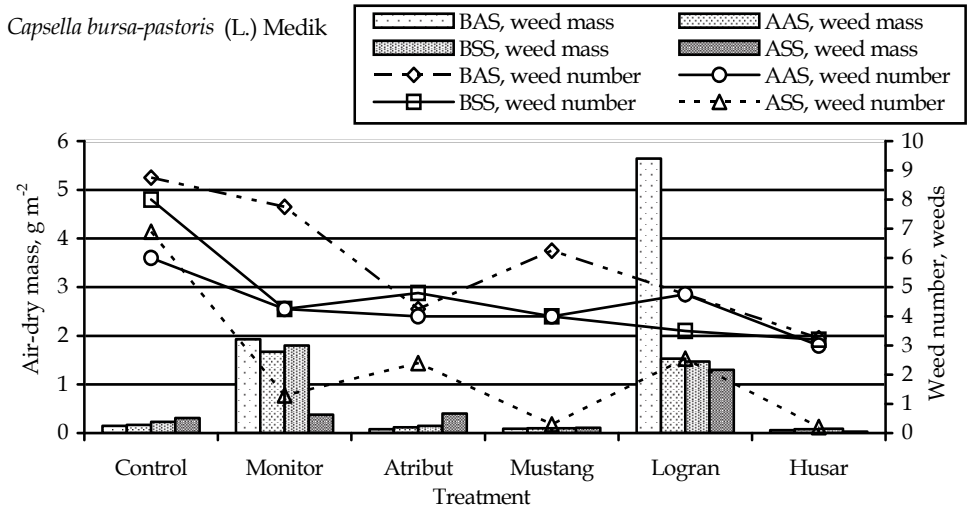
Treatment	Crop weediness average one weed plant air-dry mass, g per weed			
	Before autumn application of herbicides	After autumn application of herbicides	Before spring application of herbicides	* After spring application of herbicides *Sekator* 300 g ha^{-1} and *MCPA* 1 L ha^{-1} composite
	All annual weeds			
Control	0.10	0.11	0.11	0.14
Monitor	0.27	0.16	0.17	0.18
Atribut	0.23	0.13	0.13	0.24
Mustang	0.06	0.13	0.13	0.13
Logran	0.38	0.21	0.22	0.25
Husar	0.03	0.09	0.09	0.09
	All perennial weeds			
Control	0.20	1.72	-	0.10
Monitor	0.82	-	0.53	-
Atribut	0.68	-	-	-
Mustang	-	2.52	6.4	-
Logran	-	-	-	-
Husar	0.24	0.13	-	-
	All weeds			
Control	0.10	0.11	0.11	0.14
Monitor	0.27	0.16	0.17	0.18
Atribut	0.23	0.12	0.13	0.24
Mustang	0.06	0.14	0.14	0.13
Logran	0.38	0.21	0.22	0.25
Husar	0.03	0.09	0.09	0.09

Note. See experimental design.

Table 3. Average one weed plant air-dry mass change in winter wheat crop with autumn and spring applications of herbicides.

Separate weed species of winter wheat crop responded adequately to the autumn application of herbicides as the whole crop weed community. Winter annual weed *Capsella bursa-pastoris* (Fig. 5) was moderate in density and mass, however, it had tendency to increase in winter wheat crop without autumn application of herbicides. However, higher initial *Capsella bursa-pastoris* population in plots of *Monitor* and *Logran* treatments was inhibited and decreased with modern autumn application of herbicides (Fig. 5). Winter annual weed *Centaurea cyanus* (Fig. 6) was dominant in winter wheat crop agrophytocenoses before autumn application of herbicides. It comprised 4.4%-8.4% of weed density and 1.6%-17.3% of total weed biomass.

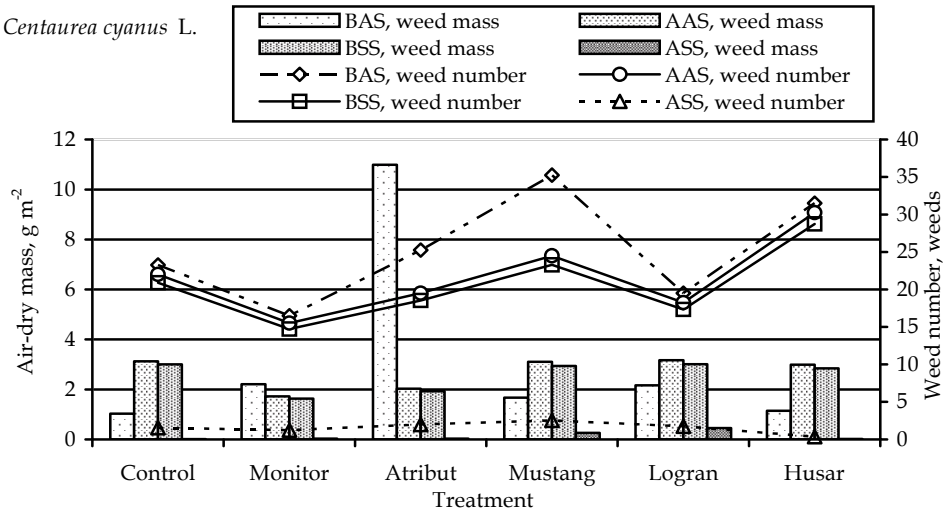
Density and mass of *Centaurea cyanus* had tendency to decrease after herbicide application in autumn. In overwintered crop *Centaurea cyanus* decreased by 5% in density and by 79.6%-19.8% in mass comparing it with the crop before winter time. Decrease of *Centaurea cyanus* density was similar in control treatment without autumn application of herbicides, however its mass decreased just by 3.8%, i.e. the *Centaurea cyanus* mass decrease after overwintering was by 5.2-20.9 times lower than in plots with autumn chemical weed control (Fig. 6). The other winter annual weed *Galium aparine* (Fig. 7) in winter wheat was less numerous and less in biomass comparing to *Centaurea cyanus* (Fig. 6), *Capsella bursa-pastoris* (Fig. 5) or *Raphanus raphanistrum* (Fig. 8). During wintering *Galiun aparine* without autumn herbicide application, density in the crop increased in mass by 56.5% and in density by 16.7%. It formed more competitive weed community against winter wheat in spring. Autumn application of herbicides subserved effectively *Galium aparine* control (Fig. 7). Summer annual weed *Raphanus raphanistrum* (Fig. 8) initial population made 3.2-9.8 weeds per square meter and 1.6-8.2 g m^{-2} of air-dry mass. It comprised 0.9%-2.6% of total weed density and 2.5%-13.1% of total weed mass. After autumn application of herbicides, *Raphanus raphanistrum* density and mass decreased by 21.9%-36.2% and 38.1%-65.5% accordingly.



Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

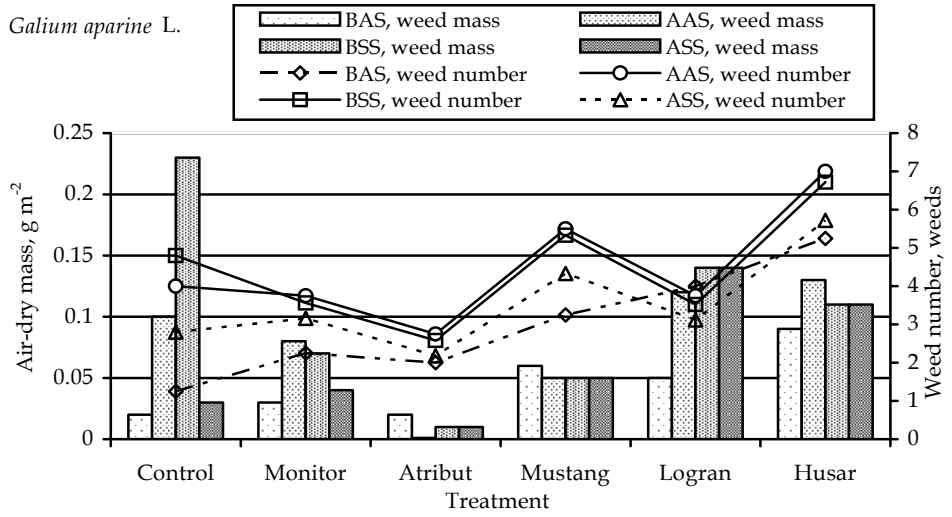
Fig. 5. *Capsella bursa-pastoris* (L.) Medik in winter wheat crop before and after autumn and spring application of herbicides.

In spring, renewing crop vegetation, *Raphanus raphanistrum* plants made 0.8%-1.9% of total weed density and 2.2%-6.2% of total weed mass. It was visible decrease of this weed population, hence, it showed that biologically summer annual weeds already can successfully survive during winter while conventionally it should not happen (Fig. 8). Other winter wheat crop weed belonging to *Brassicaceae* family, *Raphanus* genus was *Raphanus sativus* (Fig. 9). *Raphanus sativus* was present just in one treatment, was low in number and biomass. *Raphanus sativus* separately had no substantial effect on crop weediness and belonged to temporal weed flora element in the crop.



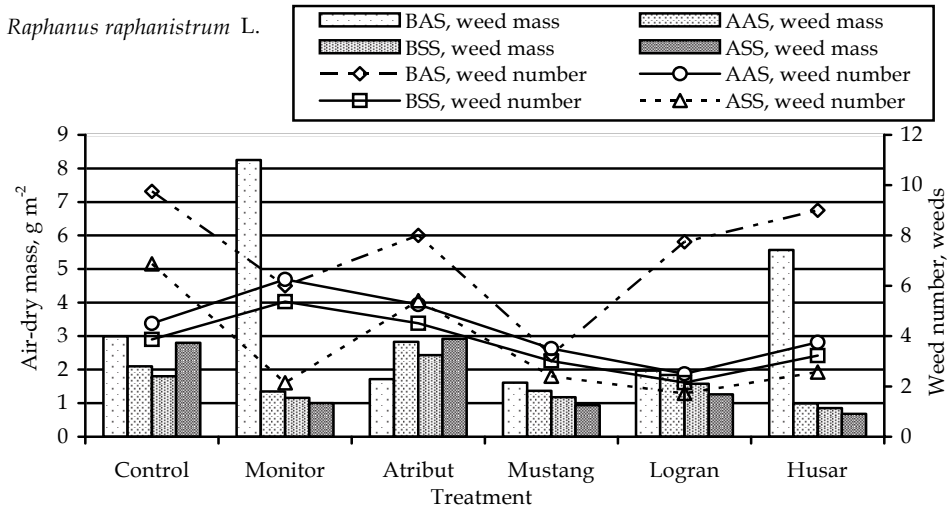
Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 6. *Centaurea cyanus* L. in winter wheat crop before and after autumn and spring application of herbicides.



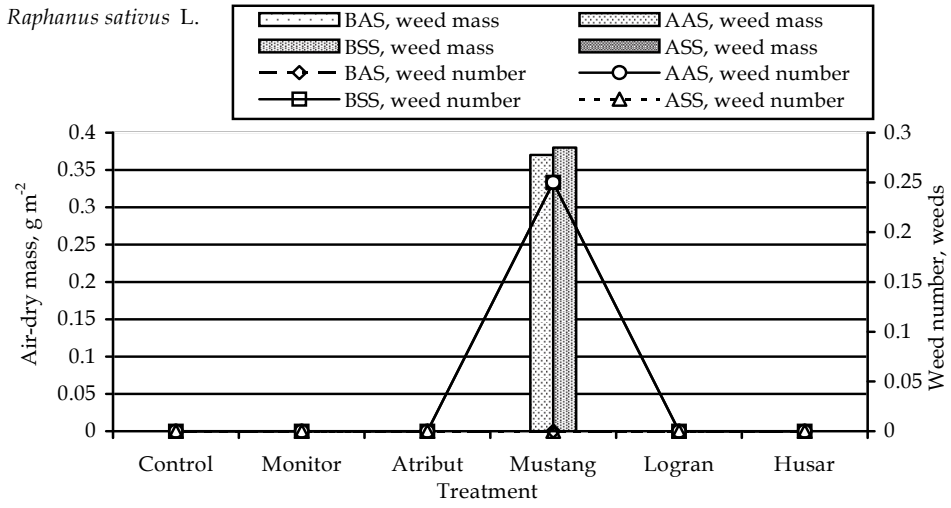
Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 7. *Galium aparine* L. in winter wheat crop before and after autumn and spring application of herbicides.



Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

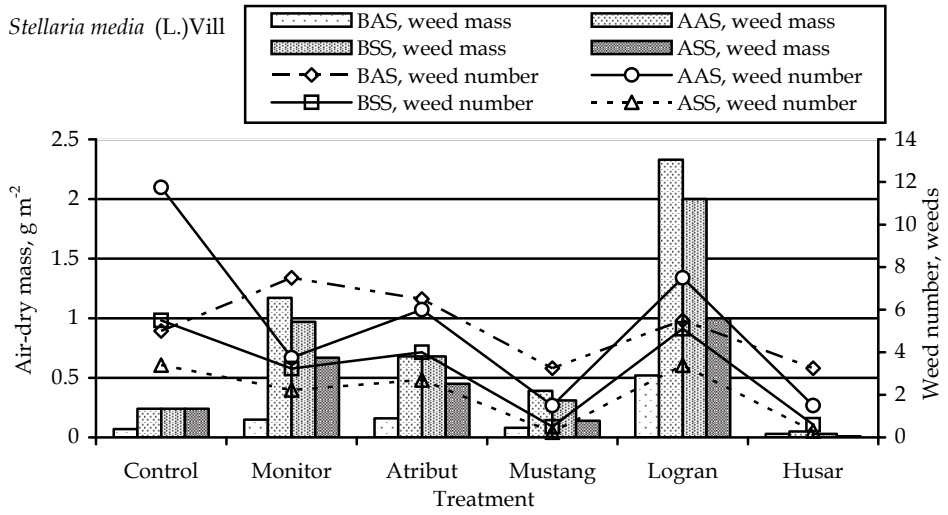
Fig. 8. *Raphanus raphanistrum* L. in winter wheat crop before and after autumn and spring application of herbicides.



Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 9. *Raphanus sativus* L. in winter wheat crop before and after autumn and spring application of herbicides.

More important winter wheat crop weed was *Stellaria media* (Fig. 10). Biologically belonging to the summer annual ephemeral weeds *Stellaria media* showed ability to survive during winter (either as *Raphanus raphanistrum*) that was not usual for the conventional Lithuanian conditions (Aleksandravičiūtė et al., 1961). *Stellaria media* overwintering was not effected even by the unfavourable wintering conditions (see meteorological conditions, subchapter 2.1). Chemical weed control, especially in autumn was not successful for control of this weed. On the contrary, *Stellaria media* was initiated to growth after herbicide application in autumn (Fig. 10). It could be influenced by the *Stellaria media* biological quality to launch new branches (stems and roots) from each damaged or fresh node. Consequently, it makes *Stellaria media* control and evaluation even more complicated.

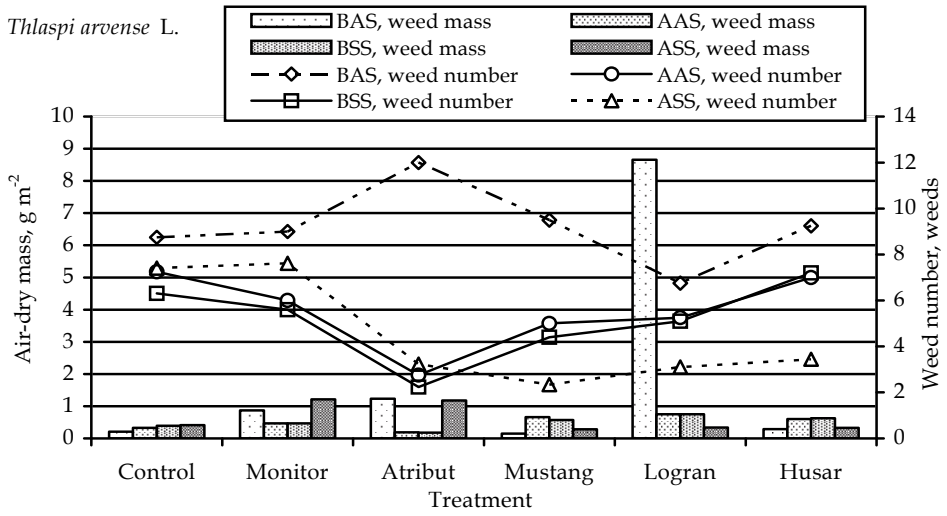


Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 10. *Stellaria media* (L.)Vill in winter wheat crop before and after autumn and spring application of herbicides.

Winter annual weed *Thlaspi arvense* made 1.8%-3.2% of total weed density and 0.23%-13.6% (the highest excess in *Logran* treatment plot) of total crop weed mass before autumn herbicide application (Fig. 11). After autumn chemical weed control applied in winter wheat crop it had tendency to decrease in number by 2.4-1.6 times and till 11 times in mass. Over wintered *Thlaspi arvense* has trivial increase in mass of control and *Husar* treatments, trivial

decrease in *Mustang* treatment and sustained autumn level in other treatment plots. Autumn application of herbicides as *Mustang*, *Logran* and *Husar* in winter wheat crop lead to decease of *Thlaspi arvense* mass after spring spraying while after autumn application of *Monitor* and *Atribut Thlaspi arvense* mass increased 1.6 and 6.6 times after spring spraying accordingly. In mentioned last two autumn treatment cases (*Monitor* and *Atribut*) standard spring application of herbicides was ineffective.

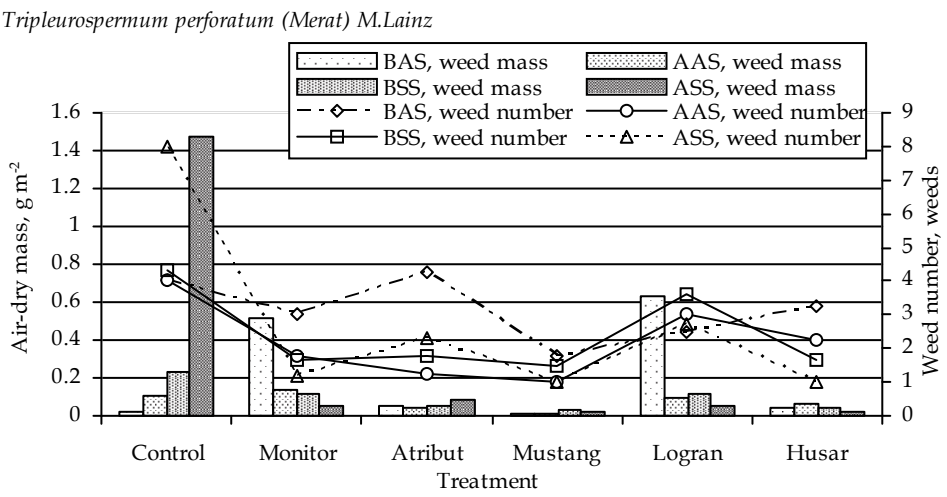


Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 11. *Thlaspi arvense* L. in winter wheat crop before and after autumn and spring application of herbicides.

Winter annual weed *Tripleurospermum perforatum* was spread enough homogenously in the experimental field except two excesses in *Monitor* and *Logran* treatments at early winter wheat crop development stages in autumn before applying chemical weed control means, that was reduced till averagely general *Tripleurospermum perforatum* weediness in the autumn crop after herbicides application (Fig. 12).

In spring *Tripleurospermum perforatum* plant development was limited by standard spring herbicide application (composite of herbicides Sekator 300 g ha^{-1} and MCPA 1 L ha^{-1} with a winter wheat growth regulator Cycocel 1.5 L ha^{-1} at BBCH 22-23) while in control treatment without autumn herbicide application it was not effective. It could be concluded that even without significant crop weediness decrease in autumn growth period of winter wheat weeds are damaged and weaken what is essentially highlighted at conventional spring application of herbicides in winter cereals.



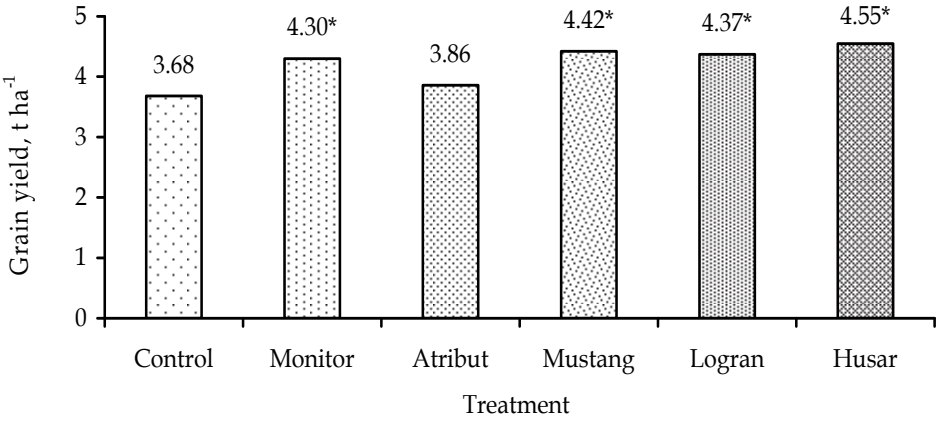
Note. BAS – before autumn spraying, AAS – after autumn spraying, BSS – before spring spraying, ASS – after spring spraying

Fig. 12. *Tripleurospermum perforatum* (Merat) M.Lainz in winter wheat crop before and after autumn and spring application of herbicides.

2.4 Crop productivity

Winter wheat grain yield was expressed in moisture of 14% and absolutely clean mass. Grain moisture was established by drying grains in a thermostat at the temperature of 105 ^{0}C until they reached the constant weight. The biggest winter wheat yield 4.55 t ha^{-1} was got using *Husar* (Iodosulphuron-methyl-sodium) in autumn and the least one 3.68 t ha^{-1} in control treatment without autumn application of herbicides (Fig. 13). Average winter wheat grain yield in Lithuania in the same year was 2.46 t ha^{-1} (Statistics Lithuania, 2011). Average winter wheat grain yield in Lithuania in 2006 comparing it with 2005 decreased by 35% and general yield by 43.6% (Market research, 2007). Modern technologies of herbicide application at early stages of winter wheat development in autumn are very promising while comparing winter wheat grain yield in our experiment with average winter wheat yield in Lithuania. During 2005-2010 the highest winter wheat grain average yield was 4.76 t ha^{-1} in 2008 (Statistics Lithuania, 2011), i.e. just by 4.4% higher than in our best treatment. Though, winter wheat vegetation 2007-2008 meteorological conditions for winter wheat growing were better than during 2005-2006 vegetation.

In our experiments (Pilipavičius et al., 2010b) essential winter wheat grain yield increase by 0.62-0.87 t ha^{-1} was established after use of *Monitor* (Sulphosulphuron), *Mustang* (Florasulam + 2.4-D 2-ethylhexyl ester), *Logran* (Triasulphuron) and *Husar* (Iodosulphuron-methyl-sodium) compared with in autumn unsprayed control. Winter wheat grain yield increase reached 25%, 20%, 19% and 24% accordingly, and after spraying by *Atribut* (Propoxycarbazone-sodium) grain yield had tendency to increase (Fig. 13).



Note. LSD_{05}=0.372; * - essential differences from control treatment

Fig. 13. Winter wheat grain yield of crop with autumn application of herbicides. (Pilipavičius et al., 2010b)

Evaluating grain chemical composition (Table 4), it was established that independently of used herbicides, nutritional composition of grain was not radically different. The amount of crude protein in winter wheat grain changed from 8.9% to 9.9% and there was found from 1.43% to 2.1% of crude fat, from 1.93% to 2.67% of crude fibre and from 1.2% to 1.7% of crude ash (Pilipavičius et al., 2010b).

Treatment	in grain dry matter							
	Crude protein		Crude fat		Crude fibre		Crude ash	
	%	t ha^{-1}	%	t ha^{-1}	%	t ha^{-1}	%	t ha^{-1}
Control	8.9	0.328	1.53	0.056	2.23	0.082	1.2	0.044
Monitor	9.4	0.404	1.43	0.061	2.7	0.116	1.5	0.064
Atribut	9.1	0.351	1.69	0.065	2.59	0.099	1.2	0.046
Mustang	9.9	0.437	2.1	0.092	2.43	0.107	1.3	0.057
Logran	9.3	0.406	2.00	0.087	1.93	0.084	1.7	0.74
Husar	9.5	0.432	1.82	0.082	2.67	0.120	1.7	0.077

Table 4. Grain chemical composition of winter wheat crop with autumn application of herbicides (Pilipavičius et al., 2010b).

A statistically reliable reverse linear correlation r = -0.565** (Pilipavičius et al., 2010b) was established between crop weed number after spring spraying by herbicides and winter wheat grain yield (2).

$$y = 4.517 - 0.091\ x;\ P = 0.004 \quad (2)$$

Evaluating dependence between weed air-dry biomass after herbicide application in spring and winter wheat grain yield (3), it was established statistically reliable reverse linear correlation r = -0.438* (Pilipavičius et al., 2010b).

$$y = 4.454 - 0.0128\ x;\ P = 0.032 \quad (3)$$

It was in conformity as dependence of winter wheat grain yield on weed density (2) and coincided (3) with the law of crop productivity (Lazauskas, 1990, 1993; Pilipavičius et al., 2009).

3. Conclusion

Winter wheat *Triticum aestivum* L. is sensitive to weed competition in early stages of growth and development. In conventional technologies herbicides in winter cereals are applied in spring. Therefore, perennial and annual (especially winter annual) weeds over-wintered successfully in cereals have favourable conditions to grow and compete with cereals when vegetation in spring is renewing.

Perennial weeds are well adapted to over-wintering biologically while their air-dry mass increases till 11% during wintering. Conventionally, it is opposite to annual weeds. However, it was established that during winter time in winter wheat crop annual weeds, even some summer annual ones, had increase adaptivity of successful surviving winter frosts and accumulated higher one plant average mass by 5-6%during winter time. Moderated increase of annual weed mass during winter time was in conformity with the research hypothesis that many annual weeds successfully survive winter time as earlier was not usual.

Separate weed species of winter wheat crop responded adequately to the autumn application of herbicides as the whole crop weed community. Standard spring spraying as conventional technology with composite of herbicides is insufficient effective while average weed plant mass increase from 0.10 gram in autumn till 0.14 gram in spring after herbicide application, i.e. increase make 40%. Weeds had tendency to spread in winter wheat crop without autumn application of herbicides and formed more competitive weed community against winter wheat in spring. It can be concluded that even without significant crop general weediness decrease in autumn by herbicides weeds are damaged and weaken what is essentially highlighted at conventional spring application of herbicides in winter cereals. Weeds left in the crop after autumn spraying by herbicides reliably by 33.5% increased crop weediness in spring before conventional spraying, as described in regression equation $y = 4.077 + 0.335\ x$.

Winter wheat yield and its agrophytocenoses weed air-dry mass in spring crop was in conformity with the law of crop productivity. Winter wheat grain yield depended on weed air-dry mass and was described by the reverse linear correlation $r = -0.438^*$ and regression $y = 4.45 – 0.013x$ analyses.

Modern technologies of herbicide application at early stages of winter wheat development in autumn are very promising while in our experiment it gives increase in grain yield till 25% and comparing it with average winter wheat yield in Republic of Lithuania during the same period it was got increase in grain yield from 50% to 85%.

For the best weed control and winter wheat yield results herbicides should be applied in autumn, especially when the weather is favourable for prolonged development of weeds even at low density of perennial ones in the crop.

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