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Rhizosphere effect on the dissipation of pesticides in a biobed system

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Rhizosphere effect on the dissipation of pesticides in a biobed system

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"El éxito es dependiente del esfuerzo"

Sófocles

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Summary and outline of this thesis

Inadequate pesticide handling in agriculture may increase the risk of environmental contamination due to the dispersion into non-target sites. To solve this problem, the biobed system is a biotechnological tool used to reduce pesticide point source contamination. The first biobed system proposed in Sweden consisted in a biologically active substrate, which retains pesticides into the organic matter and enhance their microbial degradation. In its original design, biobed consisted of a 60 cm deep pit in the ground with a clay layer in the bottom (10 cm) and the remaining volume filled with a biomixture and a grass layer covering the surface. The traditional biomixture consists of straw, topsoil, and peat in a volumetric proportion of 2:1:1. The straw stimulates the growth of lignin-degrading fungi and the activity of ligninolytic enzymes, which can degrade different pesticides. The soil provides sorption capacity and other degrading microorganisms, and the peat contributes to high sorption capacity and regulates the humidity of the system. A grass layer covering the system provides evapotranspiration, regulates humidity and reveals pesticide spillages in particular.

The traditional biomixture has been modified to alternative biomixtures in other countries for specialized purposes. In this sense, the use of readily available components in the biomixture is an important aspect to build a biobed system in farmyards. Some studies have reported that for example, sun flower crop residues, olive leaves, grape stalks, among others, can be used replacing straw (lignocellulosic material) in the biomixture to enhance adsorption and degradation of several pesticides. Therefore, in this thesis the use of readily available lignocellulosic wastes as barley husk, sawdust and oat husk, as total or partial substitutes of straw in pesticide dissipation was studied. Through degradation assay of atrazine, chlorpyrifos and isoproturon and their metabolite formation, and the adsorption of these three pesticides was also evaluated.

Moreover, microbial transformation/mineralization of pesticides is the most important process of biodegradation in biobed system. The size and the activity of the

microbial biomass affect the rate of pesticide degradation. Plants can sustain large microbial populations in the rhizosphere through their root exudates. In this sense, their use in the biobed system can be a huge advantage. In fact, plants and their associated rhizosphere microorganisms have been important in the remediation of several pesticides. Therefore, the root system can improve biomixture conditions for microorganisms, which are responsible for the degradation of these contaminants, which has not been deeply considered in studies of biobed system until now. Thus, four plant species (*Lolium perenne*, *Festuca arundinacea*, *Trifolium repens* and *Agrostis tenuis*) were evaluated to use them as grass layer on selected biomixture of a biobed system, which tolerate and enhances dissipation of atrazine, chlorpyrifos and isoproturon in a mixture.

The outline of this thesis begins with a general introduction. In Chapter 1, we reported the general objectives of our thesis in relation to the problem of pesticide contamination, biobed system to reduce point-source contamination by pesticides of ground water and the benefits of specific grass layer to enhance the degradation of pesticides compared with unplanted matrix.

In Chapter 2, we presented a literature review for the sources of pesticide contamination in non-target sites, and the bioremediation strategies for this contamination; we focused in biobed system and phytoremediation technology. In this chapter, it is hypothesized that the potential of a biobed system considering their optimal conditions to dissipate pesticide can be improved when knowing the rhizosphere effect of grass layer on this system; we discussed the benefits of plant-microorganism interaction that involves degradation of pesticides.

In Chapter 3, we demonstrated that the straw of the traditional biomixture is possible to be replaced partially or completely with other readily available lignocellulosic wastes. Overall, degradation assay showed that barley husk can be only used as a partial substitute for straw (biomixture composed of barley husk, straw, topsoil and peat in a volumetric proportion of 1:1:1:1). Indeed, biomixture composed of barley husk, topsoil and peat in a volumetric proportion of 2:1:1, promoted microbial activity but not pesticide degradation. Similarly, the use of sawdust as a lignocellulosic material in the biomixture is

only recommended as a partial substitute for straw (biomixture composed of sawdust, straw, topsoil and peat in a volumetric proportion of 1:1:1:1) because the high lignin content in this material can cause the retardation of pesticide degradation. Nevertheless, we found that biomixtures composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1, exhibited high degradation capacities comparable with traditional biomixture composed of straw, topsoil and peat in a volumetric proportion of 2:1:1.

In Chapter 4, we showed that adsorption capacity of atrazine, chlorpyrifos and isoproturon on different biomixtures was related with the physic-chemical characteristics of the biomixtures and pesticides tested. In fact, Biomixture composed with sawdust showed a higher adsorption capacity, indicating that the high lignin content of this substrate increased adsorption of the pesticides. However, biomixtures with oat husk showed an intermediate adsorption capacity of tested pesticides. Moreover, the pesticides behavior considering their chemical nature was chlorpyrifos>atrazine>isoproturon, where adsorption decreased when increase the water solubility of each pesticide.

In Chapter 5, we observed that the rhizosphere of specific plant species significantly enhances the dissipation of an atrazine, chlorpyrifos and isoproturon mixture in the biomixtures of this biobed system. The largest and most rapid dissipation of three pesticides was in the planted pots compared with unplanted pots. In fact, the enhanced dissipation of pesticides was accompanied by the increases exudation of oxalic acid and malic acid concentration, which induced the lignin-degrading enzymatic activities (phenoloxidase activity). Moreover, high relative abundance of fungal and bacterial population was detected. Therefore, the grass layer composed of *Lolium perenne*, *Festuca arundinacea* and *Trifolium repens* in the biomixture is an important factor to enhance the removal of atarzine, chlorpyrifos and isoporturon in mixtures of a biobed system.

In Chapter 6, we presented a general discussion and conclusions, where the use of a lignocellulosic waste in the biomixture and the evaluation of a grass layer in the biobed system for dissipation of pesticides were discussed. Besides, we presented future directions concerning plant-microorganism interaction of grass layer in a biobed system. The main conclusions of this thesis are: (1) The biomixture of biobed system can be prepared with

lignocellulosic wastes such as oat husk. Biomixture composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1 showed a high degradation and an intermediate adsorption capacity of atrazine, chlorpyrifos and isoproturon, comparable with the traditional biomixture (straw:topsoil:peat 2:1:1 by volume). (2) Rhizosphere effect of grass layer in the biomixture enhances dissipation of atrazine, chlorpyrifos and isoproturon compared with unplanted biomixture.

Finally, in Chapter 7 we showed in the Appendix 1, the biological activities in each biomixture tested at different maturity stages before pesticide contamination. Then, in Appendix 2, we reported the tolerance and potential of different plant species (*L. perenne* (L), *F. arundinacea* (F), *T. repens* (T) and *A. tenuis* (A)) for the bioremediation of atrazine, chlorpyrifos and isoproturon on biobed system.

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Chapter 1

General Introduction

General introduction

1.1 Introduction

Pesticide point source contamination has been identified as a significant contributor to the deterioration of the quality of natural water resources, which represents about 40 to 90% of the total pesticide load. This contamination on field can occur through the inadequate pesticide handling such as accidental spillages during tank filling or cleaning the spraying equipment (Carter, 2000; Neumann et al., 2002; Jaeken and Debaer, 2005). In fact, pesticide residues and their transformation products are frequently found in groundwater and surface waters. In this sense, atrazine (Bhagobaty et al., 2007), chlorpyrifos (Kolpin et al., 2000) and isoproturon (Stangroom et al., 1998) in water resources were detected. In addition, Palma et al. (2004) reported that three different herbicides and one fungicide were detected in surface waters of Traiguén river in Southern Chile, which is mainly an agricultural area.

On-farm biopurification systems, termed ‘‘biobeds’’, are a biotechnological tool widely distributed in Europe and, more recently, in South America as well. In fact, this system has been successfully implemented in Chile (Diez et al., 2013a). Such systems were designed and implemented to mitigate point source contamination by agriculture pesticides (Torstensson and Castillo, 1997). This system consist originally of a 60 cm deep pit in the ground filled with a biomixture and a grass layer covering the surface (Castillo et al., 2008). The principal component of this system is the biomixture typically composed of straw, peat and topsoil in a volumetric proportion of 2:1:1. Straw stimulates the growth of lignin-degrading fungi and the activity of ligninolytic enzymes (e.g. phenoloxidase activity), which can degrade many different pesticides. Topsoil provides sorption capacity and other degrading microorganisms, and the peat contributes to high sorption capacity and regulates the humidity of the system. A Grass layer covering the system provides evapotranspiration, regulates humidity and reveals pesticide spillages in particular (Torstensson and Castillo, 1997; Castillo and Torstensson, 2007; Castillo et al., 2008).

The efficiency of all biobed systems is based on the capacity of the biomixture to effectively degrade and retain the high pesticide load discharges. In this sense, Tortella et al. (2013a) reported that in biomixture composed of straw, Andisol topsoil and peat in a volumetric proportion of 2:1:1, the atrazine removal was efficient (≥78%) after repeated applications (40 mg a.i. kg^{-1}). Although the microorganisms were reduced after each application, these were rapidly recovered. In the same way, the successive carbendazim application in the biomixture had a high ≥87% dissipation capacity and microbiological robustness (Tortella et al., 2013b). Furthermore, Tortella et al. (2012) demonstrated that biomixture was contaminated with 200 mg kg^{-1} of chlorpyrifos of which 85% were degraded efficiently after 40 d incubation. In another work, Fernández-Alberti et al. (2012) demonstrated that biomixture has a higher capacity to retain chlorpyrifos (K_f = 4920) than topsoil (K_f = 3304), due to the effect of biomixture (organic matter-rich substrate) and hydrophobic character of the chlorpyrifos (log K_{ow} 4.7). However, this biomixture showed a high degradation (70%) and formation 35% of the main metabolite 3,5,6-trichloro-2-pyridinol (TCP) after 10 days degradation time. Similarly, Karanasios et al (2010a) showed a positive relationship between degradation rates and adsorption capacity, particularly biomixture composed of compost was efficient in pesticide degradation and, at the same time, showed a high adsorption capacity. Therefore, knowing the balance between adsorption and biodegradation to increase pesticide removal irreversibly is very important.

Moreover, the traditional biomixture (Swedish biomixture, see Torstensson and Castillo, 1997) has been modified to alternative biomixtures in other countries for specialised purposes. In this sense, Karanasios et al. (2010b) reported that local Mediterranean lignocellulosic materials as sun flower crop residues, olive leaves, grape stalks among others, can be used replacing the straw in the biomixture to enhance adsorption and degradation of several pesticides. Coppola et al. (2007) reported that chlorpyrifos was degraded efficiently without accumulation of their main metabolite (TCP) in the presence of straw and garden compost as lignocellulosic material of biobed system. In another study, biomixtures composed with cotton crop residues showed that promoted the degradation of hydrophilic pesticides (terbuthylazine) and the adsorption of hydrophobic pesticides (chlorpyrifos) (Kravvariti et al., 2010). In addition, vine branches,

coco chips, and willow chopping among others have been tested as replacement of straw in the biomixture showing high adsorption and degradation capacity (Vischetti et al., 2008; De Wilde et al., 2009). Therefore, to find an alternative material for biomixture of biobed system has advantages for the implementation in different countries.

On the other hand, microbial transformation/mineralization is the most important route for pesticide degradation in biobed system. Size and activity of the microbial biomass affect the rate of pesticide degradation. Plants can sustain large microbial populations in the rhizosphere through their root exudates. In this sense, their use in the biobed system can be a huge advantage. Overall, the rhizosphere can be described as the soil adjacent to plant roots; therefore, it is influenced by activity, impacting physicochemical conditions and biological activity in the surrounding rhizosphere compartment (Hinsinger et al., 2009; Neumann et al., 2009). Thus, as the rhizosphere continuously provides important sources of nutrients through their root exudates, the microbiological activity in this zone is up to four-fold greater compared with soil away from plant, resulting in a zone with higher metabolic capacities for the microbiological attack of both organic matter and contaminants (El Shatnawi and Makhadmeh, 2001; Alkorta and Garbisu, 2001; Pilon-Smits, 2005; Haichar et al., 2008). In fact, low-molecular weight compounds such as sugar and amino acids, organic acids represent one of the most labile sources of carbon in soil for microorganisms (Jones et al., 2003). Moreover, plant roots induce pH changes in the rhizosphere about as much as two pH units. Therefore, the rhizosphere can acidify this zone with the excretion of organic acids (Fageria and Stone, 2006) and benefit fungi growth in a biobed system.

A reported mechanism used by rhizospheric microorganisms to take up contaminants is the production of biosurfactant, facilitating the contaminant degradation, mainly of hydrophobic compounds, and concomitantly increasing their availability to plants (Nielsen et al., 2002; Read et al., 2003; Rajaei et al., 2013). In addition, some studies show that organic acids affect desorption of organic contaminants in soil and enhance their bioavailability significantly (White et al., 2003; Luo et al., 2006; Mingji et al., 2009). In this context, it has been reported that plant species and soil are responsible cooperatively for the structure and function of microbial diversity in the rhizosphere (Berg and Smalla,

2009). Therefore, plants and their associated rhizosphere microorganisms are an important contribution in degradation processes of contaminant in soil (Wang and Oyaizu, 2009).

Some studies have reported that plant species, such as *Lolium perenne*, *Festuca arundinacea* and *Agrostis tenuis*, with fibrous rooting systems, have shown a high potential to remove contaminants by providing a high root surface area that interacts with soil microorganisms for biodegradation. Thus, *Lolium multiflorum* has demonstrated an increased removal of atrazine, chlorpyrifos and pentachlorophenol (Banks et al., 1999; He et al., 2005; Merini et al., 2009; Korade and Fulekar, 2009; Urrutia et al., 2013a). In addition, Singh et al. (2004) demonstrated that the rhizosphere of *Pennisetum clandestinum* tolerated and improved atrazine and simazine degradation, compared with unplanted soil. In another work, Wang and Oyaizu (2009) evaluated the phytoremediation potential of four plants species for dibenzofuran-contaminated soil. The authors found that *Trifolium repens L.* not only had the highest root biomass, but also the highest dibenzofuran-degrading bacterial numbers compared with those of the other three grass species. Therefore, the rhizosphere of this grass layer and its associated microorganisms could be an important component to improve the degradation of pesticides in the biopurification system, which has not been deeply considered in studies of biobeds until now. In addition, it is important to identify the plant species capable of surviving a particular toxic contamination level in the biobed system.

1.2 Hypothesis and research objectives

1.2.1 Hypotheses

Considering the previously mentioned facts related to adapting the biomixture by greater availability of other lignocellulosic wastes in different places, and the capacity of rhizosphere of grass layer to degrade pesticides, it is possible to establish the following working hypotheses:

- The efficiency of a biobed system in the dissipation of pesticides will be increased through the incorporation of different lignocellulosic wastes into the biomixture. Different lignocellulosic wastes will improve the secretion of phenoloxidase enzymes of fungi biomass.
- On the other hand, influence of the rhizosphere of grass layer in a biobed system will enhance dissipation of pesticide; the rhizosphere will increase microbial activity for an accelerated degradation of pesticide through radical exudates production.

1.2.2 Research objectives

1.2.2.1 General objective

To study the potential use of different lignocellulosic wastes in the biomixture, besides the grass layer effect of a biobed system, on the dissipation of atrazine, chlorpyrifos and isoproturon.

1.2.2.2 Specific objectives

1. To evaluate the potential use of different lignocellulosic wastes (barley husk, sawdust and oat husk) as partial or total replacement for straw in biomixture of biobed system for the dissipation of a mixture of pesticides.
2. To evaluate the tolerance to pesticides of plant species on grass layer in the biobed system with the previously selected biomixture.
3. To evaluate the rhizosphere effect on the stimulation of microorganisms capable of degrading pesticides in biobeds.

Chapter 2

Bioremediation strategies to enhance dissipation of pesticide residues

Bioremediation strategies to enhance dissipation of pesticide residues

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Abstract

Pesticide residues have been frequently detected in non-target sites. The point source contamination is an important entry under groundwater resources. To solve this problem, over the past few decades there has been much interest in developing *in situ* strategies for bioremediation of pesticide contamination. Biopurification systems (biobeds) offer an alternative method for the treatment of pesticides from point sources. In addition, phytoremediation is a promising technology for the dissipation of several pesticides. In the present review, we have summarized the sources and detection of pesticides contamination in non-target sites and discussed evidence about the bioremediation strategies of biobed system and phytoremediation especially the mechanisms defined as rhizodegradation to degrade several pesticides. The future outlook of integrating bioremediation strategies, as a more efficient biopurification system for improving the dissipation of pesticide residues is noted.

Keywords: plants, rhizosphere, pesticides, bioremediation, biobed system.

2.1 Introduction

Pesticides play an important role in the success of modern farming and food production. However, residues of these compounds can be detected in different non-target sites such as soil, surface and ground water. The possible sources of contamination into non-target sites have been described as non-point or point sources. Therefore, it is important to identify and develop a novel process for the control and treatment of this type of contamination.

Bioremediation strategies have been applied to remove pesticides by the use of biological processes of living organisms.

For example, biobed system offers an alternative method to minimize pesticide contamination of point sources (Castillo et al. 2008). The principal component is biomixture, which is composed traditionally of straw, topsoil and peat in a volumetric proportion of 2:1:1 (Castillo et al., 2008). This composition promotes the development of several microorganisms, especially ligninolytic fungi, which can degrade pesticides through extracellular enzymes, e.g., phenoloxidases (Castillo and Torstensson, 2007).

In addition, many authors promote phytoremedation as a new technology for sustainable development, in which plants are used with the aim of removing contaminants from environment and their transformation into harmless forms. A rhizodegradation mechanism is a specific type of phytoremediation involving both plants and their associated rhizosphere microbes. This appears to be particularly effective for removal and/or degradation of pesticides compared with unplanted soil (Anderson and Coats, 1995; Korade and Fulekar, 2009; Merini, et al., 2009). Plant-microbial interaction increases microbial activity at the root-soil interface named "rhizosphere", where physical, chemical, and/or biological parameters are being modified by the presence of roots and their exudates (Anderson et al., 1993; Walton et al. 1994).

The aim of this review is to describe pesticide contamination in non-target sites and present evidence of bioremediation strategies such as biobed system and rhizodegradation to enhance the dissipation of these compounds and to discuss future perspectives about considering integrating both strategies, through evaluation of grass layer ability in the biobed system to improve pesticide dissipation.

2.2 Source of pesticide contamination and bioremediation strategies

2.2.1 Source of pesticide contamination in non-target sites

The entry of pesticides and their metabolites into large volumes of soil and surface or groundwater may be categorized as diffuse or point source. Input pathways of pesticides named diffuse source are related to the movement of pesticides from the field where they are applied to surface and groundwater. This may occur from rainfall- or irrigation-generated surface runoff, through-flow/interflow/subsurface lateral flow, leaching or spray drift. Point sources of contamination are largely the result of pesticide handling procedures, for example, tank filling, spillages, faulty equipment, washing, inappropriate waste disposal, and direct contamination (Carter, 2000; Tang et al., 2012). Studies have shown that point sources contaminations represent 40 to 90% of the entries of these contaminants into water (Jaeken and Debaer, 2005). Consequently, several studies detected pesticides in soil, surface and groundwater in different countries (Table 2.1):

Table 2.1. Pesticide residue detection in soil, superficial and groundwater in some countries

Sector	Pesticides	Detected level	Matrix	Reference
Chile	Lindane, diazinon, oxifluorphen, dicofol, azinphos-methyl, methabenzothiazuron, lenacilo, diflubenzuron and atrazine	>0.1 ugL^{-1}	Surface water	Baéz et al. (1996)
Canada	Alachlor, metolachlor, atrazine	>5 ugL^{-1}	Groundwater	Goss et al. (1998)
Europa	Isoproturon	>0.1 ugL^{-1}	Surface and groundwater	Stangroom et al. (1998)
Netherlands	Atrazine and simazine	>200 ngL^{-1}	Groundwater	Maanen et al. (2001)
United Kingdom	Isoproturon and chlorotoluron	0.1 – 0.8 ugL^{-1}	Groundwater	Johnson et al. (2001)
Greece	Lindane (γ-BHC), phorate, propachlor and	0.005 to 0.01 μgL^{-1}	Groundwater	Karasali et al. (2002)

	chlorpyrifos ethyl			
Chile	Simazine, hexazinone, 2,4-D, picloram and carbendazim	>0.3 ugL^{-1}	Surface water	Palma et al. (2004)
South Africa	DDT, and its metabolites (DDDs and DDEs), chlordane, hexachlorobenzene (HCB), heptachlor and endosulfan	>5.5 ngL^{-1}	Surface and groundwater	Fatoki and Awofolu (2005)
Hungarian	Atrazine	0.07 μgg^{-1}	Soil	Oldal et al. (2006)
Chile	Aldrin, Dieldrin, DDT, and DDE	>0.6 $mgkg^{-1}$	Soil	Henríquez et al. (2006)
Spain	Atrazine, desethylatrazine, simazine, desethylsimazine, metolachlor, desethylterbuthylazine, terbuthylazine and metalaxyl	>0.01 ugL^{-1}	Surface and groundwater	Hildebrandt et al. (2008)
Brazil	Simazine, metribuzin, metolachlor, trifluralin, atrazine, deisopropylatrazine and deethylatrazine	0.14 - 1.7 ugL^{-1}	Surface and groundwater	Dores et al. (2008)
Spain and Portugal	Terbuthylazine, cyprodinil, tebuconazole, metalaxyl and chlorpyrifos	>0.1 ugL^{-1}	Groundwater and soil	Sánchez-González et al. (2013)

On the other hand, the persistence of the pesticides depends on its physical and chemical properties and the characteristics of the environment. Therefore, it is necessary to know the characteristic of each compound when we research remediation methods of pesticides detected in non-target sites.

2.2.2 Bioremediation strategies

Bioremediation of contaminated sites is defined as the elimination, attenuation or transformation of polluting or contaminating substances by the use of biological processes

of, living organisms. This is a relatively low-cost, low-technology technique, which generally has a high public acceptance and can often be carried out on site (Vidali, 2001). For example, biopurification system known as biobed system, which has the ability to retain and degrade pesticide to prevent water contamination (Torstensson and Castillo, 1997; Castillo and Torstensson, 2007), and phytoremediation technology that uses living plants to clean contaminated sites, through contaminant removal, degradation, or containment, such as pesticides (Pilon-Smits, 2005)

2.2.2.1 Biobed system

Biobed is a biopurification system that was implemented for reducing the risk from point source contamination of water resources. Biobeds consist of a 60 cm deep pit in the ground filled with a biomixture and a grass layer covering the surface. The biomixture is traditionally composed of topsoil, peat and straw in a volumetric proportion of 1:1:2. The topsoil provides sorption capacity and is an important source of pesticide-degrading bacteria and peat contributes to sorption capacity, moisture control and pH decrease promoting fungi development (Torstensson and Castillo, 1997). Straw as lignocellulosic material is the main substrate in the biomixture as it allows the development of ligninolytic fungi that promotes pesticide degradation in the biomixture (Castillo and Torstensson 2007). In addition, the use of grass layer over the biobed regulates the moisture in this system, it reveals pesticide spillages in particular and can produce root exudates to support the cometabolic process (Castillo et al., 2008), which has not been deeply considered in studies of biobeds.

Several studies have demonstrated that biomixture of biobeds has been efficient in the adsorption and degradation of different pesticides (Henriksen et al., 2003; Vischetti et al., 2004; Spliid et al., 2006; Castillo and Torstensson, 2007). However, the efficiency of tested biomixtures in almost all biobeds was studied, but grass layer was not considered. For example, in biobed treatments at laboratory scale a strong decrease 76 % of total extractable isoproturon after 100 d was observed (von Wirén-Lehr et al., 2001). In addition, biomixture composed of straw, Andisol topsoil and peat in a volumetric proportion of 2:1:1 and contaminated with 200 mg kg^{-1} of chlorpyrifos were degraded efficiently 85 % after 40 d of incubation (Tortella et al., 2012). In another study, Tortella et al. (2013a) showed that

after successive atrazine application, the biomixture composed of straw, Andisol topsoil and peat in a volumetric proportion of 2:1:1 had a high ≥78% degradation capacity. Interestingly, Castillo and Torstensson (2007) showed that biobeds can remove complex mixtures of pesticides pertaining to different chemical classes such as triazines, pyrethrins and derivatives of ureas, among others. They demonstrated that the straw levels in biomixture are correlated positively to the content of respiration and/ or phenoloxidase content during the degradation of most pesticides in study. Coppola et al. (2007) reported that chlorpyrifos was degraded efficiently without accumulation of their main metabolite (TCP) in the presence of straw and garden compost as lignocellulosic material of biobed system. In another work, Karanasios et al. (2010b) reported that local Mediterranean lignocellulosic materials as sunflower crop residues, olive leaves, grape stalks, among others, can be used replacing straw in the biomixture to enhance adsorption and degradation of several pesticides.

In this sense, some lignocellulosic materials of the biomixtures have been replaced in some countries, for adaptation purposes. Since, this material can be gathered from wood, grass, agriculture and forestry residues as well as from municipal solid wastes are particularly abundant in nature and have a potential for bioconversion. Lignocellulosic materials consist mainly of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Jorgensen et al., 2007). The composition of these constituents can vary from one plant species to another. wheat, rice, barley and oat, of which straw, stalks, husks and cobs are among the residues from the processing and harvesting of major agricultural commodities that can be obtained (Detroy and Hesseltine, 1977).

Apart from the composition of the biomixtures, it is necessary to consider the physical and chemical characteristics of the tested biomixtures, such as pH about 5.9 suitable for fungi, low nitrogen levels in the biomixtures to favor activation of lignin-degrading enzymes, since the lignin-degrading system of many white-rot fungi is nitrogen-regulated (Castillo et al., 2007; Waldrop and Zak, 2006). At low nitrogen levels, fungi activate the production of phenoloxidases, whereas higher levels can enhance growth but

inhibit the production of enzymes. Therefore, nitrogen addition is not recommended in biobed systems (Castillo et al, 2007). The moisture level is also an important factor to promote different environments that can influence the oxygen level, the microbial activity and the amount of pesticide in solution. According to Castillo and Torstensson (2007) moisture of 60% of WHC in the biomixture of biobed may give enough water for microbial processes, solubilization of pesticides, and adequate pore space for oxygen to support aerobic processes, and the highest dissipation of most pesticides, whereas moisture at 30 and 90% WHC limited the microbial activity. In another study, biomixture with 60% of WHC showed the highest degradation of chlorpyrifos (70%) compared with 64 and 56% of removal in biomixtures at 40 and 80% WHC, respectively. (Fernández-Alberti et al., 2012). In addition, the biobed age or maturity of the biomixture before its use in the pesticide degradation is an important factor. For example, the study of Henriksen et al. (2003) demonstrated that a biobed with a fresh biomixture (without previous compost), submitted to high hydraulic loads, produces a high leaching of pesticides. Nevertheless, leaching can be minimized after 1 year of operation. Fernández-Alberti et al., 2012 reported that the biomixture pre-incubated 15 days before pesticide contamination showed the highest degradation of chlorpyrifos and formation of their main metabolite 3,5,6-trichloro-2-pyridinol (TCP) compared with biomixtures pre-incubated 0 and 30 days. These results can be attributed to the fact that during the incubation of the biomixtures, physical and chemical changes are produced due to different reactions originated from the successions of the different microbial populations (bacteria, fungi and actinomycetes, fundamentally).

2.2.2.2 Phytoremediation technology

Phytoremediation is the name given to a set of biotechnologies that uses living plants to clean contaminated sites, through contaminant removal, degradation, or containment. We will focus on the mechanism defined as rhizodegradation is based on the secretion by plants in root exudates, which support growth and metabolic activities of diverse fungal and bacterial communities in the rhizosphere capable of degrading several contaminants (Anderson et al., 1994). In fact, densities of rhizosphere microorganisms can be as much as two to four orders of magnitude greater than those in the surrounding bulk soil (Alkorta and Garbisu, 2001; Pilon-Smits, 2005). Several studies are demonstrated that

plant and their associated rhizosphere microorganisms could be important in the remediation of soils contaminated with organic chemicals compared with bulk soils, as shown in Table 2.2.

Table 2.2 Researches that report rhizosphere effect on the dissipation of several pesticides compared with unplanted soil

Plant species	Pesticide	Rhizosphere	Unplanted soil	References
Carduus nutans L., Nepeta cateria L., Kochia scoparia, Chenopodium berlandieri, Hordeum jubatum and *Panicum capillare.*	Atrazine	≥ 8.5%		Anderson and Coats (1995)
Kochia scoparia (L.)	Atrazine	62.1%	48,7%	Perkovich et al. (1996)
Zea mays L.	Atrazine	$T_{1/2}$ = 7 d	$T_{1/2}$ >45 d	Costa et al. (2000)
Zinnia angustifolia	Mefenoxam	78%	44%	Pai et al. (2001)
Zea mays L.	Atrazine	61%	40%	Marchand et al. (2002)
Triticum	Butachlor	$T_{1/2}$ = 18 d	$T_{1/2}$ = 23.2 d	Yu et al. (2003)
Panicum virgatum	Isoxaben	$T_{1/2}$ = 24 h	$T_{1/2}$ = 30 d	Drakeford et al. (2003)
P. clandestinum	Atrazine and simazine	45-52%	22-20%	Singh et al. (2004)
Zea mays L.	Atrazine	90%	-	Huang et al. (2007)

Brassica	Chlorpyrifos	>1.4–4.2 times	-	Wang et al. (2007)
Oryza sativa L.	Simazine	$T_{1/2}$ = 52.9 d	$T_{1/2}$ = 73 d	Min and Xiaomei (2008)
Cyperus iria L,C. pilosus V., C. difformis L., Fimbristylis miliacea V., Marsilea crenata P., and *Jussiaea linifolia V.*	Carbofuran	$T_{1/2}$ = 15–19 d	$T_{1/2}$ = 58 d	Plangklang and Reungsang (2008)
Lolium multiflorum	Chlorpyrifos	≥ 90%	-	Korade and Fulekar (2009)
Lolium multiflorum	Atrazine	≥ 20%	-	Merini et al. (2009)
Amaranth (*Amaranthus caudat*), Lettuce (*Lactuca sativa*), Water cress (*Nasturtium officinale*) and Kidney bean (*Phaseolus vulgaris L.*)	Dimethoate and malathion	≥ 90%	-	Al-Qurainy and Abdel-Megeed (2009)
Lolium multiflorum	Pentachlorophenol	≥ 90%	-	Urrutia et al. (2013a)

In general, phytoremediation technology application has advantages and limitations, according to Morikawa and Erkin (2003). Plants have many features that increase environmental cleanup. These advantages are: (1) It is aesthetically pleasing, energy costs and expenses are reduced and natural resources are conserved because plants use solar energy; (2) There is a minimal environmental disruption and *in situ* treatment preserves topsoil; (3) It is very useful at sites with shallow of contamination; (4) It is useful for treating a broad range of contaminants under several environmental conditions; (5) It is inexpensive (60-80%) or even less expensive than conventional physico-chemical methods (Schnoor, 1997). The limitations are; (1) It is a time consuming process, and it may take at least several growing seasons to cleanup a site; (2) Plants that absorb toxic heavy metals or persistent chemicals may pose a risk to wildlife and contaminate the food chain; (3) In addition, intermediate formed from those organic and inorganic contaminants may be cytotoxic to plants and animals including humans; (4) Understanding mass balance analyses and the metabolic fate of contaminants in plants are the keys to proving the applicability of phytoremediation.

Phytoremediation as full-scale application is currently limited to a small number of projects. However, a biopurification system used on-farm, known as modular biofilter based on several vertically stacked $1m^3$ units filled with an organic substrate mixture (Pussemier et al., 2004), was modified with some additional horizontal units on the ground containing specific plant species for additional purification of pesticides wastes and evaporation in this system (Debaer and Jaeken, 2006). Therefore, it would be interesting to evaluate the effect of specific plant species in a biobed system to improve dissipation of several pesticides by the process that occurs in the rhizosphere as shown in the following sections.

2.3 Pesticide biotransformation in plants and microorganisms

Pesticide biotransformation may occur via multistep processes known as metabolism or cometabolism.

In general, metabolism of pesticides in plant and microorganisms may involve a three-phase process: phase I, oxidation, reduction or hydrolysis to generally produce a more

water-soluble and usually a less toxic product than the original pesticide; phase II, involves conjugation of a pesticide or pesticide metabolite to a sugar, amino acid, or glutathione, which increases water solubility and reduces toxicity; phase III involves conversion of phase II metabolites into secondary conjugates, which are also nontoxic (Hatzios, 1991; Shimabukuro, 1985; Van Eerd et al., 2003).

Transformation in phase I should be converted into metabolites by different reactions when pesticides may not be conjugated directly, as shown below.

Oxidation: Oxygenation is the most common first step in biotransformation of pesticides. The most extensive reaction studied in plants and animals is oxidative enzymes that use cytochromes P450 (Baerg, et al. 1996). Cytochrome P450s are encoded by a superfamily of genes designated as CYP, which have highly conserved residues around the heme portion of the protein. The regulation and expression of the CYP genes are not well understood in plants or in microorganisms, mainly because P450 enzymes are usually present in very low levels in the cells that have not been exposed to physiochemical, physiological nor xenobiotic stress. Many of these stress conditions modulate the metabolism of an organism, thereby modifying pesticide metabolism, which may affect growth and development of the organism. Besides, other oxidative enzymes are produced by plants and microorganisms, such as peroxidase, polyphenoloxidase, laccase and tyrosinase, which catalyze polymerization of several anilines and phenols (Dec and Bollag, 2001). In addition, microorganisms have been reported that produce numerous enzymes which can oxidize nitroaromatic compounds, through oxidative reactions by enzymes such as monooxygenases, flavin monooxygenases, and dioxygenases and biotransformed halogenated compounds (Häggblom, 1992) and nitroaromatic pesticides (Van Eerd et al., 2003).

Hydrolysis: These reactions are catalyzed by hydrolytic enzymes such as esterases, phosphatases, amidases and others. These enzymes cleave bonds by adding H or OH from H_2O to each product. For example, Hoagland and Zablotowicz (2001) have investigated the role of plant hydrolytic enzymes in pesticide hydrolysis reactions. A wide array of esterases, lipases, and proteases are involved in pesticide detoxification and degradation. For instance, amide hydrolysis by amidases is important in the detoxification of alachlor

and other pesticides with amide bonds. Monooxygenase enzymes are important in the transformation of nitroaromatic pesticides such as trifluralin (2.6-dinitro-N, N-dipropyl-4-(trifluoromethylbenzenamine)). In studies with microorganisms it has been demonstrated that esterase enzyme, specifically of *Pseudomona sp.* and *Azospirillum sp*., degrades ethion (organophosphate) (Foster et al., 2004), and *Flavobacterium sp.* and *Sphingomonas paucimobilis* degrades cadusafos and ethoprophos (organophosphates) (Karpouzas et al., 2005). Topp (2001) demonstrated that *Pseudaminobacter sp., Pseudomonas sp*., and *Nocardiodes sp*. degrade atrazine by hydrolase enzyme.

Reduction: The reductive metabolism of pesticides is a process that rarely occurs in plants; Fedtke (1983) showed the reductive deamination of the s-triazines (metamitron and metribuzin) in tolerant crops and resistant weeds. On the other hand, Lusby et al. (1980) showed that trifluralin is transformed via nitroreductase by microbes.

The conjugation and storage processes in phase II and III involve the conjugation of the pesticide molecule or its metabolites with natural plant constituents. Phase II processes give rise to the formation of soluble conjugates (mainly with sugars and amino acids). The most involved plant products in conjugation are glucose, glutathione, malonic acid, and amino acids. Glutathione conjugation is important in Phase II for transformation in plants (Hatzios, 2001).

Conjugation of insoluble structures in the plant such as co-polymerization with lignin is referred to as a Phase III reaction. This results in the formation of non-extractable or bound residue. Pesticides (mainly conjugated pesticides) are often bound to plant cell walls (Van Eerd et al., 2003). On the other hand, mainly glycoside conjugates are deposited in the vacuole, where they are stored and slowly degraded (Pillmoor et al., 1984). In relation to microorganisms, the pesticide combines with cell metabolites. Conjugation or the formation of addition product is accomplished by those microorganisms catalyzing the addition reaction of an amino acid, organic acid or methyl crown to the substrate, e.g., in the microbial metabolism of sodium dimethyl dithiocarbamate, the organism combines the fungicide with an amino acid molecule normally present in the cell thereby inactivating the pesticides/chemical.

The rate of metabolic attack on a pesticide by plants varies with the species type, the time of residence in or on the plants, and the degree of entry into the plant. With some differences, microorganisms, insects, plants, and mammals metabolize foreign organic compounds by the same major pathways (Hall et al., 2001).

Cometabolism is biotransformation of a pesticide that is not used as an energy source or as a constitutive element of the organisms. According to Crowley et al. (2001), it can occur by three general mechanisms, by which the rhizosphere may act to enhance cometabolism of anthropogenic contaminants. (1) The rhizosphere may allow selective enrichment of degrader organisms compared with root-free soil. (2) The rhizosphere may enhance growth-linked metabolism or stimulate microbial growth by providing a natural substrate (root exudates), when the concentration of xenobiotics is low or unavailable. (3) The rhizosphere is rich in natural compounds that may induce cometabolism of xenobiotics in certain microorganisms, which induce initial degradation of pesticide (Crowley et al. 1997; Alexander 1999; Van Eerd et al., 2003).

2.4. Mediated process by the plants that involve degradation of pesticides

2.4.1. Effect of roots on dissipation of pesticides

Quantity and quality of root exudates are determined by plant species, age of an individual plant and external factors like biotic and abiotic stressors. Root exudation clearly represents a significant carbon cost for to the plant. In fact, the root exudates are nearly 40% of all photosynthetically fixed carbon, which is transferred to the rhizosphere (Lynch, 1990). In addition, root exudates contain released ions (i.e. H+), inorganic acids, oxygen and water (Uren 2000; Bais et al., 2006). Organic compounds can often be separated into two classes: low-molecular weight compounds, which include amino acids, organic acids, sugars, phenolic and an array of secondary metabolites, and high-molecular weight compounds such as mucilage and proteins (Marchner 1995, Badri and Vivanco, 2009). Exudation occurs in any soil horizon with root activity as a result of passive ‘loss’ of compounds from the root or as active exudation of organic compounds (Jones et al., 1996). Particularly, low molecular weight organic acids (LMWOAs) are considered to be of great importance in many soil and plant processes, these compounds are typically present in the

range of 5–50 mM internally in the root cell vacuoles (Ryan et al., 2001; Jones et al., 2003) and about 1–10 fold lower in cytosol (Jones, 1998). In addition, they are one of the most labile C sources in soil. Once released into the soil solution they may be quickly taken up and broken down by the soil microbial community, and also the sorption of exudates may occur in soil (Jones, 1998).

Therefore, plant roots can promote microbial growth in the rhizosphere mainly through their root exudates, which have C-compounds (Jones et al., 2004). Rhizosphere microorganisms use these substances as easily available C and energy sources for growth and respiration. The number of microorganisms in the rhizosphere is 19 to 32 times larger compared with root-free soil, because microorganisms are not limited by available C in this zone (Bodelier et al., 1997). Indeed, Valé et al. (2005) reported that root microbial activity was positively correlated with root soluble carbon concentration. Thus, He et al. (2005) demonstrated that pentachlorophenol degradation was the highest in the root compartment and the six separated compartments at various distances from the root surface of planted rhizobox system rather than unplanted treatments, which the researchers attributed to the beneficial effect of root exudates and their interaction with microbial activity on degradation. In fact, Walton et al. (1994) concluded that plants increase or improve root exudates under a chemical stress, which modifies microfloral composition or activity of the rhizosphere enhancing contaminant degradation.

On the other hand, in order to maintain their charge balance, roots release protons whenever they take up more cations than anions and take up protons in the opposite case. Thereby, plant roots induce pH change in the rhizosphere about as much as two pH units (Hinsinger et al., 2003; Brimecombe et al., 2001). In addition, the respiration of roots and rhizosphere microorganisms can affect pH in their environment, by contributing with a significant CO_2 build-up in the rhizosphere, which may produce carbonic acid. Root exudates directly influence availability of essential nutrients in soil and to potentially toxic metals such as Al and a range of trace metals (Hinsinger et al., 2005). In this sense, this effect may influence in the bioavailability of ionisable contaminants. In fact, about one-third of pesticides are acidic or basic and, depending on the pH of the soil and the strength of the pesticide dissociation constant, these compounds may be present mainly as anions

and cations in the soil (Arias-Estévez et al., 2008). For example; weak acid pesticides, those ionizing in soil solution to form anionic species (A−), are repelled by the negative charges of soil colloids, being more available when pH increases. On the other hand, weakly basic pesticides are adsorbed through ionic bonds and/or by physical adsorption, depending on pH of the system, because basic pesticides in acidic medium form protonated species (HB+) (Thorstensen et al., 2001). Studies have shown that root exudates may alter the availability of organic contaminants in the soil environment. White et al. (2003) and Luo et al. (2006) demonstrated that the root exudates enhanced desorption of persistent organic contaminants (POPs) (dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyltrichloroethane (DDT)). They speculated that low molecular weight organic acids cause the partial dissolution of the soil structure through the chelation of inorganic structural ions.

2.4.2. Microorganisms in the rhizosphere in dissipation of pesticides

Rhizosphere is a hot spot of microbial interactions by exudates released by plant roots. Size and composition of the microflora in the rhizosphere depend not only on soil type, but also on the plant species and even on the cultivar (Miller et al., 1989). The microorganisms play a role in ecosystem processes consisting of the nutrient biogeochemical cycles and biodegradation (Prosser, 2002). The microbial groups and other agents found in the rhizosphere include bacteria, fungi, nematodes, protozoa, algae and microarthropods (Lynch, 1990; Raaijmakers, 2001). Considering that there are 60% bacteria and 12% fungi in the rhizosphere (Kerry, 2000), many of these microorganisms include nitrogen-fixing bacteria, endo- and ectomycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) and fungi. Our focus considered microorganisms with capabilities of degradation pesticides ,e.g. preliminary studies done by Alexander (1980) demonstrated how *Bacillus spp., Pseudomonas spp., Aspergillus spp., Fusarium spp., Mucor spp., Penicillium spp.,* and *Trichoderma spp*., can use pesticides as substrates degrading them. Arbuscular mycorrhizal (AM) fungi form a symbiotic relationship with over 90% land plants (Smith and Read, 1997). These AM fungi can significantly extend the volumes of soil to which the host plant root has access, thus improving the plant growth through better nutrition uptake and alleviating contaminant toxicity (Bolan, 1991;

Marschner and Romheld, 1998). Huang et al. (2007) showed that residual concentrations of atrazine in soils decreased significantly, especially in the rhizosphere soil of *Zea mays L*. Here, mycorrhizal (*Glomus caledonium*) treatment enhanced the dissipation of atrazine in near-rhizosphere and bulk soils, where atrazine concentrations (2.0 mg kg^{-1}) 73.7% atrazine in the bulk soil with mycorrhizal was reduced, whereas 31.4% in the non-inoculated treatment was reduced. Korade and Fulekar (2009) made the isolation and identification of microorganisms in the rhizosphere selecting *P. nitroreducens* PS-2. This bacterium was cultured to degrade chlorpyrifos with the aim of performing bioaugmentation in ryegrass rhizosphere soil, where degradation of chlorpyrifos was about 1.35 times higher for 25 mg kg^{-1} and ranged from 1.38 to 2.02 for 50 mg kg^{-1} in bioaugmented soil compared with non-bioaugmented soil. On the other hand, chlorpyrifos dissipation was greater in inoculated soil during the experimentation and ranged from 1.31 to 2.64 times, when compared with non-inoculated soils for 100 mg kg^{-1} spiked soil. Sarkar et al. (2010) also studied the biodegradation of miticide propargite, it was carried *in vitro* selecting *Pseudomonas strains*, which was isolated from tea rhizosphere, showing that these organisms have adapted to this chemical, indicating that five isolations of the 13 selected isolate were capable of tolerating the highest concentration of propargite (10 mg L^{-1}) tested in the present study. Finally, pentachlorophenol (PCP) can be completely degraded by microorganism *Sphingobium chlorophenolicum* ATCC 39723 where, PCP- 4 - monooxygenase (PcpB) is the first enzyme in the PCP biodegradation pathway encoded by pcpB gene (Dams et al., 2007). Many microorganisms from the rhizosphere capable of degrading different groups of pesticides were identified in Table 2.3.

1 **Table 2.3** Microorganisms identified from the rhizosphere that enhance dissipation of pesticides.

Vegetal species	Rhizospheric microorganisms	Pesticide	Reference
Wheat (*Triticum aestivum*)	*Pseudomonas putida strains*	2,4-D	Kingsley et al. (1994)
Barley (*Hordeum vulgare*)	*Burkholderia cepacia*	2,4-D	Jacobsen (1997)
Winter wheat (*Triticum aestivum*)	*Sphingobium chlorophenolicum*	Pentachlorophenol	Dams et al. (2007)
Maize (*Zea mays L.*)	*Glomus caledonium*	Atrazine	Huang et al. (2007)
Sweet flag (*Acorus calamus*)	*Stenotrophomonas maltophilia, Bacillus licheniformis, Bacillus megaterium, Rahnella aquatilis (three strains), Umbelopsis isabellina, Volutella ciliata and Botrytis cinerea.*	Atrazine	Marecik et al. (2008)
Soybean *(Glycine max* (L.) Merr.) maize (*Zea mays* L.) resistant crops	*Fusarium spp.*	Glyphosate	Kremer and Means (2009)

Ryegrass *(Lolium multiflorum*)	*Pseudomonas spp. Penicillum spp. Bacillus firmus Aspergillus niger Clostridium spp. Aspergillus fumigatus Streptomyse spp.*	Chlorpyrifos	Korade and Fulekar (2009)
Amaranth (*Amaranthus caudat*), Lettuce (*Lactuca sativa*), Water cress (*Nasturtium officinale*) and Kidney bean (*Phaseolus vulgaris L.*)	*Pseudomonas frederiksbergensis*	Dimethoate and malathion	Al-Qurainy and Abdel-Megeed (2009)
Tea (*Camellia sinensis (L)* O. Kuntze)	*Pseudomonas strain*	Dicofol	Sarkar et al. (2009)
Tea (*Camellia sinensis (L)* O. Kuntze)	*Pseudomonas putida*	Propargite	Sarkar et al. (2010)

2

2.5. Future outlook

Contamination of surface and ground water resources by pesticides has been reported according to their agricultural use. It is important to develop novel processes for the control and treatment of this type of contamination, such as the biobed system, which has been adapted to local requirement of several countries. However, the efficiency of this biopurification system can be improved when knowing the additional effects of its grass layer used as phytoremediation/rhizodegradation technology.

The use of plant species can enhance dissipation of hazardous compounds such as pesticides which are tolerant due to their metabolic and cometabolic processes. They are enhanced by organic substances originating from roots (exudates) reflecting for prolific microbial growth (population increase) capable of degrading these contaminants, and improving bioavailability of pesticides varying according to the different physicochemical properties of soils and pesticides.

Therefore, the use of specific plant species in the biopurification systems is presented as an attractive option because of their great advantages in the degradation of many pesticides. However, it is important to understand phyto-/rhizodegradation technology according to the benefits of plant-microorganism interaction in several environments, which do not consist only of soil, elucidating aspects such as biology, biochemistry and engineering of these remediation systems.

Chapter 3

Degradation of pesticide mixture on modified matrix of a biopurification system with alternatives lignocellulosic wastes

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Degradation of pesticide mixture on modified matrix of a biopurification system with alternatives lignocellulosic wastes

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Abstract

The biobed systems were designed to retain and to degrade pesticides through the properties of a biomixture composed of straw (ST), topsoil and peat (PT) 2:1:1 v/v. The ST is the main substrate in the biomixture, as it allows the proliferation of fungi that promotes pesticide degradation. The use of readily available components in the biomixture is an important aspect to build a biobed. Therefore, potential use of readily available wastes as barley husk (BH), sawdust (SW) and oat husk (OH), as total or partial substitutes of ST were tested in pesticide degradation studies. Metabolite formation and the biological activities were also evaluated. Biomixture composed of OH was highly efficient in pesticide degradation, with $t_{½}$ values of 28.6, 58.9 and 26.8 d for atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP). On the other hand, comparable for degrading capacities with the ST based biomixture were obtained with SW and BH, but only as partial replacement. Contrarily, high $t_{½}$ values (more than 100 d) were obtained in biomixtures with total substitution of ST by SW or BH. Metabolite formation was observed in all biomixtures tested, but without clear formation patterns. Moreover, high and stable biological activity was observed in the biomixtures composed of OH. Therefore, our results demonstrated that ST can be partial or totally replaced by OH in the biomixture allowing an efficient degradation of pesticide mixture. However, it is recommended that ST can be only partially replaced by BH and SW in the biomixture to allow efficient pesticide degradation.

Keywords: Biobed, Pesticide mixture, Lignocellulosic wastes, Biological activities

3.1 Introduction

The inadequate pesticide handling in agriculture may increase the risk of environmental contamination due to the dispersion into non-target sites. Some studies have demonstrated the presence of pesticide residues such as ATZ (Bhagobaty et al., 2007), CHL (Kolpin et al., 2000) and ISP (Stangroom et al., 1998) in water resources. Pesticide contamination can occur through non-point source or point source contamination. However, studies have been demonstrated that point source contamination, during filling and cleaning of sprayers can contribute to accumulation of these compounds in surface and groundwater (Carter, 2000; Neumann et al., 2002; Castillo et al., 2008).

Biobed is a low-cost biopurification system composed of a biomixture of ST, topsoil and PT 2:1:1 v/v, which has the ability to retain and degrade pesticide (Torstensson and Castillo, 1997; Castillo and Torstensson, 2007). The soil provides sorption capacity and is an important source of pesticide-degrading bacteria and PT contributes to sorption capacity, moisture control and pH decrease promoting fungi development (Torstensson, 2000). The ST is the main substrate in the biomixture as it allows the development of ligninolytic fungi that promote pesticide degradation in the biomixture (Castillo and Torstensson, 2007). This biomixture composition has been efficient in the degradation of several pesticides (Vischetti et al., 2004; Castillo and Torstensson, 2007). However, the biomixture has had to be adapted due to the greater availability of others lignocellulosic wastes in some countries. In this sense, Karanasios et al. (2010b) reported that local Mediterranean lignocellulosic materials as sun flower crop residues, olive leaves, grape stalks among others, can be used replacing the ST in the biomixture to enhanced adsorption and degradation of several pesticides. In other work, Coppola et al. (2007) reported that the use of urban compost and citrus peel in the biomixture caused a high chlorpyrifos degradation, but an accumulation of their metabolite 3,5,6-trichloro-2-pyrinidol (TCP) was observed. In this same way, Kravvariti et al. (2010) demonstrated that cotton crop residues enhanced the degradation of hydrophilic pesticides and the adsorption of hydrophobic pesticides.

Although, several lignocellulosic residues have been evaluated as a biomixture component for pesticide degradation, the use of other readily available residues at no cost as SW, BH and OH has been less studied. Almost, most studies of pesticide degradation in modified biomixture have been performed with application of one pesticide and not with a mixture of pesticides. However, some studies (Fogg et al., 2003; Leistra and Matser, 2004; Karanasios et al., 2010b) have shown evidence that the persistence of a number of pesticides may be changed when used in combination with other pesticides.

Therefore, the aim of this research was to evaluate the potential utilization of BH, SW and OH as a replacement for partial or total ST in biomixture for the degradation of a mixture of pesticides (ATZ, CHL and ISP). These lignocellulosic residues can be found in sufficient amounts at low or no cost and hence can be potentially used in biobed biomixture for pesticide degradation.

3.2 Materials and methods

3.2.1 Chemicals

Formulated pesticides ATZ (atranex 500SC), ISP (Fuego 50SC) and CHL (chlorpyrifos S480) were purchased from Agan Chemicals Manufacturers Ltd. Deethylatrazine (DEA), deisopropylatrazine (DIA), monodesmethyl-isoproturon (MDPIPU), TCP, 3-methyl-2- benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB) were purchased from Aldrich. All other chemicals and solvents were of analytical reagent grade and were purchased from Equilab and Merck Chile.

3.2.2 Preparation of the biomixtures

For the preparation of the biomixtures, an Andisol topsoil (0–20 cm depth) belonging to Temuco series (38°42´S, 73°35´W), BH, SW, OH and ST as lignocellulosic material and commercial peat were used. Lignocellulosic wastes like BH, OH and ST were collected from crop residues; and SW was collected from sawmill waste. All lignocellulosic wastes were cut in small pieces (2–3 cm) using a food processor and soil was sieved (to 3 mm). The constituents were mixing vigorously to obtain a homogeneous biomixtures and in volumetric proportions as been described in Table 3.1. The biomixture moisture content was adjusted to 60% of the water holding capacity (WHC) by adding distilled water. All biomixtures were placed in polypropylene bags for maturation processes and stored in dark at 20 ± 2 °C for 40 d before being used in the experiments. The composition and characteristics of the biomixtures and their components were measured as shown in Table 3.1.

Table 3.1 Composition and the physical and chemical characteristics of the biomixtures and their components used in this study

Substrates or biomixtures	Symbol	Composition (v/v)	pH	Total N (%)	Organic C (%)	C/N	Lignin (%)	Cellulose (%)
Barley husk	BH	1	6.29	0.40	32.1	80.3	2.4	9.6
Oat husk	OH	1	5.79	0.75	36.7	48.9	7.5	34.3
Sawdust	SW	1	4.63	0.15	28.9	192.6	20.6	54.3
Straw	ST	1	7.06	0.56	34.3	61.3	9.9	41.8
Soil	S	1	5.22	0.47	11.1	23.6	ND*	ND
Peat	P	1	2.67	0.57	43.7	76.6	ND	ND
ST:S:P	A	2:1:1	4.83	0.54	30.8	57.0	5.6	32.3
BH:S:P	B	2:1:1	5.05	0.46	29.7	65.0	1.6	25.4
SW:S:P	C	2:1:1	5.43	0.34	28.1	82.6	17.3	43.1
BH:ST:S:P	D	1:1:1:1	5.21	0.50	30.3	60.6	5.6	32.7
SW:ST:S:P	E	1:1:1:1	5.72	0.44	29.0	65.9	13.8	37
OH:S:P	F	2:1:1	5.56	0.64	32.1	50.2	3.7	39.2
OH:ST:S:P	G	1:1:1:1	5.88	0.59	31.0	52.5	4.3	34

*Not determined

3.2.3 Degradation studies

A bulk sample (630 g dry weight) from each biomixture was separated into 21 sub-samples (30 g dw) in triplicate. These sub-samples were placed in glass flasks (500 mL) and were sprayed individually with a mixture of formulated pesticides ATZ, CHL and ISP corresponding to a dose of approximately 100 mg a.i. kg^{-1}. In addition, 21 sub-samples per each biomixture without pesticides were used as control. The biomixture moisture content was adjusted to 60% of the WHC by regular adding of distilled water. Afterwards, all biomixtures were incubated in dark at 20 ± 2 °C for 90 d. Immediately after mixing, samples of treated biomixtures and control were periodically up to 90 d for residual pesticide and their main metabolite analysis.

ATZ, CHL and ISP degradation in different biomixtures was described with the first-order kinetic equation as $C = C_0 e^{-kt}$, and from the equation, we obtained (Eq. *(1)*):

$$t_{1/2} = Ln(2)/k \qquad (1)$$

Biological activities in different biomixtures during the degradation study were determined by measuring microbial respiration, phenoloxidase activity and fluorescein diacetate (FDA) hydrolysis. All biological activities were performed in the samples with and without pesticide mixture dose (100 mg a.i. kg^{-1} approximately of each pesticide) on 0, 10, 30, 60 and 90 d of incubation.

3.2.4 Analytical procedures

3.2.4.1 Characterization of the biomixture

The pH was measured using a mixture of air-dried substrate and deionised water (1:5 w/v). The organic carbon was measured using the Walkley and Black (1934) method, the total nitrogen level was measured using digestion with H_2SO_4 according to the AOAC official method 976.06; the lignin and acid detergent fibre (ADF) content was measured

using the AOAC standard method 973.18 (AOAC, 1990a), and neutral detergent fibre (NDF) content was measured using the AOAC standard method 992.16 (AOAC, 1990b). The cellulose content was calculated indirectly from the percentage of ADF and lignin (% ADF minus % lignin) (Mani et al., 2006). The WHC was determined gravimetrically by saturating the substrate (50 g) with distilled water and allowing it to drain for 1 h.

3.2.4.2 Extraction and pesticide analysis

Residual pesticides were extracted from the biomixtures (5 g dw) by shaking (1 h, 250 rpm) with 20 mL of acetone and ultrasonication (30 min). After centrifugation (10000 rpm), 5 mL of the supernatant was collected, filtered through a PTFE membrane (0.2 µm pore size, Millipore), evaporated with fluxed N_2 to dryness, and dissolved with 1 mL of acetonitrile. They were subsequently analyzed as described below. Recovery of ATZ, CHL and ISP was >85%.

The concentrations of ATZ, CHL, ISP and their metabolites were determined by HPLC using a Merck Hitachi L-2130 pump, a Rheodyne 7725 injector with a 20 µL loop and a Merck Hitachi L-2455 diode array detector. Separation was achieved using a C18 column (Chromolit RP-8e, 5 µm 4.6 x 100 mm). Eluent A was 1 mM ammonium acetate, and eluent B was acetonitrile. The gradient conditions used for the separation of pesticides was as follows: 0–2 min of 95% A, 2–4 min of 95–70% A, 4–7 min of 70% A, 7– 12 min of 70–30% A, 12–16 min of 30% A, 16–17 min of 30–95% A and 17–20 min of 95% A. The flow rate was set as follows: 0– 12 min at 1.0 mL min^{-1}, 12–16 min of a 1.0–2.0 mL min^{-1} increase and 16–20 min at a constant 2.0 mL min^{-1}. The column temperature was maintained at 30 ± 1 °C. The detector was set at three wavelengths for data acquisition 220, 245 and 290 nm. Instrument calibrations and quantifications were performed against pure reference standards (0.1–10 mg L^{-1}) for each compound.

3.2.4.3 Biological activities in the biomixture

For measuring basal respiration 30 g dw of each biomixture (in triplicate) were weighed in a stoppered glass flask (500 mL) and sprayed with a pesticide mixture of formulated ATZ, CHL and ISP at 100 mg a.i. kg^{-1}. A plastic cup with 15 mL of NaOH (0.1 N) was placed inside each glass flask. The flasks were tightly closed and incubated at 20 ± 2 °C. The CO_2 evolved was trapped in 0.1 N KOH solution and determined by titration with a 0.1 N HCl solution after adding a saturated $BaCl_2$ solution. The production of CO_2 was measured over 90 d and expressed as the number of mg of CO_2 accumulated per g of biomixture.

Phenoloxidase activity was assessed using MBTH/DMAB method (adapted from Castillo et al., 1994). Briefly, samples (10 g dw) of the biomixtures were shaken (150 rpm, 2 h) with 25 mL of a 100 mM succinate–lactate buffer (pH 4.5). The samples were centrifuged (4000 rpm, 20 min). The supernatant of each sample was collected, filtered through 0.45 µm membrane and measured immediately. The reaction mixture contained 300 µL of 6.6 mM DMAB, 100 µL of 1.4 mM MBTH, 30 µLof 20 mM MnSO4 and 1560 µL of the filtered sample; the reaction was initiated with the addition of 10 µL of 10mM H_2O_2. The reaction was observed in a Spectronic Genesis 2PC spectrophotometer at 590 nm (ε = 0.053 μM^{-1} cm^{-1}). For the possible presence of lignin peroxidase (LiP) and Laccase (Lac) activity, no correction was made, thus measurement may represent the sum of manganese peroxidase, LiP and Lac and is expressed as phenoloxidase activity (Castillo and Torstensson, 2007).

Fluorescein diacetate hydrolysis was measured periodically during 90 d according to a protocol by Schnürer and Rosswall (1982) with slight modifications. Briefly, 1 g dw biomixture was incubated in a 30 mL conical flask with 9.9 mL of sterile 60 mM sodium phosphate buffer at a pH of 7.8. The reaction was started by adding 0.1 mL of an FDA solution (2.0 mg mL^{-1}). After 1 h of incubation at 25 ± 1 °C, 10 mL of acetone was added to stop the reaction. The A490 was measured in a spectrophotometer after the biomixture was removed using centrifugation and filtration. The concentration of the released

fluorescein was calculated using a calibration curve with standard quantities of FDA, and the results were expressed as µg FDA g^{-1} h^{-1}.

3.2.5 Statistical analysis of data

The experiments were conducted with three independent replicates. Measurements of residual pesticides in the different biomixtures during the degradation period were subjected to an analysis of variance (one-way ANOVA), and the averages were compared using the Duncan multiple range test at $p \leq 0.05$ to identify significant differences between the biomixtures at the same sampling time.

Microbial activity measurements in the different biomixtures during the degradation period were subjected to a one-way ANOVA, and the averages were compared using the Duncan multiple range test at $p \leq 0.05$ to identify significant effects of sampling time in the same biomixture.

3.3 Results and discussion

3.3.1 Degradation studies

Degradation of the three pesticides was described by first-order kinetics and the calculated half-life ($t_{½}$) values, with R^2 in the ranges of 0.95- 0.91, 0.94 - 0.85 and 0.91-0.74 for ATZ, CHL and ISP, respectively are shown in Fig. 3.1. The $t_{½}$ values of the pesticides in the different biomixtures varied markedly. Higher $t_{½}$ values for CHL and ISP (more than 150 d) were evident in biomixtures B and C, where the ST was completely replaced with BH and SW. The $t_{½}$ values for these pesticides were 3 to 6-fold higher compared to biomixture control (biomixture A) and the other biomixtures evaluated. Similarly, a relatively slower degradation rate was observed in biomixture E, where 25% of the ST was replaced with SW. The $t_{½}$ values obtained for CHL and ATZ in biomixtures D,

F and G, where the ST was partially or completely replaced with OH, were comparable to those obtained in the biomixture control (A). The biomixtures containing OH (F and G) were more efficient than the biomixture control even in the degradation of ATZ.

The $t_{½}$ values obtained in this study of different biomixtures demonstrated a low degradation rates for biomixtures where the ST was replaced completely with BH and partially or completely replaced with SW (biomixtures B, C and E, respectively). These results can be attributed to a high C/N ratio (82.6 and 65.9, see Table 3.1), found in both biomixtures containing SW (biomixtures C and E), which does not favour the growth of pesticide-degrading microorganisms or the rapid degradation of pesticides in biomixtures as has been reported by Castillo and Torstensson (2007) and Castillo et al. (2008). Moreover, the use of wood residues in the biomixture could cause an immobilization of the pesticides in the biomixtures due to the high lignin content in SW (20.6% see Table 3.1) compared to others residues evaluated (between 2.4% and 9.9%), producing a retardation of pesticide degradation. Rodríguez-Cruz et al. (2012) have reported that pesticide degradation was negatively affected by the addition of different wood residues to soil, and a high correlation with the lignin content in the wood residues was observed. On the other hand, a low degradation rate in the biomixture where the ST was completely replaced with BH (biomixture B) can be attributed to the low lignin content of both the BH and the biomixture (2.4% and 1.6%, respectively, see Table 3.1). In this sense, Castillo et al. (2008) have reported that organic substrates with no lignin or a low lignin content may not support a robust enough microbial activity for pesticide degradation in a biobed system.

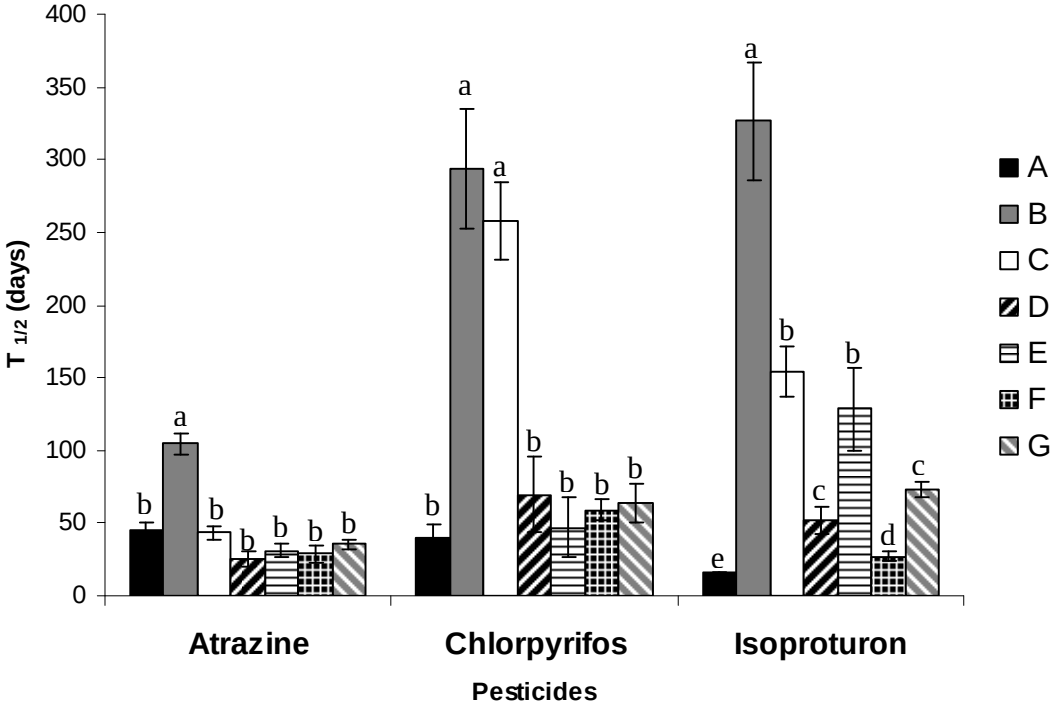

Fig. 3.1 Shown are the half-life ($t_{1/2}$) values after 90 d (b) for atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) at 100 mg i.a kg^{-1} in the biomixtures composed of varying proportions of different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) during the degradation period. The different letters refer to significant differences in mean values between biomixtures with the same pesticide (n = 3) determined using the Duncan Test ($p < 0.05$).

As was noted above, OH based biomixtures (biomixtures F and G) showed high degradation efficiency comparable to control biomixture. This may due to similar characteristics (C/N and lignin content) between the control biomixture (biomixture A) and the biomixtures containing OH that promotes the development of robust microorganisms, allowing for efficient pesticide degradation. The degradation products of ATZ, CHL and ISP were also determined. As shown in Fig. 3.2a and b, small amounts of ATZ degradation

products DIA and DEA were detected in all biomixtures evaluated. The formation of both metabolites was highly variable, but, in general, DIA was detected at all sample points (Fig. 3.2a), and an accumulation of this metabolite was evident in all biomixtures. DEA was also detected in all the biomixtures evaluated. However, at the end of the experiment, no detectable quantities of this metabolite could be observed, likely indicating a faster degradation of this compound. Variability in the formation of these degradation products can be explained by the different characteristics of the organic substrates used in the biomixtures. The addition of organic substrates has been shown to modify the pesticide degradation pathways (Houot et al., 1998; Aguilera et al., 2009).

TCP, the most important degradation product of CHL, was also detected in the biomixtures evaluated. As shown in Fig. 3.2c, a high level of TCP was observed between 10 d and 30 d after pesticide application. However, low concentrations of TCP were detected in the biomixtures after this time period, indicating a possible degradation of this metabolite. As shown in Fig. 3.2c, no detectable TCP was found in the traditional biomixture after 30 d post-application of pesticides. Similar results were reported by Tortella et al. (2012). They found an accumulation of TCP during the first days after the application of CHL in a traditional biomixture (soil, peat and straw). However, TCP degradation was evident after 10 d of incubation. Conversely, the quantity of TCP detected in biomixtures C and D was low (Fig. 3.2c). However, this cannot be attributed to the rapid degradation of this metabolite given the low CHL degradation rate observed for these biomixtures (Fig. 3.1).

MDIPU, a principal degradation product of ISP, was also determined. As shown in Fig. 3.2d, MDIPU was detected in all biomixtures evaluated. However, an accumulation of this metabolite was evident in biomixtures A and B. As with biomixture A in our assays, the accumulation of this metabolite accompanied a high ISP degradation rate in a traditional biomixture of a biobed system as reported by von Wirén-Lehr et al. (2001). Conversely, as shown in Fig. 3.2d, low MDIPU concentrations were detected in biomixtures C, D, E, F and G. This may be because these biomixtures promote the

development of MDIPU-degrading microorganisms. However, more microbiological studies are necessary to clearly explain these differences.

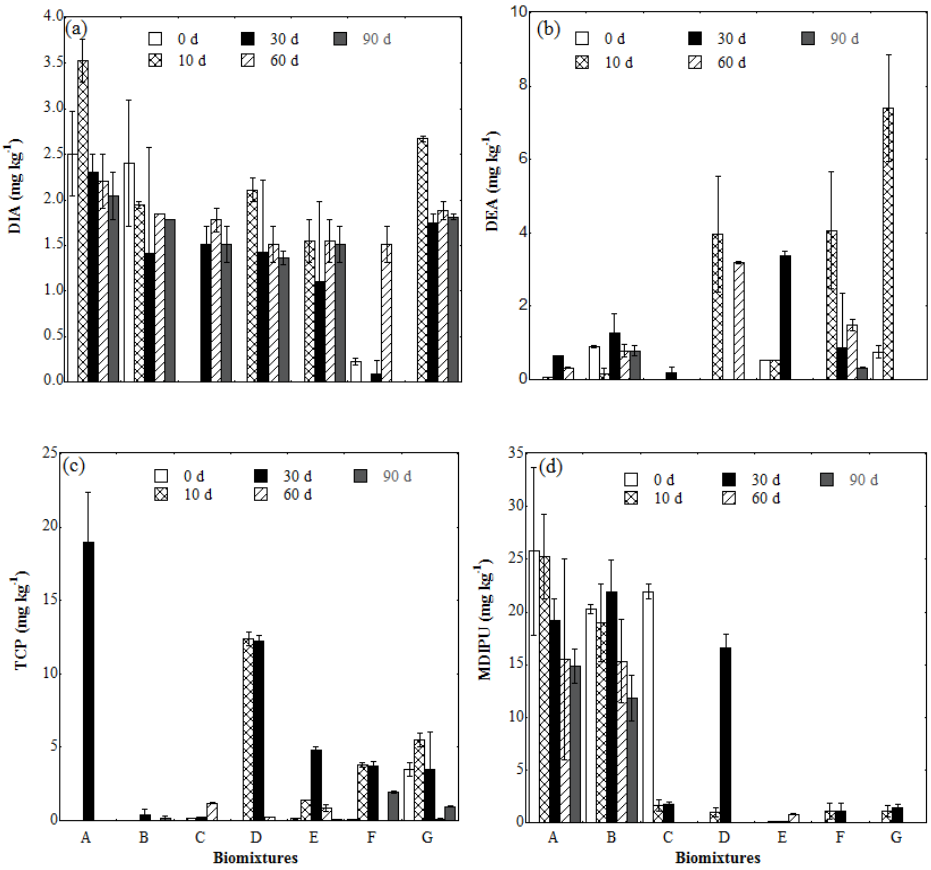

Fig. 3.2 Shown is the formation of metabolites deisopropylatrazine (DIA) (a), deethylatrazine (DEA) (b), 3,5,6-trichloro-2-pyridinol (TCP) (c) and monodesmethyl-isoproturon (MDIPU) (d) in the biomixtures containing varying proportions of different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) during the degradation period. Each value is the mean of three replicates, and the error bars show the standard deviation of the mean.

3.3.2 Determination of biological activities in the biomixtures

A basal respiration rate was used to assess the metabolic activity of each biomixture (Fig. 3.3). A high respiration rate was observed in biomixture G (1.06 mg CO_2 g^{-1} d^{-1}, on day 119), where ST was partially replaced by OH, followed by biomixture A (the traditional biomixture with 0.51 mg CO_2 g^{-1} d^{-1}, on day 119). Both of these biomixtures showed the highest degradation rates for the three pesticides evaluated, as shown in Fig. 3.1. This effect could most likely be attributed to the presence of an easily degradable carbon source for pesticide-degrading microorganisms, which is reflected in the low lignin content of this biomixture compared with the other biomixtures (see Table 3.1). Conversely, low respiration rates were observed for biomixtures C and E, where ST was replaced (totally and partially) by SW and higher lignin content was also determined (Table 3.1). The low respiration rates observed for these biomixtures coincides with the low degradation rates observed in biomixtures C and E. Similar respiration levels were observed in the biomixtures where ST was replaced by biomixtures B and D, where the respiration rates at 119 d of incubation were 0.35 and 0.40 mg CO_2 g^{-1} biomixture d^{-1}, respectively, which were higher than for biomixtures C and E. However, a low degradation rate was observed in these biomixtures. These results demonstrate that a high pesticide degradation rate does not always correlate with a high respiration rate likely because an appropriate microbial activity is required to obtain a high degradation efficiency as has been reported by Castillo et al. (2008).

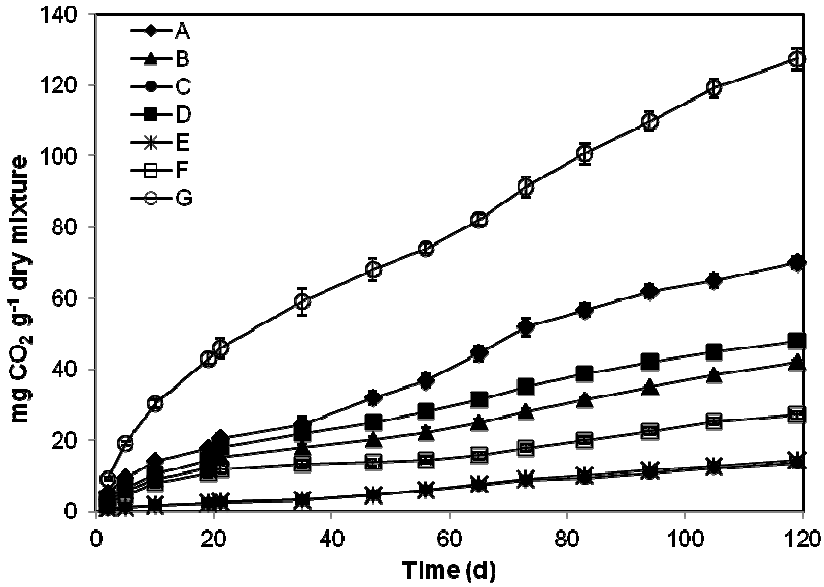

Fig. 3.3 Shown is the cumulative respiration (mg CO_2 g^{-1}) for atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) at 100 mg i.a kg^{-1} in the biomixtures composed of varying proportions of different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) throughout the degradation period. Each value is the mean of three replicates, and the error bars show the standard deviation of the mean.

The phenoloxidase activity was high in all the biomixtures evaluated except for B biomixture, where ST was completely replaced with BH (Fig. 3.4a). This low activity could be also related to the low pesticide degradation in this biomixture and may be caused by the development of other microorganisms not related to lignin degradation due to the low lignin content of BH. In this sense, as mentioned above, not low values in basal respiration levels were founded in B biomixture. An explanation for this observation is that the readily available carbon sources contained in BH can contribute to respiration without contributing to the phenoloxidase activity as has been reported by Castillo and Torstensson (2007). For this reason, the phenoloxidase activity could be higher in biomixture D than biomixture B. In biomixture D, only a fraction of the ST was replaced with BH (25%). ST contains more lignin than BH; therefore, the degradation of this substrate is linked to phenoloxidase

activity (Torstensson and Castillo, 1997), which would explain the higher phenoloxidase activity in biomixture D more than biomixture B.

As shown in Fig. 3.4b, high FDA hydrolysis values were obtained for all the biomixtures evaluated, indicating that all biomixtures were biologically active during the incubation time, and not significant negative effects were caused by the application of the pesticides. High FDA values for were obtained for B biomixture, where the ST was replaced with BH, demonstrating that high microbial activity is supported by this biomixture. However, as mentioned above, a high biological activity is not always associated with pesticide degradation because the microorganisms in the biomixture may not have the capacity to degrade pesticides.

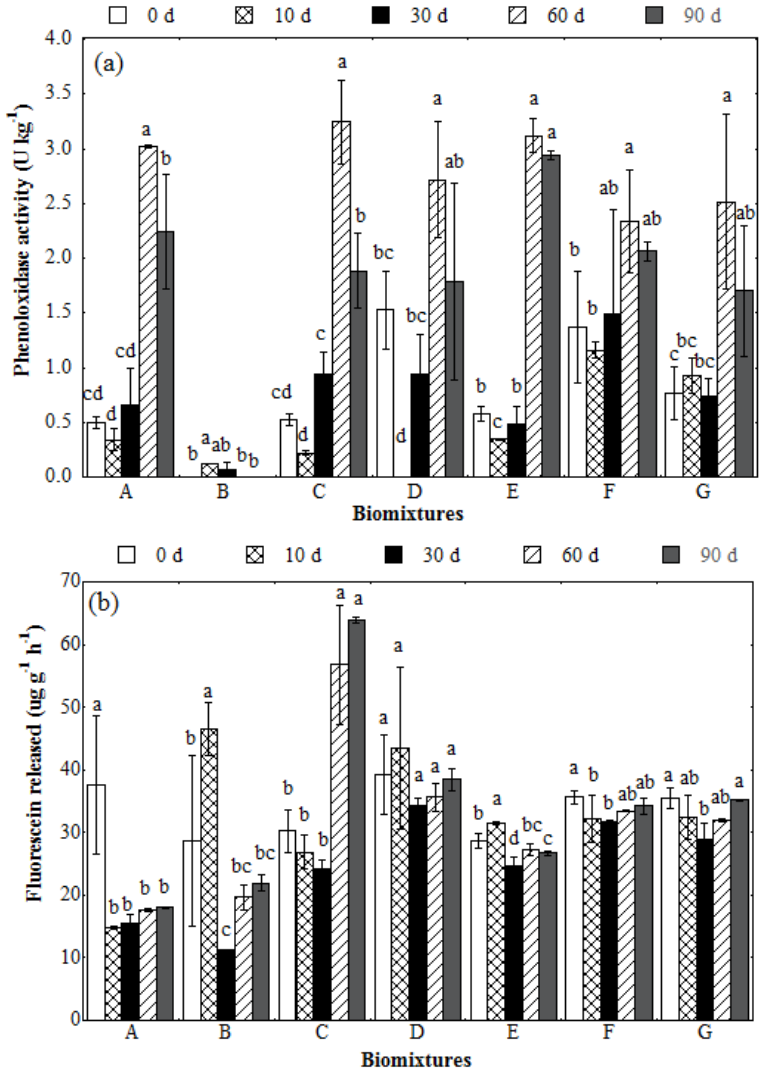

Fig. 3.4 Shown are the phenoloxidase activity (a) and hydrolytic activity (FDA) (b) in the biomixtures composed of varying proportions of different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) during the degradation period. Different letters refer to significant differences in the mean values (n = 3), assessed using Duncan Test ($p < 0.05$), among the sampling times of the same biomixture.

3.4 Conclusions

According to results obtained, it is possible to conclude that the straw can be partially or completely replaced with other readily available lignocellulosic substrates. Overall, the biomixtures containing OH (biomixtures F and G) exhibited high degradation capacities that were comparable to the traditional biomixture (biomixture A). The half-life values obtained for the biomixtures containing BH (biomixtures B and D) demonstrated that this substrate can be used only as a partial substitute for the ST; completely replacing the ST with BH promoted microbial activity but not pesticide degradation. However, the use of SW as a lignocellulosic substrate in the biomixture is only recommended as a partial substitute for the ST (25%) because the high lignin content in this substrate can cause the retardation of pesticide degradation.

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Chapter 4

Pesticide adsorption on modified matrix of a biopurification system with alternatives lignocellulosic wastes

Pesticides adsorption on modified matrix of a biopurification system with alternative lignocellulosic wastes

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4.1 Introduction

The inadequate pesticide handling in agriculture may increase the risk of environmental contamination due to the dispersion into non-target sites. Pesticide contamination can occur through non-point source or point source contamination. Pesticide point source contamination has been identified as a significant contributor to the deterioration of the quality of natural water resources, which represents about 40 to 90% of the total pesticide load. This contamination on field can occur through the inadequate pesticide handling such as accidental spillages during tank filling or cleaning the spraying equipment (Carter, 2000; Neumann et al., 2002; Castillo et al., 2008). In fact, some studies have demonstrated the presence of pesticide residues such as atrazine (Bhagobaty et al., 2007), chlorpyrifos (Kolpin et al., 2000) and isoproturon (Stangroom et al., 1998) in water resources.

On-farm biopurification systems, commonly known as “biobeds”, are a biotechnological tool used to reduce pesticides point source contamination (Torstensson and Castillo, 1997). The principal component is biomixture (Castillo et al., 2008), which is composed traditionally of straw, topsoil and peat in a volumetric proportion of 2:1:1. However, some lignocellulosic substrates of the biomixture have been replaced for adaptation purposes in some countries (Karanasios et al., 2010b; Kravvariti et al., 2010;

Urrutia et al., 2013b; Diez et al., 2013b). An efficient biomixture must have the capacity both to adsorb and degrade contaminants (Castillo et al., 2008).

The adsorption capacity in a biopurification system should be extended enough to limit the risk for rapid pesticide leaching and protect microorganism of discharges pesticide in concentration extremes. Therefore, effect of organic biomixture and chemical nature of pesticide influences the balance between adsorption and biodegradation in the biomixture. However, increasing adsorption could reduce pesticide bioavailability and limit the contribution of biodegradation of pesticides (Karanasios et al., 2012). In this sense, organic amendments added to soil often decreased atrazine mineralization due to increased sorption of the herbicide (Briceño et al., 2007). Besides, the biobed material showed enhanced ability to absorb isoproturon compared with soil (Henriksen et al., 2003). In another study, Kravariti et al. (2010) reported that biomixtures composed of composted cotton crop residues, soil and straw in various proportions increased degradation of terbuthylazine (less hydrophobic pesticide), but they increased adsorption of chlorpyrifos (more hydrophobic pesticide) compared with soil. Similarly, Karanasios et al. (2010a) demonstrated that biomixtures composed of composted residues or peat, showed higher adsorption affinity of tested pesticide, compared with soil. Besides, biomixture with olive leaf compost was the most efficient in pesticide degradation and, at the same time, showed the highest affinity for adsorption of some tested pesticides. They suggested that exogenous organic carbon might have a positive influence on both degradation and adsorption processes.

Moreover, the adsorption of linuron, isoproturon, metalaxyl, isoxaben, bentazon and lenacil was investigated on substrates commonly used in a biopurification system as lignocellulosic material, e.g. straw, willow chopping, coconut chips, garden waste compost, and others. The best fit was obtained with the Freundlich model, where it was showed that more immobile pesticides (i.e. linuron and isoxaben) tended to associate with the organic substrate, whereas more mobile pesticides partition in the water (i.e. bentazon) should be taken with care as these will easily leach through the system (De Wilde et al., 2009).

Therefore, the aim of this study was to investigate adsorption of atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) with varying physico-chemical characteristics on biomixtures composed of barley husk (BH), sawdust (SW) and oat husk (OH) as a replacement for partial or total straw (ST), topsoil (S) and peat (P) in various proportions.

4.2. Materials and methods

4.2.1 Pesticides

Commercial formulations of ATZ (atranex 500SC), ISP (Fuego 50SC) and CHL (chlorpyrifos S480) were purchased from Agan Chemicals Manufacturers Ltd. The composition of the pesticide formulations is unknown due to patent protection, but this is most likely as these formulations are both water dispersable or soluble granulates, they will contain surfactants. De Wilde et al. (2009) reported that no significant differences in K_f values between the commercial formulations and analytical standards of liuron and isoproturon pesticides in different substrates of biomixtures. In addition, Beigel et al. (1998) observed that the low concentrations employed of surfactant in most pesticides formulations not increase pesticide partitioning to soil phase.

The characteristics of pesticides used in this study are showed in Table 4.1. ATZ and ISP in form of suspension concentrate were prepared in distilled water; chlorpyrifos in form of emulsifiable concentrate was prepared in distilled water and acetone as cosolvent in a proportion of 4:1 to form a dissolved solution. The concentration of pesticides was determined by High Performance Liquid Chromatography (HPLC).

Table 4.1 Characteristics of pesticides used in this study

Active ingredient	Chemical class	Target	Log K_{ow}*	Water solubility*	pKa*
Atrazine	Triazine	Herbicide	2.5	33 mg L^{-1} (20°C)	1.7
Chlorpyrifos	Organophosphate	Insecticide and acaricide	4.7	1.4 mg L^{-1} (25°C)	nd**
Isoproturon	Phenylurea	Herbicide	2.5	65 mg L^{-1} (22°C)	nd

*(Tomlin, 2003)
**nd: no dissociation

4.2.2 Biomixtures

For the preparation of the biomixtures, an Andisol topsoil (0–20 cm depth) belonging to Temuco series (38°42´S, 73°35´W), BH, SW, OH and ST as lignocellulosic material and commercial peat were used. Lignocellulosic wastes such as barley husk (BH), and oat husk (OH) as a replacement for partial or total straw (ST) were collected from crop residues; and sawdust (SW) was collected from sawmill waste. All lignocellulosic wastes were cut in small pieces (2–3 cm) using a food processor and soil was sieved (to 3 mm). The constituents were mixed vigorously to obtain a homogeneous biomixture and prepared in volumetric proportions as been described in Table 4.2. The biomixture moisture content was adjusted to 60% of the water holding capacity (WHC) by adding distilled water. All biomixtures were placed in polypropylene bags for maturation processes and stored in dark at 20 ± 2 °C for 40 d before being used in the experiments. For isotherms assay biomixture samples were air dried, ground in a food processor to obtain a homogenous powder and then sterilized by chloroform method. The composition and characteristics of the biomixtures and their components were measured as shown in Table 3.1 Chapter 3.

Table 4.2 Composition of biomixtures composed of straw (ST), barley husk (BH), sawdust (SW), oat husk (OH), topsoil (S) and peat (P) in different volumetric proportions.

Biomixture	ST	BH	SD	OH	S	P
A	2	0	0	0	1	1
B	0	2	0	0	1	1
C	0	0	2	0	1	1
D	1	1	0	0	1	1
E	1	0	1	0	1	1
F	0	0	0	2	1	1
G	1	0	0	1	1	1

4.2.3 Adsorption study

The assay was performed in triplicate in 30 mL glass tubes containing 0.2 g sample of each biomixture and, 10 mL of ATZ, CHL and ISP (1.0, 2.5, 5.0, 7.5 and 10.0 mg L^{-1}), were added separately. The KCL 0.1 M was used as background electrolyte solution. The assay was performed at the natural pH value of the tested biomixtures, among 4.88 and 5.88 (Table 3.1). Samples were shaken overnight in an orbital shaker (200rpm) at room temperature (20 ± 2°C). Subsequently, samples were centrifuged at 10,000 rpm (Eppendorf 5804 R centrifuge) for 10 min and then passed through a membrane filter PTFE of 0.22 µm pore size. The ATZ, CHL and ISP concentration in the supernatant was determined by HPLC. A preliminary adsorption kinetic study showed that apparent equilibrium between the amount of pesticide adsorbed and the amount of pesticide in solution was reached within 6 h. However, a period of 8 h was used in order to ensure the equilibrium state in this study.

4.2.3.1 Calculation of adsorption parameters

To evaluate the sorption capacity of the different biomixtures, the experimental data were fitted to the empirical Freundlich model isotherm (Eq.[1]):

$$C_s = K_f * C_e^{1/n} \quad [1]$$

Where C_s is the mass of ATZ, CHL or ISP adsorbed per mass of substrate (mg kg^{-1}); C_e is the concentration of ATZ, CHL or ISP remaining in the solution (mg L^{-1}) after equilibration; K_f is the Freundlich distribution coefficient (L kg^{-1}); *1/n* is an exponential empirical parameter that accounts for nonlinearity in sorption behavior (Sposito, 1984). If *1/n*, the adsorption isotherm is linear, i.e. $K_f = K_d$. The K_d value was normalized to organic carbon (OC) content of each sample to calculate K_{oc} (Eq. [2]):

$$K_{oc} = K_d/f_{oc} \quad [2]$$

Where f_{oc} is the biomixture organic carbon fraction

4.3 Results and discussion

The adsorption isotherms showed different curvature for ATZ, CHL and ISP (Fig. 4.1, 4.2 and 4.3). All adsorption isotherms were described adequately by the Freundlich equation, with $R^2 \geq 0.81$, ≥ 0.85 and ≥ 0.98 for ATZ, CHL and ISP, respectively (Table 4.3). Adsorption parameters for the linear partitioning model, i.e. K_d, and the organic carbon normalized partition coefficient values calculated from Eq. [2], K_{oc}, are presented in Table 4.4. The high R^2 values ($R^2 \geq 0.87$, ≥ 0.85 and ≥ 0.97 for ATZ, CHL and ISP, respectively) suggest that the linear model fits to the adsorption isotherms quite well, in many cases better than Freundlich model.

Based on the 1/n value (Table 4.3), isotherms can be classified as an L, S or C type according to Giles et al. (1960). In general, it was observed that isotherms were of the L-type (1/n< 1), showing a convex curvature, however for some pesticide-biomixture combinations S-type (1/n>1) isotherms were observed (e.g. CHL in contact with barley husk in B- biomixture and sawdust in C-biomixture and ISP on the straw in A-biomixture, barley husk in B and D-biomixture, sawdust in C- biomixture and oat husk on F and G-biomixture). This indicates that the mutual interaction between CHL and ISP molecules is higher than the sorbate-sorbent interaction at high concentrations. Similarly, an S-type isotherm has also been reported for isoproturon on different biomixture substrates (De

Wilde et al., 2009). The 1/n values were less than 1 for ATZ in all biomixtures, CHL in A, D, E, F and G-biomixture and ISP in E-biomixture. This indicates a high affinity between the pesticide and the biomixture.

Adsorption was influenced by both physicochemical properties of the pesticides and the type of biomixtures. The Freundlich parameter K_f displayed the highest adsorption capacity by CHL, with K_f values about 918-24575 mg $^{(1-(1/n)}$ $l^{(1/n)}$ kg^{-1}, compared with ATZ and ISP, where K_f values were about 17.75-47.54 and 3.15-58.22 $mg^{(1-(1/n)}$ $l^{(1/n)}$ kg^{-1}, respectively. These results can be explained by the high organic carbon content of the biomixtures (≥28.1 %), and the pesticide characteristics. Thus, the strongest and most extensive adsorption of CHL is explained by its higher hydrophobic character (log K_{ow} 5.0) compared with more water-soluble ATZ (log K_{ow} 2.5) and ISP (log K_{ow} 2.5), which are reflected in the normalized distribution coefficient for organic carbon (K_{oc}) showed in Table 4.4, making this compound less mobile than more soluble pesticides. According to the general behavior of the adsorption capacity, the order of adsorption (CHL>ATZ>ISP) was almost inversely proportional to pesticide water solubility (Table 4.1). Comparable results were observed by Rojas et al. (2014), who found that the adsorption capacity of atrazine, alachlor, endosulfan sulfate and trifluralin, increased with the hydrophobic character of the pesticides and decreased with their water solubility. However, high variation in K_{oc} values of a pesticide in the different biomixtures (Table 4.4), suggested that other factors influencing pesticide adsorption include the nature of organic matter, the specific surface area and the particle size of the different components of the biomixture (De Wilde et al., 2009). In addition, it is also important to consider other pesticide properties in the adsorption capacity of biomixtures. For example, atrazine is a weak base (pKa = 1.7); therefore low pH in tested biomixtures could contribute to in adsorption capacity through ionic bonds and/or by physical adsorption (Briceño et al., 2007).

Adsorption capacity of biomixtures was described by linear regression for comparison purposes. Biomixtures presented different adsorption capacity according with their chemical characteristics: for ATZ (E > A > C > G > D > F > B), CHL (C > B > A > F > E > D > G) and ISP (E > A > F > G > C > D > B). Our results showed that highest

adsorption capacity for all the studied pesticides (based in K_d values) was in sawdust-based biomixtures (C and E) that contains 17.3 and 13.8% of lignin content, respectively. In this sense, Rodrígues-Cruz et al. (2012) demonstrated that a high adsorption of pesticides by soil amended with pine occurs due to the lignin content of wood.

In addition, we observed that K_d values (1068-7230) were highest for CHL in all organic biomixtures compared with ATZ (12.4-31.0) and ISP (14.8-26.2). Similarly, a high K_d value has also been reported of 2753 for CHL in traditional biomixture (24.1% organic carbon), being lowest (K_d = 602) in soil with 8.5 % of organic carbon (Fernández-Alberti et al., 2012). In another work, Kravariti et al., 2010 observed that the adsorption of CHL was higher with K_d values of 746 in compost biomixture compared with soil with Kd = 17, where the organic carbon content was of 6.7% and 0.9%, respectively.

Therefore, certain physicochemical characteristics of the organic biomixture could be used as indicators of the eligibility to be used in a biobed system. In fact, biomixture composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1 showed an intermediate adsorption capacity for tested pesticides, which can promote a positive relationship between degradation and adsorption capacity to increase pesticide removal irreversibly.

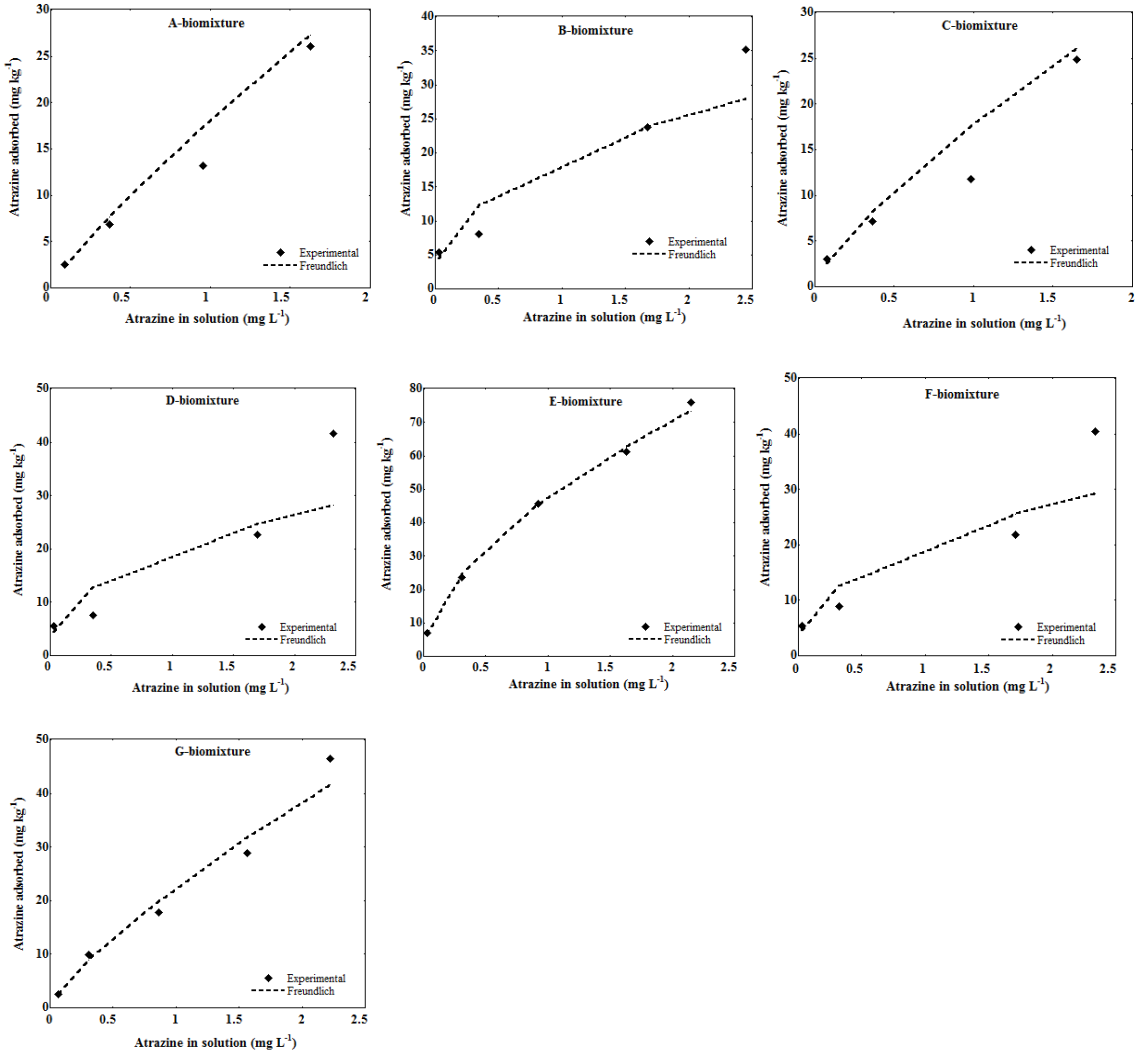

Fig. 4.1 Adsorption isotherm of atrazine in different biomixtures, adjusted to the Freundlich model.

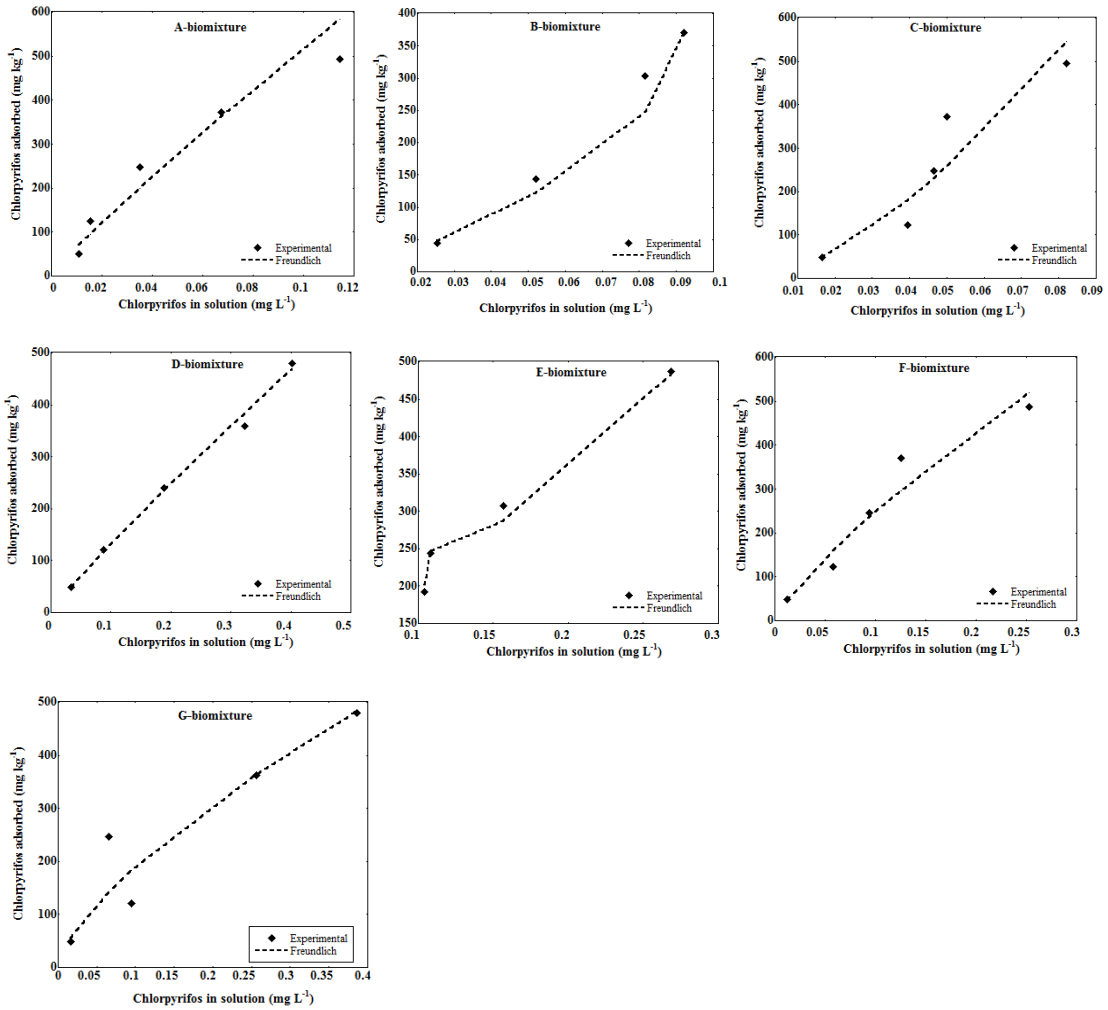

Fig. 4.2 Adsorption isotherm of chlorpyrifos in different biomixtures, adjusted to the Freundlich model.

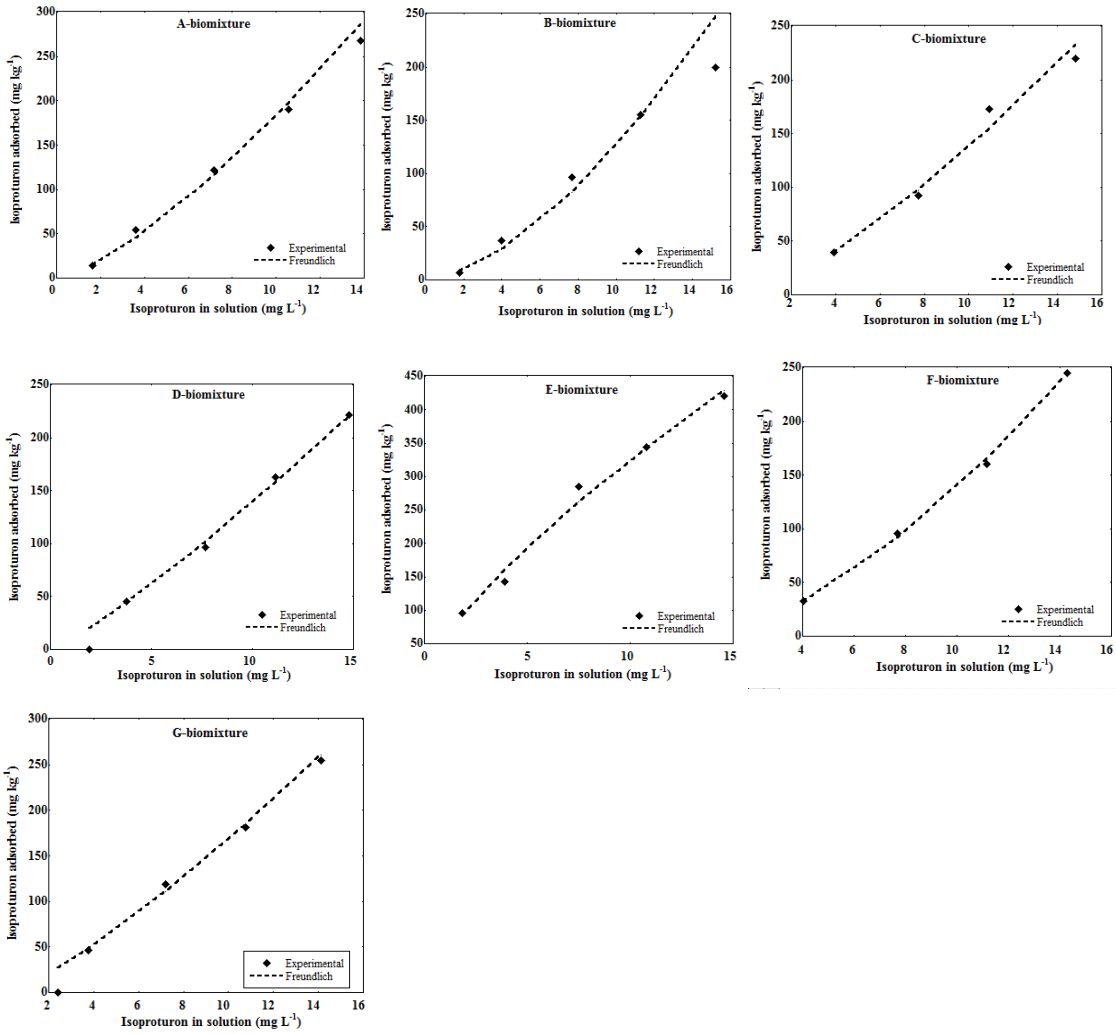

Fig. 4.3 Adsorption isotherm of isoproturon in different biomixtures, adjusted to the Freundlich model.

Table 4.3. Freundlich adsorption parameters obtained from ATZ, CHL and ISP adsorption isotherms in all biomixtures

Biomixture	ATZ			CHL			ISP		
	K_f *	1/n	R^2	K_f *	1/n	R^2	K_f *	1/n	R^2
A	18.02	0.86	0.96	4178	0.91	0.92	7.82	1.37	0.99
B	19.16	0.42	0.89	19116	1.65	0.96	3.15	1.60	0.98
C	17.75	0.77	0.92	24575	1.52	0.91	6.26	1.34	0.99
D	19.79	0.42	0.81	1069	0.90	0.99	9.31	1.17	0.99
E	47.54	0.56	0.99	918	0.63	0.85	58.22	0.74	0.98
F	20.29	0.42	0.88	1572	0.80	0.96	3.62	1.58	0.99
G	22.32	0.78	0.99	933	0.69	0.85	8.93	1.27	0.99

*($mg^{(1-(1/n)}\ l^{(1/n)}\ kg^{-1}$)

Table 4.4. Linear adsorption parameters obtained from ATZ, CHL and ISP adsorption isotherms in all biomixtures

Biomixture	ATZ			CHL			ISP		
	K_d*	R^2	K_{oc}	K_d *	R^2	K_{oc}	K_d *	R^2	K_{oc}
A	20.1	0.94	82	4073	0.94	16694	20.6	0.99	84
B	12.4	0.91	103	5681	0.85	47148	14.8	0.99	122
C	19.8	0.90	143	7230	0.88	52356	17.3	0.98	124
D	15.0	0.90	129	1124	0.99	9641	16.8	0.99	144
E	31.0	0.97	260	1778	0.89	14954	26.2	0.97	220
F	14.0	0.87	116	1878	0.91	15534	20.5	0.98	169
G	19.1	0.98	157	1068	0.87	8826	19.8	0.99	163

* $l\ kg^{-1}$

4.4. Conclusion

The adsorption capacity was related with the physic-chemical characteristics of the tested biomixtures and pesticides. Biomixture composed of sawdust showed the highest adsorption capacity, indicating that the high lignin content of this substrate increased adsorption of the pesticides. However, this behavior can reduce the availability of pesticides for chemical hydrolysis and microbial degradation. Moreover, biomixture composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1 showed an intermediate adsorption capacity for tested pesticides, which can limit the risk of leaching of this system without affecting degradation of pesticide irreversibly.

Moreover, the chemical nature of the pesticides also affects the adsorption behavior. Thus, the order of adsorption capacity was chlorpyrifos>atrazine>isoproturon, where adsorption capacity decreased when the water solubility of each pesticide increased.

Chapter 5

Influence of the rhizosphere of the grass layer in a biopurification system on the dissipation of a pesticide mixture

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Influence of the rhizosphere of the grass layer in a biopurification system on the dissipation of a pesticide mixture

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Abstract

In a biopurification system as the biobeds, the rhizosphere of grass layer may be a significant factor to promote the pesticides dissipation in the biomixture. Therefore, a greenhouse assay was performed to evaluate the effects of the rhizosphere of *Lolium perenne*, *Festuca arundinacea* and *Trifolium repens* on the dissipation of a pesticide mixture composed by atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) in an organic biomixture (oat husk:topsoil:peat) of a biobed system. The assay were performed in glass pot which were divided into two separate compartments; root surface (RC) and root-free (RFC), filled with biomixture and contaminated with pesticide mixture at 5 mg kg^{-1}. Pots glass without plants and pesticide mixture were established for control purposes. Moreover, biological activities as phenoloxidases β-glucosidase, fungal and bacterial communities by qPCR, and organic acid of root exudates were also evaluated. The results demonstrated that higher dissipation for all pesticides evaluated occurred in planted pots compared control, after 30 d of incubation. In general, phenoloxidase and β-glucosidase activities were higher $p < 0.05$ in planted biomixtures with and without pesticides compared to control. Inverse correlation between phenoloxidase activity and residual pesticide (0.684 to 0.952) was founded during all time of assay. Fungal and bacterial gene copy numbers were increased in planted biomixtures compared with the unplanted biomixtures. Indeed, fungal biomass was positively correlated with the phenoloxidase activity on day 1 ($r = 0.825$) and day 30 ($r = 0.855$). In addition, the exudation of oxalic and malic acid in contaminated treatments were higher than control, which could be involved in oxidation of pesticide mixture. Therefore, our results indicate that the microbial rhizosphere of these plant species were important for dissipation of ATZ, CHL and ISP in the biomixtures of biobed system.

Keywords: Rhizosphere, pesticide mixture, biopurification, phenoloxidase

5.1 Introduction

The rhizosphere can be described as the soil adjacent to plant roots and, therefore influenced by its activity, impacting physicochemical conditions and biological activity in the surrounding rhizosphere compartment (Hinsinger et al., 2009; Neumann et al., 2009). In this sense, as the rhizosphere continuously provides nutrient source by their root exudates, the microbiological activity in this zone is up to four fold greater compared with soil away from plant, resulting in a zone with higher metabolic capacities for the microbiological attack of both organic matter and contaminants (El Shatnawi and Makhadmeh, 2001; Alkorta and Garbisu, 2001; Pilon-Smits, 2005; Haichar et al., 2008).

A reported mechanism used by rhizospheric microorganisms to take up contaminants is the production of biosurfactant, facilitating the contaminant degradation, mainly of hydrophobic compounds, and concomitantly increasing their availability to plants (Nielsen et al., 2002; Read et al., 2003; Rajaei et al., 2013). In this context, has been reported that plant species and soil cooperatively are responsible of the structure and function of microbial diversity in the rhizosphere (Berg and Smalla, 2009). Therefore, plants and their associated rhizosphere microorganisms are an important contribution in degradation processes of contaminant in soil (Wang and Oyaizu, 2009).

Degradation of contaminants by the rhizosphere and their associated microorganisms nor only can be an important contribution in soils. In this sense, their use in on-farm biopurification system as “biobed” can be a huge advantage. Biobeds is a biopurification system used to minimize point source contamination by pesticides. Their main component is its organic biomixture, composed by straw, soil and peat (2:1:1 vv^{-1}), where pesticides are degraded (Castillo et al., 2008). In general, in the top of the biobed is installed a grass layer, that is used to keep moisture in the biomixture and to reveals pesticide spillages. Moreover, the rhizosphere of this grass layer and its associated microorganisms could be an important component to improve the degradation of pesticides in this biopurification system, which has not been deeply considered in studies of biobeds until now. Therefore, is important to identify the plant species capable of surviving a particular toxic contamination level in the biobed system.

Some studies have reported that plant species such as *Lolium perenne*, *Festuca arundinacea* and *Agrostis tenuis*, with fibrous rooting systems, have shown a high potential to remove contaminants by providing a high root surface area that interacts with soil microorganisms for biodegradation. In this sense, *Lolium multiflorum* has demonstrated increased removal of atrazine, chlorpyrifos and pentachlorophenol (Banks et al., 1999; He et al., 2005; Merini et al., 2009; Korade and Fulekar, 2009; Urrutia et al., 2013a). In addition, Singh et al. (2004) demonstrated that the rhizosphere of *Pennisetum clandestinum* tolerated and improved atrazine and simazine degradation, compared with unplanted soil. In another work, Wang and Oyaizu (2009) evaluated the phytoremediation potential of four plants species for dibenzofuran-contaminated soil. The authors found that *Trifolium repens L.* not only had the highest root biomass but also the highest dibenzofuran-degrading bacterial numbers compared with those of the other three grass species.

Therefore, the aim of the present work was to evaluate the effects of the rhizosphere of the grass layer composed by a mixture of *Lolium perenne*, *Festuca arundinacea* and *Trifolium repens* on the dissipation of a pesticide mixture in an organic biomixture of biobed system.

5.2 Materials and Methods

5.2.1 Preparation of the biomixture

The biomixture was prepared according to Tortella et al. (2013a) with some modifications. This was prepared mixing Andisol topsoil, commercial peat (43.7% organic carbon) and oat husk (OH) (36.7% organic carbon) as lignocellulosic material (Urrutia et al., 2013b) in the volumetric proportion of 1:1:2 by volume. The soil (30.7% sand, 41.8% silt, 27.4% clay, 6.4% organic carbon, pH 5.2) belonging to the Temuco series, (38°42’S, 73°35’W) was collected (0-20 cm depth) and sieved (3 mm) and OH was collected from crop residues. The constituents were mixed vigorously to obtain a homogeneous biomixture. The biomixture moisture content was adjusted to 60% of the water holding capacity (WHC) by adding distilled water. The biomixture was placed in polypropylene

bags for maturation processes and stored in the dark at 20 ± 2 °C for 40 days before being used in the experiments.

4.2.2 Experimental design

Pots were constructed from a glass box of 18 x 17 x 9 (length x width x height in cm). The pots were divided into two sections: a root compartment (RC) (6 cm in width) and root-free compartment (RFC) (3 cm in width) separated by nylon mesh (25 µm). The design successfully prevented root hairs from entering the adjacent zones as well as keeping the zones separated, allowing the transfer of microorganisms and root exudates between the compartments. One hundred forty grams of biomixture were used to fill the pots. Each treatment had three replicates. The RC contained one hundred seeds of *Lolium perenne*, *Festuca arundinacea* and *Trifolium repens* at a proportion of 50, 45 and 5%, respectively. The seeds were sown in the biomixture with a layer of soil over it (2 g), and the biomixture was maintained at 60% water holding capacity under greenhouse conditions (22±3/18±3°C day/night temperatures; 16/8 h light/dark photoperiod; 60-70% relative humidity) for 30 days. Pots were contaminated with an aqueous solution mixture of ATZ, ISP and CHL to reach a 5 mg kg^{-1} concentration of each pesticide (root compartment (RC+P) and root- free compartment (RFC+P)). The non-contaminated pots (root compartment (RC-P) and root- free compartment (RFC-P)) and without plant (contaminated (C+P) and non-contaminated (C-P)) pots were used as controls.

Pots were arranged in a randomised design. Post-application of pesticides on days 1, 15 and 30, the pesticide concentration, enzymatic activities (β-glucosidase and phenoloxidase), and total abundance of bacteria and fungi were measured in the biomixture of different compartments of the pots. In addition, root exudates of grass layer (oxalic acid, malic acid, citric acid and succinic acid) were measured.

5.2.3 Analytic Procedures

5.2.3.1 Extraction and pesticide analysis

Residual pesticides were extracted from biomixtures (5 g dry weight (dw)) by shaking (1 h, 250 rpm) with 20 mL of acetone and ultrasonication (30 min). After centrifugation (10,000 rpm), 5 mL of the supernatant was collected, filtered with a polytetrafluoroethylene (PTFE) membrane (0.2 µm pore size; Millipore), evaporated with fluxed N_2 to dryness, and dissolved in 1 mL of acetonitrile:water. Then, pesticides concentration was determined by high-performance liquid chromatography (HPLC).

Concentration of ATZ, CHL, ISP was determined by HPLC (Agilent Series 1100, Böblingen, Germany) equipped with a diode array detector. A 150 mm x 2.1 mm i.d. Zorbax Rx-C8 analytical column and a 12.5 mm x 2.1 mm i.d. Eclipse XDB-C8 guard cartridge, both packed with diisopropyl n-octyl (5 mm), were also used. Eluent A (70%) was Mili-Q water adjusted to pH 3, and eluent B was acetonitrile. The gradient condition used for the separation of pesticides was as follows: 3 min 30% B, 4–5 min 35% B, 6–8 min 40% B, 9–13 min 60% B, 14–22 min 70% B, and 23-32 min 30% B. The flow rate was constant at 0.2 mL min^{-1}. The column temperature was maintained at 30±1ºC. The detector was set at three wavelengths for data acquisition: 220, 245 and 288 nm. Instrument calibrations and quantifications were performed against pure reference standards (0.5–5 mg L^{-1}) for each compound. Recovery of ATZ, CHL and ISP was > 90%.

5.2.3.2 Enzyme activity analysis

Phenoloxidase activity was assessed using the MBTH/DMAB method (adapted from Castillo et al., 1994). Briefly, samples (10 g dw) of the biomixture were shaken (150 rpm, 2 h) with 25 mL of a 100 mM succinate-lactate buffer (pH 4.5). The samples were centrifuged (4000 rpm, 20 min). The supernatant of each sample was collected, filtered through a 0.45-µm membrane and measured immediately. The reaction mixture contained 300 µL of 6.6 mM DMAB, 100 µL of 1.4 mM MBTH, 30 µLof 20 mM MnSO4 and 1560

µL of the filtered sample. The reaction was observed in a Spectronic Genesis 2PC spectrophotometer at 590 nm (ε = 0.053 μM^{-1} cm^{-1}). No correction was made for the possible presence of lignin peroxidase (LiP) and Laccase (Lac) activity. Thus measurement may represent the sum of manganese peroxidase, LiP and Lac activity but is expressed as phenoloxidase activity (Castillo and Torstensson, 2007).

β-glucosidase activity was analysed by determining the amount of p-nitrophenol (PNP) produced from 4-nitrophenyl-β-D glucanopyranoside (PNG) as described by (Tabatabai, 1982). Enzyme activity was determined in triplicate using 0.2 g of biomixture sample.

5.2.3.3 Extraction of DNA from biomixture

Total DNA was extracted from 250 mg dw of each of the three samples taken from the biomixture of pots treatments. Each sample was mixed with a solution of 100 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, and 2% (w/v) sodium dodecyl sulphate. Glass beads of different diameters were added in a bead-beater tube and the soil solution was shaken for 30 s at 1600 rpm in a mini bead-beater cell disruptor (Mikro-Dismembrator; S.B. Braun Biotech International) before centrifugation at 12,235 rpm for 1 min. For protein precipitation, supernatants were incubated on ice for 10 min with a 1/10 volume of 3 M sodium acetate and centrifuged (12,235 rpm, 5 min, 4°C). In the last step, nucleic acids were precipitated from the collected supernatants by adding 1 volume of ice-cold isopropanol. For DNA purification, polyvinyl-polypyrrolydone (PVPP, Sigma-Aldrich, USA) columns were used and were prepared by adding 92–95 mg (i.e., 1.2 cm) of PVPP powder to Micro-Spin Chromatography columns (Bio-Rad, USA). First, the tubes were centrifuged (2 min, 3,270 rpm) with 400 µL of H_2O. This procedure was repeated twice. Then, the DNA extracts were purified using the PVPP columns (3,270 rpm, 4 min, 10°C) (Petric et al., 2011).

5.2.3.4 Real-Time PCR Quantification (Q-PCR) of bacteria and fungi

The total bacteria and fungi abundance in the biobed were quantified using the small ribosomal subunit (16S and 18Sr RNA genes). For this purpose, the primers sets 341F 5'-CCTACGGGAGGCAGCAG-3' and 518R 5'-ATTACCGCGGCTGCTGG-3' and FR1 5'-A(I)CCATTCAATCGGTAXT-3' and FF390 5'-CGATAACGAACGAGACCT-3' were selected for bacteria and fungi, respectively. Assembly of 16S rRNA and 18Sr RNA genes in pGEMT easy vector (Promega) plasmid using *Pseudomonas putida* KT2440 and *Fusarium oxysporum* JX910900, standard curves, and bacterial and fungal Q-PCR conditions were performed according to Castillo et al. (2013). Reactions were conducted in an iCycler MyiQ™ System (Bio-rad, USA) using SybrGreen® as the detection system. The reaction was performed in a final volume of 15 µL containing 7.5 µL of iQ™ SYBR® Green Supermix, 0.5 µM of each primer, 0.5 mg mL^{-1} of bovine serum albumin (BSA) and 2 ng of template DNA. The function values that describe the relationship between Ct (threshold cycle) and the number of sequences of each bacterial and fungal are -3.28 and -3.10, respectively, with a PCR efficiency of 101% and 109%. Controls without templates gave null or negligible values.

5.2.3.5 Extraction and root exudates analysis

Organic acids were collected from grass layer of each sampling days, the roots were washed with distilled water and then placed in 50 mL of distilled water for 30 min, 5 mL of the supernatant was collected, filtered through a PTFE membrane (0.2 µm pore size, Millipore). Then, organic acid concentration was determined by high-performance liquid chromatography (HPLC).

The concentrations of oxalic acid, malic acid, citric acid and succinic acid were determined by HPLC using a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with a 20 µL loop and a Merck Hitachi L-7455 diode array detector. Separation was achieved using a C18 column (Purospher Star RP-C18e, 5 µm 4.6 x 150 mm) with a guard column (LichroCART RP-C18e, 5 µm 4 x 4 mm). Eluent A was methanol, and eluent B was

phosphate buffer. The gradient condition used for the separation of organic acids was as follows: 0–15 min of 7% A. The flow rate was set as follows: 0– 15 min at 1.0 mL min^{-1}. The column temperature was maintained at 30 ± 1 °C. The detector was set at wavelength for data acquisition 210 nm. Instrument calibrations and quantifications were performed against pure reference standards (0.1–10 mg L^{-1}) for each compound. Recovery of each organic acid was > 90%.

5.2.3.6 Statistical analysis of data

The experiment was conducted with three independent replicates. The pesticide measurement, enzymatic activities and size of the total bacterial and fungus community during the dissipation period were subjected to a one-way ANOVA, and the averages were compared using the Tukey multiple range test at $p \leq 0.05$ to identify significant effects of different treatments in the same sampling day. In addition, Pearson correlations were used to assess the relationships between pesticides and phenoloxidase activity, as well as, the relationships between phenoloxidase activity and fungal biomass on days 1 and 30.

5.3 Results and Discussion

5.3.1 Dissipation of ATZ, CHL and ISP

The effect of the rhizosphere on ATZ, CHL and ISP dissipation in the biomixtures of the biobed system is shown in Fig. 5.1a, b and c. In general, the presence of grass layer increased removal of the three pesticides. The data indicates higher dissipation of the three pesticides at all sampling occasions in the RC+P compartment. At sampling day 1 the dissipation of ATZ, CHL and ISP was 15.46, 13.83 and 25.55%, respectively, in the RC+P compartment. Similar results were observed in the RFC+P compartment (12.85, 9.57 and 24.60% for ATZ, CHL and ISP dissipation, respectively). Whereas, the dissipation of ATZ, CHL and ISP was only 0, 5.4 and 3.9%, respectively, in the unplanted pot (C+P). In addition, the pesticide dissipation on day 15 was significantly higher ($p<0.05$) in the RC+P

compartment compared with the C+P treatment without plants. At the end of the trial (30 days incubation), the RC+P compartment had 20.3%, 24.4% and 22.35% higher removal compared with the unplanted biomixture (C+P) for ATZ, CHL and ISP, respectively.

These results agree with several reports that demonstrated that the rhizosphere of tolerant plant species was effective in promoting the dissipation of different contaminants. The presence of mangrove *Kandelia candel* (L.) Druce roots improved the dissipation of phenanthrene and pyrene 47.7% and 37.6%, respectively, compared with unplanted treatment values of 26.2% and 22.8%, respectively, after 60 days (Lu et al., 2001). In addition, *Lolium multiflorum* displayed 20% higher atrazine removal capacity than bulk soil after 21 days of incubation (Merini et al., 2009). In the same way, *L. multiflorum* displayed a high pentachlorophenol (PCP) removal (>96%) in a planted rhizotron system as compared with unplanted treatments that displayed approximately 36% of PCP dissipation (Urrutia et al., 2013a).

On the other hand, dissipation of the three pesticides in the root-free compartment near to the rhizosphere (RFC+P) displayed no significant differences at almost all assay times with the RC+P compartment, which could demonstrate the influence of the root exudates in microbial composition and activity, given that they are capable of degrading ATZ, CHL and ISP. Similar results were reported by He et al. (2005), who found the highest pentachlorophenol degradation in the root compartment and the six separated compartments at various distances from the root surface of planted rhizobox system rather than unplanted treatments, which the researchers attributed to the beneficial effect of roots exudates and their interaction with microbial activity on degradation.

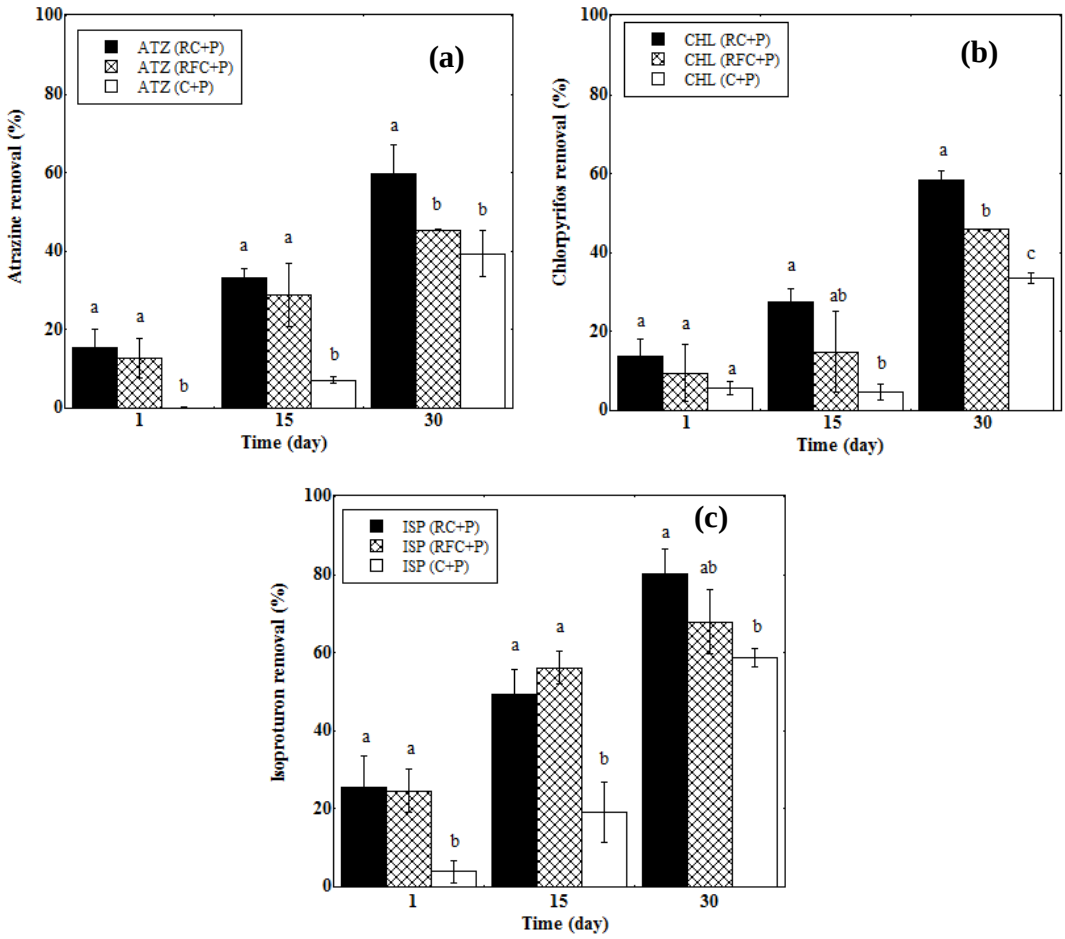

Fig. 5.1 Pesticide removal (%) after 1, 15 and 30 d for ATZ (a), CHL (b) and ISP (c) at 5 mg kg^{-1} in the biomixtures planted (root compartment (RC+P) and root-free compartment (RFC+P)) and unplanted pots (C+P). Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Different letters refer to significant differences in mean values among the different treatments of each pesticide on the same day.

5.3.2 Enzyme activity analysis

The β-glucosidase activity displayed different responses in planted compared with unplanted treatments (Fig. 5.2a). Lower values were observed in the C+P treatment for all days, which is consistent with this activity. β-glucosidase activity has been proposed as a soil quality indicator, as it indicates changes in organic matter in the soil. In addition, the presence of herbicides decreases its activity (Debosz et al., 1999; Moreno et al., 2011). However, the presence of plants significantly ($p<0.05$) increased the levels of β-glucosidase activity, which in the RC+P and RFC+P compartments were higher than the activity in the non-contaminated treatments on days 15 and 30. In this context, Hernández-Allica et al. (2006) reported that a microcosm planted with *Thlaspi caerulescens* in metal-contaminated soil, higher values of β-glucosidase activity were observed as compared with unplanted treatments. These results might be related to the organic compounds released by the plant roots, which enhance microbial biomass and activity.

In the same manner, the presence of plants in the different treatments stimulated phenoloxidase activity, as shown in Fig. 5.2b. Phenoloxidase activity has been reported as the most important biological activity for contaminants degradation in soil (Briceño et al., 2007; Diez, 2010) and in the biomixture of biobed systems (Castillo et al., 2008). Our results showed that contaminated pots in both compartments (RC+P and RFC+P) displayed a significant increase ($P < 0.05$) in phenoloxidase activity compared with unplanted treatments (C+P and C-P) on days 1, 15 and 30. These results are in agreement with those of Lee et al. (2008), who observed that the presence of plant roots in soil contaminated with phenanthrene and pyrene (PAHs) increased the amount of peroxidase activity and PAHs dissipation compared with unplanted pots.

The plant-microbial interactions in the root zone with fungi, including the formation of symbiotic mycorrhiza, may produce exoenzymes in the rhizosphere as peroxidases; these enzymes may play an important role in the detoxification of soil contaminants. However, this effect varies with plant species and environmental factors such as nutrient status of the matrix (Chaudhry et al., 2005).

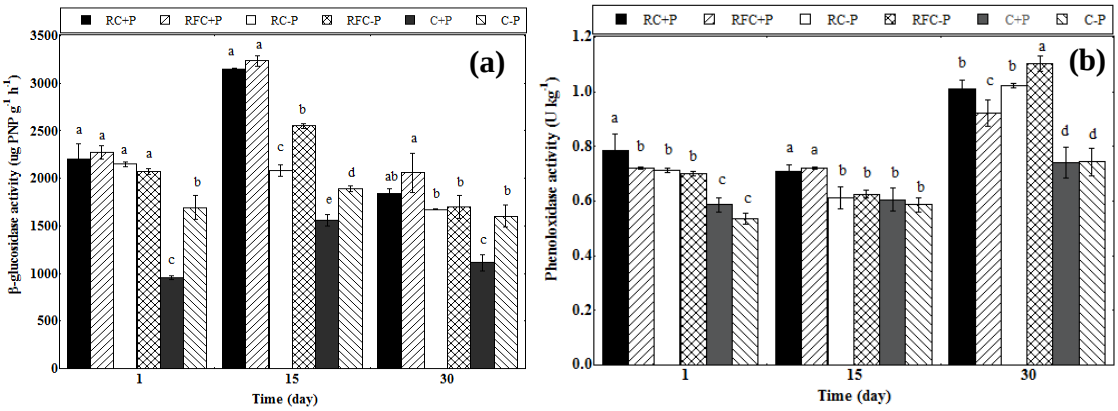

Fig. 5.2 β-glucosidase activity (a), phenoloxidase activity (b) in the biomixture contaminated with 5 mg kg^{-1} concentrations of pesticides, in planted pots (root compartment (RC+P) and root- free compartment (RFC+P)) and unplanted pots (C+P). In addition, non-contaminated pots (root compartment (RC-P) and root- free compartment (RFC-P)) and unplanted pots (C-P) were used as controls during the incubation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Different letters refer to significant differences in the mean values among different treatments of each pesticide on the same day.

A significant correlation was found between phenoloxidase activity and residual pesticide concentration for all sampling days (Table 5.1). The ATZ, CHL and ISP residual concentration had a negative correlation with phenoloxidase activity with a correlation coefficient of 0.684 to 0.952. These values suggest that these enzymes may be involved in dissipation of these pesticides. Castillo and Torstensson (2007) reported that degradation of metamitron, chloridazon, isoproturon, and linuron was correlated with phenoloxidase activity in biomixtures of a biobed system.

Table 5.1. Significant correlation between phenoloxidase activity and residual concentration of atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) in the biobed biomixtures of different treatments

	Time (day)	**ATZ**	**CHL**	**ISP**
Phenoloxidase activity	1	-0.910**	-0.691*	-0.882**
	15	-0.878**	-0.684*	-0.935**
	30	-0.818**	-0.952**	-0.840**

*correlation is significant at the 0.05 level (bilateral)
**correlation is significant at the 0.01 level (bilateral)

4.3.3 Real-Time PCR Quantification (Q-PCR) of bacteria and fungi

The fungal and bacterial population sizes were estimated by real-time PCR assay (Fig. 5.3a and b). Fungal and bacterial gene copy numbers were increased in planted biomixtures compared with that of unplanted biomixtures on day 30.

The total fungal community size was significantly higher ($p<0.05$) in the contaminated biomixtures with plants (RC+P and RCF+P) compared with the unplanted and contaminated biomixtures (C+P) on days 1 and 30, which is the same pattern in pesticide removal previously displayed by the contaminated treatments. In addition, in control treatments without pesticides higher values were detected in planted treatments in both compartments compared unplanted pots. The RFC-P compartment displayed the greater biomass size (7.34×10^6 copy number ng^{-1} DNA) on day 30, which is consistent with the high phenoloxidase activity detected on the same day. These results indicate that fungal biomass and activity were greatly increased in the biomixtures of the biobed system with a grass layer. Fungal population size was positively correlated with the phenoloxidase activity on day 1 ($r=0.825$) and day 30 ($r=0.855$). Thus, the rhizosphere effect was positive, which favors the dissipation of ATZ, CHL and ISP in the biomixtures. On the other hand,

the bacterial community size in the planted pots on day 1 displayed no significant differences ($p<0.05$), and only the C-P treatment size was higher. However, in planted pots (in RC+P, RFC+P and RFC-P), the bacterial copy number was increased compared with the other treatments on day 30. The increase of population size could have also contributed to the higher pesticide dissipation compared with unplanted pots.

Our results are comparable with those of the study of He et al. (2005), who reported that planted treatments, especially in the near-rhizosphere, contained a significantly increased and large microbial biomass that could mediate the enhanced degradation of pentachlorophenol. These differences observed between soil with and without plants, as well as among various sampling zones in proximity to roots, were expected on the basis of microbial growth and community structure modified by both pentachlorophenol and root exudates.

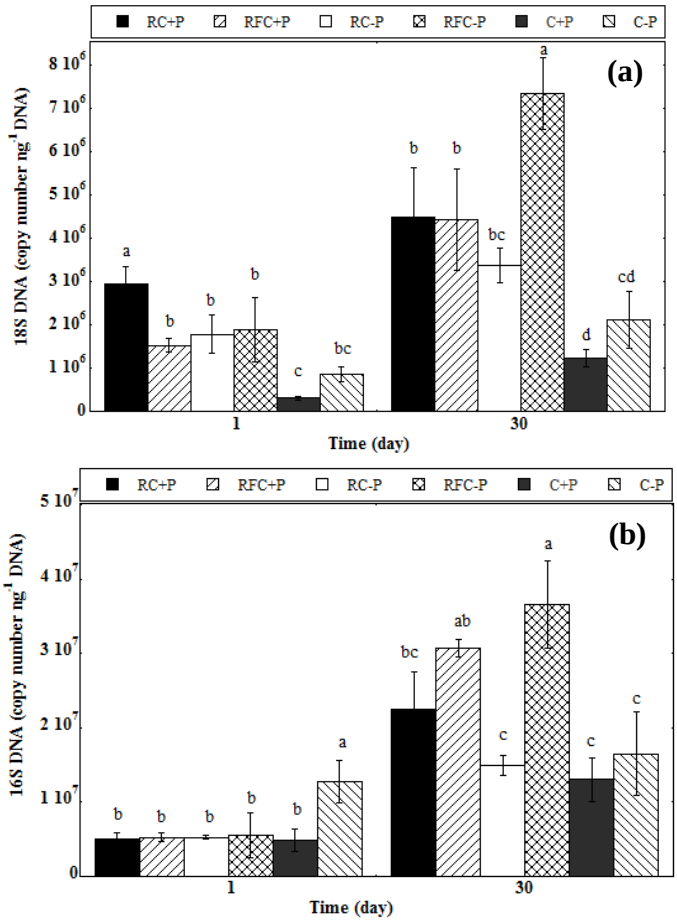

Fig. 5.3 Estimation of the fungal (a) and bacterial (b) population size in the biomixtures contaminated with 5 mg kg^{-1} concentration of pesticides, in planted pots (root compartment (RC+P) and root- free compartment (RFC+P)) and unplanted pots (C+P). In addition, non-contaminated pots (root compartment (RC-P) and root-free compartment (RFC-P)) and unplanted pots (C-P) were used as controls, during the incubation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Different letters refer to significant differences in mean values among different treatments of each pesticide on the same day.

5.3.4 Organic acids of root exudates

Oxalic acid, malic acid, citric acid and succinic acid exuded by roots of *L. perenne*, *F. arundinacea* and *T. repens* in a proportion of 50, 45 and 5 %, respectively, are shown in Fig. 5.4. Overall, the organic acids measured were detected on all sampling days. Oxalic acid was higher in contaminated pots compared with control without pesticides. Besides, in both treatments this production increases during the incubation time. Malic acid production showed higher detection in treatments with pesticides than in control treatments on day 1, 15 and 30. Citric acid concentration was the lowest compared with the other organic acids tested. Contrarily, Succinic acid production was the highest concentration detected compared with the other organic acids. However, citric acid and succinic acid showed similar behavior, being the production higher in treatments without pesticides than contaminated pots.

Exudations of organic acid depends on several environmental factors and have been implicated in many soil and plant process, such as mobilization and uptake of nutrients by plants and microorganisms, detoxification of metals by plants, microbial proliferation in the rhizosphere and dissolution of soil minerals (Ryan et al., 2001). Therefore, is difficult to determine the role of these specific organic acids in the dissipation of ATZ, CHL and ISP. However, our results showed that the concentration of oxalic acid and malic acid were higher in response to pesticide contamination. In this sense, this effect can be related with the stimulation of phenoloxidases which include manganese peroxidases, because organic chelator such as oxalate and malate produced by ligninolytic fungi (e.g. white rot fungi) can stabilize the Mn^{3+}, and thereby stimulate the lignin-degrading enzymatic activities (Marco-Urrea and Reddy, 2012; Hakala et al., 2005). Therefore, these organic acids could be involved in the enhanced oxidation of the pesticides in planted pots. In addition, microbial population size of fungi was increased in rhizosphere compartment with the pesticides addition. In this sense, Walton et al. (1994) demonstrated that plants may respond to chemical stress by increasing the exudation radical affecting the microorganism's composition or activity of the rhizosphere, being capable of degrading toxicants.

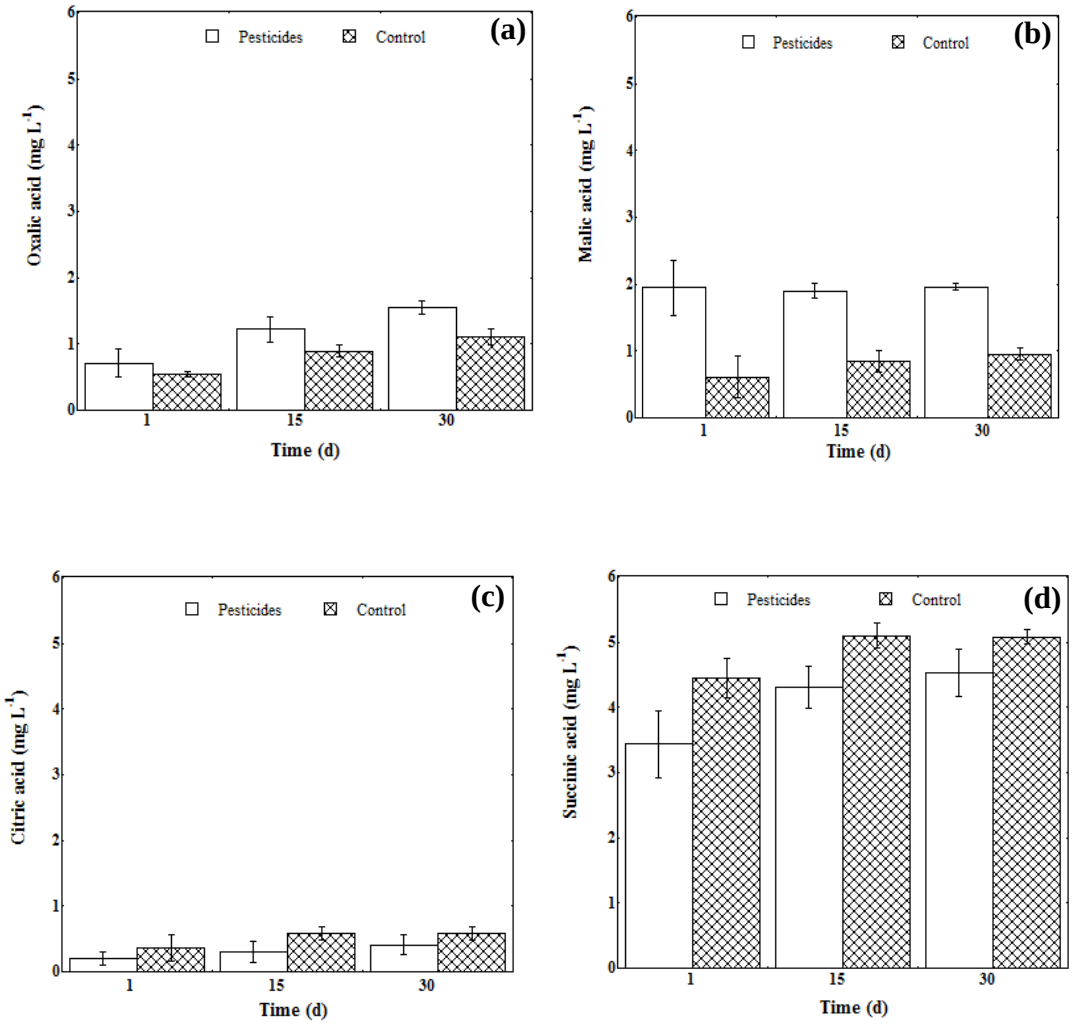

Fig. 5.4 Root exudation of oxalic acid (a), malic acid (b), citric acid (c) and succinic acid (d), under pesticide mixtures contamination (ATZ, CHL and ISP at 5 mg kg^{-1}) and control treatments without pesticides, during the incubation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean.

4.4 Conclusion

The present study demonstrated that the rhizosphere effect significantly enhances the dissipation of an atrazine, chlorpyrifos and isoporturon mixture in the biomixtures of this biobed system. The largest and most rapid loss of three pesticides in the planted pots was in the root compartment (RC) and then the root-free compartment (RFC) because of the transfer of microorganisms and root exudates between compartments, as compared with unplanted pots (C). In fact, the enhanced dissipation of pesticides was accompanied by the increases exudation of oxalic acid and malic acid concentration, which induced the lignin-degrading enzymatic activities (phenoloxidase activity). Moreover, high relative abundance of fungal and bacterial population was detected. Therefore, if pesticide spills occurs, damaging the grass cover of biobeds, the presence of roots near this zone can promote biological activity and thus dissipation of pesticides.

Our results showed that the grass layer composed of *L. perenne*, *F. arundinacea* and *T. repens* planted in the biomixture is an important factor to enhance the removal of ATZ, CHL and ISP in mixtures of a biobed system.

Acknowledgments

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Chapter 6

General discussion, concluding remarks and future directions

6.1 General discussion

In the present study we aimed to evaluate the potential use of different lignocellulosic wastes in biomixture and grass layer effect in a biobed system on the dissipation of atrazine, chlorpyrifos and isoproturon. In order to achieve the above we divided the subject on two main areas: We evaluated the efficiency of a biobed system in the dissipation (degradation in chapter 3 and adsorption in chapter 4) of pesticides through the incorporation of different lignocellulosic wastes into the biomixture. Then, in chapter 5 we analyzed the influence of the rhizosphere of a grass layer in a biobed system in the dissipation of pesticides. We used *L. perenne* and *F. arundinacea* and *T. repens* due to their tolerance capacity under pesticide contamination, which is reported in Chapter 7.

The potential use of different lignocellulosic wastes (barley husk, sawdust and oat husk) was studied as partial or total replacement for straw in biomixture of a biobed system for the dissipation of atrazine, chlorpyrifos and isoproturon. As has been described in literature, straw has been the main and most used substrate as lignocellulosic material in the biomixture of biobed system. However, the high availability of alternative lignocelullosic materials (e.g. crop wastes) in different countries has led to its potential replacement. Therefore, in chapter 3 we evaluated degradation capacity of a mixture of three pesticides in six different biomixtures with total or partial replacement of straw with barley husk, sawdust or oat husk, and the traditional biomixture composed of straw, topsoil and peat in a volumetric proportion of 2:1:1 as control.

Degradation of the three pesticides was described by first-order kinetics and the calculated half-life ($t_{½}$) values. Higher $t_{½}$ values for chlorpyrifos and isoproturon (more than 150 d) were evident in biomixtures containing barley husk and sawdust, where the straw was completely replaced with these wastes. The $t_{½}$ values for these pesticides were 3 to 6-fold higher compared to biomixture control and the other biomixtures evaluated. Similarly, a relatively slower degradation rate was observed in biomixture where 25% of the straw was replaced with sawdust. The $t_{½}$ values obtained for clorpyrifos and atrazine in biomixtures where straw was partially replaced with barley husk and oat husk, also in

biomixture where straw was totally replaced with oat husk, were comparable to those obtained in the biomixture control. The biomixtures containing oat husk were more efficient than the biomixture control even in the degradation of atrazine. Therefore, our results, demonstrated that barley husk substrate can be used only as a partial substitute for straw (barley husk:straw:topsoil:peat in a volumetric proportion of 1:1:1:1). Since, in biomixture where straw was totally replaced with barley husk promoted microbial activity but no necessarily pesticides degradation. Similarly, the use of sawdust as a lignocellulosic substrate in the biomixture is only recommended as a partial substitute for straw (sawdust:straw:topsoil:peat in a volumetric proportion of 1:1:1:1) because the high lignin content in this substrate can cause retardation of pesticide degradation. However, the biomixtures containing oat husk (oat husk:topsoil:peat in a volumetric proportion of 2:1:1, and oat husk:straw:topsoil:peat in a volumetric proportion of 1:1:1:1) exhibited the highest degradation capacities that were comparable with to the traditional biomixture. Particularly, biomixture composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1 showed lower t½ values of 28.6, 58.9 and 26.8 d for atrazine, chlorpyrifos and isoproturon, respectively.

The formation of the main metabolites in degradation assay was also quantified, deisopropylatrazine, deethylatrazine for atrazine, 3,5,6-trichloro-2-pyridinol for chlorpyrifos and monodesmethyl-isoproturon for isoproturon. Metabolite formation was observed in all biomixtures tested, but without clear formation patterns. Nevertheless, our results showed that the use of oat husk (50%) pesticides are degraded efficiently without accumulation of the different metabolites, which suggests a faster degradation of these metabolites in the presence of a well-established lignin-degrading microflora. In this sense, this biomixture (oat husk:topsoil:peat in a volumetric proportion of 2:1:1) showed a high and stable production of phenoloxidase activity, which has been reported as the most important biological activity from ligninolytic fungi for pesticide degradation in the biobed. The phenoloxidases include peroxidases (e.g., manganese and lignin peroxidases) and polyphenoloxidases (e.g., laccases), which have broad substrate specificity and are able to transform a wide range of toxic compounds, including pesticides. However, the possibility

that these pesticides may also be degraded by other microorganisms or/and abiotic processes cannot completely excluded.

In chapter 4, pesticides adsorption capacity of all tested biomixtures was evaluated. Biomixture containing sawdust showed the higher adsorption affinity for atrazine, chlorpyrifos and isoproturon compared with the other tested biomixtures. Indeed, this effect affected bioavailability for degradation process of pesticides, especially of hydrophobic compound (chlorpyrifos). Moreover, the chemical nature of the pesticides also affects the adsorption behavior. Thus, the order of adsorption capacity was chlorpyrifos>atrazine>isoproturon, where adsorption capacity decreased when the water solubility of each pesticide increased in all tested biomixtures.

According to our findings, certain physicochemical characteristics of the biomixture components could be used as indicators of the eligibility of a lignocellulosic material to be used in the biomixture. Lignocellulosic material such as straw or oat husk, characterized by high organic C content (34.3 and 36.7%), lignin content (9.9 and 7.5%), and cellulose content (41.8 and 34.3%), respectively, were favorable for development of ligninolytic fungi and phenoloxidase production. In addition, pesticide physicochemical properties also affect the balance between adsorption and biodegradation in their dissipation. The pesticides tested in this study showed that hydrophobic chlorpyrifos increased adsorption in these organic biomixtures. However, in our results a positive relationship between degradation and adsorption capacity in straw and oat husk-based biomixtures was evident. Thus, biomixture composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1 showed the highest degradation as well as an intermediate adsorption capacity for tested pesticides. Therefore, this lignocellulosic waste could be an effective alternative to straw in biobed biomixture.

Biobed system consist originally of a deep pit in the ground, filled with a biomixture and a grass layer covering the surface. Their main component is its organic biomixture; we found that oat husk-based biomixture was effective in dissipation of tested pesticide. Moreover, in the top of the biobed is installed a grass layer, that is used to keep moisture in the biomixture and to reveal pesticide spillages. As has been reported in literature, we know

that the biotechnology which uses plants, commonly termed as "phytoremediation" contributes with dissipation of different pesticide through one or more of following mechanisms such as, phytodegradation (degradation of organic contaminants absorbed by the plants), phytovolatilization (volatilization of contaminants) and/or rhizodegradation (plants roots stimulate microbial communities in the rhizosphere to degrade contaminants). In this thesis focused on the mechanism defined as rhizodegradation. Hence, plants can sustain large microbial populations in the rhizosphere through their root exudates. In this sense, their use in the biobed system can be a huge advantage. Therefore, the rhizosphere effect on the stimulation of microorganisms capable of degrading pesticides in a biobed system was evaluated.

Previously, we evaluated the tolerance of *Lolium perenne*, *Festuca arundinacea*, *Trifolium repens* and *Agrostis tenuis* to pesticides in the biobed system (Chapter 7 in Appendix 2). The conditions for the seed germination in the biomixture before pesticide contamination, includes appropriate moisture content (60%), the correct temperature (20±2°C) and soil over the selected biomixture (oat husk, topsoil and peat in a volumetric proportion of 2:1:1 that was reported in Chapter 3 and 4). Then, phytotoxicity assay was evaluated using the seed germination technique. Seed germination was tested on a petri dish with filter paper. The filter paper was moistened with aqueous solution of each pesticide (atrazine, chlorpyrifos and isoproturon) in concentrations of 0, 5, 10 and 100 mg kg^{-1} for different plant species. Our results showed a low phytotoxicity, with germination index values between 43 and 61% in *L. perenne*, *F. arundinacea* and *T. repens* for all pesticides at a concentration of 5 mg kg^{-1}. In addition, we evaluated the tolerance and potential of the four plant species (*L. perenne*, *F. arundinacea*, *T. repens* and *A. tenuis*), after 30 d germination/growth, in the cups filled with biomixture. The cups were contaminated with an aqueous solution mixture of atrazine, chlorpyrifos and isoproturon to reach a 2, 5 and 10 mg kg^{-1} concentration. The plant species *L. perenne* and *F. arundinacea* were able to survive in pesticide contaminated biomixture, whereas other plants died about 15 days after pesticide application in all tested concentrations. According to these results, a combination of 50 % *L. perenne*, 45 % *F. arundinacea* and only a 5 % *T. repens* was selected. *T. repens* was included due to its contribution to nitrogen nutrition of grass layer.

Finally, in Chapter 5 a greenhouse assay was performed to evaluate the effects of the rhizosphere of *Lolium perenne*, *Festuca arundinacea* and *Trifolium repens* on the dissipation of a pesticide mixture composed by atrazine, chlorpyrifos and isoproturon (concentration of 5 mg kg^{-1}) in an organic biomixture (oat husk:topsoil:peat in a volumetric proportion of 2:1:1) of a biobed system. The assay was performed in glass pots which were divided into two separate compartments; root surface (RC) and root-free (RFC). Glass pots without plants and pesticide mixture were established for control purposes. Moreover, biological activities as phenoloxidases β-glucosidase, fungal and bacterial communities by qPCR, and organic acid of root exudates were also evaluated.

The results demonstrated that higher dissipation for all evaluated pesticides occurred in planted pots compared with control, after 30 d incubation. The largest and most rapid loss of three pesticides in the planted pots was in the root compartment (RC) and then the root-free compartment (RFC) because of the transfer of microorganisms and root exudates between compartments, as compared with unplanted pots (C). In this sense, our results showed that the β-glucosidase activity was higher in root compartment and root-free compartment (near to the rhizosphere) compared with unplanted control. Similarly, the presence of plants in the different compartments stimulated phenoloxidase activity, which is the most important biological activity for pesticide degradation in a biobed system, as was demonstrated in the biomixture composed of oat husk, topsoil and peat in a volumetric proportion of 2:1:1 (Chapter 3). Indeed, inverse correlation between phenoloxidase activity and residual pesticide (0.684 to 0.952) was found during the entire assay. Furthermore, fungal and bacterial gene copy numbers were increased in planted biomixtures compared with the unplanted ones. In fact, fungal biomass was positively correlated with phenoloxidase activity on day 1 ($r = 0.825$) and day 30 ($r = 0.855$).

Moreover, our results showed that the root exudates particularly oxalic acid and malic acid were increased, which have the potential capability of chelation. In this sense, several researches have reported that the catalytic cycle of the enzyme manganese peroxidase (MnP) requires the presence of dicarboxylic acids to chelate and stabilize the oxidized and very unstable Mn^{3+}, which is responsible for the final oxidation of several compounds as pesticides. Hence, these root exudates enhanced oxidation of the pesticides

in the oat husk-based biomixture. Therefore, our results indicate that the rhizosphere of these plant species were important for dissipation of atrazine, chlorpyrifos and isoproturon in the biomixtures of a biobed system.

6.2 Concluding remarks

Taking into account the main results, it can be concluded that:

- Traditional biomixture composed of straw, topsoil and peat in a volumetric proportion of 2:1:1, can be completely or partially replaced by other readily available lignocellulosic wastes. Indeed, the biomixture composed of oat husk (oat husk:topsoil:peat 2:1:1 by volume) displayed a high degradation and adsorption capacity, comparable with the straw based biomixture. In addition, biomixtures partially replaced with barley husk, sawdust and oat husk showed a higher degradation of atrazine, chlorpyrifos and isoproturon.

- On the other hand, the use of barley husk as a total lignocellulosic substrate in the biomixture promoted microbial activity but not pesticide degradation. Similarly, sawdust, as a total substitute of straw, showed increase of adsorption capacity due to the high lignin content, which can cause the retardation of pesticide degradation.

- *L. perenne* and *F. arundinacea* and *T. repens* were selected to be used as grass layer in biobed system due to their tolerance capacity under pesticide contamination.

- Rhizosphere effect significantly enhances the dissipation of an atrazine, chlorpyrifos and isoproturon mixture in the biomixtures compared with unplanted biomixture of this biobed system.

- In planted pots, the enhanced dissipation of pesticides was accompanied by the increases in enzymatic activities and relative abundance of fungal and bacterial populations. Therefore, the rhizosphere effect of grass layer on biobeds promotes microorganism activity capable of degrading pesticides in biobeds.

- In addition, the concentration of oxalic acid and malic acid in root exudates increased, which could be involved in the enhanced oxidation of the pesticides mixture in planted pots.

6.3 Future directions

Results of this thesis support the hypothesis that the efficiency of a biobed system in the dissipation of pesticides was increased through the incorporation of different lignocellulosic wastes into the biomixture, specifically with husk. In addition, the effect of the rhizosphere on grass layer increased microbial biomass and activity for an accelerated degradation of pesticide through radical exudate production. However, the stimulation in dissipation of atrazine, chlorppyrifos and isoproturon in this biomixture could occur by a number of mechanisms, besides, this study provides the first evidence that the grass layer of biobed system enhances dissipation of pesticides compared with unplanted biobed system.

Therefore, more studies are necessary to evaluate the influence of this grass layer on microbial functional diversity and microbial communities such as symbiotic mycorrhizas, which have been implied in degradation of several contaminants. Besides, the mechanisms of the root exudate component impacting on the pesticides still need further elucidation.

Besides, it is necessary to evaluate the role of the different microbial components of the biomixtures microflora on pesticide dissipation and their response to pesticide exposure, because understanding the dynamics of microbial communities and their processes involved in pesticide dissipation in a biobed system can facilitate their modification towards an optimized biodepuration performance.

Moreover, more studies are needed to evaluate the effect of changing the straw by others lignocellulosic material through time, such as availability of nutrient sources; the activity and abundance of microorganisms, and others factors.

7.1 Appendix 1

The following appendix 1 show the potential of readily available wastes as barley husk (BH), sawdust (SW), and oat husk (OH), as total or partial substitutes of straw ST in the biomixture of biobed system. The biological activities in each biomixture tested at different maturity stages before of pesticide contamination were analysed.

7.1.1 Methodology

7.1.1.1 Biological activities in biomixtures of biobed at different maturity stages

For the preparation of the biomixtures, an Andisol topsoil (0–20 cm depth) belonging to Temuco series (38°42´S, 73°35´W), BH, SW, OH and ST as lignocellulosic material and commercial peat were used. Lignocellulosic wastes such as BH, OH and ST were collected from crop residues; and SW was collected from sawmill waste. All lignocellulosic wastes were cut in small pieces (2–3 cm) using a food processor and soil was sieved (to 3 mm). The constituents were mixed vigorously to obtain a homogeneous biomixtures and in volumetric proportions as described in Table 3.1 (Chapter 3). The biomixtures were put in polypropylene bags for the maturation process, moisture content was adjusted with distilled water to 60% of their water holding capacity and stored in the dark at 20 °C±2. Polypropylene bags were maintained under this condition during the following time of maturation: 0 d (fresh biomixture), 10, 20, 30 and 40 d, prior to evaluate the pesticide fate in biomixtures. The biological parameters of microbial respiration, phenoloxidase activity, and hydrolytic (FDA) activity were measured.

7.1.2 Results and discussion

7.1.2.1 Biological activities in biomixtures of biobed at different maturity stages

Biological activities in the biomixture at different maturity stages were measured according to CO_2 evolved from organic matrix as a consequence of microbial respiration, the hydrolysis of FDA related to several hydrolases (Sánchez-Monedero et al., 2008), and phenoloxidase activity, which are responsible for the degradation of several pesticides

(Castillo et al., 2008). In Fig. 7.1, the results are shown for the evolution of CO_2 for A, B, C, D, E and F-biomixture pre-incubated for 40 days. The use of barley husk-based substrates (B and D-biomixture) and oat husk-based substrates in G-biomixture caused an initial great flush of CO_2 during the first 10 d, with respiration rates of 3.92, 4.05, 3.55 mg CO_2 g^{-1} d^{-1} on day 10, respectively, followed by oat husk-based substrates F-biomixture (1.64 mg CO_2 g^{-1} d^{-1} on day 10) and straw-based substrate A-biomixture (1.52 mg CO_2 g^{-1} d^{-1} on day 10) compared with the sawdust-based substrates (C and E-biomixture), where the respiration rates were 0.34, 0.49 mg CO_2 g^{-1} d^{-1} on day 10. This effect could most likely be attributed to the presence of more easily degradable carbon sources for microorganisms as fungi. In fact, for barley husk and oat husk as total or partial substituted of straw in biomixtures, growths of fungi were evident at 10 d of maturation stage (Fig. 7.2). However, the results indicate that after 10 days the microorganisms' respiration was stabilized in all biomixtures.

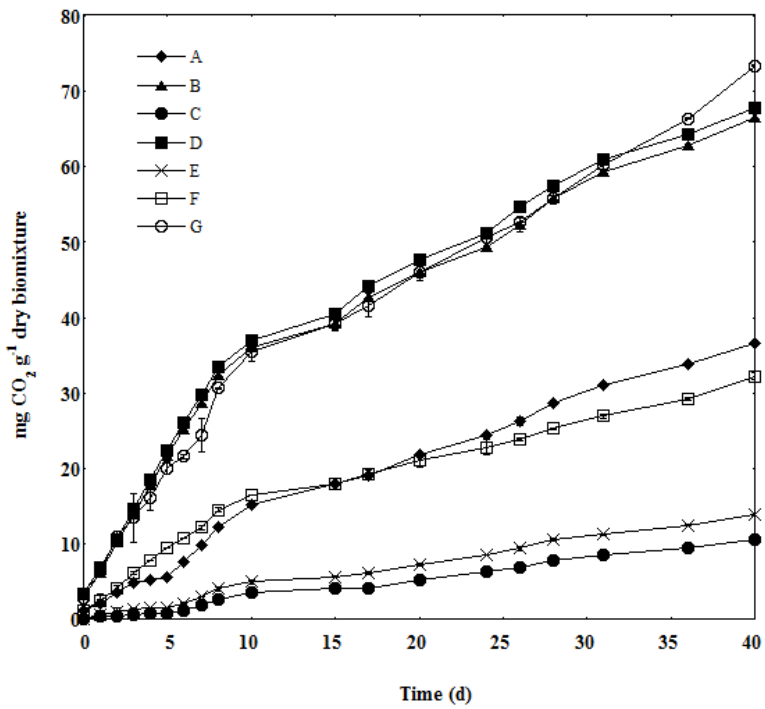

Fig. 7.1 Cumulative CO_2 production from biomixtures constituted by different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) in varying proportions during the maturation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean.

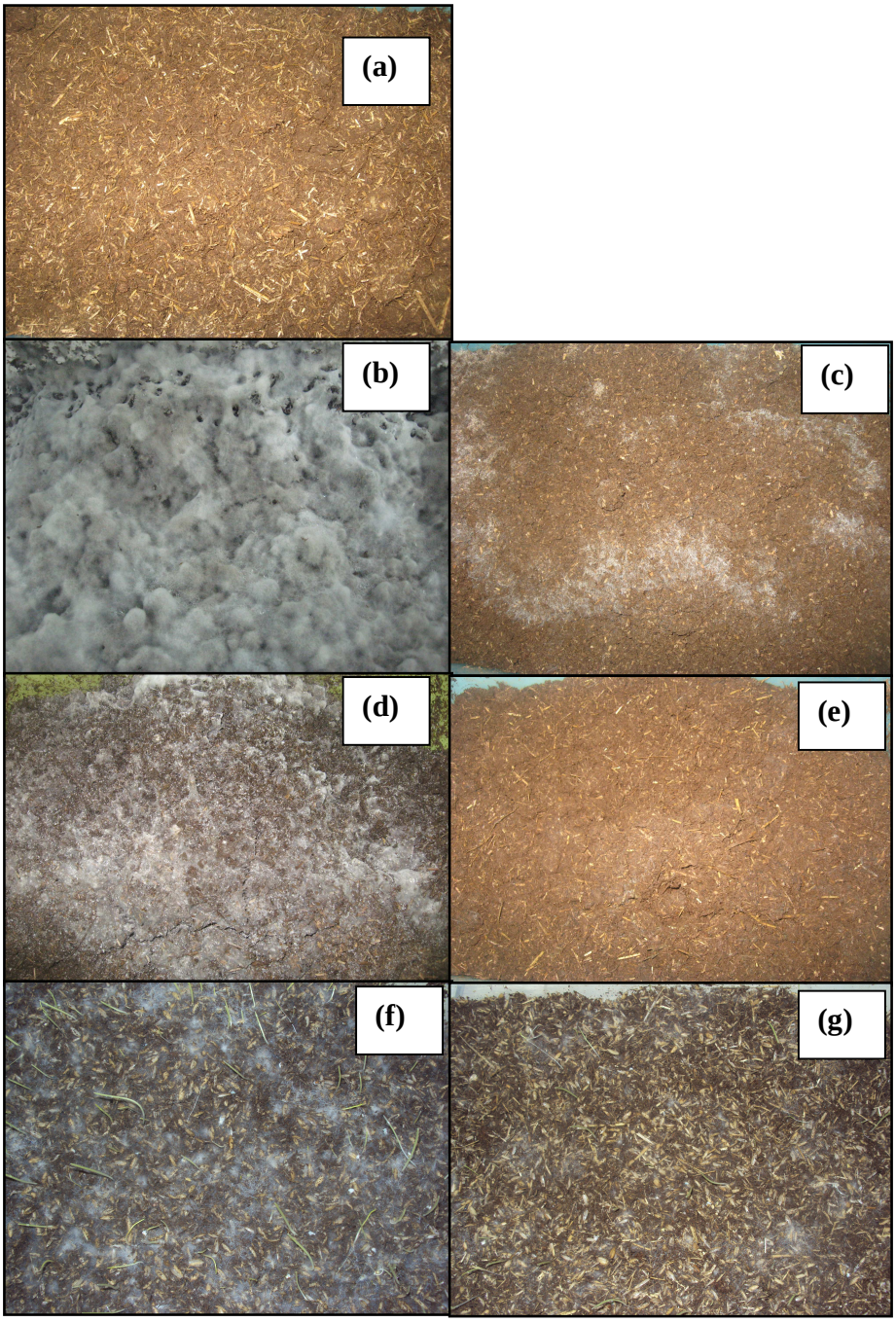

Fig. 7.2 Biomixtures composed of straw, topsoil and peat (2:1:1 v/v) (a); barley husk, topsoil and peat (2:1:1 v/v) (b); sawdust, topsoil and peat (2:1:1 v/v) (c); barley husk, straw, topsoil and peat (1:1:1:1 v/v) (d); sawdust, straw, topsoil and peat (1:1:1:1 v/v) (e); oat

husk, topsoil and peat (2:1:1 v/v) (f); oat husk, straw, topsoil and peat (1:1:1:1 v/v) (g); after 10 d maturation at 20 ±2 °C and under dark conditions.

The hydrolytic (FDA) activity in different maturity stages of biomixtures showed significant differences ($p < 0.05$), when compared among the different sampling times at each biomixture (Fig. 7.2). It was noted that the FDA activity for barley husk-based substrate in B-biomixture was the lowest compared with other biomixtures. However, for A, B, C, E, F and G-biomixture, significant differences were not observed between 30 and 40 d incubation time.

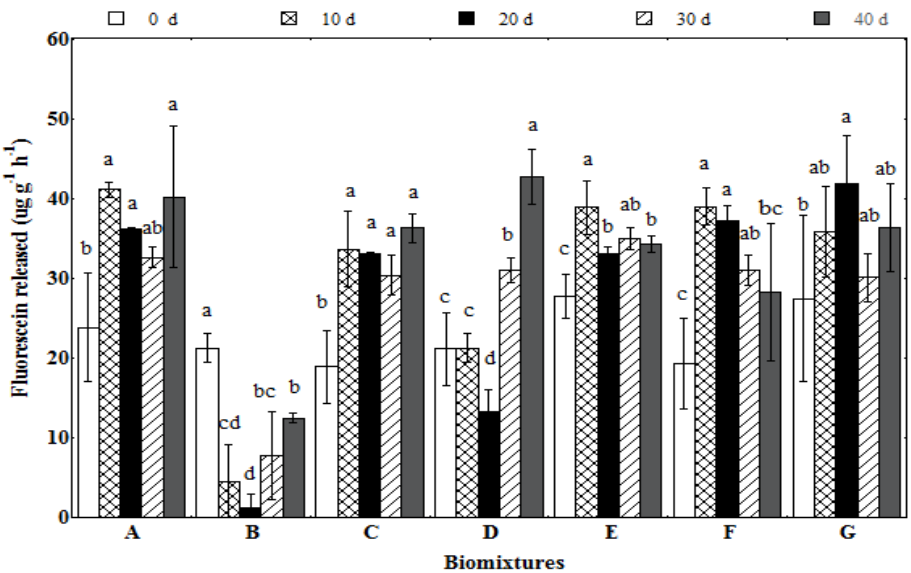

Figure 7.3 Hydrolytic (FDA) activity measured in biomixtures constituted by different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) in varying proportions during the maturation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Bars indicated by different capital letters show significant differences between sampling times of the same biomixture.

Formation of phenoloxidase was measured to observe the activity of ligninolytic fungi in the different biomixtures tested (Fig. 7.4). For the barley husk-based substrate (B and D-biomixture), high activity was observed on day 1, decreasing significantly ($p < 0.05$) on days 10, 20, 30 and 40. However, phenoloxidase content displayed high and stable values over 10 days of maturation period for (A, C, E, F and G-biomixture).

The studies of respiration, hydrolytic (FDA) and phenoloxidase activities were used as a criterion of biological stabilization for each biomixture before pesticide contamination. In fact, chlorpyrifos degradation and its main metabolite formation (TCP) were affected by different maturity stages of tested biomixture (Fernández-Alberti, 2012; Tortella et al., 2012). Our results indicate that all biomixtures require over 30 d for their stabilization.

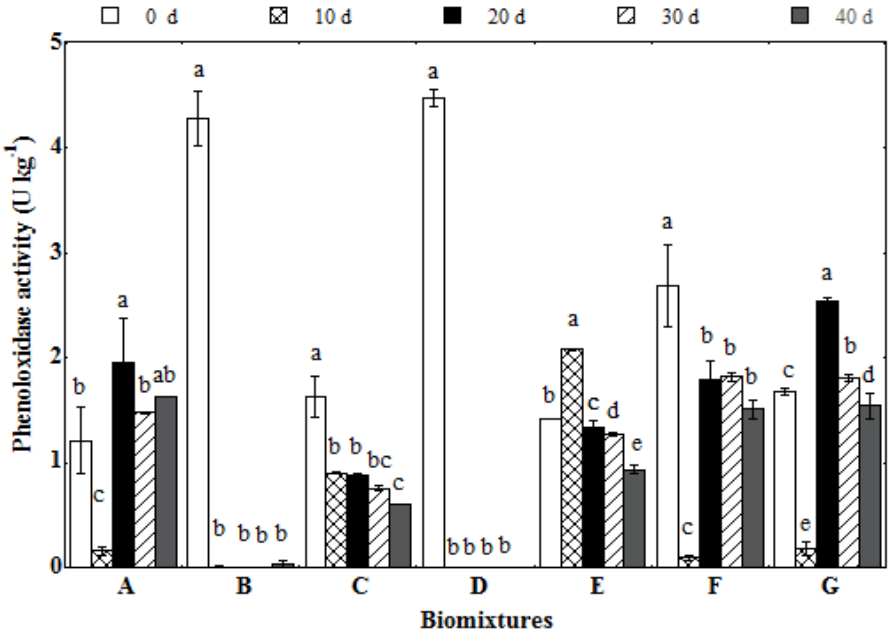

Figure 7.4 Phenoloxidase activity (b) measured in biomixtures constituted by different lignocellulosic materials (straw (A), barley husk (B, D), sawdust (C, E), and oat husk (F, G)) in varying proportions during the maturation time. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Bars indicated by different capital letter show significant differences between sampling times of the same biomixture.

7.2 Appendix 2

The present appendix 2 reported the tolerance and potential of different plant species (*L. perenne* (L), *F. arundinacea* (F), *T. repens* (T) and *A. tenuis* (A)) for the bioremediation of atrazine, chlorpyrifos and isoproturon on biobed system.

7.2.1 Methodology

7.2.1.1 Germination in the biomixtures of biobed before adding pesticides

Seeds of *L. perenne* (L), *A. tenuis* (A), *F. arundinacea* (F) and *T. repens* (T) were sterilized in 2% (v/v) sodium hypochlorite solution for 20 min and washed for 30 min with distilled water. Plastic pots containing 100 g of biomixture composed of oat husk, topsoil and peat (2:1:1 v/v), and biomixture with a layer of soil above (2g) were prepared for germination of each plant species. Ten seeds were placed in each pot, and the pots were placed inside a growth chamber (in a randomized experimental design). The experiment was carried out in triplicate for each of the plant species. The temperature of the growth chamber was maintained at 20 ±2 °C under dark conditions. Water content of the biomixture in the pots was checked daily and when necessary adjusted to field capacity, to prevent leaching. Total germination was calculated after 7 days.

7.2.1.2 Phytotoxicity assay

The phytotoxicity assay was evaluated using the seed germination technique (Zucconi et al., 1981). Four plant species; *L. perenne* (L), *A. tenuis* (A), *F. arundinacea* (F) and *T. repens* (T) were chosen to determine the phytotoxicity of pesticide concentrations. The concentrations of ATZ, CHL and ISP were 0, 5, 10 and 100 mg kg^{-1} and were prepared in distilled water. Prior to germination, seeds were sterilized in 2% (v/v) sodium hypochlorite solution for 20 min and washed for 30 min with distilled water. Seed germination was tested on wet filter paper. A piece of filter paper was placed on a petri dish and moistened with 10 mL aqueous solution of each pesticide for different plant species. Controls were set up by moistening the filter paper with 10 mL distilled water. Forty seeds were placed in each petri dish, covered by a lid and incubated under normal conditions at 20 ±2 °C. Germinated seeds and root length were determined 7 d after initiation. Seeds were considered germinated when the shoot extends to half of seed length and the radical

extends to the seed length. Each treatment was replicated three times. The percentages of relative seed germination, relative root elongation, and germination index (GI, a factor considering seed germination and root elongation) were calculated as follows:

$$\text{Relative seed germination (\%)} = \frac{\text{\# of seeds germinated in pesticides x100}}{\text{\# of seeds germinated in control}}$$

$$\text{Relative root growth (\%)} = \frac{\text{Mean root length in pesticides x 100}}{\text{Mean root length in control}}$$

$$\text{GI} = \frac{\text{(\% relative seed germination) x (\% relative root growth)}}{100}$$

7.2.1.3 Tolerance assay

To conduct plant experiment, the biomixture (70 g dw) was filled in 250 mL glass cups. Sixty seeds of each species were sown directly in the biomixture composed with oat husk, topsoil and peat (2:1:1 v/v) with a layer of soil on it (2 g), biomixture was maintained at 60% water holding capacity, under greenhouse conditions (22±3/18±3 °C day/night temperatures; 16/8 h light/dark photoperiod; 60-70% relative humidity). After 30 d germination/growth, the cups were contaminated with an aqueous solution mixture of ATZ, ISP and CHL to reach a 2, 5 and 10 mg kg^{-1} concentration. The non-contaminated cups of each plant species were used as controls. All treatments were arranged in a randomized design, each treatment had three replicates, with a total of 48 experimental units. At desired sampling time (25 days), biomixture from each planted cup was gently separated from the roots for enzymatic activity analysis (Urease, acid phosphatase, β-glucosidase, Fluorescein diacetate (FDA) hydrolysis and phenoloxidase). Roots and shoots were washed with deionized water and dried for biomass analysis.

7.2.2 Results and discussion

7.2.2.1 Germination in the biomixtures of biobed before adding pesticides

The germination condition in the biomixture before pesticide contamination was evaluated, adding a layer of soil between the seed and the biomixture the germination increased, because the seeds serve as nutrient source to the fungi as shown in Fig. 7.5.

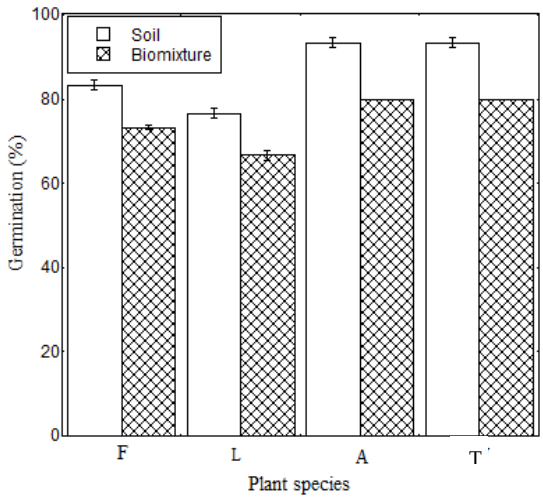

Fig. 7.5 Total germination of *F. arundinacea* (F), *L. perenne* (L), *A. tenuis* (A) and *T. repens* (T) species after 7 days in biomixture composed of oat husk, topsoil and peat (2:1:1 v/v), and biomixture with a layer of soil above.

7.2.2.2 Phytotoxicity assay

The germination percentages were affected with pesticide addition compared with control (distilled water). In different pesticide (ATZ, CHL and ISP) concentrations, a rapid decline in seed germination was found in *L. perenne*, *F. arundinacea* and *A. tenuis* seeds. However, *T. repens* maintained about 80% germination in all tested pesticide concentration. The root elongations of *T. repen*, *L. perenne* and *F. arundinacea* were ≥50% in three ATZ concentration tested, being the lowest in *A. tenuis* in all concentrations. Besides, root elongations were retarded in 50 and 100 mg kg^{-1} of CHL and ISP concentration treatments, with the exception of *L. perenne*, *F. arundicanea*, *A. tenuis and T. repens* in treatment with

5 mg kg^{-1} of CHL, and *L. perenne*, *F. arundicanea* and *T. repens* with values ≥ 60% of relative root elongation in treatment with 5 mg kg^{-1} of ISP (Fig. 7.7).

The germination index which combines seed germination and root growth, elucidated the toxicity of several chemical substances and evaluated the use of plant capabilities. In the present study, the germination index was ≥60% in *T. repens* in three concentrations of ATZ tested. And, *L. perenne*, and *F. arundinacea* showed values of ≥40% in treatment with 5 mg kg^{-1} ATZ, decreasing the germination index in treatments of 50 and 100 mg kg^{-1}. Besides, treatments with CHL showed the highest values in a concentration of 5 mg kg^{-1}, where *F. arundinacea* has 60%, *T. repens* 57%, *A. tenuis* 56% and *L. perenne* 42%. Similarly, germination index was greater with values of 54, 48, 45 and 45%, for *T. repens*, *F. arundinacea*, *L.perenne* and *A. tenuis*, respectively, in treatments with ISP at 5 mg kg^{-1}.

These results showed a lower phytotoxicity with germination index values between 43 and 61% in *L. perenne*, *F. arundinacea* and *T. repens* for all pesticides at a concentration of 5 mg kg^{-1}, compared with pesticide concentration of 50 and 100 mg kg^{-1}. The inhibitory effect of these pesticides can be related to the plants that are sensitive to contaminant application (Banks and Schultz, 2005). In fact, atrazine inhibited significantly germination and seedling growth of the six tested plant species (Burhan and Shaukat, 2000). In another study Gange et al. (1992) reported that chlorpyrifos reduced germination in the annual grass and annual forb. The germination inhibition is presumably caused by the inhibition of enzyme pathways; e.g. amylase activity thereby suppressing the mobilization of reducing sugars and also due to the impairment of respiratory metabolisms (Shaukat, 1976).

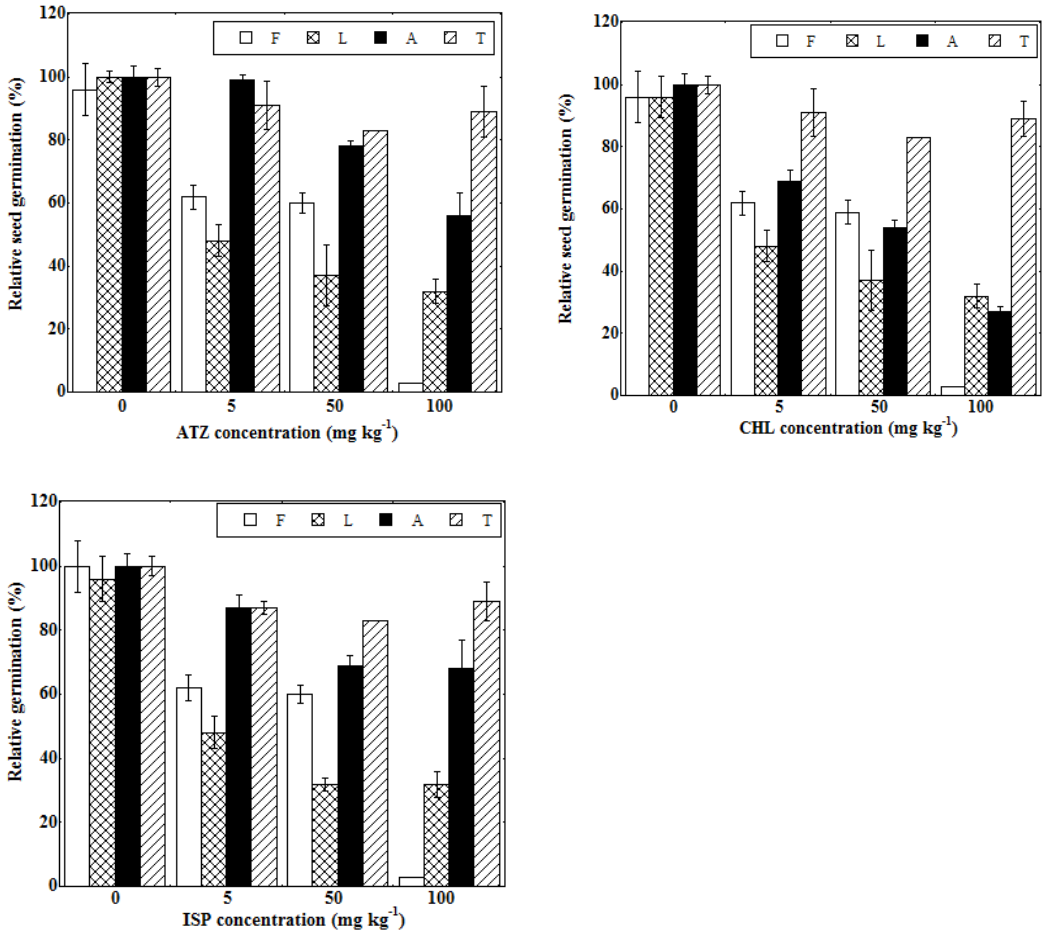

Fig. 7.6 Relative seed germination percentage of four plant species in atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) at different concentrations.

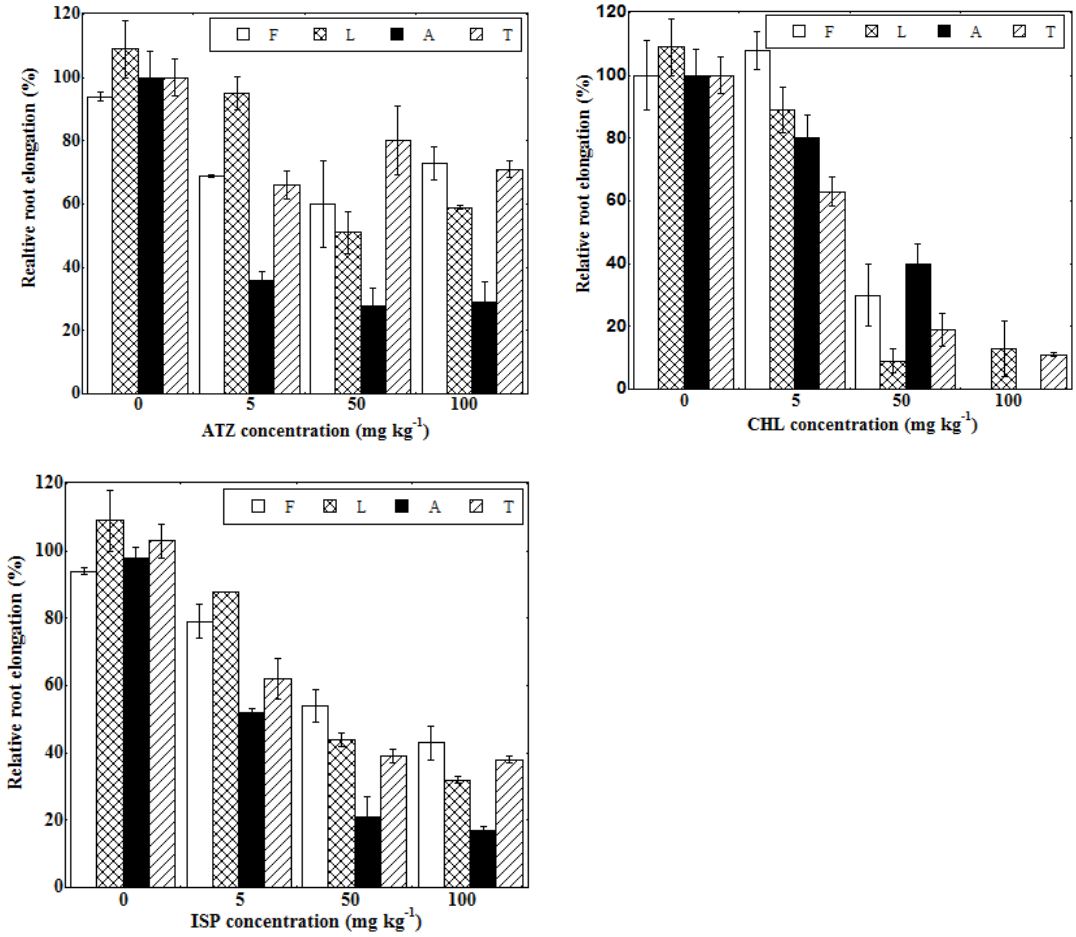

Fig. 7.7 Relative root elongation of the four plant species in atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) at different concentrations.

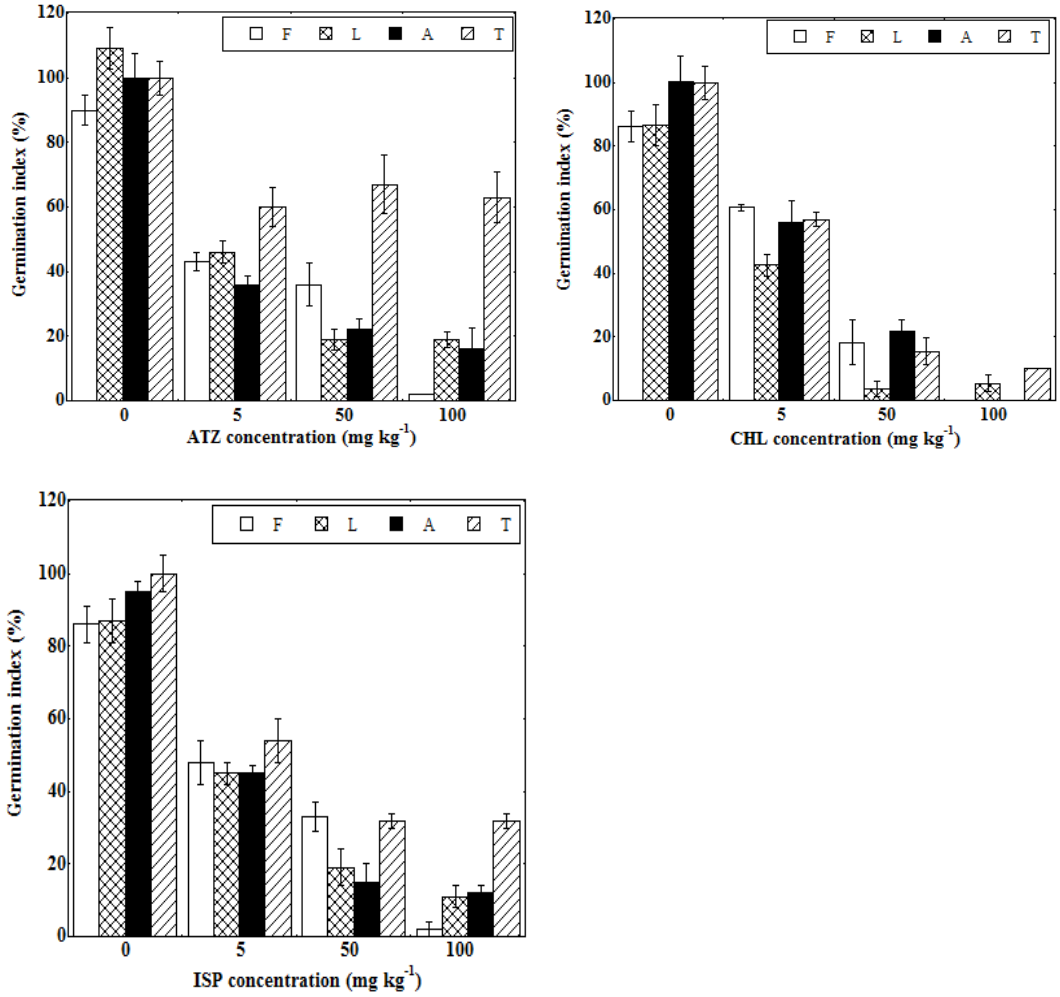

Fig. 7.8 Germination index of the four plant species in atrazine (ATZ), chlorpyrifos (CHL) and isoproturon (ISP) at different concentrations.

7.2.2.3 Tolerance assay

The present research has been carried out to evaluate the tolerance and potential of different plant species (*L. perenne* (L), *F. arundinacea* (F), *T. repens* (T) and *A. tenuis* (A)) for bioremediation of a pesticide mixture on biobed system. Shoot and root biomasses of *L. perenne*, *F. arundinacea*, *T. repens* and *A. tenuis* in the biomixture contaminated with different concentrations of ATZ, ISP and CHL mixture are shown in Table 7.3 and Fig. 7.8. This pesticide mixture has an inhibitory effect on plant biomass (shoot and root dry matter) of *T. repens* and *A. tenuis* in all concentrations used (2, 5 and 10 mg kg^{-1}), and for *F.*

arundinacea in shoot dry matter in a 10 mg kg^{-1} pesticide concentration, being significantly lower ($p<0.05$) than those of the control without pesticides. On the other hand, shoot and root dry matter of *L. perenne* were not affected in all pesticide concentrations used, root dry matter of *F. arundinacea*, was tolerant in all concentrations and shoot only up to 5 mg kg^{-1} pesticide concentration. On the other hand, shoot and root dry matter of *L. perenne* were not affected in all pesticide concentrations used, root dry matter of *F. arundinacea* was tolerant in all concentrations, but dry matter of shoot was not affected up to 5 mg kg^{-1} pesticides concentration.

Table 7.3 Dry matter (%) *L. perenne* (L), *F. arundinacea* (F), *T. repens* (T) and *A. tenuis* (A) plants (shoot and root) in biomixture planted with each plant species contaminated with different pesticide mixture concentrations, after 25 days.

	Initial concentration (mg kg^{-1})	**L**	**F**	**T**	**A**
		Dry matter of plant (%)			
Shoot	0	23	22	16	14
	2	19	10	0*	0*
	5	18	18	0*	0*
	10	13	15*	0*	0*
Root	0	15	24	18	18
	2	14	20	0*	0*
	5	14	19	0*	0*
	10	13	18	0*	0*

(*) refer to significant differences in mean values among different concentrations of pesticides with the same plant species.

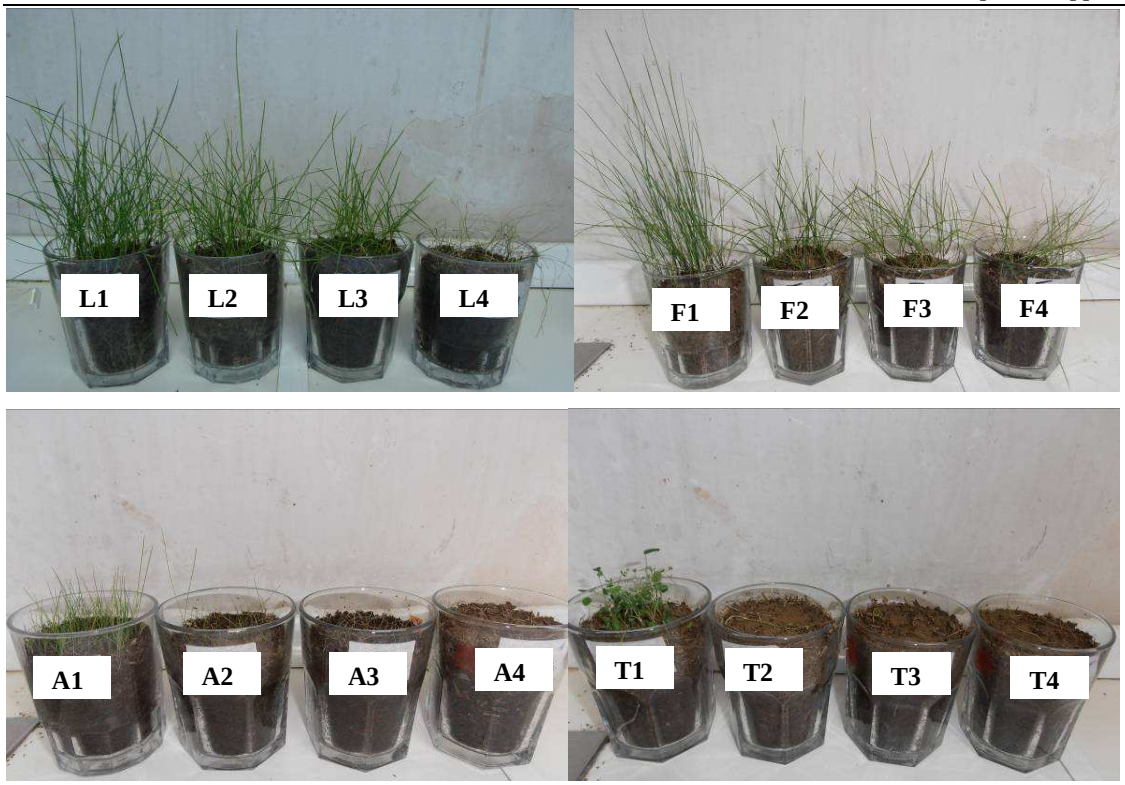

Fig. 7.8 Growth response of *Lolium perenne*, *Festuca arundinacea*, *Agrostis tenuis* and *Trifolium repens* in biomixture composed of OH, topsoil and PT 2:1:1 v/v, contaminated with ATZ, CHL and ISP mixture. *L. perenne* (L1, control; L2, 2 mg kg^{-1}, L3, 5 mg kg^{-1} and L4, 10 mg kg^{-1}); *F. arundinacea* (F1, control; F2, 2 mg kg^{-1}, F3, 5 mg kg^{-1} and F4, 10 mg kg^{-1}); *A. tenuis* (A1, control; A2, 2 mg kg^{-1}, A3, 5 mg kg^{-1} and A4, 10 mg kg^{-1}); *T. repens* (T1, control; T2, 2 mg kg^{-1}, T3, 5 mg kg^{-1} and T4, 10 mg kg^{-1}), 23 days after pesticide application.

For the rhizosphere biomixture sample analysis, phenoloxidase activity was increased in 5 and 10 mg kg^{-1} concentrations of pesticides in biomixture for each plant species (Fig. 7.9a), this effect may occur to catalyze the pesticide degradation (Castillo et al 2008). The FDA hydrolysis values are observed in Fig 7.9b, *F. arundinacea* decreased this activity when the concentration increased, being significantly lower in treatments of 5 and 10 mg kg^{-1}. On the other hand, in the different concentrations used in the biomixture planted with *L. perenne*, *T. repens* and *A. tenuis*, this activity was more stable, it should be noted that *T. repens* and *A. tenuis* survived about 15 days after pesticide application.

However, similar values of FDA hydrolysis activity to those of the treatments with more tolerant plant species were detected. This effect could be generated through their previous exudates released and plant tissues decomposition may be used as nutrient source; thus, the microorganisms of these treatments were activated. The β-glucosidase activity (Fig 7.9c) showed significant differences being the highest in 5 and 10 mg kg^{-1} concentrations of pesticides in planted biomixture, with *L. perenne* and *F. arundinacea*. This behavior might be attributed to the root exudates, but it should be noted that plant types vary widely with respect to root parameters such as morphology, root exudation (Grayston, et al., 1996), fine root turnover (Gill and Jackson, 2000), root decomposition (Van der Krift et al., 2002), and associated microbial communities (Smalla et al., 2001). Therefore, it is important to know the plant species for the rhizoremediation by different contaminants. On the other hand, the β-glucosidase values were in the range from 2107 to 3734 ug g^{-1} h^{-1} in treatments with *T. repens* and *A. tenuis*, which were similar to some values in *L. perenne* and *F. arundinacea*. These results could be attributed to plant tissue decomposition which contributed as nutrient source, as explained above. A similar pattern of phosphatase activity (Fig. 7.9d), in *L. perenne*, *F. arundinacea* and *T. repens* was detected, where the activity was increased with the pesticide concentration. Urease activity (Fig. 7.9e) showed a different behavior in each plant species. Overall phosphatase and urease activities have been indicators of soil biological status (Zhuang et al., 2007).

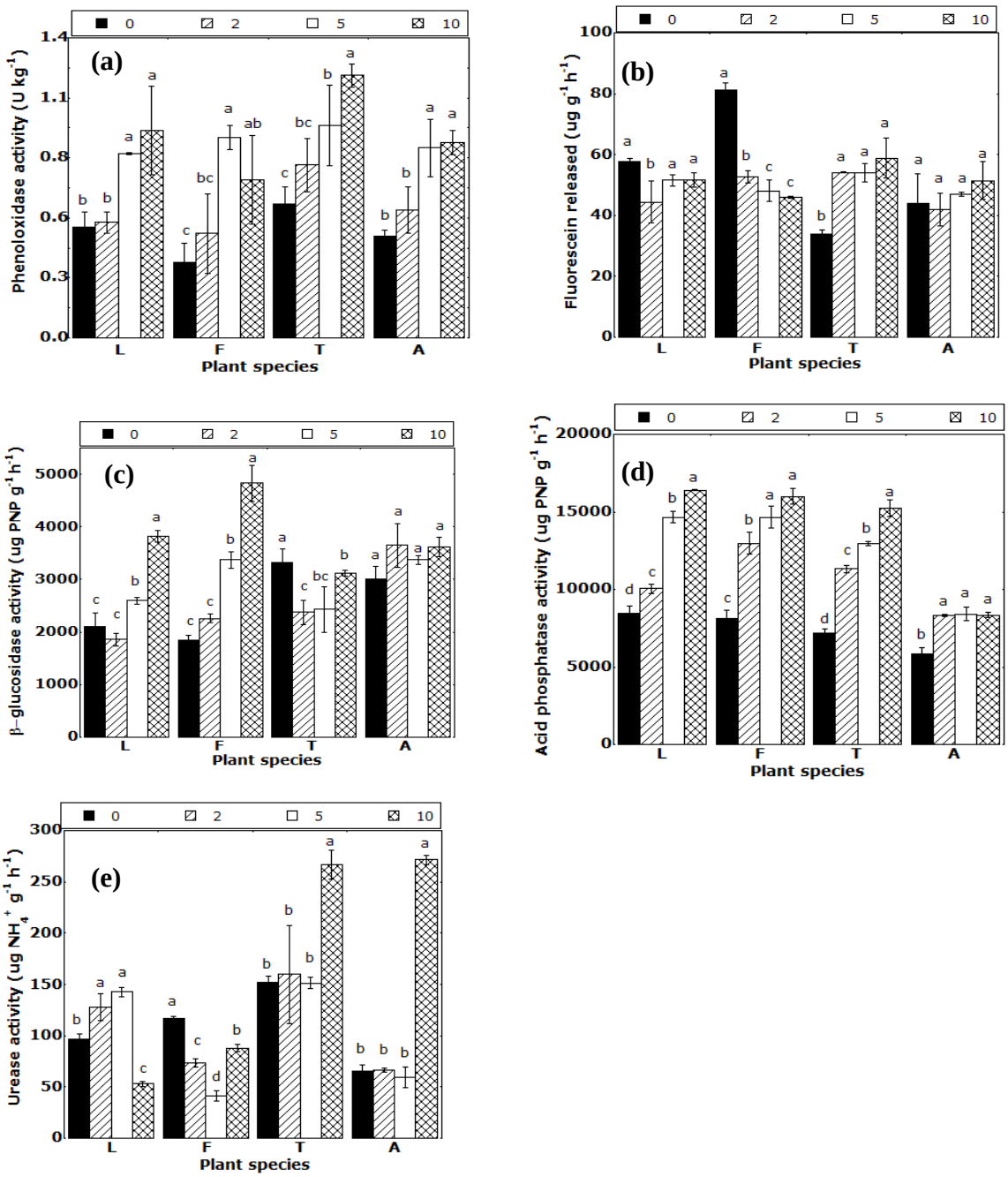

Fig. 7.9 Phenoloxidase activity (a), hydrolytic activity (FDA) (b), β-glucosidase activity (c), phosphatase activity (d) and urease activity in the biomixture contaminated with 0, 2, 5 and 10 mg kg^{-1} concentrations of pesticides planted with *L. perenne* (L), *F. arundinacea* (F), *T. repens* (T) and *A. tenuis* (A), after 25 days of assay. Each value is the mean of three replicates with error bars showing the standard deviation of the mean. Different letters refer to significant differences in mean values among different concentration of pesticides with the same plant species.

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