CRANFIELD UNIVERSITY

SOFIA GOUMA

BIODEGRADATION OF MIXTURES OF PESTICIDES BY BACTERIA AND WHITE ROT FUNGI

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BIODEGRADATION OF MIXTURES OF PESTICIDES BY BACTERIA AND WHITE ROT FUNGI

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ABSTRACT

The objective of this study was to examine the potential for degradation of mixtures of pesticides (chlorpyrifos, linuron, metribuzin) by a range of bacteria and fungi and to relate this capability to enzyme production and quantify the rates of degradation of the components of the mixture of xenobiotic compounds. Overall, although bacteria (19 *Bacillus* and 4 *Pseudomonas* species) exhibited tolerance to the individual and micture of pesticides actual degradation was not evident. Five species of white rot fungi were grown on minimal salts agar plates amended with 0, 10 and 30 mg L^{-1} of chlorpyrifos, linuron and metribuzin, individually and as a mixture with a total concentration 15 and 30 mg L^{-1}. Four of these, *T. versicolor, P. gigatea, P.coccineus* and *P.ostreatus*, exhibited very good tolerance to the pesticides. They were also grown on a nutritionally poor soil extract agar amended with a mixture of the pesticides at different concentrations (0-70 mg L^{-1}). Subsequently, the ability of *T. versicolor, P. gigatea, P. coccineus* to degrade lignin and production of laccase in the presence of mixture of the pesticides was examined as well as their capacity to degrade the pesticide mixture at different concentrations (0-50 mg L^{-1}) in soil extract broth was quantified using HPLC.

This showed that only *T.versicolor* had the ability to degrade linuron, after three weeks incubation although all tested species produced laccase. Subsequently, the temporal degradation rates of *T.versicolor* was examined in relation to temporal degradation of a mixture of the pesticides chlorpyrifos, linuron and metribuzin with total concentrations 0-50 mg L^{-1} and the temporal laccase production was quantified over a six week period in relation to ionic and non-ionic water potential stress (-2.8 MPa).

These studies showed that the test isolate had the ability to produce very high levels of laccase at -2.8 MPa water potential adjusted non-ionically by using glycerol and quite lower levels in soil extract broth without stress while

T.versicolor did not produce laccase at -2.8 MPa when the medium was modified ionically.

Finally, *T.versicolor* was able to degrade the pesticide linuron in all tested water regimes, after five weeks incubation, regardless of the concentration of the mixture. In contrast, about 50% of the metribuzin was degraded, only at at -2.8 MPa water potential adjusted non-ionically with glycerol. Chlorpyrifos and its main metabolite TCP were not detected, possibly, due to a combination of hydrolysis, photolysis and volatilization degradation. The capacity of *T.versicolor* to degrade linuron in mixtures of pesticides and the production of high levels of laccase, in a nutritionally poor soil extract broth, even under water stress suggests potential application of this fungus in bioremediation.

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ABBREVIATIONS

3, 4-DCA	3, 4-dichlooroaniline
ABTS	2, 2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
BTEX:	Benzene, Toluene Ethylbenzene Xylene)
DAD	Diode Array Detector
DETP	Diethylthiophosphate
ED	Effective dose
HPLC	High Performance Liquid Chromatography
IUCLID	Uniform ChemicaL Information Database
Koc	Partition coefficient between soil organic matter and water),
LD50	Lethal Dose
MEA	Malt Extract Agar
MMY	Minimal salts Medium Yeast
OD	Optical density
PAHs:	Polycyclic aromatic hydrocarbons
PCBs:	Polychlorinated biphenyls
PCP:	Pentachlorophenol
TCA	Trichloroacetic acid
TCP	3, 5, 6-trichloro-2-pyridinol
TSB	Trypticase Soy Blood

1 LITERATURE REVIEW

1.1 Introduction

During the past twenty years, concern has arisen as to the presence of pesticides in the environment and the threat they pose to wildlife and mankind. Certainly, pesticides have improved longevity and quality of life, chiefly in the area of public health. Insect control programs have saved millions of lives by combating diseases such as malaria, yellow fever and typhus. In addition, the use of pesticides constitutes an important aspect of modern agriculture, as they are absolutely necessary for economical pest management (Rao & Hornsby, 2001).

Since the earliest times, societies have used soil as a quick and convenient disposal route for waste (Ashman& Puri, 2002) but only recently it was it found that contaminants in the soil can find their way to other areas of the environment such as surface and groundwater, rivers and atmosphere. The conventional techniques used for remediation of pesticide-contaminated sites, such as low temperature thermal desorption, chemical treatment and incineration have certain limitations and disadvantages (Frazar, 2000). Low temperature thermal desorption is an ex situ clean up technology which is capable of removing semi-volatile and volatile organic compounds from soils and sediments. It, however, requires highly specialized facilities and carries a comparatively high cost (Gavrilescu, 2005). Incineration is also a proven technology that has frequently been used for destruction of pesticides but it has met serious public opposition, because of its potentially toxic emissions and its elevated economic cost (Kearney, 1998, Zhang & Quiao, 2002).

Hence, there is an increasing interest and need to developing safe, convenient and economically feasible methods for pesticides remediation (Kearney, 1998, Zhang & Quiao, 2002). For this reason, several biological techniques, such as bioremediation and phytoremediation, have been developed (Schoefs *et al,* 2004). Currently, bioremediation is one of the most environmentally safe and cost-effective methods of decontamination and

detoxification of a pesticide-contaminated environment (Zhang & Quiao, 2002).

1.2 Bioremediation

1.2.1 Terminology

Bioremediation is a process that uses mainly microorganisms, plants, or microbial or plant enzymes to detoxify contaminants in the soil and other environments. The concept includes biodegradation, which refers to the partial, and sometimes total, transformation or detoxification of contaminants by microorganisms and plants. Mineralization is a more restrictive term for the complete conversion of an organic contaminant to its inorganic constituents by a single species or a consortium of microorganisms. Co-metabolism is another more restrictive term referring to the transformation of a contaminant without the provision of carbon or energy for the degrading microorganisms (Skipper, 1998).

The process of bioremediation enhances the rate of the natural microbial degradation of contaminants by supplementing the indigenous microorganisms (bacteria or fungi) with nutrients, carbon sources or electron donors (biostimulation, biorestoration) or by adding an enriched culture of microorganisms that have specific characteristics that allow them to degrade the desired contaminant at a quicker rate (bioaugmentation) (Mackay & Frazar, 2000). The goal of bioremediation is at least to reduce pollutant levels to undetectable, non-toxic or acceptable levels, i.e., within limits set by regulatory agencies or, ideally, to completely mineralize organopollutants to carbon dioxide (Pointing, 2001).

1.2.2 Advantages and disadvantages of bioremediation

Bioremediation is a natural process and is therefore perceived by the public as having a reduced impact on natural ecosystems. It is typically less expensive than the equivalent physical-chemical methods. The complete destruction of target pollutants is possible on site without the need of excavation or transport quantities of waste off site (Kearney, 1998, Vidali, 2001). Finally, it requires little energy input and preserves the soil structure (Hohener *et al*., 1998).

Bioremediation, however, has a number of disadvantages. This process is limited to those compounds that are biodegradable. Moreover, there are some concerns that the products of bioremediation may be more persistent or toxic than the parent compounds. Furthermore, it is difficult to extrapolate the results from bench and pilot-scale studies to full-scale field operations. All biological processes, such as this, are often highly specific and complex and take longer than other treatment options (for example incineration) (Vidali, 2001).

Compounds in mixtures are known to interact with biological systems in ways that can greatly alter the toxicity of individual compounds (Hernando *et al.,* 2003). While studies on the capacity of fungi, particularly white rot fungi and bacteria to degrade individual pesticides is extensive (Gadd, 2001) very few have examined the capacity of microorganisms to degrade mixtures of pesticides. In most pesticide-contaminated agrochemical facilities, herbicides are found in combination with other widely used agricultural chemicals and remediation strategies must take into account of the presence of multiple contaminants (Grigg *et al.,* 1997; and Memic *et al.,* 2005). Moreover, there are hardly any studies on the use of white rot fungi to clean up mixtures of pesticides.

Grigg *et al*., (1997) reported the ability of an atrazine-mineralizing mixed culture isolated from soil subjected to repeated applications of atrazine to degrade other s-triazines in liquid culture. Cyanazine and simazine either

alone or combined with atrazine were degraded in 6 days. Metribuzin was not degraded while the microbial culture completely degraded atrazine in the presence of contaminants including alachlor, metolachlor and trifluralin. A recent study by Fragoeiro and Magan (2008) demonstrated that a mixture of dieldrin, simazine and trifluralin is differentially degraded by *Trametes versicolor* and *Phanerochaete chrysosporium* and significant extracellular enzymes are produced in soil, even at -2.8 MPa water potential. Thus, research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex contaminants (Vidali, 2001).

1.2.3 Biological mechanisms of transformation

In soil, microorganisms commonly exist in large populations. Provided with adequate supplies of carbon and energy and environmental conditions conducive to growth, microbial activity, especially the production of extracellular enzymes, can significantly assist in the amelioration of contaminated sites.

Microbial genes encode the degradative enzymes, which oxidize, reduce, dehalogenate, dealkylate, deaminate, and hydrolyze hazardous chemicals such as pesticides, in the soil environment (Skipper, 1998). Once a contaminant has been enzymatically transformed to a less complex compound, it can often be metabolized using various pathways. Although a single transformation may reduce the toxicity of a contaminant, the complete mineralization of an organic compound typically requires several degradative enzymes produced by multiple genes on plasmids or chromosomes residing in a single species or among different species. The organism(s) with such enzymes may be either indigenous to the contaminated site or added as an inoculum. The enzymes can be intracellular or extracellular, and each type of enzyme has specific conditions for optimum activity (Skipper, 1998).

1.3 Lignin degrading enzymes

Laccase

Laccase (EC 1.10.3.2) is one of the very few enzymes that have been studied since the end of 19th century. It was first demonstrated in the exudates of *Rhus versinifera,* the Japanese lacquer tree. A few years later it was also demonstrated in fungi by Bertrand in 1896. Although known for a long time, laccases attracted considerable attention only after the beginning of studies of enzymatic degradation of wood by white–rot wood–rotting fungi (Baldrian.2006). Laccases are typically found in plants and fungi. Plant laccases participate in the radical-based mechanisms of lignin polymer formation; whereas in fungi laccases probably have more roles including morphogenesis, fungal plant-pathogen/host interaction, stress defence and lignin degradation (Thurston, 1994).

Laccase (benzenediol oxygen oxidoreductase) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic centre and usually called multicopper oxidases (Baldrian.2006). Laccases catalyse the reduction of oxygen to water accompanied by the oxidation of a broad range of aromatic compounds as hydrogen donors (Thurston, 1994), like phenols, aromatic amines and diamines (Nyanhongo *et al.,* 2007*).* Further more, laccase catalyzes the oxidation of non-phenolics and anilines. This reaction is involved in transforming numerous agricultural and industrial chemicals (Tortella *et al.*, 2005).

Among fungi recently studied for producing extracellular laccase, *Trametes* species are probably the most actively investigated for the laccase production because these fungi are commonly found in many parts of the world and apparently are excellent wood decomposers in nature. Indeed, *Trametes versicolor*, a representative fungus in this genus, was among the first species, from which the production of large amounts of laccase has been reported. It has already been marketed by several companies, although the

current prices are still too high for bulk environmental application (Duran and Esposito, 2000).

The catabolic role of fungal laccase in lignin biodegradation is not well understood (Trejo-Hernandez *et al.*, 2001), but some successful applications of this enzyme in decontamination have been reported. For example, dye decolouration by *Trametes hispida* (Rodriguez *et al.,* 1999), degradation of azo-dyes by *Pyricularia oryzae* (Chivukula and Renganathan, 1995) and textile effluent degradation by *Trametes versicolor* have been attributed to laccase activity. Duran and Esposito (2000) also reported that laccase from *Cerrena unicolor* produced a complete transformation of 2,4 DCP in soil colloids. In a more recent study by Zouari-Mechighi *et al.* (2006) reported that the ability of *Trametes trogii* isolated from Tunisia to decolorize Poly R 478 and several industrial dyes in agar plates and Cu^{2+} addition stimulated decolourization, suggesting that laccase could be involved in the process. The results on dye decolourization with the crude enzyme, without peroxidase activity, and the results obtained with the purified enzyme confirmed that *Tr. trogii* laccase decolorizes industrial dyes.

Lignin peroxidase

Lignin peroxidase (once called ligninase) (EC 1.11.14) was first discovered in *Phanerochaete chrysosporium* (Glenn *et al.,* 1983; Tien and Kirk, 1983). This activity is also produced by many, but not all, white rot fungi (Hatakka, 1994). This enzyme is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum pH (Gold and Alic, 1993). It shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules (Barr and Aust, 1994).

Manganese peroxidase

Manganese peroxidase (EC 1.11.13) is also a heme peroxidase and it forms a family of isoenzymes. Similarly to lignin peroxidase (LiP) they are also glycoproteins (Glenn and Gold, 1985). This enzyme shows a strong preference for Mn (II) as its reducing substrate and is not able to complete its

catalytic cycle without the presence of Mn (II) (Wariishi *et al.*, 1990). The redox potential of the Mn peroxidase-Mn system is lower than that lignin peroxidase and it has only shown capacity to oxidize in vitro phenolic substrates (Vares, 1996). Manganese peroxidase seems to be more widespread among white rot fungi than lignin peroxidase (Hatakka, 1994).

Total ligninolytic activity

The production and activity of the ligninolytic enzymes in soil (as opposed to a ligninolytic substrate) may be a prerequisite for transformation of pollutants by wood rot fungi (Lang *et al.,* 2000a). Thus, quantification of the activity of these enzymes by white rot fungal inoculants is important. Historically, various 14C-radiolabelled and unlabelled substrates have been used to screen for ligninolytic activity. However, these assays are relatively slow and difficult. The decolourization of dyes by white rot fungi was first reported by Glenn and Gold (1983) who developed a method to measure ligninolytic activity of *P. chrysosporium* based on the decolourization of a number of sulphonated polymeric dyes. Subsequently, other workers adapted the dye decolourization test for evaluating the ability of white rot fungi to degrade dyes and other xenobiotics (Nyanhongo *et al*., 2007). The high-molecular weight dyes cannot be taken up by the microorganisms and thus provide a specific screen for extracellular activity (Gold *et al.,* 1988; Field *et al*., 1993). The decolouration of polymeric dyes has been proposed as a useful screening method for ligninolytic activity (Lin *et al*., 1991; Weissenfels *et al*., 1992). Today the polymeric dyes used are inexpensive, stable, readily soluble, have high extinction coefficients and low toxicity towards *P. chrysosporium* and other white rot fungi and bacteria tested (Gold *et al*., 1988).

Other enzymes

The other microbial enzymes involved in the pollutant transformation are hydrolases. Several bacteria and fungi produce a group of extra or ecto

cellular enzymes (enzymes acting outside but still linked to their cells of origin) that include proteases, carbohydratases, esterases, phosphatases and phytases. These enzymes are physiologically necessary to living organisms (Gianfreda & Rao, 2004). Some of them (e.g. proteases, and carbohydratases) catalyze the hydrolysis of large molecules, such as proteins and carbohydrates, to smaller molecules for subsequent absorption by cells. Due to their intrinsic low substrate specificity, hydrolases may play a pivotal role in bioremediation of several pollutants (Gianfreda & Rao, 2004).

Recently Staszczak *et al., (*2000) suggested that proteases are involved in the regulation of ligninolytic activities in cultures of *T. versicolor* under nutrient limitation. Margesin *et al.*, (2000a) showed a positive influence of naphthalene on protease activity and Baran *et al*., (2004) reported an increase in phosphatase, dehydrogenase, urease and protease activities in a site in which concentrations of PAHs were higher than 1,000 μg kg^{-1}.

Mougin *et al.,* (1996) suggested the degradation of the pesticide lindane by *P. chrysosporium* via detoxification by a cytochrome P450 monooxygenase system. Cytochrome P450s are haemethiolate proteins that have been characterized in animals, plants, bacteria and filamentous fungi (Van Eerd *et al*., 2003). Regulation and expression of P450s are not well understood in plants and microorganisms because of the very low quantities of P450s enzymes usually present in these cells, particularly if the organism has not been exposed to physiochemical, physiological or xenobiotic stress (Van Eerd *et al*., 2003).

Cellobiose dehydrogenase (CDH) may be an important enzyme in pollutant degradation (Cameron, 2000). CDH is secreted by *Phanerochaete chrysosporium* and several other white rot fungi. It has been shown to directly reduce the munitions 2,4,6-trinitro tolouene (TNT) and hexahydro-1,3,5 trinitro-1,3,5-triazine (RDX) and indirectly degrade many more chemicals (Cameron, 2000).

Whether the degradation of pesticides is carried out by lignin degrading enzymes or by other enzymatic systems, or by both, the use of fungi in bioremediation is very promising and further studies are required to understand which enzymes are involved in the process. This information could be very useful in the establishment of the best conditions for enzyme production and consequent fungal bioremediation *in situ*.

1.4 Biodegrading capacities of white rot fungi

Application of fungal technology for the cleanup of contaminants has shown promise since 1985 when the white rot species *Phanerochaete chrysosporium* was found to be able to metabolize a number of important environmental pollutants (Sasek, 2003). This ability is generally attributed to the lignin degrading enzymatic system of the fungus, and a similar degrading capacity was later described for other white rot fungal species (Sasek, 2003). White rot fungi possess a number of advantages that can be exploited in bioremediation systems. Because key components of their lignin-degrading system are extracellular, these fungi can degrade insoluble chemicals such as lignin or an extremely diverse range of very persistent or toxic environmental pollutants (Barr and Aust, 1994; Cameron, 2000). The mycelial growth habit is also advantageous as it allows rapid colonization of substrates, and hyphal extension enables penetration of soil reaching pollutants in ways that other organisms cannot do (Reddy and Mathew, 2001; Fragoeiro & Magan, 2008). This can maximize physical, mechanical and enzymatic contact with the surrounding environment (Maloney, 2001).

In addition, these fungi use relatively inexpensive and abundant lignocellulosic materials as a nutrient source. They can tolerate a wide range of environmental conditions, such as temperature, pH and moisture levels (Maloney, 2001; Magan, 2007) and do not require pre-conditioning to a particular pollutant, because their degradation system is induced by nutrient deprivation (Barr and Aust, 1994).

1.4.1 White rot fungi

To understand the ability to degrade contaminants it is important to analyze the ecological niches of white rot fungi. These fungi are a physiological rather than taxonomic grouping, comprising those fungi that are capable of extensively degrading lignin (a heterogeneous polyphenolic polymer) within lignocellulosic substrates (Pointing, 2001). The name white rot derives from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance of the substrate (Pointing, 2001). Most known white rot fungi are basidiomycetes, although a few ascomycete genera within the *Xylariaceae* are also capable of white rot decay (Eaton and Hale, 1993).

In nature, white rot fungi live on woody tissues that are composed mainly of three biopolymers: cellulose, hemicellulose and lignin. Lignin, which provides strength and structure to the plant, is extremely recalcitrant. It is mineralized in an obligate aerobic oxidative process, and its degradation yields no net energy gain (Pointing, 2001), because it cannot be degraded as a sole source of carbon and energy (Field *et al.* 1993). The physiological importance of lignin biodegradation is the destruction of the matrix it forms, so that the microorganism can gain better access to the real substrates: hemicellulose and cellulose (Field *et al.*, 1993; Canet *et al.*, 2001), from where it obtains energy.

Lignin is synthesized in plants by random peroxidase-catalysed polymerization of substituted *p*-hydroxy-cinnamyl alcohols (Field *et al.*, 1993). This polymer is three-dimensional, and its monomers are linked by various carbon-carbon and other bonds and the stereo irregularity of lignin makes it resistant to attack by enzymes. The enzymatic degradation of lignin is further complicated by the chiral carbon in this polymer that exists in both L and D configurations.

Due to its molecular size it is impossible for lignin to be absorbed and degraded by intracellular enzymes, therefore ligninolytic enzymes are extracellularly excreted by the degrading fungi, initiating the oxidation of substrates in the extracellular environment (Mester and Tien, 2000). Thus, the white rot fungi have developed very non-specific mechanisms to degrade lignin extracellularly (Bar and Aust, 1994).

The three major families of lignin modifying enzymes believed to be involved in lignin degradation are laccases, lignin peroxidases and manganese peroxidases (Reddy and Mathew, 2001). The key step in lignin degradation by laccase or the ligninolytic peroxidases (LiP and MnP) involves the formation of free radical intermediates, which are formed when one electron is removed or added to the ground state of a chemical (Reddy and Mathew, 2001). Such free radicals are highly reactive and rapidly give up or accept an electron from another chemical, which triggers oxidation or reduction of "neighbouring" compounds. These radicals can carry out a variety of reactions including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol polymerisation and demethylation (Pointing, 2001). Different white rot fungi appear to be able to achieve the same effect with different combinations of enzymes (Harvey and Thurston, 2001) with respect to wood degradation.

The common features are the random nature of the structure of lignin, which requires its degradation to function in a non-specific manner. Consequently, other compounds that have an aromatic structure, such as many xenobiotic compounds, are also highly susceptible to degradation by ligninolytic enzymes (Field *et al.*, 1993; Barr and Aust, 1994). This characteristic is the greatest advantage of the use of white-rot fungi in bioremediation, since a mixture of different pollutants is usually found in most contaminated sites (Mester and Tien, 2000).

Aside from the lack of specificity, the ligninolytic system of white rot fungi offers further advantages. It is not induced by either lignin or other related

compounds (Cancel *et al.*, 1993). Thus, it is possible to degrade pollutants at relatively low concentrations that may be lower than that required to induce the synthesis of biodegrading enzymes in other microorganisms (Mester and Tien, 2000).

Furthermore, repression of enzyme synthesis does not occur when the concentration of a chemical is reduced to a level that is ineffective for enzyme induction. This is because the induction of the degradative enzymes is not dependent on the presence of the chemical. The fungus can effectively degrade very low concentrations of a pollutant to non-detectable or nearly non-detectable levels (Bumpus & Aust, 1986). Finally these enzymes are mainly of constitutive nature. This feature prevents their originating organisms to be adapted to the chemical being degraded (Gianfreda, 2004).

1.4.2 Phanerochaete chrysosporium

Phanerochaete chrysosporium is a higher basiodiomycete belonging to the family *Corticiaceae*. It is a secondary decomposer of both hardwood and softwood branches and logs and can be found in temperate forests throughout North America, Europe and Iran. *P. chrysosporium* was the first fungus to be associated with degradation of organopollutants, in 1985, because it has been extensively studied as a model microorganism in research on the mechanism of lignin degradation (Sasek, 2003). This thermophilic basidiomycete was first considered a problem in the 1970s in self-heating wood chip piles, in its anamorphic state *Sporotrichum pulverulentm.* Although, later this fungus was the subject of many investigations on cellulase and ligninase production; because of its potential in bioremediation, its natural niche remains unknown (Evans & Hedger, 2001). *Phanerochaete chrysosporium* produces lignin peroxidase and manganese peroxidase in ligninolytic conditions and laccase only when cellulose is present as carbon source (Zouari-Mechini *et al.,* 2006).

Castillo *et al*., (2001a) studied the ability of the white-rot fungus *P. chrysosporium* and the role of the lignin degrading enzyme to degrade isoproturon (N, N-dimethyl-N-[4-(1-methyllethyl) phenyl] urea), a pre- and post-emergent herbicide. In straw cultures *P. chrysosporium* was able to degrade 91% of the herbicide in 14 days of incubation. The largest MnP activity coincided with a sharp decrease of isoproturon, although LiP activity was also present.

1.4.3 Pleurotus ostreatus

Pleurotus ostreatus is an edible species commonly known as the oyster mushroom belonging to the family *Polyporaceae*. It is wide distributed throughout the Northern Hemisphere (Hestbjerg *et al*., 2003). This species is a saprophytic basidiomycete and a natural decomposer because it secretes enzymes and acids that degrade organic polymers (Pletsch *et al.*, 1999). Its great advantage is that large-scale production of fungal biomass grown on lignocellulosic substrates has already been developed for human consumption and it is economically feasible because the substrates do not need to be sterilized (pasteurization is sufficient), (Sasek, 2003). The fungal mycelium colonizes natural soil effectively (Lang *et al.*, 2000a) and its temperature requirements are considerably lower than that of *P. chrysosporium (*Hestbjerg *et al.*, 2003), as it is active at 8^0C (Heggen & Sveum, 1999). The polyphenol oxidases produced by *P. ostreatus* include laccases and Mn-dependent peroxidases, but not lignin peroxidases (Palmieri *et al,* 1997).

1.4.4 Pycnoporus coccineus

It is a non-edible species belonging to the family *Polyporaceae* and the order *Polyporales.* The fruiting bodies of this polypore genus look like bright reddish – orange brackets and are widespread on dead wood. In Australia there are two species *Pycnoporus coccineus* and *Pycnoporus sanguineus*

with overlapping distribution. They have been used in bioremediation research mainly for their effective extra cellular laccase production (Alves *et al,* 2004; Pointing *et. al*, 2000).

1.4.5 Trametes versicolor

The basiomycete *Trametes* (syn. *Coriolus*, *Polyporus, Polystictus, Microporus*) *versicolor* belongs in the order *Polyporales* and the family *Polyporaceae. Trametes versicolor* is found ubiquitously in temperate to sub-tropical forests throughout the world. It was first studied by Dodson (1987). This fungus causes rapid white invasion of moribund or fallen trees of species such as birch, beech and oak, by a rapidly extending mycelium, which utilizes free sugars in the wood of the tree (Evans & Hedger, 2001).

Several reasons account for the attractiveness of *Trametes versicolor*, chief among them is the constitutive, extracellular secretion and the non specific nature of the ligninolytic enzymes which are laccase (Lac, EC1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13) while lignin peroxidase (LiP, EC 1.11.1.14) has only rarely been reported in *Trametes versicolor* (Nyanhongo *et a*l, 2007). This fungus has been shown to be able to metabolize a wide range of organic compounds (Gadd, 2001). This ability is generally attributed to the production of extracellular ligninolytic enzymes such as laccase, which is non specific in regard to its substrate (Thurston, 1994; Pointing, 2001; Sasek *et al*.; 2003; Baldrian, 2004).

In recent studies, *Trametes versicolor* was shown to exhibit good tolerance to water stress conditions (Mswaka and Magan, 1999; Fragoeiro and Magan, 2005). This is a great advantage for bioremediation of contaminated soils. Further, this fungus can grow on cheap media such as corn, straw and even sawdust while its hyphal growth form allow it to extend far from its original point, an attractive property in bioremediation of soils (Nyanhongo *et al.*, 2007).

A large body of evidence shows that *Trametes* species are among the most versatile of white-rotters with ongoing intensive research into applications in bioremediation, effluent treatment, the pulp and paper industry, the food industry, synthetic chemistry, biofuels, cosmetics, biosensors and the textile industry, amongst others (Nyanhongo *et al.*, 2007).

1.4.6 Phlebiopsis gigantea

Phlebiopsis (syn. *Phanerochaete, Peniophora, Phlebia*) *gigantea* is a common saprophytic fungus that causes white rot of conifer logs and stumps. It is used as a biological control of annosum root rot, caused by *Heterobasidion annosum,* in Western Europe. It was found that *P. gigantea*, when applied to cur stumps, could inhibit subsequent colonization by the pathogen. In the United States, the fungus was commercially available until 1995. Currently, the Environmental Protection Agency is reviewing the status of *Phlebiopsis gigantea* as a pesticide. At this time, *Phlebiopsis gigantea* is only commercially available in England, Sweden, Norway, Switzerland and Finland. The fungus occurs throughout North America, Central America, Europe, East Africa and Southern Asia.

Table 1.1 summarizes some of the white rot fungi and their capacity for degradation of different xenobiotic compounds. Plates 1.1 to 1.4 show examples of the main species.

Table 1.1 Degradation of typical environmental pollutants by the white rot fungi.

Fungus	Type of pollutant	Reference
Phanerochaete chrysosporium	Lindane, DDT BTEX Atrazine	Bumbus *et al.,* 1985 Yavad and Reddy, 1993 Hickey *et al.,* 1994
Phanerochaete chrysosporium *Phanerochaete eryngi* *Pleurotus florida* *Pleurotus sajor-caju*	Heptachlor Lindane	Arisoy, 1998
Phanerochaete chrysosporium *Trametes versicolor*	Pentachlorophenol Creosote, Anthracene, PAHs, PCP	Alleman *et al.,* 1992 Richter *et al,* 2002 Gianfreda & Mao 2004
Trametes versicolor	Pesticides Pesticides	Khadrani *et al.,* 1999 Morgan *et al.,* 1991 Bending *et al.,*2002
Pleurotus ostreatus	Pesticides	Khadrani *et al.,* 1999
	PCBs	Gianfreda & Mao 2004
	Dyes	Sasek *et al.,* 1998
	Catechol Pyrene Phenanthrene	Bezadel *et al.,* 1996
Pleurotus pulmonaris	Atrazine	Mazaphy *et al.,* 1996
Pycnoporus sanguineus	Azo dyes	Gianfreda & Mao 2004
Bjerkandera adusta	Pesticides	Khadrani *et al.,* 1999

BTEX: benzene, toluene, ethylbenzene and xylene; PAHs: polycyclic aromatc hydrocarbons; PCBs: polychlorinated biphenyls; PCP: pentachlorophenol.

Plate. 1.1 *Pycnoporus coccineus*

Plate 1.2. *Pycnoporus coccineus*

Plate 1.3 *Phlebiopsis gigantea*

Plate 1.4 *Pleurotus ostreatus*

Plate 1.5 *T versicolor*

Plate 1.6 *P. chrysosporium*

1.5 Biodegrading capacities of bacteria

Bacteria either utilize the pesticides as sole carbon sources or, in some cases as sole nitrogen sources, or alternatively co-metabolize them. Genetic studies of pesticide degrading bacteria have centered on cloning and characterization of specific enzymes involved in pesticide degradation. The involvement of plasmid-encoded catabolic sequences in pesticide degradation has been widely documented (Somasundaram & Coats, 1990).

The degradation of pesticides *in situ* is usually achieved by a consortium of bacteria rather than a single species. Pure culture studies, however, allow the mechanisms by which the pesticide is metabolized to be elucidated. They also allow the location of genes involved in degradation of the pesticide (Aislabie & Jones, 1995). Traditional isolation methods, involving enrichment culture and plating techniques, have been used to isolate many of the microbes that are able to degrade pesticides in pure culture.

The bacterial species *Nocardia* and *Pseudomonas* utilized one or more of the side chains of atrazine aerobically as the sole source of carbon and energy. In addition, a *Pseudomonas* species able to mineralize atrazine has been enriched from soil; this organism used atrazine as the sole source of nitrogen with sodium nitrate as the carbon source (Mandelbaum *et al*., 1995). Bacteria, belonging to the genera *Achromobacter*, *Pseudomonas* and *Flavobacterium*, are able to utilize carbofuran, a carbamate pesticide, as a growth substrate (Aislabie & Jones, 1995). Recently, Carpouzas & Walker (2000) reported the ability of *Pseudomonas putida* strains epI to degrade the organophosphate ethoprophos in soil at 20 and 35 ^{0}C and at soil water potentials of -3.3 and -10 kPa. Table 1.2 shows some examples of pesticides degraded by bacteria in pure culture.

Table 1.2 Examples of bacteria able to degrade pesticides in pure culture from Aislabie and Jones, (1995)

Pesticide	Bacteria	Reference
DDT	*Alcaligenes eutrophus*	Nadeau *et al.,* (1994)
2,4D	*Alcaligenes eutrophus*	Pemberton and Fisher (1977)
	Flavobacterium	Chaudhry and Huang (1988)
	Arthortbacter	Sandman and Loos (1988)
	Pseudomonas cepacia	Bhat *et al.,* (1994)
Atrazine	*Nocardia*	Cook (1987)
	Pseudomonas	Cook (1987)
	Pseudomonas	Mandelbaum (1995)
	Rhodococcus	Behki *et al.* (1993)
	Rhodococcus	Behki and Kham (1994)
Parathion	*Flavobacterium*	Sethunathan &Yoshida(1973)
	Pseudomonas dimuta	Serdar *et al.* (1982)
Diazinon	*Flavobacterium*	Sethunathan &Yoshida(1973)
Fenthion	*Bacillus*	Patel,Gopinathan (1986)
Carbofuran	*Achromobacter*	Karns *et al*. (1986)
	Pseudomonas	Chaudhry and Ali (1988)
	Flavobacterium	Chaudhry and Ali (1988)
	Flavobacterium	Head *et al.* (1992)
EPTC	*Arthrobacter*	Tam *et al*. (1987)
	Rhodococcus	Behki *et al*. (1993)
	Rhodococcus	Behki and Kham (1994)

1.6 Pesticides

A pesticide is “any substance or mixture of substances intended to prevent, destroy, repel or mitigate any pest. This definition by FIBRA (Federal Insecticide, Fungicide and Rodenticide Act) includes insecticides, herbicides, fungicides and antimicrobials, as well as plant growth regulators, defoliants and desiccants. In 1997, agricultural industry accounted for 77% of the total use of pesticides, 12% for industrial, commercial and government organizations and the remaining 11% for private households (Moerner *et al.,* 2002).

1.7 Environmental concerns regarding the contamination with pesticides

The range of damage associated with the application of pesticides across environmental media and different receptors is equally great, providing a particularly complex example of multidimensional environmental impact (Figure1.1). Loss of aquatic and terrestrial biodiversity, contamination of surface and groundwater and agriculture produce, and poisoning of agricultural workers are among the potential negative consequences of pesticide use in agriculture alone (Mourato, 2000).

Pesticide residues can be attributed to a number of sources including releases from fields during and after application, leakage from equipment, spillages and incorrect disposal of waste and washings (Fogg, 2004). Recent research suggests that the contribution from sources other than those originating from approved applications to agricultural land may be more significant than previously realized. Such “point source” releases can be reduced by modifying handling practices to minimize losses (Fogg, 2004). However, due to time constraints and other pressures, small drips and spills are still likely to occur (Fogg, 2004). Application of pesticides for the purpose of pest management is called “non point” source contamination and in this

case pesticide presence is widespread, but at relatively low concentrations (Gan & Koskinen, 1998).

When the concentration of a pesticide, its metabolites and its by-products is significantly excessive, remediation is necessary to avoid migration to a more sensitive area of the environment (Kearney, 1998). The concentration at which soil remediation is required is referred to as the remediation trigger level, but for many xenobiotics, no guidelines are currently established. There is also a need to define the target threshold concentrations when remediation is achieved. This target remediation concentration is generally in the range of 1mg/Kg soil or a concentration arrived at after a risk assessment analysis, usually on a site-by-site basis (Kearney, 1998).

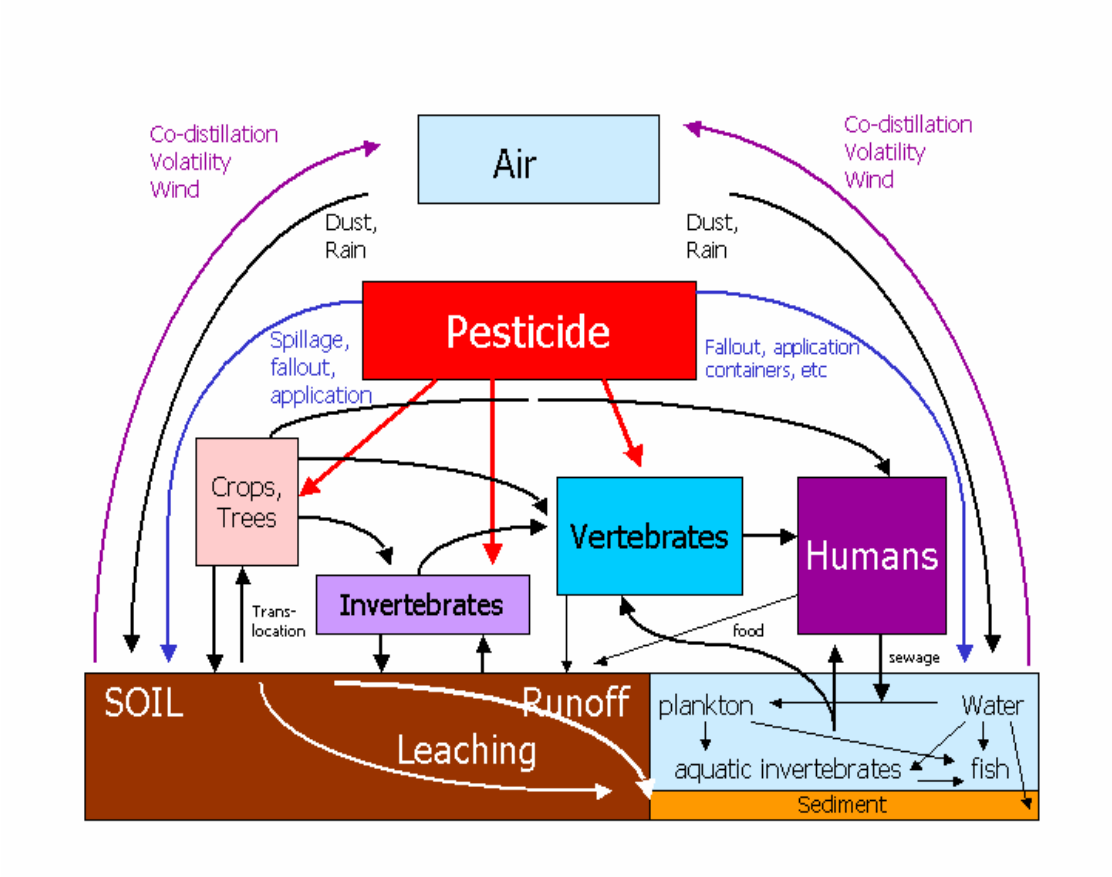


Figure 1.1 Transport of pesticides in the environment (from Gavrilescu, 2005)

Even when the level of contaminants is low and does not exceed a few μg/ kg soil, the ability of these chemicals to pass through the subsurface layers and to reach groundwater is a matter of concern, especially in areas where

drinking water is supplied from an aquifer (Muszkat *et al,* 1993). The maximum permissible concentration of pesticides in waters intended for human consumption is 0.1μg/L and 0.5μg/L in total for all substances (European Directive 80/778/EEC, 1989).

The chemicals properties of pesticides determine their retention and transport in soils (Kearney, 1998). A pesticide can reach groundwater if its water solubility is greater than 30mg/L; its adsorptivity, Koc (Koc= partition coefficient between soil organic matter and water), is less than 300-500 ml/g; its soil half life is longer than about 2-3 weeks; its hydrolysis half-life is longer than approximately 6 months and its photolysis is longer than 3 days (Barcelo, 1991).

1.7.1 Metabolic fate of pesticides

The metabolic fate of pesticides is dependent on abiotic environmental conditions (temperature, moisture, soil pH, etc.), microbial community or plant species (or both), pesticides characteristics (hydrophilicity, level of solubility*)* and biological and chemical reactions. Abiotic degradation is due to chemical and physical transformations of the pesticide by processes such as photolysis, hydrolysis, oxidation, reduction and rearrangements. Furthermore, pesticides may be biologically unavailable because of compartmentalization, which occurs as a result of pesticide adsorption to soil and soil colloids without altering the chemical structure of the original molecule. However, enzymatic transformation, which is mainly the result of biotic processes mediated by plants and microorganisms, is by far the major route of detoxification (Van Eerd, 2003).

Metabolism of pesticides may involve a three-phase process (Van Eerd, 2003). In Phase I metabolism, the initial properties of a parent compound are transformed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product than the parent. The second phase involves conjugation of a pesticide or pesticide metabolite to a

sugar or amino acid, which increases the water solubility and reduces toxicity compared with the parent pesticide. The third phase involves conversion of Phase II metabolites into secondary conjugates, which are also non-toxic. In these processes fungi and bacteria are involved producing intracellular or extra cellular enzymes including hydrolytic enzymes, peroxidases, oxygenases, etc (Van Eerd, 2003).

1.7.2 Pesticides used in the current study

Chlorpyrifos is a broad- spectrum organophosphate insecticide. It is used to control different insects on grain, cotton, fruit, nut and vegetable crops, as well as on lawns and ornamental plants (Figure 1.2A). It is also used for the control of mosquitoes and for ectoparasite control on cattle and sheep. Chlorpyrifos acts on pests primarily as a contact poison, with some action as a stomach poison. As with all organophosphates, it acts by interfering with the activities of cholinesterase, an enzyme that is essential for the proper working of the nervous system of both humans and insects (Extoxnet, 1996). It is moderately toxic to humans, moderately to very toxic to birds and very highly toxic to fish. The oral LD_{50} for chlorpyrifos in rats is 95 to 270 mg/Kg (Extoxnet, 1996). It may affect the central nervous system, the cardiovascular system and the respiratory system as well as causing skin and eye irritation (Oliver *et al.,* 2000).

Chlopryrifos is moderately persistent in soils. Its half-life in soil is usually between 60 and 120 days, but can range from 2 weeks to over 1 year depending on the soil type, climate, and other conditions. It adsorbs strongly to soil particles and it is not readily soluble in water. Its water solubility is approximately 2 mg/L. Considerable literatures indicated that a wide range of water and terrestrial ecosystems might be contaminated with chlorpyrifos (EPA, 1997; Yang *et al.,* 2006), which has increased the public concern to establish an efficient, safe and cost effective method to remove or detoxify chlorpyrifos residues in contaminated environments.

Linuron is a substituted urea herbicide (phenyl-urea) used to control annual and perennial broadleaf and grassy weeds on crop and non-crop sites (Figure 1.2B). It is used as a pre and post emergent herbicide. It works by inhibiting photosynthesis in target weed plants and it is labeled for use in soybean, cotton, pea, potato, winter wheat, carrot, corn and fruit crops.

Linuron is of low toxicity (oral LD_{50} in rats= 1,500mg/Kg) but it is highly toxic to fish and non-toxic to bees. Linuron and some of its major metabolites are suspected of being endocrine disruptors (Rasmussen *et al.*, 2005). Linuron is of relatively low acute toxicity, but is classified as an unquantifiable Group C carcinogen (that is, a possible human carcinogen for which there is limited animal evidence) and shows some evidence of developmental and reproductive toxicity (EPA, 1995).

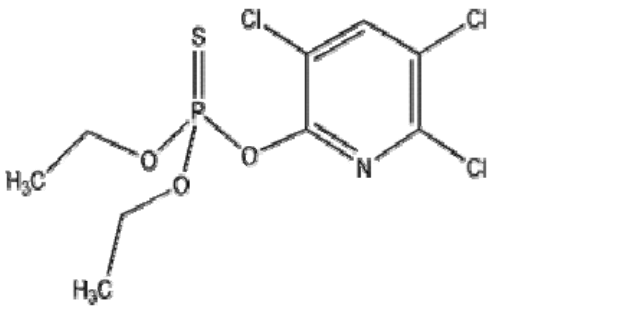
It is moderately persistent in soils. In the presence of oxygen in soil, linuron's half-life is 75 days, while it is 230 days under field anaerobic conditions. The chemical is bound in soil (especially clay) and organic matter and does not move freely. Linuron is slightly to moderately soluble in water, and it is not readily broken down in water (Extoxnet, 1993).

Metribuzin is a selective, pre and post-emergent triazinone herbicide, which inhibits photosynthesis (Figure 1.2C). It is used for the control of annual grasses and numerous broadleaf weeds in field and vegetable crops, in turf grass, and on fallow lands. It is slightly to moderately toxic to humans and mammals (LD_{50} in rats=1,090-2,300mg/Kg) and fish, while it is non-toxic to bees. This chemical is highly soluble in water and it has a moderate ability to adsorb to soil with high clay and/or organic matter content so it has great potential to leach into, and contaminate groundwater. Soil half-lives of less than one month to six months has been reported (Extoxnet, 1993). Others studies have shown that metribuzin can remain for a long time in soil (until 377days under aerobic conditions) depending on temperature, pH and soil type, accumulates in plants and interferes with other crops. The major mechanism by which metribuzin is lost from soil is microbial degradation.

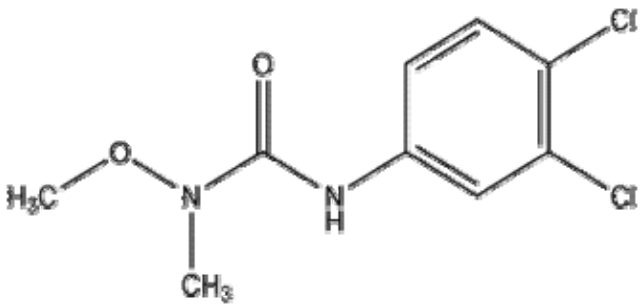
Modes of action of the pesticides used

The herbicides linuron and metribuzin act as photosynthesis inhibitors in target weed plants. Photosynthesis inhibitors shut down the photosynthetic process in susceptible plants by binding to specific sites within the plant chloroplast. Particularly, for linron and metribuzin the site of action is D_1quinone-binding protein of photosynthetic electron transport in photosystem II. Inhibition of photosynthesis could result in a slow starvation of the plant, however, in many situations rapid death occurs perhaps from the production of secondary toxic substances.

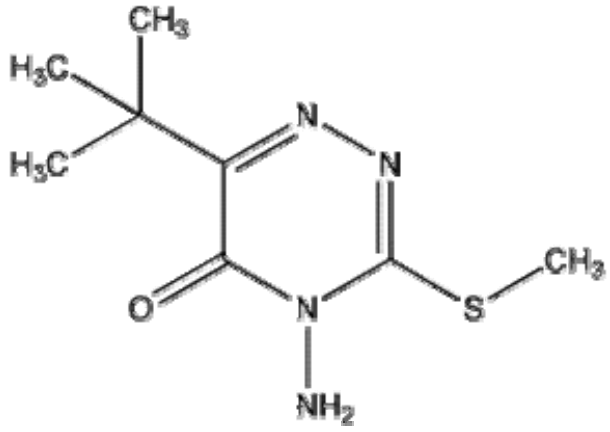
Injury symptoms include chlorosis of leaf tissue followed by necrosis of the tissue. These herbicides are taken up into the plant via roots or foliage and move in the xylem to plant leaves. As a result, injury symptoms will first appear on the older leaves, along the leaf margin.



A



B



C

Figure 1.2 Chemical structures of chlorpyrifos (A), linuron (B), metribuzin (C)

1.7.3 General review of degradation of pesticides used in current study

Chlorpyrifos

At the present time, the most widely used pesticides belong to the organophosphorus group which account for ~38% of total pesticides used globally (Post, 1998). In the USA alone over 40 million kilos of organophosphorus applied annually. Glyphosate and chlorpyrifos are the most widely used in the USA and accounts for 20 and 11% of total pesticide use, respectively (EPA, 2004). Recently, the Environmental Protection Agency (EPA) and the manufacturers of chlorpyrifos have agreed to eliminate all household applications of the insecticide, but agriculture use continues (Jones&Huang, 2003).

The environmental fate of chlorpyrifos has been studied extensively. Degradation in soil involves both chemical hydrolysis and microbial activity with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product (Singh & Walker, 2006). Chlorpyrifos has been reported to be resistant to the phenomenon of enhanced degradation (Racke *et al*., 1990). There have been no reports of enhanced degradation of chlorpyrifos since its first use in 1965 until recently. It was suggested that the accumulation of TCP, which has antimicrobial properties acts as buffer in the soil and prevents the proliferation of chlorpyrifos degrading microorganisms (Racke *et al*., 1990).

Recently, chlorpyrifos has been reported to be degraded by several bacteria. Ohshiro *et al*. (1996) reported that *Arthrobacter* sp. Strain B-5 hydrolyzed chlorpyrifos at rates depend on substrate. Mallick *et al*. (1999) reported that chlorpyrifos (10 mg L^{-1}) was completely degraded in the mineral salts medium by *Flavobacterium* sp. (ATCC 27551) for 24h and *Arthrobacter* sp for 48h, respectively. Singh *et al.,* (2004) also reported that chlorpyrifos could be degraded by *Enterobacter* sp. B-14. This bacterium degrades chlorpyrifos to diethylthiophosphate (DETP) and 3,5,6-trichloro-2 pyridinol (TCP) and utilizes DETP as a sole source of carbon and phosphorus. Many studies also

showed that chlorpyrifos in pure cultures and soil could be degraded by *Fusarium* LK. ex Fx (Wang *et al*., 2005), *Alkaligenes faecalis* DSP3 (Yang *et al*, 2005a). Li *et al.,* (2007) isolated from the polluted treatment system of a chlorpyrifos manufacturer a bacterium which was indentified as *Sphingomonas* sp. and utilized chlorpyrifos as its sole source of carbon for growth, by hydrolyzing it to 3,5,6-trichloro-2 pyridinol (TCP). Later, in 2008, the same scientists reported novel chlorpyrifos degrading bacteria which were identified as *Stenotrophomonas* sp., *Bacillus* sp. and *Brevundinomas* sp. A newly isolated *Paracoccus* sp. strain TRP, isolated from activated sludge could biodegrate both chlorpyrifos and 3,5,6-trichloro-2 pyridinol (TCP) (Xu *et al.,* 2007).

Fungal mineralization of chlorpyrifos by *Phanerochaete chrysosporium* was reported by Bumpus *et al.,* (1993). Two other white rot fungi *Hypholoma fasciculare* and *Coriolus versicolor* were able to degrade about a third of the poorly available chlorpyrifos in biobed matrix after 42 days (Bending *et al*., 2002). Also, several species of *Aspergillus, Trichoderma harzianum and Penicillium brevicompactum* were reported to utilize chlorpyrifos as sources of phosphorus and sulphur (Omar, 1998). Fang *et al.*, (2007) reported the degradation characteristics by an isolated fungal strain *Verticillium* sp., DSP in pure cultures in soil and on pakcoi (*Brassica chinensis* L.). Finally, Xu *et al.* (2007) reported mineralization of chlorpyrifos by co-culture of a bacterial strain (*Settaria* sp.) that could transform chlorpyrifos to 3,5,6-trichloro-2 pyridinol (TCP) and a TCP mineralizing fungal strain (*Trichosporon* sp.).

Linuron

Although (photo)chemical and physical processes may be involved in the removal of linuron, biodegradation is reported to be the most significant mechanism for its dissipation from soil (Caux,1998). Several enrichement cultures have indeed been obtained that use linuron as the sole source of nitrogen and carbon. Although these consortia rapidly mineralized linuron, isolation of the key organisms seemed to be difficult (Dejonghe, 2003).

Cullington &Walker (1999) isolated *Bacillus sphaericus* ATCC 12123 and *Arthrobacter globiformis* D47, respectively, which can use the alkyl chain of linuron as the sole N and C source. However these pure strains can only partially degrade linuron, since 3,4-dichlooroaniline (3,4-DCA), one of the main potential metabolites in the degradation pathway of linuron, accumulated in the medium. In 2003, Dejonghe for first time reported that the *Variovorax* strain degraded linuron further than the aromatic intermediates and was able to use linuron as the sole source of C, N and energy. In 2005, Sorensen *et al.,* reported that from a bacterial community from Danish agricultural soil which was enriched with linuron as sole carbon and nitrogen mineralized it completely. A *Variovorax* sp. was responsible for mineralization activity.

Metribuzin

Metribuzin have received a great deal of attention because of its extensive use and potential for widespread contamination of ecosystems (Lawrence *et al.,* 1993). The fate of this pesticide has been extensively studied under aerobic conditions (Pavel *et al.*, 1999). Half-lives range between 17- 377 days. In soil metribuzin is transformed to deaminated metribuzin (DA), diketometribuzin (DK), deaminated diketometribuzin (DADK) and other unidentified metabolites (Pavel *et al.*, 1999). Under anaerobic conditions the rates of degradation were more rapid in the surface than in the subsurface soil microcosms.

The major mechanism by which metribuzin is lost from soil is microbial degradation (Pavel *et al.*, 1999). The influence of soil microbiological activity on the degradation rate of metribuzin was demonstrated by several researchers (Bordjiba *et al.*, 2001). The partial transformation of metribuzin by a strain of *Streptomyces* was reported by Sheltton *et al.*, in 1996. Experiments were conducted to assess the ability of *Streptomyces* (strain PS1/5) to metabolize twelve herbicides representing several different classes including: acetanilides, triazines, ureas, uracils, and imidazoles. Incubations

in aqueous culture with dextrin as carbon source and either ammonium or casamino acids as nitrogen source resulted in transformations (> 50%) of eight of the herbicides tested: alachlor, metolachlor, atrazine, prometryne, ametryne, linuron, tebuthiuron, and bromacil; the remaining four herbicides (cyanazine, diuron, metribuzin, and imazapyr) were also transformed, but to a lesser extent.

Fungi degradation was studied in five species by Schilling *et al.* in 1985. The results obtained in liquid medium showed a total transformation by *Rhizopus japonicus* and *Cunningghamella echinulata* and a depletion rate range of 27 to 45% with *Aspergillus niger, Penicillium lilacinum* and *Fusarium oxysporum* after a 4 week incubation period. In a more recent study by Bordjiba *et al.*, in 2001, only three from 53 tested fungi species, the species *Botrytis cinerea, Sordaria superba* and *Apsidia fusca* removed more than 50% of metribuzin from liquid medium.

1.8 Environmental factors affecting bioremediation by fungi and bacteria

The control and optimization of bioremediation processes are a complex system of many factors (Vidali, 2001). These factors include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environmental factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors and nutrients). Optimum environmental conditions for the degradation of contaminants are reported in Table 1.3.

Table 1.3 Environmental conditions affecting degradation (from Vidali, 2001).

Parameters	Conditions required for microbial activity
Soil moisture	25-28% of water holding capacity
Soil pH	5.5-8.8
Oxygen content	Aerobic, minimum air filled pore space of 10%
Temperature (^{0}C)	15-45
Contaminants	Non too toxic
Heavy metals	Total content 2000 ppm
Type of soil	Low clay or silt content

1.8.1 Temperature, oxygen and nutrient availability

There is evidence that the soil environment can dramatically affect persistent organic pollutants. The soil pH, nutritional status and oxygen levels temperature vary and may not always are optimal for fungi and bacteria growth or enzyme production for pollutant transformation (Singleton, 2001).

There are many reasons for organic compounds being degraded very slowly or not at all in the soil environment, even though they are *per se* biodegradable (Romantschuk *et al.*, 2000). One reason could be strong pesticide sorption to soil and therefore decreased bioavailability (Alexander, 1994). The soils, which absorbed the chemicals to the greatest extent, showed the poorest degradation (Singleton, 2001). Another reason can be the low temperatures in soil, particularly in Northern parts of Europe and North America where soil temperatures during a large part of the year are too low for efficient microbial degradation of contaminants. The same may also be true for deeper soil layers (Romantschuk *et al.*, 2000). Most microbes isolated and studied degrade optimally at mecophilic temperatures. Their

ability to degrade xenobiotics at temperature higher or lower than 25 ^{0}C to 37^{0}C is often overlooked (Singleton, 2001).

Further, there are many environments, which are acidic or alkaline so a fruitful area of research could be the isolation and study of microorganisms, which can degrade at more extreme pH values. The solubility of compounds at different pH values will also be involved in determining the rate of degradation (Singleton, 2001).

It is now well established that organic pollutants are degraded under both aerobic and anaerobic conditions. Initially, most work concentrated on aerobic metabolism as aerobes were easier to culture than anaerobes and were considered to have a much more versatile metabolism (Singleton, 2001). Fungal degradation is very slow under oxygen restrictions resulting in partial degradation with resultant toxic intermediates being formed (Romantschuk *et al.*, 2000). In the natural environment both aerobic and anaerobic situations are commonly encountered and therefore it is essential that both anaerobic and aerobic metabolic processes have to be studied (Singleton, 2001).

1.8.2 Water availability and water potential

While soil is often measured in total water content, microbial activity depends on the fraction that is actually available for microbial growth. Thus it is important to consider the potential for bioremediation activity relevant to those conditions under which microbial activity can occur. While the wilting point of plants is about -1.4 MPa water potential microbial, especially fungal activity can occur over a much wider range of conditions (Magan, 2007).

In terrestrial ecosystems, water availability can be expressed by the total water potential (Ψt), a measure of the fraction of total water content available for microbial growth in pascals (Magan, 2007). This is the sum of three factors: (a) osmotic or solute potential (Ψs) due to the presence of ions or

other solutes, (b) matric potential (Ψm) due directly to forces required to remove bound to the matrix (e.g. soil) and (c) turgor potential of microbial cells balancing their internal status with the external environment (Jurado *et al.*, 2008).

Water activity (a_w) is an alternative measure to (Ψt) and is defined as the ratio between the vapour pressure of water in a substrate (P) and vapour pressure of pure water (Po) at the same temperature and pressure. Thus a_w = P/Po. The a_w of pure water is 1.0 and any substrate with no free water will subsequently have an a_w less than 1. It is equivalent to the equilibrium relative humidity (ERH) of the atmosphere, with pure water having an E.R.H of 100% (Magan, 1997).

All microorganisms possess a positive turgor, and maintenance of this outward-directed pressure is essential since it is generally considered as the driving force for cell expansion. Exposure of microorganisms to high osmolality environments triggers rapid fluxes of cell water along the osmotic gradient out of the cell, thus causing a reduction in turgor and dehydration of the cytoplasm. To counteract the outflow of water, microorganisms increase their intracellular solute pool by amassing large amounts of organic osmolytes, the so-called compatible solutes (Kempf and Bremer, 1998).

These have been defined by Jennings and Burke (1990) as compounds that are able to change in concentration in the cell in response to a change in external water potential, thus maintaining turgidity while having no significant effect on enzyme activity (Ramirez *et al.,* 2004).

Experimentally, variations of water potential can be created by addition of ionic solutes, such as KCl and NaCl, or non-ionically with glycerol addition to a solution or culture media.
Table 1.4 gives the relationship between the different measures for water availability.

Table 1.4 Equivalent water activity, equilibrium relative humidity and water potential at 25^0C (Magan, 1997).

Water activity	E.R.H. (%)	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.5
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.3
0.60	60	70.3

1.9 Toxicity of bioremediation products

To fully evaluate the environmental impact of pesticides, both physicochemical and toxicological analyses should be performed (EPA, 1993). Physicochemical analyses do not provide information about the toxicity of environmental samples, for interactions of the complex mix of compounds can occur, so toxicity detection is crucial in assessing environmental contamination (Ruiz *et al.,* 1997). Toxicity tests are, also, important tools to assess to what extent the bioremediation process was effective as its goal is to at least reduce pollutants levels to undetectable, non toxic levels.

Some of the tests available in the market use terrestrial organisms in their original medium and in this case the organisms are in direct contact with the contaminated soil. For example the ISO11267 standard test is used for assessing the effect of chemicals on the reproductive output of *Folsomia candida* (Collembola). Such direct tests, however are relatively time consuming (48 hrs to 30 or more days), expensive, often require extensive preparation (Perez *et al.,* 2001) and depend on the continuous culture and maintenance of the test species (Pauli and Berger, 2000).

Culture free microbiotests with organisms from different trophic and functional levels, e.g. bacteria, algae, rotifers and crustaceans, are presently available.

1.9.1 Protox

A new, ready to use is the multi-generation microbiotest with the standard ciliate *Tetrahymena thermophila,* a representative from a further ecologically relevant group - the protozoa. The latter constitute a major link between bacteria and metazoa, playing a key role in the natural microbial loop and as consumers in the artificial system of waste water treatment (Pauli and

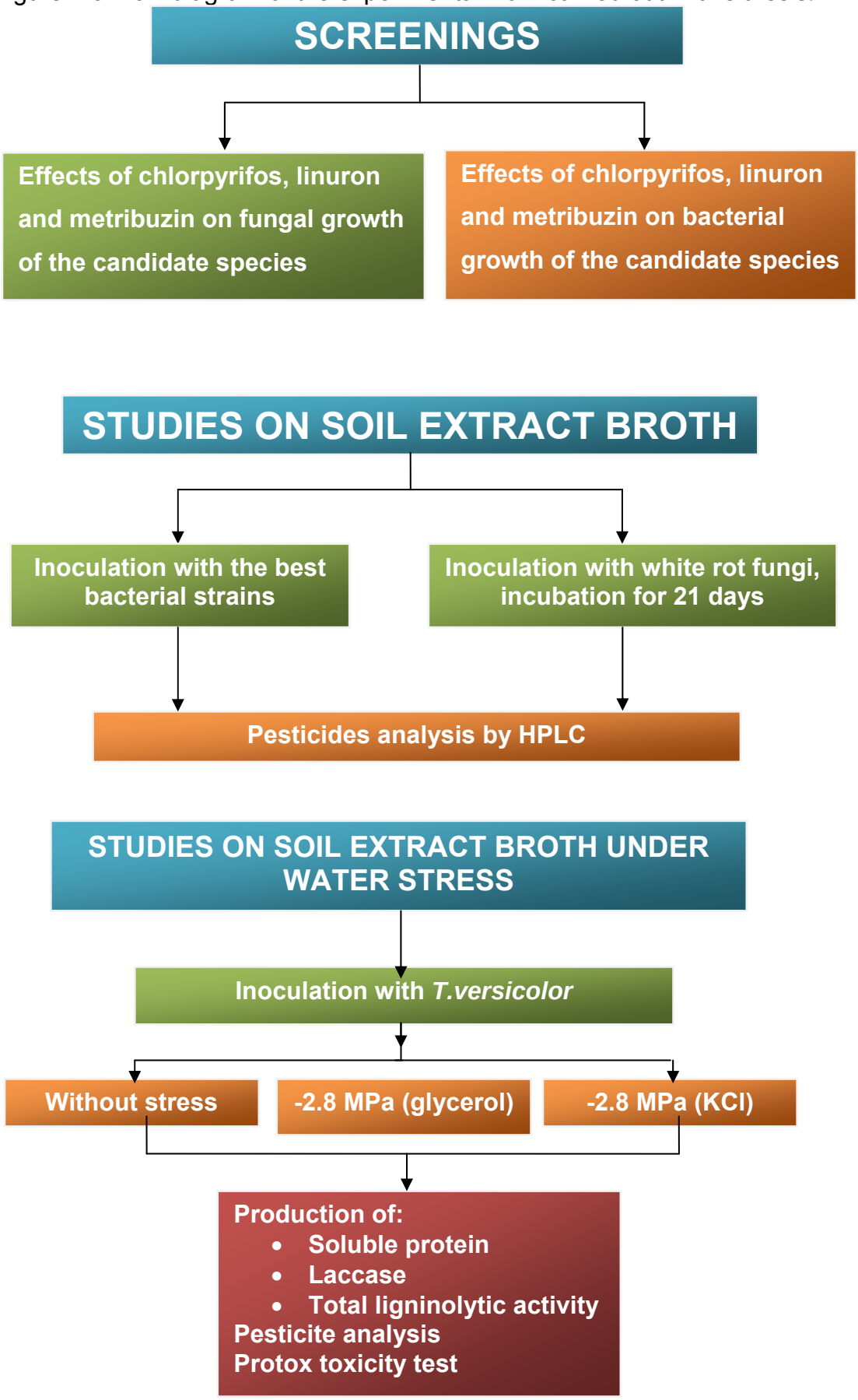
Berger, 2000). Moreover, it is a true eukaryotic cell, which can be easily cultured with a short generation time in axenic defined medium (Pauli and Berger, 2000).

This test is under consideration by Organisation for Economic Cooperation and Development (OECD) as an OECD Guideline. Fochtman *et al.,*(2000)*,* studied the potential effects of pesticides on aquatic life using thirteen widely used and registered pesticides in Poland, with potassium dichromate as a reference chemical, as well as seven different test species (ranging from protozoan to fish) among them Protoxkit. Based on their results, they suggested the use of Protoxkit as a valuable microbiotest. In another study Pauli and Berger, (2000) demonstrated based on a heterogeneous set of chemicals that toxicological results with two *Tetrahymena* species, *T. pyriformis* and *T. thermophila*, reveal a highly significant correlation (r=0.928, n=52) and nearly equal susceptibility of species. Comparisons of toxicological literature data from the International Uniform ChemicaL Information Database (IUCLID) indicate a similar toxic response even for distant ciliate relatives. Data from IUCLID also reveal that ciliate toxicity data are supplementary to those of the standard activated sludge respiration inhibition. Ciliate tests could hence serve as a powerful tool for the prediction of possible hazards to sewage treatment processes.

1.10 Aims and objectives of this project

- Examine the potential for degradation of mixtures of the pesticides chlorpyrifos, linuron and metribuzin by a range of bacteria and fungi
- Relate this capability to enzyme production
- Quantify the differential rates of degradation of mixtures of these pesticides

Figure 1.3 Flow diagram of the experimental work carried out in this thesis.



2 MATERIALS AND METHODS

2.1 Fungal inoculants

All the isolates used through these experiments were white rot fungi, which were obtained from the Applied Mycology Group, Cranfield Health (Table 2.1). Isolates were kept as solid agar Petri plate cultures on 3% malt extract agar (MEA) and were subcultured every two months. None of these strains produce toxins or other.toxic metabolites.

Table 2.1 Isolates, used in this study, and their reference number

Species	Reference
Trametes versicolor	R26
Phanerochaete chrysosporium	R170
Pleurotus ostreatus	R14
Phlebiopsis gigantea	R174
Pycnoporus coccineus	R180

2.2 Bacteria

Nineteen strains of *Bacillus* spp. were used in this study. These strains were obtained from the collection at the Laboratory of Plant Bacteriology, Technological Institute of Crete. They were all isolated from Cretan soils, and different composts. All these strains were successfully used as biological agents against bacterial and fungal diseases of plants

Moreover, the strains Pf2, Pf545 and Pf804 of *Pseudomonas fluorescens* and the strain *Pseudomonas putida* were tested in order to investigate the capability of these bacterial strains to tolerate mixture of the pesticides chlorpyrifos, linuron and metribuzin and degrade them. *Pseudomonas putida* was provided by University of Crete while the other strains by the Laboratory of Plant Bacteriology, Technological Institute of Crete. *Pseudomonas putida* is a saprophytic soil bacterium that can survive and function in the environment. It has been certified as a biosafety host for the cloning of foreign genes.

2.3 Media and substrates

2.3.1 Malt Extract Agar (MEA)

This was prepared by dissolving 20 g MEA (Lab), (Malt Extract 17 g/L, Mycological peptone 3 g/L) and the addition of 15 g agar in 1 L distilled water.

2.3.2 Minimal salts Medium Yeast (MMY)

The ingredients of the medium were: $MgSO_4$ 0.5 g, $NaNO_3$ 2 g, K_2HPO_4 0.14 g, KCl 0.5 g, $FeSO_4$ x $7H_2O$ 0.01 g, Yeast 0.5 g, Agar 20 g in 1 L distilled water.

2.3.3 MMY plus glucose

It contained the above ingredients plus 20 g glucose.

2.3.4 Trypticase Soy Blood (TSB)

(Casein peptone 17 g, Soy peptone 3 g, Sodium chloride 5 g, Dipotassium phosphate 2.5 g, Dextrose 2.5 g per litre of distilled water (Hispanlab). It was prepared according to the manufacturer's instruction.

2.3.5 Bovine Serum Albumin (BSA)

Ampules were used, each containing 1 mL of a solution consisting of 1mg/mL bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide as a preservative.

2.3.6 ABTS (2, 2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (A-1888, Sigma)

It was prepared according to the manufacturer's instruction.

2.4 Pesticides

Analytical grades of each pesticide: chlorpyrifos (O, O-diethyl1 O-3, 5,6-trichloro-2-pyridyl phos)-phorothioate) MW: 350.62, linuron (1-methoxy-1-methyl-3 (3,4-dichlorophenyl) urea) MW: 249.1 and metribuzin (1,2,4-Triazin-

5 (4H)-one,4-amino-6-(1,1-dimethylethyl)-3-(methylthio) MW:214.29 were obtained from Sigma-Aldrich Company.

Stock standard solutions, 2000ppm, were prepared by dissolving analytical standards in methanol and storing them in amber bottles at 4 ^{0}C. In this study xenobiotic concentrations are expressed in ppm (mg L^{-1}).

2.5 Fungi

2.5.1 A comparison of growth of white rot fungi in the presence of pesticide treatments on different culture media

For this purpose, malt extract agar, MMY minimal salts medium and MMY plus glucose were modified by the addition of chlorpyrifos, linuron and metribuzin individually at concentration of 10 ppm and were added to the molten agar, thoroughly mixing. A volume about 15.0 ml of each of them was poured into 9 cm diameter Petri plates. All plates were inoculated with a 4-mm agar plug from the margin of a growing colony of each fungal isolate and incubated at 25^0C. Experiments were carried out with three replicates per treatment.

2.5.2 A comparison of white rot fungi tolerance to single pesticides and mixture of them in vitro, using a minimal salts medium

In this experiment the minimal salts medium was modified by the addition of the three pesticides individually at 30ppm or as a mixture of pesticides at 15 and 30ppm. All fungi were tested at 25^0C. Growth was measured regularly for up 16 days taking two diametric measurements at right angles to each other. The temporal growth was used to obtain the growth rates from the regression lines of the radial mycelial extension, as well as the % in growth inhibition. Experiments were carried out with three replicates per treatment.

2.5.3 Screening of lignin degradation and polyphenoloxidase production

(a) Plate assay to assess lignin degradation

In order to assess which isolates could degrade lignin, the fungi were grown in a lignin medium described by Sundman and Nase (1971). The medium was prepared with 0.25 g alkaline lignin, 5 g of glucose, 5 g ammonium tartrate, 1 g malt extract, 0.5 g $MgSO_4$.7H2O, 0.01 g $CaCl_2$.2H2O, 0.1 g NaCl, 0.01 g $FeCl_3$, 1 mg of thiamine, 20 g of agar in a litre of distilled water, with pH adjusted to 4.5. To examine the enzyme production potential in the presence of the pesticides this medium was supplemented with chlorpyrifos, linuron and metribuzin, individually at 30 mg $L^{-1}$and as a mixture at 0, 15 and 30 mg L^{-1} (total concentrations). The test isolates were centrally inoculated and incubated at 25^0C for 8 days. After this period they were developed by flooding with a reagent containing equal parts of 1% aqueous solution of $FeCl_3$ and $K_3[Fe(CN)_6]$. A positive result was indicated by clear zones under or around the growth area of the lignin degrading fungi. The activity halo was measured, taking two diametric measurements at right angles to each other, for quantification of lignin degradation. The greater the clearing area, the more intense the lignin degradation.

(b) Plate assay to assess polyphenol oxidase activity

The formation of a brown halo produced by fungi on tannic acid agar reflects laccase (oxidise o- and p-phenols) and catechol oxidase (oxidise o-phenols). These activities were detected after inoculation of the isolates in culture media containing 1% tannic acid, 2% Czapek Dox modified media (containing sodium nitrate 2 g L^{-1}; potassium chloride 0.5 g L^{-1}; magnesium glycerophosphate 0.5 g L^{-1}; ferrous sulphate 0.01 g L^{-1}; potassium sulphate 0.35 g L^{-1} and sucrose 30 g L^{-1}) and 2% of bacto-agar. The tannic acid solution was adjusted to pH 4.5. Medium and acid solutions were autoclaved separately and mixed after cooling down to around 50^0C. To examine the

enzyme production potential in the presence of the pesticides this medium was supplemented with chlorpyrifos, linuron and metribuzin individually at 10 mg L^{-1} and 20 mg L^{-1} and as a mixture at 10 and 20 mg L^{-1} (total concentrations). The test fungi were centrally inoculated and incubated at 25^{o}C for 8 days. A positive activity was indicated by a brown coloration of the culture media around the colony (Rigling, 1995). The activity halo was measured, taking two diametric measurements at right angles to each other, for quantification of enzyme production. The greater the halo, the more intense the enzymatic activity.

2.6 Bacteria

2.6.1 A comparison of bacterial tolerance to single and mixtures of pesticides *in vitro* in conducive culture medium

This study assessed the tolerance level of 19 strains of *Bacillus* spp. to chlorpyrifos, linuron and metribuzin and their mixtures. These strains were obtained from the collection at the Laboratory of Plant Bacteriology, Technological Institute of Crete.

An appropriate volume of stock solution of each pesticide was added aseptically to give the final pesticide concentrations of 30, 60, 90, 120, 150 ppm. 0.2 ml aliquots of each bacterial suspension, containing approximately 1x10^{5} CFU/ ml, were used to inoculate fresh liquid cultures (5 ml) of TSB (rich medium) plus the different pesticides in tubes (10 ml). These cultures were incubated at 25^{0}C on a platform shaker at 150 rev min^{-1}. Bacterial growth was determined by measuring optical density at 600 nm after 24 hours incubation.

Subsequently, triplicate universal bottles (30ml) containing 5 ml of TSB plus the three pesticides in mixtures of 30 and 60 ppm total concentrations were inoculated with the strains that have the best tolerance to the pesticides individually and incubated as described above.

PART II STUDIES ON SOIL EXTRACT

2.7 Preparation of soil extract broth

A soil extract medium was used in this study. This medium was prepared with a sand clay loam soil from Heraklion, Crete, containing 54.6 % sand, 21.2 % silt, 24.2 % clay, 1.12% organic matter and pH 7.6 (analyzed by the laboratory of Soil Science at Technological Institute of Crete). Soil extract was prepared by mixing soil and distilled water in a 1:1 (w/v) ratio and sterilized for 30 min at 121^{0}C. The supernatant was centrifuged and then autoclaved again (30 min at 121 ^{0}C). Soil extract is a liquid broth. Technical agar (2% W/V) was added to the liquid medium in order to solidify.

Soil extract agar was modified by the addition of chlorpyrifos, linuron and metribuzin individually at concentration of 5, 25 and 625 ppm or as a mixture of pesticides in the range 0-70 ppm (total concentrations) by addition to the molten agar, thoroughly mixing and pouring into 9-cm Petri plates. All test fungi were tested at 25 ^{0}C. Growth was measured regularly for up 14 days. The effective dose (ED) for 50% inhibition of growth (EC_{50} values), relative to the control s was calculated for each fungus.

2.8 Inoculation with white rot fungi in a soil extract liquid broth

In this study only three fungal isolates were used: *Trametes versicolor, Phlebiopsis gigantea* and *Pycnoporus coccineus.*

2.8.1 Incubation conditions

A soil extract liquid broth was used in this study which was supplemented with a mixture of chlorpyrifos, linuron and metribuzin to give final concentrations 0, 10, 20, 30, 40 and 50 ppm. Aliquots of 16 ml were transferred to 9-cm Petri dishes, which were inoculated with a 6-mm agar

plug from the margin of an active fungus culture, which had been incubated on a plate of malt extract agar and active growth had commenced. Uninoculated controls were also set up for each pesticide. There were three replicates per pesticide/fungus treatment and control and incubated at 25^0C. After 21 days, the mycelium was filtered through Whatman No.1 paper filters. The fresh liquid was frozen at -20^0C and later used for pesticide quantification by HPLC.

2.8.2 Pesticide analysis

Samples were filtered through 0.2µm filters prior to HPLC quantification of all three pesticides. This was performed with an Agilent Technologies 2100 HPLC system equipped with a DAD detector an auto sampler and a C-18 column (250 mm ×4 mm). The column was operated at ambient temperature with a flow rate of 0.8 ml min^{-1} and an injection volume of 20µl.

An isocratic mobile phase system was established using acetonitrile: water at a ratio of 60: 40. This ratio enabled the separation and quantification of chlorpyrifos, linuron and metribuzin in a single HPLC run of 8 min with linuron eluting at 249 nm, chlorpyrifos at 251 nm and metribuzin at 293 nm. The retention times were 5.5, 5.2 and 3.2 minutes respectively. The limit of detection for the three pesticides was less of 0.1 mg L^{-1}. Standard curves were constructed for each standard in soil extract broth and *r*-squared values for each curve found to be> 0.99 for all three pesticides (Figure 2.1).

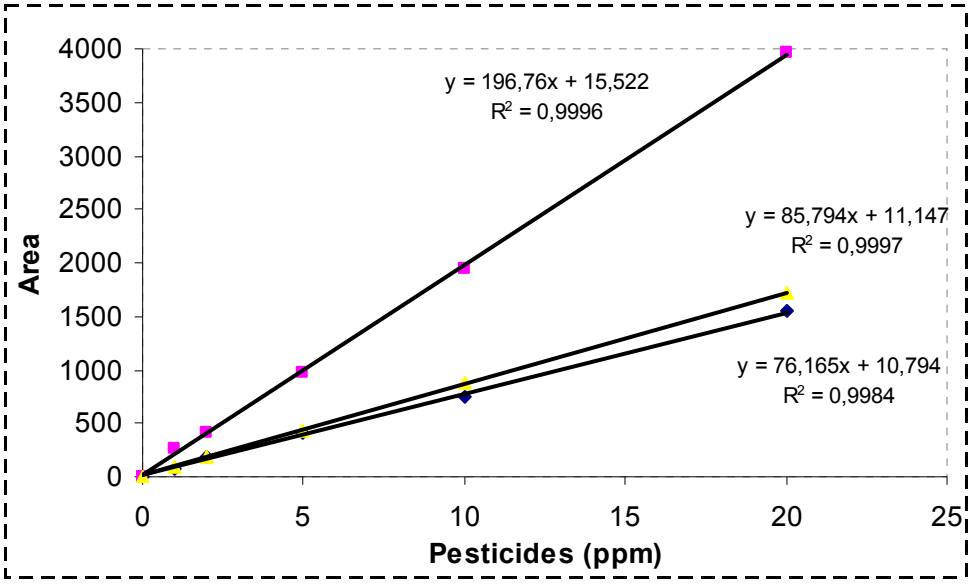


Figure 2.1 Standard curves for a mixture of chlorpyrifos, linuron and metribuzin in soil extract broth and *r*-squared values for each curve (linuron ■, metribuzin ▲ and chlorpyrifos ♦).

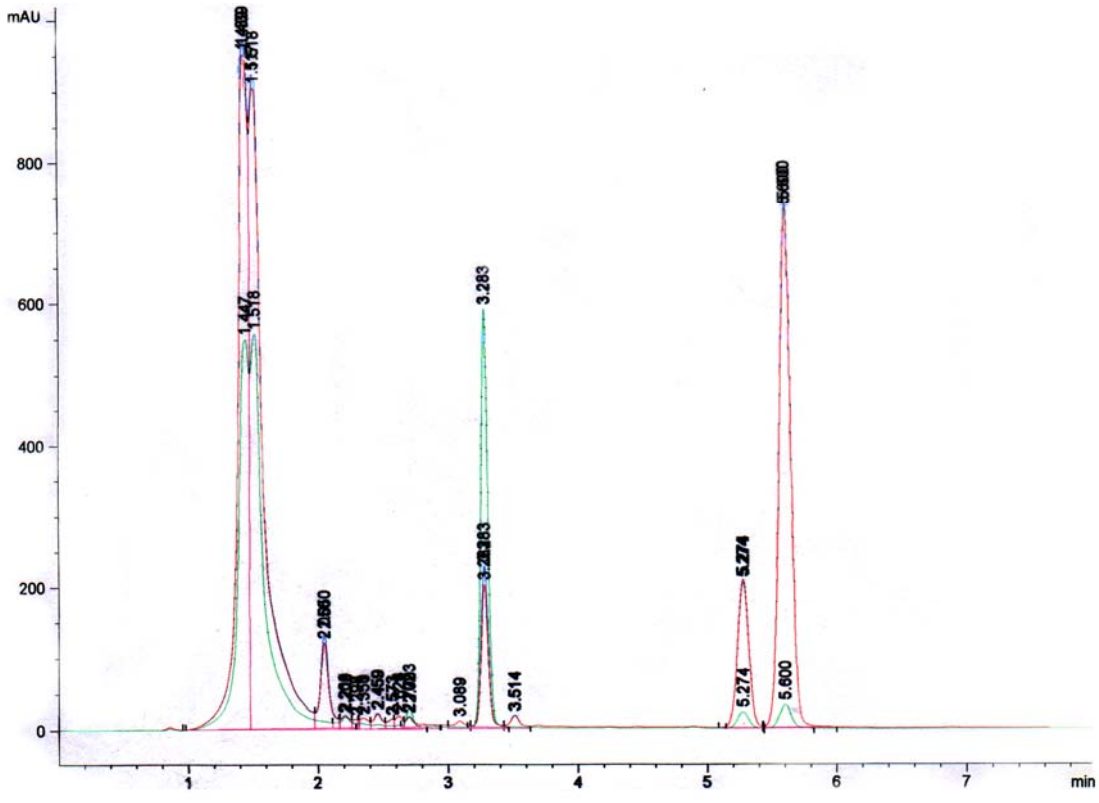


Figure 2.2 Chromatogram of metribuzin, chlorpyrifos and linuron obtained in single HPLC run with metribuzin eluting at 3.2, chlorpyrifos at 5.2 and linuron at 5.5 minutes in a mixture of 50ppm of the pesticides (equal in amount).

Moreover, chlorpyrifos was detected by HPLC using an isocratic mobile phase system with acetonitrile and water at a ratio of 80: 20 (protocol of

chlorpyrifos determination by Sigma). The column was operated at ambient temperature with a flow rate of 1.4 ml min^{-1} and an injection volume of 20µl. Standard curve was constructed for chlorpyrifos in soil extract broth and *r*-squared value found to be> 0.99 (Figure 2.2).

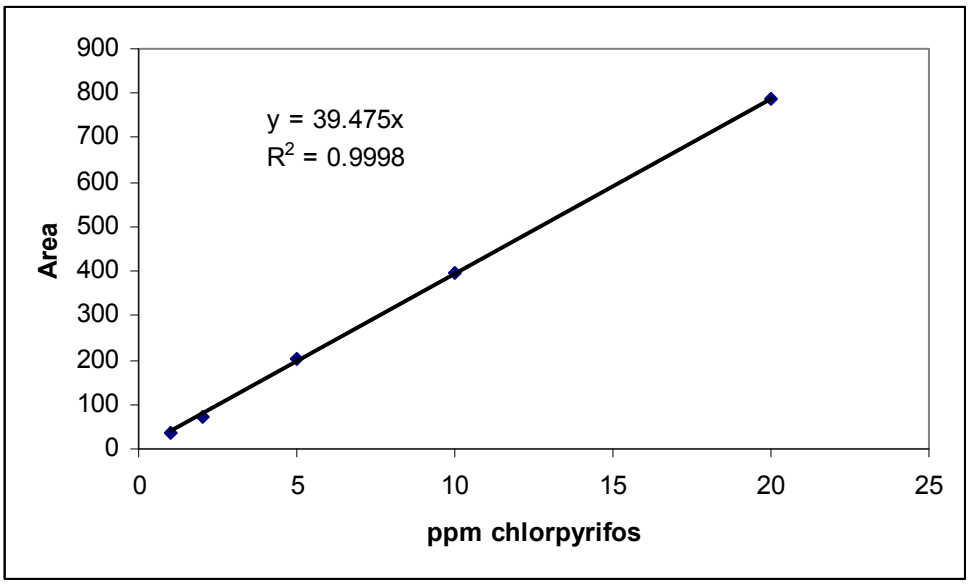


Figure 2.3 Standard curve for chlorpyrifos in soil extract broth and *r*-squared value (protocol of chlorpyrifos determination by Sigma).

Chlorpyrifos is a degradable compound, and a number of environmental forces may be active in its breakdown. In all systems (soil, water, plants and animals), the major pathway of chlorpyrifos degradation begins with cleavage of the phosphorus ester bond to yield 3,5,6-trichloro-2-pyridinol (TCP). In soils and water, TCP is further degraded via microbial activity and photolysis to carbon dioxide and organic matter (Dow Agrosciences).

TCP was detected by HPLC at 235 nm using a gradient mobile phase system mentioned below:

Eluent A: acetonitrile

Eluent B: water + 0.1% phosphoric acid

Gradient:	time (min)	% A	% B
	5	30	70
	15	80	20
	25	stop	

The column was operated at ambient temperature with a flow rate of 1.4 ml min^{-1} and an injection volume of 20µl (protocol of TCP determination by Sigma). Standard curve was constructed for TCP in soil extract broth and *r*-squared value found to be quite good (Figure 2.3).

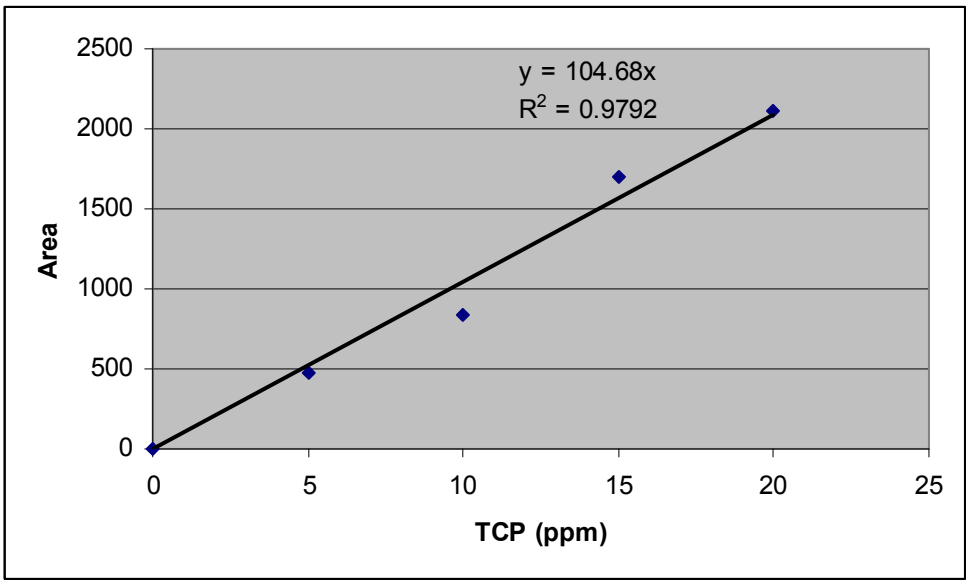


Figure 2.4 Standard curve of the main metabolite of chlorpyrifos 3,5,6-trichloro-2 pyridinol (TCP) in soil extract broth and *r*-squared value.

2.9 Inoculation with bacteria in a soil extract liquid broth

In this study, the strains 13,15 and 18 of *Bacillus subtilis,* the stains Pf2, Pf545 and Pf804 of *Pseudomonas fluorescens,* the strain *Pseudomonas putida* and mixture of all of them were tested for their potential capacity to degrade a mixture of 60 ppm (total concentration) of the pesticides chlorpyrifos, linuron and metribuzin.

2.9.1 Incubation conditions

Inoculates were prepared from cultures of the strains grown on soil extract agar medium containing the mixture 60 ppm of the pesticides for 48 hours at 25^0C, in order to maximize the degrading potential of the strains. Growths were washed from the plates and the OD_{600} of each bacterial suspension was measured. Subsequently, suitable aliquots of the cultures that

corresponded to a density of 10^8 cells ml^{-1} were transferred into soil extract liquid broth (30 ml) supplemented with a mixture of 60 ppm (total concentration) of the three pesticides and the OD_{600} was measured. Uninoculated controls were also set up. There were three replicates per pesticide/bacterial treatment and control.

Then, the cultures were incubated at 25^0C on a platform shaker at 150 rev min^{-1}. Every three hours the OD_{600} was measured and suitable aliquots were filtered through 0.2μm filters and frozen in order to HPLC quantification of the three pesticides. The pesticides were determined as described previously in section 2.8.2.

2.10 Inoculation with *Trametes versicolor*

In this study, only the fungus *Trametes versicolor* was used which was selected because it showed tolerance and ability to degrade the pesticide linuron. In order to evaluate the effect of water stress and the laccase production over a six week period, the soil extract liquid broth solute potential was adjusted in two different ways: ionically by adding potassium chloride and non-ionically by using glycerol. In order to investigate the abiotic degradation of the pesticides over a six weeks period of incubation, plates, in triplicate, with the mixtures of pesticides without inoculation with *T. versicolor* were prepared and were used as controls. In all assays Petri plates with the soil extract were prepared as described previously (2.8.1).

The water potential of the soil extract broth was adjusted to -2.8 MPa ($0.98a_w$) by adding 3.73 g of the ionic solute potassium chloride or 7.3 ml glycerol to 100 ml of soil extract.

2.11 HPLC quantification of pesticides

Pesticide working solutions (2,000 ppm) were prepared by dissolving analytical reagents in methanol and storing in amber bottles at 4^0C. An appropriate volume of stock solution of each pesticide was added aseptically to give final mixtures of the pesticide concentrations of 0, 10, 20, 30, 40, 50 ppm. The pesticides were determined as described previously in section 2.8.2.

2.12 Total ligninolytic activity

The poly R-478 (polyvinyl sulfonated backbone with anthrapyridone chromophore, violet colour) decolouration assay was used to study the overall ligninolytic activity in the soil extract, following the method described by Glenn and Gold, 1983). Dye poly R-478 (P-1900, Sigma) was added to the filtrates as an aqueous solution to a final concentration of 0.02% with gentle swirling. Directly after its addition, 1ml of the filtrates was removed and diluted 10-fold with water. The reaction mixture was kept under light (desklamp, with a 40w bulb) for 24 h for the enzyme reaction to take place.

Total ligninolytic activity was given as decolouration degree of the Poly R-478 monitoring by the percentage reduction in the absorbance at 530 and 350 nm calculated as follows:

Colour intensity = absorbance at 530 nm/ absorbance at 350 nm

% decolourisation = 100 - [(absorbance at 530 nm/ absorbance at 350 nm) sample / (absorbance at 530 nm/ absorbance at 350 nm) poly R478] x 100

Because fungal adsorption, as well as fungal transformation, reduces the intensity of the dyes in solution, it was necessary to measure soluble dye absorbance at two wavelengths, 530 and 350 nm. The two wavelengths indicated were chosen to produce the greatest change in the absorbance ratio as the dye was degraded Glenn and Gold (1983). A lower absorbance ratio means intense decolouration and higher enzymatic activity.

2.13 Laccase production in the different media

Laccase activity (EC 1.103.2) was determined using ABTS (2, 2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (A-1888, Sigma) as a substrate at 420 nm, based on the protocol described by Buswell *et al*., (1995) with

adaptations by Fragoeiro and Magan (2005). The assay was carried out at ambient temperature, with the ABTS and buffer equilibrated at 37^0C. The reaction mixture, in a total volume of 900 µl, contained 650 µl sodium acetate buffer, pH 5.0, and 100 µl of enzyme extract. The reaction was carried out directly in the spectrophotometer cuvette and initiated by adding 150 µl of 0.55 mM ABTS.

Laccase activity was determined using a spectrophotometer set in kinetic mode. Kinetic mode is a predefined analysis of the absorbance values recorded by the spectrophotometer and the output values are given as (final absorbance- initial absorbance / reaction time (min). After several tests the reaction time was set at three minutes.

Fungal laccases, among other enzymes, oxidize ABTS (green-colored molecule) to the cation radical $ABTS^+$ (dark green-colored molecule) (Pich *et al.*, 2006). The colourimetric changes can be determined by measuring the change in absorbance spectroscopy. The change in absorbance (ΔA) at a particular time interval (Δt) for a particular reaction was calculated by the Lambert-Beer equation.

Boiled enzyme was used in the control sample. One activity unit was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute, using an ε_{420} of $3.6x10^4$ moL L^{-1} $cm^{-1.}$ This assay was first optimised using commercial laccase from *Rhus vernificera*, crude acetone powder, minimum 50 units mg^{-1} solid (L-2157, Sigma), giving a positive result for laccase concentrations as low as 0.05 mg mL^{-1}, i.e.0.675 units per well.

The calibration curve obtained showed a good correlation (R^2=0.974) between the concentration of commercial purified laccase and laccase activity (U) (Figure 3.6).

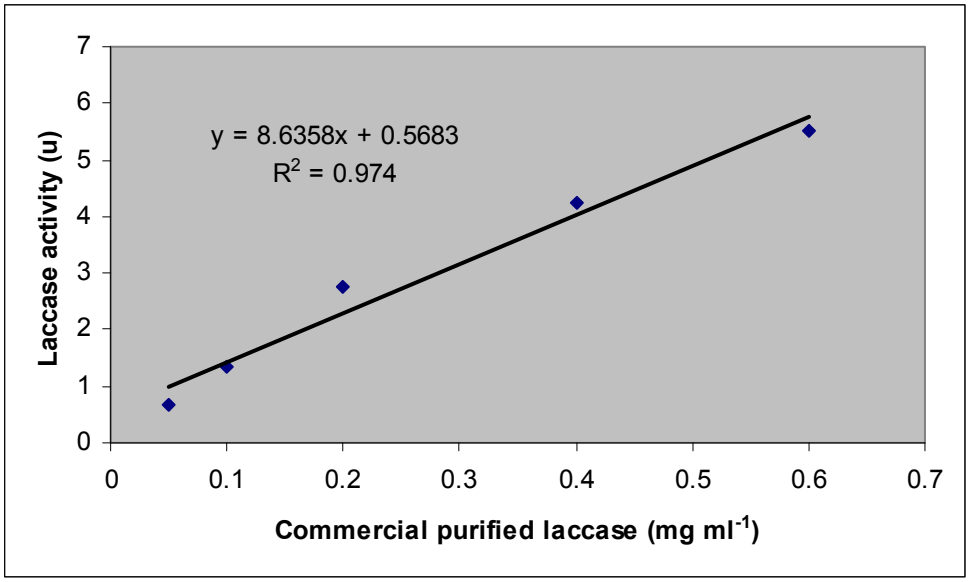


Figure 2.5 Linear regression of laccase activity against concentration of purified laccase from *Rhus vernicifera.*

2.13.1 Soluble protein

Soluble protein content of the enzyme extracts was determined with the Modified Lowry Protein Assay, after trichloroacetic acid (TCA) precipitation. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan and cysteine react with Folin-Ciocalteau reagent, a mixture of phosphotungstic and phosphomolybdic acid in phenol, to produce an unstable product that becomes reduced to molybdenum/tungsten blue. The latter can be detected colourimetrically by absorbance at 625 nm. The higher the concentration of protein, the darker the solution. A standard curve of absorbance versus micrograms Bovine Serum Albumin (BSA) protein was prepared in order to determine protein concentration.

Figure 2.5 shows the calibration curve of absorbance and Bovine Serum Albumin (BSA) protein in concentrations ranging from 200 to 1000 μg mL^{-1}.

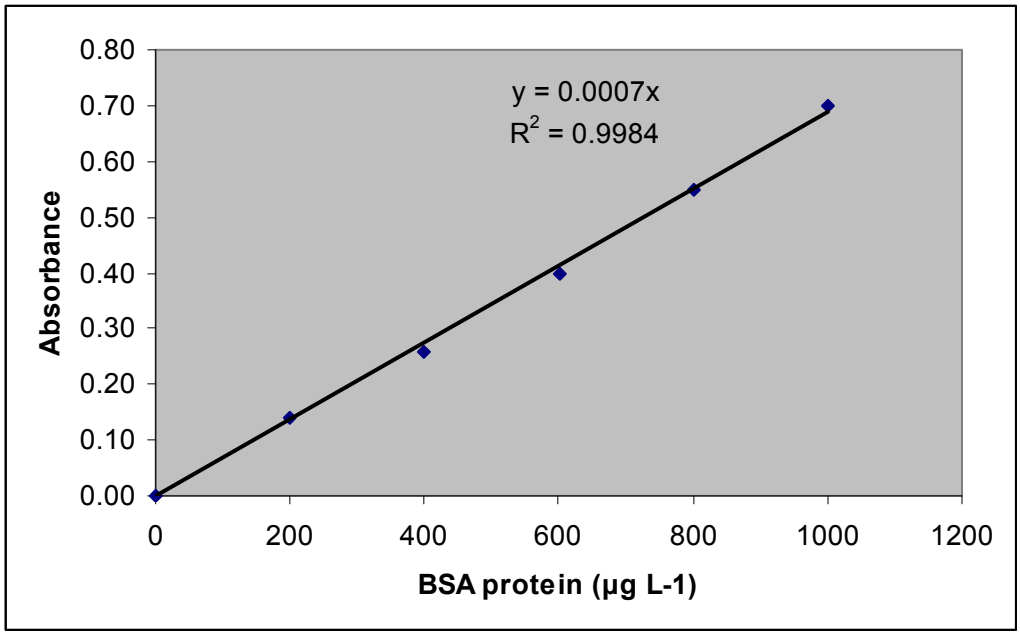


Figure 2.6 Linear regression of absorbance at 625 nm against concentration of Bovine Serum Albumin (BSA) protein (µg mL^{-1}).

2.14 Toxicity test

The toxicity measurements were performed with the PROTOXKIT F^{TM} (MicroBiotests Inc.), a 24h growth inhibition test using the ciliate protozoan *Tetrahymena thermophila.* The test is based on optical density (OD) measurement of the food substrate provided to the ciliates, in 1 cm disposable spectro-photometric cells. Ciliate growth inhibition is reflected by higher turbidity in the test cells containing the toxicant after 24h exposure, in comparison to the controls. The stock culture vial containing the ciliates in a stationary growth phase can be stored at room temperature for several months. The tests are started from the stock culture vial, without any intermediate time loss for hatching or reactivation of the test biota.

Standards solutions of the pesticides were added to soil extract for the toxicity assays. Toxicity of soil extract spiked with 1, 2.5, 5, 7.5, 10, 15 and 20 ppm of chlorpyrifos, linuron and metribuzin was determined and compared against the toxicity of these three pesticides in a mixture at total concentrations of 3, 6, 9, 12, 15, 22.5, 30 and 45 ppm.

Soil extract samples were prepared in the same way as for HPLC analysis (described in section 2.8.2).

After preparing of ciliate inoculum and the food substrate according to the detailed instructions given in the Standard Operational Procedure Manual, 2 ml of each sample, 40µl of the food substrate and 40µl ciliate inoculum were added in the test cells and the cells were closed with their lids. Subsequently and after gentle shaking (by inverting the cells a few times), the optical density (OD) of each test cell was measured at 440nm (= time T0 scorings). Finally, the cells were incubated at 30 ^{0}C for 48 hours as in some cases (which are batch dependent) it is advised to extend the incubation period to 48 hours in order to obtain enough OD decrease in the controls.

After 48 hours incubation the OD of each test cell was measured again (T28h scorings) and the percent of inhibition (%) was determined as described in the following formula:

$$\% \text{ INHIBITION} = \frac{(1 - \Delta \text{ OD sample})}{\Delta \text{ OD control}} \chi\ 100$$

2.15 Data handling and statistics

Data input, data handling/manipulation, linear regression, and graph plotting were carried out using Microsoft Excel 2003 (Microsoft Co.). Analysis of variance (ANOVA) was performed using SPSS (version 11). When required comparison between means this was carried out using ANOVA followed by Tukey Multiple Comparisons test. The 95% confidence limits (P =0.05) were used to compare treatments. Statistically significant differences among treatments are expressed by different letters.

3 RESULTS

3.1 Introduction

The objectives of this study were to assess the growth and the tolerance of five white rot fungal isolates to chlorpyrifos, linuron and metribuzin, in a minimal salts medium, individually and as a mixture. The first approach was to study if the isolates could have a good growth in this minimal medium in the presence of the pesticides in relation to rich culture media. Subsequently, the pesticides were added in higher concentrations and as a mixture and the growth rates and the % inhibition were calculated. Finally, the potential of these fungal isolates to degrade lignin and produce laccase in the presence of the three pesticides, individually and as a mixture was examined. From these studies three isolates were selected for the subsequent studies.

3.2 In vitro comparison of white rot fungi for tolerance to pesticides

3.2.1 Calculation of fungal growth rates

Colony diametric measurements were made every day throughout the incubation period by taking two readings at right angles to each other. Data were then tabulated and the linear portion of the radial extension rates used to determine growth rate as linear regression. An example is shown in Figure 3.1: the growth rate is the slope of the equation (Y=ax+b), thus for this example the growth rate was 0.4479 cm day^{-1}.

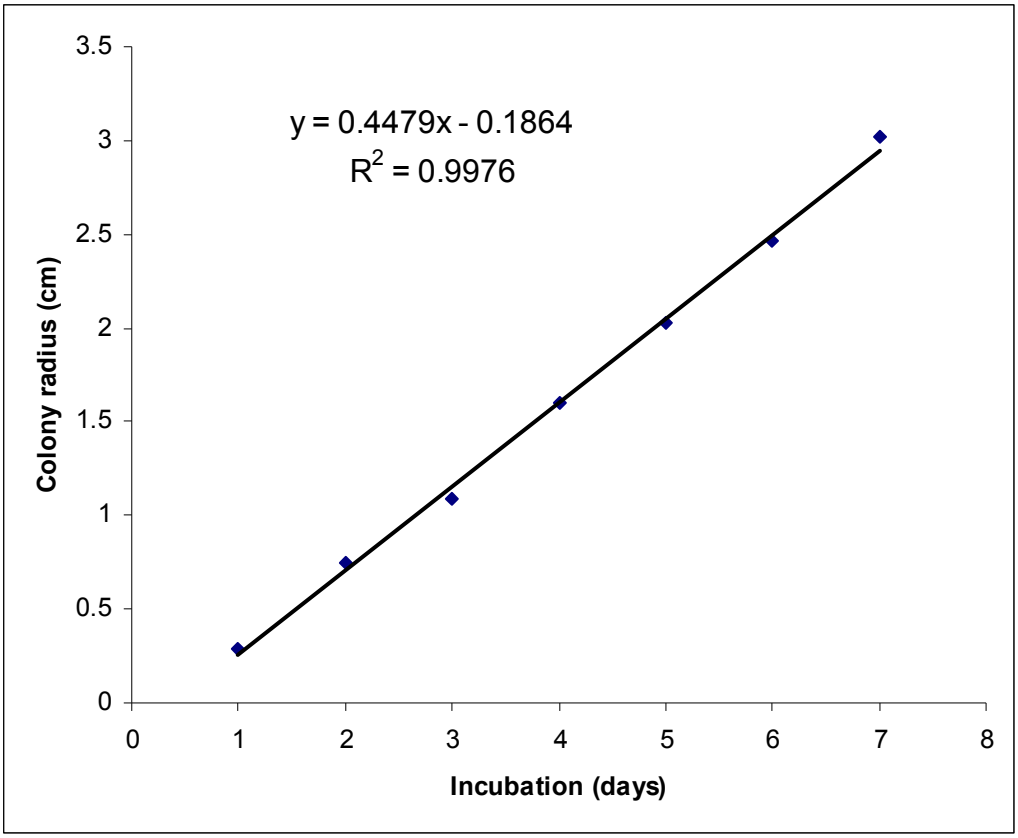


Figure 3.1 Linear regression of mycelial radial extension plotted against time in order to obtain the growth rate (cm day^{-1}).

3.2.2 In vitro tolerance to individual and mixtures of pesticides

The growth rates of the five test isolates, in different media are shown in Table 3.1. Plates 3.1 and 3.2 show examples of the effect of treatments. All test fungi had good growth on MMY, a medium that consisted of Czapek salts plus 0.5 g L^{-1} yeast broth in relation to the other two conducive media. The statistical analysis showed that the interaction between pesticides and fungi was highly significant ($p<0.001$) for all media, so separate tests were performed for each pesticide to reveal differences amongst the test fungi. In all cases the Tukey's test showed that *Phanerochaete chrysosporium* had the best growth rate in the 10 ppm treatment of the pesticides chlorpyrifos, linuron and metribuzin. Interestingly, this fungus was highly influenced by the 30ppm concentration of the pesticides individually and as a mixture. For example, the growth rate of *P. chrysosporium* was decreased from 1.09 cm day^{-1} (control) to 0.08 cm day^{-1} at 30 ppm of chlorpyrifos and to 0.10 cm day^{-1}

at 30 ppm of the mixture. This fungus showed high sensitivity to these pesticides.

The growth rate of all the test fungi was significantly decreased as the pesticide mixture was increased from 15 to 30 ppm (Table 3.2). This shows that the relative growth rates were affected in a different way depending on whether there was a single or mixture of pesticides. For example, the growth rate of *Pleurotus ostreatus* was decreased to 0.13 cm day^{-1} at the mixture of 30 ppm in relation to the control 0.38 cm day^{-1} while its growth was not affected at 30ppm of metribuzin (0.38 cm day^{-1}) (Table 3.2).

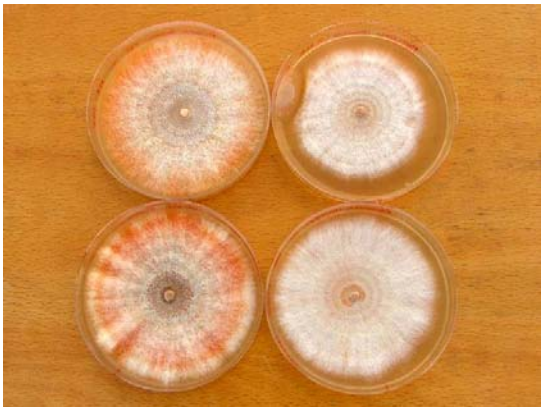


Plate 3.1 *Pycnoporus coccineus* growing on malt extract supplemented with the pesticides at 10ppm concentration.

Plate 3.2 *Pleurotus ostreatus* growing on MMY plus glucose supplemented with the pesticides at 10ppm concentration.

Table 3.1 Effect of culture medium in the presence either 10 ppm chlorpyrifos, metribuzin or linuron on growth rates (cm day^{-1}) on different nutrient media at 25^0C. They are the means of 3 replicates. The variances of all treatments were <5% of the means.
Key to media: MMY: Czapek salts modified medium, MMY plus glucose

Fungi	**Malt Broth**			
	Ctl	**Chlorpyrifos**	**Metribuzin**	**Linuron**
T. vercicolor	0,78	0,78	0,79	0,72
Ph. chrysosporium	1,55	1,42	1,57	1,35
P. ostreatus	0,69	0,52	0,68	0,33
P. gigantea	0,89	0,81	0,90	0,66
P. coccineus	0,60	0,43	0,61	0,53

Fungi	**MMY**			
	Ctl	**Chlorpyrifos**	**Metribuzin**	**Linuron**
T. vercicolor	0,77	0,52	0,70	0,78
Ph. chrysosporium	1,26	0,88	1,17	0,96
P. ostreatus	0,44	0,31	0,37	0,34
P. gigantea	0,53	0,47	0,57	0,38
P. coccineus	0,48	0,27	0,41	0,44

Fungi	**MMY plus Glucose**			
	Ctl	**Chlorpyrifos**	**Metribuzin**	**Linuron**
T. vercicolor	0,94	0,70	0,91	0,95
Ph. chrysosporium	1,59	1,65	1,77	1,57
P. ostreatus	0,61	0,28	0,61	0,57
P. gigantea	0,83	0,65	0,79	0,80
P. coccineus	0,58	0,43	0,59	0,57

Table 3.2 Growth rates (cm day^{-1}) (means ± standard deviations, n=3) in the presence 30ppm chlorpyrifos, metribuzin and linuron, individually and as mixture 15 and 30 ppm (total concentration) on a mineral salts medium at 25 ^{o}C. They are the means of 3 replicates. The variances of all treatments were <5% of the means.

	Treatments					
Fungi	**Ctl**	**Chlorpyrifos**	**Metribuzin**	**Linuron**	**mixture 15ppm**	**mixture 30ppm**
T. vercicolor	0,79	0,36	0,79	0,36	0,72	0,72
P.chrysosporium	1,09	0,08	0,15	0,04	0,03	0,09
P. ostreatus	0,38	0,34	0,38	0,14	0,18	0,13
P. gigantea	0,47	0,37	0,38	0,33	0,40	0,34
P. coccineus	0,53	0,28	0,43	0,55	0,48	0,45

Figure 3.2 shows the comparison of the relative percentage (%) inhibition between the five species examined. This shows clearly that *T. versicolor* and *P. coccineus* were least affected by mixtures of 30 ppm of the three pesticides. This was different from that observed for growth in the presence of individual pesticides. There were also marked differences between the ability of each species to tolerate the three different pesticides alone. Plate 3.3 shows the effect of mixtures of treatments on the growth of *T. versicolor* and the mycelial morphology.

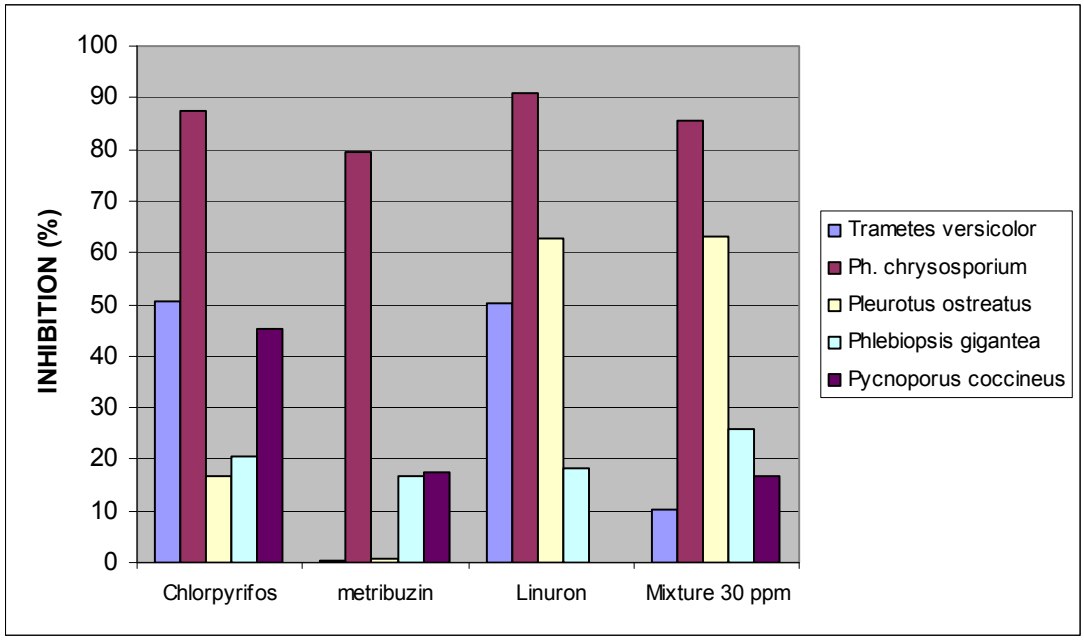


Figure 3.2 Mean growth inhibition (%) observed for five test isolates growing in a minimal medium supplemented with 30 ppm treatments (chlorpyrifos, metribuzin and linuron, individually and as a mixture). Studies were carried out on Czapek modified salts medium at 25^{o}C for a period of 15 days.

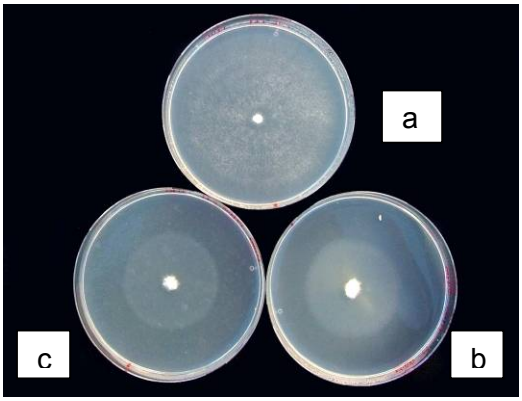


Plate 3.3 *T. versicolor* growing on MMY supplemented with mixtures of the pesticides chlorpyrifos, metribuzin and linuron at (a): 0 ppm, (b):15 ppm, (c): 30ppm.

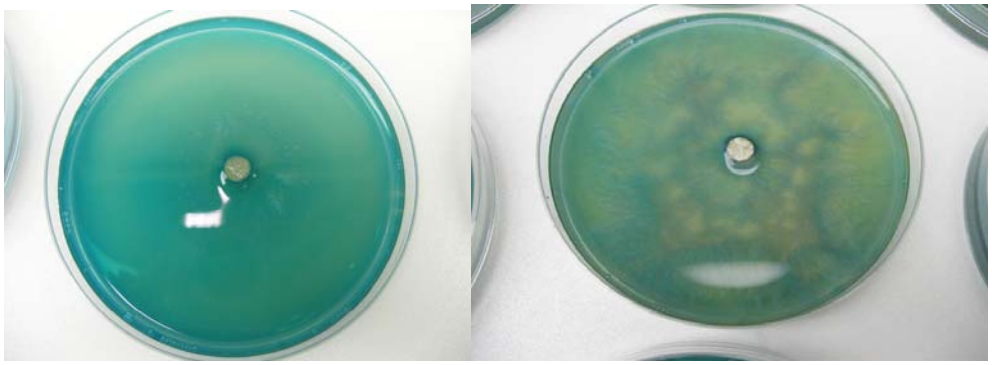
3.3 Production of lignolytic enzymes in Petri plate assays

The applicability of fungi in bioremediation of soil contaminated with pesticides depends on their capacity to grow efficiently in the presence of such compounds and their ability to produce degradative enzymes. This study was carried out to investigate the production of enzymes by the candidate fungi and the effect that pesticide treatments may have on this.

3.3.1 Potential ligninolytic activity

Phlebiopsis gigantea and *Pycnoporus coccineus* did not produce decolouration under the conditions of the assay, although these fungi grew well in the medium used in this assay.

Plate 3.4 shows the ligninolytic activity of *Trametes versicolor* expressed diffused spots so it was difficult to be quantified. This view was observed in all treatments. As conclusion, *T. versicolor* showed strong ligninolytic activity and it was not affected by pesticides concentrations and mixture of them.



A B

Plate 3.4 A positive result for plate assay to assess potential ligninolytic activity *T. versicolor* (B) in relation to the control (A).

3.3.2 Polyphenol oxidase production

The test fungi produced activity halos in all treatments (see Plates 3.5, 3.6) and showed no significant differences under different pesticide concentrations. Figure 3.3 shows polyphenol oxidase production, expressed as radius of enzymatic activity halo, by the three test fungi.

Trametes versicolor produced the highest enzymatic activity almost in all treatments. The presence of metribuzin did not affect the polyphenol oxidase production ($p=0.422$) while the presence of linuron or chlorpyrifos and mixture of the three pesticides decreased the enzymatic activity ($p<0.001$).

Phlebiopsis gigantea showed enzyme activity in the presence of the mixture and linuron treatments regardless of the pesticide concentration ($p=0.157$ and $p=0.163$, respectively). In the metribuzin and chlorpyrifos treatments the polyphenol oxidase production was not affected by the concentration of pesticides noticeably ($p=0.032$ and $p=0.018$, respectively).

Pycnoporus coccineus also produced high polyphenol oxidase activity. In chlorpyrifos, linuron and metribuzin treatments the enzyme activity was not affected by the concentration of pesticides ($p=0.77$, $p=0.2$ and $p=0.138$, respectively). In the mixture treatments there was a relative degrease ($p=0.036$).

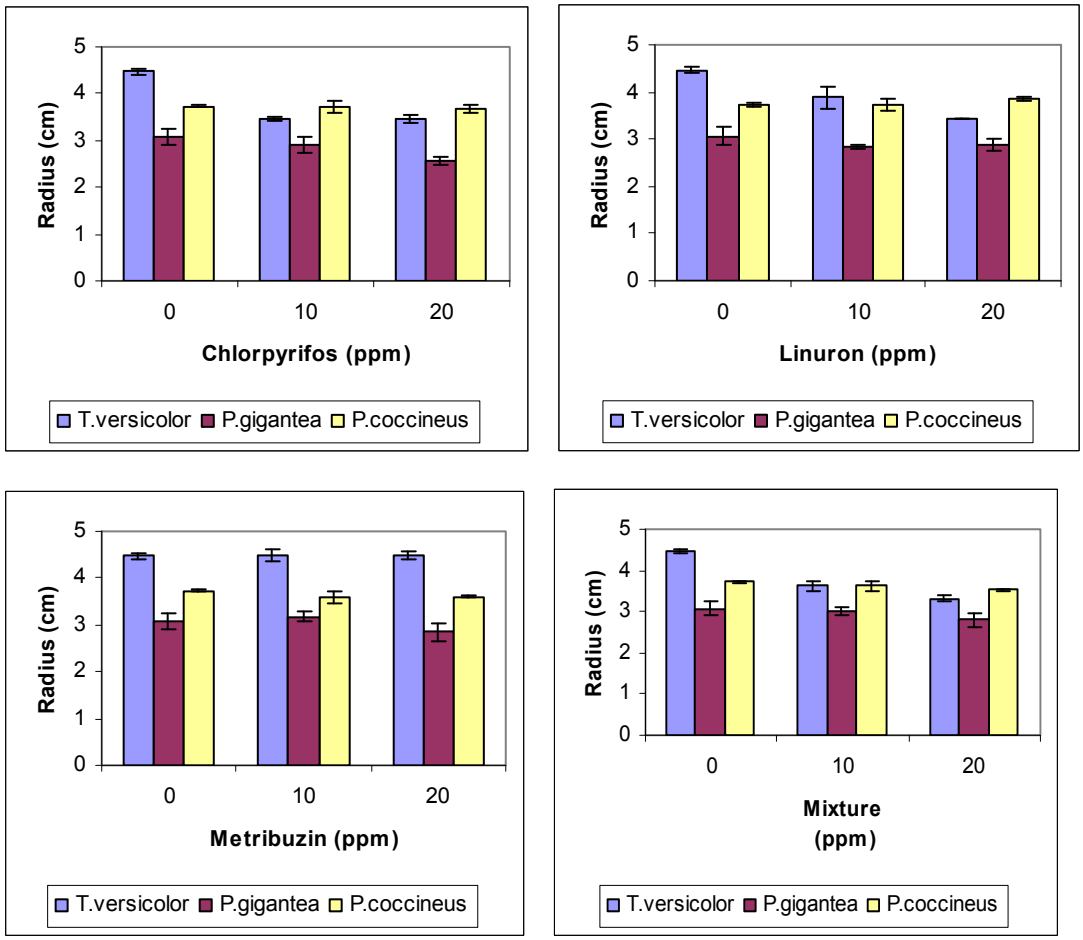


Figure 3.3 Effect of chlorpyrifos, linuron, metribuzin (0, 10 and 20 ppm) individually and as a mixture (0, 10 and 20 ppm) on polyphenol oxidase activity by *T. versicolor, P. gigantea* and *P. coccineus,* at 25^0C (expressed as radius of enzymatic activity halo ±standard deviation of the mean, n=3).

Plate 3.5 Laccase activity plate assay: Growth of *T. versicolor* in the presence of 20 ppm linuron.

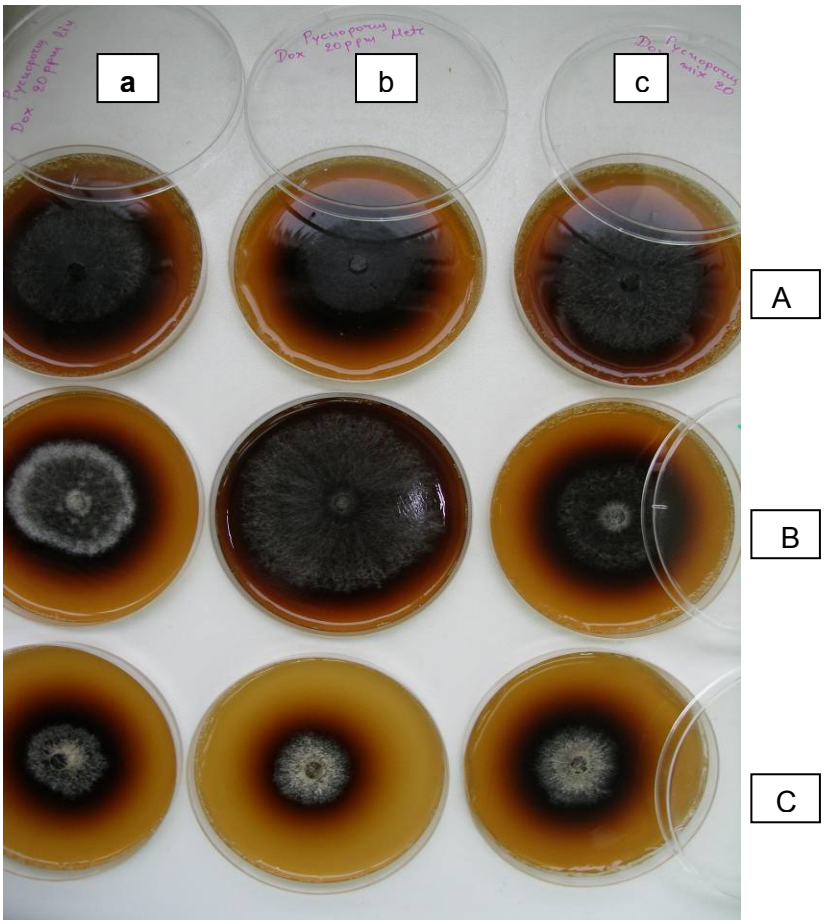


Plate 3.6 Laccase activity plate assay: Growth of *P. coccineus* (A) *T. versicolor* (B) and *P. gigantea* (C) in the presence of linuron (a), metribuzin (b) and a mixture of the pesticides chlorpyrifos, linuron and metribuzin at concentration of 20 ppm.

3.4 In vitro comparison of bacteria isolates for tolerance to pesticides

The growth of 19 strains of Bacillus sp on TSB supplemented with the pesticide chlorpyrifos was reduced as the concentrations were increased, and growth was completely inhibited at 120ppm (Table 3.3). Most of 19 strains had good growth up to the 60ppm of linuron. Above this concentration growth was completely inhibited (Table 3.4). Finally, most of the 19 strains of Bacillus sp. tolerated up to 120ppm of metribuzin. However, the pesticide metribuzin started showing inhibitory effects at 60ppm (Table 3.5). At 150ppm of this pesticide, growth was completely inhibited. Highlighted lines in tables 3.3, 3.4 and 3.5 show the stains that had the best growth.

Table 3.3 Growth of 19 strains Bacillus spp. as Optical Density (OD_{600}) at different concentrations of chlorpyrifos, after 24 hours incubation on TSB medium at 25^0C. N.G:= no growth.

Bacterial strains	Concentration of chlorpyrifos (ppm)					
	0	**30**	**60**	**90**	**120**	**150**
ctl	0	0.121	0.268	0.405	0.569	N.G
1	0.380	0.58	0.533	0.227	0.003	N.G
2	0.190	0.68	0.403	0.282	0.012	N.G
3	0.162	0.484	0.382	0.187	0.233	N.G
4	0.384	0.316	0.195	0.06	0.022	N.G
5	0.198	0.321	0.425	0.217	0.119	N.G
6	0.316	0.428	0.303	0.217	0.021	N.G
7	0.325	0.386	0.326	0.156	0.008	N.G
8	0.682	0.165	0.046	0.038	0.007	N.G
9	0.397	0.133	0.422	0.152	0.008	N.G
10	0.656	0.576	0.04	0.161	0.003	N.G
11	0.667	0.599	0.415	0.448	0.046	N.G
12	0.507	0.591	0.438	0.398	0.031	N.G
13	0.848	0.525	0.494	0.330	0.019	N.G
14	0.719	0.579	0.357	0.011	0.008	N.G
15	0.710	0.634	0.631	0.416	0.019	N.G
16	0.597	0.503	0.413	0.093	0.018	N.G
17	0.695	0.601	0.442	0.170	0.011	N.G
18	0.684	0.644	0.509	0.290	0.013	N.G
19	0.493	0.583	0.503	0.446	0.006	N.G

Table 3.4 Growth of 19 strains *Bacillus* spp. as Optical Density (OD_{600}) at different concentrations of linuron, after 24 hours incubation on TSB medium at 25^0C. N.G:= no growth

Bacterial strains	Concentration of linuron (ppm)					
	0	**30**	**60**	**90**	**120**	**150**
1	0.358	0.384	0.504	N.G	N.G	N.G
2	0.597	0.595	0.584	N.G	N.G	N.G
3	0.584	0.583	0.579	N.G	N.G	N.G
4	0.451	0.257	0.106	N.G	N.G	N.G
5	0.606	0.600	0.530	N.G	N.G	N.G
6	0.457	0.462	0.462	N.G	N.G	N.G
7	0.256	0.246	0.199	N.G	N.G	N.G
8	0.742	0.706	0.762	N.G	N.G	N.G
9	0.645	0.582	0.633	N.G	N.G	N.G
10	0.802	0.764	0.710	N.G	N.G	N.G
11	0.737	0.703	0.716	N.G	N.G	N.G
12	0.752	0.854	0.704	N.G	N.G	N.G
13	0.695	0.776	0.654	N.G	N.G	N.G
14	0.758	0.693	0.693	N.G	N.G	N.G
15	0.579	0.686	0.672	N.G	N.G	N.G
16	0.599	0.453	0.540	N.G	N.G	N.G
17	0.770	0.633	0.766	N.G	N.G	N.G
18	0.662	0.655	0.792	N.G	N.G	N.G
19	0.728	0.732	0.571	N.G	N.G	N.G

Table 3.5 Growth of 19 strains *Bacillus* spp. as Optical Density (OD_{600})* at different concentrations of metribuzin after 24 hours incubation on TSB medium at 25^0C. N.G:= no growth

Bacterial strains	Concentration of metribuzin (ppm)					
	0	**30**	**60**	**90**	**120**	**150**
1	0.380	0.405	0.569	0.236	0.001	0.007
2	0.190	0.270	0.617	0.308	0.043	N.G
3	0.162	0.221	0.427	0.225	0.027	N.G
4	0.384	0.275	0.084	0.003	0.274	N.G
5	0.198	0.198	0.299	0.589	0.536	N.G
6	0.316	0.406	0.438	0.367	0.162	N.G
7	0.325	0.349	0.267	0.083	0.006	N.G
8	0.682	0.528	0.542	0.454	0.384	0.085
9	0.397	0.403	0.356	0.115	0.621	N.G
10	0.656	0.735	0.639	0.305	0.127	0.005
11	0.667	0.676	0.631	0.600	0.395	N.G
12	0.507	0.545	0.635	0.480	0.390	N.G
13	0.848	0.630	0.594	0.586	0.563	0.026
14	0.719	0.667	0.636	0.300	0.048	N.G
15	0.710	0.625	0.775	0.608	0.275	N.G
16	0.597	0.605	0.568	0.258	0.123	N.G
17	0.695	0.640	0.576	0.693	0.335	N.G
18	0.684	0.493	0.565	0.519	0.302	N.G
19	0.493	0.699	0.652	0.495	0.181	N.G

3.5 In vitro comparison of bacterial strains for tolerance to a mixture of pesticides

In this study the *Bacillus* strains 6, 13, 15, 18 and 19 that had the best growth in the presence of high concentrations of the pesticides chlorpyrifos, linuron and metribuzin were tested for their capacity to tolerate mixtures of them at total concentrations of 30 and 60ppm. Some of the others also displayed very good growth but were excluded as their suspension was not homogeneous.

Values of OD_{600} after subtraction of the blank (soil extract + pesticides without bacteria) are shown in Figure 3.4. Strain 13 was not affected by the mixtures of 30 and 60 ppm (decrease of growth was 3 and 6%, respectively). Strains 15 and 19 had a reduction of 64 and 42%, respectively, at 60ppm mixture of the pesticides. Finally, strain 6 was very sensitive as its growth was completely inhibited.

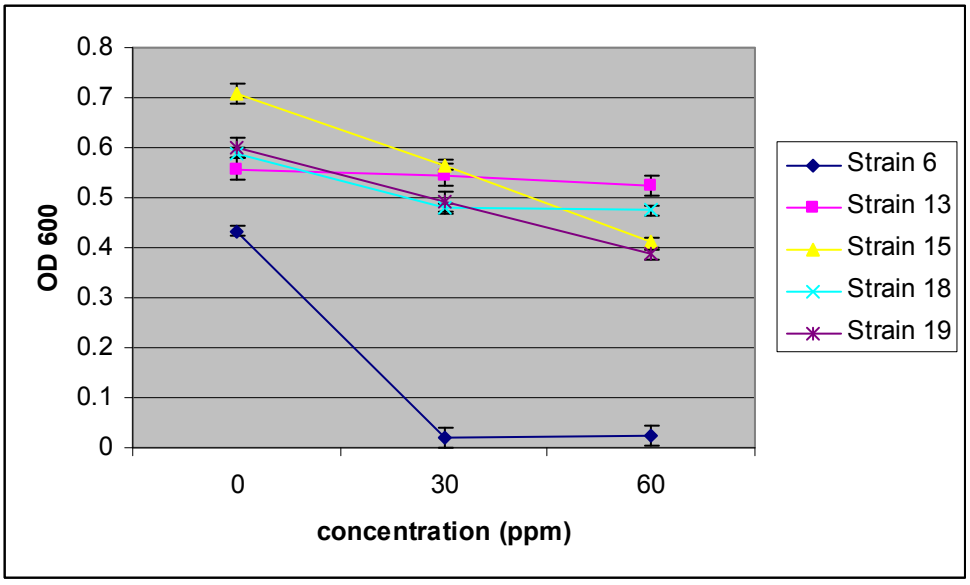


Figure 3.4 Growth (means ± standard deviations, n=3) of the best strains *Bacillus* spp. as Optical Density (OD_{600})in a mixture of 0, 30, 60 ppm of the three pesticides after 24 hours incubation in soil extract at 25^0C.

PART II STUDIES ON SOIL EXTRACT OF WHITE ROT FUNGI AND BACTERIA

3.6 Introduction

The objective of this study was to assess the growth of the best four fungi and five bacteria using soil extract as culture medium. It is a nutritionally weak medium to mimic the nutrient availability in soil. *Trametes versicolor, Pleurotus ostreatus, Phlebiopsis gigantea* and *Pycnoporus coccineus* were used as *P.chrysosporium* showed to be very sensitive to the mixture of the pesticides chlorpyrifos, linuron and metribuzin. High concentrations were used in order to calculate the effective doses (ED) for 50% inhibition of growth (EC_{50} values), relative to the controls for each fungus. The degradation of a mixture of different concentrations (0-50 ppm), also was examined, by HPLC quantification. Regarding bacteria, the growth of the best bacteria in soil extract in the presence of the mixture of pesticides was examined.

3.7 Growth of fungi in soil extract agar

Table 3.6 and 3.7 shows the growth of four fungi on the soil extract agar. There was significant decrease of the growth at 125 ppm concentration of chlorpyrifos for all tested fungi. On the contrary, at 125 ppm of metribuzin *T. versicolor* showed to be very tolerant with a EC_{50} value 292.2 ppm (Table 3.7). *P. gigantea and P. ostreatus* were very sensitive in this concentration, while *P. coccineus* had a decrease of growth almost 50%. In 125 ppm concentration of linuron growth was completely inhibited for all fungi.

In the mixtures the difference of growth was very noticeable. *P. ostreatus* had the lowest growth rate while *P. coccineus* showed to be more tolerant than all other fungi (Table 3.7). Overall, it is very clear that the relative growth rates

were affected in a different way depending on whether there was a single or mixture of pesticides.

Table 3.6 Growth rates (means ± standard deviations, n=3) expressed in cm day^{-1} for four fungal species incubated in soil extract agar supplemented with chlorpyrifos, linuron and metribuzin (0, 25,125 ppm) and as a mixture at interval of ten (0-70 ppm) at 25^0C. N.G:= no growth

		Growth rate (cm day^{-1})			
Pesticides	Concentration (ppm)	*T. versicolor*	*P. gigantea*	*P. ostreatus*	*P. coccineus*
	0	0.7±0.01	0.6±0.01	0.3±0.00	0.5±0.01
Chlorpyrifos	5	0.6±0.00	0.6±0.01	0.3±0.01	0.3±0.01
	25	0.5±0.00	0.5±0.02	0.2±0.01	0.2±0.01
	125	0.3±0.14	0.3±0.06	0.1±0.01	0.1±0.00
Linuron	5	0.7±0.00	0.6±0.01	0.3±0.03	0.5±0.01
	25	0.4±0.01	0.4±0.01	No Growth	0.4±0.00
	125	No Growth	No Growth	No Growth	No Growth
Metribuzin	5	0.7±0.02	0.6±0.01	0.3±0.00	0.4±0.00
	25	0.7±0.01	0.5±0.04	0.3±0.00	0.5±0.01
	125	0.5±0.07	No Growth	0.1±0.01	0.3±0.00
Mixture	10	0.6±0.02	0.6±0.02	0.3±0.01	0.5±0.01
	20	0.5±0.00	0.5±0.02	0.2±0.01	0.5±0.01
	30	0.5±0.01	0.5±0.00	0.1±0.01	0.4±0.02
	40	0.5±0.01	0.4±0.05	0.1±0.02	0.4±0.01
	50	0.3±0.02	0.3±0.03	No Growth	0.3±0.00
	60	0.3±0.02	0.4±0.02	No Growth	0.3±0.00
	70	0.3±0.03	0.4±0.01	No Growth	0.3±0.00

Table 3.7 Concentration (ppm) of chlorpyrifos, linuron and metribuzin individually and as a mixture that causes a 50% reduction in fungal growth (EC_{50} values) in four test fungi growing on soil extract agar.

Fungi	Pesticides			
	Chlorpyrifos	Linuron	Metribuzin	Mixture
	Concentration (ppm)			
T. versicolor	107.4	63.9	292.2	54.1
P. gigantea	106.2	62.6	95.2	51.7
P. coccineus	61.1	64.7	123.5	75.5
P. ostreatus	79.6	35.1	101.2	27.9

3.8 Growth of fungi in soil extract broth

In this study the fungi *T. versicolor*, *P. gigantea* and *P. coccineus* were incubated in Petri plates containing soil extract in order to have a first indication of their capability to degrade mixtures of the pesticides chlorpyrifos, linuron and metribuzin at total concentrations of mixture ranging from 10 to 50 ppm. The remaining concentration of the pesticides is presented as a percentage (%).

3.8.1 Pesticides analysis

Table 3.8 and Figure 3.5 show the impact of the fungi *T. versicolor*, *P. gigantea* and *P. coccineus* on degradation of the mixture of pesticides. In the treatments with chlorpyrifos there were differences in degradation rates between the fungi as well as the concentrations of pesticide. *P. coccineus* showed the highest disappearance rate at 10ppm, almost 60%. The Tukey's test showed two different subsets. The first subset included concentrations of 10, 40 and 50 ppm of chlorpyrifos, and the second the concentrations 20 and 30 ppm. The degradation rates by *T. versicolor* and *P. gigantea* were very

low at the 50 ppm chlorpyrifos. It is clear that these fungi are not able to degrade chlorpyrifos in high concentrations. Regarding metribuzin, all tested fungi were shown to be unable to degrade it, although they were tolerant and had quite high EC_{50} values for metribuzin.

In contrast, *T. versicolor* showed a great capacity to degrade linuron even in the 50 ppm concentration (Figure 3.5). The Tukey's test showed two different subsets. The first subset included 10 and 20 ppm of the mixture, and the second 30, 40 and 50 ppm. The degradation rates by *P. gigantea* were low, about 20%, in all treatments. Finally, *P. coccineus* was found to be unable to degrade linuron in all treatments ($p=0.01$).

Table 3.8 Chlorpyrifos and metribuzin remaining (%)(means ± standard deviations, n=3) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 21 days of incubation in soil extract at 25 ^{0} C.

Pesticides	Initial concentration of mixture (ppm)	Remaining pesticide (%)		
		T. versicolor	*P. gigantea*	*P. coccineus*
Chlorpyrifos	10	14.3±8.7	39.5±13.7	41.3±7.8
	20	37.4±9.9	52.3±7.8	59.4±5.0
	30	78.3±10.7	69.1±1.6	59.2±1.3
	40	84.6±0.8	75.9±2.5	45.1±7.3
	50	94.2±5.1	86.6±4.6	52.3±3.9
Metribuzin	10	62.8±10.2	83.5±2.0	57.7±3.4
	20	83.2±3.0	99.5±0.9	82.3±6.0
	30	80.1±9.21	100±0.0	84.1±1.8
	40	84.1±2.26	100±0.0	92.5±1.5
	50	100±0.0	100±0.0	91.4±3.3

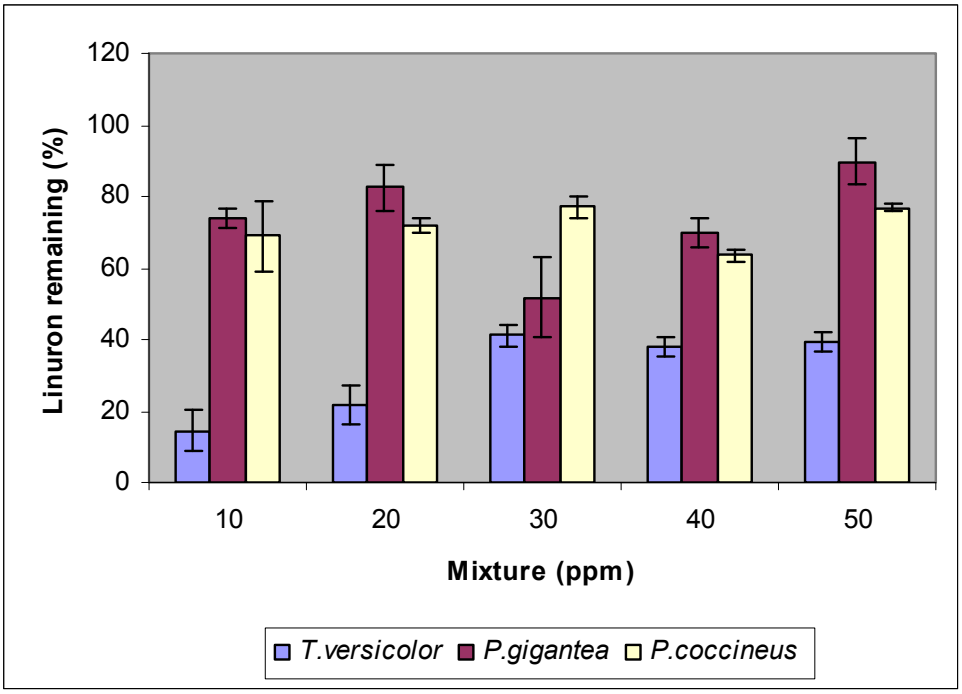


Figure 3.5 Linuron remaining (%) (means ± standard deviations, n=3) in soil extract that was initially supplemented with a mixture of pesticides at total concentrations 10, 20, 30, 40 and 50 ppm, after 21 days of incubation in soil extract at 25 ^{0} C.

3.9 Pesticides degradation by bacterial strains

3.9.1 Bacterial growth in soil extract liquid broth with the presence of mixture of pesticides

The growth of the strains *Pseudomonas putida* and *Pseudomonas fluorescens* Pfl2, Pfl804 and Pfl545 was very good, as also the mixture of all microorganisms while the strains of *Bacillus* showed a very small growth in the presence of the mixture of the pesticides at 60 ppm concentration (Figure 3.6).

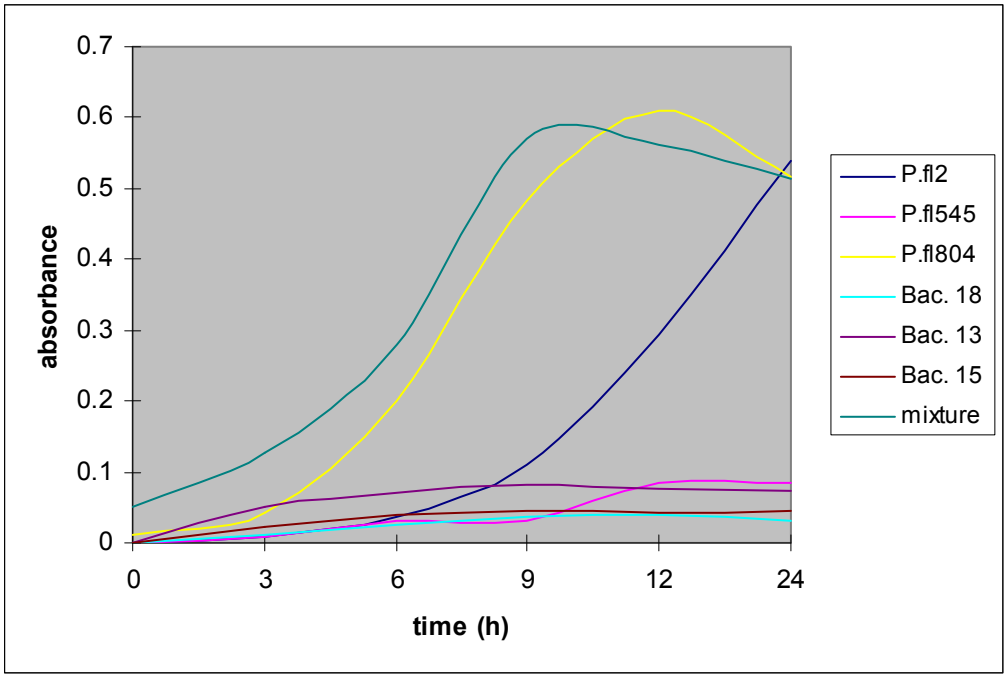


Figure 3.6 Growth of a range of single bacterial strains and a mixture of them as Optical Density at 600 nm in soil extract broth supplemented with a mixture of 60 ppm (total concentration) of the pesticides, at 25 ^{0}C.

3.9.2 Quantification of pesticides

Unfortunately, the bacterial strains and the mixture of them did not degrade any of the pesticides linuron and metribuzin that were used with chlorpyrifos in a mixture of total concentration of 60 ppm. Figures 3.7 and 3.8 show that neither linuron nor metribuzin were degraded by *Pseudomonas putida* although its growth in the mixture was very good and Figure 3.9 shows the concentration of all the other tested strains.

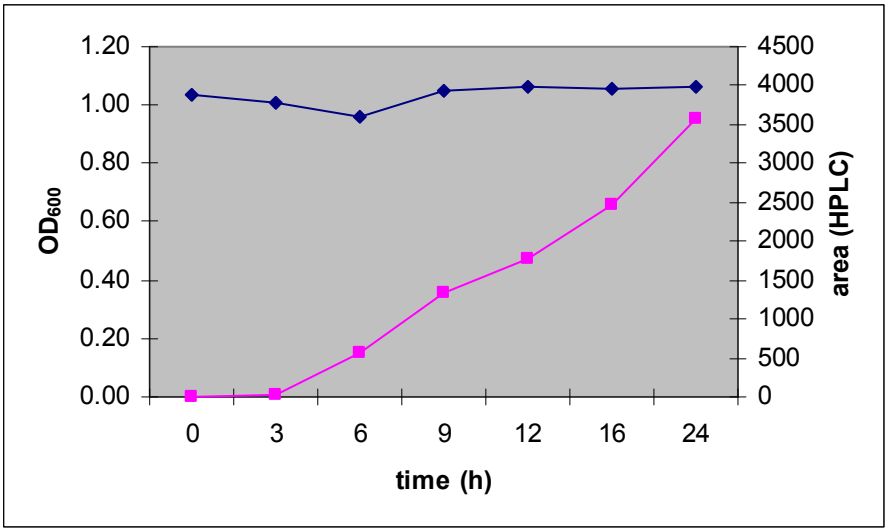


Figure 3.7 The growth of *P. putida* over a 24 hours period as Optical Density at 600nm(blue line) and linuron concentration at the same period in soil extract broth supplemented with a mixture 60 ppm of the pesticides at 25^0C.

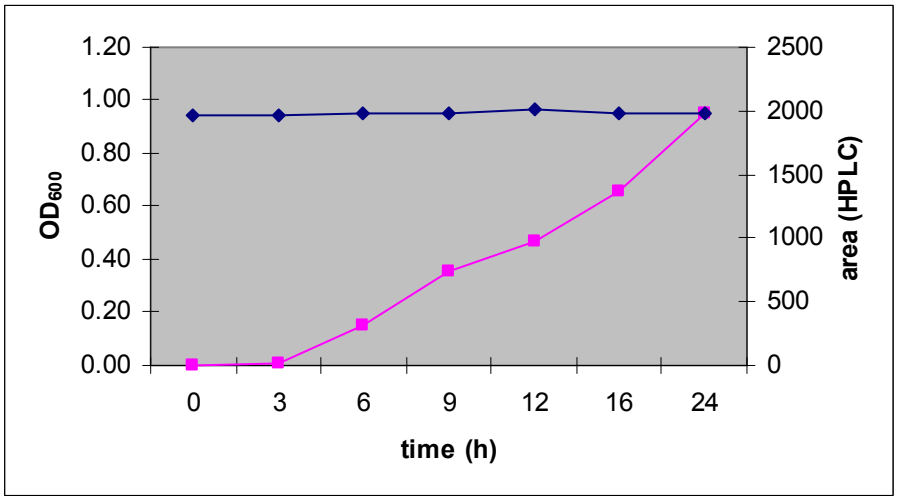


Figure 3.8 The growth of *P. putida* over a 24 hours period as Optical Density at 600 nm (blue line) and metribuzin concentration at the same period in soil extract broth supplemented with a mixture 60 ppm of the pesticides at 25^0C.

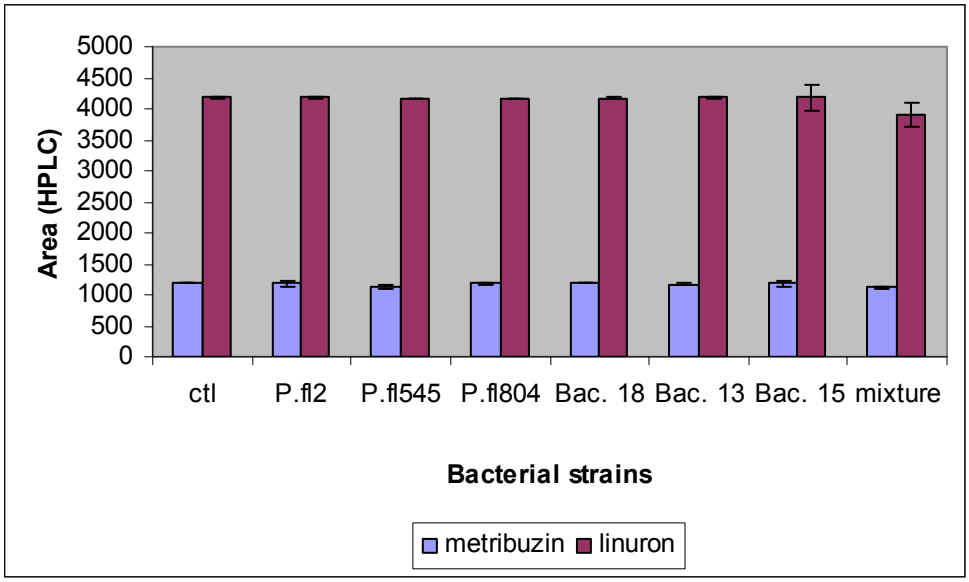


Figure 3.9 Concentrations of pesticides remaining (expressed as area by HPLC), after 24 hours incubation with bacterial strains in soil extract broth at 25 ^{0}C. Bars represent the standard deviation of the mean (n=3), per treatment.

3.10 Introduction

In this section, the capacity of *Trametes versicolor* to grow under water stress and degrade mixtures of pesticides was evaluated in order to assess its potential use as bioremediation agent. The main objectives of this study were to examine the interactions and activity of this fungus in relation to: (a) temporal degradation of a mixture of the pesticides chlorpyrifos, linuron and metribuzin at total concentrations of 0, 10, 20, 30, 40, 50 ppm (b) temporal evaluation of fungal laccase activity, over a six week period, in the soil extract liquid broth where solute potential was adjusted in two different ways: ionically by adding potassium chloride and non-ionically by using glycerol.

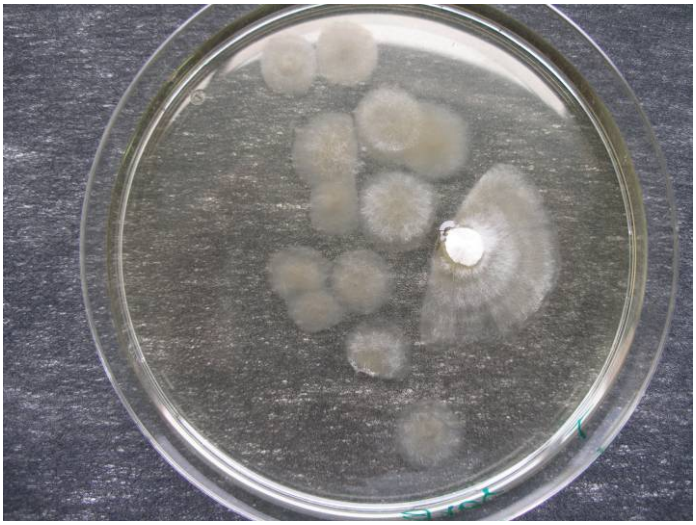


Plate 3.7 *T. versicolor* growing in soil extract liquid broth plus glycerol (-2.8 MPa) at 25^0C, seven days after inoculation.

3.11 Temporal evaluation of laccase activity

As fungal laccase has shown to be involved in biodegradation of a broad range of pollutants, the production of this enzyme is very pertinent for evaluation of the capacity for bioremediation.

Over a six week period, laccase production was determined in three different water regimes and was related to the pesticides chlorpyrifos, linuron and metribuzin remaining %, quantificated by HPLC, in order to investigate the rate of laccase production and to be estimated their possible correlation.

3.11.1 Soluble protein

The quantification of soluble protein content was essential to estimate the specific laccase activity. Moreover, this parameter can also be used as additional information on fungal activity.

The levels of extracellular protein in both cases were not high. The production of soluble protein in soil extract was not affected by pesticides concentrations and water regimes. Analysis of variance of each concentration of the mixture per week was performed and there was not significant difference. Analysis of variance of protein with two factors (weeks x water regimes) was performed in order to compare soluble protein at each concentration at the two water regimes (non-stress and non-ionic stress treatments). The results showed that there was no significant difference (see Appendix C).

The effect of mixtures of pesticides on soluble protein content in soil extract inoculated with *T. versicolor* every week over a six week period under two water regimes is shown in Figure 3.10 (without water stress) and Figure 3.11 (with -2.8 MPa water stress using glycerol).

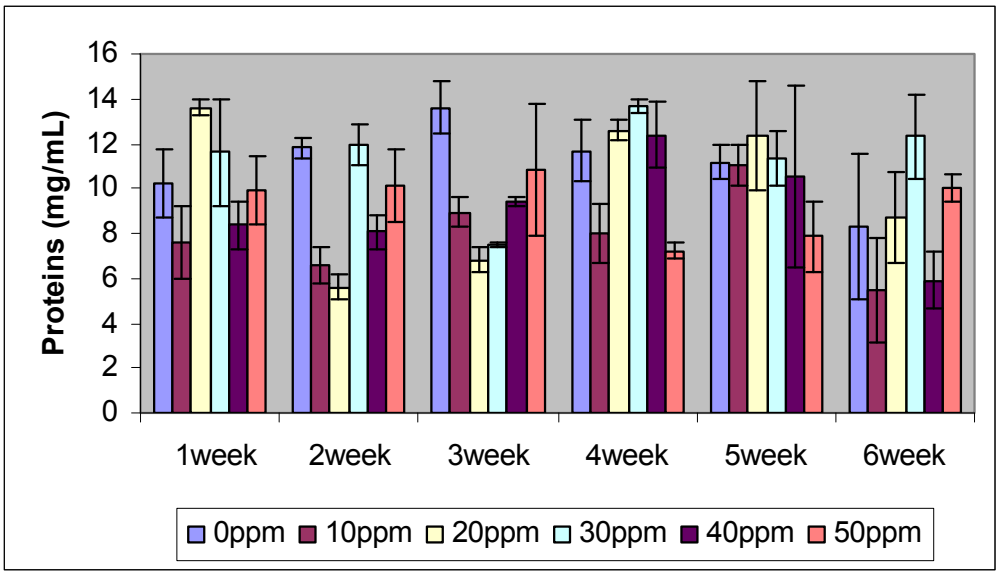


Figure 3.10 Protein concentration of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

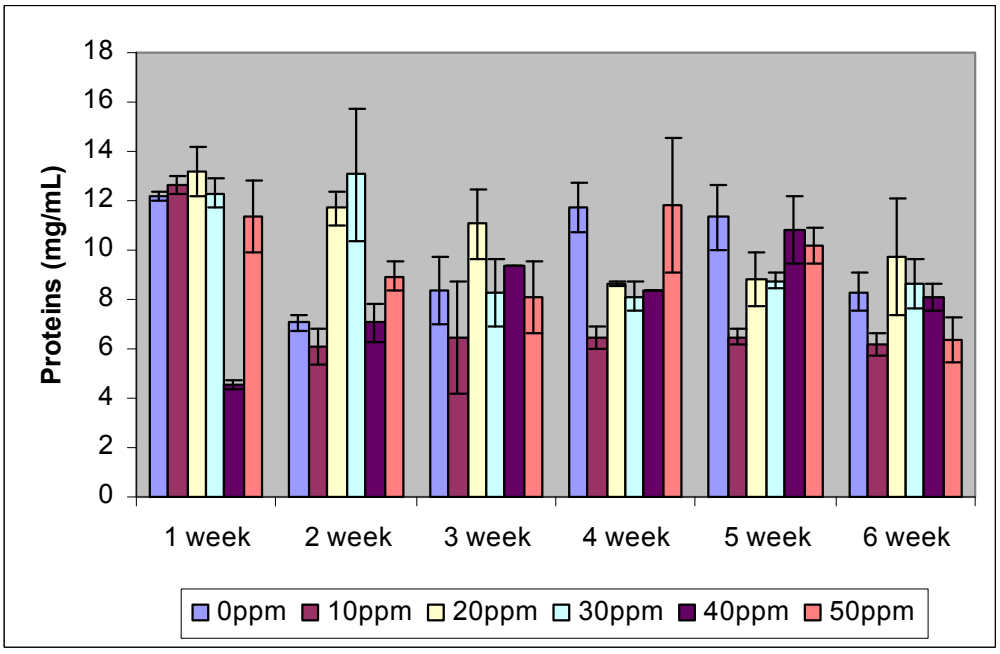


Figure 3.11 Protein concentration of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) at water potential -2.8MPa (with glycerol) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

3.11.2 Specific activity

Specific activity of laccase is the number of enzyme units per ml divided by the concentration of soluble protein in mg mL^{-1}. Specific activity values are therefore quoted as units mg^{-1}. Specific activity is of no relevance as far as setting assays is concerned, although it is an important measurement of enzyme purity and quality.

Having determined laccase units (see Appendix B) and soluble protein the specific activity was calculated as the ratio of laccase units to soluble protein the soil extract treatments and these with soil extract and glycerol. The KCl filtrates did not produce laccase in any of the treatments.

The levels of laccase produced by *T. versicolor* in soil extract broth without stress were quite high. Laccase production was shown to be induced by the pesticides mixture as at 0 ppm treatment was lower in relation to all the other treatments with the pesticides until the sixth week (Figure 3.12).

Interestingly, very high levels of laccase were produced by *T. versicolor* in soil extract broth solute potential adjusted at -2.8 MPa non-ionically by using glycerol. Laccase production was the maximum at the second and third week after the inoculation (Figure 3.13).

Statistical analysis of variance was performed for each pesticide concentration in the same water regime per week with theTukey's test. The results are shown in Tables 3.9 and 3.10. Statistically significant differences among every concentration in the same water regime per week are expressed by different letters (see Appendix D). Moreover, analysis of variance with two factors (weeks x water regimes) was performed in order to compare the specific activity of laccase at each concentration at the two water regimes (non-stress and non-ionic stress treatments). The results showed that there was significant ordered interaction between the two factors and specific activity of laccase was always the best at non ionic stress

treatments (glycerol) (see Appendix E). Finally, there was a negative correlation (R=-0.46) between specific activity and remaining linuron %, only at the fifth week and at -2.8 MPa water potential using glycerol.

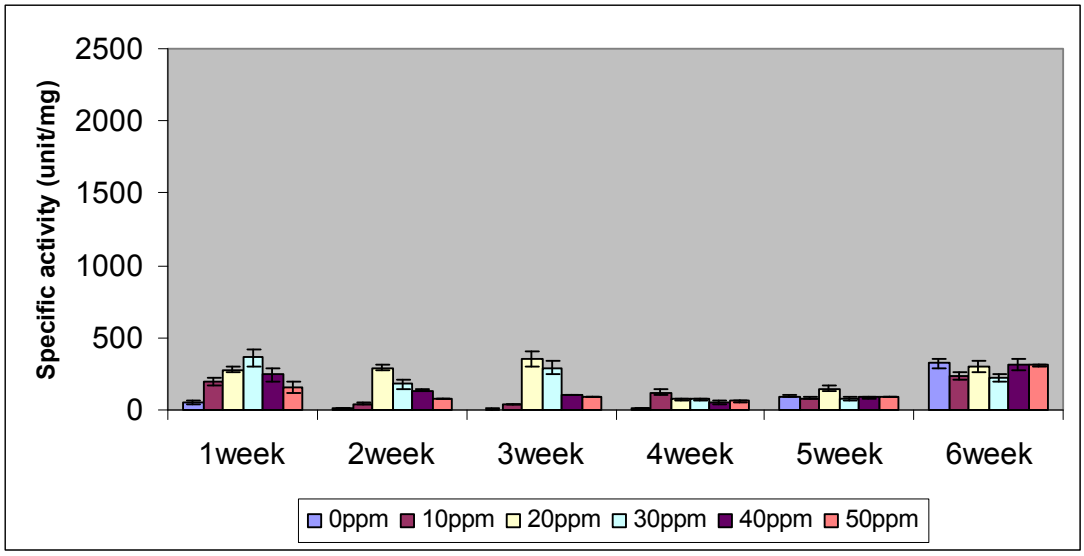


Figure 3.12 Specific activity of laccase of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

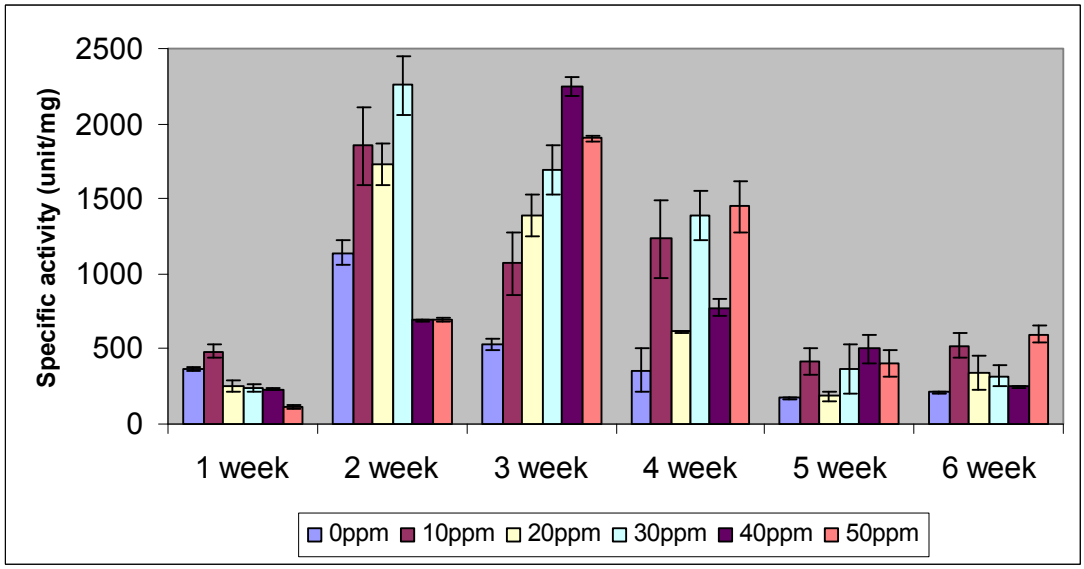


Figure 3.13 Specific activity of laccase of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) at water potential -2.8MPa (with glycerol) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

Table 3.9 Statistically significant differences of specific activity of laccase among every concentration of mixture per week, in soil extract broth without stress, expressed by different letters.

Weeks	Initial concentratiion of the mixture (ppm)					
	0	**10**	**20**	**30**	**40**	**50**
1	ab	bc	ab	c	a	a
2	a	a	ab	ab	a	a
3	a	a	b	bc	a	a
4	ab	ab	a	a	a	a
5	b	a	a	a	a	a
6	c	c	ab	abc	a	b
	p<0.001	p<0.001	p=0.016	p=0.001	p=0.233	p=0.001

Table 3.10 Statistically significant differences of specific activity of laccase among every concentration of mixture per week, at -2.8 MPa by adding glycerol, expressed by different letters.

Weeks	Initial concentratiion of the mixture (ppm)					
	0	**10**	**20**	**30**	**40**	**50**
1	ab	a	a	a	a	a
2	b	b	b	b	ab	ab
3	ab	ab	b	b	c	c
4	ab	ab	a	ab	b	c
5	a	a	a	a	ab	ab
6	b	a	a	a	ab	ab
	p=0.012	p=0.002	p<0.001	p<0.001	p<0.001	p=0.002

3.12 Total ligninolytic activity

The ability of white rot fungi to degrade contaminants has been linked to its wood degrading capabilities; therefore a strong ligninolytic activity could be advantageous in pesticide degradation.

The total ligninolytic activity of *T. versicolor* was examined in this study, under the different water regimes. The assay used is based on the quantification of the extent of decolouration of a polymeric dye (Poly R-478). The lower the ratio absorbance at 530 nm / absorbance at 350 nm the more intense is the decolouration, i.e. the higher was total ligninolytic activity.

The filtrates of the treatments 0, 30, 50 ppm at the first, fourth and fifth week under the three water regimes were tested. Overall, all treatments caused decolourazion of the polymeric dye (Figure 3.14). Table 3.11 shows the ratio absorbance at 530 nm/ absorbance at 350 nm in all treatments. The total ligninolytic activity was not affected by water availability or pesticide treatments. The latter suggest the *T.versicolor* is tolerant to the mixtures of pesticides, producing equivalents level of decolouration in the presence and absence of the xenobiotics.

Table 3.11 Total ligninolytic activity of *T. versicolor* (expressed as the ratio absorbance at 530 nm / absorbance at 350 nm ± standard deviation of the mean) in soil extract liquid broth, under three different water potential regimes: without stress, with -2.8 MPa water potential using glycerol and, with -2.8 MPa water pontential using KCl.

Water regime	Concentration (ppm)	1 week	4 week	5 week
without stress	0	0.27 ±0.03	0.27±0.01	0.12±0.00
	30	0.34±0.03	0.19±0.00	0.18±0.00
	50	0.36±0.07	0.18±0.01	0.28±0.00
Water stress with glycerol	0	0.22±0.03	0.16±0.03	0.19±0.01
	30	0.28±0.01	0.26±0.02	0.23±0.02
	50	0.27±0.01	0.27±0.02	0.27±0.01
Water stress with KCl	0	0.22±0.01	0.18±0.01	0.16±0.00
	30	0.24±0.01	0.18±0.02	0.16±0.01
	50	0.24±0.03	0.17±0.01	0.17±0.03

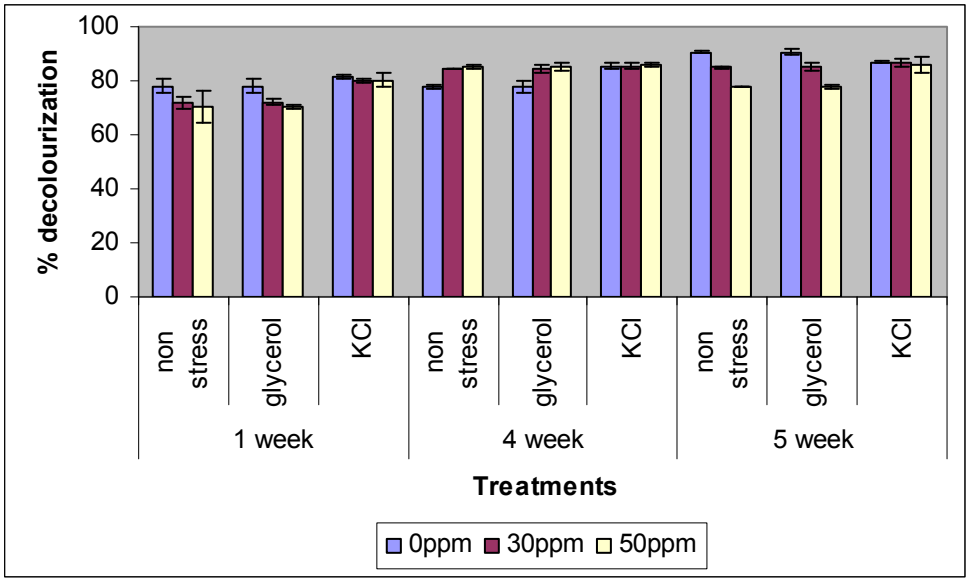


Figure 3.14 Total ligninolytic activity of *T. versicolor* (expressed as % decolouration of Poly R-478) in soil extract liquid broth, under three different water potential regimes: without stress, with -2.8 MPa water stress using glycerol and with -2.8 MPa water stress using KCl. Bars represent the standard deviation of the mean (n=3), per treatment.

3.13 Temporal evaluation of degradation of mixtures of pesticides

In order to assess the capacity of *T. versicolor* to grow under water stress and degrade mixtures of the pesticides chlorpyrifos, linuron and metribuzin their degradation rates were monitored after the first, third and fifth week. Surprisingly, chlorpyrifos was not detected in any of the treatments although it was detected in the previous study (Table 3.8). The possible explanation for this fact was investigated and is mentioned in the next section.

Uninoculated plates in triplicate were prepared for treatments in order to investigate the degradation rates of pesticides due to abiotic factors. These values are presented in red (ctl) in all the Tables.

Tables 3.12 to 3.17 show the impact of *T. versicolor* on degradation of the linuron and metribuzin in soil extract liquid broth and in soil extract liquid broth under water stress modified ionically by adding potassium chloride and non-ionically using glycerol.

Linuron degradation due to abiotic factors was quite high by the fifth week, with almost 50% in relation to the initial concentration, while metribuzin degradation was significant lower ranging from 2% to 21% in all treatments.

Linuron and metribuzin degradation in the first week was very low in all treatments and this was probably related to the slow initial growth of *T. versicolor*.

By the 3rd week higher degradation rates were evident in the treatments of linuron at both water regimes in relation to the controls and regardless of the pesticide concentrations.

Contrary to linuron, in the third week, metribuzin continued to be resistant to degradation in soil extract as well as in both water regimes (with glycerol and KCl). Moreover, in 40ppm concentration of metribuzin in soil extract there was not statistical significant difference between the controls and concentrations (p=0.281) (Table 3.19) (see Appendix F). Soil extract solute potential adjusted non-ionically by using glycerol showed a relative good degradation of metribuzin until the concentration 30ppm. Particularly, metribuzin did not detect in 10ppm concentration of the mixture while the remaining of metribuzin at 20 and 30 ppm of mixture was 40 and 65 %, respectively (Table3.16). In the concentration 40 ppm of the mixture there was not statistical significant difference in all treatments (p=0.169).

In the fifth week, it is very clear that in the inoculated plates with *T. versicolor*, linuron remaining was too low and in many cases the degradation was complete while in all controls the remaining of linuron was approximately 50% (Tables 3.12, 3.13 and 3.14).

Finally, in the fifth week and at -2.8 MPa water stress using glycerol, the percentage of metribuzin degradation reached to 40 % in the concentrations 40 and 50 ppm of the pesticides and it was highest in the concentrations 10, 20, 30 ppm of them 100, 84, 90%, respectively (Table 3.16).

The differences of degradation rates of the pesticides among all treatments for every week are presented in Tables 3.18 and 3.19.

Table 3.12 Concentration of linuron remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C. The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) linuron remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	86.4±0.8	76.6±6.9	52.3±0.9	13.8±6.9	53.9±5.0	0.00
20	86.1±1.5	84.4±3.3	67.2±0.9	18.2±9.2	55.6±0.2	0.00
30	82.2±2.8	84.4±3.3	61.1±5.1	33.2±7.0	56.6±4.4	4.6±2.3
40	87.1±1.5	81.5±0.0	84.7±1.5	8.9±1.1	70.3±0.8	3.3±1.7
50	80.2±0.4	79.2±0.0	69.9±1.6	23.4±3.9	57.9±0.8	0.00

Table 3.13 Concentration of linuron remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of glycerol ((water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) linuron remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	86.4±0.8	76.6±6.9	52.3±0.9	13.8±6.9	53.9±5.0	0.0
20	86.1±1.5	84.4±3.3	67.2±1.0	18.2±9.2	55.6±0.2	0.0
30	82.2±2.8	84.4±3.3	61.1±5.1	33.2±7.0	56.6±4.4	4.6±2.3
40	87.1±1.5	81.5±0.0	84.7±1.5	9.0±1.1	70.3±0.8	3.3±1.7
50	80.2±0.4	79.2±0.0	69.9±1.6	23.4±3.9	57.9±0.8	0.0

Table 3.14 Concentration of linuron remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of KCl (water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage(%) linuron remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	80.1±0.2	83.1±1.8	78.4±0.4	5.5±5.5	77.6±0.3	0.00
20	85.1±0.5	95.9±1.5	46.0±8.2	26.8±5.0	45.9±8.1	8.4±4.3
30	85.1±0.5	95.9±1.5	52.7±1.7	9.2±1.7	52.9±1.5	8.1±0.6
40	96.3±1.3	94.8±2.0	72.6±2.2	27.7±4.0	68.7±1.1	7.3±5.8
50	94.4±2.1	98.3±0.1	63.5±1.9	9.2±6.2	60.0±0.6	6.8±6.8

Table 3.15 Concentration of metribuzin remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^{0}C after 1, 3 and 5 weeks of incubation at 25^{0}C. The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) metribuzin remaining					
	Incubation at 25^{0}C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	77.5±0.4	77.2±1.5	79.0±1.4	70.2±6.0	80.9±2.1	69.8±1.7
20	92.3±1.5	94.4±0.4	92.1±1.1	90.0±0.8	90.5±1.7	92.9±2.8
30	89.9±0.5	90.7±0.4	88.8±2.4	82.2±0.7	78.9±3.6	92.5±4.1
40	98.6±0.6	99.2±0.7	94.5±0.3	89.2±3.2	96.5±2.6	87.2±1.8
50	91.3±2.0	94.5±1.3	91.4±0.6	80.1±3.2	86.0±4.7	69.6±6.7

Table 3.16 Concentration of metribuzin remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of glycerol ((water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) metribuzin remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	91.2±0.5	82.2±1.4	88.2±1.6	0	85.7±2.4	0
20	93.9±1.7	99.9±0.2	94.0±1.6	40.3±5.8	93.5±1.0	16.4±2.3
30	79.8±3.8	96.9±1.6	78.2±1.5	65.1±1.9	80.1±3.9	10.3±2.8
40	99.6±0.1	98.7±0.7	99.5±0.1	93.5±3.8	98.6±0.5	60.2±6.1
50	90.8±1.9	92.6±1.5	91.3±0.2	82.9±5.9	91.2±0.2	62.±4.3

Table 3.17 Concentration of metribuzin remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm (total concentrations), after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of KCl (water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage(%) metribuzin remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
3,3	78.1±3.2	82.9±0.7	72.8±5.1	67.7±4.6	69.3±1.9	71.7±3.6
6,6	93.7±5.1	90.2±1.1	88.8±5.0	84.2±6.1	94.0±3.0	90.0±6.2
10	97.2±2.1	96.0±2.4	97.7±2.2	88.3±2.8	96.8±2.0	91.8±3.9
13,3	97.6±1.3	97.4±0.3	97.5±0.4	89.4±4.1	96.8±1.5	84.1±2.0
16,6	94.0±0.1	94.6±0.6	95.5±1.0	97.6±2.0	92.±0.7	93.2±4.1

Statistical analysis

For statistical analysis, the data (% remaining pesticide) were transformed, before statistical analysis, using the following equation: $y=\arcsin\sqrt{p}$ and were back transformed for presentation.

In order to investigate the abiotic degradation of the pesticides over the six weeks period of incubation, plates, in triplicate, with the mixtures of pesticides without inoculation with *T. versicolor* and were used as controls.

Data for statistical analysis were tabulated as follows:

Treatments	Initial concentratiion of the mixture				
	10	20	30	40	50
(1) ctl non stress					
(2) Inoculated plates, non stress					
(3) ctl glycerol					
(4) Inoculated plates, plus glycerol					
(5) ctl KCl					
(6) Inoculated plates, plus KCl					

The statistical analysis showed that the interaction between pesticides concentrations per weeks was highly significant ($p<0.001$) for all treatments so separate tests were performed for each pesticide concentration in the same water regime per week with theTukey's test. The results are shown in Tables 3.18 and 3.19. Statistically significant differences among every concentration in the same water regime per week are expressed by different letters (see Appendix E).

Moreover, data obtained at the 5^{th} week were used for analysis of variance with two factors (concentrations x treatments) in order to compare the means of concentrations in the three water regimes (non-stress, ionic and non-ionic stress treatments).

For linuron, the interaction of two factors (concentrations x treatments) was significant ($p=0.002$). The interaction of different concentrations was also significant ($p=0.034$) but theTukey's test showed that the only difference was between the concentrations 6.6 and 13.3 ppm of linuron (20 and 40 ppm of the mixture). The interaction of treatments (non-stressed, non-ionic, ionic) was significant ($p <0.001$). The difference concerned controls and the inoculated plates.

For metribuzin the interaction of two factors (concentrations x treatments) was significant ($p<0.001$). The interaction of different concentrations was also

significant ($p<0.001$). TheTukey's test showed three subsets: the first concerned the concentration 3.3 ppm of metribuzin (10 ppm of the mixture), the second the concentration 10 ppm of metribuzin and the third the concentrations 6.6, 13.3 and 16.6 ppm of metribuzin. The interaction of treatments (non-stressed, non-ionic and ionic) was, also, significant ($p <0.001$). The difference was between glycerol treatments and all the others.

Table 3.18 Statistically significant differences of degradation rates of linuron among every concentration of mixture per week, in the same water regime, expressed by different letters.

Weeks	**Treatments**	**Initial concentratiion of the mixture (ppm)**				
		10	**20**	**30**	**40**	**50**
first	ctl non stress	b	b	d	c	b
	Inoculated plates, non stress	b	b	d	c	b
	ctl glycerol	b	b	d	b	c
	Inoculated plates, plus glycerol	b	c	c	b	c
	ctl KCl	a	a	b	a	a
	Inoculated plates, plus KCl	a	a	a	a	ab
third	ctl non stress	b	c	d	d	b
	Inoculated plates, non stress	a	ab	c	a	a
	ctl glycerol	b	bc	d	cd	b
	Inoculated plates, plus glycerol	a	b	b	b	a
	ctl KCl	b	bc	d	c	b
	Inoculated plates, plus KCl	a	a	a	a	a
fifth	ctl non stress	b	b	c	b	b
	Inoculated plates, non stress	a	a	ab	a	a
	ctl glycerol	c	b	c	b	b
	Inoculated plates, plus glycerol	a	a	b	a	a
	ctl KCl	b	b	c	b	b
	Inoculated plates, plus KCl	a	a	a	a	a

Table 3.19 Statistically significant differences of degradation rates of metribuzin among every concentration of mixture per week, in the same water regime, expressed by different letters.

Weeks	Treatments	Initial concentratiion of the mixture (ppm)				
		10	20	30	40	50
first	ctl non stress	a	a	ab	a	a
	Inoculated plates, non stress	a	ab	ab	a	a
	ctl glycerol	b	ab	a	a	a
	Inoculated plates, plus glycerol	a	b	b	a	a
	ctl KCl	a	ab	b	a	a
	Inoculated plates, plus KCl	a	a	b	a	a
third	ctl non stress	bc	b	b	a	abc
	Inoculated plates, non stress	b	b	ab	a	a
	ctl glycerol	c	b	ab	a	abc
	Inoculated plates, plus glycerol	a	a	a	a	ab
	ctl KCl	bc	b	c	a	bc
	Inoculated plates, plus KCl	b	b	b	a	c
fifth	ctl non stress	cd	b	b	bc	bc
	Inoculated plates, non stress	b	b	b	bc	ab
	ctl glycerol	d	b	b	c	c
	Inoculated plates, plus glycerol	a	a	a	a	a
	ctl KCl	b	b	b	c	c
	Inoculated plates, plus KCl	bc	b	b	b	c

Figures 3.15 and 3.16 show the remaining linuron and metribuzin concentrations, as percentage (%), respectively, after five weeks incubation. It is very clear that *T. vesicolor* was able to degrade linuron at all water regimes while at controls a concentration almost 50 % from the initial was remaining in the soil extract broth. In contrast metribuzin degradation was observed only at -2.8 MPa water potential adjusted with glycerol.

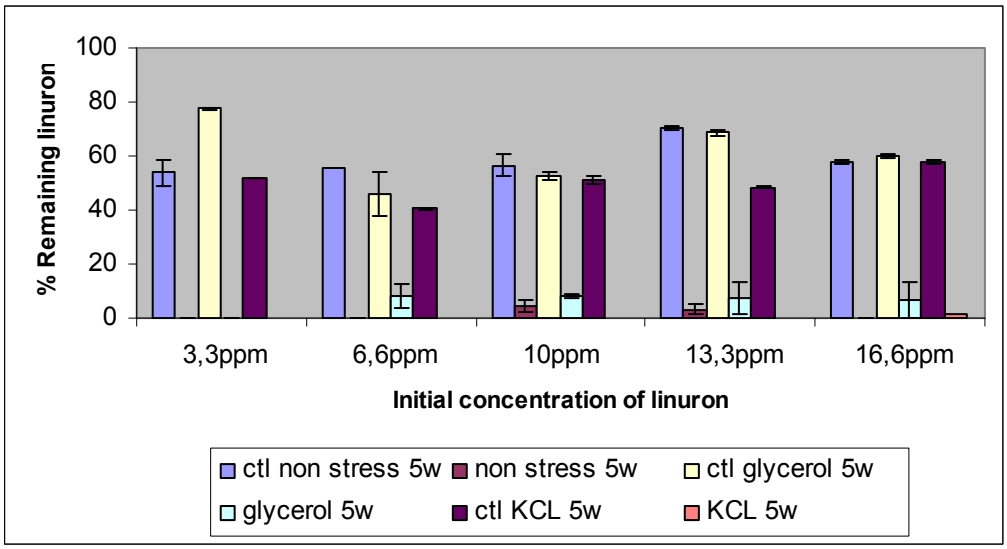


Figure 3.15 Percentage (%) linuron concentrations remaining in soil extract broth inoculated with *T. versicolor* and supplemented with a mixture of pesticides at 3.3, 6.6, 10, 13.3 and 16.6 ppm each of them, after 5 weeks of incubation at 25 ^{0}C, under three water regimes.

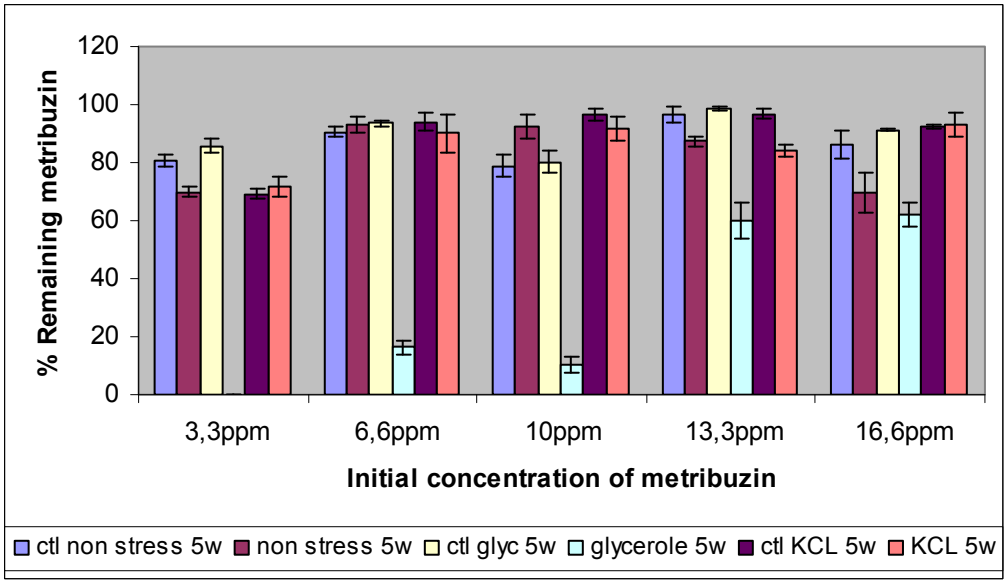


Figure 3.16 Percentage (%) metribuzin concentration remaining in soil extract broth inoculated with *T. vesicolor* and supplemented with a mixture of pesticides at 3.3, 6.6, 10, 13.3 and 16.6 ppm each of them, after 5 weeks of incubation at 25 ^{0}C, under three water regimes.

3.13.1 Chlorpyrifos degradation

No chlorpyrifos was detected in any of the treatments. Therefore the same samples were measured again using the protocol for the chlorpyrifos quantification by Sigma in order to cross-checked the results. As no chlorpyrifos was detected, TCP concentration in the samples was attempted, as TCP is considered the main metabolite of chlorpyrifos. However, noTCP was detected.

3.14 Toxicity test

In this study the goal was to assess the toxicity of the soil extract broth inoculated with *T.trametes* after 5 weeks incubation. Initially we tested the response of this toxicity to soil extract broth, spiked with different concentrations of pesticides (individually and as a mixture).

Figure 3.17 shows the response of *Tetrahymena thermophila* to to soil extract broth, spiked with different concentrations of chlorpyrifos, linuron and metribuzin, individually and as a mixture. *T. thermophila* showed high sensitivity to the mixture of pesticides with 94% and 100% inhibition in the treatments spiked with 12 ppm and 15 ppm, respectively. Chlorpyrifos and linuron in the treatment of 10 ppm caused 82 and 89% inhibition, respectively. Metribuzin was less toxic than the others, showing 86% inhibition in the 15 ppm treatment.

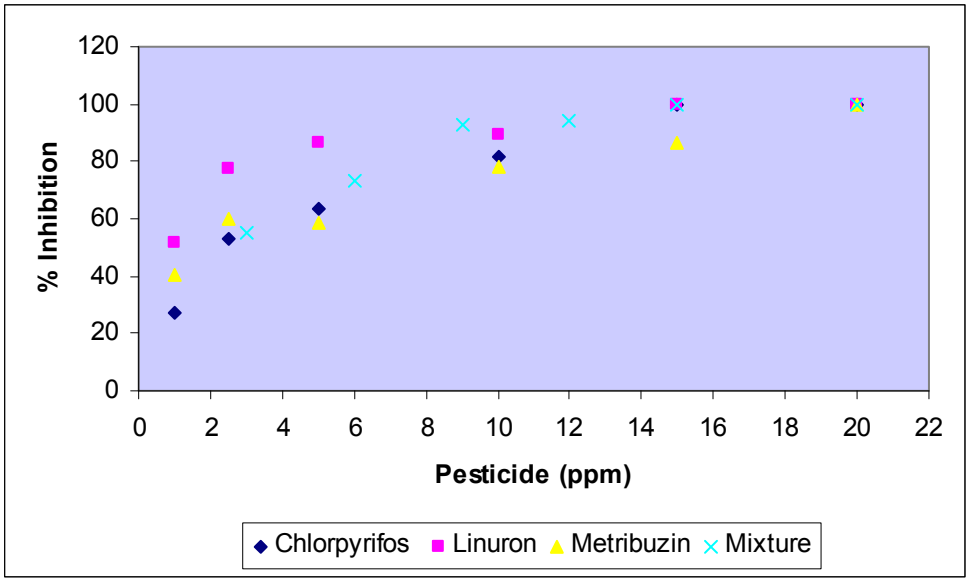


Figure 3.17 Inhibition curve for *Tetrahymena thermophila* in soil extract broth spiked with chlorpyrifos, linuron and metrbuzin individually and spiked with a mixture of the three pesticides, achieved with PROTOXKIT F^{TM}.

Only the extracts of treatments non-stressed and plus KCl at the concentrations of 0, 20 and 40 ppm after 5 weeks incubation were tested. The results obtained showed relative toxicity which, maybe, is attributed to the presence of metribuzin as this compound was not degraded. Particularly, at 20 and 40 ppm of the filtrates without stress the inhibition % was 13.4 and 18%, respectively. At the treatments with the KCl the inhibition % was 2 and 23.5 % for the 20 and 40 ppm concentrations of the mixture of pesticides, respectively.

4 DISCUSSION

4.1 General overview

Pollution of the environment has been one of the largest concerns to science and the general public in the last years. Soil and an aqueous environment are natural and preferential sinks for contamination, and their pollution represents an important concern for human and environmental health (Gianfreda & Bollag, 2002).

It is clear that microbial-based treatments of contaminated environments and industrial effluents offer an economical alternative to existing treatment methods (Singleton, 1994). Bioremediation, which involves the use of microbes to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments (Singh *et al.,* 2006).

The application of fungal technology for the clean up of polluted soils holds significant promise since 1985 when the white rot fungus *Phanerochaete chrysosporium* was found to be able to metabolise a number of important environmental pollutants (Sasek, 2003). This capacity was later described for other white rot species among them *Trametes versicolor.* Successful removal of pesticides by the addition of bacteria (bioagumentation) has been also reported for many compounds including, parathion, coumaphos, ethoprophos and atrazine (Singh *et al.,* 2006).

4.2 Fungal tolerance to pesticides

The initial studies were carried out using five fungal species. The temperature that was used in all studies was 25 ^{0}C as is considered environmentally relevant to Greece and other Mediterranean countries.

The screening experiment showed that all the tested fungi had a good growth in a mineral salts medium (MMY) in relation to the conducive media malt

extract agar and the modified MMY plus glucose. The reduction of the growth was approximately 40 % for the species *P. ostreatus* and *P. gigantea,* 20 % for the species *P. chrysosporium* and *P. coccineus.* The growth of *T. versicolor* was not affected.

The response of the test isolates to the pesticides was influenced by pesticide concentration and whether the pesticides were in a mixture or present individually. In relation to chlorpyrifos, *P. ostreatus* was more tolerant than all the others while *P. chrysosporium* showed high sensitivity to chlorpyrifos. *P. coccineus* and *P.gigantea* showed very good tolerance to linuron exhibiting no inhibition and 20 % respectively at 30ppm of linuron. Again, *P. chrysosporium* showed high sensitivity to this pesticide. Expect *P. chrysosporium,* all the other fungi have a very good tolerance at 30ppm of metribuzin and the mixture of 30 ppm of the pesticides. In the presence of the pesticide mixture all the species showed a remarkable growth decrease.

The results that were based on the growth of the fungi on the soil extract agar and the analysis of EC_{50} values confirmed the initial screenings. Particularly, all fungi were sensitive to high concentrations of linuron, while they had a very good tolerance to metribuzin. *P. ostreatus* and *P. coccineus* were more sensitive than the others to chlorpyrifos. In the mixture of pesticides *P. ostreatus* was very sensitive and was not included in subsequent studies. Overall, the results showed clearly that there was a very significance reduction of the fungal growth in the presence of mixture of pesticides in relation to their presence individually. Therefore it is very important to study of the differential effect of mixtures of compounds in mixtures as these interact with biological systems in ways that can greatly alter the toxicity of individual compounds (Hernando et al., 2003).

The study of fungal growth rates is very important for extrapolation of the potential colonization capacity in the field as it provides a good indication of the speed at which a fungus is able to colonise a substrate. Growth rates may also indicate which species may be dominant over a particular

substrate; fast growing species have an advantage over slower species as they can reach and utilise resources before their competitors (Magan and Lacey, 1984; Marin *et al*., 1998a; Marin *et al*., 1998b). Therefore, better fungal growth could help the introduced fungi to overcome competition from indigenous soil microorganisms (Singleton, 2001).

The results for laccase production, in the Petri plate assay were interesting, as all tested fungi (*T. versicolor*; *P. gigantea* and *P. coccineus*) were able to produce laccase in all the pesticide treatments. *T. versicolor* showed the highest laccase activity and it was less affected by pesticide concentration. Only *T. versicolor* was able to degrade lignin in the conditions of this assay. The fungus *P. coccineus* has been used in bioremediation research mainly for its effective extra cellular lacasse production (Alves *et al,* 2004; Pointing *et. al*, 2000). The ability of *T. versicolor* to produce a wide array of enzymes in the presence of contaminants may be advantageous in bioremediation.

4.3 Studies on the soil extract liquid broth

Studies in liquid culture are a good approach to assess an organism's ability to utilise a target compound where the compound is added to the medium either as a sole carbon source or in the presence of a growth supporting substrate (Juhasz and Naidu, 2000). Several reports have demonstrated good agreement between studies investigating optimum conditions for biodegradation in liquid culture systems and bioremediation studies in soil or natural water matrices. Lamar *et al.*, (1990) compared *P. chrysosporium* and *P. sordida* with respect to removing pentachlorophenol from liquid medium and from soil, and found that *P. chrysosporium* was more effective in soil whereas *P. sordida* was more efficient in liquid culture. This suggests that the former can provide a reliable prediction of the range of conditions in which pesticide- degrading bacteria will be active (Karpouzas & Walker, 2000).

Both success and failure have been reported when species capable of degrading pesticides in liquid culture were introduced into the soil. A strain of *Streptomyces* was able to grow on eight pesticides and also degraded them in soil (Shelton *et al*., 1996). Similar results were obtained when an iprodione degrading *Arthrobacter* strain was inoculated in to the soil (Mercadier *et al*., 1996). Several chemicals have been successfully removed from soil and aquatic environments using degrading microorganisms such as chlorinated pyridinol (Feng *et al*., 1997), coumaphos (Mulbry *et al*., 1996, 1998) and atrazine (Struthers *et al*., 1998; Topp, 2001). In contrast, MacRae and Alexander (1965) reported the failure of a 4-(2, 4-dichlorophenoxy) butyrate utilizing bacteria to degrade the chemical when introduced into a treated soil (Singh *et al.,* 2006).

Soil extract liquid broth that was used as the culture medium is a nutritionally weak medium to mimic the nutrient availability in soil. *T. versicolor, P. gigantea* and *P. coccineus* were able to grow effectively in this low nutrient status medium over a range of concentrations of a mixture of pesticides. Chlorpyrifos degradation was shown to have differences between the fungi as well as the concentrations of the pesticide. *P. coccineus* showed able to degrade chlorpyrifos almost 60%, even at 50ppm of the mixture.

Regarding metribuzin, all tested fungi were shown to be unable to degrade it. Perhaps the duration of experiment was not sufficient for the degradation as Schilling *et al*. (1985) obtained a total transformation by *Rhizopus japonicus* and *Cunninghamella echinulata* and a depletion range of 27 to 45% with *Aspergillus niger, Penicillium lilacinum*, and *Fusarium oxysporum* after a 4-week's incubation period. Finally, *T .versicolor* showed a great capacity to degrade linuron even at the 50 ppm concentration of the mixture.

Regarding bacteria, all tested bacterial strains were isolated from Cretan soils and composts and tested for their possible capability to degrade the pesticides. It was considered that they have become acclimatized well in the local conditions. Several of them demonstrated a very good growth in the

presence of high concentrations of the pesticides. However, the results showed clearly that there was a significant reduction of the growth in the presence of mixture of pesticides in relation to their presence individually. The bacterial strains and the mixture of them used were unable to degrade the mixture of pesticides. The degradation of pesticides is usually achieved by a consortium of microbes rather than a single species (Aislabie &Lloyd Jones, 1995). El-Fantroussi (2000) enriched a mixed bacterial culture able to degrade linuron and metobromuron from a previously linuron-treated orchard soil. None of the strains isolated were capable alone or in combination of degrading linuron or metobromuron. Analyzing the bacterial composition at various steps in the degradation, using denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes, strongly suggested the involvement of a bacterial consortium in the degradation. Roberts et al.,(1993) and Sørensen and Aamand, (2001) also encountered difficulties in isolating pure cultures able to degrade phenylurea compounds from different phenylurea-degrading mixed bacterial cultures enriched from soil, thus indicating a lack of single strains able to proliferate through such degradation and hence supporting the involvement of bacterial consortia.

However, several reports have demonstrated successful degradation of pesticides by *Bacillus* and *Pseudomonas* species. Particularly, Rani and Lalithakumari (1994) showed that *Pseudomonas putida* utilized methyl parathion as sole carbon and (or) phosphorus source. The bacterium elaborated the enzyme organophosphorus acid anhydrase, which hydrolyzed methyl parathion to p-nitrophenol. In 1995, a *Pseudomonas* species, isolated from an herbicide spill site, was capable of metabolizing atrazine at very high concentrations by Mandelbaum *et al*. Two strains of *P. putida* (epI and epII) isolated previously from ethoprophos-treated soil, were able to degrade ethoprophos in a mineral salts medium plus nitrogen, in less than 50 hours (Karpouzas and Walker, 2000). These researchers studied conditions such as temperature, pH and nutrient status and found out that the degradation of ethoprophos was most rapid when the bacterial culture were incubated at 25 and 37 ^{0}C at pHs ranging from 5.5 to 7.6. Addition of glucose or succinate to

the culture medium did not influence the the degrading ability of *P. putida.*The strain epI was capable of degrading the pesticide when only 60 cells mL^{-1} were used as initial inculum, while epII when inculum densities of 600 cells mL^{-1} or higher were used. Extensive biodegradation of propanil (3,4-dichloropropionanilide) by a strain of *P. putida* AF7 was demonstrated in nutrient cultures. The organism was capable of using propanil as the sole source of C, presenting up to 60% of degradation. This strain was isolated from the rhizosphere of rice grown in contaminated soil with this herbicide.

Eight bacteria were isolated by repeated subculture in liquid medium with trifluralin from a soil in which this pesticide has been used for the last four decades. In a mineral salts medium with 0.1% succinate, 0.1% yeast extract and 50 mg L^{-1} trifluralin, reductions in the level of pesticide of 24.6% for *Klebsiella* sp., 16.4% for *Herbaspirillum* sp., 25.0% and 16.0% for two strains of *Bacillus* sp. were obtained after 30 days (Bellinaso, 2003). In four other culture media that were used the degradation rates of trifluralin were very low. A bacterium strain of *Bacillus* genus isolated from cloud water was the first pure strain capable of rapidly degrading mesotrione, a new selective herbicide for control of broad-leaved weeds in maize (Durand, 2006). Finally, suspensions of soil repeatedly treated with carbofuran under glasshouse conditions enhanced the degradation of carbofuran significantly in a mineral salts medium, 96% degradation compared to 15% in unicoculated medium in 10 days. Out of the seven bacterial cultures isolated from the enrichment culture, two cultures indentified as *Pseudomonas stutzeri* and *Bacillus pumilis* enhanced carbofuran degradation, resulting in more than 98% loss of the applied pesticide in 30days (Mohapatra &Awasthi, 1997).

In all above –mentioned studies the bacterial strains were isolated from contaminated sources by enrichment cultures so the possibility to have degrading abilities was very high in contrast to the strains of *Bacillus* and *Pseudomonas* species from Cretan soils without any application of the pesticides chlorpyrifos, linuron and metribuzin. Conditions such as pH, temperature and initial inoculums density were almost the same. Several

studies failed to obtain micro-organisms capable of growing on specific chemicals or degrade them. This failure does not exclude biological involvement in degradation and could be attributed to the selection and composition of the liquid media under artificial environments, or strains requiring special growth factors (Singh &Walker, 2005). Moreover, the culture medium could have a strong influence on biodegradation and it is important to use various media when testing for xenobiotic biodegradation (Bellinaso, 2003).

4.4 Studies on differential break down of mixtures of pesticides under water stress

While studies on the capacity of white rot fungi and particularly *T.versicolor* to degrade individual pesticides is extensive (Gadd, 2001), very few have examined the capacity to degrade mixtures of pesticides as also as their activities under interacting conditions of pesticides mixtures and different water availabilities (Fragoeiro & Magan, 2004). Yavad and Reddy (1993) described co-mineralization of a mixture of the pesticides 2, 4-D and 2,4,5-T by *P.chrysosporium* wild-type and a putative peroxidase mutant in nutrient-rich broth with a small amount remaining in the mycelial fractions (5%). Bending *et al*., (2002) showed degradation rates of metalaxyl, atrazine, terbuthylazine and diuron by white rot fungi in nutrient solution of >86% for atrazine and terbuthylazine. Fragoeiro & Magan, (2004) studied the enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *T. versicolor* and *P. chrysosporium.* Their results suggested that both fungi isolates had the ability to degrade the pesticides simazine, dieldrin and trifluralin supported by the capacity for expression of a rate of extracellular enzymes at -0.7 and -2.8 MPa water potential. Particularly, *T. versicolor,* the same isolate used in current studies, was able to produce phosphomonoesterase, protease (relevant to P and N release), β- glucosidase, cellulase (carbon cycling) and laccase activity.

Water availability was considered in this study, because it plays a key role in fungal development. It was important to evaluate fungal response to pesticides at different levels of water availability, which occur naturally in soil. In this study, *T. versicolor* exhibited very high laccase activity in soil extract broth solute potential adjusted at -2.8 MPa non-ionically by using glycerol, for example in the 30 ppm treatment, at the second week, laccase activity of 2.26 units $mg^{-1}$was measured. Laccase production was the maximum at the second and third week of the incubation. In soil extract broth without stress the levels of laccase produced were quite high and they were highest at the sixth week of incubation while no laccase was observed at -2.8 MPa solute potential adjusted by adding potassium chloride. Fragoeiro & Magan, (2005) found that at -2.8 MPa solute potential adjusted by adding potassium chloride laccase production was significally reduced, but up to 20 units were still detected.

The results suggest that laccase is not only secreted in nutrient-rich substrates but is produced by mycelia growing in weak nutritional matrices. Of particular interest is the capacity of *T. versicolor* for laccase production in the presence of up a 50 ppm mixture of the pesticides. This presence was shown to stimulate laccase production as in the treatment of 0 ppm, without stress laccase production was very low related to all the other concentrations over the first four weeks period. It reached to the same levels at fifth and six week of incubation. Recent studies demonstrated induction of production of ligninolytic enzymes, particularly laccase, in the presence of copper, veratryl alcohol and a phenolic mixture (Xavier *et al.*, 2007), in the presence of mixtures of pesticides (Fragoeiro & Magan, 2005). Moreover, Mougin *et al.*, (2002) studied several agrochemicals, industrials compounds and their transformation products for their ability to enhance laccase production in liquid cultures of *T. versicolor.* Many of them enhanced laccase activity up to 20-fold having as positive control 2, 5-xylidine (35-fold enhancement of laccase activity) (Xiao *et al.*, 2004)

Cuto *et al.*, (2006) reported highly significant increases in laccase produced by *Trametes hirsuta* growing in an air-lift bioreactor after the addition of glycerol. Fragoeiro & Magan (2008) studied the impact of water potential on mixtures of the pesticides simazine, dieldrin and trifluralin, in soil inoculated with *T. versicolor* and *P. chrysosporium* in relation to different soil water potentials(-0.7 and -2.8 MPa). The reseachers showed that in natural soil the level of laccase produced by *T. versicolor* was very low, reaching the highest levels after 6 weeks incubation under both water regimes, whereas that amended with wood chips showed some laccase production, with the highest level after 12 weeks incubation. Overall, the main difference between laccase production in soil microcosms and that in soil extract broth was that in soil extract based liquid culture laccase production was much higher at -0.7 MPa while in soil microcosms the optimum was at -2.8 MPa.

Total ligninolytic activity was expressed as the capacity to decolourise poly-R478. The decolouration assay of poly-R478, with similar structure to lignin, gives information on the activity of the whole set of enzymes because the degradation of lignin is carried out by several enzymes. The results showed that decolouration of this dye, occurred in all treatments with the highest levels of decolouration after 5 weeks incubation at -2.8 MPa water potential adjusted with KCl. In all cases the decolouration rates were unaffected by pesticide treatment. The results suggest *T. versicolor* was tolerant to this mixture of pesticides, producing equivalent levels of decolouration in the presence and absence of the xenobiotics.The highest levels of decolouration that occurred in the treatments with KCl may be related to the production of other enzymes as at -2.8 MPa water potential adjusted with KCl no laccase production was observed.

There was no correlation between decolourization of the dye and degradation of the pesticide mixture in this study. A similar result was described for degradation of diuron, metalaxyl atrazine and terbuthylazine, by several fungi in liquid culture (Bending *et al.*, 2002) as well as for the degradation of simazine, trifluralin and dieldrin in soil (Fragoeiro & Magan, 2008) although

Alcalde *et al*., (2002) observed correlation with oxidation of PAHs mediated by laccases.

The present study on the degradation of the mixture of pesticides showed good capacity of *T. versicolor* to degrade linuron at all tested water regimes, regardless of the initial concentrations of mixture between 0 and 50 ppm. Metribuzin degradation occurred only in the treatments at -2.8 MPa solute potential adjusted with glycerol. A possible explanation, maybe, is that *T. versicolor* is unable to utilize this pesticide as a carbon source for its growth and as soil extract broth is a weak nutrient medium glycerol was probably used by the fungus as an additional carbon source.

Interestingly, chlorpyrifos and its main metabolite TCP were not detected. The environmental fate of this pesticide has been studied extensively. The manufacturer reports that chlorpyrifos is a degradable compound, and a number of environmental forces may be active in its breakdown. In all systems (soil, water, plants and animals), the major pathway of degradation begins with cleavage of the phosphorus ester bond to yield 3,5,6-trichloro-2-pyridinol (TCP). In soil and water, TCP is further degraded via microbial activity and photolysis to carbon dioxide and organic matter. Hydrolytic and photolytic half-lives are both around a month, at neutral pH 25° C. Under more alkaline conditions, hydrolysis proceeds more rapidly. In natural water samples, however, degradation often proceeds significantly faster; a 16-fold enhancement of hydrolysis rate has been observed in pond and canal water samples. Half-lives in the water column of less than one day are typical, due to a combination of degradation, volatilization and partitioning into sediments.

Several published studies on the biodegradation of chlorpyrifos in liquid media have reported no significant rates of abiotic degradation of chlorpyrifos. Particularly, no abiotic degradation of chlorpyrifos in uninoculated media over a four days period was concerned by Singh *et al.*, (2004) 70 hours by Singh *et al.*, (2006) over a 48 and 15 hours period by Xiaohui *et al.*, (2007)and Xu (2008) respectively.

5 . CONCLUSIONS AND FURTHER WORK

5.1 Conclusions

The main findings of this study are summarized below:

1. Screenings in a minimal salts medium (MMY) on the tolerance of five white rot fungi to chlorpyrifos, linuron and metribuzin, individually and in a mixture suggested best tolerance by *T. versicolor, P. osteatus, P. gigantea* and *P. coccineus*, while *P. chrysosporium* was very sensitive.
2. In agar-based studies *T. versicolor* was able to degrade lignin, in the presence of the pesticides, individually and as a mixture. In contrast all the others did not degrade lignin under the conditions of the assay.
3. *T. versicolor, P. gigantea* and *P. coccineus* produced laccase, in agar-based plates and in the presence of the pesticides, individually and as a mixture.
4. All three test isolates were able to grow in soil extract broth supplemented with mixture of the pesticides. They were shown to be unable to degrade metribuzin. In contrast, *T. versicolor* showed a great capacity to degrade linuron even in the 50 ppm concentration.
5. Very high levels of laccase were produced by *T. versicolor* in soil extract broth solute potential adjusted at -2.8 MPa non-ionically by using glycerol. Laccase production was the maximum at the second and third week after the inoculation.
6. The levels of laccase produced by *T. versicolor* in soil extract broth without stress were quite good and they were highest at the sixth week of incubation. This pesticides mixture was shown to induce laccase production.
7. *T. versicolor* did not produce laccase in soil extract broth solute potential when this was adjusted at -2.8 MPa ionically by adding potassium chloride.
8. The results showed that decolouration of poly R-478 dye occurred in all treatments but there was no correlation between decolourization of the dye and degradation of the pesticide mixture under the conditions of this study.

9. The results obtained on soil extract broth provide valuable background information on the abilities of *T.versicolor* to tolerate and degrade mixtures of pesticides as well as to produce high levels of laccase in soil extract even under imposed water stress at -2.8 MPa and suggest potential application of this fungus in bioremediation.

5.2 Further work

Further studies based on the findings of this study are:

- Analysis of metabolites by GC-MS

In current study linuron was degraded in soil extract broth after five weeks incubation with *T.versicolor,* while chlorpyrifos was not detected. However, these pesticides may have been completely degraded or mineralized; some of it may have been transformed in unknown metabolites. Additional analysis by GC-MS of the final products would be pertinent, as chemical or microbial co- metabolism may produce toxic intermediates.

- Studies in soil microcosms

  The current study in liquid medium has shown the potential utilisation of these pesticides by *T.versicolor* in the environment. However, the conditions in soil differ greatly from those in liquid culture, because soil is a multi-phasic, heterogeneous environment, in which the contaminants present in association with the soil particles, dissolved in soil liquids and in the soil atmosphere (Boopathy, 2000). Microcosm studies are very useful to understand the detoxifying factors associated with a soil-pesticide matrix (Shoen and Winterlin, 1987), including enzyme activities, respiratory metabolism and native microbial populations activity, as the mechanisms by which the white rot fungi degrade pollutants are still not well understood (Nerud et al., 2003) Testing in soil microcosms under Cretan conditions to examine the range of concentrations of mixture which could be broken down under different moisture and tremperature conditions.

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APPENDIX A

Chromatogram of metribuzin, chlorpyrifos and linuron obtained in single HPLC run with metribuzin eluting at 3.2, chlorpyrifos at 5.2 and linuron at 5.5 minutes in a mixture of 50ppm of the pesticides (equal in amount).

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Sample Name: without stress 1w 40a

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Acq. Operator    : sofia
Acq. Instrument  : Instrument 2                    Location : Vial 1
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Acq. Method      : C:\CHEM32\2\METHODS\PEST.M
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Analysis Method  : C:\CHEM32\2\METHODS\PEST.M
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                   (modified after loading)
Method Info      : Metribuzin, Linuron, Chlorpyrifos.
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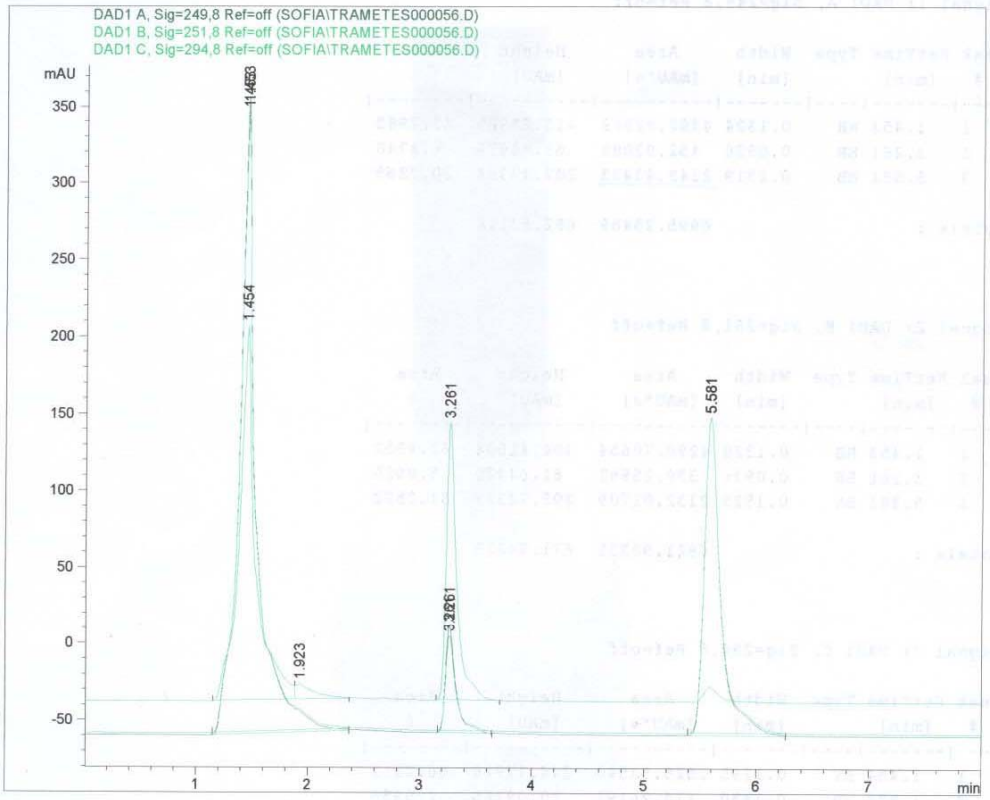


Instrument 2 9/27/2007 2:01:11 PM sofia Page 1 of 2

APPENDIX B

Laccase units

Laccase units						
without stress						
	1 week	**2 week**	**3 week**	**4 week**	**5 week**	**6 week**
0ppm	0.5±0.1	0.1±0.1	0.1±0.0	0.3±0.2	1.0±0.1	2.7±0.7
10ppm	1.4±0.2	0.3±0.1	0.3±0.2	2.1±1.0	2.5±0.8	1.2±0.3
20ppm	3.8±0.4	1.6±0.4	3.1±0.3	2.0±0.8	2.1±0.2	2.5±0.6
30ppm	2.1±0.3	2.2±0.7	0.6±0.2	0.9±0.2	2.6±	1.3±
40ppm	1.8±0.4	1.7±0.4	0.8±0.2	1.8±0.2	1.8±0.2	1.5±0.6
50ppm	1.6±0.2	1.6±0.1	1.8±0.2	1.9±0.1	2.2±0.3	2.1±0.3
glycerol						
	1 week	**2 week**	**3 week**	**4 week**	**5 week**	**6 week**
0ppm	5.7±1.2	6.9±0.5	5.9±0.5	2.9±0.4	2.6±0.6	1.8±0.3
10ppm	6.1±0.8	10.9±0.8	7.6±0.7	6.6±0.3	2.5±0.3	3.1±0.5
20ppm	3.3±0.6	20.1±0.8	14.4±0.6	6.6±0.6	4.4±0.5	2.8±0.2
30ppm	4.1±0.8	23.4±1.3	19.2±2.2	9.7±0.9	3.2±0.8	2.6±0.4
40ppm	1.5±0.6	5.4±0.1	21.0±0.9	6.5±0.9	5.3±0.9	2.8±0.5
50ppm	1.2±0.1	8.3±1.0	12.8±1.5	12.2±1.5	6.0±1.0	3.8±1.0

APPENDIX C

Statistical tests

Analysis of variance with two factors (water regime x week)

Proteins

Without stress: Substrate =1

With glycerol (-2.8MPa): Substrate =2

Concentrations: Con.

Univariate Analysis of Variance

conc = ,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	6
	4,00	6
	5,00	5
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = ,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,001[a]	11	5,75E-005	1,391	,242
Intercept	,005	1	,005	123,122	,000
week	,000	5	3,44E-005	,832	,541
substrate	2,26E-005	1	2,26E-005	,548	,467
week * substrate	,000	5	8,78E-005	2,125	,099
Error	,001	23	4,13E-005		
Total	,007	35			
Corrected Total	,002	34			

a. R Squared = ,399 (Adjusted R Squared = ,112)

b. conc = ,00

conc = 10,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	5
	3,00	5
	4,00	4
	5,00	5
	6,00	6
substrate	1,00	16
	2,00	15

a. conc = 10,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,000[a]	11	1,07E-005	1,352	,272
Intercept	,002	1	,002	203,312	,000
week	6,65E-005	5	1,33E-005	1,677	,189
substrate	2,76E-008	1	2,76E-008	,003	,954
week * substrate	4,92E-005	5	9,83E-006	1,240	,329
Error	,000	19	7,93E-006		
Total	,002	31			
Corrected Total	,000	30			

a. R Squared = ,439 (Adjusted R Squared = ,114)

b. conc = 10,00

conc = 20,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	5
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	16

a. conc = 20,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,001[a]	11	4,57E-005	1,998	,081
Intercept	,004	1	,004	172,494	,000
week	,000	5	3,42E-005	1,495	,232
substrate	4,76E-006	1	4,76E-006	,208	,653
week * substrate	,000	5	5,31E-005	2,319	,078
Error	,001	22	2,29E-005		
Total	,005	34			
Corrected Total	,001	33			

a. R Squared = ,500 (Adjusted R Squared = ,250)

b. conc = 20,00

conc = 30,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	6
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	18
	2,00	17

a. conc = 30,00

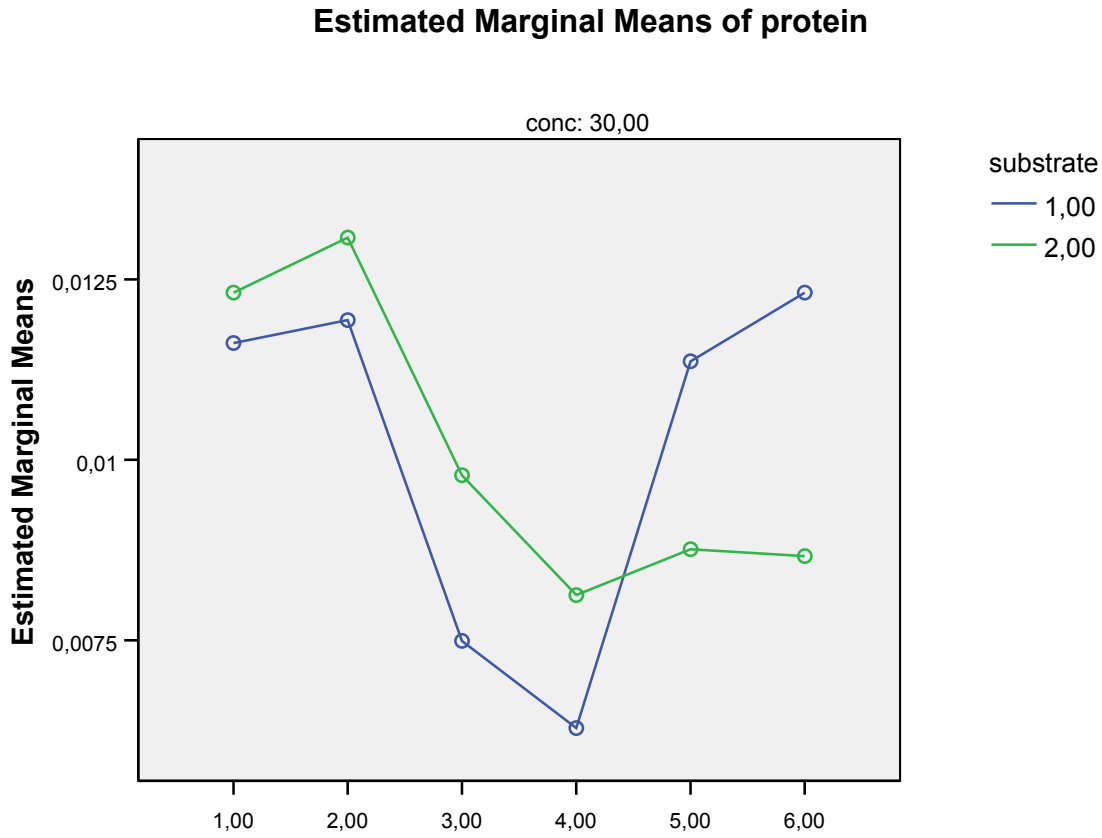
Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,000[a]	11	1,48E-005	2,821	,017
Intercept	,004	1	,004	676,493	,000
week	,000	5	2,39E-005	4,543	,005
substrate	1,83E-008	1	1,83E-008	,003	,953
week * substrate	4,18E-005	5	8,36E-006	1,589	,203
Error	,000	23	5,26E-006		
Total	,004	35			
Corrected Total	,000	34			

a. R Squared = ,574 (Adjusted R Squared = ,371)

b. conc = 30,00



conc = 40,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	4
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	17
	2,00	16

a. conc = 40,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,000[a]	11	2,06E-005	1,973	,087
Intercept	,002	1	,002	236,938	,000
week	,000	5	2,57E-005	2,459	,067
substrate	1,81E-005	1	1,81E-005	1,732	,202
week * substrate	6,90E-005	5	1,38E-005	1,322	,293
Error	,000	21	1,04E-005		
Total	,003	33			
Corrected Total	,000	32			

a. R Squared = ,508 (Adjusted R Squared = ,251)

b. conc = 40,00

conc = 50,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	6
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = 50,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9,34E-005[a]	11	8,49E-006	1,141	,377
Intercept	,003	1	,003	410,168	,000
week	1,89E-005	5	3,78E-006	,508	,767
substrate	1,51E-007	1	1,51E-007	,020	,888
week * substrate	7,36E-005	5	1,47E-005	1,978	,120
Error	,000	23	7,44E-006		
Total	,003	35			
Corrected Total	,000	34			

a. R Squared = ,353 (Adjusted R Squared = ,044)

b. conc = 50,00

APPENDIX D

Statistical tests

Specific activity

Statistical analysis of variance was performed for each pesticide concentration in the same water regime per week with the Tukey's test.

Without stress: Substrate =1

With glycerol (-2.8MPa): Substrate =2

Concentrations: Con.

Without stress

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	219447,3	5	43889,464	57,567	,000
Within Groups	9148,883	12	762,407		
Total	228596,2	17			

a. substrate = 1,00, conc = ,00

sp_act [b]

Tukey HSD [a]

week	N	Subset for alpha = .05		
		1	2	3
3,00	3	5,0872		
2,00	3	8,6117		
4,00	3	29,0258	29,0258	
1,00	3	52,3602	52,3602	
5,00	3		94,2347	
6,00	3			323,0022
Sig.		,350	,108	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = ,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	95107,758	5	19021,552	17,560	,000
Within Groups	9749,280	9	1083,253		
Total	104857,0	14			

a. substrate = 1,00, conc = 10,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05		
		1	2	3
3,00	3	29,7850		
2,00	2	44,1578		
5,00	2	84,1968		
4,00	2	124,0416	124,0416	
1,00	3		191,9263	191,9263
6,00	3			237,6563
Sig.		,090	,300	,661

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 1,00, conc = 10,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	194786,2	5	38957,237	4,458	,016
Within Groups	104875,1	12	8739,594		
Total	299661,3	17			

a. substrate = 1,00, conc = 20,00

sp_act[b]

Tukey HSD[a]

week	N	Subset for alpha = .05	
		1	2
4,00	3	121,7733	
5,00	3	187,6174	
1,00	3	280,3112	280,3112
2,00	3	289,2732	289,2732
6,00	3	302,6139	302,6139
3,00	3		456,2315
Sig.		,240	,264

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = 20,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	180447,5	5	36089,508	10,314	,001
Within Groups	41988,773	12	3499,064		
Total	222436,3	17			

a. substrate = 1,00, conc = 30,00

sp_act[b]

Tukey HSD[a]

week	N	Subset for alpha = .05		
		1	2	3
5,00	3	80,4536		
4,00	3	96,7647		
2,00	3	177,8601	177,8601	
6,00	3	219,9692	219,9692	219,9692
3,00	3		292,6933	292,6933
1,00	3			360,7950
Sig.		,108	,238	,104

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = 30,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	132448,4	5	26489,677	1,624	,233
Within Groups	179472,2	11	16315,651		
Total	311920,5	16			

a. substrate = 1,00, conc = 40,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05
		1
4,00	3	53,6842
3,00	2	103,9264
5,00	3	210,0381
2,00	3	235,8594
1,00	3	245,2956
6,00	3	314,6861
Sig.		,234

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,769.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 1,00, conc = 40,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	125129,5	5	25025,897	9,355	,001
Within Groups	32100,629	12	2675,052		
Total	157230,1	17			

a. substrate = 1,00, conc = 50,00

sp_act[b]

Tukey HSD[a]

week	N	Subset for alpha = .05	
		1	2
4,00	3	60,7572	
2,00	3	63,1456	
3,00	3	112,4375	
5,00	3	148,4546	
1,00	3	157,6662	
6,00	3		308,5250
Sig.		,267	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = 50,00

Water potential -2.8 MPa

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1551482	5	310296,411	5,097	,012
Within Groups	669664,7	11	60878,609		
Total	2221147	16			

a. substrate = 2,00, conc = ,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
5,00	2	173,8347	
6,00	3	212,0878	
4,00	3	277,7036	277,7036
1,00	3	462,0265	462,0265
3,00	3	768,3989	768,3989
2,00	3		990,3646
Sig.		,124	,051

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,769.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = ,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4380450	5	876089,986	9,872	,002
Within Groups	798733,8	9	88748,202		
Total	5179184	14			

a. substrate = 2,00, conc = 10,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
5,00	2	420,7520	
1,00	3	483,2134	
6,00	3	521,0306	
3,00	2	1067,0508	1067,0508
4,00	2	1231,3270	1231,3270
2,00	3		1850,5402
Sig.		,113	,130

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 10,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5141054	5	1028210,750	39,641	,000
Within Groups	259378,2	10	25937,820		
Total	5400432	15			

a. substrate = 2,00, conc = 20,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
1,00	3	254,8545	
6,00	3	343,3665	
5,00	3	502,2649	
4,00	2	613,8474	
3,00	2		1390,3485
2,00	3		1730,4609
Sig.		,203	,244

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 20,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6775421	5	1355084,244	9,483	,001
Within Groups	1429025	10	142902,518		
Total	8204446	15			

a. substrate = 2,00, conc = 30,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
6,00	2	320,9736	
1,00	3	339,8109	
5,00	3	370,7411	
4,00	3	1216,0820	1216,0820
3,00	2		1692,7969
2,00	3		1871,5287
Sig.		,162	,421

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 30,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5487967	5	1097593,351	63,501	,000
Within Groups	155561,9	9	17284,654		
Total	5643529	14			

a. substrate = 2,00, conc = 40,00

sp_act[c]

Tukey HSD[a,b]

		Subset for alpha = .05		
week	N	1	2	3
1,00	3	333,7396		
6,00	2	365,8393	365,8393	
5,00	3	502,9577	502,9577	
2,00	2	692,5000	692,5000	
4,00	3		775,5682	
3,00	2			2247,3214
Sig.		,112	,061	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 40,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4401525	5	880305,026	7,829	,002
Within Groups	1236794	11	112435,800		
Total	5638319	16			

a. substrate = 2,00, conc = 50,00

sp_act[c]

Tukey HSD[a,b]

		Subset for alpha = .05		
week	N	1	2	3
1,00	3	112,1766		
5,00	3	594,6697	594,6697	
6,00	3	598,6936	598,6936	
2,00	3	927,3646	927,3646	
4,00	3		1090,8834	1090,8834
3,00	2			1903,0676
Sig.		,119	,535	,121

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,769.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 50,00

APPENDIX E

Specific activity

Analysis of variance with two factors (water regime x week)

Without stress: Substrate =1

With glycerol (-2.8MPa): Substrate =2

Concentrations: Con.

Between-Subjects Factors [a]

		N
week	1,00	6
	2,00	6
	3,00	6
	4,00	6
	5,00	5
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = ,00

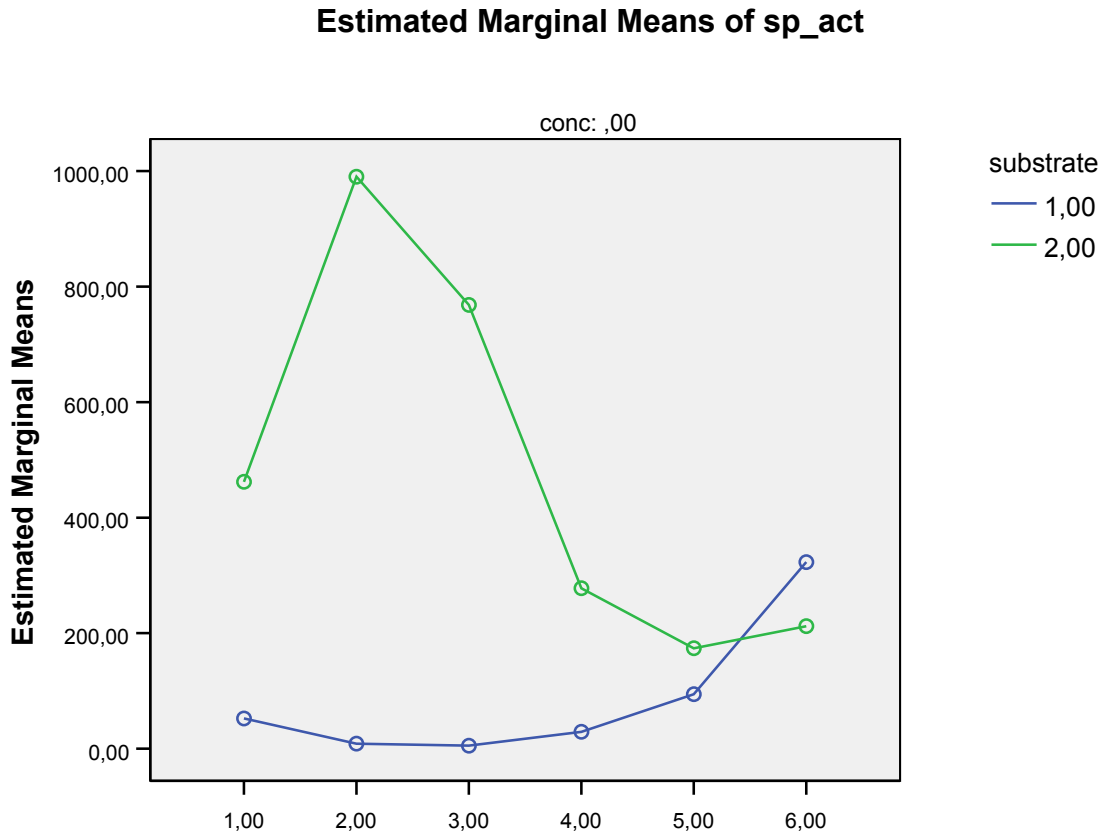
Tests of Between-Subjects Effects [b]

Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3265094,817[a]	11	296826,802	10,057	,000
Intercept	2769078,728	1	2769078,728	93,824	,000
week	557613,343	5	111522,669	3,779	,012
substrate	1350439,590	1	1350439,590	45,756	,000
week * substrate	1254534,568	5	250906,914	8,501	,000
Error	678813,583	23	29513,634		
Total	6810418,247	35			
Corrected Total	3943908,400	34			

a. R Squared = ,828 (Adjusted R Squared = ,746)

b. conc = ,00



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	5
	3,00	5
	4,00	4
	5,00	4
	6,00	6
substrate	1,00	15
	2,00	15

a. conc = 10,00

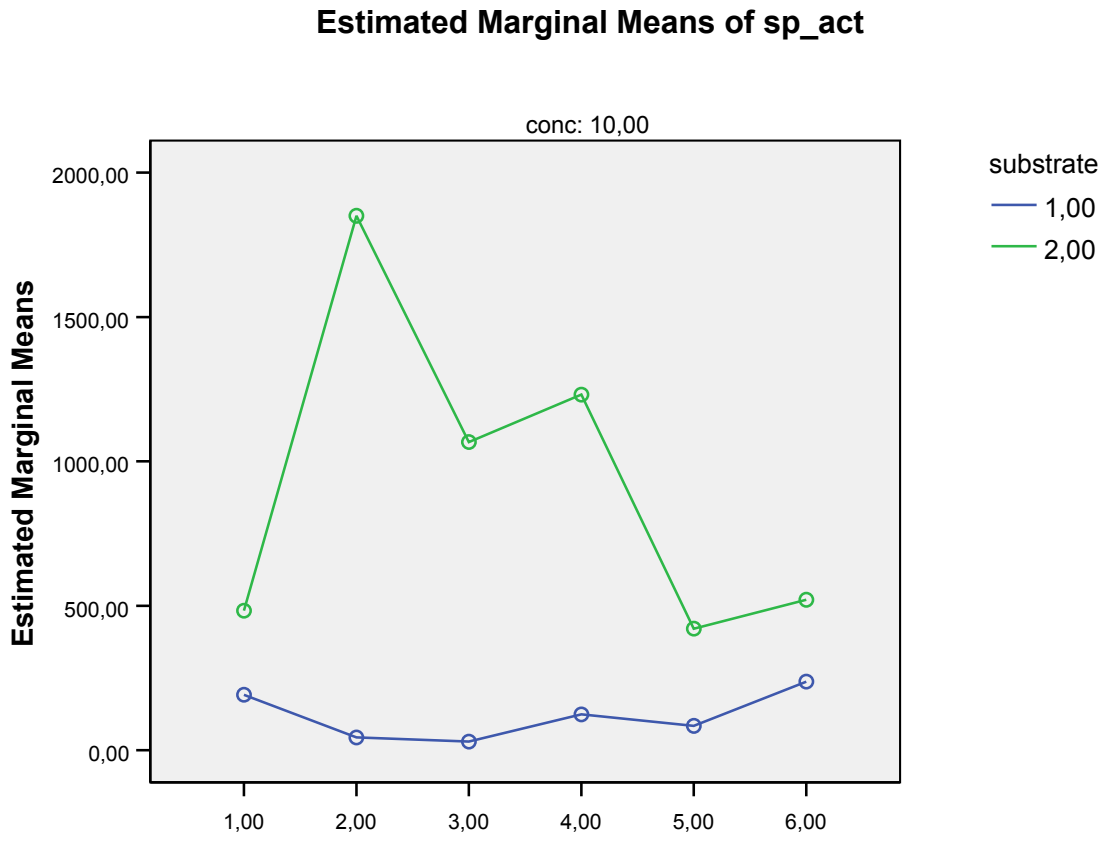
Tests of Between-Subjects Effects[b]

Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9371808,913[a]	11	851982,628	18,968	,000
Intercept	7901948,952	1	7901948,952	175,928	,000
week	1583392,059	5	316678,412	7,051	,001
substrate	4728100,721	1	4728100,721	105,266	,000
week * substrate	2375159,799	5	475031,960	10,576	,000
Error	808483,094	18	44915,727		
Total	18591936,7	30			
Corrected Total	10180292,0	29			

a. R Squared = ,921 (Adjusted R Squared = ,872)

b. conc = 10,00



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	5
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	16

a. conc = 20,00

Tests of Between-Subjects Effects[b]

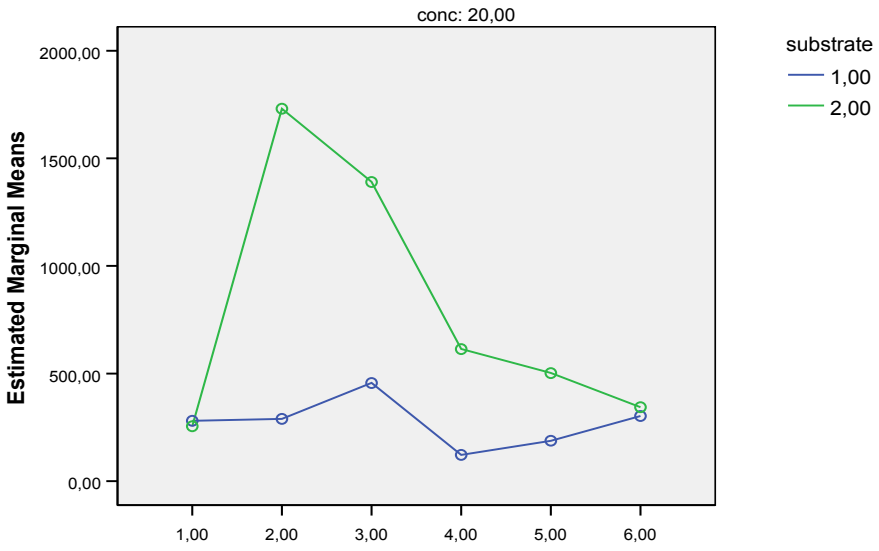
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7524866,751[a]	11	684078,796	41,317	,000
Intercept	9669058,123	1	9669058,123	583,987	,000
week	3126013,069	5	625202,614	37,761	,000
substrate	2359123,732	1	2359123,732	142,485	,000
week * substrate	2333663,583	5	466732,717	28,190	,000
Error	364253,337	22	16556,970		
Total	16808871,6	34			
Corrected Total	7889120,088	33			

a. R Squared = ,954 (Adjusted R Squared = ,931)

b. conc = 20,00

Estimated Marginal Means of sp_act



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	18
	2,00	16

a. conc = 30,00

Tests of Between-Subjects Effects[b]

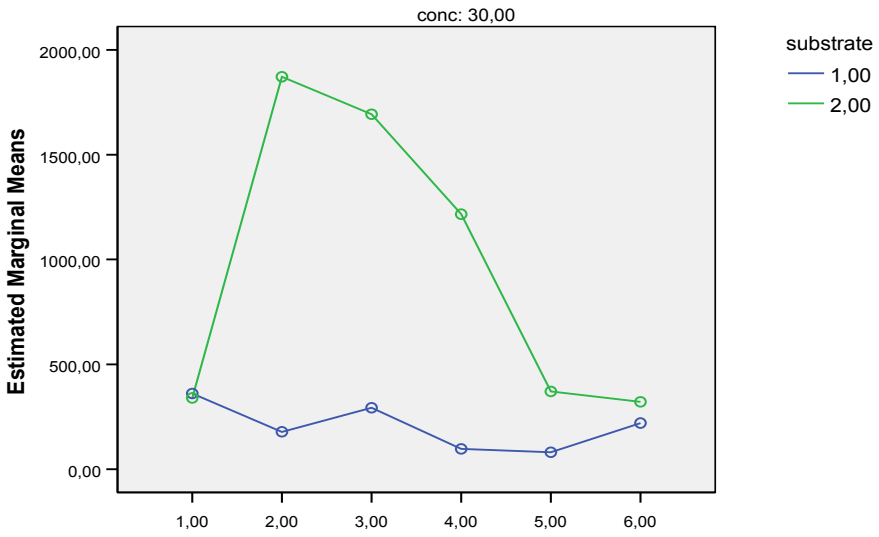
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11837167,5[a]	11	1076106,141	16,094	,000
Intercept	11438816,3	1	11438816,32	171,075	,000
week	3568921,456	5	713784,291	10,675	,000
substrate	4847891,667	1	4847891,667	72,503	,000
week * substrate	3759729,814	5	751945,963	11,246	,000
Error	1471013,951	22	66864,270		
Total	24046469,1	34			
Corrected Total	13308181,5	33			

a. R Squared = ,889 (Adjusted R Squared = ,834)

b. conc = 30,00

Estimated Marginal Means of sp_act



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	5
	3,00	4
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	17
	2,00	15

a. conc = 40,00

Tests of Between-Subjects Effects[b]

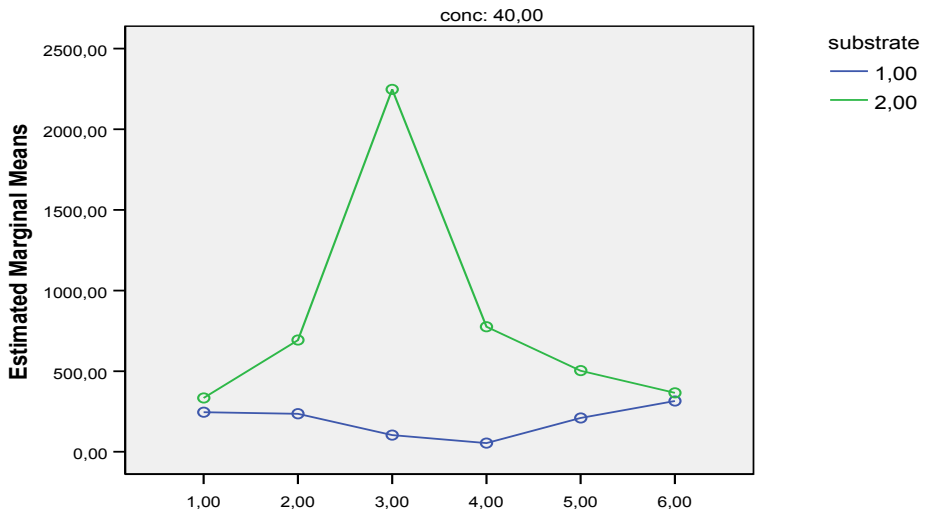
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8155237,745[a]	11	741385,250	44,257	,000
Intercept	7925061,154	1	7925061,154	473,090	,000
week	2363531,546	5	472706,309	28,218	,000
substrate	3020527,092	1	3020527,092	180,312	,000
week * substrate	3310491,114	5	662098,223	39,524	,000
Error	335034,044	20	16751,702		
Total	15367370,3	32			
Corrected Total	8490271,789	31			

a. R Squared = ,961 (Adjusted R Squared = ,939)

b. conc = 40,00

Estimated Marginal Means of sp_act



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	6
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = 50,00

Tests of Between-Subjects Effects[b]

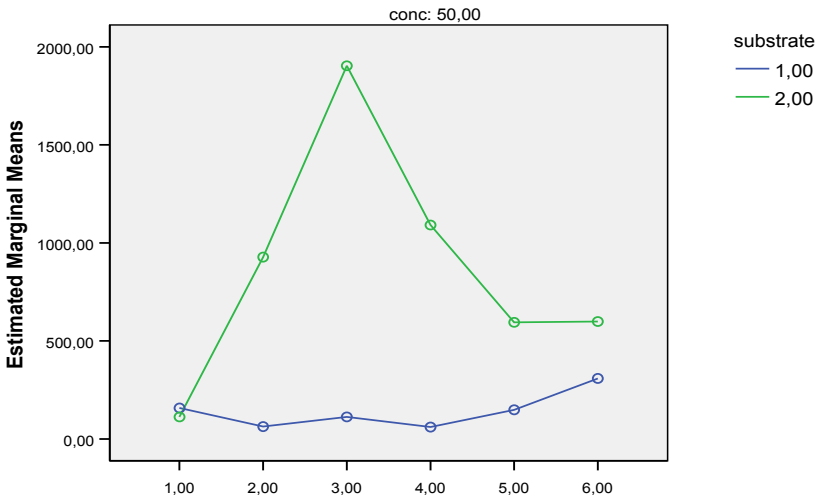
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8435056,878[a]	11	766823,353	13,899	,000
Intercept	8865638,092	1	8865638,092	160,699	,000
week	2179655,011	5	435931,002	7,902	,000
substrate	4595576,085	1	4595576,085	83,299	,000
week * substrate	2813018,652	5	562603,730	10,198	,000
Error	1268894,424	23	55169,323		
Total	17323489,4	35			
Corrected Total	9703951,301	34			

a. R Squared = ,869 (Adjusted R Squared = ,807)

b. conc = 50,00

Estimated Marginal Means of sp_act



APPENDIX F

Univariate Analysis of Variance of pesticides

LINURON = 1, WEEK = 1, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,339[a]	5	,068	15,833	,000
Intercept	19,389	1	19,389	4523,885	,000
TREATMEN	,339	5	,068	15,833	,000
Error	,051	12	,004		
Total	19,779	18			
Corrected Total	,391	17			

a. R Squared = ,868 (Adjusted R Squared = ,814)

b. LINURON = 1, WEEK = 1, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
6	3	,8216	
5	3	,8830	
2	3		1,0735
3	3		1,1086
4	3		1,1473
1	3		1,1931
Sig.		,851	,290

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,004.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 3,3333

LINURON = 1, WEEK = 1, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,746[a]	5	,149	52,124	,000
Intercept	21,510	1	21,510	7516,302	,000
TREATMEN	,746	5	,149	52,124	,000
Error	,034	12	,003		
Total	22,291	18			
Corrected Total	,780	17			

a. R Squared = ,956 (Adjusted R Squared = ,938)

b. LINURON = 1, WEEK = 1, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2	Subset 3
5	3	,7837		
6	3	,8667		
2	3		1,1683	
3	3		1,1752	
1	3		1,1892	
4	3			1,3759
Sig.		,445	,996	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,003.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 6,6666

LINURON = 1, WEEK = 1, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,373[a]	5	,075	53,639	,000
Intercept	22,029	1	22,029	15847,733	,000
TREATMEN	,373	5	,075	53,639	,000
Error	,017	12	,001		
Total	22,419	18			
Corrected Total	,389	17			

a. R Squared = ,957 (Adjusted R Squared = ,939)

b. LINURON = 1, WEEK = 1, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
5	3	,8763			
6	3		,9970		
1	3			1,1370	
2	3			1,1399	
3	3			1,1448	
4	3				1,3426
Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,001.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 10,0000

LINURON = 1, WEEK = 1, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,618[a]	5	,124	68,421	,000
Intercept	23,811	1	23,811	13178,178	,000
TREATMEN	,618	5	,124	68,421	,000
Error	,022	12	,002		
Total	24,451	18			
Corrected Total	,640	17			

a. R Squared = ,966 (Adjusted R Squared = ,952)

b. LINURON = 1, WEEK = 1, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
5	3	,8895		
6	3	,9489		
2	3		1,1258	
1	3		1,2040	
4	3			1,3490
3	3			1,3838
Sig.		,550	,283	,908

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 13,3333

LINURON = 1, WEEK = 1, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,524[a]	5	,105	66,437	,000
Intercept	24,366	1	24,366	15451,198	,000
TREATMEN	,524	5	,105	66,437	,000
Error	,019	12	,002		
Total	24,909	18			
Corrected Total	,543	17			

a. R Squared = ,965 (Adjusted R Squared = ,951)

b. LINURON = 1, WEEK = 1, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
5	3	,9587		
6	3	1,0333	1,0333	
2	3		1,0970	
1	3		1,1094	
3	3			1,3429
4	3			1,4395
Sig.		,266	,248	,094

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 16,6666

LINURON = 1, WEEK = 2, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,864[a]	5	,573	25,813	,000
Intercept	5,041	1	5,041	227,158	,000
TREATMEN	2,864	5	,573	25,813	,000
Error	,266	12	,022		
Total	8,171	18			
Corrected Total	3,130	17			

a. R Squared = ,915 (Adjusted R Squared = ,879)

b. LINURON = 1, WEEK = 2, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
6	3	,0000	
4	3	,1394	
2	3	,3152	
1	3		,8090
5	3		,8241
3	3		1,0874
Sig.		,173	,269

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,022.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 3,3333

LINURON = 1, WEEK = 2, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,699[a]	5	,340	15,430	,000
Intercept	5,500	1	5,500	249,763	,000
TREATMEN	1,699	5	,340	15,430	,000
Error	,264	12	,022		
Total	7,463	18			
Corrected Total	1,963	17			

a. R Squared = ,865 (Adjusted R Squared = ,809)

b. LINURON = 1, WEEK = 2, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
6	3	,0000		
2	3	,3666	,3666	
4	3		,5411	
5	3		,7048	,7048
3	3		,7432	,7432
1	3			,9608
Sig.		,087	,076	,342

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,022.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 6,6666

LINURON = 1, WEEK = 2, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,926[a]	5	,385	78,999	,000
Intercept	6,047	1	6,047	1239,917	,000
TREATMEN	1,926	5	,385	78,999	,000
Error	,059	12	,005		
Total	8,031	18			
Corrected Total	1,985	17			

a. R Squared = ,971 (Adjusted R Squared = ,958)

b. LINURON = 1, WEEK = 2, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
6	3	,0000			
4	3		,3052		
2	3			,6096	
3	3				,8124
5	3				,8520
1	3				,8983
Sig.		1,000	1,000	1,000	,667

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,005.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 10,0000

LINURON = 1, WEEK = 2, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,226[a]	5	,445	57,091	,000
Intercept	8,245	1	8,245	1057,253	,000
TREATMEN	2,226	5	,445	57,091	,000
Error	,094	12	,008		
Total	10,564	18			
Corrected Total	2,320	17			

a. R Squared = ,960 (Adjusted R Squared = ,943)

b. LINURON = 1, WEEK = 2, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
6	3	,2174			
2	3	,3033			
4	3		,5525		
5	3			,7966	
3	3			1,0210	1,0210
1	3				1,1700
Sig.		,833	1,000	,075	,364

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,008.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 13,3333

LINURON = 1, WEEK = 2, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,810[a]	5	,362	18,071	,000
Intercept	7,434	1	7,434	371,196	,000
TREATMEN	1,810	5	,362	18,071	,000
Error	,240	12	,020		
Total	9,484	18			
Corrected Total	2,050	17			

a. R Squared = ,883 (Adjusted R Squared = ,834)

b. LINURON = 1, WEEK = 2, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
4	3	,2462	
6	3	,2638	
2	3	,5017	
3	3		,9227
5	3		,9310
1	3		,9906
Sig.		,300	,990

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,020.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 16,6666

LINURON = 1, WEEK = 3, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,805[a]	5	,761	597,235	,000
Intercept	3,667	1	3,667	2878,022	,000
TREATMEN	3,805	5	,761	597,235	,000
Error	,015	12	,001		
Total	7,488	18			
Corrected Total	3,821	17			

a. R Squared = ,996 (Adjusted R Squared = ,994)

b. LINURON = 1, WEEK = 3, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
2	3	,0000		
4	3	,0000		
6	3	,0000		
5	3		,8058	
1	3		,8251	
3	3			1,0774
Sig.		1,000	,983	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,001.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 3,3333

LINURON = 1, WEEK = 3, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,216[a]	5	,443	40,659	,000
Intercept	3,159	1	3,159	289,810	,000
TREATMEN	2,216	5	,443	40,659	,000
Error	,131	12	,011		
Total	5,505	18			
Corrected Total	2,346	17			

a. R Squared = ,944 (Adjusted R Squared = ,921)

b. LINURON = 1, WEEK = 3, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
2	3	,0000	
6	3	,0000	
4	3	,2409	
5	3		,6889
3	3		,7421
1	3		,8414
Sig.		,120	,506

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,011.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 6,6666

LINURON = 1, WEEK = 3, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,126[a]	5	,425	82,961	,000
Intercept	4,280	1	4,280	835,176	,000
TREATMEN	2,126	5	,425	82,961	,000
Error	,062	12	,005		
Total	6,468	18			
Corrected Total	2,187	17			

a. R Squared = ,972 (Adjusted R Squared = ,960)

b. LINURON = 1, WEEK = 3, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
6	3	,0000		
2	3	,1763	,1763	
4	3		,2880	
5	3			,7950
3	3			,8143
1	3			,8522
Sig.		,088	,441	,916

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,005.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 10,0000

LINURON = 1, WEEK = 3, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,003[a]	5	,601	52,661	,000
Intercept	4,807	1	4,807	421,555	,000
TREATMEN	3,003	5	,601	52,661	,000
Error	,137	12	,011		
Total	7,946	18			
Corrected Total	3,139	17			

a. R Squared = ,956 (Adjusted R Squared = ,938)

b. LINURON = 1, WEEK = 3, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
6	3	,0000	
2	3	,1493	
4	3	,2098	
5	3		,7692
3	3		,9776
1	3		,9948
Sig.		,228	,174

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,011.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 13,3333

LINURON = 1, WEEK = 3, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,868[a]	5	,574	37,541	,000
Intercept	4,051	1	4,051	265,070	,000
TREATMEN	2,868	5	,574	37,541	,000
Error	,183	12	,015		
Total	7,102	18			
Corrected Total	3,052	17			

a. R Squared = ,940 (Adjusted R Squared = ,915)

b. LINURON = 1, WEEK = 3, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
2	3	,0000	
6	3	,0772	
4	3	,1563	
5	3		,8614
1	3		,8652
3	3		,8863
Sig.		,643	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,015.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 16,6666

LINURON = 2, WEEK = 1, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,083[a]	5	,017	14,298	,000
Intercept	23,027	1	23,027	19835,733	,000
TREATMEN	,083	5	,017	14,298	,000
Error	,014	12	,001		
Total	23,123	18			
Corrected Total	,097	17			

a. R Squared = ,856 (Adjusted R Squared = ,796)

b. LINURON = 2, WEEK = 1, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
2	3	1,0734	
1	3	1,0766	
5	3	1,0859	
4	3	1,1362	
6	3	1,1448	
3	3		1,2693
Sig.		,179	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,001.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 3,3333

METRIBUZIN = 2, WEEK = 1, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,147[a]	5	,029	4,546	,015
Intercept	32,776	1	32,776	5070,020	,000
TREATMEN	,147	5	,029	4,546	,015
Error	,078	12	,006		
Total	33,001	18			
Corrected Total	,225	17			

a. R Squared = ,654 (Adjusted R Squared = ,511)

b. LINURON = 2, WEEK = 1, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
6	3	1,2530	
1	3	1,2929	
3	3	1,3262	1,3262
2	3	1,3318	1,3318
5	3	1,3544	1,3544
4	3		1,5381
Sig.		,646	,062

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,006.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 6,6666

METRIBUZIN = 2, WEEK = 1, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,229[a]	5	,046	6,987	,003
Intercept	30,733	1	30,733	4684,189	,000
TREATMEN	,229	5	,046	6,987	,003
Error	,079	12	,007		
Total	31,041	18			
Corrected Total	,308	17			

a. R Squared = ,744 (Adjusted R Squared = ,638)

b. LINURON = 2, WEEK = 1, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
3	3	1,1079	
1	3	1,2478	1,2478
2	3	1,2604	1,2604
6	3		1,3935
4	3		1,4063
5	3		1,4243
Sig.		,264	,153

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,007.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 10,0000

METRIBUZIN = 2, WEEK = 1, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,024[a]	5	,005	1,436	,281
Intercept	38,605	1	38,605	11754,559	,000
TREATMEN	,024	5	,005	1,436	,281
Error	,039	12	,003		
Total	38,668	18			
Corrected Total	,063	17			

a. R Squared = ,374 (Adjusted R Squared = ,114)

b. LINURON = 2, WEEK = 1, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1
6	3	1,4105
5	3	1,4294
1	3	1,4585
4	3	1,4758
2	3	1,5024
3	3	1,5103
Sig.		,333

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,003.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 13,3333

METRIBUZIN = 2, WEEK = 1, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,015[a]	5	,003	1,304	,325
Intercept	30,726	1	30,726	13671,669	,000
TREATMEN	,015	5	,003	1,304	,325
Error	,027	12	,002		
Total	30,768	18			
Corrected Total	,042	17			

a. R Squared = ,352 (Adjusted R Squared = ,082)

b. LINURON = 2, WEEK = 1, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1
3	3	1,2664
1	3	1,2759
4	3	1,2973
5	3	1,3241
6	3	1,3371
2	3	1,3383
Sig.		,469

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 16,6666

METRIBUZIN = 2, WEEK = 2, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,941[a]	5	,588	108,850	,000
Intercept	14,087	1	14,087	2607,208	,000
TREATMEN	2,941	5	,588	108,850	,000
Error	,065	12	,005		
Total	17,092	18			
Corrected Total	3,005	17			

a. R Squared = ,978 (Adjusted R Squared = ,969)

b. LINURON = 2, WEEK = 2, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,0000		
6	3		,9688	
2	3		,9966	
5	3		1,0254	1,0254
1	3		1,0946	1,0946
3	3			1,2224
Sig.		1,000	,351	,057

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,005.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 3,3333

METRIBUZIN = 2, WEEK = 2, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,853[a]	5	,171	16,862	,000
Intercept	24,319	1	24,319	2404,286	,000
TREATMEN	,853	5	,171	16,862	,000
Error	,121	12	,010		
Total	25,293	18			
Corrected Total	,974	17			

a. R Squared = ,875 (Adjusted R Squared = ,823)

b. LINURON = 2, WEEK = 2, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
4	3	,6862	
6	3		1,1774
5	3		1,2480
2	3		1,2495
1	3		1,2861
3	3		1,3269
Sig.		1,000	,488

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,010.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 6,6666

METRIBUZIN = 2, WEEK = 2, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,463[a]	5	,093	13,627	,000
Intercept	25,120	1	25,120	3695,368	,000
TREATMEN	,463	5	,093	13,627	,000
Error	,082	12	,007		
Total	25,665	18			
Corrected Total	,545	17			

a. R Squared = ,850 (Adjusted R Squared = ,788)

b. LINURON = 2, WEEK = 2, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,9385		
3	3	1,0849	1,0849	
2	3	1,1437	1,1437	
6	3		1,2268	
1	3		1,2294	
5	3			1,4648
Sig.		,084	,328	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,007.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 10,0000

METRIBUZIN = 2, WEEK = 2, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,135[a]	5	,027	1,892	,169
Intercept	32,979	1	32,979	2308,039	,000
TREATMEN	,135	5	,027	1,892	,169
Error	,171	12	,014		
Total	33,286	18			
Corrected Total	,307	17			

a. R Squared = ,441 (Adjusted R Squared = ,208)

b. LINURON = 2, WEEK = 2, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1
6	3	1,2504
2	3	1,2559
1	3	1,3541
4	3	1,3574
5	3	1,3992
3	3	1,5045
Sig.		,170

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,014.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 13,3333

METRIBUZIN = 2, WEEK = 2, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,230[a]	5	,046	6,246	,004
Intercept	29,036	1	29,036	3944,660	,000
TREATMEN	,230	5	,046	6,246	,004
Error	,088	12	,007		
Total	29,354	18			
Corrected Total	,318	17			

a. R Squared = ,722 (Adjusted R Squared = ,607)

b. LINURON = 2, WEEK = 2, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
2	3	1,1085		
4	3	1,1577	1,1577	
3	3	1,2708	1,2708	1,2708
1	3	1,2816	1,2816	1,2816
5	3		1,3591	1,3591
6	3			1,4428
Sig.		,207	,111	,212

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,007.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 16,6666

METRIBUZIN = 2, WEEK = 3, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,895[a]	5	,579	266,985	,000
Intercept	13,988	1	13,988	6449,797	,000
TREATMEN	2,895	5	,579	266,985	,000
Error	,026	12	,002		
Total	16,909	18			
Corrected Total	2,921	17			

a. R Squared = ,991 (Adjusted R Squared = ,987)

b. LINURON = 2, WEEK = 3, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
4	3	,0000			
5	3		,9839		
2	3		,9888		
6	3		1,0111	1,0111	
1	3			1,1202	1,1202
3	3				1,1851
Sig.		1,000	,976	,112	,552

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 3,3333

METRIBUZIN = 2, WEEK = 3, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,982[a]	5	,396	34,431	,000
Intercept	24,016	1	24,016	2086,084	,000
TREATMEN	1,982	5	,396	34,431	,000
Error	,138	12	,012		
Total	26,136	18			
Corrected Total	2,120	17			

a. R Squared = ,935 (Adjusted R Squared = ,908)

b. LINURON = 2, WEEK = 3, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
4	3	,4153	
1	3		1,2599
6	3		1,2845
3	3		1,3150
2	3		1,3154
5	3		1,3403
Sig.		1,000	,934

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,012.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 6,6666

METRIBUZIN = 2, WEEK = 3, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,411[a]	5	,482	33,512	,000
Intercept	21,641	1	21,641	1504,303	,000
TREATMEN	2,411	5	,482	33,512	,000
Error	,173	12	,014		
Total	24,224	18			
Corrected Total	2,583	17			

a. R Squared = ,933 (Adjusted R Squared = ,905)

b. LINURON = 2, WEEK = 3, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
4	3	,3202	
1	3		1,0962
3	3		1,1121
6	3		1,2941
2	3		1,3395
5	3		1,4168
Sig.		1,000	,058

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,014.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 10,0000

METRIBUZIN = 2, WEEK = 3, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,722[a]	5	,144	16,508	,000
Intercept	28,578	1	28,578	3269,292	,000
TREATMEN	,722	5	,144	16,508	,000
Error	,105	12	,009		
Total	29,405	18			
Corrected Total	,826	17			

a. R Squared = ,873 (Adjusted R Squared = ,820)

b. LINURON = 2, WEEK = 3, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,8898		
6	3		1,1626	
2	3		1,2072	1,2072
1	3		1,4185	1,4185
5	3			1,4255
3	3			1,4567
Sig.		1,000	,051	,058

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,009.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 13,3333

METRIBUZIN = 2, WEEK = 3, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,453[a]	5	,091	9,441	,001
Intercept	24,424	1	24,424	2542,413	,000
TREATMEN	,453	5	,091	9,441	,001
Error	,115	12	,010		
Total	24,993	18			
Corrected Total	,569	17			

a. R Squared = ,797 (Adjusted R Squared = ,713)

b. LINURON = 2, WEEK = 3, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,9083		
2	3	,9911	,9911	
1	3		1,1990	1,1990
3	3			1,2706
5	3			1,2920
6	3			1,3281
Sig.		,897	,171	,606

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,010.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 16,6666

ABSTRACT

The objective of this study was to examine the potential for degradation of mixtures of pesticides (chlorpyrifos, linuron, metribuzin) by a range of bacteria and fungi and to relate this capability to enzyme production and quantify the rates of degradation of the components of the mixture of xenobiotic compounds. Overall, although bacteria (19 *Bacillus* and 4 *Pseudomonas* species) exhibited tolerance to the individual and micture of pesticides actual degradation was not evident. Five species of white rot fungi were grown on minimal salts agar plates amended with 0, 10 and 30 mg L^{-1} of chlorpyrifos, linuron and metribuzin, individually and as a mixture with a total concentration 15 and 30 mg L^{-1}. Four of these, *T. versicolor, P. gigatea, P.coccineus* and *P.ostreatus*, exhibited very good tolerance to the pesticides. They were also grown on a nutritionally poor soil extract agar amended with a mixture of the pesticides at different concentrations (0-70 mg L^{-1}). Subsequently, the ability of *T. versicolor, P. gigatea, P. coccineus* to degrade lignin and production of laccase in the presence of mixture of the pesticides was examined as well as their capacity to degrade the pesticide mixture at different concentrations (0-50 mg L^{-1}) in soil extract broth was quantified using HPLC.

This showed that only *T.versicolor* had the ability to degrade linuron, after three weeks incubation although all tested species produced laccase. Subsequently, the temporal degradation rates of *T.versicolor* was examined in relation to temporal degradation of a mixture of the pesticides chlorpyrifos, linuron and metribuzin with total concentrations 0-50 mg L^{-1} and the temporal laccase production was quantified over a six week period in relation to ionic and non-ionic water potential stress (-2.8 MPa).

These studies showed that the test isolate had the ability to produce very high levels of laccase at -2.8 MPa water potential adjusted non-ionically by using glycerol and quite lower levels in soil extract broth without stress while

T.versicolor did not produce laccase at -2.8 MPa when the medium was modified ionically.

Finally, *T.versicolor* was able to degrade the pesticide linuron in all tested water regimes, after five weeks incubation, regardless of the concentration of the mixture. In contrast, about 50% of the metribuzin was degraded, only at at -2.8 MPa water potential adjusted non-ionically with glycerol. Chlorpyrifos and its main metabolite TCP were not detected, possibly, due to a combination of hydrolysis, photolysis and volatilization degradation. The capacity of *T.versicolor* to degrade linuron in mixtures of pesticides and the production of high levels of laccase, in a nutritionally poor soil extract broth, even under water stress suggests potential application of this fungus in bioremediation.

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ABBREVIATIONS

3, 4-DCA	3, 4-dichlooroaniline
ABTS	2, 2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
BTEX:	Benzene, Toluene Ethylbenzene Xylene)
DAD	Diode Array Detector
DETP	Diethylthiophosphate
ED	Effective dose
HPLC	High Performance Liquid Chromatography
IUCLID	Uniform ChemicaL Information Database
Koc	Partition coefficient between soil organic matter and water),
LD50	Lethal Dose
MEA	Malt Extract Agar
MMY	Minimal salts Medium Yeast
OD	Optical density
PAHs:	Polycyclic aromatic hydrocarbons
PCBs:	Polychlorinated biphenyls
PCP:	Pentachlorophenol
TCA	Trichloroacetic acid
TCP	3, 5, 6-trichloro-2-pyridinol
TSB	Trypticase Soy Blood

1 LITERATURE REVIEW

1.1 Introduction

During the past twenty years, concern has arisen as to the presence of pesticides in the environment and the threat they pose to wildlife and mankind. Certainly, pesticides have improved longevity and quality of life, chiefly in the area of public health. Insect control programs have saved millions of lives by combating diseases such as malaria, yellow fever and typhus. In addition, the use of pesticides constitutes an important aspect of modern agriculture, as they are absolutely necessary for economical pest management (Rao & Hornsby, 2001).

Since the earliest times, societies have used soil as a quick and convenient disposal route for waste (Ashman& Puri, 2002) but only recently it was it found that contaminants in the soil can find their way to other areas of the environment such as surface and groundwater, rivers and atmosphere. The conventional techniques used for remediation of pesticide-contaminated sites, such as low temperature thermal desorption, chemical treatment and incineration have certain limitations and disadvantages (Frazar, 2000). Low temperature thermal desorption is an ex situ clean up technology which is capable of removing semi-volatile and volatile organic compounds from soils and sediments. It, however, requires highly specialized facilities and carries a comparatively high cost (Gavrilescu, 2005). Incineration is also a proven technology that has frequently been used for destruction of pesticides but it has met serious public opposition, because of its potentially toxic emissions and its elevated economic cost (Kearney, 1998, Zhang & Quiao, 2002).

Hence, there is an increasing interest and need to developing safe, convenient and economically feasible methods for pesticides remediation (Kearney, 1998, Zhang & Quiao, 2002). For this reason, several biological techniques, such as bioremediation and phytoremediation, have been developed (Schoefs *et al,* 2004). Currently, bioremediation is one of the most environmentally safe and cost-effective methods of decontamination and

detoxification of a pesticide-contaminated environment (Zhang & Quiao, 2002).

1.2 Bioremediation

1.2.1 Terminology

Bioremediation is a process that uses mainly microorganisms, plants, or microbial or plant enzymes to detoxify contaminants in the soil and other environments. The concept includes biodegradation, which refers to the partial, and sometimes total, transformation or detoxification of contaminants by microorganisms and plants. Mineralization is a more restrictive term for the complete conversion of an organic contaminant to its inorganic constituents by a single species or a consortium of microorganisms. Co-metabolism is another more restrictive term referring to the transformation of a contaminant without the provision of carbon or energy for the degrading microorganisms (Skipper, 1998).

The process of bioremediation enhances the rate of the natural microbial degradation of contaminants by supplementing the indigenous microorganisms (bacteria or fungi) with nutrients, carbon sources or electron donors (biostimulation, biorestoration) or by adding an enriched culture of microorganisms that have specific characteristics that allow them to degrade the desired contaminant at a quicker rate (bioaugmentation) (Mackay & Frazar, 2000). The goal of bioremediation is at least to reduce pollutant levels to undetectable, non-toxic or acceptable levels, i.e., within limits set by regulatory agencies or, ideally, to completely mineralize organopollutants to carbon dioxide (Pointing, 2001).

1.2.2 Advantages and disadvantages of bioremediation

Bioremediation is a natural process and is therefore perceived by the public as having a reduced impact on natural ecosystems. It is typically less expensive than the equivalent physical-chemical methods. The complete destruction of target pollutants is possible on site without the need of excavation or transport quantities of waste off site (Kearney, 1998, Vidali, 2001). Finally, it requires little energy input and preserves the soil structure (Hohener *et al*., 1998).

Bioremediation, however, has a number of disadvantages. This process is limited to those compounds that are biodegradable. Moreover, there are some concerns that the products of bioremediation may be more persistent or toxic than the parent compounds. Furthermore, it is difficult to extrapolate the results from bench and pilot-scale studies to full-scale field operations. All biological processes, such as this, are often highly specific and complex and take longer than other treatment options (for example incineration) (Vidali, 2001).

Compounds in mixtures are known to interact with biological systems in ways that can greatly alter the toxicity of individual compounds (Hernando *et al.,* 2003). While studies on the capacity of fungi, particularly white rot fungi and bacteria to degrade individual pesticides is extensive (Gadd, 2001) very few have examined the capacity of microorganisms to degrade mixtures of pesticides. In most pesticide-contaminated agrochemical facilities, herbicides are found in combination with other widely used agricultural chemicals and remediation strategies must take into account of the presence of multiple contaminants (Grigg *et al.,* 1997; and Memic *et al.,* 2005). Moreover, there are hardly any studies on the use of white rot fungi to clean up mixtures of pesticides.

Grigg *et al*., (1997) reported the ability of an atrazine-mineralizing mixed culture isolated from soil subjected to repeated applications of atrazine to degrade other s-triazines in liquid culture. Cyanazine and simazine either

alone or combined with atrazine were degraded in 6 days. Metribuzin was not degraded while the microbial culture completely degraded atrazine in the presence of contaminants including alachlor, metolachlor and trifluralin. A recent study by Fragoeiro and Magan (2008) demonstrated that a mixture of dieldrin, simazine and trifluralin is differentially degraded by *Trametes versicolor* and *Phanerochaete chrysosporium* and significant extracellular enzymes are produced in soil, even at -2.8 MPa water potential. Thus, research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex contaminants (Vidali, 2001).

1.2.3 Biological mechanisms of transformation

In soil, microorganisms commonly exist in large populations. Provided with adequate supplies of carbon and energy and environmental conditions conducive to growth, microbial activity, especially the production of extracellular enzymes, can significantly assist in the amelioration of contaminated sites.

Microbial genes encode the degradative enzymes, which oxidize, reduce, dehalogenate, dealkylate, deaminate, and hydrolyze hazardous chemicals such as pesticides, in the soil environment (Skipper, 1998). Once a contaminant has been enzymatically transformed to a less complex compound, it can often be metabolized using various pathways. Although a single transformation may reduce the toxicity of a contaminant, the complete mineralization of an organic compound typically requires several degradative enzymes produced by multiple genes on plasmids or chromosomes residing in a single species or among different species. The organism(s) with such enzymes may be either indigenous to the contaminated site or added as an inoculum. The enzymes can be intracellular or extracellular, and each type of enzyme has specific conditions for optimum activity (Skipper, 1998).

1.3 Lignin degrading enzymes

Laccase

Laccase (EC 1.10.3.2) is one of the very few enzymes that have been studied since the end of 19th century. It was first demonstrated in the exudates of *Rhus versinifera,* the Japanese lacquer tree. A few years later it was also demonstrated in fungi by Bertrand in 1896. Although known for a long time, laccases attracted considerable attention only after the beginning of studies of enzymatic degradation of wood by white–rot wood–rotting fungi (Baldrian.2006). Laccases are typically found in plants and fungi. Plant laccases participate in the radical-based mechanisms of lignin polymer formation; whereas in fungi laccases probably have more roles including morphogenesis, fungal plant-pathogen/host interaction, stress defence and lignin degradation (Thurston, 1994).

Laccase (benzenediol oxygen oxidoreductase) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic centre and usually called multicopper oxidases (Baldrian.2006). Laccases catalyse the reduction of oxygen to water accompanied by the oxidation of a broad range of aromatic compounds as hydrogen donors (Thurston, 1994), like phenols, aromatic amines and diamines (Nyanhongo *et al.,* 2007*).* Further more, laccase catalyzes the oxidation of non-phenolics and anilines. This reaction is involved in transforming numerous agricultural and industrial chemicals (Tortella *et al.*, 2005).

Among fungi recently studied for producing extracellular laccase, *Trametes* species are probably the most actively investigated for the laccase production because these fungi are commonly found in many parts of the world and apparently are excellent wood decomposers in nature. Indeed, *Trametes versicolor*, a representative fungus in this genus, was among the first species, from which the production of large amounts of laccase has been reported. It has already been marketed by several companies, although the

current prices are still too high for bulk environmental application (Duran and Esposito, 2000).

The catabolic role of fungal laccase in lignin biodegradation is not well understood (Trejo-Hernandez *et al.*, 2001), but some successful applications of this enzyme in decontamination have been reported. For example, dye decolouration by *Trametes hispida* (Rodriguez *et al.,* 1999), degradation of azo-dyes by *Pyricularia oryzae* (Chivukula and Renganathan, 1995) and textile effluent degradation by *Trametes versicolor* have been attributed to laccase activity. Duran and Esposito (2000) also reported that laccase from *Cerrena unicolor* produced a complete transformation of 2,4 DCP in soil colloids. In a more recent study by Zouari-Mechighi *et al.* (2006) reported that the ability of *Trametes trogii* isolated from Tunisia to decolorize Poly R 478 and several industrial dyes in agar plates and Cu^{2+} addition stimulated decolourization, suggesting that laccase could be involved in the process. The results on dye decolourization with the crude enzyme, without peroxidase activity, and the results obtained with the purified enzyme confirmed that *Tr. trogii* laccase decolorizes industrial dyes.

Lignin peroxidase

Lignin peroxidase (once called ligninase) (EC 1.11.14) was first discovered in *Phanerochaete chrysosporium* (Glenn *et al.,* 1983; Tien and Kirk, 1983). This activity is also produced by many, but not all, white rot fungi (Hatakka, 1994). This enzyme is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum pH (Gold and Alic, 1993). It shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules (Barr and Aust, 1994).

Manganese peroxidase

Manganese peroxidase (EC 1.11.13) is also a heme peroxidase and it forms a family of isoenzymes. Similarly to lignin peroxidase (LiP) they are also glycoproteins (Glenn and Gold, 1985). This enzyme shows a strong preference for Mn (II) as its reducing substrate and is not able to complete its

catalytic cycle without the presence of Mn (II) (Wariishi *et al.*, 1990). The redox potential of the Mn peroxidase-Mn system is lower than that lignin peroxidase and it has only shown capacity to oxidize in vitro phenolic substrates (Vares, 1996). Manganese peroxidase seems to be more widespread among white rot fungi than lignin peroxidase (Hatakka, 1994).

Total ligninolytic activity

The production and activity of the ligninolytic enzymes in soil (as opposed to a ligninolytic substrate) may be a prerequisite for transformation of pollutants by wood rot fungi (Lang *et al.,* 2000a). Thus, quantification of the activity of these enzymes by white rot fungal inoculants is important. Historically, various 14C-radiolabelled and unlabelled substrates have been used to screen for ligninolytic activity. However, these assays are relatively slow and difficult. The decolourization of dyes by white rot fungi was first reported by Glenn and Gold (1983) who developed a method to measure ligninolytic activity of *P. chrysosporium* based on the decolourization of a number of sulphonated polymeric dyes. Subsequently, other workers adapted the dye decolourization test for evaluating the ability of white rot fungi to degrade dyes and other xenobiotics (Nyanhongo *et al*., 2007). The high-molecular weight dyes cannot be taken up by the microorganisms and thus provide a specific screen for extracellular activity (Gold *et al.,* 1988; Field *et al*., 1993). The decolouration of polymeric dyes has been proposed as a useful screening method for ligninolytic activity (Lin *et al*., 1991; Weissenfels *et al*., 1992). Today the polymeric dyes used are inexpensive, stable, readily soluble, have high extinction coefficients and low toxicity towards *P. chrysosporium* and other white rot fungi and bacteria tested (Gold *et al*., 1988).

Other enzymes

The other microbial enzymes involved in the pollutant transformation are hydrolases. Several bacteria and fungi produce a group of extra or ecto

cellular enzymes (enzymes acting outside but still linked to their cells of origin) that include proteases, carbohydratases, esterases, phosphatases and phytases. These enzymes are physiologically necessary to living organisms (Gianfreda & Rao, 2004). Some of them (e.g. proteases, and carbohydratases) catalyze the hydrolysis of large molecules, such as proteins and carbohydrates, to smaller molecules for subsequent absorption by cells. Due to their intrinsic low substrate specificity, hydrolases may play a pivotal role in bioremediation of several pollutants (Gianfreda & Rao, 2004).

Recently Staszczak *et al., (*2000) suggested that proteases are involved in the regulation of ligninolytic activities in cultures of *T. versicolor* under nutrient limitation. Margesin *et al.*, (2000a) showed a positive influence of naphthalene on protease activity and Baran *et al*., (2004) reported an increase in phosphatase, dehydrogenase, urease and protease activities in a site in which concentrations of PAHs were higher than 1,000 μg kg^{-1}.

Mougin *et al.,* (1996) suggested the degradation of the pesticide lindane by *P. chrysosporium* via detoxification by a cytochrome P450 monooxygenase system. Cytochrome P450s are haemethiolate proteins that have been characterized in animals, plants, bacteria and filamentous fungi (Van Eerd *et al*., 2003). Regulation and expression of P450s are not well understood in plants and microorganisms because of the very low quantities of P450s enzymes usually present in these cells, particularly if the organism has not been exposed to physiochemical, physiological or xenobiotic stress (Van Eerd *et al*., 2003).

Cellobiose dehydrogenase (CDH) may be an important enzyme in pollutant degradation (Cameron, 2000). CDH is secreted by *Phanerochaete chrysosporium* and several other white rot fungi. It has been shown to directly reduce the munitions 2,4,6-trinitro tolouene (TNT) and hexahydro-1,3,5 trinitro-1,3,5-triazine (RDX) and indirectly degrade many more chemicals (Cameron, 2000).

Whether the degradation of pesticides is carried out by lignin degrading enzymes or by other enzymatic systems, or by both, the use of fungi in bioremediation is very promising and further studies are required to understand which enzymes are involved in the process. This information could be very useful in the establishment of the best conditions for enzyme production and consequent fungal bioremediation *in situ*.

1.4 Biodegrading capacities of white rot fungi

Application of fungal technology for the cleanup of contaminants has shown promise since 1985 when the white rot species *Phanerochaete chrysosporium* was found to be able to metabolize a number of important environmental pollutants (Sasek, 2003). This ability is generally attributed to the lignin degrading enzymatic system of the fungus, and a similar degrading capacity was later described for other white rot fungal species (Sasek, 2003). White rot fungi possess a number of advantages that can be exploited in bioremediation systems. Because key components of their lignin-degrading system are extracellular, these fungi can degrade insoluble chemicals such as lignin or an extremely diverse range of very persistent or toxic environmental pollutants (Barr and Aust, 1994; Cameron, 2000). The mycelial growth habit is also advantageous as it allows rapid colonization of substrates, and hyphal extension enables penetration of soil reaching pollutants in ways that other organisms cannot do (Reddy and Mathew, 2001; Fragoeiro & Magan, 2008). This can maximize physical, mechanical and enzymatic contact with the surrounding environment (Maloney, 2001).

In addition, these fungi use relatively inexpensive and abundant lignocellulosic materials as a nutrient source. They can tolerate a wide range of environmental conditions, such as temperature, pH and moisture levels (Maloney, 2001; Magan, 2007) and do not require pre-conditioning to a particular pollutant, because their degradation system is induced by nutrient deprivation (Barr and Aust, 1994).

1.4.1 White rot fungi

To understand the ability to degrade contaminants it is important to analyze the ecological niches of white rot fungi. These fungi are a physiological rather than taxonomic grouping, comprising those fungi that are capable of extensively degrading lignin (a heterogeneous polyphenolic polymer) within lignocellulosic substrates (Pointing, 2001). The name white rot derives from the appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance of the substrate (Pointing, 2001). Most known white rot fungi are basidiomycetes, although a few ascomycete genera within the *Xylariaceae* are also capable of white rot decay (Eaton and Hale, 1993).

In nature, white rot fungi live on woody tissues that are composed mainly of three biopolymers: cellulose, hemicellulose and lignin. Lignin, which provides strength and structure to the plant, is extremely recalcitrant. It is mineralized in an obligate aerobic oxidative process, and its degradation yields no net energy gain (Pointing, 2001), because it cannot be degraded as a sole source of carbon and energy (Field *et al.* 1993). The physiological importance of lignin biodegradation is the destruction of the matrix it forms, so that the microorganism can gain better access to the real substrates: hemicellulose and cellulose (Field *et al.*, 1993; Canet *et al.*, 2001), from where it obtains energy.

Lignin is synthesized in plants by random peroxidase-catalysed polymerization of substituted *p*-hydroxy-cinnamyl alcohols (Field *et al.*, 1993). This polymer is three-dimensional, and its monomers are linked by various carbon-carbon and other bonds and the stereo irregularity of lignin makes it resistant to attack by enzymes. The enzymatic degradation of lignin is further complicated by the chiral carbon in this polymer that exists in both L and D configurations.

Due to its molecular size it is impossible for lignin to be absorbed and degraded by intracellular enzymes, therefore ligninolytic enzymes are extracellularly excreted by the degrading fungi, initiating the oxidation of substrates in the extracellular environment (Mester and Tien, 2000). Thus, the white rot fungi have developed very non-specific mechanisms to degrade lignin extracellularly (Bar and Aust, 1994).

The three major families of lignin modifying enzymes believed to be involved in lignin degradation are laccases, lignin peroxidases and manganese peroxidases (Reddy and Mathew, 2001). The key step in lignin degradation by laccase or the ligninolytic peroxidases (LiP and MnP) involves the formation of free radical intermediates, which are formed when one electron is removed or added to the ground state of a chemical (Reddy and Mathew, 2001). Such free radicals are highly reactive and rapidly give up or accept an electron from another chemical, which triggers oxidation or reduction of "neighbouring" compounds. These radicals can carry out a variety of reactions including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol polymerisation and demethylation (Pointing, 2001). Different white rot fungi appear to be able to achieve the same effect with different combinations of enzymes (Harvey and Thurston, 2001) with respect to wood degradation.

The common features are the random nature of the structure of lignin, which requires its degradation to function in a non-specific manner. Consequently, other compounds that have an aromatic structure, such as many xenobiotic compounds, are also highly susceptible to degradation by ligninolytic enzymes (Field *et al.*, 1993; Barr and Aust, 1994). This characteristic is the greatest advantage of the use of white-rot fungi in bioremediation, since a mixture of different pollutants is usually found in most contaminated sites (Mester and Tien, 2000).

Aside from the lack of specificity, the ligninolytic system of white rot fungi offers further advantages. It is not induced by either lignin or other related

compounds (Cancel *et al.*, 1993). Thus, it is possible to degrade pollutants at relatively low concentrations that may be lower than that required to induce the synthesis of biodegrading enzymes in other microorganisms (Mester and Tien, 2000).

Furthermore, repression of enzyme synthesis does not occur when the concentration of a chemical is reduced to a level that is ineffective for enzyme induction. This is because the induction of the degradative enzymes is not dependent on the presence of the chemical. The fungus can effectively degrade very low concentrations of a pollutant to non-detectable or nearly non-detectable levels (Bumpus & Aust, 1986). Finally these enzymes are mainly of constitutive nature. This feature prevents their originating organisms to be adapted to the chemical being degraded (Gianfreda, 2004).

1.4.2 Phanerochaete chrysosporium

Phanerochaete chrysosporium is a higher basiodiomycete belonging to the family *Corticiaceae*. It is a secondary decomposer of both hardwood and softwood branches and logs and can be found in temperate forests throughout North America, Europe and Iran. *P. chrysosporium* was the first fungus to be associated with degradation of organopollutants, in 1985, because it has been extensively studied as a model microorganism in research on the mechanism of lignin degradation (Sasek, 2003). This thermophilic basidiomycete was first considered a problem in the 1970s in self-heating wood chip piles, in its anamorphic state *Sporotrichum pulverulentm.* Although, later this fungus was the subject of many investigations on cellulase and ligninase production; because of its potential in bioremediation, its natural niche remains unknown (Evans & Hedger, 2001). *Phanerochaete chrysosporium* produces lignin peroxidase and manganese peroxidase in ligninolytic conditions and laccase only when cellulose is present as carbon source (Zouari-Mechini *et al.,* 2006).

Castillo *et al*., (2001a) studied the ability of the white-rot fungus *P. chrysosporium* and the role of the lignin degrading enzyme to degrade isoproturon (N, N-dimethyl-N-[4-(1-methyllethyl) phenyl] urea), a pre- and post-emergent herbicide. In straw cultures *P. chrysosporium* was able to degrade 91% of the herbicide in 14 days of incubation. The largest MnP activity coincided with a sharp decrease of isoproturon, although LiP activity was also present.

1.4.3 Pleurotus ostreatus

Pleurotus ostreatus is an edible species commonly known as the oyster mushroom belonging to the family *Polyporaceae*. It is wide distributed throughout the Northern Hemisphere (Hestbjerg *et al*., 2003). This species is a saprophytic basidiomycete and a natural decomposer because it secretes enzymes and acids that degrade organic polymers (Pletsch *et al.*, 1999). Its great advantage is that large-scale production of fungal biomass grown on lignocellulosic substrates has already been developed for human consumption and it is economically feasible because the substrates do not need to be sterilized (pasteurization is sufficient), (Sasek, 2003). The fungal mycelium colonizes natural soil effectively (Lang *et al.*, 2000a) and its temperature requirements are considerably lower than that of *P. chrysosporium (*Hestbjerg *et al.*, 2003), as it is active at 8^0C (Heggen & Sveum, 1999). The polyphenol oxidases produced by *P. ostreatus* include laccases and Mn-dependent peroxidases, but not lignin peroxidases (Palmieri *et al,* 1997).

1.4.4 Pycnoporus coccineus

It is a non-edible species belonging to the family *Polyporaceae* and the order *Polyporales.* The fruiting bodies of this polypore genus look like bright reddish – orange brackets and are widespread on dead wood. In Australia there are two species *Pycnoporus coccineus* and *Pycnoporus sanguineus*

with overlapping distribution. They have been used in bioremediation research mainly for their effective extra cellular laccase production (Alves *et al,* 2004; Pointing *et. al*, 2000).

1.4.5 Trametes versicolor

The basiomycete *Trametes* (syn. *Coriolus*, *Polyporus, Polystictus, Microporus*) *versicolor* belongs in the order *Polyporales* and the family *Polyporaceae. Trametes versicolor* is found ubiquitously in temperate to sub-tropical forests throughout the world. It was first studied by Dodson (1987). This fungus causes rapid white invasion of moribund or fallen trees of species such as birch, beech and oak, by a rapidly extending mycelium, which utilizes free sugars in the wood of the tree (Evans & Hedger, 2001).

Several reasons account for the attractiveness of *Trametes versicolor*, chief among them is the constitutive, extracellular secretion and the non specific nature of the ligninolytic enzymes which are laccase (Lac, EC1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13) while lignin peroxidase (LiP, EC 1.11.1.14) has only rarely been reported in *Trametes versicolor* (Nyanhongo *et a*l, 2007). This fungus has been shown to be able to metabolize a wide range of organic compounds (Gadd, 2001). This ability is generally attributed to the production of extracellular ligninolytic enzymes such as laccase, which is non specific in regard to its substrate (Thurston, 1994; Pointing, 2001; Sasek *et al*.; 2003; Baldrian, 2004).

In recent studies, *Trametes versicolor* was shown to exhibit good tolerance to water stress conditions (Mswaka and Magan, 1999; Fragoeiro and Magan, 2005). This is a great advantage for bioremediation of contaminated soils. Further, this fungus can grow on cheap media such as corn, straw and even sawdust while its hyphal growth form allow it to extend far from its original point, an attractive property in bioremediation of soils (Nyanhongo *et al.*, 2007).

A large body of evidence shows that *Trametes* species are among the most versatile of white-rotters with ongoing intensive research into applications in bioremediation, effluent treatment, the pulp and paper industry, the food industry, synthetic chemistry, biofuels, cosmetics, biosensors and the textile industry, amongst others (Nyanhongo *et al.*, 2007).

1.4.6 Phlebiopsis gigantea

Phlebiopsis (syn. *Phanerochaete, Peniophora, Phlebia*) *gigantea* is a common saprophytic fungus that causes white rot of conifer logs and stumps. It is used as a biological control of annosum root rot, caused by *Heterobasidion annosum,* in Western Europe. It was found that *P. gigantea*, when applied to cur stumps, could inhibit subsequent colonization by the pathogen. In the United States, the fungus was commercially available until 1995. Currently, the Environmental Protection Agency is reviewing the status of *Phlebiopsis gigantea* as a pesticide. At this time, *Phlebiopsis gigantea* is only commercially available in England, Sweden, Norway, Switzerland and Finland. The fungus occurs throughout North America, Central America, Europe, East Africa and Southern Asia.

Table 1.1 summarizes some of the white rot fungi and their capacity for degradation of different xenobiotic compounds. Plates 1.1 to 1.4 show examples of the main species.

Table 1.1 Degradation of typical environmental pollutants by the white rot fungi.

Fungus	Type of pollutant	Reference
Phanerochaete chrysosporium	Lindane, DDT BTEX Atrazine	Bumbus *et al.,* 1985 Yavad and Reddy, 1993 Hickey *et al.,* 1994
Phanerochaete chrysosporium *Phanerochaete eryngi* *Pleurotus florida* *Pleurotus sajor-caju*	Heptachlor Lindane	Arisoy, 1998
Phanerochaete chrysosporium *Trametes versicolor*	Pentachlorophenol Creosote, Anthracene, PAHs, PCP	Alleman *et al.,* 1992 Richter *et al,* 2002 Gianfreda & Mao 2004
Trametes versicolor	Pesticides Pesticides	Khadrani *et al.,* 1999 Morgan *et al.,* 1991 Bending *et al.,*2002
Pleurotus ostreatus	Pesticides	Khadrani *et al.,* 1999
	PCBs	Gianfreda & Mao 2004
	Dyes	Sasek *et al.,* 1998
	Catechol Pyrene Phenanthrene	Bezadel *et al.,* 1996
Pleurotus pulmonaris	Atrazine	Mazaphy *et al.,* 1996
Pycnoporus sanguineus	Azo dyes	Gianfreda & Mao 2004
Bjerkandera adusta	Pesticides	Khadrani *et al.,* 1999

BTEX: benzene, toluene, ethylbenzene and xylene; PAHs: polycyclic aromatc hydrocarbons; PCBs: polychlorinated biphenyls; PCP: pentachlorophenol.

Plate. 1.1 *Pycnoporus coccineus*

Plate 1.2. *Pycnoporus coccineus*

Plate 1.3 *Phlebiopsis gigantea*

Plate 1.4 *Pleurotus ostreatus*

Plate 1.5 *T versicolor*

Plate 1.6 *P. chrysosporium*

1.5 Biodegrading capacities of bacteria

Bacteria either utilize the pesticides as sole carbon sources or, in some cases as sole nitrogen sources, or alternatively co-metabolize them. Genetic studies of pesticide degrading bacteria have centered on cloning and characterization of specific enzymes involved in pesticide degradation. The involvement of plasmid-encoded catabolic sequences in pesticide degradation has been widely documented (Somasundaram & Coats, 1990).

The degradation of pesticides *in situ* is usually achieved by a consortium of bacteria rather than a single species. Pure culture studies, however, allow the mechanisms by which the pesticide is metabolized to be elucidated. They also allow the location of genes involved in degradation of the pesticide (Aislabie & Jones, 1995). Traditional isolation methods, involving enrichment culture and plating techniques, have been used to isolate many of the microbes that are able to degrade pesticides in pure culture.

The bacterial species *Nocardia* and *Pseudomonas* utilized one or more of the side chains of atrazine aerobically as the sole source of carbon and energy. In addition, a *Pseudomonas* species able to mineralize atrazine has been enriched from soil; this organism used atrazine as the sole source of nitrogen with sodium nitrate as the carbon source (Mandelbaum *et al*., 1995). Bacteria, belonging to the genera *Achromobacter*, *Pseudomonas* and *Flavobacterium*, are able to utilize carbofuran, a carbamate pesticide, as a growth substrate (Aislabie & Jones, 1995). Recently, Carpouzas & Walker (2000) reported the ability of *Pseudomonas putida* strains epI to degrade the organophosphate ethoprophos in soil at 20 and 35 ^{0}C and at soil water potentials of -3.3 and -10 kPa. Table 1.2 shows some examples of pesticides degraded by bacteria in pure culture.

Table 1.2 Examples of bacteria able to degrade pesticides in pure culture from Aislabie and Jones, (1995)

Pesticide	Bacteria	Reference
DDT	*Alcaligenes eutrophus*	Nadeau *et al.,* (1994)
2,4D	*Alcaligenes eutrophus*	Pemberton and Fisher (1977)
	Flavobacterium	Chaudhry and Huang (1988)
	Arthortbacter	Sandman and Loos (1988)
	Pseudomonas cepacia	Bhat *et al.,* (1994)
Atrazine	*Nocardia*	Cook (1987)
	Pseudomonas	Cook (1987)
	Pseudomonas	Mandelbaum (1995)
	Rhodococcus	Behki *et al.* (1993)
	Rhodococcus	Behki and Kham (1994)
Parathion	*Flavobacterium*	Sethunathan &Yoshida(1973)
	Pseudomonas dimuta	Serdar *et al.* (1982)
Diazinon	*Flavobacterium*	Sethunathan &Yoshida(1973)
Fenthion	*Bacillus*	Patel,Gopinathan (1986)
Carbofuran	*Achromobacter*	Karns *et al*. (1986)
	Pseudomonas	Chaudhry and Ali (1988)
	Flavobacterium	Chaudhry and Ali (1988)
	Flavobacterium	Head *et al.* (1992)
EPTC	*Arthrobacter*	Tam *et al*. (1987)
	Rhodococcus	Behki *et al*. (1993)
	Rhodococcus	Behki and Kham (1994)

1.6 Pesticides

A pesticide is “any substance or mixture of substances intended to prevent, destroy, repel or mitigate any pest. This definition by FIBRA (Federal Insecticide, Fungicide and Rodenticide Act) includes insecticides, herbicides, fungicides and antimicrobials, as well as plant growth regulators, defoliants and desiccants. In 1997, agricultural industry accounted for 77% of the total use of pesticides, 12% for industrial, commercial and government organizations and the remaining 11% for private households (Moerner *et al.,* 2002).

1.7 Environmental concerns regarding the contamination with pesticides

The range of damage associated with the application of pesticides across environmental media and different receptors is equally great, providing a particularly complex example of multidimensional environmental impact (Figure1.1). Loss of aquatic and terrestrial biodiversity, contamination of surface and groundwater and agriculture produce, and poisoning of agricultural workers are among the potential negative consequences of pesticide use in agriculture alone (Mourato, 2000).

Pesticide residues can be attributed to a number of sources including releases from fields during and after application, leakage from equipment, spillages and incorrect disposal of waste and washings (Fogg, 2004). Recent research suggests that the contribution from sources other than those originating from approved applications to agricultural land may be more significant than previously realized. Such “point source” releases can be reduced by modifying handling practices to minimize losses (Fogg, 2004). However, due to time constraints and other pressures, small drips and spills are still likely to occur (Fogg, 2004). Application of pesticides for the purpose of pest management is called “non point” source contamination and in this

case pesticide presence is widespread, but at relatively low concentrations (Gan & Koskinen, 1998).

When the concentration of a pesticide, its metabolites and its by-products is significantly excessive, remediation is necessary to avoid migration to a more sensitive area of the environment (Kearney, 1998). The concentration at which soil remediation is required is referred to as the remediation trigger level, but for many xenobiotics, no guidelines are currently established. There is also a need to define the target threshold concentrations when remediation is achieved. This target remediation concentration is generally in the range of 1mg/Kg soil or a concentration arrived at after a risk assessment analysis, usually on a site-by-site basis (Kearney, 1998).

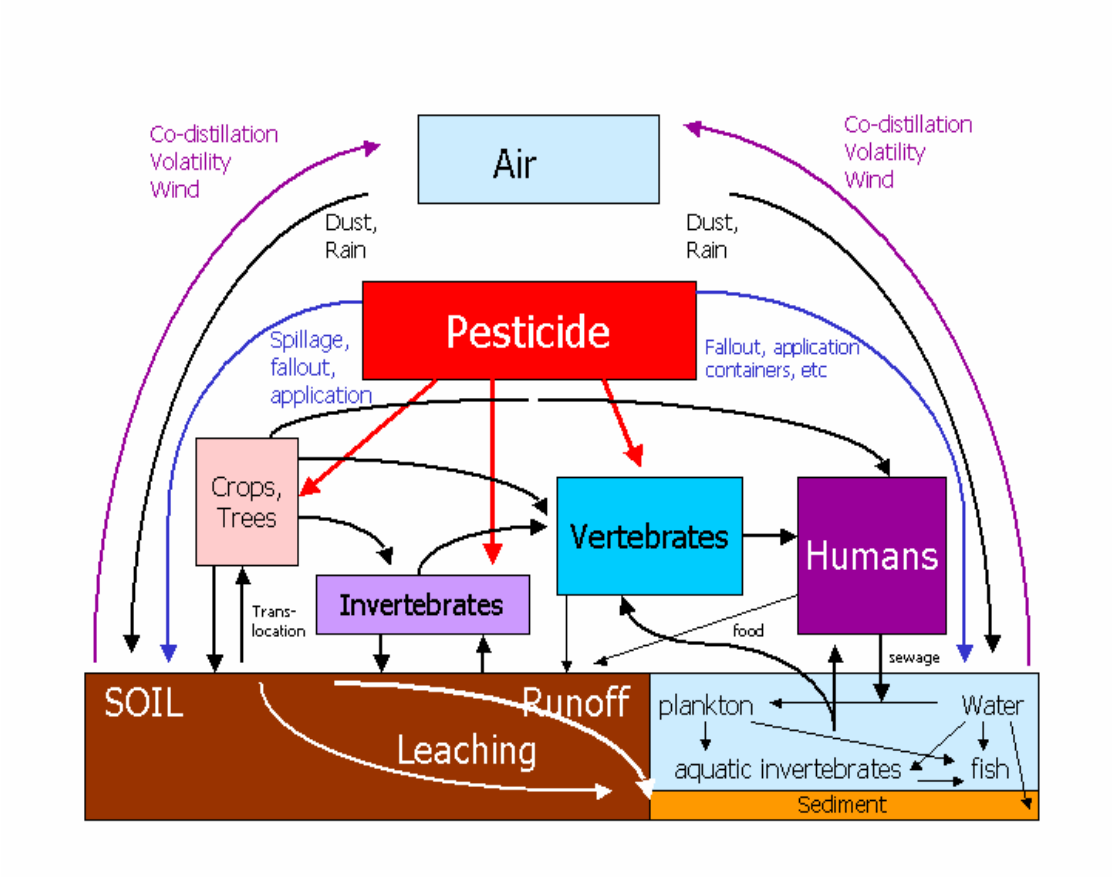


Figure 1.1 Transport of pesticides in the environment (from Gavrilescu, 2005)

Even when the level of contaminants is low and does not exceed a few μg/ kg soil, the ability of these chemicals to pass through the subsurface layers and to reach groundwater is a matter of concern, especially in areas where

drinking water is supplied from an aquifer (Muszkat *et al,* 1993). The maximum permissible concentration of pesticides in waters intended for human consumption is 0.1μg/L and 0.5μg/L in total for all substances (European Directive 80/778/EEC, 1989).

The chemicals properties of pesticides determine their retention and transport in soils (Kearney, 1998). A pesticide can reach groundwater if its water solubility is greater than 30mg/L; its adsorptivity, Koc (Koc= partition coefficient between soil organic matter and water), is less than 300-500 ml/g; its soil half life is longer than about 2-3 weeks; its hydrolysis half-life is longer than approximately 6 months and its photolysis is longer than 3 days (Barcelo, 1991).

1.7.1 Metabolic fate of pesticides

The metabolic fate of pesticides is dependent on abiotic environmental conditions (temperature, moisture, soil pH, etc.), microbial community or plant species (or both), pesticides characteristics (hydrophilicity, level of solubility*)* and biological and chemical reactions. Abiotic degradation is due to chemical and physical transformations of the pesticide by processes such as photolysis, hydrolysis, oxidation, reduction and rearrangements. Furthermore, pesticides may be biologically unavailable because of compartmentalization, which occurs as a result of pesticide adsorption to soil and soil colloids without altering the chemical structure of the original molecule. However, enzymatic transformation, which is mainly the result of biotic processes mediated by plants and microorganisms, is by far the major route of detoxification (Van Eerd, 2003).

Metabolism of pesticides may involve a three-phase process (Van Eerd, 2003). In Phase I metabolism, the initial properties of a parent compound are transformed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product than the parent. The second phase involves conjugation of a pesticide or pesticide metabolite to a

sugar or amino acid, which increases the water solubility and reduces toxicity compared with the parent pesticide. The third phase involves conversion of Phase II metabolites into secondary conjugates, which are also non-toxic. In these processes fungi and bacteria are involved producing intracellular or extra cellular enzymes including hydrolytic enzymes, peroxidases, oxygenases, etc (Van Eerd, 2003).

1.7.2 Pesticides used in the current study

Chlorpyrifos is a broad- spectrum organophosphate insecticide. It is used to control different insects on grain, cotton, fruit, nut and vegetable crops, as well as on lawns and ornamental plants (Figure 1.2A). It is also used for the control of mosquitoes and for ectoparasite control on cattle and sheep. Chlorpyrifos acts on pests primarily as a contact poison, with some action as a stomach poison. As with all organophosphates, it acts by interfering with the activities of cholinesterase, an enzyme that is essential for the proper working of the nervous system of both humans and insects (Extoxnet, 1996). It is moderately toxic to humans, moderately to very toxic to birds and very highly toxic to fish. The oral LD_{50} for chlorpyrifos in rats is 95 to 270 mg/Kg (Extoxnet, 1996). It may affect the central nervous system, the cardiovascular system and the respiratory system as well as causing skin and eye irritation (Oliver *et al.,* 2000).

Chlopryrifos is moderately persistent in soils. Its half-life in soil is usually between 60 and 120 days, but can range from 2 weeks to over 1 year depending on the soil type, climate, and other conditions. It adsorbs strongly to soil particles and it is not readily soluble in water. Its water solubility is approximately 2 mg/L. Considerable literatures indicated that a wide range of water and terrestrial ecosystems might be contaminated with chlorpyrifos (EPA, 1997; Yang *et al.,* 2006), which has increased the public concern to establish an efficient, safe and cost effective method to remove or detoxify chlorpyrifos residues in contaminated environments.

Linuron is a substituted urea herbicide (phenyl-urea) used to control annual and perennial broadleaf and grassy weeds on crop and non-crop sites (Figure 1.2B). It is used as a pre and post emergent herbicide. It works by inhibiting photosynthesis in target weed plants and it is labeled for use in soybean, cotton, pea, potato, winter wheat, carrot, corn and fruit crops.

Linuron is of low toxicity (oral LD_{50} in rats= 1,500mg/Kg) but it is highly toxic to fish and non-toxic to bees. Linuron and some of its major metabolites are suspected of being endocrine disruptors (Rasmussen *et al.*, 2005). Linuron is of relatively low acute toxicity, but is classified as an unquantifiable Group C carcinogen (that is, a possible human carcinogen for which there is limited animal evidence) and shows some evidence of developmental and reproductive toxicity (EPA, 1995).

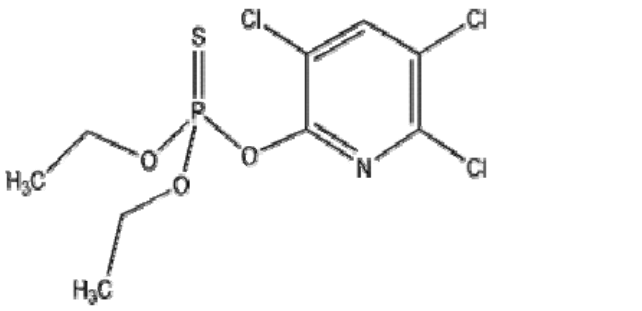
It is moderately persistent in soils. In the presence of oxygen in soil, linuron's half-life is 75 days, while it is 230 days under field anaerobic conditions. The chemical is bound in soil (especially clay) and organic matter and does not move freely. Linuron is slightly to moderately soluble in water, and it is not readily broken down in water (Extoxnet, 1993).

Metribuzin is a selective, pre and post-emergent triazinone herbicide, which inhibits photosynthesis (Figure 1.2C). It is used for the control of annual grasses and numerous broadleaf weeds in field and vegetable crops, in turf grass, and on fallow lands. It is slightly to moderately toxic to humans and mammals (LD_{50} in rats=1,090-2,300mg/Kg) and fish, while it is non-toxic to bees. This chemical is highly soluble in water and it has a moderate ability to adsorb to soil with high clay and/or organic matter content so it has great potential to leach into, and contaminate groundwater. Soil half-lives of less than one month to six months has been reported (Extoxnet, 1993). Others studies have shown that metribuzin can remain for a long time in soil (until 377days under aerobic conditions) depending on temperature, pH and soil type, accumulates in plants and interferes with other crops. The major mechanism by which metribuzin is lost from soil is microbial degradation.

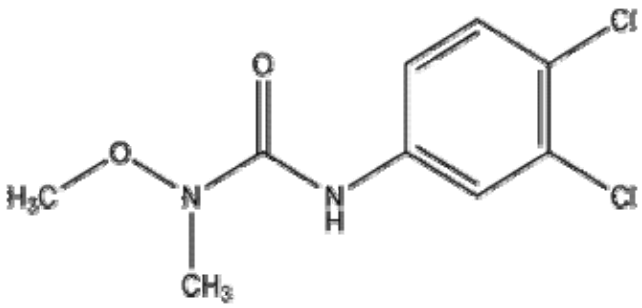
Modes of action of the pesticides used

The herbicides linuron and metribuzin act as photosynthesis inhibitors in target weed plants. Photosynthesis inhibitors shut down the photosynthetic process in susceptible plants by binding to specific sites within the plant chloroplast. Particularly, for linron and metribuzin the site of action is D_1quinone-binding protein of photosynthetic electron transport in photosystem II. Inhibition of photosynthesis could result in a slow starvation of the plant, however, in many situations rapid death occurs perhaps from the production of secondary toxic substances.

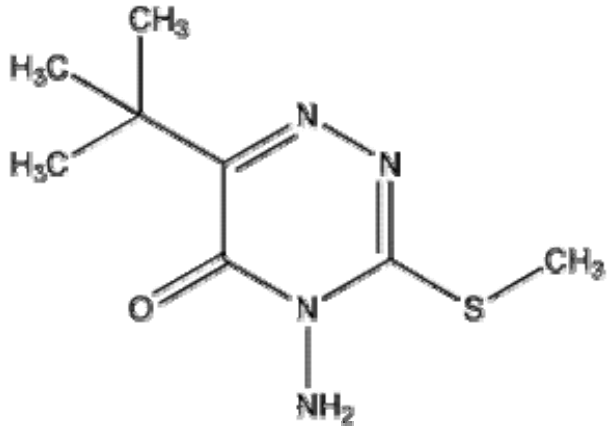
Injury symptoms include chlorosis of leaf tissue followed by necrosis of the tissue. These herbicides are taken up into the plant via roots or foliage and move in the xylem to plant leaves. As a result, injury symptoms will first appear on the older leaves, along the leaf margin.



A



B



C

Figure 1.2 Chemical structures of chlorpyrifos (A), linuron (B), metribuzin (C)

1.7.3 General review of degradation of pesticides used in current study

Chlorpyrifos

At the present time, the most widely used pesticides belong to the organophosphorus group which account for ~38% of total pesticides used globally (Post, 1998). In the USA alone over 40 million kilos of organophosphorus applied annually. Glyphosate and chlorpyrifos are the most widely used in the USA and accounts for 20 and 11% of total pesticide use, respectively (EPA, 2004). Recently, the Environmental Protection Agency (EPA) and the manufacturers of chlorpyrifos have agreed to eliminate all household applications of the insecticide, but agriculture use continues (Jones&Huang, 2003).

The environmental fate of chlorpyrifos has been studied extensively. Degradation in soil involves both chemical hydrolysis and microbial activity with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product (Singh & Walker, 2006). Chlorpyrifos has been reported to be resistant to the phenomenon of enhanced degradation (Racke *et al*., 1990). There have been no reports of enhanced degradation of chlorpyrifos since its first use in 1965 until recently. It was suggested that the accumulation of TCP, which has antimicrobial properties acts as buffer in the soil and prevents the proliferation of chlorpyrifos degrading microorganisms (Racke *et al*., 1990).

Recently, chlorpyrifos has been reported to be degraded by several bacteria. Ohshiro *et al*. (1996) reported that *Arthrobacter* sp. Strain B-5 hydrolyzed chlorpyrifos at rates depend on substrate. Mallick *et al*. (1999) reported that chlorpyrifos (10 mg L^{-1}) was completely degraded in the mineral salts medium by *Flavobacterium* sp. (ATCC 27551) for 24h and *Arthrobacter* sp for 48h, respectively. Singh *et al.,* (2004) also reported that chlorpyrifos could be degraded by *Enterobacter* sp. B-14. This bacterium degrades chlorpyrifos to diethylthiophosphate (DETP) and 3,5,6-trichloro-2 pyridinol (TCP) and utilizes DETP as a sole source of carbon and phosphorus. Many studies also

showed that chlorpyrifos in pure cultures and soil could be degraded by *Fusarium* LK. ex Fx (Wang *et al*., 2005), *Alkaligenes faecalis* DSP3 (Yang *et al*, 2005a). Li *et al.,* (2007) isolated from the polluted treatment system of a chlorpyrifos manufacturer a bacterium which was indentified as *Sphingomonas* sp. and utilized chlorpyrifos as its sole source of carbon for growth, by hydrolyzing it to 3,5,6-trichloro-2 pyridinol (TCP). Later, in 2008, the same scientists reported novel chlorpyrifos degrading bacteria which were identified as *Stenotrophomonas* sp., *Bacillus* sp. and *Brevundinomas* sp. A newly isolated *Paracoccus* sp. strain TRP, isolated from activated sludge could biodegrate both chlorpyrifos and 3,5,6-trichloro-2 pyridinol (TCP) (Xu *et al.,* 2007).

Fungal mineralization of chlorpyrifos by *Phanerochaete chrysosporium* was reported by Bumpus *et al.,* (1993). Two other white rot fungi *Hypholoma fasciculare* and *Coriolus versicolor* were able to degrade about a third of the poorly available chlorpyrifos in biobed matrix after 42 days (Bending *et al*., 2002). Also, several species of *Aspergillus, Trichoderma harzianum and Penicillium brevicompactum* were reported to utilize chlorpyrifos as sources of phosphorus and sulphur (Omar, 1998). Fang *et al.*, (2007) reported the degradation characteristics by an isolated fungal strain *Verticillium* sp., DSP in pure cultures in soil and on pakcoi (*Brassica chinensis* L.). Finally, Xu *et al.* (2007) reported mineralization of chlorpyrifos by co-culture of a bacterial strain (*Settaria* sp.) that could transform chlorpyrifos to 3,5,6-trichloro-2 pyridinol (TCP) and a TCP mineralizing fungal strain (*Trichosporon* sp.).

Linuron

Although (photo)chemical and physical processes may be involved in the removal of linuron, biodegradation is reported to be the most significant mechanism for its dissipation from soil (Caux,1998). Several enrichement cultures have indeed been obtained that use linuron as the sole source of nitrogen and carbon. Although these consortia rapidly mineralized linuron, isolation of the key organisms seemed to be difficult (Dejonghe, 2003).

Cullington &Walker (1999) isolated *Bacillus sphaericus* ATCC 12123 and *Arthrobacter globiformis* D47, respectively, which can use the alkyl chain of linuron as the sole N and C source. However these pure strains can only partially degrade linuron, since 3,4-dichlooroaniline (3,4-DCA), one of the main potential metabolites in the degradation pathway of linuron, accumulated in the medium. In 2003, Dejonghe for first time reported that the *Variovorax* strain degraded linuron further than the aromatic intermediates and was able to use linuron as the sole source of C, N and energy. In 2005, Sorensen *et al.,* reported that from a bacterial community from Danish agricultural soil which was enriched with linuron as sole carbon and nitrogen mineralized it completely. A *Variovorax* sp. was responsible for mineralization activity.

Metribuzin

Metribuzin have received a great deal of attention because of its extensive use and potential for widespread contamination of ecosystems (Lawrence *et al.,* 1993). The fate of this pesticide has been extensively studied under aerobic conditions (Pavel *et al.*, 1999). Half-lives range between 17- 377 days. In soil metribuzin is transformed to deaminated metribuzin (DA), diketometribuzin (DK), deaminated diketometribuzin (DADK) and other unidentified metabolites (Pavel *et al.*, 1999). Under anaerobic conditions the rates of degradation were more rapid in the surface than in the subsurface soil microcosms.

The major mechanism by which metribuzin is lost from soil is microbial degradation (Pavel *et al.*, 1999). The influence of soil microbiological activity on the degradation rate of metribuzin was demonstrated by several researchers (Bordjiba *et al.*, 2001). The partial transformation of metribuzin by a strain of *Streptomyces* was reported by Sheltton *et al.*, in 1996. Experiments were conducted to assess the ability of *Streptomyces* (strain PS1/5) to metabolize twelve herbicides representing several different classes including: acetanilides, triazines, ureas, uracils, and imidazoles. Incubations

in aqueous culture with dextrin as carbon source and either ammonium or casamino acids as nitrogen source resulted in transformations (> 50%) of eight of the herbicides tested: alachlor, metolachlor, atrazine, prometryne, ametryne, linuron, tebuthiuron, and bromacil; the remaining four herbicides (cyanazine, diuron, metribuzin, and imazapyr) were also transformed, but to a lesser extent.

Fungi degradation was studied in five species by Schilling *et al.* in 1985. The results obtained in liquid medium showed a total transformation by *Rhizopus japonicus* and *Cunningghamella echinulata* and a depletion rate range of 27 to 45% with *Aspergillus niger, Penicillium lilacinum* and *Fusarium oxysporum* after a 4 week incubation period. In a more recent study by Bordjiba *et al.*, in 2001, only three from 53 tested fungi species, the species *Botrytis cinerea, Sordaria superba* and *Apsidia fusca* removed more than 50% of metribuzin from liquid medium.

1.8 Environmental factors affecting bioremediation by fungi and bacteria

The control and optimization of bioremediation processes are a complex system of many factors (Vidali, 2001). These factors include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environmental factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors and nutrients). Optimum environmental conditions for the degradation of contaminants are reported in Table 1.3.

Table 1.3 Environmental conditions affecting degradation (from Vidali, 2001).

Parameters	Conditions required for microbial activity
Soil moisture	25-28% of water holding capacity
Soil pH	5.5-8.8
Oxygen content	Aerobic, minimum air filled pore space of 10%
Temperature (^{0}C)	15-45
Contaminants	Non too toxic
Heavy metals	Total content 2000 ppm
Type of soil	Low clay or silt content

1.8.1 Temperature, oxygen and nutrient availability

There is evidence that the soil environment can dramatically affect persistent organic pollutants. The soil pH, nutritional status and oxygen levels temperature vary and may not always are optimal for fungi and bacteria growth or enzyme production for pollutant transformation (Singleton, 2001).

There are many reasons for organic compounds being degraded very slowly or not at all in the soil environment, even though they are *per se* biodegradable (Romantschuk *et al.*, 2000). One reason could be strong pesticide sorption to soil and therefore decreased bioavailability (Alexander, 1994). The soils, which absorbed the chemicals to the greatest extent, showed the poorest degradation (Singleton, 2001). Another reason can be the low temperatures in soil, particularly in Northern parts of Europe and North America where soil temperatures during a large part of the year are too low for efficient microbial degradation of contaminants. The same may also be true for deeper soil layers (Romantschuk *et al.*, 2000). Most microbes isolated and studied degrade optimally at mecophilic temperatures. Their

ability to degrade xenobiotics at temperature higher or lower than 25 ^{0}C to 37^{0}C is often overlooked (Singleton, 2001).

Further, there are many environments, which are acidic or alkaline so a fruitful area of research could be the isolation and study of microorganisms, which can degrade at more extreme pH values. The solubility of compounds at different pH values will also be involved in determining the rate of degradation (Singleton, 2001).

It is now well established that organic pollutants are degraded under both aerobic and anaerobic conditions. Initially, most work concentrated on aerobic metabolism as aerobes were easier to culture than anaerobes and were considered to have a much more versatile metabolism (Singleton, 2001). Fungal degradation is very slow under oxygen restrictions resulting in partial degradation with resultant toxic intermediates being formed (Romantschuk *et al.*, 2000). In the natural environment both aerobic and anaerobic situations are commonly encountered and therefore it is essential that both anaerobic and aerobic metabolic processes have to be studied (Singleton, 2001).

1.8.2 Water availability and water potential

While soil is often measured in total water content, microbial activity depends on the fraction that is actually available for microbial growth. Thus it is important to consider the potential for bioremediation activity relevant to those conditions under which microbial activity can occur. While the wilting point of plants is about -1.4 MPa water potential microbial, especially fungal activity can occur over a much wider range of conditions (Magan, 2007).

In terrestrial ecosystems, water availability can be expressed by the total water potential (Ψt), a measure of the fraction of total water content available for microbial growth in pascals (Magan, 2007). This is the sum of three factors: (a) osmotic or solute potential (Ψs) due to the presence of ions or

other solutes, (b) matric potential (Ψm) due directly to forces required to remove bound to the matrix (e.g. soil) and (c) turgor potential of microbial cells balancing their internal status with the external environment (Jurado *et al.*, 2008).

Water activity (a_w) is an alternative measure to (Ψt) and is defined as the ratio between the vapour pressure of water in a substrate (P) and vapour pressure of pure water (Po) at the same temperature and pressure. Thus a_w = P/Po. The a_w of pure water is 1.0 and any substrate with no free water will subsequently have an a_w less than 1. It is equivalent to the equilibrium relative humidity (ERH) of the atmosphere, with pure water having an E.R.H of 100% (Magan, 1997).

All microorganisms possess a positive turgor, and maintenance of this outward-directed pressure is essential since it is generally considered as the driving force for cell expansion. Exposure of microorganisms to high osmolality environments triggers rapid fluxes of cell water along the osmotic gradient out of the cell, thus causing a reduction in turgor and dehydration of the cytoplasm. To counteract the outflow of water, microorganisms increase their intracellular solute pool by amassing large amounts of organic osmolytes, the so-called compatible solutes (Kempf and Bremer, 1998).

These have been defined by Jennings and Burke (1990) as compounds that are able to change in concentration in the cell in response to a change in external water potential, thus maintaining turgidity while having no significant effect on enzyme activity (Ramirez *et al.,* 2004).

Experimentally, variations of water potential can be created by addition of ionic solutes, such as KCl and NaCl, or non-ionically with glycerol addition to a solution or culture media.
Table 1.4 gives the relationship between the different measures for water availability.

Table 1.4 Equivalent water activity, equilibrium relative humidity and water potential at 25^0C (Magan, 1997).

Water activity	E.R.H. (%)	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.5
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.3
0.60	60	70.3

1.9 Toxicity of bioremediation products

To fully evaluate the environmental impact of pesticides, both physicochemical and toxicological analyses should be performed (EPA, 1993). Physicochemical analyses do not provide information about the toxicity of environmental samples, for interactions of the complex mix of compounds can occur, so toxicity detection is crucial in assessing environmental contamination (Ruiz *et al.,* 1997). Toxicity tests are, also, important tools to assess to what extent the bioremediation process was effective as its goal is to at least reduce pollutants levels to undetectable, non toxic levels.

Some of the tests available in the market use terrestrial organisms in their original medium and in this case the organisms are in direct contact with the contaminated soil. For example the ISO11267 standard test is used for assessing the effect of chemicals on the reproductive output of *Folsomia candida* (Collembola). Such direct tests, however are relatively time consuming (48 hrs to 30 or more days), expensive, often require extensive preparation (Perez *et al.,* 2001) and depend on the continuous culture and maintenance of the test species (Pauli and Berger, 2000).

Culture free microbiotests with organisms from different trophic and functional levels, e.g. bacteria, algae, rotifers and crustaceans, are presently available.

1.9.1 Protox

A new, ready to use is the multi-generation microbiotest with the standard ciliate *Tetrahymena thermophila,* a representative from a further ecologically relevant group - the protozoa. The latter constitute a major link between bacteria and metazoa, playing a key role in the natural microbial loop and as consumers in the artificial system of waste water treatment (Pauli and

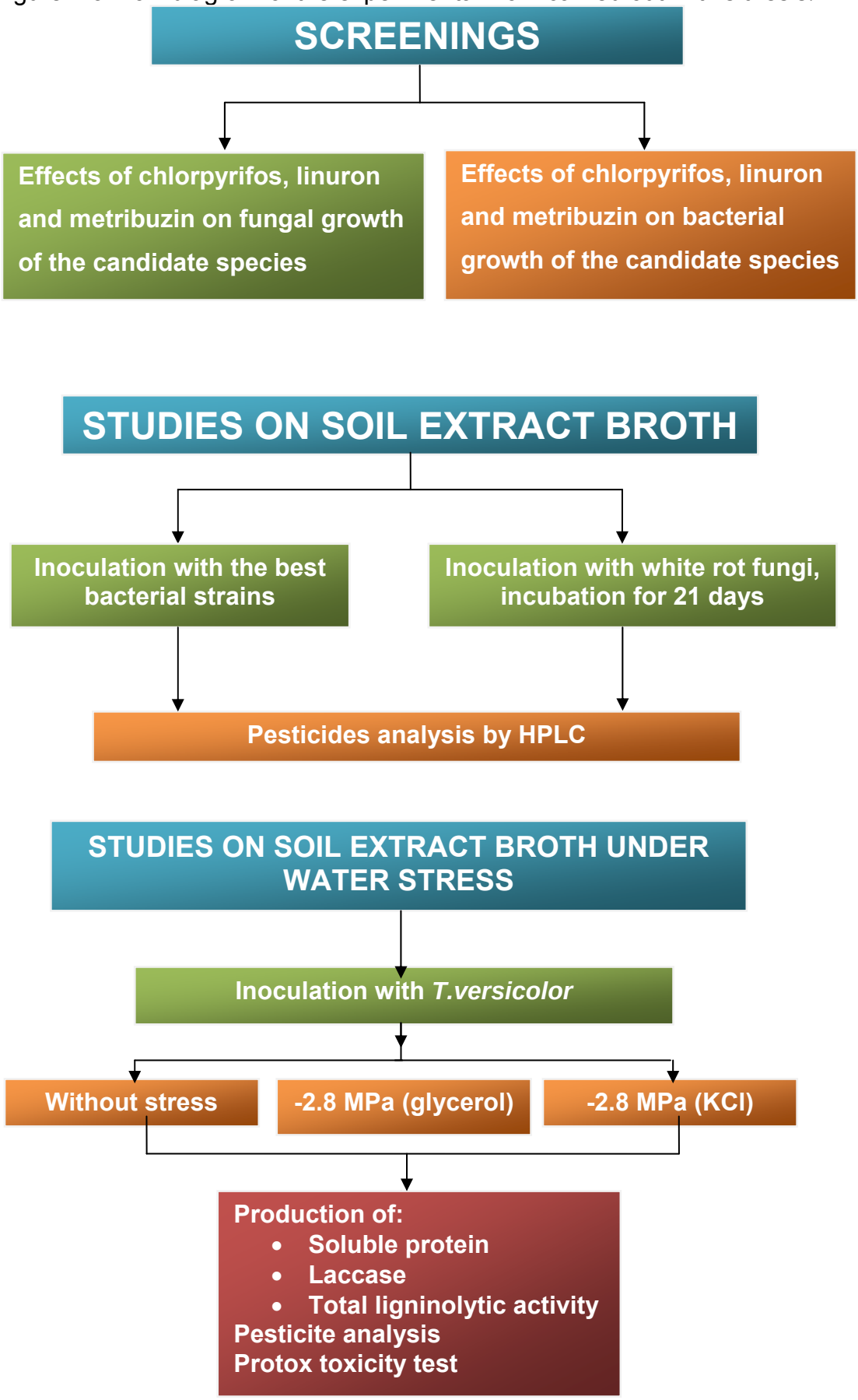
Berger, 2000). Moreover, it is a true eukaryotic cell, which can be easily cultured with a short generation time in axenic defined medium (Pauli and Berger, 2000).

This test is under consideration by Organisation for Economic Cooperation and Development (OECD) as an OECD Guideline. Fochtman *et al.,*(2000)*,* studied the potential effects of pesticides on aquatic life using thirteen widely used and registered pesticides in Poland, with potassium dichromate as a reference chemical, as well as seven different test species (ranging from protozoan to fish) among them Protoxkit. Based on their results, they suggested the use of Protoxkit as a valuable microbiotest. In another study Pauli and Berger, (2000) demonstrated based on a heterogeneous set of chemicals that toxicological results with two *Tetrahymena* species, *T. pyriformis* and *T. thermophila*, reveal a highly significant correlation (r=0.928, n=52) and nearly equal susceptibility of species. Comparisons of toxicological literature data from the International Uniform ChemicaL Information Database (IUCLID) indicate a similar toxic response even for distant ciliate relatives. Data from IUCLID also reveal that ciliate toxicity data are supplementary to those of the standard activated sludge respiration inhibition. Ciliate tests could hence serve as a powerful tool for the prediction of possible hazards to sewage treatment processes.

1.10 Aims and objectives of this project

- Examine the potential for degradation of mixtures of the pesticides chlorpyrifos, linuron and metribuzin by a range of bacteria and fungi
- Relate this capability to enzyme production
- Quantify the differential rates of degradation of mixtures of these pesticides

Figure 1.3 Flow diagram of the experimental work carried out in this thesis.



2 MATERIALS AND METHODS

2.1 Fungal inoculants

All the isolates used through these experiments were white rot fungi, which were obtained from the Applied Mycology Group, Cranfield Health (Table 2.1). Isolates were kept as solid agar Petri plate cultures on 3% malt extract agar (MEA) and were subcultured every two months. None of these strains produce toxins or other.toxic metabolites.

Table 2.1 Isolates, used in this study, and their reference number

Species	Reference
Trametes versicolor	R26
Phanerochaete chrysosporium	R170
Pleurotus ostreatus	R14
Phlebiopsis gigantea	R174
Pycnoporus coccineus	R180

2.2 Bacteria

Nineteen strains of *Bacillus* spp. were used in this study. These strains were obtained from the collection at the Laboratory of Plant Bacteriology, Technological Institute of Crete. They were all isolated from Cretan soils, and different composts. All these strains were successfully used as biological agents against bacterial and fungal diseases of plants

Moreover, the strains Pf2, Pf545 and Pf804 of *Pseudomonas fluorescens* and the strain *Pseudomonas putida* were tested in order to investigate the capability of these bacterial strains to tolerate mixture of the pesticides chlorpyrifos, linuron and metribuzin and degrade them. *Pseudomonas putida* was provided by University of Crete while the other strains by the Laboratory of Plant Bacteriology, Technological Institute of Crete. *Pseudomonas putida* is a saprophytic soil bacterium that can survive and function in the environment. It has been certified as a biosafety host for the cloning of foreign genes.

2.3 Media and substrates

2.3.1 Malt Extract Agar (MEA)

This was prepared by dissolving 20 g MEA (Lab), (Malt Extract 17 g/L, Mycological peptone 3 g/L) and the addition of 15 g agar in 1 L distilled water.

2.3.2 Minimal salts Medium Yeast (MMY)

The ingredients of the medium were: $MgSO_4$ 0.5 g, $NaNO_3$ 2 g, K_2HPO_4 0.14 g, KCl 0.5 g, $FeSO_4$ x $7H_2O$ 0.01 g, Yeast 0.5 g, Agar 20 g in 1 L distilled water.

2.3.3 MMY plus glucose

It contained the above ingredients plus 20 g glucose.

2.3.4 Trypticase Soy Blood (TSB)

(Casein peptone 17 g, Soy peptone 3 g, Sodium chloride 5 g, Dipotassium phosphate 2.5 g, Dextrose 2.5 g per litre of distilled water (Hispanlab). It was prepared according to the manufacturer's instruction.

2.3.5 Bovine Serum Albumin (BSA)

Ampules were used, each containing 1 mL of a solution consisting of 1mg/mL bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide as a preservative.

2.3.6 ABTS (2, 2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (A-1888, Sigma)

It was prepared according to the manufacturer's instruction.

2.4 Pesticides

Analytical grades of each pesticide: chlorpyrifos (O, O-diethyl1 O-3, 5,6-trichloro-2-pyridyl phos)-phorothioate) MW: 350.62, linuron (1-methoxy-1-methyl-3 (3,4-dichlorophenyl) urea) MW: 249.1 and metribuzin (1,2,4-Triazin-

5 (4H)-one,4-amino-6-(1,1-dimethylethyl)-3-(methylthio) MW:214.29 were obtained from Sigma-Aldrich Company.

Stock standard solutions, 2000ppm, were prepared by dissolving analytical standards in methanol and storing them in amber bottles at 4 ^{0}C. In this study xenobiotic concentrations are expressed in ppm (mg L^{-1}).

2.5 Fungi

2.5.1 A comparison of growth of white rot fungi in the presence of pesticide treatments on different culture media

For this purpose, malt extract agar, MMY minimal salts medium and MMY plus glucose were modified by the addition of chlorpyrifos, linuron and metribuzin individually at concentration of 10 ppm and were added to the molten agar, thoroughly mixing. A volume about 15.0 ml of each of them was poured into 9 cm diameter Petri plates. All plates were inoculated with a 4-mm agar plug from the margin of a growing colony of each fungal isolate and incubated at 25^0C. Experiments were carried out with three replicates per treatment.

2.5.2 A comparison of white rot fungi tolerance to single pesticides and mixture of them in vitro, using a minimal salts medium

In this experiment the minimal salts medium was modified by the addition of the three pesticides individually at 30ppm or as a mixture of pesticides at 15 and 30ppm. All fungi were tested at 25^0C. Growth was measured regularly for up 16 days taking two diametric measurements at right angles to each other. The temporal growth was used to obtain the growth rates from the regression lines of the radial mycelial extension, as well as the % in growth inhibition. Experiments were carried out with three replicates per treatment.

2.5.3 Screening of lignin degradation and polyphenoloxidase production

(a) Plate assay to assess lignin degradation

In order to assess which isolates could degrade lignin, the fungi were grown in a lignin medium described by Sundman and Nase (1971). The medium was prepared with 0.25 g alkaline lignin, 5 g of glucose, 5 g ammonium tartrate, 1 g malt extract, 0.5 g $MgSO_4$.7H2O, 0.01 g $CaCl_2$.2H2O, 0.1 g NaCl, 0.01 g $FeCl_3$, 1 mg of thiamine, 20 g of agar in a litre of distilled water, with pH adjusted to 4.5. To examine the enzyme production potential in the presence of the pesticides this medium was supplemented with chlorpyrifos, linuron and metribuzin, individually at 30 mg $L^{-1}$and as a mixture at 0, 15 and 30 mg L^{-1} (total concentrations). The test isolates were centrally inoculated and incubated at 25^0C for 8 days. After this period they were developed by flooding with a reagent containing equal parts of 1% aqueous solution of $FeCl_3$ and $K_3[Fe(CN)_6]$. A positive result was indicated by clear zones under or around the growth area of the lignin degrading fungi. The activity halo was measured, taking two diametric measurements at right angles to each other, for quantification of lignin degradation. The greater the clearing area, the more intense the lignin degradation.

(b) Plate assay to assess polyphenol oxidase activity

The formation of a brown halo produced by fungi on tannic acid agar reflects laccase (oxidise o- and p-phenols) and catechol oxidase (oxidise o-phenols). These activities were detected after inoculation of the isolates in culture media containing 1% tannic acid, 2% Czapek Dox modified media (containing sodium nitrate 2 g L^{-1}; potassium chloride 0.5 g L^{-1}; magnesium glycerophosphate 0.5 g L^{-1}; ferrous sulphate 0.01 g L^{-1}; potassium sulphate 0.35 g L^{-1} and sucrose 30 g L^{-1}) and 2% of bacto-agar. The tannic acid solution was adjusted to pH 4.5. Medium and acid solutions were autoclaved separately and mixed after cooling down to around 50^0C. To examine the

enzyme production potential in the presence of the pesticides this medium was supplemented with chlorpyrifos, linuron and metribuzin individually at 10 mg L^{-1} and 20 mg L^{-1} and as a mixture at 10 and 20 mg L^{-1} (total concentrations). The test fungi were centrally inoculated and incubated at 25^{o}C for 8 days. A positive activity was indicated by a brown coloration of the culture media around the colony (Rigling, 1995). The activity halo was measured, taking two diametric measurements at right angles to each other, for quantification of enzyme production. The greater the halo, the more intense the enzymatic activity.

2.6 Bacteria

2.6.1 A comparison of bacterial tolerance to single and mixtures of pesticides *in vitro* in conducive culture medium

This study assessed the tolerance level of 19 strains of *Bacillus* spp. to chlorpyrifos, linuron and metribuzin and their mixtures. These strains were obtained from the collection at the Laboratory of Plant Bacteriology, Technological Institute of Crete.

An appropriate volume of stock solution of each pesticide was added aseptically to give the final pesticide concentrations of 30, 60, 90, 120, 150 ppm. 0.2 ml aliquots of each bacterial suspension, containing approximately 1x10^{5} CFU/ ml, were used to inoculate fresh liquid cultures (5 ml) of TSB (rich medium) plus the different pesticides in tubes (10 ml). These cultures were incubated at 25^{0}C on a platform shaker at 150 rev min^{-1}. Bacterial growth was determined by measuring optical density at 600 nm after 24 hours incubation.

Subsequently, triplicate universal bottles (30ml) containing 5 ml of TSB plus the three pesticides in mixtures of 30 and 60 ppm total concentrations were inoculated with the strains that have the best tolerance to the pesticides individually and incubated as described above.

PART II STUDIES ON SOIL EXTRACT

2.7 Preparation of soil extract broth

A soil extract medium was used in this study. This medium was prepared with a sand clay loam soil from Heraklion, Crete, containing 54.6 % sand, 21.2 % silt, 24.2 % clay, 1.12% organic matter and pH 7.6 (analyzed by the laboratory of Soil Science at Technological Institute of Crete). Soil extract was prepared by mixing soil and distilled water in a 1:1 (w/v) ratio and sterilized for 30 min at 121^{0}C. The supernatant was centrifuged and then autoclaved again (30 min at 121 ^{0}C). Soil extract is a liquid broth. Technical agar (2% W/V) was added to the liquid medium in order to solidify.

Soil extract agar was modified by the addition of chlorpyrifos, linuron and metribuzin individually at concentration of 5, 25 and 625 ppm or as a mixture of pesticides in the range 0-70 ppm (total concentrations) by addition to the molten agar, thoroughly mixing and pouring into 9-cm Petri plates. All test fungi were tested at 25 ^{0}C. Growth was measured regularly for up 14 days. The effective dose (ED) for 50% inhibition of growth (EC_{50} values), relative to the control s was calculated for each fungus.

2.8 Inoculation with white rot fungi in a soil extract liquid broth

In this study only three fungal isolates were used: *Trametes versicolor, Phlebiopsis gigantea* and *Pycnoporus coccineus.*

2.8.1 Incubation conditions

A soil extract liquid broth was used in this study which was supplemented with a mixture of chlorpyrifos, linuron and metribuzin to give final concentrations 0, 10, 20, 30, 40 and 50 ppm. Aliquots of 16 ml were transferred to 9-cm Petri dishes, which were inoculated with a 6-mm agar

plug from the margin of an active fungus culture, which had been incubated on a plate of malt extract agar and active growth had commenced. Uninoculated controls were also set up for each pesticide. There were three replicates per pesticide/fungus treatment and control and incubated at 25^0C. After 21 days, the mycelium was filtered through Whatman No.1 paper filters. The fresh liquid was frozen at -20^0C and later used for pesticide quantification by HPLC.

2.8.2 Pesticide analysis

Samples were filtered through 0.2µm filters prior to HPLC quantification of all three pesticides. This was performed with an Agilent Technologies 2100 HPLC system equipped with a DAD detector an auto sampler and a C-18 column (250 mm ×4 mm). The column was operated at ambient temperature with a flow rate of 0.8 ml min^{-1} and an injection volume of 20µl.

An isocratic mobile phase system was established using acetonitrile: water at a ratio of 60: 40. This ratio enabled the separation and quantification of chlorpyrifos, linuron and metribuzin in a single HPLC run of 8 min with linuron eluting at 249 nm, chlorpyrifos at 251 nm and metribuzin at 293 nm. The retention times were 5.5, 5.2 and 3.2 minutes respectively. The limit of detection for the three pesticides was less of 0.1 mg L^{-1}. Standard curves were constructed for each standard in soil extract broth and *r*-squared values for each curve found to be> 0.99 for all three pesticides (Figure 2.1).

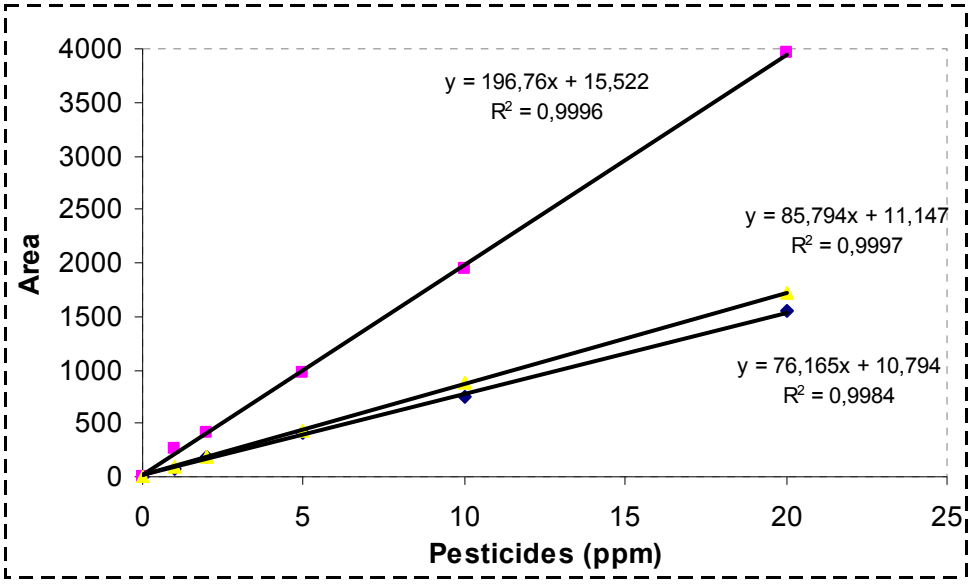


Figure 2.1 Standard curves for a mixture of chlorpyrifos, linuron and metribuzin in soil extract broth and *r*-squared values for each curve (linuron ■, metribuzin ▲ and chlorpyrifos ♦).

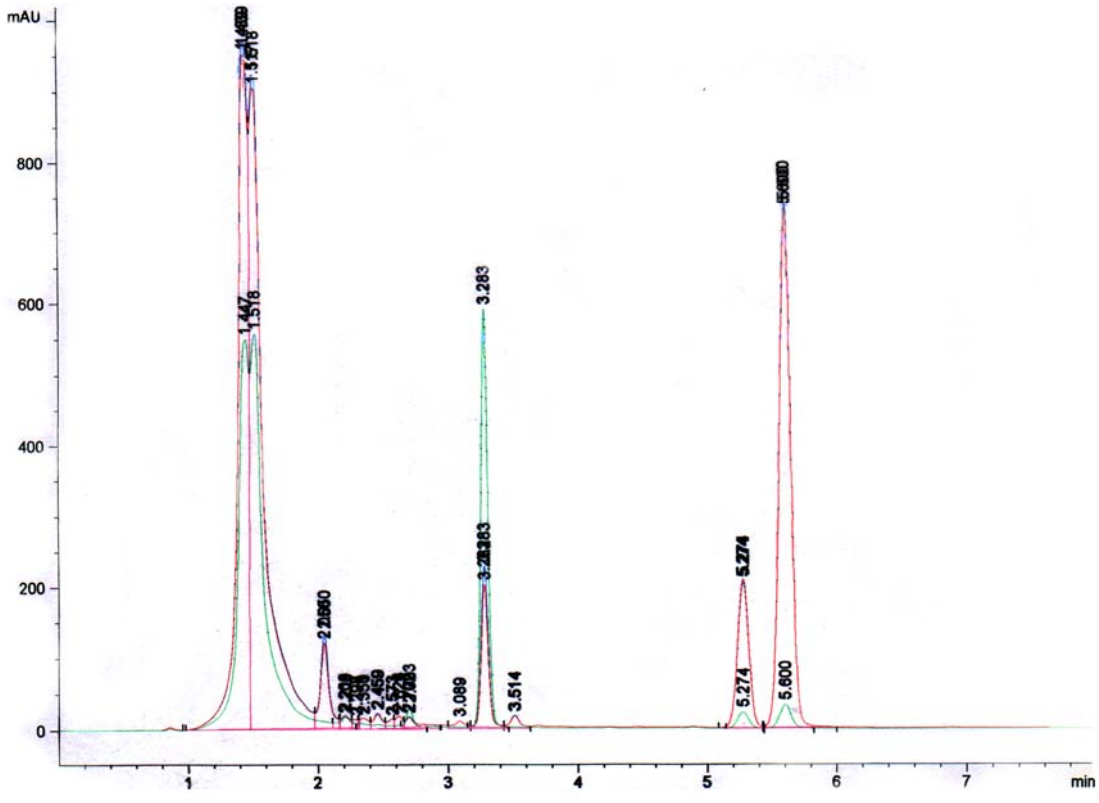


Figure 2.2 Chromatogram of metribuzin, chlorpyrifos and linuron obtained in single HPLC run with metribuzin eluting at 3.2, chlorpyrifos at 5.2 and linuron at 5.5 minutes in a mixture of 50ppm of the pesticides (equal in amount).

Moreover, chlorpyrifos was detected by HPLC using an isocratic mobile phase system with acetonitrile and water at a ratio of 80: 20 (protocol of

chlorpyrifos determination by Sigma). The column was operated at ambient temperature with a flow rate of 1.4 ml min^{-1} and an injection volume of 20µl. Standard curve was constructed for chlorpyrifos in soil extract broth and *r*-squared value found to be> 0.99 (Figure 2.2).

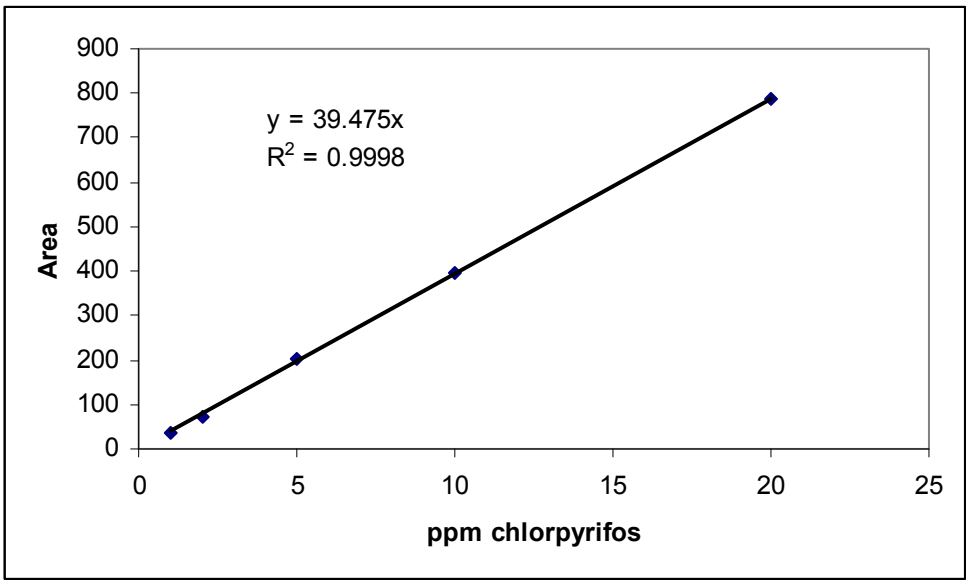


Figure 2.3 Standard curve for chlorpyrifos in soil extract broth and *r*-squared value (protocol of chlorpyrifos determination by Sigma).

Chlorpyrifos is a degradable compound, and a number of environmental forces may be active in its breakdown. In all systems (soil, water, plants and animals), the major pathway of chlorpyrifos degradation begins with cleavage of the phosphorus ester bond to yield 3,5,6-trichloro-2-pyridinol (TCP). In soils and water, TCP is further degraded via microbial activity and photolysis to carbon dioxide and organic matter (Dow Agrosciences).

TCP was detected by HPLC at 235 nm using a gradient mobile phase system mentioned below:

Eluent A: acetonitrile

Eluent B: water + 0.1% phosphoric acid

Gradient:	time (min)	% A	% B
	5	30	70
	15	80	20
	25	stop	

The column was operated at ambient temperature with a flow rate of 1.4 ml min^{-1} and an injection volume of 20µl (protocol of TCP determination by Sigma). Standard curve was constructed for TCP in soil extract broth and *r*-squared value found to be quite good (Figure 2.3).

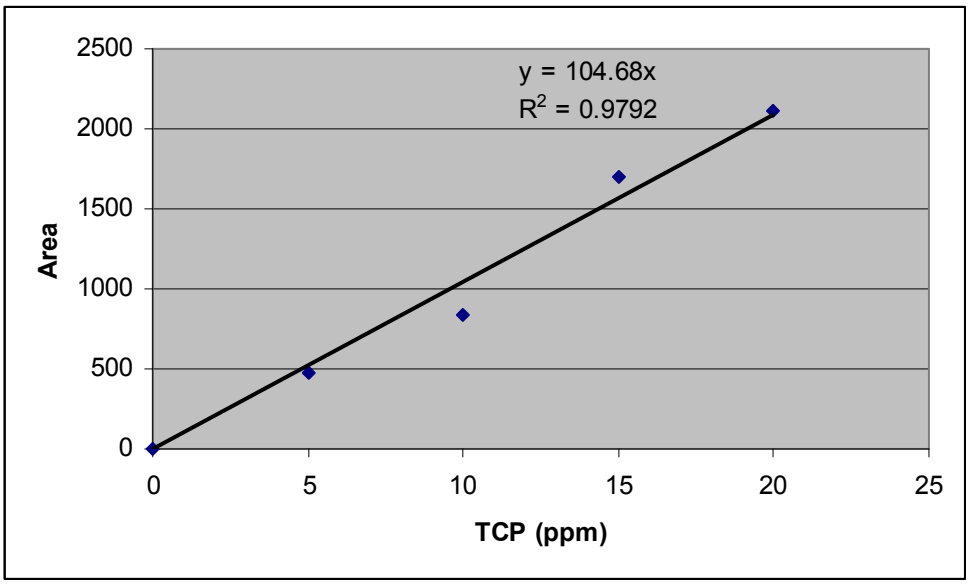


Figure 2.4 Standard curve of the main metabolite of chlorpyrifos 3,5,6-trichloro-2 pyridinol (TCP) in soil extract broth and *r*-squared value.

2.9 Inoculation with bacteria in a soil extract liquid broth

In this study, the strains 13,15 and 18 of *Bacillus subtilis,* the stains Pf2, Pf545 and Pf804 of *Pseudomonas fluorescens,* the strain *Pseudomonas putida* and mixture of all of them were tested for their potential capacity to degrade a mixture of 60 ppm (total concentration) of the pesticides chlorpyrifos, linuron and metribuzin.

2.9.1 Incubation conditions

Inoculates were prepared from cultures of the strains grown on soil extract agar medium containing the mixture 60 ppm of the pesticides for 48 hours at 25^0C, in order to maximize the degrading potential of the strains. Growths were washed from the plates and the OD_{600} of each bacterial suspension was measured. Subsequently, suitable aliquots of the cultures that

corresponded to a density of 10^8 cells ml^{-1} were transferred into soil extract liquid broth (30 ml) supplemented with a mixture of 60 ppm (total concentration) of the three pesticides and the OD_{600} was measured. Uninoculated controls were also set up. There were three replicates per pesticide/bacterial treatment and control.

Then, the cultures were incubated at 25^0C on a platform shaker at 150 rev min^{-1}. Every three hours the OD_{600} was measured and suitable aliquots were filtered through 0.2μm filters and frozen in order to HPLC quantification of the three pesticides. The pesticides were determined as described previously in section 2.8.2.

2.10 Inoculation with *Trametes versicolor*

In this study, only the fungus *Trametes versicolor* was used which was selected because it showed tolerance and ability to degrade the pesticide linuron. In order to evaluate the effect of water stress and the laccase production over a six week period, the soil extract liquid broth solute potential was adjusted in two different ways: ionically by adding potassium chloride and non-ionically by using glycerol. In order to investigate the abiotic degradation of the pesticides over a six weeks period of incubation, plates, in triplicate, with the mixtures of pesticides without inoculation with *T. versicolor* were prepared and were used as controls. In all assays Petri plates with the soil extract were prepared as described previously (2.8.1).

The water potential of the soil extract broth was adjusted to -2.8 MPa ($0.98a_w$) by adding 3.73 g of the ionic solute potassium chloride or 7.3 ml glycerol to 100 ml of soil extract.

2.11 HPLC quantification of pesticides

Pesticide working solutions (2,000 ppm) were prepared by dissolving analytical reagents in methanol and storing in amber bottles at 4^0C. An appropriate volume of stock solution of each pesticide was added aseptically to give final mixtures of the pesticide concentrations of 0, 10, 20, 30, 40, 50 ppm. The pesticides were determined as described previously in section 2.8.2.

2.12 Total ligninolytic activity

The poly R-478 (polyvinyl sulfonated backbone with anthrapyridone chromophore, violet colour) decolouration assay was used to study the overall ligninolytic activity in the soil extract, following the method described by Glenn and Gold, 1983). Dye poly R-478 (P-1900, Sigma) was added to the filtrates as an aqueous solution to a final concentration of 0.02% with gentle swirling. Directly after its addition, 1ml of the filtrates was removed and diluted 10-fold with water. The reaction mixture was kept under light (desklamp, with a 40w bulb) for 24 h for the enzyme reaction to take place.

Total ligninolytic activity was given as decolouration degree of the Poly R-478 monitoring by the percentage reduction in the absorbance at 530 and 350 nm calculated as follows:

Colour intensity = absorbance at 530 nm/ absorbance at 350 nm

% decolourisation = 100 - [(absorbance at 530 nm/ absorbance at 350 nm) sample / (absorbance at 530 nm/ absorbance at 350 nm) poly R478] x 100

Because fungal adsorption, as well as fungal transformation, reduces the intensity of the dyes in solution, it was necessary to measure soluble dye absorbance at two wavelengths, 530 and 350 nm. The two wavelengths indicated were chosen to produce the greatest change in the absorbance ratio as the dye was degraded Glenn and Gold (1983). A lower absorbance ratio means intense decolouration and higher enzymatic activity.

2.13 Laccase production in the different media

Laccase activity (EC 1.103.2) was determined using ABTS (2, 2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (A-1888, Sigma) as a substrate at 420 nm, based on the protocol described by Buswell *et al*., (1995) with

adaptations by Fragoeiro and Magan (2005). The assay was carried out at ambient temperature, with the ABTS and buffer equilibrated at 37^0C. The reaction mixture, in a total volume of 900 µl, contained 650 µl sodium acetate buffer, pH 5.0, and 100 µl of enzyme extract. The reaction was carried out directly in the spectrophotometer cuvette and initiated by adding 150 µl of 0.55 mM ABTS.

Laccase activity was determined using a spectrophotometer set in kinetic mode. Kinetic mode is a predefined analysis of the absorbance values recorded by the spectrophotometer and the output values are given as (final absorbance- initial absorbance / reaction time (min). After several tests the reaction time was set at three minutes.

Fungal laccases, among other enzymes, oxidize ABTS (green-colored molecule) to the cation radical $ABTS^+$ (dark green-colored molecule) (Pich *et al.*, 2006). The colourimetric changes can be determined by measuring the change in absorbance spectroscopy. The change in absorbance (ΔA) at a particular time interval (Δt) for a particular reaction was calculated by the Lambert-Beer equation.

Boiled enzyme was used in the control sample. One activity unit was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute, using an ε_{420} of $3.6x10^4$ moL L^{-1} $cm^{-1.}$ This assay was first optimised using commercial laccase from *Rhus vernificera*, crude acetone powder, minimum 50 units mg^{-1} solid (L-2157, Sigma), giving a positive result for laccase concentrations as low as 0.05 mg mL^{-1}, i.e.0.675 units per well.

The calibration curve obtained showed a good correlation (R^2=0.974) between the concentration of commercial purified laccase and laccase activity (U) (Figure 3.6).

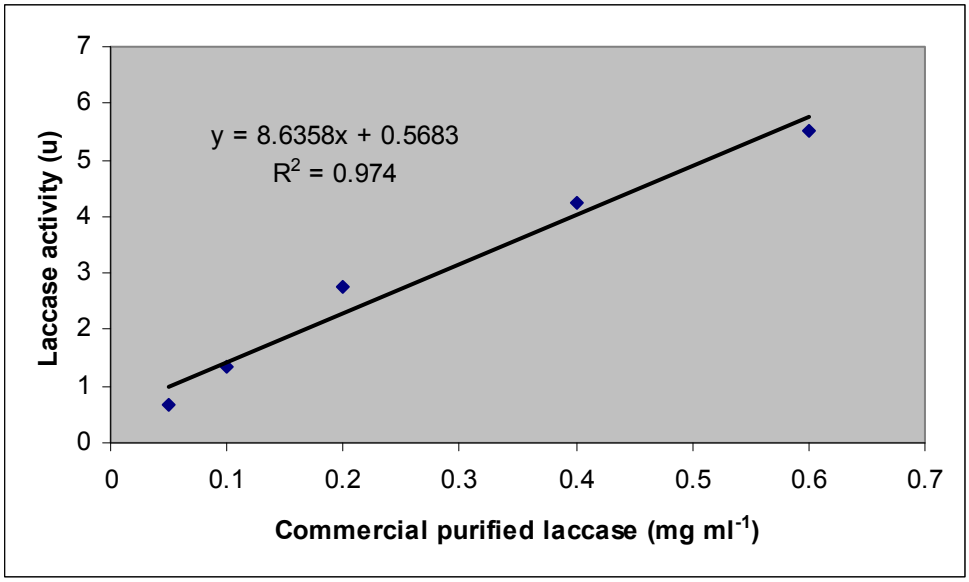


Figure 2.5 Linear regression of laccase activity against concentration of purified laccase from *Rhus vernicifera.*

2.13.1 Soluble protein

Soluble protein content of the enzyme extracts was determined with the Modified Lowry Protein Assay, after trichloroacetic acid (TCA) precipitation. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan and cysteine react with Folin-Ciocalteau reagent, a mixture of phosphotungstic and phosphomolybdic acid in phenol, to produce an unstable product that becomes reduced to molybdenum/tungsten blue. The latter can be detected colourimetrically by absorbance at 625 nm. The higher the concentration of protein, the darker the solution. A standard curve of absorbance versus micrograms Bovine Serum Albumin (BSA) protein was prepared in order to determine protein concentration.

Figure 2.5 shows the calibration curve of absorbance and Bovine Serum Albumin (BSA) protein in concentrations ranging from 200 to 1000 μg mL^{-1}.

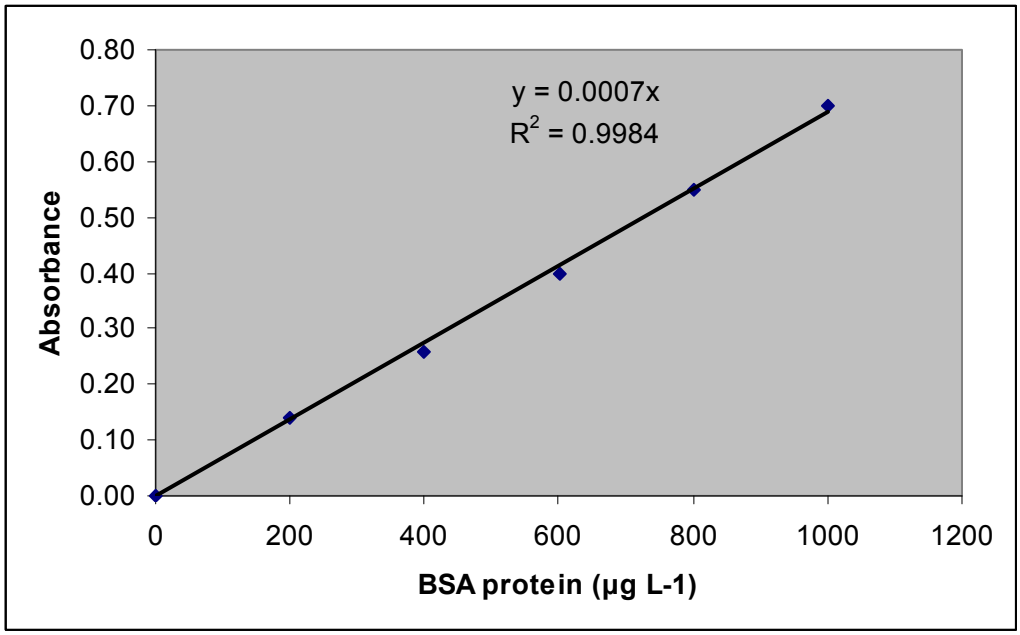


Figure 2.6 Linear regression of absorbance at 625 nm against concentration of Bovine Serum Albumin (BSA) protein (µg mL^{-1}).

2.14 Toxicity test

The toxicity measurements were performed with the PROTOXKIT F^{TM} (MicroBiotests Inc.), a 24h growth inhibition test using the ciliate protozoan *Tetrahymena thermophila.* The test is based on optical density (OD) measurement of the food substrate provided to the ciliates, in 1 cm disposable spectro-photometric cells. Ciliate growth inhibition is reflected by higher turbidity in the test cells containing the toxicant after 24h exposure, in comparison to the controls. The stock culture vial containing the ciliates in a stationary growth phase can be stored at room temperature for several months. The tests are started from the stock culture vial, without any intermediate time loss for hatching or reactivation of the test biota.

Standards solutions of the pesticides were added to soil extract for the toxicity assays. Toxicity of soil extract spiked with 1, 2.5, 5, 7.5, 10, 15 and 20 ppm of chlorpyrifos, linuron and metribuzin was determined and compared against the toxicity of these three pesticides in a mixture at total concentrations of 3, 6, 9, 12, 15, 22.5, 30 and 45 ppm.

Soil extract samples were prepared in the same way as for HPLC analysis (described in section 2.8.2).

After preparing of ciliate inoculum and the food substrate according to the detailed instructions given in the Standard Operational Procedure Manual, 2 ml of each sample, 40µl of the food substrate and 40µl ciliate inoculum were added in the test cells and the cells were closed with their lids. Subsequently and after gentle shaking (by inverting the cells a few times), the optical density (OD) of each test cell was measured at 440nm (= time T0 scorings). Finally, the cells were incubated at 30 ^{0}C for 48 hours as in some cases (which are batch dependent) it is advised to extend the incubation period to 48 hours in order to obtain enough OD decrease in the controls.

After 48 hours incubation the OD of each test cell was measured again (T28h scorings) and the percent of inhibition (%) was determined as described in the following formula:

$$\% \text{ INHIBITION} = \frac{(1 - \Delta \text{ OD sample})}{\Delta \text{ OD control}} \chi\ 100$$

2.15 Data handling and statistics

Data input, data handling/manipulation, linear regression, and graph plotting were carried out using Microsoft Excel 2003 (Microsoft Co.). Analysis of variance (ANOVA) was performed using SPSS (version 11). When required comparison between means this was carried out using ANOVA followed by Tukey Multiple Comparisons test. The 95% confidence limits (P =0.05) were used to compare treatments. Statistically significant differences among treatments are expressed by different letters.

3 RESULTS

3.1 Introduction

The objectives of this study were to assess the growth and the tolerance of five white rot fungal isolates to chlorpyrifos, linuron and metribuzin, in a minimal salts medium, individually and as a mixture. The first approach was to study if the isolates could have a good growth in this minimal medium in the presence of the pesticides in relation to rich culture media. Subsequently, the pesticides were added in higher concentrations and as a mixture and the growth rates and the % inhibition were calculated. Finally, the potential of these fungal isolates to degrade lignin and produce laccase in the presence of the three pesticides, individually and as a mixture was examined. From these studies three isolates were selected for the subsequent studies.

3.2 In vitro comparison of white rot fungi for tolerance to pesticides

3.2.1 Calculation of fungal growth rates

Colony diametric measurements were made every day throughout the incubation period by taking two readings at right angles to each other. Data were then tabulated and the linear portion of the radial extension rates used to determine growth rate as linear regression. An example is shown in Figure 3.1: the growth rate is the slope of the equation (Y=ax+b), thus for this example the growth rate was 0.4479 cm day^{-1}.

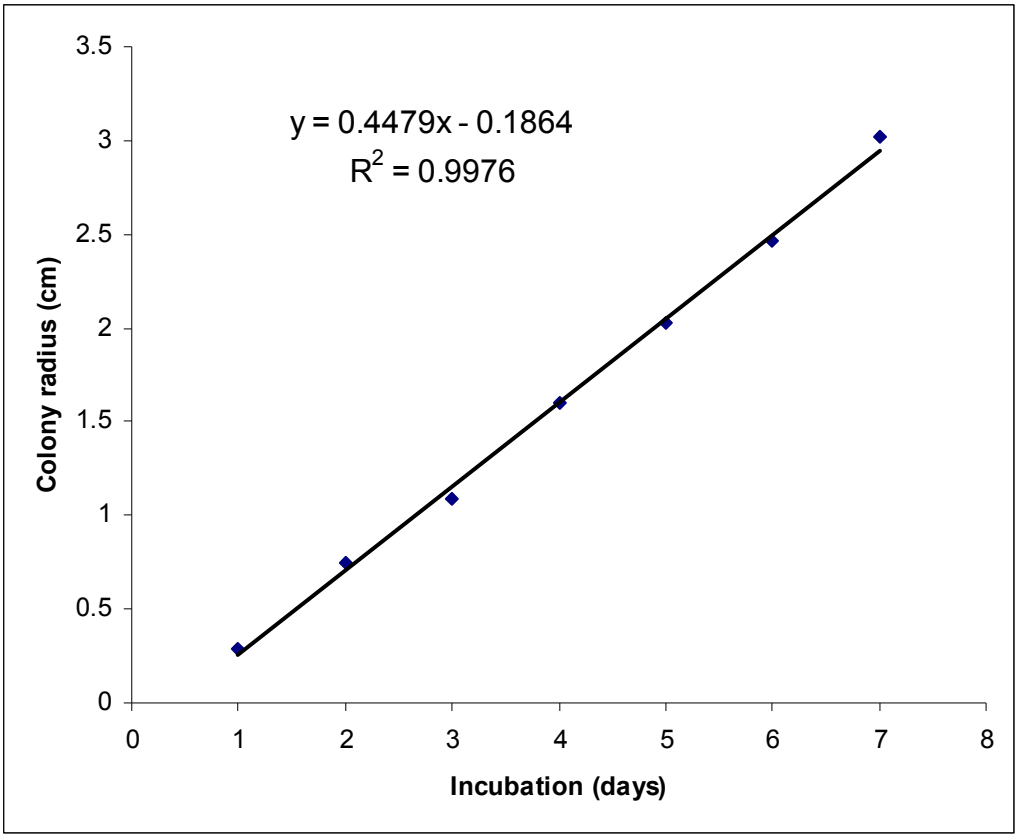


Figure 3.1 Linear regression of mycelial radial extension plotted against time in order to obtain the growth rate (cm day^{-1}).

3.2.2 In vitro tolerance to individual and mixtures of pesticides

The growth rates of the five test isolates, in different media are shown in Table 3.1. Plates 3.1 and 3.2 show examples of the effect of treatments. All test fungi had good growth on MMY, a medium that consisted of Czapek salts plus 0.5 g L^{-1} yeast broth in relation to the other two conducive media. The statistical analysis showed that the interaction between pesticides and fungi was highly significant ($p<0.001$) for all media, so separate tests were performed for each pesticide to reveal differences amongst the test fungi. In all cases the Tukey's test showed that *Phanerochaete chrysosporium* had the best growth rate in the 10 ppm treatment of the pesticides chlorpyrifos, linuron and metribuzin. Interestingly, this fungus was highly influenced by the 30ppm concentration of the pesticides individually and as a mixture. For example, the growth rate of *P. chrysosporium* was decreased from 1.09 cm day^{-1} (control) to 0.08 cm day^{-1} at 30 ppm of chlorpyrifos and to 0.10 cm day^{-1}

at 30 ppm of the mixture. This fungus showed high sensitivity to these pesticides.

The growth rate of all the test fungi was significantly decreased as the pesticide mixture was increased from 15 to 30 ppm (Table 3.2). This shows that the relative growth rates were affected in a different way depending on whether there was a single or mixture of pesticides. For example, the growth rate of *Pleurotus ostreatus* was decreased to 0.13 cm day^{-1} at the mixture of 30 ppm in relation to the control 0.38 cm day^{-1} while its growth was not affected at 30ppm of metribuzin (0.38 cm day^{-1}) (Table 3.2).

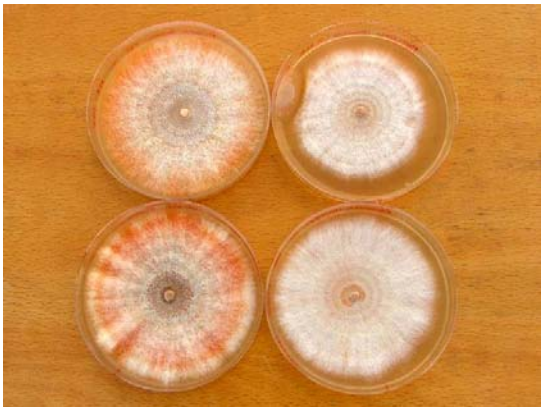


Plate 3.1 *Pycnoporus coccineus* growing on malt extract supplemented with the pesticides at 10ppm concentration.

Plate 3.2 *Pleurotus ostreatus* growing on MMY plus glucose supplemented with the pesticides at 10ppm concentration.

Table 3.1 Effect of culture medium in the presence either 10 ppm chlorpyrifos, metribuzin or linuron on growth rates (cm day^{-1}) on different nutrient media at 25^0C. They are the means of 3 replicates. The variances of all treatments were <5% of the means.
Key to media: MMY: Czapek salts modified medium, MMY plus glucose

Fungi	**Malt Broth**			
	Ctl	**Chlorpyrifos**	**Metribuzin**	**Linuron**
T. vercicolor	0,78	0,78	0,79	0,72
Ph. chrysosporium	1,55	1,42	1,57	1,35
P. ostreatus	0,69	0,52	0,68	0,33
P. gigantea	0,89	0,81	0,90	0,66
P. coccineus	0,60	0,43	0,61	0,53

Fungi	**MMY**			
	Ctl	**Chlorpyrifos**	**Metribuzin**	**Linuron**
T. vercicolor	0,77	0,52	0,70	0,78
Ph. chrysosporium	1,26	0,88	1,17	0,96
P. ostreatus	0,44	0,31	0,37	0,34
P. gigantea	0,53	0,47	0,57	0,38
P. coccineus	0,48	0,27	0,41	0,44

Fungi	**MMY plus Glucose**			
	Ctl	**Chlorpyrifos**	**Metribuzin**	**Linuron**
T. vercicolor	0,94	0,70	0,91	0,95
Ph. chrysosporium	1,59	1,65	1,77	1,57
P. ostreatus	0,61	0,28	0,61	0,57
P. gigantea	0,83	0,65	0,79	0,80
P. coccineus	0,58	0,43	0,59	0,57

Table 3.2 Growth rates (cm day^{-1}) (means ± standard deviations, n=3) in the presence 30ppm chlorpyrifos, metribuzin and linuron, individually and as mixture 15 and 30 ppm (total concentration) on a mineral salts medium at 25 ^{o}C. They are the means of 3 replicates. The variances of all treatments were <5% of the means.

	Treatments					
Fungi	**Ctl**	**Chlorpyrifos**	**Metribuzin**	**Linuron**	**mixture 15ppm**	**mixture 30ppm**
T. vercicolor	0,79	0,36	0,79	0,36	0,72	0,72
P.chrysosporium	1,09	0,08	0,15	0,04	0,03	0,09
P. ostreatus	0,38	0,34	0,38	0,14	0,18	0,13
P. gigantea	0,47	0,37	0,38	0,33	0,40	0,34
P. coccineus	0,53	0,28	0,43	0,55	0,48	0,45

Figure 3.2 shows the comparison of the relative percentage (%) inhibition between the five species examined. This shows clearly that *T. versicolor* and *P. coccineus* were least affected by mixtures of 30 ppm of the three pesticides. This was different from that observed for growth in the presence of individual pesticides. There were also marked differences between the ability of each species to tolerate the three different pesticides alone. Plate 3.3 shows the effect of mixtures of treatments on the growth of *T. versicolor* and the mycelial morphology.

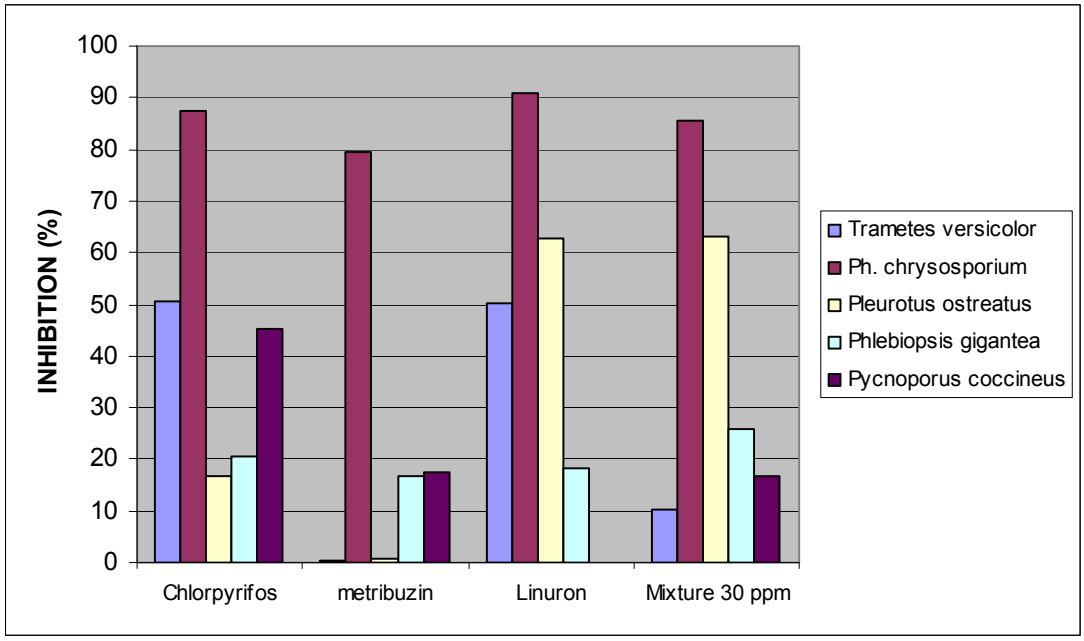


Figure 3.2 Mean growth inhibition (%) observed for five test isolates growing in a minimal medium supplemented with 30 ppm treatments (chlorpyrifos, metribuzin and linuron, individually and as a mixture). Studies were carried out on Czapek modified salts medium at 25^{o}C for a period of 15 days.

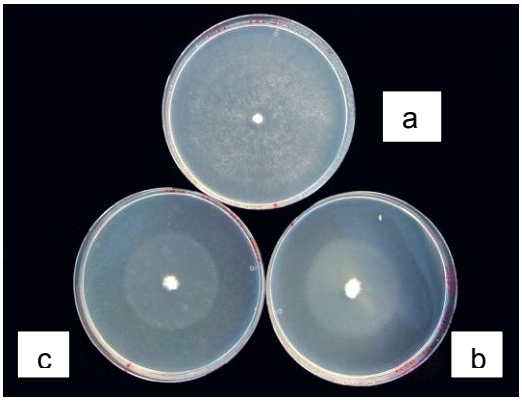


Plate 3.3 *T. versicolor* growing on MMY supplemented with mixtures of the pesticides chlorpyrifos, metribuzin and linuron at (a): 0 ppm, (b):15 ppm, (c): 30ppm.

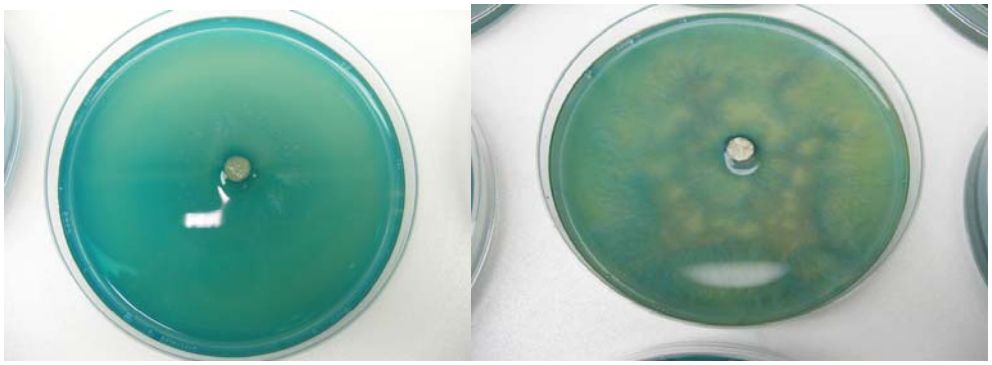
3.3 Production of lignolytic enzymes in Petri plate assays

The applicability of fungi in bioremediation of soil contaminated with pesticides depends on their capacity to grow efficiently in the presence of such compounds and their ability to produce degradative enzymes. This study was carried out to investigate the production of enzymes by the candidate fungi and the effect that pesticide treatments may have on this.

3.3.1 Potential ligninolytic activity

Phlebiopsis gigantea and *Pycnoporus coccineus* did not produce decolouration under the conditions of the assay, although these fungi grew well in the medium used in this assay.

Plate 3.4 shows the ligninolytic activity of *Trametes versicolor* expressed diffused spots so it was difficult to be quantified. This view was observed in all treatments. As conclusion, *T. versicolor* showed strong ligninolytic activity and it was not affected by pesticides concentrations and mixture of them.



A B

Plate 3.4 A positive result for plate assay to assess potential ligninolytic activity *T. versicolor* (B) in relation to the control (A).

3.3.2 Polyphenol oxidase production

The test fungi produced activity halos in all treatments (see Plates 3.5, 3.6) and showed no significant differences under different pesticide concentrations. Figure 3.3 shows polyphenol oxidase production, expressed as radius of enzymatic activity halo, by the three test fungi.

Trametes versicolor produced the highest enzymatic activity almost in all treatments. The presence of metribuzin did not affect the polyphenol oxidase production ($p=0.422$) while the presence of linuron or chlorpyrifos and mixture of the three pesticides decreased the enzymatic activity ($p<0.001$).

Phlebiopsis gigantea showed enzyme activity in the presence of the mixture and linuron treatments regardless of the pesticide concentration ($p=0.157$ and $p=0.163$, respectively). In the metribuzin and chlorpyrifos treatments the polyphenol oxidase production was not affected by the concentration of pesticides noticeably ($p=0.032$ and $p=0.018$, respectively).

Pycnoporus coccineus also produced high polyphenol oxidase activity. In chlorpyrifos, linuron and metribuzin treatments the enzyme activity was not affected by the concentration of pesticides ($p=0.77$, $p=0.2$ and $p=0.138$, respectively). In the mixture treatments there was a relative degrease ($p=0.036$).

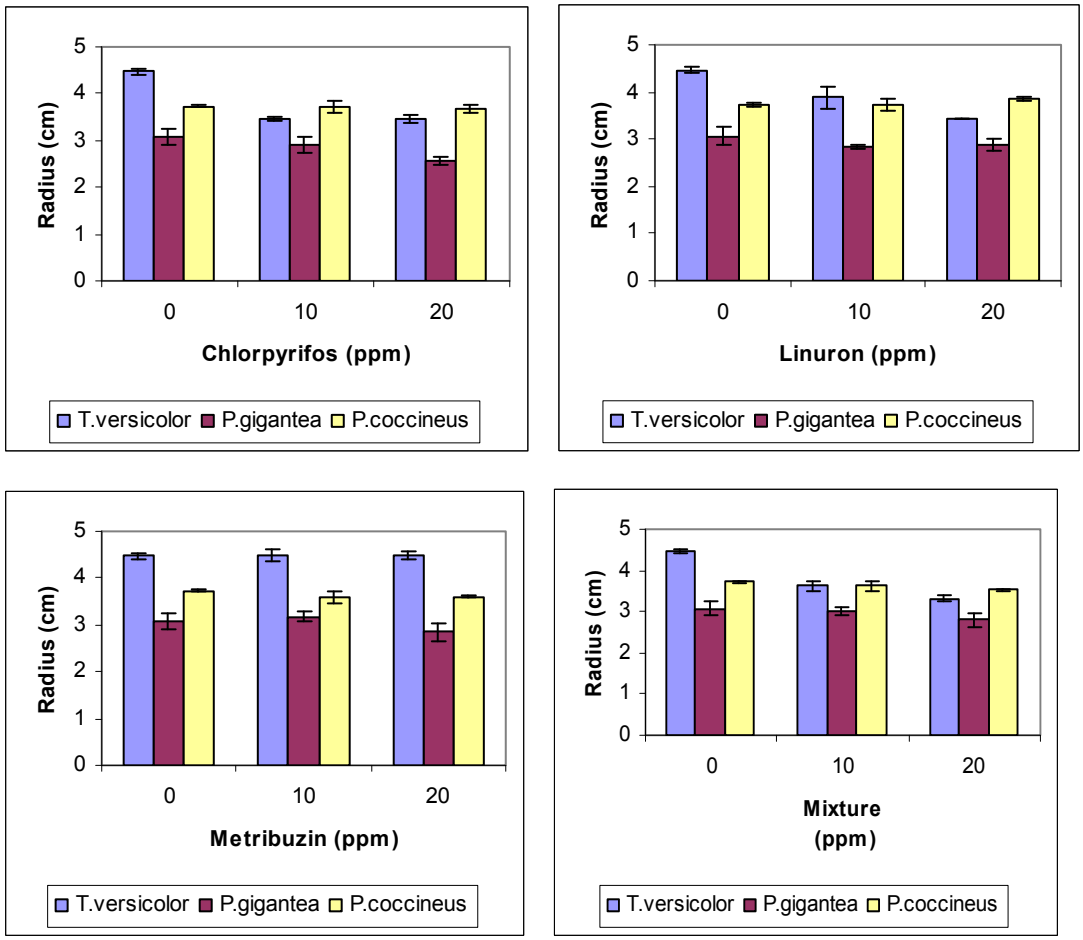


Figure 3.3 Effect of chlorpyrifos, linuron, metribuzin (0, 10 and 20 ppm) individually and as a mixture (0, 10 and 20 ppm) on polyphenol oxidase activity by *T. versicolor, P. gigantea* and *P. coccineus,* at 25^0C (expressed as radius of enzymatic activity halo ±standard deviation of the mean, n=3).

Plate 3.5 Laccase activity plate assay: Growth of *T. versicolor* in the presence of 20 ppm linuron.

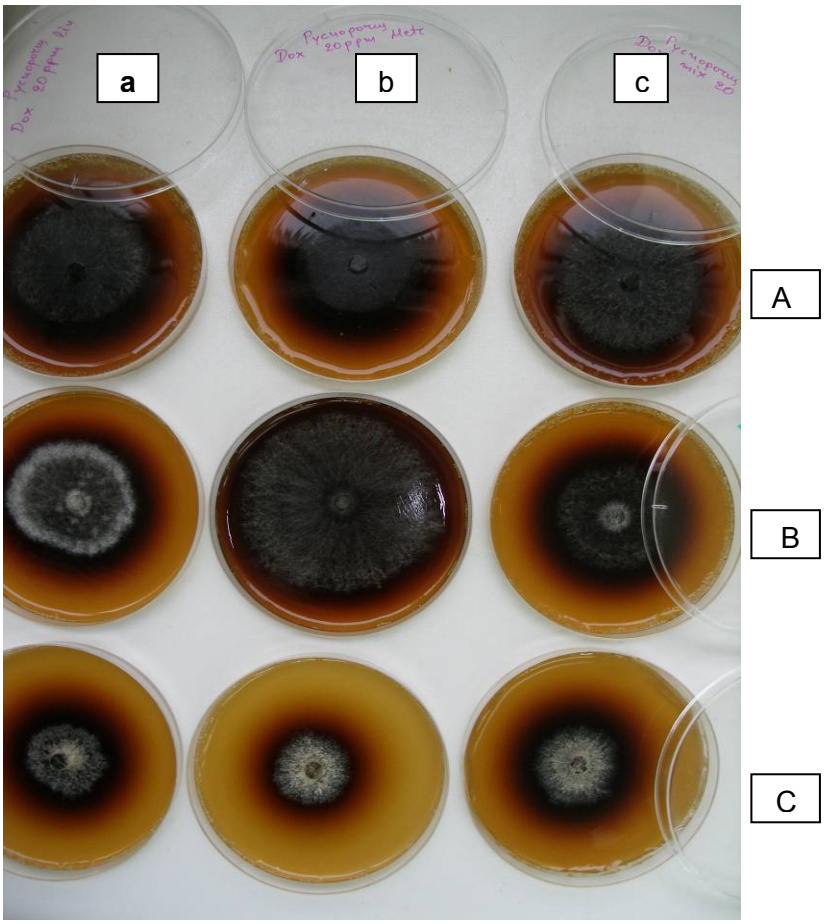


Plate 3.6 Laccase activity plate assay: Growth of *P. coccineus* (A) *T. versicolor* (B) and *P. gigantea* (C) in the presence of linuron (a), metribuzin (b) and a mixture of the pesticides chlorpyrifos, linuron and metribuzin at concentration of 20 ppm.

3.4 In vitro comparison of bacteria isolates for tolerance to pesticides

The growth of 19 strains of Bacillus sp on TSB supplemented with the pesticide chlorpyrifos was reduced as the concentrations were increased, and growth was completely inhibited at 120ppm (Table 3.3). Most of 19 strains had good growth up to the 60ppm of linuron. Above this concentration growth was completely inhibited (Table 3.4). Finally, most of the 19 strains of Bacillus sp. tolerated up to 120ppm of metribuzin. However, the pesticide metribuzin started showing inhibitory effects at 60ppm (Table 3.5). At 150ppm of this pesticide, growth was completely inhibited. Highlighted lines in tables 3.3, 3.4 and 3.5 show the stains that had the best growth.

Table 3.3 Growth of 19 strains Bacillus spp. as Optical Density (OD_{600}) at different concentrations of chlorpyrifos, after 24 hours incubation on TSB medium at 25^0C. N.G:= no growth.

Bacterial strains	Concentration of chlorpyrifos (ppm)					
	0	**30**	**60**	**90**	**120**	**150**
ctl	0	0.121	0.268	0.405	0.569	N.G
1	0.380	0.58	0.533	0.227	0.003	N.G
2	0.190	0.68	0.403	0.282	0.012	N.G
3	0.162	0.484	0.382	0.187	0.233	N.G
4	0.384	0.316	0.195	0.06	0.022	N.G
5	0.198	0.321	0.425	0.217	0.119	N.G
6	0.316	0.428	0.303	0.217	0.021	N.G
7	0.325	0.386	0.326	0.156	0.008	N.G
8	0.682	0.165	0.046	0.038	0.007	N.G
9	0.397	0.133	0.422	0.152	0.008	N.G
10	0.656	0.576	0.04	0.161	0.003	N.G
11	0.667	0.599	0.415	0.448	0.046	N.G
12	0.507	0.591	0.438	0.398	0.031	N.G
13	0.848	0.525	0.494	0.330	0.019	N.G
14	0.719	0.579	0.357	0.011	0.008	N.G
15	0.710	0.634	0.631	0.416	0.019	N.G
16	0.597	0.503	0.413	0.093	0.018	N.G
17	0.695	0.601	0.442	0.170	0.011	N.G
18	0.684	0.644	0.509	0.290	0.013	N.G
19	0.493	0.583	0.503	0.446	0.006	N.G

Table 3.4 Growth of 19 strains *Bacillus* spp. as Optical Density (OD_{600}) at different concentrations of linuron, after 24 hours incubation on TSB medium at 25^0C. N.G:= no growth

Bacterial strains	Concentration of linuron (ppm)					
	0	**30**	**60**	**90**	**120**	**150**
1	0.358	0.384	0.504	N.G	N.G	N.G
2	0.597	0.595	0.584	N.G	N.G	N.G
3	0.584	0.583	0.579	N.G	N.G	N.G
4	0.451	0.257	0.106	N.G	N.G	N.G
5	0.606	0.600	0.530	N.G	N.G	N.G
6	0.457	0.462	0.462	N.G	N.G	N.G
7	0.256	0.246	0.199	N.G	N.G	N.G
8	0.742	0.706	0.762	N.G	N.G	N.G
9	0.645	0.582	0.633	N.G	N.G	N.G
10	0.802	0.764	0.710	N.G	N.G	N.G
11	0.737	0.703	0.716	N.G	N.G	N.G
12	0.752	0.854	0.704	N.G	N.G	N.G
13	0.695	0.776	0.654	N.G	N.G	N.G
14	0.758	0.693	0.693	N.G	N.G	N.G
15	0.579	0.686	0.672	N.G	N.G	N.G
16	0.599	0.453	0.540	N.G	N.G	N.G
17	0.770	0.633	0.766	N.G	N.G	N.G
18	0.662	0.655	0.792	N.G	N.G	N.G
19	0.728	0.732	0.571	N.G	N.G	N.G

Table 3.5 Growth of 19 strains *Bacillus* spp. as Optical Density (OD_{600})* at different concentrations of metribuzin after 24 hours incubation on TSB medium at 25^0C. N.G:= no growth

Bacterial strains	Concentration of metribuzin (ppm)					
	0	**30**	**60**	**90**	**120**	**150**
1	0.380	0.405	0.569	0.236	0.001	0.007
2	0.190	0.270	0.617	0.308	0.043	N.G
3	0.162	0.221	0.427	0.225	0.027	N.G
4	0.384	0.275	0.084	0.003	0.274	N.G
5	0.198	0.198	0.299	0.589	0.536	N.G
6	0.316	0.406	0.438	0.367	0.162	N.G
7	0.325	0.349	0.267	0.083	0.006	N.G
8	0.682	0.528	0.542	0.454	0.384	0.085
9	0.397	0.403	0.356	0.115	0.621	N.G
10	0.656	0.735	0.639	0.305	0.127	0.005
11	0.667	0.676	0.631	0.600	0.395	N.G
12	0.507	0.545	0.635	0.480	0.390	N.G
13	0.848	0.630	0.594	0.586	0.563	0.026
14	0.719	0.667	0.636	0.300	0.048	N.G
15	0.710	0.625	0.775	0.608	0.275	N.G
16	0.597	0.605	0.568	0.258	0.123	N.G
17	0.695	0.640	0.576	0.693	0.335	N.G
18	0.684	0.493	0.565	0.519	0.302	N.G
19	0.493	0.699	0.652	0.495	0.181	N.G

3.5 In vitro comparison of bacterial strains for tolerance to a mixture of pesticides

In this study the *Bacillus* strains 6, 13, 15, 18 and 19 that had the best growth in the presence of high concentrations of the pesticides chlorpyrifos, linuron and metribuzin were tested for their capacity to tolerate mixtures of them at total concentrations of 30 and 60ppm. Some of the others also displayed very good growth but were excluded as their suspension was not homogeneous.

Values of OD_{600} after subtraction of the blank (soil extract + pesticides without bacteria) are shown in Figure 3.4. Strain 13 was not affected by the mixtures of 30 and 60 ppm (decrease of growth was 3 and 6%, respectively). Strains 15 and 19 had a reduction of 64 and 42%, respectively, at 60ppm mixture of the pesticides. Finally, strain 6 was very sensitive as its growth was completely inhibited.

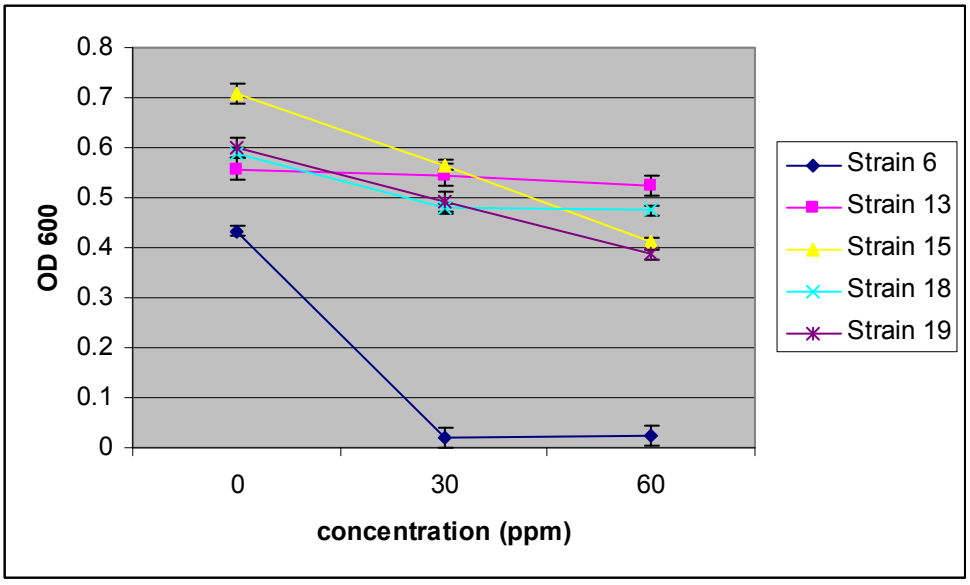


Figure 3.4 Growth (means ± standard deviations, n=3) of the best strains *Bacillus* spp. as Optical Density (OD_{600})in a mixture of 0, 30, 60 ppm of the three pesticides after 24 hours incubation in soil extract at 25^0C.

PART II STUDIES ON SOIL EXTRACT OF WHITE ROT FUNGI AND BACTERIA

3.6 Introduction

The objective of this study was to assess the growth of the best four fungi and five bacteria using soil extract as culture medium. It is a nutritionally weak medium to mimic the nutrient availability in soil. *Trametes versicolor, Pleurotus ostreatus, Phlebiopsis gigantea* and *Pycnoporus coccineus* were used as *P.chrysosporium* showed to be very sensitive to the mixture of the pesticides chlorpyrifos, linuron and metribuzin. High concentrations were used in order to calculate the effective doses (ED) for 50% inhibition of growth (EC_{50} values), relative to the controls for each fungus. The degradation of a mixture of different concentrations (0-50 ppm), also was examined, by HPLC quantification. Regarding bacteria, the growth of the best bacteria in soil extract in the presence of the mixture of pesticides was examined.

3.7 Growth of fungi in soil extract agar

Table 3.6 and 3.7 shows the growth of four fungi on the soil extract agar. There was significant decrease of the growth at 125 ppm concentration of chlorpyrifos for all tested fungi. On the contrary, at 125 ppm of metribuzin *T. versicolor* showed to be very tolerant with a EC_{50} value 292.2 ppm (Table 3.7). *P. gigantea and P. ostreatus* were very sensitive in this concentration, while *P. coccineus* had a decrease of growth almost 50%. In 125 ppm concentration of linuron growth was completely inhibited for all fungi.

In the mixtures the difference of growth was very noticeable. *P. ostreatus* had the lowest growth rate while *P. coccineus* showed to be more tolerant than all other fungi (Table 3.7). Overall, it is very clear that the relative growth rates

were affected in a different way depending on whether there was a single or mixture of pesticides.

Table 3.6 Growth rates (means ± standard deviations, n=3) expressed in cm day^{-1} for four fungal species incubated in soil extract agar supplemented with chlorpyrifos, linuron and metribuzin (0, 25,125 ppm) and as a mixture at interval of ten (0-70 ppm) at 25^0C. N.G:= no growth

		Growth rate (cm day^{-1})			
Pesticides	Concentration (ppm)	*T. versicolor*	*P. gigantea*	*P. ostreatus*	*P. coccineus*
	0	0.7±0.01	0.6±0.01	0.3±0.00	0.5±0.01
Chlorpyrifos	5	0.6±0.00	0.6±0.01	0.3±0.01	0.3±0.01
	25	0.5±0.00	0.5±0.02	0.2±0.01	0.2±0.01
	125	0.3±0.14	0.3±0.06	0.1±0.01	0.1±0.00
Linuron	5	0.7±0.00	0.6±0.01	0.3±0.03	0.5±0.01
	25	0.4±0.01	0.4±0.01	No Growth	0.4±0.00
	125	No Growth	No Growth	No Growth	No Growth
Metribuzin	5	0.7±0.02	0.6±0.01	0.3±0.00	0.4±0.00
	25	0.7±0.01	0.5±0.04	0.3±0.00	0.5±0.01
	125	0.5±0.07	No Growth	0.1±0.01	0.3±0.00
Mixture	10	0.6±0.02	0.6±0.02	0.3±0.01	0.5±0.01
	20	0.5±0.00	0.5±0.02	0.2±0.01	0.5±0.01
	30	0.5±0.01	0.5±0.00	0.1±0.01	0.4±0.02
	40	0.5±0.01	0.4±0.05	0.1±0.02	0.4±0.01
	50	0.3±0.02	0.3±0.03	No Growth	0.3±0.00
	60	0.3±0.02	0.4±0.02	No Growth	0.3±0.00
	70	0.3±0.03	0.4±0.01	No Growth	0.3±0.00

Table 3.7 Concentration (ppm) of chlorpyrifos, linuron and metribuzin individually and as a mixture that causes a 50% reduction in fungal growth (EC_{50} values) in four test fungi growing on soil extract agar.

Fungi	Pesticides			
	Chlorpyrifos	Linuron	Metribuzin	Mixture
	Concentration (ppm)			
T. versicolor	107.4	63.9	292.2	54.1
P. gigantea	106.2	62.6	95.2	51.7
P. coccineus	61.1	64.7	123.5	75.5
P. ostreatus	79.6	35.1	101.2	27.9

3.8 Growth of fungi in soil extract broth

In this study the fungi *T. versicolor*, *P. gigantea* and *P. coccineus* were incubated in Petri plates containing soil extract in order to have a first indication of their capability to degrade mixtures of the pesticides chlorpyrifos, linuron and metribuzin at total concentrations of mixture ranging from 10 to 50 ppm. The remaining concentration of the pesticides is presented as a percentage (%).

3.8.1 Pesticides analysis

Table 3.8 and Figure 3.5 show the impact of the fungi *T. versicolor*, *P. gigantea* and *P. coccineus* on degradation of the mixture of pesticides. In the treatments with chlorpyrifos there were differences in degradation rates between the fungi as well as the concentrations of pesticide. *P. coccineus* showed the highest disappearance rate at 10ppm, almost 60%. The Tukey's test showed two different subsets. The first subset included concentrations of 10, 40 and 50 ppm of chlorpyrifos, and the second the concentrations 20 and 30 ppm. The degradation rates by *T. versicolor* and *P. gigantea* were very

low at the 50 ppm chlorpyrifos. It is clear that these fungi are not able to degrade chlorpyrifos in high concentrations. Regarding metribuzin, all tested fungi were shown to be unable to degrade it, although they were tolerant and had quite high EC_{50} values for metribuzin.

In contrast, *T. versicolor* showed a great capacity to degrade linuron even in the 50 ppm concentration (Figure 3.5). The Tukey's test showed two different subsets. The first subset included 10 and 20 ppm of the mixture, and the second 30, 40 and 50 ppm. The degradation rates by *P. gigantea* were low, about 20%, in all treatments. Finally, *P. coccineus* was found to be unable to degrade linuron in all treatments ($p=0.01$).

Table 3.8 Chlorpyrifos and metribuzin remaining (%)(means ± standard deviations, n=3) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 21 days of incubation in soil extract at 25 ^{0} C.

Pesticides	Initial concentration of mixture (ppm)	Remaining pesticide (%)		
		T. versicolor	*P. gigantea*	*P. coccineus*
Chlorpyrifos	10	14.3±8.7	39.5±13.7	41.3±7.8
	20	37.4±9.9	52.3±7.8	59.4±5.0
	30	78.3±10.7	69.1±1.6	59.2±1.3
	40	84.6±0.8	75.9±2.5	45.1±7.3
	50	94.2±5.1	86.6±4.6	52.3±3.9
Metribuzin	10	62.8±10.2	83.5±2.0	57.7±3.4
	20	83.2±3.0	99.5±0.9	82.3±6.0
	30	80.1±9.21	100±0.0	84.1±1.8
	40	84.1±2.26	100±0.0	92.5±1.5
	50	100±0.0	100±0.0	91.4±3.3

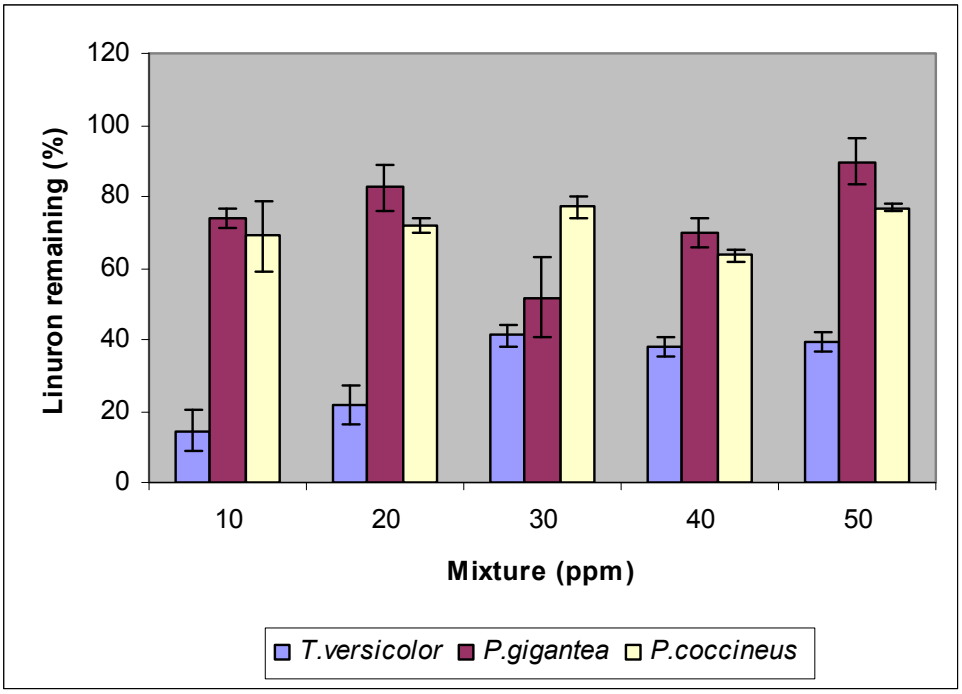


Figure 3.5 Linuron remaining (%) (means ± standard deviations, n=3) in soil extract that was initially supplemented with a mixture of pesticides at total concentrations 10, 20, 30, 40 and 50 ppm, after 21 days of incubation in soil extract at 25 ^{0} C.

3.9 Pesticides degradation by bacterial strains

3.9.1 Bacterial growth in soil extract liquid broth with the presence of mixture of pesticides

The growth of the strains *Pseudomonas putida* and *Pseudomonas fluorescens* Pfl2, Pfl804 and Pfl545 was very good, as also the mixture of all microorganisms while the strains of *Bacillus* showed a very small growth in the presence of the mixture of the pesticides at 60 ppm concentration (Figure 3.6).

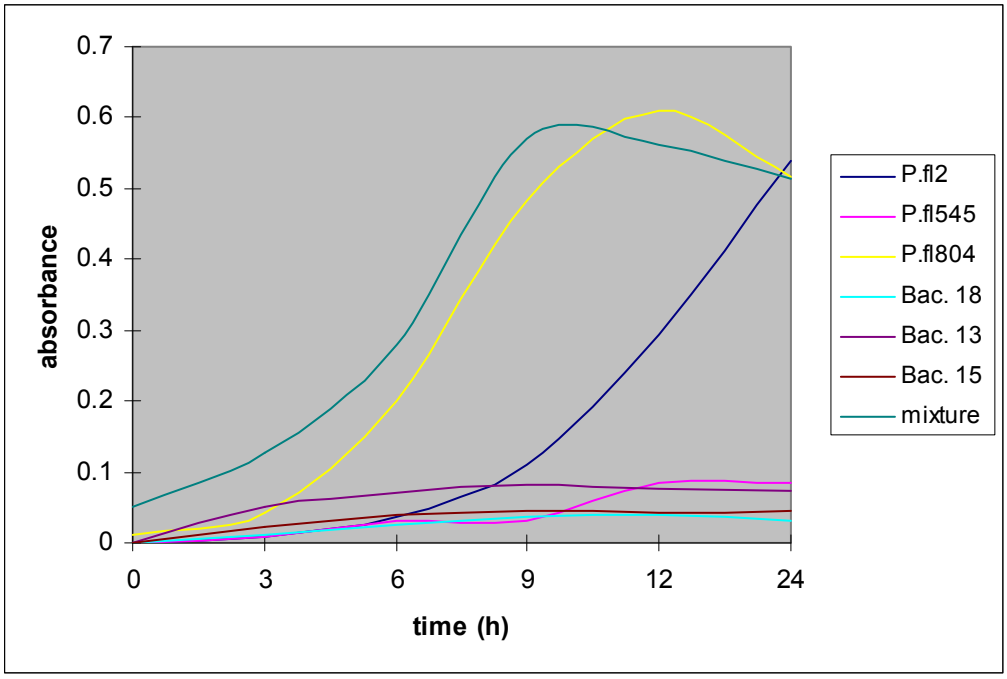


Figure 3.6 Growth of a range of single bacterial strains and a mixture of them as Optical Density at 600 nm in soil extract broth supplemented with a mixture of 60 ppm (total concentration) of the pesticides, at 25 ^{0}C.

3.9.2 Quantification of pesticides

Unfortunately, the bacterial strains and the mixture of them did not degrade any of the pesticides linuron and metribuzin that were used with chlorpyrifos in a mixture of total concentration of 60 ppm. Figures 3.7 and 3.8 show that neither linuron nor metribuzin were degraded by *Pseudomonas putida* although its growth in the mixture was very good and Figure 3.9 shows the concentration of all the other tested strains.

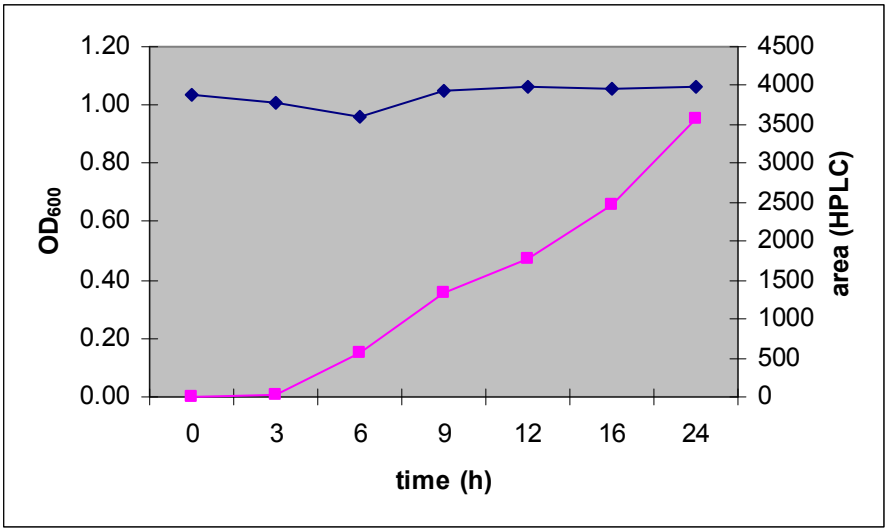


Figure 3.7 The growth of *P. putida* over a 24 hours period as Optical Density at 600nm(blue line) and linuron concentration at the same period in soil extract broth supplemented with a mixture 60 ppm of the pesticides at 25^0C.

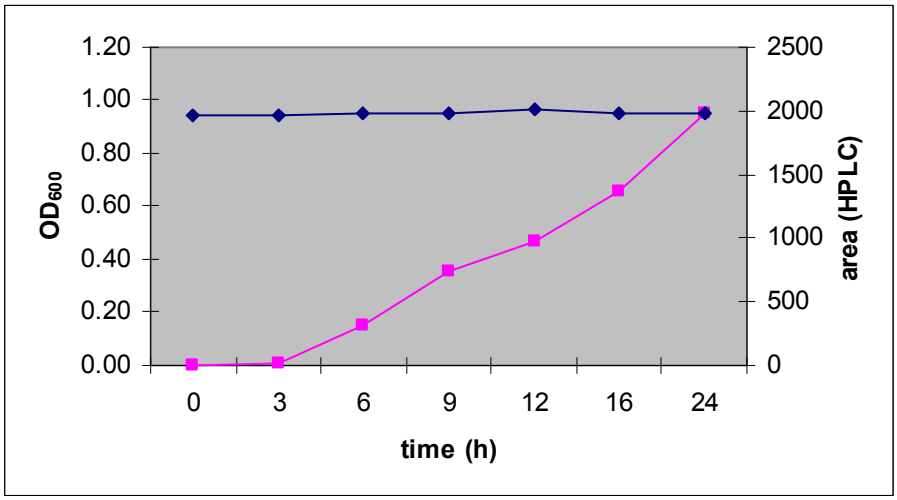


Figure 3.8 The growth of *P. putida* over a 24 hours period as Optical Density at 600 nm (blue line) and metribuzin concentration at the same period in soil extract broth supplemented with a mixture 60 ppm of the pesticides at 25^0C.

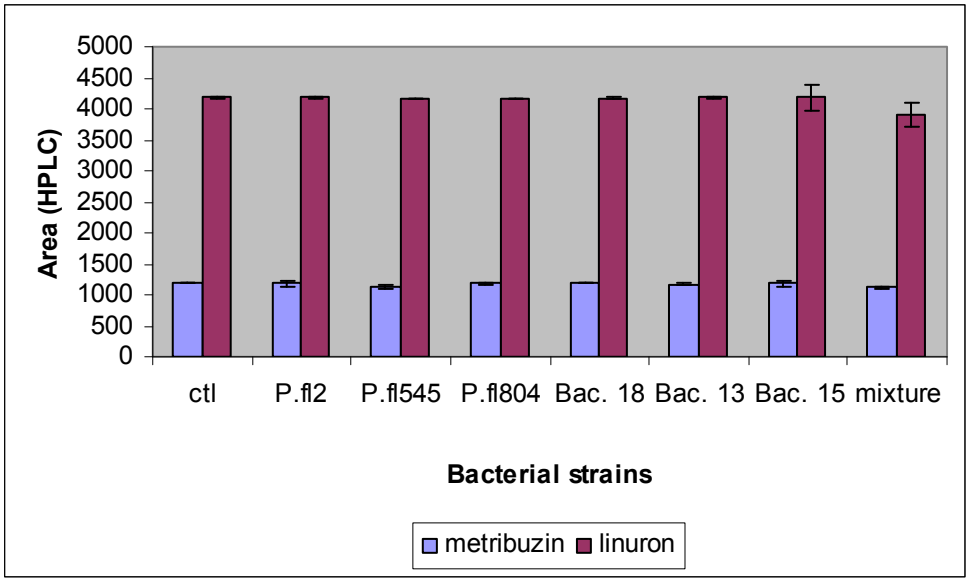


Figure 3.9 Concentrations of pesticides remaining (expressed as area by HPLC), after 24 hours incubation with bacterial strains in soil extract broth at 25 ^{0}C. Bars represent the standard deviation of the mean (n=3), per treatment.

3.10 Introduction

In this section, the capacity of *Trametes versicolor* to grow under water stress and degrade mixtures of pesticides was evaluated in order to assess its potential use as bioremediation agent. The main objectives of this study were to examine the interactions and activity of this fungus in relation to: (a) temporal degradation of a mixture of the pesticides chlorpyrifos, linuron and metribuzin at total concentrations of 0, 10, 20, 30, 40, 50 ppm (b) temporal evaluation of fungal laccase activity, over a six week period, in the soil extract liquid broth where solute potential was adjusted in two different ways: ionically by adding potassium chloride and non-ionically by using glycerol.

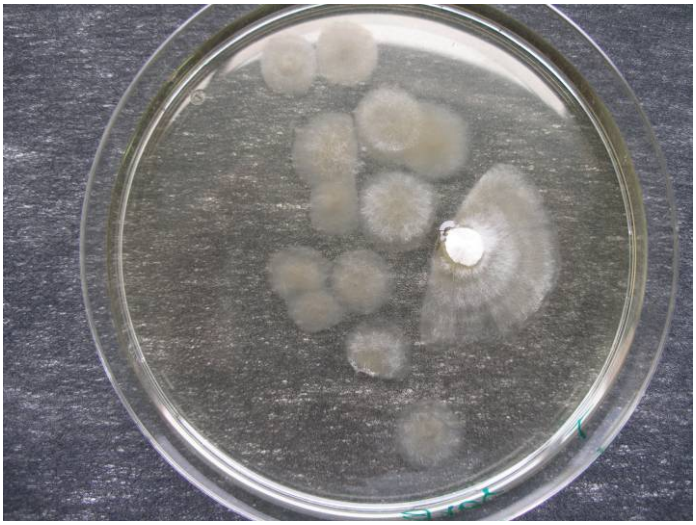


Plate 3.7 *T. versicolor* growing in soil extract liquid broth plus glycerol (-2.8 MPa) at 25^0C, seven days after inoculation.

3.11 Temporal evaluation of laccase activity

As fungal laccase has shown to be involved in biodegradation of a broad range of pollutants, the production of this enzyme is very pertinent for evaluation of the capacity for bioremediation.

Over a six week period, laccase production was determined in three different water regimes and was related to the pesticides chlorpyrifos, linuron and metribuzin remaining %, quantificated by HPLC, in order to investigate the rate of laccase production and to be estimated their possible correlation.

3.11.1 Soluble protein

The quantification of soluble protein content was essential to estimate the specific laccase activity. Moreover, this parameter can also be used as additional information on fungal activity.

The levels of extracellular protein in both cases were not high. The production of soluble protein in soil extract was not affected by pesticides concentrations and water regimes. Analysis of variance of each concentration of the mixture per week was performed and there was not significant difference. Analysis of variance of protein with two factors (weeks x water regimes) was performed in order to compare soluble protein at each concentration at the two water regimes (non-stress and non-ionic stress treatments). The results showed that there was no significant difference (see Appendix C).

The effect of mixtures of pesticides on soluble protein content in soil extract inoculated with *T. versicolor* every week over a six week period under two water regimes is shown in Figure 3.10 (without water stress) and Figure 3.11 (with -2.8 MPa water stress using glycerol).

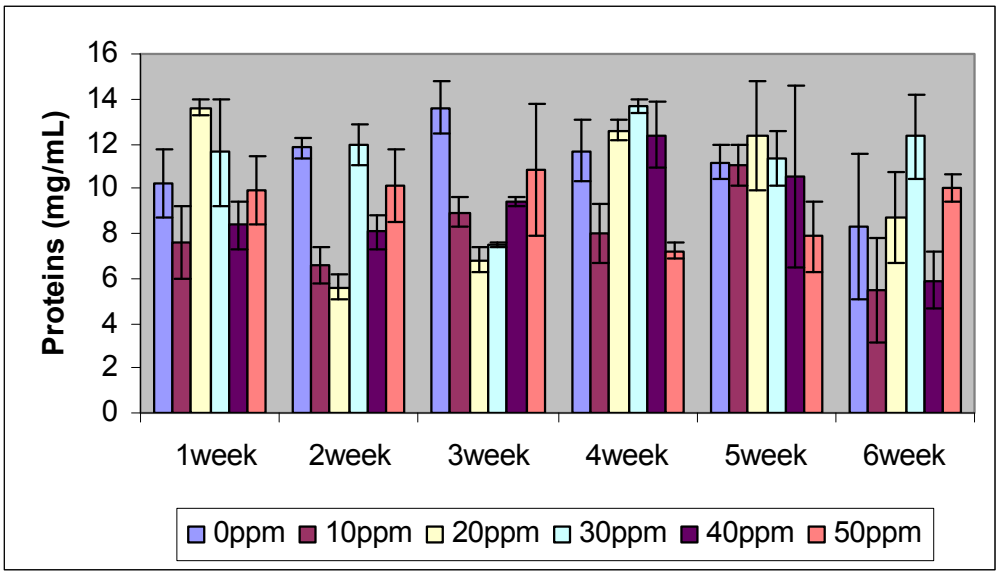


Figure 3.10 Protein concentration of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

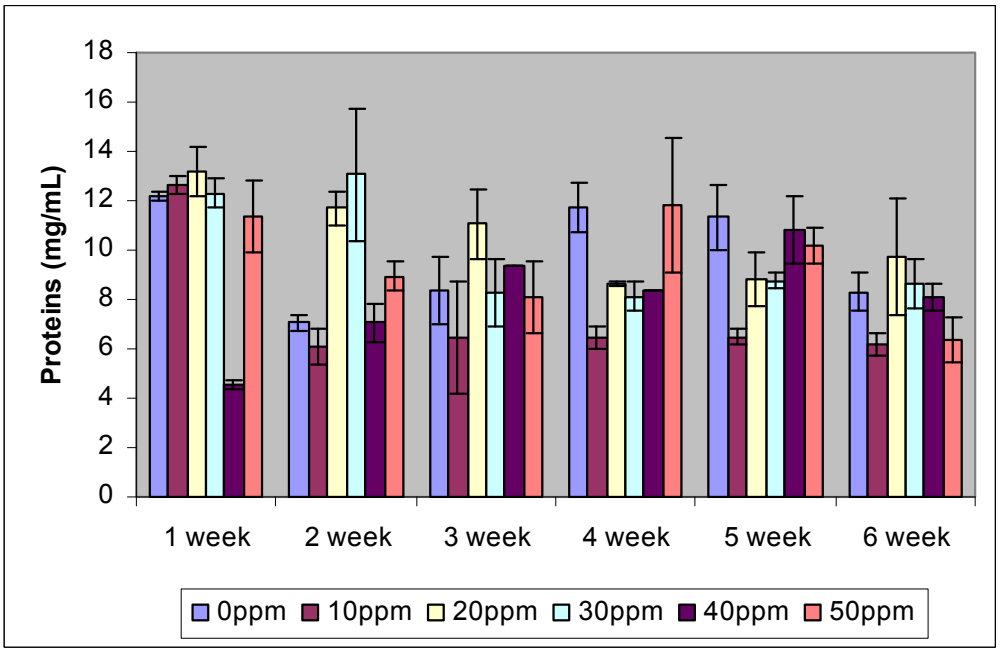


Figure 3.11 Protein concentration of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) at water potential -2.8MPa (with glycerol) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

3.11.2 Specific activity

Specific activity of laccase is the number of enzyme units per ml divided by the concentration of soluble protein in mg mL^{-1}. Specific activity values are therefore quoted as units mg^{-1}. Specific activity is of no relevance as far as setting assays is concerned, although it is an important measurement of enzyme purity and quality.

Having determined laccase units (see Appendix B) and soluble protein the specific activity was calculated as the ratio of laccase units to soluble protein the soil extract treatments and these with soil extract and glycerol. The KCl filtrates did not produce laccase in any of the treatments.

The levels of laccase produced by *T. versicolor* in soil extract broth without stress were quite high. Laccase production was shown to be induced by the pesticides mixture as at 0 ppm treatment was lower in relation to all the other treatments with the pesticides until the sixth week (Figure 3.12).

Interestingly, very high levels of laccase were produced by *T. versicolor* in soil extract broth solute potential adjusted at -2.8 MPa non-ionically by using glycerol. Laccase production was the maximum at the second and third week after the inoculation (Figure 3.13).

Statistical analysis of variance was performed for each pesticide concentration in the same water regime per week with theTukey's test. The results are shown in Tables 3.9 and 3.10. Statistically significant differences among every concentration in the same water regime per week are expressed by different letters (see Appendix D). Moreover, analysis of variance with two factors (weeks x water regimes) was performed in order to compare the specific activity of laccase at each concentration at the two water regimes (non-stress and non-ionic stress treatments). The results showed that there was significant ordered interaction between the two factors and specific activity of laccase was always the best at non ionic stress

treatments (glycerol) (see Appendix E). Finally, there was a negative correlation (R=-0.46) between specific activity and remaining linuron %, only at the fifth week and at -2.8 MPa water potential using glycerol.

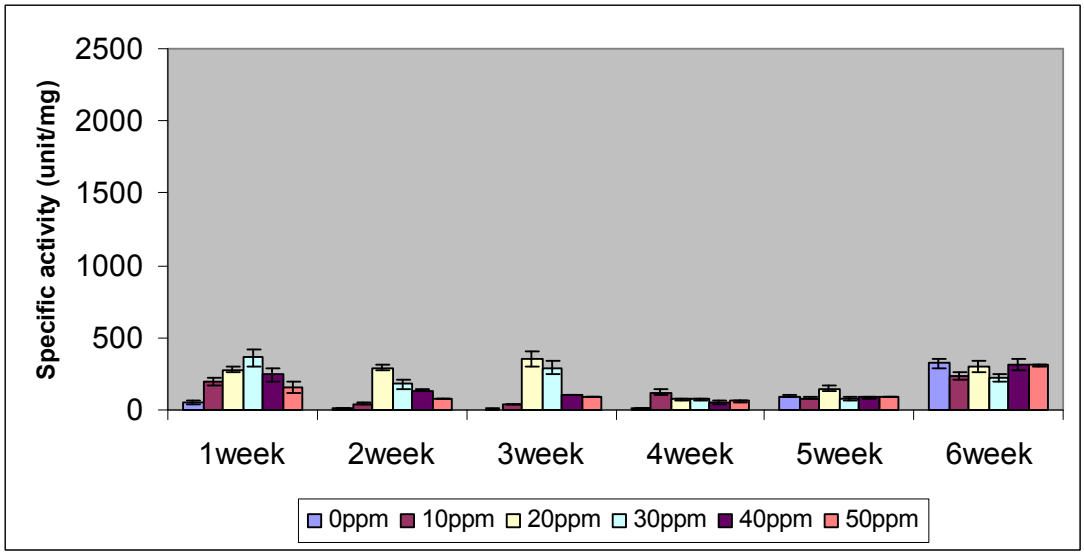


Figure 3.12 Specific activity of laccase of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

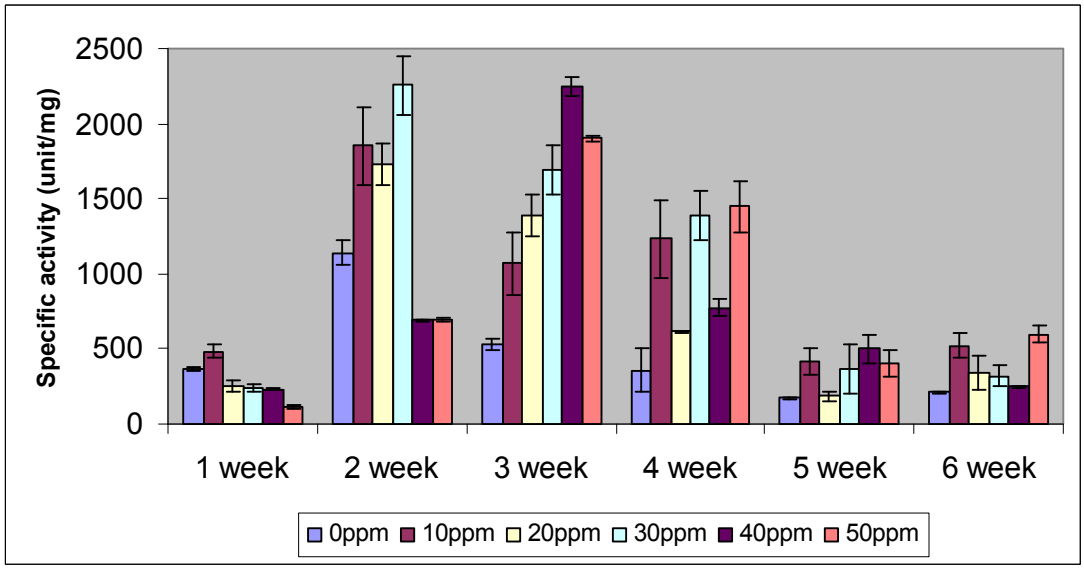


Figure 3.13 Specific activity of laccase of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 10, 20, 30, 40, 50 ppm) at water potential -2.8MPa (with glycerol) over a six weeks period, at 25^0C. Bars represent the standard error (n=3).

Table 3.9 Statistically significant differences of specific activity of laccase among every concentration of mixture per week, in soil extract broth without stress, expressed by different letters.

Weeks	Initial concentratiion of the mixture (ppm)					
	0	**10**	**20**	**30**	**40**	**50**
1	ab	bc	ab	c	a	a
2	a	a	ab	ab	a	a
3	a	a	b	bc	a	a
4	ab	ab	a	a	a	a
5	b	a	a	a	a	a
6	c	c	ab	abc	a	b
	p<0.001	p<0.001	p=0.016	p=0.001	p=0.233	p=0.001

Table 3.10 Statistically significant differences of specific activity of laccase among every concentration of mixture per week, at -2.8 MPa by adding glycerol, expressed by different letters.

Weeks	Initial concentratiion of the mixture (ppm)					
	0	**10**	**20**	**30**	**40**	**50**
1	ab	a	a	a	a	a
2	b	b	b	b	ab	ab
3	ab	ab	b	b	c	c
4	ab	ab	a	ab	b	c
5	a	a	a	a	ab	ab
6	b	a	a	a	ab	ab
	p=0.012	p=0.002	p<0.001	p<0.001	p<0.001	p=0.002

3.12 Total ligninolytic activity

The ability of white rot fungi to degrade contaminants has been linked to its wood degrading capabilities; therefore a strong ligninolytic activity could be advantageous in pesticide degradation.

The total ligninolytic activity of *T. versicolor* was examined in this study, under the different water regimes. The assay used is based on the quantification of the extent of decolouration of a polymeric dye (Poly R-478). The lower the ratio absorbance at 530 nm / absorbance at 350 nm the more intense is the decolouration, i.e. the higher was total ligninolytic activity.

The filtrates of the treatments 0, 30, 50 ppm at the first, fourth and fifth week under the three water regimes were tested. Overall, all treatments caused decolourazion of the polymeric dye (Figure 3.14). Table 3.11 shows the ratio absorbance at 530 nm/ absorbance at 350 nm in all treatments. The total ligninolytic activity was not affected by water availability or pesticide treatments. The latter suggest the *T.versicolor* is tolerant to the mixtures of pesticides, producing equivalents level of decolouration in the presence and absence of the xenobiotics.

Table 3.11 Total ligninolytic activity of *T. versicolor* (expressed as the ratio absorbance at 530 nm / absorbance at 350 nm ± standard deviation of the mean) in soil extract liquid broth, under three different water potential regimes: without stress, with -2.8 MPa water potential using glycerol and, with -2.8 MPa water pontential using KCl.

Water regime	Concentration (ppm)	1 week	4 week	5 week
without stress	0	0.27 ±0.03	0.27±0.01	0.12±0.00
	30	0.34±0.03	0.19±0.00	0.18±0.00
	50	0.36±0.07	0.18±0.01	0.28±0.00
Water stress with glycerol	0	0.22±0.03	0.16±0.03	0.19±0.01
	30	0.28±0.01	0.26±0.02	0.23±0.02
	50	0.27±0.01	0.27±0.02	0.27±0.01
Water stress with KCl	0	0.22±0.01	0.18±0.01	0.16±0.00
	30	0.24±0.01	0.18±0.02	0.16±0.01
	50	0.24±0.03	0.17±0.01	0.17±0.03

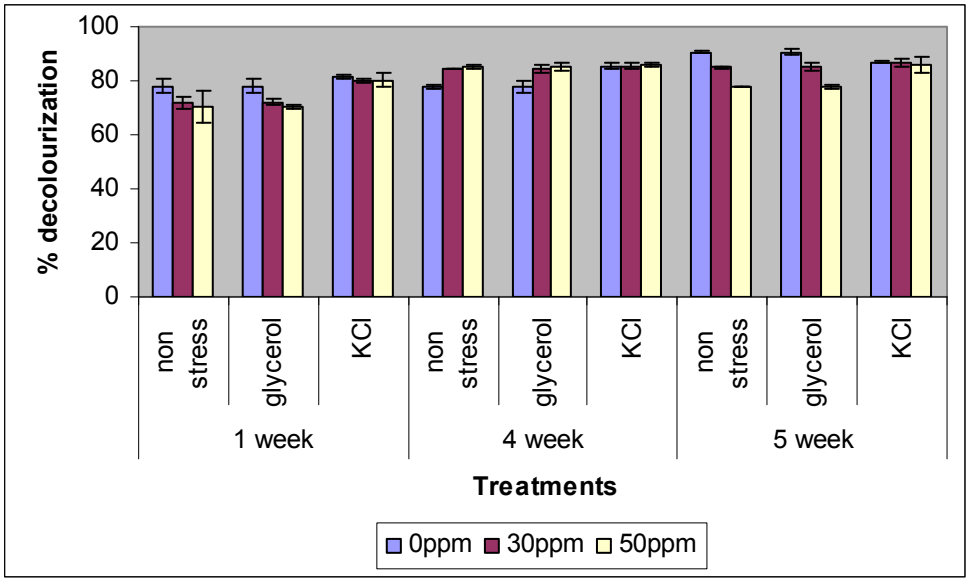


Figure 3.14 Total ligninolytic activity of *T. versicolor* (expressed as % decolouration of Poly R-478) in soil extract liquid broth, under three different water potential regimes: without stress, with -2.8 MPa water stress using glycerol and with -2.8 MPa water stress using KCl. Bars represent the standard deviation of the mean (n=3), per treatment.

3.13 Temporal evaluation of degradation of mixtures of pesticides

In order to assess the capacity of *T. versicolor* to grow under water stress and degrade mixtures of the pesticides chlorpyrifos, linuron and metribuzin their degradation rates were monitored after the first, third and fifth week. Surprisingly, chlorpyrifos was not detected in any of the treatments although it was detected in the previous study (Table 3.8). The possible explanation for this fact was investigated and is mentioned in the next section.

Uninoculated plates in triplicate were prepared for treatments in order to investigate the degradation rates of pesticides due to abiotic factors. These values are presented in red (ctl) in all the Tables.

Tables 3.12 to 3.17 show the impact of *T. versicolor* on degradation of the linuron and metribuzin in soil extract liquid broth and in soil extract liquid broth under water stress modified ionically by adding potassium chloride and non-ionically using glycerol.

Linuron degradation due to abiotic factors was quite high by the fifth week, with almost 50% in relation to the initial concentration, while metribuzin degradation was significant lower ranging from 2% to 21% in all treatments.

Linuron and metribuzin degradation in the first week was very low in all treatments and this was probably related to the slow initial growth of *T. versicolor*.

By the 3rd week higher degradation rates were evident in the treatments of linuron at both water regimes in relation to the controls and regardless of the pesticide concentrations.

Contrary to linuron, in the third week, metribuzin continued to be resistant to degradation in soil extract as well as in both water regimes (with glycerol and KCl). Moreover, in 40ppm concentration of metribuzin in soil extract there was not statistical significant difference between the controls and concentrations (p=0.281) (Table 3.19) (see Appendix F). Soil extract solute potential adjusted non-ionically by using glycerol showed a relative good degradation of metribuzin until the concentration 30ppm. Particularly, metribuzin did not detect in 10ppm concentration of the mixture while the remaining of metribuzin at 20 and 30 ppm of mixture was 40 and 65 %, respectively (Table3.16). In the concentration 40 ppm of the mixture there was not statistical significant difference in all treatments (p=0.169).

In the fifth week, it is very clear that in the inoculated plates with *T. versicolor*, linuron remaining was too low and in many cases the degradation was complete while in all controls the remaining of linuron was approximately 50% (Tables 3.12, 3.13 and 3.14).

Finally, in the fifth week and at -2.8 MPa water stress using glycerol, the percentage of metribuzin degradation reached to 40 % in the concentrations 40 and 50 ppm of the pesticides and it was highest in the concentrations 10, 20, 30 ppm of them 100, 84, 90%, respectively (Table 3.16).

The differences of degradation rates of the pesticides among all treatments for every week are presented in Tables 3.18 and 3.19.

Table 3.12 Concentration of linuron remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C. The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) linuron remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	86.4±0.8	76.6±6.9	52.3±0.9	13.8±6.9	53.9±5.0	0.00
20	86.1±1.5	84.4±3.3	67.2±0.9	18.2±9.2	55.6±0.2	0.00
30	82.2±2.8	84.4±3.3	61.1±5.1	33.2±7.0	56.6±4.4	4.6±2.3
40	87.1±1.5	81.5±0.0	84.7±1.5	8.9±1.1	70.3±0.8	3.3±1.7
50	80.2±0.4	79.2±0.0	69.9±1.6	23.4±3.9	57.9±0.8	0.00

Table 3.13 Concentration of linuron remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of glycerol ((water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) linuron remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	86.4±0.8	76.6±6.9	52.3±0.9	13.8±6.9	53.9±5.0	0.0
20	86.1±1.5	84.4±3.3	67.2±1.0	18.2±9.2	55.6±0.2	0.0
30	82.2±2.8	84.4±3.3	61.1±5.1	33.2±7.0	56.6±4.4	4.6±2.3
40	87.1±1.5	81.5±0.0	84.7±1.5	9.0±1.1	70.3±0.8	3.3±1.7
50	80.2±0.4	79.2±0.0	69.9±1.6	23.4±3.9	57.9±0.8	0.0

Table 3.14 Concentration of linuron remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of KCl (water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage(%) linuron remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	80.1±0.2	83.1±1.8	78.4±0.4	5.5±5.5	77.6±0.3	0.00
20	85.1±0.5	95.9±1.5	46.0±8.2	26.8±5.0	45.9±8.1	8.4±4.3
30	85.1±0.5	95.9±1.5	52.7±1.7	9.2±1.7	52.9±1.5	8.1±0.6
40	96.3±1.3	94.8±2.0	72.6±2.2	27.7±4.0	68.7±1.1	7.3±5.8
50	94.4±2.1	98.3±0.1	63.5±1.9	9.2±6.2	60.0±0.6	6.8±6.8

Table 3.15 Concentration of metribuzin remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^{0}C after 1, 3 and 5 weeks of incubation at 25^{0}C. The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) metribuzin remaining					
	Incubation at 25^{0}C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	77.5±0.4	77.2±1.5	79.0±1.4	70.2±6.0	80.9±2.1	69.8±1.7
20	92.3±1.5	94.4±0.4	92.1±1.1	90.0±0.8	90.5±1.7	92.9±2.8
30	89.9±0.5	90.7±0.4	88.8±2.4	82.2±0.7	78.9±3.6	92.5±4.1
40	98.6±0.6	99.2±0.7	94.5±0.3	89.2±3.2	96.5±2.6	87.2±1.8
50	91.3±2.0	94.5±1.3	91.4±0.6	80.1±3.2	86.0±4.7	69.6±6.7

Table 3.16 Concentration of metribuzin remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm, after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of glycerol ((water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage (%) metribuzin remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
10	91.2±0.5	82.2±1.4	88.2±1.6	0	85.7±2.4	0
20	93.9±1.7	99.9±0.2	94.0±1.6	40.3±5.8	93.5±1.0	16.4±2.3
30	79.8±3.8	96.9±1.6	78.2±1.5	65.1±1.9	80.1±3.9	10.3±2.8
40	99.6±0.1	98.7±0.7	99.5±0.1	93.5±3.8	98.6±0.5	60.2±6.1
50	90.8±1.9	92.6±1.5	91.3±0.2	82.9±5.9	91.2±0.2	62.±4.3

Table 3.17 Concentration of metribuzin remaining (%±SE) in soil extract that was initially supplemented with a mixture of pesticides at 10, 20, 30, 40 and 50 ppm (total concentrations), after 1, 3 and 5 weeks of incubation at 25^0C, under the impact of KCl (water potential=-2.8 MPa). The values in red correspond to the concentrations remaining in the uninoculated plates.

	Percentage(%) metribuzin remaining					
	Incubation at 25^0C					
	1week		**3week**		**5week**	
c (ppm)	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates	Ctl plates	Inoculated plates
3,3	78.1±3.2	82.9±0.7	72.8±5.1	67.7±4.6	69.3±1.9	71.7±3.6
6,6	93.7±5.1	90.2±1.1	88.8±5.0	84.2±6.1	94.0±3.0	90.0±6.2
10	97.2±2.1	96.0±2.4	97.7±2.2	88.3±2.8	96.8±2.0	91.8±3.9
13,3	97.6±1.3	97.4±0.3	97.5±0.4	89.4±4.1	96.8±1.5	84.1±2.0
16,6	94.0±0.1	94.6±0.6	95.5±1.0	97.6±2.0	92.±0.7	93.2±4.1

Statistical analysis

For statistical analysis, the data (% remaining pesticide) were transformed, before statistical analysis, using the following equation: $y=\arcsin\sqrt{p}$ and were back transformed for presentation.

In order to investigate the abiotic degradation of the pesticides over the six weeks period of incubation, plates, in triplicate, with the mixtures of pesticides without inoculation with *T. versicolor* and were used as controls.

Data for statistical analysis were tabulated as follows:

Treatments	Initial concentratiion of the mixture				
	10	20	30	40	50
(1) ctl non stress					
(2) Inoculated plates, non stress					
(3) ctl glycerol					
(4) Inoculated plates, plus glycerol					
(5) ctl KCl					
(6) Inoculated plates, plus KCl					

The statistical analysis showed that the interaction between pesticides concentrations per weeks was highly significant ($p<0.001$) for all treatments so separate tests were performed for each pesticide concentration in the same water regime per week with theTukey's test. The results are shown in Tables 3.18 and 3.19. Statistically significant differences among every concentration in the same water regime per week are expressed by different letters (see Appendix E).

Moreover, data obtained at the 5^{th} week were used for analysis of variance with two factors (concentrations x treatments) in order to compare the means of concentrations in the three water regimes (non-stress, ionic and non-ionic stress treatments).

For linuron, the interaction of two factors (concentrations x treatments) was significant ($p=0.002$). The interaction of different concentrations was also significant ($p=0.034$) but theTukey's test showed that the only difference was between the concentrations 6.6 and 13.3 ppm of linuron (20 and 40 ppm of the mixture). The interaction of treatments (non-stressed, non-ionic, ionic) was significant ($p <0.001$). The difference concerned controls and the inoculated plates.

For metribuzin the interaction of two factors (concentrations x treatments) was significant ($p<0.001$). The interaction of different concentrations was also

significant ($p<0.001$). TheTukey's test showed three subsets: the first concerned the concentration 3.3 ppm of metribuzin (10 ppm of the mixture), the second the concentration 10 ppm of metribuzin and the third the concentrations 6.6, 13.3 and 16.6 ppm of metribuzin. The interaction of treatments (non-stressed, non-ionic and ionic) was, also, significant ($p <0.001$). The difference was between glycerol treatments and all the others.

Table 3.18 Statistically significant differences of degradation rates of linuron among every concentration of mixture per week, in the same water regime, expressed by different letters.

Weeks	**Treatments**	**Initial concentratiion of the mixture (ppm)**				
		10	**20**	**30**	**40**	**50**
first	ctl non stress	b	b	d	c	b
	Inoculated plates, non stress	b	b	d	c	b
	ctl glycerol	b	b	d	b	c
	Inoculated plates, plus glycerol	b	c	c	b	c
	ctl KCl	a	a	b	a	a
	Inoculated plates, plus KCl	a	a	a	a	ab
third	ctl non stress	b	c	d	d	b
	Inoculated plates, non stress	a	ab	c	a	a
	ctl glycerol	b	bc	d	cd	b
	Inoculated plates, plus glycerol	a	b	b	b	a
	ctl KCl	b	bc	d	c	b
	Inoculated plates, plus KCl	a	a	a	a	a
fifth	ctl non stress	b	b	c	b	b
	Inoculated plates, non stress	a	a	ab	a	a
	ctl glycerol	c	b	c	b	b
	Inoculated plates, plus glycerol	a	a	b	a	a
	ctl KCl	b	b	c	b	b
	Inoculated plates, plus KCl	a	a	a	a	a

Table 3.19 Statistically significant differences of degradation rates of metribuzin among every concentration of mixture per week, in the same water regime, expressed by different letters.

Weeks	Treatments	Initial concentratiion of the mixture (ppm)				
		10	20	30	40	50
first	ctl non stress	a	a	ab	a	a
	Inoculated plates, non stress	a	ab	ab	a	a
	ctl glycerol	b	ab	a	a	a
	Inoculated plates, plus glycerol	a	b	b	a	a
	ctl KCl	a	ab	b	a	a
	Inoculated plates, plus KCl	a	a	b	a	a
third	ctl non stress	bc	b	b	a	abc
	Inoculated plates, non stress	b	b	ab	a	a
	ctl glycerol	c	b	ab	a	abc
	Inoculated plates, plus glycerol	a	a	a	a	ab
	ctl KCl	bc	b	c	a	bc
	Inoculated plates, plus KCl	b	b	b	a	c
fifth	ctl non stress	cd	b	b	bc	bc
	Inoculated plates, non stress	b	b	b	bc	ab
	ctl glycerol	d	b	b	c	c
	Inoculated plates, plus glycerol	a	a	a	a	a
	ctl KCl	b	b	b	c	c
	Inoculated plates, plus KCl	bc	b	b	b	c

Figures 3.15 and 3.16 show the remaining linuron and metribuzin concentrations, as percentage (%), respectively, after five weeks incubation. It is very clear that *T. vesicolor* was able to degrade linuron at all water regimes while at controls a concentration almost 50 % from the initial was remaining in the soil extract broth. In contrast metribuzin degradation was observed only at -2.8 MPa water potential adjusted with glycerol.

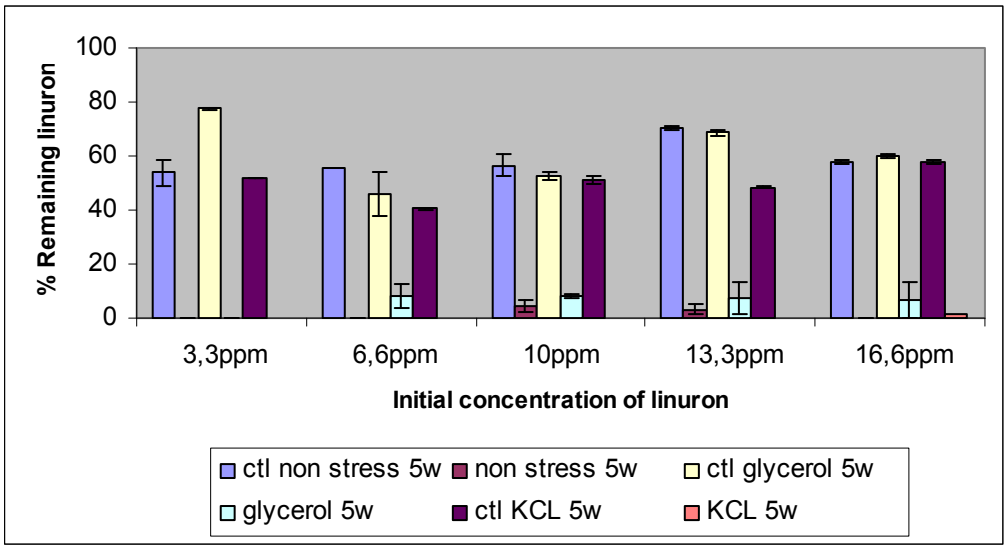


Figure 3.15 Percentage (%) linuron concentrations remaining in soil extract broth inoculated with *T. versicolor* and supplemented with a mixture of pesticides at 3.3, 6.6, 10, 13.3 and 16.6 ppm each of them, after 5 weeks of incubation at 25 ^{0}C, under three water regimes.

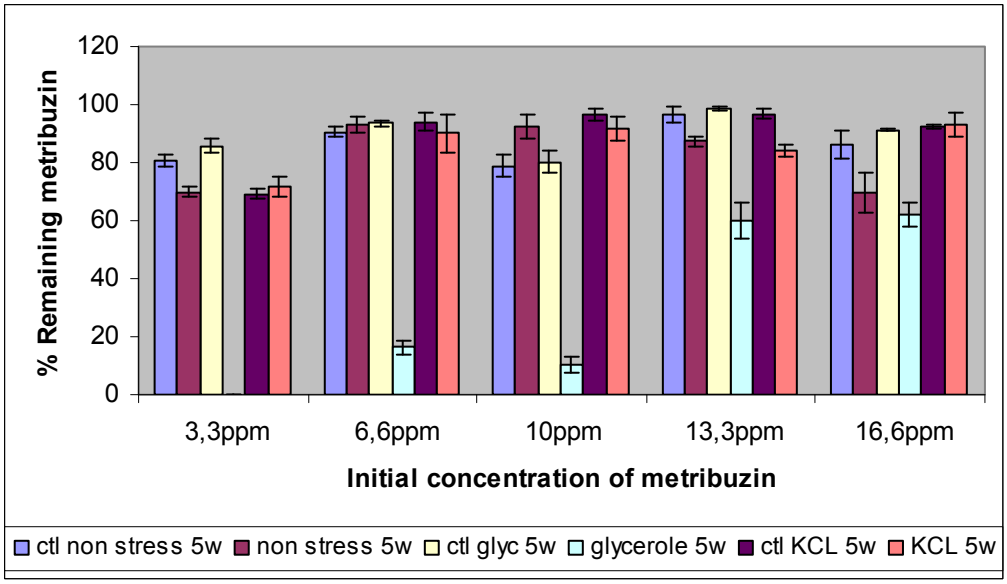


Figure 3.16 Percentage (%) metribuzin concentration remaining in soil extract broth inoculated with *T. vesicolor* and supplemented with a mixture of pesticides at 3.3, 6.6, 10, 13.3 and 16.6 ppm each of them, after 5 weeks of incubation at 25 ^{0}C, under three water regimes.

3.13.1 Chlorpyrifos degradation

No chlorpyrifos was detected in any of the treatments. Therefore the same samples were measured again using the protocol for the chlorpyrifos quantification by Sigma in order to cross-checked the results. As no chlorpyrifos was detected, TCP concentration in the samples was attempted, as TCP is considered the main metabolite of chlorpyrifos. However, noTCP was detected.

3.14 Toxicity test

In this study the goal was to assess the toxicity of the soil extract broth inoculated with *T.trametes* after 5 weeks incubation. Initially we tested the response of this toxicity to soil extract broth, spiked with different concentrations of pesticides (individually and as a mixture).

Figure 3.17 shows the response of *Tetrahymena thermophila* to to soil extract broth, spiked with different concentrations of chlorpyrifos, linuron and metribuzin, individually and as a mixture. *T. thermophila* showed high sensitivity to the mixture of pesticides with 94% and 100% inhibition in the treatments spiked with 12 ppm and 15 ppm, respectively. Chlorpyrifos and linuron in the treatment of 10 ppm caused 82 and 89% inhibition, respectively. Metribuzin was less toxic than the others, showing 86% inhibition in the 15 ppm treatment.

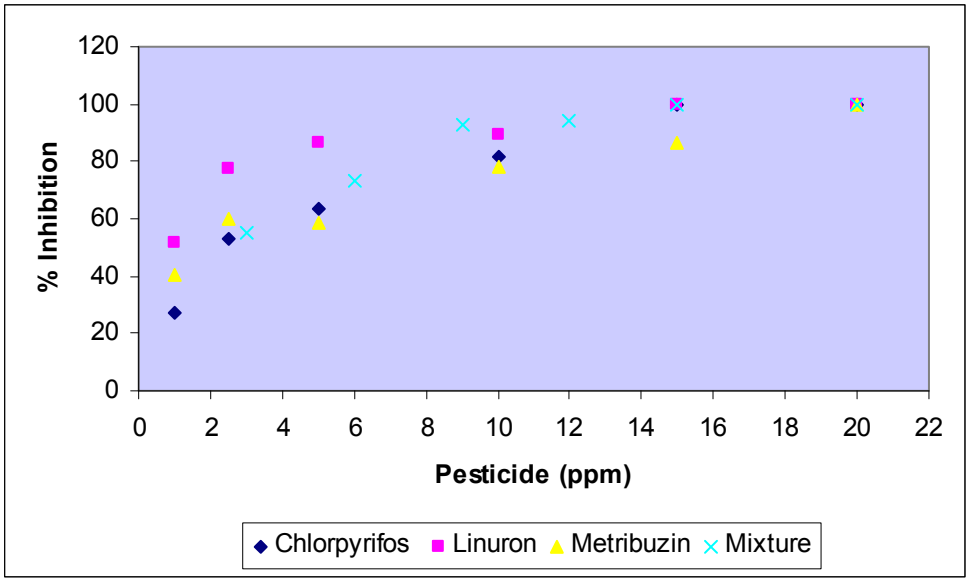


Figure 3.17 Inhibition curve for *Tetrahymena thermophila* in soil extract broth spiked with chlorpyrifos, linuron and metrbuzin individually and spiked with a mixture of the three pesticides, achieved with PROTOXKIT F^{TM}.

Only the extracts of treatments non-stressed and plus KCl at the concentrations of 0, 20 and 40 ppm after 5 weeks incubation were tested. The results obtained showed relative toxicity which, maybe, is attributed to the presence of metribuzin as this compound was not degraded. Particularly, at 20 and 40 ppm of the filtrates without stress the inhibition % was 13.4 and 18%, respectively. At the treatments with the KCl the inhibition % was 2 and 23.5 % for the 20 and 40 ppm concentrations of the mixture of pesticides, respectively.

4 DISCUSSION

4.1 General overview

Pollution of the environment has been one of the largest concerns to science and the general public in the last years. Soil and an aqueous environment are natural and preferential sinks for contamination, and their pollution represents an important concern for human and environmental health (Gianfreda & Bollag, 2002).

It is clear that microbial-based treatments of contaminated environments and industrial effluents offer an economical alternative to existing treatment methods (Singleton, 1994). Bioremediation, which involves the use of microbes to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments (Singh *et al.,* 2006).

The application of fungal technology for the clean up of polluted soils holds significant promise since 1985 when the white rot fungus *Phanerochaete chrysosporium* was found to be able to metabolise a number of important environmental pollutants (Sasek, 2003). This capacity was later described for other white rot species among them *Trametes versicolor.* Successful removal of pesticides by the addition of bacteria (bioagumentation) has been also reported for many compounds including, parathion, coumaphos, ethoprophos and atrazine (Singh *et al.,* 2006).

4.2 Fungal tolerance to pesticides

The initial studies were carried out using five fungal species. The temperature that was used in all studies was 25 ^{0}C as is considered environmentally relevant to Greece and other Mediterranean countries.

The screening experiment showed that all the tested fungi had a good growth in a mineral salts medium (MMY) in relation to the conducive media malt

extract agar and the modified MMY plus glucose. The reduction of the growth was approximately 40 % for the species *P. ostreatus* and *P. gigantea,* 20 % for the species *P. chrysosporium* and *P. coccineus.* The growth of *T. versicolor* was not affected.

The response of the test isolates to the pesticides was influenced by pesticide concentration and whether the pesticides were in a mixture or present individually. In relation to chlorpyrifos, *P. ostreatus* was more tolerant than all the others while *P. chrysosporium* showed high sensitivity to chlorpyrifos. *P. coccineus* and *P.gigantea* showed very good tolerance to linuron exhibiting no inhibition and 20 % respectively at 30ppm of linuron. Again, *P. chrysosporium* showed high sensitivity to this pesticide. Expect *P. chrysosporium,* all the other fungi have a very good tolerance at 30ppm of metribuzin and the mixture of 30 ppm of the pesticides. In the presence of the pesticide mixture all the species showed a remarkable growth decrease.

The results that were based on the growth of the fungi on the soil extract agar and the analysis of EC_{50} values confirmed the initial screenings. Particularly, all fungi were sensitive to high concentrations of linuron, while they had a very good tolerance to metribuzin. *P. ostreatus* and *P. coccineus* were more sensitive than the others to chlorpyrifos. In the mixture of pesticides *P. ostreatus* was very sensitive and was not included in subsequent studies. Overall, the results showed clearly that there was a very significance reduction of the fungal growth in the presence of mixture of pesticides in relation to their presence individually. Therefore it is very important to study of the differential effect of mixtures of compounds in mixtures as these interact with biological systems in ways that can greatly alter the toxicity of individual compounds (Hernando et al., 2003).

The study of fungal growth rates is very important for extrapolation of the potential colonization capacity in the field as it provides a good indication of the speed at which a fungus is able to colonise a substrate. Growth rates may also indicate which species may be dominant over a particular

substrate; fast growing species have an advantage over slower species as they can reach and utilise resources before their competitors (Magan and Lacey, 1984; Marin *et al*., 1998a; Marin *et al*., 1998b). Therefore, better fungal growth could help the introduced fungi to overcome competition from indigenous soil microorganisms (Singleton, 2001).

The results for laccase production, in the Petri plate assay were interesting, as all tested fungi (*T. versicolor*; *P. gigantea* and *P. coccineus*) were able to produce laccase in all the pesticide treatments. *T. versicolor* showed the highest laccase activity and it was less affected by pesticide concentration. Only *T. versicolor* was able to degrade lignin in the conditions of this assay. The fungus *P. coccineus* has been used in bioremediation research mainly for its effective extra cellular lacasse production (Alves *et al,* 2004; Pointing *et. al*, 2000). The ability of *T. versicolor* to produce a wide array of enzymes in the presence of contaminants may be advantageous in bioremediation.

4.3 Studies on the soil extract liquid broth

Studies in liquid culture are a good approach to assess an organism's ability to utilise a target compound where the compound is added to the medium either as a sole carbon source or in the presence of a growth supporting substrate (Juhasz and Naidu, 2000). Several reports have demonstrated good agreement between studies investigating optimum conditions for biodegradation in liquid culture systems and bioremediation studies in soil or natural water matrices. Lamar *et al.*, (1990) compared *P. chrysosporium* and *P. sordida* with respect to removing pentachlorophenol from liquid medium and from soil, and found that *P. chrysosporium* was more effective in soil whereas *P. sordida* was more efficient in liquid culture. This suggests that the former can provide a reliable prediction of the range of conditions in which pesticide- degrading bacteria will be active (Karpouzas & Walker, 2000).

Both success and failure have been reported when species capable of degrading pesticides in liquid culture were introduced into the soil. A strain of *Streptomyces* was able to grow on eight pesticides and also degraded them in soil (Shelton *et al*., 1996). Similar results were obtained when an iprodione degrading *Arthrobacter* strain was inoculated in to the soil (Mercadier *et al*., 1996). Several chemicals have been successfully removed from soil and aquatic environments using degrading microorganisms such as chlorinated pyridinol (Feng *et al*., 1997), coumaphos (Mulbry *et al*., 1996, 1998) and atrazine (Struthers *et al*., 1998; Topp, 2001). In contrast, MacRae and Alexander (1965) reported the failure of a 4-(2, 4-dichlorophenoxy) butyrate utilizing bacteria to degrade the chemical when introduced into a treated soil (Singh *et al.,* 2006).

Soil extract liquid broth that was used as the culture medium is a nutritionally weak medium to mimic the nutrient availability in soil. *T. versicolor, P. gigantea* and *P. coccineus* were able to grow effectively in this low nutrient status medium over a range of concentrations of a mixture of pesticides. Chlorpyrifos degradation was shown to have differences between the fungi as well as the concentrations of the pesticide. *P. coccineus* showed able to degrade chlorpyrifos almost 60%, even at 50ppm of the mixture.

Regarding metribuzin, all tested fungi were shown to be unable to degrade it. Perhaps the duration of experiment was not sufficient for the degradation as Schilling *et al*. (1985) obtained a total transformation by *Rhizopus japonicus* and *Cunninghamella echinulata* and a depletion range of 27 to 45% with *Aspergillus niger, Penicillium lilacinum*, and *Fusarium oxysporum* after a 4-week's incubation period. Finally, *T .versicolor* showed a great capacity to degrade linuron even at the 50 ppm concentration of the mixture.

Regarding bacteria, all tested bacterial strains were isolated from Cretan soils and composts and tested for their possible capability to degrade the pesticides. It was considered that they have become acclimatized well in the local conditions. Several of them demonstrated a very good growth in the

presence of high concentrations of the pesticides. However, the results showed clearly that there was a significant reduction of the growth in the presence of mixture of pesticides in relation to their presence individually. The bacterial strains and the mixture of them used were unable to degrade the mixture of pesticides. The degradation of pesticides is usually achieved by a consortium of microbes rather than a single species (Aislabie &Lloyd Jones, 1995). El-Fantroussi (2000) enriched a mixed bacterial culture able to degrade linuron and metobromuron from a previously linuron-treated orchard soil. None of the strains isolated were capable alone or in combination of degrading linuron or metobromuron. Analyzing the bacterial composition at various steps in the degradation, using denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes, strongly suggested the involvement of a bacterial consortium in the degradation. Roberts et al.,(1993) and Sørensen and Aamand, (2001) also encountered difficulties in isolating pure cultures able to degrade phenylurea compounds from different phenylurea-degrading mixed bacterial cultures enriched from soil, thus indicating a lack of single strains able to proliferate through such degradation and hence supporting the involvement of bacterial consortia.

However, several reports have demonstrated successful degradation of pesticides by *Bacillus* and *Pseudomonas* species. Particularly, Rani and Lalithakumari (1994) showed that *Pseudomonas putida* utilized methyl parathion as sole carbon and (or) phosphorus source. The bacterium elaborated the enzyme organophosphorus acid anhydrase, which hydrolyzed methyl parathion to p-nitrophenol. In 1995, a *Pseudomonas* species, isolated from an herbicide spill site, was capable of metabolizing atrazine at very high concentrations by Mandelbaum *et al*. Two strains of *P. putida* (epI and epII) isolated previously from ethoprophos-treated soil, were able to degrade ethoprophos in a mineral salts medium plus nitrogen, in less than 50 hours (Karpouzas and Walker, 2000). These researchers studied conditions such as temperature, pH and nutrient status and found out that the degradation of ethoprophos was most rapid when the bacterial culture were incubated at 25 and 37 ^{0}C at pHs ranging from 5.5 to 7.6. Addition of glucose or succinate to

the culture medium did not influence the the degrading ability of *P. putida.*The strain epI was capable of degrading the pesticide when only 60 cells mL^{-1} were used as initial inculum, while epII when inculum densities of 600 cells mL^{-1} or higher were used. Extensive biodegradation of propanil (3,4-dichloropropionanilide) by a strain of *P. putida* AF7 was demonstrated in nutrient cultures. The organism was capable of using propanil as the sole source of C, presenting up to 60% of degradation. This strain was isolated from the rhizosphere of rice grown in contaminated soil with this herbicide.

Eight bacteria were isolated by repeated subculture in liquid medium with trifluralin from a soil in which this pesticide has been used for the last four decades. In a mineral salts medium with 0.1% succinate, 0.1% yeast extract and 50 mg L^{-1} trifluralin, reductions in the level of pesticide of 24.6% for *Klebsiella* sp., 16.4% for *Herbaspirillum* sp., 25.0% and 16.0% for two strains of *Bacillus* sp. were obtained after 30 days (Bellinaso, 2003). In four other culture media that were used the degradation rates of trifluralin were very low. A bacterium strain of *Bacillus* genus isolated from cloud water was the first pure strain capable of rapidly degrading mesotrione, a new selective herbicide for control of broad-leaved weeds in maize (Durand, 2006). Finally, suspensions of soil repeatedly treated with carbofuran under glasshouse conditions enhanced the degradation of carbofuran significantly in a mineral salts medium, 96% degradation compared to 15% in unicoculated medium in 10 days. Out of the seven bacterial cultures isolated from the enrichment culture, two cultures indentified as *Pseudomonas stutzeri* and *Bacillus pumilis* enhanced carbofuran degradation, resulting in more than 98% loss of the applied pesticide in 30days (Mohapatra &Awasthi, 1997).

In all above –mentioned studies the bacterial strains were isolated from contaminated sources by enrichment cultures so the possibility to have degrading abilities was very high in contrast to the strains of *Bacillus* and *Pseudomonas* species from Cretan soils without any application of the pesticides chlorpyrifos, linuron and metribuzin. Conditions such as pH, temperature and initial inoculums density were almost the same. Several

studies failed to obtain micro-organisms capable of growing on specific chemicals or degrade them. This failure does not exclude biological involvement in degradation and could be attributed to the selection and composition of the liquid media under artificial environments, or strains requiring special growth factors (Singh &Walker, 2005). Moreover, the culture medium could have a strong influence on biodegradation and it is important to use various media when testing for xenobiotic biodegradation (Bellinaso, 2003).

4.4 Studies on differential break down of mixtures of pesticides under water stress

While studies on the capacity of white rot fungi and particularly *T.versicolor* to degrade individual pesticides is extensive (Gadd, 2001), very few have examined the capacity to degrade mixtures of pesticides as also as their activities under interacting conditions of pesticides mixtures and different water availabilities (Fragoeiro & Magan, 2004). Yavad and Reddy (1993) described co-mineralization of a mixture of the pesticides 2, 4-D and 2,4,5-T by *P.chrysosporium* wild-type and a putative peroxidase mutant in nutrient-rich broth with a small amount remaining in the mycelial fractions (5%). Bending *et al*., (2002) showed degradation rates of metalaxyl, atrazine, terbuthylazine and diuron by white rot fungi in nutrient solution of >86% for atrazine and terbuthylazine. Fragoeiro & Magan, (2004) studied the enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *T. versicolor* and *P. chrysosporium.* Their results suggested that both fungi isolates had the ability to degrade the pesticides simazine, dieldrin and trifluralin supported by the capacity for expression of a rate of extracellular enzymes at -0.7 and -2.8 MPa water potential. Particularly, *T. versicolor,* the same isolate used in current studies, was able to produce phosphomonoesterase, protease (relevant to P and N release), β- glucosidase, cellulase (carbon cycling) and laccase activity.

Water availability was considered in this study, because it plays a key role in fungal development. It was important to evaluate fungal response to pesticides at different levels of water availability, which occur naturally in soil. In this study, *T. versicolor* exhibited very high laccase activity in soil extract broth solute potential adjusted at -2.8 MPa non-ionically by using glycerol, for example in the 30 ppm treatment, at the second week, laccase activity of 2.26 units $mg^{-1}$was measured. Laccase production was the maximum at the second and third week of the incubation. In soil extract broth without stress the levels of laccase produced were quite high and they were highest at the sixth week of incubation while no laccase was observed at -2.8 MPa solute potential adjusted by adding potassium chloride. Fragoeiro & Magan, (2005) found that at -2.8 MPa solute potential adjusted by adding potassium chloride laccase production was significally reduced, but up to 20 units were still detected.

The results suggest that laccase is not only secreted in nutrient-rich substrates but is produced by mycelia growing in weak nutritional matrices. Of particular interest is the capacity of *T. versicolor* for laccase production in the presence of up a 50 ppm mixture of the pesticides. This presence was shown to stimulate laccase production as in the treatment of 0 ppm, without stress laccase production was very low related to all the other concentrations over the first four weeks period. It reached to the same levels at fifth and six week of incubation. Recent studies demonstrated induction of production of ligninolytic enzymes, particularly laccase, in the presence of copper, veratryl alcohol and a phenolic mixture (Xavier *et al.*, 2007), in the presence of mixtures of pesticides (Fragoeiro & Magan, 2005). Moreover, Mougin *et al.*, (2002) studied several agrochemicals, industrials compounds and their transformation products for their ability to enhance laccase production in liquid cultures of *T. versicolor.* Many of them enhanced laccase activity up to 20-fold having as positive control 2, 5-xylidine (35-fold enhancement of laccase activity) (Xiao *et al.*, 2004)

Cuto *et al.*, (2006) reported highly significant increases in laccase produced by *Trametes hirsuta* growing in an air-lift bioreactor after the addition of glycerol. Fragoeiro & Magan (2008) studied the impact of water potential on mixtures of the pesticides simazine, dieldrin and trifluralin, in soil inoculated with *T. versicolor* and *P. chrysosporium* in relation to different soil water potentials(-0.7 and -2.8 MPa). The reseachers showed that in natural soil the level of laccase produced by *T. versicolor* was very low, reaching the highest levels after 6 weeks incubation under both water regimes, whereas that amended with wood chips showed some laccase production, with the highest level after 12 weeks incubation. Overall, the main difference between laccase production in soil microcosms and that in soil extract broth was that in soil extract based liquid culture laccase production was much higher at -0.7 MPa while in soil microcosms the optimum was at -2.8 MPa.

Total ligninolytic activity was expressed as the capacity to decolourise poly-R478. The decolouration assay of poly-R478, with similar structure to lignin, gives information on the activity of the whole set of enzymes because the degradation of lignin is carried out by several enzymes. The results showed that decolouration of this dye, occurred in all treatments with the highest levels of decolouration after 5 weeks incubation at -2.8 MPa water potential adjusted with KCl. In all cases the decolouration rates were unaffected by pesticide treatment. The results suggest *T. versicolor* was tolerant to this mixture of pesticides, producing equivalent levels of decolouration in the presence and absence of the xenobiotics.The highest levels of decolouration that occurred in the treatments with KCl may be related to the production of other enzymes as at -2.8 MPa water potential adjusted with KCl no laccase production was observed.

There was no correlation between decolourization of the dye and degradation of the pesticide mixture in this study. A similar result was described for degradation of diuron, metalaxyl atrazine and terbuthylazine, by several fungi in liquid culture (Bending *et al.*, 2002) as well as for the degradation of simazine, trifluralin and dieldrin in soil (Fragoeiro & Magan, 2008) although

Alcalde *et al*., (2002) observed correlation with oxidation of PAHs mediated by laccases.

The present study on the degradation of the mixture of pesticides showed good capacity of *T. versicolor* to degrade linuron at all tested water regimes, regardless of the initial concentrations of mixture between 0 and 50 ppm. Metribuzin degradation occurred only in the treatments at -2.8 MPa solute potential adjusted with glycerol. A possible explanation, maybe, is that *T. versicolor* is unable to utilize this pesticide as a carbon source for its growth and as soil extract broth is a weak nutrient medium glycerol was probably used by the fungus as an additional carbon source.

Interestingly, chlorpyrifos and its main metabolite TCP were not detected. The environmental fate of this pesticide has been studied extensively. The manufacturer reports that chlorpyrifos is a degradable compound, and a number of environmental forces may be active in its breakdown. In all systems (soil, water, plants and animals), the major pathway of degradation begins with cleavage of the phosphorus ester bond to yield 3,5,6-trichloro-2-pyridinol (TCP). In soil and water, TCP is further degraded via microbial activity and photolysis to carbon dioxide and organic matter. Hydrolytic and photolytic half-lives are both around a month, at neutral pH 25° C. Under more alkaline conditions, hydrolysis proceeds more rapidly. In natural water samples, however, degradation often proceeds significantly faster; a 16-fold enhancement of hydrolysis rate has been observed in pond and canal water samples. Half-lives in the water column of less than one day are typical, due to a combination of degradation, volatilization and partitioning into sediments.

Several published studies on the biodegradation of chlorpyrifos in liquid media have reported no significant rates of abiotic degradation of chlorpyrifos. Particularly, no abiotic degradation of chlorpyrifos in uninoculated media over a four days period was concerned by Singh *et al.*, (2004) 70 hours by Singh *et al.*, (2006) over a 48 and 15 hours period by Xiaohui *et al.*, (2007)and Xu (2008) respectively.

5 . CONCLUSIONS AND FURTHER WORK

5.1 Conclusions

The main findings of this study are summarized below:

1. Screenings in a minimal salts medium (MMY) on the tolerance of five white rot fungi to chlorpyrifos, linuron and metribuzin, individually and in a mixture suggested best tolerance by *T. versicolor, P. osteatus, P. gigantea* and *P. coccineus*, while *P. chrysosporium* was very sensitive.
2. In agar-based studies *T. versicolor* was able to degrade lignin, in the presence of the pesticides, individually and as a mixture. In contrast all the others did not degrade lignin under the conditions of the assay.
3. *T. versicolor, P. gigantea* and *P. coccineus* produced laccase, in agar-based plates and in the presence of the pesticides, individually and as a mixture.
4. All three test isolates were able to grow in soil extract broth supplemented with mixture of the pesticides. They were shown to be unable to degrade metribuzin. In contrast, *T. versicolor* showed a great capacity to degrade linuron even in the 50 ppm concentration.
5. Very high levels of laccase were produced by *T. versicolor* in soil extract broth solute potential adjusted at -2.8 MPa non-ionically by using glycerol. Laccase production was the maximum at the second and third week after the inoculation.
6. The levels of laccase produced by *T. versicolor* in soil extract broth without stress were quite good and they were highest at the sixth week of incubation. This pesticides mixture was shown to induce laccase production.
7. *T. versicolor* did not produce laccase in soil extract broth solute potential when this was adjusted at -2.8 MPa ionically by adding potassium chloride.
8. The results showed that decolouration of poly R-478 dye occurred in all treatments but there was no correlation between decolourization of the dye and degradation of the pesticide mixture under the conditions of this study.

9. The results obtained on soil extract broth provide valuable background information on the abilities of *T.versicolor* to tolerate and degrade mixtures of pesticides as well as to produce high levels of laccase in soil extract even under imposed water stress at -2.8 MPa and suggest potential application of this fungus in bioremediation.

5.2 Further work

Further studies based on the findings of this study are:

- Analysis of metabolites by GC-MS

In current study linuron was degraded in soil extract broth after five weeks incubation with *T.versicolor,* while chlorpyrifos was not detected. However, these pesticides may have been completely degraded or mineralized; some of it may have been transformed in unknown metabolites. Additional analysis by GC-MS of the final products would be pertinent, as chemical or microbial co- metabolism may produce toxic intermediates.

- Studies in soil microcosms

  The current study in liquid medium has shown the potential utilisation of these pesticides by *T.versicolor* in the environment. However, the conditions in soil differ greatly from those in liquid culture, because soil is a multi-phasic, heterogeneous environment, in which the contaminants present in association with the soil particles, dissolved in soil liquids and in the soil atmosphere (Boopathy, 2000). Microcosm studies are very useful to understand the detoxifying factors associated with a soil-pesticide matrix (Shoen and Winterlin, 1987), including enzyme activities, respiratory metabolism and native microbial populations activity, as the mechanisms by which the white rot fungi degrade pollutants are still not well understood (Nerud et al., 2003) Testing in soil microcosms under Cretan conditions to examine the range of concentrations of mixture which could be broken down under different moisture and tremperature conditions.

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APPENDIX A

Chromatogram of metribuzin, chlorpyrifos and linuron obtained in single HPLC run with metribuzin eluting at 3.2, chlorpyrifos at 5.2 and linuron at 5.5 minutes in a mixture of 50ppm of the pesticides (equal in amount).

Data File C:\Chem32\2\DATA\SOFIA\TRAMETES000056.D
Sample Name: without stress 1w 40a

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Acq. Operator    : sofia
Acq. Instrument  : Instrument 2                    Location : Vial 1
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                   (modified after loading)
Analysis Method  : C:\CHEM32\2\METHODS\PEST.M
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                   (modified after loading)
Method Info      : Metribuzin, Linuron, Chlorpyrifos.
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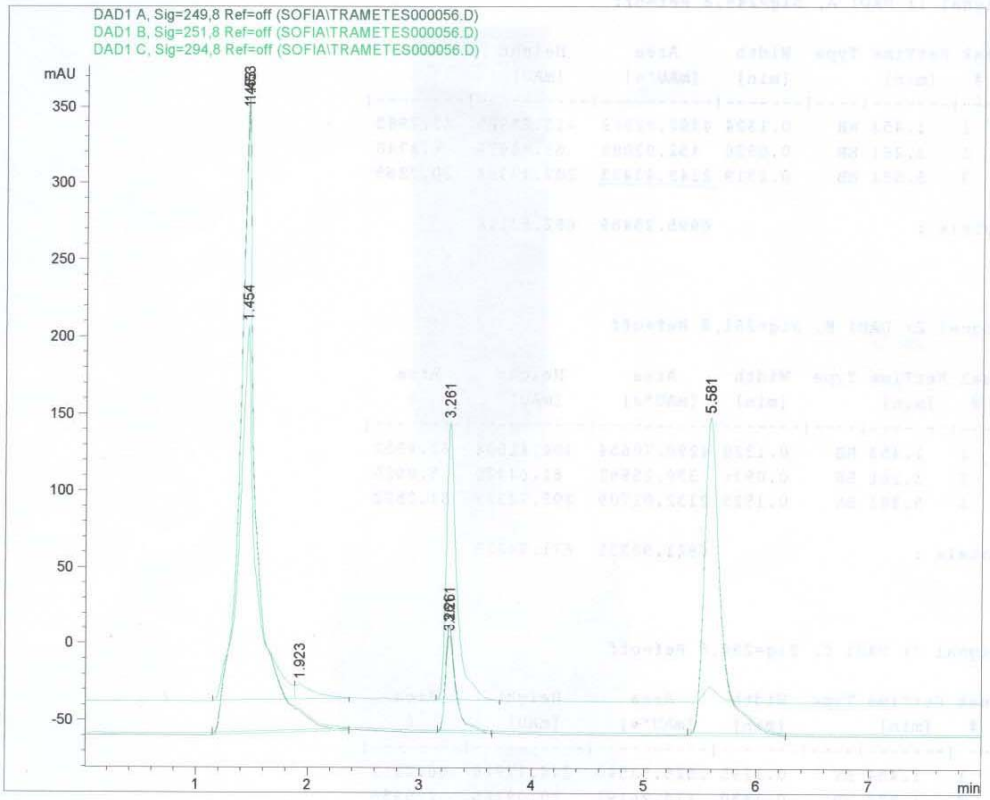


Instrument 2 9/27/2007 2:01:11 PM sofia Page 1 of 2

APPENDIX B

Laccase units

Laccase units						
without stress						
	1 week	**2 week**	**3 week**	**4 week**	**5 week**	**6 week**
0ppm	0.5±0.1	0.1±0.1	0.1±0.0	0.3±0.2	1.0±0.1	2.7±0.7
10ppm	1.4±0.2	0.3±0.1	0.3±0.2	2.1±1.0	2.5±0.8	1.2±0.3
20ppm	3.8±0.4	1.6±0.4	3.1±0.3	2.0±0.8	2.1±0.2	2.5±0.6
30ppm	2.1±0.3	2.2±0.7	0.6±0.2	0.9±0.2	2.6±	1.3±
40ppm	1.8±0.4	1.7±0.4	0.8±0.2	1.8±0.2	1.8±0.2	1.5±0.6
50ppm	1.6±0.2	1.6±0.1	1.8±0.2	1.9±0.1	2.2±0.3	2.1±0.3
glycerol						
	1 week	**2 week**	**3 week**	**4 week**	**5 week**	**6 week**
0ppm	5.7±1.2	6.9±0.5	5.9±0.5	2.9±0.4	2.6±0.6	1.8±0.3
10ppm	6.1±0.8	10.9±0.8	7.6±0.7	6.6±0.3	2.5±0.3	3.1±0.5
20ppm	3.3±0.6	20.1±0.8	14.4±0.6	6.6±0.6	4.4±0.5	2.8±0.2
30ppm	4.1±0.8	23.4±1.3	19.2±2.2	9.7±0.9	3.2±0.8	2.6±0.4
40ppm	1.5±0.6	5.4±0.1	21.0±0.9	6.5±0.9	5.3±0.9	2.8±0.5
50ppm	1.2±0.1	8.3±1.0	12.8±1.5	12.2±1.5	6.0±1.0	3.8±1.0

APPENDIX C

Statistical tests

Analysis of variance with two factors (water regime x week)

Proteins

Without stress: Substrate =1

With glycerol (-2.8MPa): Substrate =2

Concentrations: Con.

Univariate Analysis of Variance

conc = ,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	6
	4,00	6
	5,00	5
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = ,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,001[a]	11	5,75E-005	1,391	,242
Intercept	,005	1	,005	123,122	,000
week	,000	5	3,44E-005	,832	,541
substrate	2,26E-005	1	2,26E-005	,548	,467
week * substrate	,000	5	8,78E-005	2,125	,099
Error	,001	23	4,13E-005		
Total	,007	35			
Corrected Total	,002	34			

a. R Squared = ,399 (Adjusted R Squared = ,112)

b. conc = ,00

conc = 10,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	5
	3,00	5
	4,00	4
	5,00	5
	6,00	6
substrate	1,00	16
	2,00	15

a. conc = 10,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,000[a]	11	1,07E-005	1,352	,272
Intercept	,002	1	,002	203,312	,000
week	6,65E-005	5	1,33E-005	1,677	,189
substrate	2,76E-008	1	2,76E-008	,003	,954
week * substrate	4,92E-005	5	9,83E-006	1,240	,329
Error	,000	19	7,93E-006		
Total	,002	31			
Corrected Total	,000	30			

a. R Squared = ,439 (Adjusted R Squared = ,114)

b. conc = 10,00

conc = 20,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	5
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	16

a. conc = 20,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,001[a]	11	4,57E-005	1,998	,081
Intercept	,004	1	,004	172,494	,000
week	,000	5	3,42E-005	1,495	,232
substrate	4,76E-006	1	4,76E-006	,208	,653
week * substrate	,000	5	5,31E-005	2,319	,078
Error	,001	22	2,29E-005		
Total	,005	34			
Corrected Total	,001	33			

a. R Squared = ,500 (Adjusted R Squared = ,250)

b. conc = 20,00

conc = 30,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	6
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	18
	2,00	17

a. conc = 30,00

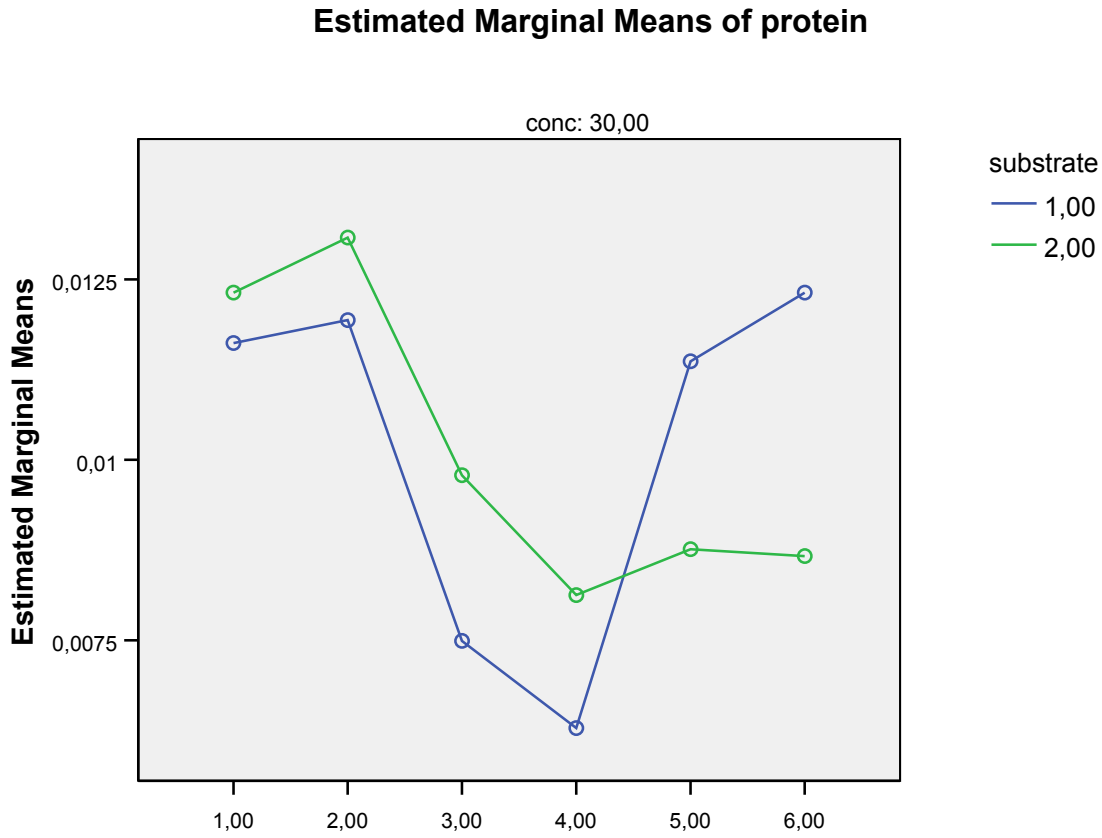
Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,000[a]	11	1,48E-005	2,821	,017
Intercept	,004	1	,004	676,493	,000
week	,000	5	2,39E-005	4,543	,005
substrate	1,83E-008	1	1,83E-008	,003	,953
week * substrate	4,18E-005	5	8,36E-006	1,589	,203
Error	,000	23	5,26E-006		
Total	,004	35			
Corrected Total	,000	34			

a. R Squared = ,574 (Adjusted R Squared = ,371)

b. conc = 30,00



conc = 40,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	4
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	17
	2,00	16

a. conc = 40,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,000[a]	11	2,06E-005	1,973	,087
Intercept	,002	1	,002	236,938	,000
week	,000	5	2,57E-005	2,459	,067
substrate	1,81E-005	1	1,81E-005	1,732	,202
week * substrate	6,90E-005	5	1,38E-005	1,322	,293
Error	,000	21	1,04E-005		
Total	,003	33			
Corrected Total	,000	32			

a. R Squared = ,508 (Adjusted R Squared = ,251)

b. conc = 40,00

conc = 50,00

Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	6
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = 50,00

Tests of Between-Subjects Effects[b]

Dependent Variable: protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9,34E-005[a]	11	8,49E-006	1,141	,377
Intercept	,003	1	,003	410,168	,000
week	1,89E-005	5	3,78E-006	,508	,767
substrate	1,51E-007	1	1,51E-007	,020	,888
week * substrate	7,36E-005	5	1,47E-005	1,978	,120
Error	,000	23	7,44E-006		
Total	,003	35			
Corrected Total	,000	34			

a. R Squared = ,353 (Adjusted R Squared = ,044)

b. conc = 50,00

APPENDIX D

Statistical tests

Specific activity

Statistical analysis of variance was performed for each pesticide concentration in the same water regime per week with the Tukey's test.

Without stress: Substrate =1

With glycerol (-2.8MPa): Substrate =2

Concentrations: Con.

Without stress

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	219447,3	5	43889,464	57,567	,000
Within Groups	9148,883	12	762,407		
Total	228596,2	17			

a. substrate = 1,00, conc = ,00

sp_act [b]

Tukey HSD [a]

week	N	Subset for alpha = .05		
		1	2	3
3,00	3	5,0872		
2,00	3	8,6117		
4,00	3	29,0258	29,0258	
1,00	3	52,3602	52,3602	
5,00	3		94,2347	
6,00	3			323,0022
Sig.		,350	,108	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = ,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	95107,758	5	19021,552	17,560	,000
Within Groups	9749,280	9	1083,253		
Total	104857,0	14			

a. substrate = 1,00, conc = 10,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05		
		1	2	3
3,00	3	29,7850		
2,00	2	44,1578		
5,00	2	84,1968		
4,00	2	124,0416	124,0416	
1,00	3		191,9263	191,9263
6,00	3			237,6563
Sig.		,090	,300	,661

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 1,00, conc = 10,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	194786,2	5	38957,237	4,458	,016
Within Groups	104875,1	12	8739,594		
Total	299661,3	17			

a. substrate = 1,00, conc = 20,00

sp_act[b]

Tukey HSD[a]

week	N	Subset for alpha = .05	
		1	2
4,00	3	121,7733	
5,00	3	187,6174	
1,00	3	280,3112	280,3112
2,00	3	289,2732	289,2732
6,00	3	302,6139	302,6139
3,00	3		456,2315
Sig.		,240	,264

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = 20,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	180447,5	5	36089,508	10,314	,001
Within Groups	41988,773	12	3499,064		
Total	222436,3	17			

a. substrate = 1,00, conc = 30,00

sp_act[b]

Tukey HSD[a]

week	N	Subset for alpha = .05		
		1	2	3
5,00	3	80,4536		
4,00	3	96,7647		
2,00	3	177,8601	177,8601	
6,00	3	219,9692	219,9692	219,9692
3,00	3		292,6933	292,6933
1,00	3			360,7950
Sig.		,108	,238	,104

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = 30,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	132448,4	5	26489,677	1,624	,233
Within Groups	179472,2	11	16315,651		
Total	311920,5	16			

a. substrate = 1,00, conc = 40,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05
		1
4,00	3	53,6842
3,00	2	103,9264
5,00	3	210,0381
2,00	3	235,8594
1,00	3	245,2956
6,00	3	314,6861
Sig.		,234

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,769.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 1,00, conc = 40,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	125129,5	5	25025,897	9,355	,001
Within Groups	32100,629	12	2675,052		
Total	157230,1	17			

a. substrate = 1,00, conc = 50,00

sp_act[b]

Tukey HSD[a]

week	N	Subset for alpha = .05	
		1	2
4,00	3	60,7572	
2,00	3	63,1456	
3,00	3	112,4375	
5,00	3	148,4546	
1,00	3	157,6662	
6,00	3		308,5250
Sig.		,267	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

b. substrate = 1,00, conc = 50,00

Water potential -2.8 MPa

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1551482	5	310296,411	5,097	,012
Within Groups	669664,7	11	60878,609		
Total	2221147	16			

a. substrate = 2,00, conc = ,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
5,00	2	173,8347	
6,00	3	212,0878	
4,00	3	277,7036	277,7036
1,00	3	462,0265	462,0265
3,00	3	768,3989	768,3989
2,00	3		990,3646
Sig.		,124	,051

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,769.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = ,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4380450	5	876089,986	9,872	,002
Within Groups	798733,8	9	88748,202		
Total	5179184	14			

a. substrate = 2,00, conc = 10,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
5,00	2	420,7520	
1,00	3	483,2134	
6,00	3	521,0306	
3,00	2	1067,0508	1067,0508
4,00	2	1231,3270	1231,3270
2,00	3		1850,5402
Sig.		,113	,130

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 10,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5141054	5	1028210,750	39,641	,000
Within Groups	259378,2	10	25937,820		
Total	5400432	15			

a. substrate = 2,00, conc = 20,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
1,00	3	254,8545	
6,00	3	343,3665	
5,00	3	502,2649	
4,00	2	613,8474	
3,00	2		1390,3485
2,00	3		1730,4609
Sig.		,203	,244

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 20,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6775421	5	1355084,244	9,483	,001
Within Groups	1429025	10	142902,518		
Total	8204446	15			

a. substrate = 2,00, conc = 30,00

sp_act[c]

Tukey HSD[a,b]

week	N	Subset for alpha = .05	
		1	2
6,00	2	320,9736	
1,00	3	339,8109	
5,00	3	370,7411	
4,00	3	1216,0820	1216,0820
3,00	2		1692,7969
2,00	3		1871,5287
Sig.		,162	,421

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 30,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5487967	5	1097593,351	63,501	,000
Within Groups	155561,9	9	17284,654		
Total	5643529	14			

a. substrate = 2,00, conc = 40,00

sp_act[c]

Tukey HSD[a,b]

		Subset for alpha = .05		
week	N	1	2	3
1,00	3	333,7396		
6,00	2	365,8393	365,8393	
5,00	3	502,9577	502,9577	
2,00	2	692,5000	692,5000	
4,00	3		775,5682	
3,00	2			2247,3214
Sig.		,112	,061	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,400.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 40,00

ANOVA[a]

sp_act

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4401525	5	880305,026	7,829	,002
Within Groups	1236794	11	112435,800		
Total	5638319	16			

a. substrate = 2,00, conc = 50,00

sp_act[c]

Tukey HSD[a,b]

		Subset for alpha = .05		
week	N	1	2	3
1,00	3	112,1766		
5,00	3	594,6697	594,6697	
6,00	3	598,6936	598,6936	
2,00	3	927,3646	927,3646	
4,00	3		1090,8834	1090,8834
3,00	2			1903,0676
Sig.		,119	,535	,121

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2,769.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. substrate = 2,00, conc = 50,00

APPENDIX E

Specific activity

Analysis of variance with two factors (water regime x week)

Without stress: Substrate =1

With glycerol (-2.8MPa): Substrate =2

Concentrations: Con.

Between-Subjects Factors [a]

		N
week	1,00	6
	2,00	6
	3,00	6
	4,00	6
	5,00	5
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = ,00

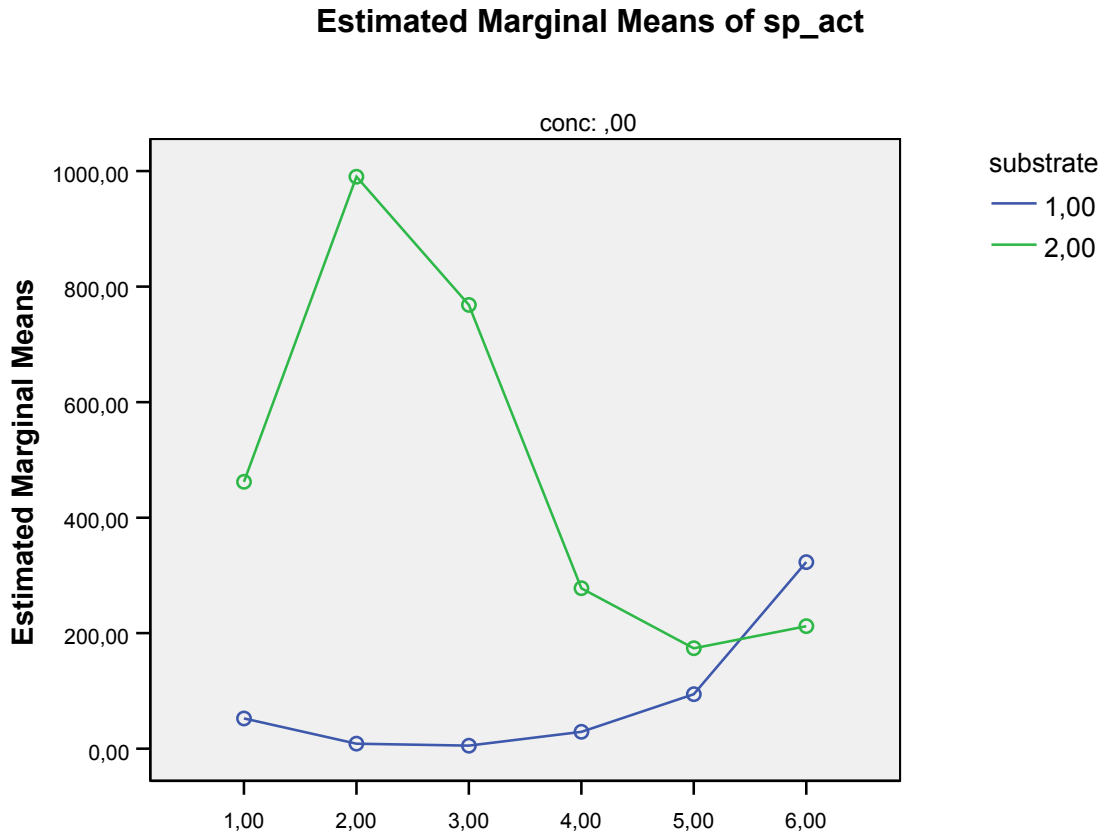
Tests of Between-Subjects Effects [b]

Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3265094,817[a]	11	296826,802	10,057	,000
Intercept	2769078,728	1	2769078,728	93,824	,000
week	557613,343	5	111522,669	3,779	,012
substrate	1350439,590	1	1350439,590	45,756	,000
week * substrate	1254534,568	5	250906,914	8,501	,000
Error	678813,583	23	29513,634		
Total	6810418,247	35			
Corrected Total	3943908,400	34			

a. R Squared = ,828 (Adjusted R Squared = ,746)

b. conc = ,00



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	5
	3,00	5
	4,00	4
	5,00	4
	6,00	6
substrate	1,00	15
	2,00	15

a. conc = 10,00

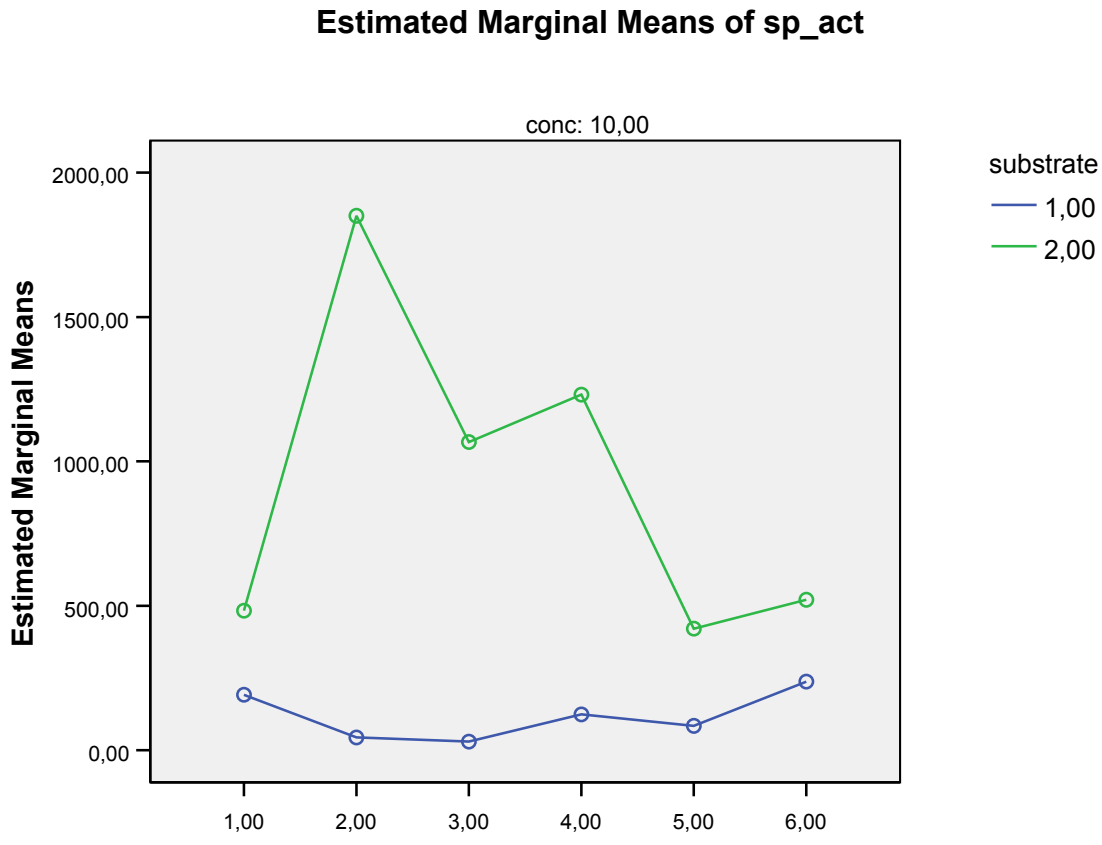
Tests of Between-Subjects Effects[b]

Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9371808,913[a]	11	851982,628	18,968	,000
Intercept	7901948,952	1	7901948,952	175,928	,000
week	1583392,059	5	316678,412	7,051	,001
substrate	4728100,721	1	4728100,721	105,266	,000
week * substrate	2375159,799	5	475031,960	10,576	,000
Error	808483,094	18	44915,727		
Total	18591936,7	30			
Corrected Total	10180292,0	29			

a. R Squared = ,921 (Adjusted R Squared = ,872)

b. conc = 10,00



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	5
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	16

a. conc = 20,00

Tests of Between-Subjects Effects[b]

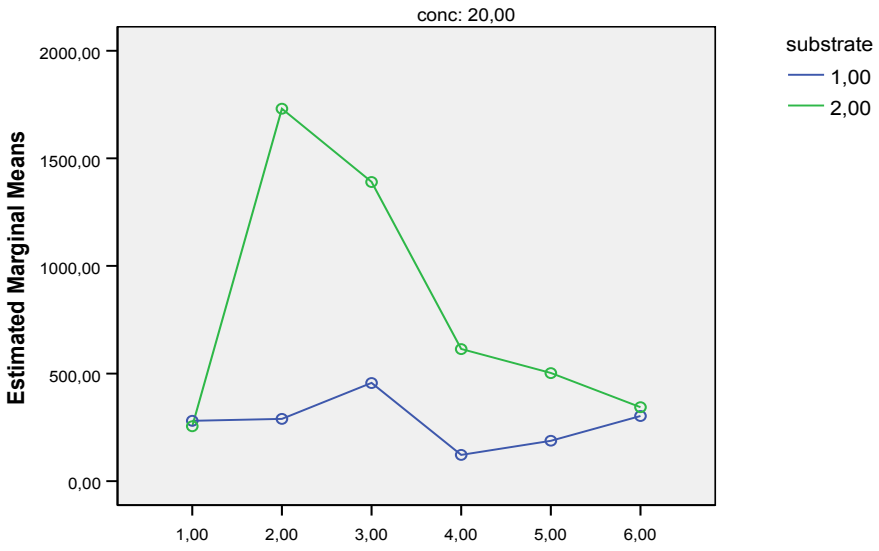
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7524866,751[a]	11	684078,796	41,317	,000
Intercept	9669058,123	1	9669058,123	583,987	,000
week	3126013,069	5	625202,614	37,761	,000
substrate	2359123,732	1	2359123,732	142,485	,000
week * substrate	2333663,583	5	466732,717	28,190	,000
Error	364253,337	22	16556,970		
Total	16808871,6	34			
Corrected Total	7889120,088	33			

a. R Squared = ,954 (Adjusted R Squared = ,931)

b. conc = 20,00

Estimated Marginal Means of sp_act



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	18
	2,00	16

a. conc = 30,00

Tests of Between-Subjects Effects[b]

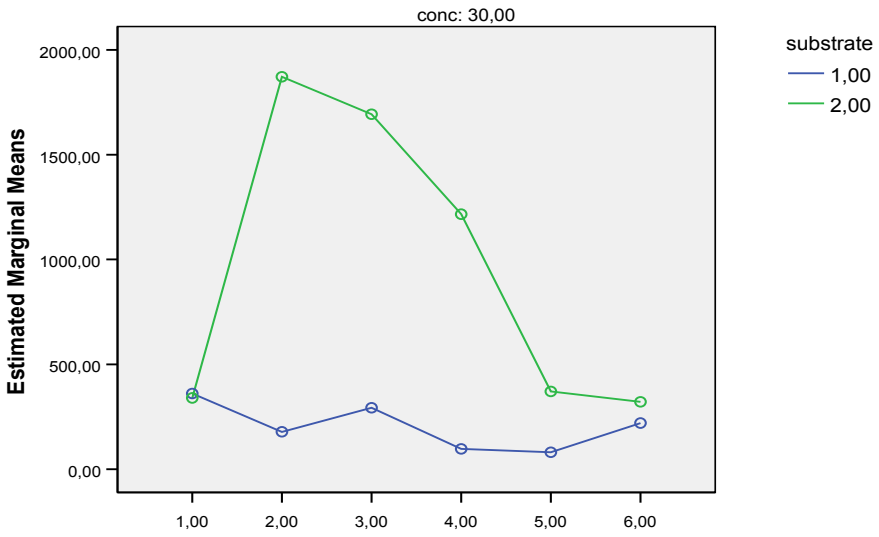
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11837167,5[a]	11	1076106,141	16,094	,000
Intercept	11438816,3	1	11438816,32	171,075	,000
week	3568921,456	5	713784,291	10,675	,000
substrate	4847891,667	1	4847891,667	72,503	,000
week * substrate	3759729,814	5	751945,963	11,246	,000
Error	1471013,951	22	66864,270		
Total	24046469,1	34			
Corrected Total	13308181,5	33			

a. R Squared = ,889 (Adjusted R Squared = ,834)

b. conc = 30,00

Estimated Marginal Means of sp_act



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	5
	3,00	4
	4,00	6
	5,00	6
	6,00	5
substrate	1,00	17
	2,00	15

a. conc = 40,00

Tests of Between-Subjects Effects[b]

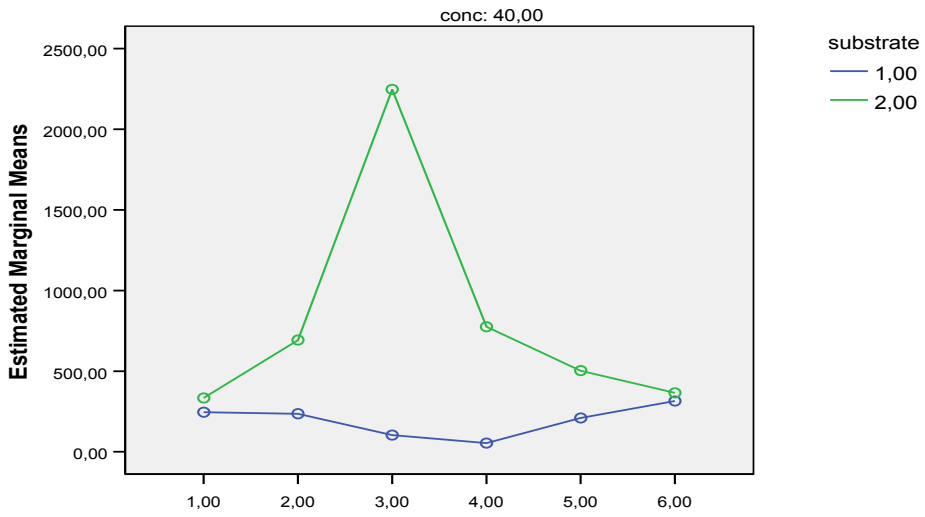
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8155237,745[a]	11	741385,250	44,257	,000
Intercept	7925061,154	1	7925061,154	473,090	,000
week	2363531,546	5	472706,309	28,218	,000
substrate	3020527,092	1	3020527,092	180,312	,000
week * substrate	3310491,114	5	662098,223	39,524	,000
Error	335034,044	20	16751,702		
Total	15367370,3	32			
Corrected Total	8490271,789	31			

a. R Squared = ,961 (Adjusted R Squared = ,939)

b. conc = 40,00

Estimated Marginal Means of sp_act



Between-Subjects Factors[a]

		N
week	1,00	6
	2,00	6
	3,00	5
	4,00	6
	5,00	6
	6,00	6
substrate	1,00	18
	2,00	17

a. conc = 50,00

Tests of Between-Subjects Effects[b]

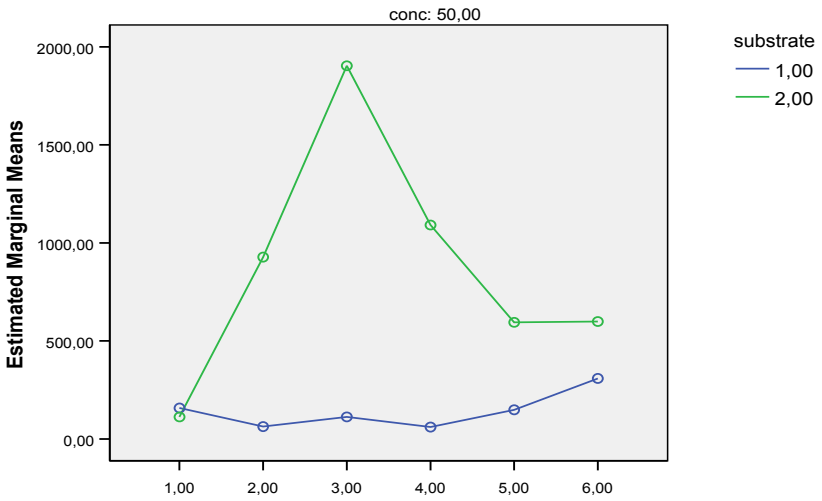
Dependent Variable: sp_act

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8435056,878[a]	11	766823,353	13,899	,000
Intercept	8865638,092	1	8865638,092	160,699	,000
week	2179655,011	5	435931,002	7,902	,000
substrate	4595576,085	1	4595576,085	83,299	,000
week * substrate	2813018,652	5	562603,730	10,198	,000
Error	1268894,424	23	55169,323		
Total	17323489,4	35			
Corrected Total	9703951,301	34			

a. R Squared = ,869 (Adjusted R Squared = ,807)

b. conc = 50,00

Estimated Marginal Means of sp_act



APPENDIX F

Univariate Analysis of Variance of pesticides

LINURON = 1, WEEK = 1, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,339[a]	5	,068	15,833	,000
Intercept	19,389	1	19,389	4523,885	,000
TREATMEN	,339	5	,068	15,833	,000
Error	,051	12	,004		
Total	19,779	18			
Corrected Total	,391	17			

a. R Squared = ,868 (Adjusted R Squared = ,814)

b. LINURON = 1, WEEK = 1, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
6	3	,8216	
5	3	,8830	
2	3		1,0735
3	3		1,1086
4	3		1,1473
1	3		1,1931
Sig.		,851	,290

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,004.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 3,3333

LINURON = 1, WEEK = 1, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,746[a]	5	,149	52,124	,000
Intercept	21,510	1	21,510	7516,302	,000
TREATMEN	,746	5	,149	52,124	,000
Error	,034	12	,003		
Total	22,291	18			
Corrected Total	,780	17			

a. R Squared = ,956 (Adjusted R Squared = ,938)

b. LINURON = 1, WEEK = 1, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2	Subset 3
5	3	,7837		
6	3	,8667		
2	3		1,1683	
3	3		1,1752	
1	3		1,1892	
4	3			1,3759
Sig.		,445	,996	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,003.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 6,6666

LINURON = 1, WEEK = 1, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,373[a]	5	,075	53,639	,000
Intercept	22,029	1	22,029	15847,733	,000
TREATMEN	,373	5	,075	53,639	,000
Error	,017	12	,001		
Total	22,419	18			
Corrected Total	,389	17			

a. R Squared = ,957 (Adjusted R Squared = ,939)

b. LINURON = 1, WEEK = 1, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
5	3	,8763			
6	3		,9970		
1	3			1,1370	
2	3			1,1399	
3	3			1,1448	
4	3				1,3426
Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,001.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 10,0000

LINURON = 1, WEEK = 1, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,618[a]	5	,124	68,421	,000
Intercept	23,811	1	23,811	13178,178	,000
TREATMEN	,618	5	,124	68,421	,000
Error	,022	12	,002		
Total	24,451	18			
Corrected Total	,640	17			

a. R Squared = ,966 (Adjusted R Squared = ,952)

b. LINURON = 1, WEEK = 1, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
5	3	,8895		
6	3	,9489		
2	3		1,1258	
1	3		1,2040	
4	3			1,3490
3	3			1,3838
Sig.		,550	,283	,908

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 13,3333

LINURON = 1, WEEK = 1, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 1, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,524[a]	5	,105	66,437	,000
Intercept	24,366	1	24,366	15451,198	,000
TREATMEN	,524	5	,105	66,437	,000
Error	,019	12	,002		
Total	24,909	18			
Corrected Total	,543	17			

a. R Squared = ,965 (Adjusted R Squared = ,951)

b. LINURON = 1, WEEK = 1, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
5	3	,9587		
6	3	1,0333	1,0333	
2	3		1,0970	
1	3		1,1094	
3	3			1,3429
4	3			1,4395
Sig.		,266	,248	,094

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 1, CONCETR = 16,6666

LINURON = 1, WEEK = 2, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,864[a]	5	,573	25,813	,000
Intercept	5,041	1	5,041	227,158	,000
TREATMEN	2,864	5	,573	25,813	,000
Error	,266	12	,022		
Total	8,171	18			
Corrected Total	3,130	17			

a. R Squared = ,915 (Adjusted R Squared = ,879)

b. LINURON = 1, WEEK = 2, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
6	3	,0000	
4	3	,1394	
2	3	,3152	
1	3		,8090
5	3		,8241
3	3		1,0874
Sig.		,173	,269

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,022.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 3,3333

LINURON = 1, WEEK = 2, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,699[a]	5	,340	15,430	,000
Intercept	5,500	1	5,500	249,763	,000
TREATMEN	1,699	5	,340	15,430	,000
Error	,264	12	,022		
Total	7,463	18			
Corrected Total	1,963	17			

a. R Squared = ,865 (Adjusted R Squared = ,809)

b. LINURON = 1, WEEK = 2, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
6	3	,0000		
2	3	,3666	,3666	
4	3		,5411	
5	3		,7048	,7048
3	3		,7432	,7432
1	3			,9608
Sig.		,087	,076	,342

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,022.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 6,6666

LINURON = 1, WEEK = 2, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,926[a]	5	,385	78,999	,000
Intercept	6,047	1	6,047	1239,917	,000
TREATMEN	1,926	5	,385	78,999	,000
Error	,059	12	,005		
Total	8,031	18			
Corrected Total	1,985	17			

a. R Squared = ,971 (Adjusted R Squared = ,958)

b. LINURON = 1, WEEK = 2, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
6	3	,0000			
4	3		,3052		
2	3			,6096	
3	3				,8124
5	3				,8520
1	3				,8983
Sig.		1,000	1,000	1,000	,667

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,005.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 10,0000

LINURON = 1, WEEK = 2, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,226[a]	5	,445	57,091	,000
Intercept	8,245	1	8,245	1057,253	,000
TREATMEN	2,226	5	,445	57,091	,000
Error	,094	12	,008		
Total	10,564	18			
Corrected Total	2,320	17			

a. R Squared = ,960 (Adjusted R Squared = ,943)

b. LINURON = 1, WEEK = 2, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
6	3	,2174			
2	3	,3033			
4	3		,5525		
5	3			,7966	
3	3			1,0210	1,0210
1	3				1,1700
Sig.		,833	1,000	,075	,364

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,008.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 13,3333

LINURON = 1, WEEK = 2, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 2, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,810[a]	5	,362	18,071	,000
Intercept	7,434	1	7,434	371,196	,000
TREATMEN	1,810	5	,362	18,071	,000
Error	,240	12	,020		
Total	9,484	18			
Corrected Total	2,050	17			

a. R Squared = ,883 (Adjusted R Squared = ,834)

b. LINURON = 1, WEEK = 2, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
4	3	,2462	
6	3	,2638	
2	3	,5017	
3	3		,9227
5	3		,9310
1	3		,9906
Sig.		,300	,990

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,020.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 2, CONCETR = 16,6666

LINURON = 1, WEEK = 3, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,805[a]	5	,761	597,235	,000
Intercept	3,667	1	3,667	2878,022	,000
TREATMEN	3,805	5	,761	597,235	,000
Error	,015	12	,001		
Total	7,488	18			
Corrected Total	3,821	17			

a. R Squared = ,996 (Adjusted R Squared = ,994)

b. LINURON = 1, WEEK = 3, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
2	3	,0000		
4	3	,0000		
6	3	,0000		
5	3		,8058	
1	3		,8251	
3	3			1,0774
Sig.		1,000	,983	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,001.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 3,3333

LINURON = 1, WEEK = 3, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,216[a]	5	,443	40,659	,000
Intercept	3,159	1	3,159	289,810	,000
TREATMEN	2,216	5	,443	40,659	,000
Error	,131	12	,011		
Total	5,505	18			
Corrected Total	2,346	17			

a. R Squared = ,944 (Adjusted R Squared = ,921)

b. LINURON = 1, WEEK = 3, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
2	3	,0000	
6	3	,0000	
4	3	,2409	
5	3		,6889
3	3		,7421
1	3		,8414
Sig.		,120	,506

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,011.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 6,6666

LINURON = 1, WEEK = 3, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,126[a]	5	,425	82,961	,000
Intercept	4,280	1	4,280	835,176	,000
TREATMEN	2,126	5	,425	82,961	,000
Error	,062	12	,005		
Total	6,468	18			
Corrected Total	2,187	17			

a. R Squared = ,972 (Adjusted R Squared = ,960)

b. LINURON = 1, WEEK = 3, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
6	3	,0000		
2	3	,1763	,1763	
4	3		,2880	
5	3			,7950
3	3			,8143
1	3			,8522
Sig.		,088	,441	,916

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,005.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 10,0000

LINURON = 1, WEEK = 3, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,003[a]	5	,601	52,661	,000
Intercept	4,807	1	4,807	421,555	,000
TREATMEN	3,003	5	,601	52,661	,000
Error	,137	12	,011		
Total	7,946	18			
Corrected Total	3,139	17			

a. R Squared = ,956 (Adjusted R Squared = ,938)

b. LINURON = 1, WEEK = 3, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
6	3	,0000	
2	3	,1493	
4	3	,2098	
5	3		,7692
3	3		,9776
1	3		,9948
Sig.		,228	,174

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,011.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 13,3333

LINURON = 1, WEEK = 3, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 1, WEEK = 3, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,868[a]	5	,574	37,541	,000
Intercept	4,051	1	4,051	265,070	,000
TREATMEN	2,868	5	,574	37,541	,000
Error	,183	12	,015		
Total	7,102	18			
Corrected Total	3,052	17			

a. R Squared = ,940 (Adjusted R Squared = ,915)

b. LINURON = 1, WEEK = 3, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
2	3	,0000	
6	3	,0772	
4	3	,1563	
5	3		,8614
1	3		,8652
3	3		,8863
Sig.		,643	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,015.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 1, WEEK = 3, CONCETR = 16,6666

LINURON = 2, WEEK = 1, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,083[a]	5	,017	14,298	,000
Intercept	23,027	1	23,027	19835,733	,000
TREATMEN	,083	5	,017	14,298	,000
Error	,014	12	,001		
Total	23,123	18			
Corrected Total	,097	17			

a. R Squared = ,856 (Adjusted R Squared = ,796)

b. LINURON = 2, WEEK = 1, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
2	3	1,0734	
1	3	1,0766	
5	3	1,0859	
4	3	1,1362	
6	3	1,1448	
3	3		1,2693
Sig.		,179	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,001.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 3,3333

METRIBUZIN = 2, WEEK = 1, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,147[a]	5	,029	4,546	,015
Intercept	32,776	1	32,776	5070,020	,000
TREATMEN	,147	5	,029	4,546	,015
Error	,078	12	,006		
Total	33,001	18			
Corrected Total	,225	17			

a. R Squared = ,654 (Adjusted R Squared = ,511)

b. LINURON = 2, WEEK = 1, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
6	3	1,2530	
1	3	1,2929	
3	3	1,3262	1,3262
2	3	1,3318	1,3318
5	3	1,3544	1,3544
4	3		1,5381
Sig.		,646	,062

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,006.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 6,6666

METRIBUZIN = 2, WEEK = 1, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,229[a]	5	,046	6,987	,003
Intercept	30,733	1	30,733	4684,189	,000
TREATMEN	,229	5	,046	6,987	,003
Error	,079	12	,007		
Total	31,041	18			
Corrected Total	,308	17			

a. R Squared = ,744 (Adjusted R Squared = ,638)

b. LINURON = 2, WEEK = 1, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
3	3	1,1079	
1	3	1,2478	1,2478
2	3	1,2604	1,2604
6	3		1,3935
4	3		1,4063
5	3		1,4243
Sig.		,264	,153

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,007.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 10,0000

METRIBUZIN = 2, WEEK = 1, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,024[a]	5	,005	1,436	,281
Intercept	38,605	1	38,605	11754,559	,000
TREATMEN	,024	5	,005	1,436	,281
Error	,039	12	,003		
Total	38,668	18			
Corrected Total	,063	17			

a. R Squared = ,374 (Adjusted R Squared = ,114)

b. LINURON = 2, WEEK = 1, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1
6	3	1,4105
5	3	1,4294
1	3	1,4585
4	3	1,4758
2	3	1,5024
3	3	1,5103
Sig.		,333

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,003.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 13,3333

METRIBUZIN = 2, WEEK = 1, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 1, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,015[a]	5	,003	1,304	,325
Intercept	30,726	1	30,726	13671,669	,000
TREATMEN	,015	5	,003	1,304	,325
Error	,027	12	,002		
Total	30,768	18			
Corrected Total	,042	17			

a. R Squared = ,352 (Adjusted R Squared = ,082)

b. LINURON = 2, WEEK = 1, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1
3	3	1,2664
1	3	1,2759
4	3	1,2973
5	3	1,3241
6	3	1,3371
2	3	1,3383
Sig.		,469

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 1, CONCETR = 16,6666

METRIBUZIN = 2, WEEK = 2, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,941[a]	5	,588	108,850	,000
Intercept	14,087	1	14,087	2607,208	,000
TREATMEN	2,941	5	,588	108,850	,000
Error	,065	12	,005		
Total	17,092	18			
Corrected Total	3,005	17			

a. R Squared = ,978 (Adjusted R Squared = ,969)

b. LINURON = 2, WEEK = 2, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,0000		
6	3		,9688	
2	3		,9966	
5	3		1,0254	1,0254
1	3		1,0946	1,0946
3	3			1,2224
Sig.		1,000	,351	,057

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,005.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 3,3333

METRIBUZIN = 2, WEEK = 2, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,853[a]	5	,171	16,862	,000
Intercept	24,319	1	24,319	2404,286	,000
TREATMEN	,853	5	,171	16,862	,000
Error	,121	12	,010		
Total	25,293	18			
Corrected Total	,974	17			

a. R Squared = ,875 (Adjusted R Squared = ,823)

b. LINURON = 2, WEEK = 2, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset	
treatment	N	1	2
4	3	,6862	
6	3		1,1774
5	3		1,2480
2	3		1,2495
1	3		1,2861
3	3		1,3269
Sig.		1,000	,488

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,010.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 6,6666

METRIBUZIN = 2, WEEK = 2, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,463[a]	5	,093	13,627	,000
Intercept	25,120	1	25,120	3695,368	,000
TREATMEN	,463	5	,093	13,627	,000
Error	,082	12	,007		
Total	25,665	18			
Corrected Total	,545	17			

a. R Squared = ,850 (Adjusted R Squared = ,788)

b. LINURON = 2, WEEK = 2, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,9385		
3	3	1,0849	1,0849	
2	3	1,1437	1,1437	
6	3		1,2268	
1	3		1,2294	
5	3			1,4648
Sig.		,084	,328	1,000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,007.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 10,0000

METRIBUZIN = 2, WEEK = 2, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,135[a]	5	,027	1,892	,169
Intercept	32,979	1	32,979	2308,039	,000
TREATMEN	,135	5	,027	1,892	,169
Error	,171	12	,014		
Total	33,286	18			
Corrected Total	,307	17			

a. R Squared = ,441 (Adjusted R Squared = ,208)

b. LINURON = 2, WEEK = 2, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1
6	3	1,2504
2	3	1,2559
1	3	1,3541
4	3	1,3574
5	3	1,3992
3	3	1,5045
Sig.		,170

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,014.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 13,3333

METRIBUZIN = 2, WEEK = 2, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 2, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,230[a]	5	,046	6,246	,004
Intercept	29,036	1	29,036	3944,660	,000
TREATMEN	,230	5	,046	6,246	,004
Error	,088	12	,007		
Total	29,354	18			
Corrected Total	,318	17			

a. R Squared = ,722 (Adjusted R Squared = ,607)

b. LINURON = 2, WEEK = 2, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
2	3	1,1085		
4	3	1,1577	1,1577	
3	3	1,2708	1,2708	1,2708
1	3	1,2816	1,2816	1,2816
5	3		1,3591	1,3591
6	3			1,4428
Sig.		,207	,111	,212

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,007.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 2, CONCETR = 16,6666

METRIBUZIN = 2, WEEK = 3, CONCETR = 3,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 3,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,895[a]	5	,579	266,985	,000
Intercept	13,988	1	13,988	6449,797	,000
TREATMEN	2,895	5	,579	266,985	,000
Error	,026	12	,002		
Total	16,909	18			
Corrected Total	2,921	17			

a. R Squared = ,991 (Adjusted R Squared = ,987)

b. LINURON = 2, WEEK = 3, CONCETR = 3,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset			
treatment	N	1	2	3	4
4	3	,0000			
5	3		,9839		
2	3		,9888		
6	3		1,0111	1,0111	
1	3			1,1202	1,1202
3	3				1,1851
Sig.		1,000	,976	,112	,552

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,002.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 3,3333

METRIBUZIN = 2, WEEK = 3, CONCETR = 6,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 6,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1,982[a]	5	,396	34,431	,000
Intercept	24,016	1	24,016	2086,084	,000
TREATMEN	1,982	5	,396	34,431	,000
Error	,138	12	,012		
Total	26,136	18			
Corrected Total	2,120	17			

a. R Squared = ,935 (Adjusted R Squared = ,908)

b. LINURON = 2, WEEK = 3, CONCETR = 6,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
4	3	,4153	
1	3		1,2599
6	3		1,2845
3	3		1,3150
2	3		1,3154
5	3		1,3403
Sig.		1,000	,934

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,012.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 6,6666

METRIBUZIN = 2, WEEK = 3, CONCETR = 10,0000

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 10,0000

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2,411[a]	5	,482	33,512	,000
Intercept	21,641	1	21,641	1504,303	,000
TREATMEN	2,411	5	,482	33,512	,000
Error	,173	12	,014		
Total	24,224	18			
Corrected Total	2,583	17			

a. R Squared = ,933 (Adjusted R Squared = ,905)

b. LINURON = 2, WEEK = 3, CONCETR = 10,0000

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

treatment	N	Subset 1	Subset 2
4	3	,3202	
1	3		1,0962
3	3		1,1121
6	3		1,2941
2	3		1,3395
5	3		1,4168
Sig.		1,000	,058

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,014.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 10,0000

METRIBUZIN = 2, WEEK = 3, CONCETR = 13,3333

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 13,3333

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,722[a]	5	,144	16,508	,000
Intercept	28,578	1	28,578	3269,292	,000
TREATMEN	,722	5	,144	16,508	,000
Error	,105	12	,009		
Total	29,405	18			
Corrected Total	,826	17			

a. R Squared = ,873 (Adjusted R Squared = ,820)

b. LINURON = 2, WEEK = 3, CONCETR = 13,3333

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,8898		
6	3		1,1626	
2	3		1,2072	1,2072
1	3		1,4185	1,4185
5	3			1,4255
3	3			1,4567
Sig.		1,000	,051	,058

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,009.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 13,3333

METRIBUZIN = 2, WEEK = 3, CONCETR = 16,6666

Between-Subjects Factors[a]

		N
treatment	1	3
	2	3
	3	3
	4	3
	5	3
	6	3

a. LINURON = 2, WEEK = 3, CONCETR = 16,6666

Tests of Between-Subjects Effects[b]

Dependent Variable: ARC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,453[a]	5	,091	9,441	,001
Intercept	24,424	1	24,424	2542,413	,000
TREATMEN	,453	5	,091	9,441	,001
Error	,115	12	,010		
Total	24,993	18			
Corrected Total	,569	17			

a. R Squared = ,797 (Adjusted R Squared = ,713)

b. LINURON = 2, WEEK = 3, CONCETR = 16,6666

Homogeneous Subsets

ARC[c]

Tukey HSD[a,b]

		Subset		
treatment	N	1	2	3
4	3	,9083		
2	3	,9911	,9911	
1	3		1,1990	1,1990
3	3			1,2706
5	3			1,2920
6	3			1,3281
Sig.		,897	,171	,606

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = ,010.

a. Uses Harmonic Mean Sample Size = 3,000.

b. Alpha = ,05.

c. LINURON = 2, WEEK = 3, CONCETR = 16,6666