CRANFIELD UNIVERSITY

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Ph.D. Thesis

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USE OF FUNGI IN BIOREMEDIATION OF PESTICIDES

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ABSTRACT

Eight isolates (7 species) of white rot fungi were grown on soil extract agar amended with 0, 5 10 and 20 mg l^{-} simazine, trifluralin and dieldrin, individually and as a mixture, under two different water regimes (-0.7 and -2.8 MPa water potential). The best isolates were *T.versicolor* (R26 and R101) and *P.ostreatus*, exhibiting good tolerance to the pesticides and water stress and the ability to degrade lignin and produce laccase in the presence of these pesticides.

As a result, the activity of those three isolates plus *Phanerochaete chrysosporium* (well described for its bioremediation potential) was examined in soil extract broth in relation to differential degradation of the pesticide mixture at different concentrations (0-30 mg l^{-1}) under different osmotic stress levels (-0.7 and -2.8 MPa). Enzyme production, relevant to P and N release (phosphomonoesterase, protease), carbon cycling (β-glucosidase, cellulase) and laccase, involved in lignin degradation was quantified. The results suggested that the test isolates have the ability to degrade different groups of pesticides, supported by the capacity for expression of a range of extracellular enzymes at both -0.7 and -2.8 MPa water potential. *P.chrysosporium* and *T.versicolor* R101, were able to degrade this mixture of pesticides independently of laccase activity, whereas *P.ostreatus* and *T.versicolor* R26 showed higher production of this enzyme. Complete degradation of dieldrin and trifluralin was observed, while about 80% of the simazine was degraded regardless of osmotic stress treatment in a nutritionally poor soil extract broth. The results with toxicity test (Toxalert®10), suggested the pesticides were metabolised. .Therefore the capacity for the degradation of high concentrations of mixtures of pesticides and the production of a range of enzymes, even under osmotic stress, suggested potential applications in soil.

Subsequently, microcosm studies of soil artificially contaminated with a mixture of pesticides (simazine, trifluralin and dieldrin, 5 and 10 mg kg $soil^{-1}$) inoculated with *P.ostreatus*, *T.versicolor* R26 and *P.chrysosporium*, grown on wood chips and spent mushroom compost (SMC) were examined for biodegradation capacity at 15ºC. The three test isolates successfully grew and produced extracellular enzymes in soil. Respiratory activity was enhanced in soil inoculated with the test isolates, and was generally higher in the presence of the pesticide mixture, which suggested increased

mineralization. Cellulase and dehydrogenase was also higher in inoculated soil than in the control especially after 12 weeks incubation. Laccase was produced at very high levels, only when *T.versicolor* R26 and *P.ostreatus* were present. Greatest degradation for the three pesticides was achieved by *T.versicolor* R26, after 6 weeks with degradation rates for simazine, trifluralin and dieldrin 46, 57, and 51% higher than in natural soil. And by *P.chrysosporium*, after 12 weeks, with degradation rates 58, 74, and 70% higher than the control. The amendment of soil with SMC also improved pesticide degradation (17, 49 and 76% increase in degradation of simazine, trifluralin and dieldrin compared with the control).

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1 Literature Review and Objectives

1.1 Introduction

Currently there are a number of possible mechanisms for the clean-up of pesticides in soil, such as chemical treatment, volatilization and incineration. Chemical treatment and volatilization, although feasible are problematic as large volumes of acids and alkalis are produced and subsequently must be disposed of. Incineration, which is a very reliable physical-chemical method for destruction of these compounds, has met serious public opposition, because of its potentially toxic emissions, and its elevated economic costs (Kearney, 1998; Zhang and Quiao, 2002). Overall most of these physical-chemical cleaning technologies are expensive and rather inefficient (Kearney, 1998; Nerud *et al.*, 2003) because the contaminated soil has to be excavated at a site and moved to a storage area where it can be processed. Due to environmental concerns associated with the accumulation of pesticides in food products and water supplies there is a great need to develop safe, convenient and economically feasible methods for pesticide remediation (Zhang and Quiao, 2002). For this reason several biological techniques involving biodegradation of organic compounds by microorganisms have been developed (Schoefs *et al.*, 2004).

The use of microorganisms (fungi or bacteria), either naturally occurring or introduced, to degrade pollutants is called bioremediation (Pointing, 2001). Microbial metabolism is probably the most important pesticide degradative process in soils (Kearney, 1998) and is the basis for bioremediation, as the degrading microorganisms obtain C, N or energy from the pesticide molecules (Gan and Koskinen, 1998).

The goal of bioremediation is to at least reduce pollutant levels to undetectable, non-toxic or acceptable levels, i.e. within limits set by regulatory agencies (Pointing, 2001) or ideally completely mineralize organopollutants to carbon dioxide. From an environmental point of view this total mineralization is desirable as it represents complete detoxification (Gan and Koskinen, 1998).

The use of bioremediation to remove pollutants is typically less expensive than the equivalent physical-chemical methods. This technology offers the potential to treat contaminated soil and groundwater on site without the need for excavation (Balba *et al.*, 1998; Kearney, 1998), it requires little energy input and preserves the soil structure (Hohener *et al.*, 1998). Perhaps the most attractive feature of bioremediation is the reduced impact on the natural ecosystems, which should be more acceptable to the public (Zhang and Quiao, 2002).

The complexity of microbial mechanisms for degradation of organopollutants as well as the time period before microbial degradation starts, requiring weeks to months, has made the technology slow to emerge as a viable method of remediation (Nerud *et al.*, 2003). It becomes apparent that more detailed studies of the principles of biodegradation, and the development of efficient methods of decontamination are needed to solve the hazardous waste problem (Nerud *et al.*, 2003).

At present bioremediation conducted on a commercial scale utilises prokaryotes, with comparatively few recent attempts to use white rot fungi. These filamentous organisms however, offer advantages over bacteria in the diversity of compounds they are able to oxidise (Pointing, 2001). In addition, they are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than bacteria (Evans and Hedger, 2001). Therefore, white rot fungi represent a powerful prospective tool in soil bioremediation and some species have already been patented (Sasek, 2003). Interestingly only a few companies have included the use of ligninolytic fungi for soil remediation into their program, for example "EarthFax Development Corp." in USA or "Gebruder Huber Bodenrecycling" in Germany.

1.2 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). The mycelial growth habit is also advantageous as it allows rapid colonisation of substrates, and hyphal extension enables penetration of soil reaching pollutants in ways that other organisms cannot do (Reddy and Mathew, 2001). This can maximise physical, mechanical and enzymatic contact with the surrounding environment (Maloney, 2001). In addition, these fungi use 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) and do not require pre-conditioning to a particular pollutant, because their degradative system is induced by nutrient deprivation (Barr and Aust, 1994).

1.2.1 White rot fungi

To understand the ability to degrade contaminants it is important to analyse 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 mineralised 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 obtain energy.

The usual biological answer to breaking down biopolymers is to use highly specific enzymes. This approach is normally extremely effective as a minimum amount of protein (enzyme) is synthesised by the organism to cleave the polymer (Evans and Hedger, 2001). However, the way white rot fungi degrade wood is rather different, because lignin is hydrophobic and highly insoluble, which poses problems for catalysis by enzymes, that tend to be water soluble (Harvey and Thurston, 2001).

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 ether 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 (Bar and Aust, 1994) extracellularly.

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 dimerisation/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 are 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 and Aust, 1986).

1.2.2 Evidence for enzyme mediated contaminant degradation by white rot fungi

There are many studies on degradation of pollutants by ligninolytic fungi, and the range of xenobiotics degraded by these microorganisms is very wide. Table 1.1 shows some examples of compounds degraded by white rot fungi.

Although the precise role of enzymes in pesticide degradation by white rot fungi is yet to be established, there is mounting evidence which suggests that the lignin degradation enzyme complex (lignin peroxidases, manganese peroxidases and laccases) is responsible at least in part for the degradative capabilities of these fungi. However, not all fungi produce all these enzymes simultaneously, and it has often been difficult to find a distinct correlation between the pollutant degradation and production of enzymes even if the enzymes have been detected (Nerud *et al.*, 2003). Previous research has shown interesting associations between contaminant degradation and enzyme activity.

Table 1.1 Degradation of typical environmental pollutants by ligninolytic basidiomycetes

Fungus	Type of pollutant	Reference
P.chrysosporium	Lindane, DDT	Bumpus *et al.*, 1985
	BTEX (benzene, toluene, ethylbenzene and xylene)	Yavad and Reddy, 1993
	Atrazine	Hickey *et al*., 1994
P.chrysosporium, *Phanerochaete eryngi*, *Pleurotus florida* and *Pleurotus sajor-caju*	Heptachlor and lindane	Arisoy, 1998
P.chrysosporium and *Trametes versicolor*	Pentachlorophenol	Alleman *et al.*, 1992
T.versicolor	Pesticides	Khadrani *et al*., 1999 Morgan *et al*., 1991
Pleurotus ostreatus	Pesticides	Khadrani *et al*., 1999
	Dyes	Sasek *et al*., 1998
	Catechol, pyrene and phenanthrene	Bezalel *et al*., 1996
Pleurotus pulmonaris	Atrazine	Masaphy *et al.*, 1996
Bjerkandera adusta	Pesticides	Khadrani *et al*., 1999

Laccase activity in biodegradation of xenobiotic compounds with lignin-like structures has already attracted considerable interest (Trejo-Hernandez *et al.*, 2001), and its biodegradative properties have been studied exhaustively for different contaminants. This enzyme is a copper-containing phenoloxidase involved in the degradation of lignin (Pointing, 2001) and it oxidises phenol and phenolic lignin sub-structures (Tuor *et al.*, 1995). The catabolic role of fungal laccase in lignin biodegradation is not well understood (Heggen and Sveum, 1999; 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 Demir (2004) biological degradation of benzene and toluene by *T.versicolor* was analyzed and the biomass determined. Within an incubation period of 48 hrs, it was observed that, removal was completed after 4 hours when initial toluene concentration was 50 mg l^{-1} and was completed in 36 hrs when this was 300 mg l^{-1}. Biodegradation was completed by the end of the 4^{th} hr at benzene concentrations of 50 mg l^{-1} while it continued for 42 hrs at 300 mg l^{-1}. With the addition of veratryl alcohol, a laccase inducer, to the basic feed medium, the operation of the enzyme system was enhanced and biodegradation completed in a shorter time period (Demir, 2004).

Han *et al.* (2004) studied the degradation of phenanthrene by *T.versicolor* and its laccase was purified. After 36 hrs incubation, about 46 and 65% of 100 mg l^{-1} of phenanthrene added in shaken and static fungal cultures were removed, respectively. Although the removal percentage was highest (76.7%) at 10 mg l^{-1} of phenanthrene, the transformation rate was maximal (0.82 mg h^{-1}) at 100 mg l^{-1} of phenanthrene in the fungal culture. When the purified laccase of *T.versicolor* reacted with phenanthrene, the compound was not transformed.

Another interesting example of contaminant degradation and enzyme activity was in the study described by Barr and Aust (1994). They described cyanide to be quite toxic to spores of *P.chrysosporium* (50% inhibition of glucose metabolism at 2.6 mg l^{-1}).

This toxicity was due to the absence of lignin peroxidases which can rapidly metabolise cyanide, as ligninolytic 6 day old cultures could tolerate considerably higher cyanide concentrations (50% inhibition of glucose metabolism at 182 mg l^{-1}).

Valli *et al.* (1992) demonstrated the mineralization of the dioxin 2,7-dichlorobenzeno-p-dioxin by *P.chrysosporium*. Their results showed that purified lignin peroxidases and manganese peroxidases were capable of mineralization in a multi-step pathway. Esposito *et al.* (1998) showed that different actinomycetes were able to degrade diuron in soil, using manganese peroxidases.

Regardless of many suggestions that the degradation of xenobiotics by white rot fungi is mediated by enzymes involved in lignin degradation some authors have found contradictory evidence. For example, Jackson *et al.* (1999) reported degradation of TNT by non-ligninolytic strains of *P.chrysosporium*. Bending *et al.* (2002) showed >86% degradation of atrazine and terbuthylazine by white rot fungi in liquid culture and found no relationship between degradation rates and ligninolytic activity. Other studies with *P.chrysosporium* in liquid culture have reported biotransformation of the insecticide lindane independently of the production of ligninolytic enzymes (Mougin *et al.*, 1996). These researchers ruled out the involvement of peroxidases in lindane biotransformation and mineralization, and they assessed the activity of the cytochrome P450 monooxygenase, an enzymatic system used by many organisms as a detoxification tool. They found that a P450 inactivator (1-aminobenzotriazole) drastically reduced pesticide metabolism, but phobarbital, a P450 inducer, did not increase lindane breakdown.

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*. It is essential to assess the production of theses enzymes in soil, because that is where bioremediation will take place in field conditions, and because there are considerably more studies on enzyme production in liquid cultures than in soil. Additionally, there is little information on the degradation of mixtures of pesticides, which in

environmental conditions are more common than contamination with single pesticides.

1.3 Feasibility of bioremediation by white rot fungi

1.3.1 Terminology

In practice bioremediation of organopollutants *in situ* generally applies to contaminated soils. Two approaches are recognised: biorestoration in which the physico-chemical nature of the soil (e.g. nutrients, aeration) is altered to encourage indigenous microorganisms to degrade the pollutant and bioaugmentation in which a known degrading microorganism is introduced in the contaminated soil (with or without physico-chemical alteration) (Pointing, 2001).

1.3.2 Soil contamination

Since the earliest times, societies have used soil as a quick and convenient disposal route for waste (Ashman and Puri, 2002), but only recently it was found that contaminants in the soil can find their way to other areas of the environment (Figure 1.1). This escape of contaminants is very serious, since other environmental niches even more fragile than soil may become contaminated. Soils are contaminated when they have elevated concentrations of chemicals (usually as a result of human activity) compared with soil that are regarded as being in pristine condition. Contamination becomes "pollution" once these elevated concentrations begin to have an adverse effect on organisms (Ashman and Puri, 2002).

Most soils are to some extent contaminated by naturally occurring harmful or toxic elements, but not all soils are polluted (Bridges, 1997). The most common soil pollutants include metallic elements and their compounds, asbestos, organic chemicals, oils and tars, pesticide residues, explosive and asphyxiant gases, and radioactive materials (Bridges, 1997). These substances often arrive in the soil as a result of intentional disposal, such as spillages and from atmospheric fallout (Bridges, 1997). Among these contaminants, pesticides are of primary importance due to their continuous entry into the soil environment (Sannino and Gianfreda, 2001).

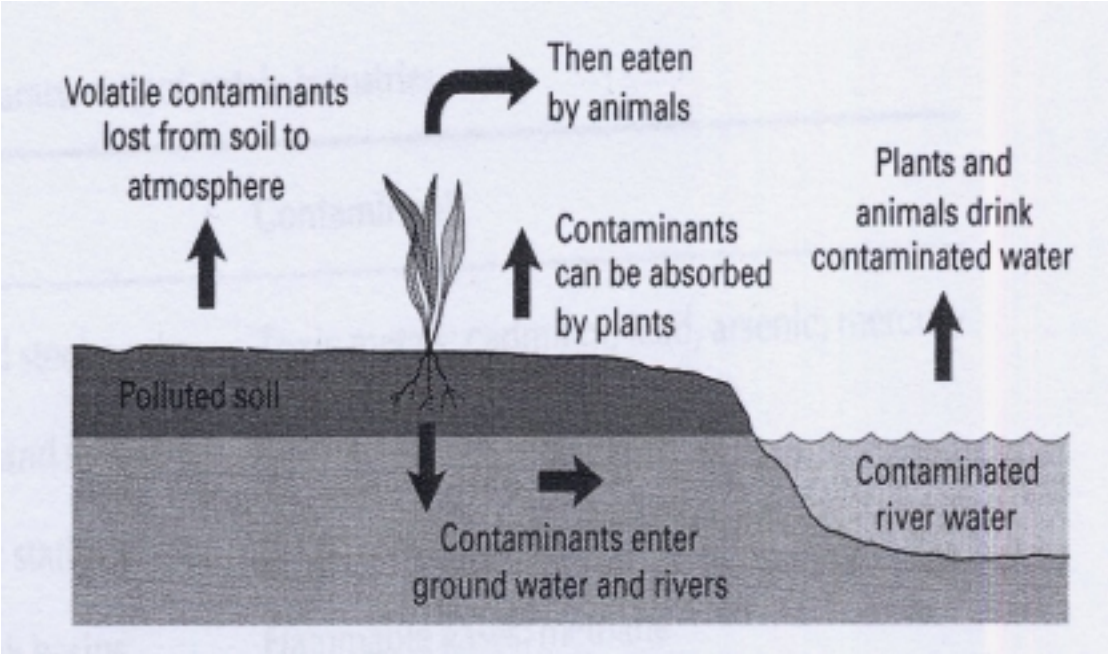


Figure 1.0.1 Contaminants in soil can find their way to other areas of the environment (Ashman and Puri, 2002).

1.3.3 Pesticides

The term "pesticides" embraces an enormous diversity of products that are used in a number of different activities (Mourato *et al.*, 2000), especially agriculture, that currently accounts for 75% of the total use of pesticides (Buyuksonmez, 1999).

Besides agricultural applications, large amounts of pesticides are used for maintaining urban plantings, hygienic handling and storage, control of vegetation beneath power lines and along railways and roadways, mosquito and fly control, preservation of wood and control of mould growth in paper mills. Moreover, pesticides have played a great role in reducing diseases such as malaria and typhus fever. It has been estimated that the use of DDT saved approximately 5 million lives and prevented a hundred million illnesses in the 1940's (Buyuksonmez, 1999).

1.3.4 Environmental concerns regarding the contamination with pesticides

Several hundred pesticides of different chemical nature are currently widely used for agricultural purposes throughout Europe and the USA (Barceló, 1991), which resulted in mixed impacts: on the one hand utilization of pesticides produces an enormous increase in agricultural productivity (Kuo and Regan, 1999). On the other hand, due to their widespread use, pesticides are currently detected in various environmental matrices such as soil, water and air (Barceló, 1991) and there is great concern about their potential environmental hazard (Sannino *et al.*, 1999).

Contamination with pesticides can lead to pollution of surface water and groundwater (Kuo and Regan, 1999; Juhler *et al.*, 2001), reduced biodiversity and depression in soil heterotrophic bacteria (including denitrifying bacteria), and fungi (Ahmed *et al.*, 1998). Inadequate management practices specifically involving the on-farm handling of pesticides appear to be a major source of pesticide contamination (Kuo and Regan, 1998). The wastes and rinsates from spray and storage equipment are sources of contamination (Kuo and Regan, 1999) and soil disposal is the most common method of handling these diluted pesticide wastes (Schoen and Winterlin, 1987). Also of great concern are pesticide spills or waste disposal sites, which are characterized by the presence of large quantities of pesticides often in mixtures in localized areas of soil (Gan and Koskinen, 1998). Contamination at these sites is described as point source contamination whereas contamination resulting from the application of pesticides for the purpose of pest management is called non-point source contamination (Gan and Koskinen, 1998). In the latter case pesticide presence is widespread, but at relatively low concentrations.

When the concentration of a pesticide, its metabolites or 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 1 mg kg soil^{-1} or a concentration arrived at after a risk assessment analysis, usually on a site-by-site basis (Kearney, 1998). Curiously, when

pesticides are applied at normal agricultural rates, roughly <1-4.5 kg ha^{-1} residues in the surface soils range in concentration from <0.5 to 2.5 mg kg $soil^{-1}$ (ppm) (Kearney, 1998).

Even when the level of contaminants is low and does not exceed a few μg kg $soil^{-1}$ (ppb), 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 0.1 μg kg $soil^{-1}$ and 0.5 μg kg $soil^{-1}$ in total for all substances (European Directive 80/778/EEC and decree of 3rd January 1989).

The chemical properties of pesticides determine their retention and transport in soils, (Kearney, 1998). A pesticide can reach groundwater if its water solubility is greater than about 30 mg l^{-1}; its adsorptivity, Koc (Koc= partition coefficient between soil organic carbon and water), is less than 300-500 ml g^{-1}; 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 (Barceló, 1991).

1.3.5 Simazine, trifluralin and dieldrin

The most common pesticides are herbicides, insecticides and fungicides, where herbicides account for nearly 50% of all pesticides used in developed countries and insecticides account for 75% of all pesticides used in developing countries (Maloney, 2001). Figure 1.0.2 compares the persistence of different pesticides in soil. In the current study, we used three pesticides, two herbicides (simazine and trifluralin) and one insecticide (dieldrin). It shows that dieldrin is the most persistent of the pesticides employed in this study, followed by simazine and then trifluralin. They are included in the "UK Red List of Substances", a list issued by the Department of the Environment for the purposes of controlling the input of dangerous substances into the aquatic environment. The chemical structure of these pesticides is shown in Figure 1.0.3.

Simazine is one of the most widely used herbicides in the world. It is the least water soluble of all triazine herbicides and is therefore very immobile in soil and very

ineffective as a foliar application to weeds (McEwen and Stephenson, 1979). It is used to control broad-leaved weeds and annual grasses in field, berry fruit, nuts, vegetable and ornamental crops, turf-grass, orchards and vineyards. At higher rates it is used for non-selective weed control in industrial areas (Extoxnet, 1996). The acute oral LD_{50} in rats is >3000 mg kg^{-1}, it is non-toxic to birds and bees (Extoxnet, 1996) and is moderately persistent. Simazine residues have been detected in groundwater at concentrations in the range of 0.02-0.1 μg l^{-1}(ppb) to 3.4 μg l^{-1}.

Trifluralin is a pre-emergence herbicide, which must be incorporated in soil within 24 hours of application. It is used in crops such as maize, rapeseed, barley, cotton, carrots, orange, grapefruit, peach, peppermint (Extoxnet, 1996). Although pure trifluralin is not acutely toxic to test animals by oral, dermal or inhalation routes, certain formulated products may be more toxic than the technical material itself (Heged *et al.*, 2000). Trifluralin volatilises easily, but once incorporated in the top 3-5 cm of soil it is strongly absorbed by soil, being of moderate to high persistence (Heged *et al.*, 2000). Trifluralin is of low toxicity to birds and mammals with an acute oral LD_{50} in rats >10000 mg kg^{-1} (Extonet, 1996).

Dieldrin is a chlorinated hydrocarbon insecticide of high toxicity (LD_{50} in rats=37-87 mg kg^{-1}) and high persistence in soil. This insecticide is very immobile in soil and its 95% disappearance rate is 5-25 years. Morrison *et al.* (2000) reported the presence of dieldrin in soil 15 years after the previous application. Volatilisation is responsible for much of the dieldrin lost from the soil surface. Persistence is also affected by soil type. Soils with high organic matter content showing higher dieldrin binding and persistence than sandy soils (Extoxnet, 1996).

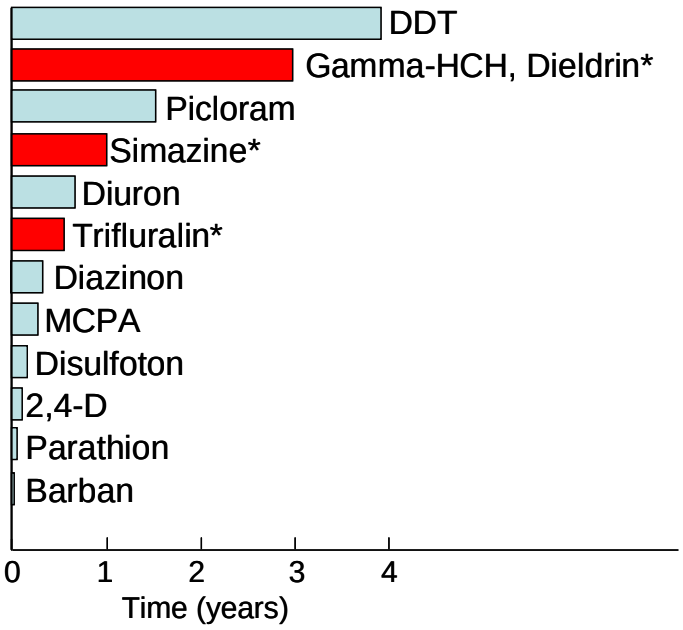


Figure 1.0.2 The persistence in soil of some common pesticides (From Ashman and Puri, 2002) *show the pesticides used in this study.

A B C

Figure 1.0.3 Chemical structure of simazine (A), trifluralin (B) and dieldrin (C).

1.4 Enhancing growth of white rot fungal growth in soil

Currently, there are many studies being carried out to optimise the biodegradation potential of white rot fungi in contaminated soil (Ryan and Bumpus, 1989; Morgan *et al*., 1993; McFarland *et al*., 1996; Meysami and Baheri, 2003). If it is accepted that the extracellular ligninolytic enzymes are at least in part responsible for the critical initial reactions of pollutant transformation, the production and activity of these enzymes in contaminated soil under field conditions are two prerequisites for successful application of white rot fungi in soil bioremediation (Lang *et al.*, 1998).

In natural soil a wide range of saprophytic microorganisms exist. Introduction of white rot fungi requires effective growth and competition with these native populations. Additionally the bioremedial fungi should be able to secrete the necessary enzymes into the soil matrix to enhance degradation of pesticide molecules that they would otherwise be unable to incorporate across cell walls (Canet *et al.*, 2001).

Most of the protocols for delivering inoculum of wood rot fungi for soil bioremediation have been adopted from mushroom growers, who have perfected the art of producing fungal spawn on lignocellulosic waste. Species used in mushroom production have been formulated on inexpensive substrates such as corn cobs, sawdust, wood chips, peat or wheat straw. When used in bioremediation these substrates are impregnated with mycelium and mixed with contaminated soil (Paszczinksi and Crawford; 2000; Reddy and Mathew, 2001). There is little information available on survival of white rot fungi in soil, especially fungi that are not used for human consumption. Several groups are investigating ways to improve the survival of wood rot fungi in polluted soils (Ryan and Bumpus, 1989; Morgan *et al*., 1993; Bennet *et al.*, 2001).

Certainly, better fungal growth could help introduced fungi to overcome competition from indigenous microorganisms, and enhance bioremediation. This is critical as native soil microorganisms may occupy the lignocellulosic substrate and restrain growth and activity of the white-rot fungus, inhibiting fungal lignino-cellulose decomposition and reducing the enzymes released (Lang *et al.*, 2000b).

Boyle (1995) reported an increase in growth and carbon dioxide production in natural soil supplemented with carbon amendments, and observed that mineralisation of [^{14}C] penthachlorophenol (degradation to $^{14}CO_2$) was much faster in soil that had been amended with alfalfa and benomyl and inoculated with *T.versicolor*. Another study showed that the addition of straw increased the hyphal length of white rot fungi in soil (Morgan *et al.*, 1993).

Besides strong growth capabilities, it is important that the inoculation conditions promote enzyme production. Moredo *et al.* (2003) investigated ligninolytic enzyme production by the white rot fungi *P.chrysosporium* and *T.versicolor* pre-cultivated on different insoluble lignocellulosic materials: grape seeds, barley bran and wood shavings. Cultures of *P.chrysosporium* pre-grown on grape seeds and barley bran showed maximum lignin peroxidase and manganese-dependent peroxidase activities (1000 and 1232 units l^{-1}, respectively). *T.versicolor* pre-cultivated with the same lignocellulosic residues showed maximum laccase activity (around 250 units l^{-1}). *In vitro* decolouration of the polymeric dye Poly R-478 by the extracellular liquid obtained in the above-mentioned cultures was monitored in order to determine the respective capabilities of laccase, LiP and MnP. It is noteworthy that the degrading capability of LiP when *P.chrysosporium* was pre-cultivated with barley bran gave a percentage of decolouration of about 80% in 100 seconds (Moredo *et al.,* 2003).

The utilisation of these solid substrates in soil may also be advantageous as a means to distribute fungal inoculum evenly in large volumes of soil (Harvey and Thurston, 2001), and according to Singleton (2001) growth amendments could also exert beneficial effects by sorbing pollutants and hence decreasing the amount of toxic pollutant that is bioavailable.

1.5 Evidence for pesticide degradation in soil by white rot fungi

Although the majority of studies on bioremediation of pesticides using white rot fungi has not been conducted in soil, these organisms have already been demonstrated to be capable of transforming and/or mineralising several individual pesticides in soil. The diversity of known pesticide compounds degraded by several white rot fungi in soil is reviewed in this section, as well as some ecophysiological features of these fungi.

1.5.1 Phanerochaete chrysosporium

P.chrysosporium was the first fungus to be associated with degradation of organopollutants, because it has been extensively studied as a model microorganism in research on the mechanism of lignin degradation (Sasek, 2003). This thermophilic basidiomycetes, was first considered a problem in the 1970s in self-heating wood chip piles, in its anamorphic state *Sporotrichum pulverulentm* (Burdsal, 1981). Although later this fungus was the subject of many investigations on cellulase and ligninase production; because of their potential in bioremediation, its natural niche remains unknown (Evans and Hedger, 2001).

Most research on *P.chrysosporium* has been conducted in liquid and/or synthetic media, less is known regarding its bioremediative capabilities in soil. The work carried out by Lamar and co-workers is invaluable in this respect (Lamar and Dietrich, 1990; Davis *et al.,* 1993; Lamar *et al.,* 1993; Lamar *et al.,* 1994). They concentrated on the use of *P.chrysosporium*, *Phanerochaete sordida* and *Trametes hirsuta* to detoxify pentachlorophenol and creosote contaminated soil on a field scale. Of the three fungi *P.sordida* proved to be the most effective inoculant as it showed the highest transformation ability, and was able to grow at lower temperatures. In one field study the temperature decreased to 8^0C, posing problems for growth of *P.chrysosporium* as this fungus grows slowly at such environmental temperatures. It has a temperature optimum of 39ºC. Tekere *et al.* (2001) and Hestbjerg *et al.* (2003) reported that field conditions did not always enable *P.chrysosporium* to achieve optimum activity and therefore it was not a good competitor in the soil environment (Sack and Fritsche, 1997; Hestbjerg *et al.*, 2003). This last point was reinforced by Radtke *et al.* (1994) who reported bacteria that from polluted and agricultural soil antagonise the growth of *P.chrysosporium* on solid media.

Nevertheless, some studies have described the successful application of *P.chrysosporium* as a bioremediation agent in soil. For example McFarland *et al.* (1996) described complete alachlor transformation by this fungus, within 56 days of treatment. Reddy and Mathew (2001) also showed that this species was able to degrade DDT, lindane and atrazine.

1.5.2 *Pleurotus ostreatus*

P.ostreatus is an edible species, commonly known as the oyster mushroom (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 sterilised (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 and Sveum, 1999).

P.ostreatus has been tested for lindane degradation, and found to be effective decreasing concentrations from 345 to 30 mg l^{-1}, within 45 days, in a bench-scale test (www.earthfax.com). Subsequent pilot-scale tests were conducted utilising macro-scale plots with capacities of about 2 cubic yards: lindane concentrations decreased from 558 to 37 mg l^{-1} in 274 days. Following performance of the pilot-scale tests, approximately 750 tonnes of contaminated soil were excavated. The contaminated soil was mixed with 16% (w/w) fungal inoculum (i.e. sawdust and cottonseed hulls thoroughly colonised with *P. ostreatus*). Initial lindane concentrations ranged from 7.1 to 37 mg l^{-1}, averaging 21 mg l^{-1}. After 24 months of treatment lindane concentrations had decreased by 97% to 0.57 mg l^{-1}, achieving the industrial treatment goal of 4.4 mg l^{-1} and almost also reaching the residential risk-based concentration of 0.49 mg l^{-1} (www.earthfax.com).

Novotny *et al.* (1999) also described *P.ostreatus* as a suitable candidate to apply for the clean-up of soils contaminated with recalcitrant pollutants because of its capability of robust growth and efficient extracellular enzyme production in soil even in the presence of pollutants such as PAHs. They suggested that mycelial growth through contaminated soil and efficient enzyme expression were the key to removal of the pollutant molecules from the bulk soil.

1.5.3 Trametes versicolor

The basidiomycete *Trametes* (syn. *Coriolus*, *Polyporus*, *Polystictus*) *versicolor* is a very efficient white rot species in nature (Staszak *et al.*, 2000), that was first studied by Dodson *et al.* (1987). This fungus causes rapid white rot invasion of moribund or fallen trees of species such as birch, beech and oak, by a rapidly extending mycelium which utilises free sugars in the wood of the tree (Evans and Hedger, 2001). *T.versicolor* has been used in bioremediation research because of its effective extracellular laccase production and high tolerance to pentachlorophenol. However, little knowledge exists on its capacity in natural soil (Sasek, 2003). One example is described by Tuomela *et al.* (1999) that showed *T.versicolor* mineralised 29% of added PCP during 42 days of growth in soil. However, soil environmental conditions were not studied in detail, which could have a big importance on degradation rates.

1.5.4 Other white rot fungi

Lentinus edodes, the gourmet mushroom has been shown to possess the capacity for removing more than 60% of pentachlorophenol from soil (Pletsch *et al.*, 1999) and appears to remain active at lower temperatures that are typical of temperate soils of central and Northern Europe (Okeke *et al.*, 1996).

Kodama *et al*. (2001) reported degradadation of simazine by *Penicillium steckii*, isolated from soil samples where the herbicide had been spread. Simazine was gradually degraded by this fungus in mineral media containing yeast extract at 25 mg l^{-1} and the pesticide at 50 mg l^{-1}. It was observed that the rate of simazine degradation was improved when assimilable carbon sources were added to the medium. They reported a breakdown of 53% simazine after 5 days of cultivation at 30ºC, when glucose was added to the basal medium.

1.6 Application of spent mushroom compost in soil remediation

Composting matrices and composts are rich sources of xenobiotic-degrading microorganisms including bacteria, actinomycetes and ligninolytic fungi, which can degrade pollutants to innocuous compounds such as carbon dioxide and water. These microorganisms can also bio-transform pollutants into less toxic substances and/or

lock up pollutants within the organic matrix, thereby reducing pollutant bioavailability (Semple *et al.*, 2001).

Spent mushroom compost (SMC) is a by-product of the mushroom production, which is produced in large amounts. For every 200g of *Pleurotus* spp produced in Malaysia, about 600 g of spent compost is produced (Singh *et al.*, 2003) or 5kg of SMC generated for every 1 kg of edible mushrooms according to Law *et al.* (2003). This resulted in 40 Mtonnes of SMC in 1999. The disposal of SMC is a major problem for mushroom farmers. They either discretely burn or discard it (Singh *et al.* 2003) and thus its exploitation as a potential bioremediation adjuvant has received significant attention (Chiu *et al.*, 2000).

Mushroom cultivation involves the pure culture of spawn, composting and pasteurization of the substrate and careful regulation of growing conditions (Ball and Jackson, 1995). The substrates are lignocellulosic residues, such as straw, horse manure, chicken manure and activators (Ball and Jackson, 1995). The purpose of composting the substrate is to exclude microorganisms that may interfere with mushroom growth. Following mushroom harvest, SMC is likely to contain not only a large and diverse group of microorganisms but also a wide range of extracellular enzymes active against wheat straw (Ball and Jackson, 1995). Singh *et al.* (2003) reported extraction of cellulase, hemicellulose, β-glucosidase, lignin peroxidases and laccase from SMC. It also contains a very high organic content (20%) including cellulose hemicellulose and lignin (Kuo and Regan, 1999), from the unutilised lignocellulosic substrate (Singh *et al.* 2003).

Previous research showed some interesting findings using this type of compost as a bioremediation adjuvant: Law *et al.* (2003) reported that SMC of *Pleurotus pulmonarius* could remove 89.0 +/- 0.4% of 100 mg PCP l^{-1} within 2 days at room temperature predominantly by biodegradation. Kuo and Regan (1999) used sterilised SMC as an adsorption medium for removal of a mixture of pesticides (carbaryl, carbofuran and aldicarb) with a concentration range of 0-30 mg l^{-1} and found that SMC was able to adsorb carbamate pesticides from aqueous solutions successfully, which was possibly related to the increased organic matter content.

With mushroom production being the largest solid state fermentation industry in the world (Lau *et al.*, 2003) and with so much waste being produced, it is extremely important to find a good use for SMC. Thus, the use of this residue as a soil amendment to improve pesticide bioremediation is an interesting area for research. Furthermore, there is no information on the effect of the addition of SMC on soil enzymes, soil respiration and soil populations, and how these metabolic parameters are affected by the presence of pesticides and water availability.

1.7 Environmental factors affecting bioremediation by fungi

1.7.1 Temperature, oxygen and nutrient availability

Fungal bioremediation is subject to the prevailing temperature, moisture and soil conditions (Kearney, 1998). The soil pH, nutritional status and oxygen levels vary and may not always be optimal for fungal growth or extracellular enzyme production for pollutant transformation (Singleton, 2001). Thus, the kinetics of pesticide degradation in the field is commonly biphasic with a very rapid degradation rate in the beginning followed by a very slow prolonged dissipation. The remaining residues are often quite resistant to degradation (Alexander, 1994).

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). 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).

Anaerobic conditions may also contribute because fungal degradation is very slow under oxygen restrictions resulting in partial degradation with resultant toxic intermediates being formed (Romantschuk *et al.*, 2000). Other factors that can contribute to pesticide degradation in soils include the chemical nature of the pesticide, amount and type of soil organic matter, microbial community structure and

activity, soil type, pH, pesticide concentration, pesticide formulation and presence of other pesticides (Shoen and Winterlin, 1987).

1.7.2 Water availability

The concept of water activity and water potential

Total water content of a substrate is not always a good indicator of water availability for microbial growth. For example, the water content of a solid substrate consists of strongly bound water and free weakly bound water. Free water is more readily available for microbial growth and how easily this can be removed depends on the water content and the type of the substrate. Water activity (a_w) is defined by the ratio between the vapour pressure of water in a substrate (P) and the vapour pressure of pure water (P_0) at the same temperature and pressure. Thus:

$$a_w = \frac{P}{P_o}$$

The a_w of pure water is 1 and any substrate with no free water will subsequently have an a_w less than 1.

Water potential (Ψ_w) is an alternative measure to a_w, and is defined as the amount of work that must be done per unit quantity of pure water in order to transport reversibly and isothermally an infinitesimal quantity of water from a pool of pure water at atmospheric pressure to a point in a system under consideration at the same point elevation (Griffin, 1981). Ψ_w is the sum of the pressure potential (Ψ_p), solute (or osmotic) potential (Ψ_s)and matric potential (Ψ_m):

$$\Psi_w = \Psi_p + \Psi_s + \Psi_m$$

Ψ_p = pressure potential and is the potential of water per unit volume as affected by external pressure. In filamentous fungi, it represents the turgor potential of the protoplasm created by plasma membranes and cell walls)

Ψ_s = solute (or osmotic) potential and is the potential of water per unit volume as affected by the presence of solutes. It is compared with the chemical potential of pure

water at atmospheric pressure, at the same temperature and height, with the chemical potential of the reference water being set at zero (Salisbury, 1992; Magan, 1997).

Ψ_m = matric potential and is the potential of water per unit volume as affected by the presence of a solid matrix. It is a measure of the tendency for a matrix to adsorb additional water molecules, for example a dry colloid or hydrophilic surface has an extremely negative matric potential (Salisbury, 1992).

Ψ_w is related directly to a_w by the following formula:

$$\Psi_w = \frac{RT}{V_w \ln\ a_w}$$

where R is the ideal gas content, T the absolute temperature and V_w is the volume of 1 mole of water. The advantage of Ψ_w is that it is possible to partition osmotic and matric components and their influence on growth and physiological functioning of microbes. Soil microbiology studies use water potential, while for solid substrates, where solute potential is the major force, a_w is commonly used. The relationship between a_w and Ψ_w is shown in Table 1.2.

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. Matric potential can be manipulated by the addition of PEG8000 (polyethylene glycol), which is a long inert, non-ionic chain polymer.

Soil total water content is often expressed as percentage moisture content. This is the ratio between dry and wet weight. However, soil moisture content does not indicate the quantity of water that is readily available to microorganisms for metabolism.

The availability of water in soil may be a very important factor affecting the success of bioremediation, since water availability affects fungal growth and enzyme production (Marin *et al.*, 1998). Boyle (1995) studied the growth response of *P.chrysosporium* and *T.versicolor* to water stress imposed by polyethylene glycol. Water potential values down to about -0.5 MPa had little effect, but growth of both fungi was progressively inhibited at lower water potential values. In natural soil enzyme activities decreased sharply at higher and lower moisture contents (Boyle,

1995). The carbon dioxide production also decreased in dry soil and remained high when the soil was wet, even though MnP and laccase activities decreased. It is likely that organisms other than white rot fungi were responsible for the production of this carbon dioxide (Boyle, 1995), which suggests that in bioremediation both the inoculant organisms and the native soil microflora are affected by water potential fluctuations.

Matric potential influences the physiological activity of soil microorganisms (Zak *et al.*, 1999) and different fungi may have optimal biodegradation rates at different water availabilities, as reported by Okeke *et al.* (1996). They showed that *L.edodes* was more effective in transforming pentachlorophenol at lower moisture, while *P.chrysosporium* was more effective at higher moisture levels.

Besides affecting microbial behaviour water availability affects pesticide binding and distribution in the soil. The behaviour of organic compounds in water plays a very significant role in their availability for microbial utilization in the environment (Atagana *et al.*, 2003). Water content can have two contrary effects on biodegradation: high water content enhances contact between the contaminant and the bioremedial microorganisms, leading to increased contaminant bioavailability. However, it can also decrease the overall biodegradation rate, because it affects contaminant and oxygen transfer through the aqueous phase (Schoefs *et al.*, 2004). Schoefs *et al.* (2004) also showed that hexadecane biodegradation rates decreased with increasing water content. They suggested that while high water content enhances microbial growth and contaminant desorption, the effect of a limitation of oxygen through the aqueous phase appears to dominate.

Few detailed studies have been carried out on the impact of water stress on fungal growth and enzymatic activity *in vitro* in the presence of pesticides, and even fewer in the presence of mixtures of pesticides.

Furthermore, little information is available on the activity and biodegradation capacities of white rot fungi in soil under different water regimes, which is very relevant to the successful application of a white rot fungus in soil where water availability fluctuates significantly, throughout the year.

Table 1.2 Equivalent water activity, equilibrium relative humidity and water potentials 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	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.30
0.60	60	70.30

1.8 Toxicity of bioremediation products: parameters to assess soil activity

Soil is a complex matrix undergoing constant change in its component parts: chemical, physical and biological. These components are significantly affected by environmental factors and anthropogenic management and influence (Harris and Steer, 2003). Pollutants introduced into the soil exert an influence on the microbiota, which manifests itself in changes in enzyme activity, soil respiration, biomass and microbial populations (Baran *et al.*, 2004). Soil biological investigations can give information on the presence of viable microorganisms as well as on the effects of pollutants on the metabolic activity of soil (Margesin *et al.*, 2000).

In some cases, contaminants have a stimulatory effect on soil enzymes that results from the gradual adaptation of microorganisms to the pollutants and the utilisation of xenobiotics as a source of carbon and energy (Baran *et al.,* 2004). After this period of stress there is an increase in respiratory intensity, an increase in enzyme activity, development of microorganisms and a gradual decomposition of pollutants (Boopathy, 2000).

Since the fungal bioremediation process depends on the extent to which the fungal inoculant succeeds in colonising the contaminated soils, an interesting approach is to assess fungal growth in the soil. Although, this is difficult, because of the problems of quantification which occur when trying to measure the growth of filamentous fungi in such heterogeneous environments. For this reason, in studies with wood rot fungi, indirect methods are often used. These include the evolution of carbon dioxide, the detection of dehydrogenase and ligninolytic enzymes or the removal of the target compound (Bennet *et al.*, 2001) Some of these methods are described bellow.

1.8.1 Soil respiration

The oxidation of organic matter by aerobic organisms results in the production of carbon dioxide (Harris and Steer, 2003). Respiration may increase in response to an increase in microbial biomass or as a result of the increased activity of a stable biomass (Harris and Steer, 2003). Mineralization studies involving measurements of total carbon dioxide production provide useful information on the biodegradability

potential of pesticides in soil. Hollender *et al.* (2003) showed that oxygen consumption and carbon dioxide production as well as the kinetics of these processes are all informative parameters characterizing the whole microbial respiration potential and their nutrient limitation in soil samples.

Soil respiration is determined on the basis of either carbon dioxide evolution or the oxygen consumption rate. The measurement of CO_2 appears to be preferable as it has the advantage of greater sensitivity due to low background concentration present in the atmosphere and enables measurements for any length of time (Dilly, 2001).

1.8.2 Enzyme production

Soil enzyme activities are major candidates as early indicators of ecosystem stress and may function as "sensors", since they integrate information on the microbial status and on soil physico-chemical conditions (Aon *et al*., 2001). Their perturbations may sensitively predict soil degradation earlier than other slowly changing soil properties, such as organic matter (Dick, 1994). Quantification of the different pools of enzyme activity in soils (intra and extracellularly) is desirable to assess the contribution that microbial communities make to production of specific enzymes and their relationship to changes in soils (Klose and Tabatabai, 1999).

Soil microorganisms (bacteria and fungi) are the main source of enzymes and despite their relatively low concentrations, they play a crucial role in nutrient cycling in soil (C, N, P and S) (Aon *et al.*, 2001). Many soil microorganisms depend on the effective production of their extracellular enzymes to supply them with nutrients (Harris and Steer, 2003). Because of their extracellular nature, these enzymes are often trapped in soil organic and inorganic colloids, and some soils will therefore have a large background of extracellular enzymes not directly associated with the microbial biomass (Harris and Steer, 2003). The overall activity of a single enzyme may depend on enzymes in different locations including intracellular enzymes from viable proliferating cells and accumulated or extracellular enzymes stabilized in clay minerals and or complexed with humic colloids (Burns, 1982).

Several studies have examined the effect of pesticides on the activity of enzymes in soils with different origins (Sannino and Gianfreda, 2001). Despite the numerous

reports on this topic and the efforts to find reliable relationships between measured effects and properties of soils, chemical characteristics of pesticides, and/or classes of enzymes, no general conclusions can be drawn (Sannino and Gianfreda, 2001). Little knowledge exists on the impact that inoculation with white rot fungi has on enzymes production especially under different water potential regimes.

Dehydrogenase activity

Biological oxidation of organic compounds is generally a dehydrogenation process, which is catalysed by dehydrogenase enzymes (Balba *et al*, 1998). Dehydrogenases are endogenous enzymes (within a cell) that catalyze the dehydrogenation of organic compounds (Harris and Steer, 2003). Their activity is linked to the respiratory and energy producing processes in the cell, and basically depends on the metabolic state of microorganisms (Guerra *et al.*, 2002). These enzymes play an important role in the oxidation of organic matter by transferring the hydrogen from the organic substrates to the electron acceptor. Several specific enzyme systems are involved in the dehydrogenase activity of the soils and they reflect to a great extent the soil biochemical activities (Balba *et al*, 1998).

Assessing the dehydrogenase activity in soil can give information on the possible inhibitory or stimulatory effect of contaminants on the microbial activities and/or on the fungal inoculant. Dehydrogenase activity is the most frequently used test for determining the influence of various pollutants (heavy metals, pesticide, crude oil) on the microbiological quality of soil (Frankenberger and Johansson, 1982; Brookes, 1995; Dick, 1994; Margesin *et al.* 2000a, b). Guerra *et al.* (2002), demonstrated that measurement of dehydrogenase activity is a simple low cost-effective and sensitive test to assess the toxic effects of heavy metals on microbial activity in contaminated soils.

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 ^{14}C-radiolabelled and unlabelled substrates have been used to screen for ligninolytic activity. However, these assays are relatively slow and difficult. More recently the development of assays utilizing polymeric dyes as substrates for the lignin degradative system has facilitated these screening procedures (Gold *et al*., 1988). 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

Other enzyme activities which are involved in key reactions of metabolic processes of soils (i.e. organic matter decomposition, nutrient cycling) are useful in order to provide a better picture of the status of soil processes when affected by pollution. Some of these have been shown to be sensitive to soil quality (Acosta-Martinez *et al.*, 2003).

For example β-glucosidase and phosphomonoesterases catalyse reactions involved in the biogeochemical transformations of C, N, P and S (Taylor *et al.*, 2002) and are likely to be an essential component of any assessment of substrate mineralization (Taylor *et al.*, 2002). β-glucosidase activity is involved in the final step of cellulose degradation, that provides simple sugars for microorganisms in soils (Costa-Martinez *et al.*, 2003). β-glucosidase is the third enzyme in a chain of three enzymes that breaks down labile cellulose and other carbohydrate polymers (Boerner and Brinkman, 2003).

Alkaline and acid phosphatase activities catalyse the hydrolysis of both organic P esters and anhydrides of phosphoric acid into inorganic P (Acosta-Martinez *et al.*, 2003). These two enzymes are frequently regarded as ecto-enzymes, i.e. enzymes acting outside but still linked to their cells of origin. Phosphomonoesterases (or acid phosphatase) are associated with the phosphorous cycle: they form an important group of enzymes catalysing the hydrolysis of organic P esters to orthophosphates

(Vuorinen and Saharinen, 1996). The activity of phosphomonoesterase or acid phosphatase is strongly correlated with the rate of release of both inorganic N and P to the soil solution (Boerner and Brinkman., 2003). Monreal and Bergstrom. (2000) reported soil enzyme beta-glucosidase as a sensitive indicator for assessing the health of microbial mineralisation processes of the C and N cycles.

As the major components of organic matter consist of cell wall polymers and reserve polysaccharides, enzymes such as cellulase are of crucial importance as primary agents for decomposition (Wirth and Wolf, 1992).

The growth of wood decay fungi, especially under natural conditions requires control of their nitrogen economy, involving regulation of proteolytic activities for intracellular protein turnover, extracellular digestion of protein sources and modification of proteins through limited proteolysis (Staszczak *et al.*, 2000). The protein turnover is involved in basic cellular functions such as the modulation of the levels of regulatory proteins and adjustment to stress. In recent years it has become clear that proteolysis plays an essential role in response to stress conditions such as high temperatures or nutrient deprivation (Hilt and Wolf, 1992).

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 1000 $\mu g\ kg^{-1}$.

1.8.3 Total soil microbial populations

Soil microbial communities are among the most complex, diverse, and important assemblages in the biosphere. Because of such a high level of diversity, soil microbial communities are among the most difficult to phenotypically and genetically characterize (Zhou *et al.*, 2004). They are a keystone of the function and structure of soil (Harris and Steer, 2003).

The soil microbial biomass has been defined as the part of the organic matter in soil that constitutes living organisms smaller than 5-10 μm^3. These microorganisms

largely bacteria, fungi, algae, and nematodes are important to soil nutrition through their role in decay of plant and other organic matter in the soil and as nitrifiers (McEwen and Stephenson, 1979). Anything that disrupts their activity could be expected to affect the nutritional quality of soils and would thus have serious consequences (McEwen and Stephenson, 1979). Therefore changes to the metabolic profiles of soil microbial communities could have potential use as early indicators of the impact of management or other perturbations on soil functioning and soil quality. Soil analyses of the total microbial counts in the contaminated soil can provide useful information on soil biological activities and the extent to which the indigenous microbial population has acclimatised to the site conditions (Balba *et al.*, 1998). In addition a comprehensive knowledge of the diversity of the autochthonous microbial communities of natural ecosystems and their degradative potential is very important when assessing the strategy and outcome of bioremediation (Stahl and Kane, 1993). It also gives information on whether the microbial populations will be capable of degrading pollutants quickly enough or whether supplementing starter cultures will be useful (Wunsche *et al.*, 1995).

1.8.4 Toxalert®10

Toxicity tests are important tools to assess to what extent the bioremediation process was effective. 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 and often require extensive preparation (Perez *et al.*, 2001). The use of bacteria as test organisms is advantageous and offers statistical advantage in using large number of bacteria instead of a small number of organisms used in other non-bacterial bioassays (Querehi *et al.*, 1984).

Previous studies investigated to what extent aquatic tests like Toxalert can be used for assessing soil contamination (Bennet and Cubbage, 1992; Johnson and Long, 1998; Doherty 2001; Kovats, *et al.*, 2003). The results obtained with the *Vibrio fischeri* test are often consistent with the results of other ecotoxicological tests and with analytical derived concentration of the contaminant (Doherty, 2001).

Theoretical considerations of this bioassay

Toxalert®10 developed by Merck uses bioluminescence of the bacterium *Vibrio fischeri* as the endpoint. Bioluminescence is a natural phenomenon in which visible light is generated by an organism as a result of a chemical reaction (Kovats, *et al.*, 2003).

There are diverse types of organisms that display bioluminescence including bacteria, protozoa, fungi, sponges, crustaceans, insects, fish, squid, jellyfish and lower plants. Bioluminescent organisms occur in a variety of habitats particularly in the deep sea where light is employed for functions including defence, reproduction and feeding. The enzymes involved in the luminescent system (lux) including luciferase, as well as the corresponding lux genes, have been most extensively studied from marine bacteria in the *Vibrio* and *Photobacterium* genera. It has been found that the light emitting reactions are very distinct for different organisms with one common component the molecular oxygen. The emission of light is a consequence of respiration and can be read by a luminometer.

$$FMNH_2 + O_2 + RCHO \xrightarrow{luciferase} FMN + RCOOH + H_2O + LIGHT$$

Chemicals or mixtures of chemicals which are toxic to bacteria can disturb some cellular functions such as the electron transport system and change some cellular structures such as cytoplasmic constituents or the cell membrane. These alterations lead to a reduction in light output, proportional to the strength of the toxic compounds.

1.9 Aims and objectives of the project

Expansion of agricultural and industrial activities in recent decades has led to pollution of soil and groundwater with pesticides and many treatment processes have been developed to reduce the environmental impacts of this contamination. Physical and chemical methods for soil clean up are very expensive, and for this reason it is of great interest to assess the potential use of white rot fungi in soil decontamination.

These organisms have been described as good bioremediation agents because of their robustness and tolerance to several xenobiotics.

Most reported research on pesticide remediation in soil has been concerned only with single pesticides. However, in contaminated soils pesticides are more commonly found in mixtures (Schoen and Winterlin, 1987). Very few studies have examined the degradation of mixtures of pesticides in soils and/or the way the native microflora enzymatically responded to such mixtures. Moreover, there are hardly any studies on the use of white rot fungi to clean-up mixtures of pesticides. It is important to examine what enzymatic changes are triggered when these fungi are introduced into contaminated soils, and how the clean-up process is affected by environmental factors. An environmental factor that may well have a crucial effect on bioremediation is soil water availability, as it varies naturally throughout the year. Nevertheless, very few studies look at the effects of water availability on bioremediation.

The initial work in this study (Part I) assessed the tolerance of eight white rot fungi to simazine, trifluralin and dieldrin, individually and in a mixture, in soil extract agar. From this screening four isolates were selected for the subsequent study: to assess the effect of matric and osmotic stress on growth and tolerance to individual and mixtures of pesticides, in media supplemented with pesticides. The potential of these four fungal isolates to produce laccase and degrade lignin in the presence of simazine, trifluralin and dieldrin, individually and in a mixture was also evaluated.

The second part of the study (Part II) focused on the interactions and activity of four white rot fungi isolates: *T.versicolor* (R26), *T.versicolor* (R101), *P.ostreatus*, and *P.chrysosporium* in soil extract broth. Degradation of a mixture of different concentrations (0-30 mg l^{-1}) of simazine, dieldrin and trifluralin and production of enzymes relevant to P and N release (phosphomonoesterase, protease) and carbon cycling (β-glucosidase, endocellulase) and laccase activity were quantified to study the degradative capacities of these fungi, under different water regimes.

The final component of work examined the activity three white rot fungi: *T.versicolor* (R26), *P.ostreatus* and *P.chrysosporium* and an organic amendment SMC in soil microcosms, under two different water potentials. Soil respiration, dehydrogenase

activity, total ligninolytic activity, production of cellulase and laccase and degradation of pesticides were assessed in order to study the interaction between inoculant, pesticide concentration and water stress.

The key objectives of this study were:

- ✓ Compare growth of eight different fungal isolates under different water availabilities and pesticide concentrations (simazine, trifluralin and dieldrin, individually and in a mixture).
- ✓ Investigate the production of laccase and ligninase in pesticide amended media
- ✓ Determine hydrolytic enzyme production for *P.ostreatus*, *T.versicolor* and *P.chrysosporium* in soil extract liquid broth.
- ✓ Determine pesticide degradation rates and toxicity in soil extract liquid broth after 25 day incubation with fungal inocula.
- ✓ Establish soil microcosms and select the parameters that best describe soil activity.
- ✓ Construct temporal profiles for enzyme activity, microbial respiration, total microbial populations and pesticide disappearance in soil microcosms inoculated with *P.ostreatus*, *T.versicolor* and *P.chrysosporium*, over a period of 24 weeks.
- ✓ Examine temporal profiles of enzyme activity, microbial respiration, total microbial populations and pesticide disappearance in soil microcosms amended with SMC, a by-product from the mushroom industry.
- ✓ Investigate the interactions between pesticide disappearance, enzyme production and total soil respiration in soil of different water potentials.

Figure 1.4 summaries the programme of this work.

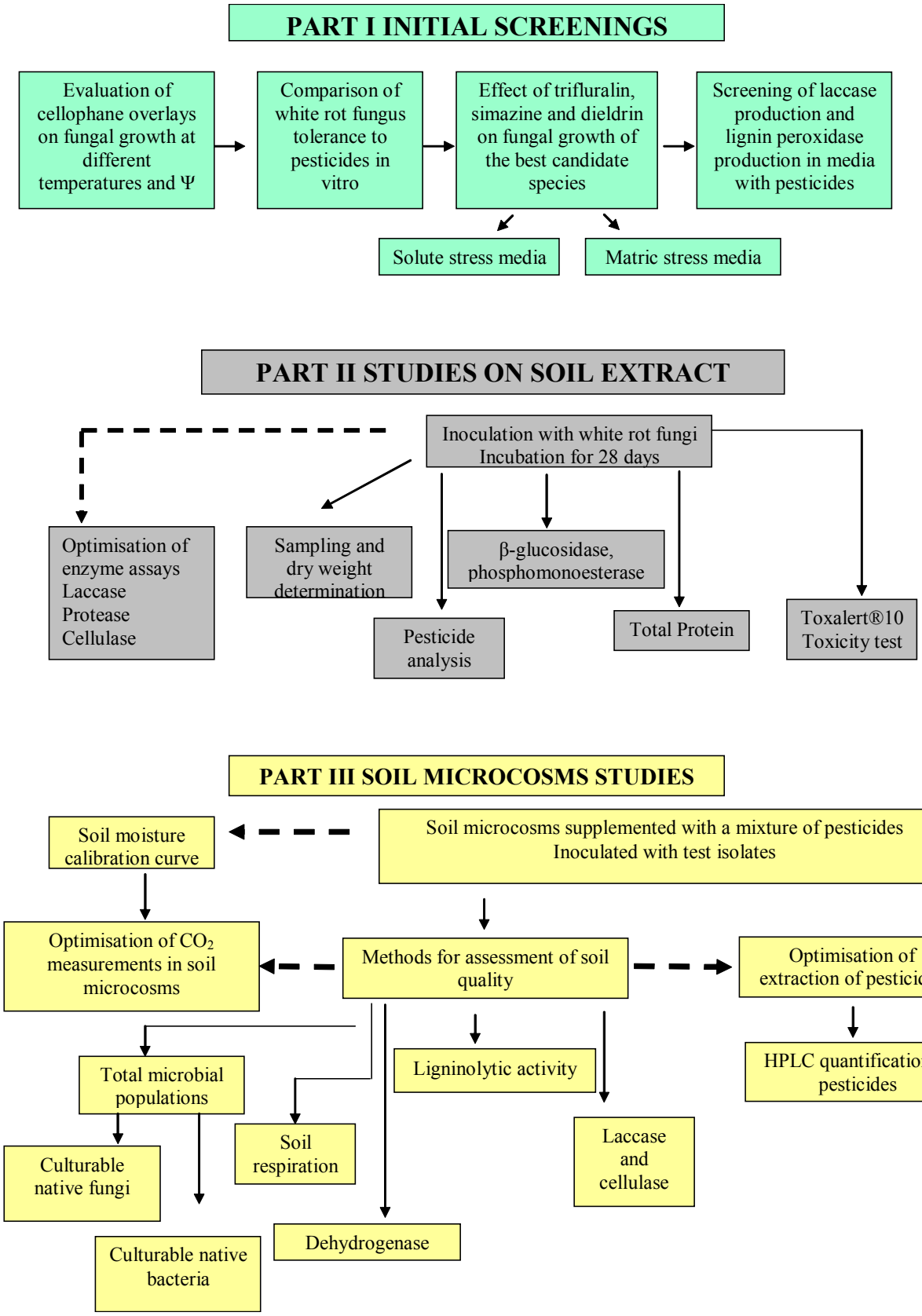


Figure 1.0.4 Flow diagram of the experimental work carried out in this thesis.

2 Materials and Methods

2.1 Fungal inoculants

All of the isolates used throughout this study were white rot fungi and their identification (name and collection reference) is listed in Table 2.1. Isolates were kept as slopes or plates on malt extract agar (MEA) for up to 3 months. Purity of storage cultures was verified by streak plating.

Table 2.1 Isolates used in this study and their reference number. All isolates marked with an * were provided by Dr Mike Challen (HRI-Warwick, Wellesbourne, Warwick, United Kingdom and those marked with ** were supplied by Dr. A. Mswaka** University of Zimbabwe, Zimbabwe.

Species	Our reference	Collection Reference
*Phanerochaete chrysosporium**	R170	ATCC 35541; ME446
*Pleurotus ostreatus**	R14	Sinden P11
*Trametes versicolor**	R26	FPRL 28 A
*Polystictus sanguineus**	R29	FPRL 150[a]
*Pleurotus cystidiosus**	R46	Cz.
*Pleurotus sajor-caju**	R139	NCP
*Trametes versicolor***	R101	TVE123
*Trametes socotrana***	R100	TSO 131

2.2 Media and Substrates

2.2.1 Modification of media and substrates water potential

A soil extract medium was used in this study. This medium was prepared with a sandy loam soil from Silsoe, Bedfordshire, containing 71.78% sand, 15.79% silt, 12.43% clay, 5.01% organic matter, 81.7 ± 4.06 mg kg^{-1} soil extractable phosphorous, 4.7 ± 0.17 mg kg^{-1} soil nitrate-N, 0.7 ± 0.035 mg kg^{-1} soil ammonium- N, organic matter: furnace 5.01%, titration 1.67% and pH of 6.07 (analysed by National Soil Resources Institute, Silsoe,

Bedfordshire). Soil extract was prepared by using 200 g of untreated field-moist soil in 400 ml of tap water. The soil/water mixture was autoclaved for 30 minutes, centrifuged at 2400 *g* for 20 minutes and filtered through filter paper (Whatman No. 1), using a vacuum pump.

The water potential of the basic medium was adjusted to -0.7 and -2.8 MPa by adding 1.15 g and 3.73 g of the ionic solute potassium chloride to 100 ml of soil extract. Soil extract is a liquid broth. For solid medium experiments technical agar No. 1 (2%) was added to the liquid medium.

2.3 Pesticides

Analytical grades of each pesticide: simazine (6-chloro-N2,N4-diethyl-1,3,5-triazine-2,4-diamine) MW: 201.66, trifluralin (a,a,a-trifluro-2,6-dinitro-N,N-dipropyl-*p*-toluidine) MW: 335.32 and dieldrin (1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene) MW: 380.9 were obtained from Greyhound, Birkenhead, UK. Stock standard solutions were prepared by dissolving analytical standards in methanol and storing in amber bottles at 4ºC. Working standard solutions were obtained by dilution with acetonitrile.

In this study xenobiotic concentrations are expressed in soil mg l^{-1} (liquid and agar studies) or mg kg^{-1} soil (soil studies). Table 2.2. shows the equivalence between concentration in SI units and molarity, for the pesticides used in the study.

Table 2.2 Pesticide concentrations expressed in mg l^{-1} and μM.

Treatment	Conc. (mg l^{-1})	Simazine (μM)	Trifluralin (μM)	Dieldrin (μM)
Single pesticide	5	24.79	14.91	13.13
Single pesticide-	10	49.59	29.82	26.25
Single pesticide	20	99.18	59.65	52.51
Mixture	5	8.27	4.97	4.38
Mixture	10	16.53	9.94	8.75
Mixture	15	24.79	14.91	13.13
Mixture	30	49.59	29.82	26.25

Part I Initial screening of fungi

2.4 A comparison of tolerance of isolates to pesticides in vitro

2.4.1 Evaluation of cellophane overlays on fungal growth at different temperatures and water availabilities

For this purpose, 9 cm diameter Petri plates containing about 15.0 ml of soil extract agar were divided in two groups, half of which were overlayed with a sterile cellophane disc and half without cellophane discs. All plates were centrally inoculated with a 4-mm agar plug from the margin of a growing colony of each fungal isolate. Care was taken to avoid puncturing the cellophane layer to prevent any direct contact between the fungal colony and the soil extract agar.

All test fungi were tested at 15ºC and 25ºC, and -0.7 and -2.8 MPa. Growth was measured regularly for up to 14 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 linear radial mycelial extension. Experiments were carried out with three replicates per treatment.

2.4.2 Growth of fungal test species in the presence of single and mixture of pesticides

Soil extract agar was modified by the addition of trifluralin, simazine and dieldrin, individually or as a mixture of pesticides in the range 0-20 mg l^{-1} by addition to the molten agar, thoroughly mixing and pouring into 9-cm Petri plates. These were overlayed with sterile cellophane discs and centrally inoculated with a 4-mm agar plug taken from the margin of a growing colony of each fungal test-isolate. The treatments used in this experiment were: control (without addition of pesticide); simazine, trifluralin and dieldrin individually at 5, 10 and 20 mg l^{-1} and a mixture of these three pesticides at 5, 10 and 20 mg l^{-1} (total concentrations). This experiment was carried out at 15ºC. Growth was measured regularly for up to 40 days. EC50 values, the pesticide concentration that caused a 50% growth reduction in relation to the control, without pesticide, were calculated for each fungus, as well as the % in growth inhibition in the 20 mg l^{-1} treatment.

2.4.3 Effect of matric and osmotic potential on fungal tolerance to pesticides

For this experiment only four fungal isolates were used: *T.versicolor* (R26 and R101) *P. ostreatus* and *P.chrysosporium*. The latter was included in this study because it is the most commonly used species in practical field-based bioremediation systems employing white rot fungi.

In order to evaluate the effect of different solute and matric stress the media were adjusted in two different ways: adding potassium chloride or polyethylene glycol (PEG 8000), respectively. In both assays the plates were incubated at 25^0C, growth was measured regularly for up to 40 days and radial growth rates were calculated.

Solute stress media

The effect of osmotic potential on radial growth was assessed by adding KCl to soil extract agar as described in section 2.2.1. For each water potential the medium was modified with the addition of trifluralin, simazine and dieldrin at 5 and 10 mg l^{-1} and a mixture of the three pesticides (total concentrations 5 and 10 mg l^{-1}).

Matric stress media

The effect of matric potential on radial extension was assessed by adding 17.5, 35.0 and 51.2 g of polyethylene glycol 8000 (Carbowax PEG8000, Fisher) to 100 ml of soil extract prior to autoclaving, to obtain the following water potentials: -0.05, -1.5 and -2.8 MPa (Steuter *et al.*,1980). The hot solutions were poured onto Petri dishes containing a solid growth support platform, which consisted of capillary matting, a black polyester fabric disc and a cellophane disc (Mswaka and Magan, 1999), because PEG 8000 does not solidify below –1.5 MPa.

For each matric potential the medium was modified with the addition of trifluralin, simazine and dieldrin at 0, 5 and 10 mg l^{-1} and a mixture of the three pesticides (total concentrations of 0, 5 and 10 mg l^{-1}).

2.5 Screening of lignin degradation and polyphenoloxidase production

2.5.1 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 $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 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 simazine, trifluralin and dieldrin, individually and as a mixture at 0, 5 and 10 mg l^{-1} (total concentrations).

The test isolates were centrally inoculated and incubated at 15^{o}C for 15 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.

2.5.2 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 50ºC. To examine the enzyme production potential in the presence of the pesticides this medium was supplemented with simazine, trifluralin and dieldrin, individually and as a mixture at 5 and 10 mg l^{-1} (total concentrations).

The test fungi were centrally inoculated and incubated at 15^{o}C for 15 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.

Part II Studies in soil extract broth

2.6 Inoculation with white rot fungi

2.6.1 Incubation conditions

A soil extract liquid broth was used in this study at -0.7 and -2.8 MPa. Erlenmeyer flasks (250 ml) containing 100 ml of soil extract were supplemented with a mixture of simazine, dieldrin and trifluralin to give final concentrations of 0, 5, 10, 15 and 30 mg l^{-1}. Four plugs of actively growing mycelium were inoculated in each flask, at 27 ± 1°C, for 25 days with constant agitation at 150 rpm. All treatments were carried out in triplicate.

2.7 Parameters evaluated

2.7.1 Sampling and dry weight determination

After the incubation period the mycelium was filtered through Whatman No. 1 paper filters and biomass determined by drying the mycelium for 48 hours at 80°C. The fresh filtrate was frozen at –20°C and used later for pesticide quantification, protein determination and various enzymatic determinations.

2.7.2 Enzyme production

We chose to monitor the activity of several enzymes, which are specific for a range of substrates. These included laccase, phosphomonoesterase, β-glucosidase, protease and cellulase. In some cases, a miniaturization of the enzyme assays was established by an adaptation to microtitre plates involving semi-automated multi-channel pipettes and a microtitre plate reader. As a consequence, minimum reagent and sample volumes were required, saving time, materials and labour. Moreover, rapid screenings of a large number

of samples was facilitated (Wirth and Wolf, 1992). The optimisation stages are described in the results section (3.4.1).

Laccase

Laccase activity (EC 1.10.3.2) was determined with ABTS (2,2- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) (A-1888, Sigma) at 405 nm, based on the protocol described by Buswell *et al.* (1995). The assay was carried out at ambient temperature, with the ABTS and buffer equilibrated at 37ºC. The reaction mixture, in a total volume of 300 µl (appropriate for 96 well microtitre plates), contained 150 µl sodium acetate buffer, pH 5.0, and 100 µl of enzyme extract. The reaction was initiated by adding 50 µl of 0.55 mM ABTS.

Laccase activity was computed from the increase in A405, recorded in a microtitre plate reader (Dinex Technologies MRX Revelation) set in the kinetic mode (reaction time of 10 minutes, 5 seconds agitation at the beginning).

Boiled enzyme was used in the control sample. One activity unit was defined as the amount of enzyme producing a 0.001 increase in the optical density in 1 min at the conditions of the assay. 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 0.03125 mg ml^{-1}, i.e. 0.375 units per well.

Protease

The protease activity (EC 3.4.24) in the filtrate was quantified using sulphanilamide azocasein substrate (Germano *et al.*, 2002), purchased from Sigma (A-2765), and the assay was optimised in 96 well microtitre plates instead of cuvettes. Azocasein is a chemically modified protein, prepared by adding sulphanamide groups to casein, which are orange and are covalently linked to the peptide bonds. When azocasein is subject to proteolytic action short peptides and amino acids are liberated from the chain and remain in solution, giving an orange colour to the solution. The greater the proteolytic activity the more intense the orange colour of the solution.

The assay was carried out using 30 µl of azocasein (1% in 0.2 M Tris-HCl buffer, pH 7.5). The reaction was started by adding 20 µl of enzyme solution, after incubation for 1 hour at

37 ° C, the enzyme was inactivated by the addition of 150 µl of trichloroacetic acid solution (10% W/V) and this solution was neutralised using 50 µl of NaOH 1M.

Trichloroacetic acid (TCA) was added to stop the reaction and to precipitate macromolecules, including the enzymes and the undigested azocasein. These were then removed by centrifuging the microtitre plate at 958 *g*, for 10 minutes, in a centrifuge equipped with a rotor for microtitre plates. Subsequently, supernatants (150 µL) were transferred to a 96-well, half-size EIA plate (175 µl cavities, Costar, 1 cm path length). The absorbance was measured spectrophotometrically at 440 nm against a blank prepared similarly but with the enzyme solution inactivated (100ºC, 10 minutes). One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – boiled sample absorbance) x 1000 x min^{-1}. This assay was first optimised using commercial protease from *Aspergillus oryzae*, 500 units g^{-1} (P-6110, Sigma), giving a positive result for concentrations as low as 0.0005 units of protease in the well.

Cellulase

Carboxymethyl-substituted (CM-) and water soluble polysaccharide derivatives labelled covalently with remazol brilliant blue R (RBB), i.e., CM-cellulose-RBB was used as a substrate for cellulase (EC 3.2.1.4) (Wirth and Wolf, 1992). The assay was performed in microtitre plates. The experimental procedure was as outlined in the Remazol Brilliant Blue R (RBB) protocol, supplied by LOEWE Biochemica.

CM-cellulose (50 µl; 4mg ml^{-1}) and buffer (50 µl 0.2M sodium acetate buffer, pH 5) were equilibrated in an incubation chamber at 37ºC. After the addition of 100 µl of enzyme sample the microtitre-plates were sealed with low evaporation lid and incubated for 30 min. The reaction was terminated by the addition of 50 µl of HCl 2N, causing the precipitation of the non-degraded high polymeric substrate. Subsequently the plates were cooled on ice (10 min) and centrifuged at 1450 *g* in a centrifuge equipped with a rotor for microtitre-plates. Supernatants (175 µl) containing soluble dye-labelled degradation products were transferred to a 96-well, half size EIA plate (175 µl, Costar, 1 cm path length) and measured spectrophotometrically at 600 nm. Blanks were prepared similarly (3 replicates per treatment) but without the addition of enzyme sample during incubation.

One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – blank absorbance) x 1000 x min^{-1}. This assay was initially optimised using commercial cellulase from *Aspergillus niger*, minimum 0.3 units mg^{-1} solid, (C-1184, Sigma), giving a positive result for concentrations as low as 0.002 units in the well.

β-glucosidase, phosphomonoesterase

β-D-glucosidase (EC 3.2.1.21) and phosphomonoesterase (EC 3.1.3.2) activities were assayed using p-nitrophenyl substrates (Keshri and Magan, 2000): 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl phosphate disodium salt, respectively, in 96 well microtitre plates. The reaction was started by adding 80 µl of enzyme extract, with 40 µl acetate buffer (0.05M pH 4.85) and 80 µl of substrate: 25 mM for 4-nitrophenyl-b-D-glucopyranoside (Acros Organics, cat. 2492-87-7) and 15 mM for 4-nitrophenyl phosphate disodium salt (Acros Organics, cat. 4264-83-9), followed by incubation at 37^0C. The control included boiled enzyme extract and was treated in the same way as the samples.

After 1h, 10 µl of 1M sodium carbonate solution (Sigma Chemical Co., UK) was added to stop the reaction and the plates were left for 3 min before reading absorbance at 405 nm. The increase in absorbance corresponded to the liberation of p-nitrophenol by enzymatic hydrolysis of the substrate. Total enzymatic activity in the enzyme extract samples was determined by extrapolation from a calibration curve made with the 4-nitrophenol standards

Calibration curve of 4-nitrophenol: Standard 4-nitrophenol solutions (Spectrophotometric grade, N/3200/48, Fisher Chemicals) of known concentrations in a range between 3.28 and 210 µg ml^{-1} were prepared using 0.05 M acetate buffer pH 4.85. The standard solutions were treated in the same way as the samples: by mixing 80 µl of p-nitrophenol solution with 120 µl of buffer, followed by incubation for 1h at 37^0C. Sodium carbonate solution (10 µl, 1M) was added and the microtitre plate was left for 3 min before absorbance was read at 405 nm. Specific enzyme activity was expressed in nmol p-nitrophenol released min^{-1} μg^{-1} protein.

Soluble protein

Soluble protein content of the enzyme extracts was determined by using the Bicinchoninic Acid Protein Assay Kit (Sigma). The kit contains bicinchoninic acid solution, copper (II)

sulphate pentahydrate 4 % solution and a protein standard BSA 1.0 mg ml^{-1} (A-3059, Sigma). The assay is colorimetric, utilizing the abilities of proteins to reduce alkaline Cu(II) to Cu(I), which forms a purple complex with bicinchoninic acid. Absorbance of the resulting solution at 550 nm is directly related to protein concentrations.

The bicinchoninic acid and the Cu(II) solution are combined at a 50:1 ratio for the working reagent. This reagent is stable for one day at room temperature. A 10 µl aliquot of a sample was placed in the appropriate microtitre plate wells. In the blank wells 10 µl of potassium phosphate buffer (10mM pH 7.2) was used in place of the samples. 200 µl of working reagent was placed in all of the wells, and plates were covered, shaken and then incubated at 37 °C for 30 min. The plates were allowed to cool after being removed from the incubator; the absorbance at 550 nm was then determined using a plate reader. Protein concentration in the enzyme extract samples was determined by extrapolation from a calibration curve made with the BSA standards.

2.7.3 Pesticide analysis

Samples were filtered through 0.2 µm filter (Fisher, FDP-466-001C) and diluted with acetonitrile (75% sample: 25% acetonitrile) prior to injection in the HPLC system. HPLC quantification of all three pesticides was performed with a Gilson HPLC system equipped with a UV detector (117 UV detector, Gilson), Gilson 401C Dilutor, Gilson 231XL Sampling injector, Gilson 306 Pump and Gilson 811C Dynamic Mixer, equipped with a Altima C18 5µm column (4 mm x 250 mm x 4.6 mm). The column was operated at ambient temperature with a flow rate of 1.5 ml min^{-1} and an injection volume of 50 µL.

An isocratic mobile phase system was established using acetonitrile:water at a ratio of 70:30. The HPLC-UV detector was monitored at 215 nm. The HPLC method used in this study was adapted from a method to quantify dieldrin described by Elyassi (1997). The HPLC method used enabled the separation and quantification of simazine, dieldrin and trifluralin in a single HPLC run of 20 min with simazine eluting at 3, trifluralin at 11 and dieldrin at 13 min (Figure 2.1). The limit of detection for the three pesticides was 0.1 mg l^{-1}. Standard curves were made for each standard in soil extract broth and *r*-squared values for each curve found to be > 0.99 for all three pesticides (Figure 2.2).

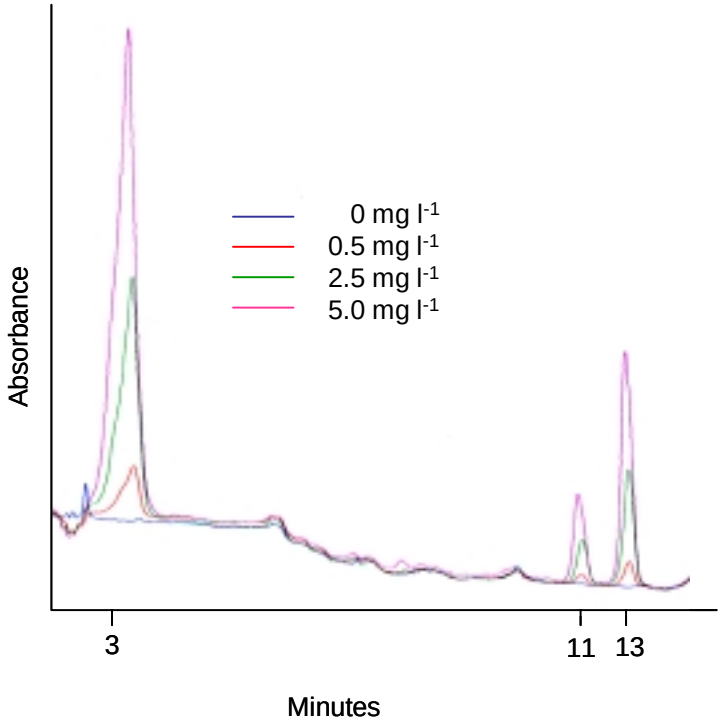


Figure 2.1 Chromatogram of simazine, dieldrin and trifluralin obtained in single HPLC runs with simazine eluting at 3, trifluralin at 11 and dieldrin at 13 min (line a: 0.5 mg l^{-1}; line b: 1 mg l^{-1} and line c: 2.5 mg l^{-1}).

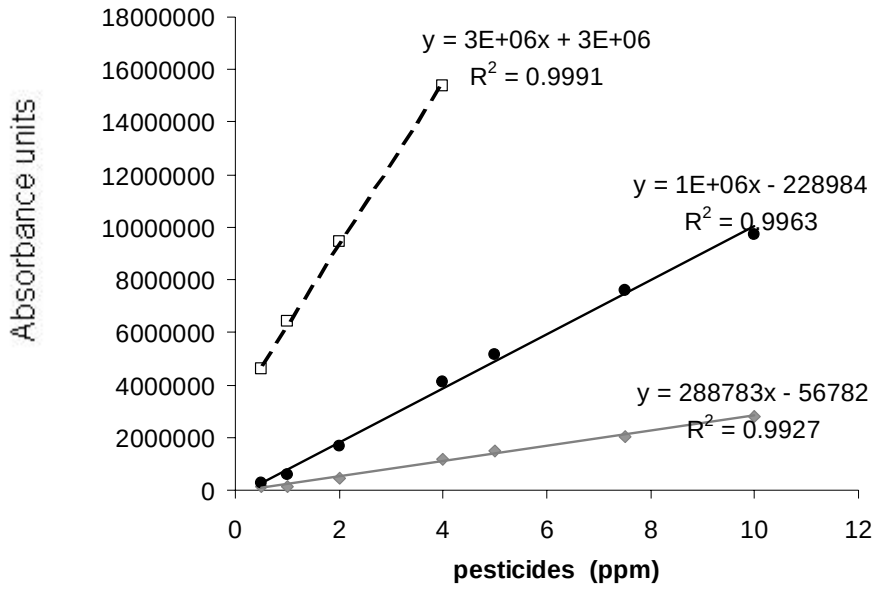


Figure 2.2 Standard curves for a mixture of simazine, trifluralin and dieldrin in soil extract broth and *r*-squared values for each curve (simazine □, dieldrin • and trifluralin ◆).

2.7.4 Toxicity test

The toxicity measurements were performed with the TOXAlert® 100 (Merck) using the liquid dried luminescent bacteria *Vibrio fischeri* (NRRL B- 11177).

Standard solutions of the pesticides were added to soil extract for the toxicity assays. Toxicity of soil extract spiked with 1, 2, 4, 6, 8 and 10 mg l^{-1} of simazine, trifluralin and dieldrin was determined and compared against the toxicity of these three pesticides in a mixture at total concentrations of 3, 6, 9, 12, 15, 18, 21, 24 and 27 mg l^{-1}.

Soil extract samples were prepared in the same way as for the HPLC analysis (described in section 2.7.3).

The osmolatily of all standards and samples were adjusted to 2% NaCl to provide optimal living conditions for the marine test organism.

1250 µl of reconstitution solution (provided in the kit) was added to each Toxalert® 10 toxicity test measurement cuvette, and the suspension was left at room temperature for 20 minutes to allow full reconstitution.

After reconstitution the content of all cuvettes was transferred to a sterile test tube and mixed thoroughly to make a homogeneous pool of bacteria. 500 µl of bacterial suspension was transferred to each measuring cuvette and the luminescence was read immediately.

The luminescence was recorded 15 minutes later and the percent of inhibition (%I) was determined as described in the following formula:

$$\% \text{ INHIBITION} = \{1- (T_{15}/ T_0)/ (C_{15}/C_0)\} \times 100$$

T_0 = Light in sample vial at 0 minutes T_{15} = Light in sample vial at 15 minutes
C_0 =Light in control vial at 0 minutes C_{15}=Light in control vial at 15 minutes

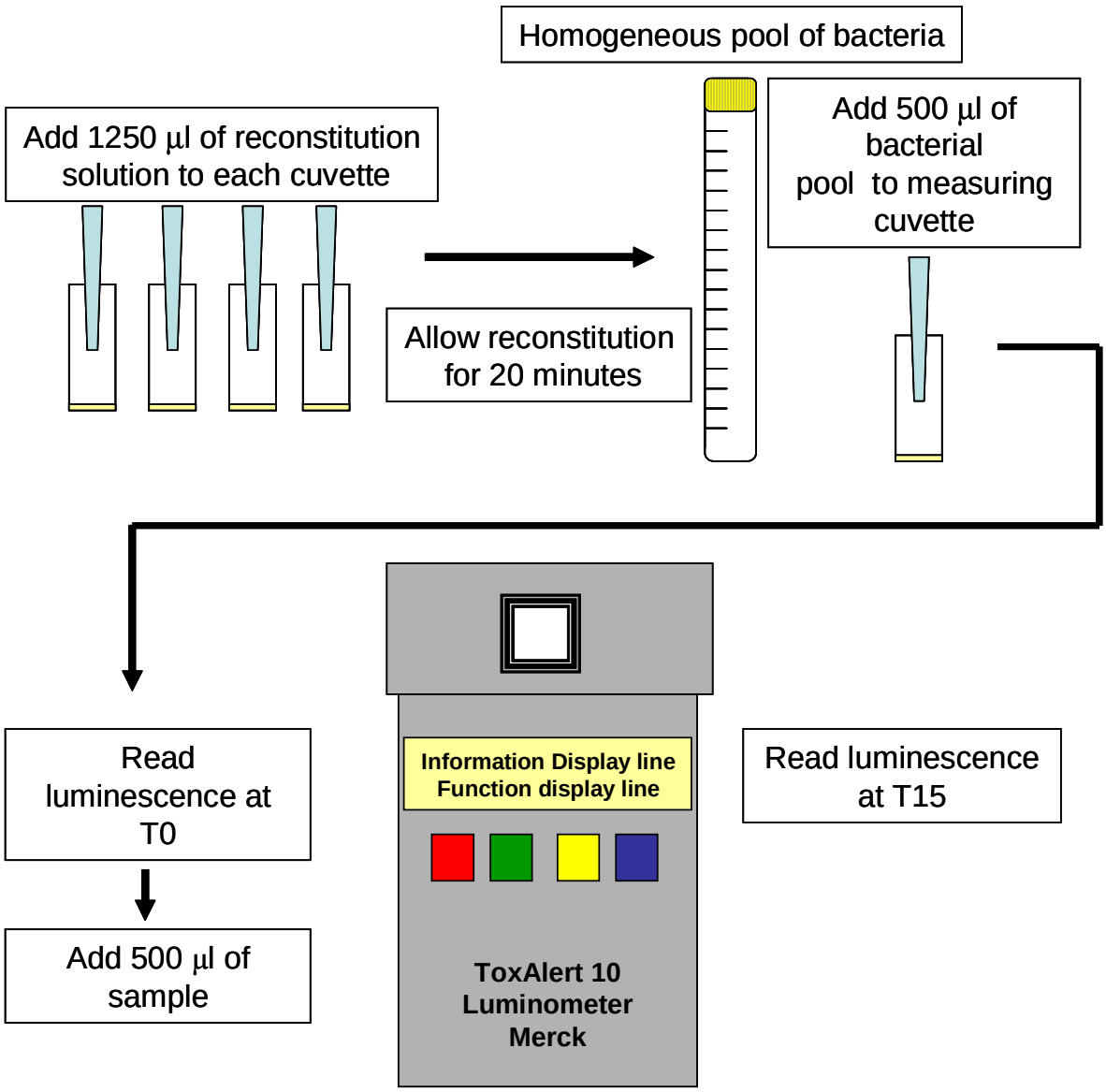


Figure 2.3 Schematic description of the protocol of ToxAlert® 10 toxicity test.

Part III Soil microcosm studies

2.8 Soil moisture calibration curve

Water availability determines the microbial activity in soil. Before the microcosm experiments were carried out, a moisture sorption isotherms was developed for soil by adding different volumes of water to 50 g of soil, in a range between 0.5 to 4.0 ml. Soil sub-samples were left to equilibrate overnight at 4^{o}C, before measuring the water potential with Aqualab-Dewpoint Potentiometer WP4. This enabled accurate modifications of water potential to be made. The relationship between the amount of added water (ml) and resultant moisture content (%) of soil is shown in Figure 2.5, and the relationship between the amount of water added and resultant water potential is shown in Figure 2.5.

2.9 Soil microcosms

The pesticide degradation potential, respiratory activity, total microbial populations and the enzyme production of soil inoculated with the selected fungi were evaluated using these microcosms. Each microcosm comprised 100 g of non-sterile soil and 5 g of inoculated carrier, the same ratio described by Boyle (1995). Since white rot fungi are obligate aerobes (Pointing, 2001) aeration was ensured by using glass vessels for plant tissue culture (V-8630, SIGMA) with vented caps, with a polypropylene membrane 0.22 μm pore size (B-3031, SIGMA). The soil used in these experiments was the same sandy loam described in section 2.2.

2.9.1 Pesticide incorporation into soil

Pesticide working solutions were prepared by dissolving analytical standards in methanol and storing in amber bottles at 4ºC. These solutions were diluted in water and added to each soil microcosms, in order to obtain the desired water potential and a final concentration of 0, 5 and 10 mg kg soil^{-1}, depending on the treatment. These pesticide solutions were added to the soil with a pipette (dripping the solution very carefully) then homogenised by grinding with a mortar and pestle and were left to equilibrate overnight at 4ºC.

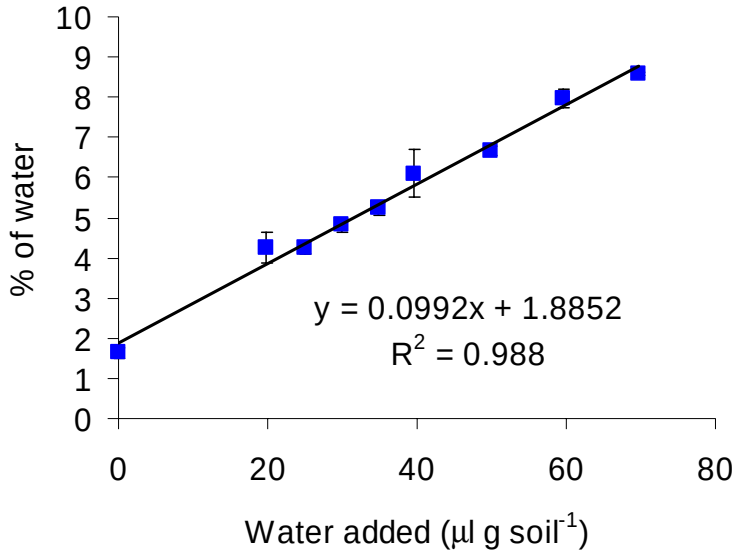


Figure 2.4 Moisture content (%) after addition of various volumes of water to 50 g of soil. Vertical bars represent the mean standard deviation (n=3).

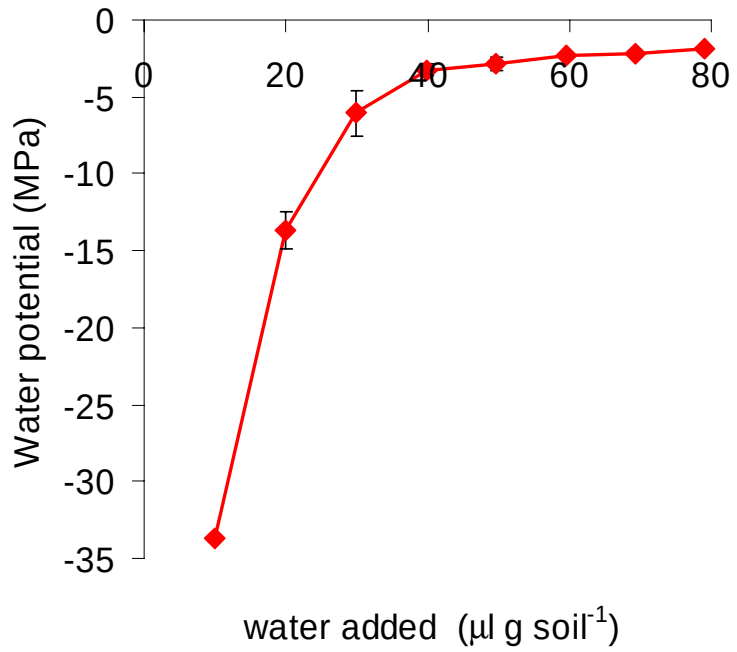


Figure 2.5 Water potential (MPa) after addition of various volumes of water to 50 g of soil. Vertical bars represent the mean standard deviation (n=3).

2.9.2 Inoculation

The water potential of each microcosm was adjusted to -0.7 and -2.8 MPa by adding 10 and 5 ml of water to each jar (respectively). Glycerol:water solutions were used to maintain the steady-state ERH equivalent to the soil treatment water potential.

The inoculum was prepared for each fungus by growing on wet (50% water content) sterile wood chips for 4 weeks at 25ºC prior to inoculation of soil. The different treatments are described in Table 2.2. The jars were inoculated and incubated at 15ºC. Three replicates of each treatment were destructively sampled after 0, 6 12 and 24 weeks. The initial soil moisture and fresh: dry weight ratio of each soil sample was determined by drying 8-10 g of fresh soil at 65^{o}C to a constant weight. At the end of each incubation period, samples from each microcosm were cultured on malt extract agar and nutrient agar using the serial dilutions technique (section 2.11.5).

Table 2.3 Different treatments used in soil microcosm experiments. Each treatment was carried out in triplicate and destructively sampled 0, 6, 12 and 24 weeks.

Substrate	**Ψ (MPa)**	**Mixture of pesticides (mg kg soil^{-1})**		
Soil	-0.7	0	5	10
	-2.8	0	5	10
Soil + wood chips	-0.7	0	5	10
	-2.8	0	5	10
Soil + Spent mushroom compost	-0.7	0	5	10
	-2.8	0	5	10
Soil + wood chips inoculated with *T.versicolor* (R26)	-0.7	0	5	10
	-2.8	0	5	10
Soil + wood chips inoculated with *P.chrysosporium*	-0.7	0	5	10
	-2.8	0	5	10
*Soil + wood chips inoculated with *P.ostreatus*	-0.7	0	5	10
	-2.8	0	5	10

*There are no data for soil inoculated with *P.ostreatus* 24 weeks incubation, due to a contamination problem.

2.10 Quantification of pesticide concentrations in soil

2.10.1 Extraction of pesticides from soil

10 ml of solvent were added directly to 5 g of wet soil in a conical flask. Two different extraction methods and five different solvent conditions were tested:

A- Methanol 100%

B- Acetonitrile 100%

C- Acetonitrile 75% water 25%

D- Methanol 50% Acetonitrile 50%

E- Acetonitrile 40% methanol 40% water 20%

And two different approaches were tested:

1- solvent-soil slurry was sonicated for 3 minutes

2- solvent-soil slurry was shaken overnight in rotatory shaker in the dark.

After extraction the solvent-soil slurry was poured through a 100 mm top diameter funnel lined with Whatman No.1 filter paper containing 1g of filtering agent Celite 545 (Aldrich, cat. 41993) and collected in a 250 ml beaker. The best extraction method was used for the samples extracted from soil microcosms.

2.10.2 HPLC quantification of pesticides

As described in section 2.7.3.

2.11 Assessment of fungal growth and metabolic activity in soil

2.11.1 Soil respiration

Soil respiration was measured by monitoring the concentration of carbon dioxide in the head-space of the microcosm jars, using a Gas Chromatographer (GC) equipped with a packed column (Porapak Q packed glass column) and a thermal conductivity detector (Carlo Erba Instruments, GC 8000 Series MFC800). The conditions of the analysis were the following: column temperature 100^0C; injector temperature: 100^0C; detector temperature 180^0C; filament temperature 230^0C; carrier gas (Helium) and flow rate 40 ml min^{-1}. CO_2 concentration was measured by injecting 3 ml headspace gas and was estimated by reference to a standard calibration gas mixture (10.3 % CO_2 in N_2).

Optimisation of carbon dioxide measurements in soil microcosms

As described in section 2.9 the microcosms jars had vented caps, to allow gas exchange. In order to obtain a detectable concentration of CO_2 the vented caps were sealed and left at room temperature, prior to CO_2 analysis. Jars were sealed for 0, 1 and 3 hours in order to optimise the procedure. Respiration rate was expressed as mg CO_2 h^{-1} g $soil^{-1}$.

2.11.2 Dehydrogenase activity

2 (p-iodophenyl)-3-(p nitrophenyl)-5-phenyl tetrazolium chloride INT (Acros Organics cat. 146-68-9) was used as substrate for soil dehydrogenase activity (Von Mersi and Schinner, 1991). The INT solution (9.88 mM) was prepared by dissolving 500 mg of INT into 2 ml of N,N-dimethylformamide, followed by the addition of 50 ml of distilled water. The solution was sonicated for 2 minutes and water was added to bring the volume up to 100 ml. The solution was stored in the dark and always used fresh.

The method is based on the incubation of 0.5 g of moist soil with 375 µl of Tris-HCl buffer (1M, pH 7.0) and 500 µl of the substrate INT at 37^0C for 2h, in the dark followed by colorimetric estimation of the reaction product iodonitrotetrazolium chloride INF (I-7375, Sigma). After the incubation every sample was mixed with 2500 µl of extraction solution ethanol: N,N-dimethylformamide (50:50), and kept in the dark. The samples were shaken

vigorously at 20 minutes intervals for 1h to extract the INF, produced in the reaction. After filtration the developed INF was measured at 434 nm against the control. To eliminate the chemical (non-microbial) INT reduction controls were prepared with autoclaved soil (121^{o}C for 20 min) and treated like the samples.

For the calibration curve of INF: standard INF (Sigma I-7375) solutions of known concentrations in a range between 0.324 and 12.96 μg ml^{-1}, were prepared in N,N-dimethylformamide. 875 μl of standard solution was added to 2.5 ml of extracting solution and the absorbance was read at 434 nm.

2.11.3 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, following the method described by Baheri and Meysami (2002). The assay consisted of mixing 1 g of wet soil with 5 ml of dye poly R-478 (P-1900, Sigma) in aqueous solution (0.02 g l^{-1}). The reaction mixture was kept under light (desklamp, with a 40w bulb) for 24 h for the enzyme reaction to take place. After 24 h the mixture was centrifuged for 4 min at 5000 rpm (eppendorf centrifuge: Beckman Microfuge ® Lite) in order to separate the soil particles. Total ligninolytic activity was given as decolouration degree of the Poly R-478, monitored by the percentage reduction in the absorbance ration at 520 nm and at 350 nm (Moredo *et al.*, 2003) , calculated as follows:

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

% Colour of a sample = (absorbance at 530 nm/ absorbance at 350 nm) $_{sample}$ x 100 / (absorbance at 530 nm/ absorbance at 350 nm) $_{poly\ R478}$

% decolouration = 100 - [(absorbance at 530 nm/ absorbance at 350 nm) $_{sample}$ / (absorbance at 530 nm/ absorbance at 350 nm) $_{poly\ R478}$]

A lower absorbance ratio, means intense decolouration and higher enzymatic activity.

2.11.4 Enzyme extraction in soil

The extraction of enzymes from soil is an ideal way to measure various soil enzyme activities. This method is especially useful for determining activities in small volumes (i.e,

microtitre plates). Compared to incubation of bulk soil with buffer, an extract is more convenient to handle and more homogenous when working with small sample volume. The following enzyme assays were performed on an enzyme extract, obtained from each soil sample, as described subsequently.

Enzymes in the soil were extracted by mixing 5 g of soil and 20 ml 10 mM phosphate buffer at pH 6.5, agitated in an incubator shaker at a speed of 250 rpm (KS501 Digital IKA Labortechnik) at 4^0C for 1 hour (Criquet *et al.* 1999). This was followed by centrifugation (Beckman Microfuge ®Lite), at 3800 rpm for 6 min, at room temperature. The supernatant obtained contained the fungal enzymes and was stored in 1.5 ml microcentrifuge tubes at – 20^{o}C.

Laccase and cellulase activities

Same procedure as described in section. 2.7.2.

2.11.5 Total microbial populations

Enumeration of culturable native bacteria

Ten fold dilutions of the soil suspensions, from 10^{-1} (1 g of soil + 9 ml of sterile water) to 10^{-7} were used to spread plate (0.2 ml) on nutrient agar supplemented with 0.5 g cyclohexamide, to prevent fungal growth. The Petri plates were incubated at 28^{o}C and observed after 3 days for quantification of the number of colonies. By knowing the number of colonies obtained for a given dilution the population density per gram of soil can be calculated. Total culturable native bacteria was expressed as LOG_{10} (CFU) g soil $^{-1}$.

Enumeration and identification of culturable fungi

Ten fold dilutions of the soil suspensions, from 10^{-1} (1 g of soil + 9 ml of sterile water) to 10^{-3} were used to spread plate (0.2 ml) on malt extract agar supplemented with 0.5 g chloramphenical, to prevent bacterial growth. The Petri plates were incubated at 28^{o}C and observed after 7-10 days for quantification of the number of colonies as well as identification of the isolated fungi. By knowing the number of colonies obtained for a given dilution the population density gram^{-1} of soil was calculated. Total culturable fungi was expressed as LOG_{10} (CFU) g soil $^{-1}$.

2.12 Data handling and statistics

Data input, data handling/manipulation, linear regression, and graph plotting was carried out using Microsoft Excel 2003 (Microsoft Co.). Other statistical tests (i.e. ANOVA and other statistical tests) were performed using XLSTAT© (Version 5.1). When required comparison between means was carried out using ANOVA followed by Tukey Multiple Comparisons test. ANOVA tables can be found in the appendix II.

3 Results

Part I Initial Screening of fungi

3.1 Introduction

The objectives of this study were to assess the tolerance of eight white rot fungal isolates to simazine, trifluralin and dieldrin, individually and as a mixture. For this purpose, soil extract agar was used as the culture medium. It is a nutritionally weak medium to mimic the nutrient availability in soil. Since white rot fungi predominantly interact with xenobiotics extracellularly (Bennet *et al.*, 1995) a cellophane overlay was used to separate the mycelium from the culture medium. The first approach was to study if this cellophane overlay had major effects on fungal growth. Subsequently, soil extract agar was supplemented with simazine, trifluralin and dieldrin (individually and as a mixture) to assess fungal tolerance to these compounds under osmotic potential conditions optimal for fungal growth (-0.7 MPa) and also at -2.8 MPa, below the wilting point of plants (-1.4 MPa) (Mswaka and Magan, 1999). From this study four isolates were selected for the subsequent studies: assessment of the effect of matric and osmotic forces on fungal growth, in media supplemented with pesticides. Finally, the potential of these fungal isolates to degrade lignin and produce laccase in the presence of simazine, trifluralin and dieldrin, individually and as a mixture, was examined.

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

The purpose of this study was to optimise the conditions for a soil extract agar study (temperature, use of cellophane overlay) and assess the ability of the selected white rot isolates to grow in this nutritionally weak medium (soil extract agar) supplemented with simazine, trifluralin and dieldrin, individually and as a mixture.

3.2.1 Calculation of fungal growth rates

Colony diametric measurements were made frequently throughout the incubation period by taking two readings at right angles to each other. Data was then tabulated and the linear portion of the radial extension rates used to determine growth rate via

linear regression. Figure 3.1 shows an example: the growth rate is the slope of the equation (Y= ax + b), thus for this example the growth rate was 0.517 mm day^{-1}.

3.2.2 Evaluation of cellophane overlays on fungal growth at two different temperatures and two different water availabilities

Table 3.1 and Table 3.2 describe the effect of cellophane overlay, incubation temperature and water potential on growth rates of eight white-rot fungi growing in soil extract agar. The use of a cellophane overlay between the medium and the test species did not impair fungal growth, and temperature had a strong impact on the relative growth rates as most isolates had higher growth rates at 25 than at 15ºC.

All eight fungal species grew effectively in soil extract agar and at 15ºC, there was little difference between growth of species on the agar surface or on the cellophane overlay. However at 25ºC the growth rates of more test isolates was affected by the cellophane overlay. Although the growth rates were significantly higher at 25 ($P<0.05$) than at 15ºC for all fungal species tested, in subsequent experiments with soil extract agar and soil microcosms 15ºC was chosen, because it was less affected by the overlay of cellophane and more realistic to *in* situ environmental temperatures.

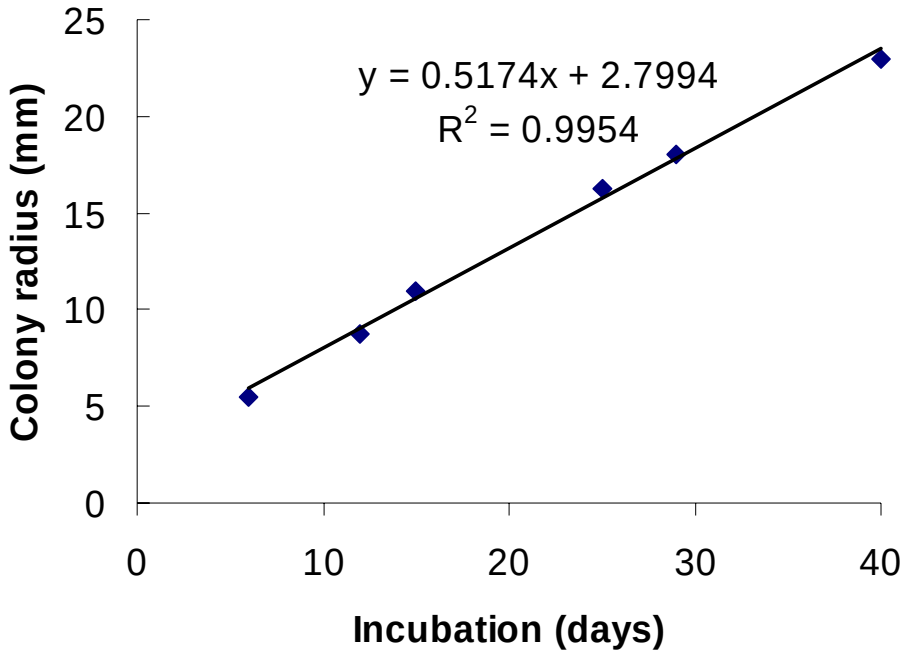


Figure 3.1 Linear regression of mycelial radial extension plotted against time in order to obtain the growth rate (mm day^{-1}).

Table 3.1 Growth rate (average of 3 replicates ± mean standard deviation) expressed in mm day^{-1} for eight fungal species subjected to two different water potentials (-0.7 and -2.8 MPa) and in the presence and absence of cellophane overlay at 15ºC.

		Growth rate (mm day^{-1})		
Fungi	Ψ (MPa)	Cellophane	No cellophane	Significance
P. sajor-caju	-0.7	2.5±0.03	2.6±0.02 *	P=0.015
	-2.8	1.1±0.12	1.2±0.04	NS
T.versicolor (R26)	-0.7	2.8±0.01	2.9±0.04	NS
	-2.8	1.3±0.06	0.6±0.01 *	P<0.001
P. cystidiosus	-0.7	0.9±0.06	1.1±0.04 *	P=0.005
	-2.8	0.2±0.07	0.2±0.02	NS
T.versicolor (R101)	-0.7	1.1±0.14	1.0±0.03	NS
	-2.8	1.5±0.44	0.9±0.05	NS
T. socotrana	-0.7	1.5±0.53	0.7±0.01	NS
	-2.8	1.7±0.30	0.7±0.02	NS
P. sanguineus	-0.7	1.5±0.27	1.7±0.52	NS
	-2.8	0.8±0.17	0.90±0.09	NS
P.chrysosporium	-0.7	3.0±0.16	3.2±0.04	NS
	-2.8	0.3±0.03	0.3±0.03	NS
P.ostreatus	-0.7	2.9±0.10	2.9 ± 0.03	NS
	-2.8	1.2±0.05	1.4±0.06 *	P=0.029

Asterisk (*) means that there was a significant difference between the two groups: with and without cellophane, for that fungus and water potential condition, according to the statistical test performed (One-way ANOVA); NS: not significantly different.

Table 3.2 Growth rate (average of 3 replicates ± mean standard deviation) expressed in mm day^{-1} for eight fungal species subjected to two different water potentials (-0.7 and -2.8 MPa) and in the presence and absence of cellophane overlay at 25ºC.

		Growth rate (mm day $^{-1}$)		
Fungi	Ψ (MPa)	Cellophane	No cellophane	Significance
P. sajor-caju	-0.7	4.7 ± 0.14*	5.3 ± 0.06 *	P=0.003
	-2.8	2.9 ± 0.09 *	3.2 ± 0.01 *	P=0.007
T.versicolor (R26)	-0.7	4.8 ± 0.23 *	3.3 ± 0.01 *	P<0.001
	-2.8	1.6 ± 0.04 *	0.8 ± 0.10 *	P<0.001
P. cystidiosus	-0.7	2.2 ± 0.11	2.2 ± 0.04	NS
	-2.8	0.6 ± 0.03 *	1.2 ± 0.04 *	P<0.001
T.versicolor (R101)	-0.7	2.0 ± 0.49	1.5 ± 0.09	NS
	-2.8	1.7 ± 0.18	1.6 ± 0.24	NS
T. socotrana	-0.7	2.3±0.09 *	1.4 ± 0.01 *	P<0.001
	-2.8	3.2 ± 1.75	1.7 ± 0.01	NS
P. sanguineus	-0.7	2.7 ± 0.21	2.7 ± 0.0	NS
	-2.8	2.3 ± 0.26	2.4 ± 0.01	NS
P.chrysosporium	-0.7	5.71 ± 0.0259	5.5 ± 0.2	NS
	-2.8	2.86 ± 0.0342	2.9 ± 0.01	NS
P.ostreatus	-0.7	3.43 ± 0.0692 *	2.3 ± 0.02 *	P<0.001
	-2.8	2.22 ± 0.0660 *	2.5 ± 0.05 *	P=0.005

Asterisk (*) means that there was a significant difference between the two groups: with and without cellophane, for that fungus and water potential condition, according to the statistical test performed (One-way ANOVA); NS: not significantly different.

3.2.3 *In vitro* tolerance to individual and mixtures of pesticides at two different water potentials

The fungi used in bioremediation must have good growth rates, in order to potentially colonise soil and overcome the competition of the native microorganisms. In this study 8 isolates were grown on soil extract agar amended with 5, 10 and 20 mg l^{-1} simazine, trifluralin and dieldrin individually and as a mixture. Overall most test isolates were tolerant to the pesticide treatments. However, their growth rates were highly influenced by water availability, and pesticide concentration. A comparison of the effect of these two factors on the growth rates of the eight test isolates is shown in Table 3.3 and Table 3.4. Table 3.3 shows the EC_{50} values (pesticide concentration causing 50% growth reduction compared to the control). Table 3.4 shows the percentage growth inhibition observed in the 20 mg l^{-1} treatment. The best isolates were *T.versicolor* (R26 and R101), and *P.ostreatus*.

Table 3.3 Concentration (mg l^{-1}) of simazine, trifluralin and dieldrin individually and as a mixture, that causes a 50% reduction in fungal growth (EC_{50}) in eight test isolates growing on soil extract agar. N.G:= no growth; N.I. = no inhibition.

		Simazine	Trifluralin	Dieldrin	Mixture
Isolates	Ψ (MPa)	EC_{50} (mg l^{-1})	EC_{50} (mg l^{-1})	EC_{50} (mg l^{-1}))	EC_{50} (mg l^{-1})
P.cystidious	-0.7	46	33.9	21.2	28.3
	-2.8	27.4	8.6	13.3	70.5
P.sajor-caju	-0.7	70.7	20	15	17.6
	-2.8	11.2	23.3	N.G.	10.8
T.socotrana	-0.7	N.I	38.3	44.4	33.1
	-2.8	25.5	15.3	31.2	38
P.sanguineaus	-0.7	N.I	47.1	17.4	22.4
	-2.8	14.7	17.6	10.8	11.9
T.versicolor	-0.7	314	213	22.6	55.8
(R26)	-2.8	N.I	30.9	1207	13.6
T.versicolor	-0.7	N.I	24.6	115	32.3
(R101)	-2.8	26.2	50.6	24.6	25
P.chrysosporium	-0.7	N.G.	14.7	14.4	2.8
	-2.8	N.G.	N.G.	N.G.	N.G.
P.ostreatus	-0.7	45	33.6	19.9	14.1
	-2.8	19.9	27.5	12.2	19.8

Table 3.4 Growth inhibition (% I) observed for eight test isolates growing in soil extract agar supplemented with 20 mg l^{-1} treatments (simazine, trifluralin and dieldrin, individually and as a mixture). N.G:= no growth; N.I. = no inhibition.

		Simazine	Trifluralin	Dieldrin	Mixture
Isolates	Ψ (MPa)	% I	% I	% I	% I
P.cystidious	-0.7	22	15	47	35
	-2.8	36	29	75	14
P.sajor-caju	-0.7	14	50	67	57
	-2.8	89	43	N.G.	92
T.socotrana	-0.7	N.I	26	23	30
	-2.8	39	16	32	26
P.sanguineaus	-0.7	N.I.	21	57	45
	-2.8	68	57	93	84
T.versicolor (R26)	-0.7	3	5	44	26
	-2.8	N.I	32	1	74
T.versicolor (R101)	-0.7	N.I	20	9	31
	-2.8	38	20	41	40
P.chrysosporium	-0.7	N.G.	34	69	94
	-2.8	N.G.	N.G.	N.G.	N.G.
P.ostreatus	-0.7	22	30	50	71
	-2.8	50	36	82	51

3.2.4 Effect of osmotic and matric potential on fungal tolerance to pesticides

After studying the responses of these eight test isolates to the three pesticides individually and as a mixture, further studies were restricted to those which had the best tolerance to the pesticides. These were *T.versicolor* R101 and R26 and *P. ostreatus*. *P.chrysosporium* was also included because is a very well studied species, used previously in many bioremediation studies.

The purpose of this study was to assess the tolerance of the four test isolates to the same pesticides individually and as a mixture, under different water regimes, achieved by changing the osmotic potential of soil extract to -0.7 and -2.8 MPa and the matric potential to -0.5, -1.5 and -2.8 MPa.

Osmotic potential effects

In this first approach different water regimes were obtained with the addition of KCl to soil extract agar. Overall, these 4 test isolates showed better growth at -0.7 MPa and the addition of pesticides to the culture medium influenced growth rates. Plate 3.1 shows an example of a test isolate growing on a mixture of simazine, trifluralin and dieldrin at 0, 5 and 10 mg l^{-1}, at –0.7 and -2.8 MPa osmotic potential, after 30 days incubation.

Overall *P.chrysosporium* showed the lowest tolerance to the pesticides used in this study and showed very high sensitivity to water stress. While *T.versicolor* (R26) and *P.ostreatus* had good tolerance to water stress and pesticides. *T.versicolor* (R101) had lower growth rates than the other two test isolates, but it was less affected by changes in water potential and pesticide treatment.

The effect of osmotic potential and pesticide treatment as growth rates for *T.versicolor* (R26) in soil extract agar are shown in Figure 3.2. Water availability had a strong impact on *T.versicolor* (R26) growth rates, as the fungus showed markedly higher growth at -0.7 MPa water potential. Interestingly, at both water regimes the growth rates were not noticeably affected by the concentration of the pesticide (or

mixture of pesticides), which suggested that this isolate can easily grow in medium supplemented with pesticides, without suffering any toxicity effects.

T.versicolor (R101) was very tolerant to water stress, as the fungus had high growth rates under imposed water stress, at -2.8 MPa (Table 3.5). The highest growth rates were observed at -0.7 MPa for soil extract agar supplemented with simazine, which once again suggest the test isolates are very tolerant to this pesticide. Additionally the fungus showed good tolerance to dieldrin, at both water potentials.

P.ostreatus showed higher growth rates under freely water available, but under water stress conditions the fungus still showed good tolerance to the pesticides (Table 3.5). Interestingly, *P.ostreatus* had very high growth rates in the mixture treatment. Its growth was noticeably higher than the growth showed by the other test isolates. For example, in the treatment mixture 5 mg l^{-1}, the growth rate was 10 times higher than that in the simazine 5 mg l^{-1} mixture treatment, at both water potentials. Even though its growth in the presence of the mixture of pesticides was much higher than in single pesticide treatments, the fungus also showed good tolerance to all single pesticides, including dieldrin, which was highly toxic to some of the other test isolates.

P.chrysosporium was highly influenced by water potential and pesticide treatments as the fungus showed growth inhibition or extremely low growth rates at -2.8 MPa (see Table 3.5). Unlike the other test species that seemed to be very tolerant to simazine, *P.chrysosporium* was extremely sensitive to this pesticide. The highest growth rates were observed for the mixture treatment, at -0.7 MPa, and at this water potential the fungus also showed good tolerance to trifluralin.

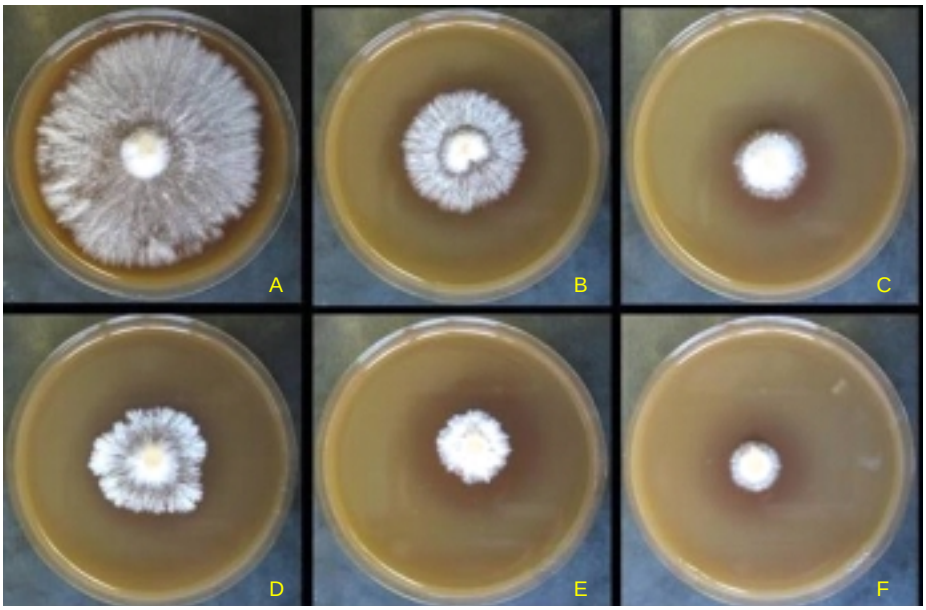


Plate 3.1 *P. ostreatus* growing on soil extract agar supplemented with a mixture of pesticides at 0, 5 and 10 mg l^{-1}, at 15ºC, at –0.7 MPa water potential (A, B, C) and –2.8 MPa water potential (D, E, F), during the course of the experiment.

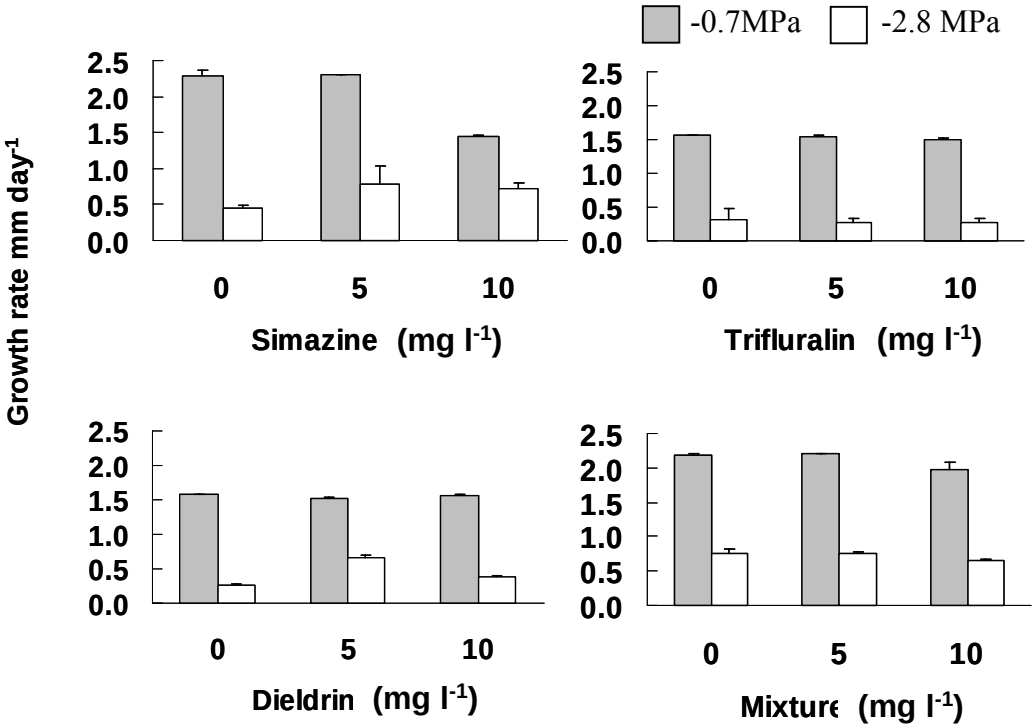


Figure 3.2 *T.versicolor* (R26) growth rates (mm day^{-1}) in soil extract agar supplemented with simazine, trifluralin and dieldrin at 0, 5 and 10, individually and as a mixture, at -0.7 and -2.8 MPa osmotic potential, incubated at 15°C. Bars indicate standard deviation of the mean (n=3).

Table 3.5 Mean fungal growth rates (± standard deviation) for *T.versicolor* (R101), *P.ostreatus* and *P.chrysosporium* in soil extract agar supplemented with three pesticides individually and as a mixture, under different osmotic water potentials, at 15ºC.

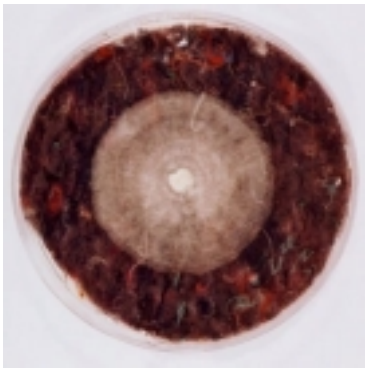
		T.versicolor (R101)		*P.ostreatus*		*P.chrysosporium*	
Ψ(MPa)		-0.7	-2.8	-0.7	-2.8	-0.7	-2.8
Simazine	0	1.4±0.01	0.9±0.22	0.5±0.03	0.5±0.10	0	0
(mg l^{-1})	5	1.4±0.05	0.8±0.17	1.0±0.03	0.5±0.02	0	0
	10	1.4±0.10	0.7±0.09	0.7±0.05	0.5±0.02	0	0
Trifluralin	5	0.9±0.51	0.4±0.03	0.9±0.04	0.4±0.06	0.7±0.34	0
(mg l^{-1})	10	0.6±0.01	0.6±0.05	1.0±0.02	0.5±0.04	0.3±0.05	0
Dieldrin	5	0.8±0.13	0.7±0.06	1.0±0.02	0.6±0.09	0.1±0.01	0.2±0.01
(mg l^{-1})	10	0.8±0.15	0.6±0.18	0.5±0.03	0.4±0.07	0.4±0.58	0
							0
Mixture	5	0.7±0.01	0.7±0.15	11.8±0.16	4.6±1.22	0.5±0.50	0
*(*mg l^{-1}*)*	10	0.7±0.22	0.5±0.00	6.1±1.13	5.5±0.10	0.2±0.12	0

Matric potential effects

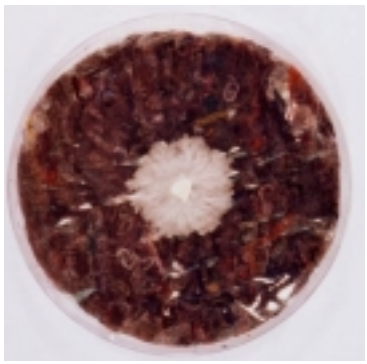
The different water regimes were attained with the addition of polyethylene glycol (PEG 8000) to the culture media. Plate 3.2 shows three of the test isolates growing on matrically modified medium. Overall, fungal growth was much lower in matric than osmotically modified media. Additionally, in matric potential treatments, the effect of the pesticides on growth was more significant. Indeed, one of the test isolates, *P.chrysosporium*, did not grow under any of the treatment conditions examined. *T.versicolor* (R101) showed the best growth rates in this study. Figure 3.3 shows the growth rates obtained for *T.versicolor* (R26) in soil extract adjusted to -0.5, -1.5 and -2.8 MPa matric potential. At the highest water availability (-0.5 MPa) *T.versicolor* (R26) was tolerant of all pesticide treatments, with the highest growth rates in the mixture of pesticides. In the mixture treatment there was a decrease in growth at 10 mg l^{-1}. In the simazine and dieldrin treatments there was no difference in growth between 5 and 10 mg l^{-1}. At the intermediate matric potential (-1.5 MPa) the fungus was not tolerant to any of the pesticides, even though it grew in the control. At -2.8 MPa growth of this species was completely inhibited.

Table 3.6 shows the effect of matric potential and pesticide on growth rates of *T.versicolor* (R101). At the -0.5 and -1.5 MPa matric potential *T.versicolor* (R101) was tolerant to all pesticide treatments. At -0.5 MPa matric potential there was no significant difference in growth in different concentrations for the simazine and the mixture treatments, which suggests *T.versicolor* (R101) was very tolerant to simazine and the mixture. At -1.5 MPa this isolate had good tolerance to all treatments. At -2.8 MPa there was complete inhibition of growth for all treatments with simazine and mixture, while some growth was observed at 10 mg l^{-1} of trifluralin and dieldrin.

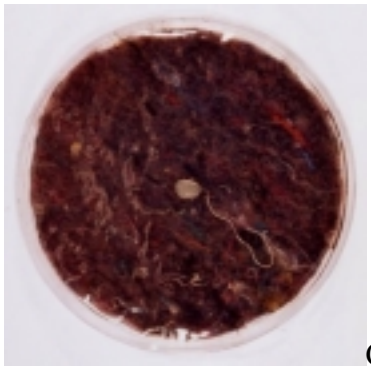
Table 3.6 shows the growth rates obtained by *P.ostreatus* in soil extract adjusted to -0.5, -1.5 and -2.8 MPa matric potential. This fungus was highly influenced by the matric potential and the presence of pesticides. At -0.5 MPa matric potential the fungus showed some growth, but some reduction was observed in the pesticide treatments, comparing with the control, which suggests *P.ostreatus* was very sensitive to the pesticides under matric imposed water. Complete growth inhibition was observed at -1.5 and -2.8 MPa.



A



B



C

Plate 3.2 *P.ostreatus*, *T.versicolor* (R26) and *P.chrysosporium*, growing on matrically modified soil extract media at –0.5 MPa, at 15^0C.

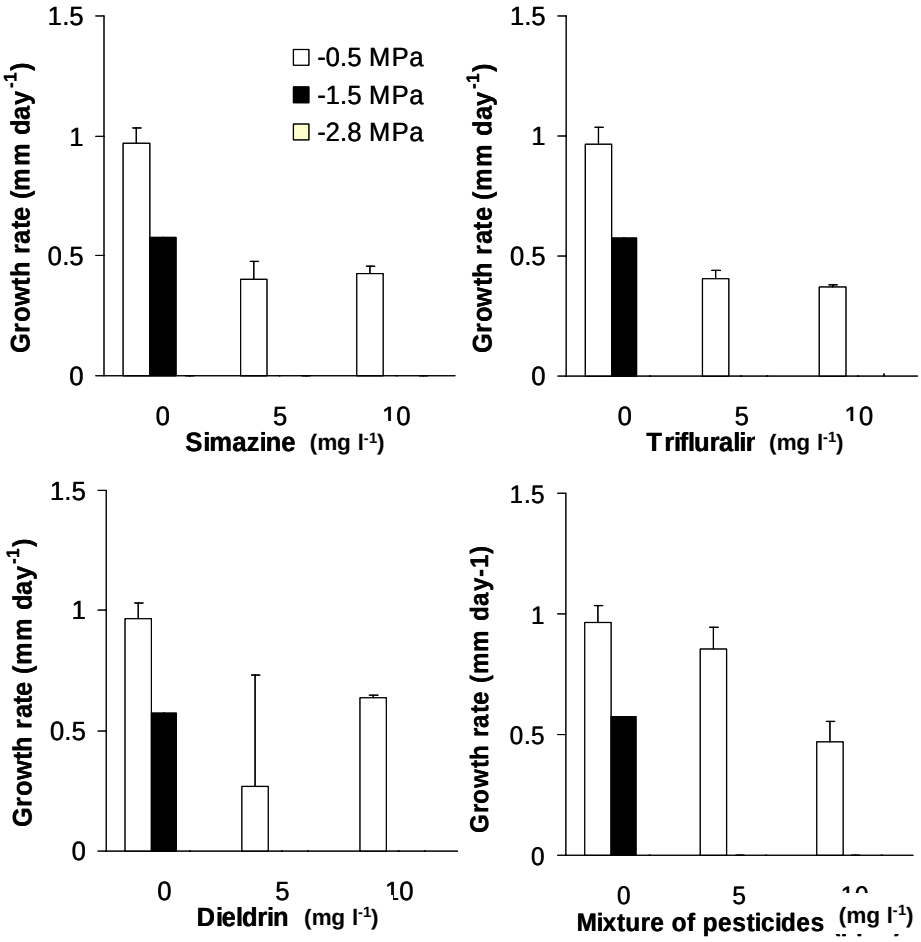


Figure 3.3 Comparison of growth of *T.versicolor* (R26) in soil extract supplemented with simazine, trifluralin and dieldrin at 0, 5 and 10, individually and as a mixture, at -0.5, -1.5 and -2.8 MPa matric potential, incubated at 15ºC (Bars are the standard deviation of the mean, n=3).

Table 3.6 Mean fungal growth rates (± standard deviation) for *T.versicolor* (R101), *P.ostreatus* and *P.chrysosporium* in soil extract supplemented with three pesticides individually and as a mixture, under different matric potentials, at 15ºC.

		T.versicolor R101			*P.ostreatus*		
Ψ (MPa)		-0.5	-1.5	-2.8	-0.5	-1.5	-2.8
Simazine	0	0.6±0.07	0.5±0.04	0	1.7±0.03	0	
(mg l^{-1})	5	0.7±0.07	0.4±0.03	0	0.6±0.09	0	0
	10	0.6±0.18	0.5±0.01	0	0.2±0.36	0	0
Trifluralin	5	0.6±0.03	0.4±0.02	0	1.0±0.09	0	0
(mg l^{-1})	10	0.4±0.01	0.5±0.06	0.1±0.01	0.4±0.07	0	0
Dieldrin	5	0.7±0.03	0.3±0.01	0	0.2±0.10	0	0
(mg l^{-1})	10	0.7±0.10	0.5±0.03	0.1±0.01	1.0±0.00	0	0
Mixture	5	0.7±0.02	0.4±0.01	0	1.5±0.00	0	0
(mg l^{-1})	10	0.8±0.05	0.4±0.04	0	0.1±0.00	0	0

3.2.5 Production of ligninolytic enzymes in Petri plate assays

The applicability of fungi in bioremediation of soil contaminated with pesticides depends on their capacity to grow 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 species and the effect that water potential and pesticide treatment may have on this.

Potential ligninolytic activity

Overall *T.versicolor* (R26 and R101) and *P.ostreatus* showed strong ligninolytic activity expressed as the radius of clearing zone (Plate 3.3). *P.chrysosporium* did not produce decolouration under the conditions of the assay, although this isolate grew well in the medium used in this assay.

Figure 3.4 shows lignin degradation by *T.versicolor* (R26) in culture medium adjusted to -0.7 and -2.8 MPa. Higher ligninolytic activity was found at -0.7 MPa. Under this water regime in the mixture and simazine treatments *T.versicolor* (R26) degraded lignin regardless of the concentration of pesticides. Similarly *T.versicolor* (R101) (Table 3.7) showed higher decolouration at -0.7 MPa but was able to degrade lignin in all pesticide treatments AT -2.8 MPa.

Ligninolytic activity by *P.ostreatus* (Table 3.7) was also higher at -0.7 than at -2.8 MPa, except in the mixture where it was not as affected by water potential as in the other treatments. At -0.7 MPa in the mixture, trifluralin and dieldrin, ligninolytic activity was higher at 0 mg l^{-1} and decreased in the 5 and 10 mg l^{-1} treatments. At -2.8 MPa in the mixture, simazine and trifluralin the same trend was observed as ligninolytic activity degradation decreased, with the increase in pesticide concentration. However, in dieldrin treatment the fungus there were no differences between 5 and 10 mg l^{-1}.

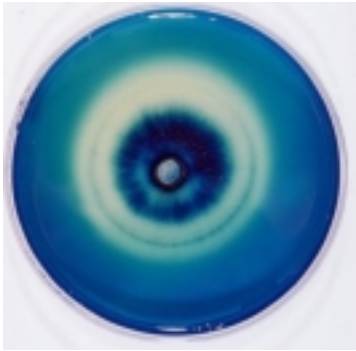


Plate 3.3 Positive result for plate assay to assess potential ligninolytic activity (*T.versicolor*).

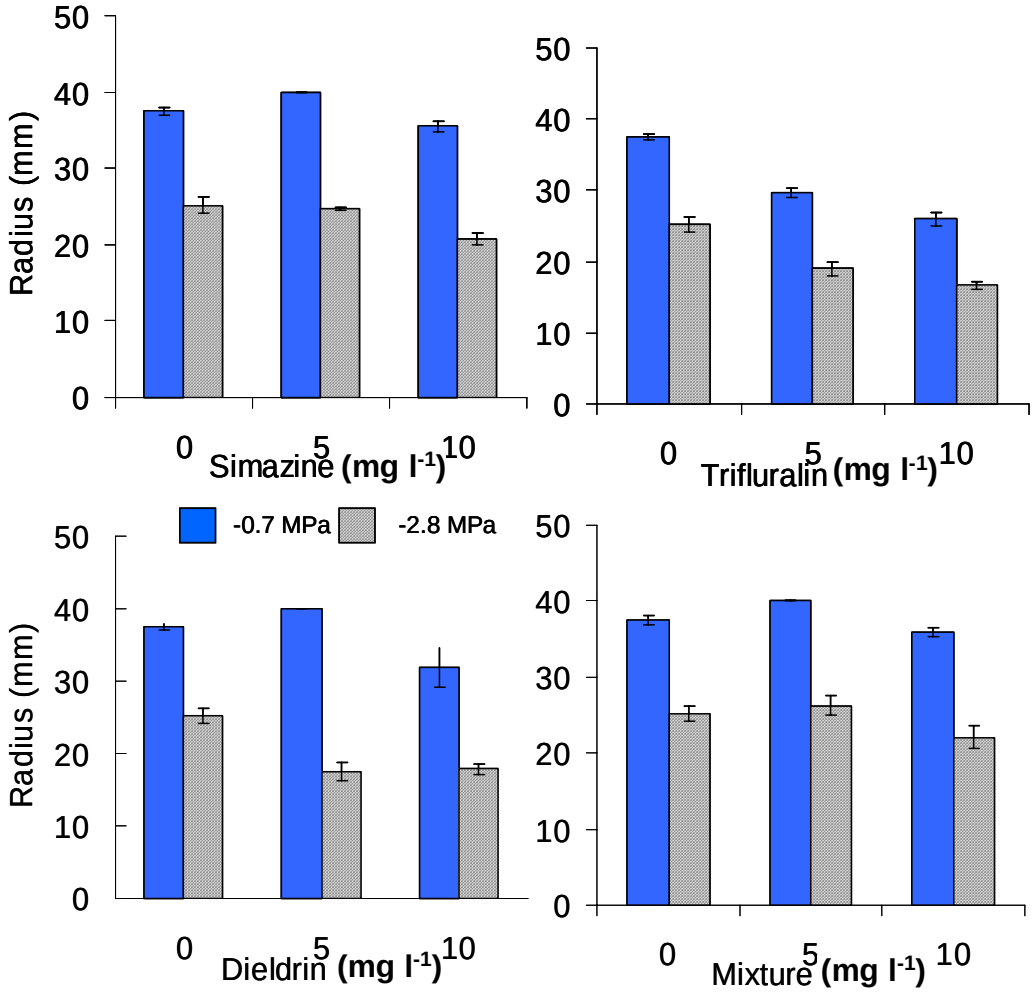


Figure 3.4 Effect of simazine, trifluralin and dieldrin (0, 5 and 10 mg l^{-1}) individually and as a mixture on potential ligninolytic activity by *T.versicolor* (R26), at 15ºC (expressed as radius of enzymatic clearing zone ± standard deviation of the mean, *n*=3).

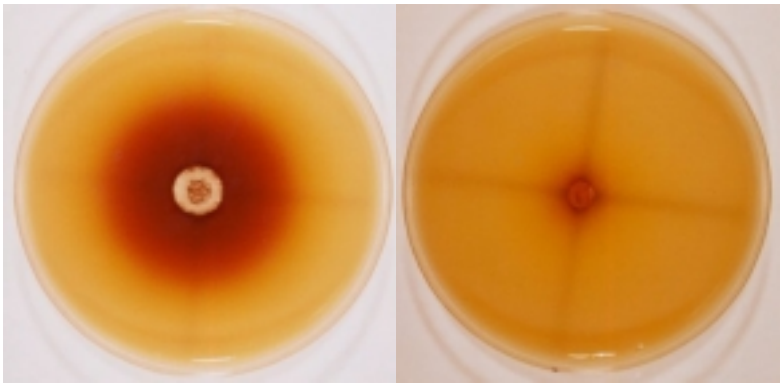
Table 3.7 Effect of simazine, trifluralin and dieldrin (0, 5 and 10 mg l^{-1}) individually and as a mixture on ligninolytic activity by *T.versicolor* (R101) and *P.ostreatus*. at 15ºC (expressed as radius of enzymatic clearing zone ± standard deviation of the mean, *n*=3).

		T.versicolor (R101)		*P.ostreatus*	
	Ψ (MPa).	-0.7	-2.8	-0.7	-2.8
Simazine	0	21±0.6	11±1.0	40±0.0	27±0.7
(mg l^{-1})	5	21±0.6	10±1.0	21±0.4	13±0.4
	10	16±0.6	13±1.0	21±1.2	10±0.1
Trifluralin	5	14±0.6	12±0.5	35±0.6	25±0.6
(mg l^{-1})	10	11±0.6	8±0.6	28±0.6	19±0.5
Dieldrin	5	15±1	10±1.5	36±1.5	13±1.9
(mg l^{-1})	10	11±1	6±1.0	25±0.2	15±0.6
Mixture	5	15±0.6	8±0.6	25±0.6	22±1.5
(mg l^{-1})	10	21±0.6	9±0.6	17±0.6	18±1.0

Polyphenol oxidase production

All test isolates, except *P.chrysosporium*, produced activity halos (see Plate 3.4) and showed different activities under different pesticide conditions. *P.chrysosporium* was unable to grow or produce activity halos under the conditions of this assay. Figure 3.5 shows polyphenol oxidase production by *T.versicolor* (R26) in medium adjusted to -0.7 and -2.8 MPa. This fungus produced a halo in all the treatment conditions but showed less enzyme activity under water stress than with freely water available. At -0.7 MPa *T.versicolor* (R26) also showed enzyme activity in the presence of the mixture, simazine and trifluralin treatments regardless of the pesticide concentration. Under water stress, *T.versicolor* (R26) was also capable of forming the activity halo, and interestingly, this was stimulated with increasing pesticide concentration. For example in the mixture there was an increase in 5 mg l^{-1} when compared with the control.

Table 3.8 shows polyphenol oxidase by *T.versicolor* (R101). Interestingly this was highly influenced by pesticides and water availability. The highest enzymatic activity was found for simazine treatment. At -0.7 MPa *T.versicolor* (R101) did not produce an activity halo in the control (0 mg l^{-1}), whereas in the treatments with a mixture, simazine and dieldrin treatments there was a stimulation at 5 mg l^{-1}. However, in the treatment with trifluralin polyphenol oxidase was only produced at 10 mg l^{-1}. Under water stress this fungus produced the activity halo in all treatments except dieldrin, suggesting high sensitivity to this insecticide. In the other treatments, there was enzyme activity at 5 and 10 mg l^{-1}. Table 3.8 shows polyphenol oxidase production by *P.ostreatus* that showed significantly less activity under water stress, at -2.8 MPa ($P<0.001$). The highest enzyme production was observed in the treatments with mixture and simazine. Interestingly in the trifluralin and dieldrin treatments there was a sharp increase in enzymatic production at 10 mg l^{-1} (at -0.7 MPa). At -2.8 MPa *P.ostreatus* produced polyphenol oxidases all pesticide treatments, except dieldrin, which has already been observed for *T.versicolor* (R101). These two test species seem to be very sensitive to this insecticide. In the mixture the production of polyphenol oxidase was not affected by the concentration of pesticides.



(a) (b)

Plate 3.4 Laccase activity plate assay: (a) positive result for the isolate *T.versicolor* R26, and (b) negative result for the isolate *P.chrysosporium*.

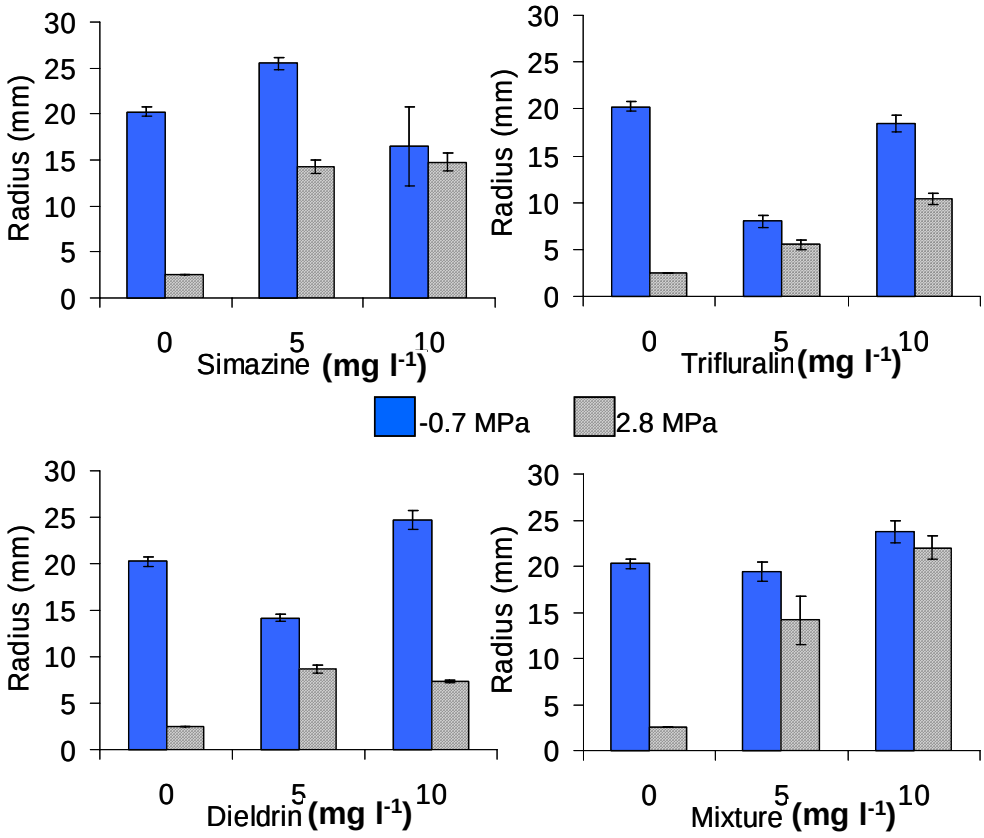


Figure 3.5 Effect of simazine, trifluralin and dieldrin (0, 5 and 10 mg l^{-1}) individually and as a mixture on laccase activity by *T.versicolor* (R26), at 15ºC (expressed as radius of enzymatic activity halo ± standard deviation of the mean, *n*=3).

Table 3.8 Effect of simazine, trifluralin and dieldrin (0, 5 and 10 mg l^{-1}) individually and as a mixture on laccase activity by *T.versicolor* (R101) and *P.ostreatus*. at 15ºC

		T.versicolor (R101)		*P.ostreatus*	
	Ψ (MPa)	-0.7	-2.8	-0.7	-2.8
Simazine	0	0	3±0	14±1.5	6±0.5
(mg l^{-1})	5	17±0.3	6±1.0	13±0.5	5±0.5
	10	7±0.6	4±1.3	5±0	3±0
Trifluralin	5	0	4±1.5	2±0.2	2±0
(mg l^{-1})	10	6±0.5	10±0	8±0	1±0.6
Dieldrin	5	3±0.2	0	3±0.2	0
(mg l^{-1})	10	4±0.8	0	7±1.3	0
Mixture	5	5±0.3	4±0.5	10±0	5±0
*(*mg l^{-1}*)*	10	6±0.2	4±0.3	7±1.5	7±0.2

Part II Soil Extract Studies

3.3 Introduction

The objectives of these studies were to examine the interactions and activity of four white rot fungi: *T.versicolor* (R26 and R101), *P.ostreatus*, *P.chrysosporium* in soil extract broth, in relation to (a) degradation of a mixture of different concentrations (0-30 mg l^{-1}) of simazine, dieldrin and trifluralin, (b) interactions with osmotic stress (-0.7 and -2.8 MPa) and (c) production of enzymes relevant to P and N release (phosphomonoesterase, protease) and carbon cycling (β-glucosidase, endocellulase) and laccase activity.

3.4 Hydrolytic enzymes

3.4.1 Optimisation of enzyme assays in 96 wells microtitre plates

The miniaturization of the enzyme assays was established by an adaptation to microtitre-plates involving semi-automated multi-channel pipettes and a microtitre plate reader. The optimisation stages are described below.

Laccase

For the assay to quantify laccase activity the protocol of Buswell *et al.* (1995) was followed. After reducing the volumes of reagents, it was necessary to choose the best incubation period for the assay. Several incubation times and two different reading modes of the microtitre plate reader (Dinex Technologies MRX Revelation): kinetic and endpoint, were tested.

Kinetic mode is a predefined analysis of the absorbance values recorded by the microtitre-plate reader, the output values are given as (final absorbance- initial absorbance)/ reaction time (min). In the endpoint mode the output values are expressed as absorbance units, at the different moments the microtitre-plate is read, and the calculations are made subsequently by subtracting the initial absorbance to the final absorbance, divided by the period of time between readings.

Figure 3.6 to Figure 3.8 show calibration curves of purified commercial laccase in concentrations ranging from 0.03 to 0.8 mg ml^{-1}. Using the kinetic mode two approaches were tested: a) the reagents were incubated at 37ºC, mixed with the enzyme solution (that had been defrost and kept in ice) and then the microtrite-plate was incubated inside the microtitre plate reader (Figure 3.6) with readings every 30 seconds. The second approach was: b) the reagents were incubated at 37ºC, then mixed with the enzyme solution (cold) followed by incubation of the reaction mixture (in the microtitre plate) at 37ºC prior to incubation in the microtitre plate reader (Figure 3.7). Endpoint mode was also tested (Figure 3.8): the microtitre plate was read at the start of the reaction and then at the end, and in the meantime it was incubated at 37ºC.

For the subsequent assays, leading to the analysis of laccase activity in the samples we chose to set the microtitre plate reader in the kinetic mode, the reaction time was 10 minutes, 5 seconds agitation at the beginning, without previous incubation. This assay was sensitive for laccase concentrations as low as 0.03125 mg ml^{-1}, using commercial laccase from *Rhus vernificera*.

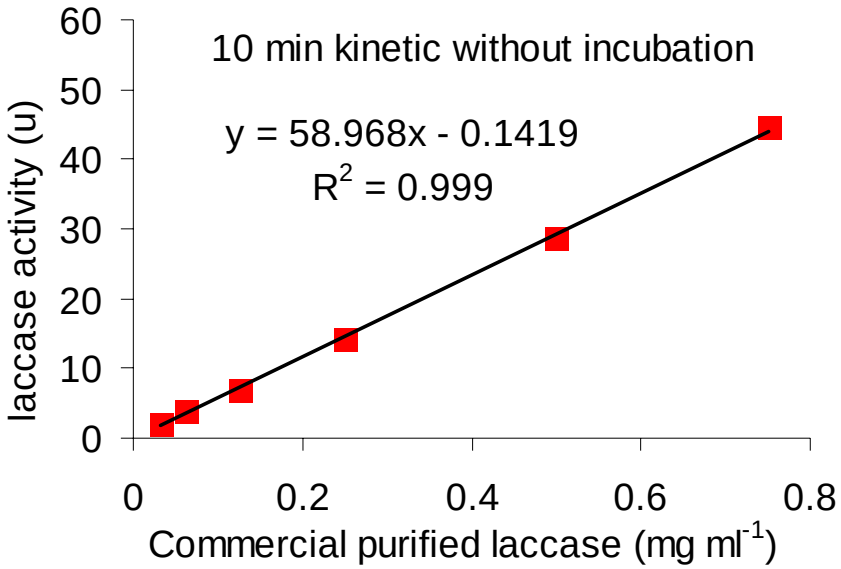


Figure 3.6 Linear regression of laccase activity against concentration of purified commercial laccase from *Rhus vernificera*. The microtitre plate reader was set in the kinetic mode (10 min) without previous incubation of the microtitre plate.

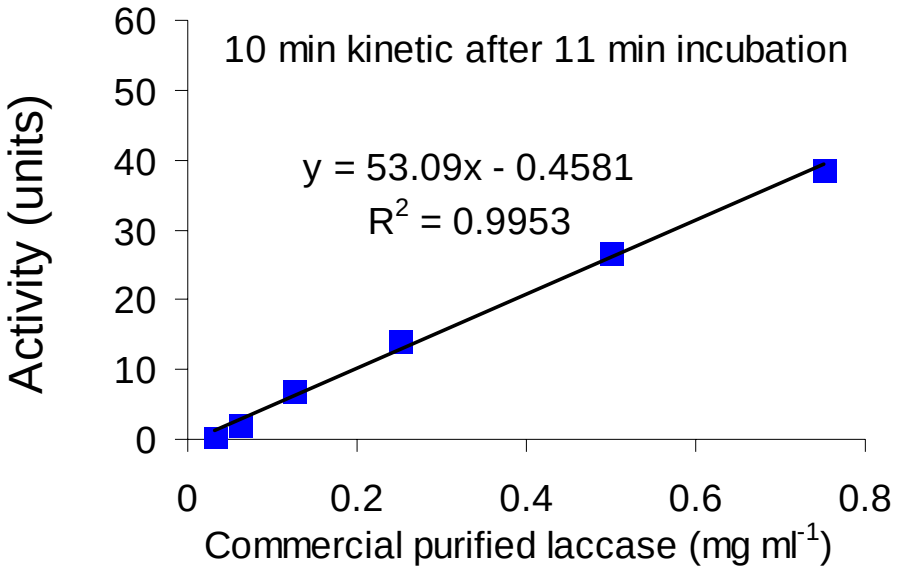


Figure 3.7 Linear regression of laccase activity against concentration of purified commercial laccase from *Rhus vernificera*. The microtitre plate reader was set in the kinetic mode (10 min) with previous incubation of the microtitre plate at 37ºC for 11 mins.

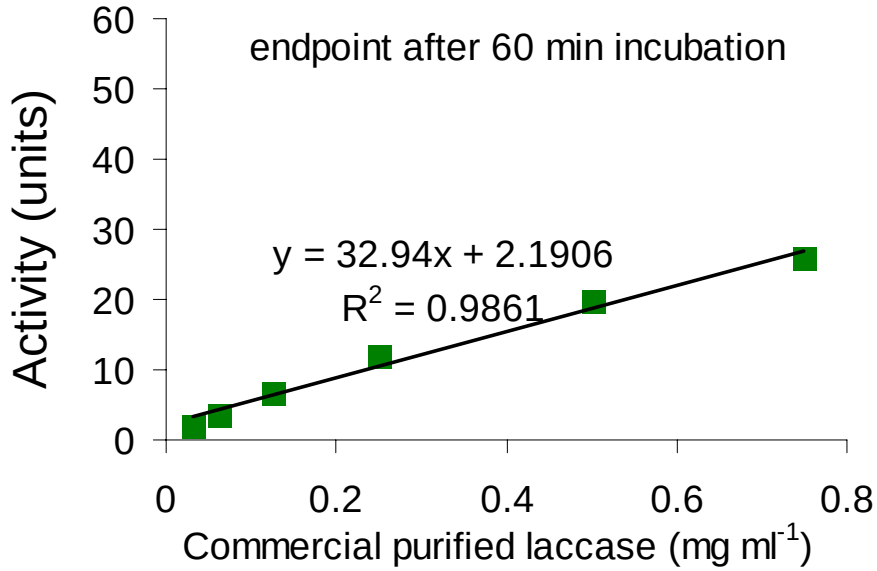


Figure 3.8 Linear regression of laccase activity against concentration of purified commercial laccase from *Rhus vernificera*. The microtitre plate reader was set in endpoint mode and the microtitre plate was incubated for 60 mins at 37ºC.

Protease

To assess protease activity we followed the assay described by Germano *et al.* (2002). This assay had to be miniaturised for a microtitre plate, by reducing the reagent volumes, keeping exactly the same concentrations referred in the literature. The optimisation consisted in trying the assay with increasing concentrations of commercial protease, from *Aspergillus oryzae*, to assess the assay's sensitivity (Figure 3.9). The assay was sensitive to concentrations as low as 0.0063 units ml^{-1} protease (Figure 3.10).

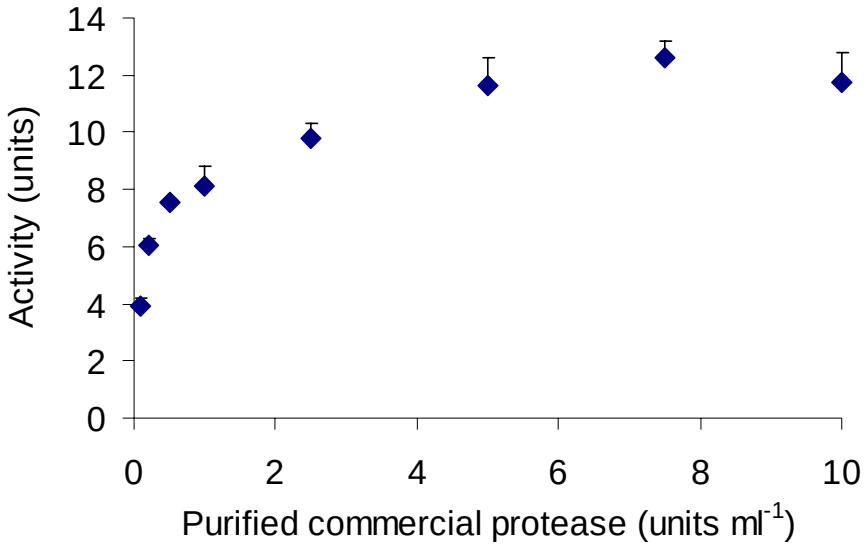


Figure 3.9 Optimisation for protease activity assay, with purified protease from *Aspergillus oryzae* in concentrations in the range 0-10 units ml^{-1}. The microtitre plate reader was set in endpoint mode and the microtitre plate was incubated for 60 mins at 37ºC.

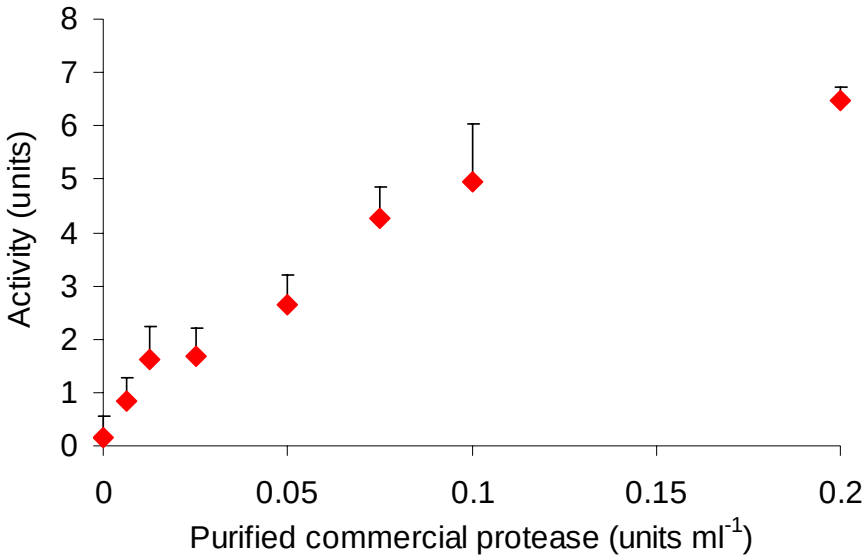


Figure 3.10 Optimisation for protease activity assay, with purified protease from *Aspergillus oryzae* in concentrations in the range 0-0.2 units ml^{-1}. The microtitre plate reader was set in endpoint mode and the microtitre plate was incubated for 60 mins at 37ºC.

Cellulase

To assess cellulase activity we followed the microtitre plate assay described by Wirth and Wolf (1992) and tested it using commercial cellulase from *Aspergillus niger*. Different incubation times (from 30 mins to 4 hrs) were examined in order to choose the most suitable incubation time for this assay. The assay was sensitive to concentrations of purified commercial cellulase as low as 0.01 units ml^{-1} (Figure 3.11).

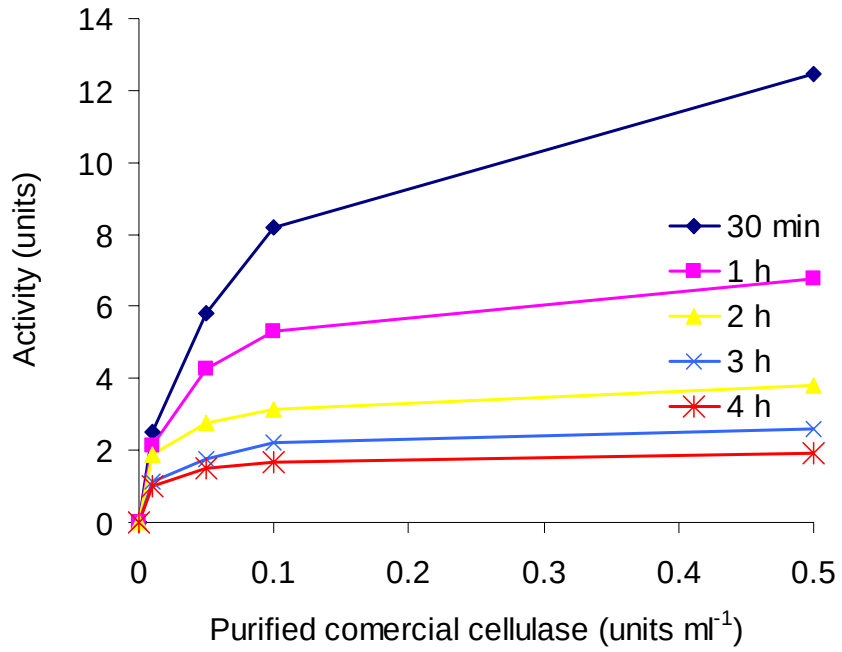


Figure 3.11 Cellulase activity of increasing concentrations of purified cellulase from *Aspergillus niger,* in concentrations ranging from 0-0.5 units ml^{-1}, with different incubation times (30 min- 4 hrs).

3.4.2 Enzyme production in soil extract samples

Having optimised the conditions for the enzymatic assays, the enzymatic activity of the four test isolates in the soil extract broth was examined. Enzyme production, relevant to P and N release (phosphomonoesterase, protease), carbon cycling (β-glucosidase, cellulase) and laccase activity were examined.

The impacts of the pesticide mixture concentration and water stress treatments on the production of five enzymes by *T.versicolor* R26 (Table 3.9) and R101 (Table 3.10), *P.ostreatus* (Table 3.11) and *P.chrysosporium* (Table 3.12) were examined.

P.chrysosporium and *T.versicolor* (R101) produced no laccase in any of the treatments. In contrast, *P.ostreatus* produced very high levels of laccase. Higher production of this enzyme was observed under osmotic stress ($P=0.001$), and all the treatments showed increased levels of laccase compared to the control ($P<0.05$). At this water regime laccase production was unaffected by the different concentrations of the pesticide mixture ($P<0.05$). At -0.7 MPa this fungus showed the highest laccase production at 10 and 30 mg l^{-1} ($P<0.05$).

T.versicolor (R26) also produced very high amounts of laccase, especially with freely available water. At -0.7 MPa there was a significant increase in laccase production between the control and the treatments (P=0.036). Under osmotic stress laccase production was significantly reduced, although up to 20 units were still detected. At -2.8 MPa osmotic stress there was no significant difference between laccase activity in the different pesticide treatments.

Cellulase production by *P.chrysosporium* and *P.ostreatus* occurred at very low levels. *P.chrysosporium* production of cellulase was low regardless of treatment (P=0.596), while *P.ostreatus* showed higher cellulase production under osmotic stress (P<0.001), but the production of this enzyme was unaffected by the pesticide treatment. *T.versicolor* R26 and R101 exhibited significantly higher activities of cellulase at –0.7 MPa than at –2.8 MPa (P=0.006 and P=0.036, respectively) in the pesticide treatments.

With regard to other hydrolytic enzymes, *T.versicolor* (R101) produced higher concentrations of protease than the other isolates. This production was higher under water stress (P<0.001) and was unaffected by the mixture concentration (P=0.139). Protease production by *P.chrysosporium* occurred at low levels regardless of treatment. *T.versicolor* (R26) produced higher protease levels at the lowest water availability (P<0.001) and showed a significant increase in protease levels at 5 mg l^{-1}, under water stress (P<0.001). Production of protease by *P.ostreatus* was not influenced by water availability (P=0.760), nor pesticide concentration at -2.8 MPa.

β-glucosidase production by *T.versicolor* (R26) and *P.ostreatus* was higher than *P.chrysosporium* or *T.versicolor* (R101). *P.ostreatus* showed high production of this enzyme regardless of water potential or pesticide treatment (P=0.076 and P=0.077, respectively). *T.versicolor* (R101) produced higher β-glucosidase under water stress (P<0.001), showing the highest β-glucosidase activity in the control under this water regime. *T.versicolor* (R26) produced higher β-glucosidase levels at 5 mg l^{-1} under water stress (P<0.05). *P.chrysosporium* showed the highest production of this enzyme in the control at -0.7 MPa (P<0.05).

Contrarily to β-glucosidase, phosphomonoesterase production by *P.ostreatus* was very low. Phosphomonoesterase activity by *P.ostreatus* was higher under water stress,

showing a significant reduction in the 15 and 30 mg l^{-1} treatments ($P<0.05$), under this water regime. *T.versicolor* (R101) also showed higher phosphomonoesterase activity at -2.8 MPa ($P=0.011$), but the activity of this enzyme was not influenced by mixture concentration. The production of phosphomonoesterase by *P.chrysosporium* was much higher than by *T.versicolor* (R26) and the activity of this enzyme by these two isolates was not affected by water potential or pesticide concentration.

Table 3.9 Extracellular enzyme activities of *T.versicolor* (R26) growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1} total concentrations), for 25 days, at 27^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		Mixture of pesticides (mg l^{-1})				
Enzyme (U)	Ψ (MPa)	0	5	10	15	30
Cellulase	-0.7	2.1	3.6	1.8	4.5	4.2
L.S.D. = 1.86	-2.8	1.6	1.9	1.9	1.8	2.4
Phosphomonoesterase*	-0.7	9.2	5.9	6.0	2.2	2.8
L.S.D. = 12.67	-2.8	8.9	8.1	13.7	4.5	5.0
β-glucosidase*	-0.7	14.8	14.2	23.2	16.4	45.1
L.S.D. = 21.18	-2.8	10.2	51.2	11.0	25.7	3.5
Protease	-0.7	0	4.1	6.1	2.1	2.3
L.S.D. = 39.23	-2.8	0.3	58.6	28.3	1.0	0
Laccase	-0.7	91.9	230.1	206.2	214.4	205.7
L.S.D. = 126.34	-2.8	9.3	3.2	11.8	6.5	20.2

*nmol PNP $min^{-1}mg^{-1}$ protein

Table 3.10 Extracellular enzyme activities of *T.versicolor* (R101) growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1} total concentrations), for 25 days, at 27°C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		Mixture of pesticides (mg l^{-1})				
Enzyme (U)	Ψ (MPa)	0	5	10	15	30
Cellulase	-0.7	1.6	1.3	2.5	1.1	0.4
L.S.D. = 2.40	-2.8	1.8	0.9	0.0	0.0	0.0
Phosphomonoesterase*	-0.7	4.7	1.0	1.5	4.0	3.1
L.S.D. = 2.45	-2.8	8.3	9.0	3.3	4.9	4.0
β-glucosidase*	-0.7	2.8	1.0	1.9	0.4	0.0
L.S.D. = 4.75	-2.8	11.4	4.3	1.7	2.8	5.3
Protease	-0.7	12.7	43.4	33.9	12.9	16.3
L.S.D. = 27.57	-2.8	45.4	44.1	47.4	49.0	64.2
Laccase	-0.7	0.0	0.0	0.0	0.0	0.0
	-2.8	0.0	0.0	0.0	0.0	0.0

*nmol PNP $min^{-1}mg^{-1}$ protein

Table 3.11 Extracellular enzyme activities of *P.ostreatus* growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1} total concentrations), for 25 days, at 27°C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		Mixture of pesticides (mg l^{-1})				
Enzyme (U)	Ψ (MPa)	0	5	10	15	30
Cellulase	-0.7	1.4	0.5	1.1	0.8	0.9
L.S.D.= 1.05	-2.8	0.4	0.0	0.2	0.2	0.1
Phosphomonoesterase*	-0.7	0.2	0.0	0.0	0.0	1.3
L.S.D.= 2.74	-2.8	7.9	6.6	4.5	0.0	0.0
β-glucosidase*	-0.7	20.1	14.2	5.4	9.0	14.7
L.S.D.= 29.81	-2.8	41.0	18.1	22.6	13.0	10.6
Protease	-0.7	2.3	1.7	44.5	0.0	12.0
L.S.D.= 31.24	-2.8	7.2	10.8	28.1	2.3	2.6
Laccase	-0.7	14.5	17.6	126.6	92.5	146.4
L.S.D.= 109.34	-2.8	28.2	206.2	146.5	192.1	138.4

*nmol PNP $min^{-1}mg^{-1}$ protein

Table 3.12 Extracellular enzyme activities of *P.chrysosporium* growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1} total concentrations), for 25 days, at 27°C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		Mixture of pesticides (mg l^{-1})				
Enzyme (U)	Ψ (MPa)	0	5	10	15	30
Cellulase	-0.7	3.4	4.4	0	1.2	1.5
L.S.D. = 2.21	-2.8	4.2	1.1	0.1	0.9	0.3
Phosphomonoesterase*	-0.7	13.0	26.1	13.9	11.5	16.0
L.S.D. = 12.72	-2.8	22.1	35.4	26.0	22.9	17.8
β-glucosidase*	-0.7	29.0	9.7	1.4	0.9	3.9
L.S.D. = 13.78	-2.8	9.3	3.0	1.1	1.0	0.3
Protease	-0.7	0.1	4.5	23.8	22.4	8.9
L.S.D. = 20.28	-2.8	6.9	1.7	1.7	0	0.1
Laccase	-0.7	0	0	0	0	0
	-2.8	0	0	0	0	0

*nmol PNP $min^{-1}mg^{-1}$ protein

3.5 Soluble protein

The quantification of soluble protein content was essential to estimate the specific enzymatic activity; moreover, this parameter can also be used as additional information on fungal activity. The effect of mixtures of pesticides, on soluble protein content in liquid media inoculated with the test isolates, after 25 days incubation, under two different water regimes is shown in Figures 3.12 and 3.13.

P.chrysosporium produced the highest levels of extracellular protein. All isolates except *T.versicolor* (R101) showed significantly higher soluble protein content at -2.8 MPa than at -0.7 MPa ($P<0.05$). *T.versicolor* (R101) filtrates contained comparable levels of soluble protein at both water potentials ($P=0.077$). *T.versicolor* (R26) soluble protein content was not significantly affected by pesticide treatments at –0.7 MPa. However, at –2.8 MPa, there was a statistically significant decrease in protein content at 15 mg l^{-1}. At -0.7 MPa *T.versicolor* (R101) protein content was unaffected by pesticide concentration ($P<0.05$). At -2.8 MPa, in the 30 mg l^{-1} there was an increase in protein content ($P<0.05$). At the two osmotic conditions tested *P.ostreatus* and *P.chrysosporium* soluble protein production was unaffected by pesticide concentration ($P<0.05$).

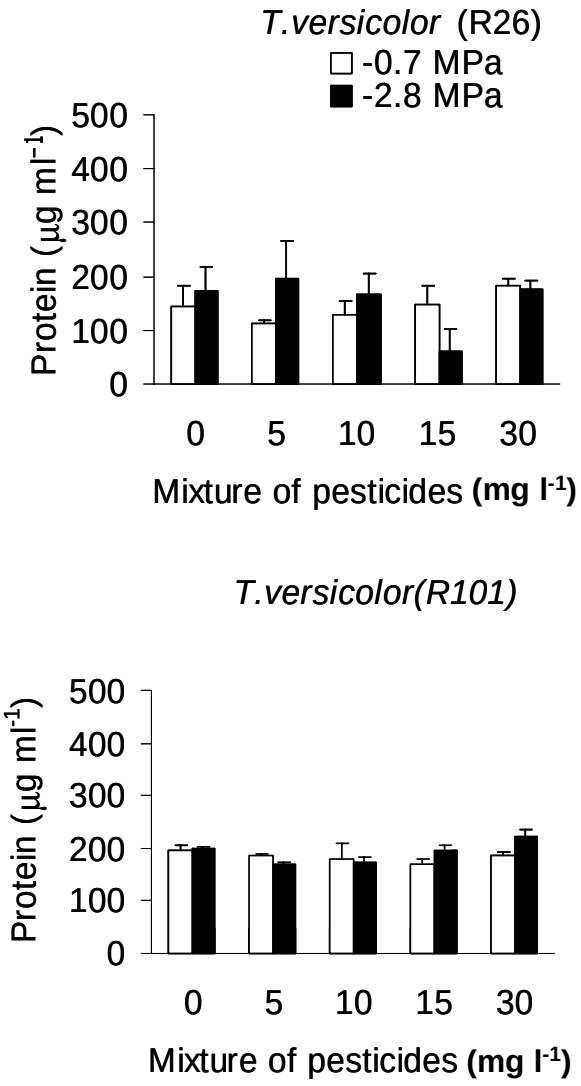


Figure 3.12 Protein concentration of *T.versicolor* (R26) and *T.versicolor* (R101) growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1}), for 25 days, at 27^0C, under two different water potential regimes. Bars represent the standard deviation of the mean (*n*=3).

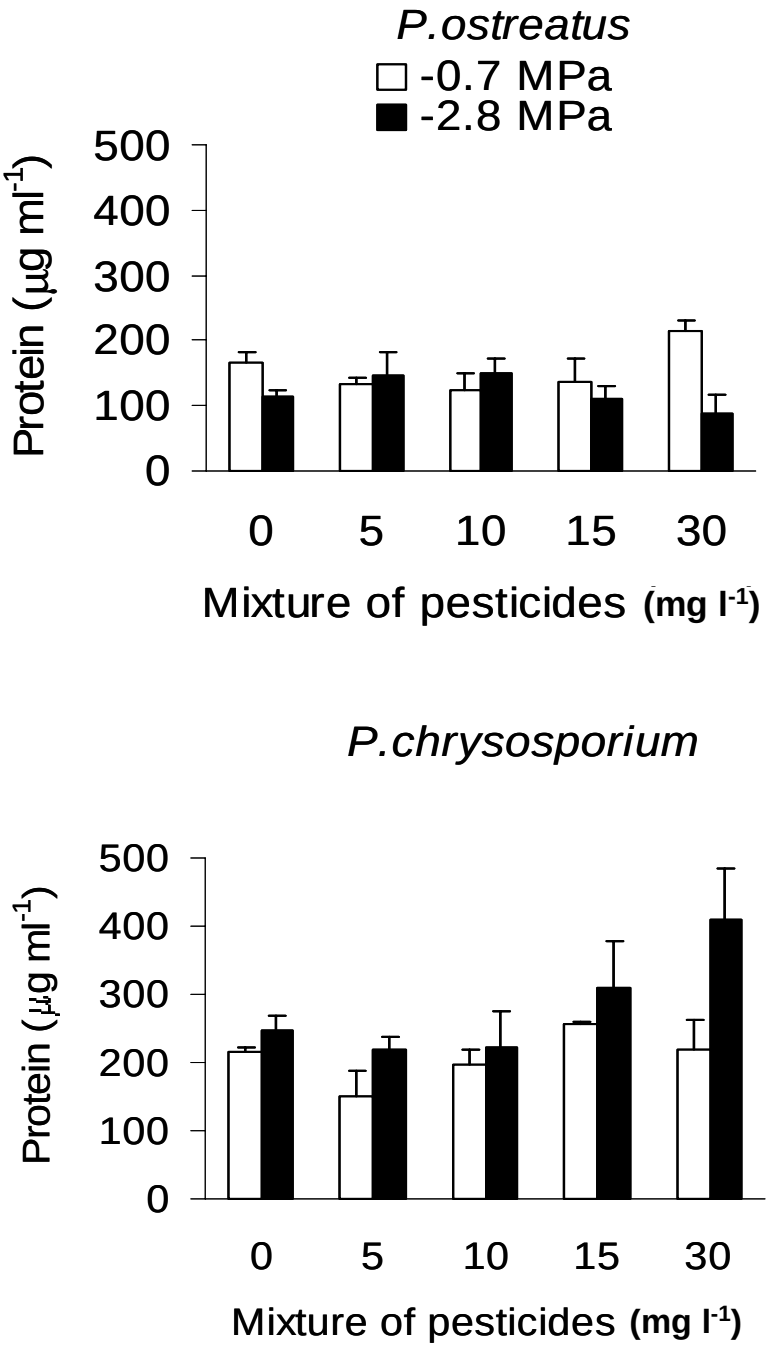


Figure 3.13 Protein concentration of *P.ostreatus* and *P.chrysosporium* growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1}), for 25 days, at 27^0C, under two different water potential regimes. Bars represent the standard deviation of the mean (*n*=3).

3.6 Biomass

The effect of mixtures of pesticides, at two different water potential, on biomass of the four test isolates, after 25 days incubation, is shown in Figures 3.15 and 3.16.

All four isolates were tolerant of and grew effectively in the presence of up to 30 mg l^{-1} of the mixture of pesticides in the soil extract broth. All species showed significantly higher biomass production at -2.8 than at -0.7 MPa ($P<0.05$). *T.versicolor* (R26) total biomass was not significantly affected by pesticide treatments at –0.7 MPa. However, at –2.8 MPa, there was a statistically significant increase in biomass when comparing the untreated control with 10 mg l^{-1} treatment ($P=0.031$), suggesting stimulation of growth. At the two osmotic conditions tested *T.versicolor* (R101) biomass production was affected by water potential ($P=0.025$) and pesticide concentration. At -0.7 MPa the treatment 10 mg l^{-1} produced the highest biomass levels ($P<0.05$). Under water stress in there was a reduction in biomass the 30 mg l^{-1} treatment ($P=0.041$). At the two osmotic conditions tested *P.ostreatus* and *P.chrysosporium* biomass production was unaffected by pesticide concentration ($P<0.05$), which again suggests good tolerance to the mixture of pesticides.

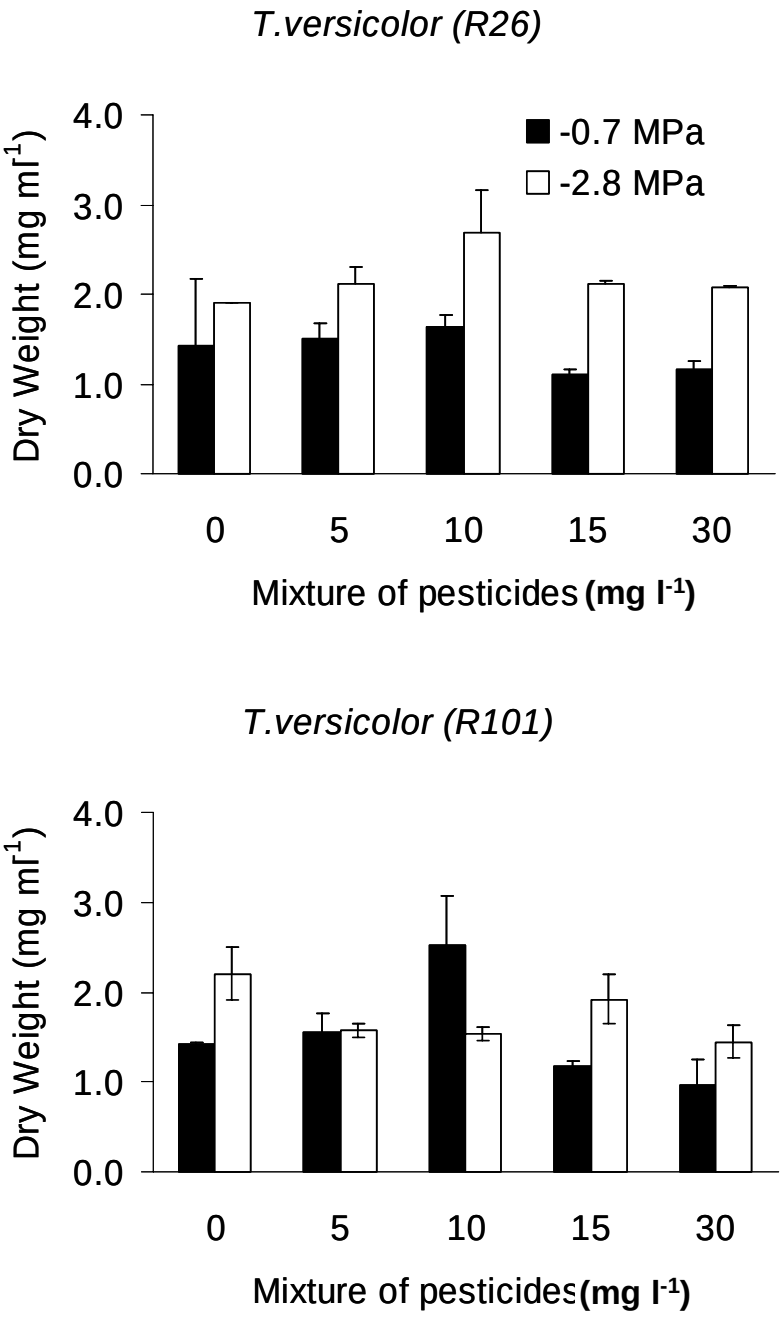


Figure 3.14 Dry weight of mycelium of *T.versicolor* (R26 and R101) growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1}), for 25 days, at 27°C, under two different water potential regimes. Bars represent the standard deviation of the mean (*n*=3).

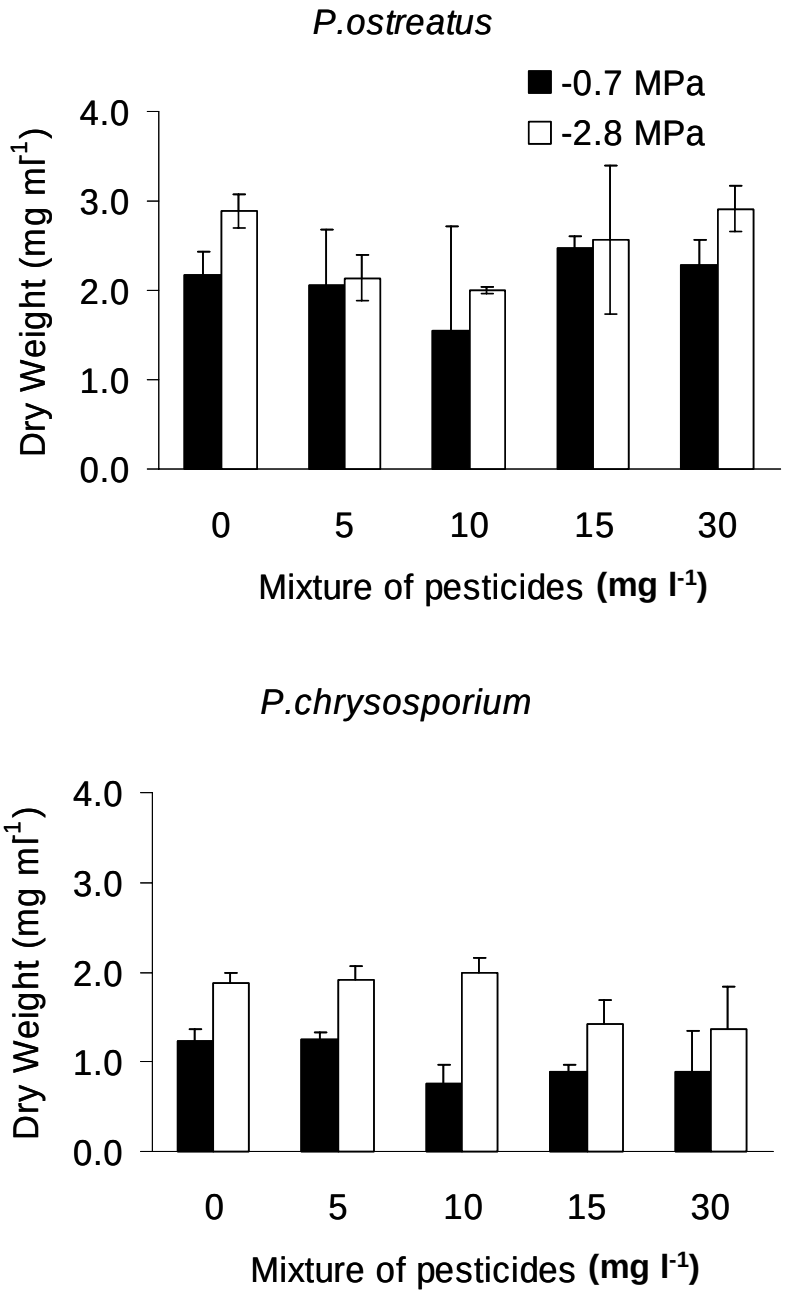


Figure 3.15 Dry weight of mycelium of *P.ostreatus* and *P.chrysosporium* growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 mg l^{-1}), for 25 days, at 27°C, under two different water potential regimes. Bars represent the standard deviation of the mean (*n*=3).

3.7 Pesticide analysis

Tables 3.13 and 3.14 show the impact of the two fungal inoculants on degradation of the mixture of pesticides. In the treatments with *P.chrysosporium* and *T.versicolor* (R26) practically no dieldrin and trifluralin were detected after 25 days incubation, regardless of initial concentration of the mixture used or the osmotic stress tested. For simazine, only 20% of the initial concentration was present even in the 30 mg l^{-1} concentration treatment. For both fungal species there was no significant effect of osmotic stress on simazine disappearance rates (*P.chrysosporium* P=0.285 and *T.versicolor* P=0.720). For *P.chrysosporium* simazine disappearance was significantly higher in the 30 mg l^{-1} treatment (P<0.050). *T.versicolor* also showed the highest disappearance rate in the 30 mg l^{-1} treatment, regardless of the osmotic potential used (P<0.050). The degradation rates by the *T.versicolor* (R101) and *P.ostreatus* were not as good, since the concentration of dieldrin remaining and trifluralin were much higher than in the treatments inoculated with the other two fungal isolates. For both fungal species there was no significant effect of osmotic stress on simazine degradation rates (for *T.versicolor*-R101 P= 0.064 and for *P.ostreatus* P=0.076). For *P.ostreatus,* simazine disappearance rates were not affected by initial concentration of the mixture, either. In contrast, *T.versicolor* showed significantly higher degradation in the 30 mg l^{-1} treatment, at -2.8 MPa (P<0.05).

The degradation rates of trifluralin and dieldrin by *T.versicolor* (R101) and *P.ostreatus* were significantly affected by water potential and initial concentration of the pesticide mixture. *P.ostreatus* showed significantly less trifluralin degradation in the 15 mg l^{-1} treatment, at both water potentials (P<0.001). *T.versicolor* (R101) showed higher degradation in the 10 and 30 pm, at -0.7 MPa (P<0.001), and in the 10 mg l^{-1} treatment, at -2.8 MPa. Regarding dieldrin, *P.ostreatus* showed the highest degradation rates in the 30 mg l^{-1} treatment (P<0.05), at -0.7 MPa and in the 15 mg l^{-1} treatment (P<0.001) at -2.8 MPa. *T.versicolor* (R101) also showed the best degradation in the 30 mg l^{-1} treatment (P<0.05), at -2.8 MPa.

Table 3.13 Concentration of pesticides remaining in soil extract that was initially supplemented with a mixture of pesticides at 5, 10, 15 and 30 mg l^{-1}, after 25 days of incubation at 27^{o}C, under two different water regimes.

			Remaining pesticide (%)		
	Ψ (MPa)	Initial conc. mixture (mg l^{-1})	Simazine	Trifluralin	Dieldrin
T.versicolor (R26)	-0.7	5	27.5±0.27	0	0
		10	20.2±7.21	0	0
		15	10.5±7.09	0	0.1±0.08
		30	13.9±0.52	0	0
	-2.8	5	15.0±2.18	0	0
		10	22.7±5.65	0	0
		15	19.7±5.28	0	0
		30	11.6±1.40	0	0
T.versicolor (R101)	-0.7	5	23.2±1.51	8.5±3.09	10.0±0.26
		10	17.3±0.57	2.5±0.57	1.7±0.36
		15	18.7±3.09	8.9±0.98	8.0±0.93
		30	12.2±2.38	1.8±0.04	1.7±0.13
	-2.8	5	22.8±1.64	19.6±1.27	18.0±0.41
		10	21.8±2.15	3.7±1.59	10.8±1.04
		15	22.7±4.23	8.0±0.25	9.0±0.19
		30	13.5±4.85	16.5±0.11	3.2±0.13

Table 3.14 Concentration of pesticides remaining in soil extract that was initially supplemented with a mixture of pesticides at 5, 10, 15 and 30 mg l^{-1}, after 25 days of incubation at 27°C, under two different water regimes.

			Remaining pesticide (%)		
	Ψ (MPa)	Initial concentration of mixture (mg l^{-1})	Simazine	Trifluralin	Dieldrin
P.ostreatus	-0.7	5	10.9±3.01	1.1±0.26	8.3±3.09
		10	16.5±6.92	1.9±0.36	8.8±0.57
		15	6.9±5.61	5.0±0.93	8.8±0.98
		30	23.51±1.83	1.7±0.13	3.9±0.04
	-2.8	5	17.6±2.14	9.0±0.41	19.3±1.27
		10	16.9±3.32	4.6±0.14	10.1±1.59
		15	13.0±0.59	6.1±0.19	7.9±0.25
		30	26.2±14.39	3.3±0.13	18.6±0.11
P.chrysosporium	-0.7	5	31.7±7.11	0	0.3±0.13
		10	23.9±0.99	0	0.1±0.02
		15	18.9±1.46	0	0.1±0.12
		30	13.0±2.19	0	0
	-2.8	5	22.8±5.22	0	0
		10	19.3±0.66	0	0
		15	25.2±9.03	0	0
		30	11.7±3.25	0	0

3.8 Toxicity test

In this study the goal was to assess the toxicity of the soil extract broth inoculated with the test isolates after the incubation period of 25 days. Initially we tested the response of this toxicity test to soil extract broth, spiked with different concentrations of pesticides (individually and as a mixture).

Figure 3.16 shows the response in luminescence emitted by *V .fischeri* to soil extract broth spiked with different concentrations of simazine, trifluralin and dieldrin, individually and as a mixture. *V.fischeri* showed high sensitivity to the mixture of pesticides with 97% luminescence inhibition in the treatments spiked with 5 mg l^{-1}. Trifluralin was less toxic than dieldrin, in the treatments over 6 mg l^{-1}. Simazine caused the lowest luminescence inhibition, showing a maximum inhibition of 20% in the 10 mg l^{-1} treatment.

Table 3.15 shows the inhibition of luminescence for soil extract liquid broth (at -0.7 and -2.8 MPa) inoculated for 25 days with *P.chrysosporium*, *T.versicolor* (R26 and R101) and *P.ostreatus*. Overall the extracts did not show toxicity, which was in accordance with the HPLC pesticide quantification, as the level of pesticides after 25 days incubation were lower than those that caused *V.fischeri* inhibition (Figure 3.16). The exceptions were the treatments 15 mg l^{-1}, -0.7 MPa and 30 mg l^{-1}, -2.8 MPa that showed 7 and 19% luminescence inhibition, even though low concentrations of pesticides were detected by HPLC.

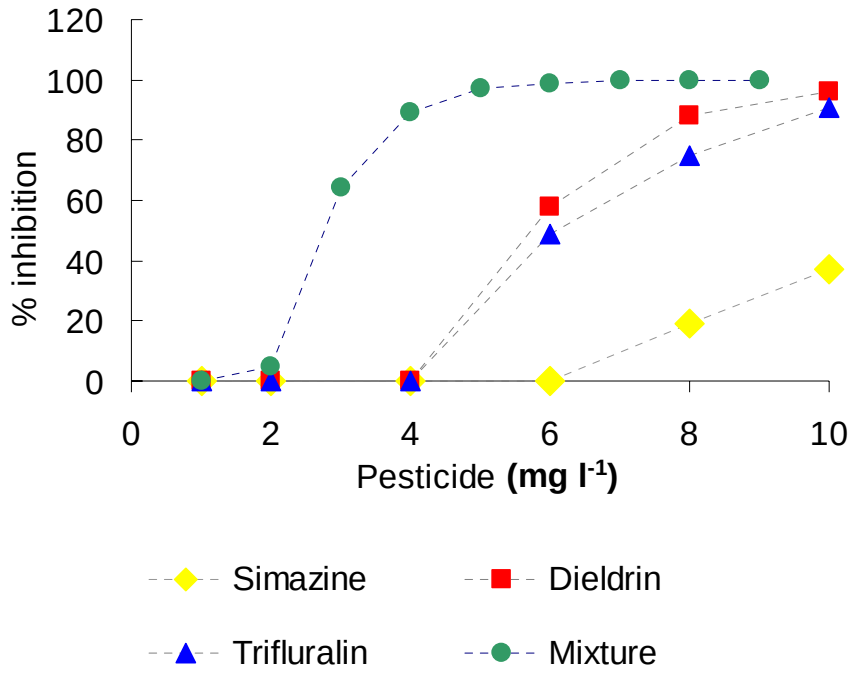


Figure 3.16 Inhibition curve of the luminescence for *V.fischeri* in soil extract broth spiked with simazine, trifluralin and dieldrin individually, and spiked with a mixture of the 3 pesticides, achieved with Toxalert®10.

Table 3.15 ToxAlert®10 toxicity test: Inhibition values for soil extract liquid broth (at -0.7 and -2.8 MPa) containing a mixture of pesticides inoculated with *P.chrysosporium*, *T.versicolor* (R26 and R101) and *P.ostreatus*, for 25 days.

Fungal inoculant	Ψ (MPa)	Mixture of pesticides (mg l^{-1})				
		0	5	10	15	30
T.versicolor (R26)	-0.7	0	0	0	7	0
	-2.8	0	0	0	0	19
T.versicolor (R101)	-0.7	0	0	0	0	0
	-2.8	0	0	0	0	0
P.ostreatus	-0.7	0	0	0	0	0
	-2.8	0	0	0	0	0
P.chrysosporium	-0.7	0	0	0	0	0
	-2.8	0	0	0	0	0

Part III Soil Microcosms Studies

3.9 Introduction

In this section, the ability of three white rot fungal isolates to grow in soil microcosms and degrade pesticides was evaluated in order to assess their potential use as bioremediation agents. The main objectives of this study were to examine the interactions and activity of these three white rot fungi in soil microcosms in relation to: (a) degradation of mixtures of simazine, dieldrin and trifluralin (b) soil respiration, (c) dehydrogenase activity, (d) total ligninolytic activity, (e) production of cellulase and laccase (f) microbial populations structure and interactions with water availability (-0.7 and -2.8 MPa).

Three test isolates were used: *T.versicolor* (R26), *P.ostreatus* and *P.chrysosporium*, because the fourth isolate, *T.versicolor* (R101), which successfully grew, produced extracellular enzymes and degraded the mixture of pesticides, in soil extract broth, did not grow on the carrier (wood chips). It was therefore not possible to use the isolate as a fungal inoculant. The other three isolates showed good growth in soil, under both water regimes examined, as shown in Plate 3.5 and Plate 3.6. The level of colonisation was affected by the pesticide treatment and water availability. *T.versicolo*r and *P.ostreatus* showed very extensive growth in soil, whereas *P.chrysosporium* showed relative less growth. Both *P.ostreatus* and *T.versicolor* R26 seem to grow less under lower water availability, and macroscopically appeared that in the pesticide treatments the colonisation was not as successful as in the control. For the microcosms inoculated with *P.chrysosporium,* such differences were not observed. Because it is very difficult to measure fungal growth in soil, and macroscopic observation is not a quantitative approach, indirect methods (respiration and enzyme activities) were used to study the success of fungal colonisation. The effect of pesticide concentration and water availability on these parameters was also assessed.

0 ppm at -2.8 MPa

0, 5

10 ppm

at -0.7 MPa

0, 5

10 ppm

at -2.8 MPa

Plate 3.5 *P.ostreatus*, growing in soil microcosm, under two different water regimes (at -0.7 and -2.8 MPa) after 12 weeks incubation.

0 ppm at -2.8 MPa

0, 5

10 ppm

at -0.7 MPa

0, 5

10 ppm

at -2.8 MPa

Plate 3.6 *P.chrysosporium*, growing in soil microcosm, under two different water regimes (at -0.7 and -2.8 MPa) after 12 weeks incubation.

3.10 Quantification of pesticide concentrations in soil

In order to assess the success of bioremediation it was important to evaluate to what extent the fungal inoculant was able to degrade the pesticide from the contaminated soil. For this purpose soil sub-samples were taken, from each soil microcosms, for subsequent pesticide extraction and HPLC analysis, to estimate the concentration of each pesticide remaining in the soil. Uninoculated control soil samples were also used to compare degradation rates by natural soil microflora.

3.10.1 Optimisation of the extraction of pesticides from soil

Figure 3.17 shows the percentage recovery of pesticides from soil spiked with a mixture of simazine, trifluralin and dieldrin, using two extraction methods: sonication and agitation as well as different solvent conditions. Best recovery rates were obtained with overnight agitation in methanol (100%). Anomalous recovery rates were observed for the samples that were sonicated, which can be explained with the temperature increase caused by sonication that may have led to evaporation of the extraction solvent, which cause an increase in pesticide concentration. In subsequent analysis pesticides were extracted from soil samples with overnight agitation, using 100% methanol.

3.10.2 Pesticide concentration remaining in soil after incubation

Pesticide analysis for simazine, trifluralin and dieldrin was carried out on all soil microcosm treatments up to 24 weeks incubation. Figure 3.18 shows as an example a comparisons of the effect of different treatments on the pesticide percentage remaining in soil in the different treatments under two different water regimes.

The results showed the treatments with fungal inoculants had higher pesticide degradation rates than the control, and the best inoculants were *P.chrysosporium* and *T.versicolor* R26.

In natural soil the percentage of pesticide degradation was low after 6-12 weeks, and after 24 weeks about 38% of simazine, 18% trifluralin and 37% dieldrin remained in

soil. In soil amended with wood chips (Table 3.16) improved degradation rates, comparing with the control were found for some treatments.

Soil inoculated with *T.versicolor* R26 (Table 3.17) showed good degradation rates for the three pesticides, at -2.8 MPa after 6 weeks (10 mg kg soil^{-1} initial mixture concentration). The increase in pesticide degradation was 46% for simazine, 57% for trifluralin and 51% for dieldrin compared to the control.

Soil inoculated with *P.ostreatus* (Table 3.18) also showed significant improvement in degradation of simazine and trifluralin. After 6 weeks incubation degradation of of these two were 100 and 60% higher, respectively, compared to the control soil, at -2.8 MPa (5 mg kg soil^{-1} treatment). In soil inoculated with this isolate the remaining concentrations of dieldrin were never significantly different of the control soil.

Soil inoculated with *P.chrysosporium* (Table 3.19) showed good degradation rates of the three pesticides at -2.8 MPa after 12 weeks incubation (5 mg kg soil^{-1} treatment). The simazine, trifluralin and dieldrin degradation rates were 58, 74, and 70% higher than in the control soil.

Soil amended with SMC (Table 3.20) also showed a significant increase in degradation at -2.8 MPa (10 mg kg soil^{-1} initial concentration). The degradation rates in soil with this amendment for simazine, trifluralin and dieldrin were 17, 49 and 76% higher than in the uninoculated control soil.

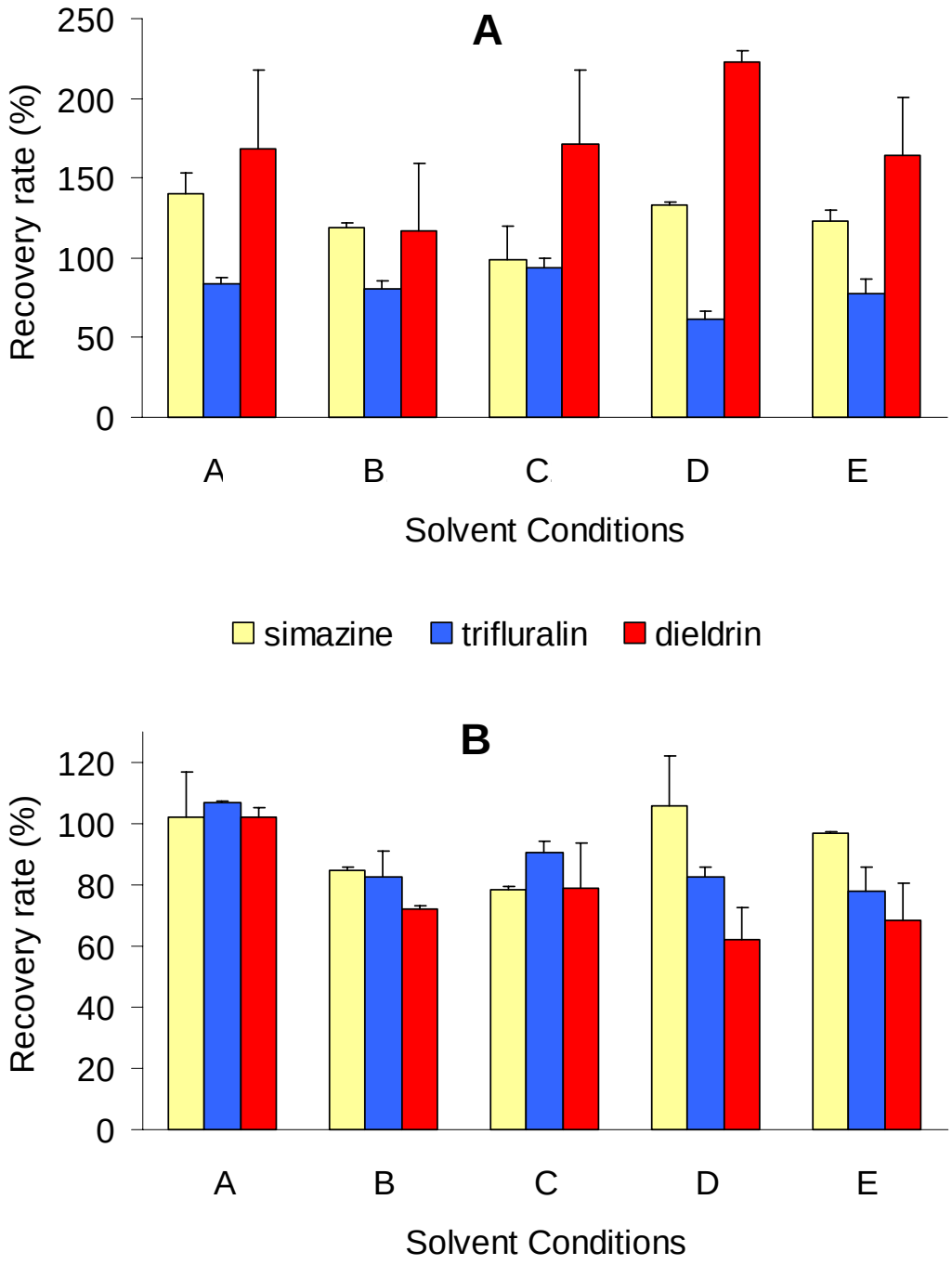
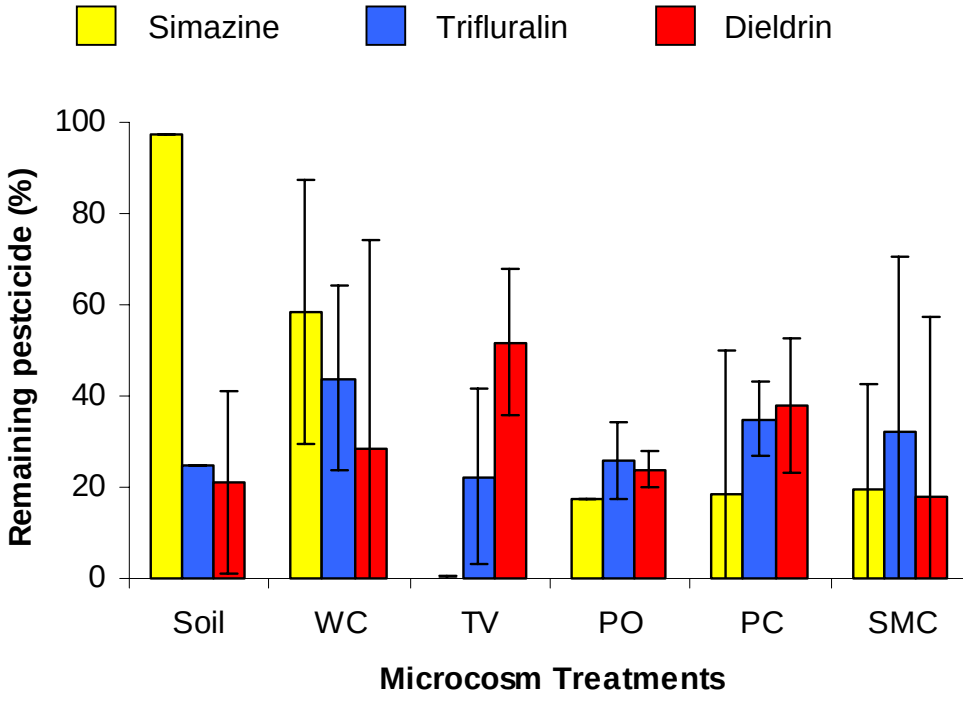


Figure 3.17 Percentage recovery of simazine, trifluralin and dieldrin from soil spiked with a mixture of the three pesticides. Extraction with sonication (A) overnight agitation (B) Different solvents: Methanol 100% (A), Acetonitrile 100% (B), Acetonitrile 75% water 25% (C), Methanol 50% Acetonitrile 50% (D), Acetonitrile 40% methanol 40% water 20% (E).

(a)



(b)

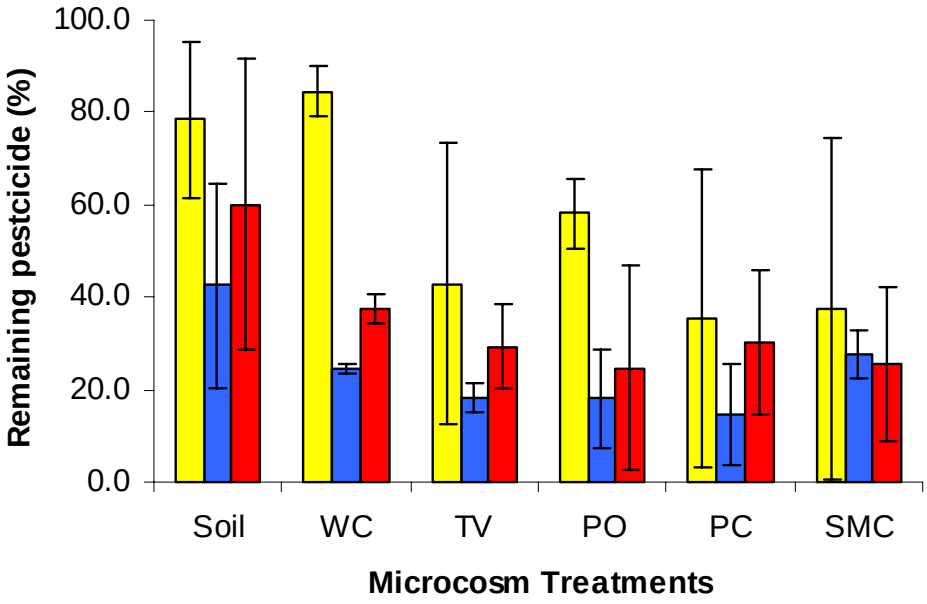


Figure 3.18 Pesticide (%) remaining in soil microcosms. (a) -0.7 MPa- and 6 weeks incubation at 15ºC and (b) at -2.8 MPa after 12 weeks incubation. The different treatments were: soil; WC, wood chips; P.c., *P.chrysosporium;* P.o., *P.ostreatus;* T.v., *T.versicolor* and SMC, spent mushroom compost.

Table 3.16 Comparison of percentage pesticide concentration remaining in soil supplemented with wood chips and a pesticide mixture 5 and 10 mg kg soil^{-1}, after 6, 12 and 24 weeks incubation at 15^{o}C, under two different water regimes. The values in red correspond to the concentrations remaining in natural soil (control). * means there was a significant difference between the control and the treatment.

Incub. (weeks)	Initial conc. (mg kg soil^{-1})	Ψ (MPa)	(% Remaining) Simazine		Trifluralin		Dieldrin	
6	5	-0.7	86.2	98.3	34.9	41.5	47.0*	76.3
	10		58.6*	97.5	44.0	24.6	28.8	20.9
	5	-2.8	99.3	97.8	48.9	51.8	41.7	40.8
	10		86.2	78.8	24.8	42.9	38.2	60.0
			L.S.D = 14.96		L.S.D = 25.63		L.S.D = 28.09	
12	5	-0.7	43.1*	95.6	16.4	29.2	34.4	33.5
	10		53.4*	72.5	32.5	37.6	21.6	46.5
	5	-2.8	92.3	99.4	28.9*	56.3	51.6	46.6
	10		24.3*	70.1	7.9*	35.8	38.4	59.8
			L.S.D.= 18.46		L.S.D.= 16.78		L.S.D.= 25.99	
24	5	-0.7	60.6	49.4	6.8	10.0	48.7	50.2
	10		26.8	29.5	4.7	7.2	13.8	24.6
	5	-2.8	76.5*	29.3	45.5*	30.1	48.9*	28.7
	10		60.1*	45.3	41.3*	22.1	57.7	44.3
			L.S.D.= 14.29		L.S.D.=8.67		L.S.D.=16.92	

Table 3.17 Comparison of percentage pesticide concentration remaining in soil inoculated with *T.versicolor* and supplemented with a pesticide mixture 5 and 10 mg kg soil^{-1}, after 6, 12 and 24 weeks incubation at 15^{o}C, under two different water regimes. The values in red correspond to the concentrations remaining in natural soil (control). * means there was a significant difference between the control and the treatment.

Incub. (weeks)	Initial conc. (mg kg soil^{-1})	Ψ (MPa)	(% Remaining) Simazine		Trifluralin		Dieldrin	
6	5	-0.7	21.9*	98.3	19.9*	41.5	26.5*	76.3
	10		0.1*	97.5	22.3	24.6	51.8*	20.9
	5	-2.8	78.1*	97.8	24.5*	51.8	20.0*	40.8
	10		42.9*	78.8	18.3*	42.9	29.3*	60.0
			L.S.D.= 16.68		L.S.D.= 17.64		L.S.D.= 26.58	
12	5	-0.7	53.7*	95.6	7.7*	29.2	0.0	33.5
	10		26.5*	72.5	23.2	37.6	47.3	46.5
	5	-2.8	59.9*	99.4	27.2*	56.3	43.8	46.6
	10		42.7	70.1	19.1*	35.8	49.0	59.8
			L.S.D.=28.45		L.S.D.= 16.20		L.S.D.=44.0	
24	5	-0.7	70.4*	49.4	27.1	10.0	20.8*	50.2
	10		40.3*	29.5	6.5	7.2	18.3	24.6
	5	-2.8	44.8*	29.3	20.0	30.1	25.1	28.7
	10		38.9	45.3	33.0	22.1	35.2	44.3
			L.S.D.= 12.48		L.S.D.= 17.36		L.S.D.= 26.9	

Table 3.18 Comparison of percentage pesticide concentration remaining in soil inoculated with *P.ostreatus* and supplemented with a pesticide mixture 5 and 10 mg kg soil^{-1}, after 6, and 12 weeks incubation at 15^{o}C, under two different water regimes. The values in red correspond to the concentrations remaining in natural soil (control). * means there was a significant difference between the control and the treatment.

Incub. (weeks)	Initial conc. (mg kg soil^{-1})	Ψ (MPa)	(% Remaining) Simazine		Trifluralin		Dieldrin	
6	5	-0.7	78.6*	98.3	26.6*	41.5	18.6	76.3
	10		0.0*	97.5	26.7	24.6	27.2	20.9
	5	-2.8	0.0*	97.8	20.7*	51.8	27.8	40.8
	10		58.2*	78.8	18.0*	42.9	24.7	60.0
			L.S.D.=18.12		L.S.D.=17.86		L.S.D.=70.91	
12	5	-0.7	28.6*	95.6	16.8	29.2	18.3	33.5
	10		29.9*	72.5	12.7*	37.6	35.4	46.5
	5	-2.8	61.6*	99.4	24.9*	56.3	34.0	46.6
	10		35.9*	70.1	9.0*	35.8	25.2	59.8
			L.S.D= 26.09		L.S.D= 16.27		L.S.D= 42.44	

Table 3.19 Comparison of percentage pesticide concentration remaining in soil inoculated with *P.chrysosporium* and supplemented with a pesticide mixture 5 and 10 mg kg soil^{-1}, after 6, 12 and 24 weeks incubation at 15^{o}C, under two different water regimes. The values in red correspond to the concentrations remaining in natural soil (control). * means there was a significant difference between the control and the treatment.

Incub. (weeks)	Initial conc. (mg kg soil^{-1})	Ψ (MPa)	(% Remaining)					
			Simazine		Trifluralin		Dieldrin	
6	5	-0.7	36.7*	98.3	22.1*	41.5	26.9*	76.3
	10		36.2*	97.5	25.3	24.6	12.7	20.9
	5	-2.8	77.2	97.8	33.8 *	51.8	32.5	40.8
	10		35.6*	78.8	14.5 *	42.9	30.1*	60.0
			L.S.D:= 20.83		L.S.D:= 18.90		L.S.D:= 17.96	
12	5	-0.7	74.1*	95.6	12.1*	29.2	16.5	33.5
	10		24.4*	72.5	42.7	37.6	0.0*	46.5
	5	-2.8	42.0*	99.4	14.7*	56.3	14.1*	46.6
	10		35.7*	70.1	6.3*	35.8	20.3*	59.8
			L.S.D.= 14.81		L.S.D.= 16.66		L.S.D.= 20.10	
24	5	-0.7	46.6	49.4	16.5*	10.0	39.2*	50.2
	10		34.4	29.5	11.1	7.2	19.2	24.6
	5	-2.8	61.6*	29.3	11.5*	30.1	25.8	28.7
	10		23.5*	45.3	13.9*	22.1	19.3*	44.3
			L.S.D= 15.73		L.S.D= 5.09		L.S.D= 13.25	

Table 3.20 Comparison of percentage pesticide concentration remaining in soil amended with SMC and supplemented with a pesticide mixture 5 and 10 mg kg soil^{-1}, after 6, 12 and 24 weeks incubation at 15^{o}C, under two different water regimes. The values in red correspond to the concentrations remaining in natural soil (control). * means there was a significant difference between the control and the treatment.

Incub. (weeks)	Initial conc. (mg kg soil^{-1})	Ψ (MPa)	(% Remaining) Simazine		Trifluralin		Dieldrin	
6	5	-0.7	100.0	98.3	49.5	41.5	80.6	76.3
	10		13.2*	97.5	40.0	24.6	39.5	20.9
	5	-2.8	89.9	97.8	53.1	51.8	46.8	40.8
	10		37.5*	78.8	27.4	42.9	25.4*	60.0
			L.S.D.=15.20		L.S.D.= 42.28		L.S.D.= 22.10	
12	5	-0.7	56.8*	95.6	14.5	29.2	3.9*	33.5
	10		18.2*	72.5	21.8	37.6	19.9*	46.5
	5	-2.8	83.6*	99.4	23.4*	56.3	25.2	46.6
	10		58.1*	70.1	18.4*	35.8	14.5*	59.8
			L.S.D.=14.17		L.S.D.=16.28		L.S.D.= 21.76	
24	5	-0.7	71.4*	49.4	14.1	10.0	7.3*	50.2
	10		16.5*	29.5	22.8*	7.2	45.3*	24.6
	5	-2.8	59.8*	29.3	14.5*	30.1	17.3	28.7
	10		22.6*	45.3	48.2*	22.1	40.0	44.3
			L.S.D.= 7.53		L.S.D.= 8.90		L.S.D.= 17.46	

3.11 Soil respiration

In this experiment, the evolution in CO_2 concentrations in soil was used as an index of microbial activity. Soil respiration is a very relevant parameter in the assessment of the success of bioremediation processes, as it gives information on microbial activity as well as mineralization intensity. An increase in carbon dioxide concentrations could indicate an increase in microbial numbers and/or a boost in microbial activity. The purpose of this study was to compare the respiratory activity in the different treatments, to assess the differences between fungal inoculant, and its interaction with water availability and pesticide concentration. Prior to the analysis of the microcosms a preliminary study was conducted to optimise the measurement of carbon dioxide concentrations, and is described below.

3.11.1 Optimisation of carbon dioxide measurements in soil microcosms

Since the microcosm jars had vented caps, to maintain an aerobic system, carbon dioxide concentrations were very low in the headspace, because there was gas exchange with the surrounding atmosphere. In order to obtain a detectable concentration of CO_2 the jars were sealed and left at room temperature, prior to the analysis for 0, 1 and 3 hrs. Detectable carbon dioxide levels were achieved by sealing the jars for 3 hours. Figure 3.19 shows an example of carbon dioxide levels in 12 different jars immediately after sealing the jar, 1 and 3 hrs later.

From each jar a single measurement was taken because it was found that there was a decrease in CO_2 concentration when multiple measurements were made. In subsequent analysis the microcosm jars were sealed for 3 hrs and left at room temperature, prior to CO_2 analysis.

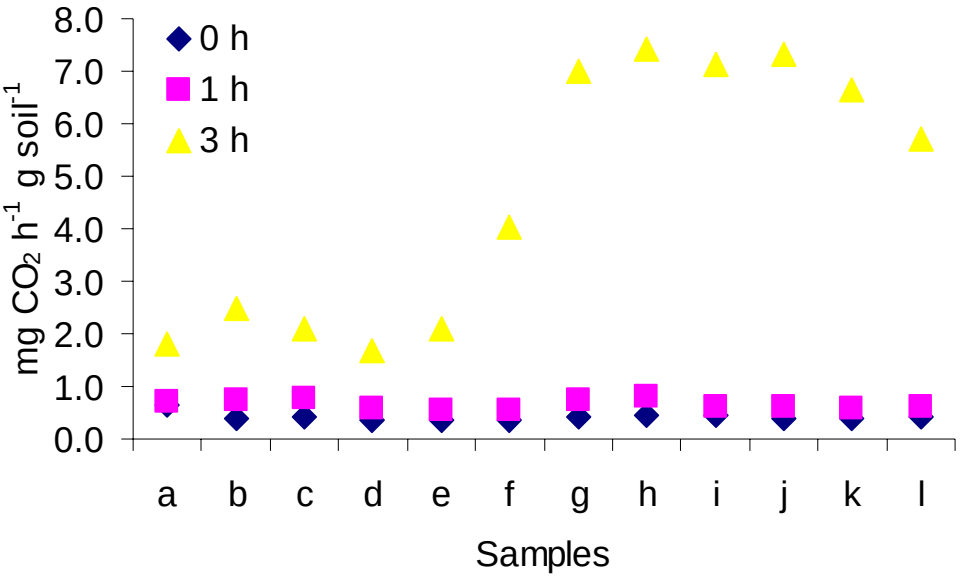


Figure 3.19 Different carbon dioxide measured in twelve different jars (a- l), at different incubation times: 0 h (CO_2 measured without sealing the jar); 1 h (CO_2 measured after jar had been sealed for one hour); 3 h (CO_2 measured after jar had been sealed for three hours).

3.11.2 Soil respiration in different treatments

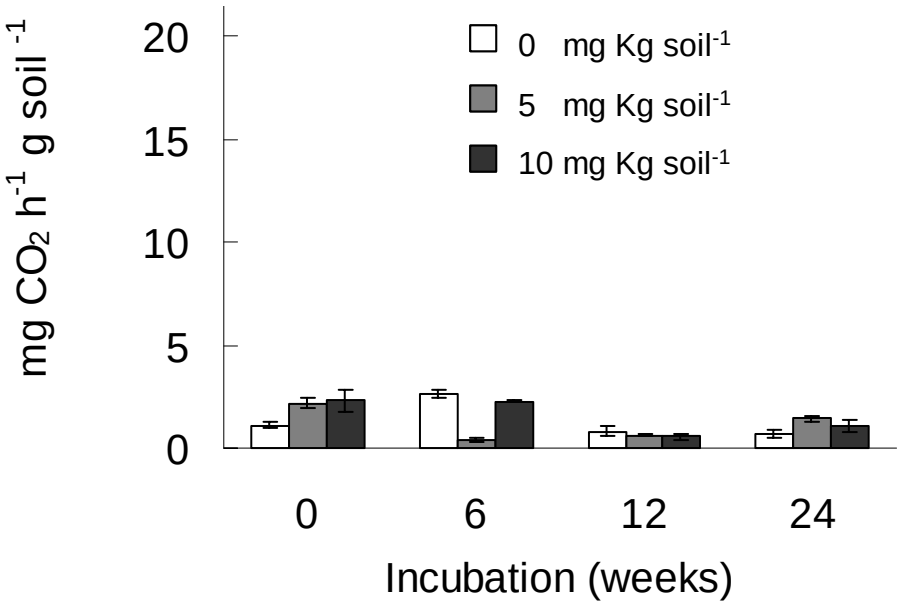
Higher carbon dioxide levels indicate higher respiration rates. In this study maximum respiration occurred after 6 weeks in the treatments inoculated with the test isolates, and decreased by the end of the experiment. Interestingly in soil inoculated with the test isolates, higher increases in respiration rates (compared to natural soil) were found in the presence of the mixture of pesticides.

In natural soil the respiratory activity was low, throughout the experiment (Figure 3.20). In soil amended with wood chips improved respiratory activity was found for all the treatments, but not as marked as in soil inoculated with the fungal isolates (Figure 3.21).

Soil inoculated with *T.versicolor* R26 (Figure 3.22) showed improved respiration rates in all the treatments. The highest increase was observed after 6 weeks, at -0.7 MPa with CO_2 concentrations 1.2, 54 and 11 times higher than in natural soil, for the treatments 0, 5 and 10 mg kg $soil^{-1}$, respectively. Soil inoculated with *P.ostreatus* (Figure 3.23) also showed an increase in respiration rates in all the treatments. After 6 weeks incubation, at -2.8 MPa, the concentration of CO_2 was 30, 6 and 5 times higher than in natural soil (for the treatment 0, 5 and 10 mg kg $soil^{-1}$ respectively). Soil inoculated with *P.chrysosporium* (Figure 3.24) showed the highest increase in respiration rates (compare to the other isolates). After 6 weeks incubation CO_2 production in the treatments 0, 5 and 10 mg kg $soil^{-1}$ was 29, 19 and 14 times higher than in natural soil (at -2.8 MPa).

Soil amended with SMC also showed a significant increase in respiratory rates (Figure 3.25), but not as intense as in the treatments with the fungal inoculants. At -2.8 MPa after 6 weeks incubation the respiratory activity in soil with this amendment was 4, 3 and 1.2 times higher than in natural soil (for 0, 5 and 10 mg kg $soil^{-1}$ treatments). However in the10 mg kg $soil^{-1}$, -0.7MPa after 0, 6 and 12 weeks, the production of CO_2 was lower 50, 50 and 10% lower than in natural soil.

(a)



(b)

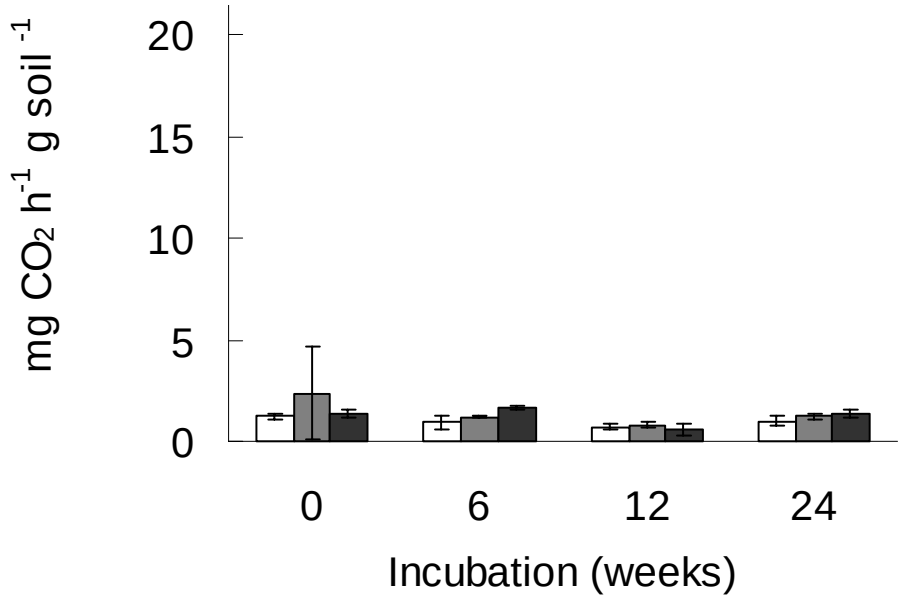
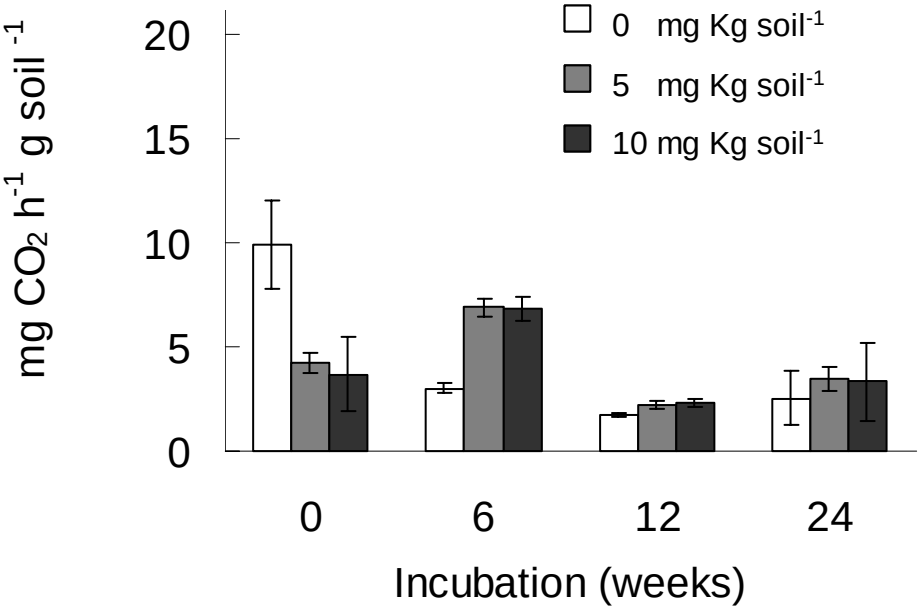


Figure 3.20 Changes in carbon dioxide concentrations in soil treated with a mixture of pesticides (0, 5 and 10 mg kg $soil^{-1}$), incubated at 15ºC for 0-12 weeks, under two different water regimes (a) -0.7 and (b) -2.8 MPa. Results are presented as means (±standard deviations) of three replicates, per treatment.

(a)



(b)

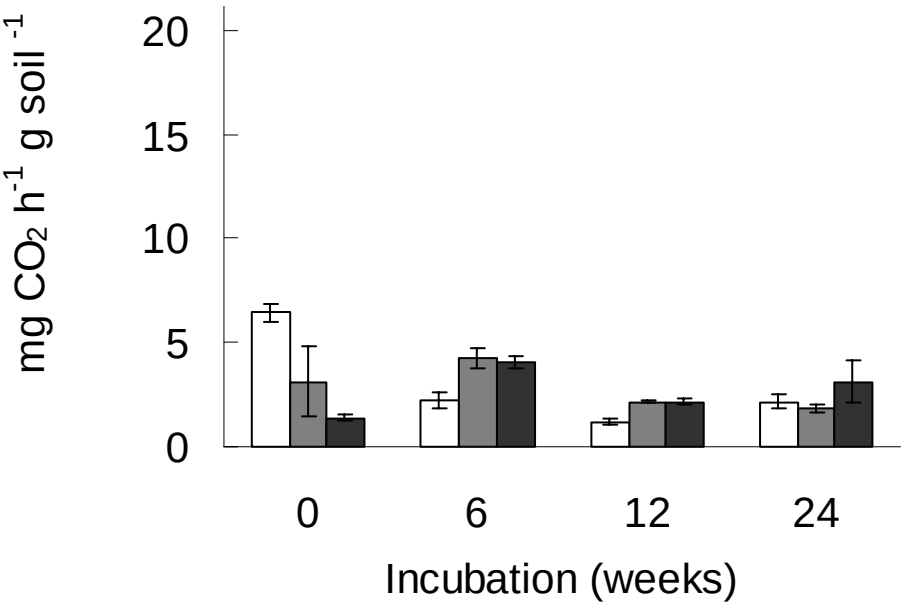
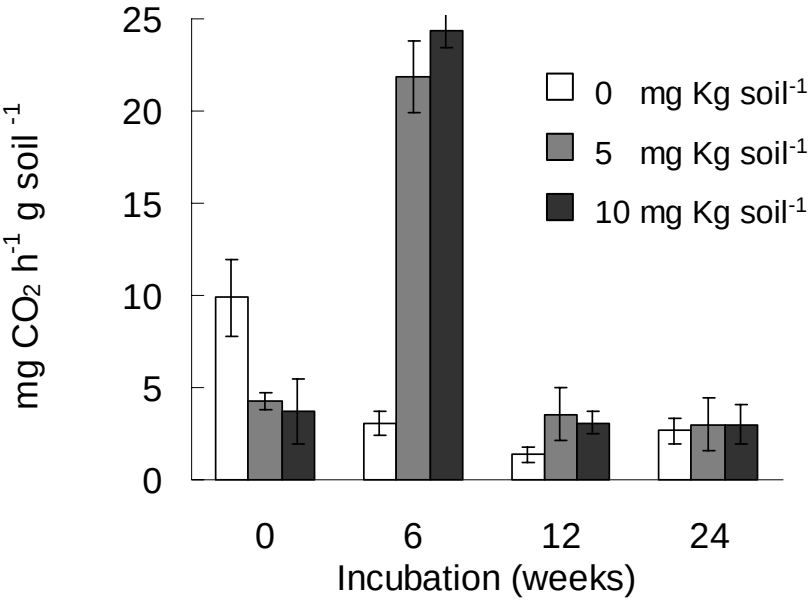


Figure 3.21 Changes in carbon dioxide concentrations in soil amended with wood chips and containing a mixture of pesticides (0, 5 and 10 mg kg soil^{-1}), incubated at 15ºC for 0-12 weeks, under two different water regimes: (a) -0.7 and (b) -2.8 MPa. Results are presented as means (±standard deviations) of three replicates, per treatment.

(a)



(b)

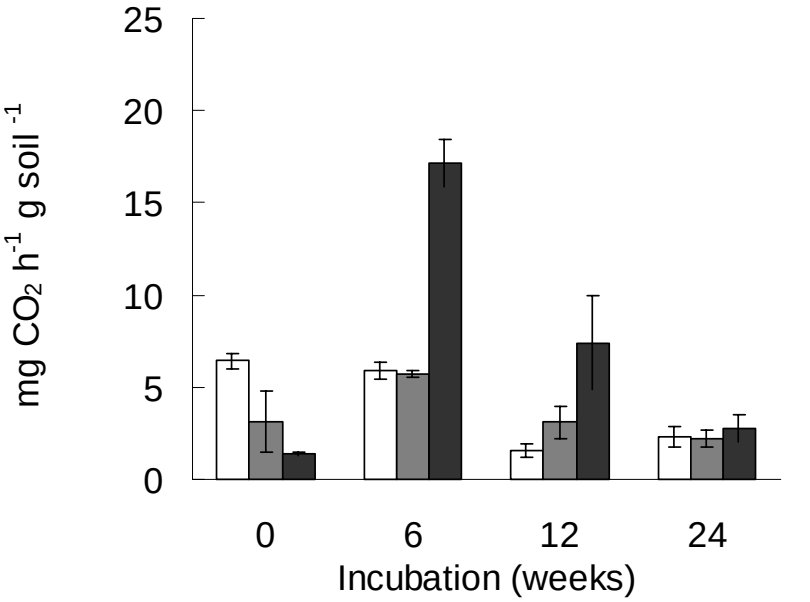
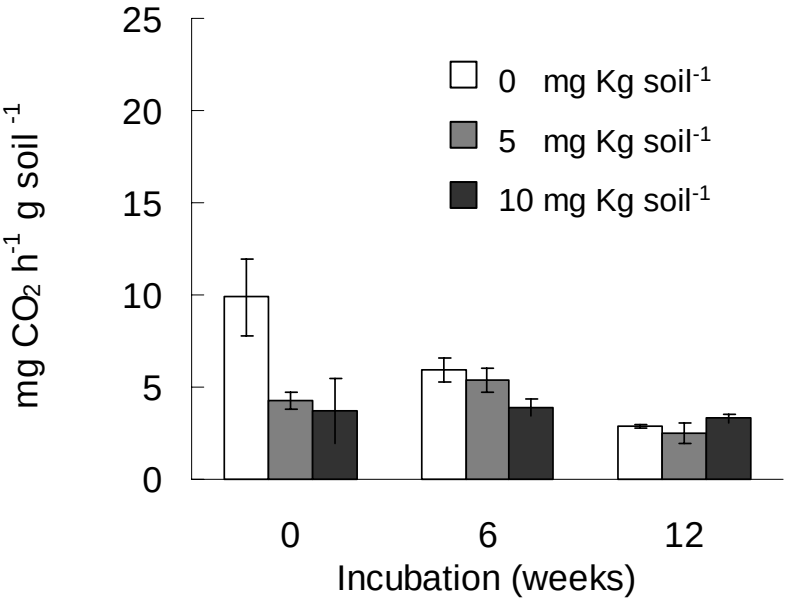


Figure 3.22 Changes in carbon dioxide concentrations in soil amended with wood chips inoculated with *T.versicolor* and containing a mixture of pesticides (0, 5 and 10 mg kg soil^{-1}), incubated at 15ºC for 0-12 weeks, under two different water regimes (a) -0.7 and (b) -2.8 MPa. Results are presented as means (±standard deviations) of three replicates, per treatment.

(a)



(b)

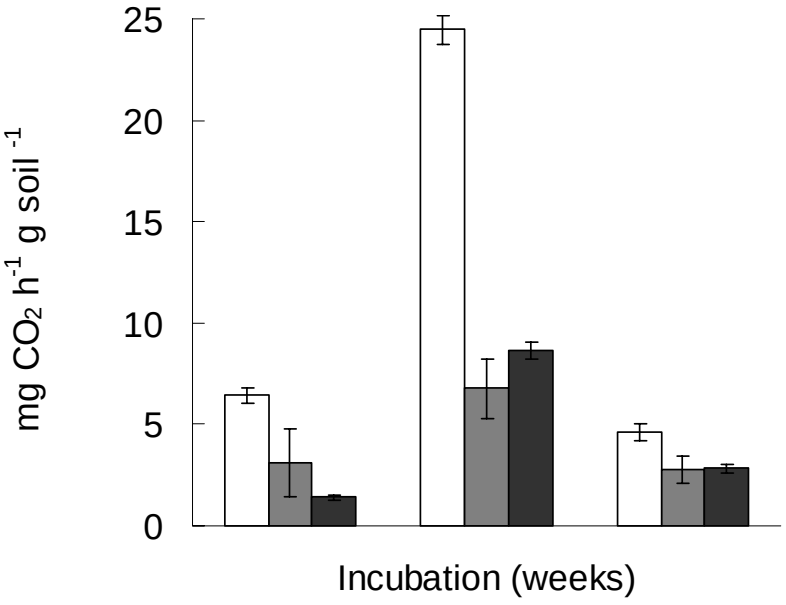
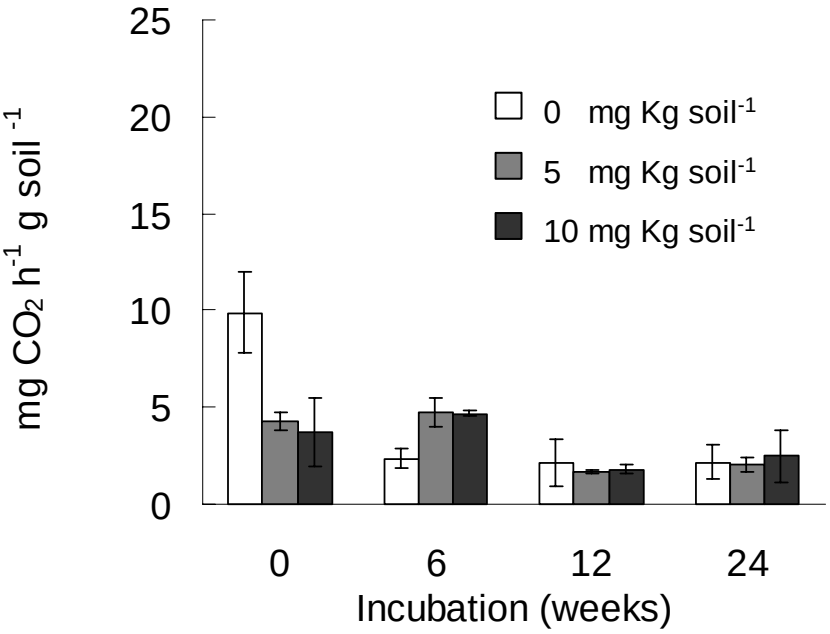


Figure 3.23 Changes in carbon dioxide concentrations in soil amended with wood chips inoculated with *P.ostreatus* and containing a mixture of pesticides (0, 5 and 10 mg kg soil^{-1}), incubated at 15ºC for 0-12 weeks, under two different water regimes (a) -0.7 and (b) -2.8 MPa. Results are presented as means (±standard deviations) of three replicates, per treatment.

(a)



(b)

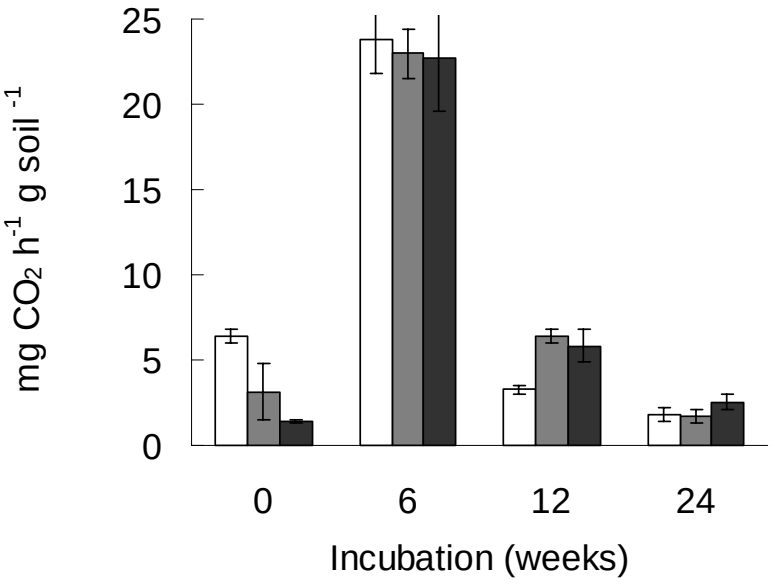
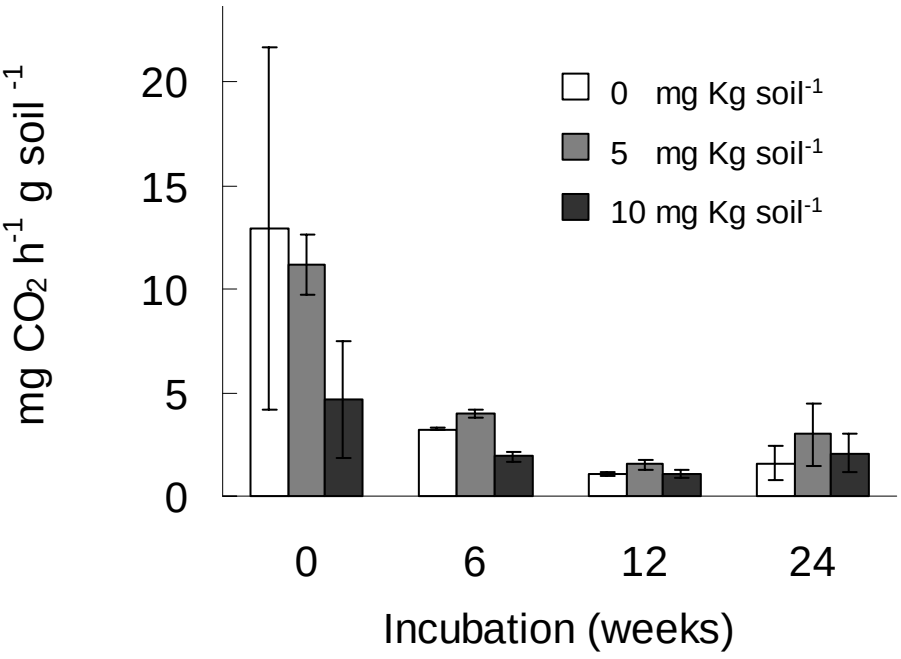


Figure 3.24 Changes in carbon dioxide concentrations in soil amended with wood chips inoculated with *P.chrysosporium* containing a mixture of pesticides (0, 5 and 10 mg kg $soil^{-1}$), incubated at 15ºC for 0-12 weeks, under two different water regimes (a) -0.7 and (b) -2.8 MPa. Results are presented as means (±standard deviations) of three replicates, per treatment.

(a)



(b)

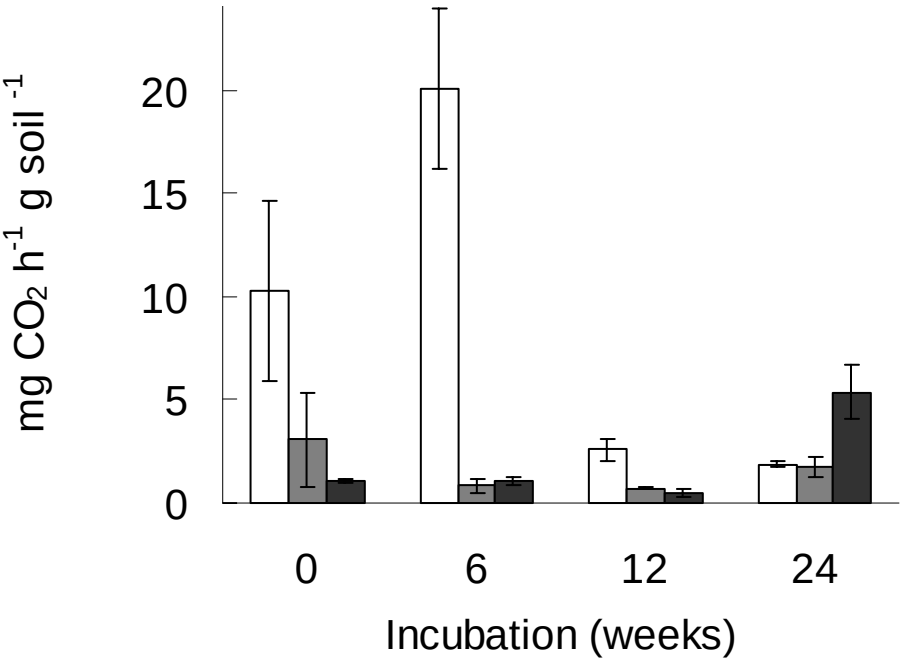


Figure 3.25 Changes in carbon dioxide concentrations in soil amended with SMC and amended with a mixture of pesticides (0, 5 and 10 mg kg soil^{-1}), incubated at 15ºC for 0-12 weeks, under two different water regimes (a) -0.7 and (b) -2.8 MPa. Results are presented as means (±standard deviations) of three replicates.

3.12 Dehydrogenase activity

For this parameter the differences between the treatments with the fungal inoculant were not as marked as for the respiratory activity. Overall the activity of this enzyme was higher in soil inoculated with the test isolates. For example *T.versicolor* dehydrogenase activity in the treatments 5 and 10 mg kg soil^{-1} mixture, was 80 and 100 % higher than in natural soil, after 6 weeks, at -2.8 MPa.

In natural soil, without inoculant or amendment (Figure 3.26) the addition of pesticides enhanced dehydrogenase activity ($P<0.001$) with higher levels being produced at -0.7 MPa ($P<0.001$). Soil amended with wood chips (Figure 3.27) produced the highest dehydrogenase levels at the beginning and after 6 weeks incubation, with increased enzyme production in the presence of the pesticide mixture.

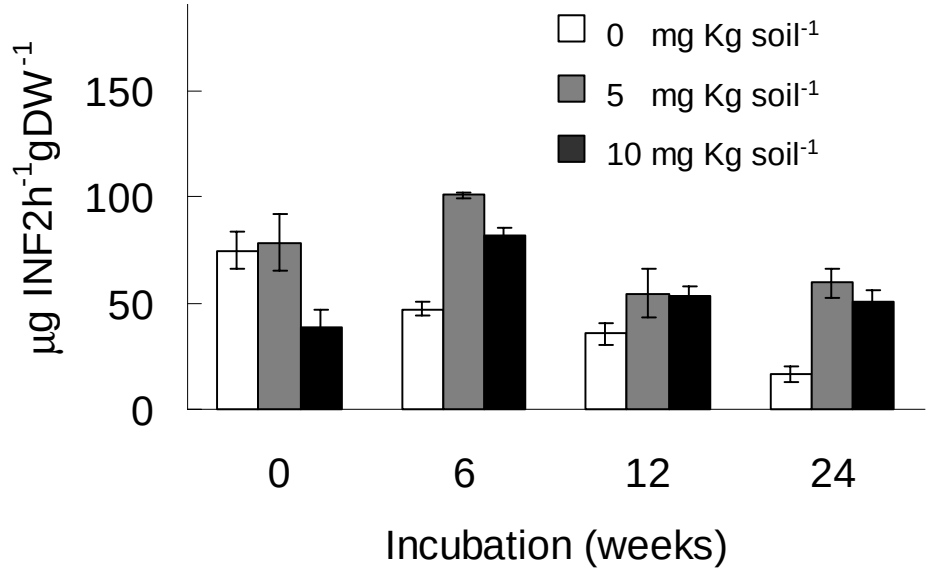
In soil inoculated with *T.versicolor* (Figure 3.28) the presence of pesticides enhanced dehydrogenase activity, after 6 and 12 weeks incubation under both water regimes ($P<0.05$). Water availability influenced on dehydrogenase activity, with higher production of this enzyme in the -0.7 MPa treatment ($P<0.001$).

In soil inoculated with *P.ostreatus* (Figure 3.29) significantly higher production of dehydrogenase at -0.7 MPa was observed at the beginning of the experiment ($P<0.001$). However, after 6 weeks incubation the highest activities were detected under water stress, in the presence of the pesticide mixture.

When *P.chrysosporium* (Figure 3.30) was used as a fungal inoculant the levels of dehydrogenase decreased with the incubation period, as the treatments showed higher activities at the beginning of the incubation ($P<0.001$). Similarly to *T.versicolor*, in this treatment dehydrogenase activity was increased in the presence of the pesticide mixture, which suggests higher microbial activity in the presence of the pesticides.

Interestingly soil amended with SMC (Figure 3.31) also showed a marked increase in dehydrogenase, regardless of water regime ($P=0.068$). Dehydrogenase levels were particularly high after 6 weeks incubation in the presence of the pesticide mixture (at -0.7 and -2.8 MPa) and after 12 weeks incubation but only at -2.8 MPa.

(a)



(b)

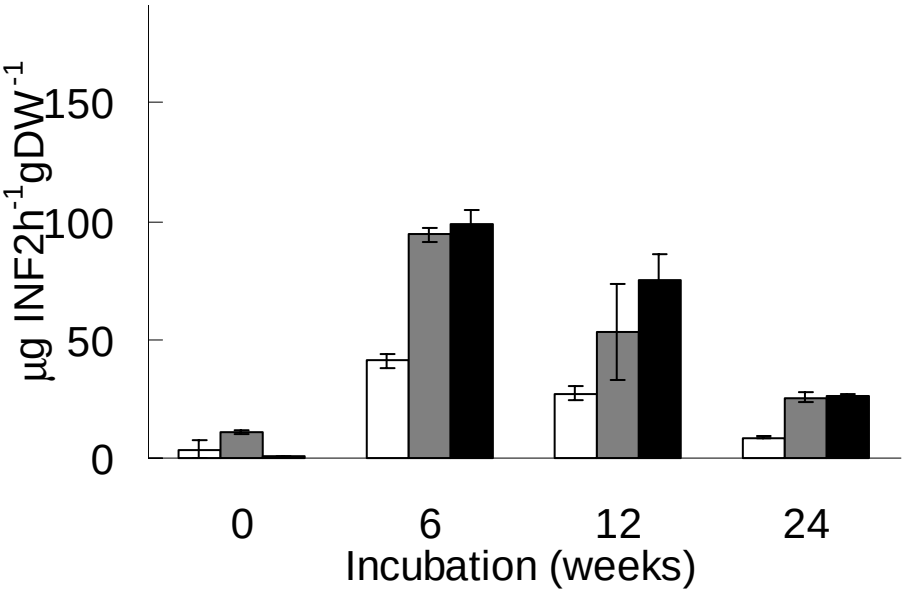
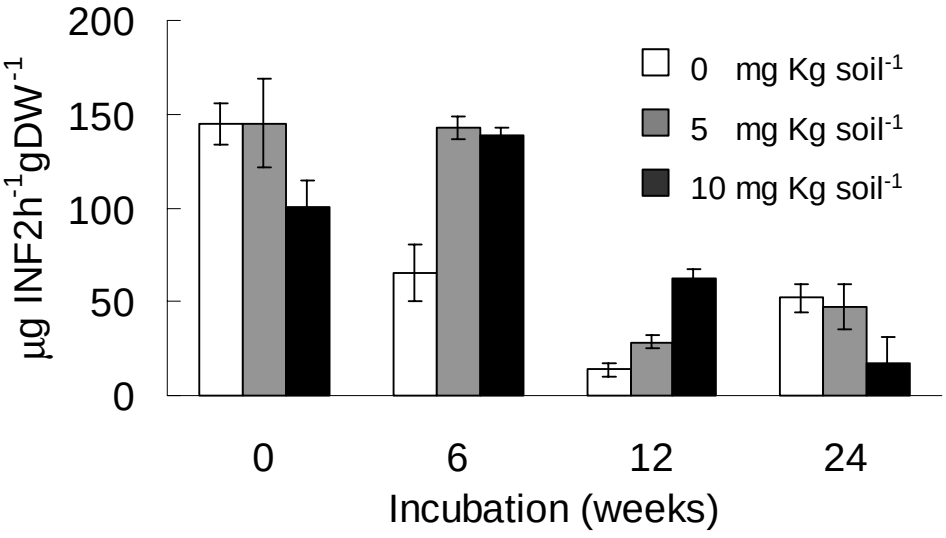


Figure 3.26 Dehydrogenase activity (expressed as µg INF produced in 2h g^{-1} dw) in natural soil supplemented with a mixture of pesticides at 5 and 10 mg kg $soil^{-1}$ incubated for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 MPa and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

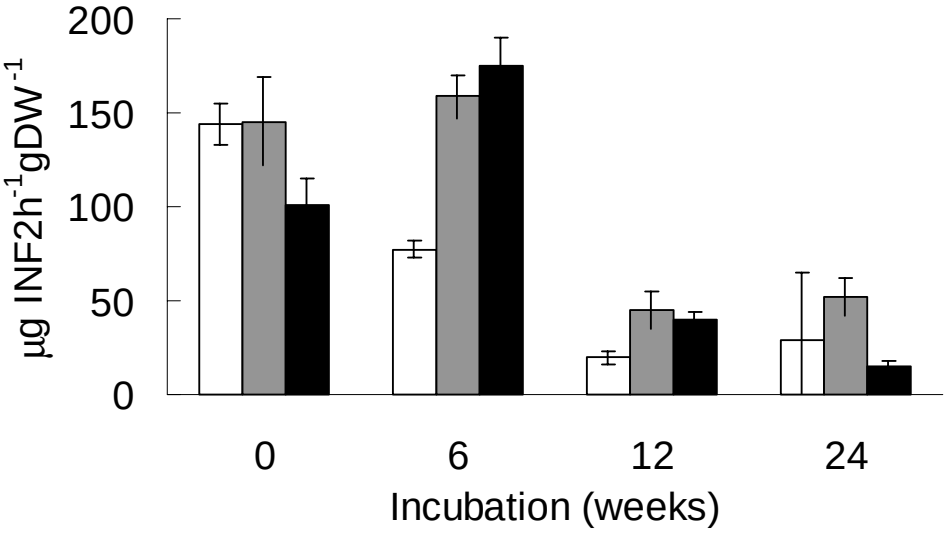
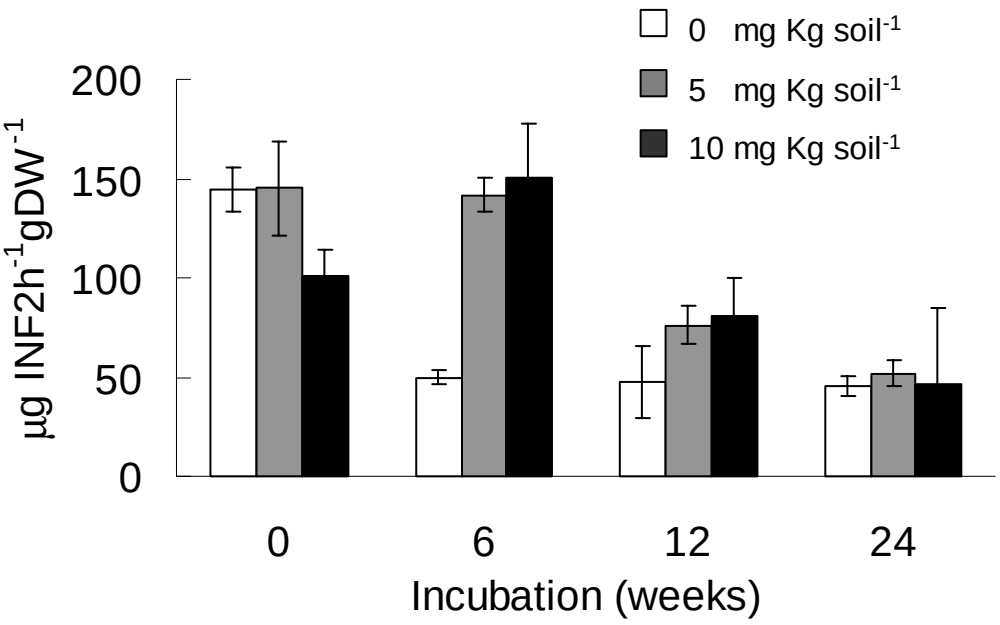


Figure 3.27 Dehydrogenase activity (expressed as µg INF produced in 2h g^{-1} dw) in non-inoculated soil microcosms supplemented with wood chips for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

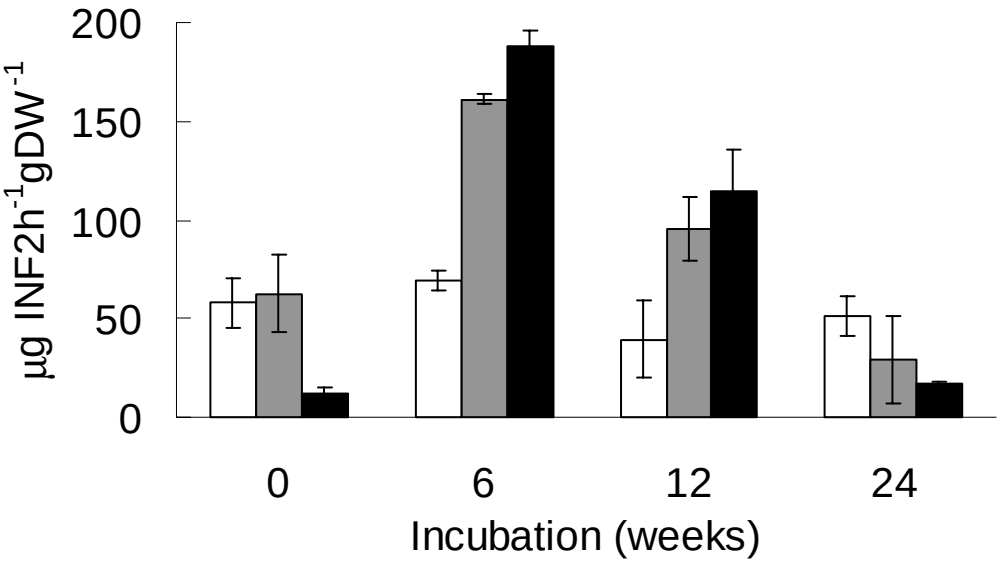
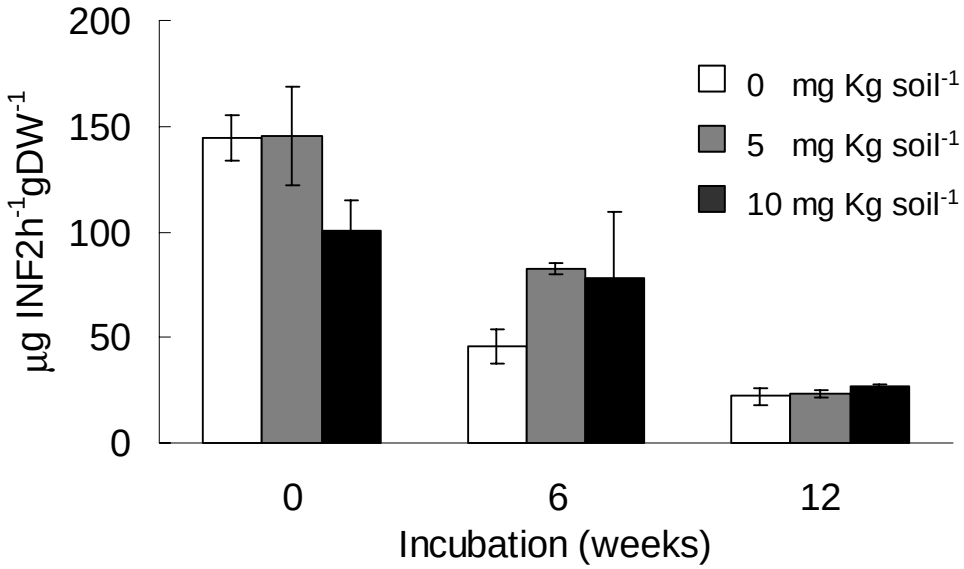


Figure 3.28 Dehydrogenase activity (expressed as µg INF produced in in 2h g^{-1} dw) in soil microcosms, inoculated with *T.versicolor* (R26) for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

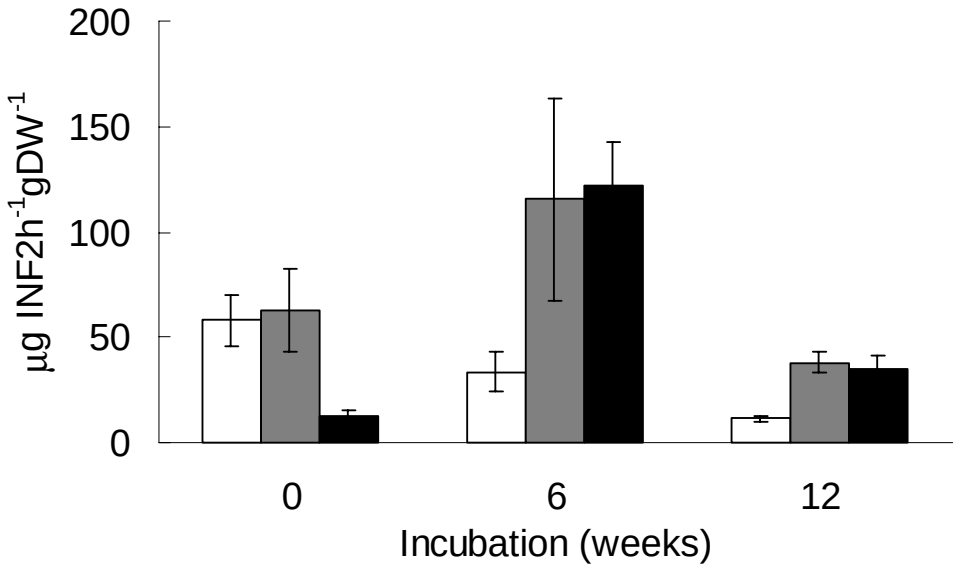
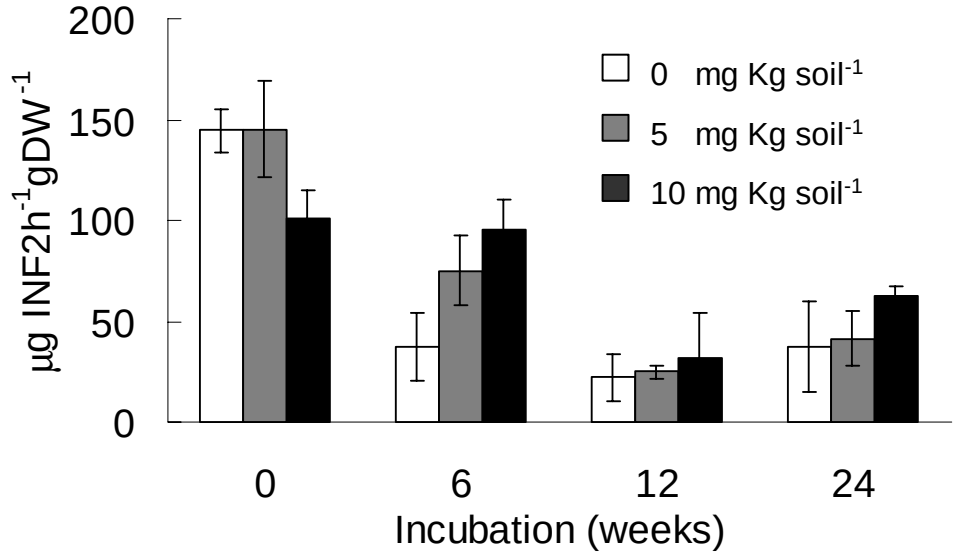


Figure 3.29 Dehydrogenase activity (expressed as µg INF produced in 2h g^{-1} dw) in soil microcosms, inoculated with, *P.ostreatus* for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

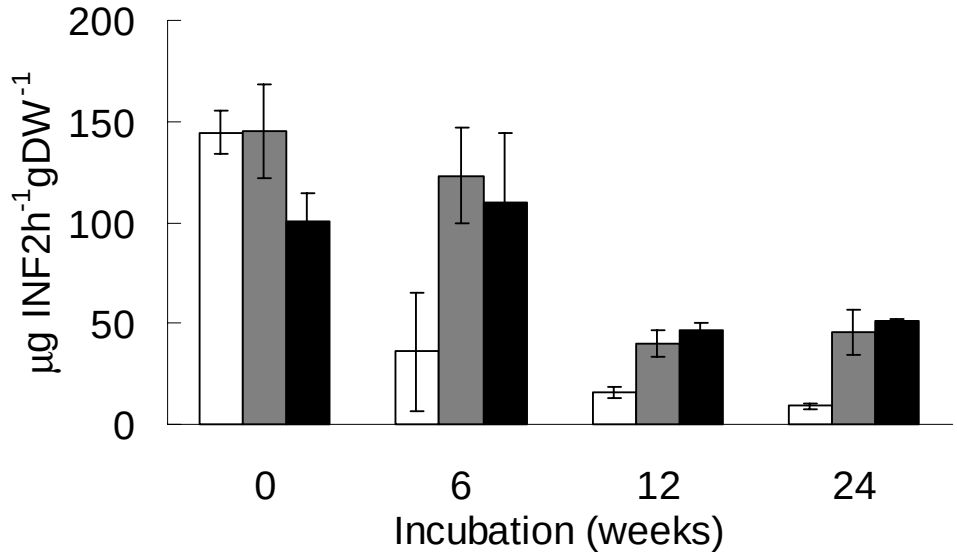
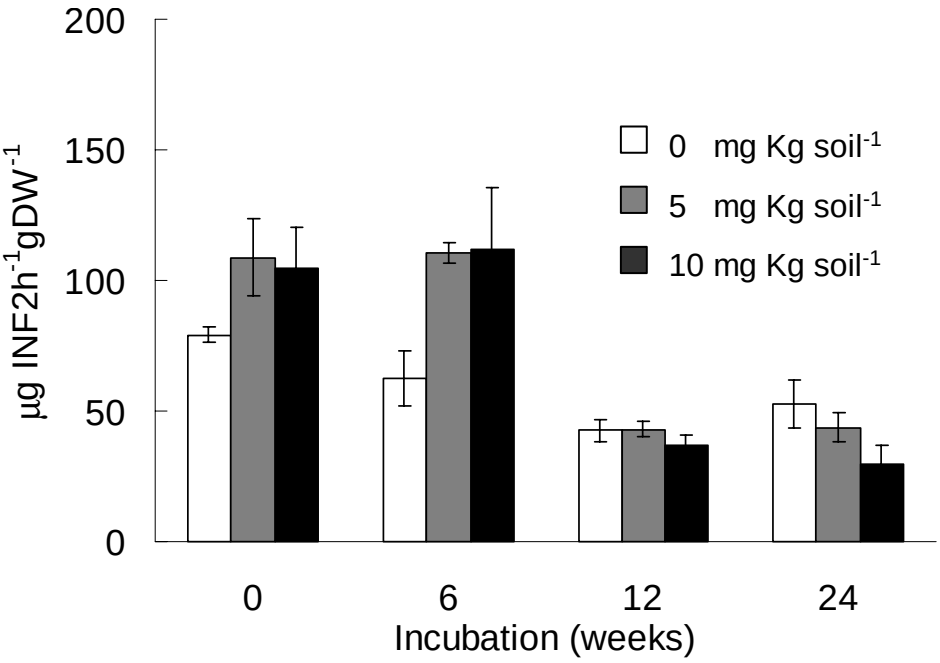


Figure 3.30 Dehydrogenase activity (expressed as µg INF produced in 2h g^{-1} dw) in soil microcosms, inoculated with *P.chrysosporium* for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

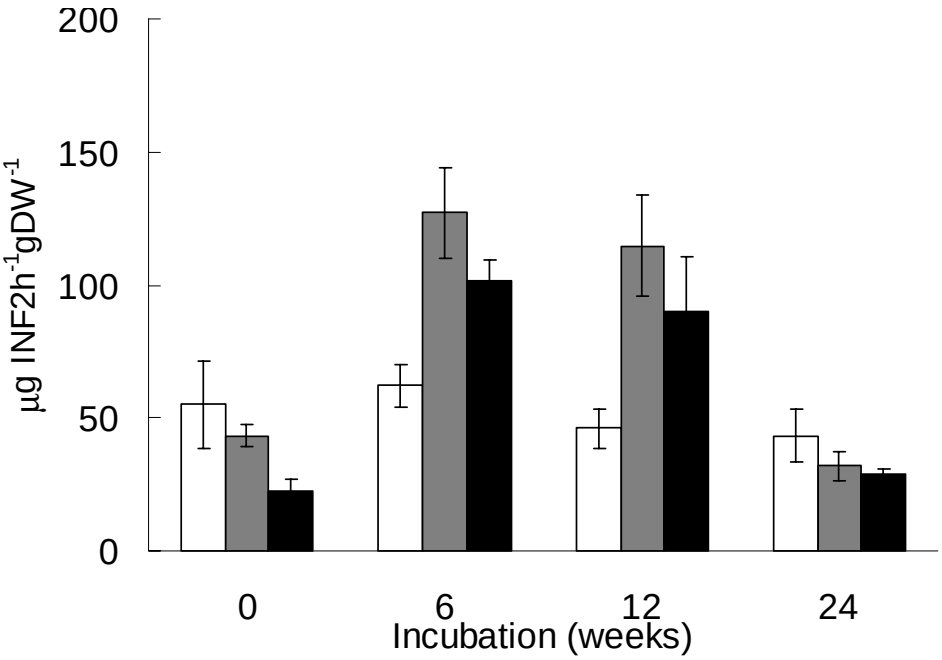


Figure 3.31 Dehydrogenase activity (expressed as µg INF produced in 2h g^{-1} dw) in soil microcosms, inoculated with SMC for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

3.13 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 three test isolates used in this study are known to be good lignin degraders. However, there is little information on their ligninolytic activity in soil. In this study, the three test isolates were tested for their ability to decolorize Poly-R478 in soil under different pesticide and water treatments, as a measure of total ligninolytic activity. Prior to total ligninolytic activity assessment in the soil microcosms a preliminary study was conducted and it is described below.

The total ligninolytic activity in different soil microcosms, under two water regimes was examined. The assay used in this study assay 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*1000 the more intense is the decolouration, i.e. the higher was the total ligninolytic activity.

The decolouration obtained in the soil treatments was compared with the natural decolouration of the dye. Figure 3.32 shows the results of a preliminary assay, where the natural decolouration of Poly-R478, and the extent of decolouration in the presence of commercial horseradish peroxidase (HRP) alone and with hydrogen peroxide (H_2O_2) were assessed. HRP is known to decolorize Poly-R478, and its activity is enhanced by hydrogen peroxide. Figure 3.33 shows the results of this test expressed as colour intensity (%), and Figure 3.34 in % decolouration.

Overall, all soil treatments caused decolouration of the polymeric dye. In natural soil (Figure 3.35) the total ligninolytic activity was not affected by water availability or pesticide treatment ($P= 0.681$ and $P= 0.454$, respectively). Interestingly water potential had an impact on decolouration % in soil amended with wood chips (Figure 3.36), with higher decolouration at -2.8 MPa ($P<0.001$), and under this water regime the decolorization rates were significantly higher in the pesticide treatments compared with the control (0 mg kg soil^{-1}) ($P<0.05$).

In soil inoculated with *T.versicolor* (Figure 3.37) the total ligninolytic activity was significantly higher at -0.7MPa ($P=0.013$). There were no significant differences

between pesticide treatment (P= 0.226), which suggest the fungal inoculant is tolerant to this mixture of pesticides, producing equivalents level of decolouration in the presence and absence of the xenobiotics.

When the inoculant used in the microcosms was *P.ostreatus* (Figure 3.38) significantly higher decolouration was observed at the highest water availability (P=0.002). As it was described for soil inoculated with *T.versicolor*, the total ligninolytic activity was unaffected by pesticide treatment (P=0.373). The same trend was found in soil inoculated with *P.chrysosporium* (P> 0.001, for water availability and P=0.801 for pesticide mixture) (Figure 3.39).

In soil microcosm amended with SMC (Figure 3.40) the treatment that showed the highest decolouration rates, and this was not affected by water availability (P= 0.532) or pesticide concentration (P=0.093).

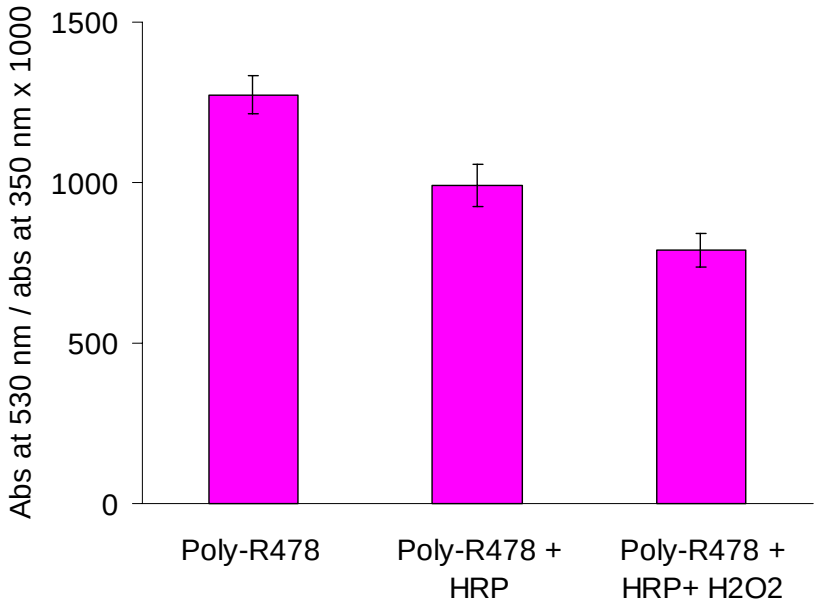


Figure 3.32 Ratio between absorbance at 530 nm and absorbance at 350 nm x 1000, for: Poly-R178=polymeric dye, HRP=commercial horseradish peroxidase and H_2O_2= Hydrogen Peroxide.

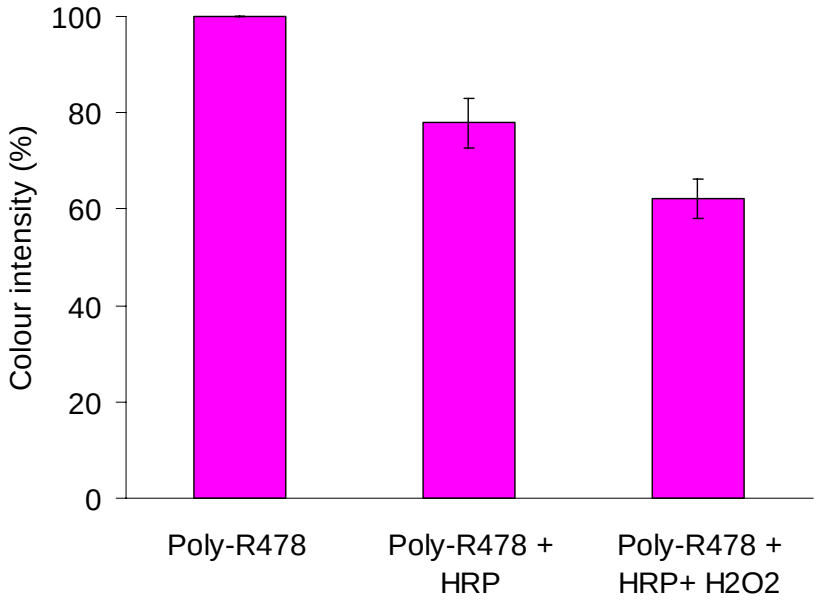


Figure 3.33 Colour intensity (%)= (absorbance at 530 nm/ absorbance at 350 nm) $_{sample}$ x 100 / (absorbance at 530 nm/ absorbance at 350 nm) $_{poly\ R478}$ for: Poly-R178=polymeric dye, HRP=commercial horseradish peroxidase and H_2O_2= Hydrogen Peroxide.

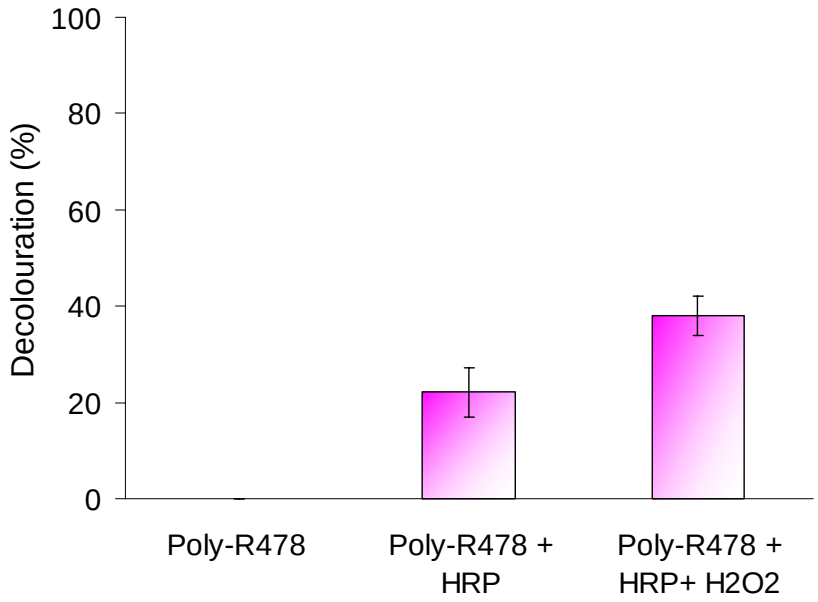
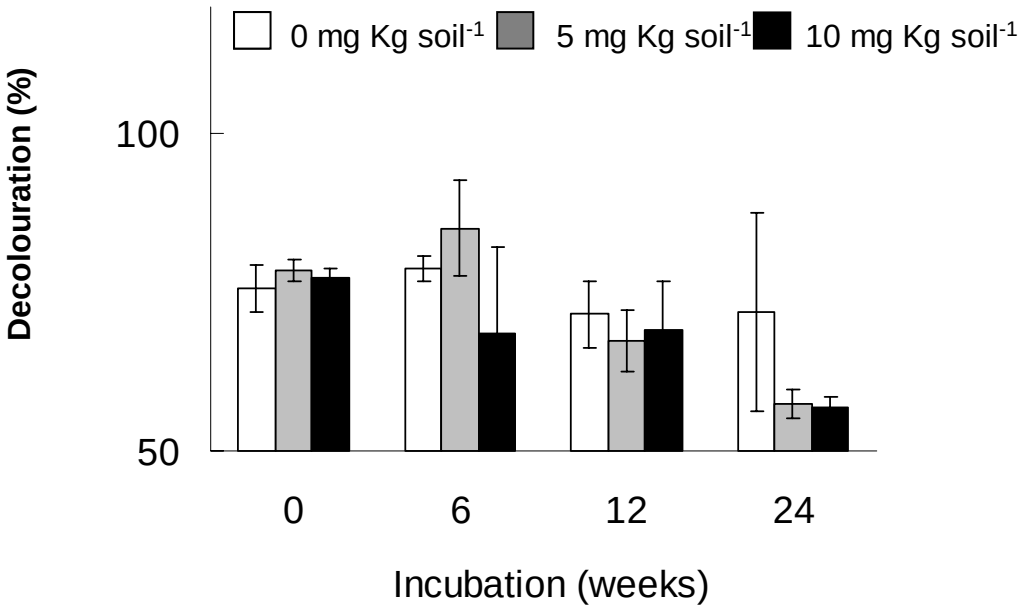


Figure 3.34 Percentage decolouration = 100 - [(absorbance at 530 nm/ absorbance at 350 nm) $_{sample}$ / (absorbance at 530 nm/ absorbance at 350 nm) $_{poly\ R478}$] for: Poly-R178=polymeric dye, HRP=commercial horseradish peroxidase and H2O2= Hydrogen Peroxide.

(a)



(b)

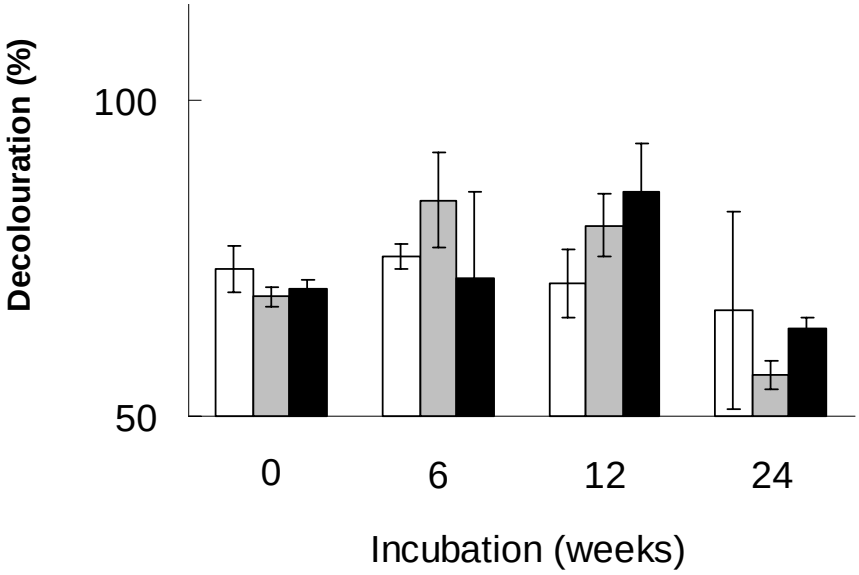
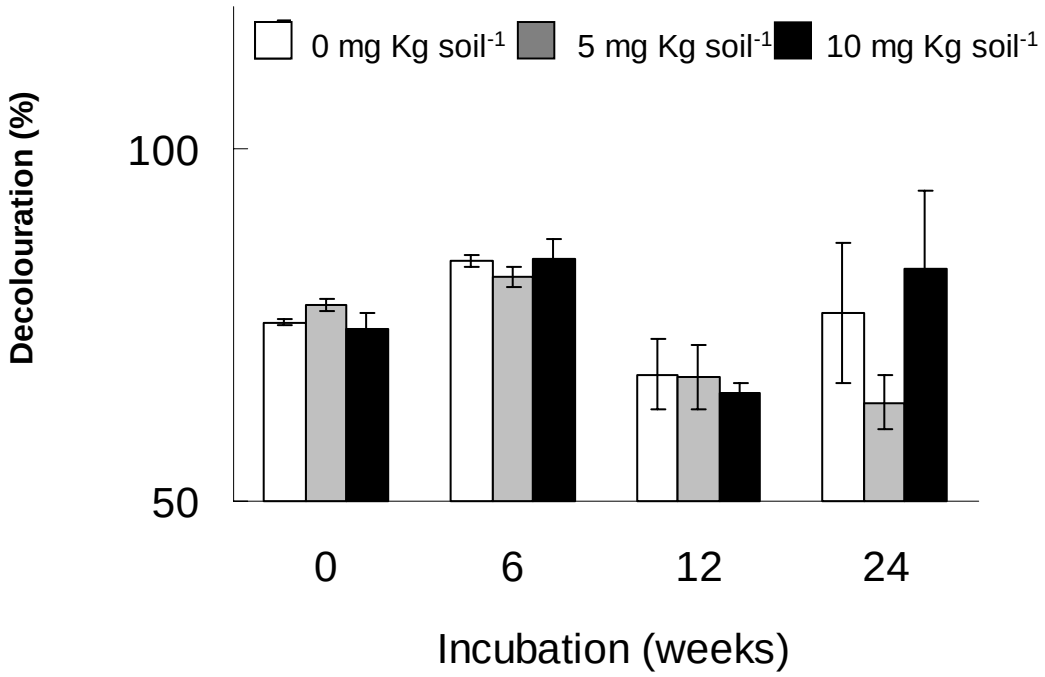


Figure 3.35 Total ligninolytic activity (expressed as % decolouration of Poly R478) in non-inoculated soil microcosms, supplemented with a mixture of pesticides at 5 and 10 mg kg soil^{-1}, incubated for 0-24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

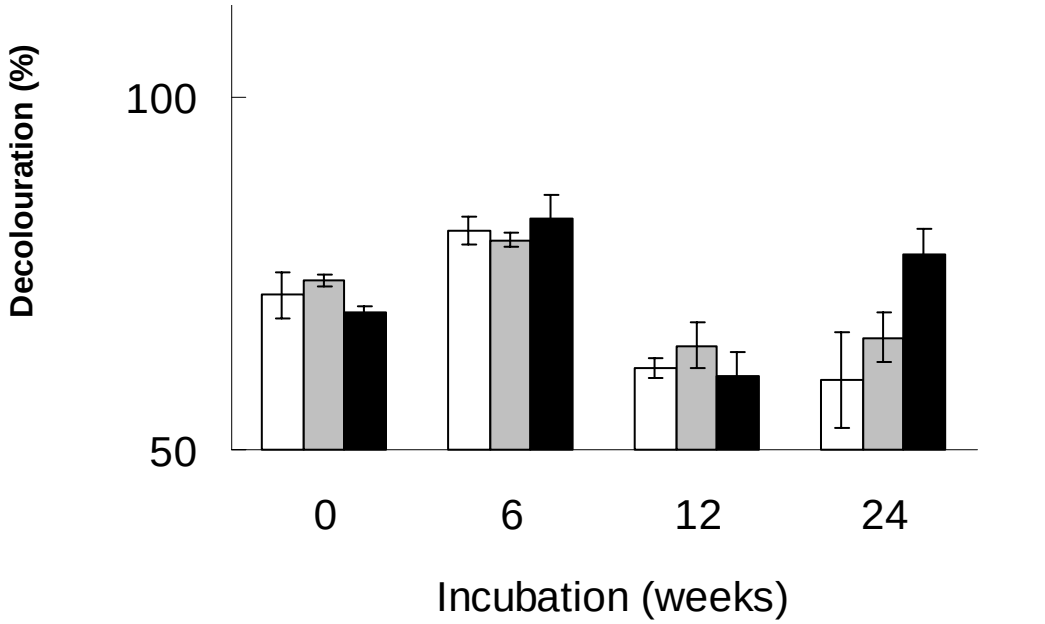
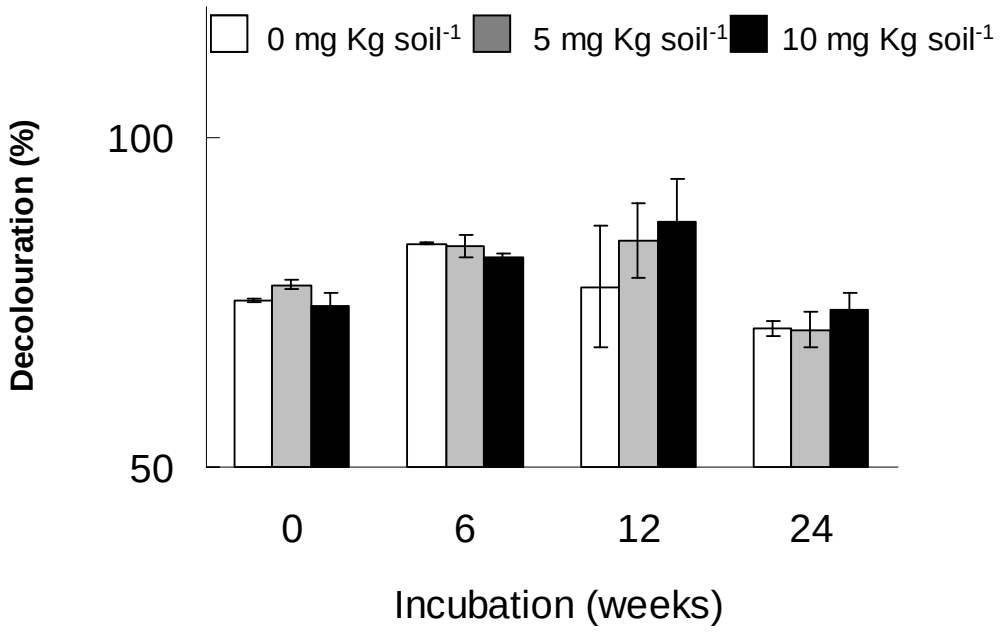


Figure 3.36 Total ligninolytic activity (expressed as % decolouration of Poly R478) in soil microcosms non-inoculated and amended with wood chips incubated for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

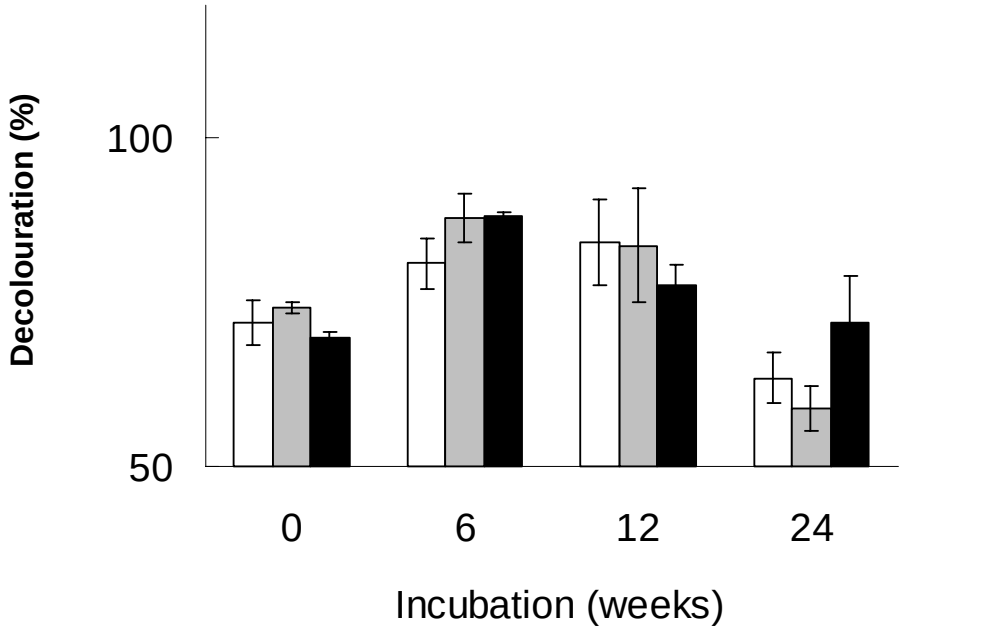
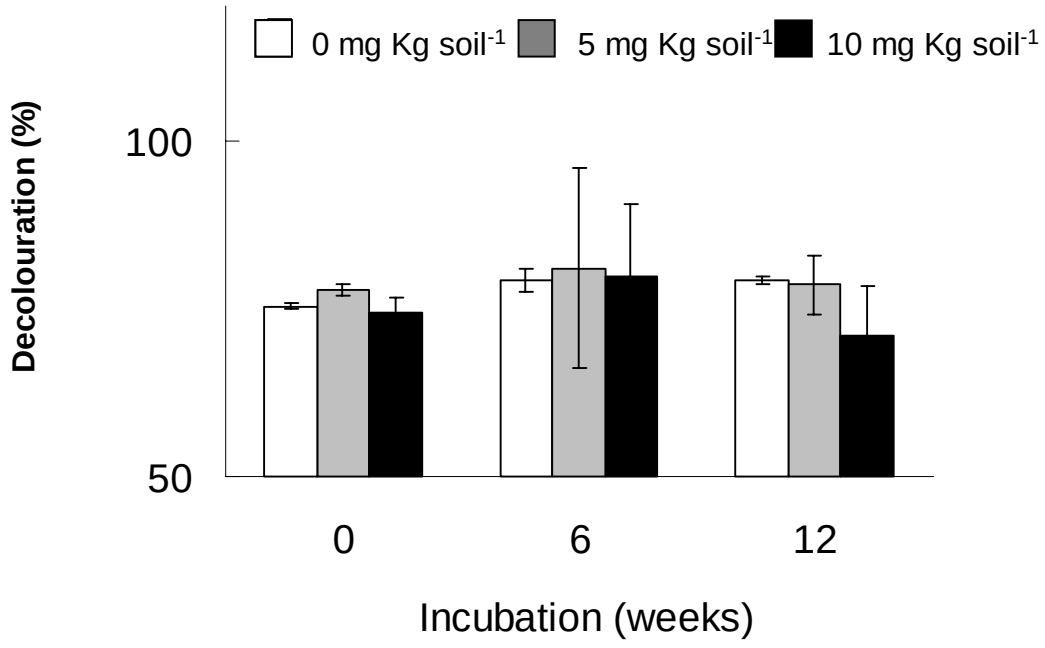


Figure 3.37 Total ligninolytic activity (expressed as % decolouration of Poly R478) in soil microcosms inoculated with *T.versicolor* (R26), incubated for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

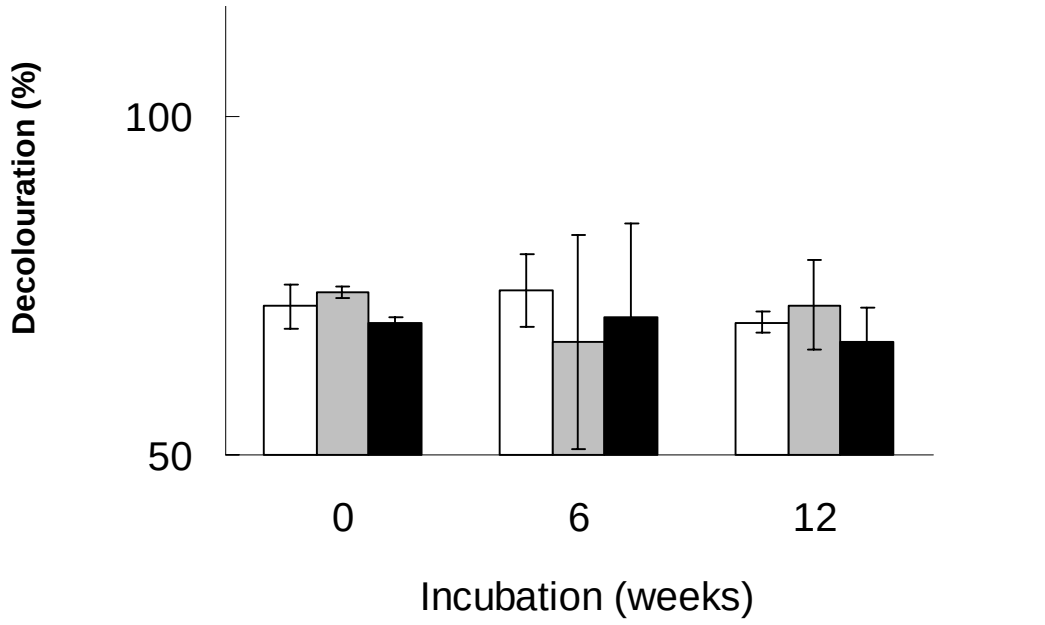
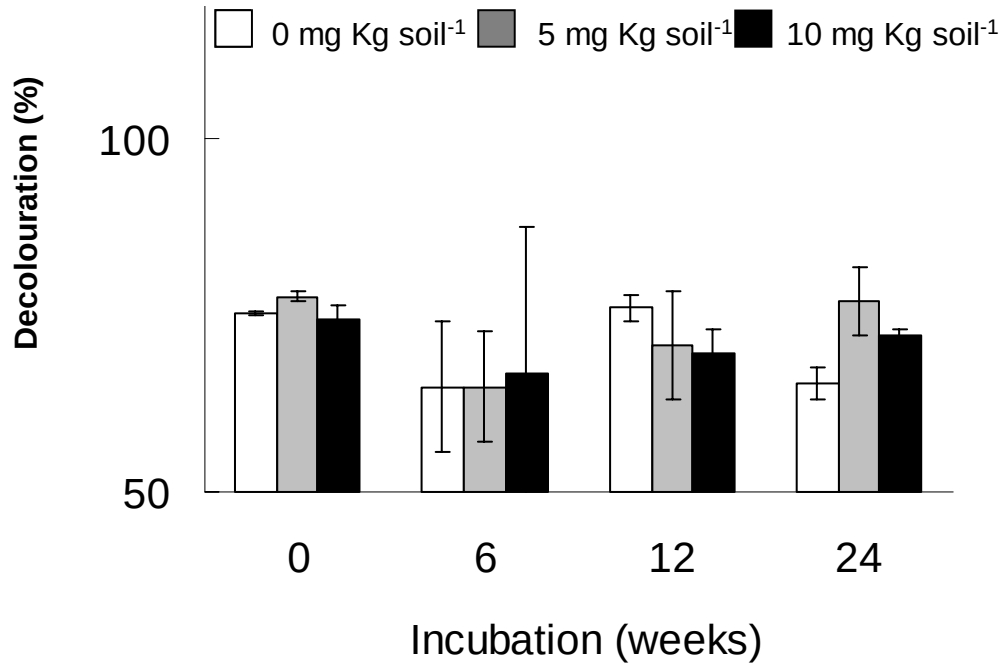


Figure 3.38 Total ligninolytic activity (expressed as % decolouration of Poly R478) in soil microcosms inoculated with *P.ostreatus*, incubated for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

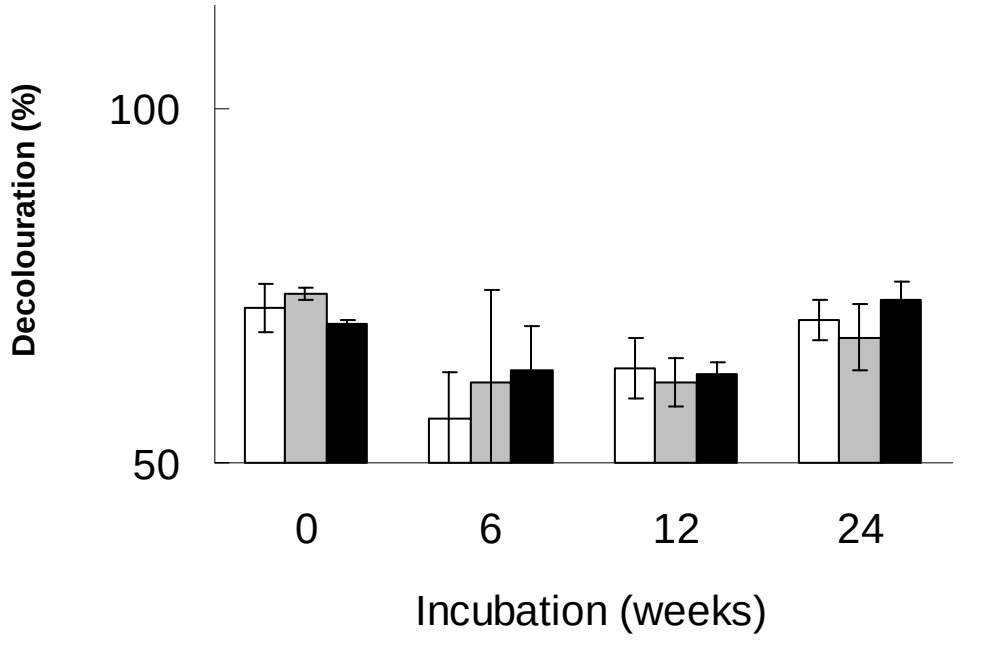
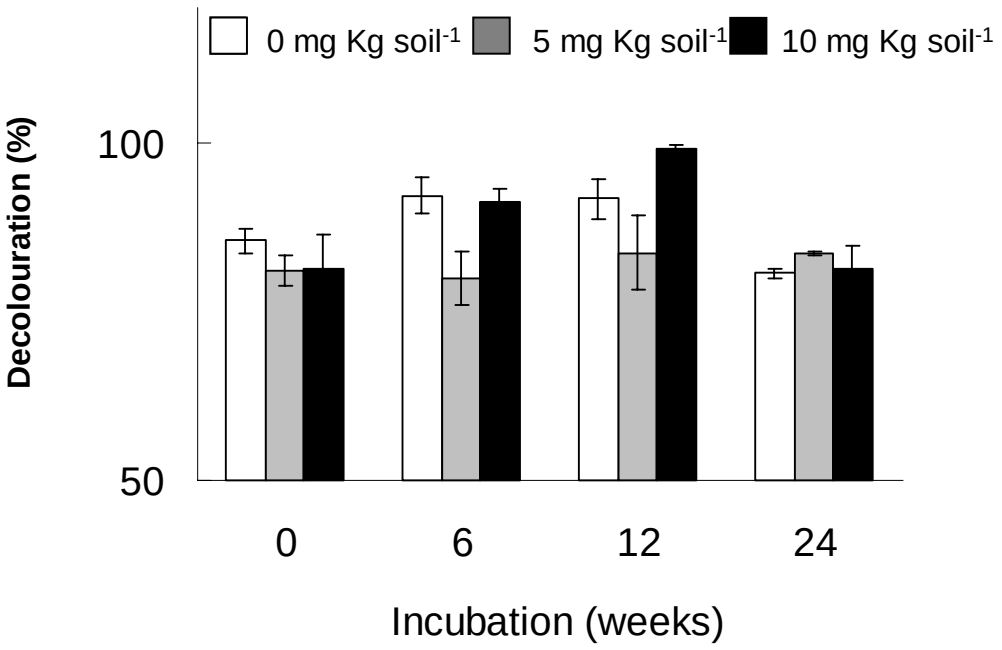


Figure 3.39 Total ligninolytic activity (expressed as % decolouration of Poly R478) in soil microcosms inoculated with *P.chrysosporium*, incubated for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

(a)



(b)

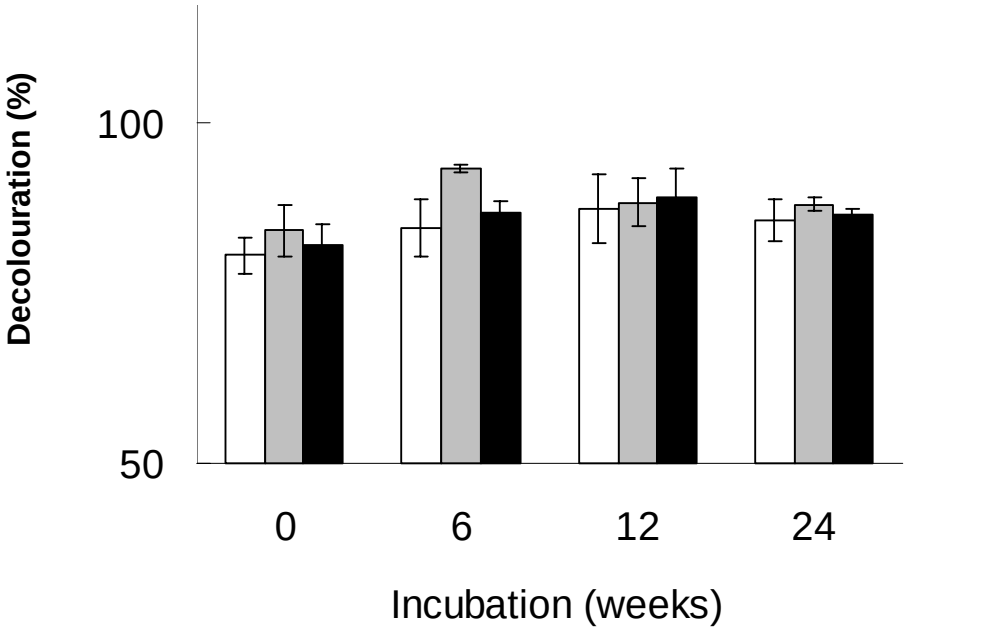


Figure 3.40 Total ligninolytic activity (expressed as % decolouration of Poly R478) in soil microcosms, inoculated with SMC for up to 24 weeks, at 15^{o}C, under two different water potential regimes (a) -0.7 and (b) -2.8 MPa. Bars represent the standard deviation of the mean (n=3), per treatment.

3.14 Temporal laccase activity in soil microcosms

Laccase is one of the enzymes involved in lignin degradation, and in contrast to respiratory and dehydrogenase activities, laccase was exclusively produced by the inoculated fungi and gave a good estimate of the ability of each isolate to colonise soil microcosms. As fungal laccase has shown to be involved in biodegradation of a broad range of pollutants, the production of this enzyme in soil is very pertinent for evaluation of the capacity for bioremediation.

In natural soil the production of laccase was very low in all treatments (data not shown). In soil amended with wood chips alone there was some laccase production in some of the treatments especially after >=12 weeks. The enzyme may have been produced by native soil wood degrading fungi that in the meantime might have colonised the wood chips. Surprisingly, the highest level of activity for soil amended with wood chips was observed in the 10 mg kg soil^{-1} treatment, under water stress (22 U g soil $^{-1}$).

Soil inoculated with *T.versicolor* (Table 3.21) showed the highest laccase activity after 6 weeks incubation, in the 5 and 10 mg kg soil^{-1} treatments, under both water regimes. After 24 weeks incubation laccase levels decreased in all treatments. Water availability did not have a significant impact on laccase production in the soil inoculated with the test isolate (P=0.154).

In soil inoculated with *P.ostreatus* (Table 3.22) lower levels of laccase were produced (comparing with soil inoculated with *T.versicolor*) and in contrast to the latter treatment, laccase production was higher in the control (0 mg kg soil^{-1}) than in the treatments containing pesticide.

Laccase production in soil inoculated with *P.chrysosporium* (Table 3.23) was very low and only occurred in some of the pesticide treatments.

In natural soil amended with SMC the production of laccase was very low in all treatments (data not shown).

Table 3.21 Laccase activity (U g soil^{-1}) in soil inoculated with *T.versicolor* (R26) and amended with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes.

		- 0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture	0	0	26.7	368.5	4.4	0	93.3	61.8	0.5
(mg kg soil^{-1})	5	0	300.0	61.8	0	0	797.8	41.2	0
L.S.D.= 165.1	10	0	74.4	21.3	8.9	0	562.2	22.5	0.6

Table 3.22 Laccase activity (U g soil^{-1}) in soil inoculated with *P.ostreatus* and amended with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes.

		- 0.7 MPa			-2.8 MPa		
	Incubation (weeks)	0	6	12	0	6	12
Mixture	0	0	96.3	133.3	0	272.4	77.0
(mg kg soil^{-1})	5	0	0	13.3	0	29.6	34.0
L.S.D.= 93.1	10	0	3.0	0	0	13.3	10.5

Table 3.23 Laccase activity (U g soil^{-1}) in soil inoculated with *P.chrysosporium* and amended with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes.

		- 0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture	0	0	0	0	0	0	0	0	0
(mg kg soil^{-1})	5	0	0	1.4	1.0	0	0	0	0.5
L.S.D.= 3.1	10	0	5.9	0	1.8	0	13.3	1.1	2.1

3.15 Temporal cellulase activity in soil microcosms studies

Production of cellulase varied with water availability and with the fungal inoculant used. The lowest production was found in natural soil (Table 3.24). Soil amended with wood chips showed high levels of cellulase in some of the treatments (5 mg kg soil^{-1}, 6 weeks and 10 mg kg soil^{-1}, 12 weeks).

Cellulase production in soil inoculated with *T.versicolor* (Table 3.26) was unaffected by water potential or pesticide treatment (P=0.924 and P=0.101, respectively). Soil inoculated with this test isolate produced less cellulase than soil inoculated with the other two isolates.

Soil inoculated with *P.ostreatus* (Table 3.27)produced significantly higher levels of cellulase at -2.8 (P=0.002), than at -0.7 MPa, with maximum activities of this enzyme in the 5 mg kg soil^{-1} treatment. Similarly, in soil inoculated with *P.chrysosporium* (Table 3.28) higher levels of cellulase were produced at -2.8 MPa (P<0.001). However, there were no differences between the pesticide treatments. With this inoculant cellulase activity was higher after 6 and 12 weeks incubation, decreasing after 24 weeks (P<0.05).

Soil amended with SMC showed very low levels of cellulase (Table 3.29).

Table 3.24 Cellulase activity in soil supplemented with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		-0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture (mg kg soil^{-1})	0	64.68	3.89	1.33	1.33	55.41	10.89	18.67	26.96
	5	81.45	3.11	2.67	4.00	95.53	11.67	8.00	7.26
	10	73.96	4.00	1.33	5.48	92.73	34.11	105.19	40.89
L.S.D.= 51.8									

Table 3.25 Cellulase activity in soil amended with wood chips supplemented with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		-0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture (mg kg soil^{-1})	0	88.0	50.7	50.4	43.4	94.8	30.0	45.2	33.6
	5	78.6	8.9	65.0	16.7	94.3	151.1	77.5	56.4
	10	102.1	1.2	5.3	31.4	62.3	10.0	107.6	70.4
L.S.D.= 74.1									

Table 3.26 Cellulase activity (U g soil^{-1}) in soil inoculated with *T.versicolor* and supplemented with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		-0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture (mg kg soil^{-1})	0	88.0	33.1	93.8	61.5	94.8	28.0	42.7	42.7
	5	78.6	12.6	25.3	51.0	94.3	32.1	25.3	43.1
	10	102.1	13.9	28.9	34.1	62.3	98.7	31.7	33.6
L.S.D.= 80.9									

Table 3.27 Cellulase activity (U g soil^{-1}) in soil inoculated with *P.ostreatus* and supplemented with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		-0.7 MPa			-2.8 MPa		
	Incubation (weeks)	0	6	12	0	6	12
Mixture (mg kg soil^{-1})	0	88.0	64.9	76.0	94.8	168.1	167.4
	5	78.6	103.7	81.6	94.3	143.7	18.4
	10	102.1	81.6	78.7	62.3	119.6	95.6
	L.S.D.= 81.8						

Table 3.28 Cellulase activity (U g soil^{-1}) in soil inoculated with *P.chrysosporium* and supplemented with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		-0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture (mg kg soil^{-1})	0	88.0	36.1	52.9	33.9	94.8	97.5	61.2	36.9
	5	78.6	80.7	61.3	33.5	94.3	128.7	92.4	13.5
	10	102.1	75.9	54.5	22.2	62.3	128.7	122.7	33.6
L.S.D.= 75.9									

Table 3.29 Cellulase activity (U g soil^{-1}) in soil amended with SMC and supplemented with a mixture of pesticides (5 and 10 mg kg soil^{-1} total concentrations), for up to 24 weeks, at 15^{o}C, under two different water potential regimes. Least significant differences (P=0.05) are for Pesticide x water potential interactions.

		-0.7 MPa				-2.8 MPa			
	Incubation (weeks)	0	6	12	24	0	6	12	24
Mixture (mg kg soil^{-1})	0	69.8	0.1	1.3	30.5	85.4	2.1	1.3	41.9
	5	67.0	0	1.3	20.6	33.7	3.2	4.0	59.6
	10	96.8	9.2	2.7	25.3	5.6	17.4	1.3	81.3
L.S.D.= 51.7									

3.16 Total microbial populations

3.16.1 Total viable bacterial populations

Overall there were few significant differences in total viable bacterial populations between treatments. Statistical analysis (three-way ANOVA for each inoculant time) showed several differences between treatments that are described below.

In natural soil and soil inoculated with *T.versicolor* (Figure 3.41 and 3.43) at 24 weeks there was a significant decrease in bacterial viable populations in the 10 mg kg soil^{-1} treatment ($P<0.05$), at -0.7 MPa. In soil amended with wood chips there were no differences between treatments. In soil inoculated with *P.ostreatus* (Figure 3.44) the total number of viable bacteria was unaffected by the water availability ($P= 0.044$) or incubation times ($P<0.05$). However at -0.7 MPa there was a decrease in bacterial populations at 5 mg kg soil^{-1} ($P=0.012$). In soil inoculated with *P.chrysosporium* (Figure 3.44) at 6 weeks incubation the number of bacterial populations was stimulated in the presence of pesticides ($P<0.05$). At 24 weeks bacterial populations were higher at -0.7 MPa in the control. In soil amended with SMC there were no differences between treatments (Figure 3.46).

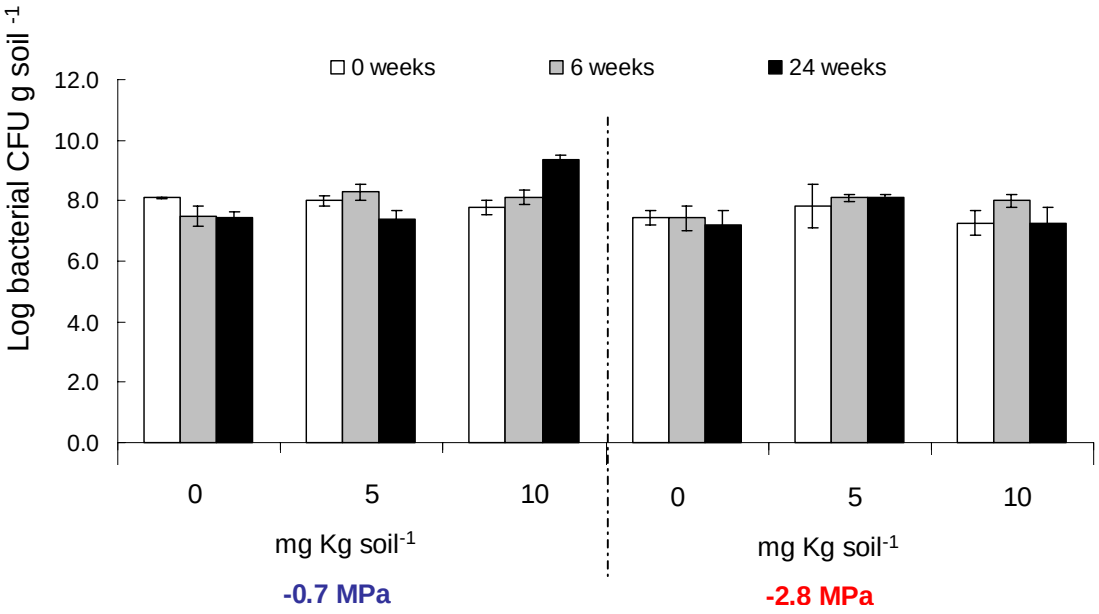


Figure 3.41 Total viable bacterial populations (log CFU g soil^{-1}) in natural soil, microcosms amended with wood chips, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

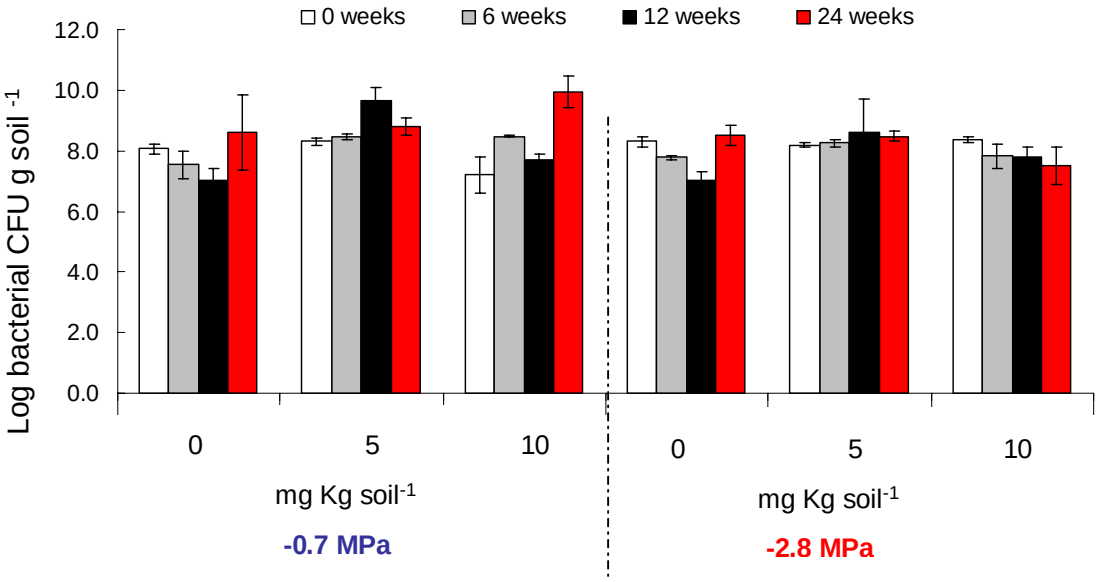


Figure 3.42 Total viable bacterial populations (log CFU g soil^{-1}) in natural soil amended with wood chips, microcosms amended with wood chips, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

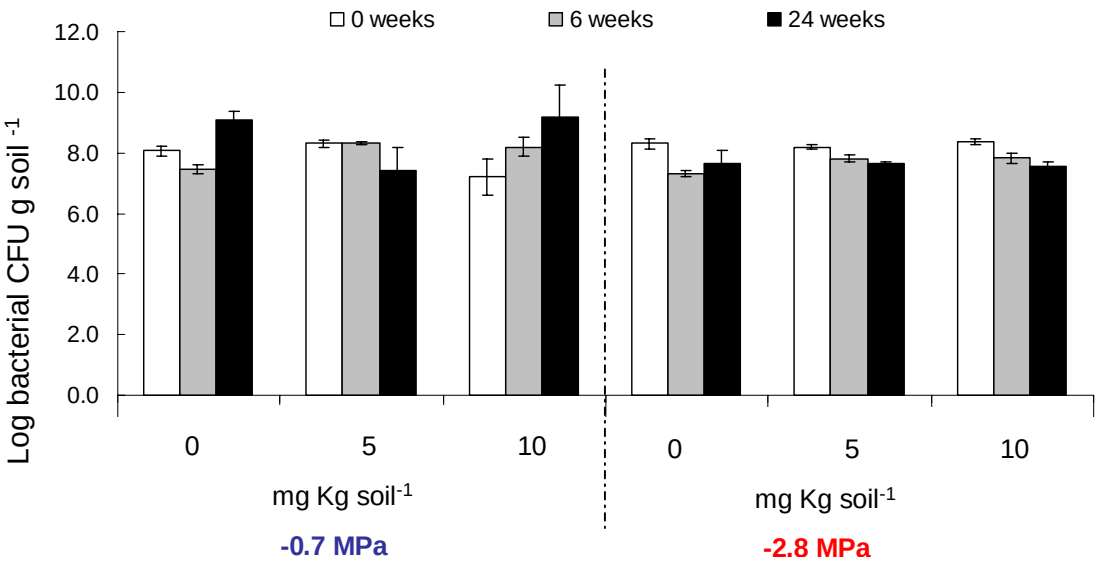


Figure 3.43 Total viable bacterial populations (log CFU g soil^{-1}) in soil inoculated with *T.versicolor* (R26), microcosms amended with wood chips, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

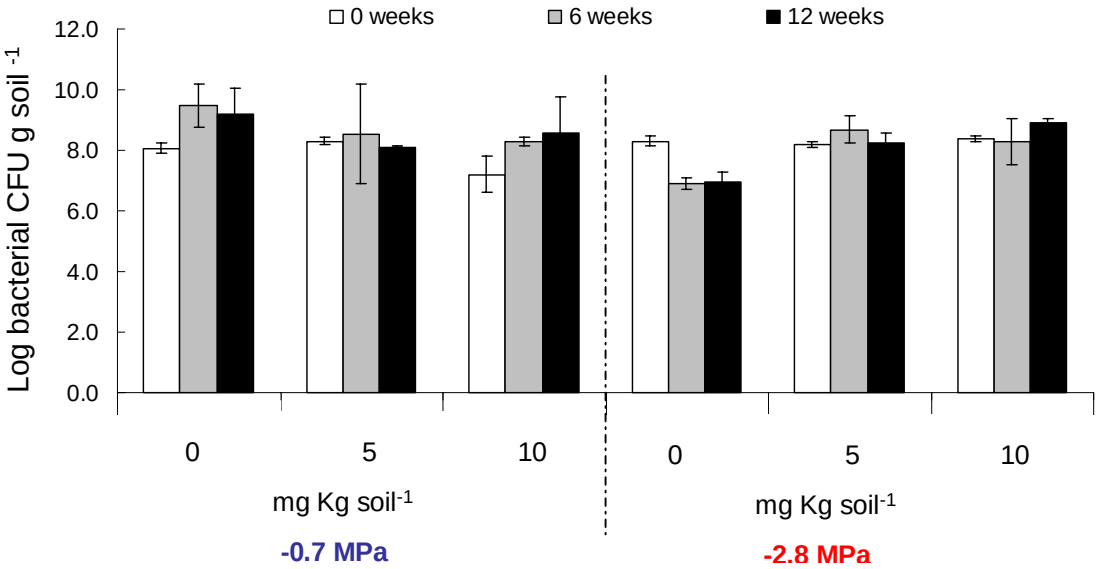


Figure 3.44 Total viable bacterial populations (log CFU g soil^{-1}) in soil inoculated with *P.ostreatus*, microcosms amended with wood chips, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

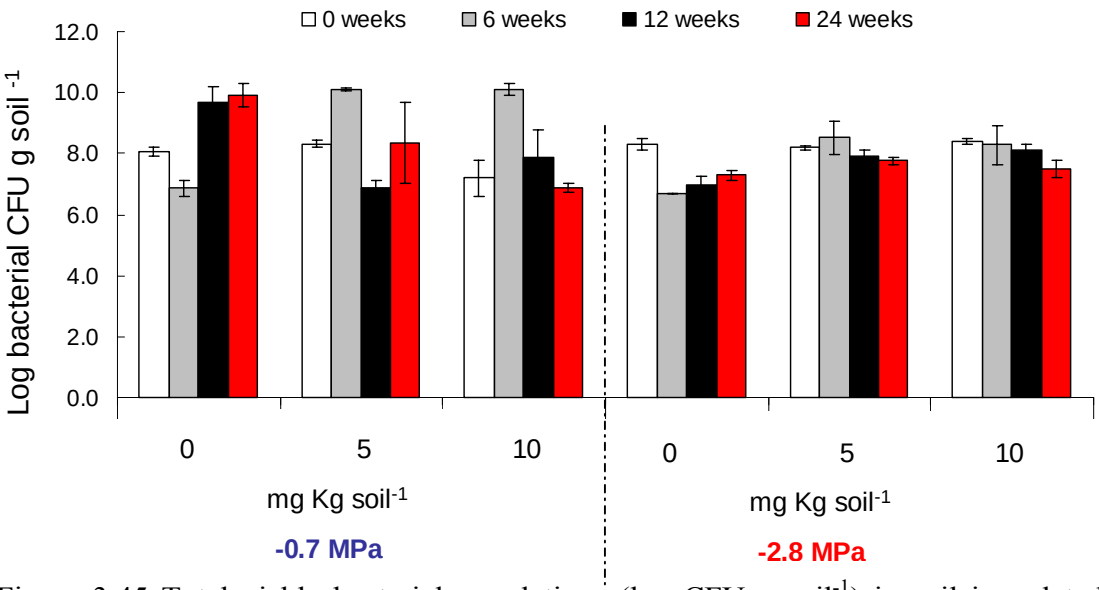


Figure 3.45 Total viable bacterial populations (log CFU g soil^{-1}) in soil inoculated with *P.chrysosporium*, microcosms amended with wood chips, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

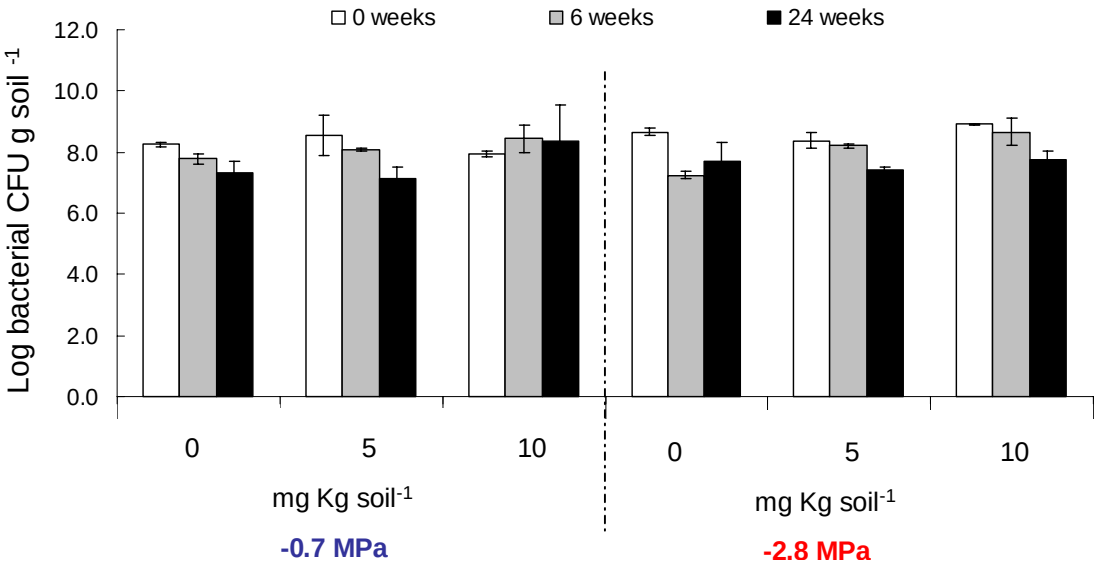


Figure 3.46 Total viable bacterial populations (log CFU g soil^{-1}) in soil microcosms amended with SMC, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

3.16.2 Total viable fungal populations in soil microcosms

Overall, there were few significant differences in total viable fungal populations between treatments. Statistical analysis (three-way ANOVA for each inoculant time) showed several differences between treatments that are described below. In natural soil (Figure 3.47) the number of fungal populations was significantly lower at the start of the experiment in the -0.7MPa water potential ($P<0.001$). In soil amended with wood chips (Figure 3.48) the number of fungal populations was higher at 24 weeks incubations ($P<0.05$). In soil inoculated with *T.versicolor* (Figure 3.49) there was an increase in fungal populations at 24 weeks in the 10 mg kg soil^{-1} pesticide treatment ($P<0.05$). In soil inoculated with *P.ostreatus* at 6 weeks incubation there was a decrease in fungal populations at 10 mg kg soil^{-1} ($P<0.005$), at 12 weeks the same decreased was observed for 5 and 10 mg kg soil^{-1} treatments ($P<0.05$). In soil inoculated with *P.chrysosporium* the number of fungal populations was significantly higher at 12 weeks incubation ($P<0.001$).

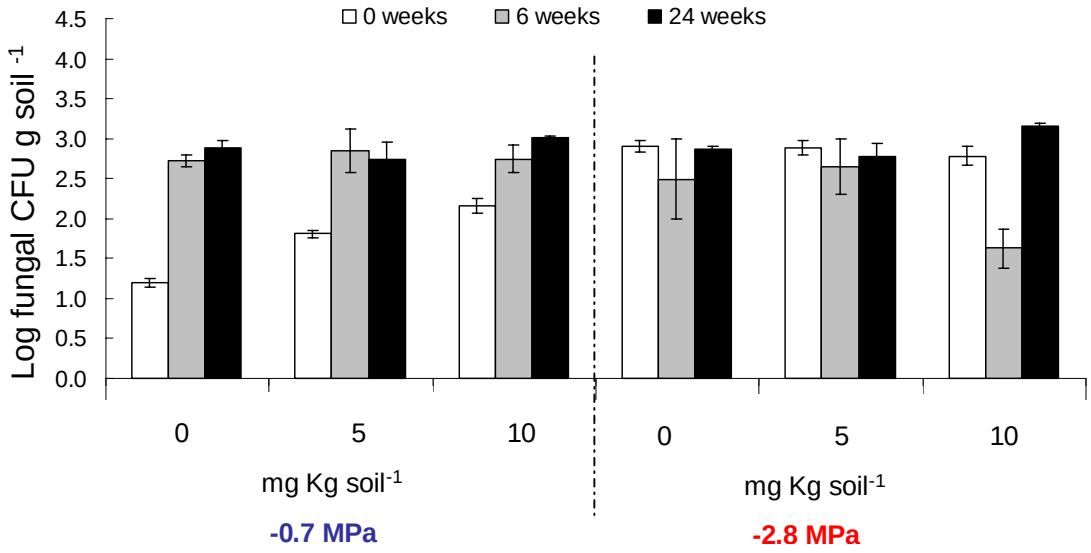


Figure 3.47 Total viable fungal populations (log CFU g soil^{-1}) in natural soil microcosms, at 0.7 and -2.8 MPa supplemented with a mixture of pesticides. Bars represent the standard deviation of the mean (n=3), per treatment.

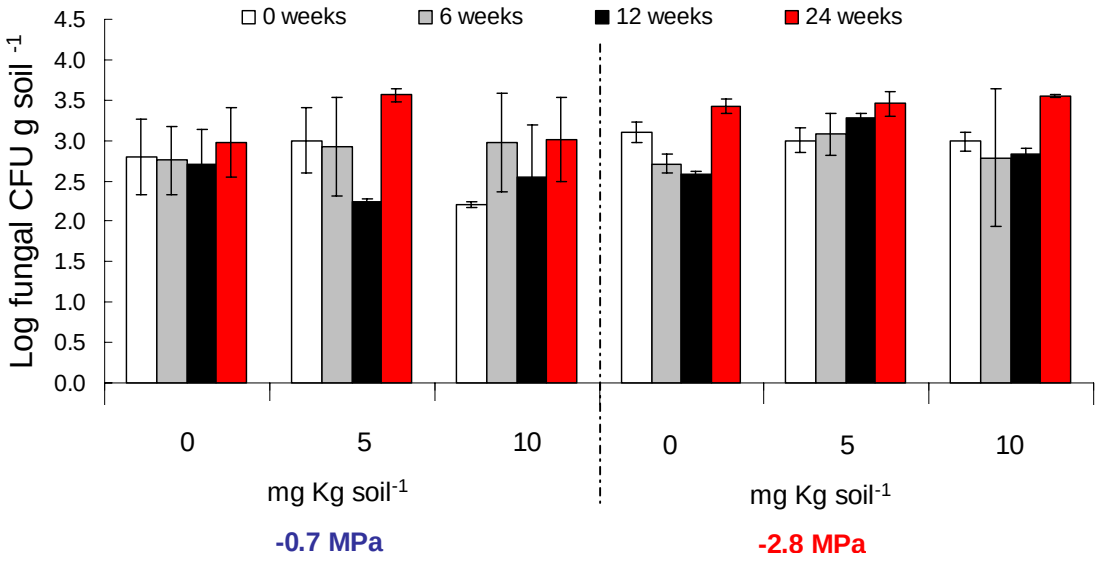


Figure 3.48 Total viable fungal populations (log CFU g soil^{-1}) in natural soil microcosms amended with wood chips, at 0.7 and -2.8 MPa supplemented with a mixture of pesticides. Bars represent the standard deviation of the mean (n=3), per treatment.

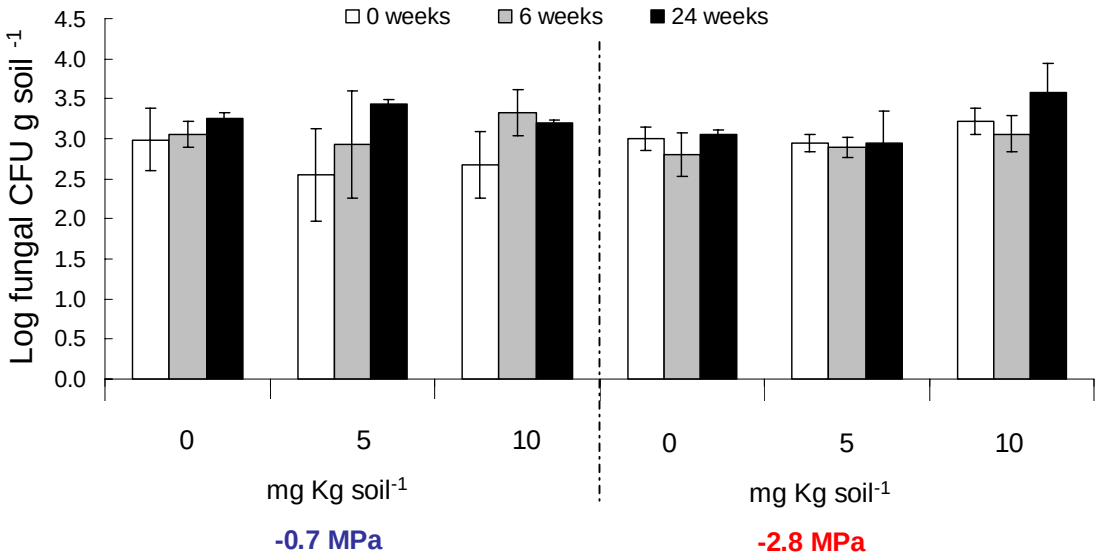


Figure 3.49 Total viable fungal populations (log CFU g soil^{-1}) in soil inoculated *T.versicolor* (R26), microcosms amended with wood chips, at 0.7 and -2.8 MPa supplemented with a mixture of pesticides. Bars represent the standard deviation of the mean (n=3), per treatment.

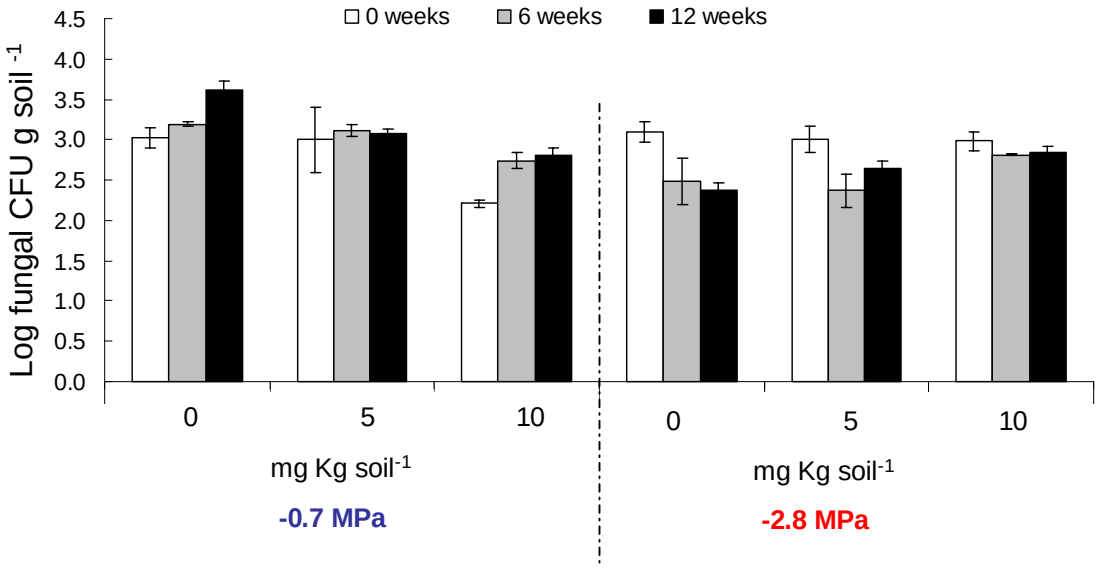


Figure 3.50 Total viable fungal populations (log CFU g soil^{-1}) in soil inoculated *P.ostreatus*, microcosms amended with wood chips, at 0.7 and -2.8 MPa supplemented with a mixture of pesticides. Bars represent the standard deviation of the mean (n=3), per treatment.

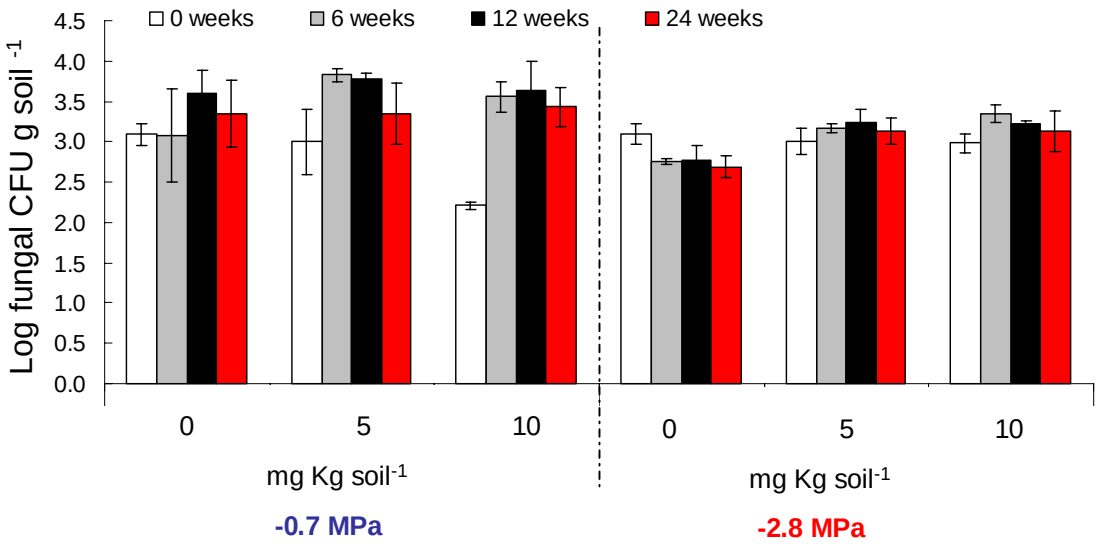


Figure 3.51 Total viable fungal populations (log CFU g soil^{-1}) in soil inoculated *P.chrysosporium*, microcosms amended with wood chips, at 0.7 and -2.8 MPa supplemented with a mixture of pesticides. Bars represent the standard deviation of the mean (n=3), per treatment.

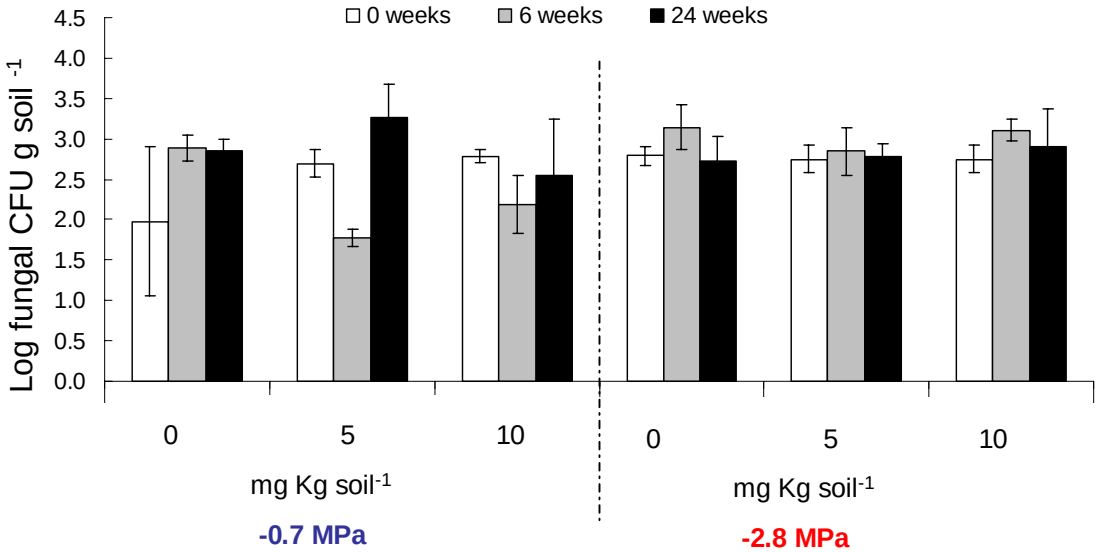


Figure 3.52 Total fungal counts (log CFU g soil^{-1}) in soil microcosms amended with SMC, under two different water regimes and pesticide concentration. Bars represent the standard deviation of the mean (n=3), per treatment.

3.16.3 Impact of treatments on fungal population structure in soil microcosms

The results showed the fungal diversity in soil microcosms varied in the different treatments. In natural soil (Figure 3.54). *Aureobasidium, Penicillium* and *Cladosporium* were the predominant genera, although in the presence of the mixture of pesticides, under higher availability *Doratomyces* was the most abundant. Interestingly yeast pink is very common in the absence of the pesticides.

In soil amended with wood chips alone *Doratomyces* was very abundant in all the treatments, independently of the water potential. In all the treatments where wood chips were used (alone and as the substrate where the fungal inoculant was pre-grown) *Aureobasidium* and *Acremonium* were very abundant.

In soil inoculated with *T.versicolor* (Figure 3.55) *Acremonium, Doratomyces* and *Aureobasidium* were very common. Whereas in soil inoculated with *P.ostreatus* and *P.chrysosporium* a great density of white yeast was observed (Figures 3.55 and 3.56).

In soil amendment with SMC the diversity profile was very similar to the one described for *T.versicolor* (Figure 3.56).

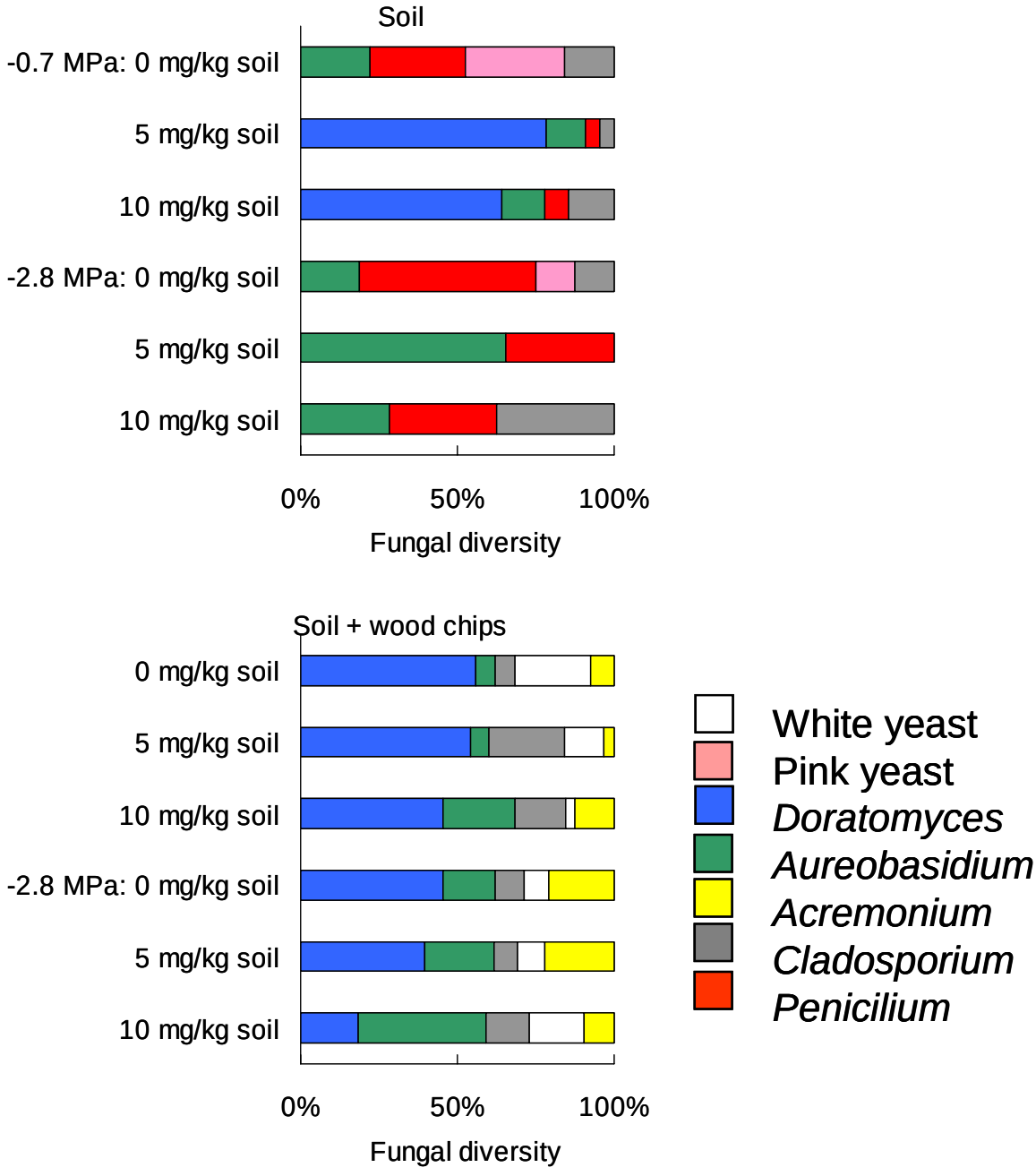


Figure 3.53 Fungal diversity in soil microcosms with natural soil and soil amended with wood chips, and supplemented with a mixture of pesticides, under two different water regimes.

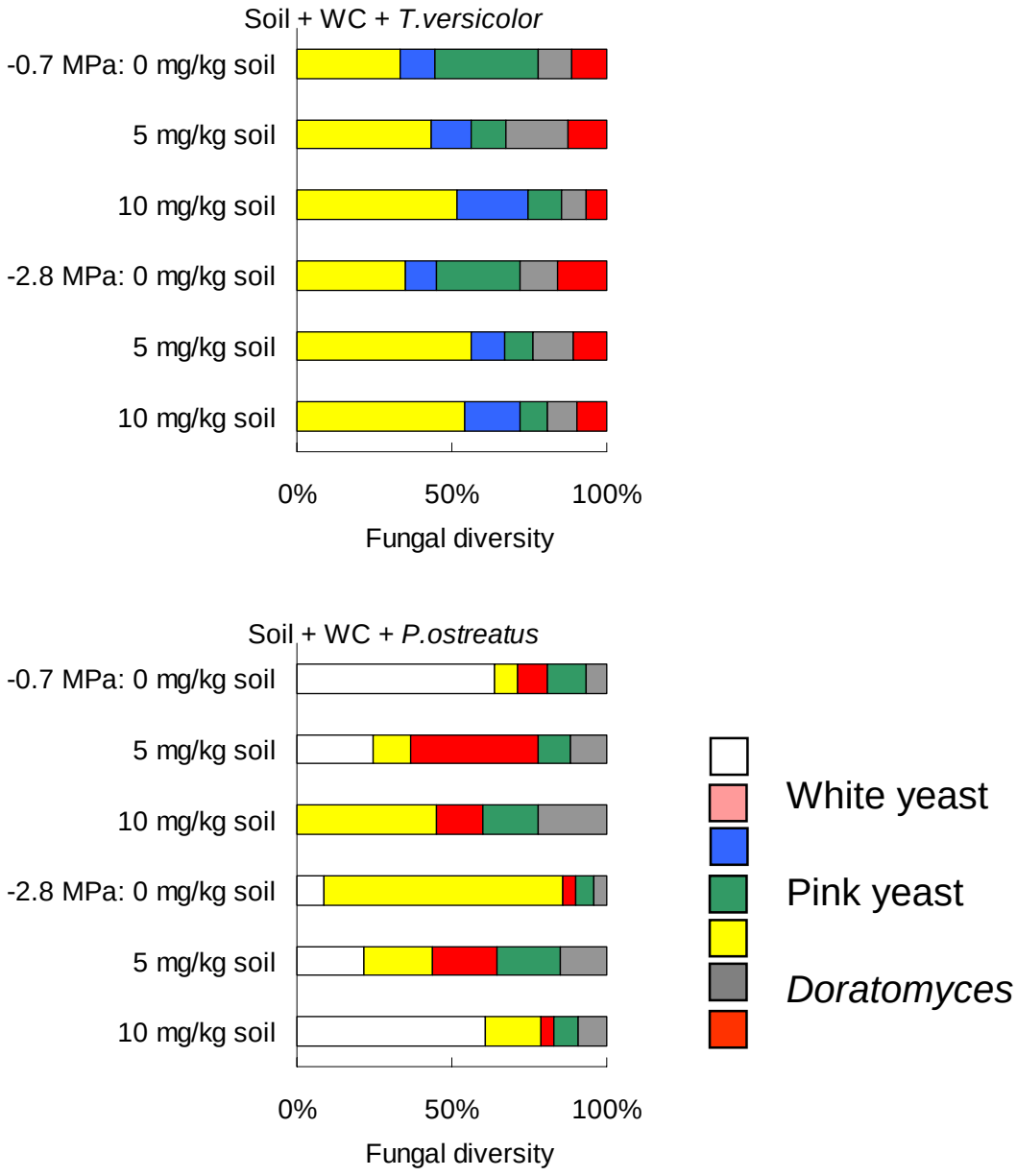


Figure 3.54 Fungal diversity in soil microcosms inoculated with *T.versicolor* and *P.ostreatus* and SMC, and supplemented with a mixture of pesticides, under two different water regimes.

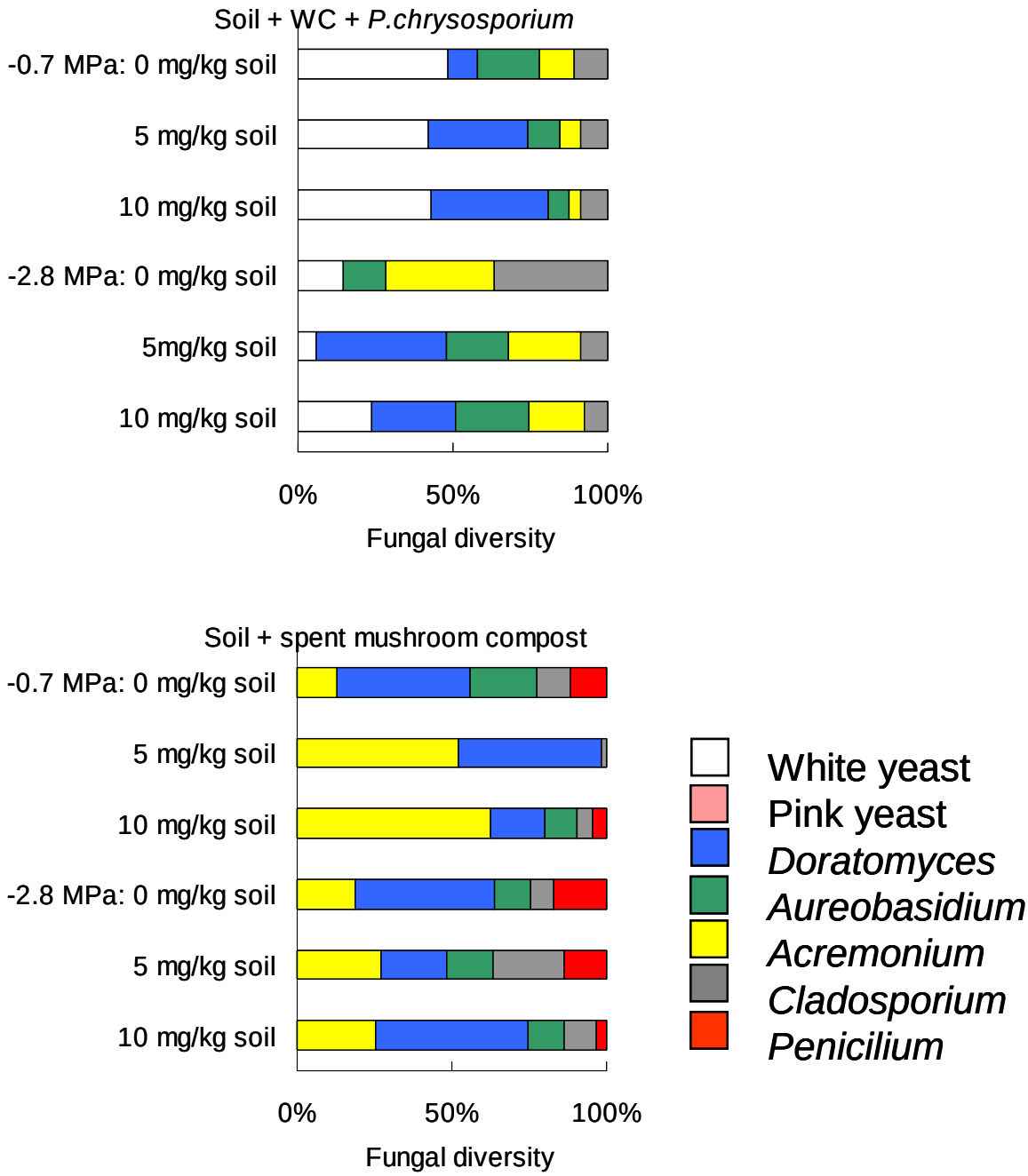


Figure 3.55 Fungal diversity in soil microcosms inoculated with *P.chrysosporium* and SMC, and supplemented with a mixture of pesticides, under two different water regimes.

3.17 Correlation between different parameters

In addition to single analytical values of the individual biological and biochemical parameters some correlations were also calculated from the results, at 6 weeks incubation, when the different biochemical, enzymological parameters suggested higher metabolic activity.

When carbon dioxide production was compared with dehydrogenase activity in soil microcosms amended with an increasing concentration of a mixture of pesticides (as shown in Figure 3.57) a correlation was found. Table 3.30 shows the correlation coefficients (r^2) between these two parameters.

When comparing soluble protein content in soil and microbial populations (Figures 3.58 and 3.59) a correlation was found in some of the treatments (Table 3.31).

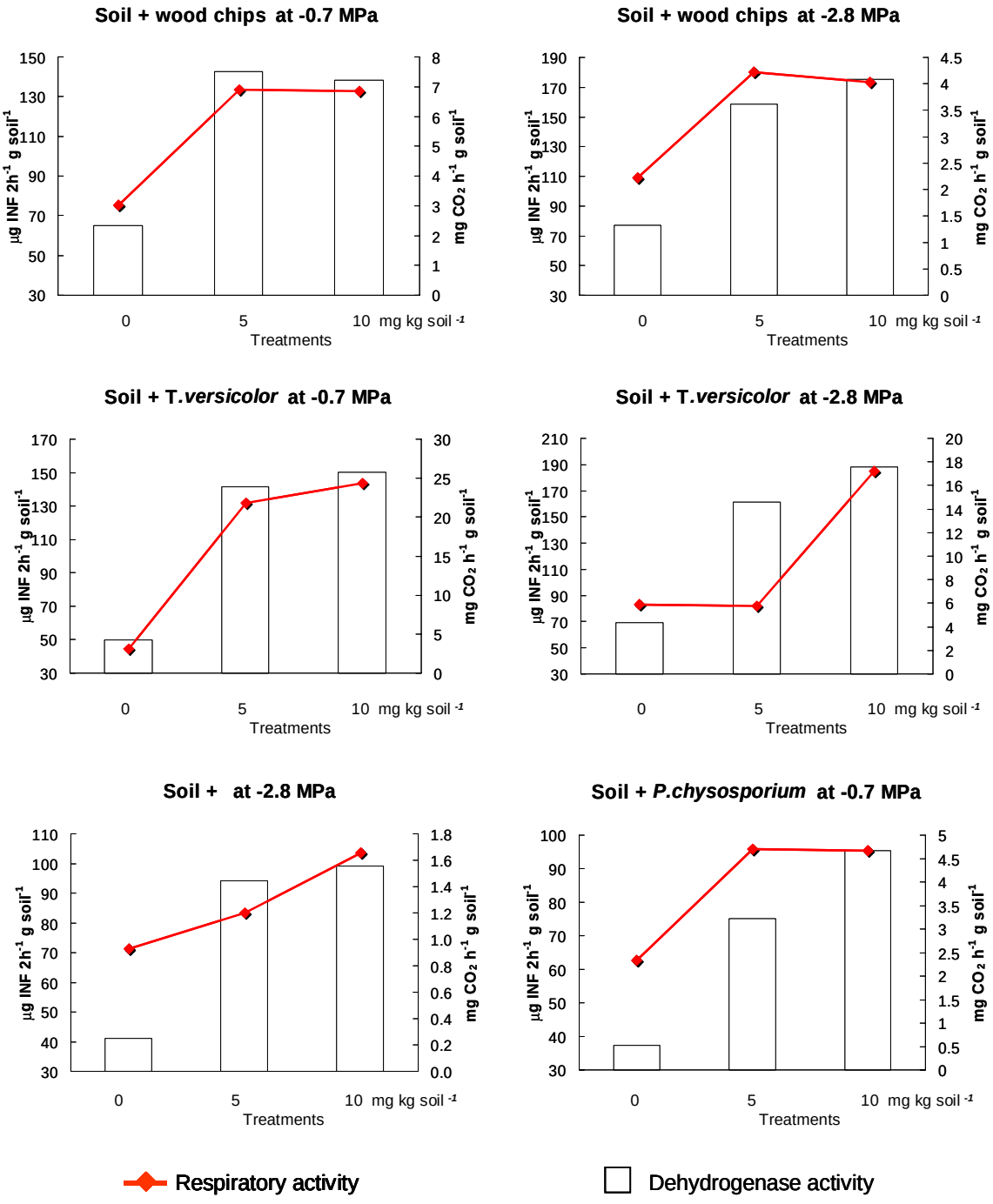


Figure 3.56 Examples of comparison between dehydrogenase production (as µg INF $2h^{-1}$ g $soil^{-1}$) and CO_2 (mg CO_2 h^{-1} g $soil^{-1}$) released in soil microcosms after 6 weeks incubation.

Table 3.30 Correlation (expressed as r^2) between carbon dioxide concentration and dehydrogenase activity, for increasing concentration of pesticides.

Treatments	Water potential	r^2
Soil + *P.chrysosporium*	-0.7 MPa	0.871
	-2.8 MPa	0.883
Soil + *P.ostreatus*	-0.7 MPa	0.417
	-2.8 MPa	0.975
Soil + *T.versicolor*	-0.7 MPa	0.999
	-2.8 MPa	0.443
Soil + SMC	-0.7 MPa	0.038
	-2.8 MPa	0.852
Soil	-0.7 MPa	0.732
	-2.8 MPa	0.694
Soil + WC	-0.7 MPa	0.999
	-2.8 MPa	0.939

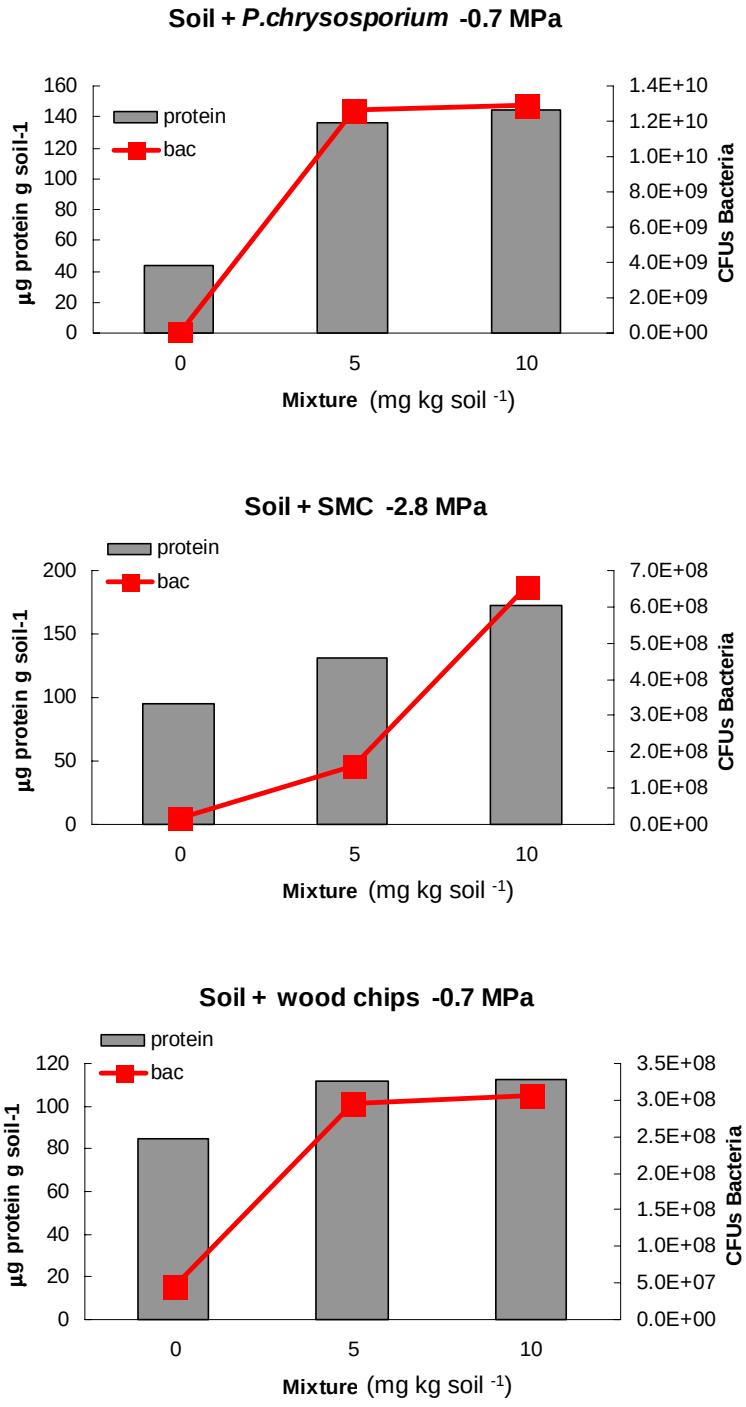


Figure 3.57 Example of comparison between soluble protein (µg g soil^{-1}) and total bacterial counts (CFUs) in soil microcosms after 6 weeks incubation (the values represent the mean *n*=3).

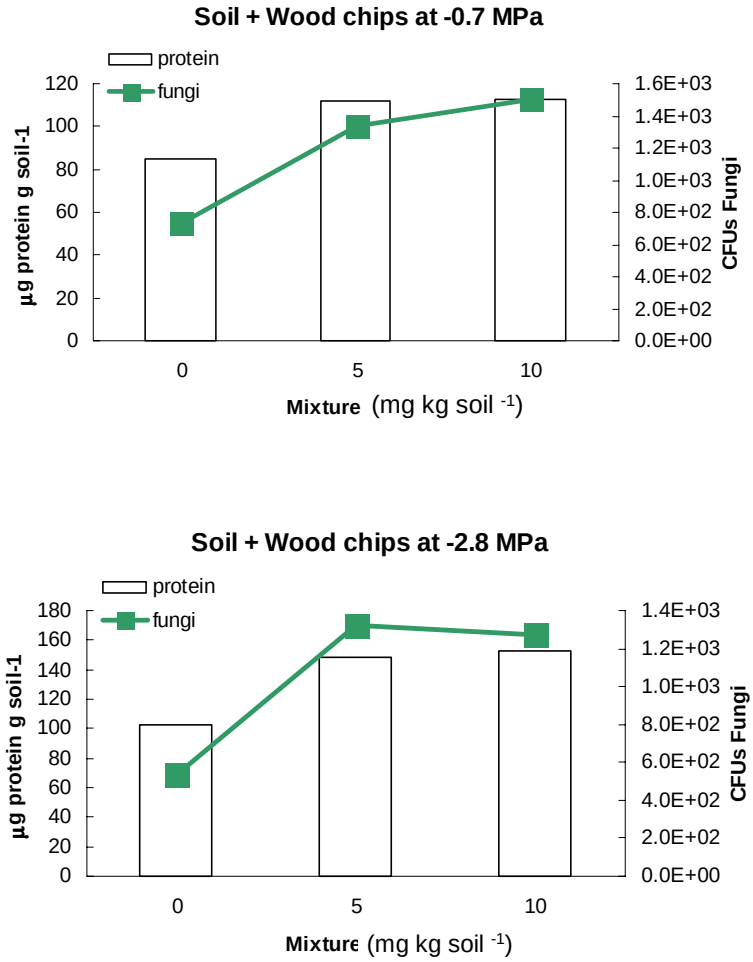


Figure 3.58 Example of comparison between soluble protein (µg g soil^{-1}) and total fungal counts (CFUs) in soil microcosms after 6 weeks incubation (the values represent the mean *n*=3).

Table 3.31 Correlation (expressed as r^2) between total microbial counts (CFUs bacteria and CFUs fungi) and soluble protein content (μg protein g $soil^{-1}$), for increasing concentration of pesticides.

	Ψ (MPa)	r^2 CFU bacteria	r^2 CFU fungi	
P.chrysosporium	-0.7	0.997	0.536	Figure 3.57
	-2.8	0.939	0.376	
P.ostreatus	-0.7	0.839	0.982	
	-2.8	0.045	0.805	
T.versicolor	-0.7	0.249	0.011	
	-2.8	0.480	0.303	
SMC	-0.7	0.365	0.145	
	-2.8	0.925	0.347	Fig.3.58
Soil	-0.7	0.980	0.042	
	-2.8	0.930	0.011	
WC	-0.7	0.999	0.970	Figs 3.58 and 3.59
	-2.8	0.406	0.980	Fig.3.58

4 Discussion

4.1 General overview

As currently soil contamination is a major environmental problem, the need to remediate contaminated sites has led to the development of new technologies that emphasize the destruction of the contaminants rather than the conventional approach of disposal (Boopathy, 2000). Bioremediation involves the use of microorganisms or microbial processes to degrade environmental contaminants, and is among these new technologies. 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 fungal species.

4.2 Fungal tolerance to pesticides

The initial studies were carried out using eight fungal isolates. Using this range of basidiomycetes, initial experiments showed that at 15ºC the use of cellophane discs to separate soil extract agar had no effect on relative mycelial extension rates. However, at 25ºC in 44% of treatments growth was different. Previous studies in relation to growth rates and water potential in media with cellophane have shown some differences in mycelial extension in presence and absence of cellophane (Ramos *et al*., 1999). This separation was important to make sure all interactions between mycelium and culture medium were extracellular.

Regarding of the effect of temperature on growth rates the isolates showed better growth at 25ºC, as the highest growth rate obtained at 15°C was 2.9 mm day^{-1}. In fact, some of the species used in this study have optimal growth at 30ºC, with growth rates at optimum conditions of 7.0 ± 0.5 mm day^{-1} (Tekere *et al*., 2001; Mswaka and Magan, 1999). But, because 15ºC is environmentally more relevant to U.K. and other European countries, where low temperature in soil can be a limiting factor to microbial degradation of soil contaminants (Romanstschck *et al.*, 2000) the subsequent screening test, to evaluate fungal tolerance to pesticides, was carried out at 15ºC. Lower growth rates do not necessarily imply lower enzyme production,

important in the context of degradation of xenobiotics, but the fungi has to grow well for colonisation establishment.

The screening experiment, at 15°C, showed that in the range of pesticide concentrations used *P.ostreatus*, *T.versicolor* R26 and R101 were the most tolerant isolates. These conclusions were based on the analysis of EC_{50} values. *P.chrysosporium* showed high sensitivity to the pesticides, however, it was included in subsequent studies because it is well known for its enzymatic capacities and has been thoroughly studied for bioremediation purposes (Reddy and Mathew, 2001).

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 simazine, *T.versicolor* (R26 and R101) were tolerant to this pesticide, with the isolate R26 having higher growth rates than R101 at –0.7 MPa, in the soil extract agar. At –2.8 MPa their growth rates were equivalent, suggesting *T.versicolor* R101 tolerated well interacting factors of pesticide concentration and water potential. Besides, in matric potential media *T.versicolor* R101 performed better than *T.versicolor* R26. *P.ostreatus* showed a good tolerance to simazine at both water potential regimes (-0.7 and –2.8 MPa), exhibiting high growth rates in the presence of this pesticide up to 10 mg l^{-1}. When matric potential was used, this microorganism was not as tolerant. Overall, the results showed that *T.versicolor* R101 was more tolerant to simazine on a wider range of conditions, than the other isolates tested in this study.

The response to trifluralin and dieldrin was rather similar to the response to simazine. Again, *T.versicolor* R101 showed a good tolerance to the pesticide at different water regimes. In contrast *T.versicolor* R26 and *P.ostreatus*, which grew well with fully availability of water, did not perform as well as *T.versicolor* R101, under conditions of water stress.

In the presence of the pesticide mixture *P.ostreatus* showed the best tolerance. Generally, the response of the isolates to the mixture showed a similar trend to the response to dieldrin and trifluralin individually, however, *P.ostreatus* showed a remarkable growth increase in the presence of this mixture of pesticides, especially at -0.7 MPa.

The study of fungal growth rates is very important for extrapolation of the potential colonisation capacity in the field as it provides a good indication of the speed at which a fungus is able to colonise and transverse 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 growth could help the introduced fungi to overcome competition from indigenous soil microrganisms (Singleton, 2001).

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 may occur naturally in soil. This parameter was modified by changing the osmotic and matric potential of the culture media. Generally, fungal germination and growth has been demonstrated to be more sensitive to matric than osmotic potential (Ramos *et al.*, 1999). McQuilken *et al.* (1992) reported that mycelial extension decreased below –0.5 MPa and ceased between –2.5 and 3.0 MPa osmotic potential. However, in media where the matric potential was adjusted with PEG6000, both growth and germination ceased at –2.0MPa (McQuilken *et al.*, 1992). Matric potential effects on growth may be of particular importance for growth and survival in soil, since it influences the physiological activity of soil microrganisms (Zak *et al*., 1999).

The results for lignin degradation and laccase production, in the Petri plate assays, were interesting, as both isolates of *T.versicolor* (R101 and R26) and *P.ostreatus* were able to degrade lignin under both water regimes and in all the pesticide treatment conditions. Laccase production also occurred, but it was more affected by water availability and pesticide concentration. Since laccase is one of the enzymes involved in lignin degradation, the results suggest that in some of the treatments (e.g. dieldrin) where there was no laccase production, but lignin degradation was detected other ligninolytic enzymes might have been produced. The ability to produce a wide array of enzymes in the presence of contaminants may be advantageous in bioremediation. Moreover, the capacity of growing and producing extracellular enzymes under different water availabilities is essential when attempting to use fungal inoculants for remediation of contaminated soil.

4.3 Soil extract broth as an approach to soil studies

This study showed that *T.versicolor* (R26 and R101), *P.ostreatus* and *P.chrysosporium*, were able to grow effectively in low nutrient status media over a range of concentrations of a mixture of pesticides. Two osmotic potential conditions were examined: -0.7 MPa which is optimal for growth of such white rot fungi, and -2.8 MPa, which is well as below the wilting point of plants (Mswaka and Magan, 1999). All species produced significantly higher biomass under water stress conditions. This ability may be particularly beneficial in the soil environment where water availability fluctuates significantly. The studies in soil extract agar media showed that *P.chrysosporium* was more sensitive to this mixture of pesticides than the other test isolates; however, in the present study in soil extract broth this was not observed.

The present study on the degradation of the mixture of pesticides showed good capacity by *T.versicolor* (R101) and *P.chrysosporium* at both osmotic potential treatments, regardless of the initial concentrations of pesticides between 0 and 30 mg l^{-1}. *P.ostreatus* and *T.versicolor* (R101) also showed good pesticide degradation, which was affected by water availability and initial concentration of the pesticide mixture. It was not possible to establish a trend explaining the way these factors affected pesticide degradation. The duration of the experiment was clearly sufficient for the bioremediation to be completed, as the concentration of pesticides by the end of the experiment was close to zero.

Interestingly, greater degradation was obtained in the treatments with higher initial concentration of pesticides. We have concentrated on the direct impact of kosmotropic solutes such as NaCl, PEG 600 (Brown, 1990). It is not known whether increasing concentrations of mixtures of pesticides can act as anthropogenic chaotropic solutes (e.g. phenols, urea, ethanol, benzyl alcohols) which may further interact with osmotic stress and influence the effects observed. However, few studies have considered these interactions.

Previously Tekere *et al*. (2002) reported degradation rates of about 82% for the pesticide lindane (an organochlorine, like dieldrin) by *P.chrysosporium* but the highest degradation rates were achieved when the initial concentrations were as low

as 5 and 10 mg l^{-1}. Lower degradation was obtained at initial concentrations of 20 and 40 mg l^{-1}. Surprisingly, few studies on the degradation of mixtures of pesticides have been carried out (Yavad and Reddy, 1993; Bending *et al.*, 2002). Yavad and Reddy (1993) described co-mineralisation 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. However, nutritionally rich media were used and water stress interactions were not considered.

A common procedure to assess the outcome of bioremediation is to measure the disappearance of the lethal effects of toxic substances (Tang *et al.*, 1998), in the current study a toxicity test was run on the soil extract after inoculation and incubation with the test isolates. The results suggested that the extracts were no longer toxic, which is in accordance with the HPLC results that showed very low concentrations of pesticides remaining in the soil extract. This is an interesting finding, because in some cases after bioremediation, some metabolites are produced and they may be as toxic or more toxic than the initial contaminants. In this case, according to the results of the toxicity test this did not occur, the final product of bioremediation was in fact harmless.

The results suggest that the hydrolytic and ligninolytic enzymes are not only secreted in nutrient-rich substrates but are produced by mycelia growing in weak nutritional matrices. Of particular interest is the capacity for production of these enzymes in the presence of up to a 30 mg l^{-1} mixture of the pesticides. In this study, *T.versicolor* R26 exhibited very high laccase activity, for example in the 30 mg l^{-1} treatment at –0.7MPa laccase activity of 680 units ml^{-1} was measured, although growing in a weak soil extract medium. *P.ostreatus* also produced very high levels of laccase, especially under osmotic stress and in the presence of pesticides. Previous studies suggest that a fungus showing laccase activity of 120 to 1000 units ml^{-1} in compost is a potential commercial source for laccase (Trejo-Hernandez *et al*., 2001). Thus, the levels of laccase produced by these test isolates may have some applications.

Under the treatment conditions used in this study *P.chrysosporium* and *T.versicolor* R101 (the tropical isolate) did not produce laccase, although previous studies with

other isolates of these species (Shim and Kawamoto, 2002) reported high activities of laccase by these fungi. The conditions of their assay were different as the fungus was grown in a bioreactor and the culture medium was enriched with veratryl alcohol, an inducer of laccase activity. Laccase is a copper-containing phenoloxidase involved in the degradation of lignin (Pointing, 2001), and its catabolic role in lignin biodegradation is not well understood (Eggen, 1999; Trejo-Hernandez *et al*., 2001) but this enzyme has already attracted considerable interest for biodegradation of xenobiotic compounds with lignin-like structures (Trejo-Hernandez *et al*., 2001).

Previously, it was assumed that degradation of xenobiotics by white rot fungi is mediated by enzymes involved in lignin degradation but Jackson *et al*. (1999) reported degradation of TNT by non-ligninolytic strains of *P.chrysosporium*. Other studies with *P.chrysosporium* in liquid culture have reported biotransformation of the insecticide lindane independently of the production of ligninolytic enzymes (Mougin *et al*., 1996), and Bending *et al.* (2002) showed >86% degradation of atrazine and terbuthylazine by white rot fungi in liquid culture. However, no relationship between the degradation rates and ligninolytic activity was found.

β-glucosidase and phosphomonoesterase are enzymes that carry out specific hydrolyses and were selected in these experiments because they catalyse reactions involved in the biogeochemical transformations of C and P and are likely to be an essential component of to assess substrate mineralization (Taylor *et al*., 2002). β -glucosidase hydrolise β-glucosides in soil or in decomposing plant residues (Hayano *et al*., 1985). The formation of orthophosphoric ions by decomposition and mineralization of the organic matter is accomplished by phosphoesterases, these enzymes can be intra or extracellular and can be effective even after cell death (Brohon *et al*., 1999).

The results show higher production of phosphomonoesterase by the *T.versicolor* R26 and *P.chrysosporium*, independently of pesticide concentration. *P.ostreatus* and *T.versicolor* R26 produced higher levels of this enzyme under osmotic stress. None of the pesticides used in this study contain phosphorous, thus this enzyme may not act directly on the pesticide mixture but might be involved in degradation metabolism. Phosphomonoesterases are associated with phosphorous cycle: they form an

important group of enzymes catalysing the hydrolysis of organic P esters to orthophosphates (Vuorinen and Saharinen, 1996).

Production of β-glucosidase by *P.chrysosporium* was strongly inhibited in the presence of the mixture of pesticides, whereas *T.versicolor* R26 showed stimulation in activity of this enzyme. *P.ostreatus* produced high levels of β-glucosidase regardless of water potential and pesticide treatment. As β-glucosidase is associated with the carbon cycle this result may suggest that *T.versicolor* R26 may have a better capacity for utilizing this mixture of pesticides as a source of carbon. β –glucosidase has been observed to be the most consistent parameter indicating the effects of different treatments among the several C cycle enzymes tested (Bandick and Dick, 1999).

Leiros *et al*. (1999) studied the effect of addition of Cu to soil on the activities of several enzymes, including β –glucosidase and phophomonoesterase. They described a sharp decrease in phosphomonoesterase activity with addition of Cu. While β –glucosidase activity showed a tendency to increase with increasing Cu concentrations.

Proteases are rate-limiting enzymes in nitrogen mineralisation processes (Horra *et al*., 2003). In this study proteases were produced at both osmotic stress levels and the range of pesticide concentrations used. These have been monitored previously in relation to soil quality status but not in relation to bioremediation aspects. They could play an important role in enhancing degradation of mixtures of xenobiotic compounds in soil systems. Staszczak *et al.* (2000) suggested that both intracellular and extracellular proteases are involved in the regulation of ligninolytic activities in cultures of *T.versicolor* under nutrient limitation. It is not clear if there is a relationship between ligninolytic activity and protease secretion in white rot fungi (Staszczak *et al.* 2000). Baran *et al.*, (2004) found high protease activity in soil contaminated with PAH, however in the present study it was not possible to find a correlation between pesticide concentration and protease activity.

In summary, the results on soil extract broth suggested that *T.versicolor* R26 and R101, *P.ostreatus* and *P.chrysosporium* have the capacity to degrade different groups of pesticides, supported by the ability for expression of a range of extracellular enzymes regardless of imposed osmotic stress at -2.8 MPa. *P.chrysosporium* and

T.versicolor R101 were also able to degrade this mixture of pesticides independently of laccase activity.

This study in liquid medium described the potential microbial utilisation of these pesticides in the environment. However, the adsorption of the pesticide molecules to soil particles might be an important factor in reducing the availability of the compounds for microbial attack. Bioavailability, defined as the ability of a compound to be freely transported across the cell membrane for intracellular metabolism or available for extracellular metabolism, is perhaps the most important factor limiting bioremediation (Atagana *et al.*, 2003). For these reasons, subsequent studies were conducted using soil microcosms, with the same pesticides to examine the impact on degradation by fungal inoculants pre-grown on a ligninocellulosic substrate.

4.4 Soil microcosms: an approach to bioremediation studies

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). However, the conditions in soil differ greatly from those in liquid culture, because soil is a multi-phasic, heterogeneous environment, in which the contaminant is present in association with the soil particles, dissolved in soil liquids and in the soil atmosphere (Boopathy, 2000). Additionally in sterile liquid culture factors such as competition with indigenous microflora, colonisation of the soil matrix or even survival are not considered. A study involving soil microcosms is a more complex approach, which requires some background information (usually from liquid culture studies) about the ideal conditions of growth for the microorganisms and their behaviour towards the pesticide. 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). This type of studies can provide valuable information to properly develop decontamination procedures to apply *in situ*.

In the current study, the physiological response of three fungal inoculants and soil microorganisms to a mixture of pesticides and water potential was evaluated through the analysis of various parameters as respiration, pesticide degradation and enzyme activities. Interestingly, most reported research on pesticide degradation in soil has been concerned only with single pesticides, however in soil-containing disposal systems, pesticides are more commonly found in complex mixtures (Shoen and Winterlin, 1987).

4.4.1 Fungal growth in soil microcosms

White rot fungi naturally grow into wood fibres secreting ligninolytic enzymes, which depolymerises the lignin (Meysami and Baheri, 2003) but they do not show any growth on contaminated soil, unless they had been pre-grown on woody materials or bulking agents for at least 10 days to establish complete growth and enzyme activity (Meysami and Baheri, 2003). These woody materials can be straw, woodchips or sawdust (Harvey and Thurston, 2001).

Soil environmental conditions such as pH, nutrient and oxygen levels may not be optimal for fungal growth or for activity of the fungal extracellular enzymes involved in pollutant transformation (Singleton, 2001). Furthermore, optimal performance of white rot fungal mycelium into soil depends especially on its survival, colonisation of the soil matrix and relation to the autochthonous soil microflora (Sasek, 2003). Once favourable conditions are established the potential for colonisation by fungi are impressive, since the domain of mycelial cords is enormous: with the ability to penetrate soils forming a network that can occupy many square meters (Pletsch *et al*., 1999).

In the present study the test isolates were grown on wood chips for 30 days prior to inoculation in soil. Very extensive colonisation of the wood chips was observed, as this woody substrate represents a source of nutrients for the fungal inoculant, but also for the indigenous soil microflora. Under the conditions used in the present study all three test isolates were viable in natural soil, overcoming the competition with the native soil microflora, under both water regimes (-0.7 and -2.8 MPa). *T.versicolor* and *P.ostreatus* showed very good colonisation in the soil microcosms, whereas *P.chrysosporium* showed relatively less growth.

In the present study a ratio 5 g inoculant: 100 g soil was used. Other authors used different ratios. For example Novotny *et al*. (2003), described dye degradation in soil, using microcosms comprised of 8 g soil mixed with 8 g of straw grown *Irpex lacteus* (a white rot fungus). Canet, *et al.* (2001) used 10 g inoculated straw in 25 g soil. Ryan and Bumpus (1989) used a ratio 1 g straw to 4 g soil; Elyassi (1997) used 1 g straw: 10 g soil and Morgan *et al.* (1993) used ground corn cobs to soil at 4 g ground corn cobs: 1 g soil.

An important point when optimising the conditions for bioremediation in soil microcosms is to predict the transfer of the technology to the field. The inoculation of fungi in soil must be a robust and cheap method, to be effective on a large-scale (Singleton, 2001). Clearly, consideration must be given to amendment cost and the space available for remediation on site (Singleton, 2001). Avoiding the use of very large amounts of ligninocellulosic substrates is important, because that would have adverse effects on the treatments economics (Boyle, 1995).

Novotny *et al.* (1999) in a study on removal of PAHs from sterile soil using the same species as those used in the present study, found the colonisation rates and mycelium density values decreased in the following order: *P.ostreatus*> *P.chrysosporium*>*T.versicolor*. However, studies like this, on sterile soil may not give a give a good estimate of the activity of the fungi in soil. Because natural soil contains a rich natural microflora, in a bioremediation system fungal inoculants face a strong competition. Supporting this, Lang *et al.* (1998) observed very pronounced differences in *Dichomitus squalens* enzyme activity between sterile and non-sterile soil. Previous studies reported isolation of microbes antagonistic to *P.chrysosporium* from soil (Ali and Wainwright, 1994; Radtke *et al.*, 1994). Interestingly *P.ostreatus* (one of the species used in the current study) showed only a slight reduction in enzyme activity between sterile and natural soil (Lang *et al.*, 1998). These findings suggest that this species has great potential to colonize soil, and is a strong competitor against the soil natural microflora.

Probably, in soil, microorganisms with a life strategy of fast growth will be favoured; however, the fast growers are not necessarily the organisms capable of degrading xenobiotics (Hestbjerg *et al.*, 2003). Moreover, an increase in white rot fungal biomass will not necessarily mean that this biomass is in an appropriate physiological

state (Boyle, 1995) to promote degradation of contaminants. The direct objective measurement of fungal growth in soil is difficult since the hyphae stick to the solid substrate (Novotny *et al.*, 1999), therefore the quantification of fungal colonisation must examine different parameters on metabolic activity and enzyme activity.

4.4.2 Pesticide degradation in soil microcosms

Pesticide degradation was estimated by quantifying the pesticide concentration remaining in soil after incubation, at 15 ºC, under two different water regimes -0.7 and -2.8 MPa (which corresponded to 5 and 10 % moisture content, respectively).

The results showed the treatments with fungal inoculants had higher pesticide degradation rates than the control, and the best inoculants were *P.chrysosporium* and *T.versicolor* R26. In soil amended with wood chips improved degradation rates, comparing with the control were found for some treatments. Soil inoculated with *T.versicolor* R26 showed good degradation rates for the three pesticides. The maximum increase in pesticide degradation was 46% for simazine, 57% for trifluralin and 51% for dieldrin compared to the control. Soil inoculated with *P.ostreatus* also showed significant improvement in degradation of simazine and trifluralin (100 and 60% higher than the control) however in soil inoculated with this isolate the remaining concentrations of dieldrin were not significantly different of those in the control soil. Interestingly this isolate showed low tolerance to dieldrin in the laccase Petri plate assay. In soil inoculated with *P.chrysosporium* simazine, trifluralin and dieldrin degradation rates were 58, 74, and 70% higher than in the control soil. In soil amended with SMC the degradation rates were also higher than in the control (17, 49 and 76% for simazine, trifluralin and dieldrin), however not as high as in soil inoculated with *T.versicolor* and *P.chrysosporium*.

In summary higher degradation was observed in the treatments inoculated with the test isolates, however the results after 24 weeks incubation showed some discrepancies, with some of the treatments having higher concentrations of pesticide than those measured after 12 weeks incubation. Because, soil is a heterogeneous environment and a destructive sampling system was used variation between replicates can occur. Boyle (1995) found bioremediation results to be variable, which may be due to the fungi not always growing well or not always expressing their degradative

system in the soil. Furthermore, the sorption of pesticide molecules to soil particles may reduce the availability of the compound for microbial attack (Atagana *et al.*, 2003).

Perhaps the most intuitively direct means of determining the potential of fungal inoculants to degrade a pesticide is to measure its levels before and after treatment, in the microcosm. However, due to the heterogeneous (physical, chemical and biological) nature of soil it is likely that a variety of microbial (aerobic and anaerobic) and chemical transformations will occur during bioremediation leading to the possible formation of many breakdown products (McGrath and Singleton, 2000). Chemical analysis of such mixtures is time consuming and also gives no indication of the possible synergetic toxic effects that may occur (McGrath and Singleton, 2000). Therefore, it appears that methods which give an indication of residual soil toxicity after bioremediation will supplement more traditional chemical analysis, and give a good estimate of the success of the reclamation process (McGrath and Singleton, 2000).

4.4.3 Respiratory activity

Higher carbon dioxide levels indicate higher respiration rates, suggesting high mineralization rates, supporting the biodegradation of pesticides by native or introduced microorganisms. In this study, maximum respiration occurred by week 6, and decreased by the end of the experiment. Soil inoculated with *T.versicolor* R26 showed the highest respiratory activity. Overall, higher respiratory activity was observed in the presence of the mixture of pesticides.

Soil inoculated with *T.versicolor* R26 showed improved respiration rates in all the treatments with CO_2 concentrations up to 1.2, 54 and 11 times higher than in natural soil, for the treatments 0, 5 and 10 mg kg $soil^{-1}$, respectively. In soil inoculated with *P.ostreatus* this increase was up to 30, 6 and 5 times compared to natural soil (for the treatment 0, 5 and 10 mg kg $soil^{-1}$ respectively). But the highest augment was observed in soil inoculated with *P.chrysosporium* with CO_2 production up to 29, 19 and 14 times higher than in natural soil to in the treatments 0, 5 and 10 mg kg $soil^{-1}$.

In this study soil amended with wood chips had a higher respiratory activity than natural soil (with CO_2 concentrations up to 17 times higher than in the control soil), even though no microbial inoculant was present. This increase was probably due to the colonisation of the wood chips by native soil microrganisms. Another reason for this increase could be the improved aeration caused by the wood chips, which may have increased the metabolic activity of the aerobic microrganisms native in soil. Similar increases in CO_2 production were found with other organic amendments such as alfalfa and bran (Boyle, 1995).

The decrease in carbon dioxide production towards the end of the treatment was possibly caused by the exhaustion of the readily degradable organic fraction. (Balba *et al.*, 1998).

In the current study, water availability did have an effect on respiration rates, but this effect varied with different inoculants. Soil moisture can limit soil respiration by limiting microbial contact with available substrate or by causing dormancy and/or death to soil microorganisms at low soil water potentials (Orchard and Cook, 1983). On the other hand, if soil is too wet the activity of aerobic soil microrganisms can be compromised, because of aneorobiosis. In fact, none of the water regimes used in the current study was too dry or too wet to impair soil microbial activity. Some studies have looked at the effect of water potential on respiratory activity. For example: Conant *et al*. (2004) determined the effect of temperature and water potential on respiration rates and reported higher rates in wetter soils (-0.03 and -0.05 MPa) than the drier (-1.0 and -1.5 MPa). They also found that the optimum moisture content for growth and enzyme activity was between 30-50% (w/w of dry soil), although the effect of the freely water available was not determined (Meysami and Baheri, 2003). Balba *et al.* (1998) reported higher levels of CO_2 when the dry weight was around 910 g kg^{-1} which was equivalent to about 9% moisture content.

The analysis of the response of respiratory activity showed a marked difference between natural soil and soil supplemented with a mixture of pesticides: respiratory activity was higher in the contaminated soil under both water regimes. In previous studies, the addition of contaminants did have an effect on the respiratory activity. Bundy *et al.* (2002) reported that the addition of diesel caused a sharp increase in respiration (against respiration rates of 100 nmol CO_2 $g^{-1}h^{-1}$ in control soils).

Soil respiration measurements are used frequently as a sensitive and easily analysable microbial parameter for the characterization of soil samples (Hollender *et al*., 2003). The respiration activity is closely related to other microbial parameters such as microbial biomass (Dilly, 2001). It gives an evaluation of the global microbial activity (Brohon *et al*., 1999). It is one of the most frequent parameters used to measure the organic decomposition rates in soil (Brohon *et al*., 1999), and its dependence on numerous biotic and abiotic factors makes it a specific tool for measuring the activity level of the soil microflora with time (Brohon *et al*., 1999).

The standard method to estimate soil respiration and mineralization of substrates is by measuring continuously or semi-continuously the production of CO_2 from small soil samples (≤100 g) treated with the test chemical, for a minimum of 30 days. The main disadvantage of soil respiration tests is that the activity of the total soil microflora is determined. When certain species are affected by the test chemical, this will often not be noticed, as other (less sensitive) species may take over the activity of the sensitive ones (Somerville and Greaves, 1987). For example Boyle (1985) reported that enzyme activities decreased sharply at higher and lower moisture contents. CO_2 production also decreased in dry soil but remained high when soil was wet even though enzyme activities decreased. This suggests that it is likely that organisms other than white rot fungi were responsible for the production of CO_2

4.4.4 Enzyme activities in soil microcosms

Enzyme activities are involved in processes important to soil function, such as organic matter decomposition and synthesis, nutrient cycling and decomposition of xenobiotics (Acosta-Martinez *et al*., 2003). In the current study dehydrogenase, total ligninolytic activity, cellulase and laccase activities were assessed, in order to study the response of fungal inoculants to a mixture of pesticides in soil. This group of enzymes should represent the responses of a diverse microbial assemblage (fungal inoculants and native soil flora) to a wide range of substrate types and more importantly to the contaminating pesticides. For biodegradation to take place the fungal inoculants introduced in the soil and/or the native soil microflora must be able to produce degradative enzymes, that remain active in the contaminated soil.

Dehydrogenase

Overall, the addition of pesticide increased dehydrogenase activity in most treatments. For example in soil inoculated with *T.versicolor* the dehydrogenase activity in the treatments 5 and 10 mg kg soil^{-1} mixture, was 80 and 100 % higher than in natural soil, after 6 weeks, at -2.8 MPa.

In the control soil the addition of pesticides enhanced dehydrogenase activity with higher levels being produced at -0.7 MPa. Soil amended with wood chips produced the highest dehydrogenase levels at the beginning and after 6 weeks incubation, with increased enzyme production in the presence of the pesticide mixture.

The results showed that in soil inoculated with *T.versicolor* the presence of pesticides enhanced dehydrogenase activity, after 6 and 12 weeks incubation under both water regimes. Higher production of dehydrogenase was observed at -0.7 MPa. In soil inoculated with *P.ostreatus* after 6 weeks incubation the highest dehydrogenase activities were detected under water stress, in the presence of the pesticide mixture. When *P.chrysoporium* was used as a fungal inoculant, the levels of dehydrogenase decreased with the incubation period. Similarly, to what was described for soil inoculated with *T.versicolor*, in this treatment dehydrogenase activity was increased in the presence of the pesticide mixture, which suggests higher metabolic activity in the presence of the pesticides.

Regarding the effect of water potential on dehydrogenase activity, it was difficult to establish a pattern on the effect of this parameter on the enzyme activity. Previous studies described an increase in soil dehydrogenase activity with increasing water content (Quilchano and Maranon, 2002)

Previous studies have also correlated pesticide degradation with dehydrogenase activity. For example Min *et al.* (2001) reported that increasing concentrations of butachlor in soil enhanced the activity of dehydrogenase with the highest activity on the 16th day after application of 22 mg kg soil^{-1} of butachlor. Baran *et al.* (2004) reported high dehydrogenase activity in soil contaminated with PAH. Previously, Felsot and Dzantor (1995) described the effect of alachlor and organic amendment on soil dehydrogenase activity and on pesticide degradation rates. Alachlor initially inhibited soil dehydrogenase in soil. Amendment of soil with corn-meal caused faster

degradation of alachlor. At very high concentrations of alachlor (750 mg kg soil^{-1}) dehydrogenase activities in amended soils surpassed levels in corresponding no-pesticide controls after 21d, with coincident alachlor degradation >50% during the same period. They suggested that stimulation of microbial activity by addition of organic amendments may enhance co-metabolism of high concentrations of pesticides in soil.

Interestingly McGrath and Singleton (2000) reported a completely different effect of pollutants on dehydrogenase activity. They assessed PCP transformation in soil, and observed that after 6 weeks remediation, soil PCP levels had decreased from an initial 250 to 2 mg kg^{-1}. However, soil dehydrogenase activity remained very low in all soils containing PCP and did not recover throughout the experiment (6 weeks) despite the decrease in PCP levels. They suggested either toxic PCP transformation products were formed or that soil microbes had not fully recovered from initial toxic responses towards PCP. They also reported that soil inoculation with *P.chrysosporium* did not improve PCP remediation over uninoculated soil.

In the current study soil amended with wood chips had higher dehydrogenase activity than the control soil. Previous studies by Moorman *et al.* (2001) reported that the addition of 0.5% sawdust stimulated dehydrogenase activity. Incorporation of corn-meal and sewage sludge into designated plots of soils contaminated with herbicides (alachlor, atrazine, metochlor and atrazine) during 100 days showed that dehydrogenase activities were highest in organic-material amended plots (Dzantor *et al*. 1993). Interestingly the levels of trifluralin had declined by 70-80% in corn-meal amended plots and by 60-75% in unamended plots.

Dehydrogenation is considered to be caused by a large group of endocellular enzymes which transfer H^+ and electrons from a substrate to an appropriate acceptor during the initial stage of organic compound oxidation. The electrons are transferred via a transporter chain to the final acceptor, oxygen, to form water. The principle of the method to quantify dehydrogenase activity is the electron acceptor is replaced by an oxido-reduction indicator (INT), that in the reduced state is transformed into formazan (INF) a red compound, insoluble in water but soluble in organic solvents (Brohon *et al*., 1999). The activity of dehydrogenase is considered an indicator of the oxidative metabolism in soils and thus of the microbiological activity because being exclusively

intracellular it is linked to viable cells (Quilchano and Maranon, 2002). Even though dehydrogenase reflects a broad range of microbial oxidative activities, and it does not always consistently correlate to microbial numbers, CO_2 evolution or O_2-consumption. Additionally, dehydrogenase activity may depend upon the nature and concentration of amended C-substrates and alternative electron acceptors (Somerville and Greaves, 1987). Since the activity of this enzyme depends on the total metabolic activities of soil microorganisms the concentrations in different soils do not always reflect the number of viable microorganisms isolated on a particular medium (Page *et al.*, 1982). Rossell and Tarradellas (1991) concluded that short-term (substrate-induced) dehydrogenase activity may reflect the impact of chemicals on the physiologically active biomass of the soil microflora.

Enzymes involved in wood degradation

The degradative capacity of white rot fungi is assumed to result from the activity of non-specific free-radical based mechanisms of ligninolytic enzymes, lignin peroxidases, manganese peroxidases and laccases (Nerud *et al*., 2003). The lignin degradation system is suitable for the elimination of not only lignin from wood but also various so-called lignin related pollutants (Mester and Tien, 2000). When white rot fungi colonise contaminated soil, lignin degrading enzymes are released into the extracellular medium, degrading large molecules that would otherwise be unable to cross cell walls (Canet *et al*, 2001). Such metabolism has the great advantage that the fungi avoid the uptake of potentially toxic substances. Furthermore, because of the non-specific action of the enzymes involved, preconditioning of the fungi to individual pesticides is not necessary. And, as the induction of the extracellular enzymes system is independent of the presence of contaminants, the fungi can degrade contaminants at extremely low concentrations (Canet *et al.*, 2001).

In this study the total ligninolytic activity in soil was assessed as well as laccase and cellulase activities.

Total ligninolytic activity

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 in soil 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 6 weeks incubation, in soil inoculated with *T.versicolor* and in soil amended with SMC. After 12 weeks incubation the highest decolouration rates were observed in soil amended with SMC. In both cases the decolouration rates were unaffected by pesticide treatment. The results suggest the fungal inoculants were tolerant to this mixture of pesticides, producing equivalent levels of decolouration in the presence and absence of the xenobiotics. Interestingly, water potential had an impact on the decolouration percentage in soil amended with wood chips, with higher decolouration at -2.8 MPa. In this water regime the decolouration rates were significantly higher in the pesticide treatments compared with the control (0 mg kg soil^{-1}).

There was no correlation between degradation 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). In contrast, Alcalde *et al.* (2002) observed that decolouration of Poly R-478 was correlated to the oxidation of PAHs mediated by laccases.

Laccase

In the current study the presence of pesticides did not impair laccase production. In natural soil the production of laccase was insignificant, whereas soil amended with wood chips showed some laccase production in some of the treatments especially after an incubation period ≥ 12 weeks. The enzyme may have been produced by native soil wood degrading fungi that in the meantime might have colonised the wood chips. The highest level of activity for soil amended with wood chips was observed in the 10 mg kg soil^{-1} treatment, under water stress (22 U g soil $^{-1}$).

Soil inoculated with *T.versicolor* had the highest laccase activity, after 6 weeks incubation, in the 5 and 10 mg kg $soil^{-1}$ treatments, under both water regimes, although after 24 weeks incubation laccase levels decreased in all treatments. Water availability did not affect laccase production in the soil by this isolate.

In soil inoculated with *P.ostreatus* lower levels of laccase were produced and this was reduced in the pesticide treatments. *P.chrysosporium* produced very low concentrations of laccase and only occurred in the pesticide treatments. The enzyme may have been produced by the native soil microflora since *P.chrysosporium* did not produce laccase under sterile conditions in soil extract broth or in the Petri plate assay.

A previous study also showed that *P.chrysosporium* did not produce laccase in soil (Novotny *et al.*, 1999), and that high laccase production by *T.versicolor* in soil occurs after 30 days incubation (Leonowicz and Bollga, 1987). In contrast, Novotny *et al*. (1999) reported this species produced very low concentrations of laccase in soil.

Like in the current study, Boyle (1995) found that laccase was only detected when white rot fungi were known to be present. This author suggested that the laccase assay could be used to measure white rot fungi colonisation of non-sterile soil, in contrast with CO_2 production which reflected the activity of the total soil microbial populations (Boyle, 1995).

Interestingly, previous studies showed a different pattern regarding the effect of pesticide on laccase activity. For example Sannino *et al.* (1999) showed that *Cerrena unicolor* did not produce laccase in the presence of simazine 0.5-7 mgl^{-1}. These authors showed that 2,4-DCP transformation by laccase is repressed when simazine is present in the reaction mixture. This was not observed in the current study, in fact *T.versicolor* R26 produced the highest levels of laccase in the presence of the pesticide mixture.

Few studies have considered the implications of water stress on the enzymatic activity. Boyle (1995) found that *T.versicolor* did not produce laccase in soil at -3.4MPa but high activities were detected in soils at -0.9MPa and -0.4MPa water potential.

Cellulase

The highest production of cellulase was found in soil inoculated with *P.ostreatus* and *P.chrysosporium* at -2.8 MPa, whereas the lowest production was found in natural soil, and soil amended with SMC. Soil amended with wood chips showed high levels of cellulase in some of the treatments (only in 5 mg kg soil^{-1}-6 weeks; 10 mg kg soil^{-1}-12 weeks). This enzyme could have been produced by native cellulolytic soil microorganisms attacking the wood chips. *T.versicolor* produced less cellulase than the other two isolates tested.

4.4.5 Microbial communities

Overall the results showed few significant differences in total viable microbial populations between treatments. This has also been reported in a previous study (Katayama *et al*., 2001). They examined the effect of 5 pesticides, individually, on the structure of microbial communities and found that simazine did not significantly affect the microbial biomass in soil.

However, other studies showed significant alterations in microbial populations in contaminated soil. For example: Min *et al.* (2001) reported that the number of actinomycetes declined significantly after the application of butachlor at different concentrations from 5.5 to 22 mg kg soil^{-1}, while that of true bacteria and fungi increased. Interestingly Ahmed *et al.* (1998) reported that very low concentrations of chlorinated hydrocarbon pesticides detected in top soil (0.5 μg kg soil^{-1}) caused a sharp decrease in nitrifying bacteria and a significant depression in soil heterotrophic bacteria and fungi.

The microbial population of a site contaminated with pesticides may be eliminated, significantly reduced or altered; but alternatively, microbes may adapt to the presence of toxic compounds and can survive by degrading them (Jonhston and Camper, 1991) as some microrganisms can utilise pesticides as a nutrient source.

As soil microrganisms are not equal resistant to xenobiotics, some of them are very sensitive and do not grow when toxic compounds are present in high concentrations or constitute a low carbon and energy source, while other are able to adapt (Guirard *et al.*, 2003) and grow well. For this reason the microbial communities within

contaminated ecosystems tend to be dominated by those organisms capable of utilising and/or surviving toxic contamination. As a result, these communities are typically less diverse than those in non-stressed systems. This diversity may be influenced by the complexity of chemical mixtures present and the length of time the populations have been exposed (Macnaughton *et al*., 1999). Since the quantification of the colony-forming units on agar plates does not give information on the bacterial diversity, similar bacterial densities do not necessarily mean the microbial communities are similar.

Furthermore, the quantification of the colony-forming units on agar plates has been criticized (Harris and Steer, 2003) because only 0.1 to 1% of the cells present in an environmental sample can actually be cultivated on nutrient media. Little is known about the remaining non-culturable portion of the microbial community. For example, fastidious microorganisms do not form colonies. On the other hand, germination of resting stages (i.e. spores) and fragmentation of fungal hyphae with each fragment producing a separate colony can lead to overestimations of the numbers of some microbial groups.

For these reasons, there is a growing interest in the development of biological and biochemical methods for the assessment of microbial structure in soil (Vepsalainen *et al*., 2001) as changes in the profiles of soil microbial communities could have potential use as early indicators of the impact of management or other perturbations on soil functioning and soil quality. Methods such as polar lipid fatty acid analyses (PLFA), based on the analysis of polar lipids present in the biological membranes yield a direct quantitative method of the biomass as well as a profile of the microbial community structure (Vestal and White, 1989), would be interesting to use in study of microbial populations during and after bioremediation.

4.4.6 Effect of spent mushroom compost in the microcosms

Overall soil microbial activity and pesticide degradation was enhanced in soil amended with SMC. For example, the degradation of pesticides after 12 weeks was 17, 49 and 76% (for simazine, trifluralin and dieldrin) higher in soil amended with SMC than in the control. An increase in CO_2 levels was found at 24 weeks in the 10 mg kg soil^{-1} treatment, and at 6 weeks in the treatment without pesticide (-2.8 MPa).

The high concentration of CO_2 towards the end of the experiment at 10 mg kg soil^{-1} (3x control soil), and after 6 weeks in the control treatment (20x control soil) could be related to the development of fungal mycelium from the SMC.

Interestingly this treatment showed higher dehydrogenase activity in the presence of the pesticide mixture (at -0.7 and -2.8 MPa) and after 12 weeks incubation (at -2.8 MPa), which suggested an increased metabolic activity in the presence of the pesticides. The total ligninolytic activity was very high in soil amended with spent mushroom compost. At -0.7 MPa after 12 weeks in the 10 mg kg soil^{-1} treatment the decolouration was close to 100%. Cellulase production varied throughout the experiment, with higher activities after 0 and 24 weeks incubation. Possibly, initially the cellulase detected was from the SMC, which is naturally rich in enzymes whereas at a later stage this activity could be linked with fungi that meanwhile may have developed, possibly from the SMC.

The increase in soil activity observed when SMC was used as a soil amendment can be linked to the microorganism and extracellular enzymes abundant in this substrate (Singh *et al.*, 2003). Furthermore, the addition of SMC increased the organic matter content in the soil microcosms and this may have been the reason for the improved soil activity observed. High organic matter content is typically associated with higher microbial numbers and a great diversity of microbial populations, as it serves as a storehouse of carbon and energy as well as a source of other macronutrients such as nitrogen, phosphorous and sulphur (Boopathy, 2000). It has been shown in previous investigations that the increase in soil organic matter enhances enzyme activities (Dick *et al*., 1988; Martens *et al*., 1992; Kandeler and Eder, 1993; Klose *et al*., 1999; Pascual *et al*., 1999). Liang *et al.* (2003) reported a significant increase in soil alkaline phosphatase and soil respiration rates in soils that were supplemented with organic manure. Other example of enhanced soil activity with the addition of organic matter was described by Moorman *et al.* (2001), showed that the addition of manure to soil contaminated with a mixture of trifluralin, atrazine and metolachlor significantly increased bacterial populations, dehydrogenase activity and respiration rates.

Besides the positive effect that SMC showed when used as a soil amendment Buswell (1994) proposed the use of this agro-waste as a source of fungal inoculum, when

using fungi for bioremediation, in cases where the availability of fungal inoculum is of practical concern (Buswell, 1994).

4.4.7 Correlation between different parameters

Some correlations were also calculated from the results, at 6 weeks incubation, when the different biochemical, enzymological parameters suggested higher metabolic activity, and a correlation between CO_2 content and dehydrogenase activity was confirmed in this study for some of the treatments. High CO_2 content was obtained in treatments with high dehydrogenase activity. The same correlation was reported in a recent stud by Garcia *et al*., (2004). Since dehydrogenase is present in all microorganisms, dehydrogenase assays are considered to be an accurate measure of microbial oxidative activity in the soil (Taylor *et al.*, 2002). As the enzymes involved in the dehydrogenase assay are mainly intracellular and related to the phosphorylation processes (Garcia *et al*., 2004), an increase in these processes leads to an increase in CO_2 production, a by-product of the phosphorilative process, which was confirmed in the current study. A correlation was also found between soluble protein content and total microbial populations for some treatments.

4.5 Differences between studies: soil extract broth and soil microcosms

Although the white rot fungi used in the soil extract broth study were exceptionally efficient degrading the mixture of pesticides in soil extract broth, in the soil microcosms the biodegradation rates were not has high. Several factors might have contributed to these differences. For example, the temperature was higher than in the soil extract broth assays. The rate of pesticide transformation can be doubled or even tripled when temperature increases by $10^{o}C$ (Gan and Koskinen, 1998). Other factors could have been competition from native bacterial and fungal populations in soil microcosms, as the soil extract broth was sterile which allowed the test isolates to grow without the limitations encountered in soil. Additionally, because white rot fungi are not native to soil some autochthonous bacteria and fungi may become predominant over the growth of fungal inoculants. The chemical sorption of the pesticide to soil particles, could have been another factor influencing the degradation rates in soil, by reducing the proportion of pesticide bioavailable for degradation.

Only occasionally have direct comparisons been made between liquid medium and soil experiments. 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. Ryan and Bumpus (1989) reported higher 2,4,5-trichlorophenoxyacteic acid mineralisation rates by *P.chrysosporium* (62%) in liquid culture after 30 days, compared to that in soil (30%).

Regarding enzyme production there were also some differences between soil extract broth and soil microcosms that are summarized in Table 4.1. Interestingly, the main differences concern the effect of water availability on laccase and cellulase activities. Generally, enzyme activities were higher at -2.8 MPa in soil, whereas in soil extract these were higher at -0.7 MPa. The comparison between biomass production, expressed as dry weight in the soil extract experiment and dehydrogenase activity in soil microcosms, the results were fairly consistent in regard of water availability, however whereas in soil the biomass increased with the concentration of the pesticide mixture, in soil extract this was only observed for the *T.versicolor* inoculant.

Table 4.1 Comparison between soil extract broth and soil microcosms for laccase, cellulase and biomass production (dry weight for soil extract broth and dehydrogenase activity for soil microcosms). T.v.= *T.versicolor* (R26), P.o.= *P.ostreatus* and P.c.= *P.chrysosporium*.

Parameter	Fungi	Soil extract broth	Soil microcosms
Laccase	*T.v.*	Higher production in presence of pesticide mixture, higher production at -0.7MPa	Higher production in presence of pesticide mixture, higher production at -2.8MPa
	P.o.	Higher production in presence of pesticide mixture, higher production at -2.8MPa	Decrease in presence of pesticide mixture, higher production at -2.8MPa
	P.c.	Not detected	Detected at very low levels at 10 mg kg soil^{-1} under both water regimes
Cellulase	*T.v.*	Higher at -0.7 MPa	Higher at -2.8 MPa
	P.o.	Higher in control, higher at -0.7 MPa	Higher at -2.8 MPa
	P.c.	Higher at lower mixture concentration, higher at -0.7 MPa	Higher at -2.8 MPa
Biomass	*T.v.*	Higher at -2.8 MPa, increase in presence of mixture	Higher at -2.8 MPa, increase in presence of mixture
	P.o.	Higher at -2.8 MPa (higher in presence of mixture at this Ψ)	Higher at -2.8 MPa, increase in presence of mixture
	P.c.	Higher at -2.8 MPa (higher in control for this Ψ)	Higher at -2.8 MPa, increase in presence of mixture

4.6 Microbiological and biochemical parameters sensitive to pesticide contamination

Successful bioremediation is dependent on an interdisciplinary approach involving such disciplines as microbiology, engineering, ecology, geology and chemistry (Boopathy, 2000). To evaluate the outcome of bioremediation it is critical to assess some microbiological and biochemical parameters all giving information on soil quality. It is difficult to choose which parameters are more reliable, as the relationship between an individual biochemical property and the total microbial activity is not always clear, in complex systems like soils where the microorganisms and processes involved in the degradation of organic compounds are highly diverse (Nannipieri *et al.,* 1990).

In the current study the comparison of some of the microbiological parameters between soil artificially contaminated with a pesticide mixture and control soil showed some interesting findings: dehydrogenase, cellulase activities and total number of bacteria were higher in contaminated soil (at 5 and 10 mg kg $soil^{-1}$), respiratory activity was also increased in the presence of 10 mg kg $soil^{-1}$ of a pesticide mixture. These results suggested an increase in metabolic activity in contaminated soil. This was also described by Ayoama and Itaya (1995) in slightly polluted soil, where conditions gradually deteriorated due to the presence of contaminants with the microorganisms increasing their metabolic rate to the detriment of biosynthesis.

The results are also confirmed by a more recent study by Filip (2002) examining the relative sensitivity of selected microbiological and biochemical parameters for the assessment of soil quality based on long–term soil analyses from 49 differently anthropogenically-affected European soil. This project involved scientists from the Czech Republic, Hungary, Russia, Slovakia and Germany that looked at samples from different sites in Europe. After evaluation of more than 20 individual parameters they concluded that N_2-fixing bacteria, total microbial biomass, soil respiration and dehydrogenase activity could serve as sensitive indicators of soil quality (a summary table of the results is shown in Table 4.2).

Similarly Visser and Parkinsson (1992) suggested that the biological and biochemical properties that are the most useful for detecting the deterioration of soil quality are

those that are more closely related to nutrient cycles, including soil respiration, microbial biomass, nitrogen mineralization capacity and the activity of soil enzymes.

Despite many studies on the use of enzymes as sensors to monitor the effects of pesticides on soil microflora, some authors are rather sceptical in relation to this approach. For example Somerville and Greaves (1987) stated that soil enzyme activities would be of little value to monitor side effects of pesticides on microflora. These authors claim that there is no universally agreed methodology, and almost any result can be achieved by varying assay conditions (temperature, pH, substrate). And even though tests on enzyme activity have been described by many authors, little data is available to judge the reproducibility of these methods. Also soil animals, such as collembola and isopods, significantly influence the activity of several enzymes, such as urease (Verhoef and Brussard, 1990), dehydrogenase, and cellulase (Teuben and Roelofsma, 1990). Therefore, discriminating between direct and indirect effects of the tested chemicals on microorganisms is difficult.

Table 4.2 Relative sensitivity of the selected microbiological and biochemical parameters for the assessment of soil quality based on long –term soil analyses from 49 differently anthropogenically-affected European soil (From Zilip, 2002).

Parameter	Relative sensitivity*
Microbial biomass	+ /++
Respiration (CO_2) release	+++
Dehydrogenase activity	+++/ ++++

*Sensitivity (relative to a control soil): (+) low; (++++) maximum

5 Conclusions and Further Work

5.1 Conclusions

The results obtained in this study provide valuable knowledge on the abilities of *T.versicolor*, *P.ostreatus* and *P.chrysosporium* to colonise soil and might serve as a sound basis for the further exploitation of these species as fungal inoculants in biological remediation processes.

The main findings are summarised below:

1. Screening in soil extract agar (a nutritionally poor medium), on the tolerance of eight white rot fungi to simazine, trifluralin and dieldrin (listed in the "UK Red List of Toxic Substances), individually and in a mixture, under two different water regimes suggested best tolerance by *T.versicolor* R26 and R101 and *P.ostreatus*.

2. When the effect of water stress was examined changing the water availability by modifying osmotic and matric potential all test isolates showed better tolerance to osmotic imposed stress.

3. In agar-based studies *T.versicolor* R26 and R101 and *P.ostreatus* were able to degrade lignin and produce laccase, in the presence of the pesticides, individually and as a mixture, under two different water regimes. In contrast *P.chrysosporium* did not produce laccase or degraded lignin under the conditions of the assay.

4. Because of the technical limitations in examining enzyme production and pesticide degradation in soil extract agar, a liquid culture study was conducted with soil extract liquid broth.

5. All four test isolates were able to grow in soil extract broth and degraded mixture of pesticides: complete degradation of dieldrin and trifluralin was observed, while about 80% of the simazine was degraded regardless of osmotic stress treatment, after 25 days.

6. The assessment of the toxicity (Toxalert®10) in the soil extract after 25 days incubation with the test isolates, suggested the final product was innocuous

7. Expression of a range of extracellular enzymes in soil extract broth at both -0.7 and -2.8 MPa was observed: *P.ostreatus* and *T.versicolor* R26 showed high production of laccase whereas *P.chrysosporium* and *T.versicolor* R101 did not produce this enzyme.

8. The capacity of tolerance and degradation of mixtures of pesticides and production of a range of enzymes in soil extract even under osmotic stress, suggested potential bioremediation applications of theses isolates in soil, which was tested subsequently in soil microcosms

9. *T.versicolor* (R26), *P.ostreatus* and *P.chrysosporium* were able to colonise soil contaminated with a mixture of pesticides

10. Inoculation with the test isolates increased soil activity, expressed as an increase in respiratory, enzymatic activities and biodegradation rates when compared with the control:

11. Very high levels of laccase were produced in soil inoculated with *T.versicolor* R26, especially in the presence of the pesticide mixture

12. Soil inoculated with *T.versicolor* R26 showed improvement in degradation rates: 46, 57, and 51% for simazine, trifluralin and dieldrin, higher than in the control soil.

13. Soil inoculated with *P.chrysosporium* showed pesticide degradation rates: 58, 74, and 70% (for simazine, trifluralin and dieldrin) higher than in the control soil.

14. Soil amended with SMC also showed improved pesticide degradation: 17, 49 and 76% for simazine, trifluralin and dieldrin higher than in unamended soil

15. Soil inoculated with *T.versicolor* R26 showed improved respiration rates in all the treatments with concentrations of CO_2 up to 1.2, 54 and 11 times higher than in natural soil (for the treatments 0, 5 and 10 mg kg $soil^{-1}$, respectively)

16. In soil inoculated with *P.ostreatus* the respiration rates were up to 30, 6 and 5 times higher than in natural soil for the treatment 0, 5 and 10 mg kg $soil^{-1}$ respectively

17. The highest respiratory activity was observed in soil inoculated with *P.chrysosporium* up to 29, 19 and 14 times higher than in natural soil (for the treatments 0, 5 and 10 mg kg soil^{-1}).

5.2 Further Work

Possible further studies based on the findings of this study are:

- ✓ Study of the microbial populations structure

The current study has shown few differences on microbial populations, therefore a more detailed approach on the effect of this mixture of pesticides, before and after bioremediation, on soil microbial structure, would be very interesting. I would suggest the analysis of polar fatty acids, a method based on the analysis of polar lipids in the biological membranes, which yields a direct quantitative method of the biomass as well as a profile of the microbial community structure. Molecular techniques, that rely on the capture and amplification of sequences of interest could also be a good method to characterise the soil microbial communities in microcosm experiments.

- ✓ Analysis of metabolites by GC-MS

The current study has shown significant decreases in detectable pesticides in soil after incubation with fungal inoculants. However, these pesticides may have been completely degraded or mineralised; some of it may have been transformed in unknown metabolites. Additional studies on the toxicity of soil after remediation as well as a thorough analysis by GC-MS of the final products would be pertinent, as chemical degradation and microbial cometabolism may produce toxic intermediates.

- ✓ Field studies in a contaminated site

The reliability of microcosm studies in the laboratory to interpret field conditions is much debated. Microcosms differ from the field situation concerning the influence of temperature and moisture dynamics, the influence of root presence, and the composition of the soil biota community. The studies described in this thesis provide an estimate of the possible hazard imposed by this mixture of pesticides on

the soil microflora and the efficiency of the test isolates in the bioremediation of these chemicals. Although the results are very promising, under field conditions the outcome may be different. First, the exposure to the contaminants may be different since the chemicals are not distributed uniformly, and bioavailability will often vary due to various sorption processes. Second, by contrast, in the laboratory the test fungus is under controlled conditions, without secondary stresses, such as cold, drought or excessive rain, which possibly gave a better performance than in the field.

The uncertainties attached to the laboratory-to-field extrapolation can be avoided by conducting experiments under semi-field or field conditions. Therefore, I would suggest further studies to examine the feasibility of using these white-rot fungi at a larger scale, first in boxes (containing around 10 kg of soil) and later in a contaminated site.

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