CRANFIELD UNIVERSITY

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BIOTECHNOLOGY CENTRE

Ph.D. THESIS

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BIOREMEDIATION OF THE PESTICIDES DIELDRIN, SIMAZINE, TRIFLURALIN USING TROPICAL AND TEMPERATE WHITE-ROT FUNGI

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Dedicated to my daughter Sheeva.

ABSTRACT

The natural breakdown of three pesticides on the UK Red List, dieldrin, simazine and trifluralin in water and soil varied with environmental conditions. In both sterile and unsterile water trifluralin was degraded to some extent at 20 and 30°C. In contrast, dieldrin and simazine were stable over the 42 days incubation period. A gradient HPLC method was developed for the simultaneous quantification of the three pesticides in soil. In field capacity soil mixtures of the three pesticides (5 and 10 ppm) showed a similar stability with limited degradation at 20°C but increased rates of degradation at 30°C. At the higher concentration the pesticides naturally degraded at a slower rate. Simazine and trifluralin degradation was significantly enhanced with increasing temperature from 20 to 30°C. Water potential (field capacity, -0.065 MPa, and -0.28 MPa) had little effect on the natural breakdown rate of dieldrin. Simazine showed a greater breakdown in the mid-wetness soil, while trifluralin was degraded rapidly in the field capacity soil, but not at all in the driest treatment over the 70 day experimental period.

In vitro studies on solid agar media overlayed with cellophane showed that of four fungi examined, *Trametes cingulata, Trametes socotrana* (tropical species) and *Phanerochaete chrysosporium* and *Polystictus versicolor* (temperate species) all except *P.chrysosporium* were able to grow in the presence of 5 ppm of any of the three pesticides at 20 and 30°C, with the latter only growing at 30°C. At 10 ppm concentration *P. chrysosporium* did not grow, regardless of temperature or time of incubation (up to 56 days). HPLC was used to quantify the temporal rates of degradation in the solid agar media and this showed that *P. versicolor* and *T. socotrana* were very effective at breaking down the three pesticides, at 20 and 30°C.

The chosen fungi were grown on chopped straw as a carrier and incorporated into soil microcosms in the ratio of 1:10 containing mixtures of the three pesticides (5, 10 ppm) at 20 and 30°C, and subsequently under different water potential regimes at 20°C only, over periods of 70 days. *P. versicolor* alone significantly increased breakdown of 5 ppm dieldrin by 26% over untreated controls, while simazine breakdown was increased by 16%. However, for simazine at 30°C there was no difference between temporal rates of natural breakdown and those containing fungal inocula, regardless of concentration. With 5 ppm trifluralin, a maximum breakdown in untreated soil was 67% after 70 days. By contrast, this pesticide was undetectable after 28 days in the presence of the inoculant *P .versicolor*. This increased to 42 days where a mixture of the two fungi were used. Generally the mixture of fungi used in this study were not as effective in bioremediation of these pesticides as a single species. Field capacity soil appeared to be the best condition for *P. versicolor* to degrade dieldrin and trifluralin added at 10 ppm. However, for simazine this occurred in the driest water potential (-0.28 MPa) used.

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Contents **Page**

CHAPTER ONE

LITERATURE REVIEW

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER THREE

RESULTS

CHAPTER FOUR
DISCUSSION

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

Literature review

1.1. INTRODUCTION

It is now recognised that there are many different ways in which pollution of the environment by toxic organic chemicals arise. For example, industrial effluents often contain phenolic compounds and solvents which are difficult to remove using conventional processes and can contaminate rivers, lakes and sediments. Coal and oil derivatives from old gas manufacturing plants, pesticides from wood treatment plants, leakage of compounds from underground storage tanks and leachates from landfill sites can cause severe soil and ground water pollution. Other sources include accidental oil spills which contaminate both marine and terrestrial ecosystems as well as pollution derived from the deliberate release of pesticides used for agricultural purposes. Therefore, the diversity of pollutants being produced via industrial, agricultural and other manufacturing sources entering the environment is continually increasing. Their broad occurrence in the environment have led to attempts being made to find systems to hasten and improve the degradation of such recalcitrant chemicals (Livingstone, 1993; Singleton, 1994).

1.2. XENOBIOTICS

Initially, the term xenobiotic was limited to compounds which were chemically synthesised by man, and which contained structural elements that were not supposed to occur naturally (xenobiotic = foreign to life). Among the groups that were thought to be special for xenobiotics were chlorine, sulphonic acid and nitro groups. This definition, however, lead to several problems. Some compounds, which were synthesised in large quantities and were called xenobiotics, were later detected in natural systems. Therefore xenobiotics have more recently been defined as

"compounds that are released into the environment by the action of man, so that their concentrations are higher than natural" (Muller, 1992).

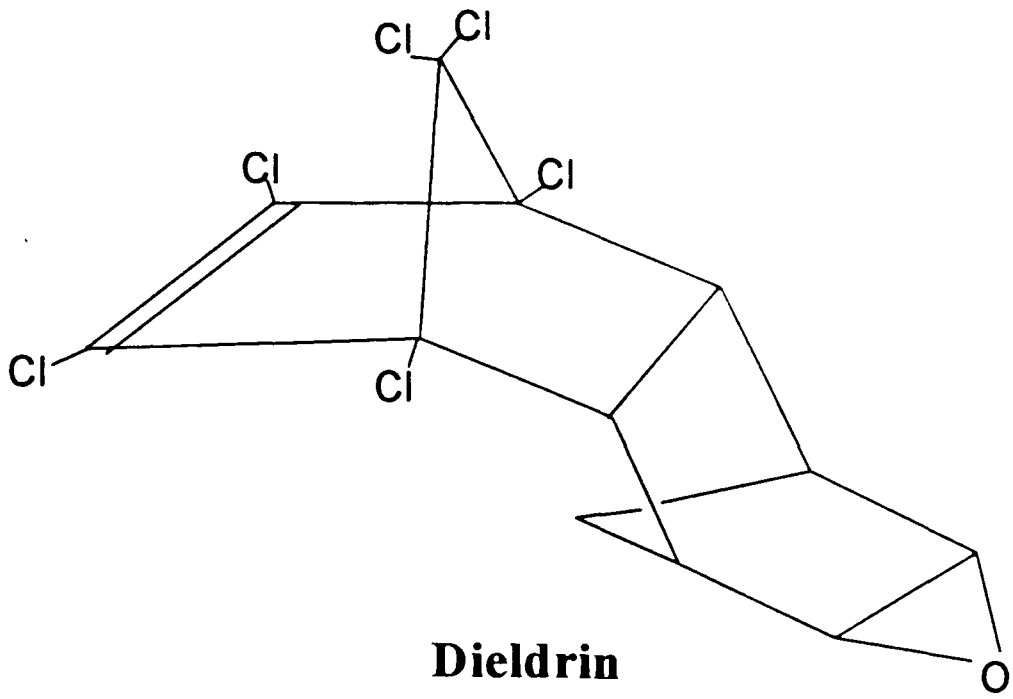
1.3. THE RED LIST AND PESTICIDES

In July 1988 the UK Department of the Environment (DOE) issued a consultation paper entitled "Input of dangerous substances to water" . In this publication the DOE identified a limited range of the most dangerous substances, which were selected according to strict scientific criteria, whose discharge to water should be minimised as far as possible. This compilation is called the "**Red List**" which follows EEC legislation, in particular the Dangerous Substances Directive (76/464/EEC) which is concerned with aquatic pollution in rivers, estuaries, ground water and the sea and agreements made at the three North Sea Conferences (Department of Environment and Welsh Office, July 1988) (Table 1.1). The list includes the three pesticides chosen for the purposes of this project. These were dieldrin (an organochlorine insecticide), simazine (a triazine herbicide) and trifluralin (a dinitroaniline herbicide) (Figure 1.1). The chemicals were also used as part of a previous study carried out by Roldan-Garcia, (1994) where fifteen species of tropical and temperate white-rot fungi were screened for their ability to tolerate these pesticides.

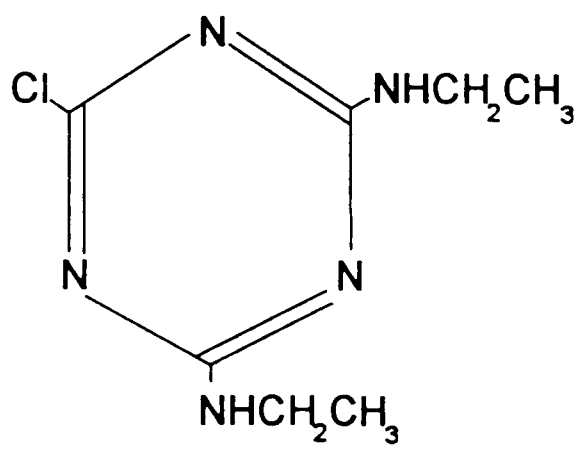
Pesticides in general have been classified as (a) "Highly persistent", (b) "Moderately persistent" and (c) "Non-persistent". Persistence times reflect the periods for 75 to 100 percent disappearance of pesticide residues from the environment. Non-persistent pesticides have persistence values of less than 3 months; moderately persistent pesticides take up to 18 months to break down; and highly persistent pesticides may last in the environment for a number of years. The three pesticides used in this study will now be described in more detail.

Table 1.1. List of the chemicals in the UK "Red List" and some of their commercial uses

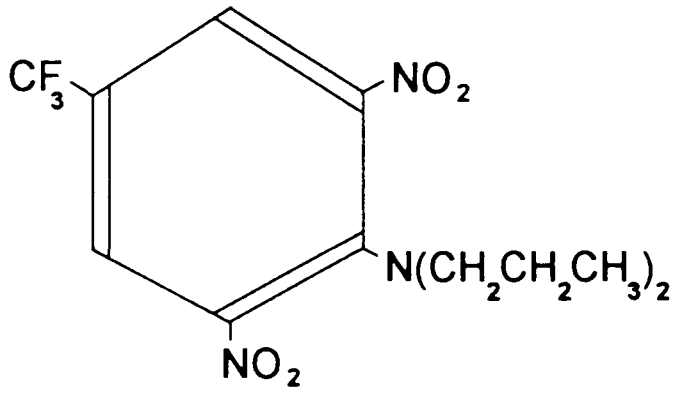
Chemicals	**Commercial uses**
Mercury and its compounds	Thermometers, barometers, insecticides, fungicides
Cadmium and its compounds	Alloys, protective layer against corrosion
γ-Hexachlorocyclohexane	Insecticide
Dichlorodiphenyltrichloroethane (DDT)	Insecticide
Pentachlorophenol (PCP)	Insecticide
Hexachlorobenzene	Fungicide, wood preservatives
Hexachlorobutadiene	Solvent for polymers, natural rubber
Aldrin	Insecticide
Dieldrin	***Insecticide***
Endrin	Insecticide
Carbontetrachloride	Raw material for the production of CFCs
Polychlorobiphenyls	Fire retardants, lubricants
Dichlorvos	Insecticide
Dichloroethane	Fruit ripening agent, Solvent
Trichlorobenzene (TCB)	Solvent in chemical manufacturing
Atrazine	Herbicide
Simazine	***Herbicide***
Tributyl and triphenyl tin and their compounds	Disinfectant, wood preservatives, fungicide
Trifluralin	***Herbicide***
Fenitrothion	Insecticide
Malathion	Insecticde
Endosulfan	Insecticide



Dieldrin



Simazine



Trifluralin

Figure 1.1. The molecular structures of the three pesticides selected from the Red List and used in this project

1.3.1. Dieldrin

Developed after the Second World War, dieldrin is a "highly persistent" pesticide. It has been suggested that dieldrin has a half-life of 15-20 years. The calculated half-life in water at 25°C (based on an evaporation rate of 5.33×10^{-5} ml hr^{-1}) is 500 days. The time for 95% disappearance from soil is 12.8 years. It has a vapour pressure of 0.4 mPa at 20°C. Due to its toxicity and high persistence dieldrin and its mother compound aldrin are amongst pesticides which have been banned in countries such as Australia, Pakistan and USA. However, little work has been done into remediation of this pesticide from soil or any other media. This is particularly surprising when one considers the fact that it is more toxic than DDT (LD_{50} in rats = 37-87 mg kg^{-1}), a compound that has been studied extensively over the past 20 to 30 years (LD_{50} in rats for DDT = 113-118 mg kg^{-1}).

These halogenated organic compounds are neurotoxins which appear to disturb the delicate balance between Na^+ and K^+ ions within the neuron. They are effective as non-systematic insecticides against most insects. They can also be incorporated into soil for the control of termites and soil-borne insects. Industrial uses include timber preservation, termite proofing of plastic, rubber, plywood and building boards (Martijn *et al.*, 1993; Nicholson & Blaine, 1993).

1.3.2. Simazine

Simazine is one of the most widely used herbicides in the world, belonging to the group called the triazines. The triazines are strong inhibitors of photosynthesis, and their selectivity depends on the ability of tolerant plants to degrade or metabolise the parent compound whereas the susceptible plants are unable to do so. Triazines are applied to the soil primarily for their post-emergence activity. There are many triazines

on the market today; they are used in greatest quantity in maize production and non-selectively on industrial sites. Simazine is the least water soluble of all the triazine herbicides. It is the pre-emergence herbicide recommended for the control of broad-leaved and grass weeds in deep-rooted crops particularly in maize production (Ware, 1982).

The acute oral LD_{50} in rats is >3000 mg kg^{-1} (technical grade). It is non-toxic to birds and honey bees. There is 100% disappearance from soils in 12 months making it a "moderately persistence" pesticide. It has a vapour pressure of 0.81 mPa at 20°C (Nicholson & Blaine, 1993; Hausewirth & Wetzel, 1996).

1.3.3. Trifluralin

The activity and selectivity of substituted 2,6-dinitroaniline compounds as herbicides were first reported in 1960. Trifluralin is by far the most widely used chemical in the dinitroaniline group, due to its selectivity, for use in two very important crops, cotton and soya beans. It has also rapidly become one of the most widely used herbicides in America.

Dinitroanilines have relatively high vapour pressure and readily volatilise from soil surfaces, particularly if the soil is warm and moist. They are also one of the most susceptible group of chemicals to photolysis. To avoid the loss of effectiveness by volatility or photolysis, most of these herbicides are applied as preplant soil-incorporated treatments. Once incorporated in the top 3-5 centimeters of soil, they are extremely immobile, owing to their low water solubility and tendency to adsorb.

The dinitroaniline herbicides are most toxic to germinating plant seedlings, particularly grasses. They are readily absorbed from soil by the penetrating shoots and

to some extent by roots of young seedlings. They inhibit growth of the active seedlings but their most obvious effect is the inhibition of lateral root formation. Perennial crops or weeds, deep seeded crops or transplanted crop plants with established root systems are the most tolerant (McEwen & Stephenson, 1979). In one study, controlled release of trifluralin was used to observe the ability of this herbicide to redirect root growth of established plants hence avoiding problems associated with unwanted root penetration in certain areas (Tworkoski, 1996). So although these herbicides may be taken up by roots of established plants they are poorly translocated from these sites of root uptake to the upper portions of the plants; metabolism in plants is very slight (McEwen & Stephenson, 1979). It is a moderately persistent herbicide.

Trifluralin is of low toxicity to birds and mammals with an acute oral LD_{50} in rats of >10 g kg^{-1}. Trifluralin shows a loss in soil of 75% to 90% in 0.5-1.0 year. It has a vapour pressure of 13.7 mPa at 25°C (Nicholson & Blaine, 1993).

1.4. BIOREMEDIATION

Bioremediation (also known as bioreclamation or biorestoration) has been defined as "the controlled use of microbiological systems to detoxify waste" (Dzantor *et al.*, 1993). There are various ways which can be employed to carry out remediation of xenobiotics. For example, soil contaminated with PCBs (polychlorinated biphenyls) can be treated by incineration, solvent or detergent washing techniques, and by bioremediation means. The problem with the first two methods is that contamination has been merely displaced rather than detoxified. In contrast, bioremediation is a low cost alternative which theoretically produces no toxic end products (Sukop & Cogger, 1992; Singleton, 1994).

Bioremediation is not an entirely new technology and has been used over the past 100 years to treat and transform waste products. The municipal waste water treatment industry is dependent on exploitation of micro-organisms to treat some of the unpleasant material from this water. What is new, is the use of micro-organisms to detoxify soils, ground water or similar environmental media from various organic material i.e. the type of matrix in which degradation may take place and the type of chemicals in such environments (Baker & Herson, 1994).

Soil and water contamination with high concentration of pesticides through improper product handling or waste disposal, or through accidents, is a major concern in many agrochemical manufacturing facilities. High concentrations of many ordinary biodegradable pesticides are more persistent and mobile in soils than low concentrations. The combination of prolonged persistence and greater mobility may increase the risk of surface or ground water contamination by high pesticide concentrations (Dzantor *et al.*, 1993). Indeed the greatest concern regarding soil contamination is the danger of leaching of toxic chemicals into the surface or ground water.

Pesticides have contributed greatly to controlling detrimental activities of species of insects, plants, bacteria and fungi, resulting in increased food production in an era of a growing world population. However, as mentioned above, because of their wide spread use, these chemicals have accumulated significantly in the environment, leading to environmental pollution.

Generally, after application of pesticides three different degradation pathways operate:

(1) physical, e.g., photolysis and temperature; (2) chemical, e.g. hydrolysis and (3)

biological, e.g., by micro-organisms (Coats, 1991).

Transformation by micro-organisms can occur in one of five ways. These include co-metabolic degradation, detoxification, polymerization and binding to naturally occurring compounds and finally mineralization (Semple & Fermor, 1995). It is therefore worth noting that microbial degradation does not always lead to total detoxification of an environmental pollutant being degraded. Clearly, the ideal situation would be the mineralization of the compounds to carbon dioxide and water, hence removing the xenobiotic completely.

Most studies are carried out in the laboratory before being conducted in the field. Laboratory studies however, can in most of cases, only give a prediction of the rates of degradation and can differ markedly from those observed under field conditions. The presence of a crop can also affect the behaviour of pesticides and influence microbial activity in the soil (Durand &Barcelo, 1992).

1.5. BIOAUGMENTATION

Bioaugmentation is the use of known microbial inoculants to enhance bioremediation. However there are a number reasons why bioaugmentation has not been successful:

(1) the pollutant concentration may be too low to support the microbial inoculant.

(2) the microbes may be susceptible to toxins or natural predators in the environment.

(3) microbes may utilise other organic materials in preference.

(4) in solids matrices, like soil, the microbes may be unable to move through the soil to sites containing the pollutants (Goldstein *et al.*,1985).

Other factors involved in determining the amount of degradation include compound

toxicity (Spiker *et al.*, 1992), solubility, strain of microbes and amount of inoculum used (Greer & Shelton, 1992; Corneau *et al.*, 1993). Small populations added as inocula into natural water may be eliminated by protozoan grazing or nutrient shortage (Ramadan *et al.*, 1990).

1.6. MICRO-ORGANISMS AND BIOREMEDIATION

In bioremediation, micro-organisms enhance the degradation of toxic material, in some cases all the way to carbon dioxide or to their mineral constituents (Table 1.2).

There are reports of complete mineralisation of toxic organic chemicals by pure cultures but these are rare, and break down products are usually found. The toxicity of these intermediates need to be elucidated if these organisms are to be used in future bioremediation programmes. This is important as in some cases the actual metabolites could be more toxic than the parent compound. The problem of toxic intermediates produced by pure cultures may be overcome by the use of mixed cultures or microbial consortia which have a wider spectrum of metabolic properties.

In these microbial communities effects such as co-metabolism may take place. Co-metabolism occurs when one organism which is growing on a particular substrate also oxidises a second substrate which is of no value to itself, in terms of assimilation or use as carbon and /or energy source. The oxidation products of the second substrate however are used by other micro-organisms in the community. In other cases co-metabolism in the strict sense may not occur and a compound may be modified in turn by a succession of microbes, each of which gain a small amount of carbon and/or energy from the transformation.

Due to very strict substrate specificities, the ability of a particular microbial species

Table1.2. Examples of some common toxic organic chemicals and the micro-organisms that metabolise them.

Chemicals	**Usage**	**Micro-organisms**
Trichloroethyl	Industrial cleaning solvent	*Methanotrophs, Escherichia coli*
Pentachlorophenol	General biocide in wood treatment	*Phanerochaete chrysosporium,* Anaerobes *Flavobacterium Rhodococcus chlorophenolicus*
BTEX compounds e.g. Benzene, toluene	Gasoline and aviation fuel, solvents used in industrial synthesis	*Phanerochate chrysosporium*; Anaerobic bacteria
Polycyclic aromatic e.g. hydrocarbons, (naphthalene, anthracene).	Fossil fuel components	White-rot fungi, *Aspergillus ochraceus, Cunninghamella elegans, Mycobacterium*
Polychlorinated biphenyls	Synthesis solvents	*Acitnitobacter,*
Chlorobenzenes	Solvents, fumigants, intermediates in pesticide manufacture	*Alcaligenes, Pseudomonas*
2,4-Dichlorophenoxyacetic acid	Herbicide	*Alcaligenes xylosoxidans*
Alkyl halides	Insecticide	*P. chrysosporium*
Trinitrotoluene(TNT)	Munitions	*P. chrysosporium*

to metabolise different toxic compounds is limited. This can be remedied by transfer of genes coding for enzymes with broader substrate specificities to develop microbes which will degrade a variety of different pollutants. It has been suggested that the use of pure cultures of genetically-manipulated organisms in degradation systems may in fact be preferable to the use of mixed cultures (Barr & Aust, 1994b; Singleton, 1994). Standard enrichment techniques tend to favour rapidly growing, versatile gram-negative bacteria, and fungi have therefore received less attention as candidates for waste treatment. Certain fungi such as the white-rot basidiomycetes, however, are able to degrade the polymer lignin (Higuchi, 1990). Lignin the second most abundant compound in nature (the first is cellulose), is a very complex, three-dimensional polymer, consisting of non-repeating phenyl propanoil units linked by various carbon-carbon and ether bonds (Figure 1.2). The lignin's irregularity, large size and chiral carbon centre all make it very difficult for this molecule to be broken down by normal enzymes and it is generally resistant to microbial degradation (Sarkanen & Ludwig, 1971). Lignin functions as support to woody plants by acting as an adhesive and binds cells and cellulose microfibrils in xylem to produce a laminated composite material. White-rot fungi which use cellulose as a carbon source, possess the unique ability to degrade lignin completely to carbon dioxide enabling access the cellulose molecule, that is towards the interior of the wood fibre. The whole process of lignin break down is energy-consuming and driven by the break down of wood components released by delignification such as cellulose and oligosaccharides. Lignin does not induce its own degradation like cellulose; the stimulus is instead nutrient starvation, i.e. nitrogen, sulphur or carbohydrate starvation, a condition known as iodophasic metabolism, also referred to as lignolysis (Kirk & Farrell, 1987; Higson, 1991). The process by which fungi degrade lignin is oxidative, probably involving enzymes such as lignin peroxidases (LiP), manganese

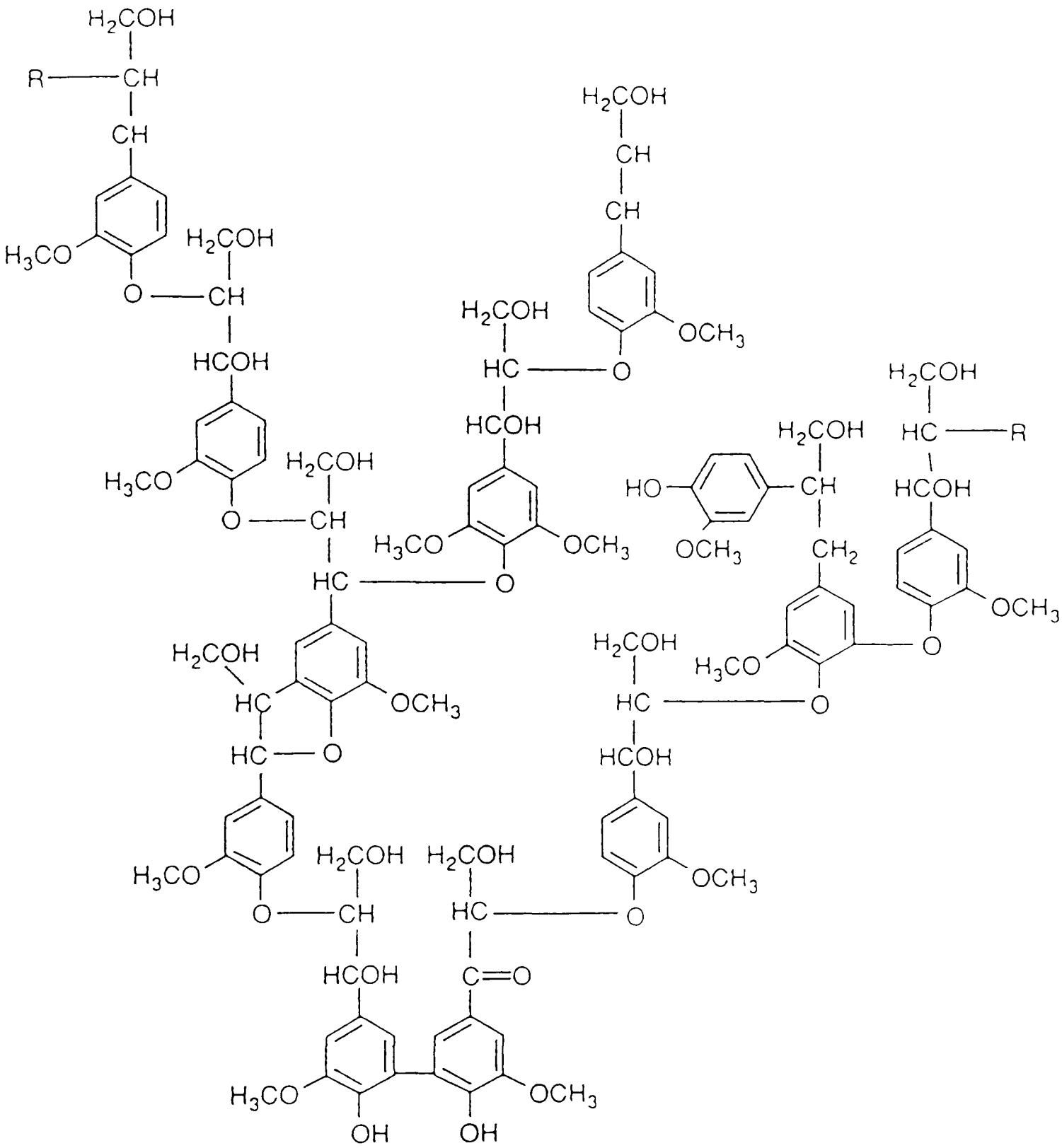


Figure 1.2. Diagrammatic structure of lignin: R indicates the chemical structure of the polymer that extends beyond what is shown in the diagram.

peroxidases (MnP), and laccases.

According to their typical production patterns of extracellular ligninolytic enzymes, white-rot fungi may be divided into three main groups:
i) LiP-MnP group, (ii) MnP-laccase group, and (iii) LiP-laccase group, although overlaps and exceptions certainly occur. A fourth group may be the laccase-AAO group , AAO denoting aryl alcohol oxidase. LiP (lignin peroxidase, ligninase) and MnP (manganese peroxidase, Mn-dependent peroxidase) are heme-containing glycoproteins which require hydrogen peroxide as an oxidant (Farmer *et al.*, 1960; Hatakka, 1994).

Some support for the involvement of these enzymes comes from comparing the effects of different conditions on their activities with the effects on the degradative process itself . For example, in studies with *Phanerochaete chrysosporium* nitrogen repressed the formation of LiP and MnP and also inhibited lignin degradation. Manganese (Mn^{2+}) increased MnP activity but decreased that of LiP. It also decreased lignin degradation, consistent with LiP playing a rate-limiting role in the process. In one study, Masaphy *et al.* (1996) found that addition of small concentrations of Mn(II) to a culture of the white-rot fungus *Pleurotus pulmonarius* enhanced its biotransformation of atrazine. It is not known, however, if the LiP, the MnP or the laccases function in the initial attack on the lignin or during later stages of degradation (Kirk & Farrell, 1987 ; Bonnarme & Jeffries, 1990; Perez & Jeffries, 1990).

The differences amongst fungi generally indicate that they may have different mechanisms for degrading lignin or it could result from the effects of the conditions on fungal growth. Altered growth could, in turn, alter the medium (e.g. carbohydrate concentration or pH), which might affect lignin degradation. It is assumed that LiP is important in the solubilization process and MnP in formation of CO_2 (Boyle *et al.*, 1992).

The lignolytic system of white-rot fungi appears to lend itself to decontamination of xenobiotics in a number of ways. The system is able to act on an insoluble and extracellular material; xenobiotics often have very limited solubility in water and are not readily available in soil for intracellular metabolism. Also the extracellular biodegradation system explains why the fungi can be quite resistant to toxic or mutagenic chemicals. Bumpus *et al.* (1985), found that inhibition of cyanide mineralisation by *P. chrysosporium* was not evident until concentrations of about 260 ppm. Table 1.3 shows a diverse range of toxic chemicals degraded by white-rot fungi. In these types of metabolism, cleavages occur at a variety of carbon-carbon and carbon-oxygen bonds regardless of the configuration or presence of any chiral centres. Here, the strict stereochemical relationships, an important feature of enzyme-substrate interactions dictated by active site conformation, no longer apply.

Degradation of a xenobiotic should be initiated like that of lignin by nutrient limitation. We would expect no requirement for prior exposure to the xenobiotic (often the case with actinomycete and bacterial processes) and degradation should continue even as the target is reduced to low concentrations. In bacterial systems, a residue generally remains owing to the target's concentration falling well below the enzyme's K_m and due to a lack of induction of enzymes synthesis. The white-rot fungal system should proceed until the concentration of the target substrate is as low as that of the activated enzyme, i.e., essentially undetectable (Barr & Aust, 1994a). In addition, introduced fungi would not be subject to protozoan attack and if lignocellulose materials were applied to the contaminated soil, the fungus should be able to compete well with other organisms, especially those sensitive to the hydrogen peroxide-peroxidases system (Higson, 1991 ; Laine & Jφrgensen, 1996; Vipulanandan *et al.*, 1996). Although environmental conditions that result in the induction of ligninase production are not entirely clear, frequently in laboratory cultures, it

Table 1.3. Various xenobiotics degraded by white-rot fungi

Reference	fungal species	Xenobiotics degraded
Bumpus et al. (1985)	*Phanerochaete chrysosporium(PC)*	DDT, Lindane, Benzo(a)pyrene, 2,3,7,8-tetrachlorodibenzo(p) dioxine, 3,4,3',4'-trichlorobiphenyl
Bezalel et al. (1996)	*Pleurotus ostreatus*	Catechol, Pyrene, Phenanthrene
Alleman et al. (1992); Valo et al. (1985)	*PC, Trametes versicolor*	Pentachlorophenol(PCP)
Masaphy et al. (1996)	*Pleurotus pulmonarius*	Atrazine
Yadav & Reddy, (1993)	*Phanerochaete chrysosporium*	Benzene, Toluene, Ethylbenzene and Xylene(BTEX)

is nitrogen limitation that brings about maximum activity. It is possible to decontaminate the soil, by inoculating strains of fungi into this contaminated soil perhaps by using some sort of carrier. Examples of carriers of fungal inocula are woodchips, wheat straw, corn cobs and other agricultural products which are thoroughly covered with fungal mycelium before inoculation into soil. These substrates provide a nutrient reserve to support the colonisation of contaminated soil. After an effective inoculation, mycelial penetration may serve to distribute the organism and the extracellular enzyme activity may permit degradation of soil bound contaminants, which would not otherwise be available for metabolism (Morgan & Watkinson 1989; Okeke, *et al.* 1993; Lestan & Lamar, 1996). However, Ali & Wainwright (1994) successfully inoculated a non-sterile agricultural loam soil with spore suspensions of *Phanerochaete chrysosporium* and reported a 60% reduction in the time taken to degrade a concentration of the fungicide benomyl compared to uninoculated soil.

The ability of white rot fungi to degrade xenobiotics is not limited to soil alone. Kennedy *et al.* (1990) did some comparative studies into biodegradation of alkyl halide insecticides by *P. chrysosporium* in both soil and aqueous cultures. They found that mineralisation rates of both [^{14}C] aldrin and [^{14}C] dieldrin were higher in liquid cultures than in soil.

Although there are a large number white-rot fungi which could prove to be potential candidates for bioremediation of various xenobiotics in the environment, *Phanerochaete chrysosporium* appears to be one of a very few white-rot fungi species which have been given an immense deal of attention in this area (Lamar *et al*, 1989; Spiker *et al*, 1992; Yadav & Reddy, 1993; Bumpus, 1993). Other white-rot fungi which have been looked at and shown some promise in tackling hazardous chemicals are *Pleurotus*

ostreatus (Bezalel *et al*, 1996) and *Trametes versicolor* (Alleman *et al*, 1992; Vyas *et al*, 1994). In fact Alleman *et al.* (1992) argued that *Trametes versicolor* was superior in degrading higher doses of PCP in liquid media than *P. chrysosporium*. Bezalel *et al.* (1996) on the other hand observed a faster mineralisation of PAHs in liquid media when using *Pleurotus ostreatus* than both *P. chrysosporium* and *T. versicolor.*

Knowledge of the potential of using other species of white-rot fungi in bioremedial systems are scarce both in *in vitro* laboratory studies and *in situ* soil studies. Furthermore much research has been concentrated on bioremediation systems for pentachlorophenols (PCPs) and polychlorinated biphenyls (PCBs), perhaps at the expense of other important xenobiotics on the UK Red List, including the three pesticides studied in this project.

1.7. ABIOTIC AND BIOTIC FACTORS AND THEIR EFFECT ON BIOREMEDIATION

Factors that affect the persistence of introduced organisms, their transport from the original application site and their effects on ecosystem functioning also need to be examined before release can be considered. Environmental factors such as pH, temperature, bioavailability, pesticide concentration, nutrient supply, oxygen availability and water potential (when contamination occurs in non-aqueous environment) can all influence degradation by preventing growth of organisms and more subtly by affecting gene expression. To improve microbial treatment processes to a satisfactory level therefore, the relevant factors need to be optimised. These factors will now be considered in more detail.

1.7.1. pH

pH value is a measure of acidity or alkalinity of an environment. Each organism thrives within a pH range and usually has a well-defined pH optimum in which it is most active. Most natural environments have pH values ranging between 5 and 9, so organisms with optima in this range are most common, although there are few organisms that grow well at low pH (acidophiles) or high pH values (alkalinophiles).

Fungi are known to prefer more acidic environments and hence may be of use under such conditions, whereas bacteria may be preferable under alkaline conditions (Brock & Madigan, 1991).

Valo *et al.* (1985) found that the optimum pH for degradation of pentachlorophenol (PCP) for mixed cultures was from 6.4 to 7.2. The fungus *Phanerochaete chrysosporium* degraded the BTEX compounds over a wide range of pH values from 4.5 to 7.0 (Yadav & Reddy, 1993). The solubility of compounds will vary with pH thus determining the rate of degradation. In soil, at high pH values, most of the pesticides and their hydrolysis products are more mobile, i.e. at high pH values there is less adsorption enabling greater mobility through the soil. In general, adsorption of chemicals is weak at neutral pH and above, leading to increased movement and faster degradation in alkaline soils. Winterlin *et al.* (1989) found that the pH of soil was a major factor influencing degradation of pesticides but that it was different for aerobic and anaerobic soils. Half-lives of most pesticides were shorter in alkaline soils under anaerobic conditions, while the opposite was true under aerobic conditions. Thus changing pH of contaminated soil/water could be a worthwhile option, to enhance the rate of microbial degradation.

1.7.2. Temperature

Temperature is one of the most important environmental factors influencing activity, growth and survival of micro-organisms. Every organism has a characteristic minimum temperature below which no growth can occur, an optimum temperature at which growth is most rapid and a maximum temperature above which growth is not possible (Magan, 1997). At maximum temperatures, denaturation of cell enzymes take place, hence cell death occurs. The minimum temperature for micro-organisms is not as clear, and could be affected by other factors in the environment, e.g. high concentrations of xenobiotics in such environments could force the minimum temperature to increase. Clearly, micro-organisms are able to degrade xenobiotics most efficiently at their optimum temperature. Most microbes isolated to date, degrade xenobiotics best at mesophilic temperatures, but some can work best at high or low temperatures and their ability to degrade such chemicals at temperatures lower than 25°C or higher than 37°C is often overlooked (Brock & Madigan, 1991 ; Singleton, 1994).

It has been shown that temperature can have more influence on herbicide degradation than soil itself, i.e. it can be the main factor influencing degradation (Holly & Roberts, 1963). According to Walker *et al.* (1983) a change in the temperature of soil containing simazine from 10 to 30°C increased the rate of loss of this herbicide by a factor of between 2 and 5. Importantly, studies on anaerobic PCB degradation found that temperature, as well as affecting the rate of degradation of a compound, can also affect the breakdown pathways and subsequent end products formed (Wiegel *et al.*, 1992).

1.7.3. Oxygen availability

It is now well established that organic pollutants are degraded under both aerobic

and anaerobic conditions (Neilson, 1990 ; RadeHaus & Schmidt, 1992). As a general rule it is regarded that the more highly chlorinated pollutants are more susceptible to degradation under anaerobic conditions (by reductive chlorination) while less chlorinated ones are more susceptible to degradation by aerobic mechanism (Fatherpure & Vogel, 1991).

If oxygen is necessary in bioremediation of pesticides in soil, it may very rapidly be consumed by the active population and oxygen availability may become the key limiting factor determining biodegradation rates (Morgan *et al.*, 1991). In such cases oxygen may be provided via air sparging in aeration bank walls, passing through oxygenated water or addition of hydrogen peroxide. Okeke *et al.* (1993) reported a significant improvement in fungal degradation of pentachlorophenol by (*Lentinula*) *edodes* in soil when small quantities of hydrogen peroxide (H_2O_2) were added. However, although H_2O_2, is highly efficient in releasing oxygen in the soil, care should be taken as too high a concentration of this chemical can prove detrimental to the bioremediating micro-organism (Ritter & Scarborough, 1995).

1.7.4. Concentration

Concentration is another factor which could have diverse effects, on the degradation of pesticides. Indeed, one study showed that many factors can influence degradation of pesticides depending on their chemical structure, but the most consistent and prominent of all factors was concentration. The higher the pesticide concentration, the more difficult it was for effective degradation (Schoen & Winterlin, 1987). The same study also showed that, high concentrations of a mixture of pesticides (atrazine, 2,4-D, trifluralin, carbaryl, diazinon, fenitrothion and captan) are known to adversely affect microbial activity,

i.e. they could prove toxic and reduce microbial populations. This was demonstrated when their numbers were investigated in contaminated soil compared to pesticide-free soil (Schoen & Winterlin, 1987). In another study Vyas *et al.* (1994) found that mineralisation of PCB 77 using the white-rot fungi *Phanerochaete chrysosporium*, *Coriolopsis polysona* and *Trametes versicolor* was higher when cultures were spiked with 30.14 nmol of the pesticide than with 513.7 nmol. This led them to conclude that higher concentration may be toxic, not only to the fungal enzyme system responsible for degradation of chlorine-bearing biphenyl, but also to the cellular metabolism in general.

1.7.5. Bioavailability

Generally, if a pesticide is mobile as a solution in soil then it is available for biodegradation by micro-organisms. One of the factors affecting bioavailability is sorption of xenobiotics to particulate matter, i.e. sorption may mean that the compound is not available for degradation. Indeed, some research has suggested that organic contaminants in the soil-bound state being unavailable for biological degradation could be a rate limiting factor in degradation by bacteria in the soil (Ogram & Jessup, 1985; Shimp & Young, 1988).

A sub-class of polyphenol oxidases (which are oxidative enzymes involved in polymerisation and depolymerisation of lignin) has the ability to bind xenobiotics to humic components present in the soils. This interaction would decrease the amount of pollutant available to organisms, thereby reducing toxicity and preventing leaching of the compound from the soils to water systems, although these xenobiotics can be released in the environment later (Singleton, 1994). Thus binding to humus could result in reduced bioavailability of contaminants, but highly hydrophobic material may become more mobile

following incorporation into humic acid fractions, as has been observed with DDT (Ballard, 1971). The general expectation is that, less pesticide will be retained by sandy soils low in organic matter than by finer textured soils with higher organic and clay matter content which tend to immobilise these compounds (Huang & Frink, 1989). In a recent study Kastner & Mahro (1996) concluded that addition of compost to pure soil contaminated with PAH, diminished the sorptive effect of the soil and intensified the biodegradation.

Water solubility is one factor responsible for movement of pesticides in soil, at least at low concentration in soil typical of field situations. At high concentration, solubility can take on more importance as the cause of pesticide mobility (Khan, 1980). Low solubility limits the transport of pollutants into the microbial cell effectively stopping its degradation (Miller & Bartha, 1989). This can be cured by adding biosurfactants or detergents (Churchill *et al.*, 1995; Tiehm *et al.*, 1995). Another way of solving the problem of low water solubility may be the use of micro-organisms such as fungi which secrete extracellular degradative enzymes (Singleton, 1994).

Bioavailability has other implications, including influencing the ability of pesticides to leach into underground water. According to Aharonson (1990) "a pesticide can reach ground water if its water solubility is greater than about 30 mg ml^{-1}; its adsorptivity (K_{oc} = partition coefficient between soil organic carbon and water) is less than 300-500 $cm^{-3}g$; its soil half-life longer than 2-3 weeks; its hydrolysis half-life longer than 6 months and photolysis half-life longer than 3 days" .

1.7.6. Nutrients

Micro-organisms can be quite diverse in the way they use nutrients available to them. According to Stanier *et al.* (1986), some species of micro-organisms such as lactic

acid bacteria can utilise a very small number of simple organic compounds, whereas *Pseudomonas* species are able to metabolise over 90 different organic compounds as a sole carbon and energy source.

Fungi may not metabolise xenobiotics as a sole carbon or energy source, and additional carbon sources for improving pollutant degradation are sometimes necessary. Addition of cheap carbon sources to contaminated sites may speed up treatment. For example, a medium can be supplemented with mineral salts, vitamins, and 3,4-dimethoxy benzyl alcohol (veratryl alcohol) to enhance mineralisation. In some cases however, nutrient nitrogen can repress degradative enzymes (Redhaus *et al.*, 1992; Baker & Herson, 1994) (Section 1.6.).

In a study of treatment of contaminated soil with atrazine and trifluralin by Winterlin *et al.* (1989) it was found that the half-life of the pesticides were greatly reduced following the addition of amendments of lime and manure. They found that altering the pH value alone had little effect on the herbicides breakdown but when combined with other treatments such as organic matter, the process could be indirectly improved through the enhancement of microbial activity. According to Semple & Fermor (1995), mushroom compost, a rich source of nutrient for a diverse population of micro-organisms such as bacteria, mesophilic and thermophilic actinomycete and lignin degrading fungi has a great potential for bioremediation of a variety of aromatic pollutants.

1.7.7. Water availability

All micro-organisms need a sufficient source of water to carry out cell function, growth, reproduction and transport of material across the cell membrane. The cell membrane of micro-organisms is semi-permeable which allows water molecules to enter the

cell through osmosis to come to equilibrium with its environment. However, often conditions dictate a water shortage, either due to presence of a high concentration of salts, a dry arid desert region or in intermediate moisture agricultural products. Decreasing water reduces water availability, solute transport and migration of unicellular organisms which require water films (Morgan & Watkinson, 1989; Magan, 1997).

Water availability is particularly important in soil as it can determine adsorption levels and significantly influence the activity of microbial remedial systems. Water content, expressed as a percentage of either fresh weight (wet weight basis) or oven dry weight (dry weight basis), is the most easily determined measure of water in a material. In a substrate some water molecules are bound tightly to the substrate by strong forces, while other molecules are free or more weakly bound. Free water is readily available for microbial activity whereas availability of the bound water molecules is very much dependent on the degree of binding. This binding force varies with the type of substrate, thus total water content is not a good indicator of water availability for microbial activity. The availability of water to micro-organisms can be described using the terminology of water activity (a_w) or water potential (ψ) (Scott, 1957). Water activity (a_w) is the ratio between the vapour pressure of water in a substrate (P) and the vapour pressure of pure water (Po), at the same temperature and pressure, and is expressed by the formula:

$$a_w = P / Po$$

In soil the water potential terminology (ψ) is used instead of a_w and the two are linked to each other by the formula:

$$\psi = RT/V \log_{10} a_w$$

where R is the Ideal Gas Constant($Jmol^{-1} K^{-1}$), V the volume of one mole of water (m^3), T the temperature (K). The unit of water potential is in pascals (Pa). The relationship

between a_w and Ψ is shown in the Table 1.4 (Magan, 1997).

Generally micro-organisms and typically fungi need water potentials of -40 to 0 MPa to survive. Bacteria tend to be very sensitive to values of less than -1.4 MPa. The wilting point of plants is -1.4 MPa (Brock & Madigan, 1991; Lacey *et al.*, 1991).

Due to the dynamic nature of environmental climate and its effect on soil, variability of soil water potential does occur and either directly or indirectly it can have a crucial effect on the degradation of xenobiotics in soil. It is indeed remarkable that relatively little work has been carried out in this area (Kostowska & Rola, 1984; Carter, 1991).

1.7.8. Combination of abiotic and biotic factors

Sometimes however, a number of factors described in Section 1.7. need to be manipulated together in order to achieve a reasonable enhancement of bioremediation of toxic materials. For example, when pollutants are in an area where the soil cannot be disturbed, or there is deep-seated contamination, then an *in-situ* process would need to adopted. This involves optimisation of environmental conditions for bioremediation, by addition of nutrients, moisture level adjustment and increased aeration. Ground water flow must also be controlled so that the pollutants do not escape the site. This is usually achieved by pumping the water with added oxygen and nutrient back into the contaminated soil (Singleton, 1994). Other factors which could affect degradation include organic matter and clay content. Ahonen & Heinonen-Tanski, (1994), attempting to improve degradation of simazine at low temperatures concluded that soil aeration as well as fertilisation with organic manure might be responsible for simazine degradation. These could be achieved by addition of chicken manure and mechanical treatment to improve aeration.

Table 1.4. Water activity, equilibrium relative humidity (E.R.H) and water potentials at 25°C

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

In a laboratory study, it was observed that the use of various organic amendments accelerated the co-metabolic degradation of alachlor and metachlor when freshly added to soil. However, the same amendments produced only marginal increases in the degradation of herbicide residues that had aged in soil (Dzantor & Felsot, 1991). A further study, showed that aged alachlor and metachlor residues degraded rapidly when they were diluted 10-fold with fresh soil and also amended with corn meal. Soil dilution alone, or amending the undiluted, contaminated soils with organic material, produced only a small increase in dissipation of the herbicides compared to the dissipation in corresponding undiluted and unamended controls. The results suggested that a combination of land farming, which the soil dilutions simulated, and biostimulation may provide an effective means for detoxifying certain herbicide contaminants in soil (Dzantor *et al.* 1993).

Ritter & Scarborough (1995) suggested that optimum conditions for biological activity at a bioremediation site include pH values between 6.5 and 8.5, temperature between 27°C and 35°C and a ratio of organic carbon to available nitrogen and phosphorus of 300: 15: 1.

1.8. OBJECTIVES

1. To determine the temporal natural breakdown rates of different concentrations of the pesticides dieldrin, simazine and trifluralin in water at 10, 20 and 30°C using High Performance Liquid Chromatography (HPLC).
2. To screen the four fungi, *Phanerochaete chrysosporium, Polystictus versicolor*, *Trametes socotrana* and *Trametes cingulata,* for their ability to tolerate and grow in the presence of different concentrations of the three pesticides *in vitro*.
3. To quantify the capabilities of the four fungi to degrade the above pesticides at different temperatures and time, *in vitro* on solid agar media.
4. To determine the temporal natural breakdown of the three pesticides in soil at different initial water potentials, concentrations and temperatures.
5. Develop an HPLC system for the simultaneous quantification of all the three pesticides in a gradient mobile phase system.
6. Determine the effect of the best single or combination of fungi for the simultaneous breakdown of the three pesticides at different temperatures and initial water potentials.

CHAPTER TWO

Materials and Methods

2.1. PESTICIDES

Dieldrin technical grade (90% a.i.) and standard grade (99% a.i.) were obtained from ICN Biochemicals. Technical grade simazine (97% a.i.) and standard grade simazine (99.8% a.i.) were both gifts provided by CIBA-GEIGY. Technical grade trifluralin (solution 95% a.i. , crystals 99.2% a.i.) and standard grade trifluralin (99.4% a.i.) were gifts provided by DOW ELANCO.

All the solvents used were of HPLC grade purity purchased from Sigma-Aldrich. The dissolving solvents for the sample stock solutions were chosen for their miscibility with water.

2.2. HPLC COLUMNS AND INSTRUMENTATION

The HPLC column for dieldrin was an Alltima C18 5µm of dimensions 250 x 4.6 mm i.d. B fitting. The column for simazine was an Ultremex C18 3 µm of dimensions 150 x 4.6 mm i.d. and was purchased from Phenomenex. Trifluralin analysis required an Envirosep-PP reversed phased 4 mm column of dimensions 125 x 3.2 mm i.d. , purchased from Phenomenex. All of the above were used in conjunction with relevant guard columns, at room temperature. A Gilson 175 HPLC system fitted with a U.V. detection unit was used to quantify pesticides. For the analysis of pesticide mixtures in soil extracts, the trifluralin column was found to be the best for resolving the three peaks.

2.3. NATURAL BREAKDOWN OF PESTICIDES IN WATER

All the pesticide samples were prepared in water and stored in amber coloured bottles in triplicate and incubated at 10, 20 and 30°C. Triplicate blank samples (water only)

were also set up. The sampling times (T) were at 0, 7, 14, 21, 28 and 42 days for all the samples. The samples were destructively taken in three replicates at the above time intervals. All samples were frozen at -28°C and kept under this condition until analysis on the HPLC system was carried out.

2.3.1. Dieldrin samples

A stock solution of dieldrin was prepared by dissolving technical grade dieldrin in acetone, producing a concentration of 1978 ppm. The working samples were made up at 3 and 6 ppm solutions in tap water, and a third set of 3 ppm solutions in sterile tap water using relevant volumes of stock solution. The pH of tap water and sterile tap water were found to be 7.25 and 5.85 respectively.

2.3.2. Simazine samples

In this case the stock solution was prepared at 2250 ppm by dissolving technical grade simazine in tetrahydrofuran (THF). Three sets of solutions were prepared, 5 and 10 ppm in tap water and 5 ppm in sterile tap water. The pH of the tap and sterile water was 7.14 and 5.72 respectively.

2.3.3. Trifluralin samples

A stock solution of technical grade trifluralin in acetone was prepared to give a concentration of 2173 ppm. The spiked sample solutions were at 5 and 10 ppm in tap water and 5 ppm in sterile tap water. Tap water had a pH value of 7.30, and sterile water, 5.79.

2.4. ANALYSIS OF WATER SPIKED WITH PESTICIDES

The concentrations of the pesticides in water were determined using High Performance Liquid Chromatography. The stock standards were prepared in their relevant dissolving solvents, i.e. simazine in THF, dieldrin and trifluralin in acetone. However to prepare the calibration standards, a calculated aliquot of the stock solution was added to a known volume of HPLC grade water producing the desired standard concentration. Each solution was then mixed with one or two organic solvents so that the final solution had the same solvent composition as the mobile phase for that analysis. To use simazine as an example, addition of the calculated amount of stock simazine solution to 20.0 ml of water produced a 2 ppm water solution. This was then made up to 50.0 ml using acetonitrile (10.0 ml, aliquot) and methanol (20.0 ml, aliquot). As will become clear later, this final solution has the same composition as the mobile phase for the analysis of simazine. A similar calibration method was carried out in all experiments, unless otherwise indicated.

2.4.1. Analysis of simazine

The mobile phase used in this analysis consisted of water/acetonitrile/methanol (40:20:40) as recommended by the column manufacturer. The detection wavelength recommended by Phenomenex was 220 nm. This was confirmed by running a U.V. scan of a 30 ppm simazine solution on a CompuSpec spectrophotometer (Figure 2.1). The HPLC sample injection volume was 50 µl and the mobile phase flow rate was at 0.5 ml min^{-1}. The Absorbance Units corresponding to Full Scale deflection on the recorder (AUFS) was set at 0.05.

Simazine solutions were defrosted at room temperature (25°C approx.) overnight.

Spectrophotometer	CompuSpec
Title	simazine
Spectrophotometer	CompuSpec
Title	simazine
Filename	SIMA-01.WS2
Operator	A Elyassi
Comments	30 ppm in methanol
Date	21 September 1994
Start Wavelength	200.0 nm
End Wavelength	400.0 nm
Temperature	OFF
Plot Step	1.0 nm
Scan mode	Abs
Scan speed	2400 nm/min

No.		Wavelength	Height
1	Peak	220.4	2.133
2	Peak	262.1	0.113

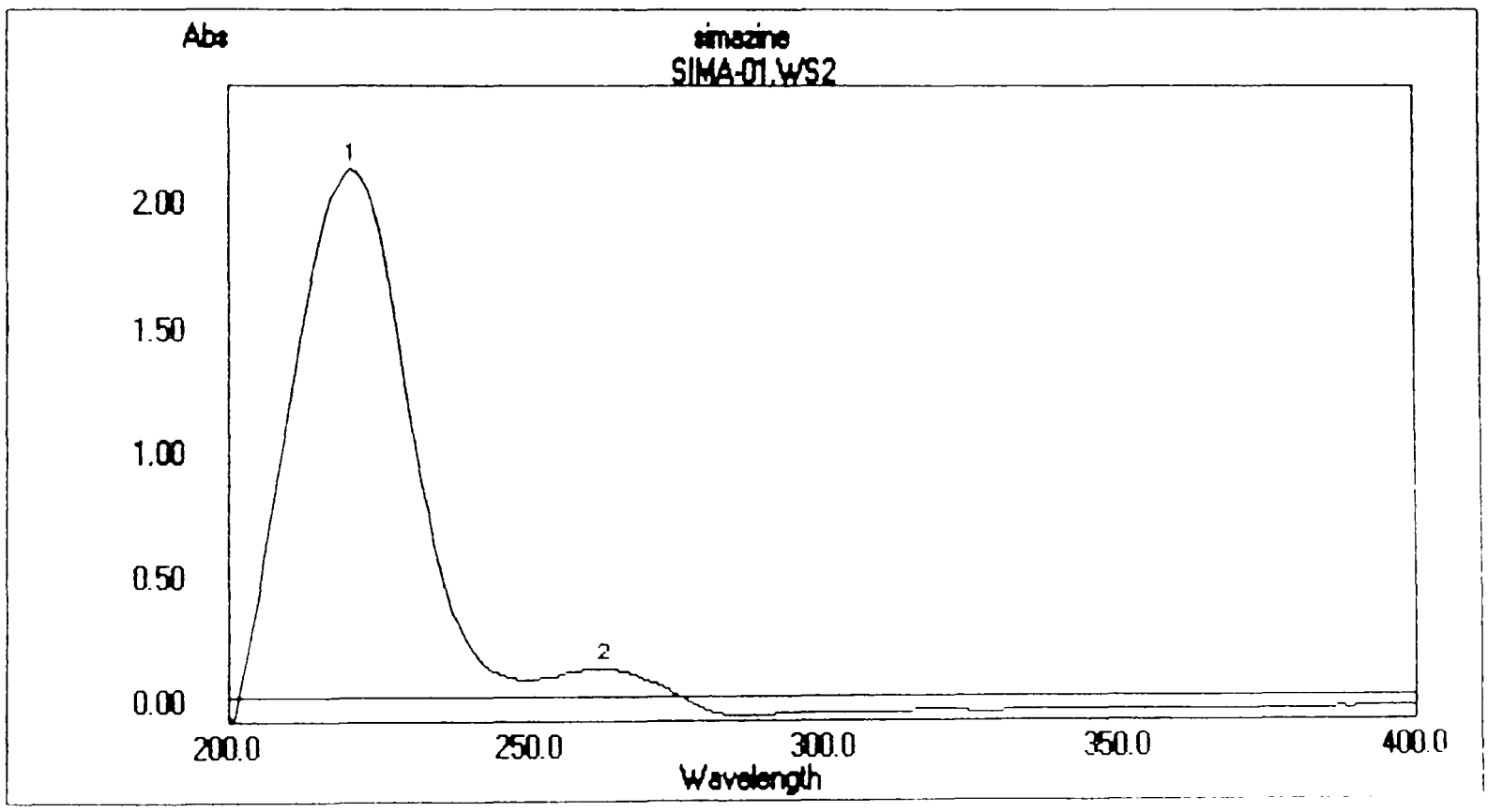


Figure 2.1. U.V. scan of simazine standard in methanol at 30 ppm.

The slight precipitate which first resulted on addition of stock solution to water, was separated out by centrifuging at 4200 rpm for about 20 minutes. The resultant clear supernatant solution was pipetted out (4.0 ml aliquots) and mixed with acetonitrile (2.0 ml, aliquots) and methanol (4.0 ml, aliquots). This produced 10.0 ml of clear solution with the same composition as the mobile phase ready to be injected on the column. All the spiked samples and controls were treated in exactly the same way.

To analyse the simazine samples quantitatively, a series of calibration solutions were prepared which ranged from 0.098-7.95 ppm using a stock standard solution of concentration 218 ppm (approx.). This was altered to a new range of 1.5-10 ppm, since the initial analysis placed the sample concentrations at a range 2.5-4 ppm. The entire analysis took around ten days during which time two stock standards were prepared. These were kept in the -28°C freezer when not in use. The typical correlation coefficient of the calibration curve had a range of 0.999 to 1.000 (Figure 2.2). The same analytical procedure was carried out for all test simazine samples. The analysis time was 12 minutes with simazine eluting at around 8.3 to 8.7 minutes.

2.4.2. Analysis of trifluralin

For this analysis the mobile phase composition was developed by manipulating the ratio of the two solvents, water and acetonitrile. Initially, the flow rate was set at 0.75 ml min^{-1} and the mobile phase ratio 63:37 (acetonitrile : water). This produced a trifluralin retention time of 9.5 to 10.2 minutes. However, to produce a shorter retention time and sharper peaks, a final flow rate of 1.1 cm^3 min^{-1} and mobile phase ratio of 60:40 (acetonitrile : water) was used. The detection wavelength was 220 nm, the injection volume 50 µl and

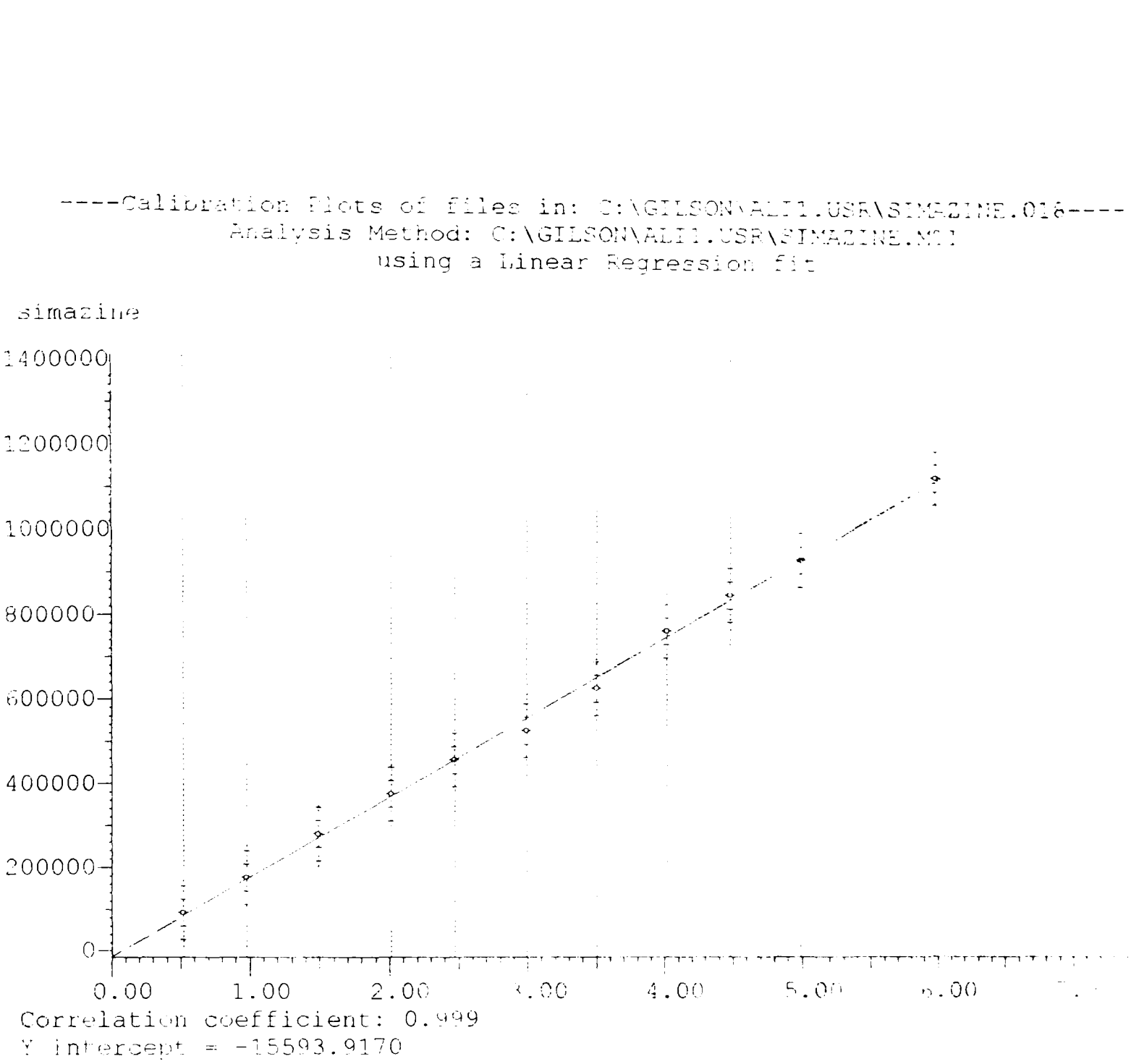


Figure 2.2. Example of a typical calibration curve of simazine standards. The x axis displays the concentration (ppm) and the y axis shows the area under the peak.

the AUFS value 0.005.

The frozen samples were defrosted over night, in the dark and the slight amount of precipitate present was centrifuged off at 4200 rpm for 20 minutes. The resultant clear supernatant solution (5.0 ml, aliquots) was mixed with acetonitrile (0.5 ml, aliquots). This solution was then ready to be injected on to the HPLC column.

The stock standard solutions, had concentrations of around 105 ppm. The calibration standards were prepared by adding calculated volumes of stock solution to HPLC grade water, and making up to volume (5.0 ml, aliquot). This was followed by addition of acetonitrile (0.5 ml, aliquot). The initial range was from 0.51- 6.5 ppm which was later modified to include 0.06 - 0.7 ppm. The correlation coefficients were between 0.990 to 0.999 (Figure 2.3). The run time was 12 minutes and trifluralin eluted at 8.5-9.0 minutes.

2.4.3. Analysis of dieldrin

In this analysis the UV detection wavelength was determined by running a UV scan of a dieldrin solution (Figure 2.4). The mobile phase composition was determined by varying the water : acetonitrile mixture ratio. The optimum value was found to be 30:70 (water: acetonitrile). The injection volume was 50 µl and the flow rate 1.5 ml min^{-1}. The AUFS value was set at 0.012.

The initial preparation was the same as for the other two pesticides. The clear supernatant solution (5.0 ml, aliquot) was diluted with acetonitrile (0.50 ml, aliquot), which was then ready to be injected on to the HPLC column. All the spiked samples and controls were treated in exactly the same way.

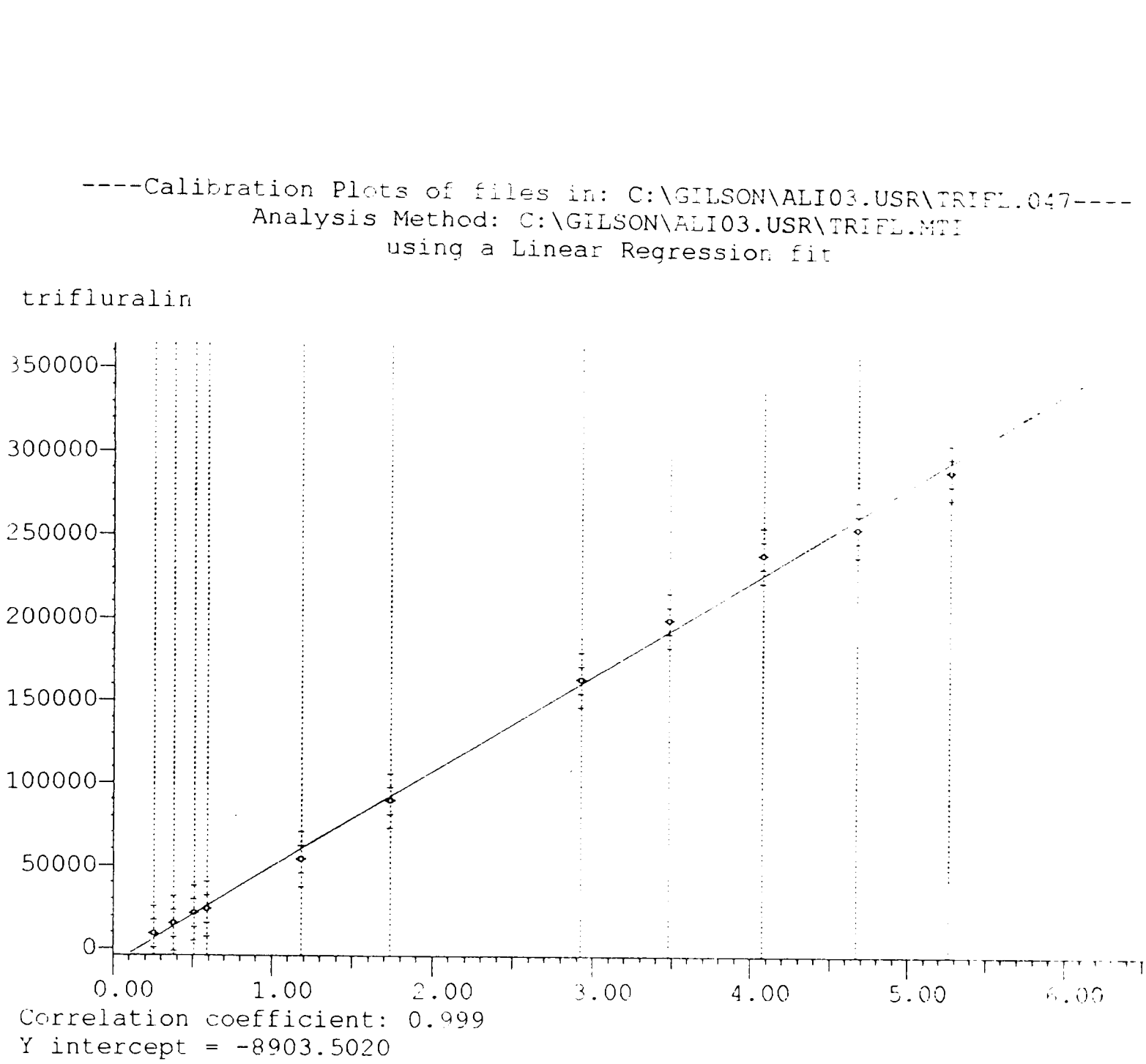


Figure 2.3. Example of a typical calibration curve of trifluralin standards. The x axis displays the concentration (ppm) and the y axis shows the area under the peak.

Spectrophotometer	CompuSpec
Title	Dieldrin
Filename	DIEL--01.WS2
Operator	A Elyassi
Comments	100ppm in methanol
Date	21 September 1994

Start Wavelength	200.0 nm
End Wavelength	400.0 nm
Temperature	OFF
Plot Step	1.0 nm
Scan mode	Abs
Scan speed	2400 nm/min

No.		Wavelength	Height
1	Peak	214.4	2.171

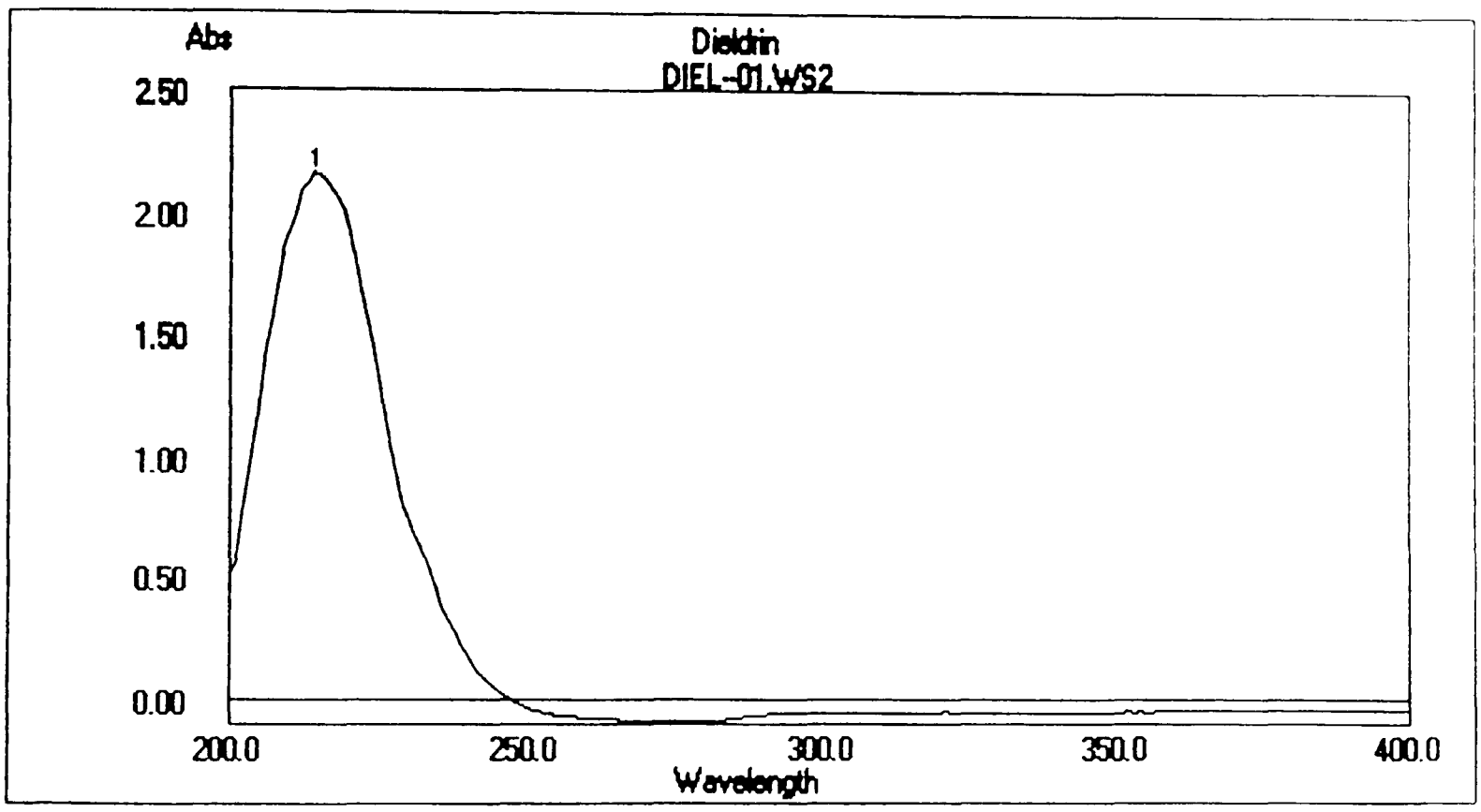


Figure 2.4. U.V. scan of dieldrin standard in methanol at 100 ppm.

The calibration standards were prepared using a stock solution of standard grade dieldrin dissolved in acetone; its final concentration was 110 ppm. The relevant volumes of stock were added to HPLC grade water and then made up to volume with HPLC grade water (5 ml, aliquot); this was then diluted with acetonitrile (0.5 ml, aliquot). A series of calibration solutions prepared ranged from 0.089-1.50 ppm proved too high for the samples. A second batch of 0.050 - 0.70 ppm were the correct range for the samples analysed.

The run time was 19 minutes with dieldrin eluting at about 15.5 minutes. The correlation coefficient of the calibration curves ranged from 0.999 to 1.000 (Figure 2.5).

2.5. IN VITRO SCREENING OF WHITE-ROT FUNGI FOR THEIR ABILITY TO TOLERATE AND DEGRADE DIFFERENT PESTICIDES

2.5.1. The fungi

All of the species chosen for the purposes of this project were white-rot fungi. Two tropical species belonging to the *Trametes* genus, *T. socotrana* and *T. cingulata* (provided by Dr. A Y Mswaka; University of Zimbabwe), and two temperate species, *Phanerochaete chrysosporium* and *Polystictus versicolor* obtained from Horticultural Research International (HRI) (kindly provided by Dr. M P Challen) were used. All four species have previously been screened for their tolerance towards the selected pesticides (see Section 2.1) at concentrations of up to 1500 ppm (Roldan-Garcia,1994).

2.5.2. Pesticide-amended agar media

In these experiments a weak Malt Extract (0.5% w/v) Agar (1.5% w/v) (MEA) was used to encourage the fungi to use the pesticide as a nutrient source.

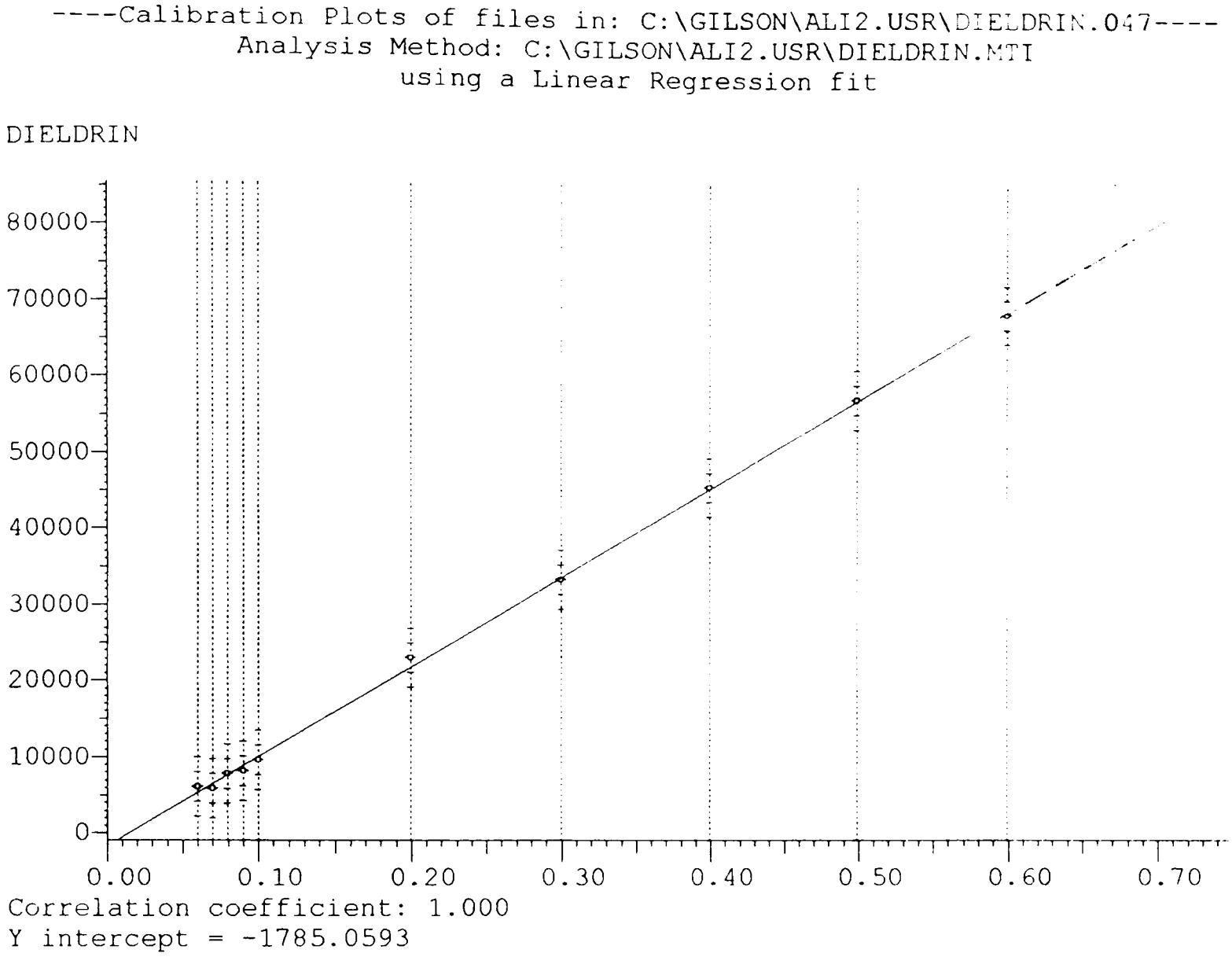


Figure 2.5. Example of a typical calibration curve of dieldrin standards. The x axis displays the concentration (ppm) and the y axis shows the area under the peak.

All the stock solutions were made up using technical grade pesticides dissolved in HPLC grade methanol. THF and acetone were both avoided (as a dissolving solvent) because of possible adverse effects on the plastic Petri plates used. After autoclaving, the agar mixture was allowed to cool to around 50 to 60°C. To this, relevant stock pesticide solutions were added to produce two treatments, one at aprox. 5.0 ppm and the other at around 10.0 ppm. The agar was immediately poured into 9 cm Petri dishes and allowed to cool. These were all stored at 4°C. The pesticide-free controls were treated in exactly the same way. Pesticide-incorporated uninoculated control agar plates, were also prepared and treated in the same way.

For simazine, the samples were prepared using stock solutions ranging from 370 - 400 ppm. Dieldrin stock solutions ranged from 380 - 400 ppm, and trifluralin stock solutions also had a range of 380 - 400 ppm.

2.5.3. Inoculations and measurements

For all the experiments sterile, water permeable sterile 85 mm cellophane discs (Cannings Ltd, Bristol) were placed on the agar medium surface before inoculation took place. The relevant fungi were then inoculated centrally as 5 mm diameter plugs taken from the margin of growing colonies. In all cases the radial growth was measured in two directions at right angles to each other, daily or as required until the end of the incubation period or until the petri plates were completely covered by the colony. It was decided to use cellophane discs to separate the mycelial matt from the agar as it would have been problematic to carry out the pesticide extraction and improve the efficiency of extraction.

Initially, simazine samples incubated at 20°C were stored for up to 42 days only,

with sampling times of 0, 14, 28 and 42 days. However, with the 10 and 30°C incubation temperatures, storage time was extended to 70 and 56 days respectively, with sampling times of 0, 42, 56 and 69 days for 10°C and 0, 14, 28, 42 and 56 days for 30°C. As no growth was observed with simazine samples at 10°C (except in the case of *P. versicolor* which showed a very thin sparse mycelial growth) the dieldrin and trifluralin incorporated agar plates were only incubated at 20 and 30°C. The sampling times for these were at 0, 28 and 56 days.

All the experiments were carried out with at least 15 replicates per treatment. Three replicates of all treatments were destructively sampled at each sampling time. During sampling, one quarter segment of the agar was cut out and weighed. The exact weight of each sample (approx. 5 grams) was recorded. The samples were frozen at -28°C until extraction and analysis at a later date.

To compare the effect of the cellophane on fungal growth, pesticide-free agar plates without cellophane were also inoculated with all the fungi at 10, 20 and 30°C. The mycelial extention was compared to culture grown on MEA in the absence of cellophane discs. This showed that there was no significant difference in growth patterns.

No growth of *P. chrysosporium* was observed at 20°C in the pesticide-treatment plates, in two fully replicated experiments.

2.5.4. Extraction method development for simazine

For this experiment MEA was prepared as described in Section 2.5.2. A batch of 6 replicate simazine-incorporated agar plates at a concentration of 10.0 ppm was prepared using a stock solution of 440 ppm.

The agar was cut out and weighed in a range of 5.0-5.3 grams. These were frozen at -28°C for 24 hours then thawed for extraction. One triplicate set of samples were homogenised (for 2 minutes) and shaken vigorously (for 2 minutes) in methanol (15.0 ml, aliquot). For the other set, acetonitrile (15.0 ml, aliquot) was used. The samples were then centrifuged at 4200 rpm for about 10 minutes. The clear supernatant solution was filtered using a 0.2µ nylon syringe filter to ensure a particulate-free solution. This clear filtered solution was stored at -28°C until analysis.

2.5.5. Extraction method development for dieldrin

For dieldrin, a stock solution of 345 ppm in methanol was prepared from which 10 ppm set of MEA plates were prepared. From these five batches of four replicate samples were weighed out, ranging from 4.9-5.1 grams. The solvents used for these extractions were 100% methanol, 10% acetone in methanol, 20% acetone in methanol, 50% acetone in methanol and 100% acetone. The rest of the procedure was as described for simazine.

2.5.6. Extraction method development for trifluralin

In this case the stock solution, had a concentration of 385 ppm, and the individual plates had a concentration of 10.03 ppm. Initially, the solvents and the weight range of the samples were exactly the same as dieldrin (Section 2.5.5.). However, due to less than satisfactory recoveries, a second batch of trifluralin samples were prepared. These consisted of three sets of four replicates and the extraction solvents used were 100% acetonitrile, 50% acetone in acetonitrile, and 100% ethyl acetate.

2.6. ANALYSIS OF PESTICIDE-TREATED MEA

For these analyses, the stock solution was prepared in the best solvent with which the pesticide was extracted, i.e. methanol for simazine, acetone for dieldrin, and acetonitrile for trifluralin.

For the calibration standards each solution was prepared in a 50 ml volumetric flask. To start with a mixture of HPLC grade water (10.0 ml, aliquot) plus the extraction solvent (30 ml, approx.) were added to a volumetric flask. This was then spiked with a calculated volume of pesticide stock solution and made up to volume using the extraction solvent, hence producing the desired concentration. The use of 20% (V/V) of water, prevented the formation of split peaks and ensures satisfactory peak shapes when HPLC analysis was carried out.

In the case of the sample preparation, after equilibration to room temperature, each extracted pesticide solution was diluted with HPLC grade water (20% V/V). This discrepancy was later corrected when calculating the results.

2.6.1. Analysis of simazine-treated MEA

For these analyses the concentration of stock solutions were about 170-180 ppm and the calibration standards ranged from 0.30-5.0 ppm. The typical correlation coefficient of the calibration curve had a range of 0.997-0.999. The analysis time was 12 minutes with simazine eluting at 8.3 to 8.6 minutes. The AUFS value was set at 0.025.

2.6.2. Analysis of dieldrin-treated MEA

For dieldrin the concentration of stock solutions were in the region of 350 ppm, producing calibration standards ranging from 0.50 to 4.50 ppm. The correlation coefficient

of the calibration curves had a range of 0.990 to 0.995. The run time for this analysis was 19 minutes and dieldrin eluted between 15.80 and 16.10 minutes. A 0.005 AUFS value was chosen for this analysis.

2.6.3. Analysis of trifluralin-treated MEA

For trifluralin the stock solution concentration was at about 255 ppm, producing calibration standards in the range 0.100 to 4.50 ppm. The correlation coefficient of the calibration curve ranged from 0.999 to 1.00. A 12 minutes run time was chosen with trifluralin eluting at about 7.6 minutes. For this analysis the acetonitrile part of the mobile phase contained a small amount of THF (2%, V/V). This was to improve the shape of the peak. The sensitivity value AUFS was set at 0.001.

2.7. MOISTURE RETENTION CHARACTERISTIC OF SOIL

2.7.1. The soil

A sandy loam soil (PT008) was supplied by Levington Agriculture (Ipswich). It contained 15% clay; 19% silt; 66% sand; 1% organic compounds. Its pH value was 6.2 .

2.7.2. Moisture absorption curve of the soil

The initial moisture content of the soil was determined by weighing four, 5 gram, soil subsamples, which were dried at 70°C over a 24 hour period. The wet weight (%) of the soil was then calculated. Samples of soil were weighed out, in 5.0 gram sub-samples and known volumes of water (0.05, 0.15, 0.25, 0.5, 0.75, 0.9 and 1.0 ml) were added to duplicate soil samples. The samples were all shaken and left at 4°C, overnight, to enable

equilibration of the soil/water mixture. One replicate was dried at 70°C for moisture content determination and the other was used to measure the water potential using a Wescor HR 33T psychrometer and a C52 sample chamber. The water potential was recorded from the microvolt output and corrected to mega pascals (-MPa).

From the results obtained, data of moisture content (%) vs. water potential (-MPa), and amounts of added water to soil (ml g^{-1}) vs. water potential were constructed.

2.8. EFFECT OF TEMPERATURE ON BIOREMEDIATION OF PESTICIDES USING FUNGI IN SOIL AT FIELD CAPACITY

In this part of the project three sets of experiments were carried out in parallel. In one the natural breakdown of a mixture of the three pesticides in soil was determined. The other two experiments, examined the potential for improving the breakdown of these pesticides in soil. These were carried out by using a single fungal species (*P. versicolor*) or a combination of two (*P. versicolor* and *T. socotrana*). These fungi were used based on the results of screening the four candidate species (see Section 2.5. and 2.6.) for their ability to tolerate and break down the three pesticides. The carrier material for incorporation of these fungi into the soil was wheat straw (cv. Riband). All the degradation experiments were carried out in triplicate in 60 ml amber bottles with Teflon-lined screw cap lids. Separate bottles were used for each of the three replicates, and for each sampling time. Also at each sampling time all the bottles were opened, shaken gently and resealed quickly.

The initial experiments involving pesticides in water, and simazine incorporated agar, showed that very little fungal growth or pesticide breakdown occurred at 10°C. Therefore, in the following experiments, the effect of fungal inoculants on breakdown of 5 and 10 ppm

of the mixture of three pesticides were carried out at 20 and 30°C.

2.8.1. Natural breakdown of the pesticides in soil

Stock solutions were prepared for each pesticide, with simazine in THF, and trifluralin and dieldrin in acetone. The three pesticides were collectively added to a known volume of sterile Reverse Osmosis (R.O.) water and stirred in. To this a calculated amount of soil was added so that the final concentration of each pesticide in the soil was at about 5 ppm for one batch and 10 ppm for the other . A water to soil ratio of 0.1 ml per gram of soil was also achieved. In addition a batch of pesticide-free control soils were also prepared. After leaving the bulk samples at 4°C for 24 hours (with occasional thorough mixing) samples were weighed out (15 grams) and incubated at the relevant temperatures.

Three replicate samples for each treatment initially (0) and after 28, 42 and 70 days were destructively taken and frozen at -80°C until extraction was carried out.

2.8.2. Preparation and inoculation of the straw

The wheat straw was ground in an electric grinder to produce individual fractured pieces, <1.0 cm in length. These were weighed in conical flasks (10 grams, approx.) and autoclaved. Water (18.0 ml, aliquots) was added to each flask and left to equilibrate at 4°C for 24 hours. After this equilibration period, some flasks were inoculated with *P. versicolor* and some with *T. socotrana* by using fifteen 5 mm agar plugs in each flask. The straw inoculated with *T. socotrana* was kept at 30°C and required only five days for sufficient mycelial growth to occur. By contrast growth of *P. versicolor* required 10 -12 days at the same temperature.

2.8.3. Incorporation of fungal inoculants into pesticide contaminated soil

Inoculated straw was incorporated into soil at a ratio of 1 gram straw to 10 gram soil. For the mixed inoculum 0.5 gram of each inoculant was used to maintain the same straw to soil ratio. In both cases the straw was thoroughly mixed into the soil and left at 30°C for 24 hours before wetting up the soil with the water/pesticide mixture. The procedure for preparation of pesticide stock solutions, spiking the water and incorporation into the soil were the same as that described in Section 2.8.1.

In all cases three replicates per treatment were destructively sampled initially (0) and after 28, 42 and 70 days. Samples were stored at -80°C until extraction and quantification.

2.9. EFFECT OF SOIL WATER POTENTIAL ON BIOREMEDIAL CAPABILITIES OF THE FUNGUS *P. VERSICOLOR*

In this experiment the effect of *P. versicolor* on breakdown of the mixture of three pesticides was carried out at -0.065 and -0.28 MPa water potentials. The data on the effect of *P. versicolor* in field capacity soil was obtained from Section 2.8.3.

The incubation temperature used was 20°C, as this was shown to be the temperature at which the bioremedial efficacy could be best observed. Each pesticides concentration in soil was at 10 ppm. The time scales and samples regime was the same as that described in Sections 2.8.2 and 2.8.3.

2.10. METHOD DEVELOPMENT FOR EXTRACTION OF PESTICIDES FROM SOIL

For these tests the concentration of each pesticide in soil was about 10 ppm. The procedure for the addition of pesticides to water and soil were the same as those adopted for real samples (Section 2.8.1.). The extraction solvents used were as follows:

1) Methanol 100 %

2) Methanol 98%, Acetone 1%, THF 1%

3) Methanol 100% saturated with KCl (KCl sat.)

4) Methanol 100% saturated with KCL (KCl sat.) *

5) Methanol (KCl sat.) 98%, Acetone 1%, THF 1%

6) Methanol (KCl sat.) 97%, Acetone 2%, THF 1%

7) Methanol (KCl sat.) 95%, Acetone 4%, THF 1%

8) Methanol (KCl sat.) 89%, Acetone 10%, THF 1%

9) Acetonitrile 100% *

10) Acetone 100% *

For every extraction procedure 10 gram pesticide spiked soil subsamples were weighed (3 or 4 replicates per treatment) and frozen at -80°C for several days. After thawing each sample overnight at 4°C, the 15 ml aliquots of selected extraction solvent was added to the soil. The mixture was sonicated for 10 minutes, and shaken at 400 revs per minutes in a circular motion shaker (IKA-LABORTECHNIK, KS 250 basic) for 10 minutes. The sonication and shaking procedure was then repeated. This mixture was left in the dark (at room temperature) for 10 minutes to allow the soil to settle down. The clear supernatant

solution was then withdrawn with a syringe and filtered using a nylon 0.2 μ syringe filter.

For those solvents marked with an asterisk, an additional 10 minutes sonication and 10 minutes shaking was carried out. The extracts were stored at -28°C until analysis.

2.11. ANALYSIS OF THE SOIL EXTRACTS

2.11.1. Method development for the analysis of soil extracts

Unlike the previous occasions, where only one analyte was under analysis at any one time, in this case three analytes needed to be quantified simultaneously (dieldrin, simazine and trifluralin). From the individual U.V. traces a common wavelength of 215 nm was chosen. The mobile phase flow rate was set in such way that the back pressure did not exceed 2500 psi at any time. This would avoid any detrimental effect on the column.

Initially, a mobile phase of 90% acetonitrile, 10% water was used. The high concentration of the organic phase ensured a rapid elution of the analytes. Each analyte would in turn be injected individually to determine its retention time. The ratio of the solvents would then be altered over a number of runs until sufficient resolution of different peaks could be achieved. In this case the columns used for the analysis of simazine and dieldrin did not produce such separations. The trifluralin (Envirosep-PP) column however produced a separation of about 3 minutes between trifluralin and dieldrin's peak at 50:50, acetonitrile/water mobile phase ratio, although simazine eluted at just under a minute making it very difficult to analyse. At 75: 25, water/acetonitrile ratio simazine eluted at just over 3 minutes but this delayed the elution of the two other peaks to over an hour, clearly not a very practical run time. To redress this, a gradient binary solvent system was developed. During this run the ratio of water to acetonitrile started at 75:25 (to elute simazine in an

appropriate time) and was gradually changed to 50:50 (to resolve and elute trifluralin and dieldrin in a reasonable time) and back to 75:25 ready for the next injection. This produced a viable method for the analysis of the three pesticides in soil extract.

The run time was 30 minutes, with simazine eluting at about 4 minutes, dieldrin at 17 minutes and trifluralin at around 20 minutes. The AUFS value was set at 0.001.

2.11.2. Analysis of the experimental soil samples

The triplicate 15 gram samples were taken and left in the 4°C fridge for thawing overnight. Each sample provided 10 grams of soil for extraction and 5 grams for determination of its moisture content. The moisture content determination method were exactly the same as that described in Section 2.7.2. The extractions were done according to the method number 10 described in Section 2.10. The extraction solvent used was HPLC grade acetone and the extracts were then stored at -28°C until analysis. The final preparation of the sample before injecting onto the HPLC column was the addition of 25% (V/V) HPLC grade water to each soil extract. This was done to improve the peak shape and prevent peak splitting due to the presence of the strong extraction solvent.

For this analysis, the three pesticides stock standard solutions were all prepared, in the extraction solvent. The calibration standards were made up in 50 ml volumetric flasks. To each flask HPLC grade water was added (12.5 ml, aliquots), which was followed by acetone (30 ml). This was spiked with calculated volumes of each pesticide stock solution, and made up to volume using the extraction solvent, hence producing the required concentration. The range of calibration standards were from around 0.20 ppm to about 5.0 ppm. The typical correlation coefficient of the calibration curves ranged from 0.990 to

0.999 (Figure 2.6). The AUFS value was set at 0.001. The acetonitrile part of the mobile phase contained 2% (V/V) THF which helped to sharpen the eluting peaks.

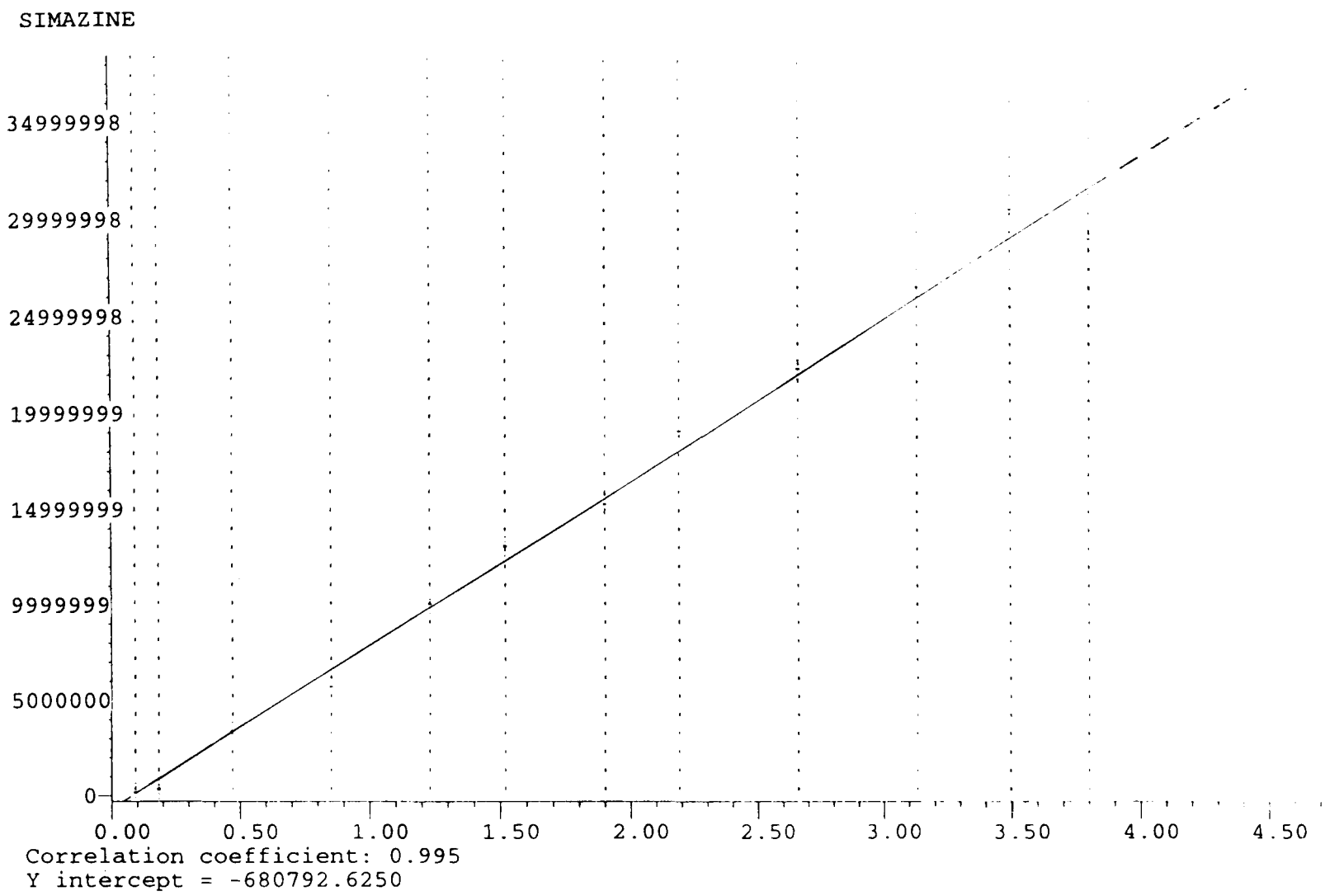


Figure 2.6. Example of a typical calibration curve used in analysis of simazine in soil extracts.

CHAPTER THREE

Results

3.1. IN VITRO SCREENING OF WHITE-ROT FUNGI FOR THEIR ABILITY TO TOLERATE DIFFERENT CONCENTRATIONS OF THE PESTICIDES

3.1.1. Effect of simazine on fungal growth

In this section, the effect of 5 and 10 ppm simazine incorporated into MEA on the growth of various fungi was investigated. These treatments were incubated at 10, 20 and 30°C. Analysis of variance (one-way) of the growth rates was carried out to determine the Least Significant Differences (LSD) to make statistical comparisons between treatments.

Figure 3.1 shows the effects of incubating *P. chrysosporium* and *P. versicolor* in the presence and absence of simazine at 10°C. At this temperature the only fungi that showed any growth were the temperate species *P. chrysosporium* and *P. versicolor*.

Figure 3.1a shows that *P. versicolor* grew best on the simazine-free medium with radial growth decreasing with increasing simazine concentration. Figure 3.1b compares the growth rates (mm day^{-1}) of *P. versicolor* and *P. chrysosporium* based on linear regressions of the temporal extension rates. In the absence of simazine the growth rate of *P. chrysosporium* was higher than *P. versicolor,* although *P. chrysosporium* was very sensitive to simazine, unable to grow in the presence of either 5 or 10 ppm simazine.

P. versicolor was tolerant of 5 ppm simazine with growth rates only marginally lower than the control treatment. This difference was not statistically significant. The growth of *P. versicolor* was inhibited by about 50% in the presence of 10 ppm simazine when compared to 5 ppm.

Figure 3.2a and 3.2b show the effect of 5 and 10 ppm simazine, incorporated

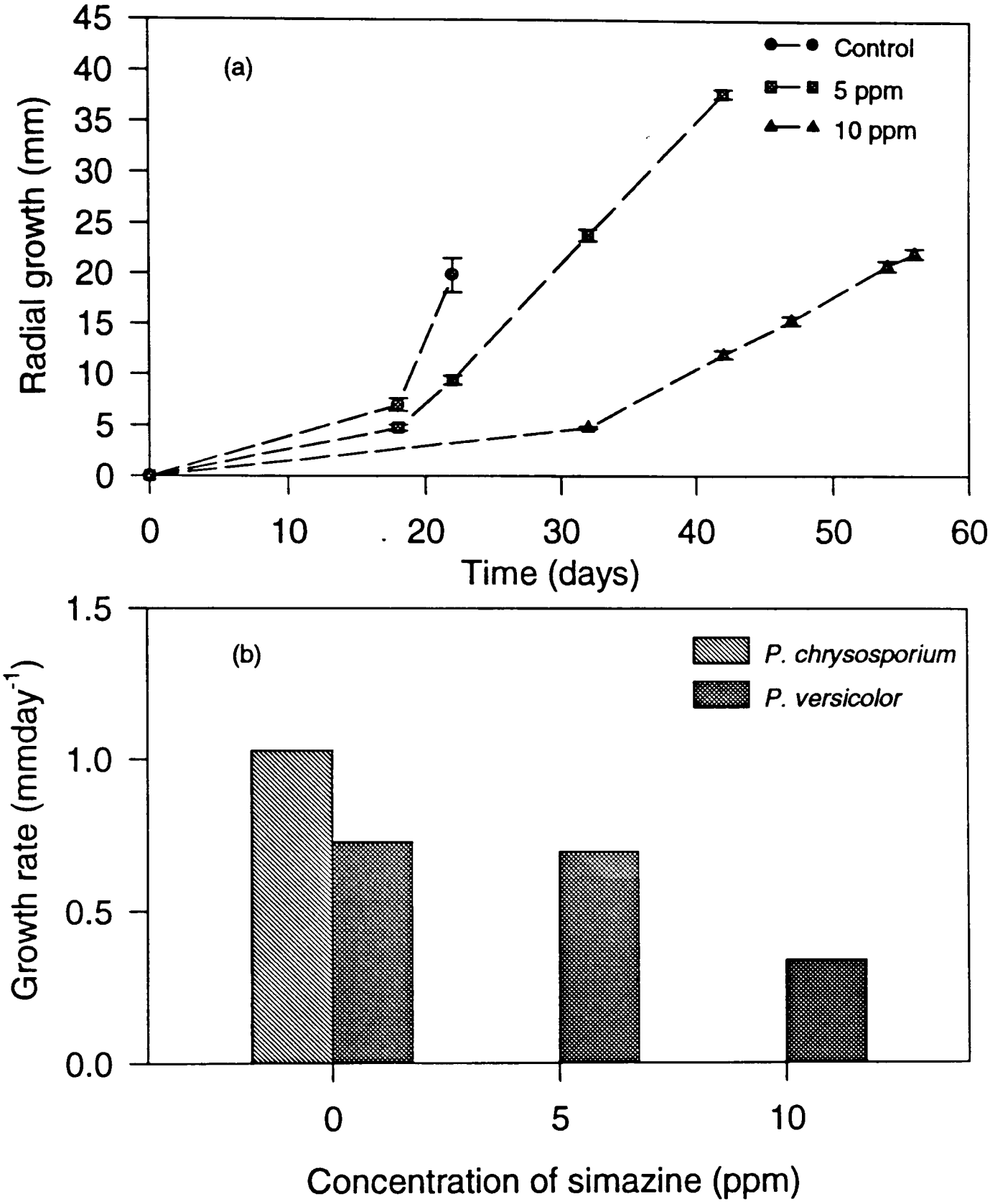


Figure 3.1. Comparison of (a) growth curves of *P. versicolor* in the absence (control) and presence of simazine incorporated into 0.5% malt extract agar incubated at 10°C (error bars indicate standard deviation of the means) and (b) growth rates ($mmday^{-1}$) of *P. versicolor* and *P. chrysosporium* at 10°C. The least significant difference ($P<0.05$) for growth rates was 0.116.

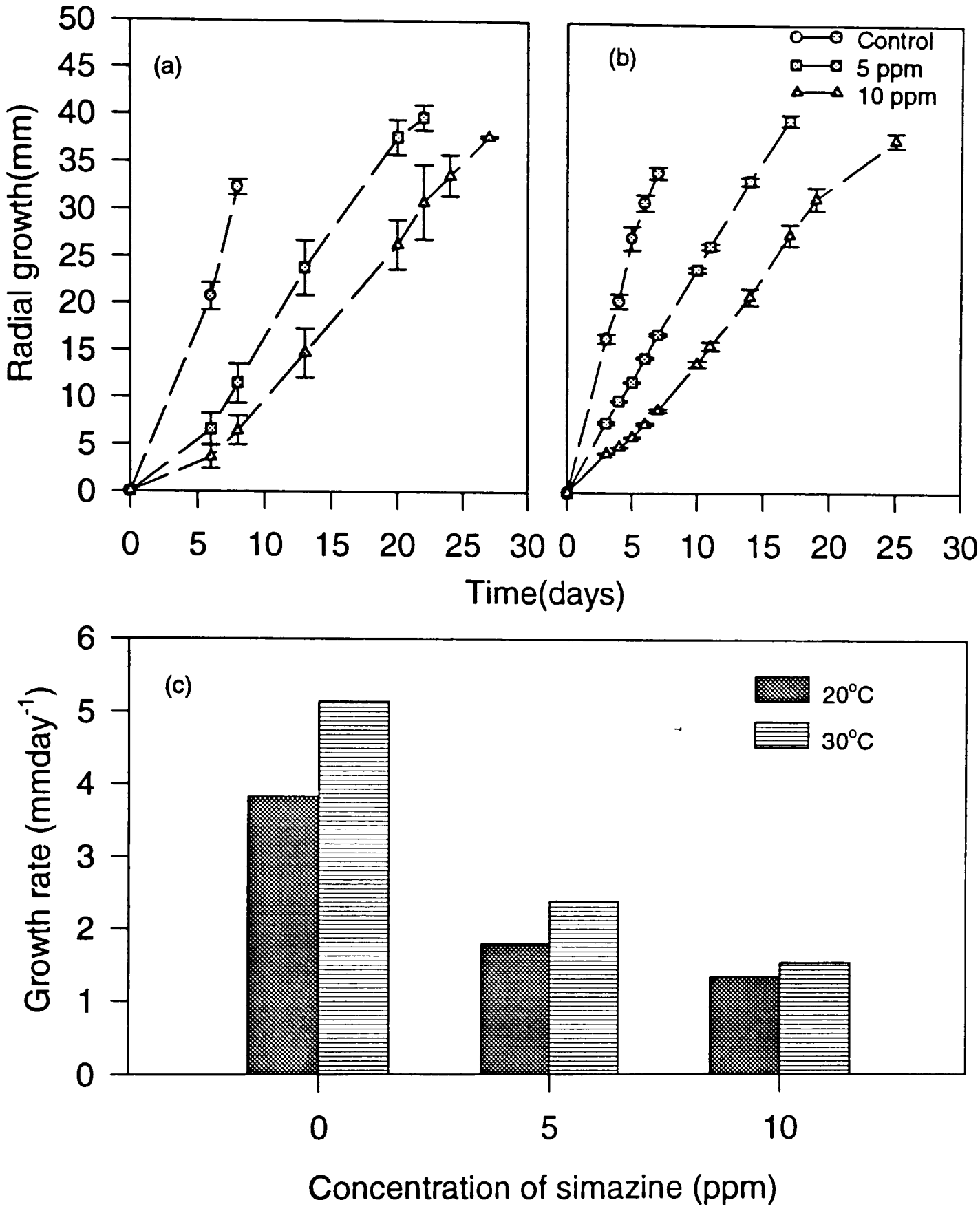


Figure 3.2. Growth curves of *P. versicolor* in the absence (control) and presence of simazine incorporated in 0.5% malt extract agar at 5 and 10 ppm at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.214, 0.123 at 20°C and 30°C respectively.

into solid agar on the temporal growth of *P. versicolor* at 20 and 30°C. This shows that the radial growth was most rapid in the control and decreased proportionally with simazine concentration. Figure 3.2c shows that *P. versicolor* grew better at 30 than 20°C, and growth rates were decreased by about 55 and 65% respectively at 5 and 10 ppm simazine concentrations. The inhibitory effect of increasing simazine concentration was shown to be statistically significant.

Figure 3.3 and 3.4 show the results obtained with *T. socotrana* and *T. cingulata* in the presence and absence of simazine. For both these fungi growth was better at 30 than 20°C in all treatments. Generally, growth rates were significantly ($P < 0.05$) decreased by about 50% with 5 ppm simazine concentration and 65% with 10 ppm.

Figure 3.5 compares the growth rates of these three fungi with *P. chrysosporium* at 20 and 30°C. This shows that in the absence of simazine the temperate fungus *P. chrysosporium* grew faster than the others at both 20 and 30°C. However, growth of *P. chrysosporium* was completely inhibited in the presence of simazine (5 or 10 ppm) in the 20°C incubation treatments (Figure 3.5a). At 30°C, limited growth was observed in the 5 ppm simazine treatment only (Figure 3.5b).

There was a general pattern in the growth rates of the three fungi at 20°C in both control and simazine treatments with growth rates in the order *P. versicolor* > *T. socotrana* > *T. cingulata.* At this temperature the difference in growth rates were statistically significant ($P < 0.05$) between all fungi (where growth was observed). However, the difference between *T. socotrana* and *T. cingulata* in untreated controls, and *P. versicolor* and *T. socotrana* at 5 ppm simazine concentration were found to be small.

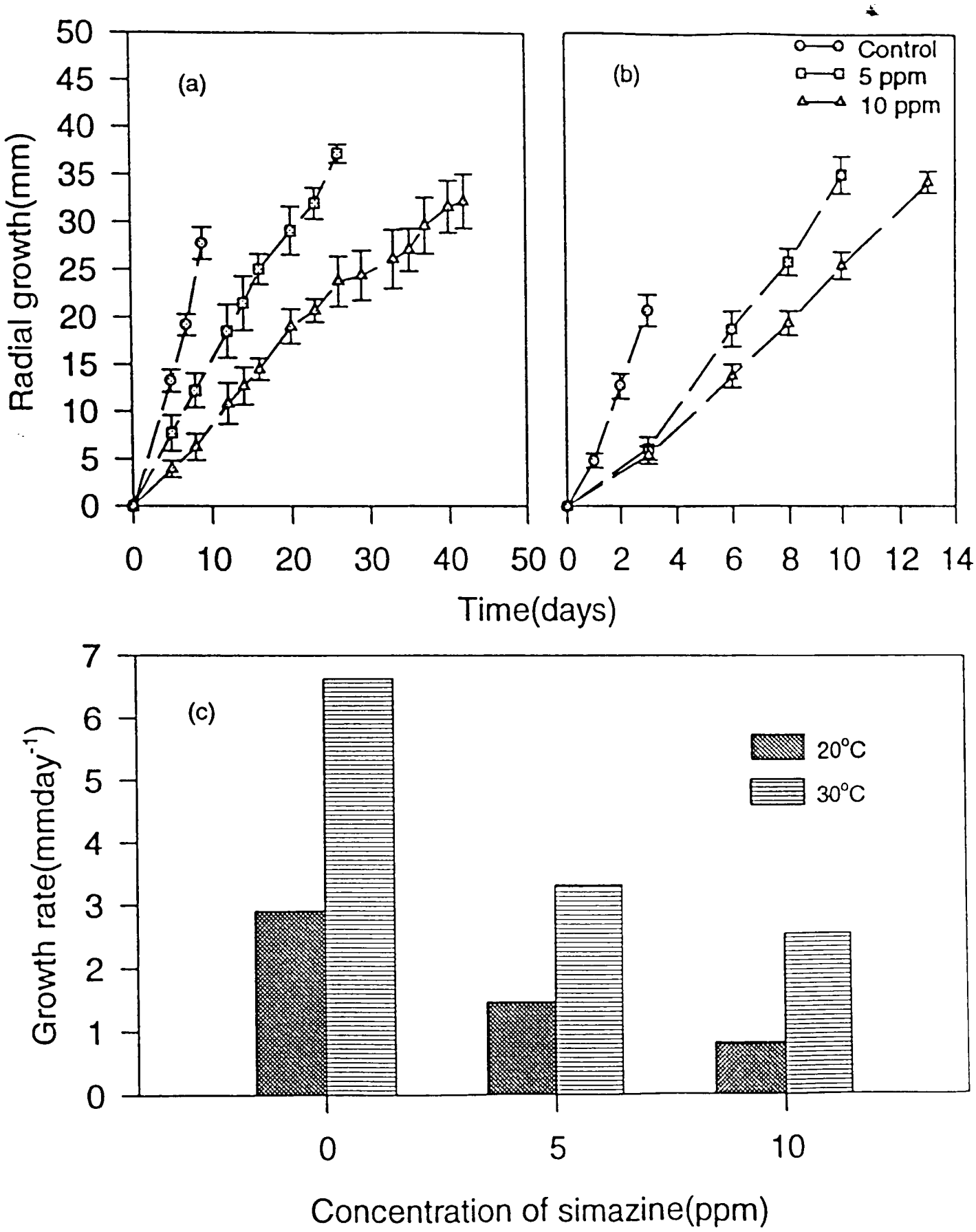
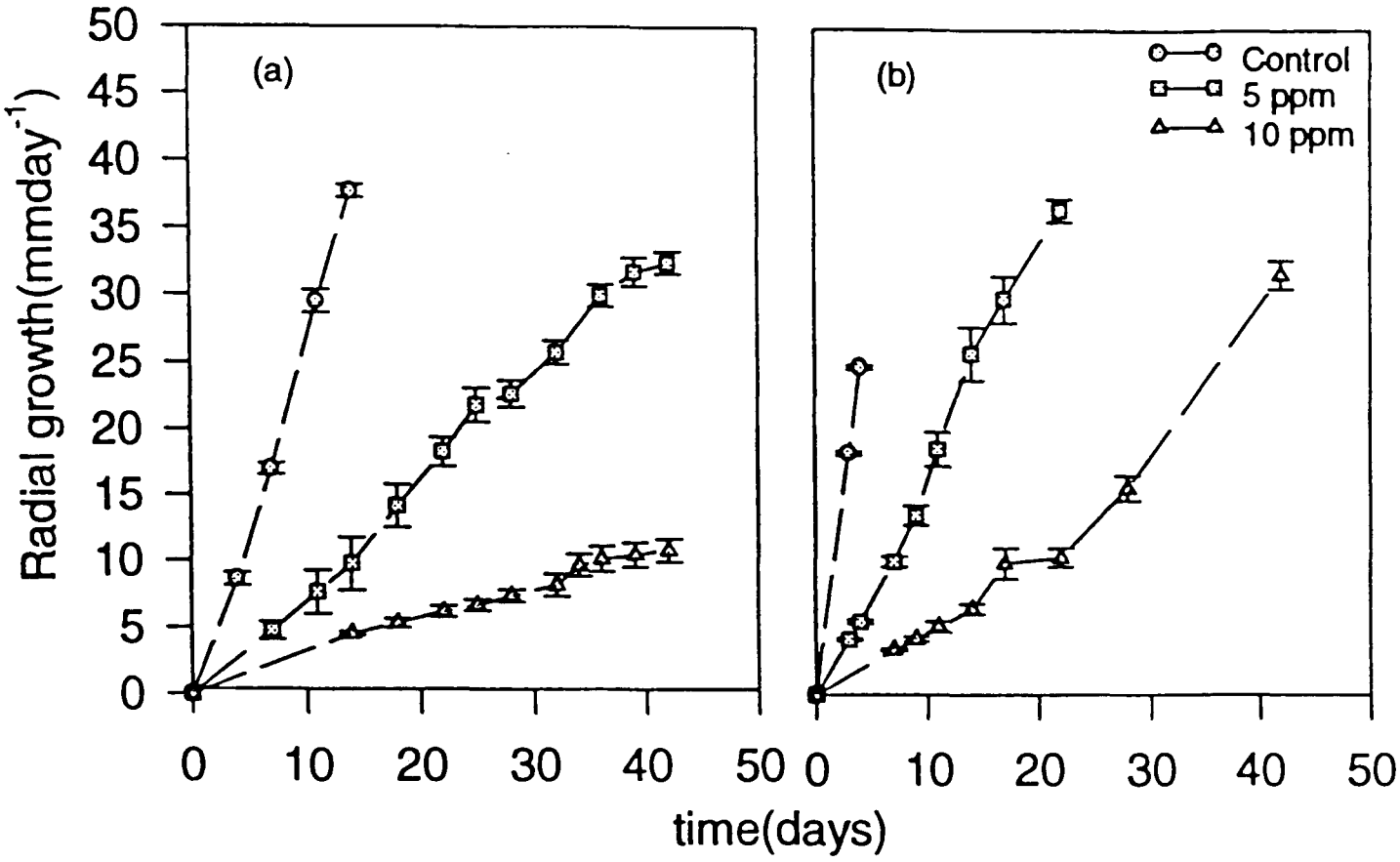


Figure 3.3. Growth curves of *T. socotrana* in the absence (control) and presence of simazine incorporated in 0.5% malt extract agar at 5 and 10 ppm at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.262, 0.184 for 20 and 30°C respectively.



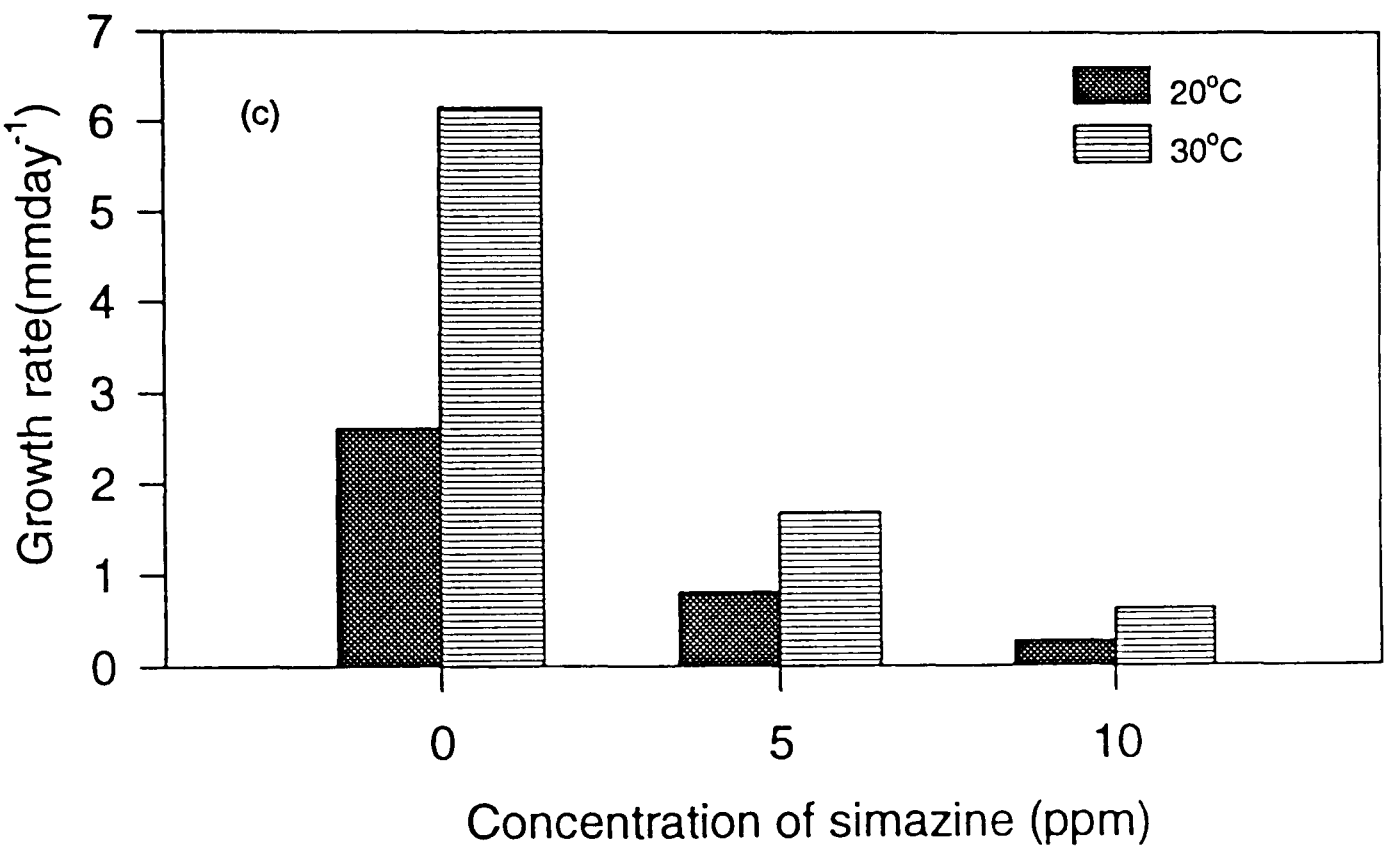


Figure 3.4. Growth curves of *T. cingulata* in the absence (control) and presence of simazine incorporated in 0.5% malt extract agar at 5 and 10 ppm at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differneces ($P<0.05$) for growth rates were 0.824, 0.111 for 20 and 30°C respectively.

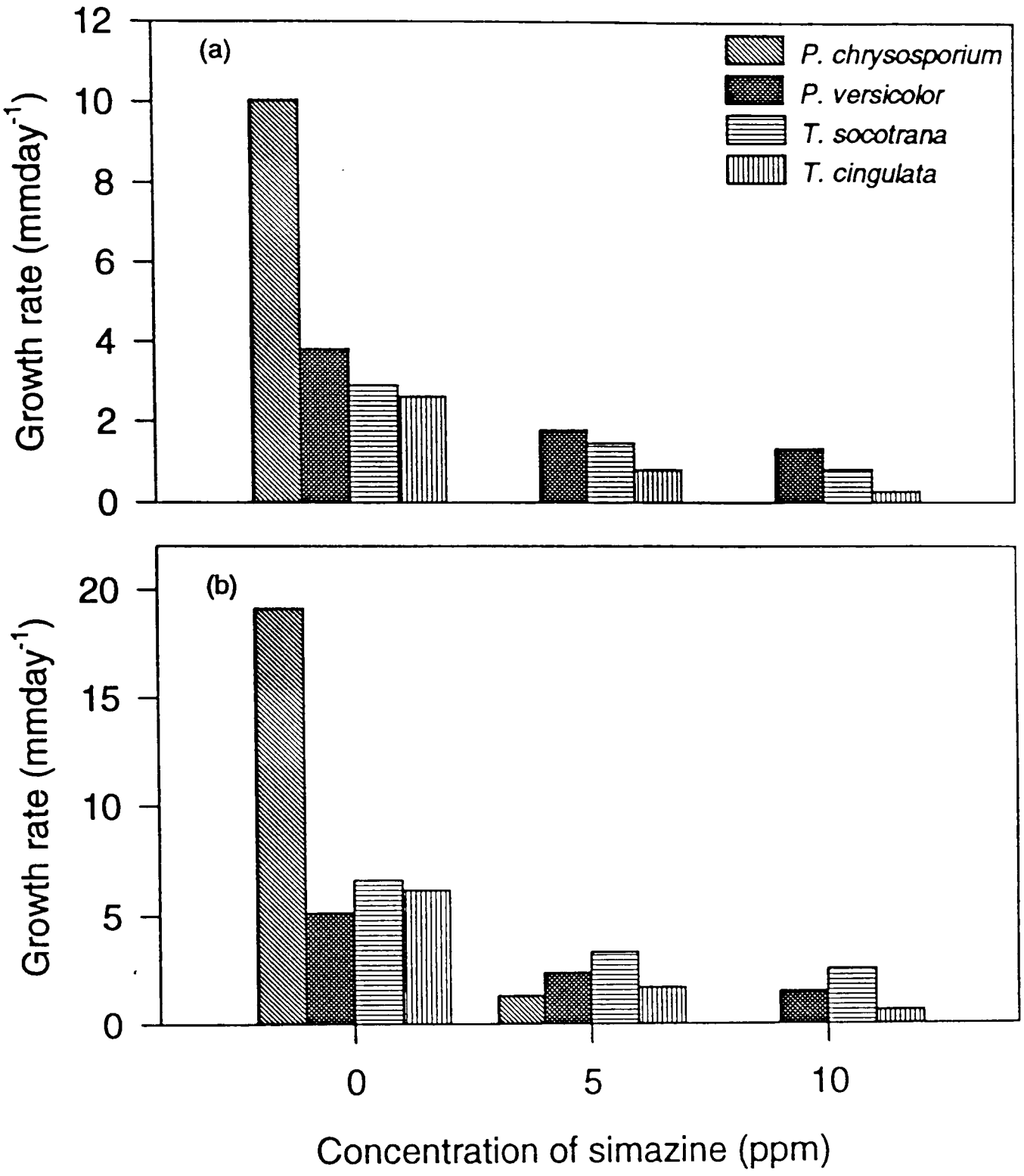


Figure 3.5. Comparison of growth rates of various fungi in the presence and absence of simazine incubated at (a) 20°C and (b) 30°C. The least significant differences ($P<0.05$) were 0.235, 0.138, 0.149 for control, 5 ppm and 10 ppm at 20°C respectively and 0.571, 0.175, 0.162 for control, 5 ppm and 10 ppm at 30°C respectively.

At 30°C the tropical *Trametes* species displayed a slightly faster growth rate on the simazine-free medium than the temperate fungus *P. versicolor*. In the presence of the pesticide (at both 5 and 10 ppm) the tropical fungus *T. socotrana* showed the fastest growth rate followed by *P. versicolor* and *T. cingulata*. Statistical analysis showed that apart from the similarities between growth rates of *T. socotrana* and *T. cingulata* in the untreated controls, all the other treatment effects had a statistically significant effect ($P < 0.05$) on growth rates.

3.1.2. Tolerance to dieldrin of the selected fungi

Figure 3.6 shows that *P. versicolor* was tolerant of both 5 and 10 ppm dieldrin. As with simazine, fungal growth was inhibited by about 50 to 70% with 5 and 10 ppm concentration respectively. Similarly, growth was better at 30 than 20°C.

Figure 3.7 and 3.8 shows that *T. socotrana* and *T. cingulata* were slightly more sensitive than *P. versicolor* to dieldrin, especially at 20°C.

Comparison of the growth rates at 20°C (Figure 3.9a) in the presence and absence of dieldrin had a similar pattern as that observed with simazine. However at 30°C, in the presence of the insecticide dieldrin, *P. versicolor* grew the fastest, followed closely by *T. socotrana* and finally by *T. cingulata* at both 5 and 10 ppm (Figure 3.9b). Again *P. chrysosporium* showed the fastest growth rate in the pesticide-free control treatments at both 20 and 30°C but displayed only slight growth in the presence of 5 ppm dieldrin at 30°C and no growth was observed in 10 ppm dieldrin. Thus the growth rate of *P. versicolor* seemed to be least affected by decreasing temperature or increasing pesticide concentration relative to the other fungi used.

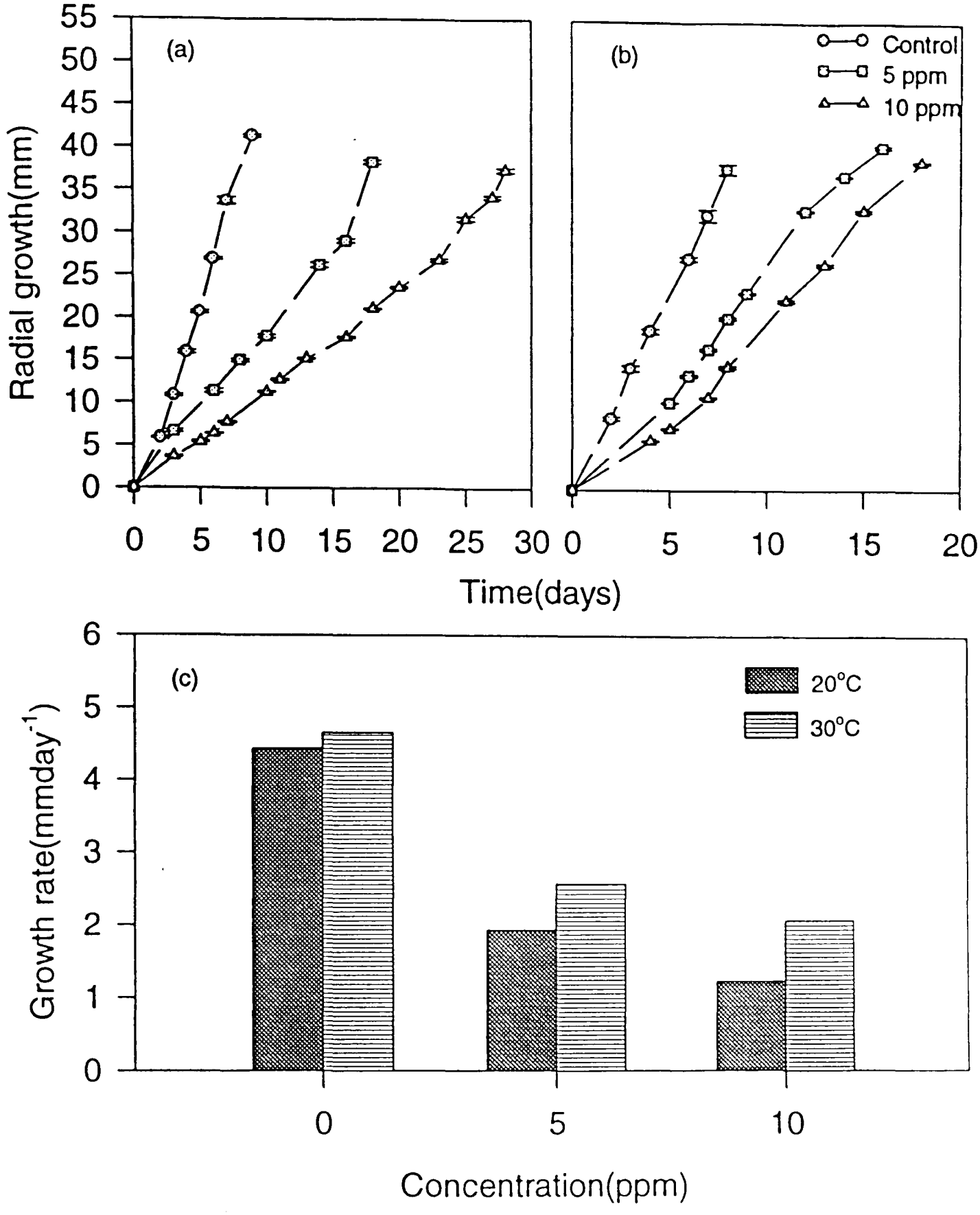


Figure 3.6. Growth curves of *P. versicolor* in the absence (control) and presence of dieldrin incorporated in 0.5% malt extract agar at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.049, 0.087 for 20 and 30°C respectively.

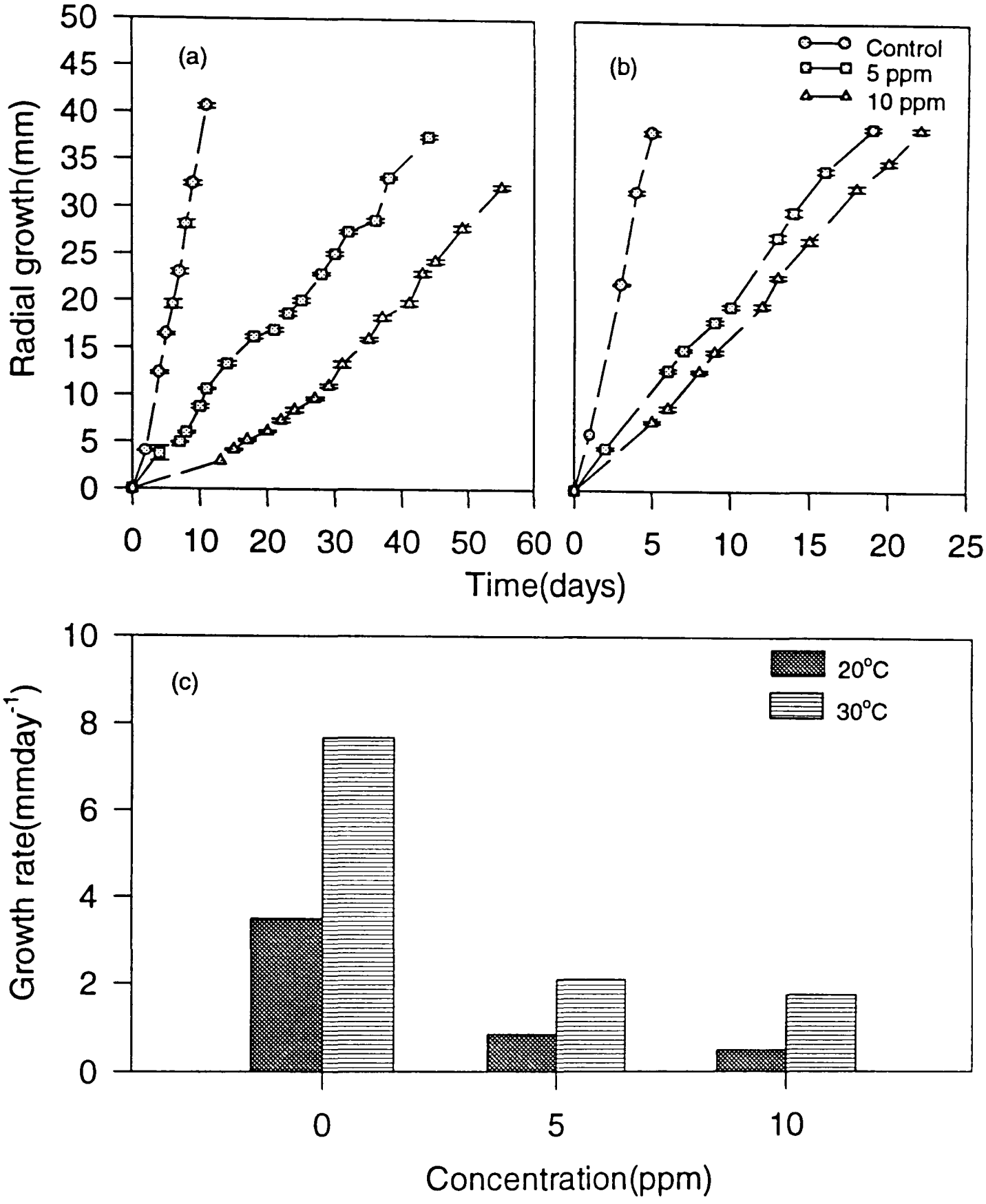
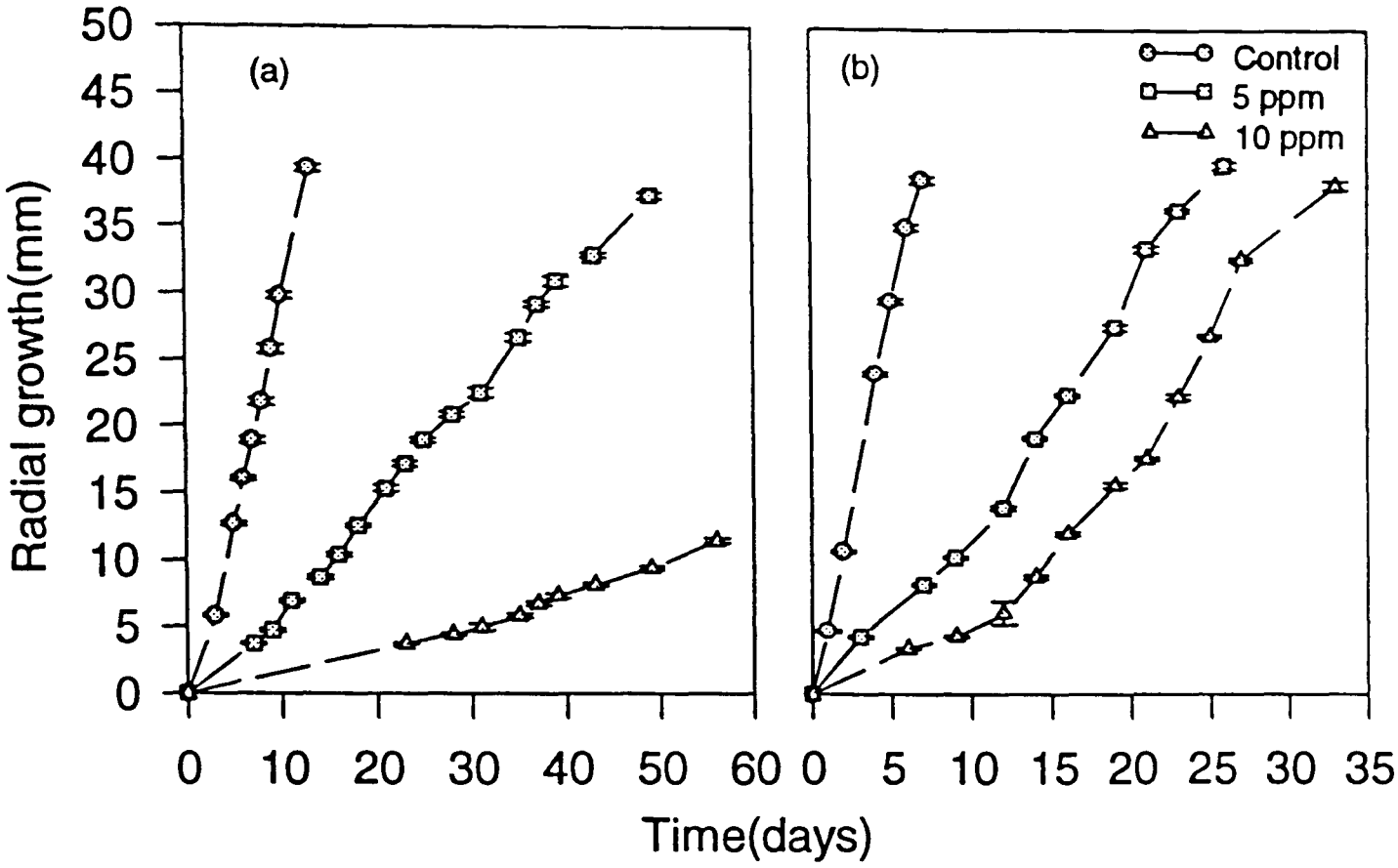


Figure 3.7. Growth curves of *T. socotrana* in the absence (control) and presence of dieldrin incorporated in 0.5% malt extract agar at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.049, 0.060 for 20 and 30°C respectively.



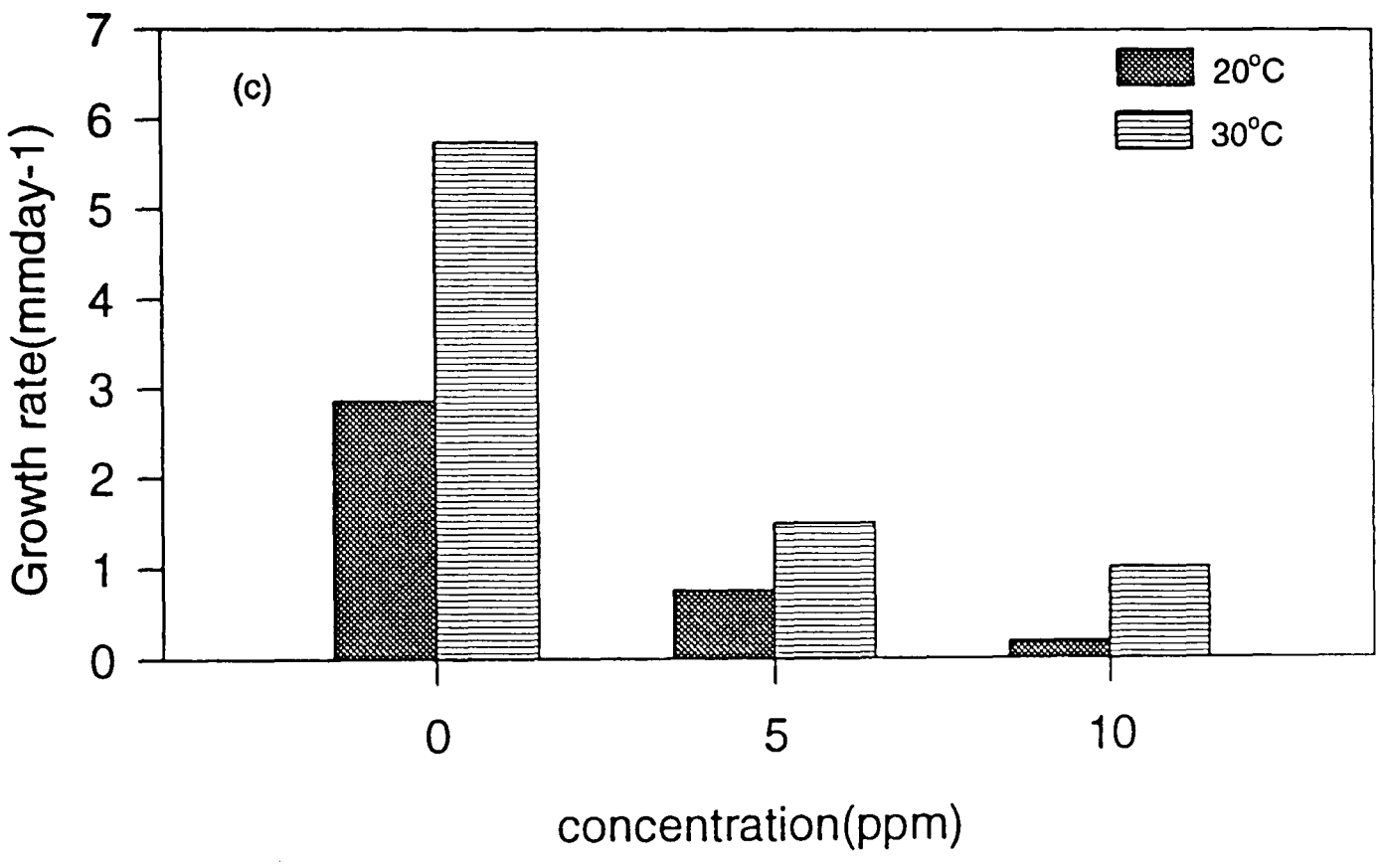


Figure 3.8. Growth curves of *T. cingulata* in the absence (control) and presence of dieldrin incorporated in 0.5% malt extract agar at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.035, 0.049 for 20 and 30°C respectively.

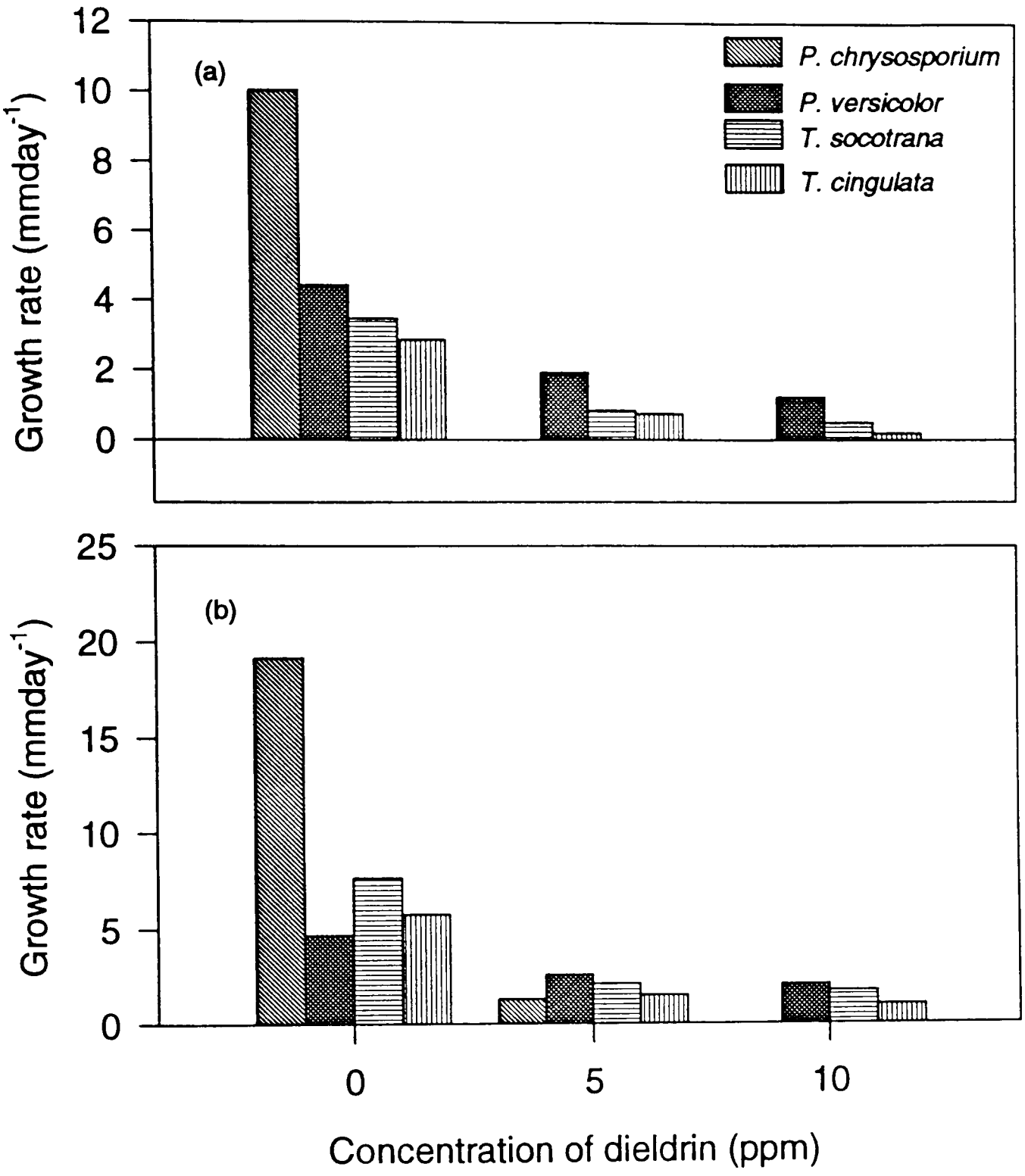


Figure 3.9. Comparison of growth rates of various fungi in the presence and absence of dieldrin incubated at (a) 20°C and (b) 30°C. The least significant differences ($P<0.05$) were 0.073, 0.028, 0.009 for control, 5 ppm and 10 ppm at 20°C respectively and 0.10, 0.053, 0.019 for control, 5 ppm and 10 ppm at 30°C respectively.

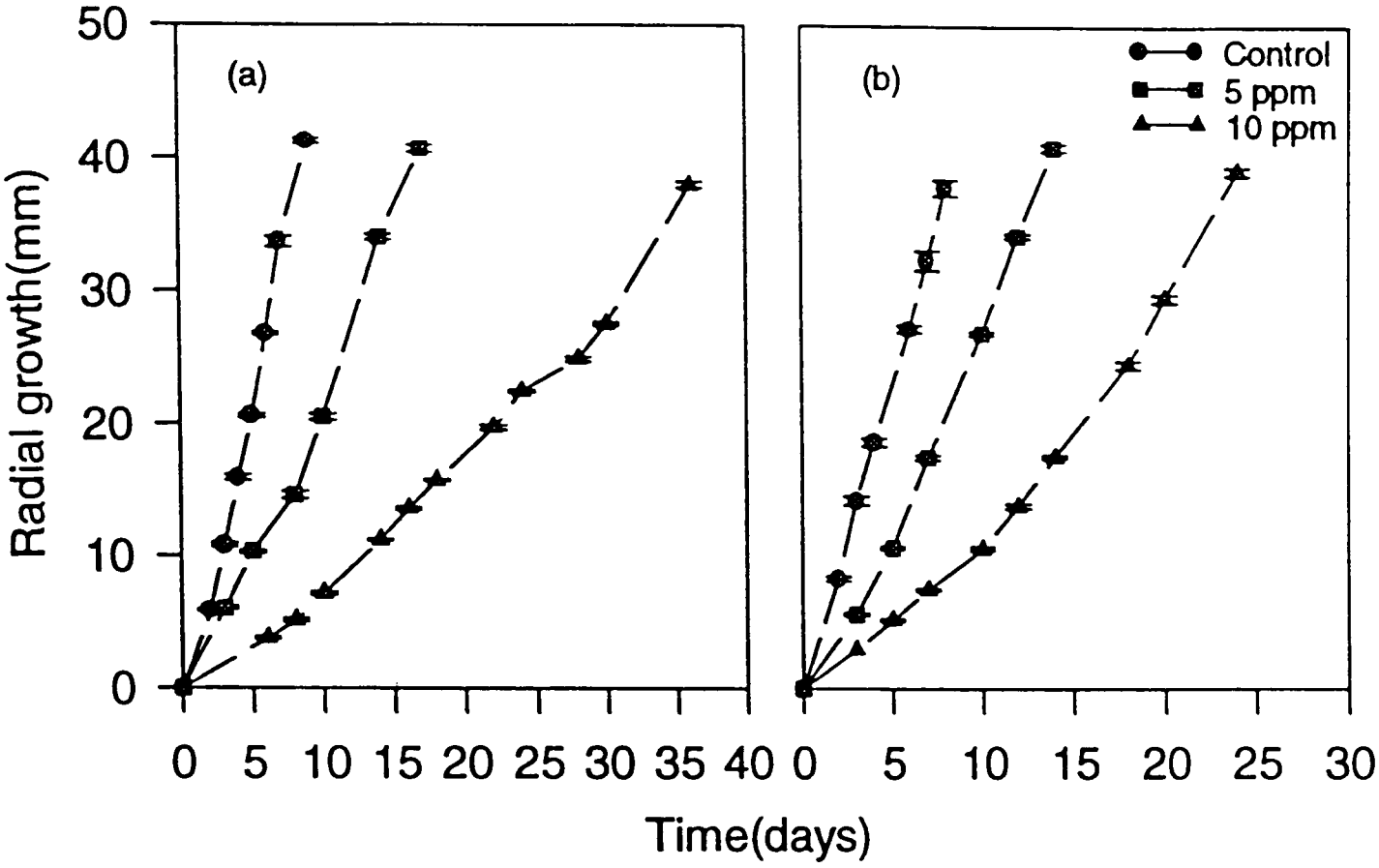
Statistically the difference in growth rates between the fungi were all significant ($P < 0.05$), except for comparisons between *T. socotrana* and *T. cingulata* in the presence of 5 ppm dieldrin, at 20°C.

3.1.3. Fungal growth in the presence of trifluralin

Both the presence of trifluralin, and an increase in the concentration of this herbicide had an inhibitory effect on the growth of the fungi tested (Figures 3.10, 3.11, 3.12). These results were consistent with those obtained with simazine and dieldrin (see Sections 3.1.1. and 3.1.2.). Once again *P. chrysosporium* only grew in the presence of 5 ppm trifluralin at 30°C, and *P. versicolor* was least affected by changes in either temperature or pesticide concentration. Statistical analysis indicated that increasing the herbicide concentration had a significant inhibitory effect ($P < 0.05$) on the growth of the fungi.

On comparing the growth rates at 20°C the pattern obtained appears to be very similar to that of simazine and dieldrin with *P. chrysosporium* showing the fastest growth rate in the absence of the pesticides (Figure 3.13a). At 30°C, in the pesticide-free controls, *P. chrysosporium* had the fastest growth rate followed by the tropical fungi *T. socotrana* and *T. cingulata*, and lastly the temperate *P. versicolor* (Figure 3.13b).

At 30°C *P. versicolor* and *T. socotrana* had the same growth rates when inoculated on agar containing 5 ppm trifluralin, followed by *T. cingulata* and then *P. chrysosporium*. At 10 ppm the growth rate order appeared to follow the trend *P. versicolor* > *T. socotrana* > *T. cingulata*, with no growth observed when the fungus



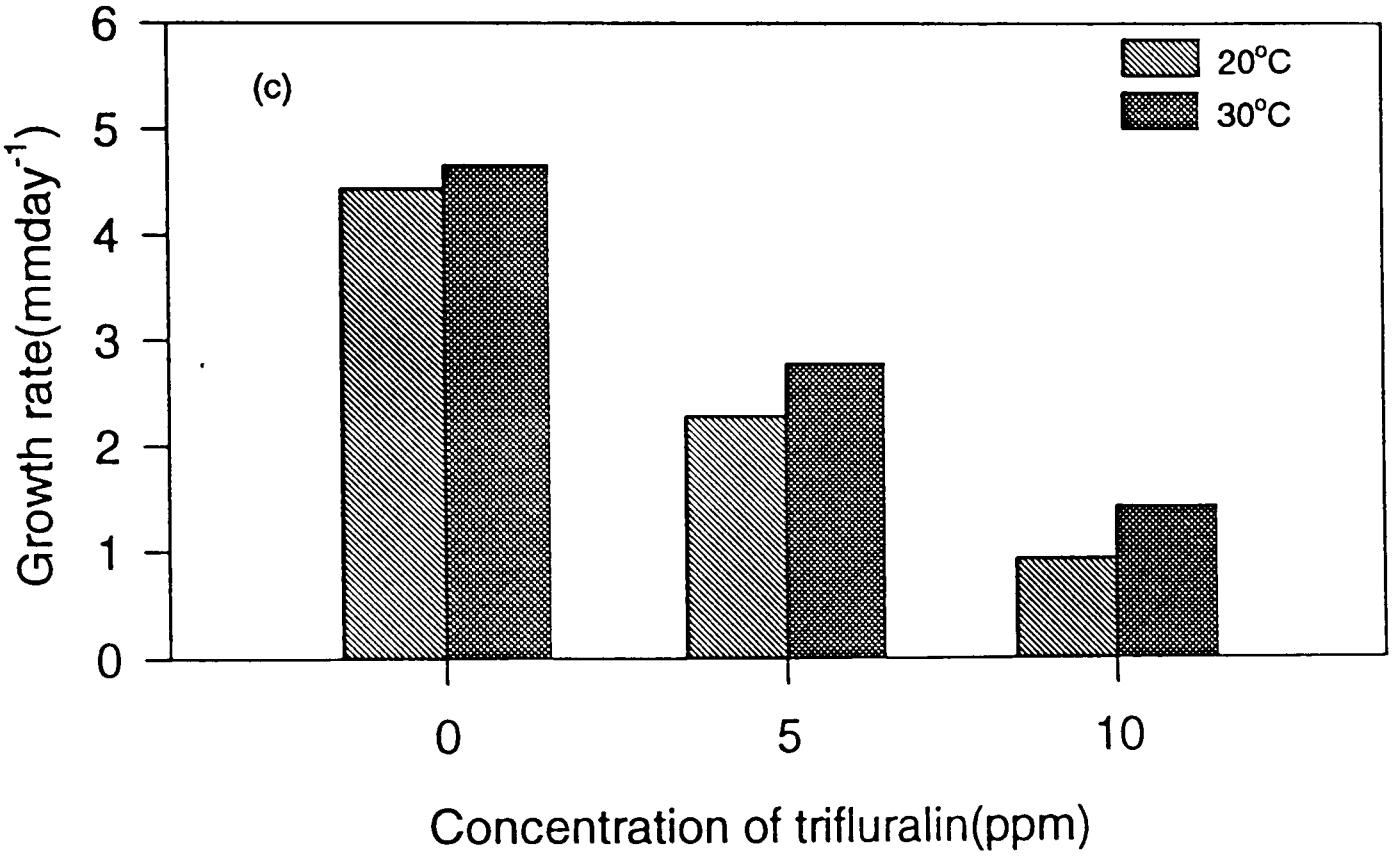
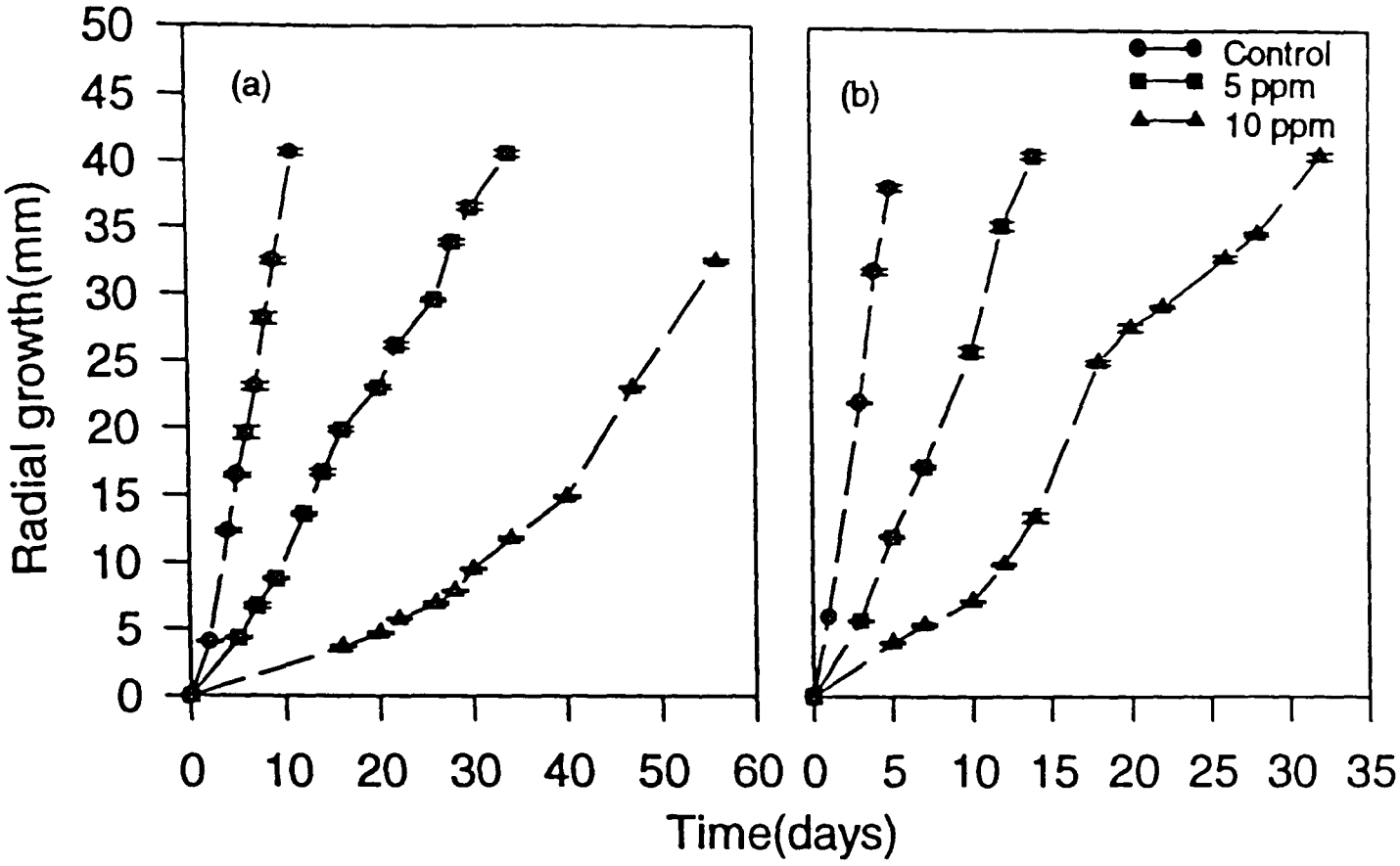


Figure 3.10. Growth curves of *P. versicolor* in the absence (control) and presence of trifluralin incorporated in 0.5% malt extract agar at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.049, 0.089 for 20 and 30°C respectively.



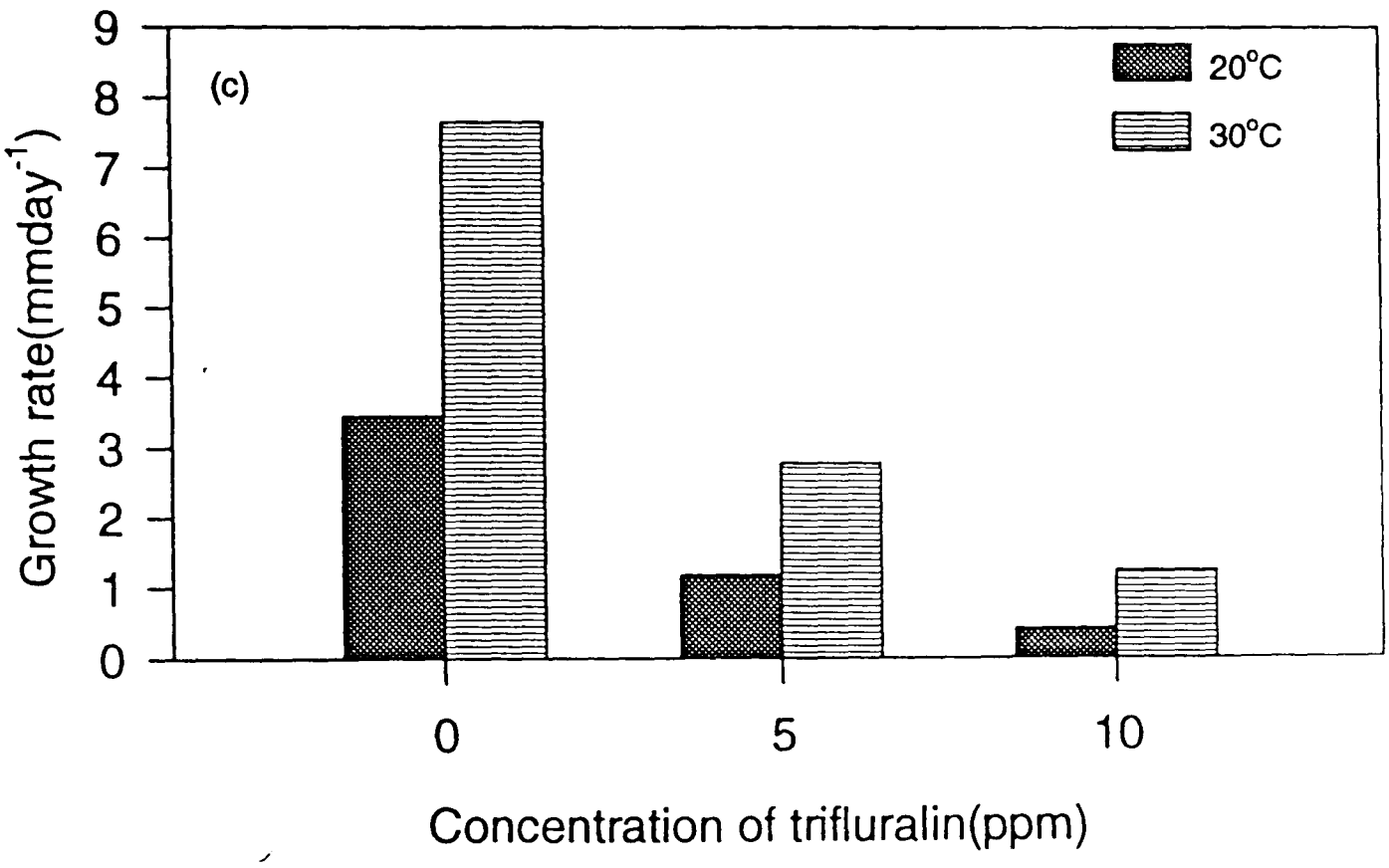


Figure 3.11. Growth curves of *T. socotrana* in the absence (control) and presence of trifluralin incorporated in 0.5% malt extract agar at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.049, 0.060 for 20 and 30°C respectively.

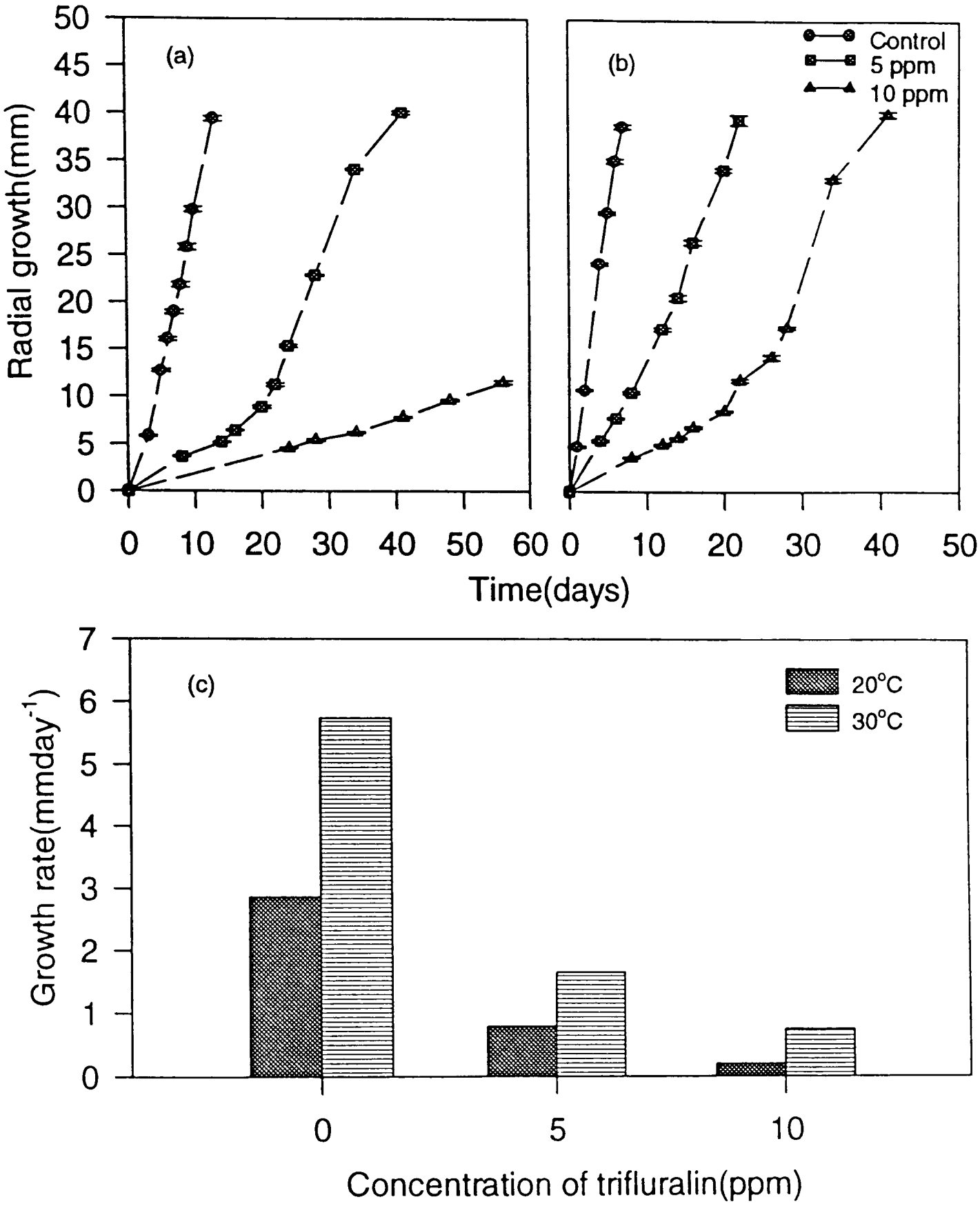


Figure 3.12. Growth curves of *T. cingulata* in the absence (control) and presence of trifluralin incorporated in 0.5% malt extract agar at (a) 20°C and (b) 30°C (error bars indicate the standard deviation of the means) and (c) comparison of growth rates between treatments. The least significant differences ($P<0.05$) for growth rates were 0.035, 0.049 for 20 and 30°C respectively.

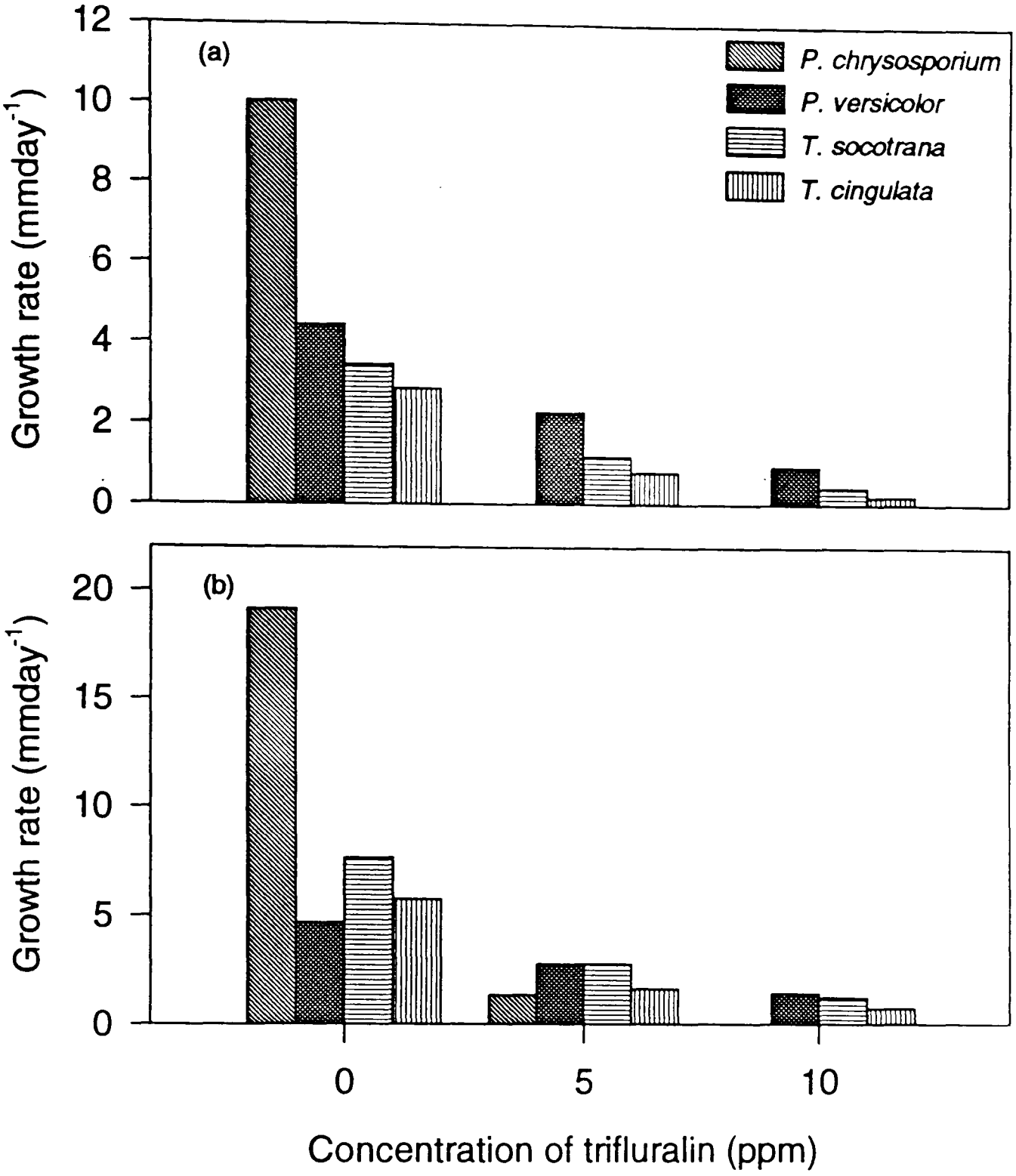


Figure 3.13. Comparison of growth rates of various fungi in the presence and absence of trifluralin incubated at (a) 20°C and (b) 30°C. The least significant differences ($P<0.05$) were 0.073, 0.028, 0.012 for control, 5 ppm and 10 ppm respectively at 20°C and 0.103, 0.057, 0.016 for control, 5 ppm and 10 ppm at 30°C respectively.

P. chrysosporium was used.

Statistical analysis of the growth rates showed a significant difference between the growth rates in each treatment except that between *P. versicolor* and *T. socotrana* in the presence of 5 ppm trifluralin incubated at 30°C, where the growth rates were similar.

3.2. QUANTIFICATION OF RATES OF PESTICIDE BREAKDOWN *IN VITRO*

In this section analysis of variance on control samples and treatments were carried out to facilitate a comparison of the means between the two conditions using LSD.

3.2.1. Method development for the extraction and analysis of pesticide in the agar media

A series of different extraction solvents with the pesticides added at 10 ppm to 0.5% MEA were compared to determine the relative extraction efficiencies for the three pesticides.

Simazine showed the best recovery when the extraction solvent was 100% methanol (Table 3.1), dieldrin with 100% acetone (Table 3.2) and trifluralin with 100% acetonitrile (Table 3.3). Extraction efficiencies were in the range 85-94%.

3.2.2. Effects of temperature and time on fungal breakdown of simazine

Figure 3.14 shows the degradation rate of simazine by *P. versicolor* at 10°C.

Table 3.1: Recovery of simazine from malt extract agar

Extraction solvent	**Mean recovery (%)**
100 % methanol	**85 ± 4.35**
100 % acetonitrile	**59 ± 3.50**

Table 3.2: Recovery of dieldrin from malt extract agar

Extraction solvent	**Mean recovery (%)**
100 % methanol	**77 ± 1.85**
10 % acetone in methanol	**78.5 ± 1.02**
20 % acetone in methanol	**80.1 ± 2.46**
50 % acetone in methanol	**87.0 ± 2.05**
100 % acetone	**88.8 ± 2.83**

Table 3.3: Recovery of trifluralin from malt extract agar

Extraction solvent	**Mean recovery (%)**
100 % methanol	**49.6 ± 1.98**
10 % acetone in methanol	**50.2 ± 2.99**
20 % acetone in methanol	**49.2 ± 2.68**
50 % acetone in methanol	**50.9 ± 2.51**
50 % acetone in acetonitrile	**78.6 ± 2.51**
100 % acetone	**56.4 ± 4.28**
100 % acetonitrile	**94.0 ± 3.03**
100 % ethyl acetate	**ND**

ND: Below detection limit of 0.2 ppm

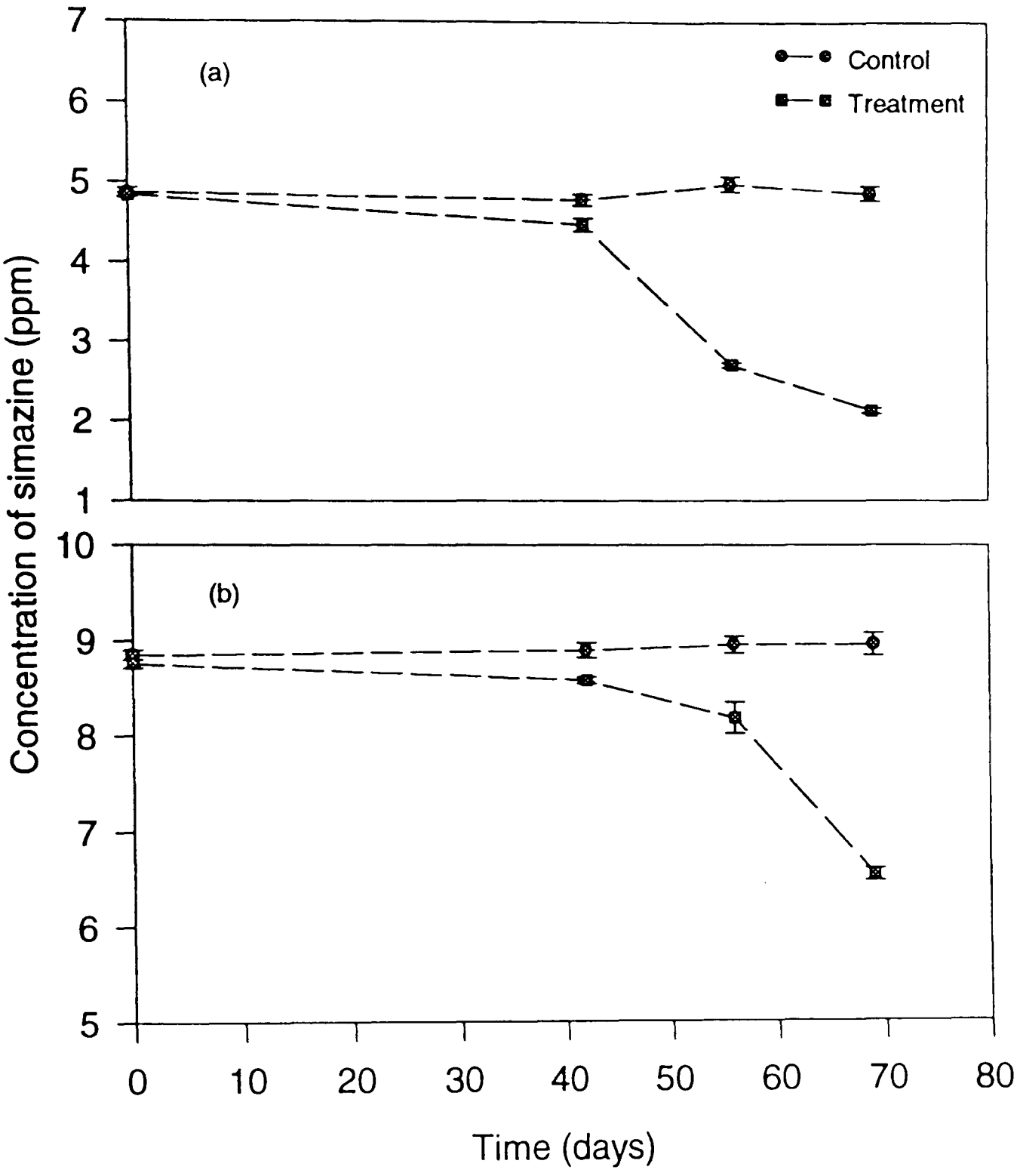


Figure 3.14. Comparison of temporal breakdown of simazine incorporated in 0.5% malt extract agar at (a) 5 ppm and (b) 10 ppm in the absence (control) and presence of *P. versicolor* incubated at 10°C. Error bars indicate the standard deviation of the means. The least significant differences ($P<0.05$) were 0.109, 0.155 for 5 and 10 ppm respectively.

Here, for both 5 and 10 ppm concentrations, the starting point of any noticeable breakdown did not begin until approximately 40 days after incubation when a significant difference was observed between the control and treatments.

Figure 3.15a and 3.15b compare the concentrations of simazine (initially 5 and 10 ppm) with the control for *P. versicolor* (treatment) over 42 and 56 days incubation period respectively. This shows little difference was observed between controls and treatments during the first 12-14 days of incubation. However, after about 28 and 40 days there were statistically significant differences between treated and untreated controls. The breakdown rate was significantly better at 30 than 20°C.

T. socotrana also demonstrated an ability to degrade simazine at both 20 and 30°C indicating a significant difference between control and pesticide treatment in both cases (Figure 3.16). For *T. cingulata* no clear degradation of simazine was apparent at 20°C even after 40 days incubation (Figure 3.17a). However at 30°C, *T. cingulata* showed an ability to significantly degrade simazine at both 5 and 10 ppm after 40 days (10 ppm) and 28 days (5 ppm) (Figure 3.17b).

By contrast, only a slight breakdown was observed in the 5 ppm treatment at 30°C for *P. chrysosporium* (Figure 3.18a). This fungus was unable to degrade any of the pesticides at 10 ppm concentration, where no growth occurred (Figure 3.18b).

Table 3.4. summarises the final percentage breakdown of simazine relative to untreated controls in the presence of the four fungi at 10, 20 and 30°C. This shows that *T. socotrana* and *P. versicolor* demonstrated the greatest ability to degrade simazine.

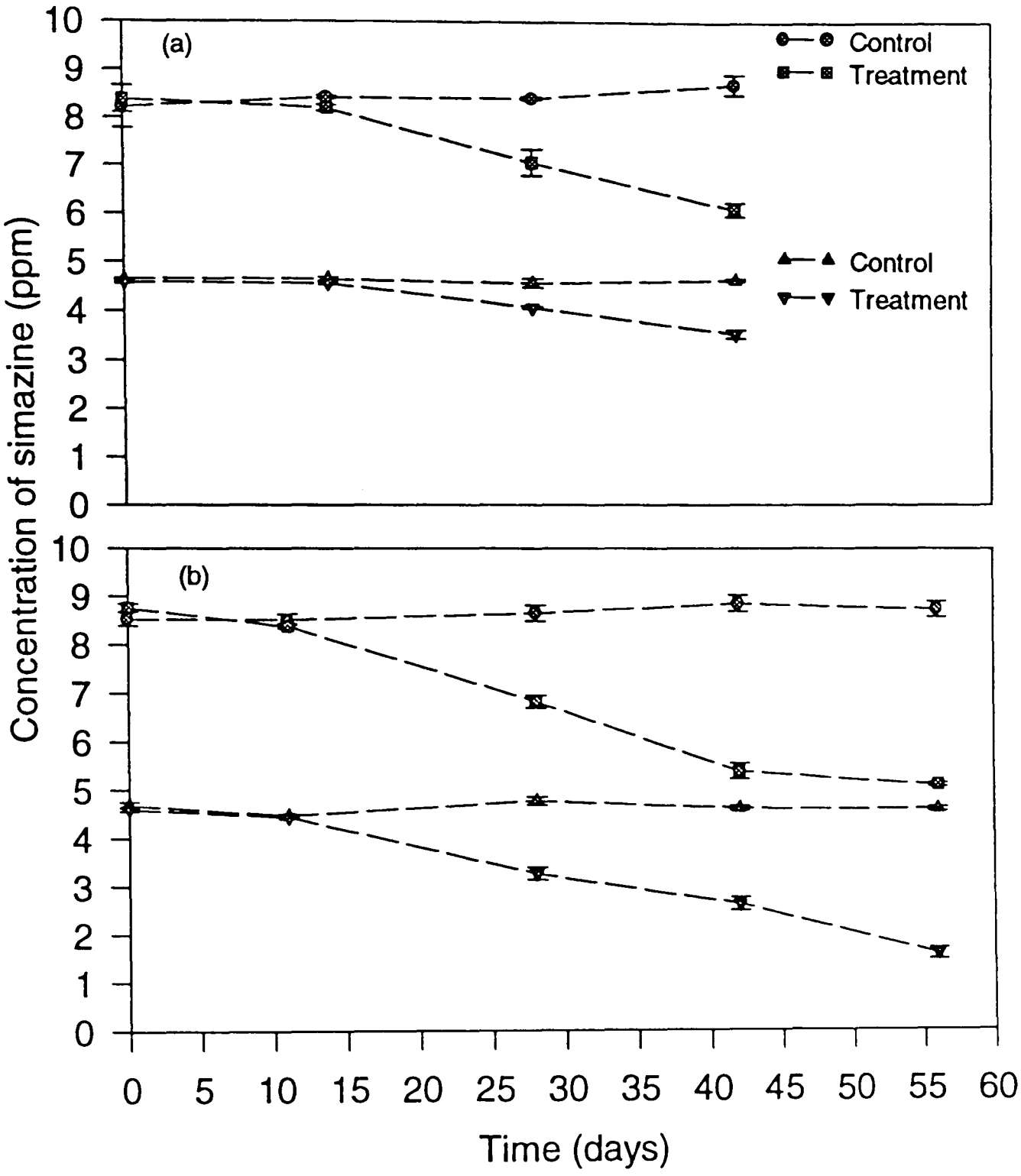


Figure 3.15. Comparison of temporal breakdown of simazine incorporated in 0.5% malt extract agar at 5 and 10 ppm in the absence (control) and presence of *P. versicolor* incubated at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. The least significant difference ($P<0.05$) were 0.077, 0.396 for 5 and 10 ppm at 20°C respectively and 0.142, 0.222 for 5 and 10 ppm at 30°C.

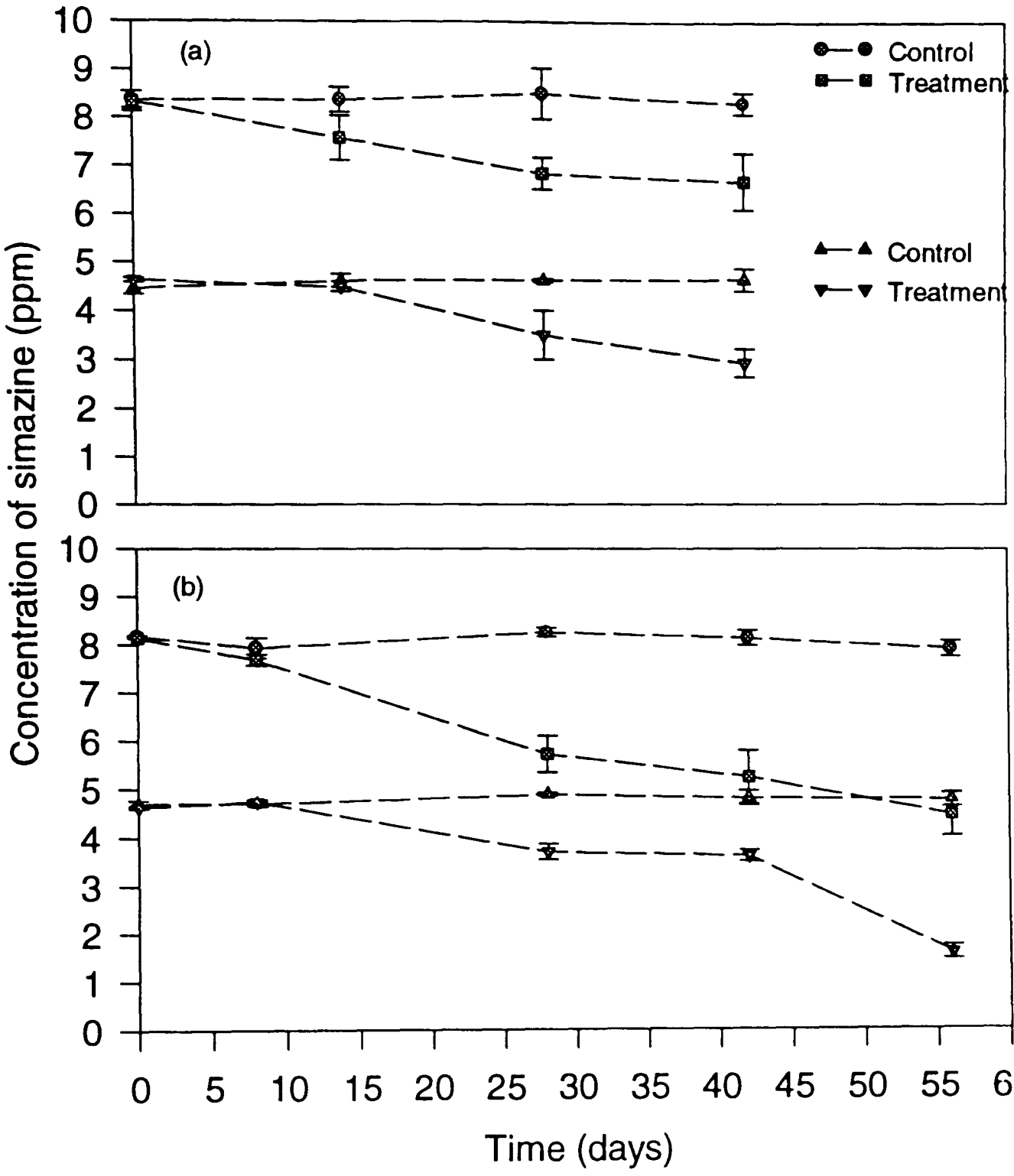


Figure 3.16. Comparison of temporal breakdown of simazine incorporated in 0.5% malt extract agar at 5 and 10 ppm in the absence (control) and presence of *T. socotrana* incubated at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. The least significant difference ($P<0.05$) were 0.406, 0.654 for 5 and 10 ppm at 20°C respectively and 0.187, 0.473 for 5 and 10 ppm at 30°C.

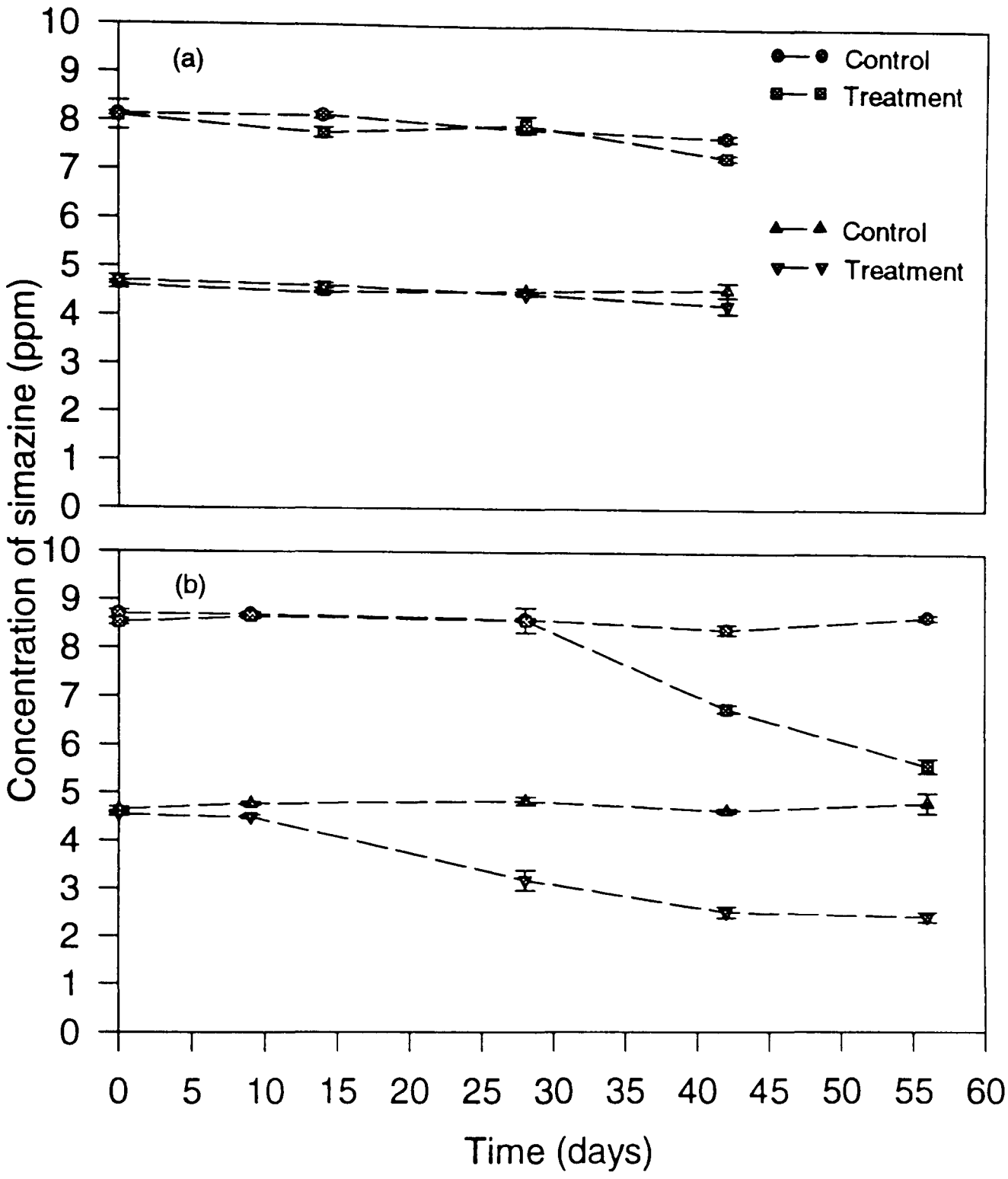


Figure 3.17. Comparison of temporal breakdown of simazine incorporated in 0.5% malt extract agar at 5 and 10 ppm in the absence (control) and presence of *T. cingulata* incubated at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. The least significant difference ($P<0.05$) were 0.173, 0.238 for 5 and 10 ppm at 20°C respectively and 0.194, 0.194 for 5 and 10 ppm at 30°C.

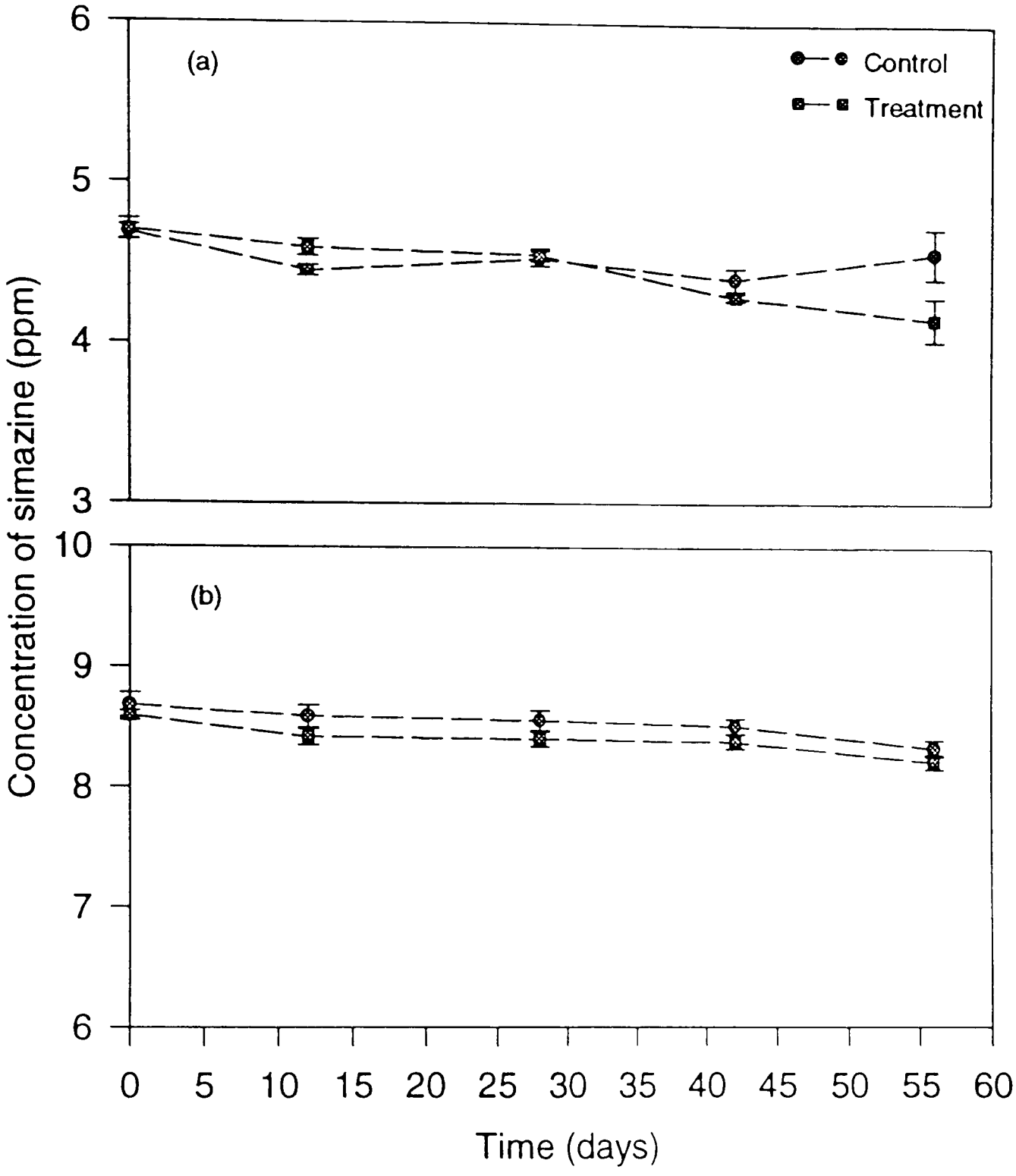


Figure 3.18. Comparison of temporal breakdown of simazine incorporated in 0.5% malt extract agar at (a) 5 ppm and (b) 10 ppm in the absence (control) and presence of *P. chrysosporium* incubated at 30°C. The error bars indicate the standard deviation of the means. The least significant differences ($P<0.05$) were 0.132, 0.103 for 5 and 10 ppm respectively.

Table 3.4. Percentage breakdown of simazine relative to untreated controls by *P. chrysosporium*, *P. versicolor*, *T. socotrana* and *T. cingulata* at 10, 20 and 30°C.

Fungi	Incubation period (days)	Incubation temperature (°C)	Initial concentration (ppm)	Percentage breakdown (%)
P. chrysosporium	**0**	**20**	**5**	**-**
P. chrysosporium	**42**	**20**	**-**	**NFG**
P. chrysosporium	**0**	**20**	**10**	**-**
P. chrysosporium	**42**	**20**	**-**	**NFG**
P. chrysosporium	**0**	**30**	**5**	**-**
P. chrysosporium	**56**	**30**	**-**	**9.2**
P. chrysosporium	**0**	**30**	**10**	**-**
P. chrysosporium	**56**	**30**	**-**	**NFG**
P. versicolor	**0**	**10**	**5**	**-**
P. versicolor	**69**	**10**	**-**	**55.8**
P. versicolor	**0**	**10**	**10**	**-**
P. versicolor	**69**	**10**	**-**	**25.3**
P. versicolor	**0**	**20**	5	**-**
P. versicolor	**42**	**20**	**-**	**27.0**
P. versicolor	**0**	**20**	**10**	**-**
P. versicolor	**42**	**20**	**-**	**22.2**
P. versicolor	**0**	**30**	**5**	**-**
P. versicolor	**56**	**30**	**-**	**63.6**
P. versicolor	**0**	**30**	**10**	**-**
P. versicolor	**56**	**30**	**-**	**41.7**
T. cingulata	**0**	**20**	**5**	**-**
T. cingulata	**42**	**20**	**-**	**9.5**
T. cingulata	**0**	**20**	**10**	**-**
T. cingulata	**42**	**20**	**-**	**4.5**
T. cingulata	**0**	**30**	5	**-**
T. cingulata	**56**	**30**	**-**	**47.1**

Table 3.4. Continued

Fungi	Incubation period (days)	Incubation temperature (°C)	Initial concentration (ppm)	Percentage breakdown (%)
T. cingulata	**0**	**30**	**10**	**-**
T. cingulata	**56**	**30**	**-**	**34.6**
T. socotrana	**0**	**20**	**5**	**-**
T. socotrana	**42**	**20**	**-**	**36.4**
T. socotrana	**0**	**20**	**10**	**-**
T. socotrana	**42**	**20**	**-**	**19.8**
T. socotrana	**0**	**30**	**5**	**-**
T. socotrana	**56**	**30**	**-**	**65.6**
T. socotrana	**0**	**30**	**10**	**-**
T. socotrana	**56**	**30**	**-**	**45.10**

NFG: No Fungal Growth

3.2.3. Effect of temperature and time on fungal breakdown of dieldrin

For this pesticide, *P. versicolor* showed little or no capability for breakdown of dieldrin at either 20 or 30°C (Figure 3.19). Surprisingly at 20°C, 10 ppm samples appeared to show a greater relative breakdown than the 5 ppm samples. However, at 30°C this was reversed and there was negligible breakdown of the 10 ppm samples but a slightly higher breakdown rate for the 5 ppm samples, where there was a statistically significant difference between the control and treatments.

At 20°C, *T. socotrana*, displayed a greater ability to degrade 5 ppm than 10 ppm dieldrin with statistically significant differences between the treatment and controls after both 28 and 56 days (Figure 3.20a). This trend was also repeated at 30°C (Figure 3.20b).

T. cingulata was unable to degrade 10 ppm dieldrin samples at 20°C over the time period of the experiment (Figure 3.21a). However at 5 ppm concentration, dieldrin was significantly degraded after 28 and 56 days. These were shown to be statistically significant. At 30°C, degradation of both 5 and 10 ppm dieldrin were more rapid, with significant breakdown achieved after 28 and 56 days (Figure 3.21b).

Table 3.5 summarises the relative percentage breakdown of dieldrin in the presence of the four fungi at 20 and 30°C. From this data *T. socotrana* had the best capability for degrading dieldrin followed closely by *T. cingulata*. However, *P. versicolor* appeared to be only slightly better than *P. chrysosporium* in degrading 5 ppm dieldrin at 30°C, the only treatment in which *P. chrysosporium* showed any growth.

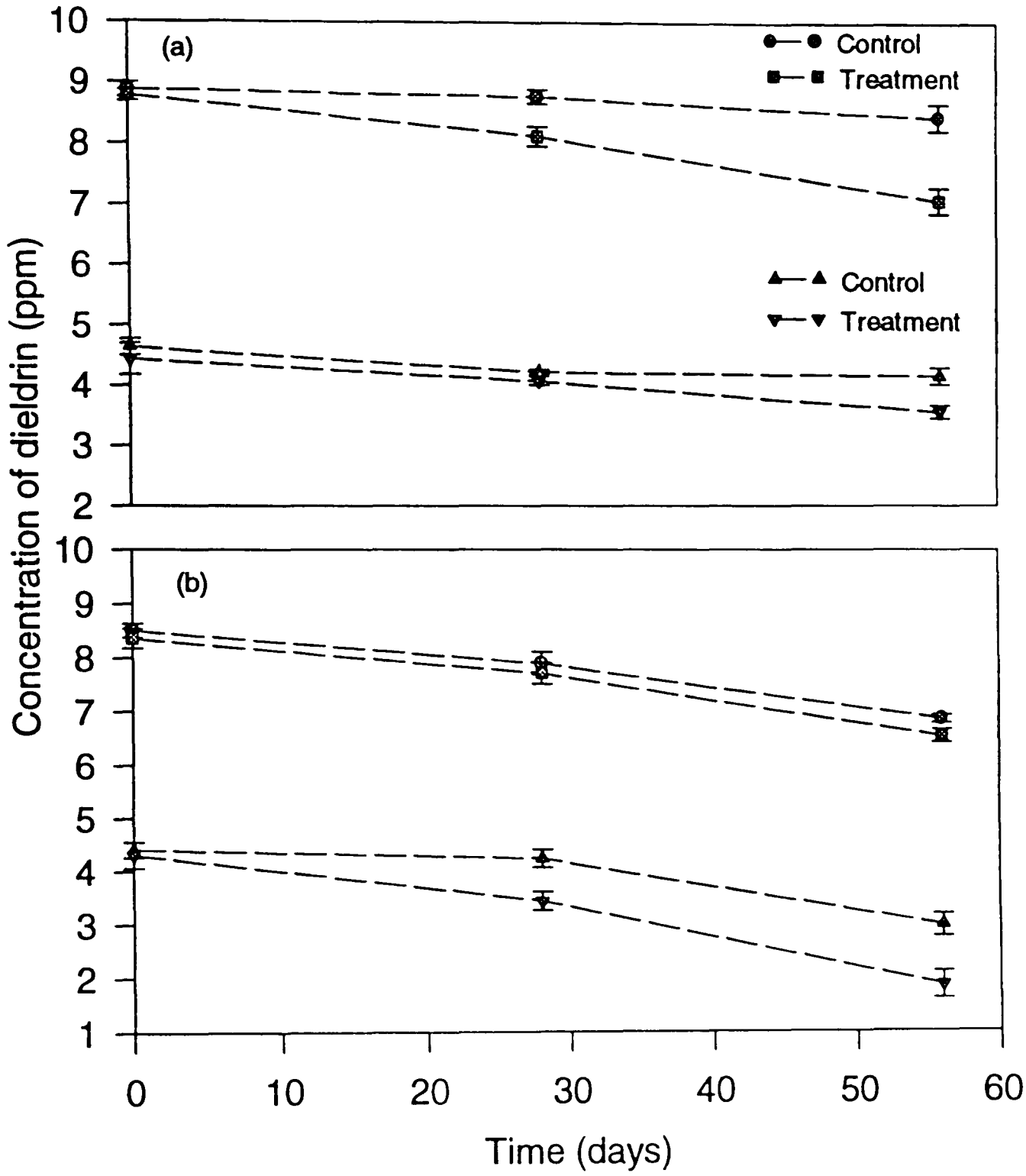


Figure 3.19. Comparison of temporal breakdown of dieldrin incorporated in 0.5% malt extract agar at 5 and 10 ppm in the absence (control) and presence of *P. versicolor* incubated at (a) 20°C and (b) 30°C. The error bars indicate the standard deviation of the mean. The least significant differences were 0.258, 0.281 for 5 and 10 ppm at 20°C respectively and 0.365, 0.287 for 5 and 10 ppm at 30°C respectively.

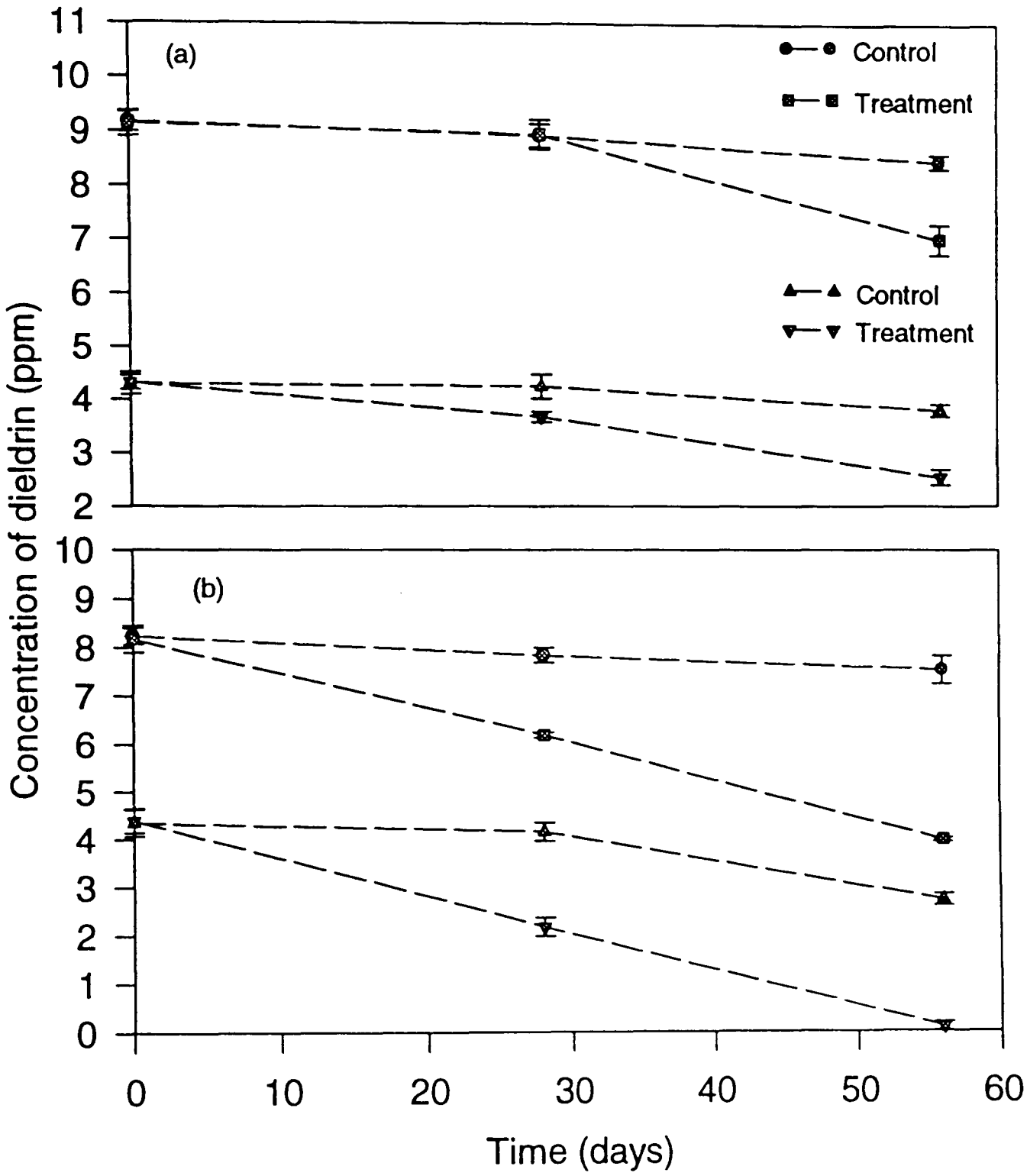


Figure 3.20. Comparison of temporal breakdown of dieldrin incorporated in 0.5% malt extract agar at 5 and 10 ppm in the absence (control) and presence of *T. socotrana* incubated at (a) 20°C and (b) 30°C. The error bars indicate the standard deviation of the mean. The least significant differences were 0.292, 0.402 for 5 and 10 ppm at 20°C respectively and 0.360, 0.342 for 5 and 10 ppm at 30°C respectively.

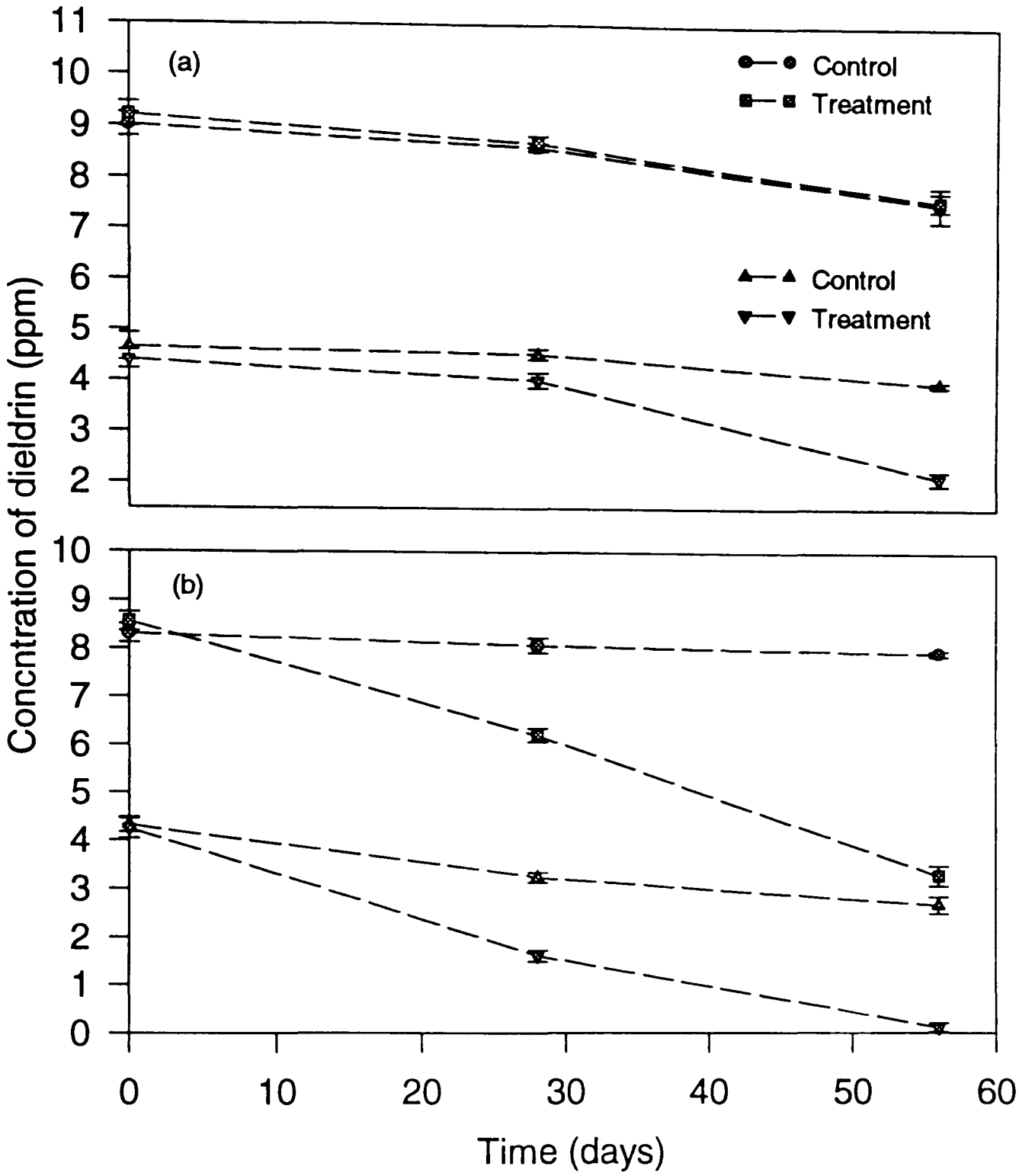


Figure 3.21. Comparison of temporal breakdown of dieldrin incorporated in 0.5% malt extract agar at 5 and 10 ppm in the absence (control) and presence of *T. cingulata* incubated at (a) 20°C and (b) 30°C. The error bars indicate the standard deviation of the mean. The least significant differences were 0.292, 0.390 for 5 and 10 ppm at 20°C respectively and 0.292, 0.270 for 5 and 10 ppm at 30°C respectively.

Table 3.5. Percentage breakdown of dieldrin relative to untreated controls by *P. chrysosporium*, *P. versicolor*, *T. socotrana* and *T. cingulata* at 20 and 30°C.

Fungi	Incubation period (days)	Incubation temperature (°C)	Initial concentration (ppm)	Percentage breakdow (%)
P. chrysosporium	0	20	5	-
P. chrysosporium	56	20	-	NFG
P. chrysosporium	0	20	10	-
P. chrysosporium	56	20	-	NFG
P. chrysosporium	0	30	5	-
P. chrysosporium	56	30	-	19.2
P. chrysosporium	0	30	10	-
P. chrysosporium	56	30	-	NFG
P. versicolor	0	20	5	-
P. versicolor	56	20	-	9.6
P. versicolor	0	20	10	-
P. versicolor	56	20	-	14.4
P. versicolor	0	30	5	-
P. versicolor	56	30	-	24.2
P. versicolor	0	30	10	-
P. versicolor	56	30	-	<3
T. socotrana	0	20	5	-
T. socotrana	56	20	-	29.5
T. socotrana	0	20	10	-
T. socotrana	56	20	-	15.6
T. socotrana	0	30	5	-
T. socotrana	56	30	-	60.0
T. socotrana	0	30	10	-
T. socotrana	56	30	-	43.0
T. cingulata	0	20	5	-
T. cingulata	56	20	-	37.4

Table 3.5. Continued

Fungi	Incubation period (days)	Incubation temperature (°C)	Initial concentration (ppm)	Percentage breakdown (%)
T. cingulata	**0**	**20**	**10**	**-**
T. cingulata	**56**	**20**	**-**	**0**
T. cingulata	**0**	**30**	**5**	**-**
T. cingulata	**56**	**30**	**-**	**58.7**
T. cingulata	**0**	**30**	**10**	**-**
T. cingulata	**56**	**30**	**-**	**57.2**

NFG: No Fungal Growth

3.2.4. Effect of temperature and time on fungal breakdown of trifluralin

Due to the volatile and heat-sensitive nature of trifluralin no pesticide was detected after 28 days in the 5 ppm samples at both 20 or 30°C. Thus no temporal data was collected regarding the ability of any of the fungi, especially *P. chrysosporium* which only showed slight growth at 30°C, and 5 ppm concentration. The only data obtained was from the 10 ppm samples incubated at 20°C with *P. versicolor*, *T. socotrana* and *T. cingulata*. These are thus presented in the form of Summary tables rather than figures.

Table 3.6 shows a relative breakdown of about 67% in the control samples, whereas in the presence of *P. versicolor* this value increased to greater than 97%. Table 3.7 shows the ability of *T. socotrana* in degrading trifluralin relative to the control samples which although very effective was not as good as *P. versicolor*. *T. cingulata* showed some ability to degrade this herbicide when incorporated into agar but was not as effective as either *P. versicolor* or *T. socotrana* (Table 3.8).

Table 3.9 summarises the relative increase in the percentage breakdown of trifluralin in the presence of the three fungi.

3.3. NATURAL BREAKDOWN OF THE TEST PESTICIDES IN WATER

Figure 3.22a and 3.22b compare the temporal natural breakdown of 3 and 6 ppm dieldrin at 10, 20 or 30°C. There was no significant difference in the natural breakdown over the 42 day period in unsterile water. Generally at both 20 and 30°C the rate of breakdown was slightly higher in unsterile than sterile water (Figure 3.23).

Simazine addition at 5 and 10 ppm to unsterile tap water showed a slight

Table 3.6. Percentage breakdown of trifluralin in the presence and absence of *P. versicolor* at 20°C over a period of 56 days (initially added at 10 ppm). Data on mean of breakdown after recovery ± standard deviation.

Fungi	Incubation period (days)	Incubation temperature (°C)	Obtained concentration (ppm)	Percentage breakdown (%)
Control	**0**	**20**	**9.30 ± 0.26**	-
Control	**28**	**20**	**3.08 ± 0.11**	**66.8**
Control	**56**	**20**	**ND**	-
P. versicolor	**0**	**20**	**9.35 ± 0.15**	-
P. versicolor	**28**	**20**	**ND**	**> 97.86**
P. versicolor	**56**	**20**	**ND**	-

ND: Below detection limit of 0.2 ppm

Table 3.7. Percentage breakdown of trifluralin in the presence and absence of T. *socotrana* at 20°C over a period of 56 days (initially added at 10 ppm). Data on mean of breakdown after recovery ± standard deviation.

Fungi	Incubation period (days)	Incubation temperature (°C)	Obtained concentration (ppm)	Percentage breakdown (%)
Control	**0**	**20**	**9.57 ± 0.15**	-
Control	**28**	**20**	**2.93 ± 0.18**	**67.62**
Control	**56**	**20**	**ND**	-
T. socotrana	**0**	**20**	**9.57 ± 0.15**	-
T. socotrana	**28**	**20**	**0.69 ± 0.10**	**92.79**
T. socotrana	**56**	**20**	**ND**	-

ND: Below detection limit of 0.2 ppm

Table 3.8. Percentage breakdown of trifluralin in the presence and absence of T. *cingulata* at 20°C over a period of 56 days (initially added at 10 ppm). Data on mean of breakdown after recovery ± standard deviation.

Fungi	Incubation period (days)	Incubation temperature (°C)	Obtained concentration (ppm)	Percentage breakdown (%)
Control	**0**	**20**	**9.40 ± 0.17**	-
Control	**28**	**20**	**2.73 ± 0.11**	**70.96**
Control	**56**	**20**	**ND**	-
T. socotrana	**0**	**20**	**9.30 ± 0.17**	-
T. socotrana	**28**	**20**	**1.10 ± 0.09**	**88.17**
T. socotrana	**56**	**20**	**ND**	-

ND: Below detection limit of 0.2 ppm

Table 3.9. Calculated relative increase in percentage breakdown of trifluralin due to the presence of fungi

Fungi	Percentage breakdown of trifluralin (%)
P. versicolor	**> 30.98**
T. socotrana	**25.17**
T. cingulata	**17.89**

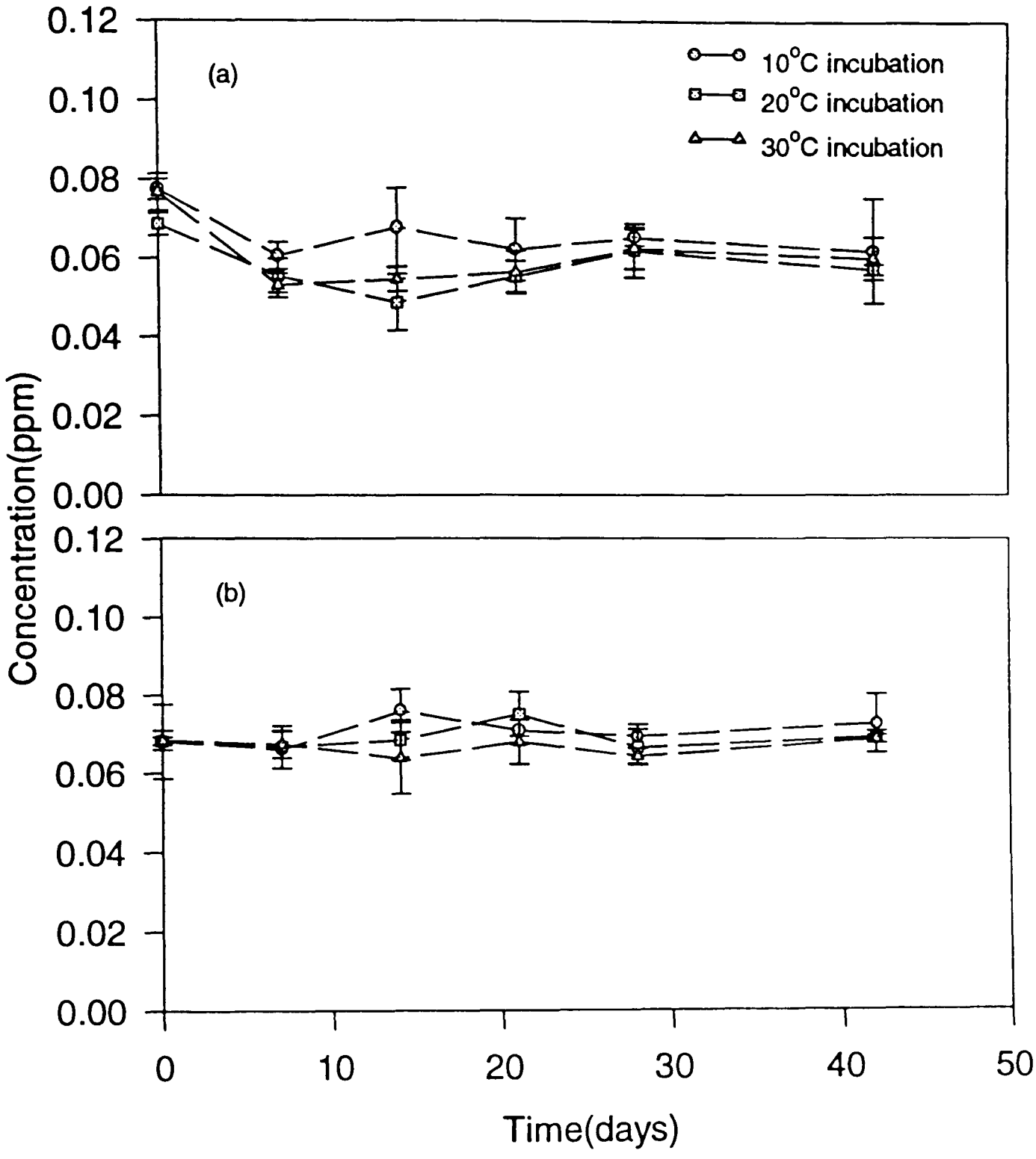


Figure 3.22. Effect of temperature and time on the natural breakdown rate of dieldrin in unsterile tap water with an initial added concentration of (a) 3 ppm (b) 6 ppm and incubated at various temperatures. Error bars indicate the standard deviation of the means.

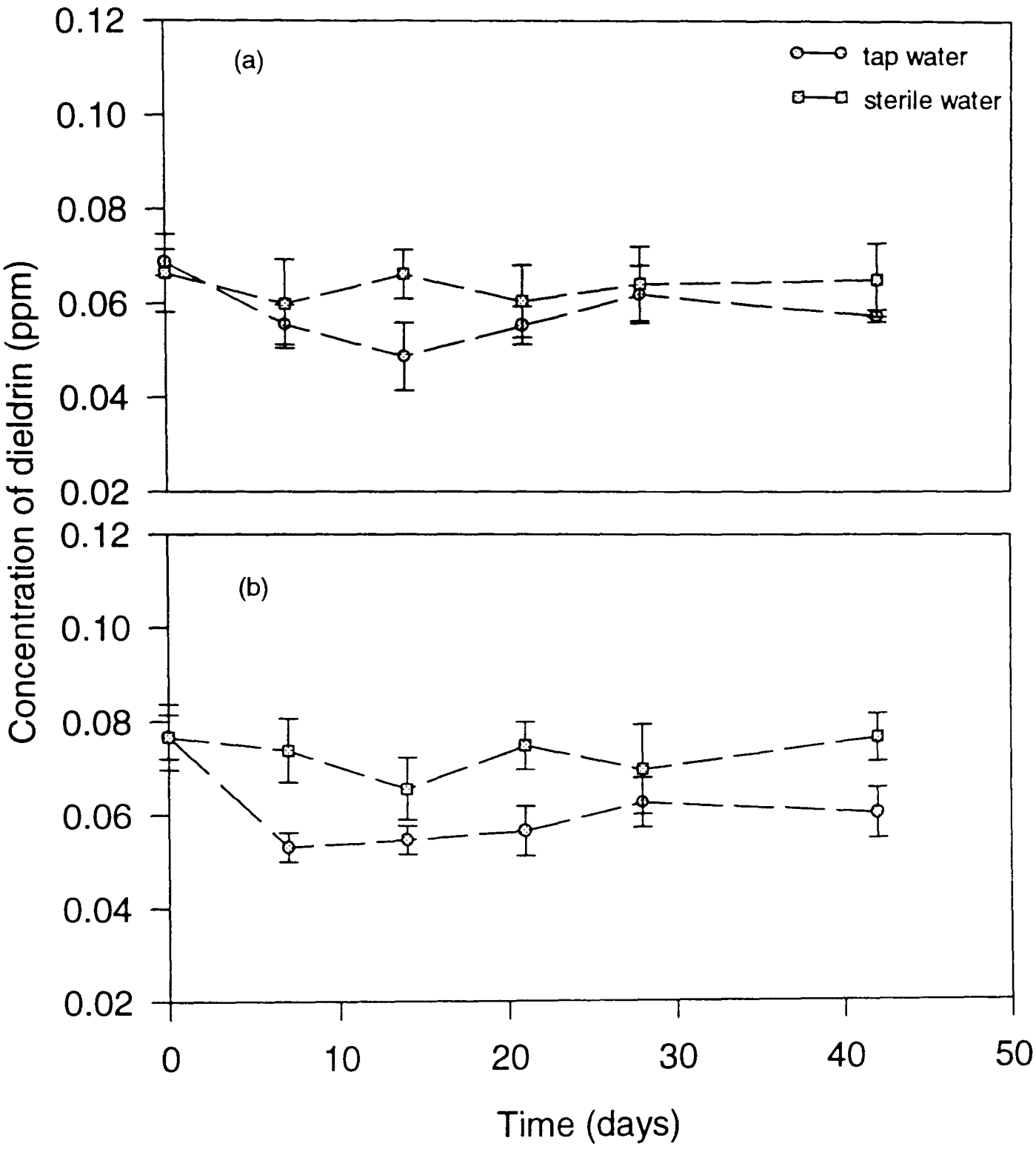


Figure 3.23. Comparison of temporal natural breakdown rates of dieldrin in sterile and unsterile tap water added at 3 ppm and incubated at (a) 20°C and (b) 30°C. Error bars indicate standard deviation of the means.

decrease in the detected concentration over the 42 days test period at all three temperatures tested (Figure 3.24). The comparison between sterile and unsterile water showed a greater breakdown of simazine in the latter than the former, especially at 30°C (Figure 3.25).

In contrast to simazine and dieldrin, trifluralin showed a more marked breakdown pattern in the unsterile tap water, specially at 20 and 30°C (Figure 3.26). However, there was only a slight difference in the rates of breakdown of trifluralin in sterile and unsterile tap water (Figure 3.27).

3.4. BIOREMEDIATION OF PESTICIDES IN SOIL USING FUNGI

3.4.1. Moisture absorption curves

To accurately control the moisture content of soil, a moisture sorption isotherm had to be constructed for the sandy loam soil used in this study. Figure 3.28 shows (a) the relationship between known amounts of water added and water potential of the soil and (b) the relationship between moisture content and water potential of the soil.

3.4.2. Method development for the extraction of pesticides from soil

An HPLC gradient method was successfully developed and used to simultaneously analyse the mixture of pesticides in soil. These were initially added at 10 ppm each, as a mixture in soil. A series of extraction solvents were examined to obtain the most efficient method for recovery of the pesticides from soil. Tables 3.10, 3.11 and 3.12 show the range of solvent systems used for extraction of dieldrin, simazine and trifluralin from spiked soil. It was found that the best solvent was 100% acetone.

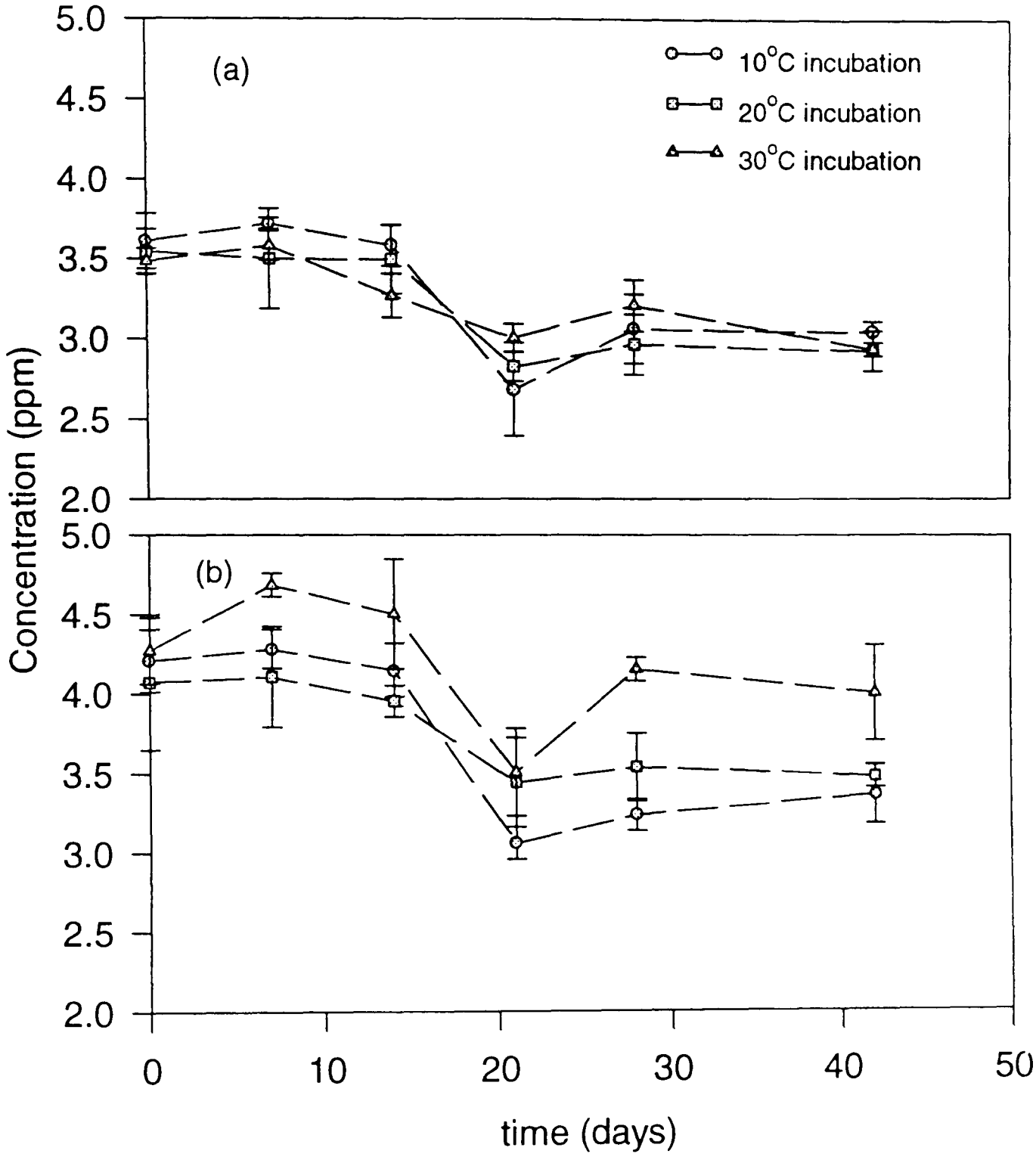


Figure 3.24. Effect of temperature and time on the natural breakdown rates of simazine in unsterile tap water with an initial added concentration of (a) 5 ppm and (b) 10 ppm and incubated at various temperatures. Error bars indicate the standard deviation of the means.

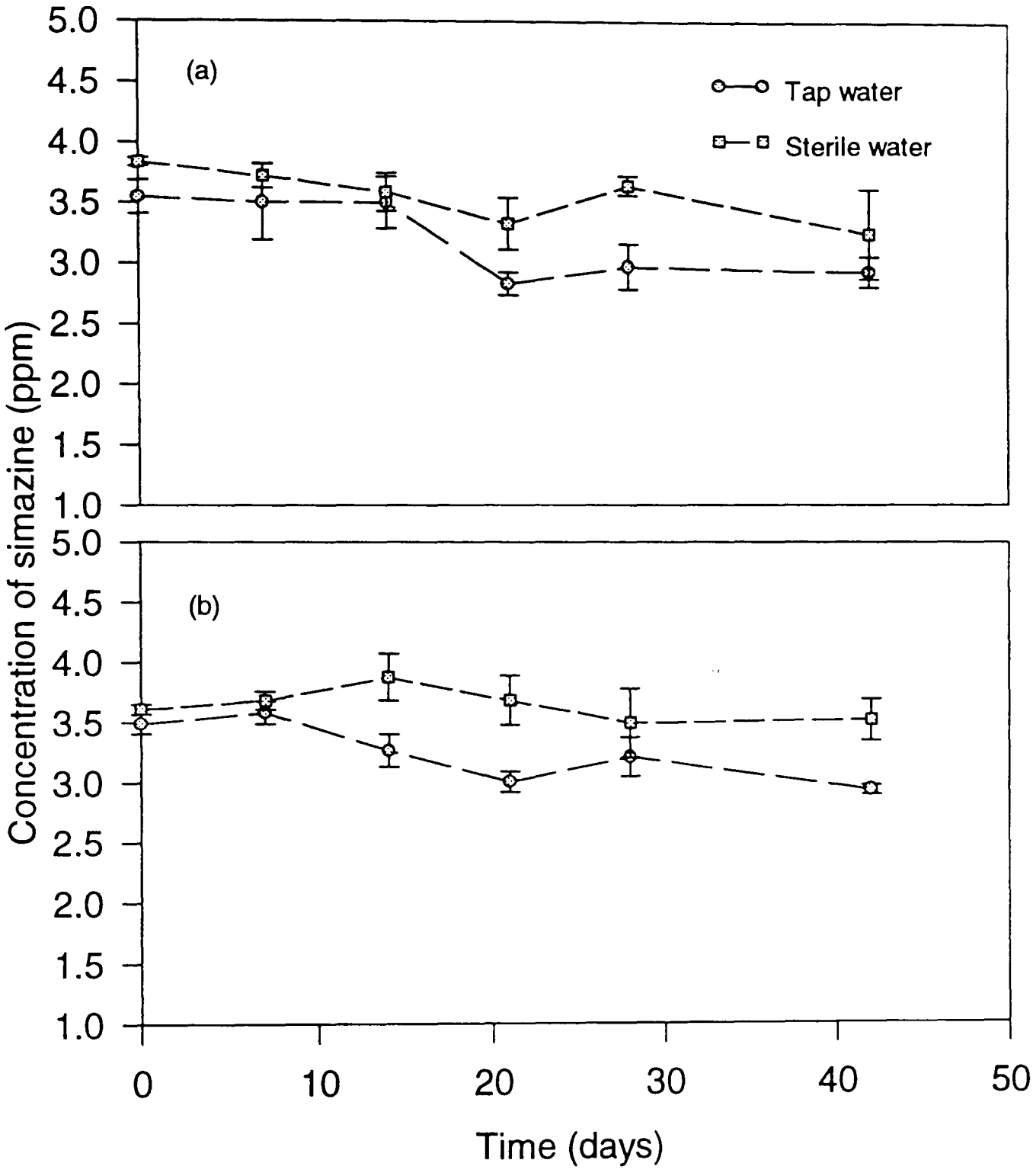


Figure 3.25. Comparison of temporal natural breakdown rates of simazine in sterile and unsterile tap water added at 5 ppm and incubated at (a) 20°C and (b) 30°C. Error bars indicate standard deviation of the means.

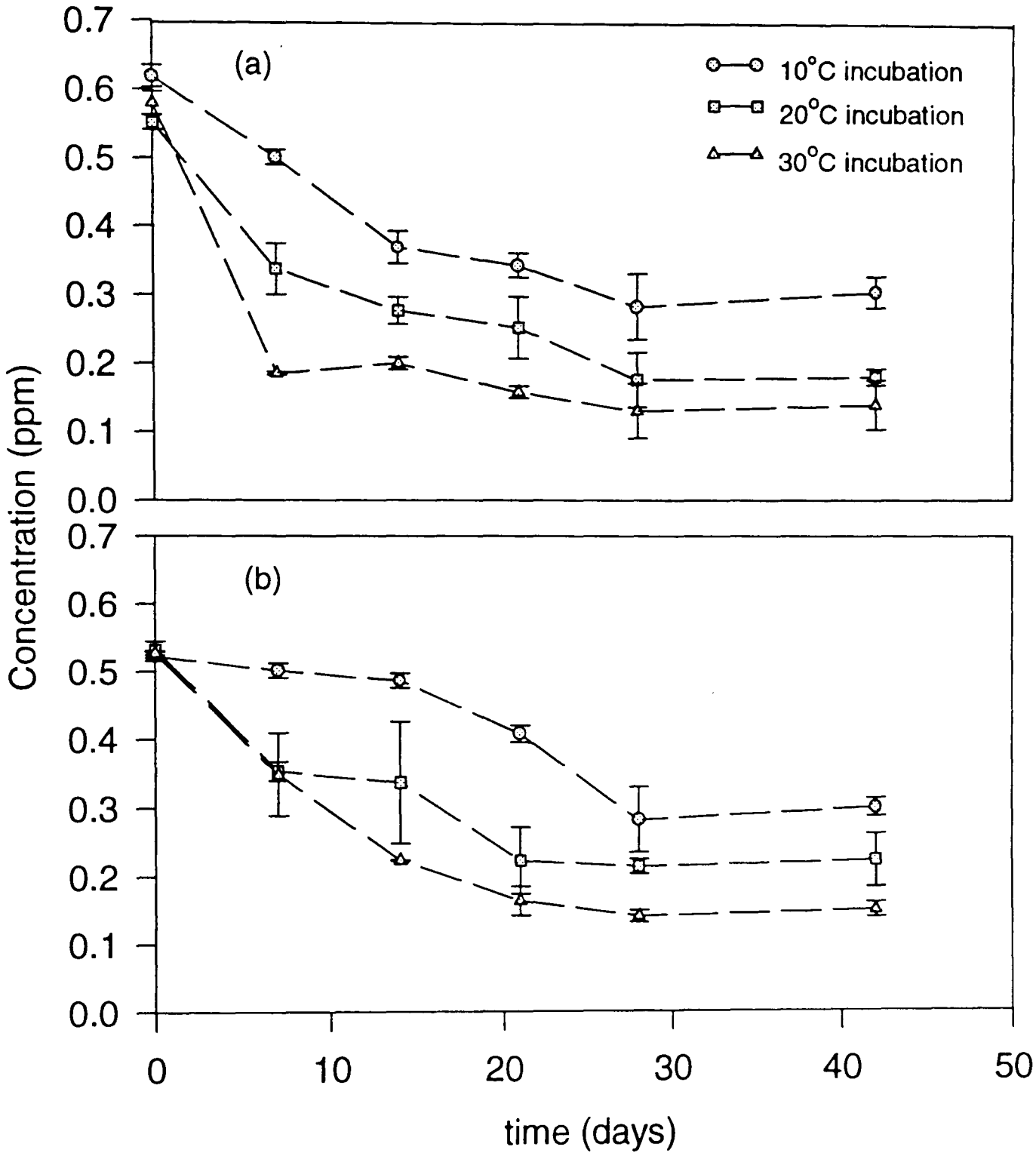


Figure 3.26. Effect of temperature and time on the natural breakdown rates of trifluralin in unsterile tap water with an initial added concentration of (a) 5 ppm and (b) 10 ppm and incubated at various temperatures. Error bars indicate standard deviation of the means.

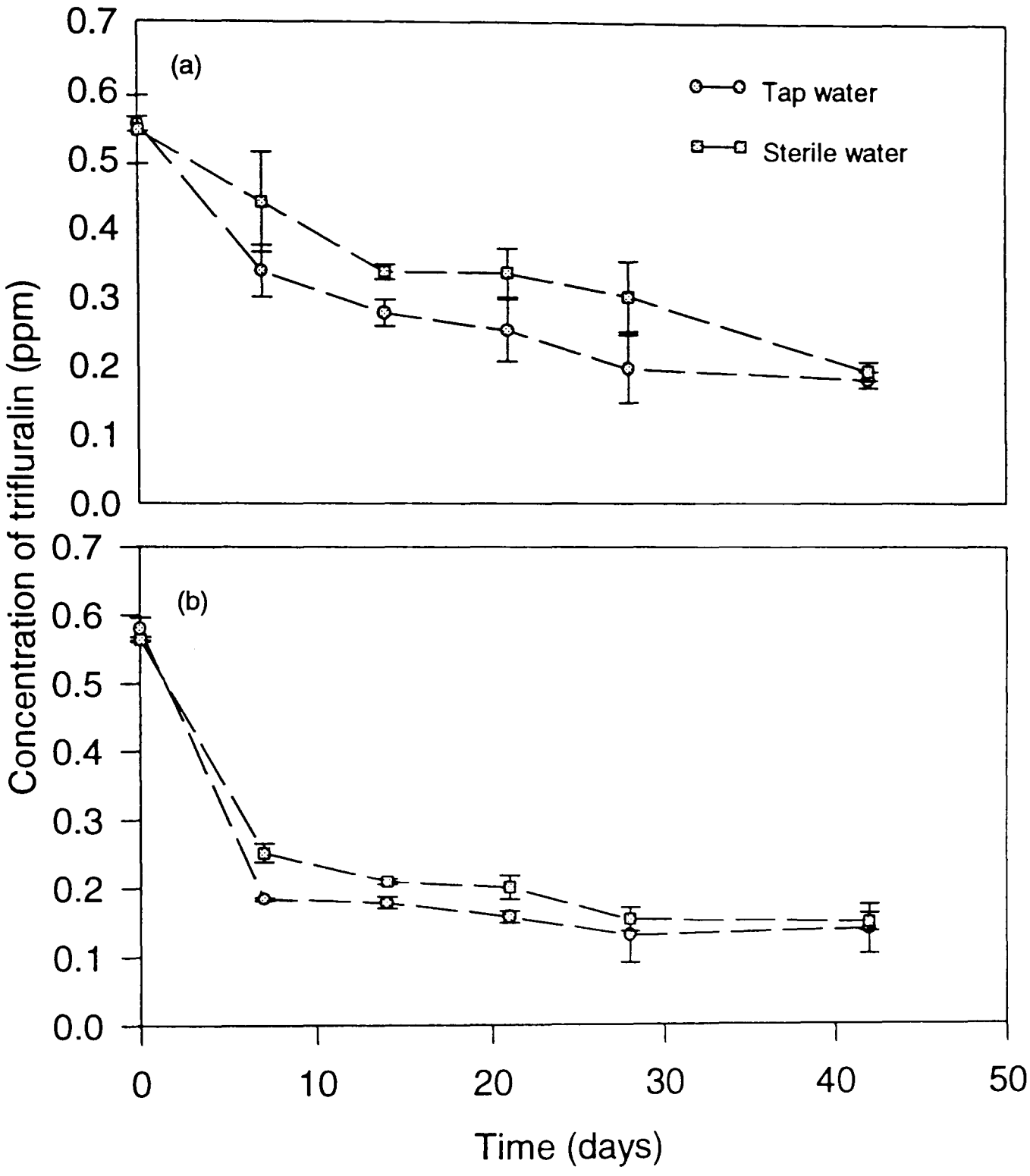


Figure 3.27. Comparison of temporal natural breakdown of trifluralin in sterile and unsterile tap water added at 5 ppm and incubated at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means.

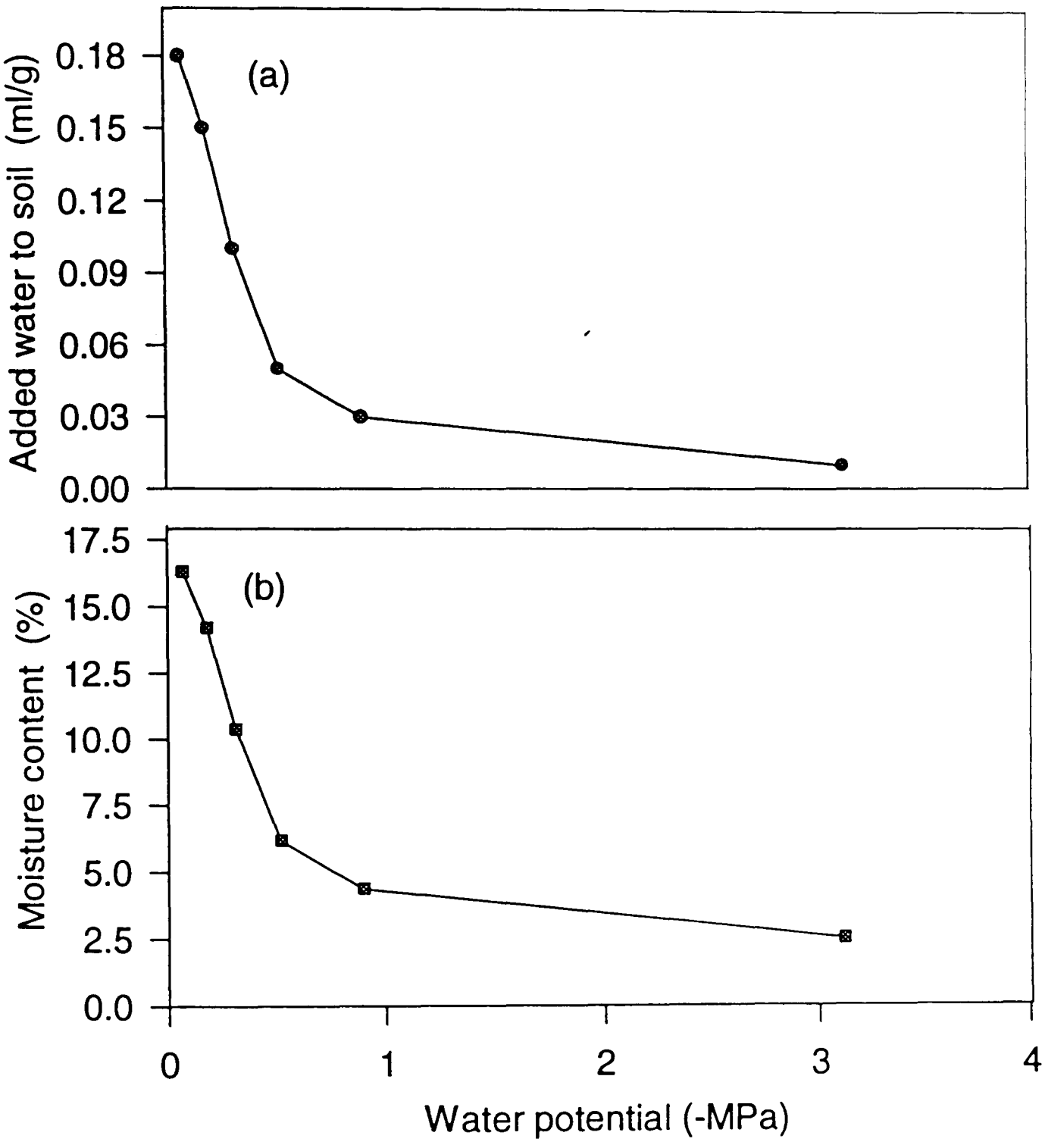


Figure 3.28. The relationship between (a) added water and (b) moisture adsorption (wet weight basis) and water potential of a sandy loam soil at 25°C.

Table 3.10. Comparison of different solvent systems for efficiency of recovery of dieldrin from soil (added at 10 ppm), mean recoveries ± standard deviation

Extraction solvent	**Extraction time (min)**	**Recovery (%)**
1) Methanol 100 %	**50**	**58.9 ± 0.55**
2) Methanol 98 % + acetone 1% + THF 1 %	**50**	**56.9 ± 2.5**
3) Methanol100 % saturated with KCl	**50**	**52.7 ± 0.64**
4) Methanol 100 % saturated with KCl	**70**	**61.4 ± 1.53**
5) Methanol (KCl sat.) 98 % + acetone 1 % + THF 1 %	**50**	**60.9 ± 1.25**
6) Methanol (KCl sat.) 97 % + acetone 2 % + THF 1 %	**50**	**60.9 ± 1.82**
7) Methanol (KCl sat.) 95 % + acetone 4% + THF 1 %	**50**	**60.9 ± 0.97**
8) Methanol (KCl sat.) 89 % + acetone 10 % + THF 1 %	**50**	**67.1 ± 1.4**
9) Acetonitrile 100 %	**70**	**72.5 ± 1.7**
10) Acetone 100 %	**70**	**86.2 ± 2.76**

Table 3.11. Comparison of different solvent systems for efficiency of recovery of simazine from soil (added at 10 ppm); mean recoveries ± standard deviation

Extraction solvent	**Extraction time (min)**	**Recovery (%)**
1) Methanol 100 %	**50**	**58.5 ± 1.79**
2) Methanol 98 % + acetone 1% + THF 1 %	**50**	**56.9 ± 0.44**
3) Methanol100 % saturated with KCl	**50**	**62.2 ± 1.86**
4) Methanol 100 % saturated with KCl	**70**	**72.8 ± 0.89**
5) Methanol (KCl sat.) 98 % + acetone 1 % + THF 1 %	**50**	**60 ± 0.47**
6) Methanol (KCl sat.) 97 % + acetone 2 % + THF 1 %	**50**	**56 ± 1.5**
7) Methanol (KCl sat.) 95 % + acetone 4% + THF 1 %	**50**	**59 ± 0.90**
8) Methanol (KCl sat.) 89 % + acetone 10 % + THF 1 %	**50**	**45 ± 0.33**
9) Acetonitrile 100 %	**70**	**77.2 ± 0.85**
10) Acetone 100 %	**70**	**84.8 ± 1.10**

Table 3.12. Comparison of different solvents systems for efficiency of recovery of trifluralin from soil (added at 10 ppm); mean recoveries ± standard deviation

Extraction solvent	**Extraction time (min)**	**Recovery (%)**
1) Methanol 100 %	**50**	**29.5 ± 1.7**
2) Methanol 98 % + acetone 1% + THF 1 %	**50**	**26.0 ± 1.22**
3) Methanol100 % saturated with KCl	**50**	**45.1 ± 1.23**
4) Methanol 100 % saturated with KCl	**70**	**55.3 ± 2.46**
5) Methanol (KCl sat.) 98 % + acetone 1 % + THF 1 %	**50**	**28 ± 1.3**
6) Methanol (KCl sat.) 97 % + acetone 2 % + THF 1 %	**50**	**23 ± 0.55**
7) Methanol (KCl sat.) 95 % + acetone 4% + THF 1 %	**50**	**25 ± 1.24**
8) Methanol (KCl sat.) 89 % + acetone 10 % + THF 1 %	**50**	**38 ± 0.95**
9) Acetonitrile 100 %	**70**	**58.6 ± 2.6**
10) Acetone 100 %	**70**	**70 ± 2.0**

This produced a recovery of around 86% for dieldrin, 85% for simazine and 70% for trifluralin. The optimum extraction time was 70 minutes.

3.4.3. Effect of temperature on natural breakdown of pesticides in field capacity soil

In this section analysis of variance (one-way) was carried out to compare the effects of changes in temperature on the breakdown of the pesticides.

Figure 3.29 shows the effect of temperature on the temporal natural breakdown of dieldrin in field capacity soil. The effect of temperature was slight on the breakdown of dieldrin. For 5 ppm dieldrin, there was a faster breakdown rate at 30 than 20°C particularly between 42 and 70 days of incubation (Figure 3.29a). With 10 ppm dieldrin, there was again a slightly more rapid breakdown at 30°C (Figure 3.29b). However, in both cases breakdown rates were only in the range 3-5%.

In contrast, Figure 3.30 shows a different pattern with simazine. At 20°C, there was very slow degradation over the whole incubation period with both 5 and 10 ppm simazine concentrations. However, at 30°C there was a significant decrease in both 5 and 10 ppm simazine, with approximately 0.5 and 2 ppm remaining, respectively in these two treatments after 70 days incubation. Statistically, there was a significant difference ($P < 0.05$) between the breakdown of simazine at 20 and 30°C.

For the third pesticide tested, the pattern of degradation of 5 and 10 ppm trifluralin at 20 and 30°C was similar to that obtained with simazine (Figure 3.31). At 20°C very little natural degradation occurred, while at 30°C just over 1.0 and 3 ppm remained in the 5 and 10 ppm treatments, respectively. This breakdown occurred after

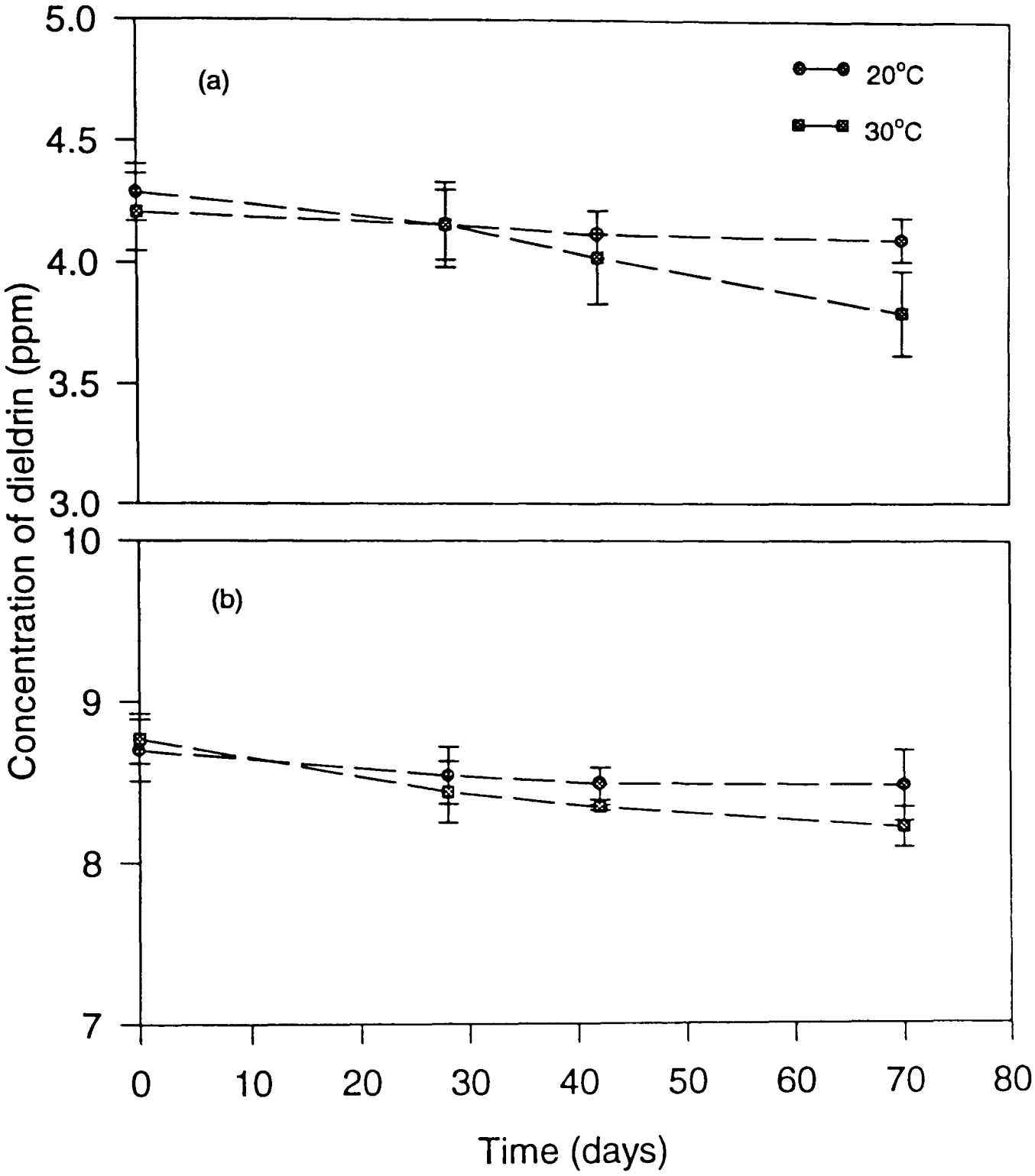


Figure 3.29. Natural temporal breakdown rates of (a) 5 ppm and (b) 10 ppm dieldrin in field capacity soil incubated at 20 and 30°C. Error bars indicate standard deviation of the means. Least significant differences ($P<0.05$) were 0.251, 0.274 for 5 and 10 ppm respectively.

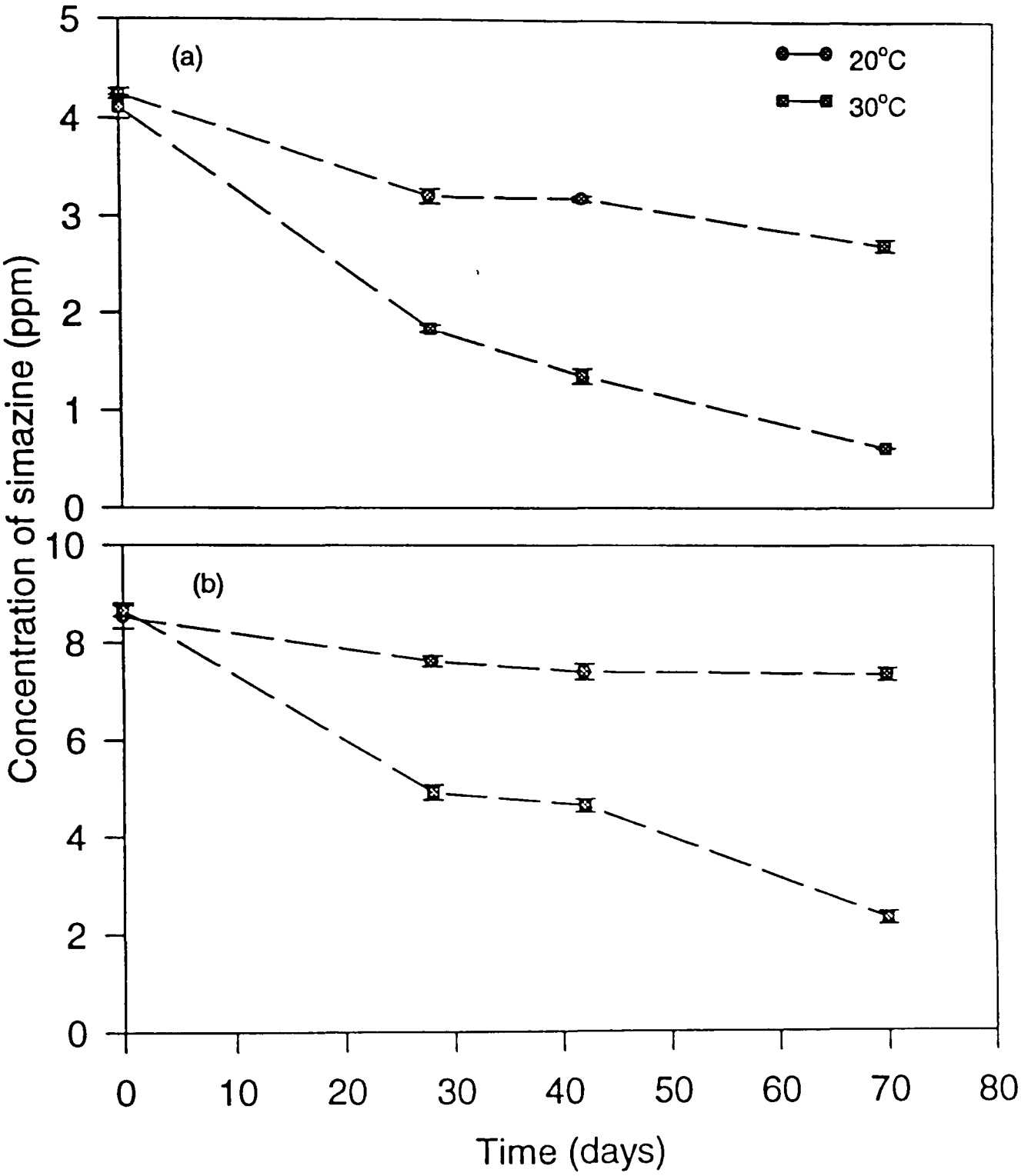


Figure 3.30. Natural temporal breakdown rates of (a) 5 ppm and (b) 10 ppm simazine in field capacity soil incubated at 20 and 30°C. Error bars indicate standard deviation of the means. Least significant differences ($P<0.05$) were 0.109, 0.262 for 5 and 10 ppm respectively.

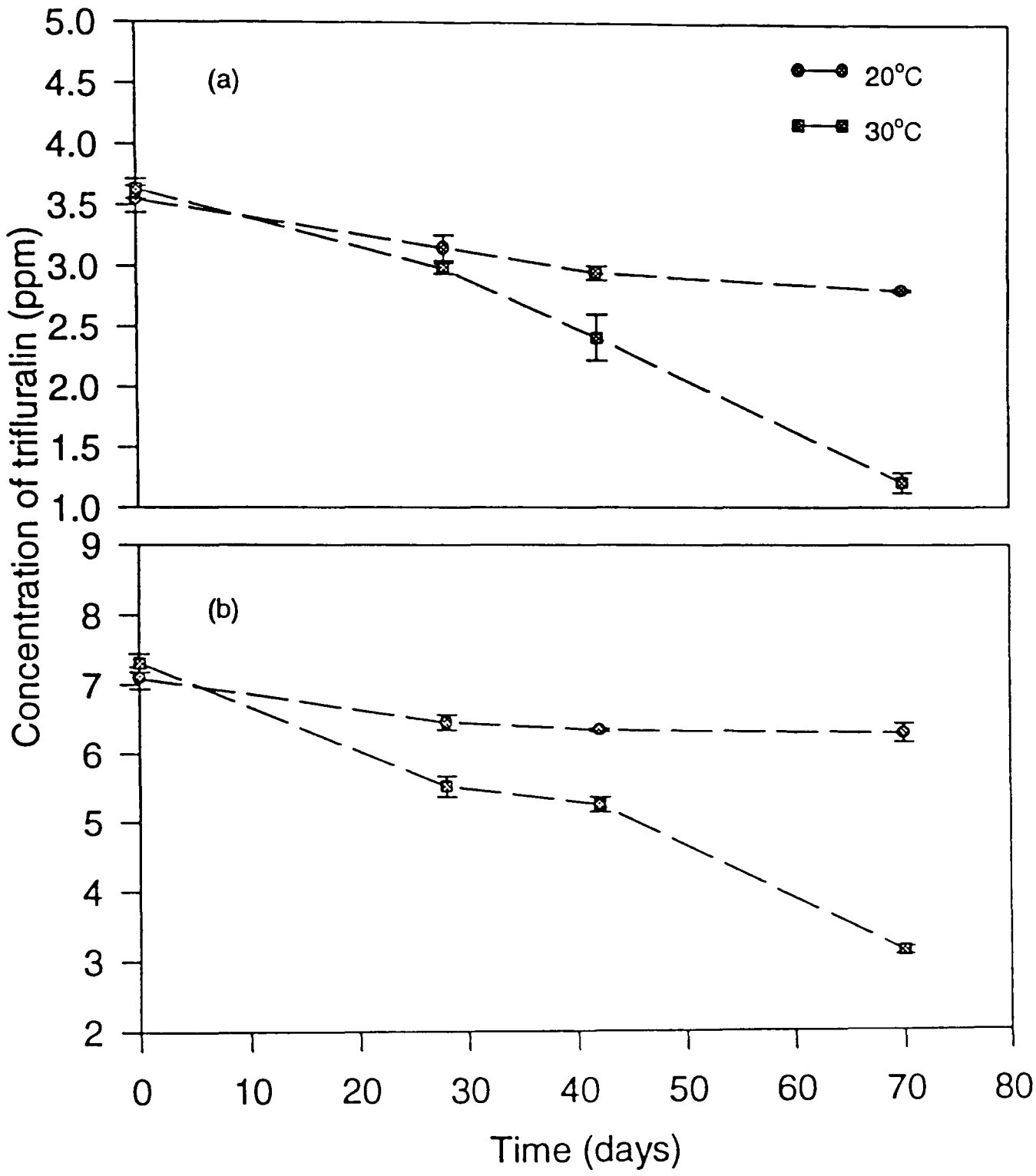


Figure 3.31. Natural temporal breakdown rates of (a) 5 ppm and (b) 10 ppm trifluralin in field capacity soil incubated at 20 and 30°C. Error bars indicate standard deviation of the means. Least significant differences ($P<0.05$) were 0.173, 0.212 for 5 and 10 ppm respectively.

28, 42 and 70 days in both the 5 and 10 ppm samples. Therefore, once again the change in temperature made a significant difference to the breakdown rate of pesticides in the field capacity soil, which was found to be statistically significant.

3.4.4. Effects of soil moisture content on natural breakdown of pesticides

In this section, analysis of variance (one-way) on different water potential soils containing a mixture of the three pesticides were carried out to determine the effect that different moisture content treatments may have on the patterns of natural breakdown.

Figure 3.32 compares the effect of soil water potential on the simultaneous breakdown of the three pesticides over 70 days at 20°C. This showed that for all three pesticides there was a significant effect of water potential on degradation rates.

Simazine: Figure 3.32a shows that there was a steady decrease in concentration of simazine over time in the field capacity soil. At a water potential of -0.28 MPa which was the driest soil treatment, initially no decrease in concentration was observed. However, between 28 and 42 days there was a sharp drop in concentration which was followed by a slower decrease, ending with a final concentration after 70 days which was not significantly different to that present in the field capacity soil. Surprisingly in the -0.065 MPa water potential soil (the mid-range wetness), the concentration displayed an eventual sharper decrease in concentration than even the field capacity soil. Statistically, this difference was shown to be significant.

Dieldrin: Figure 3.32b compares the degradation pattern of dieldrin in the three soil water potential treatments. This shows that virtually no degradation of dieldrin occurred at water potentials of -0.065 and -0.28 MPa. However, a steady breakdown

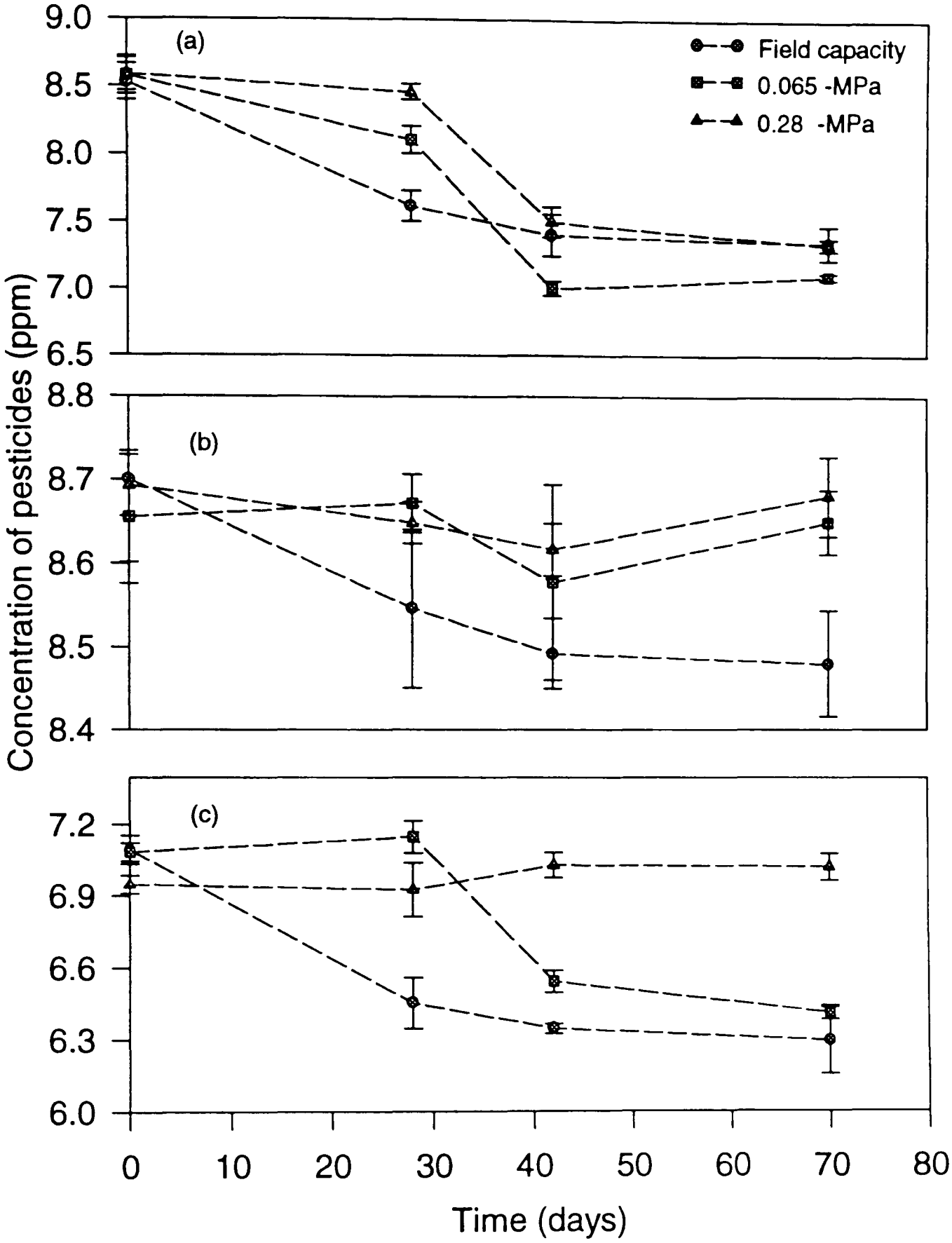


Figure 3.32. Comparison of effect of water potential on simultaneous degradation of (a) simazine (b) dieldrin and (c) trifluralin each added at an initial concentration of 10 ppm with 20°C incubation temperature. Error bars indicate standard deviation of the means. Least significant differences ($P<0.05$) were 0.185, 0.107, 0.160 for simazine, dieldrin and trifluralin respectively.

was observed in field capacity soil over the 70 day period. Statistically, there was no significant difference between final concentrations in -0.065 and -0.28 MPa treatments, although there was a significant difference between these and that of field capacity soil.

Trifluralin: (Figure 3.32c) showed no breakdown of trifluralin over the 70 day period when incorporated in the driest soil treatment (-0.28 MPa). The steady degradation of the pesticide in the field capacity soil was again observed, resulting in the lowest remaining concentration (about 6.3 ppm). In the mid-range wetness there was virtually no fall in concentration over the first 28 days (as with simazine), but the concentration present fell sharply to an eventual level similar to that in field capacity soil. The difference in the concentrations of these two treatments (field capacity and -0.065 MPa) were not shown to be statistically significant.

The initial water potential of the soil samples were determined using the data in Figure 3.28(b). Table 3.13 shows the initial water potential and the subsequent moisture content of the soil samples, determined at each sampling time. This shows that only a slight decrease in moisture content of the soil occurred throughout the experimental period.

3.4.5. Effect of temperature on the temporal bioremediation of pesticides using single and mixed inoculum in field capacity soil

In this study, the single and mixed inocula were incorporated into the soil after growth of the fungi on a chopped straw substrate. The ratio of the inoculated straw to soil was 1 : 10. In all treatments a mixture of the three pesticides were used together. However, for clarity of presentation each pesticide will be dealt with separately.

Table 3.13. Moisture content of the soil microcosm over time

Soil Medium*	Mean Moisture Content (%)			
	T = 0	*T = 28*	*T = 42*	*T = 70*
Field Capacity, Water added at 0.10 ml/g	17.3	17.2	17.0	16.8
-0.065 MPa, Water added at 0.075 ml/g	15.9	15.8	15.6	15.6
-0.28 MPa, Water added at 0.030 ml/g	12.0	12.0	11.9	11.8

T represents the sampling times in days.

* *The initial moisture content of soil alone with no added water was 10% (-0.30 MPa).*

A further point worth noting is that, due to the presence of straw mixed in soil (hence taking up some soil volume) the initial and subsequent recoveries of the pesticides were higher in soil alone than soil + inoculated straw. As a result, the analysis of variance of the curves were only carried out on the data containing the soil + inoculated straw. For the purpose of comparisons between soil and soil + inoculated straw, the slope of each curve was resolved (Figure 3.33) and analysis of variance was carried out on the relevant slopes to determine significant differences.

Dieldrin: Figure 3.34a compares the capability of *P. versicolor* alone or *P. versicolor* + *T. socotrana* to enhance the breakdown of 5 ppm dieldrin at 20°C over a 70 day incubation in soil microcosms. There was little degradation of the pesticides in the control (soil + pesticides only) but both the single and mixed inocula increased the degradation significantly. There was only a small difference between using *P. versicolor* alone or *P. versicolor* + *T. socotrana* together, with this difference not being statistically significant. At 30°C (Figure 3.34b) *P. versicolor* was more effective than *P. versicolor* + *T. socotrana* after 42 days, and the difference statistically significant. This was also shown to be the case for comparisons between the untreated controls and the fungal treatments.

The ability of *P. versicolor* and the mixed inoculum to enhance degradation of 10 ppm dieldrin is shown in Figure 3.35. Again, at 20° and 30°C both single and mixed inocula enhanced the breakdown of the pesticide. There was little difference in the patterns of degradation caused by single or mixed inocula, although after 42 days *P. versicolor* alone was slightly more effective than *P. versicolor* + *T. socotrana.*

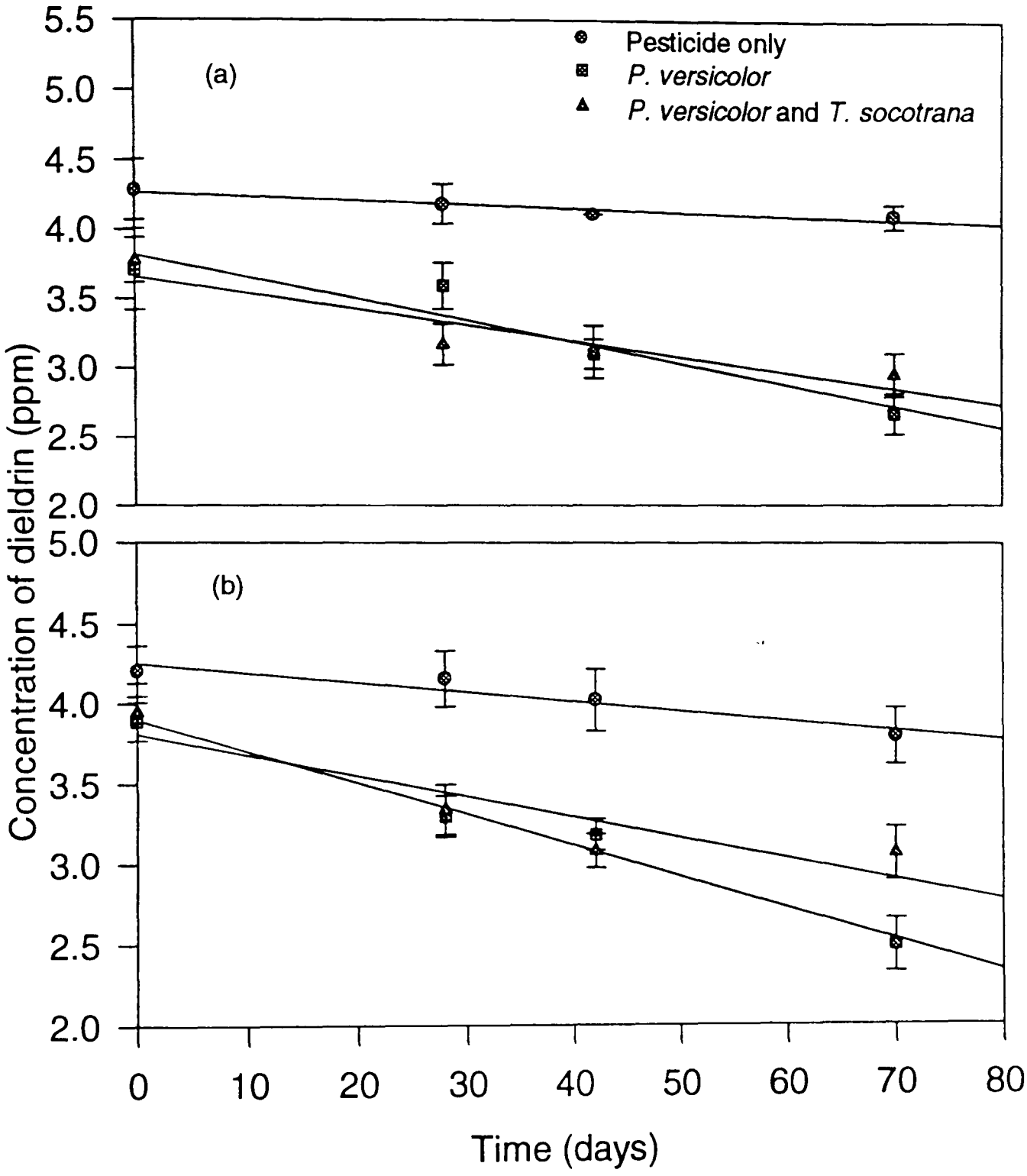


Figure 3.33. An example of the construction of best fitting lines through the means of the pesticide concentrations. The slopes of the lines were statistically analysed to compare the extent of degradation of the pesticide under the experimental conditions.

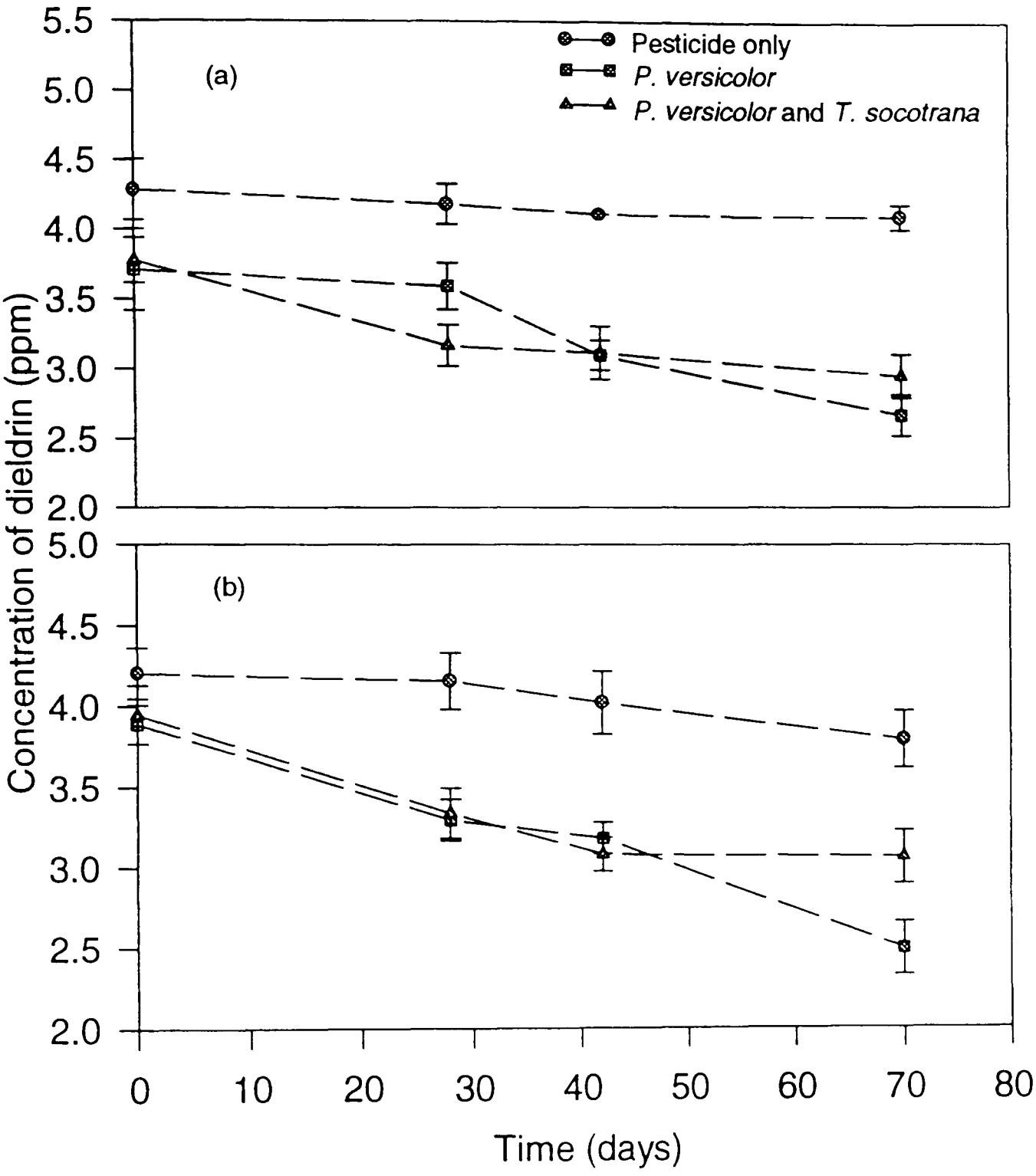


Figure 3.34. Comparison of effect of temperature and fungal treatments on temporal degradation of 5 ppm dieldrin in field capacity soil at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. Least significant differences ($P<0.05$) were 0.283, 0.261 for 20 and 30°C fungal treatments respectively.

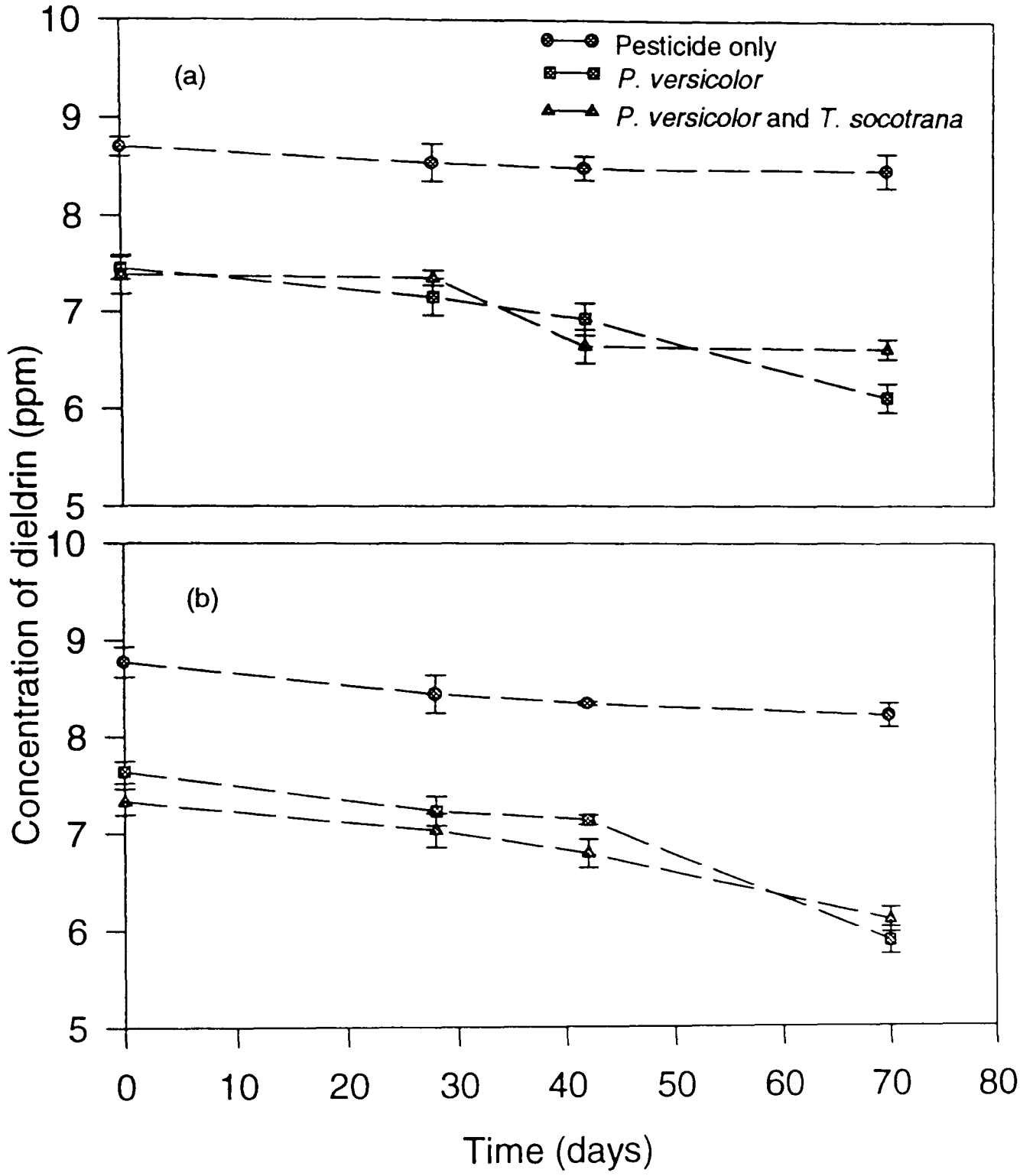


Figure 3.35. Comparison of effect of temperature and fungal treatments on temporal degradation of 10 ppm dieldrin in field capacity soil at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. Least significant differences ($P<0.05$) were 0.250, 0.226 for 20 and 30°C fungal treatments respectively.

Here the difference between the two fungi was statistically significant at 20°C but not at 30°C. However, the difference between the control and fungal treatments were statistically significant at both temperatures.

Simazine: At 20°C, 5 ppm simazine was degraded to some extent in all untreated controls and fungal treatments (Figure 3.36). However, there was a significant increase in this degradation rate when fungi were present whether singly or as a mixture (Figure 3.36a). This difference was less apparent at 30°C and all treatments showed almost identical breakdown rates of simazine. There was no statistically significant difference between these treatments (Figure 3.36b). At 20°C and with an initial concentration of 10 ppm simazine, the *P. versicolor* and *P. versicolor* + *T. socotrana* treatments were both significantly better than the control (Figure 3.37a). However, at 30°C, *P. versicolor* alone was significantly better than using *P. versicolor* + *T. socotrana* together or the untreated control (Figure 3.37b).

Trifluralin: The effect of temperature and time on degradation of 5 ppm trifluralin is shown in Figure 3.38. This shows that in the untreated controls, 5 ppm trifluralin is rapidly degraded naturally, especially at 30°C. In the presence of *P. versicolor* at 20°C, none of the initial 5 ppm concentration of trifluralin was detected at the 42 d sample (Figure 3.38a). In the presence of *P. versicolor* + *T. socotrana*, this occurred after 70 days. In contrast, at 30°C, no trifluralin was detected in the 28 d samples for either of the fungal treatments (Figure 3.38b). At both 20 and 30°C incubations, there was a significant difference between treated and untreated samples. Also at 20°C a statistically significant difference was observed between the presence of a single, and two fungi, after 28 days.

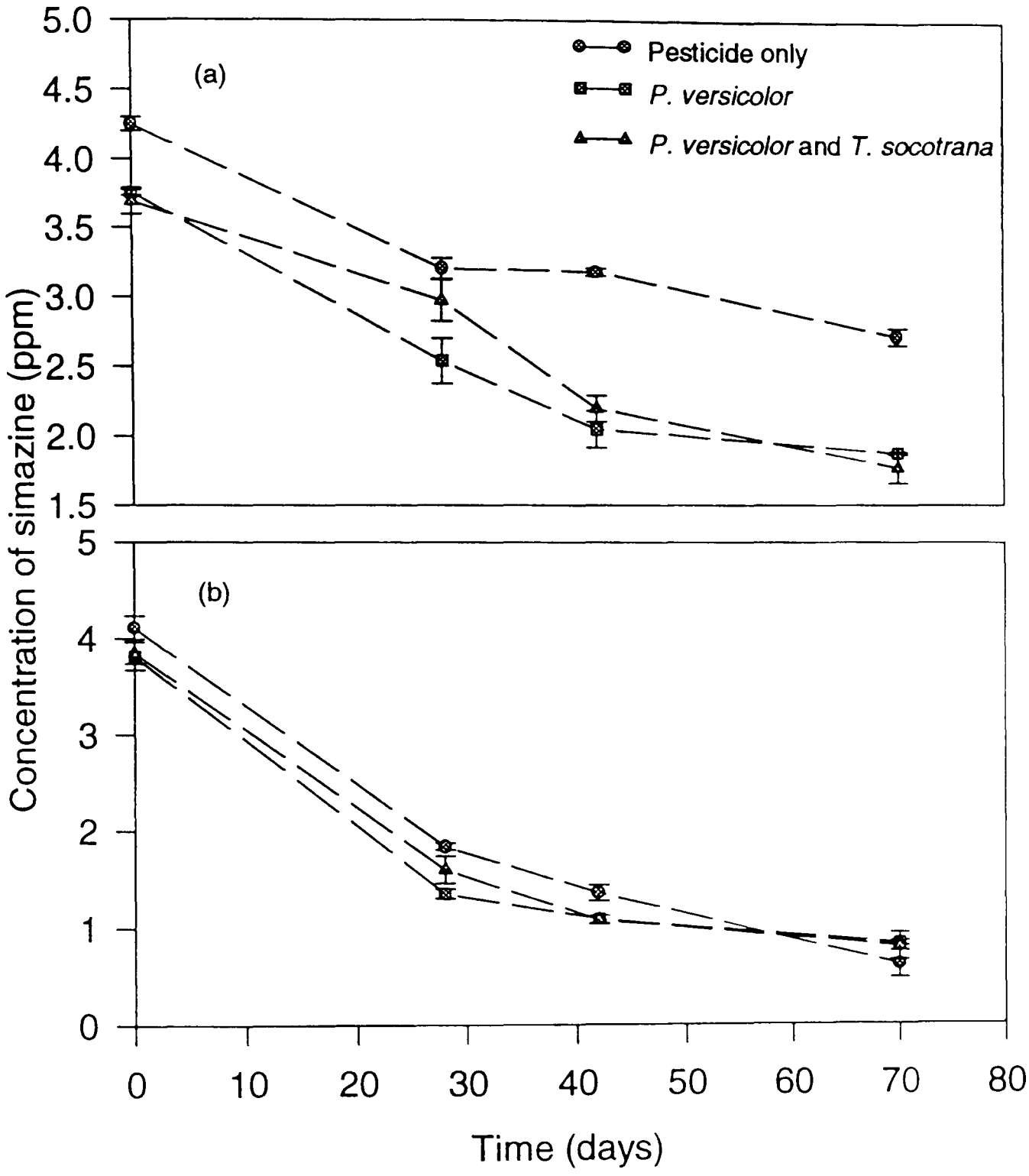


Figure 3.36. Comparison of effect of temperature and fungi on temporal degradation of 5 ppm simazine in field capacity soil at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. Least significant difference ($P<0.05$) were 0.160, 0.169 for 20 and 30°C fungal treatments respectively.

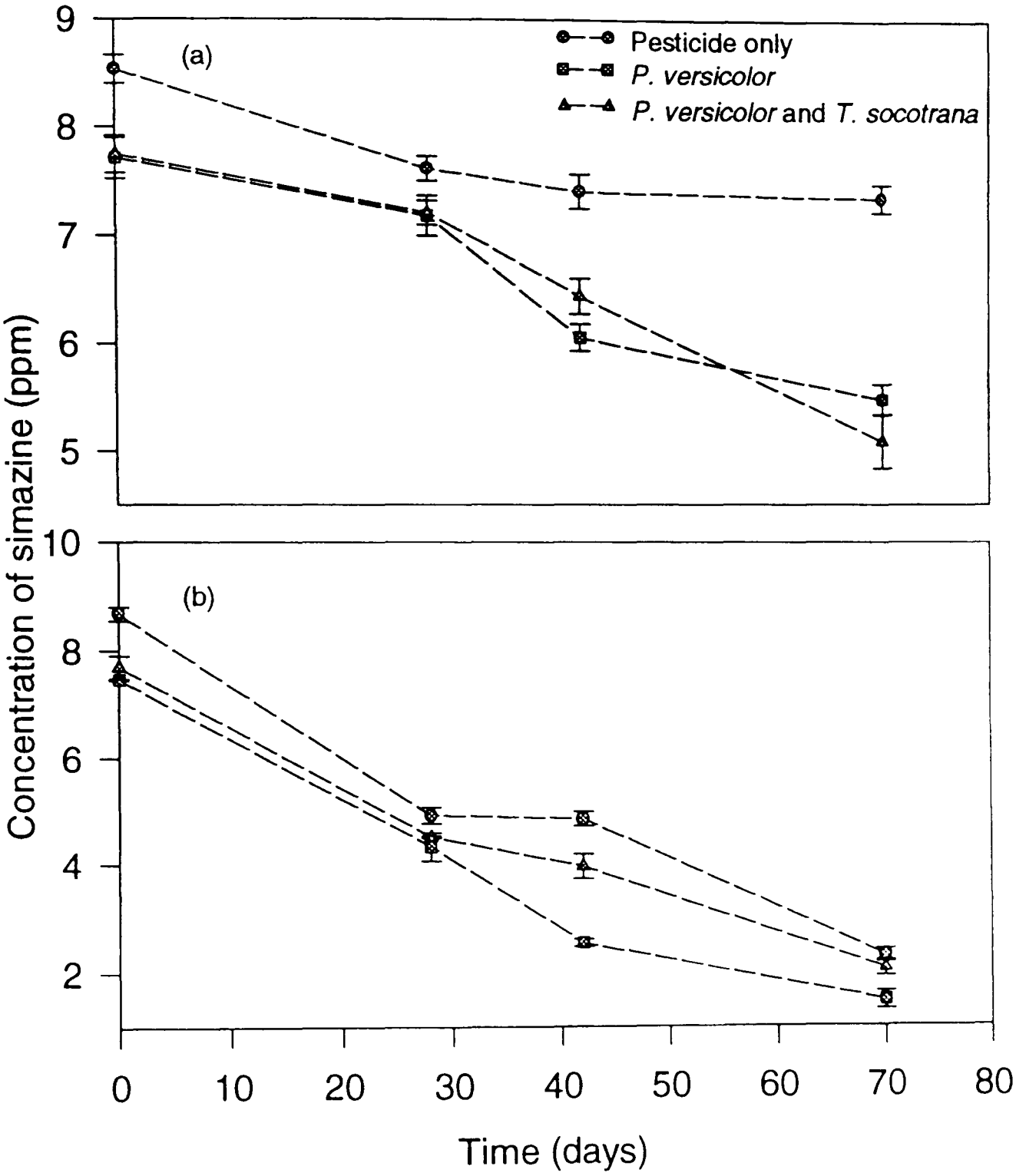


Figure 3.37. Comparison of effect of temperature and fungi on temporal degradation of 10 ppm simazine in field capacity soil at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. Least significant difference ($P<0.05$) were 0.272, 0.266 for 20 and 30°C fungal treatments respectively.

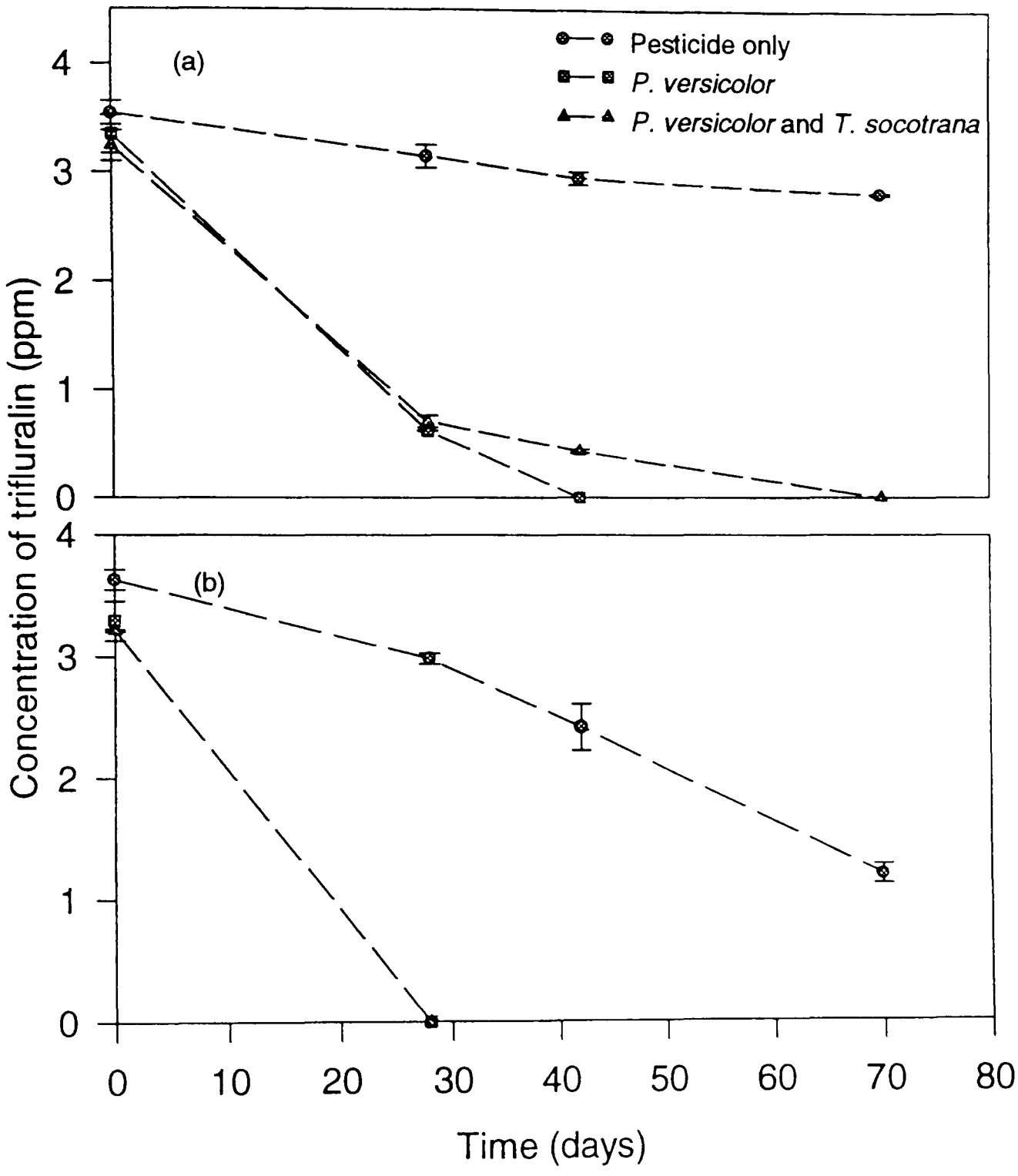


Figure 3.38. Comparison of effect of temperature and fungi on temporal degradation of 5 ppm trifluralin in field capacity soil at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. Least significant difference ($P<0.05$) were 0.142, 0.173 for 20 and 30°C fungal treatments respectively.

For 10 ppm trifluralin the degradation patterns at 20°C (Figure 3.39a) was very similar to that for the 5 ppm samples. For the samples in soil alone and incubated at 30°C significant breakdown of the trifluralin was observed. Again this degradation was significantly enhanced in the presence of the fungal inoculants. In the soil containing *P. versicolor* + *T. socotrana* no herbicide was detected after 28 days. Similar results were obtained for *P. versicolor* inoculated soil, but after 42 days (Figure 3.39b).

Table 3.14 summarises the analysis of variance of the slopes of curves regarding the temporal degradation of the three pesticides in soil in the presence and absence of fungi.

3.4.6. Effect of soil water potential on bioremedial capability of the fungi *P. versicolor*

As in Section 3.4.4. analysis of variance was carried out to determine significant differences between water potential treatments.

Figure 3.40, compares the bioremedial potential of *P. versicolor* in degrading simazine, dieldrin and trifluralin at three different water potentials.

Simazine: *P. versicolor* performed best at degrading simazine in the driest soil (-0.28 MPa) with field capacity soil also being effective (Figure 3.40a). The difference between the two treatments however was statistically significant.

In the mid-range wetness soil (-0.065 MPa), initially *P. versicolor* degraded simazine at a faster rate than in the other two conditions. However, after 28 days, the concentration remained constant until after 42 days when a slow breakdown rate of the herbicide occurred leaving a final concentration greater than the other two treatments.

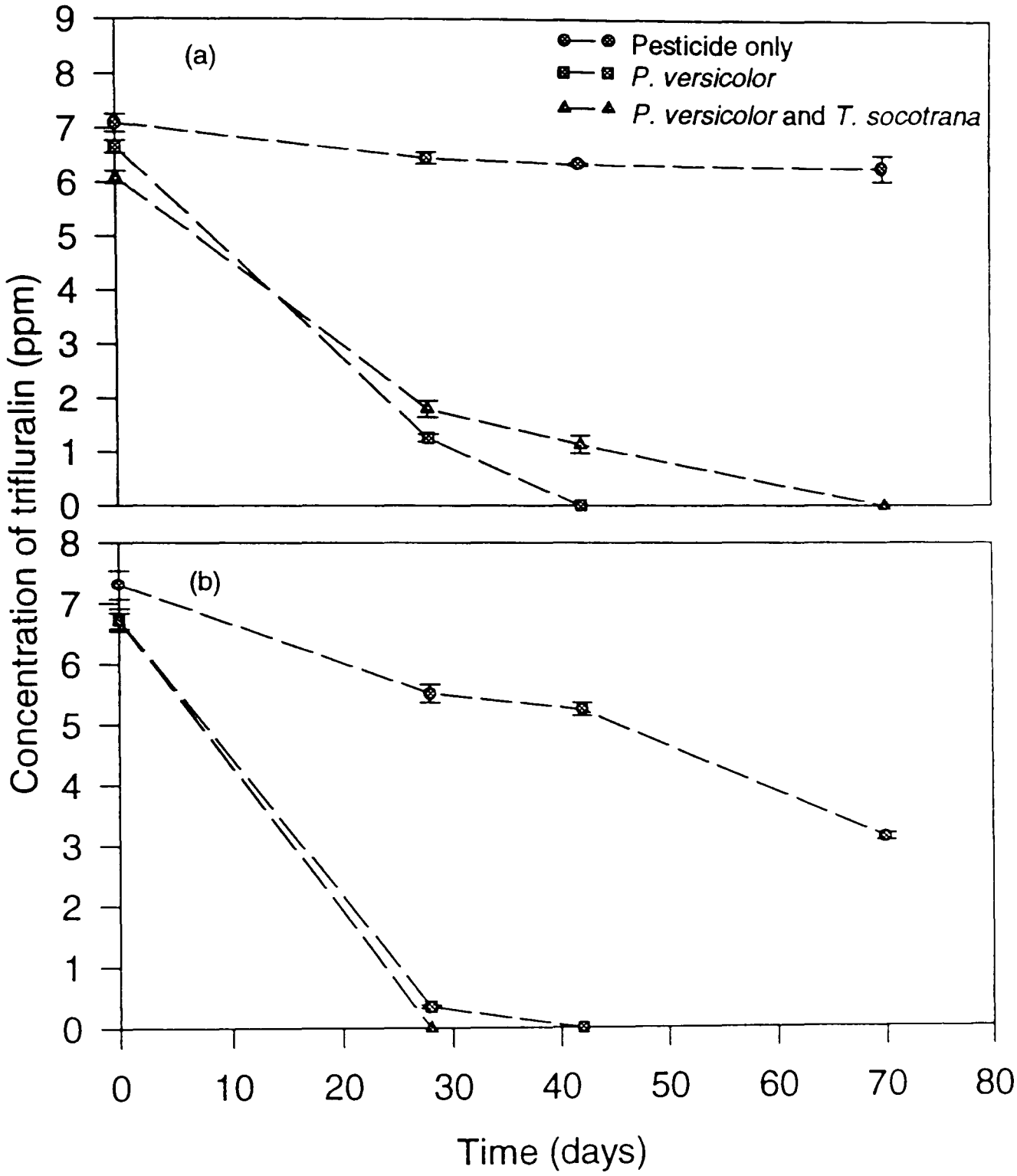


Figure 3.39. Comparison of effect of temperature and fungi on temporal degradation of 10 ppm trifluralin in field capacity soil at (a) 20°C and (b) 30°C. Error bars indicate the standard deviation of the means. Least significant differences ($P<0.05$) were 0.227, 0.209 for 20 and 30°C fungal treatments respectively.

Table 3.14. Analysis of variance of the slopes of curves regarding temporal degradation of various pesticides in soil with no inoculated fungi (control), soil plus straw inoculated with *P. versicolor* (PV) and soil plus straw inoculated with *P. versicolor* + *T. socotrana* (PV +TS) incubated at 20 and 30°C. (* significant difference $P<0.05$ relative to control) LSD = Least significant difference.

Treatment	Figure	Average slope value (ppm / day)	Incubation Temperature	LSD (ppm / day)
Dieldrin (control) (5 ppm)	**3.34**	**0.0027**	**20°C**	**0.0034**
PV + dieldrin (5 ppm)	**3.34**	**0.016***	**20°C**	
PV + TS + dieldrin (5 ppm)	**3.34**	**0.012***	**20°C**	
Dieldrin (control) (5 ppm)	**3.34**	**0.006**	**30°C**	**0.0014**
PV + dieldrin (5 ppm)	**3.34**	**0.020***	**30°C**	
PV + TS + dieldrin (5 ppm)	**3.34**	**0.013***	**30°C**	
Dieldrin (control) (10 ppm)	**3.35**	**0.0032**	**20°C**	**0.0020**
PV + dieldrin (10 ppm)	**3.35**	**0.019***	**20°C**	
PV + TS + dieldrin (10 ppm)	**3.35**	**0.012***	**20°C**	
Dieldrin (control) (10 ppm)	**3.35**	**0.0078**	**30°C**	**0.0011**
PV + dieldrin (10 ppm)	**3.35**	**0.024***	**30°C**	
PV + TS + dieldrin (10 ppm)	**3.35**	**0.017***	**30°C**	
Simazine (control) (5 ppm)	**3.36**	**0.021**	**20°C**	**0.0007**
PV + simazine (5 ppm)	**3.36**	**0.027***	**20°C**	
PV + TS + simazine (5 ppm)	**3.36**	**0.028***	**20°C**	
Simazine (control) (5 ppm)	**3.36**	**0.047**	**30°C**	**0.0019**
PV + simazine (5 ppm)	**3.36**	**0.042***	**30°C**	
PV + TS + simazine (5 ppm)	**3.36**	**0.043***	**30°C**	

Table 3.14. Continued

Treatment	Figure	Average slope value (ppm / day)	Incubation Temperature	LSD (ppm / day)
Simazine (control) (10 ppm)	**3.37**	**0.017**	**20°C**	**0.0019**
PV + simazine (10 ppm)	**3.37**	**0.034***	**20°C**	
PV + TS + simazine (10 ppm)	**3.37**	**0.039***	**20°C**	
Simazine (control) (10 ppm)	**3.37**	**0.088**	**30°C**	**0.0012**
PV + simazine (10 ppm)	**3.37**	**0.087**	**30°C**	
PV + TS + simazine (10 ppm)	**3.37**	**0.079***	**30°C**	
Trifluralin (control) (5 ppm)	**3.38**	**0.011**	**20°C**	**0.0060**
PV + trifluralin (5 ppm)	**3.38**	**0.082***	**20°C**	
PV + TS + trifluralin (5 ppm)	**3.38**	**0.045***	**20°C**	
Trifluralin (control) (5 ppm)	**3.38**	**0.035**	**30°C**	**0.012**
PV + trifluralin (5 ppm)	**3.38**	**0.11***	**30°C**	
PV + TS + trifluralin (5 ppm)	**3.38**	**0.11***	**30°C**	
Trifluralin (control) (10 ppm)	**3.39**	**0.011**	**20°C**	**0.0070**
PV + trifluralin (10 ppm)	**3.39**	**0.16***	**20°C**	
PV + TS + trifluralin (10 ppm)	**3.39**	**0.086***	**20°C**	
Trifluralin (control) (10 ppm)	**3.39**	**0.058**	**30°C**	**0.015**
PV + trifluralin (10 ppm)	**3.39**	**0.17***	**30°C**	
PV + TS + trifluralin(10 ppm)	**3.39**	**0.24***	**30°C**	

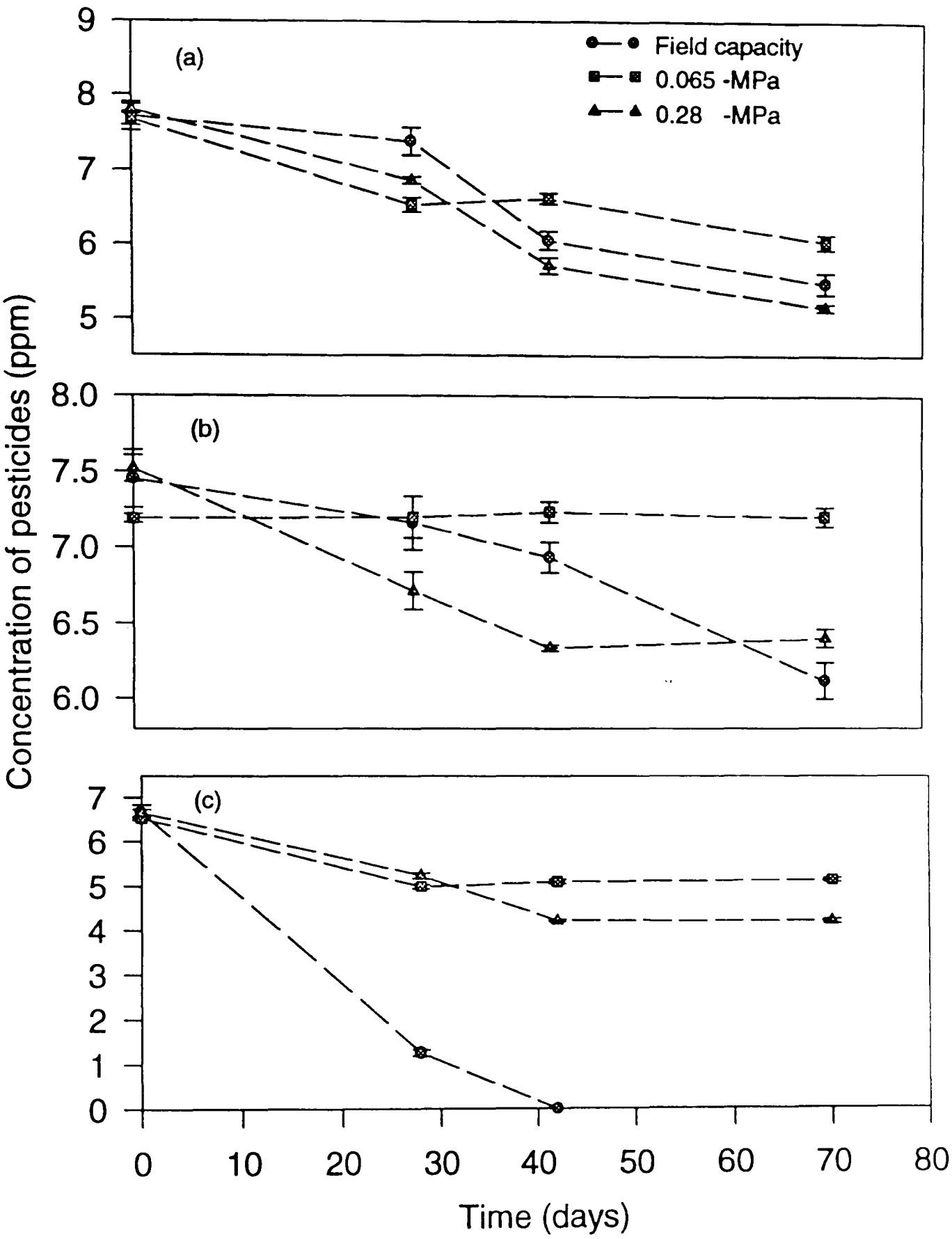


Figure 3.40. Effect of water potential on the ability of *P. versicolor* to simultaneously breakdown (a) simazine, (b) dieldrin and (c) trifluralin added at an initial concentration of 10 ppm and incubated at 20°C. Error bars indicate standard deviation of the mean. Least significant difference ($P<0.05$) were 0.192, 0.185 and 0.120 for simazine, dieldrin and trifluralin respectively.

At the end of experimental period there was a statistically significant difference between all three water potential treatments.

Dieldrin: *P. versicolor* performed best in field capacity soil, where the insecticide was degraded at a steady rate after 28, 42 and 70 days (Figure 3.40b). Surprisingly, the mid-range wetness soil (-0.065 MPa) proved to be the poorest conditions for *P. versicolor* to degrade dieldrin in soil. In fact, no noticeable degradation of the insecticide was observed at this water potential. In the driest soil, *P. versicolor* degraded dieldrin at a steady rate until after 42 days when no further breakdown was observed until the 70 d of the sample. As with simazine there was a statistically significant difference between treatments at the final sampling time (70 d) of the experiment.

Trifluralin: Similarly, *P. versicolor* degraded trifluralin most effectively in field capacity soil, where no trifluralin was detected after the 42d sample (Figure 3.40c). The next most effective treatment for *P. versicolor* to degrade trifluralin was the driest soil (-0.28 MPa). Results were similar to those for dieldrin with an initial sharp drop in the concentration of trifluralin until 42 days, after which time the concentration remained relatively constant with no noticeable degradation up to 70 days. As with the other two pesticides the mid-range wetness (-0.065 MPa) soil proved to be the poorest water potential for *P. versicolor* to degrade trifluralin. In this case an initial sharp drop in the concentration of trifluralin was observed which had a similar degradation slope to the -0.28 MPa soil. However, this decrease was only observed until the 28 d sample, after which time no obvious breakdown was observed. Statistically, there was a significant difference between the three treatments after 28, 42 and 70 days.

Table 3.15. summarises the final percentage breakdown of the pesticides relative to untreated soil controls in the presence of *P. versicolor* and *P. versicolor* + *T. socotrana* at 20 and 30°C.

Table 3.15. Percentage breakdown of pesticides relative to untreated soil controls by *P. versicolor* (PV) and *P. versicolor* + *T. socotrana* (PV + TS) at 20 and 30°C.

Fungal treatment	Incubation temperature (°C)	Final incubation period (days)	Percentage breakdown (%)
PV + dieldrin (5 ppm)	**20**	**70**	**24**
PV + TS + dieldrin (5 ppm)	**20**	**70**	**18**
PV + dieldrin (5 ppm)	**30**	**70**	**26**
PV + TS + dieldrin (5 ppm)	**30**	**70**	**12**
PV + dieldrin (10 ppm)	**20**	**70**	**15.5**
PV + TS + dieldrin (10 ppm)	**20**	**70**	**7.5**
PV + dieldrin (10 ppm)	**30**	**70**	**17**
PV + TS + dieldrin (10 ppm)	**30**	**70**	**11**
PV + simazine (5 ppm)	**20**	**70**	**14**
PV + TS + simazine (5 ppm)	**20**	**70**	**16**
PV + simazine (5 ppm)	**30**	**70**	**0**
PV + TS + simazine (5 ppm)	**30**	**70**	**0**
PV + simazine (10 ppm)	**20**	**70**	**14**
PV + TS + simazine (10 ppm)	**20**	**70**	**20**
PV + simazine (10 ppm)	**30**	**70**	**7**
PV + TS + simazine (10 ppm)	**30**	**70**	**0**
PV + trifluralin (5 ppm)	**20**	**42**	**>73**
PV + TS + trifluralin (5 ppm)	**20**	**70**	**>73**
PV + trifluralin (5 ppm)	**30**	**28**	**>76**
PV + TS + trifluralin (5 ppm)	**30**	**28**	**>76**

Table 3.15. Continued

Fungal treatment	Incubation temperature (°C)	Final incubation period (days)	Percentage breakdown (%)
PV + trifluralin (10 ppm)	**20**	**42**	**>86.5**
PV + TS + trifluralin (10 ppm)	**20**	**70**	**>86.5**
PV + trifluralin (10 ppm)	**30**	**42**	**>69**
PV + TS + trifluralin (10 ppm)	**30**	**28**	**>72.5**

CHAPTER FOUR

Discussion

4.1. NATURAL BREAKDOWN OF PESTICIDES IN WATER AND SOIL

In this section each pesticide will be looked at individually for their natural breakdown in water and soil.

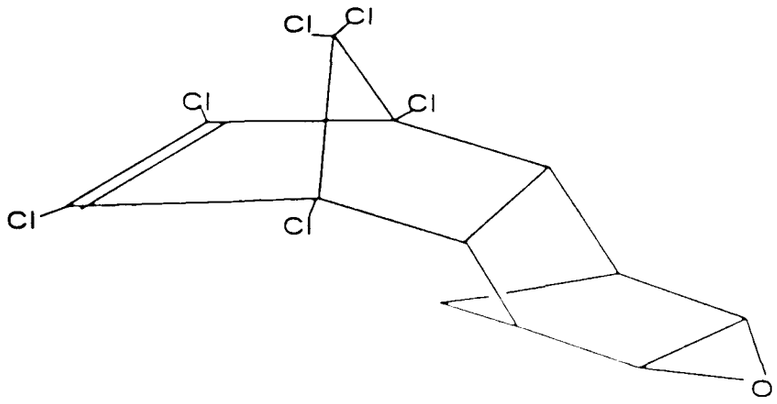
In water the solubility of the three pesticides was very low and produced precipitation after addition of each pesticide to the water. This resulted in much lower concentrations being detected in solution. However, in soil this was not the case where both 5 and 10 ppm concentrations of the pesticides were effectively distributed in the medium.

Dieldrin: In tap water this highly persistent insecticide showed very little or no breakdown at 10, 20 and 30°C. This was consistent with the findings of McDougall *et al.* (1994) where a number of pesticides including aldrin, dieldrin, lindane, chlorpyrifos and prothiofos in stored roof water in galvanised tanks, concrete tanks and also in distilled water were examined under laboratory conditions. They found that the dieldrin samples stored in the dark at 23°C showed little significant breakdown over a 36 week period. In the present study there appeared to be a slightly higher degradation rate of dieldrin in unsterile tap water when compared to sterile tap water, at 30°C. This could be due to microbial activity in unsterile tap water, although it is also likely to be partially due to the difference in the pH value between unsterile (pH=7.25) and sterile water (pH=5.85).

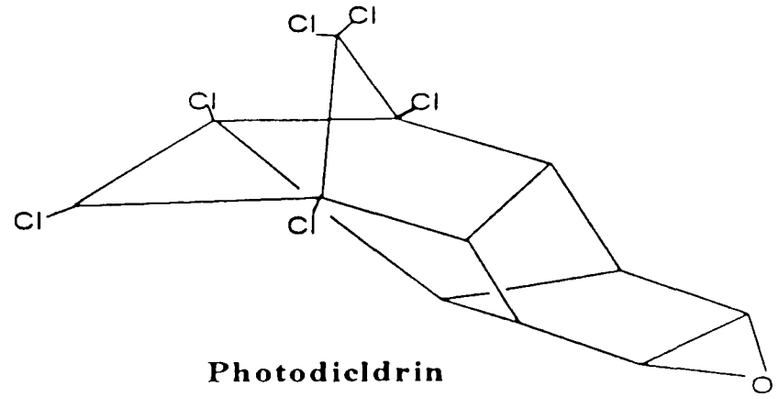
In field capacity soil dieldrin showed a similar persistency to that in water, although increasing the temperature to 30°C enhanced the degradation relative to that observed at 20°C. For example at 5 ppm concentration, dieldrin showed a 4%

degradation at 20°C but a 10% degradation at 30°C over the 70 day period. An increase in concentration of dieldrin from 5 to 10 ppm had the effect of decreasing the breakdown rate, although the degradation rate increased from 2.5% at 20°C to 6% at 30°C. It is probable that at the higher temperature the activity of the indigenous microflora would be much greater, where one could envisage greater activity of the relevant enzymes, although an increase in non-biological reactions should also be considered. Ghadiri *et al.* (1995) found that a constant soil temperature of 30°C (with constant moisture content) significantly enhanced the microbial degradation of both aldrin and dieldrin compared to outdoor storage where temperature had both daily and seasonal fluctuations, but stayed below 30°C for much of the year. It is though, possible that the variation in moisture content of the soil in the outdoor environment also could have had some effect on the degradation rates of the insecticides under experiment. However, in the present study apart from the slight increase in degradation of dieldrin in field capacity soil (3%), dieldrin showed little difference in degradation rates at the other two water potentials (-0.065 and -0.28 MPa) examined. This was again consistent with Ghadiri *et al.* (1995) who concluded a generally low sensitivity of the organochlorine pesticides aldrin, dieldrin, endrin and chlordane to soil moisture content, although they showed a slower degradation rate of these pesticides in very dry soil of -7.00 MPa, when compared to -0.32 and -0.07 MPa.

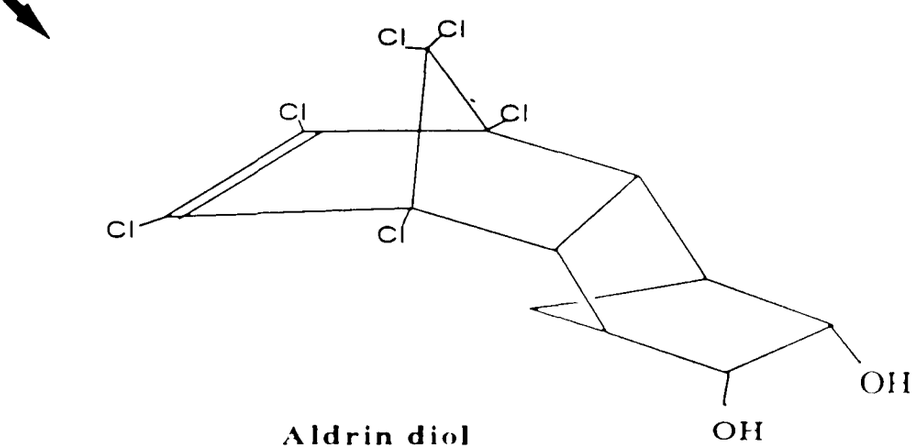
Several microbial species are known to convert dieldrin to photo-dieldrin by the formation of an intramolecular bridge (Figure 4.1). In soil, dieldrin can be converted to the aldrin diol, a less toxic compound due to its increased water solubility (Nicholson and Blaine, 1993). The type of organisms involved in such conversions are yet to be identified.



Dieldrin



Photodieldrin



Aldrin diol

Figure 4.1. The major metabolites of dieldrin

Simazine: In both sterile and unsterile tap water in the dark, simazine was degraded to a small extent although there was not a marked effect of temperature. In water and in the presence of sunlight simazine is hydrolysed to herbicidally inactive 6-hydroxy simazine (Nicholson & Blaine, 1993). However, in the present study the samples were stored in amber coloured bottles to study the effect of water alone in which the hydrolysis would be much slower.

In soil a definite difference in the degradation of this herbicide between 20 and 30°C indicated an enhancement of degradation as soil temperature conditions were increased. Kostowska & Rola (1984) who did experiments into simazine breakdown rate in various soils at 20 and 30°C concluded that simazine degradation was positively correlated only with temperature although the water content was kept at 90% of water holding capacity throughout their experiments. Walker *et al.* (1983) found that a change in temperature of soil containing simazine from 10 to 30°C increased the degradation rate of this herbicide by a factor of between 2 and 5. An important point to note is the effect of increased concentrations of this herbicide on its natural breakdown rate. In the present study using a higher concentration of simazine (10 ppm) showed a lower breakdown rate at both 20 (14%) and 30°C (74%) relative to a 5 ppm initial concentration at 20°C (36%) and 30°C (84%). Similarly, Schoen & Winterlin (1987) found that an increase in the concentration of a mixture of pesticides (atrazine, 2,4-D, trifluralin, carbaryl, diazino, fenitrothion and captan) adversely affected the microbial activity and populations in the soil under investigation. In another study of mineralisation of PCB 77, Vyas *et al.* (1994) concluded that the higher concentration of 513.7 nmol as opposed to 30.1 nmol may be toxic not only to the microbial enzyme systems but also to cellular metabolism in general.

However the effect of soil water potential was not examined in many of these studies. This factor could significantly influence the ability for pesticide degradation by micro-organisms in soil. In the present study in the field capacity soil a steady pattern of degradation of simazine was observed although this was less than that of the mid-wetness soil (-0.065 MPa) which showed an overall percentage breakdown of 18% over the 70 day test period. The steady degradation in field capacity soil was probably due to continuous presence and activity of both bacteria and fungi as well as non-biological reactions during the temporal experiments. Although one should bear in mind that at field capacity or conditions just above this level, may result in anaerobic conditions which may affect the microflora and hence rates of degradation. It is thus important to have aerobic conditions present. The higher degradation rate of simazine in the mid-wetness soil treatment over the field capacity soil could be a result of an increase in microbial activity or the presence of simazine-degrading micro-organisms which are more active at the lower moisture content over the other competing micro-organisms. Often many micro-organisms grow optimally at water potentials less than field capacity. Indeed some fungi can survive at water potentials of -40 MPa, although the wilting point of plants is about -1.4 MPa (Brock & Madigan, 1991 ; Lacey *et al.*, 1991).

Increased adsorption of pesticides to soil particles over time (aging) and reduced extraction is another aspect which can account for an apparent degradation of these chemicals. It is generally accepted that much less pesticide is retained by a sandy soil with low organic content than one with a higher organic and clay content (Huang & Frink, 1989). In the present study the sandy loam soil used had 66% sand with 1% organic content. Also according to Ogram & Jessup (1985) it is not always the case that sorption renders a pesticide unavailable for degradation. In some cases the soil micro-

organisms may also be adsorbed to soil particles and in the proximity of the pesticides being degraded. Thus the adsorption of the pesticides to soil particles might either enhance or decrease microbial degradation rates in soil.

Due to the similarities of structure between atrazine and simazine, one can predict metabolites of simazine by looking at atrazine metabolites elucidated by Kruger *et al.* (1996) (Figure 4.2). This shows that the two tertiary amine groups are dealkylated to produce more water soluble primary amines. Furthermore, in another type of reaction the Cl group is replaced by a hydroxy group producing the 6-hydroxy simazine which goes on to be dealkylated, losing the ethyl groups. This is again a more water soluble product than the mother compound.

Trifluralin: This herbicide is the most volatile compound in the dinitroaniline group (Johnston & Camper, 1991) which probably explains its greater rate of decrease in water with increasing temperature which ranged from about 45% at 10°C to about 75% at 30°C. The difference between degradation in sterile water and unsterile was minimal, especially at 30°C, which is an indication of the overwhelming effect of temperature on the degradation of this chemical, in both treatments.

In field capacity soil, there was a clear and significant difference in the rate of degradation of trifluralin between 20 and 30°C treatments. As with simazine this was probably due to a combination of microbial breakdown and non-biological reactions at the higher temperature, although volatilisation must have also played an important role in the rate of disappearance of this particular pesticide from soil.

In the driest water potential treatment (0.28 -MPa) trifluralin showed no apparent degradation over the experimental period and which contrasted with the rapid

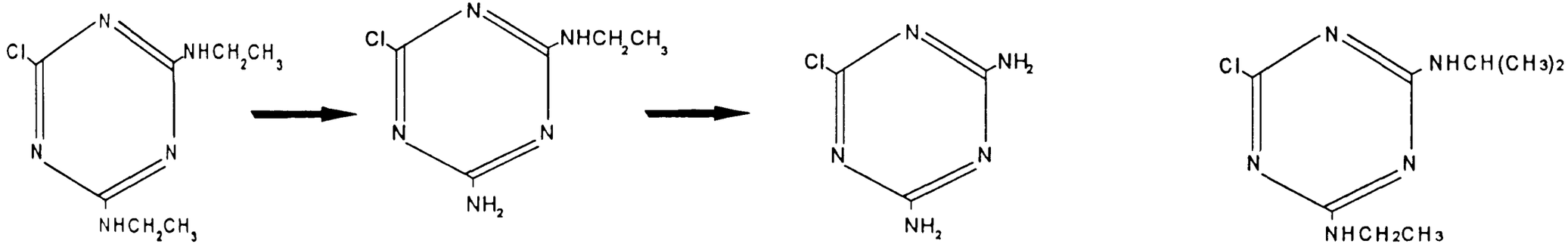


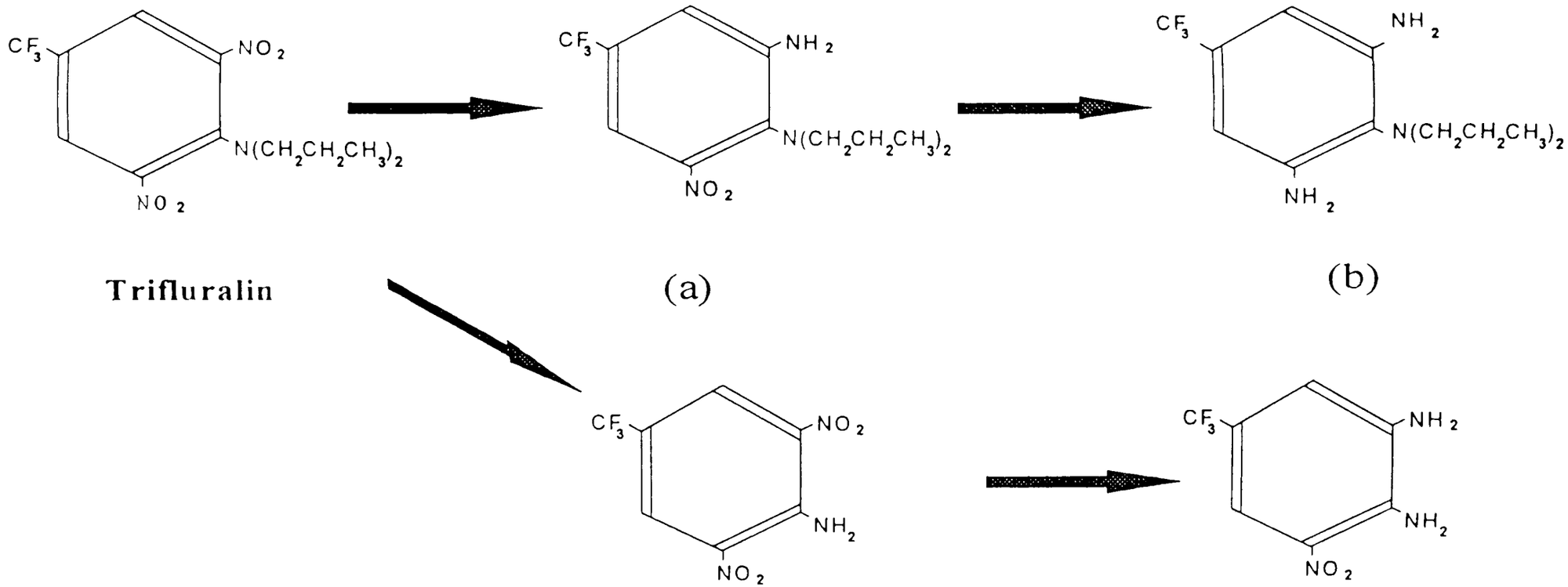
Figure 4.2. The possible metabolites of simazine, as well as chemical structure of atrazine.

disappearance in the field capacity soil. This suggests that microbial activity was markedly decreased in these conditions or that the chemical reactions were rendered inactive by adsorption to soil particles. According to Nicholls (1991) the drier the soil the stronger the adsorption, and hence less volatilisation occurs. Volatilisation is apparently greatest in wet soils. Grass *et al.* (1994) found that the lowest volatilisation of trifluralin in soil occurred at the lower (48%) than the higher (78%) range of air relative humidity. Jolly & Johnstone (1994) concluded that trifluralin degradation in soil increased with increasing both temperature and moisture but no degradation occurred in air-dried soil.

Interestingly, Jolly & Johnstone (1994) found that water added to soil with a low initial level of water present, may have more influence on degradation of trifluralin than if the same higher per cent water content was already present in the soil. This could be due to the water added to the soil being more available for degradation of trifluralin than that already present. The addition of water to air-dry soil may also affect adsorption of trifluralin to the soil, which, in turn may affect degradation.

In soil, trifluralin is degraded by hydrolysis, giving the corresponding diamino and subsequently triamino derivatives by reduction of the nitro groups (Figure 4.3a, b). In another reaction the tertiary amine entity loses the two propyl groups resulting in a primary amine though this reaction occurs to a lesser extent (Figure 4.3c) (Nicholson & Blaine, 1993). A further biotransformation product has been suggested by Zayed *et al.*, (1983) where one of the NO_2 groups is reduced producing a primary amine substituent (Figure 4.3d).

Unfortunately very little work has been carried out on the toxic nature of the metabolites of dieldrin, simazine or trifluralin. However one assumes that if these metabolites are



Trifluralin

(a) **2-amino-6-nitro-trifluoro-N,N-dipropyl toluidine**

(b) **2,6-diamino-trifluoro-N,N-dipropyl toluidine**

(c) **2,6-dinitro-trifluoro-*p*-toluidine**

(d) **2-nitro-6-amino-trifluoro-*p*-toluidine**

Figure 4.3. The major metabolites of trifluralin

produced as a result of the action of micro-organisms, it is more likely that they are less toxic to these organisms than the parent compounds.

4.2. *IN VITRO* CAPABILITY OF WHITE-ROT FUNGI FOR BREAKDOWN OF PESTICIDES

Four fungi were studied in detail for their tolerance and ability to degrade the three pesticides individually using two initial concentrations (5 and 10 ppm). These fungi were chosen based on a previous extensive screening programme carried out by Roldan-Garcia (1994). Here the effect of temperature was also examined, although experiments at 10°C were only carried out with simazine as the fungi either grew poorly (*P. versicolor*) or not at all (*T. cingulata*, *T. socotrana* and *P. chrysosporium*) in the presence of this pesticide.

A comparison of the capabilities of the selected fungi showed that *P. chrysosporium* grew faster than the other three species at 10, 20 and 30°C in the absence of pesticides. In fact its growth rate, relative to that at 10°C increased by 10 to 20 fold at 20 and 30°C respectively (1.0 mm day^{-1} at 10°C to 10 mm day^{-1} at 20°C and 18 mm day^{-1} at 30°C). In the presence of the pesticides, growth of this fungus was however drastically inhibited. Examination of the growth of the other three fungi in the absence of pesticides, the climatic source of the species was important. For example, *P. versicolor*, a temperate fungus, grew at 10°C, with an optimum at 20°C. In contrast, the tropical species (*T. socotrana and T. cingulata*) did not grow at 10°C, but grew faster than *P. versicolor* at 30°C.

P. chrysosporium commonly used in bioremediation systems was surprisingly

sensitive to the three pesticides in this study. At 10 and 20°C the isolate used in this study was completely inhibited at both 5 and 10 ppm, while at 30°C (at 5 ppm) it grew very slowly when compared to the other three species studied. This could partially be due to strain differences.

Overall, *P. versicolor* was most tolerant of trifluralin and dieldrin followed by *T. socotrana* and *T. cingulata* in the presence of both 5 and 10 ppm concentrations.

The increase in degradation of the pesticides achieved by the fungi examined in this study generally paralleled the growth pattern of the species. For example, *P. versicolor* grew best in the presence of trifluralin and was the most efficient species at degrading this herbicide. Similar patterns was observed with simazine where *P. versicolor* was again the best species.

The most variable results was obtained with the insecticide dieldrin. As mentioned previously, the growth rates showed *P. versicolor* to be the most tolerant fungus followed by *T. socotrana* and then *T. cingulata*. *P. chrysosporium* showed only poor growth at 5 ppm and 30°C. However, when the degradation patterns of the three fungi, *P. versicolor*, *T. socotrana* and *T. cingulata*, were analysed the results were quite different. With dieldrin *T. socotrana* or *T. cingulata* showed the best performance, with *P. versicolor* being less efficient. However in the presence of 10 ppm, at 20°C, *T. cingulata* was unable to degrade dieldrin to any degree, with *T. socotrana* being more efficient than *P. versicolor*.

Surprisingly, *P. chrysosporium* was ineffective at degrading 5 ppm dieldrin at 30°C and unable to degrade the other pesticides under any of the treatment conditions. Previously, Morgan *et al.* (1991) carried out experiments in nutrient rich liquid media

incubated at 30°C over a 70 day period. Using ^{14}C labelled pesticides they found that the relative degradation of 3,4-dichloroaniline, dieldrin and phenanthrene per unit biomass of the established fungi were in the order *Trametes versicolor* > *Chrysosporium lignorum* > *Phanerochaete chrysosporium.*

Overall, in the present study, *T. socotrana* and *T. cingulata* were found to be by far the most efficient fungi to degrade dieldrin. Alleman *et al.* (1992) found the *Trametes* species, *T. versicolor*, to be most the most effective fungus in degrading the polychlorinated pesticide, PCP (pentachlorophenol) among a group of six white-rot fungi which included *P. chrysosporium.* However, the results in the present study indicate that the most tolerant fungus is not always the most efficient at degrading the pesticide. Lamar *et al.* (1990c) who carried out experiments on growth rates of several *Phanerochaete* spp. found that *P. chrysosporium* showed a higher growth rate on 2% malt agar than *P. sordida.* However, *P. sordida* degraded PCP more efficiently than *P. chrysosporium* in an aqueous culture medium.

It has been shown that *P. chrysosporium* is extremely efficient in degrading DDT under both nitrogen-deficient and nitrogen-rich conditions using the major enzymes lignin peroxidases and manganese peroxidases (Bumpus & Aust, 1987; Katayama & Uchida, 1992; Fernando & Aust, 1994). Work by Mswaka (1994) has shown that isolates of *T. socotrana* and *T. versicolor* were able to degrade hydrolytic and alkali preparation of lignin, indicative of ligninase activity. Thomas *et al.* (1992) found that the highest ligninase activity was at around 33°C, when using *P. chrysosporium* to degrade PCBs added to liquid cultures, over the range 30 to 40°C. The fact that mineralisation of DDT has also occurred in non-lignolytic cultures of *P. chrysosporium* however has led to the suggestion that a second degrading system may be present although this was

not established (Lin *et al.*, 1990) and needs to be investigated. In fact, Dhawale *et al.* (1992) have argued that the two enzymes lignin and manganese peroxidases are not essential for degradation of phenanthrene by the white-rot fungus *P. chrysosporium* and that this fungus can degrade certain environmental pollutants under non-lignolytic conditions as well. In the present study a 0.5% w/w malt extract agar was used to provide a relatively low nutrient environment to ensure conditions suitable for lignolytic enzyme production.

Other enzymes such as veratryl alcohol oxidase (VAO) and aromatic alcohol oxidase (AAO) have been purified from fungi such as *Pleurotus ostreatus* but their role in lignin degradation is not known (Sannia *et al.*, 1991; Hatakka, 1994). As well as the lignin peroxidases, another enzyme which has been found in the extracellular culture fluid of *P. chrysosporium* is glyoxal oxidase (GLOX), a H_2O_2-producing enzyme which uses glyoxal and methylglyoxal as substrates to reduce molecular oxygen to H_2O_2. The two substrates were also present in the lignolytic cultures (Kersten, 1990; Barr & Aust, 1994a). Another major enzyme recognised with lignin degrading activity is laccase. Recent study has shown that *Trametes versicolor* is able to produce laccase intra- and extracellularly when grown on a substrate such as wheat straw and beech wood (Schlosser *et al.*, 1997), although the role of laccase is thought to be limited and somewhat unclear (Buswell, 1994).

In most studies mentioned previously where the capabilities of the fungi to degrade xenobiotics has been assessed (Lamar *et al.* 1990a; Yadav & Reddy, 1993; Dhawale *et al.* 1995), work has been done in nutrient rich and nutrient limited liquid media where good contact was achieved between the fungi and the test chemicals. Additionally in most cases the fungi have been grown to some extent before the pollutant

was added to the liquid culture. This produces very good *in vitro* results, but is not very realistic for subsequent testing in media such as soil. In the present study a more rigorous regime was used where the fungus was grown with a relatively low nutrient status (0.5% MEA) on top of a cellophane disc overlay on the solid agar media thus providing no direct contact between the fungal mycelia and the target pesticide. Hence for effective degradation of the test pesticides, our study depended on the capability of the fungal species to produce extracellular enzymes which could diffuse through the cellophane to degrade the pesticides. Besides direct *in situ* experiments in soil microcosms, the method used in this study may be a more useful one for comparing the potential of fungi for bioremediation systems.

4.3. BIOREMEDIATION OF PESTICIDES IN SOIL USING FUNGI

The *in vitro* studies produced two possible candidates to be examined in detail for their ability to degrade a mixture of the three pesticides in soil microcosms. These were *P. versicolor* and *T. socotrana*, which were incorporated on wheat straw as a carrier material. The carrier was, we believe, essential to ensure fungal growth and establishment before addition to the soil. Because of the low carbon status of the sandy loam soil (1%) this would not encourage effective fungal colonisation. However, Bumpus (1993) has suggested that, even in organic soils (with total organic content of 60 and 95%) the organic compounds present are often unsuitable growth substrates for most fungi.

In this study, the control treatment consisted of soil incorporated with pesticides only. Soil incorporated with pesticides/straw mixtures without fungal inoculant were not included as a control. The presence of straw (without fungal inoculant) might have

stimulated some microbial activity, resulting in enhanced degradation of the pesticides mixture.

4.3.1. Effect of temperature and concentration

In this part of the study the experiments were carried out in field capacity soil only whilst observing the effect of temperature and concentration on the capability of either *P. versicolor* alone or in partnership with *T. socotrana*. Here increasing the incubation temperature generally resulted in an increase in the rate of degradation of the pesticides mixture in soil, whilst increasing the concentration had the opposite effect.

Dieldrin: At 20°C 5 ppm dieldrin showed a breakdown of 4% in the untreated control soil, but 28% in the presence of *P. versicolor* alone and 22% in the presence of both *P. versicolor* and *T. socotrana*. At 30°C, these values increased to 10 and 36% for controls and in the presence of *P. versicolor* respectively, but remained at 22% for the mixture of the two fungi.

At the higher concentration of 10 ppm dieldrin at 20°C in the control treatment showed only a 2.5% breakdown. However this was increased seven fold to 18% in the presence of *P. versicolor* alone and four fold to 10% when both *P. versicolor* and *T. socotrana* were present. The increase in the temperature from 20 to 30°C improved the breakdown rate to 6, 23 and 17% in the control, *P. versicolor* and *P. versicolor* + *T. socotrana* treatments, respectively.

Simazine: For 5 ppm simazine at 20°C, a 36% breakdown was observed in the control treatment, which was increased to 50 and 52% in the presence of *P. versicolor* and *P. versicolor* plus *T. socotrana* respectively. At 30°C however, the breakdown was

around 80% for all three treatments.

The increase in the concentration to 10 ppm simazine at 20°C produced a natural breakdown of 14% which was enhanced to 28% in the presence of *P. versicolor* alone and 34% when both fungi were present. At 30°C, this value was increased to 73, 80 and 73% respectively.

Trifluralin: At 20°C the natural breakdown of 5 ppm trifluralin in the field capacity soil reached 21% after the 70 day incubation period. However, in the presence of *P. versicolor* no trifluralin was detected after 28 days and none after 42 days when *P. versicolor* + *T. socotrana* were present. At 30°C the natural breakdown of trifluralin increased to 67% after 70 days but no trifluralin was detected after the initial analysis in either of the fungal treatments.

When the concentration of trifluralin was increased to 10 ppm at 20°C incubation temperature, the natural breakdown of trifluralin was only about 11% after 70 days incubation, but in the presence of *P. versicolor* no trifluralin could be detected after 28 days, and none after 42 days when *P. versicolor* + *T. socotrana* were present. At 30°C the natural breakdown increased to 28% after the 70 day period. In the presence of *P. versicolor* + *T. socotrana* no trifluralin was detected after the initial analysis although this period increased to > 28 days when *P. versicolor* alone was incorporated in the soil.

The increase in the degradation rate of the pesticides mixture in soil at 30°C was probably due to the increased activity of the introduced fungi as well as non-biological transformations. The *in vitro* studies also showed an increase in fungal activity at 30 when compared to 20°C. The effect of increased pesticide concentration also reduced breakdown rates in this study. Ali & Wainwright (1994), carried out an experiment in

which a spore suspension of *P. chrysosporium* was used to breakdown three concentrations of the fungicide benomyl. Here an immediate breakdown of the benomyl (56.25 and 112.5 μgg^{-1}) was observed which was completed within 46 days. However, the degradation of the higher concentration of this fungicide ($225\mu gg^{-1}$) did not begin until 40-50 days after the addition of the inoculum, and was not completed before 85 days.

Overall, *P. versicolor* proved to have a more consistent capability for the degradation of pesticides in soil than the mixture of *T. socotrana* + *P. versicolor* together. The use of mixtures of fungal inoculants can result in either additive, synergistic or antagonistic effect on the treatment pesticides. It appears from the results that there may be some antagonism between *T. socotrana* and *P. versicolor* which make them unsuitable for use together. From the literature the majority of experiments have been carried out with *P. chrysosporium* only, without any attempt to exploit the potential use of two or more fungi. Although we found an antagonistic effect between *T. socotrana* and *P. versicolor,* other combinations and groups need to be examined for the possibility of synergetic affects.

The temperature used for the final stage of this project was 20°C. The use of lower than optimal temperature was also employed in an *in situ* study by Lamar & Dietrick (1990). They found that the inoculation of soil, which contained 250-400 μgg^{-1} of pentachlorophenol (PCP), with a wood chip inoculum thoroughly colonised with *P. chrysosporium* or *Phanerochaete sordida*, produced an overall 88 to 91% reduction of PCP in 6.5 weeks which was achieved under sub-optimal temperatures (<32°C) for both growth and activity of the fungi. In another study Lamar *et al.* (1990a) showed that the concentration of extractable PCP from sterile soil microcosms inoculated with *P.*

chrysosporium fully grown on pulpwood chips drastically decreased (ave. decrease 98%) compared to control uninoculated soil (ave. decrease 43%) over a two month period at an incubation temperature of 25°C.

4.3.2. Effect of water potential

For the final phase of this project, *P. versicolor* was used to investigate the effect of water potential on the ability of this inoculant to degrade a mixture of the three pesticides (simazine, dieldrin and trifluralin) in soil at 20°C each added at 10 ppm. As discussed in Section 4.1, field capacity was the optimal water potential for natural degradation of dieldrin and trifluralin in soil. This pattern was also followed when *P. versicolor* was incorporated into soil (using wheat straw as the carrier), although this degradation was substantially increased in the presence of the fungus, particularly for trifluralin and dieldrin. Here simazine degraded best at -0.28 MPa, the driest soil treatment.

For trifluralin and dieldrin the driest soil treatment (-0.28 MPa) was found to be the second best water availability condition for enhancing breakdown by *P. versicolor*. The fungus showed good tolerance of this water potential perhaps suggesting a wider water potential range for active growth or a lower optimum for activity.

In the mid-wetness (-0.065 MPa) soil, *P. versicolor* appeared to be unable to compete with other micro-organisms and was less active resulting in practically no increase in degradation of the three pesticides, especially of dieldrin and trifluralin. Overall, trifluralin is the pesticide which was most effectively degraded by *P. versicolor* when incorporated on straw in soil.

Bumpus (1993) has argued that fungi present in soil, e.g., *Penicillium* spp.,

Aspergillus spp. and *Trichoderma* spp., exist as quiescent spores or in a vegetative state in which minimal metabolism and growth occurs. These and other micro-organisms in soil have a competitive edge in their "ecological niche" and can sometimes prevent any credible bioremediation from taking place. To overcome this, in the case of *P. chrysosporium* for example, he suggested inoculation of the fungus on a suitable growth substance (e.g. wood chips, wheat straw) would enhance the chances of an effective bioremediation system.

In the present study *T. socotrana* and *P. versicolor* were allowed to effectively colonise the straw prior to addition to soil, thus providing them with a competitive edge over the indigenous soil micro-organisms. Furthermore, the more easily available water-soluble nutrients in the straw would have been utilised by the white-rot fungi prior to addition to the soil. Hence this component would not be available for the indigenous micro-organisms. Therefore it cannot be argued that the addition of the carrier or growth substrate to the soil might encourage some indigenous micro-organisms to utilise the added straw as a nutrient source and thus be also contributing to of the bioremediation in addition to the introduced white-rot fungus.

It can also be suggested that the addition of the pesticide-water mixtures to the soil, which had previously been dissolved in organic solvents, could have markedly decreased the number of surviving indigenous micro-organisms. For example, Nicholls (1991), has suggested that addition of organic solvents to soil could have a sterilising effect on the soil microflora. The addition of a mixture of pesticides could also have a detrimental effect on naturally inhabiting micro-organisms. However, Olson *et al.* (1984) found that the overall soil microbial populations were unaffected by 16 ppm or less trifluralin, with fungi and actinomycete population being favoured by such additions.

Harden *et al.* (1993) found that addition of straw to soil increased the size of the indigenous microbial biomass even in the presence of simazine and dinoterb (4-7 ppm) in the initial 20 days at 25°C. However, the detrimental side-effects of the herbicides on the soil microflora continuously increased during the 87 day experimental period. Mileski *et al.* (1988) found that the toxic effect of PCPs on the fungal growth of *P. chrysosporium* could be circumvented by allowing the mycelial mat to become established before adding the PCP.

Fungi have a great potential in bioremediation of xenobiotics in the environment. The use of fungal bioaugmentation on an industrial scale, however, has been hampered by several factors. Although fungi can be delivered to soil via carrier materials such as wood chip, wheat straw and corn cobs, these material have a low inoculum potential (fungal biomass per unit weight or volume of the inoculum) which necessitates the production and application of large bulks of inoculum/carrier, requiring addition ratios of 10 to 40% w/w to the soil. These can be relatively expensive and uneconomic for transport and routine use. In the present study a 10% w/w ratio of inoculated straw to soil was used. This proved to be an effective and practicable ratio both in terms of effectiveness of the inoculum in degrading the applied pesticides and ease of application. Transportation is another factor which can be difficult due to excessive metabolic heat of the inoculum during the transport causing increased use of nutrients by the fungi and a detrimental effect of the heat on biomass. Fungi are generally slow growing micro-organisms and are not as versatile as most bacteria which can a have wide spectrum of biodegradative abilities, are often easy to culture and manipulate in the laboratory. Bacteria though are very sensitive to changes in water availability and temperature in the soil environment. However, during incorporation of the fungi into the soil care is needed

to prevent the intense mechanical action which could damage the fungal mycelia and decrease the degrading ability of the fungi (Bumpus, 1993; Lestan & Lamar, 1996). The above problems have been addressed by Lestan & Lamar (1996), who have produced pelleted forms of various fungi which substantially increased the degradation of high concentrations of PCP (100 ppm) artificially added to various soils.

This study did not include the possible impact of ageing on the capability for bioremediation. This would have required much larger equilibrium periods prior to initiation of bioremediation experiments.

Furthermore, our studies could not use radiolabelling experiments to determine the rate of mineralisation of the pesticides, because of lack of facilities. However, the HPLC traces indicated that no secondary peaks of the products were formed during the experimental period (70d).

The present study shows clearly that more attention should be given to other white-rot fungi besides *P. chrysosporium* which has received a disproportionately great deal of attention. Other fungi such as *P. versicolor* and *T. socotrana* need to be studied in greater details both with regards to their enzymology and ability to biodegrade harmful chemicals in the environment. It has become clear from this study that the soil water potential could have a crucial effect on the ability of micro-organisms to degrade pesticides in soil. Also the natural environmental field conditions fluctuate markedly from region to region, it is surprising that only a few researchers have yet considered this critical issue (Walker *et al.*, 1983; Kostowska & Rola , 1984; Carter, 1991).

Indeed as discussed previously, white-rot fungi have several advantages with regards to degradation of harmful pollutants in the environment over bacteria but the complex mechanisms involved in optimising such systems has slowed down their

emergence as viable methods of remediation. Barr & Aust (1994) concluded that the best way of designing viable methods for bioremediation is through a more detailed study of these white rot fungi and their mechanisms.

This study has demonstrated that mixtures of pesticides can be simultaneously degraded effectively over a wide water potential and temperature range by fungi not previously examined in detail. The method of soil inoculation, using wheat straw as a carrier was shown to be feasible at a 1:10 ratio of inoculant:soil. Larger scale field trials would be necessary to evaluate whether this rate would be economical for further exploitation of fungi such as *T. socotrana* or *P. versicolor*.

CONCLUSIONS

1. The present study demonstrated that the tropical white rot fungi *Trametes cingulata* and *T. socotrana*, and temperate species *Polystictus versicolor* were a able to grow in the presence of 5 and 10 ppm dieldrin, simazine and trifluralin although tolerance varied with temperature and concentration over periods of 56 days. Of these *T. socotrana* and *P. versicolor* were most tolerant of the pesticides. In contrast, an isolate of the commonly used species *Phanerochaetae chrysosporium* was not very tolerant except at 30°C and in the presence of only 5 ppm of pesticide.

2. A HPLC technique was developed to efficiently analyse the extracted pesticides from solid agar media. This was done to study the effect of above screened fungi on pesticide breakdown incubated at various temperatures when added at 5 and 10 ppm.

3. By using cellophane overlays there was no contact between the test fungi and the pesticides. This showed that the fungi studied were able to produce extracellular enzymes to degrade the pesticides. *T. socotrana*, *T. cingulata* and *P. versicolor* were all able to degrade up to 65% of 5 ppm pesticides, and up to 45% of 10 ppm pesticides over periods of up to 56 days. Breakdown was optimum at 30°C relative to untreated controls.

5. The fungi best able to degrade dieldrin were *T. socotrana* and *T. cingulata*; for simazine were *P. versicolor* and *T. socotrana*, and for trifluralin were *P. versicolor* and *T. socotrana*.

6. The natural breakdown rates of dieldrin, simazine and trifluralin were very slow in both unsterile and sterile water over incubation periods of 42 days. There was no statistically significant difference between the rates in sterile and unsterile water.

7. The mixture of the three pesticides added to soil microcosms were efficiently extracted and simultaneously analysed using a unique developed HPLC gradient method. This was done to study the breakdown pattern of the pesticides mixture in soil microcosms.

8. The natural breakdown rates of the pesticides in soil was more pronounced than that in water and was highly influenced by temperature and water potential. Simazine and trifluralin degradation were significantly enhanced at 30°C when compared to 20°C. Water potential had little effect on the natural breakdown of dieldrin, while that of simazine (17%) was best in the mid-wetness soil (-0.065 MPa) and trifluralin (11%) was most rapidly degraded in field capacity soil after 70 days incubation.

9. *P. versicolor*, and *T. socotrana* were individually grown on chopped straw as a carrier before being inoculated alone or as a mixture of *P. versicolor* plus *T. socotrana* into soil microcosms containing a mixture of the three pesticides in the ratio of 1:10, straw:soil mixture. This demonstrated that as temperature was increased from 20 to 30°C, the breakdown rate was also increased. For example, the temporal breakdown of 5 ppm dieldrin in the presence of *P. versicolor* was increased from 28% at 20°C to 36% at 30°C over a 70 day incubation period.

10. There were statistically significant increases in the degradation of the three pesticides over that of untreated controls.

11. In general a mixture of *P. versicolor* plus *T. socotrana* was not found to be as effective as *P. versicolor* added alone at both 20 and 30°C, suggesting that some antagonism occurred between these species which decreased the efficacy of the individual fungi.

12. Due to increased breakdown rate of pesticides at 30°C, comparison of the effect of fungi over untreated controls to degrade the pesticides was difficult at this temperature. This was particularly apparent for simazine where at 30°C there was

little difference between untreated controls and those treated with the fungi. Therefore in the final phase of the project which was the study of the effect of water potential on the ability of the fungus *P. versicolor* to degrade the pesticide mixture, an incubation temperature of 20°C was used.

13. Soil water potential significantly influenced the breakdown of the three pesticides by *P. versicolor*. *P. versicolor* was most effective at enhancing the breakdown of dieldrin and especially trifluralin (10 ppm) in field capacity soil. However, degradation of simazine occurred best in the driest soil treatment used (-0.28 MPa) suggesting a good tolerance and activity in a wider range of water potential by this fungus.

14. This study suggests that some new tropical *Trametes* species and the temperate species such as *P. versicolor* have significant potential for enhancing the breakdown of mixtures of xenobiotic compounds.

15. Studies are now needed to examine the feasibility of using these fungi in larger field trials of the bioremedial systems in naturally contaminated sites.

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