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Biogenic volatile organic compounds as a potential stimulator for organic contaminant degradation by soil microorganisms.

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Capsule - *A amendment of soils with monoterpenes may induce organic contaminant degradation by indigenous soil microorganisms*

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Abstract

The effects of monoterpenes on the degradation of ^{14}C-2,4-dichlorophenol (DCP) were investigated in soils collected from areas surrounding monoterpene and non-monoterpene emitting vegetation. Indigenous microorganisms degraded ^{14}C-2,4-DCP to $^{14}CO_2$, after 1 d contact time. Degradation was enhanced by prior exposure of the soils to 2,4-DCP for 32 d, increasing mineralization extents up to 60%. Monoterpene amendments further enhanced 2,4-DCP degradation, but only following pre-exposure to both 2,4-DCP and monoterpene, with total 2,4-DCP mineralisation extents of up to 71%. Degradation was greatest at the higher monoterpene concentrations ($\geq$ 1 μg kg^{-1}). Total mineralisation extents were similar between concentrations, but higher than the control and the 0.1 μg kg^{-1} amendment, indicating that increases in monoterpene concentration has a diminishing enhancing effect. We suggest that monoterpenes can stimulate the biodegradation of 2,4-DCP by indigenous soil microorganisms and that monoterpene amendment in soils is an effective strategy for removing organic contaminants.

Keywords — mineralisation, biodegradability, 2,4-dichlorophenol, monoterpenes, α-pinene, limonene, ageing soils.

1. Introduction

Around 5000 structurally determined isoprenoids have been identified, including monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}) and higher molecular weight species (Geron *et al.*, 2000). Monoterpenes ($C_{10}H_{16}$) are the simplest family of isoprenoids (>1000 compounds), forming an array of acyclic and cyclic structures (Fall, 1999). Most studies investigating biogenic isoprenoids have focused on emissions from foliage to the atmosphere, and only limited attention has been paid to subsurface emissions (Hayward et al., 2001). However, isoprenoids have been detected in litter emissions, and are likely products of microbial activity (Isidorov and Jdanova, 2002; Isidorov et al., 2003) or derivatives of root exudates (Lin et al., 2007). The degradation of monoterpenes by indigenous soil microflora has also been demonstrated (Owen et al., 2007). In a review of secondary plant metabolites in phytoremediation, Singer et al (2003) summarised research by Focht (1995), who proposed that plant terpenes (particularly monoterpenes and sesquiterpene) might be natural analogues for PCB oxidation, and subsequent studies have investigated the efficacy of amending PCB-contaminated soil with biogenic terpenes in an effort to induce and/or enhance PCB degradation (Focht, 1995; Gilbert and Crowley, 1997; Hernandez et al., 1997; Tandlich et al., 2001). It may be that exogenous sources of terpenes may enhance the growth and reproduction of PCB-degrading microorganisms and induce the genes encoding enzymes involved in PCB degradation. Indeed, Leigh et al. (2007) demonstrated that there was a very active biphenyl-degrading capacity in indigenous microbes in the rhizosphere soil of *Pinus nigra*, using an innovative stable isotope probing technique. This suggestion that degradation of PCBs in soil might be enhanced by analogue enrichment was made almost forty years ago (Raymond and Alexander, 1971), and subsequently revived twenty years later (e.g. Brunner et al., 1985). More recently, it has been suggested that plant terpenes are likely co-metabolites for PCB degradation due to

their structural similarity to biphenyl (Hernandez et al., 1997; Singer et al., 2003 and references therein), which can facilitate biodegradation of PCBs through co-metabolism. However, biphenyl can not be applied to PCB-contaminated land because it is also harmful to the environment. The ability of soil microorganisms to utilize root exudates, particularly isoprenoids, as their sole carbon and energy source (Cleveland and Yavitt, 1998) may justify the hypothesis that isoprenoids emitted within the soil profile may also enhance the biodegradation of PCBs through co-metabolism, and they have the advantage of being harmless to the environment.

The terpene isoprenoids limonene and α-pinene were selected for investigation as they are plant-derived compounds that dominate woodland soil emissions (Steinbrecher et al., 1999; Hayward et al., 2001; Isidorov and Jdanova, 2002; Isidorov et al., 2003; Lin et al., 2007) , and are readily degraded by indigenous microorganisms (Misra et al., 1996; Misra and Pavlostathis, 1997; Owen et al., 2007); both possess a cyclic structure with a chemical structure closely related to 2,4-DCP. In aerated soil, 2,4-dichlorophenol (DCP) is the main catabolic metabolite of 2,4-dichlorophenoxyacetic acid (2,4-D), a widely used selective systemic herbicide (Tomlin, 1997). Several studies have demonstrated that 2,4-DCP is biodegradable under both aerobic and anaerobic conditions with several pathways of degradation identified (Haggblom, 1992). Because 2,4-DCP is considered representative of common soil and water contaminants (Xing and Pignatello, 1998), it was selected as the test contaminant for this study. While this paper focuses exclusively on terpenoid compounds as structural analogues of 2,4 DCP, it is recognised that there are thousands of other secondary metabolites, such as flavonoids which have a basic 10-carbon ring skeleton, which may also function as structural analogues and co-metabolites of 2,4 DCP and other persistent organic pollutants in soil systems.

Previous work on the influence of co-substrate concentration on contaminant mineralisation are limited, although Tandlich et al. (2001) suggested that biodegradation of PCBs is independent of the concentration of co-substrate used (10 mg l^{-1} and 20 mg l^{-1}). Aged woodland soils have also shown higher levels of [UL-^{14}C] 2,4-DCP mineralisation than fresh soils, which was significantly enhanced by the addition of monoterpenes (Rhodes et al., 2007).

In this study, the use of monoterpenes as a natural analogue for the stimulation of 2,4-DCP degradation was investigated, by quantifying (i) the ability of indigenous microorganisms in soils associated with monoterpene emitting vegetation to metabolise 2,4-DCP; (ii) the effect of pre-exposure on degradative ability; and (iii) the effect of monoterpene (co-substrate) concentration on catabolic activity.

2. Materials and methods

2.1 Materials

Non-labeled 2,4-DCP was supplied by ACROS Organics, UK, and ^{14}C-2,4-DCP in toluene was obtained from Sigma Aldrich, UK. Limonene and α-pinene were obtained from Fisher Scientific, UK. Goldstar, multipurpose liquid scintillation cocktail was supplied by Meridan. Sodium hydroxide used for the CO_2 traps and chemicals for the minimum basal salts solution were supplied by BDH Laboratory Supplies.

2.2 Soil sampling and preparation

Soil samples of ca. 3 kg were collected from sites located under three vegetation types; pine (Pinus sylvestris), oak (Quercus robur) and mixed turf grass. Quercus robur and Pinus sylvestris are isoprene- and monoterpene-emitting vegetation types (Stewart et al., 2003), respectively, and grass is a non-volatile isoprenoid-emitting vegetation. Woodland soils were collected from Lancaster University Campus, Lancashire, U.K. and grassland soil from Mycercough College, Garstang, Lancashire, UK. Soil was homogenized by sieving (2 mm) to remove stones and root material and stored at 4^{o}C in the dark for no more than a month prior to commencement of the experimental work.

2.3 Soil Characteristics

Soil pH and moisture content were determined using standard techniques. The organic matter content was determined by an acid hydrolysis followed by combustion at 450 ºC for 4 h and by a Carbo Erla 1108 Elemental Analyser . Phosphate and potassium content were determined by acid digestion with HNO_3. Phosphate reducing agent (neutralised with NaOH) was used to develop the characteristic blue colour for spectrometric determination at 882 nm (Cecil Ce 1011 UV spectrometer). Potassium

was determined directly using a Sherwood 410 Flame Photometer calibrated against the highest concentration (20 mg l^{-1}) standard solution.

2.4 Soil Spiking

Soils were spiked with non-labeled 2,4-DCP dissolved in acetone (20 ml) as the carrier solvent giving a final concentration in soil of 10 mg kg^{-1} at field moisture content (30-40%). A stainless steel spoon was used to blend the soils and 2,4-DCP spike to give a homogenous distribution (Doick et al., 2003). Soils were subject to three different preparations: (1) "Freshly spiked" soils were spiked with 2,4-DCP and amended with the isoprenoid compounds at five concentrations varying in order of magnitude (0.1, 1, 10, 100, 1000 μg kg^{-1}) and degradation monitored immediately; (2) "2,4-DCP aged" soils were spiked with 2,4-DCP and stored in amber glass jars in darkness at room temperature for 32 d before amendment with isoprenoid compounds at the start of the mineralisation assay; (3) "Isoprenoid and 2,4-DCP aged" soils were spiked with both 2,4-DCP and isoprenoid compounds and stored in amber glass jars in darkness at room temperature for 32 d before starting the mineralisation assay. For each of the three preparations "non-amended" soils were spiked with 2,4-DCP (dissolved in acetone) without isoprenoid amendment and monitored as a control. Each treatment was performed in triplicate.

2.5 Respirometry

The effects of exposure time and isoprenoid amendment on the rate and extent of mineralisation of 2,4-DCP was determined by measuring $^{14}CO_2$ production using respirometric assays (Reid et al., 2001). Soil samples (10 g), 30 ml minimal basal salts solution, and $^{12}C/^{14}C$-2,4-DCP were added to each respirometric flask, to achieve a ^{14}C-activity of approximately ~80 Bq ^{14}C-2,4-DCP g^{-1} soil and concentration/mass of 10 mg

kg^{-1}. A CO_2 trap, consisting of a 7 ml scintillation vial containing 1 ml NaOH (1 M) solution, was suspended from the lid of each respirometer to trap $^{14}CO_2$ evolved as a result of ^{14}C-2,4-DCP mineralisation. Respirometers were placed securely in an orbital incubator (SANYO® Gallenkamp orbital incubator) at 20 ^{o}C and shaken at 100 rpm to agitate and ensure adequate mixing of the slurry over the period of sampling. The ^{14}C-activity in the $^{14}CO_2$ traps was assessed at regular intervals by replacing the NaOH traps and adding scintillation fluid (5 ml) to each spent $^{14}CO_2$ trap. After storage in darkness overnight, trapped ^{14}C activity was quantified using a Packard Canberra, Tri-Carb® 2300TR liquid scintillation counter. An analytical blank (containing no ^{14}C 2,4-DCP) determined the level of background radioactivity. A blank, (autoclaved control) was used to estimate 2,4-DCP lost through volatilization. Maximum mineralisation rates were calculated from the steepest part of the $^{14}CO_2$ respirometric curve and presented as % $^{14}CO_2$ d^{-1}; total extents of mineralisation are defined as cumulative mineralization (%) at the end of the respirometric incubation; and lag time is defined as the time taken to achieve 5% of $^{14}CO_2$ evolution.

2.6 Statistical Analysis

Sigma Stat® Version 2.03 and Sigma Plot® 2000 software packages were used to analyze data. To compare the extents, rates and lag times of ^{14}C-2,4-DCP mineralisation, two and three way analysis of variance (ANOVA) were conducted. Data were ln transformed in some cases to achieve equal variance. Tukey multiple comparisons test were used to identify treatments with significant differences.

3. Results

3.1. Mineralisation of ^{14}C-2,4-DCP in freshly spiked soils

The ability of indigenous soil microflora to mineralise ^{14}C-2,4-DCP was measured in freshly 2,4-DCP spiked soils, sampled from sites under pine, oak and grassland (Figure 1). Up to 40 % of the freshly applied ^{14}C-2,4-DCP was mineralised during the 17 d incubation period in the soils freshly spiked with isoprenoid compounds. Less than 7% of $^{14}CO_2$ release was lost by volatilization (Table 2). There were significant differences in lag times between each of the soil types (>2 d in the pine and the oak soils, <1 d in the grassland soils; $P<0.05$; Table 2). Overall, isoprenoid amendment did not affect lag times ($P>0.05$). However, within the limonene amended soils, there was a significant isoprenoid concentration effect with 100 and 1000 μg kg^{-1} amendments, resulting in significantly shorter lag times than the control and the 1 μg kg^{-1} limonene amendment ($P<0.05$).

Mineralisation rates varied from 2.5 % $^{14}CO_2$ d^{-1} to 13.9 $^{14}CO_2$ % d^{-1} (Table 2). Mineralisation rates in the grassland soil were significantly greater than in the oak and the pine woodland soils ($P< 0.001$). There were no overall significant effects of isoprenoid amendment on the rates of mineralisation ($P>0.05$). However, limonene concentrations of 100 and 1000 μg kg^{-1} in the grassland soil resulted in significantly higher mineralisation rates than in the control soils, and in the soils amended with 0.1 or 1 μg kg $^{-1}$ ($P<0.05$). Mineralisation rates in the soils with limonene amendment of 1000 μg kg^{-1} also differed significantly from the 10 μg kg^{-1}amendment ($P<0.05$). No enhancement of mineralisation rates were observed in soils with α-pinene amendments ($P>0.05$).

There was an overall significant difference in the extent of mineralisation between each soil type ($P<0.001$). There were no differences in extents between the limonene

and the α-pinene amended pine soils, nor between the limonene and the α-pinene amended oak soils. However, in the grassland soil, α-pinene amendment resulted in higher extents of mineralisation than in the limonene amendment ($P<0.05$). Amendment with α-pinene resulted in higher extents of mineralisation in the pine soil than in the oak soil (Tables 2, 3; $P<0.05$). The extents of mineralisation in the control (no amendment) treatment were significantly lower than for the 100 μg kg^{-1} limonene-amended soil ($P<0.05$) and lower (but not significantly) than the 1000 μg kg^{-1} amendment ($P=0.07$).

3.2. Mineralisation of ^{14}C-2,4-DCP in aged soils

The capacity of the indigenous soil microflora to mineralise ^{14}C-2,4-DCP was measured in the woodland and grassland soils following a 32 d incubation in the presence of 2,4-DCP (Figure 2). Up to 61% mineralisation occurred in 2,4-DCP aged soils over the 17 d incubation period, with <7% of $^{14}CO_2$ release by volatilization (Table 3). Lag times were less than one day for all except the α-pinene amended oak and grassland soils, where lag times were >1 d, and up to 3 d (Table 3). There were significant differences in lag times between soil types ($P<0.05$), but there was no overall significant influence of isoprenoid amendment ($P>0.05$). Lag times in the oak soil were significantly greater than lag times in the pine and the grassland soils amended with limonene ($P<0.05$). In the α-pinene amended soils, lag times in all of the soil types were significantly different from each other ($P<0.05$), with shortest lag times in the pine soil and longest in the grassland soil.

Mineralisation rates for 2,4-DCP in aged soils ranged from 4.1 % $^{14}CO_2$ d^{-1} to 45.3 % $^{14}CO_2$ d^{-1} (Table 3). In a given soil, there were large differences in mineralisation rates between isoprenoid types, but no overall significant effect of isoprenoid amendments on the mineralisation rates ($P>0.05$). Mineralisation rates were

significantly different between each of the soil types ($P<0.05$), with the highest rates in the pine soil and the lowest in the oak soil.

Overall, isoprenoid amendment did not have a significant influence on the extents of mineralisation ($P>0.05$). However, there was a significant difference in the extents of mineralisation between each soil type ($P<0.05$). Within both limonene- and α-pinene-amended soils, the pine soil exhibited the greatest extents of mineralisation. In limonene amended soils, there was a significant influence of isoprenoid amendment ($P<0.05$) in pine soil, with the 0.1 and 1 μg kg^{-1} amendment having a significantly greater extent of mineralisation than all other concentrations of limonene amendments and the control treatment. However, no effect of isoprenoid amendment was observed in the α-pinene amended soils ($P>0.05$).

3.3. Mineralisation of 2,4-DCP in soils aged with both limonene and 2,4-DCP

The capacity of the indigenous soil microflora to mineralise ^{14}C-2,4-DCP was measured in the woodland and grassland soils following a 32 d incubation in the presence of limonene and 2,4-DCP (Figure 3). Over the incubation period of 9 d, up to 71% mineralisation was achieved in soils aged with 2,4-DCP and limonene, with <6% of $^{14}CO_2$ released due to volatilization (Table 4). Lag times were <1 d for the majority of treatments in the limonene and 2,4-DCP aged soils (Table 4). Isoprenoid concentration had a significant effect on lag times. Lag times in incubation with no limonene and the 0.1 mg kg^{-1} limonene amended soils were similar ($P>0.05$), but significantly longer than lag times in the soils with 1, 10, 100 and 1000 μg kg^{-1} limonene amendments ($P<0.05$). Lag times in soils with 1, 10, 100 and 1000 μg kg^{-1} limonene amendments were also similar ($P>0.05$). Lag times also differed significantly

between soil types ($P<0.05$); the pine and the grassland soils had similar lag times, which were shorter than those in the oak soil.

Overall, there was a significant isoprenoid amendment effect on mineralisation rates ($P<0.05$). The mineralisation rates for the control treatment and the 0.1 μg kg^{-1} amendment were not statistically different ($P>0.05$), but these rates were slower than those resulting from 1, 10, 100 and 1000 μg kg^{-1} amendments ($P<0.05$). The mineralisation rates in soils amended with 1, 10, 100 and 1000 μg kg^{-1} limonene were similar ($P>0.05$).

Generally, increasing limonene concentration enhanced the extents of mineralisation of ^{14}C-2,4-DCP ($P<0.05$; Figure 4). For the limonene amendment of 10 μg kg^{-1} in the pine soil and 100 μg kg^{-1} in the grassland soil, the extents of mineralisation were relatively low and did not follow the general trend. The absence of limonene (control) resulted in a significantly lower extent of mineralisation than all the limonene amendments ($P<0.05$). The 0.1 μg kg^{-1} limonene amendment resulted in a mineralisation extent similar to the no-addition of limonene treatment ($P=0.52$), but this was significantly lower than extents resulting from all the other limonene amendments ($P<0.01$). The 1, 10, 100 and 1000 μg kg^{-1} amendments had similarly high extents of mineralisation ($P>0.05$). There was also a significant difference between soil types ($P<0.05$); extents of mineralisation in the oak and the grassland soils were similar, and significantly lower ($P<0.05$) than in the pine soil.

4. Discussion

4.1. Adaptation of soils to ^{14}C-2,4-DCP mineralisation.

In the freshly spiked soils, between 22 and 39% mineralisation occurred after 17 d incubation, which agrees with previous studies. For example, Rhodes et al. (2007) reported 19% of ^{14}C-2,4-DCP released as $^{14}CO_2$ after 14 d (in soils spiked with 10 mg kg^{-1} 2,4-DCP). Further, Boucard et al. (2005) found 23% 2,4-DCP was mineralized after 20 d (total concentration 1.4 mg kg^{-1}) and Shaw and Burns (1998) found that 25% ^{14}C-activity was mineralized after 21 d (total concentration 20 mg kg^{-1}). Mineralisation in freshly contaminated soils indicates that soils have the potential for 2,4-DCP catabolism, thus 2,4-DCP degraders were already present in the indigenous microbial community to permit immediate utilization of 2,4-DCP (Shaw and Burns, 1998; Boucard et al., 2005). It has been shown that biodegradation of 2,4-DCP is feasible in previously uncontaminated soils, due to the presence in many soil microorganisms having the tfdB gene that encodes 2, 4-DCP hydroxylase (Vallaeys et al., 1996).

In the soils, spiked and aged with either 2,4-DCP alone, or with limonene, the catabolic activity of the indigenous microflora increases with contact time. Overall, 2,4-DCP aged soils exhibited greater extents and rates of ^{14}2,4-DCP mineralisation, with degradation of up to 60% and rates of between 4.14 and 45.34 % d^{-1}. This contrasts with the maximum 39% extent of mineralisation and rates between 2.45 and 13.95 % d^{-1} for ^{14}2,4-DCP degradation in soils freshly spiked with 2,4-DCP. This has been observed for other organic contaminants in soil, particularly that of PAHs (Hwang and Cutright, 2002; Macleod and Semple, 2002; 2006; Reid et al., 2002; Lee et al., 2003). Macleod and Semple (2002) suggested that the process of increasing degradation ability (adaptation) could be via (1) induction of specific enzymes; (2) genetic changes resulting in increased metabolic capabilities; or (3) the selective enrichment for

organisms with the metabolic capability to transform the target contaminant or a combination of these mechanisms.

The properties of the sigmoidal mineralisation curves of the inoculated ^{14}2,4-DCP observed in soils freshly spiked with 2,4-DCP show that a period of microbial growth (acclimation or lag phase) is required before significant mineralisation rates occur (e.g.Grosser *et al.*, 1991). After 32 d, most soils' mineralisation curves for inoculated ^{14}2,4-DCP had shorter lag times and earlier onset of rapid rates of mineralisation compared with freshly spiked soils. This indicates that 32 d was sufficient for adaptation and enrichment in the soils of microbial populations with 2,4-DCP degradative ability.

In contrast, similar mineralisation processes lasted >24 and ≤ 76 weeks in pyrene-contaminated pasture soil, and were not completed at all in woodland soils contaminated with pyrene even after 90 weeks of pre-exposure (Macleod and Semple, 2002). This highlights the rapidity of the development of 2,4-DCP catabolic activity in the soils used in the present study. Clearly the physico-chemical properties of the molecules and their interactions with the soil will be important contributing factors, with pyrene being a more complex and less water soluble molecule than the chlorophenol.

4.2. The effect of isoprenoid and isoprenoid concentration on ^{14}C-2,4-DCP mineralisation in soil

The overall insignificant differences between isoprenoid amendments and the no-addition of isoprenoid treatment in the freshly spiked soils are comparable with the findings of Rhodes et al. (2007). In this study, Rhodes et al. (2007) found that soils freshly spiked with 2,4-DCP and amended with α-pinene, or an isoprenoid mixture

containing limonene, showed no enhanced 2,4-DCP mineralisation relative to the no-addition of isoprenoid control. In this current study, it was found that soils aged with 2,4-DCP then amended with isoprenoid at the start of the mineralisation assay also showed an insignificant difference in biodegradation between isoprenoid amendments and the no-addition of isoprenoid control. These combined results suggest that 2,4-DCP catabolic activity is not significantly influenced by the presence of isoprenoids. It appears that the period of contact time with 2,4-DCP is more critical than the isoprenoid concentration applied; however, although not significant, within limonene amended freshly spiked soils, the 100 and 1000 μg kg^{-1} amendments resulted in a greater extent of mineralisation and shorter lag times than the no-addition of limonene control in the oak and the pine soils. In the grassland soil, freshly amended with limonene, greater rates of mineralisation were also observed in the 100 and 1000 μg kg^{-1} amendments. The greater extents, shorter lag times and faster rates observed for grass soils amended with higher concentrations of limonene implied that there may have been a stimulatory effect on the microbial 2,4-DCP catabolic activity above a minimum concentration.

Macleod et al. (2002) suggest that higher concentrations of contaminant during prior exposure result in greater catabolic potential; therefore, a greater concentration of co-substrate might result in greater catabolic potential. The results presented in this study support this hypothesis, but also indicate that the enhancing effect diminishes at higher concentrations. There is a rapid rate of increase in the extent of mineralisation with increasing limonene concentration, followed by a diminishing rate of increase in mineralisation extent at high concentrations of limonene ($\geq$ 1 μg kg^{-1}). This suggests that there might be both co-substrate and substrate (contaminant) concentration thresholds, below which catabolic activity is not maximized (Macleod and Semple, 2003). However, Tandlich et al. (2003) did not report the existence of a concentration

effect, suggesting that biodegradation of PCBs was independent of the concentration of co-substrate (10 and 20 mg l^{-1}). However, it is possible that the concentrations of co-substrate applied were high enough that the enhancing effect of co-substrate concentration had stabilized and so there appeared to be no benefit in applying higher concentrations of co-substrate. Alternatively, the concentration independence might have been a function of the reduced bioavailability of PCB molecules.

^{14}C-2,4-DCP mineralisation rate was lowest in grassland soils. This may be explained by the fact that grass is a non-monoterpene emitting vegetation. If grass roots do not emit monoterpenes, then the soil microflora may not be enriched with species possessing the enzymes to degrade these compounds which are structural analogues of 2,4-DCP. Deciduous oak trees emit isoprene rather than monoterpenes (Possell et al., 2004), and mineralisation rates in the oak soil were statistically similar to those in the grassland soils. In contrast, pine trees are monoterpene emitters (Janson, 1993), and mineralisation rates in the pine soils were higher. Hence, natural exposure of the pine woodland microbial communities to monoterpenes, which are structurally similar (natural analogue) to 2,4-DCP, appears to confer a greater inherent ability to adapt to the presence of 2,4-DCP. Natural exposure of pine soils to monoterpenes from roots and litter may also result in a larger and/or more active community of 2,4-DCP degrading microorganisms, and is an example of how different plant communities could influence the microflora in the rhizosphere.

4.3. Influence of soil characteristics on ^{14}C-2,4-DCP mineralisation

Soil organic matter (SOM) content differed significantly between the different soil types, with the greatest SOM content in woodland soils (48% in pine and 17% in oak). Sorption of 2,4-DCP is strongly correlated to SOM content, with increased sorption at

greater SOM contents (Benoit *et al.*, 1999; Yang *et al.*, 2004). It has recently been proposed that in soils with high SOM content, the rate of transfer of contaminants from solid to aqueous soil phases and hence to microorganism limits mineralisation (Macleod and Semple, 2002). However, in this present study, mineralisation was greatest in soils that contained the highest SOM content; this supported the findings Benoit *et al* (1999). A possible explanation for the greater mineralisation in soils with higher SOM content may be that SOM provides a source of energy and a reservoir of nutrients for the indigenous soil microflora. Low SOM frequently limits the growth of microbes, thus the stimulatory effects of SOM content on microbial biomass might override the possible reduced biodegradation rates as a consequence of high SOM to give a greater overall extent of 2,4-DCP mineralisation in woodland soils. Further, it has been reported that the presence of organic matter does not significantly affect the biodegradation of monoterpenes (Misra *et al.*, 1996).

All soils were acidic, reflecting their high organic matter content. The woodland soils had significantly lower pH than the grassland soil (pH 3.7 ± 0.02 and pH 3.9 ± 0.03, for pine and oak, respectively; pH 5.1 ± 0.06 for grass). Acidity affects the efficacy of some chlorinated pesticides (e.g. chlorophenols) which become less mobile in acidic soils (Jensen, 1996). This may also have contributed to mineralisation rates of ^{14}C-2,4-DCP in the different soils.

4. Conclusions

Soil-contaminant contact time is a critical factor affecting the development of catabolic activity within indigenous soil microflora. Isoprenoid amendment has an enhancing effect only when sufficient time has elapsed for microbial adaptation. Following adaptation, increasing isoprenoid concentrations enhanced biodegradation, with the

enhancing effect diminishing at higher concentrations. Degradation was greatest in 2,4-DCP and limonene aged soils. Overall, this study supports the suggestion that amendment of soils with monoterpenes induces organic contaminant degradation by indigenous soil microflora. Therefore, it is feasible to suggest that there is potential for *in-situ* remediation of contaminated soils through the stimulation of indigenous microorganisms through applications of exogenous isoprenoid such as terpene rich plant residues or the planting of isoprenoid emitting vegetation.

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List of Figures

Figure 1. Mineralisation (%) of ^{14}C-2,4-DCP by indigenous soil microorganisms in 3 soil types; (pine, oak and grass), freshly spiked with 2,4-DCP and amended with limonene (A, B, C) or α-pinene (D, E, F) at 5 different concentrations; 0.1 (○), 1.0 (□), 10.0 (Δ), 100.0 (◊) and 1000.0 μg kg^{-1}(∇). A no-addition of limonene/α-pinene control (■) is plotted, and an autoclaved control (●), represents loss of 2,4-DCP through volatilisation. Error bars are the S.E.M of the triplicates.

Figure 2. Mineralisation (%) of ^{14}C-2,4-DCP by indigenous soil microorganisms in 3 soil types (pine, oak and grass), aged with 2,4-DCP for 32 d and amended with limonene (A, B, C) or α-pinene (D, E, F) at the start of the mineralisation assay, at 5 different concentrations; 0.1 (○), 1.0 (□), 10.0 (Δ), 100.0 (◊) and 1000.0 μg kg^{-1}(∇). A no-addition of limonene/α-pinene control (■) is plotted, and an autoclaved control (●), represents loss of 2,4-DCP through volatilisation. Error bars are the S.E.M of the triplicates.

Figure 3. Mineralisation (%) of ^{14}C-2,4-DCP by indigenous soil microorganisms in 3 soil types (pine, oak and grass), aged with 2,4-DCP and limonene for 32 d, at 5 different concentrations; 0.1 (○), 1.0 (□), 10.0 (Δ), 100.0 (◊) and 1000.0 μg kg^{-1}(∇). A no-addition of limonene control (■) is plotted, and an autoclaved control (●), represents loss of 2,4-DCP through volatilisation. Error bars are the S.E.M of the triplicates.

Figure 4. Dependency of mineralisation extent (%) of ^{14}C-2,4-DCP by indigenous soil microorganisms in 3 soil types (pine, oak and grass) aged with 2,4-DCP and limonene,

on concentration of applied limonene. Note: the limonene concentration = 0 $\mu g\ kg^{-1}$ was allocated a value of 10^{-10} $\mu g\ kg^{-1}$ to facilitate plotting the logarithmic axis

Table Captions

Table 1. The physico-chemical properties of each soil type (mean ± S.E.M).

Table 2. Mineralisation extent, initial rates, and lag times (mean ± S.E.M) for ^{14}C-2,4-DCP degradation in 3 soil types; pine, oak and grass, freshly spiked with 2,4-DCP and limonene or α-pinene at 5 different concentrations; 0.1, 1, 10, 100 and 1000 μg kg^{-1}. A no-addition of isoprenoid treatment shows the effect of isoprenoid addition and an autoclaved control represents loss of 2,4-DCP through volatilisation.

Table 3. Mineralisation extent, initial rates, and lag times (mean ± S.E.M) for ^{14}C-2,4-DCP degradation in 3 soil types; pine, oak and grass, aged with 2,4-DCP (32 d) and amended with limonene or α-pinene, at the start of the mineralisation assay, at 5 different concentrations; 0.1, 1, 10, 100 and 1000 μg kg^{-1}. A no-addition of isoprenoid treatment shows the effect of isoprenoid addition and an autoclaved control represents loss of 2,4-DCP through volatilisation.

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Table 1. The physico-chemical properties of each soil type (mean ± S.E.M).

	Soil		
	Pine	**Oak**	**Grass**
Texture	sandy loam	sandy loam	sandy clay loam
Moisture content (%)	30.6 - 81.5	16.0 - 40.3	11.3 - 39.4
pH	3.72 ± 0.023	3.92 ± 0.027	5.10 ± 0.062
ΔpH	0.75 ± 0.024	0.70 ± 0.049	0.32 ± 0.072
Loss on ignition (%)	48.42 ± 1.40	17.24 ± 0.49	4.24 ± 0.37
PO_4 (ppb)	342.81 ± 1.25	249.00 ± 8.12	579.47 ± 1.26
K (ppm)	8.07 ± 0.39	7.69 ± 0.37	13.23 ± 0.79

Table 2. Mineralisation extent, initial rates, and lag times (mean ± S.E.M) for ^{14}C-2,4-DCP degradation in 3 soil types; pine, oak and grass, freshly spiked with 2,4-DCP and limonene or α-pinene at 5 different concentrations; 0.1, 1, 10, 100 and 1000 µg kg^{-1}. A no-addition of isoprenoid treatment shows the effect of isoprenoid addition and an autoclaved control represents loss of 2,4-DCP through volatilisation.

Soil Type	Treatment	limonene			α-pinene		
		Extent (%)	Rate (% d^{-1})	Lag time (d)	Extent (%)	Rate (% d-1)	Lag time (d)
Pine	Autoclaved	2.51 ± 1.79	0.49 ± 0.41	n/a	5.61 ± 2.17	0.72 ± 0.45	n/a
	0	29.25 ± 1.57	3.82 ± 0.33	5.00 ± 0.35	28.59 ± 1.12	3.45 ± 0.71	2.97 ± 0.09
	0.1	31.59 ± 0.58	4.47 ± 0.15	4.93 ± 0.11	27.06 ± 0.27	3.75 ± 0.32	3.45 ± 0.11
	1.0	28.25 ± 2.90	3.81 ± 0.33	5.36 ± 0.27	27.63 ± 0.65	4.18 ± 0.42	3.23 ± 0.09
	10.0	30.61 ± 0.73	3.69 ± 0.08	4.90 ± 0.04	27.74 ± 0.18	3.92 ± 0.29	3.10 ± 0.04
	100.0	32.50 ± 0.35	3.88 ± 0.07	4.62 ± 0.09	27.89 ± 0.90	3.49 ± 0.18	3.03 ± 0.06
	1000.0	30.96 ± 0.18	3.49 ± 0.14	4.75 ± 0.06	29.54 ± 2.99	3.85 ± 0.17	2.77 ± 0.63
Oak	Autoclaved	5.71 ± 2.52	1.02 ± 0.47	n/a	3.40 ± 0.29	0.42 ± 0.05	n/a
	0	22.20 ± 0.34	2.97 ± 0.25	4.11 ± 0.14	24.28 ± 1.47	3.94 ± 0.35	5.91 ± 0.23
	0.1	23.83 ± 0.14	2.58 ± 0.22	3.72 ± 0.07	24.57 ± 1.20	4.39 ± 0.21	6.06 ± 0.48
	1.0	25.04 ± 0.57	2.55 ± 0.08	3.90 ± 0.20	35.70 ± 4.05	6.35 ± 0.94	4.53 ± 0.78
	10.0	24.80 ± 0.67	3.26 ± 0.07	3.63 ± 0.18	23.67 ± 1.23	4.61 ± 0.35	6.13 ± 0.62
	100.0	25.17 ± 0.74	2.99 ± 0.33	3.54 ± 0.22	25.24 ± 1.66	5.02 ± 0.48	5.97 ± 0.02
	1000.0	25.20 ± 0.13	2.45 ± 0.25	3.42 ± 0.11	26.27 ± 0.75	4.48 ± 0.52	5.88 ± 0.28
Grass	Autoclaved	6.42 ± 1.61	1.32 ± 0.73	n/a	6.34 ± 0.78	3.60 ± 2.07	n/a
	0	25.24 ± 2.15	7.31 ± 0.48	0.75 ± 0.08	38.66 ± 2.45	13.95 ± 3.24	1.13 ± 0.11
	0.1	24.24 ± 1.99	7.12 ± 0.83	0.96 ± 0.10	36.52 ± 1.94	11.58 ± 2.22	1.22 ± 0.01
	1.0	25.52 ± 0.31	7.31 ± 0.07	0.85 ± 0.003	34.07 ± 1.60	9.76 ± 1.73	1.26 ± 0.13
	10.0	26.72 ± 0.85	8.11 ± 0.33	0.68 ± 0.04	35.47 ± 1.34	10.52 ± 1.79	1.13 ± 0.02
	100.0	28.13 ± 0.41	8.61 ± 0.09	0.65 ± 0.02	36.05 ± 2.31	11.20 ± 2.33	1.28 ± 0.08
	1000.0	28.55 ± 0.43	9.89 ± 0.30	0.51 ± 0.02	37.92 ± 2.26	12.25 ± 2.26	1.22 ± 0.05

* n/a indicates lag times was greater than the 17 d period of the experiment.

Table 3. Mineralisation extent, initial rates, and lag times (mean ± S.E.M) for ^{14}C-2,4-DCP degradation in 3 soil types; pine, oak and grass, aged with 2,4-DCP (32 d) and amended with limonene or α-pinene, at the start of the mineralisation assay, at 5 different concentrations; 0.1, 1, 10, 100 and 1000 μg kg^{-1}. A no-addition of isoprenoid treatment shows the effect of isoprenoid addition and an autoclaved control represents loss of 2,4-DCP through volatilisation.

Soil Type	Treatment	limonene			α-pinene		
		Extent (%)	Rate (% d^{-1})	Lag time (d)	Extent (%)	Rate (% d^{-1})	Lag time (d)
Pine	Autoclaved	0.64 ± 0.06	0.05 ± 0.01	n/a*	2.72 ± 0.13	0.30 ± 0.05	n/a
	0	44.33 ± 0.45	7.67 ± 0.05	0.66 ± 0.01	60.22 ± 2.64	45.34 ± 2.39	0.11 ± 0.007
	0.1	45.78 ± 1.82	13.48 ± 1.40	0.38 ± 0.04	57.55 ± 0.98	43.52 ± 0.58	0.11 ± 0.003
	1.0	51.26 ± 0.62	14.32 ± 0.40	0.35 ± 0.01	55.51 ± 2.91	41.96 ± 3.01	0.12 ± 0.009
	10.0	45.59 ± 5.96	8.24 ± 1.26	0.75 ± 0.15	57.24 ± 1.78	40.67 ± 2.99	0.12 ± 0.006
	100.0	48.81 ± 1.14	7.46 ± 0.10	0.69 ± 0.02	50.83 ± 4.27	38.43 ± 3.59	0.13 ± 0.012
	1000.0	48.81 ± 1.11	7.63 ± 0.08	0.69 ± 0.01	57.09 ± 2.55	43.42 ± 2.81	0.12 ± 0.007
Oak	Autoclaved	1.22 ± 0.55	0.15 ± 0.08	n/a*	4.07 ± 0.33	0.42 ± 0.05	n/a
	0	38.95 ± 0.66	7.47 ± 0.09	0.78 ± 0.03	33.26 ± 2.31	4.73 ± 0.59	1.80 ± 0.30
	0.1	41.45 ± 0.65	6.94 ± 0.60	0.93 ± 0.07	34.30 ± 1.81	5.62 ± 1.04	1.34 ± 0.28
	1.0	39.63 ± 1.20	7.36 ± 0.38	0.88 ± 0.04	35.84 ± 2.47	5.55 ± 0.61	1.38 ± 0.16
	10.0	42.83 ± 0.89	8.21 ± 0.41	0.80 ± 0.02	36.44 ± 2.45	5.24 ± 0.65	1.45 ± 0.30
	100.0	41.23 ± 2.50	7.23 ± 0.57	0.92 ± 0.06	32.79 ± 4.13	4.47 ± 1.00	2.23 ± 0.91
	1000.0	39.57 ± 1.00	7.44 ± 0.70	0.87 ± 0.11	29.86 ± 0.32	4.14 ± 0.14	2.24 ± 0.30
Grass	Autoclaved	3.89 ± 0.16	0.39 ± 0.05	n/a*	6.28 ± 0.87	0.89 ± 0.18	n/a
	0	26.66 ± 0.11	9.03 ± 0.09	0.55 ± 0.01	52.16 ± 0.35	34.51 ± 0.84	2.15 ± 0.01
	0.1	25.84 ± 0.94	8.46 ± 0.17	0.59 ± 0.01	36.72 ± 1.71	16.30 ± 1.59	2.36 ± 0.03
	1.0	25.70 ± 0.55	7.58 ± 0.50	0.67 ± 0.05	47.48 ± 2.54	26.31 ± 3.81	2.18 ± 0.03
	10.0	26.70 ± 0.19	8.97 ± 0.34	0.56 ± 0.02	44.93 ± 1.84	25.28 ± 3.01	2.22 ± 0.03
	100.0	28.17 ± 0.34	8.99 ± 0.56	0.56 ± 0.03	48.20 ± 4.33	27.83 ± 6.24	2.10 ± 0.04
	1000.0	26.08 ± 2.37	7.96 ± 0.96	0.65 ± 0.09	48.37 ± 2.61	26.81 ± 3.37	2.17 ± 0.03

n/a indicates lag times was greater than the 17 d period of the experiment.

Table 4. Mineralisation extent, initial rates, and lag times (mean ± S.E.M) for ^{14}C-2,4-DCP degradation in 3 soil types; pine, oak and grass, aged with 2,4-DCP and limonene for 32 d, at 5 different concentrations; 0.1, 1, 10, 100 and 1000 μg kg^{-1}. A no-addition of limonene treatment shows the effect of limonene addition and an autoclaved control represents loss of 2,4-DCP through volatilisation.

Soil type	Treatment	Extent (%)	Rate (% d^{-1})	Lag time (d)
Pine	Autoclaved	1.54 ± 0.12	0.20 ± 0.02	n/a
	0	40.65 ± 1.37	7.94 ± 0.17	0.77 ± 0.01
	0.1	51.60 ± 0.64	19.14 ± 0.42	0.26 ± 0.001
	1.0	70.91 ± 1.91	54.81 ± 1.76	0.09 ± 0.003
	10.0	37.58 ± 0.67	5.58 ± 0.13	1.01 ± 0.01
	100.0	64.48 ± 4.46	48.34 ± 3.55	0.10 ± 0.01
	1000.0	70.52 ± 4.38	55.03 ± 4.61	0.09 ± 0.01
Oak	Autoclaved	1.48 ± 0.04	0.23 ± 0.03	n/a
	0	19.45 ± 0.99	3.49 ± 0.55	1.95 ± 0.14
	0.1	15.74 ± 1.04	2.30 ± 0.15	2.85 ± 0.27
	1.0	54.46 ± 1.28	26.11 ± 0.51	0.19 ± 0.003
	10.0	48.84 ± 2.37	17.18 ± 0.85	0.29 ± 0.01
	100.0	57.24 ± 4.12	42.68 ± 1.19	0.45 ± 0.34
	1000.0	60.57 ± 4.33	43.81 ± 2.99	0.11 ± 0.01
Grass	Autoclaved	5.89 ± 1.14	0.90 ± 0.27	n/a
	0	24.84 ± 0.13	10.10 ± 0.43	0.50 ± 0.02
	0.1	37.48 ± 1.14	17.02 ± 0.73	0.30 ± 0.01
	1.0	38.49 ± 0.44	10.97 ± 0.76	0.47 ± 0.31
	10.0	43.55 ± 1.79	25.58 ± 1.47	0.20 ± 0.01
	100.0	31.35 ± 2.06	17.55 ± 1.31	0.29 ± 0.02
	1000.0	41.18 ± 3.83	23.04 ± 2.15	0.22 ± 0.02

* n/a indicates lag times was greater than the 9 d period of the experiment.

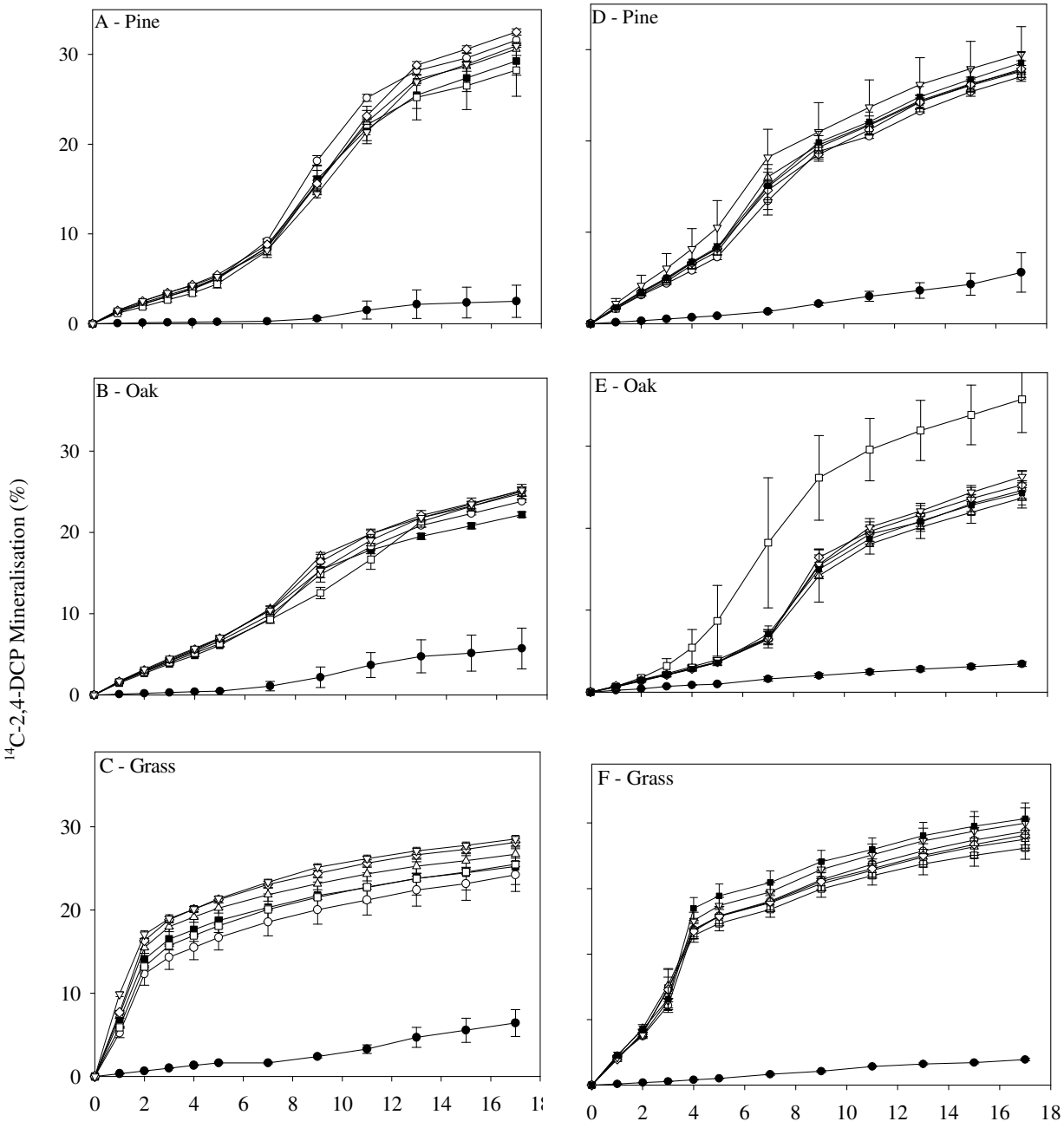
A - Pine
D - Pine
B - Oak
E - Oak
C - Grass
F - Grass
^{14}C-2,4-DCP Mineralisation (%)
0
10
20
30
0
2
4
6
8
10
12
14
16
18

Figure 1

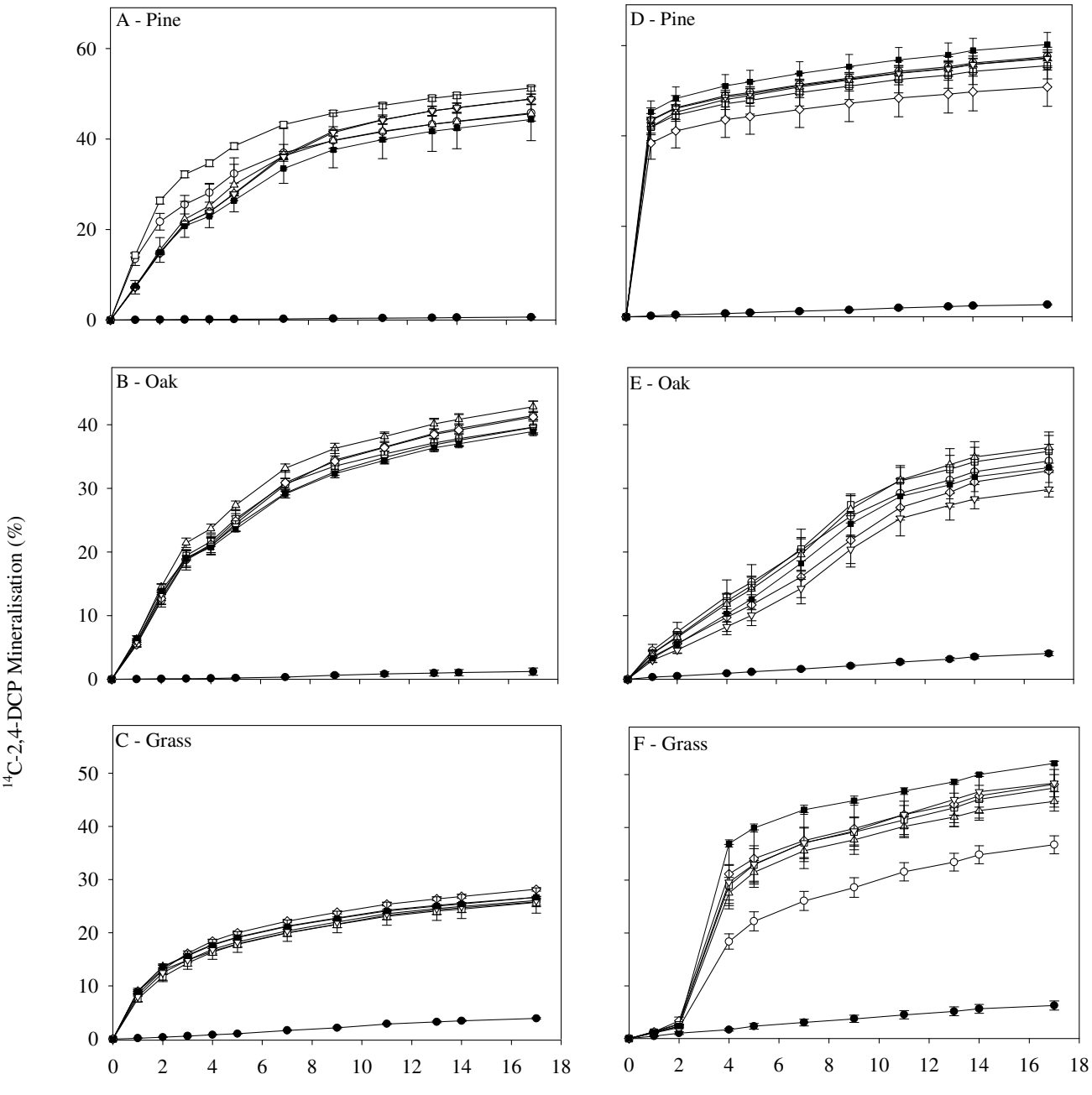
A - Pine
D - Pine
B - Oak
E - Oak
C - Grass
F - Grass
^{14}C-2,4-DCP Mineralisation (%)
0
20
40
60
0
10
20
30
40
0
10
20
30
40
50
0
2
4
6
8
10
12
14
16
18

Figure 2.

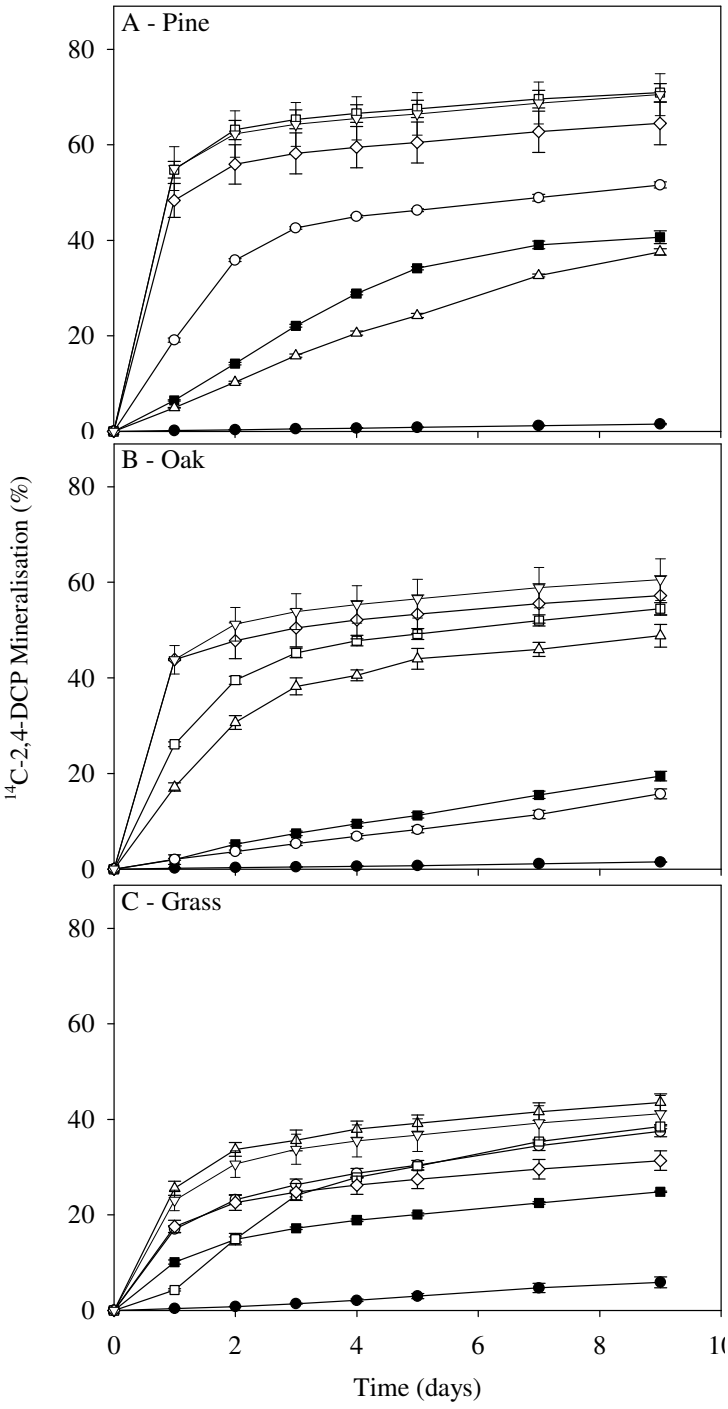
A - Pine
B - Oak
C - Grass
^{14}C-2,4-DCP Mineralisation (%)
Time (days)
0
20
40
60
80
0
2
4
6
8

Figure 3.

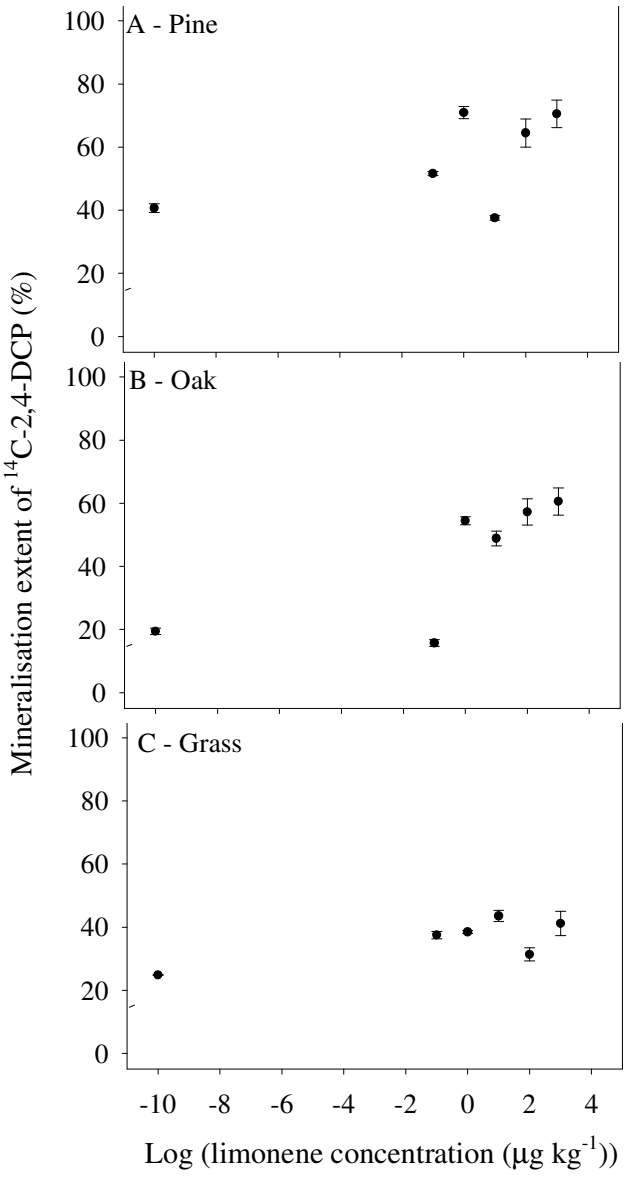
A - Pine
B - Oak
C - Grass
Mineralisation extent of ^{14}C-2,4-DCP (%)
Log (limonene concentration (µg kg^{-1}))
100
80
60
40
20
0
-10
-8
-6
-4
-2
0
2
4

Figure 4.