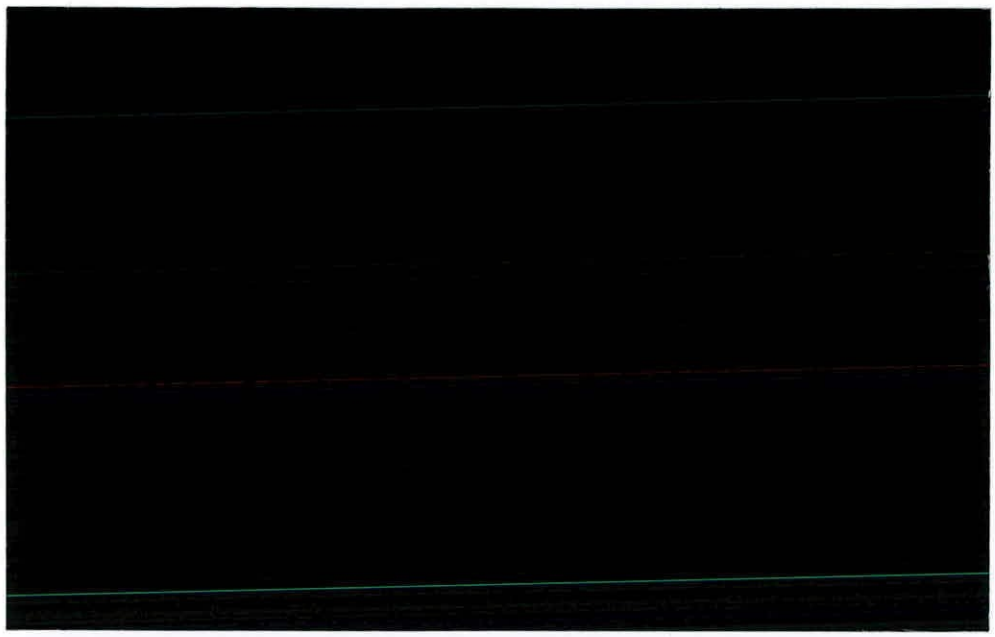

Institute of Virology and Environmental Microbiology

Centre for Ecology & Hydrology

Natural Environment Research Council

Molecular genetics and process level events in the biodegradation of xenobiotics in rhizosphere soils.

CEH Integrating fund
Progress report for 1995/1996

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1. SUMMARY

The study has focused on the chlorobenzenes (CBs) as a class of model compounds with which to investigate the microbial process level events associated with xenobiotic degradation. The chlororbenzenes are red listed, highly toxic, widely used and persist in the environment. The enzymatic processes governing the bacterial metabolism of these compounds are generally well understood, facilitating an understanding of activity *in situ*. However, the genes coding for the enzymes directly involved with the degradation of certain dichlorobenzenes have yet to be identified. Studies at Monks Wood, using mono- and di-CBs, have shown that soil microflora from pristine sites has the inherent capacity for degradation (mineralization and disappearance), and most importantly that degradation is greatly enhanced in rhizosphere soils. Rates of mineralization were greater in the rhizosphere for some congeners. Within the di-CB homologue group, 1,2 is readily mineralized while 1,4 is not, indicating a difference in their interaction with soil microbes as the physical-chemical properties of these two compounds are virtually identical. Furthermore, a 4 week lag phase in mineralization rates of 1,2 isomers in non-rhizosphere soil (but not in rhizosphere soil) has been recorded, after which mineralization increases exponentially, coinciding with the selection of microbes that carry the genes responsible for degradation. The lack of an equivalent lag time in rhizosphere soils indicates that this habitat supports a more active detoxifying microflora. Although the involvement of roots as a source of nutrients and metabolic stimuli for microbial activity is important, it is also evident that the enzymatic functions required for the catabolism of CBs are already present in the rhizosphere-soil environment. These enzymatic functions may for example include oxidases required to degrade lignin break down products and aromatic plant metabolites exuded or leached into the rhizosphere. By applying phenotypic and genotypic techniques in combination with direct analytical methods we have been able to describe changes in microbial population dynamics during xenobiotic degradation and determined how communities adapt to the impact and selective pressure imposed by pollutants.

The overall aim of this study is **to assess the microbial population and genetic events that lead to enhanced degradation of DCB in soils**. To date a number of approaches have been taken:

- **Assess the impact of DCB application on soil bacterial communities.**
  To degrade foreign organic matter the soil microbial community must first be resilient to any toxic shock associated with contaminant introduction. For example, microbial cells unable to resist toxic shock, may become moribund, inactive and eventually lost from the community. A community with diminished genetic capacity may be less capable of adapting to environmental stress.

- **Assess the influence of plant tissue on bacterial community biomass, composition and DCB degradation rates.**
  Soils low in organic matter, such as those lacking plant tissue, have reduced buffering capacity and are particularly susceptible to toxic shock. The response of microbial communities to DCB introduction, with and without plant tissue, has been compared.

- **Determining the adaptive events in the soil microbial community that leads to enhanced DCB degradative activity.**
  Develop and apply enrichment procedures for DCB utilizing bacteria and evaluate increased activity of a specific taxa and the relevance of the horizontal spread of degradative genes in community adaptation.

2. INTRODUCTION

Chlorobenzenes (CBs) are widely used as solvents, degreasers, deodorants and produced as intermediates in the synthesis of dyes and pesticides. Their extensive use in the past few decades has resulted in large quantities being released into the environment, both by accident and through routine disposal in waste treatment facilities. These releases have resulted in detection of CBs in ground waters and soil. This has lead to increasing concerns about the fate and persistence of these compounds in the environment. Although the observed levels of CBs in the environment are in general too low to cause immediate acute toxicity to organisms (Pearson, 1982), they are lipophilic and been found to cause liver and renal cancer in mice and rats (National Toxicity Program, 1987). To add to the concern of long term exposure of low levels, CBs are chemically stable in nature and not prone to photodegradation (US EPA, 1980). Most natural environments have the capacity to degrade a wide range of organic xenobiotics, such as CBs, even though they have not previously been exposed. This may be due to the genes which encode for the functional enzymes responsible for degradative pathway, being present in the indigenous microbial community, at low frequency in uncontaminated soils. Following contamination the degradative activity of the microbial community within the habitat is greatly enhanced. CBs for instance have been found to be readily mineralised under appropriate conditions in the laboratory by bacteria isolated from soil and water (de Bont *et al.*, 1986; Haigler *et al.*, 1988; Spain *et al.*, 1987). These studies have resulted in the determination of the metabolic pathway used for DCB degradation (mineralization and disappearance) (Reineke & Knackmuss, 1984; Spiess *et al.* 1995). However, what has rarely been studied in any depth are the molecular and population events that occur within the DCB degradative community that leads to increased degradative activity.

There are two possible, but not necessarily mutually exclusive, scenarios leading to the enrichment of a degradative population;

i) direct selection of a DCB degrading sub-population that are already present in low numbers in the environment or not expressing the phenotype,

ii) or transfer of DCB degradative genes that enable the recipient micro-organisms to tolerate the toxic effects and utilise the contaminant.

Since removal of DCBs from the environment can only occur via enzymatically catalysed reactions, this has stimulated considerable interest into microbial populations able to mineralise and remove the compound. These investigations included those already undertaken at ITE. Although the microbial population events that led to increased degradative activity were not originally examined, preliminary studies at Monks Wood on the degradation of mono- and di- CBs, have shown this activity is greatly enhanced in rhizosphere compared to non-rhizosphere soil. Rates of mineralization were 20-30 fold greater in the rhizosphere compared to bulk soil for some congeners but not for others. Within the di-DCB homologue group, 1,2 was readily mineralised while 1,4 is not, indicating a difference in their interaction with soil microbes, as the physical-chemical properties of these two compounds are virtually identical. Furthermore, a 4 week lag phase in mineralization rates of 1,2-DCB isomers in non-rhizosphere soil (but not in rhizosphere soil) has been recorded, after which mineralization increases exponentially, coinciding with the selection of microbes that carry the genes responsible for degradation. The lack of an equivalent lag time in rhizosphere soils indicates that this habitat supports a more active detoxifying microflora. Clearly the enzymes that catabolise DCBs are already prevalent in the rhizosphere and may include oxidases required to degrade lignin break down products and aromatic plant metabolites exuded or leached into the rhizosphere.

In recent years evidence has gradually accumulated to support the view that the rhizosphere is a site in which microbial degradation of hazardous organic compounds in soil is enhanced. The potential of phytoremediation of organic pollutants was first observed in studies of agricultural chemicals, in which the persistence of several insecticides and herbicides in soils were shown to decrease in the rhizosphere (Hsu & Bartha, 1979; Sandman & Loos, 1984;). Although this phenomenon was regarded as contrary to effective crop protection, from the prospective of environmental clean-up, the rhizosphere holds great promise for elevated rates of organic pollution degradation. Indeed accelerated rates of polyaromatic hydrocarbons, trichloroethylene and dichlorobenzoate mineralisation in the plant root in field and

laboratory has already been convincingly demonstrated (Walton & Anderson, 1990; Crowley *et al.*, 1996). Although it is generally accepted that enhanced loss of organic pollutants is due to microbial activity in the rhizosphere, it is likely that humification and translocation of organics into the plant tissues plays a role. What is more certain is that little is known of how rhizosphere communities stimulate rates of degradation. It may be that during growth on roots exudates, there is a fortuitous selection of heterotrophic micro-organisms that degrade the organics after being primed by growth on exudates or that utilise certain components of root exudates for co-metabolism of xenobiotics. In older root zones, where micro-organisms may be primed by more recalcitrant insoluble carbon sources, thus accelerating the rate of adaptation to break down of complex organic pollutants. Studies have also show that certain chlorinated aromatics that are structurally analogous to monomeric components of plant lignin can act as chemo-attractants of chlorinated benzoate degrading populations. The possible mechanisms that can be envisaged to account for the enhance rate of microbial degradation are therefore almost infinite.

To test these hypotheses it is necessary to consider changes in total biomass and in community, phenotypic and genotypic structure of the biomass and of individual species. Complete characterisation of the soil microbial biomass diversity shifts at the community, phenotypic and genotypic level in the presence of pollutants is impossible, given the overall diversity of soils and as only a small percentage of the soil microbial biomass is culturable. Therefore, it is necessary to study a model system where a culturable component of the microbial biomass is known to be:
(a) sensitive,
(b) capable of degrading the pollutant and
(c) prolific in the soil environment.

3. AIMS

To understand the mechanisms involved in the degradation of xenobiotics by bacteria in the rhizosphere at both the molecular genetic and process level. Most soil environments have the capacity to degrade a wide range of xenobiotics although the genes which encode for the functional enzymes are normally present in the indigenous microflora of uncontaminated soils at low frequency. Following contamination by xenobiotics the degradative activity of the habitat is greatly enhanced either by the direct selection of strains or by the transfer of biodegradative genes that enable recipient micro-organisms to tolerate the toxic effects, or utilize the contaminant as a substrate. Studies at Monks Wood (ITE) have shown that the rhizosphere is a site of greatly increased xenobiotic (chlorobenzenes - CBs) degradation. We propose to concentrate our efforts, during this three year study, on optimising the rhizosphere for the bioremediation of terrestrial habitats. However, before effective inocula and application strategies can be developed, we need to improve our understanding of the biotic and abiotic processes that influence the availability of substrates, bacterial population succession, and the role of microbial consortia and gene transfer in the biodegradation of pollutants in rhizosphere and non-rhizosphere soils. By combining the relevant expertise in environmental bacterial genetics and process level pollution microbiology available at IVEM and ITE we intend to determine the fate of xenobiotics in soils.

The overall aim of this study is **to assess the microbial population and genetic events that lead to enhanced degradation of DCB in soils**. To date a number of approaches have been taken:

- **Assess the impact of DCB application on soil bacterial communities.**
  To degrade foreign organic matter the soil microbial community must first be resilient to any toxic shock associated with contaminant introduction. For example, microbial cells unable to resist toxic shock, may become moribund, inactive and eventually lost from the community. A community with diminished genetic capacity may be less capable of adapting to environmental stress.

- **Assess the influence of plant tissue on bacterial community biomass, composition and DCB degradation rates.**
  Soils low in organic matter, such as those lacking plant tissue, have reduced buffering capacity and are particularly susceptible to toxic shock. The response of microbial communities to DCB introduction, with and without plant tissue, has been compared.

- **Determining the adaptive events in the soil microbial community that leads to enhanced DCB degradative activity.**
  Develop and apply enrichment procedures for DCB utilizing bacteria and evaluate increased activity of a specific taxa and the relevance of the horizontal spread of degradative genes in community adaptation.

4. THE PARTNERSHIP

Integration and complementation of expertise:

IVEM, provide the expertise required for identification and the genotypic and phenotypic characterisation of bacterial isolates to facilitate the description of bacterial community succession and adaptation in response to the selective pressures of xenobiotic contamination of soils. Evaluations of the metabolic potential and profiles of total microbial communities will also be compared against diversity estimates of community succession for isolated bacteria. Isolates, identified by gas chromatography using the Microbial Identification System on the basis of their cellular fatty acid methyl ester (FAME) component, will use the extensive databases available to accurately identify them at the sub-species level to monitoring population succession. The functionality and diversity in cellular activity was assess by comparative analysis of the substrate utilization pattern on total sampled communities derived from the different treatments. This assay, BIOLOG-GN, provides data on the ability of sampled communities to utilise any of the 95 sole carbon sources available. Detailed molecular genetic approaches will be applied to isolate and characterise genes directly involved in the catabolism of the DCBs. Efforts will target an assessment of the role that horizontal gene transfer may play in the distribution or assembly of specific pathways that enable communities to detoxify or utilise xenobiotics.

ITE, Monks Wood will take the lead in the determination of process events following exposure of rhizosphere and non-rhizosphere soils to DCBs. Effort has concentrated on the degradation of 1,2-DCB (including fate kinetic and bioavailability studies) and the changes in the dynamics of the soil microbial biomass as determined by assessment of basal respiration, total biomass Carbon and direct enumeration of the "metabolically active" component of bacteria and fungi. ^{14}C labelled 1,2-DCB and cold compounds (analysed using GC-ECD and GC-MS). All experiments compare the effects of DCBs on soil and rhizosphere microflora in microcosms developed and maintained at ITE.

5. DELIVERABLES

This collaborative study will enable the elucidation of the microbial processes responsible for the degradation of a model xenobiotic (1,2-DCB) in rhizosphere and non-rhizosphere soils. By improving our understanding of the molecular processes and biological mechanisms involved such approaches can be optimised to develop *in situ* bioremediation technologies, based on the combination of phytoremediation and soil inocula.

6. WORK PROGRAMME

0 - 12 months:	follow the development of microbial communities in polluted and non-polluted soils, with and without rhizosphere material.
12 - 24 months	develop *in vitro* culture systems for the isolation of microbes able to degrade selected 1,2-DCBs.
18 24 months	contrast 1,2-DCB impact on transformation rates, community activity and population succession in true rhizosphere systems
18 - 30 months	establish a suitable microbiological baseline to allow molecular probes to be developed for the detection of 1,2-DCB degrading genes.
24 - 36 months	assess the role of plasmids in the dissemination and reassortment of genetic information.
18 -36 months	develop predictive model systems for evaluating the utility of biodegradative inocula in plant rhizospheres.

7. EXPERIMENTAL APPROACH

7.1. Microcosm design.

Microcosms were based on 500 ml Duran flasks fitted with Teflon lined lids. Each flask contained 70 g dry wt. of agricultural soil collected at the Monk's Wood field station, Cambridgeshire. The soil was a clay silt, pH 7.4, with no previous exposure history to 1,2-dichlorobenzene. Moisture content was adjusted to typical field capacity by adding sterile distilled water (30 ml) to air dried soil. The assembled microcosm was equilibrated at 20°C for 7 day prior to experimentation. All treatments were undertaken in triplicate under rigorously controlled conditions.

7.2. Biomass estimation and vital staining.

7.2.1 *Fungal biomass.* Total fungal hyphal length (both living and dead) was determined by vital staining with fluorescein diacetate (FDA). Soil (1 g) was weighed into a sterile conical flask with 100 ml of sterile phosphate buffer (pH 7.5) and shaken by hand for 3 min to ensure aggregate dispersal. FDA (Sigma chemicals) was dissolved in acetone (Sigma chemicals) at a concentration of 2 g l^{-1}, and 50 µl of this solution was added to the soil suspension, and the suspension shaken for a further 3 min. The supernatant was then filtered through a grided Millipore membrane filter (0.45 µm). The filter was then washed with 10 ml of the phosphate buffer twice to remove excess FDA. Fluorescing (live) and non-fluorescing (dead) hyphal length was determined using a Leica Laborlux S fluorescence microscope at 40 times magnification using a BG 12 filter. Hyphal length was enumerated in 6 alternate squares across the centre of the filter using an eyepiece graticule. Though this staining technique may underestimate total soil hyphal length, potentially due to inefficient extraction of hyphae from soil particles, as only one soil type was used the differences between treatments will be consistent. The hyphal enumeration is therefore defined as extractable FDA active cells.

7.2.2 *Vital staining of bacterial component.* The number of viable and non-viable bacterial numbers was determined using the Baclight™ viability kit (Molecular Probes, Europe). The kit is composed of two DNA stains, one that only permeates intact plasma membranes (live cells) and the other that stain cells with disrupted membranes (dead cells). Living cells fluoresce green and are visible using a O-5715 UV filter, dead cells, with compromised membranes, stain red and are visible using a O-5723 UV filter in conjunction with the fluorescence microscope.

Cells were stained with Baclight by adding 1g of soil to 25 ml of sterile distilled water in a sterile test tube and mixed thoroughly for 10 min. To a 2.5 ml aliquot of this suspension, 3 µl of Baclight reagent was added and incubated in the dark for 15 min. An aliquot of the supernatant (30 µL) was then placed in a bacterial counting chamber. Numbers of dead and living bacteria were determined in 30 alternate squares across the grid etched on the base of the counting chamber. Again, like the fungal assay, the extraction procedure will quantify systematic differences between treatments, but may underestimate the total bacterial numbers.

7.3. Estimate of 1,2 DCB transformation rate in soils

Mineralization of ^{14}C 1,2 DCB was determined by inclusion of a KOH trap (1M) (supplied by Merck) in the microcosms. This trap was replaced at weekly intervals and the trap counted for ^{14}C activity by adding an aliquot of the trap to Hi-ionic-fluor liquid scintillation fluid (supplied by Packard Industries) and then determining counts per minute using a Packard 2500 TR liquid scintillation analyser. To ensure that both radiolabelled and non-radiolabelled treatments were treated identically, KOH traps were included in all microcosms and replaced at the same time as the ^{14}C treatments.

7.4. Bacterial population dynamics.

7.4.1 *Isolation and enumeration of soil bacteria* On each sampling occasion approximately 2 g of soil were removed from each microcosm. One gram was placed into a tube containing 10 ml of quarter strength Ringer solution and suspended by vigorous mechanical, vortex mixing for 1 min. The soil suspensions was serially diluted and spread onto 3% Tryptic Soy Broth Agar (TSBA, Difco, UK), amended with 50 mg l^{-1} cyclohexamide, and *Pseudomonas* Agar Base supplemented with 10 mg l^{-1}

cetrimide, 10 mg l^{-1} fucidin, and 50 mg l^{-1} cephaloridine (PSA-CFC, Oxoid, UK). The plates were incubated at 18°C for up to 10 days and those containing between 20 and 200 colonies counted every day over the incubation period. The numbers of bacteria and Pseudomonads present were expressed as colony forming units (c.f.u.) per gram (dry weight) of soil. Dry weights were determined by drying sub-samples of soil at 80°C until no further weight decrease was observed.

Daily counts assessed the rate of colony development and appearance on the agar isolation plates. These assessments provide crude index for the rate of growth and the ability of isolated cells to respond physiologically as manifest by colony development, an "index of responsive growth capacity". Such approaches were originally described by Hattori and co-workers, and more recently have been used as measures of impact after the perturbation of microbial habitats following the application of inocula (De Leij *et al.* 1993) or as a result of plant maturation (Lilley *et al.*, 1996). In the original articles Hattori argued that colony appearance on agars was indicative of the nascent growth rate of oligotrophs

The study of DCB has focused on dewscriptions of Pseudomonad populations as this group of bacteria typically represent a high proportion of the total culturable bacterial community, they are common residents of the phytosphere and, due to their ability to respond rapidly (positively and negatively) to changing environmental conditions, they serve as a well defined indicator group of impact or perturbation. Furthermore, a number of well defined and highly selective artificial media are available.

7.5. **Bacterial identification and isolate characterisation**

The original isolation plates were sampled for developed colonies. Ten colonies were picked from each replicated isolation plate used to estimate the number of colony forming units of bacteria in each sampled microcosm. Colonies were collected from dilution plates containing between 20 and 200 cfu. This precaution was taken to reduce interference and the density dependent inhibition of colony development that can occur on packed agar plates. All collected colonies were streaked to clonal purity and grown overnight on TSBA at 28°C before analysis as described below. All isolates were grown in TSB broth and stored at -70°C in glycerol:saline.

7.5.1 *Cellular fatty acid analysis of isolated bacteria.* Isolates for FAME analysis were cultured on TSB agar plates. Cells were harvested after 24 h incubation at 28 C and whole cell fatty acids were saponified, methylated and extracted as described by Miller & Berger (1985). FAME analysis was performed using a Hewlett-Packard model 5890 series II gas chromatograph. All analyses were conducted in triplicate and the mean FAME profiles were used to generate a unique library for each isolate as described previously (Thompson *et al.* 1993a; 1993b). Fatty acid peaks were named by the Microbial Identification System (MIS) software (Microbial ID. Inc., Newark, DE) and isolates identified using the MIS "Aerobe Library". All isolates were identified using "Aerobe Library" 3.50 (October 1991 update).

7.5.2 *Genomic DNA isolation.* Genomic DNA was isolated from bacteria by an adaptation of the CTAB method described previously (Bailey *et al.*, 1995). A mass of cells (10-15 mg) were removed from the surface of the agar and placed in an Eppendorf tube and either frozen at -70°C for subsequent extraction or extracted immediately. Lysis buffer (20 mg l^{-1} Proteinase K (Sigma), 0.5% (w/v) Sodium Dodecyl Sulphate) was prewarmed to 55°C and 500µl added to each tube. Cells were resuspended in the lysis buffer by gentle pipetting and incubated at 55°C for 30 minutes. One hundred microlitres of 5M sodium chloride (prewarmed to 65°C) were added and mixed with the lysed cells. To this high saline suspension was thoroughly mixed in 80µl of CTAB solution (10% (w/v) hexadecyltrimethyl ammonium bromide (Sigma Co., UK) in 0.7M NaCl) and incubated at 65°C for 10 minutes after mixing. Deproteination was performed by extraction with an equal volume of chloroform : isoamyl alcohol (24 : 1). The samples were mixed gently and the phases separated by centrifugation at 11,600g for 2 minutes. The upper aqueous phase was mixed with 0.6 volumes of isopropanol and the DNA allowed to precipitate for 10 minutes at room temperature. The DNA was collected by centrifugation at 11,600g for 5 minutes. The pellet was washed in 100µl of 70% ethanol and resuspended in 200µl TE

(10mM Tris-HCl pH 8.0, 1mM EDTA) with 10μg ml^{-1} RNase A. The prepared DNA was stored at -20°C.

7.5.3 *DNA digestion and Southern blotting.* Approximately 2μg of DNA was digested with the restriction enzyme *Kpn*I (10 Units, Boehringer Mannheim, UK) at 37°C overnight. DNA fragments were separated by Pulse Field Gel Electrophoresis (PFGE, BIORAD, UK) using a 1% (w / v) pulsed field certified agarose (BIORAD, UK) in 0.5 x Tris-borate buffer as recommended by the manufacturers. Electropheoresis conditions were selected to separate DNA fragments of 150 to 5 kbp size range; 200V, 2-6 second ramp time for 16 h. Lambda DNA molecular size markers and a standard strain, *Pseudomonas fluorescens* SBW25 (Bailey and Thompson, 1992) were included to allow between gel comparisons. Gels were stained for 30 min in 0.5 μg/ml Ethidium bromide in x0.5 TBE and visualised on a transilluminator. Images were scanned and saved as **.tif** files using BIORAD gel imager. After photographing the gel, DNA was transferred overnight to nylon Hybond N+ membrane (Amersham, UK) using 0.4M sodium hydroxide as the transfer buffer according to the manufacturers instructions. Membranes were stored wrapped in plastic film.

7.5.4 *Probe preparation and hybridization of cellular DNA rRNA-RFLP profile.* The probe used for ribotyping of all fluorescent Pseudomonads was isolated from pAC10 on a 4.5kb *Bam*HI fragment which includes both the 16S and 23S ribosomal RNA genes (Housiaux *et al.*, 1988). The fragment was purified from agarose gel and 50 ng was labelled with α^{32}PdATP by random priming as described by the manufacturers (Amersham, UK). Hybridization and autoradiography were carried out according to the manufacturers' instructions. Ribosomal RNA-DNA RFLP hybridization patterns were identified and differentiated by eye. Identical patterns were confirmed by running isolates on the same agarose gel. The number of isolates in each ribotype group (defined as all isolates sharing an identical pattern of hybridizing bands with at least one band different from any other group) were determined. The abundance of each ribotype was calculated as the number of isolates in each group divided by the total number of isolates. A frequency distribution for the number of ribotypes at each abundance was constructed

7.6. Community metabolic profiling

The total metabolic potential of the whole microbial community of soils treated with DCB was assessed as described by Ellis *et al.* (1995). Soil suspensions used to determine colony forming units of bacteria (2.2), were washed twice in 30 ml 0.85% (w/v) saline by centrifugation at 7740*g* at 4°C for 15 min (Beckman, UK) and then resuspended in 20 ml of saline. Commercially prepared microtitre plates (BIOLOG, Hayward, CA) with a total of 95 different sole-carbon sources in individual wells, together with a redox dye, tetrazolium violet, which indicates respiration via production of NADH, were inoculated with 150 μl aliquots of the suspension in each well. The plates were incubated at 28°C and scored at 24 h intervals for up to 13 days on a 4-point scale (0, 1, 2, 3) according to the extent of colouration production observed.

Table 1. Carbon source substrates available on BIOLOG-GN plates

CONTROL H2O	a-cyclodextrin	dextrin	glycogen	tween 40	tween 80	N-acetyl-D-gal	N-acetyl-D-glu	adonitol
i-erythritol	D-fructose	L-fucose	D-galactose	gentiobiose	a-D-glucose	m-inostol	a-D-lactose	lactulose
D-melibiose	B-methyl-d-glu	D-psicose	D-raffinose	L-rhamnose	D-sorbitol	sucrose	D-trehalose	turanose
acetic acid	cis-aconitic a	citric acid	formic acid	D-galac ac lac	D-galacturonic	D-gluconic aci	D-glucosaminic	D-glucuronic
p-hydroxyOacet	itaconic acid	a-ketobutyric	a-ketoglutaric	a-ketovaleric	D L-lactic aci	malonic acid	propionic acid	quinic acid
bromo-succinic	succinamic aci	glucuronamide	alaninamide	D-alanine	L-alanine	L-alanyl-glyci	L-asparagine	L-aspartic aci
L-histidine	hydrox-L-prol	L-leucine	L-ornithine	L-phenylalanin	L-proline	L-pyroglutamic	D-serine	L-serine
urocanic acid	inosine	uridine	thymidine	phenylethylami	putrescine	2-aminoethanol	2 3-butandiol	glycerol

7.7. Data analysis

Quantitative data were subjected to numerical analysis as described previously (Thompson *et al.*, 1993a). The FAME profiles of individual isolates were compared using principle components analysis as described by Cavigelli *et al.* (1995).

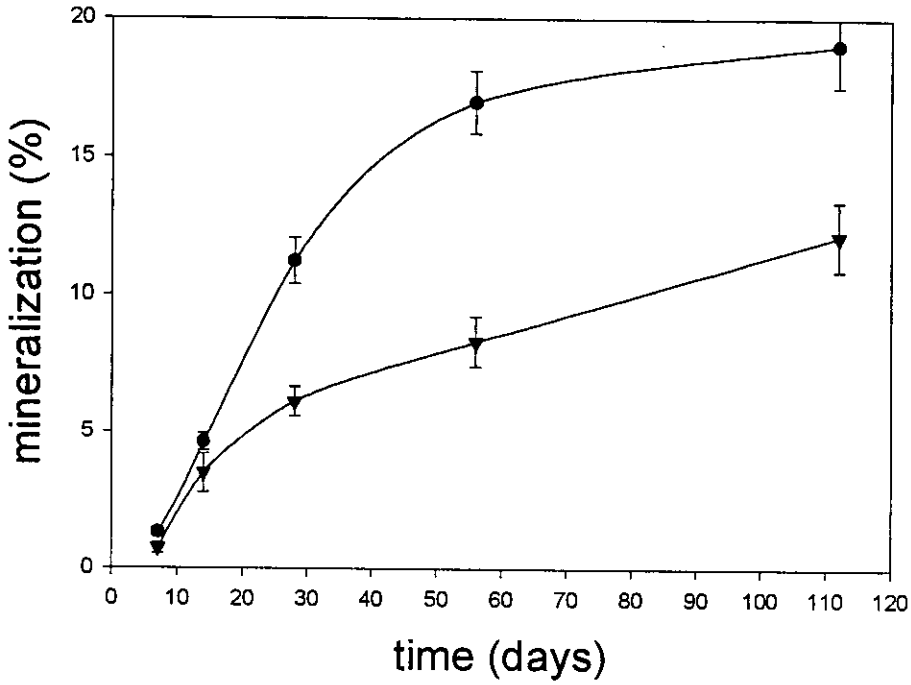
The relationship between BIOLOG metabolic profiles for sampled microbial communities was determined by calculation of Euclidean distance, a measure of dissimilarity, and clustered by the "average between groups" method (UNISTAT, UK). The actual distances calculated and the distances shown on the dendrogram were compared by Pearson product-moment correlation (cophenetic correlation) to ensure that the dendrogram gave an accurate representation of the data (Bridge, 1993). The relationships between samples taken over the season and those taken on the same day from different habitats were investigated. An estimation of the metabolic diversity was made by calculating the percentage of positive reactions in each plate. The variance in the values obtained was assessed by one way analysis of variance and the least significant difference (LSD) determined to test the significance of the differences between group means. The degree of association between BIOLOG metabolic diversity estimations and the plate count data was determined by Pearson product-moment correlation and significance tested by the one tailed *t*-test. All statistical analysis was undertaken using the UNISTAT statistical package (Unistat Ltd., London, UK.). Coeficient of variance calculations for biomass data were made using minitab software.

8. RESULTS

Experiment 1

8.1. Mineralisation rates of 1,2 dichlorobenzene in soils and rhizosphere amended soil.

FIGURE 1. Mineralisation rates of 1,2 dichlorobenzene in soils and root amended soil.

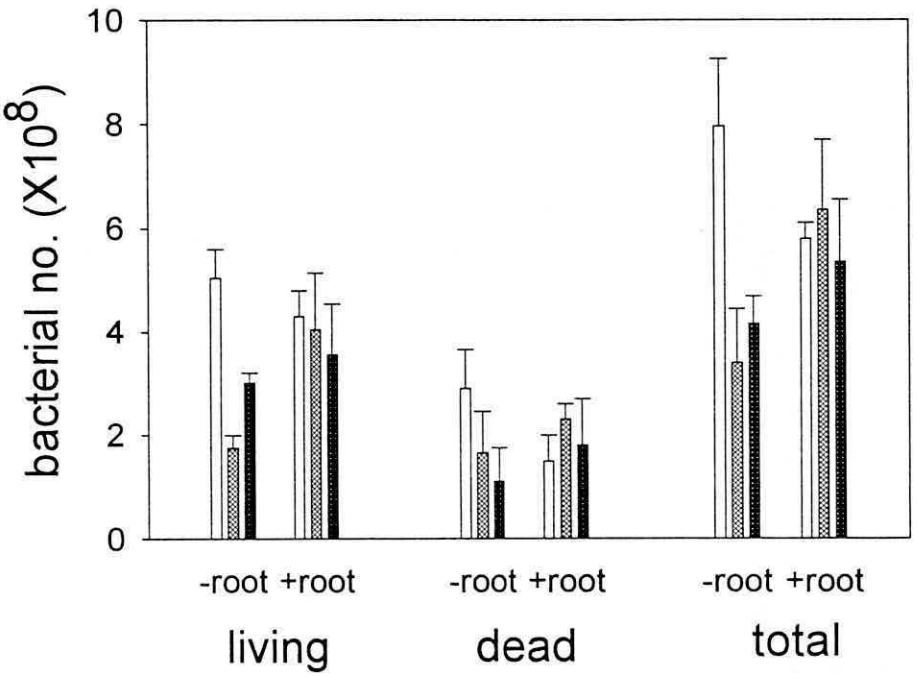


Mineralization of 1,2-dichlorobenzene in the presence (circles) and absence (triangles) of roots. Each data point is the average of 3 replicates and error bars are the standard error of the mean. Mineralization of ^{14}C-labelled 1,2-dichlorobenzene showed that the presence of decaying roots stimulated the ultimate degradation of dichlorobenzene (figure 1). Mineralization was both time dependent (P=0.007) and root dependent (P=0.000). There was no root/time interaction (P=0.807) which reflects the very similar pattern of mineralization between the two treatments. Initially, differences in mineralization rates between the two treatments were small, but increased with time, and by 28 d, the presence of decaying roots had increased mineralization by 100%, and this difference persisted to the end of the experiment. On termination of the experiment nearly 20% of the label had been mineralised in the presence of roots.

8.2. **Impact of 1,2-dichlorobenzene concentration and root addition on the size and diversity of the soil microbial biomass**

Two sets each of three microcosms of equilibrated soils were treated with either 10 or 50 μg of neat 1,2 dichlorobenzene (purchased from Sigma chemicals, purity > 99%) per gram dry wt of soil. To one set of treated microcosms, 6 g fresh wt. of 10 mm long segments of grass roots (*Holcus lanatus)* were added. Root tissue was prepared from fresh plants grown in standard potting compost. Roots were cut from the plant, adhering soil removed by washing in sterile distilled water, blotted dry with tissue paper, and mixed into the DCB treated soil. All microcosms were left to incubate for 14 d. Control microcosms of untreated soil or soil amended with root material were prepared (n = 3). Estimates of the community and population dynamics of resident microflora were made by microscopic vital staining biomass estimations, plate count estimates and community metabolic profiling.

FIGURE 2. Bacterial numbers (vital, dead, and total) response to 14d exposure to 1,2-dichlorobenzene in soil and soil amended with decaying roots



Equilibrated microcosms were untreated (control, open bar) or treated with 10 μg g^{-1} 1,2-dichlorobenzene (hatched bar) or 50 μg g^{-1} 1,2-dichlorobenzene (shaded bar) in the presence and absence of decaying roots. Bacterial numbers were determined by the Baclight assay. n=3, sem.

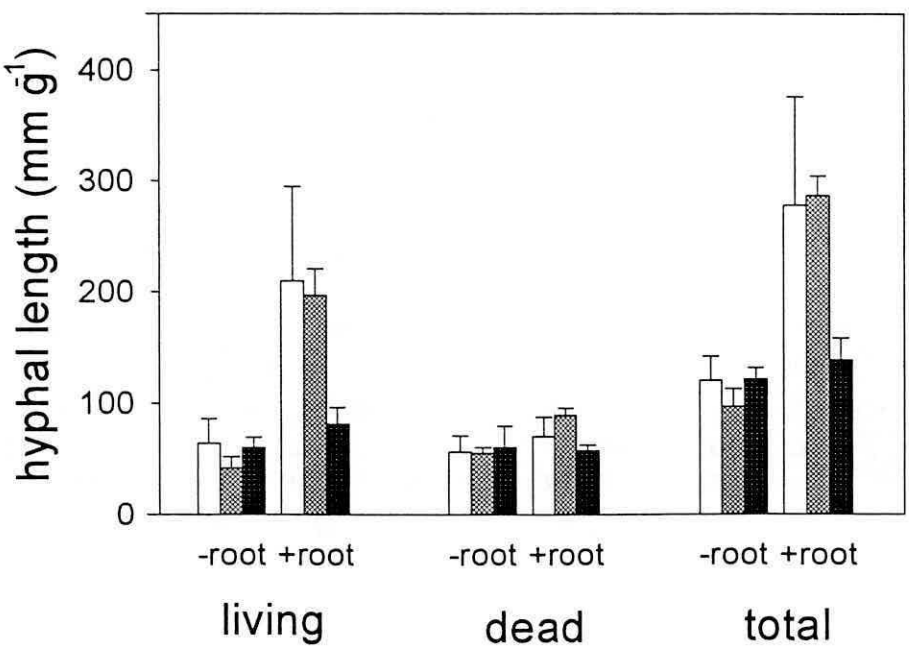
TABLE 2. Analysis of variance for the effects of plant roots and 1,2-dichlorobenzene on bacterial numbers, as assayed by Baclight.

Analysis relates to the data presented in figure 2. Probabilities of treatments not being significant are quoted.

treatment	root P value	1,2-dichlorobenzene P value	interaction P value
vital	0.249	0.056	0.0125
dead	0.970	0.584	0.262
total	0.458	0.112	0.080

8.2.1 *Impact of DCB on total bacterial count.* Over a 14 d time period, exposure to both roots and 1,2-dichlorobenzene had an effect on vital bacterial numbers (figure 2). In the absence of roots, both 10 and 50 μg g^{-1} 1,2-dichlorobenzene caused about a 50% decrease in vital counts, yet the presence of roots appeared to buffer the inhibitory effect of dichlorobenzene at both concentrations as there was little or no inhibition of counts. Analysis of variance of the vital count data (table 2) showed that the dichlorobenzene effect was bordering on significance (P=0.056) but that the interaction effect between roots and dichlorobenzene was significant, showing that the presence of roots was altering the impact of dichlorobenzene. Interestingly, the presence of decaying roots did not stimulate the vital bacterial biomass at 14 d. There were no significant effects of the treatments on the dead counts, and while the total counts mirrored the pattern of the vital counts (figure 2), non of the treatments were significant.

FIGURE 3. Fungal biomass, hyphal length (vital, dead, and total), response to 14d exposure to 1,2-dichlorobenzene in soil and soil amended with decaying roots



Equilibrated microcosms were lefty untreated (control, open bar) or treated with 10 μg g^{-1} 1,2-dichlorobenzene (hatched bar) or 50 μg g^{-1} 1,2-dichlorobenzene (shaded bar) in the presence and absence of decaying roots. Hyphal lengths were determined by FDA hydrolysis. n=3, sem.

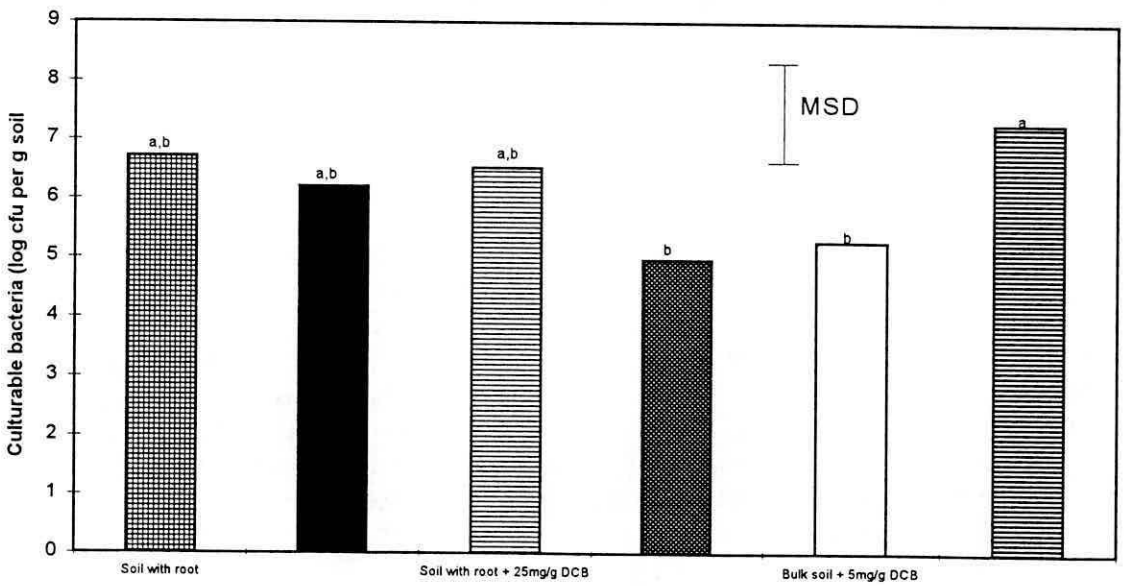
TABLE 3. Analysis of variance for the effects of plant roots and 1,2-dichlorobenzene on fungal hyphal length, as assayed by FDA hydrolysis.

Analysis relates to the data presented in figure 3. Probabilities of treatments not being significant are quoted.

treatment	root P value	1,2-dichlorobenzene P value	interaction P value
vital	0.005	0.249	0.195
dead	0.170	0.569	0.363
total	0.005	0.282	0.127

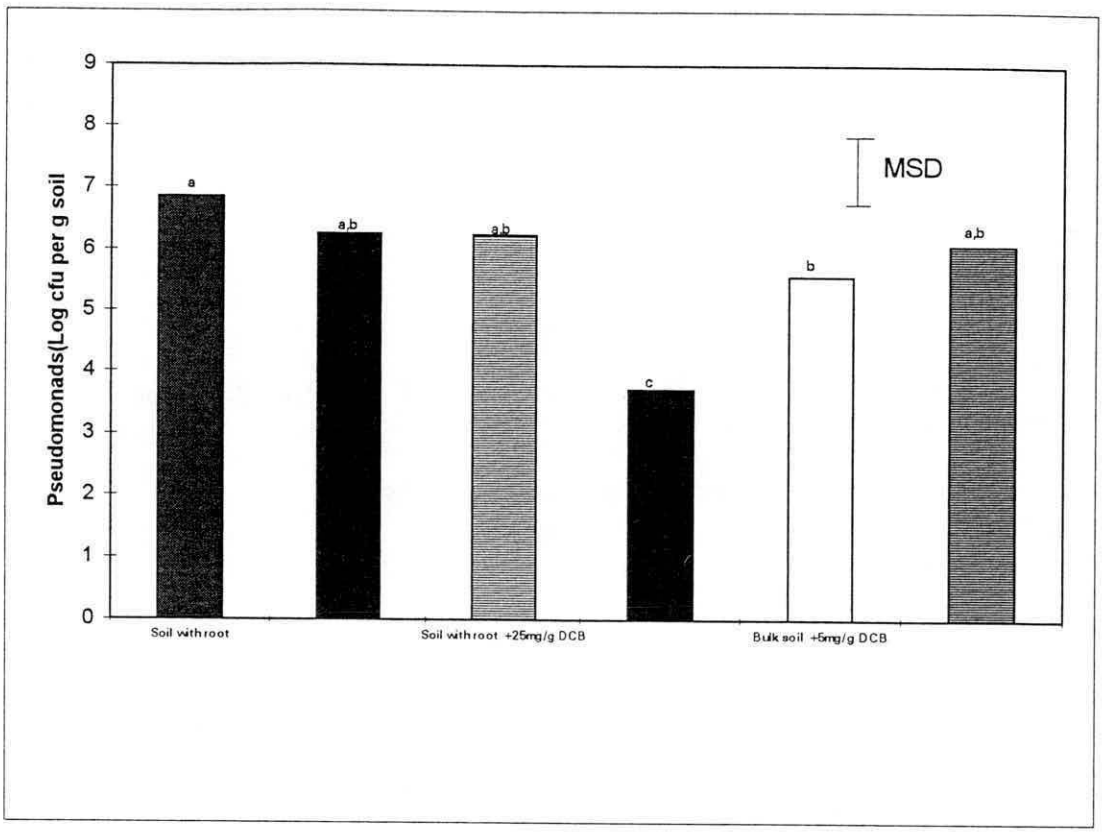
8.2.2 *Impact of DCB on fungal biomass.* Fungal hyphal lengths responded very differently to bacterial numbers in that the presence of dichlorobenzene had little or no effect on the fungal biomass in the absence of roots, figure 3, opposite to what was observed in bacteria. The presence of roots greatly increased vital hyphal lengths (by 3 fold). When decaying roots were present, 10 μg g^{-1} dichlorobenzene had no effect on hyphal lengths, but 50 μg g^{-1} dichlorobenzene inhibited vital hyphal length, with hyphal length being less than 50% of the control (figure 3). Analysis of variance showed that only the presence of roots had a significant effect on vital hyphal length (table 3). There was no effect of roots or dichlorobenzene at 14 d (no factors were significant in the analysis of variance). Total hyphal length mirrored vital hyphal length and again, only root term was significant in the analysis of variance.

FIGURE 4a. Impact of DCB concentrations on bacterial density in simulated rhizosphere and bulk soils



Estimates of culturable bacteria were made on TSBA 14 days after the treatment of soils with excised grass roots (the three columns on left) and without roots (the three columns on right), control (hatch) without DCB, in the presence of 10 (bar) or 50 (striped) μg 1,2-DCB g^{-1},. n=3, msd value shown. Letters above column demonstrate significance.

FIGURE 4b. Impact of DCB concentrations on pseudomonad density in simulated rhizosphere and bulk soils



Estimates of culturable fluorescent Pseudomonads were made on PSA-CFC 14 days after the treatment of soils with excised grass roots (the three columns on left) and without roots (the three columns on right), control (hatch) without DCB, in the presence of 10 (bar) or 50 (striped) μg 1,2 DCB g^{-1},. n=3, msd value shown. Letters above column demonstrate significance.

8.2.3 *Impact of DCB on population dynamics of culturable component of bacteria.* The impact of 10 and 50 μg DCB g^{-1} on the number of culturable bacteria was assessed in bulk soils and soils containing root material. Neither treatment had any detectable impact on the number of culturable bacteria or Pseudomonads (Fig 4a and 4b) in soils containing plant tissue. However, in bulk soil lacking root material addition of 50 μg DCB g^{-1} caused culturable counts to increase significantly ($P < 0.05$) in numbers by two orders of magnitude, compared to control soils. Addition of 10 μg DCB g^{-1} had no detectable impact on the number of culturable bacteria. Both concentrations of DCB caused a significant increase in the number of pseudomonad ($P < 0.05$), counts increasing by approximately two orders of magnitude. The response pseudomonad numbers to 10 and 50 μg DCB g^{-1} showed no significant difference (Fig 4b).

In this and all subsequent experiments described the soil microcosms were found to be highly reproducible, in terms of community activity, taxa composition and culturable numbers.

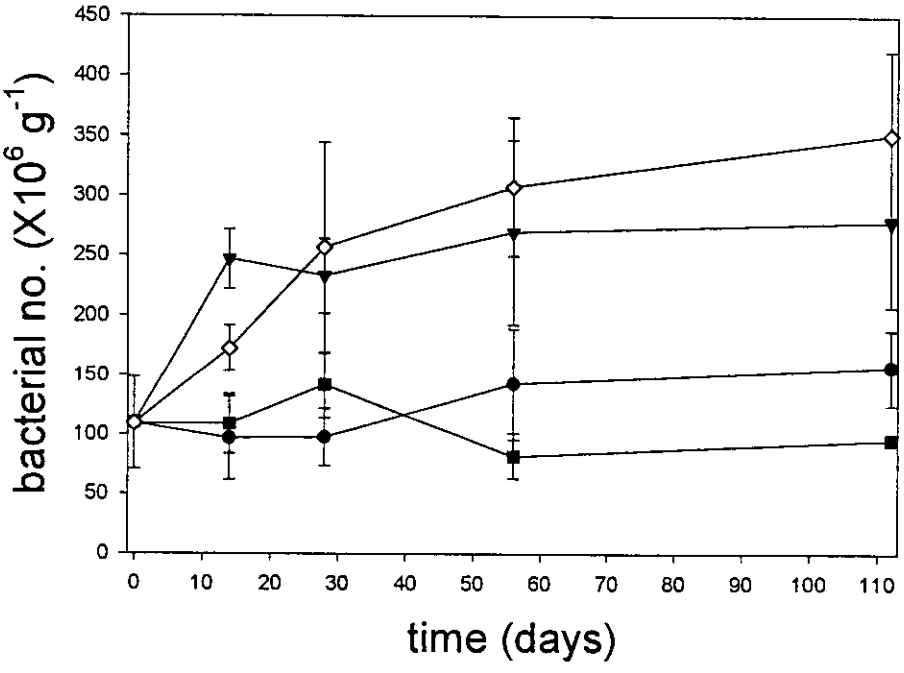
Experiment 2

8.3. Time course of microbial biomass response to 1,2-dichlorobenzene and root addition

Microcosms were established identical to experiment 1 except that they contained 100 g soil d.wt., rather than 70 g. After the 7 days pre-incubation period, 6 g fresh roots were added to half of the microcosms. Then neat 1,2-dichlorobenzene was applied to some of the microcosms at a concentration of 10 $\mu g\ g^{-1}$ soil d. wt. to give 4 experimental treatments in all, a factorial design of with and without roots and with and without 1,2-dichlorobenzene. Two sets of the experimental design were prepared. One set was labelled with uniformly labelled ^{14}C- 1,2-dichlorobenzene (supplied by Sigma at a radiochemical purity >95%). The labelled was applied as a hexane solution (100 µl) (supplied by Rathburns) which had an activity of 0.1 $\mu Ci\ ml^{-1}$. Hexane (100 µl) was added to all non-radiolabelled treatments so that all microcosms had received identical chemical applications.

Bacteria (living, direct viable count and plate count of colony forming units/ g soil) and fungi (direct living and dead count) were enumerated at each harvest, as described in experiment 1. Estimates of the community and population dynamics of resident microflora were made by microscopic vital staining biomass estimations, plate count estimates and community metabolic profiling.

FIGURE 5. Impact of 1,2 DCB on bacterial numbers (total direct count) over 112 d in the presence and absence of decaying root material



Bacterial numbers were determined by the Baclight assay. Symbols represent the following experimental treatments: circles = control; triangle = root amended; square = 1,2-dichlorobenzene amended; diamond = root and 1,2-dichlorobenzene amended. Bacterial numbers in the untreated control were unaffected by the microcosm assay throughout the entire time course of 112 days. n=3, sem.

Table 4. **Analysis of Covariance of the response of vital bacterial numbers, over time, in soil amended with 1,2-dichlorobenzene (10 μg g^{-1}) and decaying roots.**

Probabilities of treatments (Figure 5) not being significant are quoted.

treatment	**P value**
time	0.000
root	0.006
1,2 dichlorobenzene	0.464
time * root	0.010
time * 1,2-dichlorobenzene	0.130
root * 1,2-dichlorobenzene	0.681
time * root * 1,2-dichlorobenzene	0.238

8.3.1 *Impact of DCB on total bacterial count.* When vital bacterial numbers were monitored over a time period of 112 days, in the presence and absence of 10 μg g^{-1} dichlorobenzene and in the presence and absence of roots, the presence of roots greatly stimulated bacterial numbers, consistently, 2-3 fold that of the controls (figure 5). While it appears that dichlorobenzene had an effect at some time points and for some treatments (in the presence of roots at 14 days and in the absence of roots at 112 d), generally dichlorobenzene had little effect. This is reflected in the analysis of covariance of the data which showed that none of the terms that included dichlorobenzene were significant (table 4). However, both root and time had a significant effect, and the root/time interaction was also significant.

Table 5. **Analysis of Covariance of the response of fungal hyphal length, over time, in soil amended with 1,2-dichlorobenzene (10 μg g^{-1}) and decaying roots, (see figure 6)**

Probabilities of treatments (figure 6) not being significant are quoted.

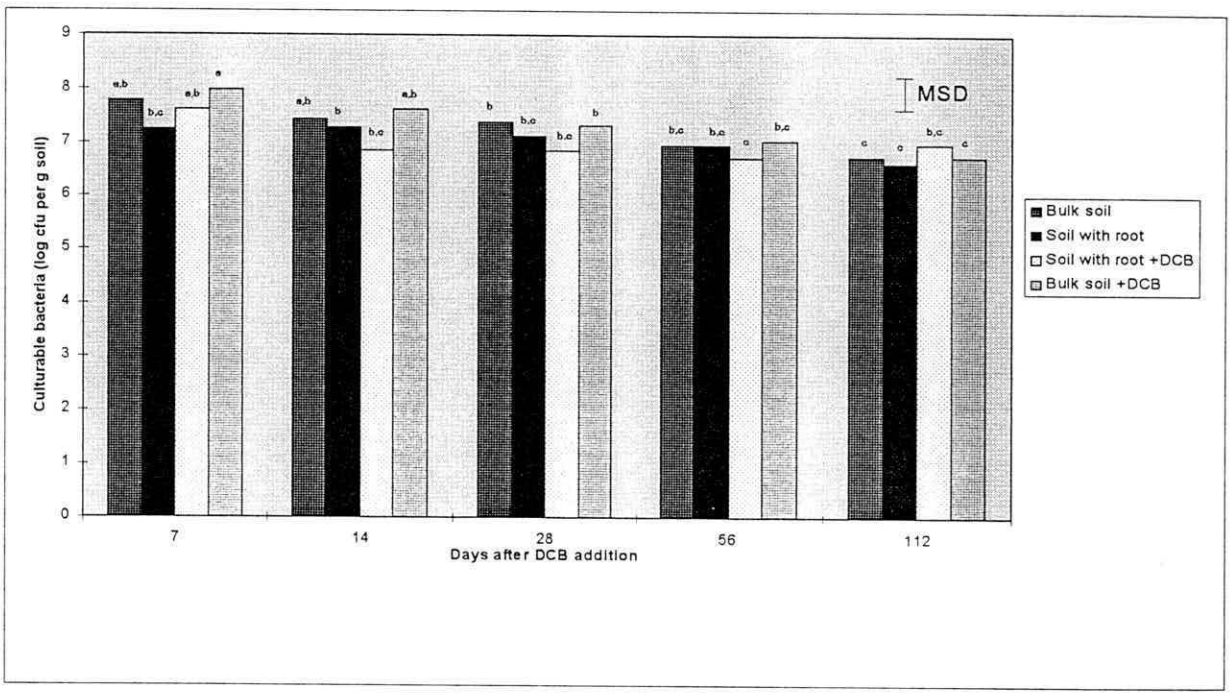
treatment	**vital** P value	**dead** P value
time	0.035	0.313
root	0.000	0.130
1,2 dichlorobenzene	0.846	0.108
time * root	0.026	0.991
time * 1,2-dichlorobenzene	0.819	0.421
root * 1,2-dichlorobenzene	0.684	0.909
time * root * 1,2-dichlorobenzene	0.836	0.841

8.3.2 *Impact of DCB on fungal biomass.* The pattern of vital fungal hyphal lengths following exposure to decaying roots (figure 6) was similar to the bacteria, although the stimulation of hyphal lengths was larger than the stimulation of bacterial numbers, with hyphal lengths being stimulated by up to 5 fold in the presence of roots. Dichlorobenzene had little effect on hyphal length, both in the presence and absence of roots. Control hyphal lengths stayed relatively constant throughout the experiment. Analysis of covariance showed that time, root and time/root all had a significant effect on vital hyphal length (table 5). There was no clear pattern in the amount of dead hyphal length present in the soil (figure 6). Analysis of covariance confirmed this as there were no significant differences in the treatment terms.

8.3.3 *Impact of DCB on population dynamics of culturable component of bacteria.* Soils with and without root material were incubated with 10 μg DCB g^{-1} soil for 112 days. Although there was a small (approximately 0.5 log) but significant ($P < 0.05$) decrease in the number of culturable bacteria throughout the study, there was no significant and consistent difference detected between treatments. On the first sampling occasion taken seven days after microcosm establishment, significantly ($P < 0.05$) greater numbers of bacteria were detected in bulk soil with DCB than soils containing root tissue but in the absences of DCB (figure 7a and 7b). No significant differences in the number of culturable bacteria were detected on any sampling day or treatment thereafter.

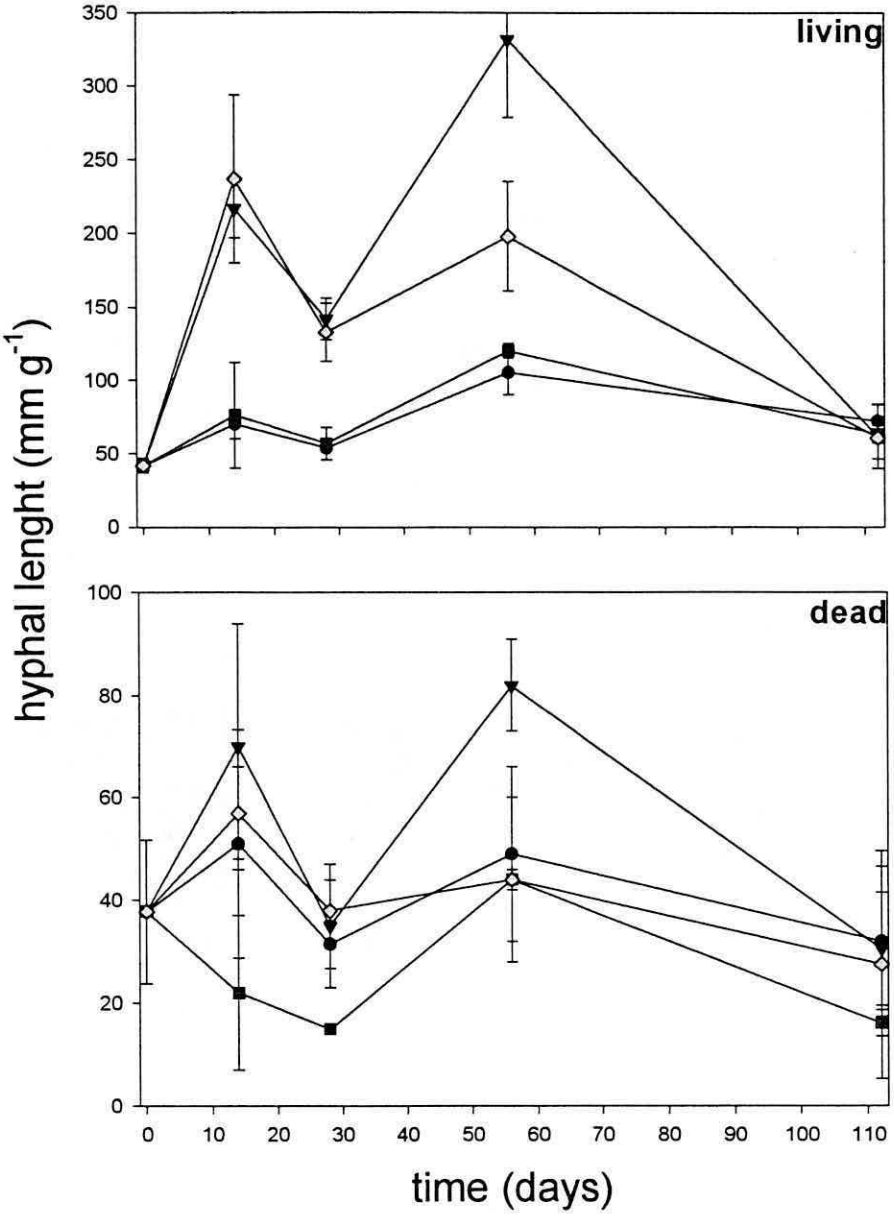
A similar pattern was observed in the pseudomonad population (Fig 7b). Apart from pseudomonad counts in bulk soils with DCB added, that were significantly ($P < 0.05$) greater that detected in the other three treatments detected on day 7, there was no significant or consist difference between treatments on any subsequent sampling days.

FIGURE 7a. Impact of 1,2 DCB on bacterial numbers (cfu) isolated on TSBA over 112 d in the presence and absence of decaying root material



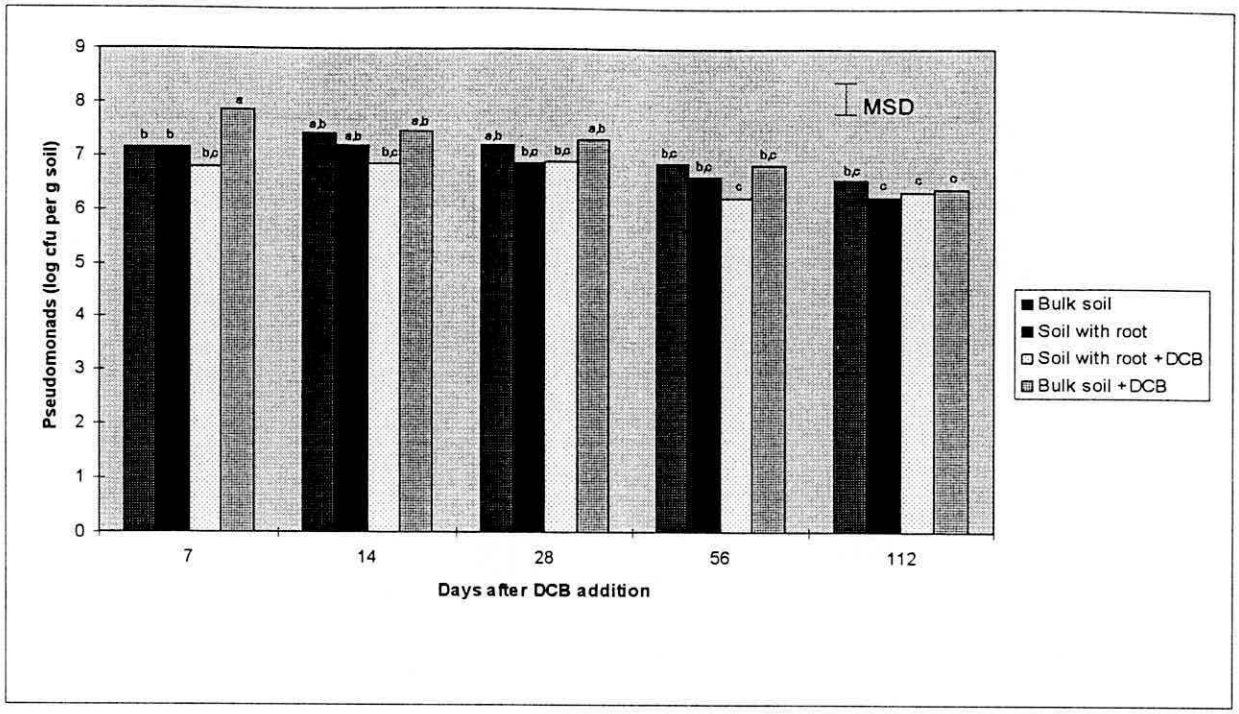
Changes in the bacterial population dynamics between treatments over time were evaluated in the microcosms with and without root material following the addition of 10 μg 1,2 DCB g^{-1} soil. Letters above histogram bars indicate significant differences in counts.

FIGURE 6. Impact of 1,2 DCB on fungal biomass in the presence and absence of decaying root material



Fungal hyphal lengths (vital, dead, and total) determined over a time course of 112 d in the presence of 1,2-dichlorobenzene (10 μg g^{-1}) and absence decaying roots. Hyphal lengths were determined by FDA hydrolysis. Symbols represent the following experimental treatments: circles = control; triangle = root amended; square = 1,2-dichlorobenzene amended; diamond = root and 1,2-dichlorobenzene amended. n=3, sem.

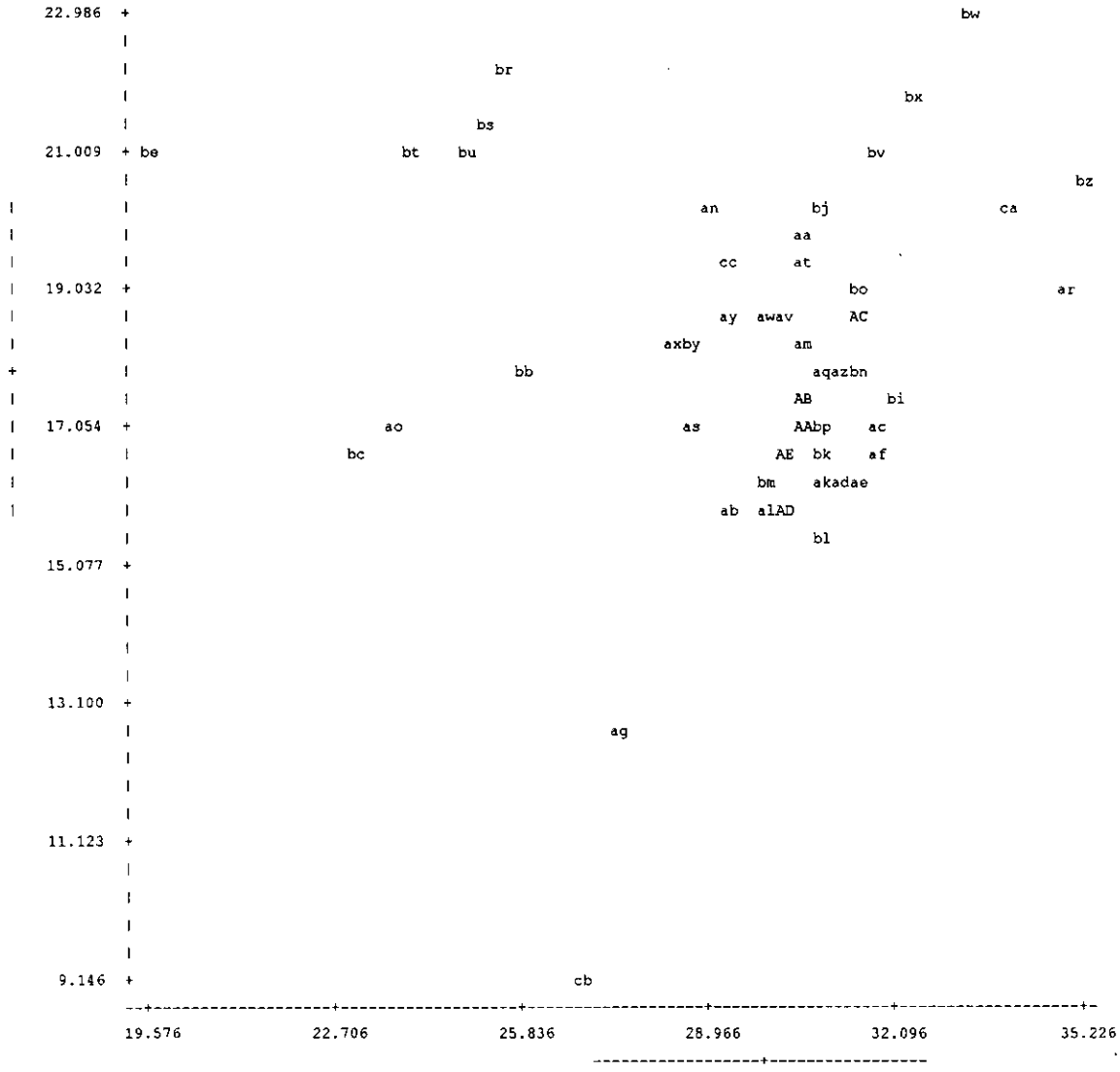
FIGURE 7b. Impact of 1,2 DCB on pseudomonad numbers (cfu) isolated on PSA-CFC over 112 d in the presence and absence of decaying root material



Changes in the pseudomonad population dynamics between treatments over time were evaluated in the microcosms with and without root material following the addition of 10 μg 1,2 DCB g^{-1} soil. Letters above histogram bars indicate significant differences in counts.

8.3.4 *Qualitative evaluation in changes in the genetic diversity of pseudomonad populations following DCB exposure.* On each sampling occasion 30 pseudomonad isolates were randomly picked from agar plates for each treatment (10 from each replicate), and on each of the 5 sampling occasions (total of 600 isolates). Isolates were phenotypically characterised and grouped according to their FAME profiles using principle components analysis. These analyses revealed there to be no grouping of isolates, based on their phenotype, according to the treatment or day of isolation (Fig 8), indicating that the DCB had no detectable impact on the composition of the pseudomonad population.

FIGURE 8. PCA analysis of FAME-MIS profiles of fluorescent pseudomonad isolates from microcosms treated fro 112d with 1,2 DCB in the presence and absence of decaying root material.



Groupings of pseudomonad isolates taken from soil treated with 1,2-DCB, control, planted and unplanted soil. Groupings are based on principle components analysis of fatty acid methyl ester composition of each isolate. Samples were taken 56 days after DCB application.

8.3.5 *Community metabolic profiles associated with DCB treatment.* The total soil microbial community ability to metabolise 95 sole carbon sources was assessed using the BIOLOG system combined with cluster analysis. This revealed that communities from bulk and soil amended with plants roots clustered apart on the basis of their metabolic profiling. However, there was no evidence that DCB addition had any detectable impact on the communities metabolic potential (figure 9).

FIGURE 9. Dendrogram following cluster analysis of total community metabolic profiles for DCB treated soils with and without decaying roots.

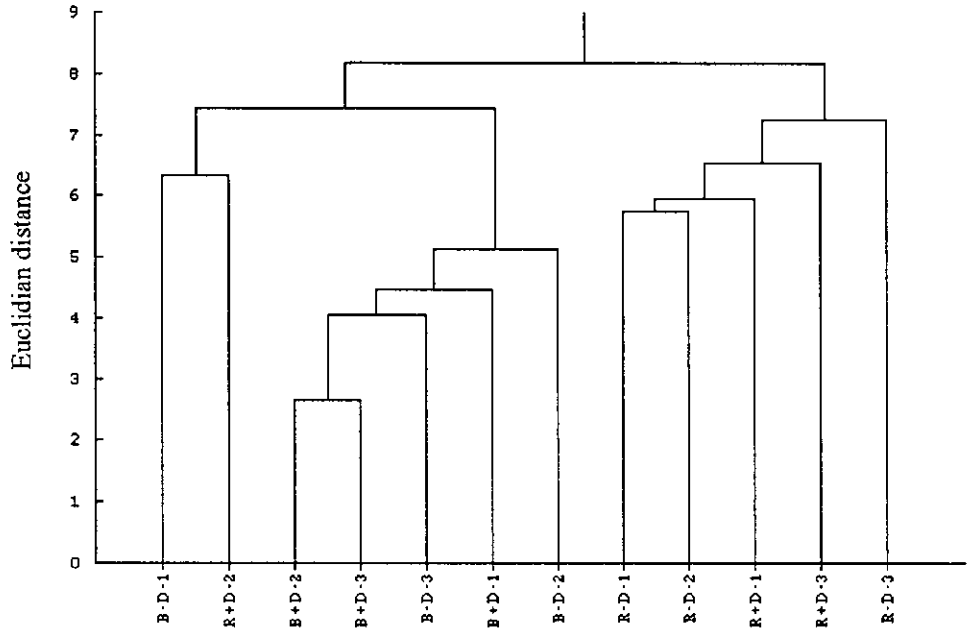


Fig. 9: Dendrogram indicting the relationship between metabolic profiles of soil microbial communities exposed to 10µg/g of 1,2-DCB for 112 days. The relationship between samples is measured in Euclids and clustered by UPGMA. Key: B-D=Bulk soil without DCB, B+D=Bulk soil with DCB, R-D= Soil with root without DCB, R+D=Soil with root with DCB, numbers indicate replicate number.

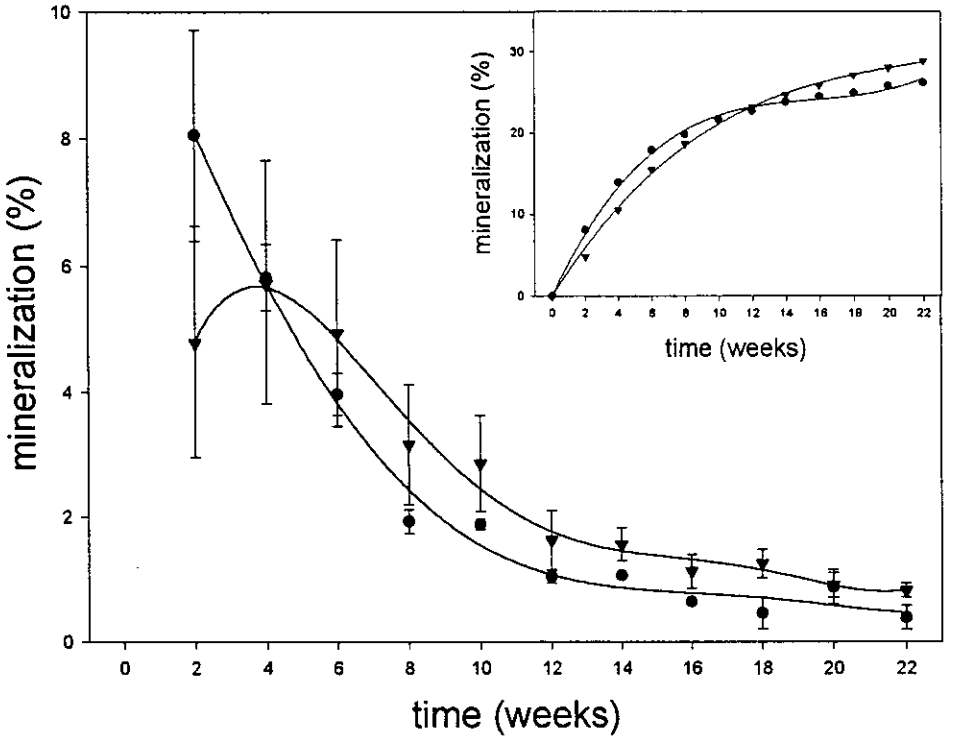
Experiment 3

8.4. **Time course of microbial response to repeated and one-off addition of 1,2-dichlorobenzene.**

This experiment was designed to investigate the effect on the soil microbial biomass of a 10 repeated exposure of low concentrations of 1,2-dichlorobenzene (10 µg g^{-1}) at fortnightly intervals with an equivalent exposure to the same total concentration (that is the sum concentration of the 10 repeated exposures, 100 µg g^{-1}) added as a single dose at the start of the experiment. Stabilised microcosms of 100g soil were treated at 14 d intervals, and all soils were thoroughly stirred at each interval. Two sets of the experimental design were prepared. One set was labelled with uniformly labelled ^{14}C-1,2-dichlorobenzene (supplied by Sigma at a radiochemical purity >95%) to assess mineralisation rates, the other to investigate the microbiology.

8.4.1. *Comparison of 1,2-DCB mineralisation in soils singly or regularly exposed.* Mineralization rates were initially greatest in the pulse exposure, as opposed to the single exposure. However, at future time points (2 and 6 weeks) mineralization in the two treatments were similar, and at all future time points mineralization was always greater in the soils that received a single addition. The pattern of cumulative mineralization also reflected the enhanced mineralization at the early stages of the experiments, and it is not until week 14 that the single dosed treatment cumulatively mineralized more label. Analysis of variance showed that for both mineralization rate and cumulative mineralization, that time and method of exposure (single verses pulsed) were significant, although the interaction term was not significant.

FIGURE 10. Mineralisation rate of 1,2 DCB in soils exposed to a single and regular addition



100 µg of 1,2 DCB were added per gram of soil either as 10 regular treatments of 10 µg g^{-1} every 14 days (triangles), or as a single treatment at day 0 (circles). n=3, sem.

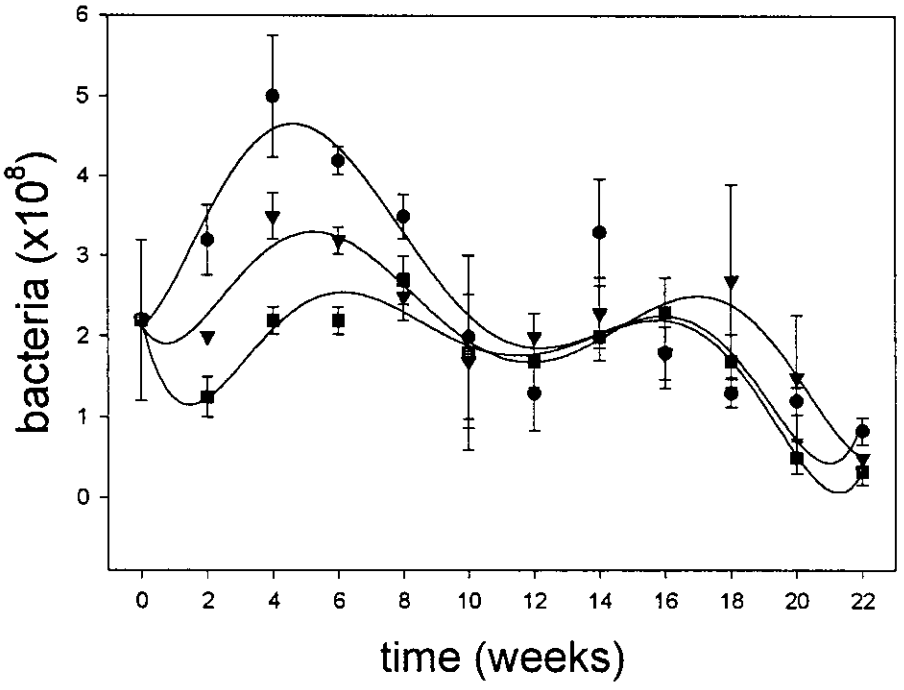
Table 6. Analysis of variance of mineralization of 1,2-dichlorobenzene over time, figure 10.

Probabilities of treatments not being significant are quoted.

treatment	**14 d** P value	**cumul.** P value
time	0.000	0.009
dose	0.011	0.013
time * dose	0.796	0.985

8.4.2 *Impact of DCB on total bacterial count.* The control, extractable, vital bacterial population varied in this experiment, rising by 2-fold by week 4 compared to initial numbers. After 4 weeks, numbers declined steadily to 50% of initial numbers. Both dichlorobenzene treatments followed a very similar pattern, although numbers were inhibited compared to controls until week 10, with the single dose have more of an inhibitory effect. After week 10, regression analysis show that the bacterial numbers converge. This suggests adaptation of the bacteria at a community level to the pollutant stresses. Analysis of variance showed that both time and dose was highly significant, and that the interaction between these two factors was also significant.

FIGURE 11. Impact on total bacterial counts in soils treated with repeated or one-off addition of 1,2-dichlorobenzene.



100 μg of 1,2 DCB were added per gram of soil either as 10 regular treatments of 10 μg g^{-1} every 14 days (triangles), or as a single treatment at day 0 (circles). Controls of untreated soils (Squares) were included. Total bacterial live counts were determined with Baclight. n=3, sem.

Table 7 Analysis of Covariance, response of vital bacterial numbers over time (figure 11).

Probabilities of treatments not being significant are quoted.

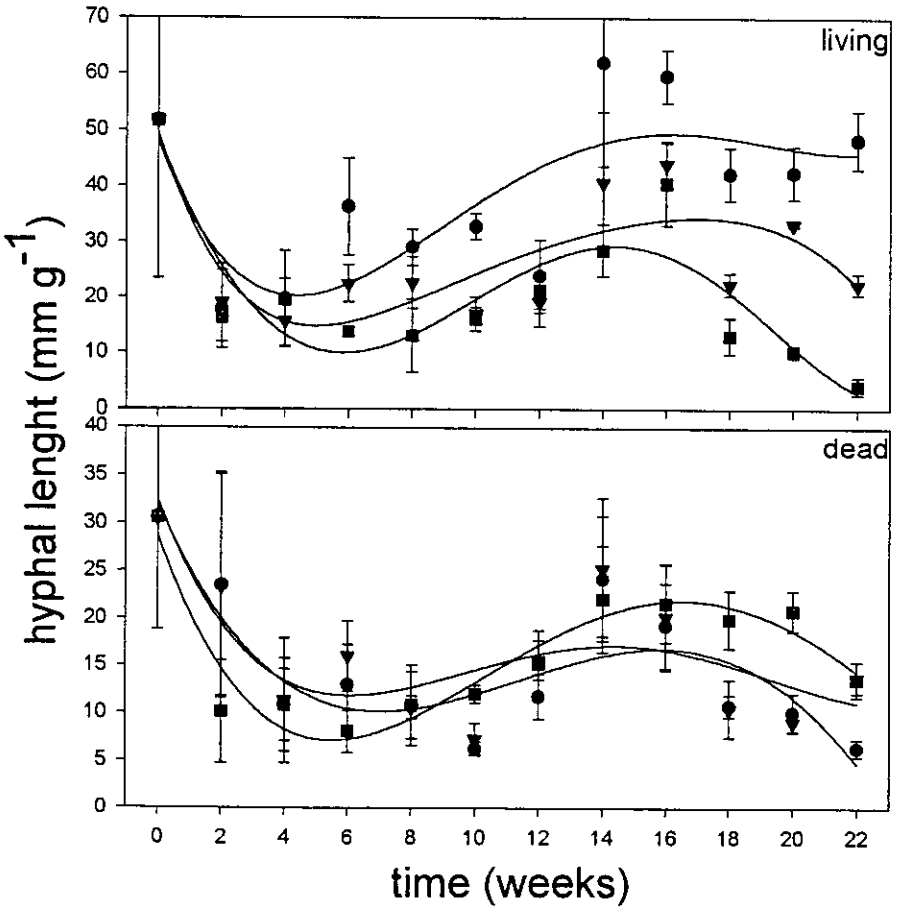
treatment	P value
time	0.000
1,2 dichlorobenzene	0.000
time * 1,2-dichlorobenzene	0.002

8.4.3 *Impact of DCB on fungal biomass.* The fungal biomass behaves very differently to the bacterial biomass. Vital fungal hyphal length initially drops markedly at week 2 for all treatments, including controls. Control lengths recover steadily throughout the experiment, exceeding their initial

value by week 14, and staying fairly constant after this time period. The single dose inhibited the recovery seen in the controls, although the lengths did increase slightly up to week 14 before declining sharply. At the termination of the experiment, hyphal lengths in the single dosed exposure were less than 10% of the control population. For the pulsed exposure, the pattern was more variable, but in general, this treatment was intermediate between the control and single exposure. Analysis of covariance revealed that both time and the time*dose interaction were significant, showing that DCB treatments effected the fungal community dynamics.

Dead (non-vital) fungal biomass was also measured during this experiment. Dead biomass was initially inhibited by the single addition treatment compared to the controls, but by week 10 biomass was similar. At the termination of the experiment dead hyphal length was very was about 100% greater than in controls, even though vital biomass was less than 10% of controls. This shows that the DCB treatments is clearly inhibiting turnover of the fungal biomass. It is not clear what mechanism is involved in this effect on turnover as fungal biomass (both vital and dead) appear to recover up to weeks 14-16 and then crash in the presence of DCB. It could be that the large shifts in the bacterial community (table 7) inhibit fungal growth rather than a direct effect of DCB. The time*dose term was significant for the dead fungal biomass.

FIGURE 12. Impact on fungal biomass in soils treated with repeated or one-off addition of 1,2-dichlorobenzene



100 µg of 1,2 DCB were added per gram of soil either as 10 regular treatments of 10 µg g^{-1} every 14 days (triangles), or as a single treatment at day 0 (circles). Controls of untreated soils (Squares) were included. Hyphal length for live and dead tissue were determined with FDA. n=3, sem.

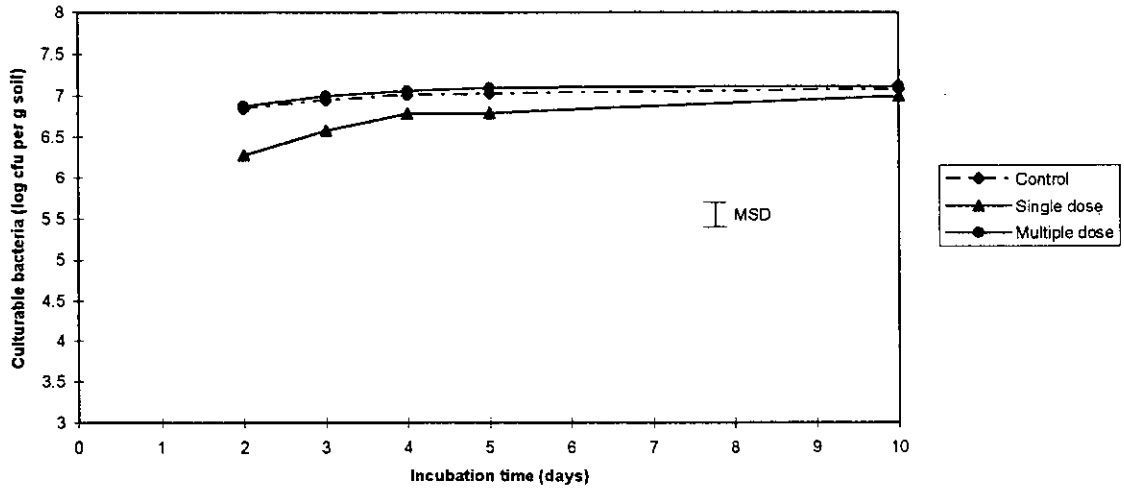
Table 8. Analysis of Covariance, response of fungal hyphal length over time (figure 12)

Probabilities of treatments not being significant are quoted.

treatment	**vital** P value	**dead** P value
time	0.000	0.739
dose	0.929	0.106
time * dose	0.032	0.029

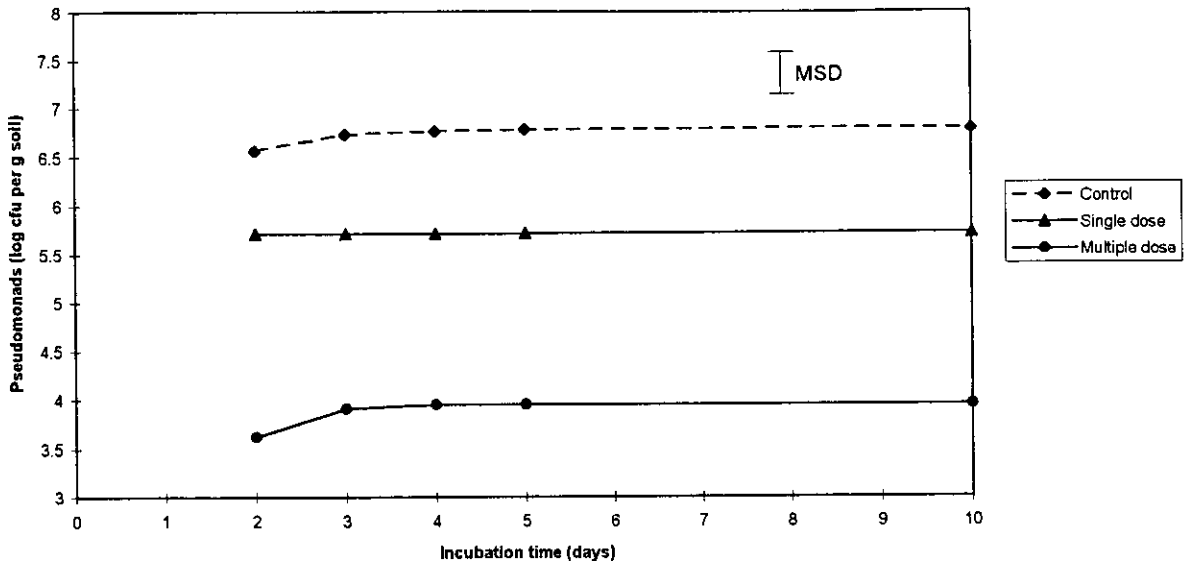
8.4.4 *Impact of single and multiple long term application of 1,2 DCB on population dynamics of culturable component of bacteria.* The impact of single (100 µl DCB g^{-1} to mimic a spillage event) and multiple doses (10 X 10 µl DCB g^{-1} to mimic chronic pollution) of the same quantity of DCB on the soil microbial community were compared. Isolation plates used to assess the total number of culturable bacteria were scored on 5 occasions for the 10 day incubation period. This revealed that after two days incubation, counts from soil treated with a single dose of DCB were significantly lower ($P < 0.05$) than detected in the control and soils treated with multiple applications of DCB. However, after two days incubation neither DCB application had any detectable impact on total number of culturable bacteria, when compared to the control (figure 13). In contrast, both single and multiple DCB doses significantly ($P < 0.05$) reduced pseudomonad counts. After 10 days incubation pseudomonad counts in exposed to single and multiple DCB doses were 10.3- and 688-fold lower than the control soil respectively (figure 14).

FIGURE 13. Community density and changes in the rate of colony development during a 10 day plate incubation period for bacteria isolated on TSB agar. Evaluation of changes in microbial communities in response to single and multiple doses of 1,2-DCB.



The total, cumulative number of colonies appearing per plate, n=3. msd value shown. Plates were incubated at 18 °C and counted on a daily basis. (see Lilley *et al.*, 1996). 1,2-DCB added as a single dose of 100 µg /g soil at time 0, or as 10 doses of 10 µg /g soil every 14 days. Microcosms were sampled at the end of the experiment, t = 140 days

FIGURE 14. **Pseudomonad population density and changes in the rate of colony development during a 10 day plate incubation period for bacteria isolated on PSA-CFC agar. Evaluation of changes in microbial communities in response to single and multiple doses of 1,2-DCB..**



The total, cumulative number of pseudomonad colonies appearing per plate, n=3. msd value shown. Plates were incubated at 18 °C and counted on a daily basis. (see Lilley et al., 1996). 1,2-DCB added as a single dose of 100 µg /g soil at time 0, or as 10 doses of 10 µg /g soil every 14 days. Microcosms were sampled at the end of the experiment, t = 140 days.

8.4.5 *Qualitative evaluation of the impact of single and multiple long term application of 1,2 DCB on the genetic diversity of soil microbial communities.* The impact of single and multiple doses of DCB on species composition of bacteria isolated from soil using TSA was assessed by FAME characterisation. Two hundred and six isolates were characterised by FAME analysis (tables 9, 10). This analyses revealed that both DCB treatments had a significant impact on the ability to maintain isolates in culture after isolation and the species composition. Only ten percent of strains isolated from control soil were lost on subsequent sub-culturing compared to 18 and 43% from the single and multiple dose treatment respectively. The most significant impact of the single DCB treatment was the decrease in the number of Gram positive isolated, most notably *Arthrobacter* and *Micrococcus* and increase in the number of Gram negative genera, particularly *Pseudomonas*. Multiple applications of DCB had a devastating impact on the Gram negative component of the community which decreased to only 8% of the identified community, compared to 54 and 32 % in the control and single dose soil. The greatest change in the community detected was decrease in the pseudomonad population, from 45% and 65% in the control and single dose soils respectively, to less than 2% of isolates. In contrast, the *Bacillus* community showed a significant increase the number detected in soils exposed to multiple applications of DCB (table 10).

Table 9 **FAME-MIS identification of bacterial isolates taken from TSBA plates. Samples collected from untreated soils and soils treated singly or multiply with 100 µg 1,2-DCB g^{-1}.**

		Control				Single dose				multiple dose		
Taxa	a	b	c	Total	a	b	c	Total	a	b	c	Total
Treatment Code												
Acidovorax facilis						2		2				
Acinetobacter calcoaceticus												
Arthrobacter oxydans	11	8	4	25	1	1	1	3	6	4	8	18
Arthrobacter globiformis			2	2	1	2		3			2	2
Arthrobacter viscosus					2			2				
Bacillus brevis	2	1	1	4						2		2
Bacillus laterosporus									2	2	2	6
Bacillus megaterium									3	4	1	8
Bacillus mycoides	2	1	1	4	4	3	4	11		2	1	3
Bacillus psychrophilus										2		2
Bacillus pumilus												
Brevibacterium linens						2		2				
Pseudomonas chlororaphis	3	5	4	12	10	3	6	19				
Pseudomonas coronafaciens		2		2								
Pseudomonas fluorescens	5	8	4	17	6	3	10	19				
Pseudomonas putida	2		4	6		3	2	5				
Pseudomonas vesicularis						4	2	6	1			1
Rathyibacter triticum							1	1				
Micrococcus luteus		4	3	7					3			3
Micrococcus lylae									3			3
Micrococcus roseus	2			2						1		1
Micrococcus varians										1		1
NM											2	2
Total	27	29	23	79	24	23	26	73	18	18	16	52
Number of taxa	7	7	8		6	9	7		6	8	6	

Values for individual replicates are given to demonstrate reproducibility of system.

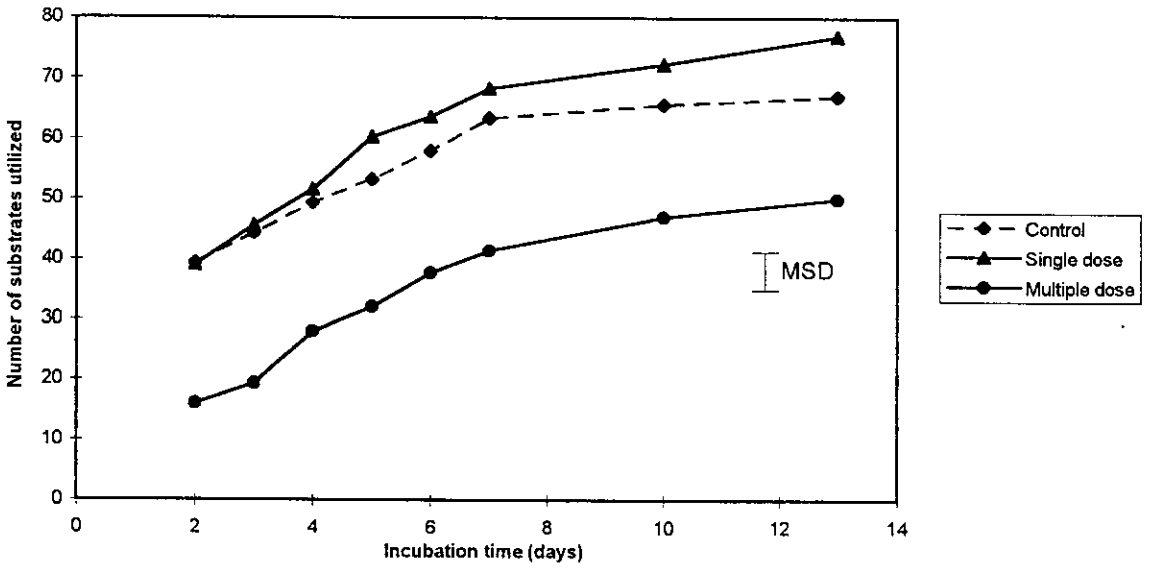
Table 10. **Summary data of predominant bacterial genera identified from Table 9.**

		Impact of DCB exposure on the diversity of culturable soil bacteria					
Treatment		Control		Single dose		Recurring dose	
Genera							
Acidivorax		0		2		0	
Arthrobacter		27		8		20	
Bacillus		8		11		21	
Brevibacterium		0		2		0	
Pseudomonas		37		50		1	
Micrococcus		9		0		8	
unknown		0		0		2	
Total		81		73		52	
Loss of isolates (%)		10		18		43	
% Gram positive		54		32		92	

8.4.6 *Community metabolic profiles associated with single and multiple long term application of 1,2 DCB.* The impact of single and multiple dose applications of DCB on the metabolic potential of total soil microbial communities was assessed by scoring BIOLOG plates on eight occasions over a 13 day incubation (figure 15). Multiple applications of DCB significantly reduced the metabolic potential of the total community. After two days of incubation only 16 sole carbon compounds were utilised by the total microbial community compared to 40 in the single application and control soils. After 13 days incubation, the number of carbon compounds utilised in the multiple exposure soil increased to 49, but was still significantly lower that the 66 positives in the control soil. Although the number of sole carbon sources utilised by the multiple application soils was lower that the controls after two days incubation, the rate of increase in the number of positives in the subsequent 11 days incubation was similar to that detected in the control soil. The number and rate of increase in the number of sole carbon sources utilised in soil exposed to single dose of DCB showed no significant difference. However, after 13 days incubation soil exposed to a single dose of DCB were significantly more metabolic diverse (76 sole carbon sources) compared to the control (67 carbon sources).

The metabolic potential data was also analysed using cluster analysis. This confirmed that the carbon utilisation pattern observed for both the single dose and control soils were very similar, since samples from both conditions clustered closely (figure 16). However, samples exposed to multiple exposures of DCB clustered away from the other two treatments confirming that this treatment had greatest impact on the metabolic potential of the soil microbial community.

FIGURE 15. Changes in the substrate utilization patterns during prolonged incubation on BIOLOG indicator plates of sampled microbial communities taken from soils treated with single or multiple doses of 1,2 DCB.



Changes in community profiles were determined over a 13 day incubation period for extracted samples applied to BIOLOG-GN microtitre plates. 1,2-DCB was added as a single dose of 100 μg /g soil at time 0, or as 10 doses of 10 μg /g soil every 14 days. Microcosms were sampled for analysis at the end of the experiment, t= 140 days.

Figure 16 **Dendrogram following cluster analysis of total community metabolic profiles for soils treated with single or multiple doses of DCB.**

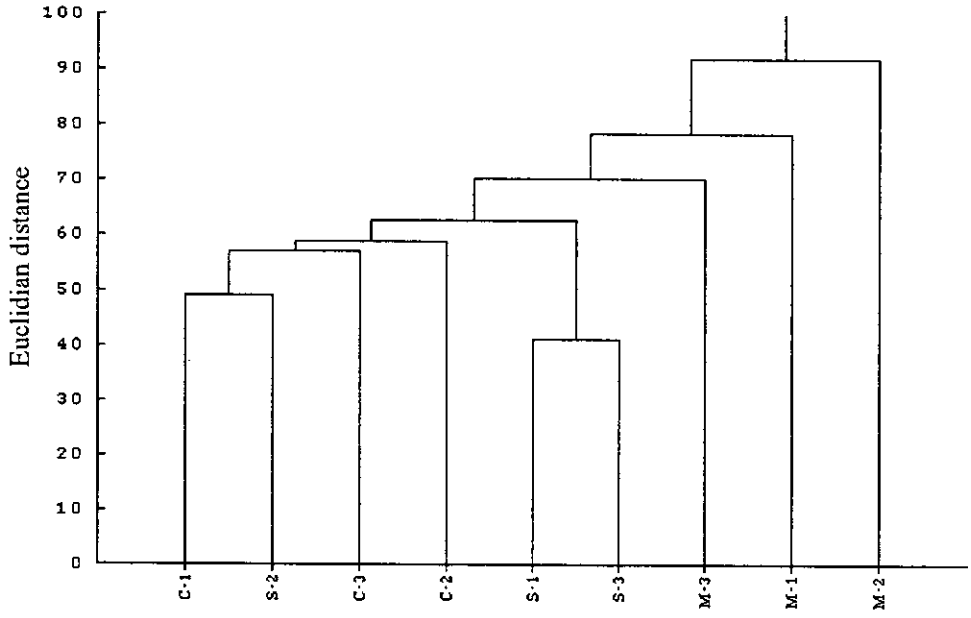


Fig. 16: Dendrogram indicting the relationship between metabolic profiles of soil microbial communities exposed to single dose (100μg/g) and ten doses (10μg/g) of 1,2-DCB. The relationship between samples is measured in Euclids and clustered by UPGMA. Key: C=control, S=single dose, M=multiple dose, numbers indicate replicate number.

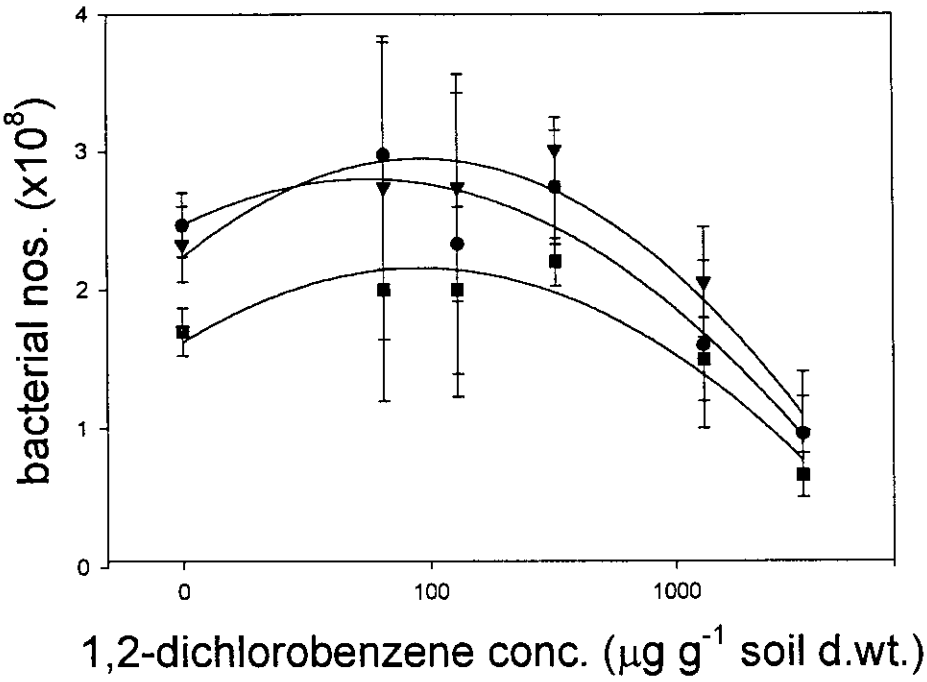
Experiment 4

8.5 Response of 1,2-dichlorobenzene concentration on the size and diversity of the soil microbial biomass

Standard microcosms as described above were established and incubated for 7 day prior to experimentation. Soil was dosed with 6 concentrations (0, 65, 130, 325, 1300 and 3250 μg g^{-1} soil d.wt.) of neat 1,2 dichlorobenzene (purchased from Sigma chemicals, purity > 99%). All microcosms were then thoroughly mixed and left to incubate before live, total bacterial numbers and fungal hyphal lengths were determined by vital staining and epiflourescence microscopy at 7, 14 and 49d. A final, end point determination of plate count numbers and bacterial diversity was determined at 56 d.

8.5.1 *Impact of DCB on total bacterial count.* Dose response for bacteria in soil exposed to DCB showed, in this case, that the bacteria were relatively insensitive to DCB concentrations below 1000 μg g^{-1}. This is in contrast to other experiments were concentrations as low as 10 μg g^{-1} caused significant inhibition of vital bacterial numbers (figure 2 and 11), although 10 μg g^{-1} had no effect in an other experiment (figure 5). In the experiment reported in figure 5, bacterial numbers were consistent over time and the same is true of this present experiment (figure 17). In the experiment reported in figure 11, the bacterial population was increasing rapidly with time. This may suggest that DCB is more toxic when bacterial biomass is increasing compared to when the population is in equilibrium or static. Increasing DCB concentration did have a significant effect on bacterial numbers ($P<0.001$), as did time ($P=0.002$).

FIGURE 17. Temporal impact of increasing DCB concentrations on bacterial biomass in soils



The impact after increasing concentrations of 1,2-dichlorobenzene on live bacterial numbers was determined after 7 (circles), 14 (triangles) and 49 (squares) days of exposure to 1,2DCB. Bacterial numbers were determined by the Baclight assay. n=3, sem.

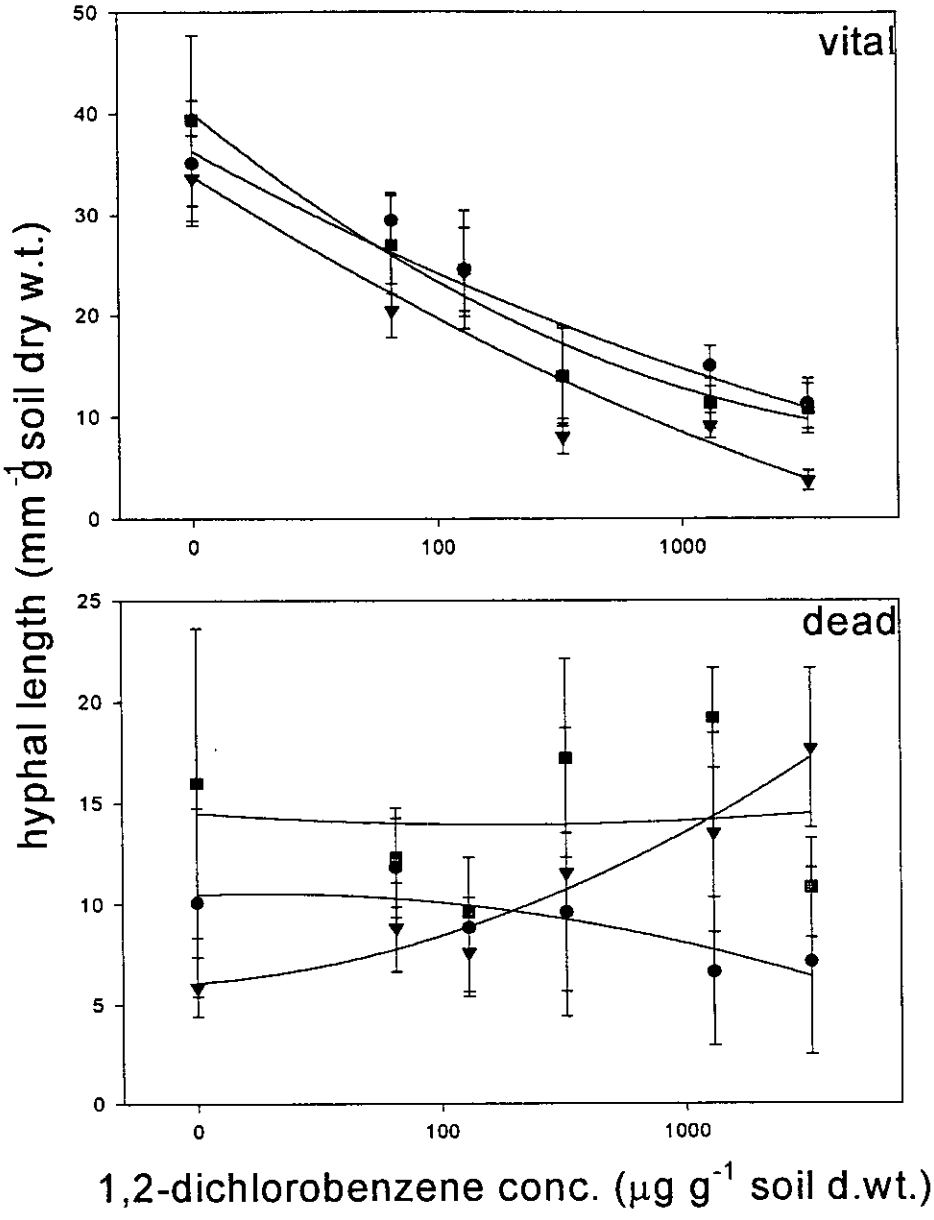
Table 11. Temporal impact of increasing DCB concentrations on bacterial biomass in soils

Analysis of Covariance, response of vital bacterial numbers, over time, (figure 17). Probabilities of treatments not being significant are quoted.

treatment	P value
time	0.002
1,2 dichlorobenzene	0.000
time * 1,2-dichlorobenzene	0.346

8.5.2 *Impact of DCB on fungal biomass.* Fungi were much more sensitive to 1,2-DCB than bacteria in this experiment, with hyphal lengths declining rapidly with increasing DCB concentration (figure 18). There was no great change in hyphal length with time (P=0.48). 1,2-DCB concentration had a very strong effect on vital hyphal lengths (P<0.001) and there was no significant interaction between time and 1,2-DCB. The dead (non-vital) fungal hyphal length did vary with time and exposure, though no clear pattern emerged and the time and time*DCB factors in the analysis of variance were only bordering on significance.

Figure 18 **Temporal impact of increasing DCB concentrations on fungal biomass in soils**



The impact after increasing concentrations of 1,2-dichlorobenzene on live and dead fungal hyphal lengths was determined after 7 (circles), 14 (triangles) and 49 (squares) days of exposure to 1,2DCB. n=3, sem.

Table 12 **Analysis of Covariance, response of fungal hyphal length, over time (figure 18).** Probabilities of treatments not being significant are quoted.

treatment	**vital** P value	**dead** P value
time	0.485	0.067
1,2 dichlorobenzene	0.000	0.542
time * 1,2-dichlorobenzene	0.914	0.057

9. DISCUSSION AND CONCLUSIONS

The experiments reported from the collaborative study undertaken as a part of the novel initiative for integrated research between member institutes within the Centre for Ecology and Hydrology, propose to utilise such a model system to investigate soil microbial biomass response to a pollutant stress. Investigations have followed the fate and mineralisation of introduced 1,2-DCB in soils and soils amended with root material. The response of the indigenous microbiota has been evaluated by direct measures of biomass, cellular activity and metabolic profiles. Extractive assessments of the microbial community have targeted those populations that can be isolated on and form colonies on well defined bacteriological agars. Specific component of the isolated community have been identified and genetically characterised to gain an over all indication of response and perturbation that resulted from the application of 1,2-DCB. As a target population of considerable ecological and economic importance the fluorescent Pseudomonads have been targeted as reliable indicator group for soil health. The fluorescent Pseudomonads, common soil and rhizosphere bacteria, are known to be capable of degrading chlorobenzenes in general, and 1,2-dichlorobenzene in particular. Degrading strains have been isolated from 1,2 dichlorobenzene contaminated soil. Conversely, fluorescent pseudomonad strains are known to be sensitive to the presence of 1,2-dichlorobenzene. Fluorescent pseudomonad are typical soil and rhizosphere competent bacteria, their population density and cellular metabolic activity is elevated in soil by the presence of both living and decaying plant roots. Therefore, the fluorescent pseudomonad soil population may be manipulated experimentally by addition of root material. These factors, and the availability of sensitive, selection agars make this family of the rRNA group I, γ-protobacteria both ideal and ecologically relevant candidates for intensive investigations of the microbial response to DCB ingress.

Enumerating bacteria. In bacterial ecology representative, random and reproducible sampling is often problematic. The common discrepancies between total counts and viable counts, typically quoted at less than 1% in soils are a particular case for concern, while the dispersal of bacteria from samples and incubation conditions (medium, nutrient strength, temperature, incubation time and colony density) exert inevitable selection pressures on the bacteria that do grow on isolation plates (Hattori, 1982, 1983, 1988; Kashara & Hattori, 1992; Miller *et al.*, 1990; Lambert *et al.*, 1987). It has often been considered that plating methods should be regarded as isolating an ecologically significant portion or a consistent physiological subset of the bacterial community which can be used to indicate trends in the wider community (Gilbert *et al.*, 1993, Olsen & Bakken, 1987, Kennedy & Smith, 1995). Hattori (1988) and more recently De Leij *et al.* (1993) and Lilley *et al.* (1996) demonstrate that the kinetics of colony formation on plates yields information regarding the physiological state of cells in the environment and facilitates classification into distinct physiological groups. Therefore the microcosm studies addressed these recognised problems by suitably adapting methodologies to define the diversity of isolates, the diversity of whole analysed communities, and by the direct microscopic assessment of microbial biomass and activity using vital stains. The comparative analyses of these data will allow the construction of suitable models that evaluate true process level activity. Further estimates hat target specific genes both for function and molecular diversity will provide the insight required to understand the microbial and biochemical kinetics of the system.

It is considered highly probable that a wide range of soil bacteria and fungi possess the genetic ability to break down, either partially or completely, chlorinated aromatics such as chlorobenzenes, polychlorinated biphenyls and organochlorine pesticides. Degrading isolates may be selected using continuous enrichment culture or by isolation from contaminated soils. However, in experiments to re-introduce degrading isolates to soil to stimulate biodegradation, indigenous isolates appear to be at least as effective at degradation as inoculants. This suggests;
(a) that the genes for degradation are present at low frequency but selected for in the presence of pollution,
(b) the stochastic mutation rate produces isolates that are able to degrade, either by catabolism or by utilizing the pollutant as a C source or
(c) that chlorinated aromatic degrading genes are present at high frequencies in the soil microbial biomass. The latter may be the case if aromatic degrading genes serve another ecological function.

It was the absence of any detailed international literature that assesses the impact of xenobiotics on the diversity or activity of soil micro-organisms that stimulated this project. In contrast to 2,4D and other related organics, 1,2-DCB is highly volatile and therefore difficult to include in the design of culture systems for the selection of DCB utilising strains from the environment. We are currently focusing on this aspect of the project, and will more fully report on our successes in this are within the next twelve months. These selection assays, where we control the availability of the DCB as a substrate are essential for the focused genetic phase of the project. To be able to isolates and characterise the genes associated with DCB utilisation sensitive and reliable assays are required. Once available specific pathways may be cloned, or we may begin the process of determining whether plasmids, or other mobile genetic elements play a significant role in the apparent enrichment of populations that we have already observed in the microcosm experiments so far undertaken. The molecular approaches will draw on ongoing investigations at IVEM and through collaborations with international partners (e.g by drawing on the network of expertise established on an EU-Biotechnology Programme in which we were involved; **EU-Biotechnology**. Bio2 CT92-0491, *Ecological and molecular study of gene mobilizing capacity of soils and related ecosystems*) by adapting an approach used to study 2,4-D (Top *et al.*, 1996; Top *et al.*, 1995). These considerations in natural environments are particularly relevant to the study of DCB impact on soils and simulated rhizosphere soils described here. By understanding the selective process and by identifying the genetic pathways responsible we will be able to combine chemical and microbial process activity with directed studies of the genetic variation associated with the divergence or convergence in diversity of challenged habitats. Such data may lead to the development of assays for predicting the rate that an introduced xenobiotic may be degraded in the environment. Such evaluations are of immense importance in sustaining and detoxifying the natural environment.

Conclusions

The study has allowed the development and testing of a suitable model systems for assessing microbial activity in soils challenged with DCB contamination. From the studies made the following general conclusions have been drawn which assist in the design of future investigations.

- Microcosms were highly reproducible and phenotypic methods used to detect community change were sensitive enough to assess response to DCB impact.
- DCB can potentially have toxic impact on some soil microbial communities, but may also act as a potential carbon source for others.
- Soil has enormous buffering capacity, high doses of DCB are required before impact is detected.
- The presence of root material masks impact of DCB on soil microbial communities.
- Application regimes significantly influenced impact on microbial communities.
- Multiple dose application of DCB had a greater impact, influencing taxa composition.
- Pseudomonads were particularly detrimentally influenced by multiple doses of DCB, allowing Bacilli to proliferate.
- Multiple dose increased physiological stress as reflected in slow rate colonies developed on plates
- FAME analysis of Pseudomonads isolated from soil with root tissues and bulk soil with and without DCB additions failed to distinguish treatments.
- Increase in rate of DCB degradation probably due to increased activity of all community rather than change in composition of community.
- Greater deleterious impact of multiple application compared to the single dose was probably due to increased bioavailability in multiple dose. In single dose DCB absorbed on to soil ion exchange sites and made unavailable.
- Single dose increased metabolic potential of community but had detrimental impact on *Arthrobacter* and *Micrococcus*.
- Fungal biomass appears to be more sensitive to DCB than the bacterial biomass.
- Bacterial response to DCB depends on whether the biomass is static or expanding with time.
- DCB is mineralized more rapidly in the presence of decaying roots.
- Mineralization is highly dependent on form of exposure

10. CURRENT AND FUTURE OBJECTIVES

- Assess impact of DCB in soils planted with a growing plant, fully functional rhizopshere. (both)
- Develop DCB based isolation media for the selection and isolation of DCB utilizing strains. (both)
- Isolate genes and assay for the presence of horizontal gene transfer for DCB utilization. (ivem)
- Assess genetic (molecular-genetic diversity) response of communities to DCB. (ivem)
- Establish field and microcosm studies to test community response to DCB addition. (ite)

11. OUTPUT

refereed publications:

Meharg A.A.[1*], Wyatt C.L.[1], Thompson I.P.[2], Bailey M.J.[2] & Maguire N.[1] Microbial factors regulating the degradation of 1,2-dichlorobenzene in soil. In Preparation.

Thompson I.P.[1*], Meharg A.A.[2], , Bailey M.J.[1] & Maguire N.[2] Soil microbial biomass dynamics in response to repeated and single additions of 1,2-dichlorobenzene to soil. In Preparation.

12. REFERENCES

Bailey, M.J. & Thompson, I.P. (1992). Detection systems for phylloplane Pseudomonads. In: Wellington, E.M. & van Elsas J.D. (eds.). *Genetic interactions between micro-organisms in the microenvironment*. pp 126-141. Pergamon Press.

Bailey, M.J., Lilley, A.K., Thompson, I.P., Rainey, P.B. & Ellis, R.J. (1995). Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet; stability and potential for marker gene transfer. Molecular ecology, 4. 755- 764.

Bridge, P.D. (1993) Classification. In: Biological Data Analysis. A Practical Approach. (Fry, J.C. Ed.), pp. 219-242. Oxford University Press, New York, USA.

Cavigelli, M.A., Robertson, G.P. & Klug, M.J. (1995). Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. Plant & Soil 170, 99-113.

Crowley, D.E, Brennerova M.V., Irwin, C., Brenner V. & Focht D.D (1996) Rhizosphere effects on biodegradation of 2,5-dichlorobenzoate by a bioluminescent strain of root-colonizing *Pseudomonas fluorescens*. FEMS Microbiology Ecology, 20 79-89.

de Bont, J.A.M., Vorage, J.A.W, Hartmans, S. (1986). Microbial degradation of 1,3-dichlorobenzene. Applied & Environmental Microbiology 52, 677-680.

De Leij, F.A.A.M., Whipps, J.M. and Lynch, J.M. (1993) The use of colony development for the characterization of bacterial communities in soil and on roots. Microb. Ecol. 27, 81-97.

Ellis, R.J., Thompson, I.P. & Bailey, M.J. (1995). Metabolic profiling as a means of characterising plant-associated microbial communities. FEMS Microbiology Ecology 16, 9-18.

Gilbert, G.S., Parke, J.L., Clayton, M.K. and Handelsman, J. (1993) Effects of an introduced bacterium on bacterial communities on roots. Ecology 74, 840-854.

Haigler, B.E, Nishino, S.F. & Spain J.C. (1988) Degradation of 1,2-Dichlororbenzene by a *Pseudomonas* sp. Applied & Environmental Microbiology 54, 294-301.

Hattori, T. (1982) Analysis of plate-count data of bacteria in natural environments. J. Gen. Appl. Microbiol. 28, 13-22.

Hattori, T. (1983) Further Analysis of Plate-Count Data of Bacteria. J. Gen. Appl. Microbiol. 29, 9-16.

Hattori, T. (1988) The viable count: quantitative and environmental aspects. Science Tech/Springer-Verlag. Madison.

Housiaux, P.J., D.F. Hill, and G.B. Petersen. 1988. Nucleotide sequence of a gene for 5S ribosomal RNA from *Pseudomonas aeruginosa*. Nucleic Acids Res. **16**:2722.).

Jacques, M-A. and Morris, C.E. (1995) A review of the issues related to the quantification of bacteria from the phyllosphere. FEMS Microbiol. Ecol. 18, 1-14.

Kasahara, Y. and Hattori, T. (1991) Analysis of bacterial- populations in a grassland soil according to rates of development on solid media. FEMS Microbiol. Ecol. 86, 95-101.

Kennedy A.C. and Smith K.L. (1995) Soil microbial diversity and the sustainability of agricultural soils. Plant and Soil 170, 75-86.

Lambert, B., Gossele, F., Leyns, F., Papon, Y., Swings, J. and van Rooyen, L. (1987) Rhizobacteria of maize and their antifungal activities. Appl. Environ. Microbiol. 53, 1866- 1871.

Lilley, A.K., Fry, J.C., Bailey, M.J. & Day, M.J. (1996). Comparison of aerobic heterotrophic taxa isolated from four root domains of mature sugar beet (*Beta vulgaris*). FEMS Microbiology Ecology. In Press

McInroy, J.A. and Kloepper, J.W. (1994) Studies on indigenous endophytic bacteria of sweet corn and cotton. In: Molecular Ecology of Rhizosphere Microorganisms (O'Gara, F., Dowling, D.N. and Boesten, B. Eds.), pp.19-28. VCH Publishers, New York.

Miller L. & Berger T. (1985) Bacteria identification by gas chromatography of whole cell fatty acids. Hewlett Packard Application Note pp. 228-241, Hewlett-Packard, Avondale, PA.

Miller, H. J., Liljeroth, E., Henken, G. and van Veen, J. A. (1990) Fluctuations in the fluorescent pseudomonad and actinomycete populations of rhizosphere and rhizoplane during the growth of spring wheat. Can. J. Microbiol. 36, 254-258.

National Toxicity Program, 1987. National Toxicity Program technical report on the toxicology and carcinogenesis studies of 1,4-dichlorobenzene (CAS No. 106-46-7) in F344/n rats and B6C3F mice (gavage studies). National Institutes of Health publication No. 86-2575. department of Health and Human Services, Triangle Park, N.C.

Olsen, R.A. and Bakken, L.R. (1987) Viability of soil bacteria - optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol. 13, 59-74.

Pearson C.R. (1982) Halogenated aromatics. p. 89-116. In O. Hutzinger (ed.). The Handbook of environmental chemistry, anthropogenic compounds, vol 3, part B. Springer-Verlag, New York.

Reineke, W. & Knackmuss, H.-J. (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene degrading bacterium. Applied & Environmental Microbiology 47, 395-402.

Sandman, E.R. & Loos, M.A. (1984) Enumeration of 2, 4-D degrading microorganisms in soils and crop plant rhizosphere using indicator media: high populations associated with sugar cane (*Saccharum officinarum*). Chemosphere 13, 1073.

Spain, J.C. & Nishino, S.F. (1987) Degradation of 1,4-dichlororbenzene by a *Pseudomonas* sp. Applied & Environmental Microbiology 53, 1010-1019.

Speiss E., Sommer C. & Gorisch H. (1995) Degradation of 1,4-Dichlorobenzene by *Xanthomonas flavus* 14p1. Applied & Environmental Microbiology, 61 3884-3888.

Thompson, I.P., Bailey, M.J., Ellis, R.J. and Purdy, K.J. (1993) Subgrouping of bacterial populations by cellular fatty acid composition. FEMS Microbiol. Ecol. 102:75-84.

Thompson, I.P., Bailey, M.J., Fenlon, J.S., Fermor, T.R., Lilley, A.K., Lynch, J.M., McCormack, P.J., McQuilken, M.P., Purdy, K.J., Rainey, P.B. and Whipps, J.M. (1993) Quantitative and qualitative seasonal-changes in the microbial community from the phyllosphere of sugar-beet (*Beta-vulgaris*). Plant and Soil 150, 177-191.

Top, E.M., Maltseva, O.V. & Forney, L.J. (1996). Capture of a catabolic plasmid that encodes only 2,4-dichlorophenoxyacetic acid: -ketoglutaric acid dioxygenase (TfdA) by genetic complementation. Applied and Environmental Microbiology 62: 2470-2476

Top, E.M., Holben, W.E. & Forney, L.J. (1995). Characterisation of diverse 2,4-dichlorophenoxyacetic acid degradative plasmids isolated from soil by complementation. Applied and Environmental Microbiology 61: 1691-1698.

US Environmental Protection Agency (1980) Ambient water quality criteria for chlorinated benzenes. EPA 400/5-8-028. Office of Water Regulation and Standards. Washington, D.C.

Walton, B.T. & Anderson, T.A (1990) Microbial degradation of trichloroethylene in the rhizosphere: potential application to biological remediation of wastes sites. Applied & Environmental Microbiology, 56 1012-1016.

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