Effect of propargyl bromide and 1,3-dichloropropene on microbial communities in an organically amended soil

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Abstract

In this study we investigated the response of microbial communities in unamended and manure-amended soil treated with the fumigants propargyl bromide (PBr) and 1,3-dichloropropene (1,3-D). The soil fumigants were applied at a rate of 10, 100, and 500 mg kg^{-1}. After treatment of the soils, the metabolic activity was assessed by monitoring the dehydrogenase activity (DHA). PBr and 1,3-D initially inhibited the DHA when applied at 500 mg kg^{-1}; however, after 8 weeks, recovery of the DHA only occurred in amended soil. Bacterial community level changes were monitored over a 12-week period after fumigation using denaturing gradient gel electrophoresis of polymerase chain reaction-amplified 16S rDNA fragments. Band numbers were drastically reduced upon application of the fumigants, but reestablished more rapidly in the amended soil. To determine changes in the community diversity, the Shannon–Weaver index of diversity, *H*, was calculated for all treatments. In unamended and amended soil, the community diversity decreased with increasing fumigant concentration. In the PBr-treated soils, the diversity was higher in amended soil at all concentrations throughout the study, while in the 1,3-D treatments, the results were mixed. At 1, 4, 8, and 12 weeks after fumigation, major bands were excised from the gels and the DNA was cloned for sequence analysis. The bacterial communities in the fumigated amended soils were dominated by *Streptomyces* spp., other genera of actinomycetales, including *Frankia*, *Cytophagales*, *Actinomadura*, and *Geodermatophilus*, and a number of unidentified bacteria. Our results suggest that it may be feasible to reduce the impact of fumigant pesticides on soil microbial populations by stimulating microbial community structure, diversity and activity through the addition of organic amendments.
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1. Introduction

Soil fumigants are extensively used to control plant-parasitic nematodes, weeds, fungi, and insects. Ideally, a pesticide should be toxic only to the target organisms; however, fumigants are a class of pesticide with broad biocidal activity and affect many non-target soil organisms. Soil microorganisms, in particular, play critical roles in sustaining the health of natural and agricultural soil systems. Their importance in nutrient cycling, especially that of N, P, and S, is essential for proper plant nutrition and, thus,

maintaining agricultural productivity [8]. Unfortunately, the impact of soil fumigation practices on soil microbial communities and their recovery following fumigation is largely unknown and has been ignored until recently [23], mainly due to the lack of appropriate methods to describe community structure [13]. Changes in the microbial community composition, for example, as a result of pesticide applications, may lead to changes that interfere with the functional diversity and, ultimately, the overall soil quality. Because of the potential relationships between microbial diversity and ecosystem sustainability, soil microorganisms are being increasingly recognized as sensitive indicators of soil health [45]. Ultimately, the linking of information between microbial community structure/diversity and crop production will likely be an important step in being able to predict soil fertility.

With the scheduled phase-out of methyl bromide (MeBr) nearing in 2005 in the United States, the use of

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a number of fumigants can be expected to increase. One such fumigant is 1,3-dichloropropene (1,3-D). Commercial formulations contain approximately equal ratios of (*Z*)-1,3-D (*cis*-isomer) and (*E*)-1,3-D (*trans*-isomer), and 1,3-D is often formulated with chloropicrin. 1,3-D is considered to be an important alternative pesticide to MeBr. Another potential alternative to MeBr is propargyl bromide (PBr, 3-bromopropyne). PBr was developed in the 1960s by the Dow Chemical Co. [15] and was used in the soil fumigant Triazone, a combination of MeBr, PBr, and chloropicrin. However, PBr is not currently registered as a pesticide. Currently, there is interest in registering PBr as a fumigant pesticide (Albemarle Corp., Baton Rouge, LA, USA, personal communication). Since little ecotoxicological information exists with respect to these and many other fumigant pesticides, it is essential that pesticide effects on bacterial diversity be examined. It should also be of interest to develop methods that minimize the long-term impacts that fumigants have on soil microbial communities.

The benefits of ameliorating soil with organic amendments are well documented. They have been shown to improve soil fertility, physicochemical conditions, and crop growth [22,34]. Increases in the soil organic matter content, gained from using organic amendments, are considered essential for maintaining long-term land use and sustainable farming systems. Organic amendments have also been found effective in reducing potentially harmful fumigant emissions [18] and controlling soilborne pathogens by stimulating antagonistic organisms [1] or by producing toxic volatile compounds [16]. Applications of organic amendments have also been shown to increase the soil microbial biomass and stimulate microbial activity [5,36,37].

Considering the many benefits of using organic amendments, especially with respect to biostimulation, the objective of this study was to evaluate the impacts of PBr and 1,3-D on microbial communities and activities in manure-amended soil. We hypothesized that the restoration of the microbial community and/or activities would occur more rapidly in soil amended with composted steer manure (CSM) after fumigation than in unamended soil. Since there is also interest in registering PBr as a fumigant, and limited information is available on its toxic effects, another interest was to compare the effects of PBr with 1,3-D, a more widely used and studied fumigant. The effect of CSM upon the rate of fumigant degradation is also addressed.

2. Materials and methods

2.1. Chemicals

1,3-D (97% pure; 48% [*Z*] and 49% [*E*]), iodonitrotetra-zolium violet (INT; 2-[4-iodephenyl]-3-[4-nitrophenyl]-5-

phenyltetrazolium chloride), and iodonitrotetrazolium formazan (INTF) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The PBr (97% pure) was obtained from Fluka (Ronkonkoma, NY, USA). Barium chloride, sodium hydroxide, and phenolphthalein (1% [w/v] in 95% [v/v] alcohol) were purchased from VWR Scientific Products (West Chester, PA, USA).

2.2. Soil and organic amendment

The Arlington sandy loam soil (coarse-loamy, mixed, thermic, Haplic Durixeralf) was obtained from the University of California, Riverside, Agricultural Experiment Station. Soil was removed from the top 30 cm with a steel shovel, passed through a 2-mm stainless sieve and then stored at 5°C for no more than 1 week prior to its use. The Arlington soil has a pH of 7.2, maximum water holding capacity (WHC_{max}) of 0.2 kg kg^{-1}, and contains 0.92% organic matter. The CSM (Hyponex Corp., Marysville, OH, USA) was purchased from The Home Depot. The CSM had a moisture content of 39% (w/w, dry wt. basis), pH of 8.8, and an organic C content of 14%.

2.3. Degradation experiment

To monitor the rate of PBr and 1,3-D degradation, 10 g (dry wt.) quantities of unamended and CSM-amended soil were weighed into 21-ml glass headspace vials. Amended soils were prepared by thoroughly mixing soil and CSM (3%, dry wt. basis) in plastic bags. The fumigants were applied as an aqueous solution at a rate of 10, 100, and 500 mg kg^{-1}. The fumigants were applied as an aqueous solution since drip application is the typical method used to distribute the 1,3-D-containing soil fumigant, Telone (Dow AgroSciences), and this would also be the method of delivery for PBr. The treated vials were immediately capped with Teflon-faced butyl rubber septa (Supelco, Bellefonte, PA, USA) and aluminum seals, and incubated at 20°C. The final moisture content of the soil was 50% of the WHC_{max}. Triplicate samples were then removed at specific time intervals and frozen at −20°C until analyzed.

To extract the fumigant residues from the soil samples, the vials were uncapped while still frozen, and then 10 ml of ethyl acetate and 10 g of anhydrous sodium sulfate were added to each vial, followed by immediate recapping. Once the soils thawed, the vials were shaken for 1 h on a horizontal shaker (200 oscillations min^{-1}) and then allowed to stand until all suspended particulate matter settled. After settling, a 1 ml aliquot of the soil extract was transferred to a GC vial. The soil extract was analyzed for PBr and 1,3-D on a Hewlett-Packard 5890 gas chromatograph, equipped with a RTX-624 capillary column (30 m, 0.25 mm × 1.4 μm, Restek Corp., Bellefonte, PA, USA, USA) and connected to a micro-electron capture detector. The operating conditions were as follows: carrier gas, He, 1.2 ml min^{-1}; inlet temperature, 230°C; detector temper-

ature, 280°C; column temperature, isothermal at 110°C for 7 min.

2.4. Substrate-induced respiration (SIR) experiment

The soil respiration rate, based on CO_2 production, was measured using modified methods of Alef [2] and Zibilske [50]. Soil (150 g, dry wt.) was placed into 500-ml Erlenmeyer flasks and treated with CSM and fumigants as described above. After the addition of the fumigants, each flask was sealed with 324A aluminum tape (Nashua, Watervliet, NY, USA) and allowed to stand for 24 h at room temperature. Next, 1.5 g glucose, 30 mg K_2HPO_4, and 225 mg NH_4Cl was added to each flask and thoroughly mixed into the soil. The flasks were then sealed with two-hole rubber stoppers fitted with Teflon tubing (i.d. 1.07 mm, o.d. 1.88 mm) and sparged with CO_2-free air at a rate of 50 cm^3 min^{-1} (approximately six flask evacuations per hour). The flask effluent air was sparged through 25 ml of 1 M NaOH for an additional 24 h to trap the evolved CO_2. The CO_2 mass of each trap was measured by adding excess barium chloride (1.5 M) and titrating with 1 M HCl. A few drops of phenolphthalein were added as an indicator. Duplicate CO_2 measurements were made for each soil treatment and the data are expressed as mg CO_2 g^{-1} dry soil per 24 h. To determine significant differences between the treatments, statistical analyses were performed using SAS/STAT software [40].

2.5. Mesocosm experiment

Arlington soil (0.5 kg, dry wt.) was added to glass Mason jars with an approximate volume of 1 l. The experiment consisted of a total of 12 treatments. PBr and 1,3-D were applied to both unamended and CSM-amended soil as described above. Non-fumigated unamended and amended soil served as untreated controls. After application of the fumigants, each of the Mason jars was tightly sealed for 24 h, wrapped in aluminum foil to prevent light penetration, and incubated at room temperature (18–22°C). After 24 h, the Mason jars were loosely capped to prevent anaerobic conditions and allow the fumigants to naturally diffuse out of the jars. The soil moisture content was maintained by the periodic addition of sterile distilled water.

2.6. Dehydrogenase assay

The dehydrogenase activity (DHA) was determined using a modified method of von Mersi and Schinner [47], where INT is reduced to INTF. The DHA assay was conducted at 1, 2, 3, 4, 6, 8, and 12 weeks after the fumigants were applied. Prior to soil sampling, the soil in each Mason jar was thoroughly mixed by vigorously inverting the jar several times. Three replicate samples were randomly collected from each Mason jar. Soil (0.5 g, dry wt.) was

placed in 21-ml glass vials and mixed with 0.75 ml of Tris buffer (1 M, pH 7) and 1 ml of INT solution (5 g l^{-1}). The vials were sealed with Teflon-lined septa and aluminum seals, and incubated for 2 h at 40°C in the dark with vigorous shaking every 20 min. After the 2-h incubation period, the solution was centrifuged ($8000 \times g$ for 2 min) and the absorbance of the supernatant was measured at 464 nm. The DHA is expressed as µg INTF g^{-1} dry wt. per 2 h.

2.7. DNA extraction from soil

After the soil was mixed in each of the Mason jars as described above, three random soil samples (about 3 g each) were aseptically removed using an ethanol disinfected spatula. The soil samples were placed into a sterile glass vial and frozen at −74°C until the DNA was extracted. Soil samples were collected 1, 4, 8, and 12 weeks after fumigant application. The total soil bacterial DNA was extracted using an UltraClean soil DNA isolation kit (MO BIO Laboratories, Solana Beach, CA, USA). The DNA extraction procedure was conducted according to the manufacturer's protocol except as follows: 0.5 g of soil was placed into a 2-ml Bead Solution tube and shaken for three 20 s intervals (speed setting of 4) using a Fast-Prep FP120 Instrument (Qbiogene, Carlsbad, CA, USA). The isolated DNA was then used as a template for polymerase chain reaction (PCR). The triplicate soil samples from each treatment were later pooled together for DNA extraction after denaturing gradient gel electrophoresis (DGGE) analysis of PCR amplification products revealed that the banding patterns between the replicates were alike.

2.8. PCR

PCR of the DNA templates was performed using the 63f (5′-CAGGCCTAACACAT GCAAGTC-3′) and 518r (3′-ATTACCGCGGCTGCTGG-5′) primers, which are located in the V3 region of the 16S rRNA genes of eubacteria [28,31]. A 40-nucleotide GC-clamp was attached to the 5′ end of 63f primer [31]. The PCR mixtures contained 10–100 ng of template DNA, 20 pmol of each primer, 2.5 µl of the thermophilic DNA polymerase $10 \times$ reaction buffer (500 mM KCl, 100 mM Tris–HCl (pH 9 at 25°C), 1% Triton X-100), 0.625 U of Taq DNA polymerase (Promega, Madison, WI, USA), 5 nmol of each deoxyribonucleoside triphosphate, 1.5 µl of 15 mM $MgCl_2$, and sterile distilled water to a final volume of 25 µl. The PCR temperature conditions were as follows: 92°C for 2 min, followed by 30 cycles of 92°C for 1 min, 55°C for 30 s, and 72°C for 1 min, and a final primer extension at 72°C for 6 min. The PCR products were visualized on 1% (w/v) agarose gels by UV transillumination with ethidium bromide staining. The PCR fragment was approximately 500 bp in length.

2.9. DGGE

DGGE was performed using the DCode Universal Mutation System (Bio-Rad, Hercules, CA). The PCR amplification products (25 µl) were loaded onto 8% (w/v) polyacrylamide gels which were prepared with a linear gradient ranging from 30 to 70% denaturant (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). Gels were electrophoresed in $1\times$ TAE buffer (Bio-Rad) for 3 h at 60°C and 200 V. After electrophoresis, the gels were stained for 15 min in distilled water containing 0.5 µg ethidium bromide ml^{-1}, and then photographed on a UV transilluminator with a Polaroid camera.

2.10. Statistical analysis of DGGE bands

Photographs of the DGGE gels were digitized using ImageMaster Labscan (Amersham-Pharmacia Biotech, Uppsala, Sweden). The gel lanes were then aligned using ImageMaster 1D Elite 3.01 (Amersham-Pharmacia Biotech), normalized to contain the same amount of total signal after background subtraction, and analyzed to give a densitometric curve. Band positions were converted to R_f values between 0 and 1, and profile similarity was calculated by determining Dice's coefficient for the total number of lane patterns. Dendrograms were constructed using the unweighted pair group method with mathematical averages. The determination of structural diversity according to the Shannon–Weaver index, H ($H = -\Sigma P_i \log P_i$, where P_i is the ratio of peak height to sum of all peak heights), was calculated on the basis of the number of DGGE bands and their relative intensities as described by Eichner et al. [12].

2.11. Cloning and sequencing of DGGE bands

To identify the dominant bacterial species, major bands were excised from the polyacrylamide gels, each with a new razor blade, placed in 20 µl of sterile distilled water, and incubated overnight at 4°C. 10 µl of the eluate was then used as template DNA in a PCR with the primers and reaction conditions described above. After PCR, the products were visualized for size on 1% agarose gels and purified using a Qiagen PCR purification kit (Valencia, CA, USA). The PCR products were then cloned into a pGEM-T Easy Vector (Promega), and the ligated plasmids were transformed into competent *Escherichia coli* JM109. Selection of transformants containing recombinant plasmids was achieved using blue/white screening. Isolated white colonies were then grown overnight at 37°C in Luria–Bertani medium containing 0.5 mg ampicillin l^{-1}. Plasmids from *E. coli* were isolated using a Qiagen plasmid purification kit and sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequence identification was performed using the BLAST database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov).

3. Results and discussion

3.1. PBr and 1,3-D degradation experiments

Fig. 1 shows the disappearance of PBr and 1,3-D in unamended and CSM-amended soil at concentrations of 10, 100, and 500 mg kg^{-1}. The degradation reactions for both fumigants are well described by the first-order ($r \geq 0.95$) kinetic model (Table 1). For 1,3-D, the concentrations used fall near those used in actual field applications (i.e. 6.3–313% of the recommended dosage of 160 mg kg^{-1}). No recommended dosages have been established for PBr; therefore, an equivalent dosage was used for comparative purposes. Compared to the unamended soil controls, the degradation of PBr and 1,3-D was enhanced by the addition of 3% CSM. In PBr-treated soil at concentrations of 10, 100, and 500 mg kg^{-1}, the degradation rate was 3.9, 6.0, and 3.5 times higher in amended soil, respectively, than in unamended soil. In amended soil treated with 1,3-D at the same concentrations, the degradation rate was 2.3, 1.4, and 1.4 times higher, respectively, than in unamended soil. Application of organic amendments to soil has been shown to significantly increase the biological and chemical degradation rate of 1,3-D [9,17] and other pesticides [11,18,30,41]. In a previous study conducted with Arlington soil, the biological degradation of 1,3-D in unamended soil accounted for 9% of the total degradation, but with the addition of as little as 5% CSM, 43% of the 1,3-D was biologically degraded [9]. With respect to PBr, the degradation reaction is primarily abiotic, and is postulated to occur via a nucleophilic substitution reaction with soil organic matter, resulting in the methylation of soil organic matter and formation of bromide and hydrogen ions [35]. Yates and Gan [49] and Ma et al. [25] reported that the degradation of PBr increased with increasing soil organic matter content. The degradation of PBr and 1,3-D was also found to be concentration-dependent; as the fumigant concentration increased, the degradation rate constant decreased (half-life increased). These results are in agreement with previous results, which showed that 1,3-D degradation was dependent upon the initial soil concentration [26]. The slower rates of 1,3-D degradation at the higher concentrations (i.e. 100 and 500 mg kg^{-1}), and the fact that the first-order coefficients are nearly the same in both unamended and amended soil, may indicate that 1,3-D posed inhibitory effects on degrading organisms. However, in the case of PBr, this effect is not nearly as dramatic since degradation of PBr is most likely a result of abiotic reactions. Regardless of the mechanisms of degradation for PBr and 1,3-D, the impact of these fumigants on the microbial communities and their recovery will be dependent upon the rate of degradation, as changes in the
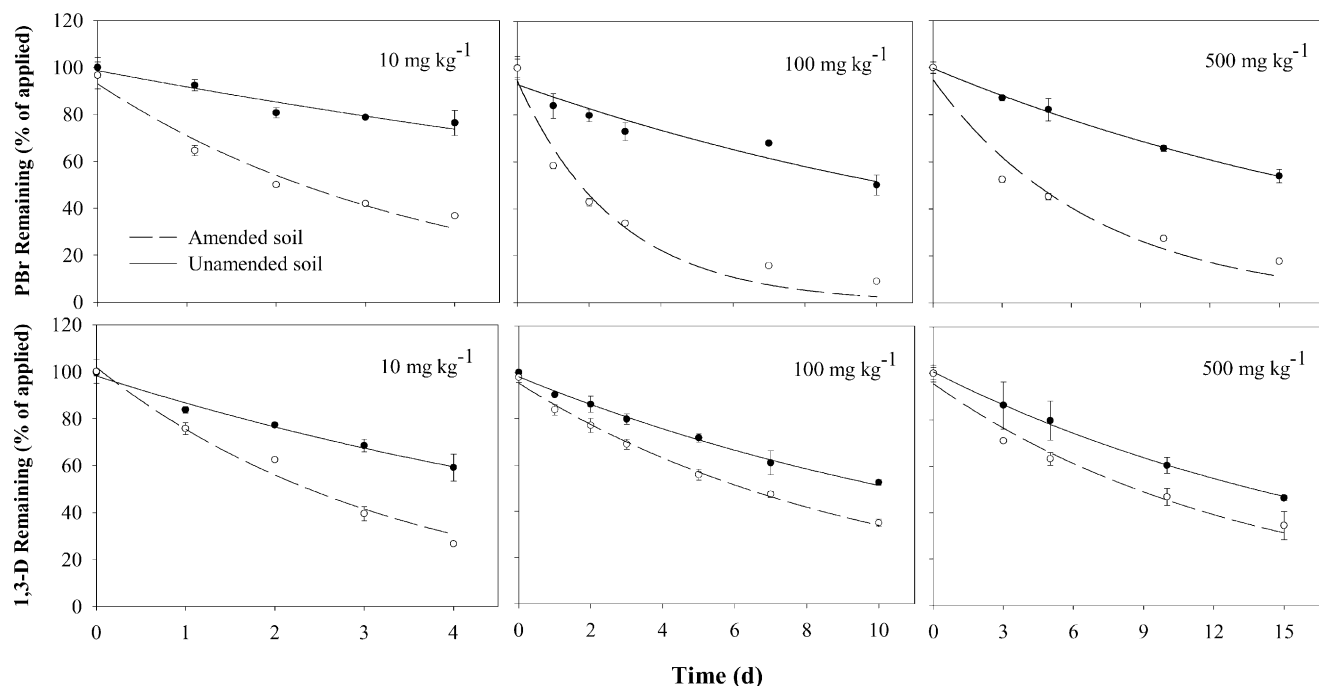
Fig. 1. Degradation of PBr and 1,3-D in unamended and manure-amended Arlington sandy loam. Vertical error bars are standard deviation ($n = 3$).

fumigant concentration will rapidly occur after application in the field.

3.2. SIR

Along with the DHA, SIR was measured to characterize the microbial activity and has been used to estimate the size of the microbial biomass [4]. The use of SIR to assess the impact of pesticides on microbial activity and biomass has been reported [6,19,20,24,43,48]. Results from the SIR experiment, evaluated as CO_2 evolved (mg g^{-1} dry soil per 24 h), are reported in Fig. 2A,B. SIR was markedly inhibited by incremental additions of PBr or 1,3-D to either unamended or CSM-amended soil in the 48 h after fumigation, but across all fumigated soils, SIR was the highest in the PBr-treated soil, except in amended soil at 10 mg kg^{-1}. In unamended soil, at 10 mg kg^{-1} of PBr or 1,3-D,

SIR was reduced to 81 and 51% of the control, respectively, while at 500 mg kg^{-1} (in the case of 1,3-D, this is three times the recommended dose), SIR was reduced to 61 and 22% of the control, respectively. Apparently, an equivalent dose of 1,3-D has a greater impact on the microbial activity in unamended soil in the 48 h immediately after fumigation. In a study where the soil fumigant metam-sodium (which primarily breaks down to methyl isothiocyanate) was used, SIR was strongly affected at concentrations of 320 and 3200 mg kg^{-1} 48 h after fumigation (with 320 mg kg^{-1} representing a recommended agricultural dose), but at a concentration of 3.2 mg kg^{-1} no effect on SIR was observed [27]. Even after 28 days, the effect of metam-sodium fumigation on SIR was similar to the results obtained after 48 h. The addition of organic amendments to soil has been found effective in reducing the impact of pesticides on microbial activity [6]. In alfal-

Table 1
First-order degradation rate constants (k), half-life values ($t_{1/2}$), and correlation coefficients of fitting (r) in Arlington soil treated with PBr or 1,3-D

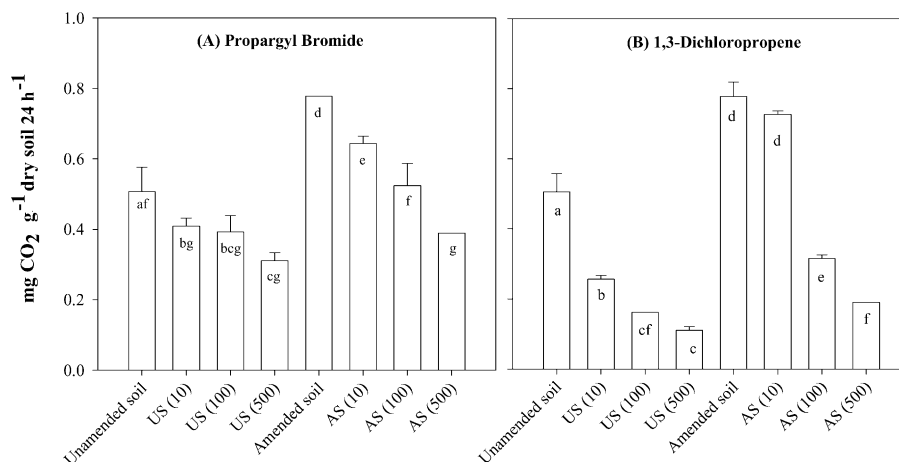| Fumigant	(mg kg^{-1})	Matrix	k (day^{-1})	$t_{1/2}$ (day)	r
PBr	10	Unamended soil	0.07 ± 0.01	9.9	0.96
	100		0.06 ± 0.01	11.6	0.95
	500		0.04 ± 0.00	17.3	1.00
	10	Amended soil	0.27 ± 0.03	2.6	0.98
	100		0.36 ± 0.06	1.9	0.98
	500		0.14 ± 0.02	5.0	0.98
1,3-D	10	Unamended soil	0.13 ± 0.00	5.3	0.99
	100		0.07 ± 0.01	9.9	1.00
	500		0.05 ± 0.00	13.9	1.00
	10	Amended soil	0.30 ± 0.00	2.3	0.99
	100		0.10 ± 0.00	6.9	1.00
	500		0.07 ± 0.01	9.9	0.97

Fig. 2. Effect of (A) PBr and (B) 1,3-D on 24-h SIR in unamended and manure-amended Arlington sandy loam. Vertical error bars represent the error between duplicate samples. Treatments with different letters are significantly different ($P < 0.05$). Values in parentheses are the fumigant application rates in mg kg^{-1}. US, unamended soil; AS, amended soil.

fa-amended soil, SIR was unaffected over a 56-day experimental period when treated with benomyl (a fungicide) at a rate of 125 mg kg^{-1}. In CSM-amended soil at 100 mg kg^{-1} of PBr and 1,3-D, SIR was reduced to 67 and 41% of the control, respectively, while at 500 mg kg^{-1}, SIR was reduced to 50 and 25% of the control, respectively. Overall, these results suggest that the impacts of fumigation on soil respiratory activity are not as severe in PBr-treated soil within the first 48 h after fumigation. However, the benefits of amending soil with CSM were greater in 1,3-D-treated soil, since SIR was 2.2 times higher (1.4 times higher in PBr-treated soil), on average, than in respective fumigated unamended soil.

3.3. Dehydrogenase activity

The addition of organic materials to soil is known to stimulate microbial and enzyme activity as a result of increased metabolism of the readily available nutrients [29,32]. The measurement of DHA is particularly useful in assessing microbial activity since it is linked to the microbial oxidation of organic substances. To determine the long-term impact of PBr and 1,3-D on the activity of the soil microbial community, DHA measurements were utilized. In this study, the incorporation of CSM into non-fumigated Arlington soil significantly increased the DHA over the 12-week incubation period compared to the un-amended soil control (Fig. 3A,B). In unamended and amended soil treated with PBr at 10 mg kg^{-1} or 1,3-D at 10 and 100 mg kg^{-1}, the DHA was similar to the non-fumigated controls. Coincidentally, the higher rate of fumigant degradation in the amended soils at these concentrations corresponds well with the increased DHA in the amended soil treatments, although, in the case of PBr, these results probably have little meaning since PBr is degraded primarily through abiotic mechanisms [35]. At 100 mg kg^{-1}, PBr is apparently more toxic to the micro-

bial biomass than 1,3-D, as indicated by the reduced DHA at this concentration. However, by week 4 in the amended soil only, the DHA had returned to levels observed in the untreated control. In soils treated with 500 mg kg^{-1} of PBr or 1,3-D the DHA was repressed to its lowest level, but by week 8 in the amended soil treatments it had recovered to levels similar to that of the control. In CSM-amended soil treated with 100 mg kg^{-1} of PBr, recovery of the DHA occurred by week 4. In contrast, DHA in un-amended soils to which 500 mg kg^{-1} of PBr (also 100 mg kg^{-1}) or 1,3-D was added did not demonstrate significant recovery in 12 weeks. Suppression of the DHA, in both unamended and amended soils, is presumably a direct result of the adverse effects of PBr and 1,3-D on soil microbial populations. Although there is little or no literature available on the effects of 1,3-D and PBr on soil DHA, the application of numerous fungicides and herbicides has been shown to have an inhibitory effect on DHA [3,42,44].

3.4. PCR-DGGE and analysis of microbial community structure

The V3 region of 16S rDNA was amplified from the community DNA to examine the effect of different concentrations of PBr and 1,3-D in unamended and CSM-amended soil on the microbial community. DGGE analysis of the PCR amplification products from weeks 1, 4, 8, and 12 are shown in Fig. 4A–D, respectively. The numbers of bands detected in the DGGE gel for each of the treatments are given in Table 2. A visual observation of the DGGE gel banding patterns reveals that a shift in the structural composition of the communities occurred during the 12-week study. To analyze the structure of the microbial community, based on the DGGE patterns of the 16S rDNA fragments, we used two different methods: (i) cluster analysis, and (ii) the Shannon–Weaver index of diversity, H. Cluster analysis of the DGGE patterns for
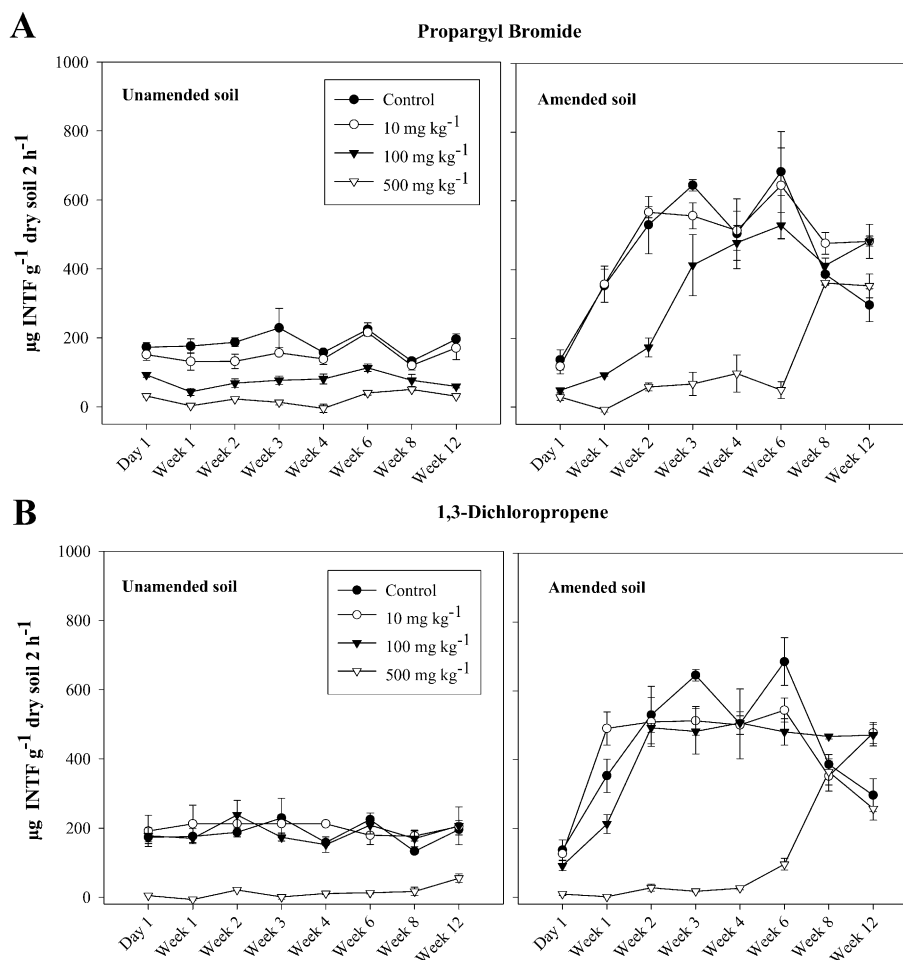
Fig. 3. Effect of (A) PBr and (B) 1,3-D on DHA in unamended and manure-amended Arlington sandy loam over a 12 week period after fumigation. Vertical error bars are standard deviation ($n = 3$).

weeks 1, 4, 8, and 12 are shown in Fig. 5A–D, respectively. The effect of the fumigants on the microbial community structure was most dramatic 1 week after application, as unamended and amended soils treated with PBr or 1,3-D clustered away from the controls (Fig. 5A). No bands were discernable in unamended soil treated with

Table 2
Number of bands detected in the DGGE gel for each of the treatments

Treatment	Week 1	Week 4	Week 8	Week 12
Unamended soil	19	27	32	15
PBr (10[a])	10	6	24	7
PBr (100)	10	1	6	3
PBr (500)	1	1	2	0
1,3-D (10)	6	26	34	23
1,3-D (100)	4	15	5	26
1,3-D (500)	3	11	6	5
Amended soil	18	26	39	37
PBr (10[a])	19	28	36	28
PBr (100)	16	20	35	23
PBr (500)	5	5	30	17
1,3-D (10)	6	24	40	17
1,3-D (100)	5	29	39	21
1,3-D (500)	2	1	23	18

[a]Concentration of fumigant in mg kg^{-1} of dry soil.

100 mg kg^{-1} of PBr and 500 mg kg^{-1} of 1,3-D, and in amended soil at 100 and 500 mg kg^{-1} of 1,3-D (Fig. 4A). By week 4, PBr and 1,3-D treatments began to cluster closer to the control in amended soils and in unamended soil treated with 1,3-D only (Fig. 5B), which corresponds to the formation of new bands in these treatments as shown in Fig. 4B. In contrast, unamended soil treated with PBr, at all concentrations, clustered farthest from the control (Fig. 5B). Furthermore, by week 8, unamended soil treated with PBr and 1,3-D at 100 and 500 mg kg^{-1} clustered away from the controls (Fig. 5C). In amended soil, visible bands appeared at all fumigation rates (Fig. 4C). Apparently, the microbial community derives added benefits from the CSM, either by enhanced degradation of the fumigants, which likely reduces the amount of pesticide that comes in contact with the microorganisms, or by stimulation of microbial growth as a result of the added nutrients or both. In addition, the amended soils treated with PBr began to cluster away from the amended soil control as the fumigant rate increased as during week 1. PBr-treated (at 10 and 100 mg kg^{-1}) and 1,3-D-treated (at 500 mg kg^{-1}) unamended soils were similar to each other, and also clustered away from the unamended soil control
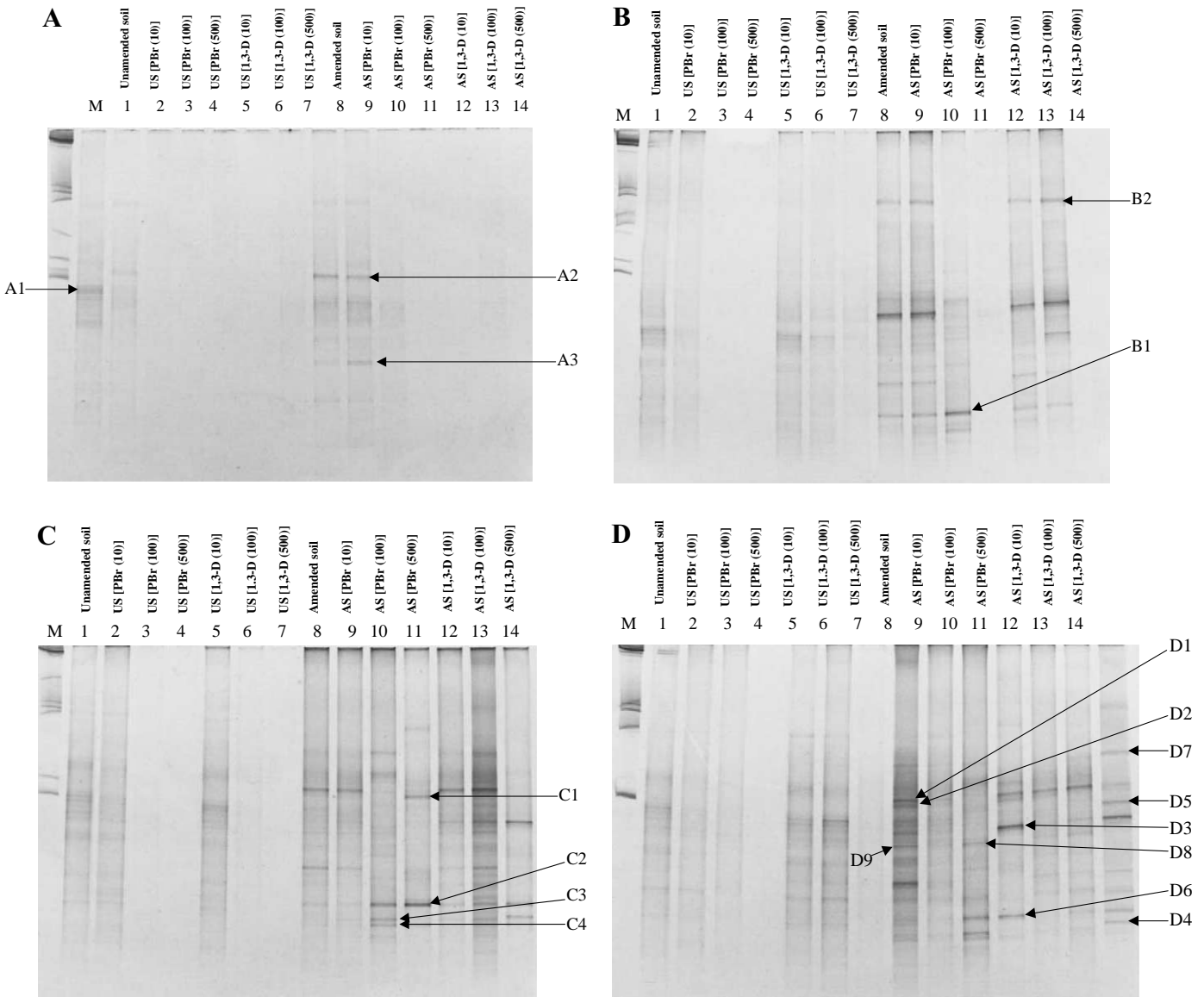
Fig. 4. DGGE analysis of 16S rDNA fragments amplified from unamended and manure-amended Arlington sandy loam treated with PBr or 1,3-D. The PCR fragments were separated on a linear gradient of 30–70% denaturant (A) 1, (B) 4, (C) 8, and (D) 12 weeks after the fumigants were applied. Values in parentheses are the fumigant application rates in mg kg^{-1}. US, unamended soil; AS, amended soil. Lane: M, molecular marker (1-kb ladder). Bands designated with a letter and number were removed for sequence analysis (see Table 4).

(Fig. 5D). Overall, during the course of the experiment, the impact of both fumigants on the microbial community was less dramatic in the CSM-amended soils. In unamended soil, PBr was more damaging to the community structure than 1,3-D, as fewer bands were detected in the PBr treatments (Table 2) and a longer time was required for recovery. Our results are in agreement with Ibekwe et al. [23], who found that 1,3-D had less of an impact on soil microbial communities than other soil fumigants; the other fumigants tested were methyl bromide and methyl isothiocyanate.

The diversity values, H, from fumigant-treated unamended and amended soil are shown in Table 3. Increasing H values indicate increased diversity of the dominant microbial community. In general, the structural diversity of the microbial community decreased with increasing fumigant concentration, regardless of the treatment. During the course of the experiment, H values ranged from zero (which indicates that one or no bands were found; see Table 2) in some of the high-concentration treatments to about 1.4 in some of the controls and 10 mg kg^{-1} treatments. In unamended soil, the PBr treatments had a stronger impact on the structural diversity of the microbial community than 1,3-D, especially at the highest concentration tested. In amended soil treated with 10 mg PBr kg^{-1} the H differed little from the control, whereas this was not the case in unamended soil. Compared with PBr-treated amended soil, the H values were substantially low-
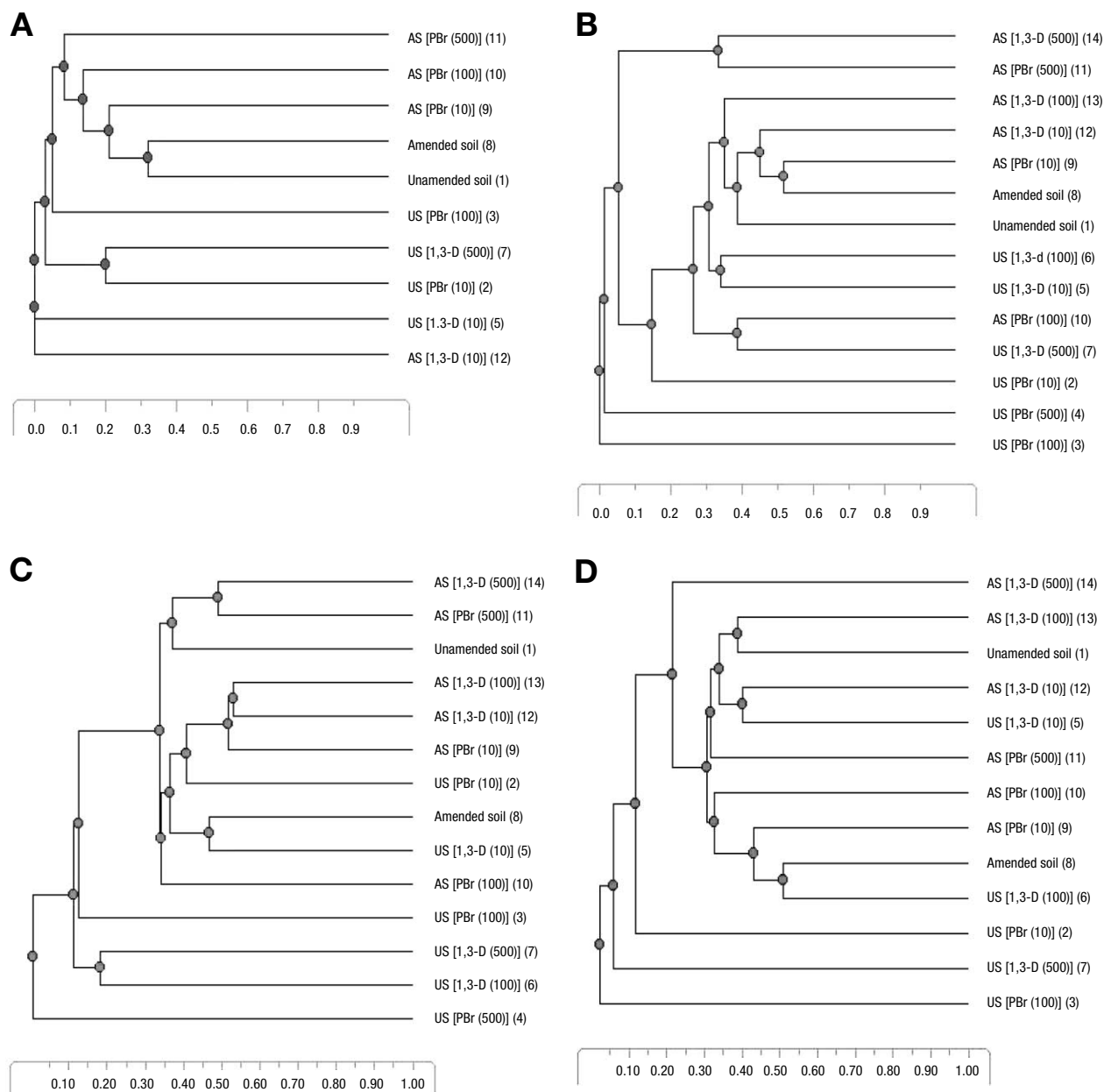
Fig. 5. Cluster analysis of DGGE patterns of 16S rDNA fragments, generated from the data in Fig. 4A–D, at (A) 1, (B) 4, (C) 8, and (D) 12 weeks after fumigation with PBr and 1,3-D. US, unamended soil; AS, amended soil. Values within the brackets are the fumigant application rates in mg kg^{-1}, while values in parentheses, outside the brackets, refer to the lane numbers in the DGGE gels. Absence of a treatment indicates that there were no discernable bands.

er at all concentrations of 1,3-D in amended soil 1 week after application. At week 4, the H values at 1,3-D concentrations of 10 and 100 mg kg^{-1} were similar to the amended soil control, but the H value of the 500 mg kg^{-1} treatment was zero. However, by weeks 8 and 12, the H values at all concentrations of 1,3-D were similar to that of the PBr treatments. Based on the information presented in Table 3, the structural diversity of the dominant microbial community was the highest in fumigated unamended soil at week 4 (1,3-D) and week 8 (PBr), and in amended soil at week 8. For amended soil controls, H increased with time, while in unamended soil controls,

maximum H was observed at 8 weeks. Across all weeks, the structural diversity in the PBr treatments was, on average (when compared to their respective controls), higher in soil amended with compost manure; however, the structural diversity in the 1,3-D treatments was similar in unamended and amended soil at week 1, lower in amended soil at weeks 4 and 12 (10 and 500 mg kg^{-1} treatments), and higher at week 8 (100 and 500 mg kg^{-1} treatments). Compared with 1,3-D, our results indicate that PBr is most damaging to the microbial community diversity in unamended soil, but not in soil when amended with CSM.

Table 3
Effect of PBr and 1,3-D on the Shannon–Weaver index of diversity, H [a]

Treatment	Week 1	Week 4	Week 8	Week 12
Unamended soil	1.21	1.32	1.41	1.11
PBr (10[b])	0.95	0.76	1.34	0.82
PBr (100)	1	0	0.76	0.48
PBr (500)	0	0	0.3	0
1,3-D (10)	0.77	1.32	1.42	1.28
1,3-D (100)	0.6	1.12	0.69	1.26
1,3-D (500)	0.47	1.01	0.75	0.66
Amended soil	1.13	1.21	1.38	1.42
PBr (10[b])	1.13	1.29	1.41	1.4
PBr (100)	1.19	0.99	1.3	1.19
PBr (500)	0.7	0.69	1.06	0.98
1,3-D (10)	0.76	1.25	1.37	1.07
1,3-D (100)	0.69	1.27	1.42	1.17
1,3-D (500)	0.29	0	1.04	1.05

[a] H values equal to zero indicate that one or no bands were detected.
[b] Concentration of fumigant in mg kg^{-1} of dry soil.

3.5. Bacterial identification

Phylogenetic analysis of the sequences derived from the gel bands is shown in Table 4. In this study, 31 clones were sequenced, but four pairs were identical, so that only 27 of the 31 clones were distinctively different. Clone sequences were affiliated with the following phyla: β- and γ-subdivision of the Proteobacteria, high G+C Gram-positive bacteria, *Cytophaga/Flexibacter/Bacteroides*, and green non-sulfur bacteria. The effect of the fumigants on microbial community structure was most dramatic 1 week after the fumigants were applied (Fig. 4A), as bands were only visible in the controls and in soils treated with 10 mg PBr kg^{-1}. The lack of bands in all of the other fumigant-treated soils at week 1 indicates that the predominant bacterial species were highly affected by fumigation. Only three dominant bands (A1, A2, and A3) and numerous faint bands were observed at week 1 (Fig. 4A). The sequences within band A1 were 99% similar to an uncultured copper smeltery bacterium, and in band A2 the sequences matched 95% to an unclassified bacterium and 100% to a β-proteobacterium. Band A3 sequences were 91 and 100% similar to a γ-proteobacterium and an uncultured eubacterium, respectively (Table 4). The uncultured eubacterium has previously been detected in a hydrocarbon- and chlorinated-solvent-contaminated aquifer [10]. The ability of one DGGE band to produce more than one sequence match arises from the fact that certain 16S rRNA sequences are indistinguishable between species from certain groups of phylogenetic bacteria [46]. Consequently, one organism may produce more than one DGGE band because of multiple heterogeneous DNA

Table 4
BLAST search matches of 16S rDNA sequences amplified from DGGE bands

Week	Band	BLAST sequence match	% Similarity	Accession no.
1	A1	Uncultured copper smeltery bacterium D104	99	AF337854
	A2	Unclassified bacterium	95	Z95737
		Uncultured β-proteobacterium E18_pH4/5/FL	100	AF178102
	A3	γ-Proteobacterium EHK-1	91	AF228694
		Uncultured eubacterium WCHB1-02	100	AF050593
4	B1	Uncultured rumen bacterium	100	AY006525
		Streptomyces sp. CHR3	98	AF026080
	B2	Uncultured *Cytophagales* sp.	100	U70709
		Uncultured bacterium #0319-6J10	93	AF234076
8	C1	*Bacillus chitinolyticus*	95	AB021183
		Uncultured bacterium #0319-6J10	93	AF234076
		Virgibacillus proomii	100	AJ012667
	C2	*Streptomyces* sp. IM-1760	97	AF131506
		Streptomyces cyaneus	100	AJ310927
	C3	*Frankia* sp.	99	AF131894
	C4	*Actinomadura* sp. IM-5508	98	AF131305
		Unclassified Pseudomonas group	94	AF102866
12	D1	γ-Proteobacterium EHK-1	91	AF228694
	D2	Uncultured bacterium	100	AB062687
		Uncultured *Cytophagales* sp.	100	U70709
	D3	Uncultured bacterium SBR1108	100	AF269004
		Flavobacterium-like sp. oral clone AZ105	100	AF385548
	D4	*Streptomyces cyaneus*	100	AJ310927
	D5	Uncultured bacterium	100	AF390909
	D6	*Geodermatophilus* sp.	97	X92358
		Streptomyces bikiniensis	99	U72174
	D7	Metal-contaminated soil clone K20-90	93	AF145874
		Uncultured bacterium #0319-6J10	92	AF234076
	D8	Uncultured bacterium	94	AF379697
	D9	Uncultured bacterium	100	AF390909
		Uncultured bacterium	100	AB062687

templates [7,33,38] or bands at similar positions may have originated from different sequences with similar melting points.

At weeks 4, 8, and 12 there was a noticeable increase in the number of bands, especially in the CSM-amended soils (Fig. 4B–D and Table 2). In amended soil, bands (B1 and B2) were present in the 10 and 100 mg kg^{-1} treatments by week 4, but not at the highest concentration tested (Fig. 4B). Sequences within bands B1 and B2 matched 100% with an uncultured rumen bacterium and uncultured *Cytophagales* sp., respectively (Table 4). Band B1 also matched 98% to *Streptomyces* sp. CHR3, which has been shown to be resistant to both mercuric chloride and phenylmercuric acetate [39]. By week 8 many additional bands were present in amended soil, even those treated with 500 mg kg^{-1} of PBr or 1,3-D (Fig. 4C). Novel bands appeared as C1, C3, and C4. Band C1 sequences matched 100% to *Virgibacillus proomii* and band C2 sequences were 97% and 100% similar to *Streptomyces* sp. IM-1760 and *Streptomyces cyaneus*, respectively (Table 4). Streptomyces species are widely distributed in soil, litter, and composts and make up about 90% of all actinomycete isolations (which encompass 10–50% of the total microbial population in soil). Sequences within bands C3 and C4 were 99% and 98% similar to the actinomycetes *Frankia* sp. and *Actinomadura* sp. IM-5508, respectively (Table 4). Actinomycetes may represent a large portion of the bacteria detected in CSM-amended soils because they were added with the manure. During composting the intense heating of the manure generally leaves actinomycetes as the dominant organism. In unamended soil, no DGGE gel bands were discernable by week 12 in the 500 mg kg^{-1} treatments; however, in amended soil treated with 500 mg 1,3-D kg^{-1}, numerous faint bands were visible between weeks 8 and 12 (Fig. 4D). No novel bands appeared in the unamended and amended soils at week 12. Band D6, which was also present during week 8 (as band C3), matched two genera of actinomycetes, *Streptomyces bikiniensis* (99% similar), a producer of and resistant to streptomycin [21], and *Geodermatophilus* sp. (97% similar), which is a strictly aerobic, chemoorganotrophic, and mesophilic bacterium [14], while sequences with band D4 matched 100% to *Streptomyces cyaneus*. High sequence similarities also occurred with four clones (bands D2, D3, D5, and D9), which matched 100% to unidentified bacteria.

4. Conclusions

In conclusion, we have shown that the effect of PBr and 1,3-D on soil microbial activity can be observed using SIR and DHA assays. In unamended soil treated with PBr (10 mg kg^{-1}) or 1,3-D (10 and 100 mg kg^{-1}), the microbial activity was not inhibited during the 12-week study. At these concentrations it is possible that specific microbial

groups were not harmed by the fumigants. However, the addition of PBr at 100 and 500 mg kg^{-1} and 1,3-D at 500 mg kg^{-1} was effective in suppressing the microbial activity, which did not exhibit significant recovery. In contrast, the amelioration of soil with CSM enhanced the long-term recovery of the microbial activity at these higher concentrations. Regarding the CSM application rate used in this study, 3% (w/w) represents a feasible field application rate. For example, the incorporation of 3% CSM into the top 10-cm soil layer (assuming the bulk density is 1.3 g cm^{-3}) translates to 39 tonne of amendment (dry wt.) per ha. The use of organic amendments is also useful in stimulating the degradation of PBr and 1,3-D. As the fumigants move through the soil profile, enhanced degradation of the fumigants will occur within the amended soil layer, which should help reduce atmospheric emissions to acceptable levels.

In addition, we were able to identify a diverse number of bacteria by amplifying and cloning 16S rDNA from the total community DNA. Overall, PBr exhibited the greatest impact on the microbial community structure and diversity in unamended soil, but these impacts were reduced in soil amended with CSM. Discrepancies, however, probably exist between the microbial communities in laboratory-controlled studies and environmental samples, and an effort should certainly be made to conduct comparative studies under field conditions. With respect to pesticide impacts on microbial community structure in the field, attention should be applied to the spatial and temporal distribution of the pesticides, as community changes will certainly differ around the point of application. Additional environmental variables that will impact microbial communities in the field are wind, rain, and temperature. Therefore, the impact of pesticides on the microbial community in the field may or may not be as significant as under laboratory conditions. Our results suggest that it may be feasible to reduce the impact of fumigant pesticides on soil microbial populations by stimulating the microbial community structure, diversity, and activity through the addition of organic amendments, such as CSM.

References

[1] Akhtar, M. and Malik, A. (2000) Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. Bioresource Tech. 74, 35–47.

[2] Alef, K. (1995) Microbial characterization of contaminated soils. In: Methods in Applied Soil Microbiology and Biochemistry, 1st edn. (Alef, K. and Nannipieri, P., Eds.), pp. 503–505. Academic Press, New York.

[3] Anderson, J.R. (1978) Pesticide effects on nontarget soil microorganisms. In: Pesticide Microbiology (Hill, I.R. and Wright, S.J.L., Eds.), pp. 313–533. Academic Press, London.

[4] Anderson, J.P.E. and Domsch, K.H. (1978) A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem. 10, 215–221.

[5] Bandick, A.K. and Dick, R.P. (1999) Field management effects on soil enzyme activities. Soil Biol. Biochem. 31, 1471–1479.

[6] Chen, S.-K., Edwards, C.A. and Subler, S. (2001) A microcosm approach for evaluating the effects of the fungicides benomyl and captan on soil ecological processes and plant growth. Appl. Soil Ecol. 18, 69–82.

[7] Cillia, V., Lafay, B. and Christen, R. (1996) Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. Mol. Biol. Evol. 13, 451–461.

[8] Coyne, M.S. (1999) Soil Microbiology: An Exploratory Approach. Delmar, New York.

[9] Dungan, R.S., Gan, J. and Yates, S.R. (2001) Effect of temperature, organic amendment rate, and moisture content on the degradation of 1,3-dichloropropene in soil. Pest. Manag. Sci. 57, 1107–1113.

[10] Dojka, M.A., Hugenholtz, P., Haack, S.K. and Pace, N.R. (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer. Appl. Environ. Microbiol. 64, 3869–3877.

[11] Dzantor, E.K., Felsot, A.S. and Beck, M.J. (1993) Bioremediating herbicide-contaminated soils. Appl. Biochem. Biotechnol. 39/40, 621–630.

[12] Eichner, C.A., Erb, R.W., Timmis, K.N. and Wagner-Döbler, I. (1999) Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. Appl. Environ. Microbiol. 65, 102–109.

[13] Elliot, L.F., Lynch, J.M. and Papendick, R.T. (1996) The microbial component of soil quality. In: Soil Biochemistry, vol. 9 (Bollag, J.M. and Stotzky, G., Eds.), pp. 1–21. Marcel Dekker, New York.

[14] Eppard, M., Krumbein, W.E., Koch, C., Rhiel, E., Staley, J.T. and Stackebrandt, E. (1996) Morphological, physiological, and molecular characterization of actinomycetes isolated from dry soil, rocks, and monument surfaces. Arch. Microbiol. 166, 12–22.

[15] FCH (1997) Farm Chemical Handbook. Meister, Willoughby, OH.

[16] Gamliel, A. and Stapleton, J.J. (1997) Improvement of soil solarization with volatile compounds generated from organic amendments. Phytoparasitica 25S, 31–38.

[17] Gan, J., Yates, S.R., Crowley, D. and Becker, J.O. (1998) Acceleration of 1,3-dichloropropene degradation by organic amendments and potential application for emissions reduction. J. Environ. Qual. 27, 408–414.

[18] Gan, J., Yates, S.R., Papiernik, S. and Crowley, D. (1998) Application of organic amendments to reduce volatile pesticide emissions from soil. Environ. Sci. Technol. 32, 3094–3098.

[19] Harden, T., Joergensen, R.G., Meyer, B. and Wolters, V. (1993) Soil microbial biomass estimated by fumigation extraction and substrate-induced respiration in two pesticide-treated soils. Soil Biol. Biochem. 25, 679–683.

[20] Hart, M.R. and Brookes, P.C. (1996) Soil microbial biomass and mineralisation of soil organic matter after 19 years of cumulative field applications of pesticides. Soil Biol. Biochem. 28, 1641–1649.

[21] Huddleston, A.S., Cresswell, N., Cristina, M., Neves, P., Beringer, J.E., Baumberg, S., Thomas, D.I. and Wellington, E.M.H. (1997) Molecular detection of streptomycin-producing Streptomycetes in Brazilian soils. Appl. Environ. Microbiol. 63, 1288–1297.

[22] Hungalle, N., Lal, R. and Terkuile, C.H.H. (1986) Amelioration of physical properties by Mucuna after mechanized land clearing of a tropical rainforest. Soil Sci. 141, 219–224.

[23] Ibekwe, A.M., Papiernik, S.K., Gan, J., Yates, S.R., Yang, C.-H. and Crowley, D.E. (2001) Impact of fumigants on soil microbial communities. Appl. Environ. Microbiol. 67, 3245–3257.

[24] Lin, Q. and Brookes, P.C. (1999) Comparison of substrate induced respiration, selective inhibition and biovolume measurements of microbial biomass and its community structure in unamended, ryegrass-amended, fumigated and pesticide-treated soils. Soil Biol. Biochem. 31, 1999–2014.

[25] Ma, Q.L., Gan, J., Becker, J.O., Papiernik, S.K. and Yates, S.R. (2001) Evaluation of propargyl bromide for control of barnyardgrass

and *Fusarium oxysporum* in three soils. Pest. Manag. Sci. 57, 781–786.

[26] Ma, Q.L., Gan, J., Papiernik, S.K., Becker, J.O. and Yates, S.R. (2001) Degradation of soil fumigants as affected by initial concentration and temperature. J. Environ. Qual. 30, 1278–1286.

[27] Macalady, J.L., Fuller, M.E. and Scow, K.M. (1998) Effects of metam sodium fumigation on soil microbial activity and community structure. J. Environ. Qual. 27, 54–63.

[28] Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. and Wade, W.G. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64, 795–799.

[29] Martens, D.A., Johanson, J.B. and Frankenberger Jr, W.T. (1992) Production and persistence of soil enzymes with repeated addition of organic residues. Soil Sci. 153, 53–61.

[30] Moorman, T.B., Cowan, J.K., Arthur, E.L. and Coats, J.R. (2001) Organic amendments to enhance herbicide biodegradation in contaminated soils. Biol. Fertil. Soils 33, 541–545.

[31] Muyzer, G., Dewaal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

[32] Nannipieri, P., Muccini, L. and Ciardi, C. (1983) Microbial biomass and enzyme activities: Production and persistence. Soil Biol. Biochem. 15, 679–685.

[33] Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wleshuber, A., Amann, R., Ludwig, W. and Backhaus, H. (1996) Sequence heterogeneities of genes encoding 16S RNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.

[34] O'Hallorans, J.M., Muñoz, M.A. and Colberg, O. (1993) Effect of chicken manure on chemical properties of a Mollisol and tomato production. J. Agric. Univ. P.R. 77, 181–191.

[35] Papiernik, S.K., Gan, J. and Yates, S.R. (1999) Mechanism of degradation of methyl bromide and propargyl bromide in soil. J. Environ. Qual. 29, 1322–1328.

[36] Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M. and White, D.C. (2001) Soil microbial community response to dairy manure or ammonium nitrate applications. Soil Biol. Biochem. 33, 1011–1019.

[37] Perucci, P. (1990) Effect of addition of municipal solid waste compost on microbial biomass and enzyme activities. Biol. Fertil. Soils 10, 221–226.

[38] Rainey, F.A., Ward-Rainey, N.L., Janssen, P.H., Hippe, H. and Stackebrandt, E. (1996) Clostridium paradoxum DSM 7308T contains multiple 16S rRNA genes with heterogeneous intervening sequences. Microbiology 142, 2087–2095.

[39] Ravel, J., Amoroso, M.J., Colwell, R.R. and Hill, R.T. (1998) Mercury-resistant actinomycetes from the Chesapeake Bay. FEMS Microbiol. Lett. 162, 177–184.

[40] SAS Institute Inc. (1998) SAS/STAT software, Version 8.0. Cary, NC.

[41] Schoen, S.R. and Winterlin, W.L. (1987) The effects of various soil factors and amendments on the degradation of pesticide mixtures. J. Environ. Sci. Health B 22, 347–377.

[42] Smith, S.N. and Pugh, C.J.F. (1979) Evaluation of dehydrogenase as a suitable indicator of soil microflora activity. Enzym. Microbiol. Technol. 1, 279–281.

[43] Smith, M.D., Hartnett, D.C. and Rice, C.W. (2000) Effects of long-term fungicide applications on microbial properties in tallgrass prairie soil. Soil Biol. Biochem. 32, 935–946.

[44] Tu, C.M. (1992) Effect of some herbicides on activities of microorganisms and enzymes in soil. J. Environ. Sci. Health B. 27, 695–709.

[45] Turco, R.F., Kennedy, A.C. and Jawson, M.D. (1994) Microbial indicators of soil quality. In: Defining Soil Quality for a Sustainable Environment (Doran, J.W., Coleman, D.C., Bezdicek, D.F. and

Stewart, B.A., Eds.), SSSA Spec. Publ. 35, pp. 73–90. SSSA, Madison, WI.

[46] Vallaeys, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigaud, A. and Soulas, G. (1997) Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in the rhizobia and methanotrophs. FEMS Microbiol. Ecol. 24, 279–285.

[47] von Mersi, W. and Schinner, F. (1991) An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol. Fertil. Soils 11, 216–220.

[48] Wardle, D.A. and Parkinson, D. (1990) Effects of three herbicides on soil microbial biomass and activity. Plant Soil 122, 21–28.

[49] Yates, S.R. and Gan, J. (1998) Volatility, adsorption, and degradation of propargyl bromide as a soil fumigant. J. Agric. Food Chem. 46, 755–761.

[50] Zibilske, L.M. (1994) Carbon mineralization. In: Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties, Book Series no. 5, pp. 835–863. SSSA, Madison, WI.