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Effect of organic mulches on soil bacterial communities one year after application

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Abstract The application of organic mulches as a soil cover is effective in improving the quality of soil. However, very little information is available on the effect of mulches on the soil microbial community. In this study, we investigated the effect of various organic mulches on soil dehydrogenase activity (DHA) and microbial community structures in the top 1 cm and 5 cm below the soil surface 1 year after application of the mulches. DHA was stimulated at both depths in plots mulched with grass clippings (GC), but was not significantly different from the control for the other mulch treatments. Fatty acid methyl ester (FAME) analysis and denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction-amplified 16S rDNA fragments were used to assess changes in the soil microbial community structure. Cluster analysis and principle component analysis of FAME profiles showed that only soil mulched with pine chips distinctively clustered from the other treatments. At the soil surface, bacterial DGGE profiles revealed that distinct shifts in several bacterial populations occurred in soils mulched with GC and eucalyptus yardwaste (EY), while DGGE profiles from soil at the 5 cm depth revealed no distinct changes. Changes in bacterial diversity at the soil surface under different mulches were calculated based on the number of bands in the DGGE profile using the Shannon-Weaver index of diversity (H). Compared to the control (H =0.9), the GC- and EY-treated soils showed slightly increased bacterial diversity, with an H of 1.1 and 1.0, respectively. These results indicate that the long-term effect of organic mulches on the soil microbial activity and community structure is highly dependent upon the type of mulch and is mostly exerted in the top few centimeters of the soil profile.

Keywords Dehydrogenase activity · Denaturing gradient gel electrophoresis · Fatty acid · Organic mulch · Soil bacterial community structure

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Introduction

Recent attention on the ecotoxicological impacts of agrochemicals and their high cost has promoted the need to develop reliable alternative methods to protect and conserve soils (Abawi and Widmer 2000). One such method is the land application of organic waste materials. With the over abundance of waste materials generated daily by human activities, there is great interest in using organic residues, which are generally difficult to manage in an environmentally acceptable way (Casale et al. 1995; Albiach et al. 2000). The addition of organic residues to soils has been found to be effective in reducing potentially harmful fumigant emissions (Gan et al. 1998) and controlling soilborne pathogens by stimulating antagonistic organisms or by producing toxic volatile compounds (Akhtar and Malik 2000). The proper use of many types of organic residues as mulches on agricultural soils is known to provide various minerals (e.g., N, P, and S) essential for plant nutrition, increase the soil organic matter content, and influence soil structure and many other related physical, chemical, and biological parameters. In addition, organic amendments also increase the size, biodiversity, and activity of microbial populations (Perucci 1990; Bandick and Dick 1999; Peascock et al. 2001).

Changes in microbial community activities and structure, as a result of agricultural practices can be used as early indicator of soil "health" and "quality" since soil microbial communities play a critical role in the recovery

of a soil from a disturbance (Bending et al. 2000). Using culture-independent methods, the composition of communities can be inferred based on extraction, quantification, and identification of molecules—cellular identifying markers such as fatty acid methyl esters (FAMEs) or environmental nucleic acids directly from soil that are specific identifiers of microorganisms or microbial groups (Morgan and Winstanley 1997; Lawlor et al. 2000). Since molecular methods analyze the entire bacterial community, covering those bacteria that cannot be cultured, such methods are becoming especially important tools in soil microbial ecology (Pickup 1991; Stackebrandt et al. 1993).

The analysis of FAMEs from phospholipids has been used as an effective method to describe microbial strains and communities or to differentiate among environmental samples by their fatty acid "fingerprint" (Zelles et al. 1992). Since there are many different kinds of fatty acids in the lipids of microorganisms and different organisms have different combinations of these fatty acids, FAME profiles of soils impacted by different agricultural practices can indicate management-induced changes in the composition of microbial communities (Ibekwe and Kennedy 1999). In addition, FAME profiles are relatively inexpensive and can be easily analyzed by gas chromatography (Cavigelli et al. 1995).

The primary objective of this study was to investigate the effects of various organic mulches on soil bacterial activities and community structure at the soil surface and 5 cm below the soil surface. Dehydrogenase activities (DHAs), using the iodonitrotetrazolium chloride (INT) method, were measured. We also determined differences in bacterial community structure based on polymerase chain reaction (PCR) amplification of 16S rDNA followed by denaturing gradient gel electrophoresis (DGGE). Finally, we evaluated whether there were differences in the whole soil microbial community structure among the various mulched soils based on FAME profiles. Identification of NH_3-oxidizing bacteria was also addressed. Autotrophic NH_3-oxidizing bacteria play a significant role in the cycling of N and maintaining soil quality. They oxidize NH_3 produced during the decomposition of organic matter to NO_2^- and subsequently to NO_3^- (Prosser 1989).

Materials and methods

Experimental design and soil sampling

Soil samples of a Hanford sandy loam (coarse-loamy, mixed, thermic Typic Xerothent, pH 8.1, C 0.2%, C/N ratio 10.64) were collected in August 2000 from an on-going field experiment at the University of California, Riverside, Agricultural Experiment Station. Samples were taken both at 0–5 cm and 5–10 cm depth. The field experiment was initiated in September 1999 and consisted of microplots (0.5×0.5 m) that were amended (surface application only) with various organic waste materials to a thickness of 10 cm. The amendments used were grass clippings (GC), alfalfa hay stems (AH), composted steer manure (CM; Kellog Supply, Carson, Calif.), wheat straw stems (WS), shredded redwood (SR), eucalyptus yardwaste (EY), pine chips (PC), oleander yardwaste (OY), and chipped construction waste (CW) (Table 1). As a result, there were a total of ten treatments, including the control, which where laid out in a randomized block design with three replicates. No crops were grown on the plots, but sprinkler irrigation was used to moisten the mulches. Irrigation was applied for about 2 h daily, 5 days a week, except during the winter months when water was added only 3 days per week. During the study the soil moisture tension (measured with a tensiometer at 10 cm below the soil surface) varied (on average) from 12.7 kPa in plots mulched with OY to 16.4 kPa in the control plots.

Table 1 Mean percent of C and N in the mulches

Mulch	C (%)	N (%)	C:N ratio
Grass clippings	40.3	3.7	10.9
Alfalfa hay stems	42.9	2.9	14.8
Composted manure	30.1	1.4	21.5
Shredded redwood	48.1	0.33	145.8
Eucalyptus yardwaste	47.2	1.3	36.3
Pine chips	48.8	0.74	65.9
Oleander yardwaste	45.4	0.76	59.7
Chipped construction waste	46.2	0.85	54.4

Soil samples (surface and 5 cm below) from each microplot were collected using an ethanol-disinfected stainless steel spatula. The soil samples were placed in sterile glass vials and stored at −20°C unless indicated otherwise.

Dehydrogenase activity assay

The dehydrogenase activity (DHA) was measured on fresh soil samples to assess the metabolic microbial activity of the soil at the soil surface and 5 cm below in the microplots. To measure the DHA, the INT method was employed (von Mersi and Schinner 1991). This method is based on the reduction of INT to iodonitrotetrazolium formazan. Soil (0.5 g dry weight) was weighed into 20-ml test tubes and mixed with 0.75 ml of 1 M TRIS buffer and 1 ml of 10 mM INT solution. The test tubes were tightly sealed with rubber stoppers and incubated at 37°C in the dark for 2 h. The controls contained autoclaved soil (121°C for 20 min) and were treated like the samples. After the 2-h incubation period, each sample was mixed with 2.5 ml of an extraction solution (1:1 solution of *N*, *N* -dimethylformamide and 100% ethanol) and kept in the dark. The samples were then vigorously shaken every 20 min for 1 h. After centrifugation, the supernatant was measured at 464 nm (DU 640 Spectrophotometer; Beckman, Calif.).

FAME analysis

Soil samples (1 g dry wt.) were placed in Teflon-lined screw cap culture tubes. The FAME extraction consisted of four-steps (Sherlock Microbial Identification System 1999):

1. Saponification: lysis solution was added to the soil to liberate the fatty acids from the cellular lipids by adding 1 ml NaOH solution (45 g NaOH in 150 ml methanol), and heating the samples to 100°C in a water bath for 30 min, then cooling to room temperature.
2. Methylation: formation of methyl esters of the fatty acids by adding 2 ml HCl solution (6 N; 325 ml HCl added to 275 ml CH_3OH), placing in an 80°C water bath for 10 min, and then immediately cooling to room temperature.
3. Extraction: transfer of the FAMEs from the aqueous phase to an organic phase by adding 1 ml hexane/methyl *tert* -butyl ether (1:1; v/v) and rotating the samples end-over-end for 10 min.
4. Base wash: aqueous wash of the organic extract with 3 ml NaOH (1.2%; w/v) by rotating the tubes end-over-end for 5 min.

Finally, the organic phase (i.e., upper phase) containing the FAMEs was removed from the tubes and placed in gas chromatograph vials. The FAME were then identified and quantified by gas chromatography-flame ionization detection (HP 6890; Hewlett-Packard, Wilmington, Del.).

Soil DNA extraction

Total soil bacterial DNA was extracted from frozen samples to determine the effects of the various mulches on the bacterial community structure. DNA was extracted from 0.5 g soil using a FastDNA Spin kit for soil (BIO 101, Vista, Calif.). The amount of DNA was estimated visually by electrophoresis in 1% agarose gels after ethidium bromide staining.

PCR-DGGE analysis

The PCR mixture contained 10 pmol of each primer, about 40 ng template DNA, 50 mM TRIS buffer (pH 8.3), 2.5 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates, 12.5 μg bovine serum albumin, 0.125 U Taq polymerase, and sterile distilled water to a final volume of 25 μl. The primers used for PCR were PRUN518r (5′ATT ACC GCG GCT GCT GG-3′) and PRBA338f (5′ GC clamp-ACT CCT ACG GGA GGC AGC AG-3′) located in the V3 region of the 16S rRNA genes of bacterioplankton (Øvreås et al. 1997). PBRA338f contains a region that is conserved among the domain *Bacteria*, and PRUN518r is located at a universally conserved region. The GC-rich sequence attached to the 5′-end of primer PBRA338f prevents the PCR products from completing melting during separation via DGGE (Muyzer et al. 1993). PCR amplification was performed at 92°C for 2 min, followed by 35 thermal cycles of 92°C for 1 min, 55°C for 30 s, and 72°C for 1 min, and final single extension at 72°C for 6 min. The size of the PCR product was visualized by electrophoresis in 1% agarose gels after ethidium bromide staining. Strong bands of approximately 200 bp were subject to DGGE analysis.

DGGE analysis was conducted using a DCode system (Bio-Rad Laboratories, Hercules, Calif.). Samples of PCR product (20 μl) were loaded onto 8% polyacrylamide gels in 1×TAE buffer. The polyacrylamide gels were made with a linear denaturing gradient ranging from 30% denaturant at the top of the gel to 70% denaturant at the bottom (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). The electrophoresis was run for 3 h at 60°C and 200 V. After electrophoresis, the gels were stained with ethidium bromide, and photographed on a UV transilluminator with a Polaroid camera. The photographs were scanned into a computer digital image analysis and band intensities were measured for each sample.

Statistical analysis

Statistical differences ($P<0.05$) in the dehydrogenase activity and percentage of fatty acids were determined using SAS statistical software (1998). The digitized DGGE gel data were analyzed to investigate microbial community structure as described by Ibekwe et al. (2001). The Shannon-Weaver index of diversity (H) was used to compare changes in the diversity of the bacterial community structure within the mulched soils. H was calculated by using the function $H = -\sum P_i \log P_i$, where $P_i = n_i / N$, where n_i is the height of a peak and N is the sum of all peak heights in the curve (Eichner et al. 1999). Principle component analysis of FAME profiles was performed using the software package CANOCO (Microcomputer Power, Ithaca, N.Y.). Cluster analyses were performed using MiniTab 12.0 (MiniTab, State College, Pa.).

Identification of NH_3 oxidizers

DNA was directly extracted from mulched soils as described above. Two sets of PCR primers were used to recover NH_3-oxidizing bacterial communities. Primers pA and pH (eubacterial in origin), were used to amplify the entire 16S rDNA sequence (1.5 kb) as described by Kowalchuk et al (1999). The 1.5-kb fragment was excised from a 0.8% agarose gel with a clean razor blade, placed in 20 μl sterile distilled water, and incubated overnight at 4°C. One milliliter of the eluate was used as template DNA in a second PCR with the primers CTOf189-GC and CTO654r for DGGE analysis. These primers specifically amplify a 465-bp fragment of the 16S rRNA gene from β-proteobacteria NH_3-oxidizing bacteria (Kowalchuk et al. 1997). PCR amplification was performed at 94°C for 1 min, followed by 25 thermal cycles of 92°C for 30 s, 57°C for 1 min, and 68°C for 45 s, and a final extension at 68°C for 45 s. PCR products (465 bp) recovered with the CTO primers were then subjected to DGGE under the same conditions as described above, except the gels contained a linear gradient of 35–45% denaturant.

Bands chosen for sequence analysis were excised from the DGGE gel with a clean razor blade, placed in 20 μl sterile distilled water, and incubated overnight at 4°C. One milliliter of the eluate was reamplified with the second primer set (CTO primers). After the PCR products were visualized for size on 1% agarose gels, they were purified using a Qiagen PCR purification kit (Valencia, Calif.). The PCR products were then cloned into a pGEM-T Easy vector (Promega, Madison, Wis.), and the ligated plasmids were transformed into competent *Escherichia coli* JM109. Selection of transformants containing recombinant plasmids was done using blue/white screening. 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, Calif.). Sequence identification was conducted using the BLAST database [National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)].

Results and discussion

Dehydrogenase activity

Addition of organic materials to soil is known to stimulate microbial and enzyme activity (Nannipieri et al. 1983). Often the level of soil enzyme activity increases with increasing soil organic matter content, and this may be correlated to the population dynamics of the soil microorganisms (Speir and Ross 1976; Speir 1977). The measurement of DHA is particularly useful in assessing the microbial activity since it is linked to the microbial oxidation of organic substances. In this study, the DHAs of three of the treatments, at the soil surface, were significantly different from the control (bare soil) 1 year after the mulches were applied (Fig. 1a). Those mulches were GC, CM, and SR; however, GC was the only treatment which had a stimulatory effect on the DHA, while the DHA in the SR plots was the lowest of all the treatments. At 5 cm below the soil surface, GC once again had a stimulatory effect on the DHA, and none of the other treatments were found to be significantly different from the control (Fig. 1b). Overall, these results indicate that the effect of the mulches on the DHA is mostly confined to the soil surface layer.

Although DHA activities were not measured during the course of the experiment, it appears that GC produced a long-term stimulatory effect on the soil DHA. After

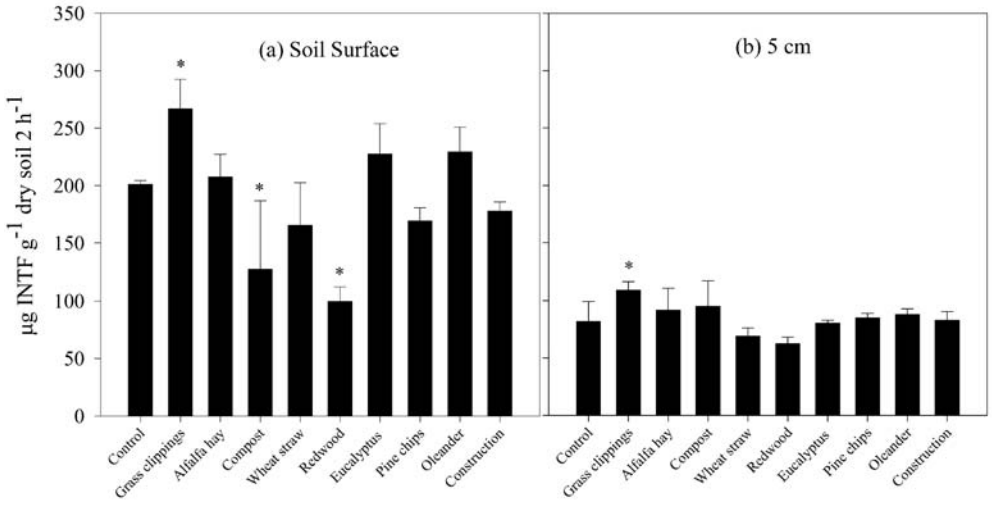

Fig. 1 Soil dehydrogenase activity at **a** the soil surface and **b** 5 cm below the soil surface 1 year after the application of the mulches. *Error bars* represent the SD of three replicates. * $P < 0.05$ (significantly different from the control). *INTF* Iodonitrotetrazolium formazan

1 year, a visual inspection of the microplots revealed that a substantial fraction of the GC was degraded when compared to the other mulches. The biodegradation of the GC may have released nutrients into the soil which could have stimulated the microbial activity and been responsible for the increased DHA. It has been recognized that GC, when freshly cut, are useful mulch materials because they contain a very large percentage of N (Rodale 1974). The GC studied here contained 3.7% N, the highest N content of any of the mulches used in this study, followed by AH at 2.9% N and CM at 1.4% N (Table 1).

The above results are in agreement with those of several studies which reported that the activity of many soil enzymes was dependent on the type of organic material applied (Speir and Ross 1976; Ladd 1978). In addition, it cannot be ruled out that the other treatments influenced the physicochemical conditions of the soil which in turn had an impact upon the microbial activity and, hence, the DHA. Perucci et al. (1984) found that the addition of organic residues, such as maize and wheat straw, decreased the activity of several soil enzymes including DHA. In other studies, it was reported that soil DHA was significantly inhibited by the toxic effects of heavy metals present in composted manure, particularly by Pb and Cu (Bonmati et al. 1985; Marzadori et al. 1996).

Not many soil enzymes are useful as general indicators of the viable soil microbial biomass or activity (Dick et al. 1996). DHA has been attractive as a biological indicator since these enzymes are an integral part of microorganisms and because of their role in the oxidation of organic matter (Casida et al. 1964). However, some researchers have reported that DHA cannot always be consistently correlated with other biological properties such as O_2 uptake and CO_2 evolution (Frankenberger and Dick 1983). Chander and Brookes (1991) found that there was an abiotic reaction between the end product measured in DHA, triphenylformazan, and Cu, which may result in an underestimation of the DHA in soils that have been contaminated with soil pollutants. Also, DHA may not be appropriate to project permanent changes in soil quality because dehydrogenase cannot accumulate in a complexed form in soils and its activity is best used only as an indication of the viable microbial population. Nevertheless, many studies have applied DHA to estimate the microbial biomass and biological activity under various soil management systems (Stevenson 1959; Skujins 1973; Ladd and Paul 1973).

FAME analysis

Analysis of FAMEs extracted directly from soil has the unique ability to characterize whole soil microbial communities rapidly and inexpensively (Cavigelli et al. 1995). In this study, the FAMEs were analyzed to investigate the composition of the whole microbial community structure (predominantly fungi and bacteria) in each of the mulched soils. Soil samples were only collected from the GC, CM, SR, EY, and PC microplots at the soil surface. The mean percent distributions of fatty acids are shown in Table 2. These include fatty acids with carbon lengths of 12–19, which is consistent with other studies that reported fatty acids of 12–19 carbons in length are the most dominant in bacterial lipids (Haack et al. 1994). Palmitic acid (16:0) appeared to be the most common and dominant fatty acid in all of the samples. Zelles et al. (1992) found that the presence of palmitic acid was strongly correlated with a number of measures of microbial biomass. In addition to palmitic acid, other commonly found fatty acids were 14:0, i15:0, 16:1ω5c, and 18:1ω9c. These fatty acids were present in all of the samples and made up about 63–78% of the total fatty acid content. The presence of certain fatty acids can be used as a marker to identify certain microbial types (Table 3). The i15:0, a15:0 and i16:0 signature fatty acids for Gram-positive bacteria (Ratledge and Wilkinson 1988), 18:1ω9c for eukaryotes including fungi (Vestal and White 1989), and 16:1ω5c for CH_4-oxidizing bacteria (Tunlid and White 1992) were present in all of the mulched soils,

Table 2 Mean percent distribution of fatty acids[a] in the soil surface samples

Fatty acid	Mean percent distribution of fatty acids					
	Control	Grass clippings	Composted manure	Shredded redwood	Eucalyptus yardwaste	Pine chips
12:00	4.5	5.9	9.5	4.7	3.1	10.0*
14:00	6.3	6.0	7.7	5.9	10.6*	5.0
i15:0	7.1	7.4	8.7	8.1	9.0	2.9
a15:0	3.1	4.1	1.9	2.1	2.1	1.5
15:1w8c	0.0	0.7	2.8	0.0	0.3	13.5*
16:00	33.5	28.6	29.3	30.6	34.0	12.6*
i16:0	4.5	3.3	0.6	2.8	1.8	1.1
N-Aol16:0	0.0	1.2	2.4	9.5*	1.0	1.0
10Me16:0	6.4	0.0	0.0	2.6	0.0	17.9*
16:1w5c	5.8	16.4*	2.6	8.2	19.8*	7.1
10Me17:0	3.7	5.2	0.8	0.0	4.8	1.1
i17:1w10c	0.0	0.0	0.3	0.0	0.0	5.5*
17:1w7	0.0	0.9	4.5*	0.0	1.0	7.2*
18:00	7.2	3.6	2.5	3.1	2.2*	1.8*
18:1w9c	18.0	7.0	24.6	18.5	8.6	9.2
18:3w6c	0.0	1.9	1.3	4.1*	0.3	2.5
10Me19:0	0.0	8.0*	0.4	0.0	1.4	0.0

* $P < 0.05$ (significantly different from the control)

[a] Fatty acid nomenclature: the prefixes i, a and Me refer to isomethyl, anteisomethyl and mild-chain methyl branching, respectively; the suffixes c and t for *cis* and *trans*, respectively, refer to geometric isomers

Table 3 Marker fatty acids in the phospholipids of several microorganisms

Fatty acids	Microorganisms
i15:0, a15:0, i16:0	Gram-positive bacteria
16:1w5c	CH_4-oxidizing bacteria
10Me 16:0	SO_4^{2-}-reducing bacteria, Actinomycetes
16:0, 18:3w6c	Fungi
18:1w9c	Fungi, green algae

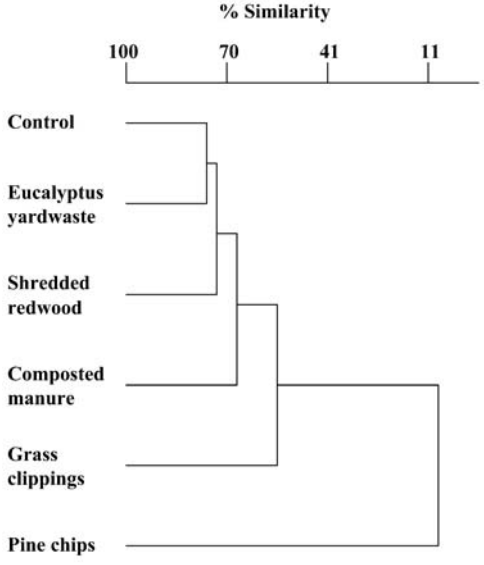

Fig. 2 Cluster analysis of fatty acid methyl ester signatures for microbial communities treated with different organic mulches

including the control. In contrast, 18:3ω6, the signature fatty acid for fungi (Hill et al. 2000), was not present in the control, but was present in all of the mulched soils. In particular, the fatty acid 10Me16:0, which was dominant only in the PC-treated sample, is a signature fatty acid for sulfate-reducing bacteria, specifically for *Desulfovibrio* (Morgan and Winstanley 1997). Other dominant signature fatty acids include 12:0, 14:0, 15:1ω8c, and 16:1ω5c. Fatty acid 14:0 was dominant in the EY-treated soil, while 16:1ω5c was dominant in both the GC- and EY-treated soils. These results are consistent with those of Bossio et al. (1998), who found that organically managed soils had significant amounts of 14:0 and 16:1ω5c compared to soils receiving synthetic fertilizers and pesticides. Signature fatty acids for Gram-negative bacteria were not found in any of the samples. Gram-positive bacteria, as identified by i15:0, a15:0 and i16:0, were similarly distributed among the samples regardless of the treatment, except in the PC treatment.

When FAME profiles were utilized to assess community structure between rhizosphere and non-rhizosphere soil (Ibekwe and Kennedy 1999), it was reported that Gram-positive bacteria were equally distributed between the rhizosphere and non-rhizosphere, while Gram-negative bacteria were higher in rhizosphere soil than non-rhizosphere soil. In another study, Zelles et al. (1997) found that Gram-positive bacteria were less affected by chloroform fumigation than Gram-negative bacteria. Apparently, the cell wall of Gram-positive bacteria and their ability to form spores increases survival after abrupt environmental changes.

Cluster analysis of the FAME profiles showed that the PC-treated soil was the most different from the other treatments and the control (Fig. 2). Overall, the PC-treated soil was <11% similar to the other treatments. This observation was supported by the percentage of fatty acids in Table 2, which shows distinct microbial community shifts in PC-treated soil (i.e., 16:0 for fungi and i15:0 for Gram-positive bacteria distinctively de-

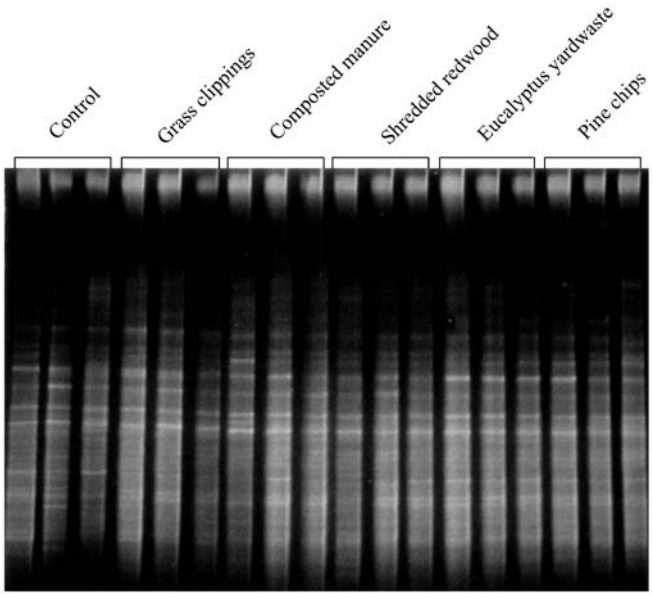

Fig. 3 Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA fragments of triplicate soil samples taken from the soil surface. Samples were collected 1 year after the mulches were applied. The gradient of the urea and formamide ranged from 30 to 70%

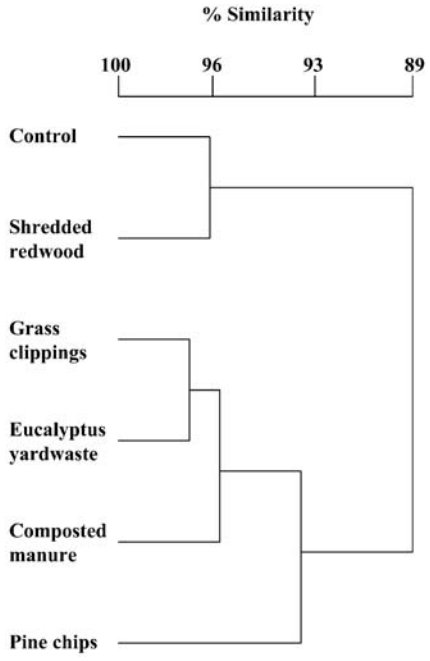

Fig. 4 Cluster analysis of 16S rDNA profiles of bacterial communities in soil plots receiving different organic mulches

creased, while 15:1ω8c and 10Me 16:0 for sulfate-reducing bacteria distinctively increased). The EY treatment was the most similar to the control (at about 75%), followed by SR, CM, and GC at about 72, 66, and 55%, respectively.

Bacterial community structure

A comparison of soil bacterial community structure can be obtained by DGGE analysis of 16S rDNA fragments (Jensen et al. 1998). Here, soil samples were analyzed only from the plots mulched with GC, CM, SR, EY, and PC. Bacterial DGGE profiles generated from the universal eubacterial primers (338f and 518r) revealed the structural composition of communities in each of the organically mulched soils at the surface (Fig. 3). The profiles between the triplicate microplot soil samples were very similar with respect to complexity and band composition. Each of the distinguishable bands in the separation pattern is assumed to represent an individual bacterial species or group which is part of the microbial community. One DGGE band can produce more than one sequence match since certain 16S rRNA sequences are indistinguishable between species from certain phylogenetic groups of bacteria (Vallaeys et al. 1997). Likewise, one organism may produce more than one DGGE band because of multiple heterogeneous DNA templates (Cillia et al. 1996; Nübel et al. 1996; Rainey et al. 1996) or two different PCR products may migrate together as a result of having the same melting behavior.

At the soil surface, bacterial DGGE profiles revealed that distinct shifts occurred in several bacterial populations. In particular, certain DNA bands became more intense in soils mulched with GC and EY, when compared with the bare-soil control. This suggests that these treatments had a stimulatory effect upon some bacterial genera or species. On the other hand, in soils mulched with CM, no additional bands were detected which did not exist in the control. DGGE profiles generated from 5 cm below the soil surface appeared very similar regardless of the mulching treatment (data not shown). Overall, our results suggest that the effect of the mulches was more distinctive at the soil surface, which is consistent with the results obtained with the FAME analysis and the DHA assay. In general, numbers of all microbial populations decrease with increasing depth from the soil surface (Konopka and Turco 1991). Since DGGE profiles generated from the 5 cm depth appeared very similar, regardless of the treatment, only data from the soil surface were then used to compare the similarity of bacterial communities among the different treatments. Cluster analysis, based on the number of bands and their intensity, revealed that the control and SR, which is a slowly decomposable substrate, clustered together, while GC and EY, which are both easily decomposable substrates, and CM formed similar communities (Fig. 4). Soil mulched with PC produced a unique bacterial community.

Using both the number and relative intensities of the bands in the DGGE gel, H , which indicates the diversity of abundant ribosomal gene sequence types within the community (Eichner et al. 1999), was calculated at the soil surface. Organically mulched soils showed slightly

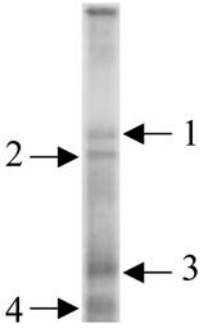

Fig. 5 DGGE analysis of polymerase-chain reaction-amplified 16S rDNA fragments from NH_3-oxidizing bacteria using CTO primers 189f and 654r. The gradient of the urea and formamide ranged from 30 (*top*) to 45% (*bottom*)

different levels of diversity, ranging between 0.8 and 1.1. The average *H* from the bare-soil control was 0.9. Compared to the control, the GC- and EY-treated soils showed slightly increased bacterial diversity, with an *H* of 1.1 and 1.0, respectively, while CM-treated soil showed slightly deceased diversity, with an index value of 0.8. The *H* values of the SR- and PC-treated soils were similar to the bare-soil control.

Recovery of NH_3 oxidizers

DNA extracted from soil was first amplified with eubacterial primers prior to specific amplification with the β-subgroup NH_3-oxidizer-specific primers, CTO189f-GC and CTO654r. Soil samples were only collected from the control, GC, CM, SR, EY, and PC microplots. After PCR, the products were analyzed by DGGE using a gel with a linear gradient of denaturant from 30 to 45%. All samples produced identical banding patterns with four dominant bands grouped as doublets (Fig. 5). The appearance of a doublet band is consistent with other researchers' results using the CTO primers, and has been reported to be the result of an ambiguous position in the CTO654r primer (Kowalchuk et al. 1997). The ambiguous site is located at position 647 (*E. coli* numbering) (Brosius et al. 1981). Since the DGGE banding patterns were all alike, the four dominant bands were randomly excised from the DGGE gel regardless of treatment. The excised bands were reamplified and sequenced for identification (Table 4). Bands 1 and 2 were found to be 99% similar to uncultured *Nitrosospira* spp. These results are in agreement with a number of studies that suggest *Nitrosospira* is the most common of the β-subgroup of NH_3 oxidizers in soil environments (Hastings et al. 1997; Kowalchuk et al. 1998; Stephen et al. 1998). Band 3 was 99% similar to an uncultured β-proteobacterium, and band 4 matched 100% to an unidentified soil organism.

NH_3 oxidation is often the rate-limiting step during the loss of N from systems due to denitrification. Therefore, conditions which are favorable for the activity of NH_3-oxidizing bacteria in natural environments may grossly affect the net N balance (Kowalchuk et al. 1999). The ability to identify and track the presence and activity of specific groups of NH_3-oxidizing bacteria should help in the overall understanding of the N balance. Our results have shown that DGGE of specifically amplified 16S rDNA fragments, using CTO primers, and coupled with sequence analysis, can be used to detect specific NH_3 oxidizers of the β-subgroup in environmental soil samples. This study also confirmed the potential value of using molecular approaches to study ecologically important groups of organisms that are particularly difficult to isolate using culture-dependent methods.

In conclusion, application of different mulches resulted in overall shifts in microbial community structure in the top centimeter of the soil which were readily distinguished by analysis of the FAME profiles and 16S rDNA profiles of the microbial communities. In all cases, the major effect of the mulches was exerted in the top centimeter of the soil immediately under the mulch. Using the combination of methods for analyzing the microbial communities, the data suggest that mulches primarily affected the fungal species composition. When the bacterial communities were specifically examined using PCR-DGGE of 16S rDNA, the data showed that with the exception of PC, all of the other mulches had relatively minor effects on bacterial community structure. Bacterial species diversity of the microbial communities, was slightly greater in soils mulched with GC and EY, but there were no effects of the different mulches on the species composition of NH_3-oxidizing bacteria. Although the mulches serve as substrates for microbial growth, most of the materials that were used were relatively recalcitrant and had no observable effect or relatively minor effects on microbial activity. Plots mulched with PC, AH, WS, EY, OY, and CW were no different from the unmulched control soil at the soil surface 1 year after application of the mulches, whereas microbial activity was negatively impacted in plots that were mulched with CM or SR. The rapid biodegradation of GC, when compared to the other mulches, may explain why the microbial activity was stimulated in the plots mulched with this material.

Table 4 Sequence analysis of denaturing gradient gel electrophoresis bands derived using CTO primers

Band	BLAST search match	% Similarity	Accession no.
1	Uncultured *Nitrosospira* rt16S-9	99	AJ271103
2	Uncultured *Nitrosospira* rt16S-4	99	AJ271098
3	Uncultured beta proteobacterium CSP19	99	AJ272566
4	Unidentified soil organism R6–143	100	U42970

Over the long term, it may be possible that repeated applications of mulches may lead to continued changes in microbial community structure and species diversity as soil organic matter increases. Mulches will change the cation exchange of the soil, and lead to improved soil structure as the soil organic matter is increased. The soil depth that is affected by mulching may also increase as earthworms and soil animals facilitate soil mixing. Nonetheless, the results of this study suggests that even under irrigation, this is a slow process which is initially constrained to the top few centimeters of soil.

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