

The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis

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Abstract

The impact of the fungicide chlorothalonil on dominant bacterial and fungal populations following application to turfgrass, forest, and agricultural soils was investigated. Chlorothalonil was applied to each soil at three rates, representing 0.2, 1 and 5 times the recommended label rate for turfgrass, and incubated for a 2-week period. Changes to the microbial community caused by the chlorothalonil application were assessed following DNA extraction, PCR-amplification using both bacteria domain- and fungal-specific primers, then separation with denaturing gradient gel electrophoresis (DGGE). Digitized DGGE images were used to determine two parameters: the number of bands per lane and the Shannon–Wiener index of diversity (H'), both of which were used only for comparison of the different treatments, and not as true diversity measurements. Bands appearing to be either enhanced or inhibited as a result of the chlorothalonil treatment were excised and sequenced. Increased rates of chlorothalonil impacted eight bacterial populations (two inhibitions, four enhancements, and two non-specific responses) and four fungal populations (all inhibitions). Band number and H' indicated an altered but not significantly different ($P < 0.05$) bacterial and fungal community structure following chlorothalonil application. Sequencing of excised DGGE bands indicated an impact on several groups of bacteria (*Cytophaga–Flavobacterium–Bacteroides*, α -, β -, γ -, and δ -proteobacteria) and two fungal taxa (zygomycota and ascomycota). Although changes to the overall community structure of dominant species were non-significant, we conclude that following a single chlorothalonil application and a short incubation period, community changes including both enhancement and inhibition of a variety of dominant organisms can occur.

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1. Introduction

Chlorothalonil (tetrachloroisophthalonitrile) is a widely used fungicide for the control of foliar pathogens in agronomic systems. The United States Geological Survey (USGS) has estimated that yearly

in excess of 4.8×10^6 kg of chlorothalonil are applied to crops in the United States (United States Geological Survey, 1997). Although these fungicide applications are primarily targeted to plant leaf surfaces, a thin plant canopy, over-application, or application followed by irrigation will allow the fungicide to contact the soil (Cisar and Snyder, 1996; Petrovic et al., 1996). The estimated half-life of chlorothalonil in soil varies from 5 to 90 days, depending on the

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soil type (Jury et al., 1987; Nelson, 1996). Thus, the potential exists for chlorothalonil to remain in the soil environment for several weeks following application. The environmental fate of applied fungicides, including chlorothalonil, has been recently reviewed (Sigler et al., 2000) and this review showed that an understanding of the impact of fungicides on the soil microbial community is lacking. The persistence of chlorothalonil, along with its frequency of use warrants investigation of the fungicide's short-term impacts on the soil bacterial and fungal populations.

Traditional methods have been previously used to demonstrate that fungicides, including chlorothalonil, could increase (Suyama et al., 1993; Ganesan and Mathivanan, 1996), decrease (Tu, 1993; Shukla and Mishra, 1996), or not affect numbers of soil organism (Revellin et al., 1993). However, it is well known that community structure results obtained using methods based on the culturing of organisms isolated from treated vs. untreated environmental samples may misrepresent the true impact on the soil microbial community (Ward et al., 1990; Zuberer, 1994; Dunbar et al., 1997). Furthermore, complications involved with traditional fungal population characterization include limited sample size to accurately survey the high number of species present and the lack of appropriate expertise and protocols (Cannon, 1997). To our knowledge, no study exists that describes the impact of fungicide application on soil bacterial and fungal community structure evaluated from the molecular standpoint. To avoid some of the biases presented by traditional methods, we adopted the use of denaturing gradient gel electrophoresis (DGGE), which has been used to assess the community structure of microbial communities through the separation of PCR-amplified partial 16S rRNA gene (Ovreas and Torsvik, 1998; Tiedje et al., 1999; Muyzer et al., 1993). More recently, the conversion of visual DGGE images to numerical data has been useful in determining various indices of diversity including species richness (S) and the Shannon–Wiener diversity index (H') (Konopka et al., 1999; Eichner et al., 1999). By performing band detection with the help of computer software and predetermined detection parameters, biases in band detection and discrimination can be limited.

Previous studies using molecular fingerprinting have shown that herbicide application to soil resulted in structural changes to bacterial communities (El

Fantroussi et al., 1999; Engelen et al., 1998). It is assumed that herbicides indirectly impact soil microbial populations by eliminating vegetation, including plant roots, which are an important source of harborage and nutrients for microbial survival (Badalucco et al., 1996; Maloney et al., 1997). The influence of fungicides on soil populations may be quite different from that of herbicides. Fungicides may encourage bacterial survival by serving as a source of nutrition and energy, eliminating potential fungal antagonists or by indirectly increasing the amount of available nutrients following the death of sensitive fungi. In addition to the obvious biocidal properties, non-sensitive fungal populations may acquire nutrients from sensitive fungi that have died as a result of exposure to the fungicide. This is analogous to bacterial populations where parts of the population are subsequently stimulated by the release of cellular materials from the killed biomass.

In this study, we describe the short-term or acute effects of chlorothalonil on both the bacterial and fungal communities of soils from three different management settings representing three soil types; sand, silty clay loam, and loam. Due to the sorptive nature of chlorothalonil (Winkler et al., 1996), we have concentrated on this short time period in order to maximize detection of acute effects on the microbial community, as it is this time period when the fungicide will be in solution at the highest level.

2. Materials and methods

2.1. Soils and chlorothalonil treatments

Soils from three management systems without a history of chlorothalonil application were used in this study (Table 1), a United States Golf Association-specified sand taken from the root-zone of a 1-year-old putting green, a forest soil (loam), and a silty clay loam from a no-till maize plot, hereafter referred to as “turfgrass rhizosphere”, “forest”, and “agricultural” soils, respectively. Samples from the forest and agricultural sites were taken from the “A” horizon to a depth of approximately 10 cm with a flame-sterilized spade. The turfgrass rhizosphere soil was also collected to a 10 cm depth using a 2.5 cm diameter flame-sterilized soil core sampler. Several sub-samples (5–10) were immediately pooled through sieving (2 mm), homogenized

Table 1
Physical properties of soils used in this study

Soil	Soil type	Organic matter (%)	Sand (%)	Silt (%)	Clay (%)	pH
Turfgrass rhizosphere	Sand	0.9	73.6	13.4	12.9	7.6
Forest	Loam	5.5	16.0	49.0	35.0	7.0
Agricultural	Silty clay loam	5.5	36.0	45.0	19.0	6.7

by hand to generate composite samples and stored at 4 °C until analyzed. Technical grade chlorothalonil (Chem Service Inc., West Chester, PA) was applied to triplicate soil samples (75 g each) at 2.85, 14.25, and 71.25 mg chlorothalonil per gram of dry soil. The rates represent 0.2, 1 and 5 times concentrations of the highest recommended label rate for chlorothalonil application to turfgrass sites. To generate a carrier free application process, chlorothalonil was first precipitated onto acid washed, autoclaved silica sand (Turco and Konopka, 1990). This approach eliminated any potential confounding effects that could result from the use of organic carriers. Work in our laboratory has shown the release of the chemical from the sand surface to be nearly 100%. After adding the fungicide-coated sand to the soils at the desired rates, the soils were transferred to plastic bags, incubated in the dark at room temperature, and sampled as described later. The plastic bags were not sealed in order to allow for adequate air exchange; thus, periodic adjustments of water content were performed following moisture content analysis. Control soils (no fungicide treatment) were treated with sand prepared as described previously but without fungicide amendment.

2.2. Soil nucleic acid extraction and PCR-DGGE

Three c. 0.5 g samples of soil from each plastic bag were taken immediately following fungicide application and again following 2 weeks of incubation.

DNA extraction was performed as described previously (Sigler et al., 2001) and the concentration of the extracted DNA was determined through measuring absorbance at A_{260} and purity determined by A_{260}/A_{280} .

To facilitate the efficient handling of samples and data, each of the 36 DNA samples (three samples taken from four treatments prepared in triplicate) was screened with PCR-DGGE. The DGGE profiles were identical, not only within triplicate samples, but also among all samples of a treatment triplicate. Thus, all PCR products derived from a common treatment triplicate were pooled, yielding three PCR samples per treatment for analysis. Furthermore, a comparison of the pooled samples from common treatments also revealed identical profiles (not shown). Therefore, one sample representing each treatment was randomly chosen for the final analysis in order to accommodate the electrophoresis of all samples on a single gel.

Four primers were used in this study for the amplification of bacterial 16S rRNA genes and fungal 28S rRNA genes (Table 2). For DGGE, a 40 bp GC-rich sequence was attached to the forward primers (Muyzer et al., 1993). PCR reactions were performed in a Pro-gene thermal cycler (Techne Inc., Princeton, NJ) with primers 341-f and 534-r-GC according to the method of Muyzer et al. (1993) for 16S rRNA gene amplification (approximately 194 bp product from bacteria) and primers 403-f and 662-r-GC for 28S rRNA gene amplification (Sandhu et al., 1995) (two possible product sizes of approximately 200 or 420 bp from fungi).

Table 2
PCR primers used in this study

Primer ^a	16S or 28S rRNA gene target (position) ^b	Sequence (5′–3′)	References
341-f	Bacteria 16S (341–357)	ATT ACC GCG GCT GCT GG	Muyzer et al. (1993)
534-r	Bacteria 16S (534–518)	(GC)-CCT ACG GGA GGC AGC AG	Muyzer et al. (1993)
403-f	Fungi 28S (403–422)	GTG AAA TTG TTG AAA GGG AA	Sandhu et al. (1995)
662-r	Fungi 28S (662–645)	(GC)-GAC TCC TTG GTC CGT GTT	Sandhu et al. (1995)

^a f, forward primer; r, reverse primer; (GC), G + C rich sequence attached at 5′ end as noted in text.

^b *E. coli* numbering for 16S rRNA gene primers, *S. cerevisiae* numbering for 28S rRNA gene primers.

Electrophoresis of the PCR products was performed as described previously (Sigler et al., 2001) in gels made of 8% acrylamide in a gradient of denaturants ranging from 45 to 60%. Gels were stained by gently shaking for 12 min in 50 ml of $1 \times$ TAE containing a 1:5000 dilution of SYBR green (Molecular Probes Inc., Eugene, OR).

2.3. Image analysis

The resulting banding patterns were visualized with UV transillumination and photographed using the Gel Doc 2000 gel documentation system (Bio Rad Laboratories, Hercules, CA). DGGE gels are composed of several lanes containing multiple bands of DNA with varying intensities. Band patterns and average intensities of individual bands were analyzed using Quantity One image analysis software, version 4.0 (BioRad Laboratories). After applying a rolling disc background subtraction (setting nine) and a sensitivity setting of 4.667, the software performed an analysis of each lane, acquiring information concerning each band's position and average intensity relative to the cumulative intensity value of all bands in the given lane. A band of DNA was detected if it accounted for greater than 0.2% of the total lane intensity. The same software settings were used in the analysis of all lanes in each gel. Identification of bands occupying common positions in multiple lanes was also performed.

A matrix was constructed using this information and used to calculate numerical values describing the community structure of the microbial communities. The number of bands detected (an index of the number of dominant members) and the Shannon–Wiener index

$$H' = \sum_{i=1}^s \left(\frac{n_i}{N} \right) \ln \left(\frac{n_i}{N} \right)$$

where n_i is the area of each peak and N the sum of all peak areas (Magurran, 1988) were used as numerical descriptors to facilitate the comparison of microbial community structures among the treated samples.

The results of the analyses are reported as averages over the replications described previously. Comparisons of Shannon diversity indices were performed first by estimating the variance in H' of each forefield

sample:

$$\text{Var}H' = \frac{\sum \rho_i (\ln \rho_i)^2 - (\sum \rho_i \ln \rho_i)^2}{N} + \left(\frac{S-1}{2N^2} \right)$$

where ρ_i is the proportional abundance of the i th species (band intensity in pixel density), N is the total number of species (bands), and S the total number of individuals detected (summed band intensities). A t -test allowed the comparison of two fingerprint lanes by

$$t = \frac{H'_1 - H'_2}{(\text{Var}H'_1 + \text{Var}H'_2)^{1/2}}$$

where H'_1 is the diversity of sample 1 and $\text{Var}H'_1$ is its variance. The associated degrees of freedom were calculated by

$$\text{d.f.} = \frac{(\text{Var}H'_1 + \text{Var}H'_2)^2}{((\text{Var}H'_1)^2/N_1) + ((\text{Var}H'_2)^2/N_2)},$$

(Magurran, 1988).

2.4. 16S rRNA gene sequence determination

DGGE bands that appeared visually to be either lost or gained due to the application chlorothalonil, were excised, eluted in 100 ml water, re-amplified as described above, and electrophoresed with DGGE. Band excision, PCR, and DGGE were repeated until a single band was present. In general, two to three additional runs were necessary for isolation of a single band. For cloning and sequencing, PCR products generated from DGGE bands were amplified with primers 341-f and 534-r or 403-f and 662-r (primers 534-r and 662-r were without the GC clamp), ligated into pGEM-T vector (Promega Corp., Madison, WI) and transformed into *Escherichia coli* DH5- α (Sambrook et al., 1989). Sequences were determined using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ.) and a Pharmacia ALF express automated sequencer (Amersham Pharmacia Biotech, Piscataway, NJ). At least five transformed cells per band were subjected to sequencing in order to determine the sequence dominating the excised band. In each case, sequences generated from each of the five cloned plasmid insert sequences were >99% similar. The derived sequences were compared to 16S rRNA gene sequences in

the National Center for Biotechnology Information database using the BLAST 2.0 algorithm (Altschul et al., 1997).

2.5. Nucleotide sequence accession numbers

The partial environmental 16S rRNA and 28S rRNA gene sequences recovered in this study from the chlorothalonil treated soils have been deposited in the Genbank database under accession numbers AF427081-A91.

3. Results

3.1. Denaturing gradient gel electrophoresis

As evidenced by the number of DNA bands detected in the control lanes of the denaturing gradient gels,

the three soils displayed abundant bacterial (Fig. 1) and fungal (Fig. 2) community complexity prior to the application of chlorothalonil. Between 11 and 13 dominant bacterial DNA bands and between 12 and 18 fungal DNA bands were detected in the three soils along with a background DNA smear, denoting a high level of microbial diversity in each of the soils tested.

Many of the visually intense DGGE bands were unique to each of the individual soils suggesting that the three soils harbored differing dominant bacterial and fungal populations. Additionally, there were several similarities in banding position among common soils receiving differing chlorothalonil rates indicating many common microbial members were present in each soil regardless of the fungicide rate. Each soil sample that was incubated for 2 weeks without chlorothalonil treatment displayed initial ($t = 0$ weeks) DGGE fingerprints similar in terms of band number and intensity to fingerprints assessed follow-

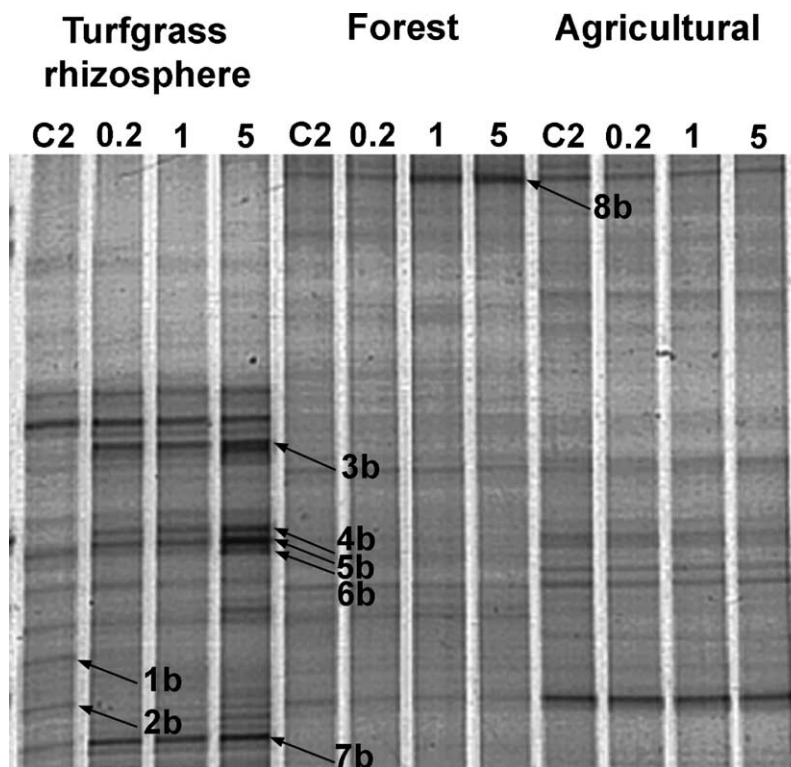


Fig. 1. Denaturing gradient gel electrophoresis separation of PCR-amplified partial 16S rRNA genes from chlorothalonil treated turfgrass rhizosphere, forest soil, and agricultural soil. All lanes represent samples analyzed following 2 weeks of incubation as described in the text. Chlorothalonil treatments were as follows: C-2, control following 2 weeks of incubation (no chlorothalonil application); 0.2, 0.2 times label application rate; 1, 1 time label application rate; 5, 5 times label application rate. Labeled arrows represent bands excised for sequencing.

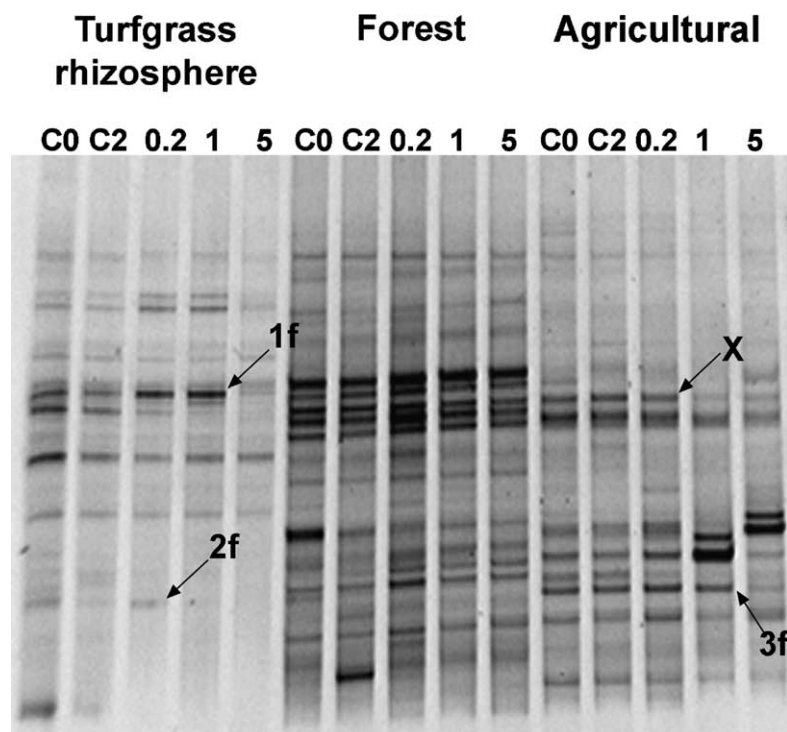


Fig. 2. Denaturing gradient gel electrophoresis separation of PCR-amplified 28S rRNA genes from chlorothalonil treated turfgrass rhizosphere, forest soil, and agricultural soil. With the exception of lanes labeled with C-0, all lanes represent samples analyzed following 2 weeks of incubation as described in the text. Chlorothalonil treatments were as follows: C-0, control at $t = 0$ weeks (no chlorothalonil application); C-2, control at $t = 2$ weeks; 0.2, 0.2 times label application rate; 1, 1 time label application rate; 5, 5 times label application rate. Labelled arrows represent bands excised for sequencing. Band labelled with "X" was not clonable, as described in text.

ing 2 weeks of incubation, indicating that the incubation conditions had little impact on the detectable bacterial (not shown) and fungal populations (Fig. 2, lanes C-0 and C-2 for each soil type). Any DGGE band pattern changes that occurred in the control treatments over the 2 week incubation were assumed to be caused by non-fungicide related processes. Since our goal was to characterize the impact of the fungicide application on the microbial populations, these bands were not considered for further analysis.

Changes in dominant bacterial populations following fungicide treatment were evident only in the turfgrass rhizosphere and forest soils (Fig. 1). For the agricultural soil, no visible changes in DNA banding patterns were detected. Eight bands, seven excised from turfgrass rhizosphere DNA profiles and one from the forest soil DNA profiles represented the extent of the detectable changes following incubation with

chlorothalonil at the described rates. All changes in the bacterial community profiles were in response to chlorothalonil application, as the untreated controls displayed no changes in their patterns following the 2-week incubation period (not shown). Six bacterial populations represented by clones 3b, 4b, 5b, 6b, 7b, and 8b displayed a positive dose-response to the chlorothalonil application as shown by a successive increase in band intensity as the chlorothalonil rate increased. As assessed by observation of DGGE band intensity, two populations (clones 1b and 2b) were completely inhibited after the 2-week incubation with chlorothalonil regardless of the application rate.

While changes in fungal DNA band patterns and intensities were detected in the turfgrass rhizosphere and agricultural soil, the impact was limited to the inhibition of two dominant populations in each soil (Fig. 2). Specifically, the band representing clone 1f

was enhanced at the 0.2 and 1 times application levels (clone 1f) but disappeared in the 5 times treatment while another band displayed slight enhancement at the 0.2 times application level (clone 2f). In the agricultural soil, two fungal populations displayed an inhibition in response to the chlorothalonil applications, however only one of the DGGE bands representing these populations could be sequenced despite several attempts at cloning the DNA. The unsequenced band represented a population that exhibited inhibition following the 1 and 5 times application rates while clone 3f exhibited inhibition only at the 5 times rate.

3.2. Assessment of community structure

Although DGGE band intensities revealed that several bacterial and fungal community members were affected by the fungicide treatments, the calculation of band number and Shannon–Wiener diversity index (H') values indicated that all soil samples experienced non-significant ($P < 0.05$) changes in overall richness and diversity of dominant bacteria (Table 3) and fungi (Table 4) following the incubation with chlorothalonil. Although none of the detected microbial communities were significantly impacted by the fungicide applications, the increase in fungicide rates from 0.2 to 5 times seemed to decrease bacterial and fungal band number and H' in almost every sample. The lone exception

Table 3

Band number (Num) and Shannon–Wiener diversity index (H') values for bacteria in response to chlorothalonil applications to the three soils investigated in this study

Soil	Chlorothalonil rate			
	C-2	0.2 times	1 time	5 times
Turfgrass rhizosphere				
Num	13	13	17	21
H'	2.56	2.55	2.82	3.03
Forest				
Num	10	10	7	7
H'	2.28	2.23	1.60	1.57
Agricultural				
Num	10	10	9	8
H'	1.87	2.19	1.99	1.78

C-2 refers to the control treatment (no chlorothalonil applied) at 2 weeks following incubation. All differences are non-significant ($P < 0.05$).

Table 4

Band number (Num) and Shannon–Wiener diversity index (H') values for fungi in response to chlorothalonil applications to the three soils investigated in this study

Soil	Chlorothalonil rate				
	C-0	C-2	0.2 times	1 time	5 times
Turfgrass rhizosphere					
Num	12	12	8	8	8
H'	1.93	1.81	1.24	1.26	1.19
Forest					
Num	18	18	20	17	16
H'	3.60	3.47	4.10	3.30	3.05
Agricultural					
Num	14	14	13	9	10
H'	2.47	2.39	2.25	1.60	1.70

C-0 and C-2 refer to control treatments (no chlorothalonil applied) at 0 and 2 weeks following incubation, respectively. All differences are non-significant ($P < 0.05$).

to this trend was the turfgrass rhizosphere bacteria population, in which band number and H' were observed to increase as fungicide rates were increased. DGGE fingerprints from all bacterial populations in addition to turfgrass rhizosphere and agricultural soil fungal populations revealed that the number of bands as well as the population composition representing the non-treated soils were the same as those from soils treated at the 0.2 times rate. Despite the similar number of bands, H' values from each of these samples indicated changes in community structure.

3.3. Nucleotide sequencing and analysis

PCR-DGGE of amplified 16S/28S rRNA genes from chlorothalonil treated soils resulted in eight bacterial bands and three fungal bands excised for sequence analysis. Comparison of our cloned sequences with published sequences in the NIH Genbank database resulted in similarities ranging from 86 to 100% (Table 5). With the exception of clones 1b (86%), 2b (92%), 3b (90%), 7b (93%), and 8b (92%), the sequences were greater than 95% identical to organisms in the NIH database. The sequence of cloned DNA derived from excised bands was most similar to organisms in three different groupings of bacteria: the *Flexibacter–Cytophaga–Bacteroides* phylum (one band), and the division *Proteobacteria* (seven

Table 5

Phylogenetic relationships of clones sequenced in this study

Clone (Genbank accession number)	Number of bp sequenced	Kingdom	Phylogenetic group ^a	Soil ^b	Closest identified relative (Genbank accession number)	Identity (%)
1b (AF427081)	192	Bacteria	CFB	Turf	<i>Flexibacter flexilis</i> (M62794)	86
2b (AF427082)	193	Bacteria	δ-Proteobacteria	Turf	<i>Polyangium cellosum</i> (AY039304)	92
3b (AF427083)	193	Bacteria	γ-Proteobacteria	Turf	<i>Nevskia ramosa</i> (AJ001010)	90
4b (AF427084)	188	Bacteria	γ-Proteobacteria	Turf	<i>Lysobacter antibioticus</i> (AB019582)	94
5b (AF427085)	189	Bacteria	β-Proteobacteria	Turf	<i>Leptothorix discophora</i> (L33975)	95
			β-Proteobacteria		<i>Rubrivivax gelatinosus</i> (D19623)	95
6b (AF427086)	169	Bacteria	α-Proteobacteria	Turf	<i>Blastomonas natatora</i> (AJ299222)	100
			α-Proteobacteria		<i>Agrobacterium rhizogenes</i> (D13945)	100
			α-Proteobacteria		<i>Blastomonas ursinicola</i> (AB024289)	100
			α-Proteobacteria		<i>Erythrobacter citreus</i> (AF227259)	100
7b (AF427087)	194	Bacteria	γ-Proteobacteria	Turf	<i>Pseudomonas cichorii</i> (Z76658)	93
8b (AF427088)	193	Bacteria	γ-Proteobacteria	Forest	<i>Nevskia ramosa</i> (AJ001010)	92
1f (AF427089)	422	Fungi	Zygomycota	Turf	<i>Glomus claroideum</i> (AF235007)	96
2f (AF427090)	255	Fungi	Ascomycota	Turf	<i>Chaetomium murorum</i> (AF286405)	89
3f (AF427091)	255	Fungi	Ascomycota	Agri	<i>Fusarium epistroma</i> (AF228352)	100

^a CFB, *Cytophaga-Flavobacterium-Bacteroides*; α, alpha; β, beta; γ, gamma; δ, delta.

^b Turf, turfgrass rhizosphere; forest, forest soil.

bands). Each subdivision of the *Proteobacteria* was represented by at least one of the seven sequences that grouped within the *Proteobacteria* with most of the sequences (four) similar to sequences within the gamma subdivision.

The three fungal 28S rRNA gene sequences were found to be most similar to two of the four major fungal phyla, the Zygomycota (clone 1f) and the Ascomycota (clone 2f and 3f, Table 3). A Genbank search revealed that the sequence of clone 1f was most similar to that of *Glomus claroideum* (97%) while the sequences of clones 2f and 3f were most similar to *Chaetomium murorum* (89%) and *Fusarium epistroma* (100%), respectively.

4. Discussion

Most of the changes to the bacterial community following chlorothalonil treatments at 0.2, 1 or 5 times the high-recommended label rate (2.85, 14.25, and 71.25 mg g⁻¹ soil, respectively) were observed in the turfgrass rhizosphere (seven visual changes) with only one change detected in the forest soil and

none in the agricultural soil (Fig. 1). DGGE fingerprinting revealed that six bacterial populations were enhanced by the chlorothalonil treatments (represented by clones 3b, 4b, 5b, 6b, 7b, and 8b) while no fungal populations were enhanced. It is possible that the enhancement resulted from the direct use of the chlorothalonil by bacteria as a nutrient substrate. Although degradation data were not obtained in this study, chlorothalonil was easily degraded in soil and was often associated with a concomitant increase in bacterial biomass (Katayama et al., 1991b). It is also plausible that bacterial populations increased based on carbon liberated from dead fungal and/or bacterial biomass (Jenkinson, 1966) as the responses were associated with increased application rates.

Suppression of two turfgrass rhizosphere bacterial populations was evident following application of chlorothalonil at all rates (clones 1b and 2b). Although negative responses of bacteria to pesticide applications have been shown to occur in culture-based experiments using soils treated with chlorothalonil (Tu, 1993), the mechanism of bacterial inhibition in the current study is unclear. However, it has been shown that suppression of chlorothalonil-degrading

bacterial populations was associated with repeated chlorothalonil applications, suggesting that a toxic effect results when a minimum inhibitory concentration of chlorothalonil is reached in the soil (Katayama et al., 1991a).

Fungal DGGE band patterns included two detectable population changes in both the turfgrass rhizosphere and agricultural soil. All of the impacted fungal populations resulted in inhibitions, although each was affected at differing chlorothalonil rates. An obvious mechanism of fungal inhibition by chlorothalonil involves the chemical's fungicidal properties. Chlorothalonil is used in a large variety of applications and it impacts a broad spectrum of fungal targets; thus, an inhibitory effect was predicted. The reasons for the lack of detectable impact are numerous. Primarily, we suggest that the fungicide was suppressing new growth but leaving the living biomass in place. Although the current study did not address the issue of fungicide target selectivity, future research should focus on this area in order to more completely understand the impact of fungicides on microbial communities. It is also possible that while the chemical possesses a wide spectrum of activity, the target species might not have been present (or at least present in sufficient numbers to allow detection) in the investigated soils. This seems highly unlikely, as all soils were derived from sites characterized by intense vegetation inputs and thus it is assumed that many fungal targets, especially those associated with turfgrass and maize culture, would be present. On the other hand, while the soils were obtained from agronomic settings, our laboratory incubations were performed in the absence of plants, thereby decreasing the potential detection of plant associated fungi. Furthermore, DGGE is an effective method for the detection of dominant members of a microbial community and large population shifts associated with those dominating communities. Although there were many intense fungal bands detected with our DGGE process, less abundant members that were potentially impacted by the chlorothalonil treatment may have been undetected especially if community changes were slight.

It must be noted that in the current study, the use of H' was not intended to describe the true diversity of the treated soils, but merely to provide a numerical indicator to compare changes in the structure of dominant members in the microbial community. Since H'

is based on the summed proportional abundances of individual organism types (DGGE bands and their intensity), a decrease in H' either implies a decrease in the number of organism types (bands) or a decrease in the proportional abundance of a given type (band intensity). Despite the general trend of decreased bacterial and fungal richness and H' associated with increased chlorothalonil rates, the most impacted community was that of the turfgrass rhizosphere bacteria, in which the number of bands increased from 13 detected bands at $t = 0$ weeks to 21 detectable bands in the 5 times treatment 2 weeks after chlorothalonil application. Although the chlorothalonil application had a strong positive impact on the turfgrass rhizosphere bacterial community, the fungal community was negatively impacted, with band number decreasing from 12 to 8 detectable bands in the $t = 0$ and $t = 2$ weeks (5 times application rate) samples, respectively. The indices calculated in this study are based partially on the intensity of bands in DGGE fingerprints and rely on the assumption that band intensity is related to initial DNA template amount in the extracted sample, and thus, original population size. Although biases are often associated with PCR-amplification of mixtures of DNA templates (Casamayor et al., 2000; Nübel et al., 1996; Suzuki and Giovannoni, 1996; von Wintzingerode et al., 1997), band intensity will often relate to initial cell numbers (Gelsomino et al., 1999) and has been frequently related to relative dominant phylotype abundance (Konopka et al., 1999; Eichner et al., 1999; Schauer et al., 2000; Murray et al., 1996; Ovreas et al., 1997; Nubel et al., 1999). However, assessing a general mode of action of the impact is difficult as the impacts on the three investigated soils were dependant on application rate, soil type, and organisms investigated. Furthermore, it is important to note that due to the tremendous bacterial richness in soil systems (Torsvik et al., 1990), some impacts, either positive or negative, may go undetected especially if minor members of the population are affected by a given treatment.

Differences in the observed response among these three soils may reflect the higher organic matter content in the forest and agricultural soils (5.5%) versus the turfgrass rhizosphere soil (0.9%). Chlorothalonil reacts quickly with the organic carbon component of soil and water systems and is sorbed (Dell et al., 1994). We suggest sorption/retention may explain

the difference in population changes in the turfgrass rhizosphere soil (sand), versus the forest (loam) and agricultural (silty clay loam) soils and may play a key role in limiting the effects of fungicides on bacteria and fungi (Castro et al., 1997; Katayama et al., 1995). Changes in the bacterial community were evident following treatment with chlorothalonil at lower rates (0.2 times) than would normally be applied, further illustrating the enhanced availability of the fungicide in the turfgrass rhizosphere, even at reduced application rates. Currently, no direct information exists to describe the effect of sorption on fungicide availability to fungi. However, it was apparent in this study that band number and H' indicated a stronger fungal community change in the sandy, low organic matter environment of the turfgrass rhizosphere soil than in the higher organic matter environment of the forest and agricultural soils (Table 4), suggesting that sorption may impact chlorothalonil availability to fungi as well as bacteria.

The comparison of excised band sequences to those published in the Genbank database (Table 5) suggested that the impact of the fungicide treatments was not confined to any one group of bacteria or fungi. Sequencing revealed that the bacteria affected by the chlorothalonil treatment were most similar to members of three divisions that include the *Proteobacteria* (seven clones) and the *Cytophaga–Flexibacter–Bacteroides* group (1 clone). Within the *Proteobacteria*, four subdivisions were represented by at least one bacterial population impacted by chlorothalonil applications. Likewise, 28S rRNA gene sequences revealed that two phyla of fungi were impacted by the fungicide treatment. Fungal sequences were most similar to those of the vascular arbuscular mycorrhizal *Glomus* spp. and *Chaetomium* spp., both common terrestrial fungi genera (Dodd et al., 2000), and *Fusarium* spp., members of which are known maize pathogens (Soonthornpoc et al., 2000). Although several of the 16S/28S rRNA sequences were highly similar (>95%) to published Genbank sequences, short rRNA gene sequences (approximately 194 bp for bacteria and either 200 or 420 for fungi) contain too little information to confirm organism identity. Nevertheless, it is clear that all chlorothalonil application rates impacted a variety of bacteria and fungi especially in the sandy turfgrass rhizosphere where sorption appeared to play a role in the impact on the community structure.

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