Molecular Analysis of Soil Bacterial Communities Following Application of 2,4-Dichlorophenoxyacetate (2,4-D)

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Abstract

Soil microcosms were constructed from conventional till, zero till and successional field soils to investigate the effect of the management history of the soil on bacterial communities after 2,4-D application. After amended 2,4-D had been degraded, the bacterial community was analyzed by most probable number (MPN) counts of total heterotrophs and 2,4-D degraders and by Small subunit ribosomal DNA (SSU rDNA) analysis using Amplified Ribosomal DNA Restriction Analyses (ARDRA) and Terminal Restriction Fragment Length Polymorphism (TRFLP). The microcosms observed from each of the different field plots showed shifts in the community by both culture and SSU rDNA analyses. The same dominant ARDRA pattern appeared from the SSU rDNA genes amplified from the community DNA from each of the field soils. 2,4-D degrading isolates were also obtained from terminal MPN tubes showing growth on 2,4-D. Some of the isolates also had this ARDRA pattern. Analyses of the partial sequence of the SSU rDNA genes from these isolates identified them as close relatives of the *Burkholderia* genus. Since dominant microbial members selected in each treatment appeared to be the same, the management history of the soil did not influence the selection of dominant 2,4-D degraders. The diversity index measured before and after 2,4-D addition by Shannon-Weiner equation using the ribotype number as species number and peak area the species abundance showed that management history of the soil did influence ribotype diversity.

Key words: Degradation, diversity, herbicide, gene probe, microbial community, ribosomal DNA

Introduction

The use of pesticides such as 2, 4-di-chlorophenoxyacetate (2,4-D) continues to increase annually. 2,4-D has been found to be environmentally safe because of the ease by which it is degraded by soil microorganisms (Foster & Mckercher, 1973; Loos *et al.*, 1979). Since the half-life of the herbicide range from 4 to 31 days depending on soil environmental condition and soil type, it is generally accepted that 2,4-D does not persist in the soil beyond one growing season (Smith & Hayden, 1981). However, Thompson *et al.*, (1984) showed that after applying 2,4-D at the normal field rate (1 mg/kg), they observed a chemical residue close to detection limit (approximately 5 $\mu g\ kg^{-1}$) by the end of the growing season. Even though residues of this magnitude were unlikely to have any biological significance, no studies have been done to elucidate the persistence of high 2,4-D concentrations in sites where the herbicide has been applied for sometime. Microbial community could be different in sites that have a prior 2,4-D history as compared to sites that had not been exposed to the herbicide. Ou (1984) observed higher concentrations of 2,4-D degrading organisms at sites that had been contaminated with 2,4-D compared to sites that had not been exposed to the herbicide. Also, 2,4-D degraders have been isolated from agricultural or Industrial sites exposed to xenobiotic chemicals with success ((Bhat et al., 1994; Chaudry & Huang, 1988; Don &

Pemberton, 1981; Hartman et al., 1979) while others have experienced difficulty in isolating 2,4-D degraders from non-agricultural soils (Takami et al., 1994) suggesting that microorganisms responsible for 2,4-D degradation in pristine sites are different. Thus, Kamagata *et al.* (1997) isolated 2,4-D degraders from pristine environments and found their growth rates slower than the slow growing 2,4-D degrading isolates obtained from agricultural soil studied by Ka *et al.* (1994). Holben *et al.*, (1994) and Xueqing *et al.* (1995) did not observe differences in the response of soil microbial community that had a prior 2,4-D application history and that of the control soil with no such treatment when subjected to 2,4-D selection. One would expect that soil communities with history of exposure to 2,4-D would have populations adapted to the herbicide while communities with history of limited or no exposure to 2, 4-D might be more sensitive. However, this was not observed. Thus, it is unknown the extent 2,4-D history of a soil influences the microbial community.

Soil management practices such as conventional till and no till could also affect the microbial community. Generally no-till plots have better aggregate structure, being platy near the surface and having interconnection of fine pores throughout the profile whilst the conventionally tilled soil is composed of granular and fragmented structural units with no evidence of earthworm activity (Drees *et al.* 1994). Even though these differences exist, the primary question is how both the prior management and the 2,4-D histories of the soil will effect the microbial community when 2,4-D selection is exerted. To investigate this further, soils similar in soil type, origin and climate history but which had been exposed to different land practices and different 2,4-D application history were chosen. Significant differences in the structures of the resident communities were expected in the samples of soil used and their responses to 2,4-D application to be different.

The objective of this study was to investigate the effect of 2,4-D selection on microbial population of soils with different tillage practices and 2,4-D prior history.

Materials and methods

Peptone-tryptone-yeast-extract-glucose (PTYG) medium, which contained 0.25 g of peptone (Difco Laboratories, Detroit, MI.), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate and 0.003 g of calcium chloride, was used to culture and determine the total viable counts of microorganisms. Most probable number of 2,4-D degraders in soil samples was determined by using the minimum medium amended (Stanier *et al.*, 1966) with 300 ppm 2,4-D.

Soils

Surface soil samples were collected from the Long Term Ecological Research (LTER) study area at the Kellogg Biological Station, Michigan, USA. The soil treatments were T1- the conventional tilled soil, T2- zero tilled soil and T8- which is a successional field that had not been exposed to 2,4-D for the past 40 years. The relevant histories of the soils used in the study are given in Table 1. For each treatment, a 2.5 cm diameter core was taken from 0-15 cm depth of the soil after removing the organic litter to expose the mineral soil. Ten such cores were taken randomly from the plot and mixed to obtain one composite sample per treatment replicate. Three replicate plots of each treatment were sampled. The composite samples were placed in plastic bags, kept on ice and stored at 4 °C until used. Inter sample contamination was avoided by

cleaning the core with border soils between each plot to be sampled. Soil moisture content was determined by weight difference after drying the soil overnight at 105 °C. All soils are classified as Typic Hapludalfs, fine loamy, mixed mesic.

Soil microcosm

The microcosm consisted of 300 g of soil that had been sieved through a 2 mm sieve and placed in a polyethylene bag. Each microcosm received the same concentration of phosphate and either 0 ppm or 100 ppm 2,4-D which had been dissolved in 0.1 *M* phosphate buffer. The moisture content was adjusted to 25 % (wt/wt) with sterile distilled water and the bags were incubated at 25 °C in the dark. The 2,4-D concentration in the soil was monitored by taking samples at given time periods (3 days intervals) and analyzing the extract by high pressure liquid chromatography (HPLC). The soil was amended with 2,4-D whenever the concentration of 2,4-D fell below 10 ppm. Such an amendment of 2,4-D per treatment was done five times. Three replicate microcosms constructed from each of the three replicate plots soils were incubated for each treatment.

Enumeration of bacteria

The enumeration of 2,4-D degrading bacteria and total heterotrophs was done by MPN for each soil sample before and after soil microcosm studies. MPN analyses were performed by inoculating 1.8 ml of 2,4-D medium with 0.2 ml of the final dilution of the serially diluted soil suspensions. Five replicate sets of tubes were assayed for each soil sample at each dilution. The inoculated tubes were incubated at 25 °C in a shaker for 3-4 weeks prior to analysis, after which 1 ml of the MPN medium was cleared of cells by centifugation for 5 min. High pressure liquid chromatography (HPLC) was performed on the supernatant, with positive tubes being scored as those with less than 30 ppm of 2,4-D remaining. Total heterotroph count was scored from the MPN tubes of PTYG medium that became visually turbid after 3 days. The most probable number of the culturable hetero-trophs and 2,4-D degraders in the soil sample was determined according to Cochran (1950).

Isolation of 2,4-D degraders

2,4-D degraders were isolated from soil samples of plots with different management

TABLE 1.
Management history of soil samples used

Soil treatment	*Soil management history*	*History of 2,4-D application*
T1	Conventional tilled. Under cultivation for 40 years prior to 1989, under high input corn/soybean rotation since 1989	2,4-D has been applied at the normal rate for 10 years
T2	Zero tilled. As treatment 1, but No till management since 1989	2, 4-D has been applied at the normal field rate for 10 years
T8	Successional field. Unfertilized field left to regrowth of plants for the past 40 years. Never tilled.	No 2,4-D has been applied for the past 40 years

practices, T1 (conventional till), T2 (zero till) and T8 (successional field). Isolates were obtained from the MPN culture tubes containing the highest dilution that exhibited 2,4-D degradation. These were enriched further by two additional transfers into fresh 2,4-D mineral medium. Each enriched culture was then plated onto 2,4-D agar medium (2,4-D mineral medium plus 0.1 % casamino acids and 1.5 % agar) and incubated at 30 °C for 2 -7 days. Single colonies were tested for 2,4-D degradation in fresh 2,4-D mineral liquid medium. The purity of the isolates was confirmed by streaking a 2,4-D agar medium or R2A agar medium.

Quantization of 2,4-D biodegradation

The biodegradation of 2,4-D was measured as the disappearance of the compound as determined by HPLC. At appropriate time points (normally at three day intervals 1g samples were taken from the 2,4-D treated soils and combined with 1 ml of sterile distilled water in a micro centrifuge tube. The soil slurry was mixed vigorously for 1 minute and then pelleted by centrifugation for 5 min. The supernatant was filtered through 0.45 mm Acrodisc filters (Gelman, Ann Arbor, MI) and then analyzed by HPLC on a Lichrosorb RP-18 column (Anspec Co., Ann Arbor, Mich.) with methanol and 0.1 % H_3PO_4 (60: 40) as the eluant.

Purification of microbial community DNA from soil

Microbial community DNA was extracted and purified from 5 g of each soil as described by Zhou *et al.* (1996). The extracted DNA was run through 0.8 % low melting agarose gels overnight in order to separate the humates. The excised DNA was purified further by using the DNA Wizard kit following the manufacturer's protocol. DNA was quantified spectrophotometrically by measuring absorption at 260 nm.

Small subunit ribosomal DNA (SSU rDNA) restriction analysis

Amplified ribosomal DNA restriction analysis (ARD RA) was carried out following PCR amplification of SSU rDNA using Eubacteria primers 49F (the sequence is as follows, 5' TNANACATGCAAGTCGR RCG 3') where N=dK/dP and R=dK and 1510R (5' RGYTACCTTGTTACGACTT 3) where R=dK and Y=dP' as described by Moyer (1997). For each 50 µl reaction 1 µl (50 -100 ng) of soil DNA was used as template. Approximately 10 µl of PCR product was subsequently used for each restriction digestion. Amplified 16S rDNA was double digested with MSP1 and RSA1, Hha 1 and Hae III according to the manufacturer's recommendation. Restriction fragments were resolved by gel electrophoresis in 3 % Metaphor agarose (FMC) and later visualized in ethidium bromide.

SSU rDNA Terminal Restriction Fragment Length Polymorphism (TRFLP)

PCR amplification of SSU rDNA was performed using 8F (5'- TCTGGTT-GATCCTGCCAGAG-3') Hex and 1392R (5' -ACGGGCGGTGTGTACA- 3') primers labeled with a fluorescent dye at the 5' end. DNA amplification was verified by electrophoresis of the PCR mixture (3 µl) in 1.0 % agarose in TAE buffer. The PCR products were purified using Wizard PCR purification columns (Promega, Madison, Wis.) and were eluted in a final volume of 50 µl. Aliquots of the products were digested separately with each of the above restriction enzymes. The TRFLP fingerprint of each community was determined by using the 373A automated sequencer (Applied Biosystems

Instruments, ABI, Foster City, Calif). From the resulting electropherograms, the ribotype diversity indices before and after 2,4-D addition were calculated using the Shannon-Weiner diversity index which is expressed as $H=\Sigma pi \ln pi$, pi is the relative density, ribotype number is the species number and the peak area is the species abundance.

Results

Bacterial numbers

The total viable bacterial count before and after 2,4-D application remained relatively constant at approximately 10^8 cells /g soil, (Fig. 1). The density of 2,4-D degraders on the other hand was lowest for treatment T8, which had no prior exposure to 2,4-D (21 degraders before 2,4-D addition whilst treatments T1 and T2, which had a previous 2,4-D exposure, had high initial numbers of 10^4/g soil). After the five additions of 2,4-D, the number of 2,4-D degraders increased to 10^8 / g in all soil treatments indicating the equivalent enrichments of 2,4-D degraders in all soils.

Dominant 2,4-D degraders

Dominant 2,4-D degraders present in the terminal MPN tubes were isolated from all soils. The strains were distinguishable by cell or colony morphology and most of them were gram negative. Partial SSUr RNA analysis showed that they were all β-protoebacteria (Table 2). It is possible that some 2,4-D degrading micro-organisms were diluted out in the batch enrichment procedure so that not all the dominant species in soil were isolated. The 2,4-D degrading taxon found most frequently in the three treatments soils (T1, T2, and T8) after 2,4-D application was *Burkholderia* sp.(Table 2). All the isolates were screened for *tfdA* by PCR amplification using conserved *tfdA* primers (Fig. 2). The

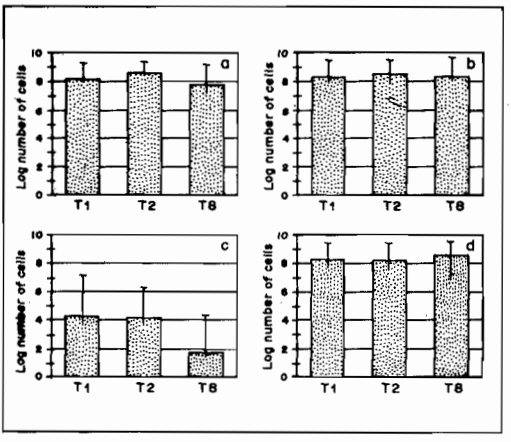

Fig. 1. MPN of 2, 4-D degraders and total viable comb (or total heterotroph) before and after 2, 4-D selection (a) Total viable count before 2, 4-D selection, (b) Total viable count after 2, 4-D selection. (c) MPN or 2, 4-D degraders before 2, 4-D selection (d) MPN of 2, 4-D degraders after 2, 4-D selection.

tfdA gene encodes the first enzyme of the 2,4-D degradative pathway. This gene is specific for most 2,4-D degraders and it transforms 2,4-D to 2,4 dichlorophenol and glyoxylate (Fukumori & Hausinger, 1993). Hybridization of the amplicon to a *tfdA* gene probe of the pJP4 was done. A high stringency wash was used (60 C, 0.1X SSC) to decrease the likelihood of detecting false positives from unknown genes that may have common sequences with the probes but having no activity against 2,4-D. This high degree of stringency would also decrease the likelihood of detecting homology with forms of the target genes that were divergent. Almost all the isolates from the treatments had the *tfdA* gene, (Fig. 2). Thus most of the strains isolated from these had the *tfdA* sequences that shared high degree of similarity to that of pJP4, a plasmid of *Ralstonia eutropha* JMP134 that has been extensively characterized for 2,4-D degradation genes.

TABLE 2

Characteristics of 2, 4-D degrading isolates from soils of differet management history

Treatment	*Isolate*	*Putative identification*	*Similarity in %*	*Presence or absence of tfdA*	*Morphology description on agar medium*	*Lag period when grown on 2, 4-D medium in hrs*
0 ppm plot	*001	*Burkholderia graminis*	100	+	very small tiny spots transparent, Gram negative short rods	19
100 ppm plot	012	*Variovorax paradoxus*	99	-	yellowish gummy colony which are spreading > 2.0 mm. Gram negative scattere cocci	19
	023	*Ralstonia eutropha*	97	+	almost whitish translucent colonies of size 2 mm. Gram negative cocci	36
	026	*Alcaligenes sp.* str. M91-3	97	-	very whitish, gummy, opaque and irregular spreading colonies	26
	*029	*B. carophylli*	95.6	+	Colony whitish yellow, 2 mm in diameter, roundish in size, smooth edges, translucent and Gram negative cocci to short rods	12
T1	*007	*B. graminisi*	100	+	translucent, whitish yellow colonies, colony about 2 mm in diameter, Gram negative cocci to short rods	12
	010	unidentified	-	+	yellow opaque colonies of 1.6 mm in diameter. Gram negative short rods scattered	
	022	*Rhodopseudomonas palustris*	98	-	whitish yellowish colony Gram negative scattered cocci	40
T2	017	*R. uetropha*	99	+	Gram positive light yellow colony, transparent and 1.5 mm is the diameter of the is the diameter of the colony	20
	*028	*B. graminis*	96	+	Colony are whitish yellow and 1.5 mm in size, colonies have smooth edges, translucent, Gram negative and clustered.	10

TABLE 2 continued

Treatment	*Isolate*	*Putative identification*	*Similarity in %*	*Presence or absence of tfdA*	*Morphology description on agar medium*	*Lag period when grown on 2, 4-D medium in hrs*
T8	019	*Burkholderia pseudomallei*	96	+	Whitish roundish colonies with sommth edges, colony size about 1.8 mm. Translucent, Gram negative cocci to short rods.	24
	*027	*Burkholderia caryophylli*	97	+	Whitish yellowish colony, 1.5-2.0 mm in size, Gram negative cocci to short rods.	18
	034	unidentified	-	-	small whitish colonies of size less than 2 mm	40

*common genus in all the treatments

SSU rDNA analysis

Community ARDRA pattern of the microcosm soil showed an initial population where none of the banding patterns showed dominance, i.e., before the addition 2,4-D (Fig 3). However, after the 2,4-D selection, certain bands disappeared and few others were dominant (increased in intensity). These dominant bands were observed in all the soil treatments suggesting that irrespective of the soil history the same dominant 2,4-D degraders were selected. Some of the 2,4-D isolates from the different soils had a similar ARDRA after 2,4-D selection (Fig. 4), especially isolates of lanes 2, 7, 9, 15, 18, 20 and 31.

The similarity of the isolate and community ARDRA patterns was confirmed by running the restricted digested products of some of these isolates side by side with that of the community DNA with the band distribution pattern of the isolates coinciding with that of the community ADRA pattern. Partial SSU rDNA sequencing analysis showed that these isolates (strains 001, 007, 027, 028 and 029, Table 2) belonged to the genus *Burkholderia*. The lag phase of these dominant ones appeared shorter than the rest of the isolates, < 12 hrs (Table 2), and this might be a

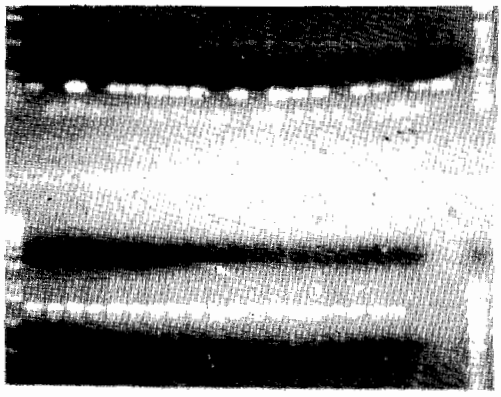

Fig. 2. tfdA amplification of isolates from soils of different management histories (Top part). Size of fragment is 360 bp. Bottom part is 16 s rDNA of isolate to act as control for the above reaction.

competitive advantage to the isolates. ARDRA pattern of isolates of Ka *et al.* (1994) who had done a previous isolation from the plots were compared to the recent isolates to observe differences in the banding pattern with time. Slight differences in banding pattern were observed when one compares lanes 26-30 to lanes 2,7,9 and 15.

Electropherograms of the TRFLP also showed a shift in the microbial community after 2,4-D addition (Fig. 5). A shift in the terminal restriction fragments (TRF) from

the 400 and 550 bp region to the 100 to 150 bp region was observed (Fig. 5). TRF of 298, and 545 bp were no longer seen after 2,4-D addition indicating that such microbial populations might be susceptible to high rates of 2,4-D application. On the otherhand after applying 2,4-D, TRF of 141, 151, and 408 bp increased in peak intensity. A soil TRF of 140-141 bp with *Msp1* coincided with the TRF of *Burkholderia* sp. After 2,4-D addition to the different soils, the TRF 141 bp, 151 bp products became the primary and secondary dominant peaks in T1 and T2. In soil T8, only a TRF of 141 bp was the primary dominant, the secondary dominant was an 834 bp fragment.

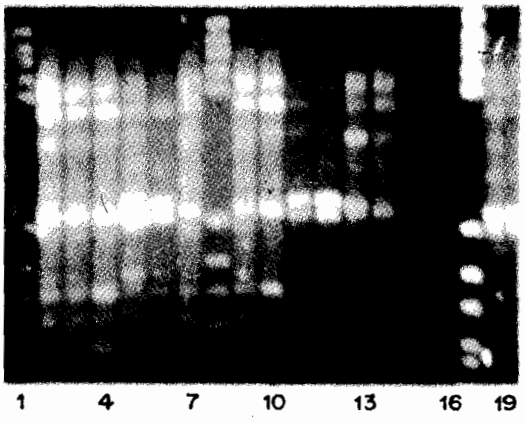

Fig. 3. Community ADRA pattern of soil treatments before and after 2, 4-D addition. Lanes 1, 8, 16, and 17 are marker DNA V; Lanes 2 to 4, T1 before 2, 4-D addition, Lanes 5 6 T1 after 2, 4-D addition, Lanes 7, 9, 10, T2 before 2, 4-D; Lanes 11 to 12, T2 after 2, 4-D, Lanes 13 to 15, T8 before 2, 4-D, and lanes 18 and 19, T8 after 2, 4-D.

In all the treatments the ribotype diversity decreased after 2,4-D addition (Table 3) and there was no significant difference in the diversity indices of the soil treatments after 2,4-D addition. Prior to 2,4-D selection, the diversity index of T8 was the highest being significantly different from the other treatments. An analysis of the changes of the diversity indices before and after 2,4-D selection (Table 3) demonstrated that the effect of 2,4-D was drastic on T8 where the decrease in the ribotype diversity was 34 % and this was significantly different from all the other treatments. Changes in the diversity index for the zero tilled soil was not as dramatic as that of T1 even though both soil treatments had prior exposure to 2,4-D before

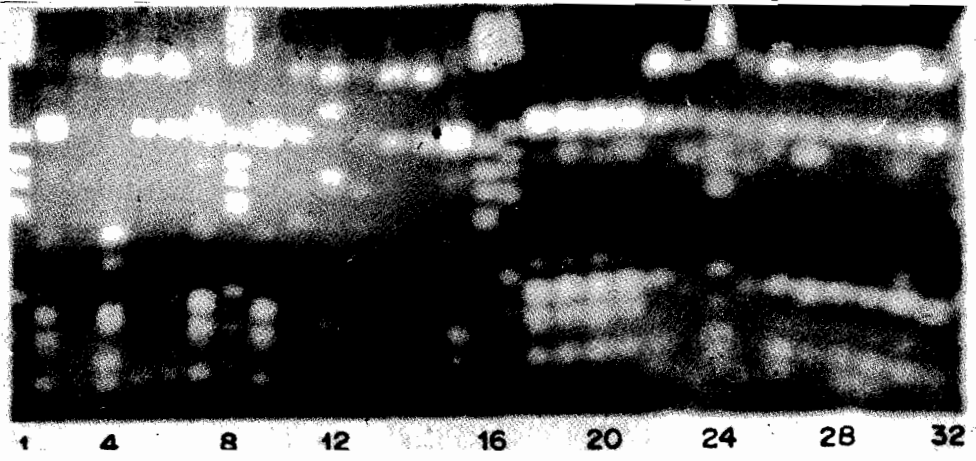

Fig. 4. ARDRA pattern of 2, 4-D isolates from soil samples of different management history. Lanes 1, 8, 16, 17, 24, and 32 are marker DNA V. Lanes 2 and 3 are restriction fragments of isolates from 0 ppm 2, 4-D Gene Transfer Plots, lanes 4 to 7 are isolates from 100 ppm plot 2, 4-D plots, lanes 9 to 12 are isolates from T1, lanes 13 to 15 are isolates from T2 and lanes 18 to 23 are isolates from T8 and lanes 25 to 31 which are strains 1443, 9112, 712, 1173, 9157, 912-2, and 9226 isolated by Ka et al. (1994).

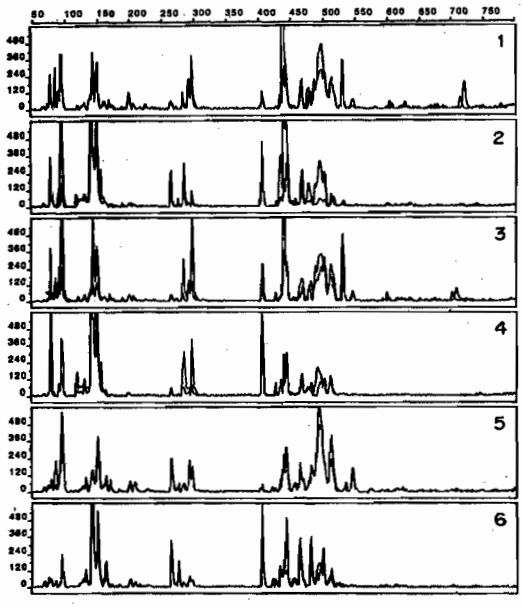

Fig. 5. Electropherograms of Terminal Restrictions Fragments of soil samples before and after 2, 4-D addition in soil microcosm experiments. 1 & 2 represent T1 before and after 2, 4-D addition. 3 and 4 represent T2 before and after 2, 4-D addition, 5 and 6 represent T8 before and after 2, 4-D addition. Electropherograms are overlaps of 2 replicates.

the experiment. This difference observed between T1 and T2 might be due to the better aggregate structure of T2 which had interconnection of fine pores. The better aggregate structure is likely to create more pores for microorganisms to escape the toxic effect of 2,4-D.

Discussion

Three methods, viz cultural method, and two types of SSU rDNA analysis were used to analyze the microbial community of a soil microcosm and all showed shifts in microbial community structure. The combination of both the MPN of 2,4-D degraders and the total heterotroph count showed that the culturable component of the microbial community had shifted. ARDRA resolves from the subgenera to the species level and it detects the more dominant community members. For a given soil treatment, differences in the ARDRA patterns observed before and after 2,4-D addition suggest that there has been a shift in the microbial community where new members of the microbial community were dominant. The similarity of the ARDRA pattern after 2,4-D addition irrespective of the treatment suggested that similar members of the community were selected when 2,4-D was the primary carbon source.

ARDRA pattern of some isolates obtained from the various soil treatments matched that of community ARDRA pattern, suggesting that the dominant population in all the

TABLE 3

Changes in diversity index of treatments before and after 2,4-D applications

Soil treatment	*Ribotype number before 2,4-D*	*Ribotype number after 2,4-D*	*Diversity index before 2,4-D*	*Diversity index after 2,4-D*	*% Change in diversity index*
T1	52a	35a	3.88a	2.86a	26b
T2	50a	37a	3.68a	3.04a	17a
T8	62b	37a	4.10b	2.71a	34b

Means followed by the same letter are not significantly different at the 5% level of significance according to the Duncan's Multiple Range Test.

treatments might be culturable and that the isolates obtained were not different but members of the existing soil microbial community. Matching ARDRA band patterns cannot be used to unequivocally establish the members of the community (Massol-Deya *et al.*, 1995). For T1 and T2, at least two dominant populations were observed by TRFLP. This means the coexistence of the dominant populations under 2,4-D selection. The possible explanation for coexistence of these populations might be that competition for nutrients might not be direct thus one population might adhere on surfaces of the soil particles and the other population might be in soil solution. The differential exploitation of any of these dimensions may explain regional coexistence of different species (Frenchel, 1987).

Previous studies by Xueqing *et al.* (1995) did not show differences in the resident microbial communities after applying 2,4-D at the normal field rate. By adding to the soil microcosm 100 times the normal 2,4-D application rate might lead to more selection so that the desirable effects are observed but this was not the case since dominant members selected appeared to be the same. The consistency of the viable counts before and after applying 2,4-D suggests that they might be the keystone species of the community and the members are easily replaced when a member dies. Treatment T8 had the lowest 2,4-D degraders before 2,4-D addition because this plot had never been plouged at the Kellogg Biological Station and provides a historical reference point for below ground processing and population. The undisturbed soil profile of this site contains twice the soil organic matter (ca. 2 %) of the historically-tilled main site (Robertson *et al.*, 1997).

The presence of a high number of species (high diversity) in T8 especially allows for many interspecies relationships before 2,4-D addition. The decrease of the ribotype diversity after 2,4-D selection of T8 might be due to toxic effect of 2,4-D on the microbial populations and also partly due to new biomass which dilutes the original biomass such that they are not as abundant as before 2,4-D application.

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