CHAPTER TWO

A MOLECULAR ANALYSIS OF THE SHORT TERM RESPONSE OF SOIL BACTERIAL COMMUNITIES TO 2,4-D SELECTION

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 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 isolate 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 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. Rather, 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 this parameter.

Introduction

The use of pesticides, such as 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. Since the half life of the herbicide ranged from 4 to 31 days depending upon soil environmental condition and soil type, it is generally accepted that 2,4-D did not persist in the soil beyond one growing season. 2,4-D was used as a model compound in this study because of the large body of knowledge available concerning the ecology (2, 23, 24, and 25) biochemistry (12, 15) and genetics (6, 16, 17, 26, and 27) of its biodegradation. Biodegradation of 2,4-D in soil, whether at high or low concentrations is influenced by other factors apart from the environmental ones. One of such factor is the prior 2,4-D application history of the soil.

Sites that had been contaminated with 2,4-D had higher concentrations of 2,4-D degrading organisms compared to sites that had not been exposed to 2,4-D, (20). Also, 2,4-D degraders have been isolated from agricultural or industrial sites exposed to xenobiotic chemicals with success (1,3, 6, 13, 14, and 30) while others have experienced difficulty in isolating 2,4-D degraders fom non- agricultural soils (29) suggesting that microorganisms responsible for 2,4-D degradation in pristine sites are different. This become obvious when Kamagata et al. (19) isolated 2,4-D degraders from pristine environments and found that their growth rates were slower than the slow growing 2,4-D degrading isolates obtained from agricultural soil studied by Ka et al. (18). Also, isolates obtained from contaminated sites or by the conventional enrichment with 2,4-D had genes of the canonical 2,4-D degradation pathway whilst sites without prior exposure to

the herbicide or with selection for low concentrations of 2,4-D without enrichment yielded other combinations of 2,4-D alleles (9).

One would expect that communities with long histories of exposure to 2,4-D would have populations adapted to the herbicide while communities with history of limited or no esposure to 2,4-D might be more sensitive. However this was not observed when soil that had history of direct 2,4-D application were compared to controls with no direct application of the herbicide (16, 33). Thus it is not clear whether the extent of prior 2,4-D use affects the soil microbial community. To investigate this further, three soils were chosen that were similar in soil type, origin and climate history but had been exposed to different land practices namely conventional till, zero till and successional vegetation following abandonment of agriculture. The latter soil had never been directly exposed to 2, 4-D. It was thought that the different land use practices and differences in prior exposure to 2,4-D would result in significant differences in the structures of the resident communities and that their responses to the experimental application of high concentrations of 2,4-D might be different. This study elucidates whether the prior management history of the soil affects the selection of the dominant microbial population and/or changes in the diversity index following short term 2,4-D application.

2.0 Materials and Methods

Media and reagents: 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 for MPN counts of

heterotrophs. Most probable number of 2,4-D degraders in soil samples was determined by using the MMO medium amended with 300 ppm 2,4-D (28).

Soils: Surface soil samples were collected in August 1996 from three sites in the LTER study area (Long Term Ecological Research) at the Kellogg Biological Station (KBS). For each treatment, a 2.5-cm diameter core was taken from 0-15 cm depth of the soil after removing the organic litter and exposing 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 use. Intersample 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. The relevant history of the soils are given in Table 1. All soils are classified as Typic Hapludalfs, fine loamy, mixed mesic.

Soil microcosms: 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 identical concentrations 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 soil samples at given time periods 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. Five sequential amendments of 2,4-D were done per treatment. 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 serially diluted soil suspensions. Five replicates sets of tubes were assayed for each soil sample at each dilution. The inoculated tubes were incubated at 25° C with shaking for 3-4 weeks prior to analysis, after which 1 ml of the MPN medium was cleared of cells by centrifugation 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 heteroph count was scored from the MPN tubes of PTYG medium that became visually turbid after 3 days. The most probable number of the culturable heterotrophs and 2,4-D degraders in the soil sample was determined according to Cochran (4).

Isolation of 2,4-D degraders: 2,4-D degraders were isolated from soil samples of plots with different management practices, T1 (conventional till), T2 (zero till) T8 (successional field), and the 0 & 100 ppm 2,4-D treated subplots at KBS. 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 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 to 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 broth sample or R2A agar medium.

Quantitation of 2,4-D biodegradation: 2,4-D biodegradation was measured as the disappearance of the compound as determined by HPLC. At appropriate time

points, 1 g samples were taken from the 2,4-D treated soils and combined with 1 ml of sterile distilled water in a microcentifuge 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. (34). 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.

SSU rDNA restriction analysis: Amplified ribosomal DNA restriction analysis (ARDRA) was carried out following PCR amplification of SSU rDNA using eubacterial primers 49F and 1510R as described by Moyer (22). For each 50 ul reaction, 1 ul of purified soil DNA was used as template. Approximately 10 ul of PCR product was subsequently used for each restriction digestion. Amplified 16S rDNA was double digested with *Msp*1 and *Rsa*1, *Hha* 1 and *Hae* III according to the manufacturer's recommendation. Restriction fragments were resolved by electrophoresis in 3 % Metaphor agarose (FMC) gels containing TAE and later visualized in ethidium bromide.

SSU rDNA TRFLP: PCR amplification of SSU rDNA was performed using 8F-Hex and 1392R primers labelled with a fluorescent dye at the 5' end. DNA amplification was verified by electrophoresis of aliquots of the PCR mixture (3 ul) in 1.0 % agarose in TAE buffer. The PCR products were purified using Wizard

PCR purification colums (Promega, Madison, Wis,) and were eluted in a final volume of 50 ul. Aliquots of the products were digested separately with each of the above restriction enzyme. The TRFLP fingerprint of each community was determined by using the 373A automated sequencer (Applied Biosystems Instruments, ABI, Foster City, Calif).

Results

Bacterial numbers: The total viable 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 otherhand was lowest for treatment T8, which had no prior exposure of 2,4-D (21 degraders) before 2,4-D addition whilst treatments Tl 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 enrichment 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. All strains were distinguishable by cell or colony morphology. All were gram negative. Partial SSUr RNA analysis showed that they were all Beta Protoebacteria. 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 *tfd*A by PCR amplification using conserved *tfdA* primers (Fig. 1) and then by hybridization of the amplicon to a *tfdA* gene probe of the pJP4. 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 *tfd*A gene; only few did not, (Table 3). Thus most of the strains isolated from these had *tfdA* sequences that shared high degree of similarity to the *tfd*A of pJP4.

SSU rDNA analysis: Community ARDRA analysis of the microcosm soil showed an initial complex community where no population was dominant before 2,4-D addition (Fig. 3). However, after 2,4-D had been applied dominant members were selected in all treatments (T1, T2, & T8) and the banding pattern suggested that, irrespective of the soil history, the same dominant 2,4-D degraders appeared. Many of the isolates from soils of different management practices also showed the same ARDRA pattern, and thus this pattern matched that from the soil community DNA. The similarity of the isolate and community ARDRA patterns was confirmed by running the restricted digested products of these isolates side by side with that of the community DNA (Fig. 5). Partial SSU rDNA sequencing analysis showed that these isolates (strains 001, 007, 027, 028, and 029) belong to the *Burkholderia* genus. The lag phase of these dominant ones appeared shorter than the rest of the isolates, < 12 hrs (Table 2).

Electropherograms of the TRFLP also showed a shift in the microbial community after 2,4-D addition (Fig 6). A shift in the terminal restiction fragments (TRF) from the 400 and 550 bp region to the 100 to 150 bp region was observed (Fig. 6). TRF of 298, and 545 were no longer seen after 2,4-D addition indicating that microbial populations might be susceptible to high rates of 2,4-D application. On

the otherhand after applying 2,4-D, TRF 141, 151, and 408 increased in peak intensity. A soil TRF of 140-141 bp with *Msp1* coincided with the TRF of *Burkholderia* sp. A plot of the % peak area and the size of the ribotype showed that 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 (Figs 7a &7b) and T2 (Figs 8a & 8b). In soil T8 (Figs 9a & 9b), only a of TRF 141 bp was the primary dominant:, the secondary dominant was an 834 bp fragment.

In all the treatments the ribotype diversity decreased after 2,4-D addition (Table 4) but the effect of 2,4-D was drastic on T8 where the decrease in the ribotype diversity was 34 %, even though T8 had the highest ribotype diversty before 2,4-D application. Similarly, diversity index was high for all the treatments before 2,4-D application but it decreased after 2,4-D application especially in T8 (Table 4). 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 the experiment. This difference observed between T1 and T2 might be due to the better aggregate structure of T2 which has interconnection of fine pore (8). The better aggregate structure is likely due to higher carbohydrate concentration in this soil possibly creating room for microorganism to escape the toxic effect of 2,4-D.

Discussion

Initially it was hypothesized that the dominant members selected in the agricultural soils will be different from these in the non- agricultural soils because it was expected that years of applying 2,4-D may have selected variants in the natural population that were more fit (better adapted) to use 2,4-D as substrate than the organisms in the T8 soil which had never been exposed to 2,4-D. Since

there is a low probability of a given variant having an enhanced fitness coefficient and there is not a natural means of rapid microbial dissemination in soil, it is perhaps not surprising that no such variants were encountered given the small sample size taken from the field. A second means by which prior history could affect community structure is the different populations could be favored by different plant cover, tillage or fertilizer treatments. This could result in different 2,4-D degraders becoming dominant.

MPN method was useful for determining the number of 2,4-D degrading organisms present in the samples, but reveals nothing about the diversity of the populations nor about the unculturable part of the microbial community. Dunbar (9) showed that MPN of total bacterial counts and of 2,4-D degraders were one log unit higher than plate counts on a given agar meduim. ARDRA on the otherhand provided an alternative method that does not rely on culturing for evaluation of dominant microbial community members. ARDRA suggested that a similar dominant community structure was selected when 2,4-D was the primary carbon source. Since the ARDRA pattern of the community matched that of some of the isolates it suggests tdhat the dominant population in all the treatrments might be culturable. 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 explaination for coexistence of these populations might be that competition for nutrients might not be direct. For example one population might adhere on surfaces (10) and the other population might be in soil solution. Also the niche is considered to have three dimensions: resources, habitat, and time. The differential exploitation of any of these dimensions may explain regional coexistence of different species (11)

The presence of a high number of species (high diversity) in T8 especially allows for many interspecies relationships before 2,4-D addition. A community that has a complex structure, rich in information as reflected by high species richness needs a low amount of energy for maintaining such structure (21). This lowered energy requirement is reflected in a lower primary productivity rates per unit biomass (20, 27). while a stable diversity level is maintained. Although stability is associated with high diversity (31) in a biologically acclimated microbial community, that does not imply that the community is able to cope with severe disturbance. The drastic effect of 2,4-D on T8 such that the decrease in the ribotype diversty after 2,4-D addition might be due to growth of the new biomass which dilutes the original biomass such that they are not as abundant as before 2,4-D application.

This study demonstrates that the prior management history of the soil does not play a critical role in detemining which members dominate after 2,4-D addition. A critical trail may be that the microbial populations with short a lag phase are favoured and hence dominate early. Such populations might be r - strategist with high Ks and high U max values. Management history instead helps to determine the changes in the diversity index when a single substrate is imposed.

Table 1. Management history of soil sample used

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

Table2. Characteristics of 2,4-D degrading isolates from soils of different management history

Treatment	Isolate	Putative identification	Similarity in %	Presence or absence of *tfdA*	Morphology description on R2A 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 scattered 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	36

					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. eutropha*	99	+	Gram positive light yellow colony, transparent and 1.5 mm 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
T8	019	*Burkholderia pseudomallei*	96	+	Whitish yellow roundish colonies with smooth edges, colony size about 1.8 mm. Translucent, Gram negative cocci to short rods.	24
	*027	***Burkholderia caryophylli***	97	+	Whitish yellow 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

Table 4. 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 the diversity index
T1	52	35	3.88	2.86	26
T2	50	37	3.68	3.04	17
T8	62	37	4.10	2.71	34

Shannon-Weiner diversity index is expressed as $H = \sum pi \ In \ pi$

Where pi is the relative density, Ribotype number is species number and peak area is species abundance.

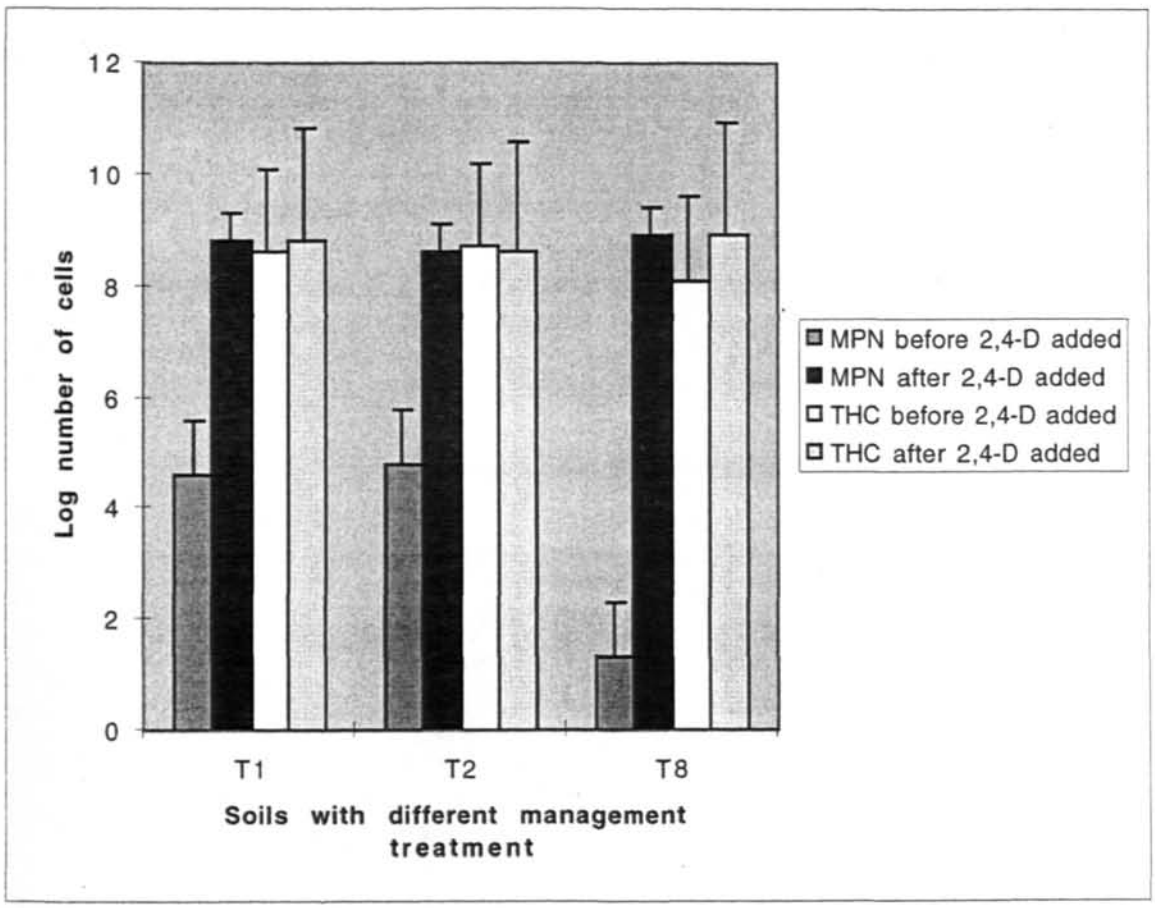


Fig. 1. MPN of 2,4-D degraders and of total heterotroph (THC) before and after 2,4-D addition in soil microcosm experiment. Bars are representative of three replicates i.e from three field plot replicates.

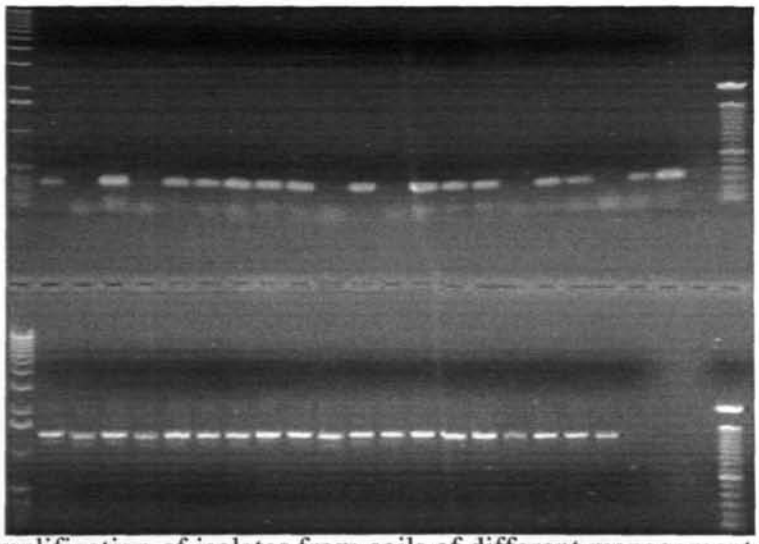


Fig. 2. *tfd*A amplification of isolates from soils of different management histories (Top part) . Size of fragment is 360 bp. Bottom part is 16 s rDNA of isolate to act as control for the above reaction.

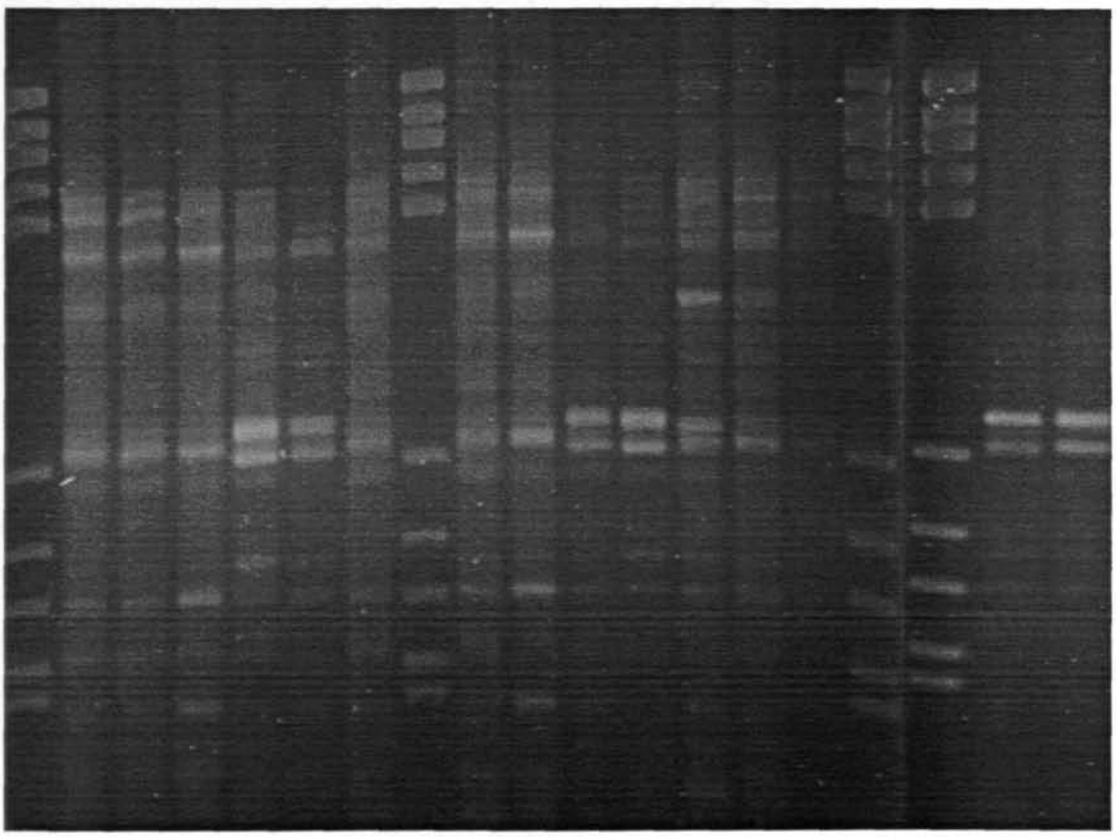


Fig.3. Community ARDRA pattern of soil treatments before and after 2,4-D addition. Lanes 1, 8, 16, and 17 are marker DNA V; Lanes 2 to 4, T1 before 2,4-D addition, Lanes 5 to 6 T1 after 2,4-D addition, Lanes 7, 9, 10, T2 before 2,4-D; Lanes 11 to 12, T2 after 2,4-D, Lanes 13 to 15, T8 before 2,4-D, and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

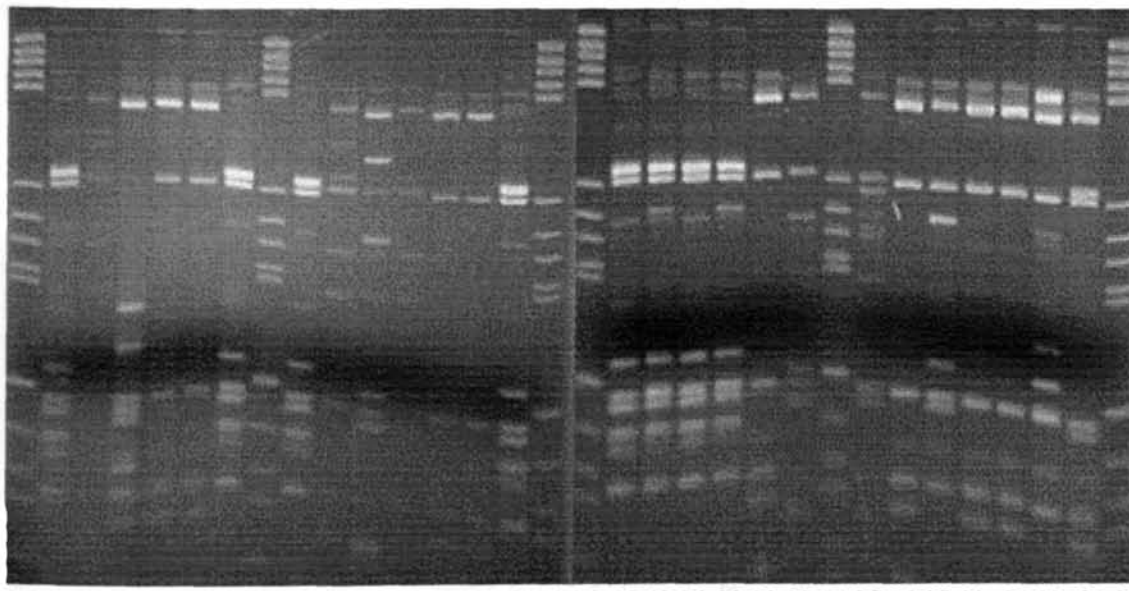


Fig 4. ARDRA pattern of 2,4-D isolates from soil samples of different management history. Lanes 1, 8, 16, 17, 24, and 32 are marker DNA V. Lanes 2 and 3 are restriction fragments of isolates from 0 ppm 2,4-D Gene Transfer Plots, lanes 4 to 7 are isolates from 100 ppm plot 2,4-D plots, lanes 9 to 12 are 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. (17).

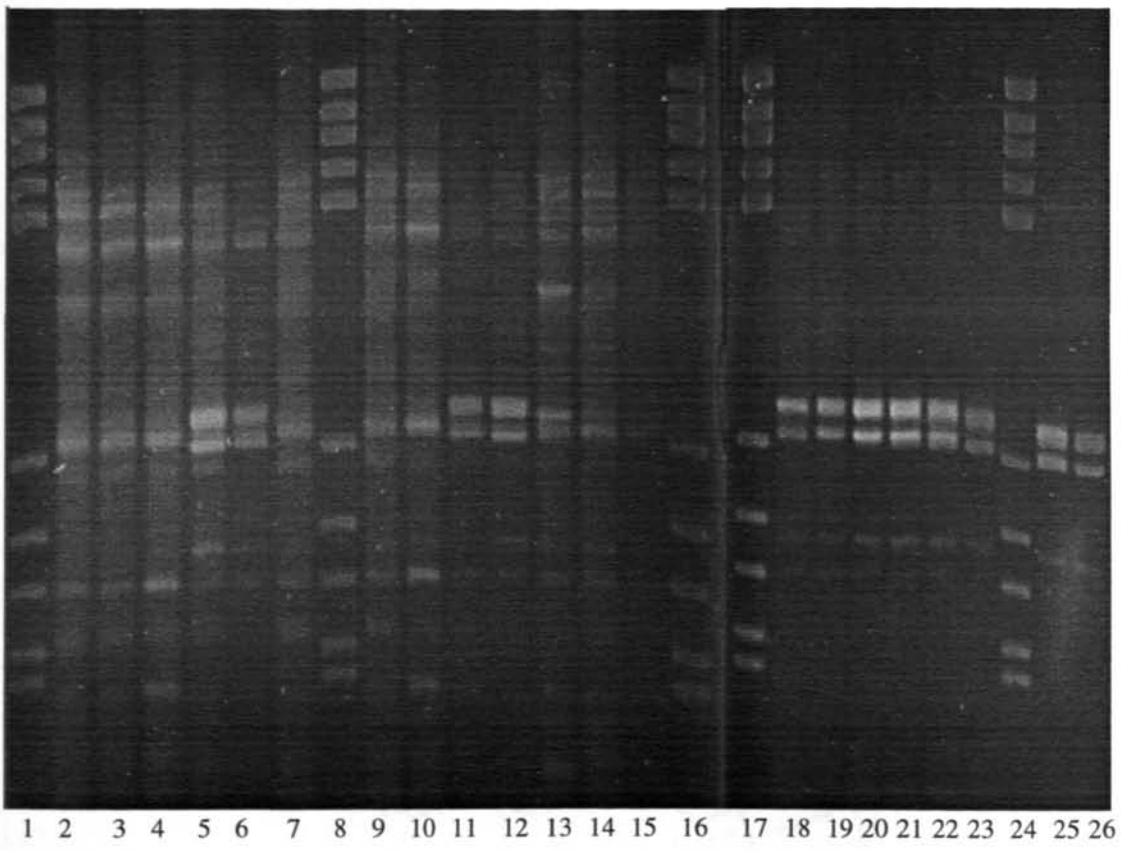


Fig.5. Effect of 2,4-D and soil management treatments on the selection of microbial communities as measured by community and isolate ARDRA. Lanes 1, 8, 16, and 17 are marker DNA V; Lanes 2 to 4, T1 before 2,4-D addition, Lanes 5 to 6 T1 after 2,4-D, Lanes 7, 9, 10, T2 before 2,4-D; Lanes 11 to 12, T2 after 2,4-D, Lanes 13 to 15, T8 before 2,4-D, and lanes 18 and 19, T8 after 2,4-D. Lanes 20, to 26 are isolates from T1, T2, and T8 strains # 001, 007, 019, 027, 028 and 029 respectively.

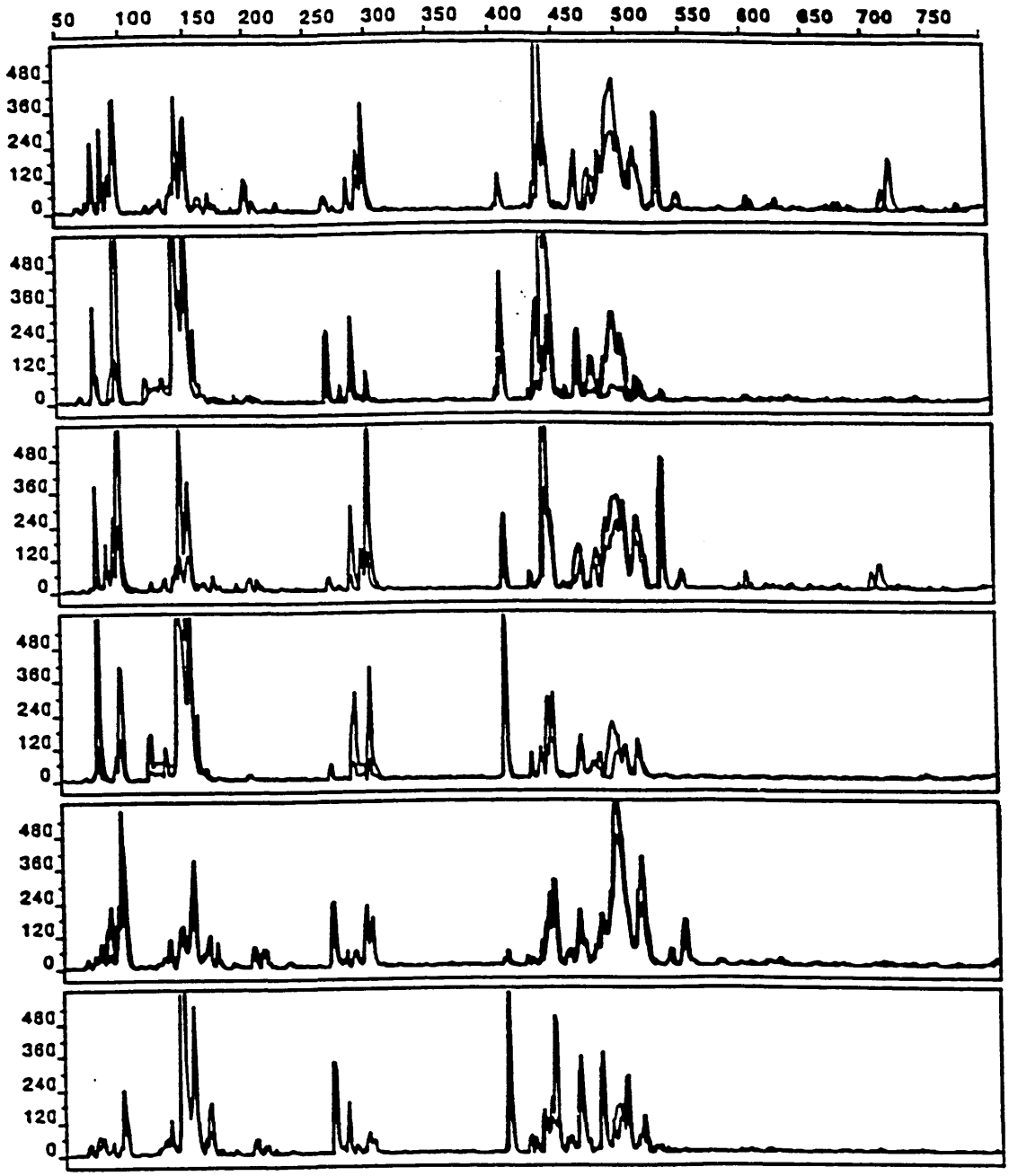
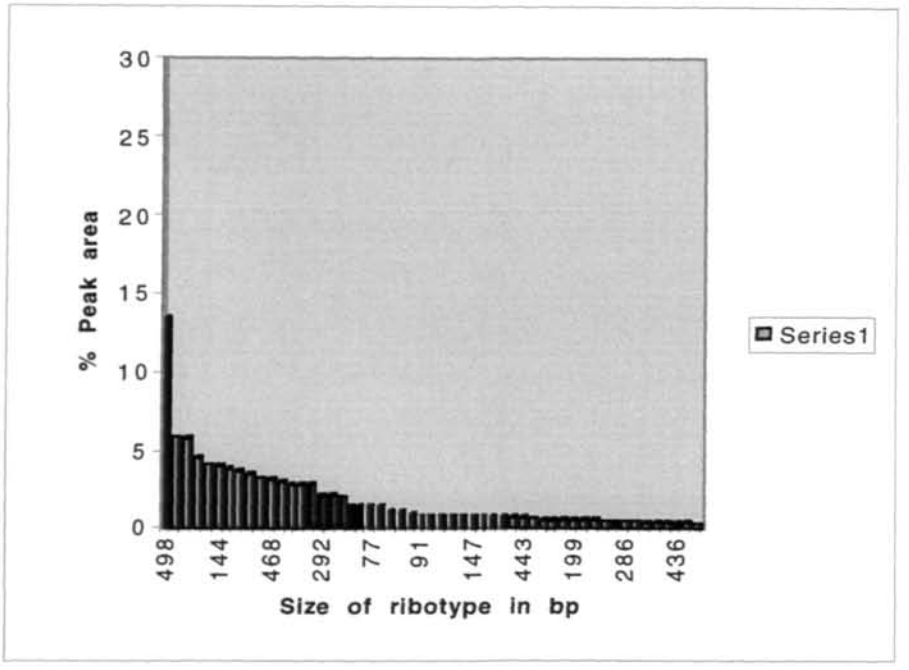


Fig. 6. Electropherograms of Terminal Restriction Fragments of Soil samples before and after 2,4-D addition in soil microcosm experiments. 1& 2 represent T1 before and after 2,4-D addition. 3 &4 represent T2 before and after 2,4-D addition, 5&6 represent T8 before and after 2,4-D addition. Electropherograms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



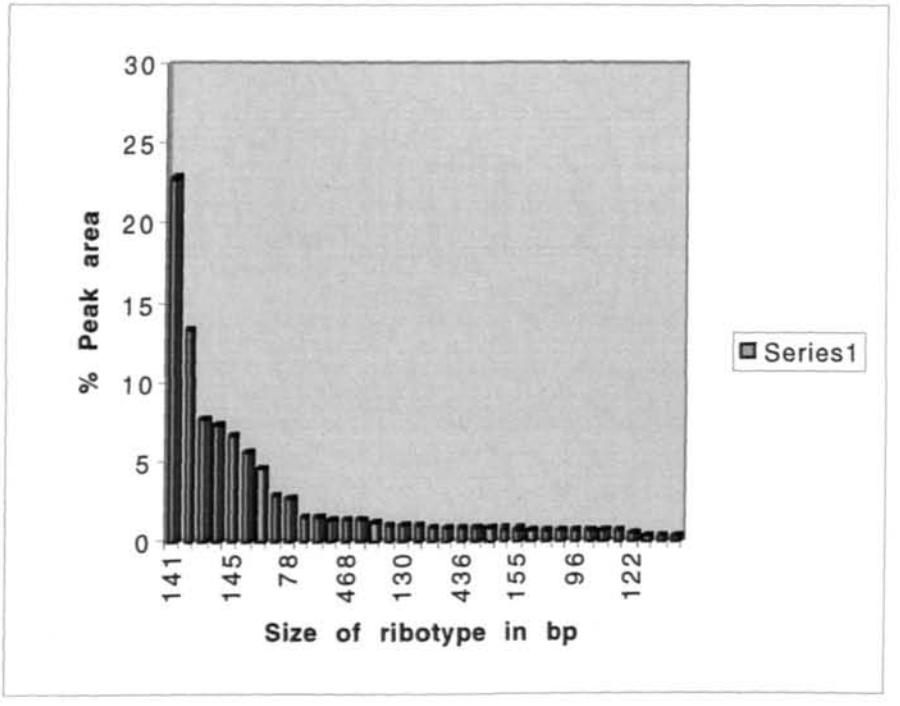
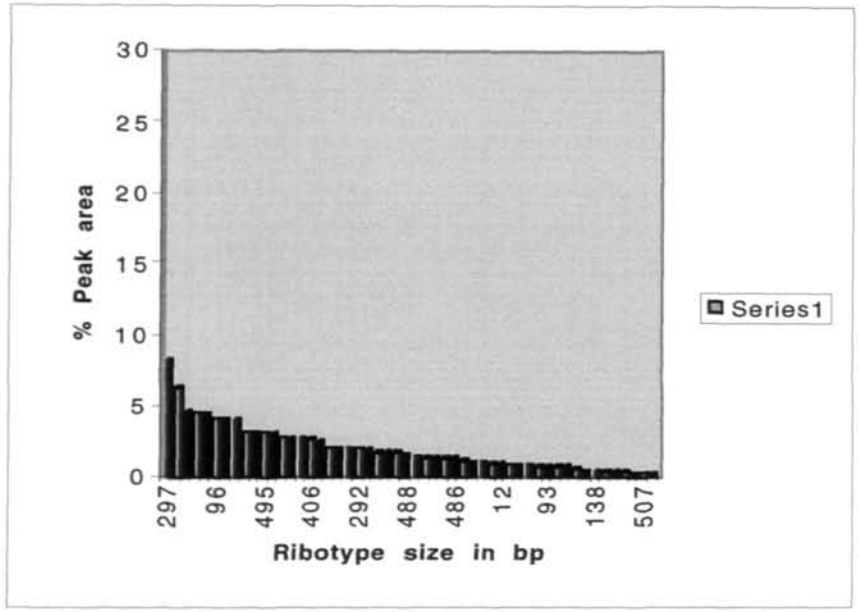


Fig. 7 a & 7 b. Relationship between the percentage peak area and size of ribotype of T1 before (top) and after (bottom) 2,4-D addition in soil microcosm experiment.



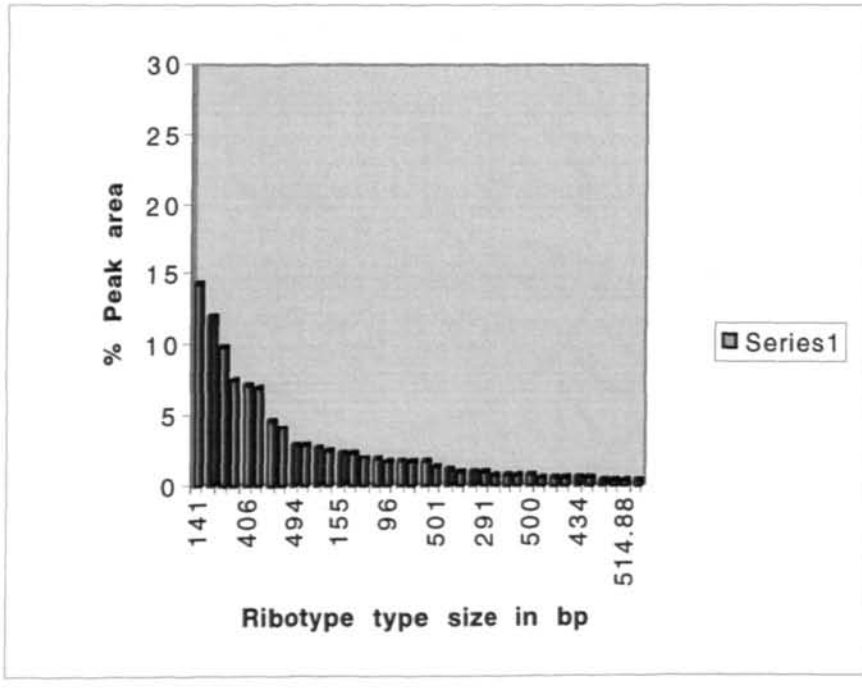
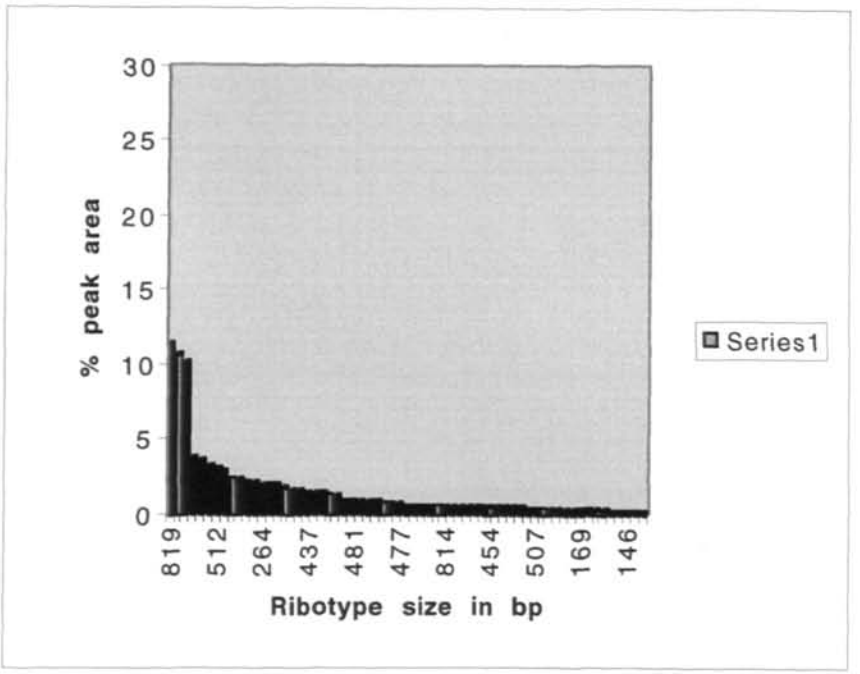


Fig. 8 a & 8 b. Relationship between the percentage peak area and size of ribotype of T2 before (top) and after (bottom) 2,4-D addition in soil microcosm experiment.



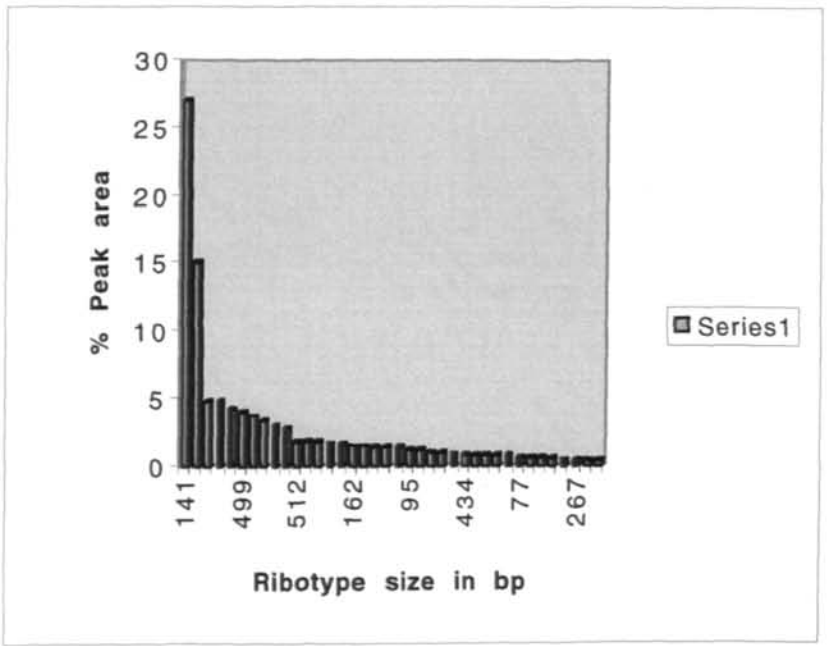


Fig. 9 a & 9 b. Relationship between the percentage peak area and size of ribotype of T8 before (top) and after (bottom) 2,4-D addition in soil microcosm experiment.

References

1. **Bhat, M.A., M. Tsuda, K. Horike, M. Naozaki, C.S. Vaidyabatgabm and T. Nakazawa.** 1994. Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. Appl. Environ Microbiol. **60**: 307-312

2. **Biederbeck, V.O., C.A. Campbell and A.E. Smith.** 1987. Effects of long term 2,4-D application on soil biochemical processes. J. Environ. Qual. **165**: 257-262.

3. **Chaudry, G.R. and G.H. Huang**. 1988. Isolation and characterization of a new plasmid from a *Flavobacterium* sp. which carries the genes for degradation of 2,4-dichlorophenoxyacetate. J. Bacteriol. **170**. (90); 3897-3902.

4. **Cochran, W.G**. 1950. Estimation of bacterial densities by means of the "most probable number". Biometrics **6**: 105-116.

5. **Crews, T.E. K. Kitayuama, J.H. Fownes, R.H. Riley, D.A. Herbert, D. Mueller-Dombois, and P.M. Vitousek**. 1995. Changes in soil phosphorus fractions and ecosystem dynamics across a long chronosequence in Hawaii. Ecology **76:** 1407-1424.

6. **Don and Pemberton. 1981**. Properties of six pesticide degradation plasmids from *Alcaligenes eutrophus*. J. Bacteriol. **145**: 681-686.

7. **Don R. H., A.J. Weightman, H.J. Knackmuss, and K.N. Timmis**. 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **161**: 85-90.

8. **Drees, L., R., A. D. Karathanasis, L. P. Wilding, and R.L. Blevins.** 1994. Micromorphological characteristics of long-term no-till and conventionally tilled soils. Soil Sci. Soc. Am J,. **58**. 508-517.

9. **Dunbar J.** 1995. Effect of selection on the structure of a 2,4-dichlorophenoxyacetate (2,4-D) degrading guild in soil. PhD thesis. Michigan State Univ. E. Lansing.

10 **Dykhuizen, D.E., and D.L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47**: 150-168.

11. **Fenchel T.** (1987) Ecology- potentials and limitations. p. 35-41. *In* Kinne, O. (ed.), Excellence in ecology, **1**. Ecological Institute, Olendorf/Luhe.

12. **Fukumori, F. and R.P. Hausinger.** 1993. Purification and characterization of 2,4-dichlorophenoxyacetate/ alpha-ketoglutarate dioxygenase. J. Biol. Chem., **268**: 24311-24317.

13. **Haller, H.D., and R.K. Finn.** 1979. Biodegradation of 3-chlorobenzoate and formation of black color in the presence and absence of benzoate. Eur. J. Appl. Microbiol. **8**: 191-205.

14. **Hartmann, J., W. Reineke, and H.-J. Knackmuss.** 1979. Metabolism of 3-chloro-, 4-chloro, and 3,5-dichlorobenzoate by a pseudomonad. Appl. Environ. Microbiol. **37**: 421-428.

15. **Hausinger, R.P., F.Fukumori, D.H. Hogan, T.M. Sassanella, Yoichi Kamagata, H. Takami and R.E. Saari.** 1996. Biochemistry of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation : Evolution implication. *In* Microbial diversity and genetics of biodegradation (K. Horikoshi, M. Fukuda, and T. Kudo, eds,), Japan Scientific Societies Press, Tokyo.

16. **Holben, W.E. , B.M. Schroeter, V.G.M. Calabrese, R.H. Olsen, j.K. Kukor, V.O. Biederbeck, A.E. Smith and J.M. Tiedje.** 1994. Gene probe analysis of a microbial populations selected by amendment with 2,4-D. Appl. Environ. Microbiol. **58**: 3941- 3947.

17. **Ka, J.O. , W.E. Holben and J.M. Tiedje.** 1992. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)- degrading bacteria isolated from 2,4-D -treated field soils. Appl. Environ. Microbiol. **60**:1106-1115.

18. **Ka, J.O., W.E. Holben, and J.M. Tiedje.** 1994. Analysis of competition in soil among 2,4-D degrading bacteria. Appl. Environ. Microbiol. **60**:1121-1128.

19. **Kamagata, Y. R.R. Fulthorpe, K. Tamura, H. Takami, L.J. Forney and J.M. Tiedje**. 1997. Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid degrading bacteria. Appl. Environ. Microbiol. **63**: 2266-2272.

20. **Loos M. A.** 1975. Phenoxyalkanoic acid. p. 1-128. *In* P.C. Kearney and D.D. Kaufman (eds.) Herbicides, Vol. 1. Marcel Dekker, New York.

21. **Margelaf, R.** 1967. Perspectives in Ecological Theory. University of Chicago Press , Chicago.

22. **Moyer C. L.** 1997. The definitive step-by-step protocols for ARDRA (Amplified Ribosomal DNA Restriction Analysis) of cultured bacterial isolates. A handout given to the Centre for Microbial Ecology, June 97.

23. **Olson, B.M. and C.W. Lindwall** (1991) Soil microbial activity under chemical fallow conditions: effects of 2,4-D and glyphosate. Soil Biol. Biochem, **23**: 1071-1080.

24. **Ou L.T.** (1984). 2,4-D degradation and 2,4-D degrading microorganisms in soils. Soil Sci. **137**: 100-107.

25. **Ou L.T. , D.F. Rothwell, W.B. Wheeler, J.M. Davison**. 1978. The effect of high 2,4-D concentrations on degradation and carbon dioxide evolution in soils. J. Environ. Qual. **7**: 241-246.

26. **Perkins E.J. and P.F. Lurquin** 1988. Duplication of a 2,4-dichlorophenoxyacetic acid monoxygenase gene in *Alcaligenes eutrophus* JML 134 (pJP4). J. Bacteriol. **170**: 5669-5672.

27. **Perkins E.J., M.P. Gordon, O. Caceres, and P.F. Lurquin.** 1990. Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J.Bacteriol. **172**. 2351-2359.

28. **Revelante, N. and M. Gilmartin.** 1980. Microplankton diversity indices as indicators of eutrophication in the northern Adriatic Sea. Hydrobiologia. **70:** 277-286.

29. **Stanier, R., N. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**: 159-271.

30. **Takami, H., Y. Kamagata, R. Fulthorpe, L.J. Forney, and J.M. Tiedje.** 1994. Microbial communities degrading 2,4-dichlrophenoxyacetate in pristine soils, abstr. N-45. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.

31. **Tilman, D.; D. Wedin and Johannes Knops.** 1996. Productivity and sustainability by biodiversity in grassland ecosystem. Nature **379**: 718-720.

32.**Willems, A., J. de Ley, M. Gillis and K. Kersters.** 1991. *Comamonadaceae,* a new family encompassing the acidovorans rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenes paradoxus* (Davis 1969). Int. J. Syst. Bacteriol. **41**: 445-450.

33. **Xueqing X., J. Bollinger and A. Ogram.** 1995. Molecular genetic analysis of the response of three soil microbial communities to the application of 2,4-D. Mol. Ecol.**4**: 17-28.

34. **Zhou J., M.A. Bruns and J.M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62**: 316 322.