CHAPTER FOUR

An analyses of a soil bacterial community subjected to long term 2,4- D selection in field plots by the Reverse Sample Genome Probing.

Abstract

The 2,4-D-degrading microbial population selected by 2,4-D amendments to soil were analyzed by culture methods, the use of a 2,4-D pathway gene probe, and by reverse sample genome probing (RSGP) in a three year field study. Total soil bacterial DNA was extracted from the subplots of the 2,4-D gene transfer plot area and analyzed by PCR amplification using *tfdA* primers followed by hybridization on slot blots using the *tfdA* gene probe of pJP4 under high stringency. Slot blot analyses revealed that the *tfdA* hybridization signal was high for the higher 2,4-D treated plots especially for 1998.

To monitor the microbial dynamics of 2,4-D degraders in the field, 26 2,4-D degraders were used as standards. These 2,4-D degraders were mostly isolated from terminal MPN dilutions from these plots. Their denatured genomic DNAs were immobilized on a master filter which was hybridized with the labeled community DNA extracted from the subplots. RSGP showed that 2,4-D degraders were enriched in plots where 2,4-D had been applied. In the 100X plots of 1998, standard strains with higher relative hybridization intensity were mostly of the genera *Burkholderia* and *Ralstonia* and these had *tfdA*. The non-*tfdA* standard strains had a lower hybridization intensity than the *tfdA* standards indicating a competitive disadvantage of such strains. In the 10X 1998 plots, *Alcaligenes* sp. appeared abundant whilst in the 1X plot *Burkholderia graminis* was abundant. Little or no hybridization intensity was obtained for the control plots using RSGP. For the 1997 samples, no particular taxonomic dominance was observed in any of the treatments.

Introduction

The soil is a complex environment and has large numbers of microorganisms that constitute up to 1-3 % of soil organic carbon (6). Because the solid phase is composed of particles from less than 0.2 um to greater than 2 mm diameter, the soil contains a network of pores with a similar range of dimensions. Soil microorganisms are neither randomly nor uniformly distributed through the soil fabric but congregate in pores which are more than large enough to comfortably contain them. Kilbertus (17) showed that there was a consistent ratio of 3:1 between the diameter of pores and the diameter of the bacteria or colonies therein. Microorganisms also congregate near suitable food sources such as cell remnants, fecal materials and amorphous organic matter. The physical position of the microbes affect the level of diversity and function. Differences in function have been found as a result of microbe proximity to the rhizosphere (21), position in landscape (37), depth in soil profile (8), and arrangement in macro-and micropore spaces (18). These environmental differences likely provide a wide array of different microhabitats for soil microbes that would support a high degree of microbial diversity.

Several measures suggest that microbial diversity in soil is high. Estimates of genotypic diversity in these communities based on DNA renaturation experiments suggest that there are $4x10^3$ to $7\ x10^3$ different genomic equivalents per 30 g of soil (36). Culture-based methods also suggest that there is high microbial diversity in soil, even though these methods underestimate the community (24, 29) recovering less than 1 % of the viable community (4, 19, 25, 38). Molecular approaches in which rRNA sequences are used to determine the composition of natural communities have also confirmed that there is a high level

of bacterial diversity in soil communities (5, 19, 34) even though these approaches suffer from some biases and lack resolution at the species level. Recent studies by Zhou et al. (42) showed an unusual diversity pattern of equally abundant species observed in surface and vadose soil microbial communities. On the otherhand in the plant and animal kingdoms (20), no communities consisting of species of equal abundance are rare. Zhou et al. (42) attributed such high microbial diversity to spatial isolation i.e the habitat is subdivided into many separate pockets of resources and thus populations can avoid competition by being physically isolated. Such spatial isolation could enhance the probability of successful colonization of an alien microbe and make it difficult for dominance to be seen.

The diversity of microbial communities generally decrease in response to environmental stress or disturbances which upset the ecological balance of that ecosystem (1). The populations that develop in communities subjected to disturbance exhibited increased physiological tolerances (2), being able to grow over a wide range of temperatures, pH values etc. compared with populations from undisturbed controls. Hence, disturbance selects for generalists. Similar responses have been observed in acid mine drainage. Populations in polluted streams were exposed to low pH and the populations that became dominant had a high broad growth range and other physiological characteristics that clearly made them generalists (22).

Ka et al. (16) studied the response of a soil community to 2,4-D application for four years at three different application rates. At the highest 2,4-D application rate (100 mg/kg), Southern blots probed by 16S rRNA gene probe showed that the total soil microbial community had been shifted to one or two dominant strains presumably 2,4-D degraders (16). Since the probe used could detect all

eubacteria it is not certain that the dominant bands detected were really that of 2,4-D degraders. The study reported here follows up the population analysis of Ka et al. seven to ten years after applying 2,4-D to field plots and with newly developed methods that allow better monitoring of the dynamics of 2,4-D degraders selected.

The objectives of this study therefore were as follows,

i) to determine if a few dominant members are selected by the 2,4-D treatments and whether they are the same across the spatial scale of the study plots and

ii) to determine whether the RSGP technique was suitable for monitoring the response of soil microbial communities to long term effects of 2,4-D.

Materials and Methods

Biochemical reagents: Hybond-N^+ hybridization transfer membrane was purchased from Amersham Life Science Inc. Reagent grade chemicals were from BDH, Gibco and Sigma. ECL direct nucleic acid labeling and detection systems were also purchased from Amersham Life Science Inc.

Isolation of 2,4-D degraders: The isolation procedure has been described elsewhere in Chapter 2. In addition some isolates of Ka et al. (14) were used. These were isolated from the same plots in 1992.

Soil sampling. 2,4-D was applied to the 2,4-D Gene Transfer plot at the Kellogg Biological Station, Hickory Corners, MI. during 1996 to 1998. The plot consisted of eight subplots, each 3.6 by 9.1 m (16). Each subplot was separated by a buffer zone 4.5 m wide. The first application of 2,4-D occurred in October of 1988, and was continued each year. The levels of 2,4-D application were 0X (for the control plot), 1X (which is the rate of good agricultural practice - 1.1 kg/ha), 10X the normal field rate of application and 100X the normal field rate of application.

Each application rate was replicated twice. Beginning in 1996, the dimethylamine ester form of 2,4-D was applied from August each year to early October in the form of five applications every other week. One week after the last application, soil samples were collected, kept on ice and brought to the laboratory and immediately sieved through a 2 mm sieve. The same 2,4-D application scheme was followed in August 1997. However in August 1998 instead of five applications of 2,4-D, seven applications were made so that it might be possible to see differences in microbial communities not previously detectable. Soil samples were stored at -20°C °C for experimental use.

Enumeration of bacteria. The enumeration of 2,4-D degrading bacteria and total heterotrophs was done by MPN for each soil sample. MPN analyses of 2,4-D degraders was performed by inoculating 1.8 ml of 2,4-D medium with 0.2 ml serially diluted soil suspensions. Five replicate sets of tubes were assayed for each soil sample at each time point. 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. 2,4-D was measured by analysing of the supernatant by high pressure liquid chromatography (HPLC) with positive tubes being scored as those with less than 30 ppm of 2,4-D remaining. For the total heteroph count, the MPN tubes of PTYG medium that became visually turbid after 3 days were considered positive. The most probable number of the culturable heterotrophs and 2,4-D degraders in the soil sample was determined according to Cochran (4).

Extraction of genomic DNA from isolates. All the isolates were maintained on a medium of MMO + 300 ppm 2,4-D. Isolates were cultured overnight in PTYG medium. The genomic DNA was extracted using the Quiagen kit following the

manufacturer's protocol. The yield of the DNA was checked at 260 nm whilst the purity of the DNA was determined by the ratio of 260/ 280 nm. The purity ranged from 1.5 to 1.7. The product was stored at - 20°C.

Total bacterial community DNA. Soil DNA was extracted using the method of Zhou et al. (41) from soil samples from the 2,4-D Gene Transfer Plots.

Genomic diversity of isolates: Denatured chromosomal DNA of 27 isolates was immobilized on a master filter (Hybond N^+) by slot blotting following the manufacturer's protocol. Covalent linkage of DNA to the filter was done by UV irradiation. One lane of each membrane contained standards of λ DNA (or the marker DNA lane) . Preliminary experiments had shown that λ DNA did not cross hybridize to any of the strains as expected (40). Chromosomal DNA of individual isolates was mixed with λ DNA, labelled with horse radish peroxidase using the ECL direct nucleic acid labeling and detection kit and hybridized with the master filter to evaluate the extent of cross hybridization to the other isolates. The same procedure was repeated with the next isolate, thus requiring 27 replicate filters and 27 different probes. The position of the isolates and the weights of the chromosomal DNA spotted on the filter were maintained throughout the experiment (Table 1). Isolates which strongly cross hybridized to other isolates were discarded. Bacterial standards, defined as bacteria with genomes showing relatively little genomic cross-hybridization, were selected. The genome complexity value kλ/kx, was calculated according to (40) for each bacterial standard as follows;

$$k\lambda/kx=(fx/f\lambda) \times (I\lambda/c\lambda) \times (Ix/cx)^{-1}$$

Where fx is the weight fraction of standard x in the probe
cx is the weight of denatured DNA x spotted on the filter
cλ is the weight of denatured DNA λ spotted on the filter

$f\lambda$ is the weight fraction of bacteriophage λ DNA in the probe
Ix is the observed net hybridization intensity for standard x in integrated volume (optical density)
$I\lambda$ is the observed net hybridization intensity for bacteriophage λ in integrated volume (optical density).

Reverse genome probing. After selecting the bacterial standards, chromosomal DNA from these standards was immobilized on the 2,4-D master filter. Denatured bacteriophage λ DNA (1, 5, 10, 20, 30, 40, 50, 100 ng) was included on one side of the filters as markers. Following covalent linkage of the DNAs to the filters, the filters were stored at -20°C. Sample DNA (soil community DNA at least 100 ng) and bacteriophage λ DNA (20 ng) were randomly labelled with horse radish peroxidase using the ECL direct nucleic acid labeling and detection kit from Amersham Life Sci. Inc. The λ DNA served as an internal standard. An equivalent volume of glutaraldehyde was added and mixed thoroughly. The probe mixture was then incubated for 10 min. at 37°C to complete the labelling. Labeled probe was then hybridized to the master filter for at least 16 h at 42°C. Prior to that the filter had been prehybridized for at least 40 min with Amersham golden buffer containing 0.5 M NaCl and 5 % (w/v) blocking agent. The golden buffer contains 6M urea which is equivalent to 50 % formamide in reducing the Tm of hybridization. Post hybridization washes were done at high stringency using 0.1X SSC following the manufacturer's protocol. Hybridization signal was detected by autoradiography using X-Omat AR film (Kodak, Rochester, N.Y.) exposed at room temperature for 30 minutes. The time of exposure to film was the same for all treatments so that the results could be compared. A background signal was determined for all hybridization spots which was subtracted from the hybridization intensity to obtain the relative hybridization intensity.

Quantification of the blot signals was done using Image Quant, a computer software of the Plant Biology Building.

SSU rRNA sequence. A partial 16S rRNA gene sequence was determined for all bacterial standards. A 1.5 kb fragment was amplified by PCR with primers 49F and 1510 R (23). The PCR product was visualized in 1.0 % Agarose gel and purified further with the Wizard Kit PCR Prep following the manufacturer's protocol. The PCR products were sequenced with primer 529R targeting the conserved regions of the 16S rRNA. Sequencing by fluorescently-labeled dye termination was performed at Michigan State University Sequencing facility using the Applied Biosystems Model 373A automatic sequencer (Perkin Elmer Cetus).

Sequence analyses. Bacterial strains with the most similar rRNA sequences were obtained by searching the GenBank data program using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (3). Ambiguous nucleotides were deleted from the sequence alignment leaving at least 350 bp for sequence analysis.

PCR amplification and hybridization of the *tfdA* gene. Total DNA was isolated from the field soil samples of 1996, 1997, and 1998 from the various 2,4-D treated plots. *tfdA* primers were those of Vallaey's et al. (39) that had been derived from the conserved regions of sequence in the *tfdA* genes of pJP4 and *Burkholderia* strain RASC (79 % identical to *tfdA*-pJP4). The sequence of the forward primer TVU was 5' ACG GAG TTC TG(C/T) GA (C/T) ATG-3'. The sequence of the reverse primer, TVL was 5'AAC GCA GCG (G/A) TT (G/A)TC CCA-3'. The PCR mix contained 5 ul 10X PCR buffer (GIBCO, Gaithersberg, MD), 1.5 mM $MgCl_2$ 1 uM primer TVU, 1uM primer TVL, 1 mM dNTPs (GIBCO), 1.5 units of Taq

polymerase (GIBCO), 10-100 ng template DNA and sterilized distilled water, to bring the final volume to 50 ul. The predicted size is 360 bp. Amplified products were separated on a 1 % agarose gel, and compared to PCR products generated from JMP134 and RASC genomic DNA as templates.

PCR products were applied to Hybond N^+ and UV cross linked to the membrane with a Stratalinker. To create probes, PCR products obtained using DNA from JMP134 were labeled with horseradish peroxidase using ECL direct nucleic acid labeling and detection systems according to the manufacturer's instructions. Probing was done under high stringency conditions.

Results

MPN of 2,4-D degraders and total viable count The populations of both 2,4-D degraders and total viable microorganisms in the field plot were determined for 1996 and 1997 (Fig. 1). The 2,4-D degrading population was approximately 10,000 cells /g soil in the subplots not treated with 2,4-D but it was higher in the 2,4-D -amended subplots. Linear regression analyses of the data showed significant correlation (r= 0.96) between the 2,4-D degrading population and the 2,4-D application rate for both 1996 and 1997 sampling dates. The indigenous 2,4-D degrading populations in the control subplots were relatively stable throughout the two years. The total viable counts were stably maintained around 10^8 to 10^9 cells / g soil and were not affected by 2,4-D application, however the 1997 values were 1/2 log higher.

Probing total soil bacterial DNA with the tfdA probes. The total soil bacterial DNA isolated from eight subplots in 1996, 1997, and 1998 was analyzed by *tfdA* PCR amplification followed by hybridization to the *tfdA* gene probes of the pJP4 plasmid under high stringency (Fig. 3). Hybridization signal was obtained in all

treatment plots and even for some of the control plots in all the three years (Fig.3). Generally, hybridization signal increased with increase in 2,4-D application and the highest signal was obtained for 100X plots in 1998.

Genome diversity of 2,4-D degrading isolates: Genomic DNA derived from purified strains was tested for cross-hybridization (32, 40) and genomes that showed little cross-hybridization with each other were selected as standards. The 26 standards are listed in Table 1 in the order in which they were spotted on the master filter. Sequencing of the rRNA gene of the standards and comparison of the sequence obtained with those in GenBank suggested an identification for 24 of the standards (Table 1). The genus *Burkholderia* was most prevalent among the isolates while six standards had *Ralstonia* strains as the closest homology. The genera *Rhodopseudomonas* and *Variovorax* (standards 4 & 9) were also among the standards. *Alcaligenes* (now likely *Ralstonia sp.*) was represented in Ka's isolates as was one *Sphingomonas* strain. Some standards showed less cross hybridization to other standards on the master filter whilst others showed some cross hybridization. Cross-hybridization was generally low relative to self hybridization which was taken as 100 %. For example standard 3 (Fig. 4) showed up to 23 % cross hybridization with standards 5, 23 and 25. Standard 16 on the other hand showed strong cross hybridization to standard 2 (> 50 %) standard 3, and standard 23. Standard 23 showed significant cross hybridization with standard 14 and 25 (Fig. 5). The genome complexity $k\lambda/kx$ values ranged from 17 for standard 12 to 367 for standard 19. Where values were unusually high, hybridization was done again. to obtain a better value. These values reflect different apparent genome complexity.

RSGP profile of bacterial community in 1998 2,4-D plots. Strong hybridization signals were obtained between soil DNA and bacterial standards (Fig 7). The hybridization was strong particularly for 100X 2,4-D plots in 1998 (Fig. 7a). Standards 9, 11, 15, 17, and 18 seemed to be the rarer members of the community in all treatments. On the otherhand, standards 6, 7, 8, 10, 13, 14, 16, 23, 24, & 25 were equal in dominance. These standards were mostly *Burkholderia* and *Ralstonia* species. Previous experiments have shown that *Sphingomonas* sp. and *Pseudomonas picketti* (now *Burkholderia*) appeared to be dominant members in the community in the same plot (16). *Sphingomonas* (standard 19) was not particularly dominant in this analysis. The standards that seemed dominant mostly had the *tfdA* gene. Bacterial standards (10, 15, 17, 18, and 20) which did not have the *tfdA* gene had a lower hybridization intensity.

The hybridization intensity by RSGP in 1998 was less on the 10X plots than for the 100X plots. Standard 25, an *Alcaligenes* sp., appeared to be dominant in this microbial community (Fig. 7b). Standard 2, which is *Burkholderia graminis,* was abundant in the 1X plot (Fig. 7c). For the control plots it was difficult detecting any hybridization signal for the bacterial standards indicating that even if the genomes were present they must be in low numbers (Fig.7d).

RSGP profile of bacterial community in 1997 2,4-D plots.

Compared to that of 1998, the relative hybridization intensity is lower for each of the 2,4-D treated plots (Figs. 8 a-c). No strain or taxonomic group appeared to be particularly dominant. Even though there was an enrichment for 2,4-D degraders as seen by MPN (Fig. 1) the signal intensity was not as high in 1997 as in 1998. Significant correlation was observed between hybridization intensity of 100X 98

standards and 100X 97 standards (r = 0.70) indicating that the effect on 2,4-D selecting for these isolates continued in a strain specific manner in 1998 (Fig.9).

Discussion

Reverse sample genome probing can be used to monitor the response of individual strains in microbial communities to environmental changes such as loss of substrate. Its advantage is the ability to rapidly track the abundance of multiple microbial genomes in a natural sample. The present collection of 26 genomes on the master filter is modest relative to the microbial diversity that is present in the soil (36). The master filter created in this study covered only a small fraction of the resident community although it possibly represented a significant portion of the 2,4-D degraders in the plots. The dominant communities as determined by the RSGP were not *Sphingomonas* sp. as Ka et al. (14) found but members of the *Burkholderia* sp. and *Ralstonia* class. Strains of these closely related genera were especially prominent in the 100X plots of 1998. The possible reason for this observed difference with Ka's earlier data might be due to the type of plasmid and plasmid host interactions that are key determinants of competitive outcome (13). When *P. cepacia* DBO1 harbored plasmid pKA4, it resulted in slower growth in a 2,4-D medium than the rapid growth observed when this strain harbored pJP4. It was not surprising that dominant strains in the 100X plots of 1998 had the *tfdA* gene which encodes the first enzyme in the 2,4-D cannonical degradative pathway. This might indicate the competitive advantage of strains with the *tfdA* gene over the non- *tfdA* strains. *Sphingomonas* sp. does not encode this gene of the pathway (9, 30). Ka et al. (13) noted that the rate of growth of *Sphingomonas* was slow and the lag period was 60 h which he suggested would affect the competitive ability of this strain.

Little or no RSGP signal was observed for the control plots with the bacterial standards even though the DNA from some of the control community hybridized to the *tfdA* probe. The *tfdA* assay, however was much more sensitive since the hybridization was of PCR products from *tfdA* specific primers. The wide variation in kλ/kx (genome complexity) values is likely due to the differences in the genome sizes although there might be other reasons. Values at the high end of the scale may result if a standard is not a pure culture, while values at the low end may reflect the presence of repetitive sequence of a small plasmid in high copy number (33). In this study high kλ/kx values were obtained when the bacterial standard probe hybridized less to itself than to the other standards on the master filter but this unusual observation has been noted by others (27, 33). Moreover, the purity of the strains were checked on several occasions on R2A agar medium. Although correction for cross-hybridization is meaningful for a closed system that consists of a mixture of known strains (e.g a synthetic microcosm) its use for analysis of samples obtained from the environment (open system) is doubtful because not all component chromosomes of the "environmental genome" can be obtained in pure form by culturing (32). Thus the values presented were not corrected for cross -hybridization.

RSGP has good potential for use to analyze the dynamics of the soil microbial community. The method is quantitative, a feature lacking in many of the molecular methods now popular in microbial ecology. It also provides a means for uncovering rare members of the community. The disadvantage of this method is that individual genomes are separated from the target environment by culturing. Thus, although the RSGP assay does not require culturing and can be performed quickly, it describes the microbial community only in terms of the culturable

component. It also avoids the several PCR related biases such as variations of G+C content of templates (31), variations of DNA template concentration, chimera formation, and biases in primer hybridization. The genome probes can easily distinguish species from different genera and can to a degree distinguish species within the same genus (27). RSGP can shed light on some of the tractable problems encountered in soil microbial ecology. For instance, the relationship between species diversity and the functioning of the soil ecosystem is not well understood. Attempts have been made to link these major characters inorder to predict changes in ecosystems functioning such when diversity is altered due to disturbance (35). If different master filters are prepared for genera of bacteria, eg. a master filter for only Burkholderia sp, and another for Pseudomonas sp. etc differences in hybridization intensity after probing with community DNA can be correlated with the ecosystem function.

Previous studies in this plot by Ka et al. (15), showed little or no hybridization intensity of *tfdA* with total DNA extracted from the KBS soils prior to exposure to 2,4-D but it was not so in the present study. The possible reason for this disparity is that in the present study the PCR primers anneal to conserved regions in at least three of the different alleles, and may detect genes with lower sequence similarity missed by other methods. Thus the PCR method is more sensitive than the method used by Ka et al. (15). The detection of the *tfdA* gene in the control plots might be due to surface water flow or leaching, especially if there was a heavy rain few days after 2,4-D application. It could also be that the presence of the *tfdA* gene in the control plot is due to selection on naturally occurring compounds that bear structural resemblance to 2,4-D. Some examples include the numerous aryl-ether compounds released during fungal degradation

of lignin (26) or different halogenated aromatic metabolites produced by fungi and bacteria (11, 12, 28). Alternatively, it is possible that the natural substrate for TfdA bears little resemblance to 2,4-D and the ability to degrade this compound is fortuitous. It might also be that the *tfdA* gene or closely related homologs widespread among some of the microbial populations are likely to exist in them for a purpose other than the degradation of 2,4-D (12).

The physical, chemical and biological complexity of soil provide a multitude of microenvironments for growth of different bacterial populations (7, 10). With such heterogeneity, dominance of a single species following addition of a growth limiting nutrient would be expected only on a microscale. Thus greater diversity might be observed in undisturbed soil following selection and this might explain why many different strains appeared equally dominant after the various 2,4-D treatments. It is possible that moderate mixing of the soil prior to construction of the microcosm disrupted most of the heterogeneity which exists in situ and forced many populations which would otherwise have been physically or ecologically segregated to compete directly. Hence, community structure data from microcosm studies should be interpreted with caution.

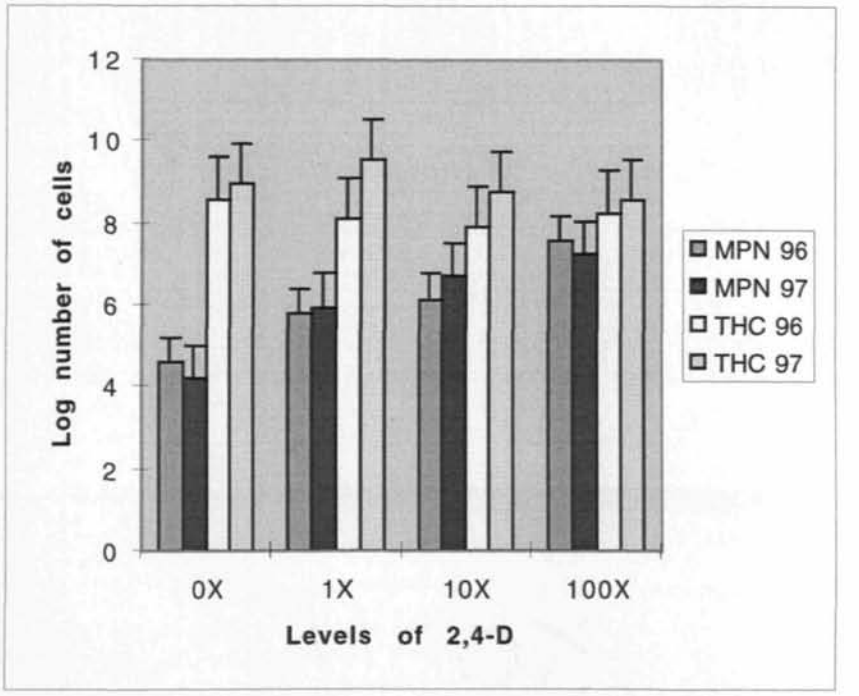
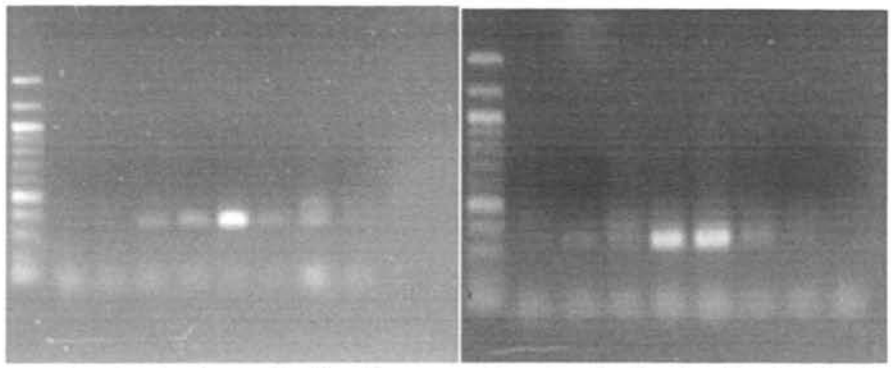


Fig.1. MPN of 2,4-D degraders and total heterotroph count (THC) for field soil samples in 1996 & 1997. Amount of 2,4-D applied shown relative to normal agricultural practice = X. Bars are from 2 MPNs, one of each replicate of the two field plot replicates.



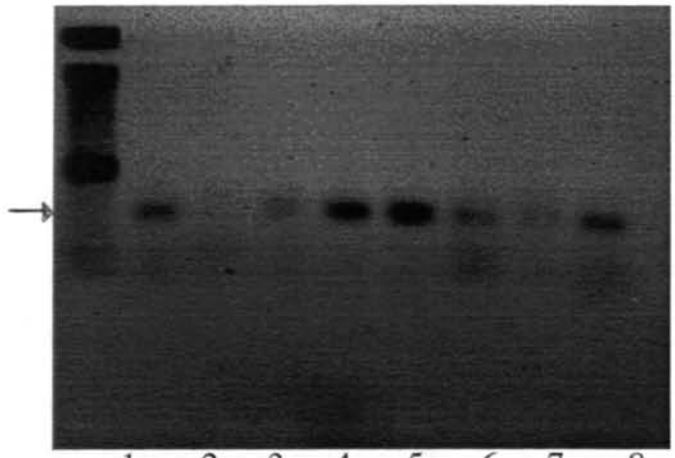


Fig. 2. Soil DNA for 1996 (a), 1997 (b), and 1998 (c) amplified with conserved *tfdA* primers. Size of the *tfdA* fragment is approximately 360 bp. The lanes 1 through 8 are as follows 0X, 1X, 10X 100X, 100X, 10X, 1X, 0X.

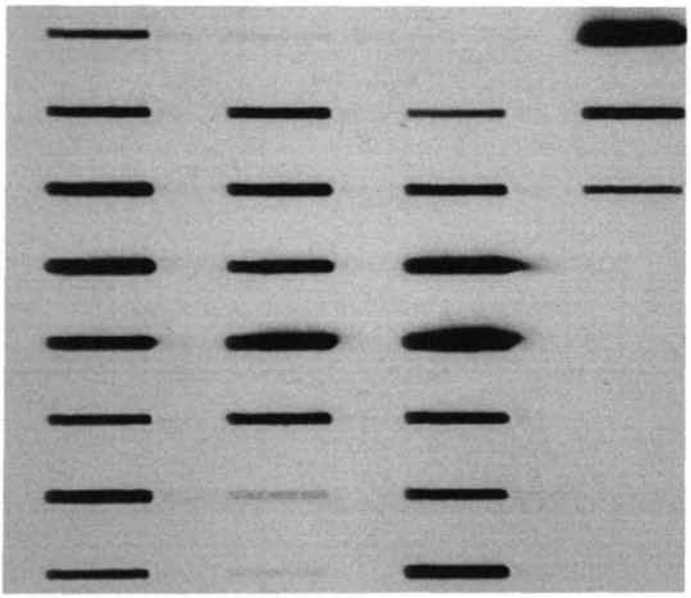


Fig. 3. Community DNA from field subplots (0X, 1X, 10X, and 100X) amplified with conserved *tfdA* primers and probed with *tfdA* of the pJP4 under high stringency. Lanes 1 ,2 ,3 are for 1996, 1997,and 1998. Lane 4 is 100 ng, 10ng, and 1 ng of pJP4 hybridized with tfdA of pJP4 under high stringency. The rows are for treatments from 0X, 1X, 10X, 100X, 100X, 10X, 1X, 0X. All these treatments represent the northern side of 2,4-D plots where 2,4-D application was continued.

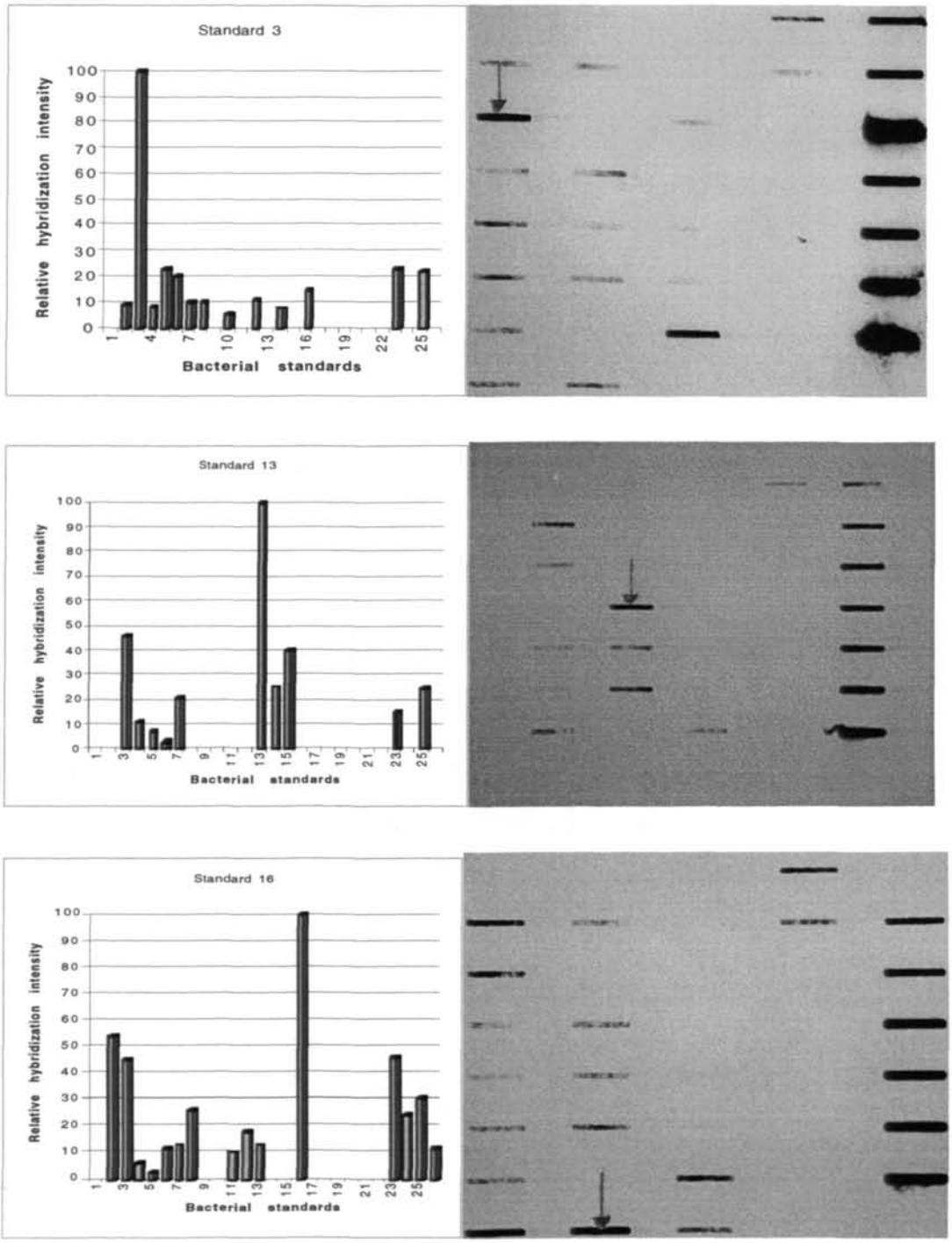


Fig. 4. Examples evaluating cross -hynridization of the 26 2,4-D degrading isolates to each other.The chromosomal DNAs for standards 1 to 26 present on the master filter as indicated in Table 1. The right lane side of the filter are λ DNA markers. A mixture of 20 ng of bacteriophage λ DNA and 100 ng of chromosomal DNA was labeled and hybridized with master filter. Relative hyridization intensity Ix (%) is plotterd on the y -axis, self hybridization is taken as 100 %. The standard number is on x- axis. Cross -hybridization data are shown for standards 3, 13 & 16. Arrow indicates self hybridization.

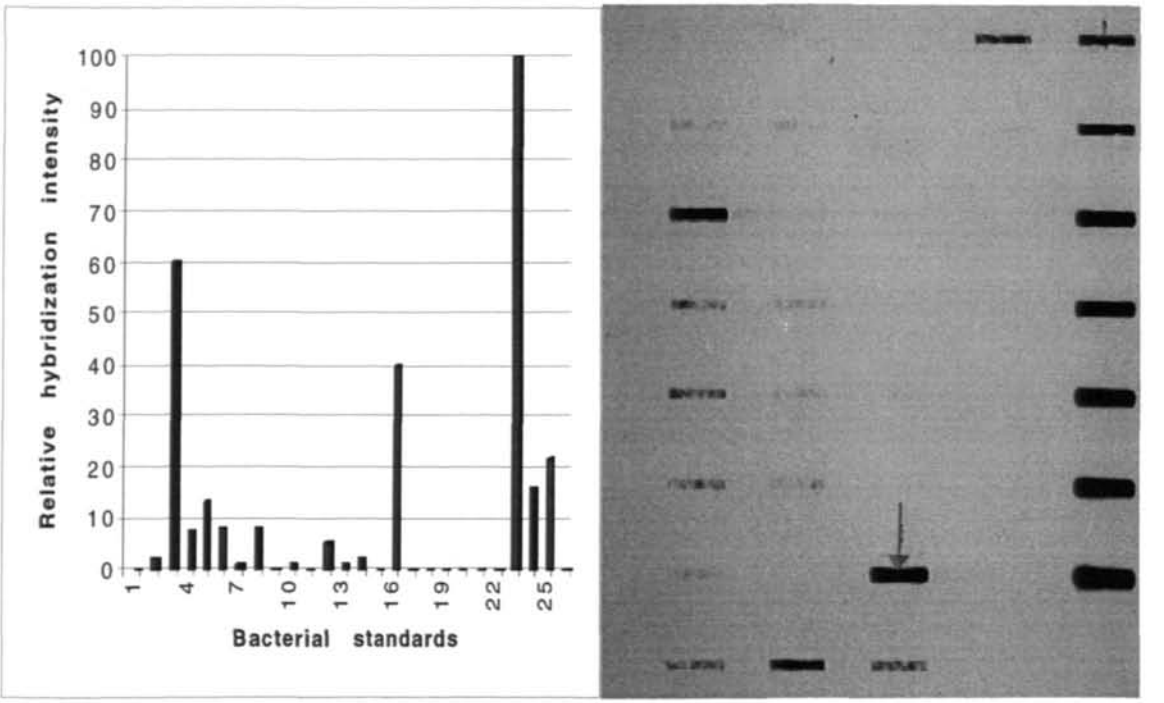


Fig. 5. Hybridization of standard 23 on the master filter. Self hybridization is 100 %. Relative hybridization Ix is on the y-axis, and standard number is on the x-axis. Arrow indicates self hybridization.

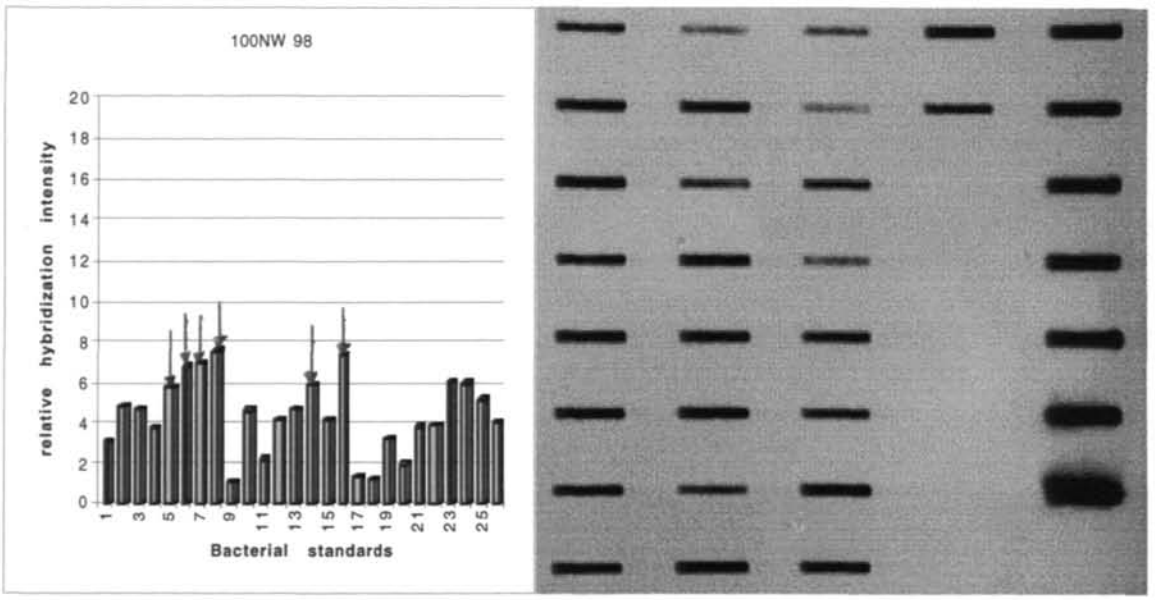
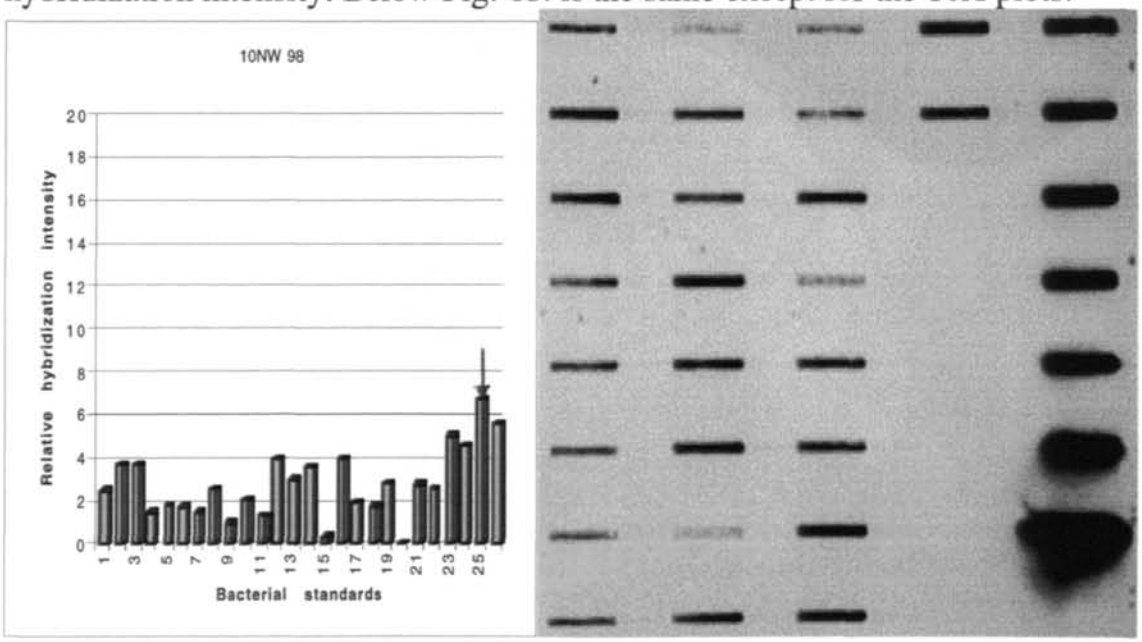


Fig. 6a. Influence of long term 2,4-D application on the microbial community in field plot (100X plots where 2,4-D was applied 100X the normal field rate for 10 yr.) as measured by RSGP. The community DNA from the plot was labeled and hybridized to the master filter. Quantitation of the hybridizations are displayed as bar diagrams (Ix relative hybridization intensity in integrated optical density (10^5 on the vertical axis). The standard number is on the horizontal axis; data are not corrected for cross-hybridization). Arrows indicate standards with high relative hybridization intensity. Below Fig. 6b. is the same except for the 10X plots.



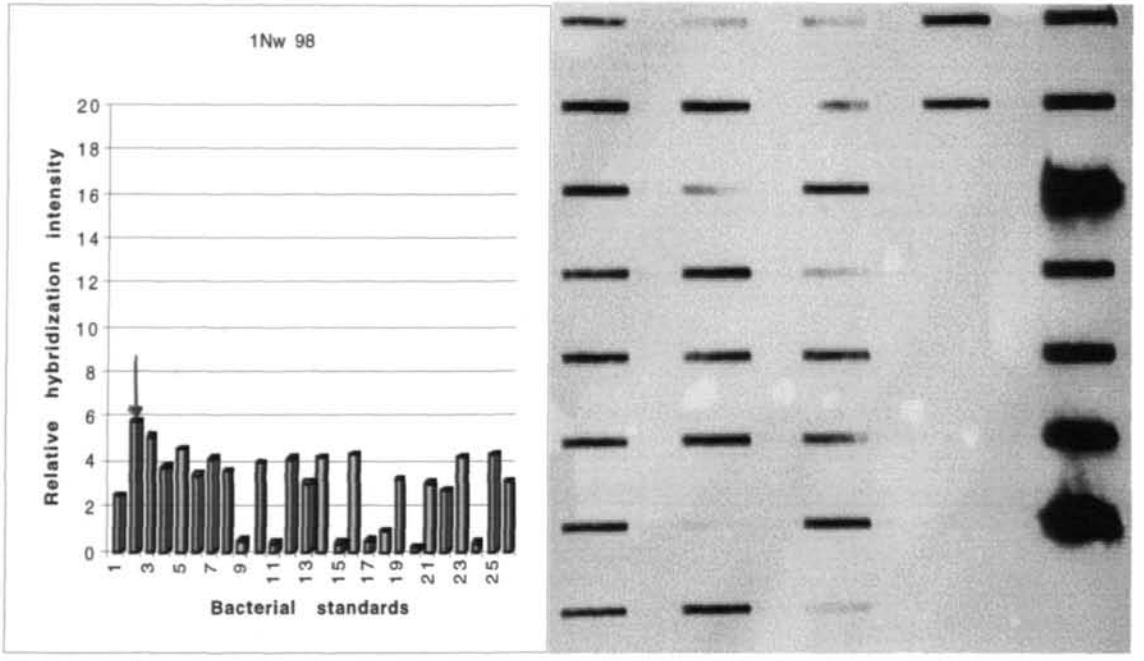


Fig 6c. Long term effect of 2,4-D selection on microbial community in the 1X plots as measured by RSGP. Figure features the same for Fig. 7a.

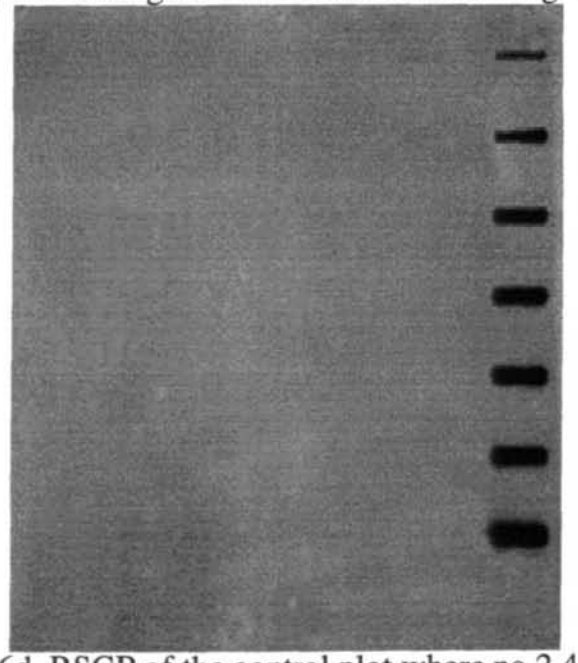


Fig 6d. RSGP of the control plot where no 2,4-D was added.

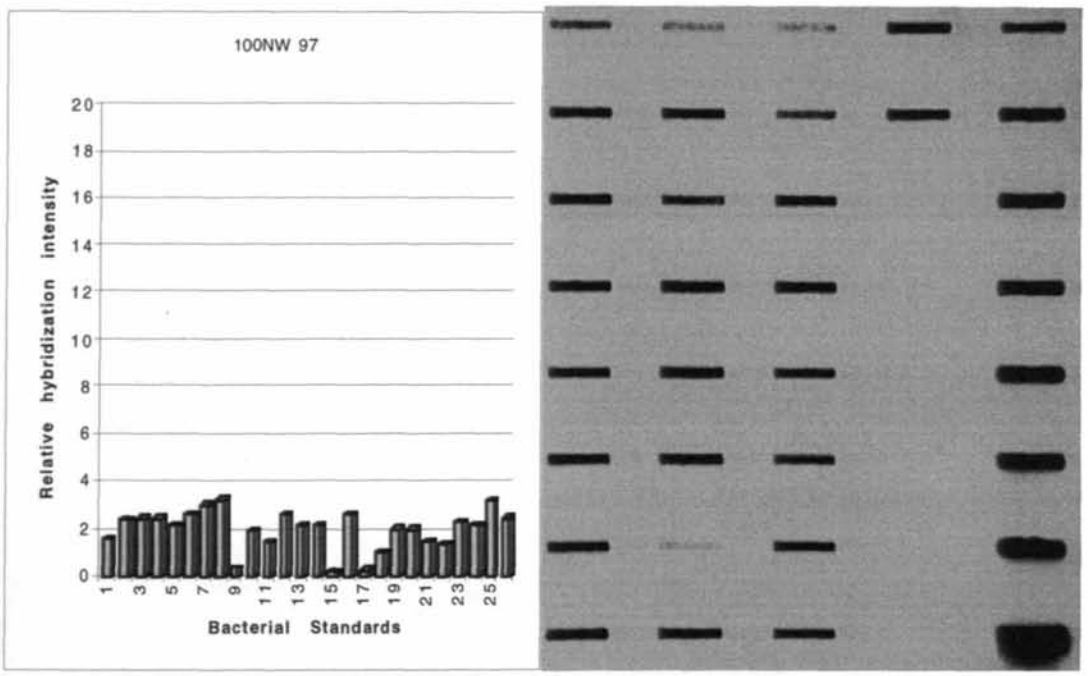


Fig.7a. Effect of long term 2,4-D application on microbial community in soil samples from 100X plots in 1997. See Fig. 7a for details of figure.

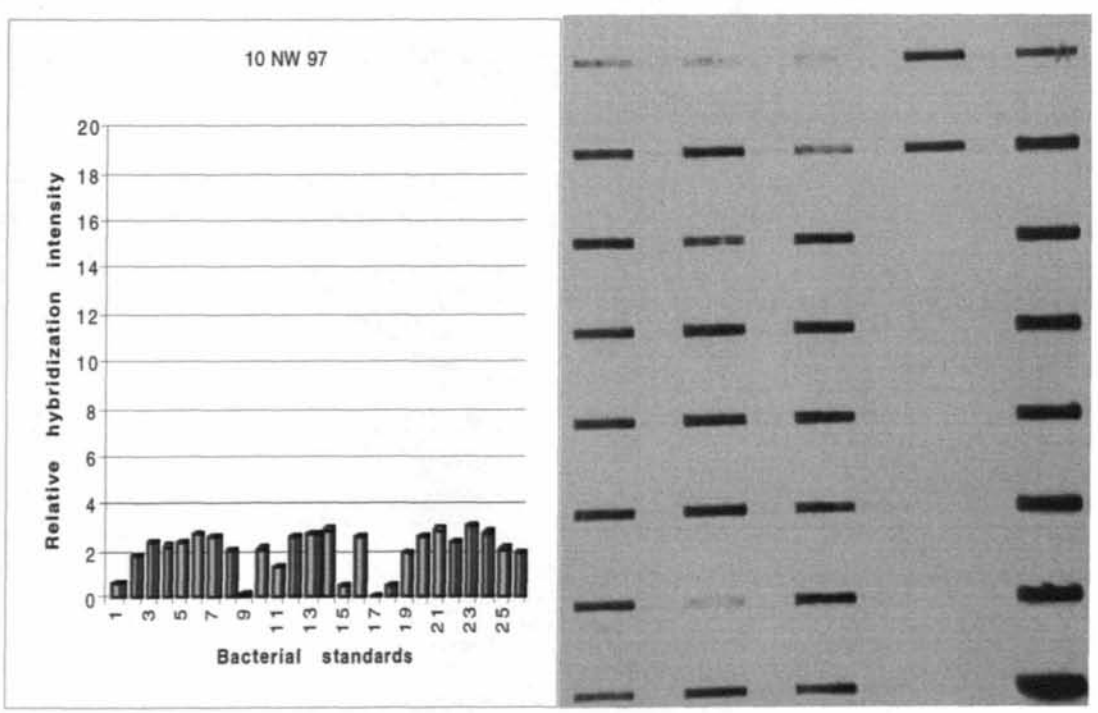


Fig.7b. RSGP showing the 2,4-D effect on microbial community sampled in 1997 from the 10X plots.

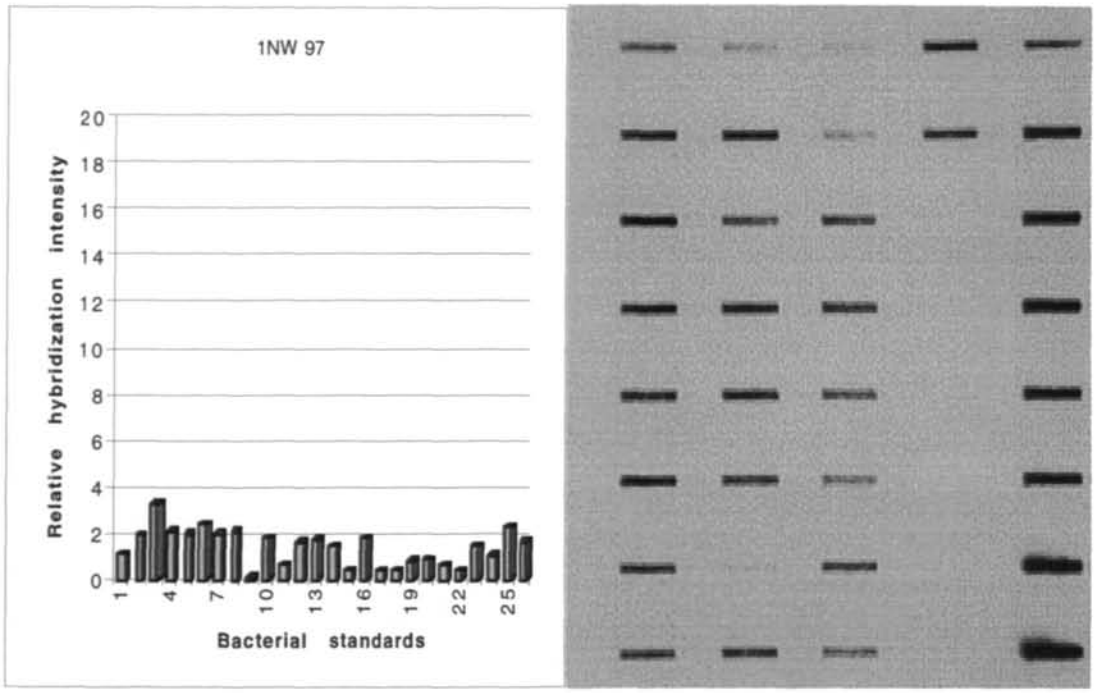


Fig. 7c. RSGP showing 2,4-D effect on microbial community sampled in 1997 from the 1X plots.

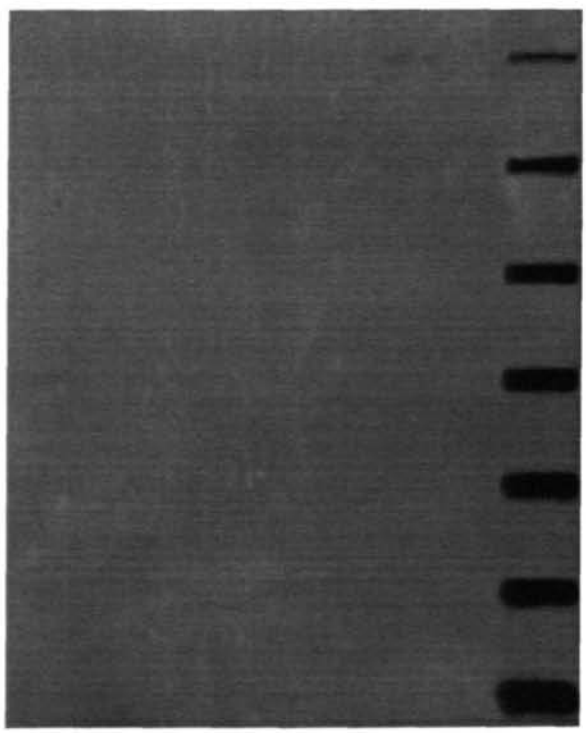


Fig 7d. RSGP showing control plot where no 2,4-D was applied. Little or no hybridization intensity detected on the master filter.

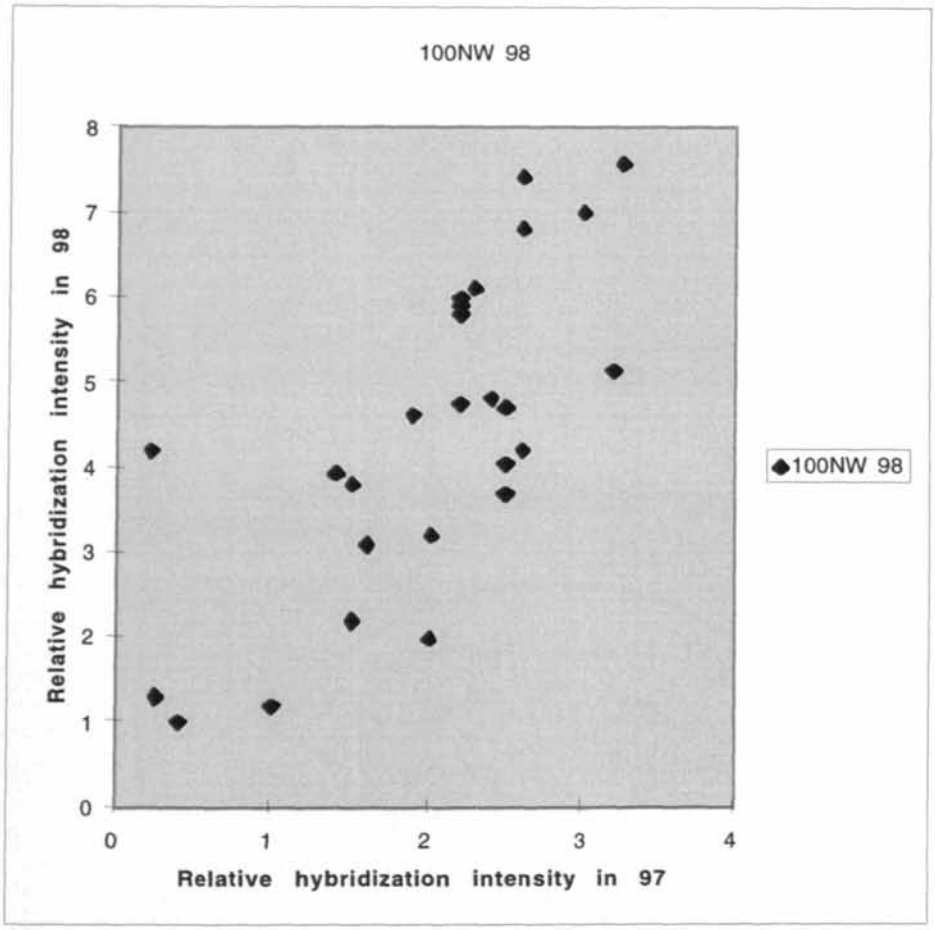


Fig. 8. Relationship between relative hybridization intensity of each strain to DNA from 100X plots in 1998 and 100X plots in 1997. Correlation coefficient was 0.70.

Table 1. 2,4-D isolates selected as standards for RSGP

Position on blot	Strain #	cx in ng [a]	cλ in ng [b]	Nearest homolog	Genome complexity [c] kλ/kx	Presence or absence of *tfdA*	[d] S_{ab} value %
1	001	31	10	*Burkholderia graminis*	35	+	100
2	007	66	10	*B. graminis*	78	+	100
3	010	79	10	unidentified	60	-	-
4	012	55	10	*Variovorax paradoxus*	35	+	99
5	017	65	10	*R. eutropha*	54	+	99
6	018	57	10	"	43	+	96
7	019	57	10	*B. pseudomallei*	367	+	97
8	021	58	10	*Burkholderia sp.*	107	+	96
9	022	25	10	*Rhodopseudomona palustris*	110	_	98
10	023	46	10	*R. eutropha*	67	+	97
11	026	52	10	*Alcaligenes eutropha*	189	-	97
12	027	65	10	*B. caryophylli*	17	+	97
13	028	63	10	*B. graminis*	67	+	96
14	029	63	10	"	74	+	96
15	030	19	10	unidentified	81	-	-
16	031	64	10	*Burkholderia glathei*	112	+	97
17	033	20	10	*R. eutropha*	116	+	95
18	034	19	10	*Pseudomonas aeruginosa*	165	-	96
19	1443	39	10	*Sphingomonas sp.*	30	-	-
20	9112	26	10	*Ralstonia sp.*	32	+	96
21	9226	63	10	*Ralstonia sp. TFD41*	80	+	98
22	712	35	10	*Ralstonia sp.*	57	+	96
23	524	58	10	*Pseudomonas cissicola*	37	-	92
24	1172	47	10	*Alcaligenes eutrophus*	69	+	
25	912-2	90	10	*Alcaligenes sp.*	86	_	99
26	9157	52	10	*Alcaligenes eutropha*	89	+	97

[a] Amount of denatured chromosomal DNA (ng) spotted on the filter.

[b] Amount of denatured λ DNA (ng) spotted on the filter. Other amounts were spotted but this was used for the calculation of genome complexity. [c] $k\lambda/kx=(fx/f\lambda) \times (I\lambda/c\lambda) \times (Ix/cx)^{-1}$ See text for definition of terms.

[d] S_{ab} value similarity coefficient for query by using the Genbank data program.

Isolates of Ka et al. (14) are from # 19 - 26. Nearest homolog of isolate was identified by partial sequencing of the 16S rRNA genes.

References

1. **Atlas, R.M.** (1983) . Diversity of microbial communities. Adv. Microb. Ecol. **7**:1-49.

2. **Atlas, R.M., A. Horowitz, Krichevsky and A.K. Bej.** 1991. Response of microbial populations to environmental disturbance. Microbial Ecol. **22**:249-256.

3 **Altschul, S.F., W.Gish, W. Miller, E.W. Myers and D.J. Lipman.** 1990. "Basic Local Alignment Search Tool." J. Mol. Biol. **215**:403-410.

4. **Biovin-Jahns, V..; A. Bianchi, R. Ruimy, J. Garan, S. Daumas and R. Christen.** 1995. Comparison of phenotypical and molecular methods for the identification of bacterial strains isolated from a deep subsurface environment. Appl. Environ. Microbiol. **61**:3400-3406.

5. **Borneman, J. P.W. Sroch, K.M. O 'Sullivan, J.A. Paulus, N.G. Rumjanek, J.L. Jansen, J. Nienhus and E.W. Triplet.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. **62**:1935-1943.

6. **Clarholm, M.** 1985. Possible roles for roots bacteria, protozoa and fungi in supplying nitrogen to plants. p. 355-365. *In*: Ah Fitter , D.Atkinson, D J.Read, MB Usher (ed). Ecological interactions in soil: Plants, microbes and animals. Blackwell, Oxford.

7. **Foster, R.C.** Microenvironments of soil microorganisms. 1988. Biol. Fert. Soils. **6**:189-203.

8. **Fredrickson, J.K., D.L. Balkwill, J.M. Zachara, S.M.W. Li, F.J. Brockman and M.A. Simmons.** 1991. Physiological diversity and distributions of heterotrophic bacteria in deep cretaceous sediments of the Atlantic coastal plain. Appl. Environ. Microbiol. **57:** 402-411.

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

10. **Fulthorpe, R. R. and R.C. Wyndham. 1992.** Involvement of a chlorobenzoate catabolic transposon, Tn 5271, in community adaptation to chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem. Appl. Environ. Microbiol. **58**:314-328.

11. **Gribble, G.W.** 1992. Naturally occurring organohalogen compounds a survey. J. Nat. Prod. **55**: 1353-1395.

12. **Hogan, D.A. D.H. Buckley, C.H. Nakatsu, T.M. Schmidt and R.P.Hausinger.** Distribution of the *tfdA* gene in soil bacteri that do not degrade 2,4-dichlorophenoxyacetic acid -degrading bacteria. Microbial Ecol.. **34**:90-96.

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

14. **Ka, J.O., W. E. Holben and J.M. Tiedje.** 1994. Genetic and phenotypic diversity of 2,4-Dichlorophenoxyacetic acid (2,4-D) - degrading bacteria isolated form 2,4-D treated field soils. **60** : 1106 - 1115.

15. **Ka, J.O., W.E. Holben and J.M. Tiedje.** 1994. Use of gene probes to aid in recovery and identification of functionally dominant 2,4-dichlorophenoxyacetic acid degrading populations in soil. Appl. Environ. Microbiol. **60**: 1116-1120.

16. **Ka, J.O. P.Burauel, J.A. Bronson, W.E. Holben and J.M. Tiedje.** 1995. DNA probe analysis of microbial community selected in field by long term 2,4-D application. Soil Sci. Soc. Am. J. **59**: 1581-1587.

17. **Kilbertus, G. O. Reisinger.** 1975. Degradation du materiel vegeta activite invitro et in situ de quelques microoganismes. Rev. Ecol. Biol. Sol. **12**:363-374.

18. **Lee, K.E. and R.C. Foster**. 1991. Soil fauna and soil structure, Aust. J. Soil Res: **29**:745-775.

19. **Liesack, W. and Stdackebrandt.** 1992. Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol.**174**: 5072-5078.

20. **Magurran A.E.** 1988. Ecological diversity and it measurement (Princeton University Press, Princeton, New Jersey).

21. **Metting, B.F**. 1993. Soil microbiology ecology. Marcel Dekker. Inc, New York. pp. 350.

22. **Mills, A.C. and L.M. Mallory.** 1987. The community structure of sessile heterotrophic bacteria stressed by acid mine drainage. Microbiol. Ecol. **14**: 219-232.

23. **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 Center for Microbial Ecology, June 97.

24. **Nannipier, P., S. Greco and B. Ceccanti.** 1990. Ecological significance of the biological activity in soil, p 293-356. *In* J.-M. Bollag and G. Stotzky (ed), Soil biochemistry, vol. 6. Marcel Dekker, Inc., New York, N.Y.

25. **Nubel, U. B. Engelen, A. Felske, J. Snaidr, A. Weishuber, R.I. Amann, W.Ludwig, and H. Badkhars.** 1996. Sequence heterogeneity of genes encoding 16S rRNA in *Paenibacillus polymyxa* detected by temperature gradient gel electrophjoresis. J. Bacteriol. **178**: 5636-5643.

26. **Ribbons, D.W.** 1987. Chemicals from lignin. p. 485-494. *In* B .C. Hartley, P.M.A Broda, P.J Senior (ed), Technology in the 1990s: utilization of lignocellulosic wastes, Royal Society , London, England U.K.

27. **Shen, Y. L. G.Stehmeier, and G.Voordouw.** 1998. Identification of hydrocarbon -degrading bacteria in soil by reverse sample genome probing. Appl. Environ. Microbiol. **64**: 637-645.

28. **Siuda, J.F, and J.F.de Bernardis,** 1973. Naturally occurring halogenated compounds. Lloydia **36**:107-143.

29. **Sorheim, R., V.L. Torsvik and J. Goksoyr.** 1989. Phenotypical divergences between populations of soil bacterial isolated on different media. Microbiol. Ecol. **17**: 181-192.

30. **Suwa, Y. W.E. Holben, and L.J. Forney**. 1994. Cloning of a novel 2,4-D catabolic gene isofunctional to *tfdA* from *Pseudomonas* sp. TFD3 Q-403, p293. In Abstracts of the 94th GeneralMeeting of the America Society for Microbiology .1994. American Society for Microbiology, Washington, D.C.

31. **Suzuki, M.T. and S.J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ Microbiol. **62**. 625-630.

32.**Telang, A.J., G. Voordouw, S.Ebert, N. Sifeldeen, J.M. Foght, P.M. Fedorak, and D.W.S. Westlake,** 1994. Characterization of the diversity of sulfate-reducing bacteria in soil and mining waste water environment by nucleic acid hybridization technique. Can. J. Microbiol. **40**:955-964.

33. **Telang, A.J. S.Ebert, J.M. Foght, D.W.S.Westlake, G.E. Jenneman, D. Geventz, and G. Voordouw.** 1997. Effect of nitrate injection on the microbial

community in an oil field as monitored by reverse sample genome probing. Appl. Environ. Microbiol. **63**:1785-1793.

34. **Tiedje, J.M. J.-Z Zhou, K. Nusslein, C.L. Moyer and R.R. Fulthorpe.** 1997. Extent and patterns of microbial diversity, p. 35-41. *In* M.T. Martins et al. (ed.), Progress in microbial ecology. Brazilian Society for Microbiology, Sao Paulo, Brazil.

35. **Tilman, D.** 1996. Biodiversity: population versus ecosystem stability. Ecology. **77**:350-363.

36. **Torsvik, V., J. Goksoyr, F.L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782-787.

37. **Turco, R.F. and D.F. Bezdicek.** 1987. Diversity within two serogroups of *Rhizobium leguminosarium* native to soils in the Palouse of Eastern Washington . Ann. Appl. Biol. **11**:103-114.

38. **Ward, D. M. Bateson, R.Weller, and A. Ruff-Roberts.** 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microbiol. Ecol. **12**: 219-286.

39.**Vallaeys, T. , R.R. Fulthorpe, A.M. Wright, G. Soulas.** 1990. The metabolic pathway of 2,4-dichlorophenoxyacetic acid degradation involves families of tfdA and tfdB genes according to PCR-RFLP analyses. FEMS Microbiol. Ecol. **20**: 163-172.

40. **Voordouw, G., Y.Shen, C.S. Harrington, A.J. Telang, T.R. Jack and D.W.S. Westlake.** 1993. Quantitative reverse sample genome probing of microbial communities and its application to oil field production waters. Appl. Environ. Microbiol. **59**:4101-4114.

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

42. **Zhou, J. Xia, R.V. O' Neill, L.-Y. Wu A.V. Palumbo and J.M. Tiedje.** 1998. Unusual diversity in soil microbial communities , Intl. Ecol. Congress, Italy.