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THE EFFECT OF 2,4-DICHLOROPHENOXYACETATE SELECTION ON MICROBIAL COMMUNITIES IN MICROCOSM AND FIELD STUDIES AND THE IMPACT ON ECOSYSTEM FUNCTION

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By

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ABSTRACT

THE EFFECT OF 2, 4- DICHLOROPHENOXYACETE ON MICROBIAL COMMUNITIES IN MICROCOSM AND FIELD STUDIES AND THE IMPACT ON ECOSYSTEM FUNCTION

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The effect of long term 2,4-D selection on the microbial community and its impact on nutrient transformation was studied in the field and microcosms. 2,4-D was applied at (0X which is the control), the normal field rate of 1.1 kg/ha (1X), 10X and 100X the normal field rate for ten years. The microbial community was analyzed by cultural methods, reverse sample genome probing (RSGP) and functional gene probing. RSGP showed that the microbial community in the 100X plots had been altered such that there was an enrichment in some 2,4-D degrading populations compared to the control. In such altered microbial communities, microbial biomass, and microbial respiration were reduced. Carbon and nitrogen mineralization were also depressed and the depressive effect lasted for a 30 day incubation. Effect of long term high 2,4-D application did not significantly interfere with nutrient transformation although it did reduce plant productivity which may have contributed to the reduction in soil microbial biomass.

Microcosm studies demonstrated that management history of the soil (40 years plant succession, 10 years no - till, conventional agriculture) did not influence which 2,4-D degraders became dominant members in the 2,4-D

amended soil. Dominance by *Burkholderia* sp. was seen in the microcosm studies whilst in the field studies *Burkholderia* and its close relative *Ralstonia* sp. were dominant providing evidence of niche partitioning of 2,4-D degrading bacteria in the field. Discontinuing 2,4-D treatment in the southern part of the plots for three years resulted in a decrease in culturable and RSGP detectable population of 2,4-D degrading bacteria although 2,4-D enriched populations were still above control levels three years after 2,4-D added had been suspended. Hence it appears to take many years for perturbed populations to return to their normal densities one the perturbation is removed.

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CHAPTER ONE

Literature Review

1.0. Introduction

Synthetically produced chemicals such as insecticides, fungicides, herbicides and other pesticides have become an integral part of modern agriculture. Although complex in nature, many pesticides are readily degraded by microorganisms that are present in soil and water. Among them, 2,4-D has had widespread use as a herbicide during the past several decades. It is selectively toxic to broadleaf plants but not to monocotyledons, which include cereal crops. Application sites range from lawns, gardens and golf courses, to cereal crops, and pastures. The rate of application is 0.2-2.0 kg active ingredient (acid equivalent) per hectare. Because of the millions of hectares involved, interest in the environmental distribution and effect of 2,4-D has intensified in recent years, especially regarding impacts on nontarget plant species in an ecosystem. 2,4-D could also influence soil microorganisms and microbial processes that play a vital role in regulating nutrient cycling and availability. The runoff and leaching of herbicides applied to fields could contaminate rivers, lakes and groundwater, which is a potential danger to humans and other organisms. There is a concern that the trend of increasing herbicide use may eventually result in alteration of the soil biological equilibrium. Thus it is imperative to study the long term effect of pesticide 2,4-D applications on soil ecosystems and to determine whether changes in the soil microflora and their processes occur.

1.1 2,4-D and the microorganisms that degrade it

2,4-D is commonly prepared by the condensation of 2,4-dichlorophenol with monochloroacetic acid in a strongly alkaline medium at moderate temperature, or by chlorination of phenoxyacetic acid. The chemical structure is a modification of a naturally occurring plant hormone, auxin, and hence it is the chlorinated form of

auxin that kills dicotyledonous plants (13). At low application rates, 2,4-D serves as a growth regulator in apple trees to reduce premature fruit drop. Because of the ease by which soil microorganisms degrade 2,4-D at low concentrations, e.g. less than 50 ug/g soil, it is not considered a serious environmental contaminant. Audus (3), through soil perfusion experiments, showed that the detoxification of 2,4-D in garden loam soil was almost entirely due to the activity of microorganisms. Later he isolated *Bacterium globiforme* from 2,4-D enriched soils and established its ability to degrade 2,4-D. Since then a number of 2,4-D degrading bacteria have been isolated from agricultural or industrial sites, probably often from where 2,4-D or related chemicals have been applied (7, 12). Even in uncontaminated, pristine ecosystems, uninfluenced by human activity 2,4-D degraders have been isolated (42). Hence microorganisms that degrade 2,4-D are widely distributed in nature and presumably a part of the normal microbial community.

Organisms that have been reported to be capable of degrading 2,4-D belong to a number of genera including *Arthrobacter, Alcaligenes, Corynebacterium, Flavobacterium, Pseudomonas* etc. (Table 1). Most of the strains that degrade 2,4-D studied in the 1990s have been categorized into phylogenetic groups. One group comprises representatives from the beta and gamma subdivisions of the Proteobacteria. They contain 2,4-D degrading genes similar to the *tfd* genes of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) the first 2,4-D degrader studied at the genetic level (15). 2,4-D degraders in the beta and gamma subdivisions of the Proteobacteria usually carry a gene with 60. % or more sequence similarity to the canonical *tfdA* of pJP4 in *R. eutropha* JMP134.

Table 1. Selected list of 2,4-dichlorophenoxyacetate degrading organism:

Organism	Pathway location	Size	Origin	Reference
Alcaligenes eutrophus JMP134	pJP4	89kb	Australia	Don and Pemberton1981
Alcaligenes paradoxus JMP134	pJP1	58mD	Australia	Fisher, et al. 1978
Alcaligenes paradoxus	pJP2	53 kD	Australia	Don and Pemberton 1981
Alcaligenes eutrophus	pJP9	37 kb	Australia	Don and Pemberton
Alcaligenes sp.	pEML159	56 kD	Oregon	Amy et al.;1985
Arthrobacter	*			Tiedje et al, 1969.
Corynebacterium	*			Leather & Fox 1977
Flavobacterium	pRC10	45 kb		Chaudry and Huang, 1989
F. peregrinum	*			Steenson and Walker, 1957.
Mycoplana	*			Walker and Newman 1956
Nocardia	*			Loos,1975
Pseudomonas sp.	*			Evans & Smith, 1971.
Xanthobacter sp.	*			Ditzelmuller et al, 1989
Pseudomonas sp.	pEST4001	78 kb	Estonia	Asumees & Heinaru, 1990.
P. cepacia BR16001	chromosone		Nova Scotia	Greer et al, 1990.
Bordatella				Greer et al; 1990
Phanerochaete crysosporium				Yadav and Reday, 1997.
Strain RASC	chromosome		Oregon	Amy et al. 1985
TFD6	chromosome		Michigan	Tonso et al. 1994.

* Plasmids encoding 2,4-D degradation have not yet been identified for these strains.

The other group is composed of strains in the alpha subdivision of the Proteobacteria. The DNA of this group does not hybridize to *tfd* genes nor do the strains contain the alpha-ketoglutarate- dependent 2,4-dioxygenase enzyme (TfdA) found in the former group (28) .The pathway and genes involved in 2,4-D degradation in this group are unknown. *Sphingomonas* strains are the primary example in this latter group. 2,4-D degraders of this group are of ecological importance since they are easily isolated from lakewater, sewage sludge, and soil systems.

1.2 Some factors affecting the rate of 2,4-D degradation

The ability for microbes in the soil to degrade 2,4-D depends on its bioavailability, which is somewhat higher than for other pollutants which usually have lower water solubility. Most pesticides reversibly partition between soil solution and soil organic matter making the bioavailability of the pesticide a function of the partition coefficient (kd) which is also a function of the water solubility and organic matter content (67). Since bacteria themselves may be sorbed to soil colloids, it is conceivable that bacteria and pesticide may be sorbed on adjacent locations, thereby facilitating scavenging of the chemical by the sorbed bacteria. Thus pesticide sorption might either enhance or decrease microbial degradation. Environmental factors such as soil moisture, temperature, pH, and soil organic matter content also affect the bioavailability of pesticides, including 2,4-D, and their degradation in soil.

Soil moisture and temperature:

Soil moisture and temperature both significantly affect microbial activity. The optimum moisture tension and temperature of 2,4-D degradation was 0.1 bar (0.01 MPa) and 27°C, respectively, in a sandy loam soil (63). Moisture and temperatures

above the optimum resulted in slower rates of 2,4-D degradation in another study (44). Degradation of 2,4-D decreased progressively down to a water potential of -5.5 MPa with no breakdown observed at water potential at -22MPa (33).

pH:

The optimum pH range for 2,4-D degradation in broth cultures was 6.2- 6.9 (84). The 2,4-D degradation in soil was slower in the acid range (pH 3.8-5.6) than in the neutral range (pH 6.6 to 7.4) (72). In the neutral pH range, more 2,4-D is thought to be present in its dissociated form, which could be less toxic. However, fungi degrade 2,4-D in acid soils, while degradation in neutral soil is catalyzed mainly by bacteria and actinomycetes. Torstensson (83) measured the degradation of 2,4-D in cultures of soil microorganisms at different pH values and found half lives of 5 to 8 days at pH 8.5 to 5.0. At pH 4.5 and 4.0 the half-life increased to 21 and 41 days respectively.

Soil organic matter:

The adsorption of 2,4-D and microorganisms to soil particles was found to be controlled by the soil organic content. Sorbed 2,4-D was completely protected from biological degradation (60). Clay was observed to have low adsorption capacity for 2,4-D (32).

2,4-D formulation:

The 2,4-D formulation used can affect the rate of degradation and may have differential effects on the soil microflora. The commercial formulations are typically esters or dimethylamine salts of 2,4-D. It has been observed that degradation occurred at higher rates for the formulated 2,4-D than the technical

grade (62). At high application rates the common commercial formulations of 2,4-D have a much less depressive effect on soil microbial populations and processes than does the technical grade (74). This may be attributed to the fact that additional carbon or nutrients were present in the formulation or that the solubility is increased. For instance, the solubility of the dimethlamine salt of 2,4-D in water is higher than that of the technical grade. Also the 2,4-D in the formulation is neutralized by dimethylamine, implying that the local soil pH did not change appreciably. The pH in the soil receiving technical grade 2,4-D decreased by as much as 3 pH units depending upon soil type and 2,4-D concentration. This decrease in pH may have an inhibitory effect on soil microbial activity. Newman (56) found that the depressive effects of Na-salt forms of 2,4-D and other phenoxy acids on soil bacteria and fungi increased sharply with increasing soil acidity while the ester forms were equally inhibitory at all pH values and were always more deleterious than the Na-salt forms. He concluded that the phenoxy acids were inhibitory and were taken up by microbial cells only in the undissociated form.

1.2 Adaptation of organisms to 2,4-D

After a small amount of 2,4-D has been removed by adsorption on soil colloids, a lag period follows before noticeable degradation is seen (4). The length of the lag period varies according to the chemical; for example 2,4-D, MCPA (2-methyl-4-chlorophenoxyacetic acid), and 2,4-5-T (2,4,5- trichlorophenoxyacetic acid) characteristically exhibit short, long and extremely long lag phases, respectively. A phase of rapid substrate disappearance follows the lag phase.

Subsequent addition of substrate results in rapid disappearance without a lag. The lag phase is a result of the time required for the development in soil of an

effective population of herbicide-degrading organisms. The lag phase may also involve the induction of appropriate enzymes in the degrading organism and the selection of mutants with altered enzymatic specificity or novel metabolic activities. Other genetic mechanisms of adaptation may involve gene transfer, point mutation, DNA rearrangement or gene duplication.

Gene transfer: Transfer of genes has been shown to occur by transformation (25), transduction, and conjugation of plasmids and transposons on these plasmids (26, 55,). Gene transfer resulting in adaptation of host cells to new compounds has been demonstrated when the biodegradative capacity of *Pseudomonas* sp. strain B13 was expanded from 3-chlorobenzoate to 4-chlorobenzoate and 3,5-dichlorobenzoate by transfer of the TOL plasmid pWWO from *P. putida* m-2 (39). Transfer of catabolic plasmids can however lead to regulatory or metabolic problems for the cell so that additional mutations in the primary transconjugants are often required to construct strains with the desired metabolic activities.

Gene transfer techniques were further applied to construct a novel metabolic pathway for degradation of chlorinated benzene by mating *P. putida* F1, a strain which contains the *tod* genes encoding a broad-substrate-specificity benzene dioxygenase and benzene glycol dehydrogenase, with *Pseudomonas* sp. strain B13, which carries a modified ortho cleavage pathway on a plasmid. Oltmas et al (61) obtained transconjugants which could completely metabolize 1,4-dichlorobenzene. There is evidence that similar gene transfer processes occurred in nature in the evolution of the chlorobenzene pathway in *Pseudomonas* sp. strain p51 (85). This strain harbors a catabolic plasmid containing two catabolic operons, one encoding a modified *ortho* cleavage

pathway (86, 87) and the other encoding chlorobenzene dioxygenase and chlorobenzene glycol dehydrogenase (88). The complete chlorobenzene dioxygenase gene cluster is located on a transposable element, suggesting that the pP51 plasmid was formed by the transposition of the chlorobenzene dioxygenase transposon onto an ancestral plasmid of pP51 that contained only the modified ortho cleavage pathway genes (88).

Point mutations: Single-site mutations arise continuously and at random locations as a result of errors in DNA replication. It is also possible that a diversity of stress factors, including chemical pollutants (9), stimulate error- prone DNA replication and hence accelerate evolution. The accumulation of single base pair changes may not be the sole mechanism for the divergence of properties in contemporary catabolic enzymes. Other mechanisms such as gene conversion or slipped-strand mispairing would allow faster divergence of DNA sequences (36, 48).

DNA rearrangements: Gene rearrangements are also evident between the different operons for the modified ortho pathway enzymes. The *clc*ABD and *tcb* CDEF operons contain an extra open reading frame with 53.3% deduced amino acid sequence identity and which has no apparent function. This open reading frame is missing from the *tfd*CDEF (23).

The similarity between *xyl*XYZL genes and *ben*ABCD gene sequence (34, 35) suggests that these genes form a DNA module which was inserted in a different catabolic operon.

Gene duplication: Gene duplication has been considered an important mechanism for the evolution of microorganisms. Once duplicated, the extra gene copy could essentially be free of selective constraints and thus diverge much

faster by accumulating mutations. pJP4 appears to have undergone several gene duplications (15, 65). By DNA-DNA hybridization, 2 copies of *tfd*C and *tfd*A and of the putative regulatory genes *tfd*S and *tfd*R have been found in *Alcaligenes eutrophus* (*Ralstonia eutropha*).

1.3 Genes for 2,4-D degradation

Genes for 2,4-D degradation have been extensively characterized in *Ralstonia eutropha* JMP134 (pJP4). The 2,4-D catabolic genes in strain JMP134 are encoded on plasmid pJP4 that is an 80 kb, Inc P1, broad host range, self-transmissible plasmid that also carries genes for mercury resistance (14). It encodes *tfd*A BCDEF, six of the seven genes required for the conversion of 2,4-D to products of intermediary metabolism (15). This plasmid can transfer to and be maintained in members of the alpha, beta, and gamma subdivision of the Proteobacteria (including *E. coli, Rhodopseudomonas sphaeroides, Agrobacterium sp., Rhizobium sp., Pseudomonas fluorescens P. putida, Ralstonia eutropha,* and *Alcaligenes paradoxus*) at frequencies ranging from 10^{-1} to 10^{-7} (14). The phenotype for 2,4-D degradation is observed only in *R. eutropha*, *A. paradoxus*, *P. cepacia* and *P. putida*. This phenotype is not detected in pJP4 transconjugants of *Agrobacterium*, *Rhizobium*, *E. coli, Rhodopseudomonas*, *Acinetobacter*, *P. fluorescens* and *P. aeruginosa.* One possible explanation for the limited host range of expression of 2,4-D degradation is that the missing seventh gene, maleylacetate reductase (*mar*), is chromosomally encoded, so that only strains with this gene can metabolize 2,4-D as their sole source of energy (43).

Other 2,4-D plasmids

Other 2,4-D degraders have been found with 2,4-D genes located on plasmids (1, 7, 11, 16). Pemberton et al. (64) found that of 22 colonies isolated on 2,4-D after only 15 to 20 generations in non-selective broth culture, only 5 colonies still contained plasmids conferring ability to catabolize 2,4-D. Three of these were similar to pJP4 in size and restriction pattern and incompatibility but had the ability to degrade phenoxyacetic acid (when not induced by 2,4-D) which is not found for pJP4. Chaudry and Huang (11) found another plasmid, pRC10, encoding a 2,4-D pathway in *Flavobacterium*. This 45 kb plasmid has sequences that hybridize to *tfd*A and *tfd*B of the pJP4, but has no sequences that hybridze to the region of pJP4 involved in conjugation, incompatibility and maintenance. Plasmid pEML159, discovered in Oregon sewage sludge, is similar to pJP4 in size, restriction pattern and ability to transfer the 2,4-D degrading phenotype to other bacteria. This similarity is perhaps surprising since pJP4 was isolated on the other side of the world, in Australia, suggesting that pJP4 is globally distributed.

Chromosomally encoded 2,4-D genes:

Although the best characterized genes for 2,4-D degradation are plasmid encoded (1, 7, and 22), there is evidence that other 2,4-D degraders have chromosomally encoded 2,4-D genes, including strains RASC (76), TFD 6 (54) and BR16001 (32), which appears to be identical to TFD 6. Recently the *tfd*A and *tfd*B genes from *Burkholderia* sp RASC have been cloned and sequenced (76). The RASC *tfd* genes share > 77 % DNA sequence similarity with the corresponding pJP4 genes and encode isozymes for the modified ortho-cleavage pathway. Despite their chromosomal location, the RASC genes are transmissible between species, although the mechanism of transfer is unknown. In

hybridization studies of a collection of 47 dominant 2,4-D degraders from the Kellogg Biological Station site, Ka et al. (41) demonstrated that the *tfd*A gene was on a typical sized plasmid (< 100 kb) in 75 % of the strains and on the chromosome (or megaplasmid) in the other 25 %. Furthermore, they showed that the entire 2,4-D degrading plasmid could be incorporated into the chromosome of strain *Alcaligenes paradoxus* 2811P under non-selective conditions. Hence the location of 2,4-D genes can be quite fluid (40).

The *tfd*A (75) gene encodes the first enzyme of the 2,4-D degradation pathway. This enzyme transforms alpha-ketoglutarate to succinate and carbon dioxide concomitant with conversion of 2,4-D to 2,4 dichlorophenol and glyoxylate (28). 2,4-dichlorophenol hydroxylase, encoded by *tfd*B gene also located on the pJP4 plasmid (15) catalyzes 2,4-dichlorophenol to yield 3,5-dichlorocatechol (65). This product then enters the modified ortho pathway, which is catalyzed by the *tfd*C gene product, an intradiol dioxygenase that yields 2,4-dichloro-cis, cis-muconate (15, 66). Subsequent steps in the pJP4 pathway involve the conversion of 2,4 dichloro-cis-cis-muconate to 2-chlorodiene-lactone with elimination of chloride anion by the enzyme chloromuconate cycloisomerase, encoded by the *tfd*D gene located on pJP4. The pJP4-derived *tfdE* gene encodes a dienelactone hydrolase (65) that hydrolyzes 2-chlorodienelactone to form 2-chloromaleylacete. The chromosomally-encoded enzyme (chloromalelyl reductase) converts 2-chloromaleylacete to malelylacetate and finally to succinate and acetylcoenzyme A, which enters the tricarboxyylic acid cycle.

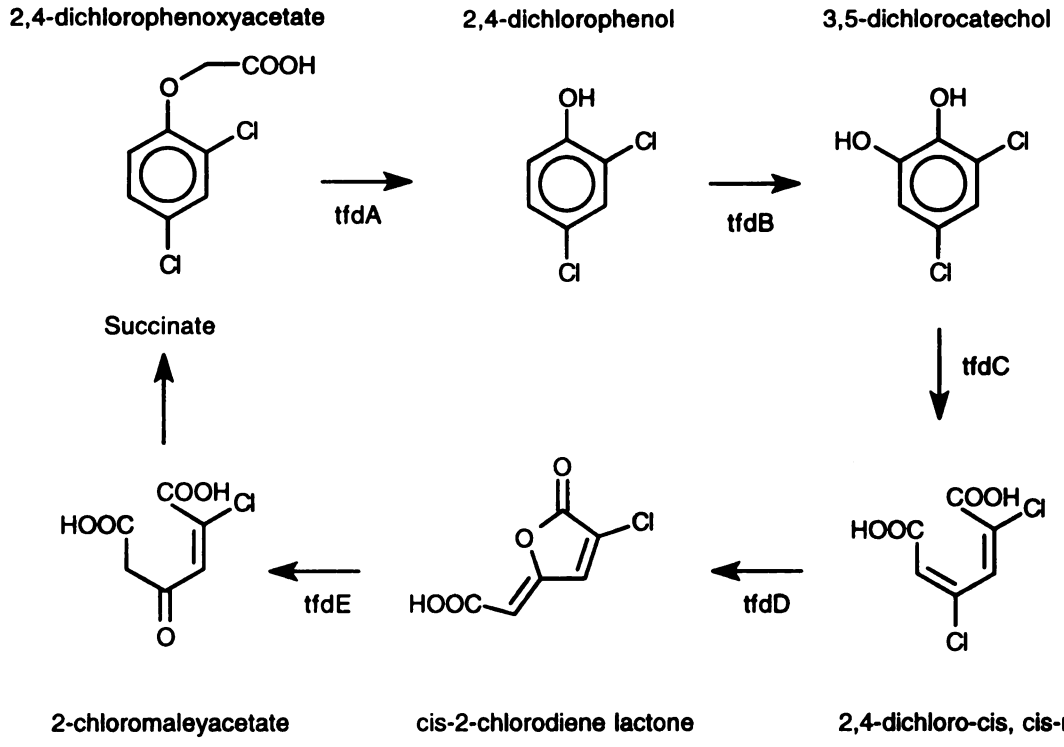

Fig. 1. 2,4-D degradative pathway encoded by plasmid pJP4 in Ralstonia eutropha JMP134. The genes *tfd*A,B,C,D, and E encode 2,4-dichlorophenoxyacetic acid/ alpha-ketoglutarate dioxygenase, 2,4-dichlorophenol hydroxylase, 3,5-dichlorocatechol dioxygenase, chloromuconate cycloisomerase and chlorodiene-lactone hydrolase, respectively

The *tfd* genes on pJP4 are organized into 3 transcriptional units consisting of *tfd*A, *tfd*B and *tfd*CDEF operons and are regulated by *tfdR/tfdS* genes. The *tfd*A gene is located 13 kb away from the *tfd*CDEFB gene cluster (28). These *tfd* genes do not always occur together in the beta Proteobacteria but appear as distributed mosaics of dissimilar 2,4-D pathways and genes (27). They have varying degrees of DNA sequence similarities to the pJP4- borne genes. The diversity of associations between low and high similarity elements (*tfd*A, *tfd*B, *tfd*C) suggests that some strains have recruited individual genes from different

sources rather than a preassembled whole gene. This hypothesis is consistent with the observation that *tfd*A appears to have been recruited independently, based on its distance from the other *tfd* genes as they occurred on pJP4.

The appearance of highly similar *tfd* genes in different bacterial strains is most parsimoniously explained by horizontal genetic events. For instance the *tfd*B gene is similar in sequence to the phenol monoxygenase gene, *phe*A of *Pseudomonas* sp. EST1001 (57, 65). The *tfd*CDE gene sequences are also similar to those of the chlorocatechol degradative genes *clc*ABD and *tcb*CDE (23, 49). The *tfd*A gene, on the otherhand, exhibits no significant similarity to other known gene sequences in chloroaromatic metabolism, and the reaction catalyzed by 2,4-D- alpha ketoglutarate dioxygenase (TfdA) appears to be unique to 2,4-D catabolism. The *tfd*A gene was first sequenced from *Ralstonia eutropha* JMP134. Two more *tfd*A genes were cloned from chromosomal locations in the *Burkholderia* strain RASC and *Burkholderia* strain TFD6. These proved to be identical to each other and 78.5 % similar to the original. An alignment of the two variants allowed conserved areas to be identified and primers to be designed for the amplification of *tfd*A-like genes. Genes carried by RASC do not hybridize to those found on pJP4 under high stringency conditions (27).

Biochemical pathways for 2,4-D degradation

There are at least three different biochemical pathways for 2,4-D degradation. The first pathway involves the reductive dechlorination reaction that has been identified in some microorganisms, such as *Azotobacter chroococcum,* which dissimilate 2,4-D via 4-chlorophenoxyacetic acid, 4- chlorophenol, and 4-chlorocatechol (6). The elimination of chloride from the aryl C-2 position in this aerobic organism is similar to the transformation of 2,4-D observed in many

anaerobic environments (6). The second pathway involves ring hydroxylation prior to side chain removal. This mechanism has been observed for *Pseudomonas* strain NCIB 9340 which can convert a fraction of 2,4-D to 2,4- dichloro-6-hydroxyphenoxyacetic acid (21). Third is the TFD pathway, in which the modified ortho-cleavage and an initial aryl ether cleavage yield glyoxylate and 2,4-dichlorophenol (80). Subsequent cleavage of the aromatic ring results in the formation of chlorocatechol that undergoes several additional enzymatic transformations to yield acetate and succinate. 2,4-D can also be co-metabolized by enzymes with low substrate specificity resulting in the accumulation of 2,4-dichlorophenol.

1.5 Microbial communities and related interactions

The microbial community is an assemblage of populations of microorganisms occurring and interacting at a given location called a habitat. Populations within the community tend to interact with each other and not with populations of other communities (78). One such interaction is competition which is a negative relationship between two populations that utilize the same resource. These resources can be space, or nutrients such as carbon, nitrogen, phosphorus, oxygen, or water, etc.

Competition tends to bring about ecological separation of closely related populations. This is the principle of competitive exclusion (24), i.e two populations cannot occupy exactly the same niche, because one will win the competition and the other will be eliminated. Thus competition is a fundamental mechanism acting in natural selection. Populations may coexist, however, if populations can avoid absolute direct competition by using different resources at different times.

Current views of competition dynamics in physically complex environments such as soil are extrapolated from mixed liquid environments such as stirred batch cultures and chemostats. In serially-transferred batch cultures and chemostats, competition for a single growth- limiting nutrient typically results in the dominance of one population and extinction of other competitors. However, coexistence of competing populations in batch cultures or chemostats can be achieved by introducing factors such as surfaces for bacterial attachment (20), chemical gradients (92), resource fluctuations (72), chemical inhibitors (47), predators (13) or multiple growth- limiting nutrients (29, 81). These factors are natural components of soil microbial communities. Consequently, the view has arisen that coexistence may be common in natural environments such as soil (29). Dominance of one or a few competing populations in the microbial community may occur only at microscales that are difficult to observe while coexistence of a large number of populations might be observed at scales of millimeters and greater.

A critical factor in determining the persistence of any population within a community is its genetic fitness, i.e, the contribution of one or more alleles of the population to succeeding generations (46). Therefore the totality of genes within the individual populations determines the stability of the community. Genes can be transferred to a new population within the community to form new allelic combinations with differing degrees of fitness (18, 49) Differences in fitness between alleles or genotypes reflect systematic differences in either mortality or reproduction, which in turn reflect systematic difference in ecological properties such as ability to compete for limiting resources, susceptibility to predation, etc.

Processes that bring about a systematic change in the frequency of alleles include mutation, recombination, and genetic drift (46). Genes that contribute to the fitness of populations are usually maintained within the community and those that do not are lost from that communty. Gene transfer however can result in the maintenance of an allele or extra chromosomal element in the population in the face of opposing selection (73). One strain that is less fit than another can be maintained by recurring mutation or by migration from another source population. One allele that is less fit than another may also be maintained in a population by virtue of its association with a favourable allele elsewhere in the genome.

1.6. Effects of 2,4-D on microbes in soil

The soil microbial community interacts with the other components of the ecosystem such as the soil minerals and the above ground plant community (79). Soil management may modify the factors controlling soil microbial activity and shift the equilibrium of the ecosystem. The application of 2,4-D to the soil, at both the normal field rate of application (1.1 kg/ha) and experimentally at higher rates is an example of such a management practice.

At a normal field rate of 2,4-D application, the growth of bacteria and fungi was stimulated because of the ease by which microbes degraded 2,4-D and benefitted from it as a carbon and an energy source. The stimulation of non-filamentous bacterial and fungal growth rates was believed to be due to the elimination of some of the actinomycete populations by the pesticide, thus decreasing competition from these groups of organisms. At normal rates of 2,4-D application, nitrification rates were temporarily reduced (30), whereas ammonification and microbial respiration were unaffected. The activity of urease

and of acid and alkaline phosphatase were reduced only for a short time (10). 2,4-D also inhibits other microorganisms such as *Sporocytophaga*, *Cellvibrio* sp. Aerobic and anaerobic spore formers were affected when the concentration in soil was less than 0.1%. Despite these specific reports, the effect of 2,4-D was found not to be ecologically significant when evaluated in the field after thirty-five years of consecutive application at normal field rates (10).

Application of 2,4-D at higher- than- normal field rates affects some soil microbes and the general microbial community structure. Lenhard (45) found that 100 to 1000 ppm of 2,4-D decreased dehydrogenase activity as well as the total microbial population in the soil. Rates above 500 ppm caused autolysis of the bacteria and decreased nitrogen fixation by *Azotobacter*. Growing cells of *A. chroococuum* and *A. agile* were more resistant to the harmful effects of 300 ppm of 2,4-D triethanolamine salt than were resting cells (52). In vitro studies showed that the growth of *Aspergillus niger* and several other soil fungi were affected. Biodegradation of high rates of 2,4-D application in soil was accomplished by a few microbial species, probably because of their greater metabolic specialization for this chemical (2). Shifts in the microbial community from the more diverse to a less diverse, primarily 2,4-D- degrading populations, was observed in soil microcosms and in field studies (37, 41). In their study, 2,4-D degrading strains of *Sphingomonas* sp.(an alpha *Proteobacteria*) and *Pseudomonas picketii* (a beta *Proteobacteria*) became dominant members of the microbial community.

High rates of 2,4-D application tend to disturb the ecosystem as was shown by an increase in the metabolic quotient (qCO_2) when 200 ug/g of 2,4-D was applied to the soil. An increase in qCO_2 indicates a disturbed ecosystem or those in earlier successional stages (58, 59). In such a disturbed state a reduction

in efficiency of the microbial biomass was observed. A higher percentage of substrate is respired as carbon dioxide than is incorporated into biomass production or maintenance of microbial tissue (38). The use of the qCO_2 value as a bioindicator of disturbance in the ecosystem has been criticized as not being sensitive enough to distinguish between the effects of disturbance and stress. It does not decline predictably in response to ecosystem development when stress increases along successional gradients (90). Even though the use of qCO_2 has been criticized, it could be used as an index of adversity of environmental conditions for soil microflora. It is also relatively easy to use.

Other indications of a disturbed ecosystem are an increase in community respiration, hence the R/B ratio (the maintenance respiration to biomass ratio) increases. Also P/R (production/respiration) ratio tends to become unbalanced in a disturbed ecosystem. Margelaf (53) pointed out that the drain of productive energy "opens up" the ecosystem so that auxiliary energy from the outside results in an increase in unused resources that may be stored within the system or exported. Nutrient turnover and horizontal transport increases, while vertical cycling of nutrients decreases. Also nutrient loss increases and the system becomes" leaky". In mature ecosystems, competition for resources tends to favour k-selected species, wheras in stressed ecosystems there is an increase in opportunistic or r-selected species. Species diversity is reduced by toxic inputs (insecticides, for example) and dominance increases, At the ecosystem level, redundancy of parallel processes theoretically declines. Autogenic successional trends reverse (succession reverts to earlier stages), and the equilibrium of the ecosystem may be altered (59).

1.7. Soil management and the microbial community

Other forms of soil management in ecosystem development may involve different cultivation practices, such as conventional till, no- till and successional fields left to regrowth. These cultivation practices could result in the modification of factors controlling soil microbial activity. Generally no- till plots have better aggregate structure, being platy near the surface and having interconnection of fine pores throughout the profile. The better aggregate structure is due to the higher carbohydrate concentration in the soil, resulting from an increase in microbial activity, increase in biomass and enhanced synthesis of polysaccharides which stabilize the aggregates. Earthworm channels with excretment infillings are abundant in no-till plots at all depths, but are absent in conventionally tilled plots (19). Pores in no- till plots were more abundant than in the conventionally tilled plots especially pores of size < 100 um in diameter (19). Absence of tillage was important in increasing N mineralization capacity (91). Conventionally tilled soil, on the other hand, was composed of granular and fragmented structural units with no evidence of earthworm activity (20). A higher degree of humification of organic matter was observed in non tilled grass pasture (70). A successional field which has never been plowed at the Kellogg Biological Station 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 (68).

1.8 Research focus

Most previous studies have used 2,4-D concentrations equivalent to field application rates of 1-2 ppm. For these concentrations it is generally accepted that

2,4-D did not persist more than for one season. Short and long term effects on the ability of soil microorganisms to degrade pesticide at higher concentrations, upto 100 ppm are not well documented. At high application rates, the soil microbial community shifts such that a few members become dominant. When such selection has been exerted for a long time it is not known whether communities with a few members will perform the normal nutrient cycling and ecosystem functional processes. My research was focused on the following hypotheses:

1. The previous management history of the soil affects which dominant members in the bacterial community will be selected during 2,4-D treatment.

2. Long term selection by 2,4-D affects the microbial community thereby disturbing ecosystem equilibrium and decreasing the rate of nutrient transformation, particularly of carbon and nitrogen.

3. Upon termination of a long- term 2,4-D selection, the microbial community structure gradually reverts to its former status.

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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 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 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 *tfdA* 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.

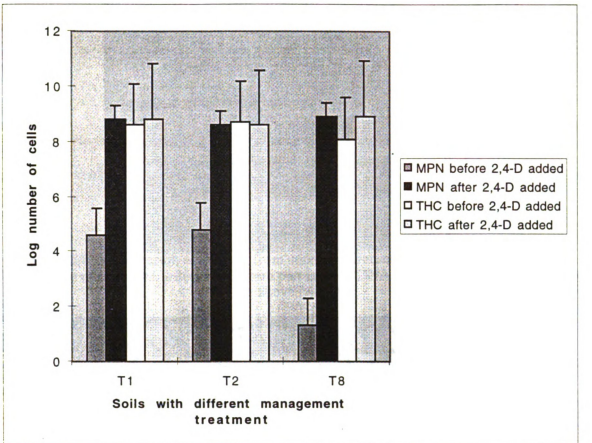


Fig. 1. MPN of 2,4-D degraders and of total heterotroph (THC) before and after 2,4-D addition in soil microcosm experiment. Bars are representative of three replicates i.e from three field plot replicates.

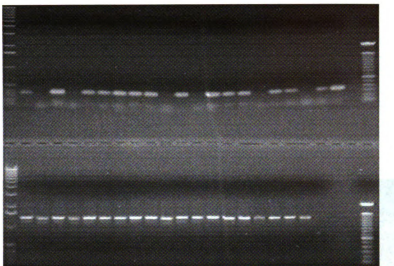


Fig. 2. *tfd*A amplification of isolates from soils of different management histories (Top part) . Size of fragment is 360 bp. Bottom part is 16 s rDNA of isolate to act as control for the above reaction.

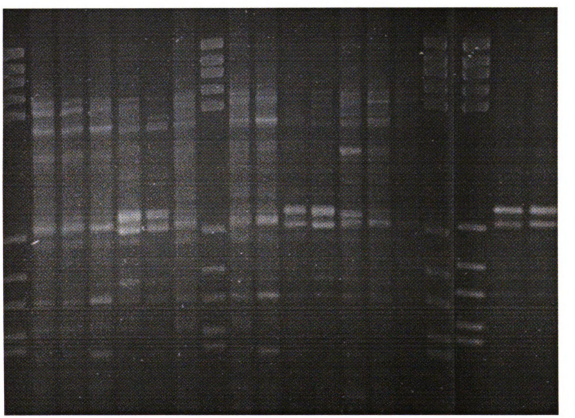


Fig.3. Community ARDRA pattern of soil treatments before and after 2,4-D addition. Lanes 1, 8, 16, and 17 are marker DNA V; Lanes 2 to 4, T1 before 2,4-D addition, Lanes 5 to 6 T1 after 2,4-D addition, Lanes 7, 9, 10, T2 before 2,4-D; Lanes 11 to 12, T2 after 2,4-D, Lanes 13 to 15, T8 before 2,4-D, and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

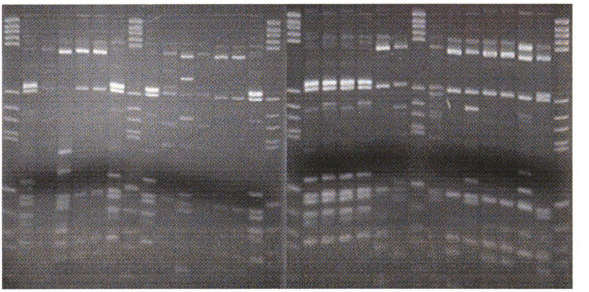


Fig 4. ARDRA pattern of 2,4-D isolates from soil samples of different management history. Lanes 1, 8, 16, 17, 24, and 32 are marker DNA V. Lanes 2 and 3 are restriction fragments of isolates from 0 ppm 2,4-D Gene Transfer Plots, lanes 4 to 7 are isolates from 100 ppm plot 2,4-D plots, lanes 9 to 12 are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

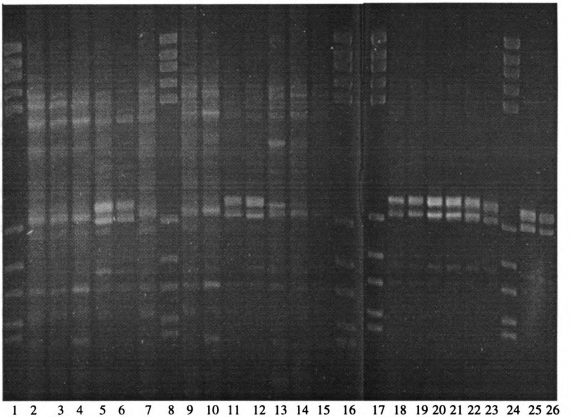


Fig.5. Effect of 2,4-D and soil management treatments on the selection of microbial communities as measured by community and isolate ARDRA. Lanes 1, 8, 16, and 17 are marker DNA V; Lanes 2 to 4, T1 before 2,4-D addition, Lanes 5 to 6 T1 after 2,4-D, Lanes 7, 9, 10, T2 before 2,4-D; Lanes 11 to 12, T2 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.

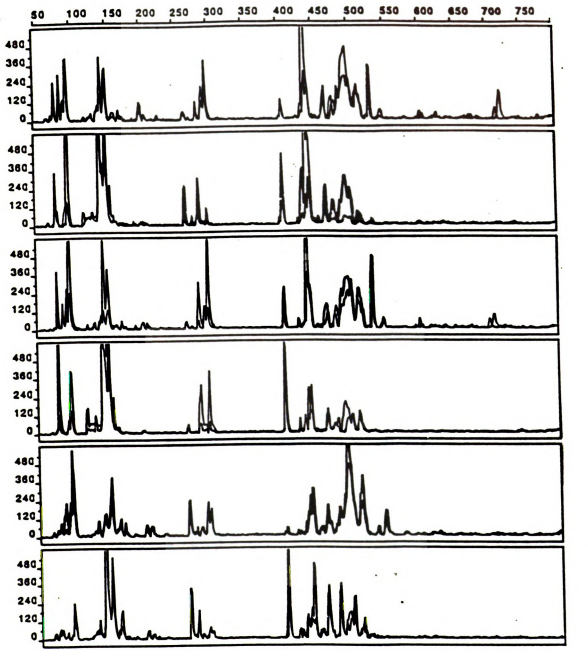
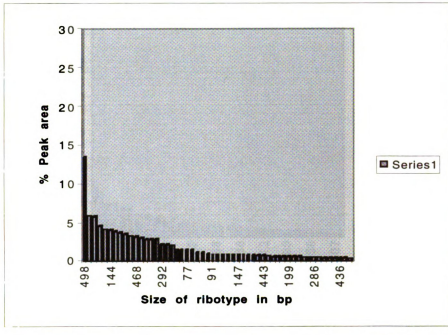


Fig. 6. Electropherograms of Terminal Restriction Fragments of Soil samples before and after 2,4-D addition in soil microcosm experiments. 1& 2 represent T1 before and after 2,4-D addition. 3 &4 represent T2 before and after 2,4-D addition, 5&6 represent T8 before and after 2,4-D addition. Electropherograms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



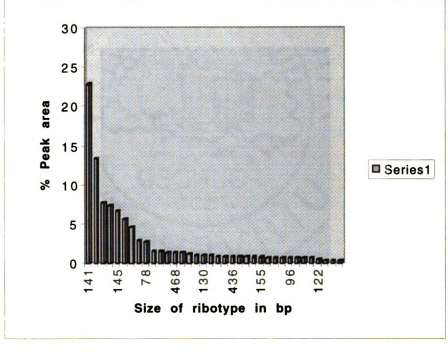
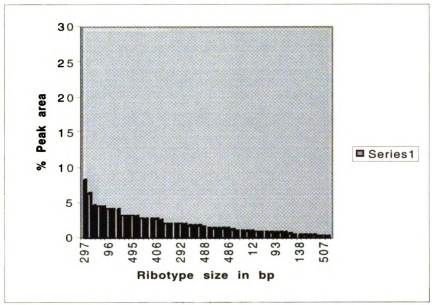


Fig. 7 a & 7 b. Relationship between the percentage peak area and size of ribotype of T1 before (top) and after (bottom) 2,4-D addition in soil microcosm experiment.



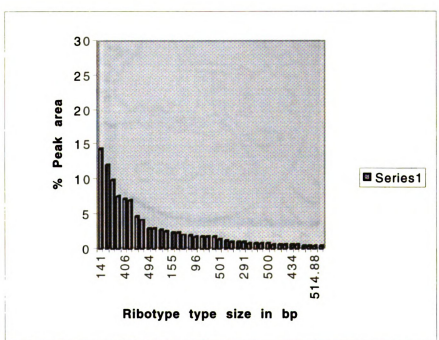
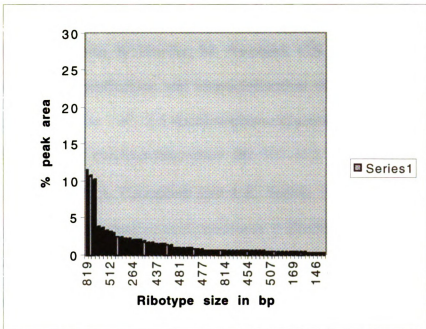


Fig. 8 a & 8 b. Relationship between the percentage peak area and size of ribotype of T2 before (top) and after (bottom) 2,4-D addition in soil microcosm experiment.



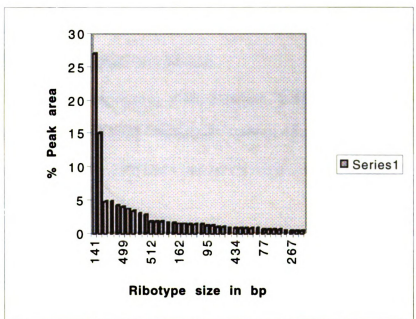


Fig. 9 a & 9 b. Relationship between the percentage peak area and size of ribotype of T8 before (top) and after (bottom) 2,4-D addition in soil microcosm experiment.

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CHAPTER THREE

THE LONG TERM EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON ECOSYSTEM FUNCTIONING OF FIELD PLOTS

Abstract

The effects of annual application of 2,4 -D on the soil biosphere was studied in plots that received technical grade or the amine formulation of 2,4-D at rates of 0 kg/ha (control plot), 1X (the normal field rate of application of 1.1 kg/ha), 10X, and 100X that amount for ten consecutive years. The soils were sampled to determine the effect of herbicide on microbial C and N processes, and the structure and the metabolic status of microbial community. In the top 15 cm of soil, microbial biomass, microbial nitrogen, and total phospholipid fatty acids (PLFA) were depressed with an increase in 2,4-D concentration while rates of nitrification increased. Both nitrogen and carbon mineralization were depressed with increased 2,4-D concentration. After one month of incubation the depressive effect of 2,4-D on C mineralization was significant but this was not the case at subsequent sampling times.

The Gram negative community in the 100X plots appeared to be in the inactive phase based on PLFA signatures whilst that of the other treatments were in the active phase. The rate of microbial turnover in these plots was low and the microbial community was showing signs of adaptation. Although some herbicide -induced changes were significant ($P < 0.05$) all the depressive and stimulatory effects were relatively small (< 50 % change from the control). The effects of long term 2,4-D application were not ecologically significant to the soil microbial community nor did they significantly interfere with nutrient cycling to adversely affect soil fertility. The results suggest a trend towards disturbance of the ecosystem equilibrium if 2,4-D were to be continually and repeatedly applied to the plots at high rate.

Introduction

The role of microorganisms in agroecosystems is often understated (23) yet without microbes and their function, no other organism could be supported by the soil. Microorganisms constitute a source of and sink for nutrients in all ecosystems and play a major role in plant litter decomposition and nutrient cycling (5, 9, 39), soil structure (26), dinitrogen fixation (40), mycorrhizal associations (1), reduction in plant pathogens (10) and other alterations in soil properties that influence plant growth.

Soil microorganisms also are sensitive biological markers (31. 43) and useful for classifying disturbed or contaminated systems since diversity can be affected by minute changes in the ecosystem. Early investigators such as Waksman (42) noted that the use of microorganisms coupled with physical and chemical parameters could indicate the fertility of a soil. The microbial criteria identified then were numbers of microbes, nitrification, carbon dioxide evolution, cellulose decomposition, dinitrogen fixation, etc. Domsch et al. (13), on the otherhand, suggested other microbial parameters be measured such as those populations with greater sensitivity to perturbation including nitrifiers, Rhizobium, actinomycetes and those of moderate sensitivity, which were algae, bacteria, fungi and soil respiration, denitrification and ammonification. It is also possible that individual species can function as indicators of the status of an ecosystem. These are the keystone species in that they should reflect the response of the community they represent. There is, however, concern over the use of single populations to identify stress response of a system (6, 24). It is argued that the effect of stress on single species cannot predict what will happen

throughout the community nor identify all the interactions that may be altered (7).

Management practices influence microbial activities over the long term in agricultural lands (3, 27, 32). One such agricultural practice is the use of herbicides to control the weeds which pose a major threat to crop yields (34, 36). Therefore, there has been considerable interest in the side effect of these chemicals on non-target organisms, including soil microorganisms (16, 35). Most of the studies on this subject have focused on herbicide effects on microbially mediated processes eg. nitrification, denitrification, soil respiration and soil enzyme activity (8, 20, 46). Process level measurements, although critical to understanding the ecosystem, may be insensitive to community level changes due to the redundancy of these functions and the complexity of relationships within particular communities. This measure also does not indicate the diversity and location of the organisms responsible for such alteration. The majority of studies have used laboratory incubation techniques for investigating such side-effects (35) but there are certain difficulties in attempting to extrapolate such information to the field, especially relating it to the importance of a detected herbicide side-effect relative to the background spatial and temporal biotic variability in the field (11). The authors generally conclude that detailed field data on spatial and temporal variation in environmental variables could provide a background of natural variability against which pesticide side-effects could be assessed.

In recent times, field experiments have been conducted such as that of Wardle and Parkinson (44) who found that 2,4-D significantly influenced all microbial variables investigated but that they were transient being detectable only within the first 1-5 days of herbicide addition. Similarly, Biederbeck (2)

found that the effect of 2,4-D after 40 years of field application was not of ecological significance. Domsch et al. (13) also concluded that most detected side -effects of pesticide applications on the soil microflora were relatively unimportant because variability in natural factors may depress soil processes by over 90 %. Few have done ecosystem level studies monitoring ecosystem processes and their relation to community structure and activity.

The objective of this study was to elucidate any long term effect of 2,4-D of soil ecosystem processes carried out by the microbial community.

Materials and Methods

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 (15). 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 2,4-D the dimethylamine ester form 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.

Microbial parameter measurements.

The potential nitrification rate is the enzymatic oxidation of ammonia to nitrates by the nitrifiers (viz Nitrosomonas and Nitrobacter) and this rate was determined by the shaken soil- slurry method for assessing the maximum rate (Vmax) of nitrification for a soil sample (18). Samples were incubated under laboratory conditions that had been optimized with respect to water content, ammonium, aeration and P availability. Fifteen gram of sieved, field-most soil was put into a 250-ml Erlenmeyer flask. with 100 ml of combined solution (0.3 mM KH_2PO_4, 0.7 mM K_2HPO_4 and 0.75 mM NH_4SO_4) and the flask covered with a vented cap. All flasks were placed on an orbital shaker and shaked at approximately 180 rpm for 24 h. At 2 h intervals aliquot samples (5 ml) were collected, 1 ml of 2N HCL was added and centrifuged. The supernatant was then stored at -20°C and later analyzed for NH_4^+ and NO_3^- contents.

The N mineralization rate is the microbial release of organically bound nitrogen to inorganic mineral forms and it was determined using the sieved field-moist soil that was placed in plastic caps and put in a Mason jar sealed with a lid, Hart et al., (18). The soil was then incubated for at least 100 days at room temperature (25° C) in the dark. Ammonium and nitrate concentrations were determined by extracting soil subsamples with 1N KCl before the experiment began and every tenth day of incubation.

Carbon mineralization was measured by incubation of soil for > 100 days at room temperature in the dark in Mason jars. Accumulated CO_2 was collected in a plastic vial containing 1N KOH that was placed near the soil sample in the jar. Every ten days the CO_2 released was determined and fresh vial containing 1N KOH was placed in the Mason jar.

Microbial biomass was determined by the chloroform - fumigation incubation method (CFI) as described by Howarth and Paul (21). Chloroform was used to lyse living microbial cells in a soil sample whilst an identical sample remained unfumigated. The soil samples were then incubated for a period of 10 days in sealed Mason jars. The temporary flush of carbon dioxide was primarily due to decomposition of microorganisms (22). In addition, an increase in the NH_4^+ pool occurred as a result of mineralization of nitrogenous constituents from lysed microorganisms. The increase in CO_2 evolution and extractable NH_4^+ from fumigated samples were used to estimate the size of soil microbial biomass carbon and microbial biomass nitrogen, respectively. The microbial respiration rate was determined from the CO_2 evolved from the unfumigated soil after the ten day period of incubation. Thus differences in incubation period distinguished microbial respiration from carbon mineralization.

Statistical analyses. Analyses of variance (ANOVA) were performed with the general STATA program for the microbial biomass, microbial respiration, microbial nitrogen and carbon mineralization. The number of replicates was three. To determine the standard error of the metabolic status of microorganisms n was two.

PLFA analyses. Samples of soil for phospholipid fatty acid analysis (PFLA) were removed as soon as soils were sieved and kept frozen at -20 °C. The samples were then sent on dry ice to the Microbial Insight Incorporated at Rockford, Tennesee. The **metabolic status** of the Gram negative population was determined as follows;

$$\frac{\text{cy 17:0}}{\text{16:1w7c}} + \frac{\text{cy19:0}}{\text{18:1w7c}}$$

which is based on the fact that in Gram negative bacteria 16:1w7c and 18:1w7c are converted to cylopropyl fatty acids (cy 17:0 and cy 19:0) as microbes move from log to a stationery phase (17).

Turn over rate= $\frac{1}{\text{cy 17:0/16:1w7c + cy19:/18:1w7c}}$

Stress indicator of microbes: Gram negative bacteria generate trans fatty acids to minimize the permeability of their cell membranes as protection against changes in the environment such as toxicity or starvation. Hence

$$\frac{\text{16:1w7t}}{\text{16:1w7c}} + \frac{\text{18:1w7t}}{\text{18:1w7c}}.$$

if the sum of the two is > 0.1 then the starvation state is indicated (19).

Results

Effect of 2,4-D on non-target organisms: The total PFLA for the control was the highest (29,182 pm/g soil, Table 1) suggesting that this plot had the highest viable cells. This value decreased by 42 % in the plots with the highest 2,4-D. Generally as the 2,4-D concentration increased the total PLFA also decreased. 2,4-D depressed the Gram negative population (represented as the monoenoics) more than the other populations (Fig. 1 a and Fig. 1d). In this comparison the 4 % decrease in the Gram negatives was compensated by a slight increase in the Gram positive (mostly represented by terminally branched saturated fatty acid) and the actinomycete populations (also represented by the mid chain branched saturated fatty acid). The % Gram positive bacteria on the otherhand increased slightly in the 10X 2,4-D treatment and then decreased again. The proportions of the

actinomycete and the eukaryote populations remained relatively unchanged in the rest of the treatments.

Effect of 2,4-D concentrations on microbial parameters: High 2,4-D concentrations reduced the microbial biomass as measured by the chloroform fumigation method by 22 % in the 100X plots as compared to the control plots and the other treatments (Table 1), Similarly microbial respiration declined indicating that there was a reduction in microbially related activities with an increase in 2,4-D concentration although no difference was statistically significant. Microbial biomass nitrogen on the otherhand increased with 2,4-D concentration and the differences in treatment were significant. The low C:N ratio especially in the 100X treated plot suggest that nitrogen was being immobilized into the microbial cells rather than being released into the envinronment for the plant community. The C:N ratio for the other treatments remained relatively high (Table 1).

The Gram negative population contributed more to microbial respiration with increasing 2,4-D (r 58, Table 2) whilst the converse was observed for the Gram positive population and eukaryote populations (Table 2). A negative correlation coefficient was observed between microbial nitrogen and the % of Gram negative population in the microbial community implying that an increase in microbial nitrogen was not favorable to the Gram negative population. Generally microbially biomass carbon was negatively correlated with microbial biomass nitrogen (Table 2).

Transformation of carbon was depressed in the 100X plots but not in the other treatments (Fig. 2). Significant differences in the treatments were observed for carbon mineralization on the 30th day of incubation but not on the fiftieth

and seventy days of incubation (Table 1). Likewise, the rate of carbon transformation declined with an increase in 2,4-D. The Gram negative population played a significant role in C mineralization as 2,4-D concentration increased (r being 98%) as compared to the Gram positive and the eukaryote populations (Table 2). Net nitrogen mineralization also declined in the other plots. This is reflected in the decrease in nitrogen mineralization rate as 2,4-D concentration increased (Fig.3). However, there were no significant differences in the treatment after 30, 60 and 70 days of incubation . At all levels of 2,4-D treatment, correlation between the rate of carbon and nitrogen mineralization was not high and was even negative in the 10X 2,4-D treated plots (Table 2) implying that the two processes were not closely related. Beyond 70 days of incubation, N immobilization was observed in some of the higher 2,4-D treated plots, for instance in the 10X and the 100X plots.

The nitrification rate, on the otherhand, was slightly higher (4.57 ug/g soil) with the highest 2,4-D concentration. A graphic plot of the nitrification vs time revealed a biphasic activity (Fig. 4). The control, 1X 10X plots had 4.46, 3.27, 3.71 ug/g soil as their nitrification rates, respectively. The rate of N mineralization for all 2,4-D treatments was similar, however, the rate of C mineralization for the control plot was higher than the rest of the treatments (Table 3). This indicates that long term 2,4-D application has the tendency to affect the carbon cycle.

Metabolic status of the microbial community: According to the PLFA analysis, the gram negative community was in the inactive phase whilst for the other treatments they were in the active phase (Table 1). Moreover the microbial turnover rate declined from the control plots to the higher 2,4-D treated plots. The turnover rate in such higher treated plots was 0.43 as compared to 0.74 for the

control plots. Also the microbial community showed signs of adaptation to the environmentally induced stress from either toxicity or starvation. Ammonification on the otherhand was not affected by the increase in the 2,4-D concentrations, hence the rate of ammonification remained the same in all the plots.

Discussion

With increase in 2,4-D concentration, microbial respiration declined which might not be beneficial to soil fertility since it can reduce the rate of herbicide dissipation leaving a herbicide concentration which might be injurious to more sensitive 2,4-D degraders. The effect of high 2,4-D concentrations on the microbial community might be due to the accumulation of 2,4-dichlorophenol especially when 2,4-D is applied at high rates (28). Chlorophenols, are classic cytochrome uncouplers and are not always readily used as sole carbon source because of toxicity at low substrate concentration e.g. 100 ppm, (33). Recently 2,4-dichlorophenol was isolated by Smith (38) from laboratory-incubated soils and identified as a soil degradation product of 2,4-D.

Another reason why high 2,4-D concentration appear toxic to the microbial community might instead be due to the decrease in available soil organic matter or altered soil conditions due to the lack of plant growth. The higher 2,4-D treated plots were virtually weed free even after the planting season and this was observed at least for 2 years. This could possibly lead to decrease in organic matter, causing significant changes in the microbial populations hence affecting nutrient transformation. Significant changes in the microbial populations were observed in a dwarf apple tree orchard treated annually with atrazine at 4 kg/ha for fifteen years (44). In particular anaerobic sporeforeming bacteria and cellulolytic microorganisms were permanently reduced. The authors ascribed

these changes to the long-term elimination of direct vegetative cover and concomitant loss of organic matter input to the atrazine treated soil. Also lack of substrates in the 100X plots for the microorganisms could have contributed to some of the observed side effects. If carbon in the form of crop residue from surrounding plots had been addded to that plot, the C:N ratio would likely not have been as low as in the 100X plots. The microbes would have used the carbon for growth and microbial biomass might not have been depressed to that extent.

Any interpretations of antimicrobial herbicide effects on soil fertility is difficult, because herbicide induced stress in the surface soil can cause changes which can be both inhibitory and stimulatory to microbial populations and their activities which although statistically significant, are neither of ecological consequence nor of practical agricultural significance. The microbial community in these Gene transfer plot mnay have been sustainable by adding crop residue to the plot from time to time so that the effects of 2,4-D could be seperated from the effect of carbon loss.

The absence of any significant effect of 2,4-D concentration on nitrogen mineralization suggests that over the many years of 2,4-D application the major groups of organisms within the soil microflora have become adapted to 2,4-D . Similar soil microbial adaptations in response to repeated applications have also been reported from studies of long-term effects of other herbicides (14, 15, 44). The stimulatory effect of 2,4-D on nitrification was unexpected because it had been reported that phenoxy herbicides significantly, albeit temporarily, reduce nitrification, (16, 25, 28, 37). The increase in nitrification I observed was not relevant to the maintenance of soil fertility because the nitrate could easily be leached from the ecosystem and burden the receiving waters. In the current

study, the nitrifier population might have adopted to the 2,4-D application an explanation noted by other authors who saw adaptation of nitrifiers to phenoxy herbicides after repeated application (41). The slightly higher nitrification rate observed might also be due to the effect of higher organic N added through the usage of the dimethylamine form of 2,4-D. This form of 2,4-D is 20 % N by weight and organic N released could stimulate growth of the nitrifier population. The biphasic form of NO_3^- production suggested that nitrifiers initially used soil N and later used the N from the dimethylamine salt of the 2,4-D for growth. The low C:N ratio of the microorganisms might have a negative impact on the ecosystem because the nitrogen is not made available for plant growth but immobilized by microorganisms. The low microbial turnover rate also suggests that it takes a long time for this N to be released to the environment. It is not surprising that after 70 days of incubation that N immobilization was observed especially in the 100X and the 10X soils.

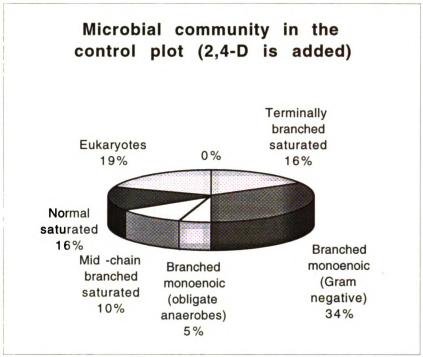
Through C mineralization, energy is released for other ecosystem functions. Hence any decrease in energy released in an ecosystem causes the system to degrade. The reduction in the microbial biomass with increasing 2,4-D concentration could mean that the net primary productivity and soil organic matter levels are affected because of the high correlation of microbial biomass and net primary productivity (30). But, in the present study such a reduction was obvious only in the Gram negative populations possibly because of the differences in cell wall composition. The Gram positive bacteria have a thicker cell wall peptidoglycan with no outer membrane and have techoic acids whilst the Gram negative bacteria have a thin layered peptidoglycan layer. Zelles et al (47)

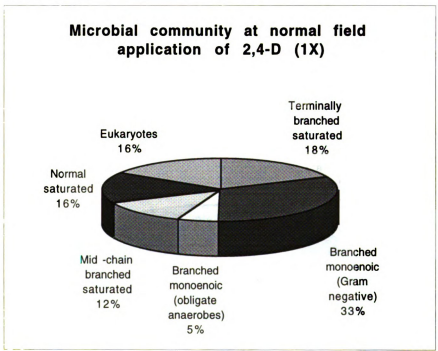
also observed that Gram negative populations were heavily affected during chloroform fumigation whilst the Gram positive only was slightly affected.

Because the effects of a toxic chemical are a function of both dose and time, the possibility of harm to the soil microflora is greater in situations where repeated applications are used over a long period. The use of PLFA in the current studies instead of soil enzymes as a measure of the microbial status is advantageous because it also gives more information on "health" of the microbial community. Domsch (12) stated that the duration and magnitude of the response to herbicide induced stress should be compared to that of the naturally occurring stress situations or "catastrophes" in soil microhabitats such as drought, flooding, freezing etc. From the result of many soil biological studies it was observed that a depression of 50 % or more in biomass or biochemical process that persist for 30 days or longer frequency occurs in surface soils under natural conditions (16). Hence Domsch (12) states that ecologically critical situations occur when the herbicide-induced depressions of soil microbial biomass or function last for more than 60 days and the reduction is > 50 % of the control. Assessment of our data according to this European system for chemical stress evaluation shows that that none of the herbicide-induced depressions of soil microbial biomass or functions met the criteria. Further, these 2,4-D effects were generally confined to the surface 10-15 cm and, as stated by Greaves and Malkomes (16), side- effects that are restricted to the surface layer may be ignored since a large volume of underlying rooted soil will maintain normal biological processes.

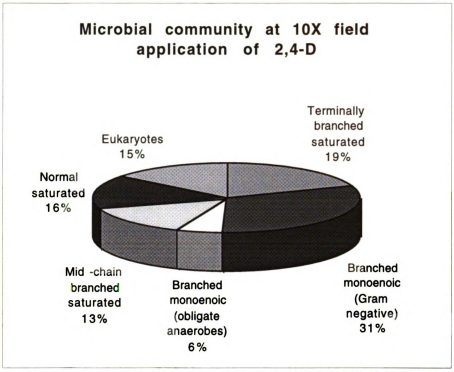
Since 10 years of 2,4-D at four different concentrations viz 0X, 1X, 10X, and 100X, produced temporary and minor soil side effects that did not significantly interfere with the normal cycling of C and N in surface soil under

field conditions, we conclude that there was no agronomically significant effect of long-term 2,4-D applications on soil fertility. However, it is possible that with time the effect of 2,4-D especially on the 100X plot might be of ecological significance since the microbial populations were not "healthy". These findings show that the repeated use of 2,4-D at high concentration (100X) might result in a disturbance of the biological equilibrium in the soil and possibly lead to eventual loss of fertility. While the microbial ecosystem was not seriously perturbed, the plant - soil ecosystem was since primary productivity (plant growth) was seriously reduced. Hence the plant community was much more sensitive to herbicide-induced effects.





Figs. 1a-d. Distribution of microbial community components as determined by PLFA . 2,4-D additions are as follows a=0X, b= 1X, c=10X, d=100X. Data are mean values of 2 replicates.


Microbial community at 10X field application of 2,4-D
Terminally branched saturated 19%
Eukaryotes 15%
Normal saturated 16%
Mid -chain branched saturated 13%
Branched monoenoic (obligate anaerobes) 6%
Branched monoenoic (Gram negative) 31%

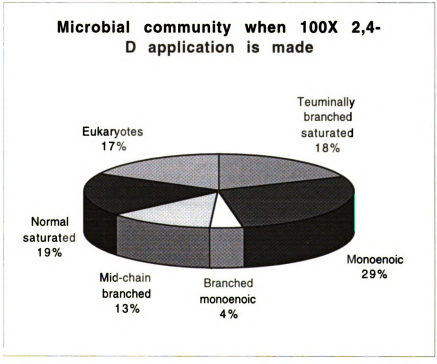

Microbial community when 100X 2,4-D application is made
Teuminally branched saturated 18%
Eukaryotes 17%
Normal saturated 19%
Mid-chain branched 13%
Branched monoenoic 4%
Monoenoic 29%

Fig. 1c and 1d.

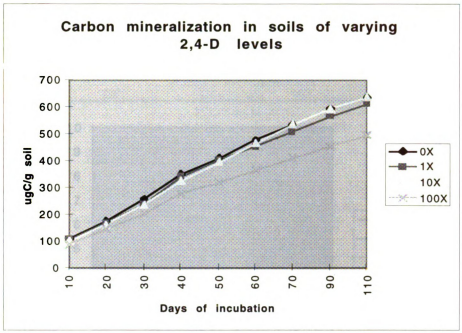


Fig. 2. Cumulative C mineralization with time. The plots were amended with the indicated 2,4 -D concentrations.

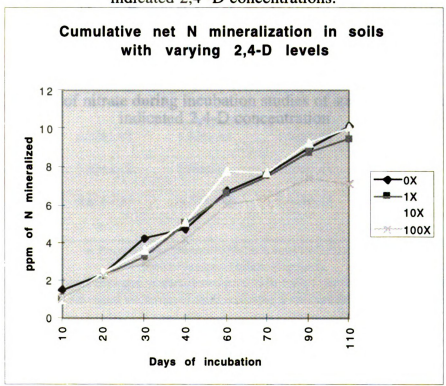


Fig. 3. Cumulative net N mineralization with time amended with different 2,4-D concentrations. The plots were amended with the indicated 2,4 -D concentrations.

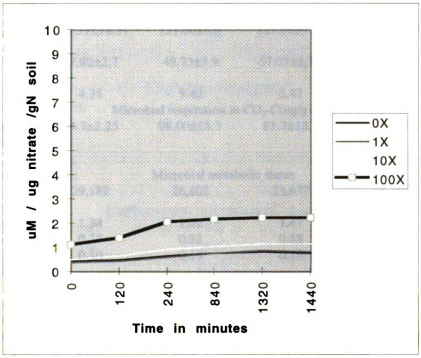


Fig. 3. Release of nitrate during incubation studies of soils amended with the indicated 2,4-D concentration

Table 1. Effect of long-term use of 2,4-D on microbial parameters and ecosystem functioning

Variables measured	2,4-D Treatment levels				Significance of F ratio (probability)
	0X	1X	10X	100X)	
			Microbial biomass		
Carbon ugC/g soil	113.39±18.31	121.00±6.9	121.58±9.94	84.04±2.31	Prob>F (0.00001)
Nitrogen ug/g soil	47.92±2.7	48.73±3.9	57.07±2.76	61.11±1.65	Prob >F (0001)
C/N Ratio	4.38	5.42	5.47	2.49	
			Microbial respiration in CO_2-C(ug/g soil)		
10 day incubation	94.7±2.25	98.00±13.3	85.7±18.7	66.00±15.87	Prob >F (0.1227)
			Microbial metabolic status		
Total PLFAs pm/g soil	29,182	26,802	23,637	18,166	
[a]Growth phase	1.34	1.02	1.47	2.35	
[b]Turn over rate	0.74	0.98	0.68	0.43	
[c]Signs of adaptation	0.10	0.10	0.11	0.17	
			Carbon mineralization ($ugCO_2$/g soil)		
30 day incubation	83.36±2.1	73.01±3.46	72.27±8.19	59.42±10.12	Prob>F (0.0001)
50 day incubation	82.43±21.91	64.16±7.27	75.18±9.91	50.13±13.7	Prob.F (0.1358)
70 day incubation	64.12±7.84	59.03±14.8	71.34±4.195	46.24±4.95	Prob>F (0.3676)
			Nitrogen mineralization (ppm)		
30 day incubation	6.26±1.99	5.56±1.64	4.54±0.05	4.62±0.081	Prob>F (0.3778)
60 day incubation	7.53±0.42	7.49±0.73	8.75±1.44	8.313±1.26	Prob>F (0.427)
70 day incubation	9.81±0.35	10.53±0.89	9.64±0.13	11.29±0.57	Prob>F (0.025)

a. Phase of growth=cy17:/16:1w7c+cy19:0/18:1w7c

b. Turn over rate= 1/phase of growth

c. Stress indicator of microbes=16:1w7t/16:1w7c+18:1w7t/18:1w7

All values are means of three replicates ± standard deviation

Table 2. Effect of increasing 2,4-D on relationships of the microbial parameters

Variable	r (% Correlation coefficient)	Regression equation
	Microbial Respiration	
Gram negative vs microbial respiration	58	y=-80+5.36 % Gram negative in population
	Microbial Nitrogen	
Microbial carbon vs microbial nitrogen	-65	y=79.87-0.24x
Gram negative vs microbial nitrogen	-81.5	y=134.2-2.54x
	Carbon mineralization (C min)	
Gram negative vs C min	98	y=-81.7+4.86x
Gram positive vs C min	-66	y=170.3+5.48x
	n=8	

Table 3. Effect of 2,4-D concentration on nutrient transformation

2,4-D treatments	nitrification rate	N mineralization rate within the first 30 days of incubation in ug/g soil	C mineralization rate within the first 30 days of incubation in ug/g/day
0X	4.46 ±1.5	0.14	7.5
1X	3.27 ±63	0.1	6.8
10X	3.71 ±.68	0.12	6.7
100X	4.75 ±1.3	0.97	6.0

All values are means of three replicates.

$$\text{rate of N min} = \frac{(NH_4^+\text{-N} + NO_3^-\text{-N in ppm})_{t+1} - (NH_4^+\text{- N} + NO_3^-\text{-N})_{to}}{30 \text{ days (incubation period)}}$$

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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;

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

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

fλ 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)
Iλ 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.

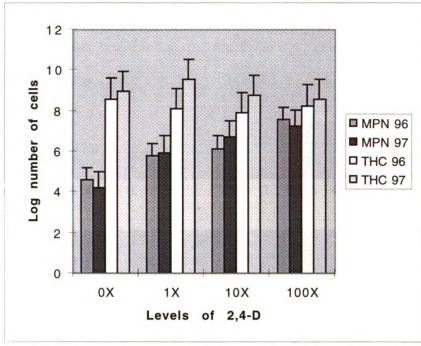
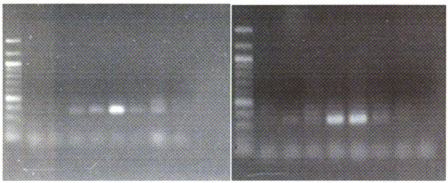


Fig.1. MPN of 2,4-D degraders and total heterotroph count (THC) for field soil samples in 1996 & 1997. Amount of 2,4-D applied shown relative to normal agricultural practice = X. Bars are from 2 MPNs, one of each replicate of the two field plot replicates.



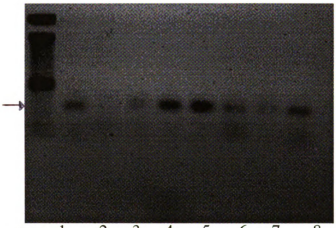


Fig. 2. Soil DNA for 1996 (a), 1997 (b), and 1998 (c) amplified with conserved *tfdA* primers. Size of the *tfdA* fragment is approximately 360 bp. The lanes 1 through 8 are as follows 0X, 1X, 10X 100X, 100X, 10X, 1X, 0X.

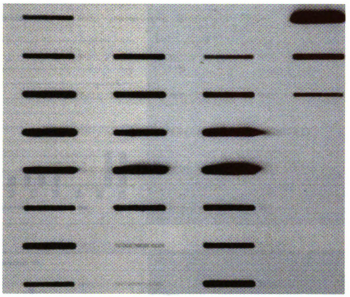


Fig. 3. Community DNA from field subplots (0X, 1X, 10X, and 100X) amplified with conserved *tfdA* primers and probed with *tfdA* of the pJP4 under high stringency. Lanes 1 ,2 ,3 are for 1996, 1997,and 1998. Lane 4 is 100 ng, 10ng, and 1 ng of pJP4 hybridized with tfdA of pJP4 under high stringency. The 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.

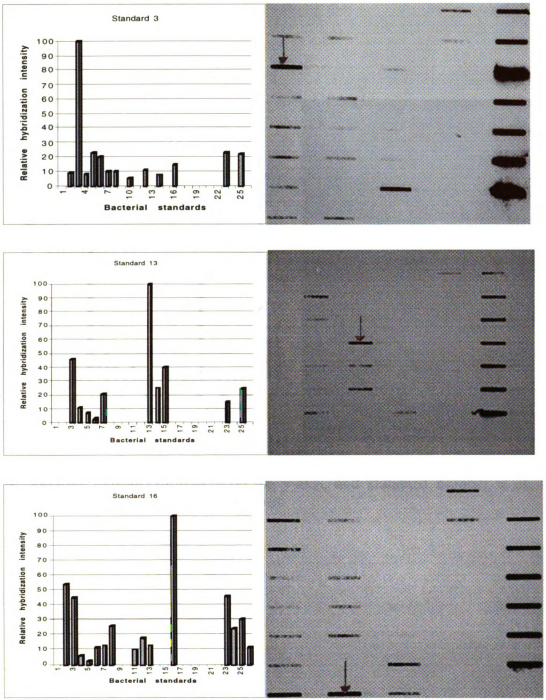


Fig. 4. Examples evaluating cross -hynridization of the 26 2,4-D degrading isolates to each other.The chromosomal DNAs for standards 1 to 26 present on the master filter as indicated in Table 1. The right lane side of the filter are λ DNA markers. A mixture of 20 ng of bacteriophage λ DNA and 100 ng 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.

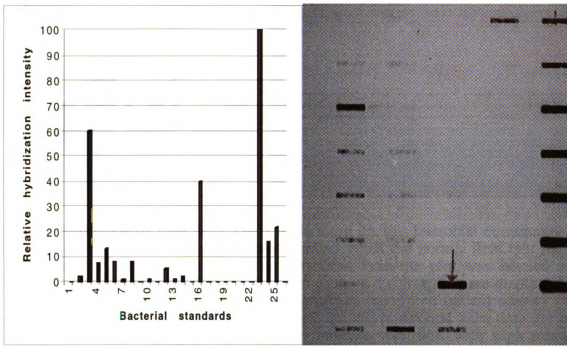


Fig. 5. Hybridization of standard 23 on the master filter. Self hybridization is 100 %. Relative hybridization Ix is on the y-axis, and standard number is on the x-axis. Arrow indicates self hybridization.

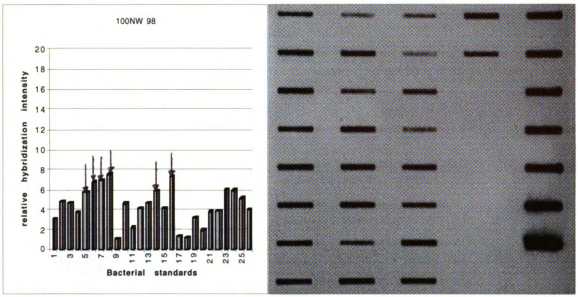
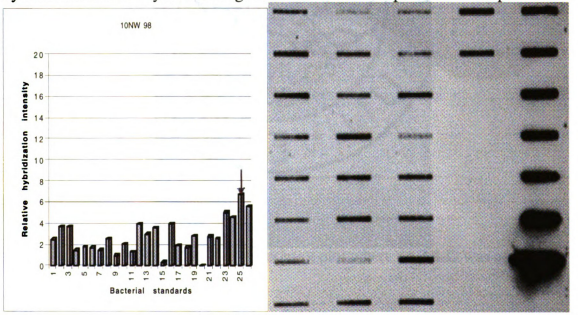


Fig. 6a. Influence of long term 2,4-D application on the microbial community in field plot (100X plots where 2,4-D was applied 100X the normal field rate for 10 yr.) as measured by RSGP. The community DNA from the plot was labeled and hybridized to the master filter. Quantitation of the hybridizations 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.



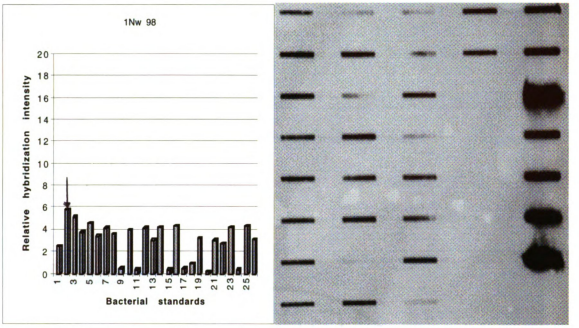


Fig 6c. Long term effect of 2,4-D selection on microbial community in the 1X plots as measured by RSGP. Figure features the same for Fig. 7a.

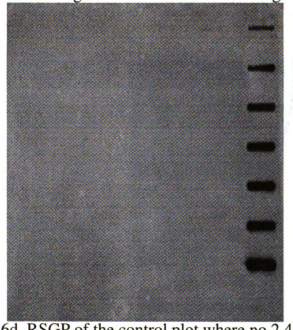


Fig 6d. RSGP of the control plot where no 2,4-D was added.

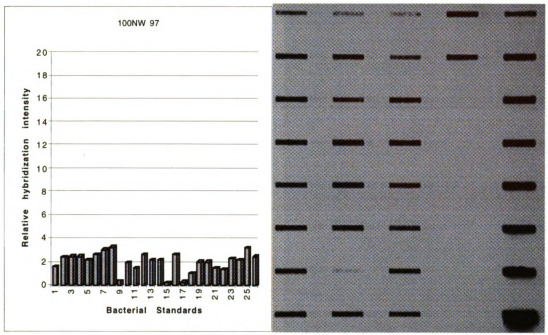


Fig.7a. Effect of long term 2,4-D application on microbial community in soil samples from 100X plots in 1997. See Fig. 7a for details of figure.

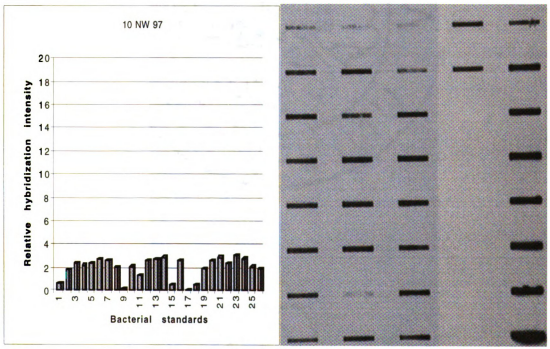


Fig.7b. RSGP showing the 2,4-D effect on microbial community sampled in 1997 from the 10X plots.

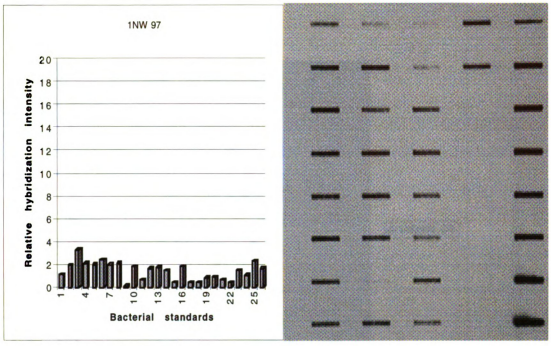


Fig. 7c. RSGP showing 2,4-D effect on microbial community sampled in 1997 from the 1X plots.

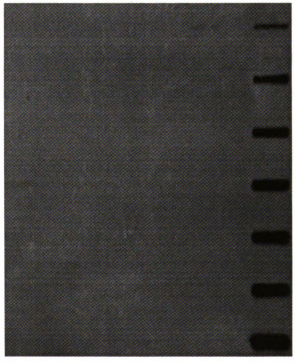


Fig 7d. RSGP showing control plot where no 2,4-D was applied. Little or no hybridization intensity detected on the master filter.

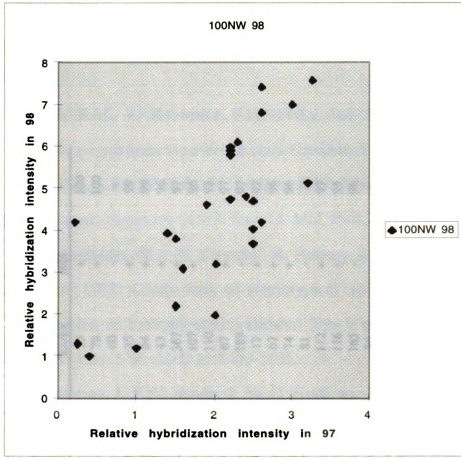


Fig. 8. Relationship between relative hybridization intensity of each strain to DNA from 100X plots in 1998 and 100X plots in 1997. Correlation coefficient was 0.70.

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.

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CHAPTER FIVE

RESILIENCE OF THE 2,4-D DEGRADING BACTERIAL POPULATION IN THE FIELD FOLLOWING TERMINATION OF 2,4-D AMENDMENT

Abstract

The rebound of the soil microbial community was investigated over a three year period following termination of 2,4-D application on field plots that had received 2,4-D at various concentrations for 7 years. Methods used to analyze the microbial community included viable counts by MPN, 2,4-D degrader strain density by reverse sample genome probing (RSGP) and community analysis by PLFA . The number of culturable 2,4-D degraders declined by 1/2 log unit each year in each of the treatment plots. Two years after terminating 2,4-D application to the plots 2,4-D degraders in the 10X and 1X plots were close to the numbers of the control plot. This suggests that the magnitude of perturbation might determine the resilience of that ecosystem. Amplifying the *tfdA* gene in the terminated plots and probing with the *tfdA* of the pJP4 under high stringency showed high hybridization signal especially in the terminated 100X plots suggesting the residual effect of 2,4-D in these plots. Reverse sample genome probing (RSGP) also showed a decline in the hybridization intensity of the bacterial standards on the master filter. No signal was recorded for about 40 % of the standards in all treatments 3 years after terminating 2,4-D application. Microbial populations in a secondary succession seemed to decay at differential rates as the ecosystem reverts back to its normal condition.

Introduction

There are many kinds of perturbations such as the introduction of new substrates into ecosystems, volcanic eruptions, pesticide applications to soil etc. that may overwhelm the ecosystem's homeostatic control and disrupt the existing community. Once the disrupting factor is removed, homeostasis acts to restore the disturbed community through secondary succession (8). Some perturbations may involve changes in species abundance as observed by Peele et al (12) who noted a decline in the abundance of gram negative bacteria while gram positive bacteria increased at a marine site impacted by pharmaceutical dumping. Other perturbations may also involve the removal of some or all of the species requiring recoveries of a much longer duration (13).

The time taken for a perturbation to diminish to a given percentage of its initial value, however may be relatively independent of the size of the perturbation and also the systems resistance (13. Whether the system returns to normal i.e individual species abundance and number of species are restored after perturbation is not clear. Alternatively species composition may remain the same while densities of the species change, or vice versa. A rapid recovery after a stress is a measure of the resilience of the system. One measure of recovery is defined by O'Neill (11) as the square root of the sum of squares of the deviations between the perturbed transient behavior and the equilibrium. The response of soil trophic systems to stress and recovery following stress is highly variable with communities of similar structure.

Application of 2,4-D at higher than the normal field rate to soil can be a form of perturbation since it can be toxic for microbial populations. Lenhard (6) found that 100 to 1000 ppm of 2,4-D decreased dehydrogenase activity as well as

the total microbial population. Rates above 500 ppm caused autolysis of the bacteria and decreased nitrogen fixation by *Azotobacter*. Growing cells of *A. chroococuum* and *A. agile* were more resistant to the harmful effects of 300 ppm of 2,4-D triethanolamine salt than were resting cells (7). In vitro studies showed that the growth of *Aspergillus niger* and several other soil fungi were affected. Shifts in the microbial community from the more diverse to less diverse, primarily 2,4-D- degrading populations, was observed in soil microcosm and in field studies (3). High rates of 2,4-D application tend to disturb the ecosystem as was shown by an increase in the metabolic quotient (qCO_2) when 200 ug/g of 2,4-D was applied to the soil. An increase in qCO_2 indicates a disturbed ecosystem or those in early successional stages (8, 10). In such a disturbed state, a reduction in efficiency of the microbial biomass is observed. A higher percentage of substrate is respired as carbon dioxide than is incorporated into biomass production or maintenance (4).

Many studies focus on describing microbial communities but few study the rate and extent of return of the microbial community to its original state after a perturbation. This study attempts to characterize the recovery of soil microbial community shifted by long term 2,4-D application after that treatment had been terminated.

Materials and methods

Media and reagents The treatment plots were as described in Chapter 4.

In June 1996 the subplots were divided into two, a northern side and a southern side. 2,4-D treatment was continued on the northern side and discontinued on the southern side. Thus for the northern side 2,4-D had been applied for 10 yr as of August 1998 whilst on the southern side, 2,4-D was applied for 7 yr, through

1995. MPN counts, soil DNA extraction, reverse sample genome probing, *tfdA* amplification were as described in chapter 4. PLFA analysis was described in Chapter 3. Sampling of soil from the various plots was usually done in September but in 1997, the sampling was done in early October.

Results.

Enumeration of bacteria. Total viable counts remained constant in 1996 at approximately 5x 10^8 cells / g whilst in 1997 they were slightly higher at 10^9 cells/g irrespective of the level of 2,4-D application (Fig 1). MPN counts of 2,4-D degraders was generally 1/2 log higher in 1996 than in 1997 but they had already dropped 0.4- 0.8 log in 1997 from the counts found in the comparable treated plots (Chapter 4). Two years after terminating 2,4-D application, the number of 2,4-D degraders in all the treatments were higher than that of the control indicating that it takes at least 2 years for the enriched microbial population to die back to its normal carrying capacity.

Reverse sample genome probing. Relative hybridization intensity was generally low, but plots of higher 2,4-D treatments had a slightly higher hybridization signal than plots that had lower 2,4-D treatment (Fig. 2). Hybridization signals for about 40 % of the standard strains were non- detectable over all treatments. The detection limit for the method was determined to be 10 pg of target DNA in a genomic Southern blot (ECL Kit detection). This corresponds to a relative hybridization intensity of approximately 0.1 %. *Sphingomonas* (standard 19) was not detected. Strains which were detectable were mostly *Burkholderia* sp, *Variovorax* sp. and *Alcaligenes* sp and an unidentified standard 23 which maintained a high hybridization intensity in almost all the treatments. Bacterial

standards which were non- detectable were mostly non- *tfdA* containing strains already noted to be rare members of the community (Chapter 4).

PLFA Analysis. The total PLFA in the 100X plots was higher than that in the control plots by 6 % whilst the total PLFA in 1X plot was the highest for the all treatment plots (Table 1). The percent monoenoics and the percent eukaryotes in the 100X plots were slightly higher than that in the control plots. Microorganisms in all treatment plots were in the active phase of growth as indicated by the cy 17:0/16:1w7c +cy19:/18:1w7c which ranged from 1.0 to 1.2 in 100X plots. The health status of the gram negative community as measured by 16:1w7t/16:1w7c + 18;1w7t/18:1w7c, ranged from 0.9 in the control plots to 0.11 in the 100X plots. The slightly higher ratio for the 100X plots indicates that the gram negative community in the 100X plots had not fully recovered from the 2,4-D application.

***tfdA* analysis.** The first year after terminating 2,4-D application (1996), *tfdA* hybridization signal was detected in the 100X plot and one of the control plots. In 1997, hybridization signal was detected in 100X plots and one of the 10X plots (Fig. 3). Increased *tfdA* signal was obtained for 1998 which I suspected was due to runoff from north to south following heavy rainfall. Cross contamination was confirmed by finding *tfdA* amplification from the border areas. Hence 2,4-D and / or 2,4-D degraders on the south plots might have come from the north plots, especially in 1998.

Discussion

The higher total viable counts for the 1997 plot versus 1996 might be due to the differences in the time of sampling. The 1997 sampling was early October versus September for the 1996 sample. In October the maize had been harvested and the stover left on the field likely created a conducive microenvironment for

microorganisms to flourish. MPN counts of 2,4-D degraders declined by 1/2 log /year suggesting that it will take 2 more years for the MPN of 2,4-D degraders in the 100X plots to reach the level of the control plots. Thus to some extent the magnitude of the perturbation determines the resilience of the system. PLFA analysis also confirmed that the microorganisms in the 100X plots needed time to recover. The MPN values for the terminated side of the plots in 1998 could not be included because of the suspected contamination of these subplots from the treated plots.

The presence of the *tfdA* gene was monitored in the plots because it is the only gene in the 2,4-D pathway known to be used exclusively in 2,4-D degradation, unlike *tfdB* and *tfdC*, which have homologs found in other degradative pathways. The *tfdA* gene encodes an alpha-ketoglutarate-dependent 2,4-D dioxygenase (3) and is the first enzyme in the pathway which converts 2,4-D into 2,4-dichlorophenol and glyoxylate. Detecting the presence of the gene in terminated field plots one or two years after 2,4-D application, especially in the 100X plots, suggests either that there might be some residual effect of 2,4-D, or that the 2,4-D populations persistent for several years without substrate.

Many possible reasons contribute to the higher total PLFA observed for the 100X plots than in the control plots. One reason might be the release of nutrients from microorganisms which were killed when high 2,4-D concentrations were applied. The surviving microorganisms make use of the released microbial nitrogen. Van Veen and Paul (17) noted that with a field population of $5x10^8$ cells/g soil, death by lysis releases about 9 ug nitrogen g^{-1} soil. A similar observation was made by Allen-Morley et al., (1) who noted an increase in enumerated bacteria following freezing.

RSGP showed that bacterial populations as represented on the master filter declined at different rates, some at fast rates and some at slow rates. This is because there is less substrate available to maintain their growth after 2,4-D application had been terminated and some of the populations enter into a stable stationary phase whilst others die. Released plasmids to such as those carrying *tfd* genes might be taken up by indigenous strains before they are mineralized. Standard 23 is one of isolate that maintains relatively high population levels in all the treatment plots and its decline rate appears to be slower than that of the other isolates even though it does not contain *tfdA*. The exact reason for the observed differences in decline rates of the microbial populations is not known and would need further investigation.

This study demonstrates that three years after terminating 2,4-D application, microbial populations decay at differential rates and that the magnitude of the perturbation determined the resilience of the ecosystem.

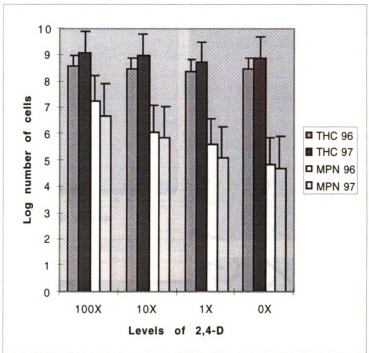


Fig.1. MPN of 2,4-D degraders and total heterotroph count (THC) for field soil samples in 1996 & 1997. Amount of 2,4-D applied shown relative to normal agricultural practice = X. The bars are from 2 MPNs, one of each field plot replicates.

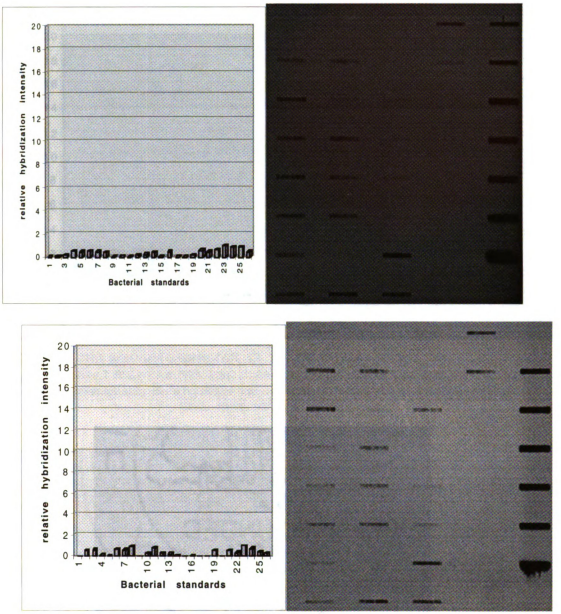
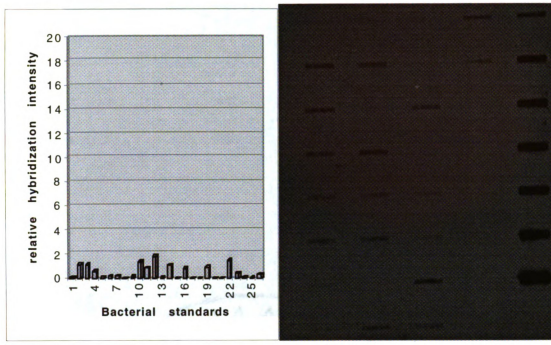


Fig. 2a and 2b. top and bottom. The effect of terminating 2,4-D application on the microbial community in field plots (100X and 10X where 2,4-D application was terminated for 3 years) as measured by RSGP. The community DNA from the plot was labeled and hybridized with master filter. Hybridization intensity in integrated optical density (10^5 on the vertical axis). The standard number on the horizontal axis: data not corrected for cross-hybridization.



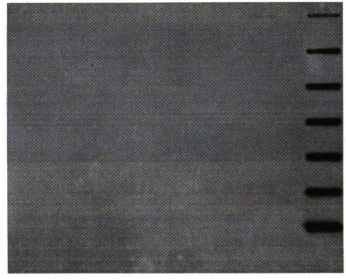


Fig. 2c and 2d. top and bottom. The effect of terminating 2,4-D application on the microbial community in field plots (1X and 0X i.e the control plot where 2,4-D application was terminated for 3 years) as measured by RSGP. The community DNA from the plot was labeled and hybridized with master filter. Hybridization intensity in integrated optical density (10^5 on the vertical axis). The standard number on the horizontal axis: data not corrected for cross-hybridization.

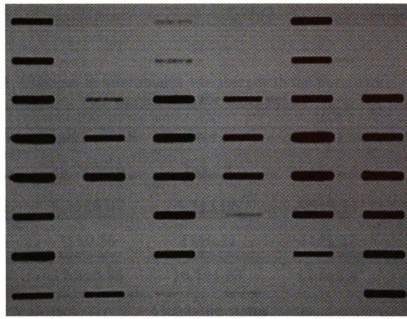
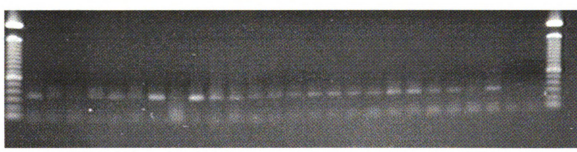


Fig3 Community DNA from field subplots (0X, 1X, 10X and 100X) probed with *tfdA* of the pJP4 under high stringency. Lanes 1 & 2 are for 1996, lanes 3 ,4 are for 1997 and lanes 5 and 6 are for 1998. Lanes 2, 4 and 6 are the terminated side. For a particular lane, eg. Lane 1 from top the treatments are 0X, 1X, 10X, 100X, 100X, 10X, 1X, and 0X.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Fig.4. Soil community DNA from all treatment plots from both the terminated and the unterminated side amplified with the conserved *tfdA* primers (size of fragment is 360 bp) to demonstrate the leaching of 2,4-D to the southern section of terminated plots.Lanes 1 to 15 are Soil DNA from the northern side , 1,3,5,7,9,11,13,and 15 are from 0X, 1X, 10X, 100X, 100X, 10X, 1X, 0X plots, whist 2,4,6,8,10,12,and 14 are the border plots in between the 2,4-D plots, thus lane 2 is a border plot between plot 1 and plot 3.Lanes 16 to 26 are soil DNA from southern side. Lanes 16,17,18,20, 22, 24, 25 and 26 are)X, 1X, 10X, 100X, 100X, 10X, 1X, 0X, whilst 19, 21, 23, are border plots. Marker DNA 100 bp ladder are at the sides of the PCR products.

Table 1. Changes in microbiable variables with the termination of 2,4-D application for three years

	0X	1X	10X	100X
Total PFLA pM/ g dry soil	26,023±2305	29,841±2817	24839±1740	29532±2805
Monoenoics %	33.7±0.49	34±1.2	33.15±0.64	34.25±0.78
Branched Monoenoic %	5.35±0.07	5.3±1.06	4.9±0.14	5.7
MidBrSats %	11±0.56	11±0.21	11±1.27	10.5±0.57
Eukaryotes %	17.8±0.85	16.7±1.33	17.6±1.4	17.9±1.22
		Metabolic status		
cy:0/16:1w7c +cy19:0/18:1 w7c	1.1±0.34	1.2±0.03	1.0±0.02	1.2±0.25
16:1w7t/16:1 w7c + 18:1w7t/18:1 w7c	0.9±0.04	0.11±0.04	0.08	0.11±0.03

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