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Evaluation of a soil-based pesticide wastewater disposal system using a combination of chemical parameters and microbial biomarker techniques

Aaron Dean Peacock

To the Graduate Council:

I am submitting herewith a thesis written by Aaron Dean Peacock entitled "Evaluation of a soil-based pesticide wastewater disposal system using a combination of chemical parameters and microbial biomarker techniques." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biosystems Engineering Technology.

William E. Hart, Major Professor

We have read this thesis and recommend its acceptance:

Thomas L. Mueller, Arnold M. Saxton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Evaluation of a Soil-Based Pesticide Wastewater Disposal System Using a Combination of Chemical Parameters and Microbial Biomarker Techniques

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Aaron Peacock

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For Atina, Emerson, and Aislyn,

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ABSTRACT

Non-point source pollution from agricultural pesticides is a growing problem in surface and groundwater contamination. Even though an operator may follow best management practices during application, there remains the residual pesticide contaminated wastewater (tank rinsates).

Mixed waste streams of commonly used East Tennessee herbicides at various concentrations were tested in soil columns which simulated a Soil Bed Bioreactor, and then repeated with the addition two other insecticides. Pesticide dissipation, soil chemistry, and microbial community response were of prime interest.

Results indicate that pesticide dissipation behavior in the bioreactor is similar to that in the field, and that of the seven pesticides analyzed in this experiment, six significantly dissipated at low and moderate concentrations within 30 days. As pesticide concentration in the bioreactor increased the microbial community structure shifted to one that was resistant, a shift that may ultimately be involved in detoxification.

These data demonstrate and validate the utility of this technology to concentrate and dissipate pesticide rinsates, and show that these methods could become an important tool for farmers and custom applicators.

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Background and Introduction

Background and Justification

The growth of global agriculture is slowing, and at the same time 90 million people are being added to the population each year (Hammond, 1996). The Food and Agriculture Organization of the United Nations estimates that the arable land available worldwide to sustain each person will decline from 0.3 ha in 1981 to 0.13 ha by 2050 (Machi, 1997). This situation is placing pressure on producers of food and fiber to increase yields and preserve gains in productivity, without the luxury of increasing acreage. Most of the increase in productivity over the last 40 years can be attributed to the improvement of cultivars and the increased use of fertilizers and pesticides. Tremendous progress has been achieved in productivity, but this achievement has come with environmental consequences.

One of the potential consequences of modern agriculture is the contamination of surface and groundwater with pesticide residues. The term pesticide includes any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest, or intended as a plant regulator, defoliant, or desiccant (U.S. EPA, 1992). Pesticide residues present in surface or groundwater may enter into the ecosystem as a result of

normal application practices. Additional potential sources of pesticide contamination include spray drift during application, spills, leaks, and discharges associated with mixing, handling, storage, and disposal of empty containers.

Pesticides in aquatic environments have been shown to destroy food sources of higher organisms, resulting in the starvation of certain species. Pesticide residues may also reduce the available vegetation that provides cover for small fish, and contribute to eutrophication of affected waterways (U.S. EPA, 1992). Concurrently, half of the drinking water in the U.S. comes from groundwater sources (Cohen *et al.*, 1984), and pesticide residues in groundwater can lead to restrictions on human and livestock consumption and require expensive measures to remove and reclaim.

Pesticide-contaminated surface and groundwater have caused some public concern and resulted in closer government scrutiny of conventional farming and pesticide handling practices. To address the problem, government agencies and pesticide manufacturers have set strict label guidelines for the handling and application of pesticides. However, even though a producer may follow the label guidelines and employ all the best management practices, the potential of pesticide-contaminated surface and groundwater remains a significant problem in production agriculture or anywhere that pesticide use is common.

Tank Rinsates

Perhaps the most common way unwanted pesticide is generated is via tank rinsates; wastewaters that contain pesticide at low concentrations. Rinsates are generated when cleaning the interior of the supply tank or the exterior of the spray rig prior to loading, when changing from one chemical to another, from spills during the mixing or loading of the pesticide, and by improperly handling concentrate containers (Glover, 1998). Taken on an individual basis, rinsates may not appear to be a serious problem, but they have been identified as a considerable contributor of non-point source pollutants.

The sheer volume of pesticide rinsates produced by commercial aerial applicators illustrates this problem. Approximately half of the commercial application of pesticides each year is by aircraft. Each day one spray-plane can generate 30 gallons of aqueous rinsate, principally from tank washing yielding residual materials that contain approximately 500 ppm of pesticide residue. With 6000 aircraft applying pesticides each day, it has been estimated that a volume of 60 million gallons containing 12,000 kg of pesticide residue are being produced by this single source each year. (Whittaker *et al.*, 1979).

To date there has been little guidance for the producer or commercial applicator in regards to the treatment or disposal of pesticide contaminated wastewater. In contrast, the EPA has set strict guidelines on the pesticide

manufacturing and repackaging industry in reference to their waste streams. In recent years the EPA has produced one technical development document "Technical Development Document For The Pesticide Formulating, Packaging, And Repackaging Effluent Guidelines, Pretreatment Standards, And New Source Performance Standards" (Zuskin *et al.*, 1996) and one final report, "Pollution Prevention (P2) Guidance Manual for the Pesticide Formulating, Packaging and Repackaging Industry: Implementing the P2 Alternative" (Zuskin *et al.*, 1998). In these two documents the EPA lists six acceptable technologies for the pretreatment and treatment of pesticide contaminated wastewater. These technologies are, emulsion breaking, chemical oxidation, membrane filtration, activated carbon adsorption, hydrolysis, and chemical precipitation/separation. We will examine each of these in turn.

Emulsion Breaking

The majority of pesticide products are a mixture of active ingredients, surfactants, oils, and emulsifiers designed to make the "formulated product" as easy to apply as possible. Emulsions result when these inert substances come in contact with water. Emulsions reduce the effectiveness of many treatment technologies. To ameliorate this problem producers need to employ methods such as temperature control, acid addition, or chemically assisted clarification before the use of other final treatment technologies.

Such systems are not suitable or recommended for use by the average producer or commercial applicator. In some instances they require sophisticated equipment and knowledge of general chemistry. Moreover, while they work well in industry, it would be unrealistic to think they could be employed economically on a small scale.

Chemical Oxidation

The addition of an oxidizing agent to pesticide wastewater brings about chemical oxidation. The oxidizing agent transfers one or more electrons to the target pesticide, causing its destruction. Oxidizing agents used in the industry include chlorine, ozone, and hydrogen peroxide. Chemical oxidation, however, has the potential to produce chlorinated organic compounds when treating some types of pesticide wastes, e.g., dithiocarbamates. This would in effect produce compounds more toxic than the pesticide itself. Another drawback to chemical oxidation is that other finishing technologies may be required to complete the treatment process. As sophisticated monitoring and process operation would need to be implemented, such technologies would not be recommended for producers or commercial applicators.

Membrane Filtration

In theory, membrane filtration is the simplest treatment method for the removal of pesticide in contaminated wastewater. Membrane filtration

techniques use a pressure-driven semi-permeable membrane to separate and suspend pesticide from contaminated wastewater. The size of the membrane filter determines the type and amount of contaminant that may be removed from the system. Membrane filtration systems include microfiltration, reverse osmosis, and ultrafiltration. However, high-pressure pumps can result in leaks and associated containment problems if not properly maintained. Moreover, these systems work best for a single contaminant and must be optimized for such use. A mixed waste stream would require several filtration steps and would entail complicated management issues.

Activated Carbon Adsorption

Carbon materials such as wood or coal that are processed through dehydration, carbonization, and oxidation produce a filter that is highly adsorbent. These filters have a large surface area per unit mass and a proportional number of internal pores. Organic contaminants with certain chemical properties are filtered from pesticide contaminated wastewater by physical and chemical forces. Activated carbon filters are limited, in that they are appropriate only for certain pesticides. Moreover, they may be adversely affected by other organics in the waste stream that are competitors for the filters' active sites. These systems also require the use of extensive calibration to discover the breakthrough or saturation point of the filter, which

must be calculated for each pesticide used. Once a carbon filter is spent, it requires disposal as toxic waste, which may be expensive and time consuming.

Hydrolysis

By reacting with a base or water, residual pesticide is broken down into smaller less toxic compounds. In most cases a hydroxyl group (OH) is introduced into the reactant, displacing another group. Hydrolysis is straightforward, but is appropriate for only a limited number of pesticides. Again, extensive testing is required to ensure that the pesticide is being destroyed.

Chemical Precipitation/Separation

Chemical precipitation/separation utilizes techniques by which insoluble precipitates are formed from the organic or inorganic compounds in the wastewater. This is accomplished by the addition of chemicals during treatment, with filtration used to remove the products of the reaction. This technology also requires the use of expensive machinery and intensive process management that is difficult for a producer to employ.

Each one of these six systems has been shown to be effective in the removal of pesticide from wastewater streams. However, these technologies

have certain characteristics that preclude their use by producers or custom applicators on a small scale. First, the technologies presented are not zero discharge systems, but return the remediated water back into the environment from which it came. Secondly, they require extensive calibration and continued testing to ensure proper performance, and in addition to a thorough understanding of the chemistry involved, some of the technologies require sophisticated and expensive equipment. Finally, these systems were designed with pesticide manufacturers in mind, and as a result are not easily applicable to mixed waste streams, such as those produced on farms or by commercial applicators. It is apparent that a new paradigm for rinsate disposal is necessary if we are to seriously consider onsite treatment .

The technologies and problems described above bring up several issues that need to be addressed when considering onsite treatment methods of pesticide-contaminated wastewater for the commercial applicator or small scale producer.

- An end producer will be using many different pesticides throughout the season based on the crops grown. The technology must be able to handle multiple waste streams.
- The system must be easy to use, and calibration and testing of the system must be kept at a minimum.
- Given that onsite users will generally not be chemists, the system should not require an extensive knowledge of pesticide chemistry.

- It should be zero discharge, with none of the pesticde wastewater returned to the environment in a potentially damaging form.
- The system should not only remove (filter) the pesticide from the wastewater, but should also degrade or destroy the contaminants so as to minimize the need for disposal of contaminated containers or substances.
- It must be inexpensive.

Onsite Pesticide Removal Systems

The first component of comprehensive onsite pesticide management is the proper storage and disposal of pesticide concentrate containers. The EPA and the pesticide manufacturing industry have established guidelines (labels) on the proper storage and disposal of pesticide concentrate containers. This includes storage options that are contained and meet certain requirements for temperature and ventilation. In addition, when pesticide concentrate containers are empty they must be triple rinsed and disposed of by burning or by disposal in the proper facility.

The second component of comprehensive onsite pesticide management is frequent calibration of the spray apparatus. This ensures that label laws are being complied with and that enough active ingredient is being applied to the target without the risk of over-application and the associated environmental consequences. Third, is the use of a properly designed

rinsepad. Use of such a rinsepad can eliminate or reduce the amount of wastewater that is released into the environment.

In the early 1990's managers from The University of Tennessee Agricultural Experiment Stations were using all the aforementioned best management practices that pertain to the use of pesticides. They felt, however, that there was still a problem with rinsates, and subsequently asked The University of Tennessee Agricultural and Biosystems Engineering Department if a system could be developed to handle the 1000 to 1500 gallons of pesticide rinsates generated each year at the Knoxville and Highland Rim sites (Corwin, 1996) (Yoder Pers. Comm.,1998). In response to the inquiry a broad research approach was developed by investigators at The University of Tennessee Agricultural and Biosystems Engineering Department. Six steps were outlined (Yoder Pers. Comm., 1998):

1. "To address the rinsate disposal problem through a thorough understanding of not only the waste itself, but also of the currently available disposal options/methods both in and out of the field.
2. To choose the available rinsate disposal technology deemed best.
3. To investigate ways of optimizing the chosen rinsate disposal option.
4. To develop an economical mixing/loading facility design that would effectively collect the rinsate from rinse operations and deliver it to the rinsate disposal system.

5. Ensure compliance with all pertinent federal and state regulations and important safety practices.
6. To install pilot- and full-scale units for use on the experiment stations while making the facility as practical and inexpensive as possible, without compromising safety or disposal effectiveness."

To implement this plan three tracks of research were initiated. The first was to attack the problem of reducing rinsate waste in the field, the second was to optimize and resolve the chosen rinsate disposal option, and the third was to design a complete rinsate facility that would house the chosen disposal technology. This approach was intended to address every aspect of rinsate management, and to provide sound solutions for the experiment stations and eventually for private farmers and custom applicators.

A product of the first research track was the Tennessee In-Field Sprayer Mounted Rinse System (TISMRS) (Hart, 1991; Wills and Burcham, 1992). This system allowed producers to modify existing spray rigs with an additional wash tank and associated plumbing and a cleaning nozzle, effectively reducing the amount and concentration of rinsate requiring disposal. TISMRS was a significant advance in rinsate management and has been thoroughly integrated into the rinsate management scheme of the experiment stations in Tennessee. This system alone can decrease rinsate waste requiring final disposal by 90% (Ellis, personal comm.).

The second research track has also produced significant results due to the efforts of several graduate students and their advisors. Corwin (1996), conducted a thorough survey of all the technologies in use at that time to handle rinsate wastes. Corwin's research concluded that a soil-based system could be used to both concentrate and degrade pesticide-contaminated rinsates. This system employed a soil bed or lined pit in which pesticide contaminated rinsate was placed. The water evaporated and the residual pesticide was sorbed to the soil and then degraded by soil chemical reactions or by soil microorganisms. Up until that time no researchers had attempted to optimize such a system. Corwin designed a soil column experiment that simulated a soil bed and tested the effect of the soil temperature, soil texture, mode of application, and airflow on the evaporation/degradation rates of two selected pesticides. A "substantial volume reduction (water evaporation) and decontamination (herbicide dissipation)" of the pesticide-contaminated rinsate was achieved in the soil columns. This led to a scale-up of the procedure in the next study.

Glover (1998) began research on the design of a full-scale soil-bed bioreactor to determine the relationship between the lab-scale soil columns and full-scale soil beds. Once the soil bed bioreactor was designed and built, Glover used Corwin's management plan to operate and assess the function of the soil bed bioreactor, showing that the lab-scale results were applicable to

the full-scale soil bed bioreactor (SBBR) in both evaporation and degradation rates.

The most recent research at The University of Tennessee in the area of rinsate disposal was completed by King (1999). King took the SBBR design of Glover and incorporated it into a full-scale rinsate disposal facility design, which was developed specifically for the Plant and Soil Science unit of The University of Tennessee Agricultural Experiment Station in Knoxville. A rinsepad structure was also designed for use during loading and rinsing of spray equipment. A full-scale SBBR system that was capable of containing all the rinsate generated at the site was also laid out. Additionally, the facility has been designed to comply with all pertinent federal and state regulations.

Introduction

Why use soil as the matrix?

Soil bioreactors have been used to treat organic pollutants (Karamanev et al., 1998), and soil is the logical matrix for the filtration and dissipation of pesticide. Among the reasons for this assessment are that the soil contains different types of clay and organic matter to which pesticide will sorb, that soil contains an almost infinite amount of genetic and phenotypic diversity within its microbial population capable of the transformation of

pesticide (Torsvick et al., 1990), and that many pesticides have been specifically designed to break down in soil.

The soil has three basic functions in the SBBR directly related to performance for evaporation and pesticide dissipation. Figure 1-1 illustrates an overview of these functions. The first function of the soil is as a biodiversity matrix. This may be the most useful benefit the soil provides, that is, an environment for the microbial population. It is this microbial population that will determine the ultimate fate of the pesticide in the SBBR. According to Bollag and Liu (1990), soil microbes use five processes in the transformation of pesticides. First is biodegradation, in which the pesticide serves a substrate for growth. Second is cometabolism, in which a pesticide is transformed by a metabolic reactions but is not used by the microbes as an energy source. Third is polymerization or conjugation, in which case pesticide molecules are linked together with other pesticides or with naturally-occurring compounds such as organic matter. Fourth is accumulation, in which the pesticide is incorporated into the microbe, and fifth is the secondary effects of microbial activity, which may include changes in the pesticide because of changes in pH, redox conditions, reactive compounds etc., that are facilitated by microorganisms (Bollag and Liu, 1990).

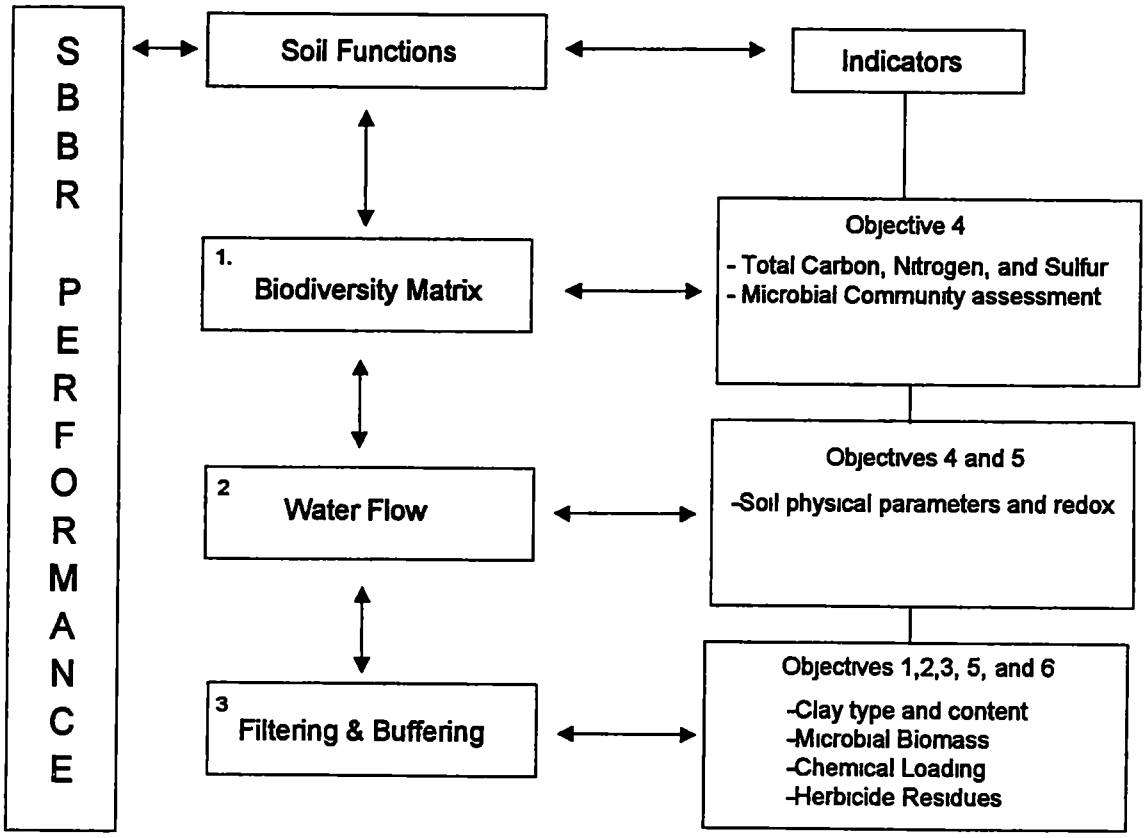

Figure 1-1 Soil functions and indicators in the SBBR.

The second function of the soil is to allow water to flow through and evaporate from the surface while adsorbing the pesticide. The research for optimizing this function was accomplished by Corwin in the first phase of the study (Corwin, 1996), and this is indeed the most pertinent function of the SBBR. The evaporation of the water precludes its storage, thus eliminating the major problem of rinsate management. The third function of the soil involves filtering and buffering. In this capacity the soil serves to sorb pesticide from the soil water and also provide a buffering capacity that may inhibit a lethal dose of pesticide from destroying the soil microbial community (Welp and Brummer, 1999). For each of these functions there are several parameters that can be measured, and by assessing these in a prudent experimental design insight can be gained into the operational effectiveness and versatility of the SBBR. These parameters are broken into three categories; physical, chemical, and biological.

Research Objectives

Corwin's research was responsible for the optimization of soil type and water application scheme for the SBBR (Corwin, 1996). Now that these parameters have been established a more detailed investigation into the suitability and function of the SBBR must take place. It has been noted that a

producer will use more than a single pesticide throughout the growing season, and that any system such as the SBBR must be resilient enough to handle multiple waste streams. In addition, the pesticides added will not be pure active ingredients, but formulated products that may complicate the dissipation of pesticide. Moreover, some producers will require the SBBR to handle higher concentrations of pesticides than others. The foregoing statements produced several questions that have served as the basis for this next phase of SBBR research and led to the following study objectives:

1. The evaluation of the dissipation of a combination of commonly used East Tennessee herbicides as formulated products in a batch waste-stream.
2. Assessment of the impact of insecticide formulated product addition to the batch waste-stream.
3. Exploring the effects of high contaminant levels on the SBBR.
4. Defining and correlating the microbial community biomass, structure and metabolic status with contaminant level and dissipation.
5. Exploring the general soil chemistry of the system at the established parameters.
6. The comparison of results from this study with previous research by Corwin and Glover.

In order to accomplish these objectives a three-tiered approach consisting of physical, chemical, and biological parameters were used to assess the impact of pesticide addition and water management on the soil columns

(simulated SBBR) exposed to different levels of pesticide concentrations. In the first experiment a mixture of five herbicides (atrazine, dicamba, fluometuron, metolachlor, and sulfentrazone) as formulated products were added in four concentrations each; 0-control, 10-ppm, 100-ppm, and 1000-ppm for a total concentration of 0, 50, 500, and 5000-ppm in each of the respective treatments. The water management program consisted of a five-day flood-dry cycle, with five days at saturated conditions followed by five days of drying. Replicated columns were destructively sampled at days 0, 30, 60, and 90. A second experiment repeated the first but omitted the control and 1000-ppm concentration and included two insecticide (chlopyrifus and λ-cyhalothrin) formulated products.

Pesticide residue assay

Perhaps the most obvious question addressed by this study was to what degree residual pesticide dissipated within the soil columns. This is the ultimate test of the SBBR at this level of the study. In order to accomplish objectives one, two, and three, each pesticide was extracted from the soil and analyzed by LC, GC, or GC-MS, and the rates of dissipation were calculated for each time point.

Phenotypic assay

The ultimate fate of pesticide in the soil bed bioreactor is in large part dependent upon the reactor's soil microbial community. Understanding the impact of pesticide addition and column management on this community is ultimately vital to the operational efficiency and sustainability of the system. Phospholipid fatty acid (PLFA) analysis was used to provide information about the microbial community biomass, metabolic status and community structure (White *et al.*, 1997; Stephen *et al.*, 1999; Frostegard *et al.*, 1996). This assay was used to partially accomplish the fourth objective.

Genotypic assay

To further explore the fourth objective a nucleic-acid-based assay was also used. Nucleic-acid-based molecular biological methods have risen in popularity among scientists interested in assessing the diversity of microbial communities and their responses to environmental change. This assay provided information at the family level of classification and enabled the elucidation of function in the system.

Chemical analysis

In addition to the previous assays, a suite of chemical analyses was performed. This addressed objective five by providing information regarding

the impact of pesticide addition on the soil chemistry, and was used to correlate the resultant pesticide dissipation and microbial community response.

Comparison of results

One important question that developed was how the system would respond to the addition of the pesticides as formulated products rather than technical grade active ingredients. This was addressed by comparing the results of this study with the results of previous research by Corwin and Glover, who used only active ingredient in there respective experiments.

Chapter organization

The objectives of this study are fairly diverse and required a broad approach; therefore each chapter deals with particular objectives separately. In the second chapter soil chemistry and pesticide dissipation data are presented. Chapters three and four deal with the PLFA and DNA assays for the assessment of the SBBR microbial community. Chapter five uses a new data analysis tool (Neural Networks) to further analyze the microbial response to water management and pesticide addition. Chapter six is a synopsis of all of the data and attempts to bring together in a brief and concise fashion the most important findings and conclusions.

Chapter 2

Chemical Data

Introduction

During this study soil columns were used to simulate a soil-bed bioreactor, a system designed for the concentration and dissipation of pesticide-contaminated rinsates. In order to address objectives one, two, three, five, and six, we compared the affect of contamination level and type of pesticide on the ability of the system to dissipate the chemicals in addition to the affect of pesticides on column soil chemistry. As stated earlier, the study was divided into two experiments. In the first, a mixture of five herbicides from different chemical families was applied to the columns at various concentrations (Table 2-1). In the second experiment the same five herbicides were used, along with two insecticides from different chemical families.

There are many factors involved in determining the recovery of pesticides in soils. Essington et al. (1995) showed that recovery of fluometuron and norflurazon from fortified soils were significantly influenced by equilibration time, loading rates, and soil type. Most significant was that as the herbicide contact time with the soil increased, recovery decreased, reflecting an equilibrium state

Table 2-1. Pesticides used in the soil column experiments

Common name	Trade Name	Used In Exp	Formulation	Chemical Family
Atrazine	Atrazine	1,2	4L	Triazine
Clhorpyrifus	Lorsban	2	4EC	organophsphate
Dicamba Salt	Banvel	1,2	4L	Benzoic acid
Fluometuron	Cotoran	1,2	4L	Urea
Lambda-Cyhalothrin	Karate	2	1 EC	pyrethroid
Metolachlor	Dual	1,2	7 8EC	Chloroacetamide
Sulfentrazone	Spartan	1,2	75DF	Aryl Triazolinone

of the pesticide with the soil. Essington et al. concluded that pesticide recoveries in the laboratory may not provide a true measure of pesticide in the soil.

Equally important is the question of pesticide volatilization. These soil columns have been designed to minimize the amount of volatilization by application of the water through the bottom of the column. The pesticides must pass through the entire length of the column before volatilization can take place. Junk and Richard (1984) state that volatilization of pesticides from a soil pit containment system appeared to be minimal, and Winterlin et al. (1989) had similar findings with their system. Theoretical work by Baker and Johnson (1984) appears to confirm the practical experiments of the previous researchers indicating there is little danger of large amounts of pesticides moving into the atmosphere with evaporating water in a soil system.

For purposes of this study, dissipation is defined as the disappearance of parent compound or pesticide, regardless of fate. Daughter or degradation products from atrazine, dicamba, fluometuron, and metolachlor were observed and identified by GC-MS (data not shown), but there was no attempt to quantify these daughter products.

Materials and Methods

Soil

A Whitwell loam soil containing 41% sand, 39% silt, and 20% clay was collected from The University of Tennessee Agricultural experiment station Plant and Soil Science Unit, and air dried. The soil was sieved through a 1/4" screen (6.35-mm openings), then through a 1/8" screen (3.2-mm openings).

The soil was mixed with the formulated product of 5 herbicides; (atrazine 4L, dicamba 4EC, fluometuron 4L, metolachlor 7.8EC, and sulfentrazone 75DF) to achieve final concentrations of active ingredient of 0-control, 10, 100, and 1000 ppm for each herbicide. The amount of active ingredient in each formulated product was calculated, and that number used as a divisor to discover the amount of formulated product to add. For the 4L formulations 40g active ingredient * 1ml FP/0.4798g active ingredient = 83.36 ml, so 86.36ml of 4L formulated product was applied to 40kg of soil in order to achieve a concentration of 1000 ppm. This approach was repeated for each concentration and each formulation. The pesticide formulated products were placed in 250-ml amber jars, and enough water was added to bring the total volume to 200 mls. The soil was then weighed into 8 pans of 5 kg each. Each of the bottles of that selected pesticide concentration was added to each pan (*i.e.,* 25 ml of each pesticide of one concentration were added to each of the soils in the pans that were to receive that treatment). This was repeated for all treatments. The soil pans were

allowed to dry for three days and then all pans of a given concentration were combined in a mixing drum and homogenized for approximately 1 hour prior to column loading. Just prior to loading the columns with soil, quadruplicate samples of each treatment were collected from the mixing drum and stored for future analysis.

The second experiment was a repeat of the first but without the 0-control and 1000-ppm concentrations, and with the addition of the two insecticide formulated products chlorpyrifus 4EC and λ-cyhalothrin 1EC.

Column Design and Packing

Soil columns designed and built by Corwin were used (Corwin, 1996). Briefly, a 10-cm diameter by 25-cm tall thin-walled PVC pipe fitted with two polypropylene barbs was used as the column housing. The hose barbs were placed at the bottom and top of the column, with hoses attached to the barbs and a water supply bottle. When active, the water was fed through the siphon tube to the bottom fitting. The water was gravity, and once the water level reached the top fitting it entered the vacuum tube which prevented any more water from entering the column (Figure 2-1). Corwin's Standard operating procedure #BKC-1 (Corwin, 1996) was used to pack the columns with from 2.77-2.88 kg of soil to yield a uniform bulk density in the range of 1.34-1.4 g/cm^3.

So as not to bias evaporation and subsequent dissipation results, soil columns were placed randomly in dark environmental chambers with a continual

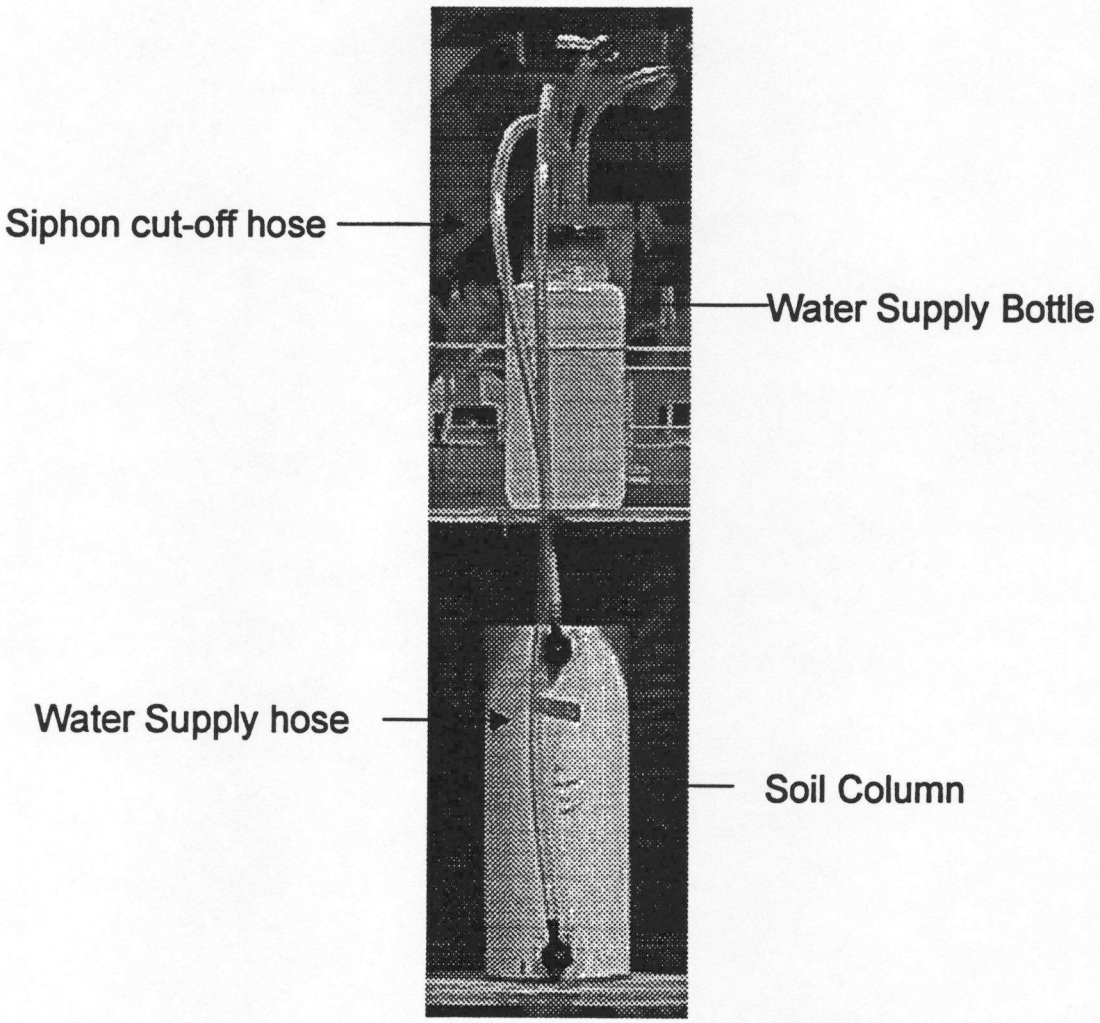

Figure 2-1. Soil Column apparatus

airflow of ≈ 3.8 meters/second and ambient temperature. Columns were alternated in a 5-day flood-dry cycle to simulate soil bed bioreactor (SBBR) conditions (Corwin, 1996; Glover, 1998). At 30, 60, and 90 days, columns of each concentration were sacrificed and the contents transferred to drying pans. The soil was air-dried, homogenized, and transferred to sample bags for future analysis.

Pesticide analysis

Pesticide extraction from the soil was accomplished according to procedures outlined by Gallaher and Mueller (1996). Soil (40g) and methanol (80 ml) were added to 250 ml low density polyethelene bottles. The bottles were placed on a gyrotary shaker for 18 hrs at 120 cycles/min. Once removed from the shaker, the bottles were centrifuged at 2000 rpm for 10 minutes. The supernatant was filtered through qualitative paper and placed into amber autosample vials for analysis. Samples from the 1000-ppm concentration were diluted with the appropriate amount of Methanol to achieve a concentration within the dynamic linear range of our instrument.

Atrazine, chlorpyrifus, λ-cyhalothrin, and metolachlor were quantified using a Hewlett Packard 5890 series 2 gas chromatograph interfaced with a Hewlett Packard 5971 mass selective detector with electron impact at 70 eV, and using a Zebron ZB-1 100% polydimethylsiloxane 60-m (0.25 mm ID X 0.25 µm film thickness) column (Phenomenex INC. Torrance, CA). The injector and

detector temperatures were 280°C and 300°C, respectively. The initial chromatographic conditions consisted of a temperature of 100°C (held for 2 minutes), then ramped at 5°C/minute to 220°C, then ramped to 280°C at the same rate and held for 5 minutes. The ions (m/z) monitored for atrazine were 58, 200, and 215; for chlorpyrifus 97, 199, and 314; for λ-cyhalothrin 181,197, and 208; and for metolachlor 152, 238, and 240. Analytes were quantified by multi-point external calibration curves made from pure standards with a range of 0.1 to 250 ppm.

Dicamba, fluometuron, and sulfentrazone were separated using a Phenomenex C18 (250 mm X 3.2 mm, 100 Å and 5 µm) column, and were quantified using a Hewlett Packard Series 1100 HPLC interfaced with a L1315A diode array detector operated at 254 nm with a bandwidth = 16, reference 360 = nm and bandwidth = 100. The slit width was 4 nm and peak response time was greater than 0.4 minutes. Isochratic runs were 30 minutes each at a flow rate of 0.5 ml/minute, and the mobile phase was 35% acetonitrile, 65% 0.01% H_3PO_4 in water. A 10 µl injection was used. Peaks were identified by elution times by comparison to known standards and were quantified by either six- or eight-point external calibration curves. The ranges of the calibration curves were from 0.1 to 170 ppm and were derived from pure standards.

Soil Analytical Methods

All chemical analyses were conducted using dry soil. The soil was crushed and homogenized with a mortar and pestle prior to analysis. Total soil C, S, and N were measured by dry combustion using a LECO CNS2000 analyzer (LECO, St. Joseph, MI) as described by Matejovic (1997). Soil pH was measured with a Corning pH meter 440 (Corning, NY) on a 1:2 soil-water paste. Soil was extracted with 1M KCl and the extracts were assayed for ammonium and nitrate by the indophenol blue method adapted for microtiter plates by Sims et al. (1995). Color intensity was measured using a 7250 microplate reader (Cambridge Technology Inc., Watertown, MA) with a 650 nm filter. Soils were also extracted with Mehlich I extractant (Mehlich, 1953), and the extracts were analyzed for exchangeable Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn using an inductively-coupled argon plasma-optical emission spectrometer (ICAP61, Thermo Jarrell Ash Corp., Franklin MA).

Statistical Analysis

A completely randomized design (CRD) with factorial treatments and replication was used. For the first experiment there were 4 treatments (0-control, 10 ppm, 100 ppm, and 1000 ppm herbicides), 4 timepoints (0, 30, 60, and 90 days) and 4 replicates, for a total of n=64. The model was:

$$y = \mu + \text{Treatment} + \text{Time} + \text{Treatment*Time} + \text{error},$$

where y = the observed effect and μ is the estimate of the overall mean. The second experiment repeated the first, except that two insecticides were added to the soil and the 0-control and 1000 ppm concentrations were omitted, resulting in 32 soil columns for analysis. Analysis of variance was conducted using the General linear model Statistica procedure (Statistica 1998, Tulsa, OK). Effects were considered significant at a $P \leq 0.05$. Fisher's Least Significant Difference was used for mean separation. Factor analysis was conducted on the soil chemical data using MINRES as the extraction procedure and Varimax as the rotation. Pesticide half-life estimation was performed using the SAS (SAS Institute Cary, NC.) NONLIN procedure as applied by Stiles (1999).

Results

Dissipation of pesticides: Experiment 1

Pesticide dissipation in the soil columns varied with concentration. Figures 2-2, 2-3 and table 2-2 summarize these findings. At the 10 ppm concentration (50 ppm total pesticide load), over the 90-day study period there was a reduction in all pesticide levels except sulfentrazone. The level of atrazine decreased 84%, with an estimated half-life of 18 days. The dicamba level was reduced by 100%, and fluometuron by 51% with an estimated 83 day half-life. Metolachlor levels declined by 82% with an estimated half-life of 35 days.

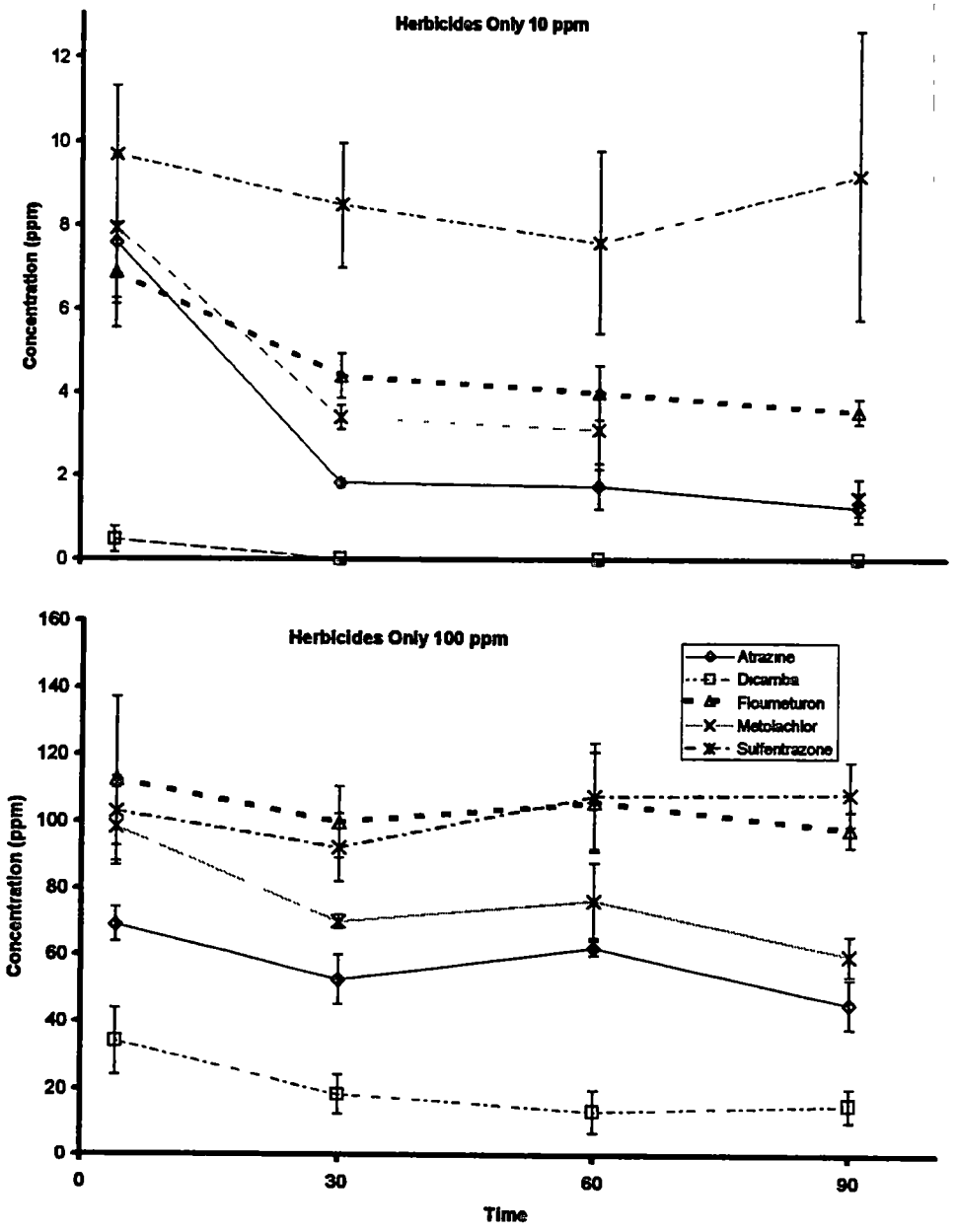

Figure 2-2. Concentration over time for herbicide dissipation in columns from experiment 1 10 and 100 ppm. N=4 and error bars represent 1 standard deviation about the mean

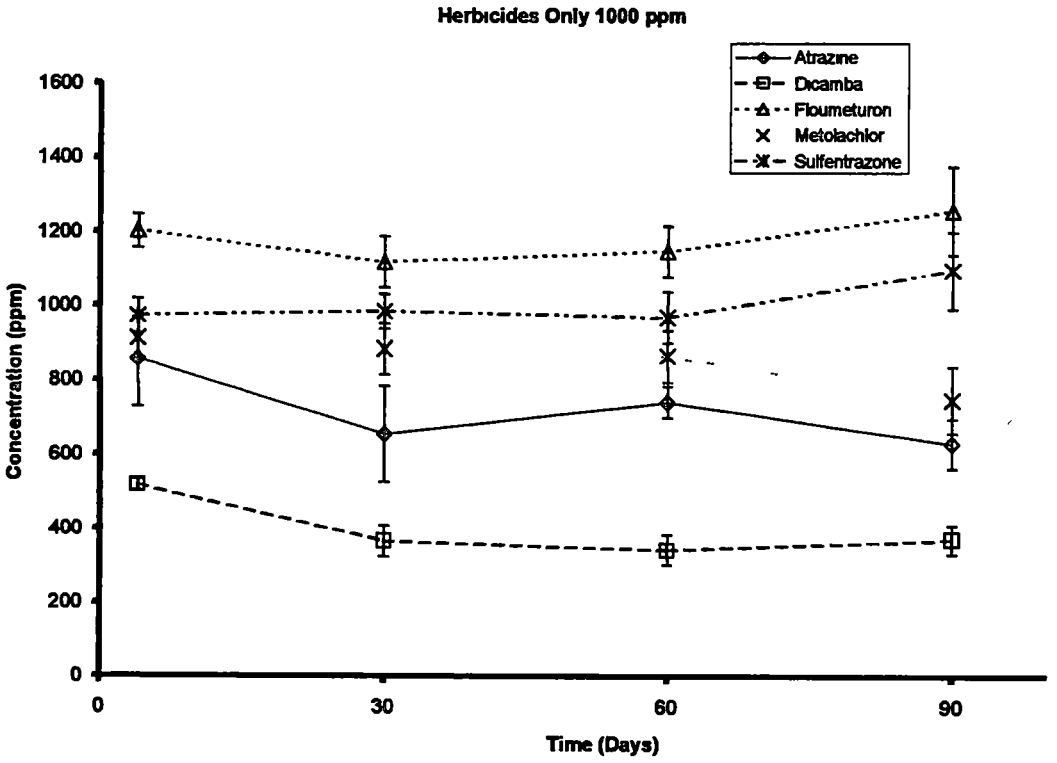

Figure 2-3. Concentration over time for herbicide dissipation in soil columns for experiment 1 1000-ppm. N=4 and error bars represent 1 standard deviation about the mean.

Table 2-2. Mean pesticide dissipation (ppm) over time Experiment 1.

Pesticide Dissipation 10 ppm

Herbicide	Day 4	Day 30	Day 60	Day 90	% Dissipation	*Half-Life (D)
Atrazine	7 6a	1.8b	1 8b	1 2b	84%	18
Dicamba	0.5a	0.0b	0 0b	0.0b	100%	<30
Fluometuron	6 9a	4 4b	4.0b	3.5b	51%	83
Metolachlor	7.9a	3 4b	3.1b	1 5c	82%	35
Sulfentrazone	9 7a	8 5a	7 6a	9 2a	0%	ND

Pesticide Dissipation 100 ppm

Herbicide	Day 4	Day 30	Day 60	Day 90	% Dissipation	*Half-Life (D)
Atrazine	69.2a	52.6b	62 3a	45 5b	35%	193
Dicamba	34 0a	18 3b	13 5b	15 4b	55%	55
Fluometuron	162 7a	99.8a	106 1a	97 9a	0%	ND
Metolachlor	98.4a	70 0b	76.6b	59 8c	40%	139
Sulfentrazone	103 1a	92.3a	107 8a	108 4a	0%	ND

Pesticide Dissipation 1000 ppm

Herbicide	Day 4	Day 30	Day 60	Day 90	% Dissipation	*Half-Life (D)
Atrazine	853 9a	652 6b	739 3ab	626 7b	27%	240
Dicamba	516 9b	364 5b	339 6b	367 3b	29%	156
Fluometuron	1201 6a	1116 2a	1144 8a	1255.0b	0%	ND
Metolachlor	910 7a	881 3a	860 9a	744 4a	18%	330
Sulfentrazone	970 9a	982 4a	965 6a	1092 1b	0%	ND

Within row means followed by the same letter are not significantly different according to LSD ($\alpha = 0.05$, n=4).

* Estimated half-life.

At the 100 ppm concentration (500 ppm total load), atrazine was reduced by 35% with a corresponding estimated half-life of 135 days, the dicamba reduced by 55% with an estimated half-life of 55 days, and metolachlor dissipated by 40% with an estimated half-life of 139 days. At this concentration there was no significant dissipation of fluometuron or sulfentrazone. In the heavily-contaminated columns with 1000-ppm loadings (5000 ppm total load), there was a 27% reduction in the amount of atrazine with an estimated 240 day half-life, a 29% reduction in the amount of dicamba with an estimated 156 day half-life, and an 18% reduction in the amount of metolachlor with an estimated 330 day half life. Floumeturon and sulfentrazone showed no reduction over time.

Soil Chemical data

Soil chemical results are summarized in Table 2-3. Total C, S, N, and extractable Ca, Cu, Mg, Na, and Zn did not change significantly over the course of the experiment within any treatment. Extractable K declined with time, while extractable P increased then decreased over the 90-day experiment. The addition of pesticides at the 1000-ppm level also caused significant increases in total soil C, S, N, Na, and NH_4^+.

Figures 2-4 thru 2-7 show the redox sensitive indicators Fe, Mn, NO_3^- and NH_4^+. Over time at all pesticide concentrations extractable Fe first increased then decreased. Mn levels increased with time at all pesticide concentrations, but most acutely at the100-ppm concentration. The NO_3^- level dropped over time

Table 2-3. Mean soil chemical values over time for experiment 1.

Time and Treatment	CARBON % by wt	SULFUR % by wt	NITROGEN % by wt	pH	EC	CA ppm	CU ppm	FE ppm	K ppm	MG ppm	MN ppm	NA ppm	P ppm	ZN ppm	Ammonia ppm	Nitrate ppm
Control T=0	1 09	0 01	0 10	5 31	0 10	1098 44	1 67	108 28	106 54	129 84	36.90	18.84	8 66	11.82	8 42	22.90
Control T=30	1 10	0 01	0 10	5 48	0 07	1131 64	2 40	134 92	95 43	130 45	114 07	23.47	9 29	9 13	6 09	11.82
Control T=60	1 11	0 02	0 08	5 63	0 19	1142 53	1 83	182 21	94.03	128 76	156 90	26.34	9 78	7 49	10.88	8 79
Control T=90	1 12	0 01	0 10	5 48	0 08	1063 45	1 78	149 93	82.00	130 13	137 55	25.90	8 51	8 20	7 80	13.31
10 ppm T=0	1 07	0 01	0 09	5 30	0 22	1083 57	1 57	108 60	94.33	127 41	39.80	19 46	9 11	7 70	9 48	18.60
10 ppm T=30	1 16	0 01	0 11	5 60	0 06	1135 56	1 93	136 75	99 12	128 06	134 34	21.96	9 71	5 59	14.26	10.91
10 ppm T=60	1 16	0 02	0 07	5 58	0 06	1102 81	1 68	160 50	91.38	128 48	129 23	29 16	9 25	5 55	7 39	9 80
10 ppm T=90	1 10	0 01	0 10	5 58	0 07	1036 00	1 99	115 68	83.20	128 43	122 20	25.65	8 03	4 92	5 12	9 05
100 ppm T=0	1 08	0 01	0 10	5 28	0 24	1062 15	1 47	107 03	97.94	124 20	46.61	26 71	8 62	5 31	16 16	17.89
100 ppm T=30	1 12	0 01	0 10	5 84	0 07	1115 16	1 89	159 07	100 10	125 37	310 11	25.89	10.67	3 48	31 72	9 31
100 ppm T=60	1.16	0 02	0 09	5 84	0 08	1093 92	1 76	171 68	93.26	122 94	322 39	27.61	10.57	3 17	33.62	8 94
100 ppm T=90	1 12	0 01	0 10	5 85	0 07	1045 78	1 59	167 55	83.55	123 33	293 65	29.00	9 55	2 65	35 14	6 68
1000 ppm T=0	1 25	0 04	0 13	5 21	0 20	1301 91	1 46	110 83	100 42	125 72	124 11	58 78	8 60	4 99	34.86	24.90
1000 ppm T=30	1 44	0 03	0 17	5 38	0 26	1114 40	1 67	127 58	104 28	128 15	227 20	67.99	10.01	4 27	30.07	13.81
1000 ppm T=60	1 44	0 04	0 14	5 50	0 22	1100 11	1 35	129 69	92.92	122 96	269 78	58.54	9 88	3 58	24 19	12.72
1000 ppm T=90	1 36	0 02	0 16	5 45	0 23	995 43	1 43	96.53	82.38	117 53	227 60	55.65	8 19	3 29	38.39	9 68

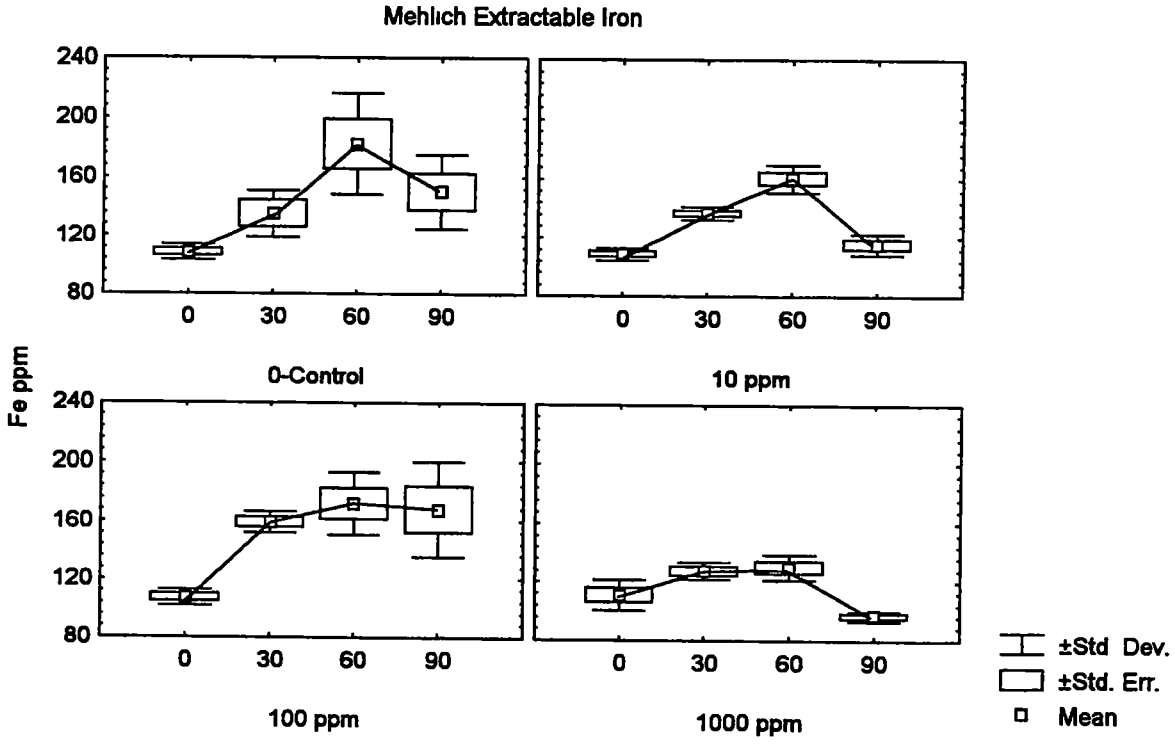

Figure 2-4. Concentration over time of Mehlich extractable Fe from experiment 1, n=4 for each point and the x-axis is days.

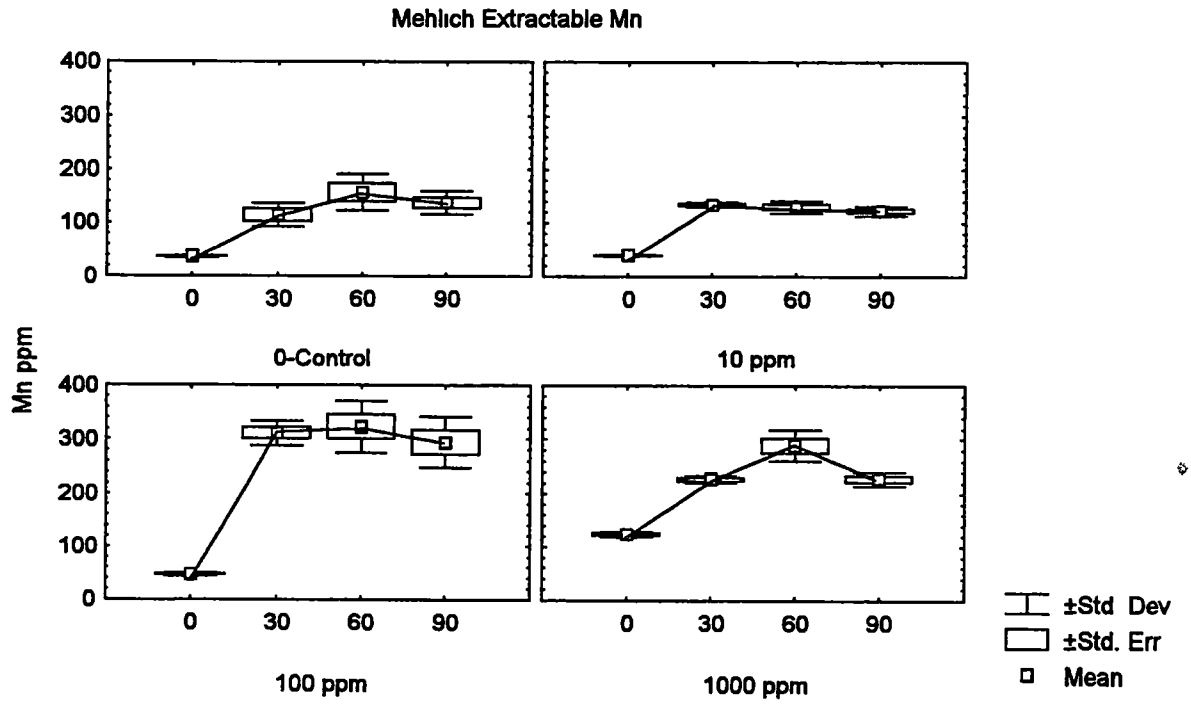

Figure 2-5. Concentration over time of Mehlich extractable Mn from experiment 1, n=4 for each point and the x-axis is days.

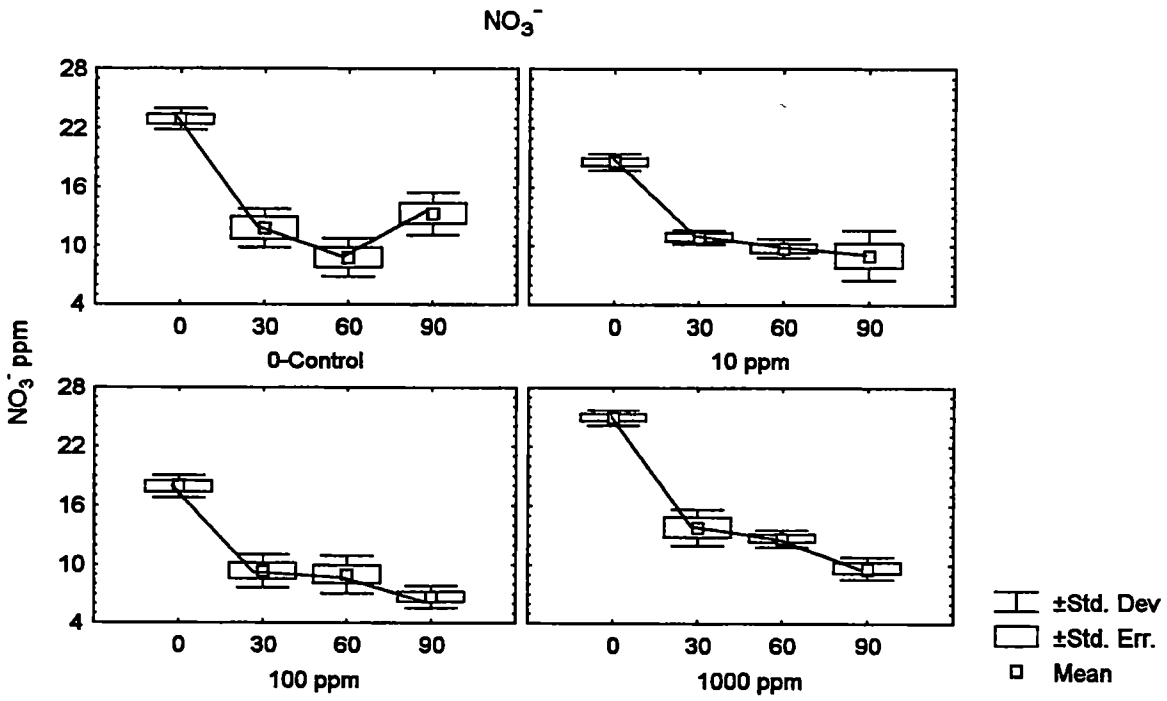

Figure 2-6. Concentration of Nitrate (ppm) over time for experiment 1, n=4 for each point and the x-axis is days.

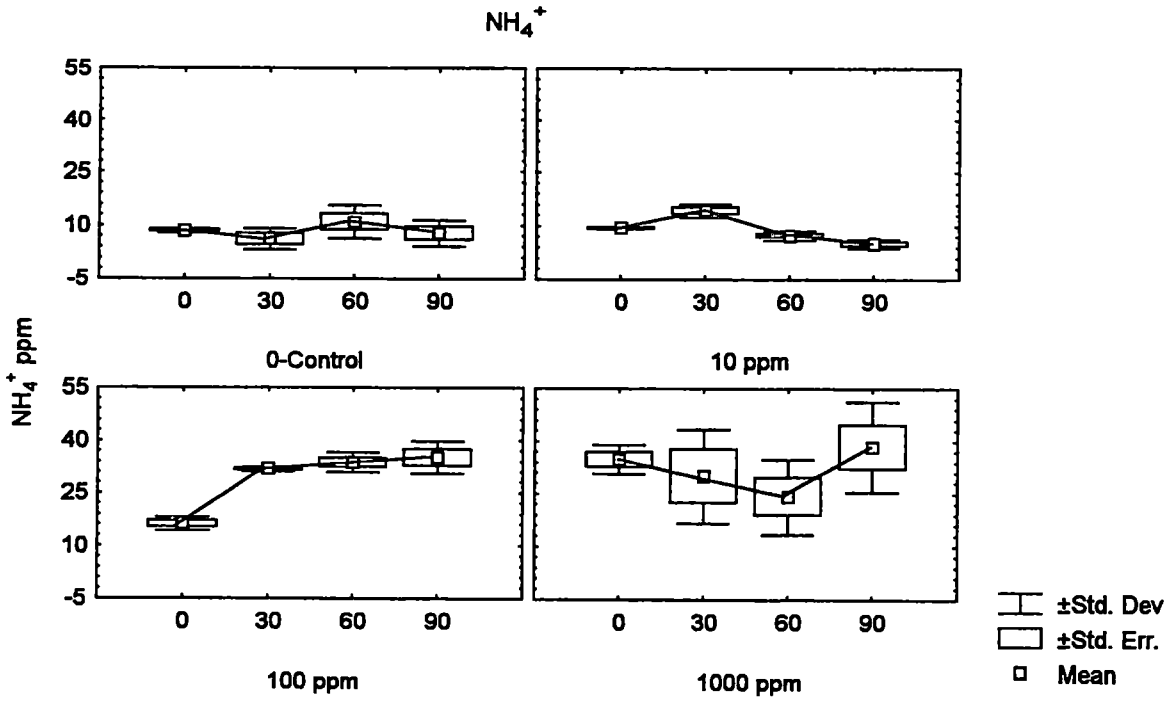

Figure 2-7. Concentration of Ammonia (ppm) over time for experiment 1, n=4 for each point and the x-axis is days.

at all concentrations of pesticides, while the NH_4^+ level was static in the control, 10, and 1000-ppm treatments while rising in the 100-ppm columns during the first 30 days of the study.

The electroconductivity (EC) of the system was relatively static throughout the experiment at all concentrations, but was consistently higher at the 1000-ppm level (Figure 2-8). At all concentrations the soil pH initially rose before leveling off (Figure 2-9). The most dramatic pH swing was in the 100-ppm columns, wherein over the first 30 days the pH increased from 5.3 to 5.9 before leveling off (Figure 2-9).

A factor analysis of the soil chemical species showed that the variables were correlated and could be described by two factors. Variables highly correlated with factor 1 were NH_4, N, C and Na and are describing the effect of the addition of pesticides. Variables highly correlated with factor two were pH, Mn, Fe, and NO_3^- and describe the developing anoxic conditions in the system. The factor scores are plotted in figure 2-10 and the factor loadings are presented in table 2-4.

Dissipation of Pesticides Experiment 2

Results of the second experiment were comparable to that seen previously. At 10 ppm there was a 60% reduction in atrazine with an estimated 37day half-life, a 33% reduction in fluometuron with a corresponding estimated

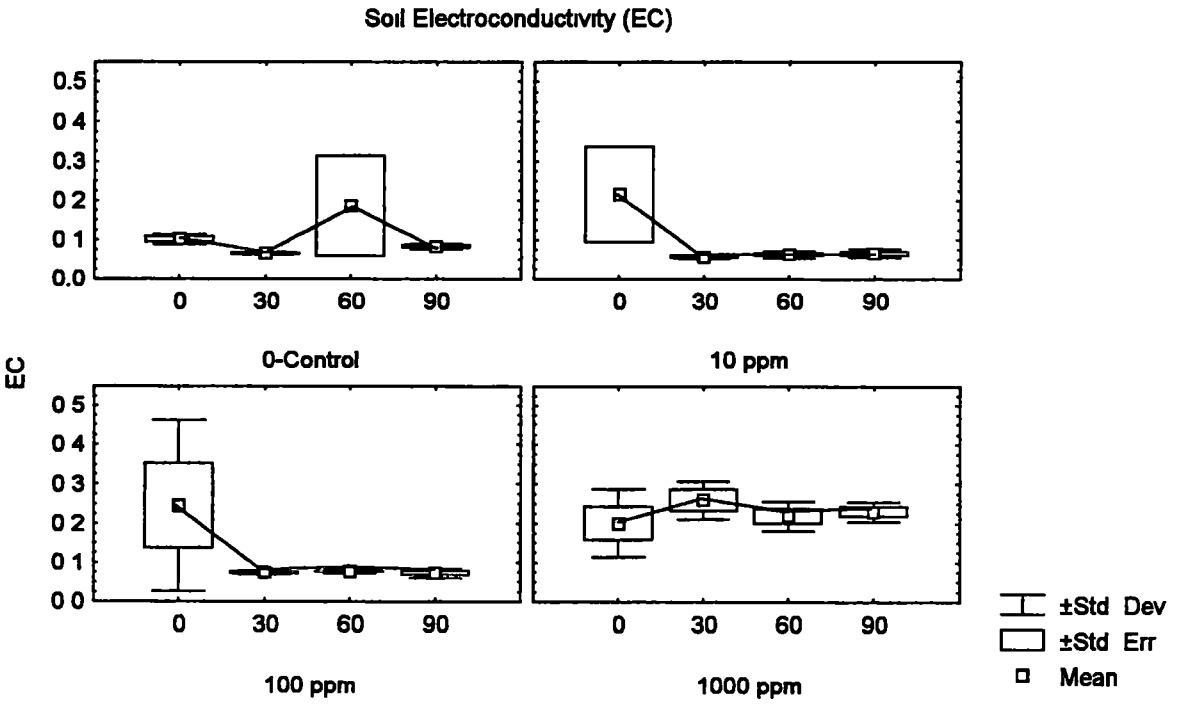

Figure 2-8. Electroconductivity over time for experiment 1, n=4 for each point and the x-axis is days.

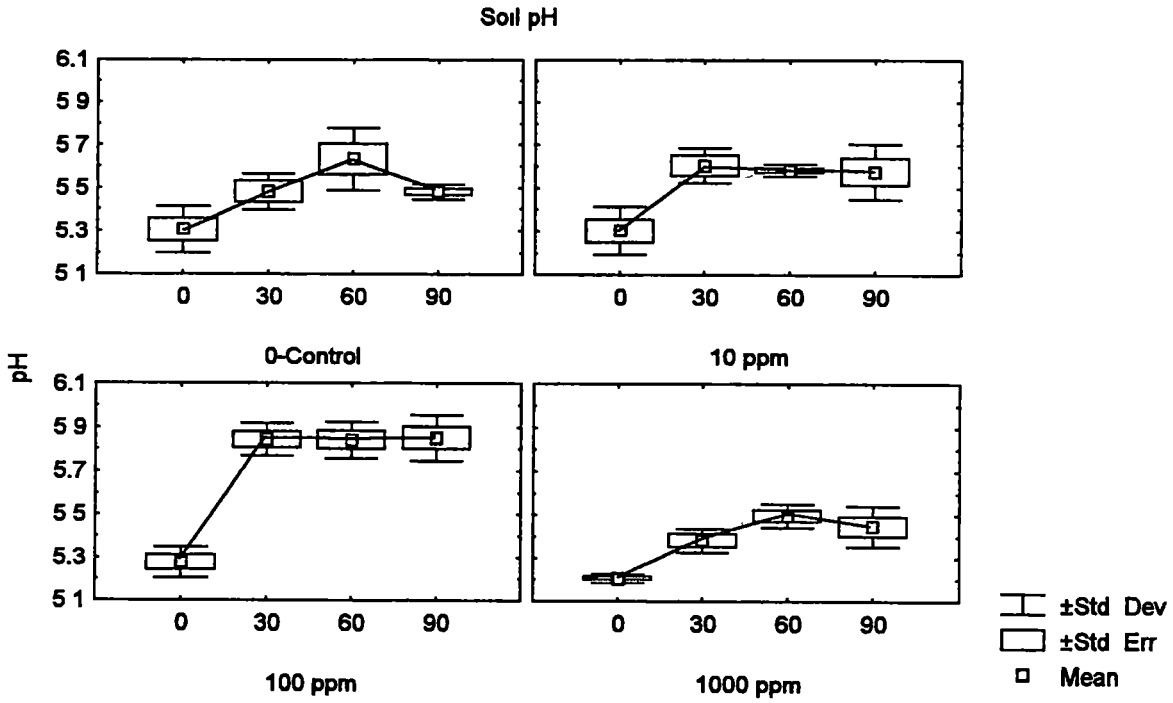

Figure 2-9. pH over time for experiment 1, n=4 for each point and the x-axis is days.

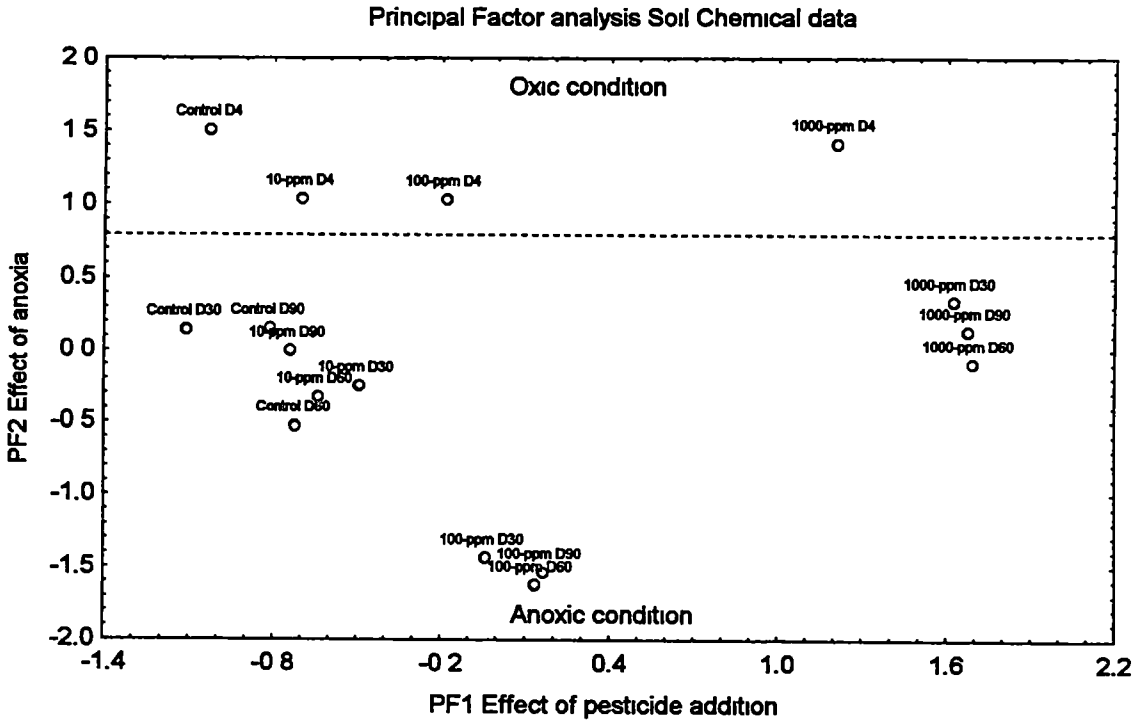

Figure 2-10. Factor analysis of soil chemical data. The variance explained by the two rotated factors are 28 and 24% respectively, with n=4 for each point. This graph illustrates the effect of pesticide addition and increasingly anoxic conditions in the system.

Table 2-4. Major components (loading >|0.70|) of the two factors contributing to treatment differences in the columns as determined by principal factor analysis using soil chemical data.

Positive and negative loadings for each factor are given.

Positive Contributors		Negative Contributors	
Variable	Loading	Variable	Loading
Factor 1: Primary effect of pesticide treatment on soil chemical variables			
Na	0.88		
C	0.87		
N	0.83		
ammonia	0.70		
Factor 2: Effect of oxidation state			
nitrate	0.76	pH	-0.97
		Mn	-0.82
		Fe	-0.75

126 day half-life, and an 87% reduction in the amount of metolachlor with a estimated half-life of 19 days (Table 2-5). For the insecticides, a 96% reduction in chlorpyrifus with an estimated 8 day half-life, and an 87% reduction in the amount of λ-cyhalothrin with an estimated 20 day half-life were determined. Dicamba was not detected, and there was no change in sulfentrazone levels.

At 100 ppm, atrazine levels dropped by 60% with an estimated 46 day half-life, fluometuron dropped by 32% with an estimated 141 day half life, metolachlor was reduced by 52% with an estimated half-life of 62 days, chlorpyrifus dropped by 83% with an estimated 25 day half life, while λ-cyhalothrin levels were reduced by 44% with an estimated half-life of 85 days. Dicamba and sulfentrazone showed no dissipation.

Discussion

Pesticide dissipation

Table 2-6 (compiled from the USDA-ARS pesticide properties database 1990) lists the pesticides used in this study and selected chemical properties. Perhaps what is of most value is the field half-life. This is the time it takes for half of the pesticide to dissipate under field conditions. By using the half-life, direct comparisons between pesticide dissipation rates can be examined. However since this experiment was run for 90 days it was not enough time to determine true half-lives for the pesticides. In order to compensate a mathematical model

Table 2-5. Mean pesticide dissipation values over time (ppm), experiment 2.

Herbicide and Insecticide Dissipation 10 ppm

Herbicide	Day 4	Day 30	Day 60	Day 90	% Dissipation	*Half-Life (D)
Atrazine	8 5a	4 7b	2.0c	3.0c	65%	37
Dicamba	0a	0a	0a	0a	ND	ND
Fluometuron	14.2a	11.3b	8 9b	9.6b	33%	126
Metolachlor	11 2a	4 1b	1 3c	1.4c	87%	19.3
Sulfentrazone	12 0a	9 9a	8.1a	12 9a	0%	ND
Chlorpyrifus	12.0a	1.0b	0.6b	0 5b	96%	8
Lambda-Cyhalothrin	3.6a	1 4b	0 6b	0.5b	87%	20

Herbicide and Insecticide Dissipation 100 ppm

Herbicide	Day 4	Day 30	Day 60	Day 90	% Dissipation	*Half-Life (D)
Atrazine	100.7a	49.7b	35.4b	40 4b	60%	46
Dicamba	17 7a	12 9a	15 1a	19 0a	0%	ND
Fluometuron	122 6a	72 9b	76 1b	83 8b	32%	141
Metolachlor	98.1a	55.2b	42.6b	47 3b	52%	62
Sulfentrazone	105 1a	80.3b	91.1ab	93 5ab	0%	ND
Chlorpyrifus	144.2a	56.1b	34.7c	24.7c	83%	25
Lambda-Cyhalothrin	100 6a	60 8b	52.7b	56 0b	44%	85

Within row, means followed by the same letter are not significantly different according to LSD ($\alpha = 0.05$, n=4).

* Estimated half-life.

Table 2-6. Selected pesticide properties (USDA-ARS, 1990)

Common name	Solubility mg/l	Field Half-Life days	Sorption mL/g	Vapor Press mm Hg	pKa	pKb
Atrazine	33	60	100	2.89E-07		12.32
Chorpyrifus	0.4	30	6070	1.70E-05		
Dicamba Salt	400000	14	2	0	1.91	
Fluometuron	110	85	100	9.38E-07		
Lambda-Cyhalothrin	0.005	22-84	180000	1.50E-09	NA	
Metolachlor	530	90	200	3.14E-05		
Sulfentrazone	780	548		1.00E-09		

was used (SAS NONLIN, SAS Institute, Cary Ind.) to extrapolate the results and subsequently provided estimated half-lives.

Atrazine

Published half-lives for atrazine in soil range from a low of 15 days for anaerobic conditions to a high of 330 days for aerobic conditions, with an average field half-life of 60 days (USDA-ARS, 1990). Estimated half-life data from the current experiments (18 to 240 days) fall within the range of observed values for this pesticide. An 85% dissipation of atrazine was observed in the previous column experiment (Corwin, 1997), with 73% dissipation in the full sized bed (Glover, 1998). Here an 84% reduction in atrazine was observed in experiment 1 (10-ppm) concurring with the findings of the previous researchers. From this agreement of results a couple of observations can be stated, first, the addition of the atrazine as formulated product did not significantly impact the column dissipation. Previous researchers used technical grade atrazine that did not contain any inert ingredients, and until this current experiment it was not known how the system would perform under "real world" conditions. Increasing the concentration of pesticide in the columns caused the rate of atrazine dissipation to decline, however this may be expected.

Dicamba

Dicamba proved to be the most easily dissipated among the pesticides applied to the columns. In both experiments and in all concentrations, the detectable dicamba concentration was significantly lower than originally applied. Dicamba is very mobile in soil, and has high water solubility, low sorption coefficient and a vapor pressure of 0. The half-life for dicamba in soils is 14 days (USDA-ARS, 1990). The process of preparing and loading the soil columns may have caused sufficient disturbances to dissipate the dicamba.

Fluometuron

The data generated from the previous experiments (Corwin, 1996 and Glover, 1998) again agree with the data from this study with respect to fluometuron. The additional herbicides or insecticides did not appear to inhibit the ability of the columns to dissipate fluometuron, especially at low concentrations. Fluometuron did not show significant dissipation in experiment 1 at 100 ppm, but did in experiment 2. This may be due to high variance recorded in the fluometuron dissipation data from experiment 1 at the 100 ppm concentration, or it may be that the addition of the insecticides at this concentration created some type of synergistic effect that allowed for a reduction in the amount of this herbicide in the higher concentration. An example of this type of reaction in soil systems was researched by Nkedi-Kizza and Brown

(1998), who found an enhanced mineralization of carbaryl when it exists in a mixture with atrazine and diuron.

Metolachlor

Metolachlor demonstrated significant dissipation in both experiments and at all concentrations. The published half-life for this pesticide is 90 days aerobic and 81 days anaerobic, when applied at label rates (USDA-ARS, 1990). Here a faster rate of dissipation for this pesticide at estimated half-lives of 19-35 days for low concentrations were demonstrated. As with all the previous herbicides, half-life increased with concentration.

Sulfentrazone

Sulfentrazone did not significantly dissipate at any concentration in any experiment. The published half-life for sulfentrazone is 1.5 years aerobic and 9 years anaerobic. As the column studies were conducted for a period of 90 days, this was not long enough to gauge the efficacy of the soil to dissipate this pesticide. The primary routes of dissipation for this pesticide are through photolysis and leaching. Direct photolysis is not possible once the pesticide is in the soil matrix, and leaching was not possible in this closed system.

Chlorpyrifus and λ-Cyhalothrin

Chlorpyrifus dissipation in the soil columns was consistent with that published in the literature (USDA-ARS, 1990) and there were no negative interactions with other pesticide. λ-cyhalothrin dissipation in the soil columns was also consistent with the published literature and appeared to have no negative effect on the dissipation of any other pesticide.

Soil Chemical data pH and EC, Experiment 1

The soil pH in the control and pesticide columns rose significantly in all treatments, from an initial value of ≈ 5.3 to a high of ≈ 5.89 in the 100-ppm concentration. Since all the treatments experienced the increase in pH, it is most likely a soil phenomena and not tied directly to the addition of the pesticide. One possible reason for the elevation in pH is that the soil was screened through a 1/8" screen prior to the loading of the columns. This homogenization of the soil may have broken up the existing structure and allowed the dissolution of various soil salts and other soil buffering constituents into the matrix.

The EC of the system was static across all treatments except at 1000 ppm, where the value was double that of the lower concentrations. A possible explanation for this would be the addition of the pesticide and the associated formulated product served to increase the amount of soluble salts in the soil. Moreover, the addition of the pesticides at the high concentration significantly

increased the Na content of the system, which would also increase the EC of the system.

Soil Chemistry Redox indicators

Visual inspection of the soil in the columns indicated gleying from the bottom of the columns up to ≈ 10 cm, and reduced conditions were suspected. One of the benefits of performing the Mehlich extraction (Mehlich, 1953) was that information could be gained on the redox state of the redox sensitive metals Fe and Mn. The susceptibility of analyzed markers to reduction fall in the range of NO_3^->Mn>Fe, with NH_4^+ being a marker for oxidation. That is, after O_2 is exhausted respiring microbes will preferentially use first NO_3^-, then Mn, and finally Fe as their terminal electron acceptors. The NH_4^+ is oxidized in aerobic systems, so if there is no disappearance of this chemical then oxidation may not be occurring. At all pesticide concentrations extractable Fe (as $FeII^+$) rose through the initial 60 days of the experiment before decreasing again between 60 and 90 days at 10 and 1000 ppm. Mn levels increased at all concentrations through the course of the experiment, and most dramatically at 100 ppm.

The increase in metals, along with the drop in NO_3^- and a static NH_4^+ level, suggest that the soil in the columns was becoming relatively more reduced through the first 60 days. This conclusion was also supported by the genetic data (See chapter four). This can have implications for the dissipation potential of the SBBR, since more reduced conditions may lead to longer half-lives of

some pesticide residues. Factor analysis presented a two-dimensional snapshot of what took place in the soil columns. First, the addition of pesticides increased the Na, N, C, and NH_4^+ levels, as was described as the first factor (Figure 2-10). The second factor was formed from the redox-indicating species, and showed the relative level of anoxia in the soil columns.

Conclusions

- At low pesticide concentrations there was significant dissipation of all pesticides except Sulfentrazone (Objectives One and Two).
- At higher concentrations, fluometuron did not show any significant dissipation (Objective Three).
- Half-lives of pesticides increased with concentration (Objective Three).
- The addition of insecticides did not significantly impact pesticide dissipation in these column experiments (Objective Two).
- The soil columns became relatively more anoxic with time (Objective Five).
- The addition of large amounts of pesticide to the soil significantly increases the soil C, N, S, and Na levels (Objective Five).
- Results from these experiments agree with past research of Corwin and Glover with respect to dissipation of atrazine and fluometuron (Objective Six).
- Addition of atrazine and fluometuron as formulated products did not significantly effect the dissipation of their active ingredients (Objective Six).

Chapter 3

Community Analysis

Phospholipid Fatty Acid Data

Introduction

The ultimate fate of PAI in the soil bed bioreactor is in large part dependent upon the reactor's soil microbial community. Understanding the impact of pesticide addition and column management on this community is then vital to the ultimate operational efficiency and sustainability of the system. Until recently the tools required to investigate complex systems such as the SBBR did not exist. Largely dependent on advances in molecular procedures and instrumentation, recent developments have led to enormous improvements in the analysis of microbial communities in natural environments. Phospholipid fatty acid (PLFA) analysis is one method that is currently available and provides information about the microbial community biomass, metabolic status and community structure (White and Ringelberg, 1996; Stephen *et al.*, 1999; Frostegard *et al.*, 1996).

The PLFA are the major lipid components in the bacterial cell membrane, are not found in storage products (Kates, 1986), and are labile upon cell death (White, *et al.*, 1979). Microorganisms can be categorized depending on the fatty-acid synthetic pathway (Kaneda, 1991). If the specific PLFA components can be related to certain subsets of the microbial community, then the PLFA patterns can be used to define changes in the community composition (Ringelberg *et al.*,

1989). Examples of PLFA that are routinely used as indicators of community composition are listed in Table 3-1. As products of biosynthetic pathways, PLFA reflect the phenotypic response of microorganisms to environmental conditions (White and Ringelberg, 1996). For example, cyclopropane fatty acids (cy17:0 and cy19:0) are formed preferentially over the monoenoics 16:1ω7c and 18:1ω7c as bacteria switch from the log to the stationary growth phase (Findlay *et al.*, 1990; Guckert *et al.*, 1986; White *et al.*, 1996). Although varying between organisms, ratios of cyclopropane to monoenoic fatty acids generally fall within the range of 0.05 (log phase) to 2.5 or greater (stationary phase; Guckert *et al.*, 1986; White, 1983). Also, bacteria make *trans* - monounsaturated fatty acids, thereby increasing membrane ordering and decreasing fluidity (Sekkema *et al.*, 1995) in response to changes in the environment such as desiccation (Kieft *et al.*, 1994; Macnaughton *et al.*,1999a) and exposure to hydrocarbons (Pinkart *et al.*, 1996; Stephen *et al.*, 1999).

In this study we used PLFA to assess the viable biomass, community structure, and metabolic status of the soil column (simulated SBBR) microbial communities at different levels of pesticide concentrations (objective four). A mixture of five herbicides (atrazine, dicamba, fluometuron, metolachlor, and sulfentrazone) as formulated products were added in four concentrations each, (0-control, 10 ppm, 100 ppm, and 1000 ppm) for a total concentration of 0, 50, 500, and 5000 ppm in each of the respective treatments. Quadruplicate columns were destructively sampled at days 0, 30, 60, and 90.

Table 3-1. Selected PLFA biomarkers

PLFA	Functional Group	Reference
i15 0, a15 0, i17 0, a17.0	Gram-positive bacteria	Ringelberg etal., 1997
i15 0, a15.0, i16 0, i17 0, a17.0	Gram-positive bacteria	Frostegård & Bååth, 1996
i15·0, a15.0, i16.0, i17.0	Gram-positive bacteria	Bardgett et al , 1996
10me16, i15 0, a15.0, i16.0 i17:0, a17 0	Gram-positive bacteria	Zogg et al., 1997
i15 0, i16:0, 10me16.0	Gram-positive bacteria	Zak et al , 1996
16 1ω7c, 18·1ω7c, cy 17.0, cy19.0	Gram-negative bacteria	Ringelberg et al., 1997
16:1ω9,16:1ω7t, cy 17:0,18.0ω7,cy19.0	Gram-negative bacteria	Frostegård & Bååth, 1996
cy 17 0,18 1ω7,cy19 0	Gram-negative bacteria	Bardgett et al , 1996
16.1ω5c,16:1ω7c,16:1ω7t,16:1ω9c, 18:1ω5c,18 1ω7c,cy17:0,cy19.0	Gram-negative bacteria	Zogg et al , 1997
16·1ω7c, cy17.0, 18:1ω7c, 18.1ω7t cy 19 0	Gram-negative bacteria	Zak et al , 1996
10me18 0	Actinomycetes	Ringelberg et al., 1997
Mid-chain branched fatty acids	Actinomycetes	White et al , 1997
10me16.0	Sulfate-reducers (*Desulfobacter)*	Ringelberg et al, 1997
i17:1ω7c	Sulfate-reducers (*Desulfovibrio)*	Ringelberg et al, 1997
i17·1ω7c, i15·1ω7c, i19.1ω7c	Sulfate-reducers (*Desulfovibrio)*	White et al., 1997
16 1ω8c, 18.1ω8c	Methane oxidizers	Ringelberg et al , 1997
18.2ω6, 18.3ω6, 18 3ω3	Fungi	Ringelberg et al , 1997
18 1ω9, 18 2ω6, 18·3ω3	Fungi	Zogg et al , 1997
18.1ω9, 18:2ω6	Fungi	Zak et al., 1996
20.3ω6, 20.4ω6	Protozoa	Ringelberg et al., 1997
20.2ω6, 20.3ω6, 20:4ω6	Protozoa	White et al , 1997
18.1ω9c, 18.1ω11c, 18 3ω3, 20.5ω3	Higher plants	Ringelberg et al , 1997

Taken from Kirchner (1999)

Materials and Methods

PLFA Analysis

Solvents used were GC grade from Fisher Scientific, Fairlawn, NJ. Each lot was tested for purity before use. Silicic acid was Unisil (Clarkson Chemical Company). Potassium hydroxide and the internal fatty acid standard 19:0 were from Sigma Chemical Co., St. Louis, MO. Glassware for lipid analysis was freed of lipid contaminants by washing with non-phosphate detergent and rinsing with ten volumes of clean tap water, then ten volumes of deionized water, and heating in a muffle furnace at 450°C for 4 hours.

Lyophilized soil was extracted with the single-phase chloroform-methanol-buffer system of Bligh & Dyer (1954), as modified by White et al. (1979). Five mL of chloroform, 10 mL of methanol, and 5 mL of phosphate buffer (50 mM, pH = 7.4) were added to the soil, mixed, and allowed to equilibrate for 3 hours. The single phase extractant was separated from the solid material by centrifugation at 2000 rpm for 20 minutes and decanted. Five mL of chloroform was used to wash the pelletted solids, which were then re-centrifuged, and the chloroform added to the extractant. An additional 5 mL of water was added to the extract to force the separation of the aqueous from the organic phases (ratio is now 1:1:0.8 MeOH:$CHCl_3$:buffer, v:v:v). After separation for approximately 12 hours, the organic phase was pipetted to a new test tube and the solvent removed with a stream of dry nitrogen at <37°C.

The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (Guckert et al., 1985). Pasteur pipets (1 cm diameter) partially plugged with glass wool were prepared, and 0.5 g silicic acid added as a slurry in 30 Mm ammonium acetate n MeOH. The columns were pre-eluted with 5 mL of acetone and 5 ml of chloroform, and the sample transferred to the column with 3 100-μL washes of chloroform. Neutral lipids were eluted with 5 mL chloroform, glycolipids with 5 mL acetone, and polar lipids with 10mL of methanol. The solvent was removed from the polar lipids under a stream of dry nitrogen at <37°C. All results presented are for the polar lipid fraction.

The polar lipids were transesterified to the fatty acid methyl esters by a mild alkaline methanolysis (Guckert et al., 1985). The polar lipid extract was dissolved in 1 mL of chloroform/methanol 1:1(v/v), 1 mL of methanolic KOH was added, and the mixture heated at 60°C for 30 minutes. Fatty acid methyl esters (FAMES) were recovered from the organic fraction of the sample after adding 2 ml of hexane and 2 ml of water to break phase.

The FAMES were analyzed by capillary gas chromatography with flame ionization detection on a Hewlett-Packard 5890 Series 2 chromatograph with a 50 m non-polar column (0.2 mm I.D., 0.11 μm film thickness). The injector and detector were maintained at 270°C and 290°C, respectively. The column temperature was programmed from 60°C for 2 minutes, then ramped at 10°C per minute to 150°C, then ramped to 312°C at 3°C per minute. Preliminary peak

identification was by comparison of retention times with known standards. Detailed identification of peaks was by gas chromatography/mass spectroscopy of selected samples using a Hewlett-Packard 5890 series 2 gas chromatograph interfaced to a Hewlett –Packard 5971 mass-selective detector using the same column and temperature program as previously described. Mass spectra were determined by electron impact at 70 eV. Methyl nonadecanonate (c19:0) was used as the internal standard, and the PLFA expressed as equivalent peak response to the internal standard.

Fatty acids are named according to the convention X:YωZ, where "X" stands for the number of carbon atoms in the chain, "Y" for the number of unsaturations, and "Z" the number of carbon atoms from the methyl end of the molecule to the first unsaturation encountered. Prefixes include the following: "i" ≡ iso-branched, "a" ≡ anteiso-branched, "10Me" ≡ methyl branch on the tenth carbon from the carboxylate end, "Br" ≡ branched at unknown location, and "Cy" ≡ cyclopropyl. The suffixes "c" and "t" stand for the cis-and trans-geometric isomers of the unsaturation, respectively. When different fatty acids had the same designation, they were distinguished by lower case letters; a, b, etc. A subtle ambiguity in this naming convention is that the number of carbons given for iso, anteiso, unknown branched, and cyclopropyl fatty acids includes all of the carbons in the molecule, except the esterified methyl group, but 10Me16:0 indicates a methyl group attached to a 16-carbon side chain (Kates, 1986).

Statistical analysis of PLFA profiles

The relative proportion (percentage mole fraction) or biomass (pmol/g) of PLFA was used to test the null hypothesis that adding PAI to the columns would not influence the composition of the soil microbial communities. To test that hypothesis, an analysis of variance (ANOVA) using the General Linear Model SAS procedure (SAS Institute Cary, NC.) for a completely randomized design with four treatments was used. The values reported are least square means of 4 replicates and standard of the means were determined. Differences in the mean proportions of PLFA in each treatment were tested using a Fisher's Least-Significant-Difference procedure. MANOVA procedures were also conducted to analyze PLFA profiles. Data (percentage mole fraction) were arc-sine transformed prior to analyses to meet assumptions of normality. The PLFA compositions (expressed in mole percent) of different samples were subjected to unconstrained ordination (a principal-components analysis, PCA) and a constrained ordination (CCA). The PCA and CCA were performed on arc-sine transformed relative proportion (mol%) data using the statistical package "Statistica 1997" (StatSoft Inc., Tulsa OK) for the PCA and "Canoco 4" (Microcomputer power, Ithica NY) for the CCA. The plot generated from a PCA illustrates profile differences and also identifies which PLFAs contribute to the differences in pesticide levels. Significance level for all statistical tests was $P = 0.05$.

Results

Viable Biomass

Control Columns

The viable microbial biomass was stable throughout the first 60 days of the study (Figure 3-1). Between day 60 and day 90 there was a significant 38% reduction of total biomass in the control columns. The biomass of all PLFA functional groups (normal sats, mid-chain branched sats, terminally branched sats, branched monounsaturates and monounsaturates) mirrored the total biomass, and experienced a 35-43% reduction in biomass within the last 30 days of the study (Figures 3-2 through 3-7). The exception to this trend was the polyunsaturates, which experienced a steady decline in biomass over the complete time-course (Figure 3-7).

10-ppm Concentration

Where pesticides had been added to the soil at 10-ppm concentration there was a significant decrease of 14% in the total viable microbial biomass by the 60th day of the experiment (Fig. 3-1). Only normal saturated and terminally branched saturated showed no change in amount over time. The mid-chain branched saturates, branched monounsaturated, monounsaturated,

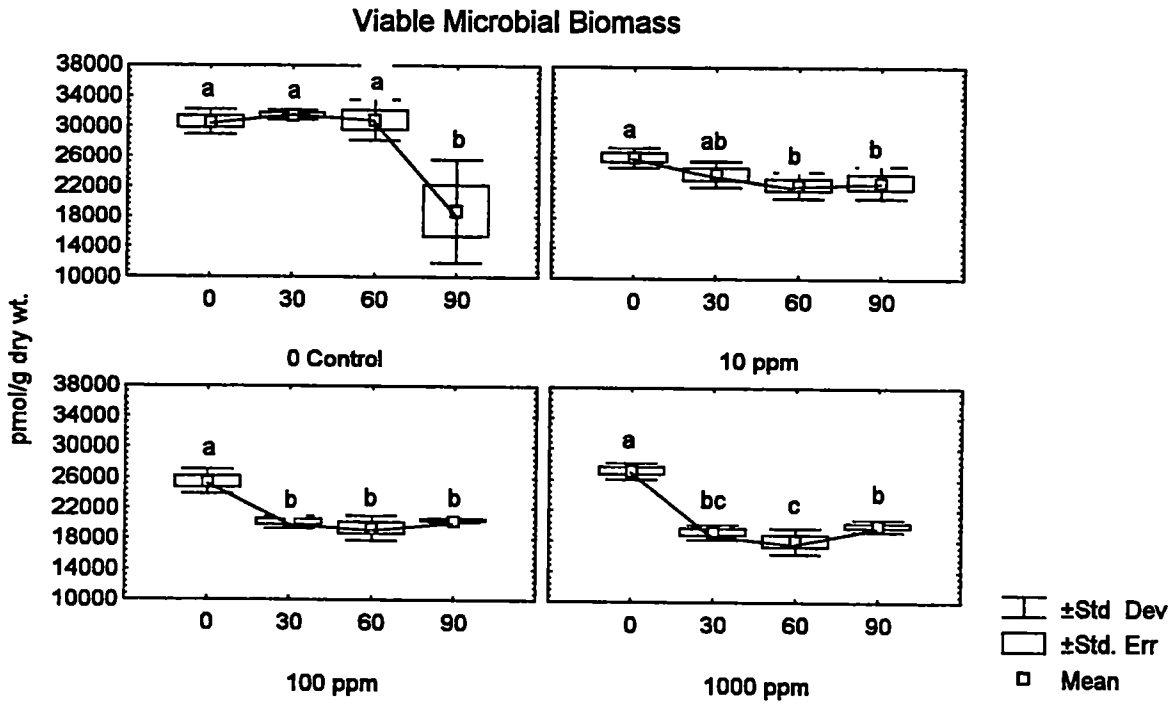

Figure 3-1. Biomass over time of total PLFA.
N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

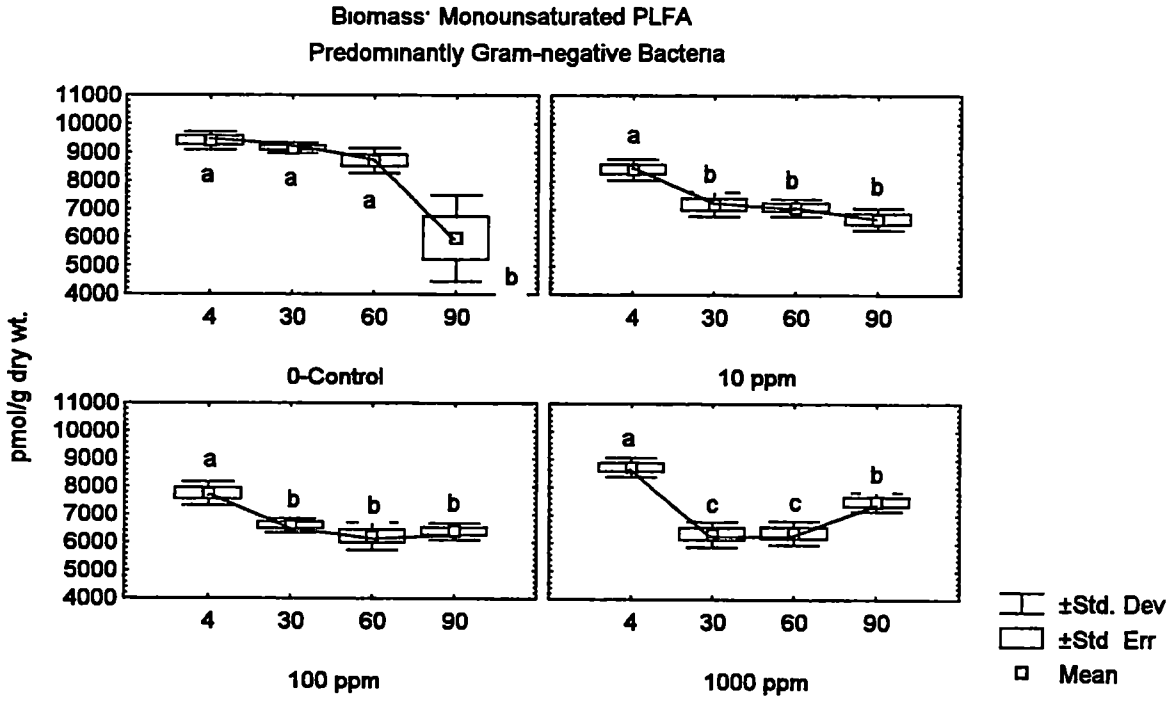

Figure 3-2. Biomass over time of monounsaturated PLFA. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

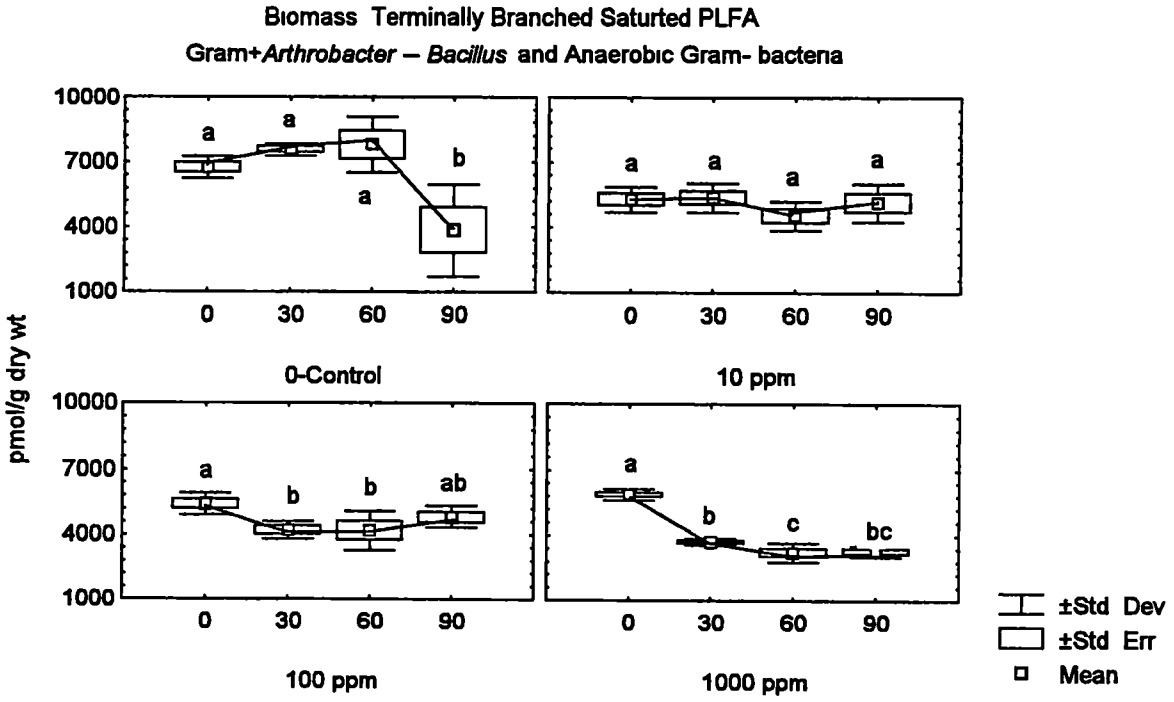

Figure 3-3. Biomass over time of terminally branched PLFA. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

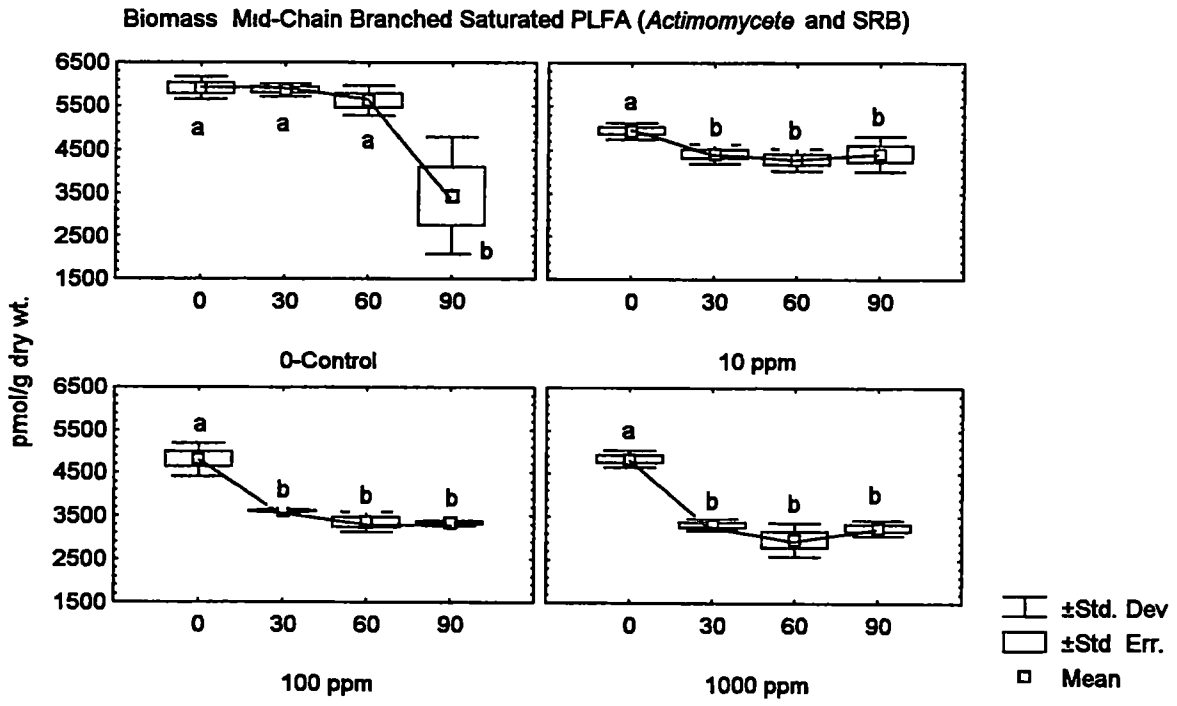

Figure 3-4. Biomass over time of mid-chain branched PLFA. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

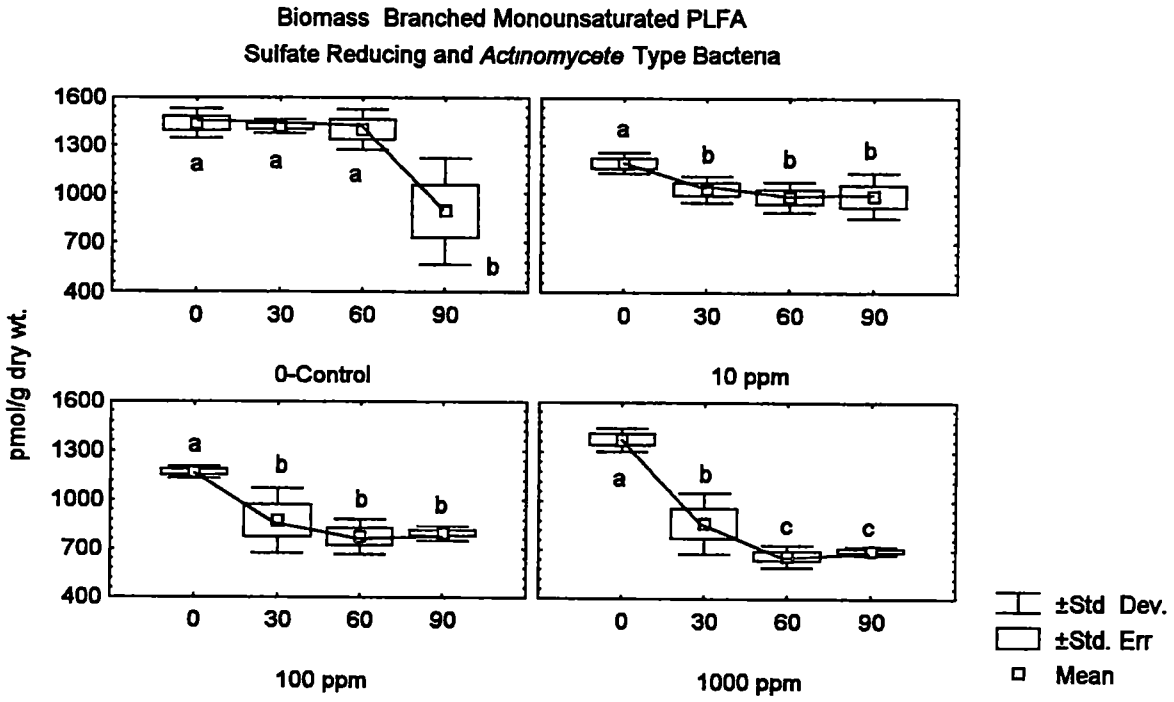

Figure 3-5. Biomass over time of branched monounsaturated PLFA. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

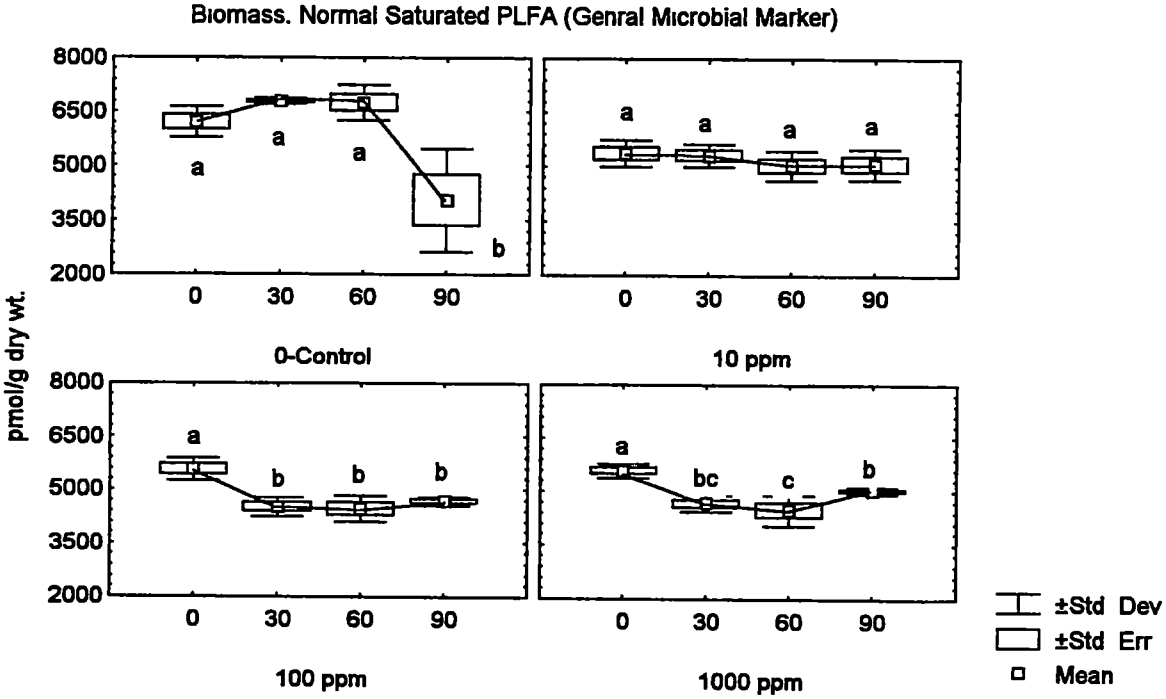

Figure 3-6. Biomass over time of normal saturated PLFA. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

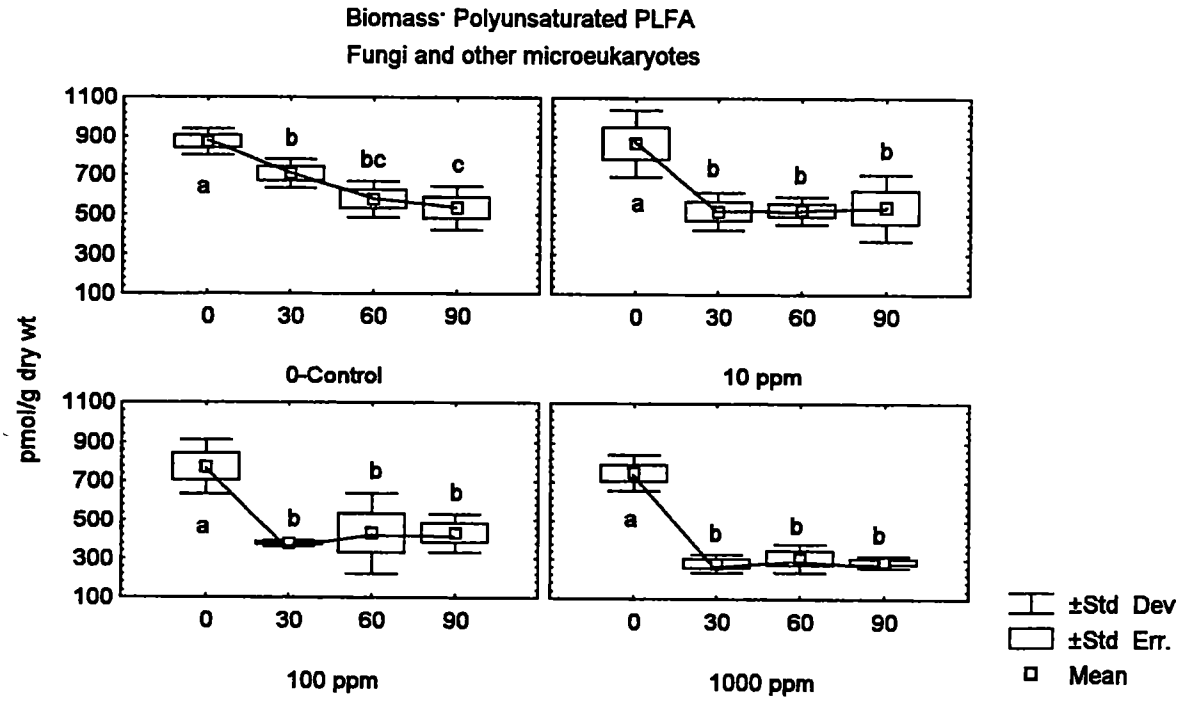

Figure 3-7. Biomass over time of polyunsaturated PLFA. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

and polyunsaturated PLFA all declined significantly between day 0 and day 30 and were steady thereafter.

100-ppm Concentration

There was a significant 21% reduction in total microbial biomass within the first 30 days of the experiment. With the exception of the terminally-branched saturates, all the PLFA functional groups were affected. Terminally-branched saturates biomass declined in the first 30 days but recovered by the end of the experiment (Figure 3-3).

1000-ppm concentration

There was a significant 30% reduction in the amount of microbial biomass through the first thirty days of the experiment that leveled off by day sixty. From day 60 to day 90 there was a significant 11% increase in the total biomass. Of the PLFA groups, all declined within the first 30 days in proportions similar to the overall biomass. The only groups to recover from day 60 to 90 were the normal saturates and monounsaturated PLFA.

Metabolic Status

The 16:1ω7t/c isomer ratio showed no significant change over time in the control, 10, and 100-ppm treatments (Figure 3-8). In the 1000-ppm treatment, however, this ratio showed a significant increase between day 4 and

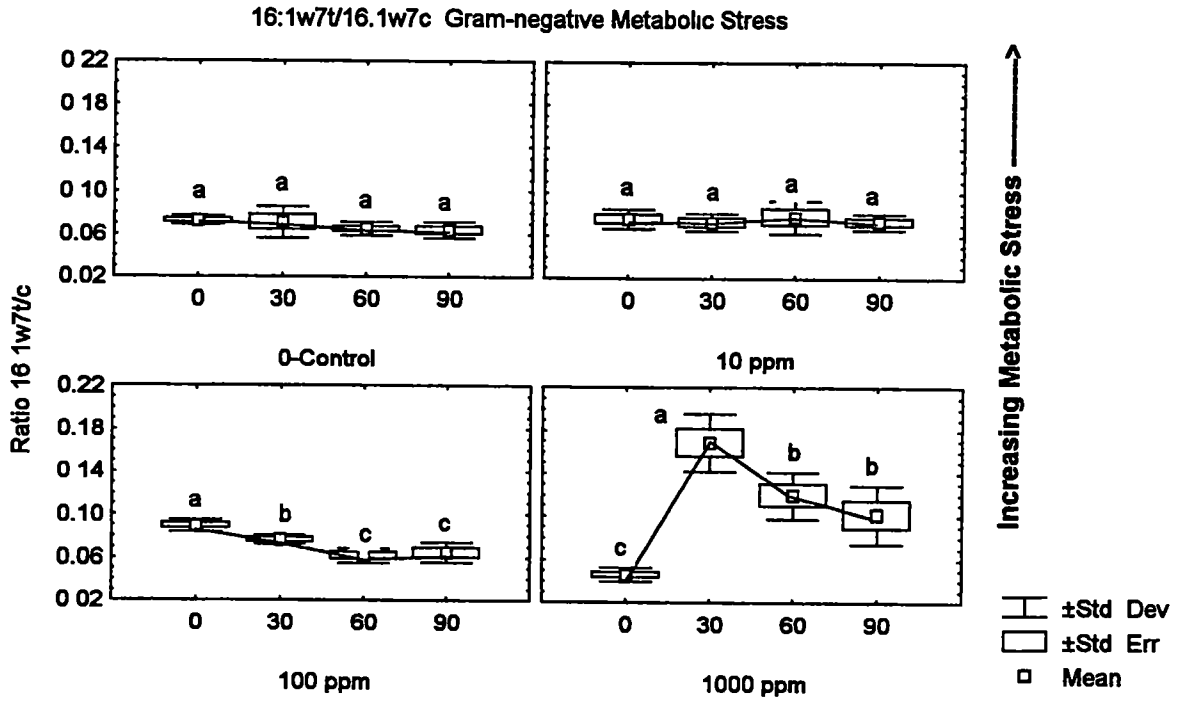

Figure 3-8. 16:1w7t/c isomer ratio over time, an index of Gram-negative metabolic stress. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

day 30 before declining through 90 days. The 18:1ω7t/c isomer ratio showed a significant decline in the 100-ppm concentration in the first 30 days. There were no other significant changes over time at any concentration.

The i10me16:0/i17:1ω7c ratio showed a significant increase at 1000 ppm and continued for the entire experiment (Figure 3-9). The control columns showed the opposite effect, with this ratio significantly decreasing. There were no significant changes in this ratio over time in either the 10 or 100-ppm concentrations.

Over the 90 days of the experiment the ratio of cy17:0/16:1ω7c increased significantly at all concentrations except 100-ppm, where there was a decrease over the first 30 days (Figure 3-10). The cy19:0/18:1ω7c isomer ratio increased significantly over time in the 0-control and 10-ppm concentrations, while dropping significantly in the first 30 days in the 100 and 1000-ppm treatments (Figure 3-11).

Community Structure

Factor analysis identified PLFA and biomass that were significantly correlated (loading > |0.7|) in describing similarities and differences between treatment PLFA profiles (Figure 3-12). The analysis identified two factors that accounted for 27 and 20% of the variance, respectively. The most significant PLFA for the two factors are listed in Table 3-2. Factor one was attributable to

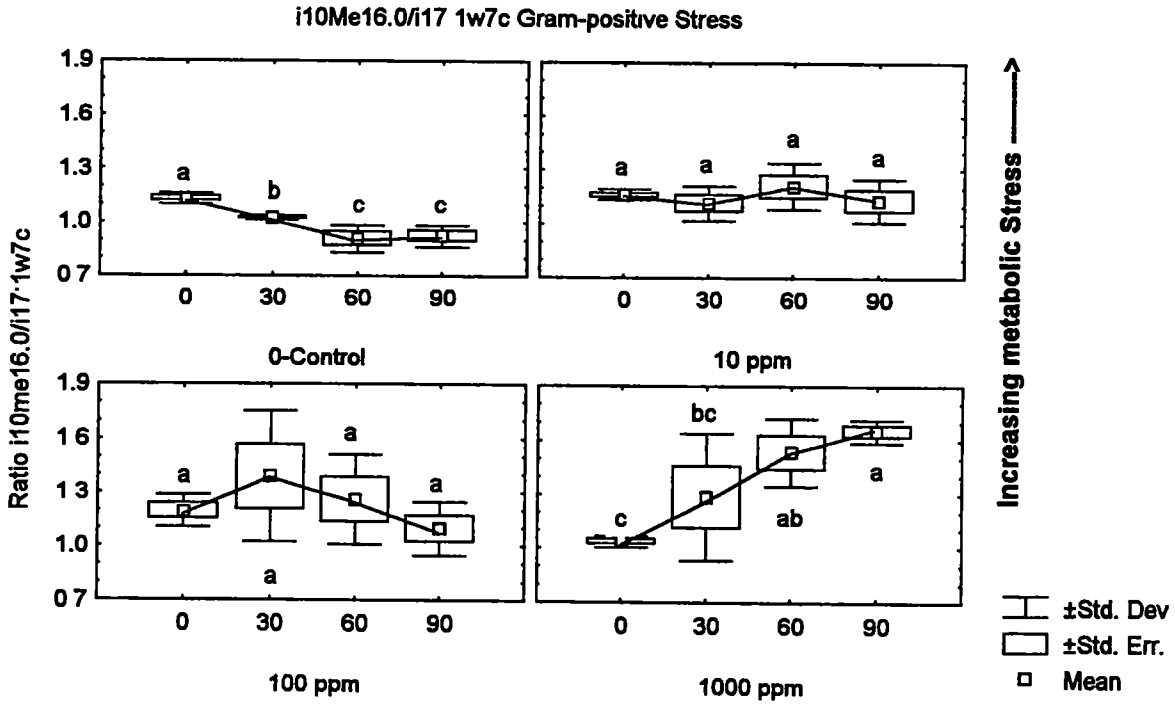

Figure 3-9. I10me16:0/i17:1w7c isomer ratio over time, an index of Gram-positive metabolic stress. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

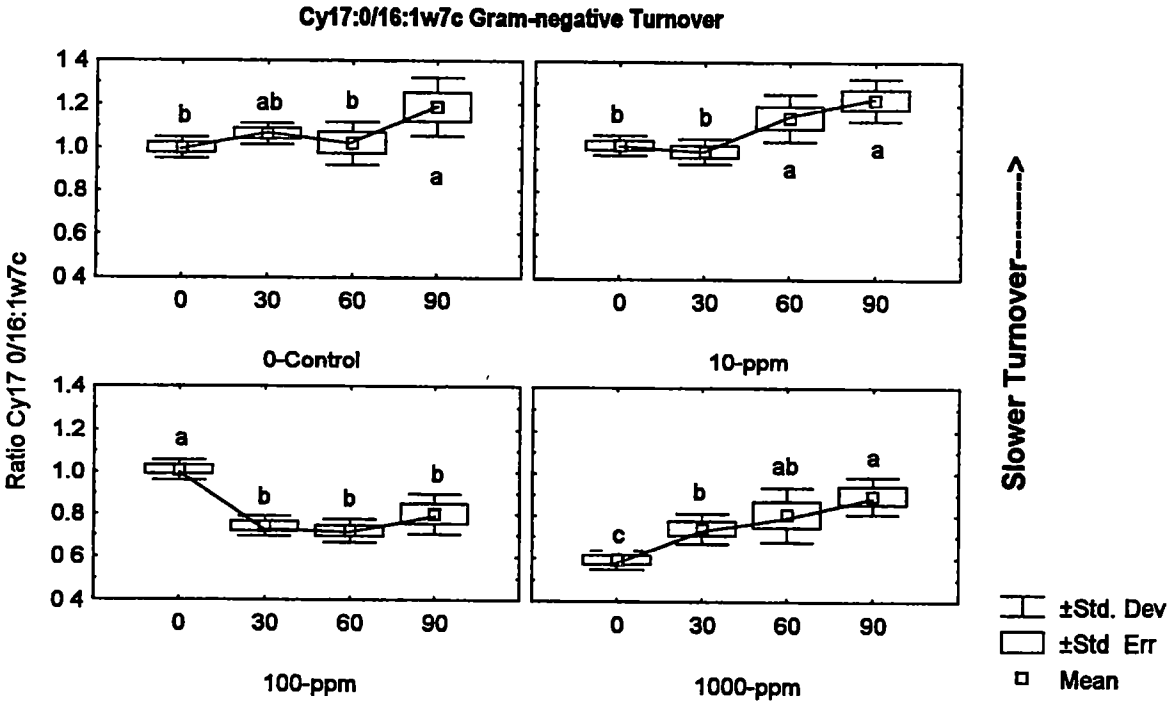

Figure 3-10. Cyclopropyl17:0/16:1w7c ratio over time, an index of Gram-negative turnover. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

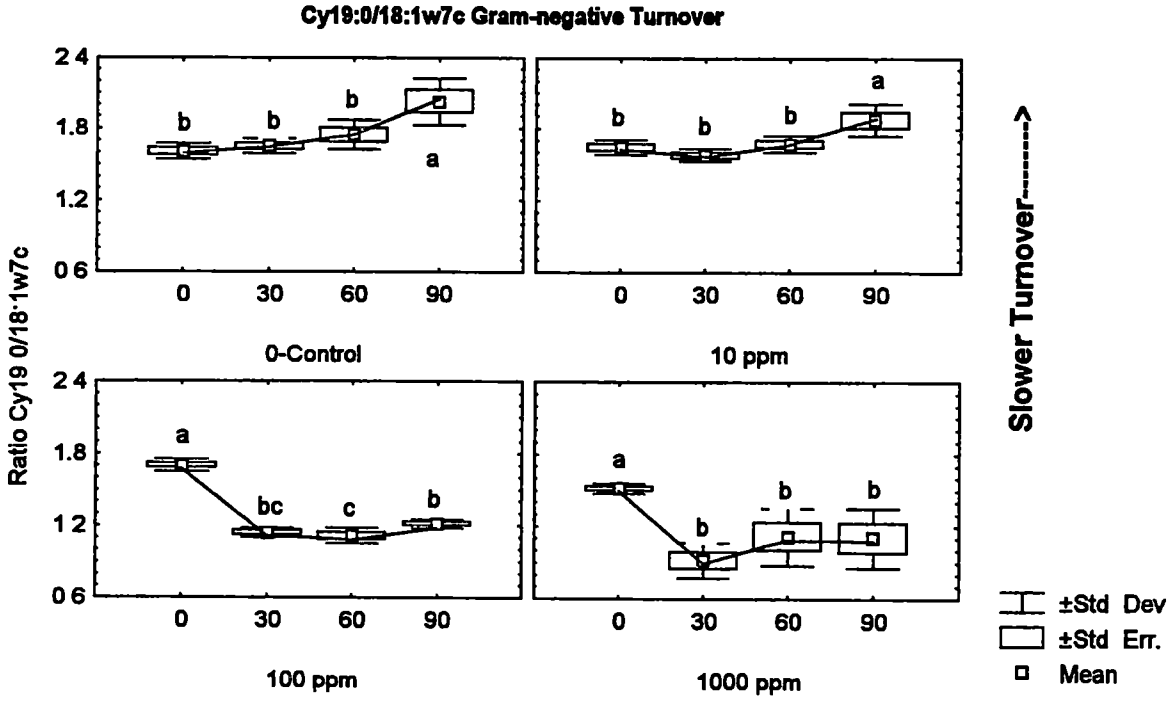

Figure 3-11. Cyclopropyl19:0/18:1w7c ratio over time, an index of Gram-negative turnover. N=4 and x-axis is days. Within box same letters are not significantly different according to LSD (α=0.05).

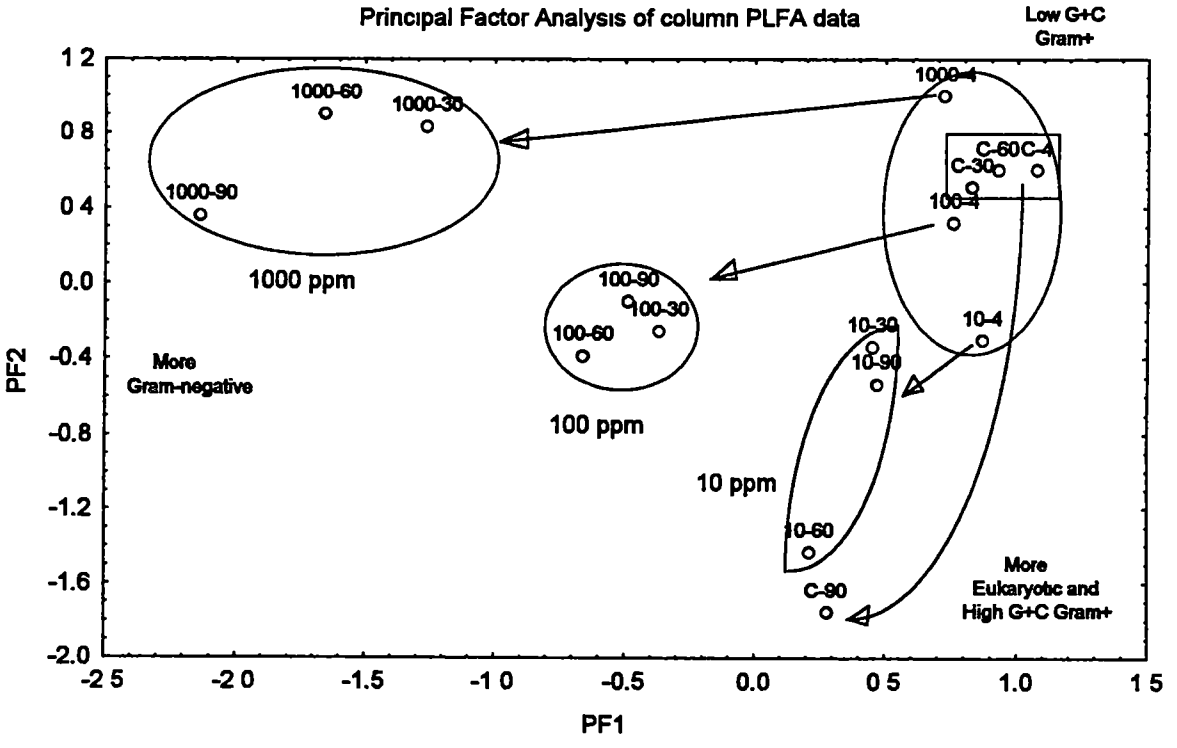

Figure 3-12. Factor analysis of column PLFA data. Variance explained by factors 1 and 2 are 27 and 20% respectively. Each point represents the mean of 4 samples.

Table 3-2. Major components (loading>|0.7|) of the two factors contributing to treatment differences in the columns as determined by principal factor analysis using the arcsin squareroot transformed PLFA mol percent and biomass data. Positive and negative loadings for each factor are given.

Positive Contributors		Negative Contributors	
Variable	Loading	Variable	Loading
Factor 1: Primary effect of pesticide treatment on microbial community			
i17:1w7c	0.71	18:1w7c	-0.93
i17:0	0.72	cy17:0	-0.91
i16:0	0.72	16:0	-0.89
biomass	0.75	16:1w7c	-0.85
br16:0b	0.76	16:1w7t	-0.82
18:1w9c	0.79	18:1w7t	-0.80
a17:0	0.80	24:0	-0.79
10me16:0	0.82		
16:1w5c	0.83		
16:1w9c	0.87		
Factor 2: Effect of time in the control columns			
i15:1w10	0.72	18:0	-0.76
br14:0a	0.80		
14:0	0.84		
i10me14:0	0.86		
i14:0	0.87		

pesticide addition, and positive contributors included mid-chain branched saturates, terminally branched saturates, monounsaturates and total biomass, while negative contributors to factor one were primarily monounsaturates. Factor 2 was attributable to time. Positive contributors to this factor were short mid-chain and terminally branched saturated PLFA; 18:0 was the only negative contributor for factor 2. Constrained ordination or CCA analysis of the PLFAs was essentially identical to the PCA. In regions with high pesticide concentrations 16 and 18 carbon monounsaturated PLFA were the most correlative.

The within-group analysis of the 16 and 18 carbon monounsaturated fatty acids showed a significant increase in both biomass and the relative proportion of 16:1ω7c in the 100 ppm treatment , while cy17:0 showed a decreasing biomass trend with a low point at day 60 (P=0.051) (Figure 3-13). The relative proportion of cy17:0 increased significantly in the first 30 days of the experiment and then leveled off. In the 1000-ppm treatment the biomass level of 16:1ω7c remained constant throughout the 90 day study, while its relative proportion increased significantly. The level of cy17:0 significantly increased in both measures (biomass and relative proportion) over time in the 1000-ppm concentration columns (Figure 3-14).

The 18-carbon fatty acid 18:1ω7c increased significantly in both biomass and relative proportion at 100 ppm, while cy19:0 biomass declined and its relative proportion remained constant (Figure 3-13A). At 1000-ppm biomass

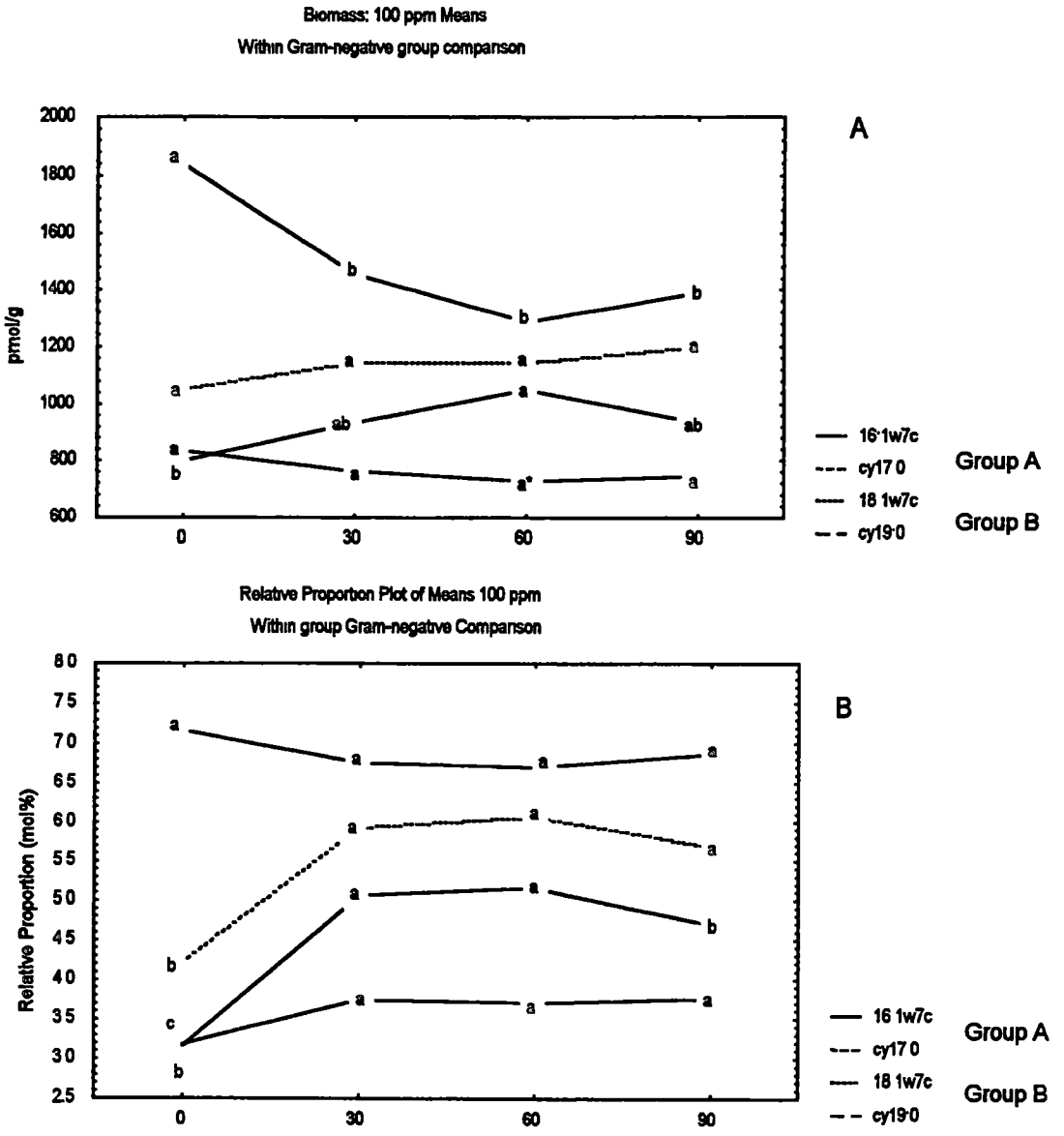

Figure 3-13. A: Biomass levels over time at 100-ppm for 16 and 18 carbon monounsturated PLFAs. B: Relative proportion (mol%) levels over time at 100-ppm for 16 and 18 carbon PLFAs. Within- line same letters are not significantly different according to LSD (α=0.05, n=4). X-axis is measured in days.

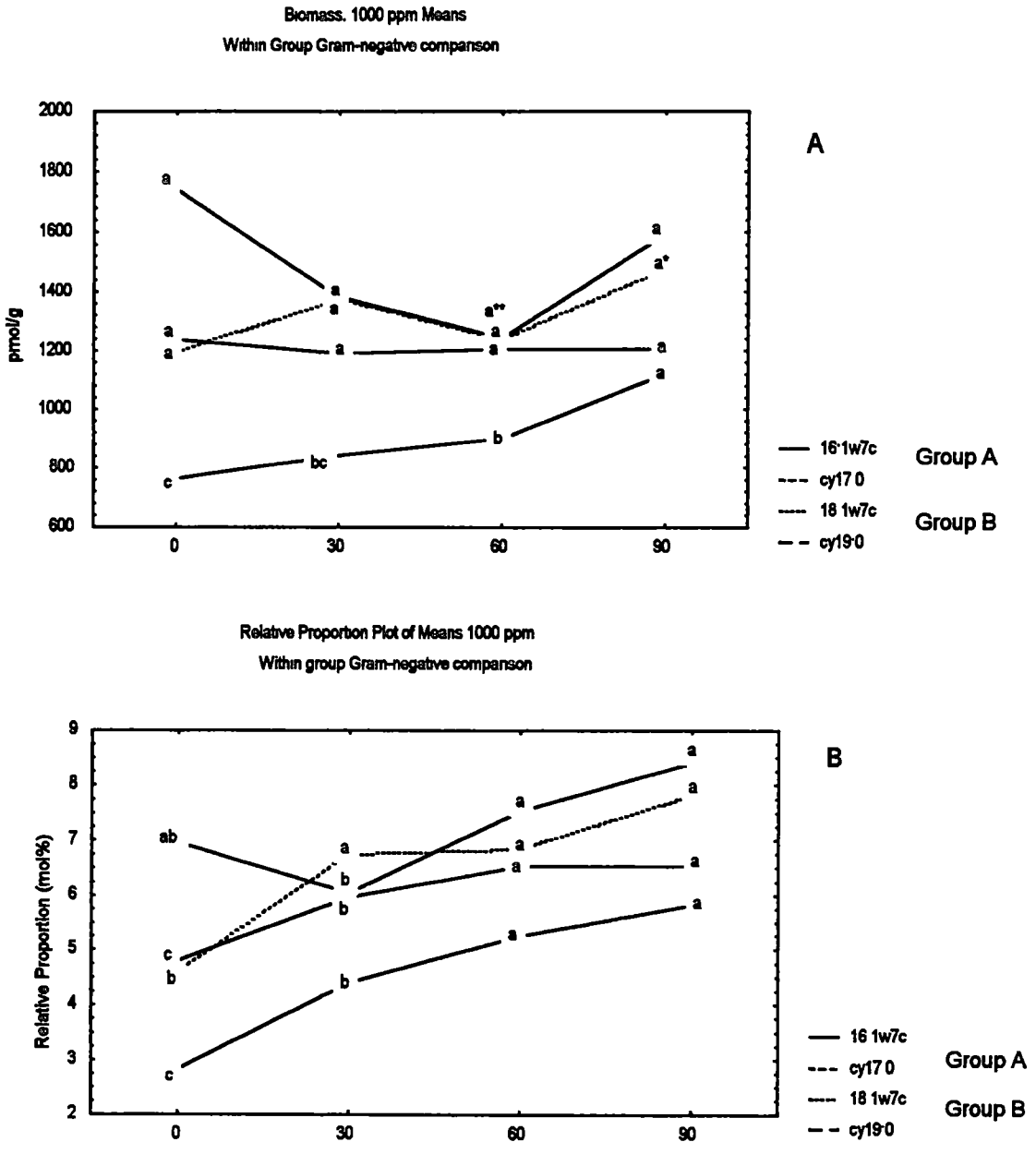

Figure 3-14. **A**: Biomass levels over time at 1000-ppm for 16 and 18 carbon monounsaturated PLFAs. **B**: Relative proportion (mol%) levels over time at 1000-ppm for 16 and 18 carbon PLFAs. Within line same letters are not significantly different according to LSD (α=0.05, n=4). X axis is measured in days.

18:1ω7c increased, as did the relative proportion. Biomass cy19:0 declined between day 4 and day 60 before rising again, and the relative proportion of this fatty acid declined between day 4 and 30, then rose through the rest of the experiment.

Discussion

Control Columns

The flooding of the soil columns increased diffusion of solutes (Campbell, 1977), and lowered osmotic potential and oxygen availability. In the desiccation part of the cycle, diffusion was impeded, and the osmotic potential and oxygen availability was increased. Over the first 60 days of this 5-day cycle, the biomass by PLFA was constant, while biomarkers for eukaryotes decreased. This reduction in eukaryotic biomarkers may indicate a greater sensitivity of these organisms to the wet/dry cycle. However, the factor plot showed that changes in the microbial community over the first 60 days were subtle (Figure 3-12). Bacterial markers for stress and/or starvation (Kieft *et al.*, 1994) were high in the native soil at the beginning of the experiment due to the preparation regime (drying and screening) of the soil for the columns, and did not change significantly over the first 60 days. In this way the native soil community was selected to survive low carbon availability, wetting, and drying.

Adapting to the SBBR water management regime by the soil microbial community required the expenditure of cellular energy, for example to synthesize

osmolytes and exopolysaccharides (Roberson and Firestone, 1992; Brown, 1990). Facultative anaerobes must make use of different enzyme systems under anaerobic versus aerobic conditions. The loss of half of the biomass by PLFA between 60 and 90 days is consistent with the exhaustion of cellular energy substrates, and subsequent cell lysis and degradation of polar lipids (Harvey *et al.*, 1986).

10-ppm Concentration

In this treatment the total soil microbial biomass did not drop over time, but biomarkers for Gram-negative bacteria (Ringelberg *et al.*, 1997), actinomycetes (White *et al.*, 1997), and soil micro-eukaryotes (Zak *et al.*, 1996) did significantly decrease in the first 30 days. In comparison to the control columns it was clear that some of the indigenous soil microorganisms were sensitive to low amounts of pesticides. It is also important to consider that pesticides as they are used in the field include not only active ingredients, but also a mixture of oils, soaps, solvents, and other formulation products that aid in the mixing and dispersal of the PAI. This fact has many implications for the SBBR soil microbes. The surfactants used in the formulated products can disrupt membrane potentials, and contribute to cell damage or lysis. It was not possible to discover if the immediate response of the SBBR microbial community is due to the effect of the PAI or of the associated formulations. It is assumed, however that over time the

chemistry of the PAI will dictate the reaction of the SBBR microbial community and the ultimate fate of the PAI (Hornsby *et al.*, 1996).

There were no changes in ratios indicating metabolic stress, but there was a slow-down in the turnover rate of the Gram-negative portion of the SBBR microbial community. One reason there was no biomass crash in these columns may be due to added substrate via the pesticides. The subsequent slower turnover may be an indication of the exhaustion of that added substrate.

Factor analysis showed that there was a shift in the microbial population at 10 ppm (Figure 3-12), but this shift was subtle in nature and was perhaps more an adaptive response by the indigenous soil microbes rather than wholesale exchange of dominant species (Zogg *et al.*, 1997).

100-ppm Concentration

The addition of pesticides at 100 ppm had a pronounced negative effect on total soil microbial biomass as well as on all individual microbial groups in the first thirty days. Excepting the Gram-positive *Arthrobacter* type bacteria, none of the individual microbial groups recovered the biomass levels they had at day 4. Although there was a significant drop in biomass, there was no rise in the measures of toxicity or slowed turnover rates. In fact the opposite occurred, with a significant rise in the rate of Gram-negative turnover and a drop in the measured metabolic stress (Figure 3-6 through 3-9). Factor analysis showed

that this treatment caused a shift in the microbial community from diverse to one that was more heavily dominated by Gram-negative bacteria.

Analysis of the individual fatty acids 16:1ω7c, 18:1ω7c, cy17:0 and cy19:0 showed that a portion of the Gram-negative community was responding positively to the addition of the pesticides. This conclusion was supported by the data in that the biomass level of 16:1ω7c significantly increased while the level of cy17:0 decreased (P=0.051), and when normalized for biomass the trend for 16:1ω7c was accentuated while cy17:0 showed a slight increase. A more pronounced but similar trend was observed for the ratio of 18:1ω7c to cy19:0. These results could be interpreted as indicating that there were two separate groups of Gram-negative bacteria, group A; containing higher proportions of the 16 carbon fatty acid moiety and group B, with more of the 18 carbon moiety.

1000-ppm Concentration

The total biomass level decreased significantly in the first 30 days of the experiment, a decrease that was mirrored in all specific microbial subgroups (Figures 3-2 through 3-7). However, unlike with the 100-ppm treatment, there was an 11% increase in total biomass between day 60 and 90 that was attributable to the predominantly Gram-negative bacterial biomarkers. Moreover, data from the factor analysis also suggested a community shift toward one that was more Gram-negative in structure. The Gram-positive stress marker showed

a significant rise throughout the time-course, indicating that this portion of the community was impaired by the high concentration of pesticides

The growth phase indicators for the Gram-negative community showed that the previously established group A (bacteria consisting mostly of 16 carbon PLFAs) was inhibited by the high levels of pesticides. This is indicated by the rise in the ratio of cyclopropyl 17:0 to 16:1ω7c (Figure 3-10 and 3-11). The group A metabolic stress indicator also showed a significant increase in the first 30 days of the experiment (Figure 3-8). Conversely the group B (bacteria that contain more 18 carbon PLFAs) ratio of cyclopropyl 19:0 to 18:1ω7c declined in the first 30 days of the experiment, indicating that this group of bacteria were not negatively impacted by pesticide addition at 1000 ppm. In addition, the group B metabolic stress indicator showed no increase in the relative amount of stress to these organisms.

At this level of pesticide contamination, all of the major groups of microorganisms were negatively affected by the addition of the pesticides. A segment of the Gram-negative community which primarily contains the 18:1ω7c pathway, denoted as Gram-negative group B, responded positively to the contaminant, while those that contained the 16:1ω7c pathway were inhibited. The data from the factor analysis shows the most highly loaded variables that correlated with pesticide level were 18:1ω7c (|0.93|) and cy17:0 (|0.91|), confirming the results from the biomass and stress indicators.

Figure 3-15 shows the classic contaminant species response model as presented by Tate (1995). In this model the ultimate importance of any biotic or abiotic trait is its contribution to community stability and resilience, that is, the capacity of the ecosystem (in this case the SBBR) to withstand increasing amounts of PAI. At the low level of contamination only the most sensitive organisms were affected and the accompanying shift in community structure was subtle. As the concentration of the contaminants was increased selection was intensified and a greater proportion of the microbial community was affected. This was observed in the 100-ppm level, where there was a documented rise in a resistant or contaminant degrading consortia of Gram-negative organisms (Figure 3-16). At the highest level of contamination this degrading or resistant consortia was further narrowed to a select group of Gram-negative organisms (Figure 3-17).

Conclusions

- Operation (wet-dry cycle) of the control soil columns caused a substantial decrease in the total biomass of the system due to substrate exhaustion and bacterial desiccation.
- Application of pesticides even at low levels was toxic to certain portions of the soil microbial community, and mortality increased with increased PAI concentration.

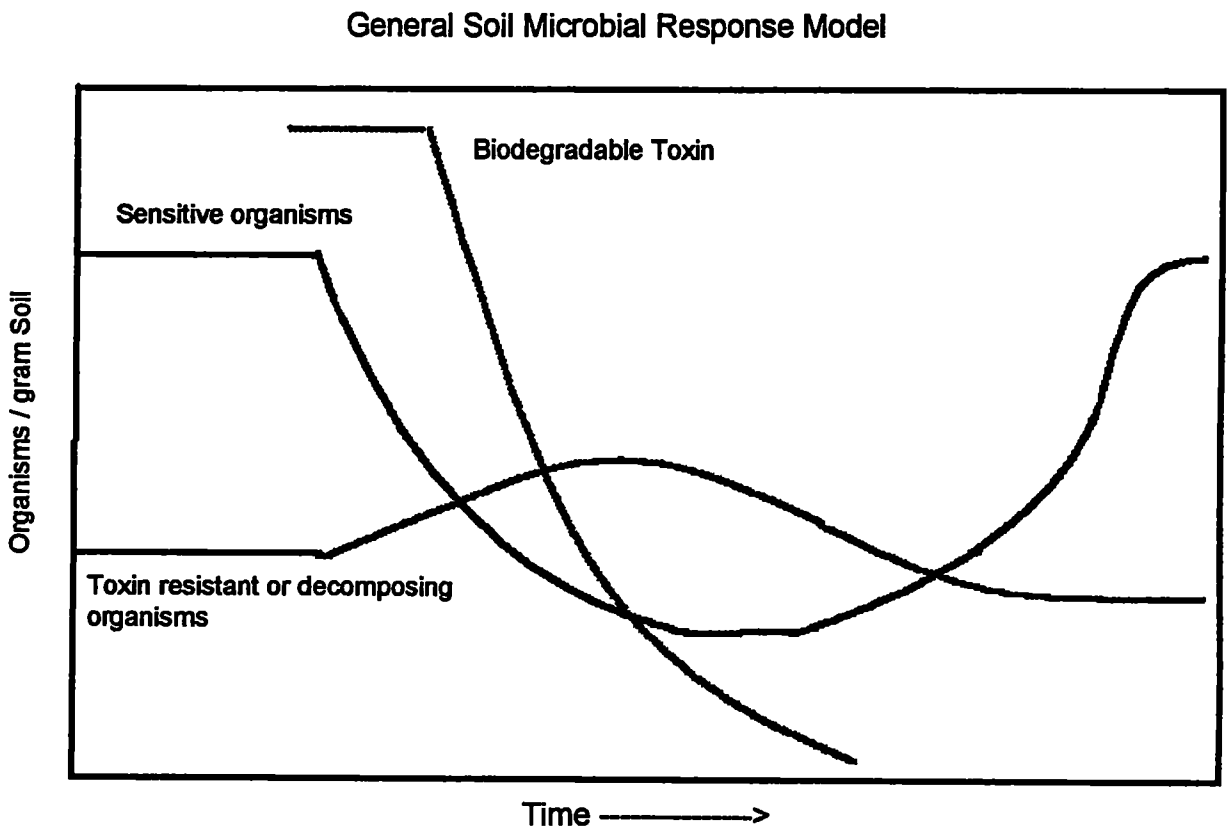

Figure 3-15. Model of classic microbial response to a biodegradable toxin over time adapted from Tate (1995) Sensitive organisms die off and are replaced by resistant and or toxin degrading organisms.

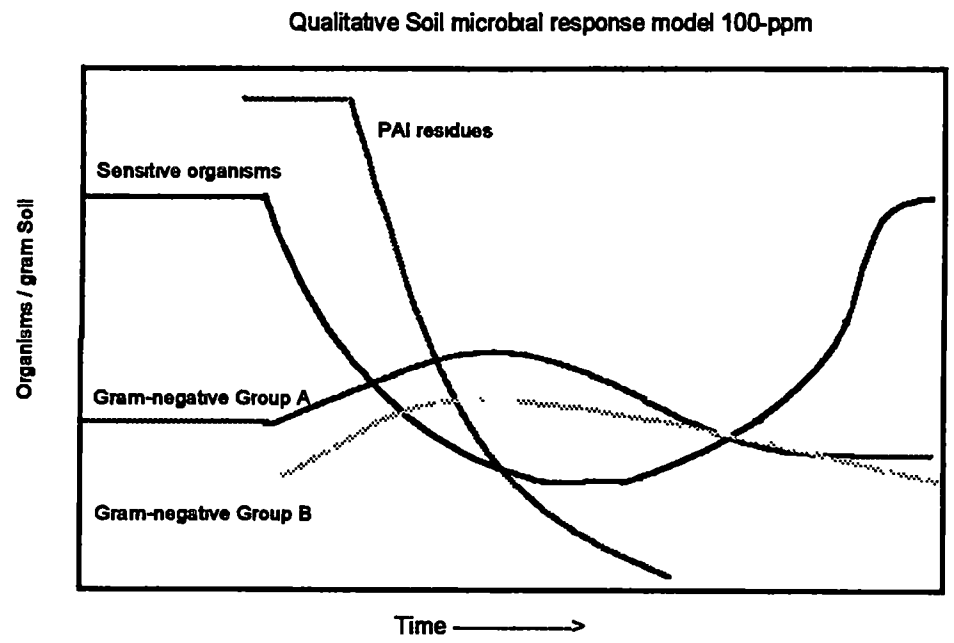

Figure 3-16 Soil microbial response model over time of Gram-negative group A and B organisms at 100-ppm Both sub-populations are able to increase relative percentage and biomass

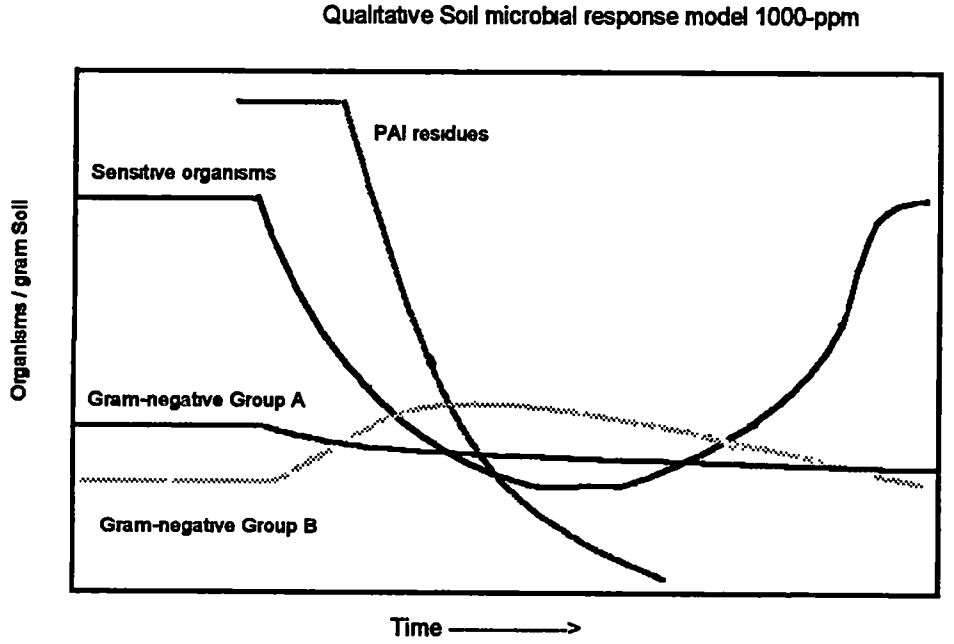

Figure 3-17 Soil microbial response model over time of Gram-negative group A and B organisms at 1000-ppm. At this concentration only the group B sub-population responds positively while group A is inhibited

- Total biomass was not affected at low PAI concentration and was moderately affected at moderate concentrations.
- Higher pesticide concentrations resulted in slower turnover and increased stress in the system.
- Higher pesticide concentrations stimulated a subset of pesticide resistant or degrading microbial organisms.
- At the highest concentrations the subset of resistant or pesticide degrading organisms was narrowed by selection pressures.
- Constrained and unconstrained ordination algorithms agreed with the biomass and relative proportion data and documented the microbial community responses to PAI contamination.

Chapter 4

Community Analysis

Nucleic Acid data

Introduction

As stated in the previous chapter, the ultimate fate of pesticides in the soil bed bioreactor is in large part dependent upon the reactor's soil microbial community. Understanding the impact of pesticide addition and column management on this community is then vital to the ultimate operational efficiency and sustainability of the system. Until recently the tools required to investigate complex systems such as the SBBR have not existed. Largely dependent on advances in molecular procedures and instrumentation, recent developments have led to enormous improvements in the analysis of microbial communities in natural environments.

Nucleic-acid-based molecular biological methods have risen in popularity among scientists interested in assessing the diversity of microbial communities and their responses to environmental change. Polymerase chain reaction (PCR) and facilitated recovery of specific nucleic acid fragments, followed by chromatographic separation of the product on the basis of melting point, provides a "fingerprint" of the microbial community. DGGE (denaturing gradient gel electrophoresis) is one such method and is achieved by forming a gradient of chaotropic agents in the gel, increasing towards the base. The temperature of the

gel is held constant throughout the run. The resultant banding pattern formed in the gel is characteristic of the microbial community from which it originated (Figure 4-1). These patterns can be compared and contrasted between treatments to discover differences and similarities (Muyzer *et al.*, 1993; Stephen et al., 1998).

Sequencing of prominent bands from the gel following excision (Kowalchuk *et al.*, 1997; Stephen *et al.*, 1999; Macnaughton *et al.*, 1999a,b), can reveal the presence of specific groups or can be used to infer the importance of previously unknown organisms in the process under investigation. The sequence of a 16S rDNA molecule from an "unknown" organism can always place it within the framework provided by 16S rDNA sequences of cultured organisms. The strength of the placement is generally dependent on its similarity to known sequences, and the length of the sequence recovered. The framework of known sequences is extensive, due to the efforts of many researchers and culture collections, and is formalized by the efforts of the Ribosomal Database Project (Maidak *et al.*, 1999) at the Center for Microbial Ecology, Michigan State University.

In this experiment PCR of 16s rDNA followed by DGGE was used to asses the impact of pesticide addition and water management on the soil column (simulated SBBR) microbial communities exposed to different levels of pesticide concentrations. A mixture of five herbicides (atrazine, dicamba, fluometuron,

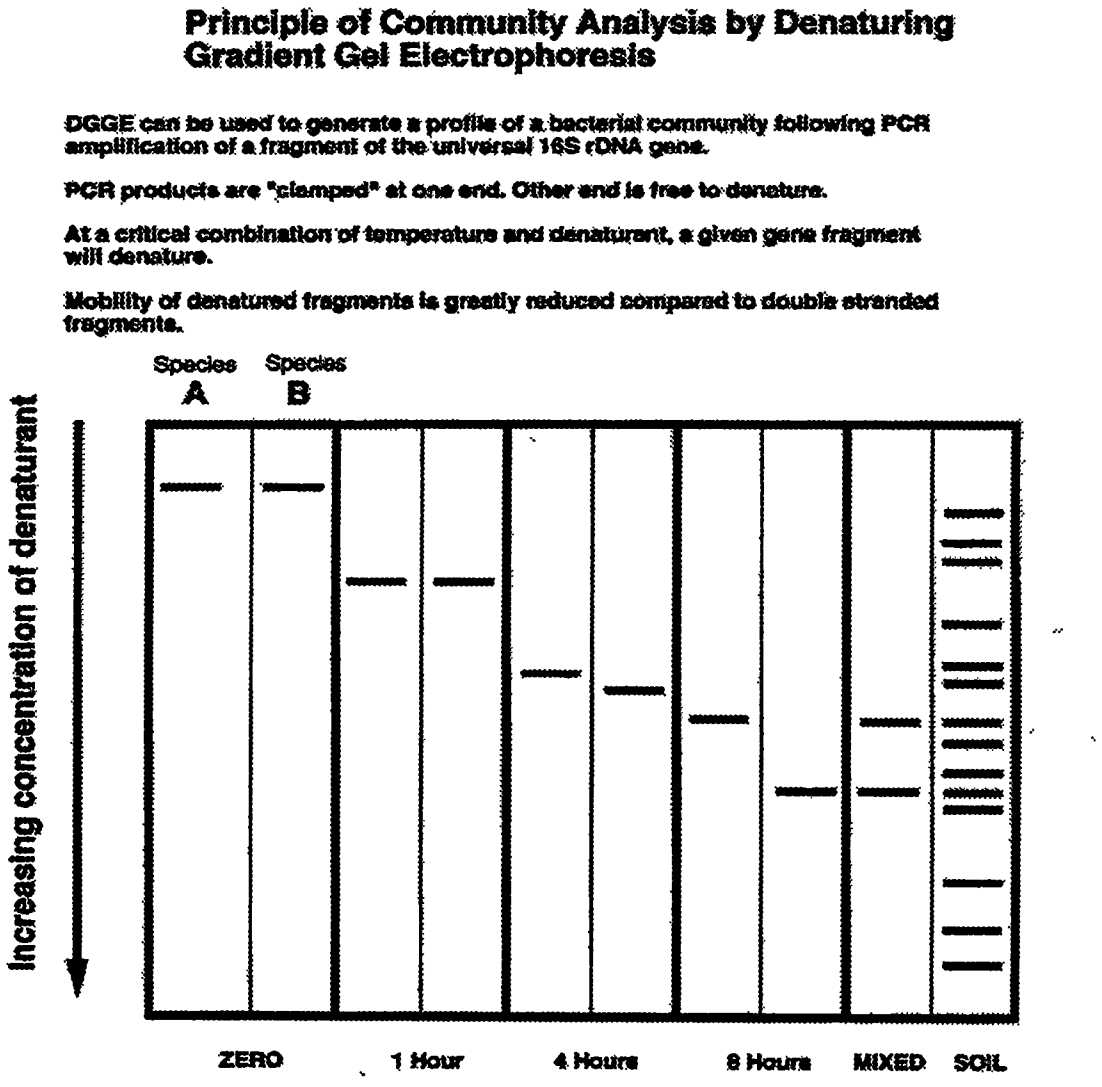

Figure 4-1 Principle of community analysis by DGGE

metolachlor, and sulfentrazone) as formulated products were added in four concentrations each, 0-control, 10 ppm, 100 ppm, and 1000 ppm for a total concentration of 0, 50, 500, and 5000 ppm in each of the respective treatments. Quadruplicate columns were destructively sampled at day 4, 30, 60, and 90. For this analysis triplicate samples from Day 4 and duplicate samples from day 90 for each treatment are presented.

Materials and methods

Mechanical Lysis

DNA extraction proceeded as described by Stephen *et al.* (1999). Soil (0.5 g) was transferred to a 1.5 ml microcentrifuge tube, 425 μl of phosphate buffer was added, and the sample was vortexed briefly. To the centrifuge tube was added 0.5 g of sterile 0.17 mm glass beads and 175 μl of CRSR solution, and again the sample was vortexed briefly. The soil was then placed into a Bio 101 "Fastprep" FP120 shaker for 45 sec at a speed of 6.5 m/s, and then centrifuged for 3 minutes at 13,000 rpm.

DNA extraction

The supernatant from the samples was drawn off and transferred to a sterile 1.5 ml Eppendorf tube and put on ice. Chloroform (300 μl) was added to the tube that contained the glass beads, and the sample was vortexed briefly

then centrifuged again for 3 minutes at 13,000 rpm. The remaining aqueous layer was again removed from the sample and combined with the previous sample. To the tube was added an equal amount of isopropanol, the sample was mixed, and placed on ice for 30 minutes. The samples were again centrifuged for 15 minutes at 13,000 rpm and the resulting supernate discarded, then 1 ml of ethanol (80%) was added to the sample and the sample was vortexed briefly and centrifuged for 5 minutes at 13,000 rpm twice more. After the ethanol wash the remaining ethanol was removed with a pipette and the sample was dried for approximately 25 minutes or until visibly dry.

Once the tubes were dry the pellet was resuspended and dissolved in 100 µl of pH 8.0, 0.25M tris buffer, and 100 µl of Sigma phenol: Chloroform: isoamyl alcohol 25:24:1was added. The samples were then centrifuged for 3 minutes at 13,000 rpm and the upper (aqueous) layer was transferred to a new 1.5 ml Eppendorf tube. This last step was repeated as advised in the Geneclean ® Protocol. The DNA extract was further purified by using the Geneclean ® Spin kit (Bio 101, Inc., Alta Vista, CA) according to the manufacturer's instructions. The final DNA was then eluted into 2 X 50 µl deionized water.

PCR

PCR was performed as described by Muyzer *et al.*, (1993). Thermocycling consisted of 35 cycles of 92°C for 45s, 55°C for 30s and 68°C for 45s, using 1.25 units of Expand HF polymerase (Boehringer) and 10 pmol each of the primers

described by Muyzer *et al.* (1993) (the forward primer carried the 40 bp GC clamp required for DGGE) in a total volume of 25 μl. Thermocycling was performed using a 'Robocycler' PCR block (Stratagene). The primers targeted eubacterial 16S regions corresponding to *Escherichia coli* positions 341-534 (Brosius *et al.*, 1981). A portion (20%) of each PCR product was analyzed by agarose gel electrophoresis (1.5% agarose, 1x TAE buffer) and ethidium bromide flourescence. The amount of DNA used for DGGE analysis was standardized to 600 ng by comparison with molecular weight standards (1kb+ ladder; Gibco BRL) using ALPHA IMAGER ® software (Alpha Innotech).

DGGE analysis

DGGE analysis was performed using a D-code 16/16 cm gel system with 1.5 mm gel width (Bio-Rad) maintained at a constant temperature of 60 °C in 6L of 0.5x TEA buffer (20 mM Tris acetate, 0.5 mM EDTA, pH 8.0). Gradients were formed between 20% and 55% denaturant with 100% denaturant defined as 7M urea plus 40% (v/v) formamide. Gels were run at 35V for 16 h. Gels were stained in purified water (Milli-Ro; Millipore) containing ethidium bromide (0.5 mgl^{-1}) and destained twice in 0.5x TAE for 15 min each. Images were captured using ALPHA IMAGER ® software (Alpha Innotech).

Extraction of DNA from acrylamide gels

The central 1mm^2 portion of strong DGGE bands were excised using a razor blade and soaked in 50 μl of purified water (Milli-Ro; Millipore) overnight at 4°C. A portion (15 μl) was removed and used as the template in a PCR as above. The products were purified by electrophoresis through a 1.2% agarose/TAE gel followed by glass-milk extraction (Geneclean kit; BIO 101).

Cloning of PCR-amplified products

Amplification of products that failed to generate legible sequences directly were cloned into the PCR-TOPO 2.1 cloning vector (Invitrogen) according to the manufacturer's instructions. Recombinant (white) colonies were screened by a two-stage procedure to ensure recovery of the DGGE band of interest. First, plasmid inserts (n=12 for each band) were reamplified by PCR using vector specific primers (M13 reverse and T7; Invitrogen). The products were digested with restriction endonuclease *Msp*I and analyzed by agarose electrophoresis (2% agarose, 1x TAE buffer). Two products from each digestion pattern group were reamplified using the 16S-specific PCR primers described above (Muyzer *et al.*, 1993) and subjected to DGGE analysis to select the sequences that co-migrated with the original band of interest. Sequences that were of high frequency in clone libraries (as defined by digestion pattern) and co-migrated with the original environmental band were selected for sequence analysis (two clones per band) and used as probes in confirmatory membrane hybridization analysis.

Sequence analysis

PCR products from excised bands and cloned products were sequenced using the primer 516r (GWATTACCGCGGCKGCTG; W=A or T, K=G or T; Lane *et al.*, 1985) and an ABI-prism model 373 automatic sequencer with dye terminators (Perkin-Elmer). Sequences were compared with the Genbank database using the Blast N facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov). Sequences were classified using the RDP release of 31 July 1998 (Maidak *et al.*, 1997).

Results

DGGE analysis of bacterial microbial diversity

Triplicate samples from day 4 and duplicate samples from day 90 were processed for each pesticide concentration and analyzed by PCR- DGGE of 16s rDNA. The day 90 control and 10-ppm samples and all samples from day 4 carried complex communities in which few major bands were observed (Figure 4-2). Analysis of the 90-day samples contaminated at 100 and 1000-ppm showed strong and reproducible banding (Figure 4-2).

Five sequences were recovered from the 100-ppm samples (Figure 4-2). Table 4-1 presents the results and compares the sequences with databases.

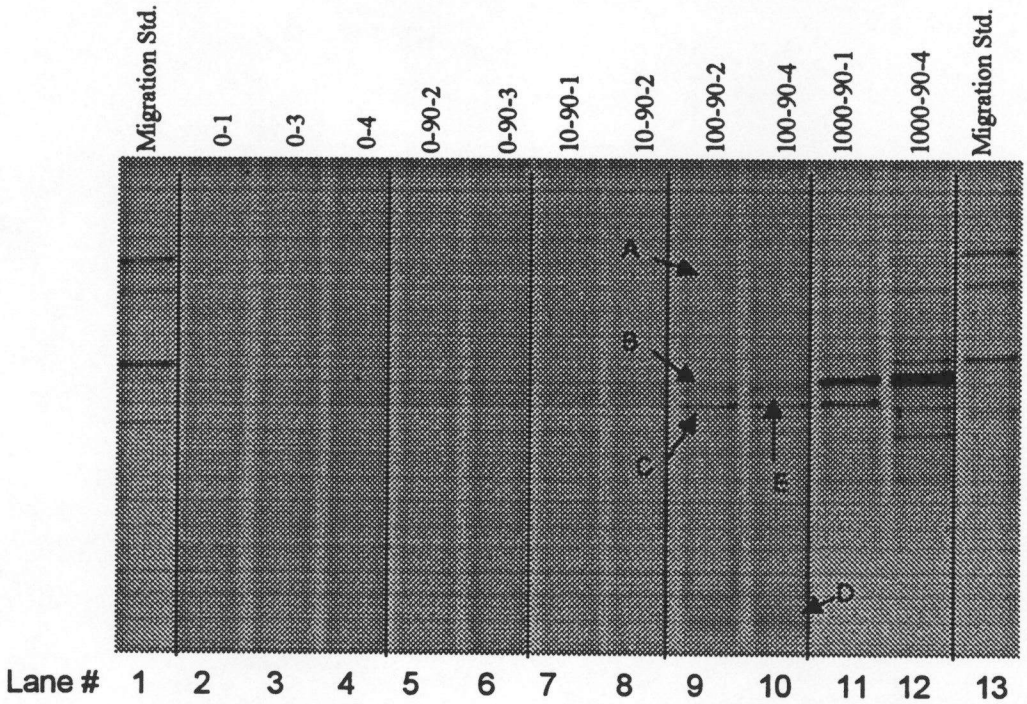

Figure 4-2. DGGE bacterial community profile of soil from the SBBR. Lanes 2-4 day 0 control, lanes 5-6 day 90 control, lanes 7-8 day 90 10-ppm, lanes 9-10 day 90 100-ppm, and lanes 11-12 day 90 1000-ppm. Lanes 1 and 13 are migration standards. The gel shows community profile changes from day 0 to 90 in the 100 and 1000-ppm samples. The letters represent bands identified in Table IV-C1

Table 4-1 Comparison of sequences derived from DGGE bands with databases.

Band	clone # (Closest match)	Where found
A	K20-64	Metal Contaminated Super-fund site
B	WR1113	Metabolically active members in PCB cont Moorland soil
C	*Burkholderia sp*. LB400	Biphenyl and PCB degrader
D	SJA-172 (*Syntrophobacter wolinii*)	As part of an anaerobic trichlorobenzene degrading consortia
E	SJA-68 (*Dehalococcoides ethenogenes*)	As part of an anaerobic trichlorobenzene degrading consortia

References: Band A (unpublished), Band B (Nogales *et al* , 1999), Band C (Lau and Bergon Submitted), Band D (von Wintzingerode *et al*., 1999), and band E (von Wintzingerode *et al*., 1999)

There were eleven bands excised from the 1000-ppm sample lanes. Sequence analysis showed that all of the bands were very similar and were all part of the genus *Eneterobacteriaceae*. All bands had a better than 98% homology with *Pantoea agglomerans*, a facultative anaerobe capable of dissimilatory metal reduction and the degradation of complex organic matter (Francis *et al.*, 2000).

Discussion

Control and 10-ppm treatments

There was no shift in banding pattern during the experiment for the control and 10-ppm samples. These sample lanes (2-8) were basically a smear, and are the result of diverse community profiles in which no specific genus was dominant. It is possible that at low pesticide concentration the soil may buffer any negative effect caused by the applied herbicides (Welp and Brummer, 1999), and that the resultant selection pressure is not strong enough to support or cause a shift in the phylogenetic community structure that can be detected by DGGE.

100-ppm treatment

Five sequences were recovered from the DGGE gel. Two of the clones (SJA-68 and SJA-172) are members of an anaerobic trichlorobenzene-degrading consortium (von Wintzingerode *et al.*, 1999) used in a fluidized bed reactor that had been seeded with Saale river sediment (Norwalk *et al.*, 1996). It is important to note that soil used for this column study was collected just 6 meters away from

the Tennessee river at The University of Tennessee Agricultural experiment station. This may indicate that these organisms are fairly ubiquitous in certain environments. SJA-68 showed a loose affiliation with *Dehalococcoides ethenogenes*, an anaerobic Green nonsulfur bacterium that is capable of the complete reduction of PCE and TCE to ethene gas (Maymó-Gatell *et al.*, 1997). Clone SJA-172 is a close relative of *Syntrophobacter wolinii*, a Gram-negative rod described by Boone and Bryant (1980). *Syntrophobacter wolinii* is capable of degrading propionate in coculture with an H_2-using organism and in the absence of light and electron acceptors such as O_2, sulfate, or nitrate. This organism was originally isolated from an anaerobic sewage digester.

Another of these sequences, *Burkholderia sp.* LB400, is a Gram-negative facultatively anaerobic prototype biphenyl and PCB degrading organism (Lau and Bergeron, unpublished). *Burkholderia sp.* have been shown to degrade many types of pollutants and have also been shown to have a tolerance for heavy metal contamination (Macnaughton *et al.*, 1998). Clone WR1113 was originally found as a metabolically active member of a PCB polluted moorland soil (Nogales *et al.*, 1999). Sequence K20-64 was first recorded at a metal-contaminated superfund site by Marsh (unpublished); this site was contaminated by predominantly inorganic materials (Macnaughton pers. comm.).

Bacterial isolates which closely resemble the clone sequences have two traits in common. They were detected in anaerobic environments, and they are all capable of reductive dechlorination. This suggests an explanation for the

presence of these microbes, in that they may be using the pesticides as a substrate or a terminal electron acceptor. This would be consistent with the pesticides used; of the five, three (atrazine, dicamba, and sulfentazone) have chlorinated ring structures, one has a chlorine terminal group (metolachlor) and the last (fluometuron) consists of a fluorinated ring structure. Stamper and Tuovinen (1998) have suggested that metolachlor and other acetanilide herbicides may be initially broken down by reductive dechlorination, in a process that proceeds by the formation of glutathione-acetanilide conjugates.

1000-ppm treatment

At this pesticide concentration the microbial community was dominated by members of the genus *Enterobacteriaceae*. All eleven excised bands were homologous to *Pantoea agglomerans*. This organism is a Gram-negative facultative anaerobe capable of decomposition of complex organic matter, and has been shown to be able to use several terminal electron acceptors in anoxic systems (Francis *et al.*, 2000). It is unclear why this organism so dominated the community. The evidence suggests, however that it was the only bacterial genus that could survive at this level of pesticide concentration. This data also agrees with the PLFA data from chapter 3 that indicated a strong rise in the level of Gram-negative bacterial biomass.

Conclusions

- The phylogenetic community structure was diverse and changes were not detectable by DGGE in the control or low-level pesticide contaminated columns.

- The phylogenetic community structure shifted in the moderately contaminated columns from one that was diverse to one dominated by a consortium of sequences that are closely related to anaerobic dechlorinators.

- At the highest level of contamination the soil microbial community was dominated by a single genus *Enterobacteriaceae*.

Chapter 5

Neural Network Analysis of PLFA data

Introduction

In order to further investigate the SBBR microbial community response to the addition of pesticides a Neural Network analysis was used. Neural networks (NN) have been defined as the mathematical models represented by a collection of simple computational units interlinked by a system of connections (Cheng and Titterington, 1994). The number of these units can be very large and the connections intricate. Neural networks are rapidly becoming the most important tools in bioinformatics research and have been applied to a wide variety of biological data such as clustering like samples (Gyllenberg and Koski, 1995), and genomic sequence analysis (Baldi and Brunak, 1998). Neural networks resemble the brain in a couple of ways; first, knowledge is acquired by the network through a learning process, and second, interlinked connection strengths known as synaptic weights are used to store knowledge (Haykin, 1994). Once a network of unit processors is assembled it is trained by changing the connection weights through back-propagation of predictive errors. The resulting solution is regularized to ensure its general nature, and the previous steps are repeated through the domain of NN topologies until the optimal extraction of signal versus noise is found.

The ability of NN to identify and produce models without the need for mechanistic assumptions and which closely fit complex non-linear data is ideally suited for soil microbial assemblages. If properly applied the resulting model may distinguish signal from noise in associations among an arbitrary number of variables. In one case, neural networks were used with PLFA to detect the effects of rising atmospheric CO_2 on soil microbial assemblages associated with White Oak, and produced a more interpretable signal than did conventional multivariate statistics (Ringelberg *et al.*, 1997). PLFA data has been analyzed successfully by NN to map changes in the soil microbial community signaling bioremediation (Almeida *et al.*, 1998). Most recently, NN have been used as an aid to interpreting PLFA profiles of natural microbial communities (Noble *et al.*, 2000).

In an effort to further characterize microbial community response to the addition of pesticides, a NN analysis was applied to PLFA profiles from pesticide contaminated soil columns. The output from the resulting model was used in conjunction with other linear models to elucidate the impact of pesticides on the soil microbial community. The NN was also used to predict pesticide concentration in the soil.

Materials and Methods

Data for Neural Network

Neural network analyses was performed and optimized as established by Noble *et al.*, (2000). Briefly, the complete input data consisted of 16 PLFA profiles, each as a mean of the four replicates from each time. Each PLFA profile consisted of 62 fatty acids. The concentration of each PLFA was rescaled, with a minimum value of the data being set at 0.1 and a maximum value of 0.9 to improve scalability of NN training (Masters, 1993). NN analysis was performed for each pesticide individually, i.e., a separate NN was developed to associate the PLFA profile and each corresponding pesticide concentration.

Sensitivity analysis

The relative importance of each input parameter to predict the target values was calculated by performing sensitivity analysis on the trained NN (Masters, 1993). In this study, sensitivity of an output parameter $Out_{j=1,2,...nj}$ (there are nj output parameters) to an input parameter $In_{i=1,2,...ni}$ (there are ni input parameters) was defined as the normalized ratio between variations caused in Out_j by variations introduced in In_j and is represented by the following equation:

$$NS_{i,jc} = (dOut_{j,c} / d\ In_{i,c})(In_{i,c} / Out_{j,c})$$
$$S_i = [\ \Sigma_{j=1,2,\ldots nj;\ c=1,2,\ldots nc}\ (NS_{i,jc})\ ] / [\ \Sigma_{i=1,2,\ldots ni,\ j=1,2,\ldots nj;\ c=1,2,\ldots nc}\ (NS_{i,jc})\ ]$$

(eq. 1)

$i = 1, 2, \ldots ni$; input index
$j = 1, 2, \ldots nj$; output index

c= *1, 2, ... nc*; sample (case) index

The normalized sensitivity for an individual profile c, NS_{ijc} was calculated for every single combination of input, i, and output parameters, j, and for every single profile (there are *nc* profiles). The overall sensitivity to an input, S_i, was determined by taking the average over all profiles and all binary outputs used to classify them. Finally, the sensitivity values obtained are represented as relative values, calculated as a percent value of the sum of all sensitivities (Eq1, S_i).

Multivariate statistical analysis

Multiple regression analysis was performed using PLFAs with cumulative sensitivities greater than 0.04 for each individual pesticide (sum of all sensitivities is 1). This was repeated for each pesticide with all PLFAs that had sensitivities above 0.04. Principal factor analysis of arcsin-transformed mol% data was performed using principal components as the extraction method and varimax rotation.

Statistical software

The NN and sensitivity analysis software were implemented with BrainCel version 3.0 (Promised Land Tech.) and Matlab version 5.2 equipped with the NN toolbox (Math Works Inc.). For the NN analysis, the logsig equation logsig(x) = $1/(1+e^{-x})$, was used as a transfer function, and error back-propagation was used to optimize the connection weights. Full interconnection between the layers was

used. Learning and momentum rates are self-adjusting in Mat-lab and BrainCel. The input and output architecture consisted of 62 input neurons representing the 62 PLFA's and one output neuron for the predicted concentration of pesticide. For training 90% of the data was used, and then the result was validated with the other 10%. In order to minimize the effective number of degrees of freedom in the network, training was stopped when the error measured with independent test data started to increase (Bishop, 1995). This criterion was used to select the optimum number of hidden neurons.

The multiple regression analysis was performed using SAS (SAS institute Cary, NC), and the factor analysis by Statistica (StatSoft Inc., Tulsa OK).

Results

The ability of the NN analysis to predict the amount of contamination varied according to the particular pesticide. The r^2 for atrazine was 0.999; for dicamba, 0.906; fluometuron, 0.997; metolachlor, 0.985; and for sulfentrazone, 0.840 (Figure 5-1). Only 12 fatty acids were observed with sensitivities (what a particular variable is contributing to the NN weights) over 0.04 (refer to Table 5-1). The choice of a sensitivity signal of 0.04 was based on the relative noise of the background sensitivities, since a value of 0.04 was determined to be above the noise threshold.

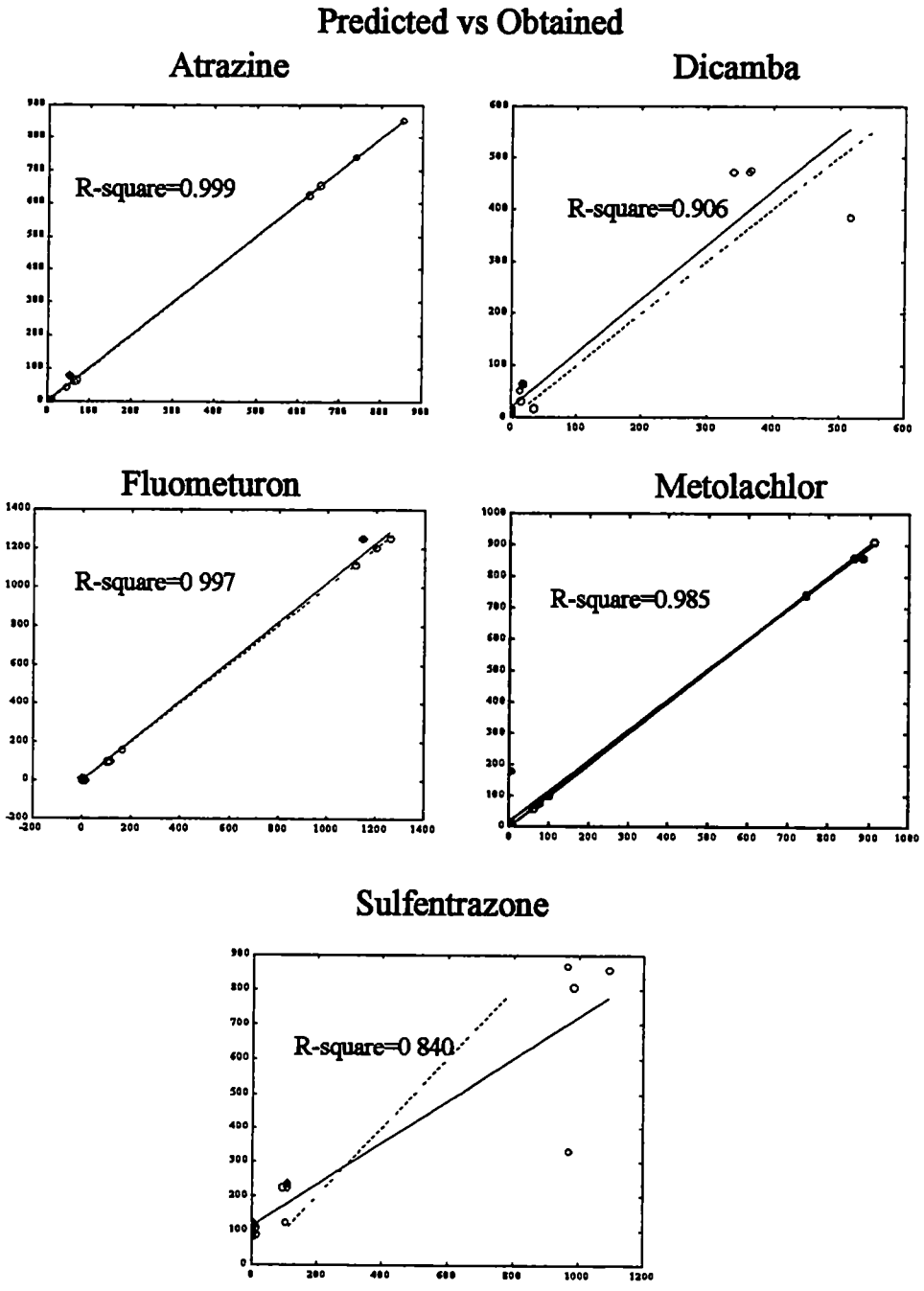

Figure 5-1. Predicted vs obtained values (ppm) for a trained Neural Network to predict pesticide concentration.

Table 5-1. Sensitivity analysis of PLFAs.

Fatty Acid	Atrazine	Dicamba	Fluometuron	Metolachlor	Sulfentrazone
17:0				0.061	
18:0					0.062
i10me15:0	0.1		0.043		
i10me16:0	0.098		0.049		0.062
a10me16:0					0.058
i10me18:0		0.04			
br16:0b		0.04			
10me16:0			0.057		
10me17:0	0.045			0.045	0.085
i16:0		0.059			
i17:0		0.057	0.049		
a17:0			0.045		

Cummulative sensitivities for each pesticide equal 1.

Multiple regression analysis

Each individual pesticide produced a suite of PLFAs that generated sensitivity indicia above 0.04. The most sensitive PLFAs from the NN analysis were used as variables in a multiple regression to ascertain if the PLFAs had an inherent linear quality, and to discover if the model was able to predict pesticide concentration as well as the NN. The table 5-2 shows the r^2 values and significant ($P \leq 0.05$) PLFAs for the model when all sensitive PLFAs were used. Table 5-3 shows the r^2 values and significant ($P \leq 0.05$) PLFAs when only those PLFAs specific to a certain pesticide were used.

Discussion

The performance or ability of the NN to predict the level of contamination in this study was dependent on the behavior of the pesticide used and the quality of the input data. The dicamba chemical data had several zero or near-zero values, which possibly skewed the predicted values from the NN. Sulfentrazone showed no dissipation, actually increasing (due to experimental error or de-sorption) in all concentrations slightly over time. Although there was not complete agreement, the lowest r^2 value was 0.840, large enough to produce useful prediction (Saxton per. comm.)

Of most interest in this analysis are the sensitivities of the individual PLFAs. This information provides a non-linear estimation of how important each individual fatty acid is in relation to the concentration of pesticides. In statistical

Table 5-2. R-square values and significant (at $p \leq 0.05$) PLFAs from MRA. In this analysis all PLFAs with sensitivities greater than 0.04 for any pesticide were included in the model.

	Atrazine	Dicamba	Fluometuron	Metolachlor	Sulfentrazone
r-square	0.77	0.73	0.82	0.8	0 82
			Significant PLFAs		
17:0			X	X	
18:0					
i10me15:0					
i10me16:0					
a10me16:0					
i10me18:0					
br16:0b	X	X	X	X	X
10me16:0					
10me17:0	X			X	
i16:0					
i17:0	X	X	X	X	X
a17:0					

Table 5-3. R-square values and significant (at $p \leq 0.05$) PLFAs from MRA. In this analysis only PLFAs with sensitivities greater than 0.04 for the specific pesticide were included in the model.

	Atrazine	Dicamba	Fluometuron	Metolachlor	Sulfentrazone
r-square	0.54	0.64	0.74	0.42	0.61
			Significant PLFAs		
17:0				X	
18:0					
i10me15:0			X		
i10me16:0	X		X		X
a10me16:0	X				X
i10me18:0					
br16:0b		X			
10me16:0					
10me17:0				X	
i16:0		X			
i17:0		X	X		
a17:0					

terms this can be thought of as a variable weight or variance explained. In this case only 12 PLFAs produced a sensitivity score above 0.04 (Table 5-1). The sensitive fatty acids could likely be from the *actinomycetes* or other high G+C Gram-positive bacteria (Holt *et. al.*, 1994). Two lines of evidence would support this. First, the fatty acid signature is typical of this group of soil bacteria, i.e., 10me16:0, 10me17:0, i16:0, i17:0 and a17:0 (Holt *et. al.*, 1994). Secondly, if the normal saturates 17:0 and 18:0 (ubiquitous) are excluded, the remaining iso and antiiso methyl branched fatty acids are proposed *actinomycetal* or Gram-positive stress markers (Ringelberg per.comm.).

Data from the multiple regression analysis showed that when all of the most sensitive PLFAs are used, an adequate linear model can be generated. In addition, two PLFAs (br16:0b and i17:0) were significant contributors in every pesticide model. It was also observed that only PLFAs indicative of community change were significant in the model, while those associated with metabolic stress were not. When only the sensitive PLFAs from a particular pesticide were used however, the predictive ability of the model was hampered. This information allowed an inspection of the character of the data. It appears that as a group these PLFAs (or the group of microorganisms represented by these PLFAs) reacted in a somewhat linear fashion to the addition of pesticides; in this case, the higher the concentration the greater the impact. However, the assessment of the impact of individual pesticides on particular PLFAs appeared to be more nonlinear in nature.

One possibility is that the NN analysis may have been detecting the stress response of the soil *actinomycetal* or Gram-positive community induced by the addition of pesticides, whereas the Multiple regression analysis was only able to detect differences in community structure. Previous linear ANOVA analysis showed a significant increase in the proportion of i17:1ω7c/i10me16:0, a ratio that has been shown to increase in contaminated soil and is attributable to the *actinomycetal* population (White, unpublished data). In addition, previous linear PCA analysis produced ten highly negatively correlated variables due to pesticide addition (fatty acids negatively impacted by pesticide addition). Half of these ten fatty acids were also the most sensitive (most important) in the NN analysis.

The analysis of compositional data is a zero sum. If one part of the microbial community is able to cope with a stress better than others, the relative proportion of that community is increased at the expense of the remaining microbiota. In this case it appears that the NN analysis was able to detect fatty acids representing a portion of the soil microbial community, possibly the *actinomycetes* or other high G+C Gram-positive bacteria, that were negatively impacted by the addition of pesticides. This conclusion was supported by factor analysis, ANOVA, and multiple regression. The factor analysis attempted to explain all of the variance in the data, and the multiple regression the variance from all independent to dependent variables. The NN was able to parse a portion of the data that could explain the impacts of pesticide addition and be

used to predict concentration. By performing the NN analysis, confidence in the previous analyses was enhanced and a better understanding of the soil microbial community response to the impacts pesticide addition was achieved.

Conclusions

- Neural Network analysis was able to use PLFAs to predict pesticide concentration.
- Overall Gram-positive and *actinomycte* response to pesticides appeared to be linear while stress response was relatively non-linear.
- Neural Network analysis was supported by other linear data analysis techniques and provided a "closer examination" of the PLFA data.

Chapter 6

Summary

This study was designed to gain a better understanding of the operational capability, performance, and stability of the soil bed bioreactor. This was accomplished by using soil columns to simulate a functioning SBBR under differing amounts and types of pesticides. Pesticide dissipation, soil chemistry, and contaminant impact on the SBBR microbial community were of prime interest. A suite of chemical and biological assays (Figure 6-1) were performed, and the resultant data used to address research objectives. Specific research objectives were set forth as follows:

1. To evaluate the dissipation of commonly-used East Tennessee herbicides as formulated products in a batch waste-stream.
2. To assess the impact of the addition of insecticide formulated products on the batch waste-stream.
3. To explore the effects of high pesticide levels on the SBBR.
4. To define and correlate the microbial community biomass, structure and metabolic status with contaminant level and dissipation.
5. To investigate the general soil chemistry of the system at the established parameters.
6. To compare the results from this study with previous research by Corwin and Glover.

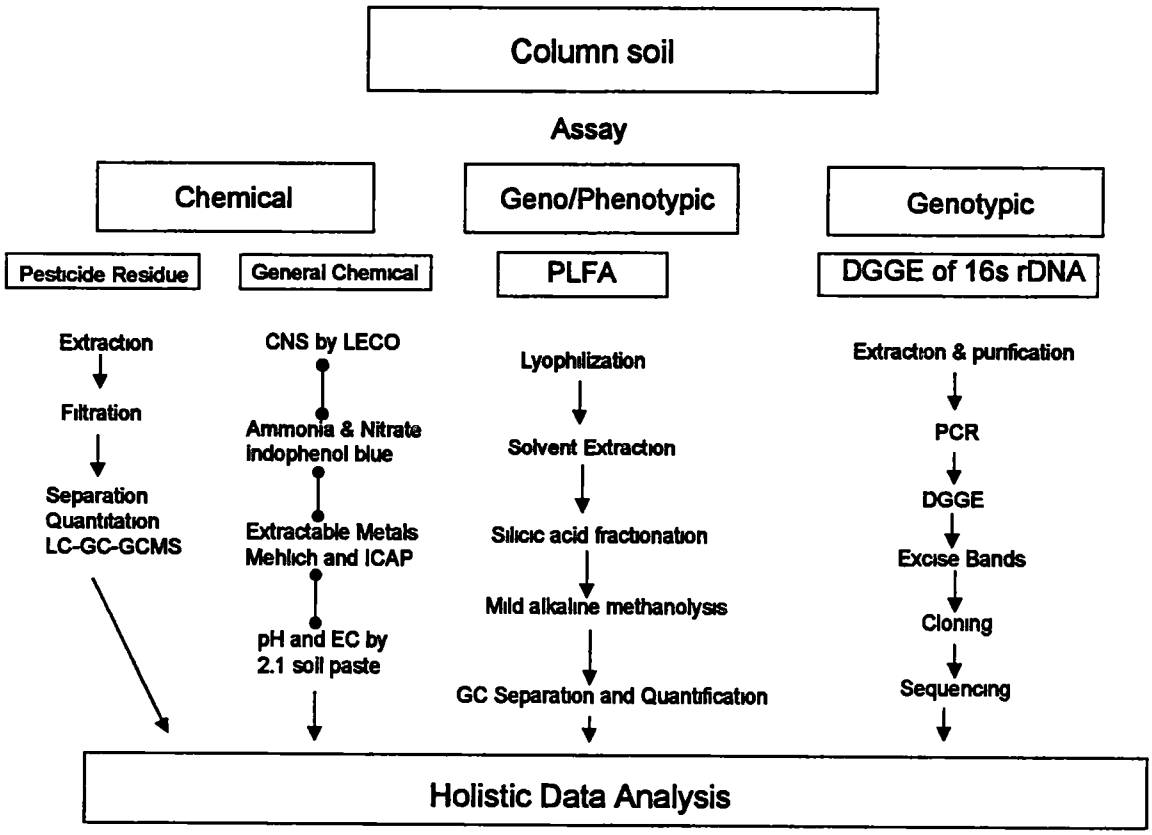

Figure 6-1 column soil assay plan

Dissipation

Four of the five added herbicides showed significant dissipation at low concentrations. Sulfentrazone was the only compound that did not dissipate at any concentration. This study showed that the addition of insecticides did not significantly impact the performance of the SBBR. Moreover, the dissipation rates from the SBBR were comparable to field rates in all cases, and thus it may be possible to use published field dissipation rates as a guide for how a given pesticide may behave in the SBBR. As the concentration of pesticides in the SBBR was increased the ability of the system to dissipate the pesticides was slowed, leading to the conclusion that an upper operating limit of somewhere under 1000 ppm total pesticide concentration should be recommended.

At 10 ppm, where an appropriate comparison could be made, the dissipation results from Corwin and Glover were consistent with those achieved in this study. This is important in that this study used formulated pesticides while in the former studies technical active ingredients were used.

Soil Chemistry

There were two main effects on the column soil chemistry due to the experimental design. These effects were correlated and could be described by a factor analysis. The main effect of pesticide addition was to increase the soil carbon, nitrogen, and sodium content. The other effect described the process of reduction that developed in the system over time. The reduced conditions in the

columns may be a cause for concern since many pesticides are more readily dissipated in aerobic systems. However, the reduced conditions in the SBBR did not appear to slow dissipation. The results of this data were also supported by the DGGE analysis that showed a rise in anaerobic bacteria.

SBBR Microbiology

PLFA analysis of the SBBR microbial community detected a loss of biomass at the lowest pesticide concentration, and this loss was more pronounced with higher concentrations. In addition, as the concentration of pesticides in the system increased, the metabolic stress in the microbial community increased. This was monitored through linear and nonlinear techniques. Increasing the pesticide concentration also magnified selection pressure on the microbial community and resulted in a shift of community assemblage. Figures 6-2 and 3 illustrate the response of the microbial community. At the100-ppm concentration, PLFA and DGGE analyses indicated an increase in an anaerobic dechlorinating consortia of microorganisms (Table 6-1). One hypothesis would be that this microbial consortia was using the chlorinated pesticides as either a substrate or terminal electron acceptor. This sort of phenomena has been documented in several cases in soil microbial ecology, and is the basis for natural attenuation and bioremediation of biodegradable pollutants (Tate, 1995). At 1000 ppm the selection pressure on the microbial community was increased and only *Eneterobacteriaceae* were detected. This genus has been shown to be capable of reductive dechlorination,

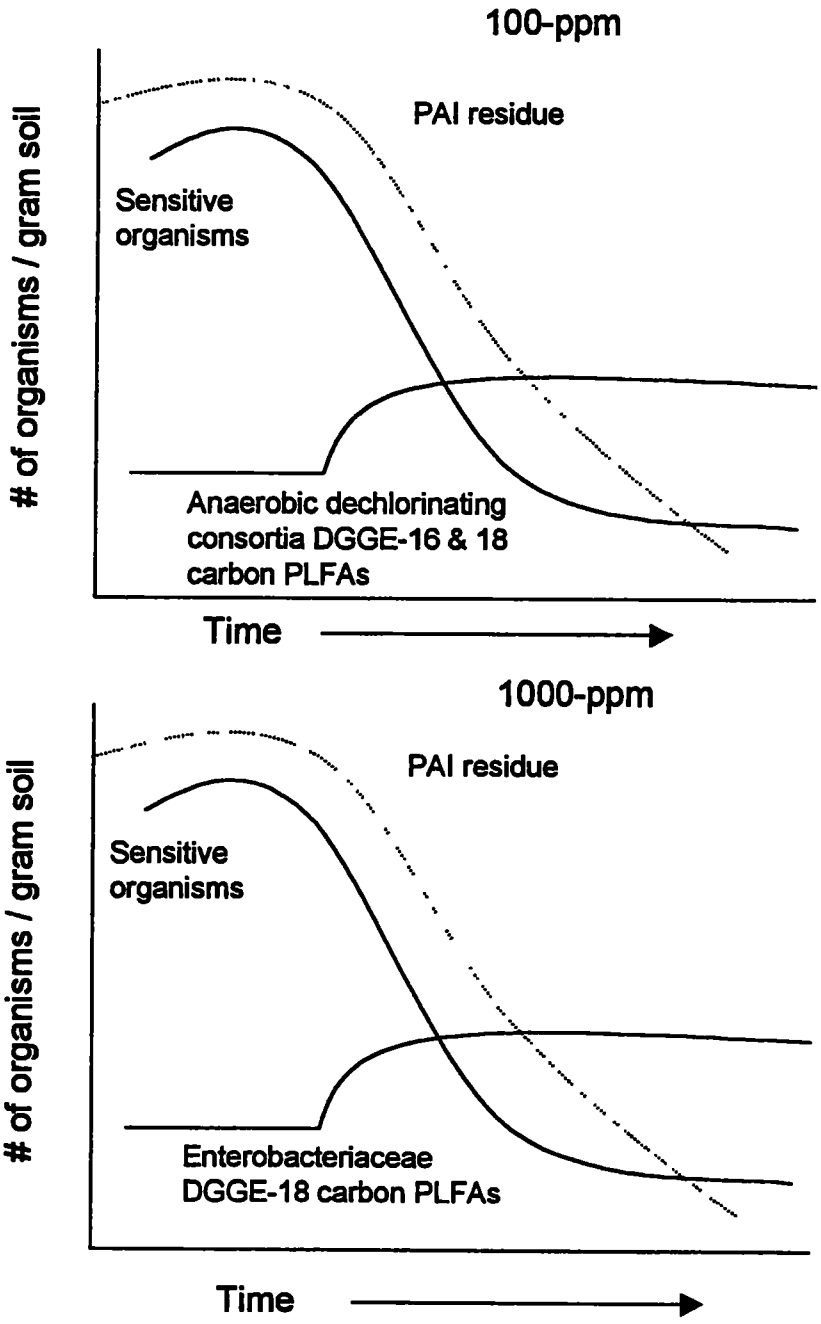

Figure 6-2. Qualitative microbial community structure models for 100 and 1000-ppm pesticide concentrations.

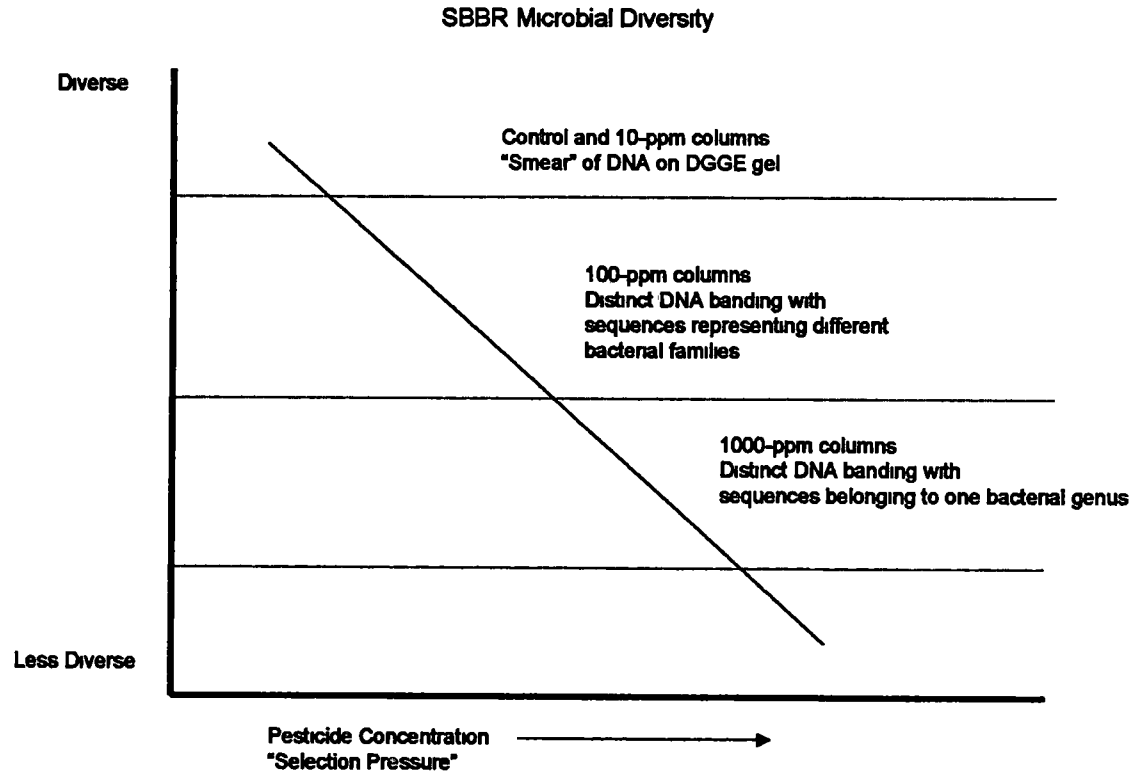

Figure 6-3 SBBR microbial diversity model.

and in addition the recovered sequences show a close relationship to facultative anaerobes which are capable of the decomposition of complex organic compounds (Francis *et al.*, 2000), among which some of these pesticides may be included.

The chemical, PLFA, and DNA data were complimentary and confirmatory. These analyses used in tandem provided a way to investigate the SBBR soil (the ultimate matrix) response to the addition and eventual dissipation of pesticide wastes.

These data lead to an important conclusion: if pesticides are added to the SBBR in sufficient quantities the microbial community will shift to one that may be resistant to the pesticides and ultimately detoxify them. If this is indeed the case, then the SBBR design and management plan put forth by researchers in department of Agriculture and Biosystems Engineering should be considered validated and the next phase begun.

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Vita

Aaron Dean Peacock was born on April 6, 1966 in Auburn, California. In 1984 he graduated from Delta High School in Delta, Utah. In 1987 Aaron married Atina Eve Thomas. He currently has two children, Emerson 10, and Aislyn 3. In the fall of 1997 he was awarded a Bachelor of Science Degree in Agriculture from The University of Tennessee Knoxville.

He returned to The University of Tennessee in 1998 and in May of 2000 was awarded a Master of Science degree in Biosystems Engineering Technology.