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A LABORATORY STUDY ON THE DEVELOPMENT AND TESTING OF A BIOAUGMENTATION SYSTEM FOR CONTAMINATED SOILS

By

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A MICROBIAL BIOAUGMENTATION TECHNIQUE FOR CONTAMINATED SOILS

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ABSTRACT

Ph.D. Reza Mehmannavaz **Agricultural and Biosystems Engineering**

The primary objective of this study was to investigate the use of water table management (WTM) as a microbial delivery system for in-situ bioaugmentation of contaminated soils. In addition, the use of Rhizobium (*R.*) for PCB degradation in soils was evaluated.

First, the presence and isolation of a variety of strains of *Rhizobium meliloti* was demonstrated using plant nodulation tests on alfalfa plants in soils that were contaminated for over 15 years with PCBs, PAHs and heavy metals. Next, *R. meliloti,* strain A-025, was selected based on its membrane (hydrophobicity, adhesion) characteristics and its potential to transform PCBs. This strain was delivered and implanted in soil columns, 200 mm in diameter x 1000 mm in length, packed with a sandy loam soil, using surface and subirrigation. The results of this study showed that subirrigation led to a higher number and a more uniform distribution of the bacterial cells in the soil at 60, 300, 500, and 700 mm depths, than surface irrigation.

In a different setup, similar columns were packed with a PCB contaminated soil. These soil columns were bioaugmented with three bacterial cultures, i.e., *R. meliloti* (strain A-025), *Comomonas testosteroni* (strain B-356) and an indigenous bacterial consortium using subirrigation. The results indicated that bioaugmentation of the PCB contaminated soil was possible by using subirrigation. Bioaugmentation with the indigenous culture was observed to be

more effective in the biodegradation of PCBs than with A-025 and B-356 cultures at 140 and 340 mm depths. However, at 590 mm depth, bioaugmentation with strain A-025 was observed to be better than the other treatments. Sequential aerobic and anaerobic cycles appear to be of significance for effective dechlorination of PCB congeners to lower chlorinated congeners.

In a separate exploratory study, the rhizospheric effects of alfalfa plants on *R. meliloti* for PCB depletion were investigated. The results suggest that the growth of alfalfa plants and bioaugmentation of soil with *R. meliloti,* strain A-025, increased the depletion of PCB congeners in the soil as compared to bioaugmentation alone. In other preliminary studies, the results showed that the presence of PCBs in a sandy loam soil increases the filtration of bacterial cells. Also, soil type and the presence of PCBs affected water infiltration, moisture, and hardness of the soil. Furthermore, water table management system along with bioaugmentation of soil columns with *R. meliloti,* strain A-025, decreased the concentration of atrazine by 31% during anaerobic and aerobic cycles and reduced the concentration of nitrate by 87% and 78% in the absence and presence of atrazine, respectively, in the drainage water.

The overall results of this work indicate that water table management (subirrigation) can be used for bioaugmentation of contaminated soils. Also, use of *R. meliloti* may prove to be an interesting option for soils contaminated with PCBs, atrazine and nitrate.

RÉSUMÉ

Mehmannavaz Reza, Ph.D. **Génie agricole et biosystèmes**

L'objectif principal de ce travail est d'étudier l'utilisation du système d'irrigation souterrain comme un moyen d'apport de bactéries en vu d'une bioaugmentation des sols contaminés et d'évaluer le potentiel de dégradation des BPC par le Rhizobium. En utilisant le test de nodulation, la présence et l'isolement de souches différentes de *Rhizobium meliloti* à partir de sols contaminés pendant plus de 15 ans avec les hydrocarbones aromatiques et chloro-aromatiques et les métaux lourds ont été démontrés.

La souche A-025 de *R. meliloti* a été sélectionnée de la collection des isolats bactériens en se basant sur les caractéristiques de sa membrane (hydrophobie et adhésion) et sa capacité de dégrader les BPC. Cette souche a été amendée aux colonnes de sol par irrigation de surface ou par subirrigation. Cette étude a été réalisée dans des colonnes en acier ayant un diamètre de 200 mm et une longueur de 1000 mm et qui ont été remplis d'un sol sablo-limoneux . Les résultats de cette étude ont montré que la subirrigation a donné un nombre plus élevé de cellules bactériennes avec une distrubution plus uniforme mesurée à différents niveaux de profondeur du sol : 60, 300, 500 et 700 mm.

Dans une autre expérience, les mêmes colonnes ont été remplis par un sol contaminé par des BPC. Le sol contenu dans ces colonnes a été bioaugmenté en utilisant : la souche A-025 de *R. meliloti*, la souche B-356 de *Comomonas testosteroni* et une microflore bactérienne indigène (un consortium de bactéries indigènes). Il a été démontré que la bioaugmentation avec la microflore indigène

était plus efficace dans la biodégradation des BPC qu'avec A-025 et B-356 aux profondeurs de 140 et 340 mm. Cependant, à 590 mm de profondeur, la souche A-025 a présentée une meilleure bioaugmentation par rapport aux autres traitements. Des cycles alternés aérobiques et anaérobiques ont été démontrés essentiels pour une déchlorination efficace des congénères de BPC pour donner des produits moins chlorinés.

Dans une étude préliminaire, l'effet de la rhizosphère des plantes de luzerne sur l'activité de dégradation des BPC par *R. meliloti* a été étudié. Les résultats ont indiqué que la présence des plantes de luzerne a participé à l'augmentation de la disparition des BPC dans le sol bioaugmenté par la souche A-025 en comparaison à un traitement bioaugmenté uniquement par la même souche. D'autres études préliminaires ont montré que la présence du BPC dans un sol sablo-limoneux augmente la filtration des cellules bactériennes plus que dans un sol argileux. D'autre part, le type de sol et la présence de contaminants affectent l'infiltration de l'eau, le taux d'humidité et la dureté du sol. D'autre part, le système d'irrigation souterrain utilisé pour la bioaugmentation avec *R. meliloti* (A-025) a permis une diminution de la concentration de l'atrazine à plus de 31% durant des cycles aérobiques et anaérobiques et de 87% et de 78% de la concentration des nitrates en présence et en absence d'atrazine respectivement.

En conclusion, on peut dire que le système d'irrigation souterrain pourrait être utilisé pour la bioaugmentation avec *R. meliloti* des sols contaminés pour contrôler des polluants environnementaux comme le BPC, l'atrazine et le nitrate.

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LIST OF SYMBOLES AND ABBREVIATIONS

BP	Biphenyl
B.	Bradyrhizobium
C.	Comomonas
cfu	Colony forming units
E.	Escherichia
ECD	Electron capture detector
GC	Gas chromatography
N	Nitrogen
NA	Nitrate & atrazine
ND	not determined
P	plants
PAH	Polyaromatic hydrocarbons
PB	Plant & bacteria
PCB	Polychlorinated biphenyl
R.	Rhizobium
SAC	Surface active compounds
SD	Standard deviation
SDW	Sterilized deionized water
WFPS	Water filled pore space
WTM	Water table management

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The following is a statement from the faculty of Graduate Studies and Research concerning manuscript-based theses:

"Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

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Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscript co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the

accuracy of such statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for the thesis."

CONTRIBUTIONS TO KNOWLEDGE

Within the scope of the thesis, the following contributions to knowledge have been made:

1. A new and effective method for bioaugmentation of soils has been developed. The method uses a water table management system to deliver and distribute bacterial cells with upward water flow (subirrigation) in a soil profile. This method was compared to the more traditional bioaugmentation method, surface irrigation. The results indicated that subirrigation provided significantly better implantation of bacteria than surface irrigation in soil columns.
2. Different species of Rhizobium were isolated from soils that were contaminated for over 15 years with PCBs, PAHs, and heavy metals. This indicates that these organisms exist and can tolerate and survive in such contaminated areas. Furthermore, hydrophobicity and adhesion of these cells seem to be different than in non-rhizobial strains.
3. Two of the isolated *Rhizobium meliloti*, strains A-025 and A-029, seem to dechlorinate more chlorinated PCB congeners in Aroclor 1242 to lower chlorinated congeners. The survival of the bioaugmented strains and dechlorination (biodegradation) of Aroclor 1242 congeners were enhanced in presence of $CaCO_3$. Aerobic followed by anaerobic conditions with *R. meliloti* strain A-025 after 388 days, showed that biodegradation of peaks 54 (congeners 106, 123, 149), 37 (congener 74), 30 (congeners 37, 42), and 17

(congener 26) seem to be possible only under anaerobic conditions by this strain.

4. The possible creation of subsequent aerobic and anaerobic conditions seem to allow dechlorination of the highly chlorinated PCB congeners into lower chlorinated congeners in all three treatments bioaugmented with bacterial cultures, *R. meliloti* and *C. testosteroni*, and by the indigenous organisms. PCB dechlorination patterns were different at 140, 340, and 590mm depths and for each bacterial cultures used to bioaugment the soil.
5. Growth of alfalfa plants and bioaugmentation of the soil with *R. meliloti*, strain A-025, increased the depletion of PCB congeners in the soil as compared to bioaugmentation alone. Growth of alfalfa plants can be an effective method in the removal of PCBs in a contaminated soil.
6. Bioaugmentation of PCB contaminated soils with *R. meliloti* strain A-025 resulted in the hardening of the soil. The PCB contamination of a sandy soil seems to affect the transport of bacteria more than the clay content of the soil by increasing the filtration of bacterial cells. However, the surface infiltration of water was influenced more by the clay content than by the soil PCB content.
7. Bioaugmentation may be used as a biofiltration system for agricultural pollutants. Bioaugmentation of soil columns with *R. meliloti*, strain A-025, decreased the concentration of atrazine up to 31% during anaerobic and aerobic cycles and reduced the concentration of nitrate up to 87% and 78% in

absence and presence of atrazine, respectively, during a saturated period (anaerobic) in the drain water.

CHAPTER 1

INTRODUCTION

1.1 STATEMENT AND NATURE OF THE PROBLEM

Today's societies generate a large amount of waste annually. In the last few decades, a large amount of hazardous organic waste such as polychlorated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) has been released in terrestrial ecosystems. This has resulted in widespread contamination of ecosystems, particularly soil systems (Shann and Boyle, 1994). The USA produces 300 million metric tons of hazardous waste each year, or approximately 1.3 metric tons per capita. This includes wastes from households, various industries including the food, textile, lumber, petroleum, chemical, and transportation (Bollag and Bollag, 1995). Bollag and Bollag (1995) estimated that the chemical industry alone generates over 5 million tons of waste and more than half of these chemicals are released into the ecosystem.

Since many organic contaminants such as PCBs are carcinogenic, recalcitrant, and tend to bioaccumulate, their presence in the environment poses a significant hazard to human health (Shann and Boyle, 1994). While releases of such chemicals have serious environmental impacts on their immediate surroundings, many more ecotoxicological problems might not be foreseen and people are likely to be affected by today's spills in the distant future. Growing public awareness of the impacts of toxicants released into the environment has created political pressure to minimize the problem. With today's technology and

sensitive analytical tools, the regulatory agencies are able to demand more efficient and stringent cleaning of contaminated sites. Some traditional methods such as excavation and shipment of hazardous waste sites are being viewed as merely relocation and not as a solution. For instance, incineration of PCB contaminated soils removes the wastes, but disposal of ash or residues remains a problem and new concerns over air pollution are created. Society has therefore turned to technology to develop better methods of disposal. One such method is bioremediation, in which microorganisms are used to biodegrade or biotransform, and thus detoxify wastes and minimize the environmental impact.

Although bioremediation has been successfully used for decades to treat waste water, the application of this technique to soil and groundwater pollution control is fairly new (Bollag and Bollag, 1995). It was the grounding of the Exxon Valdez during which Exxon and the US Environmental Protection Agency, through a Technology Transfer Cooperative Agreement, began a research program to determine whether bioremediation could play a role in the cleanup of the spill site. Initial experiments confirmed the presence of oil-degrading microorganisms at the accidental site, so the focus of the research was on biostimulatory remediation and no "non-indigenous bacteria" were used in the cleanup. The effort to remediate the oil spill included fertilizer applications to accelerate natural microbial degradation of the oil. In this case, indigenous hydrocarbon-degrading species were present in relatively high concentrations, and fertilization alone stimulated populations five to tenfold (Forsyth et al., 1995; Prince, 1992; Turco and Sadowsky, 1995).

Conventional soil remediation technologies, which will be discussed in Chapter two, are costly, unpredictable, and usually insufficient. In recent years, land treatment approaches that increase natural biodegradation rates have been investigated. A number of these techniques involve ex-situ treatments where the contaminated soil must be brought to the cleanup site, thus, increasing the cost and the environmental impact of the treatment. Alternatively, in-situ remediation has been attempted in different ways, including both biostimulation of the indigenous microbial population by adding nutrients and/or bioaugmentation of the contaminated site with degrading microorganisms.

The in-situ cleanup of contaminated soil has met with varying degrees of success (Shann and Boyle, 1994). There is still a lack of knowledge regarding the functioning of complex microbial communities. Little is known about the environmental conditions required for the optimal functioning of a microbial ecosystem or about interactions between microorganisms (Verstraete and Top, 1992).

Even though research in bioremediation technology has expanded rapidly over the past several years and bioremediation has been accepted as an effective means for decontamination of soils, bioaugmentation faces a few problems. Due to the novelty of the technology, it is not viewed as a positive alternative by the public and regulatory agencies because bioaugmentation requires inoculation of a contaminated site with non-indigenous microorganism. Also, basic research is still needed to better understand the biological, chemical, and physical factors affecting bioaugmentation and survival of the inoculants. However, bioaugmentation of

sites contaminated with complex contaminants (such as those with PCBs) is necessary. Because PCBs seem to require different groups of organisms for their complete degradation and since it is possible that the indigenous microorganisms may not have the ability to degrade such chemicals, bioaugmentation could serve as an alternative to biostimulation in such situations.

There is no evidence of bioaugmentation for in-situ treatment of contaminated soils. A major problem for in-situ bioaugmentation of contaminated soils is that delivery of the desired microorganism to depths greater than 0.5 m has not met with much success. The more traditional method for bioaugmentation has been to inoculate a contaminated site at the soil surface, and either plow and turn the soil or use surface irrigation to inoculate the deeper zones. Both methods have shown to be costly, time consuming and not very efficient. We propose an alternative to these traditional methods based on water table management (WTM) technology.

In particular, we forward a hypothesis that a water table management system could deliver bacteria efficiently and uniformly throughout the soil profile and maintain optimal conditions in the subsoil, thereby enhancing microbial activity and consequently improving in-situ bioremediation of contaminated soils. This WTM based bioaugmentation system will be designed and tested to simulate field conditions for distributing bacterial cells and creating conditions conducive to optimal growth conditions to enhance microbial metabolism of PCBs in the subsoil.

1.2 OBJECTIVES

The overall objective of this study was to develop a biological pollution control system for contaminated soils, using subirrigation as the delivery system to bioaugment and, consequently, to evaluate the biotransformation of PCBs in a contaminated soil. More specifically, the objectives were to:

1. Evaluate the use of a water table management system for in-situ bioaugmentation and consequently biodegradation of PCBs in a contaminated soil,
2. Isolate indigenous *Rhizobium meliloti* from soils contaminated with PCBs and investigate the hydrophobicity and adhesion of these bacterial cells as parameters in order to select an appropriate strain to be used with our system, and
3. Evaluate the ability of *R. meliloti* in biotransformetion of PCBs in a sterile soil microcosm and in weathered contaminated soil.

The preliminary tests for bacterial delivery and transport were carried out using columns packed with a sterilized sandy loam soil. This study confirmed the use of subirrigation in the delivery and implantation of bacteria in the soil. Therefore, a subirrigation system was used to deliver *R. meliloti* (strain A-025) and *C. testosteroni* (strain B-356) and an isolated uncharacterized microflora into different locations of columns packed with a PCB contaminated soil. Saturated (anaerobic) and unsaturated (aerobic) conditions were created in the soil in a cyclical manner to enhance the biotransformation of PCB congeners.

The rhizobial strain, A-025, was isolated from a soil contaminated with PCBs, PAHs, and heavy metals. It was selected to be used in the transport study based on its membrane characteristics (hydrophobicity and adhesion to soil). It was also used with another newly isolated *R. meliloti*, strain A-029, and compared to a laboratory strain of *R. meliloti*, strain Zb57, for PCB biotransformation in sterile soils contaminated artificially with Aroclor 1242 in a soil microcosm study.

Water table management has proven to be an effective method for biostimulatory remediation (Ugwuegbu, 1997). As part of the previous research done in our laboratory, subirrigation was used to deliver nutrients and air to biostimulate the indigenous microbial population and thus enhance the bioremediation of a soil contaminated with nitrate and diesel. In the present study, water table management delivery system has been extended to distribute bacteria into the subsoil.

1.3 THESIS ORGANIZATION

The general introduction and literature related to the aforementioned objectives are presented in chapters 1 and 2. The results of this research are reported in Chapters 3 – 6 in paper format with connecting texts, while chapter 7 includes the general summary and conclusions and Chapter 8 includes the recommendations for future research. For the sake of completeness, some sections in various chapters are repetitive.

Chapter 3, titled "Isolation and characterization of symbiotic N_2-fixing *Rhizobium meliloti*", describes how different strains of Rhizobia were isolated and

tested for their hydrophobicity and adhesion to a sandy soil. Both of these characteristics of bacterial cells influence the fate of inoculants and are therefore important factors to consider in bioaugmentation of soils by an irrigation system.

Based on the results of Chapter 3, one of the newly isolated strains (A-025) was selected. It was used to test and compare subirrigation with surface irrigation as bacterial cell delivery techniques in a sandy soil as described in Chapter 4, titled "Comparison of surface and subsurface irrigation as microbial delivery tools in soil columns". In addition, two of the strains (A-025 and A-029) were tested for their ability to biodegrade PCBs in sterilized soil microcosms contaminated with Aroclor 1242. This study is described and discussed in Chapter 5, titled "Biotransformation of Aroclor 1242 by three implanted *Rhizobium meliloti* strains in sterile soil microcosms".

The WTM (subirrigation) system described in Chapter 4 was used to bioaugment a PCB contaminated soil in a column study. Three different bacterial cultures (*Rhizobium*, *Comomonas*, and an Indigenous microflora) were used to bioaugment the soil columns. Dechlorination and biotransformation of PCBs were compared for the different bacterial cultures. This study is discussed in Chapter 6, titled "Water Table Management in biotransformation of PCBs by bioaugmentation with *Rhizobium*, *Comomonas* and an Indigenous Cultures".

Appendix A describes four preliminary studies that relate to the use of bioaugmentation, WTM, Rhizobium, and PCBs. Because the primary goal of this research project was to develop a bioaugmentation system for contaminated soils,

these four studies may help in the further development of this technology. These studies were performed to: 1) Investigate microbial diversity in contaminated soils and during a bioremediation period; 2) Investigate the rhizospheric effects of alfalfa plants on *R. meliloti* in PCB biotransformation; 3) Investigate the effect of contaminants and bioaugmentation on soil-water properties; and 4) Investigate whether bioaugmentation using a water table management system and Rhizobia could be used to control agricultural pollution. These studies are presented in Appendix A as follows: A1) "Change in bacterial population density and diversity in contaminated soils and in a bioremediation treatment"; A2) "Rhizospheric effects of alfalfa on biotransformation of PCBs in a soil bioaugmented with *Rhizobium meliloti*"; A3) "Microbial transport, water infiltration, moisture loss, and hardness in clay, sandy loam, and PCB contaminated sandy loam soils"; and A4) "Biofiltration of atrazine and nitrate by *Rhizobium meliloti* A-025 using a water table management system".

1.4 SCOPE OF THE PROJECT

In this study, a water table management system was used to enhance bioremediation through bioaugmentation in soil columns packed with a PCB contaminated soil. It involved a subirrigation system to deliver two species of bacteria and a mixed microbial population, and to provide aerobic and anaerobic conditions at different locations and depths of soil columns contaminated with PCB. As a whole, this research includes many different experiments. The results

of each experiment apply to the conditions and parameters used in that study. Therefore, generalization of these experiments and their results should be done with care, and particular attention must be given to the type of soil used and the type of contaminants and organisms present in the soil. The use of *Rhizobium meliloti* in bioremediation is novel. Therefore, further investigations are needed to better understand the role of Rhizobium in bioremediation technologies.

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CHAPTER 2

LITERATURE REVIEW

This dissertation is based on a multidisciplinary approach that includes microbiology, soil and water engineering, and chemistry. Because the main objective of this research is to develop a system for bioaugmentation of contaminated soils, it is important to understand the fate and behavior of pollutants and microorganisms in soils. It is also equally important to understand how the organisms can play a role in biodegradation of pollutants such as PCBs. Therefore, this chapter is divided into six major sections. It covers the literature on: fate of chemicals and microorganisms in soils, bioremediation methods and biodegradation of PCBs, use of Rhizobium in bioremediation, transport of bacteria by water in soil, and the general remarks.

2.1 SOIL STRUCTURE, MICROBIOLOGY AND FATE OF CHEMICALS

Soils vary widely with regard to geology, hydrology, climate, fertility, and physical attributes. The geophysiochemical properties of soil are important in determining the fate of a contaminant in a soil profile (Huysman and Verstraete, 1993). Soils play an important role in attenuating the toxic effects of a contaminant through binding and sorption properties and also in providing a solid, physical support to help protect and stabilize microorganisms and their cellular components. As shown in Figure 2.1a, soil is an irregular mixture of different phases: 1) inorganic and organic solids, 2) soil solution containing dissolved

organics and inorganics, and 3) gases (Knaebel and Vestal, 1994). Soils are generally considered to become nutrient poor as they tend to be depleted of microbially available carbon and nitrogen. Changes in temperature and moisture have a great impact on the soil environment and resident microbial populations.

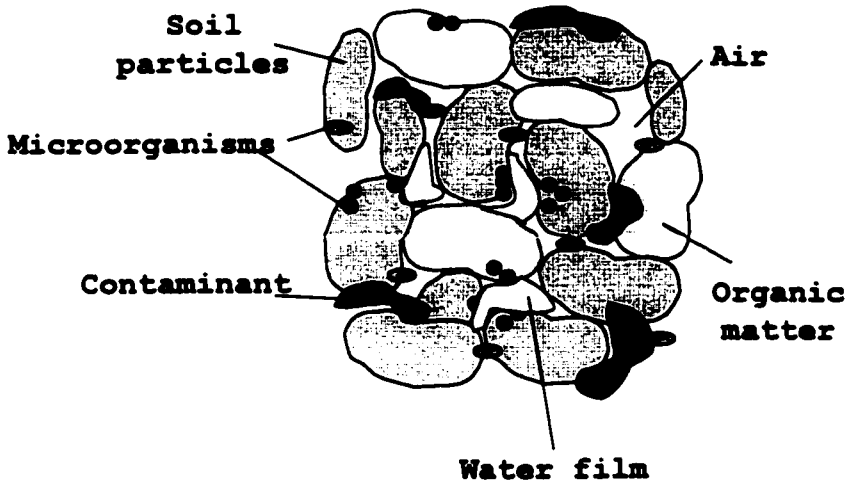
The fate of contaminants in soils depends on a combination of abiotic and biotic processes, including photo-degradation, leaching, immobilization, and biodegradation. The biodegradation of chemicals in soils depends upon the chemical, physical, microbial, and climatic characteristics of a particular soil (Knaebel and Vestal, 1994). Most soils are multi-phasic systems, containing a mineral matrix and associated organic matter that is surrounded by a water film. A gas phase occupies the pore spaces in unsaturated soils; whereas, pore spaces are part of the aqueous phase in saturated soils (Huysman and Verstraete, 1993). When chemicals are introduced onto the surface of a soil, a number of physical phenomena impact their removal or fate in the environment. The four primary processes involved in contaminant transport are decay, advection, dispersion, and adsorption (Yates and Yates, 1990). Adsorption is the chemical binding of a contaminant to the surface of a solid medium such as soil. Such binding may be reversible or irreversible depending upon the properties of the contaminant and the subsurface medium.

Volatilization and/or photo-oxidation may be important in removing some spilled chemicals from the soil surface and this would vary with the permeability of the soil surfaces (Figure 2.1b). In addition to biological degradation on the surface, photo-oxidation mechanisms, may partially oxidize the contaminants and

make them more water soluble and perhaps more bioavailable, or create a greater movement through the underlying unsaturated soil and eventually to the water table (Figure 2.1b). Chemicals with lower water solubility can also move down through the soil, but more slowly, thereby introducing an additional layer phase to a multiphasic matrix which may change the water holding capacity of a soil (Huysman and Verstraete, 1993) and even effect the membrane characteristics of the microorganisms.

Degrading microorganisms add yet another dimension to an already complex soil system (Figure 2.1a). In soil, microorganisms can either be free-living or associated with other organisms, such as plants. There is growing evidence that the degradation rate of hazardous organic compounds in the rhizosphere is greater than in root-free soil due to increased microbial activity. In addition to degradation, xenobiotics deposited in soil may also undergo synthetic processes, such as polymerization or binding to humus. Various interactions may be responsible for adsorption or binding of xenobiotics to humic substances, such as covalent bonds, van der Waal's forces, H-bonding, ion exchange, hydrophobic bonding, ligand exchange, or entrapment in the molecular net of humus (Dec and Bollag, 1994).

Type and concentration of chemicals on surfaces of soil particles could influence the fate of microorganisms in a soil matrix (e.g., higher nutrient concentrations stimulate bacterial growth and cell yield). The fate of microorganisms depends on two main components: survival and transport (Yates and Yates, 1990).



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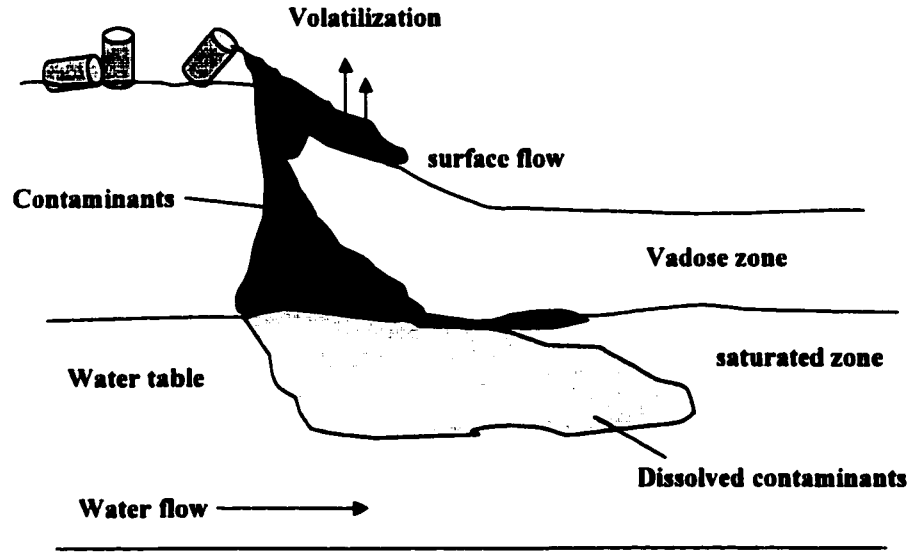


B

Figure 2.1. Schematic representation of a soil, A; Representation of contaminant spills distributed through a soil matrix, B.

Transport is necessary for the microorganisms to reach the adsorbed nutrients or to move to different locations in the medium. Van Loosdrecht et al. (1990) suggest that bacteria can reach a soil particle surface through either: 1) diffusive transport, which exhibits a Brownian motion with a velocity slower than convective transport or transport of motile cells and is responsible for crossing layers where convective transport does not take place, 2) convective transport, which is due to liquid flow and is diffusion controlled at diffusive layers, 3) active movement, which is an accidental contact with a surface or a response to chemotaxis.

Soil texture, organic matter, moisture, pH, temperature, and the chemical compositions of the soil are very important factors affecting the survival of microorganisms (Yates and Yates, 1990). Van Gestel et al. (1996) showed that biomass C is higher than organic C in lower fractions of soil particles and that N level were higher than organic C in finer fractions. They concluded that the spatial distribution of microorganisms in sandy-loam soil structure was determined by clay particles and organic matter. They also suggested that clay particles and organic colloids ensure microbial growth and survival in soils by their capacity to buffer the nutrient supply to microorganisms closely associated with their surfaces. However, their results also indicated that close association with clay particles does not protect microorganisms against severe soil drying. Another important affect soil structure can have on bacterial populations is to offer protection from protozoan predation through properties of charged surfaces or by increasing the distances between predator and prey.

2.2 SOIL DECONTAMINATION

As mentioned previously, there are many factors involved in the fate of chemicals in the environment. Since contaminated sites exhibit different characteristics, cost-effective remediation plans are site-specific. There are a variety of treatment technologies available for contaminated soils: physico-chemical techniques, solid-phase biotreatment (land-farming, biopiles), slurry phase biotreatment (bioreactors), biotreatment in-situ, and a combination of the biological with the physical or chemical techniques. The ex-situ treatments such as physico-chemical techniques include excavation followed by incineration or chemical treatment. These processes can be effective but they are expensive and destructive. In addition, the transport of contaminated soil is in itself a hazard.

Some in-situ physical and chemical treatment methods, such as vapor-phase stripping of volatiles and extraction of polluted ground water for treatment with activated charcoal, resins or chemical agents, may not result in total decontamination of the soil. More recalcitrant pollutants such as PCBs, are very stable due to their low volatility and solubility and the above mentioned methods are not very effective in complete remediation of soils contaminated with PCBs. Treatments such as incineration of such soils only create other chemical residues which are released into the air or remain in the ashes which are then contained permanently or temporarily in land fills.

The use of microorganisms to remediate contaminated sites ex-situ or in-situ, is an innovative technology. Although biological technology has been used

for decades (Walton et al., 1994a) and has been commercially available since the 1970s, it has only gained acceptance and publicity since the U.S. Environmental Protection Agency (EPA) and Exxon Company, USA, demonstrated its effectiveness on Alaskan beaches contaminated by the Valdez oil spill (Forsyth et al., 1995). More recent examination of the cost-effectiveness of this technology has led to its application to hazardous chemicals at waste sites. Success obtained by using the natural microflora to clean up soil, sediment, and water have gained continued interest and research in bioremediation (Walton et al., 1994a).

2.2.1 Techniques in Bioremediation

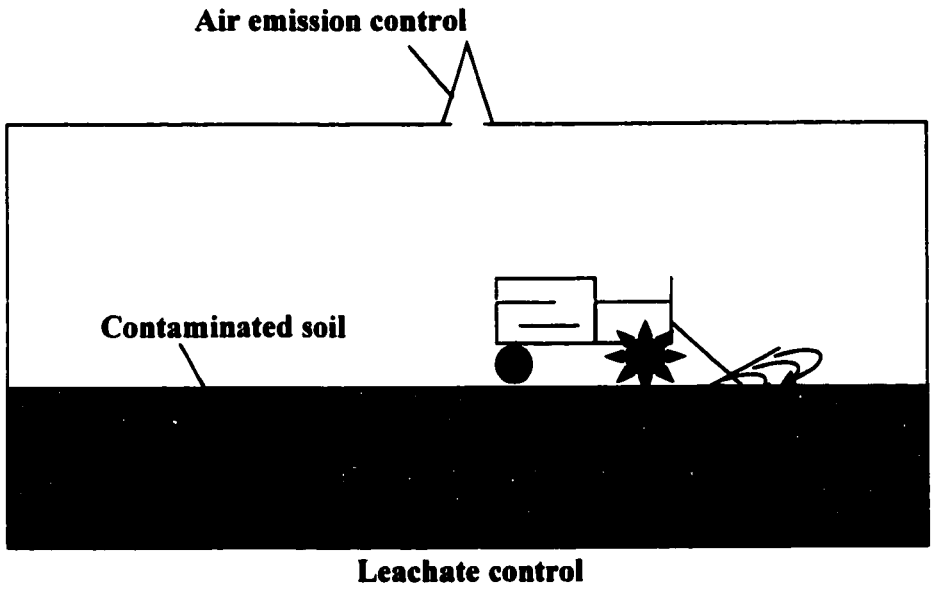
Bioremediation is the use of microorganisms or plants to detoxify an environment by transforming or degrading pollutants. There are four basic techniques that could be used for both ex-situ or in-situ bioremediation of soils: 1) Biostimulation: enhancement of activities of indigenous microorganisms by addition of nutrients, regulation of redox conditions, pH change, or removal of other limiting conditions; 2) Bioaugmentation: inoculation of microorganisms with specific biotransforming abilities into contaminated environments; 3) Enzyme Treatment: application of immobilized enzymes to transform or degrade specific pollutants; 4) Phytoremediation: use of plants to remove, contain, or transform pollutants (Walton et al., 1994b). This review will focus on bacterial bioremediation techniques.

Several methods have been and are being developed for bioremediation of contaminated soil. These include ex-situ treatments such as composting or solid-phase treatments and in-situ treatments such as land farming (Figure 2.2a). Composting bioremediation is a method where biodegradable materials are

combined in a pile for the growth of microorganisms and it has been a popular method used by farmers as a way of removing natural organic wastes while generating soil organic matter (Figure 2.2b). In solid-phase treatment (biopile), the contaminated soil is placed in an impermeable lined treatment bed and nutrients are added to stimulate growth and activity of microorganisms (Figure 2.3a). Land farming is an on-site treatment method where the degradation of pollutants is enhanced by supplementing the soil with nutrients and oxygen, followed by tillage and irrigation to create an optimal environment with special consideration given to the moisture content and nutrient availability for microbial activity and to increase the chance of contact between contaminants and microorganisms. In-situ bioremediation involves the use of organisms to remove pollutants at the site of contamination.

Often, these organisms are indigenous to the area and are adapted to that particular environment. These microorganisms transform the compound present until one nutrient reaches a limiting concentration. Therefore, in biostimulation of organisms for in-situ bioremediation (Figure 2.3b), the limiting nutrient is added to the environment to allow the organisms to continue degrading the pollutant (Bollag and Bollag, 1995). Table 2.1 shows some of the technologies used in remediation of contaminated soils.

In the mentioned techniques, the degrading populations are present in the contaminated soil. However, for more recalcitrant pollutants at sites where local environmental conditions have prevented the development of a degradative microbial community, inoculation with specific populations of microorganisms or consortia (bioaugmentation) may be necessary. It is possible to develop microbial inocula with specific process conditions that result in an increase with the overall bioremediation efficiencies, and lower costs (Verstraete and Top, 1992).



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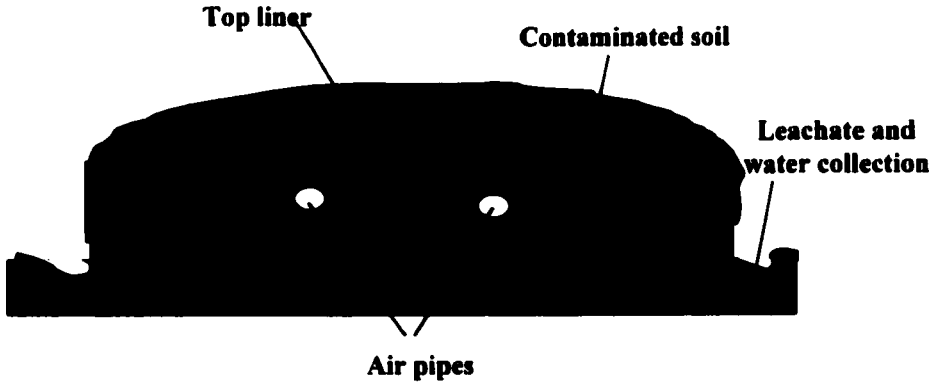

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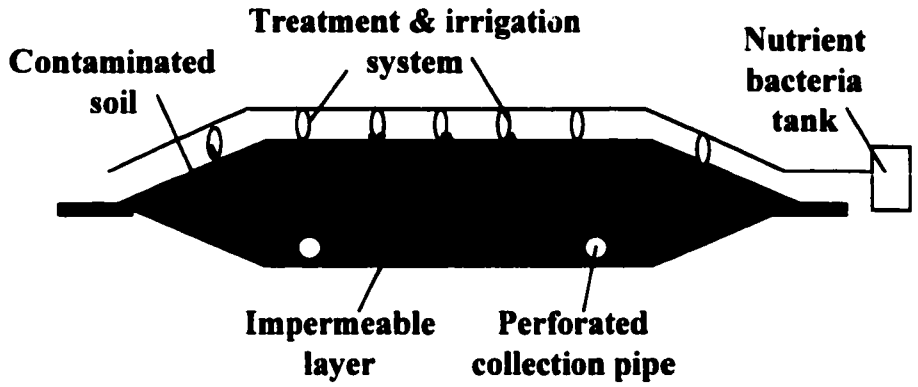
Figure 2.2. Schematic diagrams of surface biological treatments, A) land farming, B) composting.



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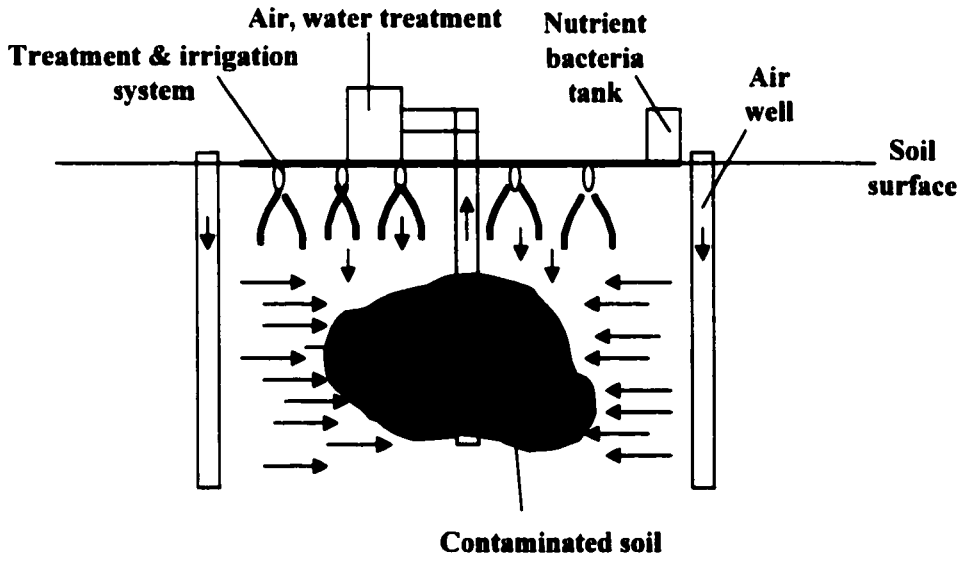

B

Figure 2.3. Schematic diagrams of current A) ex-situ, B) in-situ bioremediation technologies.

Table 2.1. Comparison of bioremediation technology performance on organohalide contaminated soils.

Treatment Technology	Compound	Removal (%)	Comments
Physical-Chemical			
Aeration	Chlorinated solvents	99.9	Limited by soil types and to volatile compounds; limited application
Soil flushing and washing	Organohalides	75-89	Proven effective; higher cost than bioremediation alone; not effective on clay or silt
In situ bioremediation	TCE	20-30	Limited by soil permeability
Bioventing			
	t-DCE	80-85	Up to 20% due to microbial
Inoculation stimulation	Aroclor 1242	37-52	Oxygen, biphenyl, nutrients
	Aroclor 1260	>20	Bromobiphenyl addition
Ex situ bioremediation	TCE	90	TCE-co-metabolizer added;
Bioreactors			methane stimulation
Composting	Freons	94	Applicable to wide
	TCE	89-99	range of organohalides
Bioslurry	TCE	86-100	Nutrient addition

(Reference: Adriane and Vogel, 1995)

2.2.1.1. Bioaugmentation

An alternative to the enhancement of bioremediation by indigenous microorganisms (biostimulation) is the use of an inoculum (bioaugmentation) of an appropriate pure or mixed culture of degrading microorganisms. In bioaugmentation, as is in biostimulation, nutrient amendment might be necessary for maximal removal of the pollutants (Bollag and Bollag, 1995). Certain situations could require manipulation of the environment in favor of the particular

microorganisms used in the bioaugmentation. Creation of favorable conditions may be accomplished by addition of inducer compounds, co-substrates, or commercial microbial inocula, biosurfactant producing strains or by manipulation of pH or nutrient balance or bioavailability (Forsyth et al., 1995). Bioaugmentation with proven contaminant-degrading microorganisms, whether indigenous or exogenous with respect to the contamination site, leads to a higher degree of confidence in remediation success and could save time and money. For example, acenaphthene, a compound common to creosote and coal tar wastes, can be biodegraded slowly by indigenous populations that adapt to its presence. However, it can be degraded more rapidly by selectively adapted, enriched cultures (Forsyth et al., 1995). Bioremediation of soils contaminated with such mixed compounds usually requires bioaugmentation at some point in the bioremediation process. Even though many of the degrading microorganisms used in bioaugmentation are naturally occurring and are members of stable microbial communities, recent bioremediation research has focused on the use of genetic engineering and selection techniques for the construction or isolation of microorganisms with efficient degradation capabilities (Turco and Sadowsky, 1995).

Isolation of degradative organisms is relatively straightforward. Soil samples from a contaminated site that might contain degradative bacteria are collected. If the degradation of PCBs is desired, soils near an electrical plant or other sites likely to be contaminated with the materials of interest are sampled. The bacteria are then isolated through selective processes for the ability to degrade

PCBs by increasing concentrations of the contaminant in the growth media. Once the bacteria are isolated, they can be adapted to different concentrations of the contaminant (Landis and Yu, 1995).

If the newly isolated organisms are to be used in a field situation, basic survival traits must also be determined. Following selection of strains with desirable characteristics, additional improvement of strains might be possible through adaptation and/or mutagenesis to obtain even more favorable characteristics; "All of these processes are considered natural selection processes"(Forsyth et al., 1995).

The bioremediation community is about evenly divided between companies that practice bioaugmentation and those that rely only on biostimulation. Bioaugmentation has been widely practiced in the controlled environments of wastewater treatment plants, especially those handling, toxic, inhibitory, or difficult-to-degrade wastes. With a better understanding of the microorganisms and isolation of non-pathogenic organisms, bioaugmentation will be a practical technique for in-situ bioremediation of some contaminated soils at the field level.

2.2.2 Advantages and Disadvantages of Bioremediation

The main reason for the popularity of bioremediation is its potential for significantly reducing hazardous waste site cleanup costs. Costs as low as $75 to $200 per cubic yard are reported, compared with conventional technology costs for incineration or secured land filling of $200 to $800 per cubic yard (Bollag and

Bollag, 1995). Because bioremediation is a natural process and often results in complete degradation of the contaminants, waste products are usually not generated and there is minimal site disruption, thus reducing public concern and minimizing environmental impact. Another important characteristic is the relative simplicity of the technology compared with many other on-site treatment technologies. Operational requirements may be lower than on-site incineration, solidification/stabilization, or soil washing systems, possibly resulting in fewer mechanical problems and lower costs (Bollag et al., 1994). These characteristics make bioremediation techniques ideal for clean up of chemical pollutants (Bollag and Bollag, 1995).

However, bioremediation also has some problems. In order for organisms to successfully reduce pollutant levels, their populations must increase or be maintained at a proper level. At some locations, control of growth conditions might be difficult. Other nutrient sources at a site might be more preferred by the organisms or be more bioavailable than the contaminant. The toxicity of other substances, such as heavy metals, or predator/prey interactions could have a great influence on the number of organisms present, particularly when bioaugmentation is the method of choice. Bollag and Bollag (1995) suggest that despite the benefits and relative simplicity of bioremediation techniques, a number of problems exist. Some of the most notable problem areas and issues are: "(a) regulatory barriers; (b) scale-up from bench/pilot level to the field; (c) failure of regulatory agencies to consider the full range of remediation options or configurations; (d) liability for failure to achieve goals; and (e) development time and costs"(Bollag et al., 1994).

2.2.3 Factors in Bioremediation

Successful bioremediation of soils is a function of independent but interrelated factors: the contaminant, microorganisms, and the environment (Turco and Sadowsky, 1995). Concentration of the toxicant is essential in determining the success of a bioremediation attempt. Too low a concentration will not stimulate the growth of the degradative organism. At low concentrations, degradation may not occur due to the lack of nutritive content of the xenobiotic as substrate or the concentration may be too low for the induction of the degradative enzymes (Muller, 1992). At too high a concentration, the chemical could become toxic and the culture dies (Landis and Yu, 1995). In addition to the level of toxicant, its bioavailability is important. The characteristics of a contaminated site, such as the clay content, moisture level, pH, and temperature are important in determining the availability of the contaminant and the survival of microorganisms. In situations where it may be necessary to attempt the in-situ bioremediation of a toxicant, in particular when bioaugmentation is preferred, factors such as competitors and predators also become important. Not only do the degradative organisms have to be able to degrade the toxicant, they must be able to compete effectively with other micro flora and escape predation.

2.3 BIODEGRADATION

Aerobic and anaerobic biodegradation processes may account for both contaminant concentration reduction and loss of pollutants. Aerobic biodegradation relies on dissolved oxygen as the electron acceptor used by the

microorganisms. Since, oxygenases and dioxygenases are involved in many degradation pathways, oxygen is essential for the degradation of pollutants such as aromatic hydrocarbons which are easily degraded aerobically (Muller, 1992). In many cases, oxygen can become a limiting factor. In several remediation processes, the degradation of a compound was initiated by simply supplying enough oxygen (Rifai et al., 1995). Many organic compounds are completely biodegraded under aerobic conditions to CO_2 and water through the TCA cycle and the electron transport chain. However, aromatic compounds are more challenging metabolically. The 3-ketoadipic acid pathway is used for metabolism of such compounds to produce acetyl-CoA and succinic acid that can easily enter into the TCA cycle. This involves formation of catechol or protocatechuic acid (Landis and Yu, 1995). The regulation of enzymes involved in such pathways depends on the group of organisms.

Regardless of the availability of C, microorganisms must utilize an electron acceptor if degradation is to occur. In contrast to aerobic respiration, anaerobic organisms can make use of a number of alternate electron acceptors. The favored reductant is often a reflection of the availability of the material, the biochemical abilities of populations and the amount of carbon substrate. The type of electron acceptor favored will reflect the redox status of the system, Therefore, the sequence of reduction, O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2 reflects the redox potential and oxidizing capacity of the chemical (Turco and Sadowsky, 1995). Under oxygen-limited conditions strict and facultative anaerobic bacteria are able

to metabolize and grow if supplied with one of a number of the aforementioned electron acceptors.

Since mixed microbial populations have a greater possibility of degrading more complex compounds (such as chlorinated compounds) due to their larger catabolic gene pool, the complete biodegradation of xenobiotics in nature by a single organism is rare rather than the general rule. This applies especially to the aerobic degradation of chlorinated hydrocarbons, which requires a number of oxidase and hydrolase enzymes, each with their own substrate specificities. This is exemplified in the degradation of PCBs, which requires inducible biphenyl dioxygenases for the initial co-metabolic transformation steps, resulting in the formation of chlorobenzoate. Since biphenyl dioxygenases do not recognize these intermediates, a different set of oxygenases is required to degrade these substrates further, thus accomplishing mineralization of the PCB. Similarly, degradation of highly chlorinated compounds has been recognized to be accomplished only by sequential anaerobic dehalogenation, followed by aerobic oxidative degradation (Adriaens and Vogel, 1995). Also, a community of microorganisms may be required to remove toxic intermediates generated during degradation of a compound. This is the case during anaerobic degradation of halogenated compounds, where build-up of hydrogen by one of the methanogenic community members is growth inhibitory to acetogens and thus needs to be removed (Adriaens and Vogel, 1995).

2.4 POLYCHLORINATED BIPHENYLS (PCB)

Polychlorinated biphenyls (PCBs) are a class of compounds with low chemical reactivity, heat stability, non-flammability, and high electrical resistance. For over 60 years, they have been used as insulating fluids, hydraulic and lubricating fluids, heat exchange fluids, and additives in adhesive inks and paints (Fiebig et al., 1993). Some 1.4 billion pounds have been manufactured, and several hundred million pounds have been released into the environment, where they have persisted and have become ubiquitous environmental contaminants (Table 2.2) (Bedard and Quensen III, 1995). The very properties that made PCBs attractive to industry, such as resistance to fire and persistence, are the same properties that have resulted in their ecotoxicological problems. Since the mid 1970s production has been banned and use of PCBs have been regulated due to their chronic toxicity and concern about their environmental persistence. Due to the lipophilic and hydrophobic nature of these compounds, they tend to bioaccumulate in living tissues and the food Chain (Swoboda-Colberg, 1995).

Table 2.2. Example of areas considered to be significantly contaminated with polychlorinated aromatic compounds.

General Area	PCAs	Vertebrates Affected
Baltic and North Seas	PCBs > DDT	Fish, marine mammals, piscivorous birds
Great Lakes	PCBs>DDT>cyclodienes	Fish, birds
Hudson River, NY	PCBs	Fish
Seveso, Italy	PCDDs	Livestock, wildlife
Sheboygan River, WI	PCBs	Fish, birds
St. Lawrence Seaway	PCBs > DDT	Fish, whales

Reference: Hansen, 1994

There are four major routes by which PCBs enter the environment: (1) industrial accidents, (2) incomplete destruction of PCB-containing products, (3) weathering of PCB-containing products, and (4) leaking from landfills (Swoboda-Colberg, 1995). PCBs from any of the above sources can enter municipal sewage systems and can spread throughout the environment via diffusion, rain, snow, dust, and wind.

PCBs are commercially produced by chlorinating biphenyls with anhydrous chlorine in the presence of a catalyst such as ferric chloride (Hansen, 1994). This reaction occurs at very high temperatures and results in a crude product that requires further purification. The final commercial product is a mixture of PCBs with varying chlorine concentrations (Figure 2.4) and various impurities such as polychlorinated dibenzofuran (PCDF) and polychlorinated quaterphenyls (PCQs) that are formed during the production of PCBs (Swoboda-Colberg, 1995).

There are 209 theoretically possible PCB molecules, referred to as congeners that differ in the number (from 1 to 10) and position of chlorines (Figure 2.4). Most commercial mixtures contain from 60 to 90 different congeners. In the United States and Great Britain, nearly all PCBs were manufactured by Monsanto under the trade name Aroclor (Hansen, 1994). Most formulations were distinguished by a four digit number; the first two digits were usually 12 for 12 carbon atoms, and the last two digits usually indicated the percent chlorine by weight. Thus Aroclor 1242 contains 42% chlorine by weight. Trade names for PCB formulations manufactured in other countries include

Fenclor (Caffaro, Italy), Phenoclor and Pyralene (Prodelec, France), Clophen (Bayer, Germany), and Kanechlor (Kanegafuchi, Japan) (Bedard and Quensen III, 1995; Rice and O'Keefe, 1995) (Table 2.4).

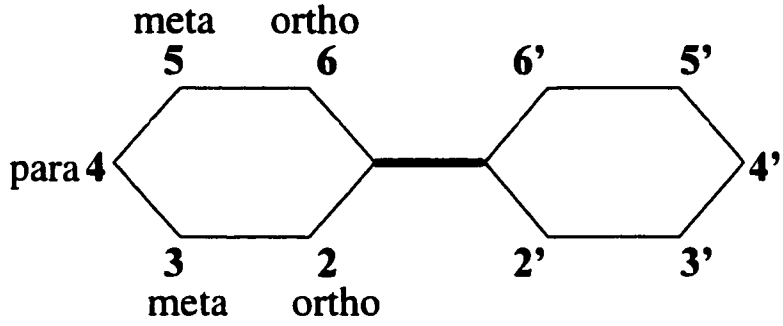

Figure 2.4. Structure of a PCB congener showing the numbering system for chlorine positions.

Table 2.3. Approximate molecular composition (w/w%) of Aroclors.

Isomer	Aroclor 1242	Aroclor 1254	Aroclor 1260
Mono-CI	1.0	<0.1	ND
Di-CI	16.0	0.5	ND
TriCl	49.0	1.0	<0 5
Tetra-CI	25.0	21.0	1.5
Penta-CI	8.0	48.0	12.0
Hexa-CI	1.0	23.0	38.0
Hepta-CI	<0.1	6.0	41.0
Octa-CI	ND	ND	8.0
Deca-CI	ND	ND	<0.1

Reference: Hansen, 1994

In 1929, the Monsanto Company began production of PCBs and they were used in many capacities until the regulation of their use in the late 1970s. Use of PCBs is classified into: closed uses, which include electrical insulation for wires, cables, and condensers, as well as coolant/dielectric in transformers and capacitors; nominally closed uses, which include hydraulic fluids, heat transfer

fluids, and high pressure lubricants; and the open ended uses, which include addition of PCBs to paints, ink dyes, plasticizers, protective coating for woods, dedusting agents, adhesives, pesticide extenders, and microencapsulation of dyes for carbonless duplicating paper. However, since the 1970s PCB applications have been limited to closed end systems (Rice and O'Keefe, 1995).

Table 2.4. Commercial PCB products and producers.

Trade Name	Producer and Country
Aroclor	Monsanto (USA)
Aroclor, Santotherm	Mitsubishi-Monsanto (Japan)
Chlophen	Bayer (Germany)
Delor	Chemko (Czechoslovakia)
Fenclor	Caffaro (Italy)
Kanechlor	Kanegafuchi (Japan)
Phenoclor, Pyralene	Prodelec (France)
Soval	Sovol (USSR)

Reference: Hansen, 1994

2.4.1 Complexities of PCBs

The polychlorinated biphenyls have presented a major problem to both analysts and environmental risk professionals. When the first analytical techniques became available for measuring the concentration of these compounds, the values obtained represented all the congeners of PCB in a particular formulation such as Aroclor 1254. Due to inability to separate and detect each of the 209 congeners, it was difficult to differentiate toxicity or environmental fate of each congener. It is now known that each congener differs in the number and position of chlorine atoms on the biphenyl ring, as well as in its toxicity and persistence in the environment (Shane, 1994).

The adsorption of PCB congeners to many surfaces increase as their water solubility decreases. In soil, adsorption depends on the organic carbon and clay content of the soil; the higher the organic carbon content the greater the degree of adsorption. PCBs are among the best known group of persistent environmental toxicants. This group of congeners exhibit varying degrees of metabolism, induction potential, toxicity and environmental persistence depending on the degree and position of chlorination on the molecule. For example, the hexachlorinated biphenyl 2,4,5,2', 4', 5' is recalcitrant to metabolism and very persistent in the environment. Whereas with only two chlorines less than the hexachlorinated biphenyl, the tetrachlorinated biphenyl 3,4,3', 4' is metabolized and less persistent in the environment (Kleinow and Goodrich, 1994). Hansen (1994) suggests that the melting point and vapor pressure of PCBs both depend on number and position of the chlorine atoms. Alcock et al. (1995) suggest that water solubility also has a profound effect on individual congener disposition; however, this is more closely correlated with the degree of chlorination rather than the position. Vanier et al. (1996) state that potential toxicity is not always a function of PCB concentration and their study showed that PCB congeners, IUPAC number 101, 105, 118, 138, 153, and 183, had the highest toxicity potential on the induction of mixed-function oxidases.

There is no evidence that PCBs occur naturally; therefore, all PCBs present in the environment must have originated from anthropogenic sources. Since PCBs have a tendency to bioaccumulate, there has been a great concern about PCB incorporation into the food chain and their subsequent

bioaccumulation. As a result, the uppermost trophic levels have the most probable concentration of PCBs in the environment (Rice and O'Keefe, 1995). There has been PCB effects on aquatic plankton and invertebrates at water concentrations below 0.1 ppm which can modify populations and cause ecological changes (Hansen, 1994). These effects may not be readily apparent since exposures have occurred gradually and the ecosystems may have adapted and changed long before cause/effect relationships were examined. The important effects on human health are those associated with chronic exposure, those which develop slowly after a pulse of exposure, and subtle effects on development which may occur following prenatal exposure.

2.4.2 Microbial Biodegradation of PCBs

In PCB biodegradation, the number and position of substituted chlorines governs the rate of the degradation. Degradation decreases as chlorine numbers increase. PCBs containing more than four chlorines are more difficult to biodegrade. Furukawa et al. (1979) state that PCBs containing two chlorines on either the ortho position of a single ring (i.e., 2,6-) or both rings (i.e., 2,2'-) show very similar resistance to biodegradation. If the chlorine atoms are on only one ring, biodegradation of PCBs is faster than containing the same number on both rings. In general, preferential ring fission of molecules occurs with non-chlorinated or lesser chlorinated rings (Furukawa et al., 1979). Another factor that is likely to decrease the rate of PCB degradation, is accumulation of toxic metabolites from biphenyl and chlorobiphenyls that interact with the enzyme

activities involved in PCB transformation. This effect of interfering metabolites on the flux of biphenyl-PCB degradation pathway in *C. testosteroni* B-356 has recently been shown in liquid media by Sandossi et al. (1992) and in soil by Guilbeault et al. (1994).

Aerobically, the PCB degradation pathway is a four-step process that is generally limited to congeners with four to five chlorines (Bedard et al., 1986). This begins with a dioxygenase attack at carbons 2 and 3 resulting in the formation of chlorinated 2,3-dihydro-2,3-dihydroxybiphenyl and subsequent metabolism through the *meta* fission pathway to chlorobenzoic acid (Figure 2.5). The *mono-* and *di*-chlorobenzoic acids that are generated from the oxidation of dechlorinated PCBs are in turn mineralized by aerobic and anaerobic bacteria (Bedard et al., 1986; Bedard and Quensen III, 1995). Aerobic chlorine removal does not occur until after 1) ring fission of the biphenyl molecule or the chlorocatechol intermediates or 2) dehalogenase activity on the chlorobenzoate intermediates (Adriaens and Vogel, 1995).

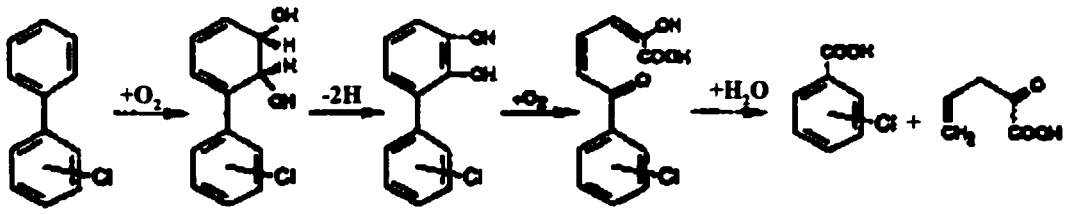

Figure 2.5. Biodegradation of chlorobiphenyls by the 2,3-dioxygenase pathway.

The reductive dechlorination of PCBs in anaerobic environments is a newly discovered and potentially important environmental fate of PCBs. It has significant implications for both risk assessment and bioremediation strategies. Dechlorination is expected to reduce the toxicity of PCB mixtures and should also make them more aerobically degradable (Bedard and Quensen III, 1995); therefore, a sequential anaerobic/aerobic microbial process has the potential to degrade PCBs biologically. Numerous bacterial strains with the ability to degrade mono- through tetra-chlorobiphenyls have been isolated from soils and aquatic sediments (Bedard et al., 1986; Abramowicz, 1990). Many studies have demonstrated that PCB dechlorination occurs in-situ in aquatic sediments and is biologically regulated. There have been suggestions that several distinct dechlorination processes can be recognized on the basis of the congener specificity observed at each site and in laboratory experiments. In studies with mixed soil microflora, meta-isomer substitution of various groups on the benzene ring led to slower degradation than the ortho-chloro substitution (Furukawa et al., 1979). PCBs at sites such as the Hudson River sediments have been shown to have undergone extensive dechlorination. Of all the mono- and dichlorobiphenyls present in the sediment (62%-73% of total), 55%-60% were *o*- or *o*, *p*-chlorinated (Adriaens and Vogel, 1995). Dechlorination of Aroclor 1242, Aroclor 1254, and Aroclor 1260 was first demonstrated in the laboratory by Quensen et al. (1990) using Hudson River (NY) sediment. All mixtures dehalogenated to biphenyl isomers with an average chlorine substitution of two to three chlorines. In this study, Aroclor 1260 degraded more slowly than the others did.

The specificity of microbial dechlorination varies widely, even within the same sediment. Bedard and Quensen III (1995) state there are five possible factors that determine whether a chlorine will be removed from any particular congener in each sediment: 1) the microbial populations present, 2) the position (*ortho*, *meta*, or *para)* of the chlorine relative to the opposite phenyl ring, 3) the surrounding chlorine configuration, 4) the chlorine configuration on the opposite ring, and 5) the incubation conditions (the Aroclor added, temperature, carbon nature and availability, electron acceptors present, salinity, and other contaminants.

Bedard and Quensen III (1995) proposed that PCBs could be totally degraded by anaerobic dechlorination followed by aerobic oxidation. Furthermore, they demonstrated that environmentally dechlorinated Aroclor 1242 extracted from Hudson River sediment could be extensively degraded by *Alcaligenes eulrophus* H850, a bacterium isolated from the Hudson River. Their experiments demonstrated that the constituents of every congener peak in Aroclor 1242 were transformed by dechlorination, oxidation, or a combination of both. The net effect was that 81% of the dechlorinated PCBs, including all of the 2-CB, were degraded in 48 hours and that the combination of dechlorination and oxidation resulted in decreases of 76%, 78%, 93%, and 80%, respectively, for the total di-, tri-, tetra-, and pentachlorobiphenyls originally present in Aroclor 1242. The work of Kohler et al. (1988) showed the ability of *Acinetobacter sp.* strain P6 to transform the components of 25 of the 40 major peaks (congeners) of Aroclor 1254. This makes it one of the most versatile PCB-transforming organisms. In comparison, Bedard

and Quensen III (1995) report that *A. eutrophus* H850 degrades the components of 15 of the 44 largest peaks in Aroclor 1254.

Anaerobes from the Hudson River have shown to remove virtually all meta and para chlorines from Aroclors 1242 and 1248, leaving predominantly ortho- substituted mono- and dichlorobiphenyls (Van Dort and Bedard, 1991). Van Dort and Bedard (1991) suggest that the dechlorination of 2,3,5,6-CB occurs in two separate stages. In the first stage (21-29 weeks), approximately 92% of the 2,3,5,6-CB was dechlorinated; of this total, 79% was converted to 2,3,6-CB by loss of a meta chlorine and 21% was converted to 2,5-CB, via 2,3,5-CB, by sequential loss of an ortho chlorine and then a meta chlorine. In the second stage, which began at 28 weeks, the 2,3,6-CB was rapidly dechlorinated to 2,6-CB. These two stages may reflect a shift in the microbial population. The first population has a long acclimation time and can dechlorinate 2,3,5,6-CB and 2,3,5-CB but not 2,3,6-CB. The second population has a shorter acclimation time and can dechlorinate 2,3,6-CB to 2,6-CB, but it has no activity against 2,3,5,6-CB or 2,3,5-CB.

The enhancement of the rate of PCB bioremediation has been attempted by adding carbon sources (Focht and Brunner, 1985; Barriault and Sylvestre, 1993). The ability to co-metabolize PCB congeners varies significantly from species to species. Although complete mineralization of monochlorinated biphenyls by single strains has been reported, no cultures with the ability to completely mineralize more highly chlorinated biphenyls or to grow on any congener that has one or more chlorines on each ring has been reported (Kohler et al., 1988). The

reason could be that if the anaerobic biotransformation of PCBs is limited to reductive dechlorination, then the responsible microorganisms must use compounds other than PCBs as their source of carbon for energy and growth. Therefore, by providing an appropriate carbon source, it is possible to increase the reducing equivalents available for PCB reduction and/or to support growth of the dechlorinating population and in turn increase PCB dechlorination activity. The metabolism of ^{14}C-labelled PCBs, which comprise the Aroclor 1242 mixture, was greatly enhanced by biostimulation with addition of biphenyl to soil (Focht and Brunner, 1985). Focht and Brunner (1985) also suggest that the rate limiting step in the co-metabolic-commensal metabolism of PCBs to CO_2 had to be the initial oxidation, since the rate of $^{14}CO_2$ production was directly related to the population density of BP oxidizers. They also mention that, there is a limit to the extent that compounds in Aroclor 1242 can be degraded. 2,5,2',5'-tetrachlorobiphenyl, a component in peak 6, is not co-metabolized in pure cultures by *Acinetobacter* strain P6. Since strain P6 can neither dehalogenate nor utilize any of the PCBs contained in this mixture for growth, the production of $^{14}CO_2$ must be effected by another population that oxidizes the cometabolites (Focht and Brunner, 1985). PCB-dechlorinating microorganisms do not metabolize the biphenyl skeleton of PCBs. They must therefore require a source of carbon other than PCBs. This is usually provided by the organic matter present in sediments (Bedard and Quensen III, 1995). A study by Fish (1996) also concluded, reductive dechlorination and aerobic biodegradation of PCBs occurred when Aroclor 1242 was added to upper Hudson River sediments in a test tube microcosms study. At

greater concentrations, biotransformations were accelerated after a lag time of 4 to 8 weeks.

2.5 RHIZOBIA

According to Bergeys' Manual, bacteria in the genus Rhizobia, are aerobic, gram negative, normally rod shaped, without endospores, motile, one polar or sub-polar flagellum, or two to six flagella and capable of utilizing many carbohydrates. Their size ranges from 0.5 - 0.9 by 1.2 - 3.0 μm. Optimal growth of most strains occurs in the range of 25-30°C and pH of 6.0-7.0 (Somasegaran and Hoben, 1994).

The Rhizobial population is not uniformly distributed through the soil matrix. Its density is highest in the legume rhizospheres because of their symbiotic association with leguminous plants. Usually, their population is about 10 to 200 times higher, and in exceptional cases 10000 times higher than in the surrounding soil of legume rhizosphere. Rhizobial densities in soils are usually below 10^6 cells/g dry soil (Garcia-Plazaola et al., 1993a).

Rhizobia are used as inocula in many different countries for agricultural purposes. These symbiots, fix N_2 in association with roots of leguminous plants, such as beans, clover or alfalfa where they cause formation of nodules on the roots. They live within these nodules (as bacteroids) in a symbiotic relationship with the plants and fix nitrogen from the air. N_2 fixation in legume root nodules requires biochemical cooperation between the plant and Rhizobia. Bacteroids contribute the N_2-fixing system and haem for leghaemoglobin and the plant

furnishes anaerobic conditions and contributes to production of the globin and the assimilation and export of the NH_3 produced. The plant may also regulate the type and/or quantity of carbon compounds supplied to the Rhizobium bacteroids (Tortora et al., 1992).

Rhizobia are not only N_2-fixers but also denitrifiers, and they are capable of utilizing nitrate respiration to support anaerobic growth (Zablotowicz and Focht, 1979; Knowles, 1982). Garcia-Plazaola et al. (1993a) have suggested that free-living Rhizobium has the potential to remove fixed nitrogen from the soil by denitrification. Oxygen, nitrate, temperature, moisture and labile organic matter availability are the main factors controlling denitrification in the soil by Rhizobium. Even though denitrification activity by some microorganisms in different soils has been detected at temperature as low as 0 - 10 °C, denitrification by Rhizobium in soil is enhanced by temperatures from 15 to 25 °C and by high water filled pore space (WFPS) which helps regulate oxygen (Garcia-Plazaola et al., 1993a). These anaerobic conditions are easily achieved in flooded soils after rainfall or irrigation. The ability of aerobic bacteria to utilize nitrogenous oxides as terminal electron acceptors enables them to survive and grow during periods of anoxia, which may be advantageous for their survival.

2.5.1 Use of Rhizobia in Bioremediation

Only a limited number of Rhizobium strains have been investigated for aromatic metabolism. Chen et al. (1984) and Chen et al. (1985) have demonstrated that Rhizobium is capable of metabolizing aromatic substrates as sole source of

carbon and energy for growth. But, they also indicated that even closely related species may differ in their pathways of aromatic catabolism. Hussein et al. (1974) have shown *R. meliloti* is capable of degrading up to 90-94% of catechol.

A few people have investigated the biodegradation of aromatic compounds under anaerobic and denitrifying conditions. In contrast with nitrogen fixation, denitrification can be carried out in both symbiotic and free-living cells under low oxygen tensions. Al-Bashir et al. (1990) have illustrated that Naphthalene mineralisation under denitrifying conditions occurs in many different soil setups with unidentified microorganisms. They also indicate that it would be easier and probably more economical to supply the needed amounts of electron acceptors for the decontamination reaction to take place in the soil-water systems in the form of nitrate, rather than as molecular oxygen. Chen et al. (1993) suggest that the ability to catabolize aromatic compounds and fix atmospheric N_2 is advantageous to bacteria in the soil environment. Garcia-Plazaola et al. (1993b) suggest that the capacity to denitrify may enhance the survival of these bacteria in soils under anaerobic conditions, as well as support the anaerobic growth of the free living cells. A recent study by Damaj and Ahmad (1996) in our laboratory showed transformation of PCB congeners containing up to five chlorine atoms and a degradation of 40% was observed for Aroclor 1242.

2.6 TRANSPORT OF BACTERIA BY WATER IN SOIL

Movement of bacteria in soil is a function of water content and water potential relationships and is influenced by their sorption onto soil particles or

transport through small soil pores (Huysman and Verstraete, 1993). Huysman and Verstraete (1993) also suggest that microorganisms generally adhere to soil surfaces by electrostatic interactions, Van der Waals forces, and hydrophobic interactions. Therefore, the ionic strength of the medium and the soil surface properties are the predominant factors affecting bacterial transport and cell retention in soil is a function of the type of bacterium (cell surface properties) as well as of the soil surfaces. They reported that the migration of a hydrophobic bacterial strain was 2 to 3 times slower than that of a hydrophilic strain. They were able to correlate this with an increased adhesion of the hydrophobic strains to soil particles. Turco and Sadowsky (1995) have shown that the contribution of the flagella to movement declines as the soil dries and the water film thickness decreases. They also suggest that the presence of flagella may enhance cell sorption to soil surfaces. Contrary to the above suggestions, Gannon et al. (1991) conducted a study to relate the properties of different bacterial strains to their transport with water moving through soil. The bacteria differed markedly in their extent of transport. Transport of 19 strains for their retention by soil was not correlated with hydrophobicities or net surface charges of the cells. Among 10 strains tested, the presence of flagella was also not correlated with transport. Retention was statistically related to cell size, with bacteria shorter than 1.0 μm usually showing higher percentages of cells being transported through the soil. They suggest that more than one characteristic of bacterial cells determines whether the organisms are transported through soil with moving water. Huysman et al. (1993b) suggest that transport of the bacteria in a soil profile depends not

only on hydrophobicity of the bacteria but on clay content and bulk density of the soil. Transport is also more influenced by the surface characteristics of the bacteria than by their size. They also report that pores with diameters of 16-30 μm are particularly important for the migration of the bacteria into the soil during percolation with water. Yates and Yates (1990) suggest that a large microorganism may be excluded from the smaller pores and are thus forced to travel only through the larger pores where their average velocity would be greater than that of the medium as a whole. General starvation and consequent decrease in the size of the bacteria prior to injection has been used as a strategy to improve penetration (Macleod et al., 1988).

Movement of bacteria in soil can occur via passive transport with water or by active transport with use of energy (Huysman and Verstraete, 1993). In general, passive movement will carry bacteria greater distances, over several centimeters, than active movement. Some bacteria have been shown to move through soil columns at pore velocities of 3-30 m/day (McCanlou et al., 1994). Harvey et al. (1989) state that indigenous bacterial populations are transported through contaminated aquifers substantially greater than for non indigenous populations. This is because growth of native bacteria during transport would compensate for their removal due to sorption/biological adhesion to particle surfaces, filtration, predation, and lysis.

Adsorption is the chemical binding of a microorganism to the surface of a solid medium such as soil. Such binding to a soil surface may be reversible or irreversible and equilibrium or diffusion controlled, depending upon the properties

of microorganism and the subsurface medium (Yates and Yates, 1990). Microbially produced surface-active compounds (SACs) such as fatty acids, lipids, peptides, and polysaccharides, may interact with interfaces and affect the adhesion and deadhesion of bacteria. SACs may also play a role in the movement of gliding bacteria across interfaces and regulation of bacterial cell surface hydrophobicity (Neu, 1996). In a study by Shingaki et al. (1994), under acidic conditions, the negative cell surface charge became lower and at the most acidic condition tested (pH 2.4), 34 out of 40 strains of bacteria showed a positive surface charge. This change in the cell surface charge caused by pH-shift from neutral to acidic was considered to be a result of loss of negative charge of functional groups of the proteins and lipids existing or exposed at the cell surface.

The reversibility of bacterial adsorption and detachment are important factors in estimating transport for long-term transport systems. Time scales for detachment may be of the order of weeks. Bacterial growth may therefore be more important than detachment near the soil surface where doubling times are high. In deeper environments, detachment time scales may be large relative to doubling times, suggesting that the rate of detachment may be the most important transport process (McCanlou et al., 1994). Number of bacteria that attache in a few hours may take weeks to completely detach and move with the ground water.

The influence of hydrophobicity on adhesion is in agreement with reports that hydrophobicity is an important factor in bacterial adhesion to polystyrene, fat tissue, minerals, and stainless steel (Huysman and Verstraete, 1993; Huysman and Verstraete, 1993b). McCanlou et al. (1994) indicate that hydrophilic bacteria

could move further before being removed by attachment to soil, but once attached, would be resuspended at a slower rate. Relatively hydrophobic strains have migration times shorter than those of hydrophilic strains (Harkes et al., 1992). Highly negatively charged strains had shorter migration times than less negatively charged strains. When the fastest migrating strain with respect to glass was allowed to migrate along solid surfaces differing in hydrophobicity and charge, no differences in migration times were observed. Stenstrom (1989) suggested that high hydrophobicity always coincided with enhanced adhesion to the mineral particles that negative charge of the bacterial surfaces appeared to play no role in the adhesion event. However, the positive charges on the cell surface contributed to the adhesion process. This was particularly evident for cells exhibiting a high degree of hydrophobicity. Kasahara et al. (1993) suggest that slow growing isolates of bacteria have more hydrophobic cell surface than fast-growing isolates.

Huysman and Verstraete (1993) state that the inoculation and irrigation method greatly influence the degree of transport. At high irrigation rates the water flow through macro pores would increase, thus minimizing filtering of the bacteria by the soil matrix. When the water flows at a slower rate, bacteria move through the soil in a thinner water film and are drawn nearer to the soil particles. This increases the potential for adhesion of bacteria and decreases the transport. They also suggest that in soil columns, most of the bacteria adhere after 10 min exposure and can not be transported to deeper soil layers with the percolating water. Decreasing the flow rate, even at saturation, should decrease the cell movement in the soil (Lance and Gerba, 1984). A study by Trevors et al. (1990)

showed that transport of cells introduced into 1 cm of the vertical soil microcosms was dependent on the flow rate of water and the number of times microcosm were flushed with ground water. The introduced cells were detected in the effluent water samples even after three flushes of ground water and 10 days of inoculation. They suggest that parameters such as soil type, type of water used for irrigation, rate of water flow and the number of applications and volumes of water applied, the type of bacteria and its density, and the application method are important factors and should be considered depending on the type of information required. In a study by Hekman et al. (1995), two percolating treatments applied daily were used to distribute bacterial cells in soil. They observed that transport was generally similar between the two water regimes (0.9 and 4.4 mm/h). In a loamy sand soil, there was no change in the bacterial distribution during the 51 days of experiment. However, in a silt loam, cell numbers in the lower soil layers were significantly reduced to levels at or below the limit of detection. Pre-incubation of the inoculated soil before starting percolation reduced the inoculant transport in the loamy sand soil measured after 5 days, but not that determined after 54 days. Delayed percolation in the silt loam soil affected bacterial transport only after 54 days.

Breitenbeck et al. (1988) showed that movement of *Bradyrhizobium japonicum* in soil was dependent upon water movement and that both percolating water and an advancing wetting front readily transported bacteria in coarse-textured soils. Percolation with equivalent of 10 cm of rainfall dispersed *B. japonicum* throughout 40 cm columns containing sand and silt loam soils.

Percolation with 5 cm of water was sufficient to disperse *B. japonicum* throughout 20 cm columns of these soils but did not transport these bacteria below the surface 4 cm of a sand amended with 12% kaolin. These findings indicated that the cells of *B. japonicum* are readily transported by an advancing wetting front and that non-saturated flow of soil water contributes to dispersal of inoculum in soils. At lower initial moisture contents (higher water potential), only the narrowest pores are filled with water and the inoculum is expected to penetrate into narrower pores and more cells will penetrate aggregates than in soil with higher initial moisture contents. When water fluxes are not too large, water in pores will prevent introduced cells from penetrating these pores. The results of a study by Natsch et al. (1996) suggest that a heavy rainfall occurring after application of bacteria to field soil leads to the transport of a significant number of the bacteria through the channels of preferential flow (macropores) to depths of 150 cm.

Huysman and Verstraete (1993b) observed similar filtration coefficients when different concentrations of bacteria were added to the soil. This suggests that independent of the concentration of bacteria, a soil layer of equal depth is necessary to reduce the number of bacteria by a proportion and that, upon percolation of the water through the soil column, a constant proportion of the bacteria adheres to the soil or is filtered out by the soil matrix.

2.7 GENERAL REMARKS

Water table management (WTM) has been suggested to be the best management system to control agricultural pollution (Munster et al., 1996; Liaghat and Prasher, 1996; Liaghat et al., 1996; Jebellie and Prasher, 1996) and in a recent soil column study, it was shown to be an effective technique for biostimulation of indigenous populations to remediate a soil contaminated with diesel (Ugwuegbu, 1997). The research presented here is focused on the use of WTM as a technique to bioaugment and bioremediate a soil contaminated with PCBs. The microorganisms used include indigenous microflora in addition to a known PCB degrader *C. testosteroni*, strain B-356, and a newly isolated *R. meliloti*, strain A-025.

Despite the work of the past few decades, the fate of PCBs is not clearly defined. However, it is obvious that the environmental factors that are important to microbial ecology in general may be expected to influence PCB dechlorination and ultimately biodegradation. Most of the experiments to date have used culture conditions quite different from the environmental situations. To better understand what is important in controlling environmental dechlorination and bioremediation of PCBs, investigations using more relevant conditions are essential.

The survival and transport of bacteria through soil are of major importance in bioremediation and particularly bioaugmentation and are partly dependent on characteristics of the bacterial cells. Increased hydrophobicity may be an important strategy in the survival of some bacteria during transport in water. Starving bacteria with a higher cell surface hydrophobicity may adhere to surfaces

and scavenge the adsorbed nutrients or contaminants, thereby enhancing their chances of survival and increasing their efficiency as biodegraders.

It is difficult to predict whether repeated inoculations are necessary for bioaugmentation of contaminated soils. If the inoculated organism is tolerant to the toxicants present, the chances of survival and establishment of an effective population size is feasible. However, if there is some toxic effect, then it might be necessary to introduce bacteria repeatedly until the concentration of the chemical decreases below the toxic level.

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PREFACE TO CHAPTER 3

Rhizobia are found as free living soil bacteria and in symbiotic relationship with leguminous plants. As symbiots, these bacteria use the organic aromatics that are produced by the plants as their energy source. Therefore, we attempted to determine whether these organisms were capable of degrading some of the aromatic hydrocarbons that are considered as pollutants with adverse affects in the environment. Presence of Rhizobia was tested in soils contaminated with PCBs, PAHs, and diesel. Existence of these bacteria in these soils would indicate: 1) these bacteria are tolerant to toxic chemicals, and 2) these bacteria may use these contaminants as energy source and may be capable of degrading them.

Chapter 3 describes the isolation of Rhizobia and preliminary tests that were preformed with the isolated Rhizobial strains. Among these tests were hydrophobicity and adhesion of the bacterial cells. Behavior of bacteria in water and soil are important parameters when transport and delivery of bacteria in the soil are considered. Hydrophobicity and adhesion of bacteria cells are good indicators of the behavior of the cells in a soil environment. Therefore, we measured these two factors to guide us in the selection of a bacterial strain from our isolated collection to be used in future studies.

A part of this work has been published in the Inter. Biodet. Biodeg. (Ahmad, D., R. Mehmannavaz, and M. Damaj. 1997. Isolation and characterization of symbiotic N_2-fixing *Rhizobium meliloti* from soils contaminated with Aromatic/Chloroaromatic hydrocarbons: PAHs and PCBs. Internat. Biodet. Biodeg. 39(1):33-43). Dr. M. Damaj was responsible for the

molecular biological work of the published paper. The hydrophobicity and adhesion results were presented at the annual meeting of the Canadian Society of Microbiology (CSM): Reza Mehmannavaz, Darakhshan Ahmad, Shiv O. Prasher. 1998. Variation in cell surface properties among rhizobial strains isolated from soils contaminated with aromatic hydrocarbons and metals: hydrophobicity and adhesion to sandy soil. 1998 Annual Meeting of the Canadian Society of Microbiology held in Guelph, Ontario in June 1998, also will be submitted for publication as: Mehmannavaz, R., Ahmad, D. and Prasher, S.O. Variation in hydrophobicity and adhesion among rhizobial strains isolated from soils contaminated with aromatic hydrocarbons and metals.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF SYMBIOTIC N_2-FIXING *Rhizobium meliloti*

ABSTRACT

Using plant nodulation tests on *Medicago sativa* (alfalfa), the presence of a variety of strains of *Rhizobium meliloti* was demonstrated in soils contaminated with aromatic/chloroaromatic hydrocarbons and with no history of indigenous alfalfa presence. We purified some of these strains and characterized their colony morphologies. Twelve isolates of *Rhizobium meliloti* were studied for their behavior in soil and water matrices by determining their hydrophobicity, as measured by bacterial adherence to *n*-octane, and adhesion, as measured by their retention on sandy soil particles. Two non-rhizobial strains, *E. coli* JM105 and *C. testosteroni* B-356, were also included in this study. From these results it was concluded that the population of *R. meliloti* in each of these soils was not homogeneous and was composed of several phenotypically distinct strains. Strains obtained from the soils were all effective in symbiotic N_2-fixation, irrespective of the nature or level of contamination. Both non-rhizobial strains, *E. coli* and *C. testosteroni,* showed higher level of adhesion, cell retention being respectively 85% and 78%, than those found among rhizobial strains, varying around 55%. However, the hydrophobicity values for *E. coli* was similar to those found among rhizobial strains (around 15%), where as, *C. testosteroni* showed a significantly higher level (33%). Also, the variability among the hydrophobicity values for

rhizobial strains was lower than for adhesion. Both cell surface properties studied were also influenced by the composition of growth media used. These findings indicate that at least some strains of *R. meliloti* are capable of inhabiting contaminated soils that have not been previously planted with a compatible host plant in the past. Also, they suggest a possible role of rhizobia, in decontamination and recycling of organic compounds; a role which could lead to the utilization of these agronomically important microbes for environmental clean up. To the best of our knowledge, this is the first report on the isolation of rhizobia from soils contaminated with aromatic and chloroaromatic compounds.

3.1 INTRODUCTION

The worldwide extensive use of aromatic and chloroaromatic hydrocarbons has led to ubiquitous contamination of aquatic and terrestrial ecosystems. The gathering agreement on their toxicity, bioaccumulation and recalcitrance has caused worldwide concern. The extreme persistence of some of these xenobiotics in the environment, such as PAHs, PCBs, PCP, etc., led to the early notion of the incapability of microorganisms to degrade and recycle these compounds in nature. However, the isolation of a variety of microorganisms, from a wide range of habitats, capable of transforming these pollutants has stimulated interest in using "biodegradation" for the recycling of these pollutants. "Bioremediation" is in theory the ideal choice where large volumes with low-to-moderate levels of contamination are concerned, more so if done on-site and even better if in-situ. However, bioremediation as a technology is not yet sophisticated

enough to provide consistent results, because of lack of understanding of the biological processes involved and of the biotic and abiotic factors that influence these processes (Broda, 1992; Providenti et al., 1993; Abramovicz, 1994; Colwell, 1994; Gutnick, 1994; Atlas and Cerniglia, 1995).

Bacterial strains isolated so far for their ability to metabolize, aerobically, chloroaromatic pollutants belong to different genera but Rhizobium (Atlas and Cerniglia, 1995; Wilson and Jones, 1993; Damaj and Ahmad, 1996). A few reports also mention a group of unidentified degrader strains (Kiyohara et al., 1992; Maue et al., 1994). Rhizobia, by definition and classification, are associated symbiotically with plant roots and the rhizosphere. Hence, these microbes are naturally exposed to a variety of aromatic and phenolic exudates of roots. Moreover, they are known to possess catabolic systems for simple aromatic (Chen et al., 1985, 1989, Chen and Lovell, 1990) and some complex plant compounds such as flavones (Tepfer et al., 1988; Peters and Verma, 1990; Rao and Cooper, 1994). Therefore, they may prove to possess interesting aromatic catabolic pathways and capabilities. However, to our knowledge, they have not been considered and studied for use in bioremediation of aromatic pollutants. This may be because the isolation, enumeration, characterization and identification of rhizobial species and strains, in the past, has called for elaborate and time consuming protocols (Buchanan and Gibbons, 1974 (*Bergey's manual of Determinative Bacteriology*); Somasegaran and Hoben, 1994). Moreover, they are presumed to occur at low numbers and to be relatively inactive, except when associated with nodules, and the focus has always been oriented toward their N_2-

fixing abilities. Thus, their contribution to aromatic degradation for bioremediation purposes is not readily apparent, and the well established catabolic versatility of Pseudomonads, since the work of deJong on the growth of *P. putida* on 200 substrates, has attracted much attention (Gottschalk, 1979).

Recently, in our laboratory, the degradation of PCBs (Aroclor 1242) in *R. meliloti* Zb57 was shown. Also, hybridization of genomic DNA dot blots of four species of N_2-fixing symbiotic rhizobia, *Bradyrhizobium japonicum, R. trifolii, R. leguminosarum*, including *R. meliloti* Zb57, to the BP/PCB degradation genes of the well-studied *C. testosteroni* strain B-356 (Ahmad et al., 1990; Damaj and Ahmad, 1996) were shown. Considering the possible impact of rhizobium in the field of bioremediation, we attempted to find out whether rhizobial species can be isolated as an indigenous constituent member of the microbial communities in soils contaminated with aromatic/ chloroaromatic pollutants.

The fate of microorganisms in sub surface environment depends on two main components: survival and transport. Movement of bacteria in soil is a function of water potential and content and is influenced by their sorption onto a soil particle or transport into small soil pores. Bacterial movement in soil can occur via passive transport with water or by active transport through the expenditure of energy. In general, passive movement will carry bacteria greater distances, over several centimeters, than active movement.

Microorganisms generally adhere to soil surfaces by electrostatic interactions, Van der Waals forces, and hydrophobic interactions (Huysman and Verstraete, 1993). Therefore, the ionic strength of the suspending solutions and

the soil surface properties are the predominant factors affecting bacterial movement. In many studies (Shingaki et al., 1994; Gannon et al., 1991; Gross and Logan, 1995; Johnson et al., 1996), synthetic surfactants in solution have been used in investigating the microbial cell surface properties as well as the interaction of microorganisms with interfaces. Adsorption to a soil surface has shown to be reversible or irreversible and equilibrium or diffusion controlled, depending upon the properties of microorganisms and the subsurface media (Yates and Yates, 1990). The evidence from published research (Neu, 1996) suggest that there is a significant role for microbial surface active compounds (SACs) in adhesion to and deadhesion from interfaces. SACs of microorganisms can be grouped by the species of the producing organisms or by the type of carbon source used to produce them, such as hydrocarbons, water-soluble molecules, or both.

The survival and transport of bacteria through soil are of major importance in bioremediation and particularly bioaugmentation and are partly dependent on characteristics of the bacterial cell surface properties. Increased hydrophobicity may be an important strategy in the survival of some bacteria during transport in water. Reports (Huysman and Verstraete, 1993; Huysman and Verstraete, 1993b) suggest that hydrophobicity is an important factor in bacterial adhesion to polystyrene, fat tissue, minerals, and stainless steel. Stenstrom (1989) reported that high hydrophobicity always coincides with enhanced adhesion to the mineral particles.

The work presented here provides evidence for the presence of symbiotic N_2-fixing *R. meliloti* in all samples of soils used in this study. The soils were from

sites that were contaminated either with PAHs or PCBs, each with a different contamination history and that have apparently not been previously planted with a compatible host plant in the past. N_2-fixing strains of *R. leguminosarum* have been isolated from heavy metal contaminated sewage sludge and mine spoils (Smith and Giller, 1992); however, to our knowledge, this is the first report on the isolation of rhizobia from soils contaminated with aromatic and chloroaromatic compounds. Also, this work presents the hydrophobicity and adhesion of some of these isolated strains and a laboratory strain of *R. meliloti* strain Zb57, *E. coli*, strain JM109, and *C. testosteroni*, strain B-356.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, Culture Media and Growth Conditions

The bacterial strains used were: 1) *R. meliloti*, strain Zb57, provided by D. P. S. Verma (Ohio State Biotechnology Center, Ohio State University, Columbus, OH, USA); 2) *Escherichia coli* JM109 and *C. testasteroni* B-356 (Ahmad et al., 1990). The media used, either as broth or solid with agar (15 g L^{-1}), were tryptone yeast-extract (TY) supplemented with calcium chloride (TYc) (Beringer, 1974) for rhizobial strains and Luria-Bertani (LB) (Sambrook et al., 1989) for *C. testosteroni, E. coli* and *P. putida*. For total microbial counts, serial dilutions (1 to 10^{-10}) were plated on TY and TY10 (ten fold diluted TY) plates. For biphenyl (BP) and 4-chlorobiphenyl (4-CB) degradation assays, minimal medium MM30 (Sylvestre and Fauteux, 1982) supplemented with vitamins (biotin 0.5 µg mL^{-1}, calcium pantothenate 2 µg mL^{-1} and thiamin hydrochloride 1 µg mL^{-1}) and mineral

mix 1000XM (Bhuvaneswari et al., 1977) was used. All cultures were grown at 29°C. BP and 4-CB were from the Aldrich Chemical Company (Milwaki, WI, USA) and 2,6-dichlorophenol indophenol was a gift from C. Greer (the Biotechnology Research Institute of the National Research Council of Canada, Montreal, Quebec, Canada).

3.2.2 Soils used for Isolation of Rhizobia

Details of the source, site, characteristics and designations of the soils used for isolation of bacteria in this study are presented in Table 3.1.

Table 3.1. The origin and history of soils used for isolation of rhizobia.

Soil Sample	Site History	Soil characteristics	Contamination (ppm)
S-I	Glass production factory, spillage of diesel and bunker fluids, over 15 years contamination	Heterogeneous mixture of gravel, sand, wood, rocks, glass, bricks, pH (6.50)	PAHs, 5000-85000, oil and grease
S-II	na	Heterogeneous mixture of gravel, sand, clay, pH (7.88)	PAHs
S-III	Manufacturing of boat motors and marine equip., PCB spillage from broken hydraulic lines on machines in 1960's and 1970's	Homogenous mixture of wood, sandy clay, grain size <13 mm, pH (7.75)	PCBs > 730, range 0-1400 Aroclor 1242, 1254, oil and grease
S-IV	Contaminated with diesel over 20 years	homogenous mixture of sand and silt	range 600-700 total diesel residues
S-V	Community garden, possible fertilizer, herbicide, pesticide contamination	homogenous mixture of sand and silt	
S-IX	Lysimeter of an atrazine leaching experiment, 3 years old	homogenous mixture of sand and silt	Atrazine, Metalachlor

na- not available

3.2.3 Plant Preparation and Inoculation for Nodulation and N_2-fixation Test

Seeds of *Medicago sativa* (Algonquin), clover, and pea (Lenca) obtained from the seed farm of Macdonald Campus of the McGill University (Ste. Anne de Bellevue, Quebec, Canada), were surface sterilized by soaking in 95% ethanol for 5min, followed by a 20min treatment with 2.8% NaOCI (household bleach, Javel), thorough rinsing 4-5 times with sterile distilled water, another soaking of at least 5 h at room temperature or 18 h at 4°C in sterile water and finally rinsed again several times. Seeds (2-10) were then placed either onto agar plates or slants, or on a sterilized vermiculite-perlite mixture (3:1) in plastic plant pots (Somasegaran and Hoben, 1994) or in sterile test tube assembly system (Bromfield et al., 1994; R. Wheatcroft, pers. comm) (Figure 3.1), all containing nitrogen-free plant nutrient solution (Somasegaran and Hoben, 1994), for germination in a plant growth environmental chamber (Model no. I25L CPM3000, Conviron Controlled Environ. Ltd., Winnipeg, Manitoba, Canada) under a constant temperature of 23°C and a 16 h day^{-1} light setting (1000W metal halide lamps that produced 250 µEinstein m^{-2} sec^{-1} at plant height). After two days, the germinated seedlings were inoculated with 300 µL of a range of dilutions (1 to 10^{-7}) of soil suspension or bacterial cultures following the experimental protocols. Soil suspensions were obtained by extracting 20 g of soil with 180 mL of phosphate buffered saline, PBS (Somasegaran and Hoben, 1994), either by vortexing for 5 min or shaking for 1 h at 1500 rpm, followed by a settling period of 1.5 h. Four replicates were inoculated for each experiment. Strain Zb57 was used as inoculum for a positive

control for nodulation assays. Uninoculated controls were included to verify that the seeds used were rhizobium-free by the absence of nodule formation (Figure 3.2). Observations were recorded after 6-8 weeks for nodule formation and for up to 3-4 months for establishing the efficacy of nodules for N_2-fixation as judged by the continued healthy growth of plants with nodules as compared to the deteriorating or dead plants resulting from uninoculated nodule-free controls. All experiments were repeated at two different times. Most probable number (MPN) counts for *R. meliloti* in the soil were determined using the nodulation test results following the method described in the Handbook of Rhizobia (Somasegaran and Hoben, 1994).

3.2.4 Isolation of *Rhizobium meliloti* from Root Nodules

A few of the nodules (Figure 3.2), developed with inoculum from each of the soils, were individually harvested, surface sterilized with ethanol for a few seconds, followed by a 5-10min treatment in 30% (v/v) of household bleach and thorough rinsing of at least ten times with sterile distilled water. The nodules were then crushed in 100µL of TYc in an eppendorf tube using flamed glass rods. The nodule suspension containing bacteroids was plated on TYc plates and the plates were incubated at 29°C for a few days till colonies appeared. The colonies were purified, characterized for their morphology and antibiotic sensitivity on TYc agar plates, retested for their ability to develop effective nodules on alfalfa, designated with a number and stored at –70°C in glycerol.

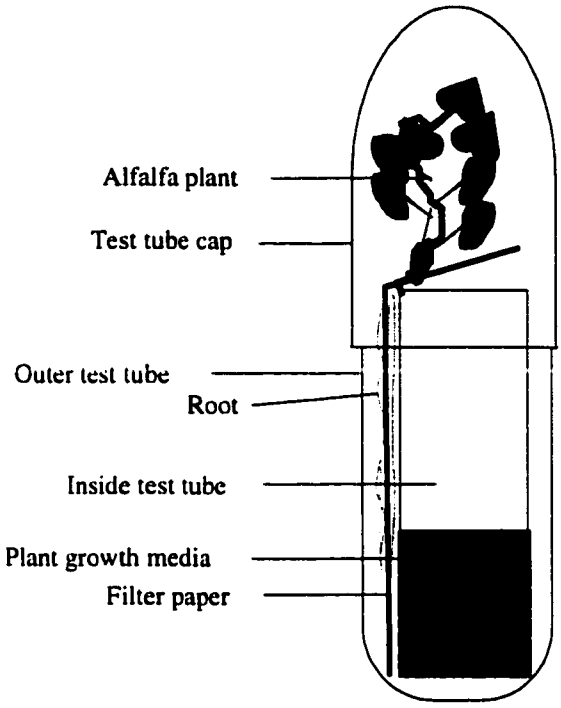

Figure 3.1. Schematic diagram of a test tube assembly system for plant growth and isolation of nodules.

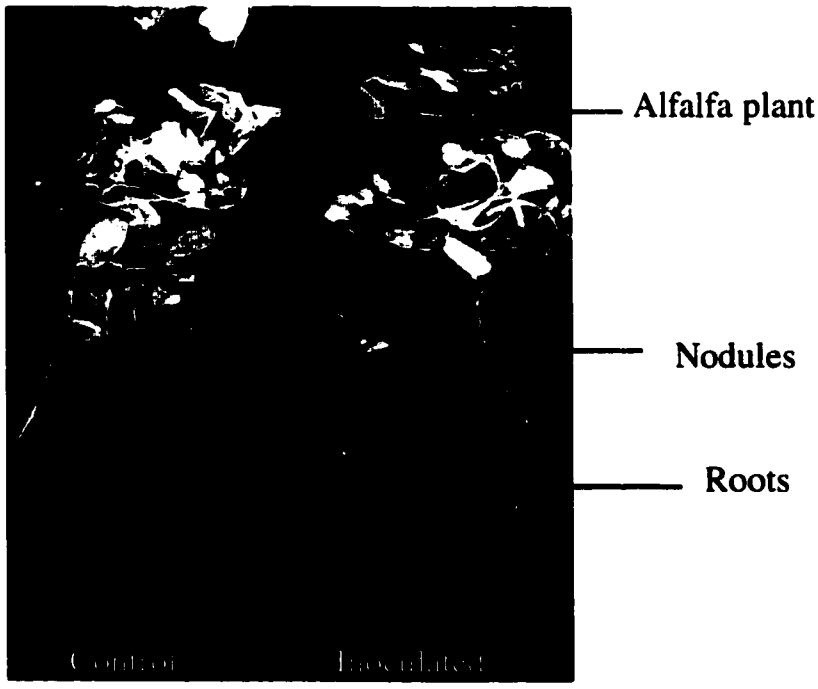

Figure 3.2. A photograph of non-inoculated and inoculated alfalfa plants with nodules.

3.2.5 Biphenyl/PCB Degradation Test using Microtest Plates

Preliminary testing for the potential of PCB degradation in resting cell suspension was carried out following the technique of Hanson et al. (1993) using redox color indicator, 2,6-dichlorophenol indophenol (DCPIP). Cells were grown for two days in rich media, TYc for rhizobia and LB for B-356, washed thoroughly twice and resuspended in minimal medium MM30, supplemented with vitamins and minerals to obtain an OD_{600} between 1.0 and 1.5. Crystals of BP or 4-CB were added to the wells of a 96-well microtest plate to which 300 µL of the suspension and 2 µL of DCPIP (15 µg mL^{-1} in sterile water) was added. Plates were incubated 29°C in the dark and change in color was recorded daily for up to seven days. Control plates were setup similarly except that BP or 4-CB was omitted from the assay mixture. Change of color from blue to white or yellow was scored as positive for the degradation of BP/4-CB. Each strain was assayed in seven wells of the microtest plate and strain B-356 was used a positive control which was always inoculated in wells with at least one row of empty wells in between to prevent cross contamination.

3.2.6 Assay for Hydrophobicity of Bacteria

The hydrophobicity of bacteria was determined with a modified version of the method described by Huysman and Verstraete (1993b). The rhizobial cells (strains shown on Fig. 3.4) were grown over night on TYc (Bromfield et al. 1994) or YEMP (Vincent, 1970) and the non rhizobial strains, *E. coli* JM105 and *C.*

testosteroni B-356, were grown on Luria-Bertani (LB) broth (Sambrook et al. 1989). The bacterial cultures were then centrifuged for 10 min. at 8000 rpm, washed twice (each time vortexed for 30 s) in PBS and resuspended to an optical density (OD_{600}) of approximately 1.0. Five mL of the washed cell suspension and 0.5 mL of Octane (a gift from PAPRICAN Research Laboratories in Pointe-Claire, Québec) were added to a test tube and mixed with a vortex mixer for 60 s. After 10 min of equilibration, the aqueous phase was transferred with a pipette to a cuvette and the OD was measured at 600 nm. The following equation was used to calculate the hydrophobicity of bacterial strains: [ODi - (Ave.ODf - Ave. ODc)] / ODi x 100, where ODi, ODf, and ODc are the OD_{600} of initial, final, and control respectively. All the tests were done in triplicates. The control tubes contained only Octane and PBS. All tests were done in triplicates.

3.2.7 Assay for Adhesion of Bacteria to Soil

The adhesion of bacteria was determined with a modified version of the method described by Huysman and Verstraete (1993b). Overnight cell cultures (strains shown on Fig. 3.5) were grown as for the hydrophobicity assay and centrifuged for 10 min at 8000 rpm, washed twice (vortexed for 30 s) in physiological saline (150 mM NaCl) and resuspended to an OD_{600} of approximately 0.7. Ten mL of the washed bacterial suspension was added to 1.0 g of sterilized garden soil, S-VI (78% sand, 3% silt, 19% clay, 3.59% organic matter, pH 6.17) in a test tube. The mixture was vortexed for 60 s and allowed to settle for 15 minutes. One mL of the aqueous layer from the top was collected and

the OD_{600} was measured. All tests were done in triplicates. A set of tubes containing 10 mL of NaCl and 1.0 g of soil was used as control for adjustment of the ODs obtained. The following equation was used to calculate the adhesion of bacterial cells to the soil: [ODi - (Ave.ODf - Ave. ODc)] / ODi x 100 where ODi, ODf, and ODc are the OD_{600} of initial, final, and control respectively.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of Contaminated Soils

All soil samples were from historically different sites subjected to previous long-term contamination as described in Table 3.1. Soil samples S-I and S-III were provided by Cintec Environment Inc. (LaSalle, Quebec) and S-II by SNC-Lavalin (Montreal, Quebec). All soil samples were collected during fall season and after removal of discrete large particles, stored in capped glass jars at 4°C until used. Soil S-I and S-II were in storage for two years and soil S-III was collected the same year in which it was used, soil S-IV was provided by Imperial Oil. Thus, these soils came from lands that had been used for industrial purposes and have been contaminated for a long time (>15 Years) with no recent (15-20 years) history of plantation or presence of the host plant. The overall microbial count and colony types on TY and TY/10 plates were much lower as compared to a normal garden soil. Rarely was any fungal or actinomycetes growth, which was significantly high in garden soil, observed, and there was no need to add any fungicide to the media used for the contaminated soil.

3.3.2 Isolation of Rhizobium Strains from Soils

Thirteen strains of *R. meliloti* from alfalfa, 3 strains of *R. leguminosarum bv. viceae* from pea, and 12 strains of *R. leguminosarum bv. trifolii* from clover plant roots for a total of 28 Rhizobium strains were isolated from soils S-I, S-II, S-III, S-IV, and S-IX as indicated in Table 3.2. Plant nodulation tests were positive with all four soils used, however, isolation of bacteroids from the nodules formed in soil S-IV were not as successful. The nodules formed as early as 2 weeks and as late as 8 weeks after inoculation in the tube assemblies. Nodulation tests, performed in pots with soil S-III, showed that when inoculation was done with the shaken soil suspension, more (37/49 or 76%) plants were nodulated as compared to those from the unshaken suspension (18/41 or 44%). Also, the number of nodules per plant was higher in pots receiving shaken soil suspension than with unshaken soil suspension. It was evident that not only more plants were infected, but also nodules from the shaken sample looked more mature and the sizes of the nodules ranged from 1 mm to 4 mm in length whereas, the nodules from the unshaken sample showed a maximum size of 2 mm. Also, the plants were healthier and showed better root systems in the shaken sample. These results indicate that bacterial cells become more available to infect the host plants and form nodules in shaken soil.

Nodules were usually rod shaped and pinkish and dark in color and the isolation of the bacteroids from these nodules was more successful compare to the

large, white, and branched nodules that were present in high numbers on plants that were inoculated with S-IV.

Table 3.2. Source and characteristics of different isolated rhizobia.

Strain code	Soil source	Plant source	Nodule Shape	Nodulation Test	N_2 Fixation	Comments
A-010	S-II	Alfalfa	ND	+	+	
A-011	S-II	Alfalfa	ND	+	+	
A-012	S-I	Alfalfa	ND	+	+	
A-013	S-I	Alfalfa	ND	+	+	
A-014	S-I	Alfalfa	ND	+	+	
A-015	S-I	Alfalfa	ND	+	+	
A-021	S-III	Alfalfa	ND	+	+	
A-022	S-III	Alfalfa	ND	+	+	
A-023	S-III	Alfalfa	ND	+	+	
A-025	S-III	Alfalfa	ND	+	+	
A-029	S-III	Alfalfa	Vshaped,large, black and white	+	+	
A-036	S-III	Alfalfa	Rod shaped	+	+	
A-041	S-IV	Alfalfa	ND	ND	ND	
A-109	S-III	Clover	ND	ND	ND	
A-111	S-III	Clover	ND	ND	ND	
Atz1	S-IX	Clover	Small, black-pink	ND	ND	
Atz2	S-IX	Clover	Small, black-pink	ND	ND	No growth on TYct
Atz3	S-IX	Clover	Small, black-pink	ND	ND	No growth on TYct
Atz4	S-IX	Clover	Small, black-pink	ND	ND	
Atz5	S-IX	Clover	Small, black-pink	ND	ND	No growth on TYct
Atz6	S-IX	Clover	Small, black-pink	ND	ND	
Atz7	S-IX	Clover	Small, black-pink	ND	ND	
Atz8	S-IX	Clover	Small, black-pink	ND	ND	
Atz9	S-IX	Clover	Small, black-pink	ND	ND	
Atz10	S-IX	Clover	Small, black-pink	ND	ND	
A-207	S-III	Pea	ND	ND	ND	
A-216	S-I	Pea	ND	ND	ND	
A-217	S-I	Pea	ND	ND	ND	
PB1-25				+	+	See Appendix A2
PB2-25				+	+	See Appendix A2
Atz1-25				+	+	See Appendix A4
Atz2-25				+	+	See Appendix A4

ND-not determined

The white color could be an indication of these bacteroids not being effective symbiots or of their low number in the nodules (Somasegaran and Hoben, 1994). All *R. meliloti* were assigned codes less than 100, *R. leguminosarum bv. trifolii* the 100 series, and *R. leguminosarum bv. viceae* the 200 series. All the purified strains were preserved at -70 °C. All plants used for retesting grew well for over 4 months, indicating that the nodulating strains were effective N_2-fixing Rhizobium.

Among the purified isolates, some had distinct colony morphologies and antibiotic sensitivities indicating that populations of *R. meliloti* existing in each of these soils were not homogenous and consisted of several kinds of *R. meliloti* (Ahmad et al. 1997). Analysis done at Agriculture Canada labs have confirmed the identity of some of the isolates of *Rhizobium meliloti* and also suggest that, strains A-012 with A-013 and A-014 with A-015 could be identical. Therefore, more than one genetically distinct *R. meliloti* were present in each soil.

3.3.3 BP/PCB Degradation in Microtitre Plates

During oxidation of hydrocarbons, oxygen, nitrates or sulfates function as electron acceptors. In this experiment, 2,6-dichlorophenol indophenol (DCPIP) was used as an electron acceptor and its color change from blue to colorless (reduced stage) would indicate the utilization of Biphenyl (BP) and 4-chlorobiphenyl (PCB) by the different bacteria strains (Hanson et al, 1993). As shown on Figure 3.3, all strains tested showed different levels of color change for both substrates used. Even though these results were not conclusive to demonstrate the mechanism of substrate biotransformation, they do indicate that

some type of oxidation was taking place. B-356 was used as a positive control and the color change usually occurred in one day, whereas, the other strains needed up to 7 days. *R. meliloti* strain A-025 showed the highest color change (88%) for 4-CB and strain A-029 had the highest color change (65%) for BP. All controls showed no color change and remained blue throughout the experiment. These results indicate that strains A-025 and A-029, are possibly the most effective degraders of these two substrates, although the mechanism of such transformation has not yet been elucidated.

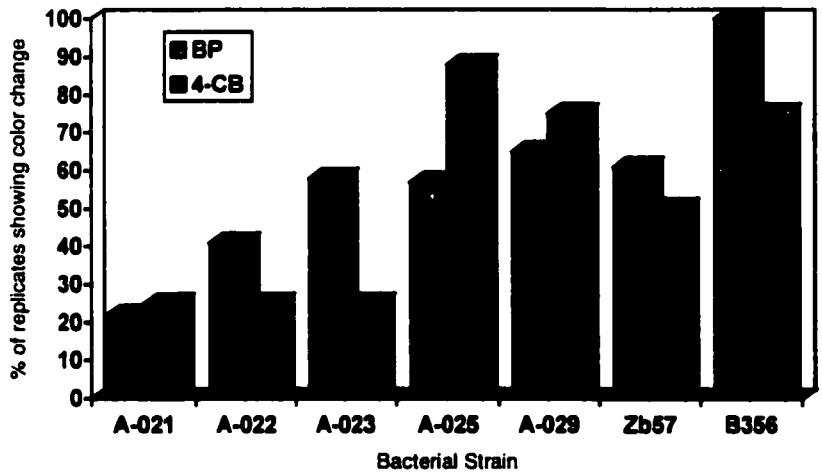

Figure 3.3. Transformation of BP/4-CB indicated by color change using DCPIP.

3.3.4 Hydrophobicity of Bacteria

As shown in Figure 3.4, of the thirteen strains studied, strain A-025 had the lowest (4.8%) and B-356 had the highest (32.6%) hydrophobicity values. This indicates that strain A-025 with the lowest hydrophobicity value, may remain in the water for longer periods than the other strains and therefore, be the most mobile in a soil profile through passive transport with water. In contrast, strain B-356 has the highest hydrophobicity which suggests that this strain would remain in the water for the least period of time and due to the likelihood of binding to soil particles or other surfaces it would be the least mobile through passive transport with water.

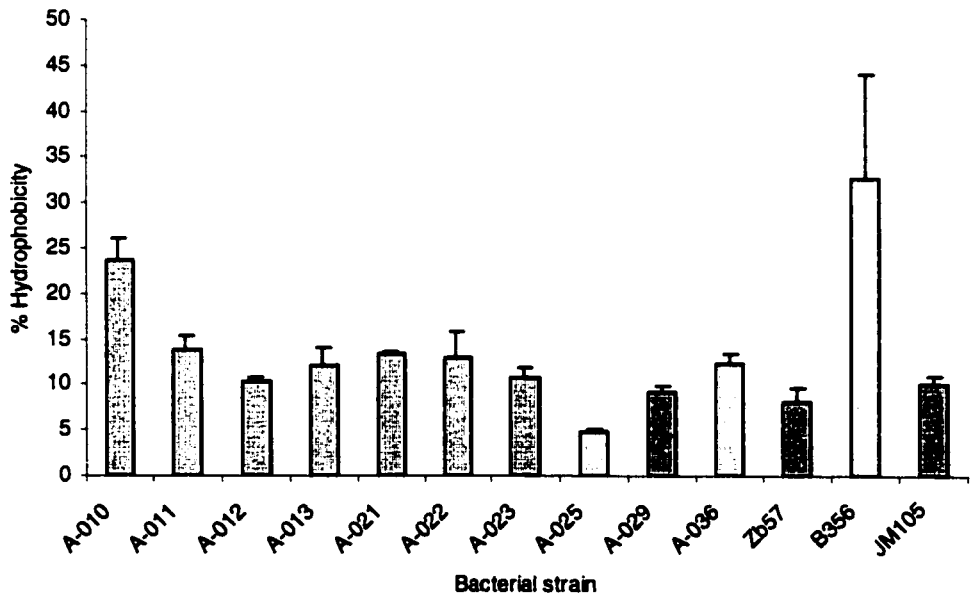

Figure 3.4. Hydrophobicity of bacterial strains. Average and standard deviation of triplicates.

3.3.5 Adhesion of Bacteria to Soil

Strain JM105 showed the highest adhesion value of 83.7% and thus, the highest affinity to bind to the tested soil (Figure 3.5). It would therefore, remain in water for the least period of time among all other strains studied. Due to this shorter retention time period, it would be the least mobile in a soil profile through passive transport with water and would travel the least distance. Strain A-013 with 29.5% adhesion value, would probably be the last strain to adhere to the surfaces of soil particles and therefore, would stay in water for a longer period than all the other strains studied and be transported to a longer distance as well.

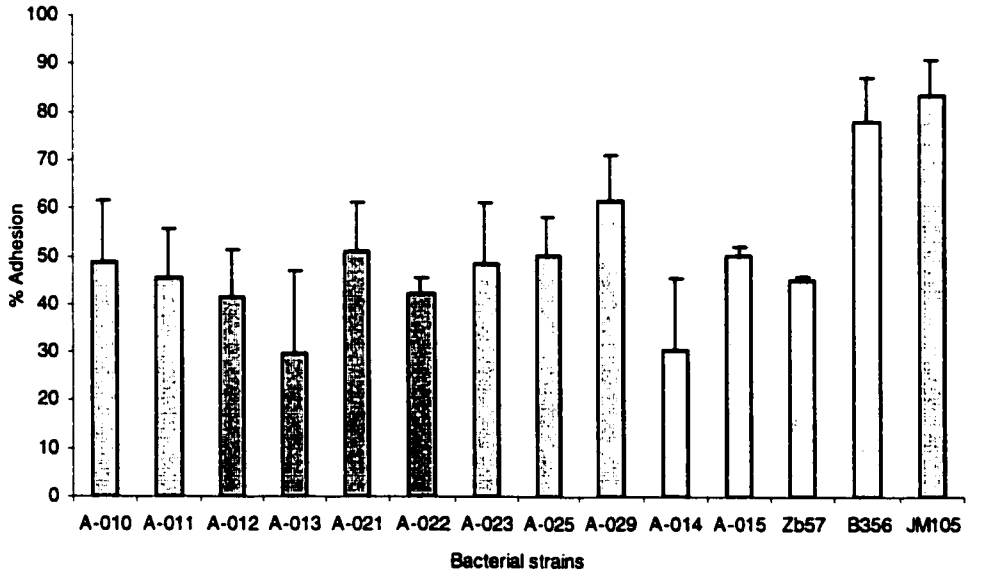

Figure 3.5. Adhesion of bacterial cells to soil S-VI. Average and standard deviation of triplicates.

Huysman and Verstraete (1993) have also reported similar results and formulated that cell retention in soil is a function of the type of bacterium as well as the soil surfaces. They reported that the migration of a bacterial cell with hydrophobic surface properties was 2 to 3 times slower compared to that with hydrophilic properties, correlating this with an increased adhesion of the hydrophobic strains to soil particles. In the study presented here, both non-rhizobial strains, *E. coli* (JM109) and *C. testosteroni* (B-356), showed higher level of adhesion than those found among rhizobial strains. However, the hydrophobicity values for *E. coli* was similar to those found among rhizobial strains where as, *C. testosteroni* showed a significantly higher level (33%). Also, lower variability among the hydrophobicity values for rhizobial strains was observed than those for adhesion. Furthermore, the two non-rhizobial strains were outliers to the cluster that was formed by the rhizobial strains (Figure 3.6).

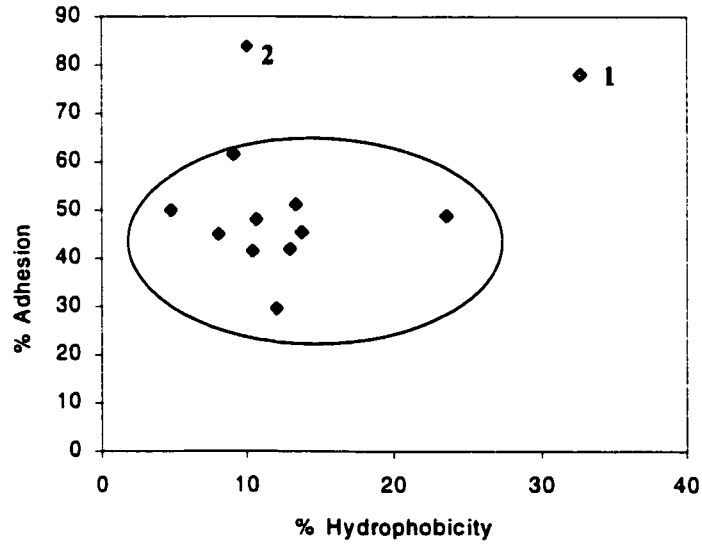

Figure 3.6. Distribution pattern of bacterial strains representing the relationship between hydrophobicity and adhesion. The rhizobia are clustered within the circle. 1 and 2 represent B-356 and *E. coli*, respectively.

3.3.6 Effect of Growth Media on Hydrophobicity and Adhesion of Cells

Both hydrophobicity and adhesion were significantly different for the rhizobial strains grown on TYc or YEMP. The hydrophobicity of A-023, A-025, A-036, and Zb57 decreased by 91, 79, 87, and 80% respectively when the strains were grown on YEMP (Figures 3.7). The adhesion of strains A-023, A-025, and Zb57, decreased by 85, 97, and 58% respectively when grown on YEMP (Figure 3.8).

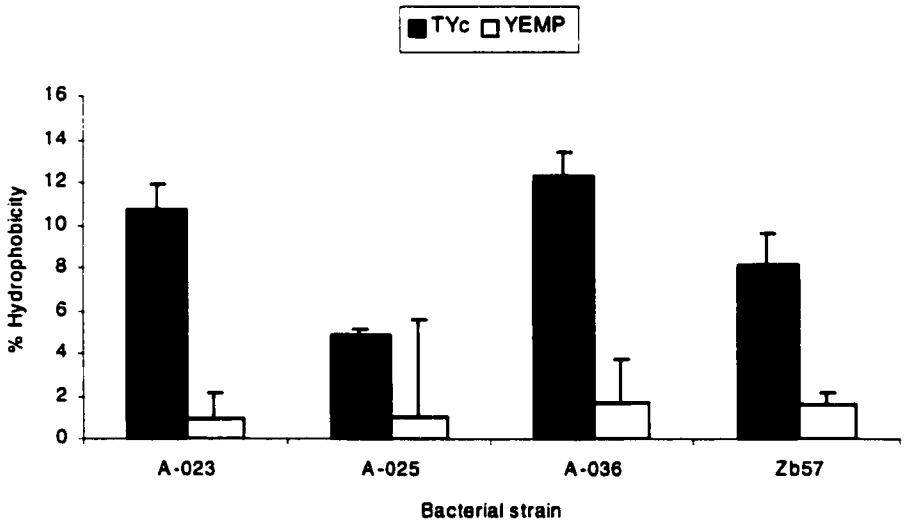

Figure 3.7. Hydrophobicity of *R. meliloti* strains grown on different media. Results represent the average of three replicates and the standard deviation.

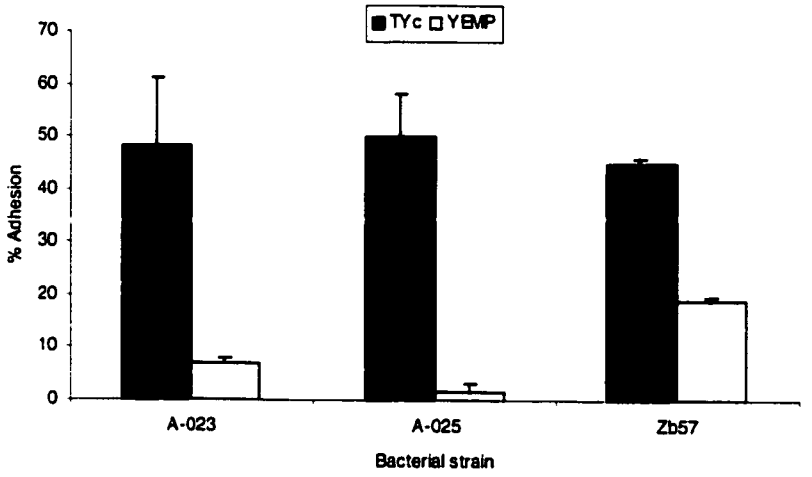

Figure 3.8. Adhesion of different *R. meliloti* strains grown on different media. Results represent the average of three replicates and the standard deviation.

The difference in the hydrophobicity and adhesion of these bacterial strains when grown on different media could be explained by previous work done on membrane characteristics of bacteria. Neu (1996) reported that microbially produced surface-active compounds (SACs) such as fatty acids, lipids, peptides, and polysaccharides, may interact with interfaces and affect the adhesion and deadhesion of bacteria. In a study by Shingaki et al. (1994), under acidic conditions, the negative cell surface charge became lower and at the most acidic condition tested (pH 2.4), 34 of the 40 strains of bacteria studied showed a positive surface charge. This change in the cell surface charge caused by pH-shift from neutral to acidic was considered to be a result of loss of negative charge of functional groups existing or exposed at the cell surface. Some microorganisms are capable of excreting fatty acids, lipids, and biosurfactants into the surrounding

media and thus creating a microbially generated conditioning film at the interface. On a hydrophobic interface, this conditioning film will change the interface from hydrophobic to hydrophilic. This means that the hydrophilic cells but not the hydrophobic cells can interact with the interface (Neu, 1996). In the present study, the rhizobial strains produced large amount of mucus when grown on YEMP agar plates whereas when grown on TYc agar plates, distinct, non-mucoid single colonies were formed. This indicates that the growth media had an influence on the physio-chemical properties of the membranes of the bacterial strains studied.

The hydrophobicity and adhesion values are involved in the ability of the microorganisms to stay in water and have a lesser chance of binding to soil particles or other chemical compounds that might be present in the soil. These characteristics of the surface of the bacteria can help in decision making when bacterial transport by irrigation is to be considered. Some bacterial cells prefer high moisture levels and are able to remain in water for longer periods of time; i.e. hydrophobicity of the cells can determine which bacterial strain would stay detached in water longer and thus be transported to longer distances by water. Most bacteria adhere to soil particles within a short time after they are introduced into the soil. By measuring their adhesion to soil particles, a reasonable estimate of how the introduced cells would behave in a soil matrix can be made. Factors such as the type of contaminants in the soil, the clay content, and the bulk density of the soil, play an important role in the adhesion of the cells to the soil particles.

The present report provides evidence for the presence of a variety of Rhizobial strains in soils contaminated for a long time with

aromatic/chloroaromatic hydrocarbons, irrespective of the nature and amount of contaminant, and where no indigenous compatible host plant was present, using alfalfa as a trap plant for nodulation. All the new isolates from the contaminated soils, although different in their colony morphology, antibiotic sensitivity, megaplasmid numbers and profiles, species-specific IS*Rm*5 RFL hybridization patterns and random genomic DNA amplification patterns, were effective for symbiotic N_2-fixafion and carried *bph*-homologous sequences (Ahmad et al., 1997).

Expecting Rhizobia to inhabit environments with aromatic pollutants is not surprising since they have long been known to metabolize simple aromatic compounds (Chen et al., 1985, 1989) and to possess ring-cleavage enzymes (Chen and Lovell, 1990). Neither is the fact that rhizobia, as natural degraders of aromatic pollutants, remained unidentified for such a long time, as the focus of all such rhizobial studies has always been oriented toward their agricultural significance, e.g. in competition and survival as inocula, in nodulation and chemotaxis toward plants, in the search for inducer molecules for N_2-fixation and as signal transduction agents of microbe-plant cross-talks, etc. Several other explanations may be added. They may have been selected against and remained unidentified as degraders of aromatic compounds on traditional selection media because of their slow growth rates and fastidious growth requirements (Somasegaran and Hoben, 1994; Amann et al., 1995; Encarnacion et al., 1995). The traditional host plant nodulation method seems to under represent the number and the type of rhizobia actually present in the soil (Somasegaran and Hoben,

1994; Van Rhijn and Vanderleyden, 1995) and the frequency of different genotypes obtained by the nodulation method differs markedly from that obtained directly from soil (Bromfield et al, 1995). Furthermore, several recent reports indicate the possibilities of the presence of a large and diverse soil population of uncharacterized rhizobia, of a non-nodulating non-symbiotic population of known rhizobia (Soberon-Najera and Najera, 1989; Segovia et al., 1991; Laguerre et al., 1993) and of free-living rhizobium-related bacteria (Jarvis et al., 1989). In conclusion, there may be a significant number of rhizobial species, subspecies, or related bacteria present in contaminated soils that are unknown. The Rhizobial strains isolated in this study may well be representing only a fraction of all rhizobial types actually present in the contaminated soil. A detailed study of this nature will be of interest for its ecological, environmental, and economical implications, and feasible to carry out using molecular biological tools.

In a contaminated site, the pollutant concentration and soil organic content can vary a great deal at different locations, and accordingly the bioavailability and exposure of pollutants to the microorganisms present. Thus, the distribution of rhizobia for that matter of any microorganism may vary accordingly at different locations. PCBs and PAHs are in general not inherently toxic to bacteria. This study provides evidence for the presence of PCB and PAH tolerant strains of rhizobia in soils. Several reports demonstrate the tolerance of rhizobial species to environmental stresses, such as heat and desiccation (Trotman and Weaver, 1995), high/low oxygen tension (Encarnacion et al., 1995), soil acidity (Chen et al., 1993), osmolarity, (Smith et al., 1994), metal contamination (Chaudri et al., 1992;

Smith and Giller, 1992), etc. Smith and, Giller (1992) have isolated effective *R. leguminosarum* from soils contaminated with heavy metals. Several other reports, as well, show the tolerance of different species of rhizobia to heavy metals (Chaudri et al., 1992; Kinkle et al., 1994). Thus, it seems that tolerant and effective strains of rhizobia may be quite well spread.

The maintenance of the nodulation and N_2-fixation ability in the absence of a compatible host plant in all new isolates is interesting. However, the nature of the selective pressure is not obvious at the moment. One far fetched explanation could be that the ability of free-living rhizobia to fix atmospheric N_2 under microaerophilic conditions (Tjepkema and Evans, 1975; Takahashi, 1993) may be selectively advantageous for its survival under the low N environment prevalent at the contaminated sites. Indeed, several free-living N_2-fixing bacteria have been shown to grow and fix N_2 under microaerophilic conditions using aromatic hydrocarbons (Chen el al., 1993). If rhizobial PCB degradation is indeed related to its ability to catabolize certain aromatic plant products, these compounds may be useful as biostimulators in soil decontamination operations using rhizobia. The fact that some PCB degraders have been shown to be able to grow on a variety of compounds that are plant products (Donnelly et al., 1994) lends support to this hypothesis. Work in our laboratory is underway to explore this possibility.

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PREFACE TO CHAPTER 4

Attempts to deliver bacteria to deeper levels of a contaminated soil have proven to be very difficult with the conventional methods such as surface application of the microorganisms followed by surface irrigation or tilling. However, subirrigation has proven to be an effective method for the delivery of nutrients (Ugwuegbu, 1997). Because subirrigation leads to a higher degree of saturation and frees the soil of air pockets, it could also serve as an efficient system for the delivery of microorganisms to deeper zones of contaminated sites. Bacteria can be distributed more efficiently into the soil by using water as a transport medium. Because of the heterogeneity of soils at different sites, it might be necessary to force water into the soil by a pump and thus increase the flow or the volume of water. Furthermore, such water table management (WTM) systems can also be used to create aerobic (drained soil) and anaerobic conditions (saturated soil). Often, both functions are required for a complete bioremediation process.

Chapter 4 describes the procedures and results of the bacterial transport study. This experiment helped us to investigate whether subirrigation could be used as a technology to transport and implant bacteria at different location and depths of soil columns. This technique was compared to the more traditional method of bacterial inoculation, surface irrigation.

The results will be submitted for publication as: Mehmannavaz, R., Prasher, S.O. and Ahmad, D. Comparison of Surface and Subsurface Irrigation as Microbial Delivery Tools for In Situ Bioaugmentation: Transport, Distribution and survival in Large Packed Saturated Soil Columns.

CHAPTER 4

COMPARISON OF SURFACE AND SUBSURFACE IRRIGATION AS MICROBIAL DELIVERY TOOLS IN SOIL COLUMNS

ABSTRACT

This study describes the testing of a subirrigation method for delivering bacteria capable of degrading contaminants to unsaturated soil zones. Delivery, transport and bacterial distribution were studied in large stainless steel columns 1000 mm long and 200 mm in diameter, packed with a sandy loam soil. Transport, implantation and survival of *Rhizobium meliloti* (strain A-025) after surface and subsurface irrigation were compared. Results showed that the transport of bacterial cells was related to adsorption/desorption of the cells to the soil particles. Even though, transport of cells was slower by subirrigation than surface irrigation, higher numbers of bacteria were implanted at different depths using subsurface irrigation. The number of bacterial cells distributed for subsurface irrigation were $1.6x10^5$, $1.3x10^5$, $2.6x10^5$ and $2.9x10^5$ cell·g^{-1} of soil and for surface irrigation, they were $2.0x10^4$, $3.0x10^4$, $1.9x10^5$, $9.0x10^2$ cell·g^{-1} of soil at 60, 300, 500 and 700 mm depths, respectively. These results were a clear indication that subirrigation could be used to bioaugment a sandy loam soil.

4.1 INTRODUCTION

Bioremediation techniques for soils include bioaugmentation (introduction of more efficient organisms, manipulated exogenous or indigenous strains) or biostimulation (improvement of growth conditions for indigenous organisms). The success of biostimulation is primarily guaranteed by the affinity of indigenous microflora to a given contaminant. When this is not the case, the option of bioaugmentation is a viable alternative. Bioaugmentation involves the introduction of a species or community of specialized bacteria to the soil. However, to be effective, the bacteria should be delivered throughout the contaminated zone and adequate conditions should be provided for their subsequent proliferation and survival.

Bioaugmentation with bacterial strains that are better performers or adapted than the indigenous flora has been attempted for several ex situ- on site or off site soil biodecontamination operations (Cattaneo et al., 1997; Trevors et al., 1990; Venosa et al., 1992; Duba et al., 1996; Geerdink et al., 1996). However, there appears to have been no attempts at *in-situ* bioaugmentation treatments of contaminated soils. Because contaminated sites are usually inoculated at the soil surface, which is then plowed, turned over or simply surface irrigated to induce downward transport of the microorganisms (Cattaneo et al., 1997; Duba et al., 1996; Venosa et al., 1992; Trevors et al., 1990; Natsch et al., 1996; Lovins et al., 1993), success in delivering desired microorganisms to depths greater than 0.5 m has been difficult (Breitenbeck et al., 1988; Gannon et al., 1991).

Downward transport of bacterial cells is limited due to a number of phenomena including adsorption and desorption of the cells onto soil particles, soil moisture distribution, and water movement (Cattaneo et al., 1997; Wan et al., 1995; Zyman and Sorber, 1988; Lance and Gerba, 1984; van Elsas et al., 1991; Thomas and Phillips, 1979; Natsch et al., 1996). Adsorption or cell retention is affected by soil chemical status (pH, CEC, organic matter, mineral content, etc.), surface properties of soil particles and soil structure (Huysman and Verstraete, 1993a; Zyman and Sorber 1988; Trevors et al., 1990; Madsen and Alexander, 1982; Cattaneo et al. 1997). Soil moisture distribution and water movement are interrelated and depend on the soil structural profile, position of drainage system, irrigation and climate conditions.

In this study, we propose an alternative to traditional surface inoculation and transport induction. The proposed system is based on water table management (WTM), a technology that was developed primarily to improve agricultural productivity (Broughton et al., 1987; Madramootoo et al. 1993) (Figure 4.1). WTM is essentially an application of subsurface drainage that is modified to also permit water flow upward through the soil profile when needed for subsurface irrigation requirements. Such a system might be quite effective at delivering necessary microorganisms to any level in the soil profile, as well as provide a system to maintain proper conditions for their effective transport, distribution, implantation and survival. Subirrigation has already proven to be an effective method for delivery of nutrients to different depths of 2000 mm soil columns (Ugwuegbu et al., 1997, unpublished results). Because subirrigation leads to a

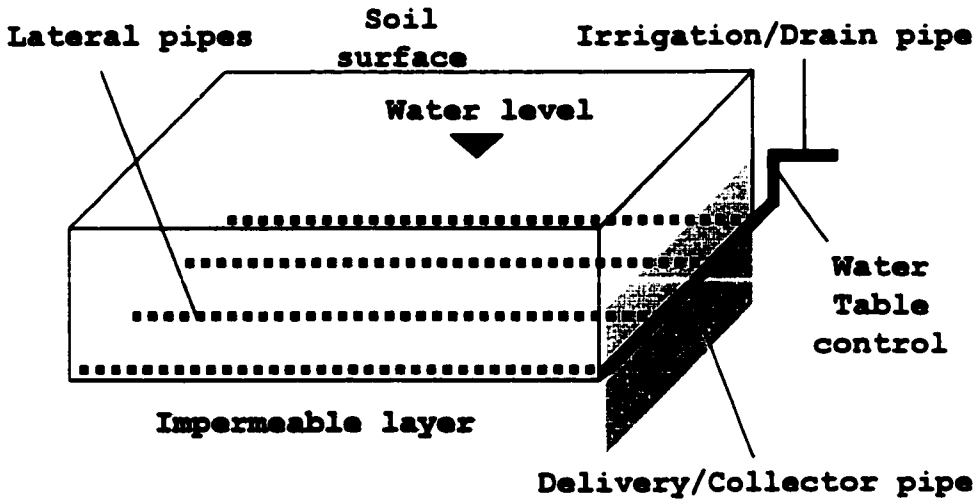

Figure 4.1. Schematic diagram of a Water Table Management system.

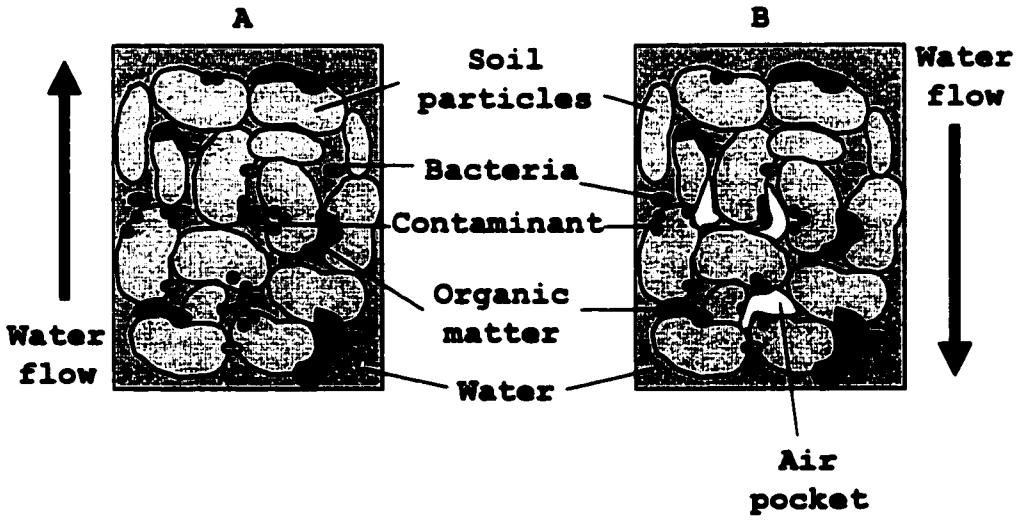

Figure 4.2. Schematic representation of a soil with A) Subirrigation and B) Surface irrigation.

higher degree of saturation and frees the soil of air pockets (Figure 4.2), bacteria can be distributed more efficiently into the soil, using water as a transport medium.

The main objective of this study was to evaluate transport and distribution of *R. meliloti* (strain A-025) (Ahmad et al., 1997), a strain with potential to degrade PCBs, to any location throughout the soil profile in large soil columns.

4.2 MATERIALS AND METHODS

4.2.1 Soil Used in the Bacterial Transport

The soil (S-VI) used was excavated from Macdonald Campus Farm after the topsoil layer was scraped off. It contained 78% sand, 3% silt, and 19% clay, as determined by the Hydrometer method (Liu and Evett, 1984). Organic matter was 3.59% by weight and the pH was 6.17.

4.2.2 Soil Column Fabrication and Packing

The soil was homogenized and packed into twelve stainless steel columns, 1000 mm long x 200 mm diameter. The columns were fitted with sampling ports on the side at 60, 300, 500 and 700 mm depths. At 980 mm, a subirrigation port was connected to a delivery pipe that had 2 mm wide slits at 25 mm intervals to supply water and bacterial cells into the column and to maintain the water table at desired levels (Figure 4.3). At the bottom, columns were packed up to 70 mm to create a gravel filter. The first layer of gravel was 30 mm deep with grain size >9.5 mm, the second layer was 20 mm with >4.75 mm particles and the third layer

was 20 mm with >2.36 mm particles. The soil was well mixed and packed on top of the gravel filter with 4.54 kg of soil for each 100 mm, for a total of 39 kg of soil and a height of 860 mm. The bulk density was 1,400 kg m^{-3} and the porosity was 39.6%. After packing, perforated Teflon tubes were connected to the sampling ports for drawing liquid samples. The columns were then flushed for a period of 8 days, by continuous saturated flow, using surface or subsurface irrigation. Flushing by surface irrigation was done by maintaining a 10 mm pond on top of the soil. The saturated hydraulic conductivity was measured at the end of the flushing period by the constant head method, which involved determining the time to collect 400 mL of water at the bottom of the columns (Liu and Evett, 1984). The columns were then drained. The final soil density in the columns was found to be around 1,600 kg m^{-3} due to settling of the soil.

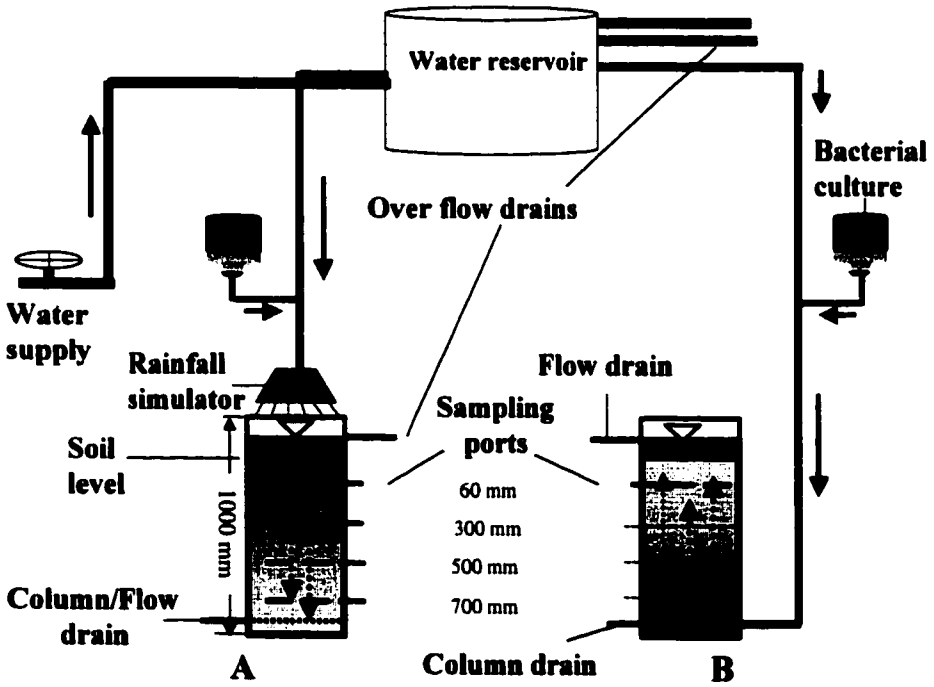

Figure 4.3. Schematic diagram of the experimental system used for bacterial transport with a) surface irrigation, and b) sub surface irrigation. (not to scale)

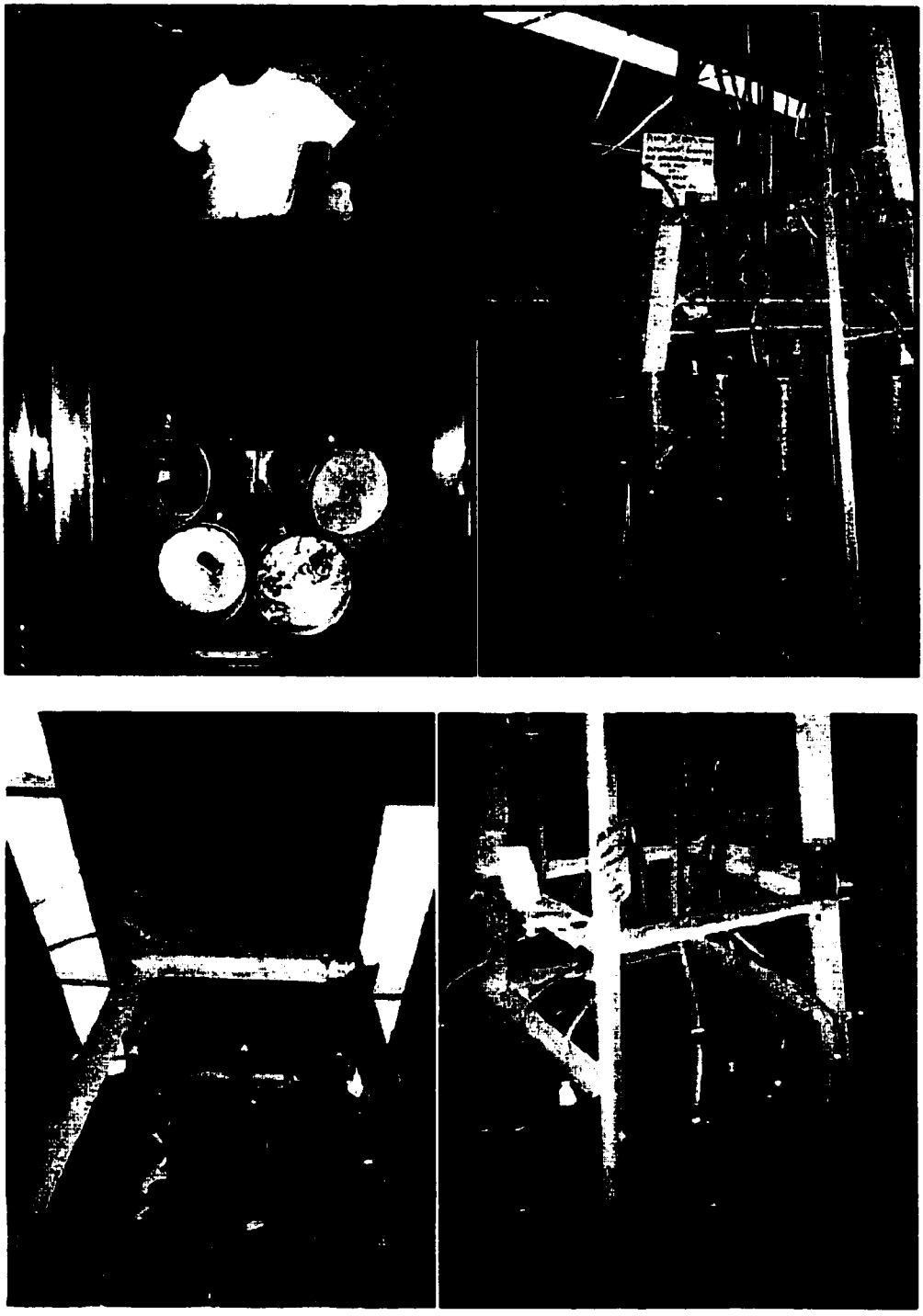

Figure 4.4. Photographs of the sterilization (autoclaving) and the experimental setup.

4.2.3 Conditioning of Soil Columns before Inoculation

Seven days after packing and flushing, the columns were sterilized by autoclaving for 1 hour each day for 3 consecutive days. All the pipes and tubes were sterilized by washing in 6.0% Sodium hypochlorite (household bleach) and then rinsed with the same tap water which was used in the experiment. Thus, after the initial sterilization, sterile conditions were not maintained. The columns were then saturated for 12 days with continuous saturated water flow to ensure maximum saturation. Samples were taken from the soil columns after 3 days of saturation period and plated on TYc (tryptone-yeast-calcium) and TYct (TYc with tellurium 400 mg L^{-1}) (Ahmad et al., 1997), selective medium for *R. meliloti*, then incubated at 29°C to test for microbial contamination and presence of *R. meliloti*.

The hydraulic conductivities of the surface irrigated columns were measured once again and the average hydraulic conductivity was used to adjust and control the flow rate for each column to about 1.8×10^3 mL h^{-1} for both surface and subirrigation systems.

4.2.4 Experimental Stages

Four treatments each with 3 replicates included: 1) subsurface irrigation with bacterial inoculum, 2) subsurface irrigation without bacterial inoculum, 3) surface irrigation with bacterial inoculum, and 4) surface irrigation without bacterial inoculum. The experiment was carried out at 25 - 27°C. Table 4.1 summarizes the experimental stages, their durations and treatments.

4.2.5 Preparation of Bacterial Cells for Inoculation

R. meliloti, strain A-025 (Ahmad et al., 1997) was inoculated into 50 mL of TYc in six 125 mL Erlenmeyer flasks, each with 50 mL from a two day old pre-culture, and incubated at 29 °C in a controlled environment incubator shaker (Psycrotherm, New Brunswick Scientific) for 24 h. It was then sub-cultured in 1 L of fresh TYc medium in six 2 L flasks and incubated for another two days. All six 1 L cultures were pooled (approximately 2.3×10^8 cell mL^{-1}), and a sample was plated on TYct agar plate to determine initial population counts. Bacterial cells were then harvested by centrifugation (Sorvall Instuments Dupont model RC5C) for 5 min at 8000 rpm in 250 mL centrifuge bottles, washed with 100 ml of 0.9% sterile saline, resuspended in 400 mL (to about 5.8×10^8 cell mL^{-1}) of sterilized deionized water and mixed thoroughly by vortexing before inoculation of the soil columns. The prepared bacterial suspension was then added to the columns at a flow rate of 2.4×10^4 mL h^{-1} (time of inoculation 1 min). After 140 hours (about 6 days), continuous saturated flow to all columns was stopped and columns were kept saturated for two more days to allow the bacterial cells to adhere and stabilize, and then drained.

4.2.6 Sampling and Microbial Analysis

Before inoculation of the soil columns with bacteria, soil-water samples were taken from each column to determine the initial microbial composition and status. Samples of 10 mL were taken from each sampling port at intervals of 14, 28, 56, and 84 h after inoculation to determine bacterial transport by percolating,

ascending and circulating water flows. The samples were kept at 4 °C in sterilized glass tubes till the next day, at which point they were plated for microbial analysis and count. Eight days after inoculation, the columns were drained and 10 g soil samples were taken for analysis of bacterial distribution in soil columns.

Bacteria were extracted from soil particles by vortexing 2 g of soil in 10 mL of sterilized 0.9% saline and serial dilutions were plated on TYct agar plates for counts of viable population. After the 10^{th} cycle of pore volume (6 days after inoculation), the columns were drained and soil samples were taken from four different depths to determine the distribution of implanted bacteria in soil. In addition to using TYct as a selective growth media, the identity of the transported bacteria, *R. meliloti,* was also confirmed by DNA hybridization tests (Results not shown).

4.2.7 Statistical Analysis

Bacterial transport was monitored by viable bacterial counts on selective nutrient agar plates various times after inoculation of the soil columns. The GLM (General Linear Model) Procedure and Repeated Measures Analysis of Variance (SAS, 1989) were used to analyze the data. Data from the control soil columns (not inoculated) was not used since plating showed that *R. meliloti* was never present.

Table 4.1. Various stages in the transport of *R. meliloti* in soil column study.

Stage & duration	Treatment	Subirrigation		Surface irrigation	
		Treated columns 6,10,11	Control columns 3,9,12	Treated columns 1,5,7	Control columns 2,4,8
1	Column packing	Packed with 39 kg of sandy soil to a bulk density of 1400 kg $(m^3)^{-1}$	Packed with 39 kg of sandy soil to a bulk density of 1400 kg $(m^3)^{-1}$	Packed with 39 kg of sandy soil to a bulk density of 1400 kg $(m^3)^{-1}$	Packed with 39 kg of sandy soil to a bulk density of 1400 kg $(m^3)^{-1}$
2 8 days	Soil column flushing	Columns flushed with continuous saturated flow	Columns flushed with continuous saturated flow	Columns flushed with continuous saturated flow maintaining 10 mm ponding	Columns flushed with continuous saturated flow maintaining 10 mm ponding
	Hydraulic conductivity	Hydraulic conductivity measured at the bottom of columns	Hydraulic conductivity measured at the bottom of columns	Hydraulic conductivity measured at the bottom of columns	Hydraulic conductivity measured at the bottom of columns
3 7 days	Drain columns	Soil columns let to drain	Soil columns let to drain	Soil columns let to drain	Soil columns let to drain
4 3 days	Sterilization	Soil columns autoclaved, 1 hour a day	Soil columns autoclaved, 1 hour a day	Soil columns autoclaved, 1 hour a day	Soil columns autoclaved, 1 hour a day
5 12 days	Saturation	Soil columns saturated	Soil columns saturated	Soil columns saturated	Soil columns saturated
	Flow rate	Flow rate adjusted to $1.8x10^3$ ml h^{-1}	Flow rate adjusted to $1.8x10^3$ ml h^{-1}	Flow rate adjusted to $1.8x10^3$ ml h^{-1}	Flow rate adjusted to $1.8x10^3$ ml h^{-1}
6 6 days (140 h)	Inoculation	400 ml bacterial suspension with continuous saturated flow	continuous saturated flow	400 ml bacterial suspension with continuous saturated flow	continuous saturated flow
	Sampling	Soil/water samples were taken at 14, 28, 56, 84 h after inoculation	Soil/water samples were taken at 14, 28, 56, 84 h after inoculation of treated columns	Soil/water samples were taken at 14, 28, 56, 84 h after inoculation	Soil/water samples were taken at 14, 28, 56, 84 h after inoculation of treated columns
7 2 days	Flow rate	Stopped water flow	Stopped water flow	Stopped water flow	Stopped water flow
8 7 days	Drain columns	Drained columns, Hydraulic conduct. measured	Drained columns, Hydraulic conduct. measured	Drained columns, Hydraulic conduct. measured	Drained columns, Hydraulic conduct. measured
9	Sampling	Soil samples taken after columns were drained	Soil samples taken after columns were drained	Soil samples taken after columns were drained	Soil samples taken after columns were drained

4.3 RESULTS AND DISCUSSION

Bacterial cells in the inoculum are transported primarily by water movement, a phenomenon which is inhibited by the adhesion of cells to soil particles, which in turn, is limited by the number of available sites. Therefore, the number of cells present at any depth is a function of initial cell numbers, soil structural and chemical characteristics, the extent of water movement, and the time period involved. Thus, at any given time, the bacterial population is partitioned into three categories: cells that have adhered to the soil matrix, cells that are free in the soil-water but will be implanted at a farther site in the profile, and cells that will simply be leached through the entire profile to the drainage water. In this study, it was hypothesized that the bacterial distribution in these categories depends on whether the soil is inoculated at the surface and water applied at the surface, or whether delivery of bacteria is done through a subsurface irrigation system. In both cases, the systems were open, i.e., water flow was continuous and drainage water was not recycled to the soil matrix.

4.3.1 Bacterial Transport

Bacterial transport was studied by sampling the soil water at various depths and times (Figure 4.5), and the drainage water at various times (Figure 4.6). The main conclusions that one may draw from Figure 4.5 is that bacterial counts in soil-water are generally higher for the surface irrigation system than for the subirrigation system, particularly over the first 28 hours. At the same time, the number of cells reaching drainage outflow is initially four times greater from the

subirrigation system than from the surface irrigation system. However, leached cells represent only a small proportion of the total cells applied, essentially 1-4% of the total number of cells applied. These figures indicate that there are more free cells in the soil-water when surface inoculation and irrigation is used than when a subirrigation delivery approach is taken.

It is also clear that subirrigation delivery is effective in transporting cells upward, as indicated by the number of cells present in soil-water at 60 mm below the surface 14 hours after inoculation. About 18% of the cells (1.33×10^6 cells) had been transported up wards from the inlet to this level. This agrees with Breitenbeck et al.'s (1988) observations that cells of *B. japonicum* were readily transported by an advancing wetting front and that the non-saturated flow of soil water also contributed to the dispersal of inoculum in the soil.

As one would expect, the number of cells in the soil-water at any depth, eventually decreases to very low levels as compared to the initial 14-hour period regardless of the system used. This reflects adhesion and leaching together, whereas the continued presence of cells in the soil-water is likely due to desorption. At such low cell numbers, few cells can be leached through the system, as indicated by the extinction of cell presence in the drainage water with time (Figure 4.6). The statistical significance of treatments is shown in Table 4.2.

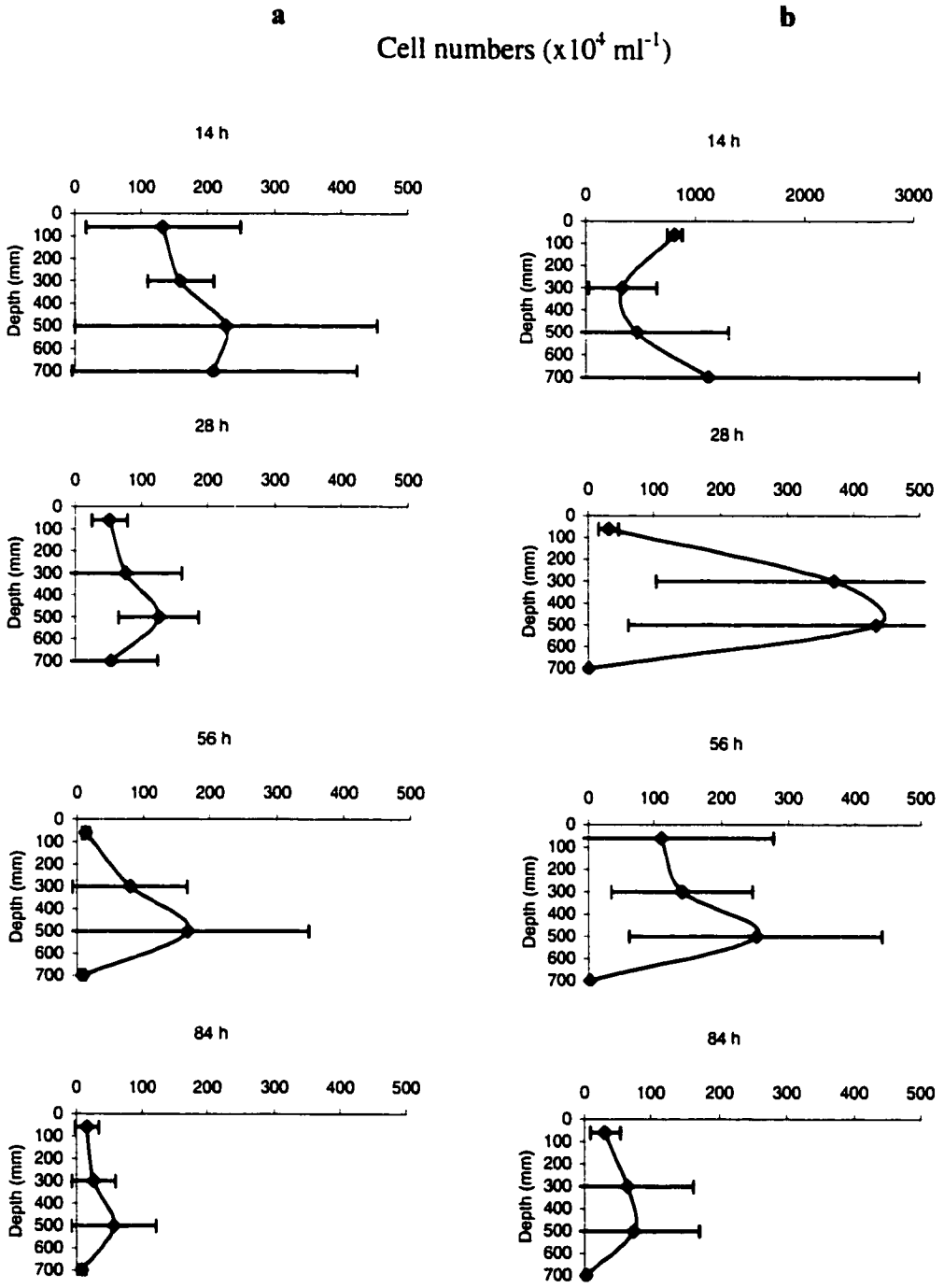

Figure 4.5. Transport of *R. meliloti*, A-025, by water in soil columns by a) subirrigation, b) surface irrigation at various times and depths. The time indicated represents the period after inoculation. Mean and standard deviation of triplicates.

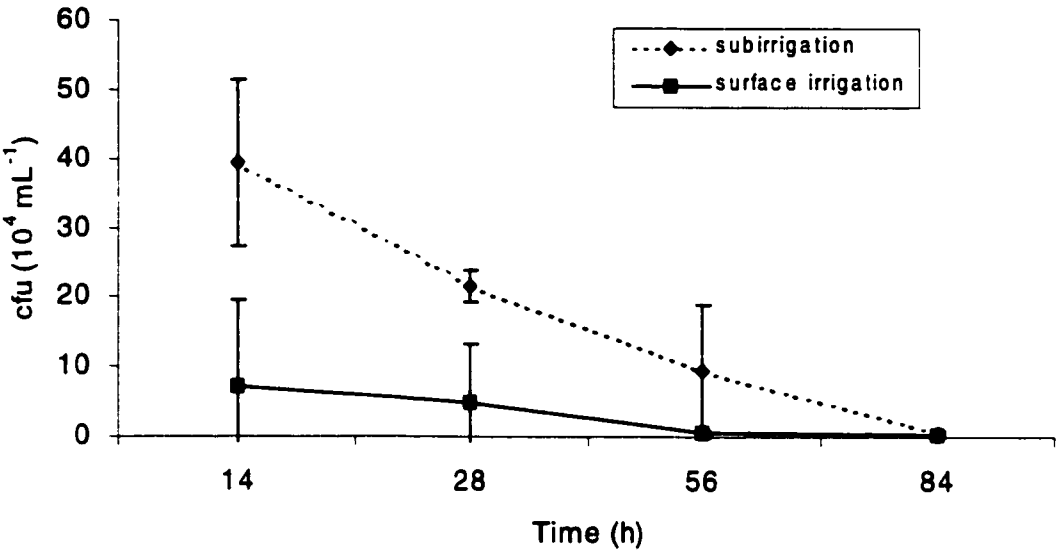

Figure 4.6. Number of bacterial cells in the drain water from the top (subirrigation) and bottom (surface irrigation) flow drains. Mean and standard deviation of triplicates. Time represents the period after inoculation.

4.3.2 Adherence, Implantation and Survival

Microbial analysis of soil samples taken after the soil columns were drained (14 days after inoculation), shows that much higher numbers of bacteria were implanted at different depths of the soil columns and more evenly distributed with subirrigation than with surface irrigation (Figure 4.7). The number of bacterial cells at 60, 300, 500 and 700 mm depths were $1.6x10^5$, $1.3x10^5$, $2.6x10^5$ and $2.9x10^5$ cell g^{-1} of soil with subsurface irrigation and $2.0x10^4$, $3.0x10^4$, $1.9x10^5$ and $9.0x10^2$ cell g^{-1} of soil with surface irrigation, respectively.

Differences in implantation due to treatments were significant at all depths (Table 4.3) except at 500 mm, the depth at which the number of free cells was highest at all times in surface-irrigated columns. Although populations in the surface-irrigated columns would be expected to increase with time given appropriate conditions, it is not clear as to how long it may take for them to reach the same levels as populations in subirrigated columns.

Huysman and Verstraete (1993b) state that the inoculation and irrigation method greatly influences the degree of transport. At high irrigation rates, water flow through macropores would increase, thus minimizing filtering of bacteria by the soil matrix. When the water flows at a slower rate, bacteria move through the soil in a thinner water film and are drawn nearer to the soil particles. This increases the potential for adhesion of bacteria and decreases transport. Decreasing the flow rate, even at saturation, should decrease cell movement in the soil (Lance and Gerba, 1984). A study by Trevors et al. (1990) showed that transport of cells introduced into 1 cm of the vertical soil microcosms was dependent on the flow rate of water and the number of times microcosms were flushed with water. Introduced cells were detected in the effluent water samples even after three flushes of ground water and 10 days of inoculation.

Table 4.2. Statistical analysis of the bacterial transport in the water.

Source	Pr > F	Source	Pr > F
Time	0.0454	Treatment	0.1425
Time*Treatment	0.5339	Depth	0.5905
Time*Depth	0.6050	Treatment*Depth	0.8599
Time*Treatment*Depth	0.7826		

Table 4.3. Statistical analysis of the bacterial implantation in the soil.

Source	Pr > F
Treatment	0.0253
Depth	0.3034
Treatment*Depth	0.5809

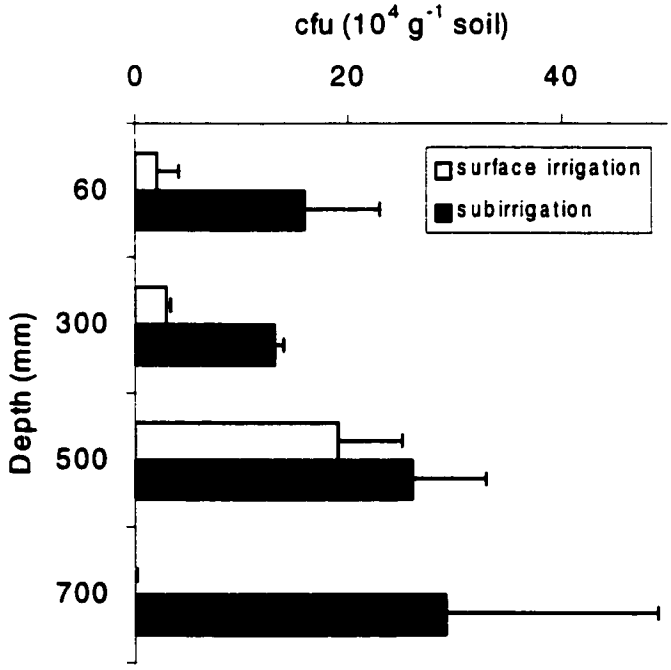

Figure 4.7. Implantation and survival of *R. meliloti*, A-025, at different depths of soil columns 14 days after inoculation. Mean of three replicates and the SD.

Considering the above findings, subirrigation could have other advantages over surface irrigation. The hydraulic conductivity of soil columns was found to be 9.52×10^3 mL hr^{-1} for subirrigation and 2.33×10^3 mL hr^{-1} for surface irrigation at an initial bulk density of 1,400 kg m^{-3}. This indicates that a higher flow rate could be used in the subirrigation system than in the surface irrigation. Thus, higher numbers of cells can be transported by subirrigation in the same time interval. Also, because of the heterogeneity of soils at contaminated sites, it might be necessary to force water into soil by a pump, an impossibility using surface irrigation due to infiltration limits at the soil surface. Furthermore, such WTM systems can also be used to create aerobic (drained soil) and anaerobic conditions (saturated soil), as needed, to ensure survival of the bacterial population and, in turn, a complete bioremediation process.

As shown in Figure 4.8, the flow rate of drainable porosity was also measured before and after treatment for both surface and subirrigation systems to determine if the flow rate would differ due to soil microbial inoculation. It is evident that the flow rate in subirrigation (Figure 4.8a) was higher than in surface irrigation (Figure 4.8b). Furthermore, the before and after effect in subirrigation is higher than for surface irrigation. Most of the difference is due to the settling of soil, causing an increased bulk density and a concomitant decrease in the flow rate. In both systems, even though statistically insignificant, a higher flow rate was maintained in the inoculated columns than in the respective control columns, as also observed by Ugwuegbu et al. (1997). However, this is contrary to previous observations (Shouche et al. 1994; Jennings et al., 1995; Vandevivere and Baveye,

1992; MacLeod et al., 1988; Vandevivere, 1995; Vandevivere et al., 1995) that suggested an increase in the population of micro-organisms or biomass might cause clogging and thus decrease the flow rate. Discrepancies in results from one study to another suggest that the effect of proliferation of bacterial populations is not unique. We suggest that the influences on water movement depend on soil structure and pore distribution, since it is possible that clogging of micropores could divert water from matrix flow to macropores, such that the overall effect in some situations is to increase water movement. Alternatively, increased macropore flow may not make up for reduced matrix flow.

It has been suggested that WTM is the best management system for controlling agricultural pollution (Munster et al., 1996; Liaghat and Prasher, 1996; Liaghat et al., 1996; Jebellie and Prasher, 1996) and to supply nutrients and air to enhance bioremediation of a diesel contaminated soil (Ugwuegbu et al. 1997, submitted). In this study, it was found that such WTM based on subirrigation provides better transport and implantation of bacteria in the soil profile than surface irrigation. WTM should, therefore, be extremely versatile and useful for efficient and economical bioremediation of soils contaminated with agricultural or industrial pollutants. Furthermore, this technology can be easily used for supplying beneficial microorganisms for promoting crop production and biological pest control management, as well as for injecting air, solvents and other required elements.

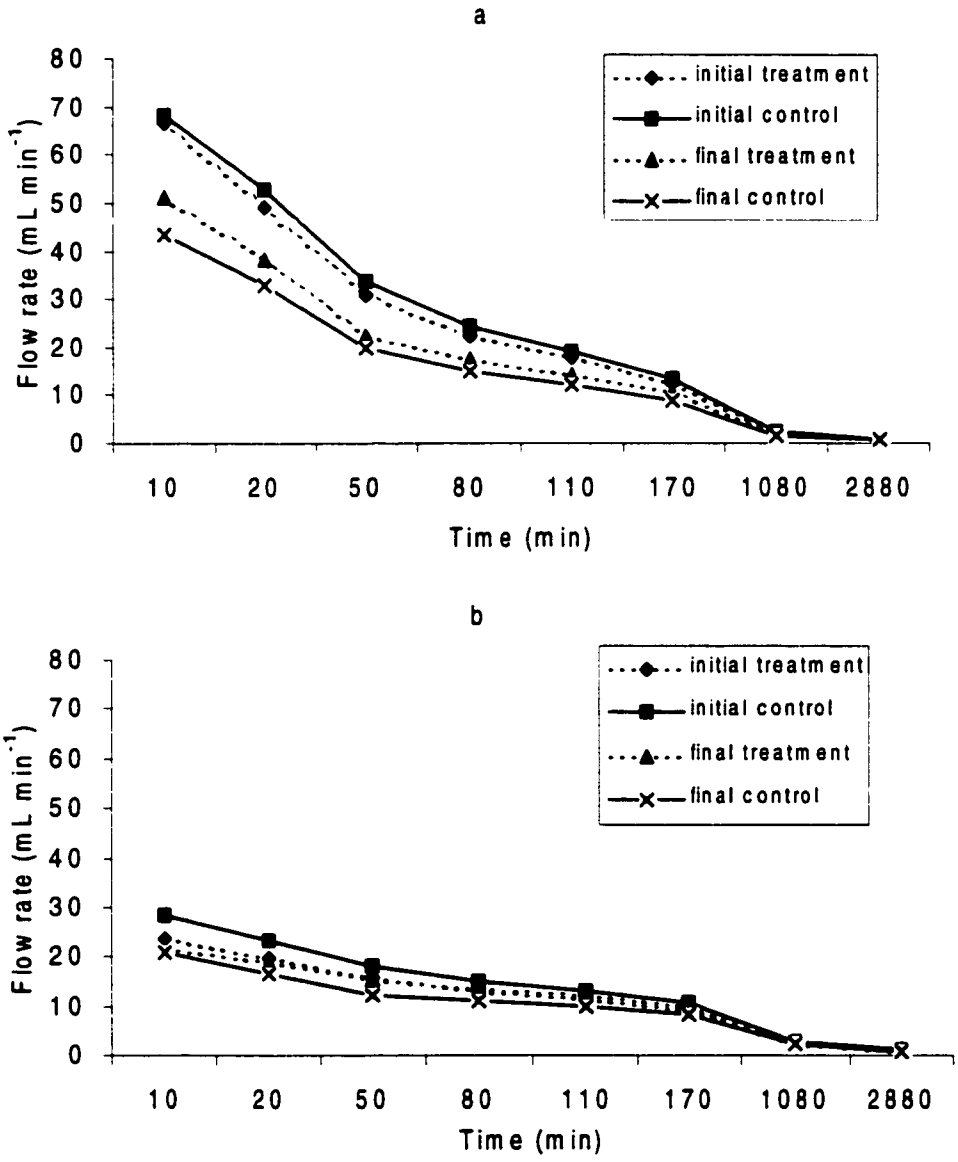

Figure 4.8. Flow rate of drainable porosity with a) subirrigation, b) surface irrigation.

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PREFACE TO CHAPTER 5

The isolation of *R. meliloti*, their membrane characteristics (hydrophobicity and adhesion), and transport of strain A-025 by water was investigated in Chapters 3 and 4. In Chapter 3, biodegradation of biphenyl and 4-chlorobiphenyl by the isolated strains assayed in microtitre plates was demonstrated. However, the evidence was based only on the color change of the redox indicator and thus the certainty in the mechanism of PCB degradation by these organisms was not clear. Therefore, it was necessary to investigate the role of these organisms in PCB biodegradation.

In this chapter, dechlorination and degradation of PCB congeners present in Aroclor 1242, by the two newly isolated strains of *Rhizobium meliloti*, strains A-025, A-029, was investigated. These strains were compared to *R. meliloti* strain Zb57, a laboratory strain that has shown some potential to degrade some chloroaromatic organics (Damaj and Ahmad, 1996). These bacterial cultures were inoculated in sterile sandy loam soil microcosms that were artificially contaminated with 100 ppm of Aroclor 1242, with and without $CaCO_3$. Dechlorination of congeners was investigated over an experimental period of 388 days. Chapter 5 describes the experimental procedures and the results obtained in this study.

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CHAPTER 5

BIOTRANSFORMATION OF AROCLOR 1242 BY THREE IMPLANTED *RHIZOBIUM meliloti* STRAINS IN STERILE SOIL MICROCOSMS

ABSTRACT

Biotransformation of PCBs (Aroclor 1242) by certain strains of *Rhizobium meliloti* isolated from PCB contaminated soils was investigated. *R. meliloti* strains A-025, A-029, and Zb57 were inoculated in sterile sandy loam soil microcosms that were artificially contaminated with 100 ppm of Aroclor 1242, with and without $CaCO_3$. Transformation of congeners was investigated through out the experimental period of 388 days. Strain A-025 microcosm was subjected to 165 days of aerobic (unsaturated) conditions and a subsequent period of 265 days of anaerobic (saturated) conditions. In presence of $CaCO_3$, all strains showed better survival and the rates of biotransformation were observed to be higher. The results indicated that the presence of $CaCO_3$ increased the soil pH to 7.5 from the initial pH of 6.17. Most of the degradations was associated with the *di-*, *tri-*, and *tetra-*chlorobiphenyls. GC-ECD analysis indicated *meta* and *para* dechlorinations to be the major pathways with all three strains. The degradation pattern for different congeners was variable. In the presence of $CaCO_3$, congeners 6, 7, and 3 were depleted up to >20% by strains A-025, A-029, and Zb57, respectively. Congeners 2,2' and 2,6 (peak 4) were the highest depleted congeners with strain A-025 up to

50% and strain Zb57 up to 40%. Congener 2,2',3,3',5 (peak 47) was decreased up to 80% by strain A-029. In the absence of $CaCO_3$, reduction was observed mostly in penta- and hexa- congeners with strains A-029 and Zb57. However, strain A-025 was able to transform 9 congeners >20% with peaks 11 (congener 2,2',5) and 30 (congeners 3,4,4' and 2,2',3,4') to more than 40% in periods of aerobic followed by anaerobic conditions. The results indicated that all tested strains were able to transform Aroclor 1242 differently and the rates of transformations were influenced by $CaCO_3$.

5.1 INTRODUCTION

Only a limited number of rhizobial strains have been investigated for aromatic metabolism. Chen et al. (1984) and Chen et al. (1985) demonstrated that Rhizobia are capable of metabolizing simple aromatic substrates as the sole source of carbon and energy for growth. They also indicated that even closely related species may differ in their pathways of aromatic catabolism. Hussein et al. (1974) showed that *R. meliloti* is capable of degrading up to 90-94% of catechol.

A recent study in our laboratory showed that the genomic DNAs from four different strains of rhizobia studied, hybridized with that of a DNA probe made from a PCB degradation gene (*bph*) from *Comomonas testosteroni* (Damaj and Ahmad, 1996). The results suggested the presence of a similar genetic system in the tested rhizobial strains similar to the BP/PCB dioxygenase system found in Pseudomonads. A short term study also showed degradation of PCB congeners containing up to five chlorine atoms and a degradation of 40% was observed for

Aroclor 1242. However, metabolic intermediates could not be identified to establish a pathway. Thus, the link between PCB degradation, the metabolic and genetic identity of the implicated system remains to be explained and defined.

In general, aerobic biodegradation of PCBs has been well studied. It has been suggested that preferential ring fission of PCBs depends on the number and position of chlorines on the biphenyl rings and that it occurs in non-chlorinated or lesser chlorinated rings (Furukawa et al., 1979). Another factor that is likely to decrease the rate of PCB degradation is accumulation of toxic metabolites from chlorobiphenyls that interact with the enzyme activities involved in PCB transformation (Sandossi et al., 1992; Guilbeault et al., 1994).

Relatively few studies have investigated the dechlorination and biodegradation of PCBs under anaerobic conditions (Van Dort and Bedard, 1991; Brown et al., 1987; Berkaw et al., 1996; Ofjord et al., 1994; Nies and Vogel, 1990). The reductive dechlorination of PCBs in anaerobic environments is a recently discovered phenomenon that is potentially important for environmental fate of PCBs. It has significant implications for both risk assessment and bioremediation strategies. Dechlorination is expected to reduce the toxicity of PCB mixtures and should also make them more aerobically degradable. Therefore, a sequential anaerobic/aerobic microbial process has the potential to degrade PCBs more effectively. Dechlorination of Aroclor 1242 was first demonstrated in the laboratory by Quensen et al. (1990) using Hudson River (NY) sediment. Reductive dechlorination of PCBs is suggested to be a two-electron transfer reaction that involves the release of chlorine as a chloride ion and its replacement

on the aromatic ring by a hydrogen atom from water (Nies and Vogel, 1991). Bedard and Quensen III (1995) stated that there are five possible factors that determine whether a chlorine will be removed from any particular congener: 1) the microbial populations present, 2) the position (*ortho*, *meta*, *para*) of the chlorine relative to the opposite phenyl ring, 3) the surrounding chlorine configuration, 4) the chlorine configuration on the opposite ring, and 5) the incubation conditions (the Aroclor added, temperature, carbon nature and availability, electron acceptors present, salinity, and presence of other contaminants). In studies with mixed soil microflora, *meta*-isomer substitution on the benzene ring has led to slower degradation than the *ortho*-chloro substitution (Furukawa et al., 1979). It has been suggested that PCB-dechlorinating microorganisms do not metabolize the biphenyl skeleton of PCBs. They must therefore require a source of carbon other than PCBs. The organic matter present in the soil or sediment usually provides this carbon source (Bedard and Quensen III, 1995).

In this study, two newly isolated *Rhizobium meliloti* strains, A-025 and A-029 (Ahmad et al, 1997) and a laboratory strain Zb57 (Damaj and Ahmad, 1996) were implanted in a sterile sandy loam soil microcosm artificially contaminated with 100 ppm of Aroclor 1242. Biotransformation of different PCB congeners in Aroclor 1242 was followed for more than a year in the presence and absence of $CaCO_3$.

5.2 MATERIALS AND METHODS

5.2.1 Soil Preparation

A sandy loam soil (78% sand, 3% silt, 19% clay, 3.7% organic matter, 6.17 pH) excavated from the Macdonald Campus Farm of McGill University was fractionated on a 425 µm sieve to obtain fine particles. The fraction retained by the 425 µm sieve was used for addition of Aroclor 1242. The soil was sterilized through three successive autoclaving periods of 30 min each on 3 consecutive days. Soil fractions (<825 µm) of 590 or 600 g (dry weight) were transferred into 1L glass jars and, where required, 6 g $CaCO_3$ was added and mixed. An appropriate volume of Aroclor 1242 in hexane was mixed with 10 g of sterilized (>425 and <825 µm fraction) soil. The hexane was allowed to evaporate and the preparation was mixed with the soils in the jars that contained 590 g of soil, 24 h before bacterial inoculation. The final concentration of Aroclor 1242 in the soil was 100 ppm. The pH of the soil was measured after vortexing 5 g of soil with 5 mL of distilled deionized water for 60 s and settling for 10 min (Somasegaran and Hoben, 1994).

Three treatments were setup for each of the three strains, Zb57, A-025, and A-029. The three experimental setups were as follows: 1) Soil + Bacteria + Aroclor 1242 + $CaCO_3$, 2) Soil + Bacteria + Aroclor 1242, and 3) Soil + Aroclor 1242, as the abiotic control. The sampling and analysis of PCBs were done in duplicate for each microcosm.

5.2.2 Preparation of Bacterial Cells

R. meliloti strains A-025, A-029, and Zb57 were inoculated from precultures into 50 mL of TYc, in 125 mL flasks, which were subsequently sub-cultured two days later in 1 L of TYc, in 2 L flasks. The cultures were incubated at 29 °C in a controlled environment incubator shaker (Psycrotherm, New Brunswick Scientific) for 24 h. The bacterial cells from the 1L cultures (about $2.3x10^8$ cell mL^{-1}) were then collected by 5 min centrifugation (Sorvall Instuments Dupont model RC5C) at 8000 rpm. The cultures were pooled before centrifugation and plated on TYct (Kinkle et al., 1994) agar plates for population count. The cells collected were washed with 100 mL of 0.9% saline and resuspended in 100 mL of sterilized deionized water (about $2.3x10^{11}$ cell mL^{-1}) and mixed well by vortexing before inoculation into the soil jars (about $3.8x10^{10}$ cell g^{-1} of soil). The microcosms were incubated for 388 days at room temperature in jars with loosely screwed tops and were mixed with a spatula at sampling times. The microcosm with strain A-025 in absence of $CaCO_3$ was reinoculated, 123 days after the first inoculation, with about $7.1x10^9$ cell g^{-1} of soil. The second inoculum was added in 50 mL of sterilized deionized water which was enough to saturate the soil and create a 5 mm pond on top of the soil, thus creating anaerobic conditions.

5.2.3 Soil Sampling and Analysis

20 g soil samples were taken periodically from each microcosm while mixing the soil in the jars (smaller samples from several places in the microcosm

were pooled). The experimental period was 388 days after inoculation, except microcosm with strain A-025 that underwent 123 days of aerobic (after the 1st inoculation) followed by 265 days of anaerobic conditions (after the 2nd inoculation). Therefore, the conditions at 123 days were considered to be the initial conditions when analyzing the results of the anaerobic period. For all other microcosms, the total loss of PCBs was compared between the initial and 388th day. However, to eliminate the loss of PCB congeners due to adhesion to soil particles, the pattern of biotransformation during the 388 days was compared to the abiotic control for all microcosms.

5.2.4 PCB Extraction and Analysis

Two 3 g soil samples from the 20 g soil were mixed in 125 mL flasks with 6 mL of sterilized deionized water and Triton X-100 (30 µL) to enhance desorption of Aroclor from soil particles (Barriault and Sylvestre, 1993). Anhydrous sodium sulfate (6 g) was added to prevent the formation of stable emulsion (Bedard et al., 1986). Hexane (15 mL) was then added, and the flasks were shaken on a rotary platform for 20 min. The hexane fraction was collected on ammonium sulfate and the aqueous phase was extracted two more times. The pooled organic fractions was then evaporated to a final volume of 3 mL, equal to the weight of soil sample in grams (Barriault and Sylvestre, 1993).

The samples were analyzed with a gas chromatograph (Hewlett Packard 5890, series II) equipped with an electron capture detector (GC/ECD) and a HP-5 (phenyl methyl silicone) capillary column of 25 m by 0.32 mm. Helium was used

as a carrier gas at a flow rate of 1 mL min^{-1}. The initial temperature (60 °C) was held for 2 min, then raised to a final temperature of 290 °C at a rate of 5 °C min^{-1} and held for 15 min. Injector and detector temperatures were 275 and 350 °C, respectively.

A late eluting peak (51, IUPAC congener no. 110) of Aroclor 1242 resistant to biodegradation was used as an internal standard. The ratio between this peak and other congener peaks at different times of the experiment were compared to determine the changes in the concentration of each congener (Barriault and Sylvestre, 1993; Damaj and Ahmad, 1996). These ratios were then compared either to the peak ratios obtained from the abiotic control or to the ratios at the initial time. Table 5.1 shows the list of congeners assigned to each peak of the GC plot observed with our system for Aroclor 1242. The assignment of congeners to peaks was based on previously published results (Damaj and Ahmad, 1996; Barriault and Sylvestre, 1993; Ballschmiter and Zell, 1980).

5.2.5 Microbial Populations Counts

The bacterial cells were extracted from the soil particles by vortexing 2 g of soil in 10 mL of sterilized 0.9% saline. Serial dilutions up to 10^{-5} were plated on TYct agar plate medium selective for *R. meliloti* (Kinkle et al. 1994) and incubated at 29 °C for viable population counts.

Table 5.1. Assignment of congeners to peaks of Aroclor 1242 analyzed on GC-ECD.

[o]Peak number	[IU] Congener	Chemical formula	[o]Peak number	[IU] Congener	Chemical formula
4	**4**,10	2,2' & 2,6	30	**37**,42	3,4,4' & 2,2',3,4'
7	7,9	2,4	31	**41**,64, 71	2,2',3,4 & 2,3,4',6 & 2,3',4',6
8	6	2,3'	33	40	2,2',3,3'
9	5,**8**	2,3 & 2,4'	35	67	2,3',4,5
10	19	2,2',6	36	58,63	2,3,3',5' & 2,3,4',5
11	18	2,2',5	37	74	2,4,4',5
12	15,**17**	4,4' & 2,2',4	38	70	2,3',4',5
13	24,27	2,3,6 & 2,3',6	39	66,95	2,3',4,4' & 2,2',3,5',6
14	16,32	2,2',3 & 2,4',6	40	91	2,2',3,4',6
15	34	2',3,5	41	**56**,60, 89	2,2',3,4,6' & 2,3,4,4' & 2,3,3',4'
16	29	2,4,5	43	90,101	2,2',3,4',5 & 2,2',4,5,5'
17	26	2,3',5	44	99	2,2',4,4',5
18	25	2,3',4	45	112	2,3,3',5,6
19	**28**,31	2,4,4' & 2,4',5	47	83	2,2',3,3',5
21	**20**,**33**, 53	2,3,3' & 2',3,4 & 2,2',5,6'	48	97	2,2',3',4,5
22	**22**,51	2,3,4' & 2,2',4,6'	49	87	2,2',3,4,5'
23	45	2,2',3,6	50	85	2,2',3,4,4'
24	46	2,2',3,6'	51	110*	2,3,3',4',6
25	52	2,2',5,5'	53	82,151	2,2',3,3',4 & 2,2',3,5,5',6
26	47,49	2,2',4,4' & 2,2',4,5'	54	106,123,149	2,3,3',4,5 & 2,3,4,4',5 & 2,2',3,4,5',6
28	48	2,2',4,5	55	105	2,3,3',4,4'
29	44	2,2',3,5'	58	138,160,163,164	2,2',3,4,4',5' & 2,3,3',4,5,6 & 2,3,3',4',5,6 & 2,3,3',4',5',6

[o]Aroclor 1242 peaks that were identified in our chromatographic system.
[IU]- Numbers refer to IUPAC convention per Ballschmiter and Zell (1980). The congeners in bold are major components of the peak.
*- Congener 110 used as internal standard.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Biotransformation of Aroclor 1242 as Compared to Initial

The degradation of Aroclor 1242 by the three strains, A-025, A-029, and Zb57 was higher in presence of $CaCO_3$ (Figure 5.1). The addition of $CaCO_3$ increased the pH of the soil to 7.5 from the initial pH of 6.17. Chen and Lovell (1990) demonstrated higher activity for several aromatic dioxygenase enzymes in *R. leguminosarum biovar viceae* at higher pH than neutral. Also, Focht and Brunner (1985) indicate that the PCB degradation rate is higher at pH 7.2 than at 6.0.

All microcosms with $CaCO_3$ exhibited highest degradations for the *di-*, *tri-*, and *tetra*-chlorobiphenyls (Figure 5.1). Peak 4 (congeners 2,2' and 2,6) was degraded to more than 40% and peaks 10, 11, 12, 23, 25, 28, and 47 (tri- to penta-congeners) all degraded close to or more than 20% in all the three microcosms. Peaks 15 and 45 with around 20% and 70% enrichment showed the highest increase with all three strains. These two peaks do not appear to have any dechlorination products (Table 5.2) with the congeners identified in Aroclor 1242. There does not appear to be a parent congener for peak 15 (IUPAC congener 34). However, peak 58 which consistes of congeners 138, 160, 163, and 164, can be the parent compound of peak 45 (IUPAC congener 112) by *para* dechlorination of congener 160 at position 4. The most depleted peaks, all had *ortho* substitutions at positions (2 and 2'). Furthermore, these positions are not present together in peaks

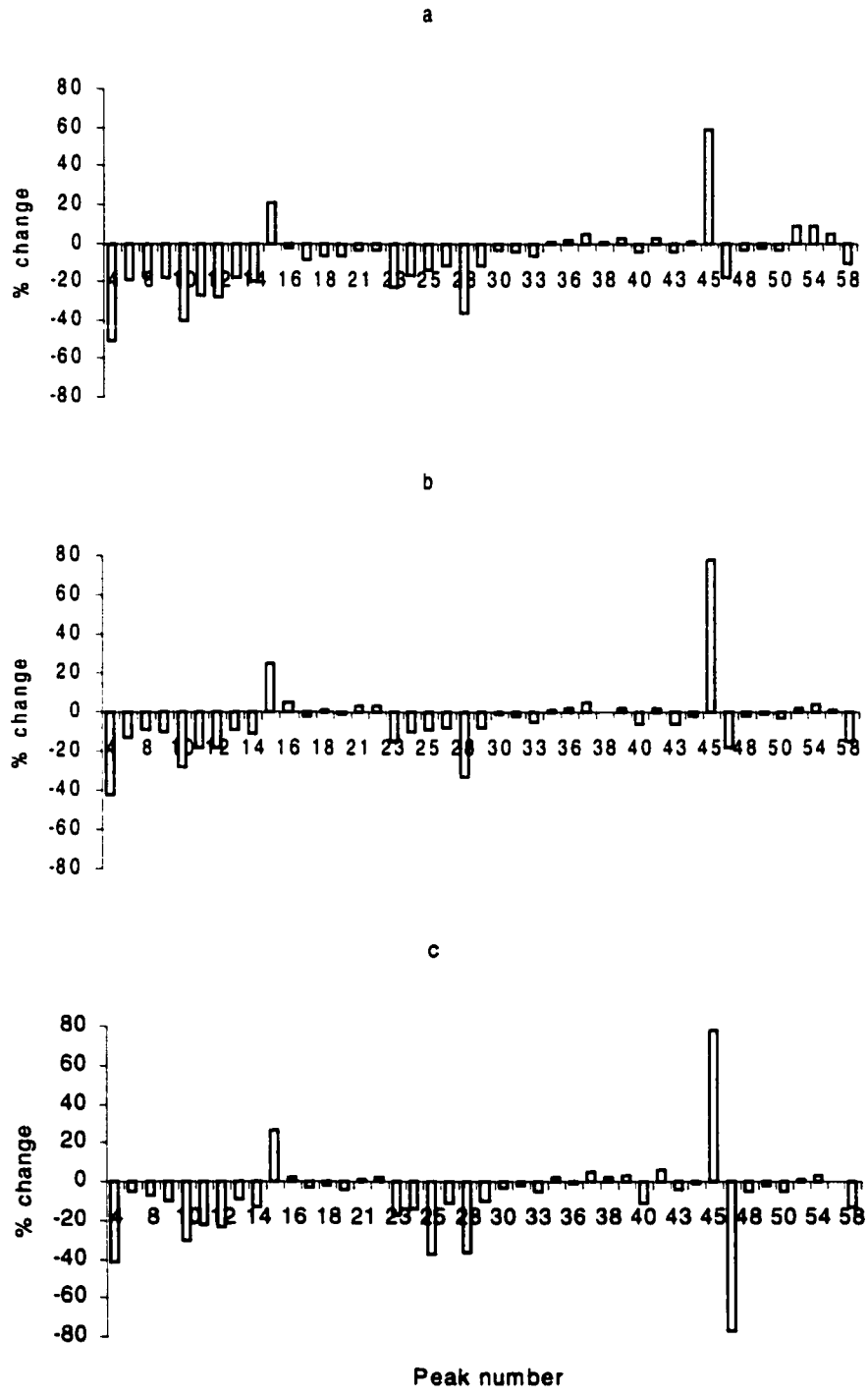

Figure 5.1. Total change in concentration of Aroclor 1242 congeners after 388 days compare to initial in presence of $CaCO_3$ for strains a) A-025, b) Zb57, and c) A-029. Average of duplicates.

15 and 45. However, the *meta* positions 3 and 5 are chlorinated in peaks 15 and 45.

In the absence of $CaCO_3$, strains A-029 and Zb57 showed close to 20% reduction in the pentachlorobiphenyls, peaks 50, 53, 54, and 55. In addition, lower chlorinated congeners, peaks 4, 10, and 28 were reduced in both of these strains (Figure 5.2). Almost all other peaks showed an increase and peaks 15 and 45 showed highest increase after 388 days.

After 123 days under aerobic conditions, strain A-025 (Figure 5.3a) showed a different transformation profile than those observed for A-029 and Zb57 after 388 days (Figure 5.2). Most of the transformed peaks belonged to *di-* and *tetra-* congeners of Aroclor 1242 (Figure 5.3a) where peaks 4, 11, and 43 were degraded more than 60, 40, and 40% respectively. Six other peaks showed almost or over 20% degradation (Figure 5.3a). During the subsequent 265 days under anaerobic conditions, strain A-025 showed the highest increase for most of the *penta-* congeners. However, peaks 7, 8, 17, 22, 30, 37, 41, and 55 showed degradations of more than 20% and peak 54 was degraded up to 60% (Figure 5.3b). Other than peaks 8 and 17, all peaks have *para* chlorine in common. Furthermore, degradation of these peaks was only observed in this microcosm. Depletion of almost 20% or more in this microcosm was observed for 11 peaks after 388 days (including both aerobic and anaerobic periods) with most of them belonging to the *di-* congeners. However, 13 peaks accumulated to more than 20% (Figure 5.3c).

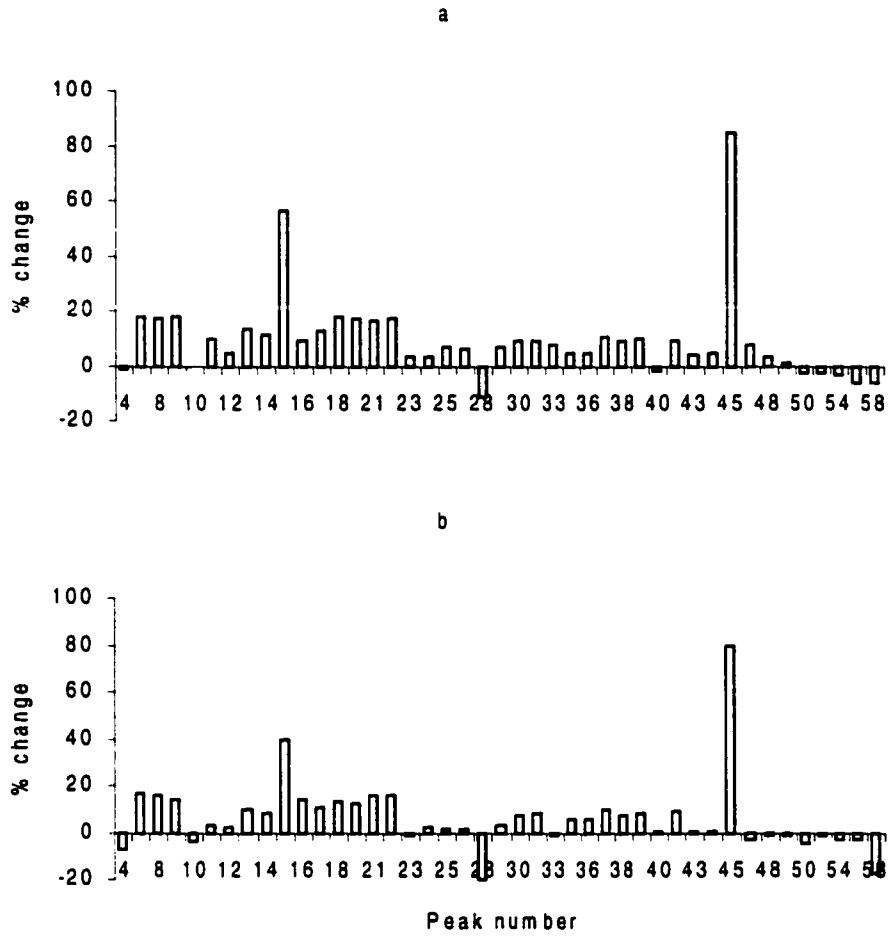

Figure 5.2. Total change in concentration of Aroclor 1242 congeners after 388 days compare to initial for strains a) Zb57 and b) A-029.

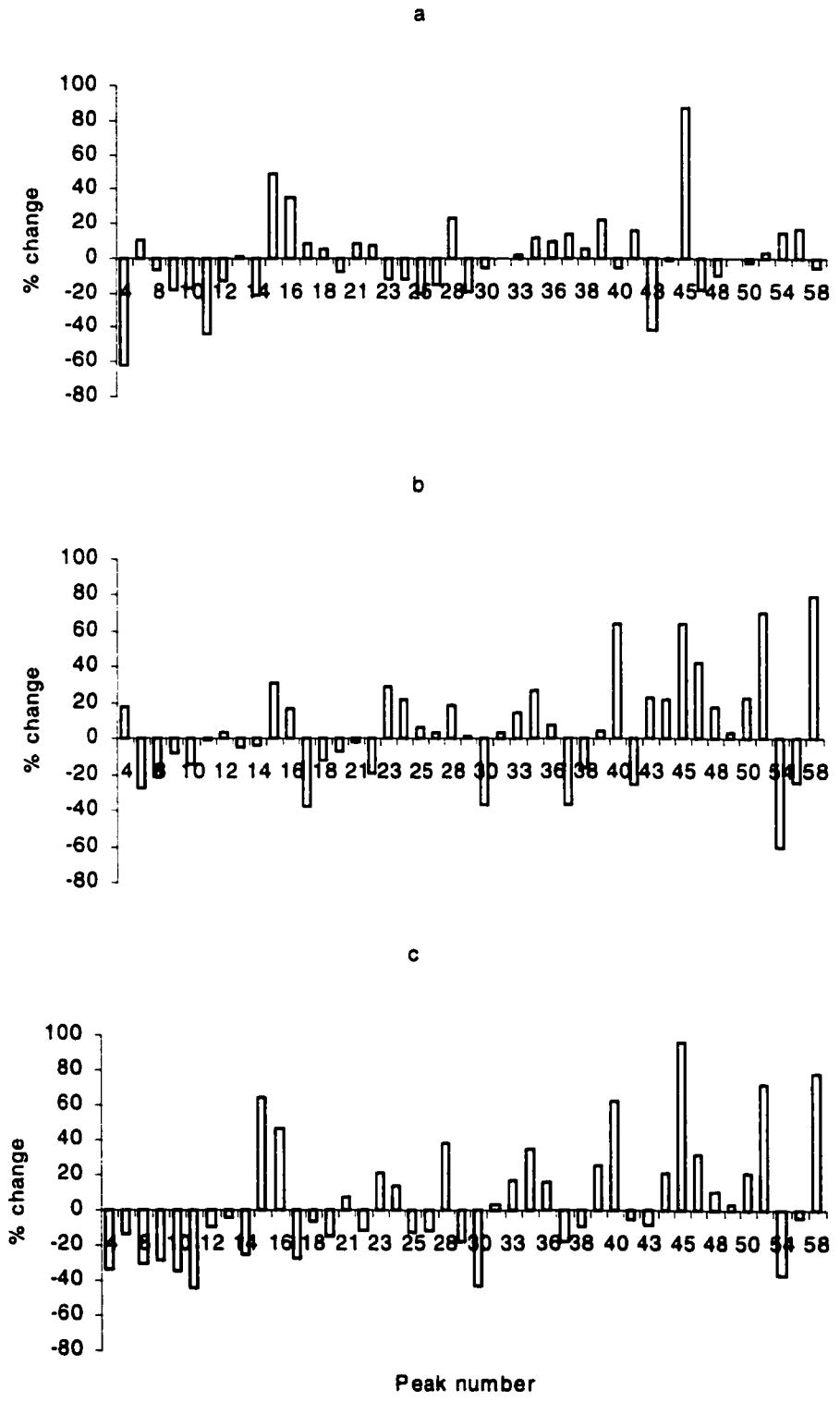

Figure 5.3. Change in concentration of Aroclor 1242 congeners with strain A-025 a) after 123 days aerobic, b) 265 days anaerobic, and c) 388 days of aerobic and anaerobic as compared to the initial. This microcosm was reinoculated after 123 days and kept saturated. Average of duplicates.

5.3.2 Biotransformation and Dechlorination as Compared to Abiotic control

When the results of microcosms with $CaCO_3$ (treatment 1) were compared to treatment 3 (the abiotic control), dechlorination of higher chlorinated congeners into lower chlorinated congeners by all three strains was observed (Figures 5.4, 5.5, and 5.6). After 39 days, strains A-025 and A-029 showed very similar results. All the *di-* congeners were depleted by more than 60% and peak 7 (IUPAC congeners 7 and 9) was depleted up to 100% by A-029 (Figures 5.4a and 5.5a). Lower degradation levels were observed for the *tri-* and *tetra-* chlorinated congeners. This pattern of dechlorination was observed for strain A-025 all through the experiment and the rate appears to be more rapid than the other two strains. Strain Zb57 showed the lowest rate of degradation for the lower chlorinated congeners, as they appear to increase up to 165 days after that their degradation is observed (Figure 5.6). Even though the pattern of biotransformation of the congeners by the three strains is different, the results obtained after 388 days look very similar.

In the absence of $CaCO_3$ the rates of transformation by strains A-029 (Figure 5.8) and Zb57 (Figure 5.9) appeared to be slower than for strain A-025 (Figure 5.7). All three strains dechlorinated highly chlorinated congeners. However, this was not as extensive as in the presence of $CaCO_3$. The microcosm with strain A-025 was reinoculated after 123 days and it was kept under anaerobic conditions for 265 days. The results of this period (Figure 5.7c and 5.7d) were compared to the abiotic control. Clearly, they turned out to be very different than the results of all other microcosms when compared to the abiotic control.

Higher chlorinated congeners present in Aroclor 1242 were dechlorinated by A-025 under aerobic and anaerobic conditions. The peaks that showed the highest transformation during the experiment and are in common with all the treatments, were peaks 4, 8, 9, 38, 47, and 58. Table 5.2 shows the possible dechlorination of congeners present in Aroclor 1242. The congeners that might be dechlorinated aerobically and anaerobically are shown in bold or underlined, respectively. If the aerobic dechlorination pathways that involve these congeners are followed, it is observed that *meta* dechlorination is the major pathway. The *ortho* dechlorination occurs at the 6 and 6' positions and *meta* dechlorination occurs at 5 and 5' positions rather than 2, 2' (*ortho*) and 3, 3' (*meta*) respectively (Figure 5.10). Aerobically, the PCB degradation is suggested to be generally limited to congeners with four to five chlorines (Bedard et al., 1986). Commandeur et al. (1996) showed that congeners 22'-, 24'-, 244'-, 24'5-, 22'33'-, 22'35'- were the most degraded under aerobic conditions. These congeners are also present in the dechlorination pathways proposed for *R. meliloti* (Figure 5.10). Furthermore, the results found by Damaj and Ahmad (1996) are in agreement with the results of this study. They showed that strain Zb57 degraded 14 peaks by 30 to 60% in liquid cultures containing 100 ppm of Aroclor 1242. All the *tetra-* and lower congeners that are shown in the anaerobic pathways (Figure 5.11) and 11 of 17 congeners suggested in the aerobic pathways (Figure 5.10), were significantly reduced in their study. These congeners are indicated by (*) in Figures 5.10 and 5.11.

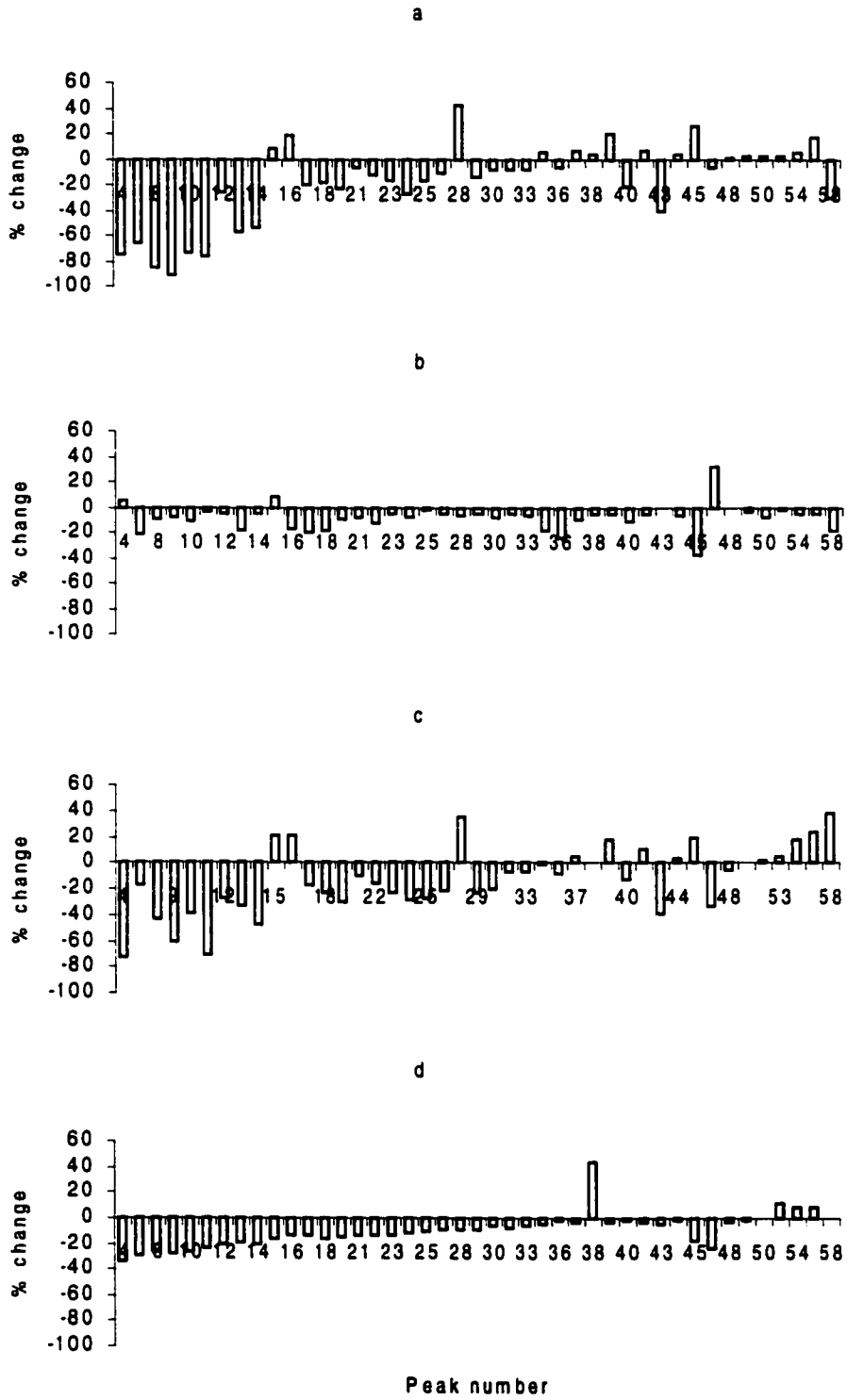

Figure 5.4. Change in concentration of Aroclor 1242 congeners with strain A-025 in presence of $CaCO_3$ compare to abiotic control after a) 39, b) 73, c) 165, and d) 388 days. Results represent the average of duplicates.

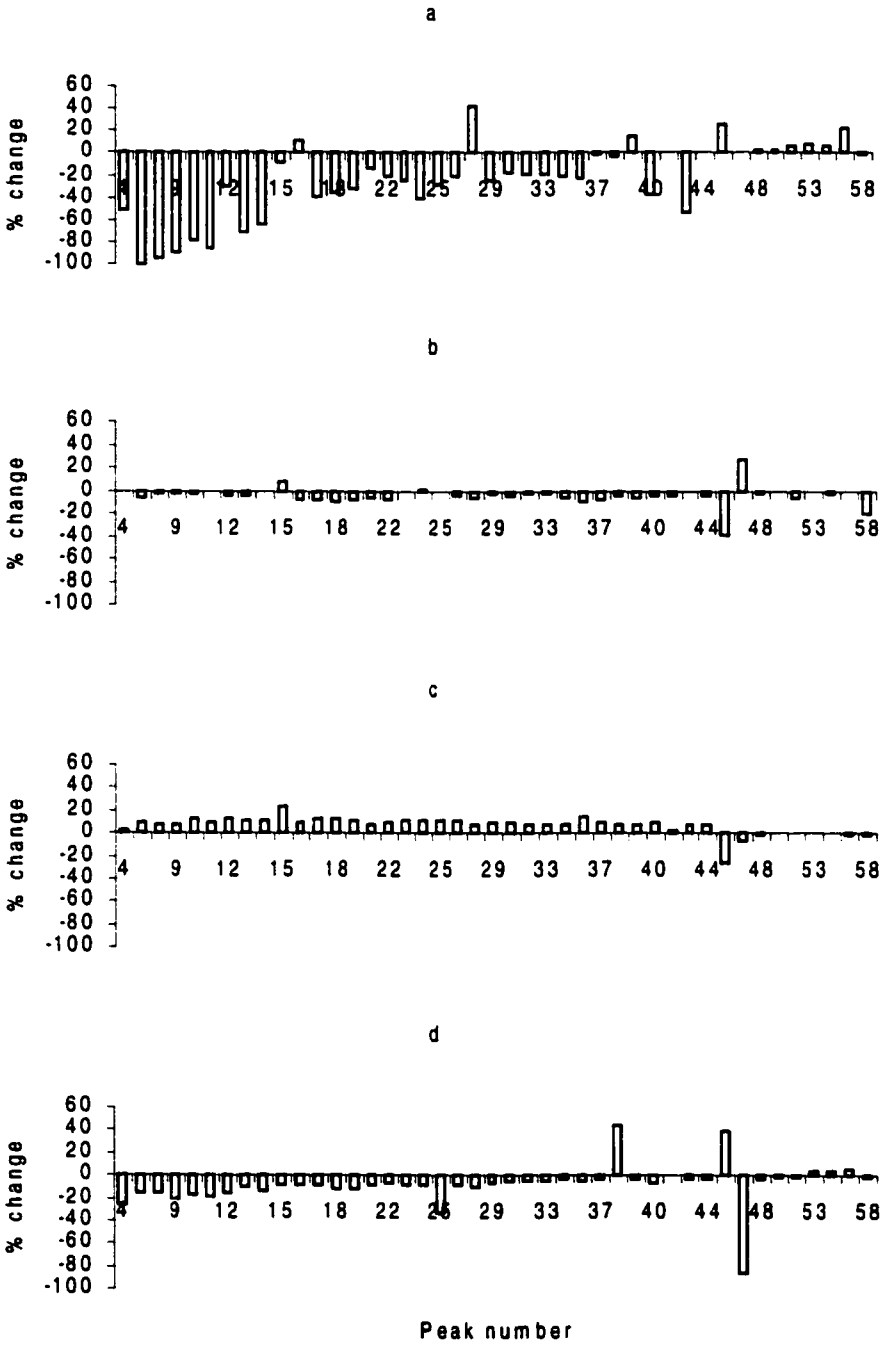

Figure 5.5. Change in concentration of Aroclor 1242 congeners with strain A-029 in presence of CaO_3 compare to abiotic control after a) 39, b) 73, c) 165, and d) 388 days. Results represent the average of duplicates.

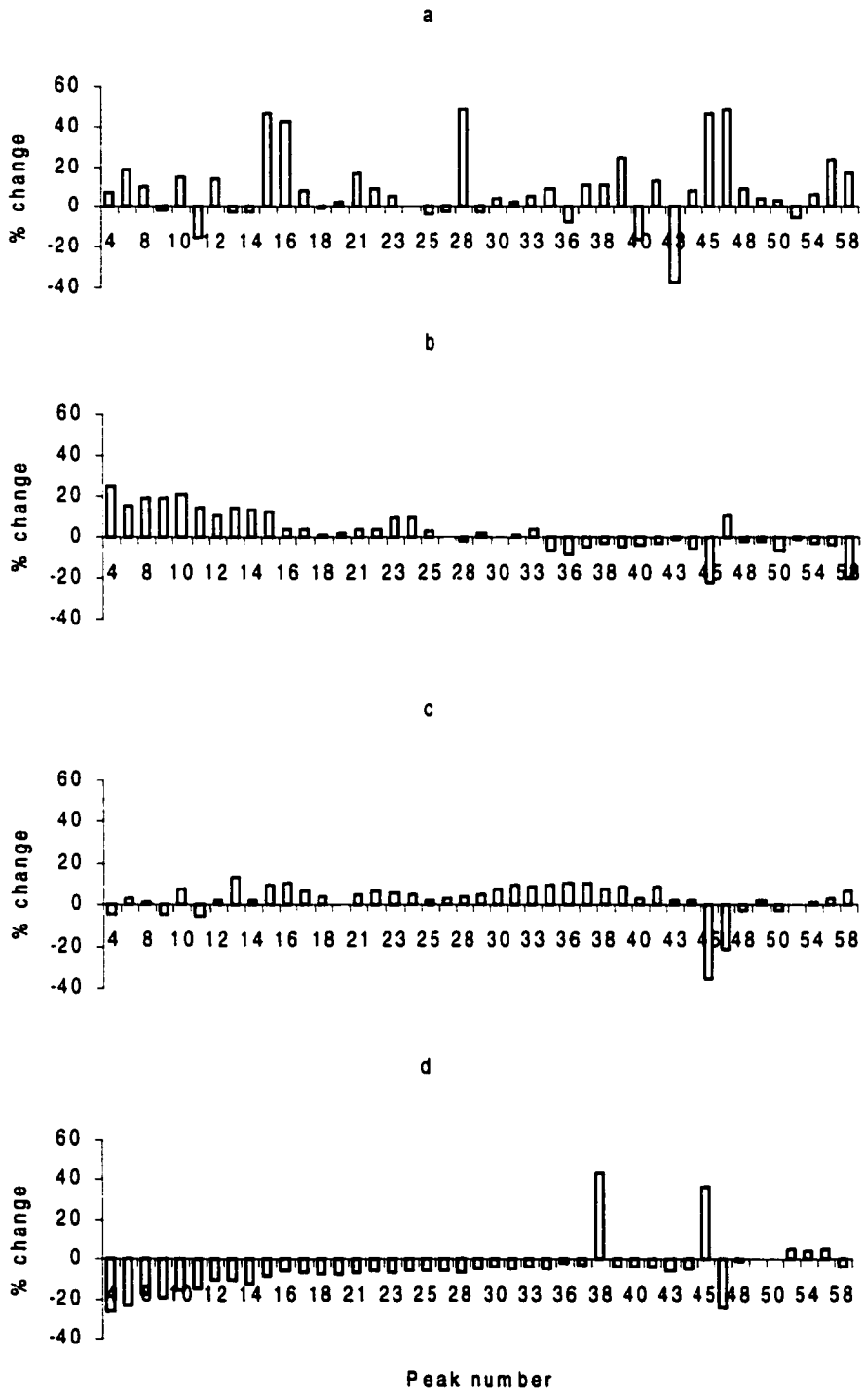

Figure 5.6. Change in concentration of Aroclor 1242 congeners with strain Zb57 in presence of $CaCO_3$ compare to abiotic control after a) 39, b) 73, c) 165, and d) 388 days. Results represent the average of duplicates.

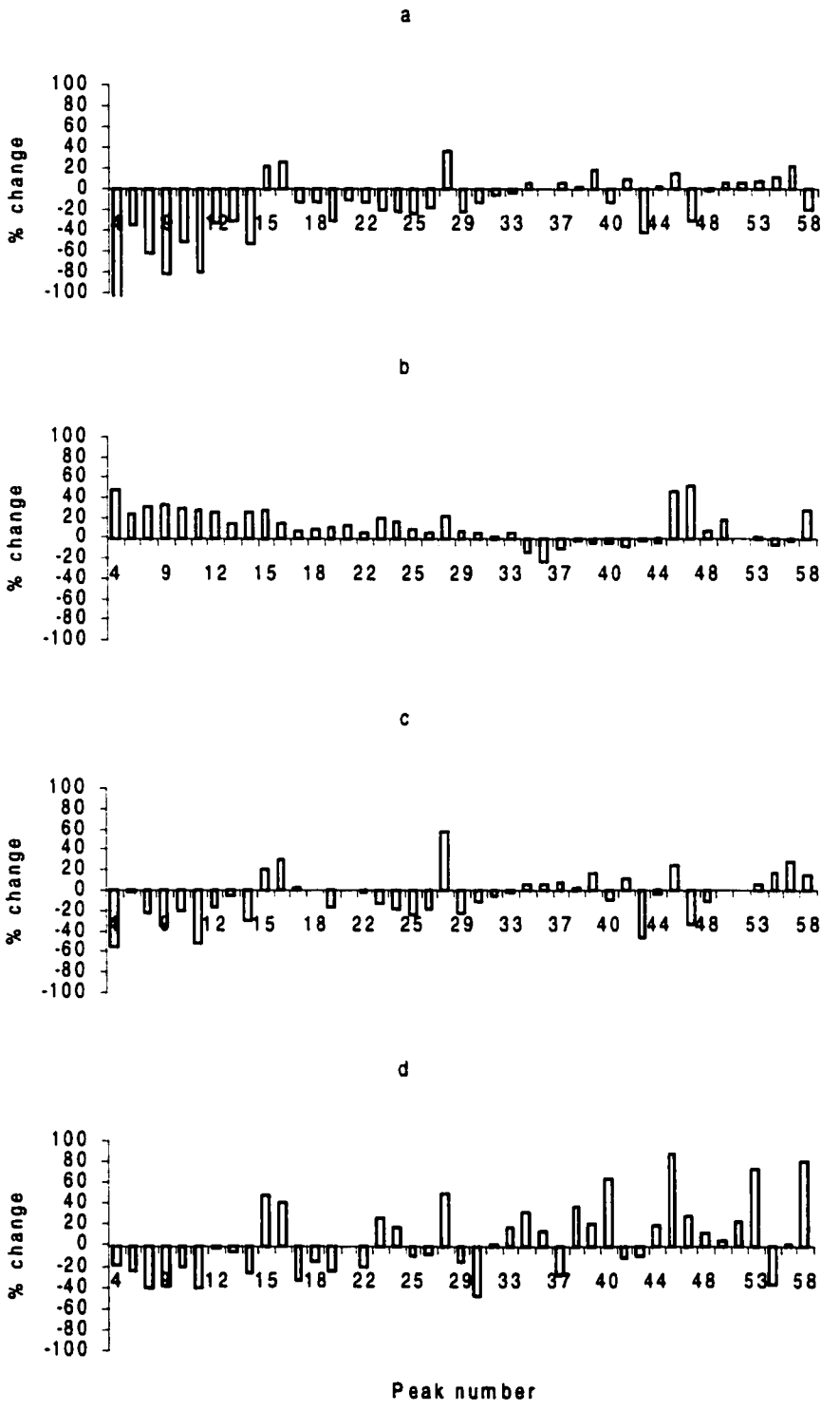

Figure 5.7. Change in concentration of Aroclor 1242 congeners with strain A-025 compare to abiotic control after a) 39, b) 73, c) 165, and d) 388 days. This microcosm was reinoculated after 123 days.

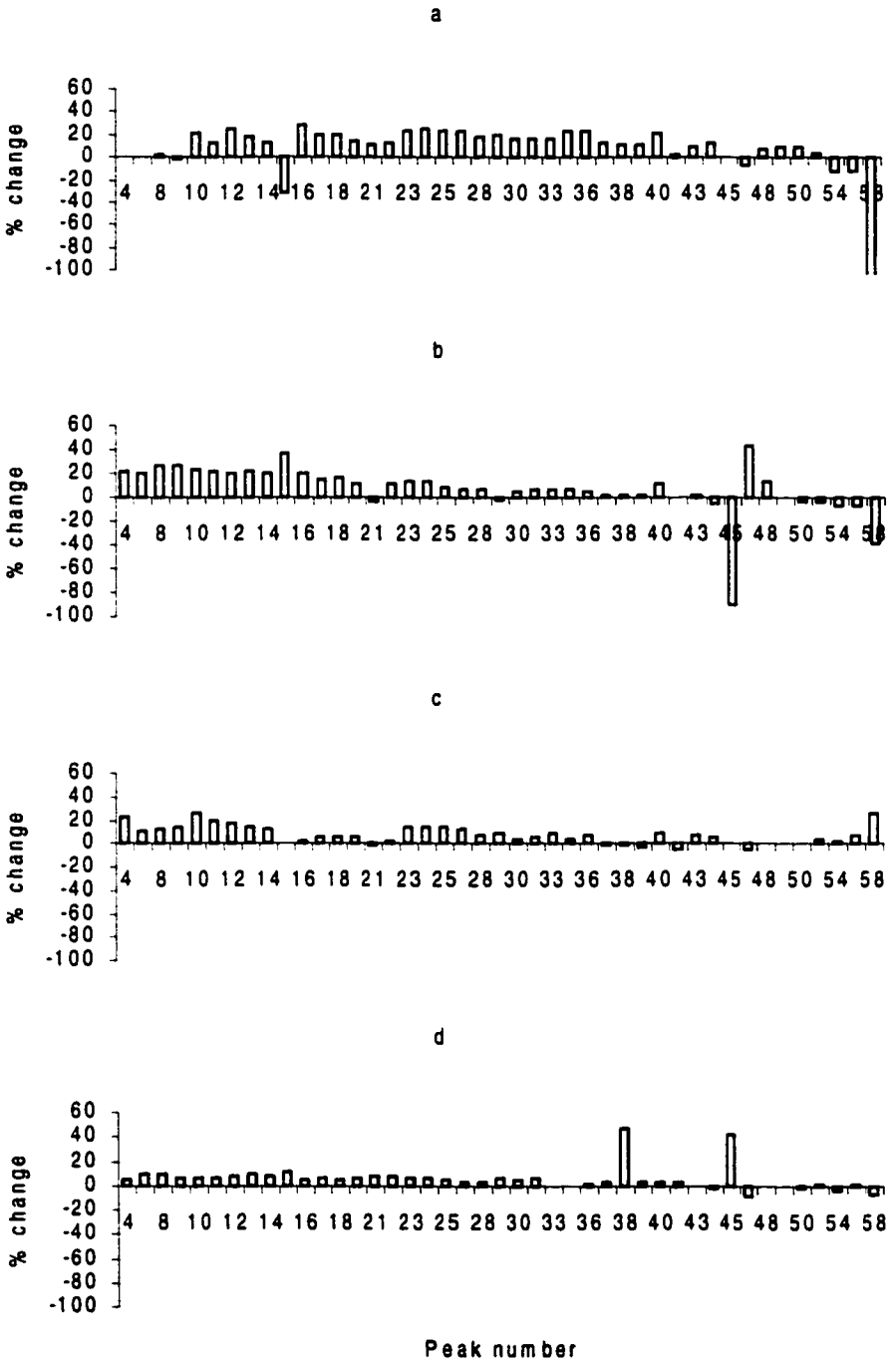

Figure 5.8. Change in concentration of Aroclor 1242 congeners with strain A-029 compare to abiotic control after a) 39, b) 73, c) 165, and d) 388 days. Results represent the average of duplicates.

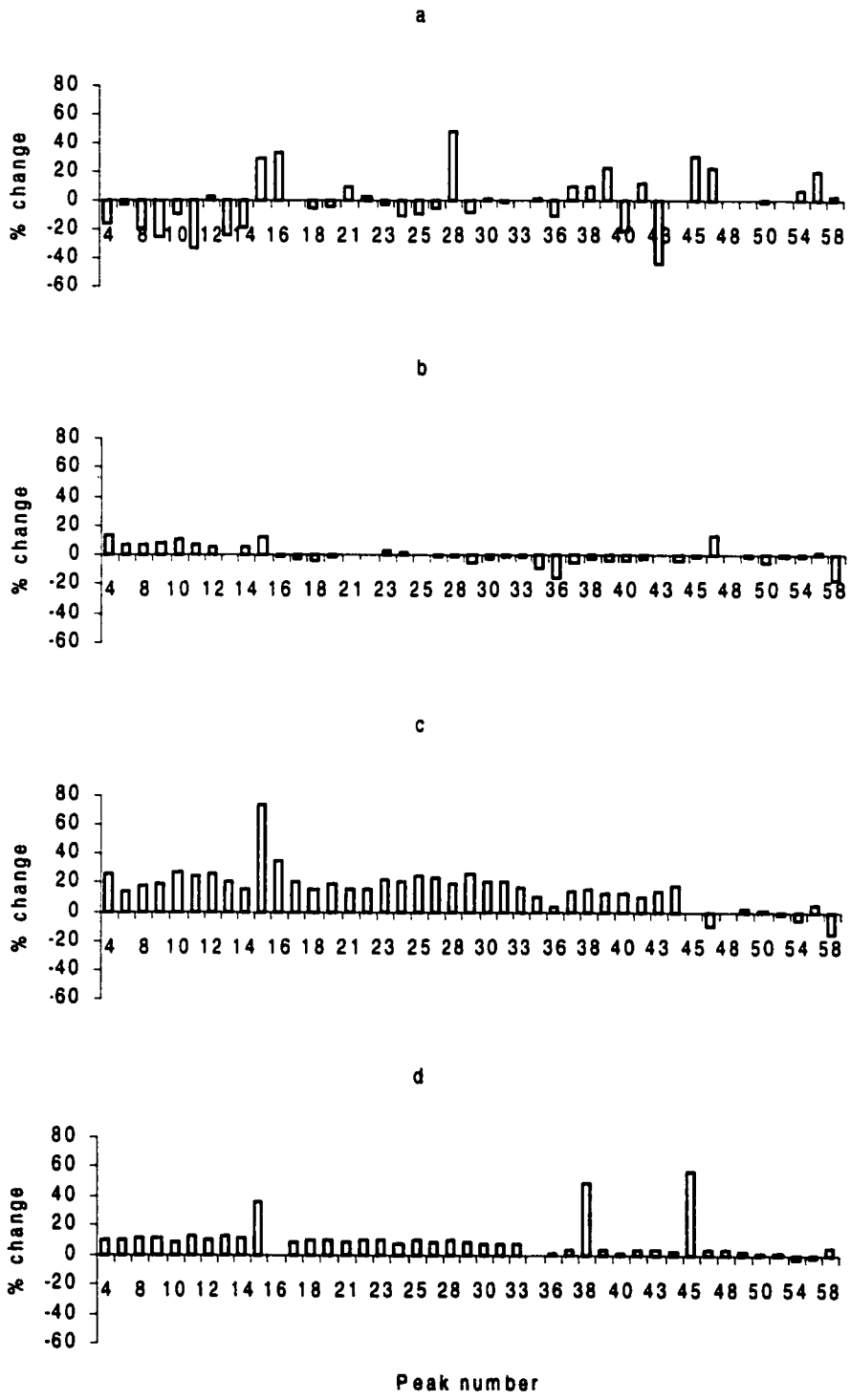

Figure 5.9. Change in concentration of Aroclor 1242 congeners with strain Zb57 compare to abiotic control after a) 39, b) 73, c) 165, and d) 388 days. Results represent the average of duplicates.

Table 5.2. Possible dechlorinations in Aroclor 1242. Suggested dechlorination routes for *R. meliloti* are shown in bold for aerobic and underlined for anaerobic.

Initial Congener	Final congener	Position	Initial congener	Final congener	Position	Initial congener	Final congener	Position
19	**4**	*o*	47	17	*p*	**90**	**42**	*m*
	10	*o*		26	*o*		63	*o*
18	4	*m*	49	17	*m*	101	48	*m*
	9	*o*					49	*m*
							52	*o*
17	4	*p*	48	18	*p*	99	48	*o*
	7	*o*		17	*m*		47	*m*
				29	*o*		74	*o*
24	10	*m*	**44**	**16**	*m*	112	none	
	5	*o*						
27	10	*m*	37	15	*m*	**83**	**40**	*m*
	6	*o*						
16	**4**	*m*	**42**	**16**	*p*	97	48	*m*
	5	*o*					67	*o*
32	10	*p*	**41**	**16**	*p*	87	41	*m*
	8	*o*		17	*m*		44	*p*
				33	*o*			
34	none		64	22	*o*	85	41	*p*
				24	*p*		42	*p*
							60	*o*
29	7	*m*	71	27	*p*	110	89	*o*
	9	*p*					64	*m*
26	6	*m*	**40**	**16**	*m*	82	40	*p*
	9	*m*		**20**	*o*		41	*m*
25	6	*p*	67	29	*m*	151	95	*m*
	7	*m*		25	*m*			
				26	*p*			
28	7	*p*	58	20	*m*	106	67	*m*
	8	*p*						
	15	*o*						
31	**8**	*m*	63	22	*m*	123	60	*m*
	9	*p*		26	*m*		63	*p*
							74	*m*
20	5	*m*	74	28	*m*	149	87	*o*
	6	*m*		31			95	*p*
33			**70**	**31**	*m*	105	89	*p*
							60	*m*
53	19	*m*	66	25	*p*	138	85	*m*
	18	*o*					87	*p*
22	5	*p*	**95**	**44**	*o*	160	106	*o*
	8	*m*		**45**	*m*		112	*p*
51	17	*o*	91	42	*o*	163	110	*m*
				45	*o*		112	*p*
				64	*o*			
45	**19**	*m*	**56**	**41**	*o*	164	110	*m*
	16	*o*		**46**	*o*			
46	**16**	*o*	60	22	*o*			
				37	*o*			
52	18	*m*	89	20	*o*			
				22	*m*			

Anaerobically, the pathways observed with strain A-025 involve *meta* and *para* dechlorination except in the case of congener 2,3,4,4'- that goes through an *ortho* dechlorination to 3,4,4' (Figure 5.11). However, this pathway might not be the major route in the biodegradation and it is reasonable to suggest that the dechlorination would be through the *para* route to 2,3,4' because it would in turn dechlorinate to 2,4'-dichlorobiphenyl which is in common in both of the suggested pathways (Figures 5.10 and 5.11). Berkaw et al. (1996) showed that only *meta* and *para* dechlorination was possible in freshwater sediments. Nies and Vogel (1990) also suggest that dechlorination occurs primarily on the *meta* and *para* positions of the highly chlorinated congeners. Anaerobes from the Hudson and St. Lawrence Rivers have been shown to remove virtually all *meta* and *para* chlorines from Aroclors 1242, leaving predominantly *ortho*- substituted *mono*- and dichlorobiphenyls (Van Dort and Bedard, 1991; Sokol et al., 1994). Fish and Principe (1994) also suggest such pattern and two of the potential pathways they have shown are the dechlorination of 2,3,3',4'- to 2,3,4'-, and to 2,4'-, and 2,4,5,4'- to 2,4,4'- and to 2,4'-. Both pathways are also proposed to be possible with strain A-025 (Figure 5.11).

None of the monochlorobiphenyls were detected through the study. Other metabolites that would be formed after ring cleavage were not tested for. However, Damaj and Ahmad (1996) have shown that strain Zb57 degrades both monochlorobiphenyls and metabolites. The *mono*- and *di*-chlorobenzoic acids that are generated from the oxidation of dechlorinated PCBs are generally mineralized by aerobic and anaerobic bacteria (Bedard and Quensen III, 1995; Bedard et al.,

1986). Furthermore, Abramowicz (1990), Bedard and Quensen III (1995) proposed that PCBs could be totally degraded by anaerobic dechlorination followed by aerobic oxidation. Their experiments demonstrated that the constituents of every congener peak in Aroclor 1242 were transformed by dechlorination, oxidation, or a combination of both.

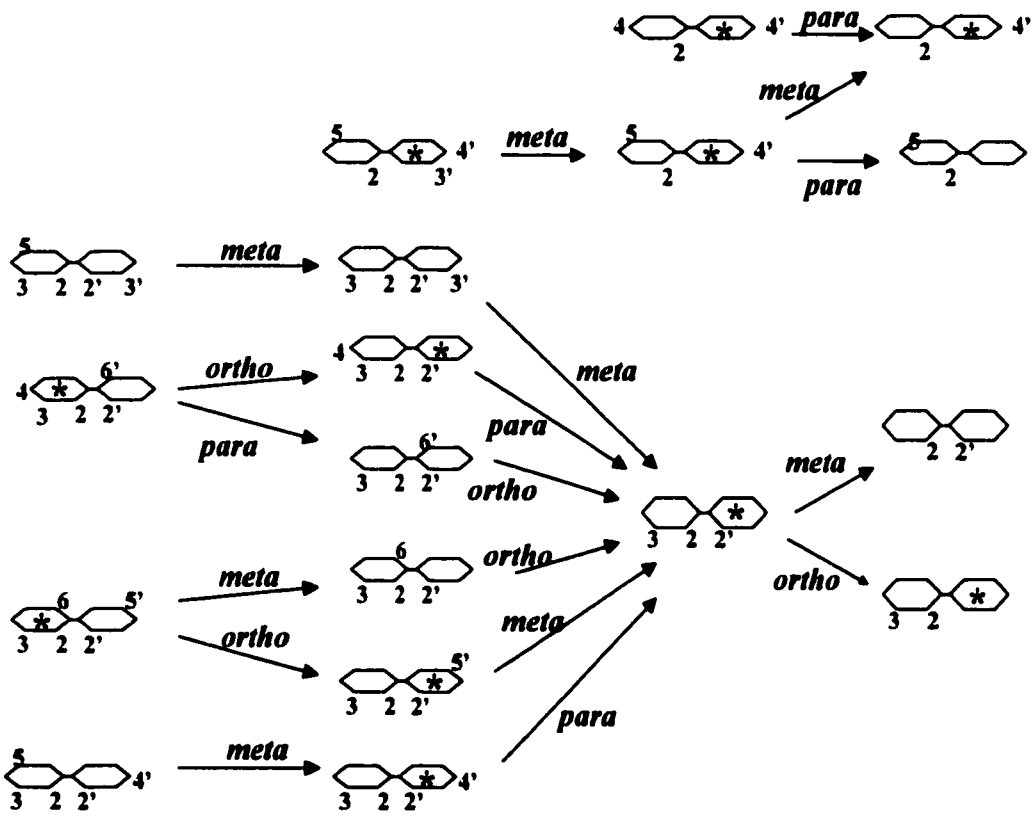

Figure 5.10. Proposed aerobic dechlorination pathways by *R. meliloti*. *- corresponds to those congeners that showed significant degradation by Zb57 in the study by Damaj and Ahmad (1996).

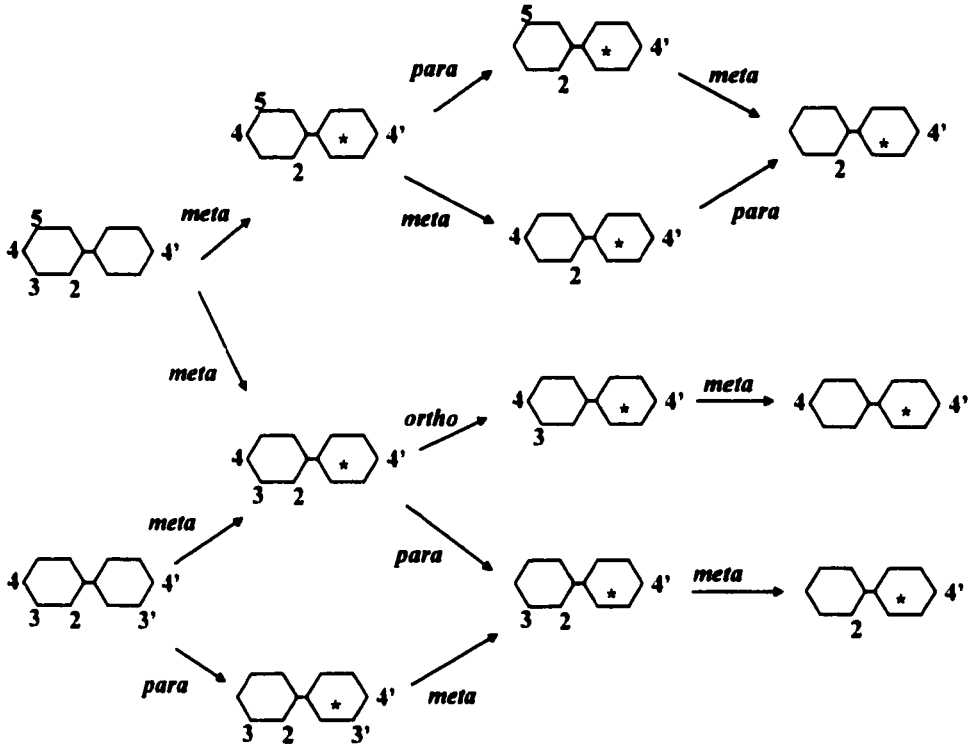

Figure 5.11. Proposed anaerobic dechlorination pathways by *R. meliloti* strain A-025. *- corresponds to those congeners that showed significant degradation by Zb57 in the study by Damaj and Ahmad (1996).

5.3.3 Bacterial Populations

The survival of the bacterial cells was determined for the bioaugmented microcosms. In microcosms with $CaCO_3$, the populations of the three strains decreased from the initial population of 3.8×10^{10} cell g^{-1} soil to less than 2×10^8 cells g^{-1} of soil during the first 7 days (Figure 5.12a). After this point, strains A-025 and Zb57 stabilized and remained at about 5×10^7 cell g^{-1} of soil. After the initial decrease, strain A-029 showed a large increase to over 3.5×10^8 cell g^{-1} of soil by the 24^{th} day, followed by a large decrease for the remainder of the experiment with a final population lower than those observed for strains A-025 and Zb57.

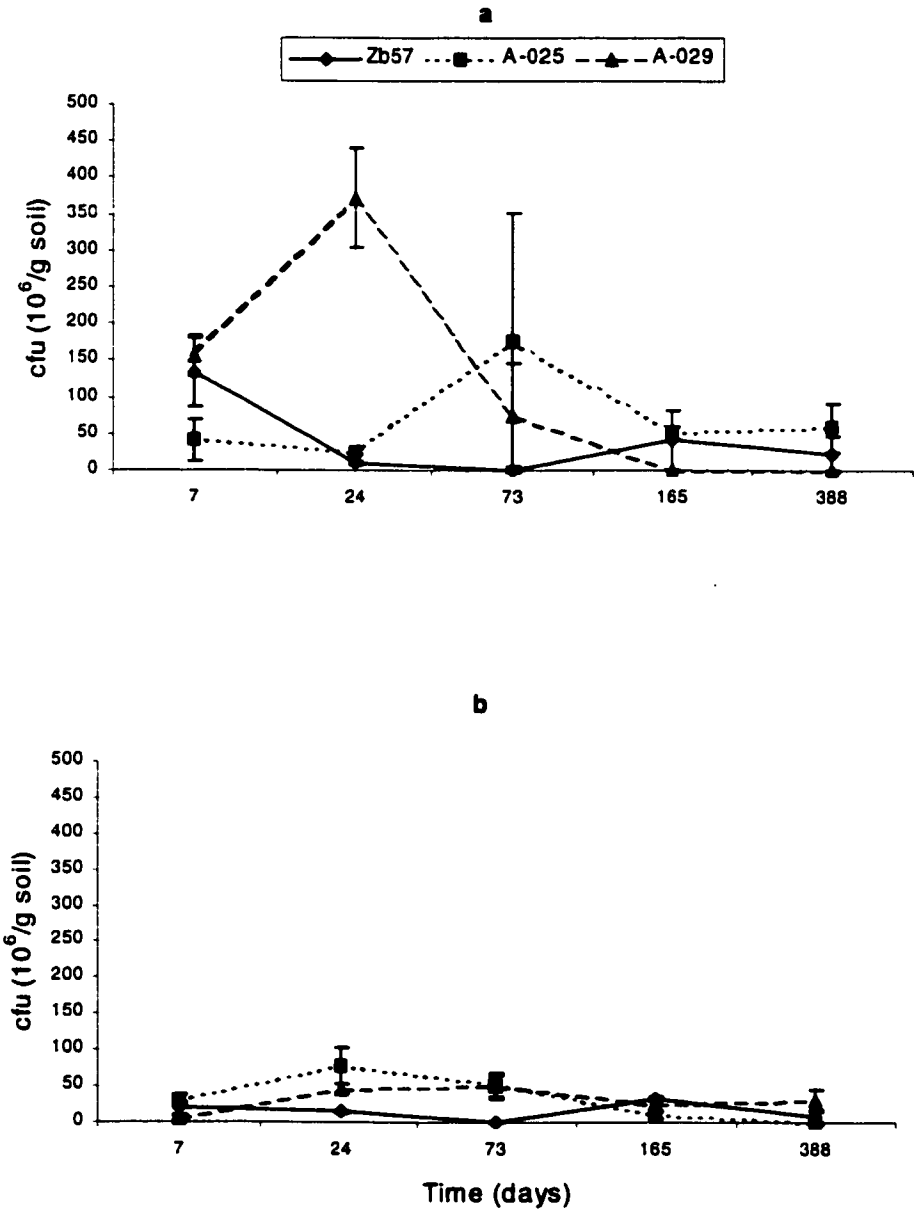

Figure 5.12. Bacterial populations during the experimental period a) with $CaCO_3$, b) without $CaCO_3$. Strain A-025 was reinoculated on the 123rd day in b. Average of duplicates and SD.

In the absence of $CaCO_3$, the initial decrease in the cell numbers was much higher than with $CaCO_3$ (Figure 5.12b). Strains A-029 maintained a slightly higher number of cells than Zb57 for the remainder of the experiment. Strain A-025 showed higher numbers of cells than the other two strains, up to 73 days. However, the numbers decreased to levels lower than the other two strains after reinoculation on the 123rd day with 7.1×10^9 cells g^{-1} and induction of anaerobic conditions for 265 days. This result is in agreement with Fochet and Brunner (1985) who found, lower cell numbers in populations inoculated at higher levels after a period of 10 and up to 25 days. The overall survival patterns of bacterial cells are also very similar to those observed in Chapter 6 for strain A-025.

From these results, it is evident that the Rhizobial strains tested have the ability to dechlorinate the more chlorinated congeners to lower chlorinated congeners present in Aroclor 1242 under both aerobic and anaerobic conditions. This process appears to be more rapid in the presence of $CaCO_3$ than without it. The total biodegradation was also greater in the presence of $CaCO_3$. Peaks 54 (congeners 106, 123, 149), 37 (congener 74), 30 (congeners 37, 42), and 17 (congener 26) could only be degraded anaerobically by strain A-025.

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PREFACE TO CHAPTER 6

In Chapter 4, we demonstrated that subirrigation was an effective method for bioaugmentation of a sandy loam soil. In Chapter 5, we demonstrated that inoculation of freshly contaminated soils with three *R. meliloti* strains (A-025, A-029 and Zb57) lead to biotransformation of PCBs present in Aroclor 1242 in sterilized soil microcosms. However, due to the volatilization, biodegradation and adsorption, the bioavailability and presence of different PCB congeners in weathered contaminated soils is different than those in a freshly contaminated soil. Therefore, it is important to investigate the use of subirrigation and *R. meliloti* in a weathered contaminated soil.

In this study, a water table management system was used to deliver and distribute bacteria to the subsoil in soil columns, and subsequently to enhance bioremediation of PCBs. The columns were packed with weathered PCB contaminated soil. A well studied PCB degrader *Comomonas testosteroni*, strain B-356, and *Rhizobium meliloti*, strain A-025, and an uncharacterized indigenous microflora were used to bioaugment these soil columns. In this study, we compared the biodegradation efficiency of these bacterial cultures by bioaugmentation using a water table management system for bacterial delivery. In addition to delivery of bacterial cells, the water table management system was used to create aerobic and anaerobic conditions in the columns to enhance degradation of PCB congeners. Chapter 6 describes the experimental protocol and the results obtained in this study.

The results will be submitted for publication as: Mehmannavaz, R., Ahmad, D. and Prasher, S.O. PCB Biotransformation by Rhizobium and Comomonas Cultures under Aerobic and Anaerobic Cycles in Soil Columns. Also as: Mehmannavaz, R., Prasher, S.O. and Ahmad, D. Water Table Management in Biotransformation of PCBs by Bioaugmentation with an Indigenous Culture under Sequential Aerobic and Anaerobic Cycles in Soil Columns.

CHAPTER 6

WATER TABLE MANAGEMENT IN BIOTRANSFORMATION OF PCBS BY BIOAUGMENTATION WITH *RHIZOBIUM, COMOMONAS* AND INDIGENOUS CULTURES

ABSTRACT

The use of water table management to deliver and distribute bacteria to the subsoil and subsequently to enhance dechlorination and/or biotransformation of PCBs was investigated. In this study, the columns (1000 x 200 mm) were packed with a soil contaminated with PCBs (Aroclors 1242, 1248, 1254, and 1260) for over 17 years. A known PCB degrader *Comomonas testosteroni* strain B-356, *Rhizobium meliloti* strain A-025, and an uncharacterized isolated indigenous microflora were used to bioaugment the soil columns. The one-year study included cycles of 10 days of aerobic and anaerobic periods for 8 months, followed by 30 day cycles for another 4 months. The dechlorination and biotransformation of PCB congeners were determined at 140, 340, and 590mm depths. The results indicated that treated columns bioaugmented with the indigenous microflora were more effective in dechlorination and total biotransformation of PCB congeners at 140 and 340 mm depths, whereas, B-356 and A-025 ranked 2nd and 3rd respectively at these depths. However, strain A-025 was more effective at 590 mm. The overall dechlorination and biodegradation of

PCB congeners differed between the treatments and the depths. Different possible aerobic and anaerobic pathways were proposed for strain A-025. *Meta*, *para*, and *ortho* dechlorination were involved aerobically and only *meta* and *para* were the possible anaerobic dechlorination pathways. Cell counts of the bioaugmented *R. meliloti* strain A-025 were highest at 340mm, followed by 590, and 140mm depths. Also, the 30 day aerobic cycles seem to be more effective than the 10 day cycles. Thus, a longer period of aerobic and a shorter period of anaerobic cycles were suggested to be more adequate for dechlorination and total biodegradation of PCBs.

6.1 INTRODUCTION

The proposed system is based on water table management (WTM), a technology that was developed primarily to improve agricultural productivity (Broughton et al., 1987; Madramootoo et al. 1993). WTM is essentially an application of controlled subsurface drainage, modified to also permit water flow upward through the soil profile when needed for subsurface irrigation requirements and hence, creating saturated conditions (anaerobic) and drained conditions (aerobic). This system is quite effective in delivering necessary microorganisms to any level in the soil profile as shown in Chapter 4 and Appendix A4, as well as in providing a system to maintain proper conditions for their survival. Subirrigation has already proven to be an effective method for delivery of nutrients to different depths of 2000mm soil columns (Ugwuegbu, 1997, unpublished results). Because subirrigation leads to a higher degree of

saturation and frees the soil of air pockets, bacteria are distributed more efficiently into the soil as compared to surface irrigation (Chapter 4). Use of water as the transport medium, increases the bioavailability of the contaminants by increasing their desorption and by transporting the organisms to the contaminant's site.

It is estimated that approximately 2 million tons of polychlorinated biphenyls (PCBs) have been produced world-wide and close to half of these have been released into the environment (Mousa et al., 1996). Although PCB use has been restricted since the 1970s, these recalcitrant compounds are ubiquitous environmental contaminants. PCBs are primarily deposited in soils, rivers and lake sediments. In the Hudson River (Chen et al., 1988), Esthwaite Water sediments (Gevao et al., 1997) and in Saint Lawrence River (Vanier et al., 1996) they are found as deep as 60 cm from the sediment-water interface and mostly from 10 to 40 cm depending on the mixtures. Some congeners undergo anaerobic dechlorination in sediments and the more soluble congeners are lost due to volatilization or are transported to other locations by water. PCBs are persistent environmental contaminants due to inability of native organisms to degrade many of the highly chlorinated congeners in aerobic environments. However, the limited anaerobic dechlorination of highly chlorinated PCBs is now seen as an important environmental fate of these compounds.

The recent discoveries of PCB dechlorination in freshwater and estuarine sediments (Berkaw et al., 1996; Sokol et al., 1994) have raised hopes for biorestoration of sites contaminated with PCBs. The reductive dechlorination is a two-electron transfer where chloride ion is released and replaced by hydrogen on

the aromatic ring (Nies and Vogel, 1991). This makes PCBs more degradable and less persistent (Van Dort et al., 1997). Naturally occurring organisms that can degrade PCBs are quite common in the environment and these organisms belong to several different microbiological types (Abramowicz, 1990).

The most common first step in the aerobic degradation of PCBs is a dioxygenase activity (Furukawa et al., 1979; Flanagan and May, 1993). For this, two adjacent non-chlorinated carbons are required. The dioxygenase attack on the 2,3- or 3,4- positions is faster than at the 2,6- positions (Quensen III et al., 1988; Bedard et al., 1987; Masse et al., 1984). Therefore, congeners with *ortho*-chlorines on the same ring are known to degrade more slowly. The aerobic biodegradation of PCBs is usually limited to penta- or less chlorinated congeners and it is less effective as the number of chlorines increases (Quensen III et al., 1990; Commandeur et al., 1996). Nevertheless, the more highly chlorinated congeners that can be dechlorinated, the more completely the lower chlorinated PCB products can be degraded by a sequential anaerobic and aerobic system (Quensen III et al., 1990).

In remediation of PCBs, microbial degradation might be a final disposal option (Erickson, 1997). However, the kinetics of degradation as well as organism's type and population, nutrients, temperature, and a host of other environmental parameters, in both scientific and practical aspects need to be better understood. This PCB degradation study was used to investigate the different known bioaugmentation strategies used to remediate a contaminated soil. Soil

columns were used to design and develop a subirrigation system as a bioaugmentation technology for bioremediation of PCB contaminated soils.

The present study was carried out to evaluate the subirrigation system as a bioaugmentation technology and the Water Table Management systems to enhance bioremediation of soils contaminated with PCBs. A well studied PCB degrader *Comomonas testosteroni*, strain B-356 (Barriault et al., 1997), *Rhizobium meliloti*, strain A-025 (Ahmad et al., 1997) and an uncharacterized mixed indigenous microflora (culture ABVII) were used in this study.

6.2 MATERIALS AND METHODS

6.2.1 Soil Column Preparation and Treatments

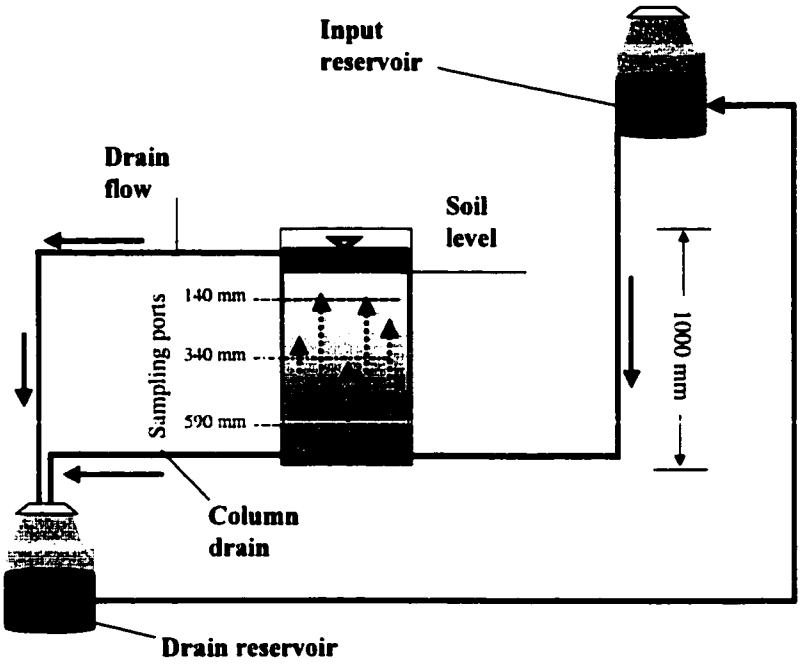
Stainless steel columns, 1000mm in length x 200mm in diameter fitted with sampling ports on the side at 140, 340, and 590 mm depths were used in this study. At the bottom of each column, a subirrigation port was connected to a delivery pipe that had 2 mm wide slits at 25 mm intervals to supply water and bacterial cells into the column and to maintain water table at desired levels as shown in Figure 6.1. The columns were packed at the bottom 70 mm to create a gravel filter. The first layer of gravel was 30 mm deep with grain size >9.5 mm, the second layer was 20 mm with >4.75 mm particles and the third layer was 20 mm with >2.36 mm particles. The PCB contaminated soil, S-VII, was sieved through a 5 mm screen and mixed well. This soil was packed on top of the gravel filter with 4.56 kg of soil for each 100 mm and for a total of 29.48 kg of soil in each column. The final height of the soil above the gravel filter in the columns

was 650 mm with a bulk density of 1500 kg m^{-3}. The history and characteristics of the soil are shown in Table 6.1.

Table 6.1. Characteristics and history of the soil, S-VII.

Sand (%)	Silt (%)	Clay (%)	Organic Matter (%)	pH	History
74	8	18	4.8	7.38	Industry Heavy metals; PCB mixtures: Aroclor 1242, 1248, 1254, 1260

After 41 days of fabrication, the columns were saturated with 24 L of tap water at room temperature. The water was recycled back into the columns several times to create a continuous saturated flow for 24 h and then were kept saturated until inoculation with the bacterial cells. A 300mL bacterial suspension was added to the input water bottles. The water was collected, recycled back into the soil columns for a period of 7 h, and the columns were kept saturated until a 2^{nd} reinoculation. After the second inoculation, the water was recycled for a period of 7 h and the columns were left saturated for 5 days after which they were drained and left dry for a period of 10 days. After this period the columns were saturated again, but for a period of 10 days and the B-356 treatment columns (treatment 2) received 15 g of biphenyl (BP) in the irrigation water. All treatments but the passive control (treatment 5), underwent a dry period (10 days) and a saturated period (10 days) for approximately 8 months and then the duration of the cycles were increased to 30 days each for the following 4 months. Approximately, two days were given as saturating and draining periods between each cycle. A schematic diagram and a photo of the setup are shown in Figure 6.1.



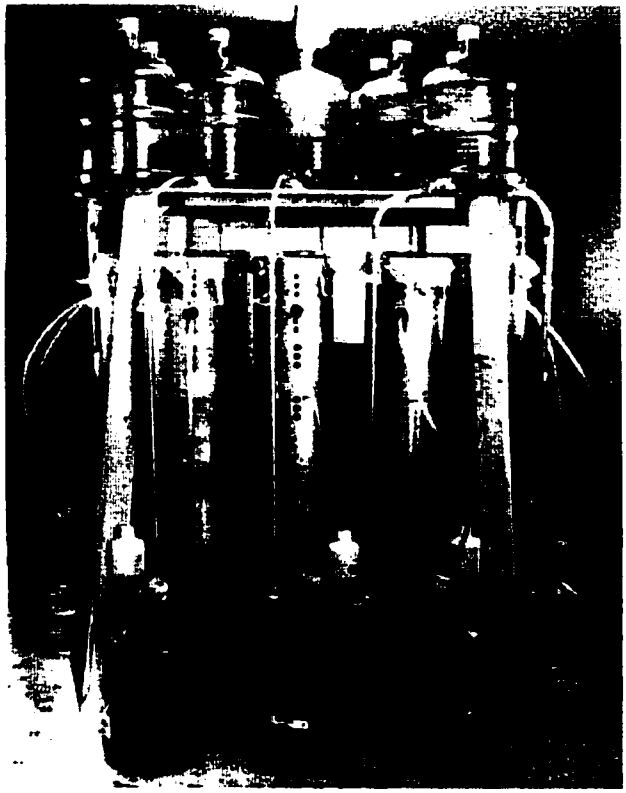


Figure 6.1. Schematic diagram and a photograph of the experimental setup.

6.2.2 Experimental Setup

Five sets of treatment columns were prepared for this experiment. Treatment sets 1-3 were designed to evaluate WTM for bioaugmentation of three different microbial cultures for biodegradation of PCBs. The 4th treatment set demonstrated the effect of WTM and this set was used as a water control since the bioaugmentation was carried out using subirrigation (WTM). The 5th treatment set represented the passive control and did not receive any treatment. More specifically, the treatments were as follows:

1- Bioaugmentation with *Rhizobium meliloti*, strain A-025,

2- Bioaugmentation with *Comomonas testosteroni*, strain B-356,

3- Bioaugmentation with uncharacterized indigenous microflora, ABVII,

4- Water treatment, non-augmented, used as a water control,

5- No treatment, used as a passive control.

6.2.3 Preparation of Bacterial Cells

R. meliloti strain, A-025, was precultured for two days in 5mL of TYc (Bromfield et al., 1994) and inoculated into 50mL of TYc in six 125mL flasks each of which was subsequently inoculated into 1 L of TYc, in 2 L flasks.

Strain B-356 was inoculated in 5mL of TY for two days and transferred into 50 mL of TY supplemented with 0.05% BP. This subculture was added to 1L of TY supplemented with 0.05% BP.

The indigenous microflora (ABVII) was cultured as follows: 5 g of soil S-VII was added to 50mL of MM30 (Sylvestre and Fauteux, 1982) supplemented

with vitamins, 100 ppm Aroclor 1242, 0.05% BP, and 0.05% yeast extract and incubated on a rotary shaker at 29 °C. Two days after, 2mL of the culture was inoculated in 50mL of TY supplemented with 100 ppm Aroclor 1242 and 0.05% BP and grown for 4 days. This culture was then filtered through glass wool and inoculated as a subculture, in 50 mL of TY supplemented with 100 ppm Aroclor 1242 and 0.05% BP. This subculture then was transferred to a 1L TY medium supplemented with 100 ppm Aroclor 1242 and 0.05% BP and grown overnight.

All the cultures, in 2 L flasks were incubated at 29°C in a controlled environment incubator shaker (Psycrotherm, New Brunswick Scientific) for 24 h. The bacterial cultures were plated on TYct plates (for Rhizobium) (Kinkle et al., 1994) and TYc for (B-356) agar plates to determine the population count. The bacterial cells were then harvested by centrifugation for 5 min at 8000 rpm at 4°C in 250mL centrifuge bottles. The collected cells, 5.066 g ($2.3x10^8$ cell mL^{-1}) of Rhizobium, 3.535 g ($3.0x10^8$ cell mL^{-1}) of B-356, and 5.816 g of ABVII were washed with 100mL of 0.9% saline and resuspended in 300mL ($7.7x10^8$ cell mL^{-1} for A-025, $1.0x10^9$ cell mL^{-1} for B-356) of sterilized water. The washed cultures were mixed by vortexing the inoculum before inoculation of the soil columns. Soil columns were reinoculated the next day for the 2nd time with 3.971, 2.368, and 4.483 g of A-025, B-356, and ABVII, respectively. Summaries of the experimental stages are presented in Tables 6.2 and 6.3.

Table 6.2. Stages of bacterial culture preparations for the PCB degradation in the soil column study.

Bacterial culture strain A-025	Bacterial culture strain B-356	Bacterial culture ABVII
Starter culture from frozen cultures, 5mL TYc (2 days)	Starter culture from frozen cultures, 5mL TY (2 days)	5g soil, S-VII, added to 50mL MM30 with 100 ppm Aroclor 1242 and 0.05% BP and 0.05% YE, incubated at 29 °C (2 days)
Sub-cultured into 50mL TYc (2 days)	Sub-cultured into 50mL TY with 0.05% BP (2 days)	2mL sub-cultured into 50mL TY with 100 ppm Aroclor 1242 and 0.05% BP, repeated twice (4 days)
Inoculated into 1L TYc (1 day)	Inoculated into 1L TY with 0.05% BP (1 day)	Sub- cultured into 1L TY with 100 ppm Aroclor 1242 and 0.05% BP, (1 day)
Cells collected, washed (in 0.9% saline) and resuspended in 300mL sterilized water	Cells collected, washed (in 0.9% saline) and resuspended in 300mL sterilized water	Cells collected, washed (in 0.9% saline) and resuspended in 300mL sterilized water

Table 6.3. Experimental stages of PCB biodegradation in the soil column study.

Period	Column Treatments
(Nov. 16-Dec.26, 1996)	Packing and preparation of columns (PCB contaminated soil, S-VII)
(Dec.26, 1996)	Initial soil samples taken before saturation of soil columns
(Dec.26, 1996)	Soil columns saturated with 24L of water (except the passive control column)
(Dec.28, 1996)	Inoculated with strain A-025, 5.066 g culture or 1L ($2.3x10^8$ cell/mL), strain B-356, 3.535 g culture or 1L ($3.0x10^8$ cell/mL), culture ABVII, 5.816 g culture
(Dec.29, 1996)	Reinoculation with strain A-025, 3.971 g of cell culture, strain B-356, 2.368 g of cell culture, culture ABVII, 4.483 g of cell culture
(Jan. 3-5,1997)	Complete drainage of columns
(Jan. 5,1997)	Water and soil samples taken
(Jan. 15, 1997)	15g of BP was added to treatment columns with strain B-356
(Jan. 5-Sep. 6, 1997)	10 days of dry (unsaturated, aerobic) and 10 days of wet (saturated, anaerobic) cycles. Water and soil samples taken after each cycle
(Sep. 6- Jan. 5, 1998)	30 days of dry (unsaturated, aerobic) and 30 days of wet (saturated, anaerobic) cycles. Water and soil samples taken after each cycle

6.2.4 Sampling

Soil samples were taken from each column at 140, 340, and 590mm depths before inoculation of the soil columns with the bacteria, to represent the initial conditions. Soil (20g) and water samples (20mL) were collected after each wet and dry cycle and analyzed for the PCB concentration, pH, and viable population count, periodically.

6.2.5 Microbial Analysis

The bacteria were extracted from the soil of the treatment bioaugmented with *R. meliloti*, strain A-025. The bacterial extraction was performed by vortexing 2 g of soil in 10mL of sterilized 0.9% saline. Serial dilutions were plated on TYct agar plates for counts of viable population of strain A-025. The colonies formed from samples taken on the 377^{th} day were tested for nodule formation on alfalfa roots using the procedure described in Chapter 3.

6.2.6 PCB Extraction and Analysis

Three grams of the 20g soil samples taken from the columns were mixed in a 125mL flask with 6mL of sterilized deionized water and Triton X-100 (30µL) to enhance desorption of Aroclor from soil particles (Barriault and Sylvestre, 1993). Anhydrous sodium sulfate (6g) was added to prevent the formation of stable emulsion (Bedard et al., 1986). Hexane (15mL) was then added and the flasks were shaken on a rotary platform for 20 min and the hexane fraction was collected on ammonium sulfate. The aqueous phase was extracted two more

times. The pooled organic phases were passed through florisil (5g) column filters and concentrated to 3mL by nitrogen gas. Ten μL of 2,3,4,5,6,2',3',4',5',6'-chlorobiphenyl (333 ppb) were added as an internal standard. Samples were diluted before their injections. The ratio of each peak against the internal standard was determined and these ratios were compared to those of the passive control. Table 6.4 shows the list of congeners assigned to each peak of the GC plot observed with our chromatography system. The assignment of congeners to peaks was based on previously published references (Ballschmiter and Zell, 1980; Larsen et al., 1992; Erickson, 1997). A chromatogram of the assigned congeners is presented in Figure 6.2. The experimental data obtained and the standard deviations for different replicates are presented in tabular form in Appendix C.

The samples were analyzed with a gas chromatograph (Hewlett Packard 5890, series II) equipped with an electron capture detector (GC/ECD) and a HP-5 (crosslinked 5% Ph Me silicone) capillary column of 25m x 0.2mm x 0.33μm film thickness. Helium was used as a carrier gas at a flow rate of 1mL/min. The initial temperature (60 ^{o}C) was held for 2 min, then raised at a rate of 5 ^{o}C/min to a final temperature of 290 ^{o}C held for 15 min. Injector and detector temperatures were 275 and 350 ^{o}C, respectively.

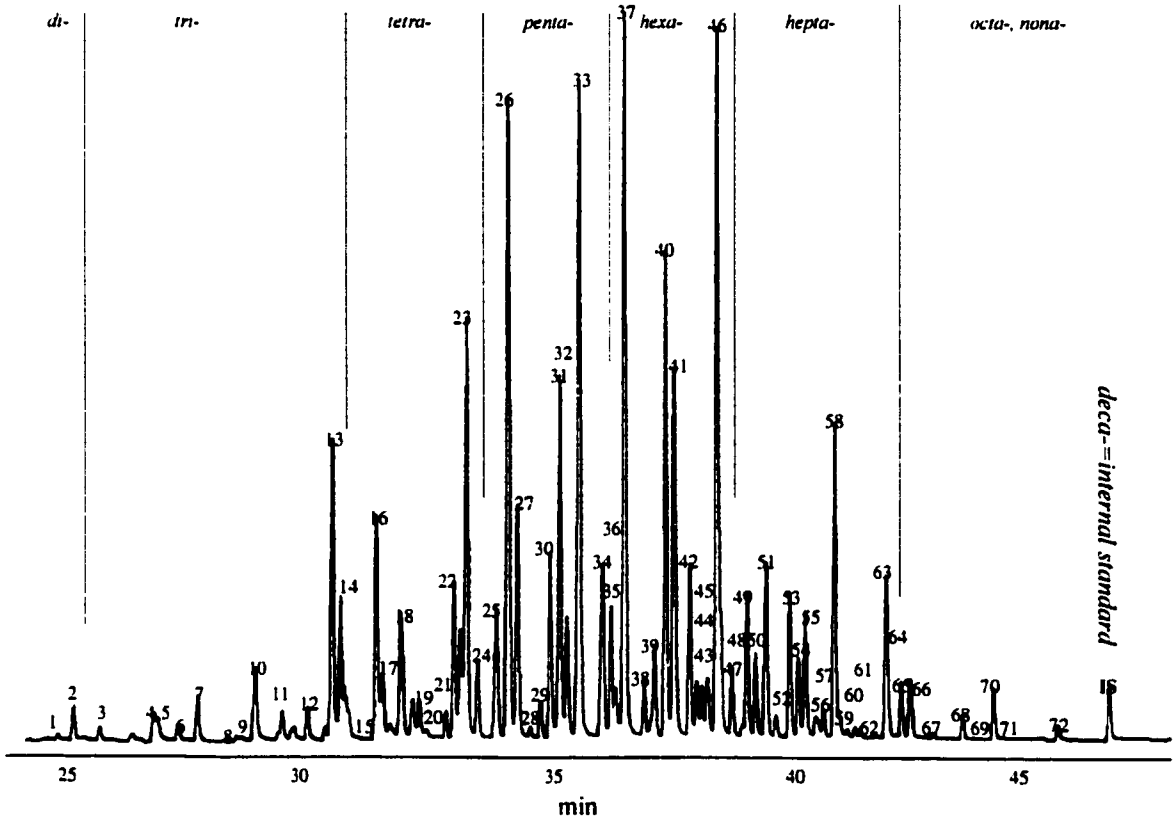


Figure 6.2. Chromatogram of the assigned PCB congeners present in the soil produced by our GC/ECD method.

Table 6.4. Assignment of congeners to peaks analyzed on GC-ECD.

peak	IUPAC PCB	Chemical formula	peak	IUPAC PCB	Chemical formula	peak	IUPAC PCB	Chemical formula
1	6	23'	26	101, 84	22'455'& 22'33'6	51	128, 167	22'33'44' & 23'44'55'
2	5,8	23 & 24'	27	99	22'44'5	52	185	22'3455'6
3	19	22'6	28	119	23'44'6	53	174	22'33'456'
4	18	22'5	29	83	22'33'5	54	177	22'33'4'56
5	15, 17	44' & 22'4	30	97	22'3'45	55	156, 171	233'44'5 & 22'33'44'6
6	24, 27	236 & 23'6	31	87, 81	22'345'& 344'5	56	157, 173	233'44'5' & 22'33'456
7	16, 32	22'3 & 24'6	32	85	22'344'	57	172	22'33'455'
8	34	2'35	33	110, 77	233'4'6 & 33'44'	58	180	22'344'55'
9	29	245	34	151, 82	22'355'6 & 22'33'4'	59	193	233'4'55'6
10	26	23'5	35	135, 144, 124	22'33'56' & 22'345'6 &2'3455'	60	191	233'44'5'6
11	31, 28	24'5 & 244'	36	107, 147	233'4'5 & 22'34'56	61	199	22'33'4566'
12	33, 20, 53	2'34 & 233' & 22'56'	37	149, 123, 118	22'34'5'6 & 2'344'5 & 23'44'5	62	169	33'44'55'
13	22	234'	38	134	22'33'55'	63	170, 190	22'33'44'& 233'44'56'
14	45	22'36	39	146	22'34'55'	64	198	22'33'455'6
15	46	22'36'	40	153	22'44'55'	65	201	22'33'4'55'6
16	52	22'55'	41	132, 105	22'33'46' & 233'44'	66	196, 203	22'33'44'5'6 & 22'344'55'6
17	49	22'45'	42	141	22'3455'	67	189	233'44'55'
18	44	22'35'	43	179	22'33'566'	68	195	22'33'44'56
19	64, 41, 71	234'6 &22'34 & 23'4'6	44	130	22'33'45'	69	207	22'33'44'566'
20	40	22'33'	45	176, 137	22'33'466' & 22'344'5	70	194	22'33'44'55'

21	74	244'5	46	138, 163, 160	22'344'5' & 2334'56 & 233'456	71	205	233'44'55'6
22	70	23'4'5	47	178, 129, 126	22'33'55'6 & 22'33'45 & 33'44'5	72	206	22'33'44'55'6
23	66,95	23'44' & 22'35'6	48	175, 159	22'33'45'6 & 233'455'	73	*209	22'33'44'55'6 6'
24	91	22'34'6	49	187	22'34'55'6			
25	56, 60, 92	233'4' & 2344' & 22'355'	50	183	22'344'5'6			

Peaks that were identified in our chromatographic system
[IU]- PCB congeners refer to IUPAC convention per Ballschmiter and Zell (1980). *- Congener 209 used as internal standard.

6.2.7 Statistical Analysis

The concentration of the internal standard (decachlorobiphenyl) was used to determine the concentration of every observed peak for each sample and then these were summed as the total PCB concentration. The total PCB concentrations for the initial time and for different treatments after 345 days at 140, 340, and 590 mm depths were determined using this procedure. The initial total PCB concentration was included in the statistical analysis as a covariable. However, no significant effect was found for the initial concentration and it was therefore not used in the model. The repeated measures ANOVA was performed using the General Linear Model (GLM) procedures of SAS version 6 (SAS Institute Inc., 1989).

6.3 RESULTS AND DISCUSSIONS

6.3.1 Biotransformation of PCBs under Aerobic and Anaerobic Cycles

In the water treatment columns, most of the di- tri-, and more than half of the hepta- chlorinated congeners showed enrichment compared to the passive control during the first 10 day dry cycle (Figure 6.3a). Increases were observed for peaks 15, 64, and 69, whereas, peaks 43, 48, 59, 61 depleted. This pattern was observed to be very different during the subsequent saturation period of 10 days (Figure 6.3b) where almost all peaks enriched compare to the passive control. The only peaks with depletion of >20% were 1, 18, and 35. During the following 10 days of dry cycle (Figure 6.3c), depletions were observed for the penta- to hepta-chlorinated congeners. No enrichment of any of other congeners was seen. The peaks that depleted the most were 51, 56, and 64. During the following 10 days of saturation (Figure 6.3d), all the di- tri- and some of the tetra- congeners showed enrichment and most of the penta- to hepta- chlorinated congeners depleted slightly less than in the previous dry cycle. Peaks 21, 36, 41, 44, 51, 55, and 56 showed depletions of up to >60%.

In the columns bioaugmented with indigenous microflora, all the di- to hexa- and more than half of the hepta- congeners were depleted during the 10 days of dry cycle (Figure 6.4a). Peaks 43, 48, and 61 depleted >40% and peaks 64 and 69 enriched, similar to the water treatment (Figure 6.3a). During the following wet cycle of 10 days (Figure 6.4b), the di- to tetra- congeners also showed results similar to that of the water treatment. The same was observed with peaks 61 and 67. However, in this treatment, peak 71 showed a significant enrichment that was

not observed in the water treatment. Peaks that depleted (>40%) were 1, 35, and 64. Peak 64 also showed the greatest depletion (>50%) during the following dry cycle (Figure 6.4c). Most of the hexa- and hepta- congeners also showed decreases of > 20% and peak 71 showed a large decrease from the previous wet cycle. This peak further depleted during the next wet cycle (Figure 6.4d). Also, the pattern was quite similar to that observed with the water treatment during the same wet cycle (Figure 6.3d). All the di-, tri- and more than half of the tetra- congeners enriched whereas, the higher chlorinated congeners, peaks 21, 35, 44, 51, and 56 depleted up to >40%.

Results from bioaugmentation with B-356 were more pronounced compared to the water and the other two bioaugmented treatments. During the 1st dry cycle (Figure 6.5a), most of the peaks enriched or only slightly depleted except peak 61 where the depletion was >60%. Most of the enrichments were of the tri- tetra-, and the hepta- congeners. During the subsequent wet cycle (Figure 6.5b), there was not much difference between this treatment and the control except that the peaks 8, 9, 20, and 21 enriched to >50% and peaks 1 and 35 depleted to >50%. During the next dry cycle (Figure 6.5c), the over all pattern was more or less similar to that of indigenous treatment. The di-, tri- and most of the tetra- congeners enriched or slightly changed, whereas, the penta- to hepta- congeners showed decreases of to >20%. Peaks 44, 51, and 56 depleted to >40%. The next wet cycle (Figure 6.5d) showed similar pattern to that observed with indigenous microflora. All the di- tri- and most of the tetra- congeners enriched and the more chlorinated congeners depleted. Peaks 21, 28, 32, 36, 41, 44, 51, 55, and 56

depleted to >40%. Peak 15 depleted only during the 2nd wet period while peak 71 enriched during the same period. Peak 64 depleted only during the two wet cycles.

The addition of BP to the B-356 bioaugmented treatment on the 20th day, could explain why there was little effect in this treatment up to this date (Figure 6.5a) that was followed by the next wet cycle (Figure 6.5b). Strain B-356 ability to degrade PCBs in soils has shown to increase in presence of BP (Barriault and Sylvestre, 1993). It has been suggested that aerobic degradation of PCBs is enhanced by addition of growth substrates such as biphenyl (Quensen III et al., 1990; Kohler et al., 1988) and of organic substrates such as glucose, methanol, or acetone in anaerobic sediments has shown to enhance dechlorination (Nies and Vogel, 1990).

Augmentation with *R. meliloti* A-025 resulted in pronounced depletion in most of the peaks and especially in the di- and tri- congeners during the 1st dry cycle (Figure 6.6a). Peaks 64 and 69 enriched and peaks 43, 48, and 59 depleted similar to that of the water and indigenous microflora treatments. Peaks 1, 4, 5, 8, 9, 67, and 71 depleted to >60%. During the next wet cycle (Figure 6.6b), the tetra- and higher chlorinated congeners showed smaller changes as compared to the passive control. However, most of the tri- chlorinated congeners enriched. The pattern during the next two periods of dry and wet were similar to those of the water treatment and the di-, tri- and most of the tetra congeners enriched, whereas, the higher chlorinated congeners depleted. During the dry cycle (Figure 6.6c), peaks 56 and 64 depleted the most and during the wet cycle (Figure 6.6d), peaks 56 and 61 depleted to >90% and peaks 21, 51, and 64 depleted to >40%.

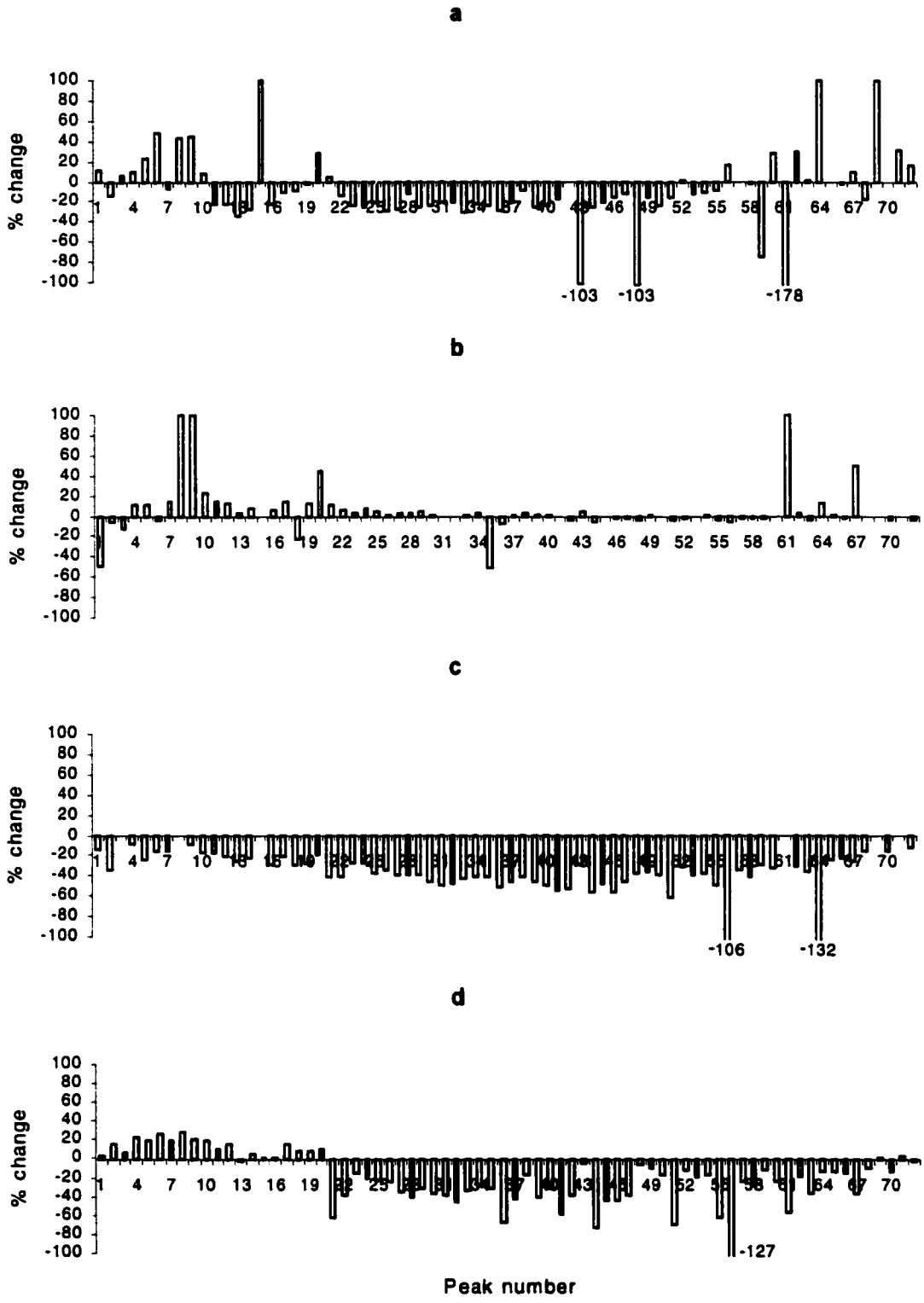


Figure 6.3. Concentration change of PCB congeners in the water treatment as compared to the passive control after a) 20 days, dry cycle, b) 32 days, wet cycle, c) 44 days, dry cycle, d) 56 days, wet cycle. Means of triplicates.

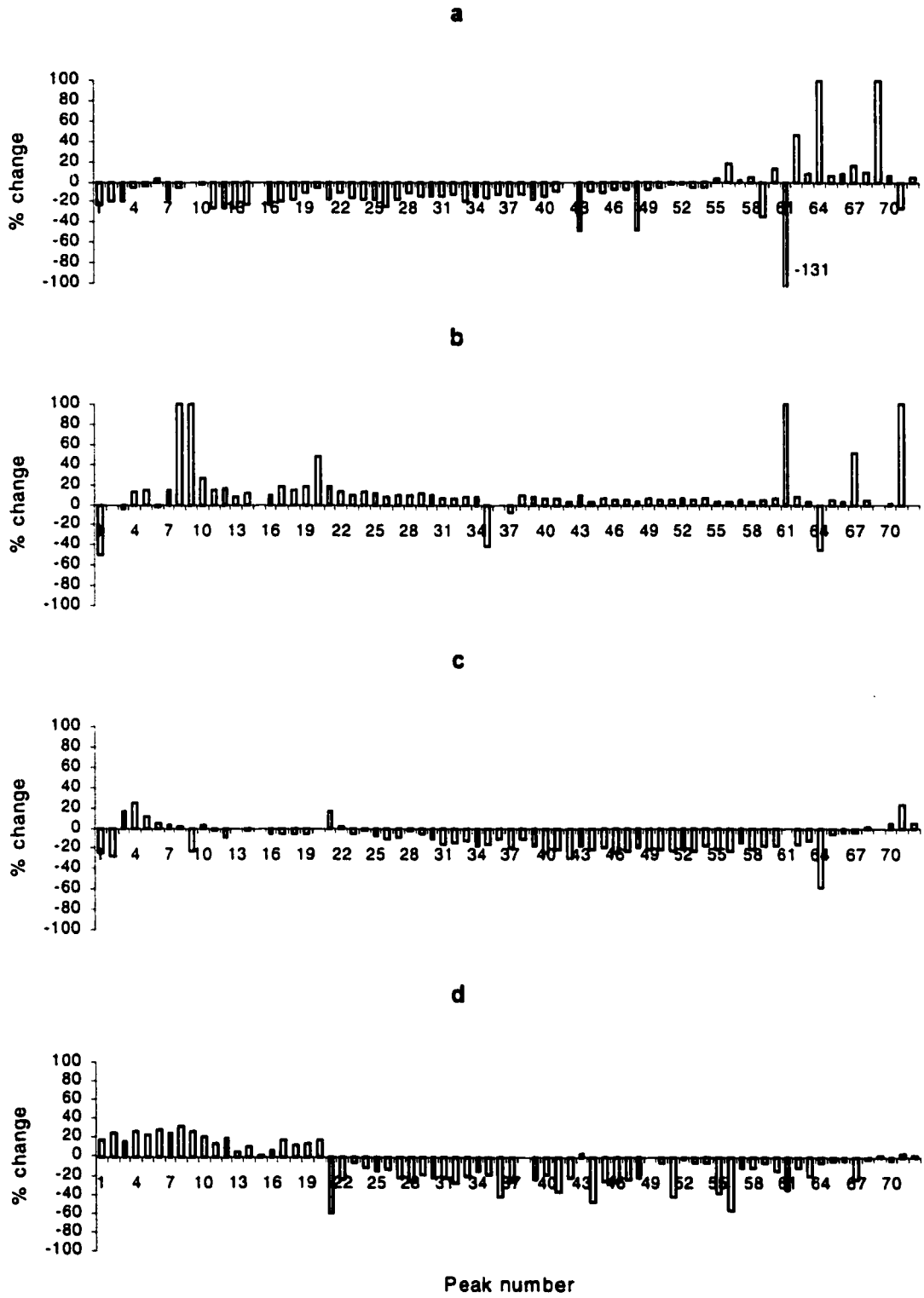


Figure 6.4. Concentration change of PCB congeners in columns augmented with indigenous microflora as compared to the passive control after a) 20 days, dry cycle, b) 32 days, wet cycle, c) 44 days, dry cycle, d) 56 days, wet cycle. Means of three replicates.

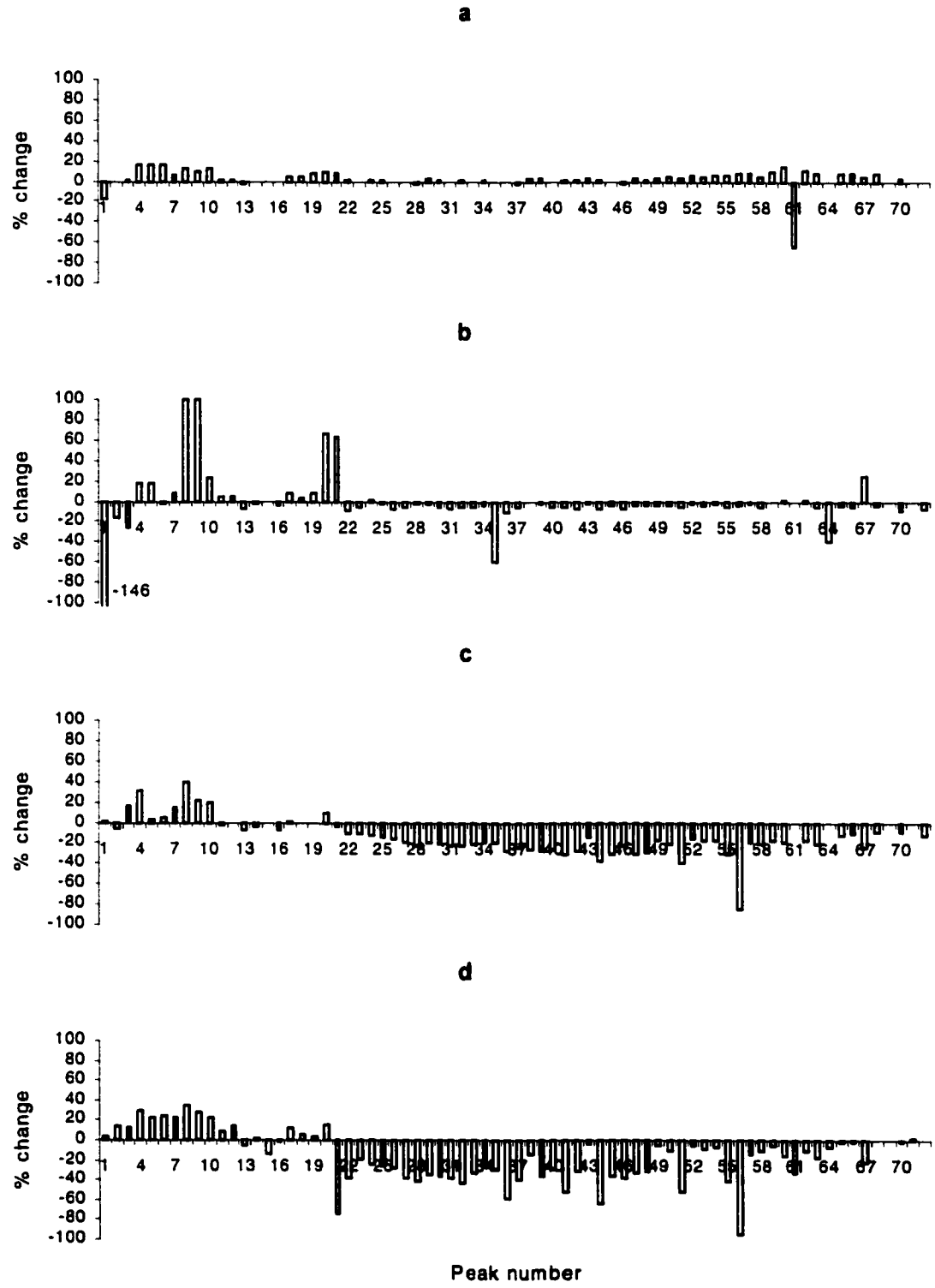


Figure 6.5. Concentration change of PCB congeners in columns augmented with B-356 as compared to the passive control after a) 20 days, dry cycle, b) 32 days, wet cycle, c) 44 days, dry cycle, d) 56 days, wet cycle. Means of three replicates.

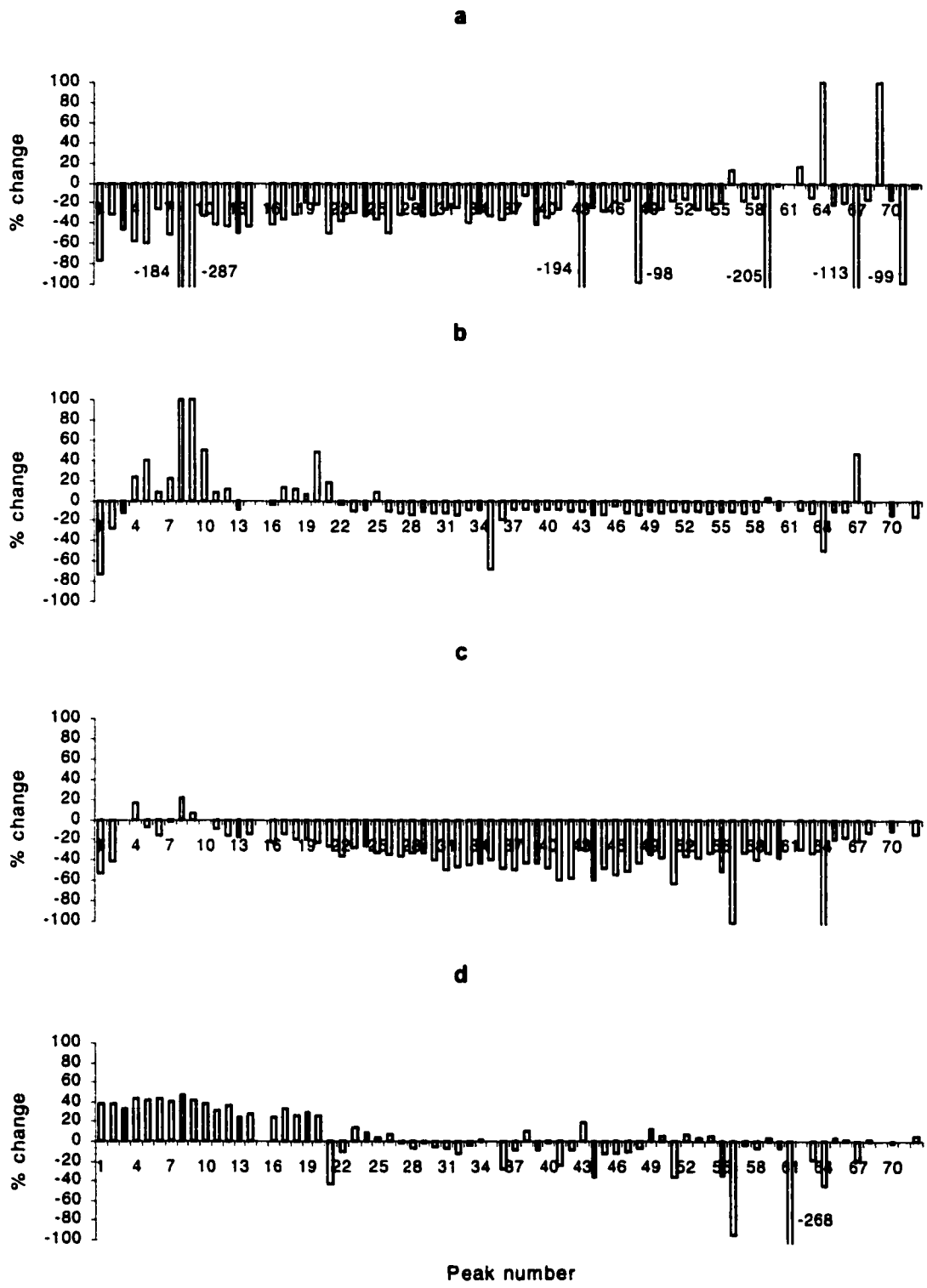


Figure 6.6. Concentration change of PCB congeners in columns augmented with A-025 as compared to the passive control after a) 20 days, dry cycle, b) 32 days, wet cycle, c) 44 days, dry cycle, d) 56 days, wet cycle. Means of triplicates.

During the 30-day cycles, the water treatment showed most transformations for the congeners during the 1st two cycles after that stabilized during the 3rd and 4th cycles (Figure 6.7). During the first dry cycle of 30 days (Figure 6.7a) congeners up to hexa- chlorination were depleted to >40%. However, during the following 30 days of saturation (Figure 6.7b), every congener except congener 49 enriched as compared to the passive control. In the following two cycles (Figure 6.7c,d), all congeners depleted except for few of the di-, tri-, and the octa- congeners.

Augmentation with the indigenous microflora was influenced the most by the dry and wet cycles (Figure 6.8). During both dry cycles, most peaks depleted to >40% and for some >60%. During both wet cycles the di- and tri- congeners enriched and during the last wet cycle most of octa- and nona- congeners also enriched. Peak 61 enriched during both wet cycles and no change during the dry cycles.

Augmentation with B-356 showed enrichment in the di- and some of the tri- congeners (Figure 6.9). The depletions were higher for the tri-, tetra-, penta-, hexa- and most of the hepta- congeners during the dry cycles. This pattern was slightly shifted to higher chlorinated congeners (penta- and higher) during the wet cycles.

Augmentation with A-025 resulted in the least effect of the different cycles. Nevertheless, more depletion was observed during the dry cycles (Figure 6.10a,b). During the 1st dry cycle the depletions were observed to be higher in the

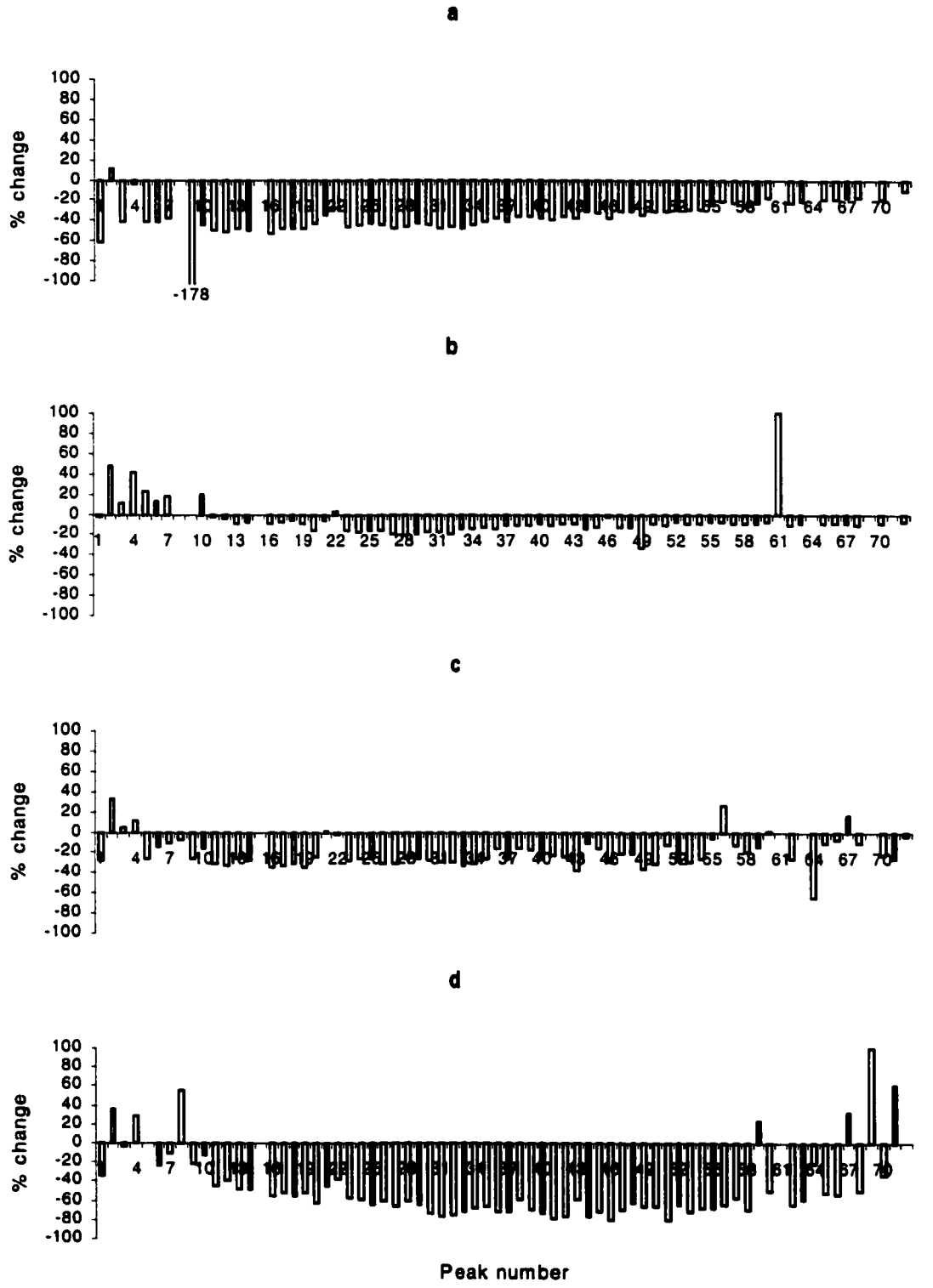


Figure 6.7. Concentration change of PCB congeners in the water treatment as compared to the passive control after a) 282 days, dry cycle, b) 314 days, wet cycle, c) 345 days, dry cycle, d) 377 days, wet cycle. Means of triplicates.

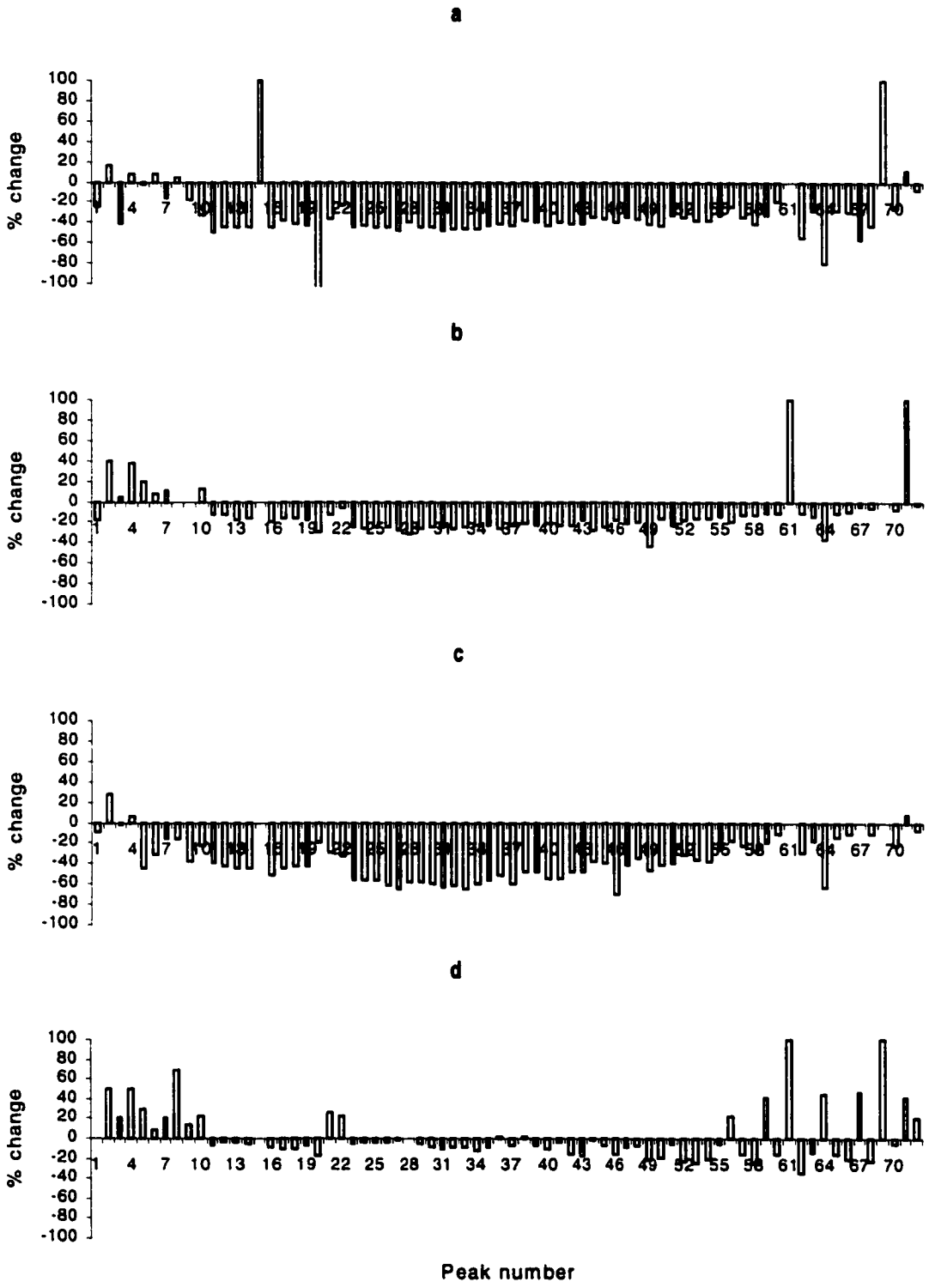


Figure 6.8. Concentration change of PCB congeners in columns augmented with indigenous microflora as compared to the passive control after a) 282 days, dry cycle, b) 314 days, wet cycle, c) 345 days, dry cycle, d) 377 days, wet cycle. Means of three replicates.

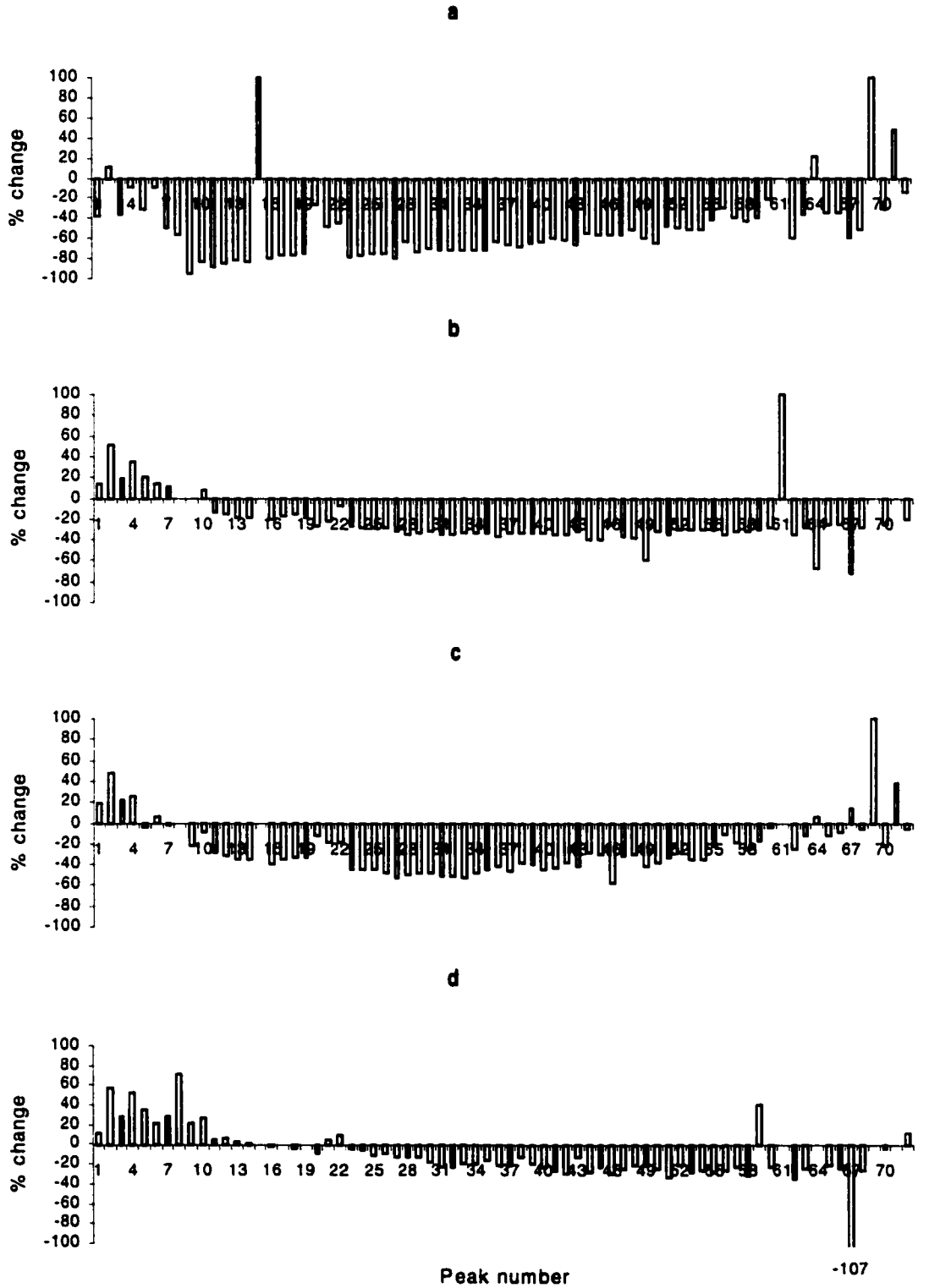


Figure 6.9. Concentration change of PCB congeners in columns augmented with B-356 as compared to the passive control after a) 282 days, dry cycle, b) 314 days, wet cycle, c) 345 days, dry cycle, d) 377 days, wet cycle. Means of triplicates.

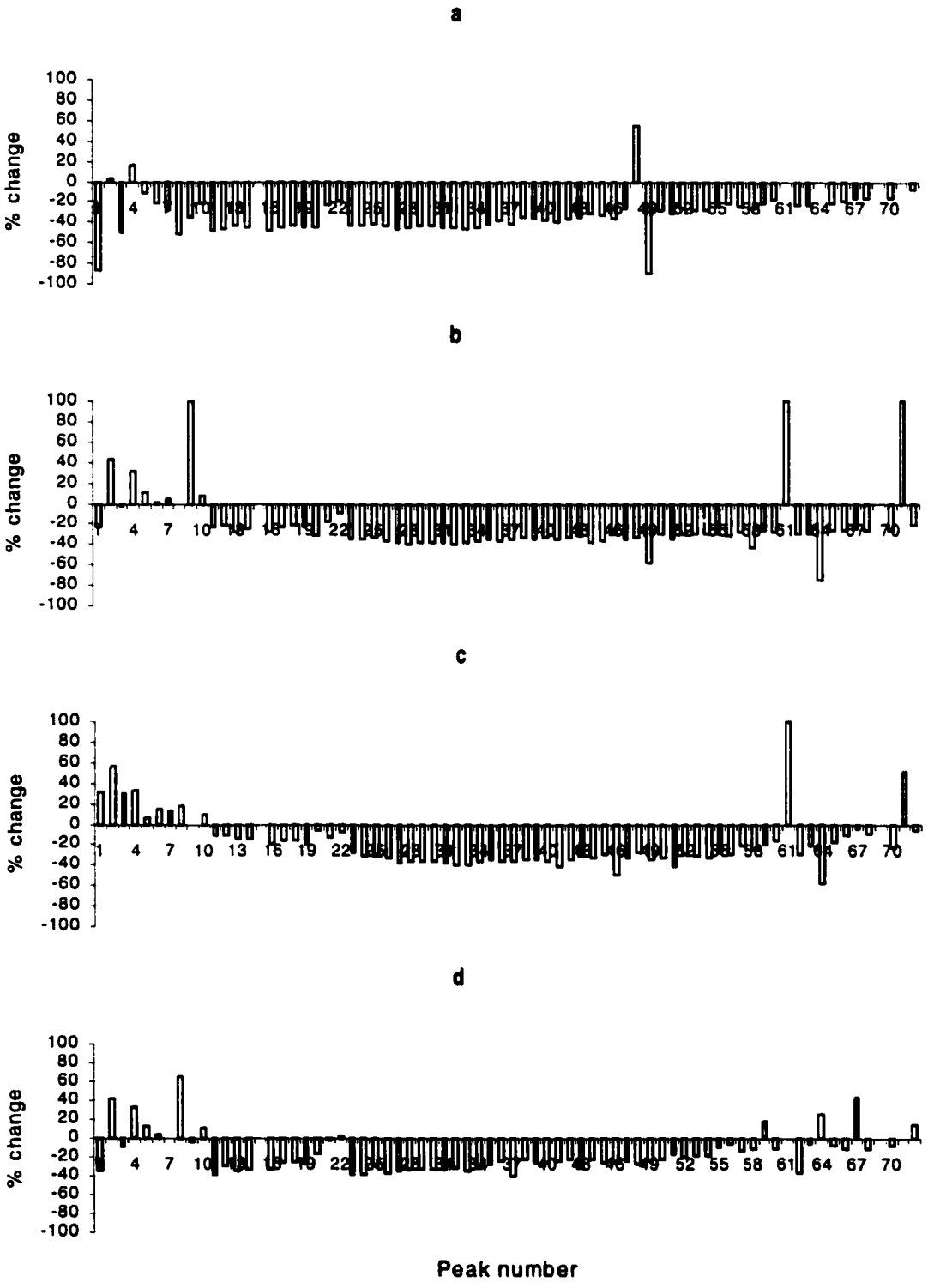


Figure 6.10. Concentration change of PCB congeners in columns augmented with A-025 as compared to the passive control after a) 282 days, dry cycle, b) 314 days, wet cycle, c) 345 days, dry cycle, d) 377 days, wet cycle. Means of triplicates.

tri- to hexa- chlorinated congeners. However, during the next wet cycle (Figure 6.10b) the di- and some of the tri- congeners showed increases that continued during the following dry (Figure 6.10c) and wet cycles (Figure 6.10d).

The period of the initial 4 cycles (10 days each cycle) covers the first 56 days after inoculation with the bacteria. Therefore, it was important to see the patterns of transformation because the number of augmented bacterial cells were the highest during these periods. Furthermore, These periods can show how the systems or the different treatments stabilized as time progressed.

The 30 day cycles represent the last 120 days of this study. This period was compared to the 10 day cycles to see if PCB degradation in each cycle would be influenced by its duration. As Figures 6.3-6.10 show, the influence of the 30-day cycle was more significant between the dry and wet cycles. The dry cycles were more effective at 30-day periods rather than 10-day periods. Furthermore, it is difficult to evaluate the changes (biotransformation) for the wet periods. These changes could have been affected by desorption of the PCBs from the soil medium in presence of water rather than it being biological.

6.3.2 Final Biotransformation at Different Depths

In addition to periodic analysis of PCB biotransformation at the depth of 340 mm at different times, samples from the 590 and 140 mm depths were also analyzed. These results were used to compare changes in the biotransformation of PCB congeners at different depths after 345 days.

590 mm Depth

The transformations in the ratio of the PCB peaks in the water treatment near the bottom at 590 mm after 345 days showed enrichment with almost all peaks and more significantly with the di- and tri- PCB congeners (Figure 6.11a). Similar results were observed for the di- and tri- congeners in the indigenous treatment (Figure 6.11b). However, peaks higher than tetra- chlorinated congeners showed slight depletions. The B-356 treatment enriched in the di- and tri- congeners mostly with peaks 5, 6, and peak 9 showed no change at all (Figure 6.11c). However, congeners higher than the tetra- chlorination depleted more than those observed in the water and the indigenous microflora treatments. The A-025 treatment showed the most depletions in the congeners with higher than the tetra- chlorination and peaks 56 and 67 showed >55% depletion (Figure 6.11d). The A-025 treatment was the only treatment where peak 67 showed such depletion. Furthermore, similar to strain B-356 (Figure 6.11c), peak 56 depleted to >50% with A-025. However, peaks 9 and 64 enriched in both water and A-025 treatments and showed no change in the indigenous and B-356 treatments.

340 mm Depth

The water treatment at the 340mm depth showed the highest levels of over all transformation compared to the same treatment at the 590 (Figure 6.11a) and 140mm (Figure 6.13a) depths. The most enrichment was observed in peak 56 (IUPAC congeners 157 and 173) (Figure 6.12a). Also at this depth, peak 71 (IUPAC congener 205) depleted, whereas, no changes were observed at the other two depths. Most decreases were observed in the tri-, tetra-, penta-, and hexa-

congeners. The columns with the indigenous microflora showed the most depletion as compared to the other treatments at the 340 mm depth (Figure 6.12b). The most depletion (>50%) was observed in the penta- and hexa- congeners with peak 46 (IUPAC congeners 138, 163 and 160) depleted to >60%. Peak 2 (IUPAC congeners 5 and 8) enriched as was observed at the other two depths. The B-356 treatment showed similar patterns but at lower depletion for the tetra- to hepta-congeners (Figure 6.12c) compared to that observed in the indigenous microflora treatment (Figure 6.12b). However, peak 69 (IUPAC congener 207) enriched at this depth in the B-356 treatment. The accumulation of the di- and tri- congeners differed from those observed at the 590 (Figure 6.11c) and 140mm (Figure 6.13c) depths with similar treatments. The A-025 treatment showed an over all transformation (Figure 6.12d) similar to those observed in the B-356 (Figure 6.12c) and to the indigenous microflora (Figure 6.12b) treatments with which the most depletion was observed in the tetra- to hepta- congeners. The most depletion (>40%) were observed in peaks 41, 46, and 51.

140 mm Depth

At 140mm depth, the water treatment showed different pattern of PCB transformation (Figure 6.13a) than those observed at the 340 and 590mm depth. The di- and most of the tri- congeners showed similar enrichment to that observed at 590mm, but peaks 8 and 9 showed significant smaller peaks than those at 590 mm. The most depletion was observed to be >20% in peaks 62 (IUPAC congener 169), 68 (IUPAC congener 195), and 70 (IUPAC congener 194). The indigenous treatment at depth 140mm (Figure 6.13b) showed the highest levels of depletion

within all treatments (Figure 6.13). Peak 64 (IUPAC congener 198) showed 193% lower concentration than that was observed in the passive control treatment and peak 71 (IUPAC congener 205) enriched the most. The B-356 treatment showed the most depletion in peaks 1 and 11 with 30 and 25% respectively (Figure 6.13c). Peak 9 showed the most enrichment (>55%) and both peaks 2 and 4 showed less enrichment than observed at the 590mm depth. As a whole, some of the tri- and most of the tetra- congeners showed the most depletion in the concentration as compared to the passive control. The A-025 is the only treatment that enriched in peak 61 (IUPAC congener 199) (Figure 6.13d) and similar depletion in peak 64 (IUPAC congener 198) as that observed with the indigenous microflora (Figure 6.13b). Furthermore, peak 20 (IUPAC congener 40) enriched only in this treatment. Even though the depletion in the congeners with higher than tetra-chlorination was similar or insignificantly different than that observed at the 590mm depth of this treatment (Figure 6.11d), some of the tri- and tetra-congeners showed higher concentrations at 590mm than at 140mm.

The overall biotransformation in the treatments as compared to the passive control was highest at 340mm depth and lowest at 590mm depth. The most depletion at depth 340mm was observed to be in the indigenous microflora treatment followed by B-356, A-025 and water treatments, respectively. Although smaller but similar pattern of depletion in the changes were observed at the 140mm depth, whereas, at the 590mm depth augmentation with strain A-025 showed the most depletion followed by B-356, indigenous microflora, and water treatments, respectively.

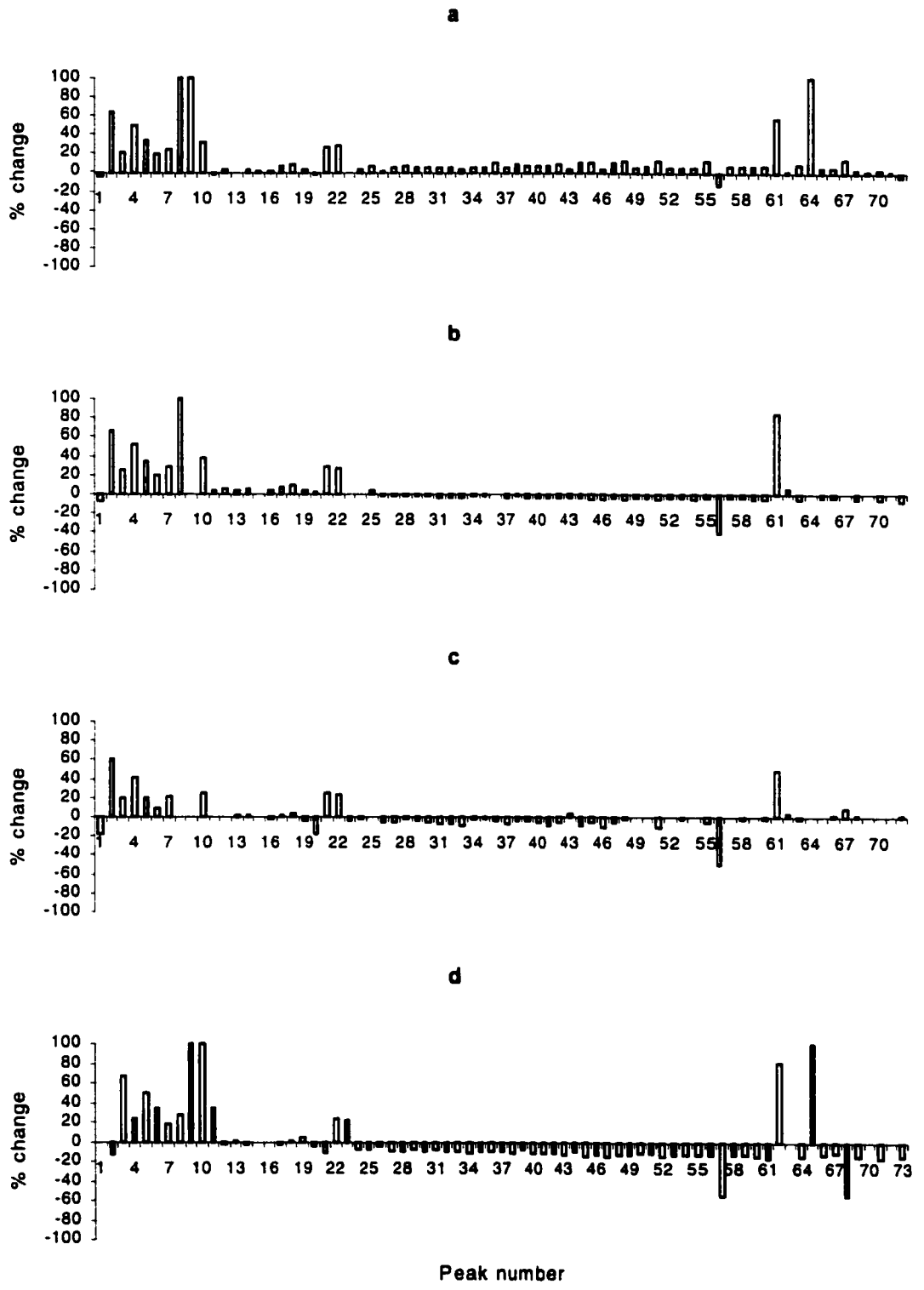


Figure 6.11. Concentration change of PCB congeners after 345 days at 590 mm depth in treatments with a) water, b) indigenous microflora, c) B-356, d) A-025 as compared to the passive control. Means of three replicates.

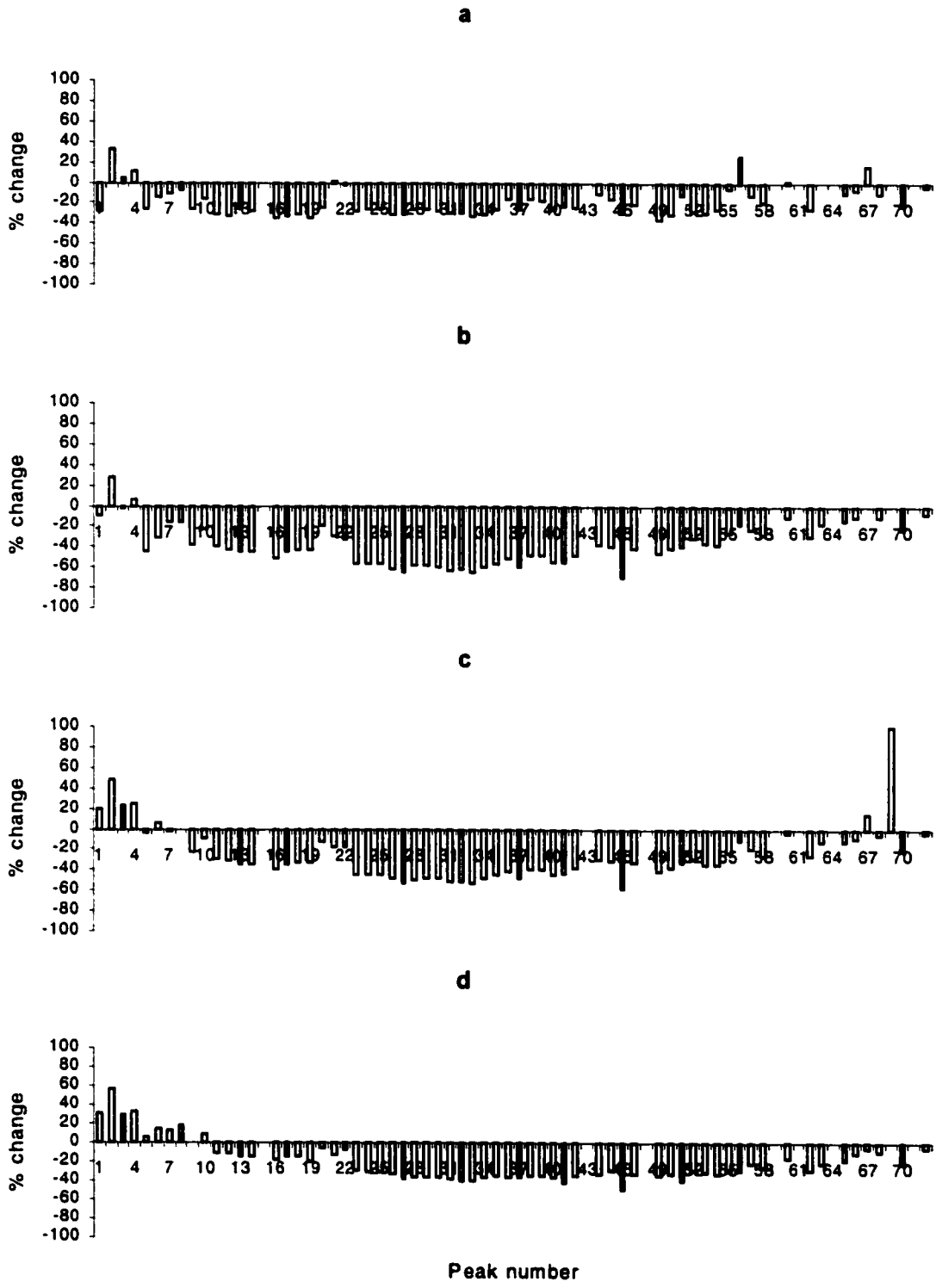


Figure 6.12. Concentration change of PCB congeners after 345 days at 340mm depth in treatments with a) water, b) indigenous microflora, c) B-356, d) A-025 as compared to the passive control. Means of three replicates.

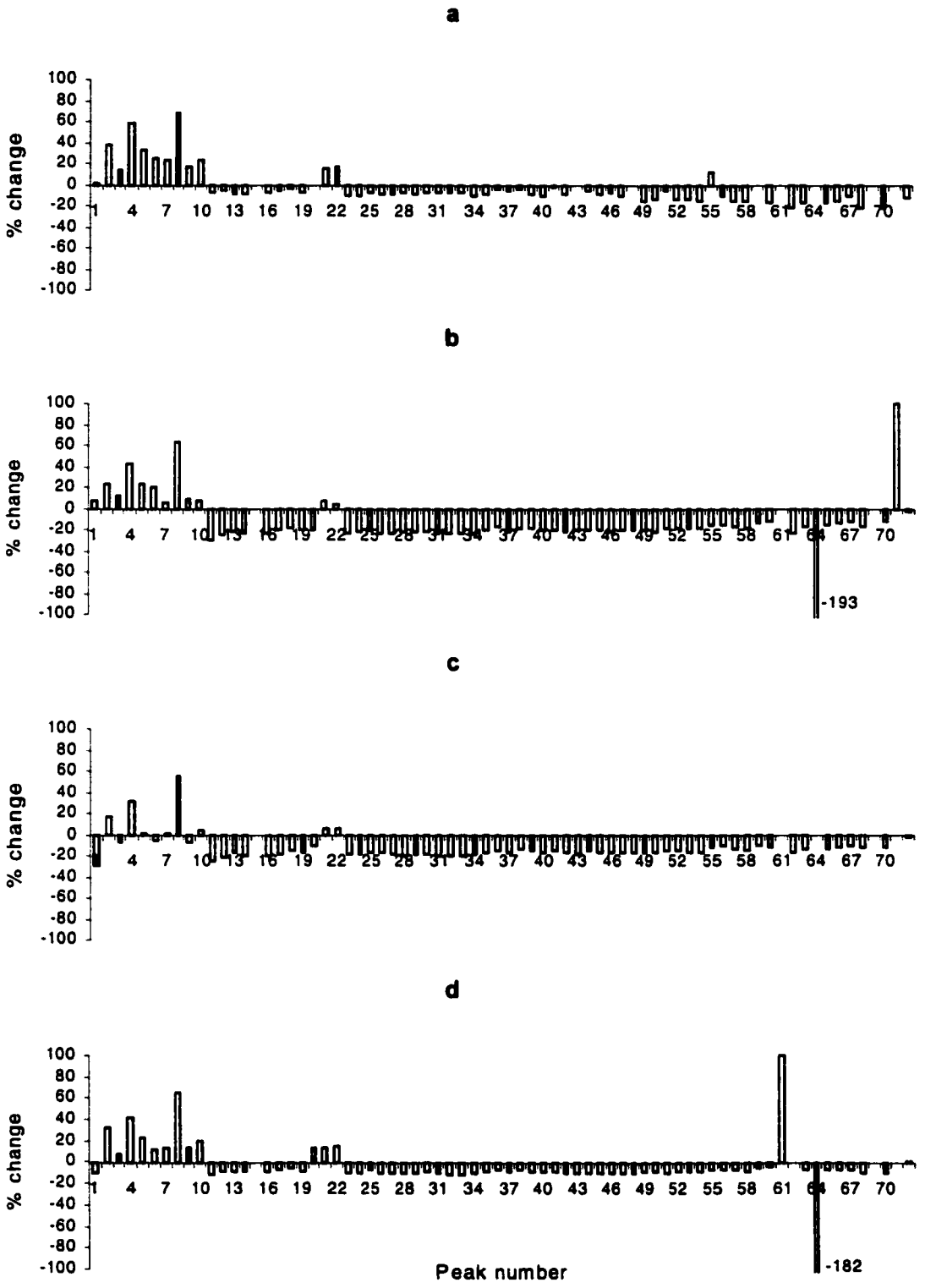


Figure 6.13. Concentration change of PCB congeners after 345 days at 140 mm depth in treatments with a) water, b) indigenous microflora, c) B-356, d) A-025 as compared to the passive control. Means of three replicates.

6.3.3 Total PCB Concentration at Different Depths

The two previous sections demonstrated the biotransformation of PCB congeners in time and depth. These results were a comparison of treatments to the passive control to determine the effect of bioaugmentation on PCBs. However, in such a system where the complexity of the soil, PCBs, and the microorganisms are combined, the passive control alone can be effected due to both biological and chemical interactions in time and depth. Therefore, the actual PCB concentrations and losses might not be evident when treatments are compared to the passive control. In this section the total PCB concentrations are discussed and statistically analyzed to demonstrate the effect of bioaugmentation on PCB concentrations at different depths.

The results of the bioaugmented treatments with strains A-025, B-356, and the indigenous culture were statistically compared to the passive control and water treatment samples. The initial samples showed the least total concentration of PCBs (63.11±19 ppm) when compared to the total PCB concentration at any depth or treatment (Tables 6.7 and 6.9) after 345 days. This indicates that the period and local parameters (time, temperature) of the experiment alone influenced the PCB levels that were extractable and analyzed. This is most evident when the initial samples were compared to the passive control for the 340 mm depth that had the highest total PCB concentration (Table 6.9). As shown in Table 6.5, both treatment and depth played a significant role in total PCB concentrations and the three bioaugmented treatments were determined to be significantly different when compared to the passive control and less significant from the water

treatment (Table 6.7). The pattern of PCB concentrations at different depths for all treatments showed to be more quadratic than linear (Table 6.6 and Figure 6.14).

The lowest total PCB concentrations, as an average of the three depths, were determined to be in the indigenous bioaugmented treatment (Table 6.7) followed by B-356 and A-025. The least total PCB concentrations were observed to be at the 590 mm depth when compared to the 140 and 340 mm depths. However, there was no significant difference between the bioaugmented treatments and the passive control at this depth as was observed for the other two depths (Table 6.9). The highest concentrations were observed to be at the 340 mm depth for all treatments.

Table 6.5. Univariate procedure of repeated measures ANOVA for total PCB concentrations (α=0.05).

Source	Pr > F
Treatment	0.0214
Depth	0.0001
Depth*Treatment	0.0397

Table 6.6. Polynomial contrast among different treatments over various depths.

Effect	Pr > F
Linear	0.1059
Quadratic	0.0994

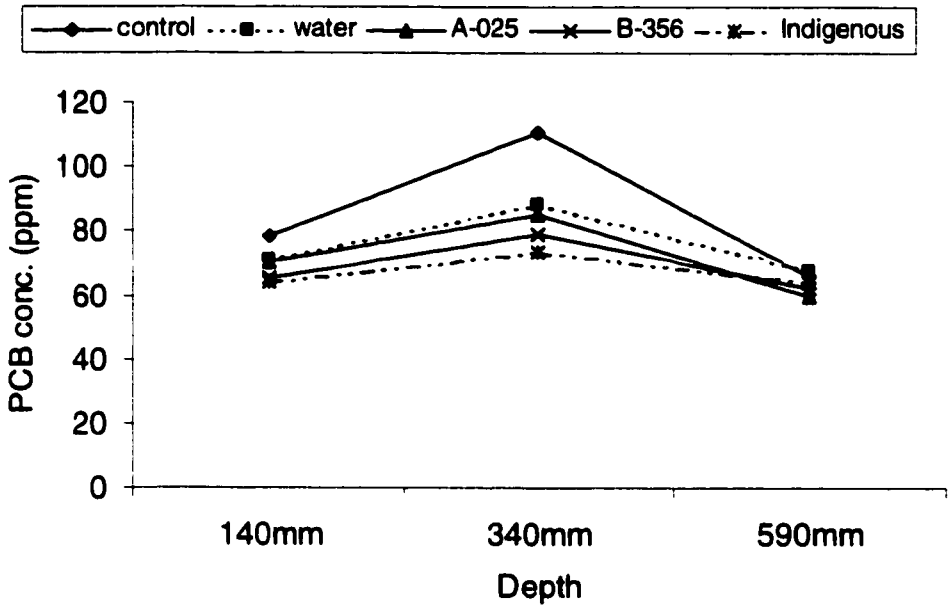


Figure 6.14. The polynomial contrast of pattern in total PCB concentrations over various depths for different treatments.

Table 6.7. Multiple pairwise comparisons of overall mean in depth among treatments for total PCB concentration.

Treatment	Mean*
Control	85.17 a
Water	75.71 ab
A-025	71.58 bc
B-356	68.62 bc
Indigenous	67.13 bc

*- Treatment means with different letters are significantly different (α=0.05), based on protected LSD test.

Table 6.8. General Linear Model procedures for total PCB concentrations at different depths (α=0.05).

Source		140mm	340mm	590mm
	C.V.	6.13	14.45	8.75
Treatment	Pr > F	0.0131	0.0369	0.4080

C.V.-coefficient of variation

Table 6.9. Multiple pairwise comparisons of means among treatments for total PCB concentration.

	Mean (ppm)		
Treatment	140mm*	340mm*	590mm*
Control	78.57 a	110.73 a	66.17 a
Water	71.11 ab	88.22 ab	67.80 a
A-025	70.23 b	85.12 b	59.40 a
B-356	65.20 b	78.80 b	61.87 a
Indigenous	63.88 b	73.23 b	64.27 a

*- Treatment means with different letters are significantly different (α=0.05), based on protected LSD test.

The complexities of PCB transformation make the explanation of the observations in this study highly speculative. Several possible explanations can be forwarded. The indigenous microflora was isolated aerobically. Therefore they showed to be the best treatment closer to the surface due to better access to O_2. The 140 and 340mm depths could have become more aerobic than the 590mm depth. Saturating and draining of the soil columns from the bottom could have created a longer and a more anaerobic environment at the 590mm depth. Moreover, strain A-025 was not enriched as B-356 and indigenous microflora were enriched with biphenyl and Aroclor 1242 (Table 6.2). Bedard et al. (1997) have shown enrichment of the inoculum can increase the dechlorination ability of the organisms. Furthermore, transformations in the B-356 treatment may not solely be due to augmentation with this strain, since, this treatment was spiked with biphenyl as well (Table 6.3). Biphenyl is suggested to be a requirement for maximal PCB degradation ability as an inducer of the dioxygenase pathway (Abramowicz, 1990). Focht and Brunner (1985) have reported an enhancement of nearly 60% in degradation of Aroclor 1242 with addition of biphenyl.

6.3.4 Risk Assessment

The health effect of PCBs is related to the structure of each of the possible 209 congeners based on the number and position of the chlorines present. Most studies involving the toxicity of PCBs have been based on dose of Aroclors, mixtures of many different PCB congeners. However, some studies such as that by Metcalfe et al. (1995) has shown the effect of individual congeners. They

found that the *meta-* and *para-* congeners showed to be more toxic than the *ortho-* congeners. The lack of *ortho-* substituted chlorine minimizes the steric hindrance and allows the phenyl rings to rotate more freely and thus creating the co-planar configuration.

The concentration of the co-planar congeners (15, 77, 81, 126, 169) present in the soil of each treatment were determined and compared to the passive control. The results indicated an overall decrease of the five congeners in the three bioaugmented treatments (Table 6.10). Congener 15 showed to increase in concentration in most cases and mostly at the 590 mm depth. This is expected, since congener 15 could be the product of the dechlorination of higher chlorinated congeners. However, this congener would be degraded rapidly by the aerobic organisms since the *meta-* and *ortho-* positions are available to be attacked by such organisms.

Another method used to interpret PCB's environmental effects is to group the dioxin-like congeners together. Wolff et al. (1998) have suggested one possible set of PCB groupings based on the persistence, hormonal effect and enzyme induction. These congeners and their groupings are presented in Figures 6.15 to 6.17. The concentrations of all congeners, potentially antiestrogenic, immunotoxic (Figure 6.15), enzyme inducers (Figures 6.16 and 6.17) and persistent and dioxin-like PCB congeners showed lower concentration in the three-bioaugmented treatments than the passive control.

Table 6.10. The changes in concentration of co-planar PCB congeners as compared to the passive control after 345 days.

Depth (mm)	Congener	Concentration (ppb)	% reduction			
		Control	Water	A-025	B-356	Indigenous
140	15	171	+40	+23	3	+24
	77	6349	10	15	20	23
	81	3116	10	14	20	23
	126	568	12	14	18	20
	169	71	21	5	18	22
340	15	319	20	+5	5	31
	77	9257	25	29	35	39
	81	4547	22	28	34	39
	126	756	17	25	24	29
	169	78	20	23	20	23
590	15	289	+47	+48	+20	+49
	77	5112	1	12	11	7
	81	2473	+2	12	10	5
	126	493	+7	15	8	7
	169	68	2	4	+1	+3

+- increase in concentration as compared to the passive control.

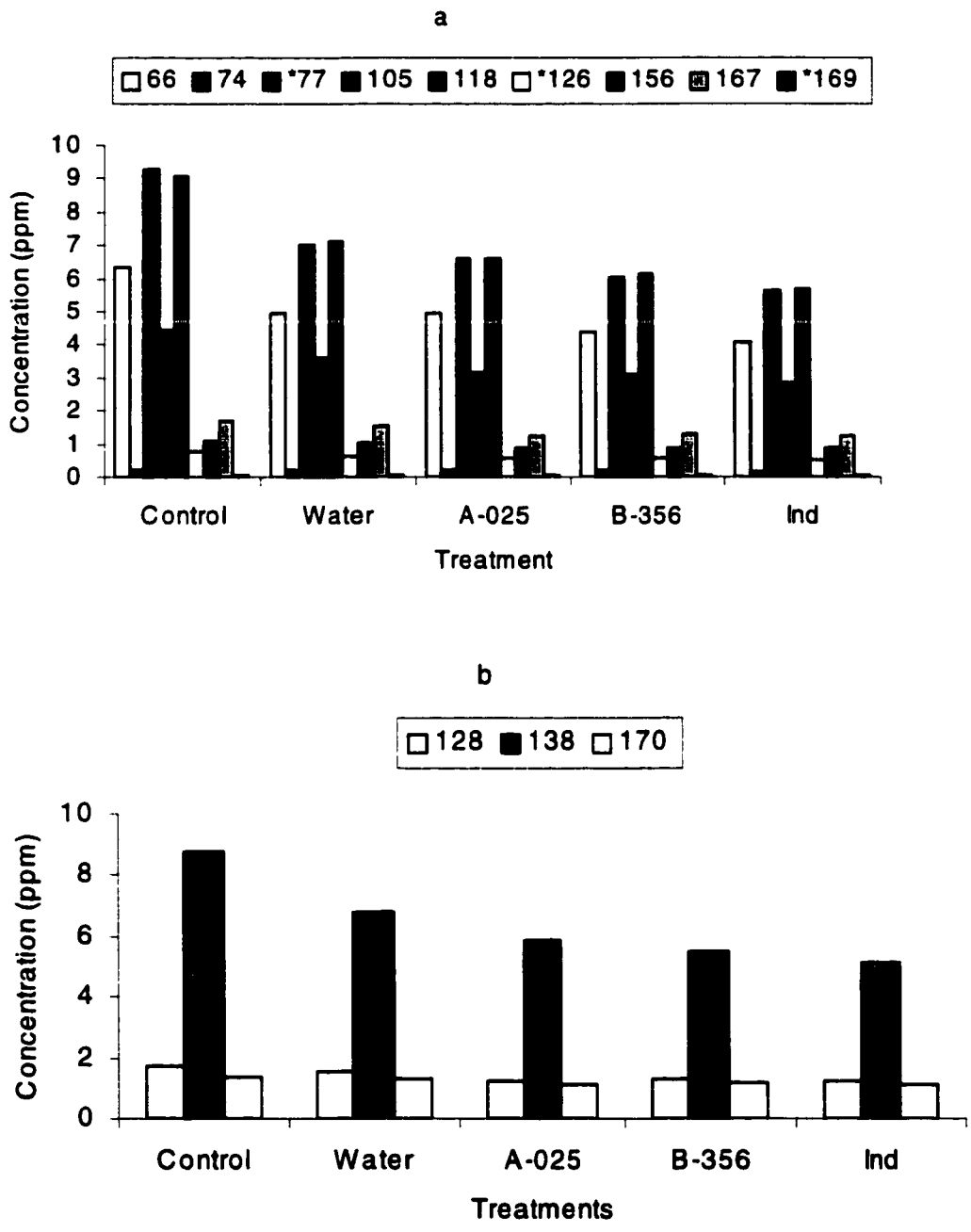


Figure 6.15. The concentrations of potentially antiestrogenic, immunotoxic and dioxin-like PCB congeners in different treatments after 345 days at 340 mm depth. a) non-*ortho* and mono-*ortho* substituted, moderately persistent, b) di-*ortho* substituted, persistent. *-planar congeners.

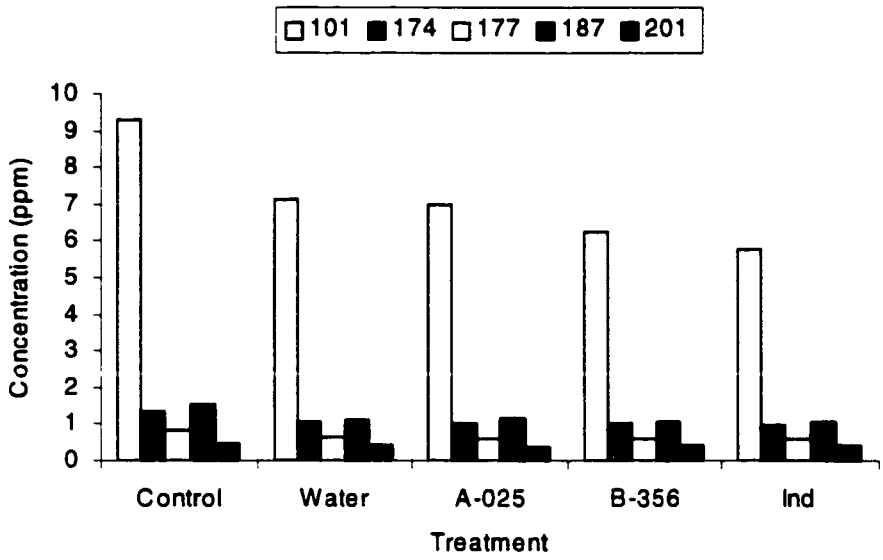


Figure 6.16. The concentration of potentially estrogenic (weak phenobarbital inducer, persistent) PCB congeners in different treatments after 345 days at 340 mm depth.

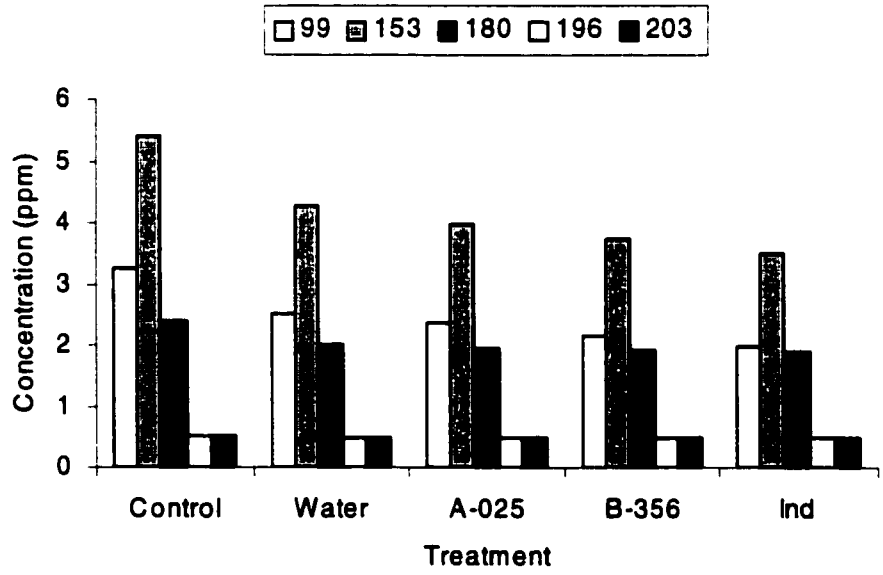


Figure 6.17. The concentration of phenobarbital, CYP1A and CYP2B inducers and biologically persistent PCB congeners in different treatments after 345 days at 340 mm depth.

6.3.5 Distribution and Survival of Strain A-025

R. meliloti, strain A-025, population was followed by plating on selective media (TYct). Soil samples from columns augmented with B-356 and indigenous microflora were analyzed for general microbial population count. These samples were plated on different nutrient media and they showed a change in population diversity and density. These results are discussed in Appendix A1.

Analysis of samples taken at 20 days after bacterial augmentation of strain A-025 showed a large decrease to 10^4 cell g^{-1} from the estimated initial number of 10^9 cell g^{-1} soil (Figure 6.18). Also, The results show even distribution of the bacterial cells through out the soil columns indicating that the bacterial cells were transported and distributed evenly at different depths since the system allowed for recycling of the drain water. The results in Chapter 4 showed an open system would allow bacterial cells to be washed out in the drain water and even distribution would result as further desorption and washing out of the cells.

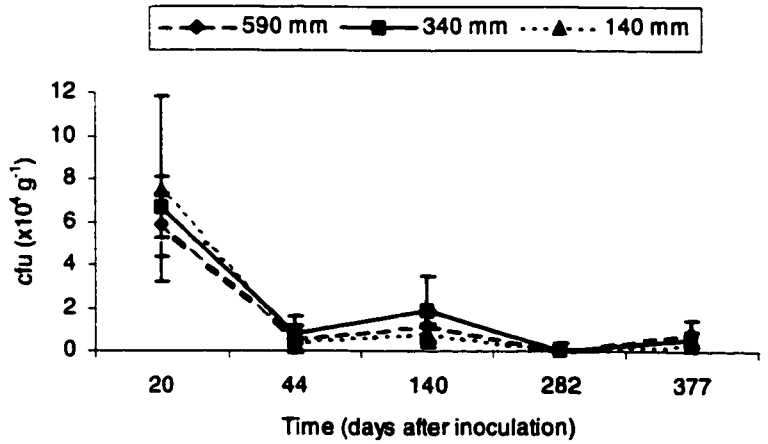


Figure 6.18. The population of *R. meliloti* strain A-025 at different depths during the experimental period. Means of triplicates and SD.

The colonies formed on TYct agar plates that were used for strain A-025 viable population count in this study were positive in nodulation tests on alfalfa roots. As shown in Appendix A2, nodulation of alfalfa roots were negative when the same soil (S-VII) was used to grow alfalfa plants.

6.3.6 Drain Water pH

The pH of the drain water collected after every wet cycle indicated a rise in the pH from approximately 7 to 7.5 during the experimental period (Figure 6.19). In reach soil, usually under flooded/anaerobic conditions, the pH increases as fermentative products are produced. This soil was highly polluted and had much less microbial population and microbial activity. However, the shift in the number and diversity could have initiated more activity in these soil columns.

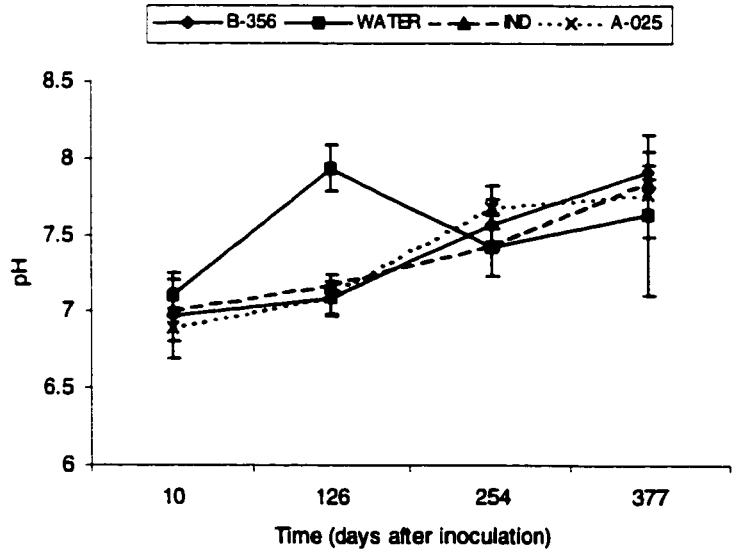


Figure 6.19. The measured pH in the drain water through the experimental period. These samples were taken after wet cycles. Means of triplicates and SD.

6.3.7 Water Flow

The influence of the bioaugmentation by different bacterial cultures on water flow was determined at the end of the experiment. The results indicated that bioaugmentation of the soil with strain A-025 did not effect the water flow through the soil columns as compared to the water treatment (Figure 6.20). These results could be both supported and be supportive of those results observed in Appendix A3 where bioaugmentation of a PCB contaminated soil did not influence the water infiltration, whereas bioaugmentation of a pristine sandy loam soil resulted an increase in water infiltration rate. However, the flow rate did increase in the bioaugmented treatment columns with the indigenous and the B-356 cultures.

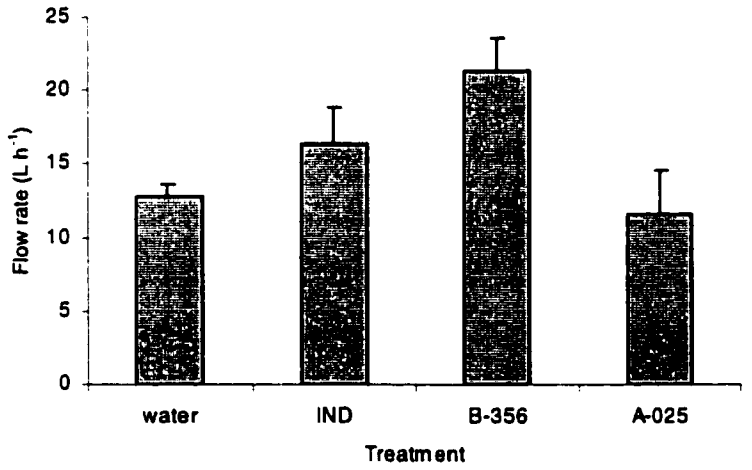


Figure 6.20. Flow rate in different treatment columns at the end of the experiment. Means of triplicates and SD.

Strain B-356 has shown to have a high hydrophobicity and adhesion to soil particles in Chapter 3. These characteristics may increase the binding of these

cells to soil medium and thus increase the ratio of macroporosity to microporosity of the soil.

6.3.8 PCB Dechlorination

Dechlorination of higher chlorinated congeners present in the soil, S-VII, into lower chlorinated congeners were observed in all the bioaugmented and water treatments. Therefore, it is difficult to assume that the transformation in the PCBs would be the result of the augmented bacterial culture alone. Bioaugmentation with different bacterial cultures not only had effects on PCB dechlorination but also on the changes in the population diversity during the experimental period as is discussed in Appendix A1. Furthermore, Van Dort and Bedard, (1991) reported a possible shift in the microbial population during an anaerobic system and Hill et al., (1989) showed that colony forming units were significantly different in different treatments of a column. Nevertheless, some congeners in the present study were affected by *R. meliloti* strain A-025 that were not in the other treatments. Considering these congeners and their possible dechlorination products, different dechlorination pathways could be suggested for both aerobic and anaerobic dechlorination due to augmentation with strain A-025. No predominant pathway or step is indicated since these pathways may vary with different PCB mixtures and environmental conditions. The dechlorinated final congener is not a non-*ortho* congener in either of the aerobic nor anaerobic pathways. This could be an advantage of dechlorination seen by augmentation with strain A-025 because the non-*ortho* congeners such as IUPAC congeners 77,

126, and 169 that are coplanar are considered to be the most toxic congeners in mammals and fish (Willman et al., 1997).

Aerobic dechlorination pathways seem to involve all the three *meta*, *para*, and *ortho* dechlorinations (Figure 6.21). If the possible pathways are compared to the suggested pathways in Chapter 5 then the initial dechlorination of 2,2',3,3',4,5,5',6-octachlorobiphenyl into 2,2',3,3',5,5',6-heptachlorobiphenyl would be *para* and this congener would go through 4 *meta* dechlorinations to produce 2,2',3-trichlorobiphenyl which subsequently would be dechlorinated on the *ortho* position to free a ring to produce 2,3-dichlorobiphenyl. Bedard et al. (1986) also showed aerobic degradation of congeners chlorinated at both *ortho* positions on a single ring was enhanced by chlorination of the second ring at positions 2',5'. This could also be the case with A-025 where 2,2',3,5',6-pentachlorobiphenyl could be a dechlorination product of 2,2',3,3',4,5,5',6-octachlorobiphenyl and a parent congener to 2,3-dichlorobiphenyl (Figure 6.21). The degradation of the *ortho* congeners during the aerobic period can be the result of the *meta* and *para* dechlorination of highly chlorinated congeners during anaerobic conditions (Bedard et al., 1987).

Anaerobic pathways seem to involve *meta* and *para* dechlorination except for the *ortho* dechlorination of 2,4,4'-trichlorobiphenyl into 4,4'-dichlorobiphenyl (Figure 6.22). Perhaps, one could suggest that *ortho* dechlorination by A-025 would be more aerobic than anaerobic since it does not fit in any of the other suggested pathway. If the possible routes from the results in this chapter are compared to those observed in Chapter 5, one could suggest that 2,3,3',4,4',5,5'-

heptachlorobiphenyl would go through two consecutive *meta* dechlorinations to form 2,3,3',4,4'-pentachlorobiphenyl which could go through *para* and *meta* or *meta* and *para* to form 2,3,4'-trichlorobiphenyl which would subsequently undergo a *meta* dechlorination to form 2,4'-dichlorobiphenyl. Congener 2,3,3',4,4'-pentachlorobiphenyl has also shown to be part of the dechlorination pathway in the Hudson River sediments (Abramowicz, 1990). Mousa et al. (1996) suggested a general anaerobic *meta* and *para* dechlorination of Aroclor 1242 and 1254 congeners to di- congeners containing chlorines at *ortho* and *para* position. Although, anaerobic *ortho* dechlorination has been reported (Van Dort and Bedard, 1991), anaerobic dechlorination at *meta* and *para* positions have been the most observed pathways (Quensen III et al., 1990; Quensen III et al., 1988) and have been suggested to be the main pathways (Abramowicz, 1990). Berkaw et al., (1996) found that only *meta* and *para* dechlorinations occurred in freshwater sediments; however, *ortho* dechlorination was observed in estuarine conditions although this was always preceded by *para* dechlorinations. The *meta* and *para* dechlorinations have been observed in methanogenic and sulfidogenic marine sediment cultures (Ofjord et al., 1994).

In general, due to the variability in volatility and insolubility of different PCB congeners, it is crucial to determine the results of depletion in more controlled assays. Since contaminated sites are not usually homogenous, and since mixtures of pollutants are present, bioremediation efforts have to account for the activities of diverse aerobic and anaerobic communities at different depths of a soil profile. Even though anaerobic dechlorination of PCBs by anaerobic and

methanogenic bacteria has been reported (Ye et al., 1995), this is the first study showing *R. meliloti* to dechlorinate the highly chlorinated PCB congeners.

The results of this study provide evidence that the water table management system could be used for bioaugmentation of contaminated soils. The possible creation of subsequent aerobic and anaerobic conditions not only for bioaugmented bacterial cultures but also for the indigenous organisms has (treatment 4) allowed dechlorination of the highly chlorinated PCB congeners into lower chlorinated congeners that could be degraded more easily and faster by many different organisms. Nevertheless, the dechlorination patterns were different for every bacterial culture and at different depths. *R. meliloti,* strain A-025, has shown the ability to survive at different depths and in the presence of the >73 congeners present in soil S-VII. Although, the exact dechlorination pathways of PCB congeners by strain A-025 needs to be confirmed, it is clear that this strain can play a major role in biodegradation of PCB congeners and more importantly at deeper zones of a soil contaminated with PCBs.

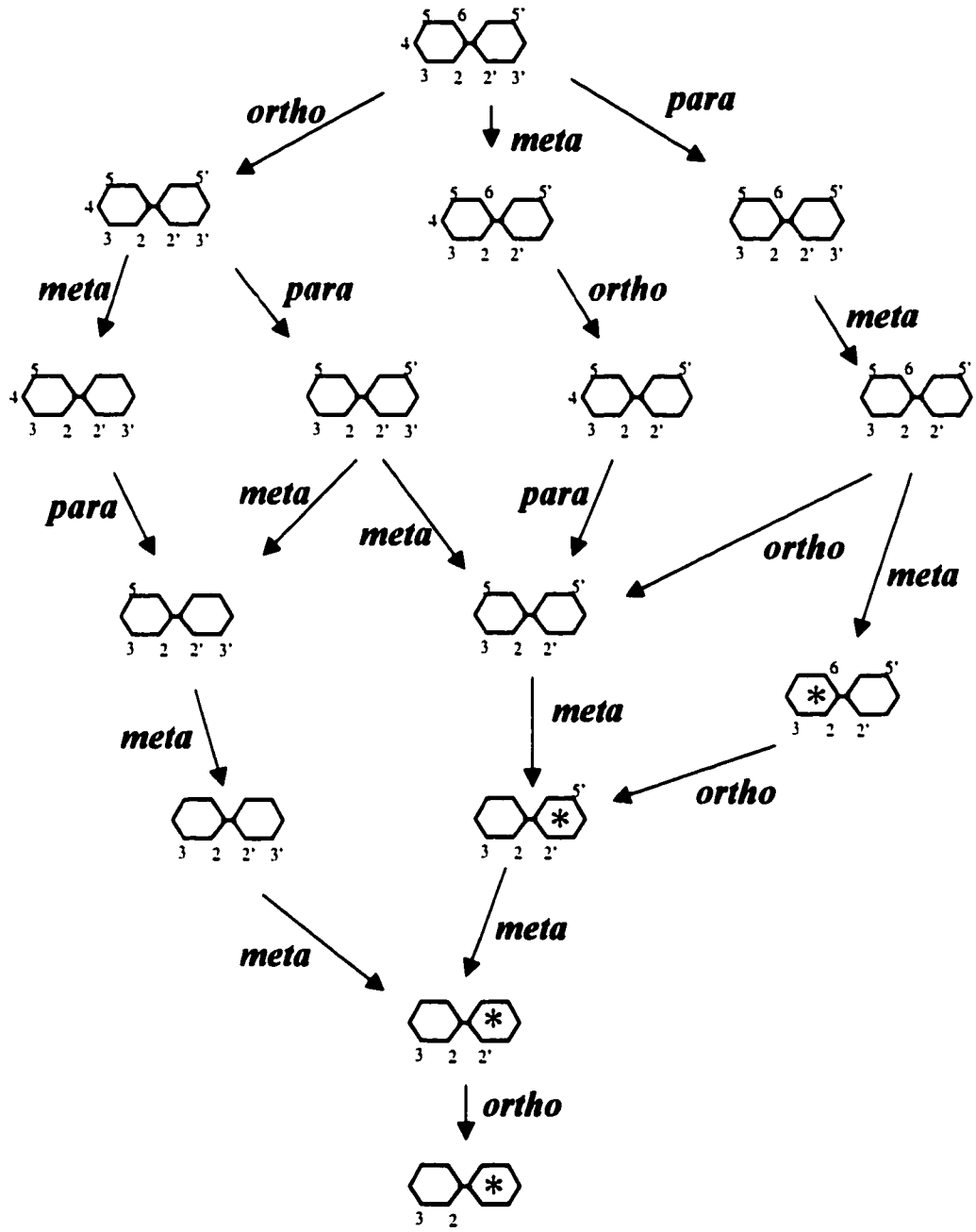


Figure 6.21. Possible aerobic dechlorination pathways by *R. meliloti* strain A-025. *- Indicate those PCB congeners that were included in the dechlorination pathways of Aroclor 1242 in Chapter 5.

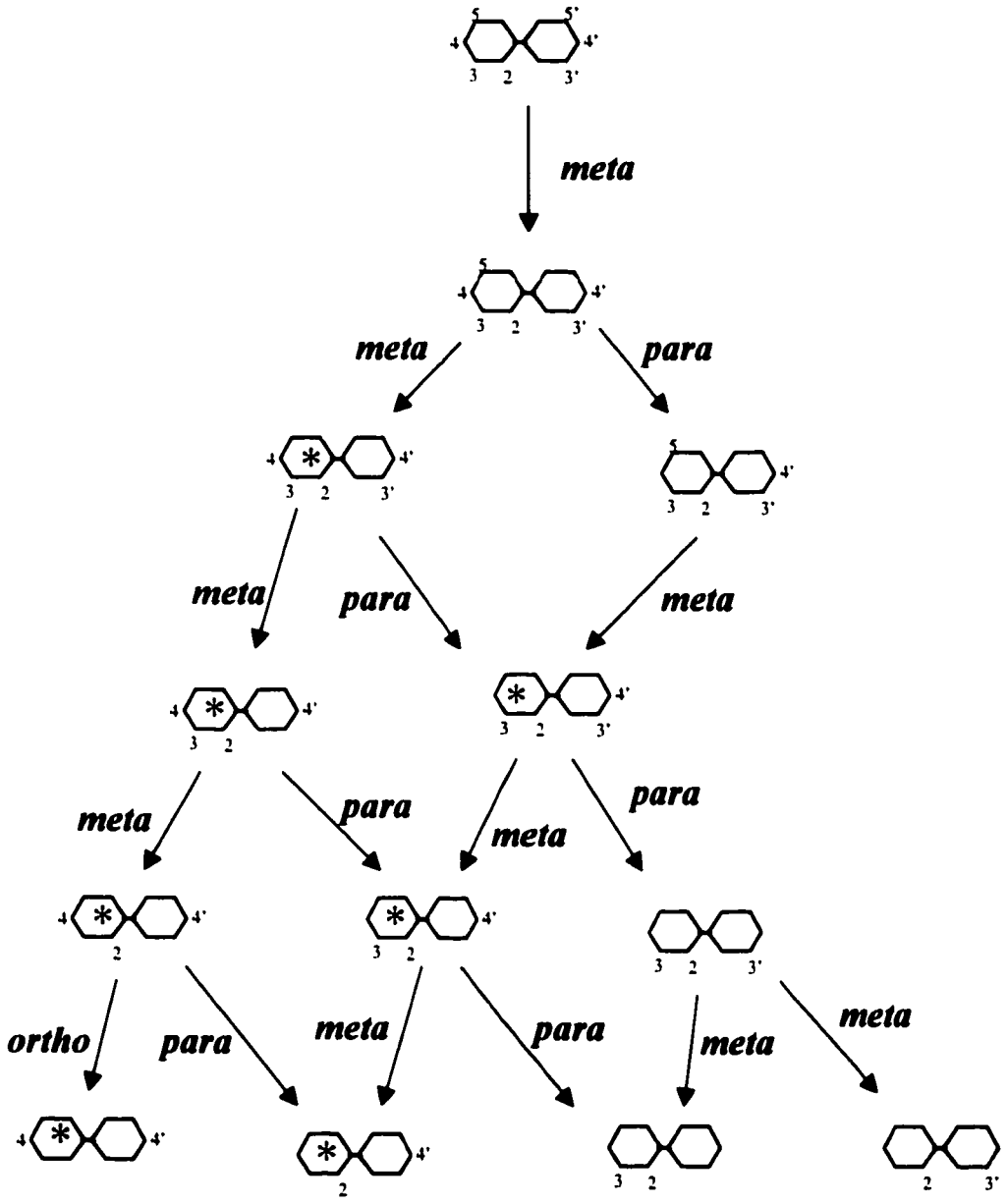


Figure 6.22. Possible anaerobic dechlorination pathways by *R. meliloti* strain A-025. *- Indicate those PCB congeners that were included in the dechlorination pathways of Aroclor 1242 in Chapter 5.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

This research was undertaken to investigate and develop an innovative in-situ bioaugmentation system for contaminated soils. This was initiated by isolating several naturally existing Rhizobia from soils contaminated with PCBs, PAHs, and heavy metals. This was followed by investigating the use of a water table management system to deliver bacteria to enhance biodegradation of PCBs in soils. In other exploratory studies presented in Appendix A, we investigated: 1) the diversity in microbial populations in contaminated soils and during a bioremediation treatment, 2) influence of alfalfa plants on *Rhizobium meliloti* in biodegradation of PCBs, 3) the effect of bioaugmentation on soil characteristics, and 4) use of WTM with Rhizobium in biofiltration of atrazine and nitrate.

7.1 BIOAUGMENTATION SYSTEM

To determine whether subirrigation could be used to deliver and distribute bacterial cells in the soil, we compared the more traditional way of soil inoculation, i.e., surface irrigation, with the proposed method, subirrigation, in saturated sterilized soil columns. The results indicated that even though both systems were able to transport the bacteria, the subirrigation system was a more efficient method to distribute a higher number of bacterial cells at different depths of the soil columns, specially at lower depths, 500 and 700 mm below the soil surface. Therefore, subirrigation seems to be a promising technology for in-situ

bioaugmentation of contaminated soils. Based on the findings from this study, the following conclusions were drawn:

1. Subirrigation can provide better transport and implantation of bacteria in the soil profile than surface irrigation.
2. Water table management system can be used to bioaugment a sandy loam soil up to a depth of 1 m.

7.2 ISOLATION OF RHIZOBIA AND MEMBRANE CHARACTERISTICS

Over 30 strains of Rhizobium were isolated from soils with history of PCB, PAH, heavy metal, and herbicide contamination. These strains were positive for N_2-fixation. Some of these isolated bacteria were tested for their behavior in soil and water by determining their hydrophobicity and adhesion to a sandy soil. The range of hydrophobicity values for the strains tested were smaller than the adhesion values observed. Both these characteristics were influenced by type of nutrient used for the bacterial cultures. The adhesion of rhizobial cells was lower than the non-rhizobial strains of *E. coli* and *C. testosteroni.* The following conclusions were drawn from this study:

1. Different species of Rhizobium were present in soils contaminated with PCBs, PAHs, and heavy metals.
2. Behavior of Rhizobial cells in water and soil is influenced by the surface characteristics of the bacteria. Both hydrophobicity and adhesion characteristics were influenced by the composition of the growth media used.

7.3 SOIL MICROCOSMS

Two of the isolated Rhizobial strains, A-025 and A-029, were evaluated for their ability to degrade PCBs in sterilized soil microcosms contaminated artificially with Aroclor 1242. These strains were compared to *Rhizobium meliloti* strain, Zb57. Results showed that the tested Rhizobial strains had different patterns in dechlorination and subsequent transformation of the PCB congeners. Also, the observed patterns in PCB biotransformation and bacterial survival were different for microcosms that were spiked with $CaCO_3$ from those that were not. The conclusions drawn from this study are as follows:

1. *R. meliloti* strains, A-025 and A-029, were found to dechlorinate highly chlorinated congeners to lower chlorinated congeners, present in Aroclor 1242.
2. The dechlorination and biotransformation of Aroclor 1242 congeners appeared to be more rapid in the presence of $CaCO_3$ than without it.
3. The survival of the bioaugmented bacterial cells were greater in the presence of $CaCO_3$.
4. With strain A-025, an aerobic cycle followed by an anaerobic cycle, caused depletion of peaks 54 (congeners 106, 123, 149), 37 (congener 74), 30 (congeners 37, 42), and 17 (congener 26) after 388 days.

7.4 BIOAUGMENTATION OF PCB SOIL COLUMNS

The use of water table management to deliver and distribute bacteria to the subsoil and subsequently to enhance biotransformation of PCBs was investigated.

Soil columns packed with a PCB contaminated soil, a well studied PCB degrader *Comomonas testosteroni*, strain B-356, a newly isolated *Rhizobium meliloti*, strain A-025 and an uncharacterized microflora isolated from the contaminated soil (indigenous to the soil used in the experiment) were used to bioaugment the soil. The results of this study were a clear evidence that the water table management system could be used to bioaugment contaminated soils. The possible creation of subsequent aerobic and anaerobic conditions not only for bioaugmented bacterial cultures but also for the indigenous organisms allowed the dechlorination of the very high chlorinated PCB congeners into lower chlorinated congeners that could be degraded much easier and faster by many different organisms. The dechlorination patterns differed for every bacterial culture and at different depths. Even though, the exact dechlorination pathways of PCB congeners by strain A-025 need to be confirmed, it is clear that this strain can play a role in biodegradation of PCB congeners and more importantly at deeper zones of a soil contaminated with PCBs. Based on the findings from this study, the following conclusions were drawn:

1. Water table management system can be used to bioaugment a sandy loam PCB contaminated soil.
2. The possible creation of subsequent aerobic and anaerobic conditions, not only for bioaugmented bacterial cultures but also for the indigenous organisms, allowed dechlorination of highly chlorinated PCB congeners into lower chlorinated congeners.

3. The PCB dechlorination patterns in the soil columns differed for treatments bioaugmented with *R. meliloti*, *C. testosteroni*, and the indigenous cultures. Also, different dechlorination patterns were observed at different depths.
4. *Rhizobium meliloti* strain A-025 showed the ability to survive at different depths of the soil columns and in the presence of PCBs for over a year.

7.5 EFFECTS OF BIOAUGMENTATION AND PCBS ON SOIL

Transport of the *R. meliloti* strain A-025 was investigated in three different soils (sandy loam, clay, sandy loam PCB contaminated) and the effect of bioaugmentation on these soils was determined. The results indicated that transport of these bacterial cells was influenced more by the presence of PCBs than by the soil's clay matter. In contrast, infiltration of surface water was influenced more by clay content than by the soil PCB content. Furthermore, the least difference in surface water infiltration between augmented and non-augmented soil columns was observed in the PCB soil. Presence of PCBs influenced the infiltration time period when bioaugmented with bacterial cells. Frequency and irrigation periods were found to be important factors when soils were microbially augmented with water. In general, the pattern of moisture loss was different between soils and the biggest difference between augmented and non-augmented soils was observed with the PCB contaminated soil. Soil hardness showed common patterns between soils, with augmented treatments becoming harder at the end of the experiment for all soils tested and the biggest difference between soils was the clay soil. The conclusions drawn from this study are:

1. Transport of *R. meliloti* strain A-025 was influenced more by the presence of PCBs than by the soil's clay matter.
2. Infiltration of surface water was influenced more by the clay content than by the soil PCB content.
3. Bioaugmentation with bacterial cells influenced water infiltration in the PCB contaminated soil.
4. Frequency and irrigation intervals were found to be important factors in the transport of bacterial cells in the soils.
5. The pattern of moisture loss was different between soil types and the biggest difference between augmented and non-augmented soils was observed in the soil contaminated with PCBs.
6. Soil hardness showed common patterns between soils, with augmented treatments becoming harder at the end of the experiment for all three soils, and the biggest difference between the soils was observed in the clay soil.

7.6 RHIZOSPHERIC EFFECTS ON PCB BIODEGRADATION

The influence of alfalfa plants on strain A-025 in biodegradation of PCBs was investigated. The results indicated that growth of alfalfa plants and bioaugmentation of the soil with *R. meliloti,* strain A-025, increases the loss of PCB congeners as compared to bioaugmentation alone. However, the loss was more attributed to the presence of the plants rather than the bioaugmentation. The plants alone were the most effective in the loss of PCBs. Bioaugmentation of the soil played a significant role in the hardness of the soil and the growth of alfalfa

plants. Based on the findings from this study, the following conclusions were drawn:

1. Growth of alfalfa plants and bioaugmentation of soil with *R. meliloti*, strain A-025, increased the loss of PCB congeners when compared to bioaugmentation alone.
2. Alfalfa plants were effective in the loss of PCBs. Bioaugmentation of soil caused the soil to harden up and affected the growth of alfalfa plants.

7.7 BIOFILTATION OF ATRAZINE AND NITRATE

R. meliloti strain A-025 was used to bioaugment soil columns to evaluate biofilteration of two agricultural pollutants, atrazine and nitrate. Results indicated that it is possible to use bioaugmentation as a biofiltration system for agricultural chemicals before they reach aquatic systems. Soil columns bioaugmented with *R. meliloti*, strain A-025, allowed lower concentrations of atrazine (30%) to be leached into the drainage waters. However, more evidence is required concerning the biotransformation of atrazine in both anaerobic and aerobic conditions with this strain of Rhizobium. Also, results indicated bioaugmentation reduced nitrate-N leaching during the saturation (anaerobic) period significantly. However, it was increased during the unsaturated (aerobic) period. Although, total nitrate-N levels were lower in the bioaugmented soil as compared to the abiotic control, it was higher in the presence of atrazine. The following conclusions were drawn from this study:

1. The use of bioaugmentation as a biofiltration system reduced the concentration of atrazine by more than 30% in drainage waters.
2. The use of bioaugmentation as a biofiltration system significantly reduced nitrate-N concentration in the drainage water during anaerobic conditions. It was found to increase during aerobic conditions.

7.8 POPULATION DIVERSITY

The use of different growth media demonstrated the diversity of bacterial populations in different soils. Also, using this technique allowed us to demonstrate the variation in the microbial populations before and after a bioremediation process. Furthermore, not only dilution in the media but also in the suspension samples demonstrated different colony type numbers. The conclusions from this study are:

1. The microbial population density and diversity varied in different soils.
2. The bacterial bioaugmentation of the soil created a shift in the indigenous microbial populations. The change was dependent on the bioaugmented culture.

CHAPTER 8

RECOMMENDATIONS FOR FUTURE WORK

The use of water table management system for bioaugmentation of contaminated soils is a novel idea and technology. As such they raise several questions. I would like to give in this section, recommendations to those who are interested to pursue the investigations. Some of these suggestions are:

1. The cooperation of groups in different disciplines is strongly recommended. I found the multidisciplinary aspect of this work to be very interesting, challenging, and rewarding. The success of this work can be attributed to this aspect of the project.
2. The TLVs (threshold limit values) by OSHA (U.S. occupational safety and health administration) for PCBs is recommended to be 0.5 mg/m^3 through skin exposure. Through out the period of this work, maximum precautions were taken to minimize exposure through any route by working under activated fume hoods and by wearing proper clothing. However, exposure to PCBs during chemical extractions of the soil samples, accompanies exposure to other chemicals such as solvents. This might increase the health risks. Therefore, I like to recommend that work such as that done in Chapter 6, should minimize the accumulative exposure to such chemicals by perhaps reducing the daily exposure to 4-5 h day^{-1} and 2-3 non-consecutive days wk^{-1}.
3. The work in Chapter 6 involved bioaugmentation of the soil with a mixed bacterial culture that was isolated aerobically. This culture was a more effective PCB degrader during the aerobic cycles of the experiment. Therefore,

there is a need for isolation of mixed cultures anaerobically that could be bioaugmented with the aerobic culture to enhance biodegradation of PCBs.

4. The bioaugmentation in the soil columns was done once at the start of the experiment. Even though, the bacterial cells seemed to survive, there was a large decrease in the population a short time after bioaugmentation. Therefore, multiple bioaugmentations with lower cell numbers might prove to be more effective for maintaining a uniform population during the treatment and thus more effective in enhancing the bioremediation process.
5. Two different time periods of consecutive aerobic and anaerobic cycles were tried in this work. The results indicated that a longer aerobic cycle than anaerobic might be more effective in PCB dechlorination. Thus, there is a need to determine the optimum time periods for the aerobic and anaerobic cycles.
6. The PCB biodegradations in this study involved mixtures of PCB congeners. Therefore, the exact dechlorination and total biodegradation pathways by *R. meliloti* were difficult to determine. It is important that single individual PCB congeners be used as the parent compound and its metabolic pathways and byproducts be determined.
7. The growth of alfalfa plants was more successful in soils contaminated with lower chlorinated PCB congeners than the soil used in Appendix A2. Therefore, there is a need to determine the phytotoxicological effects of different PCB congeners or mixtures on alfalfa seed germination and plant growth.
8. The use of *R. meliloti* strain A-025, reduced the concentration of atrazine in the drain water of the column study. However, the pathway(s) of the loss of

atrazine by this strain is still unknown. The pathway in atrazine degradation by strain A-025 needs to be determined.

9. The nitrate-N showed a significant reduction in the drain water of the bioaugmented saturated soil columns as compared to the abiotic control. However, during the aerobic period the nitrate-N increased. Nitrification by *R. meliloti* has never been reported. Therefore, it is important to determine the reasons for the increase in the nitrate-N during aerobic conditions.

APPENDIX A

PRELIMINARY STUDIES WITH WTM AND RHIZOBIUM

PREFACE TO A1

Techniques used to examine the community structure and size in a contaminated soil microbial ecosystem play a crucial role when isolation and/or use of microorganisms capable of degrading pollutants in the environment is intended or when ecotoxicological impact of pollutants, agrochemicals or implanted microorganisms is to be evaluated. During bioremediation of contaminated soils, the number and type of indigenous microorganisms play an important role.

Appendix A1 describes the methods and the results of a preliminary study where different nutrient agar plates of different nutritional compositions were used to determine the general effect on the microbial population diversity in three contaminated soils. Furthermore, the effect of microbial bioaugmentation performed in Chapter 6, on the microbial population diversity and size after the treatments were determined.

The contents of Appendix A1 have been presented at the annual meetings of the International Symposium on Microbial Ecology (ISME-8) and Society of Environmental Toxicology and Chemistry (SETAC). Experiments 1 and 2 were presented at ISME-8 in Halifax, Nova Scotia: Reza Mehmannavaz, Darakhshan Ahmad, Shiv O. Prasher. 1998. Population diversity of bacteria in soils contaminated with PCBs, PAHs, and heavy metals. 1998 Annual Meeting of the International Symposium on Microbial Ecology held in Halifax, Nova Scotia in Aug. 1998. Experiment 3 was presented at SETAC in Charlotte, North Carolina:

Reza Mehmannavaz, Darakhshan Ahmad, Shiv O. Prasher. 1998. Diversity pattern of microbial populations in a PCB contaminated soil during a bioremediation treatment. 1998 Annual Meeting of the Society of Environmental Toxicology and Chemistry in Charlotte, North Carolina in Nov. 1998.

APPENDIX A1

CHANGE IN BACTERIAL POPULATION DENSITY AND DIVERSITY IN CONTAMINATED SOILS AND IN A BIOREMEDIATION TREATMENT

ABSTRACT

The classical microbiological techniques such as plate counts of colony forming units (cfu), only represent and account for less than 10% of the total microbial populations in the soils. However, it is still frequently used and is considered to be an indispensable tool for enumeration and classification of bacteria in soil. In the present study, we attempted to establish the profile of microbial population diversity in three different soils contaminated for over 20 years and in a fourth soil during a bioremediation process. Six different selective and nonselective rich nutrient media, MH, TY, TYc, TYct, TYg and YEMP, and their lower concentrations (1/10 and 1/100) were used. The results showed that while full strength media allowed growth of fast growing bacteria, the dilution of the media used, allowed growth of slow growing bacteria. Strength of the media affected the scoring of diversity positively, but the population size negatively. Furthermore, the indigenous microbial populations were affected differently by the different bacterial cultures used to bioaugment the soil and the population size and diversity varied during the bioremediation process.

A1.1 INTRODUCTION

Soil is a heterogeneous and structured environment that is composed of multitude of microhabitats and small environmental changes can alter their microbiological composition significantly (Postma et al., 1989). Due to ecotoxicological impacts of contaminants, microbial populations change continuously. As the contaminants are transformed through biological or chemical processes, the microbial populations in parallel change and adapt to the new conditions (Kiyohara et al., 1992). Fulthorpe (1998) reported that adaptation of soils to higher concentrations and more constant levels of chlorinated organic inputs results in the loss of detectable diversity. Therefore, microbial communities play a significant role in the ecosystem function and are useful indicators of soil health (Ellis et al., 1998). Acclimation of microorganisms on different contaminants can be attributed to several mechanisms, including the induction or derepression of enzymes, mutation or genetic exchange, multiplication of smaller populations, an insufficient supply of inorganic nutrients, adaptation to the toxins or inhibitors present, and predation (Cabrera and Lebeault, 1993).

Over 3648 bacterial species belonging to 659 genera and 224 genera of soil borne bacteria have been isolated and described (Akinov and Hattori, 1996; Amann et al., 1995). However, attempts to isolate strains of many genera of microorganisms that use different substrates as growth media even from an enrichment culture have not always been successful (Cabrera and Lebeault, 1993). Akinov and Hattori, (1996) suggest that preliminary evidence show that only less

than 5-12% of all microbial species on Earth are known. The lack of a universally applicable technique complicates the problem of detection and isolation of new microorganisms (Akinov and Hattori, 1996).

Viable plate count or most-probable-number techniques have been, and still are, used for quantification of active cells in environmental samples for different purposes (Amann et al., 1995). A significant problem also exists because viable but non-culturable microorganisms are present in all environmental samples. These may be indigenous or introduced microbial cells that have been subjected to environmental conditions which may make it difficult for them to form colony forming units (cfu) on different agar media (Trevors and Cook, 1992). Nevertheless, even without considering recent molecular technologies, with the availability of innovative techniques, many more microorganisms will become culturable as was the isolation of "ultramicrobacteria" by dilution culture (Amann et al., 1995). In this study, we attempted to follow the microbial population diversity in three different soils contaminated with PCBs, PAHs, and heavy metals and also during a bioremediation through microbial bioaugmentation, using different media of varying strength.

A1.2 MATERIALS AND METHODS

A1.2.1 History of Soils

The history and characteristics of the soils used are presented in Table A1.1. Soil pH was measured after vortexing 5 g of soil with 5 mL of distilled water for 60 s and settling for 10 min (Somasegaran and Hoben, 1994).

Table A1.1. History of sites and characteristics of soils used.

Soil	*Site History	Soil characteristics	pH	*Contamination (ppm)
S-I	Glass production factory, spillage of diesel and bunker fluids, over 15 years contamination	*Heterogeneous mixture of gravel, sand, wood, rocks, glass, bricks	6.50	PAHs, 5000-85000, oil and grease
S-II	na	*Heterogeneous mixture of gravel, sand, clay	7.88	PAHs
S-III	Manufacturing of boat motors and marine equip., PCB spillage from broken hydraulic lines on machines in 1960's and 1970's	*Homogenous mixture of wood, sandy clay, grain size <13 mm	7.75	PCBs > 730, range 0-1400 Aroclor 1242, 1254, oil and grease, heavy metals
S-VII	Rail road industry, over 17 years contamination	74% sand, 8% silt, 18% clay, 4.8% organic matter	7.38	Heavy metals; PCB mixtures: Aroclor 1242, 1248, 1254, 1260

na- not available; *- As provided by the suppliers.

A1.2.2 Growth Media and Conditions

A1.2.2.1 Enumeration on different media

1.0 g of soil (S-I, S-II and S-III) was added to 10 mL of NaPP buffer and vortexed for 30 s. Serial dilutions were spread on TYc (Bromfield et al. 1994), TYg (Trevors and Cook, 1992), TYct (Kinkle et al. 1994), MH and YEMP (Vincent, 1970) in duplicate. The serial dilutions were also spread on MM30 (Sylvestre and Fauteux,1982) agar plates supplemented with vitamins and minerals. Approximately, the same amounts of naphthalene, anthracene, meta-toluate and biphenyl were placed in the cover of the petri dish. The plates were

sealed with parafilm and incubated at 29 °C. The number and types of colonies were recorded daily for 10 days.

A1.2.2.2 Enumeration on diluted media

20 g of soil S-III was shaken in 180 mL of PBS for 1.0 h at 1500 rpm. Then it was allowed to settle for 1.5 h.. Serial dilutions of the suspension were prepared. The dilutions were spread on TY, TY/10, TY/100, TYc, TYc/10, TYc/100, TYct, TYct/10, TYct/100, YEMP, YEMP/10, YEMP/100 agar plates. The plates were incubated at 29 °C and the number and types of colonies were recorded daily for 23 days.

A1.2.2.3 Microbial diversity during a bioremediation treatment

The experimental setup was as described in the column study in Chapter 6 and in Tables A1.2 and A1.3. The microbial diversity was determined as follows: Soil samples (20 g) were collected before and 377 days after bioaugmentation and treatment of the soil columns. Soil samples were taken from soil columns at 140, 340, and 590mm depths. 1 g of soil from each depth was pooled and added to 10mL of NaPP buffer and vortexed for 30 s. Serial dilutions were spread on TYc, TYc/100, TYct, and MH agar plates in duplicate. The plates were sealed with parafilm and incubated at 29 °C. The number and types of colonies were recorded daily for 20 days.

Table A1.2. Protocol followed in bioremediation experiment in the soil column study.

Bacterial culture strain A-025	Bacterial culture strain B-356	Bacterial culture ABVII
Starter culture from frozen cultures, 5mL TYc (2 days)	Starter culture from frozen cultures, 5mL TY (2 days)	5g soil S-VII added to 50mL MM30 with 100 ppm Aroclor 1242 and 0.05% BP and 0.05% YE, incubated at 29 ^{o}C (2 days)
Sub-cultured into 50mL TYc (2 days)	Sub-cultured into 50mL TY with 0.05% BP (2 days)	2mL sub-cultured into 50mL TY with 100 ppm Aroclor 1242 and 0.05% BP, repeated twice (4 days)
Inoculated into 1L TYc (1 day)	Inoculated into 1L TY with 0.05% BP (1 day)	Sub- cultured into 1L TY with 100 ppm Aroclor 1242 and 0.05% BP, (1 day)
Cells collected, washed (in 0.9% saline) and resuspended in 300mL sterilized water	Cells collected, washed (in 0.9% saline) and resuspended in 300mL sterilized water	Cells collected, washed (in 0.9% saline) and resuspended in 300mL sterilized water

Table A1.3. Stages in the PCB bioremediation study.

Period	Column Treatments
(Nov. 16-Dec.26, 1996)	Packing and preparation of columns (PCB contaminated soil, S-VII)
(Dec.26, 1996)	Initial soil samples taken before saturation of soil columns
(Dec.26, 1996)	Soil columns saturated with 24L of water (except the passive control column)
(Dec.28, 1996)	Inoculated with strain A-025, 5.066 g culture or 1L ($2.3x10^8$ cell/mL), strain B-356, 3.535 g culture or 1L ($3.0x10^8$ cell/mL), culture ABVII, 5.816 g culture
(Dec.29, 1996)	Reinoculation with strain A-025, 3.971 g of cell culture, strain B-356, 2.368 g of cell culture, culture ABVII, 4.483 g of cell culture
(Jan. 3-5,1997)	Complete drainage of columns
(Jan. 5,1997)	Water and soil samples taken
(Jan. 15, 1997)	15g of BP was added to treatment columns with strain B-356
(Jan. 5-Sep. 6, 1997)	10 days of dry (unsaturated, aerobic) and 10 days of wet (saturated, anaerobic) cycles. Water and soil samples taken after each cycle
(Sep. 6- Jan. 5, 1998)	30 days of dry (unsaturated, aerobic) and 30 days of wet (saturated, anaerobic) cycles. Water and soil samples taken after each cycle

A1.3 RESULTS AND DISCUSSIONS

A1.3.1 Enumeration on Different Media

The population diversity of the bacteria in the contaminated soils was determined and their growth compared on agar plates with nutrients of different compositions and strength. All growth media tested (Table A1.4), are considered as rich media. Within the soils, the number of different colony types (population diversity), did not vary significantly. The MH media was the least selective and allowed 7, 6, and 10 types of colonies from soils S-I, S-II, and S-III, respectively. Among the soils, S-III showed up to 10 different types of colonies on MH media and soil S-II showed the least, only 1 colony type on TYct. Among the media, TYct showed to be the most restrictive growth media with 2 different colonies types from soils S-I and S-III and 1 colony type from soil S-II. Tyct, supplemented with potassium tellurite, restricts the growth of many different bacteria. Kinkle et al. (1994) have suggested this media can be an effective way of isolating *R. meliloti* directly from the soil.

Samples from soil S-II, showed the most growth on minimal medium (MM30) agar plates supplemented with different substrates (Table A1.5). The growth of cultures from this soil was estimated to be >1000 colonies g^{-1} with all the substrates except naphthalene which showed slightly lower (<1000 colony g^{-1}) numbers. Samples from soil S-I also showed better growth than from soil S-III even when supplemented with biphenyl. Considering the history of these soils, It was expected for S-I and S-II to have better growth than S-III on anthracene, naphthalene, and meta-toluate. However, samples from S-III did not show any

growth on plates supplemented with biphenyl. This was unexpected, because S-III is the only soil with a history of PCB contamination.

Table A1.4. Types of bacterial colonies from different contaminated soils on various agar plates.

Soil	Growth medium				
	MH	TYg	TYc	TYct	YEMP
S-I	7	5	5	2	6
S-II	6	6	6	1	6
S-III	10	5	6	2	5

Table A1.5. Growth on minimal medium supplemented with different substrates.

Substrates	Soils		
	S-I	S-II	S-III
Anthracene	+++	+++++	+++
Naphthalene	++++	++++	+++
Meta-Toluate	++	+++++	+++
Biphenyl	++++	+++++	-
Control	-	-	-

Growth- + is >0, ++ is >50, +++ is >100, ++++ is >500, +++++ is >1000, - is no growth per gram of soil.

A1.3.2 Enumeration on Diluted Media

Soil samples on media with the lower concentration of nutrients were plated and observed up to 23 days (Table A1.6). The full strength rich media were covered with the bacterial colonies by the second day and it was impossible to distinguish between colonies, i.e. the results presented are those observed after 1 day of incubation. Due to lower concentration of nutrients, the populations that are fast growing, due to their slow growth, do not limit the growth of the slower growing populations. In all of the media tested, the diluted plates showed more colony types. Furthermore, not only dilution of the media but also in the suspension samples demonstrated different colony type numbers. Most colony types (up to 5) were observed to be from plates spread with dilutions 10^{-2} and 10^{-3}. However, TYct and its dilutions and YEMP/100, showed 3 –5 colony types, respectively, on lesser-diluted suspensions. The diluted nutrient plates also minimized the mucus production and it was easier to distinguish between the different colonies, specially, with YEMP. Therefore, it seems that diluted media were better for total microbial counts and that the diversity of bacteria increased as the concentration of the nutrients decreased in the selection media.

Table A1.6. Growth and diversity of bacterial colonies in soil S-III samples, grown on media of different strengths.

Media	Dilutions					
	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
TY	ND +++++	4 +++++	4 +++++	3 +++++	ND	2 +++++
TY/10	4 +++++	4 +++++	5 +++	3 +++	3 +++	1 +++
TY/100	2 +++++	4 ++++	5 +++	5 +++	3 ++	0
TYc	3 +++++	4 +++++	4 +++++	3 +++++	ND	1 ++++
TYc/10	2 +++++	4 +++++	5 ++++	4 ++++	2 +++	2 +++
TYc/100	3 +++++	4 ++++	5 ++++	4 +++	2 ++	0
TYct	3 +++++	3 ++	1 +	0	0	0
Tyct/10	3 ++++	3 ++	2 +	0	0	0
Tyct/100	3 ++++	2 +++	2 +	1 +	0	0
YEMP	2 +++++	3 +++++	3 +++++	4 +++++	ND	1 +++++
YEMP/10	3 +++++	4 ++++	5 +++	3 +++	2 +++	1 +++
YEMP/100	5 ++++	5 ++++	4 +++	3 +++	1 +++	0

Numbers represent different colony types.
Growth- + is >0, ++ is >50, +++ is >100, ++++ is >500, +++++ is >1000 per gram soil. ND- not determined.

A1.3.3 Microbial Diversity during Bioremediation

The population diversity of the bacteria in the contaminated soil was determined before and 377 days after bacterial inoculations and their growth compared on agar plates with nutrients of different compositions (Table A1.7). Furthermore, the total population variation showed to be different among the treatments and media (Figures A1.1 to A1.4). Total diversity was observed to be 9, 7, 7 and 7 types on MH, TYc, TYc/100, and TYct, respectively (Table A1.7). Within the treatments, columns augmented with the indigenous microflora (ABVII) gave up to 7 different types of colony on TYc/100 media. Within the media, TYct showed to be the most restrictive that allowed 2 colony types for each of initial and water treated samples and 3 for ABVII, B-356, A-025, and 4 for the passive control samples. The TYc/100 media allowed the most types of colonies to grow with 6, 6, 7, 5, and 6 for the initial, water, ABVII, A-025, and passive control samples, respectively. Columns augmented with B-356 showed the most population diversity on TYc plates.

In conclusion, the plate count method with different nutrients and their different concentration effectively show the changes in the diversity and size of microbial population during a bioaugmentation based bioremediation method.

Table A1.7. Diversity of bacterial colonies on various media of varying concentrations in bioaugmented soils before and after 377 days of experimental period.

Media	Dilutions	Initial	Treatments					Total Diversity
			water	ABVII	B-356	A-025	control	
MH	10^{-1}	6	5	2	4	5	4	
	10^{-3}	4	5	6	1	4	6	9
	10^{-5}	1	3	1	1	2	-	
TYc	10^{-1}	1	5	2	2	3	4	
	10^{-3}	5	4	3	5	5	6	7
	10^{-5}	-	3	3	1	1	1	
TYc/100	10^{-1}	5	6	7	2	5	6	
	10^{-3}	6	6	5	4	5	2	7
	10^{-5}	2	1	1	1	1	-	
TYct	10^{-1}	2	2	3	3	3	4	
	10^{-3}	-	-	-	-	1	-	7
	10^{-5}	-	-	-	-	-	-	

Numbers represent different colony types. – is no growth

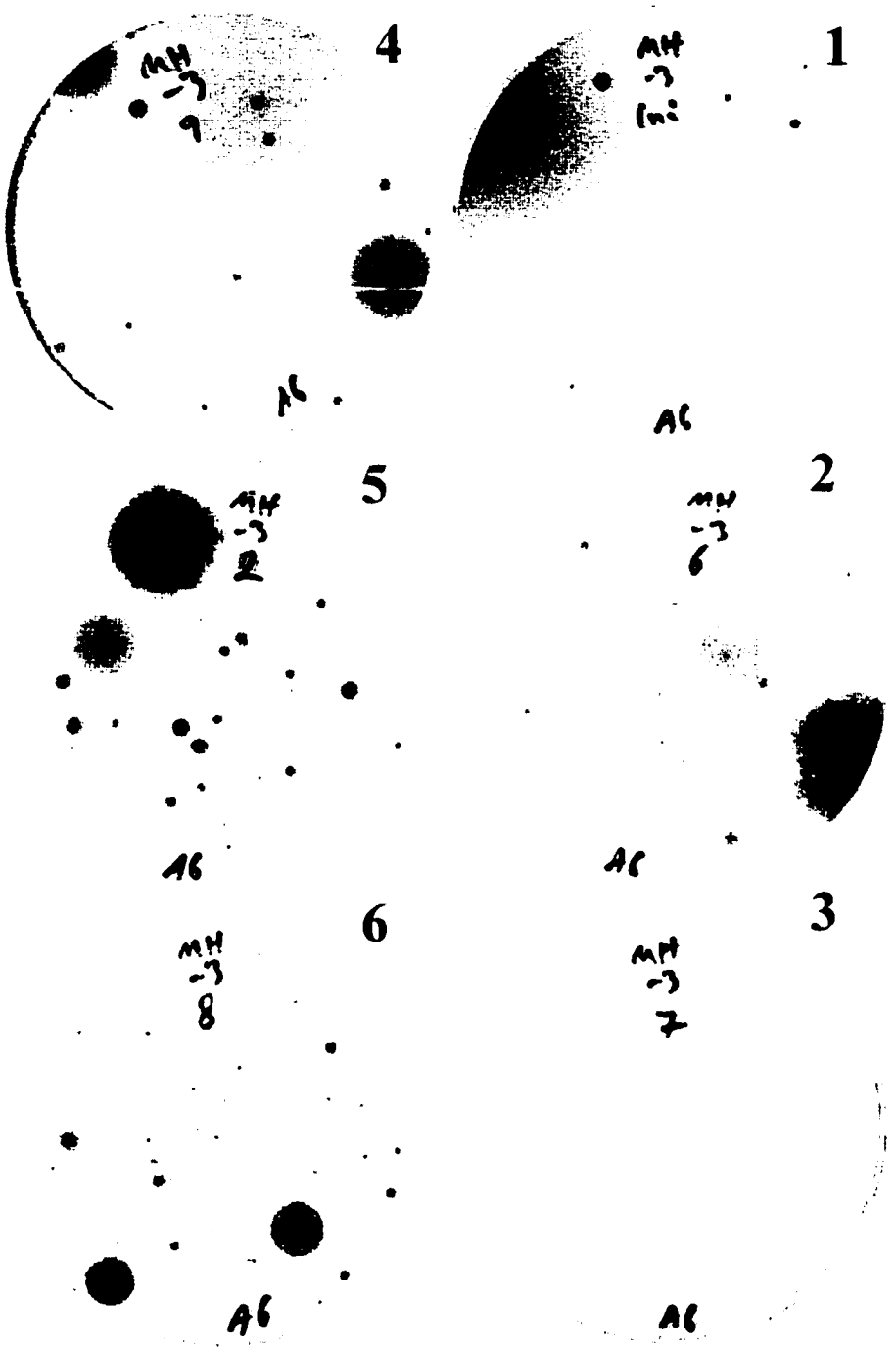

Figure A1.1. Bacterial colonies on MH agar plates: 1) initial and after 377 days of treatments with 2) A-025, 3) B-356, 4) passive control, 5) water, 6) indigenous microflora.

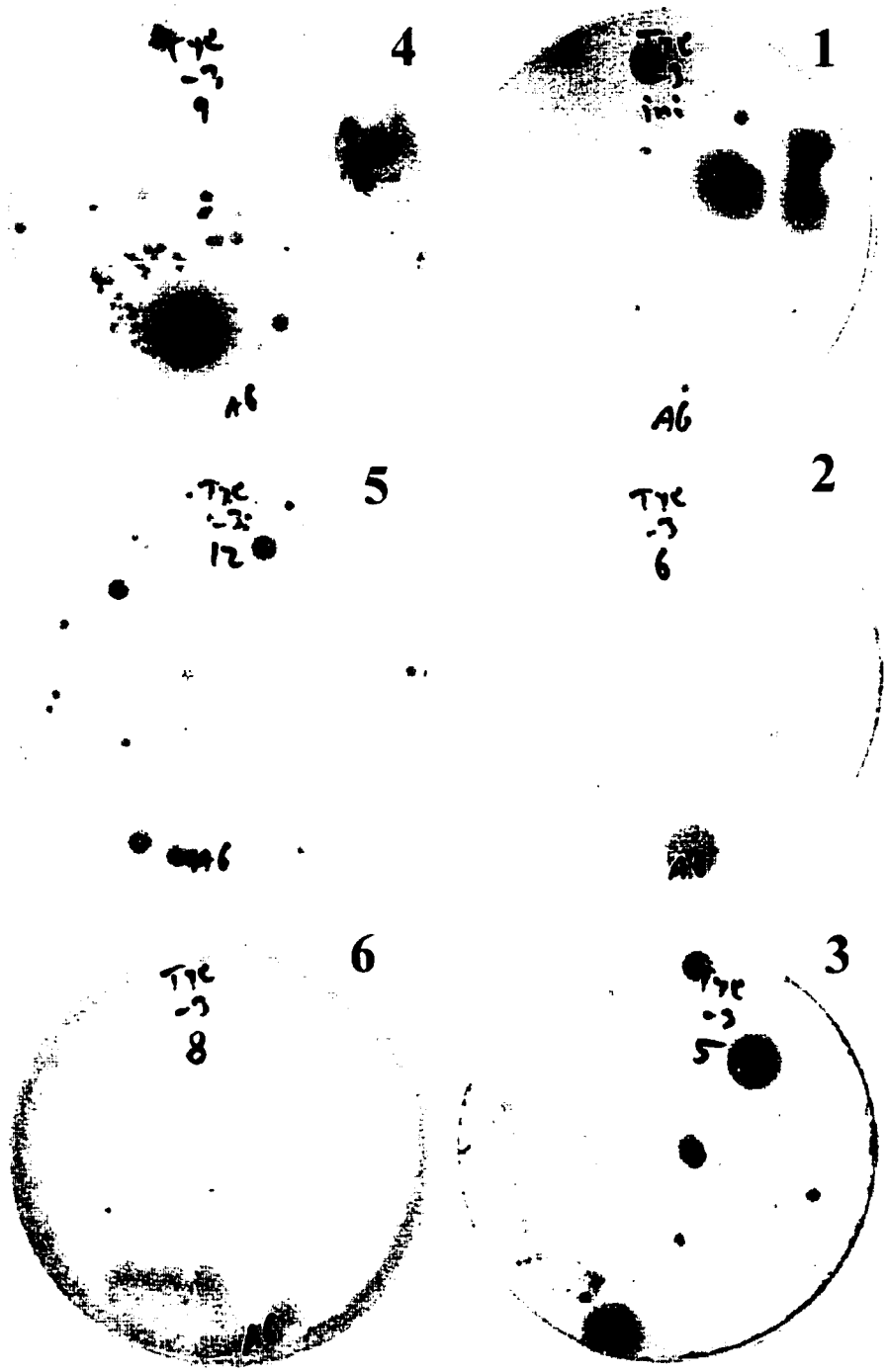

Figure A1.2. Bacterial colonies on TYc agar plates: 1) initial and after 377 days of treatments with 2) A-025, 3) B-356, 4) passive control, 5) water, 6) indigenous microflora.

Figure A1.3. Bacterial colonies on TYct agar plates: 1) initial and after 377 days of treatments with 2) A-025, 3) B-356, 4) passive control, 5) water, 6) indigenous microflora.

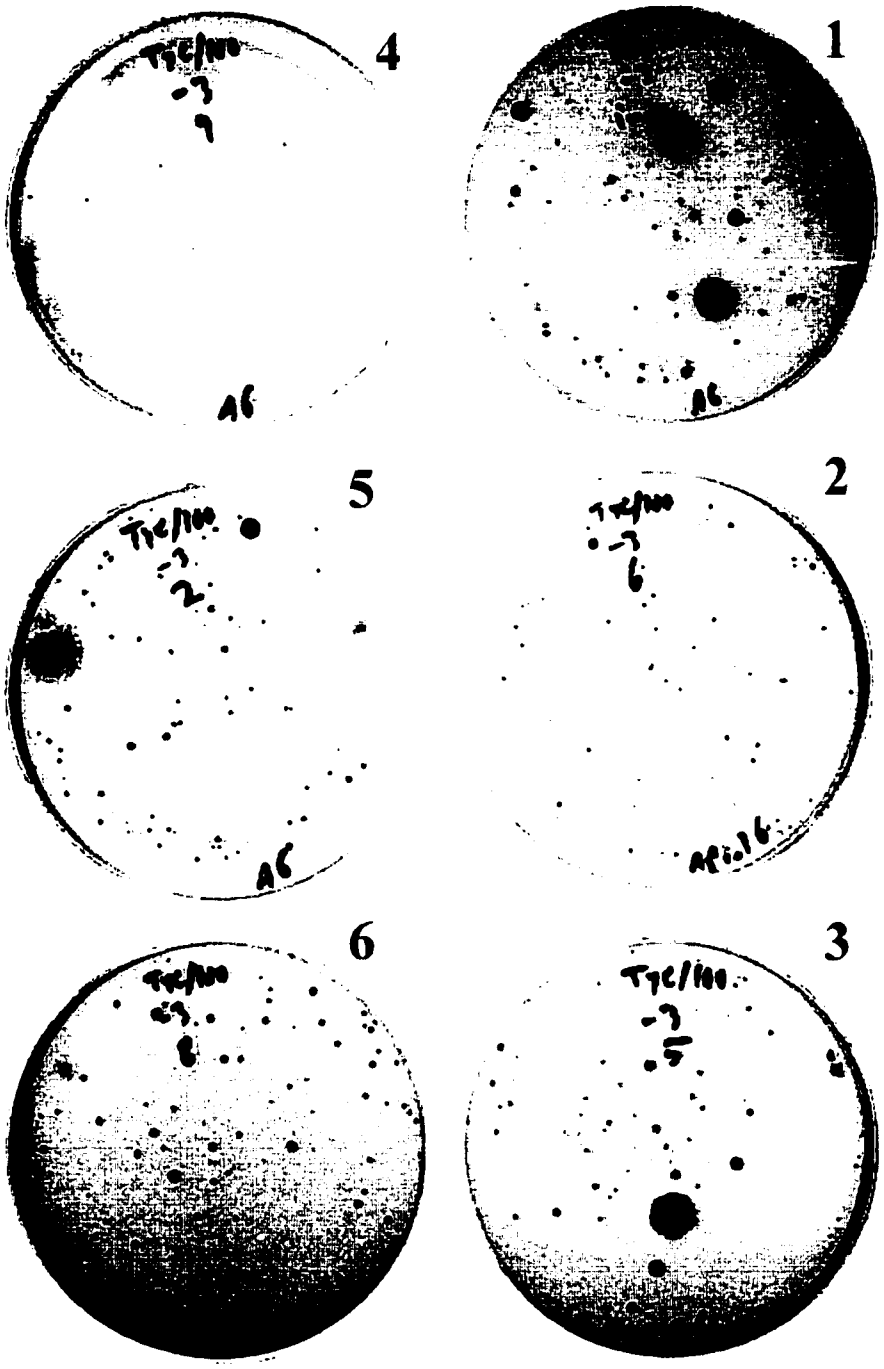

Figure A1.4. Bacterial colonies on TYc/100 agar plates: 1) initial and after 377 days of treatments with 2) A-025, 3) B-356, 4) passive control, 5) water, 6) indigenous microflora.

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PREFACE TO APPENDIX A2

Rhizobium meliloti can be found either as free living bacteria in soil or in a symbiotic relationship with alfalfa plants as bacteroids. As bacteroids, these microorganisms fix nitrogen for the plant and, in return, the plant provides nutrients (organic carbon) for the bacteria. The aromatic organic chemicals produced by the plants can be similar to some soil contaminants in their chemical structure. Rhizospheric bacteria are exposed to such plant exudates and may have evolved to degrade aromatic hydrocarbons. Thus, presence of these exudates could have biostimulatory effects on the bacteria to enhance biodegradation of contaminants such as PCBs and PAHs. Furthermore, these exudates can also play a role in bioavailability of recalcitrant pollutants by binding to the chemicals or by competing for the adherence sites to the soil particles. This in turn may enhance the biodegradation of these contaminants due to higher bioavailability.

Appendix A2 covers a preliminary study where a PCB contaminated soil was bioaugmented with a strain of *R. meliloti*, A-025, and biotransformation of PCBs was followed in the presence and absence of alfalfa plants, the host plant for *R. meliloti*. This Appendix describes the experimental procedures and the results of this study.

The results will be submitted for publication as: Mehmannavaz, R., Ahmad, D. and Prasher, S.O. Rhizospheric Effects of Alfalfa Plants on *Rhizobium meliloti* in Biotransformation of PCBs in Contaminated Soils.

APPENDIX A2

RHIZOSPHERIC EFFECTS OF ALFALFA ON BIOTRANSFORMATION OF PCBS IN A SOIL BIOAUGMENTED WITH *RHIZOBIUM meliloti*

ABSTRACT

In this study, we investigated the effect of plant-microbe interaction on bioremediation of PCBs in a contaminated soil. Eight square shape plastic containers (680mL in size) were packed with 350 g of soil (S-VII) that was contaminated for over 17 years with Aroclors 1242, 1248, 1254, and 1260. Four treatments were setup: 1) soil bioaugmented with *R. meliloti*, strain A-025, and planted with alfalfa, 2) soil bioaugmented with *R. meliloti*, strain A-025, 3) soil planted with alfalfa, and 4) control, soil with neither plantation nor microbial bioaugmentation. The results indicated that treatment with alfalfa plants (treatment 3) was the most effective in the PCB loss. Biotransformation of PCBs in the soil was more effective in presence of the alfalfa plants, however, soil hardness and moisture contents were effected by bioaugmentation of the soil. Also, the plant growth and yield were lower in the bioaugmented soils. At this point, it is difficult to attribute these losses of PCBs to alfalfa plants. Therefore, further investigations are needed to determine whether the PCBs were taken up and broken down by the plants or the loss was only through adsorption to the root system. Also, to determine whether the presence of plants had a positive effect on

bacterial degradation of PCBs or the bioaugmentation. Either possibility seeks optimism in the use of plant and bioaugmentation in remediation of soils contaminated with PCBs.

A2.1 INTRODUCTION

There are a variety of treatment technologies for contaminated soils: physico-chemical techniques, solid-phase biotreatment (land-farming), slurry phase biotreatment, in-situ biotreatment, and a combination of the biological and physical or chemical techniques. Bioremediation includes the use of microorganisms or plants to detoxify an environment, by transforming or degrading the contaminant (Walton et al., 1994). For bioremediation of recalcitrant pollutants at sites where local environmental conditions have prevented the development of a degradative microbial community, inoculation with specific populations of microorganisms or consortia (bioaugmentation) may be advantageous (Turco and Sadowsky, 1995). Such cases may as well require nutrient amendment for maximal removal of the pollutant (Bollag and Bollag, 1995). Bioaugmentation with proven contaminant-degrading microorganisms can lead to a higher degree of confidence in remediation success, and for certain sites has shown to save time and money over alternative approaches (Forsyth et al., 1995; Verstraete and Top, 1992).

Soil bioremediation research has, for the most part, focused on the role of microorganisms. However, it has been shown that plants may also play an important role in the direct and indirect removal of pollutants such as TNT by

duckweed (Schnoor et al., 1995), PAHs by alfalfa and switch grass (Pradhan et al., 1998), atrazine by corn (Alvey and Crowley, 1996) and chlorobenzoic acid by wild rye (Siciliano et al., 1998). Plants can physically remove pollutants from soil by absorbing or translocating them into plant tissue. There, metabolic processes may transform or mineralize pollutants. Plants can also indirectly remove pollutants by increasing the biological activity in soil through rhizospheric interactions through: a) relationships with nitrogen-fixing bacteria, b) biosurfactant producing bacteria, biostimulation and bioaugmentation of the degrader populations, c) the contribution of dead plant material, and d) the provision of suitable habitat for the many other organisms that inhabit the soil (Bollag et al., 1994). Rhizosphere is a zone of unique and dynamic interaction between plant roots and soil microorganisms. This specialized region is characterized by enhanced microbial biomass and activity (Walton et al., 1994; Haby and Crowley, 1996). The greater density and diversity of microorganisms commonly observed in the rhizosphere, often results in greater rates of contaminant metabolism. Higher microbial metabolic activity results from plant root exudates and plant debris including organic acids, sugars, and other organic materials. Plants benefit from increased solubilisation of minerals, synthesis of vitamins, and other growth-stimulating materials and at times protection from pathogens, mediated by microorganisms (Shann and Boyle, 1994).

The physical dimensions of microbial activity in the rhizosphere depend on many plant- and site-specific factors. These include the species and age of the rooted plant, soil properties, and climatic conditions. Site-specific soil parameters,

such as temperature, aeration, salinity, texture, and nutrient availability, affect the microbial community in the rhizosphere. Moisture, temperature, and oxygenation are especially critical factors that influence the microbial community and the metabolic pathways found in the rhizosphere (Walton et al., 1994). Root surfaces support active bacterial biofilms that significantly augment soil-surface contact, metabolic capacities, and can consequently alter most measurable soil physical and chemical parameters (Cunningham and Lee, 1995; Shann and Boyle, 1994; Walton et al., 1994a).

The plant microbial interactions and the species of plant host are critical factor in the development of a rhizosphere community. The best-characterized bacterial associations with plant roots are those of nitrogen-fixing bacteria (rhizobia) and leguminous plants (e.g., peas, soybean, and alfalfa). These symbiotic associations are unique for both their nutrient contributions to soils and the biochemical-physiological relationship between bacteria and host plant (Fisher and Long, 1992; Walton et al., 1994). Furthermore, in our previous studies (Chapters 3, 5, 6), we have shown that Rhizobia can also survive in and seem to have potential to biotransform aromatic contaminants such as PCBs and possibly atrazine (Appendix A4).

Past research on microbial transformations of organic compounds in the rhizosphere has focused primarily on agricultural chemicals, such as insecticides and herbicides. A number of researchers have described an increase in pesticide degradation in the rhizospheres of a variety of plant species (Anderson and Coats, 1995). The potential for vegetation to be used to cleanup soil remains largely

unknown. The vast majority of organic toxicants that are candidates for bioremediation have not been studied for degradation in the rhizosphere. For those compounds that have been examined in plant-microbe systems, the results differ from plant to plant, soil to soil, and laboratory to laboratory. Yet the benefits to be realized from using vegetation to support microbial degradation of toxicants in soils are considerable.

In the present study, we attempted to investigate the role of alfalfa plants in PCB biodegradation in a soil bioaugmented with *R. meliloti* strain A-025. Furthermore, plant growth and soil moisture and hardness were as well examined both for bioaugmented and planted systems.

A2.2 MATERIALS AND METHODS

A2.2.1 Experimental Design

Eight square shape plastic containers (Rubbermaid, 680 mL in size) with sampling ports on the opposite sides, were washed with NaOCl and packed with 350 g of homogenized soil. The characteristics of the soils are shown in Table A2.1. Four treatments were setup:

1) BP PCB contaminated soil with bacterial inoculation and plant

2) B PCB contaminated soil with bacterial inoculation

3) P PCB contaminated soil with plant

4) Control PCB contaminated soil

All treatments were done in duplicates.

A2.2.2 Soil Analysis

The soil texture and moisture were determined using the protocol described by Liu and Evett (1984). Hardness on the surface of the soil was measured using a Penetrometer (CL-700, SOILTEST Inc), 220 and 270 days after commencement of the experiment.

Table A2.1. Characteristics and history of the soil.

Sand (%)	Silt (%)	Clay (%)	Organic Matter (%)	pH	History
74	8	18	4.8	7.38	Industry Heavy metals; PCB mixtures: Aroclor 1242, 1248, 1254, 1260

A2.2.3 Seed Preparation and Plantation

Seeds of *Medicago sativa* (Algonquin) obtained from the seed farm of Macdonald Campus of the McGill University (Ste. Anne de Bellevue, Quebec, Canada), were surface sterilized by soaking in 95% ethanol for 5 min, followed by a 20 min treatment with 2.8% NaOCl (household bleach, Javel). After thorough rinsing 4-5 times with sterile distilled water, the seeds were soaked over night at room temperature. The next day seeds were rinsed again several times before plantation.

A2.2.4 Preparation of Bacteria for Inoculation

R. meliloti, strain A-025 (Ahmad et al., 1997) was inoculated into 50 mL of TYc in two 125 mL Erlenmeyer flasks, from a two day old preculture. It was

then incubated at 29 °C in a controlled environment incubator shaker (Psycrotherm, New Brunswick Scientific) for 24 h, and then transferred into 1 L of fresh TYc medium in two 2 L flasks and incubated for another two days. Both 1 L cultures were pooled. Bacterial cells were then harvested by centrifugation (Sorvall Instuments Dupont model RC5C) for 5 min at 8000 rpm in 250 mL centrifuge bottles, and resuspended in 200 mL sterilized deionized water (SDW) (to about 1.82×10^9 cells·mL^{-1}) and mixed thoroughly by vortexing before inoculation of the soils.

A2.2.5 Experimental Setup

The soil containers for treatments 1 and 2 were inoculated with 50 mL of the cell suspension (approximately 2 g of cell) and the soil was well mixed with a sterilized spatula. The non-augmented treatments (3 and 4) received 50 mL of SDW and were well mixed.

Prepared seeds (1g dry weight) were planted in the soil (treatments 1 and 3). All soil containers were covered with their tops and incubated in a controlled incubator chamber (Model no. I25L CPM3000, Conviron Controlled Environ. Ltd., Winnipeg, Manitoba, Canada) under a constant temperature of 23 °C and a 16 h day^{-1} light setting (1000 W metal halide lamps that produced 250 μEinstein m^{-2} sec^{-1} at plant height). After 10 days the covers were taken off and every container was watered with the same volume of water (50-70 mL) every 5 days. After 30 days the containers were taken out of the incubator and kept in the laboratory.

A2.2.6 Sample Collection and Analysis for PCBs

Soil samples (approximately 5 g) were collected through sampling ports on the side of the containers carefully without disturbing the plant root system and analyzed as follows:

Soil samples of 3g were mixed in 125mL flasks with 6mL of sterilized deionized water and Triton X-100 (30μL) to enhance desorption of Aroclor from soil particles (Barriault and Sylvestre, 1993). Anhydrous sodium sulfate (6g) was added to prevent the formation of stable emulsion (Bedard et al., 1986). Hexane (15mL) was then added and the flasks were shaken on a rotary platform for 20 min. After the two phases had separated, the hexane fraction was collected on ammonium sulfate. The aqueous phase was extracted two more times. The pooled organic phases were passed through florisil (5g) column filters before they were concentrated to 3mL. 10μL of 2,3,4,5,6,2',3',4',5',6'-chlorobiphenyl (333 ppb) was added as an internal standard. These samples were diluted before injection. The ratio of each detected peak against the internal standard was determined and ratios were compared to those of the control (treatment 4). Table A2.2 shows the list of congeners assigned to each peak of the GC plot observed with our system. The assignment of congeners to peaks were based on previously published references (Ballschmiter and Zell, 1980; Larsen et al., 1992; Erickson, 1997). A chromatogram of the assigned congeners is presented in Chapter 6 (Figure 6.2).

The samples were analyzed with a gas chromatograph (Hewlett Packard 5890, series II) equipped with an electron capture detector (GC/ECD) and a HP-5 (crosslinked 5% Ph Me silicone) capillary column of 25m x 0.2mm x 0.33μm film

thickness. Helium was used as a carrier gas at a flow rate of 1mL /min. The initial temperature (60 ^{o}C) was held for 2 min, then raised at a rate of 5 ^{o}C/min to a final temperature of 290 ^{o}C held for 15 min. Injector and detector temperatures were 275 and 350 ^{o}C, respectively.

A2.2.7 Reisolation and DNA Manipulation of Strain A-025

Soil samples from the soil containers were taken at the end of the experimental period (270 days). 2 g of soil from treatment 1 (plant and bacteria) was vortexed in 10 mL of sterilized 0.9% saline and the suspensions were spread on TYct agar plates selective for *R. meliloti*. Two of these colonies from two different TYct plates name coded as PB1-25 and PB2-25, were further purified on TYct plates. These isolates were tested for alfalfa root nodulation using the procedure described in Chapter 3.

Further identification and confirmation was done using molecular biological techniques and comparing randomly amplified polymorphic DNA (RAPD) profiles by PCR, using the protocol described by Harrison et al. (1992), Lawrence et al. (1993), and Ahmad et al. (1997). A single oligonucleotide, A1-08, was selected from our laboratory collection of oligonucleotides as the primer in PCR. The PCR was performed in a DNA thermocycler (Perkin-Elmer, Cetus) using DNA *Taq* polymerase (Pharmacia) in a reaction volume of 50 µL. The PCR program used had the following parameters: one cycle of denaturing at 94°C for 5 min, annealing at 36 °C for 5 min, synthesis at 72 °C for 2 min, 40 cycles of 44 s at

Table A2.2. Assignment of congeners to peaks analyzed on GC-ECD.

peak	IUPAC PCB	Chemical formula	peak	IUPAC PCB	Chemical formula	peak	IUPAC PCB	Chemical formula
1	6	23'	26	101, 84	22'455'& 22'33'6	51	128, 167	22'33'44' & 23'44'55'
2	5,8	23 & 24'	27	99	22'44'5	52	185	22'3455'6
3	19	22'6	28	119	23'44'6	53	174	22'33'456'
4	18	22'5	29	83	22'33'5	54	177	22'33'4'56
5	15, 17	44' & 22'4	30	97	22'3'45	55	156, 171	233'44'5 & 22'33'44'6
6	24, 27	236 & 23'6	31	87, 81	22'345'& 344'5	56	157, 173	233'44'5' & 22'33'456
7	16, 32	22'3 & 24'6	32	85	22'344'	57	172	22'33'455'
8	34	2'35	33	110, 77	233'4'6 & 33'44'	58	180	22'344'55'
9	29	245	34	151, 82	22'355'6 & 22'33'4'	59	193	233'4'55'6
10	26	23'5	35	135, 144, 124	22'33'56' & 22'345'6 &2'3455'	60	191	233'44'5'6
11	31, 28	24'5 & 244'	36	107, 147	233'4'5 & 22'34'56	61	199	22'33'4566'
12	33, 20, 53	2'34 & 233' & 22'56'	37	149, 123, 118	22'34'5'6 & 2'344'5 & 23'44'5	62	169	33'44'55'
13	22	234'	38	134	22'33'55'	63	170, 190	22'33'44'& 233'44'56'
14	45	22'36	39	146	22'34'55'	64	198	22'33'455'6
15	46	22'36'	40	153	22'44'55'	65	201	22'33'4'55'6
16	52	22'55'	41	132, 105	22'33'46' & 233'44'	66	196, 203	22'33'44'5'6 & 22'344'55'6
17	49	22'45'	42	141	22'3455'	67	189	233'44'55'
18	44	22'35'	43	179	22'33'566'	68	195	22'33'44'56
19	64, 41, 71	234'6 &22'34 & 23'4'6	44	130	22'33'45'	69	207	22'33'44'566'

20	40	22'33'	45	176, 137	22'33'46 6'& 22'344'5	70	194	22'33'44'55'
21	74	244'5	46	138, 163, 160	22'344'5' & 2334'56 & 233'456	71	205	233'44'55'6
22	70	23'4'5	47	178, 129, 126	22'33'55' 6 & 22'33'45 & 33'44'5	72	206	22'33'44'55'6
23	66,95	23'44' & 22'35'6	48	175, 159	22'33'45' 6 & 233'455'	73	*209	22'33'44'55'66'
24	91	22'34'6	49	187	22'34'55' 6			
25	56, 60, 92	233'4' & 2344' & 22'355'	50	183	22'344'5' 6			

Peaks that were identified in our chromatographic system
[IU]- Numbers refer to IUPAC convention per Ballschmiter and Zell (1980).
*- Congener 209 used as internal standard.

94 °C, 2 min at 36 °C, 2 min at 72 °C and a final extension period of 5 min at 72 °C. Fifteen µL of the amplified product was resolved by electrophoresis on a 1.6% agarose gel in TBE (Tris-borate EDTA) buffer at 100 V for 5 min followed by 50 V for 2 h, staining with ethidium bromide for 30 min, destaining overnight in water, visualized under a UV transilluminator.

A2.2.8 PCB Statistical Analysis

The concentration of the internal standard (decachlorobiphenyl) was used to determine the concentration of every observed peak for each sample and then these were summed as the total PCB concentration. The General Linear Model (GLM) procedures of SAS 6 (SAS Institute Inc., 1989) was used for analysis.

A2.3 RESULTS AND DISCUSSIONS

A2.3.1 PCB Transformation

The small size of the soil was chosen to maximize the interactions between the alfalfa plant roots and the augmented *Rhizobium meliloti,* strain A-025. To better understand the effect of these interactions in bioremediation of PCBs, treatments 2 (bacterial augmentation) and 3 (alfalfa plants) were also observed to determine the effect of each treatment that could be compared to treatment 1 (bacterial augmentation and alfalfa plant). These treatments were also compared to the control (treatment 4), to count for PCB levels due to volatilization and desorption effects in the extraction procedures. The duration of the experiment was 270 days and sampling was done on day 44 and 270.

In soil samples from the 44^{th} day planted with alfalfa and soil augmented with strain A-025 five congeners that showed >20% depletion belonged to the *tri-*, *tetra-*, *octa-*, and *nona-* chlorinated biphenyls (Figure A2.1a). In addition, significant depletion in peak 15 (2,2',3,6') and enrichment of the di- congeners was only observed with the bacterial augmentation and not with the plant treatment (Figure A2.1b). In soil with bacterial augmentation and plant, all peaks showed to deplete where congeners with higher than hepta chlorination depleted >20% in addition to the congeners that had decrease to the same levels in the other two treatments (Figure A2.1c). However, peaks 1 (2,3'), 3 (2,2',6) and 61 (2,2',3,3',4,5,6,6') were enriched. Peak 61 (2,2',3,3',4,5,6,6') is in common in the three treatments in this study, on the 44^{th} day. It was also observed to be enriched in the results obtained in Chapter 6.

In soil samples from the 270th day planted with alfalfa, showed the most depletion among the three treatments as compared to the control. Peaks 1 (2,3') and 15 (2,2',3,6') showed to be enriched and almost all other peaks depleted to >20% with mostly in the di- to hexa- congeners (Figure A2.2a). Soil augmented with bacteria alone, showed the least depletion and is the only treatment that showed enrichment in peak 61 (2,2',3,3',4,5,6,6') and mostly in the di- and some of the tri- congeners (Figure A2.2b). The enrichment and depletion were observed to be more selective with bacteria than the other two. Soil with bacteria and plant, showed patterns that resembled a combination of the results observed in treatments 2 and 3 (Figure A2.2c). However, it seems that the general depletion was lower than with plant only and more than bacteria alone and the depletion in congeners higher than octa- chlorination was more than plant alone.

On 44th day the plant size and growth was not high and therefore the PCB congeners may not have been effected much in treatment with plants. Treatment with bacteria also did not show much change in PCB pattern. However, the treatment with bacteria and plants together, the effect was more extensive. As the plants increased in number and growth, the loss of PCB congeners increased as indicated for the 270th day (Figure A2.2). At this point, it is difficult to stipulate whether the PCB loss is due to uptake and transport by plants or just adsorption to the plant roots. The patterns indicate a general depletion in total PCB congeners by plants since there are no enrichment of congeners as seen in treatment with bacteria where dechlorination plays a major role in the loss of higher chlorinated PCB congeners. Furthermore, these results indicate that microbial PCB

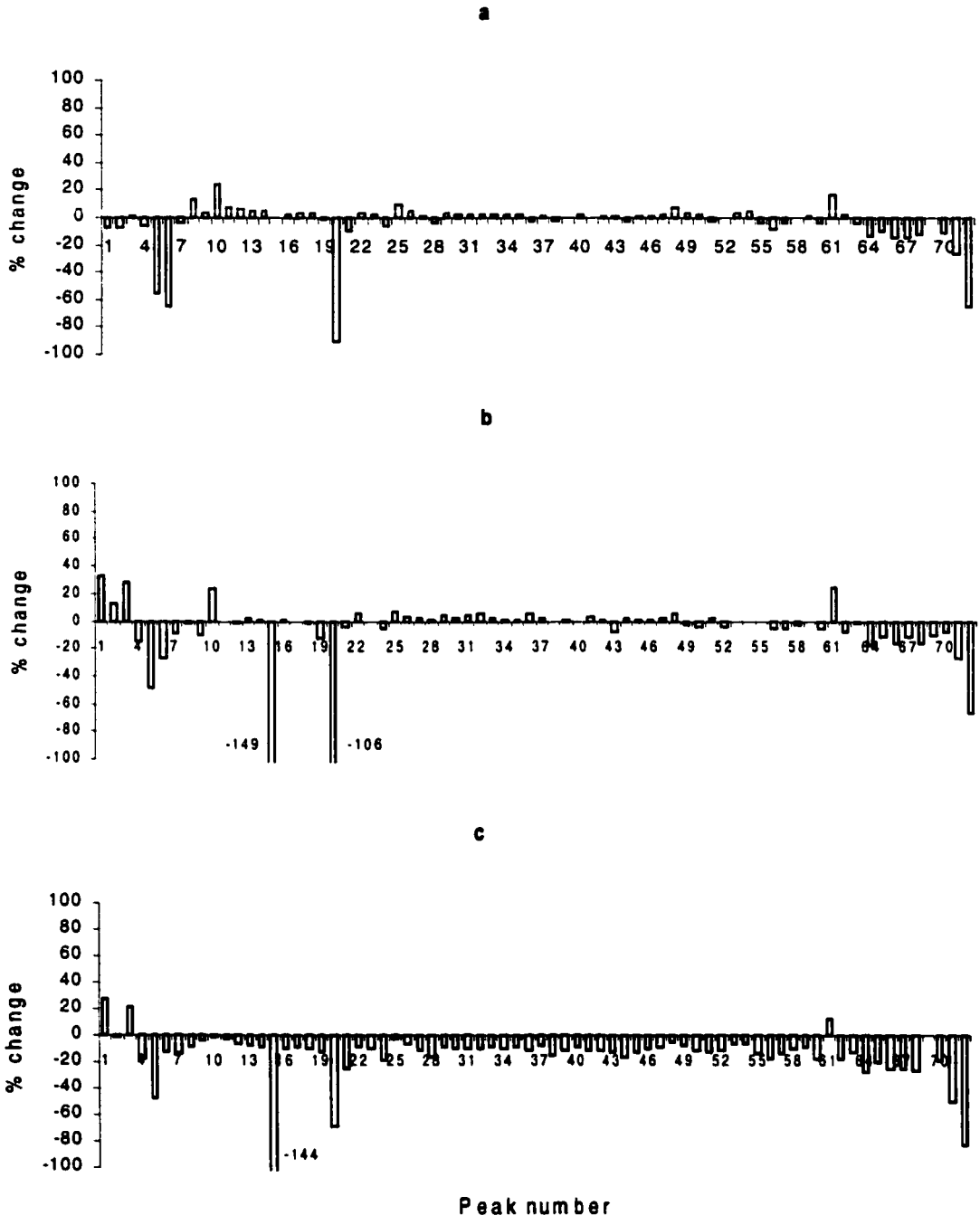

Figure A2.1. The concentration changes of PCBs after 44 days of treatment with a) P (plant), b) B (bacteria), c) BP (bacteria and plant) as compared to the control treatment. The results represent average of two replicates.

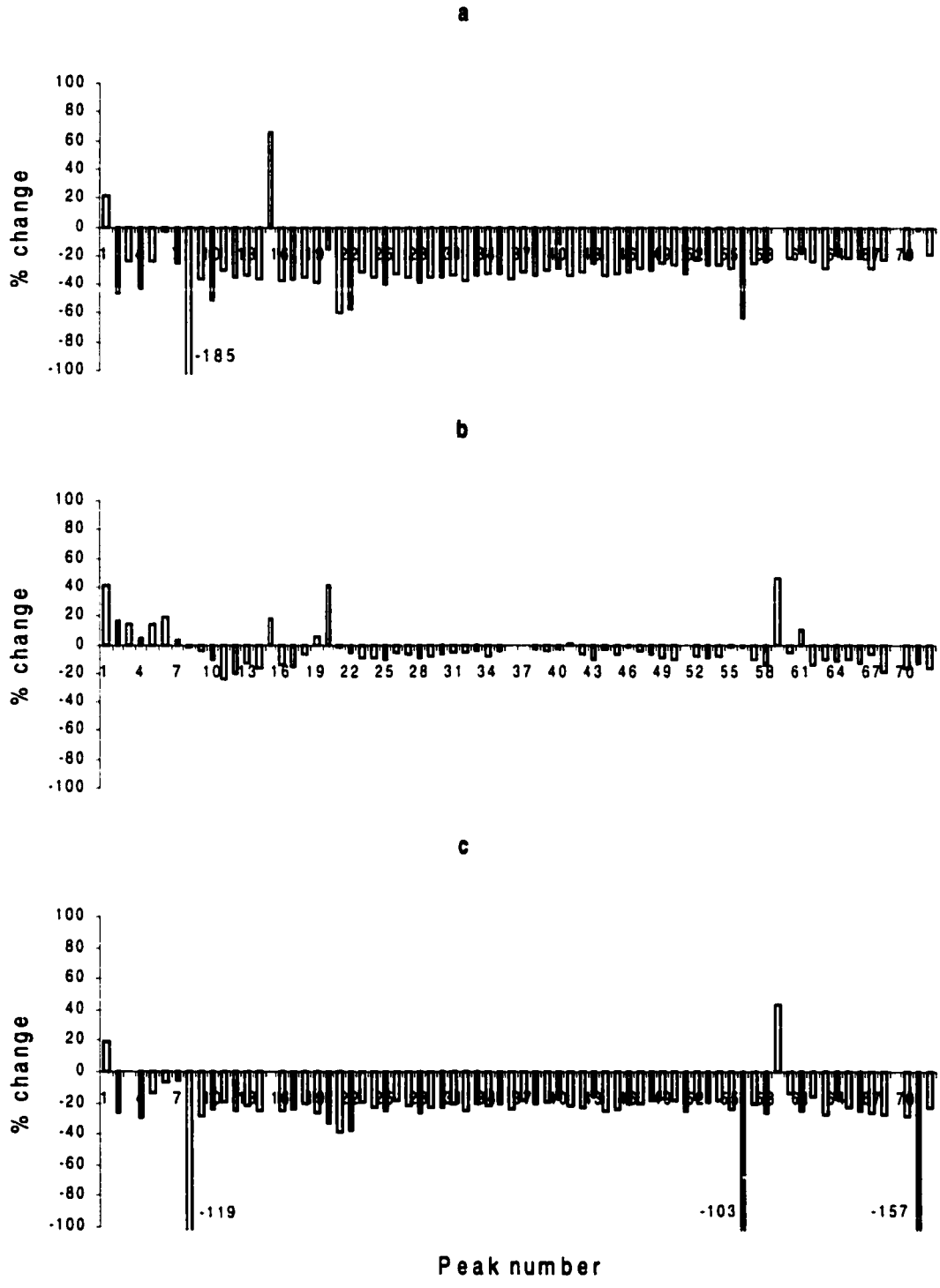

Figure A2.2. The concentration changes of PCBs after 270 days of treatment with a) P (plant), b) B (bacteria), c) BP (bacteria and plant) as compared to the control treatment. The results represent average of two replicates.

degradation requires sequential anaerobic and aerobic cycles as the total depletion in PCB congeners in this study were less significant than those observed in Chapter 6 for the same period of experimental time.

A2.3.2 Total PCB Concentration

The previous section demonstrated the biotransformation of PCB congeners in time. These results were a comparison of treatments to the control to determine the effect of bioaugmentation and alfalfa plants on PCB loss. However, in such a system where the complexity of the soil, PCBs, and the microorganisms are combined, the control alone can be effected due to both biological and chemical interactions in time. Therefore, the actual PCB concentrations and losses might not be evident when treatments are compared to the control. In this section, the total PCB concentrations are discussed and statistically analyzed to demonstrate the effect of bioaugmentation and alfalfa plants on different PCB groups based on the number of chlorines present on the biphenyl rings.

As shown in Table A2.3, there was a over all significant difference in treatments and the plant treatment was significantly different from the control and the bacterial treatment and less significant with plant and bacteria together (Table A2.4). The lowest total PCB concentration was determined to be in the plant treatment (Figure A2.3). This difference was mainly due to depletion in concentration of the penta- and hexa- congeners as compared to control (Figure A2.4).

Table A2.3. General Linear Model procedures for total PCB concentrations (α=0.05).

Source	C.V.	Pr > F
Treatment	10.6	0.0463

C.V.-coefficient of variation

Table A2.4. Multiple pairwise comparisons of overall mean among treatments for total PCB concentration.

Treatment	Mean*
Control	79.86 a
Bacteria	75.66 a
Plant & Bacteria	65.88 ab
Plant	60.47 b

*- Treatment means with different letters are significantly different (α=0.05), based on protected LSD test.

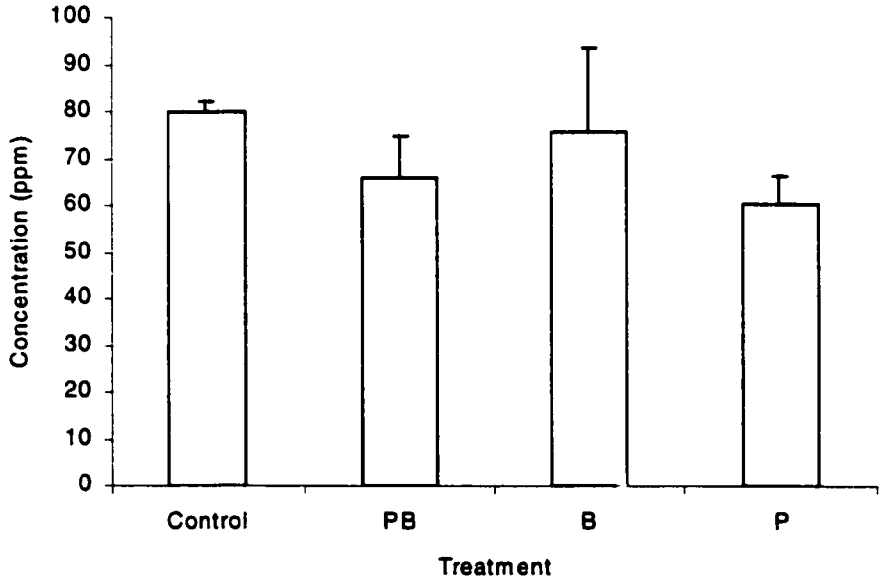

Figure A2.3. Total PCB concentration in the soil of different treatments after 270 days.

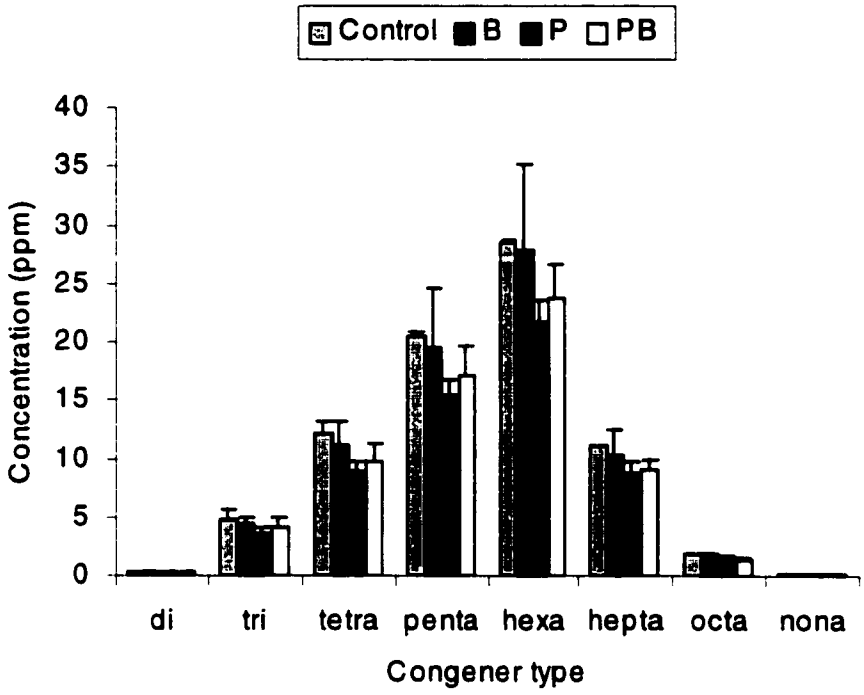

Figure A2.4. Total PCB concentration in soil of different treatments after 270 days based on the number of chlorines.

A2.3.3 Alfalfa Plant Growth

All soil containers for treatments 1 and 3 were planted with 1 g (dry weight) of alfalfa seeds and seed germination and plant growth were followed through the experimental period. Although, it was expected that the plants in treatment 1 (microbial inoculation and plant) would have better growth because of the bioaugmentation with *R. meliloti*, strain A-025, seed germination and plant growth were observed to be much higher in the non-augmented treatment. Furthermore, the root formation and growth was observed to be much better in the non-augmented plant containers (Figure A2.5). The over all alfalfa plant growth for both planted treatments were lower than expected (Figure A2.6).

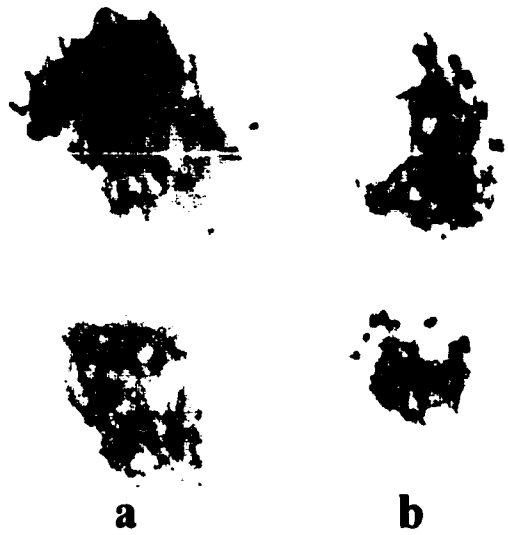

Figure A2.5. Photograph of the washed live plant tissues after 270 days for the replicates of a) plant and b) plant + bacteria treatments.

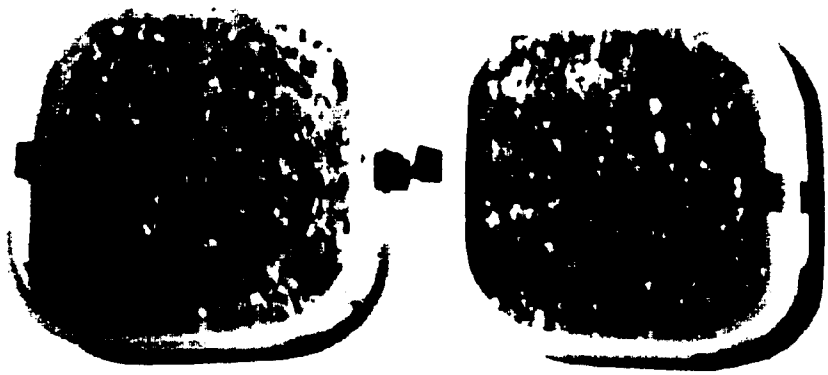

Figure A2.6. A photograph of plant containers showing plant growth after 130 days.

At the end of the experiment the number of nodules and total weight of the plants in each treatment was determined (Table A2.5). There were 12 ± 5 nodules per container in the bioaugmented treatment much lower than observed in a previous study (Chapter 3). However, the inoculum size was not the same. The nodules were also smaller in size (1.3 mm maximum) than previously observed. The non-augmented treatment with plant did not show any nodule formation indicating that *R. meliloti* was not present in this soil (S-VII).

The formation of the nodules did not effect the health and size of the plants positively as was expected. The total weight of the plant tissue (including leaf, stems, and roots) were 0.4 ± 0.2 and 1.6 ± 0.9 for treatments with plant and bacteria and with plant alone, respectively (Table A2.5). Furthermore, the plants that did grow, never reached maturity (stopped growth after 1st or 2nd trifoliate leaf) and died after a few months in both planted treatments (Figure A2.6). Therefore, the total organic matter in the soil at the end of the experiment was determined to represent the plant growth during the entire experimental period. Even though the results were not significantly different, treatment 3 (plant) showed the highest level of organic matter (Figure A2.7). Considering these results and the total weight of live plant tissue (Table A2.5), it is reasonable to suggest that bioaugmentation of the soil had a negative effect on the plant growth and health.

Table A2.5. Total number of nodules and weight of plant tissue in different treatments.

Treatment	**PB**	**P**
Total number of nodules	12±5	0
*Weight of plant tissue (g)	0.4±0.2	1.6±0.9

The results represent the mean of duplicates and SD, *- The weight includes leaf, stems, and roots of the plants.

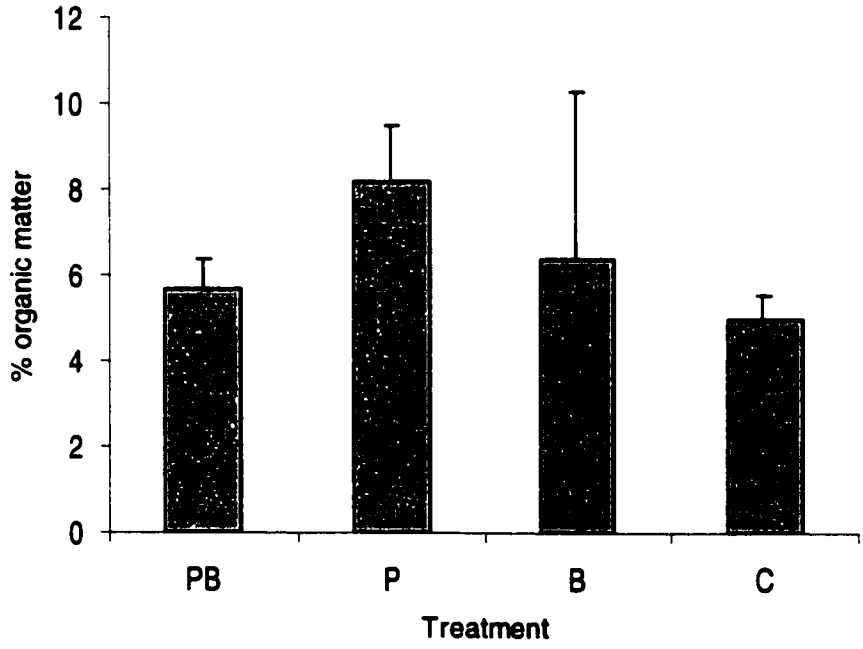

Figure A2.7. The percent organic matter in the soil of different treatments after 270 days. The results represent the average and SD of two replicates.

A2.3.4 Soil Hardness and Moisture

As time progressed during the experiment, it was observed that the bioaugmented soil treatments (1 and 3) became harder and dryer at the surface and when watered, the infiltration was not as fast as the non-augmented treatments (2 and 4). As the hardness and moisture level of the soil could have influenced the growth and health of the plants, these two parameters were measured and determined. The soil surface hardness (penetration resistance) was determined on the 237 and 270th day of the experiment (Figure A2.8). The results showed that both soils bioaugmented with bacteria had significantly higher penetration resistance than the non-augmented soils. These results are in agreement with those observed in Appendix A3, where bioaugmentation of a PCB contaminated soil increased the surface penetration resistance.

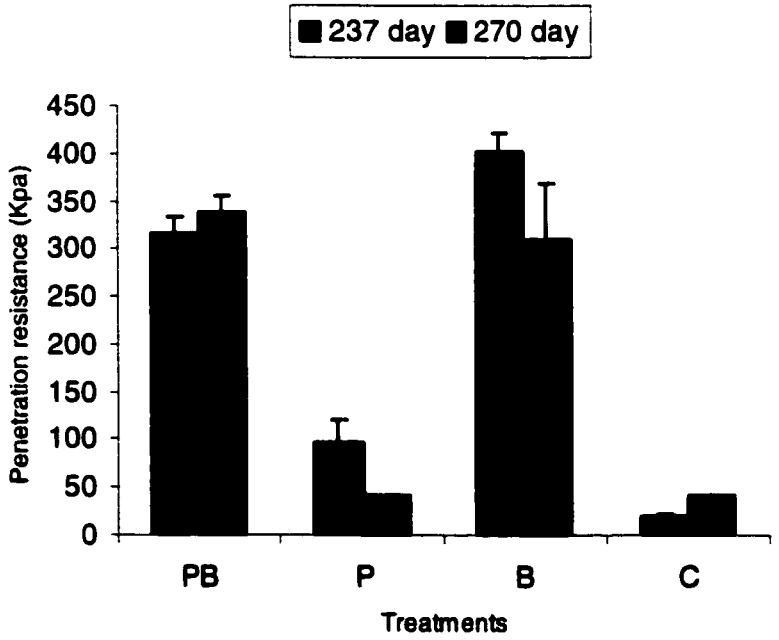

Figure A2.8. The hardness of soil surface measured as penetration resistance of the soil in different treatments at different times. Means and SD of duplicates.

The moisture levels in the soil at the end of the experiment (Figure A2.9) was highest (18%) in the treatment with plant only, while soil with bacterial bioaugmentation with plant decreased to approximately 11%. Bioaugmentation alone showed slightly higher moisture level (6%) as compared to the control and lower than both planted treatments.

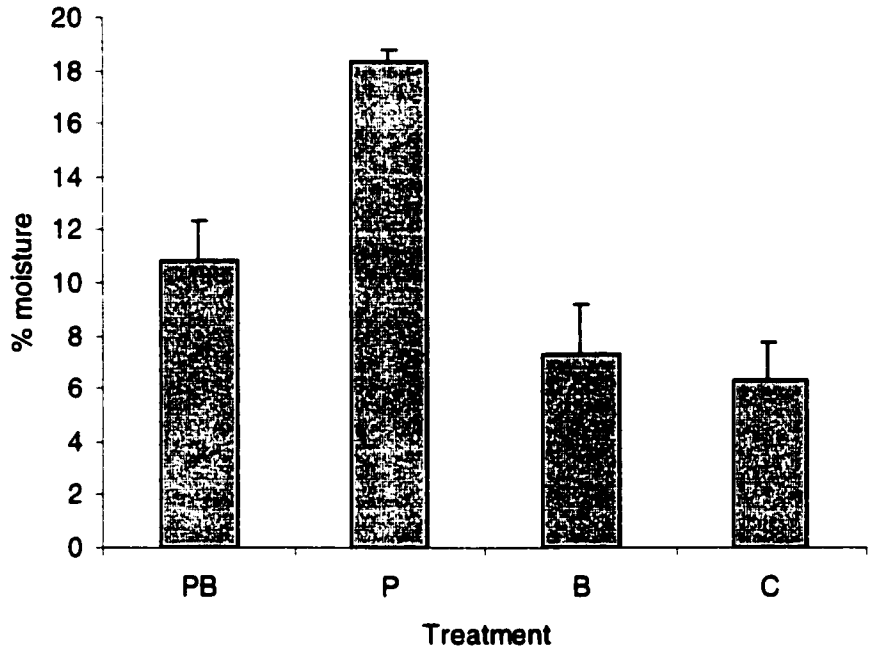

Figure A2.9. The percent of moisture in the soil after 270 days. Average and SD of two replicates.

These results indicate that soil hardness could have influenced the moisture content and even more importantly, the growth of the plants. Soil hardness has been reported to decrease crop yield (Ohu et al., 1985). Pandy (1987) has suggested soft and moist soil is the most adequate for growth of leguminous plants. Roper et al. (1995) have reported that when the tops of lucerne plants were removed, production of exudates by the plant was decreased. In this experiment, since the plants never passed the first or second trifoliate leaf growth, the production of exudates essential in the chemotactic signaling of the plant to the rhizobial cells for infection and formation of nodules could have been minimized and consequently, lower number of nodules were formed.

A2.3.5 Survival of Strain A-025

The survival of bioaugmented strain A-025 was determined in Chapters 5 and 6. Therefore, the population pattern and survival was not obtained in this experiment. Nevertheless, reisolation of the bioaugmented strain A-025 was attempted from treatment 1 (plant and bacteria) at the end of the experiment after 270 days. The population count based on the colony forming units on TYct agar plates demonstrated a population of approximately 3.0×10^3 cells g^{-1} soil present. Two of these colonies (PB1-25 and PB2-25) were positive for nodule formation and N_2-fixation. The RAPD of these strains were also determined and compared to the strain A-025 that was used to bioaugment the soil (Figure A2.10). Even though, the patterns observed for PB2-25 are not as strong as that for A-025, there are some similarities between the two. At this point it is difficult to speculate

whether both these strains are the same. However, the fact that the nodulation tests were positive for this strain and no nodule formation was seen in the non-augmented plant treatment suggests the absence of *R. meliloti* in this soil (S-VII). With more specific molecular identification, the differences might be attributed to mutations during the experimental period.

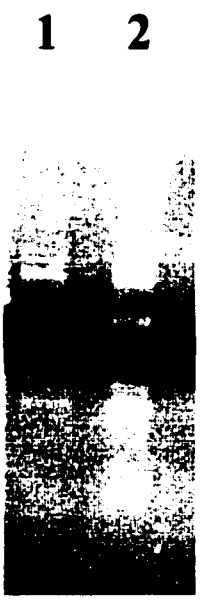

Figure A2.10. RAPD profiles produced using randomly selected primer, A1-08. Lane 1) reisolated strain PB2-25 and 2) bioaugmented strain A-025.

In conclusion, growth of alfalfa plants and bioaugmentation of the soil with *R. meliloti,* strain A-025, increased the loss of PCB congeners as compared to bioaugmentation alone. However, the loss is more attributed to the presence of the plants rather than the bioaugmentation, since plants alone were the most effective in the loss of PCBs. Plant roots could have increased the air circulation in the soil, thus, increased the volatilization of the PCBs.

Bioaugmentation of the soil played a significant role in the hardness of the soil and indirectly affecting the growth of alfalfa plants. At this point, it is difficult to predict whether PCBs as such or their biotransformation products resulted in any phytotoxicity on germination and plant growth. Perhaps, the difference of plant growth in bioaugmented and non-augmented treatments is related to bioaugmentation and soil hardness, however, growth and crop yield for 1 g of seed planted, was lower than expected in the non-augmented treatment. Therefore, it is important for further research to determine the direct phytotoxicological effects of PCBs on alfalfa seed germination and growth in order to understand the role of phytoremediation of PCBs.

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PREFACE TO APPENDIX A3

Microbial bioaugmentation of contaminated soils is a promising technology. Even though, there have been work done on survival and transport of bacteria in soil matrix, there is no evidence of work to demonstrate the effect that bioaugmentation or contaminants can have on soil's structure and properties/quality. Bioaugmentation involves addition of exogenous or indigenous bacteria to the soil. Thus, it certainly influences the biological and biochemical properties of the soil environment and in turn will effect some of the physico-chemical properties of the soil. Therefore, development of any bioaugmentation technology should include investigation of the changes that might occur as a result of bioaugmentation of contaminated soils. It should further include the effects that the contaminants can have on the soil structure and its properties. In Chapter 4, we investigated whether subirrigation could be used to transport and distribute bacterial cells at different depths of soil columns packed with a sandy loam soil. It is also important to determine how the type of soil or presence/type of contaminants could affect the transport of bacteria with water.

To answer some of the above mentioned concerns, three different soils, a clay, a sandy loam and a PCB-contaminated sandy loam, were used to determine the influence of clay content and PCBs on the transport of *Rhizobium meliloti*, strain A-025, in unsaturated soils, using water as a transport medium. Surface water infiltration, moisture loss, and soil hardness were also measured after bacterial bioaugmentation of these soils and compared to non-bioaugmented soils.

The results will be submitted for publication as: Mehmannavaz, R., Ahmad, D. and Prasher, S.O. Bioaugmentation Effects and Microbial Transport, Water Infiltration, Moisture, and Hardness in Clay, Sandy Loam and Sandy Loam PCB contaminated Soils.

APPENDIX A3

MICROBIAL TRANSPORT, WATER INFILTRATION, MOISTURE LOSS, AND HARDNESS IN CLAY, SANDY LOAM, AND PCB CONTAMINATED SANDY LOAM SOILS

ABSTRACT

Three different soils, a clay, a sandy loam and a PCB-contaminated (>100ppm) sandy loam, were used to determine the influence of clay content and contaminants on the transport of bacteria in unsaturated soils, using surface irrigation water as a transport medium. Furthermore, soil surface water infiltration, moisture loss, and hardness were measured after bacterial bioaugmentation of these soils and compared to non-bioaugmented soils. Results indicated that transport of *Rhizobium meliloti* strain, A-025, was influenced more by the presence of PCBs than by clay content. After four irrigations, a total of 4.35×10^6, 7.44×10^6 and 1.23×10^7 cells·mL^{-1} were leached out from the PCB, clay and sandy soils, respectively. Transport of the strain was directly related to the period and frequency of irrigation, making them important factors for consideration when applying bioaugmentation through water transport. Soil clay content had a greater influence on surface water infiltration than the PCB content. Infiltration was significantly influenced by bioaugmentation in clay and sandy soils, to a lesser extent with PCB contaminated soil. The greatest difference in moisture loss between bioaugmented and control soils was observed with the PCB

soil. Due to moisture loss, the clay soil became the hardest soil for surface penetration.

A3.1 INTRODUCTION

An alternative to the enhancement of bioremediation of environmental pollutants by indigenous microorganisms (biostimulation) is the use of an inoculum (bioaugmentation) having an appropriate pure or mixed culture of degrading microorganisms. Bioaugmentation with proven contaminant-degrading microorganisms, whether indigenous or exogenous with respect to the contamination site, leads to a higher degree of confidence in remediation success and can save both time and money (Forsyth et al., 1995). Biological treatment of contaminated sites, through biostimulation of the indigenous microflora, has received a great deal of attention. However, bioremediation through bioaugmentation has faced certain problems at the application level. Bioaugmentation requires techniques that allow for the delivery, transport, distribution and survival of desired organisms at different depths for various soil types and contaminants. However, knowledge concerning the influences of bioaugmentation on the characteristics of a contaminated site, such as moisture loss or soil hardness, make this technology somewhat troublesome and limited for transport and survival of bioaugmented microorganisms.

Studies in the past have concentrated mostly on soil parameters and characteristics that do not include the effects of bioaugmentation. The effects of moisture on the hardness of different soils has been investigated (Ohu et al., 1988)

and a study by Hekman et al. (1995) showed that bacterial transport was generally similar between two water regimes (0.9 and 4.4 mm/h). In a loamy sand soil, there was no change in bacterial distribution during the 51 days of experiment. However, in a silt loam, cell numbers in the lower soil layers were significantly reduced to levels at or below the limit of detection.

Bacteria, as negatively charged biocolloids, interact with soil particles, thus, clay content, organic matter, pH, cations, and the types of contaminants present in the soil influence these interactions. Bacterial movement in soil is a function of water content and water potential relationships, and is influenced by their sorption onto soil particles (Yates and Yates, 1990; McCanlou et al., 1994) or transport into small soil pores (Huysman and Verstraete, 1993a). In a study by Shingaki et al. (1994), under acidic conditions, the negative cell surface charge became lower and, at the most acidic condition tested (pH 2.4), 34 out of 40 bacterial strains showed a positive surface charge. This change in cell surface charge, caused by a pH-shift from neutral to acidic, was the result of lost negative charge on functional groups existing at or exposed to the cell surface.

Bacterial transport through saturated and unsaturated soils has been well documented (Huysman and Verstraete, 1993a,b; Lance et al., 1984; Lovins et al., 1993) and was investigated using a subirrigation system in saturated soil columns in Chapter 4. Furthermore, the influence of different soils, irrigation rates, moisture levels and different bacteria have been investigated in various studies (Hekman et al., 1995; MacLeod et al., 1988; McCanlou et al., 1994; Harkes et al., 1992; Blackburn et al., 1994; Cattaneo et al., 1997; Harvey et al., 1989). However,

to the best of our knowledge, there is no evidence of any work investigating some of the above parameters in a single study. Therefore, to better understand the effects of contaminants and soil types on bacterial transport, three soils, i.e. a sandy, a clay and a PCB-contaminated soil were bioaugmented with *R. meliloti* A-025 (Ahmad et al., 1997) and transport of these bacterial cells were compared. In addition, influence of this bacterial bioaugmentation on water infiltration, moisture loss, and hardness of these soils was also investigated.

A3.2 MATERIALS AND METHODS

A3.2.1 Soil Preparations

Three different soils, S-VI, S-VII and S-VIII, were sieved through a 0.05mm mesh and their textures were determined following the hydrometer protocol described by Liu and Evett (1984). They were categorized as sandy loam for soils S-VI and S-VII, and as clay for soil S-VIII. The characteristics and history of these soils are given in Table A3.1.

Table A3.1. Characteristics and history of soils.

Soil	Sand (%)	Silt (%)	Clay (%)	Organic Matter (%)	pH	History
S-VI	78	3	19	3.7	6.17	Macdonald Campus Farm Pristine soil
S-VII	74	8	18	4.8	7.38	Industry Heavy metals; PCB mixtures: Aroclor 1242, 1248, 1254, 1260
S-VIII	22	19	59	4.9	6.68	Macdonald Campus Farm Pristine soil

In the first experimental setup, a silicon (Kwik Seal, DAP Canada Corporation) ring was created inside eighteen syringe columns (60 cc, Becton Dickinson & Co.), at the 40 cc level, to minimize water flow at the column edges. Two layers of cheesecloth were used at the bottom of the columns to prevent loss of soil through drain water. Six columns were packed with 70 g of each soil to the 50 cc level, for a bulk density of 1,380 kg m^{-3}. These soil columns were used for the microbial transport and water infiltration tests. Experiments were done in triplicates for both bioaugmentation and controls. A schematic diagram and photo of soil columns is shown in Figures A3.1 and A3.2.

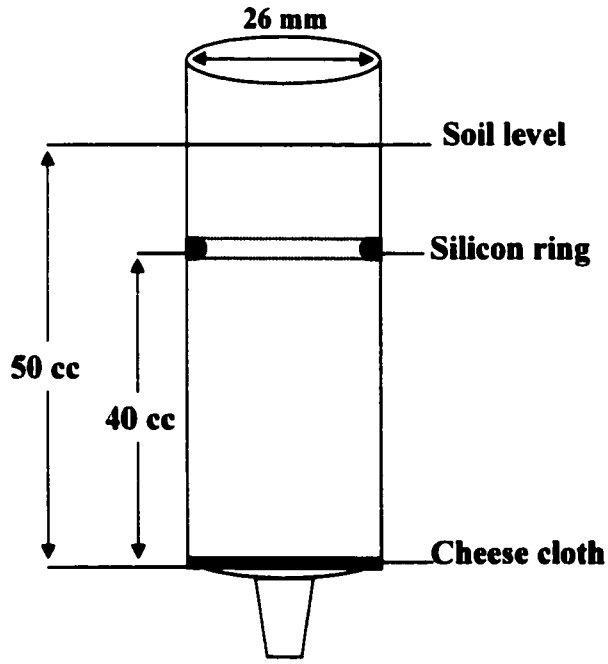

Figure A3.1. Schematic diagram of a soil column used in bacterial transport and water infiltration tests.

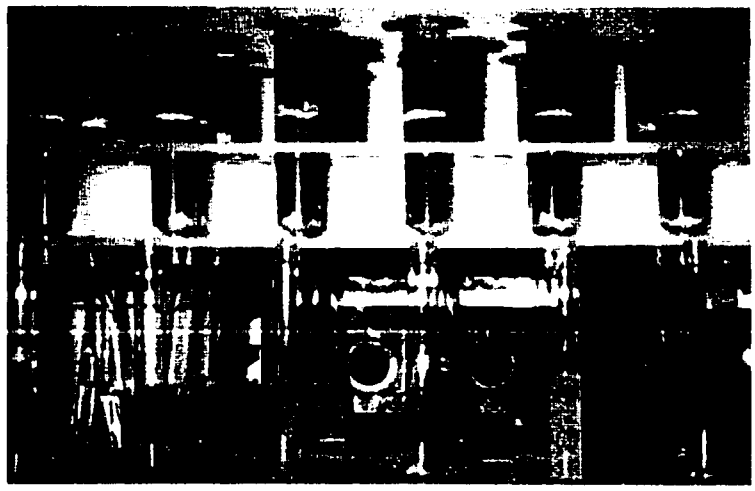

Figure A3.2. A photograph of soil columns used in bacterial transport and water infiltration tests.

In the second experimental setup, eighteen square shape plastic containers (Era Seal, microwaveable, 1020 mL in size, 120 mm in height x 105 mm in width) were used for moisture loss and soil hardness tests. For each soil six containers were packed up to 90mm from the bottom with 1000 g of soil to a bulk density of 1,008 kg m^{-3}. Every container was equiped with 3 metal rods horizontally, at 25, 45 and 65 mm from the bottom, to be used with the Time Domain Reflectrometer (TDR). A schematic diagram of a soil container is shown in Figure A3.3. Every soil was treated in triplicates for both bioaugmentation and controls.

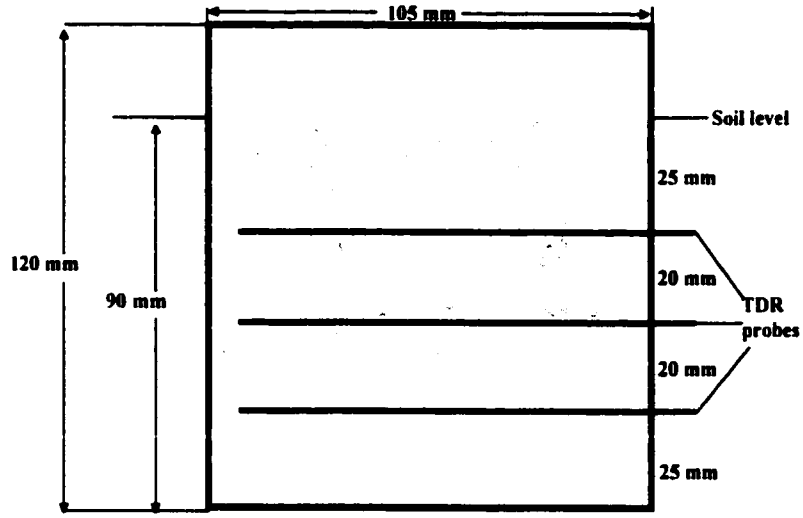

Figure A3.3. Schematic diagram of a soil container used in moisture and hardness tests.

A3.2.2 Bacterial Preparation & Inoculation

Rhizobium meliloti, strain A-025 (Ahmad et al., 1997) was inoculated into 50mL of TYc in six 125mL Erlenmeyer flasks, from a two day old preculture, and incubated at 29°C in a controlled environment incubator shaker (Psycrotherm, New Brunswick Scientific) for 24 h. Each culture was then transferred into 1.5L of fresh TYc medium in 2L flasks and incubated for another two days. All six 1.5L cultures (9L in total) were pooled. Bacterial cells were then harvested by centrifugation (Sorvall Instuments Dupont model RC5C) for 5 min at 8000 rpm in 250 mL centrifuge bottles, and resuspended in 1000 mL sterilized deionized water (SDW) (to about 6.9×10^9 cell mL^{-1}) and mixed thoroughly by vortexing before inoculation of the soils.

In the first experimental setup, soil in each treatment column was bioaugmented with 7mL of cell suspension, poured onto the soil surface and immediately followed by 10mL (SDW) surface irrigation. Non-augmented soil

columns each received 7mL SDW followed by a subsequent surface irrigation with 10mL of SDW.

In the second experimental setup, each soil in containers was inoculated with 100mL of the cell suspension, poured onto the soil surface and mixed well. Immediately after, 50mL of SDW was poured onto the surface. Non-augmented soils each received 100mL of SDW and were mixed well, followed by 50mL of SDW. The soil containers each received 150 and 275 mL of SDW, on the 1^{st} day (as 1^{st} cycle) and 18^{th} day (as 2^{nd} cycle), respectively, after bacterial inoculation.

A3.2.3 Bacterial Transport and Surface Water Infiltration

In the first experiment, each column was surface irrigated with 10mL of SDW immediately as the 1^{st} irrigation and with 15mL of SDW, on 18, 20, and 23 days after bacterial inoculation of the soil columns, as 2^{nd}, 3^{rd}, and 4^{th} irrigations. The time required for water to infiltrate the soil surface was measured as the infiltration period. Two days after every irrigation, drained water was collected and samples from the drain water were used to prepare serial dilutions. These dilutions were plated on TYct agar plates (Ahmad et al., 1997) for bacterial population counts.

A3.2.4 Soil Moisture and Hardness

Periodically, the moisture level of each soil container was measured using a TDR (Tektronix, 1502B metallic cable tester) by the methods described by Topp

and Davis, (1995) and Bonnell et al. (1991). Soil hardness, on the surface, was measured using a Penetrometer (CL-700, SOILTEST Inc).

A3.2.5 Statistical Analysis

Statistical analysis were done using the General Linear Models (GLM) Procedure, Repeated Measures Analysis of Variance, Tests of Hypotheses for between Subjects Effects using the SAS System for windows release 6.12, (SAS Institute, 1989).

A3.3 RESULTS AND DISCUSSIONS

A3.3.1 Microbial Transport

In this section of the present study, we investigated, whether, microbial transport by water could be influenced by soil type and/or presence of the contaminant, PCBs in the soil. As shown in Figure A3.4, soil S-VI, the sandy loam soil, allowed the greatest amount of bacteria (1.23×10^7 cells mL^{-1}) to be transported through the soil columns while the PCB-contaminated soil, S-VII, permitted the least number (4.33×10^6 cells mL^{-1}) of bacterial cells. Even though these two soils are very similar in texture, being mostly sandy (Table A3.1), the PCB-contaminated soil maximized bacterial filtration. These results are an indication that the lipophilicity of PCBs increases the rate of adhesion of bacterial cells in the soil profile. It is evident that the adhesion and filtration of bacterial cells due to the PCB content of soil S-VII is even higher than that of the clay soil, where 7.40×10^6 cells mL^{-1} were leached after the first irrigation.

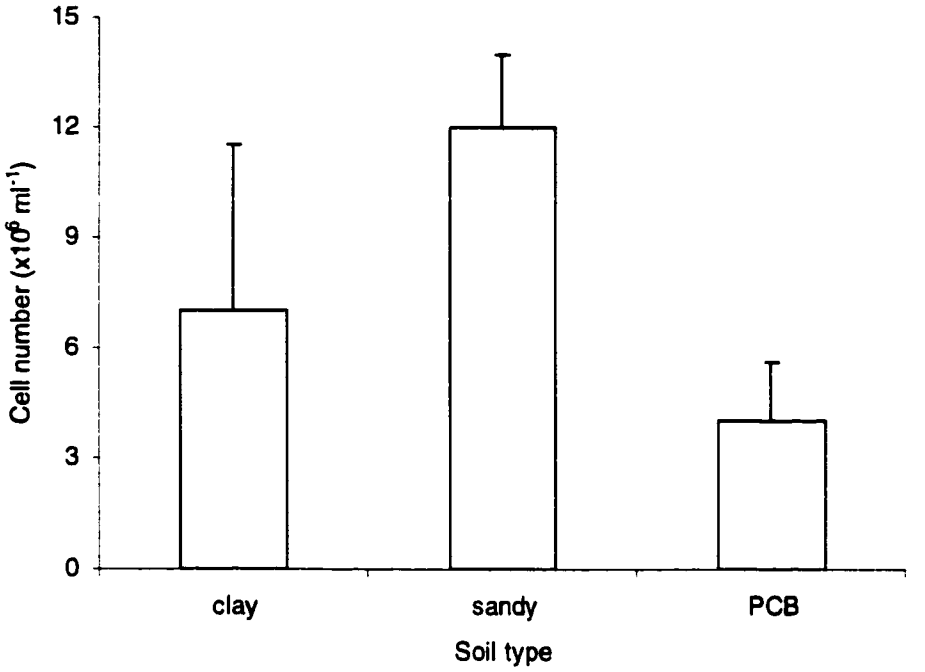

Figure A3.4. Number of *R. meliloti*, A-025, cells leached through different soils after the first irrigation. The results represent three replicates and SD.

The second irrigation, eighteen days after bacterial inoculation, demonstrated that in the PCB soil, more bacterial cells were free to be leached out ($1.16x10^4$ cells mL^{-1}) than observed for the sandy and clay soils with $6.34x10^3$ cells mL^{-1} and $7.34x10^3$ cells mL^{-1} respectively (Figure A3.5). Eighteen days represent the period required for these soils to reach almost total dryness (Figure A3.7), as is presented later. Columns packed with the clay soil would have allowed more than the observed amount of bacteria to leach if the water had passed through the soil. However, shrinkage of the clay soil, due to dryness, made

water flow around the columns very significant. Therefore, water did not pass through the soil, thus maximizing the adhesion/filtration and minimizing the leaching of cells ($7.34x10^3$ cells mL^{-1}). The shorter time period between the 2nd, 3rd, and 4th irrigations decreased the edge effect of water flow, due to higher moisture levels and subsequent swelling of clay particles. Therefore, a greater amount of bacteria ($2.15x10^4$ cells mL^{-1}) leached with the third irrigation than with the second. The sandy and PCB soils showed a decrease in the number of leached bacterial cells with subsequent irrigations (Figure A3.5).

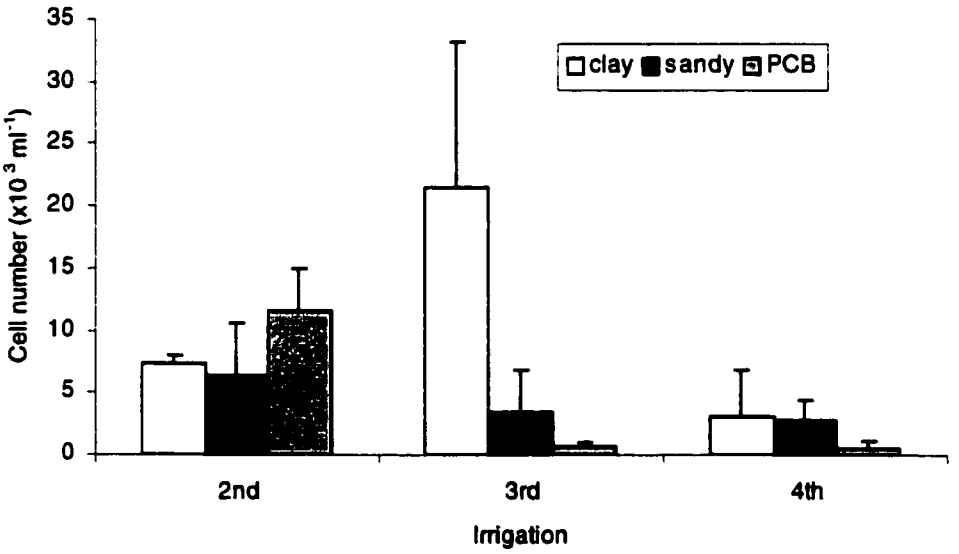

Figure A3.5. Number of *R. meliloti*, A-025, cells leached through different soils after second, third and fourth irrigations on the 18th, 20th and 23rd day. The results represent three replicates and SD.

The total leached bacterial cells for the four irrigation times were found to be $1.23x10^7$ cells mL^{-1}, $4.35x10^6$ cells mL^{-1} and $7.44x10^6$ cells mL^{-1} for soils S-IV

(sandy), S-VII (PCB), and S-VIII (clay), respectively. These patterns of total cells leached were very similar to those observed in the initial irrigation. Therefore, one can estimate the pattern of bacterial transport by water based on results obtained by immediate irrigation after bacterial inoculation. Furthermore, for a higher and more efficient distribution of bacterial cells, the period of the initial irrigation can play a significant role. Because the bacterial cells in the water would be transported by the water to different locations before having the chance to adhere to particles during a dry period. Even though, the influence of clay content and PCB levels present in the soils compared to the soil S-VI (sandy) were significant (Table A3.3) in microbial transport by water, the bacterial cells were leached (transported) through each of the soil types.

Bacterial membrane composition and characteristics change with the growth medium and environment. This, in turn, can influence interactions of bacterial cells with soil particles or other organic material that is present in the soil. Jenkins and Lion (1993) suggest that sorption of hydrophobic compounds to microorganisms is generally a passive process and can be reversible. Camper et al. (1993) observed that adsorption rates were better predictors of microbial transport than individual characteristics, such as size or motility. Gross and Logan (1995) found the number of bacteria retained by porous media was decreased by treatments that made the bacterial cell more hydrophobic and less electrostatically charged. Also, as demonstrated in Chapter 3, type of bacteria or nutrient media can influence the adhesion of bacteria cells to soil or other surfaces present. As the above results show, soil moisture levels play a significant role in bacterial

transport within the soil profile and this is most evident in soil profiles with high clay content. Furthermore, it was observed that bacterial cells were available for leaching or further transport even after a long dry period of 18 days.

A3.3.2 Soil Surface Water Infiltration

In any bioaugmentation attempt, it is important to assess the changes that can occur in a soil profile due to the bacterial augmentation of the soil. One major change could be on the surface water infiltration capacity of the soil. Such a change can ultimately play an important role in the motility and distribution of the bioaugmented cells and the pollutants present in the soil. In this study, water infiltration at the surface of the clay soil showed significant differences, between bioaugmented and non-augmented soil columns (Table A3.3), and also with the other two soils studied (Table A3.2). The first irrigation (10mL) infiltrated the clay soil in a time period of 2580 minutes in the control columns, whereas it took only 864 minutes for the bioaugmented ones (Figure A3.6). As mentioned earlier, due to the edge effect, results from the 2^{nd} to 4^{th} irrigations for the clay soil would not be reflective of a real field situation and therefore are not discussed.

Contrary to the clay soil for the 1^{st} irrigation, inoculation of the sandy soil showed higher infiltration time periods with 20.6, 15.7, 22.4 and 22.1 minutes, as compared to control columns with 3.2, 2.4, 5.9 and 5.6 minutes for the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} irrigations, respectively. Furthermore, inoculated soil columns showed a slight decrease in infiltration time for the 2^{nd} irrigation, as was observed in both augmented and control PCB soil columns.

The PCB contaminated soil with 23.8, 4.3, 8.8 and 8.6 minutes for augmented, and 18.1, 2.3, 6.0 and 4.9 minutes for control columns, for the 1st to 4th irrigations, respectively (Figure A3.6c), showed patterns similar to inoculated sandy soil columns (Figure A3.6b) for surface water infiltration time periods. Inoculated PCB soil columns showed a higher time period for water infiltration than the controls, as observed in the sandy soil columns. However, differences were not statistically significant, once again similar to that obtained for the sandy soil columns. Statistical analysis showed that there was not a significant difference between PCB control columns and sandy soils (Table A3.3), bioaugmented soil columns also did not show a significant difference (α=0.05) in these two soils. Therefore, soil PCB content and bacterial inoculation/bioaugmentation do not play a significant role in surface water infiltration.

A study by Ohu et al., (1985a) showed that increasing the organic matter of a soil would increase the hydraulic conductivity and therefore, decrease the infiltration time period. However, Minkas and Sharma (1986) found a reduction in hydraulic conductivity, related to the salinity of irrigation water such as observed in this study where bioaugmentation of the soils with live and metabolically active cells increased the surface water infiltration time period. This may suggest that metabolic by-products of bioaugmented living cells can play a significant role in the properties of both sandy and clay soils.

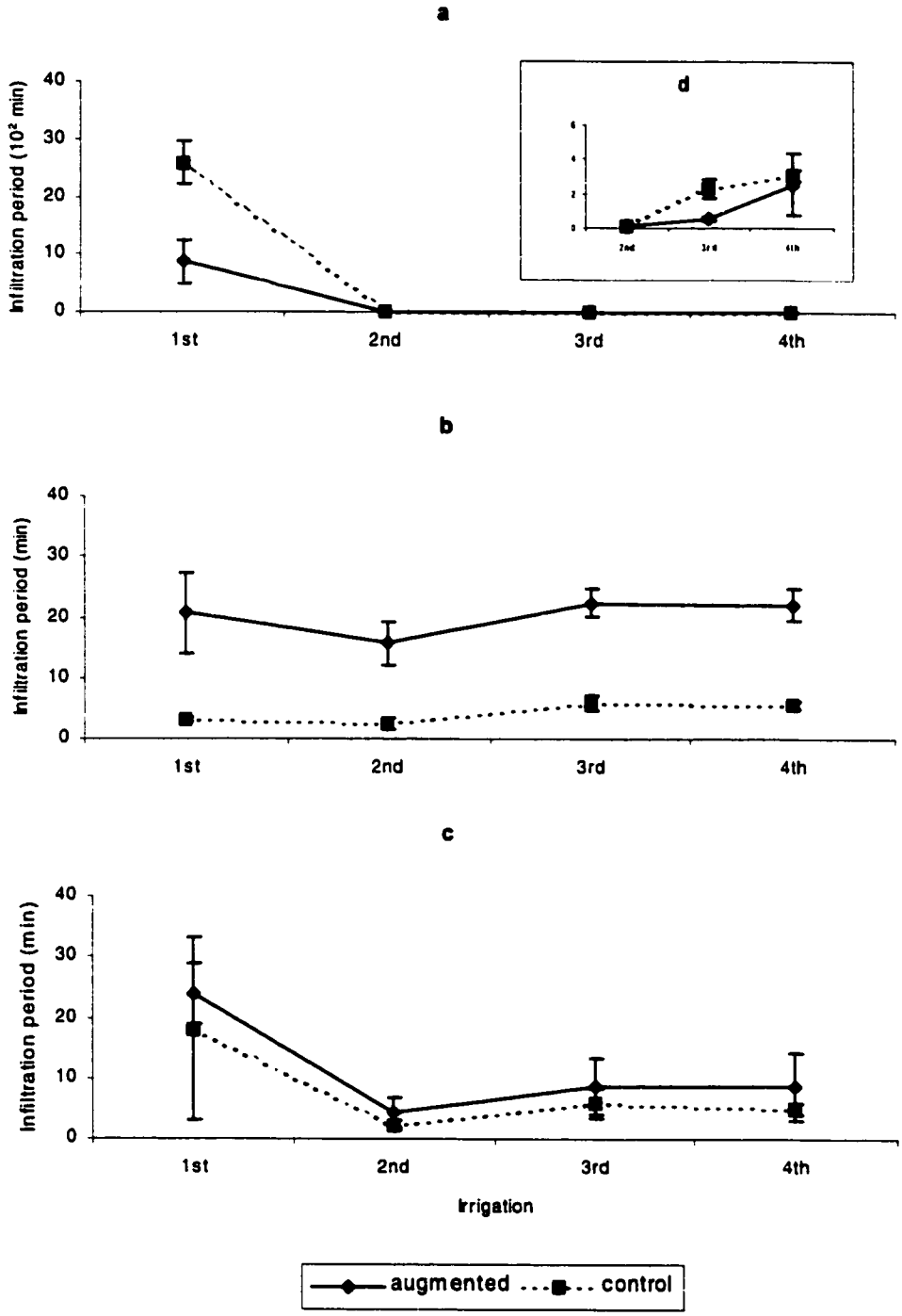

Figure A3.6. Surface water infiltration period at different irrigations for a) clay b) sandy c) PCB soils, d) is smaller scale of the infiltration period for the 2nd to 4th irrigations in the clay soil. The results represent three replicates and SD.

However, interactions of these compounds with soil particles are minimized in presence of contaminants, such as PCBs that are already bound to the soil surfaces.

The general increasing pattern in surface water infiltration time periods after the 2nd irrigation, for both augmented and control soil columns, was in agreement with results observed by Bonnell and Broughton (1993), Frenkel et al. (1978), Harker and Mikalson (1990), and Broughton et al. (1987). They noted that with subsequent irrigations, the hydraulic conductivity of soils would decrease. The likely causative factors were thought to be the migration of small particles and air bubbles to the interface of soil and drain outlets.

A3.3.3 Soil Moisture Loss

Soil moisture level plays a significant role in the transport, survival and activity of microorganisms and is crucial in bioaugmentation and bioremediation of soils contaminated with pollutants such as PCBs. Therefore, it is important to determine how such contaminants or bioaugmentation could influence the moisture content of a soil profile. In this study, the PCB soil showed the greatest difference in moisture loss with augmented treatments, with respect to the controls (Figure A3.7). The augmented soil's moisture remained higher than that of the control treatments all throughout the experiment, especially during the 2nd cycle of testing (after day 18), where the moisture decreases in controls were similar to that obtained for the 1st cycle (before day 18). However, augmented treatments had a slower trend in moisture loss. This effect was reversed with the clay soil, where

augmented treatments maintained slightly less moisture than the controls for every measured time. The sandy soil did not show any significant difference between treatments, although augmented soils showed more moisture at certain points (Figure A3.7).

The difference in moisture contents was more significant between the three soils, rather than their augmented and non-augmented setups (Tables A3.2 and A3.3). In the non-augmented soils (Figure A3.7b), the PCB soil showed highest moisture levels at both water applications, days 1 and 18. However, the moisture levels decreased sharply to reach those levels obtained for the sandy soil by the end of each cycle. The sandy soil maintained moisture levels between those of the clay and PCB soils. These trends were slightly reversed with the augmented treatments (Figure A3.7a). In this case, the PCB soil maintained the highest moisture levels throughout both cycles. The most significant difference was observed during the 1^{st} cycle, between the PCB and sandy soils. However, these two soils showed similar moisture levels during the 2^{nd} cycle. These results indicate that soil type plays a more important role in moisture loss than bioaugmentation. Even though, presence of PCBs tend to increase the water holding capacity of a sandy loam soil after an irrigation/rainfall event, the moisture level could decrease sharply through time. This change can be drastic to the indigenous and more importantly to the bioaugmented organisms that might need longer periods to adapt to changes. The results also indicated that bioaugmentation can slow down the moisture loss in a soil contaminated with PCBs.

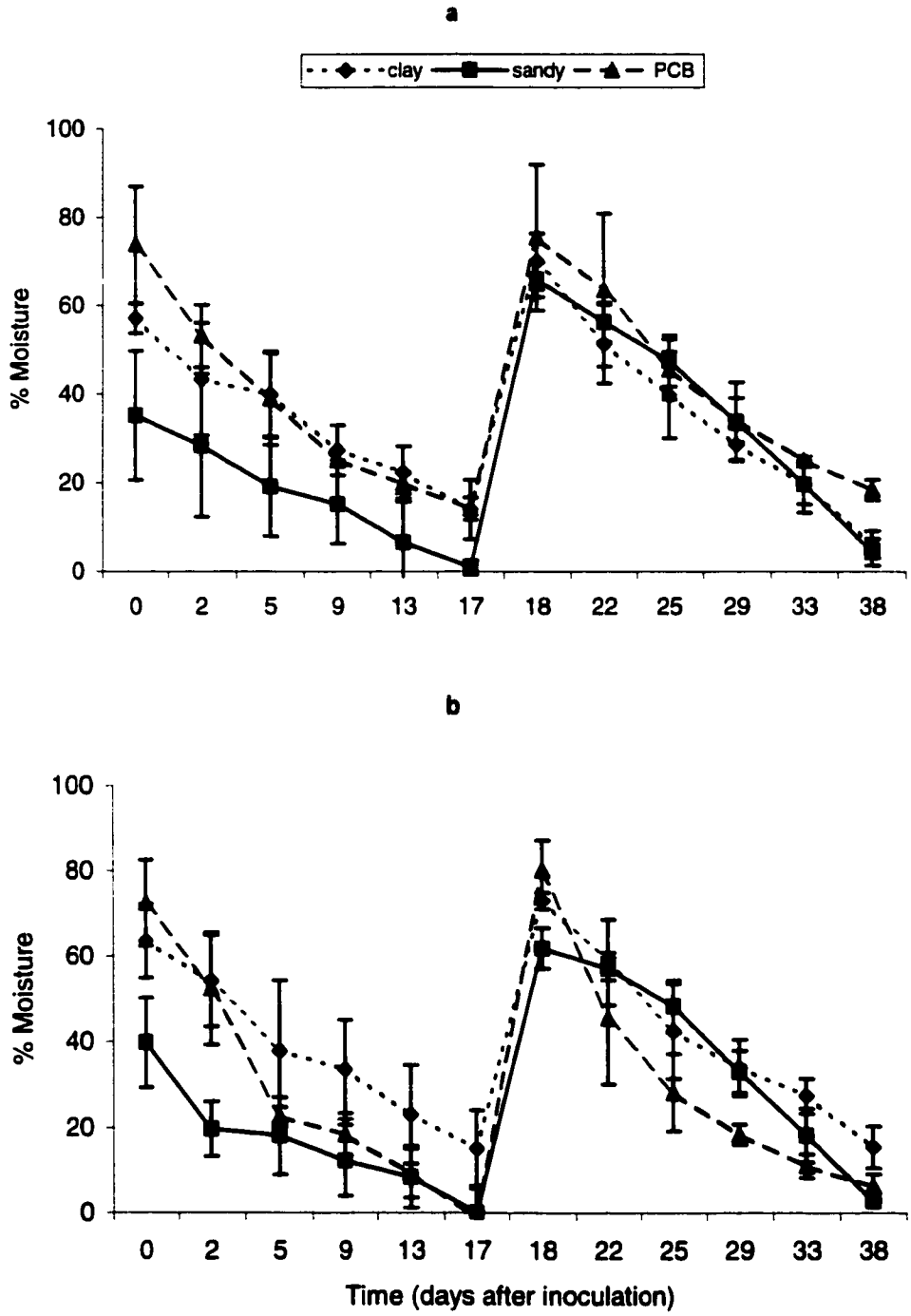

Figure A3.7. Moisture loss in different soils: a) with bioaugmentation and b) without bioaugmentation. Water was added on the 18th day for 2nd cycle. The results represent three replicates and SD.

A3.3.4 Soil Surface Hardness

The soil hardness can play an important role in the survival of microorganisms by affecting the soil moisture (less water infiltration) and air exchange. Also, it can be a significant factor for growth of plants especially when rhizo/phytoremediation is attempted. In this study, the surface hardness of the three soils was measured after the 2nd water application on day 18th. During the period of 11 days (24 to 35 days after inoculation), the augmented clay soil was found to be harder than the non augmented (Figure A3.8), whereas the sandy soil showed an insignificant difference in the results. Augmented sandy soil treatments showed a lower hardness, up to the 35th day, when it became harder than the non-augmented treatments. Hardness was similar for the PCB soils, up to the 28th day, when augmented treatments started to become harder than the controls (Figure A3.8). Soil surface hardness was also observed in the Rhizosphere study (Appendix A2) where soil S-VII was augmented with strain A-025.

Similar to the results obtained from the moisture loss study, the difference in hardness was also more significant between soils, rather than augmented and non-augmented treatments. This difference was mainly due to the clay content of the soil, which became much harder than the other two soils as moisture levels dropped. Ohu et al. (1986) also observed similar results for clay hardness and moisture levels. However, their results did not show much difference between soil types, as found in this study. The sandy soil showed the least hardness, however, hardness levels reached those observed for the PCB soil by the 35th day. The PCB

soils reached a plateau for hardness on the 30th day (6 days after 2nd water application) and then this hardness was maintained for the remainder of time.

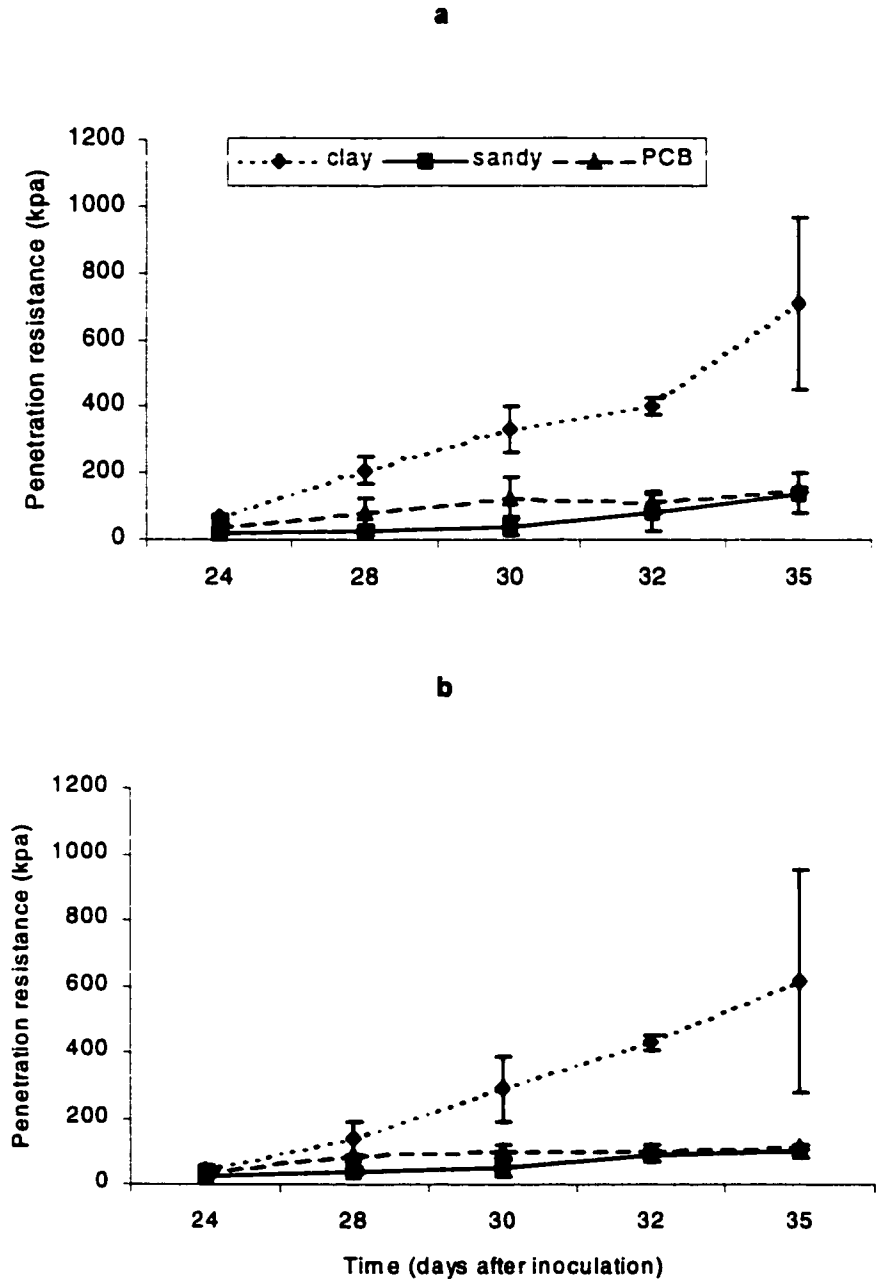

Figure A3.8. Penetration resistance of different soils: a) with bioaugmentation b) without bioaugmentation.

Table A3.2. Univariate procedure of repeated measures ANOVA for different parameters tested (α=0.05).

Source	Pr > F			
	Bacterial Transport	Infiltration	Hardness	Moisture
Treatment	0.0454	0.0001	0.0001	0.0483
Time	0.0001	0.0001	0.0001	0.0001
Time*Treatment	0.0024	0.0001	0.0001	0.0001

Table A3.3 Multiple pairwise comparisons of overall mean in time among treatments for bacterial transport, soil surface infiltration, soil surface hardness and soil moisture loss.

Treatment	Pr > F			
	Bacterial Transport	Infiltration	Hardness	Moisture
Clay+ vs Clay		0.0001	0.0322	0.3253
Clay+ vs Sand+	0.0922	0.0007	0.0001	0.1605
Clay+ vs PCB+	0.2548	0.0005	0.0001	0.2646
Clay vs Sand	-	0.0001	0.0001	0.0175
Clay vs PCB	-	0.0001	0.0004	0.0759
Sand+ vs Sand	-	0.7189	0.9703	0.8202
Sand+ vs PCB+	0.0172	0.8415	0.2822	0.0206
Sand vs PCB	-	0.9349	0.4278	0.4329
PCB+ vs PCB	-	0.9369	0.7373	0.0589

+- indicates the bioaugmented treatment.

This was more evident for PCB controls than augmented PCB soils. These informations will be very helpful when phytoremediation systems are being considered as discussed in Appendix A2. Ohu et al. (1985b) have shown that soil hardness through soil compaction can decrease crop yield up to 50%, in some cases.

In conclusion, transport of the *R. meliloti* strain A-025 was influenced more by the presence of PCBs than by the soil's clay matter. In contrast, infiltration of surface water was influenced more by clay content than by the soil PCB content. Furthermore, the least difference in surface water infiltration between augmented and non-augmented soil columns was observed in the PCB soil. Also, presence of PCBs influenced the infiltration time period when bioaugmented with bacterial cells. Results indicate that clay content and PCB levels in soil affect bacterial transport and infiltration. Frequency and irrigation periods were found to be important factors when soils were microbially augmented with water. In general, the pattern of moisture loss was different between soils and the largest difference between augmented and control soils was observed with the PCB soil. Soil hardness showed common patterns between soils, with augmented treatments becoming harder at the end of the experiment for all three soils and the largest difference between soils was the clay soil. These results demonstrate the influence of contaminants and bioaugmentation on different soil types. Hopefully, these findings will be of assistance when bioremediation systems (bioaugmentation, phytoremediation) are considered for clean up of contaminated soils/sites.

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PREFACE TO APPENDIX A4

Up to this point, it has been demonstrated that water table management system can be used to bioaugment contaminated soils and bioaugmentation with *R. meliloti* has shown to be effective in PCB transformation through dechlorination of congeners. In addition to these novel findings, *R. meliloti* is known to be a denitrifier under anoxic condition. A major source of pollutants in the environment is the extensive use of herbicides and nitrogen fertilizers in agriculture. Some of the ecotoxicological impacts of the herbicides such as atrazine have been attributed to their chlorinated chemical structures, and for fertilizers, the high leachability of nitrate into the aquatic systems. Therefore, a dechlorination mechanism that could also limit the leachability of fertilizers, could minimize the environmental impact of such pollutants.

Because, *R. meliloti* has shown to dechlorinate PCBs and is a known denitrifier, we investigated the possibility of using this organism as a biofilter to control the leaching of atrazine and nitrate in a soil column study using water table management to bioaugment the soil columns. Appendix A4 describes the experimental methods and the results of this study where strain A-025 was bioaugmented in sterilized soils in a soil column study using the subirrigation method discussed in Chapters 4 and 6.

The results will be submitted for publication as: Mehmannavaz, R., Prasher, S.O., Ahmad, D. and Markarian, N. Biofiltration of Atrazine and Nitrate by *Rhizobium* using a Water Table Management System in Sterile Soil Columns. Markarian, N. played a role in setting up the experiment and also in the extraction of atrazine from the drain waters collected in this study.

APPENDIX A4

BIOFILTRATION OF ATRAZINE AND NITRATE BY *RHIZOBIUM meliloti* A-025 USING A WATER TABLE MANAGEMENT SYSTEM

ABSTRACT

In this work, we have investigated bioaugmentation using a water table management system as a biofiltration/biocontrol technology for two agricultural pollutants, atrazine and nitrate. Plexy glass columns, 458 mm long x 139 mm diameter, were packed with a sterilized sandy loam soil. The columns were bioaugmented with *R. meliloti* strain A-025 using sub surface irrigation and to maintain aerobic and anaerobic periods. Three different treatments were setup where atrazine and nitrate were applied on the surface of the soil and the concentration of these two chemicals were analyzed in the drain water after rain simulations during 80 days of saturated and 70 days of unsaturated periods. Soil samples were also analyzed for the residues remaining in the soil. The delivery and implantation of strain A-025 using sub irrigation was determined by isolating strain A-025 from the surface soil and its identity was confirmed through root nodulation and random primer generated DNA fragment amplification pattern with PCR. The results indicated that the bioaugmented soil columns had >40% lower concentrations of atrazine in the drainage water and >30% lower in total atrazine concentration in the drainage water and the soil, as compared to the

abiotic control treatment. The nitrate-N was detected to be significantly lower in bioaugmented treatments during the saturated period with 81% and 86% lower nitrate concentrations in the presence and absence of atrazine, respectively. However, the total nitrate-N in the drain water and the soil was higher in the bioaugmented treatments when atrazine was present with nitrate. The overall results indicate that water table management system can be used for bioaugmentation of agricultural soils and *R. meliloti* strain A-025 could be a candidate for biofiltration of atrazine and nitrate.

A4.1 INTRODUCTION

Agricultural chemicals play a significant role in food, as well as feed production and protection. It is estimated that corn yields in the second half of the 20^{th} century quadrupled due to the use of fertilizers and pesticides (McRae, 1989). However, agricultural sources of environmental pollutants, such as pesticides and fertilizers, account for almost half of the earth's land (Pimental, 1993).

One of the most commonly used herbicide is atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine). It has been used as a selective herbicide on agricultural crops such as corn, sugar cane, pineapple and fruit trees (Radosevich, et al., 1995). Its ecotoxicological impact has been well documented (Solomon et al., 1996; Baturo et al., 1995; Crawford et al., 1998). Various processes, including hydrolysis, adsorption, volatilization and photodegradation, govern the fate of atrazine in the environment. However, the primary dissipation of atrazine is known to be through biological degradation at neutral pH and by

chemical processes in acidic soils (Blumhorst and Weber, 1994), therefore, the soil's chemical constituents play a significant role (Fadullon et al., 1998). Clay, organic matter, temperature, and pH are important in the adsorption phenomena as well. Atrazine adsorption increases as clay content or organic matter content of the soil increases whereas increasing temperature, soil water content and pH reverses atrazine adsorption (Harris and warren 1964). Burkhard and Guth (1981) reported that the rate of atrazine degradation by hydrolysis increases as adsorption rate increases. Wenk et al., (1998) showed that the rate of atrazine removal is proportional to water content.

Different metabolic pathways by various microorganisms (*Klebsiella*, *Pseudomonas*, *Rhizobia*) under diverse conditions, have been suggested for degradation of atrazine (De Souza et al., 1998; Crawford et al., 1998; Hapeman et al., 1995). Dealkylation, deisopropylation and dehalogenation of atrazine appear to be the major mechanisms in the microbial biodegradation pathways reported (Radosevich, et al., 1995; Behki and Khan, 1986). Various bacterial strains have been reported to use atrazine as a source of carbon (Jessee et al., 1983; Crawford et al., 1998) or/and N source (Cook and Hutter, 1981,1984; Mandelbaum et al., 1993a,b; Hapeman et al., 1995; Crawford et al., 1998).

Among agricultural fertilizers, nitrate is used very extensively and it is estimated that 20-60% of the fertilizer applied by farmers is lost through runoff, leaching and denitrification (Liaghat and Prasher, 1996), and that 30 to 60% of the nitrogen fertilizer, applied in Quebec, is leached out to waterways and ground water after rainfall and surface runoff (Miller and Mackenzie, 1978). With

improved drainage, such as water table management systems (subirrigation), nitrate-N losses can decrease substantially from 34 to <20 kg/ha/yr (Skaggs et al., 1994).

In subirrigation where water is pumped into the field, subsurface drains are used to control drainage and subsequently, a water table system is created. The effects of water table management on agricultural chemicals have been well documented (Evans et al., 1995; Madramootoo et al., 1993; Kalita and Kanwar, 1993; Munster et al., 1996). It has been suggested that controlling drainage, increases the exposure time of chemicals to degrading organisms and prolongs chemical leaching periods. However, to the best of our knowledge, bioaugmentation has not been used in previous studies.

In the last decade, microbial inoculations of soils, for pest control and fertilizers, have attracted significant attention (Selenska-Pobell, 1994; Thies et al., 1991). Rhizobia are used as inocula in many different countries for agricultural purposes since these symbiots, in the form of bacteroids, fix N_2 to the roots of leguminous plants, such as bean, clover or alfalfa. The population of free living Rhizobia is not uniform throughout the soil profile and its density is highest in the legume rhizosphere, where their population is usually about 10 to 200 times greater, and in exceptional cases 10,000 times higher than in the surrounding soil. Rhizobial densities in soils are usually below 10^6 cells g^{-1} dry soil (Garcia-Plazaola et al., 1993).

Besides the ability to fix N_2, Rhizobia are also denitrifiers as they are capable of utilizing nitrate respiration to support anaerobic growth (Zablotowicz

and Focht, 1979; Garcia-Plazaola et al., 1993). Garcia-Plazaola et al. (1993) have suggested that free-living Rhizobium have the potential to remove fixed nitrogen from soil through denitrification. Oxygen, nitrate, temperature, moisture and labile organic matter availability are the main factors controlling denitrification by Rhizobium. Even though denitrification activities by some microorganisms in different soils have been detected at temperatures as low as 0 - 10 ^{o}C, denitrification by Rhizobium is enhanced by temperatures from 15 to 25 ^{o}C and by high water-filled pore space, which helps to regulate oxygen (Garcia-Plazaola et al., 1993). These anaerobic conditions are easily achieved in flooded soils after rainfall or irrigation.

To date the only evidence of partial transformation of atrazine by a *Rhizobium* sp. (strain PATR) in liquid cultures, under aerobic conditions, has been documented by Bouquard et al. (1997). It was suggested that atrazine was transformed to hydroxyatrazine as the only metabolite through a dechlorination pathway. The objective of this study was to use water table management for bioaugmentation and to evaluate a strain of *Rhizobium meliloti*, A-025, isolated from a contaminated soil (Ahmad et al., 1997), as a biofiltration/biocontrol agent for atrazine and nitrate.

A4.2 MATERIALS AND METHODS

A4.2.1 Soil Column Design and Setup

Nine plexy glass columns, 458 mm long x 139 mm internal diameter, were packed with a sandy loam soil, S-VI (78% sand, 3% silt, 19% clay, 3.7% organic

matter, pH 6.17), excavated from the Macdonald Campus Farm of McGill University. The columns had a sampling port on the side (298 mm from the top) and were equipped with a delivery port at the bottom to supply water and bacterial inoculum into the columns. A schematic diagram of the setup is shown in Figure A4.1.

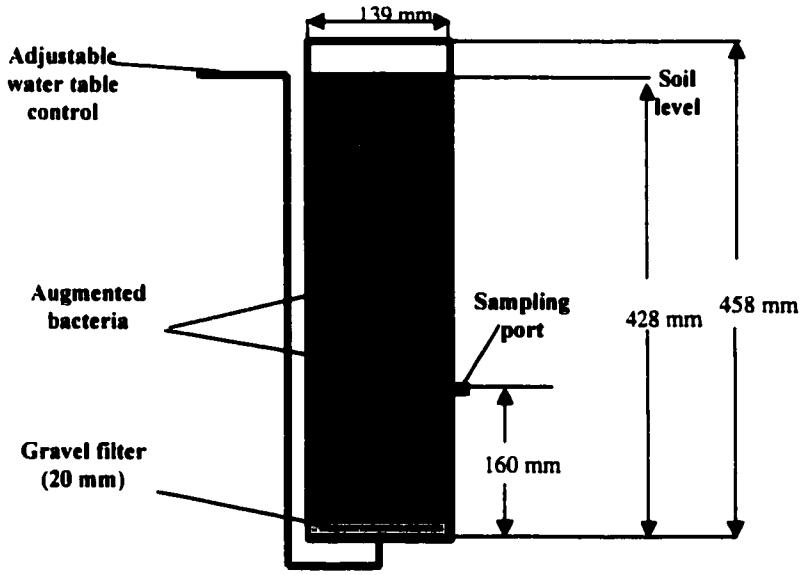

Figure A4.1. Schematic diagram of a soil column.

The soil was well mixed and autoclaved three times for one hour at 121°C every two consecutive days, before packing the columns. A 20 mm sterilized gravel filter (size between 9.5 and 2.36 mm) was packed at the bottom of the columns. The soil was then packed on top of the filter with 1.973 kg of soil every 100 mm, for a total of 8.443 kg of soil and a bulk density of 1300 kg/m^3. Cheesecloths were placed on top of the soil to minimize surface erosion during rain simulations. Pipes and tubes were sterilized by first washing in 6.0% Sodium hypochlorite (household bleach) and then rinsing with the same tap water that was used in the experiment for irrigation.

A4.2.2 Preparation of Bacterial Inoculum

R. meliloti strain A-025 (Ahmad et al., 1997) was inoculated in 5 mL of TYc as a starter culture and incubated at 29 °C on a horizontal shaker. Four-day-old cultures were sub-cultured into six 50 mL of fresh TYc in 125 mL flasks, followed by reinoculation in 1 L of fresh TYc in six 2 L flasks after 24 hours. The flasks were incubated at 29 °C in a controlled environment incubator shaker (Psycrotherm, New Brunswick Scientific) for 24 hours. The bacterial cells from the 1L cultures (approximately $3.3x10^8$ cell mL^{-1}) were then collected by centrifugation (Sorvall Instuments Dupont model RC5C) for 5 minutes at 8000 rpm in 250mL centrifuge bottles. Cultures were pooled before centrifugation and plated on TYct agar plates for microbial population counts. Cells collected were washed with 100mL of 0.9% saline and resuspended in 200mL of sterilized

deionized water and mixed well by vortexing the inoculum before application to soil columns.

A4.2.3 Experimental Design

Three treatments with three replicates were setup with treatment 3 representing the abiotic control. The treatments were as follows:

1) NA bacterial inoculation, atrazine, nitrate,

2) N bacterial inoculation, nitrate,

3) Control no bacterial inoculation, atrazine, nitrate.

The experiment was carried out in the laboratory at room temperature.

All soil columns first received 300 mL of tap water from the bottom (subirrigation), followed by inoculation of the 200mL of cell suspensions for columns assigned to bacterial inoculation, i.e. treatments 1 and 2. The columns were then saturated from the bottom by adding 2400 and 2600 mL of water to the treatment and the abiotic control columns, respectively.

Eight days after bacterial augmentation of the soil columns, 300 µL of atrazine (1000 ppm stock solution, 90% active), and 300 mg of Calcium Nitrate 4-hydrate, representing approximately 180 Kg hectare^{-1} of fertilizer, were uniformly applied to the soil surface. During the first 44 days after atrazine and nitrate inoculation, 20 mm (450 mL) of water was applied as rain simulations, on the 9th and every 7 days thereafter, to all columns for a total application of 120 mm of rainfall. This was equivalent to a rainfall once every 25-years in the month of May in Montreal, Canada. In order to observe the effect of a heavy rainfall, on the 80th

day after chemical inoculation, the equivalent of 60 mm of rainfall (1,350 mL), such as that which occurred in Montreal on July 14th, 1987, was applied. On the 87th day, the columns were drained and three more rain simulations of 40 mm were applied on the 104th, 112th and 150th day. Stages of experimental setup and, amount and time of water applications are shown in Tables A4.1 and A4.2.

Table A4.1. Experimental stages.

Stage	Application	Treatment		
		1	2	3
1	Soil packing (kg)	8.443	8.443	8.443
2	Subirrigation (mL)	300	300	300
3	Sub surface Bacterial inoculation (mL)	200	200	0.0
4	Subirrigation (mL)	2400	2400	2600
5	Surface application: Atrazine (mL of 1000ppm)	0.3	0.0	0.3
	Nitrate (mg)	300	300	300
6	Rain simulations	See Table A4.2		

Table A4.2. Schedule simulated rainfall events.

Day	Water Applied (mL)	Rainfall (mm)	Soil Condition
9	450	20	Saturated
16	450	20	Saturated
23	450	20	Saturated
30	450	20	Saturated
37	450	20	Saturated
44	450	20	Saturated
80	1350	60	Saturated
104	900	40	Unsaturated
112	900	40	Unsaturated
150	900	40	Unsaturated

A4.2.4 Sample Collection and Analysis

Water samples were collected at the bottom of the columns after every water application (simulated rainfall) for analysis of leached atrazine, nitrate, and microbial count. Soil samples (20 g) were collected through sampling ports on the side of the column before each water application during the unsaturated period to analyze atrazine and nitrate residues. Nitrate was measured by the Soil Testing Laboratory of Natural Resources Science Department of Macdonald Campus of McGill University, using a Quikchem Automated ion Analyser.

Atrazine analysis was performed as described by Liaghat et al. (1996), Liaghat and Prasher (1996), and Masse et al. (1994). Water samples were extracted by mixing 200mL of the sample with 50mL of methylene chloride in a separatory funnel. The mixture was hand shaken for 5 min and the organic layer was collected. The process was repeated three times and extracts were pooled and evaporated to dryness. Residues were dissolved in 10mL of hexane and analyzed by gas chromatography (GC). Soil samples were extracted by shaking 10 g of soil in 100 mL of methanol for 60 minutes and then filtered under suction. The filtrate was then evaporated to dryness in a rotary evaporator at 35 °C. Residues were dissolved in 10mL of hexane and analyzed with a GC. The extraction efficiency of samples was estimated to be 88% ± 5% (Liaghat and Prasher, 1996).

The GC was a varian, model 3400, equipped with a TSD detector, an autosampler, and an integrator. The column was a 0.53mm i.d. fused silica Megabore DB-5. The detector and injector were kept at 290 °C and 190 °C, respectively; the column was maintained at 150 °C for 10 min and then the

temperature was raised to 180 °C at a rate of 2.5 °C min^{-1}; the helium carrier gas flow was 15mL min^{-1}.

A4.2.5 Bacterial Population Count

Serial dilutions of the drain water, up to 10^{-4}, were spread on TYct agar plates (restrictive media for *R. melilcti*) and incubated at 29°C. The plates were incubated for a period of 15 days and the number of colonies determined periodically. The plates from the control treatment showed no growth of bacterial colonies.

A4.2.6 Reisolation and DNA Manipulation of Strain A-025

Soil samples from the surface of the soil columns were taken at the end of the experiment. 2 g of soil was vortexed in 10 mL of sterilized 0.9% saline and the suspensions were spread on TYct agar plates. Two colonies from separate plates were streaked on TYct for further purification and they were name coded as Atz1-25 and Atz2-25. These two strains were then grown in TYc liquid media for root nodulation test and DNA preparation.

To obtain randomly amplified polymorphic DNA (RAPD) profile by PCR, the protocol described by Harrison et al. (1992), Lawrence et al. (1993), and Ahmad et al. (1997) were employed. A single oligonucleotide, A1-08, was selected from our laboratory collection of oligonucleotides as the primer in PCR. The PCR was performed in a DNA thermocycler (Perkin-Elmer, Cetus) using DNA *Taq* polymerase (Pharmacia) in a reaction volume of 50 µL. The PCR

program used had the following parameters: one cycle of denaturing at 94°C for 5 min, annealing at 36 °C for 5 min, synthesis at 72 °C for 2 min, 40 cycles of 44 s at 94 °C, 2 min at 36 °C, 2 min at 72 °C and a final extension period of 5 min at 72 °C. Fifteen μL of amplified product was resolved by electrophoresis on a 1.6% agarose gel in TBE (Tris-borate EDTA) buffer at 100 V for 5 min followed by 50 V for 2 h, stained with ethidium bromide for 30 min, destained overnight in water, visualized under a UV transilluminator and photographed.

A4.2.7 Statistical Analysis

Statistical analysis for atrazine and nitrate loss were done using the General Linear Models (GLM) Procedure, Repeated Measures Analysis of Variance, Tests of Hypotheses for between Subjects Effects using the SAS System for windows release 6.12, (SAS Institute, 1989).

A4.3 RESULTS AND DISCUSSIONS

A4.3.1 Microbial Bioaugmentation

The delivery and implantation of the bacterial cells for strain A-025 were based on the bioaugmentation system, using subirrigation, described in Chapters 4 and 6. The presence of strain A-025 was determined by using PCR (RAPD) for two different colonies that were isolated from the soil at the surface of the columns, at the end of the experiment (150 days). The DNA profile of these two isolated strains, were compared to the original A-025 and the results indicated similar DNA pattern for all three strains (Figure A4.2). Furthermore, the two

colonies isolated from the soils were tested for nodulation (as described in Chapter 3) and both formed nodules on alfalfa roots. These results confirmed that the bacterial cells were transported and implanted at the soil surface, using the subirrigation method. Based on the results of Chapter 4, it was assumed approximately 20% of the inoculated cells would be transported to the surface of the soil using subirrigation.

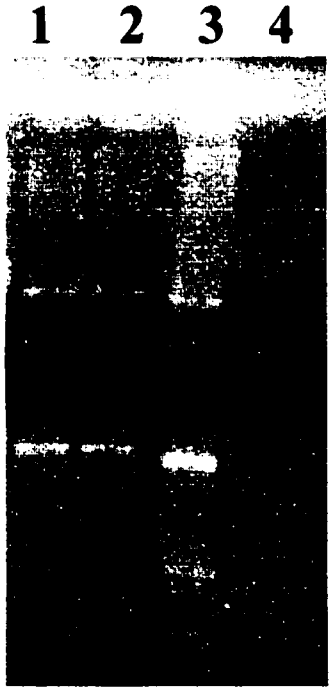

Figure A4.2. RAPD profiles produced using randomly selected primer, A1-08. Lanes: 1) Atz1-25, 2) Atz2-25, 3) A-025, and 4) negative control.

The microbial population in the drain water was determined on TYct agar plates (Figure A4.3). The results indicated that more cells were leached during the saturation period in treatment 1 (atrazine and nitrate) than that of treatment 2 (nitrate), albeit, the declining slop in treatment 1 was higher than in treatment 2. On the 104th day the number of cells in treatment 1 were even lower than that observed in treatment 2. However, there was a large increase in the population in treatment 1 on the 112th day (unsaturated, aerobic). The overall cells in the drain water were observed to be higher in treatment 1 than in treatment 2.

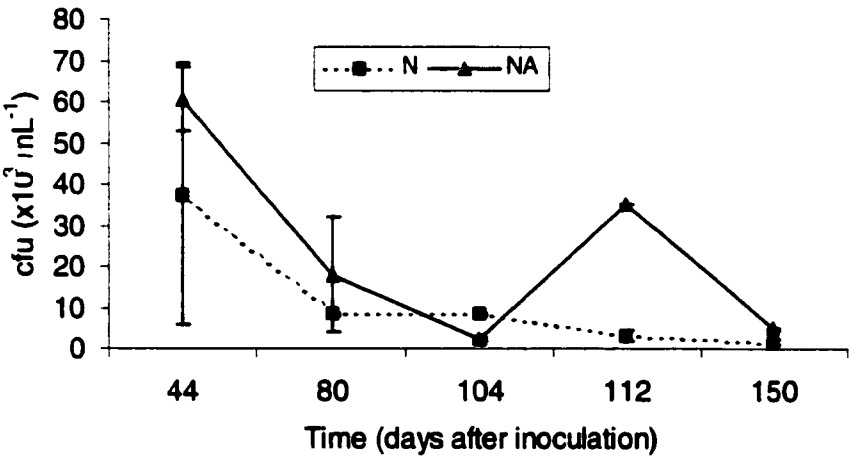

Figure A4.3. Leached population of bacterial cells, strain A-025, in the drain water during the experiment.

A4.3.2 Biofiltration of Applied Atrazine

During the saturation period of 87 days (anaerobic conditions), the atrazine concentration in drained water of the bioaugmented soil columns was shown to be lower than those found in the abiotic control columns, except on the 37th day after chemical inoculation of the columns (Figure A4.4a). The total concentrations of 7.3 ppb for the control and 5.9 ppb for the bioaugmented soil columns were observed for the 1st to 7th rain simulations, respectively. Even though, the most common atrazine degradation pathway in anaerobic conditions is nitrate respiration where nitrogen components of atrazine are used as electron acceptors (Radosevich et al., 1995; Jessee et al., 1983), also, atrazine can serve as a sole carbon source in anaerobic conditions (Crawford et al., 1998). In this study, soil columns inoculated with atrazine were also inoculated with Calcium Nitrate 4-hydrate that could also have been used for respiration. Therefore, in addition to possible biotransformation, it is conceivable that a reduction in the leaching of atrazine in bioaugmented soil columns might also have been due to adsorption of atrazine to bacterial membranes. Changes in the composition of bacterial membranes due to adhesion and saturation of the fatty acids by atrazine, organic solvents and aromatic compounds have been documented (Laura et al., 1996).

Following this saturation period, during an unsaturated period of 63 days (aerobic conditions), differences in the concentrations of leached atrazine were more significant than those observed during the saturated period. Leached total concentrations of 10.7 ppb for the control and 4.2 ppb for the bioaugmented soil columns were observed. The biggest difference was on the 112th day after atrazine

application (Figure A4.4b). During this period, lower concentrations of atrazine were also observed in the soil samples (Figure A4.4c) and the population showed a large increase (Figure A4.3). The total concentrations of atrazine were 24.5 ppb for the control and 18.6 ppb in the soil samples of the bioaugmented soil columns during the aerobic period. These results suggest that *R. meliloti*, strain A-025, could be a possible degrader of atrazine, similar to the results observed by Bouquard et al. (1997). Statistically, the differences between bioaugmented and control soil columns were significant, with 98% and 86% for atrazine concentrations in water and soil samples, respectively (Table A4.3).

In this study, the loss of atrazine could have been different in the bioaugmented soil columns if nitrate was not added or if the full experimental period had been aerobic. Wilber and Parkin (1995) have shown lesser transformation of atrazine in the presence of nitrate, and Topp et al. (1995) reported that atrazine was not degraded under anaerobic or denitrifying conditions. The dealkylation of atrazine is known to be the most frequent reaction in atrazine degradation (Behki and Khan, 1986; Mandelbaum et al., 1993b). Radosevich et al. (1995) observed dealkylation, dechlorination, and deamination in addition to ring cleavage by a strain that was isolated from agricultural soils. However, dechlorination was found to be the major atrazine degrading reaction with rhizobium strain PTAR (Bouquard et al., 1997). Radosevich et al. (1995) also observed that both C and N of atrazine were used by a single strain of bacteria.

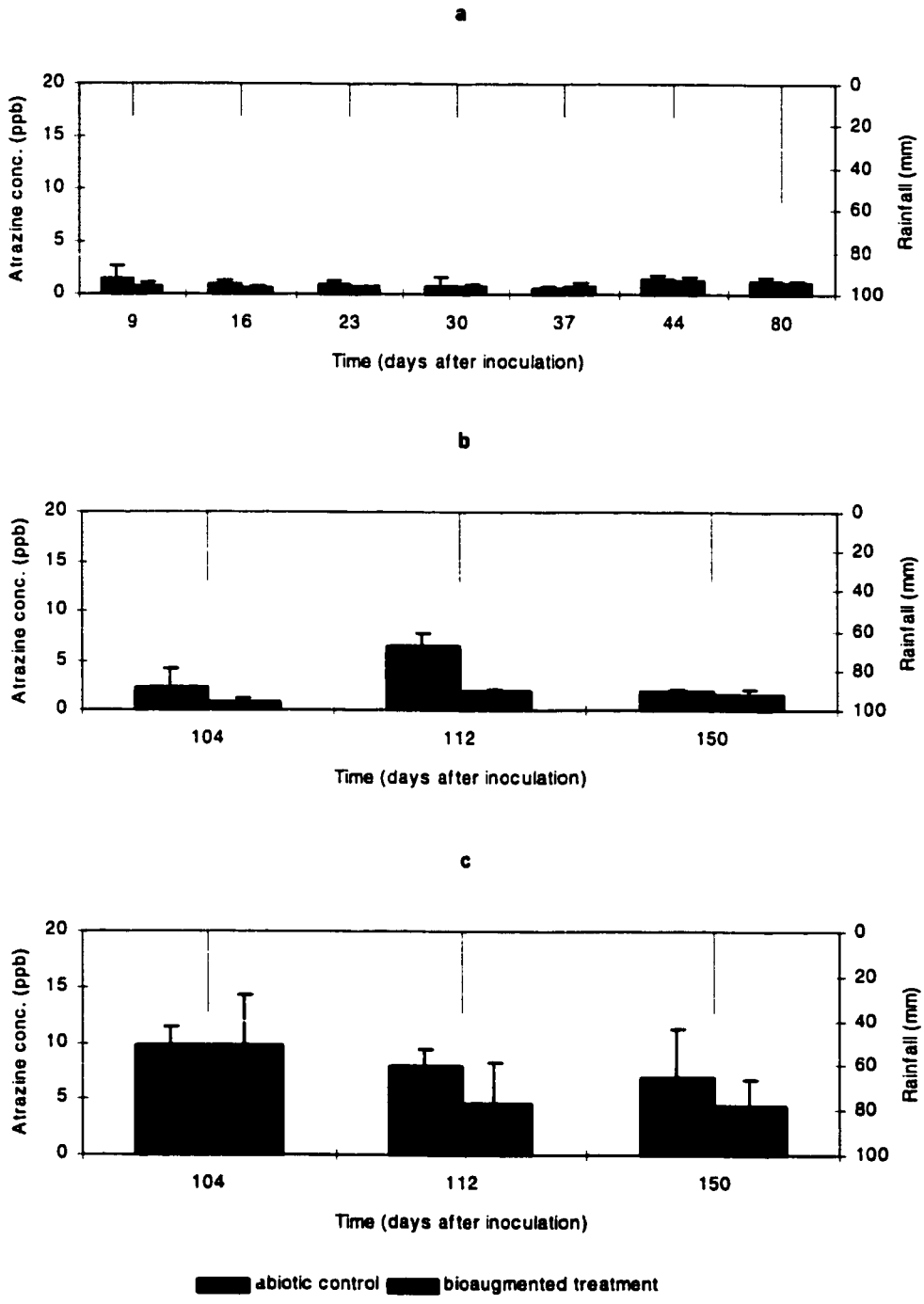

Figure A4.4. Concentration of atrazine leaching in the drain water of a) saturated period, b) unsaturated period and c) in the unsaturated soil following the saturated period. Results represent the mean and the SD of three replicates.

A4.3.3 Biofiltration of Applied Nitrate

During the saturated period, with both bioaugmented treatments (1 and 2), lower concentration of nitrate-N leached than the abiotic control (Figure A4.5a). This difference was statistically significant at 85% level (Table A4.3). The total leached nitrate concentrations were 17.95 ppm for the control columns (treatment 3), 4.03 ppm for the atrazine-nitrate soil columns (treatment 1), and 2.31 ppm for the nitrate soil columns (treatment 2), after the 1^{st} to 7^{th} rain simulations, respectively (Table A4.4). These results suggest that anaerobic conditions during the saturated period may have initiated denitrification. The fact that the total loss of nitrate-N in treatment 2 (absence of atrazine) was higher than in treatment 1 (presence of atrazine) may suggest that atrazine served as a nitrogen source.

Concentrations of leached nitrate during the unsaturated period (aerobic conditions) were contrary to the above (Figure A4.5b). Total nitrate-N concentrations of 85.49 ppm for the control, 109.43 ppm for the atrazine-nitrate soil columns, and 98.39 ppm for the nitrate soil columns were observed for rain simulations on days 104, 112 and 150 after chemical application, respectively. The results of soil analysis for the three sampled times, 104, 112 and 150 days after chemical application, showed that the control soil columns had the least total concentrations of nitrate-N with 1.66 ppm, and treatments 1 and 2 with 2.6 and 2.26 ppm respectively.

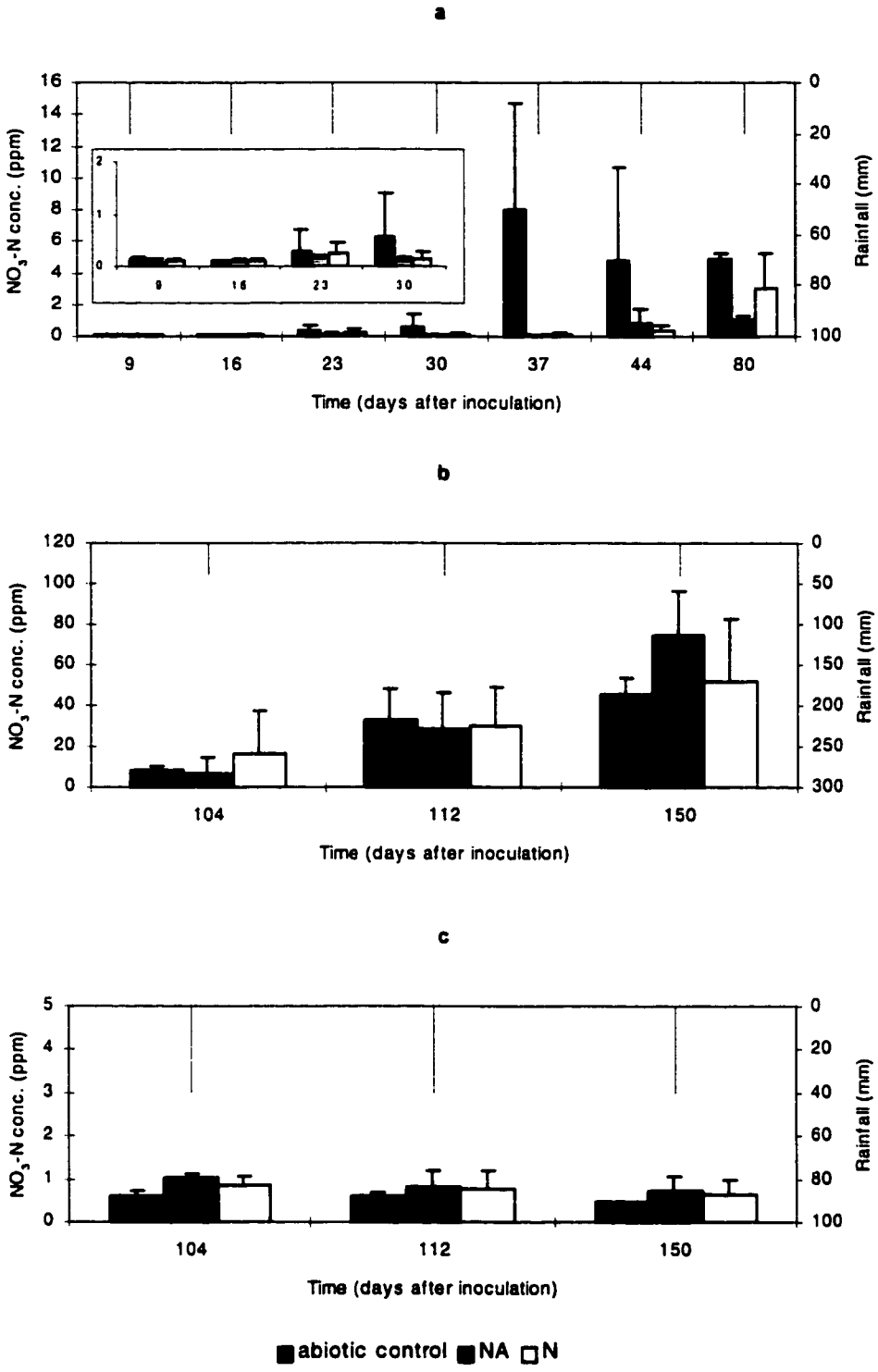

Figure A4.5. Concentration of nitrate-N in the drain water during a) saturated, b) unsaturated periods and c) in the unsaturated soil after the saturated period. Results represent the mean and SD of three replicates. NA- treatment 1 (bacteria, nitrate, atrazine), N- treatment 2 (bacteria, nitrate)

Table A4.3. Results of statistical analysis for atrazine and nitrate loss.

Condition	Treatment	Source	Pr > F
Saturated	Atrazine in drain water	Treatment	0.1167
Unsaturated	Atrazine in drain water	Treatment	0.0269
Unsaturated	Atrazine in soil	Treatment	0.1455
Saturated	Nitrate-N in drain water	Treatment	0.1490
Unsaturated	Nitrate-N in drain water	Treatment	0.4889
Unsaturated	Nitrate-N in soil	Treatment	0.4971

Zablotowicz et al. (1979) and Garcia-Plazaola et al. (1993) have suggested that the sequence of: Nitrate ion (NO_3)→Nitrite ion (NO_2)→Nitric Oxide (NO) →Nitrous oxide (N_2O) →Nitrogen gas (N_2) is the likely pathway of denitrification in anoxic environments by different species of Rhizobia. However, there is no evidence of these bacteria to oxidize nitrite ion (NO_2) into nitrate ion (NO_3) in a nitrification process in oxic conditions. Therefore, it is difficult to explain the higher concentration of total nitrate-N (Table A4.4) in the drain water of treatment 1 in presence of atrazine. Nevertheless, the measured pH of the drain water indicated lowest for treatment 1 and highest for the control (Figure A4.6). One would assume higher concentrations of nitrate-N would increase the pH and if this were the case then the measured pH during the saturated period (1st 80 days) would confirm lower levels of nitrate-N in the bioaugmented soils. However, this pattern continued into the saturated period. Therefore, other phenomena must be involved in the pH variations. Perhaps, one possibility could be that in the

bioaugmented treatments some organic acids were produced as metabolic end products that lead to a decrease in the pH.

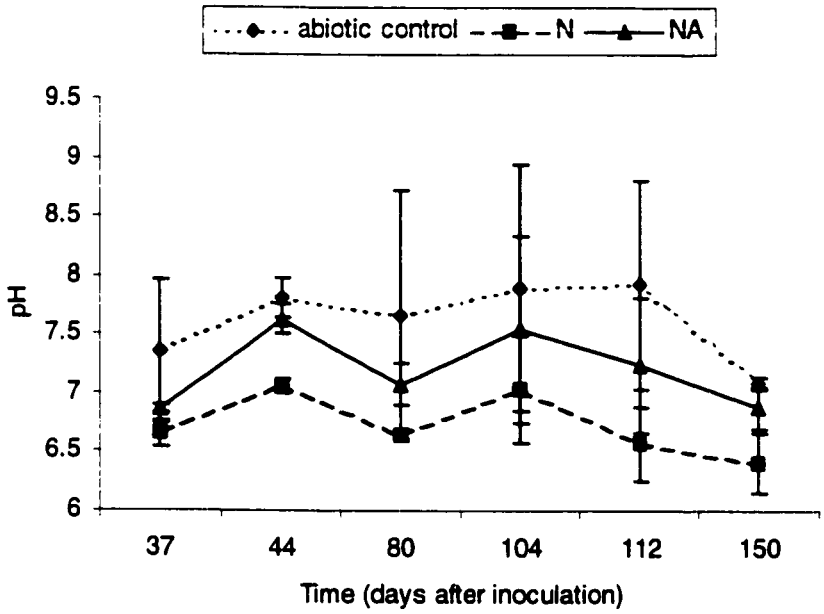

Figure A4.6. Measured pH of the drain water at different times for the three treatments.

Many studies have been conducted to investigate the transport and fate of agricultural chemicals through saturated and unsaturated soil profiles (Kanwar et al., 1985, 1988; Gish et al., 1991). Saturated conditions are the worst-case scenario for chemical movement in ground water systems (Azevedo et al., 1996). Therefore, to maximise leaching of atrazine and nitrate, the initial stage of this experiment was performed in saturated soil columns. Furthermore, in these worst-case scenarios, concentrations of atrazine detected were low, with rain simulations of 20 mm. Atrazine has shown to have a very low leachability through soil

columns (Liaghat et al., 1996a,b). Smith et al. (1992) suggested that long periods of water applications is required to have an effect on the atrazine concentration in a soil profile. Therefore, higher concentrations of both atrazine and nitrate in drain water collected after the 80th day could be the result of higher rain simulations applied after this date.

Table A4.4. Recovered concentrations of atrazine and nitrate-N during different periods of the experiment.

Chemical	Treatment	Recovered during different periods			
		Saturated	Unsaturated	Soil	Total
Atrazine (ppb)	1 (NA)	5.89±0.9	4.19±0.8	18.57±4	28.65
	3 (control)	7.30±1	10.64±3	24.47±4	42.41
Nitrate (ppm)	1 (NA)	4.03±3	109.43±11	2.60±0.6	116.06
	2 (N)	2.31±1	98.39±25	2.26±0.8	102.96
	3 (control)	17.95±14	85.49±10	1.66±0.4	105.10

In general, the total concentration of atrazine in both drain water and soil of the bioaugmented soil columns (treatment1) with *R. meliloti*, strain A-025, were 31% lower (Table A4.4) than those obtained from the control soil columns (treatment 3). However, the nitrate-N concentration was 9.7% higher than the control soil columns for treatments 1 and 1% lower for treatment 2 (Table A4.4). Although further investigation into the fate of atrazine in the presence of Rhizobia is needed, we present that water table management systems can be used for bioaugmentation of agricultural lands with desired microorganisms in a setup shown in Figure A4.7. Even more, if Rhizobia prove to be effective degraders of

atrazine in future studies, then these bacteria can also be used as biofertilizers since these organisms are nitrogen fixers as symbiots, thus, through crop rotation with leguminous plants, fertilizer application could be an unnecessary practice.

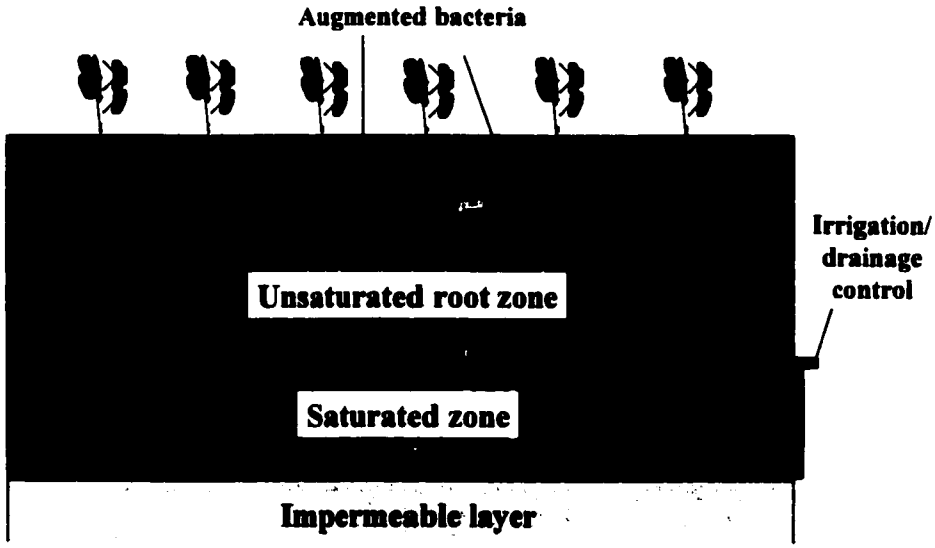

Figure A4.7. A schematic diagram of the proposed biofiltration field application.

In conclusion, results indicate that it is possible to use bioaugmentation as a biofiltration system for agricultural chemicals before they reach aquatic systems. Furthermore, soil columns bioaugmented with *R. meliloti,* strain A-025, allowed lower concentrations of atrazine to be leached into the drain. However, more evidence is required concerning the biotransformation of atrazine in both

anaerobic and aerobic conditions with this strain of Rhizobium. Also, results indicated bioaugmentation reduced nitrate-N during the saturation (anaerobic) period significantly whereas it was increased during the unsaturated (aerobic) period. Even though, total nitrate-N levels were lower in the bioaugmented soil (treatment 2) compare to the control, it was higher in presence of atrazine. Therefore, until further investigations are conducted, these data should not be extrapolated to predict the fate of nitrate-N in systems such as the one presented here.

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APPENDIX B

DIFFERENT BACTERIAL GROWTH MEDIA

Minimal Medium, (MM30): 1L preparation

A.

$(NH4)_2 SO_4$	1 g
KH_2PO_4	3 g
Na_2HPO_4	6 g

Mix in 500 ml water and adjust pH to 7.1 then autoclave. If needed add minerals and vitamins when solution cools down.

B.

Agar 15 g

Add agar to 500 ml water, dissolve and autoclave.

Add A to B and pour in plates (Sylvestre and Fauteux, 1982).

HM salt medium: 1 L preparation

Na_2HPO_4	0.125 g
Na_2SO_4	0.25 g
NH_4Cl	0.32 g
$MgSO_4 . 7H_2O$	0.18 g
FeCl3	0.004 g
$CaCl_2 . 2H_2O$	0.013 g
HEPES	1.3 g
MOPS	1.1 g

Add to 1 L water, dissolve, pH 6.6, and autoclave.

H medium: 1 L preparation

Trypton	10 g
NaCl	5 g
Agar	15 g

Add to 1 L water, dissolve, autoclave.

Modified Fahreus medium: 1 L preparation

$CaCl_2 . 2H_2O$	0.1 g
KH_2PO_4	0.1 g
$Na_2HPO_4 . 12 H_2O$	0.15 g
$MgSO_4. 7H_2O$	0.12 g
ferric Citrate	0.005 g

Agar	15 g

Add to 1 L water, dissolve, adjust pH to 6.5, autoclave. If used for watering plants, make solution without agar and add 1 ml Gibsons trace elements.

Plant Growth medium: 1 L preparation

1.5 M $MgSO_4$-$7H_2O$	1.0 ml
1.5 M $CaCl_2$ - $2H_2O$	1.0 ml
1.5 M KH_2PO_4	1.0 ml
0.8 M H_2HPO_4	1.0 ml
Solution F	1.0 ml
Solution E	2.0 ml

(Wheatcroft, personel communication)

TYg medium: 1L preparation

Tryptone	1 g
Yeast Extract	0.5 g
Agar	15 g
Glycerol	10 ml

Add to 990 ml water, dissolve, adjust pH to 7.2 and autoclave. (Trevors and Cook, 1992)

TYc medium: 1 L preparation

Tryptone	5 g
Yeast Extract	3 g
$CaCl_2$	0.84 g
Agar	15 g

Add to 1L water, dissolve and autoclave (Bromfield et al. 1994).

TYct medium: 1 L preparation

A.

Tryptone	5 g
Yeast Extract	3 g
CaCl2	0.84 g

Agar	15 g

Add to 950 ml water, dissolve and autoclave.

To isolate Rhizobium meliloti, dissolve 0.4 g potassium Tellarite in 50 ml distilled water and filter sterilize. Add to A when cooled. (Kinkle et al. 1994)

YEMP medium: 1 L preparation

Mannitol	10 g
Yeast extract	1 g
$K2HPO_4$	0.5 g
Agar	15 g

Add to 1L water, dissolve adjust pH to 7.0 and autoclave (Vincent, 1970).

APPENDIX C

RAW EXPERIMENTAL DATA

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Ind	SD	Initial	SD
1	3891	1662	4406	712	4023	1017	3492	436	3541	355	13926	5921
2	4659	1269	15396	1904	16208	4741	12519	2652	13262	1358	49619	15477
3	5551	699	8188	23	8512	2159	7277	1478	7213	250	23521	5893
4	5462	1295	12507	1491	12768	1684	9837	1837	11247	1828	35924	4297
5	7008	1425	12486	1163	12541	1569	9244	1906	10500	1908	30421	4954
6	4583	688	6662	18	6613	1137	5291	1171	5640	1045	22254	3763
7	15145	2023	23449	1885	24314	5107	20559	3976	21035	2290	68012	10027
8	0	0	1017	1438	1531	1336	0	0	668	1158	9615	1677
9	0	0	1118	1581	1545	1361	0	0	0	0	9066	4004
10	18114	2606	30804	2907	31997	6472	25572	4885	28405	4185	129191	30786
11	15049	1803	17170	1433	17028	3247	15890	2831	15331	714	51463	6460
12	14615	1467	17509	1386	17142	3331	15477	2747	15115	1275	48826	5365
13	134401	10392	157216	11242	153933	28169	143723	24891	136879	8433	402937	58096
14	59827	4332	71636	4529	69424	13318	64427	11198	62054	4791	202086	27045
15	0	0	0	0	0	0	0	0	0	0	1245	2156
16	86774	7669	103060	8199	99079	19674	90656	16405	87891	6801	299928	38387
17	25150	1999	31296	2122	29661	5704	27119	4675	26760	2573	93485	10781
18	45401	3842	57945	4361	54965	10945	50172	8831	49173	4719	188859	29451
19	14719	1076	17830	1196	16386	3013	15014	2785	14959	1292	54121	8500
20	2867	246	3269	175	3007	624	2555	444	2848	463	11347	1687
21	5770	302	9193	1018	8848	1576	8152	2097	8019	899	32192	3667
22	35022	2894	57412	5309	52790	8390	48258	10240	47199	4601	199150	22857
23	189520	9262	221721	17103	205988	37111	194755	30691	185446	10819	668865	82110
24	33664	1382	40868	2785	36708	6302	35112	5663	33160	2248	112318	12270
25	54956	2053	69197	4218	61465	11139	58084	9607	55837	4646	214934	17171
26	274674	13042	326653	24986	294487	54658	274102	43408	265699	17396	883558	50544
27	92021	2764	112291	8019	98569	18170	92598	14817	88942	6609	330653	36159
28	3794	149	4760	261	4114	726	3922	703	3652	328	18064	1517
29	14021	433	17170	1054	14993	2582	14245	2355	13493	1046	49638	6144
30	67911	1899	83668	5472	73128	13470	68391	11271	65725	4967	247799	30477
31	128746	4091	158520	11099	137436	27300	126950	21394	122974	9693	497877	62581
32	41889	1270	51634	3040	44440	8178	41672	7196	39938	3306	161053	24031
33	266143	8361	319876	23712	280663	56202	258703	45152	250534	17899	906182	125448
34	75598	2191	92423	5322	80732	15713	78064	13614	72374	5656	311050	21502
35	51307	994	63657	3640	54946	9905	53000	8780	49598	3921	204574	12598
36	16000	43	20660	1199	17164	2850	16263	3001	15649	1318	63309	9168
37	278348	4035	342355	20941	293808	57307	274705	46900	265363	20268	1131751	82225
38	22747	304	28994	1445	24462	4243	23391	4067	21994	1838	101171	12020
39	40425	80	51148	1488	42294	7502	41055	6508	38610	3195	147207	7593
40	175806	297	219822	9518	183832	35127	176678	29131	167262	13164	714378	78558
41	136814	283	173090	7269	143427	27289	133691	24417	129720	10849	578048	36262
42	54542	656	69276	1464	56763	10971	54795	9418	51729	4774	328977	105315
43	21474	929	26082	728	22836	4094	23479	4555	20494	1685	25075	43432
44	16951	349	22303	245	17350	2928	16429	2667	15934	1432	68223	8587
45	25265	223	32765	245	26246	4442	25577	4289	23710	2062	112937	9271
46	286719	626	345785	36130	293246	57493	275730	47906	267113	22905	1166725	159058
47	25679	144	33329	470	26454	4663	25781	4314	24214	2154	121208	22115
48	3734	92	2490	3521	3864	649	3856	729	3494	344	4950	8574
49	52458	2089	64466	2205	55054	9915	55918	10787	49839	4382	259456	86812
50	30841	1114	38563	933	32250	5587	32686	6254	29288	2696	149781	49768
51	59987	963	79229	303	60785	10531	57007	10082	55969	5254	264528	24299
52	8379	404	10317	141	8681	1463	8878	1698	7991	743	42361	16736
53	50127	1794	61568	1556	51884	9046	52538	10190	47395	4319	240574	73976
54	29834	1069	36689	909	30797	4975	31432	6240	27948	2580	141822	44843
55	42692	136	57042	415	44218	6860	43199	7683	40592	3933	211030	38608
56	9455	3565	9816	139	7170	988	6653	1309	6605	668	47443	17080
57	10677	201	13420	151	11017	1748	11224	2136	10040	884	53644	17875
58	96417	2897	120945	3064	99604	17184	100146	19986	90674	8783	562015	230851
59	6215	165	7740	108	6337	969	6553	1247	5838	509	6526	11303
60	3395	11	4262	62	3420	551	3532	688	3171	246	17475	7841
61	1053	1489	2861	1129	6420	6198	2131	3692	6152	5422	42916	39025
62	3558	170	4224	4	4132	909	3925	356	3707	748	3340	5785
63	56931	956	73132	107	58210	9147	58664	11525	53130	5360	324970	120765
64	0	0	1026	1450	671	1162	0	0	0	0	8068	2598
65	19702	862	24118	497	20462	3187	20977	3840	18594	1684	105291	43532
66	22885	842	28189	110	23930	3812	24637	4560	21745	2014	126967	56658
67	2200	28	2962	148	1654	1441	1718	1493	1431	1240	12180	3919
68	10151	240	12346	264	10351	1553	10868	2079	9524	894	54623	24662
69	0	0	0	0	0	0	0	0	0	0	5844	1838
70	19027	491	23042	380	19199	2719	20058	3846	17646	1660	113046	56962
71	0	0	0	0	0	0	0	0	0	0	9516	3962
72	6501	204	7362	110	6658	650	7060	1097	5922	1110	29353	9942
73	17335	174	21000	330	20891	2565	18917	896	17552	1193	78961	25801

Table C1. Experimental data for different treatments at 590mm after 345 days. /327

peak	control	SD	Water	SD	A-025	SD	B-356	SD	Ind	SD
1	7890	2087	6183	3784	10245	3023	8943	581	7282	1157
2	12351	2589	18346	9182	25350	6908	22144	1657	17384	1648
3	11413	2118	11861	5368	14519	4522	13754	41	11473	2139
4	13084	3009	14383	5757	17437	4798	16400	2126	14377	2971
5	21181	7711	16154	5508	19631	7243	18587	1159	14853	3743
6	12116	4687	10160	3307	12238	4963	11818	453	9171	2236
7	36609	8226	32163	11255	37745	8381	33868	5798	32004	4610
8	3243	555	2963	1102	3601	961	3108	861	2960	756
9	4016	698	3144	1126	3642	786	3165	803	3023	641
10	47671	7295	40682	15573	47708	10369	42120	9454	40360	7950
11	30942	5110	23334	8393	25726	2823	23062	4572	22848	2835
12	32247	5422	24031	8403	26503	4357	23698	5133	23356	3552
13	282626	48235	222468	93081	226888	25832	202315	42370	200908	30553
14	130824	22287	100667	39815	104837	13060	93636	19548	93084	15229
15	0	0	0	0	0	0	0	0	0	0
16	200395	36368	147451	59102	154560	23450	137839	28043	135731	20983
17	57535	9463	42607	14252	45709	7453	41268	8405	41152	6718
18	106730	20731	79552	29236	84608	14014	77260	16848	76901	13491
19	32853	5770	24154	8222	25074	5221	23829	5596	23658	4038
20	4702	1322	3664	1254	4009	627	4023	1213	4018	729
21	15012	3620	15222	9904	12044	2046	12050	2379	12086	3170
22	89623	20667	86709	49106	76198	12647	72532	14264	70107	14849
23	410578	79499	317730	149436	293101	34604	272608	48776	270571	43348
24	75769	14124	59623	28277	53171	7121	50311	8810	50074	8447
25	125947	24842	99762	50221	87474	12017	82968	14047	83647	16098
26	604593	132053	459863	230344	414146	51119	387240	59846	386017	65730
27	212001	47060	161764	84936	140827	18082	132360	19641	132335	24120
28	8858	1934	6991	3601	5900	844	5633	885	5785	1163
29	31447	6054	24774	12121	21151	3027	20302	2908	20612	3833
30	151914	30794	119230	60111	101837	14196	97355	13555	98352	18444
31	296538	68741	228275	120891	195603	28565	185702	25911	186740	35583
32	96452	22038	74278	37764	62585	9458	60620	8321	61267	11873
33	604290	144243	452772	234577	391494	58710	373610	52584	375758	69427
34	167066	35784	125998	59382	111689	13792	106902	15535	107295	18220
35	108803	23059	86276	41878	73951	8264	71502	9110	71811	12151
36	34351	7488	29819	16542	22990	3483	23000	2542	23289	4560
37	591373	142544	463074	244339	392847	47632	379473	44365	379279	65577
38	47673	10318	40910	21934	32243	4320	32396	3120	33046	5741
39	80027	16401	67332	34025	54201	5195	54214	5423	55364	9188
40	350956	77097	275415	134158	236808	18122	230248	27355	233065	37855
41	289580	72672	235712	128473	185896	27448	189941	18755	190877	35056
42	107755	26094	86070	41519	73189	6214	73677	8595	74497	12693
43	42857	8478	30375	9844	30168	1083	29052	4593	29701	4473
44	32039	7594	28794	15176	22107	2671	23405	2025	23733	4313
45	49535	10810	42043	19874	35014	3304	35809	4025	36482	6422
46	571595	145074	442165	258679	347890	48646	339282	36360	342389	60781
47	49343	11508	40182	17604	33892	2666	35155	3531	35958	5938
48	7001	1546	5684	2401	5013	242	5107	610	5338	890
49	99856	21753	71345	23590	68811	3311	66971	10295	69819	10575
50	56441	12357	42109	15022	39252	2125	38964	5845	40908	6381
51	111749	26544	98828	50854	72323	11016	78965	6886	82481	15483
52	14178	2966	10720	2840	10177	651	10478	1507	11143	1614
53	87801	18325	66591	21071	61648	4032	62175	8953	66217	10292
54	52853	10662	40843	12289	36575	3104	37233	5336	39647	6204
55	71294	15803	66217	27571	50909	4781	54906	5041	57939	10311
56	11077	2423	15145	10373	7821	1412	9306	640	9708	1972
57	17073	3784	14727	4590	12980	715	13570	1926	14193	2345
58	156568	36391	126774	37339	115945	10332	118441	19849	126349	20776
59	9531	2253	8028	1862	7342	851	7764	1299	8124	1273
60	4879	1426	4713	1406	3825	448	4406	626	4486	762
61	0	0	0	0	936	1621	0	0	0	0
62	5089	1447	3907	1098	3631	743	3854	844	4031	823
63	86830	19764	84154	33931	66185	6397	73777	11717	76082	13306
64	2447	605	1637	2314	1511	1308	2471	412	1756	1523
65	29973	6718	26382	7389	23649	2586	25411	4697	26982	4208
66	33815	8048	30270	8306	27927	2219	29477	5451	31279	4886
67	3049	824	3525	1571	2671	198	3304	409	3113	688
68	14400	3777	12553	3451	12099	965	12969	2502	13199	2281
69	0	0	0	0	0	0	1364	1181	0	0
70	30343	8528	23645	5831	22777	1305	23642	4342	25006	3992
71	1477	2088	1074	1519	2346	140	1815	1582	1561	1353
72	8829	2775	8087	2145	7538	347	7885	1168	8291	1277
73	21828	5858	20875	4244	20125	2234	20781	4128	22583	5487

Table C2. Experimental data for different treatments at 340mm after 345 days. /328

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Ind	SD
1	5108	721	6069	633	4566	1267	3871	540	5913	3690
2	8889	1473	16571	2006	12930	3120	10487	158	12522	3703
3	8335	1322	11314	1848	8849	1584	7636	454	10137	3865
4	7325	655	20674	11646	12431	2010	10546	868	13774	2166
5	10757	799	18874	8275	13608	2869	10746	1160	15206	4220
6	7194	1253	11202	4017	8013	1269	6710	388	9739	4396
7	23842	2430	36333	11457	27163	3745	23785	1494	26978	1985
8	1014	1434	3637	1618	2869	896	2252	93	2972	454
9	2459	120	3444	1154	2766	539	2259	119	2880	402
10	31713	2451	48300	17104	38924	7713	32472	1470	36634	3461
11	22442	1481	24451	3378	19661	1691	17657	1066	18614	1249
12	22541	1823	25032	4740	20323	1978	18354	1261	19299	1597
13	195434	12393	210002	31169	177101	19416	164052	8379	173468	15027
14	90681	6304	97694	15726	82027	8789	74827	4083	79389	6309
15	0	0	0	0	0	0	0	0	0	0
16	135767	9446	147304	24287	121785	12754	110944	5953	118318	11123
17	39910	2898	44017	7548	36612	3911	33262	1884	35966	3021
18	74643	6215	84257	14496	69164	7511	63801	4244	67609	6317
19	22849	1604	24858	3889	20722	2574	19254	1428	20367	2209
20	3893	305	4527	1025	4431	1244	3486	354	3437	572
21	9134	781	12615	2118	10263	1098	9545	557	10500	546
22	56438	3060	78739	10341	65348	7329	59276	2822	63440	5405
23	276419	12856	290965	36305	245142	23223	230348	9497	241311	23116
24	49941	2218	53133	6104	44724	4139	41593	1710	44168	4319
25	82974	3580	89861	9300	75584	6878	70199	2594	74716	7204
26	399525	14600	426852	45613	355887	31469	335451	11676	349871	35382
27	137183	5158	147799	13517	122069	10921	113689	3706	119484	12604
28	5580	111	6081	538	4967	512	4610	172	4920	535
29	20645	590	22186	1959	18400	1640	17182	606	18276	1889
30	101933	3240	110978	9642	91561	7927	85248	2667	90405	9702
31	196291	7129	215272	19687	174112	15837	161845	5287	170601	19477
32	63142	2049	69253	5366	55695	4814	51872	1792	55032	6046
33	399893	13960	436620	34923	351836	31436	331147	12012	346942	38126
34	113230	2930	120747	10351	100406	9289	93790	3166	99250	10465
35	74354	1631	79980	6562	66894	5942	62718	1583	66564	7257
36	23164	434	26059	979	21287	2065	19842	415	21178	2431
37	404572	9928	445407	24723	364632	30759	343183	9515	358808	39371
38	32377	503	36187	2117	29690	2546	28115	961	29496	2995
39	56638	413	60920	3603	51142	4054	48312	1186	51167	5296
40	251370	2952	268031	17685	226157	18057	212732	5643	224237	24351
41	198818	4441	225454	8727	179146	15665	169217	5686	178595	19898
42	77647	996	83484	6863	68892	5620	65342	2036	68598	7736
43	30849	226	16711	23632	27591	2202	25829	976	27561	2603
44	23043	177	25427	895	20538	1680	19614	510	20544	2432
45	35568	185	38021	2434	31780	2631	29939	919	31865	3540
46	409844	5064	446121	27072	363360	29707	344667	11015	362048	42563
47	35752	246	38090	2580	31820	2526	30302	950	32130	3488
48	5077	20	2825	3994	4542	419	4289	138	4576	499
49	74669	34	75937	7893	67045	5098	62905	2447	67167	6865
50	43028	88	43988	4192	38687	2986	36529	1378	38255	4233
51	82796	174	91044	3917	74099	6361	70557	2242	75013	8776
52	11257	3	11518	1224	10111	690	9654	371	10187	1015
53	69467	30	71063	7345	62561	4759	59106	2227	62935	6440
54	41494	346	42146	4236	37501	2915	35225	1475	37824	3643
55	57314	470	75890	25022	52738	4343	50260	1715	53334	5667
56	9056	173	9640	228	8349	752	8090	169	8452	904
57	14070	142	14224	1324	12883	1055	12142	454	13013	1323
58	131193	1202	132236	13707	118568	9237	112452	4711	119713	13213
59	7844	84	4213	5957	7311	582	6971	286	7403	727
60	4385	74	4395	354	4159	636	3855	135	4184	378
61	0	0	0	0	2134	3696	0	0	0	0
62	4486	37	4304	421	4380	683	3806	120	3890	567
63	74346	838	74509	6877	67767	5350	64696	2546	68839	7453
64	2036	4	1171	1656	708	1226	0	0	747	1294
65	25883	276	25825	2664	23562	1486	22614	896	24225	2619
66	29367	271	29664	3173	26840	1489	25899	969	27618	3272
67	2680	57	2847	273	2457	80	2385	113	2563	421
68	12724	256	12270	1205	11369	836	11162	586	11683	1375
69	0	0	0	0	0	0	0	0	0	0
70	23317	323	22462	2156	20882	1366	20456	936	22335	2160
71	0	0	0	0	0	0	0	0	968	1677
72	7631	750	7962	829	7564	761	7307	337	7992	1095
73	20999	1452	25718	4118	21594	1985	21715	157	23642	2966

Table C3. Experimental data for different treatments at 140mm after 345 days. /329

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	7055	74	15482	10212	16281	11383	5426	1220	16558	21205
2	21984	1691	36928	25657	63910	46521	18538	789	40434	40511
3	11007	116	24732	18720	27428	17697	10025	1099	19831	18030
4	15660	2461	23235	4687	27584	15293	14882	726	24191	15667
5	15437	2119	24866	554	25001	12274	15076	503	24353	15314
6	7102	75	19550	2435	19953	12649	7576	883	15492	13994
7	27955	488	42299	15165	61751	37316	26522	1109	39781	20801
8	2650	64	8804	5589	5637	4899	2666	288	4420	2706
9	2500	26	9169	6508	3930	3407	2473	243	4895	3841
10	37647	3714	75046	49159	95002	62117	36159	4300	64873	45114
11	18999	200	31628	22834	46345	28581	17069	765	24744	11771
12	19725	614	30400	19168	46496	28393	17656	755	25532	12272
13	178645	13737	227154	120301	380736	226927	149601	7198	234157	133271
14	78315	824	114147	65822	191111	118414	69361	3705	114461	72217
15	0	0	3079	#DIV/0!	0	0	0	0	0	0
16	112603	1185	165413	88723	281078	177369	99607	5184	165545	106342
17	33604	236	53926	27552	87592	55236	31266	1874	51122	32719
18	62260	655	107981	64631	176327	116398	58390	4966	99966	72192
19	18992	200	35543	22388	60076	40377	18485	2125	32716	24512
20	3460	266	8396	4762	10902	7580	3297	859	6369	5542
21	9454	119	15143	3928	20938	13255	9112	793	13109	6012
22	59938	1458	96763	55384	156542	102743	54050	4872	104731	81158
23	223264	2350	356630	243834	622953	401136	199621	12887	362475	263777
24	41011	432	63102	40671	109782	68878	37313	2520	63684	42035
25	68235	718	107769	67788	177499	112298	61737	4633	103585	66330
26	316751	3335	487078	327473	710326	420731	280666	19577	436303	246179
27	113507	8728	170967	119096	300235	193722	95692	7255	173767	125726
28	4763	34	9769	8589	16631	11537	4174	341	8692	7276
29	16641	405	25936	17345	44526	28141	15180	1140	26884	18661
30	79474	837	127951	87483	218444	140240	71703	5564	132642	97832
31	148566	1564	251590	181123	448326	296930	132551	10408	255081	198071
32	48442	510	81506	58098	144742	94935	44049	3418	83385	63242
33	301087	3170	450374	296394	766665	483369	265712	20411	469235	326957
34	87576	922	146054	105790	250210	162822	79249	5903	148715	113217
35	62199	4783	95925	67493	162575	104522	53058	4063	95951	68653
36	19073	201	28968	19325	49907	32035	17096	1567	32315	23828
37	309731	2179	542854	417590	919545	616033	273585	22147	542774	437706
38	26427	333	53261	43181	93853	64889	24457	1914	44068	31198
39	47305	3638	68287	42531	113499	70796	41172	3287	68593	43178
40	192077	2022	324144	243839	536002	351722	170954	13780	330855	258894
41	152846	2669	278580	221198	476154	325533	136499	11586	279352	230690
42	62207	3180	148867	144164	263620	193168	54325	4732	138294	139391
43	25583	1475	9821	13888	5921	10255	22660	2004	15988	14077
44	19752	879	33847	27828	60653	41160	17289	1590	34629	27488
45	29940	315	53892	43372	90094	60359	26780	2465	53480	42557
46	276705	2913	549115	479377	940780	660395	242377	21447	545855	494776
47	29328	713	59684	51943	99513	68811	26575	2370	54628	45457
48	4384	31	1745	2468	6276	8435	3956	394	2864	2525
49	58605	1824	112264	93837	172252	114226	52823	4925	108789	91328
50	34111	1518	62786	55642	101617	67894	31125	2922	64228	53879
51	64945	2021	126359	108489	219426	153217	59072	5696	132032	118013
52	8891	94	21410	19539	30584	20974	8500	804	18004	15635
53	54123	2409	104814	87340	160282	107297	49643	4335	100420	82658
54	31696	771	63174	52751	94679	63134	29995	2672	58291	45783
55	46740	2695	95197	83386	152883	106929	42816	4097	99594	92274
56	7446	232	24784	27162	39199	29884	7146	569	22652	25651
57	11340	505	25711	23124	37639	25942	10679	1020	23439	20551
58	100378	3124	234598	221073	361820	257921	93277	8968	230948	226159
59	6365	155	2907	4111	1452	2515	6192	597	4476	3944
60	3153	98	10731	10251	12710	9026	3254	355	7568	6864
61	9322	477	2618	3702	0	0	4952	851	3674	3192
62	3646	64	17274	21575	20670	16060	3652	451	21732	30266
63	57499	605	141964	133720	211557	151182	55758	5182	141088	139375
64	0	0	4403	3337	7135	13	0	0	3292	3846
65	20474	216	47886	42904	67579	46684	19892	1926	48265	45984
66	23825	416	55554	50695	79564	55016	23186	2226	57354	55368
67	2501	144	6517	6098	6927	6058	2278	256	6553	6538
68	11036	849	24932	27225	34477	22811	10121	826	25628	24385
69	0	0	3736	5284	2248	3894	0	0	3198	3736
70	22550	3095	50755	48993	69490	47495	18911	1543	50598	51881
71	2355	181	6356	4069	6900	6008	0	0	5682	7616
72	7175	552	17128	13111	27501	19823	6106	498	16331	16513
73	22355	1434	53099	50635	92618	67691	19253	816	49264	49400

Table C4. Experimental data for different treatments at 340mm after 20 days. /330

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	3950	384	3313	119	3044	676	2048	1774	3070	494
2	11628	2758	13068	1742	11526	1965	10829	2963	12700	2610
3	6840	711	7398	1039	7839	2866	6027	1676	7409	1535
4	8697	2192	11590	2102	14076	6447	11595	5419	11150	3191
5	8490	2275	11373	2502	17514	11737	11310	5147	11017	3011
6	5120	18	6161	1638	7241	2387	5667	2006	5735	1278
7	16585	5053	22680	3990	26104	12060	19397	5730	21252	5643
8	0	0	1294	1829	3059	3395	1791	1582	1076	1864
9	0	0	1170	1654	2823	3154	1519	1330	981	1700
10	21650	8219	32690	4377	52435	41107	29879	10558	32062	11708
11	12962	6161	17164	1742	16885	4786	13946	3274	16211	4335
12	12801	5702	16826	2597	17463	5580	13939	3797	16290	4429
13	125381	54221	147309	24841	140339	22970	120292	30404	147043	42380
14	56304	23861	69732	11466	68168	16646	57362	14926	69020	20419
15	0	0	0	0	0	0	0	0	0	0
16	80998	32694	98679	17174	94349	18785	80844	21600	97876	29264
17	23624	8894	31732	5082	33195	11309	26723	7127	31159	9250
18	44315	16922	42716	30980	60386	21250	48126	12979	56566	17307
19	13403	5129	17858	3227	17540	4557	15078	4127	17623	4927
20	1768	2500	3019	522	3306	731	3984	1619	3015	784
21	7541	3305	9805	2115	10984	4130	17867	13357	10138	4378
22	45292	18205	56117	11244	53467	9363	44658	19458	56732	19128
23	170444	72146	202837	35089	188431	21710	166962	42255	203187	56686
24	31346	13959	38680	6659	34813	4212	32417	8276	38908	10922
25	53414	21080	64784	11971	70244	21422	54751	14783	65251	19861
26	244050	98213	287109	50658	270226	26411	237536	63644	287984	81843
27	85277	34343	100812	17738	92345	8794	84142	22502	101505	29768
28	3751	1525	4420	899	4001	391	3804	1074	4462	1306
29	13282	5246	15923	2741	14622	1399	13497	3585	16089	4521
30	64256	24363	75784	13224	70268	6663	63753	16753	76752	22194
31	120467	46892	138186	24426	131817	13431	116575	31732	140467	41740
32	40279	14671	46164	7951	43581	4295	39728	10446	47055	13475
33	234922	88354	276236	48544	262685	26278	231856	63711	279020	81552
34	69609	26096	82479	14918	78140	7456	70046	18907	82480	22904
35	66408	7471	55701	9723	52949	4878	47565	12611	55704	14982
36	16401	4872	17899	3199	17070	1740	15697	4392	18158	5208
37	247645	94205	288223	50314	277038	27179	244530	66937	257198	123686
38	21186	8170	25292	4468	23902	2360	22154	6111	25425	6579
39	37824	14418	44071	7591	41921	3845	38397	10275	44219	11179
40	158574	61238	184722	32277	176873	16436	156297	42047	184334	48308
41	124099	47028	142866	25375	139705	15435	123098	34117	143709	39268
42	51691	19968	57679	10591	56838	5854	50337	13657	57680	15032
43	20221	7553	24429	4604	22357	1877	20810	5487	23984	5836
44	16745	6311	18380	3173	17892	1915	16411	4339	18656	4832
45	24891	9163	28692	4989	26906	2701	25065	6730	28700	7378
46	229085	91469	260152	46154	266489	35728	223046	62464	261787	73343
47	25061	9301	28544	5021	27317	2587	25148	6548	28674	7135
48	3799	1435	4268	807	4071	429	3843	1060	4265	1071
49	49551	18399	57864	11008	54700	4473	49867	13225	57236	14250
50	29752	11056	34181	6365	32573	2820	29920	8045	33898	8247
51	56156	20138	63005	10938	62045	6849	55933	15387	63899	16859
52	8142	3097	9293	1688	9041	791	8343	2194	9307	2179
53	47656	16997	54719	10288	52619	4565	47703	12787	54269	13203
54	28353	9837	33216	6253	31341	2666	29002	7826	32934	7971
55	41220	14868	46309	8231	45674	4442	41234	11262	46457	11730
56	6770	2646	7447	1217	7450	805	6776	1900	7583	1970
57	10439	3868	11934	2234	11464	1008	10670	2906	11864	2885
58	93269	35531	104934	20353	103294	9129	92059	26043	104049	26837
59	6194	2263	7078	1228	7845	1748	6442	1646	7033	1541
60	3275	1300	3759	699	3689	299	3445	905	3754	874
61	0	0	1041	1471	0	0	0	0	1473	1276
62	3303	1341	3950	745	3702	235	3477	1026	3887	925
63	55902	19997	63074	11753	61698	5566	55563	16580	62586	15917
64	1013	1432	1124	1590	674	1168	677	1172	787	1364
65	19601	6908	22916	4435	21675	1775	19935	5666	22474	5427
66	23185	8243	26594	5304	25613	2109	23236	6514	26060	6176
67	1390	1966	2556	653	2528	286	1721	1516	2475	572
68	10193	3675	11743	2197	11256	769	10384	3205	11511	2778
69	0	0	0	0	0	0	0	0	0	0
70	19405	6761	21724	3961	21109	1616	18704	4884	21259	5107
71	0	0	0	0	0	0	0	0	723	1252
72	6249	2314	7027	1232	6621	487	6129	1558	6735	1842
73	15821	5503	18423	1086	19563	331	16607	3594	17289	4308

Table C5. Experimental data for different treatments at 340mm after 32 days. /331

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	5343	1404	4861	455	3536	166	4777	1490	4737	1859
2	19891	2123	15453	1307	14296	1239	16756	3081	17302	7128
3	8301	747	8526	77	8286	803	8798	1422	10823	3976
4	12091	68	11591	1041	14668	2262	15528	8182	17453	6305
5	15916	1298	13335	2949	15052	3040	14441	4429	19666	8927
6	8592	653	7772	2032	7528	1869	7912	1713	9712	3660
7	27042	897	24237	2580	26728	3198	27975	8595	30392	10146
8	2296	205	2383	327	2903	448	3392	1684	2779	2423
9	2476	71	2345	319	2666	417	2785	965	2444	2137
10	40750	1631	35776	2891	40530	3854	44697	17027	45834	15079
11	19885	1246	17535	1372	18522	1515	17359	2675	21115	6264
12	21565	1335	18658	1420	18874	1499	18874	2824	21585	6757
13	193139	9057	159492	11508	166462	9641	159946	12480	207050	66680
14	88688	3715	75489	5530	78030	4907	75292	7857	93799	27602
15	0	0	0	0	0	0	0	0	0	0
16	135863	4779	109189	8218	111902	7484	112284	10960	138603	46095
17	39952	1051	34353	2377	35298	2339	35599	5028	40894	12120
18	75124	1211	60574	4229	63847	3764	65642	8313	77489	24559
19	23250	224	18839	1336	19566	1403	20520	1623	23641	7118
20	3777	76	3296	336	3126	315	3680	514	4001	1078
21	13872	1636	10212	1068	11011	890	11751	2420	17983	9544
22	85725	5349	63121	6569	63609	4582	68245	7884	93932	44756
23	286172	498	233567	15029	226389	10201	228375	7200	290109	98726
24	53208	630	43300	2959	42583	1874	41946	1170	55593	19774
25	94253	4282	71243	5219	71862	3526	73491	5590	94114	32389
26	427085	13132	328001	22056	321679	14302	327600	11506	415973	148201
27	152203	7046	112916	8426	112359	4991	112949	4241	150176	57531
28	6300	344	4693	404	4764	264	4582	162	6570	2544
29	23136	1322	17249	1242	17469	790	17241	562	23463	8955
30	117573	7017	83704	5926	84794	4125	86116	4430	114288	45119
31	228066	14646	158485	12476	153631	6782	164373	9011	211412	86526
32	73714	5727	51966	4025	50603	2167	53113	2615	69868	28359
33	438091	33356	316447	23974	306944	13470	319142	12124	416779	168473
34	127807	7908	93979	6264	90119	3332	94697	3321	116785	40643
35	84333	6284	62067	4283	60824	2343	62501	1865	77804	26011
36	28632	4110	19547	1954	19498	960	19735	899	27660	12412
37	471402	52786	334518	26819	318653	12348	336351	14131	426322	168575
38	38427	5467	28099	2274	27241	1083	27024	303	37067	14672
39	67438	8566	47785	3856	47580	1494	47079	1142	61644	21270
40	295872	32445	204192	15178	201421	6671	208524	7651	254961	84084
41	245637	41670	164457	14979	155319	6583	166348	6017	218744	96051
42	95511	11554	64903	4914	61017	2158	65873	2443	79142	25732
43	33231	544	26747	1466	26076	1012	25824	1393	30385	7679
44	30293	5863	20100	1911	19088	790	19394	394	26632	10832
45	43901	6213	30765	2353	29920	1372	29714	609	39154	13377
46	449630	71879	298146	25201	293003	14331	323659	28890	388737	142732
47	44421	6424	31503	2402	29734	1330	30082	499	38506	12776
48	6119	716	4591	381	4309	199	4214	188	5462	1600
49	82080	3485	62471	3881	61499	1819	61968	2238	72289	18641
50	48646	3359	36288	2277	35638	1019	35836	1217	42738	11078
51	107286	25234	68824	7092	66503	2388	68369	2470	93672	39625
52	12648	626	9952	581	9431	267	9633	216	11236	2734
53	77960	5650	58219	3784	57134	1443	58553	1464	68527	18125
54	45799	3945	34631	2326	34786	924	34497	778	41864	11476
55	72922	15291	50344	4918	48477	1621	49101	802	64765	23232
56	15660	8592	7845	1017	7749	442	7448	314	13737	9359
57	16015	1576	12308	857	12234	318	11931	224	15061	3994
58	151689	12969	111871	8430	109231	2790	111470	2129	133623	37459
59	9521	670	7658	460	7219	113	7235	271	8593	2058
60	5266	682	4119	258	3872	71	3911	121	4785	1241
61	0	0	0	0	0	0	0	0	0	0
62	4953	187	3923	161	3884	152	3738	247	4582	1130
63	84693	11684	64477	5257	64492	1604	62274	566	80411	23098
64	2291	99	1063	1503	672	1163	0	0	1723	1506
65	27708	1041	23041	1587	23470	609	21923	767	27998	7615
66	31641	1004	26840	2037	27127	760	25262	992	32273	8489
67	3163	607	2615	346	2615	38	2240	103	3244	1174
68	13051	525	11664	955	11686	314	10634	836	14157	3582
69	0	0	0	0	0	0	0	0	0	0
70	23954	967	21572	1753	21696	502	19568	1367	26703	7457
71	1087	1537	1130	1597	0	0	0	0	1650	1436
72	7803	40	7151	537	6925	252	6185	645	8798	2630
73	18883	644	19601	1066	19099	1116	16778	571	20197	4782

Table C6. Experimental data for different treatments at 340mm after 44 days. /332

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	5736	2334	4534	467	6491	2301	6308	1422	6332	684
2	21897	6541	20050	3816	24609	8129	27598	6439	27222	3420
3	10375	2872	8795	63	10768	3137	12713	3068	11420	1017
4	14129	5018	14375	421	17176	5256	21357	5114	18049	3837
5	16013	4661	15746	47	19143	5273	22201	5710	19515	4080
6	10182	2978	10788	1498	12591	3739	14360	3784	13193	2386
7	30196	8825	29362	1092	35508	10227	41751	8295	37243	5976
8	3066	1477	3298	251	3944	1139	4950	1049	4156	878
9	3044	1190	3011	112	3643	980	4447	652	3841	673
10	50824	18813	49648	4031	56766	13702	69165	13406	59069	10093
11	23052	4926	20137	1101	23793	5292	26943	4265	25109	3422
12	23679	5822	21970	1216	25862	5968	29406	5069	27410	3656
13	246380	87461	188835	4693	204959	13219	248761	47675	239995	23299
14	110716	37112	91006	1348	99268	10792	119872	21769	113473	13408
15	1650	2333	0	0	0	0	1462	1268	0	0
16	176411	65999	138491	4687	148799	13259	185117	33124	176069	18959
17	47304	13900	44462	793	49215	10640	57550	8681	53055	7074
18	98135	37592	83071	477	90014	14760	109688	17556	102040	12392
19	28811	9018	24464	882	28782	5664	32076	4602	30730	3575
20	6119	1752	5314	398	5859	719	7709	792	6856	1198
21	33238	28403	15879	4228	15584	2677	19323	1948	18321	736
22	160641	110611	88608	4454	96594	12711	120574	18018	115875	7511
23	402343	186204	272640	6959	298282	18824	358339	67220	347212	25716
24	78116	40881	50191	1124	58489	10128	66424	12063	64189	4597
25	147711	83081	93961	6037	105309	16923	123113	20215	116536	9796
26	638264	334466	399754	4457	453359	48791	528618	101000	511216	37217
27	243665	145531	139401	313	163633	26455	186444	36957	180167	13195
28	10160	6488	5536	35	6509	1061	7535	1602	7303	627
29	35936	21285	20904	338	24382	3902	28131	5259	27437	1965
30	191121	115376	107920	1227	121794	13715	146574	27216	142055	10042
31	382213	238457	210229	3304	245220	39165	289908	56865	280447	20978
32	128281	84767	67566	895	77597	12263	93174	17780	89954	6848
33	714292	423624	409843	10822	460248	49751	560658	105616	540075	42262
34	194876	107213	118163	1355	135830	22685	163505	32387	154507	14901
35	130348	72576	76176	214	89129	14155	106039	21886	100384	10067
36	53496	39148	24366	366	28256	4406	35036	7071	33733	3020
37	786788	482102	424455	546	470079	40777	591663	112418	565879	48909
38	49580	22042	33051	799	38384	6176	46527	9192	44803	4295
39	105915	62754	58291	669	66822	8847	81521	16742	77660	8393
40	451341	243385	264059	3176	303540	41194	367140	74404	347771	37226
41	450521	315264	215909	5382	247220	37642	310101	57910	294260	20015
42	153988	90773	85482	354	97143	13193	123718	26471	114074	10903
43	40034	13777	29941	364	34688	5265	41625	8778	38021	4410
44	53468	39548	23563	69	26652	3161	33982	7490	32289	2148
45	66175	41205	35338	94	40304	5650	50675	11007	47568	4053
46	817742	507727	434935	2915	474909	38568	624536	126433	584573	48361
47	67371	40871	37144	120	41791	5532	53055	10770	49194	4016
48	8672	5083	6435	2120	5529	756	7002	1493	6410	530
49	108120	40692	77009	1699	86202	11631	110405	22572	98745	11022
50	67014	29078	44672	831	49296	6169	64873	13133	57767	6334
51	195947	140468	88305	1934	93729	7359	134404	24663	123549	7304
52	17214	6543	12066	179	12906	1433	17547	3047	15500	1584
53	109094	45892	71923	1007	78992	9711	107242	19880	94711	10498
54	62957	26609	41953	28	45807	5753	62167	11176	54911	6209
55	129934	87036	61234	246	65675	7400	95468	16841	84551	7668
56	28588	24775	9486	4	9838	1104	15051	2201	15828	3463
57	22897	10635	14388	378	15263	1682	21432	3673	18750	1953
58	227665	103395	138246	5175	148007	18127	217928	40692	187640	21533
59	12824	4948	9061	270	9303	932	12983	2095	11221	1045
60	7978	3944	4958	122	5110	477	7241	1045	6311	691
61	4735	1894	2405	477	940	1628	3830	1453	3237	808
62	6978	3002	4590	200	4828	616	6702	1239	5794	681
63	131969	68021	74367	1534	76931	8127	119097	19190	100482	10965
64	3185	1258	2185	43	1602	1388	3184	491	2764	284
65	37345	13683	25810	968	26903	2978	38933	6524	33182	3186
66	43076	15879	29422	1420	30404	3145	44828	7662	37880	3868
67	4609	2476	2612	76	2679	290	3979	672	3368	306
68	17281	6042	12419	796	12200	1166	18273	2970	15657	1395
69	0	0	0	0	0	0	0	0	0	0
70	32965	11803	22868	1459	22470	2143	34280	5573	29327	2708
71	2806	1008	2286	401	0	0	3035	492	2677	162
72	9813	3145	7544	614	7212	812	10551	1945	9269	757
73	23928	3552	19349	3270	16932	1524	25856	3733	22571	2520

Table C7. Experimental data for different treatments at 340mm after 56 days. /333

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	5612	1966	10405	10148	19380	2730	22693	11539	26184	10068
2	15233	7857	27100	21586	49301	5285	69541	36116	65524	21344
3	8631	3244	14925	11684	25187	3258	29246	11872	31123	4774
4	10534	4789	13137	4291	30887	7269	38883	27757	36938	6940
5	10410	4991	12612	4255	28038	4795	36855	27171	34733	10612
6	6078	1373	9274	6041	21620	4211	28110	15471	26345	9906
7	20311	10322	31282	16156	62810	6938	78886	42564	75474	15828
8	1302	1841	5092	7200	11495	6291	10706	6766	10489	115
9	1241	1754	2788	3943	6494	7035	6644	#DIV/0!	6210	#DIV/0!
10	25429	13564	57159	38981	156995	80892	147274	90144	149073	10568
11	13732	7250	27684	16620	60334	23612	53527	22316	54233	3699
12	14030	7440	27000	16985	50736	9794	53665	22222	53138	6348
13	127986	57905	215951	121915	390454	8037	458745	187276	467599	135740
14	58582	27082	106466	63416	206564	13521	237921	103008	236647	60390
15	0	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
16	84823	38102	155816	97977	301068	16314	356545	151154	356671	92385
17	25386	11386	48248	28440	100911	18984	112746	49240	112612	24047
18	47073	20823	82483	87220	206539	43258	232246	108496	232576	55539
19	14314	6417	29041	19042	65748	5603	92652	66043	130526	107279
20	1636	2314	5761	4400	13078	1766	14019	6472	11614	#DIV/0!
21	7287	2946	12571	6027	39985	25406	34557	22843	20429	19289
22	45663	18730	90988	60559	210185	36557	240501	120427	250978	72674
23	170516	72247	354438	249485	691486	13551	812708	327810	816066	215115
24	31091	13598	62687	40611	120627	6352	134066	50044	134031	34017
25	53118	20661	104185	67692	220702	33551	253783	113344	252220	56043
26	244498	98846	424557	245039	810535	116415	1099407	421514	1026794	396939
27	84164	32769	172472	119080	330149	22315	392698	147312	393648	103974
28	3706	1461	10001	8792	18847	1969	21391	7831	21803	7197
29	12964	4796	26437	17610	48874	4377	60868	24757	58832	16650
30	62955	22524	128921	88371	244938	19297	301143	119891	296550	78700
31	117385	42533	255462	190279	491755	44237	604611	246208	598505	164731
32	38993	12852	82404	59201	162024	16775	200187	85144	194639	53961
33	235646	89378	456873	304003	842556	71417	1048003	416202	1029511	266224
34	69040	25292	145460	103987	282204	27107	357633	142853	341778	82948
35	65254	9103	95774	66395	184947	15971	232393	87487	185439	#DIV/0!
36	15944	4225	29739	19943	60078	8986	75485	29387	60229	#DIV/0!
37	244611	89914	556858	430221	1084968	67179	1329033	481359	1312189	295455
38	20800	7624	50802	40546	103093	8421	112026	26039	99895	#DIV/0!
39	36181	12094	65876	38428	127532	10563	152274	50810	126907	#DIV/0!
40	152960	53298	327305	233919	628267	32068	790327	298767	777145	185563
41	120902	42507	285350	226878	556865	44479	695548	254476	686210	154648
42	48765	15831	142471	130504	310152	18755	389121	144525	379792	87446
43	19710	6831	38318	24246	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
44	15706	4842	33093	23980	69612	6190	83973	30901	106056	52683
45	24065	7994	51336	37013	104112	9328	128896	47114	104532	#DIV/0!
46	219526	77950	552966	460256	1110536	61118	1408122	533386	1381829	325001
47	23654	7311	54888	42278	119445	9066	143096	48933	137937	27900
48	3573	1116	16911	18687	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
49	46928	14689	103364	75355	205312	3924	267997	92660	260248	56487
50	27486	7851	59038	41518	120780	7087	160954	64100	150361	34240
51	52716	15274	126993	101430	259430	26905	338266	131008	326288	74673
52	7389	2032	15830	10933	40426	4260	46738	16719	43438	7891
53	44028	11867	99041	72969	189925	10498	254245	99336	240284	53541
54	26274	6897	59092	42847	110339	6256	150367	57747	139235	30469
55	37771	9990	93416	74850	187486	17449	257072	108288	243286	58660
56	6091	1685	21849	21584	50628	4524	64795	25637	61254	13049
57	9344	2319	22062	16558	46402	51	58602	23438	55368	9967
58	83157	21231	221687	185466	446617	17224	618071	245866	585573	129683
59	5425	1176	21232	21245	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
60	2720	515	6452	4509	16925	453	20973	8389	18464	2985
61	4493	6353	2081	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
62	2978	882	7958	6413	22828	6816	34197	7760	35281	17755
63	49417	10825	137474	116970	258146	18392	365132	145970	340995	74655
64	0	0	12738	18014	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
65	17519	3964	46439	37701	82814	5056	120719	50408	105733	22025
66	20444	4366	55981	46864	97692	4703	145059	62362	126348	27461
67	1200	1696	10262	11551	9701	1845	16777	10059	12041	2249
68	9015	2010	27891	25034	39972	2200	63118	26630	45993	#DIV/0!
69	0	0	8163	11544	0	#DIV/0!	8621	4268	#DIV/0!	#DIV/0!
70	17493	4057	50237	44284	82512	3718	124432	49843	112238	21529
71	1114	1575	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
72	5699	1537	24939	26563	24001	1652	36525	13460	31768	2594
73	16636	6655	38189	29039	78519	6266	127416	32823	116467	3596

Table C8. Experimental data for different treatments at 340mm after 104 days. /334

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	33178	8109	20178	3672	12681	6502	5432	2122	10945	11538
2	50808	4214	46544	5912	29546	4404	15930	2917	31665	25602
3	34819	1064	22909	393	14119	679	9303	1847	16204	12550
4	22691	6197	19904	5279	16362	3505	12974	3475	18965	7178
5	29622	10735	21144	7810	16282	5347	12802	3018	18471	8494
6	24114	7524	17413	5470	12969	896	6440	1815	12061	8411
7	47651	2709	47439	6693	35595	626	24205	5362	38572	20299
8	11167	5706	12215	2874	11435	1058	1849	1605	4749	3114
9	9917	#DIV/0!	7396	2573	6483	951	1669	1461	5238	4485
10	83156	10776	98785	19888	77329	10573	30215	7316	60044	39119
11	38542	12232	41473	2881	30499	6310	15662	3113	28574	17953
12	40026	9501	39599	832	29881	5260	16064	3206	28750	16602
13	327146	6505	317039	21044	258610	28824	136346	26204	225788	125703
14	166413	3367	159882	12125	124970	7859	62331	12361	110788	67131
15	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
16	242449	1932	234257	12955	186821	17749	89522	17747	161909	99811
17	72830	49	68763	572	50868	2773	27676	5553	49965	28853
18	153070	4258	150796	9389	117013	9075	50030	9846	98129	64675
19	50685	4989	45754	4593	38540	10566	15714	3203	29431	18607
20	10829	10	9820	1341	7619	205	2097	1821	5573	4892
21	12779	2022	17729	1266	24603	19033	7671	1724	11358	4438
22	130893	14515	145961	17184	156946	84729	46270	9001	82327	55049
23	558668	779	550072	27183	488837	128825	179038	37457	332783	239955
24	96803	744	92845	2039	82141	24977	31716	6153	59636	41395
25	149206	13547	156905	6866	145005	48908	52964	11164	93960	63440
26	614076	19735	614094	23006	633906	284051	244242	52655	405538	241783
27	264806	3461	262100	7673	256850	104391	79293	15734	157169	116847
28	15094	625	15375	1193	15057	6938	3488	723	8090	7306
29	40420	977	40237	1906	37488	15680	12598	2465	23591	16355
30	198757	1394	197621	8784	194262	81813	59650	11760	115921	83975
31	393625	1193	397857	11099	388631	167629	110203	22589	223644	171479
32	125841	1020	127237	4202	127274	61518	36188	7094	72380	53818
33	694055	5850	693175	30179	690209	288024	229396	52143	414683	284860
34	231885	1464	227320	11780	204331	67165	76843	27707	130480	98756
35	146644	6157	147643	6959	137730	49445	50849	17374	85634	63468
36	45290	885	46282	3454	49542	27326	14083	2856	25658	17479
37	873193	13407	883624	31895	859209	342188	249952	73390	340161	157607
38	76968	10431	79473	1	73860	24332	21625	5596	150905	222082
39	94703	3099	93576	745	83850	25546	37588	12015	60348	40599
40	511665	8404	505384	17923	468998	156736	166556	64853	280370	215077
41	448342	4341	455990	14443	484350	242301	120166	31736	240912	199478
42	250305	11230	241017	8861	210578	58071	53200	21182	119913	122231
43	59279	1347	56364	1275	38690	3197	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
44	51575	3531	50841	1120	56699	31119	13903	2757	28846	23151
45	83115	4694	79998	3521	74931	26110	25271	8726	45449	36796
46	890017	22014	892079	19322	870899	349689	233528	76899	467454	397837
47	91432	11189	86216	2027	82053	24708	24864	9549	48465	43291
48	33582	2217	31344	1725	26312	1033	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
49	177688	10209	157446	1128	120022	10661	61186	35009	90239	72973
50	101121	5758	91297	4104	75821	11823	34730	18440	51078	40455
51	201791	5328	200523	2557	216569	112765	50492	12136	104366	89807
52	31150	7068	24217	928	22731	2379	9631	5253	15390	13894
53	164076	7519	154229	5078	119945	15290	56274	31162	81580	64878
54	95410	9692	90675	1818	70466	7715	33850	18861	48787	38437
55	151334	7042	147647	1843	154206	76579	41584	16544	72347	60719
56	41405	5044	37445	472	44811	25436	5131	1169	16921	19054
57	40045	8334	34151	538	33038	10448	11449	6456	18594	16515
58	379672	16618	356958	5836	284591	48604	106660	62675	168658	150294
59	36404	1765	35858	560	30431	1463	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
60	15520	6650	11837	3107	13197	6662	3722	1614	8597	5583
61	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
62	22300	10631	17626	7259	128217	143815	61810	24217	20361	24928
63	226397	8690	220775	836	182011	46865	62526	33578	97892	85033
64	29348	1269	26008	754	24692	995	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
65	75333	682	73377	396	57381	7962	23040	13558	32218	26024
66	89140	2970	88406	1007	65867	10040	26997	16153	38157	28960
67	12451	6415	17468	1361	9736	8017	1248	2162	2972	3044
68	43021	7574	42844	3887	32832	6653	11656	6444	16824	11440
69	10695	3397	13153	4486	9945	2826	685	1186	872	1510
70	82886	2003	75746	8209	55675	2752	22376	12811	30886	20890
71	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
72	37223	1438	34245	13403	38502	30345	7226	3425	10586	7196
73	49472	887	59245	740	86159	18522	16652	1154	42683	38915

Table C9. Experimental data for different treatments at 340mm after 140 days. /335

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	8908	919	4915	870	4448	605	8900	597	7025	1246
2	13224	2718	13063	2633	12485	1181	20168	3002	15491	4417
3	12856	1399	8047	1723	7984	433	12972	2210	9065	3023
4	10899	1901	9209	1538	12061	1752	13595	2610	11624	2296
5	14836	2021	9327	1491	12525	1047	15365	1593	14263	1491
6	8908	1991	5514	916	6801	1112	10891	1421	9019	1541
7	32213	5735	20629	2888	23348	2006	29506	7965	26988	4462
8	2700	381	0	0	1645	1426	2404	2096	2741	158
9	3350	474	1058	1496	2296	113	2405	2095	2743	27
10	40582	2505	25057	4757	31318	3464	31050	11799	31033	6135
11	26830	7444	15504	1508	16458	923	19213	5283	17325	3422
12	26828	6573	15489	2073	16675	913	19549	5284	17957	3602
13	229012	65293	134405	14243	145554	8659	169942	49651	152478	29231
14	105258	28937	61101	7529	66572	3600	77277	23443	70549	13495
15	1	0	0	0	0	0	2568	2224	728	1260
16	155151	46653	87591	10640	94938	6067	115596	35701	103618	19195
17	45469	11558	26641	3759	28824	1305	34685	10193	31705	5426
18	82662	23677	48437	6646	52715	2907	63338	18915	56745	11209
19	25671	6869	15112	1989	16171	736	19804	5230	17538	3630
20	4290	1012	2603	447	2725	125	4578	1267	2198	1920
21	11113	3112	7169	1187	8166	276	10140	4236	7931	1604
22	64570	21616	43313	6301	48639	3631	59807	22924	50727	9188
23	303597	94025	179418	15092	192611	17455	229200	72822	201661	36556
24	55356	17082	33027	3248	35287	3275	41968	13822	37016	6765
25	90460	28998	54263	6446	57734	5352	69506	24732	60001	11526
26	429221	145680	253480	19513	270566	28014	329173	109830	282748	53308
27	147829	52344	85647	7963	91267	9596	110011	38964	94908	18774
28	6355	2222	3710	434	3954	419	5186	1893	4375	794
29	22552	7655	13468	1340	14307	1488	17399	6062	14961	2907
30	107897	37467	64260	6175	68122	7067	84492	29592	70912	14035
31	207190	77474	119697	11804	128040	14943	161249	59355	132767	28243
32	67535	24702	39508	4116	42017	4456	52495	18957	43801	9056
33	418687	158223	241774	19632	257322	30065	324678	115503	271127	58177
34	120272	43918	71165	6548	75134	8074	93667	32958	78630	15853
35	79349	28481	48156	4311	50812	5478	61893	20875	52762	10657
36	25121	9409	15452	1689	16319	1959	20520	7236	16942	3870
37	417129	157970	250468	19728	266264	31223	332006	114740	276837	61794
38	34931	13324	21905	1913	23363	2635	27722	9755	24093	5474
39	59631	21536	37443	3298	39508	4186	48320	16890	40474	8572
40	254415	93332	157576	12504	166469	18073	206799	72843	169739	36710
41	204364	81425	124078	9986	131897	16092	169182	61050	139484	33671
42	80354	30906	49945	4565	52991	5682	66342	24079	53926	11771
43	32565	11256	20280	1363	21807	1815	26060	9034	21939	3671
44	24928	9376	16195	1409	17003	1949	21329	7621	17578	4389
45	38672	14262	24721	2145	26206	2772	32924	11680	27090	6075
46	366666	146020	226756	16698	240475	27620	309635	113770	248865	60865
47	37812	14046	24543	1904	26988	4146	32217	11366	26666	5938
48	5617	2084	3669	308	11832	13989	4949	1853	3955	856
49	75234	27222	47950	3174	34282	26180	63077	23025	50768	9040
50	43453	15940	28421	2161	30442	2483	35153	12788	28928	6028
51	84206	33132	54717	4447	57663	6524	75701	28042	60687	15672
52	11566	3991	7702	532	8240	645	10334	3475	8233	1420
53	69036	25069	45349	2989	48310	4095	60980	22202	47927	8984
54	41250	14820	27202	1790	28969	2394	36466	13060	28240	5599
55	57615	21497	39518	3046	41510	3956	54399	19793	41133	10404
56	9315	3454	6607	546	6883	748	9593	3334	7039	1976
57	14304	5116	9914	839	10419	960	13700	4823	10054	1829
58	128381	48429	86789	7101	91340	8376	119654	46182	86162	17607
59	8205	2839	5740	506	6093	543	7837	2623	5803	819
60	4318	1501	3142	264	3305	292	4740	1379	3365	158
61	0	0	0	0	0	0	0	0	0	0
62	4189	1641	2906	173	3044	256	3497	1292	2595	448
63	74051	27954	52006	4062	54130	5246	72368	26878	54708	11630
64	1413	1998	0	0	0	0	2017	1774	686	1188
65	25929	9294	18553	1220	19404	1615	25660	9905	19058	3240
66	29965	10741	21567	1486	22520	1634	29617	11352	21789	3630
67	2951	1182	2145	171	2263	41	2484	2171	1605	1447
68	12837	5011	9253	616	9680	482	11334	4564	8531	1966
69	0	0	0	0	0	0	707	1225	1018	1764
70	23863	8913	16984	1078	18531	1251	24381	8935	17638	2361
71	1311	1854	0	0	0	0	2794	577	850	1472
72	7522	2704	5771	366	6241	431	8834	3060	6509	569
73	19601	5705	16933	173	17810	1383	26128	1445	19583	5577

Table C10. Experimental data for different treatments at 340mm after 282 days

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	3311	17	3450	106	3263	108	4095	645	2930	397
2	5041	45	10286	805	10684	1432	11353	1022	8693	822
3	5820	26	6902	100	6905	662	7729	110	6222	469
4	5416	32	9894	229	9664	1180	9192	1506	9043	335
5	6838	241	9523	21	9442	1218	9281	841	8718	317
6	4690	63	5738	314	5803	829	5898	243	5202	665
7	16087	294	20839	133	20549	2235	19908	3306	18672	801
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	679	1177	0	0	0	0
10	20891	98	27435	665	27544	2700	24669	4872	24759	1669
11	16384	59	16998	176	16312	1235	15733	2898	14924	1358
12	15884	174	16094	70	15949	1680	15180	2666	14494	970
13	149403	1005	146367	1397	142910	12320	138290	23301	131589	10916
14	67121	319	66154	827	65363	6210	62493	10672	59435	4325
15	1	0	0	0	0	0	0	0	0	0
16	98056	922	95342	257	93951	9324	90083	15532	84735	6813
17	29105	161	28816	137	28947	3090	27276	4345	25858	1524
18	53106	305	53298	6	53648	5915	50475	8567	47563	3398
19	16564	421	16158	201	16420	1733	15356	2576	14535	918
20	3330	103	3052	183	3071	255	2889	469	2645	184
21	8161	234	8226	365	8498	733	7414	1488	7531	442
22	45528	987	49735	1417	50544	4891	46457	9000	44440	4237
23	221991	925	205009	1022	200149	16661	191141	32677	184020	18012
24	40724	144	36944	164	36653	3360	34681	5855	33336	3620
25	67219	343	61503	552	61619	6045	57373	10296	55714	5794
26	320664	2407	294751	800	286629	25251	273247	50503	265830	32637
27	111206	620	99324	66	97850	9416	92558	17182	89582	11461
28	4715	134	4150	11	4094	477	3835	759	3743	523
29	17032	486	15229	126	14966	1488	14109	2761	13957	1922
30	81800	2251	74074	648	72158	7057	68316	13559	68169	10292
31	155873	5173	140534	2086	136895	12992	127205	24378	129357	19929
32	51284	1789	45829	631	44851	4632	41677	7785	41976	6851
33	312671	10316	290216	9806	274955	28470	257104	49593	260254	40314
34	90890	3106	84326	1828	81421	9342	74880	12806	76459	13364
35	61013	2170	57168	660	54779	5917	49989	8263	51420	8533
36	19386	733	17883	298	17305	2090	15526	2801	15874	2595
37	322113	13698	307356	6549	289236	31644	264228	46697	272358	45888
38	27035	1230	25797	775	24838	2859	22275	3838	23016	3863
39	48029	2501	45823	357	43396	4986	39279	6370	40495	6881
40	204467	10418	197904	1112	185978	21304	168682	28171	174899	31004
41	161226	8295	155138	3886	145108	17732	130964	23177	135079	23033
42	65151	3623	62829	93	59212	7504	53118	8537	55148	10770
43	25491	1110	24723	751	23683	2915	21161	3105	22646	5219
44	20856	1207	19434	208	18368	2547	16201	2322	16988	2737
45	31199	1623	29245	950	27953	4017	24453	3338	26092	5013
46	298796	18356	306827	25406	279414	32761	262325	59633	262562	45543
47	31588	1909	29973	839	28520	3951	25242	3521	26860	5203
48	4664	291	4411	235	4265	698	3674	529	4031	903
49	76695	24308	60393	1169	57809	7370	51755	8265	55238	13093
50	36615	2111	35796	741	34350	4574	30445	4887	32563	7548
51	71269	5635	68318	1018	64469	9254	57671	10076	59635	9939
52	10131	549	9953	319	9491	1291	8499	1270	8974	2123
53	59193	3613	57797	849	55545	7449	49723	8036	52631	12105
54	35230	2183	34253	540	33125	4580	29566	4760	31359	7044
55	50180	3916	49403	255	47349	6587	42149	7146	45626	6544
56	8227	744	8054	40	7579	1177	6676	1310	7065	403
57	12710	826	12433	237	12027	1638	10598	1589	11673	2213
58	115149	7882	112486	2571	98453	29644	95505	15218	105354	22004
59	7577	607	7376	93	7285	942	6345	823	7026	1418
60	4070	319	4024	51	3873	529	3464	475	3773	605
61	0	0	1122	1586	1028	1781	968	1677	1482	1284
62	4233	326	4082	126	3963	546	3439	605	3943	879
63	68519	5903	66344	1225	64664	9775	58663	11482	62240	12093
64	1070	1513	0	0	749	1297	722	1251	826	1431
65	23713	1761	23029	734	22745	3229	20649	3766	22289	5200
66	27818	2188	27031	865	26792	3846	24457	4512	26322	6208
67	2630	252	2567	74	2572	386	1821	1594	2609	550
68	12350	962	11827	417	11822	1804	10586	2165	11978	2272
69	0	0	0	0	0	0	0	0	0	0
70	23046	1953	22256	845	22197	3333	20094	3802	22345	4444
71	0	0	0	0	733	1270	0	0	688	1191
72	7058	733	6980	314	7092	1074	6447	1465	7102	1173
73	18402	2117	19596	2307	22232	2042	20070	3259	18895	1764

Table C11. Experimental data for different treatments at 340mm after 314 days

peak	Control	SD	Water	SD	A-025	SD	B-356	SD	Indigenous	SD
1	5489	1504	5810	352	5078	1143	5364	475	6060	1395
2	8683	1348	19485	1960	19216	4471	17933	1507	19110	3834
3	7952	1302	11141	680	9251	1915	9846	384	11091	2375
4	7651	1339	15307	816	14356	3246	14068	605	16445	1952
5	11498	1532	16709	1182	16706	1849	15922	1116	17717	1445
6	7935	1068	9384	1295	10438	2281	9003	872	9616	1909
7	25959	3929	33909	3258	32944	6391	31847	898	36100	6103
8	1240	1753	3172	322	3657	918	3028	266	3556	677
9	2786	540	3280	323	3408	715	3140	158	3571	713
10	35559	5378	45700	5540	50712	9896	43069	2786	50299	9013
11	24145	3113	24307	1875	22142	3807	22329	384	25268	4475
12	24972	4436	25746	2245	24431	4045	23347	418	26637	4578
13	221172	36847	214825	17880	207252	33435	198949	2574	237654	54294
14	103610	17973	101087	9294	99389	16850	92798	996	108847	23562
15	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
16	161686	29362	149752	15365	153559	26551	137868	2143	164800	37524
17	47715	8806	45430	4815	47831	8240	41664	406	48039	8904
18	91157	16737	84744	10526	91443	15242	77520	1215	91930	19576
19	27495	5173	26076	2708	27282	4376	23863	77	28312	5796
20	4147	519	3699	474	4540	912	3370	140	4008	915
21	12265	2503	12215	1849	15276	2472	11210	513	19009	9739
22	73583	15167	77111	9790	94836	15755	71368	1157	106519	44775
23	322555	60330	294379	26556	293995	46839	270868	2797	346513	108685
24	60683	11927	54912	5037	55683	9001	50286	247	66413	23079
25	104026	22002	90687	9090	101573	17117	81984	268	112798	42478
26	470474	94003	419773	41864	435306	69580	379711	5860	516239	193154
27	168345	35420	144799	15342	156867	26285	129956	1088	187397	80893
28	7081	1604	6286	542	6734	1223	5454	5	8110	3691
29	25775	5501	22476	1783	24270	4078	19925	85	28059	11643
30	129198	27446	106721	9774	123275	20699	95444	657	135626	58200
31	251012	55695	203878	20266	239323	42173	179100	2725	258771	117313
32	82106	18830	67086	5933	78644	13804	58281	987	86298	40856
33	492496	108607	411207	41739	460282	77196	362314	7629	516304	227877
34	140190	32117	118976	10486	133666	22994	103222	1313	141551	57938
35	91173	21121	78583	6900	89766	14994	68215	531	95394	40872
36	29619	7177	24535	2630	30238	5526	21368	64	34603	19147
37	493327	113624	409951	54686	449710	139294	357677	5796	534022	260784
38	39432	9355	35294	3770	40525	7424	30637	368	46305	22747
39	69438	16750	58342	6399	69626	12219	50579	606	74702	35401
40	301647	73413	247758	32028	299982	49872	215314	3270	312111	144630
41	250157	59856	199836	26290	252531	45532	172991	3488	278514	153829
42	96503	24548	77575	10950	99660	17975	64837	1622	95782	45835
43	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
44	29079	7326	23480	2821	30602	6620	19804	409	33473	20085
45	43494	10970	36201	4173	43583	7491	30801	713	46805	23846
46	501198	128252	396299	55317	501745	88841	334935	7235	495422	232383
47	43222	11090	36069	4302	43629	7516	30271	729	45504	22435
48	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
49	85638	22677	73152	8692	86887	13319	61386	2087	79512	26966
50	49677	13445	42344	4859	50728	7909	34916	1360	46717	17334
51	103080	28085	80893	8986	109842	20448	67011	2120	114131	68784
52	12593	3256	10932	1249	13146	2068	9039	465	11449	3674
53	79991	21198	66423	6931	83991	12768	54681	2516	71946	25433
54	47780	13098	40218	3951	49989	7285	33137	1697	43953	15410
55	65773	18599	55534	4622	74954	12801	44814	2339	72062	39391
56	10158	2813	8818	554	12024	2104	7005	593	15476	12856
57	15111	4411	13573	977	16697	2616	10789	899	14733	5796
58	143985	42986	119820	10699	161795	26339	95041	6980	130337	50854
59	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
60	4475	1224	5892	2162	5002	866	3201	563	4396	1946
61	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
62	4749	1143	4122	314	4339	845	3103	416	4039	1595
63	76789	21831	68591	5777	90892	15029	53682	4226	76524	32298
64	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
65	25844	7078	24280	2281	30196	5096	18793	1648	25087	8913
66	30423	10414	27818	2689	33629	5459	21045	1896	27939	9746
67	1605	2270	2736	203	2901	527	670	1160	2809	1400
68	12831	3713	12043	1372	14321	2177	8842	904	11761	4106
69	0	0	1432	2024	0	0	0	0	1032	583
70	22323	6797	23534	4996	25787	3826	18509	1492	23341	5740
71	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
72	5610	1959	7828	181	8062	1635	5458	695	7923	3804
73	16607	5051	23465	275	20515	2446	14609	2431	18349	4893

Table C12. Experimental data for different treatments at 340mm after 377 days

Initial	SD
13926	5921
49619	15477
23521	5893
35924	4297
30421	4954
22254	3763
68012	10027
9615	1677
9066	4004
129191	30786
51463	6460
48826	5365
402937	58096
202086	27045
1245	2156
299928	38387
93485	10781
188859	29451
54121	8500
11347	1687
32192	3667
199150	22857
668865	82110
112318	12270
214934	17171
883558	50544
330653	36159
18064	1517
49638	6144
247799	30477
497877	62581
161053	24031
906182	125448
311050	21502
204574	12598
63309	9168
1131751	82225
101171	12020
147207	7593
714378	78558
578048	36262
328977	105315
25075	43432
68223	8587
112937	9271
1166725	159058
121208	22115
4950	8574
259456	86812
149781	49768
264528	24299
42361	16736
240574	73976
141822	44843
211030	38608
47443	17080
53644	17875
562015	230851
6526	11303
17475	7841
42916	39025
3340	5785
324970	120765
8068	2598
105291	43532
126967	56658
12180	3919
54623	24662
5844	1838
113046	56962
9516	3962
29353	9942
78961	25801

Table C13. Experimental data for the initial PCB content of the soil.