THE INFLUENCE OF SOIL PARTICLE SURFACES AND SOIL POROSITY ON THE BIODEGRADATION OF KEY REFUSE LEACHATE ORGANIC MOLECULES

by

Chris André du Plessis

Bsc Agric (Hons)

Submitted in fulfilment of the academic
requirements for the degree

Doctor of Philosophy

in the
Department of Microbiology and Plant Pathology,
University of Natal

Pietermaritzburg

1995

THE INFLUENCE OF SOIL PARTICLE SURFACES AND POROSITY ON BIODEGRADATION

by

Chris André du Plessis

Bsc Agric (Hons)

Submitted in fulfilment of the academic

requirements for the degree

Doctor of Philosophy

in the

Department of Microbiology and Plant Pathology,

University of Natal

Pietermaritzburg

1995

DECLARATION

The experimental work described in this thesis was carried out in the Department of Microbiology and Plant Pathology (International Centre for Waste Technology, Africa), University of Natal, Pietermaritzburg, from January 1992 to October 1995, under the supervision of Professors Eric Senior and Jeff Hughes.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others it is duly acknowledged in the text.

Chris A. du Plessis   B.Sc. Agric (Hons)

"To pursue a clue, we must study the fates of species present in, or added to, soils under monitored or controlled (alas, unnatural) environmental conditions. Be assured that mother nature will never violate any of her laws but she will stretch, fold, spindle, mutilate and disguise them in costumes unrecognizable to the orderly minded scientist" (Anon.).

"I will destroy the wisdom of the wise; the intelligence of the intelligent I will frustrate. Where is the wise man? Where is the scholar? Where is the philosopher of this age? Has God not made foolish the wisdom of the world?" (1 Corinthians 1:19-20).

ACKNOWLEDGEMENTS

ABSTRACT

Many studies have been undertaken to determine the effects of soil and soil properties on migrating metal pollutants. Organic pollutants, however, in addition to their interactions with soil components, are also susceptible to degradation (catabolism) by microorganisms.

Soil-microorganism-pollutant interactions have, traditionally, been studied in soil columns (microcosms). One of the shortcomings of column and *in situ* studies is that the identity and specific effect(s) of the soil component(s) affecting or influencing attenuation are not known and cannot readily be determined. Attenuation effects of the soil components are, therefore, difficult to interpret. *("Attenuation" in this context is the combined effects of both soil adsorption and microbial catabolism).* Attenuation studies often only consider the physical conditions such as aeration, permeability, flow rate, temperature, etc. This approach assumes the soil to be a homogeneous matrix with no specific physico-chemical properties attributable to different components within the matrix. Soil physical factors suspected of influencing pollutant attenuation could be misleading without consideration of the physico-chemical interactions between soil components, microorganisms and pollutants. Adhesion of pollutants and microorganisms seems to be most important in this regard.

The initial phase of this study was undertaken to examine the effects of three different soil materials on attenuation of key landfill leachate molecules. Examination of the effects of soil surface type on attenuation focused on adsorption / desorption of the pollutant molecules and microorganisms. These experiments sought to investigate the physico-chemical effects of soil, microorganism, pollutant interactions and were done as batch slurry experiments as well as in soil columns. Two soil horizons from the Inanda soil form (humic A and red apedal B) and the topsoil (vertic A) from a Rensburg soil form were used. The Inanda topsoil had a high organic matter content and both the topsoil and subsoil had a kaolinitic clay mineralogy; the Rensburg topsoil clay mineralogy was predominantly smectitic with a relatively low organic matter content .

From the batch experiments, the adsorption of a hydrophobic molecule (naphthalene) and a

heavy metal (cadmium) were found to be influenced to a significant extent by soil characteristics.

Adsorption of naphthalene was due to the soil organic matter (SOM) content whereas cadmium adsorption was due to the cation exchange capacity (CEC) of the soil. Soil characteristics did not seem to have a significant influence on the adsorption of a water soluble compound such as phenol at the concentrations used. Attenuation of naphthalene was found to be affected by adsorption of the pollutant molecule (related to SOM) as well as the CEC of the soil. The attenuation of hydrophobic molecules can possibly be ascribed to the influence of CEC on the microbial population responsible for attenuation. This would seem to indicate interaction between the soil surfaces and the catabolizing microbial population. Desorption of the pollutant (and possibly also of the microbial population) was achieved by the addition of acetonitrile and methanol both of which reduced the polarity of the water. These solvents were also found to be toxic to the catabolizing microbial population at high concentrations. The toxicity thresholds of both solvents for catabolizing microorganisms differed significantly between soil- (> 15 %, v/v) and soil free (< 5 %, v/v) treatments. This discrepancy cannot be accounted for by adsorption and is ascribed to physico-chemical interaction between microorganisms and the soil surfaces. This interaction probably affords protection from, otherwise, toxic concentrations of solvents or metals. The important effects of soil surfaces on attenuation processes were thought to be due to the strong adsorption of naphthalene. Surface attachment of microorganisms was, however, also inferred from results obtained with phenol. This seemed to indicate that microbial attachment to soil surfaces was an important aspect in attenuation and did not occur only because of pollutant adsorption.

Soil column experiments were made with both naphthalene and phenol. The naphthalene, which was adsorbed to the soil, did not leach from the columns to any appreciable extent. This was despite the addition of acetonitrile to some columns. This was probably due to greater microbial catabolism caused by desorption and, subsequent, increased soluble concentrations of the molecule. After extraction from the soil at the end of the experiment it was clear that the sterile controls held much higher concentrations of naphthalene than the experimental columns. The soil type and treatments showed little difference in the naphthalene

concentration extracted from the soil columns. This did not reflect the differences found between soil materials in the batch experiments and was probably due to the masking effect of the soil physical factors on attenuation processes. Unlike naphthalene, phenol, because of its high solubility, was detected in the column leachates at relatively high concentrations. The phenol concentrations were much higher for the Inanda subsoil (approximately 4 mM) than the Inanda topsoil (approximately 2 mM) and Rensburg topsoil ($<$ 1 mM). The Rensburg topsoil produced the lowest phenol concentrations in the leachate and this can probably be ascribed to the larger quantity of micropores in this soil. Thus, it seems that the soil physical features had a pronounced influence on attenuation. Whether this effect was directly on the studied molecule or indirectly, because of the effects on the microbial population, is not known. Inoculation of the columns with a phenol catabolizing population had only a slight increased effect on leachate phenol concentrations from all columns. This increased effect was, however, only prolonged in the case of the Inanda subsoil. The flow rate through the columns affected leachate phenol concentration which was lower with a slower flow rate and, thus, longer retention time.

From the column experiments soil physical parameters were suspected of influencing, and possibly overriding, the soil surface effects on microbial activity (capacity to catabolize a organic molecule of interest). Soil porosity, as caused by different soil materials, was suspected of being the most important soil physical parameter influencing microbial activity. To investigate the potential effect of soil porosity, relatively homogeneous porous media i.e. chromatography packing material and acid washed sand were used. These materials had more defined and distinct porosities and were considered to be suitable for investigating the fundamental influence of porosity on microbial activity. Saturated continuous flow columns were used and three types of packing configurations were tested: chromatography packing (CHROM) material (porous particles); acid washed sand (non-porous) (AWS); and a 1:1 (w/w) mixture of chromatography packing and acid washed sand (MIX). Only a single water soluble molecule, phenol, was used in this phase of the investigation.

Bacterial filtration (*"filtration" as a component of "attenuation"*) was found to be highest for the CHROM and lowest for the AWS materials. This difference in microbial retention

affected the phenol catabolism in response to increased column dilution rates. The CHROM and MIX materials had distinctly different porosities than that of the AWS, due to the internal porosity of the chromatography packing. This greater pore size distribution in the MIX and CHROM packing materials created pores with different effective pore dilution rates within the microcosms at similar overall flow rates. The greater pore size distribution in the MIX and CHROM packing materials facilitated pore colonization since some pores did not participate, or conduct, mass flow as occurred in macropores. This led to different microcolonization effects in the macro- vs micropores. Since the MIX and CHROM packing materials had more micropore colonization sites these packing materials showed a greater range of substrate affinities (i.e. K_s values) for the phenol substrate.

The extent to which micropore colonization occurred could be detected by the effect it had on phenol breakthrough curves. In the MIX and CHROM materials, microbial colonization caused blocking of micropores with a subsequent effect on the phenol breakthrough curves. The AWS material, however, which had a low inherent microporosity, showed microbially induced microporosity probably due to biofilm development. The fact that the MIX and CHROM packing materials facilitated micropore colonization was also responsible for the greater resistance to, and the recovery from, potentially inhibitory cadmium concentrations. This effect was also apparent in the presence of acetonitrile, although this effect was not identical to that observed with cadmium. Finally, column pressure build up as a function of pore clogging was determined and was found to occur in the order AWS > MIX > CHROM. This was most likely due to fewer potential liquid flow paths with a higher blocking potential in the AWS.

Extrapolation of the fundamentals of the above findings led to the conclusion that soil surface- and soil porosity effects are extremely important factors in determining the behavior of soils as bioreactors.

TABLE OF CONTENTS

INTRODUCTION

Many studies have been undertaken to determine the effects of soil and soil properties on migrating metal pollutants (Fuller, 1978; 1980). Organic pollutants, however, in addition to their interactions with soil components, are also susceptible to catabolism by microorganisms. *(Throughout this thesis the collective effects of both adsorption and microbial catabolism is termed "attenuation").* There are several important reasons for studying the interactions between pollutants, microorganisms and soil:

- Pollutants in the soil environment often migrate to the groundwater or open water bodies. This cannot be afforded in, especially, a country with a limited water supply such as South Africa;

- Understanding the mechanisms of soil-microorganism-pollutant (SMP) interactions will facilitate risk assessment in various threatened ecosystems and should be of great importance for environmental impact studies. This may be applied to potential pollutants, including agricultural and industrial chemicals (Knezovich *et al.*, 1988);

- Under certain conditions, soil filtration of pollutants, including industrial effluents, may be a viable treatment option (Gilmour *et al.*, 1977). Thus, the study of SMP interactions could be useful in evaluating the use of soil filtration as a treatment option. The most suitable soil for any particular effluent could be identified by such studies;

- Bioreclamation of polluted soils is greatly dependent on the characteristics of the soils, microorganisms and pollutants as well as their interactions (Piotrowski, 1991). These factors will, therefore, also be of importance in landfarming operations;

- Pesticides added to soil are subject to microbial catabolism as well as soil adsorption. These interactions affect pesticide activity and environmental safety (Boesten & van der Linden, 1991; Boesten *et al.*, 1991);

- Microorganism-soil interactions are relevant to inoculants added to soils (van Elsas & Heijnen, 1990) and may also be important because of their effects on soil borne plant pathogens (Stotzky, 1986); and

- Soil components, in the form of sediment or suspended particles, are also present in these environments where they may, thus, influence the attenuation of pollutants in open waters.

Research into SMP interactions is recent in South Africa. Even on a global scale, experience in this particular field of study is very limited. The present study initially focused on the SMP interactions to gain a better understanding of their influence in the soil of the final and intermediate cappings of landfills. A better understanding of these interactions can be of great practical importance for optimal use and management of landfills and, subsequent, reclamation. Soil, potentially, has a great buffering capacity so that the landfill as a bioreactor may be protected against surges of certain chemical or even physical challenges. Soils also differentially adsorb compounds. These interactions need to be understood so that the effects of various soil types on the landfill can be predicted.

The principal objectives of this study were to determine the interactions between soil characteristics, microorganism and model landfill molecules in the attenuation of these molecules. In order to create a more defined experimental environment certain variables had to be eliminated to facilitate accurate interpretation of results. All experiments were conducted under aerobic (or micro-aerobic) conditions. Although anaerobic conditions mostly prevail in landfills and at soil depths at which many pollutant molecules occur, soil remediation processes are more effectively implemented in the aerobic (closer to the surface) zone. Microbial variability was eliminated by using a constant inoculum. The influence of soil on two of the components of attenuation i.e. desorption and catabolism was studied. Volatilization was not considered because the studies were conducted in sealed containers. Although the containers were sealed the headspace allowed for sufficiently aerobic conditions. Scanning electron microscopy was the method chosen to reveal the nature and mechanisms of microbial attachment to soil particles. The effects of solvents on the SMP system had to be determined because of the adsorption of, particularly, the hydrophobic compounds and the fact that solvents could cause desorption of such compounds. The effects of a heavy metal, Cd, which can be found in landfills, was also studied.

Following elucidation of the physico-chemical interactions, soil columns were used to more closely approximate *in situ* conditions. Because of the complex nature of the study and the anticipated difficulties with soil related microbiology, it was decided to use various different experiments to investigate single variables. It is important to note that the adopted approach was to reduce replication in favour of more variables. The rationale behind this decision was

that it would be difficult to gain an understanding of the SMP system by examining only limited experimental variables and replicating such experiments for statistical significance. Because of the scarcity of information combining all the elements of the SMP system it was felt that this approach was most suitable.

The aim of the initial phase of the study was to investigate the physico-chemical interactions of soils, microorganisms and selected organic molecules to gain a better understanding of landfill covering soils. It was, however, soon realized that an even more fundamental approach was needed and that an in-depth study of the relevant interactions would necessitate experimental simplification. After the initial phase of investigation into the surface interactions of soils, microorganisms and two selected organic molecules, it was found that these surface effects did not account for all the observed differences between soils and their effect on microbial processes. This was particularly true under conditions where the soils were studied in microcosms (columns) rather than in slurries (where the porosity effects are eliminated). Soil microcosms were used since they more closely (although not precisely) simulate *in situ* conditions. This led the study to further investigate the effects of soil porosity in addition to soil surface effects. To facilitate such investigation further simplification of the experimental procedures was required which was even further removed from the original objective of understanding the role of landfill covering soils in landfills. The suspected (later proven) effects of porosity were considered to be of such importance, however, that this simplification and focus on the fundamental influences of soil parameters on microbial activity were considered to be justified. Such simplification did, however, cause the study to become experimentally distant from the original aim of understanding the influence of landfill covering soils on the behaviour of a landfill as a bioreactor. The findings of such experiments could, thus, not easily be extrapolated to landfill covering soils. The implications of the results were, therefore, discussed with regard to soil in general and not primarily with regard to landfill covering soils. Certain fundamental findings were, however, made which were applicable to soils in general and, therefore, by extrapolation also to landfill covering soils.

Due to the extremely variable nature of soil porosity and the experimental error which this factor introduces it was decided to use more homogeneous porous media. Chromatography

packing material and acid washed sand were used for this purpose. Although this could be considered to be too far removed from actual soil particles, the porosity of these porous media can be related to actual soil porosity.

The experimental sequence of this study is, therefore, divided into two distinct sections. Part I deals with the fundamental effects of soil surfaces on the potential behaviour of soil as a bioreactor. Part II deals with the fundamental effects of porous media on microbial activity, and is extrapolated to the potential effects in soils.

PART I
CHAPTER 1. THE INTERACTIONS BETWEEN SOIL, MICROORGANISMS AND POLLUTANTS

1.0   LITERATURE REVIEW

This review is by no means comprehensive enough to include all aspects which impinge on this particular field of study. It is, however, designed to cover the major principles which are important to an understanding of the topic and for interpretation of the results.

1.1   The importance of adhesion

The soil provides surface area for interaction with microorganisms and pollutants (Scott *et al.*, 1982; Fontaine *et al.*, 1991). Although soil coverings (intermediate and final) constitute only a small fraction of the landfill volume, the total surface area per unit volume of the soil greatly exceeds that of refuse. It is, therefore, reasonable to assume that the soil will have a significant influence on both microorganisms and pollutants migrating through the refuse mass. Upward migration of leachate, by capillary rise, takes place mainly through the micropores of the soil. The surface area (surrounding the pore) to pore space ratio, in these micropores, is much higher than the average for the pores that would normally be involved in downward (gravitational) migration of leachate. Migration through micropores will, thus, increase the effect of surface interaction. (Vertical migration may also be via saturated landfill gas). Interactions (adhesion) of microorganisms with surfaces have been found to influence metabolic activity and, therefore, also catabolic capabilities (biodegradation) in many instances (Fletcher, 1985; Bar-Or, 1990; van Loosdrecht *et al.*, 1990). Adhesion may also afford microorganisms greater resistance / protection against toxic substances (Kefford *et al.*, 1982). Research indicates that not only the microorganisms but also their enzyme activities are influenced by adhesion to different soil components (Gianfreda *et al.*, 1992).

It is well known that soil components have an affect on the overall nutrient status of the soil environment including the soil solution (Foth, 1984). Natural soil often has relatively low

nutrient concentrations (electron donor poor) and bacterial inhabitants have to adopt starvation responses (i.e. endospores, cysts, low maintenance energy) to survive (Lappin-Scott & Costerton, 1990). These starvation responses also include the utilization of non-essential intracellular materials to meet the energy requirements of the cell, changes in cell morphology and adhesion, and, in extremes, metabolic arrest (Morita, 1990). Oligotrophy may also influence adhesion to soil particles (Marshall, 1988). Soil cappings of landfills may, initially, have low nutrient concentrations but as the carbon rich (electron donor rich) leachate migrates vertically through the soil, nutrient concentrations increase (Harmsen, 1983). The recovery process of the previously starved cells will be important at this stage (Morita, 1990) and is likely to influence organic chemical catabolism in the leachate. The ratio of nutrient elements in solution may be influenced by soil properties through selective adhesion of the different nutrient elements. Furthermore, nutrient element ratios will influence microbial growth, adhesion and organic pollutant catabolism. These ratios are influenced *inter alia* by cation exchange phenomena and pH, which influence the amount of nutrient cations on the exchange complex.

The adhesion of microorganisms, together with adsorption of organic molecules to soil components, are particularly important in pollutant attenuation in soil (Giles *et al.*, 1986). The major components and properties of the SMP system will, therefore, be discussed with specific reference to adhesion/adsorption.

1.2   Soil components and some of their properties

1.2.1 Clays

The nature of clays is such that they differ in their adsorption capacities (cation and anion) and specific surface areas (Harter, 1977, 1986; Burns, 1979). The effects of these properties on microorganism and pollutant interactions are, unfortunately, not well understood. Some of the main properties of clays which are relevant to adhesion of organic molecules and microorganisms are discussed below. For the sake of simplicity and comparison, two of the extremes in clay mineralogy, kaolinite (a 1:1 clay) (Dixon, 1989) and montmorillonite (a 2:1

clay) (Borchardt, 1989) will be considered.

In the silicate clay minerals the characteristic negative charge originates mainly from isomorphous substitution of Si^{4+} by Al^{3+} in the tetrahedral sheets, and Al^{3+} by Mg^{2+} and other divalent cations in the octahedral sheets (Foth, 1984). The negative charge can be balanced by adsorbed cations so that the clays are electrically neutral. The differences between clay minerals in their specific surface area and cation exchange capacity (CEC) are reflected in their mean surface charge density, which is an approximate indication of the closeness of the negative charges and is probably important in adhesion (Stotzky, 1985). Even though the specific surface area and CEC of montmorillonite (800 m^2 g^{-1} and 98 $cmol_c$ kg^{-1}, respectively) greatly exceed those for kaolinite (10 m^2 g^{-1} and 6 $cmol_c$ kg^{-1}), the mean surface charge density of kaolinite (6×10^{-3} $cmol_c$ kg^{-1} m^{-2}) is greater than that of montmorillonite (1.23×10^{-3} $cmol_c$ kg^{-1} m^2). Negative charges are, thus, closer together on kaolinite than on montmorillonite. The distance between point charges on clay surfaces does appear to affect the adsorption of, particularly, organic cations. Organic chemicals have been shown to be preferentially adsorbed on clays which have distances between surface charge sites similar to the charge separation on the organic molecule (Harter, 1977). Clustering of surface charges is also important. If the surface charge density is spatially located primarily in a cluster on only one side or on the broken edges of a clay unit, as is the case with kaolinite, then the probability of surface interactions between net negatively charged clays and cells is increased (Stotzky, 1985). The difference in isoelectric points for both the clays and micribial cell is an important determining factor in this regard. These aspects have not been evaluated sufficiently in the surface interactions between clays and microorganisms. It is also possible that greater surface acidity may favour fungal growth on surfaces (Van Demark & Batzing, 1987).

Some clay-sized components in soils possess a positive electrical charge which is balanced by anions so that the system as a whole is again electrically neutral. Anion exchange capacity (AEC) is due to protonation of OH^- groups on the broken edges of clays and on oxide or hydroxide minerals. The AEC of soils may have a particular influence on soil microorganisms and soil chemicals with negative surface charges (Stotzky, 1985). Anion exchange capacity is more prone to fluctuation due to pH changes than CEC (since CEC is

7

mainly due to permanent charges in most clays) and is also greatly dependent on soil mineral composition (Foth, 1984; Stotzky, 1985). The CEC:AEC ratio is probably just as important in adhesion of microorganisms to soil as CEC or AEC alone. The CEC:AEC ratio indicates the relative net negativity of different clays and the ability of negatively charged organisms and molecules to be attracted or repulsed by these clays (Stotzky, 1985). The average CEC:AEC ratio is 6.7 for montmorillonite and 0.5 for kaolinite. (The word "average" was not defined by Stotzky (1985)). *In situ* soil CEC:AEC ratios may be quite different from the determinations made under laboratory conditions where clays are normally analyzed in a dispersed state (The Non Affiliated Soil Analysis Work Committee, 1990). In natural soil systems many of the positively charged edge sites may be unavailable as a result of aggregation of the clay particles and blockage by negatively charged inorganic or organic molecules. This aspect will be particularly important in extrapolating results from single component dispersed systems to real soil systems.

1.2.2   Oxides and hydroxides

Together with hydrous aluminosilicates, many soils also contain other oxides, oxyhydroxides and hydroxides of Fe^{3+}, Fe^{2+}, Al^{3+}, Mn^{4+}, Mn^{2+}, Ti^{4+} and Si^{4+}. Only the iron and aluminium oxide minerals will be considered here since they constitute the major reactive oxide components in most soils. The basic characteristics of the aluminium hydroxides and oxyhydroxides, and iron hydroxides were given by Hsu (1989) and Schwertmann & Taylor (1989), respectively. Unlike silicate clays, where the inherent charge is mostly permanent because of isomorphous substitution, the exchange capacity of the oxide minerals is pH dependent (Foth, 1984). Oxide minerals at their isoelectric point or zero point of charge (ZPC) are neutral. Increasing pH favours reaction with OH^- and creation of CEC. By contrast, lowering pH favours reaction with H^+ and creation of AEC. Landfill leachate, which is often acidic, may, thus, increase AEC which, in turn, may increase initial adhesion of microorganisms to these surfaces. In general, microorganisms have negative surface charges at pH values above 3. Charge characteristics of some clay and oxide minerals are given in Table 1.1.

TABLE 1.1: Charge characteristics of some soil clays and oxide minerals (Foth, 1984).

Mineral	Permanent	pH dependent	Total	<--AEC-->
	< -------------CEC----------->			
	< ------------$cmol_c kg^{-1}$------------>			
Montmorillonite	112	6	118	1
Vermiculite	85	0	85	0
Hydrous mica	11	8	19	3
Kaolinite	1	3	4	2
Gibbsite, $Al(OH)_3$	0	5	5	5
Goethite, $FeO(OH)$	0	4	4	4

The soil oxide minerals often form coatings on the surfaces of other soil minerals such as silicate clays and sand particles. These coatings alter the surface characteristics of the minerals (Hendershot & Lavkulich, 1983) and may influence microbial and organic compound interactions with these minerals. The oxide minerals have been found to be of importance in landfill leachate attenuation (Lucas & White, 1983). Although the information on microbial interactions with soil oxides and hydroxides is sparse, workers have reported interactions of soil oxides with organic compounds (Sibanda & Young, 1986) and microbially produced enzymes (Gianfreda *et al.*, 1992). These reports are mainly of sorption and crystallization reactions with the oxides and hydroxides (Huang & Violante, 1986). Organic acids have also been found to dissolve iron oxides, depending on the pH and crystallinity of the oxides (Miller *et al.*, 1986). The common crystalline iron oxides, goethite and hematite, were found to be solubilized to a much lesser extent than the non-crystalline oxides, presumably due to lower surface areas and differences in type and arrangement of surface hydroxyl and oxygen groups. This difference between crystalline and non-crystalline oxides could, possibly, also influence microbial adhesion and interaction with such surfaces. To complicate the issue, ferric iron (Fe^{3+}) and manganese (Mn^{4+}) may also be used as electron acceptors under certain conditions and be transformed to Fe^{2+} and Mn^{2+} which are more mobile in soil (Miller *et al.*, 1986).

1.2.3   Organic matter

The carbon component of soil organic matter (SOM) is composed of: 10-20% (w/w) carbohydrates, primarily of microbial origin; 20% (w/w) nitrogen-containing constituents, such as amino sugars and amino acids; 10-20% (w/w) aliphatic fatty acids, alkanes, etc.; with the balance present as aromatic molecules (Paul & Clark, 1989). Soil organic matter is commonly separated from the mineral colloidal matrix of clays and sesquioxides, and dispersed in a liquid before being studied. The classical fractionation technique involves dispersion by NaOH or sodium pyrophosphate ($Na_4P_2O_7$) (Paul & Clark, 1989). The fraction not dispersable by the peptizing action of the Na^+, the chelating action of pyrophosphate and the hydrogen bond-breaking activity of very alkaline pH regimes is known as humin. The dispersable materials precipitated at acidic pH values are known as humic acids and the material that remains in solution as fulvic acids. Fulvic acids (MW = 1,000-30,000) are composed of a series of highly oxidized aromatic rings with a large number of side chains. The building blocks of fulvic acids are benzene carboxylic acids and phenolic acids which are held together primarily by hydrogen bonding or van der Waals forces and ionic bonding. This configuration is also apparent for humic acids which are composed of higher molecular weight materials (10,000-100,000) which contain aromatic rings and nitrogen in cyclic forms and peptide chains. The functional group analyses of humic and fulvic acid are given in Table 1.2. The humates are adsorbed on clay minerals by polyvalent cations such as Ca^{2+} and Fe^{3+} and by association with hydrous oxides, either through coordination (ligand exchange) or through anion exchange on positive sites which exist on iron and aluminium oxides.

TABLE 1.2: Functional group analysis of humic and fulvic acids (Paul & Clark, 1989).

	Functional groups			
	OCH_3	COOH	Phenolic OH	Total acidity
		< ---- $cmol_c$ kg^{-1} ---- >		
Fulvic	50	10	330	1240
Humic	100	450	210	660

Most soils contain comparable amounts of fulvic acids, humic acids and humin (Paul & Clark, 1989). Lignin, because of its recalcitrance, forms a major constituent of soil humus. Humus fractionation studies play an increasingly important role in research into the behaviour of pollutant chemicals in the soil environment (Andreux *et al.*, 1991; Gu *et al.*, 1995). Lafrance *et al.* (1990) found that the dissolved organic matter in groundwater could bind hydrophobic contaminants to form "complexes" and, possibly, affect their subsurface transport. The numerous functional groups of humus (Table 1.2) cause it to have a very high CEC; up to 250 cmol$_c$ kg^{-1} (Foth, 1984). The less refractile component of SOM may also serve as a carbon source for microorganisms growing in the soil. The implications of having readily degradable SOM in a SMP environment are that it may be used as a preferential carbon source so that organic pollutant catabolism does not occur. In the context of leachate migration through soil, readily degradable SOM in the leachate could reduce the catabolic attenuation of more recalcitrant organic molecules. Many components of the SOM are adsorbed to clay and other soil components (Stotzky, 1980) so that the surface characteristics of soil mineral components may be modified. Soil organic matter also affects the soil's physical characteristics. Bulk density, porosity, aeration as well as water and heat movement are all influenced by the SOM (Marshall & Holmes, 1988). Soil organic matter has a direct effect on soil structure and aggregation (Sullivan, 1990). The significance of soil structure and aggregation to SMP systems is discussed in Section 1.6.

All of the above lead to the conclusion that SOM, especially humus, is of great importance in SMP systems (Hayes, 1984) and, particularly, in interactions of toxic organic chemicals with humic substances (Senesi & Chen, 1989). Rebhun *et al.* (1992) studied the sorption / partitioning of several organic contaminants with a wide range of hydrophobicities on clay-humic complexes [10% (w/w) clay and 0-2% (w/w) humus]. The adsorption constants of the humic fraction were found to be 8-20 times higher than on the "pure" clays which were used. In soils with low to medium organic matter contents, however, the contribution of the clay minerals to adsorption was shown to be quite significant, in spite of the fact that half of the sorption sites on the mineral surfaces were blocked by humic substances. Most of the SOM is usually situated in the surface horizon. Soils used in landfills are likely to, initially, have very low concentrations of organic matter since these soils will have been excavated and will, for the most part, be subsoil. It should, however, be recognised that the organic

content will increase with time as organics from refuse leachate accumulate on soil particles and result in biofilm development.

1.3 Pollutants

Pollutant characteristics of importance to SMP systems are: recalcitrance, adsorption characteristics, and solubility, and these are discussed below.

1.3.1 Metals

The attenuation of metal pollutants in municipal landfill leachate by soil and clay minerals has been investigated (Chan *et al.*, 1978; Griffen & Shimp, 1978). Although the focus of the present study was on the organic components of leachate, the effects of heavy metals cannot be ignored. Campbell *et al.* (1983) found that under intensive leaching with a mixed metal/carboxylic acid solution, microbial colonization of the unsaturated zone was inhibited by the presence of heavy metals. It is, however, difficult to determine whether microbial resistance would have been less if the microorganisms had been unattached. Soil component surfaces can adsorb heavy metal ions and, thus, increase metal concentration at the soil-water interface where the high concentrations may be detrimental to surface attached bacteria. The increased surface sorption of the metal ions to soil components may, however, also reduce the concentrations of the metals in solution to sub-critical levels. Finally, microorganisms may also be protected against possible toxicity due to surface attachment (Kefford *et al.*, 1982).

1.3.2 Partitioning

The vast array of organic landfill leachate components (Harmsen, 1983; Reinhard *et al.*, 1984; Sawhney & Kozloski, 1984; Venkatarami *et al.*, 1984) complicates the study of the SMP system. The interactions of soil and pollutants (and, thus, also bioavailability) are

related to the partitioning of the chemical compounds between the liquid and solid phases in the soil, as well as to the kinetics of desorption (Wilson *et al.*, 1981; Senesi & Chen, 1989; Harmsen, 1991; Lui *et al.*, 1991).

According to Green *et al.* (1981), the octanol/water partitioning coefficient (K_{ow}) is probably the single most important laboratory determined parameter for predicting the movement and adhesion of organic compounds in soils. This parameter can, unfortunately, only be measured over long periods of time (of the order of a month). The coefficient of permeability can, however, be related to more easily measured parameters of soils and organics. Green *et al.* (1981) found that the hydrophobic or hydrophilic nature of the organic compounds, as measured by the octanol/water partitioning coefficient (or, approximately, by the dielectric constant) were important in predicting the solvent's rate of flow through soils. The octanol/water partitioning coefficient measures the tendency of molecules to escape from the aqueous phase. Hydrophobic substances, such as benzene, xylene and carbon tetrachloride (log octanol/water partitioning coefficients of 2.13, 3.15 and 2.64, respectively; water = -1.15) would be expected to adhere more strongly to the soil solid phase than water. The octanol/water partitioning coefficient was found to be more important than both the organic compound density and viscosity in predicting flow rate (and adhesion) through soil (Green *et al.*, 1981). While the partitioning coefficient (K_p) for a chemical has been observed to vay significantly from soil to soil (with varying fraction organic content, f_{oc}), it was observed that normalization of the K_p values by the respective values of f_{oc} resulted in a parameter (K_{oc}) that was much less variable (independent of the soil and a function only of the chemical) (Knox *et al.*, 1993). Another way of expressing this is that K_p was observed to be proportional to f_{oc} with K_{oc} being the proportionality constant. The definition of K_{oc} is:

$$K_{oc} = K_p / f_{oc}$$

Several expressions are available for estmating K_{oc} based on aqueous solubility (S) and K_{ow} (Knox *et al.*, 1993). One such an expression is:

$$K_{om} = -0.621 \log S \text{ (mg/L)} + 3.95$$

13

where,

$$K_{om} = K_p / f_{om} \quad \text{and} \quad f_{om} = 1.72 \times f_{oc}$$

where f_{om} = fraction organic matter

Another interesting aspect of hydrophobic interactions (and the octanol/water partitioning concept) is that hydrophobic interaction reversal may occur when the soil solution contains not only water but also a non-polar cosolvent(s), as may occur in landfills. This reversal affects hydrophobic molecules (Lane & Loehr, 1992; Knox *et al.*, 1993) as well as hydrophobic microorganisms. Cosolvency results in a lowering of the polarity of water which allows desorption of hydrophobic entities. This is of particular importance when co-disposal is employed which could result in cosolvency. Through desorption of these compounds, the leachate concentrations of molecules previously adsorbed due to hydrophobicity will increase. Sorption and low solubility are major factors preventing biodegradation of hydrophobic molecules. (Weissenfels *et al.*, 1992; Barriault & Sylvestre, 1993). Cosolvency, therefore, not only increases the water soluble concentration but also increases biodegradation of hydrophobic molecules. Leachate quality is affected by the ratio of desorption to biodegradation (Section 3.1.5). When practising co-disposal with cosolvents it should, however, be kept in mind that most cosolvents are toxic to microorganisms at elevated concentrations. These toxicity threshold concentrations in soil have been found to be much higher in the presence of soil than in soil-free systems. The tolerance of elevated concentrations in the presence of soil cannot be accounted for by adsorption of cosolvents to soils and is most probably because of the microbial interactions and protection at soil surfaces.

1.3.3 Molecular weight

Steric hindrance is always a consideration when organic molecules react with minerals (Harter, 1977). The ability of a mineral to adsorb a maximum of any given organic compound depends upon whether individual molecules can fit each adsorption site and

whether they can move into interlayer positions. The former is the interaction between molecular size and adsorption site density. The latter is an interaction between molecular size and maximum expansion of, for example, a clay mineral. Other organic molecule characteristics which influence interactions with soil components are: charge, lone pair electrons, pK_a and isoelectric points (Harter, 1977). These characteristics of pollutant chemicals are shared with microorganisms and are discussed below (Section 1.4.1).

1.3.4 Biodegradability of pollutants

Recalcitrance of organic molecules to microbial catabolism is often related to the substitution of different groups on the molecule (Alexander, 1981; Morgan & Watkinson, 1989). Substitution with halogens is especially important in this regard. Not only the identity of the halogen but also the position of substitution have been found to be of importance (Alexander, 1981). The capacities for biodegradation of selected halogenated aliphatics have also been found to differ under different redox potentials with dehalogenation favoured by more anaerobic conditions (Morgan & Watkinson, 1989). Increased hydrocarbon resistance to biodegradation has also been ascribed to increased chain branching and polyaromaticity, both of which are often correlated with decreased solubility and increased soil adsorption (Mihelcic & Luthy, 1988a; Cerniglia, 1993; Wilson & Jones, 1993). The biodegradation properties of the pollutants will, thus, be significantly influenced by adhesion to soil components.

1.3.5 Concentration and alternative substrates

The concentration of the pollutant will have an effect on its biodegradation as well as its adsorption onto soil (Hutchins *et al.*, 1984; Scow *et al.*, 1989). Concentrations of other (alternative) organic compounds will also have an influence on pollutant catabolism. In soil, a potential source of alternative substrates is the SOM. In a study of naphthalene biodegradation, Mihelcic & Luthy (1988b) found that if soil organic carbon was available for uptake by microorganisms, it could be utilised instead of the naphthalene. Knezovich *et al.*

(1988) found that a significant lag phase preceded phenol catabolism in soils with organic matter contents of 0.09% (w/w) and 1.1% (w/w). It is likely that the phenol degraders preferentially utilised the available SOM before initiating phenol catabolism. This was possibly due to the fact that soil microorganisms would be acclimated to SOM compounds which are intrinsic components of the environment.

I.1.3.6   Aerobic vs anaerobic conditions

Gourdon *et al.* (1989) found the organic load of a leachate from a mixed industrial and urban waste landfill to be low and to consist mainly of compounds with molecular weights < 500 (over 95% of the total organic carbon), although volatile fatty acids were not present. It was found that the majority of the organics initially present in the leachate were catabolized under both aerobic and anaerobic conditions. The leachate fraction exhibiting recalcitrance under both aerobic and anaerobic conditions consisted of the same molecules. All of these molecules, except one, had a molecular weight below 500 and were probably industrial chemicals. These were particulary recalcitrant under anaerobic conditions but were 50% biodegraded under oxic conditions.

Apart from biological transformations, anaerobic conditions may also directly influence landfill leachate components. Sawhney & Kozloski (1984) detected (the presence of) 0.1 to 1.5 mg l^1 of substituted phenols in a landfill leachate after passage through soil. Under aerobic conditions, in laboratory tests, it had been shown that substituted phenols not only sorbed irreversibly to clays and soils but were also transformed into polymerized moieties. This suggests that substituted phenols leach through soil more readily under the anaerobic conditions of the landfill and that such transformations may be inhibited under aerobic conditions. This may also apply to other compounds.

1.3.7 Landfill stage

The composition of landfill leachate also depends on the stage of refuse decomposition.

(Refuse decomposition can be roughly characterized as an acidogenic stage which is characterized by organic acid production, and a methanogenic stage during which the pH increases because of catabolism of organic acids under anaerobic conditions). Harmsen (1983) found the organic load of leachate from a domestic sanitary landfill in the acidification stage to be high and to consist mainly of volatile fatty acids (95% of the total organic carbon (TOC)), although amines and ethanol were also present (0.8 and 0.7% of the TOC, respectively). By contrast, leachate from the methane fermentation stage was found to have a relatively low organic load but 32% of the organic carbon present consisted of compounds with a molecular weight of more than 1000. Acids, amines and alcohols could not be detected. This means that the organic compounds present were the end products of degradation (catabolic) processes.

1.3.8 Naphthalene and phenol

Because of the complexity of the SMP system, two organic molecules (phenol and naphthalene) were chosen for this study. Phenol and naphthalene, as aromatic compounds, were also chosen as model molecules not only because of their relevance to landfills but also to industrial waste streams. Phenol is a common constituent of waste streams in the energy industry and in coal carbonization (Rozich & Colvin, 1986) and is considered a priority pollutant by the United States Environmental Protection Agency (Dobbins *et al.*, 1987). Naphthalene is also listed as a priority pollutant by the US EPA (Mihelcic & Luthy, 1988a) and is an important neutral hydrophobic organic contaminant (Lui *et al.*, 1991).

These two molecules share the basic metabolic pathways involved in general biodegradation of aromatic compounds. Aromatic degradation (catabolism) generally converges at catechol or protocatechuate, then diverges via *ortho* or *meta* cleavage of these intermediates. The metabolic pathways for phenol and naphthalene catabolism have been elucidated and have been demonstrated in a wide variety of soil organisms (Franklin *et al.*, 1981) (Figure 1.1). Because the catabolic pathways of these two compounds converge at a common intermediate, phenol could be used preferentially as an alternative substrate.

1.4   Microorganisms

1.4.1 Bacterial adhesion

The importance of adhesion in the SMP system has been discussed earlier (Section 1.1). The mechanism of general microbial adhesion (not adhesion to soil surfaces specifically) will be discussed here. Although the information is of a fundamental nature it may be extrapolated to microbial adhesion in the SMP system.   Attachment can be divided into two steps. Firstly, organisms adhere in a process which is governed by physico-chemical surface properties of the bacteria and the solid, and the type of solute (van Loosdrecht *et al.*, 1990). In the second step, microorganisms anchor themselves to a surface using specific appendages or cell surface structures. The process depends on the type of bacterium in combination with the type of surface.   The adhesion step can be divided into two separate types, namely, reversible and irreversible adhesion. Reversible adhesion is similar to the deposition of a bacterium on a surface so that two-dimensional Brownian motion can still take place and the bacterium can be removed from the surface. Irreversible adhesion is where bacteria can no longer be removed from the surface and so are incapable of exhibiting Brownian motion. Bacterial adhesion is a complicated process because cells are not "ideal" particles. Furthermore, internal chemical reactions can lead to changes in molecular composition, both intercellularly and at the surface of the bacterium. These chemical processes continue after adhesion. Adhered cells are, therefore, rarely in complete physico-chemical equilibrium with their environment.

The total long-range interactions between bacteria (living colloidal particles) and a surface over a distance of more than 1nm is, according to the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory, a summation of van der Waals and coulombic interactions (van Loosdrecht & Zehnder, 1990).   Generally, surfaces of particles and microorganisms are charged. Because of electroneutrality in water, the charge on the surface is neutralized by a countercharge that is diffusely distributed around the particle. The thickness of the diffuse double layer is a function of the ion charge and ion concentration. The Gibbs free energy (G_E) of the electrostatic interaction is determined by the electrokinetic (or zeta) potential of the surfaces. Most natural surfaces and bacteria are negatively charged (van Loosdrecht *et*

al., 1987b).
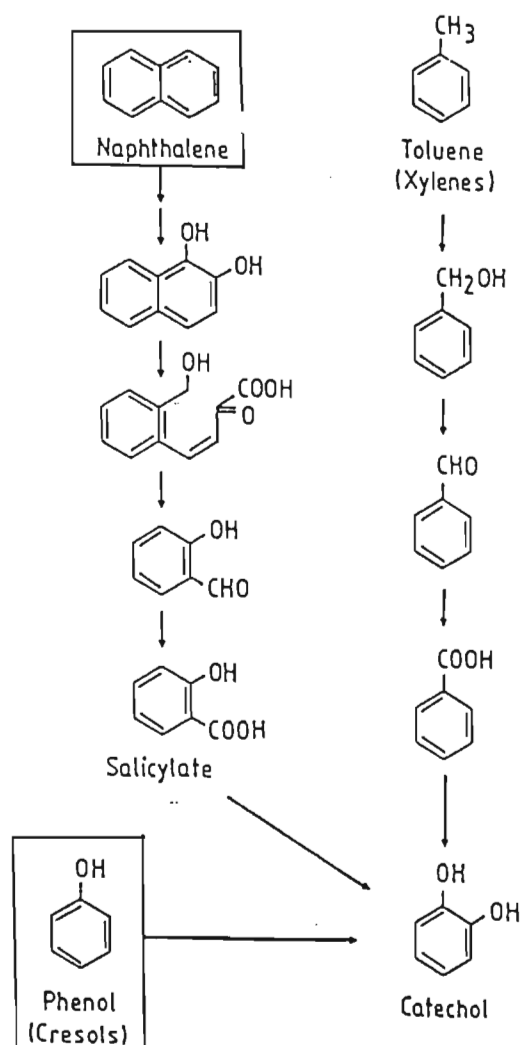
FIGURE 1.1: Biodegradative (catabolic) pathways for phenol and naphthalene in soil bacteria (Franklin *et al.,* 1981).

Figure 1.2 shows a characteristic plot of the total interaction Gibbs energy (G_{tot}, which is a summation of G_A, free energy of the van der Waals forces, and G_E, free energy of the electrostatic interaction) as a function of the separation distance (H) between a bacterium and a negatively charged surface. Figure 1.2 also shows two minima. If a bacterium reaches the primary minimum, short range forces will dominate the adhesive interaction and the

DLVO theory cannot be used to predict the interaction energy. The secondary minimum is never greatly negative so that particles captured in this minimum generally show reversible adhesion.
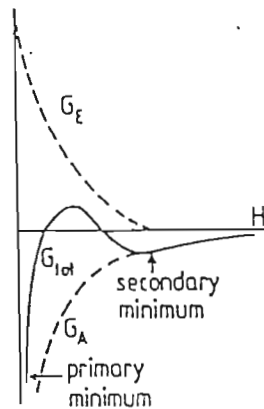
FIGURE 1.2: Gibbs energy of interaction between a sphere and a flat surface having the same charge sign (G_{Tot}), according to the DLVO theory. G_A = free energy of the van der Waals forces. G_E = free energy of the electrostatic interaction. H = separation distance. (van Loosdrecht *et al.*, 1990).

When bacteria make direct contact with a surface (separation distance H = 0) the interaction energy can be calculated from the assumption that the interfaces between solid/liquid and bacterium/liquid are replaced by a solid/bacterium interface (van Loosdrecht *et al.*, 1990). If the Gibbs free energy for adhesion is negative the adhesion is thermodynamically favoured and will proceed spontaneously. Energy values $<4 \times 10^{-20}$J cell^{-1} result in irreversible adhesion (primary minimum) which is much stronger than adhesion in the secondary minimum (Figure 1.2). Short-range interaction can, however, only become effective when long-range interactions allow a particle to approach a surface. A high maximum of G_{tot} would prevent such an approach.

The hydrophobicity of a compound is an indication of its tendency not to interact with water.

Hydrophobic interactions result from the fact that water-water contacts are thermodynamically more favourable than contacts between two non-polar groups or between a non-polar group and water (van Loosdrecht & Zehnder, 1990). Non-polar groups tend to be rejected from aqueous medium. In a "normal" soil system, this should effectively mean that hydrophobic microorganisms should adhere to the soil and organic matter particles rather than stay in suspension. Generally, the Gibbs free energy decreases with increasing hydrophobicity and results in higher adhesion strength.

There is agreement between experimental observations and theory that primary minimum adhesion (irreversible) is to be expected in the case of very strong Van der Waals attraction, i.e. both surfaces are hydrophobic, or when electrostatic forces are attractive or only weakly repulsive (van Loosdrecht & Zehnder, 1990). Normally, however, adhesion is found to be reversible. This indicates that, in thermodynamic terms, the interaction between a bacterium and a surface is relatively weak (Gibbs free energy $> 4\times10^{-20}$J cell^{-1}). Bacteria become more hydrophobic and show increased adhesion during the exponential growth phase and at high dilution rates in a chemostat. The reason for this phenomenon is not yet known (van Loosdrecht *et al.*, 1987a; van Loosdrecht & Zehnder, 1990). The reason for cell wall hydrophobicity is also not totally clear but it appears that thin fimbriae and fibrils play important roles as hydrophobins (Marshall & Cruickshank, 1973; Fletcher, 1979; Rosenberg & Kjelleberg, 1986; Marshall, 1988). Bacteria, initially adhering in the secondary minimum may, with time, simply pass through the energy barrier (if it is not too high) or penetrate the energy barrier by protruding fibrils or fimbriae. The electrostatic repulsion energy depends more strongly on the particle radius than the van der Waals forces. Fimbriae (because of their small radii) can readily adhere in the primary minimum and thus bridge the gap between the surface and bacterium. There are indications that surface polymers may sterically hinder close approach of two surfaces and force the particles to adhere in the secondary minimum. The occurrence of secondary minimum adhesion is, thus, not necessarily due to electrostatic repulsion. Most of the work on hydrophobic adhesion has been done in simulated and not natural environments such as soil. Furthermore, the soil environment is often a relatively low nutrient environment so that starvation responses occur which could greatly alter hydrophobicity due to the possible loss of fimbriae and many other factors (Marshall, 1988). Hydrophobic adhesion has also been shown to be influenced by both chromosomal and

plasmid control (Marshall, 1985) and is not only dependent on the hydrophobicity of the bacterial cells but also on that of the adhering surface (Mozes *et al.*, 1987). Soil components differ in their hydrophobicity. The SOM in particular has been shown to be more hydrophobic than the mineral components (Sullivan, 1990). This may be very important for microbial adhesion. Another interesting aspect of hydrophobic interactions (and the octanol/water partitioning concept) is that reversal of a hydrophobic interaction may occur when the soil solution is not water but a non-polar solvent, as may occur in landfills (Skoog *et al.*, 1991).

1.4.2 Biofilms

Biofilm development and its importance have been well studied and a vast amount of literature is available on most aspects of biofilms (Characklis & Marshall, 1990). Only a few aspects relevant to the SMP system will be discussed here. Biofilm accumulation is the net result of the following physical, chemical and biological processes (Cunningham *et al.*, 1990):

- Organic molecules are transported from the bulk solution to the substratum where some of them adsorb, resulting in a conditioned substratum (Figure 1.3A);

- A fraction of the planktonic microbial cells is transported from the bulk water to the conditioned substratum (Figure 1.3B);

- A fraction of the cells that strike the substratum adsorb to the substratum for some finite time and then desorb, i.e. reversible adsorption (Figure 1.3B);

- Desorption occurs because of shear forces but may also be influenced by physical, chemical or biological factors (Figure 1.3B);

- A fraction of the reversibly adsorbed cells becomes irreversibly adsorbed (Figure 1.3B);

- The irreversibly adsorbed cells grow to produce microcolonies and, ultimately, increase biofilm cell numbers while utilizing nutrients and substrate from the bulk solution (Figure 1.3C). The cell may also produce significant amounts of secretion products. One class of products is the extracellular polymeric substances (EPS) which hold the biofilm together. Polysaccharides have been shown to be essential for

the development of surface films but not for the initial adhesion of bacteria. Biofilm accumulation is, thus, increased at the expense of substrate energy (in solution) due to microbial metabolism;

- Cells and other particulate matter attach to the biofilm, increasing biofilm accumulation (Figure 1.3D); and

- Portions of the biofilm are desorbed and released into the bulk solution (Figure 1.3D). Cell multiplication also leads to the release of daughter cells into the bulk solution (Figure 1.3C).

The progression of biofilm accumulation, like so many other biological processes, frequently takes the form of a sigmoidal curve. Biofilm accumulation within porous media such as soil can substantially reduce the hydraulic conductivity (Cunningham *et al.*, 1990). The increased resistance to flow is due to the reduction of the effective pore space caused by attached cells and their extracellular matrix. Microorganisms growing as biofilms in the subsurface (or soils) have an advantage over suspended species in that they can remain near the source of fresh substrate and nutrients contained in the groundwater which flows by them. The rate of biofilm growth and, thus, the rate of biotransformation is, therefore, strongly influenced by transport characteristics, including distribution of velocity within pores, dispersivity, surface roughness (all of which are dependent on soil composition), molecular diffusivity and other variables that affect the delivery rate of substrate and nutrients to the growing cells. Hydraulic flow rate has an influence on retention time which, in turn, has an influence on biotransformations and biodegradation of organic pollutant chemicals. The longer an organic molecule is retained within a soil pore, the greater the probability that the molecule will be adsorbed or catabolized. The removal of various organic compounds from solution in biofilm columns under different conditions was reported by Cunningham *et al.* (1990) and will be further investigated in Part II.

1.4.3 Microbial interactions

Biodegradation studies are complicated by microbial interactions. The interactions may be varied and dependent on environmental circumstances (Slater, 1981; Atlas & Bartha, 1987).
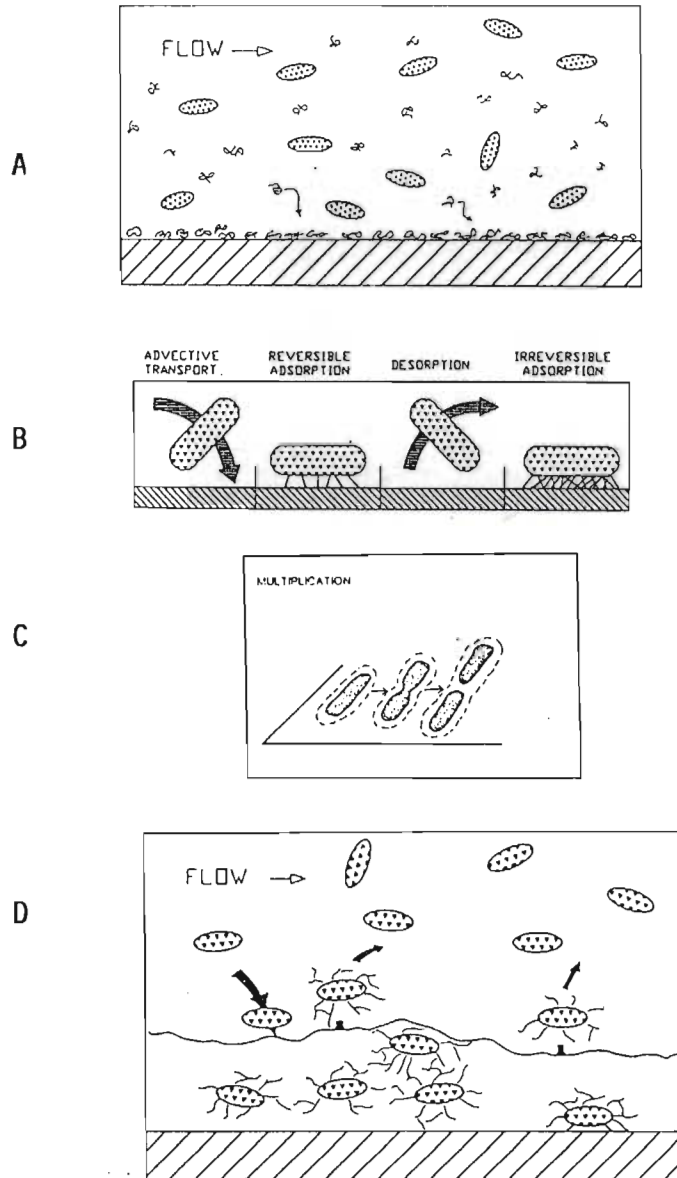
FIGURE 1.3: Transport of organic molecules to, and adsorption on, a clean substratum, forming a conditioning film (A). Transport of microbial cells to the conditioned substratum, and adsorption, desorption and irreversible adsorption of cells on the substratum (B). Growth and multiplication of microbial cells on the substratum at the expense of substrate in the solution (C). Attachment and detachment of cells and other particles to and from the biofilm (D) (Cunningham *et al.*, 1990).

Generally, it seems as if inter- and intra-species interactions are poorly understood and that little is known of the physiological changes occurring after adsorption. It is also difficult to predict what effects surface attachment to the various soil components will have on these interactions.

1.5   Physico-chemical interactions of adhesion

As stated earlier (Section 1.2.1) the properties of clay minerals which affect surface interactions with microorganisms have not been studied extensively but attempts have been made to identify the properties that affect the adsorption of soluble organic materials (Mortland, 1986). Some soluble organic compounds are similar to components of the surface structures of microbial cells and, therefore, the properties of these compounds may be relevant to adhesion of microorganisms. Before making extrapolations, it should be noted that free soluble organic compounds are not confined to the more rigid structures as is the case with compounds in cell surfaces. Hydrophobicity, which could also be included under this heading, has been discussed in relation to microorganisms (Section 1.4.1).

On the whole, soil has a great buffering capacity for hydrophobic contaminants and charged molecules. Soil is, thus, able to reduce the environmental risk of migrating adsorbable pollutants until biodegradation (if possible) occurs. This is particularly important in soils where such pollutants (organic or inorganic) may be potentially toxic at high aqueous concentrations. The soil could, thus, act as a buffer to lower the soluble concentration to below the toxicity threshold. This would also allow more time for microbial adaptation to occur. It should, however, also be recognised that every soil type has a finite buffering (adsorptive) capacity which could be exceeded. At that point it would no longer adsorb any of the challenging molecules. Thus, an increase in the equilibrium aqueous concentration would not result in increased adsorption. This point of saturation can, however, be determined with adsorption isotherms. With most hydrophobic compounds, the solubility of the molecule is the most limiting factor and not the soil adsorption capacity (Wilson & Jones, 1993). Increased adsorption occurs not only because of the sorption capacity of the soil but also because of the hydrophobic nature of the compound. These compounds are repelled

from the aqueous phase and, thus, adsorb to soil. In this case, the adsorption isotherm would show that an increase in the adsorbed concentration does not increase the equilibrium aqueous phase concentration. As the compounds are catabolized, usually from the aqueous phase, the soil adsorbed concentration replenishes the soluble concentration so that it is kept at its solubility maximum. This continues until the adsorbed amount is insufficient to sustain the solubility maximum. Water (leachate) analysis, therefore, gives only limited information about the state of contamination and should be carefully interpreted.

1.5.1 Charge interactions

The cations on the clay surfaces and their interactions with microorganisms have been well studied (Santoro & Stotzky, 1968; Gordon & Millero, 1984; Stotzky, 1986; Fontes *et al.*, 1991). In general, it was found that if a net positively charged biological entity (an organic molecule, particle or microorganism) was involved, surface interactions were usually greater when the charge compensating cations had a low valency (i.e. monovalent). The most probable explanation for this phenomenon is that monovalent cation replacement is easier than replacement of multivalent cations (Harter & Stotzky, 1971).

Most biological entities have a net negative charge under normal soil conditions. Surface interactions between these entities and clays are usually greater when the valence and the ionic strength of the exchangeable cation solution are greater. This is probably because of a decrease in the thickness of the diffuse double layer (van Loosdrecht & Zehnder, 1990). This enables the clays and the net negatively charged biological entities to approach each other more closely so that H-bonding and van der Waals interactions become effective. The above process, called the Schulze-Hardy rule, must be distinguished from the process of cation bridging where multivalent exchangeable cations on the surfaces of the clays act as bridges between the clay and the negatively charged biological entity (Stotzky, 1985). Cation bridging is not, however, a viable theory since the cations would be hydrated so that adhesion would rather be the indirect result of protonation or water bridging. Multivalent cations also have the capacity to reduce the expansibility of swelling 2:1 layer clays which reduces their total available surface area. This can result in the reduction of macromolecule

26

binding i.e. proteins (Harter & Stotzky, 1971; 1973).

The net negativity of biological entities is dependent on the pK of the dissociable functional groups and the ambient pH (Harter & Stotzky, 1971; Stotzky, 1985). The pI (pH at the isoelectric point) of the entity, which is an empirical summation of the pK values of all components capable of accepting or releasing protons (Bohinski, 1987) and the pH of the ambient solution, will determine the net charge of the entity. It is apparent that any H^+ producing process could enforce an unnatural pH condition on the soil. Adhesion characteristics of the microorganisms may, therefore, be different under such conditions. This may be particularly significant for relatively small molecules, since pI values are only important for such molecules. Adhesion of large molecules and particulate entities (and microorganisms) are not as dependent on pI values since they may have positively charged sites, even at pH values above their pI when their net charge is negative.

Some heavy metals such as Cd, Cu, Cr, Ni (and Zn), which have been found in landfill leachate (Griffen & Shimp, 1978), can cause reversal of the charge of bacterial cells (and clays) in monocultures to positive values, at pH levels above their pI values (Collins & Stotzky, 1992). This may be of importance in landfill environments where heavy metals may be present and, thus, cause increased attachment of microorganisms to soil particles because of the charge reversal. Attachment, as explained above, may significantly influence microbial catabolism of pollutant compounds.

1.5.2 Hydrogen bonding

In some instances H-bonding might be the most important type of adhesion mechanism. In contrast to coulombic and covalent bonds, H-bonding is relatively weak but the number of H-bonds may be so great that cumulative H-bonding may be extremely strong (Burns, 1979; Lipson & Stotzky, 1983). This is, however, mostly the case for situations other than soils. The formation of H-bonds between some functional groups (e.g. COH, COOH and NH) of organic moeities, and the surface O of the tetrahedral sheets of clays is reported not to be favoured, although there is some evidence of its occurrence (Harter, 1977; Oades, 1989).

The formation of H-bonds between structural OH^- groups of clays and functional groups (e.g. CO, N) of biological entities is probably of minor importance, as exposed OH^- groups are primarily located at the broken edges of clays and are usually dissociated at the pH of most soils (Stotzky, 1985). In 2:1 clays, the OH^- groups are in the octahedral sheet and are, therefore, not exposed. In 1:1 clays, most octahedral sheets are not exposed because the clay does not expand. Only the OH^- groups on the external surface of these clay domains are exposed. The mechanisms of H-bonding, protonation, van der Waals interaction and coordination may all occur in landfill soil cappings. The extent to which they occur and their importance relative to the two major mechanisms of interaction, i.e. electrostatic interaction and hydrophobic interaction, are not known.

Protonation seems to be the most important feature of interactions between clays and biological entities. Protons from the clay surfaces or, more likely, from the associated water, are transferred to the biological entity. This transfer usually takes place to O and N and, to a lesser extent, S and P. This protonation adds a positive charge to the biological entity so that it may become less negative (or neutral, or positively charged) and surface interaction with the surface of the clay can then occur via H-bonding, van der Waals forces or coulombic mechanisms (Stotzky, 1985). Protonation is a function of surface pH (pH_s) and the pH of the adjacent bulk solution (pH_b). The pH_b is usually higher than the pH_s due to the formation of the diffuse double layer around the clay. The ΔpH, which is the difference between pH_s and pH_b, depends on the charge compensating cations (ΔpH, in this context, should not be confused with its normal use where $\Delta pH = pH_{water} - pH_{KCl}$, although there may be a correlation). The higher the concentration of basic charge compensating cations, the fewer the protons on the exchange surface and, thus, the higher the pH_s. The relatively low pH and high cationic load of leachate migrating through the soil could affect the ΔpH. Although the pH_b may be above the pI value of the bacteria, the pH_s may be below it so that bacteria close to the surfaces of the clays may be protonated, thereby increasing their interaction with the clay surfaces. The pH_s may also be below the pK_a value of organic chemicals although the pH_b might be above the pK_a value. This could facilitate cationic adsorption of the organic compound to the surface (Senesi & Chen, 1989). Organic conditioning films may also be important in adhesion and act as intermediates in H-bonding between cells and clays (Section 1.5.2).

1.6 Particle aggregation, pollutant diffusivity and microbial catabolism

Douben & Harmsen (1991) showed a very strong correlation between organic chemical adsorption and desorption. Although their study was aimed at soil clean-up, it also has relevance to the study of SMP systems. Substrates such as benzene which were easily adsorbed were also readily desorbed. The opposite was found to be true for hexylbenzene. Sorption and desorption properties of the organic chemicals have a significant influence on the diffusivity of the pollutant, especially where different degrees of soil aggregation (structure formation) occur. This concept is illustrated in Figure 1.4.

The concentration of the pollutant on the surface of the aggregate is in equilibrium with the concentration of the pollutant in the aqueous phase (Figure 1.4A) although the concentration of the substance is higher at the aggregate surface than in the water phase. This is due to the high octanol/water partitioning coefficient ($K_{o/c}$). Further diffusion occurs so that the concentration of the substance becomes equal throughout the aggregate (Figure 1.4B). Highly soluble substances in soil, such as benzene, will rapidly attain equilibrium while less soluble substances, such as hexylbenzene, will require more time to do so. If soil clean-up is commenced before equilibrium within the aggregate is attained, the diffusion distance to the surface is short and the clean-up should proceed rapidly (Figure 1.4C). (Diffusion to the aggregate surface occurs because of the replacement of the contaminant water phase by contaminant-free soil solution or because of microbial catabolism at the aggregate surface). Diffusion from soil aggregates that have already attained an equilibrium level of contamination (Figure 1.4B) will proceed over a longer time period because of the greater distance of diffusion (Figure 1.4D). The diffusivity of the substance, microbial catabolism at the surface and substance concentration at the soil surface will all influence decontamination of the soil aggregate. In this context "aggregates" could be soil structural units (macro-aggregates) with a diameter of up to a few centimetres, or micro-aggregates of clay layers only a few micrometres in diameter. Microbial growth on macro-aggregates would most probably be on the outer surface of the aggregate but need not be entirely restricted to this surface. Microbial growth on micro-aggregates, however, would probably be restricted to the outer surface of the aggregate, since inter-layer spacings are often too small for microbial penetration.
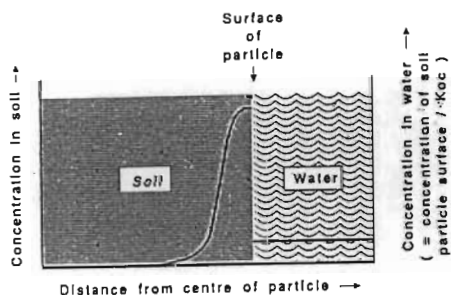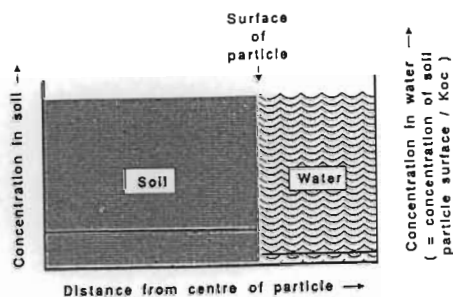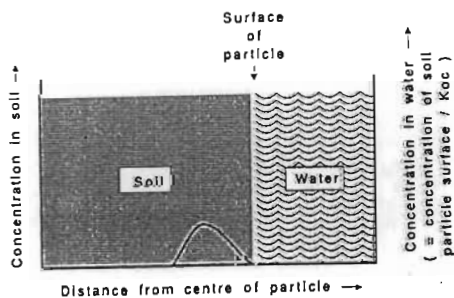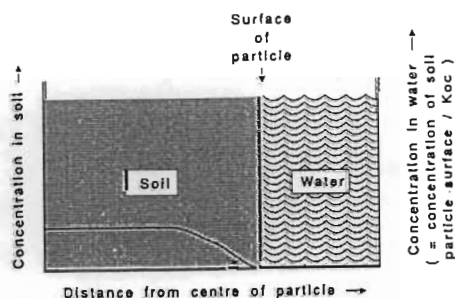
FIGURE 1.4: Contaminant diffusion and removal (catabolism) for a single soil particle (or aggregate). The particle (or aggregate) is assumed to be spherical. Recent exposure to pollutant (A). Long term exposure to pollutant (B). Recent exposure to pollutant, with "clean up" (C). Long term exposure to pollutant, with "clean up" (D). (Douben & Harmsen, 1991).

CHAPTER 2. MATERIALS AND METHODS

2.1   Experimental procedures

2.1.1   Aqueous phase naphthalene attenuation in a soil slurry

The attenuation experiments were made in 200ml sterile glass bottles closed with parafilm-lined caps. Sterile medium (Section 2.4.1) (120ml) and sterile soil (6g) were used with 10% (v/v) inoculum (soil:liquid ratio was 1:20). The bottles were incubated at 30°C on a rotational shaker (120rpm). A soil-free control was used. Samples (4ml) were centrifuged at 11000xg for 30 minutes before HPLC analysis (Section 2.3.4).

2.1.2   Scanning electron microscopy of a naphthalene catabolizing population

Soil and supernatant samples were taken from samples prepared as in Section 2.1.1 after 7 days incubation. Both soil and supernatant samples were viewed for the presence of microorganisms (Section 2.7). During inoculum preparation (Section 2.5), septa (9mm diameter) were placed in the culture and viewed after 2 weeks incubation (Section 2.7). The septa were coated with teflon (hydrophobic) on one side.

2.1.3   Adsorption of molecules to soil

Naphthalene adsorption isotherms were determined on the three sterile soils. A fixed volume (230ml) of naphthalene of different concentrations (10, 20, 25 and 30 mg l^{-1}) was added to the individual soils (12g) in 230ml sterile glass bottles with parafilm lined caps (no head space). Microbial catabolism was inhibited by the addition of 1ml of $CdCl_2$ (10 g l^{-1}). The preparations were maintained at 30°C (120rpm) for 24 h. Samples were then centrifuged at 11000xg for 30min before HPLC analysis (Section 2.3.4).

Methanol, acetonitrile and cadmium adsorption isotherms were determined by a similar

procedure before analyses (Sections 2.3.2-2.3.6)

Phenol (10 mM) supplemented nutrient medium (250 ml) [(Section 2.4.2), concentrations of KH_2PO_4 and K_2HPO_4 reduced to 0.25 g l^{-1} to prevent excessive complexation with cadmium] was incubated (30°C) individually with three sterile soils (20 g) in sealed glass bottles (500 ml) with shaking (120 rpm) for 24 h. The supernatants were then centrifuged at 11000xg for 30 min, filtered (0.2 μm) and diluted, where necessary, before analysis (Section 2.3.2).

2.1.4 Growth of naphthalene catabolizing organisms in the presence of various solvents and a heavy metal (Cd)

Each of the treatments was made in 150ml glass bottles which contained 35ml of medium (Section 2.4.1), 5ml inoculum (Section 2.5) and 10ml of various treatments i.e. acetonitrile, methanol and dissolved cadmium. The different concentrations tested are shown in Figure 3.5A-C. The bottles were incubated at 30°C in the dark without shaking. Optical density readings (Section 2.3.7) were taken at 1 - 2 day intervals and the samples returned to the experimental container. In parallel, naphthalene analyses of the supernatants were made (Section 2.2.5). The controls contained all chemicals excluding solvents or cadmium.

2.1.5 Catabolism of adsorbed naphthalene

The experiment was made in sealed glass containers (150ml) which each contained 5g of naphthalene supplemented soil (2000 mg kg^{-1}, Appendix 3, Section A.3.1), 35ml of nutrient solution (Section 2.4.1), 5ml of inoculum (Section 2.5) and 10ml of various treatments i.e. 15% (v/v) acetonitrile, 15% (v/v) methanol, 100 mg l^{-1} cadmium or distilled water (control). The bottles were incubated at 30°C on a rotational shaker at 120rpm and opened daily for 3 minutes to facilitate aeration. The experiments were run concurrently for the three different soil materials. Individual bottles were destructively sampled (Appendix 3) at regular intervals.

2.1.6 Growth of phenol catabolizing organisms in the presence of solvents and a heavy metal (Cd)

Each treatment was made in a 150ml glass bottle which contained 35ml of nutrient solution (Section 2.4.2 but with the lower phosphate concentration to avoid possible Cd precipitation), 5ml of phenol catabolizing inoculum (Section 2.5) and 10 ml of the various treatments to give final concentrations of: 15% (v/v) acetonitrile, 15% (v/v) methanol and 30 mg l^{-1} naphthalene. The individual cadmium concentrations were 0 (control), 50, 80 and 100 mg l^{-1}. The bottles were incubated stationary at 30°C in the dark. Optical density readings (Section 2.3.7) were taken at two-day intervals and the samples returned to the experimental container.

2.1.7 Phenol attenuation in soil with an acclimated microbial population

Unlike the equivalent experiment with naphthalene (Section 2.1.5), desorption of phenol was not necessary prior to analysis. Phenol was added in solution rather than directly to the soil. These experiments were made in sealed glass bottles (500ml) to which 20g of sterile soil, 30 ml of various solvents, a heavy metal molecule (cadmium), 150ml nutrient solution containing 5mM phenol (as in Section 2.4.1, with the lower phosphate concentrations) and 20ml of a phenol catabolizing inoculum (Section 2.5) were added. The bottles were incubated under aerobic conditions at 30°C, shaken at 120 rpm and briefly opened daily to facilitate aeration. Samples of the supernatant were centrifuged for 30 min at 11000xg before analysis (Section 2.3.2). The variables which were studied were: acetonitrile, 15% (v/v) (as a toxic solvent); methanol, 15% (v/v) (as a less toxic solvent); cadmium, 300, 600 mg l^{-1}; and naphthalene, 2000 mg kg^{-1} soil. The controls contained all nutrient chemicals excluding acetonitrile, methanol, cadmium and naphthalene.

2.1.8 Naphthalene column attenuation studies

The three experimental soil materials used previously (450 g) supplemented with naphthalene (Appendix A.3.1) were individually packed into PVC columns (4.5 cm diameter, length 30 cm). Tensiometers were installed near the bottom of the columns (Appendix A.3.7) prior to adding the soil. The columns each had three ports at the base, two of which were fitted with tensiometers. The other port was used to saturate the soil from the bottom during immersion in water and for draining to field capacity but was plugged during experimentation. Duplicate columns (all at a bulk density of approximately 1.3 g cm^{-3}) were immersed in tap water to saturate the soils (from the bottom) and displace the air. Glass wool plugs were used to prevent soil loss and evaporation. Leachate samples were collected regularly, centrifuged (11000xg for 30 min) and analyzed by HPLC (Section 2.3.4).

Due to the naphthalene supplementation and the potential toxicity of the carrier solvent (Appendix A.3.1) the columns were inoculated. The inoculum used was cultured in a similar way to that described in Section 2.5 but in this case the source of inoculum was kept separate i.e. Inanda subsoil, Inanda topsoil, Rensburg topsoil and was not batched together. The inoculum (including medium, Section 2.4.1) was added to each column to raise the soil water content to field capacity. The columns were set up and incubated at 30ºC as shown in Table 2.1. The control column soils were sterilized by gamma radiation (Section 2.2). The other soils were supplemented with nutrient medium containing 10% (v/v) inoculum as described above. Columns C,E and G were also supplemented with acetonitrile. The acetonitrile supplement concentration (applied every seven days) was increased by 10% at each supplementation until the concentration reached 70% (v/v) on day 65 (Figure 4.2 C, E & G). In each case, the volume of acetonitrile/nutrient/inoculum solution added was equivalent to the leachate volume collected. The columns not supplemented with acetonitrile produced sufficient leachate through the tensiometers and were only supplemented with a nutrient/inoculum mixture (as above) on day 37. After 82 days incubation the soil columns were destructively sampled. Soil samples were taken from the top (5 cm), middle (15 cm)

TABLE 2.1: Naphthalene supplemented soil columns, treatments and controls.

Column	Soil material	Treatment
A	Inanda topsoil	Nutrients plus inoculum (oil contaminated soil)
B	Inanda topsoil	Nutrients plus inoculum (Inanda topsoil)
C	Inanda topsoil	Nutrients plus inoculum (Inanda topsoil) and acetonitrile gradient
D	Inanda subsoil	Nutrients plus inoculum (same soil)
E	Inanda subsoil	Nutrients plus inoculum (same soil) and acetonitrile gradient
F	Rensburg soil	Nutrients plus inoculum (same soil)
G	Rensburg soil	Nutrients plus inoculum (same soil) and acetonitrile gradient
H	Inanda topsoil	Sterile nutrient medium
I	Inanda subsoil	Sterile nutrient medium
J	Rensburg soil	Sterile nutrient medium

and bottom (25 cm) of each column. Naphthalene was extracted with 60% (v/v) acetonitrile (Appendix A.3.3). Soil samples (approximately 3 g) were accurately weighed and added to 10 ml 60% (v/v) acetonitrile and shaken for 15 minutes (orbital shaker, 120rpm) at ambient temperature. The supernatants (1.5 ml) were centrifuged for 30 min at 11000xg and analyzed by HPLC (Section 2.3.4). The uncentrifuged soil was
air dried at room temperature (approximately 25°C). The naphthalene concentrations obtained were, thus, related to the soil dry mass.

2.1.9 Phenol column studies

The three soil materials (200 g) were individually packed into replicate PVC pipes (4.5 cm diameter, 30cm length) to an approximate bulk density of 1.3 g cm^{-3}. The Rensburg soil was diluted with acid washed sand (Section 2.2.6). One set of the packed columns was supplemented with 2 ml of 10 mM phenol solution at the sampling times indicated in Figure 4.7. Each column was fitted with two tensiometers near the bottom (Appendix A.3.7) as an insurance against tensiometer failure. The use of tensiometers was necessary to extract leachate from the soil columns which were unsaturated and, therefore, aerobic. The second set of columns was supplemented with 5 ml of the same phenol solution as above. These columns were each fitted with an end cap in which the glass wool prevented the soil particles from moving through the outlet. These columns drained freely and did not require tensiometers for leachate collection. The columns were maintained at 30°C in a temperature controlled cabinet. Leachate was collected from the outlets and tensiometers and analyzed by gas chromatography (Section 2.3.2) after recording the volumes.

Column sets 3 and 4 were the same as sets 1 and 2 but, in addition to the phenol supplements, were inoculated with a phenol catabolizing population (I.2.5). This inoculum constituted 50 % of the volumes added. Inoculation was, however, only made at the first supplementation point.

2.2   Soil characterization

Two soil types (three soil materials) were used in this soil-microorganism-pollutant interaction study, namely, Inanda and Rensburg (Soil Classification Working Group, 1991). The Inanda soil, located near Bruyn's Hill, Natal, was sampled from both the humic A and red apedal B horizons. The vertic A horizon of the Rensburg soil was collected from Ukulinga Farm near Pietermaritzburg, Natal. These soils were chosen because of the wide spectrum of general soil characteristics which they represent (Appendix 1). The Rensburg was chosen because of its high smectite clay content. The Inanda soil was chosen because of the clay mineral uniformity (kaolinitic) throughout the profile and a marked difference

between the top and subsoil in organic carbon content.

All soil samples were air dried at ambient temperature and ground to pass a 2 mm sieve before experimentation. Microbial inocula were obtained from the A horizon (0 - 10 cm) of an Oakleaf soil (Soil Classification Working Group, 1991), sampled on the banks of the Umzinduzi river at Duzi Bridge, Lincoln Meade, Pietermaritzburg. The soil was immediately passed through a 2mm sieve, well mixed and stored at 4°C in a plastic container (50 *l*). The soil was then mixed by hand every two weeks to prevent chemical gradients developing due to evaporation.

In order to relate the interactions of the various soil materials to microbial catabolism of pollutant molecules it was important to determine the characteristics of the various soil samples. The following analyses were subsequently made and the results are given in Appendix 1: surface area, particle size, exchangeable cations, clay mineralogy and organic carbon. All soils were sterilized prior to experimentation by gamma irradiation, 25 kGy (Gamwave).

All of the following soil analyses were made by the Institute for Soil Climate and Water, Pretoria, South Africa. Determination of organic carbon content was made by the Soil Physics Laboratory of Cedara Agricultural College, Pietermaritzburg, South Africa.

2.2.1 Surface area

Surface area determinations were made with a Micrometrics Flow sorb II 2300 apparatus according to the method of Brunnauer, Emmet and Teller (BET-method) (Mortland & Kemper, 1973).

2.2.2 Particle size

Particle size analyses were carried out by means of the pipette method (The Non Affiliated Soil Analysis Work Committee, 1990).

2.2.3 Exchangeable bases

Exchangeable cations were measured using LiCl as extractant (0.5N LiCl, pH 8.0), with Ca and Mg determined by atomic absorption spectrophotometry, and Na and K by flame emission (The Non Affiliated Soil Analysis Work Committee, 1990).

2.2.4 Clay mineralogy

The $<2\mu$m fractions (clay fractions) were separated by centrifugation (5000xg for 20 minutes) and rendered homoionic by shaking in a solution of 1M KCl or $MgCl_2$ for 1 hour and standing to equilibrate for 12 hours at 25°C. The flocculated clay was freed of excess salt by repeated washing with distilled water. Orientated specimens were prepared according to the suction-through method of Gibbs (1965). Expansion tests for the identification of swelling phyllosilicates (ethylene glycol vapour at 60°C for 16 hours and glycerol vapour at 90°C for 16 hours) were carried out according to the method of Novich and Martin (1983). Potassium saturation/heat treatment tests to differentiate between vermiculite, chlorite and smectite followed the protocol of Machajdik and Cicel (1981). X-ray diffraction data were obtained using a Philips X-ray diffractometer (PW1710) with graphite monochromated Co $K\alpha$ radiation, generated at 45kV and 40mA, with a 1° divergence slit and a 1° receiving slit. Philips PC-APD PW1817 software was used to analyze the data. Clay mineral quantification was based on peak area percentages and may be regarded as semi-quantitative only (Brime, 1985). Clay nomenclature was according to AIPEA recommendations (Bailey, 1980).

2.2.5 Organic carbon

The Walkley-Black method, with minor modifications, was used for organic carbon analysis (Nelson & Sommers, 1982). The analyses were done in triplicate. The method used comprised the following reagents:

1. $K_2Cr_2O_7$ - see below, not as per method;

2. H_2SO_4 as per method (the presence of Cl was not taken into account);

3. H_3PO_4 as per method;

4. Diphenylamine $(C_6H_5)_2$ was used instead of the published indicators. The end point colour change in this case was from navy blue to bright green; and

5. $FeSO_4.7H_2O$ as per method.

The procedure was similar to the published procedure except that an extra 0.5ml of $K_2Cr_2O_7$ was added (final volume 10.5 ml). In this determination no compensation was made for the incomplete oxidation of carbon. This factor (f = 1.30) was, however, incorporated in the final results.

2.2.6 Field capacity and pore volume

Pore volume and field capacity were determined for the three soil materials at an average bulk density of 1.3 g cm^{-3}. (The field capacity for the Rensburg soil was determined after dilution with acid washed sand at a ratio of 1:2 (w/w), Rensburg : acid washed sand). Soil (100 g) was placed into a water filled measuring flask and the displacement measured after shaking (to displace air bubbles). This gave the particle volume and was subtracted from the total volume occupied by soil in columns at a bulk density of 1.3 g cm^{-3} to give the total pore volume.

The soil columns were submerged in water containers so that water was forced up the columns. The water level in the containers was level with the top surface of soil in each column. This was done until the soil was saturated up to the surface and the columns were then weighed. Upward water movement was necessary to expel any air bubbles. The

columns were allowed to drain for 48 h, to reach field capacity, after which they were weighed and the water holding capacity calculated.

2.3   Analytical techniques

2.3.1 Sample preparation

All liquid samples were collected by hypodermic needle and syringe from the culture supernatants and centrifuged at 11000xg for 30 minutes before dilution if necessary. The supernatants were then filtered through a 0.2 μm millipore filter and stored in sterile Eppendorf tubes at 4°C before analysis.

2.3.2 Phenol

Analyses were made with a Varian 3600 Gas Chromatograph equipped with flame ionization detector. The column, injector and detector temperatures were maintained at 120°C, 150°C and 180°C, respectively. The flow rate of the carrier gas (oxygen-free nitrogen) was 30 ml min^{-1}. Hydrogen and air flow rates were 30ml min^{-1} and 300ml min^{-1}, respectively. Delta chromatography software was used to process the data. Duplicate aqueous samples (1 μl) were injected directly onto the 2m glass column (i.d. 3 mm) packed with Chromosorb W-HPkS support (mesh 80-100) and a 5% polyphenylether liquid phase. Solutions (10 mM) of phenol were used as standards and after standard curve construction concentrations of experimental samples were calculated by peak height comparison.

2.3.3 Acetonitrile

Acetonitrile was determined using a Varian Vista 4600 Gas Chromatograph equipped with thermal conductivity detector. A 2 m stainless steel column was used (i.d. 3 mm) packed with Chromosorb 108 (mesh 80 - 100). The column temperature was 140°C (isothermal).

The detector, filament and injector temperatures were 200°C, 350°C and 200°C, respectively. Helium was used as carrier gas at a flow rate of 30 ml min^{-1}. External standard construction was made after which experimental sample concentrations were calculated by peak height comparison.

2.3.4 Naphthalene

Analyses were made by High Performance Liquid Chromatography with a Waters 600E systems controller and Waters 486 tuneable absorbancy detector. Separation was achieved using a Nova-Pak®C$_{18}$ column (3.9mm x 150mm) with a mobile phase of 65% acetonitrile-35% water (1.5ml min^{-1}). The UV detector was set at 276 nm (Weissenfels *et al.*, 1992; Millipore Chromatography Applications Data Bank, WB059-4 & B83016). The standard curves were linear in the < 40 mg l^{-1} concentration range. Apex chromatography software was used to process the data. Concentrations of experimental samples were again calculated by peak height comparison.

2.3.5 Cadmium

The equipment and calibrations were similar to those used for naphthalene, above. A Bondapak®C$_{18}$ column (3.9mm x 300 mm) was used with a mobile phase which contained 2% acetonitrile, 2mM sodium octanesulphonate and 50mM tartaric acid (1ml min^{-1}) and a post-column dye reagent which contained 0.2mM PAR [4-(2-pyridylazo) resorcinol], 1M acetic acid and 3M ammonium hydroxide (0.5ml min^{-1}). The UV detector was set at 520nm.

2.3.6 Methanol

The equipment, column and calibrations were similar to those used for naphthalene, above. The mobile phase in this case, however, was 100% water (1.5ml min^{-1}). The detector was

set at 190 nm.

2.3.7 Optical density of microbial suspensions

Optical densities, were determined by measuring absorbancy at 590nm with a Milton Roy Spectronic 301.

2.4 Nutrient media

The various growth media for catabolism of the different model pollutants are given below.

2.4.1 Naphthalene

The nutrient medium used contained: K_2HPO_4, 1.5g; KH_2PO_4, 0.5g; $(NH_4)_2SO_4$, 0.5g; $MgSO_4.7H_2O$, 0.2g (all from Unilab); trace element solution 1ml; and 30 mg naphthalene (0.23 mM) per litre of deionized water. Two 100ml solutions containing the K_2HPO_4 + KH_2PO_4 and $(NH_4)_2SO_4$ + $MgSO_4.7H_2O$ respectively were autoclaved (121°C, 15 minutes) separately to prevent precipitation of magnesium phosphates. The final pH of the medium was 7.25 at 24°C. The medium was stored at 4°C until use.

The trace element solution was made up and stored as two separate solutions (A and B) which were mixed in a 1:1 ratio before use. Trace element solution A contained $FeCl_2.H_2O$, 1.5g; NaCl, 1.5g; $MnCl_2.4H_2O$, 0.197g; $CaCl_2.6H_2O$, 0.238g; $CuCl_2.H_2O$, 0.017g; $ZnSO_4$, 0.287g; $AlCl_3$, 0.050g; H_3BO_3, 0.062g; $NiCl_2.6H_2O$, 0.024g and concentrated HCl, 10ml per litre of distilled water.

Trace element solution B contained: Na_2MoO_4, 0.0484g; $Na_2SeO_3.5H_2O$, 0.0025g; and $NaNO_3$, 0.0033g per litre of distilled water. Both trace element solutions were filter sterilized (0.2μm) and stored at 4°C before use.

2.4.2 Phenol

The nutrient medium for phenol catabolism was similar to that used in Section 2.4.1 but phenol (0.941 g l^{-1}, 10 mM) was used instead of naphthalene.

2.5   Microbial inoculum

Erlenmeyer flasks (100ml) containing 10g of the Oakleaf soil and 10g of a crude oil contaminated soil (inoculum source, Appendix 1) and 50ml growth medium (Section 2.4.1) were incubated in a rotational incubator (New Brunswick, PhycroTherm) at 30°C and 120rpm for a week. Inocula, with soil particles in suspension, were taken from these flasks. The hydrocarbon contaminated soil from an old oil refinery site was added due to the presence of recalcitrant aromatic molecules and the probable presence of a microbial association capable of catabolizing naphthalene (and phenol).   The nutrient medium (Section I.2.4.1) to soil ratio was 100:1 (v/w). A semi-continuous aerobic culture was used as a constant source of inoculum for subsequent studies. The medium (500ml), through which air was bubbled (5 ml min^{-1}) was contained in a 1000ml bottle which was maintained at 30°C in the dark. The medium was replenished daily by removing and replacing 200ml. The inoculum bottle was also hand shaken daily for 5 minutes. This inoculum was cultured for 30 days (late exponential phase) before use. The maximum optical density (A_{590}) of the culture after sediment settling was 0.188. The inoculum was maintained at this optical density throughout the study. Naphthalene catabolizing bacteria were preserved by freeze drying and by soil adsorption and dry storage at 4°C. This method of inoculum acclimatization was also used for the phenol catabolizing bacteria. In the case of phenol the maximum optical density was 0.43 and the population was maintained at this optical density. (Nutrient medium as in Section 2.4.2).

2.6   Plate counts

2.6.1 Nutrient agar

Nutrient agar (pH 7.1) plates were prepared by dissolving: meat extract, 1.0g; peptone, 5.0g; yeast extract, 2g; sodium chloride, 8.0g and agar, 15.0g in glass- distilled water and diluting to 1 litre. After autoclaving at 121°C for 15 minutes the medium was cooled to 50°C before pouring. Following setting, the plates were stored inverted at 4°C before use to prevent water condensing on the agar surface.

2.6.2 Soil extract agar

The soil extract agar was prepared by autoclaving (121°C, 30 minutes) 1 kg of soil with 1 litre of deionized water. Agar (15g) and $CaCO_3$ (3g) were added to the supernatant after decanting and filtering (final filtration through 0.2 μm millipore). The plates were inverted as before.

2.7   Scanning electron microscopy

A Hitachi S-570 Scanning Electron Microscope was used. The supernatant (free living) microorganisms were examined after filtering through a 0.2μm millipore filter. The sediment samples were taken directly. Both soil and supernatant were prepared for viewing by critical point drying. The samples were fixed in 3% (v/v) buffered glutaraldehyde for 8 h and then washed twice in 0.05M cacodylate buffer for 30 minutes. The specimens were then dehydrated by sequential washing with alcohol: 30% for 10 minutes; 50% for 10 minutes; and 100% for 10 minutes (twice).

CHAPTER 3. RESULTS AND DISCUSSION

3.0   BATCH ATTENUATION STUDIES

The aim of these experiments was to examine the interactions between organic pollutant chemicals, different soil materials and microorganisms. The experimental approach adopted was to use a system in which the purely physico-chemical interactions were not obscured by physical effects and, thus, possible experimental artifacts were eliminated. This was achieved by determining the microbial catabolism of the various organic compounds in the presence of a growth medium and different soil materials. These initial studies, which were carried out in containers with a high liquid:soil ratio, were later related to soil column studies. It was decided that the soil column studies, where physical interactions play a significant role, could be interpreted more satisfactorily after the container (batch) studies had been completed.

3.1   Naphthalene

Naphthalene and phenol were chosen as key molecules. The reasons for these choices were given earlier (Section 1.3.8). At first, it was decided to use phenol and naphthalene simultaneously but, from preliminary analysis, it appeared that naphthalene was attenuated with a concomitant increase in phenol concentration. This occurred in the absence of microorganisms over a period of 2 - 3 days. Although an interaction between phenol and naphthalene should not occur (C. Southway, personal communication), HPLC analysis suggested a chemical reaction was taking place. It was, therefore, decided to continue the experiments with these compounds separately.

3.1.1 Scanning electron microscopy of soil surfaces and the naphthalene catabolizing population

The aim of using scanning electron microscopy was to examine the surfaces of the various soil particles to determine whether microbial surface growth of naphthalene catabolizing bacteria was significant and whether these cells differed morphologically from those in the supernatant (free living organisms). The micrographs revealed no bacterial or fungal presence on the soil surfaces (Plate 3.1) although the supernatant did contain bacterial growth. Microbial presence in the supernatant was found (Plate 3.2), although these microbial numbers were relatively low. The lack of microbial presence on the surfaces of the various soil materials suggested that the critical point drying (CPD) method (in which alcohol is used) might have removed attached cells. Scanning electron microscopy with cryo-fixing was, subsequently, employed to eliminate this possibility and the results are shown in Plate 3.3. These electron micrographs, although not as clear as those taken after fixing by the CPD method, also revealed no microbial growth. The reason for the lack of visible microorganisms could not be explained. Because of the difficulty in observing microbial attachment on soil by this technique, it was not pursued further. These difficulties could possibly be overcome by the use of other techniques such as the use of the plasmid borne fluorescence genes, which would make the bacterial population visible by direct light microscopy (Prosser *et al.*, 1994).

The hydrophobicity of the cell walls of microorganisms capable of naphthalene catabolism were also presumed to be of importance in this study. By viewing microbial attachment to hydrophilic vs hydrophobic surfaces, morphological and hydrophobic differences within the population could be demonstrated. It was observed that the bacteria growing on the teflon coated septa (Plate 3.4B) differed in morphology and/or size from the bacteria growing on the uncoated section of the septa (Plate 3.4C). Although only single electron micrographs of each section are shown, these observations were similar for many viewing fields and different septa. These results can be ascribed to the fact that the hydrophobic teflon attracted bacteria with a high percentage of non-polar groups in their cell walls, whereas the bacteria attached to the

46
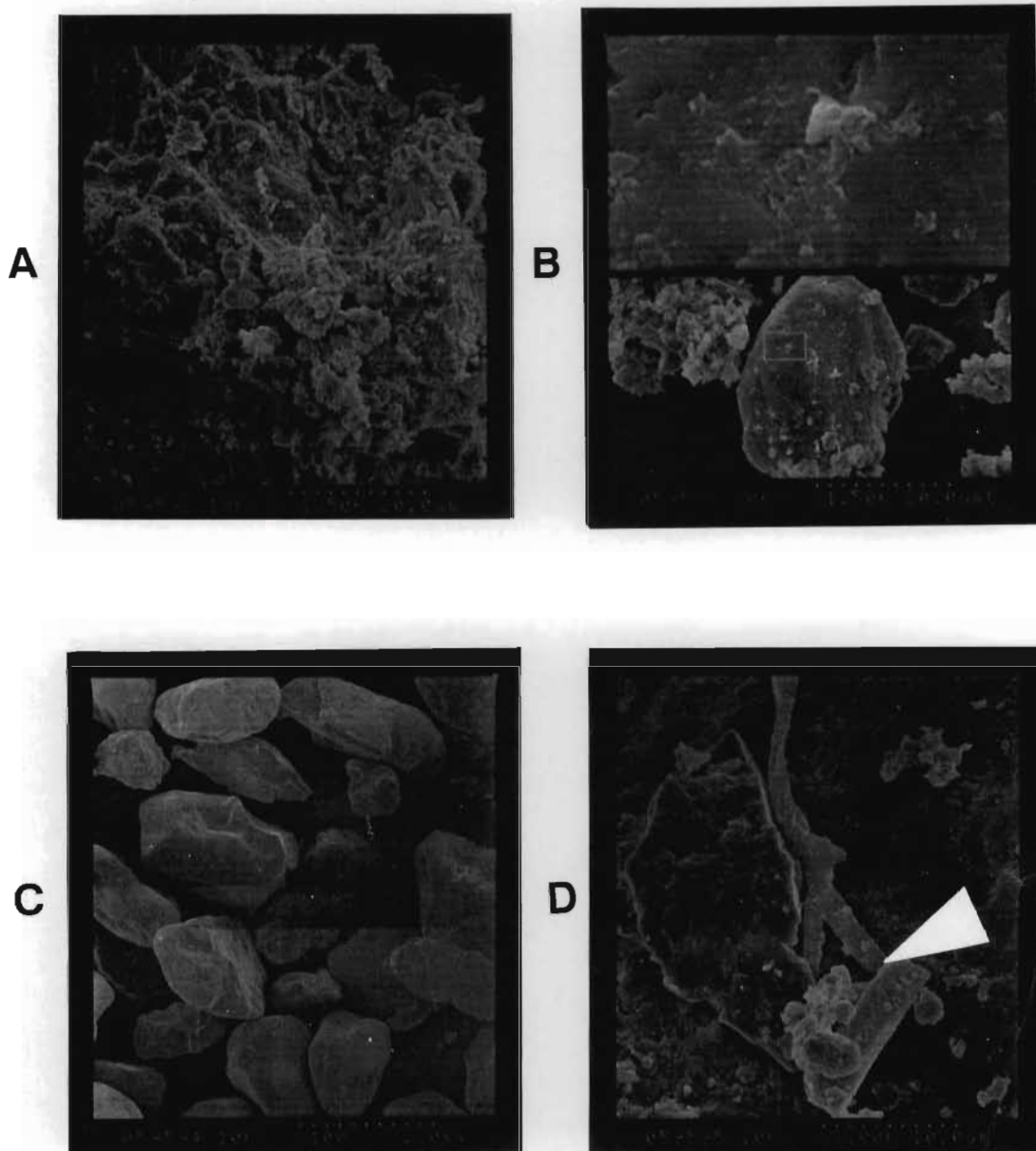
PLATE 3.1: Micrographs from naphthalene soil slurry sediments: Rensburg soil sediment, irregular surface (A). Rensburg soil sediment, smooth surface (with close-up) (B). Acid washed sand sediment (C). Inanda topsoil sediment with organic material (indicated by the arrow) (D).
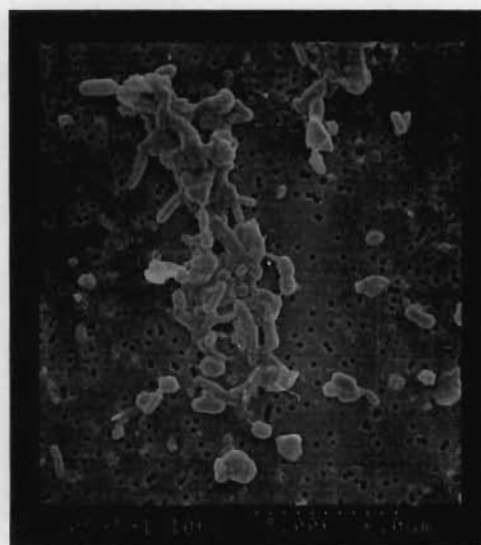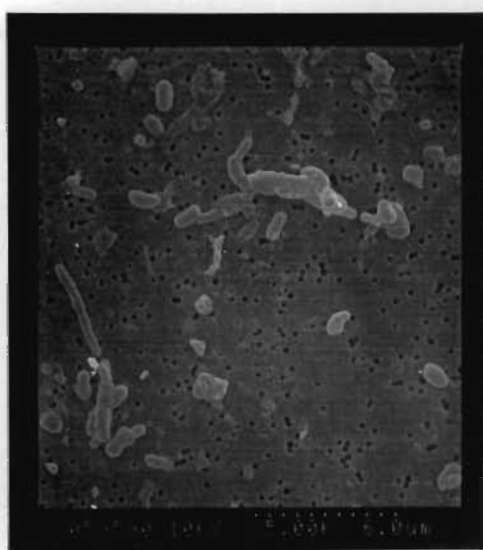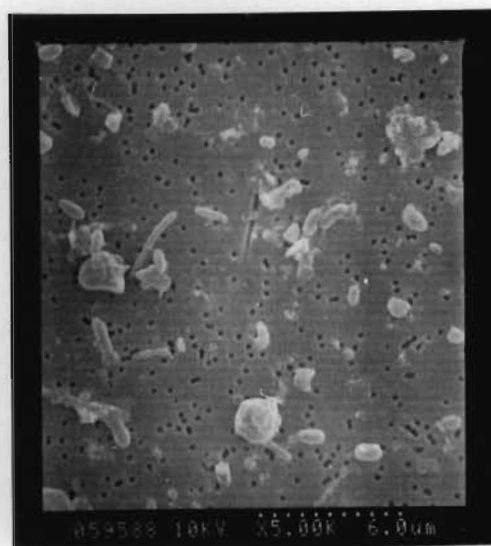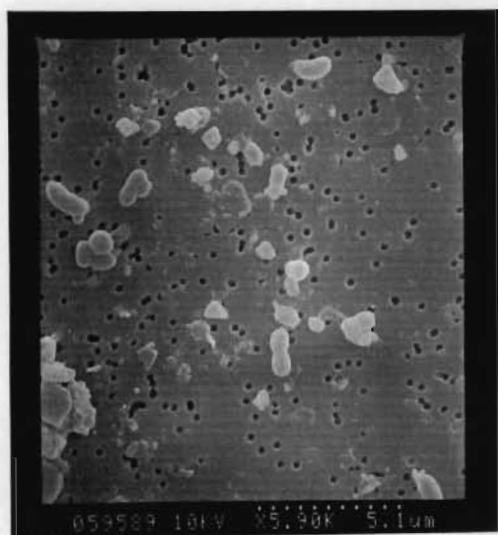
PLATE 3.2: Naphthalene catabolizing association in soil slurry supernatant. (Only the Inanda topsoil supernatant is shown here, but similar micrographs were obtained for the other soils).
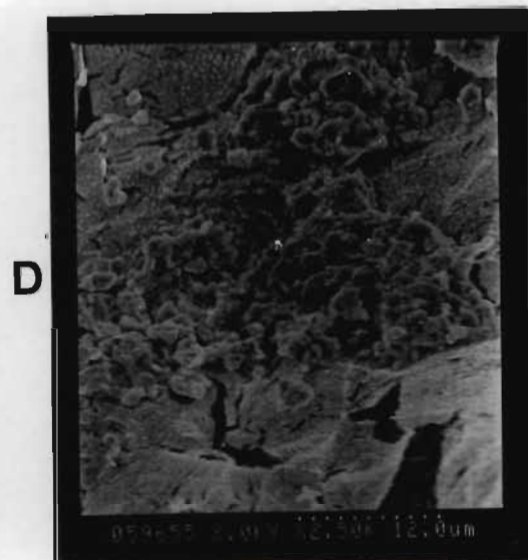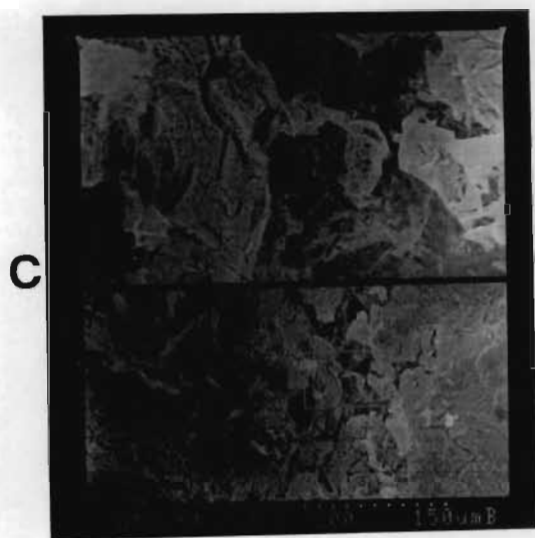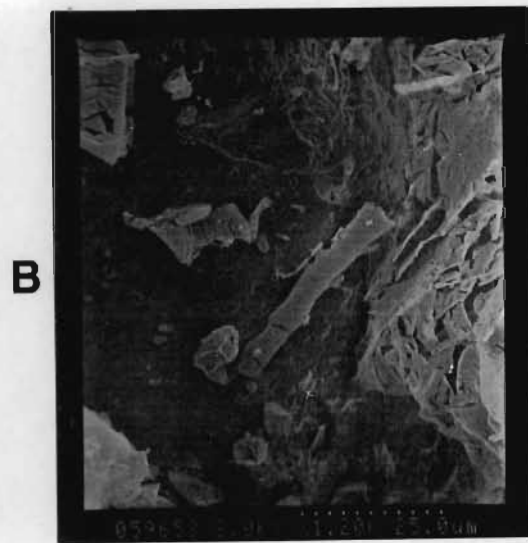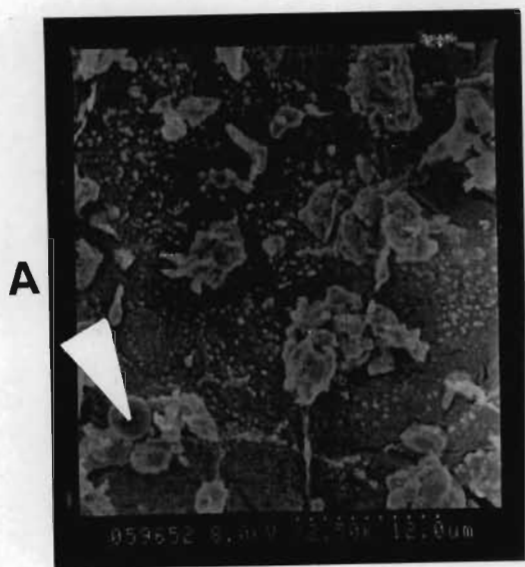
PLATE 3.3: Inanda topsoil sediment (A). The globular structure indicated by the arrow is possibly a fungal spore or sporeforming bacterium. (Fine spherical structures on background surface were found not to be bacteria when viewed at higher magnification). Inanda topsoil sediment with organic material or fungal hyphae (B). Inanda topsoil (two different magnifications) (C). Inanda topsoil irregular surface (D).
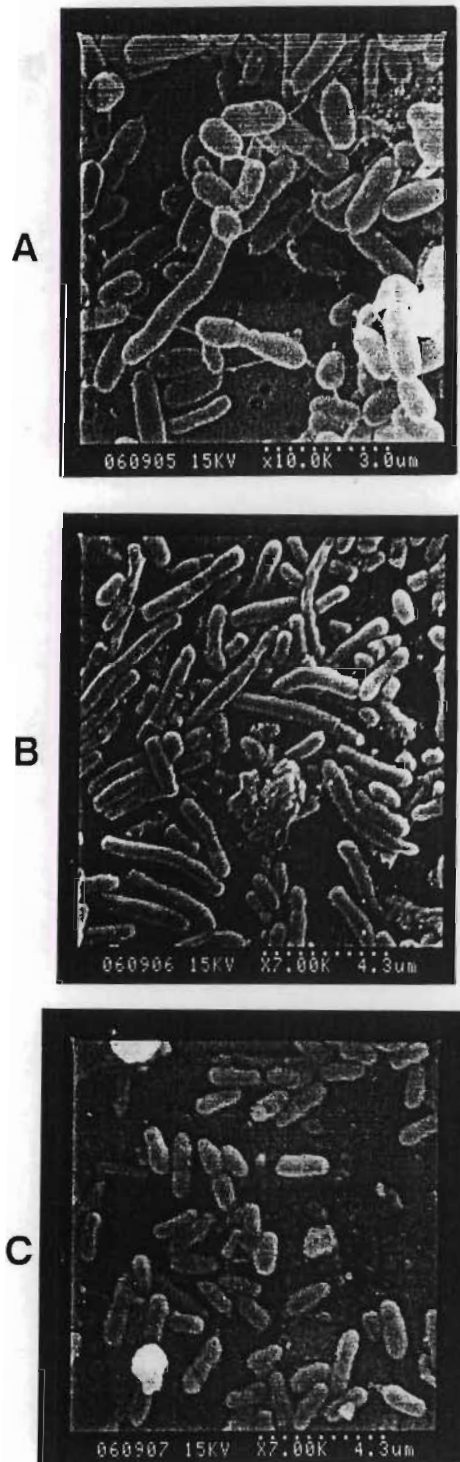
PLATE 3.4: Electron micrographs of naphthalene catabolizing bacteria; in supernatant (A), attached to teflon coated septa (B), and attached to uncoated septa (C).

uncoated surface may have had a lower non-polar group content in their cell walls. Although these results were obtained on synthetic surfaces, they may indicate the preference of certain bacteria for more hydrophobic surfaces *in situ*. These hydrophobic surfaces in soil are mostly found on the organic matter fraction (Wilson & Jones, 1993). These observations also indicate that the microbial population was indeed an association and not a monoculture (Plates I.3.4 A-C).

3.1.2   Aqueous phase naphthalene attenuation in a soil slurry

The objective of this experiment was to determine the attenuation of aqueous phase naphthalene in the presence of three soil materials. The rapid initial reduction in naphthalene concentration in the aqueous phase (Figure I.3.1) could probably be ascribed to adsorption onto the soil and, possibly, the glass bottles. Rapid adsorption is clearly indicated by the zero minute reading which was taken immediately after intiation of the experiment. This conclusion is further supported by the fact that the Inanda topsoil curve decline was the most rapid followed by that of the Rensburg soil. The Inanda subsoil curve decline was much flatter than for the other two soils. This is in accordance with the adsorption isotherms for the three soil materials (Section 3.1.3). The unexpected decline in the control curve could possibly have been due to the fact that the inoculum, which was taken from a soil suspension, contained small amounts of suspended sediment to which naphthalene could have adsorbed. Some adsorption on the surfaces of glass containers was also possible and was found on numerous occasions (Appendix 3, A.3.2-3).

Although further catabolism of naphthalene could have occurred on the soil surfaces or in the supernatant this could not be detected by monitoring the aqueous phase concentration. This problem cannot simply be eliminated by using a higher concentration of naphthalene since the maximum solubility of naphthalene in water is 32 mg l^{-1}. Although the solubility of naphthalene in soil can be increased by using organic solvents, these solvents could be toxic to the organisms or be used as an alternative carbon and energy source. Extractions from soil samples, in which the adsorbed concentration is determined, are probably more useful in cases where the compound of interest is hydrophobic. Soil extractions are,

however, extremely cumbersome and time consuming, and, perhaps more importantly, destructive. Some of these problems were overcome by using partial extraction methods (Appendix 3).
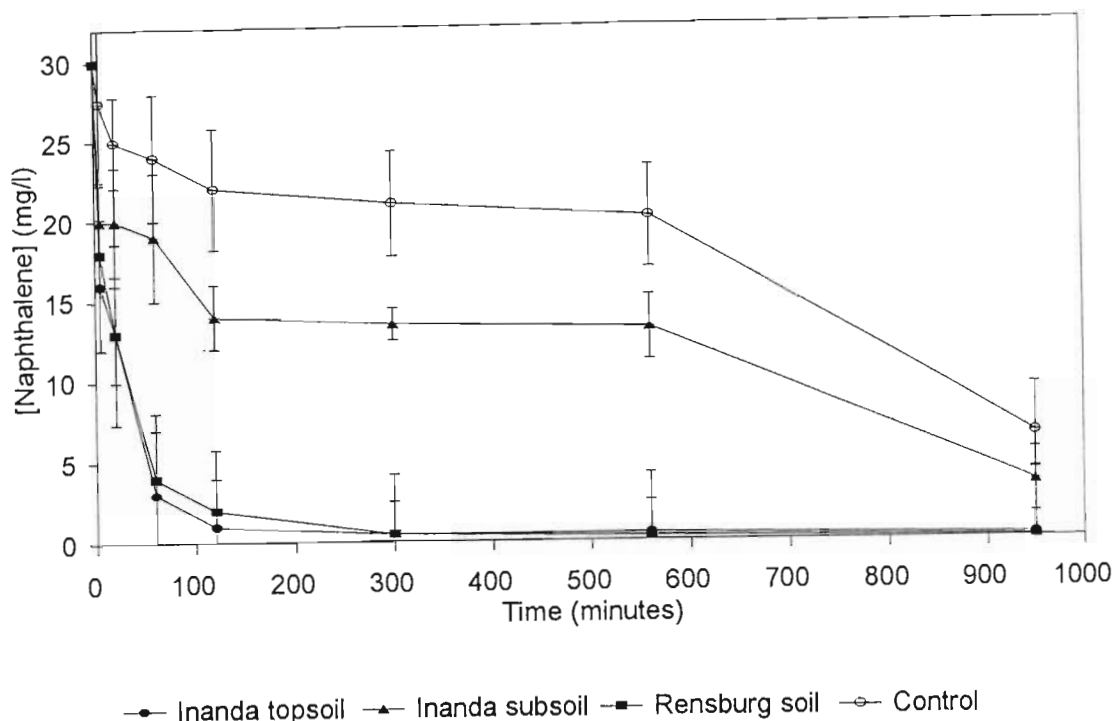
FIGURE 3.1: Aqueous phase naphthalene attenuation (degradation) in the presence of various soil materials. (Error bars in all figures represent standard deviation from mean).

3.1.3 Adsorption of molecules to soil

The aim of this experiment was to estimate the physico-chemical adsorption of naphthalene, acetonitrile, methanol and cadmium to various soil materials. From the previous experiment (Section 3.1.2), it was apparent that attenuation from the aqueous phase was masked by the rapid adsorption of the added naphthalene. It was, thus, important to determine the exact effect of this physico-chemical adsorption in order to distinguish between adsorption and catabolism in subsequent experiments. Adsorption isotherms are also very useful in that they

can be used to determine the concentration of a particular compound on the soil surface by quantifying the concentration of that compound in the aqueous phase.

Figures 3.2 A & B show naphthalene adsorption isotherms for the three soil materials as well as the partitioning of adsorbed and aqueous phase naphthalene at an original concentration of 30 mg l^{-1} (at a soil : liquid ratio used in subsequent growth experiments). The regression was assumed to be linear in accordance with comparable studies (Lui *et al.*, 1991). The adsorption maxima recorded in this study are, however, considerably higher than those reported by Lui *et al.* (1991). The differences could, possibly, be ascribed to the higher organic matter contents of the soil materials as well as to experimental artifacts (Appendix 3, Section A.3.3). From the adsorption isotherms it seemed that the adsorbed naphthalene concentration strongly correlated with the organic matter content of the soil materials and not with the clay content (Appendix 1). Adsorption of metals, however, is more closely related to the clay content rather than the organic matter (Lo *et al.*, 1992). This effect may later be of importance in determining the effect of a heavy metal (i.e. cadmium) on the SMP system. Since adsorption of naphthalene is believed to be the rate-limiting step in its catabolism (Scow & Hutson, 1992; Wilson & Jones, 1993), it seems most likely that the Inanda topsoil, with the highest adsorption capacity, would retard naphthalene catabolism to the highest degree in the SMP system. This was, subsequently, confirmed (Section 3.1.4). The relatively high liquid to soil ratio used, i.e. 230:12 (v/w) was necessary in order to detect naphthalene in the aqueous phase after adsorption. It should, however, be kept in mind that in adsorption studies such as these the contact between the sorbed molecule of interest and the soil is probably much greater than would be the case *in situ*. Characteristically, *in situ* liquid:soil ratios are much lower so that adsorption would probably not occur to the same extent. These adsorption isotherms should, therefore, be used as indicators only, rather than absolute values. The great advantage of adsorption isotherm determinations is that the adsorbed concentration of any organic molecule can be calculated by measuring the aqueous phase equilibrium concentration of that molecule. This negates the necessity for the rather tedious extraction of the molecules. This is, however, only true when the concentration is below the solubility limit of the molecule in question.
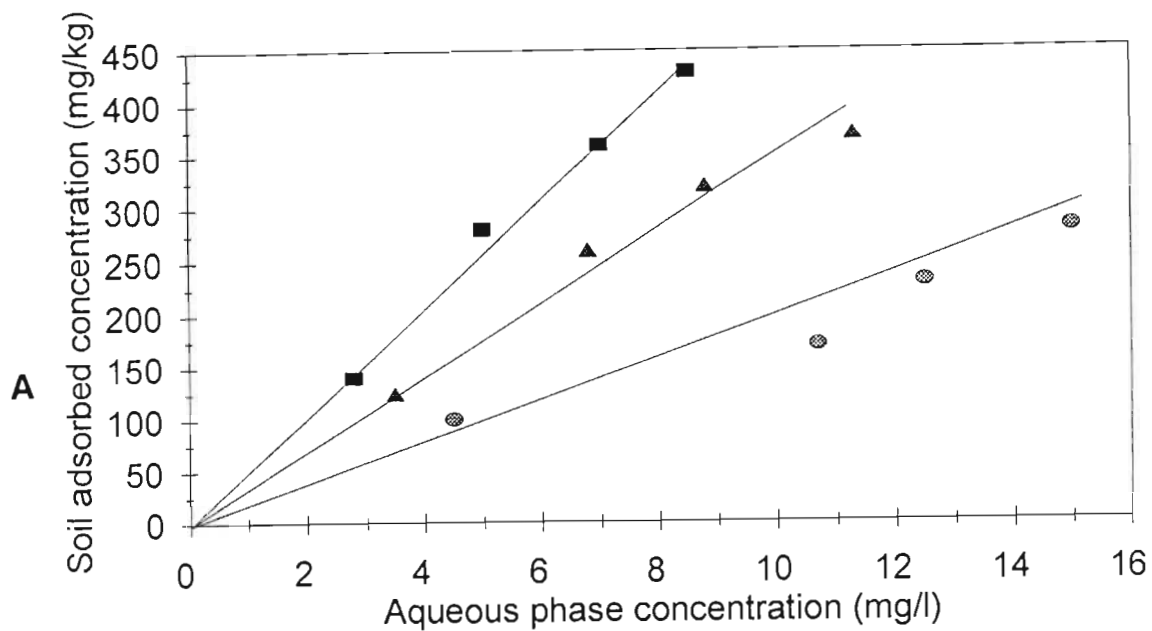
FIGURE 3.2: Adsorption isotherms for naphthalene onto Inanda topsoil, Inanda subsoil and Rensburg soil (A). Partitioning of adsorbed and aqueous phase naphthalene at an original aqueous phase concentration of 30 mg l^{-1} (B).

Adsorption isotherms also give useful information about the type of sorption which is occurring as well as the capacity of the sorbent for the sorbed molecule (Giles *et al.*, 1986). The adsorption isotherms were forced linear because of evidence in the literature that this was the type of adsorption which was found for naphthalene for most soils similar to those used here (Lui *et al.*, 1991). This indicates that the maximum adsorption capacity of the soils had not been reached at even the highest concentrations used.

The adsorption of acetonitrile was found to be negligible and the results are, therefore, not given here. Figure 3.3A shows the adsorption isotherms for methanol on the different soil materials. The adsorption of methanol (Figure 3.3B) indicates that, for this particular soil : liquid ratio and methanol concentration of 15 % (v/v) (which was used in subsequent growth experiments), more of the added methanol was adsorbed onto the Inanda topsoil than remained in the aqueous phase. This was, however, not found for the Inanda subsoil and Rensburg soils. This observation could be ascribed to adsorption onto the organic fractions of these soils (Appendix 1).

The cadmium adsorption isotherms show the great affinity of the Rensburg soil for cadmium (Figure 3.4A). Figure 3.4.B indicates that more of the cadmium was adsorbed on all the soil materials than remained in the supernatant at this particular soil : liquid ratio. Adsorption was most pronounced for the Rensburg soil, probably due to the high CEC of that soil (Appendix 1). The results of these adsorption experiments are particularly important for interpretation of subsequent results.
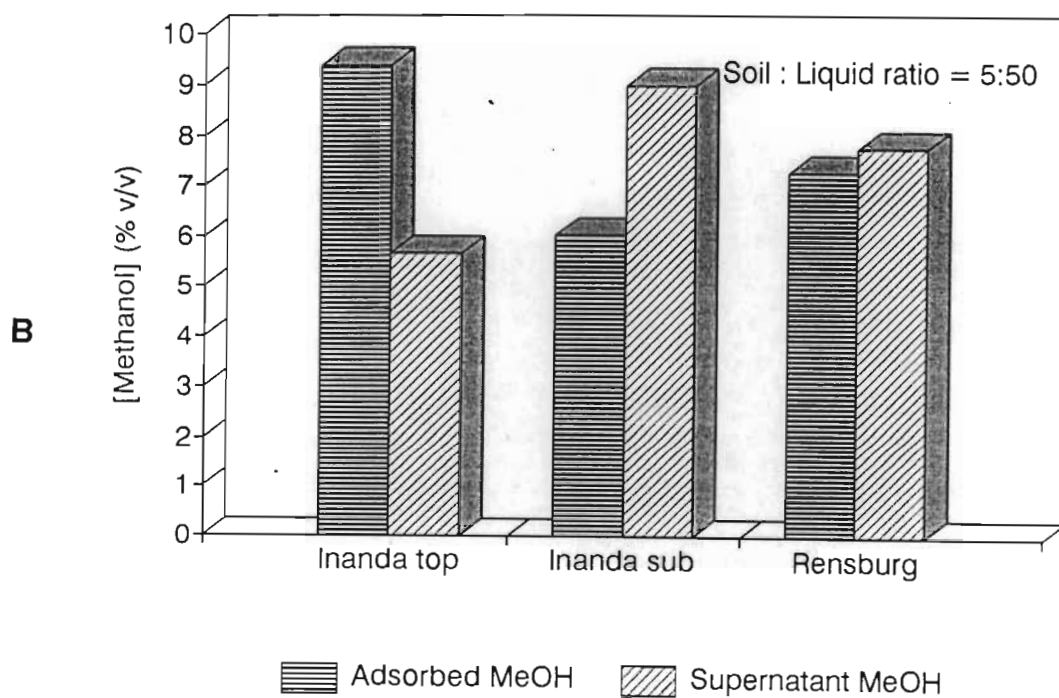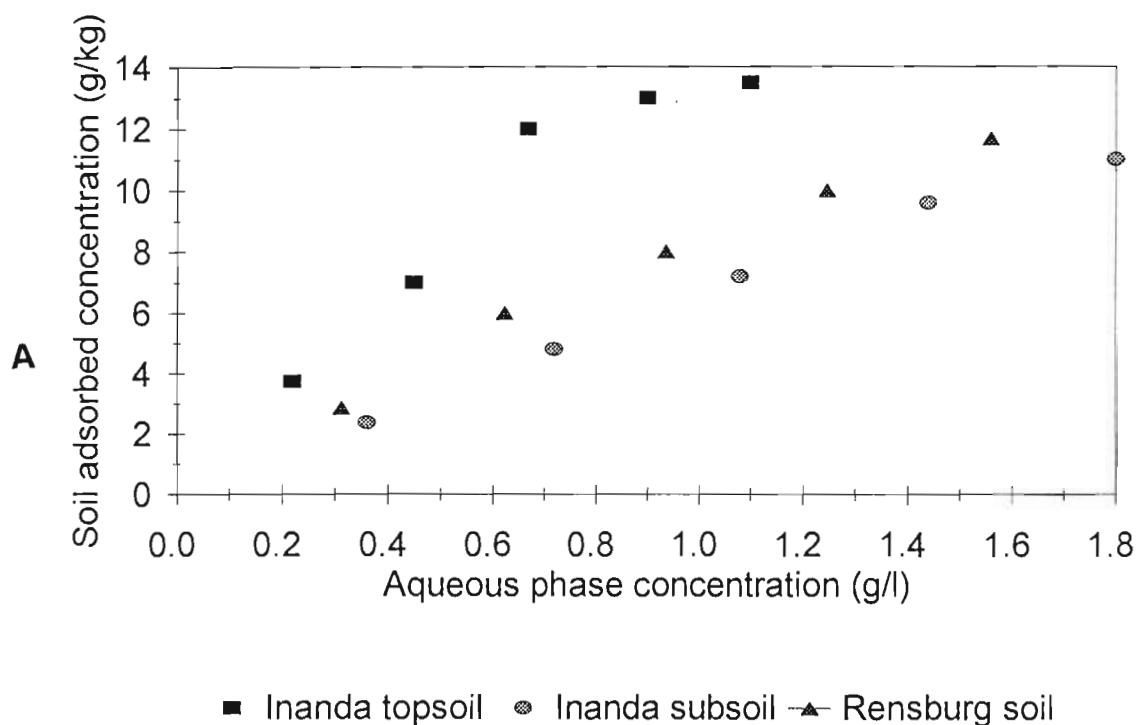
FIGURE 3.3: Adsorption isotherms for methanol onto Inanda topsoil, Inanda subsoil and Rensburg soil (A). Partitioning of adsorbed and aqueous phase methanol at an original aqueous phase concentration of 15% (v/v) (B).
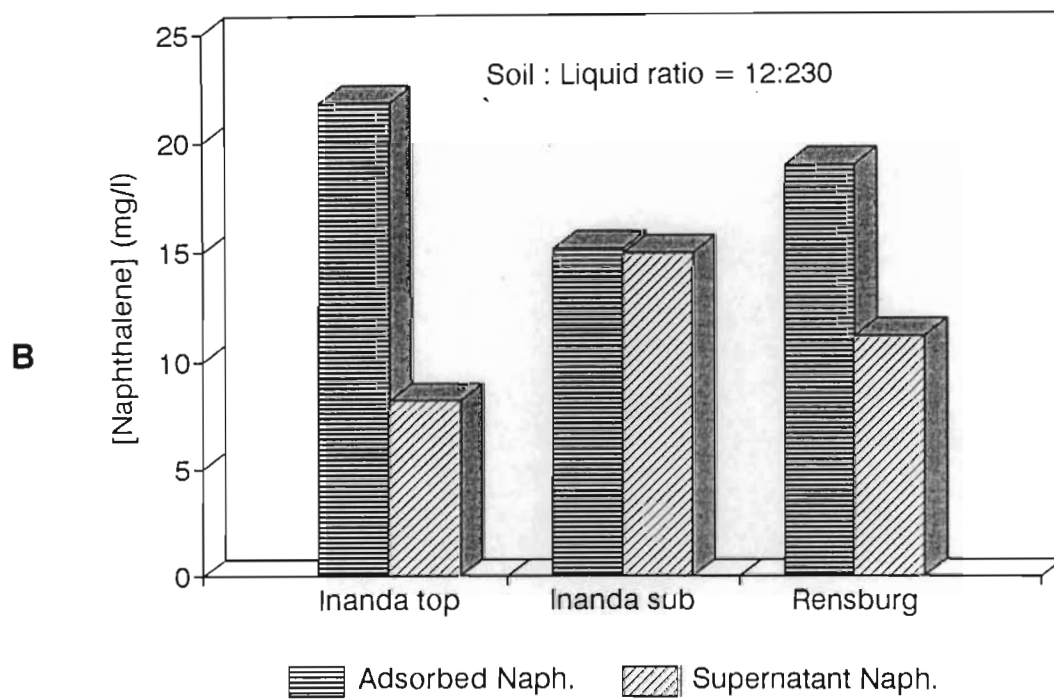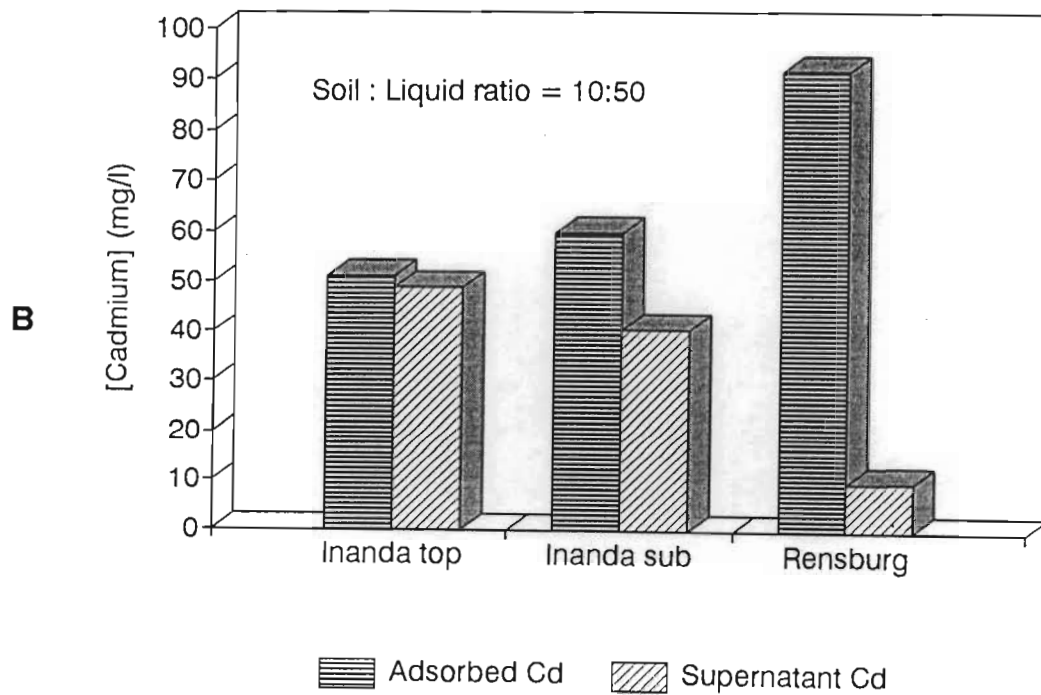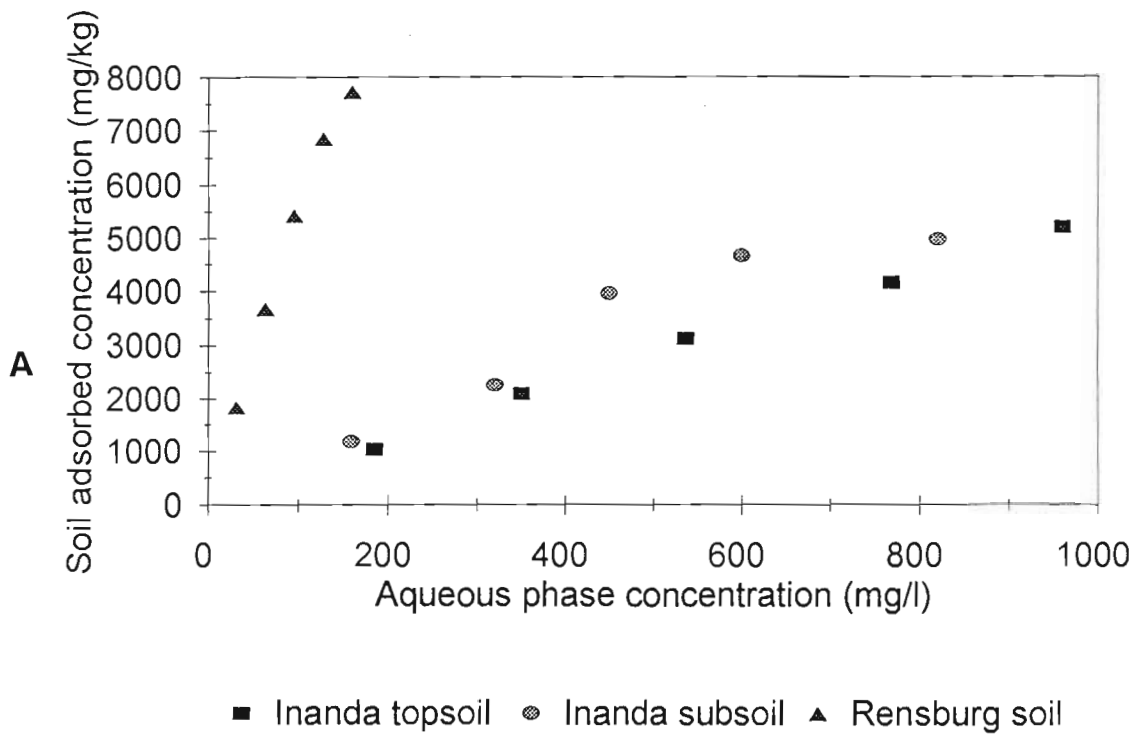
FIGURE 3.4: Adsorption isotherms for cadmium onto Inanda topsoil, Inanda subsoil and Rensburg soil (A). Partitioning of adsorbed and aqueous phase cadmium at an original aqueous phase concentration of 100 mg l^{-1} (B).

3.1.4 Growth of naphthalene catabolizing organisms in the presence of various solvents and a heavy metal (Cd) and in the absence of soil

The aim of this study was to determine both to what extent different solvents could be used as alternative substrates and the threshold bactericidal / bacteriostatic concentrations of each solvent in the absence of soil. The effects of a model heavy metal (cadmium) on the microbial population in the soil-free systems were also examined. From Figure 3.5 it can be seen that the bactericidal / bacteriostatic thresholds for acetonitrile and methanol were approximately 5 and 10% (v/v), respectively. The 5% (v/v) methanol supplemented cultures exhibited significant microbial growth compared with the controls which indicated that the methanol was used as an alternative carbon and energy source. The optical density increases in the presence of cadmium (Figure 3.3C) indicated microbial growth or, more likely, the presence of precipitates.

Microbial inhibition (Figure 3.3C) was, however, not consistent with an increase in Cd concentration. This was probably due to flocculation/precipitation. It is thought that the flocculation resulted from complexation between Cd and phosphate, although microbial growth could have been a contributory factor. To confirm naphthalene catabolism, residual substrate analyses were made on days 4 and 8. Naphthalene concentrations were, however, below the detection limit in all samples (including the control).

3.1.5 Catabolism of adsorbed naphthalene

In the previous experiment the effects of various challenges in the absence of soil were examined. The rate-limiting steps in the catabolism of a hydrophobic compound such as naphthalene are its adsorption to soil and its low solubility (Scow & Hutson, 1992; Volkering *et al.*, 1992). Adsorption can, however, be reversed by the use of solvents which reduce the
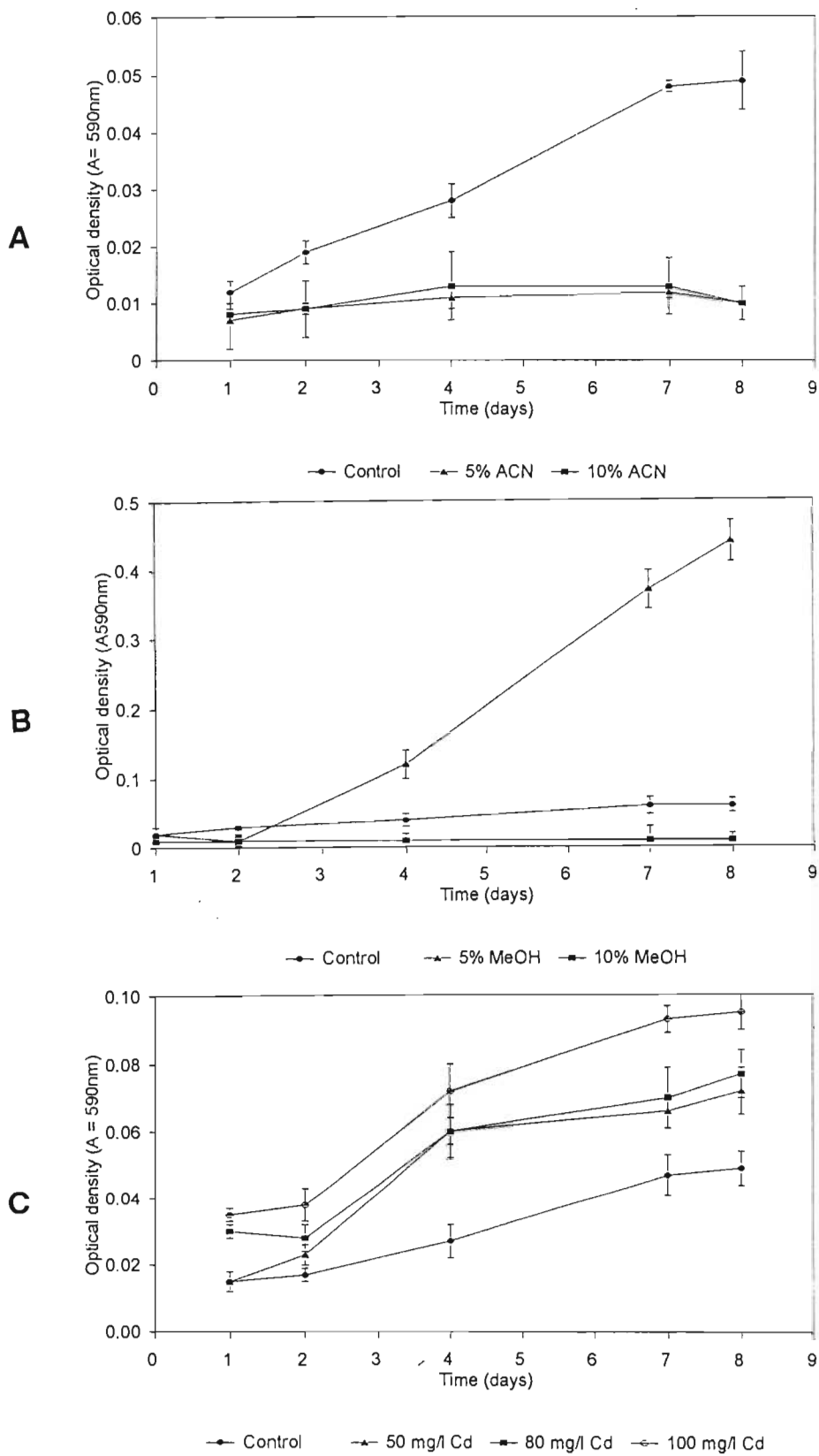
FIGURE 3.5: Growth of naphthalene catabolizing organisms in the presence of acetonitrile (A), methanol (B) and cadmium (C).

polarity of water. Solvents can solubilize and detach naphthalene (Lane & Loehr, 1992) thereby making it more susceptible to microbial catabolism. It is also possible, however, that the solvents may be toxic to the microbial population or may be used as an alternative carbon and energy sources. This motivated the use of two solvents which could differ in their toxicity thresholds as well as their potential as alternative carbon and energy sources. It should be noted that organic solvents or surfactants which often occur in landfills could decrease the polarity of water (Lane & Loehr, 1992; Wilson & Jones, 1993) thereby affecting solubilization and degradation of hydrophobic compounds. A model element, cadmium, was used to determine its effects on naphthalene catabolism in the presence of various soil materials. The reason for such a study was to determine to what extent the different soils decreased the toxicity threshold concentration of the heavy metal. This experiment, together with the previous optical density study (Section 3.1.4), were designed to answer these questions. To effectively interpret the data from this experiment it was necessary to make various comparisons as shown in Figures 3.6 and 3.7. The datum points in these figures are expressed as a percentage of the original extractable naphthalene. Figure 3.6 shows the effects of soil on naphthalene catabolism in the presence and absence of solvents and a toxic heavy metal. It is clear that in no treatment was catabolism significantly retarded. In all soils and with all treatments the naphthalene concentration significantly decreased ($>12\%$ of the original). There were, however, great differences between the soil types, particularly between days 4 and 8. The Rensburg soil showed higher residual naphthalene concentrations in this period for all but the acetonitrile treatment. It should be noted that the differences in naphthalene concentration between the soils in real terms (before adjustment for original recoverable concentration) were even greater than those given here. The higher concentration in the Rensburg soil during the 4 - 8 day period was unexpected since the adsorption isotherms indicated that the Inanda topsoil with its high organic matter content should have adsorbed most naphthalene and, therefore, retard microbial catabolism to the largest extent. It is, thus, unlikely that adsorption alone is the only relevant factor in microbial catabolism of adsorbed naphthalene. The fact that the higher concentration sequence for the soils (in all but the acetonitrile treatment) was in the order: Rensburg > Inanda topsoil > Inanda subsoil, indicates some mechanism which is directly related to a particular soil characteristic(s).
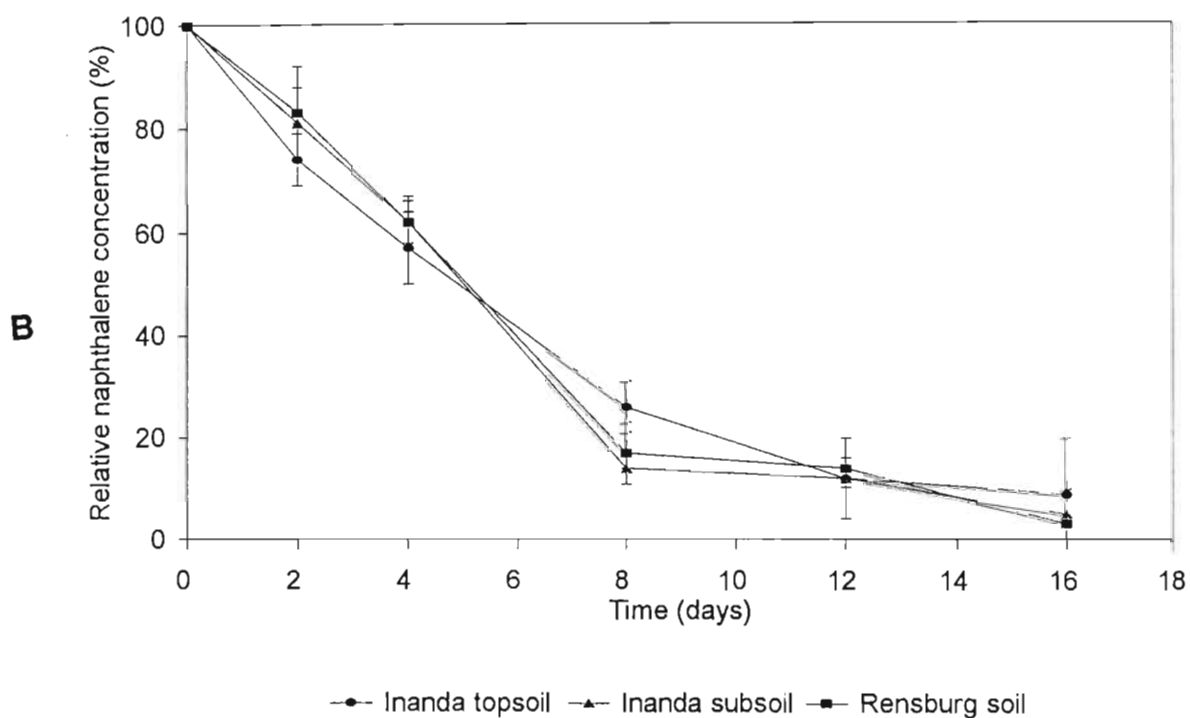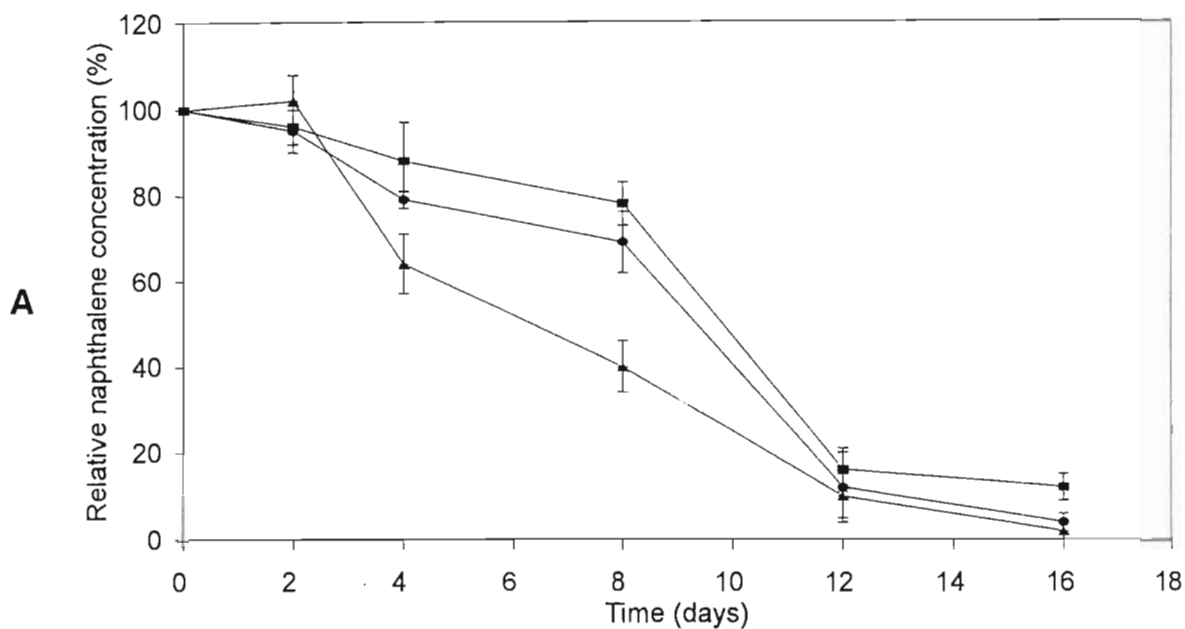
FIGURE 3.6: Catabolism of naphthalene adsorbed to soil in the: absence of inhibitory substances (A) and presence of acetonitrile (B).
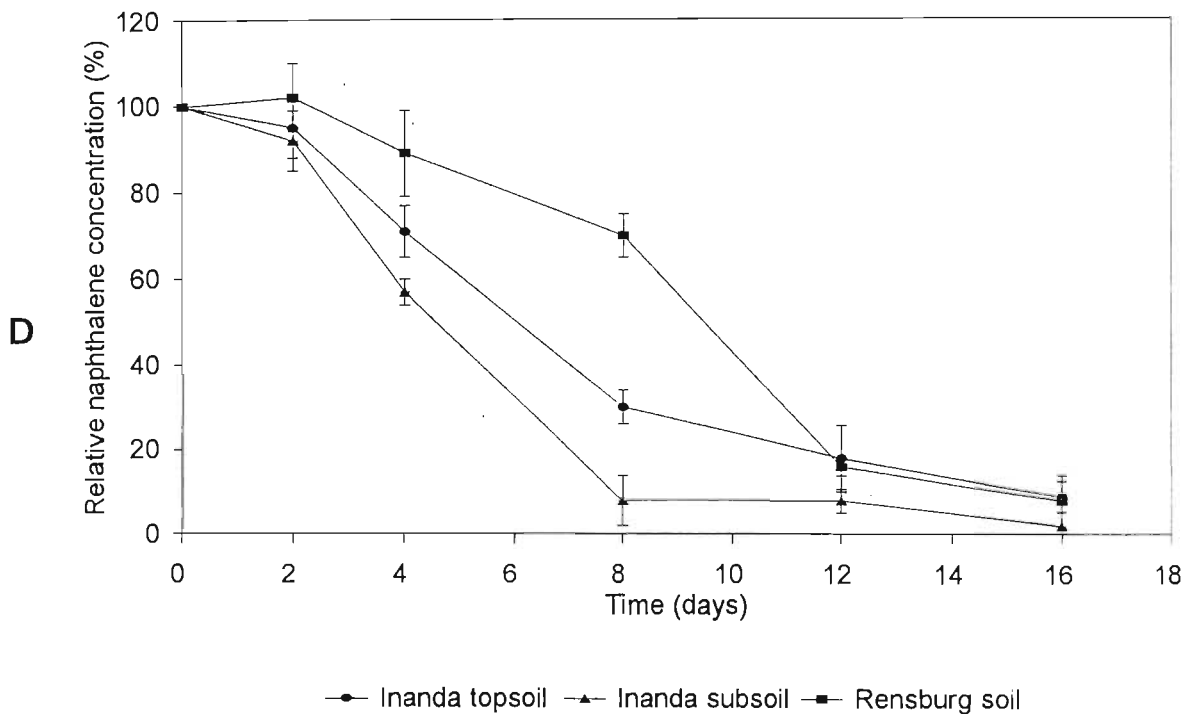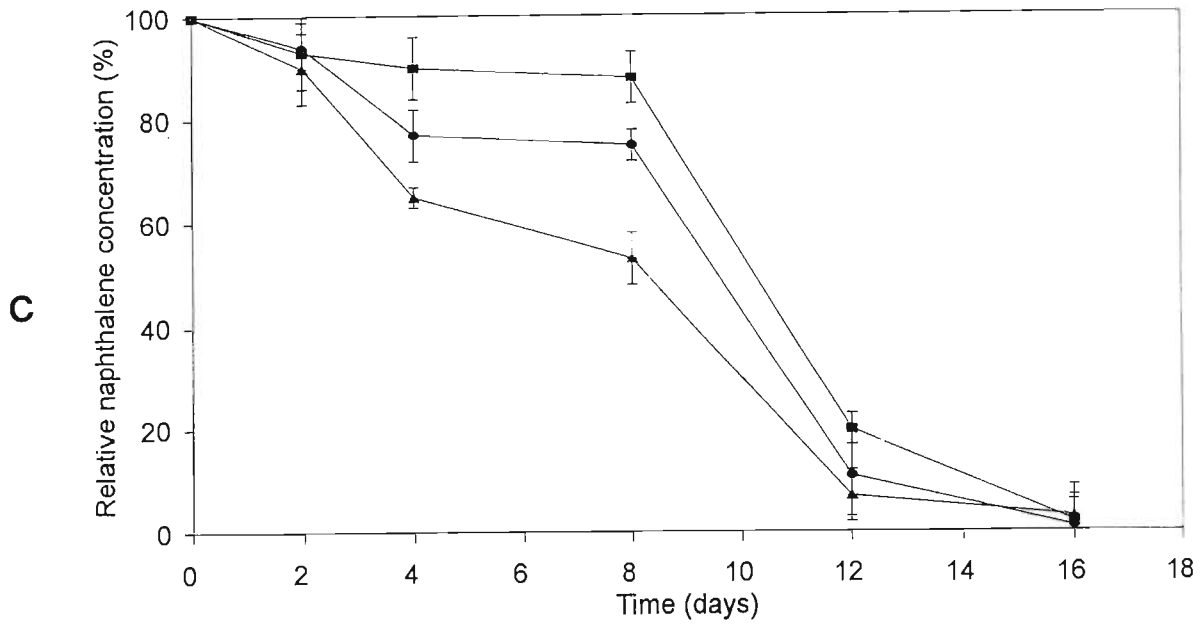
FIGURE 3.6 (continued): The effects of methanol (C) and cadmium (D) on adsorbed naphthalene catabolism.
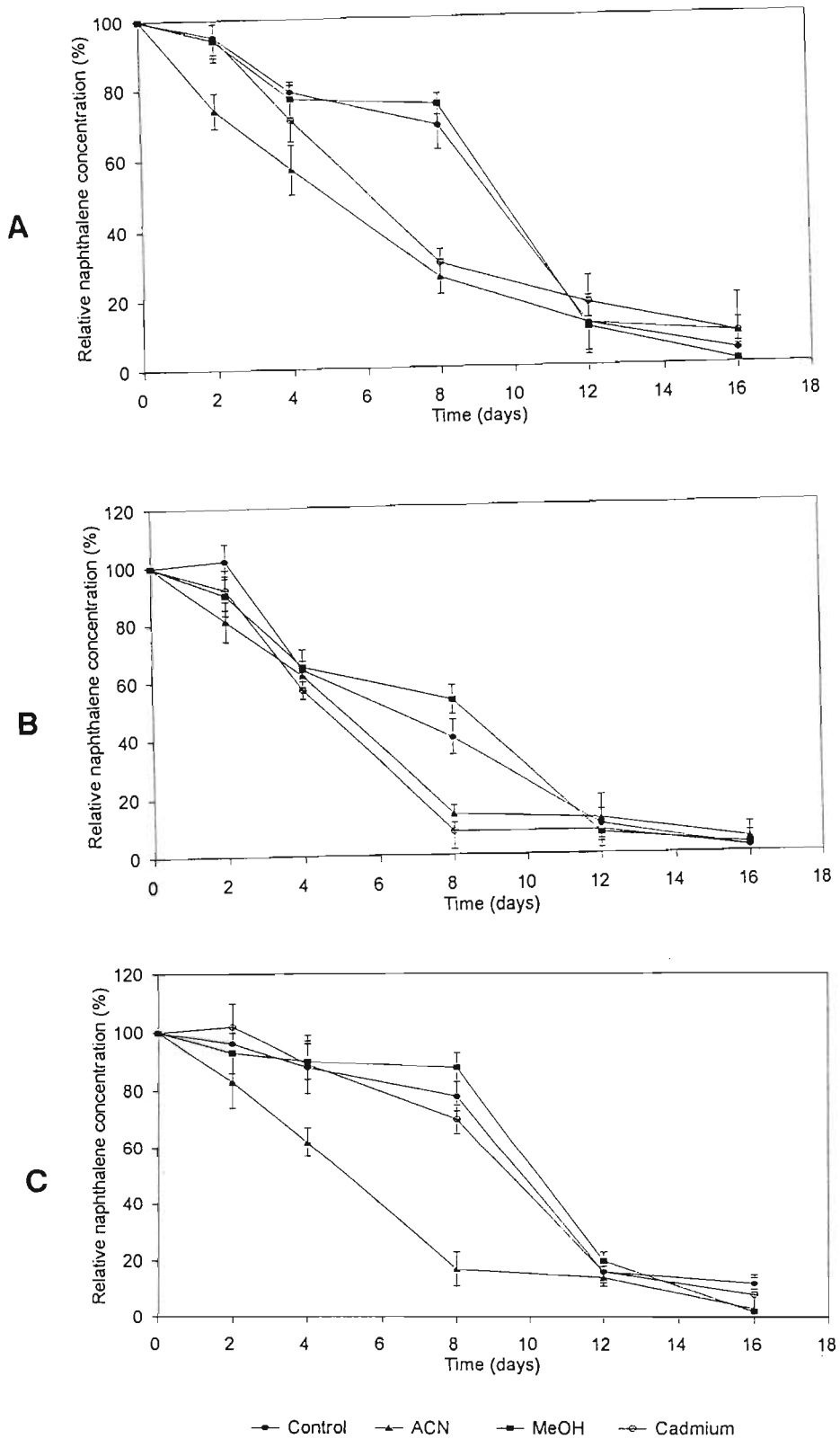
FIGURE 3.7: Effects of acetonitrile, methanol, cadmium and distilled water (control) on the catabolism of adsorbed naphthalene in the presence of Inanda topsoil (A), Inanda subsoil (B) and Rensburg soil (C).

Neither surface area (Appendix 1) nor cation exchange capacity (Appendix 1) adequately account for the observed results. A possible explanation may be found in a combination of the organic carbon content (hydrophobicity) and the cation exchange capacity (Huysman & Verstraete, 1993a). By determining which had the highest relative value for both these two soil characteristics the order of Rensburg, Inanda topsoil, Inanda subsoil is reached. The effect exerted by the organic carbon component is due to the high adsorption capacity of the organic carbon rich soil. The effect of the cation exchange component is, however, more difficult to defend. Cation exchange, as discussed in Section 1.2.1, could be responsible for limiting the catabolism of naphthalene but the exact mechanism is difficult to determine. It is unlikely that the pH, and, therefore, the CEC of the OM, was changed during naphthalene metabolism due to the buffering effect of the two phosphates included in the nutrient medium. The comparable attenuation curves in Figure 3.6B indicate that the solubilizing effect of acetonitrile on naphthalene (Appendix A.3.4) overrode the differential adsorption effects of the three soil materials. The solubilizing effect of acetonitrile was greater than that of methanol (Figure 3.6C) but neither of the solvents, nor the cadmium, seemed to be toxic, although the concentrations used were much higher than the threshold bactericidal/ bacteriostatic concentrations determined in Section 3.1.4. It should be realized that the differential effects of soil materials which were recorded during attenuation were attained under high liquid to soil ratios. These effects would, therefore, probably be even more significant in *in situ* soil conditions. In Figures 3.7A-C the effects of various challenges are shown for each soil material. For cadmium, very little effect was exerted on the Rensburg soil. In contrast, cadmium affected both Inanda soil horizons (days 4 - 8). The most likely reason is that Cd was adsorbed to the Rensburg topsoil material, due to the high CEC, and was removed from the aqueous phase.

The presence of acetonitrile did, however, result in lower naphthalene concentrations (days 4 - 8) in all three soil materials which, possibly, indicated a solubilization and desorption effect and, hence, greater microbial catabolism. The acetonitrile also seemed to solubilize the naphthalene to a much greater extent than did the methanol. Although not proven, methanol seemed to limit naphthalene catabolism to some extent. This effect cannot be ascribed to toxicity since acetonitrile is bactericidal/ bacteriostatic to the naphthalene catabolizing population at a lower concentration than methanol (Section 3.1.4). The most

probable explanation is that the methanol was used as an alternative carbon and energy source.

3.2   Phenol

3.2.1 Soil adsorption

As with naphthalene, this experiment was designed to quantify the adsorption of phenol onto soil which would occur for the concentrations planned for subsequent experiments.   Figure 3.8A shows the phenol adsorption isotherms and Figure 3.8B shows that most of the applied phenol remained in the aqueous phase at the soil : liquid ratio of the subsequent experiment. The concentration adsorbed by the different soils did not vary significantly and this could be ascribed to the high solubility of the molecule (Appendix 2, A.2.2).

3.2.2 Growth of phenol catabolizing organisms in the presence of solvents and a heavy metal (Cd)

Although adsorption is not a growth limiting phenomenon in the catabolism of phenol in soil it is important to determine the effects of solvents and heavy metals, as used in the previous experiment, for comparative purposes. The reasons for this are twofold. Phenol catabolism was used as a "control" to determine physico-chemical adsorption phenomena for naphthalene since phenol is relatively soluble in water and, thus, does not adsorb to soil to the same extent.   It is also important to consider the roles of other adsorptive molecules in the catabolic processes.   Methanol and acetonitrile were both inhibitory to the phenol catabolizing population in the absence of soil (Figure 3.9A).   Microbial growth in the presence of naphthalene was less than the control which seemed to indicate a slight inhibitory effect of naphthalene on the population.   It is unlikely that naphthalene was used as an alternative substrate since phenol is more readily catabolized than naphthalene (Section 1.3.8) and the inoculum used was acclimatized to phenol catabolism.   Figure 3.9B shows that inhibition of growth occurred at all cadmium concentrations and was not concentration

65

dependent. The highest cadmium concentration (100 mg l^{-1}) showed a slightly higher optical density at all time intervals which was most likely due to precipitation.

A

B
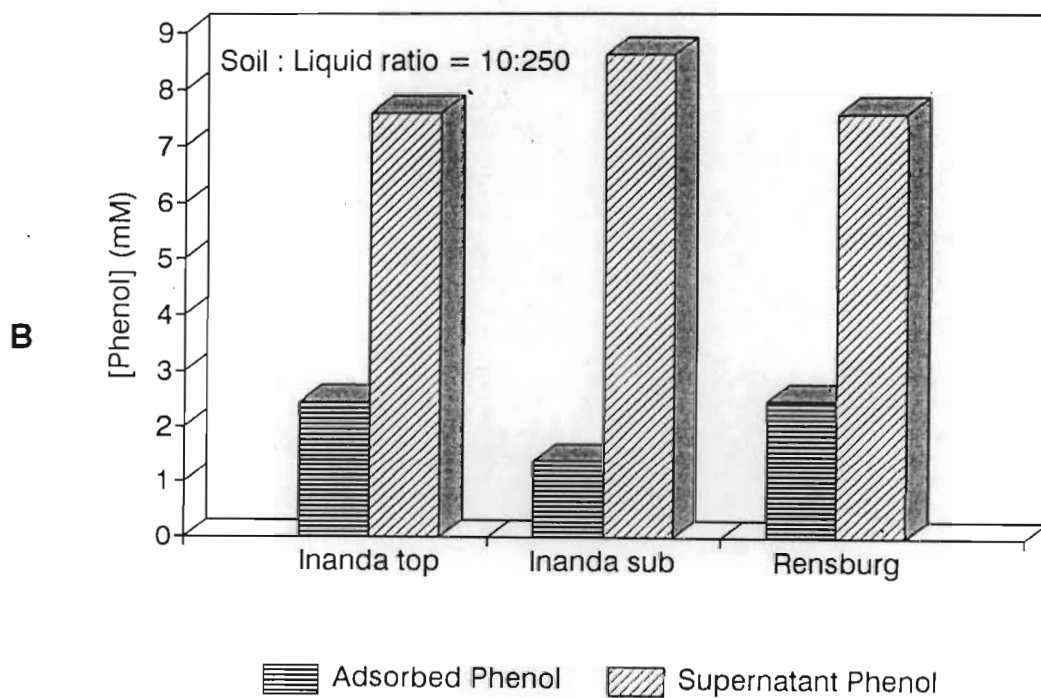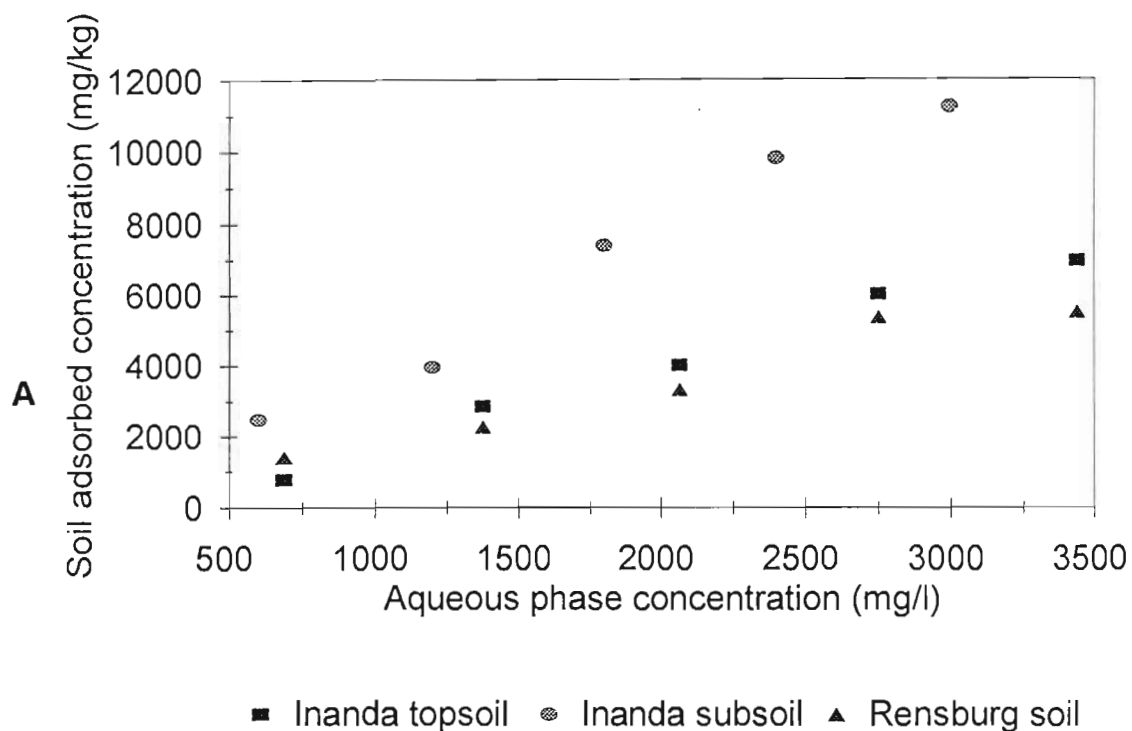
FIGURE 3.8: Adsorption isotherms (A) for phenol onto Inanda topsoil, Inanda subsoil and Rensburg soil. Partitioning of adsorbed and aqueous phase phenol (B) at an original aqueous phase concentration of 10mM.
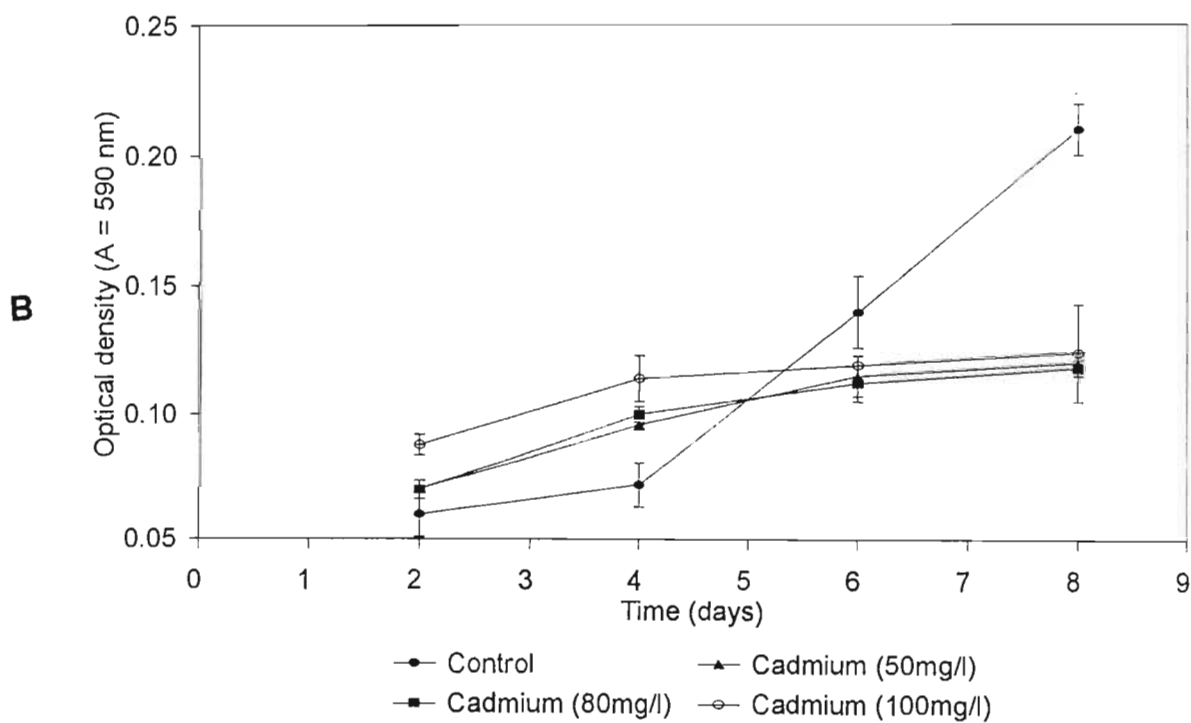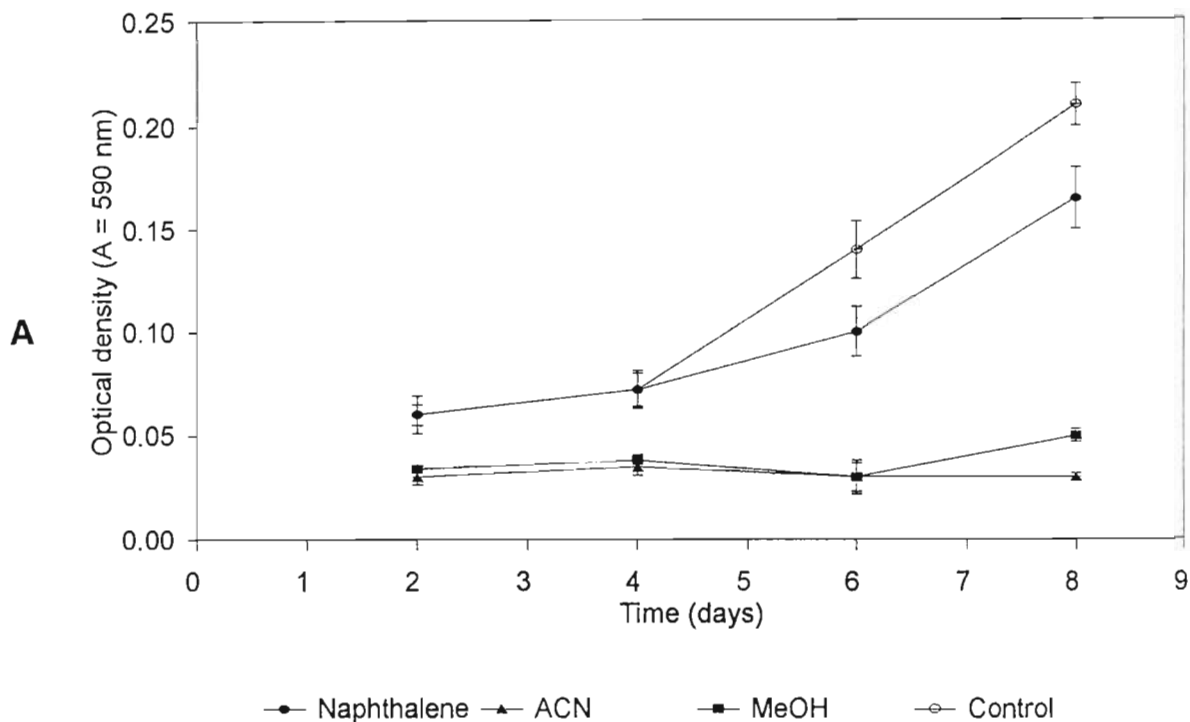
FIGURE 3.9: Effects of acetonitrile, methanol and naphthalene (A) and different cadmium concentrations (B) on the growth of the phenol catabolizing population.

3.2.3 Phenol attenuation in soil with an acclimated microbial population

The objective of this experiment was similar to that conducted for naphthalene (Section 3.1.5). In this case, however, the model pollutant compound (phenol) was not adsorbed to the same extent as the naphthalene. Thus, it was anticipated that the major effect of soil would be either directly on the microbial population or indirectly through the other variables such as metals and solvents which may influence the attenuation process. The soil material used had little effect on phenol catabolism (Figure 3.10A) which is contrary to what was found by Knezovich *et al.* (1988) where soil type was important. For their experiment, however, only *Pseudomonas putida* was used. There was, however, a slightly more rapid decrease in phenol concentration after day 10 for the Inanda subsoil. This small difference became more significant after examination of Figures 3.10B-D. In the presence of Inanda topsoil and Inanda subsoil, phenol catabolism was not inhibited by cadmium (≤ 600 mg l^{-1}), as was expected, but was inhibited by naphthalene. If the adsorption isotherms for cadmium onto these soils are assumed to be straight lines, it can be assumed from Figures 3.4 A & B that, due to adsorption, approximately half of the initial cadmium content would be present in the supernatant. This would give an effective cadmium concentration of ≤ 300 mg l^{-1} in the supernatant which should be inhibitory to microbial growth in the supernatant (Figure 3.10B). It could, thus, be argued that microbial catabolism was due to attached organisms and that the microorganisms were protected from cadmium toxicity due to close association with the soil surfaces. The fact that all cadmium concentrations were inhibitory to phenol catabolism in the presence of the Rensburg soil (Figure 3.10D) supports this argument. If the microorganisms involved in phenol catabolism were mainly free-living, cadmium would have the least effect in the Rensburg soil. This soil has the greatest CEC (Appendix 1) and, therefore, also the highest adsorption capacity for Cd (Figure 3.4) which should result in a relatively low aqueous phase cadmium concentration. Inhibition could, therefore, be ascribed to the relatively high concentration of cadmium at the soil surface in close proximity to the adsorbed catabolic species.

69
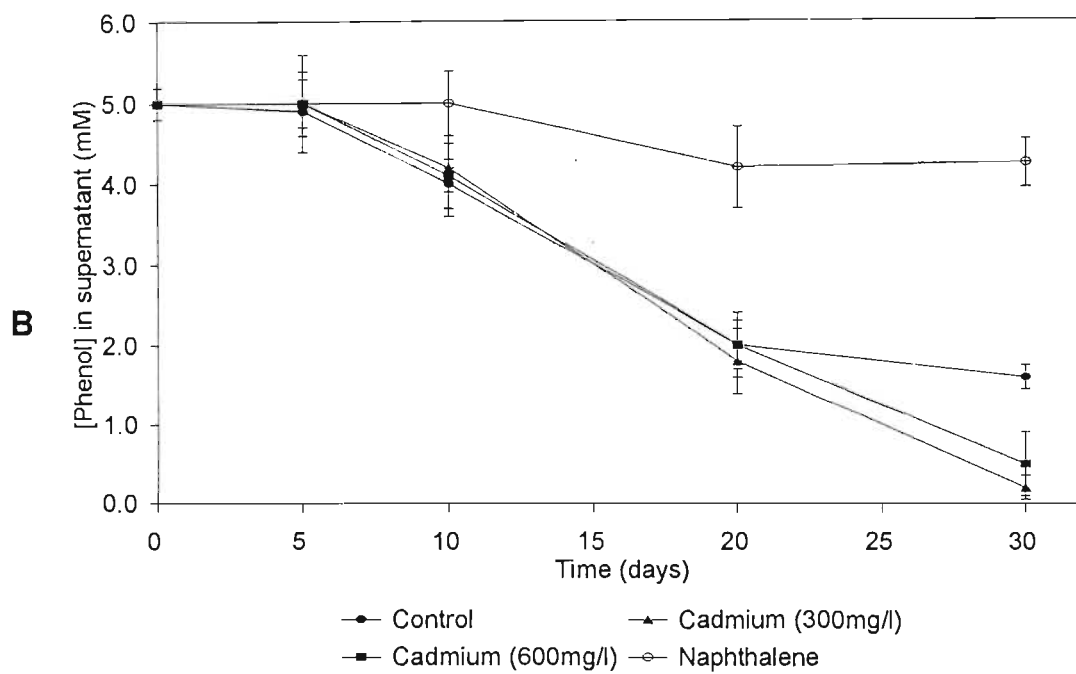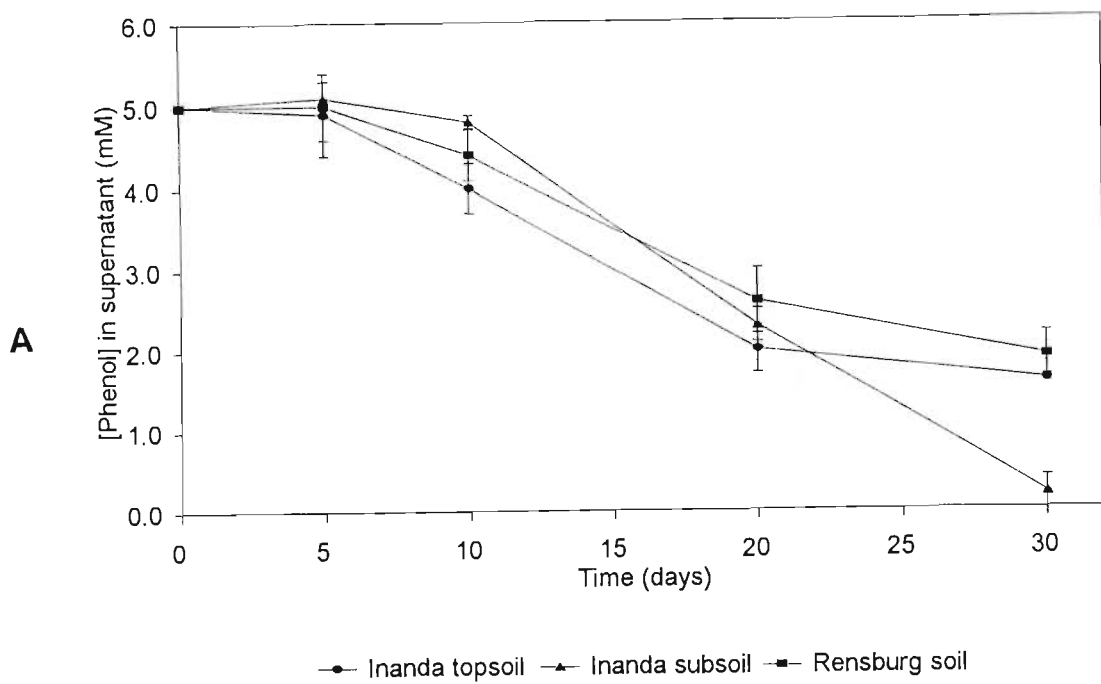
FIGURE 3.10: Phenol catabolism in the presence of the three soils (control, A). Phenol catabolism in the presence of naphthalene and different cadmium concentrations for Inanda topsoil (B).
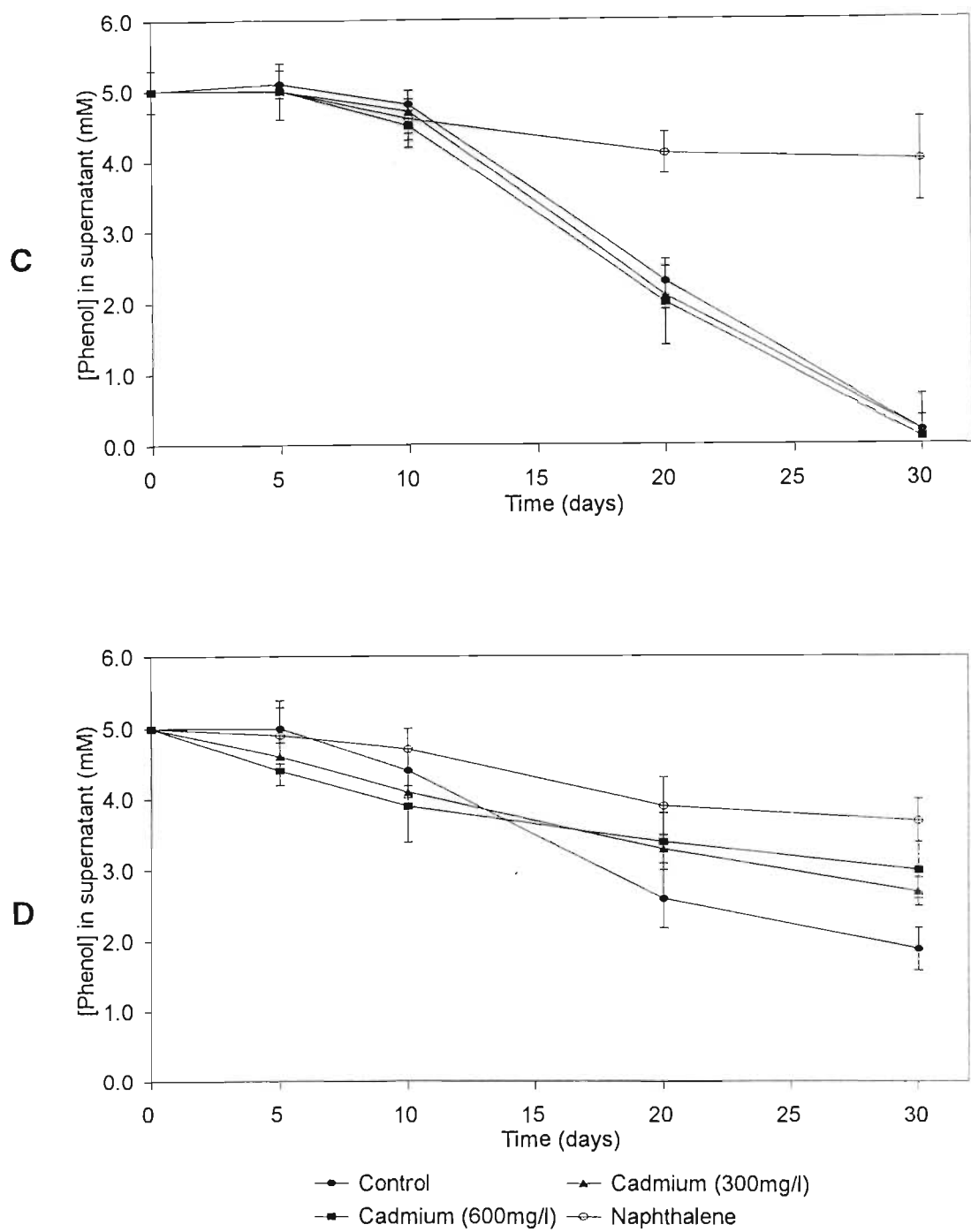
FIGURE 3.10 (continued): Phenol catabolism in the presence of naphthalene and different cadmium concentrations for Inanda subsoil (C) and Rensburg soil (D).

Although naphthalene (30 mg l^{-1}) was found to be only slightly inhibitory, if at all, to phenol catabolism in solution (Figure 3.9A), adsorbed naphthalene did cause considerable inhibition (Figures 3.10B-D). This observation supports the argument that phenol catabolizing organisms were attached or associated with soil surfaces. If these organisms were solely in the aqueous, non-attached phase they would encounter naphthalene at a maximum concentration of 32 mg l^{-1} (Appendix 2, A.2.1). The fact that naphthalene was inhibitory seems to indicate that the high concentration of adsorbed naphthalene (2000 mg kg^{-1}) in close proximity to the attached microorganisms was inhibitory.

Thus, it would appear that this concentration was so high that its effect could not be alleviated by the association of the organisms with the soil surfaces. It is unlikely that naphthalene can be used as an alternative carbon source to phenol. The catabolic pathways of these two compounds converge at a common intermediate, catechol, from which point the catabolic pathway is identical. The pathway for phenol catabolism to catechol is, however, much shorter than for naphthalene as shown in Figure I.1.1 (Franklin *et al.*, 1981).

CHAPTER 4. RESULTS AND DISCUSSION

4.0   SOIL COLUMN ATTENUATION STUDIES

The earlier stages of this study focused on the physico-chemical interactions between the soils, microorganisms and pollutant chemicals. In order to approach more closely the field situation it was decided to set up some soil columns. Under these conditions the physico-chemical interactions, as well as the physical influences of each soil type, are of importance. From an environmental safety point of view, these studies could indicate to what extent the pollutants could be degraded before migrating out of the soil profile and into the groundwater or any other water body.

I.4.1   Naphthalene

This study was designed to determine the effect(s) of a migrating nutrient solution on the catabolism of soil adsorbed naphthalene. Soil column leachate analysis was particularly important to determine whether catabolism of naphthalene occurred at a faster rate than desorption. A second aim was to determine whether mobilization of adsorbed naphthalene would occur if the polarity of the aqueous phase was reduced by a solvent such as acetonitrile. From Figure .4.1 it can be seen that all the columns did not generate equal leachate volumes. This can be attributed to differences in the unsaturated hydraulic conductivities of the soils. The aim was to keep the columns aerobic which necessitated the use of tensiometers to facilitate unsaturated flow. The effect of soil water content on the volume of leachate generated can be seen in Figure 4.1. For Columns A-D,F,G and J the curve gradient of leachate vs time increased with increased water content of the column because of the addition of nutrient solution on day 37 (indicated with an arrow). This gradient, which was more pronounced in some columns, is interesting since it is an indication of the water availability or water activity and could have an influence on microbial activity.
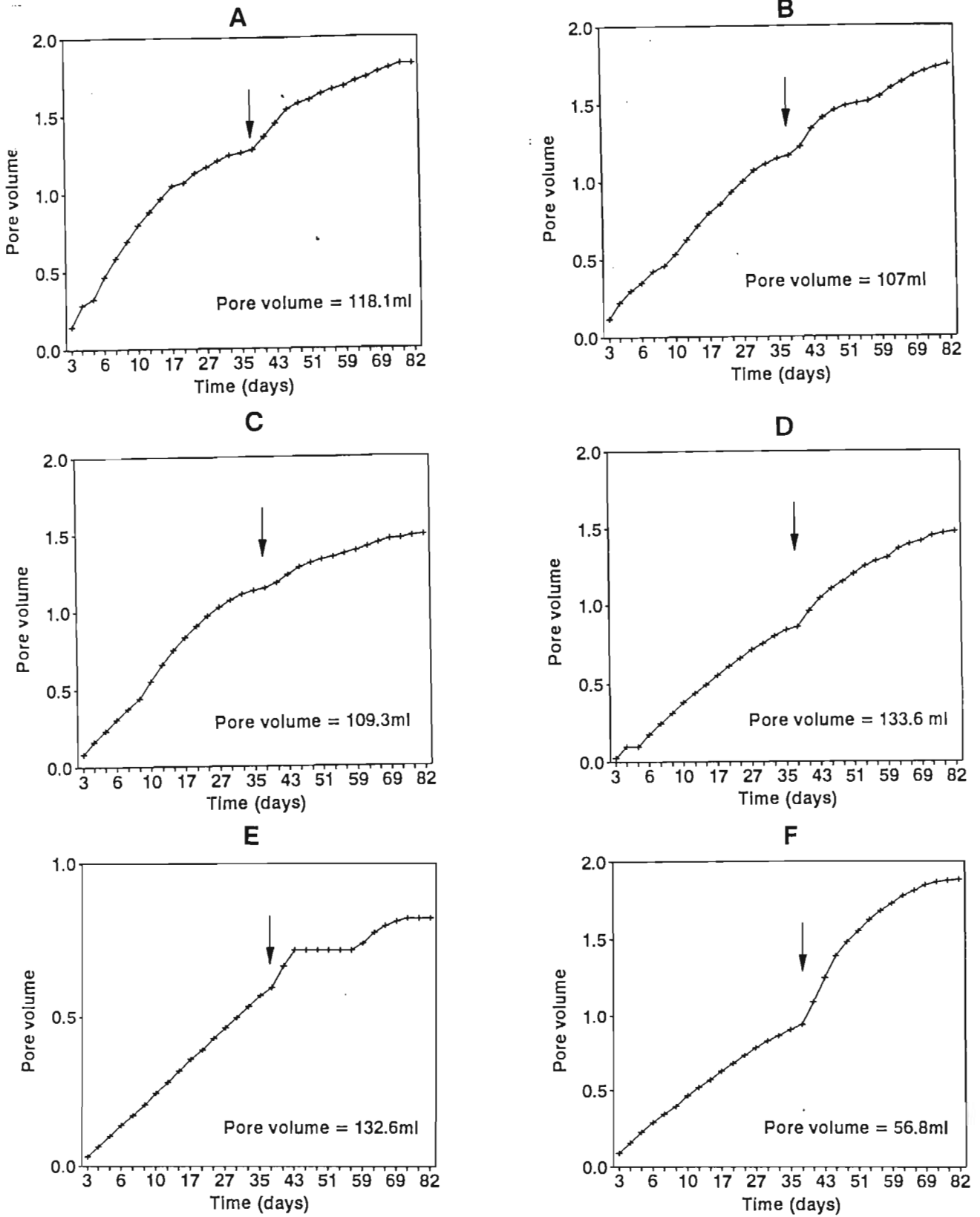
FIGURE 4.1: Cumulative leachate volumes extracted from Columns A - F. Arrows indicate the addition of nutrient/inoculum mixture on day 37. (See Table 2.1, p35 for details of different columns).

74
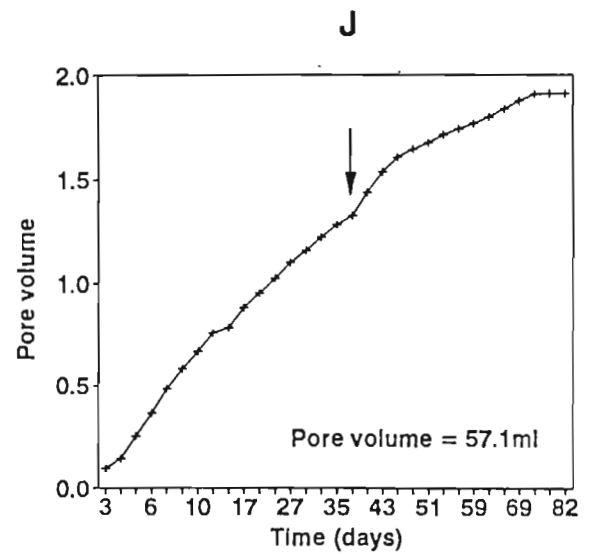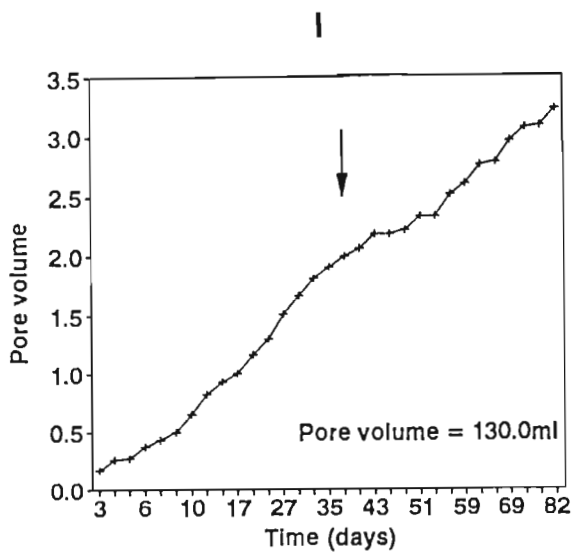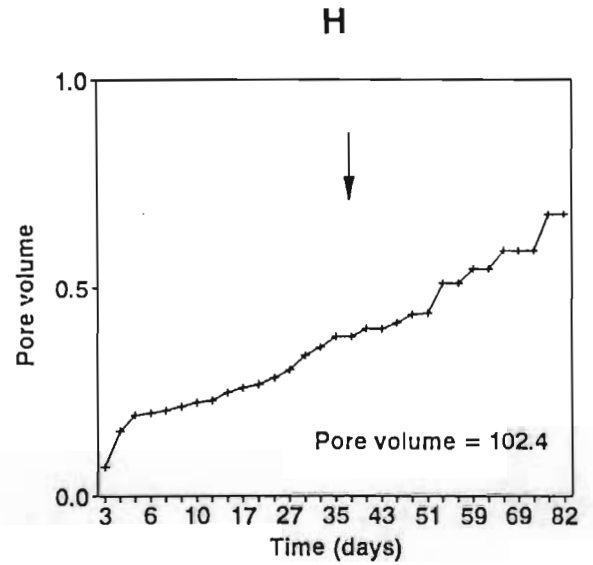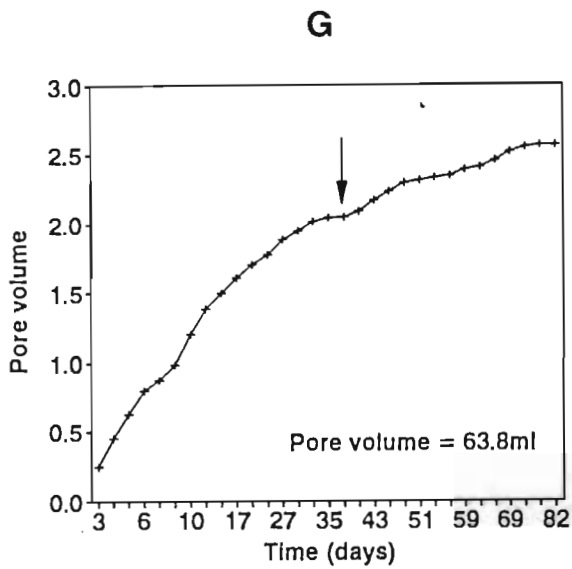
FIGURE 4.1 (continued): Cumulative leachate volumes extracted from Columns G - J. Arrows indicate the addition of nutrient/inoculum mixture on day 37. (See Table 2.1, p35 for details of different columns).

The naphthalene concentrations in the leachates were very low for all columns throughout the experiment (Figure 4.2). The columns supplemented with increasing concentrations of acetonitrile (Columns C, E and G) generated higher concentrations of naphthalene in the leachate, as was anticipated (Knox *et al.*, 1993). The low leachate naphthalene concentrations were probably due to the high adsorption capacity of the soil (Section 3.1.3). Catabolism of naphthalene is most limited by adsorption and low solubility. It seems probable, therefore, that microbial catabolism of the soluble fraction of naphthalene occurred at a faster rate than desorption. The acetonitrile should have accelerated the desorption of naphthalene but again catabolism seemed to have occurred at a fast enough rate to result in low leachate concentrations. The sterile control columns were also generally characterised by low leachate naphthalene concentrations. It, thus, appeared that adsorption was a major factor in these columns.

There is probably a correlation between hydraulic conductivity and microbial catabolism of both adsorbed and soluble compounds (Knox *et al.,* 1993). In fermentation studies the important factor of dilution rate is a measure of the medium flow rate into a vessel divided by the volume of the vessel. This could in some cases be assumed to be applicable to soil systems. The dilution rate gives an indication of the rate at which medium should be applied to a vessel (or the pore space occupied by soil microorganisms) in order to facilitate optimum microbial growth. If this flow is too great it leads to washout of the microorganisms, and if too low it leads to starvation, unless the concentration of the limiting carbon or energy source is sufficient to satisfy the maintenance energy requirement of the microorganisms. In the soil system, however, there are several complicating factors. The first problem, in using dilution rates to understand the dynamics of microbial degradation of pollutants, is that the soil volume occupied by microorganisms is difficult to determine. Under saturated conditions this volume approximates to the total pore space.
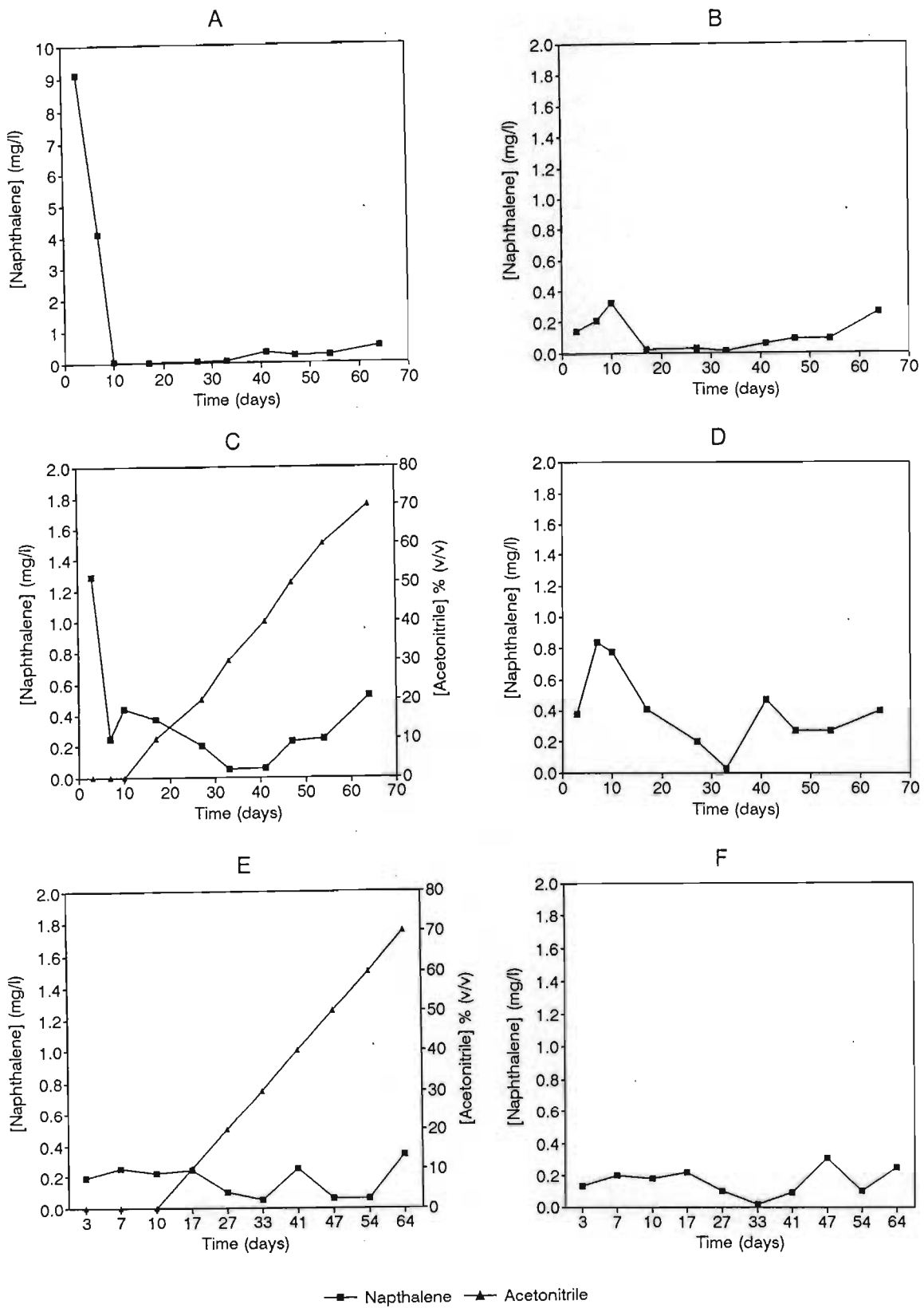
FIGURE 4.2: Changes in leachate naphthalene concentrations of Columns A-F. Acetonitrile supplementation shown for Columns C and E. (See Table 2.1, p35 for details of different columns).
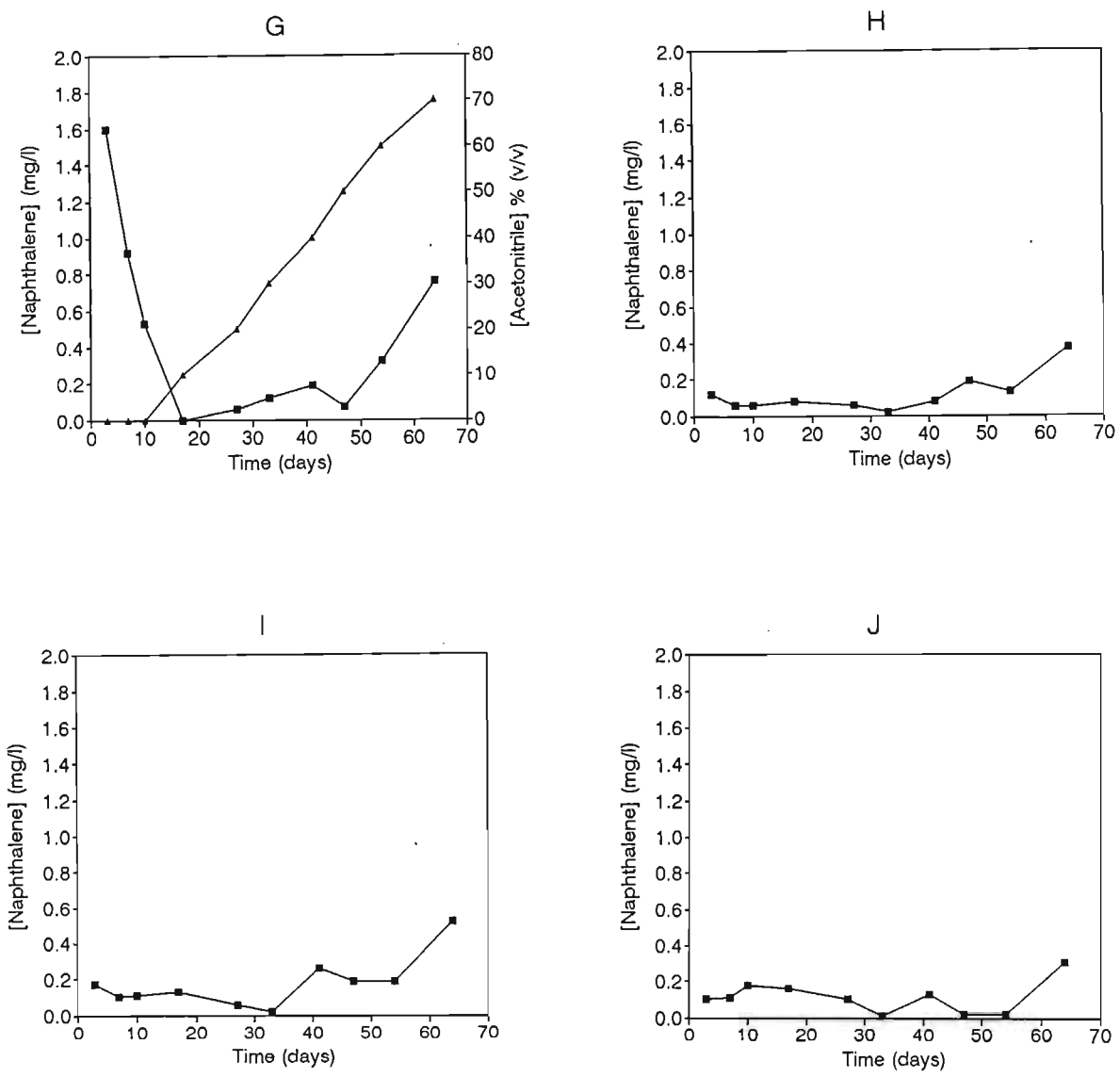
FIGURE 4.2 (continued): Changes in leachate naphthalene concentrations for Columns G -J. Acetonitrile supplementation shown of Column G. (See Table 2.1, p35 for details of different columns).

A further problem in soil is that the surface area for attachment, relative to the pore volume, is much greater than in other reactors. Under unsaturated conditions, as used in this experiment, estimation of the volume occupied by the microorganisms becomes even more difficult since it cannot simply be determined by calculating or measuring the soil water content. The water closely associated with the soil surfaces does not behave as free water and assumes the character of crystalline water (Foth, 1984). This type of water, relative to the free water, increases with a decrease in the water content. Crystalline water also causes a sharp decline in the water activity which, in turn, results in a decline in microbial activity (Stotzky, 1985).

To complicate the problem of understanding the dynamics of the SMP system there is also the factor of adsorption of compounds such as naphthalene. Because of the equilibrium between adsorbed and solution naphthalene, the hydraulic conductivity can greatly influence the microbial catabolism of this and other hydrophobic pollutants. This is related to factors which have already been discussed in Section 1.6. Figure 4.3 is a diagrammatic representation of a soil aggregate which is composed of several soil particles. The spaces between these aggregates are mainly macropores and facilitate mass flow (bulk flow). Microbial catabolism mainly occurs at the surface of the aggregate or in the micropores if the bulk flow (related to hydraulic conductivity) is such that microbial washout occurs. The macropores are usually drained at field capacity so that microbial activity is restricted to the micropores to take place. Replenishment of the micropores with nutrients or pollutants only occurs if there is sufficient time for diffusion between the bulk solution (in the macropores) and the micropores. Because of the slight solubility of most hydrophobic molecules, diffusion also occurs from the micropores, where most of the compounds are adsorbed, to the bulk solution. If the hydraulic conductivity is low (or zero), the bulk solution will soon reach the maximum solubility concentration so that no more diffusion out of the micropores into the bulk solution will occur. If, however, bulk flow does occur to an appreciable extent, the bulk solution will be constantly below the maximum solubility concentration so that the concentration gradient between micro- and macropores will stay intact, thus sustaining diffusion. Because of this effect on the concentration in the bulk solution the microbial activity in the bulk solution is also affected. The extent to which migration of pollutants (particularly hydrophobic pollutants as discussed here) occurs in soil and, therefore, also

affects microbial catabolism, depends greatly on the diffusion distance between the micropores in the aggregate and the macropores in which most of the bulk flow occurs (Knox *et al.*, 1993).
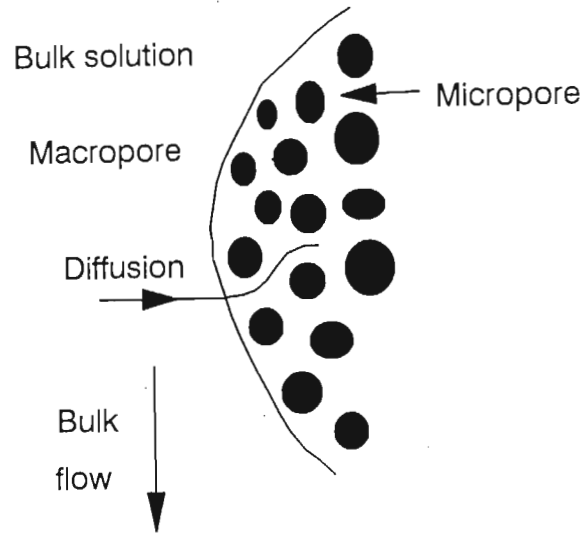
FIGURE 4.3: Diagrammatic representation of the bulk flow in macropores and diffusion into micropores.

(It should be noted that macropores are not only limited to pores between soil structural units, as indicated by the simple diagrammatic representation in Figure 4.3, but also occur in apedal soil). This phenomenon is greatly affected by soil structure, and the presence of both macro-aggregates and micro-aggregates. These micro-aggregates may also be present in relatively homogeneous soil such as that used in this study. All of these intricate variables make the interpretation of soil column studies very complex and justify the approach of using a combination of soil columns and soil batch studies to determine the interactions of the SMP system at soil surfaces.

Figure 4.4 shows the concentrations of naphthalene extracted from the control and experimental columns after 82 days incubation. There was little difference between the solvent and nutrient treated soils and between the different soil materials. The addition of a "foreign" inoculum (oil contaminated soil) to Column A also had little effect. The slight
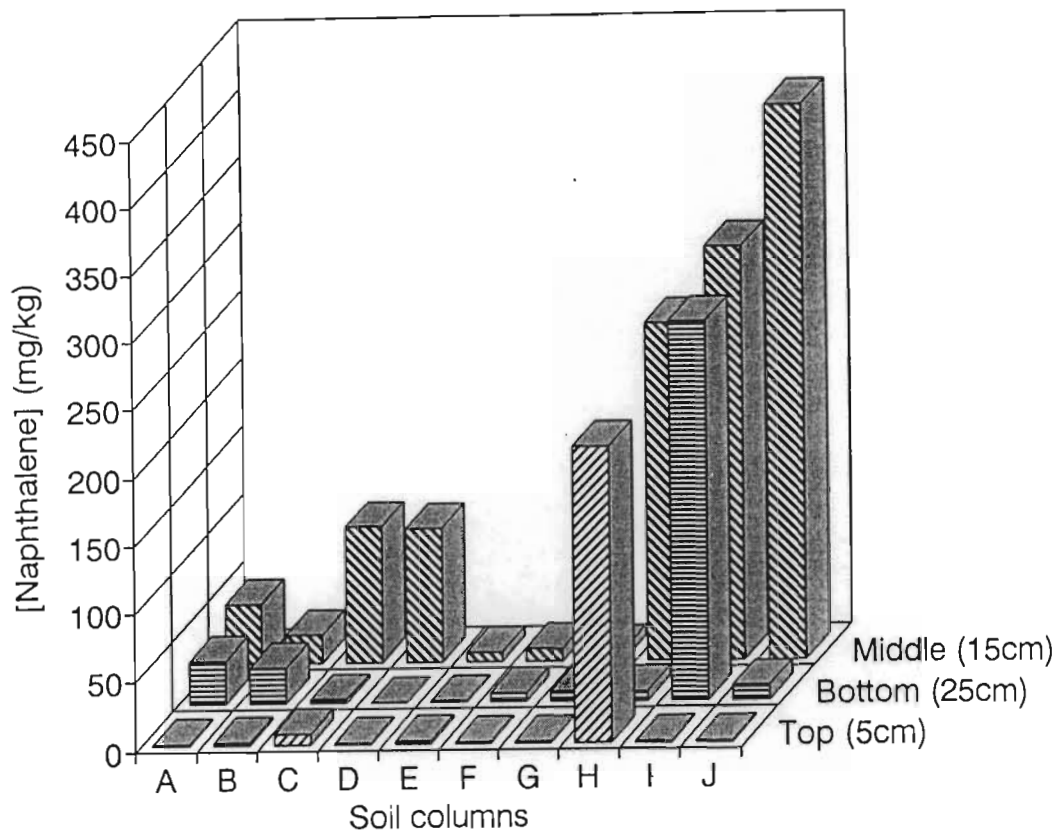
FIGURE 4.4: Naphthalene concentrations recorded in the soil after 82 days incubation and following destructive sampling.

differences which were recorded were not correlated to earlier treatment or soil material. It seemed, therefore, that any differential effect due to the physico-chemical variables at the soil surfaces, as determined in the batch studies, was masked by the purely physical soil properties (as discussed above). In comparing the sterile controls with the treated columns it seems that the middle sampling position (15 cm) holds the key. The naphthalene concentrations in the top (5 cm) and bottom (25 cm) of most columns and treatments were comparable and were, therefore, possibly accounted for by mechanisms other than microbial catabolism. The mid-column-located soil, thus, appeared to approximate more closely to the majority of a soil body. Comparisons between the sterile controls (columns H,I & J) and experimental columns (A-G) showed that microbial catabolism was responsible for naphthalene attenuation. The results highlight the importance of an active microbial

population in catabolism of soil pollutants. The results also show that leachate quality alone, particularly in the presence of soil adsorbed pollutants, does not give an effective indication of the level of soil contamination. This can be seen from the leachate concentrations of the control columns which were very similar to the treatment columns (Figure 4.2), although the total adsorbed naphthalene concentrations were much higher in the former (Figure 4.4).

These results also show that soil has a considerable buffering capacity for hydrophobic contaminants. Soil would, by adsorption, thus be able to reduce the environmental risk of migrating pollutants until biodegradation could occur. This would be particularly important in landfills where such pollutants may be potentially toxic at high aqueous concentrations. The soil could act as a buffer to lower the aqueous concentration to below the toxicity threshold. This would also allow more time for microbial adaptation to occur. This capacity is of great importance where landfills are viewed not only as dumps but, increasingly, as bioreactors which facilitate breakdown of compounds to exploitable products. It should, however, also be recognised that every soil type has a finite buffering (adsorptive) capacity which could be exceeded. At that point it would no longer adsorb any of the challenging molecule(s) i.e. an increase in equilibrium aqueous concentration would not result in increased adsorption (Knox *et al.*, 1993). This point of saturation can, however, be estimated by adsorption isotherms (Figure 4.5A). In the case of naphthalene the solubility of the compound is the most limiting factor and not the soil adsorption capacity. Increased adsorption occurs not only because of the sorption capacity of the soil but also because of the hydrophobic nature of the compound. Naphthalene is repelled from the aqueous phase and, thus, adsorbs to soil. In this case the adsorption isotherm would show that an increase in adsorbed naphthalene does not increase the equilibrium aqueous phase concentration (Figure 4.5B). This is not only true for naphthalene but also for other hydrophobic compounds. As the compounds are catabolized, usually from the aqueous phase, the soil adsorbed naphthalene replenishes the soluble concentration so that it is kept at its solubility maximum. This continues until the adsorbed amount is insufficient to sustain the solubility maximum. Water (leachate) analysis, therefore, gives only limited information about the state of contamination and should be carefully interpreted.

A

Soil adsorbed concentration

Equilibrium aqueous phase concentration

B

Soil adsorbed concentration

Maximum solubility

Equilibrium aqueous phase concentration
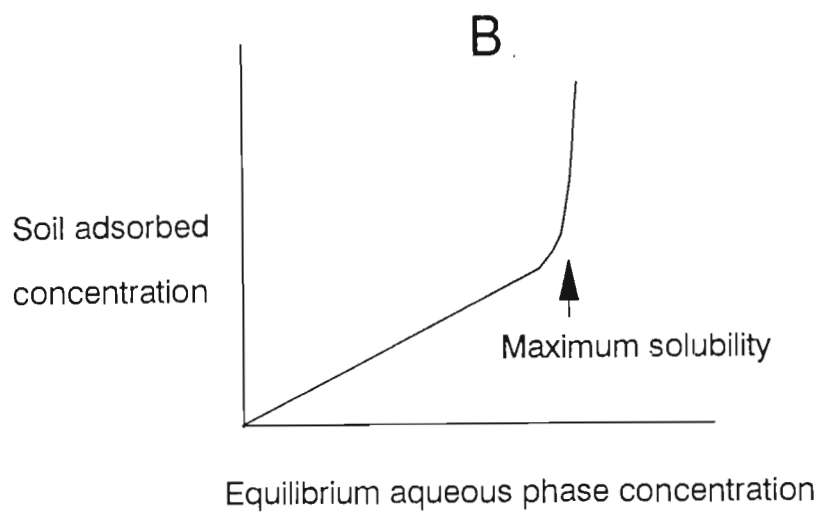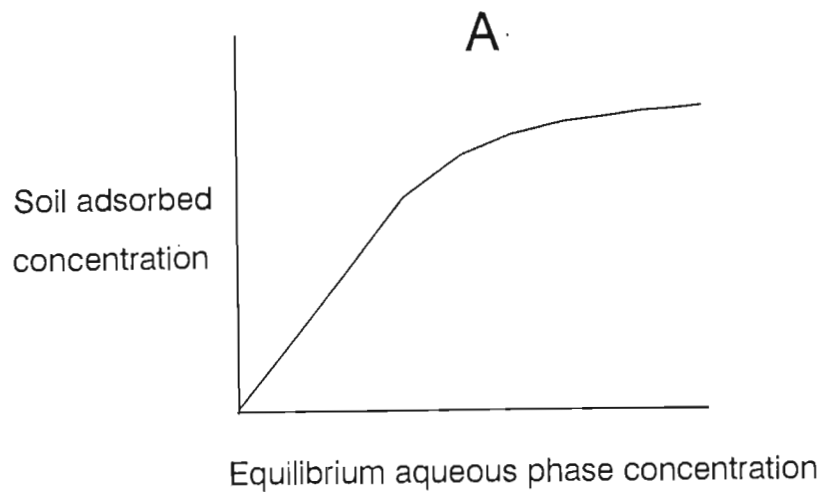
FIGURE 4.5: Diagrammatic representation of adsorption isotherms where: the adsorption capacity of the soil is exceeded (A); the solubility of the pollutant compound in water is exceeded (B).

4.2 Phenol

This study was made to determine the effects of soil materials on the attenuation of migrating phenol. Other variables investigated were the time taken for different indigenous soil microorganisms to acclimatise to the phenol and the effects of flow rate (residence time) on

catabolism of this compound in the soils. The experiment was designed to highlight the combined effects of the physico-chemical interactions and physical interactions (i.e. bulk density, aeration and water content) on the degradation of phenol. Figures 4.6 and 4.7 show that for each of the three soil materials and for both the 5 ml and 2 ml treatments the phenol concentrations at the first sampling time were higher for the non-inoculated columns. (The initial sample for the 2ml treatment took longer to retrieve due to the lower rate of liquid application). This indicates that a phenol catabolizing population takes time to develop in the soil. A pulse of phenol solution migrating through a soil would, thus, be less effectively attenuated if the soil had a high hydraulic conductivity. As discussed earlier (Section 4.1), the balance between adsorption of the compound(s) of interest and the microbial population's ability to catabolize the compound(s) in relation to the hydraulic conductivity determines the leachate concentration(s). This issue is further investigated in Part II.

For the 5 ml supplemented columns (Figure 4.6), the Inanda topsoil inoculated and non-inoculated columns (Figure 4.6A) produced very similar leachate phenol concentrations for most of the experiment. Non-acclimatization of the microbial population of the columns did not, therefore, seem to have a prolonged effect. The reason for the relatively low phenol concentrations in the leachates during the period 11 to 18 days could not be satisfactorily explained. For the Inanda subsoil (Figure 4.6B), however, the difference between the inoculated and non-inoculated columns was apparent throughout the experiment. This could possibly be ascribed to either a small indigenous microbial population which did not enrich under the selection pressures of the experiment or to inadequate protection against potentially toxic phenol concentrations provided to the microbial population. It is possible that the phenol concentration used (10 mM) was slightly inhibitory to the microbial population and that the soil did not lower the effective concentration by adsorption to a concentration below the inhibitory threshold. Alternatively, the Inanda subsoil did not provide adequate protection to the microorganisms against inhibitory concentrations of phenol. This could possibly have been due to the relatively low CEC and / or the relatively low organic matter content since the two characteristics are thought to be important in microbial adsorption / protection (Section 3.2.5).
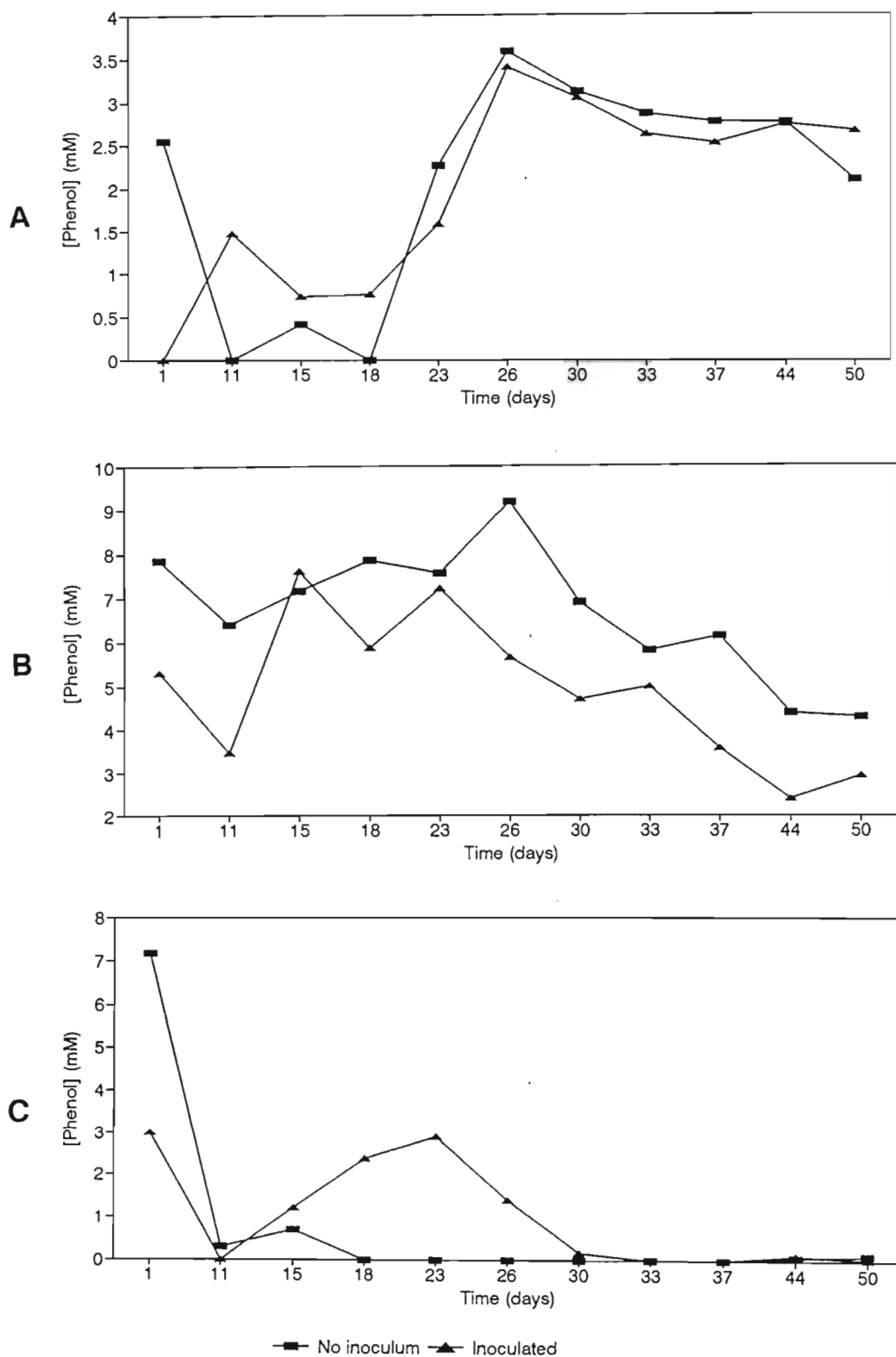
FIGURE 4.6: Phenol attenuation in Inanda topsoil (A), Inanda subsoil (B) and Rensburg (C) soil columns supplemented with 5 ml aliquots of phenol (10 mM) coincident with each sampling time.

The differences for the Rensburg soil (Figure 4.6C), observed between days 15 and 26 seemed anomalous and could not be satisfactorily explained. (Recorded differences in adsorption between the soil materials are given in Section 3.2.1).

Because of the relative similarity of the results for the inoculated and non-inoculated treatments during most of the experimental period, the averages of the two treatments for every datum point were combined and plotted (Figure 4.8A). This gave an indication of the relative attenuations of phenol by the three soils. It is clear that the Inanda subsoil effected the least phenol attenuation during the experimental period. The possible reasons for this have already been discussed. An interesting observation from Figure 4.8A is that the attenuation by the Inanda subsoil improved from day 26 possibly due to an enriched microbial population or phenol acclimatization. No clear conclusion could be made for the attenuation differences recorded for the Inanda topsoil and the Rensburg soil during the first 23 days. Subsequently, however, the Rensburg soil seemed to be more effective than the Inanda topsoil. It is important to note here that the recorded differences cannot be ascribed to hydraulic conductivity since the column flows were regulated by the addition of specific volumes of phenol solution. The total leachate volumes from the columns are given in Table 4.1. For the 2 ml treatments (Figure 4.7), after 23 days the degree of attenuation was very similar for the Inanda topsoil (Figure 4.7A) and the Rensburg soil (Figure I.4.7C) and little phenol was detected in the leachates. The results for the Inanda subsoil (Figure 4.7B) showed slight differences relative to the initial phenol concentration, after day 23.

The phenol concentrations for the inoculated and non-inoculated columns were combined as before and plotted (Figure 4.8B). The plot was similar to that of the 5 ml treatments (Figure 4.8A). The Inanda subsoil again exhibited the least attenuation capacity. For the other two soil materials, however, there seemed to be little difference after day 23. This was probably because of the slow flow rate which facilitated adsorption and microbial catabolism.

Figures 4.9A-C are comparisons of the 5 ml and 2 ml treatments which represent different hydraulic flow rates and, therefore, retention times for the phenol solution in the columns. There was considerable difference in the leachate concentrations of the two treatments for the Inanda topsoil (after 23 days) (Figure 4.9A) and the Inanda subsoil (Figure 4.9B) but

86
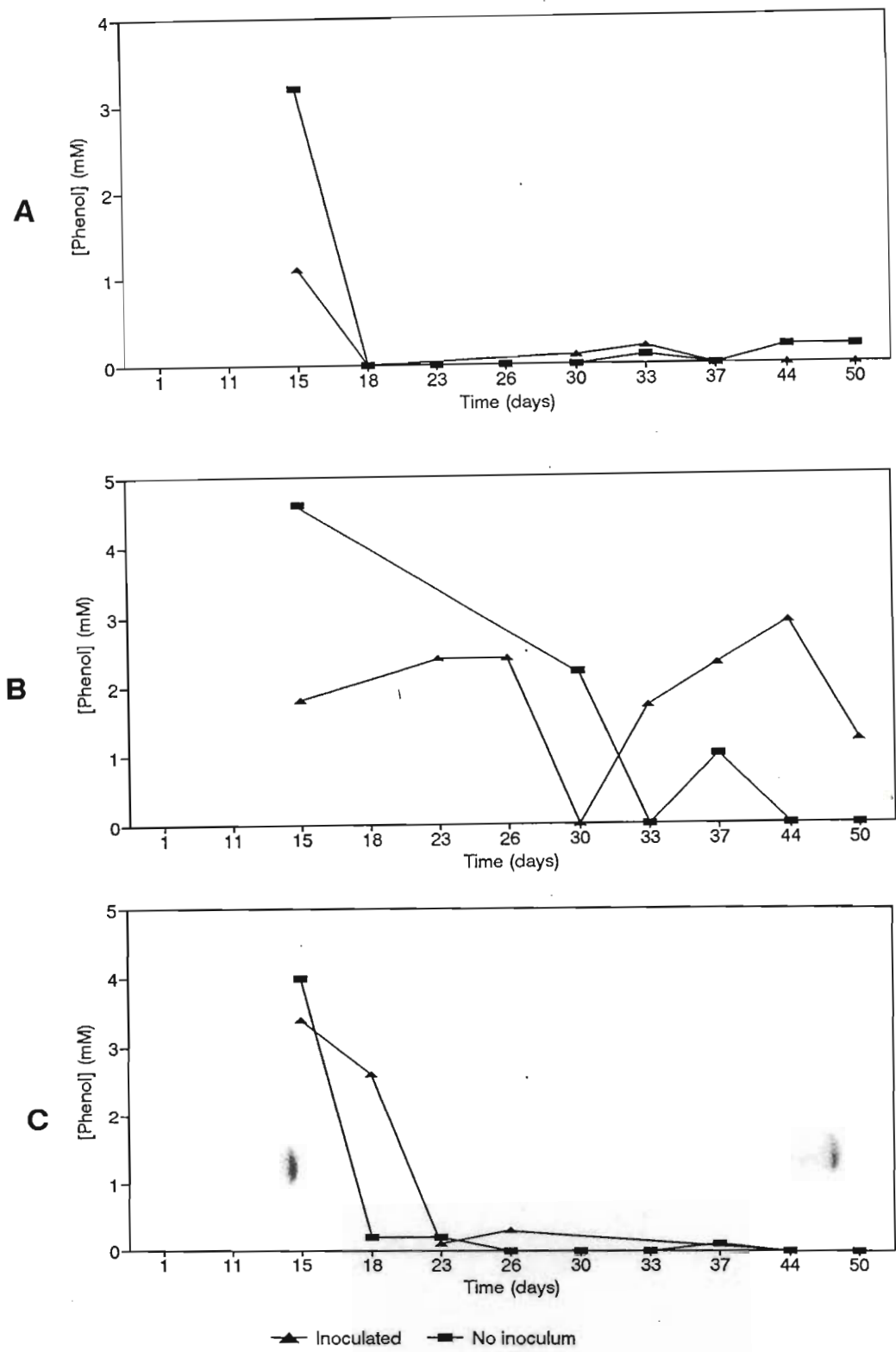
FIGURE 4.7: Phenol attenuation in Inanda topsoil (A), Inanda subsoil (B) and Rensburg (C) soil columns supplemented with 2 ml aliquots of phenol (10 mM) coincident with each sampling time. (Leachate only emerged after 15 days of treatment).
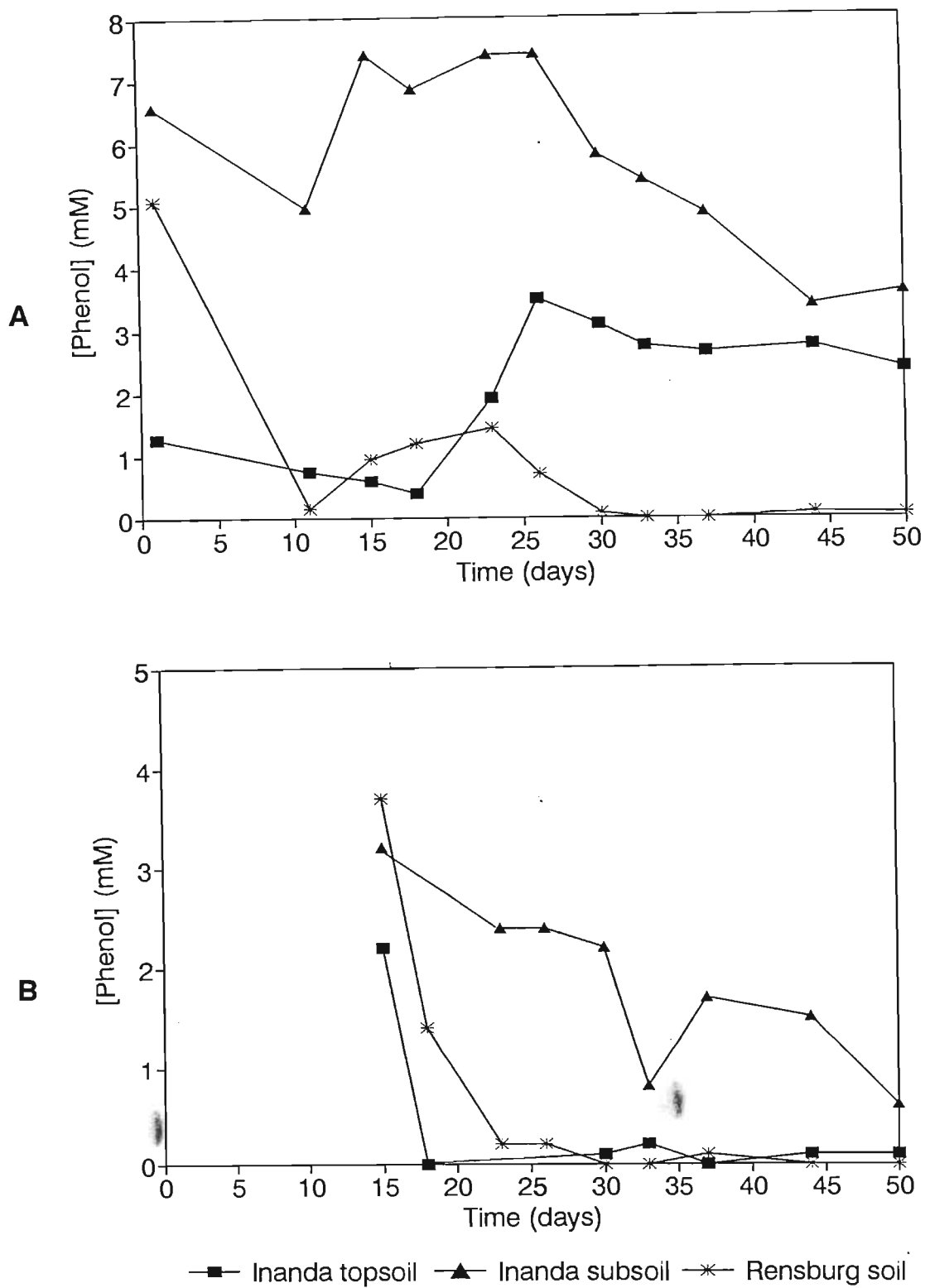
FIGURE 4.8: Phenol (10 mM) attenuation by the different soils with the 5 ml (A) and 2 ml (B) treatments. (The combined results for inoculated and non-inoculated columns were used.). (Leachate only emerged after 15 days of the 2ml treatment).

TABLE 4.1: Total leachate volumes collected from the soil columns

Soil Type	Treatment		Volume (ml) (Leachate)	Volume (ml) (Added)
Inanda topsoil	Inoculum	5 ml	37.6	55
		2 ml	1.3	22
	No inoculum	5 ml	35.0	55
		2 ml	1.3	22
Inanda subsoil	Inoculum	5 ml	38.5	55
		2 ml	1.3	22
	No inoculum	5 ml	31.9	55
		2 ml	1.8	22
Rensburg soil	Inoculum	5 ml	37.2	55
		2 ml	0.9	22
	No inoculum	5 ml	37.6	55
		2 ml	4.2	22

not for the Rensburg soil (Figure 4.9C). Although microbial activity and microbial acclimatization seem to be important, as discussed above, the observed results were probably due to a combination of adsorption capacities and microbial activities. Figure 4.10 shows the average percentage attenuations for the different treatments and soils during the experimental period. The figure does not, however, correlate with the results of phenol adsorption in a batch situation (Section 3.2.3) in terms of the expected relative adsorption capacities of the different soils. The differences here seem to be much greater than those determined in the adsorption study. This is particularly true for the Inanda topsoil and the Rensburg soil, the more so since the Rensburg soil was diluted with acid washed sand in this experiment. It thus appeared that adsorption was not the only factor controlling attenuation. Before ascribing the differences to microbial activity, it is important to consider the maximum potential adsorption which could result if there was full contact between the soil (in the columns) and the phenol solution. Calculated from the batch adsorption experiment (Section 3.2.1), the maximum potential millimoles of phenol which could be adsorbed to the soil (200 g in the columns) was: 6.1, 3.5 and 6.1 for the Inanda topsoil, Inanda subsoil and Rensburg soil, respectively. The columns, however, received only 0.55 and 0.22 millimoles phenol, for the 5 ml and 2 ml treatments, respectively during the experimental period. Since this was much less than the potential adsorbable millimoles it appears that there was not full
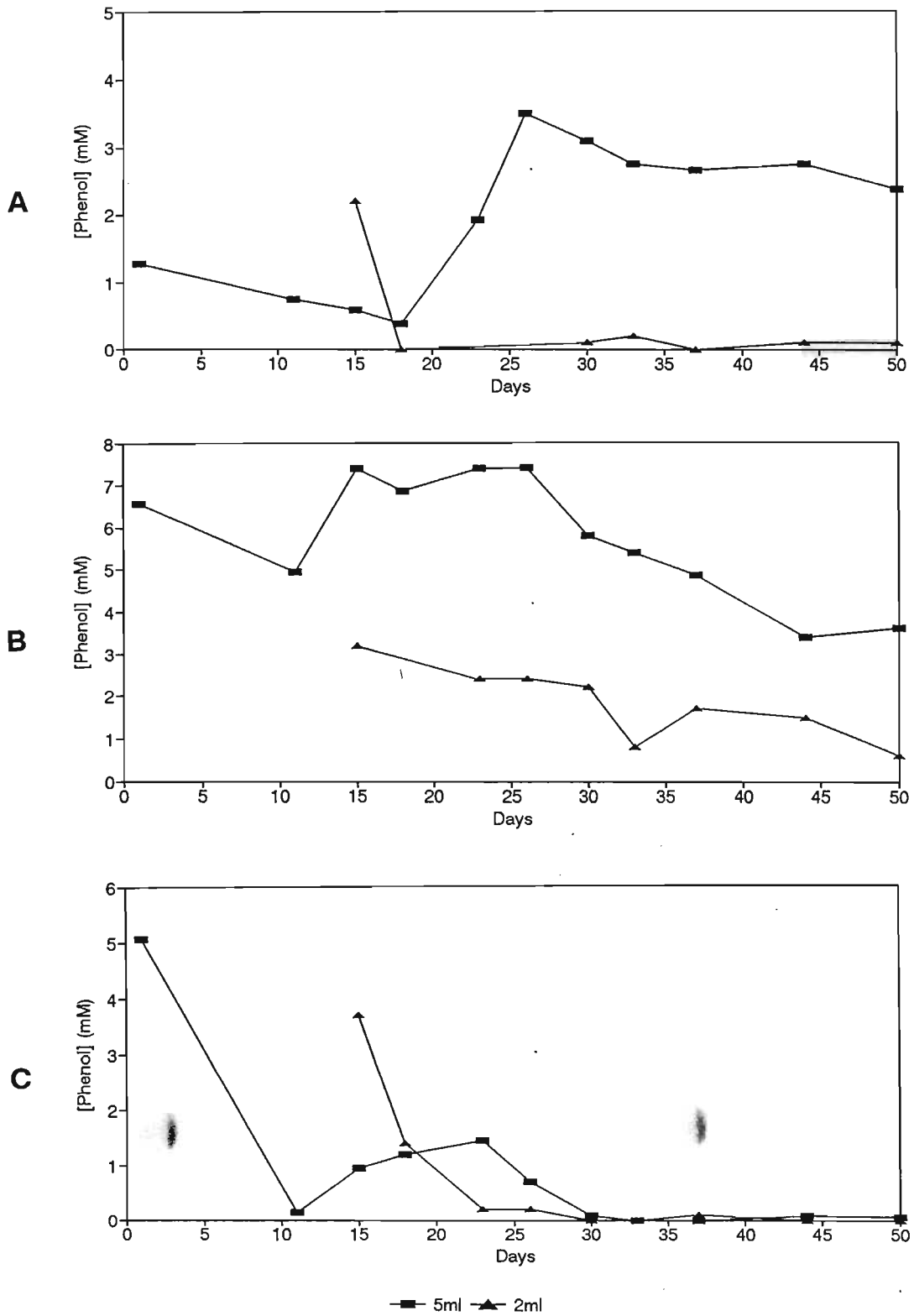
FIGURE 4.9: Changes in leachate phenol concentrations of Inanda topsoil (A), Inanda subsoil (B) and Rensburg (C) soil columns subjected to two hydraulic loading rates.
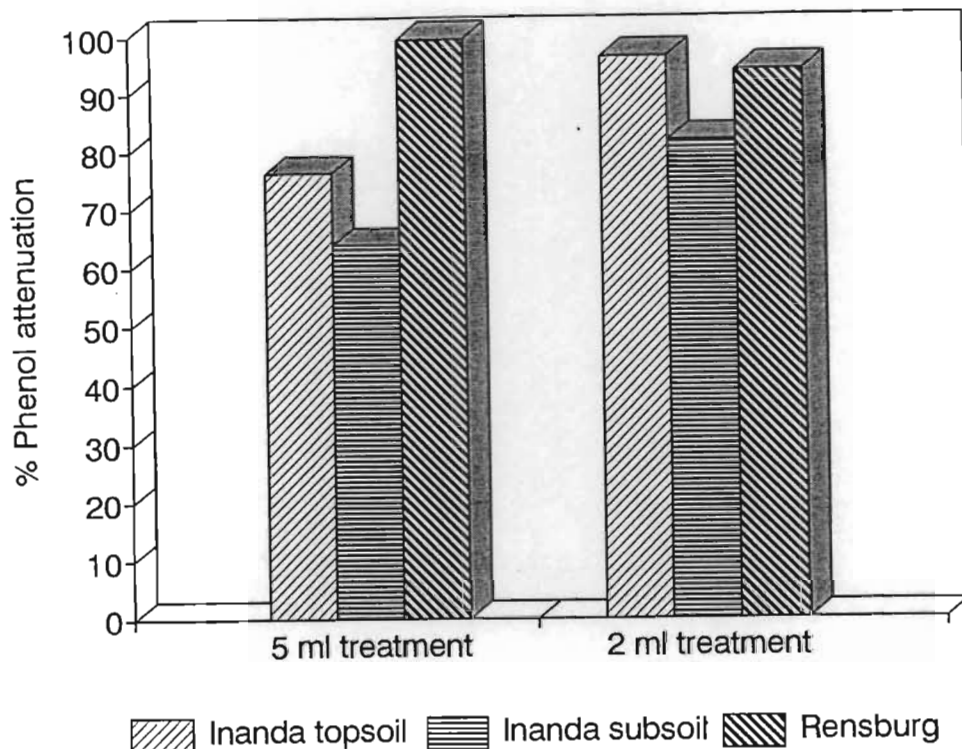
FIGURE 4.10: Average attenuations recorded for the different treatments and soils during the experimental period.

contact between the added phenol and all of the available soil surfaces. Saturation adsorption had, thus, not been reached in any of these columns. It is, therefore, difficult to determine whether the lower phenol concentrations in the leachates for the 2 ml-treated columns (Inanda topsoil and subsoil) were due to improved catabolism or greater adsorption. Increased microbial catabolism was possible due to the longer retention time in the presence of a catabolizing population, although the longer retention time could also have resulted in improved migration and adsorption of the phenol (or any other molecule) into the micropores (see discussion Section 4.1). The differences in the phenol attenuations in the 5 ml treatments did not reflect either the similarities in adsorption (Section 3.2.1) or the similarities in attenuation recorded in the equivalent batch studies. This seems to emphasise the importance of the physical properties of soil and the effect they have on *in situ* attenuation. The Rensburg soil, with its higher smectite content, probably had more

micropores (as reflected by its greater field capacity, Appendix A.1.6) than the Inanda soil materials so that the total surface available for adsorption in this soil *in situ* should be much higher.

The findings from this experiment correspond with those of Dobbins *et al.* (1987) and also confirmed the difficulties in ascribing attenuation to a particular soil characteristic. From these experiments it seemed that Rensburg soil would be the most efficient in attenuating the phenol (and possibly other phenolics) content of landfill leachate migrating through a capping. This would be particularly true for the initial phase of leachate migration when the soil adsorption sites were not yet saturated with phenol. It is, however, difficult to determine whether this superior attenuation characteristic of the Rensburg soil would continue beyond the point of saturation of the soil adsorption sites. If the soil served not only as adsorption sites for the phenol but also for the active microbial population, the superior attenuation affect would be prolonged indefinitely beyond the point of phenol saturation. Based on the results of the phenol batch studies (Section 3.3.3) this seemed to be the case and it, therefore, seems likely that a soil such as the Rensburg soil would continue to have a better attenuation capacity for the phenol in landfill leachate than the other two soil materials used in this study. A high smectite clay content soil would, thus, seem to be a better choice for landfill covering soil (intermediate and final cappings) provided that leachate attenuation was desired and the lower hydraulic conductivity, usually associated with this soil (or similar highly smectitic soils), was not problematic for reasons other than attenuation. It should also be kept in mind that the attenuation potential for a single compound such as phenol might be significantly altered by other leachate components.

CHAPTER 5. GENERAL CONCLUSIONS OF PART I

One of the difficulties encountered during this research programme was the lack of published research results from comparable studies. Many of the basic principles of this study (e.g. adsorption/desorption controlled by polarity of the mobile phase) originate from liquid chromatography studies and are relevant to the chemical interactions which occur in soils. However, the interactions of the soil physico-chemical and pollutant factors, including the microbial component, are not accounted for in the literature. Despite this, some definite conclusions can be made. The type of soil material does have an effect on the attenuation of specific pollutants which may migrate through, or be present in, the soil. So far in this research, only the physico-chemical and microbiological interactions have been evaluated under aerobic conditions with two molecules. These interpretations are, thus, expressed mindful of the risk of extrapolating limited data.

Attenuation factors relate to the mobility and adhesion of pollutants and microorganisms and are controlled, *in situ*, *inter alia* by bulk density, soil redox potential and soil structure. However, it was initially important to determine the physico-chemical interactions without the complicating variables of the physical soil factors. Once interaction on the physico-chemical level had been established it became important to incorporate column studies in which the combined effects of the physico-chemical and indirect soil physical factors were present. Figure 5.1 summarizes the findings of, primarily, the naphthalene batch culture studies with regard to toxicity of solvents (and, probably, heavy metals such as Cd (Walker *et al.*, 1989; Hiroki, 1992)). The lowest concentration is the threshold bactericidal / bacteriostatic concentration of the solvents to the naphthalene catabolizing population in the absence of soil (Section 3.2.4). It was found that the soil adsorbed the solvents and metals to a certain extent (Section 3.2.2) but this adsorption did not reduce the concentration to below the toxicity thresholds found in the soil-free systems. The apparent difference (X-factor) could not be due to volatilization since the culture vessels were sealed. A possible explanation is that there was some interaction(s) at the soil surfaces which either protected the microbial population against toxic concentrations (van Loosdrecht *et al.*, 1990) or the effective concentration was reduced by surface specific mechanisms.
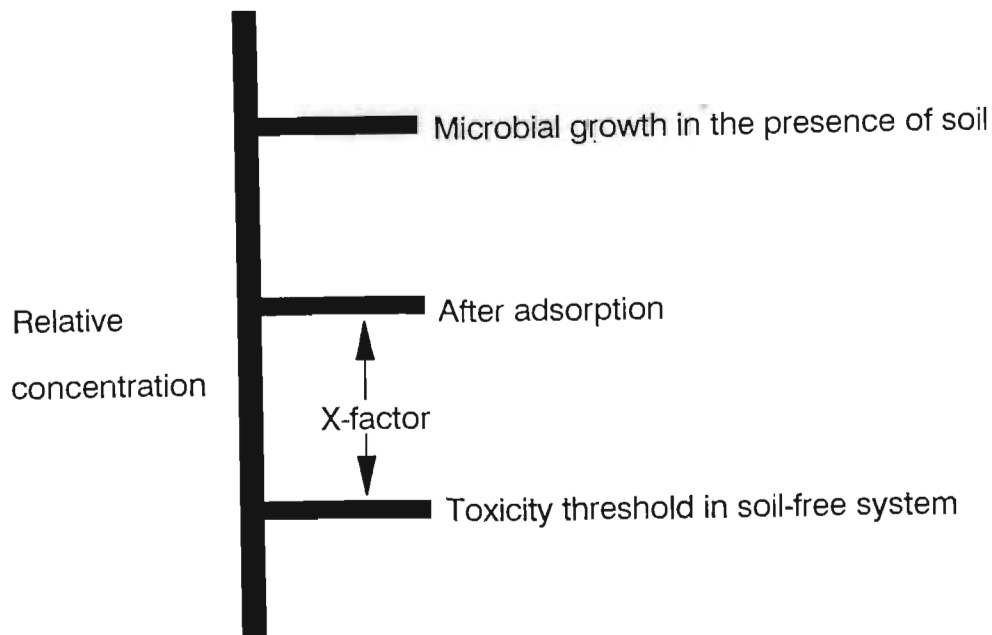
FIGURE 5.1: Diagrammatic representation of the discrepancies found in the determination of rate-limiting concentrations of, potentially, toxic compounds in the SMP system.

The concept of microbial attached catabolism and protection from toxicity was also confirmed by phenol batch attenuation studies (Section 3.2.3). In these experiments, cadmium was found to have much less effect on phenol catabolism in the presence of soil than in the absence of soil (Section 3.2.2). High concentrations of adsorbed naphthalene were found to be inhibitory to phenol catabolism which could only be possible if the microorganisms were associated with the soil surfaces. It seems likely that the soil surface / soil particle associations that influence toxicity are the same as those which determine biodegradation.

The soil column studies showed the differences in leachate concentrations of naphthalene and phenol. Hydrophobic compounds seem to be less problematic with regard to leaching from polluted soil than more soluble compounds, such as phenol. The adsorption effect, while reducing leachability of naphthalene, also limits the microbial catabolism thereof. Not only pollutant type but also soil material and hydraulic loading seemed to be important with regard to attenuation of the phenol leachate.

The results obtained from both batch and column experimentation could explain the events occurring in the intermediate and final cappings of sanitary landfills. On the whole it seems as if soil has a great "buffering" capacity in that it adsorbs potentially toxic chemicals, thus reducing the effective aqueous phase concentration of each compound. Soil also seems to have a significant effect on the microbial populations by affording protection against chemical challenges by, probably, attachment to surfaces. In this study, although soil material seemed to be an important factor in adsorption of organic (particularly hydrophobic) chemicals and cadmium, it did not have a direct (differential) effect on the attached microorganisms. The indirect effect of soil material on microbial growth due to adsorption of chemicals was, however, found to be very important. From a physico-chemical point of view soil *per se* seemed to be beneficial for the landfill as a whole. Thus, the soil layers (cappings) should protect both microbial populations and, ultimately, covering vegetation against chemical challenges. With respect to this, the clay content, clay type and organic matter content of the soil are important variables. Although it may not always be possible to use a specific soil type for landfill purposes it is nevertheless important to understand the effects of soil characteristics on the SMP system. This is probably even more important in environmental clean-up and risk assessment and should under no circumstances be ignored. The importance of the *in situ* soil physical conditions should never be assumed to override the physico-chemical SMP effects at the soil surfaces although this seemed to occur in this part of the study. The physico-chemical interactions would probably become more important as the soil and aqueous phase become more saturated with pollutants. Under such saturation conditions the physical soil factors would probably have a lesser overriding effect on the physico-chemical mechanisms.

From this study it would seem as if a soil with a high smectite clay content and, preferably, a high organic matter content would be an ideal landfill covering soil. This is, however, only true if leachate attenuation is of prime importance. It must be kept in mind that such a soil will have a relatively low hydraulic conductivity which might not always have the most beneficial effect on the landfill processes, depending on what is important to the landfill operators. This soil would, for instance, be less conductive to gas transfer than more sandy or kaolinitic soils.

The results and conclusions of Part I of this study lead to the notion that although the soil surface effects were important in affecting attenuation processes of organic chemicals, the physical effects, such as porosity, needed to be investigated in more detail. From a practical point of view soils should not only be considered for their surface effects since these caracteristics could potentially be influenced also by their physical characteristics which in turn could influence hydraulic conductivity and effective soil dilution rates. It is from this basis that Part II of the study was initiated to gain a better understanding of the soil physical effects, particularly porosity, on the behaviour of soil as a bioreactor.

One of the major shortcomings of this particular study was that microbial enumeration was unsuccessful. However, the work of Picard *et al.* (1992), where enumeration of bacteria in soil was made by direct DNA extraction and polymerase chain reaction, seems very encouraging. The use of immuno- and fluorescent redox probes (Rodriguez *et al.*, 1992; Roslev & King, 1993) could also be of great benefit in future studies. Another interesting, and probably most practical, development is the detection of luminescence-marked bacterial cells in soil by image-enhanced microscopy (Prosser *et al.*, 1994). Since microbial attachment seems to be such an important aspect in attenuation it would also be of value to elucidate the effect of the diffuse double layer of soil components on the SMP system. The thickness of the diffuse double layer, as affected by the ionic strength of the soil solution (leachate), should have a significant effect on, not only microbial attachment (Section 1.5.1) and pollutant adsorption, but also microbial catabolism of the pollutants (leachate). The implementation of the recent techniques for microbial detection at soil surfaces, combined with existing knowledge regarding the diffuse double layer, should shed new light on many of the issues considered in this work.

PART II

CHAPTER 6.   POROUS MEDIA AS A BIOREACTOR

6.0   LITERATURE REVIEW

6.1   Effect of porosity on porous media as a bioreactor: Hypothesis

From the previous experimentation (Part I) it became clear that different soil materials had significantly different effects on the biological degradation of naphthalene and phenol. These effects were not simply due to chemical adsorption but also included microbial adsorption (X Factor). The differences in attenuation between soil materials became more apparent with soil column studies (Chapter 4) compared to batch slurry systems (Chapter 3). This was believed to be due to soil physical effects such as pore size and, as a consequence, microbial and chemical migration through the soil. A proposed hypothesis to explain the physical effect is that different soils have different particle size distributions which give rise to different pore size ranges and geometries. These pore size ranges and geometries are important in establishing different microbial niches and in affecting pollutant flow patterns which, in turn, influence microbial catabolism of migrating compounds and microbial resistance to potentially toxic chemicals. Figure 6.1 shows a diagrammatic representation of pore flow.

Bulk flow mainly occurs through B while chemical and microbial migration into the pore areas marked A (secondary or micropores) occurs at a slower rate by diffusion rather than mass flow. From Figure 6.1 it is clear that microbial growth at B could be vastly different than that at A, due to lower nutrient supply (Sharma & McInerney, 1994). Retention times of compounds transported in the liquid phase are also greater at A than at B, which could result in greater microbial degradation (Abu-Ashour *et al.*, 1994). The difference between macro- and micropores is not always distinct and the transition is mostly gradual. These two terms are, however, used here to simplify the discussion.
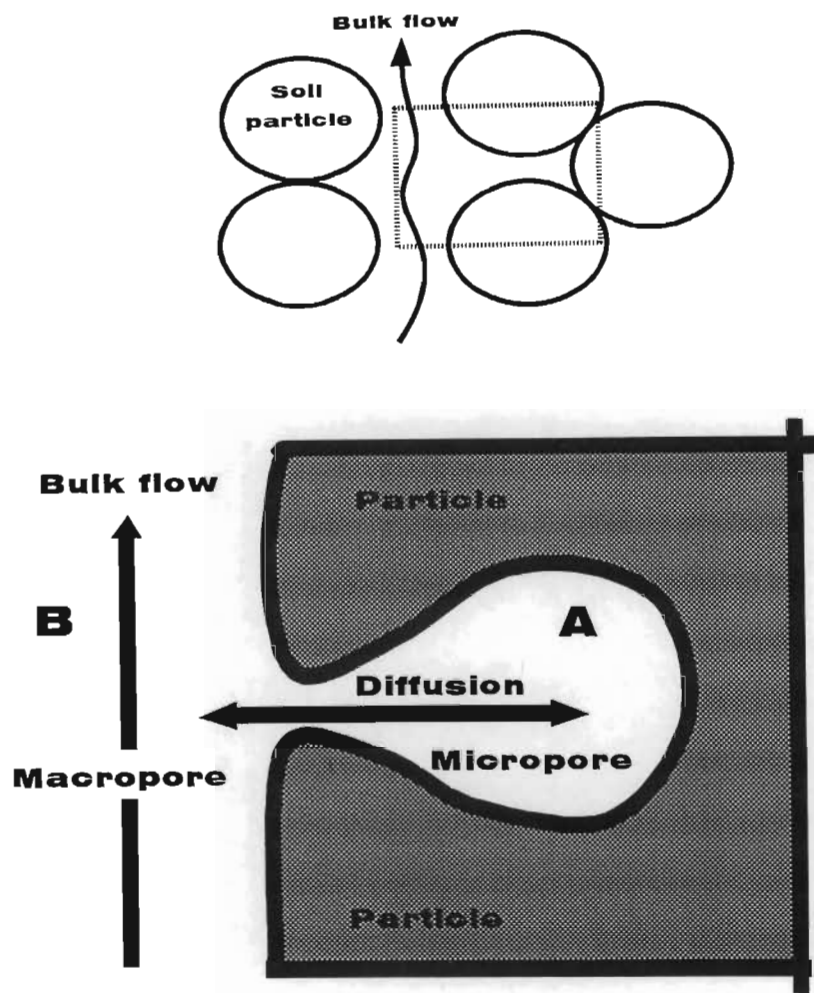
Figure 6.1: Simplified diagram of different niches in a porous medium (soil).

A represents micropore region and B represents macropore region through which bulk flow (mass flow) occurs. With a sudden release of toxic compound(s) into a porous medium the microorganisms at B will be more prone to inhibition or death due to the sudden and direct change in concentration. Microorganisms at A will be less exposed and less prone to toxic shock because of a dilution effect and relatively slow diffusion into this niche. Any compound migrating from B to A will do so by diffusion which, in turn, would result in a dilution effect of that compound at A. Due to the relatively greater surface area for attachment at A, microorganisms could be further protected from potentially toxic chemicals as a result of surface adhesion (Heynen *et al.*, 1988; Bar-Or, 1990; Morita, 1990). Less microbial attachment to pore walls in B is also due to the higher flow rates and greater shear forces in

these pores. If the concept of different pores types, as discussed here, is valid, it follows that porous media with different macro- to micropore ratios will behave differently as bioreactors.

It is well known that different soils have different microbial filtration properties (Yates & Yates, 1988; Huysman & Verstraete, 1993a, 1993b, 1993c) from which it can be inferred that at least some of the foregoing statements will apply. Microbial retention in soil is a function of microbial adsorption to particles and the filtration effect of the pores (Harvey & Garabedian, 1991; Huysman & Verstraete, 1993a). The adsorption effect in the SMP system has already been considered and found to not fully account for observed attenuation phenomena (Part I). The high ratio of micropores to macropores in clay soils is widely known to affect water retention in comparison with sandy soils (Marshall & Holmes, 1988). It is, therefore, likely that the difference in behaviour of a clay soil as a bioreactor when compared to a sandy soil, can be ascribed partly to different macro- / micropore ratios. Such differences are magnified under unsaturated conditions (Osa-Afiana & Alexander, 1982; Wan *et al.*, 1994). When a porous medium becomes unsaturated the ratio of participating micro- to macropores, as locations/venues for microbial processes, increases (Fontes *et al.*, 1991; Imhof & Jaffe, 1994). If microbial growth in micropores is vastly different to that in macropores, unsaturated conditions will increase the overall effect governed by, and within, micropores (Steele & Nieber, 1994a, 1994b). The experimental approach adopted was, therefore, to determine the different effects of macro- vs micropores in the SMP system under saturated conditions as these are easier to reproduce experimentally. It is assumed that this effect, if found to occur in saturated conditions, would be even more pronounced for unsaturated conditions. To determine the macro- / micropore effect on porous medium as a bioreactor, the adsorption effect was reduced by using inert packing material of known sizes and porosities.

6.2   Porosity and microbial exclusion from internal aggregate pores

Before proceeding with discussion of the topic it is important to illustrate how this study differed from other work related to porosity, microbial kinetics and substrate degradation. The

present study focused on those pores which are large enough to allow microbial penetration and growth. This concept and research direction differs from that of other workers who studied the effect of soil pores which exclude microorganisms (Chung *et al.*, 1992; Scow & Alexander, 1992; Scow & Hutson, 1992; Fry & Istok, 1994; Moldrup *et al.*, 1994). Exclusion of microorganisms from micropores within aggregates has been found to reduce the contact time with the substrate and, subsequently, degradation rates. Microbial growth on the compound of interest is, thus, diffusion- and sorption-dependent (Figure 6.2). Diffusion into, and sorption onto internal pore surfaces, where microorganisms cannot reach, was found to control microbial catabolism of the compound (Scow & Alexander, 1992). This model was not, however, pursued to explain the principal differences in behaviour of microorganisms between clay and sandy soils. The model shown in Figure 6.2 is possibly applicable to some *in situ* porous media conditions, such as structured clay soils, where microbial exclusion from intra aggregate pores occurs, but does not adequately explain the differences observed in non-structured soils. The model which is investigated here is one where pore sizes are such that bacterial penetration does certainly occur.

**Bacteria excluded from
micropores in aggregate**

**Inner solution
in aggregate**

**Biodegradation
in outer solution**

**Diffusion &
Sorption / Desorption**
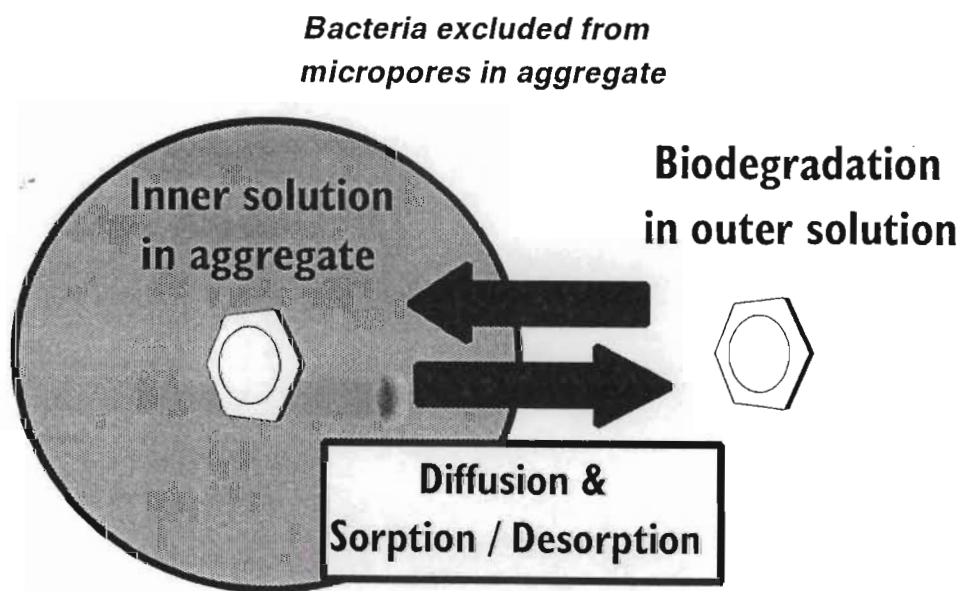
Figure 6.2: Schematic diagram of the diffusion-sorption biodegradation (DSB) model (modified from Scow & Hutson, 1992).

6.3 Effect of porosity on microbial growth and kinetics

In order to gain a clear understanding of the effects of pore size on soil as a bioreactor it is important to consider the fundamentals of growth kinetics in continuous culture experiments. It is believed that the behaviour of soil as a bioreactor, firstly under saturated conditions, is greatly affected by the controlling effect of porosity on dilution rate. In the pure sense dilution rate is given by:

$$\frac{F}{V} = D \tag{1}$$

where F = flow rate ($l\ h^{-1}$); V = culture volume (l); and D = dilution rate (h^{-1}), (Poole *et al.*, 1992; Middelbeek & Jenkins, 1992).

The flow rate can be regulated by controlling the pump speed of the influent medium and/or tube diameter. In normal continuous culture experiments the rate of change of biomass in the vessel is given by the increase in biomass growth minus the amount lost via the overflow of the vessel. It should be kept in mind that the loss of biomass in the porous medium columns used in this study is governed mainly by filtration/retention caused by pore entrapment which differs between soil types (Corapcioglu & Haridas, 1985, Shales & Kumarasingham, 1987; Gannon *et al.*, 1991). In non-porous medium containing vessels the specific growth rate (μ) is given by:

$$\frac{1}{X}\frac{dx}{dt} = \mu \tag{2}$$

which becomes:

$$\frac{dx}{dt} = \mu X - Fx \tag{3}$$

where X = total amount of biomass; x = biomass concentration; μ = specific growth rate, t = time, and F = flow rate (Poole *et al.*, 1992).

In normal continuous culture vessels steady state is reached when $\mu = D$. It is practically assumed to be steady state when the biomass concentration has not changed during two volume changes and at least a total of five volume replacements has occurred since a new dilution rate was set. At such a steady-state condition the number of cells leaving the vessel (Fx) equals the number of cells which have developed by growth (μX), i.e.

$$\frac{dx}{dt} = 0 \qquad \text{or} \qquad \mu X = Dx \tag{4}$$

Thus, $\mu = D$ where dx/dt = growth rate (Poole *et al.*, 1992).

The situation in a column containing porous media obviously differs from that in an ideal continuous culture vessel. The first, and probably most important, difference is that the number of cells leaving the vessel (column) will equal the number of cells that develop by growth minus the amount of cells filtered or retained by the packing material (or soil), i.e.

$$\text{Fx (leaving)} = \mu X \text{ (develop by growth)} - R \text{ (filtered)} \tag{5}$$

The units for these parameters are as follows:

F	=	$l\ h^{-1}$
x	=	cells l^{-1}
μ	=	h^{-1}
X	=	cells
R	=	cells

R is also referred to as a retardation factor and will be expanded later (Section 6.7.2). From the Monod equation the following basic principles apply:

$$\mu = \mu_{max} \left(\frac{S}{K_s + S} \right) \tag{6}$$

μ_{max}	=	The maximum possible growth rate (at saturating substrate concentration);
S	=	Substrate concentration in the culture liquid; and

K_s =   Monod constant (numerically equivalent to the substrate concentration with μ = 0.5 μ_{max}) also known as the saturation constant

At steady state μ = D, and thus Equation 6 becomes:

$$D = \mu_{max} \left(\frac{\bar{S}}{K_s + \bar{S}} \right) \tag{7}$$

where $\bar{S}$ = Steady state substrate concentration in the culture liquid.

If the steady-state concentration ($\bar{S}$) is much greater than K_s (say $\bar{S}$ > 10 K_s) then D ≈ μ_{max}. It is know that at steady state μ = D and, therefore, μ = μ_{max}. Rearranging the previous equation gives;

$$\bar{S} = K_s \left(\frac{D}{\mu_{max} - D} \right) \tag{8}$$

and, thus, it is clear that $\bar{S}$ can only be greater than K_s at high dilution rates. The K_s value is an indicator of the affinity of an organism for the substrate (particularly at low substrate concentrations). A low K_s value is indicative of a high affinity of the particular organism for the substrate. In order to understand the washout kinetics, and in particular the effects porous medium filtration and/or retention have on microbial kinetics, it is important to consider the basic principles shown in Figure 6.3 for a simple continuous vessel (without microbial retention).

The parameters in Figure 6.3 are related in the following equation:

$$\bar{S} = K_s D/(\mu_{max} - D) \tag{9}$$

where $\bar{S}$ will increase as D increases. The reason for this is that washout of microbial cells increases with increased dilution rate so that the contact time between organisms and substrate

(S) is reduced. The critical dilution rate (D_c) is the point at which D exceeds the maximum growth rate so that total washout occurs. Figure 6.3A shows the relationship between x and $\bar{S}$ for an organism with a low K_s value i.e. a high affinity for the substrate. An organism with a low affinity for the substrate (high K_s value) gives the plot shown in Figure 6.3B.
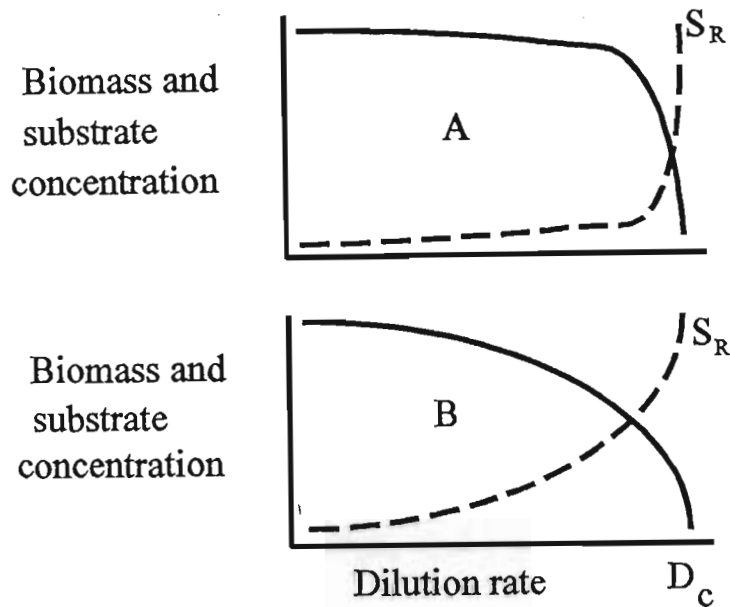
Figure 6.3: Relationship between biomass concentration (x) and substrate concentration (S) plotted against dilution rate (D). SR = substrate concentration. Dc = critial dilution rate (and D = μ at steady state). For explanation see text.

6.3.1 Determination of growth constants

A relatively simple and accurate determination of the growth constants μ_{max} and K_s can be made with the use of a continuous culture experiment and by applying Equation 7 (Middelbeek & Jenkins, 1992). This equation can be converted to a straight line (y = mx + c) by taking the reciprocals:

$$\frac{1}{D} = \frac{K_s}{\mu_{max}} \frac{1}{S} + \frac{1}{\mu_{max}} \tag{10}$$

104

By plotting $1/D$ against $1/\bar{S}$, the values of K_s and μ_{max} can be determined as shown in Figure 6.4. It is important to note that K_s and μ_{max} are constant for a particular organism since these constraints are genetically determined. When determinations of μ_{max} and K_s are made in a mixed culture the values reflect the average values for each particular set of conditions. It is well known that the population complements in continuous cultures change with increased dilution rates (Jones *et al.*, 1973; Coutts *et al.*, 1987). These population shifts occur due to the differences in growth rates and subsequent washout kinetics. When comparing the μ_{max} and K_s values for a mixed population, as determined in a continuous culture vessel, with that of a porous medium column, the following will apply. In a continuous culture vessel only a single dilution rate occurs in a well mixed vessel at any one time so that, in achieving steady state at that dilution rate, a particular species may be washed out from the vessel due to its lower specific growth rate. However, a saturated porous medium column at a particular overall dilution rate (flow rate through the column / total pore volume) could have several **"effective"** dilution rates in the various pores.
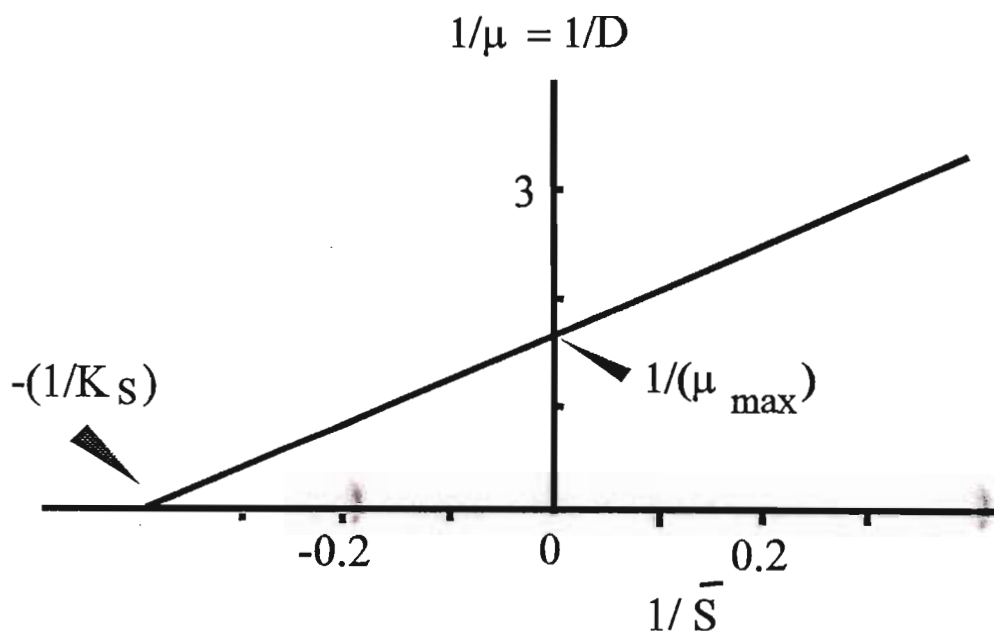
Figure 6.4: Double reciprocal plot for the determination of growth constants (Middlebeek & Jenkins, 1992).

This is due to the differences in flow rate through macro- and micropores of differing sizes and geometries. Under these conditions, population shifts are less likely to occur since a range of

effective dilution rates exists in various pores. When determining μ_{max} and K_s using the method shown in Figure 6.4 for a saturated porous medium column, the differences in the plot (μ_{max} and K_s values), when compared to a normal continuous culture vessel, could be ascribed to the following:[1]

- Filtration and/or retention effects (to be discussed in Sections 6.3 & 6.5),

- A lesser population shift in the porous medium column than in the vessel due to the greater range of effective dilution rates in the various pores; and

- A change in the effective reactor volume, which in the case of the porous medium column would relate to clogging of pores. This will decrease the volume factor in the dilution rate equation (Equation 1).

All of the above will be operative to a greater or lesser extent in different soil types, thus, influencing their efficiencies as bioreactors. It is possible that much of the differential effects of porous medium microbial activity could be explained by these factors. The range of effective dilution rates maintains microbial populations in various growth phases which, in turn, may affect their resistance to toxic chemicals. The range of dilution rates is possibly also an important factor in allowing the soil to host such a great diversity of microorganisms.

6.3.2 Filtration effect

As shown above, $\mu = D$ for steady-state conditions in continuous culture vessels (without porous medium). If the biomass is retained by some mechanism, e.g. culture feedback, the equation becomes:

$$\mu = A\,D \tag{11}$$

---

[1] *Contemplating such determinations in a soil column might seem rather crude but it illustrates the effects caused by the packing materials (or soil) and is, therefore, valid for comparison purposes.*

106

where A is the biomass concentration factor. It then follows that $\mu_{max} \approx A \, D_c$ which infers that D_c is extended beyond μ_{max}. This can (although crudely) be applied to continuous culture-type flow through a saturated porous medium column, where the biomass concentration factor is related to the filtration (or retardation) of microorganisms by the porous medium (or packing material). This value (A) can be estimated by microbial breakthrough curves (Section 8.2) and is, thus, an important part of the present study.

All of the above explanations for soil-determined effects on continuous culture are somewhat theoretical, although they highlight the various principles which make the soil such a complex bioreactor. A more accurate description of the soil would be to view it as a series of interconnected continuous culture vessels (i.e. each pore representing a separate unit) although this is made impossible by the practical analytical limitations.

6.4   Porosity and biofilms

The conceptual model of Zysset *et al.* (1994) splits the groundwater-biofilm system into three compartments, namely, the aqueous, the solid and the biofilm compartments. Each of these compartments is characterized by the nature of a dominant transport process, namely, advection for the aqueous compartment, diffusion for the biofilm compartment, and negligible transport for the solid compartment. These three compartments generally contain a mixture of phases. The aqueous compartment is usually a solution or suspension, and the solid compartment is a mixture of different solid phases. The biofilm compartment consists of an aqueous phase, and solid phases formed by bacteria, dissolved substances and, possibly, extracelluar polymers. The biofilm need not be continuously extended from the solid surface. In fact, a majority of the bacteria in such systems adheres to the solid matrix. On the basis of this, Zysset *et al.* (1994) assumed the microbial activity of bacteria suspended in the aqueous compartment to be negligible.

Their conceptual model for the development of a growing biofilm catalyzing a redox reaction with dissolved substrates (electron donor and acceptor) is as follows. Biofilm growth is

107

supposedly limited by the supply of these molecules to the bacteria within the biofilm, i.e. by diffusion into the biofilm compartment. This diffusion depends mainly on two factors. The first is the length of the diffusional path from the boundary between the aqueous and the biofilm compartments to the individual bacteria within the biofilm, averaged over the considered part of the system. This factor increases with a growing biofilm. The second factor is the diffusion surface, in other words, the boundary between the aqueous and the biofilm compartment. At an early stage of a growing biofilm, this diffusion surface, summed over the considered part of the system, increases. In later stages, however, when the biofilm is clogging certain pores, the diffusion surface is decreased. The clogging of pores may additionally cause increased intrapore velocities and, therefore, an increased detachment of the biofilm by shear forces. It may at the same time decrease the supply of substrate and, therefore, cause an increased death rate of bacteria.

A changing biofilm compartment influences not only its own transport field but also that of the aqueous compartment. This double action stems from the fact that both the aqueous and the biofilm compartments compete for the same pore space, which remains relatively constant with time. A growing biofilm compartment results in a diminishing volume of the aqueous compartment. In a system with a fixed hydraulic gradient, decreasing the volume of the aqueous compartment most probably leads to a decrease in the permeability of the system (Shaw *et al.*, 1985) and subsequent reduction of the flow velocity in the aqueous compartment. Thus, an increasing biofilm initially increases the area of the boundary while, in contrast, a further increase of the biofilm decreases the area of the boundary. For the purpose of this study a fixed flow rate was used to eliminate some of the above effects.

6.5   Clogging of pores and hydraulic conductivity

It has been suggested that bacterial reduction of saturated hydraulic conductivity (KS)[2] of natural porous media is linked to the development of anaerobic/microaerophilic conditions in these media (Shaw *et al.*, 1985).   This is not, however, always the case (Vandevivere & Baveye, 1992c).   Vandevivere and Baveye (1992b) found a strictly aerobic strain, *Arthrobacter* AK19, which was able to reduce the KS in sand columns by three orders of magnitude in 7 days.   Glucose concentrations as low as 10 mg l^{-1} were sufficient to lead to such severe clogging.   This rapid reduction in KS was associated with the formation of a thick bacterial mat at the inlet end of the sand columns used.   Dissolved nutrients which penetrated the mat by convective flow were readily metabolized and steep chemical gradients ensued.   When the formation of such a mat was prevented, reduction in KS occured at an appreciably slower rate. It was, furthermore, found that the production of extracellular polymers did not seem necessary to induce severe bacterial clogging as found in other studies (Vandevivere & Baveye, 1992a).   Extracellular polymers were produced and appeared to cause additional KS reduction when the C/N ratios were high but at lower C/N ratios they were absent from the clogged layers.   The mechanism responsible for most of the reduction in KS was thought to be the accumulation of cells themselves.   It has also been found that a decrease in hydraulic conductivity under methanogenic conditions could be due to pore blocking due to the entrapment of methane bubbles (Sanchez *et al.*, 1994).

6.6   Oxygen utilization

Due to the importance of the aerobic environment and the supply of oxygen into the experimental column, a brief discussion is warranted.   The amount of oxygen (C) required to produce 1g of dry biomass can be calculated by the following equation (Jenkins, 1992):

---

2   *Saturated hydraulic conductivity is usually denoted by "K_s". This notation is, however, also used for substrate affinity. The fact that the same notation is used for both concepts is indicative of the lack of interdisciplinary research in these two fields. In order to avoid ambiguity saturated hydraulic conductivity will be denoted by KS.*

$$C = A / (Y_s - B) \tag{12}$$

where:

A   =   amount of oxygen required for the oxidation of 1g substrate to CO_2 and H_2O;

Y_s =   yield coefficient for carbon substrate (g g^{-1}); and

B   =   dry biomass (g).

Although this equation determines the total amount of oxygen required, it does not provide any information about when oxygen is needed during cultivation. This is given by the specific O_2 uptake rate (q_o), and, typically, has units of mM O_2 g^{-1} dry weight biomass h^{-1}. The value of $\dot{q_o}$ is dependent on the specific growth rate (μ) and the biomass yield coefficient for oxygen (Y_o): $q_o = \mu / Y_o$. When q is correlated with the biomass concentration (X), the oxygen uptake rate or the oxygen transfer rate (OTR) is obtained:

$$OTR = q_o X \tag{13}$$

$$OTR = (\mu / Y_o) X \tag{14}$$

The OTR typically increases during the exponential growth phase because of increased biomass. This continues until the culture becomes limited by the OTR or by another nutrient. In the latter case, the OTR then decreases since the specific growth rate (μ) is lowered. Together with the OTR, the concentration of oxygen in the culture medium is an important consideration. The dissolved oxygen concentration also directly influences the OTR. In addition, the dissolved oxygen concentration must be above a minimum value to permit unhindered respiration. This minimum value is termed the critical oxygen concentration and is typically 5-20% of the oxygen saturation value for the aqueous medium (Jenkins, 1992). The maximum solubility of oxygen in water is very low (i.e. 8.8 mg l^{-1} at 20°C and 7.5 mg l^{-1} at 30°C) relative to the concentration that can be used by a culture. Other factors affecting the dissolved oxygen concentration are partial pressure and the presence of solutes in the medium. The effect of oxygen pressure in the gas phase (p_o) is given by Henry's Law

(Jenkins, 1992):

$$C^* = p_o / H \tag{15}$$

where
C*   =   dissolved oxygen saturation concentration (mg l^{-1});
H   =   Henry's constant which is specific for the gas and liquid phase (bar l g^{-1} oxygen); and
p_o =   partial pressure (bar), also known as the oxygen tension factor.

Henry's Law shows that as the oxygen concentration increases in the gas phase the dissolved oxygen concentration increases in the aqueous medium. Conversely, oxygen solubility decreases with an increase in temperature and with an increase in the dissolved salts.

It has also been found that a *Bacillus* species consumed more oxygen, utilized glucose at a faster rate, and was able to maintain oxygen uptake for longer periods of time when it was cultured in a medium in the presence of small glass beads (diameters of 29 and 53 μm), than when it was grown in the presence of larger beads or in bead-free medium (Sharma & McInerney, 1994). The activity of *E. coli* under fermentative conditions has been found to be inhibited in porous materials compared with its activity in liquid culture. This inhibition was more pronounced as the bead size decreased. This finding is consistent with the theory that the portion of the biomass supplied with nutrients, by diffusion, increases with a decrease in the overall pore size. Transport of oxygen through an aquifer is usually the limiting factor for *in situ* bioremediation (Wilson *et al.*, 1981). Oxygenation of porous media can be achieved by applying hydrogen peroxide. When hydrogen peroxide decomposes, the resulting oxygen may be present as dissolved oxygen or as free oxygen gas (Graves *et al.*, 1994). The movement of oxygen through porous media depends on the persistence of hydrogen peroxide and the concentration of dissolved oxygen. Graves *et al.* (1994) supplemented a 9 parts water to 1 part soil slurry with 868 mg l^{-1} hydrogen peroxide and quantitatively tracked the oxygen concentration until changes in the oxygen distribution between the gaseous and dissolved phases became asymptotic. After about 4 hours, the dissolved oxygen content of the slurry was about 70 mg l^{-1}, and the release of gaseous oxygen, as a result of hydrogen peroxide

decomposition, was asymptotic. This method, therefore, seems effective in increasing the oxygen concentration in the water provided that the costs are not prohibitive.

6.7   Microbial transport models

6.7.1   Model concepts

In most cases, the person researching transport of microorganisms in porous media is trying to answer the question: "If we apply some quantity of microorganisms to the soil at point A, how many will make it to point B?" Point B may be a source of drinking water such as a well, where the presence of microorganisms in sufficient numbers would pose a potential threat to the health of people consuming the water (Yates & Yates, 1991). Another area of application is in bioremediation of a site contaminated with a microbially degradable compound. When microbial numbers are increased at the, surface, by inoculation or alleviation of limiting factors, it is important that these migrate downwards to deeper levels of contamination. Models can provide useful information about the extent of movement as well as the time required for the microorganisms to arrive at a specified destination. In the soil environment, two major factors control microbial fate i.e. survival and movement. Both these factors are dealt with in most microbial transport models (Yates & Yates, 1988, 1991). When the soil is viewed as a bioreactor, however, the "microbial survival" part of the equation is often not applicable. The reason for this is that "survival" in most models is assumed to be a gradual decrease or a net microbial decay rate. When using the soil as a bioreactor, as in bioremediation, the main consideration is microbial growth (net increase) rather than survival (net decrease). The microbial transport aspects in most of the literature and models are, however, applicable because of their description of niches in the porous system and, therefore, warrant a summary. The transport process can be described in terms of three factors i.e. advection, dispersion and adsorption.

112

Advection and dispersion

Advection is the process by which the microorganisms are transported with the bulk flow of the groundwater (Gerba *et al.*, 1991; Sarkar *et al.*, 1994). In a simple model, advection is considered as the average velocity of the groundwater. This is obviously not the case in most porous media due to the filtration / retention effect which will be discussed later in this section. Since water does not normally flow through porous media in a straight path, dispersion is very important in describing transport. Two distinct processes operate in dispersion, namely diffusion and mechanical mixing. Diffusion is the spreading out of solute or microorganisms due to concentration gradients. This process is relatively unimportant except under conditions of low velocities. In most circumstances, dispersion caused by the motion of water (i.e. mechanical mixing) is the main process of interest. Mechanical mixing occurs when water moving through the porous medium diverges around the porous medium particles. After travelling around a number of particles, the initial concentrated flow pattern will have spread out as it moves through the porous medium. The amount of spreading depends upon the type of porous medium involved as well as on the velocity of the liquid medium (Jussel *et al.*, 1994; Simulnek & Suarez, 1994; Steenhuis *et al.*, 1994).

Adsorption

Adsorption is the third factor needed to describe the transport process and has been discussed earlier (Section 1.4.1). It is important to note that all the processes involved in adsorption will affect transport. A few particular aspects with regard to the physical environment and with specific reference to transport models are discussed here. For the purposes of developing a quantitative relationship for the adsorption process, both Langmuir and Freundlich isotherms have been used (Gerba *et al.*, 1991). Adsorption equilibria of bacteria are established within 1 to 24 hours. Flow velocities below 1 m day^{-1} probably result in equilibrium being attained. At higher flow velocities the adsorption process may not be at equilibrium, which causes lower adsorption as compared to the theoretical equilibrium values. Microorganisms may also desorb from porous medium surfaces in response to changes in pH and lower salt concentrations,

caused by rainfall (Sarkar *et al.*, 1994). Generally, adsorption is quantified by the use of equilibrium adsorption isotherms which relate the concentration of the solute in the liquid to the concentration of the solute on the adsorbing particles under equilibrium conditions. It is obvious that most microorganisms will not approximate the behaviour of solutes due to their relatively large size. Although adsorption, as characterized by the Freundlich isotherm, only applies to viruses and not to bacteria the isotherms are given here since they demonstrate the relationships between concentration, retardation, bulk density and water content. The isotherm used is as follows:

$$A = K_A C^{1/n} \tag{16}$$

where K_A and n are empirical constants. A is the virus concentration adsorbed to the solid phase and C is the virus concentration in solution. Since reported values for 1/n are statistically close to 1, 1/n can often be replaced by 1 in the Freundlich equation, yielding:

$$A = K_A C, \text{ the linear isotherm.} \tag{17}$$

In modelling microbial transport, K_A can be used to determine the retardation factor (R). Retardation is the slowing of the movement of a contaminant (virus) relative to the bulk mass of the water due to adsorption onto solids. It is related to the bulk density of the porous medium and the volumetric water content by:

$$R = 1 + (\rho_b/\Theta).K_A \tag{18}$$

where ρ_b is the bulk density (g ml^{-1}); Θ is the volumetric water content (cm ml^{-1}); and K_A is the linear adsorption coefficient.

An interesting phenomenon is that viruses and even bacteria have, under some conditions, been found to travel faster than the average pore water velocity (Gerba *et al.*, 1991). One possible explanation is pore size exclusion in which the microorganisms can only be transported through large pores where the average pore water velocity is greater than the average for the

114

entire porous medium. A second explanation is based on the mechanism of anion exclusion whereby the negatively charged microbial particles are pushed to the centre of the pore where the velocity is, on average, higher than that of the bulk medium. The effects of anion exclusion may result in an R value of less than 1. The driving force for anion exclusion is that most soil particles under "normal" soil conditions are negatively charged which induces electrostatic repulsion on the negatively charged bacteria (Section 1.4.1).

Filtration

The other main factor that governs microbial transport, apart from survival (sometimes referred to as microbial decay), is filtration. Filtration mechanisms include straining, sedimentation, inertial impingement and diffusion (Gerba *et al.*, 1991; Hekman *et al.*, 1994). Straining occurs when the microorganism in suspension in the porous matrix cannot pass through a pore opening or constriction (i.e. the wedge between two porous media particles) smaller than itself and, thus, its transport is impeded. The extent to which this occurs depends on porous medium type, microbial species, size etc. In general, under high flow velocities, the amount of bacteria filtered is less than for low flow velocities (Gerba *et al.*, 1991; Huysman & Verstraete, 1993a). Sedimentation in the pores occurs where there is a density difference between the microorganisms and water. If the microorganisms are more dense than the liquid phase and the flow properties are such that the tendency for gravitational settling is greater than the tendency to be resuspended into the flow stream, the bacteria may settle into certain parts of the porous medium. The filtration efficiency of a porous medium aquifer can be simply defined as the removal to a certain flow length:

$$C = C_o \exp(-\lambda_f X) \tag{19}$$

where C is the observed concentration of organisms; C_o is the initial concentration of organisms; X is the distance; and λ_f is the filtration coefficient. Filter mechanisms depend on hydraulic conditions (flow velocity and flow direction). When these parameters change, the bacteria may be remobilized. The filtration coefficient (λ_f) may change with time. As the

bacteria accumulate they can further reduce the diameter of the pores available for microbial movement. Very high microbial concentrations will also induce flocculation and aggregation, which will further enhance clogging.

6.7.2 Mathematical models

Percolation model

Loehle and Johnson (1994) developed a model which not only accounted for decay but also for microbial growth in porous media. They proposed a spatially explicit percolation model as a framework for interrelating microbial transport studies done at different scales, for interpreting field correlation studies, and for suggesting improved experimental designs. Their model is not a single model but is rather a framework for the development of models, for relating models at different scales, and for planning and interpreting experiments. To identify and analyze the physical, geological and biochemical parameters which affect microbial transport in subsurface porous media they built on the colloidal model of Harvey and Garabedian (1991). A one-dimensional equation for the transport of bacteria following a pulse injection, with terms for storage, reversible and irreversible adsorption, dispersion, and advection, is given by:

$$\frac{dc}{dt} + \rho_b \frac{ds}{dt} = D\theta \frac{d^2c}{dx^2} - v\theta \left(\frac{ds}{dt} + k_p c \right) \tag{20}$$

where
θ	=	effective porosity
c	=	concentration of bacteria in solution
t	=	time after injection
ρ_b	=	sediment bulk density
s	=	concentration of reversibly adsorbed bacteria on the solid surface
v	=	velocity
x	=	spatial coordinate
k_p	=	the irreversible adsorption constant

116

D   =   dispersion coefficient, which is equivalent to the product of two measurable parameters, longitudinal dispersivity (a_L) and fluid velocity (v).

A pulse injection is usually well defined and allows transport parameters to be identified. It is practically found to occur during the introduction of genetically engineered microorganisms into the subsurface for groundwater remediation. For a constant source term, Equation 20 could be suitably modified (Loehle & Johnson, 1994). The k_p parameter may be described by the colloid filtration model used to explain the removal of colloidal sized material during filtration in packed-bed systems, as follows:

$$k_p = \frac{3}{2}\left(\frac{1-\theta}{d}\right)\alpha\,\eta$$

(21)

where
d   =   diameter of the porous medium grains
α =   is the collision efficiency '
η =   is the single-factor collector efficiency

The reversible adsorption term in Equation 20 may be of two types. The first type assumes a linear isotherm and relatively rapid adsorption with respect to advection:

$$s = K_d c$$

(22)

If the adsorption constant (K_d) does not vary with time, the time variation of bacteria on surfaces and in solution can be related directly as follows:

$$\frac{ds}{dt} = K_d\left(\frac{dc}{dt}\right)$$

(23)

The second type of reversible adsorption term assumes a first-order kinetic reaction for the rates of adsorption and desorption as follows:

$$\frac{ds}{dt} = k_f c - k_r s \tag{24}$$

where k_f and k_r are the forward and reverse adsorption rate constants, respectively. These two adsorption relations give different results (Harvey & Garabedian, 1991). Water velocity, relative to equilibration time for adsorption, can be used to determine which adsorption relation should be used.

Microbial growth model

In cases where microbial species actually grow in porous media the model has to be modified. In general, organisms introduced into an unoccupied habitat exhibit exponential growth until they reach a saturation level or carrying capacity that is a function of available nutrients. Genetic selection at these two stages is quite different. A typical function describing this relation is the logistic, here including predation of bacteria by protozoa and viruses (Loehle & Johnson, 1994):

$$\frac{dN}{dt} = rN \left(\frac{(N_{eq} - N)}{N_{eq}} \right) - p(N) \tag{25}$$

where
N   =   population size
N_{eq} =   the sustainable (equilibrium) level
r   =   growth rate
p(N)   =   nonlinear function for predation
N   =   C + S [or total population equals the sum of two subpopulations, solution (C) and sorbed (S)]

It may be necessary to model the growth of C and S separately if they grow at different rates. This relation or a similar one, therefore, needs to be added to Equation 20 to describe changes in population (or mass) of microorganisms at each x location. This addition introduces a major change in the solution to Equation 20. If r is large enough relative to the velocity and

118

dispersion terms, the effect of incorporating Equation 25 into Equation 20 is to change the wave shape for bacteria passing a downstream point after a pulse injection into a front-type function.

Another complication is that when N becomes high, pore blockage due to microbial mass and slime may reduce water movement. Thus, velocities in Equation 20 become:

$$v = v(N) \tag{26}$$

a decreasing, nonlinear function of N. These two complicating factors introduce a new twist on standard transport and filtration theory. This effect represents a scaling relation where microbial mass rescales the dynamic parameters of the system. Similarly, effective porosity is a function of N

$$\Theta = \Theta(N) \tag{27}$$

and so is the filtering coefficient

$$k_p = k_p(N) \tag{28}$$

Furthermore, the dispersion coefficient, D, is in reality a function of microbial type, T,

$$D = D(T) \tag{29}$$

The D values for microorganisms differ substantially from the value measured for nonliving tracers. D must be determined experimentally for different microorganisms and environmental conditions, and cannot be estimated from inanimate tracers or assumed to be constant for all microorganisms. In fact, dispersivity resulting from active movement could even be larger in magnitude than the water velocity term and lead to movement against water flow (Loehle & Johnson, 1994).

Incorporating the complexities of Equations 25-29, the advection-dispersion-percolation Model 1 becomes:

$$\theta(N)\,\frac{dc}{dt} + \rho_b\,\frac{ds}{dt} + \frac{dN}{dt} = D(T)\,\theta(N)\,\frac{d^2c}{dx^2} - v(N)\,\theta(N)\left(\frac{dc}{dx} + k_p(N)c\right) \tag{30}$$

Because of the nonlinearities involved, this equation will almost certainly generate pulsations and cycles along a flow path, even in a homogenous medium, making it very difficult to fit from experimental data (Loehle & Johnson, 1994). For example, clogging of pores can reduce the concentrations of nutrients and oxygen in the clogged zone, which might cause bacterial death or inhibition in the clogged area. The resulting pulsations would move repeatedly through the system. Predation would also set up cycles (Atlas & Bartha, 1987). Extending Equation 30 to the additional complexity of heterogeneous cases by experimentally estimating effective parameters for the flow path as a whole is probably unworkable. For this reason Loehle and Johnson (1994) proposed the use of spatially explicit percolation models at several scales of resolution to represent heterogeneous flow paths. For certain cases, these percolation simulations might allow effective parameters to be derived for Equation 30, in which case it can be used as a simpler version of the model. In many cases (e.g. colonization of new zones, injection of nutrients) effective parameters do not exist. Equations 20 and 30 can, thus, be used as a frame of reference for transport although the actual model must be spatially explicit.

Linked transport and population dynamics

In the same way that population dynamics are a critical element in microbial transport, microbial transport is a critical element in subsurface population dynamics. Transport affects nutrient availability, dilution rates, microbial species selection, colonization rate etc. Loehle and Johnson (1994), therefore, developed a framework for linking these processes across scales. Although models for bacterial movement through porous media have been developed (Harvey & Garabedian, 1991), population dynamics in subsurface environments have not in themselves been studied, probably due to the lack of substantial data (Fontes et al., 1991).

Microbial habitats in porous media are basically microhabitats located at microsites such as microcolonies or biofilms attached to particle surfaces. Aggregates in the pore spaces are also microhabitats (Loehle & Johnson, 1994; Sarkar *et al.*, 1994). The pore space itself can also be viewed as a microhabitat to be referred to as the transport habitat of a subsurface region. In general, bacteria in porous media are either in the transport habitat, being transported to the next zone, or within a microhabitat. To address these complexities Loehle and Johnson (1994) proposed an approach that combines percolation modelling with multi-chemostat (porous media) models. Since groundwater as a growth medium generally flows continuously through regions in the subsurface, it was assumed that each habitat in a region is an open system with inputs and outputs of microorganisms and resources. In addition, they assumed that input into a habitat H_j from habitat H_i reflects the relative densities of the different types of organisms and resources in H_i. It is furthermore assumed that the entire system of habitats for a subsurface region is, in itself, open, in that input into the transport habitat comes from other transport habitats (regions upstream in the flow path), and output from the region goes from the transport habitat to one or more transport habitats or regions downstream. These assumptions can then be applied to sets of interconnected chemostats. A sequence of chemostats (porous media environments) can be illustrated by two chemostats with volumes V_1 and V_2, connected by a flow of water, organisms and substrates from chemostat 1 into chemostat 2. (Porosity and microhabitats are neglected here for the sake of simplicity). The rates at which cells and substrates emerge from chemostats 1 and 2 are $w_1 X_1$, and $w_2 X_2$, respectively where the respective washout rates are: $w_1 = W/V_1$ and $w_2 = W/V_2$; W is the rate of flow through both chemostats; and X1 and X2 are the concentrations of the microorganisms in chemostats 1 and 2. The following differential equations relate the concentrations of resource (S) and microorganism (X) in chemostats 1 and 2:

$$\frac{dX_1}{dt} = \left(\frac{r_1 S_1}{K_1 + S_1} \right) X_1 - w_1 X_1 \tag{31}$$

$$\frac{dS_1}{dt} = w_1 (S_1^0 - S_1) - q_1 \left(\frac{r_1 S_1}{K_1 + S_1} \right) X_1 \tag{32}$$

$$\frac{dX_2}{dt} = \left(\frac{r_2 S_2}{K_2 + S_2}\right) X_2 - w_2(X_1 - X_2) \tag{33}$$

$$\frac{dS_2}{dt} = w_2(S_1 - S_2) - q_2\left(\frac{r_2 S_2}{K_2 + S_2}\right) X_2 \tag{34}$$

The substrate enters chemostat 1 at concentration S_1^0. S and S_2 are the substrate concentrations in chemostats 1 and 2 at time t. X_1 and X_2 are the concentrations of cells in the two chemostats. When resources are abundant in chemostat 1, the population of cells grows at a maximum rate r_1; in chemostat 2 the population grows at maximum rate r_2. At substrate concentrations K_1 in chemostat 1 and K_2 in chemostat 2, growth occurs at half the respective maxima. An amount q_1 of substrate is taken up per bacterium produced in chemostat 1 and an amount q_2 per bacterium produced in chemostat 2. The simple-chemostat-network model described above illustrates the basic concept for simulating dynamics and transport, including colonization of new habitats. The model tracks the quantities and locations of all types of masses (i.e. the mass of each resource and each cell type). Further, since the overall microbial mass can be monitored, it gives the information required to calculate microbial effects on the velocity and dispersion terms in the transport Equation 30. For more elaborate cases, the chemostat framework can be extended to model a network of regions whose connections are determined by a percolation model representation of subsurface heterogeneities and flow paths based on field data. For heterotrophic microorganisms, a realistic chemostat-network model for population dynamics in the subsurface should include transport not only of a carbon source but also of an electron acceptor, e.g. O_2, NO_3^-. An electron acceptor can be included by using the modified Monod relationship for microbial growth kinetics as follows:

$$YR_s = \mu_{max} \rho V \left(\frac{S}{K_s + S}\right) \left(\frac{O}{K_o + O}\right) \tag{35}$$

Here, R_s is the rate of substrate utilization, μ_{max} is the maximum specific growth rate of heterotrophic microorganisms, ρ is the cell mass of organisms per unit volume, Y is the yield

coefficient for the microorganisms, S is the substrate concentration, O is the oxygen concentration, K_s is the substrate saturation constant, K_o is the oxygen saturation constant, and V is the volume of the chemostat.

Lensing *et al.* (1994) derived a similar equation:

$$\frac{X_1}{dt} = [1 - F(O_2)] \frac{[C_{org}]}{K_{Corg} + [C_{org}]} \frac{O_2}{K_{O_2} + [O_2]} \qquad (36)$$

where X_1 is the biomass of heterotrophic aerobic microorganisms and $F(Q)$ is a weighting function. This equation was further expanded by Lensing *et al.* (1994) to include organisms growing under various redox potentials which will not be considered here due to the fact that the present experimentation was conducted in aerobic environments.

6.8   Experimental rationale

From the above it is clear that in the literature soil pores are considered, in their most advanced form, as connected micro-bioreactors. What seems to be ignored though is the fact that some pores are not connected and do not have both an inflow and outflow end. The aim of the following experimentation was to investigate the importance of microbial growth in non-connected pores (usually micropores). This was done by looking, principally, at their effects on microbial growth kinetics and, secondly, microbial resistance and/or recovery from toxic chemicals. It will be noted that these discussions make use of relatively few references. This particular approach to the effects of soil porosity on microbial activity is seemingly poorly documented.   In fact, it seems to be an area which has received virtually no attention whatsoever. The probable reason is that it lies at the intercept of disciplines which are very seldom combined, i.e. microbiology, soil physics and soil hydrology. This study should, therefore, be seen as a first attempt at breaking new ground in this field.

CHAPTER 7. MATERIALS AND METHODS

7.1   Experimental procedures

7.1.1   Growth kinetics in continuous culture vessel

Specific growth rate determination for the microbial catabolizing association was made at 30°C with a 480 ml Scott bottle equipped with a magnetic stirrer bar (Figure 7.1). The bottle cap was fitted with two stainless steel tubes, one of which extended to the bottom of the vessel (inflow) and the other (outflow) which served to drain excess liquid from the top. Three reservoirs containing phenol, mineral salts and hydrogen peroxide, were linked to the bottle by peristaltic pumps (Watson Marlow 205S). The dissolved oxygen concentration of the inflow to the bottle was kept at 15 mg l^{-1} (Brubaker *et al.*, 1994; Govind *et al.*, 1994). This was much lower than the 70 mg l^{-1} at which the release of gaseous oxygen is likely (Graves *et al.*, 1994). The release of gas bubbles of any sort was undesirable since this would reduce the liquid filled pore volume and, hence, increase dilution rate. The tubing from the three reservoirs converged just before entering the reactor via a three-pronged glass manifold (Figure 7.3). The phenol reservoir contained a 10 mM (0.91 g l^{-1}) phenol solution while the mineral salts reservoir contained (l^{-1}): 1.0g KH_2PO_4, 3.0g K_2HPO_4 , 2.0g NH_4NO_3 , 0.4g $MgSO_4.7H_2O$ and 2ml trace element solution as used in Section 2.4.1. The tubing from these two reservoirs was led through the same peristaltic pump and the same diameter tubing, thus, effectively halving their concentrations on mixing. The tubing from the hydrogen peroxide vessel was passed via a separate peristaltic pump. This was necessary to achieve a final mixing ratio of 1:200 (phenol + mineral salts : hydrogen peroxide, v/v). The solutions were pumped from the three separate reservoirs to prevent microbial growth in the tubing and reservoirs. The Scott bottle was sealed so that the only oxygen inflow into the column was from dissolution of hydrogen peroxide. The continuous culture vessel was inoculated with a 10% (v/v) phenol catabolizing association (Section 2.5). The initial flow rate was set to achieve a low dilution rate. Each incremental increase in flow rate was only made after steady state was achieved, which generally occurred after approximately 3-4 pore volume flushes of the experimental container. The optical density (A_{590nm}) and phenol concentration (Appendix A.1)

were monitored daily.
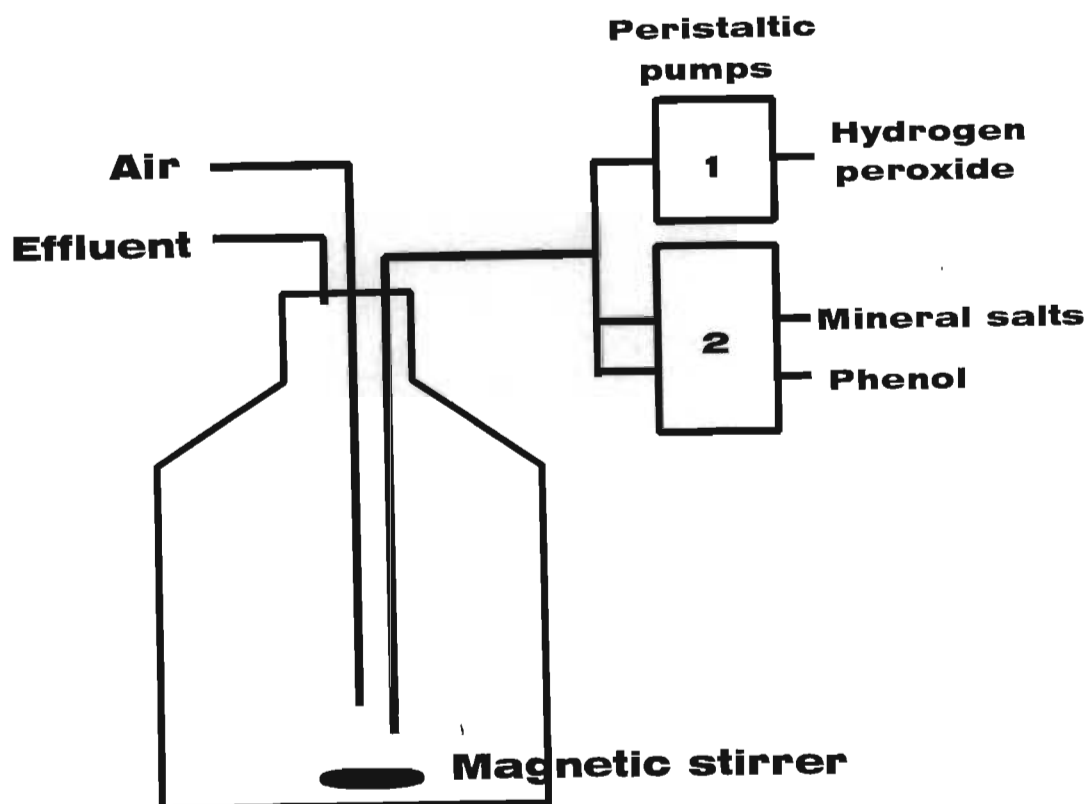
Figure 7.1: Continuous culture vessel, pump and reservoir configuration.

7.1.2 Column configuration and packing materials

Due to the variability of porous media particle sizes, three standard packing materials were used for the columns. Details of the packing materials and packed columns are given in Table 7.1. Particle size distributions of the packing materials are shown in Figures 7.2 (A-C). The abbreviations AWS (acid washed sand), MIX (50 % w/w mixture of acid washed sand and chromatography packing material) and CHROM (chromatography packing material) are subsequently used.

Table 7.1: Some physical characteristics of the packing materials.

	AWS	MIX	CHROM
Particle density (g ml^{-1})	2.78	1.82	1.75
Bulk density (g ml^{-1})	1.66	0.72	0.43
Total pore volume (ml)	100.27	150.32	188.4

The bulk densities for the three different packing materials were determined by calculating methanol displacement in a measuring cylinder over a 2 hour period. This method was used instead of the usual water displacement under vacuum. Although air displacement could easily be achieved by the vacuum method during a 24 hour period using loose porous media particles, this method could not be used in the packed columns under high vacuum due to air ingress occurring at the end caps of the columns. The low viscosity of methanol is ideal as a displacement liquid and can be used in the columns without a vacuum for particle density determinations from which pore volumes were calculated.

The particle size distribution was determined by measurement under light microscopy (Nikon Optiphot-POL Petrographic microscope). The maximum diameter of the particles was measured using a graded eyepiece lens. The particle diameter of 100 particles of each packing material was measured for this purpose. The column and experimental design are illustrated in Figure 7.3 and was similar to that used by Chen et al. (1994). The polyvinyl chloride (PVC) columns were 4.35 cm in diameter and were packed to a depth of 16.7 cm. Silicone rubber stoppers and glass wool filters were used to prevent packing displacement. All columns were fitted with a tap, sampling port and three-way glass manifold where the inflow from the three reservoirs converged. The sampling port was used to facilitate comparison of influent and effluent phenol concentrations (Appendix A.3.8). During sampling from the port the tap was closed so that the inflow was diverted to the port while preventing downward flow of the nutrient medium in the columns. The term "relative phenol concentration" was used to express the phenol eluent concentration as a percentage of the inflow concentration.
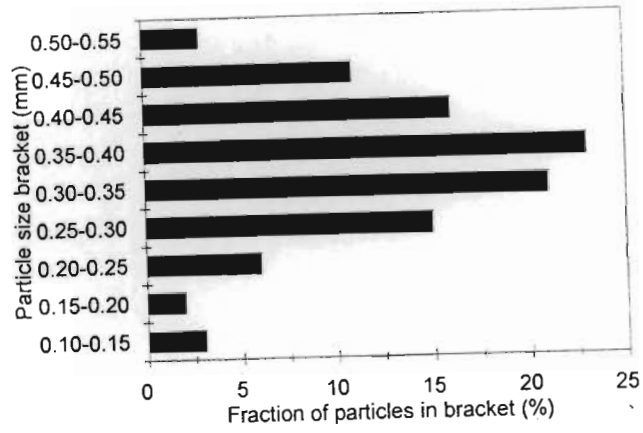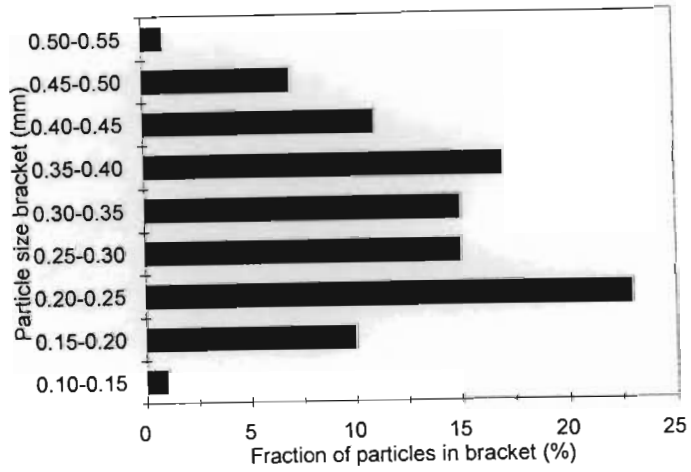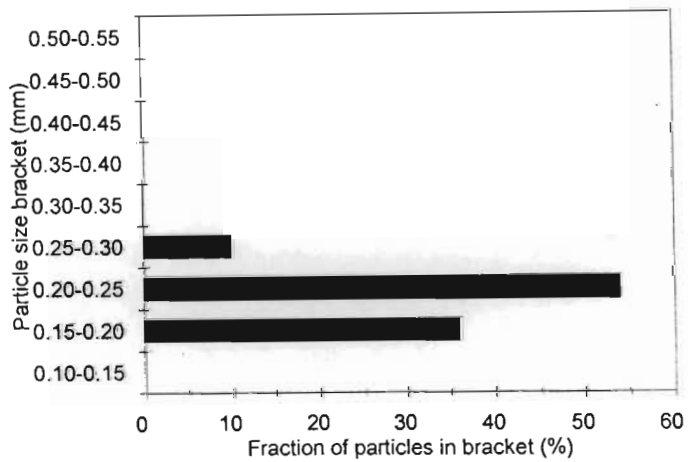
Figure 7.2: Particle size distribution for (A) AWS, (B) MIX and (C) CHROM.
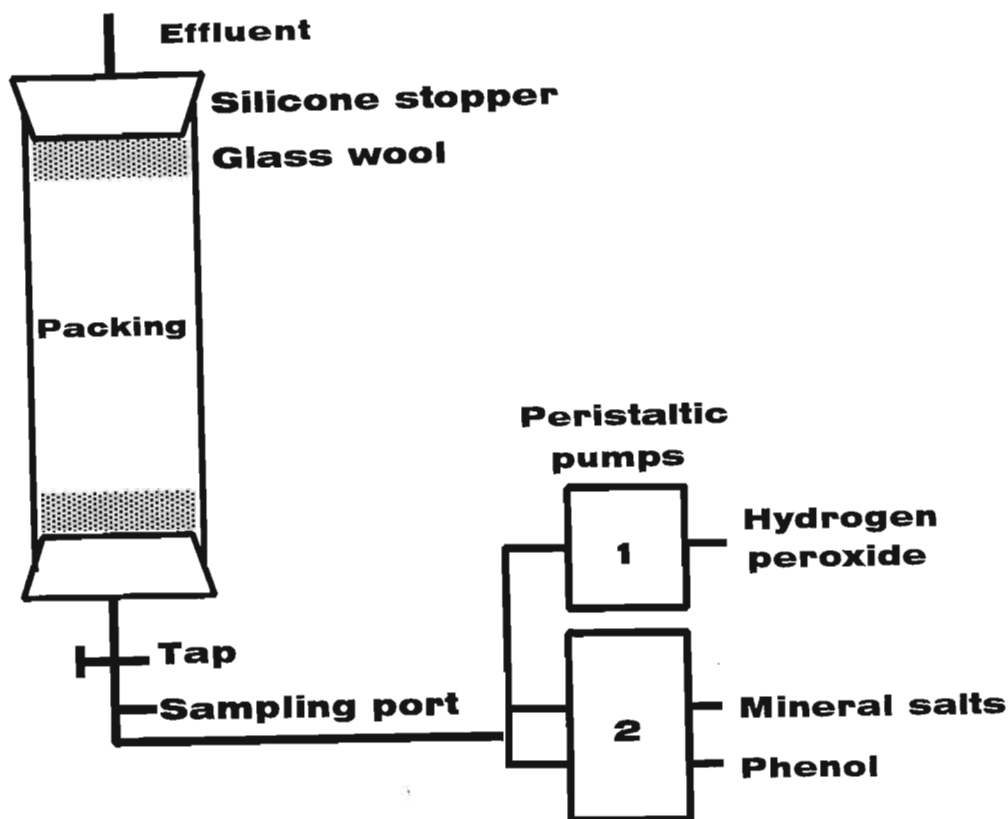
Figure 7.3: Column, pump and reservoir configuration.

All the columns were flushed with liquid medium from the bottom (upwards) in order to expel all air bubbles which were either trapped or produced. The solutions were again pumped from three separate reservoirs. The volume of liquid medium pumped through each column, the influent and effluent concentrations of phenol (UV Spectrophotometer, Philips PYE Unicam SP6-550 UV/VIS) and oxygen (Hanna Instruments HI 91410), together with the optical density of the outflow (A_{590nm}, Milton Roy Spectronic 301 spectrophotometer), were determined daily. An oxygen measuring chamber was constructed for continuous monitoring of the column effluents (Figure 7.4). At the start of each experiment the columns were inoculated (Section 7.1.2) by pumping a microbial suspension into the columns for approximately 30 minutes at a flow rate of 1 ml min^{-1}.
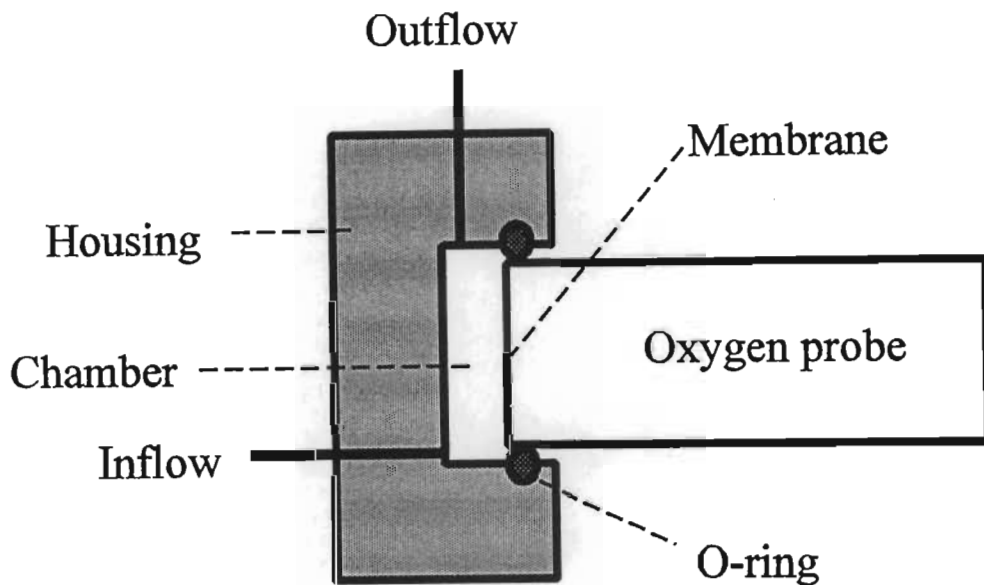
Figure 7.4: Diagrammatic illustration of the oxygen measuring chamber for the continuous flow system.

It is important to note that these constant flow experimental conditions did not simulate actual soil conditions. In real soil conditions it is more likely that constant pressure conditions exist although even these would rarely occur. The main difference between a constant flow and a constant pressure head is that the hydraulic conductivity of the constant flow columns is constant whereas it could possibly decrease due to clogging of pores under constant pressure head conditions (Vandevivere & Baveye, 1992b; 1992c). For these particular experiments, constant flow rates were used to allow constant dilution rates to be established. The fact that this would not occur under actual soil conditions does not detract markedly from the validity of the experiments to determine the effect of porosity on the soil as a bioreactor.

7.1.3 Microbial breakthrough curves

Microbial breakthrough curves were determined for the different packing materials by making use of the column configuration as discussed in Section 7.1.2. A phenol catabolizing association was cultured at 30°C in 5 mM nutrient medium (Section 2.4.2). When sufficient growth had occurred ($A_{590nm} = 0.26$), the microbial suspension was introduced into each column with a peristaltic pump (Watson Marlow 205S) at a flow rate of 1 ml min^{-1}. This relatively fast flow rate was used in order to avoid the potential interference microbial growth could effect in the determination of the filter capacity of the different packing materials. The effluent was collected with a fraction collector (Bromma LKB 2112 Redirac). The optical density readings were plotted against dilution rate rather than flow rate since the pore volume for each column was different. After 10 pore volume changes the columns were flushed with tap water and the effluent collected and analyzed for microbial presence. Microbial desorption was also plotted against pore volume flushes. Microbial breakthrough was determined for 10 pore volume flushes (PVF) only since it was considered unlikely that more PVF would be examined in subsequent column experiments.

7.1.4 Phenol degradation in response to increased column dilution rates

After inoculation of the columns (Section 7.1.3) the flow rates were individually set at rates equivalent to 1.2 pore volume displacements per day. The column effluent phenol concentrations were monitored until a state of equilibrium (or semi-equilibrium) was reached. The dilution rate for each column was increased every 4 days until no further phenol catabolism was observed.

7.1.5 Phenol breakthrough curves as a measurement of pore size distribution

Phenol breakthrough curves were determined with the columns after a period of microbial growth (Section 7.1.3). The curve shapes gave an indication of pore clogging for the different packing materials. The columns subjected to microbial growth and, subsequent, biofilm development (Section 7.1.3) were, firstly, flushed with 3 pore volumes of distilled water to remove excess phenol. A 1mM phenol solution was then introduced into the columns and the outflow concentrations were continuously monitored until they equalled the inflow phenol concentration. The columns were then flushed with 4% (w/v) sodium hypochlorite (Shaw *et al.*, 1985) equivalent to 3 pore volumes at a flow rate of 7ml min^{-1}. This was followed by flushing with distilled water for approximately 10 pore volumes at a rate of 10ml min^{-1}. A 1mM phenol solution was then introduced into the columns while the outflow concentrations were continuously monitored by UV spectrophotometry. Due to its relatively high viscosity it was probable that the sodium hypochlorite did not penetrate into, particularly, the micropores. It was, therefore, assumed (later confirmed) that the sodium hypochlorite treatment induced only partial biofilm removal. To remove any bacteria which were lodged in the micropores the columns were flushed with methanol, which has a low viscosity, for 5 pore volumes (7ml min^{-1}). The optical density (A_{590nm}) of the outflow was continuously monitored to determine whether any further microbial material was dislodged. The methanol was once again replaced by distilled water (5 pore volumes) and the phenol breakthrough curves determined as before. The procedures described here, thus, gave individual breakthrough curves for the columns with biofilm, with partial biofilm and no-biofilm.

7.1.6 Cadmium breakthrough curves

Columns were once again set up as described in Section 7.1.2 after a microbial growth period of 7 days with a flow rate equivalent to 1.2 pore volumes per day. The columns were then flushed with distilled water (1.5 pore volumes) at a flow rate of 220 ml day^{-1}. This was done to remove, particularly, non-adsorbed bacteria from the macropores as well as excessive nutrient elements such as phosphate which could interfere with Cd adsorption by the biofilm

due to precipitation of Cd phosphate. A CdCl$_2$ solution (150 mg l^{-1} Cd) was then pumped through the columns and the outflow Cd concentration monitored by atomic absorption spectrophotometry (Section 2.3.5).

7.1.7 Recovery of Cd-challenged microorganisms

After breakthrough of Cd in all columns was achieved (Section 7.1.6), nutrient medium flow was restored to 1.2 pore volume changes per day. After 2 days, the recovery of phenol degradation capabilities in the different treatment columns was again monitored and plotted against pore volume.

7.1.8 Challenging phenol catabolizers in columns with acetonitrile

Columns were set up as in Section 7.1.2 and maintained at a flow rate equivalent to 1.2 pore volumes per day. Acetonitrile was added to the nutrient and hydrogen peroxide reservoirs in 1% (v/v) increments every 3 days. The effect of acetonitrile concentration on phenol catabolism was monitored until phenol catabolism ceased. At that point the columns were again supplied with the acetonitrile-free medium to determine recovery in phenol catabolism.

7.1.9 Column pressure build up as a function of pore clogging

Columns containing the three packing materials were configured as in Section 7.1.2. In this experiment though, the columns were placed horizontally (Figure 7.5) while a phenol catabolizing microbial suspension (Section 2.5) was pumped through each at a flow rate of 1 ml min^{-1}. The pressure indicator was simply a vertical glass tube (3 mm internal diameter) in which the height of the meniscus was measured. Since the peristaltic pump ensured a constant flow rate, microbial clogging of pores caused increased pressure build up at the inlet end of

each column.
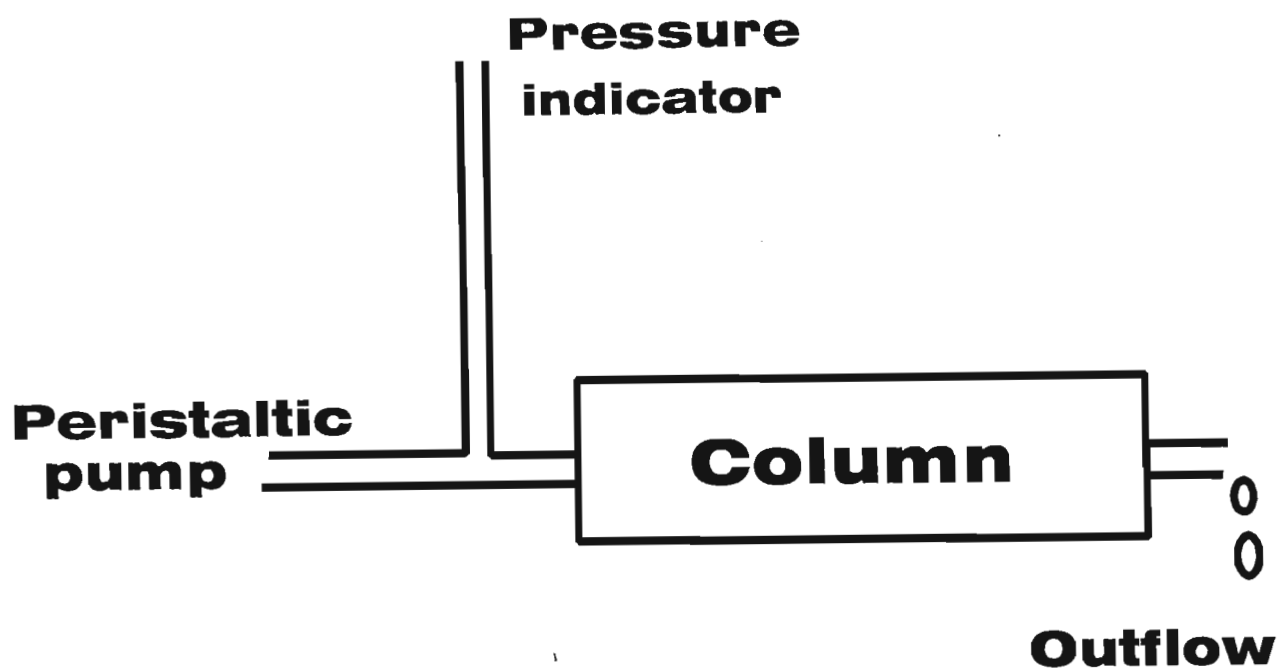
Figure 7.5: Column configuration for the determination of pressure buildup.

7.2   Analytical techniques

7.2.1 Dissolved oxygen measurement

A dissolved oxygen meter (Hanna Instruments HI 91410) was used to determine the concentration of dissolved oxygen at the effluent port of all the columns. The particular model used, has the capacity for continuous dissolved oxygen measurement and for printing readings at desired intervals. Data were directly transferred via an infra-red transmitter (Hanna Instruments HI 9200) to a personal computer for further examination. For the purpose of continuous measurement a chamber was made from PVC into which the oxygen probe was inserted (Figure 7.4). This was necessary to allow continuous measurement of dissolved oxygen in the presence of constant flow through the chamber and absence of atmospheric

interference. Since three different types of packing were used in this study it necessitated the simultaneous measurement of dissolved oxygen of the effluent of each column. Due to the high cost of oxygen meters, a timed switching mechanism was designed to allow the use of a single oxygen meter for measurement of all three columns. A timer was attached to each of the switching solenoids to direct the flow from a particular column to the chamber. Tubing (1mm diameter) leading from the columns to the chamber was kept as short as possible (15 cm) to minimize dead volume. By reducing the chamber volume to 0.7 ml the total dead volume (tubing and chamber) was reduced to 1.8 ml. Although three columns were used the timed switching mechanism was equipped with four channels. The fourth channel was used to determine the influent dissolved oxygen concentration which was maintained at 15 mg l^{-1}.

7.3   Identification of component species of a phenol catabolizing association

A bacterial association capable of phenol catabolism was aerobically cultured and maintained as discussed in Section 2.5. This was used as a source of inoculum in all subsequent experiments. In order to identify the component species of the bacterial association isolates were repeatedly subcultured until four distinct (colony colour and morphology) monocultures were obtained. Two distinct microorganisms were determined and it is suspected that the other colonies were different species and/or strains of the two genera: *Micrococcus* and *Pasteurella*. The following biochemical tests were used for preliminary identification:

***Micrococcus*:**
Gram character: Gram +ve cocci
    Tests:
    Motility   -ve
    Growth in air   +ve
    Catalase   +ve
    Oxidase   -ve
    Glucose   +ve
    O/F Test   Oxidative

***Pasteurella*:**
Gram character: Gram -ve rods
Biochemical tests: API 20E (bioMerieux SA)

Unknown organism:

Gram character: Gram +ve rods

    Tests:

Motility	+ve
Growth in air	+ve
Catalase	+ve
Oxidase	-ve
Glucose	+ve
O/F Test	No result

    API Listeria (bioMerieux SA)

    API CH50 B (bioMerieux SA)

7.4   Thin sections and microscopy

Columns containing the three different packing materials were configured as in Section 7.1.2. Microbial colonization was allowed to proceed for 2 weeks. After incubation the columns were dried at 40°C in the presence of silica gel for 72 hours. The columns were then impregnated with resin and allowed to harden at 110°C for 24 hours. After hardening, the columns were cut and a single 2mm section taken from the centre of each column before mounting onto glass microscope slides. The 2mm sections were ground to approximately 70μm. Thin sections are usually ground to 35μm, in the case of rock samples, but these samples were too brittle for such thin sectioning. The thin sections and loose packing material were viewed with a Nikon Optiphot-POL Petrographic microscope. Subsamples of the packing material were also viewed, before resin impregnation, under SEM (Section 2.7).

The scannig electron micrographs in Plates 7.1 A - C show the multi-layer configuration of the chromatography packing material. These plates also show microbial colonization and give an indication of possible pore blockage which could occur. Plate 7.1 D is an electron micrograph of the acid washed sand surface. This plate also shows bacteria lodged in small depressions on the surface. It is, however, clear that intra-porosity does not exist with this packing material. Despite several attempts it was not possible to capture images of microbial colonization of the pores between individual particles. The destructive nature of SEM sample preparation precluded this. Although integrity of particles could be achieved using fixing resins, these resins obstructed visualizing inter-particle colonization. An interesting result is

that far fewer microorganisms than expected were observed in the micrographs. The relatively low numbers of microorganisms observed in porous media seem to be a common phenomenon in SEM of such materials (Section 3.1.1).

Plates 7.2A&B are photographs of thin sections of the AWS and CHROM packing materials. It was expected that any organic material, such as biofilm growth, would show as dark areas in the pore spaces under polarized light microscopy. This was, however, not the case, probably due to the thickness of the thin sections, as indicated by the high interference colours in particularly Plate 7.2A. The dark areas in Plate 7.2A are air bubbles in the resin and not microbial growth. Although the information from the thin section is limited, it does give an indication of the types of pores and pore shapes which were present in the packing material.

The relative sizes and shapes of the different packing material particles are shown in Plate 7.2. Plate 7.2C shows the loose MIX packing material under polarized light. In this plate the AWS particles are semi-transparent while the chromatography particles are isotropic.
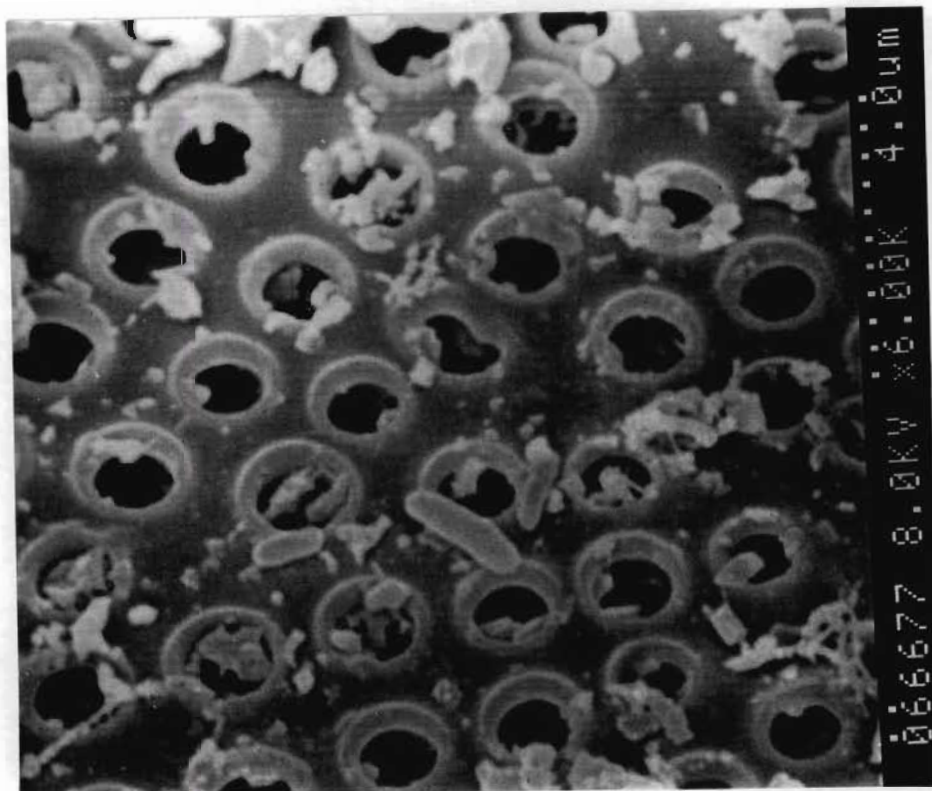
Plate 7.1: Scanning electron micrographs of (A,B) chromatography packing material.
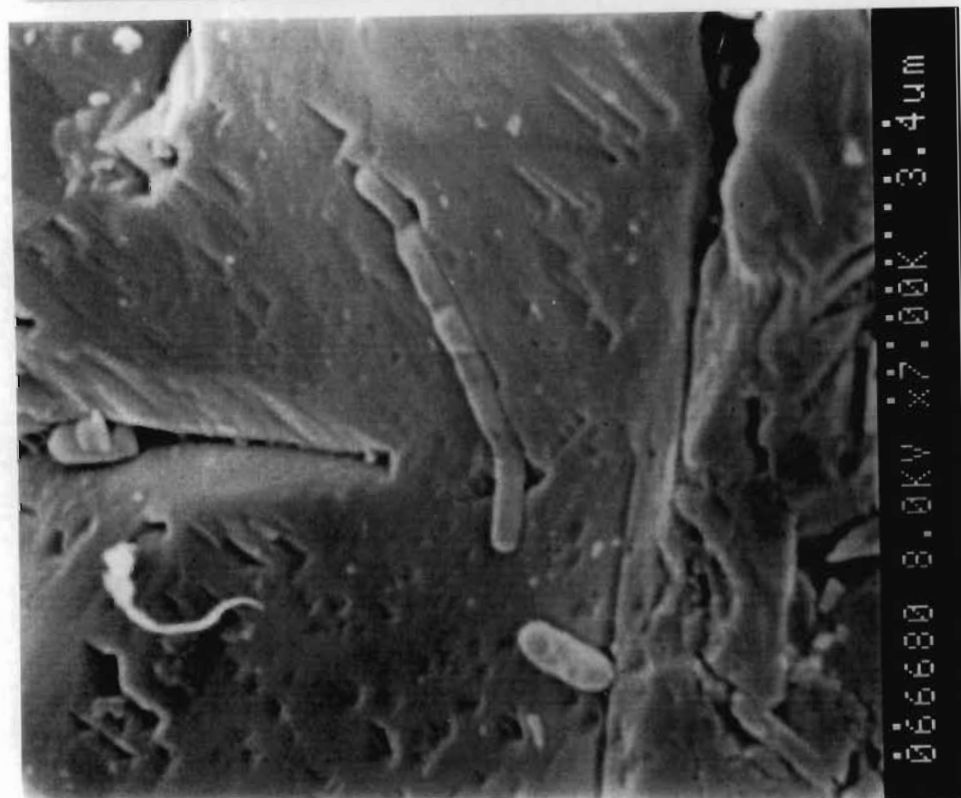
Plate 7.1 (continued): Scanning electron microscopy of (C) chromatography packing material and (D) acid washed sand.
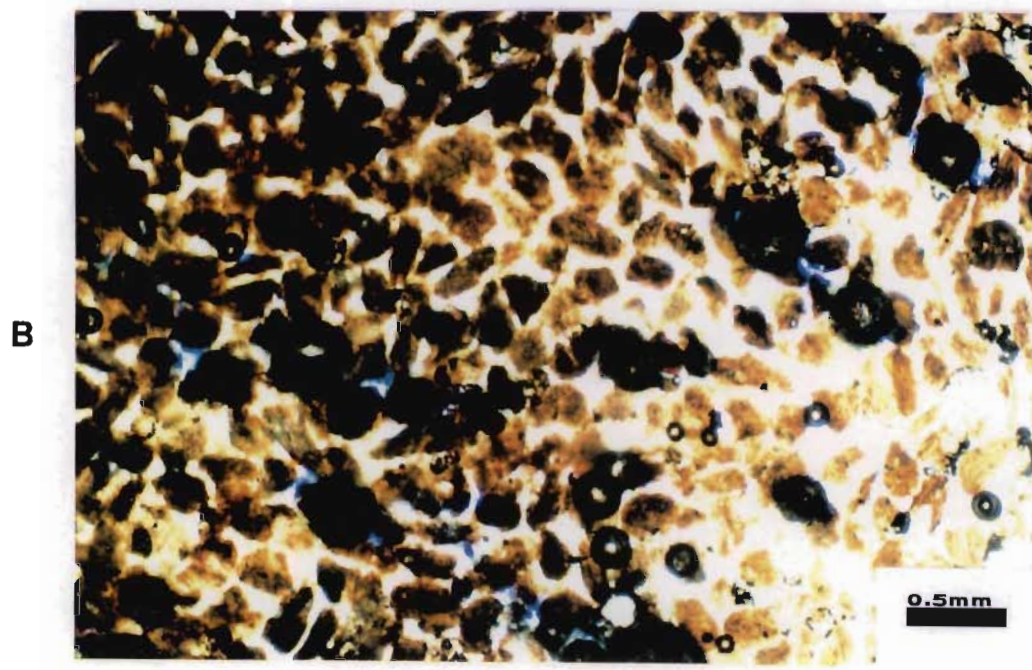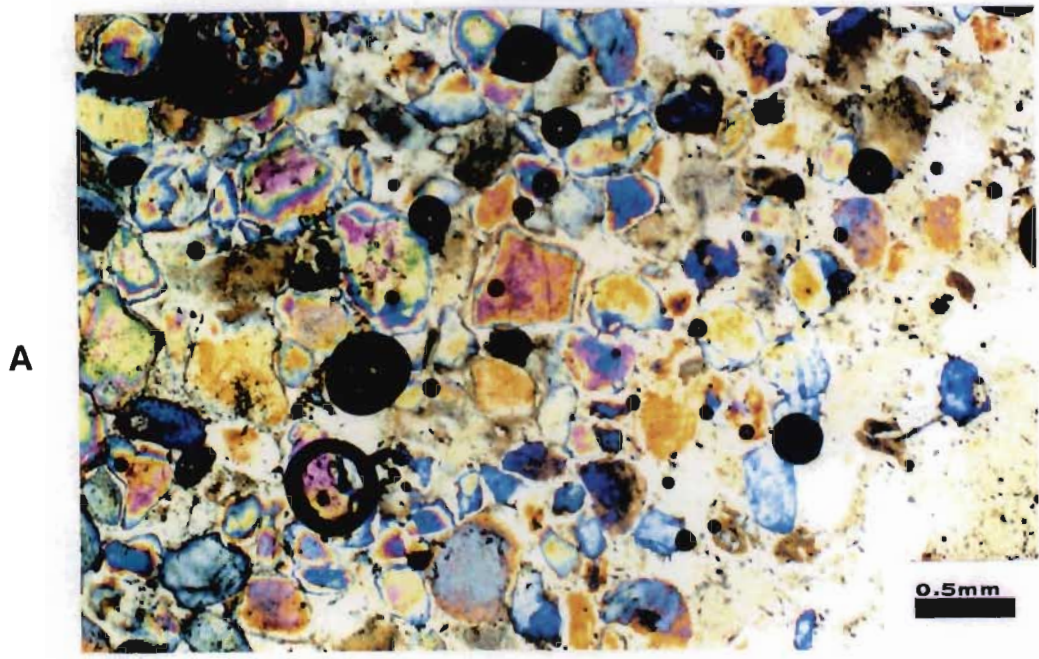
Plate 7.2: Thin section photograph of (A) AWS packing material and (B) CHROM packing material.
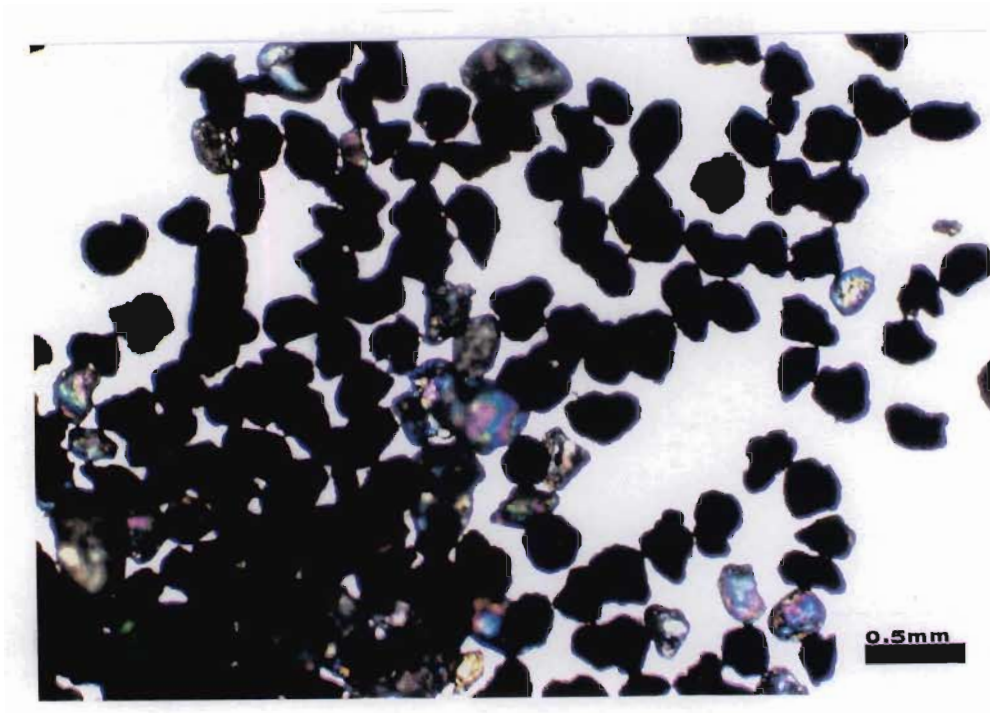
Plate 7.2 (continued): Polarized light photograph of MIX packing material.

CHAPTER 8. RESULTS AND DISCUSSION

8.3   Growth kinetics in continuous culture vessel
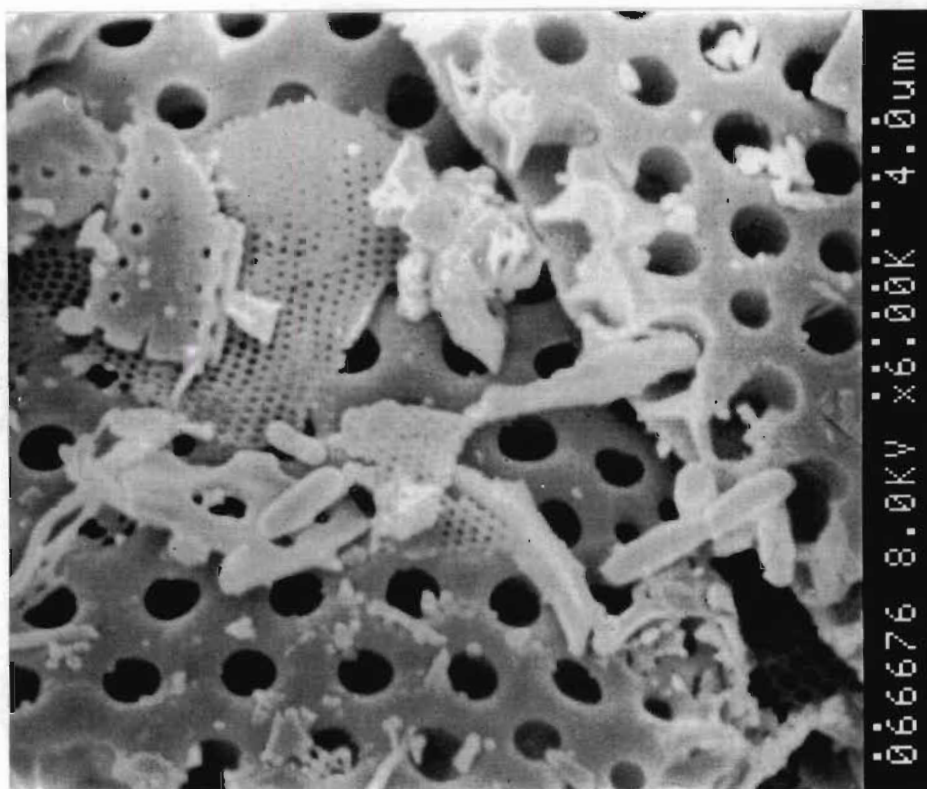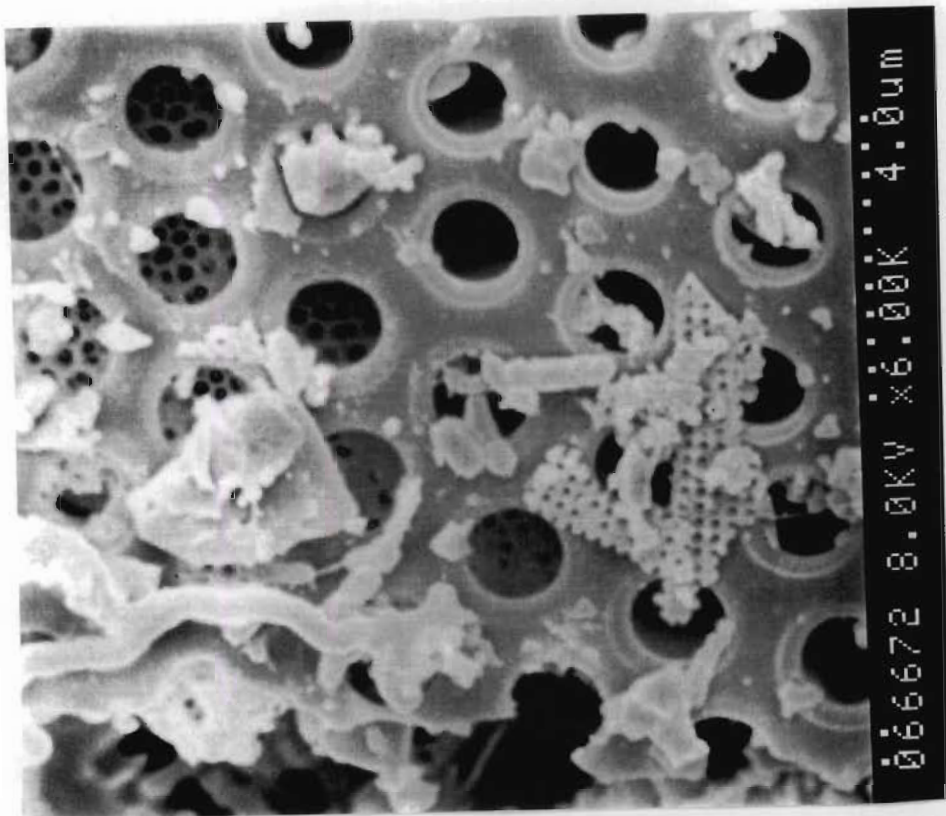
Dilution rates and the interaction with washout of microbial cells is a very important aspect of this study. Particularly useful is the comparison of dilution rate vs substrate degradation in a continuous culture vessel and in porous medium columns. The effect of dilution rate on phenol catabolism can be clearly seen in Figure 8.1. The very low dilution rates (units given in day^{-1} rather than the conventional h^{-1}) were indicative of the relatively slow biodegradation and growth of this phenol catabolizing association. The shape of the curve when compared to that in Figure 6.3 further indicated the low affinity of the bacteria for the substrate. Determination of the growth constants K_s and μ_{max} by the method described in Section 6.3 was made as shown in Figure 8.2. The K_s and μ_{max} values for the association were calculated as 8.2 mM and 0.136 h^{-1}, respectively. These values indicated a low affinity and a low growth rate of the bacteria in the phenol supplemented nutrient medium. These values were used for qualitative comparisons of porous media with the packing-free reactor to distinguish between the effects of different packing materials on growth parameters.

8.2   Microbial breakthrough curves

The microbial breakthrough curves for the different packings (Figure 8.3) show that the different packing materials had different microbial filtration effects. The acid washed sand (AWS) caused the least filtration effect while the chromatography packing (CHROM) showed the greatest microbial filtration. Retention of microbial populations in the columns is of great importance since with greater retention the contact time between the organisms and the migrating pollutant chemicals is increased. This should effect a greater degradation of a particular chemical (Jenkins, 1992), particularly at flow rates approaching D_{Crit}. The greater filtration by the CHROM than the AWS was possibly indicative of both the pore geometry and the pore size. The micropores (i.e. intraparticle pores of the CHROM) possibly accumulated bacteria to a greater extent than the macropores in both this packing and those of the AWS.

141
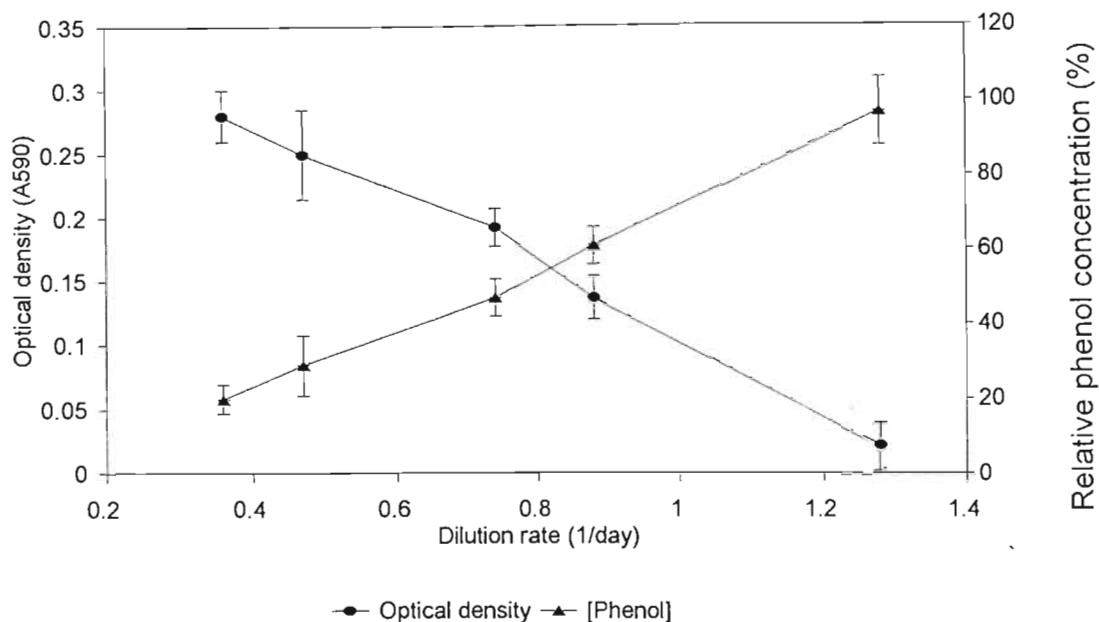
Figure 8.1: Effect of dilution rate on phenol catabolism (expressed as a percentage of the inflow concentration, 5mM) and optical density in an aerobic bioreactor (without packing material).
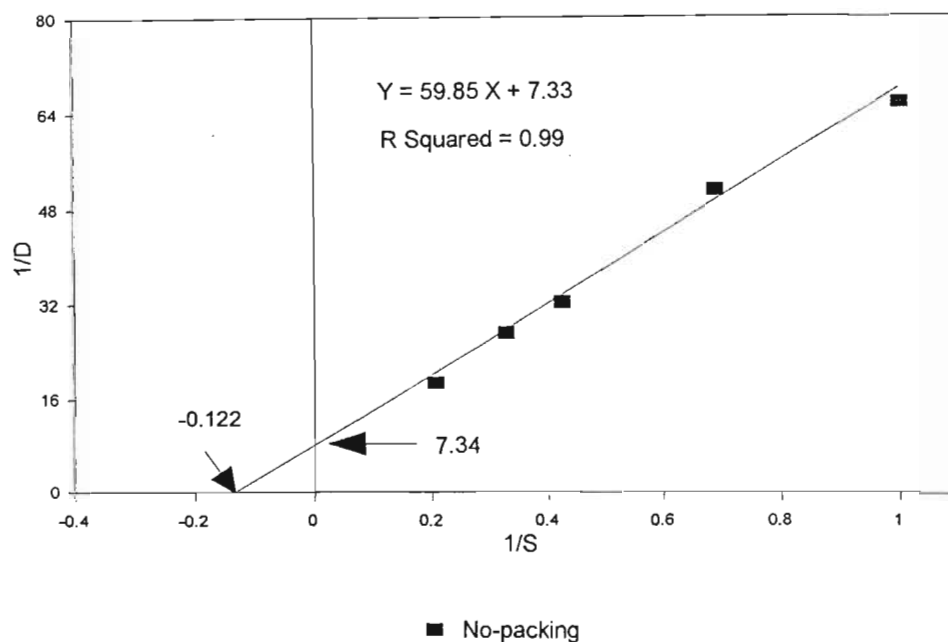
Figure 8.2: Double reciprocal plot for the determination of growth constants in the reactor vessel.
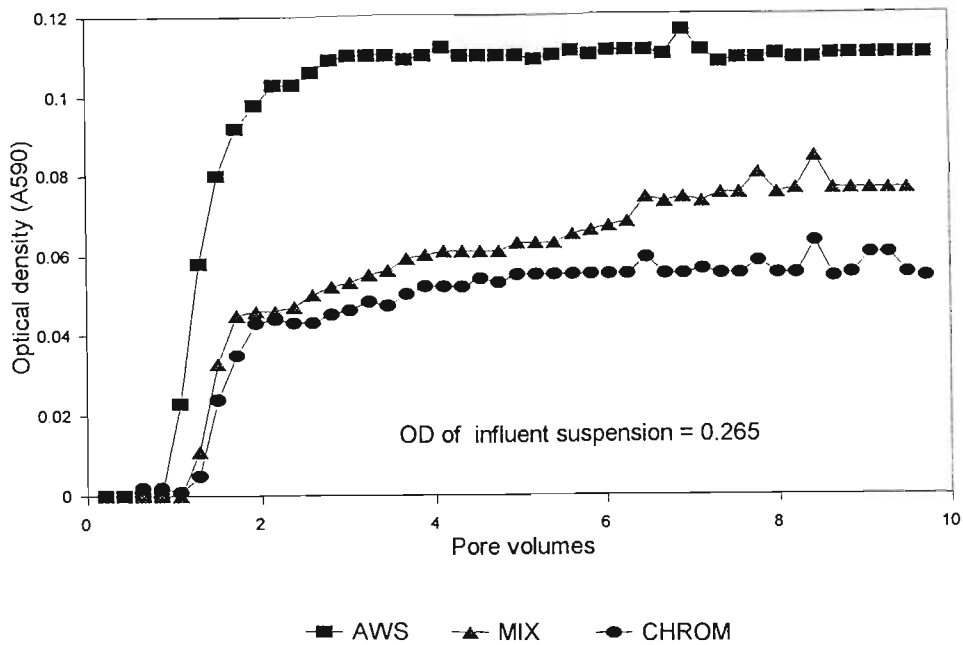
Figure 8.3: Microbial breakthrough curves for the different packing materials.

A slight inaccuracy of this method was that the same flow rate was maintained through the different columns. This caused the actual intrapore velocities to be different with the effect of greater shearing force by liquid movement occurring in the column with the smallest total pore volume. Greater washout from the acid AWS was recorded in the desorption/washout study (Figure 8.4). In the absence of micropore retention it would be expected that the packing material with the greater bacterial filtration (and retention) would take longer to reach total washout. Thus, it seemed strange that the optical density (A_{590nm}) of the bacterial suspension displaced from the CHROM column reached zero before that of the other packing materials. This seemingly strange effect was due to the fact that the AWS has only macropores from which the bacteria are easily displaced (Yates & Yates, 1988). The chromatography packing probably retains few bacteria in the macropores relative to those in the micropores (Section 7.4). For this reason, the bacteria residing in the macropores, in the chromatography packing, were easily displaced while the organisms retained in micropores were only displaced from their positions at a very slow rate giving optical density readings in the effluent below the detection limit. It, thus, created the illusion that no more bacteria were retained on the chromatography packing. The observed secondary washout maximum (Figure 8.4) was also found by other workers although the reason for this is not fully understood (Wollum & Cassel, 1978).
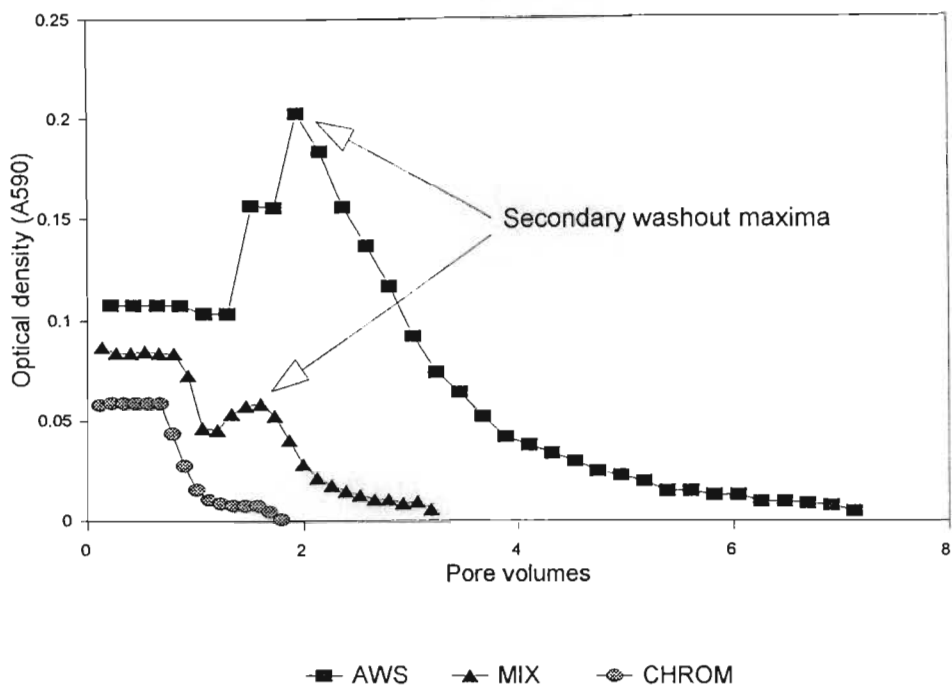
Figure 8.4: Microbial desorption/washout from the different packing materials.

8.3   Phenol degradation in response to increased column dilution rates

Since the experimental reactor was a fixed flow rate system and the total pore volumes for each packing material were different (Table 7.1), direct comparisons of phenol degradation could only be made at equivalent dilution rates. From Figure 8.5 it can be seen that the initial phenol degradation in the columns increased with increased dilution rate from 1 to $5d^{-1}$. This unusual phenomenon was most likely due to clogging of some pores. With low column flow rates it is likely that pore clogging occurred causing higher flow rates (higher pore dilution rates) through the remaining pores. This would account for the relatively low phenol degradation of the columns subjected to low dilution rates. As the column flow rates were increased, and since the remaining pores started restricting flow, the internal hydraulic pressures could have displaced material from the blocked pores (Vandevivere & Baveye, 1992a, 1992b). The displacement could have accounted for the increase in phenol degradation in the AWS column. Pores in the AWS column were mainly limited to macropores which are involved in the mass flow of liquid. The significance of the pore size distribution became
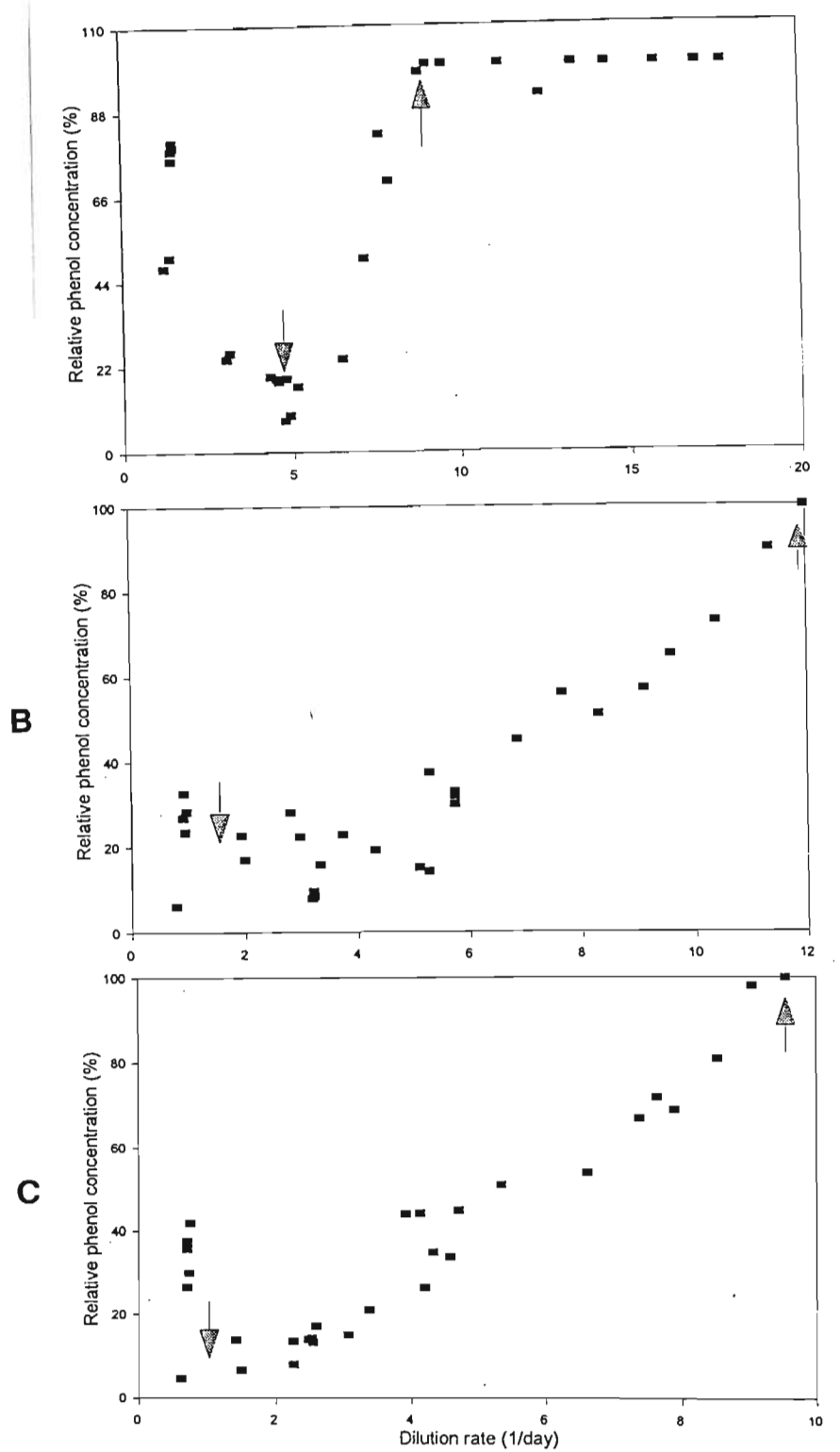
Figure 8.5: Phenol catabolism in the presence of increased dilution rates and AWS (A), MIX (B), and CHROM (C) packing materials.

more apparent when Figure 8.5A was compared with Figure 8.5B. The MIX column did not show the same phenomenon as was observed with the AWS. The greater pore size variation in the MIX column was responsible for the presence of pores participating in mass flow as well as those which were only reached by diffusion. In the case of biofilm development the micropores (not participating in mass flow) are more prone to blockage. Increased dilution rates, in this case, would not effect declogging to the same extent as in the AWS where the pores were mainly macropores. The same phenomenon occurred in all the columns (Figures 8.5 A-C) although to a lesser extent in the MIX and CHROM columns. It should be pointed out that oxygen consumption had a very low correlation with phenol catabolism ($R^2 = 0.43$) and was, therefore, not used for data interpretation in this experiment.

Phenol degradation in the presence of different dilution rates is shown in Figure 8.6. The figure shows an exponential fit of data from Figure 8.5 (between the arrows), excluding the regions of increase of phenol degradation with increased dilution rate. A plot of substrate concentration against dilution rate for the columns, was used as an indication of the affinity of the microbial population for the substrate (Figure 6.3). The curves in Figure 8.6 are indicative of the low affinity of the microbial population for the substrate. Although the K_s value for a particular microbial population is constant, the change in perceived affinity (K_s) is not due to population difference but due to microbial contact with the phenol. The curves in Figure 8.6 indicate higher affinities of the microbial populations held in the AWS and CHROM than in the MIX columns. Again this can be related to the pore size distribution and microbial colonization of the pores. The highest affinity indicated by the AWS curve was probably due to this packing material offering little micropore colonization opportunities for the bacteria. A greater proportion of the total population was, thus, in direct contact with the phenol and, hence, the seemingly higher affinity. The lower affinities of the other two packings were probably due to the presence of micropores for microbial colonization which effected decreased phenol contact with the total population and resulted in a lower perceived affinity. The greater pore size variation of the MIX column compared to the CHROM column was probably responsible for the lower affinity. Thus, the greater preferential flow paths gave more exclusion of micropore colonies and, thus, lower affinity.
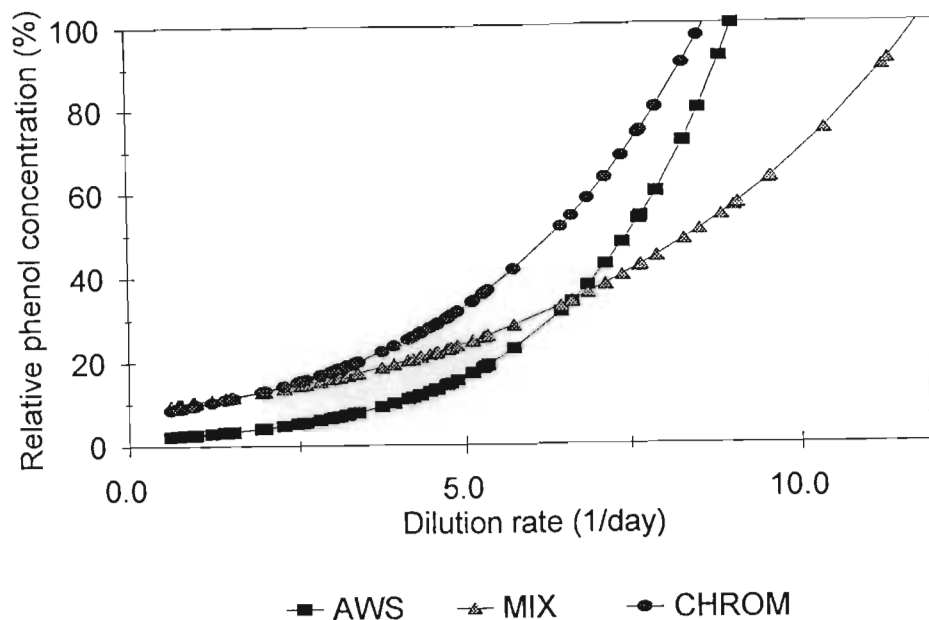
Figure 8.6: Exponential fit and comparison of data in Figure 8.5 (excluding negative slope areas).

Figure 8.7 shows the double reciprocal plots of residual substrate concentrations (effluent concentrations) against the column dilution rates (data points from Figure 8.6). The straight line data points of the AWS were similar to those which would be expected in a bioreactor without packing material (Figure 8.1). The curves represented by the MIX and CHROM data points indicated that there was more than one affinity factor involved. Several factors could, potentially, cause different affinity values (K_s) and, thus, a curve instead of a straight line. One is the presence of different populations with different inherent growth parameters. From Figure 8.1 and the straight line for the AWS in Figure 8.7 it was apparent that this factor was not responsible for the curved lines for the MIX and CHROM columns. If the presence of different populations or a population shift was, indeed, the cause, it would be apparent in the three columns. The most probable explanation for the curved MIX and CHROM lines is microbial colonization of micropores in which substrate contact and, thus, substrate affinity, are vastly different from those of microorganisms in macropores though which mass flow occurs. This possibility was substantiated by subsequent experiments (Sections 8.4 -8.7).

147
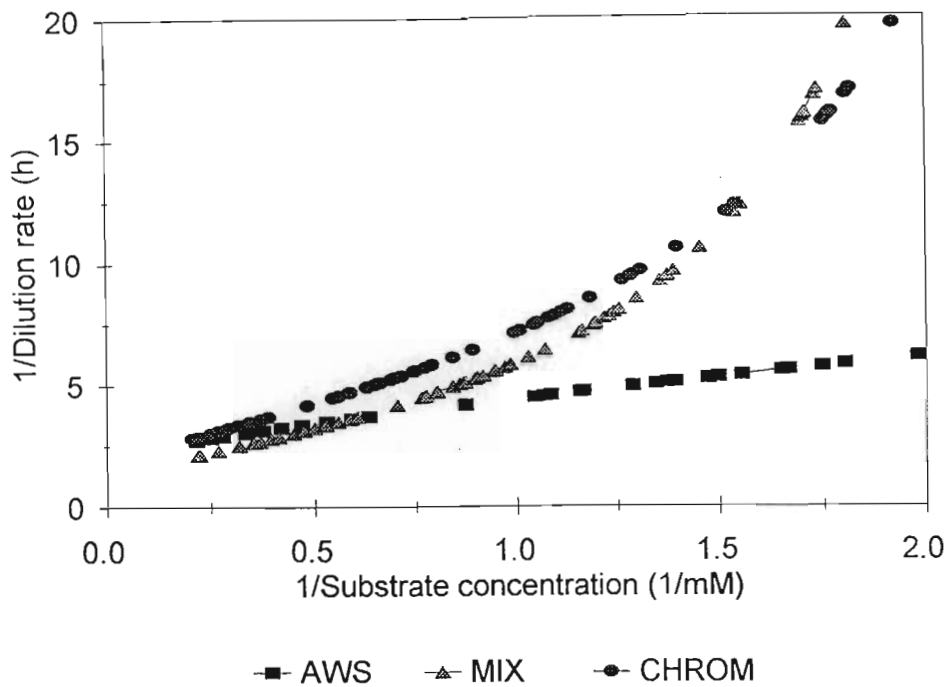
Figure 8.7: Double reciprocal plots for the determination of growth constants in the presence of AWS, MIX and CHROM packing materials.

A comparison of the line slope of AWS of Figure 8.2 with that of Figure 8.7 also revealed interesting results. The slope of the AWS (Figure 8.7) in a 1/Dilution rate vs 1/Substrate plot was much lower than that of the same microbial population in the absence of packing material. If the linear AWS line is extended to the $1/S = 0$ position the intercept would be at approximately 3 which is not too dissimilar to that in Figure 8.2, i.e. 7.34. However, if the AWS line was extended to a point at which $1/D = 0$, the X-axis intercept would be far greater (more negative) than the value of -0.122 in Figure 8.2. The more negative this X-axis intercept the smaller the K_s value, which indicates a higher microbial affinity for the AWS treatment than in the packing-free vessel. It could be argued that this was due to the greater microbial retention or retardation caused by the AWS than would be the case in a packing-free vessel. For this reason several workers have modelled processes in soil by considering the microbial retardation factor of a particular soil as reflected by pore diameters (Gerba et al., 1991; Harvey & Garabedian, 1991; Abu-Ashour et al., 1994). If microbial retardation was, however, the governing factor in regulating affinity lower gradient slopes for the MIX and CHROM columns could be expected. The greater microbial retention in the MIX and CHROM columns (Figure 8.3) would then give lower gradient slopes in the order: Packing-

148

free vessel (no retention) > AWS > MIX > CHROM. From Figure 8.7 it is clear that no part of either the MIX or CHROM slope gradients was lower than that of the AWS. This leads to the conclusion that some other factor, apart from simple microbial retention, e.g. the different colonization niches as discussed above, was the governing process.

8.4 Phenol breakthrough curves as a measurement of pore size distribution

Non-retained solute flow through a tube or pipe, at a flow rate at which no turbulence is caused, should produce a breakthrough curve with a near vertical line from 0 to 100% (Y-axis) at the 1 pore volume (X-axis) position (Figure 8.8A). This is a phenomenon known as plug flow. If the pipe, however, contains a porous medium of some sort the breakthrough curves should resemble those in Figure 8.8B. Plug flow no longer prevails and the solute of interest diffuses into the pores. Macropore flow of a portion of the solute causes detection of the solute before one pore volume has passed through the column (the curve region to the left of A in Figure 8.8B). As the solute outflow concentration approaches the inflow concentration, a certain amount of tailing is evident (position B in Figure 8.8B). This is due to the diffusion of the solute into the micropores. This process will continue until diffusion processes have reached equilibrium, i.e. the concentration of solute in the micropores is equal to that in the macropores where mass flow takes place. This tailing effect is dependent on the relative number of micropores in a particular porous medium and should, thus, increase with greater microporosity (position C in Figure 8.8B). For a non-retained solute, such as phenol on acid washed sand or chromatography packing, the breakthrough curves should intercept the 50% (Y-axis) at the 1 pore volume (X-axis) point (position A in Figure 8.8B). For retained (adsorbed) solutes the 50% (Y-axis) point will be reached at a pore volume greater than 1 depending on the adsorption of the solute on the porous medium.
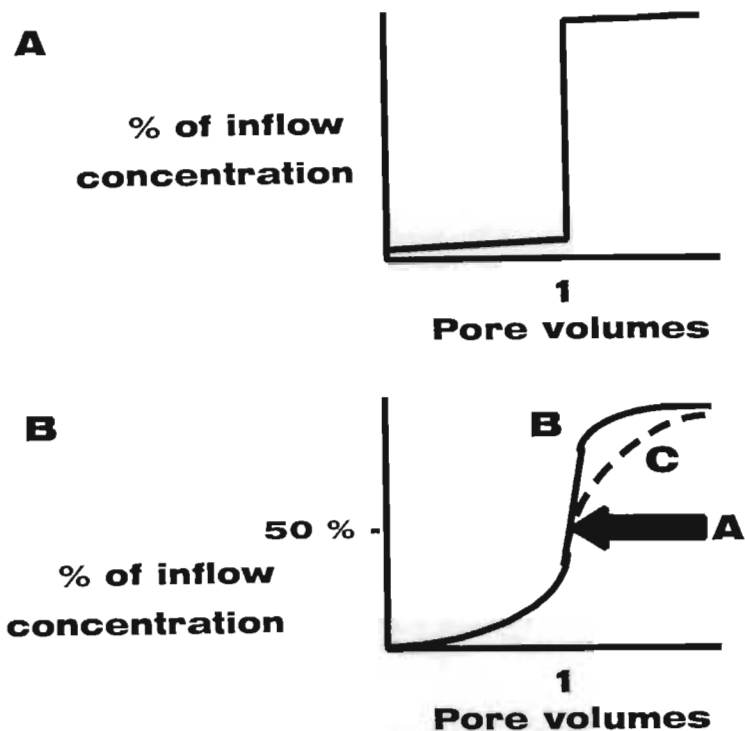
Figure 8.8: Diagrammatic representation of breakthrough curves for (A) plug flow (absence of porous medium) and (B) flow through porous medium.

The results of the breakthrough curves determined in the presence of biofilm, partial biofilm and without biofilm are shown in Figure 8.9. It is clear that phenol was not retained. It must also be pointed out that the data points preceding 0.8 pore volumes show phenol concentrations which can, for all practical purposes, be ignored. These values were caused by residual phenol and other compounds in the columns from the clean up procedures (Section 7.1.5) and should be regarded as background interference of the analytical procedure employed. The greatest amount of solute tailing found in the breakthrough curve of AWS in the presence of biofilm (Figure 8.9A) indicates an increased microporosity due to bacterial growth (Figure 8.8). The curves indicated by "partial biofilm" and "no-biofilm" represent breakthroughs after the sodium hypochlorite and methanol treatments. From these two curves it is clear that most of the biofilm was removed by the first treatment (sodium hypochlorite). The relatively few micropores in the AWS probably allowed the sodium hypochlorite to remove most of the biofilm. Unlike the AWS column (Figure 8.9A), the MIX column (Figure 8.9B) showed decreased microporosity, i.e. more tailing in the "no-biofilm" curve than in the other two curves where the biofilm was present. This indicates blocking of the micropores by microbial growth. There was only a slight difference in tailing between the "partial biofilm" and "with

150

biofilm" curves indicating that the hypochlorite was unable to remove most of the bacteria, thus, confirming preferential growth in the micropores. The results for the MIX column (Figure 8.9B) were echoed in Figure 8.9C for the CHROM column, although to a lesser extent. The most probable reason for the greater differences in "no-biofilm" vs "with biofilm" curves for the MIX column compared with the CHROM columns may be found in the pore size distribution. The MIX column had a greater pore size variation than the CHROM column due to the mixture of acid washed sand and chromatography packing in the former. Microbial blockage of micropores is, thus, to be expected since more macropore alternatives for mass flow would be available in the MIX column. A comparison of the phenol breakthrough curves for the different column packing materials in the absence of biofilm is shown in Figure 8.10A. This figure shows that the MIX and CHROM columns had the greatest microporosity with subsequent diffusion into these pores, resulting in a greater tailing effect in the absence of biofilm. The AWS showed the most rapid breakthrough. In the presence of biofilm (Figure 8.10B) the MIX column showed the most rapid breakthrough. This confirms earlier findings (Section 8.3) that microporosity in the MIX column was reduced by microbial colonization while it was promoted by biofilm development in the AWS.

Figure 8.11 shows the optical densities of the experimental column effluents which are an indication of the bacterial removal by methanol. It is important to note that the columns were flushed with 4% (w/v) sodium hypochlorite and water (Section 7.1.5), until the optical density reached zero, before methanol flushing was initiated. Due to the different pore volumes used for each treatment column, the bacterial populations as estimated by optical density were expressed as a fraction of the pore volume (Figure 8.12). Figure 8.12 shows the cumulative absorbance expressed as a fraction of pore volume. From this comparison it can be deduced that in the MIX column, the microbial biomass occupied a greater portion of the total pore volume (after flushing with sodium hypochlorite and water) than in the two other packing materials. This serves as further evidence of greater microbial colonization in the micropores of the MIX packing.
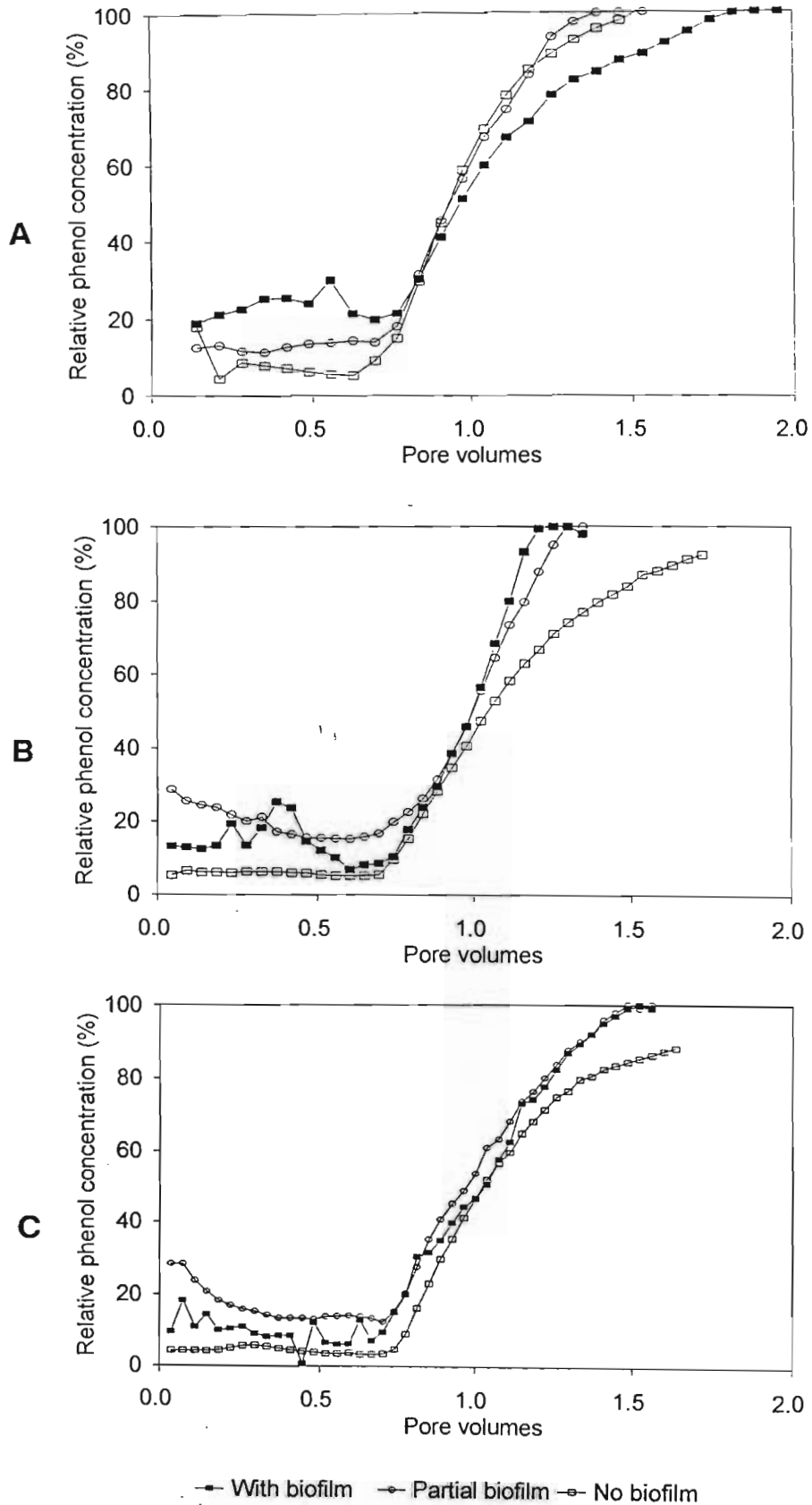
Figure 8.9: Phenol breakthrough curves under different states of biofilm growth in the (A) AWS, (B) MIX and (C) CHROM columns.
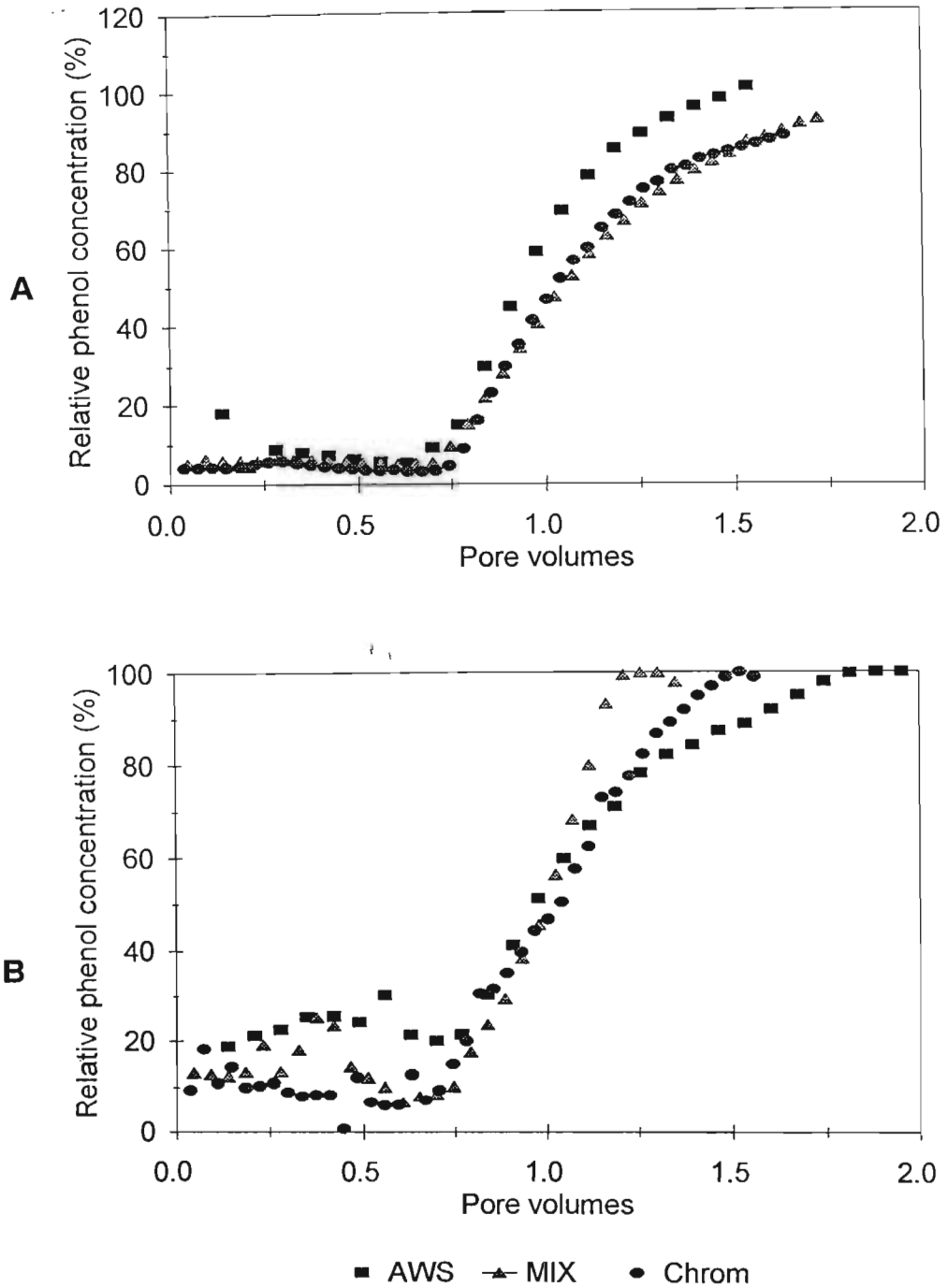
Figure 8.10: Phenol breakthrough comparisons between different column packing materials (A) without biofilm and (B) with biofilm.
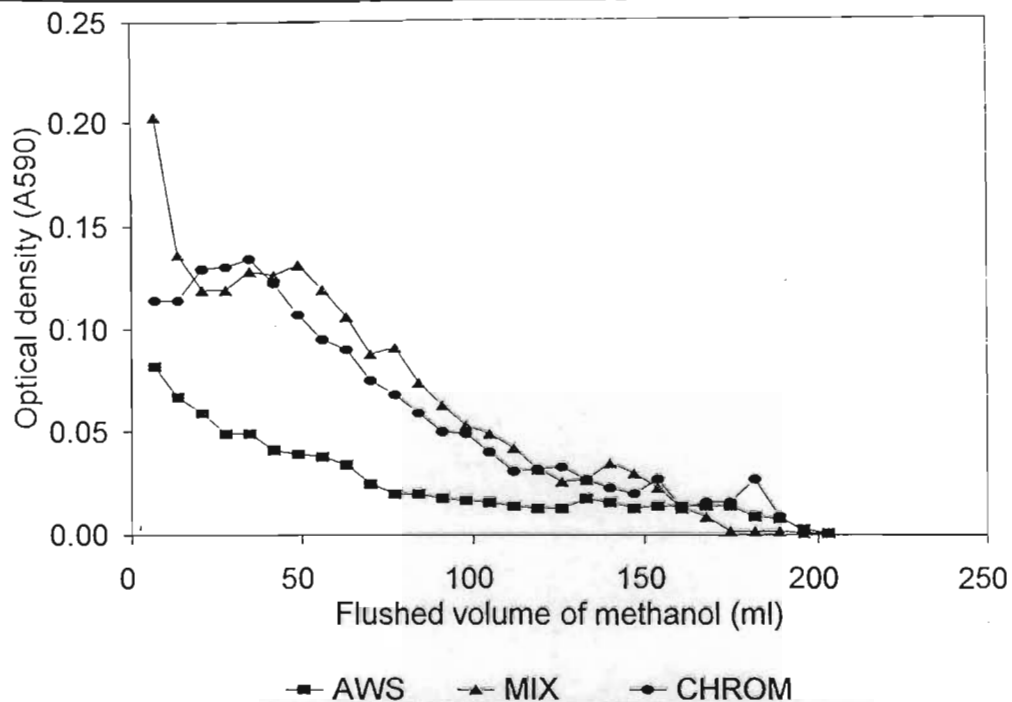
Figure 8.11: Optical density of microbial suspension flushed from columns with methanol after previous partial biofilm removal with sodium hypochlorite.
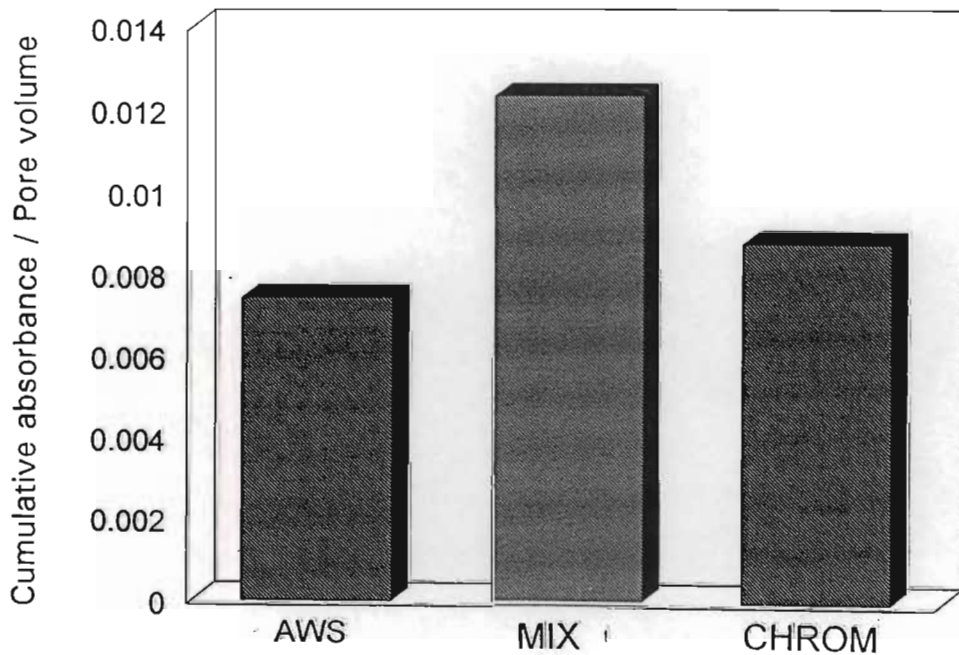
Figure 8.12: Cumulative optical density values (A_{590nm}) of Figure 8.11 expressed as a fraction of pore volumes.

8.5 Cadmium breakthrough curves

It is apparent from Figure 8.13 that the MIX and CHROM columns showed greater retention of Cd than the AWS column. This was most probably due to the greater microbial biomass, and microbial retention (Berthelin *et al.*, 1995), of the MIX and CHROM columns (Sections 7.1.6 and 7.1.7) which facilitate Cd adsorption (Walker *et al.*, 1989; Gagnon *et al.*, 1992). As shown before (Figure 8.9A), the microporosity (and subsequent microbial growth in the micropores) of the MIX and CHROM columns seemed to be much greater than that of the AWS column. This could explain why the microorganisms in some pores were excluded from direct mass flow contact with Cd and, therefore, showed less Cd adsorption. This, in turn, could have affected the functioning of the column as a bioreactor in comparison with the other two columns, as was subsequently observed (Section 8.6). The crossover of the MIX and CHROM curves in Figure 8.13 can be explained by considering both the nature of cation adsorption by microbial cells as well as the microporosity. The CHROM column had a greater percentage of micropores due to the higher percentage of chromatography packing material. The diffusion of Cd into micropores, as indicated by the tailing effect of the CHROM curve, is aided by the electrostatic charge interactions between the cell surfaces and the Cd^{2+} cations (Flemming *et al.*, 1990; Gagnon *et al.*, 1992). The lack of these interactions between phenol and microbial cells was probably responsible for the differences observed between Figures 8.13 and 8.9B. This is further illustrated in Figures 8.14 (A to C). The similarity in breakthrough curves between phenol and cadmium in Figure 8.14A, compared with Figures 8.14 (B and C), could not be fully explained. To some extent, it could be accounted for by the lack of micropore colonization in the AWS and the fact that access to microbial adsorption sites was similar for phenol and cadmium. Given the equal access of phenol and Cd to adsorption sites, Cd adsorption would still be expected to be slightly greater than for phenol, i.e. more than is shown in Figure 8.14A.

It is most likely that a greater percentage of the total biomass was affected by a potential inhibitory substance such as Cd in the packing materials with a low ratio of micro- to macroporosity. This phenomenon was further examined by monitoring the recovery of the column reactors after exposure to the Cd. The recovery of a porous medium with a lower microporosity was expected to be less rapid than that of porous media with higher microporosities since the latter have a greater "supply" of uninhibited (or less inhibited)

bacteria which could reactivate the process. Even though diffusion processes, aided by electrostatic interaction onto cell surfaces, cause migration of Cd into the micropore inhabiting microbial colonies, it is unlikely that the total adsorption of Cd onto these microorganisms would be similar to that of cells in the proximity of Cd mass flow.
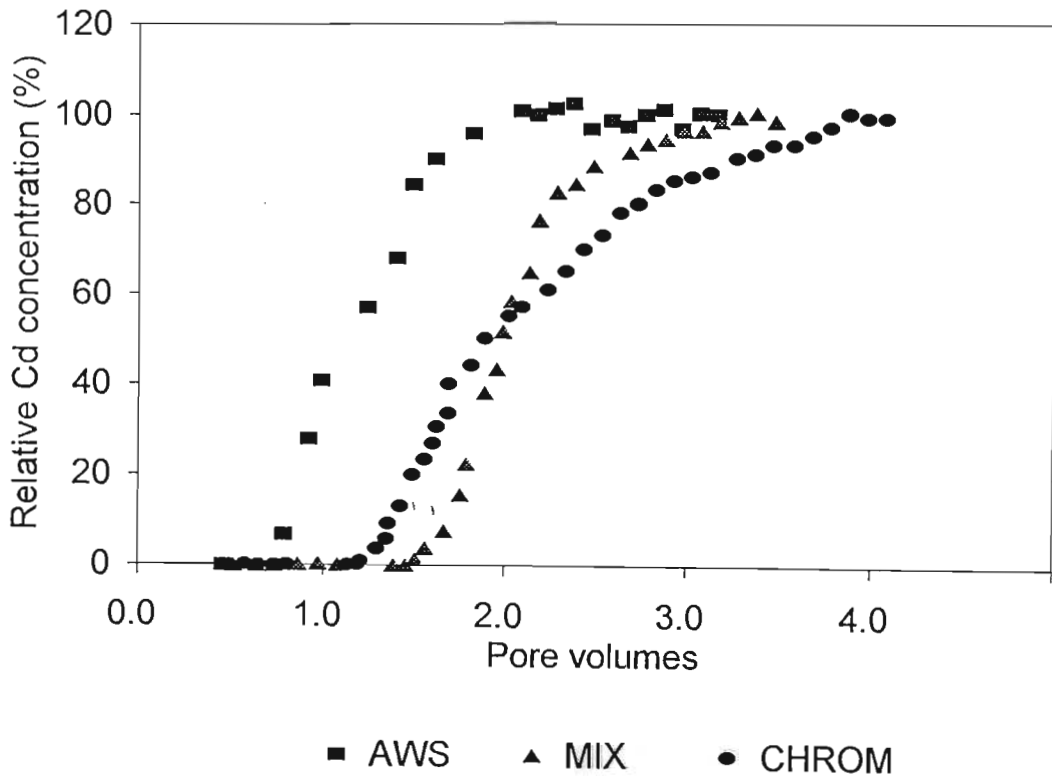
Figure 8.13: Cadmium breakthrough curves for the different packing materials in the presence of microbial biofilm.
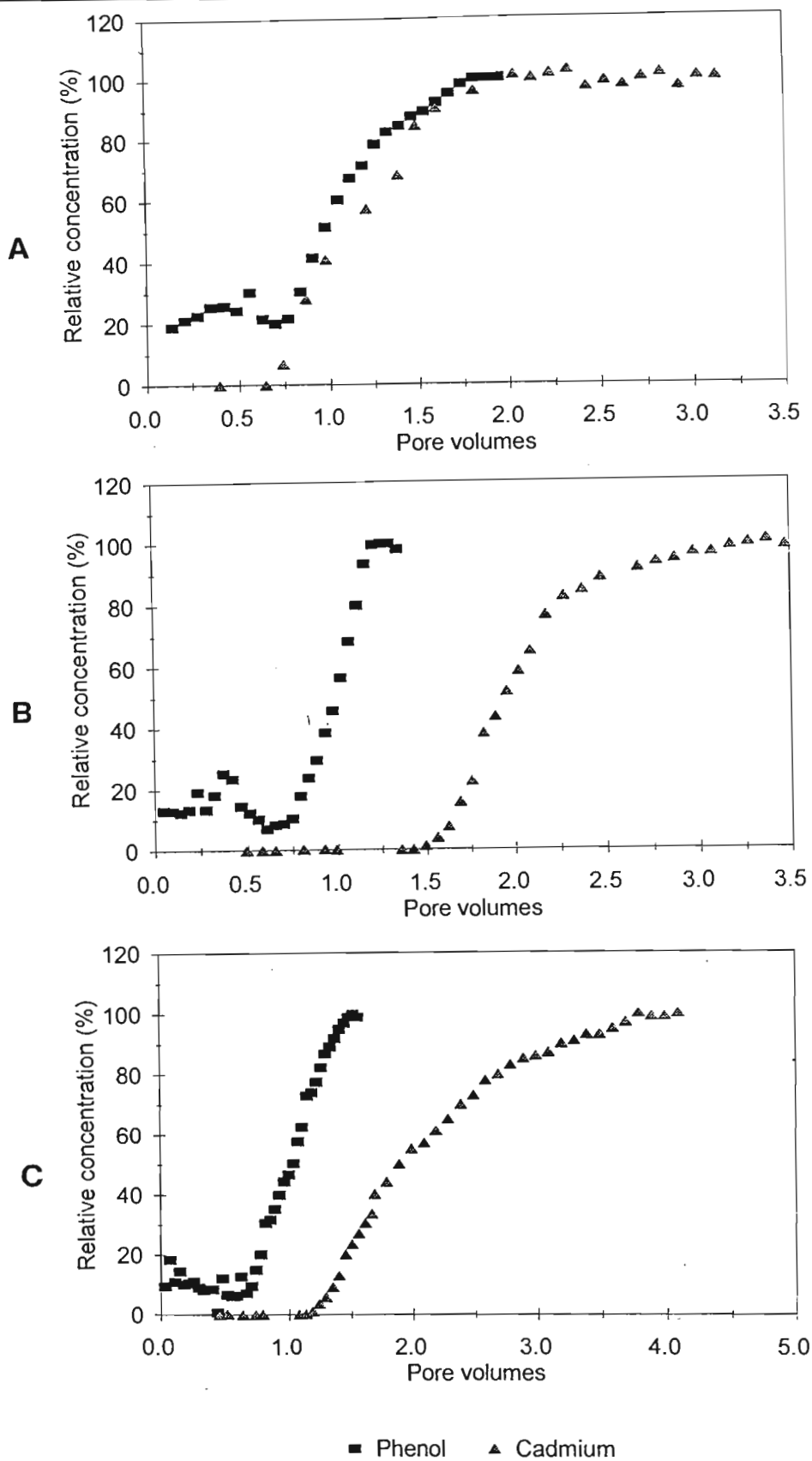
Figure 8.14: Comparison of phenol and cadmium breakthrough curves in the presence of microbial biofilm for the (A) AWS, (B) MIX and (C) CHROM columns.

8.6   Recovery of Cd challenged microorganisms

The recovery of Cd challenged columns is shown in Figures 8.15 (A & B). Recovery in phenol catabolism of the MIX and CHROM columns was found to be more rapid than that of the AWS. This became more pronounced when the recovery was considered in terms of pore volumes (Figure 8.15B). These results corresponded with previous results of micropore colonization (Section 8.4). Micropore colonization caused subsequent protection (or partial protection) against the inhibitory or toxic effect of Cd.
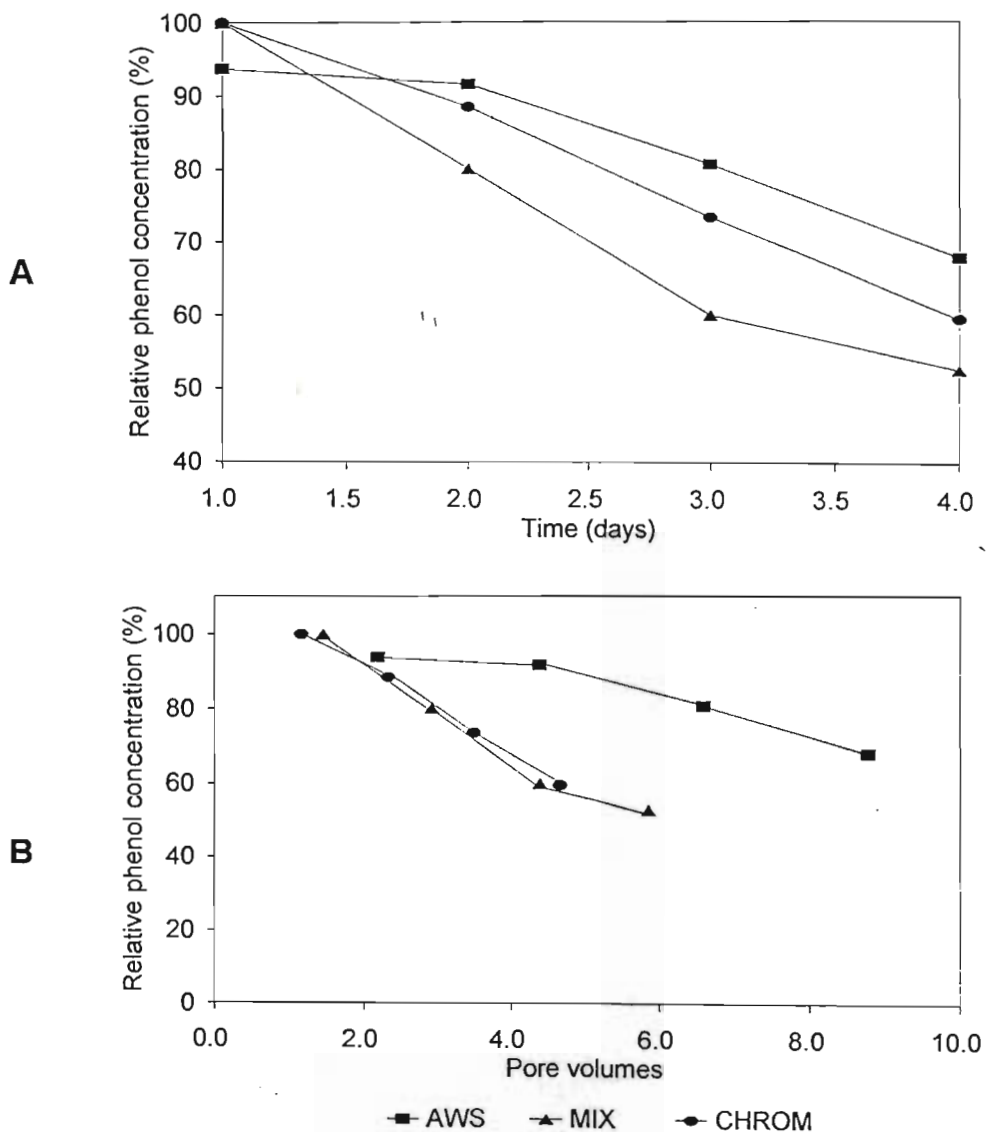
Figure 8.15: Recovery of phenol catabolism after Cd breakthrough. Expressed in (A) days and (B) pore volumes.

The survival of microbial communities in clay soils, in the presence of toxic chemicals, has often been ascribed to the high adsorption affinity of the clay on the metals (Mehlich, 1981; Bohn *et al.*, 1985). The results of this experiment[3], however, showed that adsorption was not the only mechanism involved. Since clay soils have a much greater microporosity (Marshall & Holmes, 1988) than sandy soils the effects of protection by micropore colonization are probably an important contributing factor in microbial survival and recovery.

8.7 Challenging phenol catabolizers in columns with acetonitrile

Microbial recovery in the porous media was further tested by exposing the bacteria to various acetonitrile concentrations. Figure 8.16 shows the influence of increased acetonitrile concentration on phenol catabolism for the different packing materials. From this figure it is clear that phenol catabolism in the MIX treatment was reduced or inhibited at a lower concentration than in the other two treatments. The bacteria in the CHROM column were the most tolerant to increasing acetonitrile concentrations, while the AWS column also seemed more tolerant than the MIX treatment. On careful examination, in relation to assumed microbial growth in the various pores, these results became clear and were consistent with previous findings. Due to the two size fractions of the packing materials in the MIX column it is likely that preferential macropore flow-paths would develop. This effect became more pronounced with increased microbial growth which could, potentially, clog smaller pores. The pores which do allow liquid flow through them will, thus, have a relatively high flow rate and, subsequently, high effective dilution rate. It is likely that inhibition effects would become more pronounced at high dilution rates, which was the most probable explanation for the MIX column having decreased phenol catabolism. The AWS and CHROM treatments, on the other hand, both have fairly homogenous packing material (and less pore size variation) with less chance of preferential flow paths developing. The actual effective pore dilution rate for most pores in these two columns would be less than that of the MIX treatment. The difference in phenol degradation ability as affected by 3 % acetonitrile, between the AWS and CHROM treatments, can be ascribed to the smaller particle size of the CHROM. The smaller particle size gives rise to more potential flow paths resulting in a lower dilution rate for each individual

---

3   *The porous medium used in the present study was inert which, therefore, eliminated the effect of metal adsorption.*

flow path (pore). At a lower pore dilution rate the inhibitory effect of acetonitrile could be expected to be less severe.
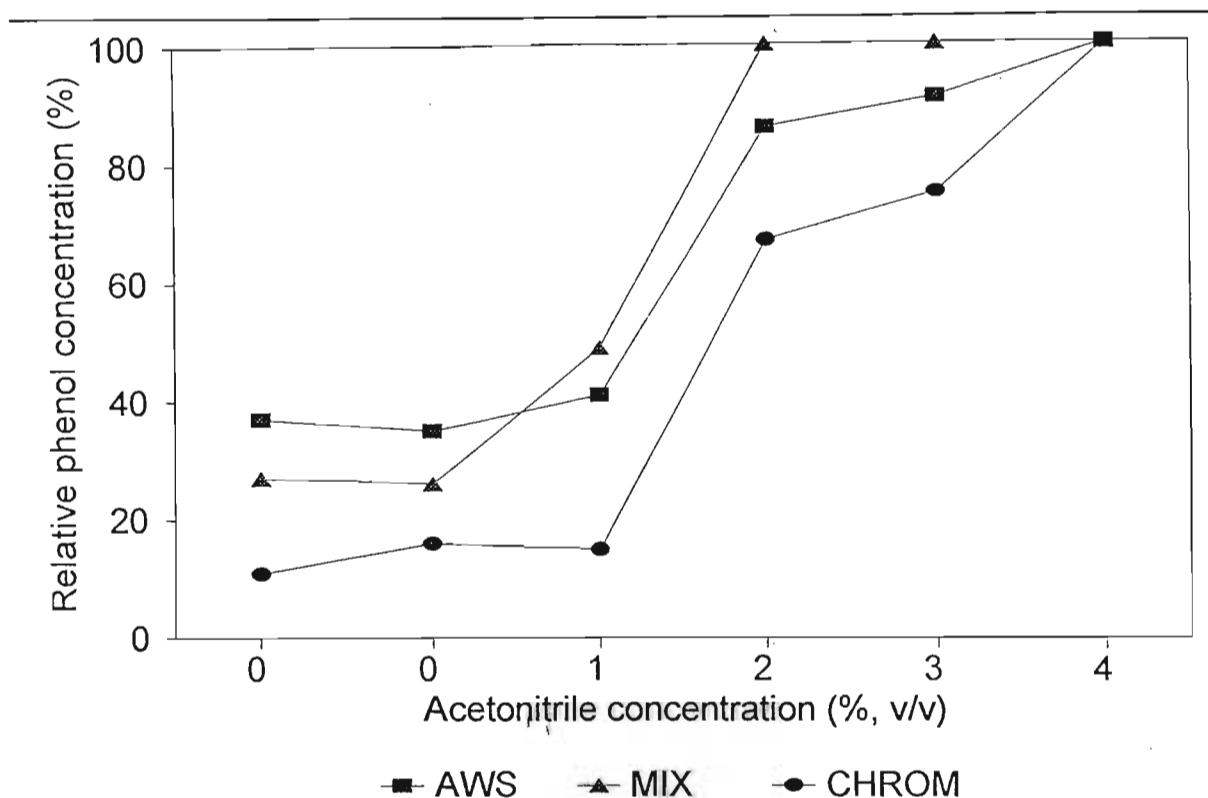
Figure 8.16: Effect of increased concentrations of acetonitrile on phenol degradation efficiency.

The recovery of phenol catabolism in the CHROM and MIX treatments was found to be more rapid than that of the AWS (Figure 8.17). The most probable explanation for this was, again, linked to the population of bacteria in the micropores from which mass flow was excluded. At the acetonitrile concentration (4%, v/v) where phenol degradation was inhibited, cell damage or removal would occur. Cell damage due to potentially toxic concentrations of acetonitrile would occur particularly in those bacteria in close contact (or close proximity) to the pores in which mass flow occurred. Cells lodged in micropores, secluded or shielded from direct mass flow (high dilution rate) would, thus, have been protected from toxic damage. The bacteria lodged in such micropores would, on restoration of optimal growth conditions, contribute considerably to the recovery of the porous system, as was observed in the CHROM and MIX columns.
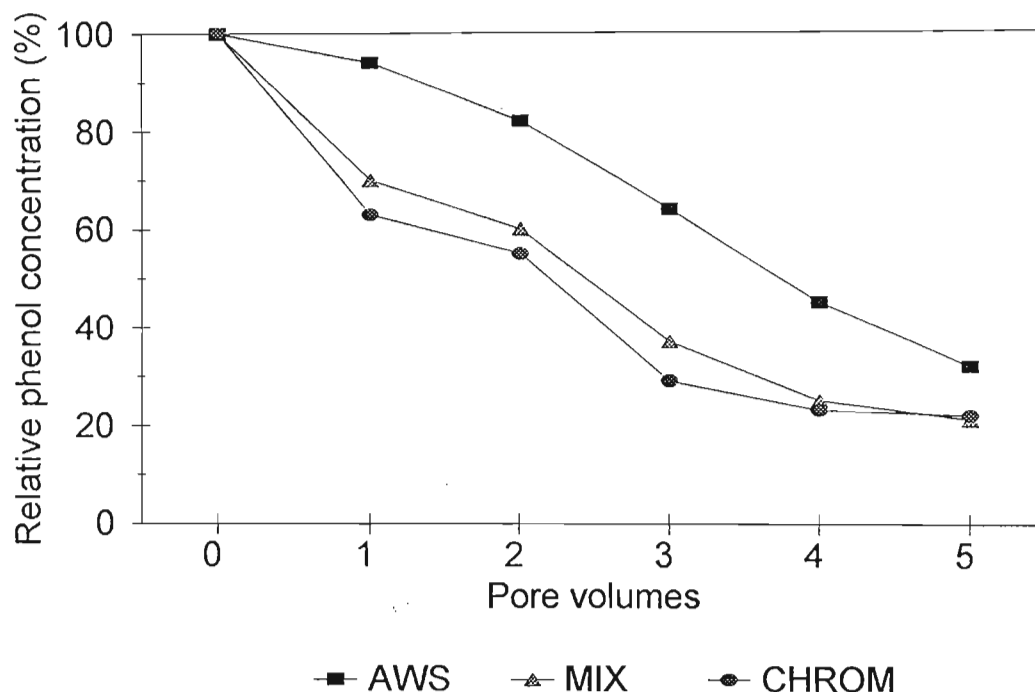
Figure 8.17: Recovery of phenol degradation efficiency following acetonitrile treatment.

8.8 Column pressure build up as a function of pore clogging

The pressure increases for the three packing materials are shown in Figure 8.18. The microbial breakthrough curves in Figure 8.3 show that microbial retention increased in the order: CHROM > MIX > AWS. It could, therefore, be expected that the pressure build up for the columns would follow the same order. This was, however, not the case (Figure 8.18) and the pressure build up was in the reverse order, i.e. AWS > MIX > CHROM. Although the AWS probably contained more pores in the macropore range it is important to note that most pores in this packing material are connected pores, i.e. one pore usually leads into the next via a bottle neck transition. Such pore bottle necks can easily be plugged by microbial filtration effects. In the case of the CHROM and MIX, to a lesser extent, micropores exist within the packing material (Section 7.4) into which microoganism can be channelled. A greater number of pores within the individual particles imply less potential blocking effect of the connected flow paths in the CHROM and MIX than in the AWS with an identical microbial suspension. Although the CHROM and MIX packing materials are capable of retaining greater

microbial numbers they are capable of maintaining flow through certain pores. This is further evidence for the theory that a certain portion of the microbial population was held in pores through which mass flow did not occur. This experiment was conducted under microbial filtration conditions and insufficient time (maximum of 8 hours) was allowed for microbial growth to significantly contribute to this process. It is, however, conceivable that microbial growth would cause even greater colonization of the micropores than was possible with this microbial filtration experiment.
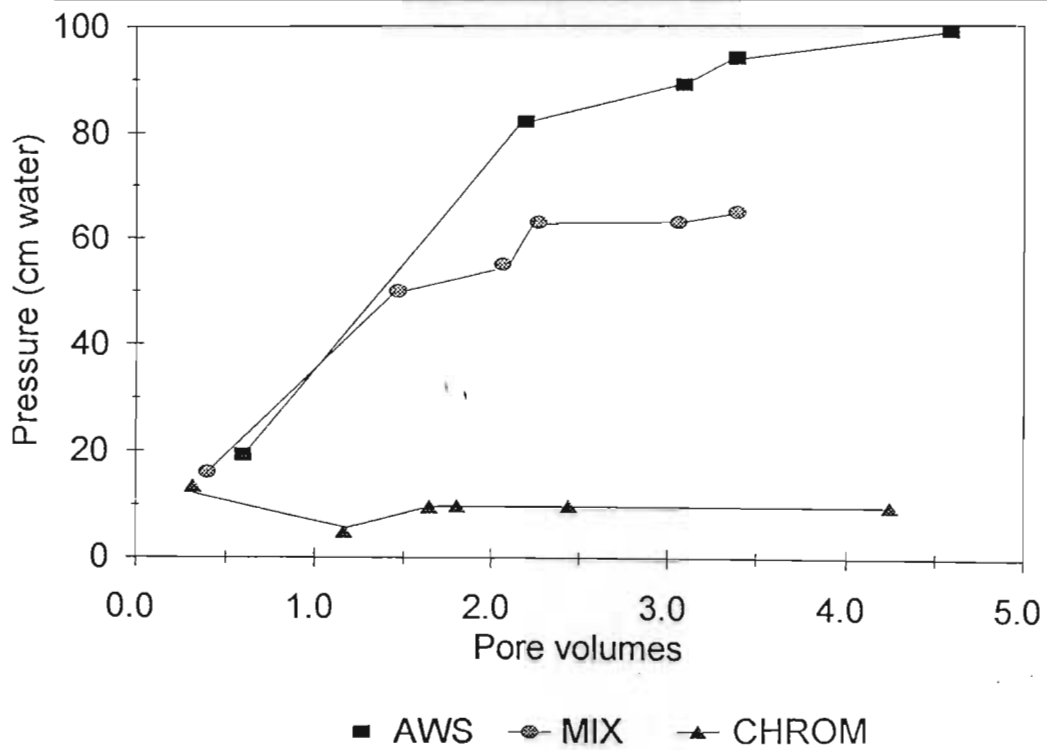
Figure 8.18: Pressure build up in columns due to microbial clogging.

CHAPTER 9. GENERAL DISCUSSION AND CONCLUSIONS OF PART II

9.1 Overview of results

In many ways the present study is unusual. The individual experiments did not provide conclusive evidence for the postulated hypothesis. The rationale followed was to find the most plausible theoretical explanation for each experiment. The fact that the most plausible theoretical explanations for the different experiments are very similar, is the strongest argument for confirmation of the initial hypothesis. The present study has, however, raised many questions which open up new avenues for potential research. The aim of this section is to discuss the potential impacts of these findings under different environmental conditions.

There are, however, conclusions which can be drawn with a reasonable degree of certainty. There was sufficient evidence to suggest that pore size, pore geometry, and pore size distribution did, indeed, have an effect on the soil, when considering it as a bioreactor, under aerobic, saturated, phenol-catabolizing conditions. There was also evidence to suggest that microbial colonization did influence either pore size or pore size distribution or a combination of both these parameters. This was concluded due to the effect of pore colonization on growth parameters and resistance to, and recovery from, growth inhibitory substances (e.g. cadmium and acetonitrile) (Sections 8.3, 8.6 & 8.7).

All of the above findings would have been substantially strengthened by visual electron microscopy evidence of physical clogging of pores at different stages. The three dimensional nature of the mechanism of pore colonization, however, posed serious limitations to existing SEM techniques. Two dimensional transmission electron microscopy (TEM) observations were also made impossible by the fact that the porous media were not naturally coherent which caused loss of integrity upon removal from the column reactors. Although the integrity of the porous media could be retained by impregnation with resins, these created artifacts which confused the interpretations.

9.2 Practical implications of pore colonization

At this point it is important to relate the effects of pore colonization, as found in this study, to possible practical implications. It is obvious that microbial growth in the experimental configuration used here was of much greater magnitude than would be encountered in most soil environments. There are, however, some features of the experimental configuration which could occur in soils, although to a lesser extent. One such feature is that microbial growth and resistance to inhibitory substances are not only affected by the physico-chemical effect of soil surfaces, as often quoted (Stotzky, 1980, Walker *et al.*, 1989), but is also affected by soil porosity. This implies that soils cannot simply be compared on clay content by texture analysis, when using them as bioreactors. Features such as soil structure should also be considered which require the use of *in situ* parameters for predictions. The reason for this is that soil structure development, more so than texture, plays an important role in determining *in situ* pore size distribution and preferential flow paths. These two features play an important role in determining the separation of pore regions, through which mass flow occurs, from non-participating pores which are more prone to microbial colonization. The implications of pore colonization have been clearly demonstrated (Sections 8.2-8.7). In real soil conditions, pore colonization would probably take a considerable time to establish. As shown in this study, the effects associated with this phenomenon should, therefore, not be assumed to be operational immediately after induction of bioremediation processes.

9.2.1 Enumeration of soil microorganisms

The results of this study also have implications for the interpretation of soil microbial enumeration attempts. Microbial enumeration of soil microorganisms is often used to determine the effects of certain treatments such as fertilization or supplementation with potentially hazardous compounds (Hiroki, 1994). The methods of enumeration employ extraction techniques which disrupt soil structure and porosity. The differences in activity between organisms occupying different pore types are negated by this type of procedure. The following scenario can be used as an example. Assume that soil A has very little pore colonization and that most bacteria are in close contact with the migrating compounds in the soil water. Soil B, however, has a structure and porosity which is such that it is conducive to

micropore colonization. A smaller proportion of the total microbial population is, therefore, in close contact with the migrating compounds in the soil water than is the case with soil A. Assume that the compounds dissolved in the soil water are toxic to the organisms and that they caused a 50% decrease in the total microbial numbers in each soil. Without considering the results of this study, it could be deduced that the toxic compounds in the soil water had an equal effect on the microbial population in both soils. If the results of this study are, however, taken into account, it could be argued that the overall effect was not similar in both soils. A greater proportion of the organisms in soil B could be assumed to be somewhat protected from the toxic chemicals in solution, due to pore colonization, than in soil A. It then follows that a 50% reduction in the microbial population in soil B implies a greater detrimental effect than a 50% reduction of the less protected population in soil A. Although this is a simplified example where all factors are not considered it illustrates the implications of this study for microbial enumeration.

9.2.2 Soil inoculation

Pore colonization also has implications for soil inoculation which is often used in bioremediation (Paul & Clark, 1989). Soil inoculation for bioremediation is often accompanied by irrigation and the addition of mineral nutrients and electron acceptors. If the results of this study are applied, inoculation concentrations should not be calculated from contaminant, and microbial adsorption data alone. Pore colonization, as well as the fact that it probably occurs to different degrees in different soils, should also be considered. Although most microbial transport models view pore colonization as a potential sink for supplemented and migrating inoculum (Yates & Yates, 1988), the subsequent effect of pore colonization on microbial activity is not considered. An additional interesting phenomenon which seriously affects the performance of microbial supplements to soil is protozoan predation (Heynen *et al.*, 1988). Pore colonization could, potentially, inhibit protozoan predation. Because this process is time dependent, soil- supplemented organisms are more prone to predation than indigenous microorganisms.

165

9.2.3 "The most suitable soil?"

One of the questions often asked when considering soil environmental risk assessment, soil bioremediation, landfill covering, landfarming or soil biofiltration is: "What is the most suitable soil?" Considerations such as aeration, hydraulic conductivity, presence of an appropriate microbial population, soil adsorption effects, the nutritional status, pH etc. are often used in making such decisions (Wilson & Jones, 1993). This discussion will, however, be limited to the effects of pore colonization since the effect of the above mentioned factors are well documented.

All other factors being equal, under saturated conditions, a soil with greater microporosity and microbial colonization of such pores would probably show lower rates of biodegradation of catabolizable compounds. The main reason for this is the reduced contact between the microorganisms within the colonized pore areas and the migrating (mainly through macropores) compounds. The situation for unsaturated soil conditions is, however, likely to be reversed. Micropores retain an increasing proportion of the total soil solution as the soil becomes dryer. It then follows that if a major proportion of the soil bacteria, under unsaturated conditions, is present in these pores, a soil with a greater number of such pores will show greater biodegradation of an organic compound. Microbial populations in soils which allow significant micropore colonization will also probably be more resistant to, and will show more rapid recovery after, the challenges of hazardous chemicals.

9.3   Parameters for predicting pore colonization phenomena

It is clear that pore colonization did, indeed, have certain effects on the behaviour of a particular porous medium as a bioreactor (Sections 8.3-8.7). It is, therefore, essential to be able to predict under which conditions this will apply in real soils and which parameters should be used for such predictions. From the present study, the following suggestions are made as a means of assessing the extent to which micropore colonization, with its subsequent ramifications, will occur under real soil conditions. In order for the phenomenon to occur it is essential for a soil to have a suitable porosity. The pore size distribution of soil (or any porous medium) is best determined by the use of water retention curves (Marshall & Holmes,

1988). These curves show the amount of water retained at different matric potentials, which is a function of (micro)porosity. It does not, however, automatically follow from the presence of pores suitable for colonization, that this phenomenon will occur. Colonization is further dependent on the soil nutrient status and other environmental factors as well as microorganism-specific factors. Water retention curves could, possibly, also be used to determine the extent to which pore colonization under a particular set of conditions has occurred. As discussed in Section 8.4 microporosity was induced by microbial colonization in the AWS (and this could also be the case in many sandy soils), while microporosity was reduced in the CHROM and MIX (as could occur in finer textured soils). The increased or decreased porosity due to these phenomena and the subsequent effects can be inferred, although not quantified, by water retention curves since changes in porosity affect water holding capacity.

A second method for determining the extent to which micropore colonization occurs in real soil conditions would be to determine breakthrough curves (Section 8.4) before and after the onset of the conditions suspected of causing microbial growth.

9.4   Hydrophobic molecules

This study and related discussions have dealt, as far as porosity is concerned, mainly with a water soluble compound i.e. phenol. Since soil pollutants are often non-polar and, thus, hydrophobic compounds with low water solubilities (Nyer *et al.*, 1993), the implications and potential applications of the phenomena found in this study, should be considered. One of the main findings of this study was that a single affinity factor (K_s) could not be used to define the affinity of a soil microbial population for a particular organic compound (Section 8.3). This was particularly true under those soil conditions where pore colonization occurred to such an extent that physical contact between the compound of interest and these organisms was limited to diffusion (Section 8.3). The further a certain section of the population was physically removed from the participating macropore flow, the lower the perceived affinity became. If this theoretical basis is used for extrapolation, one would have to conclude that this effect would be reduced when using hydrophobic molecules. Hydrophobic molecules have, by definition, a low affinity for the water phase. This causes them to be adsorbed to soil and

167

other surfaces as a result of repulsion from the water phase. Although soil adsorption of these molecules often occurs (Reddy & Locke, 1994), this adsorption is increased when more hydrophobic surfaces are available (Paul & Clark, 1989; Scheunert & Mansor, 1992). The non-polar cell wall constituents of many microorganisms (Van Loosdrecht *et al.*, 1987a, 1987b), thus, will facilitate this adsorption process. In the case of a hydrophilic organic molecule the movement of the molecule into the colonized micropore is mainly diffusion dependent. For hydrophobic molecules the diffusion process is aided by the adsorption onto microbial cell wall constituents.

9.5   Proposed modifications to mathematical models

A number of mathematical models have been developed which combine unstructured growth kinetics with the transport of bioactive components in groundwater systems. These models can be broadly grouped into two categories : (1) those that include mass transfer limitations between the fluid phase and the microorganisms (referred to as multi-phase models), and (2) those that assume that the mass transfer limitation between the biomass and the aqueous phase can be neglected (referred to as single phase models) (Wood *et al.*, 1994). The multiphase models separate the biomass and aqueous phase and assume that there is a mass transfer limitation between them (Chen *et al.*, 1992). Most models incorporate a microbial kinetics component which is incorporated into the more sophisticated transport component of the model (Wood *et al.*, 1994). Models used to describe microbial kinetics can be categorized as being either structured or unstructured. Structured models are generally more mechanistic and represent physiological changes in the cell by expressing the kinetics in terms of variables that are both internal (e.g. concentrations of enzymes, DNA, RNA, ATP) and external (e.g. environmental variables) to the cell. Unstructured models describe microbial growth as a function of environmental variables and cell concentrations only, and internal changes in cellular biochemistry are not accounted for in a mechanistic sense. Application of structured models tends to be somewhat complicated, however. Models applied to problems in the environment have been mainly of the unstructured type.

As shown in Section 6.5 most models include a Monod-type term where K_s, μ and specific growth rate parameters are used. These parameters are mostly determined in liquid culture or

porous media that are totally dissimilar to those to which the models are to be applied (Wood *et al.*, 1994). The use of a single K_s value for these models (Moldrup *et al.*, 1994) was shown to be invalid (Section 8.3). It is postulated that the accuracy of models can be improved by determining K_s factors in the actual porous media under discussion. The change in K_s caused by micropore colonization should also be taken into account (Section 8.3). If this recommendation is followed it will probably lead to models which incorporate multiple K_s values for each substrate of interest and which will be dependent on micropore colonization.

9.6   Future work

Recent work with the use of etched glass offers much potential for facilitating visual observation of pore colonization (A.B. Cunningham - Oral presentation at the *In Situ* and On-Site Bioreclamation Symposium, April 1995, San Diego, California). These techniques involve laser etching of glass slides to very precisely create macro- and micropores (or connected- and dead-end pores). These slides are covered with a second glass slide to give a transparent, three dimensional configuration of pores. This is used as a porous growth chamber where the flow rates, substrate concentrations and pore colonization can be accurately monitored. The use of image analysis with this technique further increases the potential information to be derived by this method.

Further work could also include the determination of pore colonization in real soils and the use of a greater variety of molecules. It would also be necessary to determine the extent to which the phenomenon occurs under anaerobic and low nutrient environments, as are found in soils.

REFERENCES

Abu-Ashour, J., Joy, D.M., Lee, H., Whiteley, H.R. & Zelin, S. 1994. Transport of microorganisms through soil. Water, Air and Soil Pollution 75, 141-158.

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Science 211, 132-138.

Andreux, F., Portal, J.M., Schiavon, M., Bertin, G. & Barriuso, E. 1991. The usefulness of humus fractionation methods in studies about the behaviour of pollutants in soils. Toxicological and Environmental Chemistry 31/32, 29-38.

Atlas, R.M. & Bartha, R. 1987. Microbial Ecology (Second Edition). Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.

Bailey, S.W. 1980. Summary of recommendations of AIPEA nomenclature committee. Clays and Clay Minerals 28, 73-78.

Barriault, D. & Sylvestre, M. 1993. Factors affecting PCB degradation by an implanted bacterial strain in soil microcosms. Canadian Journal Microbiology, 39, 594-602.

Bar-Or, Y. 1990. The effect of adhesion on survival and growth of microorganisms. Experientia 46, 823-826.

Berthelin, J., Munier-Lamy, C. & Leyval, C. 1995. Effect of microorganisms on mobility of heavy metals in soils. In P.M. Huang, J. Berthelin, J-M. Bollag, W.B. McGill & A.L. Page (eds), Environmental Impact of Soil Component Interactions, Lewis, London, 3-18.

Boesten, J.J.T.I. & van der Linden, A.M.A. 1991. Modelling the influence of sorption of and transformation on pesticide leaching and persistence. Journal of Environmental Quality 20, 425-435.

Boesten, J.J.T.I., van der Pas, L.J.T., Smelt, J.H. & Leistra, M. 1991. Transformation rate of methyl isothiocyanate and 1,3-dichloropropene in water-saturated sandy subsoils. Netherlands Journal of Agricultural Science 39, 179-190.

Bohinski, R.G. 1987. Modern Concepts in Biochemistry (Fifth Edition). Allyn and Bacon Inc., Boston.

Bohn, H.L., McNeal, B.L. & O'Connor, G.A. 1985. Soil Chemistry, Second Edition. John Wiley & Sons, New York.

Borchardt, G.A. 1989. Smectites. In J.B. Dixon and S.B. Weed (eds.), Minerals in Soil Environments, Second Edition. Soil Science Society of America, Inc., Madison, Wisconsin, 675-718.

Brime, C. 1985. The accuracy of X-ray diffraction methods for determining mineral mixtures. Mineralogical Magazine 49, 531-538.

Brubaker, G.R., Lane, W.F., English, D.A. & Hargens, D. 1994. Laboratory evaluation of *in situ* bioremediation for polycyclic aromatic hydrocarbon-contaminated aquifers. In R.E. Hinchee, D.B. Anderson, F.B. Metting and G.S. Sayles (eds.), Applied Biotechnology for Site Remediation, Lewis, London, 213-228.

Budavari, S., O'Niel, M.J., Smith, A. & Heckelman, P.E. 1989. The Merck Index, An Encyclopedia of Chemicals, Drugs and Biologicals, Eleventh Edition. Merck & Co., Inc., Rahway, U.S.A.

Burns, R. G. 1979. Interaction of microorganisms, their substrates and their products with soil surfaces. In D.C. Ellwood, J. Melling and P. Rutter (eds.), Adhesion of Microorganisms to Surfaces. Special Publication of the Society for General Microbiology, 2. Academic Press, New York, 193-231.

Campbell, D.J.V., Parker, A., Rees, J.F. & Ross, C.A.M. 1983. Attenuation of potential pollutants in landfill leachate by Lower Greensand. Waste Management and Research 1, 31-52.

Cerniglia, C.E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. Current Opinion in Biotechnology 4, 331-338.

Chan, K.Y., Davey, B.B. & Geering, H.R. 1978. Interaction of treated sanitary landfill leachate with soil. Journal of Environmental Quality 7, 306-310.

Characklis, W.G. & Marshall, K.C. 1990. Biofilms. John Wiley & Sons, Inc., New York.

Chen, Y.M., Abriola, P.J.J., Alvarez, P.J., Anid, P.J. & Vogel, T.M. 1992. Modelling transport and biodegradation of benzene and toluene in sandy aquifer material: Comparisons with experimental measurements. Water Resource Research 28, 1833-1847.

Chen, C.I., Mueller, R.F. & Thomas, G. 1994. Kinetic analysis of microbial sulfate reduction by *Desulfovibrio desulfuricans* in an anaerobic upflow porous media biofilm reactor. Biotechnology and Bioengineering 43, 267-274.

Chung, G., McCoy, B.J. & Scow, K.M. 1992. Criteria to assess when biodegradation is kinetically limited by intraparticle diffusion and sorption. Biotechnology and Bioengineering 41, 625-632.

Collins, Y.E. & Stotzky, G. 1992. Heavy metals alter the electrokinetic properties of bacteria, yeasts, and clay minerals. Applied and Environmental Microbiology 58, 1592-1600.

Corapcioglu, M.Y. & Haridas, A. 1985. Microbial transport in soils and groundwater: A numerical model. Advances in Water Resources 8, 188-200.

Coutts, D.A.P., Balba, M.T.M. & Senior, E. 1987. Acetogenesis and acetotrophy in a hexanoate-catabolizing microbial association isolated from anoxic landfill. Journal of Applied Bacteriology 63, 343-353.

Cunningham, A.B., Bouwer, E.J. & Characklis, W.G. 1990. Biofilms in porous media. In W.G. Characklis and K.C.Marshall (eds.), Biofilms. John Wiley & Sons, Inc., New York, 697-732.

Dixon, J.B. 1989. Kaolinite and serpentine group minerals. In J.B. Dixon and S.B. Weed (eds.), Minerals in Soil Environments, Second Edition. Soil Science Society of America, Inc., Madison, Wisconsin, 467-519.

Dobbins, D.C., Thornton-Manning, J.R., Jones, D.D. & Federle, T.W. 1987. Mineralization potential for phenol in subsurface soils. Journal of Environmental Quality 16, 54-58.

Douben, P.E.T. & Harmsen, J. 1991. Diffuse verontreiniging van het landelike gebied - Sanering: doen of niet doen? Bodem 4, 157-160.

Flemming, C.A., Ferris, F.G., Beveridge, T.J. & Bailey, G.W. 1990. Remobilization of toxic heavy metals adsorbed to bacterial wall-clay composites. Applied and Environmental Microbiology 56, 3191-3203.

Fletcher, M. 1979. The attachment of bacteria to surfaces in aquatic environments. In D.C. Ellwood, J. Melling and P. Rutter (eds.), Adhesion of Microorganisms to Surfaces. Special Publication of the Society for General Microbiology, 2. Academic Press, New York, 87-108.

Fletcher, M., 1985. Effect of solid surfaces on the activity of attached bacteria. In D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Mechanisms and Physiological Significance. Plenum Press, New York, 339-363.

Fontaine, D.D., Lehmann, R.G. & Miller, J.R. 1991. Soil adsorption of neutral and anionic forms of a sulphonamide herbicide, flumetsulam. Journal of Environmental Quality 20, 759-762.

Fontes, D.E., Mills, A.L., Hornberger, G.M. & Herman, J.S. 1991. Physical and chemical factors influencing transport of microorganisms through porous medium. Applied and Environmental Microbiology 57, 2473-2481.

Foth, H.D. 1984. Fundamentals of Soil Science (Seventh Edition). John Wiley & Sons, New York.

Franklin, F.C.H., Bagdasarian, M. & Timmins, K.N. 1981. Manipulation of degradative genes of soil bacteria. In T. Leisinger, R. Hutter, A.M. Cook and J. Nuèsch (eds.), Microbial Degradation of Xenobiotics and Recalcitrant Compounds. Academic Press, London, 109-130.

Fry, V.A. & Istok, J.D. 1994. Effects of rate-limited desorption on the feasibility of *in situ* bioremediation. Water Resources Research 30, 2413-2422.

Fuller, W.H. 1978. Investigation of Landfill Leachate Pollutant Attenuation by Soils. US EPA-600/2-78-158.

Fuller, W.H. 1980. Soil modification to minimize movements of pollutants from solid waste operations. Critical Reviews in Environmental Control 9, 213-270.

Gagnon, C., Arnac, M. & Brindle, J.R. 1992. Sorption interactions between trace metals (Cd and Ni) and phenolic substances on suspended clay minerals. Water Research 26, 1067-1072.

Gannon, J.T., Mingelgrin, M & Alexander, M. 1991. Bacterial transport through homogenous soil. Soil Biology and Biochemistry 23, 1155-1160.

Gerba, C.P., Yates M.V. & Yates, S.R. 1991. Quantitation of factors controlling viral and bacterial transport in the subsurface. In C.J. Hurst (ed.), Modelling the Environmental Fate of Microorganisms, American Society for Microbiology, Washington DC, 77-88.
for Site Remediation, Lewis, London, 229-239.

Gianfreda, L., Rao, M.A. & Violante, A. 1992. Adsorption, activity and kinetic properties of urease on montmorillonite, aluminium hydroxide and $Al(OH)_x$-montmorillonite complexes. Soil Biology and Biochemistry 24, 51-58.

Gibbs, R.J. 1965. Error due to segregation in quantitative clay mineral X-ray diffraction mounting techniques. American Mineralogist 50, 741-751.

Giles, C.H., Smith, D. & Huitson, A. 1986. A general treatment and classification of the solute adsorption isotherm. In R.D. Harter (ed.), Adsorption Phenomena. Van Nostrand Reinhold Company Inc., New York, 323-333.

Gilmour, C.M., Broadbent, F.E. & Beck, S.M. 1977. Recycling of carbon and nitrogen through land disposal of various wastes. In L.F. Elliot and F.S. Stevenson (eds.), Soils for Management of Wastes and Waste Waters. Soil Science Society of America, American Society of Agronomy, Crop Science Society of America, Madison, Wisconsin, 324-356.

Gordon, A.S. & Millero, F.J. 1984. Electrolyte effects on attachment of an estuarine bacterium. Applied and Environmental Microbiology 47, 495-499.

Gourdon, R., Comel, C., Vermande, P. & Vernon, C. 1989. Fractionation of the organic matter of a landfill leachate before and after aerobic or anaerobic biological treatment. Water Research 23, 167-173.

Govind, R., Gao, C., Lai, L., Yan, X., Pfanstiel, S. & Tabak, H.H. 1994. Development of methodology to determine the bioavailability and biodegradation kinetics of toxic organic pollutant compounds in soil. In R.E. Hinchee, D.B. Anderson, F.B. Metting and G.S. Sayles (eds.), Applied Biotechnology

Graves, D.A., Lang, C.A. & Rightmyer, J.N. 1994. Biodegradation of bis(2-ethylhexyl)phthalate, ethylbenzene, and xylenes in groundwater: Treatability study supporting *in situ* aquifer bioremediation. In R.E. Hinchee, D.B. Anderson, F.B. Metting and G.S. Sayles (eds.), Applied Biotechnology for Site Remediation, Lewis, London, 167-185.

Green, W.J., Lee, G.L. & Jones, R.A. 1981. Clay-soils permeability and hazardous waste storage. Journal of Water Pollution Control Federation 53, 1347-1354.

Griffen, R.A. & Shimp, N.F. 1978. Attenuation of pollutants in landfill leachate by clay minerals. Illinois State Geological Survey, Environmental Geological Notes, Number 78, US EPA - 600/2-78-157.

Gu, B., Schmitt, J., Chen, Z., Liang, L. & McCarthy, J.F. 1995. Adsorption and desorption of different organic fractions on iron oxide. Geochemica et Cosmochimica Acta, 59, 219-229.

Harmsen, J. 1983. Identification of organic compounds in leachate from a waste tip. Water Resources 16, 699-705.

Harmsen, J. 1991. Possibilities and limitations of landfarming for cleaning contaminated soils. In E. Hinchee and R.F. Olfenbuttel (eds.), On Site Bioreclamation. Butterworth-Heinemann, Stoneham, 255-272.

Harter, R.D. 1977. Reactions of minerals with organic compounds in the soil. In J.B. Dixon and S.B. Weed (eds.), Minerals in Soil Environments, Second Edition. Soil Science Society of America Inc., Madison, Wisconsin, 709-740.

Harter, R.D. 1986. Adsorption Phenomena. Van Nostrand Reinhold Company, New York.

Harter, R.D. & Stotzky, G. 1971. Formation of clay-protein complexes. Soil Science Society of America Proceedings 35, 383-389.

Harter, R.D. & Stotzky, G. 1973. X-ray diffraction, electron microscopy, electrophoretic mobility and pH of some stable smectite-protein complexes. Soil Science Society of America Proceedings 37, 116-123.

Harvey, R.W. & Garabedian, S.P. 1991. Use of colloid filtration theory in modelling movement of bacteria through a contaminated aquifer. Environmental Science and Technology 25, 178-185.

Hayes, M.H.B. 1984. Chemical nature and reactivities of soil organic polymers. In B. Yaron, G. Dagan and J. Goldshmid (eds.), Pollutants in Porous Media. Springer-Verlag, Berlin, 77-99.

Hekman, W.E., Heijnen, C.E., Trevors, J.T. & Van Elsas, J.D. 1994. Water flow induced transport of *Pseudomonas fluorescens* cells through soil columns as affected by inoculant treatment. FEMS Microbial Ecology 13, 313-324.

Hendershot, W.H. & Lavkulich, L.M. 1983. Effect of sesquioxide coatings on surface charge of standard mineral and soil samples. Soil Science Society of America Journal 47, 1252-1260.

Heynen, C.E., Van Elsas, J.D., Kuikman, P.J. & Van Veen, J.A. 1988. Dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil: The effect of bentonite clay on predation by protozoa. Soil Biochemistry 20, 483-492.

Hiroki, M. 1992. Effects of heavy metal contamination on soil microbial populations. Soil Science and Plant Nutrition 38, 141-147.

Hiroki, M. 1994. Population of Cd-tolerant microorganisms in soils polluted with heavy metals. Soil Science and Plant Nutrition 40, 515-524.

Hsu, P.H. 1989. Aluminium oxides and oxyhydroxides. In J.B. Dixon and S.B. Weed (eds.), Minerals in Soil Environments, Second Edition. Soil Science Society of America Inc., Madison, Wisconsin, 331-371.

Huang, P.M. & Violante, A. 1986. Influence of organic acids on crystallization and surface properties of precipitation products of aluminium. In P.M. Huang and M. Schnitzer (eds.), Interactions of Soil Minerals With Natural Organics and Microbes. Soil Science Society of America Special Publication No. 17, Madison, Wisconsin, 159-221.

Hutchins, S.R., Tomson, M.B., Wilson, J.T. & Ward, C.H. 1984. Microbial removal of waste-water organic compounds as a function of input concentration in soil columns. Applied and Environmental Microbiology 48, 1039-1045.

Huysman, F. & Verstraete, W. 1993a. Water-facilitated transport of bacteria in unsaturated soil columns: Influence of cell surface hydrophobicity and soil properties. Soil Biology and Biochemistry 25, 83-90.

Huysman, F. & Verstraete, W. 1993b. Water-facilitated transport of bacteria in unsaturated soil columns: Influence of inoculation and irrigation methods. Soil Biology and Biochemistry 25, 91-97.

Huysman, F. & Verstraete, W. 1993c. Effect of cell characteristics on the adhesion of bacteria to soil particles. Biology and Fertility of Soils 16, 21-26.

Imhof, P.T. & Jaffe, P.R. 1994. Effect of liquid distribution on gas-water phase mass transfer in an unsaturated sand during infiltration. Journal of Contaminant Hydrology 16, 359-380.

Jenkins, T.G. 1992. Environmental factors influencing growth. In *In Vitro* Cultivation of Microorganisms, T.G. Cartledge (ed.), Butterworth-Heinemann, London, 177-214.

Jones, G.L., Jansen, F. & McKay, A.J. 1973. Substrate inhibition of the growth of bacterium NCIB 8250 by phenol. Journal of General Microbiology 74, 139-148.

Jussel, P., Stauffer F. & Dracos, T. 1994. Transport modelling in heterogeneous aquifers: 1. Statistical description and numerical generation of gravel deposits. Water Resources Research 30, 1803-1817.

Kefford, B., Kjelleberg, S. & Marshall, K.C. 1982. Bacterial scavenging : Utilization of fatty acids localized at a solid-liquid interface. Archives of Microbiology 133, 257-260.

Knezovich, J.P., Hirabaya, J.M., Bishop, D.J. & Harrison, F.L. 1988. The influence of different soil types on the fate of phenol and its biodegradation products. Chemosphere 17, 2199-2205.

Knox, R.C., Sabatini, D.A. & Canter, L.W. 1993. Subsurface Transport and Fate Processes. Lewis Publishers, London.

Lafrance, P., Banton, O., Campbell, P.G.C. & Villeneuve, J.P. 1990. A complexation-adsorption model describing the influence of dissolved organic matter on the mobility of hydrophobic compounds in groundwater. Water Science Technology 22, 15-22.

Lane, W.F. & Loehr, R.C. 1992. Estimating the equilibrium aqueous concentrations of polynuclear aromatic hydrocarbons in complex mixtures. Environmental Science and Technology 26, 983-990.

Lappin-Scott, H.M. & Costerton, J.W. 1990. Starvation and penetration of bacteria in soils and rocks. Experientia 46, 807-812.

Lensing, M., Voght, M. & Herrling, B. 1994. Modelling of biologically mediated redox processes in the subsurface. Journal of Hydrology 159, 125-143.

Lipson, S. & Stotzky, G. 1983. Adsorption of reovirus to clay minerals: effects of cation exchange capacity, cation saturation and surface area. Applied and Environmental Microbiology 46, 673-682.

Lo, K.S., Yang, W.F. & Lin, Y.C. 1992. Effects of organic matter on the specific adsorption of heavy metals by soil. Toxicological and Environmental Chemistry 34, 139-153.

Loehle, C. & Johnson, P. 1994. A framework for modelling transport dynamics in the subsurface. Ecological Modelling 73, 31-49.

Lucas, J.L. & White, C.C. 1983. Design and instrumentation of semi-permeable attenuation blanket beneath a domestic waste landfill. International Land Reclamation Conference and Exhibition, Conference Proceedings, Grays, England. Industrial Seminars Ltd., Tunbridge Wells, 299-314.

Lui, K.H., Enfield, C.G. & Mravik, S.C. 1991. Evaluation of sorption models in the simulation of naphthalene transport through saturated soils. Groundwater 29, 685-692.

Machajdik, D. & Cicel, B. 1981. Potassium and ammonium treated montmorillonites. II. Calculation of characteristic layer charges. Clays and Clay Minerals 29, 47-52.

Marshall, K.C. 1985. Mechanisms of bacterial adhesion at solid-water interfaces. In D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Mechanisms and Physiological Significance. Plenum Press, New York, 133-161.

Marshall, K.C. 1988. Adhesion and growth of bacteria at surfaces in oligotrophic habitats. Canadian Journal of Microbiology 34, 503-506.

Marshall, K.C. & Cruickshank, R.H. 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Archives of Microbiology 91, 29-40.

Marshall, T.J. & Holmes, J.W. 1988. Soil Physics (Second Edition). Cambridge University Press, Cambridge.

Mehlich, A. 1981. Charge properties in relation to sorption and desorption of selected cations and anions. In M. Stelly, Chemistry in the Soil Environment, American Society of Agronomy Special Publication Number 40, American Society of Agronomy, Madison, Wisconson, 47-72.

Middelbeek, E.J. & Jenkins, T.G. 1992. Environmental factors influencing growth, In T.G. Cartledge (ed.), *In Vitro* Cultivation of Microorganisms, Butterworth-Heinemann, London, 145-176.

Mihelcic, J.R. & Luthy, R.G. 1988a. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Applied and Environmental Microbiology 54, 1182-1187.

Oades, J.M. 1989. An introduction to organic matter in mineral soils. In J.B. Dixon and S.B. Weed (eds.), Minerals in Soil Environments, Second Edition. Soil Science Society of America Inc., Madison, Wisconsin, 89-153.

Osa-Afiana, L.O. & Alexander, M. 1982. Clays and the survival of *Rhizobium* in soil during desiccation. Soil Science Society of America Journal 46, 285-293.

Paul, E.A. & Clark, F.E. 1989. Soil Microbiology and Biochemistry. Academic Press Inc., New York.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X. & Simonet, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Applied and Environmental Microbiology 58, 2717-2722.

Piotrowski, M.R. 1991. Bioremediation of hydrocarbon contaminated surface water, groundwater and soils : The microbial ecology approach. In P.T. Kostecki and E.J. Calabrese (eds.), Hydrocarbon Contaminated Soils and Groundwater, Volume 1. Lewis Publishers, Chelsea, Michigan, 203-238.

Poole, R.K., Bazin, M.J. & Keevil, C.W. 1992. Microbial Growth Dynamics. Special Publication of the Society for General Microbiology Volume 28, IRL Press, Oxford.

Prosser, J.I., Rattray, E.A.S., Silcock, D & Kilham, K. 1994. Spatial characterization of luminescence-marked bacterial cells in soil by CCD image-enhanced microscopy. Paper presented at the Society for General Microbiology (127th Meeting), University of Warwick, 5 - 7 January 1994.

Qualls, R. & Haines, B.L. 1992. Measuring adsorption isotherms using continuous, unsaturated flow through intact soil cores. Soil Science Society of America Journal 56, 456-460.

Rebhun, M., Kalabo, R., Grossman, L., Manka, J. & Rav-Acha, C. 1992. Sorption of organics on clay and synthetic humic-clay complexes simulating aquifer processes. Water Research 26, 79-84.

Reddy, K.M. & Locke, M.A. 1994. Prediction of soil sorption (K_{oc}) of herbicides using semi-empirical molecular properties. Weed Science 42, 453-461.

Reinhard, M., Goodman, N.L. & Barker, J.F. 1984. Occurrence and distribution of organic chemicals in two landfill leachate plumes. Environmental Science and Technology 8, 953-961.

Rodriguez, G.G., Phipps, D., Ishiguro, K. & Ridgeway, H.F. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Applied and Environmental Microbiology 58, 1801-1808.

Rosenberg, M. & Kjelleberg, S. 1986. Hydrophobic interactions: Role in bacterial adhesion. Advances in Microbial Ecology 9, 353-393.

Roslev, P. & King, G. 1993. Application of a tetrazolium salt with a water-soluble formazan as an indicator of viability in respiring bacteria. Applied and Environmental Microbiology 59, 2891-2896.

Rotman, B. & Papermaster, B.W. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proceedings of the National Academy of Science, U.S.A. 55, 134-141.

Rozich, A.F. & Colvin, R.J. 1986. Effects of glucose on phenol biodegradation by heterogeneous soil populations. Biotechnology and Bioengineering 28, 965-971.

Sanchez D., Vandevivere, P., Baveye, P. & Zinder, S. 1994. Decrease of the hydraulic conductivity of sand columns by *Methanosarcina barkeri*. World Journal of Microbiology and Biotechnology 10, 325-333.

Santoro, T. & Stotzky, G. 1968. Sorption between microorganisms and clay minerals as determined by the electrical sensing zone particle analyzer. Canadian Journal of Microbiology 14, 299-307.

Sarkar, A.K., Georgiou, G. & Sharma, M.M. 1994. Transport of bacteria in porous media: 1. An experimental investigation. Biotechnology and Bioengineering 44, 489-497.

Sawhney, B.L. & Kozloski, R.P. 1984. Organic pollutants in leachates from landfill sites. Journal of Environmental Quality 13, 349-352.

Scheunert, I. & Mansor, M. 1992. Binding of organic pollutants to soil organic matter. International Journal of Analytical Chemistry 46, 189-199.

Schwertmann, U. & Taylor, R. 1989. Iron oxides. In J.B. Dixon and S.B. Weed (eds.), Minerals in Soil Environments, Second Edition. Soil Science Society of America Inc., Madison, Wisconsin, 379-395.

Scott, H.D., Wolf, D.C. & Lavy, T.L. 1982. Apparent adsorption and microbial degradation of phenol by soil. Journal of Environmental Quality 11, 107-112.

Scow, K.M. & Alexander, M. 1992. Effect of diffusion on the kinetics of biodegradation: Experimental results with synthetic aggregates. Soil Science Society of America Journal 56, 128-134.

Scow, K.M. & Hutson, J. 1992. Effect of diffusion on the kinetics of biodegradation: Theoretical considerations. Soil Science Society of America Journal 56, 119-127.

Scow, K.M., Schmidt, S.K. & Alexander, M. 1989. Kinetics of biodegradation of mixtures of substrates in soil. Soil Biology and Biochemistry 21, 703-708.

Senesi, N. & Chen, Y. 1989. Interactions of toxic chemicals with humic substances. In Z. Gerstl, Y. Chen, U. Mingelgrin and B. Yaron (eds.), Toxic Organic Chemicals in Porous Media. Springer Verlag, Berlin, 37-84.

Shales, S.W. & Kumarasingham, S. 1987. Bacterial transport through porous solids: Interaction between *Micrococcus luteus* cells and sand particles. Journal of Industrial Microbiology, 2, 219-229.

Sharma, P.K. & McInerney, W.B. 1994. Effect of grain size on bacterial penetration, reproduction, and metabolic activity in porous glass bead chambers. Applied and Environmental Microbiology 60, 1481-1486.

Shaw, J.C., Bramhill, B., Wardlaw, N.C. & Costerton, J.W. 1985. Bacterial fouling in a model core system. Applied and Environmental Microbiology 49, 693-701.

Sibanda, H.M. & Young, S.D. 1986. Competitive adsorption of humus acids and phosphate on goethite, gibbsite and two tropical soils. Journal of Soil Science 37, 197-204.

Simulnek, J. & Suarez, D.L. 1994. Two-dimensional transport model for variably saturated porous media with major ion chemistry. Water Resources Research 30, 1115-1133.

Skoog, D.A., West, D.M. & Holler, F.J. 1991. Fundamentals of Analytical Chemistry, Sixth Edition. Saunders College Publishing, New York.

Slater, J.H. 1981. Mixed cultures and microbial communities. In M.E. Bushell and J.H. Slater (eds.), Mixed Culture Fermentations. Academic Press, London, 1-24.

Soil Classification Working Group, 1991. Soil Classification: A Taxonomic System for South Africa. Department of Agricultural Development, Pretoria, South Africa.

Steele, D.D. & Nieber, J.L. 1994a. Network modelling of diffusion coefficients for porous media: 1. Theory and model development. Soil Science Society of America Journal 58, 1337-1345.

Steele, D.D. & Nieber, J.L. 1994b. Network modelling of diffusion coefficients for porous media: 2. Simulations. Soil Science Society of America Journal 58, 1346-1354.

Steenhuis, T.S., Boll, J., Shalit, G., Selker, J.S. & Merwin, I.A. 1994. A simple equation for predicting preferential flow solute concentrations. Journal of Environmental Quality 23, 1058-1064.

Stotzky, G. 1980. Surface interactions between clay minerals and microbes, viruses and soluble organics, and the probable importance of these interactions to the ecology of microbes in soil. In R.C.W. Berkeley, K.M. Lynch, K. Milling, P.R. Rutter and B. Vincent (eds.), Microbial Adhesion to Surfaces. Ellis Horwood, Chichester, 215-249.

Stotzky, G. 1985. Mechanisms of adhesion to clays, with reference to soil systems. In D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Mechanisms and Physiological Significance. Plenum Press, New York, 195-253.

Stotzky, G. 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses. In P.M. Huang and M. Schnitzer (eds.), Interactions of Soil Minerals With Natural Organics and Microbes. Soil Science Society of America Special Publication No. 17, Madison, Wisconsin, 305-427.

Sullivan, L.A. 1990. Soil organic matter, air encapsulation and water-stable aggregation. Journal of Soil Science 41, 529-534.

Tabatabai, M.A. 1982. Soil enzymes. In A.L. Page, R.H. Miller & D.R. Keeney (eds.), Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties, Second Edition, Soil Science Society of America Inc., Madison, Wisconsin, 937-952.

The Non Affiliated Soil Analysis Work Committee, 1990. Handbook of Standard Soil Testing Methods for Advisory Purposes. Soil Science Society of South Africa Publication, Pretoria, South Africa.

Van Demark, P.J. & Batzing, B.L. 1987. The Microbes: An Introduction to Their Nature and Importance. Benjamin/Cummings Publishing Company, Menlo Park, California.

Vandevivere, P. & Baveye, P. 1992a. Effect of bacterial extracelluar polymers on the saturated hydraulic conductivity of sand columns. Applied and Environmental Microbiology 58, 1690-1698.

Vandevivere, P. & Baveye, P. 1992b. Relationship between transport of bacteria and their clogging efficiency in sand columns. Applied and Environmental Microbiology 58, 2523-2530.

Vandevivere, P. & Baveye, P. 1992c. Saturated hydraulic conductivity reduction caused by aerobic bacteria in sand columns. Soil Science Society of America Journal 56, 1-13.

van Elsas, J.D. & Heijnen, C.E. 1990. Methods for introduction of bacteria into soil : A review. Biology and Fertility of Soils 10, 127-133.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. & Zehnder, A.J.B. 1987a. The role of bacterial cell wall hydrophobicity in adhesion. Applied and Environmental Microbiology 53, 1893-1897.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. & Zehnder, A.J.B. 1987b. Electrophoretic mobility and hydrophobicity as a measure to predict the initial step of bacterial adhesion. Applied and Environmental Microbiology 53, 1898-1901.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W. & Zehnder, A.J.B. 1990. Influence of interfaces on microbial activity. Microbiological Reviews 54, 75-87.

van Loosdrecht, M.C.M. & Zehnder, A.J.B. 1990. Energetics of bacterial adhesion. Experientia 46, 817-822.

Venkatarami, E.S., Ahlert, R.C. & Corbo, P. 1984. Biological treatment of landfill leachates. CRC Critical Reviews in Environmental Control 14, 333-377.

Volkering, F., Breure, A.M., Sterkenburg, A. & van Andel, J.G. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Applied Microbiology and Biotechnology 36, 548-552.

Walker, S.G., Flemming, C.A., Ferris, F.G., Beveridge, T.J. & Bailey, G.W. 1989. Physiological interaction of *Escherichia coli* cell envelopes and *Bacillus subtilis* cell walls with two clays and ability of the composite to immobilize heavy metals from solution. Applied and Environmental Microbiology 55, 2976-2984.

Wan, J., Wilson, J.L. & Kieft, L.T. 1994. Influence of the gas-water interface on transport of microorganisms through unsaturated porous media. Applied and Environmental Microbiology 60, 2, 509-516.

Wang, Y. 1992. Effects of chemical oxidation on anaerobic treatment of phenols. Water and Environment Research 64, 268-273.

Weissenfels, W.D., Klewer, H-J. & Langhoff, J. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Applied Microbiology and Biotechnology 36, 689-696.

Wilson, J.T., Enfield, C.G., Dunlop, W.J. & Cosby, R.L. 1981. Transport and fate of selected organic pollutants in a sandy soil. Journal of Environmental Quality 10, 501-506.

Wilson, S.C. & Jones, K.C. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. Environmental Pollution 81, 229-249.

Wollum, A.G. & Cassel, D.K. 1978. Transport of microorganisms in sand columns. Soil Science Society of America Journal 42, 72-76.

Wood, B.D., Dawson, C.N., Szecsody, J.E. & Streile, J.P. 1994. Modelling contaminant transport and biodegradation in a layered porous media system. Water Resources Research 30, 1833-1845.

Yates, M.V. & Yates, S.R. 1991. Modelling microbial transport in the subsurface: A mathematical discussion. In C.J. Hurst (ed.), Modelling the Environmental Fate of Microorganisms, American Society of Microbiology, Washington DC, 48-76.

Yates, M.V. & Yates, S.R. 1988. Modeeling microbial fate in the subsurface environment. CRC Reviews in Environmental Control, Volume 17, Issue 4, Lewis, London, 307-344.

Zysset, A., Stauffer, F. & Dracos, T. 1994. Modelling of reactive groundwater transport governed by biodegradation. Water Resources Research 30, 2423-2334.

APPENDIX 1

A.1 Soil characterization results

TABLE A.1.1: Surface area.

Sample		Surface area $(m^2\,g^{-1})$	
Rensburg topsoil	(Rg)	31.15	
Inanda topsoil	(Ia)	13.23	
Inanda subsoil	(Ib)	44.99	
Oakleaf topsoil	(Oa)	6.11	

TABLE A.1.2: Particle size as a percentage (w/w).

Size fraction		Rensburg	I.top	I.sub	Oak
Coarse sand	(2-0.5mm)	1.7	2.0	2.2	3.7
Medium sand	(0.5-0.25mm)	1.8	15.7	10.7	18.8
Fine sand	(0.25-0.1mm)	2.1	24.8	16.8	26.6
Very fine sand	(0.1-0.05mm)	2.8	6.0	5.1	14.8
Coarse silt	(0.05-0.02mm)	16.3	6.4	4.9	5.1
Fine silt	(0.02-0.002mm)	25.6	8.9	7.8	13.8
Clay	(<0.002mm)	49.7	36.2	52.5	17.2

TABLE A.1.3: Exchangeable cations.

Sample	Na	K	Ca	Mg	S*
	<-----cmol$_c$ kg^{-1}----->				
Rensburg topsoil	1.36	0.12	6.00	5.60	13.08
Inanda topsoil	0.04	0.36	0.28	0.20	0.88
Inanda subsoil	0.04	0.04	0.24	0.18	0.50
Oakleaf topsoil	0.18	0.12	1.92	2.38	4.60

* The sum of exchangeable Na, K, Ca and Mg ions

185

TABLE A.1.4: Clay mineralogy.

Sample	Mineral	% (w/w)
Rensburg topsoil	Smectite	71
	Kaolinite	20
	Quartz	7
	Lepidocrocite	2
Inanda topsoil	Quartz	35
	Kaolinite	32
	HIV*	19
	Feldspar	9
	Gibbsite	5
Inanda subsoil	Kaolinite	48
	HIV	28
	Quartz	12
	Gibbsite	9
	Feldspar	3
Oakleaf topsoil	Kaolinite	30
	Mica	23
	Quartz	23
	Vermiculite	15
	Smectite	9

* Hydroxy interlayered vermiculite

TABLE A.1.5: Organic carbon.

Soil	Estimated# organic carbon (%, w/w)
Inanda top	5.00
Inanda sub	0.57
Rensburg	2.48
Oakleaf	2.08

After method calibration

TABLE A.1.6: Pore volume and field capacity*.

Soil	Pore volume (ml g^{-1})	% Water (ml g^{-1}) at field capacity
Inanda topsoil	31	23
Inanda subsoil	37	27
Rensburg soil	17	31

* Values are the average of three replicates at an average bulk density of 1.3 g cm^{-3}

APPENDIX 2

A.2 Characteristics of chemicals used

A.2.1 Naphthalene

The chemical formula for naphthalene is $C_{10}H_8$ (Budavari *et al.*, 1989). Naphthalene is one of the polyaromatic hydrocarbon group of molecules. Other characteristics (Nyer *et al.*, 1993) include:
- Solubility in water at 25°C, 32 mg l^{-1};
- Specific gravity at 20°C referred to water at 4°C, 1.145;
- Octanol/water partitioning coefficient (K_{ow}), 2.8×10^3;
- Henry's Law constant, 20;
- Adsorption capacity (mg compound per g carbon at 500ppb), 5.6;
- Melting point, 80°C;
- Boiling point, 218°C; and
- Molecular weight, 128.16.

A.2.2 Phenol

The chemical formula for phenol is C_6H_6O. Other characteristics (Nyer *et al.*, 1993) include:
- Solubility in water at 25°C, 93 g l^{-1};
- Specific gravity at 20°C referred to water at 4°C, 1.071;
- Octanol/water partitioning coefficient (K_{ow}), 29;
- Henry's Law constant, 0.017;
- Adsorption capacity (mg compound per g carbon at 500ppb), 161;
- Melting point, 43°C;
- Boiling point, 182°C;
- Molecular weight, 94.11; and
- pKa value, 10.0.

APPENDIX 3

A.3 Development of methods

A.3.1 Supplementation of soil with naphthalene

As discussed in Section 3.1.2, the combined effect of adsorption and catabolism of naphthalene in the aqueous phase was so rapid that to follow the depletion path (attenuation) was extremely difficult. The maximum solubility (32 mg l^{-1}) of naphthalene is the most limiting factor. To overcome this problem it was decided to supplement the soil with naphthalene before experimentation. In this way the initial concentration could be much higher and, therefore, facilitate a greater concentration range through which naphthalene catabolism could be followed. A problem which had to be overcome, however, was to evenly supplement (coat) the soil with the chemical. This was achieved by dissolving the naphthalene (2g) in acetone (60ml) or acetonitrile (60ml) before mixing with 1kg soil and allowing the carriers to volatilize leaving the soil "coated" with naphthalene. The concentration used was similar to the concentrations of polyaromatic hydrocarbons examined in other studies (Weissenfels *et al.*, 1992). It was, however, found that if the supplemented soil was left undisturbed to facilitate volatilization, a gradient developed in which some of the naphthalene migrated with the carrier to the surface. It was, therefore, necessary to mix the soil regularly (every 30 minutes) until volatilization was complete.

Acetonitrile was found to be a particularly good carrier since it did not interfere with subsequent analyses because the HPLC UV detector is "blind" to the molecule at the selected wavelength of 276nm. However, a problem with acetonitrile as a carrier is that it has a residual toxicity (at very high concentrations in the initial stage) in the soil. This was determined by monitoring the CO_2 evolution from soil columns by trapping it in 0.3M $Ba(OH)_2$ and observing the formation of a precipitate. Soil columns supplemented with naphthalene via acetonitrile were found not to produce CO_2 even after inoculation with naphthalene catabolizing species. Conversely, residual acetone was found to be non-toxic but did cause interference in analyses. The acetone peaks for some of the soil samples were so

great that they masked the relatively smaller peaks on the chromatogram. This problem was resolved by using a longer volatilization time. Rapid microbial catabolism of the acetone, which seemed to be preferentially degraded before naphthalene, also minimized this problem. Therefore, in all soil supplementations acetone was used as the solvent carrier.

A.3.2 Total analysis of the experimental container

With most of the naphthalene on the soil surfaces (Section 3.1.3) and, therefore, limited information gained from analyzing the aqueous phase (Section 3.1.2), analysis of the adsorbed naphthalene was particularly important. Soil extraction procedures are usually tedious and only give results for the adsorbed molecule. To determine the concentration of naphthalene in both the aqueous and adsorbed phases requires two separate analyses. This motivated the development of a method by which the total naphthalene could be determined.

The ratio of the mobile phase for naphthalene analysis is 65% acetonitrile and 35% water (Section 2.3.4). Since this ratio is sufficient to elute the hydrophobic naphthalene off the C_{18} separation column it was assumed that a similar concentration would also desorb most soil-adsorbed naphthalene. It must be stressed, however, that this method does not remove all naphthalene from adsorbed sites but, for comparative analysis, i.e. the identical soil, the use of this method is justified.

With this method 5g of each of the 2 g kg^{-1} naphthalene supplemented soil types were added to 50ml nutrient solution (Section 2.4.2) plus inoculum in 150ml bottles. After incubation (7 days), destructive sampling was employed by adding 75ml acetonitrile to each bottle. The final acetonitrile : aqueous ratio was, thus, 60:40 which approximates to the ratio needed to elute naphthalene from a hydrophobic separation column. The whole bottle was then ultrasonicated for 5 minutes and the suspension centrifuged at 11000 x g for 30 minutes before filtration through a 0.2μm millipore filter, to remove sediment, prior to HPLC analysis.

A.3.3 Recovery efficiency

To test the recovery efficiency, a 60:40 acetonitrile : water mixture subsample was compared with an 82:18 mixture subsample of the same soil. This was achieved by taking a sample at the 60:40 ratio and diluting with acetonitrile to give the higher ratio (82:18). The results of this recovery efficiency test are given in Figure A1.
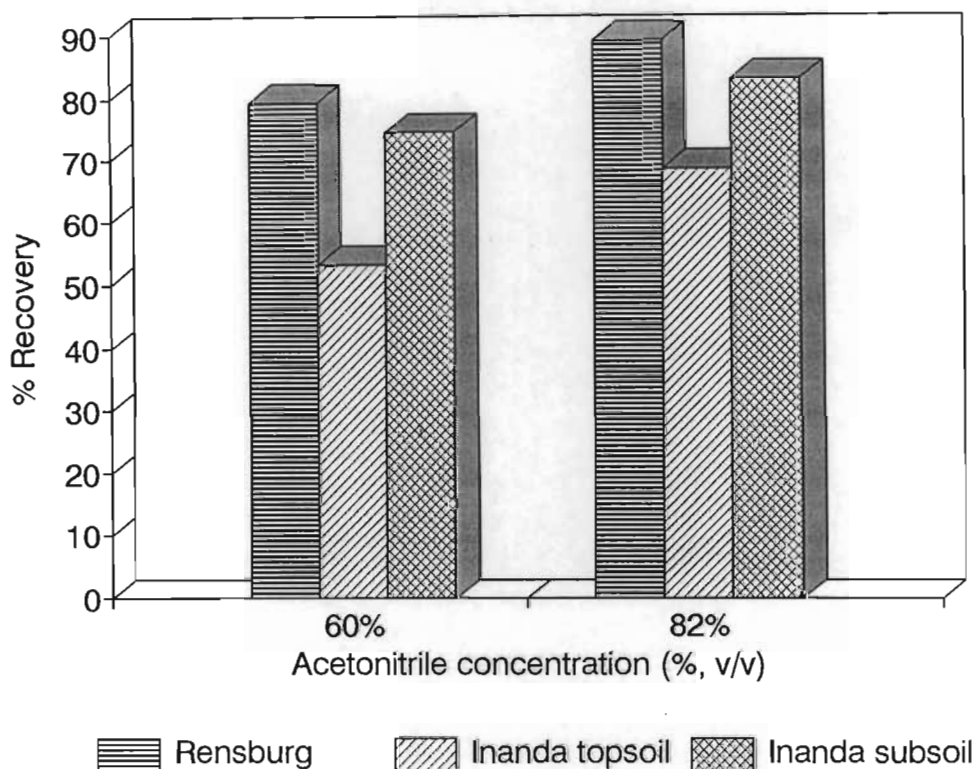
FIGURE A1: Recovery efficiency for naphthalene from different soils using two acetonitrile concentrations.

The different recovery efficiencies recorded for the three soil materials (Figure A1) indicated that the soils were either not contaminated equally or, more probably, the recovery efficiency was affected by the soil materials. For all soils, the naphthalene recovery with 82% acetonitrile was greater than with 60%. This indicates that the extraction method gives a partial recovery only. In all experiments naphthalene was recovered from soil using a 60% acetonitrile extraction.

The recovery efficiencies of the various analytical steps were also evaluated. A tensiometer attached to a syringe was inserted into a reference container which contained a naphthalene solution of 30 mg l^{-1}. After drawing the solution through the tensiometer the concentration was determined by HPLC analysis. A 31% loss of naphthalene was recorded (Figure A2). This was assumed to be due to adsorption on the tensiometer. (Adsorption on the syringe was assumed to be negligible relative to adsorption on the tensiometer due to the much smaller relative surface area of the syringe). With subsequent extractions through the tensiometer the adsorptive loss was reduced. Exact data in this regard are not given but this phenomenon seems to be related to the amount of solution sampled through the tensiometer.

The effect of volatilization/adsorption within the sealed experimental containers (glass bottles) was also determined by using containers which were 20% and 99% full. These bottles contained the same naphthalene solution and analysis was carried out after incubation at 30°C for 24 h. The results indicated that a considerable loss occurred probably due to both volatilization and adsorption on the glass containers (Figure A2). The fact that the 20% full bottles showed greater loss seemed to indicate volatilization whereas the 9% loss from the bottles with negligible head space (airspace) indicated adsorption onto the glass. The latter effect was also confirmed as the solution was transferred from glass bottles to Eppendorf tubes (average loss of approximately 4% for every 1.5ml Eppendorph transfer). These findings highlight the intrinsic error in quoting absolute values for experimental results unless these losses are quantitatively accounted for. The results quoted in this study have not been corrected for the losses recorded here. All experiments were made with the aim of determining the effects of the various soil materials. All factorial errors, such as those discussed here, could be expected to be present to similar extents in comparisons of the soils. Because of the aim of the study these errors were not corrected in the final results. They were, however, considered in interpretations of results and, subsequent, conclusions. The recovery efficiency for phenol was not determined since its adsorption was assumed to be much lower due to its high solubility.

Reference Standard

(30mg/l)
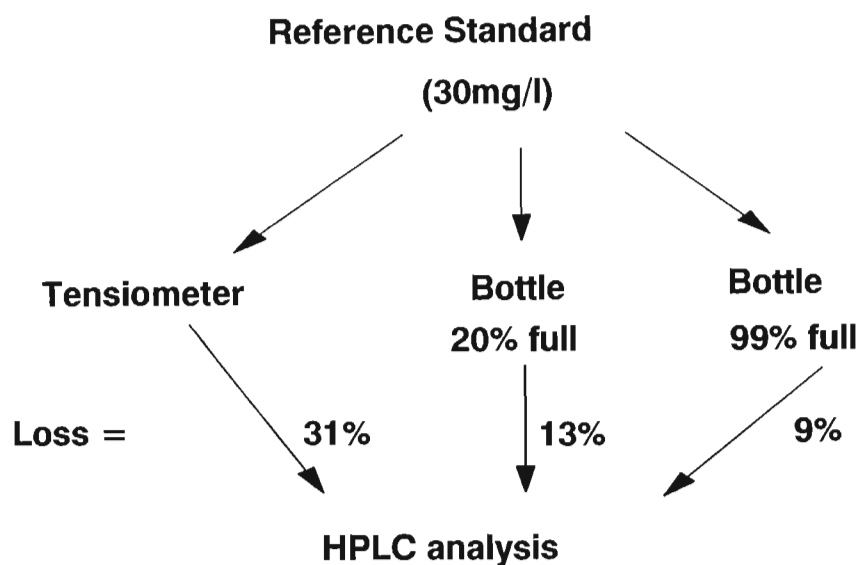
FIGURE A2: Non recovery of naphthalene from a reference container via a tensiometer and from a 20% and 99% full, closed bottle after 24h incubation (30°C).

A.3.4 Analytical techniques

Although methods are available for all of the compounds analyzed, most of these were modified to suit specific needs. The methods for naphthalene and phenol analysis by HPLC were optimized to determine phenol and naphthalene in the same chromatograph run using a solvent gradient. The UV detector wavelength was set at 254nm. All other parameters were as given for naphthalene analysis (Section 2.3.4). The gradient was as follows:

Time (min)	Flow rate (ml min⁻¹)	Water (%, v/v)	Acetonitrile (%,v/v)
0.00	1.5	75	25
5.00	1.5	75	25
7.00	1.5	30	70
9.00	1.5	30	70
11.00	1.5	0	100
13.00	1.5	0	100
20.00	1.5	75	25
30.00	1.5	75	25

A.3.5 Microbial enumeration

Plate counts (colony forming units) of a dilution series (10^{-3} to 10^{-6}) were made on nutrient agar (Section 2.6.1) and soil extract agar plates (Section 2.6.2) following incubation at 37°C for 48 h. The counts for most plates were found to be too variable for accurate quantification.

A second approach to estimate the total number of microorganisms in the suspension after extraction was to utilize viable cell staining techniques. The first staining technique tested was with acridine orange ($20\mu g$ ml⁻¹ sample). It was soon apparent, however, that this was not a useful technique to estimate microbial numbers. Not only did both the sediment and microorganisms fluoresce but the differences in dimensions of the two types of particles were too large to make accurate counts possible. These findings were later confirmed by the experiences of other workers in similar studies (F. Goede, personal communication).

A second viable cell staining technique evaluated involved the use of fluorescein diacetate. The method used was modified from Rotman and Papermaster (1966). The stain was prepared by dissolving 5mg in 1ml acetone. Aliquots ($20\mu l$) were then diluted to 100ml with distilled water. Different volumes between 1 and $5\mu l$ were added to a drop of sample on a glass slide prior to viewing. The same problem as with the acridine orange was, however, encountered. Organic and inorganic non-living particles (sediment) had dimensions both larger and smaller than those of the live organisms. It should be stressed that approximately 5 minutes was

allowed for sedimentation after the extraction procedures but this had little effect on the sediment in the sample. Centrifugation was not used to alleviate this problem since the sediments (including organic matter) are of both lighter and heavier densities than those of the organisms so that such an exercise would, probably, have been futile. This assumption was later confirmed by observations of a microbial cell layer sandwiched between a coarser soil particle layer (below) and a very fine particle size layer (above) after centrifugation (11000 x g for 30 min).

Despite the above problems, these two techniques could, possibly, be successfully employed in a sandy soil where particle size is more uniform and the particles are of a larger size and the organic matter content is very low.

It was thought that an enzyme activity determination would be a reliable method to estimate the size of the microbial population present in the extract. After an investigation into the literature (Tabatabai, 1982) and discussions with colleagues (C. Carliel, personal communication) it was, however, decided that this method was also not suitable because of poor reproducibility.

A.3.6 Bottle washer

Although not directly related to experimental procedure the design of a semi-automatic bottle washer was of great importance in automation of sample analysis. Autosampler bottles used for both HPLC and GC analysis have an extremely small neck diameter of 3mm. This does not facilitate free flow of liquids with a viscosity greater, or equal, than water. This phenomenon, thus, necessitated tedious manual washing by syringe of each individual sample bottle. A semi-automatic bottle washer was, subsequently, designed which was capable of washing 50 bottles simultaneously. The washer is shown in Plate A1 and consists of fifty "jets" onto which the sample bottles (1.8ml) are fitted. The reservoir contains a pump which discharges liquid through each "jet" at a rate of 1.7 ml sec^{-1}. A washing protocol was also tested which consisted of sequential washes with the following: tap water for 30 minutes

(reservoir is emptied after every wash); tap water with Extran (5%, v/v) for 1 hour; tap water for 30 min; tap water with 5% (v/v) methanol for 30 min; tap water for 30 min; distilled water for 30 min; and a second wash with distilled water for 30 min. The bottle caps were also washed by the same method. The effectiveness of the washing protocol was tested by HPLC using blank samples and was found to be effective for the chemicals analyzed in this study (i.e. volatile fatty acids, naphthalene and phenol).
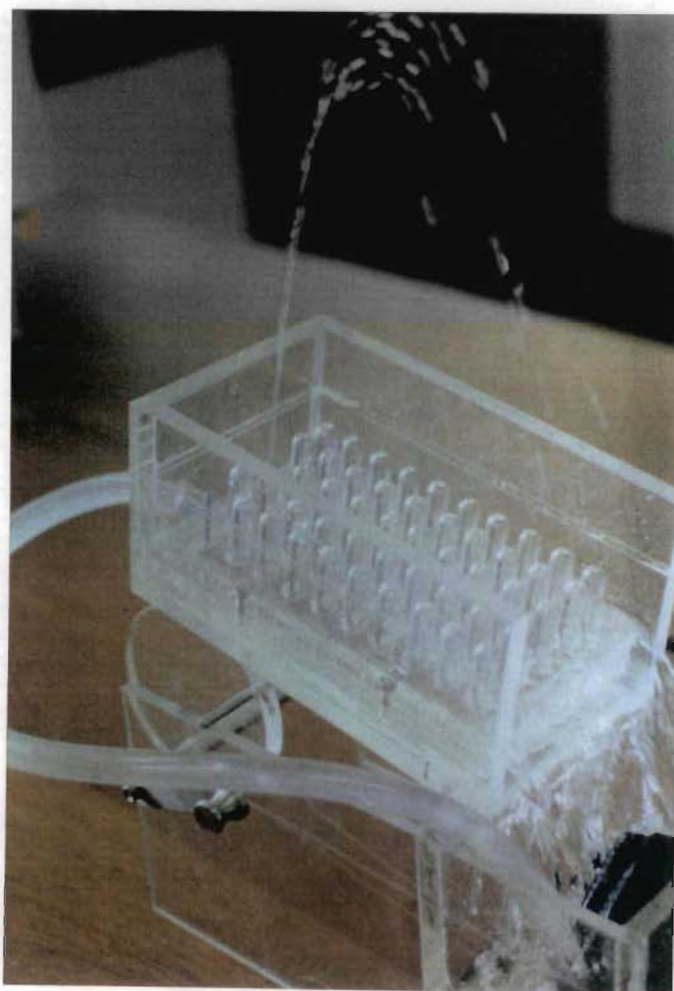

PLATE A1: Autosampler bottle washer.

A.3.7 The use of tensiometers to obtain unsaturated flow through soil columns

In most comparable studies, soil column research has been conducted under saturated conditions (Hutchins *et al.*, 1984). One of the main reasons for using saturated columns is the ease of extracting leachate (effluent) and maintaining a constant flow rate. In many European situations it is probably also an accurate simulation of natural soil conditions. In the South African context, however, most soils are usually unsaturated. It is, therefore, important to make soil column studies under both unsaturated and saturated conditions (Qualls & Haines, 1992). The problem with unsaturated columns is that it is extremely difficult to take liquid samples and equally difficult to maintain an unsaturated steady-state flow rate. This was overcome by the use of mini tensiometers. Tensiometers are ceramic cups which are usually used to measure the suction of the soil on the water in the cup. This is then registered and is used as an indication of the soil matric potential which is related to the soil water content. This principle was employed in reverse to facilitate liquid extraction from unsaturated soil. Tensiometers were installed at either the bottom or at various depths in the soil columns to extract leachate. The use of tensiometers was made possible by the fact that liquid, but not air, permeates the cups. The use of tensiometers also facilitated sample clean-up by filtration through the small pores of the tensiometer cups.

By placing these devices at the bottom of a column and applying suction by means of a syringe, a known volume of liquid can be extracted daily. The suction in the syringe is held by drawing the plunger out and placing a pin through it to prevent retraction. Steady-state flow is thus achieved by drawing liquid from the bottom of the column and then adding the same volume of liquid (ideally of the same composition) to the top. Although it could be argued that this is, strictly speaking, pulsating flow, the volumes are usually much lower than the total liquid volume in the column so that equilibrium should be attained rapidly without appreciably altering the bulk soil water content. Tensiometers were constructed by the Ceramic Studio of the Fine Arts Department, University of Natal. The clay used had the following constituents: 40% B13 ball clay; 40% G1 kaolin; 13% V clay; and 7% potassium feldspar. The bisque temperature was 700°C for 7 h and the subsequent firing temperature was 990°C for 9.5 h. Several of these tensiometers were examined by electron microscopy. The

surface pore types (Plate A2) were found to be very similar. It should be noted that the surfaces of the tensiometers do not have discrete pores but are composed of clay "flakes" which constitute a labyrinth. This configuration is probably responsible for the fact that the tensiometers do not easily become blocked.
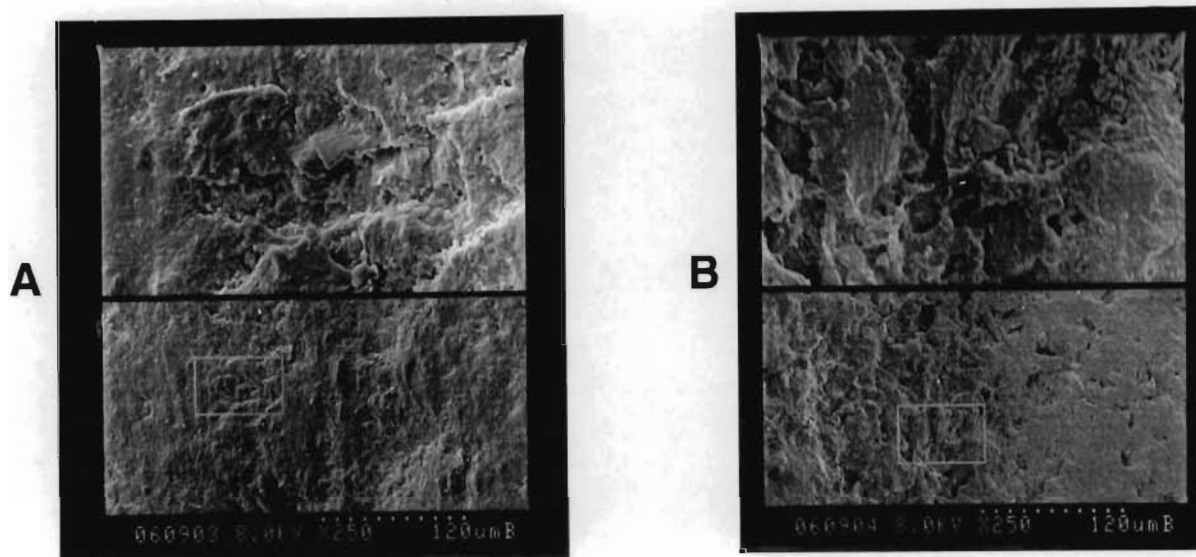

PLATE A2: **A.** Electron micrograph of the surface of a tensiometer produced by Ceramics Studio, Fine Arts Department, University of Natal. **B.** Electron micrograph of the surface of a commercially available tensiometer.

A.3.8 UV spectrophotometric analysis for phenol

Due to the variability of soil particle sizes, three standard packing materials were, initially, used in the columns. The packing materials, i.e. acid washed quartz sand (AWS), MIX and chromatography packing material (CHROM) are chemically inert and, therefore, had the potential for simplifying the analysis procedure. High performance liquid chromatography (HPLC) analysis with UV detection is necessary when analyzing phenols in soil since some of the indigenous soil organic material absorbs UV light at the same wavelength as phenol. This necessitated the separation capabilities of a HPLC-type analysis. Since the packing materials used are inert, no interferences were observed. It was, however, possible that

microbial growth could cause the production and release of organic chemicals into solution which could absorb at the same UV wavelength as phenol.

This was tested by aerobically culturing an inoculum (10 % (v/v) phenol catabolizers) in 5mM phenol-supplemented mineral salts medium (Section 2.4.2) in closed culture (250 ml Erlenmeyer flask) for 7 days at 30°C at which point $A_{590nm} = 0.170$. The sample was centrifuged (11000xg, 30 minutes) before HPLC analysis. The HPLC method involved a C_{18} column with mobile phase: MeOH: EtOH: Water: 40: 15: 45, 0.01% H_3PO_4, low UV Pic® A reagent and the UV detector wavelength set at 277 nm. Quantification was made by using external standards. The phenol concentration was determined to be 7 mg l^{-1} and the amount of interference, i.e. other peaks caused by microbial degradation products, was negligible (< 2% of the phenol peak height in the chromatogram). Simple phenol analysis by UV detection (A_{277nm}) with a UV spectrophotometer (Milton Roy Spectronic 301) could, thus, be made after centrifugation of the samples.

A.3.9 Hydraulic flow through columns

The aim of the column study, as discussed in Sections 6.11 and 6.6, was to determine the effects of porosity on microbial attenuation of migrating organic chemicals. In order to eliminate secondary effects, such as aeration due to entrapment of air bubbles, upward flow of the medium through the columns was necessary. The first approach was the use of a Mariotte bottle (constant pressure head) system. This established a constant pressure head in the vessel containing the medium. The medium was introduced via the bottom of each column and collected at the top. Although an even pressure head in the Mariotte bottle assured even flow rates through all tubes, the situation changed when the columns were connected. Different flow rates were recorded through the different columns and were ascribed to the different back pressures effected by the various packing materials. A peristaltic pump (Watson Marlow 205S) was, therefore, used to achieve equal flow rates through the individual columns. The variable back pressures from the columns did not significantly alter the flow rates which remained within 1% of each other.

198

Although equal flow rates could be achieved, this situation was not ideal. Even when the flow rate through each column was the same the actual flow rate through the total pore volume differed depending on the total pore volume of each column. The flow rates were, therefore, individually adjusted so that the pore flux for each packing material was equal. This has the most important effect on microbial growth rates.

A.3.10   Gas removal from columns

A very important aspect of the column study is that all the pores in each column participate and must not be filled with air. To facilitate this, the upward flow of liquid is useful since it minimises air entrapment. The small diameters of micropores in the packing materials, however, made it necessary to ensure liquid penetration into these pores. This was achieved by flushing the columns with methanol (upward flow). Due to the low viscosity of methanol, penetration of micropores and displacement of air bubbles was achieved. Flushing with methanol also served a secondary purpose in that the chromatography packing contained a small amount of yellow-coloured impurities which was displaced. After flushing with 10 pore volumes of methanol, distilled water was gradually added to the eluent until it was eventually distilled water only.

Although the columns were initially free of air bubbles it was possible that gases released by microbial processes in the columns could have been trapped by the packing. To eliminate the build up of such gases the columns were subjected daily to a degassing procedure. This was achieved by attaching a slightly larger diameter tubing, which was connected to the pump, to the column outlet port until no more air bubbles were detected. It should be noted that it was extremely difficult to detect the point at which all bubbles from the columns were extracted. When the procedure was continued for too long (> approximately 3 minutes) a vacuum was established in the column which resulted in air entry between the silicone stoppers and the column walls.

A.3.11 Determination of a suitable column diameter

Initially glass columns (15 mm diameter, 15 cm length) were used. These relatively small columns were used to minimize the amount of packing material. This was desirable because of the high cost of, particularly, the chromatography packing material. In the liquid culture experiments (Section 8.1) it was determined that a dilution rate of 1.4 pore volume changes per day was the maximum flow rate at which significant phenol degradation occurred. The small size of these columns, thus, necessitated only a very low flow rate. A full 24 hours was necessary to collect enough sample liquid from each column for analysis. The high microbial activity in the liquid and the fact that it had to remain in the collection vessel for a relatively long period, caused phenol catabolism in the collection vessel. Addition of bactericidal chemicals into the collection vessel was considered but it is possible that this would have caused a dilution effect or interfered with the analysis (UV absorbency at the same wavelength as phenol). Chemicals such as methanol which would not interfere with the absorbency reading are highly volatile and would, therefore, create uncertainty about the dilution factor. A thermocirculator (Fisons Haake D1) was used to lower the temperature in the collection vessels to 2°C at which level microbial catabolism of phenol ceased. A 3 mm diameter vinyl tube, connected to the thermocirculator was coiled into each collection vessel to cool the effluent. A problem encountered with this method was that condensation liquid formed on the tubing due to the low temperature and effectively diluted the small volume of sample liquid in the collection vessel. This amount could not be accurately accounted for. Due to the above problems it was decided to use larger diameter columns (4.5 cm diameter, Section 7.1.2). With the higher volume column, a higher inflow and outflow rate could be achieved, thereby eliminating most of the above problems.

A.3.12 Sterilizing medium inflow of columns

Initially medium to be pumped into the columns was sterilized by autoclaving (121°C, 15 minutes). All influent medium and tubing was sterilized by flushing with methanol. Continued changing of the influent medium reservoirs and tubing as well as the fact that the

medium had to be aerated (by sparging) made it difficult to maintain sterility. The medium was subsequently split so that the phenol was applied separately from the mineral salts. A Y-piece glass insert was used to converge the phenol and mineral salts influents just before entry into the bottom of the columns. In this way the medium was kept sterile and bacterial growth in the tubing leading to the columns was eliminated.

It was later found that the mixing ratio in each of the glass Y-pieces was not absolutely comparable (5% deviation). For this reason simple comparisons of the effluents from each column could not be made. Greater accuracy was, therefore, achieved by determining the influent concentration at the base of each column and expressing the outflow concentration as a percentage of this, i.e. relative phenol concentration (Figure 8.1).

A.3.13   Satisfying microbial oxygen demands

The objective of the column studies was to determine the effect of packing material porosity on microbial attenuation of organic chemicals migrating upwards through the column. Thus, it was essential that not only the organic chemical of interest (phenol) but also other parameters, which could be indicators of microbial activity, were, measured. Oxygen consumption in the columns was used as an additional measure of microbial activity. The rationale behind this was that if the influent medium contained the maximum amount of water soluble oxygen, any depletion of oxygen, detected at the outflow, could be taken to indicate microbial activity. This was, however, only true if oxygen disappearance was not due to any other factors.

A microbial inoculum (Sections 2.5 & 7.3) was pumped through the test column for 4 hours at a flow rate of 5 pore volume flushes per day (PVFD) to ensure an adequate microbial population. The nutrient medium was then supplied to the test column after a period of 24 hours in which no flow occurred through the column. The influent medium was a split application (Sections 7.1.1-7.1.2) and was pumped into the test column (30°C) at a rate of approximately 1.2 PVFD. The split medium contained 10mM phenol (0.91 g l^{-1}) in the first

reservoir. The second reservoir contained (l^{-1}) 1.0g KH_2PO_4, 3.0g K_2HPO_4, 2.0g NH_4NO_3, 0.4g $MgSO_4.7H_2O$ and a 2ml trace element solution (Section 2.4). By convergence of these two pump lines at the glass Y-piece the nutrient concentration was diluted by 50 % to give the desired concentration in the column.

It was found that dissolved oxygen concentrations in the mineral salts reservoir could be elevated to approximately 7.5 mg l^{-1} by sparging with filtered air at a rate of 200 ml min^{-1}. After passing through the 1 mm diameter, 30 cm length, teflon tubing to the base of the column, the oxygen concentration was reduced to 3.8 mg l^{-1}. The maximum concentration of dissolved oxygen in the effluent of the column, containing phenol catabolizing bacteria, was initially approximately 2.1 mg l^{-1}. This was reduced to zero mg l^{-1} within two hours of column flow. This was thought to be due to microbial utilization but the control readings taken from a second tube of approximately 60 cm length directly linked to the oxygen meter showed the dissolved oxygen concentration to decrease to zero mg l^{-1} after eight hours (flow rate set a 30 ml min^{-1}). This decline in dissolved oxygen concentration could not be ascribed to microbial growth on the walls of the tubing since similar results were found with sterile distilled water. Although not fully understood, it seemed likely that the disappearance of dissolved oxygen along the tubing was probably due to dissolution and/or permeability effects since the oxygen concentration could immediately be increased by increasing the flow rate to 50 ml min^{-1}. This was obviously problematic for the proposed experiments since a lack of oxygen would inhibit microbial activity in the columns. An increase in flow rate was also not desirable because of the unacceptable reduction in phenol residence time due to the high dilution rate. At this point, the use of hydrogen peroxide to release dissolved oxygen into the medium was considered.

A.3.14   The use of hydrogen peroxide to deliver O_2 into solution

Before using hydrogen peroxide as a means to provide increased concentrations of dissolved oxygen in the nutrient medium it was important to determine the chemical effect of this compound on phenol. According to Wang (1992) chemical decomposition of phenol can occur

202

in the presence of hydrogen peroxide. To determine the extent to which this would occur six 1 mM solutions were placed in 50 ml sealed bottles containing 0, 3, 6, 9, 12 and 15 % (v/v) hydrogen peroxide, respectively, and incubated at 30°C for 24 hours. After 24 hours the phenol concentrations were compared by gas chromatography analysis (Section 2.3.2).

As shown in Table A.3.1 chemical phenol decomposition was very limited. It is also important to note that the hydrogen peroxide concentrations used here were considerably higher than the 200 mg l^{-1} used during experimentation.

TABLE A.3.1: Effect of hydrogen peroxide concentration on chemical decomposition of phenol after 24 hours incubation.

H_2O_2 % (v/v)	0	3	6	9	12	15
[Phenol] % of original concentration	100	92	96	96	93	92

The addition of hydrogen peroxide to the medium necessitated a modification to the Y-piece configuration at the inflow position of each column. The Y-pieces were then fitted with a third channel (Figure 7.1) to allow the continuous inflow of hydrogen peroxide to give a 200 mg l^{-1} concentration after mixing with the other two lines.

Hydrogen peroxide was also found to absorb light at a wavelength of 277 nm which is the UV wavelength at which phenol analyses were made with a UV spectrophotometer. With a hydrogen peroxide concentration of 200 mg l^{-1} the absorbence (A_{277nm}) was 0.03 in comparison with the 0.987 for 1mM phenol solution. Due to the relatively small interference caused at this wavelength it was ignored in the UV spectrophotometeric analysis.