DEVELOPMENT OF A LABORATORY RIVER MODEL TO DETERMINE THE ENVIRONMENTAL IMPACTS OF KEY XENOBIOTIC COMPOUNDS

by

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ABSTRACT

Microorganisms are increasingly used in toxicological studies to determine potential environmental impacts of xenobiotic compounds. A multi-stage laboratory model was developed to facilitate the examination of environmental impacts of selected pollutants on fundamental cycling processes inherent to aquatic ecosystems, namely, the degradation of organic substances and nitrogen transformations under aerobic conditions. A microbial association representative of riverine ecosystems was enriched for, isolated and cultured within the model. Characterisation of the microbial association were undertaken. Scanning electron microscopy and bright field microscopy revealed that a diverse heterogenous community of microorganisms had established within the model. Successional metabolic events, namely organic carbon catabolism, ammonification of organic nitrogen and the process of nitrification were differentiated in time and space with the microbial association integrity still being retained. The establishment of a microbial association within the model was primarily dependent on: dilution rates, specific growth rates and interactions between microorganisms and the prevailing environmental conditions. Growth-rate independent populations of microorganisms established within the model and were thought to contribute significantly to the metabolic processes within the model. Nitrifying activity was identified as a rate-limiting process within the model.

Following separation of metabolic events, the ecotoxicological impacts of phenol and 2,4-dichlorophenol on the association were assessed. The biological oxidation of ammonia through

to nitrate (nitrification) was found to be a sensitive indicator of perturbation. The model was found to be suitable for testing both acute and chronic intoxication by pollutant compounds as well as for biodegradation testing and the possible evaluation of ecotoxicological impacts of wastewater treatment plants. The main disadvantages of the model arose from its operational complexity, its empirical nature and its impracticality for screening large numbers of compounds. A bioassay based on the inhibition of ammonium oxidation was developed in order to fulfil the requirements for a simple and rapid test protocol for the initial screening of perturbant compounds.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations.

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C.H. Hunter

February 1996

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CHAPTER 1: BIOMONITORING AND THE USE OF MICROORGANISMS IN ENVIRONMENTAL IMPACT STUDIES: LITERATURE REVIEW

1.1 INTRODUCTION

In recent times, increasing pollution by synthetic organic chemicals (xenobiotics) has highlighted the need for determining the environmental impacts of these compounds. Attention has focused on aquatic environments due to their importance as natural resources and their susceptibility to pollutant contamination. Many xenobiotic compounds resist natural degradation and accumulate within the environment where they pose a hazard to biotic life and can seriously impair water quality (Leisinger, 1983). Chemical and physical variables, alone, have proved insufficient in monitoring water quality (Cairns and Pratt, 1989). On the other hand, biological communities have been found to more accurately reflect the prevailing environmental conditions (Herricks and Cairns, 1982).

Biological monitoring has, thus, developed as a means for assessing ecotoxicological impacts of xenobiotic compounds (Roux, van Vliet and van Veelen, 1993). Battery test approaches using representative organisms from different trophic levels have been developed (Herricks and Cairns, 1982). Traditionally, organisms from the higher trophic levels have been considered and used in standardised single species testing protocols (*Standard Methods*, 1989). There are, however, still major shortcomings related to laboratory ecotoxicological testing such as poor extrapolation of laboratory data to field conditions and the lack of representativeness of a single test species to the functioning of an ecosystem as a whole (Dallas and Day, 1993). This has led to continued research into ecotoxicological testing.

Microorganisms have increasingly gained acceptance as test organisms in ecotoxicological testing (Blessing and Submuth, 1993). They play an integral role in ecosystem dynamics and are major determinants of the ultimate fate of xenobiotic compounds in the environment (Grady, 1985; Cairns, Mc Cormack and Niederlehner, 1992). Because of their ubiquitous nature, short life cycles, ease of culturing and rapid response to changes in the environment, they offer a rapid and inexpensive means of ecotoxicological testing (Bitton and Dutka, 1986). In this chapter an introduction to biomonitoring and the various approaches of hazard assessment using microorganisms will be made.

1.2 XENOBIOTIC COMPOUNDS

The term "xenobiotic" is given to synthetically derived organic chemicals. This term has also been used to describe natural compounds that occur in the environment in unnaturally high concentrations due to man's activities (e.g.mining and heavy metal extraction) (Leisinger, 1983).

Before 1984, the United States Environmental Protection Agency (U.S. E.P.A.) had identified 129 quantitatively significant pollutant compounds (Leisinger, 1983). A summary of these is given in Table 1.1. This total constitutes a mere fraction of the 8.5 million chemical compounds registered by the American Chemical Abstract Service (CABS) as of April 1988. Some estimates indicate that approximately 63 000 chemicals are in common use worldwide (Martell, Motekait and Smith, 1988). The annual production of xenobiotic compounds has been estimated to be in excess of 300 million tonnes (Ghisalba, 1983). This figure can be expected to increase with new compounds being developed and marketed every year.

Few of these compounds have ever been tested for either their potential fates in the environment or toxicity to biotic systems (Ghisalba, 1983). It is estimated that only 5 to 10% of known chemicals have been tested for toxicity and $< 1\%$ of the compounds manufactured in the U.S.A have been tested for their toxicity to aquatic organisms (Martell *et al*, 1988). Hazards arising from environmental exposure to xenobiotic compounds and their degradation products are, therefore, of grave concern.

Table 1.1. Ecotoxicologically significant organic pollutants listed by the U.S. E.P.A. (After Leisinger, 1983)

Chemical Class	Number of Compounds
Aliphatics	3
Halogenated aliphatics	31
Nitrosamines	3
Aromatics	14
Chloroaromatics	16
Polychlorinated biphenyls (PCB,s)	7
Nitroaromatics	7
Polynuclear aromatic hydrocarbons	16
Pesticides and metabolites	17

1.2.1. Pollutant Sources and Pathways

Pollution of the aquatic environment has been defined as the introduction by man, directly or indirectly, of substances or energy sources which are potentially hazardous to man and aquatic life and which can impair water quality for domestic, industrial, agricultural and recreational use (Meybeck and Helmer, 1992). Increased industrial and technological advances in developed and developing nations have resulted in xenobiotic compounds becoming significant environmental contaminants in recent years.

Environmental contamination can be differentiated into point source or diffuse (non-point) source pollutant discharges. Point source discharges describe single or contained inputs such as effluent discharge points and rainwater drains. In contrast, diffuse source discharges describe pollutant inputs which are spread over a wide area and cannot be ascribed to a single input and include: runoff from land; groundwater contamination; and atmospheric deposition (Meybeck and Helmer, 1992). An important difference between the two pollutant sources is that point source pollution offers a means of implementing a degree of control where discharges may be subject to collection, treatment or monitoring. Potential pollutant pathways relating to the aquatic environment are shown in Figure 1.1.

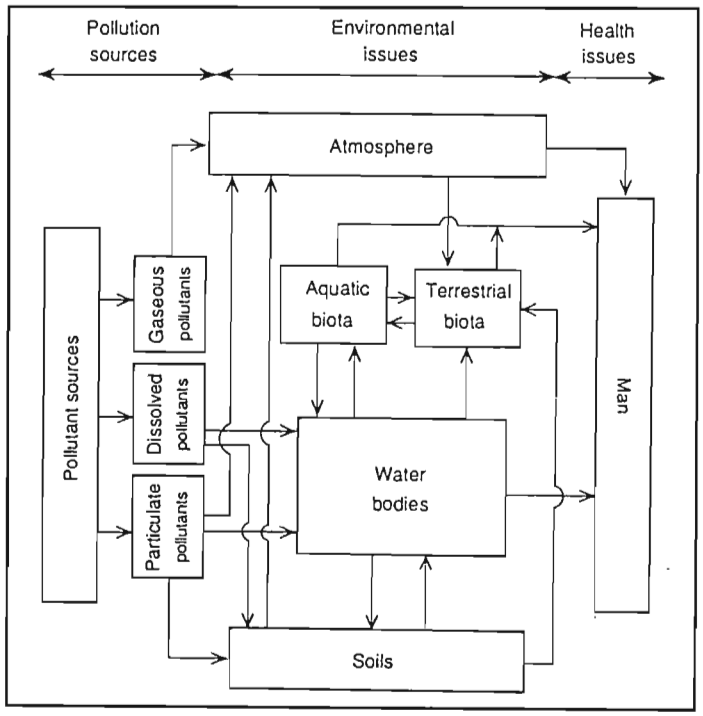


Figure 1.1. Potential pollutant pathways related to the aquatic environment. (After Meybeck and Helmer, 1992).

Pollutants may enter the aquatic environment in a gaseous state, as dissolved solute or in particulate form (Meybeck and Helmer, 1992). Consumer utilization of various chemical products such as pesticides and aerosol propellants, is an important pathway for contaminants entering the environment.

Other pollutant sources include: chemicals entering the environment in the effluents of municipal sewage treatment systems (e.g. hard detergents, solvents); chemicals resistant to biological degradation (recalcitrant) in industrial waste treatment systems and; direct discharges resulting from illegal dumping, spills and accidents (Leisinger, 1983).

Pollution and water quality degradation effectively limit the potential use of a body of water as a resource whether it be at a local, regional or national scale (Lusher and Ramsden, 1992).

Blum and Speece (1992) advocated that, for the implementation of successful pollution control strategies, certain questions must be answered. Firstly, why do certain xenobiotic chemicals manifest toxicity and what characteristics contribute to this? Is this toxicity predictable for compounds found singularly or in mixtures with other chemicals? Are these compounds potentially biodegradable? If so, what conditions are required to successfully degrade potentially toxic chemicals? Lastly, what is the relative toxicity to different treatment processes such as aerobic and anaerobic wastewater treatment processes? The ability to address these questions will facilitate the implementation of suitable water quality standards and related legislation to maintain and manage a desired water quality suited to user requirements.

1.3 HAZARD ASSESSMENT

Concerns for environmental safety resulted in an initiative in the 1970's to establish international protocols for risk assessment of chemicals (Dickson, Maki and Cairns, 1980). Increased awareness of the threats posed by environmental pollution has resulted in extensive research into determining the toxic effects of these pollutants. The concept of exposure and effect of a particular compound, integrated into a hazard assessment has been developed as the basis for a water quality approach to toxic control (Lee and Jones, 1980). Figure 1.2. illustrates the overall concept whereby, through increasing degrees of testing, greater confidence levels can be ascribed to determining environmentally "safe" concentrations of the compound in question.

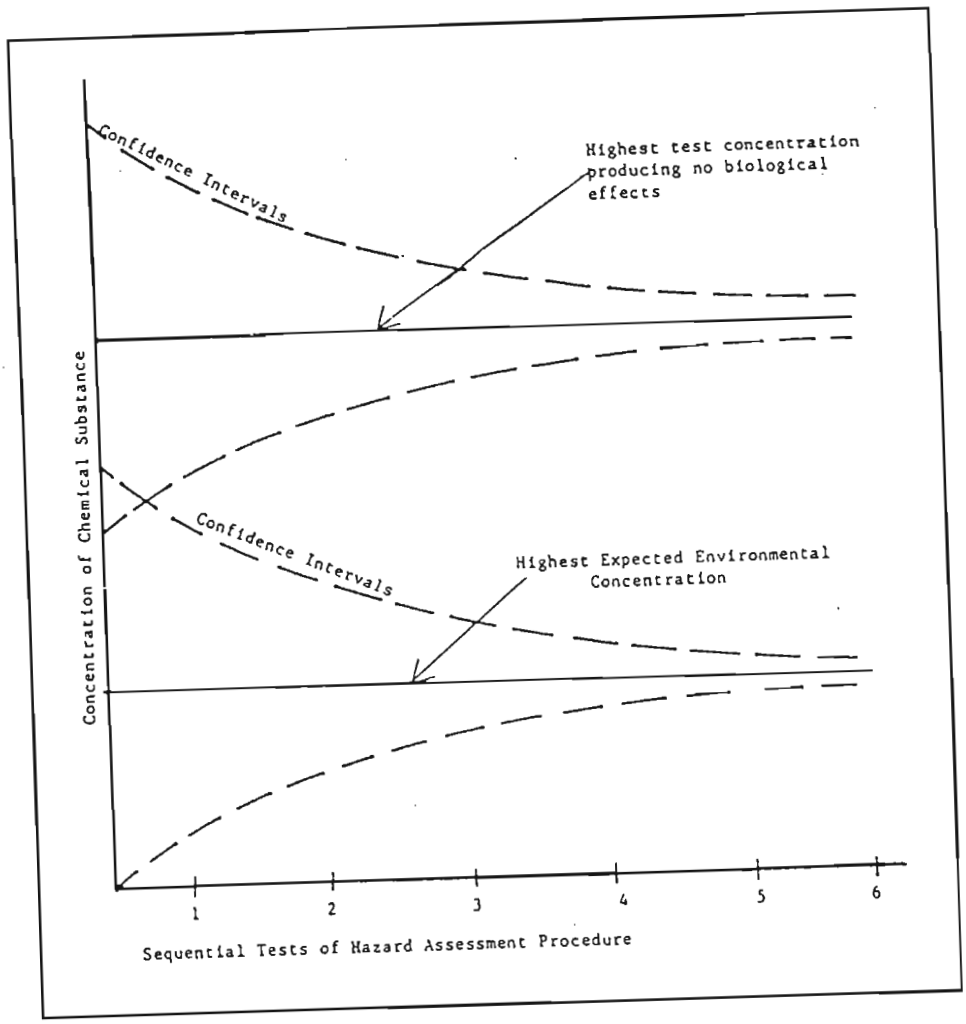


Figure 1.2. Diagrammatic representation of a sequential hazard assessment procedure demonstrating increasingly narrow confidence limits for a no-biological-effect concentration and expected environmental concentration of chemical compoud. (After Lee and Jones, 1980).

Several types of information are required for making hazard assessments. These include: the determination of dose-response relationships; the relationship between structure and activity of the toxicant; the site of toxic action; and the mechanism of the toxic effect (Trevors, 1986). For example, the Organisation for Economic Community Development (O.E.C.D.,

1982) recommended the following quantitative and qualitative information as requisites for evaluating the potential impacts of a xenobiotic compound:

1) Chemical class and structure;
2) Basic physical and chemical properties of the compound;
3) Biodegradability;
4) Degradation products resulting from biodegradation of the original pollutant and their antagonistic or synergistic effects;
5) Anticipated total production;
6) Sources and channels of distribution, purposes and patterns of use;
7) Toxic or other adverse effects of the compounds on water quality and ecology (ie. persistence and bioaccumulation); and
8) Practical situation of discharge to water bodies.

The above example illustrates the importance of a multi-disciplinary approach to assessing the potential hazards of xenobiotic compounds. For effective assessment of the ecological impacts of xenobiotic molecules to be made, rapid screening of chemicals for their treatability, environmental persistence, and toxicity is required so that resources for testing can be focused (or channelled) on potentially hazardous chemicals (Babeu and Vaishnav, 1987).

1.3.1 Quantitative Structure Activity Relationships (QSAR's)

In many instances, predicting and modelling chemical toxicity is hampered by the mediating influence of environmental variables. It is worth noting that in addition to toxicity bioassays, QSAR's have been considered (Nirmalakhandan and Speece, 1988). Based on fundamental physical and chemical properties, QSAR's seek to predict/model chemical toxicity. Various approaches have been explored and include: Linear Solvation Energy Relationships (LSERS); molecular connectivity; and log P-octanol/water partitioning. Such approaches hold promise for the initial screening of large numbers of compounds although the importance of toxicological biomonitoring to corroborate such QSAR findings/predictions cannot be understated.

1.3.2 Biological Monitoring

The assessment of potential ecotoxicological impacts of pollutant compounds is recognized as being vital for evaluating the risk of environmental contamination (U.S. E.P.A., 1985; O.E.C.D., 1987; *Standard Methods*, 1989). National water quality monitoring in South Africa previously focused on physical and chemical parameters to measure ecosystem "health" (Roux *et al*, 1993). Chemical information alone can be misleading in water quality monitoring (Cairns and Pratt, 1989). Environmental variables (e.g. water hardness, pH, dissolved organic matter) can mediate the toxic effect of a chemical (Samiullah, 1990). Chemicals may produce toxicological effects at concentrations below analytical capability (Alexander, 1981). The chemical may be subject to various transformation processes resulting in the generation of intermediates which are more, or less, toxic than the parent

compound (Grady, 1985). Chemicals undergo partitioning in the environment which, in turn, can result in misleading assumptions and measurements being made (Samiullah, 1990).

Biological communities, on the other hand, have been shown to more accurately reflect prevailing environmental conditions (Herricks and Cairns, 1982; Cairns *et al*, 1992). As a result, biological monitoring, including ecotoxicological testing, bioassessments, behavioural bioassays and bioaccumulation determinations have all developed as important tools for assessing environmental conditions and monitoring effluent discharge regulations (*Standard Methods*, 1989; Roux *et al*, 1993). In this study, the primary focus was on the role of toxicity bioassays in water quality monitoring and thus the discussion is restricted to this aspect of biological monitoring.

1.3.3 Ecotoxicological Bioassays

Aquatic ecotoxicology has developed as an interdisciplinary science, incorporating toxicology with environmental chemistry and ecology (van der Gaag, 1991). The term "bioassay" (toxicity testing) is used to describe laboratory-based monitoring of pollutant compounds (Roux *et al*, 1993). Toxicological bioassays have been developed as a means to evaluate, describe, monitor and predict the environmental impacts of pollutants to organisms and ecosystems (Cairns and Pratt, 1989). Biological systems of varying complexity are used (Maltby and Calow, 1989) to quantify the toxic effects of pollutant compounds, singly or in mixtures, (Enserinck, Maas-Diepeveen and van Leeuwen, 1991) and also to serve as biological indicators of effluent and receiving water quality (Cairns and Pratt, 1989).

Representatives from most trophic levels have been used in toxicity screening tests (Cairns and van der Schalie, 1980). The principal methods for assessing toxicity in aquatic environments are summarised in Table 1.2. Standardised bioassay tests have been extensively reviewed in the literature (U.S. E.P.A., 1985; O.E.C.D., 1987; *Standard Methods*, 1989). In South Africa, three-tier toxicity testing has been recommended involving a bacterium or cyanobacterium, an alga and a daphniid (Roux *et al*, 1993). Testing procedures have also been developed with bacteria, protozoans, macro-invertebrates and fish (Dallas and Day, 1993). The use of a series of "battery test" protocols with a number of different test organisms is advocated to ensure that the toxicity of a compound is not underestimated (Dutka and Kwan, 1981).

Different approaches are used in ecotoxicological testing depending on the information required from the bioassay. These requirements were summarised by Friedrich, Chapman and Beim (1992) and include: studies of the toxic effects of pollutants on specific organisms, populations and biocenoses; determination of the minimum inhibitory concentration of a compound within the environment; and studies of the modes of action and transformation of toxic substances in organisms in the environment.

Table 1.2. Methods for assessing toxicity in aquatic environments. (After Freidrich *et al,* 1992)

Aquatic Organisms Affected		Methods for Assessing Toxicity	
Trophic Level	Principal Organisms	Principal Methods	Additional Methods
Decomposers	Bacteria, fungi, protozoa	Biological oxygen consumption, nitrification	Decomposition of cellulose, lignin and other organic matter
Primary producers	Algae, macrophytes	Growth rates, reproductive capacity, oxygen consumption, chlorophyll fluorescence	Photosynthesis and respiration rates, morphology, histology and growth
Secondary producers	Invertebrates and some fish species	Survival rate, reproductive capacity, survival of progeny	Feeding, growth and respiration rates, morphology
Secondary consumers	Most fish and some invertebrates	Fertilisation rate, embryological development, larval survival, life cycle success	Feeding,growth and respiration rates. Biochemical analyses e.g.,hormones, haemoglobin, morphology and histology

The choice of organism(s) used in a bioassay should be based on its/their sensitivity and representativeness of the ecosystem under consideration (Friedrich *et al*, 1992). No one group should be viewed as the overall indicator organism (Cairns *et al*, 1992). Sensitivities can vary between organisms, groups and populations (Dallas and Day, 1993). Organisms, typically, exhibit different toxicity thresholds to the same or similar compounds. Toxicity

tests often exhibit variability when replicated. This has been attributed to factors such as species type, test conditions and the number of organisms used in the test. The threshold concentrations for specific compounds cannot be universally applied due to complex interactions between toxicants, test organisms and the ambient test conditions (Lankford, 1992).

Questions have arisen with regards to the suitability of using single species as test organisms since their response to a toxic dose does not necessarily reflect the functioning of the ecosystem as a whole (Freitsch, 1991). Cairns *et al* (1992) advocated that test systems incorporating biological communities should be used since they incorporate ecologically important elements such as species interactions and energy flow. The value of such an approach must be weighed against unfavourable elements such as difficult interpretation of results, increased cost, increased variability and decreased reproducibility.

Data from bioassays are commonly expressed as $IC_{50's}$ or $EC_{50,s}$ which are respectively the inhibitory or exposure concentrations, at which a 50% reduction in organism activity occurs when compared to a control under identical test conditions (Blum and Speece, 1992). The toxic effect produced by the test chemical or chemicals may be lethal, mutagenic or inhibitory to physiological activity. Bioassays may be made as acute (short-term) or as chronic (full life-cycle or long-term) tests (*Standard Methods*, 1989). Chronic effects on organisms are usually manifest at concentrations lower than those obtained from acute (short-term) bioassays (Savino and Tanabe, 1989). As a result, acute testing should be viewed as a rapid means of screening pollutants and for providing an estimate of toxicity and not for setting concentration limits for the environment (*Standard Methods*, 1989).

For bioassays to be of value, Tebo (1985) suggested that they fulfil certain criteria:

1) They should be easy to interpret where a laboratory response can be related to a potential environmental hazard;
2) Responses in the laboratory should have an extrapolative value which relates to the natural state;
3) The sensitivity of the bioassay should be a function of the objectives of the study;
4) The variability of a bioassay must be determined to ensure that its sensitivity is sufficiently high so that impacts can be detected; and
5) There must be sufficient replicability to facilitate inter-laboratory and intra-laboratory levels of standardisation.

1.4 MICROBIAL TOXICOLOGY

The field of microbial toxicology is a relatively new discipline and has evolved over the last two decades (Bitton and Dutka, 1986). Microbial communities exhibit structural and functional complexities which make them useful for studying the dynamics of ecological communities, both in the presence and absence of an anthropogenic stress (Cairns *et al*, 1992). The use of microbial communities offers a number of advantages over using organisms from higher trophic levels. These include: the significant role that microorganisms play in ecosystem dynamics; their ubiquitous nature; sensitivity to anthropogenic stress; greater species diversity; minimisation of scale effects; potential for standardisation; and their amenability to animal rights organisations (Cairns *et al*, 1992). Many of these tests are rapid, reproducible, cost effective and require little space making them useful screening tools for aquatic toxicologists and microbial ecologists (Blum and Speece, 1992). Typically,

microorganisms have been used to determine the impacts of a wide range of toxic organic and inorganic compounds in natural waters, soils and in sewage treatment processes (Eckenfelder and Musterman, 1992). Extensive reviews of the application of microorganisms in toxicity testing have been given by Pritchard and Bourquin (1984) and Bitton and Dukta (1986). Toxicity screening tests have been developed with bacteria, fungi (including yeasts) and algae.

1.4.1 Choosing a Suitable Bioassay

Rapid methods for assessing toxicological effects of chemicals are needed to simplify the task of screening large numbers of potentially toxic chemicals. The test organisms used in a bioassay should be both sensitive to and representative of the environment being studied (Trevors, 1986). Points which should be considered include: the choice of test organism; use of pure or mixed cultures; inoculum size; use of adapted or non-adapted inoculum; the growth medium used and the concentration of the test compound (Bitton and Dukta, 1986).

No single species can be expected to be an indicator of all microbial activities or potential problems. Natural isolates lack standardisation with different strains of varying sensitivities often being present. Experiments using monocultures have the advantage of being easier to standardise, replicate and interpret the experimental data. These are usually at the cost of being less representative (Cairns *et al*, 1992). Monoculture studies tend to be made under unrealistic test conditions which do not incorporate the mediating effect of the natural environment and associated organisms (Cairns *et al*, 1992). Mixed cultures on the other hand are more likely to approximate the heterogeneous conditions found in natural environments.

However, standardisation and replicability become considerably more complicated as in the case of interspecies interactions (Cairns *et al*, 1992). One way of circumventing this problem is to simulate natural environmental conditions using microcosms where it is possible to incorporate different environmental variables under controlled conditions (Porcella, Adams, Medine and Conwan, 1982).

Natural isolates lack standardization with different strains with varying sensitivities being present. Environmental impacts of pollutants on a test organism are mediated by physico-chemical factors prevalent in the conditions under which the bioassay is made. To incorporate these factors microcosms and laboratory model systems have been developed (Wimpenny, 1988)

In natural environments, microorganisms usually survive under nutritionally poor conditions (Jannasch, 1969). It has been suggested that testing protocols for bioassays should be carried out under similar nutrient conditions (Trevors, 1986). The use of adapted or non-adapted microorganisms for the bioassay poses problems in interpreting the experimental data (Bitton and Dutka, 1986). Xenobiotics which are, or can potentially be, released into the environment on a regular basis can justify the use of preadapted inocula. Conversely, compounds which are likely to contaminate the environment infrequently lend themselves to bioassays in which non- adapted inocula are used (Bitton and Dutka, 1986).

1.4.2 Impacts and Fates of Xenobiotics

Few chemicals entering the environment remain completely inert. They may be subject to a variety of biotic and abiotic transformation processes which affect the toxicity and partitioning of different forms of the compound (Baughman and Burns, 1986).

The environmental impacts and fates of a xenobiotic compound are, therefore, functions of the properties of the compound and its interaction with physical, chemical and biotic elements of the ecosystem (Lankford, 1992). Environmental parameters such as pH, temperature, redox potential (E_h), water activity (a_w), and the presence of ions, clay minerals and particulate organic matter, all contribute to the toxicity of a particular compound.

Environmental pH affects the metabolic state of a microorganism and influences the chemical form of the toxicant which may exert a differential toxicity. Temperature will influence compound solubilities and have an effect on the metabolic activities of microorganisms present. The E_h can influence the valencies of heavy metals which affect solubility and mobility. Inorganic anions and cations may reduce or enhance toxicity by affecting the chemical form of the toxicant or may compete with toxicants for adsorption sites on microbial cells. The presence of clay minerals and particulate organic matter may reduce toxicity through adsorption (Lankford, 1992).

1.4.3 Mechanism of Toxicity for Various Organic Compounds

Microorganisms appear to be sensitive sensors to chemical toxicity since they respond relatively quickly to changes in the environment (Cairns *et al*, 1992). Various mechanisms have been proposed which result in inhibitory effects to bacteria (Bitton and Dutka, 1986; Sikkema, De Bont and Poolman, 1995).

These include:

1) Damage to genetic material which may lead to protein denaturation;
2) Disruption of cell membranes causing leakage of cellular material;
3) Displacement of cations (eg. Na^+, Ca^+) from absorption sites on bacterial cell walls (eg. by acids and alkalis);
4) Blockage of bacterial chemoreceptors with resulting inhibition in degradative processes; and
5) Inhibition of enzyme activity.

Inhibition of enzyme activity can be categorised into three types, namely competitive, non-competitive, and uncompetitive, based on their effects on the kinetic parameters, maximum growth rates (μ_{max}) and growth rate constants (K_s) of the microorganisms (Bitton, 1994).

Several categories of xenobiotic compounds are known for their toxicity to microorganisms (Hedgecock, 1967):

1) Halogen compounds have long been recognised for their disinfectant qualities and represent an important group of toxic compounds. Chlorine disrupts sulphydral groups of enzymes and causes alterations in protein structure. Iodine reacts with organic compounds forming iodophores which are then able to disrupt protein structure through direct iodination or oxidation of constituent groups. Bromine has been found to act in a similar fashion;

2) Heavy metals are a second important category of inhibitory compounds. Inhibitory concentrations of heavy metals (e.g. Cu, Zn, Cd, Mg) can lead to interference with cell wall synthesis, decreased enzyme activity, and deactivation of DNA and RNA;

3) Phenols and phenolic compounds also constitute an important group of anti-microbial compounds. Unsubstituted phenols exhibit a high degree of surface activity and act by disrupting cell membranes and inhibiting oxidase enzymes associated with surface membranes. Protein precipitation and the uncoupling of oxidative phosphorylation arise when phenols enter cellular tissue;

4) Alcohols and quaternary ammonium compounds have also been found to disrupt cell membranes. Alcohols are thought to act in much the same way as phenols causing inhibition to respiration and phosphorylation. Quaternary ammonium compounds are also thought to inhibit bacterial oxidase and dehydrogenase systems causing protein denaturation and enzyme suppression; and

5) Changes in pH resulting from the presence of acids or alkalis also have an inhibitory effect on bacterial growth. High concentrations of H^+ or OH^- ions are known to displace essential ionic species such as Na^+ and Ca^+ from their adsorption sites on the cell. This displacement results in a deficiency of essential ions required for bacterial growth. Hydrolytic reactions involving the hydrogen or hydroxyl ions have also been shown to damage cell surfaces.

Toxicant action is, generally, concentration dependent. For example, phenol, which is metabolised at low concentrations, becomes toxic at higher concentrations (Bitton and Dutka, 1986). Another consideration is the presence of other chemicals in solution. Mixtures of xenobiotic compounds may show synergistic effects, i.e. exhibit greater impacts than the sum total of the individual constituents (Nirmalakhandan, Argulgnanendran, Mohson, Sun and Cadena, 1994).

A summary of factors controlling the fate of xenobiotics in the environment is listed in Table 1.3.

Table 1.3. Factors controlling the fates of xenobiotics in the environment. (After Bitton, 1994).

Factors	Consequences
Chemical Factors	
Molecular weight or size	Limited active transport
Polymeric nature	Extracellular metabolism required
Aromaticity	Oxygen requiring enzymes
Halogen substitution	Lack of dehalogenating enzymes
Solubility	Competitive partitioning
Toxicity	Enzyme inhibition, cell damage
Environmental Factors	
Dissolved oxygen	O_2-sensitive and O_2-requiring enzymes
Temperature	Mesophilic temperature optimum
pH	Narrow pH optimum
Dissolved carbon	Concentration-dependence of organic/pollutant complexes for growth
Particulates, surfaces	Sorptive competition for substrate
Light	Photochemical enhancement
Nutrient and trace elements	Limitations on growth and enzyme synthesis
Biological Factors	
Enzyme ubiquity	Low frequency of degradative species
Enzyme specificity	Analogous substrates not metabolised
Plasmid-encoded enzymes	Low frequency of degradative species
Enzyme regulation	Repression of catabolic enzyme synthesis, required acclimation or induction
Competition	Extinction of low density populations
Habitat selection	Lack of establishment of degradative populations

1.4.4 Biodegradation of Xenobiotics

Biodegradation is a major determinant of the fate of organic chemicals in the aquatic environment (Alexander, 1981).

Microorganisms are especially suited to biodegradation due to their abundance, species diversity, catabolic versatility, high metabolic activity and their ability to adapt to a wide variety of environmental conditions (Grady, 1985). Furthermore, mineralization of an organic compound to inorganic products can normally be attributed entirely to microbial activity (Alexander, 1981).

Resistance of chemicals to biodegradation arises from a number of factors (Alexander, 1985; Bitton,1994):

1) Molecular structure (e.g. halogen substitution);
2) Absence of suitable permeases to allow for compound uptake into a cell;
3) Unavailability of the compound to microbial action resulting from insolubility or adsorption;
4) Unfavourable environmental conditions (e.g. temperature, light, pH, O_2 or Eh potential);
5) Insufficient nutrients or growth factors available for microbial growth;
6) Toxic properties of pollutant compounds interfering with biodegradability; and
7) Low substrate concentrations whereby certain microorganisms in the environment may not be able to assimilate and grow on organic substances below a threshold concentration.

Biodegradation can be divided into several categories based on the response(s) of the microbial population exposed to the compound:

1) The compound may be readily utilised as carbon and energy sources. This results when the necessary enzymes for metabolism are constitutive or are readily induced (Grady, 1985);

2) The compounds are utilised following a lag or acclimation period. Several authors (Spain, Pritchard and Bourquin, 1980; Wiggins, Jones and Alexander, 1987) have suggested that this lag phase can be attributed to: the induction or derepression of enzymes specific to the degradative process; the time required for a population capable of biodegradation to increase sufficiently in size to be able to have a significant effect; or, the acclimation to a compound as a result of genetic changes through selective pressures;

3) The chemicals are subject to degradation without being used as a carbon or energy source. This is thought to be attributable to the action of enzymes with broad substrate specificity and has been termed co-oxidation or co-metabolism (Raymond and Jamison, 1971; Alexander, 1985); and

4) The organic compounds which resist degradation are termed persistent or recalcitrant (Grady, 1985). Compounds which are inherently biodegradable may persist in the environment due to unfavourable environmental conditions or variables which can render the compound biologically unavailable to degrading populations (Alexander, 1981).

Metabolic intermediates may lose toxicity or, in some instances, result in increased toxicity to microbial populations. Xenobiotics may be present in concentrations which are inhibitory resulting in potentially degradable compounds being classified as non-biodegradable (Reynoldson, 1987). To avoid toxicity influence on biodegradability, Reynoldson (1987) recommended that biodegradability testing should be undertaken at 10% of the EC_{50} value for the compound.

Biodegradation is of primary importance in removing organic pollutants, arising from wastewater effluents, from rivers and inland waters. Disturbance of this amelioration process through the direct introduction of toxic substances (eg. heavy metals, cyanides an organic toxicants) can result in environmental impairment.

1.4.5 Microbial Bioassays

Microbial toxicity bioassays have been developed to serve a number of functions (Bitton, 1994):

1) The screening of wastewaters for toxicity prior to wastewater treatment;
2) The monitoring of effluent discharges and aquatic toxicity;
3) The evaluation of process control in the pretreatment and detoxification of industrial wastes;
4) The detection and assessment of toxic inhibition of biological treatment processes; and
5) As assays in toxicity reduction evaluations (TRE) to characterise and screen for problem toxic chemicals.

Bitton and Dutka (1986) divided the various toxicity screening tests into four main categories: assays based on bacterial bioluminescence; assays based on enzyme activity; assays based on viability or growth of bacteria; and "ecological" effect assays.

a) Assays Based on Bacterial Luminescence

The ability of bioluminescent bacteria to emit light has resulted in their application in bioassay testing procedures. These bacteria are usually associated with marine environments, and live freely or in association with higher organisms. Three major types have been reported, *Photobacterium fisheri, P. phosphoreum* and *Beneckea harvery* (Hastings and Nealson, 1978). A toxicity assay, commercialised under the name Microtox™, utilises freeze-dried cultures of the bioluminescent marine bacterium *Photobacterium phosphoreum* (Bulich, 1986). Microtox™ has been used widely for determining the toxicity of wastewater effluents, complex industrial wastes, fossil fuel process water, sediment extracts and landfill leachates (Bulich, 1986). Good correlations with fish, *Daphnia* sp. and algal bioassays have indicated that this bioassay is one of the more sensitive microbial bioassays available (Giesy, Graney, Newsted, Rosiu, Benda, Kreis and Horvath, 1988). However, it appears that it is not as sensitive to toxic heavy metals. This bioassay has been the most widely used commercial test for assessing toxicity in wastewater treatment processes (Bitton, 1994).

b) Assays Based on Enzyme Activity

Inhibition of enzyme activity by pollutant chemicals has been suggested as one of the main causes of toxicity to microbial cells (Iverson and Brinkman, 1978). This has led to numerous studies being made to determine the effect of pollutant toxicity on enzyme activity. Enzymic assays such as ATPase, esterase, urease, luciferase, ß-galactosidase, ß-glucosidase and phosphatase have all been used for assessing toxicity in aquatic environments (Bitton and Koopman, 1986). Dehydrogenase enzymes, which catalyse electron transport system (ETS) activity in the cell, have received much attention (Bitton and Dutka, 1986). The toxicity assays are based on colour reactions involving the reduction of specific dyes which act as indicators of ETS activity. Indicator dyes which have been used include: methylene blue; triphenyl tetrazolium chloride (TTC); tetrazolium blue; reasazurin; and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl) tetrazolium chloride (INT) (Bitton and Koopman, 1986). Table 1.4. lists short-term toxicity assays, based on enzyme activity or biosynthesis, which have been developed.

Once developed, enzyme assays offer simple and rapid methods for assessing chemical toxicity. However, their application is limited because enzyme assays have, generally, been found to be less sensitive when compared to other assays such as Microtox™ and bioassays involving higher trophic levels (Bitton and Koopman, 1986).

Table 1.4. Short-term toxicity assays based on enzyme activity or biosynthesis. (After Bitton, 1994)

Enzyme	End Point Used
Dehydrogenases	Measure reduction of oxido-reduction dyes such as INT or TTC.
ATPase	Measure phosphate concentration using ATP as a substrate.
Esterase	Nonfluorescent substrates degrade to fluorescent products.
Phosphatase	Measure inorganic phosphate.
Urease	Measure ammonia production from urea.
Luciferase	Measure light production using ATP as a substrate.
ß-galactosidase	Measure hydrolysis of o-nitrophenyl-ß-D-galactoside.
α-glucosidase	Measure hydrolysis of *p*-nitrophenyl-α-D-glucoside.

c) Assays Based on the Measurement of Growth Inhibition, Respiration and Viability

Microorganisms play an integral role in ecosystem dynamics. Pollutants which adversely effect natural populations of microorganisms can potentially disrupt these important roles (Trevors, 1986). Hence, bacterial toxicity assays for aquatic environments have been developed based on the measurement of growth inhibition, respiration and viability of the cells (Trevors, 1986). Representative methods used as bacterial inhibition bioassays are listed in Table 1.5.

Table 1.5. Representative methods used in bacterial inhibition bioassays. (After Bitton, 1994)

Bioassay	Basis for the Test
Spirillum volutans	Toxicants cause loss of motility.
Growth inhibition	Measure growth inhibition of pure (e.g. *Pseudomonas*) or mixed cultures.
Viability assays	Measure the effect of toxicants on the viability of bacterial cultures on agar plates.
ATP assay	Measures inhibitory effect of toxic chemicals on ATP levels in microorganisms.
Respirometry	Measures effect of toxicants on microbial respiration in environmental samples.
Nitrobacter bioassay	Measures inhibition of nitrite oxidation to nitrate.
Microcalorimetry	Measures decreases in heat production by microbial activity.

Respiration rate, or rate of oxygen uptake, are useful parameters for assessing the metabolic state of aerobic microorganisms. They are easily measured and cultures respond rapidly to the presence of toxic inhibitors. Measurements of respiration rate are made by manometric methods, electrolytic respirometers, or through the direct measurement of dissolved oxygen by titrimetric methods or oxygen electrodes (King and Dutka, 1986).

d) Ecological Effect Assays

Pollutants which adversely affect natural populations of microorganisms can, potentially, disrupt essential nutrient and mineral cycling processes such as the carbon, nitrogen, phosphorus and sulphur cycles (Pritchard and Bourquin, 1984; Bitton, 1994). In 1977 the U.S. Environmental Assessment Act proposed that ecological effect assays should be developed to assess the impacts of environmental pollutants on the functioning of the ecosystem as a whole (Stern, 1980).

Heterotrophic activity, nitrogen transformations, sulphate reduction and methanogenesis have all been considered (Pritchard and Bourquin, 1984). Blum and Speece (1992) developed a toxicity data base using a common set of industrial organic chemicals assayed by aerobic heterotrophs, *Nitrosomonas* sp., *Nitrobacter* sp., methanogens and Microtox™. Nitrification has been identified as one of the processes most sensitive to environmental toxicants. Toxicity bioassays based on the inhibition of both *Nitrosomonas* sp. and *Nitrobacter* sp. have been developed in relation to wastewater samples (Williamson and Johnson, 1981; Alleman, 1986; Blum and Speece, 1991).

Ecologically important groups of bacteria have thus found application as biological indicators in toxicity bioassays. Toxicity studies made with these groups of organisms should directly reflect the ecological impact that will occur in a perturbated system.

Comparative studies of microbial and aquatic organism toxicity testing systems have, generally, shown that microbial assays are less sensitive than those for invertebrates, fish and

algae (Chang, Taylor and Leach, 1981; McFeters, Bond, Olson and Tchan, 1983; Yoshioka, 1987). Comparisons of different microbiological screening tests (*Spirillum* sp. motility test, nitrification, methanogenesis, inhibition of respiratory activity and dehydrogenase activity in activated sludge, and Microtox™) all identify Microtox™ as the most sensitive (Dutka, Nyholm and Peterson, 1983; Walker, 1988; Blum and Speece, 1992). Blum and Speece (1992) found that significant correlations occurred between the Microtox™ and *Nitrosomonas* sp. sensitivity to toxicant exposure. They concluded that toxicity to nitrification in many cases would correlate with aquatic toxicity.

1.4.6 Laboratory Micro-Ecosystems Approach

Laboratory micro-ecosystems have been used and developed to study microbial growth and interactions as they occur in natural habitats (Wimpenny, 1988). Since the early 1970's micro-ecosystems have gained importance for testing the fate and behaviour of xenobiotic compounds in the environment (Isensee, 1986). This form of testing has arisen from the need to generate ecotoxicological data which have a better extrapolative value to the environment. Environments are complex and dynamic systems which are subject to constantly changing variables which make *in situ* investigations difficult in terms of data interpretation (Parkes, 1982). Investigations into the effects of pollutant stress are of particular concern since it is difficult to differentiate natural variations from changes induced by pollutant stress (Fry, 1982).

Models of the aquatic environment have been used in ecotoxicological studies to determine the potential impacts of xenobiotics under simulated *in situ* conditions (Porcella *et al*, 1982;

Pratt, Bowers and Cairns, 1990; Freitsch, 1991; Scholz and Müller, 1992). Periphyton communities, algae, diatoms, macroinvertebrates, protozoa and microbial communities have all been used in this context (Elstad, 1986).

Laboratory micro-ecosystems may be differentiated into two types, namely, microcosms and model systems (Parkes, 1982). Microcosms are laboratory-based systems which attempt to replicate the environment, or part thereof, as closely as possible. Examples of microcosms include soil columns, sediment cores, artificial streams, aquaria and ponds. Model systems, on the other hand, operate under conditions which are functionally or fundamentally similar to the whole or part of the environment under consideration. Such model systems, typically, range in complexity from simple batch flasks to multi-stage and multi-phase chemostats (Parkes, 1982; Wimpenny, 1988). These models are then used to provide simple analogues of the natural ecosystems in which characteristic structural and functional properties can be simulated and studied (Porcella *et al*, 1982).

1.5 SUMMARY AND OBJECTIVES

Microorganisms have increasingly found application as toxicological bioassays to determine the environmental impacts of xenobiotic compounds (Blessing and Submuth, 1993). The advantages of using microorganisms as test species include their ubiquitous nature, short life cycles, rapid response to changes in the environment, stability and ease of culturing and the significant role they play in ecosystem dynamics (Bitton and Dutka, 1986).

In a move away from standardized single species testing protocols, several schools of thought

have advocated the use of microbial communities to assess potential impacts of pollutant compounds (Cairns *et al*, 1992). The rationale for using microbial communities for impact assessments is that they provide information not available from standard single species tests. In particular, such approaches can incorporate ecologically important elements such as species interactions and energy flow. As a result, many of the end points of testing give a closer approximation to events as they occur *in situ* (Cairns *et al*, 1992).

To incorporate these dimensions, laboratory model ecosystems have increasingly found application in ecotoxicological studies to determine the environmental impacts of anthropogenic substances on aquatic ecosystems (Porcella *et al*, 1982; Freitsch, 1991; Scholz and Müller, 1992). Ranging in size and complexity, laboratory models have sought to provide simple analogues of natural ecosystems in which inherent characteristic structural and functional properties can be simulated (Wimpenny, 1988).

In this research programme the principal objective was to develop a multi-stage laboratory model system to examine the environmental impacts of selected priority pollutants on a representative microbial associations found in aquatic ecosystems. Fundamental cycling processes, inherent to aquatic ecosystems, namely, the degradation of organic substances and nitrogen transformations under aerobic conditions, were chosen for the study. In the laboratory model, qualitative and quantitative responses of the component species of the microbial association to perturbations were the criteria chosen to assess and quantify toxicity, adaption to and degradation of the selected xenobiotic molecules.

CHAPTER 2: DESIGN AND CONFIGURATION OF A MULTI-STAGE LABORATORY RIVER MODEL

2.1 INTRODUCTION

In this chapter the configuration of a continuous-flow model system, used to culture a microbial association capable of fundamental cycling processes inherent to aquatic ecosystems, namely, the degradation of organic substances and nitrogen transformations under aerobic conditions, is described. A continuous-flow model system was chosen since it can incorporate important features of aquatic ecosystems, namely, the dynamic changes that occur in time and space with continuous inputs and outputs occurring simultaneously (Wimpenny, 1988). Various characteristics such as low dilution rates coupled with low nutrient concentrations can be employed to enrich for autochthonous organisms (Gottschall and Dijkhuizen, 1988). To incorporate spatial and temporal heterogenic components into the model, so that changes in space and time could be physically differentiated, a continuous multi-stage system was chosen. The model described here was adapted from a design used to examine self-purification in aquatic ecosystems (Freitsch, 1991). Previous use of this model type has allowed successional changes in microbial associations occurring during self-purification processes to be elucidated (Freitsch, 1991).

As previously stated (1.5) the aim of developing the multi-stage model was to facilitate examination of the impacts of selected pollutants on the representative association established within the model. Phenol was chosen as a model molecule for the study.

Phenol is classified as a hazardous substance and is listed by the United States Environmental Protection Agency and the United Kingdom's Department of the Environment (D.O.E.) as a priority pollutant (Watson-Craik, 1987). It is a common constituent of effluents from oil refining factories, paper pulp processing and coal liquefaction industries (Tibbles and Baecker, 1989). It forms the basic structure of many synthetic organic compounds and is also used as a precursor in the synthesis of more complex molecules such as medicinal polymers, resins, photodevelopers, perfumes, explosives, disinfectants and pesticides (Artiola-Fortuny and Fuller, 1982).

Phenol is a relatively water soluble and non-volatile aromatic compound which add to its pollutant properties. When present in sufficient concentrations it has a detrimental effect on the quality and ecological conditions of water bodies. The W.H.O. guideline for permissible phenol concentrations in drinking water is 0.001 mg l^{-1}. The toxic effects on fish have been observed at concentrations of $\geq$0.01 mg l^{-1} (Chapman and Kimstach, 1992).

2.2 LABORATORY MODEL CONFIGURATION

The overall design of the multi-stage system has been described previously (Hunter, Howard, Bailey and Senior, 1995). The model differs from that of Freitsch (1991) in configuration, length and arrangement.

The model (Figures 2.1 A and B) consisted of four identical channels (A, B, C, and D), 3 m in length and 36 mm wide, each consisting of 75 chambers. The chamber vessels had an operational volume of 122 m*l* (36 x 36 x 95 mm) and, thus, the total volume of each channel

was 9.15 *l*. The channels were constructed from 5 mm Plexiglass and built in 6 unit blocks each consisting of 2 x 25 chambers. The units were supported by a steel framework and were arranged in tiers (Figure 2.1A and Plate 2.1). Each unit was angled at 15° to create a weir flow effect to ensure mixing of the nutrient medium within the individual chambers. Construction of the model was undertaken by the Scientific Workshop of the University of Natal, Pietermaritzburg.

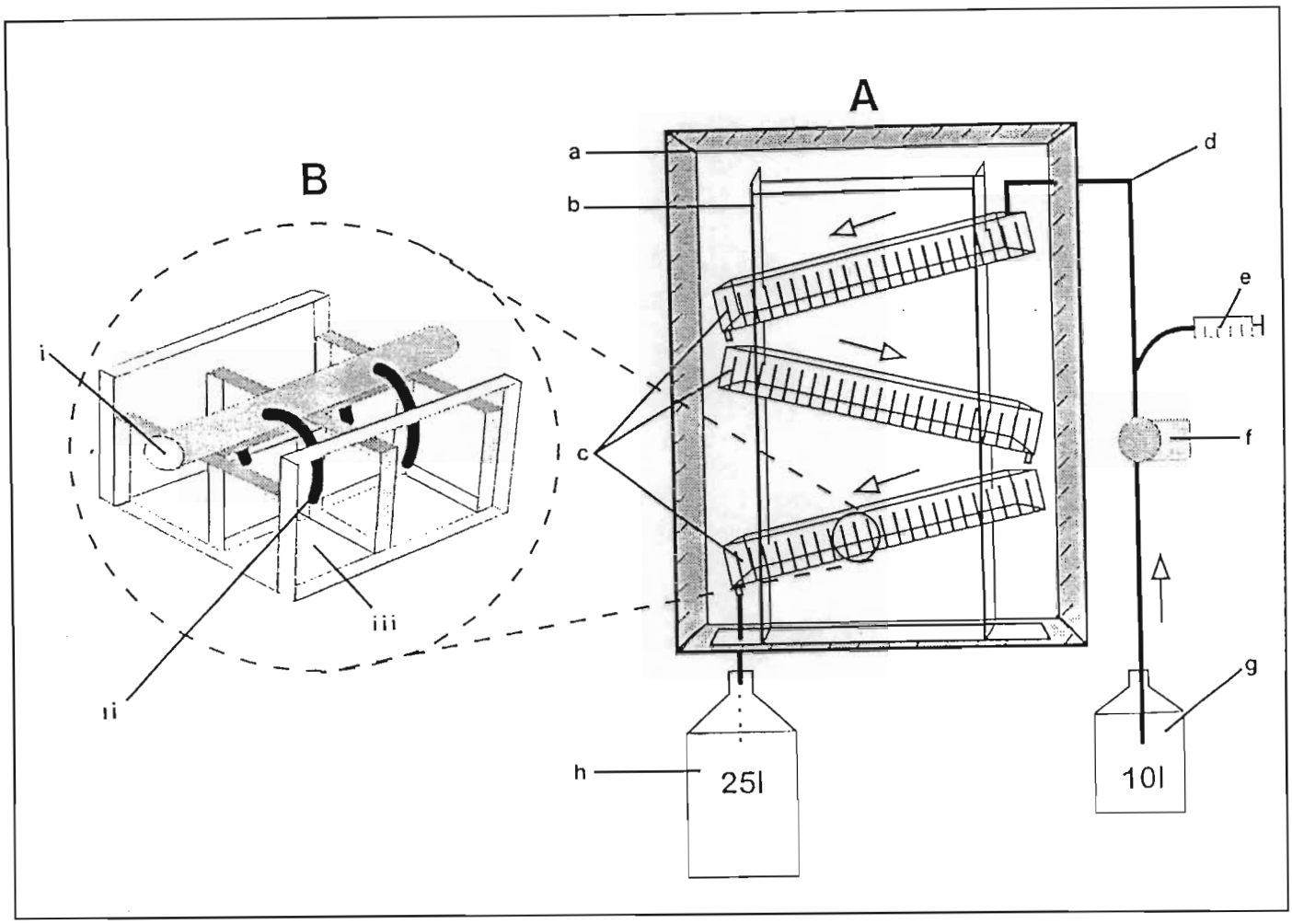


Figure 2.1 A. Diagram illustrating side view of the multi-stage laboratory model consisting of:
a - insulated dark box;
b - steel frame;
c - unit blocks of 2 x 25 chambers;
d - silicone tubing (i.d. 2.8 mm);
e - flow meter;
f - flow inducer;
g - influent medium; and
h - effluent reservoir

Figure 2.1 B. Three-dimensional view through two adjacent channels illustrating the system of interconnected tubing used to aerate each chamber with:
i - central hose pipe (i.d. 15 mm);
ii - irrigation tube (i.d. 3.5 mm); and
iii - chamber (36 x 36 x 95 mm)

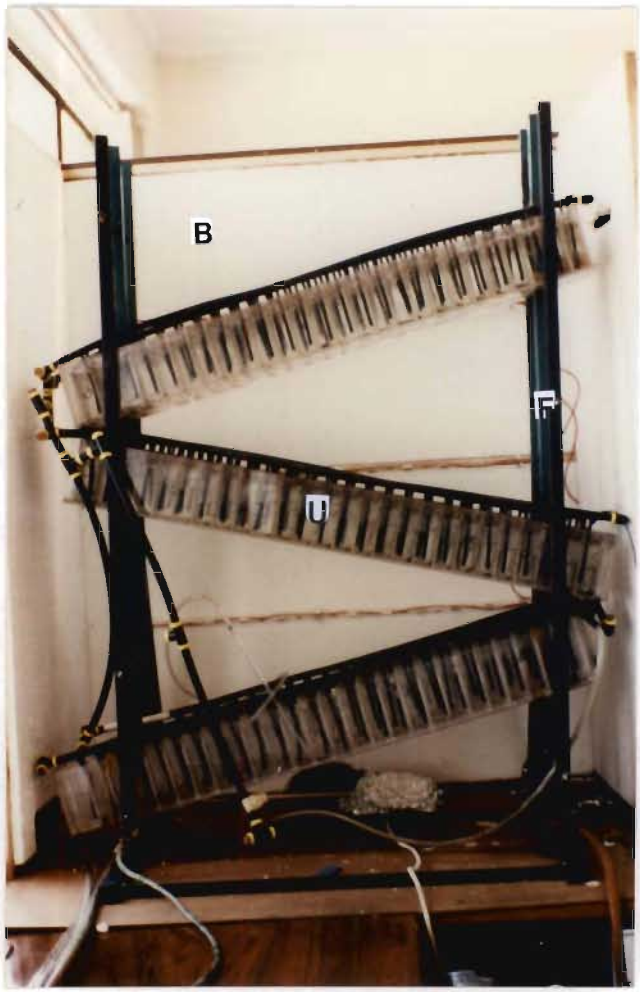


Plate 2.1. Multi-Stage Laboratory River Model housed in its insulated box (B) showing unit blocks (U), of 25 chambers each, supported by a steel framework (F) and arranged in a step-wise fashion.

2.3 OPERATIONAL CRITERIA

To limit the number of variables, the model was operated under conditions of constant darkness, temperature and aeration. Thus, the model was housed within an insulated dark box (1.2 x 1.6 x 0.6 m) constructed from masonite boards lined with polystyrene sheets (20 mm thick). Three thermostatically controlled heating elements (60 W) situated within the box were used to maintain an ambient liquid temperature of 20° C ± 2° C. These heating elements were constructed by the Scientific Electronics Workshop of the University of Natal, Pietermaritzburg.

The model was operated as a continuous open-flow system with the influent medium pumped (Watson-Marlow 503U flow inducer, Plate 2.2) into the first chamber of each channel. The medium flowed from one chamber to the next in a weir fashion down the course of the model.

An industrial blower (Regenair R2103, Gast Corp.) was used to avoid oxygen limitation by bubbling air into each chamber via a system of interconnected irrigation tubes (i.d. 3.5 mm) terminating in sparges and attached to a central hose pipe (i.d. 15 mm)(Figure 2.1A). The air was first bubbled through a water trap to humidify it and thus minimise liquid loss in the chambers.

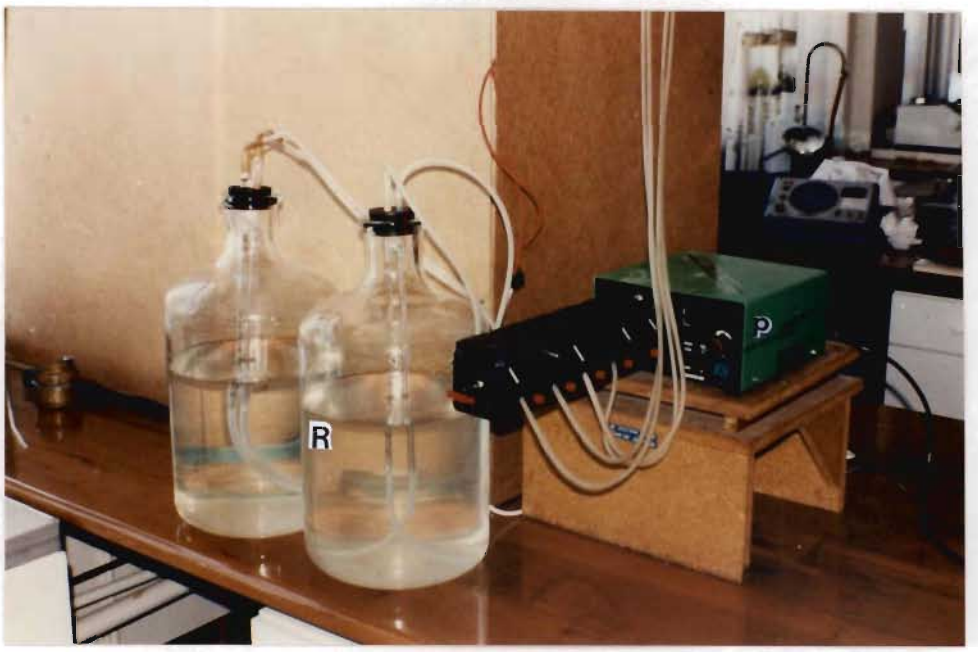


Plate 2.2. Influent reservoirs (R) (12 *l* each), connected to a Watson-Marlow 503 peristaltic pump (P).

2.4 DISCUSSION

Model ecosystems have been used to investigate under laboratory conditions, fundamental properties of aquatic environments such as community metabolism, trophic level interaction, community succession, nutrient mineralization and recycling, and stability (Elstad, 1986). Such systems afford the opportunity to simulate some portion of the natural ecosystem under manipulatable and replicable conditions for experimental purposes (Isensee, 1986; Wimpenny, 1988).

In continuous-flow model systems, selection pressures can be kept constant resulting in a

reproducible enrichment of desired organisms or groups of organisms (Gottschall and Dijkhuizen, 1988). The dilution rate of a system determines the growth rate of the free-living enriched organisms (Parkes, 1982). For non-surface attached microorganisms steady-state conditions can be achieved where growth rates equal dilution rates and where biomass concentration and environmental parameters remain constant (Gottschall and Dijkhuizen, 1988).

Aquatic ecosystems are often characterised by nutrient poor conditions (Jannasch, 1969). As a consequence, the microorganisms usually grow and compete for limiting nutrients at sub-optimal growth rates (Gottschall, 1992). Thus, with low dilution rates, coupled with low nutrient concentrations, continuous-flow systems can be employed to enrich for autochthonous populations of organisms (Parkes, 1982).

Heterogeneous microbial communities are characteristic of most environments (Bull and Slater, 1982). To incorporate this heterogeneity, multi-stage models have been developed which facilitate the separation of mixed microbial populations with retention of overlying microbial interactions (Wimpenny, 1988; Parkes and Senior, 1988). By running the multi-stage in a plug-flow mode spatial and temporal changes can be physically differentiated.

Biodegradation in aquatic environments involves the actions of diverse microbial populations (Klecka, 1986). More specifically, some complex chemicals may be degraded through the combined activities of different organisms (Alexander, 1985). Diversity thus provides greater potential for the degradation of organic compounds in the environment.

The constant removal of metabolites in continuous flow culture prevents the accumulation of potentially inhibitory compounds. This is useful in enriching for microorganisms capable of degrading inhibitory or toxic compounds and for investigating long-term effects of potentially toxic compounds (Fry, 1982). Multi-stage systems afford the opportunity for the sequential breakdown of the toxic substrate without potentially toxic substrates or intermediates accumulating (Parkes, 1982).

Continuous-flow culture systems enable ecotoxicological studies to be undertaken where low concentrations of toxic substances are applied for relatively long exposure periods to determine chronic toxicity effects (Parkes, 1982). This feature is more relevant to the natural state than prescribed lethal dose batch (acute) toxicity assays.

CHAPTER 3: MATERIALS AND METHODS

3.1 MEDIA

3.1.1 R_2A Non-Selective Growth Medium

R_2A contained (mg l^{-1} of distilled water): yeast extract, 10; proteose peptone, 10; casamino acid, 10; glucose, 10; soluble starch, 10; sodium pyruvate, 6; NaCl, 5.2; $CaCl_2$, 3; $MgSO_4$, 1.5; and $K_2HPO_4.3H_2O$, 21 with the pH adjusted to 7.3 with 5M NaOH (Reasoner and Geldreich, 1985). Stock solutions of the medium were made up in 100-fold concentrations. The influent medium for the model was then prepared by diluting 100 ml of stock medium to 10 l with distilled water. This medium was autoclaved for 15 minutes at 15 Kpa (121° C).

3.1.2 R_2A Agar Medium

R_2A agar medium was prepared as above and was set with 15 gl^{-1} agar (Reasoner and Geldreich, 1985). This medium was also sterilized by autoclaving for 15 minutes at 15 Kpa (121° C).

3.1.3 Enrichment Media for Autotrophic Nitrifiers

a) Ammonium Oxidizer Enrichment Medium

Enrichment medium for ammonia oxidizers (Soranio and Walker, 1968) was prepared by dissolving the following compounds in distilled water and diluting to 1 litre: $(NH_4)_2SO_4$, 0.5 g; KH_2PO_4, 0.2 g; $CaCl_2.2H_2O$, 40 mg; $MgSO_4.7H_2O$, 0.5 mg; $FeSO_4.7H_2O$, 10 mg chelated with Na_2-EDTA (0.5 mg); and Phenol Red, 0.5 mg.

The medium was then autoclaved at 15 Kpa (121° C) for 15 minutes. The pH after autoclaving (pH 6) was then adjusted to pH 7.5-8.0 by the addition of sterile 5% ($^{w}/_{v}$) Na_2CO_3.

b) Nitrite Oxidizer Enrichment Medium

Enrichment medium for nitrite oxidizers (Smith and Hoare, 1968), was prepared by dissolving the following compounds in distilled water and diluting to one litre: $NaNO_2$, 1.38 g; $Na_2HPO_4.2H_2O$, 2.55 g; KH_2PO_4, 0.27 g; $CaCl_2.2H_2O$, 2.5 mg; $MgSO_4.7H_2O$, 20 mg; $FeSO_4.7H_2O$, 10 mg chelated with Na_2-EDTA (10 mg); and 1 m*l* of trace element stock solution.

The trace element stock solution was prepared separately in distilled water and contained (μgml^{-1}): H_3BO_3, 20; $CuSO_4.5H_2O$, 100; $MnSO_4.2H_2O$, 20; $(NH_4)_6Mo_7O_{24}.4H_2O$, 20; $ZnSO_4.7H_2O$, 150; $CoCl_2$, 10.

The enrichment medium was autoclaved at 15 Kpa (121° C) for 15 minutes. The pH after autoclaving was $\pm$ 7.65.

3.1.4 Quarter-Strength Ringers Saline Solution

Quarter strength Ringers solution was prepared by dissolving one Ringers tablet (Oxoid) in distilled water and diluted to 500 m*l*.

The solution was then sterillized by autoclaving (15 Kpa, 121°C) for 15 minutes.

3.1.5 Mineral Salts Medium

Mineral salts medium, adapted from Coutts, Senior and Balba (1987), was prepared by dissolving in distilled water and diluting to 1 litre: K_2HPO_4, 1.5g; KH_2PO_4, 0.5g; NH_4NO_3, 0.5; $MgSO_4.7H_2O$, 0.2g; 0.5 m*l* trace element solution A; and 0.5 m*l* trace element solution B. To sterilise, the medium was autoclaved at 15 Kpa (121° C) for 15 minutes.

Trace Element Solution A contained the following (gl^{-1} distilled H_2O):

$FeCl_2.H_2O$, 1.5; NaCl, 1.5; $MnCl_2.4H_2O$, 0.197; $CaCl_2.6H_2O$, 0.238; $CuCl_2.H_2O$, 0.017; $ZnSO_4$, 0.287; $AlCl_3$, 0.05; H_3BO_3, 0.062; $NiCl_26H_2O$, 0.024; Conc. HCl, 10 m*l*

Trace Element Solution B contained the following (gl^{-1} distilled H_2O):

Na_2MO_4, 0.05; $Na_2SeO_3.5H_2O$, 0.0025; $NaNO_3$, 0.0033

Trace element solution A and B were prepared separately, filter sterilised (0.2 μm Millipore) and stored at 4°C until use.

3.1.6 Eluent Solution for High Pressure Liquid Chromatography (HPLC)

a) Borate/Gluconate Eluent Solution for Ion Chromatography (IC)

1) Borate/Gluconate Concentrate

To a one litre volumetric flask the following analytical grade reagents were added: sodium gluconate, 16 g; boric acid, 18 g; and sodium tetraborate decahydrate, 25 g. Approximately 500 m*l* of Milli-Q ultra-pure water were then added and the mixture shaken to facilitate dissolution.

Subsequently, 250 m*l* of glycerin were added before dilution to one litre with Milli-Q ultra-pure water. This concentrate was then stored under refrigerated conditions (4°C) for a maximum of 6 months.

2) Borate/Gluconate Eluent (pH 8.5)

To a one litre volumetric flask approximately 500 m*l* of Milli-Q ultra-pure water were added followed by: 20 m*l* borate/gluconate concentrate; 20 m*l* n-butanol; and 120 m*l* acetonitrile.

After diluting to one litre with Milli-Q ultra-pure water and mixing thoroughly, the solution was filtered through a 0.45 μm Millipore HA membrane filter. The eluent was stored in polyethylene containers (not glass), to prevent the leaching of ions from the glass into the solution.

b) Eluent for Reverse Phase Paired Ion Chromatography - Phenol Analysis

To a one litre volumetric flask the following were added: 400 m*l* methanol, 150 m*l* ethanol, and 20 m*l* PIC A Reagent (low UV) (Waters).

After mixing thoroughly, the solution was diluted to one litre by adding 0.01 % ($^{v}/_{v}$) H_3PO_4 in Milli-Q ultra-pure water. Where crystallization of the PIC A Reagent resulted the solution was gently warmed to facilitate solubilization. Before use, the solution was filtered through a 0.45 μm Millipore HA membrane filter.

3.2 ANALYSES

3.2.1 pH

pH was measured with a Crison pH meter (Micro *pH* 2002) fitted with an Ingold (U402 S7/120) pH probe. The pH meter was calibrated with two standards (pH 7.02 and pH 4) prior to use.

3.2.2 Organic Carbon

Organic carbon catabolism were monitored by the dissolved organic carbon (DOC mg C l^{-1}) assay (*Standard Methods*, 1989) which incorporated infrared spectrophotometry (Skaler CA-10). Samples (20 m*l*) were filtered through cellulose acetate membrane filters (0.45 μm, Sartorius) and stored at 4°C until analysis. All analyses for DOC were made by the Umgeni Water Quality Control Laboratory, Pietermaritzburg.

3.2.3 Ammonia, Ammonium, Nitrite and Nitrate

The processes of ammonification and nitrification was quantified by determination of ammonium, nitrite and nitrate (mg l^{-1}).

a) Ammonium

1) Ammonia Electrode

Dissolved ammonium concentrations were measured with an ammonia electrode (Orion 95-12) in conjunction with a pH meter (Crison). The concentrations were determined after adjusting the pH of filtered (0.45 μm cellulose acetate) aqueous samples (5ml) to >11 with 5M NaOH. This step converted the ammonium in solution to ammonia prior to measurement with the ammonia electrode (as a millivolt reading, mV). The addition of 5M NaOH to the samples was made shortly before analysis to reduce ammonia loss through vaporisation. During analysis each sample was stirred continuously with a magnetic stirrer and bar and the millivolt readout allowed to stabilise (1-2 minutes) before recording. Concentrations of ammonium were determined by comparison with an ammonium chloride standard curve (concentration range 0.1-1000 mg l^{-1} as $NH4^{+}$) (APPENDIX A).

2) Photometric Determination

In the latter stages of the study use was made of a Spectroquant photometer (Merck) to determine ammonium ion concentrations. Reagent kits (Spectroquant 14752, Merck) were used for the photometric analysis of ammonium (0.03-3.0 mg l^{-1}).

b) Nitrate and Nitrite

1) High Performance Liquid Chromatography (HPLC)

Analyses of nitrates and nitrites were made with a Waters Liquid Chromatography system with Waters 600E System Controller, Autochrome 162 CSI, Rheodyne sample injector and Waters 486 tunable absorbance detector.

i) Conditions for Anion Analysis:

Eluent: borate/gluconate (3.1.7)(pH 8.5) sparged with helium (25 m*l* min^{-1});

Pump: Waters Liquid Chromatography System with Waters 600 System Controller;

Injector: Rheodyne;

Column: IC-Pak A and Anion Guard Column (C_{18});

Data: Apex Chromatography Work-Station Version 2.10;

Flow rate: 1.2 m*l* min^{-1};

Injection volume: 100 μl; and

Detection: Direct UV at 214 nm.

ii) Sample Preparation

Samples (5-10 m*l*) for HPLC analysis were filtered through 0.45 μm cellulose acetate membrane filters to remove microorganisms and particulate matter. To remove residual

organic material, the samples were then filtered through a Sep-Pak C_{18} cartridge (Millipore). Prepared samples were stored at 4°C in HPLC sealed glass vials (2.5 m*l*) prior to analysis.

iii) Standard Preparation

Nitrate and Nitrite Concentrated Standard

Concentrated standards for nitrite and nitrate (4000 mg l^{-1}) were prepared separately by dissolving sodium nitrite ($NaNO_2$), 0.6 g and sodium nitrate ($NaNO_3$), 0.548 g in Milli-Q water and diluting to 100 m*l*. The concentrated standards were maintained at 4° C and were replaced every two months.

Working Standard

To prepare a working standard, aliquots (100 μl) of concentrate were pipetted into 100 m*l* volumetric flasks and diluted to 100 m*l* with Milli-Q ultra-pure water. A range of working standards, covering the expected concentration range of the experimental samples was prepared for calibration curve construction (APPENDIX B). Working standards were prepared weekly and were stored at 4°C.

2) Photometric Determination

A Spectroquant photometer (Merck) was used to determine nitrate and nitrite concentrations. Reagent kits, Spectroquant 14773 and 14776 (Merck), were used for the photometric analyses of nitrate (1-90 mg l^{-1}) and nitrite (0.03-3 mg l^{-1}) respectively.

3.2.4 Phenol and 2,4-Dichlorophenol

1) Reverse Phase Paired Ion Chromatography

i) Conditions for Paired Ion Chromatography:

Eluent: (3.1.7) sparged with helium (25 ml min^{-1});

Pump: Waters Liquid Chromatography System with Waters 600 System Controller;

Injector: Rheodyne;

Column: 3.9 x 150 mm Nova-Pak C_{18} (Millipore);

Data: Apex Chromatography Work-Station Version 2.10;

Flow rate: 1.0 m*l* min^{-1};

Injection volume: 20 μl; and

Detection: Direct UV at 214 nm.

ii) Sample Preparation.

Samples (5-10 m*l*) for HPLC analysis were centrifuged (Hermle Z380, BHG) for 15 minutes at 10000 rpm x g. The supernatants were filtered through 0.45 μm regenerated cellulose membrane filters to remove remaining suspended microorganisms and particulate matter. The samples were then stored ($<$ 1 week) at 4° C in HPLC sealed glass vials (2.5 m*l*) until analysis.

iii) Standard Preparation

Concentrated Standard

Concentrated standards of phenol and 2,4-dichlorophenol (1000 mg l^{-1}) were prepared separately by dissolving 0.1 g phenol and 0.1 g 2,4-dichlorophenol in Milli-Q water and diluting to 100 m*l*. The standards were maintained at 4° C and were replaced every month.

Working Standard

To prepare a working standard, aliquots (100 μl) of concentrated standard were pipetted into a 100 m*l* volumetric flasks and diluted to 100 m*l* with Milli-Q ultra-pure water. A range of working standards covering the expected concentration range of the experimental samples was prepared for each molecule for standard curve construction (APPENDIX C). Working standards were prepared daily and were kept refrigerated.

CHAPTER 4: ENRICHMENT, ISOLATION AND CHARACTERISATION OF AN AQUATIC MICROBIAL ASSOCIATION

4.1 INTRODUCTION

Microorganisms incorporating bacteria, algae and fungi constitute a major portion of the total biomass in all aquatic environments (Klecka, 1986). These organisms play an integral role in ecosystem dynamics and are major determinants in the degradation and removal of organic compounds (Cairns *et al*, 1992). Microbial communities exhibit various degrees of structural complexity and interdependence between the component populations (Rheinheimer, 1985). The compositions of these communities differ between aquatic environments depending on the prevailing selection pressures. However, in general, these communities will have similar functional groups in terms of energy flow and nutrient cycling (Cairns *et al*, 1992).

This chapter describes how functional groups of microorganisms responsible for carbon and nitrogen transformations were enriched for, isolated and cultured within the multi-stage laboratory model. Particular emphasis was placed on establishing a population of nitrifying bacteria within the model since this group of organisms was chosen as sensitive indicators for subsequent perturbation experiments.

4.2 EXPERIMENTAL PROCEDURE

4.2.1 Enrichment and Isolation of an Aquatic Microbial Association for the Multi-Stage Laboratory River Model

Riverine water samples (200 m*l*) were used as inocula for the model and were taken from various points along the Umzinduzi River in the Pietermaritzburg, Kwazulu-Natal area. Samples were taken from regions of slow- and fast-flowing waters, from the sediment water interface and from the water column. The samples were pooled and subjected to an enrichment/isolation step to provide a diverse microbial population for the study.

The enrichment/isolation was made in continuous chemostat culture (350 m*l*) (Bioflow C30 Bioreactor, New Brunswick Scientific Co. Inc.) stirred at 150 rpm with growth medium (3.1.1) introduced at a dilution rate of 0.01 h^{-1}. After seven days the culture was added to a 10 litre reservoir of growth medium and then inoculated into the first chamber of each Channel (A,B,C and D) of the laboratory model at a dilution rate of 0.5 h^{-1}. The model was then maintained as a batch culture overnight before initiating continuous flow. Subsequently, it was found that effective enrichment/isolation of heterotrophs and autotrophic nitrifiers could be achieved within the multi-stage model by adding a 10 % ($^{v}/_{v}$) inoculum of composite river water samples (filtered through fibre glass wool) to the growth medium before inoculation into the model allowing the population to establish as described above.

To facilitate temporal and spatial separation of the component species within the system a flow rate of 100 m*l*h^{-1} was chosen. The dilution rate for each individual chamber was 0.82 h^{-1}. The overall dilution rates at different points along the multi-stage model are given in Table 4.1.

Table 4.1. Overall dilution rates at various sampling points of the multi-stage laboratory model (flow rate 100 m*l*h^{-1}).

NUMBER OF CHAMBERS	TOTAL VOLUME (LITRES)	DILUTION RATE (h^{-1})
1	0.122	0.82
3	0.366	0.273
5	0.610	0.164
10	1.220	0.082
15	1.830	0.055
20	2.440	0.041
25	3.050	0.033
30	3.660	0.027
40	4.880	0.0205
50	6.100	0.016
70	8.540	0.012

4.2.2 Characterisation of Nutrient Cycling within the Multi-Stage Laboratory River Model

Qualitative and quantitative characterisation of the physiological processes occurring within the multi-stage model were determined by monitoring the dissolved organic carbon (DOC)(3.2.2), ammonia, nitrite and nitrate concentrations (3.2.3) and pH (3.2.1).

a) Preliminary Investigation

A preliminary study was undertaken whereby the model was inoculated with an association enriched and isolated from river water and allowed to establish. The model was operated for one month prior to sampling and chemical analyses. Samples were taken from Chambers 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60 and 70. In this experiment the temperature within the insulated dark box was not controlled and the study was made at ambient room temperature.

b) Investigation to Establish Steady-State Conditions for Nitrification

Nitrification was identified as a rate-limiting process for establishing steady-state conditions within the multi-stage model. Two Channels (A and B) were inoculated with a composite water sample (4.2.1) and continuous flow conditions were initiated with a flow rate of 100 $ml h^{-1}$. During a 7 week study period, starting at week 2 , samples were taken on a weekly basis to determine nitrifying activity. The first 45 chambers of each channel were used and samples were taken from Chambers 1,3,5,10,15,25,35 and 45. The temperature was regulated and maintained at 20°C $\pm$ 2°C.

c) Investigation to Establish Whether Nitrifying Bacterial Populations were Present as Free-Living or Surface-Attached Populations

At the termination of the above experiment (**b**) Channel A was flushed with 12*l* of distilled water at an overall dilution rate of 0.5 h^{-1}. The influent flow of the nutrient medium was then reinstated at the original flow rate. Samples for nitrifying activity determination were taken one week later after the channel had undergone two complete culture volume changes. The temperature was regulated and maintained at 20°C ± 2°C.

4.2.3 Characterisation of the Isolated Microbial Association

a) Isolation and Enumeration of Heterotrophs

Dilution series viable counts on R_2A agar medium (3.1.2) were used to isolate and enumerate heterotrophic bacteria from aqueous samples taken from selected individual chambers along the multi-stage model. Serial dilutions up to 10^{-7} were made by aseptically pipetting 10 m*l* samples into 90 m*l* of sterile quarter-strength Ringers solution (3.1.4). Aliquots (0.1 m*l*) of the 10^{-4} to 10^{-7} dilutions were inoculated onto duplicate sterile R_2A agar plates before spreading with a flame sterilised glass "hockey stick". After incubation under dark conditions at 27° C for 1 week the number of colonies were counted. Plates with 30-200 colonies were selected and counted. The mean count per plate was expressed as colony forming units per millilitre (c.f.u's ml^{-1}) of original sample.

b) Scanning Electron Microscopy (SEM) and Bright Field Microscopic Examination of the Isolated Microbial Association

Flocculant biomass, biofilm scrapings, precipitated biomass and aqueous samples were taken from selected points along control Channel D and were examined by SEM and bright field microscopy.

Samples of aqueous medium and biomass for SEM examination were filtered through 0.22 μm cellulose acetate membrane filters which were then fixed in 3% ($^{v}/_{v}$) gluteraldehyde for at least 12 hours. Preparation for SEM examination involved two rinses in 0.05M cacodylate buffer followed by washes in alcohol (10 minutes each with 30,50,70,80 and 90 % and three times with 100% alcohol) to dehydrate the samples. The samples were critical point dried in a Hitachi HCP-2 CPD and then splutter coated with gold-palladium before examination with a Hitachi S-570 SEM.

Wet mount slide preparations of samples taken from selected chambers along Channel D were made and viewed using bright field microscopy (Zeiss, Axiophot Photomicroscope).

c) Enrichment and Isolation of Autotrophic Nitrifying Bacteria

Liquid cultures were used to isolate the autotrophic nitrifying bacteria from the laboratory river model. Ammonia oxidisers were selected with ammonium sulphate as the sole energy source in the inorganic enrichment medium (3.1.3), while sodium nitrite was used to isolate the nitrite oxidisers (3.1.3).

A 10 m*l* sample was taken from Chamber 10 of Channel A of the river model. This sample was vortexed in a test tube for 30 seconds to effect mixing and disperse cell aggregates and detach adhered microorganisms. The sample was then serially diluted (10^{-2}, 10^{-4}, and 10^{-5}) by adding 10 m*l* aliquots to 90 m*l* of quarter-strength Ringers solution (3.1.4). A 10% ($^{v}/_{v}$) inoculum from each dilution was then added to individual Erlenmeyer flasks (250 m*l*) which contained 100 m*l* of enrichment medium for isolating ammonia or nitrite oxidising autotrophs. The cultures were incubated aerobically at 27° C and shaken in the dark at 150 rpm in a rotary shaker incubator (New Brunswick Scientific Co., Inc.). Over three weeks, samples (2 m*l*) were periodically taken and analysed for the presence of nitrite and nitrate (3.2.3) respectively.

4.3 RESULTS AND DISCUSSION

4.3.2 Characterisation of Nutrient Cycling within the Multi-Stage Laboratory River Model

a) Preliminary Investigation

Data from the preliminary investigation illustrated the spatial separation of organic carbon degradation (Figure 4.1.) and nitrogen transformation (Figure 4.2.) along the course of the model. Changes in pH resulting from these interactions were also apparent (Figure 4.3.).

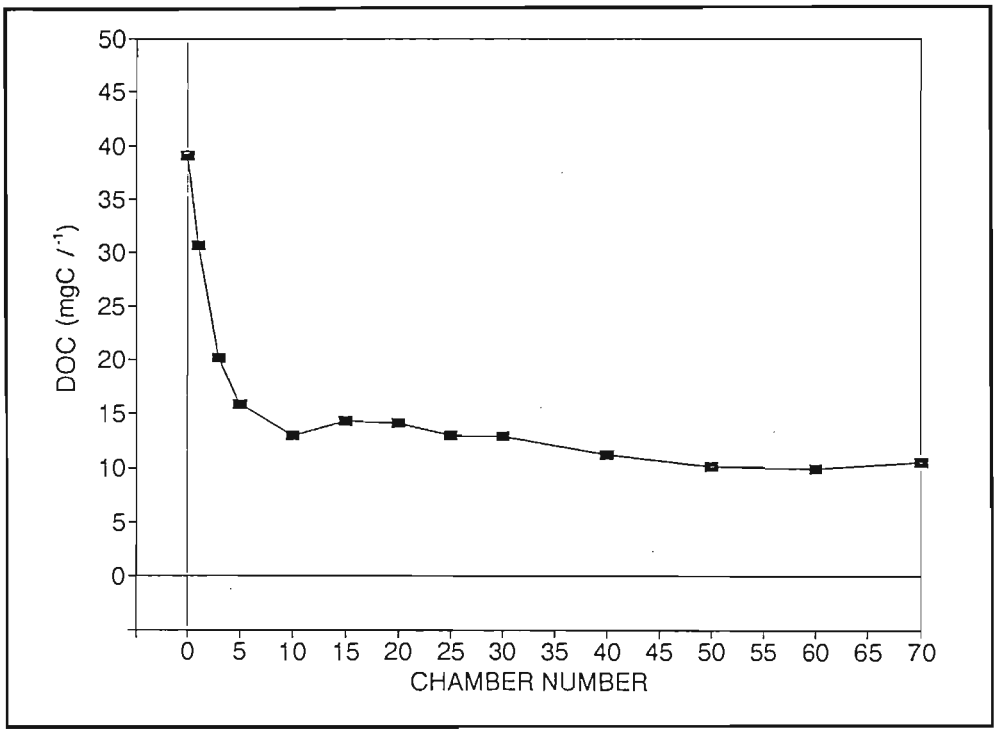


Figure 4.1. Residual dissolved organic carbon concentrations in selected chambers of the multi-chamber model after maintenance under continuous-flow conditions for one month.

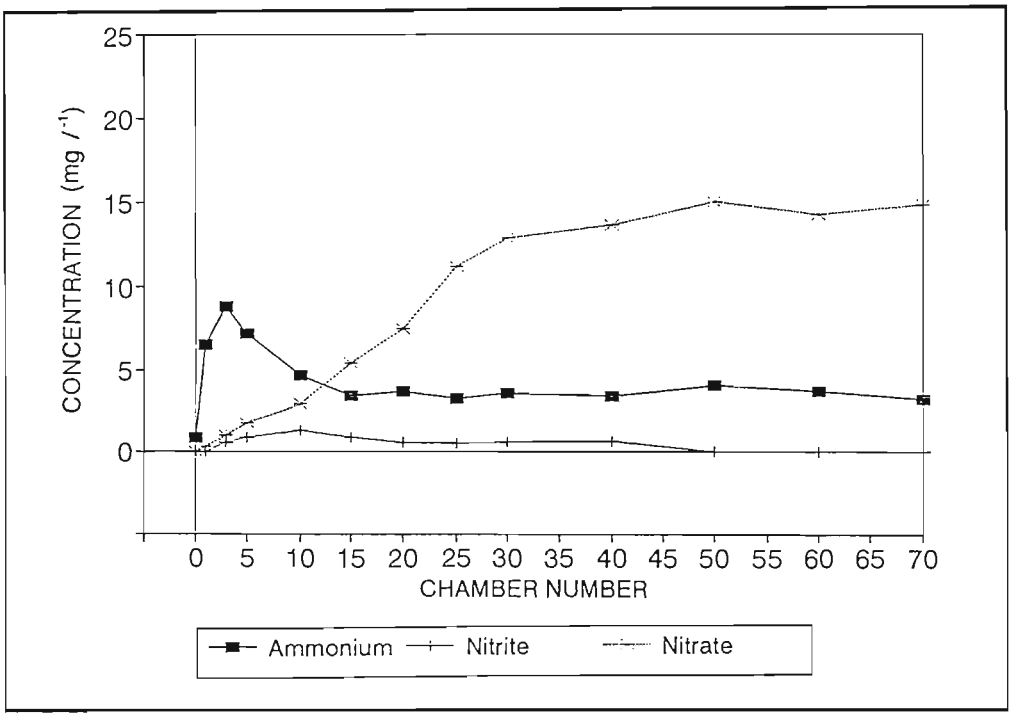


Figure 4.2. Course of nitrification along the multi-stage laboratory model after maintenance under continuous-flow conditions for one month.

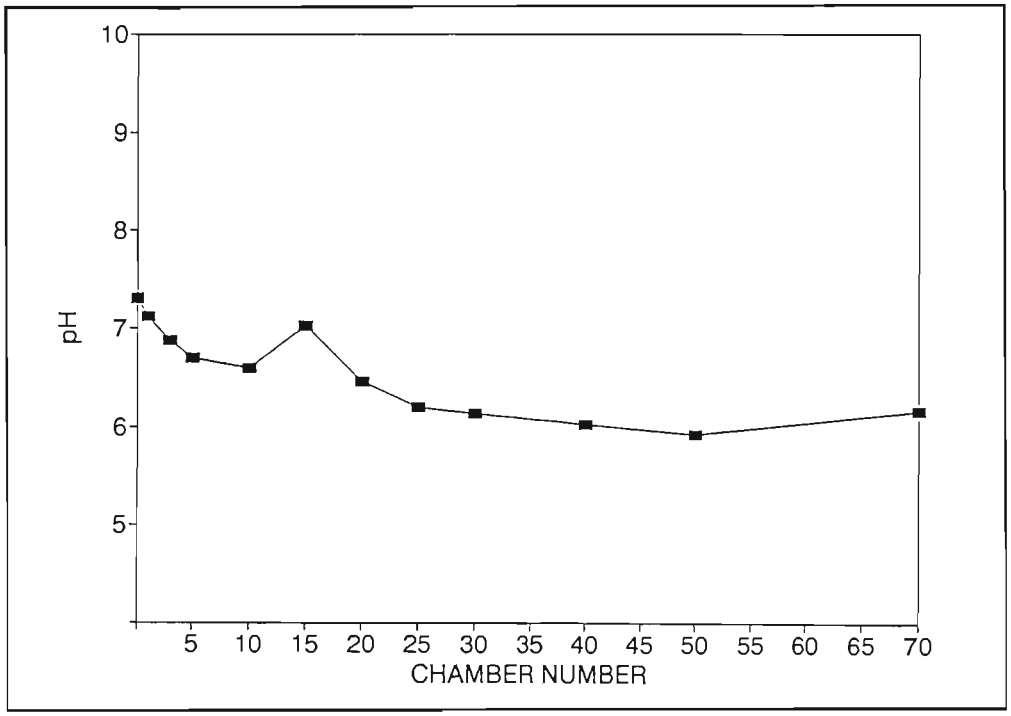


Figure 4.3. pH values recorded in selected chambers of the multi-chamber model after maintenance under continuous-flow conditions for one month.

The DOC concentrations decreased sharply in the first 5 chambers which indicated that organic carbon was catabolized early within the system (Figure 4.1.). This decrease in DOC was attributed to heterotrophic activity and was associated with increased microbial biomass observed in Chambers 3 to 5 of the model.

Figure 4.2. illustrates the course of nitrogen transformations within the system. Increases in ammonium concentration resulted from the conversion of organic nitrogen compounds to inorganic forms. Ammonification is associated with heterotrophic activity and is carried out by a wide variety of heterotrophic bacteria, actinomycetes and fungi (Bitton, 1994). The subsequent decrease and stabilisation of the ammonium concentration in downstream chambers with a concomitant increase in nitrate suggested that a nitrifying population had established.

Nitrification has been defined as the biological conversion of reduced nitrogen (ammonia) to nitrite or nitrate (Alexander, 1977). Nitrification in the environment is attributed primarily to two distinct groups of chemolithotrophic bacteria, namely, the ammonia and nitrite oxidizers which derive energy from oxidation of the inorganic nitrogen compounds (Underhill, 1990). These bacteria have been grouped into the family Nitrobacteraceae which comprises by Gram-negative organisms categorised by their shape, size, arrangement of cytoplasmic membrane, DNA base ratios and metabolic capabilities (Underhill, 1990; Brock, Koops, Ahlers and Harms, 1991). *Nitrosomonas* (ammonia oxidiser) and *Nitrobacter* (nitrite oxidiser) are the two most commonly studied genera (Brock *et al*, 1991). Various heterotrophic microorganisms, including bacteria, algae, fungi and actinomycetes have also been reported to have nitrifying activity (Focht and Chang, 1975; Focht and Verstraete, 1977). However, autotrophic nitrification usually occurs at higher rates than those found with heterotrophic nitrifiers and is, thus, thought to play a more important role in nature (Kuenen and Robertson, 1988; Brock *et al*, 1991; Stensel and Barnard, 1992). Nitrification differs from the heterotrophic oxidation of carbonaceous material on the basis that smaller quantities of biomass is generated and, secondly, that nitrifiers have relatively slow growth rates compared to heterotrophs (Abeliovich, 1987).

From Figure 4.2. it can be seen that low concentrations of nitrite accumulated between Chambers 5 and 15. When populations of nitrifying bacteria establish themselves under steady-state conditions it is often found that nitrite concentrations are low (Gray, 1990). Nitrite does not accumulate due to its concomitant oxidation to nitrate. This arises since nitrite oxidisers are able to utilise substrates at higher rates than ammonia oxidizers (Stensel and Barnard, 1992). The oxidation of ammonia to nitrite is, thus, considered an important rate-limiting step in the process of nitrification (Gray, 1990).

With the onset of nitrification a drop in pH resulted (Figure 4.3.) due to the release of protons (H^+) during the oxidation of nitrite to nitrate. Similar findings have been reported for closed systems, wastewater treatment processes with long retention times, and in waters with low buffering capacity (Gray, 1990; Underhill, 1990). The reported effects of pH on nitrification vary between investigators. pH ranges between 7.0 and 8.4 have been cited as optimal (Gray, 1990; Underhill, 1990; Bitton, 1994). At pH values below 7.2, Downing, Painter and Knowles (1964) found that a linear decrease in the nitrification rate was obtained. In general, inhibition of nitrification occurs at pH values below pH 6 (Underhill, 1990). Acclimation of nitrifying populations to acidic conditions has, however, been reported (Antoniou, Hamilton, Koopman, Jain, Holloway, Lyberatos and Svoronos, 1990; Underhill, 1990). Haug and McCarty (1972), for example, reported that a pH shift from 7.0 to 6.0 required a 10 day acclimation period for nitrification to return to its original rate.

Temperature has also been reported to be an important factor controlling the rates of nitrification in active nitrifying systems. Liquid temperatures ranging between 13 and 18°C were recorded during the course of this experiment. Diurnal fluctuations in room temperature during winter months were found to be sufficient to effect the liquid temperatures within the insulated box. Temperature limits for nitrification have been set between 5 and 40° C (Underhill, 1990). Optimal growth rates are usually obtained with temperatures between 25 and 30°C. Below 20°C and above 30°C the growth rate decreases rapidly in monocultures (Loveless and Painter, 1968). Since fluctuating temperatures can influence the overall rates of nitrification it was decided to install thermostatically controlled heating elements into the insulated box. An ambient temperature of 20°C was chosen for subsequent investigations.

Other important factors which affect the rate of nitrification are light intensity and dissolved oxygen concentration. The multi-stage model was operated under constant dark conditions to facilitate nitrification which has been shown to be photosensitive (Hooper and Terry, 1973; Yoshioka and Saijo, 1985). This photoinhibition is attributed to the photo-oxidation of cytochrome C.In this regard *Nitrobacter* has been found to be more sensitive than *Nitrosomonas* (Alleman, Keramida and Pantea-kiser, 1987). In aquatic environments, nitrification is associated with regions which are not readily exposed to light. Such environments include aerobic layers of sediment, and wastewater treatment processes where light penetration into, for example, activated sludge is poor. Continuous dark conditions were also required to prevent algal contamination of the model system. Nitrate is readily assimilated by algae thus posing a potential interference for examining nitrate assimilation metabolism.

Nitrifying microorganisms have an obligate requirement for oxygen. With low dissolved oxygen concentrations nitrification does not proceed efficiently and inhibition occurs under anoxic conditions (Underhill, 1990; Stensel and Barnard, 1992). The half saturation constant (Km) for oxygen (the dissolved oxygen concentration at which the specific growth rate is half that obtained when oxygen is not limiting) for nitrification has been found to range from 0.15 to 1 mg l^{-1} (Goreau, Kaplan, Wofsy, McElroy, Valois and Watson, 1980). To ensure that excess dissolved oxygen was present within the model each chamber was aerated. Oxygen limitation was thought to be a potential problem only when the aeration tubes became clogged with accumulating biomass resulting in localised microaerophilic regions.

Nitrogen concentrations in the medium must be in excess of the requirements of the heterotrophs present if residual nitrogen is to stimulate autotrophic activity (Gray, 1990). Only small proportions of ammonia-nitrogen are assimilated into heterotrophic biomass. The catabolism of nitrogenous compounds determines the rate at which ammonia is made available for ammonia oxidation and is, thus, also an important controlling factor in the establishment of a active nitrifying population. Taking this into consideration it was decided to add urea (15 mg l^{-1}) to the medium in later investigations. Urea is readily hydrolysed by the enzyme urease releasing ammonia and, thus, providing a rapid supply of ammonia for subsequent nitrification.

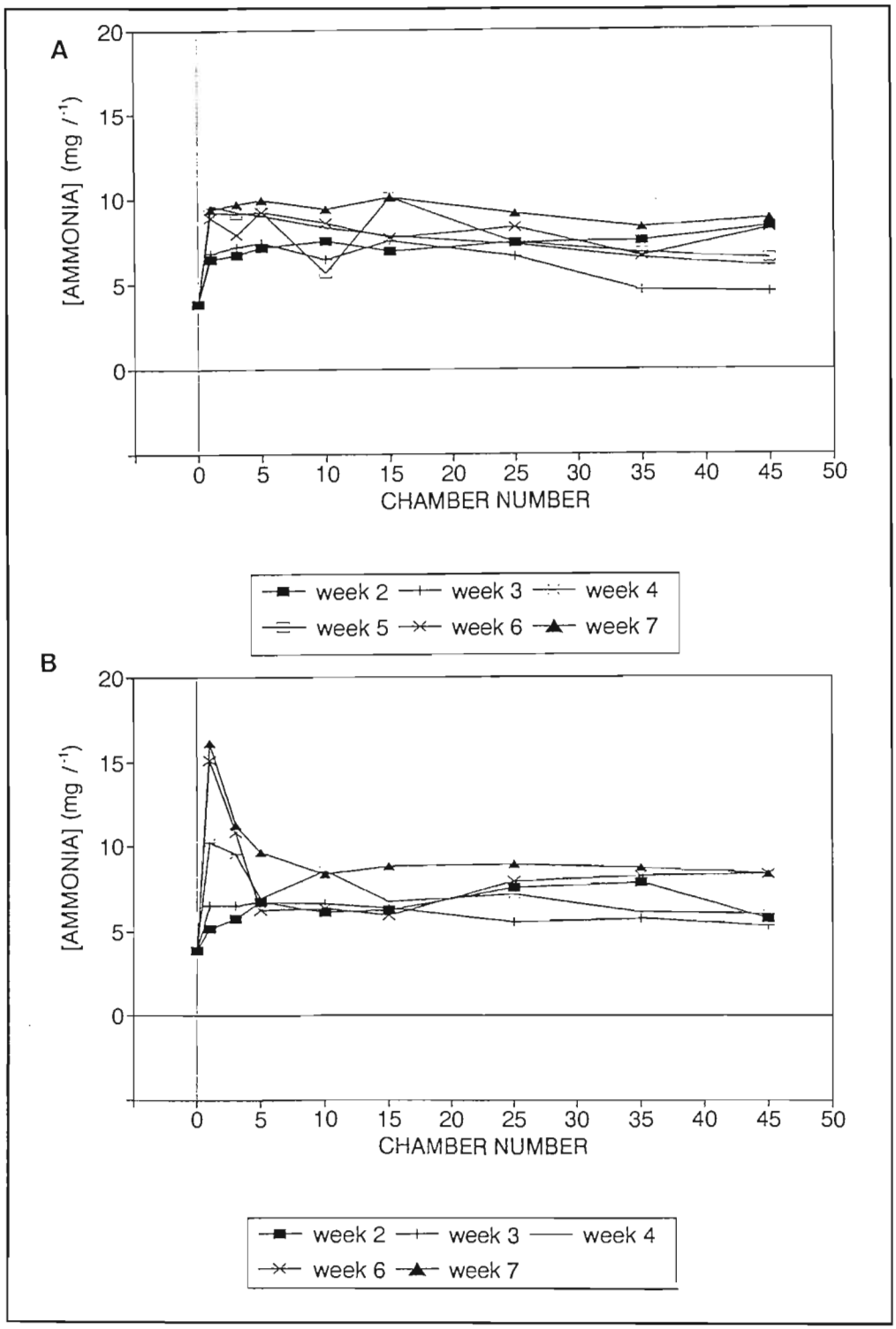
Separation of heterotrophic and nitrifying activity within the multi-stage model occurred as a result of successional changes in environmental conditions within consecutive chambers along the course of each channel. Heterotrophic microorganisms were expected to be selected for early within the multi-stage model on the basis of substrate affinities and high specific growth rates (Parkes, 1982). The subsequent mineralisation of nitrogenous compounds releases ammonia which then undergoes biological oxidation to nitrate via nitrite and leads to the establishment of populations of nitrifiers. Autotrophic nitrifiers are characteristically found to have much lower maximal specific growth rates ($\mu_{max)}$ when compared to mixed cultures of heterotrophs (Bitton, 1994). Nitrification would, thus, be expected to establish within the multi-stage model in regions or chambers along the channel with lower overall dilution rates.

At a flow rate of 100 mlh^{-1} carbon and nitrogen cycling were found to occur within the first 25 chambers of the channel. To reduce the residence time in each channel the operational

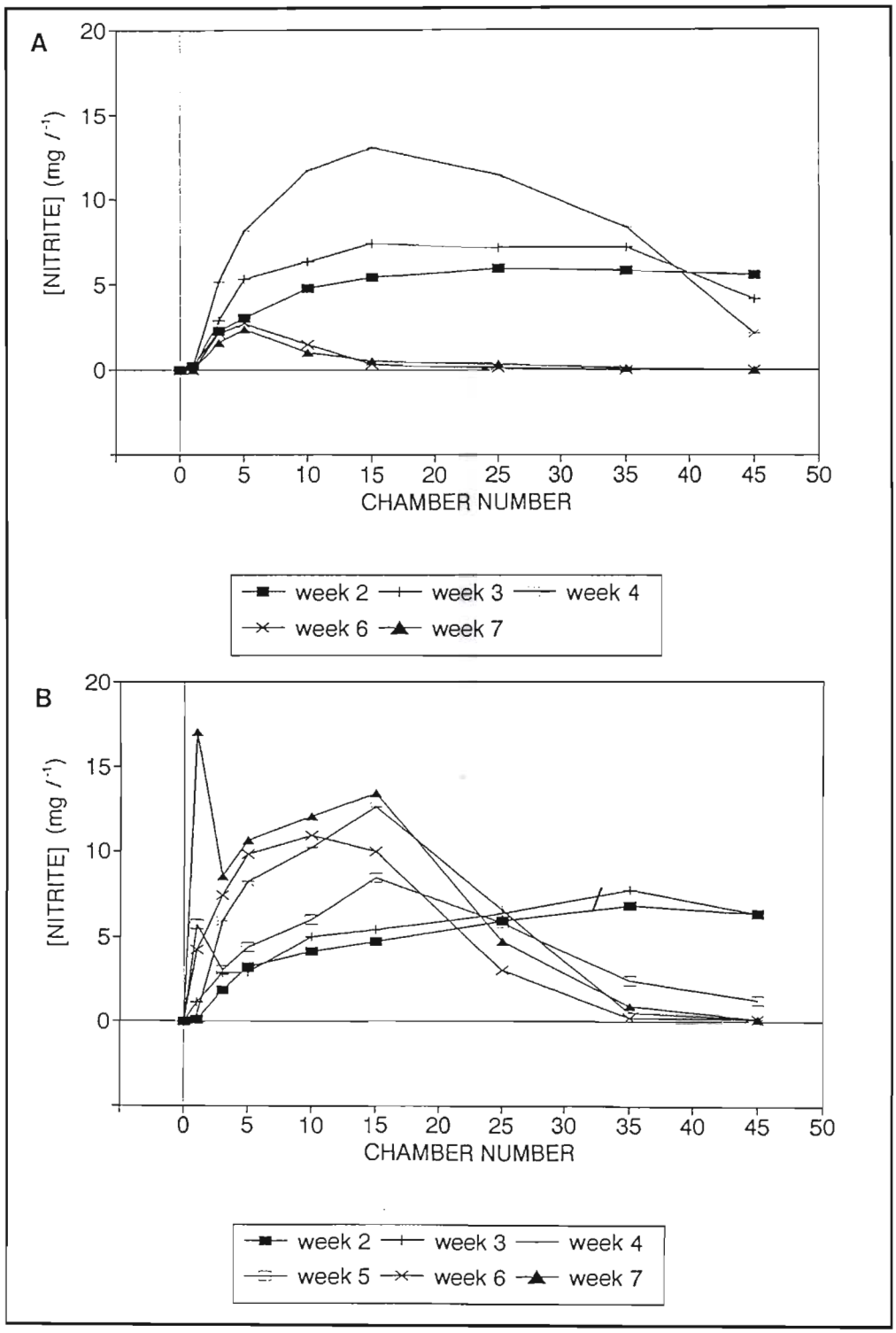
length was effectively shortened to 50 chambers for subsequent investigations.

b) Investigation to Determine Steady-State Conditions for Nitrification

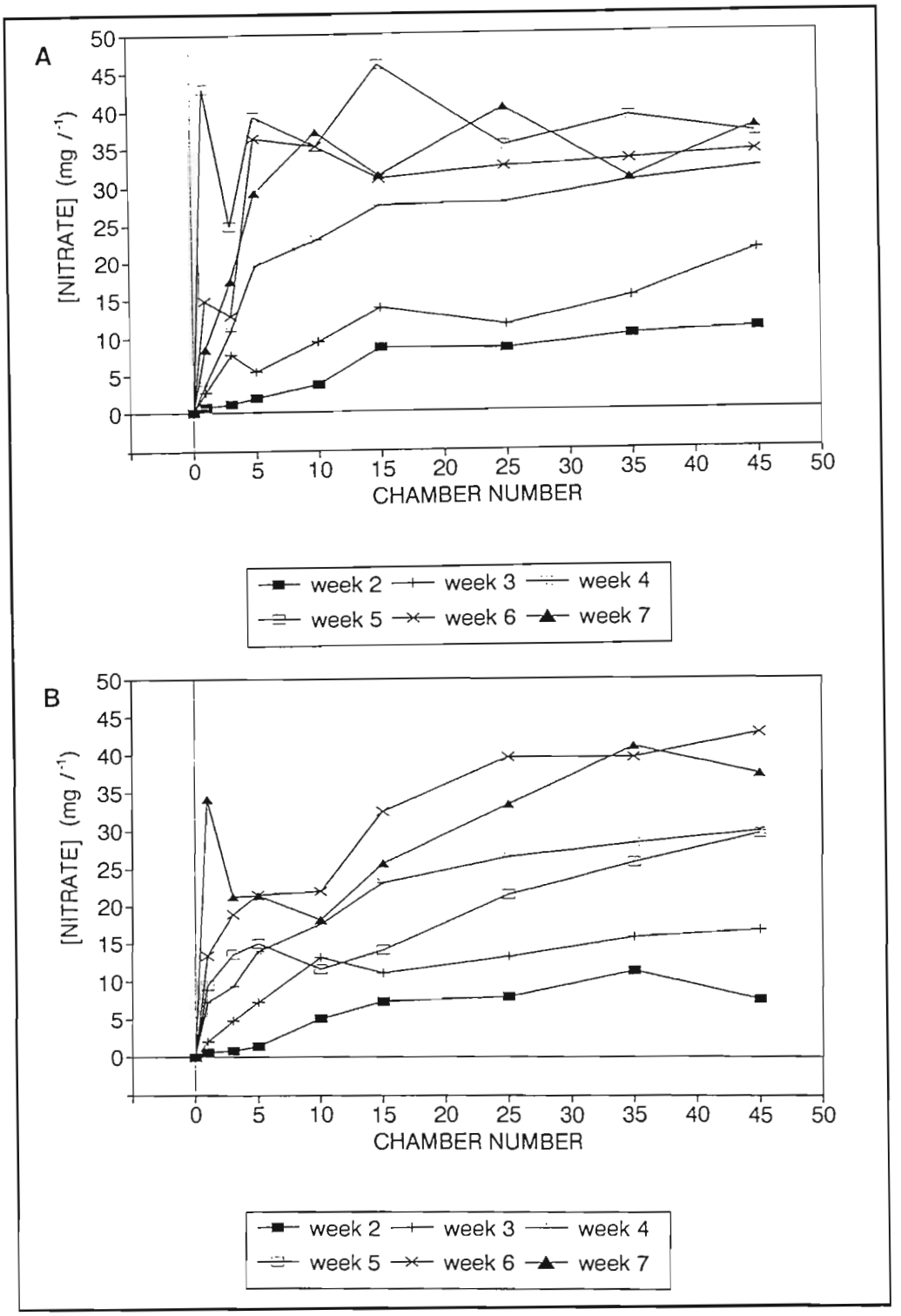
The changes in nitrifying activity which occurred within the multi-stage model during a 7 week period were recorded for Channels A and B. Changes in the ammonium, nitrite and nitrate concentrations within the selected chambers of each channel are shown in Figures 4.4. (A and B), ammonium; Figures 4.5. (A and B), nitrite and Figures 4.6. (A and B), nitrate. After 5 weeks it was found that the nozzle of the influent medium for Channel A had been displaced and was effectively feeding into Channel B. It was, however, estimated that Channel A was stagnant for less than 48 h. This problem was rectified and continuous flow was reinstated for the remainder of the experiment. Due to this pooling effect sampling of Channel A was not undertaken at week 5. Sampling was, therefore, reinstated at week 6 after two complete volume changes had resulted.



Figures 4.4 A and B. Changes in ammonium concentrations recorded in selected chambers of duplicate channels, A and B, of the multi-chamber model, during a 7 week study period.



Figures 4.5 A and B. Changes in nitrite concentrations recorded in selected chambers of the duplicate channels, A and B, of the multi-chamber model, during a 7 week study period.



Figures 4.6 A and B. Changes in nitrate concentrations recorded in selected chambers of the duplicate channels, A and B, of the multi-chamber model, during a 7 week study period.

Figures 4.4. A and B show the ammonium concentrations within the two channels during the study period. Discrete concentrations of ammonium at different points along the multi-chambered channel are regulated by the rates of mineralisation of nitrogenous compounds, the incorporation of ammonium-nitrogen into heterotrophic biomass and the non-assimilative use of nitrogen as an energy source during nitrification. With a carbon to nitrogen loading ratio of $< 2:1$ a small proportion of ammonium-nitrogen would be expected to be assimilated into heterotrophic biomass. Biological oxidation of ammonium to nitrate via nitrite was thus thought to be the main regulating factor controlling ammonium concentrations within the model. After the initial increases in ammonium concentrations within the two duplicate channels the concentrations remained relatively unchanged over the length of each channel. This suggested that this portion was unavailable for nitrification. Possible explanations for this include: deficiencies of key nutrients; low K_N saturation constants for ammonium oxidation (K_N 0.5-2.0 mg l^{-1}); and or inhibition of ammonium oxidation due to the lowered pH. In Channel A at weeks 6 and 7 ammonium peaks were recorded in Chamber 1 which suggested that increased ammonification activity had occurred in this region. This correlated with the interruption of medium flow to Channel A as previously described, and suggested that increased microbial activity resulted and was maintained even after continuous flow had been reinstated. This phenomenon could possibly be explained by the establishment of a larger population of heterotrophic ammonifiers following the stagnation period.

The course of nitrite assimilation is shown in Figures 4.5. A and B. The results of weeks 2 and 3 show that initial increases in nitrite concentrations occurred in the first 5 to 10 chambers of each channel, thus illustrating that ammonium oxidisers had established in this region in the presence of overall dilution rates ranging between 0.164 and 0.082 h^{-1}. Subsequently, a gradual accumulation of nitrite was recorded throughout the channel. Week 4 was characterised by increased concentrations of nitrite up to Chamber 15 which were

followed by decreased nitrite concentrations indicative of nitrite oxidation. At week 5 Channel B, effectively, had an increased flow rate due to the consolidation of two influents. This appeared to result in lower concentrations of nitrite. Subsequently, nitrite accumulation during weeks 6 and 7 was similar to the preconsolidated concentration. The isolated increase in nitrite concentration in Chamber 1 (week 7) of Channel A was thought to be related to flow impairment into the chamber due to the development of microbial growth at the surface of the chamber. Channel B showed an interesting change with the reinstatement of flow after week 5. For weeks 6 and 7 comparatively low concentrations of nitrite were recorded and the concentrations were significantly lowered in Chamber 15 which suggested that a concomitant oxidation of nitrite to nitrate had occurred.

Figures 4.6. A and B show the course of nitrite oxidation within the duplicate channels during the study period. Increasing concentrations of nitrate illustrated the successional changes which occurred within the model with the establishment of a population of nitrite oxidisers. At the beginning of the study period substrate availability (nitrite) appeared to be the limiting factor effecting nitrite oxidiser establishment (weeks 2 and 3). Up to week 4 the duplicate channels exhibited very similar trends with the oxidation of nitrite to nitrate following ammonia oxidation. At weeks 6 and 7 ammonia and nitrite oxidation in Channel B were differentiated temporally and spatially along the course of the channel. Accumulation of nitrite within this channel preceded nitrite oxidation which suggested that the nitrite oxidisers were separated on the basis of their specific growth rates or as a result of substrate inhibition. In Channel A at weeks 6 and 7, the nitrate concentrations plateauxed from Chambers 5 to 10 onwards, indicating the consolidation of nitrite oxidation activity.

The findings of this experiment demonstrated the slow rate at which the nitrifying populations established within the two channels of the multi-stage model. Maximum specific growth rate of nitrifiers from 0.023 to 0.057h^{-1} have been reported in the literature (Underhill, 1990; Bitton, 1994). Gray (1990) reported that steady-state conditions for nitrifying activity within activated sludge treatment processes can take anything up to 6 weeks to establish.

Start up of nitrifying systems is often characterised by nitrite accumulation until nitrite oxidizing populations reach equilibrium (Stensel and Barnard, 1992). This is attributed to ammonia oxidation generating greater amounts of energy (66 to 84 kcal $mole^{-1}$ of ammonia) than nitrite oxidation (17.5 kcal $mole^{-1}$ of nitrite) (Painter, 1970). Ammonia oxidisers achieve higher cell yields and thus greater amounts of biomass are produced (Stensel and Barnard, 1992; Bitton, 1994). Thus, in nitrifying environments, ammonia oxidisers have been found in higher numbers than nitrite oxidisers. However, *Nitrobacter* spp have been reported to have higher growth rates than *Nitrosomonas* spp thus explaining why nitrite seldom accumulates in established nitrifying systems (Bitton, 1994).

The two channels differed considerably with regards to regions of nitrifying activity and nitrite accumulation. These can be attributed to the interruption in flow which occurred in Channel A during week 5. Under batch culture conditions increased amounts of nitrifier biomass can be expected to develop in the early chambers and this leads to higher rates of nitrifying activity. The establishment of biofilm layers at the weir overflows of the chambers could, possibly, have restricted the washout of biomass from the chambers and resulted in biomass accumulation thus maintaining nitrification activity in the first 5 to 10 chambers of Channel A with the reinstatement of the influent flow. It is hypothesised that given time and

with increased build-up of biomass, Channel B would have eventually followed the same trends to those of Channel A whereby ammonia and nitrite oxidation occurred concurrently and were not separated in time and space along the course of the model.

The investigation was terminated after 7 weeks when near steady-state conditions had been reached in both channels. In practice, steady-state conditions are reached when biomass or residual substrate concentrations remain constant for at least two volume changes (Middelbeek and Drijver-de Haas, 1992). A feature of mixed microbial communities is their inability to establish true steady state conditions within a continuous culture. Loosely associated members may be washed out, genetic mutation may change the community structure, and wall growth may develop which may be physiologically very different from the free-living community (Parkes, 1982). Steady-state conditions are characterised by a stable oscillating state which becomes less pronounced with time (Senior, 1977). These oscillations have been attributed to members of a community competing for growth-limiting substrate for which none of the competitors has a great selective advantage. Small changes in culture conditions can cause considerable fluctuations in biomass and its activity (Parkes, 1982). Therefore, mixed microbial communities grown in continuous culture are in continuous transient growth phases.

The establishment of nitrifying activity within the two multi-chambered channels was consistent with growth-rate independent growth. Nitrifying activity was found to occur in regions with dilution rates that were much greater than the μ_{max} values reported for autotrophic nitrifiers. Increasing nitrifying activity found at high dilution rates appeared to be associated with the attachment of the nitrifying populations to flocculent biomass or

biofilms which developed with time within the discrete chambers of the model. This biomass build-up and biofilm formation appeared to result from insufficient agitation and mixing within each chamber. This problem was exacerbated with the clogging of the aeration tubes, through biofilm growth, which resulted in reduced bubbling and agitation of the medium.

c) Investigation to Establish whether the Nitrifying Populations were Present as Free-Living or Surface-Attached Populations.

The results of this experiment are presented in Figures 4.7. and 4.8. which show nitrogen transformation within one channel before and after flushing with distilled water.

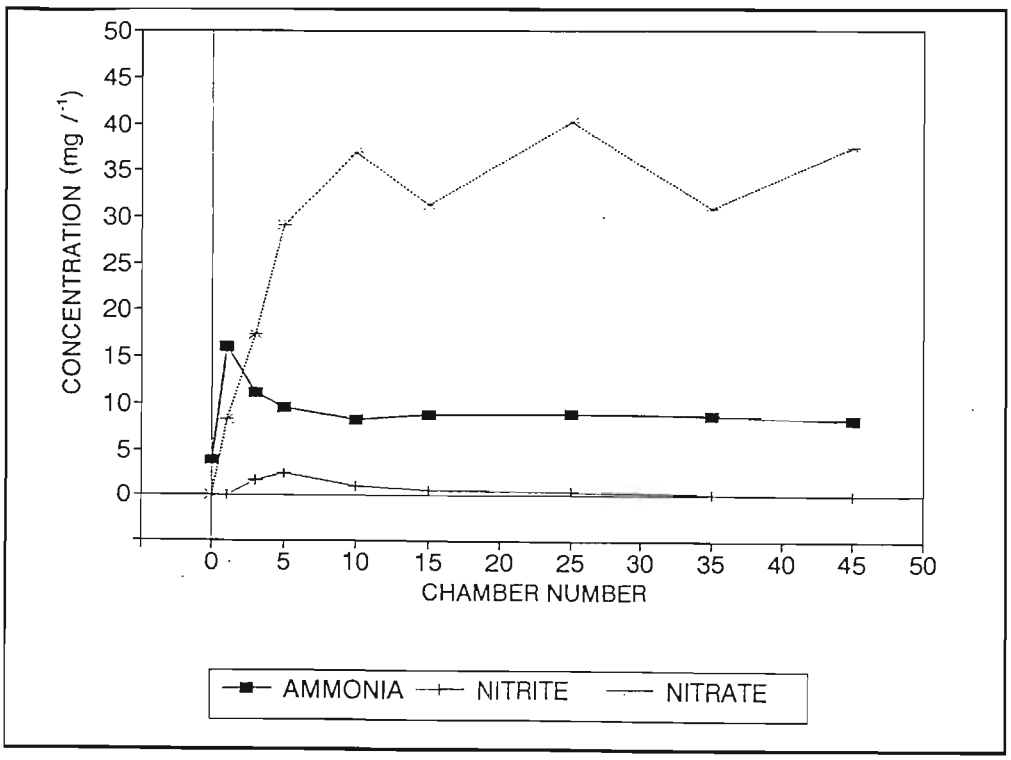


Figure 4.7. Course of nitrification along the multi-stage laboratory model prior to flushing with distilled water.

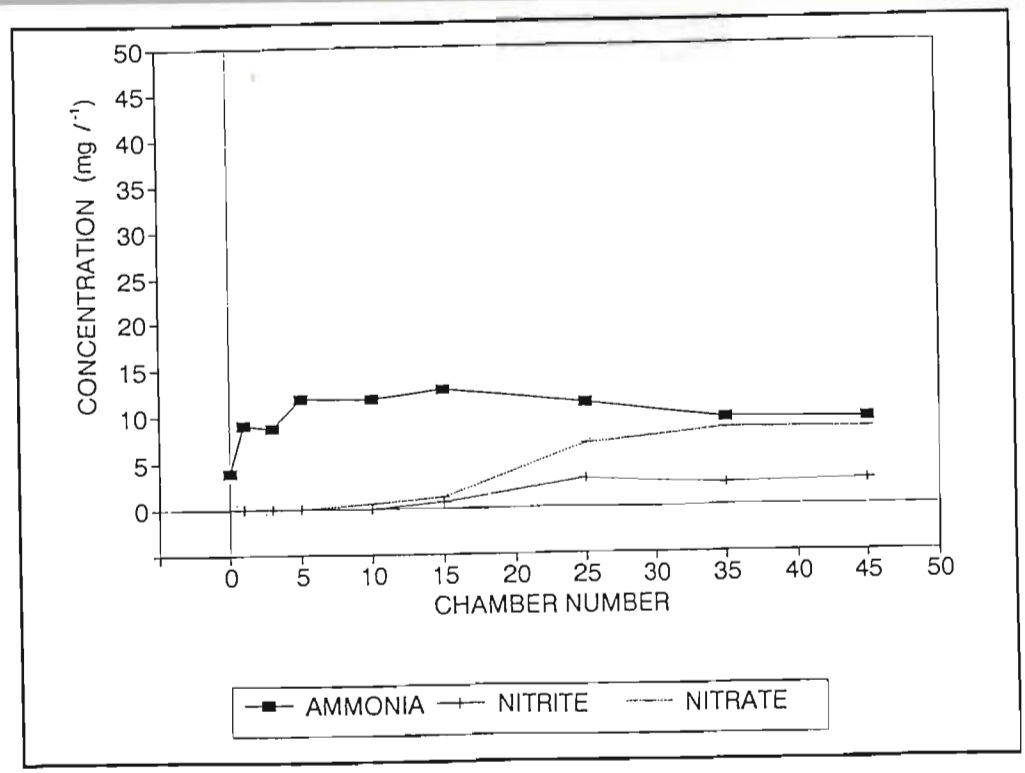


Figure 4.8. Course of nitrification along the multi-stage laboratory model subsequent to flushing with distilled water.

Comparison of Figures 4.7. and 4.8. illustrates that nitrifying activity had been displaced down the course of the multi-stage channel which suggested that the resident nitrifiers were not wall surface attached within the model. However, the presence of nitrifiers as free-living populations is difficult to explain since nitrifying activity occurred in regions with comparatively high dilution rates when compared to the μ_{max} values reported for nitrifying populations. This suggests that the nitrifying populations were possibly attached to aggregates of suspended matter found within the chambers. Retention of this biomass within the chambers under continuous flow conditions would be expected to be longer than the applied dilution rates thus allowing slow-growing populations of nitrifiers to establish themselves. This assumption is supported in the literature with reports that nitrifiers readily attach to flocculent biomass aggregates in activated sludge in wastewater treatment processes (Gray, 1990). Attachment of nitrifiers has also been reported to increase nitrifying activity thus affecting the efficiency of a nitrifying system (Diab and Shilo, 1988).

The original aim of developing the multi-stage model was to use continuous-flow to establish a mixed population of free-living bacteria within the system. Separation of component species along the course of each channel would then be based on successional metabolic events and the specific growth rates of resident populations of organisms. To achieve this, there would be a requirement for homogeneous mixing of the culture within each chamber.

4.3.3 Characterisation of the Isolated Microbial Association

a) Isolation and Enumeration of Heterotrophs

The numbers of colony-forming microorganisms in the selected chambers of Channel A are presented in Table 4.2.

Table 4.2. Numbers of heterotrophs present in selected chambers of Channel A of the multi-stage model

Chamber Number	CFU ml^{-1}
1	5.1×10^8
5	9.6×10^7
15	2.7×10^5
20	2.3×10^5
40	3.2×10^5
60	1.3×10^6

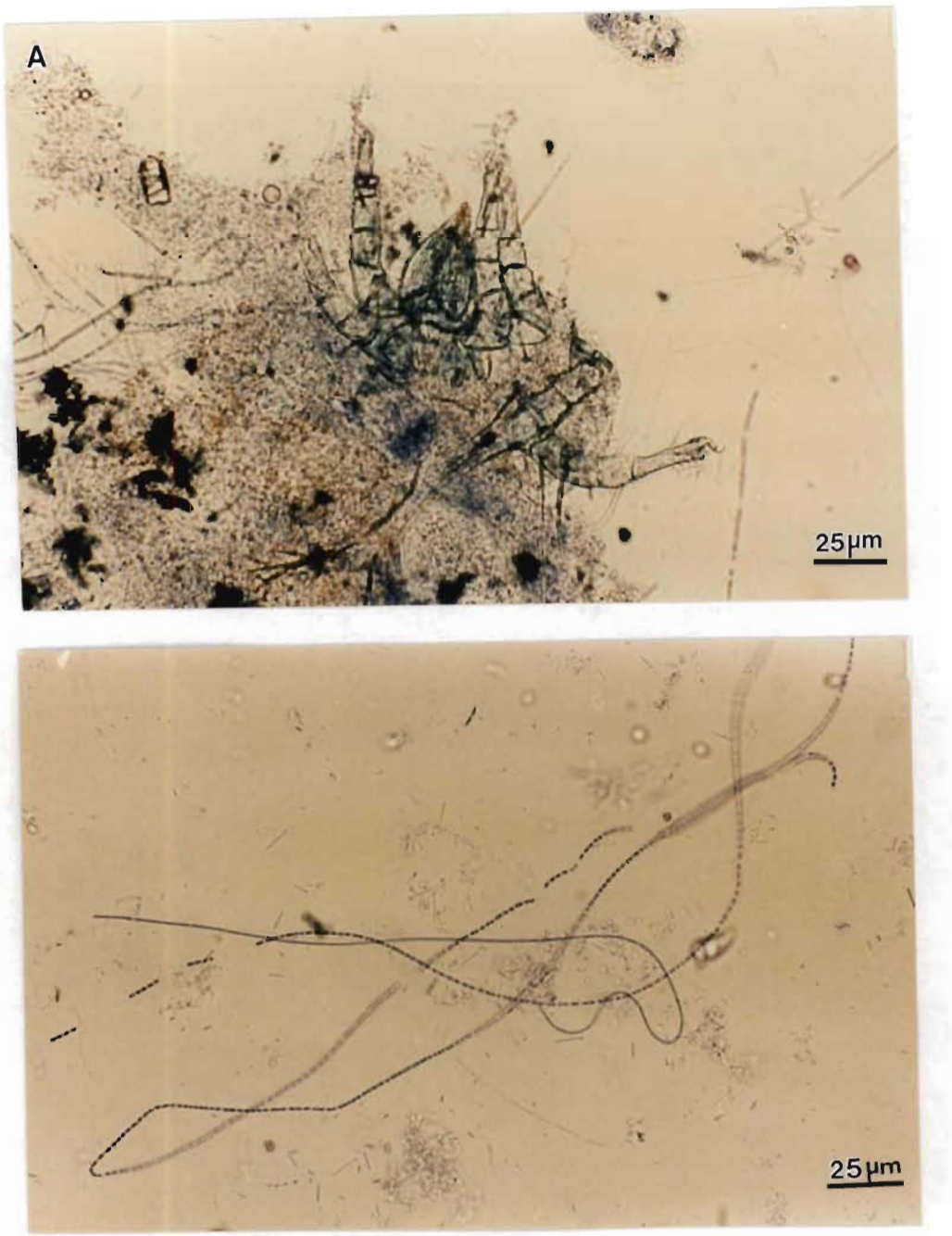
The CFU plate counts on R_2A medium were found to be highest in the chambers nearest to the influent feed, thus indicating the main region of heterotrophic activity within the model. This correlated to microbial biomass development which could be visually differentiated in time and space along the course of the multi-chambered channels. This build-up of biomass was associated with the expected heterotrophic activity occurring in the first 1-5 chambers. Microbial development was characterised by the build-up with time of biofilms and floc-like biomass within the chambers nearest the influent entry.

The colonies isolated on the non-selective R_2A agar medium were found to exhibit heterogeneity in terms of morphology and colour. This illustrated that mixed populations of heterotrophs had established. R_2A agar medium was chosen because of its relative low nutrients concentration and because of its use as a medium for culturing autochthonous bacteria from potable water (Reasoner and Geldreich, 1985).

Contrary to Monod kinetics, mixed cultures can exist and stabilise under conditions in which a single population would be expected to predominate (Parkes, 1982). Mediating factors such as the availability of other substrates, the actual growth rates of the microorganisms present and synergistic and or antagonistic interactions between members of the microbial community all contribute to stable mixed cultures arising (Parkes, 1982).

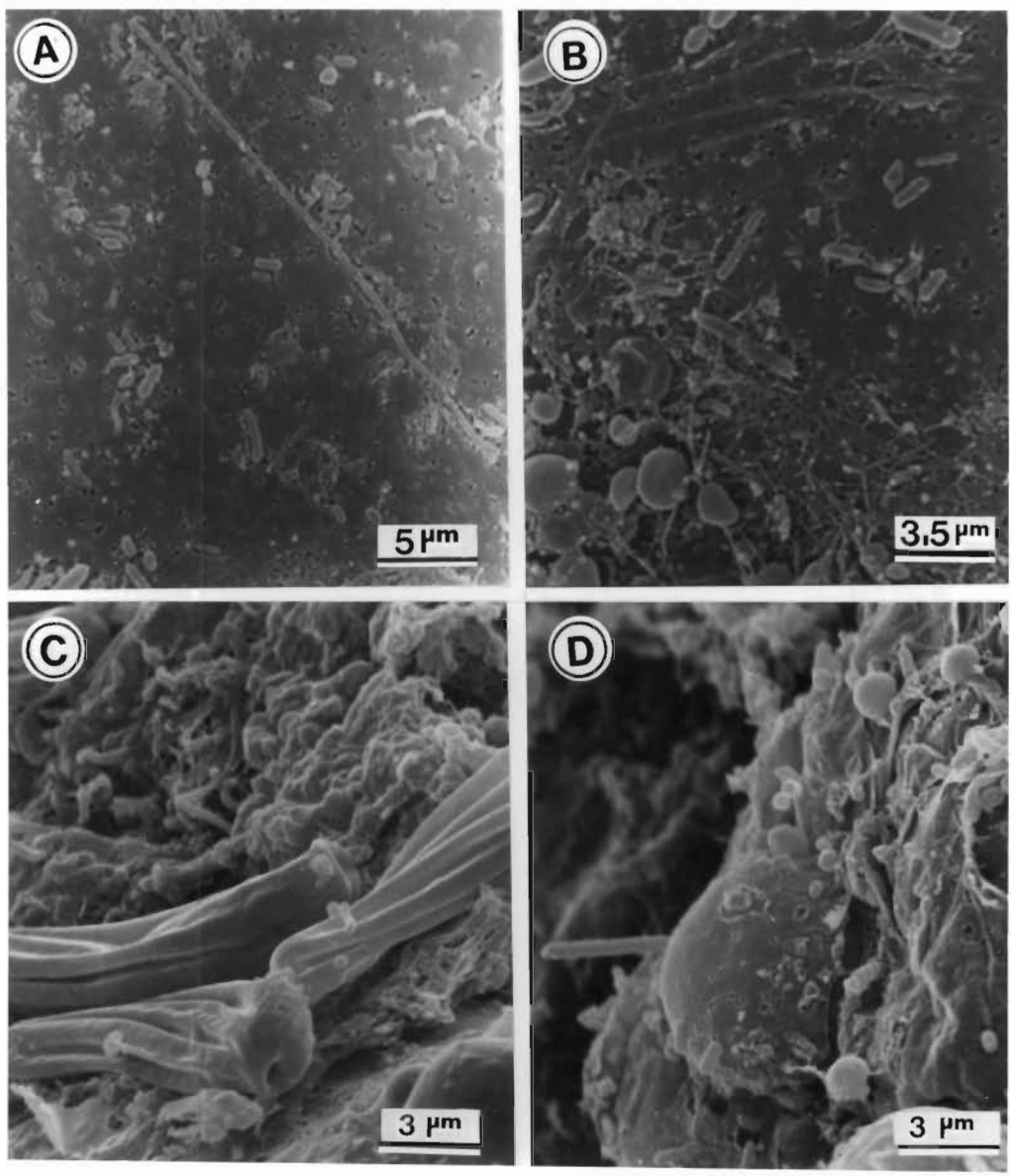
b) Scanning Electron Microscopy (SEM) and Bright Field Microscopic Examination of the Isolated Microbial Association

Microbial associations found within the laboratory model were viewed by bright field microscopy and scanning electron microscopy (SEM) (Plates 4.1. to 4.6.).



Plates 4.1 A and B. Bright field photographs of a microbial association found in Chamber 1 of control Channel D. A - Rotifers grazing on floc-like biomass; and B -filamentous bacteria.

Plates 4.2 A, B, C and D. Scanning electron micrographs of a microbial association present in Chamber 1 of control Channel D. A - free-living association; B - microbial aggregates displaying exopolymer threads; C - protozoan like organisms in association with microbial biomass; and D - floc-like aggregates of cellular biomass.


A
5 µm
B
3,5 µm
C
3 µm
D
3 µm

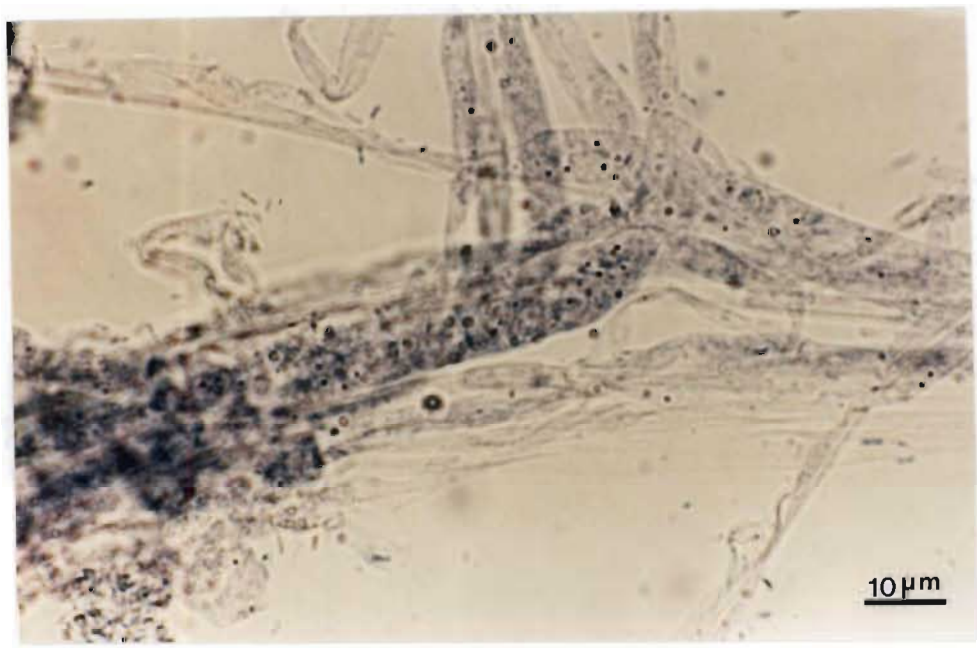
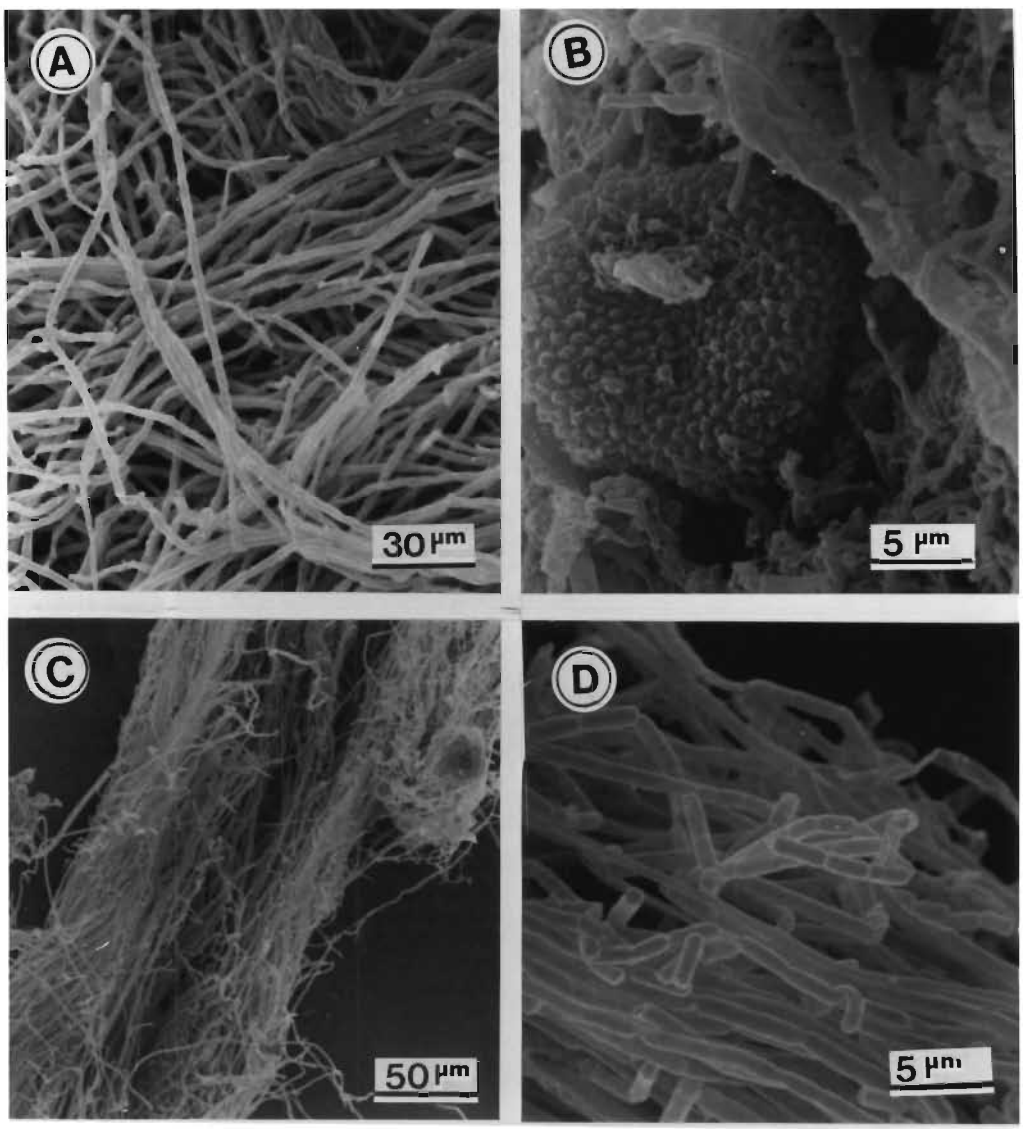
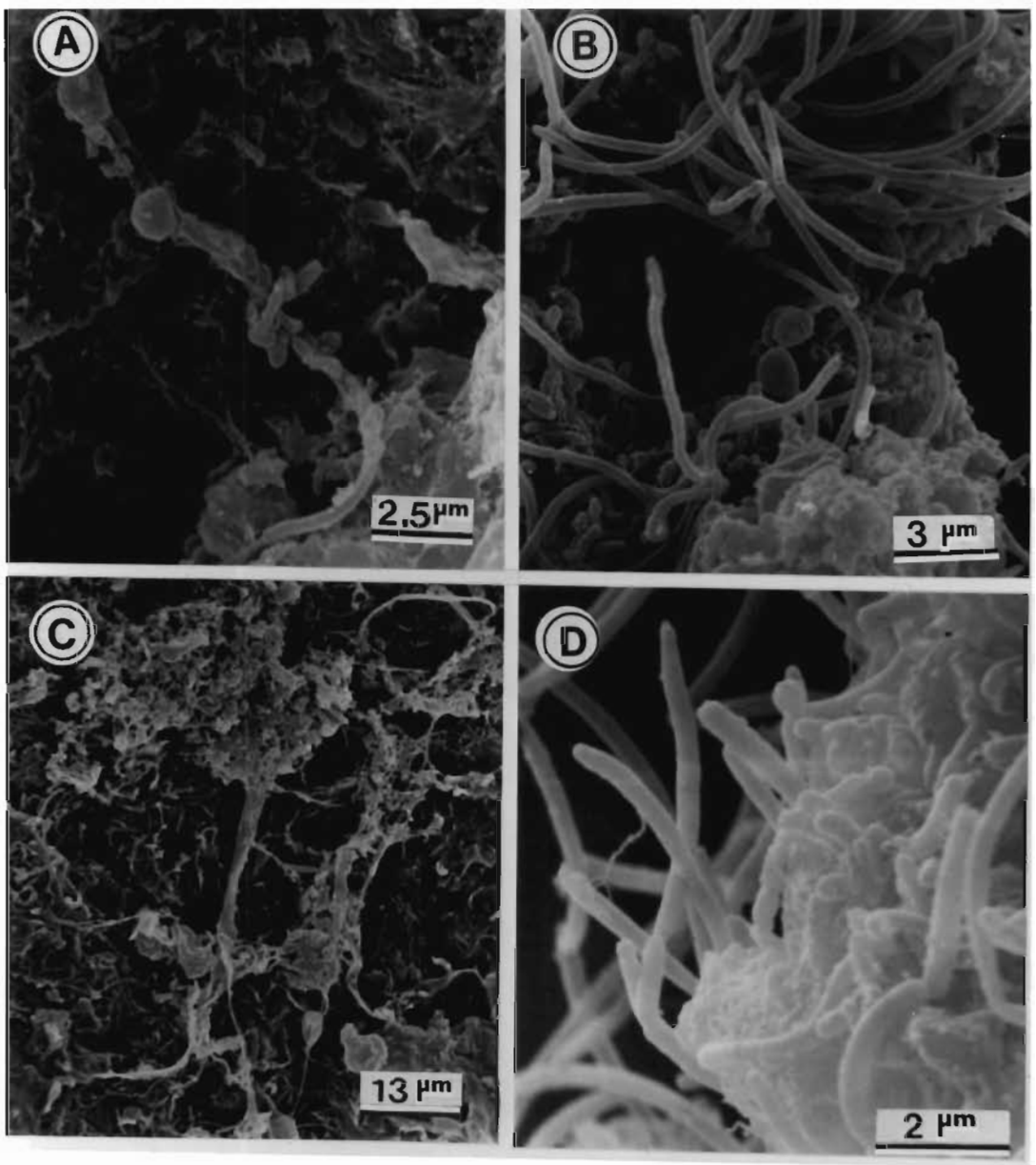


Plate 4.3. Bright field photograph of fungal hyphae found at the liquid/air interface of Chamber 1 of control Channel D.

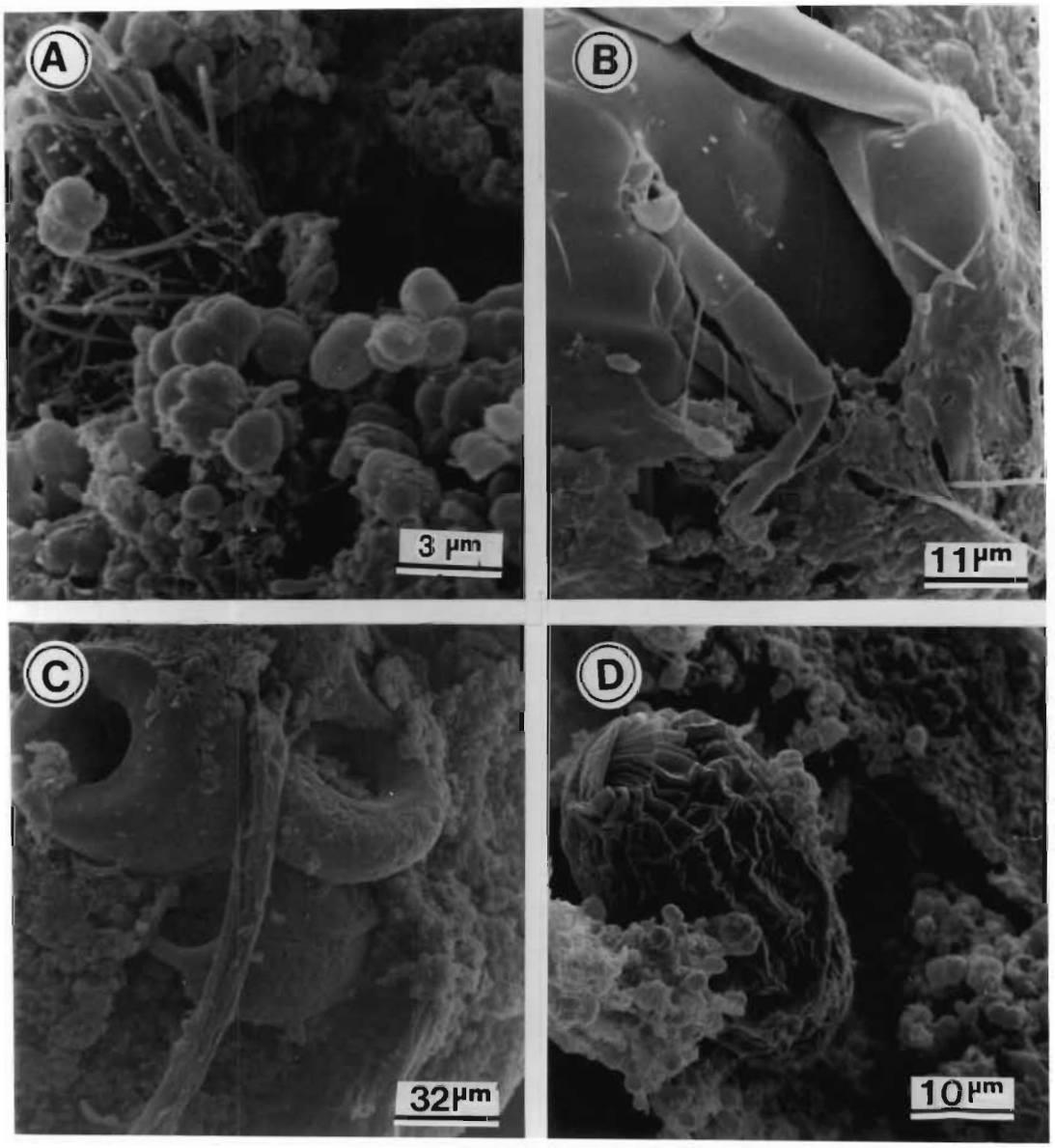
Plates 4.4 A, B, C and D. Scanning electron micrographs of filamentous biofilm growth. A and B - hyphae and associated microbial aggregates found at the liquid/air interface of Chamber 1; C and D - filamentous wall growth on Chamber 5.


A
30 µm
B
5 µm
C
50 µm
D
5 µm

Plates 4.5 A, B, C and D. Scanning electron micrographs of biofilm scrapings taken from the surface of an aeration tube from Chamber 3 (A and B) and from the wall surface of Chamber 5 (C and D).


A
2,5µm
B
3 µm
C
13 µm
D
2 µm

Plates 4.6 A, B, C and D. Scanning electron micrographs of a microbial association present in cellular material precipitated to the bottom of Chamber 15 of control Channel D. A - a ciliated protozoa in association with microbial biomass; B - a macroinvertebrate; C - algal cells; and D - rotifer.


A
3 µm
B
11µm
C
32µm
D
10µm

Scanning electron microscopy and bright field microscopy revealed that a diverse range of microorganisms had established within Channel D. Free-living bacteria, microbial aggregates, filamentous microorganisms, fungi, protozoa, rotifers, macroinvertebrates and algal cells were all present.

Floc-like assemblages of microbial biomass were found to establish with time within the first five chambers. The surfaces were colonised by biofilms and, in chambers with biomass build-up, clogging and blocking of aeration tubes sometimes resulted. This impacted on mixing and aeration within a chamber and potentially affected dissolved oxygen concentrations and associated microbial activity. In chambers where aeration had been impeded or halted, membranous biofilm growth at the liquid/air interface occurred, resulting in a pooling effect.

After the model had been operated for several months a fungal-like biomass was found to develop in Chamber 1 of each channel. This biomass was characterised by its "wet cotton-wool"-like appearance and its ability to "grow" towards the influent tube. In certain instances, the influent tube was completely enveloped. Examination of this biomass revealed that it consisted of fungal hyphae (Plate 4.3). This micro-environment was also found to support an assortment of attached bacteria and a number of protozoal grazers. With the continuous washout of cells from one chamber to another it was found that suspended biomass settled and collected in the dead space at the bottom of each chamber. The aeration tubes extended to within 10-15 mm of the bottom of each chamber and the region below this was not subjected to the continuous mixing effect caused by aeration. Representative microorganisms associated with this region are shown in Plate 4.6.

The findings of this study illustrated that a broad spectrum of heterogeneous microorganisms established within the model. Although continuous flow conditions were maintained it became apparent that growth rate-independent microorganisms were present and contributed significantly to the processes within the model. Microbial biomass and activity were effectively independent variables and were subject to changes in time and space. Such factors could be expected to influence near steady-state conditions and also have a significant effect on reproducibility and variability within such a system.

c) Isolation of Autotrophic Nitrifiers from the Multi-Stage Laboratory River Model

Isolation of the nitrifiers from the model was undertaken to demonstrate that autotrophic populations were present. After a three week incubation period microbial biomass was discernible within the two liquid cultures used to enrich the ammonia and nitrite oxidisers. Assays for nitrite and nitrate were tested positive which indicated that oxidation of ammonia and nitrite had occurred. Samples of microbial biomass from each culture were both Gram stained (Plate 4.7.) and prepared for SEM examination (Plate 4.8.).

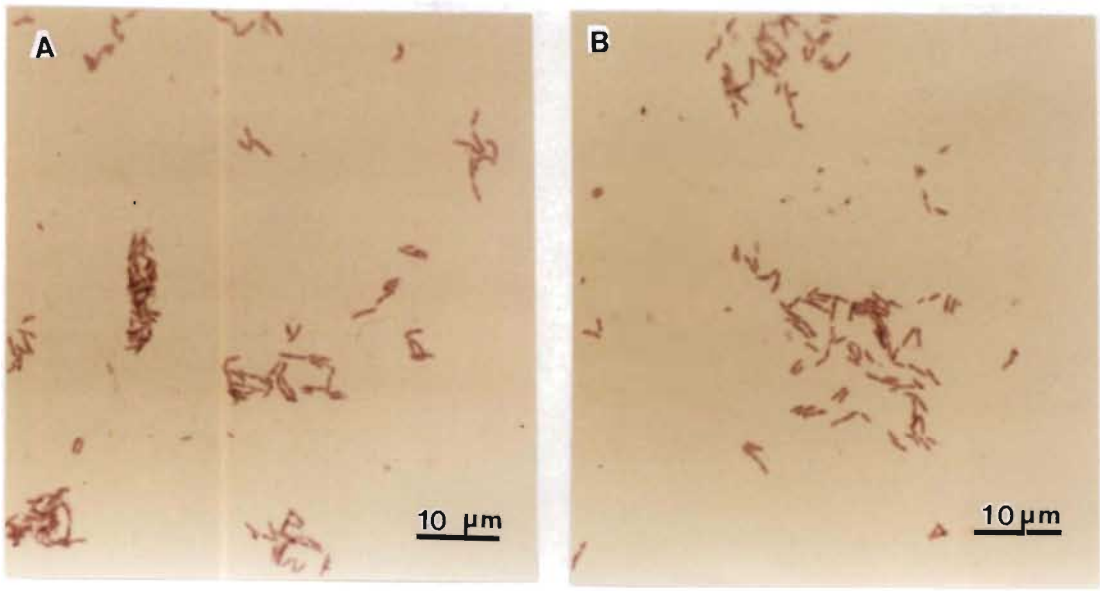
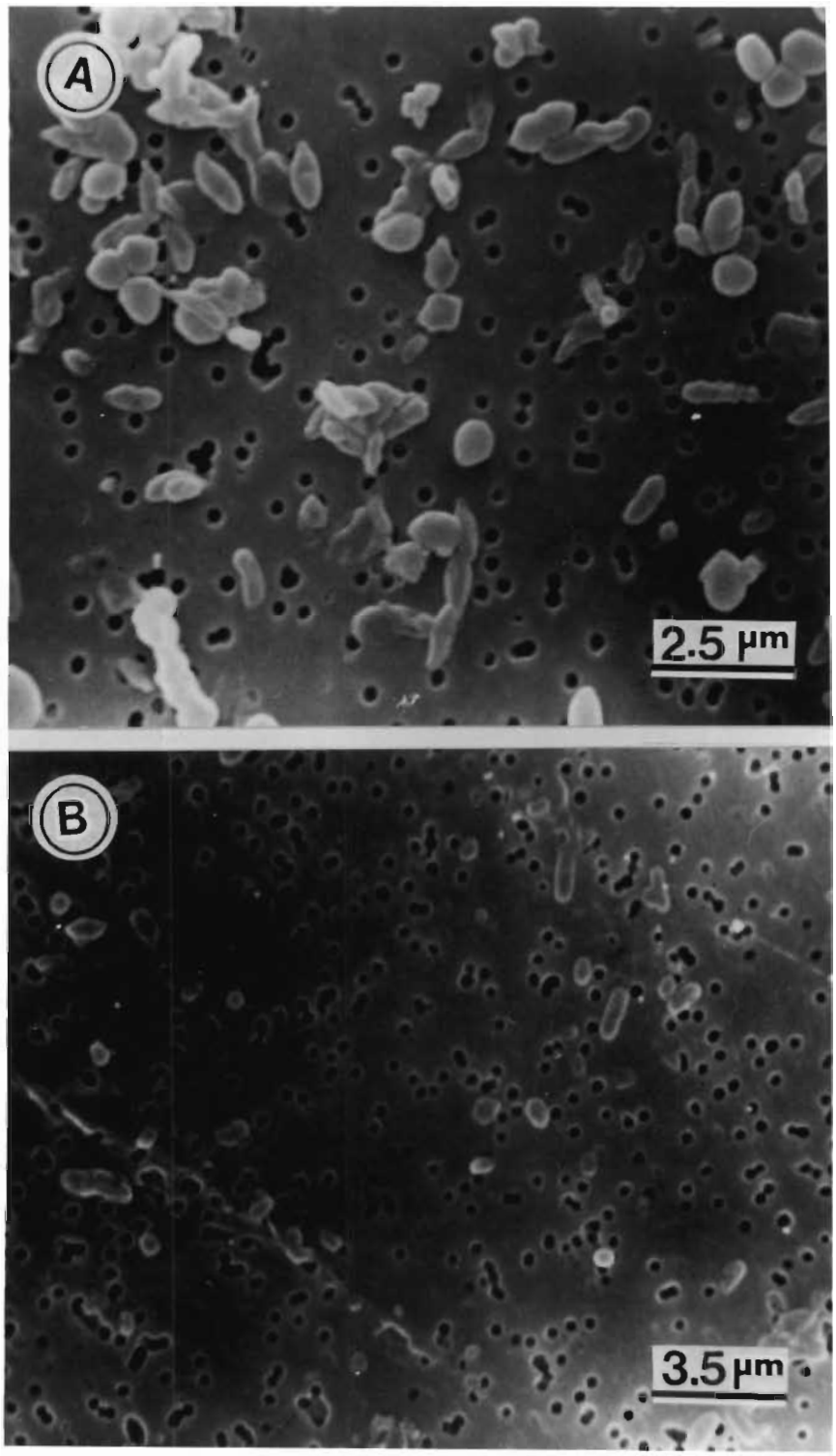


Plate 4.7. Gram stain of autotrophic nitrifiers isolated from cultures of ammonia oxidising microorganisms (**A**) and nitrite oxidising microorganisms (**B**).

Plate 4.8. Scanning electron micrographs of autotrophic nitrifiers isolated from cultures of ammonia oxidising microorganisms (**A**) and nitrite oxidising microorganisms (**B**).


A
2.5 µm
B
3.5 µm

Gram-negative rods were seen in both cultures when the Gram stained cultures were viewed by light microscopy. Scanning electron microscopy revealed that rod-shaped bacteria were present in the culture of the nitrite oxidisers. Rods and cocci were seen in the culture of ammonia oxidisers which suggested that a mixed population had established.

On the basis of these findings the assumption was made that autotrophic nitrifiers were responsible for nitrifying activity within the model. It has been reported that the growth rates of autotrophic nitrifying bacteria are usually higher than those of the heterotrophic nitrifiers and are, thus, thought to play a more important role in nature (Kuenen and Robertson, 1988; Brock *et al*, 1991; Stensel and Barnard, 1992). Heterotrophic nitrification is only thought to be a contributing factor to overall nitrification rates under environmental conditions which are adverse to autotrophic nitrifiers. Since the multi-stage model was operated under conditions within the environmental parameters reported for autotrophic nitrifiers this was unlikely to have arisen.

4.4 CONCLUSIONS

Within the model, representative microbial associations responsible for the catabolism of natural molecules were established. Successional changes in the interrelated processes of organic carbon catabolism and nitrogen transformation were separated spatially. The establishment of a microbial association within the model was primarily dependent on: specific growth rates, the overall dilution rate at various points along the model; and the interactions between the microorganisms and the prevailing environmental conditions. Nitrifying activity appeared to be growth rate independent and was identified as a rate-

limiting process for the establishment of near steady-state conditions within the multi-stage model. True steady-state conditions were not achieved due to the presence of heterogeneous microbial populations and the gradual build-up of flocculent biomass and biofilms. It was, thus, expected that growth-rate independent populations of microorganisms contributed significantly to the processes within the model.

The significance of these the findings with regards to subsequent perturbation studies are:

1) Representative microbial associations were isolated from regions of interest;
2) The slow rate at which the nitrifying populations established within the model did not facilitate short-term impact studies (to be undertaken);
3) The model was operationally complex and increased variability may have been introduced through factors such as temperature fluctuations and impairments of airflow;
4) Standardisation and reproducibility was limited due to the build-up with time of flocculent biomass and biofilm growth; and
5) The relatively large number of analyses required had a direct bearing on the cost effectiveness of the model.

CHAPTER 5: ASSESSMENT OF PHENOLIC COMPOUND PERTURBATIONS ON A MICROBIAL ASSOCIATION MAINTAINED WITHIN THE MULTI-STAGE LABORATORY MODEL

5.1 INTRODUCTION

Evaluation of the effects of pollutants on mineral and nutrient cycles has been proposed as a means of assessing the impacts of compounds released into the environment (Bitton and Dutka, 1986). Organic matter decomposition, nitrogen transformations, sulphate reduction and methanogenesis have all been considered (Blum and Speece, 1992). The inhibition of microorganism-mediated processes will have a direct bearing on functioning of an ecosystem as a whole.

In aquatic ecosystems the nitrogen cycle plays an essential role in the regulation of water quality (Welch, 1992). In unperturbated aquatic environments the nitrogen cycle usually maintains a balanced state (Welch, 1992). Disruption of this cycle can occur through inputs of increased concentrations of nitrogenous wastes from agricultural, domestic or industrial sources. Microbial transformation processes such as ammonification, nitrification and denitrification are the regulating mechanisms which are required to deal with such inputs and thus contribute to the overall self-purification capacities of the aquatic environment (Welch, 1992). Inhibition of these transformation processes can result from severe organic loading (substrate inhibition) and/or toxic pollutant compounds and can lead to the accumulation of intermediates such as ammonia, nitrite and nitrate. All of these molecules have been found to be either toxic or to impair water quality when allowed to accumulate (Dallas and Day, 1993). The elucidation of the factors controlling and inhibiting these regulatory processes will thus be of importance in understanding and predicting potential impacts of perturbant

compounds on the environment.

Nitrification, incorporating specialised autotrophic nitrifiers, has proved to be a sensitive ecological effect assay (Williamson and Johnson, 1981; Powell, 1986; Blum and Speece, 1992). This is attributed to the specialised nature of nitrifying populations, their contribution to nitrogen cycling in the aquatic environment and the relevance of nitrification to wastewater treatment processes (Blum and Speece, 1991; Eckenfelder and Musterman, 1992).

This chapter describes the impacts and fates of phenol and a halogenated substituted phenol, 2,4-dichlorophenol, on the nutrient cycling processes operating within the multi-stage laboratory model.

5.2 EXPERIMENTAL PROCEDURE

5.2.1 Dispersion/Dilution Effects

A 25 chamber uninoculated control channel (Channel D) was used to determine the dispersion/dilution of phenol when introduced into the first chamber of the model on a continuous basis. Distilled water was used as the diluent medium. Channel D was operated and sampled by the same method as described earlier (4.2.2). In a series of separate experiments different concentrations (20,60 and 100 mg l^{-1}) of phenol were continuously introduced into the first chamber of Channel D for 48 h. Concentrations of phenol were monitored 24 and 48 hours thereafter.

5.2.2 Biodegradation Testing

Biodegradation tests were made to establish whether the microorganisms cultured in the multi-stage model contributed significantly to phenol removal. Batch cultures were established with mineral salts medium (3.1.5) supplemented with phenol (20, 60 or 100 mg l^{-1}) as the sole carbon and energy source. A 10% ($^{v}/_{v}$) inoculum from Chamber 1 of Channel A was added to individual Erlenmeyer flasks (250 m*l*) which contained 90 m*l* of medium. Uninoculated phenol-supplemented medium was used as a control. The cultures were incubated aerobically at 27°C and shaken in the dark at 150 rpm in a rotary incubator (New Brunswick Scientific Co., Inc.). Samples (5 m*l*) were taken every 24 h for four days and the residual phenol concentrations were determined by HPLC analysis (3.2.4).

5.2.3 Perturbation Studies

A series of experiments was undertaken to determine the individual perturbation effects of phenol and a halogen-substituted phenol, 2,4-dichlorophenol on the nutrient cycling processes operative in the multi-stage laboratory model. Nitrification was chosen as the criterion for assessing the inhibitory effects of phenol on the established microbial association and was monitored by determining nitrite and nitrate concentrations (3.2.3). Residual phenol concentrations were assayed (3.2.4) to determine its fate within the model.

For this study three Channels (A,B and C) were used as experimental channels while the fourth, Channel D, was maintained as a control. The perturbation studies were made, after first allowing the population to establish over a period of five weeks, by introducing the

perturbant molecule into Chamber 1 of each experimental channel in the influent medium. The required weights of perturbant compound for a given concentration were dissolved in distilled water and diluted to 20 m*l*. Each solution was added aseptically to the influent medium by filter sterilization (0.45 μm regenerated cellulose filter membranes).

The individual inhibitory effects on nitrification were assessed by comparing the nitrifying activities before and after addition of the perturbant and by comparing the results with those of the control.

a) 14 Day Perturbation

An initial study was made to determine the potential long-term (chronic) impacts of 20 mg l^{-1} phenol (0.21 mM) perturbation for a continuous period of two weeks. Samples for analysis were taken on days 0 and 14.

b) 24 h Perturbation

To determine short-term (acute) impacts of phenol on the nutrient cycling processes a 24 h perturbation study was made. Phenol, in concentrations of 5 mg l^{-1} (0.053 mM) and 20 mg l^{-1} (0.21 mM) was added continuously to Channels B and C, respectively while Channel D was used as the control. Samples for analysis (nitrite, nitrate and phenol) were taken at times 0h and 24h. Because of the plug-flow nature of the multi-chambered model only the first 25 chambers were sampled.

Both of the perturbation studies were made at ambient temperature while urea (15 mg l^{-1}) was omitted from the influent medium.

c) 72 h Perturbation

With standardisation of the operational parameters of the model system (i.e. temperature control and urea supplementation), as detailed in Chapter 4, the study was extended to further develop the procedures for screening and assessing the effects of perturbant compounds. The model was allowed to establish under continuous flow conditions over a 4 to 5 week period. Inocula (10 m*l*) from the first 15 chambers of control Channel D were transferred into the corresponding chambers of Channels A,B and C and were allowed to re-establish.

Perturbation studies (72h) were undertaken as before with sampling times of 0, 24 and 72 h. Following cessation of phenol addition, samples were taken at 72 hour intervals to monitor potential recovery. To reduce the number of individual analyses, preselected chambers between Chambers 1 and 50 were sampled. Individual perturbation studies were made with phenol concentrations of 60 mg l^{-1} (0.63 mM) and 20 mg l^{-1} (0.21 mM) and with 2,4 dichlorophenol concentrations of 10 mg l^{-1} (0.37 mM) and 20 mg l^{-1} (0.0.12 mM).

5.3 RESULTS AND DISCUSSION

5.3.1 Dilution/Dispersion Effects

Residual concentrations of phenol in the multi-chambered model after periods of 24 and 48 hours are shown in Figure 5.1.

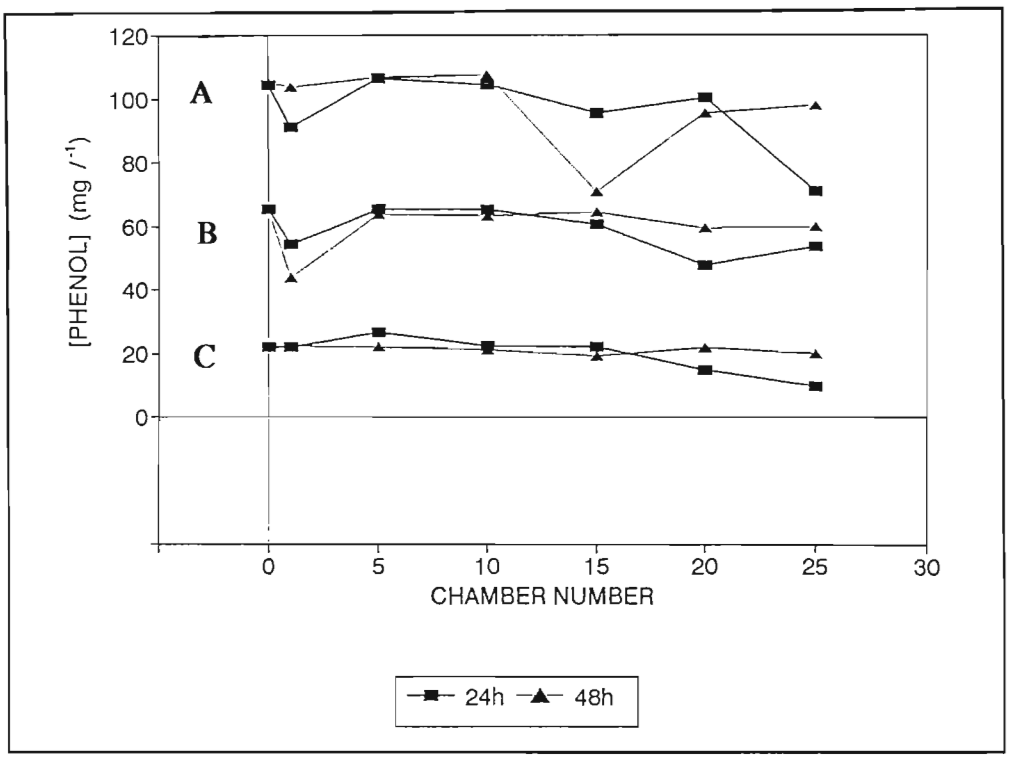


Figure 5.1. Residual phenol concentrations in selected chambers Channel 1, 24 and 48 hours after the introduction of the perturbant phenol in concentrations of 100 (A), 60 (B) and 20 (C) mg l^{-1}.

The phenol concentrations were found to equilibrate in the control channel within 24 hours. Because of the near plug-flow nature of operation of the model it was anticipated that spatial and temporal changes in residual phenol concentrations would initially occur until a complete volume change had resulted. With a flow rate of 100 ml^{-1} a complete volume change was effected after 24 hours.

This illustrates that the flow characteristics of the model did not significantly effect the overall phenol concentrations in individual chambers. Potential abiotic removal factors such as dilution and dispersion and volatilisation were not found to be significant in the model. These results were expected since within the aquatic environment phenol is readily transported in the aqueous phase due to its relatively high water solubility (6.7 gl^{-1}) (Weast, 1989). Also, phenol has a low volatility (i.e. a vapour pressure of 0.8 mm at 20°C).

5.3.2 Biodegradation Testing

Phenol degradation in batch culture over a four day period is shown in Figure 5.2. while the control results are shown in Figure 5.3.

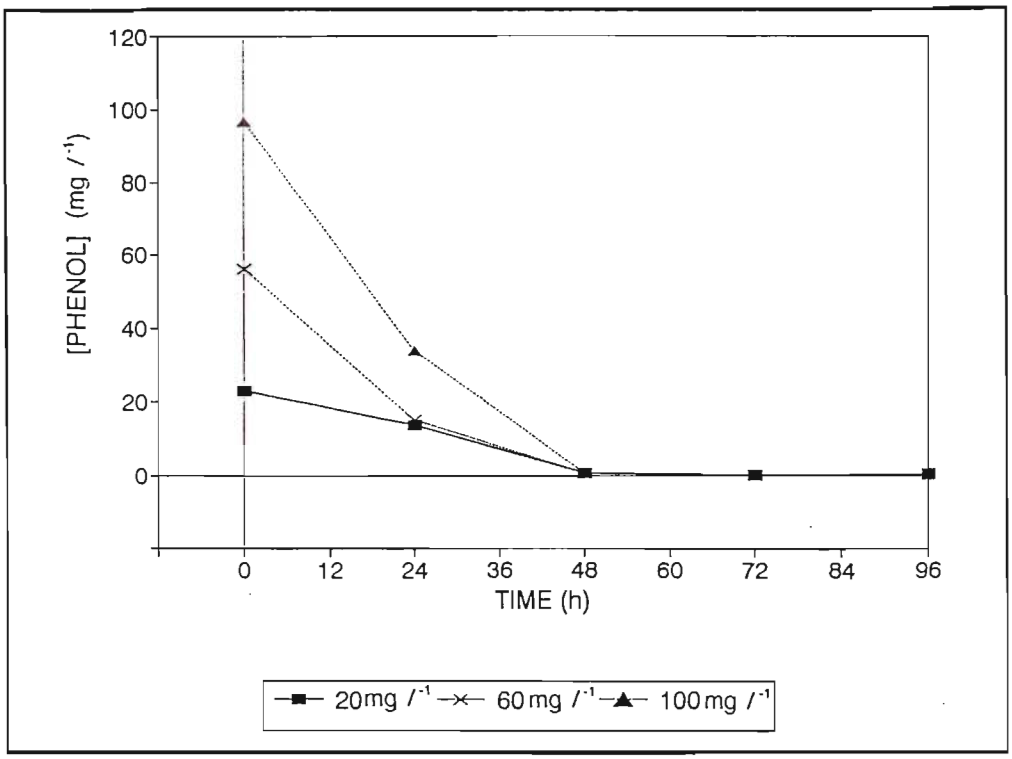


Figure 5.2. Phenol degradation in batch culture over a four day test period. Phenol was added as the sole carbon source.

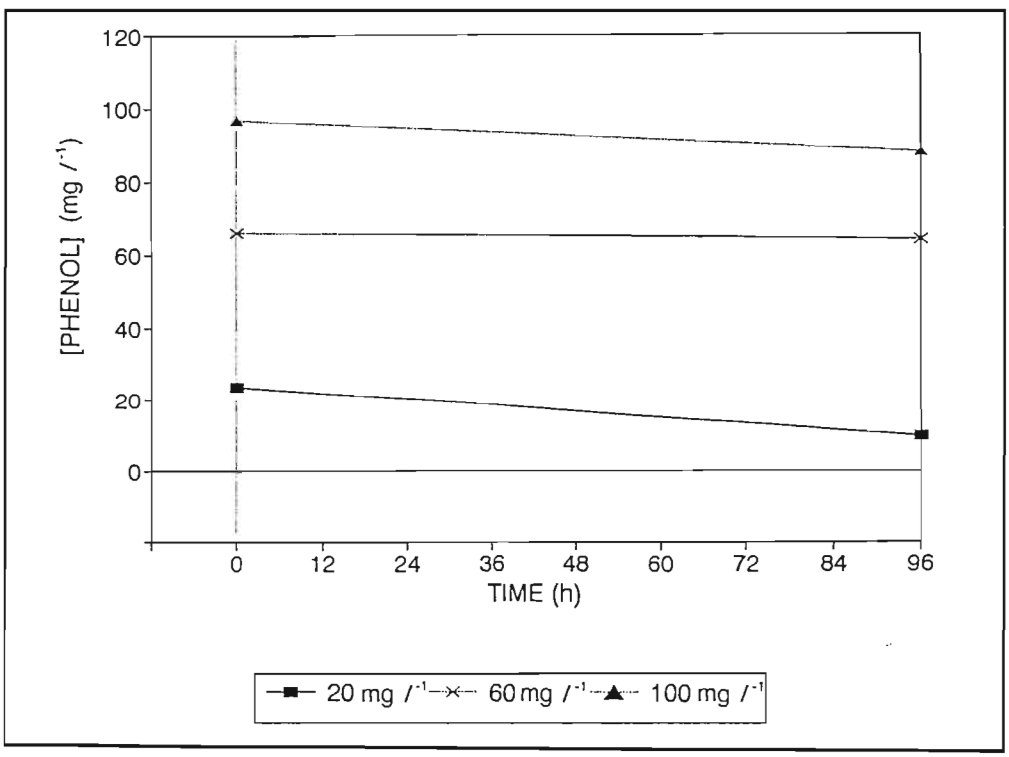


Figure 5.3. Batch culture controls for the same four day test period as Figure 5.2.

Biodegradation evaluation with different concentrations of phenol as the sole carbon and energy source showed that for the concentrations tested, the molecule was degraded during the four days test period (Figure 5.2.). Thus, it was apparent that an active phenol-catabolizing population was present in the model. The residual phenol concentrations in the controls were found not to change significantly. The drop in concentration of the 20 mg l^{-1} control was attributed to microbial contamination rather than abiotic removal factors.

Phenols are widely used as disinfectants and preservatives and have good antimicrobial activity with rapid bactericidal effects (Scott and Gorman, 1992). The activity of phenol is markedly diminished by dilution and is also reduced by the presence of organic matter. Below bacteriostatic concentrations phenol is readily degraded by a number of heterotrophic microorganisms (Bitton and Dutka, 1986; Bitton, 1994). Phenol has proved amenable to degradation via biological wastewater treatment processes (Rozich, Gaudy and D'Adamo, 1983; Bitton, 1994). Capestany *et al* (1977), for example, reported that phenol concentrations in an activated sludge plant were lowered from 1000 mg l^{-1} to 0.5 mg l^{-1} after a hydraulic retention time of 24 hours. However, the presence of phenols has been found to increase the susceptibility of biological treatment processes to periodic inhibition. Nitrification, in particular, is sensitive to phenolic inhibition (Holladay, 1978).

In batch culture studies with either monocultures or heterogeneous populations it has been found that the relationship between specific growth rate and initial phenol concentration follows a bell-shaped curve which can be described by Haldane kinetics (Rozich *et al*, 1983). Essentially, this plot follows a substrate inhibition pattern whereby increasing substrate concentrations result in decreasing specific growth rates when an optimal substrate

concentration is exceeded.

Phenol degradation in aquatic environments has been reported by a number of authors (Jones and Alexander, 1986; Hwang, Hodson and Lee 1986; Gladyshev, Gribovskaya and Adamovich, 1993). Hwang *et al* (1986) reported that microbial degradation was the primary process for the attenuation of phenol and *p*-chlorophenol in surface estuarine waters. Several schools of thought indicate that information on biodegradation is of paramount importance in predicting the persistence and concentrations of toxic chemicals in natural environments (Jones and Alexander, 1986). Attention has been given to modelling the kinetics of biodegradation in aquatic environments (Paris, Wolfe, Steen and Baughman, 1983; Rubin, Subba-Rao and Alexander, 1982). Gladyshev *et al* (1993) concluded from degradation studies of phenol in river water that the rates of phenol disappearance varied between water bodies and that experimental findings were only valid for the ecosystem under investigation. For example, changes in phenol degradation kinetics have resulted from seasonal variations (Jones and Alexander, 1986). Furthermore, Lewis, Kollig and Hodson (1986) showed that mineralization rates of organic compounds differed in water bodies with different nutrient statuses. Biodegradation of phenol and phenolic compounds is known to increase with inorganic nutrient enhancement, especially phosphorus (Jones and Alexander, 1986). Total Organic Carbon is another factor influencing degradation rates (Gladyshev *et al*, 1993). Alternative carbon sources may be used by phenol-degrading bacteria in preference to phenol and inert organic matter may act as an adsorbent (Chiou, Malcolm, Brinton and Kile, 1986). Pre-exposure to a compound or the presence of adapted or acclimated populations may also increase the rates of phenol degradation (Pritchard, O'Neile, Spain and Ahern, 1987; Wiggins *et al*, 1987). Several studies have shown that biodegradation of xenobiotic

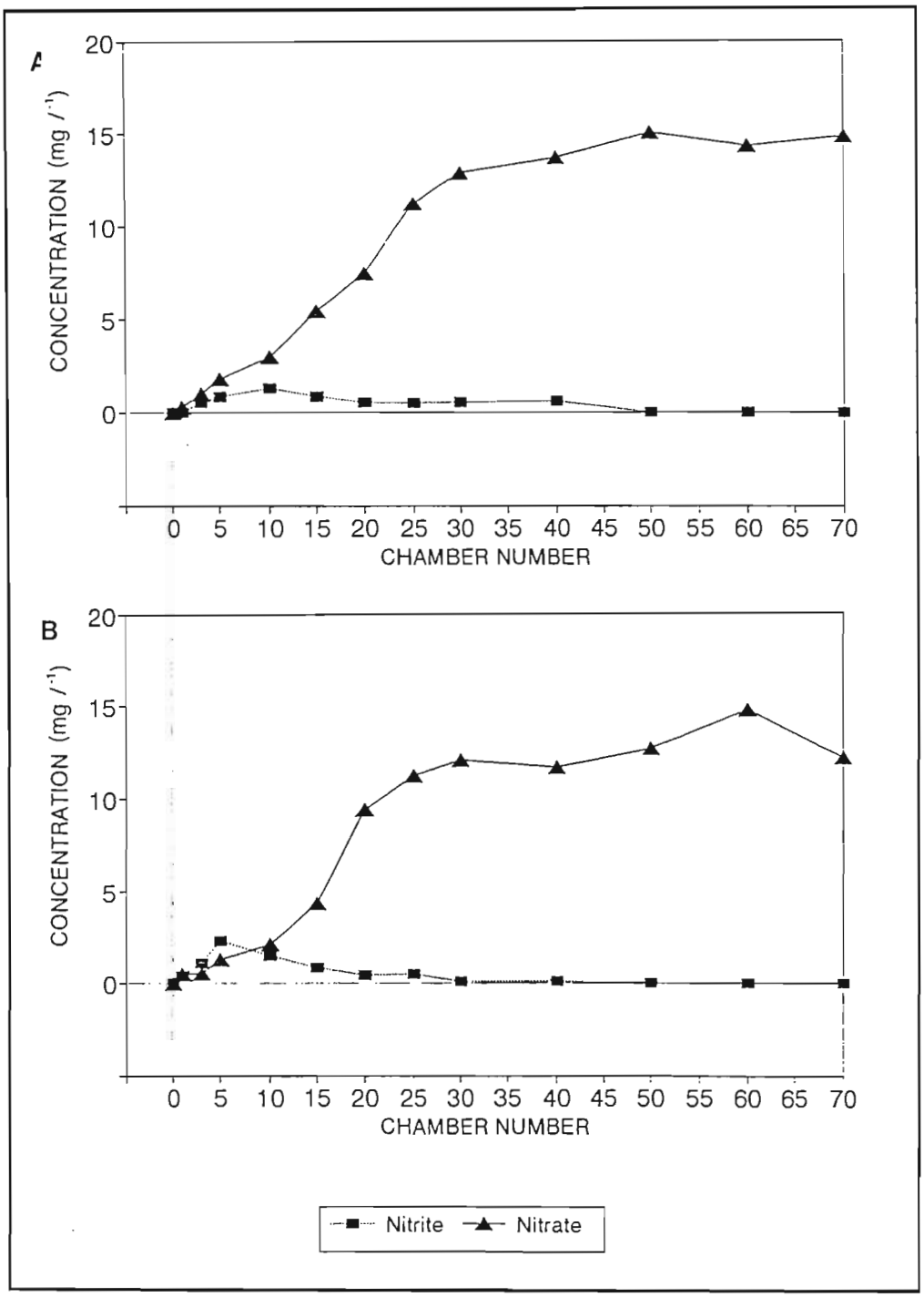
compounds is promoted in environmental water samples when detrital sediments are present (Spain, Van Veld, Monti, Pritchard and Cripe, 1984; Pritchard *et al*, 1987). Sediment-enhanced degradation may result from the presence of higher numbers of degraders on the sediment surface or from increased specific activity of the attached cells. Conversely, it has been reported that sorption of certain chemical compounds to sediments or suspended organic matter may reduce their availability (Subba-Roa, Rubin and Alexander, 1982).

5.3.3 Perturbation Studies

a) 14 Day Perturbation

For the study a 14 day perturabation period was chosen to allow two complete culture volume changes to occur within each channel to enable potential long-term perturbant effects to be determined.

A comparison of Channels A and D prior to perturbation showed that nitrification in each channel followed similar trends (Figures 5.4. A and B). Since each channel was inoculated with the same inoculum and was subjected to identical selection pressures, it was expected that the replicate channels would closely approximate to each other.



Figures 5.4 A and B. Course of nitrification in Channel A (A) and Control Channel D (B) prior to perturbation with phenol.

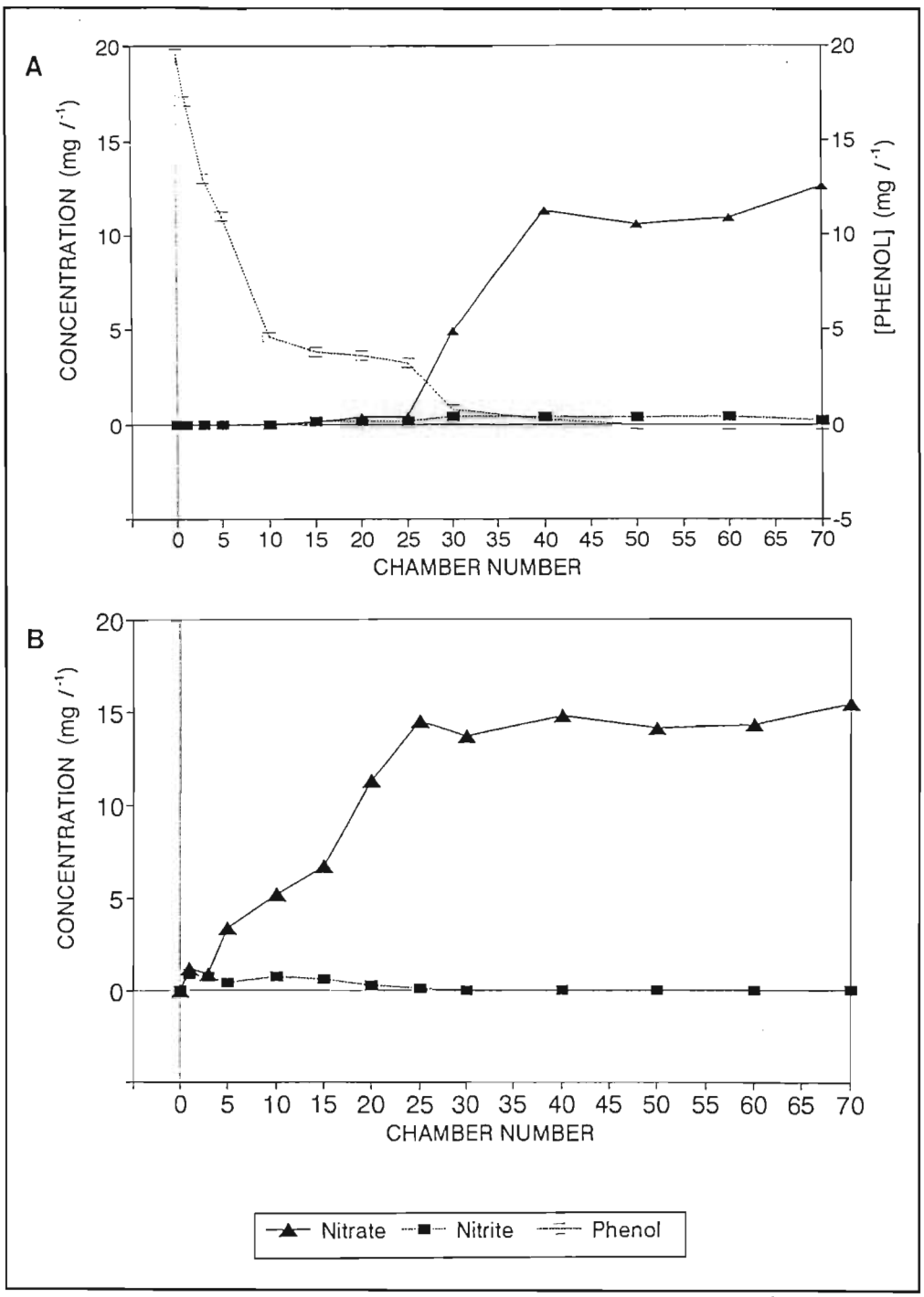


Figure 5.5 A. Course of nitrification and residual phenol concentrations in Channel A following a continuous perturbation (14 days) with 20 mg l^{-1} phenol to Chamber 1.

Figure 5.5 B. Course of nitrification in Control Channel D.

The addition of 20 mg l^{-1} phenol (0.21 mM) via the influent medium of Channel A effected a spatial shift in nitrification activity over a two week period (Figure 5.5A). It was evident that nitrification was inhibited until the residual phenol concentration had been reduced to < 4 mg l^{-1} (34 μM). Control Channel D did not exhibit any marked changes in nitrification activity during the same period (Figure 5.5B). This shift in nitrification activity indicated the sensitivity of the nitrifying populations to low concentrations of phenol. Similar observations have been reported in the literature. Progressive inhibition of the respiration of nitrifiers was found to occur in the presence of concentrations of 4 to 10 mg l^{-1} of phenol (Stafford, 1974). Hockenbury and Grady (1977) found that 5.6 mg l^{-1} of phenol resulted in a 75% inhibition of nitrification in activated sludge. An IC_{50} for phenol of 21 mg l^{-1} was indicated from a *Nitrosomonas* sp. bioassay (Blum and Speece, 1991).

It is generally accepted that, with the exception of the Microtox™ test, autotrophic nitrifying populations of bacteria are more sensitive to inhibitors than their heterotrophic counterparts (King and Dutka, 1986; Gray, 1990; Blum and Speece, 1991). For example, Blum and Speece (1991) determined an IC_{50} of 1100 mg l^{-1} for aerobic heterotrophs in activated sludge. A comparison of four microbial bioassays: the inhibition of respiration of activated sludge; *Pseudomonas fluorescens;* and *Bacillus subtilis;* and the *Spirillum volutans* motility test all indicated that the inhibitory concentrations reported for phenol (300 to 5000 mg l^{-1}) were markedly higher than those reported for nitrification inhibition (King and Dutka, 1986).

Since nitrate is the end product of nitrification it's absence was considered a suitable indicator of nitrification inhibition. Several reports have indicated that *Nitrosomonas* spp are more sensitive to toxic inhibitors than *Nitrobacter* spp (Hockenbury and Grady, 1977; Blum and Speece, 1992). Ammonia oxidation is, thus, usually considered the rate-limiting step in

nitrification inhibition. Blum and Speece (1991) reported that *Nitrobacter* spp had similar sensitivities to toxic molecules as aerobic heterotrophs. However, *Nitrobacter* sp. was found to be more sensitive to phenols.

The sensitivity of nitrifiers to inhibitors is related, in part, to their relatively slow growth rates. Toxic effects at low concentrations are more marked since recovery through the growth of resistant or adapted individuals is slow (King and Dutka, 1986). Unsubstituted phenols display a high degree of surface activity and act by both disrupting cell membranes and inhibiting oxidase enzymes associated with the surface membranes (Hedgecock, 1967). Nitrifiers derive energy solely from the oxidation of reduced inorganic nitrogen mediated by such oxidase enzymes and, thus, exhibit a marked sensitivity to phenol (Brock *et al*, 1991).

In the context of the objectives of this study, it was felt that a two week perturbation period was too long to satisfy the criteria for a rapid ecotoxicological assessment protocol for determining impacts on aquatic ecosystems. The exposure of an inhibitory compound for prolonged periods to a mixed association of microorganisms may result in increased levels of tolerance to the inhibitor and/or the ability to degrade it. Individuals in a population which are less sensitive, or are able to degrade the perturbant compound, will have a selective advantage in the presence of the inhibitor and may, thus, predominate. This acclimation is one of the main factors which determines the inhibitory effects of a chemical to populations of microorganisms in the environment (Klecka, 1986). By implication, this could lead to the underestimation of the toxic impacts to the aquatic environment since organisms from higher trophic levels do not adapt as readily to changes in their immediate environment. In a perturbation study similar to the one described here, Freitsch (1991) found that over a two year period, 4-chloro-3-cresol concentration increases from 2 to 50 mg l^{-1} mediated

acclimation of the resident microbial association. Prolonged exposure of the test system to phenol could, thus, be expected to result in the selection of phenol degraders and/or tolerant/resistant organisms. Such an approach would, therefore, not be expected to be suitable for assessing minimum inhibitory concentrations of a particular compound.

Factors which can affect apparent toxicity of an inhibitor to microbial populations are the presence of inert organic and inorganic matter and the overall ratio of microorganisms to inhibitor (King and Dutka, 1986).

The presence of inert organic or inorganic matter may substantially reduce the toxicity of some chemicals through adsorption, chemical bonding or chelation. Removal of the compound from solution can effectively lead to a reduction in the actual exposure concentration of the test compound. It has been suggested by King and Dutka (1986) that for cultures with high levels of biomass (e.g. activated sludge) the inhibitor compound reacts with the inert organic materials present so that the biomass has a certain "titre" above which the inhibitor becomes available to the bacteria. Furthermore, binding sites on the cells may be used up so that there is a reduction of toxic material in the aqueous phase. Below a minimum concentration of bacteria, sensitivity to a particular concentration of toxic chemical is, therefore, expected to be higher.

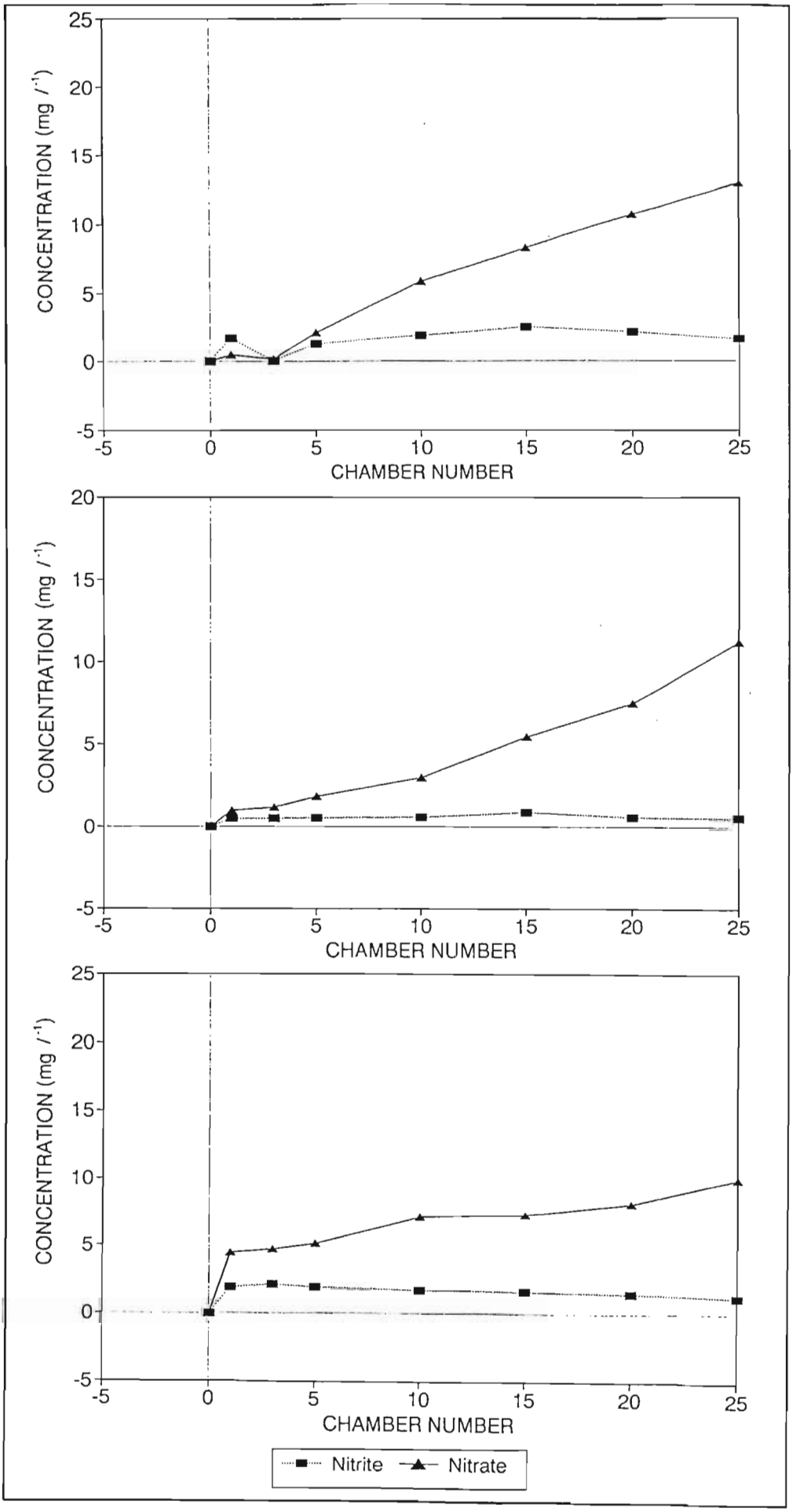
These factors can potentially lead to the underestimation of the perturbant impacts of the compound, especially to organisms from higher trophic levels. This emphasises the importance of determining the bioavailability of a compound when it is introduced into the environment.

A potential application of long-term perturbation studies in the model lies in its ability to determine the effects of organic loading on nutrient cycling processes. The relationship between the removal of the test compound from the multi-chambered model and the process of nitrification can then be assessed. Maximum inhibitory loads, adaption to, and biodegradation of, perturbant compounds can, potentially, be assessed. Such information would prove useful in defining the operational parameters for discharging potentially inhibitory compounds to biological wastewater treatment processes.

Subsequent to this experiment it was decided that a shorter perturbation period should be assessed to determine the acute response of the resident microbial populations to an inhibitor.

b) 24 h Perturbation

The courses of nitrification prior to the 24 hours perturbation study for Channels B,C and D are shown in Figure 5.6, while the residual concentrations of phenol in relation to nitrification for the two experimental channels are shown in Figure 5.7. The course of nitrification in the control Channel D in the absence of phenol is shown in Figure 5.8.



Figures 5.6 A-C. Course of nitrification in Channels B (A) and C (B) and Control Channel D (C) prior to perturbation with phenol.

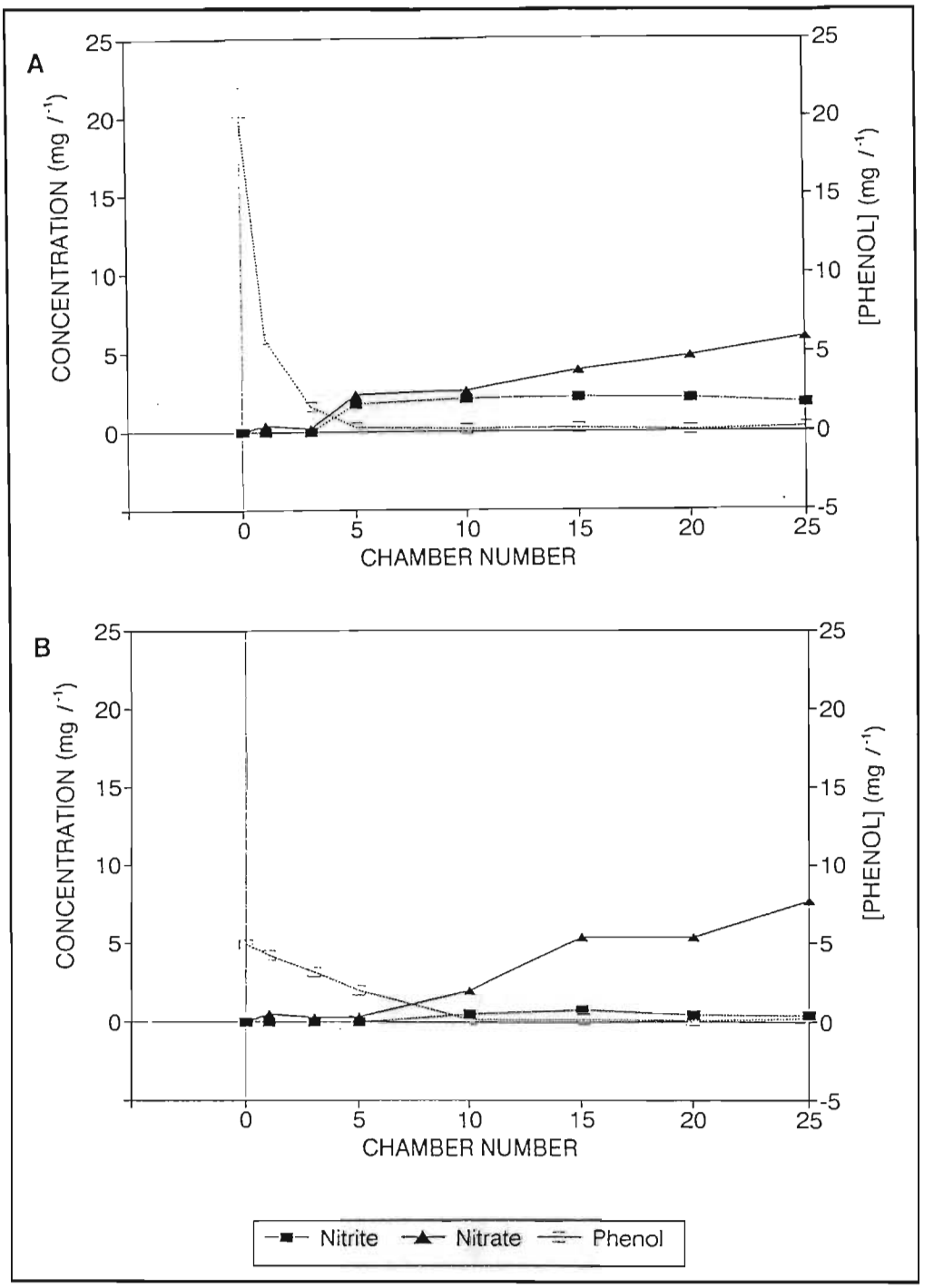


Figure 5.7 A and B. Course of nitrification and residual phenol concentrations in Channels B (A) and C (B) following continuous perturbation (24 h) of 20 mg l^{-1} and 5 mg l^{-1} phenol to Chamber 1 of Channels B and Channel C, respectively.

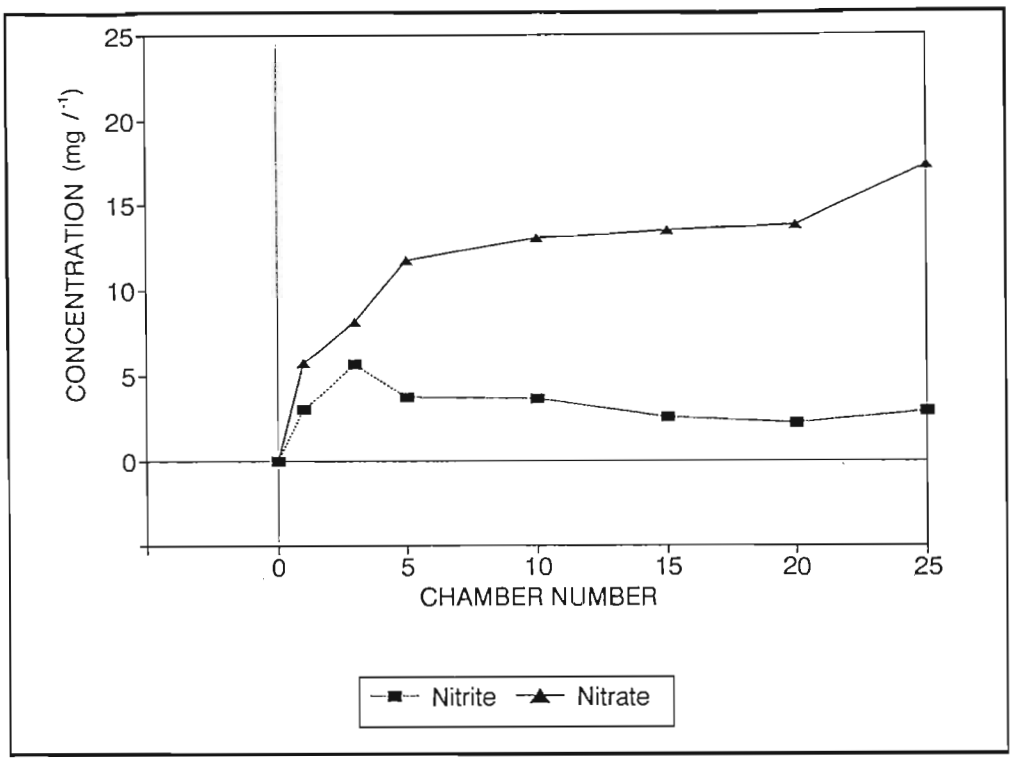


Figure 5.8. Course of nitrification in control Channel D.

After a 24 hour perturbation period it was found that for both of the concentrations of phenol tested, nitrification only proceeded when the phenol concentration was sufficiently lowered within each experimental channel (Figure 5.7 A and B).

The actual inhibitory effect of phenol at the concentrations tested is difficult to quantify since the removal of phenol effectively reduced the actual exposure concentration of the perturbant compound along each channel. The toxicological impact of phenol in the multi-stage system thus appeared to be a direct function of the removal or transformation of the molecule. This factor must be an important consideration when screening test compounds at relatively low concentrations. Removal of the perturbant compound from solution early in the test system can thus, possibly, lead to an underestimation of the potential toxicological impacts of the molecule on nitrification.

A problem which quickly became apparent was the reinstatement of the experimental channels for further perturbation trials. Because these channels had previously been subjected to different selection pressures, the microbial association would be expected to have changed accordingly. At the outset of the study the intention was to reinoculate the experimental channels from control Channel D. In theory, this would have suited a free-living association where the incumbent population could be washed out and the original one reinstated. However, in practice, this procedure did not take into consideration the flocculent biomass or the surface-attached populations which also had to be removed. This involved the cleaning and reinoculation of each experimental channel which proved impractical and time consuming. As a result, increased variability between channels was expected to occur with subsequent perturbation trials.

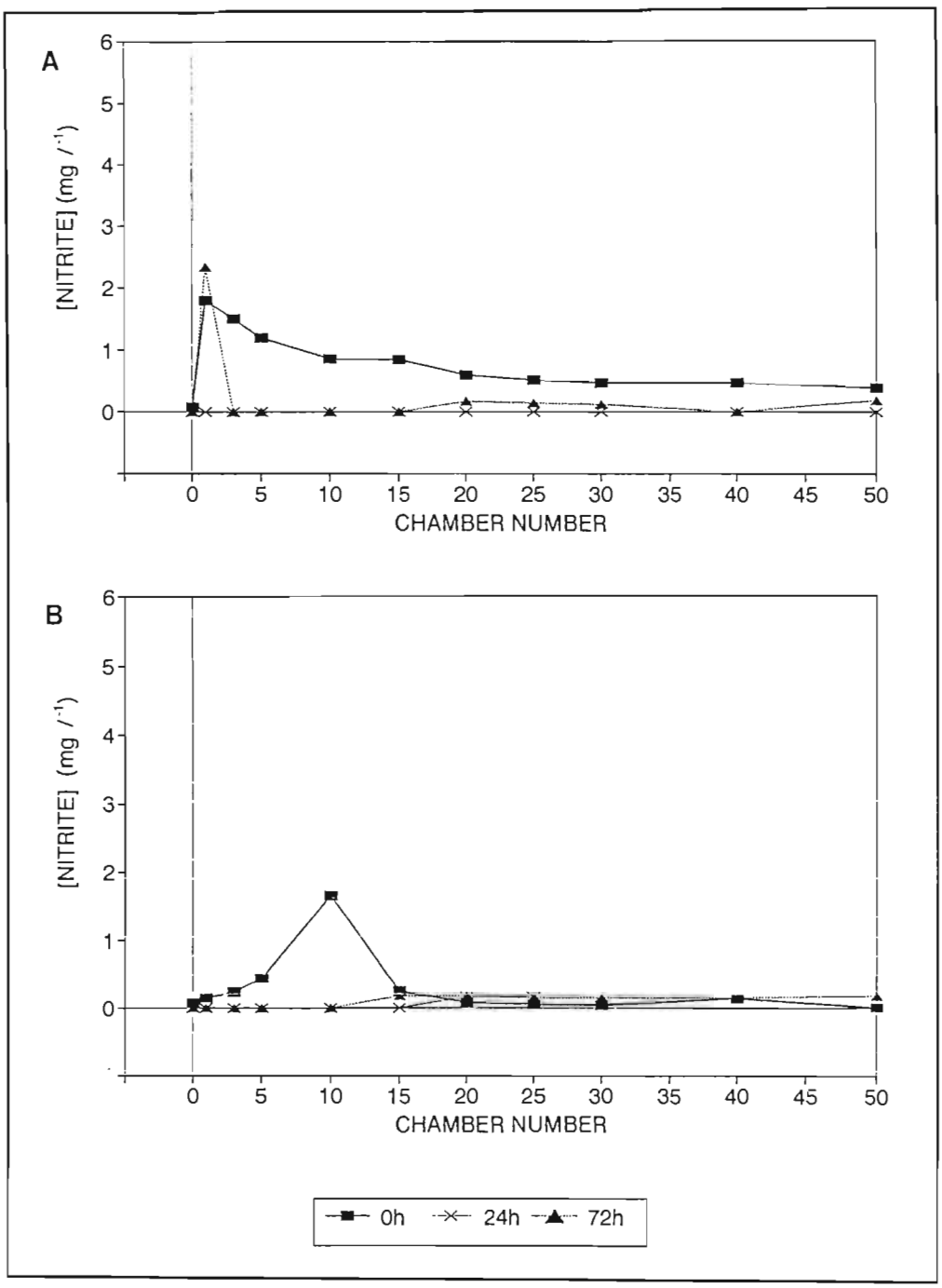
At this stage, further changes were made to the operational parameters of the model to further standardise the experimental protocol. These changes are described in Chapter Two (2.3). The establishment of a nitrifying population within the first five chambers of each experimental channel became a priority.

c) 72 h Perturbation

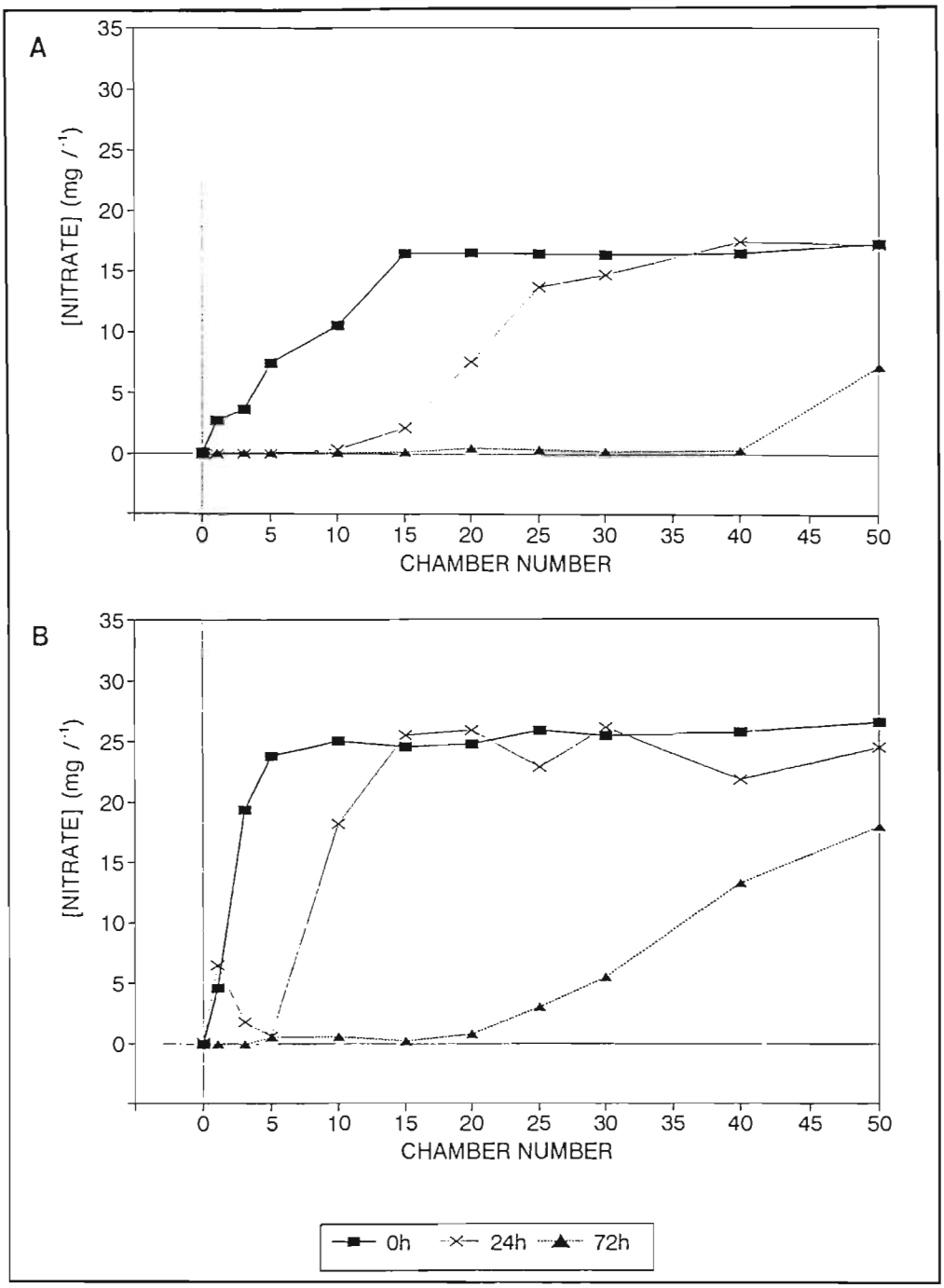
60 mg l^{-1} Phenol Perturbation Experiment

The first 72 hour perturbation exposure was made with a 60 mg l^{-1} phenol introduction to Channels A and B while Channel D was maintained as a control. The inhibitory effects of phenol on nitrite and nitrate assimilation in the two experimental channels are shown in Figures 5.9 (nitrite) and 5.10 (nitrate), respectively. The residual concentrations of phenol in the same channels for the same period are shown in Figure 5.11. Figures 5.12 and 5.13

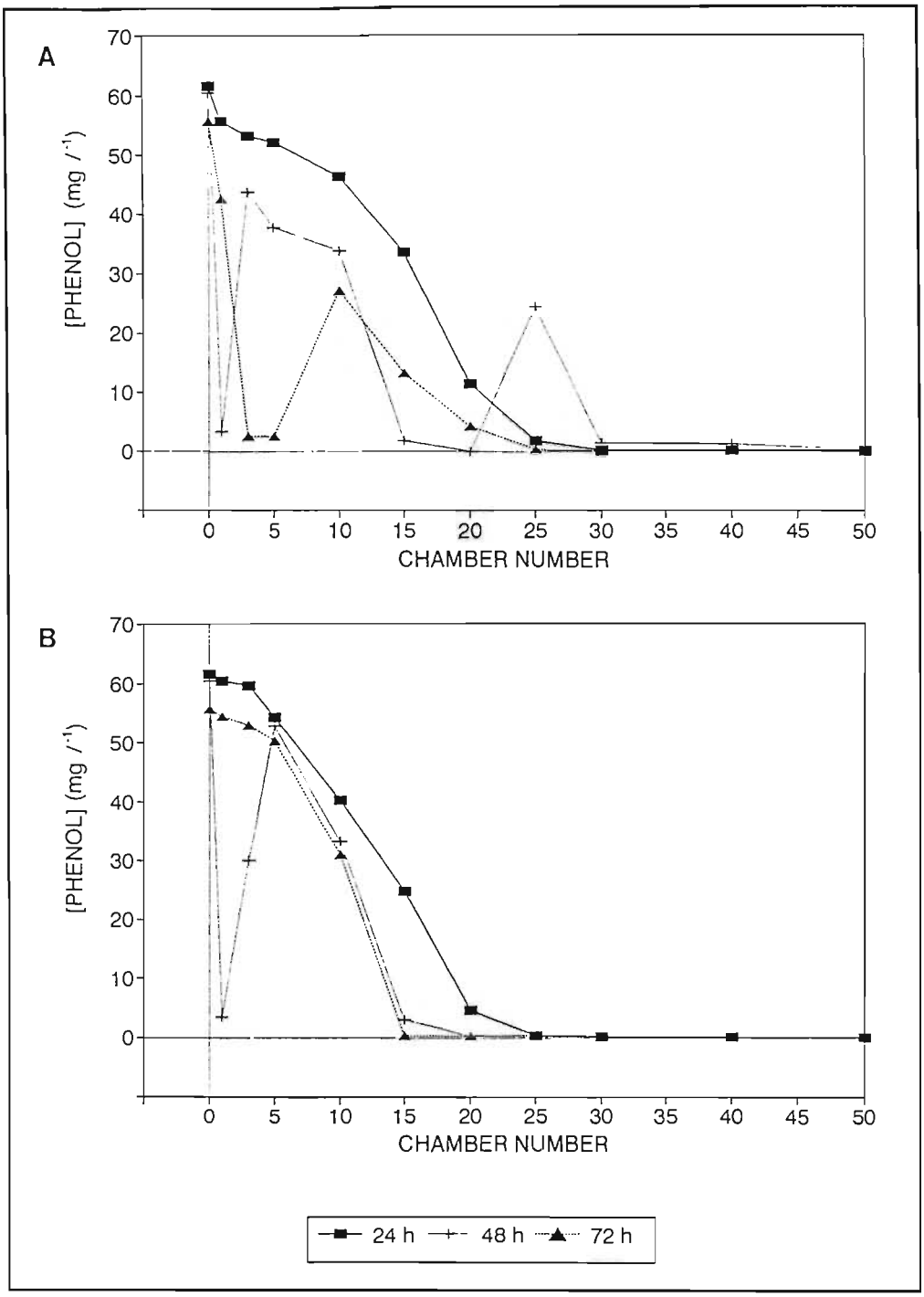
show the concentrations of nitrite and nitrate after a 216 hour recovery period. Variability within the control channel with regards to nitrite and nitrate assimilation during the course of the experiment are shown in Figures 5.14 and 5.15 respectively.



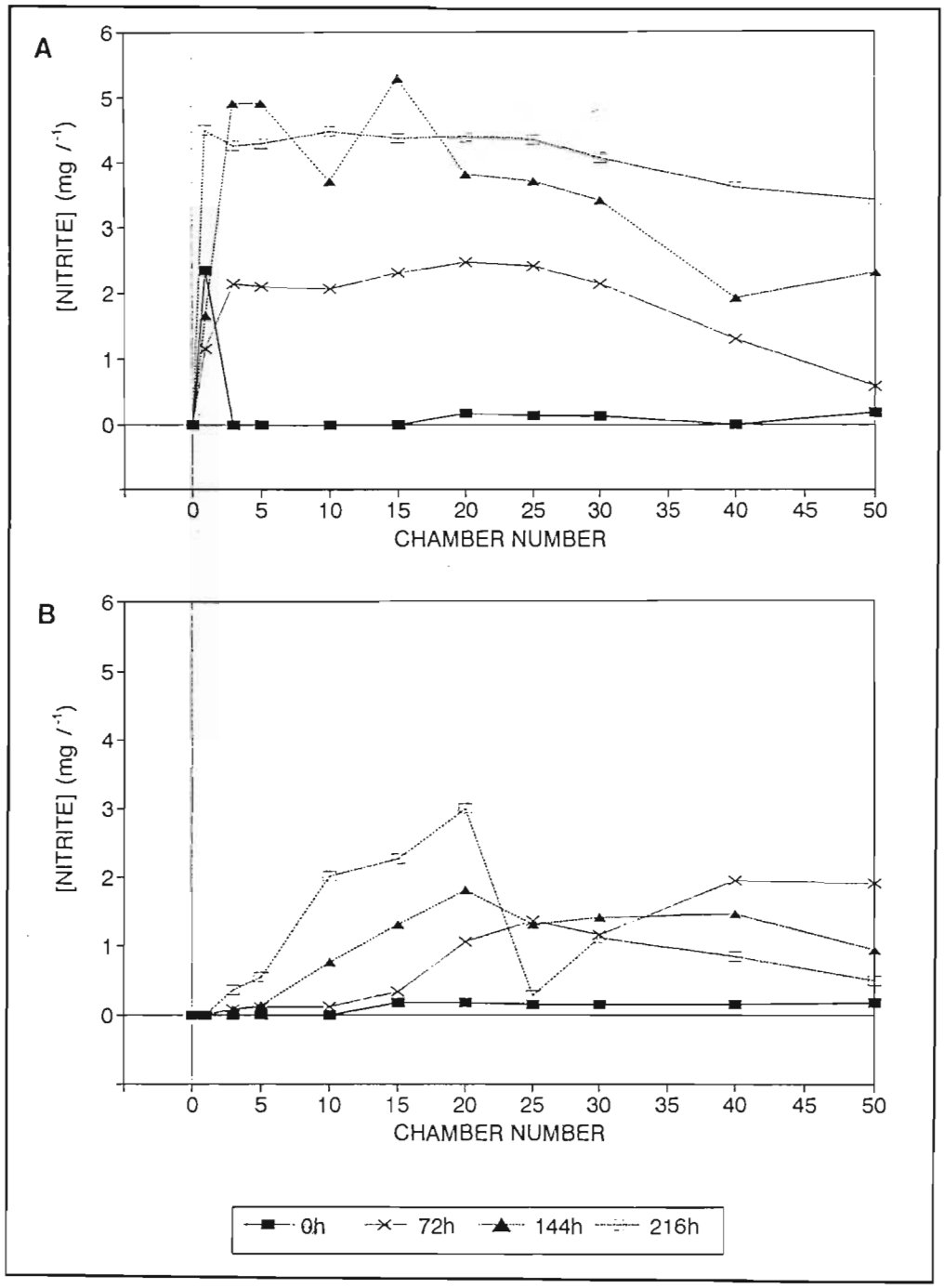
Figures 5.9 A and B. Changes in nitrite concentrations in response to phenol (60 mg l^{-1}) perturbation of Channels A (A) and B (B) of the river model.



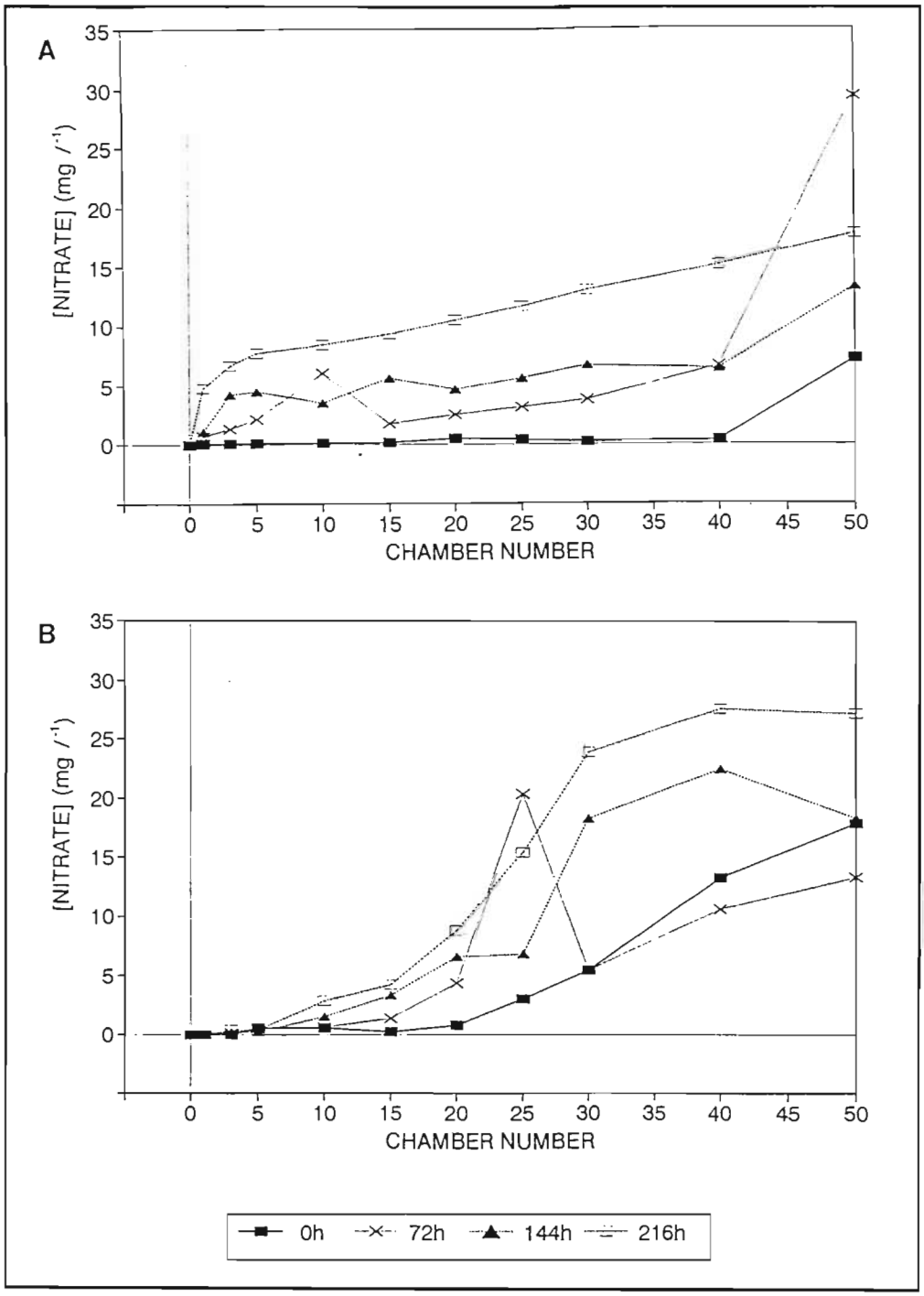
Figures 5.10 A and B. Changes in nitrate concentrations in response to phenol (60 mg l^{-1}) perturbation of Channels A (A) and B (B) of the river model.



Figures 5.11 A and B. Residual phenol concentrations in Channels A (A) and B (B) after a 72 hour perturbation period.



Figures 5.12 A and B. Changes in nitrite concentrations in Channels A (A) and B (B) after a 216 hour recovery period, subsequent to a 72 hour phenol perturbation (60 mg l^{-1}).



Figures 5.13 A and B. Changes in nitrate concentrations in Channels A (A) and B (B) after a 216 hour recovery period, subsequent to a 72 hour phenol perturbation (60 mg l^{-1}).

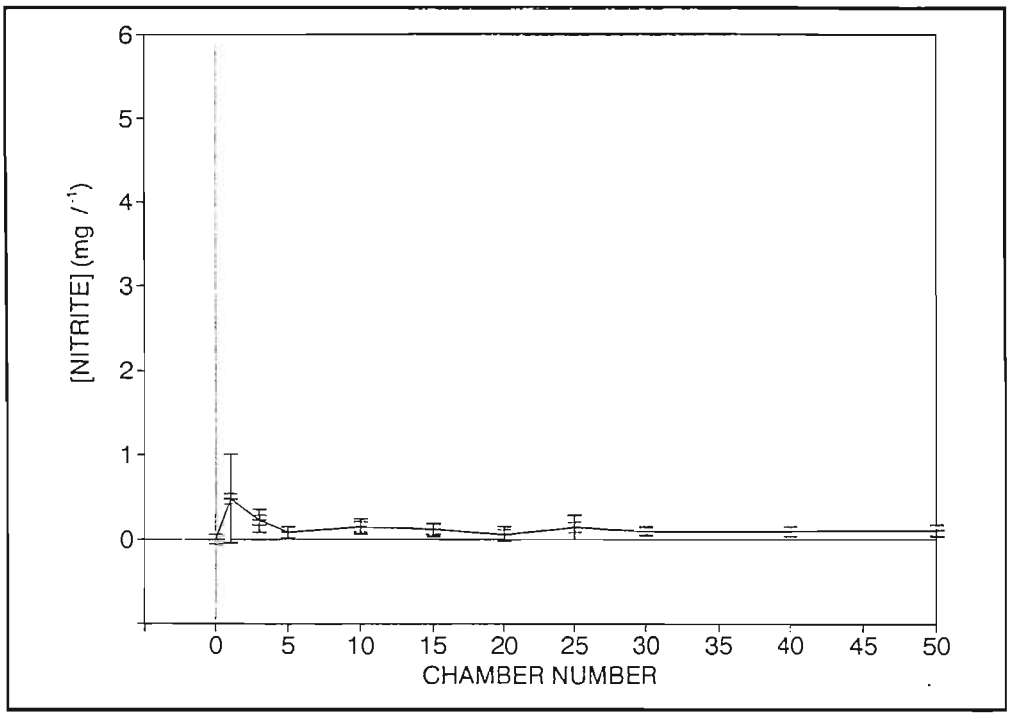


Figure 5.14. Discrete concentrations of nitrite in Control Channel D at the end of the 216 hours experimental period. Values represent means of each sampled chamber. Vertical bars depict 95% confidence intervals.

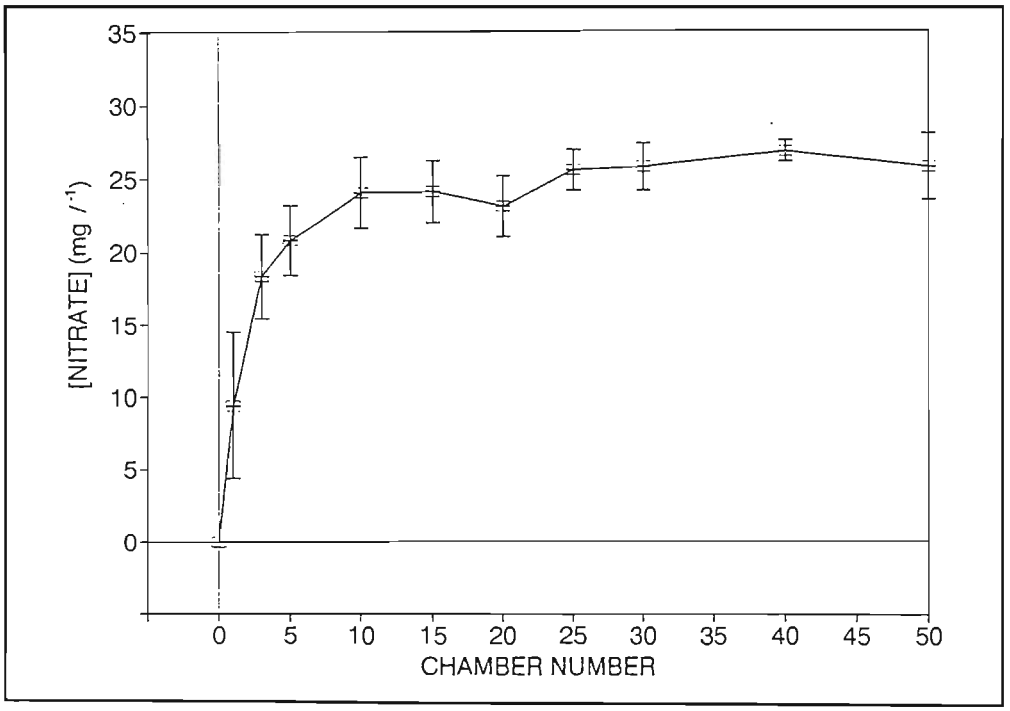


Figure 5.15. Concentrations of nitrate in selected chambers of Control Channel D at the end of the 216 h experimental period. Values represent means of each sampled chamber. Vertical bars depict 95% confidence intervals.

Prior to the perturbation experiment, the replicate channels were found to follow similar trends in terms of nitrification activity. The nitrite concentrations in both experimental channels were low indicating the rapid or concurrent oxidation of nitrite to nitrate. Higher concentrations of nitrate were, however, present in Channel B at the beginning of the experiment when compared to Channel A. The overall accumulation of nitrate appeared to occur at a faster rate in Channel B with nitrate concentrations stabilising after Chamber 5, in comparison with Chamber 15 of Channel A. These results illustrated the intrinsic variability within the experimental channels prior to the perturbation.

The addition of phenol to the experimental channels effected a population shift in nitrification activity during the 72 h perturbation period. Nitrite did not accumulate (Figures 5.9 A and B) which indicated the sensitivity of the ammonia oxidisers to the perturbant. In both channels the inhibition of nitrate production was found to be more pronounced after 72 h than at 24 hours (Figures 5.10 A and B). This was attributed to the protracted exposure time which effectively reduced the "masking effect" of the associated biomass and organic matter. This finding illustrates one of the major limitations of short-term perturbation studies whereby long-term chronic effects are not considered.

It was evident that nitrifying activity limitation was a direct function of the removal of phenol (Figures 5.11 A and B). Residual phenol concentrations were found to be significantly lowered at Chamber 30 in each channel ($< 1 mg\ l^{-1}$). For the first 24 hours this could be attributed to dilution whereas subsequent low concentrations were attributed to biodegradation.

It has been reported that the degradation of xenobiotic compounds occurs only after more labile compounds are catabolized (Alexander, 1981). This consideration is relevant to biological treatment processes with high nutrient loads but has limited relevance to aquatic environments which are usually characterised by nutrient poor conditions. This warrants further investigation to determine whether the selective degradation of a molecule such as phenol occurs either concurrent with or subsequent to the degradation of more labile compounds.

The toxic effect of a perturbant compound may manifest in various ways. There may be an inhibitory effect on nitrifiers so that they continue to grow and oxidise at significantly reduced rates. The toxicity may be bactericidal to nitrifiers and thus stop nitrification until the toxic compound is removed. Alternatively, a bacteriostatic effect may be reversible where nitrification stops temporarily but resumes when the molecule is removed or diluted (Stensel and Barnard, 1992).

After the perturbation period, nitrification was found to recover, albeit at a slow rate. Increased concentrations of nitrite were recorded which indicated the re-establishment of the ammonia-oxidising populations (Figures 5.12 A and B). For both channels, nitrate production was, however, found to occur at lower overall rates than the pre-perturbation populations, after the 9 day recovery period (Figures 5.13 A and B). Recovery in the two channels differed markedly. Channel B showed increased nitrite oxidising activity from Chamber 5 whereas Channel A exhibited nitrite oxidising activity from Chamber 1. These reinstatements of nitrification activity were consistent with the recovery of populations of nitrifiers which were resistant to or protected from the inhibitory phenol during the perturbations.

The slow recovery rates of nitrification are of particular significance if one takes into consideration the impacts of a pollutant shock load on nutrient cycling processes such as nitrification within both wastewater treatment plants and aquatic environments. Since inhibition of nitrifying activity can lead to prolonged recovery periods with lowered nitrification rates this can have detrimental effects on nutrient removal processes and, thus, impair wastewater treatment efficiency. An important result of ammonia oxidiser inhibition is the expected increase in ammonia concentrations. Thus, inhibition of nitrification may have a serious impact on ammonia removal in biological treatment processes. In relatively low concentrations (0.033 to 2.64 mg l^{-1}), free ammonia is toxic to many aquatic organisms and can have many adverse effects on the aquatic environment (Dallas and Day, 1993). Ammonia represents an oxygen demand in aquatic environments and its presence in streams can lead to lowered dissolved oxygen concentrations (Underhill, 1990). When present with phosphorus it can cause unwanted algal growth and lead to eutrophication of natural waters (Bitton, 1994).

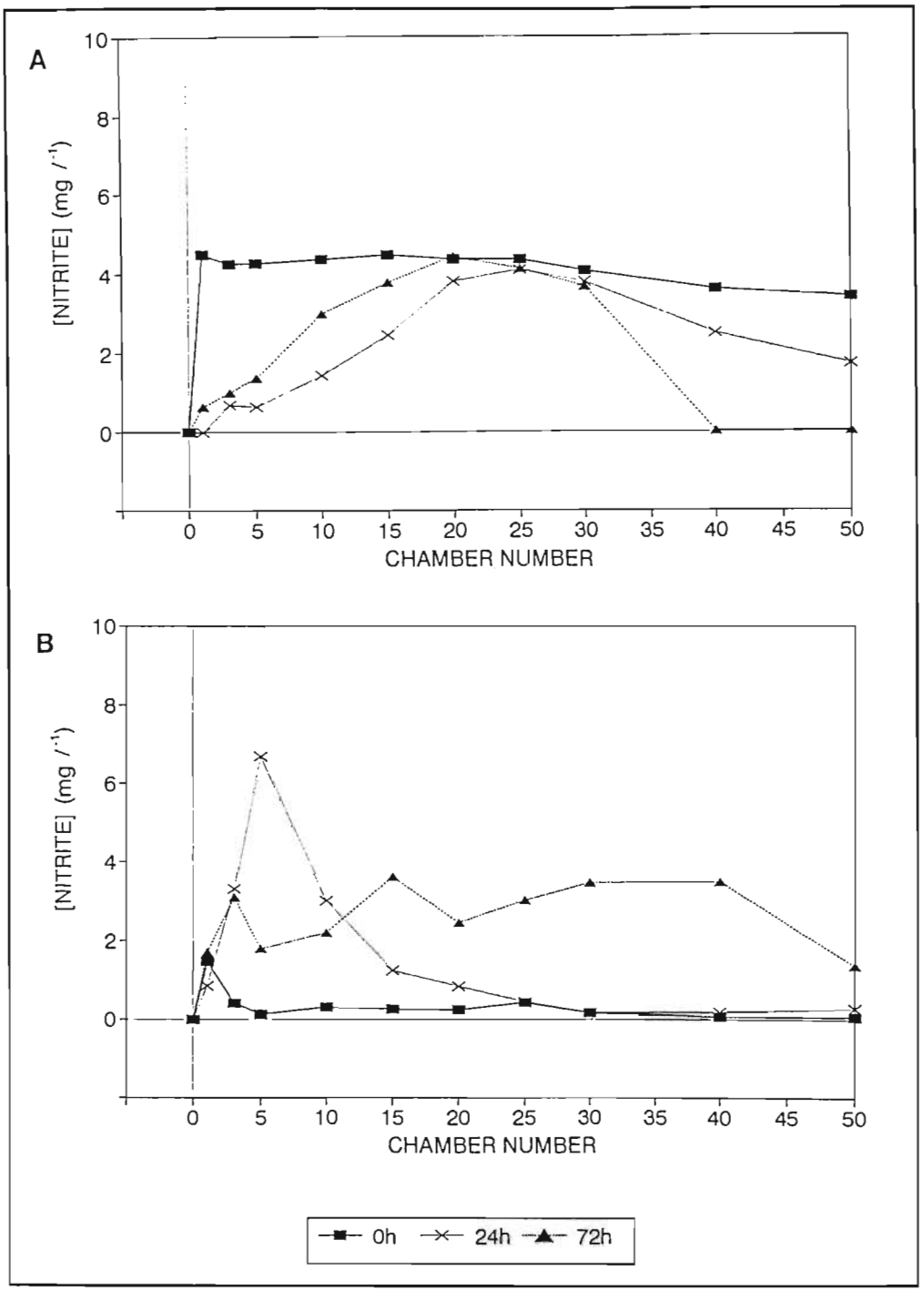
Variabilities in nitrite and nitrate concentrations within the control channel during the experimental period are shown in Figure 5.14 and Figure 5.15, respectively. The results show little variability and hence were not considered in relation to interpretation of the experimental results.

The results confirmed that a 72 hour perturbation period was sufficient to illicit an inhibitory response on nitrification within the experimental channels with the phenol concentration tested. Differences were, however, apparent in the relative impacts of phenol in the two experimental channels. While both channels displayed a population shift in nitrification activity, the degree and extent to which this occurred differed (Figures 5.9 and 5.10, A and

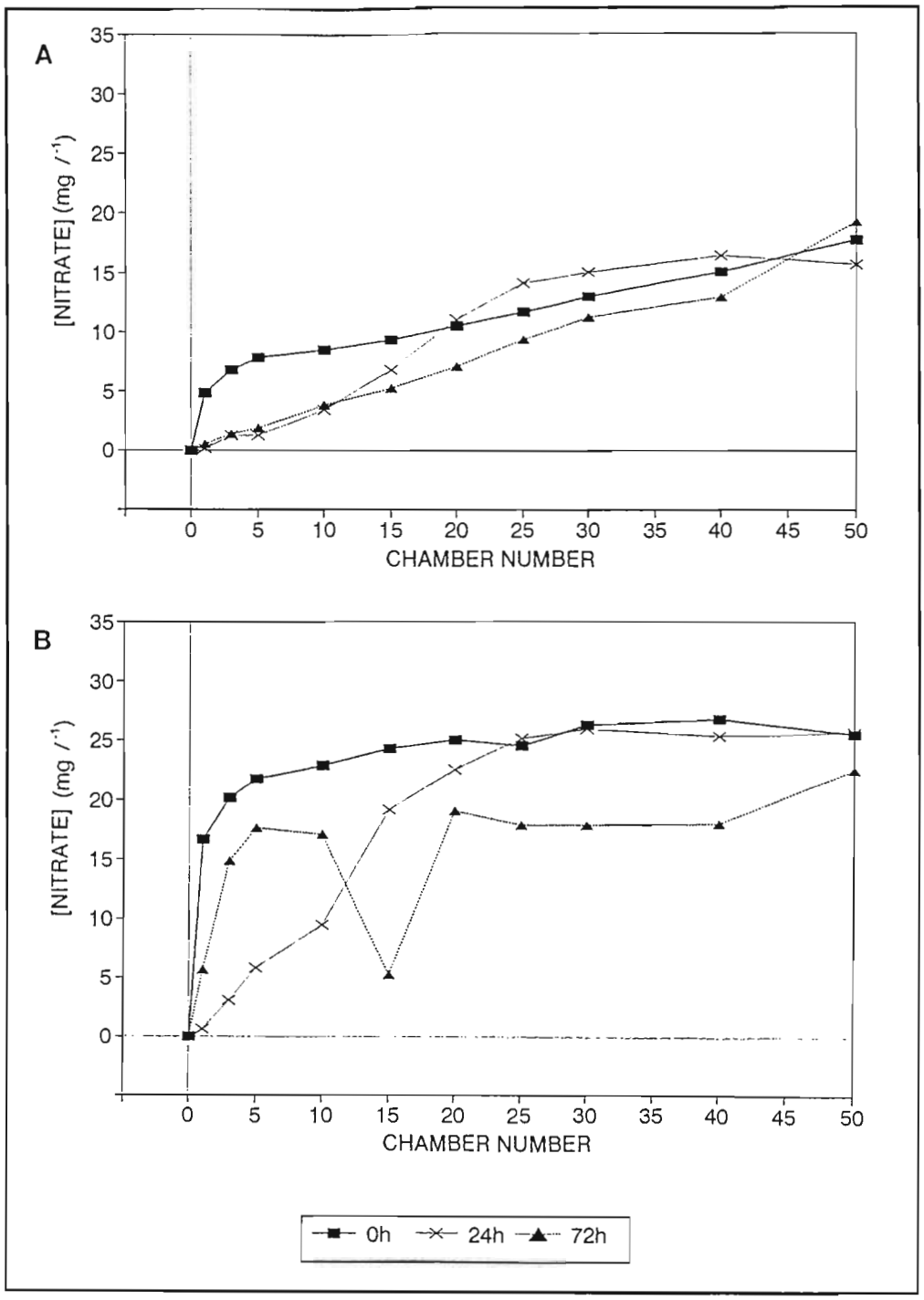
B). Channel A was characterised by a more marked response to the phenol perturbation than Channel B. This finding emphasised the variability and difficulty of reproducibility within the model system and, thus, the problems of assessing ecotoxicological impacts of a particular compound with such a model. Apart from key variables such as temperature, pH and aeration which may affect the nitrifying population, other factors have to be taken into consideration. These include competition with resident heterotrophs, acclimation of nitrifiers to phenol, and differing sensitivities of the nitrifying populations within the two channels. Other possible important contributing factors to the variability of the results were the discrepancies that arose in the aeration and mixing of the individual chambers which resulted from airflow impairment by aeration nozzle clogging.

20 mg l^{-1} Phenol Perturbation Experiment

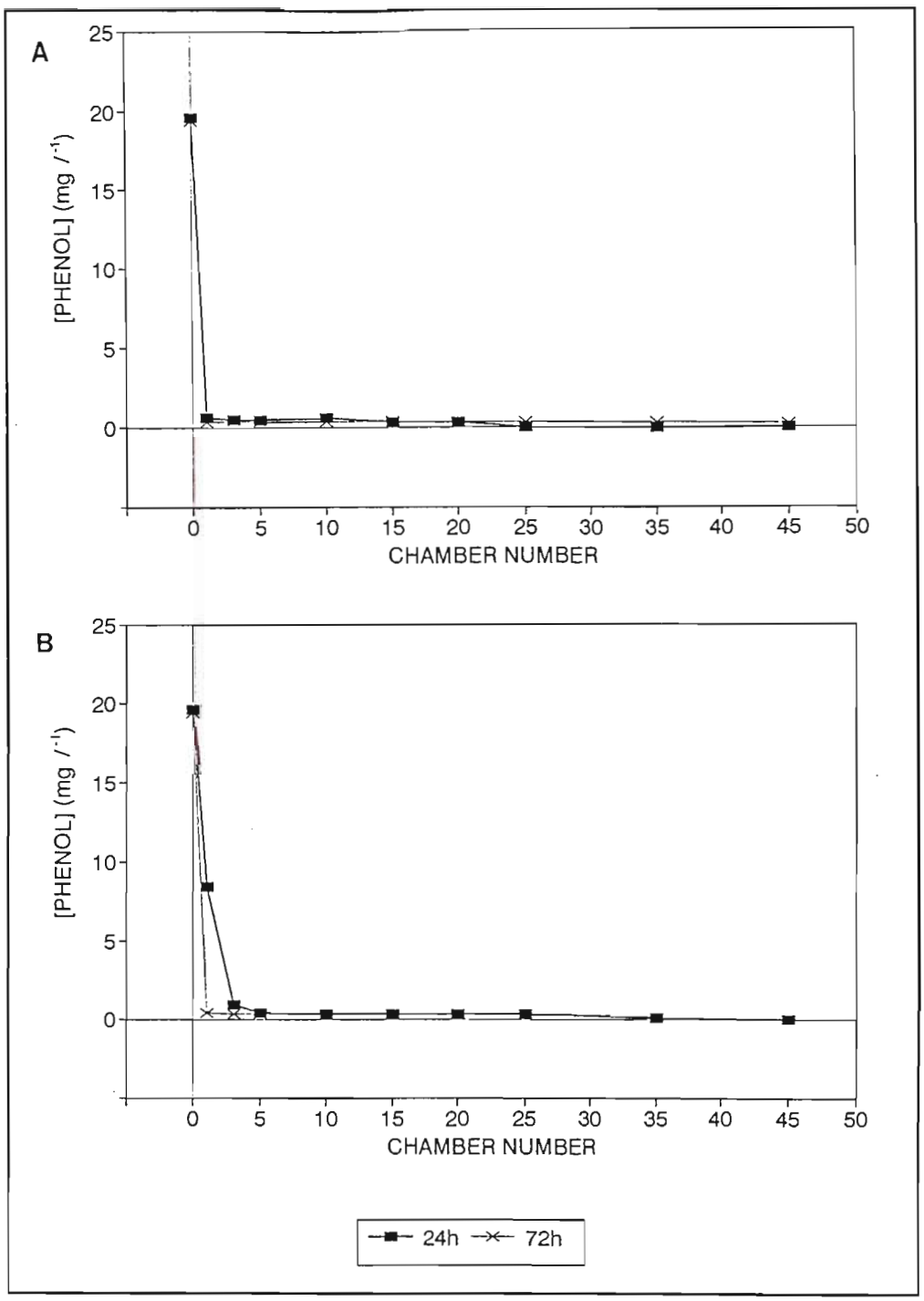
Following a 10 day recovery period a second experiment was undertaken whereby 20 mg l^{-1} phenol was added in the influent medium of Channels A and C. The inhibitory effects of phenol on the concentrations of nitrite and nitrate in selected chambers of the two experimental channels are shown in Figures 5.16 A and B, and 5.17 A and B, respectively. The residual concentrations of phenol in the same channels during the same time interval are shown in Figures 5.18 A and B. Figures 5.19 and 5.20 show nitrite and nitrate concentrations, respectively in Channel A after a 144 hour recovery period. Channel C was not monitored during this period. Channels B and D were maintained as controls and variability within each channel with regards to nitrite and nitrate concentrations during the course of the experiment are shown in Figures 5.21 A and B and 5.22 A and B, respectively.



Figures 5.16 A and B. Changes in nitrite concentrations in response to phenol (20 mg l^{-1}) perturbation of Channels A (A) and C (B) of the river model.



Figures 5.17 A and B. Changes in nitrate concentrations in response to phenol (20 mg l^{-1}) perturbation of Channels A (A) and C (B) of the river model.



Figures 5.18 A and B. Residual phenol concentrations in Channels A (A) and C (B) after a 72 hour perturbation period.

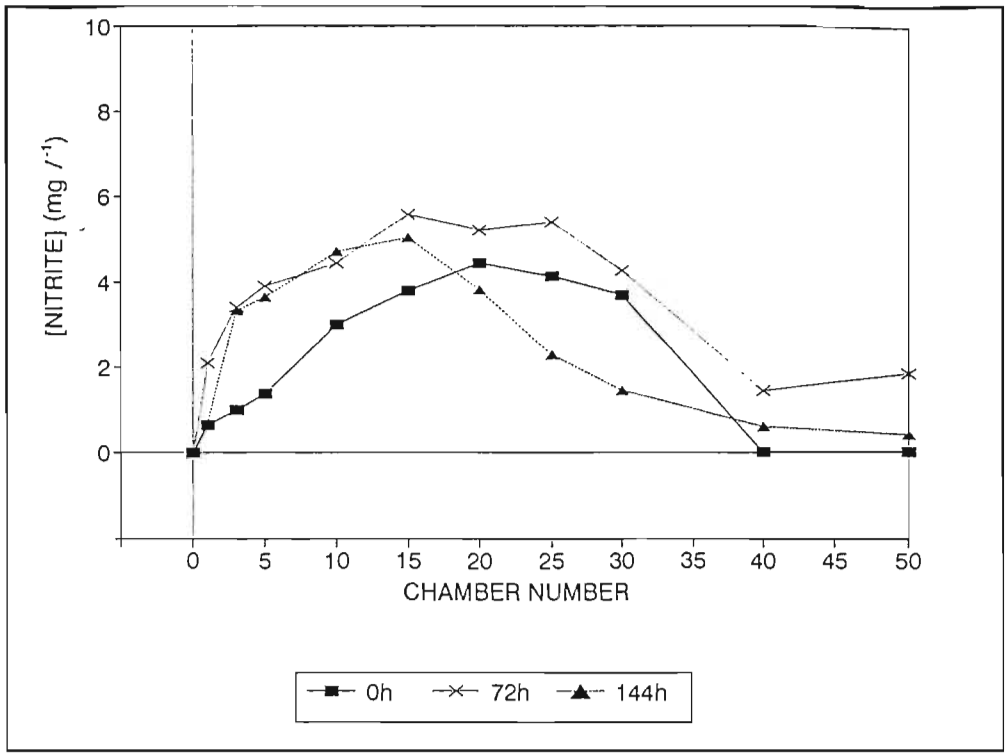


Figure 5.19. Changes in nitrite concentration in Channel A after a 144 hour recovery period, subsequent to a 72 hour phenol perturbation (20 mg l^{-1}).

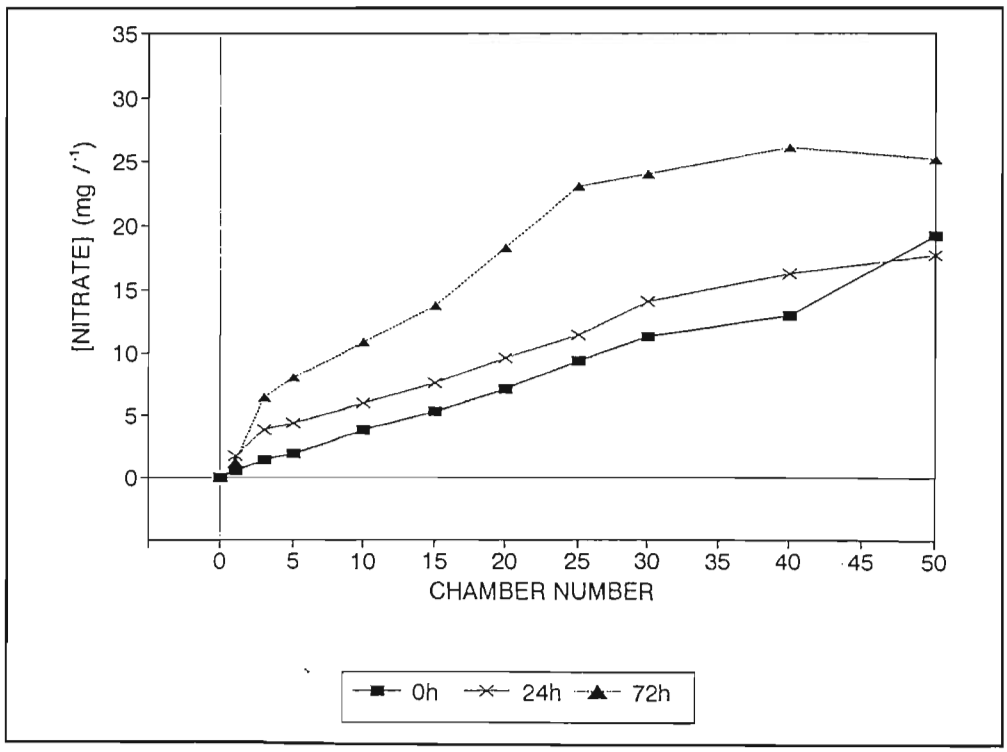
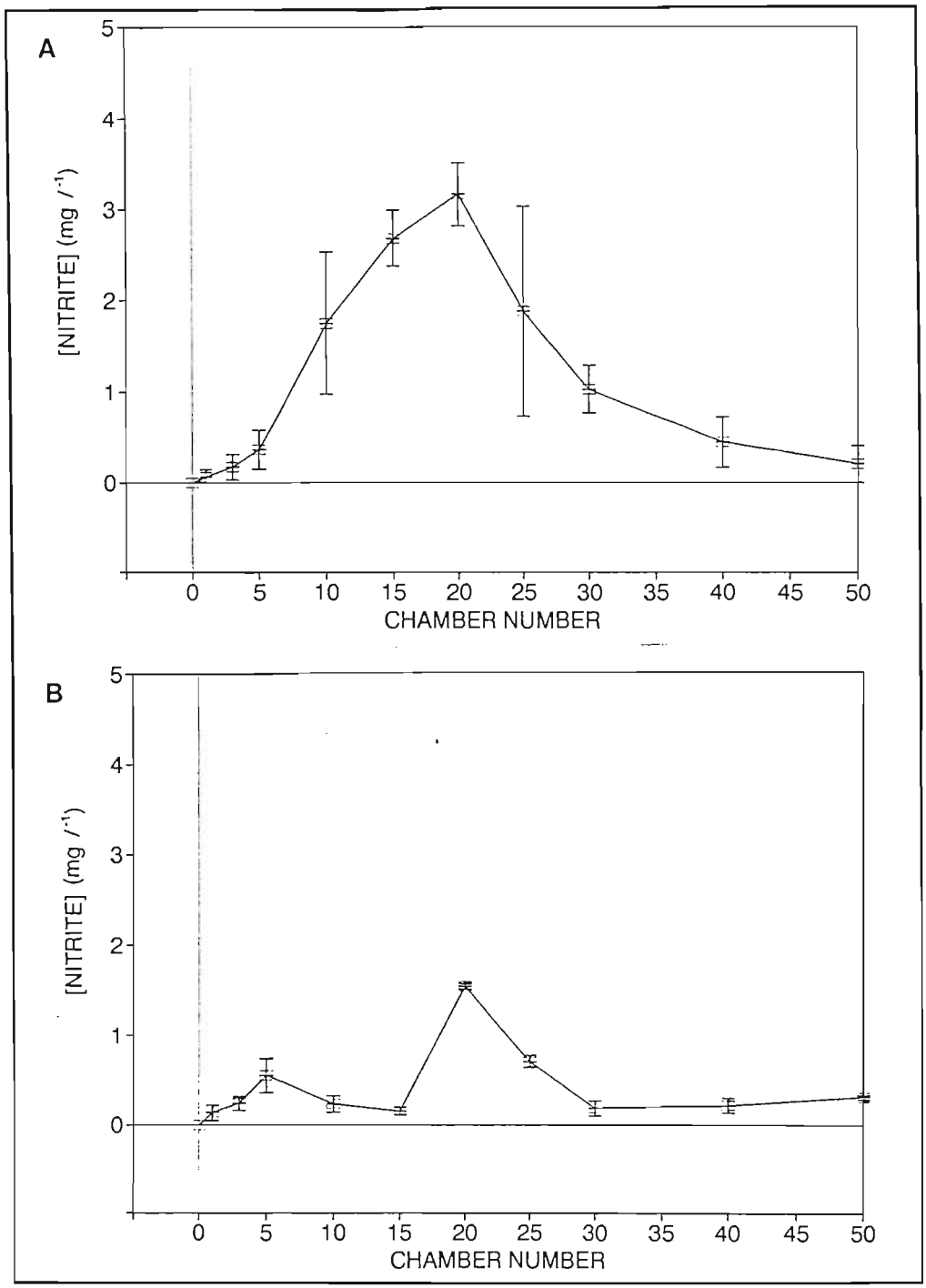
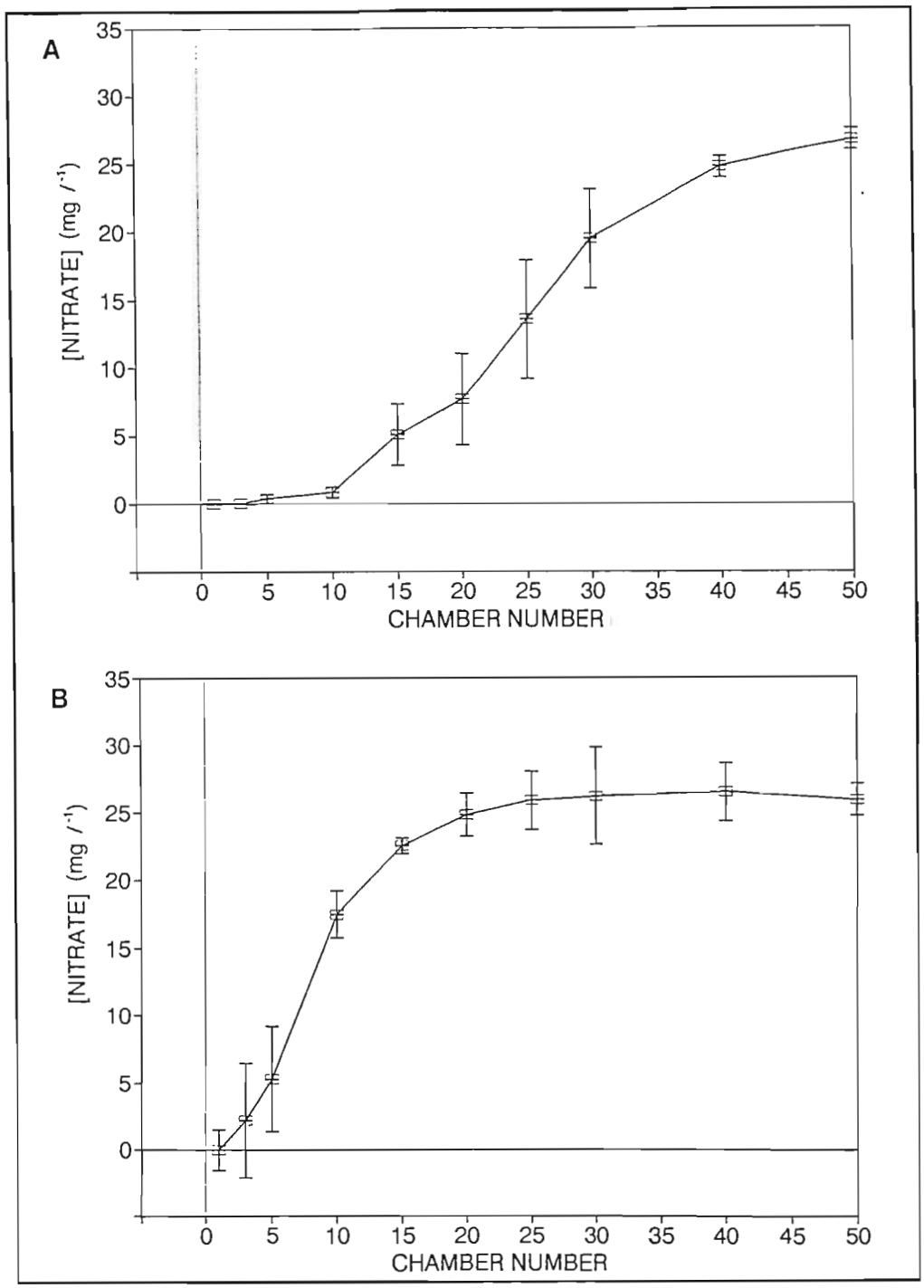


Figure 5.20. Changes in nitrate concentration in Channel A after a 144 hour recovery period, subsequent to a 72 hour phenol perturbation (20 mg l^{-1}).



Figures 5.21 A and B. Concentrations of nitrite in selected chambers control Channels B (A) and D (B) at the end of the 216 hour experimental period. Values are means of each sampled chamber. Vertical bars depict 95% confidence intervals.



Figures 5.22 A and B. oncentrations of nitrate in selected chambers control Channels B (A) and D (B) at the end of the 216 hour experimental period. Values are means of each sampled chamber. Vertical bars depict 95% confidence intervals.

Differences in the responses of the populations of nitrifiers to the perturbant were expected. This was due to the different selection pressures which the microbial associations in the two experimental channels were subjected to prior to the perturbation. Channel C exhibited nitrifying activity early within the channel indicating that a well-established population of nitrifiers was present (Figures 5.16 B and 5.17 B). Channel A exhibited relatively higher nitrite concentrations which suggested that the population of nitrite oxidisers had not established to the same extent (Figure 5.16 A). Cognisance of this variability was taken into consideration when interpreting the data.

Prior to the phenol perturbation, nitrifying activity was associated with the first 5 chambers of each channel (Figures 5.16 A and B, and 5.17 A and B). Perturbation with 20 mg l^{-1} phenol resulted in increased nitrite concentrations (Channel C) and the lowering of nitrate concentrations in the first 20 to 25 chambers of each channel (Figures 5.16 B and 5.17 A and B). This equated to a reduction in nitrification rates due to toxic/bacteriostatic inhibition in these regions, but did not effect distinct population shifts in nitrifying activity. Quantification of the inhibitory effect was difficult due to concurrent phenol attenuation. Phenol concentrations of < 1 mg l^{-1} were recorded by Chamber 3 in both channels after 24 hours and, therefore, the impacts of the molecule on nitrification were minimised (Figures 5.18 A and B).

The impact in Channel A appeared to be more pronounced than in Channel C and this was attributed to the possibility that the nitrifying population was not as well established. In Channel C, phenol perturbation resulted in increased nitrite concentrations which indicated that an interruption of nitrite oxidation to nitrate had occurred. At time 24h this corresponded to a lowered rate of nitrate accumulation up to Chamber 25 after which the nitrate

concentrations stabilised. At the end of the perturbation period (72h), nitrification activity appeared to have partially recovered in Channel C (Chambers 1 to 5), although nitrite still accumulated and this resulted in a lowered overall conversion of nitrite to nitrate. This was attributed to the slower growth rates of a recovering population of nitrite oxidisers.

After a protracted recovery period (144h), Channel A exhibited nitrification activity that exceeded that of the pre-perturbation population (Figures 5.19 and 5.20). This illustrated that the bacteriostatic effect of phenol was temporary and that nitrification activity was restored when phenol was removed or diluted. Channel C was not monitored during this period since this channel had already exhibited a recovery of nitrification activity at the end of the 72 hour perturbation period.

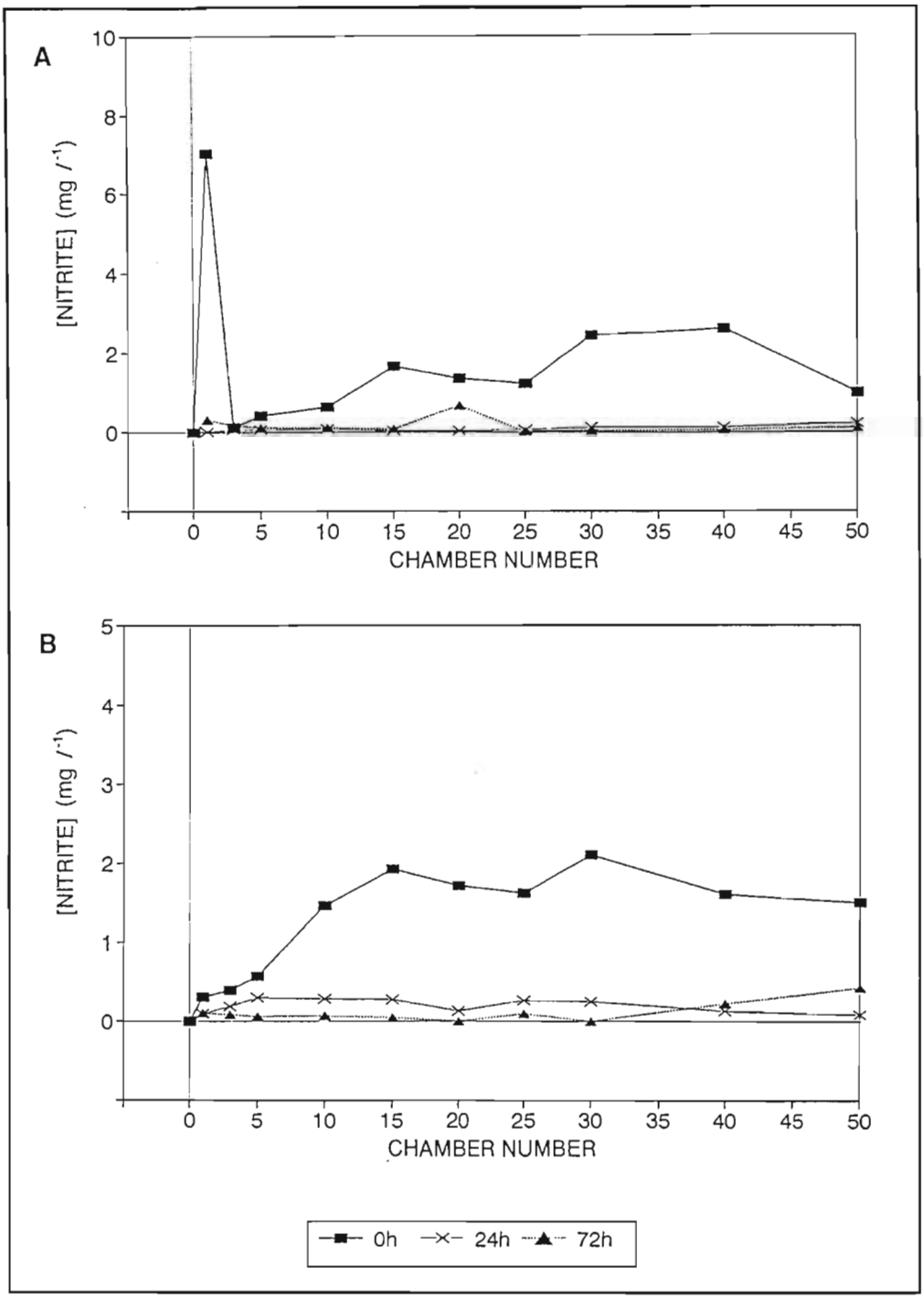
The inherent variabilities in nitrite and nitrate concentrations within the two control channels during the experimental period are shown in Figures 5.21 A and B, and 5.22 A and B, respectively. Temporal and spatial differences in nitrification were found between the two channels and these were attributed to the "age" of the nitrifying population in each channel. Channel B had previously been used as an experimental channel for the 60 mg l^{-1} phenol perturbation study (5.3.3) and as a result the nitrifying population was not as well established as the Channel D population even after an 18 day recovery period. A consequence of this finding was that the reproducibility of the experimental channels for subsequent perturbation studies would be impaired and this would, therefore, impact on the interpretation of subsequent hazard assessment data.

The rapid attenuation of phenol within each experimental channel illustrated the possible effect of acclimation of the selected microbial association. Factors such as nutrient

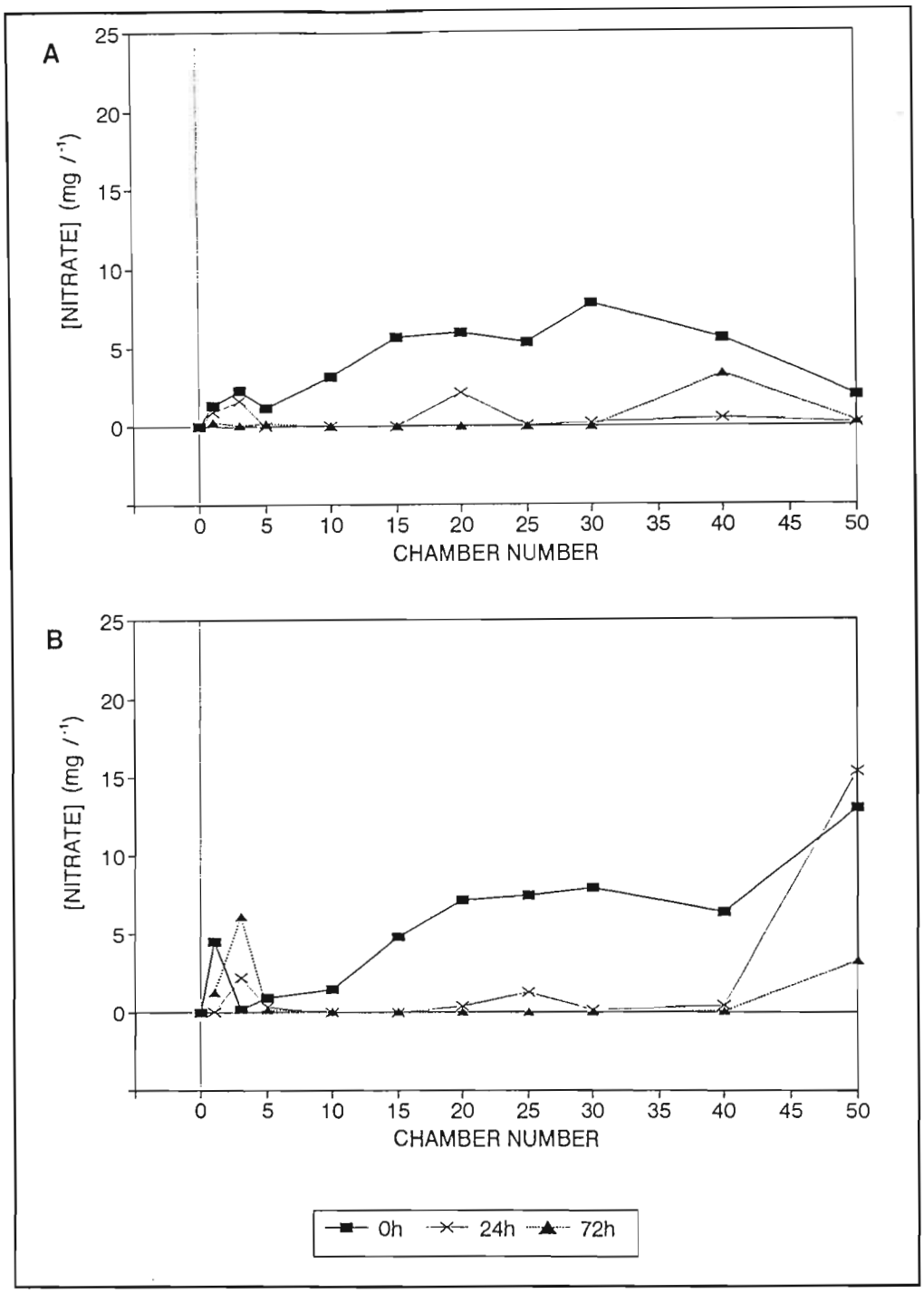
availability, environmental conditions and previous exposure to the perturbant compound all contribute to phenol degradation and its overall toxicity. At relatively low concentrations, phenol attenuation may mask the potential impacts on nitrification, thus rendering it difficult to quantify the toxicological impacts. These findings emphasised the importance of using unacclimated associations to assess potential impacts to aquatic environments. They also illustrate the relevance of making biodegradation studies in conjunction with environmental impact assessments. Compounds which are labile will have lower impacts than compounds which are recalcitrant and may become biomagnified.

2,4-dichlorophenol Perturbation Experiment

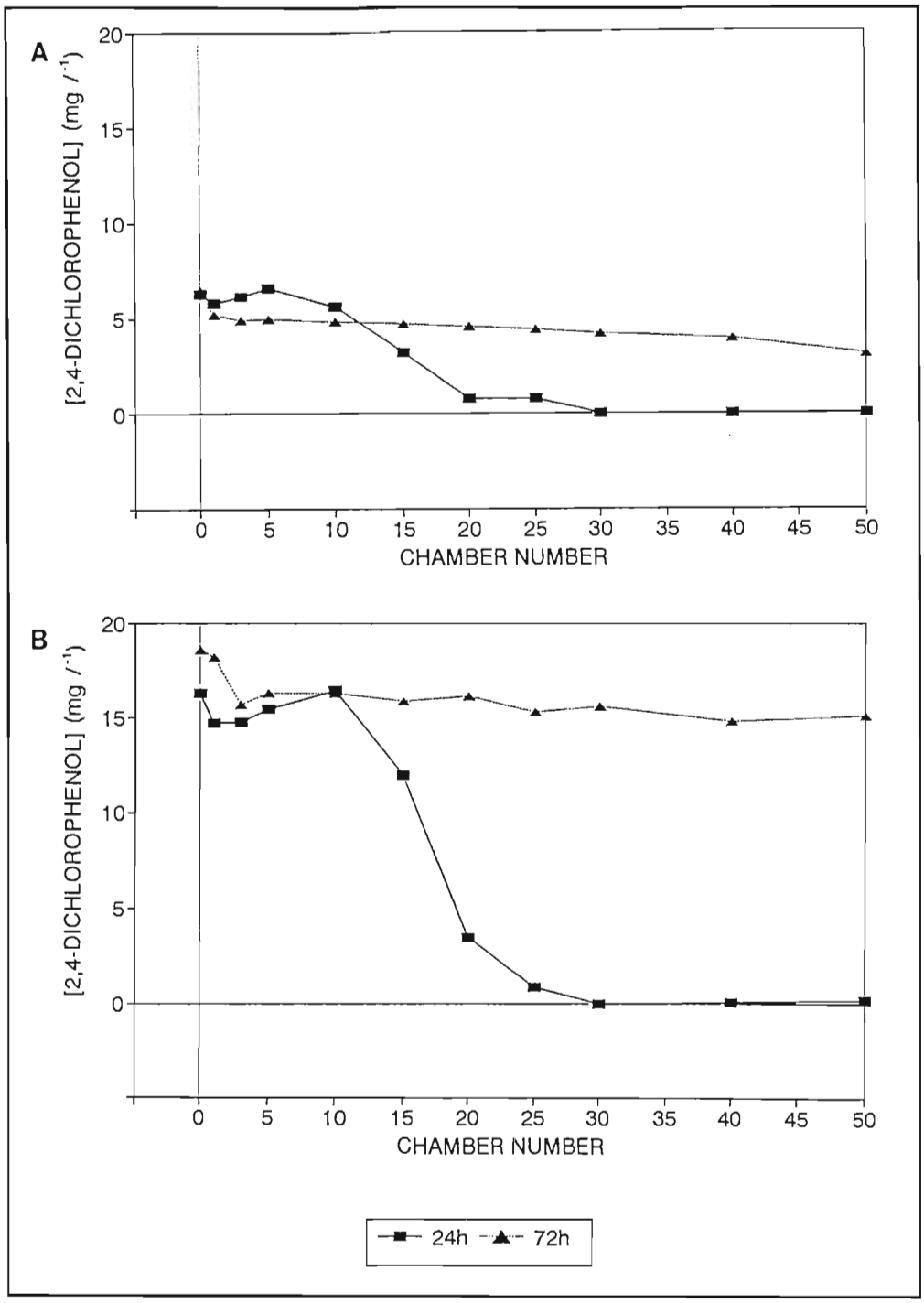
The impacts of a substituted halogenated phenol, 2,4-dichlorophenol in concentrations of 10 mg l^{-1} (Channel A) and 20 mg l^{-1} (Channel B) were investigated. The model was operated for three weeks under continuous flow conditions prior to the perturbation experiments (72 h). The effects of 2,4-dichlorophenol on the concentrations of nitrite and nitrate in selected chambers in the two experimental channels (A and B) are shown in Figures 5.23 A and B, and 5.24 A and B, respectively. The residual concentrations of 2,4-dichlorophenol in the same channels are shown in Figures 5.25 A and B. Figures 5.26 A and B and 5.27 A and B show the nitrite and nitrate concentrations after a 144 hour recovery period in the two channels. Variability, of nitrite and nitrate concentrations within the control channel during the course of the experiment are shown in Figures 5.28 and 5.29.



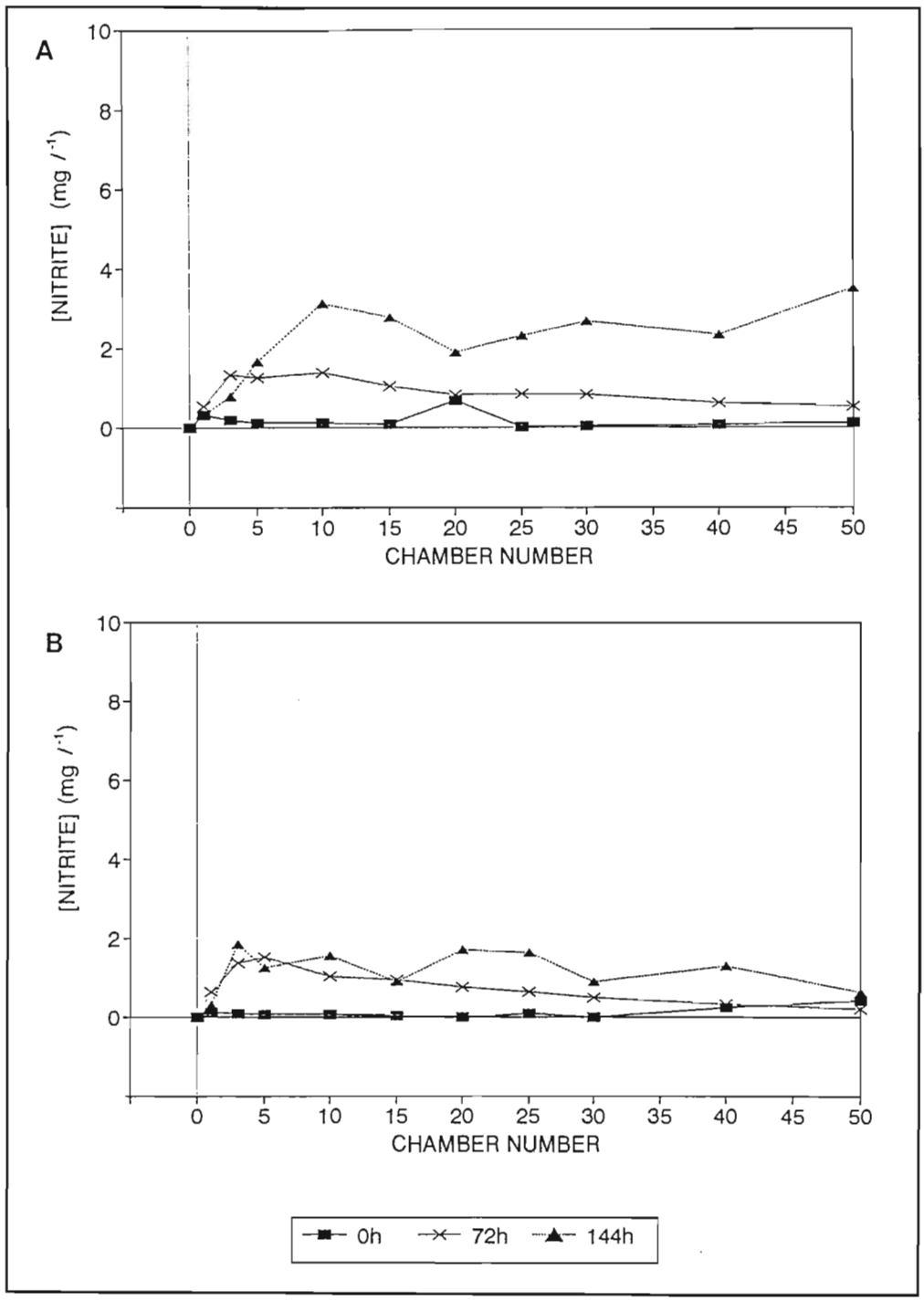
Figures 5.23 A and B. Changes in nitrite concentrations in response to 2,4-dichlorophenol perturbations of Channel A (A) (10 mg l^{-1}) and Channel B (B) (20 mg l^{-1}) of the river model.



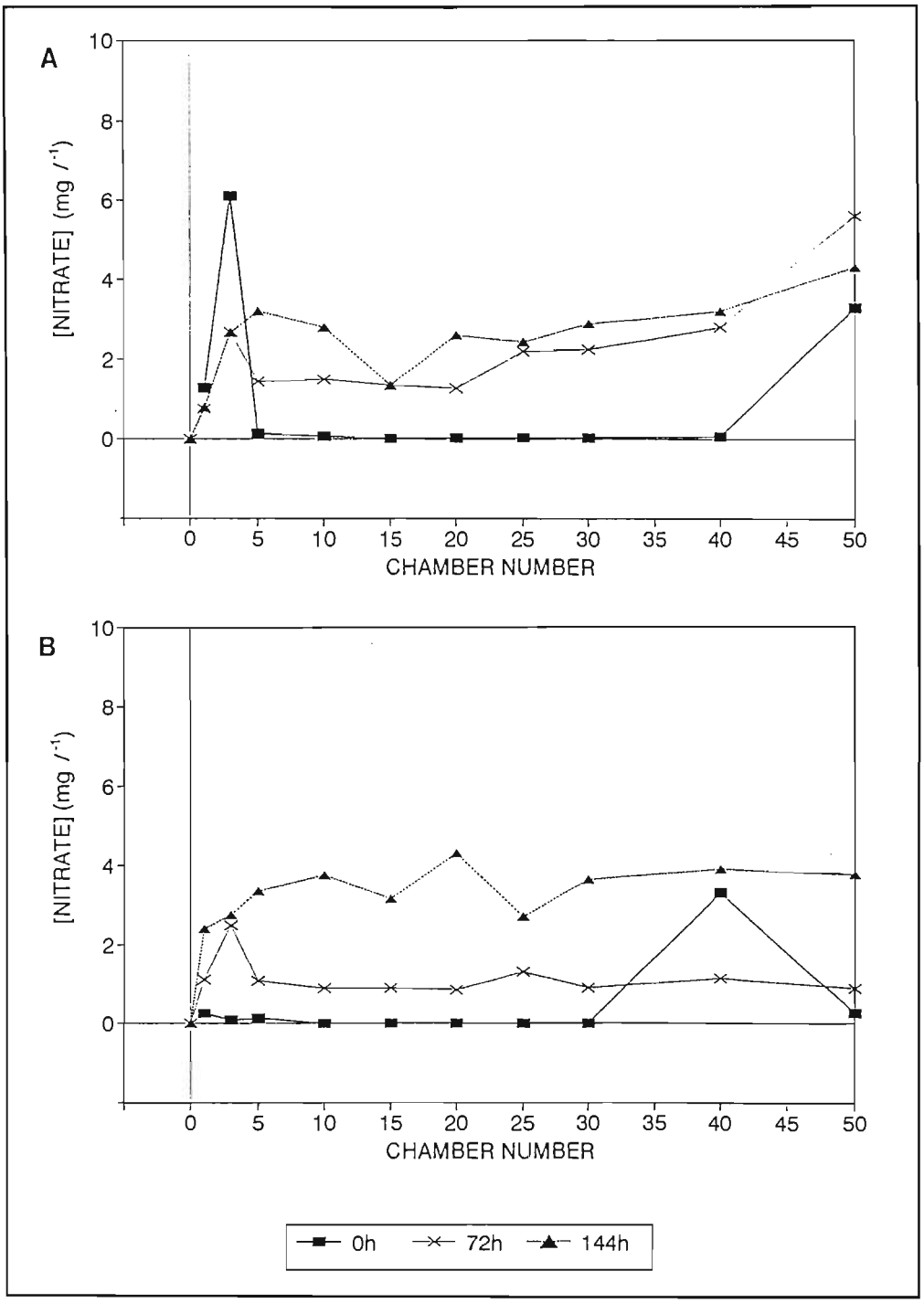
Figures 5.24 A and B. Changes in nitrate concentrations in response to 2,4-dichlorophenol perturbations of Channel A (A) (10 mg l^{-1}) and Channel B (B) (20 mg l^{-1}) of the river model.



Figures 5.25 A and B. Residual 2,4-dichlorophenol concentrations in Channels A (A) and B (B) after a 72 hour perturbation period.



Figures 5.26 A and B. Changes in nitrite concentrations in Channels A (A) and B (B) after a 144 hour recovery period, subsequent to 72 hour perturbations with 10 mg l^{-1} (A) and 20 mg l^{-1} (B) 2,4-dichlorophenol.



Figures 5.27 A and B. Changes in nitrate concentrations in Channels A (A) and B (B) after a 144 hour recovery period, subsequent to 72 hour perturbations with 10 mg l^{-1} (A) and 20 mg l^{-1} (B) 2,4-dichlorophenol.

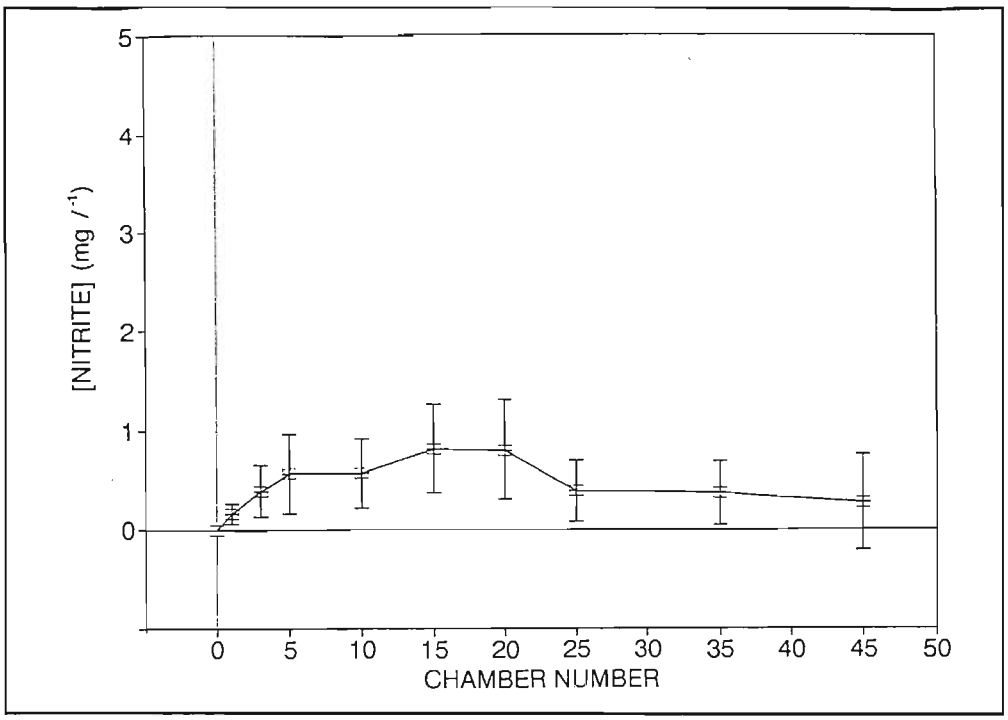


Figure 5.28. Concentrations of nitrite in Control Channel D at the end of the 216 hours experimental period. Values are means of each sampled chamber. Vertical bars depict 95% confidence intervals.

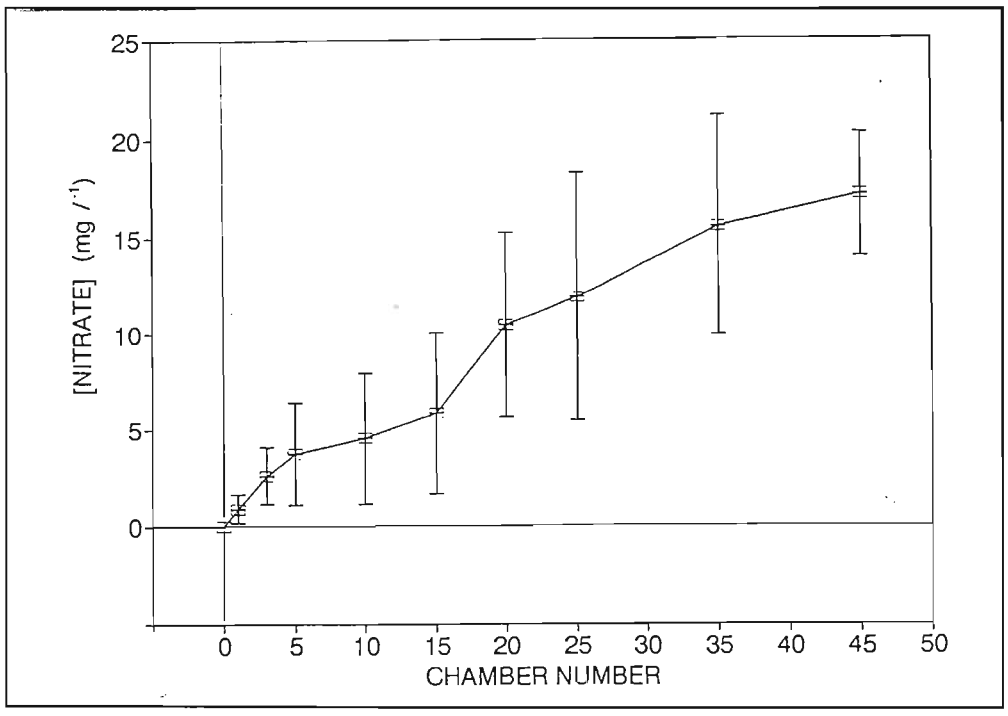


Figure 5.29. Concentrations of nitrate in Control Channel D at the end of the 216 hours experimental period. Values are means of each sampled chamber. Vertical bars depict 95% confidence intervals.

2,4-dichlorophenol was chosen for the perturbation experiment as an example of a recalcitrant halogen-substituted phenol. The actual test concentrations introduced into each channel were found to be lower than anticipated (6 mg l^{-1} and 16 mg l^{-1}) and this was attributed to either dilution errors or possible adsorption to the filter membranes during sterilization.

Marked inhibition of nitrifying activity resulted in both channels in response to both concentrations of the perturbant (Figures 5.23 A and B, and 5.24 A and B). Figures 5.25 A and B show that the residual concentrations of the perturbant compound were not significantly lowered within each channel after a 72 hour exposure period. The lowered residual concentrations after 24 hours can be attributed to dilution within the model.

Following removal of the perturbant, the recovery of nitrification within the experimental channels proved to be very slow over the 144 hours recovery period thus illustrating the lasting impacts of the molecule (Figures 5.26 A and B, and 5.27 A and B).

The variabilities in Control Channel D (Figures 5.28 and 5.29) were indicative of a nitrifying population which was not well established. This was attributed to an interruption in flow that occurred prior to the initiation of the perturbation study. Increased variability thus impacts on the result interpretation.

In this experiment biodegradation was found to play an insignificant role in attenuating the perturbant compound within the model. This emphasised one of the major limitations of the model for perturbant assessment and illustrated the empirical nature of the assessment protocol. The initial choice of concentration of the xenobiotic molecule for the perturbation

assessment is, therefore, important if time and resources are to be optimised. Thus, there is a requirement for an initial screening protocol to be used to determine an appropriate concentration range for testing in the model. For example, the LC_{50} of 2,4-dichlorophenol for the inhibition of nitrifying activity has been reported to be 0.74 mg l^{-1} (Blum and Speece, 1991).

5.4. CONCLUSIONS

By use of a multi-stage laboratory model it was shown that the inhibition of nitrification is a sensitive indicator of both phenol and 2,4-dichlorophenol perturbations. The fate of the perturbant compound within the multi-stage model was determined by direct analysis and the relationship between the residual concentrations of the test compound and the nitrifying activity could be assessed. Phenol, in concentrations 5, 20 and 60 mg l^{-1}, was found to attenuate within the model and it was apparent that low concentrations of phenol (<4 mg l^{-1}) must be reached before nitrification would proceed. 2,4-dichlorophenol, in concentrations 6 and 16 mg l^{-1}, was found to persist within the model and inhibition of nitrification resulted. Biodegradation data specific to a perturbant compound was, thus, considered an important requisite for assessing the potential impacts of a molecule on the aquatic environment. Other important considerations include an understanding of the partitioning of a compound within the environment, its susceptibility to transformation processes and its final sink within the environment.

The value of the model lies in the fact that it incorporates ecologically relevant elements which adds to the extrapolative value of information gained during ecotoxicological testing. The model was found to be suitable for both acute and chronic testing of pollutant

compounds and also holds potential for evaluating ecotoxicological impacts on wastewater treatment processes. The main disadvantages of the model arise from its operational complexity, its impractical application in screening large numbers of compounds, its empirical nature, its variability and its lack of standardisation. The slow rate at which nitrification established under pseudo-steady-state conditions was, perhaps, one of the most important limitations of this model design. This would impact on the turnaround time required for reusing the model system for subsequent investigations and would also possibly introduce increased degrees of variability. For toxicological impact assessments to be made which are pertinent to aquatic environments, the impact must be determined before acclimation arises. In the absence of this potential underestimation of the overall toxicological impacts may result. Practical application of the multi-stage model appears to be suited to a higher tier of ecotoxicological testing where the inhibitory effects of perturbant compounds on ecologically important elements can be related to their potential biodegradability within an aquatic ecosystem.

CHAPTER 6: DEVELOPMENT AND TESTING OF A CHEMOAUTOTROPHIC NITRIFIER TOXICITY BIOASSAY

6.1 INTRODUCTION

Short-term bioassays provide a valuable means for estimating the acute toxicity of inhibitory compounds (Calow, 1989). Whilst data derived from such tests are not usually suitable for regulatory purposes, they can provide a useful "first approximation" method for screening potentially inhibitory compounds. Time and resources can then be focused on compounds which have inhibitory properties. With the knowledge that nitrifiers are sensitive to phenol perturbation a nitrifier bioassay was developed with the aim of providing a means of rapidly screening perturbant compounds.

Tebo (1985) summarized the requirements for a bioassay by stating that it should be "relevant, reliable and reproducible", with the data being easily interpreted and statistically verifiable. Ideally, the test organism(s) used in such bioassays should be available from a reliable stock all year round (Tebo, 1985). Many toxicity tests involving microorganisms are rapid, reproducible, cost effective and require limited operating space (Bitton and Dutka, 1986). In recent years, microbial bioassays have, therefore, been considered with increasing interest.

Williamson and Johnson (1981) identified *Nitrobacter* sp. as a suitable bioassay for screening industrial and domestic wastewater for inhibitory compounds. However, the observation that ammonia oxidation usually fails before nitrite oxidation in wastewater treatment processes has led to the conclusion that ammonia oxidisers are more sensitive to inhibitory compounds than are nitrite oxidisers (Hockenbury and Grady, 1977; Blum and Speece, 1992). The

assessment of nitrification inhibition has been based on the evaluation of inhibition to respiration (Arbuckle and Alleman, 1992), ammonia oxidation (Hooper and Terry, 1973) and overall nitrification rates (Oslislo and Lewandomski, 1985). Such studies have been used to detect and assess toxic inhibition of biological treatment processes and to monitor effluent discharges (Bitton, 1994).

This chapter describes the establishment of a nitrifying population under steady-state conditions and its application as an inoculum for subsequent bioassays. Inhibition of ammonia oxidation was chosen as the criterion for assessing toxicological impacts.

6.2 EXPERIMENTAL PROCEDURE

6.2.1 Enrichment and Isolation of Nitrifying Bacteria from Activated Sludge and Establishment of Steady-State Culture Conditions

A Bioflow C30 bench top chemostat (New Brunswick Scientific Co., Inc.) with a working volume of 350 m*l* was used as the bioreactor to enrich and isolate autotrophic nitrifiers under continuous-flow conditions. The enrichment medium for the selection of ammonia oxidisers is described in 3.13. An initial flow rate of 2 m*l*h^{-1} was used for the experiment. After 31 days the flow rate was increased to 8.0 m*l*h^{-1}. The dilution rates for the two flow rates were 0.006 h^{-1} and 0.023 h^{-1}, respectively. Activated sludge (Darvill Sewage Works, Pietermaritzburg) (4 g wet weight) was used to inoculate the reactor at the start of the experiment. The temperature was maintained at 25°C and light was excluded by wrapping the bioreactor in aluminium foil. The pH was monitored on a daily basis (3.2.1) and was adjusted to the range 7-7.5 with sterile 5% ($^{w}/_{v}$) Na_2CO_3. Triplicate samples (5 m*l*) were

taken at regular intervals over a period of 8 weeks and assayed for ammonium by photometric analysis (3.2.3).

6.2.2 Establishment of a Suitable Protocol for a Short-Term Bioassay

Suspended biomass (10 m*l*) was taken from the Bioflow bioreactor and added to 10 and 20 m*l* of enrichment medium (3.13) in 100 m*l* Erlenmeyer flasks. The batch cultures were incubated (27°C) for 24 h in a rotary shaker incubator (New Brunswick Scientific Co., Inc.) in the dark and agitated at 150 rpm. Residual ammonium concentrations were determined at times 0,12 and 24 h. Substrate conversion was determined by calculating the difference in ammonium concentrations (3.2.3) at time 0h and at times 12 and 24h. This conversion was then expressed as a percentage of the original ammonium concentration at time 0h.

6.2.3 Establishment of a 12-hour Bioassay with Phenol as the Perturbant Molecule

The Bioflow C30 culture, operated under steady-state conditions, served as a constant source of nitrifier biomass for the perturbation experiments. These were made over a 12 h period under batch culture conditions (6.2.2). Suspended biomass (10 m*l*) was added to 10 m*l* of enrichment medium and then individual cultures were diluted to 30 m*l* with 10 m*l* of phenol solution, in concentrations of 1, 10, 20 and 50 mg l^{-1}. Taking into consideration the 1:2 dilution factor, the overall phenol concentrations tested were 0.33, 3.33, 6.66 and 16.66 mg l^{-1}, respectively. The controls were diluted with 10 m*l* of distilled water. For each concentration of phenol tested triplicate batch cultures were established. The ammonium concentrations were determined at times 0h and 12h. Each ammonium conversion was determined by calculating the difference in ammonium concentration at time 0h and time 12

h and by expressing the value as a percentage of the ammonium concentration at time 0h. The inhibitory effect of the perturbant molecule was then determined using the following equation:

$$\frac{(X_{CON}-X_{TEST})}{X_{CON}} \times 100 = \% \text{ inhibition} \qquad (6.1)$$

where:

X_{CON} is the mean percentage ammonium conversion for the control; and

X_{TEST} is the mean percentage ammonium conversion for a given concentration of perturbant molecule.

6.3 RESULTS AND DISCUSSION

6.3.1 Enrichment and Isolation of Nitrifying Bacteria from Activated Sludge and Establishment of Steady-State Culture Conditions.

The chemostat was established to enrich and isolate a population of nitrifying bacteria and to then maintain the culture under steady-state conditions so that it could be used as a standard inoculum source for subsequent bioassays. With ammonium sulphate as the sole nitrogen and energy source, ammonium oxidisers were isolated. Figure 6.1 shows the course of ammonium conversion during the eight week study period.

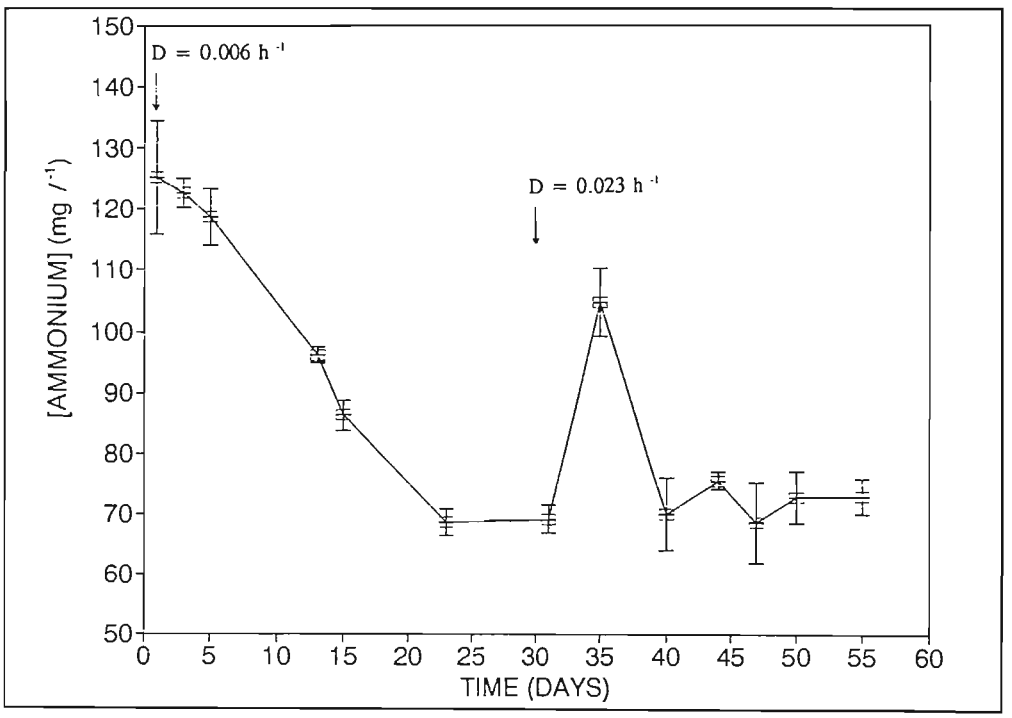


Figure 6.1. Changes in residual ammonium concentrations of a continuous-flow culture of nitrifying bacteria over an eight week period. Data points are expressed as the means of triplicate samples. The vertical bars represent 95% confidence limits.

The residual ammonium concentration decreased over the first 23 days before stabilizing, thus indicating the progressive establishment of the nitrifying bacteria. An increase in flow rate to 8 ml/h^{-1} on day 31 resulted in an initial increase in ammonium concentration before returning to steady-state conditions. Nitrifiers, characteristically, have low maximum specific growth rates and it was, therefore, important to impose a low overall dilution rate for the enrichment. Maximum specific growth rates for nitrifiers ranging from 0.006 to 0.035 h^{-1} have been reported in the literature (Bitton, 1994). To facilitate nitrifier growth and prevent washout within the chemostat an overall dilution rate of 0.006 h^{-1} was initially chosen.

To establish a microbial population within the chemostat the specific growth rate (μ) of the nitrifiers must be greater than the dilution rate (D) at the onset of open culture conditions. This should then result in a decrease in the substrate concentration until steady-state conditions are reached whereby the biomass and residual substrate concentrations level off and remain constant. When these steady-state conditions are reached the specific growth rate equals the dilution rate. If the dilution rate is increased (i.e. $\mu < D$), as in the case of increasing the flow rate, then the biomass will temporarily decrease. This in turn will cause the specific growth rate to increase again until a new balance is reached.

The benefits of using a chemostat to provide a source of microorganisms for bioassay testing are many fold. Firstly, continuous culture provides an effective means of enriching and isolating indigenous populations of nitrifiers pertinent to a particular location or environment. The cultures can be maintained almost indefinitely to provide a ready source of organisms for bioassays. This ensures a constant supply of test organisms from a reliable stock. At steady state the number of cells leaving the vessel should equal the number of cells which develop by growth and thus constant amounts of biomass with similar activities can,

potentially, be maintained. This factor contributes favourably to the reproducibility and eventual standardisation of testing procedures.

One of the major limitations associated with the use of nitrifying populations in continuous culture is the slow rate of regeneration that occurs when biomass is removed from the bioreactor. The volume of the bioreactor will, thus, dictate the number of bioassays that can be made at any one time. The generation time of the incumbent nitrifying population will determine the rate at which steady-state condition is reinstated. This problem could, possibly, be overcome or minimised by increasing the volume of the reactor vessel, or by increasing the number of reactor vessels used.

6.3.2 Establishment of a Suitable Protocol for a Short-Term Bioassay

Appreciable ammonium conversion was achieved for both concentrations of ammonium tested after 12 and 24 hours (Table 6.1). Although higher percentages of ammonium conversion were achieved after 24h it was felt that a 12h perturbation period was sufficient to establish a rapid test procedure.

Table 6.1. Ammonium conversions (%) in batch cultures after 12h and 24h

Inoculum volume (ml)	Enrichment medium volume (ml)	Substrate conversion (%)	
		12h	24h
10	10	66	72
10	20	62	83

6.3.3 Establishment of a 12-hour Toxicity Bioassay with Phenol as the Perturbant Molecule

Perturbation testing was carried out by determining the effects of increasing concentrations of phenol on ammonium conversion in batch cultures over a 12 hour period. The results of two sets of investigations are presented in Figures 6.2 and 6.3. The inhibitory effects of phenol on ammonia oxidation for the second set of experiments are presented graphically as percentage inhibition (Figure 6.4).

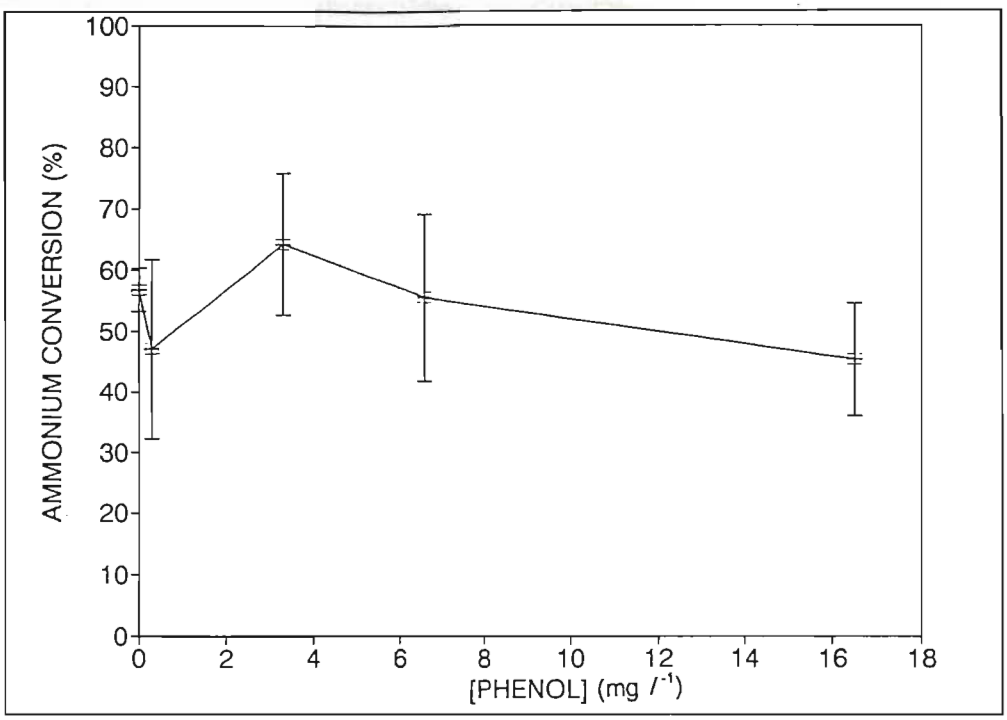


Figure 6.2. Ammonium conversions in the presence of increasing concentrations of perturbant phenol for an initial set of toxicity bioassays. Data points are expressed as the means of triplicate samples. The vertical bars represent 95% confidence limits.

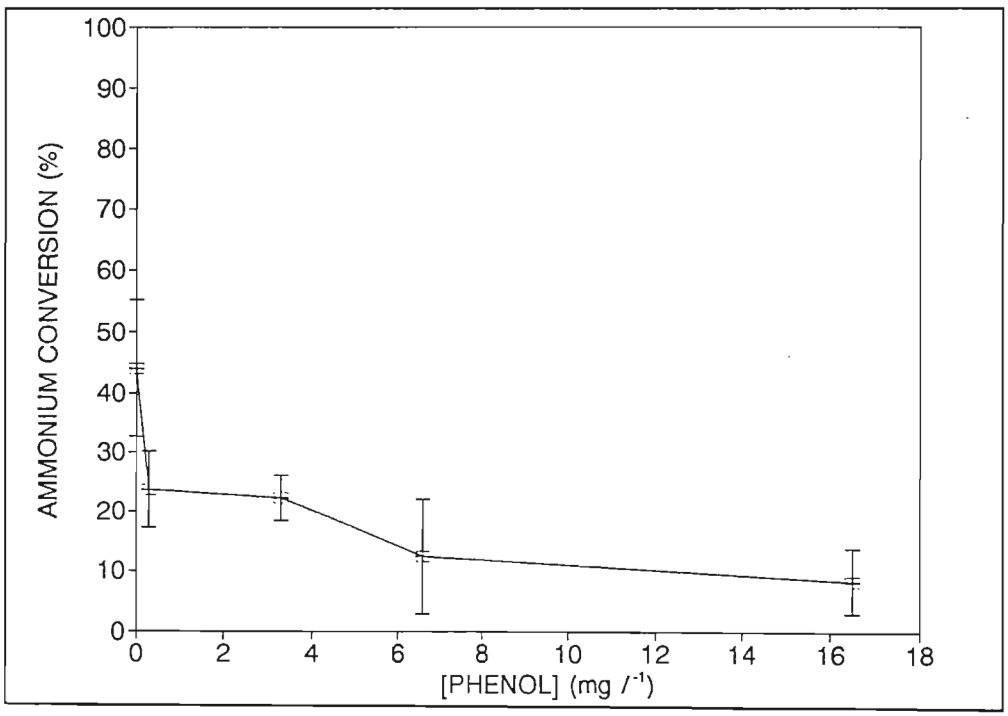


Figure 6.3. Ammonium conversions in the presence of increasing concentrations of perturbant phenol for a repeated set of toxicity bioassays. Data points are expressed as the means of triplicate samples. The vertical bars represent 95% confidence limits.

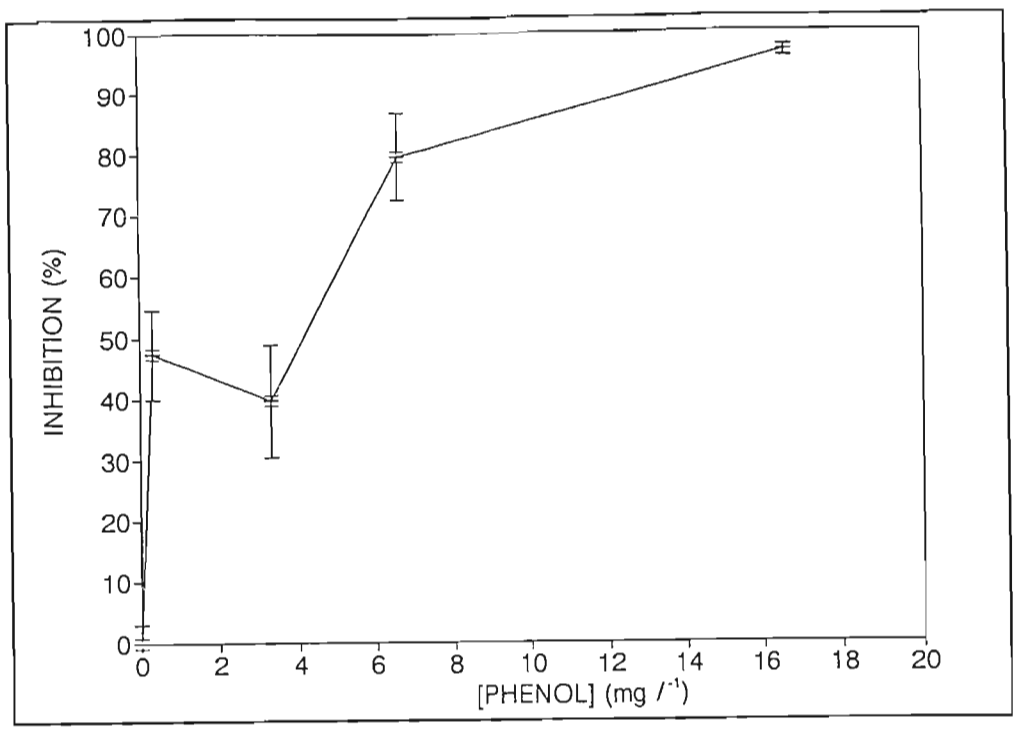


Figure 6.4. Percentage inhibition of ammonium conversion in response to increasing concentrations of phenol from the second set of toxicity bioassays.

The intrinsic variability of replicates are represented in Figures 6.2 and 6.3 by the vertical bars which depict the 95% confidence levels. The initial series of bioassays showed that no significant differences in ammonium oxidation occurred for the range of phenol concentrations tested (Figure 6.2). This was contrary to previous findings obtained with the multi-stage model where nitrification was found to be inhibited at phenol concentrations < 4mgl^{-1} (5.3.3). The experiment was repeated with freshly prepared phenol standards and the results were found to be significantly different with ammonium oxidation markedly inhibited by increasing phenol concentrations (Figure 6.3). It was, thus, thought that the anomalous findings from the first bioassay resulted either from experimental error or from the possible attenuation of phenol through biotic or abiotic means. Ideally, these bioassay should have been further replicated to verify the results.

Figure 6.4 shows the relationship between phenol concentration and substrate conversion. For the range of phenol concentrations tested it was found that marked inhibition occurred with phenol concentrations as low as 0.33 mgl^{-1} where a 47% inhibition was recorded. With 16.66 mgl^{-1} phenol a 95% inhibition resulted and, thus, this concentration appeared to approach threshold bactericidal concentration of ammonium oxidation. Taking into account the inherent variability of the results an EC_{50} in the range 0.33-4 mgl^{-1} was ascribed to phenol.

Overall, this study showed that the inhibition of ammonium oxidation was sensitive to relatively low concentrations of phenol. Potentially, this type of bioassay provides a useful means for rapidly screening compounds for toxicity and for estimating the magnitude and range of inhibitory concentrations. The added benefits of using this type of bioassay are that ammonium oxidation is easily assayed for, replicates allow for statistical verification, and it is amenable to standardisation. Theoretically, cultures can be maintained indefinitely in continuous culture and can thus be used as a constant reproducible source of suspended biomass for perturbation studies.

6.4 CONCLUSION

With low overall dilution rates continuous culture can be used to enrich and isolate representative populations of nitrifiers from environmental samples. Under steady-state conditions a microbial population can be maintained indefinitely thus facilitating the provision of suspended biomass for subsequent perturbation studies in a reproducible and replicatable manner. Under batch culture conditions ammonium oxidation proved to be a sensitive indicator of phenol perturbation and, potentially, batch culture bioassays can provide a simple and rapid means for screening large numbers of perturbant compounds or mixtures thereof.

CHAPTER 7: GENERAL DISCUSSION

The ultimate goal of ecotoxicological testing is to predict the ecological effects of chemicals and other anthropogenic substances in order to establish a basis for protecting "environmental health" (Cairns and Pratt, 1989; Scholz and Müller, 1992; Roux *et al*, 1993). The usefulness of a test system is usually based on its sensitivity and its representativeness to the ecosystem under consideration.

In a move away from standardised single species testing, ecological testing at the biological community level has been advocated (Cairns *et al*, 1992). Biological communities and ecosystems exhibit properties which result from the presence and functioning of many interacting species (Cairns and Pratt, 1989). Many of these properties may only have meaning in the community or ecosystem context and are not predictable from the properties of lower levels of organisation (i.e. population or single species). Ecologically important elements such as species interactions, nutrient cycling and energy flow have, thus, been considered as pertinent criteria for assessing ecotoxicological impacts (Schindler, 1987).

Microbial communities exhibit structural and functional complexity which make them useful in studying such properties and were, thus, chosen for the study. The aim was to simulate fundamental properties inherent to aquatic ecosystems, namely the degradation of organic substances and nitrogen transformations under aerobic conditions. A continuous-flow multi-stage model was developed in order to establish a practical means of investigating such cycling processes under manipulatable and reproducible conditions so that subsequent perturbation studies could be made.

Ecologically important groups of nitrifying bacteria have been used in ecotoxicological testing and nitrification activity was, thus, chosen as a relevant indicator of perturbation. Nitrification is essential in the cycling of nitrogen in the environment and, thus, inhibition has a direct bearing on the functioning of the ecosystem as a whole. This was thought to be of particular relevance to environments which have high nitrogen/ammonium loads such as biological wastewater treatment plants and polluted riverine systems.

Difficulties arise when attempting to analyze such mechanisms or properties under natural conditions. Natural ecosystems are extremely complex and are continuously subjected to unpredictable changes in environmental conditions (Shikano and Kurihara, 1985). Laboratory model ecosystems have sought to provide simplistic models of the environment which can then be used to assess the impacts of anthropogenic substances (Wimpenny, 1988). The value of this form of ecotoxicological testing must ultimately be weighed against unfavourable elements such as complex interpretations, increased costs and increased variability and decreased reproducibility, which are often associated with such test systems.

From the findings of this study the following observations were made:

1) Ecologically relevant processes, namely, carbon catabolism, ammonification of organic nitrogen and the process of nitrification were differentiated within the model. Spatial and temporal components were built into the design of the model and this facilitated the separation of successional metabolic events while at the same time retaining the integrity of the microbial association;

2) Selection pressures were kept constant which, theoretically, favoured the establishment of near steady-state conditions within the model. However steady-state conditions were impaired as a result of the establishment of growth-rate independent biomass. The "age" of the culture was a prime determinant contributing to the increased levels of variability in the model system. This impacted on the interpretation of data and, ultimately, limited the model's potential for standardisation and reproducibility;

3) Nitrifying activity was identified as a rate-limiting process within the model. This was perhaps one of the most important limitations of the model design and ultimately contributed to the impractical application of the model system;

4) Added to this, the model was found to be operationally complex and the large numbers of analyses made during the course of an experiment had a direct bearing on the cost effectiveness of the model. Ideally, the model should have been operated in a controlled environment to limit the role of external variables. This in itself would require specialised facilities and would, thus, limit the model's potential for wide-scale application. Refinement of the model design appeared to be feasible with reduction in size and improved aeration delivery being important end points;

5) The model was found to be suitable for short- and long-term ecotoxicological testing. The inhibition of nitrification was found to be a sensitive indicator of both phenol and 2,4-dichlorophenol perturbations;

6) Biological variables such as changes in cell density, temporal and spatial distribution of

component populations within the model, and the ability of microbial associations to acclimate to a perturbant molecule, were all thought to be important factors which affected the bioavailabilty and toxicological impacts of the compound. These factors had to be taken into consideration when interpreting data or when trying to extrapolate the information to field conditions;

7) The fates of perturbant molecules within the model were determined by direct analysis so that the relationship between the residual concentrations of the test compound and nitrifying activity could be assessed. Environmental impacts and the fates of xenobiotic compounds are determined by their interactions with physical, chemical and biological elements of the environment. It was, therefore, apparent that an assessment of the fate of a perturbant compound within the environment as well as its potential for assimilation should be integral components of any ecotoxicological impact study. Limitations to attenuation studies made within the model were: the need for sophisticated analytical equipment to assay for a perturbant compound; potentially toxic degradation products or intermediates were not taken into consideration; and, in certain instances, the inhibitory effect of a perturbant compound may have been below the detection limits of the analytical procedures used;

8) Perturbation studies within the model were essentially empirical in nature and it was recognised that there was a requirement for a simple and rapid protocol for the initial screening of perturbant molecules. A bioassay based on the inhibition of ammonium oxidation was developed to fulfil these requirements. This, potentially, enables time and resources to be focused on compounds that have inhibitory properties;

9) Potential applications for the multi-stage model include:

a) Evaluating environmental impacts of anthropogenic substances (perturbation and recovery);

b) Acute and chronic toxicity testing;

c) Defining the operational parameters for wastewater treatment processes; and

d) Determining the environmental fates of xenobiotic compounds and their susceptibility to biodegradation.

The sensitivity of ecotoxicological tests should be a function of the objectives of the study. Comparative studies have generally shown that the toxicity responses of bacterial bioassays are less sensitive than those of higher levels of biological organisation (Yoshioka,1987; Arbuckle and Alleman, 1992). As a result, microbial bioassays have become recognised as useful tools for screening for toxicity rather than for establishing lower exposure limits for the environment. Some researchers (King and Dutka, 1986) have suggested that because of the variability inherent in measuring and using toxicity data, the results should be expressed in orders of magnitude rather than placing undue emphasis on arbitrary values. Further investigation is required to assess the sensitivity of the test protocol in relation to the toxicity responses of organisms from higher trophic levels.

There is relatively little information available on the toxic effects of perturbants to different groups of environmental bacteria (Dutka *et al*, 1983; McFeters *et al*, 1983; Bitton and Dutka, 1986; Blum and Speece, 1991). Nitrifiers has been identified as being generally more sensitive to toxic inhibition than other groups of environmental bacteria (e.g. heterotrophs and methanogens) (Blum and Speece, 1991). Because of their relevance to wastewater treatment processes nitrifying bioassays have been used as rapid and convenient methods for

screening perturbation within wastewater treatment plants and in monitoring effluent discharges. Extensive toxicity data exist with regards to nitrification inhibition in wastewater treatment processes (Tomlinson, Boon and Trotman, 1966; Hooper and Terry, 1973; Hockenbury and Grady, 1977; Oslislo and Lewandowski, 1985; Blum and Speece, 1991). These data serve as a useful guideline for discharging pollutant compounds to biological wastewater treatment plants and have been used to define environmental parameters for successful operation thereof. Most of the data available are expressed as acute responses (e.g. EC_{50}'s) and the value of such information for extrapolating chronic impacts to aquatic environments must still be evaluated.

In situ biomonitoring of nitrification has been suggested for routine monitoring of environmental impacts and for evaluating ecosystem recovery in perturbated riverine ecosystems (Boterman and Admiraal, 1989). The behaviour of nutrients in ecosystems is difficult to monitor because of low concentrations and fluctuating environmental variables and, therefore, biomonitoring would appear to be suitable only in environments which have high inputs of nitrogen/ammonium (e.g. wastewater treatment plants and polluted riverine systems).

The majority of data used in evaluating the potential hazards of xenobiotic molecules have been based on single species acute toxicity testing (Cairns and Niederlehner, 1987). Such methods have provided valuable information on the sensitivities of organisms to the relative toxicities of specific chemicals or effluents. In many instances, this information has been used to derive the limits of exposure to protect entire ecosystems and there is growing concern that single species acute tests do not demonstrate the effects of long-term chronic impacts on complex ecosystems (Cairns *et al*, 1992). There is, thus, a need for establishing methods for

assessing the potential long-term impacts of pollutant compounds on biocommunities. Open continuous-flow model systems enable ecotoxicological studies to be undertaken where low concentrations of toxic substances are applied for relatively long exposure periods. The application of these types of model systems, thus, appear favourable for the future of ecotoxicological investigations.

It is important to know the responses of complex systems to perturbations if sound regulatory and management decisions on environmental protection are to be made. It is inevitable that simple, cost-effective methodologies for assessing ecotoxicological impacts will be used in preference to those that are structurally complex and require sophisticated and expensive equipment and skilled operators (Robinson, 1989). It follows that model ecosystems have an important role to play in ecotoxicological testing, especially in determining chronic toxicity exposure limits. The resulting data, if prudently used and interpreted as part of an array of data, can further enhance our ability to protect natural ecosystems from the threat of pollutant wastes and compounds.

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APPENDIX A

Example of an ammonium chloride standard curve used to determine ammonium concentrations.

Table 1. Milli-volt readings of ammonium chloride standards for standard curve.

Standard Number	Ammonium Concentration (mgl^{-1})	Milli-volt Reading (mV)
1	0.1	-138.9
2	1	-126.9
3	10	-75.6
4	100	-22.1
5	1000	37.3

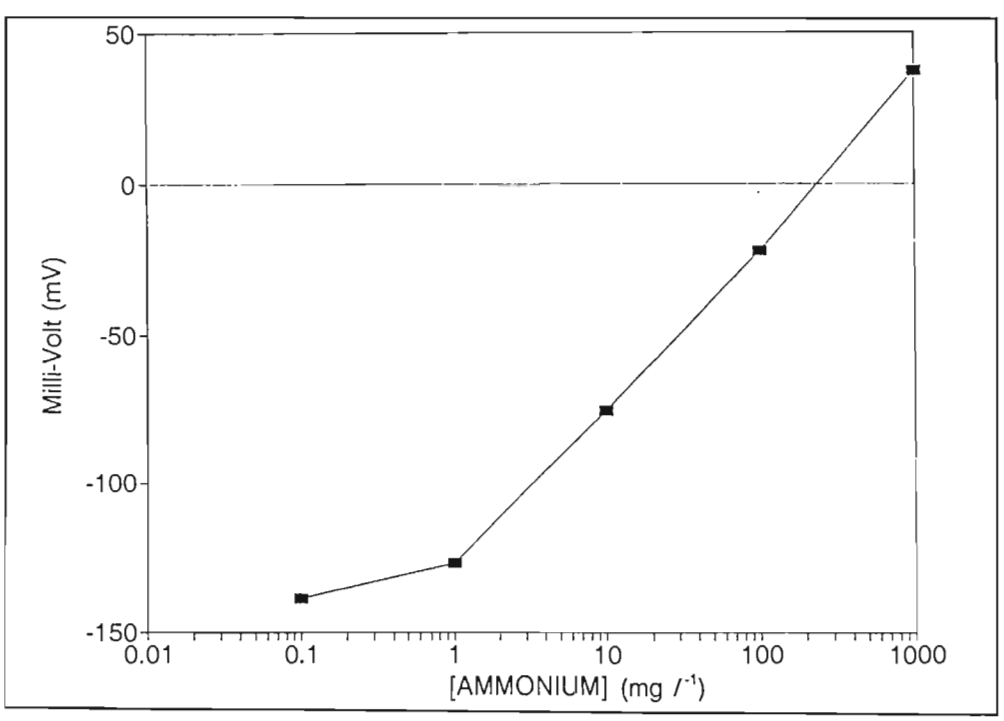

Figure 1. Ammonium chloride standard curve used to determine ammonium concentrations in solution.

APPENDIX B

Examples of standard curves used in HPLC anion analysis:

Nitrite (NO_2^-) and Nitrate (NO_3^-):

Table 1. Concentrations of nitrite and nitrate standards and peak areas for calibration curves.

Standard Number	Nitrite Concentration (mgl^{-1})	Peak Area (μS)	Nitrate Concentration (mgl^{-1})	Peak Area (μS)
1	4	925041	4	416021
2	4	918908	4	417634
3	8	1654810	8	822791
4	8	1836653	8	745216
5	12	2873041	12	1181624
6	12	2723541	12	1245146
7	16	3697366	16	1672099
8	16	3698365	16	1667412

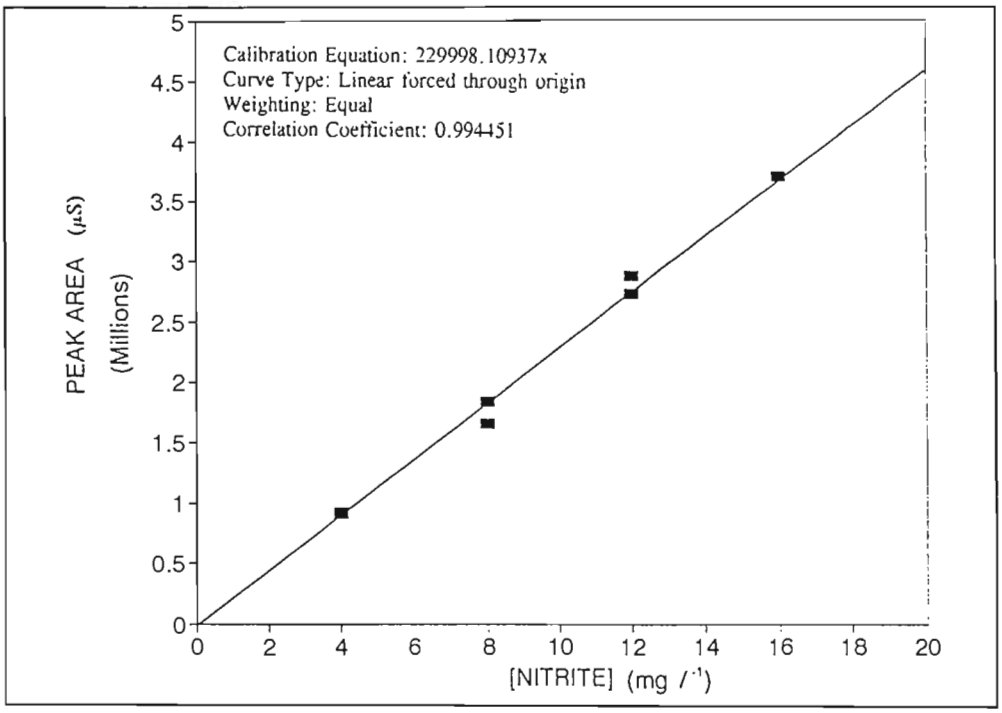

Figure 1. Calibration curve used for determining concentrations of nitrite in solution.

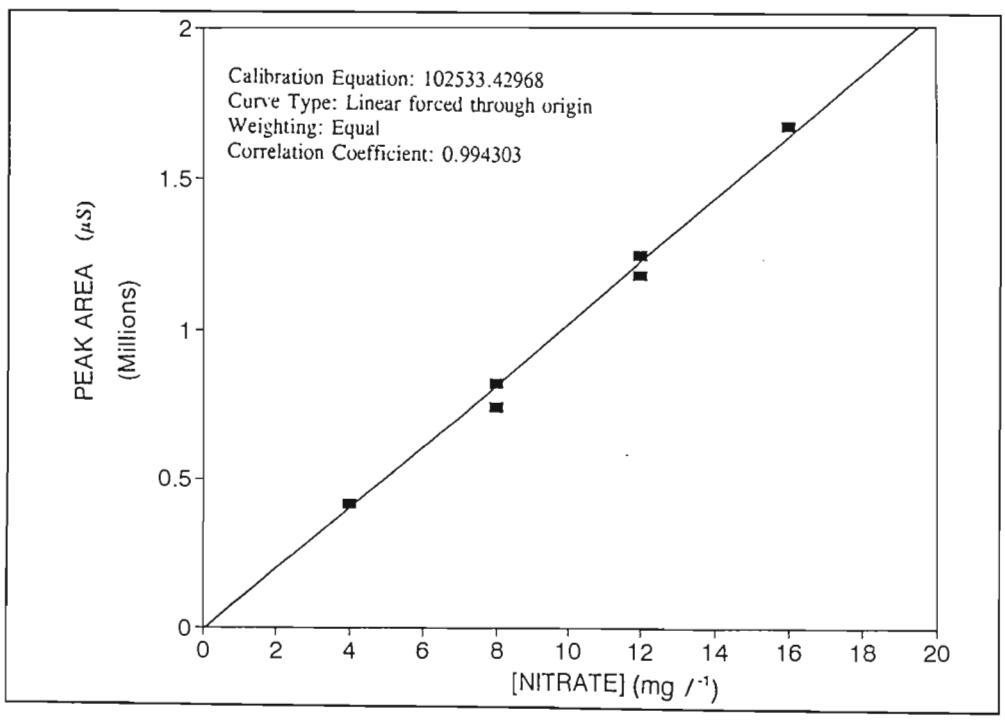

Figure 2. Calibration curve used for determining concentrations of nitrate in solution.

APPENDIX C

Examples of standard curves used in reverse phase paired ion chromatography:

Phenol and 2,4-dichlorophenol:

Table 1. Concentrations of phenol and 2,4-dichlorophenol standards and peak areas for calibration curves

Standard Number	Phenol Concentration (mgl^{-1})	Peak Area (μS)	2,4-Dichloro-phenol Concentration (mgl^{-1})	Peak Area (μS)
1	10	112054	10	34080
2	10	106814	10	35966
3	20	221197	20	65886
4	20	225937	20	66857
5	40	439041	30	100442
6	40	434702	30	97891

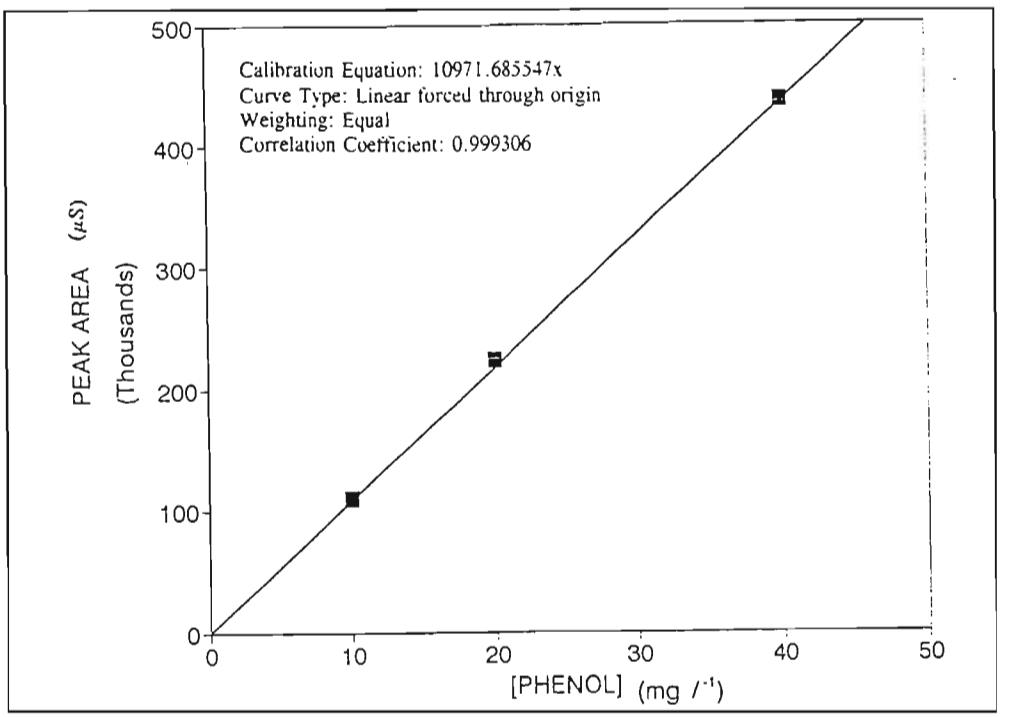

Figure 1. Calibration curve used for determining concentrations of phenol in aqueous samples

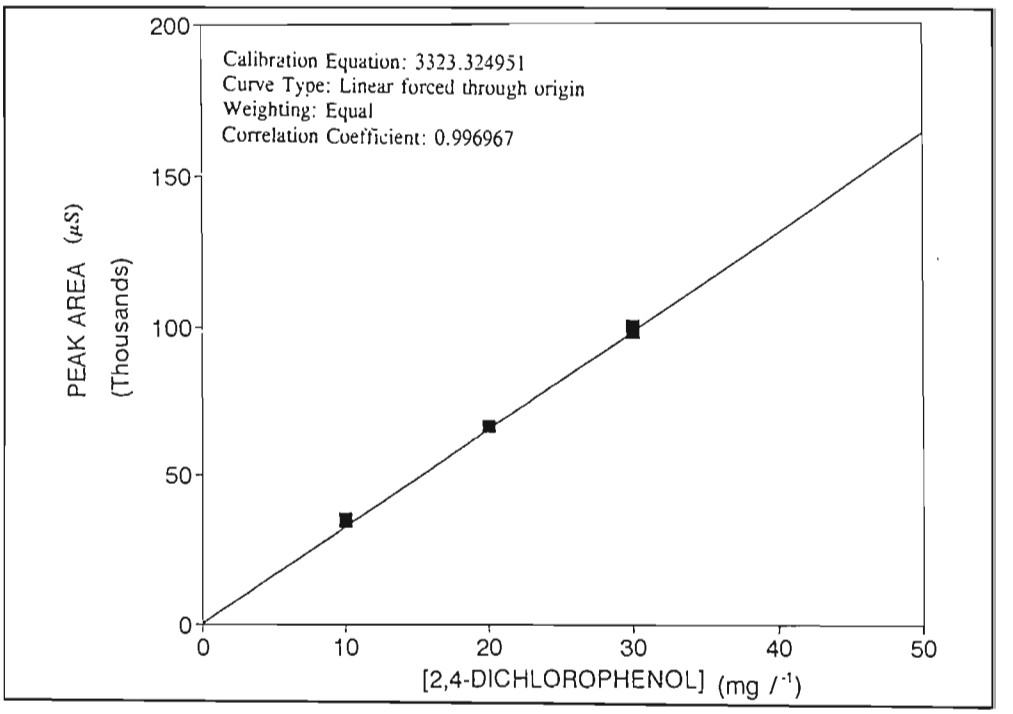

Figure 2. Calibration curve used for determining concentrations of 2,4-dichlorophenol in aqueous samples.