Development of a Multi-Stage Laboratory Model to Determine the Ecotoxicological Impacts of Selected Xenobiotic Compounds, Phenol and 2,4-Dichlorophenol, on a Population of Nitrifiers Enriched and Isolated from an Aquatic Ecosystem

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by the
International Centre for Waste Technology (Africa)
University of Natal

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DEVELOPMENT OF A MULTI-STAGE LABORATORY MODEL TO DETERMINE THE ECOTOXICOLOGICAL IMPACTS OF SELECTED XENOBIOTIC COMPOUNDS, PHENOL AND 2,4-DICHLOROPHENOL, ON A POPULATION OF NITRIFIERS ENRICHED AND ISOLATED FROM AN AQUATIC ECOSYSTEM

by

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Report to the Water Research Commission on the Project

"Development of a Laboratory River Model to Determine the Environmental Impacts of Key Xenobiotic Compounds"

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EXECUTIVE SUMMARY

1. INTRODUCTION

Synthetic organic compounds (xenobiotics) have become significant environmental contaminants in recent years. Few of these compounds have ever been tested for their ecotoxicological properties and, hence, hazards arising from their environmental exposure are of grave concern.

Microbial ecotoxicology has increasingly been used to determine the potential environmental impacts of xenobiotic compounds . This is due to the important roles that microorganisms play in ecosystem dynamics, their ubiquitous nature, rapid response to changes in environment and the ease with which they can be cultured.

In addition to standardized single species testing protocols, microbial communities have been advocated to assess potential impacts of pollutant compounds. Such approaches can incorporate ecologically important elements such as species interaction and energy flow.

To incorporate such features, laboratory model ecosystems have increasingly found application in ecotoxicological studies. Laboratory models have sought to provide simple analogues of natural ecosystems in which inherent characteristic structural and functional properties can be simulated.

1.1 OBJECTIVES

A research programme was initiated in which the principal objectives were to:

1) Develop a multi-stage laboratory model to determine the environmental impacts of selected priority pollutants on a microbial association, enriched and isolated from an aquatic ecosystems;
2) Qualitatively and quantitatively characterize the microbial association established within the laboratory model; and to
3) Determine the response of the microbial association to xenobiotic compounds introduced to the model, so as to assess and quantify toxicity and adaption to, and degradation of, key xenobiotic compounds.

Phenol and 2,4-dichlorophenol were chosen as model molecules for the perturbation study.

1.2 ECOTOXICOLOGICAL TESTING

Many xenobiotic contaminants have the potential to accumulate within the environment where they pose a hazard to life. Biological monitoring has proved to be an effective means for assessing prevailing environmental conditions and potential toxicological hazards. The concept of exposure and effect of a particular compound has been developed as the basis for a water quality approach to ecotoxicological testing. Toxicological testing has been developed as a means to evaluate, describe, monitor and predict the environmental impacts of pollutants to organisms and ecosystems. To be of value they should be sensitive, easy to interpret, have an extrapolative value and be sufficiently reproducible to facilitate inter-laboratory and intra-laboratory standardization.

1.3 MICROBIAL BIOASSAYS

Microorganisms have been used to determine the toxicological impacts of xenobiotic compounds in natural waters, soils and in biological treatment processes. They are sensitive indicators of chemical toxicity and they respond relatively quickly to changes in the environment. Many of the tests are rapid, reproducible, cost effective and require little space which makes them useful for toxicity screening.

Microbial bioassays can be broadly divided into three main categories: assays based on enzyme activity; assays based on viability or growth of bacteria; and "ecological" effect assays.

Ecologically important groups of bacteria (e.g nitrifiers) have found application as biological indicators in toxicity bioassays. Toxicity studies made with these groups of organisms can directly reflect the ecological impacts occurring in a perturbated system. Nitrification has ben identified as one such process which is sensitive to toxicants.

1.4 LABORATORY MODEL ECOSYSTEM APPROACH TO ECOTOXICOLOGICAL TESTING

Laboratory model ecosystems have been developed to simulate some portion of the natural ecosystem under manipulatable and reproducible conditions. Such systems have increasingly gained importance in testing the fate and behaviour of xenobiotic compounds in the environment.

For the research project a multi-stage continuous-flow model system was chosen.

Multi-stage systems incorporate spatial and temporal heterogenic components which allow for successional metabolic events to be differentiated with the retention of overall integrity of a microbial association.

Continuous-flow culture systems allow for selection pressures to be kept constant resulting in a reproducible enrichment of organisms. 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 the long term effects of potentially toxic compounds.

1.5 PHENOL

Phenol was chosen as a model pollutant molecule for the study. Phenol is a water soluble and non-volatile aromatic compound and forms the basic structure of many synthetic organic compounds. When present in sufficient concentrations it has a detrimental effect on water quality. The World Health Organization guideline for phenol concentrations in drinking water is 0.001 mg.l^{-1}.

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

The design and configuration of the model is detailed in chapter two of the report.

The model was constructed from plexiglass and consisted of four identical channels, 3 m in length and 36 mm wide, each consisting of 75 chambers. The channels were built in 6 unit blocks each consisting of 2 x 25 chambers. Each chamber had a volume of 122 m*l* and the total volume for each channel was 9.15 *l*. The units were arranged in tiers within a steel framework and were angled at 15° to create a weir-flow effect in each chamber.

The model was housed within an insulated dark box and operated under conditions of constant darkness, temperature and aeration. An ambient liquid temperature of 20°C ± 2° C was maintained. Air was sparged into each chamber via a system of interconnected irrigation tubes to avoid oxygen limitation. The model was operated as a continuous open-flow system with the influent medium pumped into the first chamber of each channel.

3. ENRICHMENT, ISOLATION AND CHARACTERIZATION OF AN AQUATIC MICROBIAL ASSOCIATION

Chapter three details the enrichment, isolation and characterization of a microbial association within the laboratory model.

3.1 EXPERIMENTAL PROCEDURE

Functional groups of microorganisms responsible for carbon and nitrogen transformations were enriched for, isolated and cultured within the multi-stage laboratory model.

Riverine water samples were used as inocula for the study. The samples were pooled and subjected to an enrichment/isolation step in a continuous-flow chemostat culture to provide a diverse microbial population for the study. The inoculum was introduced into the model and allowed to establish under continuous-flow conditions.

Qualitative and quantitative characterization of the physiological processes occurring within the multi-stage model were determined by monitoring the pH, dissolved organic carbon (DOC), ammonia, nitrite and nitrate concentrations. Colony Forming Units (CFU's) on non-selective (R_2A) agar were used to isolate and enumerate heterotrophic bacteria from the model. Biomass samples were taken from discrete points along the model and were examined by scanning electron microscopy (SEM) and bright field microscopy. Using inorganic nitrogen (ammonium sulphate and sodium nitrite) as an energy source, liquid cultures were used to enrich and isolate for autotrophic nitrifying bacteria from the model.

3.2 RESULTS AND DISCUSSION

The establishment of a microbial association within the model was found to be primarily dependent on: specific growth rates; overall dilution rates at various points along the model; and the interactions between the microorganisms and the prevailing environmental conditions. The findings illustrated that spatial and temporal separation of heterotrophic and nitrifying activity occurred as a result of successional changes in environmental conditions within consecutive chambers. 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 model. With the onset of nitrification a drop in pH resulted due to the release of protons (H^+) from the oxidation of nitrite to nitrate.

CFU counts of samples taken from the model were found to be the highest in the chambers nearest to the influent feed, thus indicating the main region of heterotrophic activity. Autotrophic nitrifiers were successfully enriched and isolated from the model and on the basis of this finding the assumption was made that they were responsible for nitrifying activity.

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

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. Clogging of aeration tubes sometimes resulted and inevitably impacted on mixing and aeration within individual chambers, thus, potentially, affecting the dissolved oxygen concentrations and associated microbial activity.

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

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

4. ASSESSMENT OF PHENOLIC COMPOUND PERTURBATIONS ON AN ISOLATED MICROBIAL ASSOCIATION MAINTAINED WITHIN THE MULTI-STAGE LABORATORY MODEL

In chapter four, the impacts and fates of phonol and 2,4-dichlorophenol, on the nutrient cycling processes operating within the multi-stage, are addressed.

4.1 EXPERIMENTAL PROCEDURE

Dilution and dispersion effects on phenol (20,60 and 100 mg.l^{-1}), after introduction into the model, was determined with a 25-chamber control channel. Distilled water was used as the carrier medium for the study. The residual concentrations of phenol were then monitored after 24 and 48 hours.

Biodegradation tests were made to establish the contribution of the microorganisms to the attenuation of phenol. Tests were carried out in batch cultures over a four day period and phenol was used as the sole carbon and energy source.

A series of experiments were made to determine the impacts and fate of phenol and a halogen substituted phenol, 2,4-dichlorophenol, on the operative nutrient cycling processes. An initial study was made to determine the potential long-term impacts of a 20 mg.l^{-1} phenol perturbation for a continuous 14 day period. To determine short-term (72 h) impacts, individual perturbation studies were made with phenol concentrations of 20 and 60 mg.l^{-1} and with 2,4-dichlorophenol concentrations of 10 and 20 mg.l^{-1}.

Nitrification was chosen as the criterion for assessing the inhibitory effects of perturbant molecules on the established microbial association. The inhibitory effects on nitrification were assessed by comparing the nitrifying activity before and after addition of the perturbant compound and by comparing the results with those of a control. Residual phenol concentrations were assayed to determine its fate. Recovery of nitrifying activity was also investigated subsequent to the omission of the perturbant compound from the influent medium.

4.2 RESULTS AND DISCUSSION

The flow characteristics of the model did not significantly effect the overall phenol concentrations in individual chambers. Phenol concentrations were found to equilibrate in the control channel within 24 hours. Potential abiotic removal factors such as dilution and

dispersion and volatilization were not found to be significant.

Biodegradation testing showed that for the concentrations tested, phenol was degraded during a four-day test period. Phenol concentrations in the control batch cultures were not found to change significantly.

The inhibition of nitrification was found to be a sensitive indicator of both phenol and 2,4-dichlorophenol perturbation. Phenol attenuated within the model for each concentration tested (20 and 60 mg.l^{-1}) and it was apparent that low concentrations (<4 mg.l^{-1}) must be reached before nitrification would proceed. 2,4-dichlorophenol in concentrations 10 and 20 mg.l^{-1} was found to persist with a subsequent inhibition of nitrification resulting.

It was found that phenol and 2,4-dichlorophenol exhibited a temporary bacteriostatic effect and that nitrification activity recovered at a slow rate when these compounds were removed or diluted.

It was evident that the toxicological impacts of a perturbant compound appeared to be a direct function of the removal or the transformation of the molecule. Prolonged exposure of phenol to the test system was thought to result in the selection of phenol degrading and/or tolerant /resistant organisms. Acclimation was , thus, thought to be a major contributing factor in determining the inhibitory effects of a chemical to populations of microorganisms in the environment.

Several factors such as cell density, the presence of inert organic matter, nutrient availability and environmental conditions were all thought to contribute to the potential degradation of a particular compound and its overall toxicity.

5. DEVELOPMENT AND TESTING OF A CHEMOAUTOTROPHIC NITRIFIER TOXICITY BIOASSAY

To optimise time and resources there was a requirement for an initial screening protocol to determine an appropriate concentration range for testing in the model. Chapter five of the report details the development of a nitrifier bioassay.

5.1 EXPERIMENTAL PROCEDURE

A nitrifier bioassay was developed with the aim of providing a means of rapidly screening perturbant compounds. Inhibition of ammonia oxidation was chosen as the criterion for assessing toxicological impacts.

A bench-top chemostat was used to enrich and isolate autotrophic nitrifiers under continuous-flow conditions from an inoculum of activated sludge. Ammonium sulphate was used as the sole energy source in the inorganic enrichment medium. The culture from the bioreactor, operated under steady-state conditions, served as a constant source of nitrifier biomass for perturbation experiments which were made over a 12-h period under batch culture conditions. The ammonium concentrations were determined at times 0h and 12h.

5.2 RESULTS AND DISCUSSION

With low overall dilution rates (0.006 h^{-1}) continuous culture can be used to enrich and isolate populations of nitrifiers from an inoculum of activated sludge. Under steady-state conditions a microbial population was maintained, thus facilitating the provision of suspended biomass for subsequent perturbation studies in a reproducible manner. Under batch culture conditions ammonium oxidation proved to be a sensitive indicator of phenol perturbation. Potentially, batch culture bioassays can provide simple and rapid means for screening large numbers of perturbant compounds or mixtures there of.

An EC_{50} in the range 0.33-4 $mg.l^{-1}$ was ascribed to phenol. The results compared favourably with those derived from the multi-stage model.

6. GENERAL DISCUSSION

The usefulness of a test system is based on its sensitivity and the representativeness of the ecosystem under consideration.

Microbial communities exhibit a structural and functional complexity which makes them useful for studying ecologically important elements such as species interactions, nutrient cycling and energy flow. Such properties have been considered as pertinent criteria for assessing ecotoxicological impacts.

Nitrification is essential in the cycling of nitrogen in the environment and, thus, inhibition will have a direct bearing on the functioning of the ecosystem as a whole. This is of particular relevance to environments which have high nitrogen/ammonium loads such as biological wastewater treatment plants and polluted riverine systems. The value of this form of ecotoxicological testing must, ultimately, be weighed against unfavourable elements such as complex interpretations, increased costs and variability and decreased reproducibility, which are often associated with such test systems.

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

1) Selection pressures were kept constant which favoured the establishment of near steady-state conditions within the model.
2) 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 and contributed to the increased levels of variability in the model.
3) Nitrifying activity was identified as a rate-limiting process within the model. This was perhaps one of the most important limitations of the design and, ultimately, contributed to the impracticality of the model;
4) 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 its cost effectiveness.
5) The model was 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 bioavailability and toxicological impacts of the compound.
7) Perturbation studies within the model were found to be empirical in nature.
8) 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.
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 South African Department of Water Affairs and Forestry (DWAF) recognises that biological monitoring is needed in any comprehensive water quality monitoring programme.

Challenges that need to be addressed include:

1) Evaluating the feasibility of different biomonitoring approaches/techniques and their scope for potential application;
2) Developing the necessary infrastructure from which to implement these approaches/techniques; and
3) Integrating biological data with chemical and physical data in order to provide meaningful information to resource managers.

6.1 FUTURE RESEARCH

1) Further investigation is required to assess the sensitivity of the test protocol in relation to the toxicity responses of organisms from higher trophic levels.
2) There is relatively little information available on the toxic effects of perturbants on different groups of environmental bacteria
3) Extensive toxicity data exist with regards to nitrification inhibition in wastewater treatment processes. 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.
4) *In situ* biomonitoring of nitrification has been suggested for routine monitoring of environmental impacts and evaluating ecosystem recovery in perturbated riverine ecosystems.
5) There is a need for establishing methods for assessing the potential long-term impacts of pollutant compounds. Continuous-flow model systems enable ecotoxicological studies to be undertaken where low concentrations of toxic substances are applied for relatively long exposure periods. The use of these types of models thus appears favourable for future ecotoxicological investigations.

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The Steering Committee responsible for this project, consisted of the following persons:

Mr HM du Plessis	Water Research Commission (Chairman)
Mr ME Mosia	Water Research Commission (Secretary)
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LIST OF ABBREVIATIONS

CFU	Colony Forming Unit
D	Dilution rate
DOC	Dissolved Organic Carbon
DOE	Department of Environment
DWAF	Department of Water Affairs and Forestry
EC_{50}	Exposure concentration which results in a 50% reduction of metabolic activity
K_n	Saturation constant for ammonia oxidation
SEM	Scanning Electron Microscopy
TKN	Total Kheldahl Nitrogen
TOC	Total Organic Carbon
WHO	World Health Organization
US.E.P.A	United States Environmental Protection Agency
μ	Specific growth rate
μ_{max}	Maximum specific growth rate

1.1 INTRODUCTION

Increased industrial and technological advances have resulted in synthetic organic compounds (xenobiotics) becoming significant environmental contaminants in recent years. It has been estimated that approximately 63 000 chemicals are in common use worldwide (Martell *et al.*, 1988). Few of these compounds have ever been tested for either their potential fates in the environment or their toxicity to living organisms. It has been 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). The hazards arising from environmental exposure to xenobiotic compounds and their degradation products are, therefore, of grave concern.

If an effective assessment of the ecological impacts of xenobiotic molecules is to be made, there is a requirement for chemicals to be screened for their environmental persistence, treatability and toxicity.

Microorganisms are increasingly used in toxicological bioassays to determine the potential 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 environment, ease of culturing, and the significant roles they play in ecosystem dynamics (Bitton and Dutka, 1986).

In addition to standardised single species testing protocols, several workers 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. These elements can be used to determine the end points of testing and should give closer approximations to events as they would occur *in situ* (Cairns *et al.*, 1992).

To incorporate these dimensions, laboratory model-ecosystems have increasingly found

application in ecotoxicological studies to determine the potential 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).

1.2 OBJECTIVES

A research programme was initiated in which the objectives as stated in the original proposal were to:

1) Build a multi-stage physical model to determine the specific environmental impacts of key xenobiotic compounds discharged into riverine ecosystems;
2) Qualitatively and quantitatively characterize one selected microbial population responsible for catabolism of natural organic molecules in river water;
3) Make similar characterizations of one selected sediment population with the use of image analysis technology; and
4) Determine the responses of these associations to environmental perturbations by key selected molecules to evaluate the inimical challenge of each.

During the course of the project the objectives were rationalized and ammended and were finalised with the approval of the steering committee. The ammended objectives were to:

1) Develop a multi-stage laboratory model to determine the environmental impacts of selected priority pollutants on a microbial association enriched and isolated from an aquatic ecosystem;
2) Qualitatively and quantitatively characterize the microbial association established within the laboratory model; and
3) Determine the response of the microbial association to xenobiotic compounds introduced to the model so as to assess and quantify toxicity and adaption to, and degradation of, key xenobiotic compounds.

Phenol and 2,4-dichlorophenol were chosen as representative model molecules for the perturbation study.

Once fully developed, it was proposed that the model would be used to evaluate the environmental impacts of pollutant molecules, singly and in combination. Quantitative information gained with regards to toxicity, adaption to, and biodegradability of xenobiotics could then be used to underpin the future development of a computer-based model.

1.3 ECOTOXICOLOGICAL TESTING

In recent times, pollution caused by synthetic organic chemicals has highlighted the need to determine 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 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 and, thus, biological monitoring has developed as a means for assessing the ecotoxicological impacts of xenobiotic compounds (Herricks and Cairns, 1982; Roux *et al.*, 1993).

Aquatic ecotoxicology has developed as an interdisciplinary science, incorporating toxicology with environmental chemistry and ecology (van der Gaag, 1991). The concept of exposure to, and effect of, a particular compound, integrated into a hazard assessment has been developed as the basis for a water quality approach to toxic molecule control (Lee and Jones, 1980).

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 various complexity are used to quantify the toxic effects of pollutant compounds, singly or in mixtures (Enserink *et al.*, 1991) and also to serve as indicators of effluent and receiving water quality (Cairns and Pratt, 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 reproducibility to facilitate inter-laboratory and intra-laboratory levels of standardisation.

Traditionally, representative organisms from different trophic levels have been used to develop standardised single species testing protocols (Herricks and Cairns, 1982; *Standard Methods*, 1989). 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 include ecologically important elements such as species interactions and energy flow.

1.4 MICROBIAL BIOASSAYS

Extensive reviews of the applications of microorganisms in toxicity testing have been made by Pritchard and Bourquin (1984) and Bitton and Dutka (1986). Toxicity screening tests have been developed with bacteria, fungi (including yeasts) and algae. Microorganisms appear to be sensitive indicators of chemical toxicity since they respond relatively quickly to changes in the environment (Cairns *et al.*, 1992). Many of the tests

are rapid, reproducible, cost effective and require little space which makes them useful screening tools for aquatic toxicologists and microbial ecologists (Blum and Speece, 1992). Typically, microorganisms have been used to determine the toxicological impacts of a wide range of organic and inorganic compounds in natural waters, soils and in sewage treatment processes (Eckenfelder and Musterman, 1992).

Biodegradation is a major determinant of the fates of organic chemicals in the aquatic environment (Alexander, 1981). Microorganisms are particularly important in organic chemical catabolism 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 often be attributed entirely to microbial activity (Alexander, 1981).

Microbial communities exhibit a structural and functional complexity which makes 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 associations offers a number of advantages compared with organisms from higher trophic levels. These include: the significant role that microorganisms play in ecosystem dynamics; their ubiquitous nature; their sensitivity to anthropogenic stress; their greater species diversity; and their acceptability to animal rights organizations (Cairns *et al.*, 1992).

Microbial bioassays can be broadly divided into three main categories: assays based on enzyme activity; assays based on viability or growth of bacteria; and "ecological" effect assays. Representative assays based on enzyme activity and viability or growth of bacteria are summarised in Tables 1.1 and 1.2, respectively.

Table 1.1 Short-term toxicity assays based on enzyme activity or biosynthesis (After Bitton, 1994).

Enzyme	End point used
Dehydrogenases	Measure reduction of oxidoreduction dyes such as INT or TTC.
ATPase	Measure phosphate concentration with ATP as a substrate.
Esterase	Non-fluorescent substrates degraded to fluorescent products.
Phosphatase	Measure inorganic phosphate.
Urease	Measure ammonia production from urea.
Luciferase	Measure light production with ATP as a substrate.
ß-galactosidase	Measure hydrolysis of *o*-nitrophenyl-ß-D-galactoside.
α-glucosidase	Measure hydrolysis of *p*-nitrophenyl-α-D-glucoside.

Table 1.2 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 mono- (e.g. *Pseudomonas*) or mixed cultures.
Viability assays	Measure the effects of toxicants on the viability of bacterial cultures on agar plates.
ATP assay	Inhibitory effects of toxic chemicals on ATP levels in microorganisms.
Respirometry	Measures the inhibitory effects 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.

1.4.1 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). Heterotrophic activity, nitrogen transformations, sulphate reduction and methanogenesis have all been considered (Pritchard and Bourquin, 1984). Nitrification has been identified as one of the most sensitive processes to environmental toxicants (Blum and Speece, 1992). Bioassays based on the inhibition of both *Nitrosomonas* spp and *Nitrobacter* spp have been developed for determining the toxicity of wastewater samples (Williamson and Johnson, 1981; Alleman, 1988; 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 impacts which will occur in a perturbated system.

1.5 LABORATORY MODEL ECOSYSTEM APPROACH TO ECOTOXICOLOGICAL TESTING

Since the early 1970's microecosystems have gained importance for testing the fate and behaviour of xenobiotic compounds in the environment (Isensee, 1986). Such systems offer the opportunity to simulate some portion of the natural ecosystem under manipulatable and reproducible conditions for experimental purposes (Porcella *et al.*, 1982; Wimpenny, 1988; Pratt *et al.*, 1990; Freitsch, 1991; Scholz and Muller, 1992). 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).

For the study 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). To incorporate spatial and temporal heterogeneic components into the model, so that changes in space and time could be physically differentiated, a multi-stage system was chosen.

In continuous-flow model systems, selection pressures can be kept constant resulting in a reproducible enrichment of desired organisms or groups of organisms (Gottschal and Dijkhuizen, 1988). Aquatic ecosystems are often characterized by nutrient poor conditions (Jannasch, 1969). As a consequence, microorganisms usually grow and compete for limiting nutrients at sub-optimal growth rates (Gottschal, 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). Conversely, by increasing nutrient concentrations, polluted aquatic ecosystems can be simulated.

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.

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. To incorporate this heterogeneity, multi-stage models have been developed which facilitate the spatial separation of mixed microbial populations with retention of overlying microbial interactions (activity domains)(Parkes and Senior, 1988).

Multi-stage systems afford the opportunity for the sequential breakdown of the toxic substrate without the toxic substrate or catabolic intermediates accumulating (Parkes, 1982). 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 the long-term effects of potentially toxic compounds (Fry, 1982).

The model described here (Hunter *et al.*, 1995) 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 of microbial associations occurring during self-purification processes to be elucidated (Freitsch, 1991).

1.6 PHENOL

Phenol was chosen as a representative pollutant molecule for the study. Phenol is classified as a hazardous substance and is listed by the United States Environmental Protection Agency (US.E.P.A) and the United Kingdom's Department of Environment (DOE) 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, photo-developers, perfumes, explosives, disinfectants and pesticides (Artiola-Fortuny and Fuller, 1982).

Phenol is a water soluble and non-volatile aromatic compound which adds to its pollutant properties. When present in sufficient concentrations it has a detrimental effect on the quality and ecological conditions of water bodies. The World Health Organisation (WHO) guideline for phenol concentrations in drinking water is 0.001 $mg.l^{-1}$ (Chapman and Kimstach, 1992). The South African Department of Health guidelines for drinking water quality (1994) stipulated that phenol at concentrations 0.0009-0.009 $mg.l^{-1}$ present a low to insignificant health risk. The toxic effects on fish have been observed at concentrations of ≥ 0.01 $mg.l^{-1}$ (Chapman and Kimstach, 1992).

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

2.1 LABORATORY MODEL CONFIGURATION

The model consisted of four adjacent channels (A, B, C and D), 3 m in length and 36 mm wide, each consisting of 75 chambers. Figure 2.1 A shows the side view of a single channel and Figure 2.1 B presents a three-dimensional view through two adjacent channels.

Each chamber vessel had an operational volume of 122 m*l* (36 x 36 x 95 mm) and, thus, the total volume for 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. 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 Science Workshop of the University of Natal, Pietermaritzburg.

2.2 OPERATIONAL CRITERIA

To limit the number of variables, the model system 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 achieve an ambient liquid temperature of 20°C ± 2°C. These heating elements were constructed by the Electronics Workshop of the University of Natal, Pietermaritzburg.

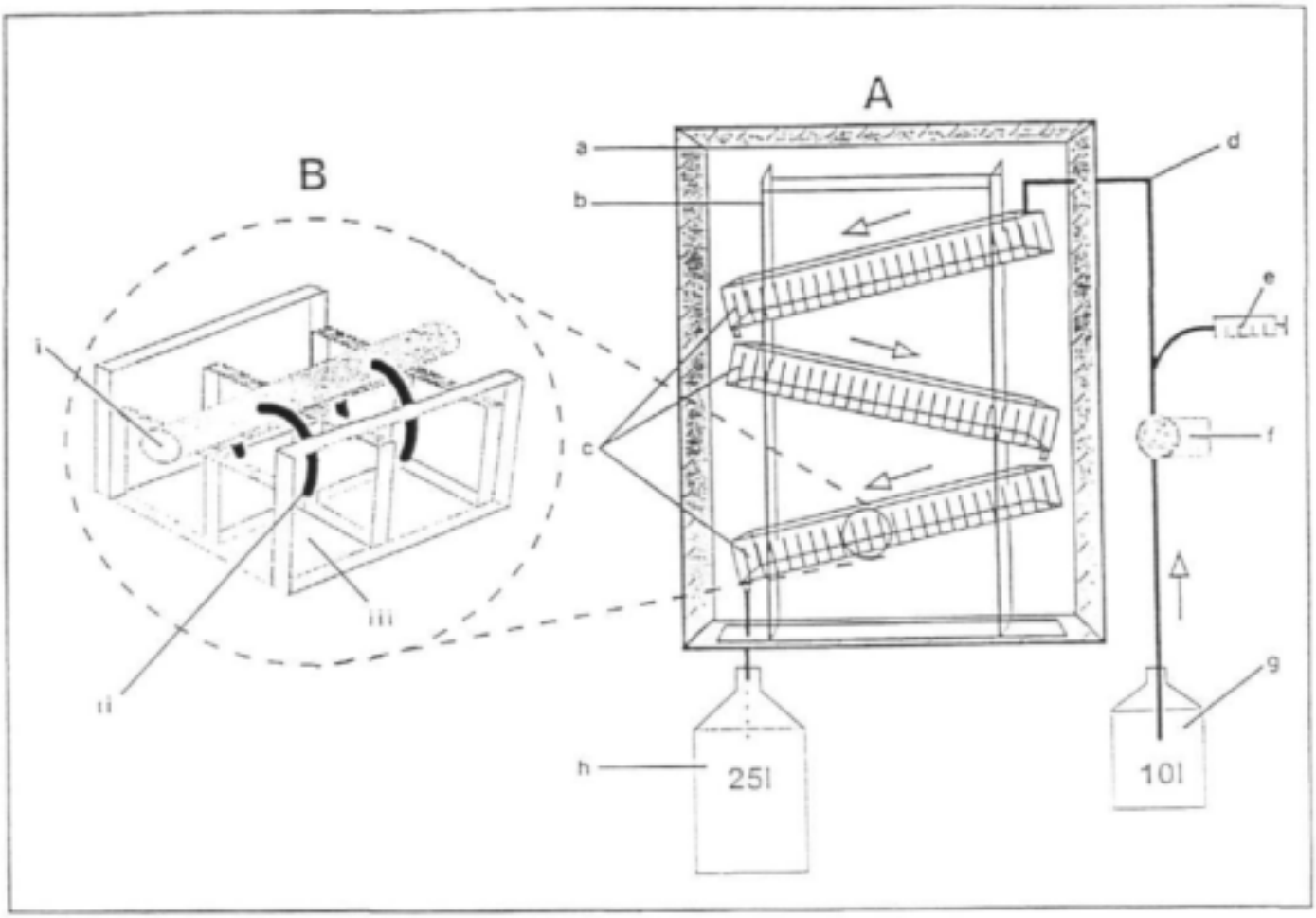


Figure 2.1 A. Diagram illustrating side view of the multi-stage laboratory model consisting of:
a - insulated dark box;
b - steel frame;
c - unit blocks of 2 x 25 chambers;
d - silicone tubing (i.d. 2.8 mm);
e - flow meter;
f - flow inducer;
g - influent medium; and
h - effluent reservoir

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)

The model was operated as a continuous open-flow system with the influent medium pumped (Watson-Marlow 503U flow inducer) 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 sparging air into each chamber via a system of interconnected irrigation tubes (i.d. 3.5 mm) attached to a central hose (i.d. 15 mm)(Figure 2.1 A). The air was first bubbled through a water trap to humidify it and thus minimise liquid loss in the chambers.

2.3 DISCUSSION

The model was originally designed and intended for bench-top use within the laboratory. Subsequent development and testing of the model indicated that variables such as light and temperature play important roles. To control these variables, adaptations were made and the model was housed in an insulated dark box and thermostatically controlled heaters were added. These adaptations, whilst adequate for preliminary investigations and for characterization of the microbial association, proved to be far from ideal. It is recommended that future work with the model requires that it should be housed within a light and temperature controlled room.

3. ENRICHMENT, ISOLATION AND CHARACTERIZATION OF AN AQUATIC MICROBIAL ASSOCIATION

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.

3.1 EXPERIMENTAL PROCEDURE

3.1.1 Enrichment/Isolation of an Aquatic Microbial Association for the Multi-Stage Laboratory Model

Riverine water samples (200 m*l*) were used as inocula and were taken from various points along the Umzinduzi River in the Pietermaritzburg, KwaZulu-Natal area. 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 flow chemostat culture (350 m*l*) (Bioflow C30 Bioreactor, New Brunswick Scientific Co., Inc.), stirred at 150 rpm, with a modified non-selective growth medium, R_2A (Reasoner and Geldreich, 1985), introduced at a dilution rate of 0.01 h^{-1}.

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 ; urea, 15; NaCl, 5.2 ; $CaCl_2$, 3; $MgSO_4$, 1.5; and $K_2HPO_4.3H_2O$, 21. A Total Organic Carbon (TOC) of 38.6 mgC.l^{-1} and a Total Kheldahl Nitrogen (TKN) of 14.2 mgN.l^{-1} were determined for the medium.

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 (control)] of the laboratory model at a dilution rate of 0.5 h^{-1}. Once filled, the model was maintained as a batch culture overnight before initiating continuous flow. To facilitate temporal and spatial separation of the component species within the system a flow rate of 100 $ml.h^{-1}$ was chosen. The dilution rate for each individual chamber was thus 0.82 h^{-1}.

3.1.2 Characterization of Nutrient Cycling within the Multi-Stage Laboratory Model

Qualitative and quantitative characterization of the physiological processes occurring within the multi-stage model were determined by monitoring the dissolved organic carbon (DOC), ammonia, nitrite and nitrate concentrations. Changes in pH were determined with a Crison pH meter fitted with an Ingold (U402 S7/120) pH probe. Dissolved organic carbon was assayed by infra-red spectrophotometry (Skaler CA-10) (*Standard Methods*, 1989). Dissolved ammonium concentrations were measured with an ammonia electrode (Orion 95-12) in conjunction with a pH meter (Crison) and by photometric analysis (Spectroquant 14752, Merck). Nitrite and nitrate were measured by anion exchange ion chromatography (Waters, Millipore) and by photometric analysis (Spectroquant 14773 and 14776, Merck).

3.1.3 Characterization of the Isolated Microbial Association

1) Isolation and Enumeration of Heterotrophs

Dilution series viable counts on R_2A agar (Reasoner and Geldreich, 1985) were used to isolate and enumerate heterotrophic bacteria from aqueous samples taken from discrete chambers along the multi-stage model.

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

Flocculent biomass, biofilm scrapings, precipitated biomass and supernatant samples were taken from discreet points along control Channel D and were examined with SEM and bright field microscopy.

3) *Enrichment/Isolation of Autotrophic Nitrifiers from the Multi-Stage Laboratory Model*

Liquid cultures were used to enrich and 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 (Soranio and Walker, 1968). Sodium nitrite was used to enrich the nitrite oxidizers (Smith and Hoare, 1968).

3.2 RESULTS AND DISCUSSION

3.2.1 Characterization of Nutrient Cycling within the Multi-Stage Laboratory Model

Initial findings from a preliminary study conducted over a month illustrated the spatial separation of organic carbon degradation (Figure 3.1) and nitrogen transformations (Figure 3.2) along the course of the model. Changes in pH resulting from these interactions were also apparent (Figure 3.3).

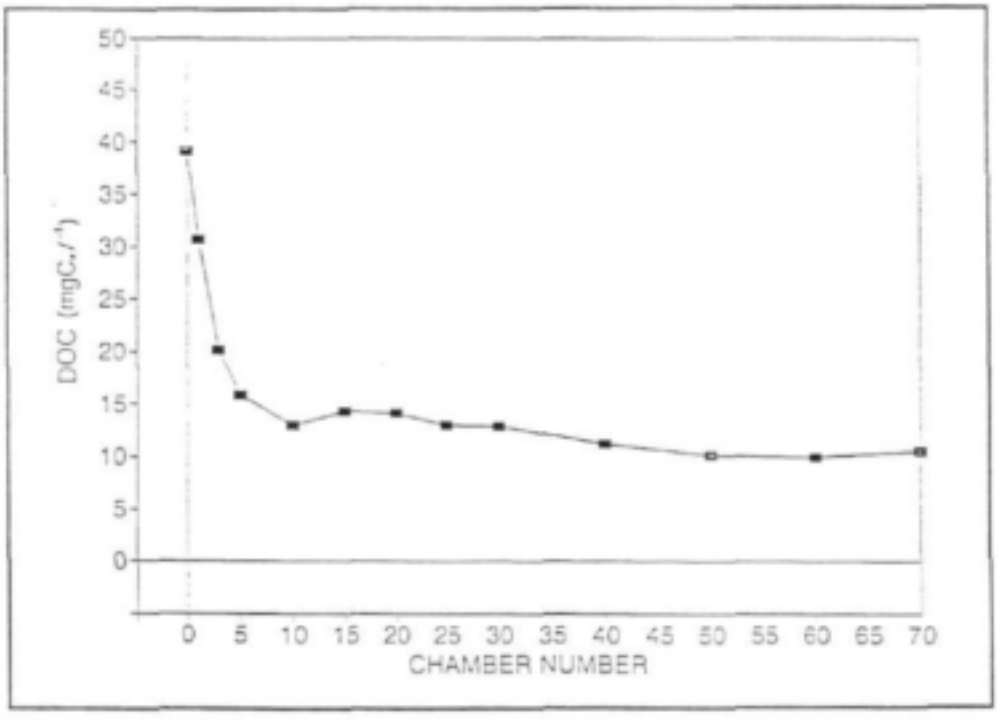


Figure 3.1 Residual dissolved organic carbon concentrations in discrete chambers of one channel of the model after maintenance under continuous-flow conditions for one month.

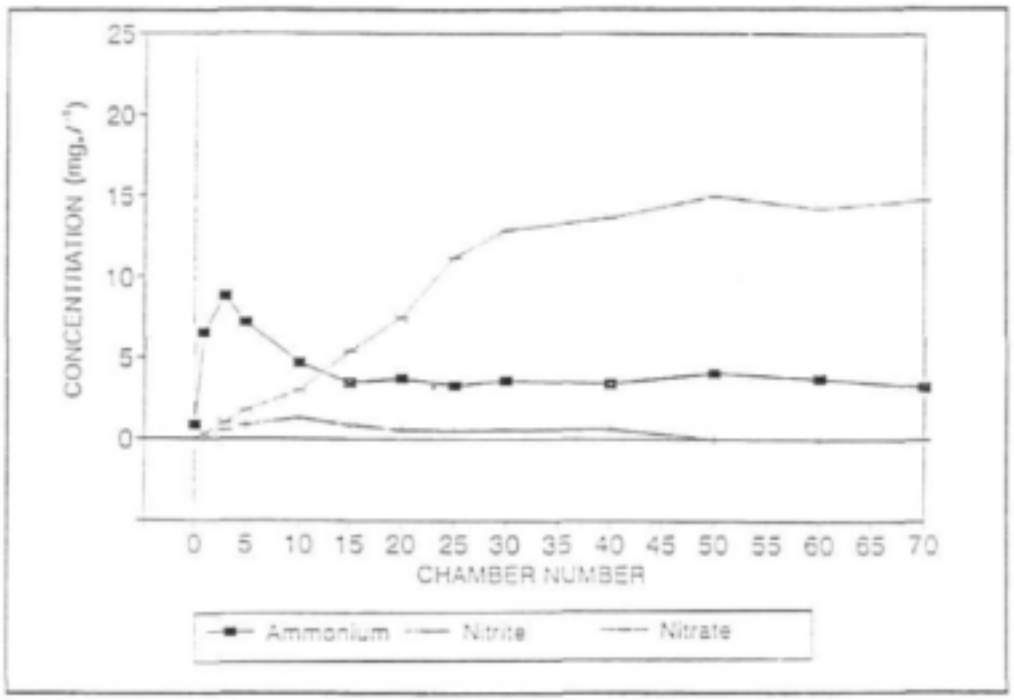


Figure 3.2 Course of nitrification along one channel of the multi-stage laboratory model after maintenance under continuous-flow conditions for one month.

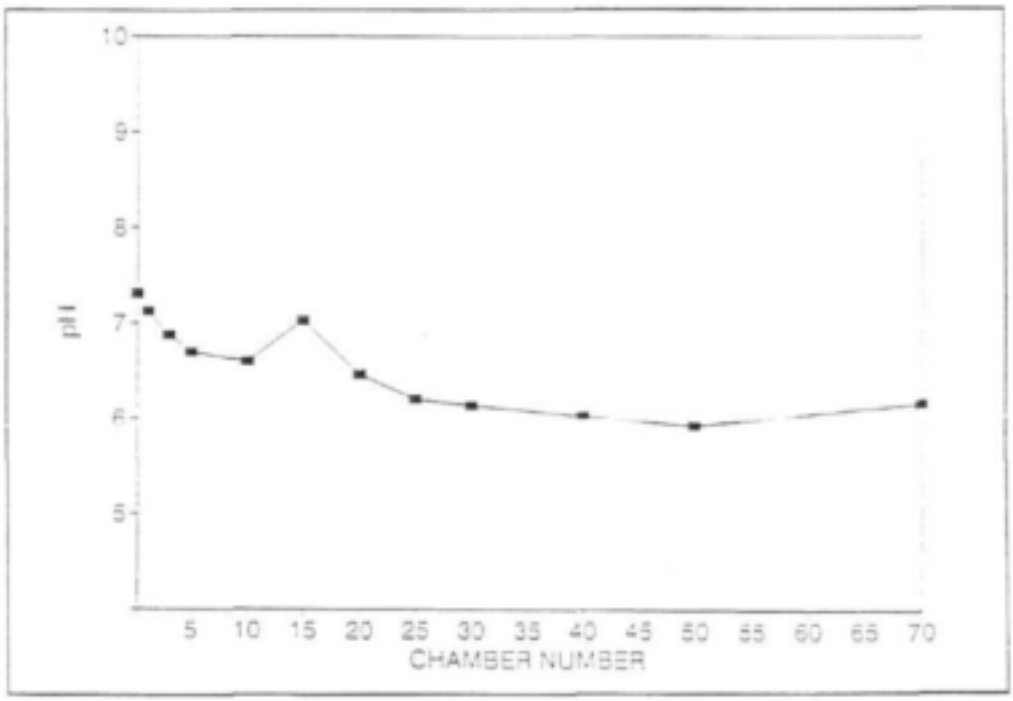


Figure 3.3 pH values recorded in discrete chambers of one channel of the model after maintenance under continuous-flow conditions for one month.

In each channel, separation of heterotrophic and nitrifying activity occurred as a result of successional changes in environmental conditions within consecutive chambers. 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). Subsequent mineralization of nitrogenous compounds released ammonia which then underwent biological oxidation to nitrate via nitrite and led to the establishment of populations of nitrifiers (Figure 3.2). Autotrophic nitrifiers have, characteristically, much lower maximum specific growth rates (μ_{max}) than mixed cultures of heterotrophs (Bitton, 1994). Nitrification was, thus, expected to establish within the multi-stage model in regions or chambers along the channel with lower overall dilution rates.

Nitrite did not accumulate due to its oxidation to nitrate 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 3.3) 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).

With a flow rate of 100 $ml.h^{-1}$ carbon and nitrogen cycling were found to occur within the first 25 chambers of each channel. To reduce the residence time in each channel the operational length was effectively shortened to 50 chambers for subsequent investigations.

To determine whether steady-state conditions for nitrification occurred within the multi-stage model, changes in nitrifying activity were recorded during a 7-week period. Changes in the ammonium, nitrite and nitrate concentrations within discrete chambers of a single experimental channel are shown in Figures 3.4, 3.5 and 3.6, respectively.

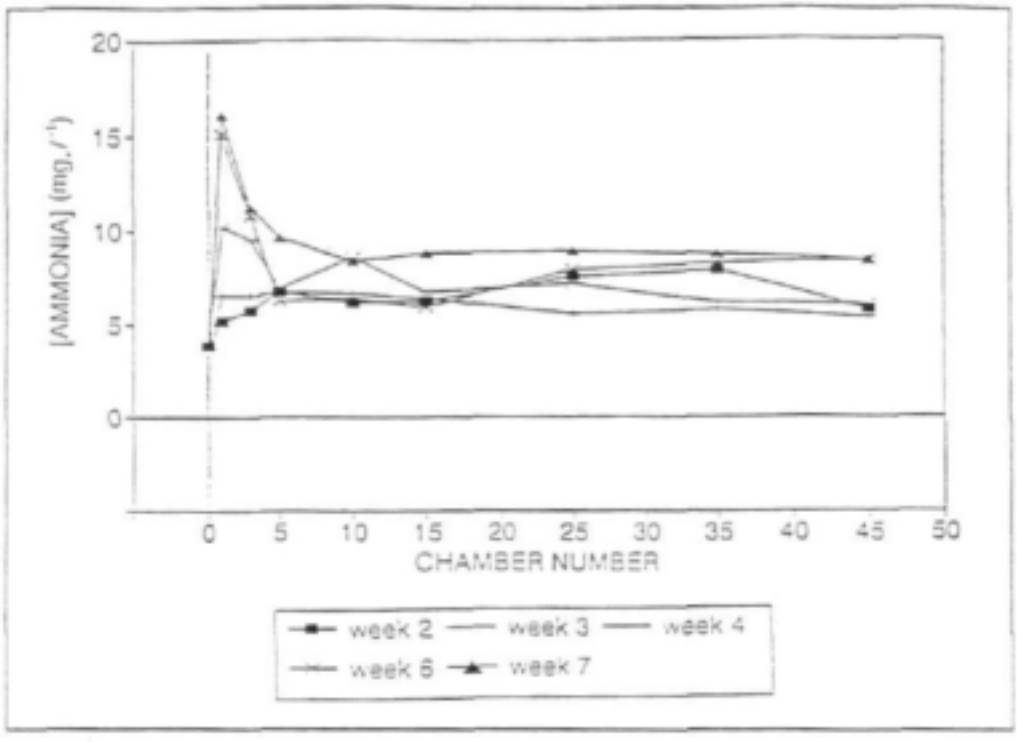


Figure 3.4 Changes in ammonium concentrations recorded in discrete chambers of Channel A of the model, during a 7-week study period.

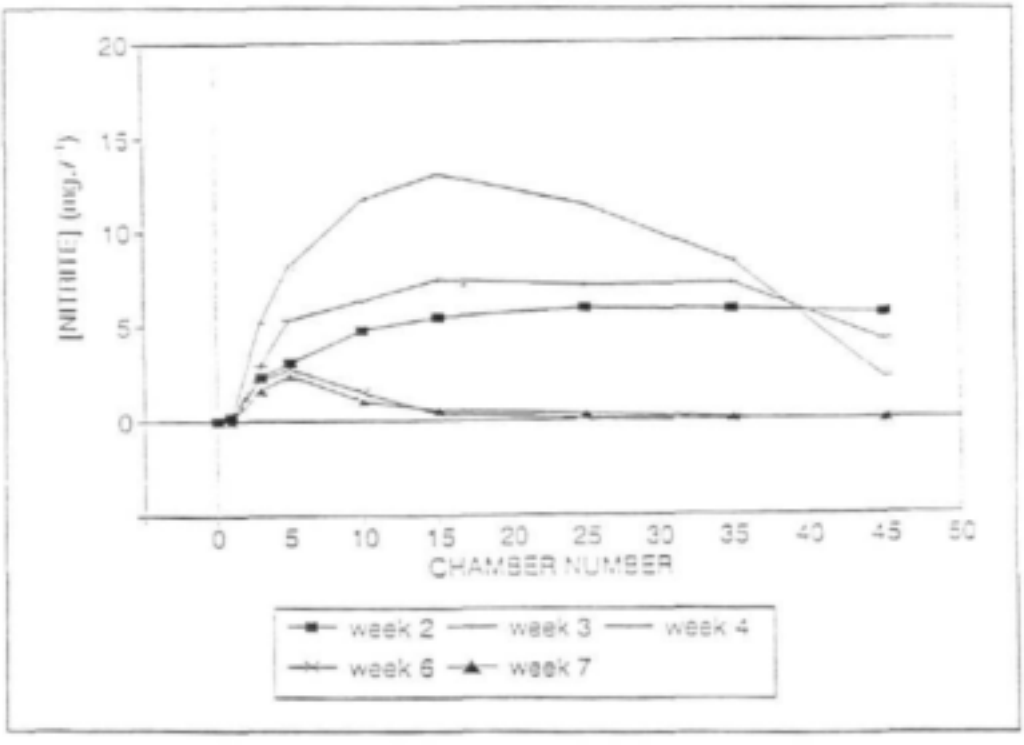


Figure 3.5 Changes in nitrite concentrations recorded in discrete chambers of Channel A of the model, during a 7-week study period.

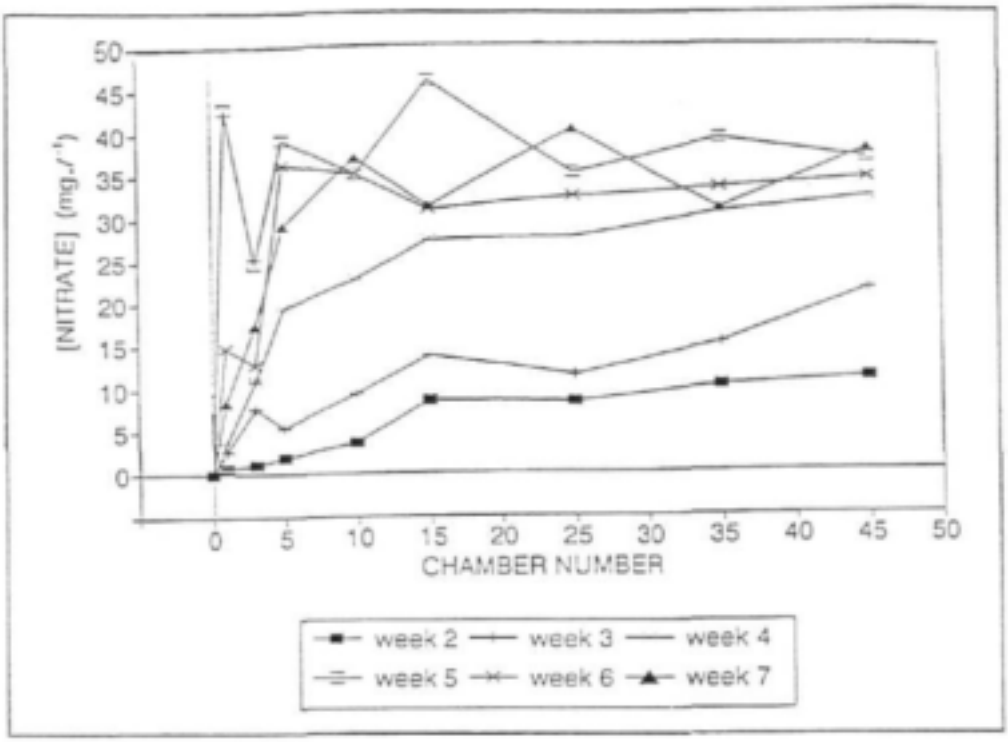


Figure 3.6 Changes in nitrate concentrations recorded in discrete chambers of Channel A of the model, during a 7-week study period.

The results of this experiment demonstrated the slow rate at which the nitrifying populations established within the channels of the multi-stage model. Maximum specific growth rates (μ_{max}) for nitrifiers from 0.023 to 0.057 h^{-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.

Ammonium concentrations at different points along the multi-chambered channel (Figure 3.4) were thought to be regulated by the rates of mineralization of nitrogenous compounds, the incorporation of ammonia-nitrogen into the heterotrophic biomass and the non-assimilative use of nitrogen as an energy source during nitrification. Biological oxidation of ammonia to nitrate via nitrite was thought to be the main regulator of ammonia concentrations in the model. After the initial increases in ammonium concentrations within the channel the concentrations remained relatively unchanged. This suggested that the residual portion was unavailable for nitrification. Possible explanations for this include: nutrient deficiencies; low saturation constants (K_N) for ammonia oxidation

(K_s 0.5-2.0 mg.l^{-1}); and/or inhibition of ammonia oxidation due to the lowered pH.

With the start-up of nitrifying systems nitrite is often found to accumulate (Figure 3.5) until the 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 oxidizers achieve higher cell yields and, thus, greater amounts of biomass are produced (Stensel and Barnard, 1992; Bitton, 1994). Thus, in nitrifying environments, ammonia oxidizers have been found in higher numbers than nitrite oxidizers. 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 increasing concentrations of nitrate illustrated the successional changes which occurred within the model with the establishment of a population of nitrite oxidizers (Figure 3.6). At the beginning of the study period substrate availability (nitrite) appeared to be the limiting factor effecting nitrite oxidiser establishment (weeks 2 and 3). Accumulation of nitrite within each channel preceded nitrite oxidation which suggested that the nitrite oxidizers were separated on the basis of their specific growth rates or as a result of substrate inhibition. At weeks 6 and 7, the nitrate concentrations plateaued from Chambers 5 to 10 onwards, indicating the consolidation of nitrite oxidation activity.

The experiment was terminated after 7 weeks when near steady-state conditions had been reached. A feature of mixed microbial communities is their inability to establish true steady-state conditions within a continuous culture. This has been attributed to members of a community competing for growth-limiting substrate for which none of the competitors has a great selective advantage. 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). Small changes in culture conditions can cause considerable fluctuations in biomass and its activity. Therefore, mixed microbial communities grown in continuous culture are in continuous transient growth phases.

The establishment of nitrifying activity within each multi-chambered channel 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. The increased 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's channels. This biomass build-up and biofilm formation appeared to have resulted from insufficient agitation and mixing within each chamber. This problem was exacerbated by the clogging of the aeration tubes through biofilm growth which resulted in reduced bubbling and agitation of the medium.

To test the assumption that the nitrifiers were growth rate independent an experiment was made whereby a single channel was flushed with two volumes of distilled water and then allowed to re-establish under normal flow conditions for two weeks. The results of this experiment are shown in Figures 3.7 and 3.8.

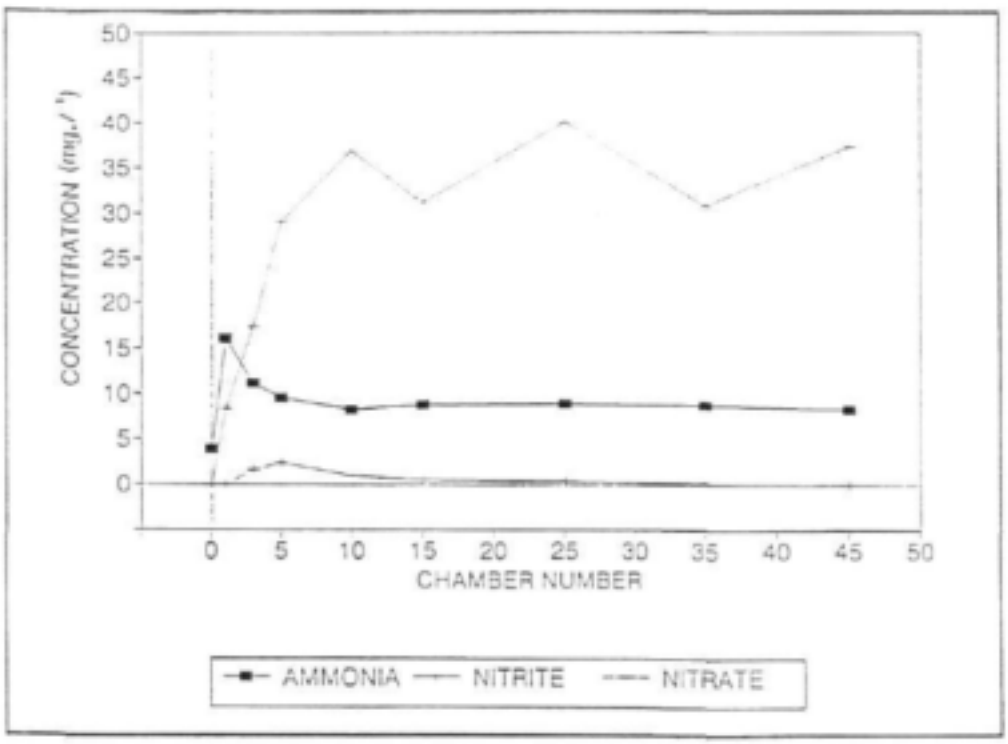


Figure 3.7 Course of nitrification along one channel of the multi-stage laboratory model prior to flushing with distilled water.

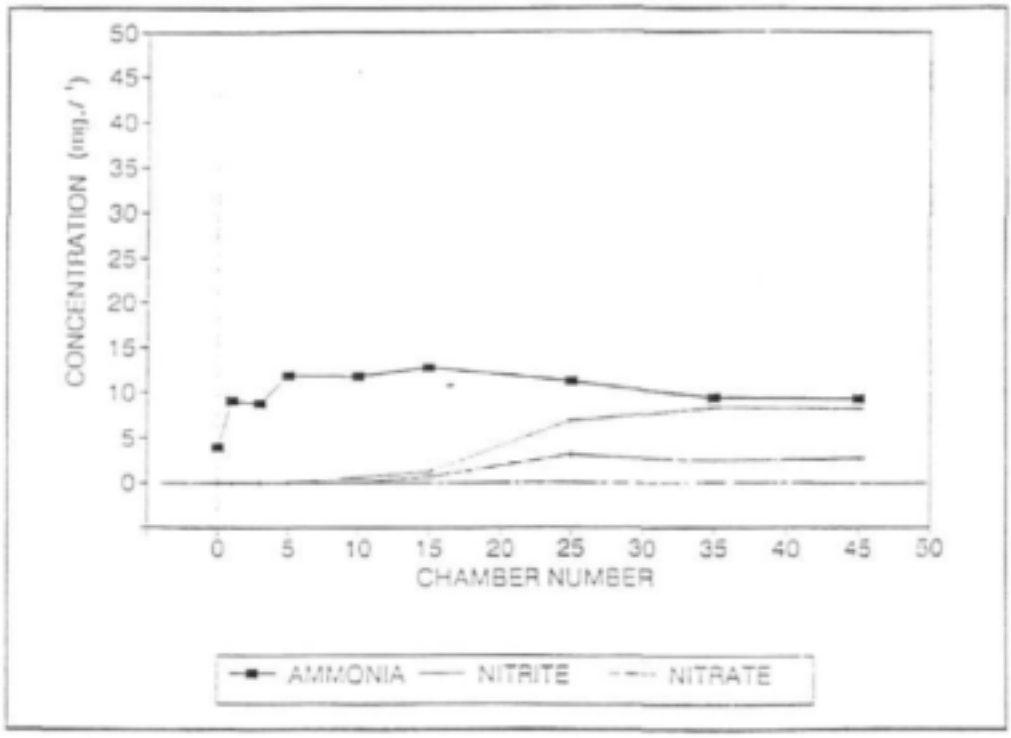


Figure 3.8 Course of nitrification along one channel of the multi-stage laboratory model subsequent to flushing with distilled water.

Comparison of Figures 3.7 and 3.8 shows that nitrifying activity was displaced down the course of the multi-stage channel which suggested that the resident nitrifiers were not surface attached. However, the presence of free-living nitrifying populations was also difficult to justify since nitrifying activity occurred in regions with comparatively high dilution rates compared to the μ_{max} values reported for these species. This suggested that the nitrifying populations were possibly attached to aggregates of suspended matter found within the chambers. This assumption is supported in the literature with reports that nitrifiers readily attach to flocculent biomass aggregates in activated sludge wastewater treatment processes (Gray, 1990).

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

3.2.2 Characterization of the Isolated Microbial Association

1) Isolation and Enumeration of Heterotrophs

The numbers of colony-forming microorganisms in discrete chambers of Channel A are presented in Table 3.1.

Table 3.1 Numbers of heterotrophs present in discrete chambers of Channel A of the multi-stage model after continuous flow operation for 7 weeks

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 counts were found to be the highest in the chambers nearest to the influent feed thus indicating the main region of heterotrophic activity within the model. The colonies isolated on the non-selective agar medium were found to exhibit heterogeneity in terms of morphology and colour. This illustrated that mixed populations of heterotrophs had established.

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

Scanning electron microscopy and bright field microscopy revealed that a diverse range of microorganisms had established within the control Channel D of the multi-stage model. Free-living bacteria, microbial aggregates, filamentous microorganisms, fungi, protozoa, rotifers, macroinvertebrates and algal cells were all present (Plates 3.1 - 3.3).

Microbial development was characterised by the build-up, with time, of biofilms and floc-like biomass within the chambers nearest the influent feed. The surfaces were covered with biofilms and in the chambers with biomass build-up, clogging and blockage of aeration tubes sometimes resulted. This impacted on mixing and aeration within an individual chamber and potentially affected the 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.

With the continual washout of cells from one chamber to another it was found that suspended biomass precipitated and collected in the dead space at the bottom of each chamber. Although the aeration tubes extended to within 10-15 mm of the bottom of each chamber, the region below this was not subjected to the continuous mixing effect caused by sparging and, thus, cellular material sedimented.

Plate 3.1 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.

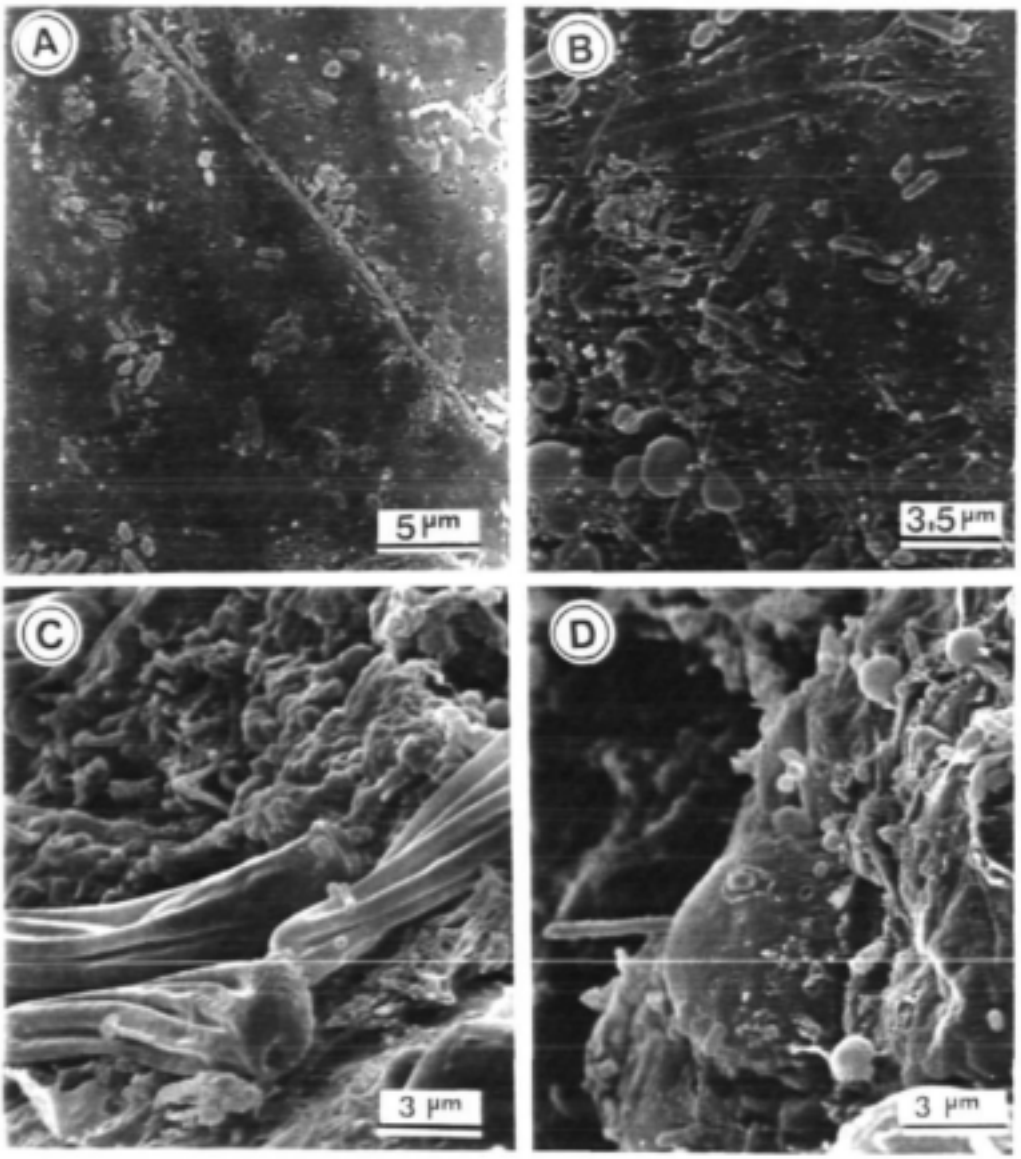

A
5 µm
B
3,5 µm
C
3 µm
D
3 µm

Plate 3.2 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).

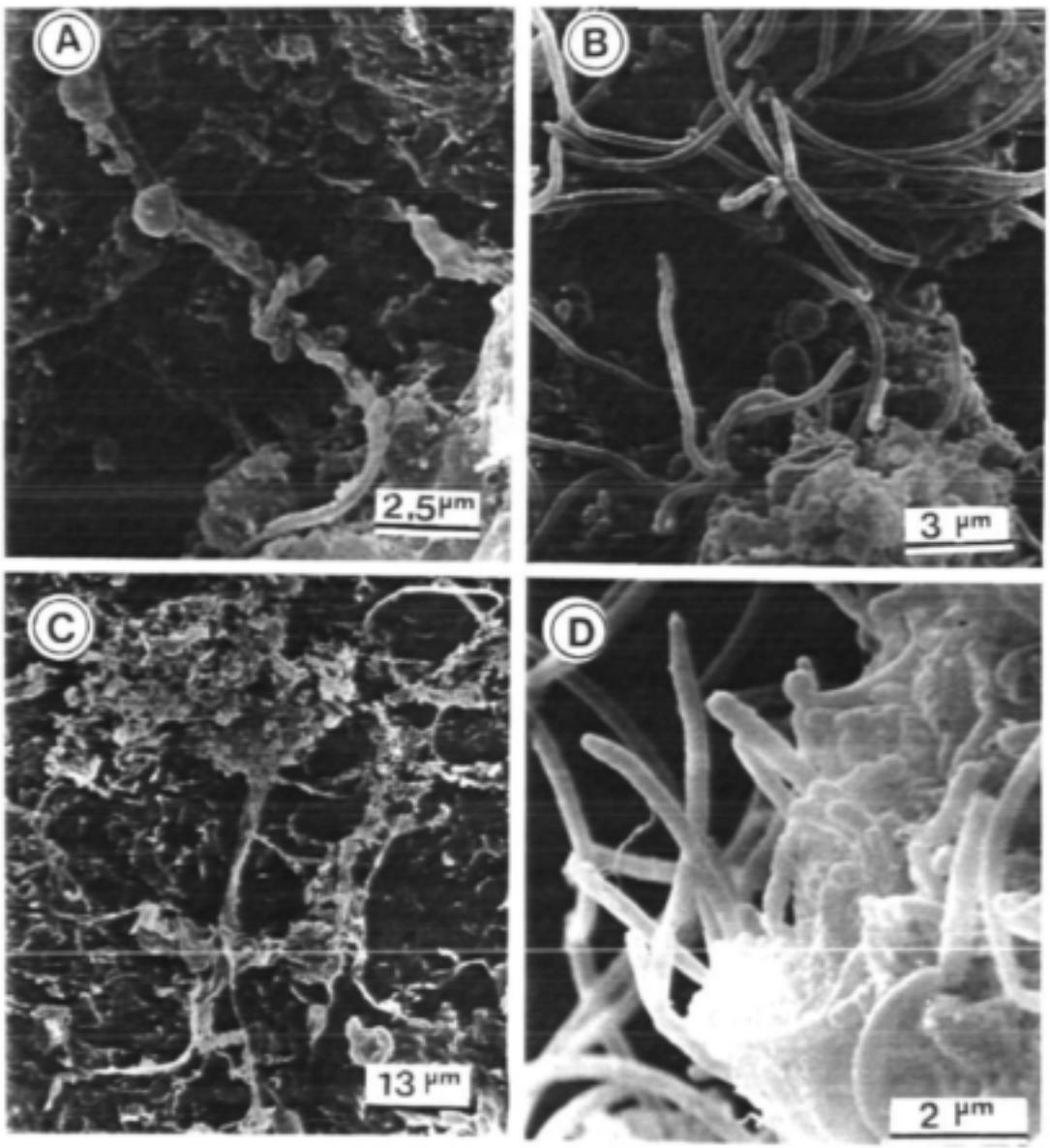
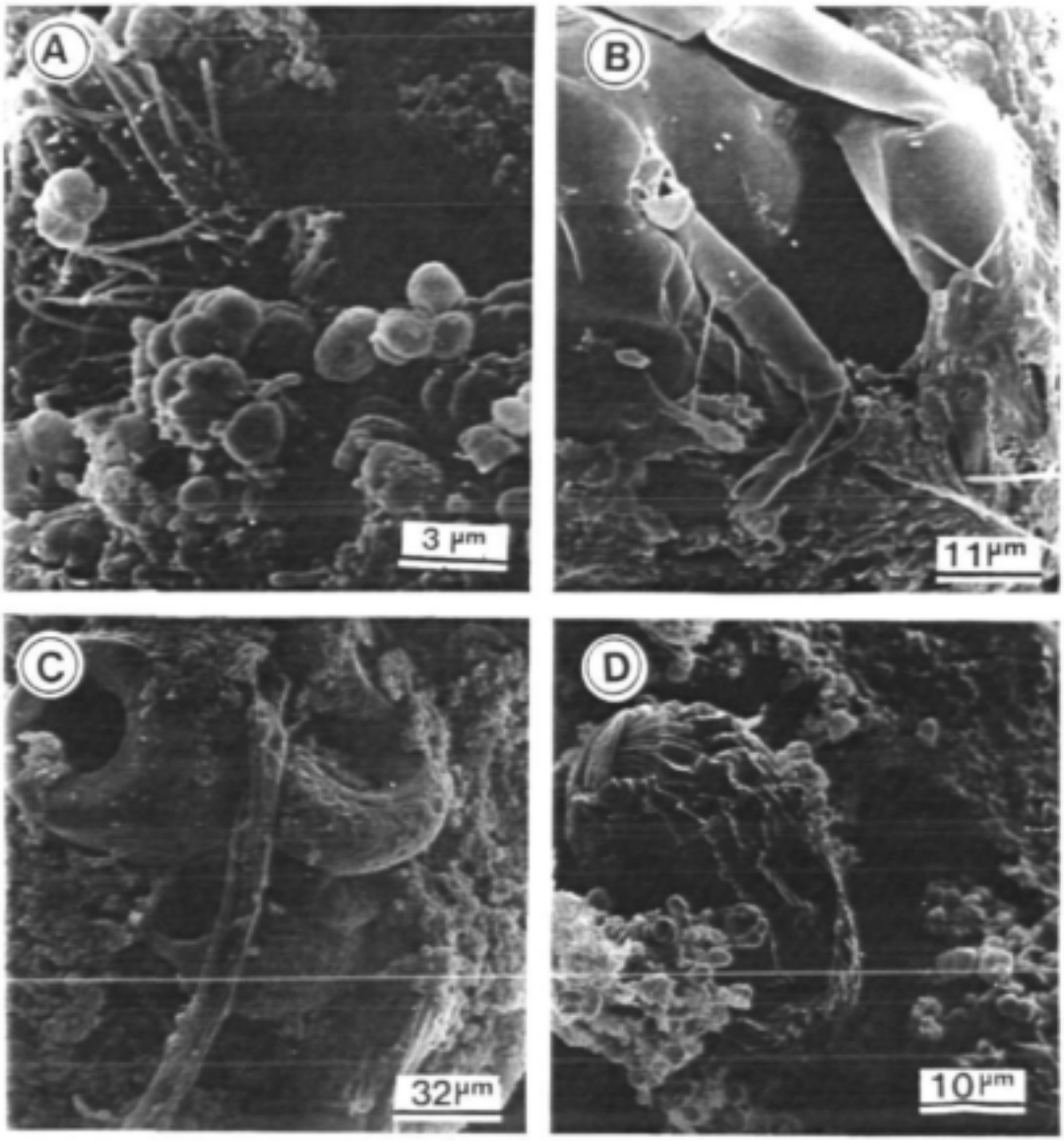

A
2,5µm
B
3 µm
C
13 µm
D
2 µm

Plate 3.3 A,B,C, and D Scanning electron micrographs of a microbial association present in cellular material precipitated at the bottom of Chamber 15 of control Channel D: A - a ciliated protozoan in association with microbial biomass; B - a macroinvertebrate; C - algal cells; and; D - rotifer.


A
3 µm
B
11µm
C
32µm
D
10µm

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 subjected to changes in time and space. These factors could be expected to influence near steady-state conditions and also have significant effects on the reproducibility and variability within the model.

3) Enrichment/Isolation of Autotrophic Nitrifiers from the Multi-Stage Laboratory Model

Enrichment/isolation of nitrifiers from the model was undertaken to demonstrate that autotrophic populations of nitrifiers were present. After a three-week incubation period microbial biomass was discernible within the two liquid cultures used to individually enrich/isolate ammonia and nitrite oxidizers. Assays for nitrite and nitrate confirmed that oxidation of ammonia and nitrite had occurred. Gram-negative rods were found in both cultures when the Gram stained cultures were viewed by light microscopy. On the basis of these findings the assumption was made that autotrophic nitrifiers were responsible for the nitrifying activity within the model.

3.3 CONCLUSIONS

Within the model, 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 in space and time. The establishment of a microbial association within the model was primarily dependent on: the 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 but 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 findings with regards to subsequent perturbation studies are:

1) Representative microbial associations were enriched and isolated from selected chambers of the model;
2) The slow rate at which the nitrifying populations established within the model did not facilitate numerous impact studies to be undertaken;
3) The model was operationally complex and increased variability was 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 numbers of analyses required ultimately had a direct bearing on the cost-effectiveness of using the model.

4. ASSESSMENT OF PHENOLIC COMPOUND PERTURBATIONS ON AN ISOLATED MICROBIAL ASSOCIATION MAINTAINED WITHIN THE MULTI-STAGE LABORATORY MODEL

The impact and fate of phenol and a halogen substituted phenol, 2,4-dichlorophenol, on the nutrient cycling processes operating within the multi-stage laboratory model were determined.

4.1 EXPERIMENTAL PROCEDURE

4.1.1 Dilution/Dispersion Effects

To determine the effect of dilution and dispersion on phenol, after introduction into the model system, a 25 chamber control channel was established. Distilled water was used as the carrier medium for the study. In a series of investigations different concentrations (20,60 and 100 mg.l^{-1}) of phenol were individually introduced into the first chamber of the control channel for 48h. The concentrations of phenol were then monitored in discrete chambers along the control channel after 24 and 48 hours.

4.1.2 Biodegradation Testing

Biodegradation tests were made to establish the contribution of the microorganisms cultured in the model to the attenuation of phenol. Batch cultures were established with mineral salts medium (Coutts *et al.*, 1987) 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 a single channel (Channel A) was added to individual Erlenmeyer flasks (250 m*l*) which contained 100 m*l* of medium. Uninoculated phenol-supplemented media were used as controls.

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.). The residual phenol concentrations were determined by reverse phase paired ion chromatography (Waters, Millipore).

4.1.3 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 laboratory model. Nitrification was chosen as the criterion for assessing the inhibitory effects of the perturbant molecules on the established microbial association and was monitored by determining nitrite and nitrate concentrations (3.1.2). Residual phenol concentrations were assayed (4.1.2) to determine its fate within the model.

Perturbation studies were made by introducing the perturbant molecule into Chamber 1 of each experimental channel in the influent medium. 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 a control.

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.

To determine the short-term (72 h) impacts of the perturbant compounds on the nutrient cycling processes, individual perturbation studies were made with phenol and 2,4-dichlorophenol as pollutant compounds in various concentrations. Recovery of nitrifying activity was also investigated subsequent to the omission of the perturbant compound from the influent medium.

4.2 RESULTS AND DISCUSSION

4.2.1 Dilution/Dispersion Effects

Residual concentrations of phenol in the multi-chambered model after periods of 24 and 48 hours are shown in Figure 4.1.

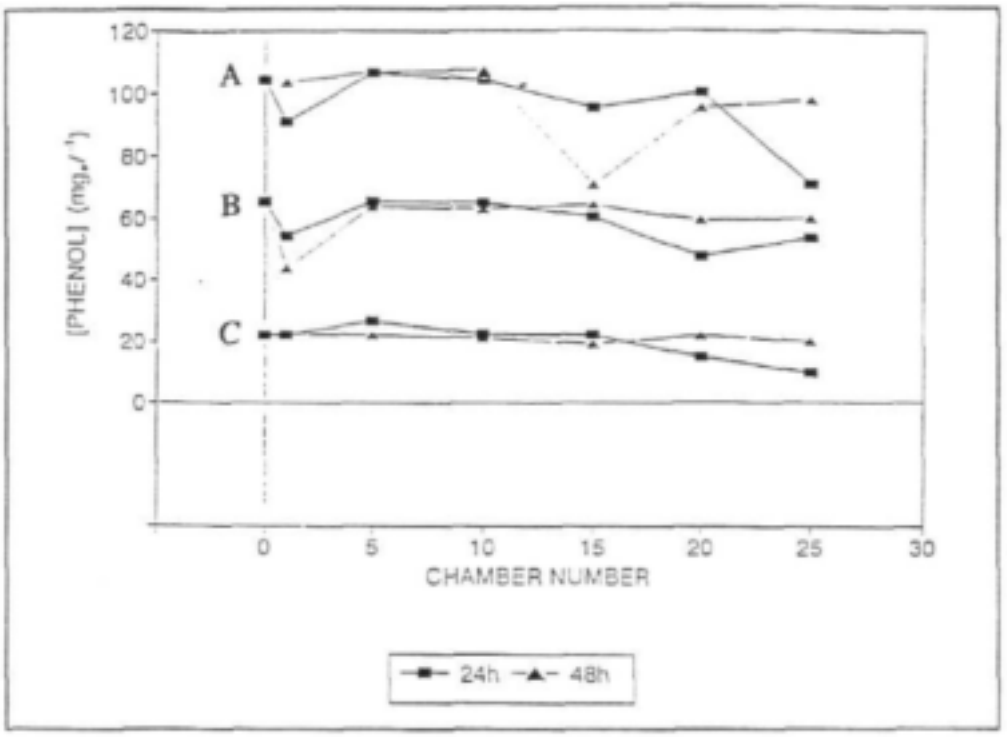


Figure 4.1 Residual phenol concentrations of Channel 1, 24 and 48 hours after the introduction of the perturbant phenol in concentrations of 100 (A), 60 (B) and 20 (C) mg.l^{-1}.

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

This illustrated 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 volatilization 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 g.l^{-1}, 0°C) and low volatility (i.e. a vapour pressure of 0.8 mm at 20°C).

4.2.2 Biodegradation Testing

Phenol degradation in batch culture over a four-day period is shown in Figure 4.2. while the control results are shown in Figure 4.3.

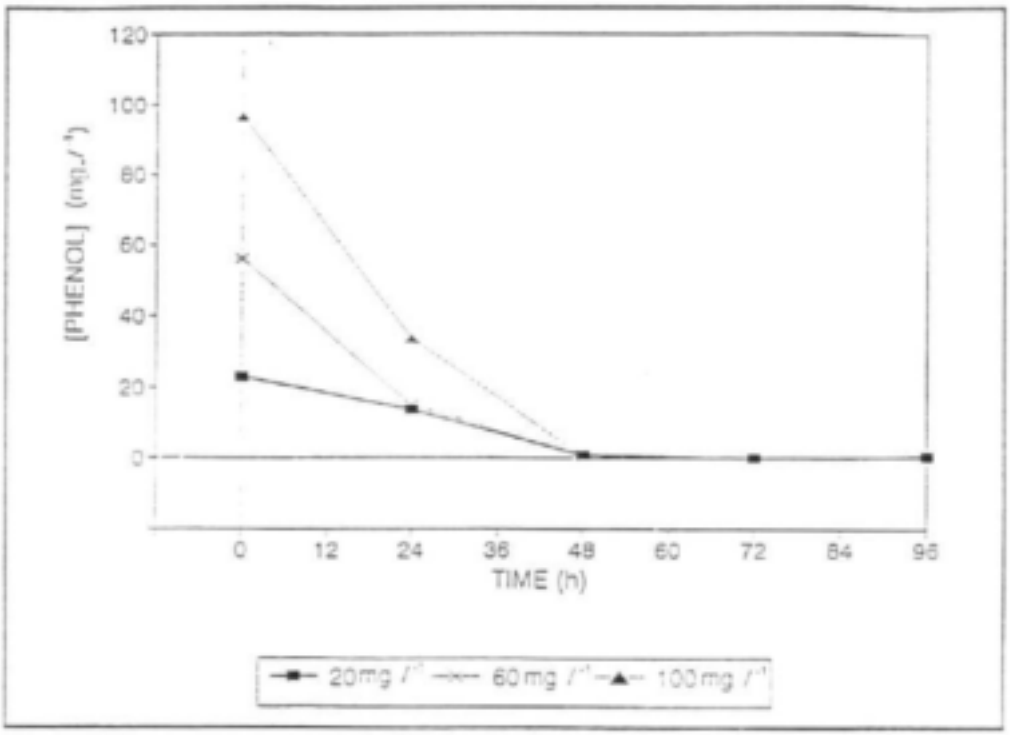


Figure 4.2 Phenol degradation in batch culture over a four- day test period. Phenol was added as the sole carbon source.

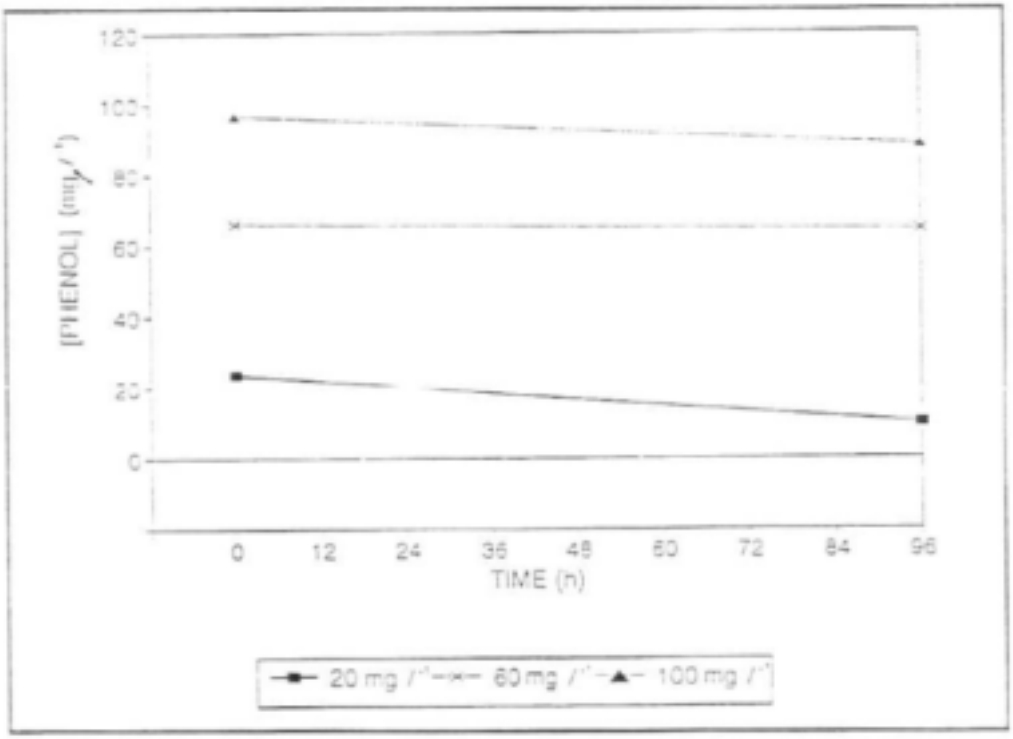


Figure 4.3 Phenol controls in batch culture for the same four-day test period as Figure 4.2.

Biodegradation testing with different concentrations of phenol as the sole carbon and energy source showed that for the concentrations tested, the molecule was degraded during a four-day test period (Figure 4.2). It was, thus, apparent that an active phenol catabolizing population was present in the model.

Phenol concentrations in the control batch cultures were not found to change significantly. The drop in phenol concentration of the 20 mg.l^{-1} control was attributed to microbial contamination rather than abiotic removal factors.

Phenol degradation in aquatic environments has been reported by a number of authors (Jones and Alexander, 1986; Hwang *et al.*, 1986; Gladyshev *et al.*, 1993). 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).

4.2.3 Perturbation Studies

1) Chronic Impacts

In a preliminary perturbation study, the addition of 20 mg.l^{-1} phenol (0.21 mM) to Channel A effected a spatial shift in nitrification activity over a two-week period (Figures 4.4 A and B). The control channel (Channel D) did not exhibit any marked changes in nitrification activity during the same period (Figures 4.5 A and B).

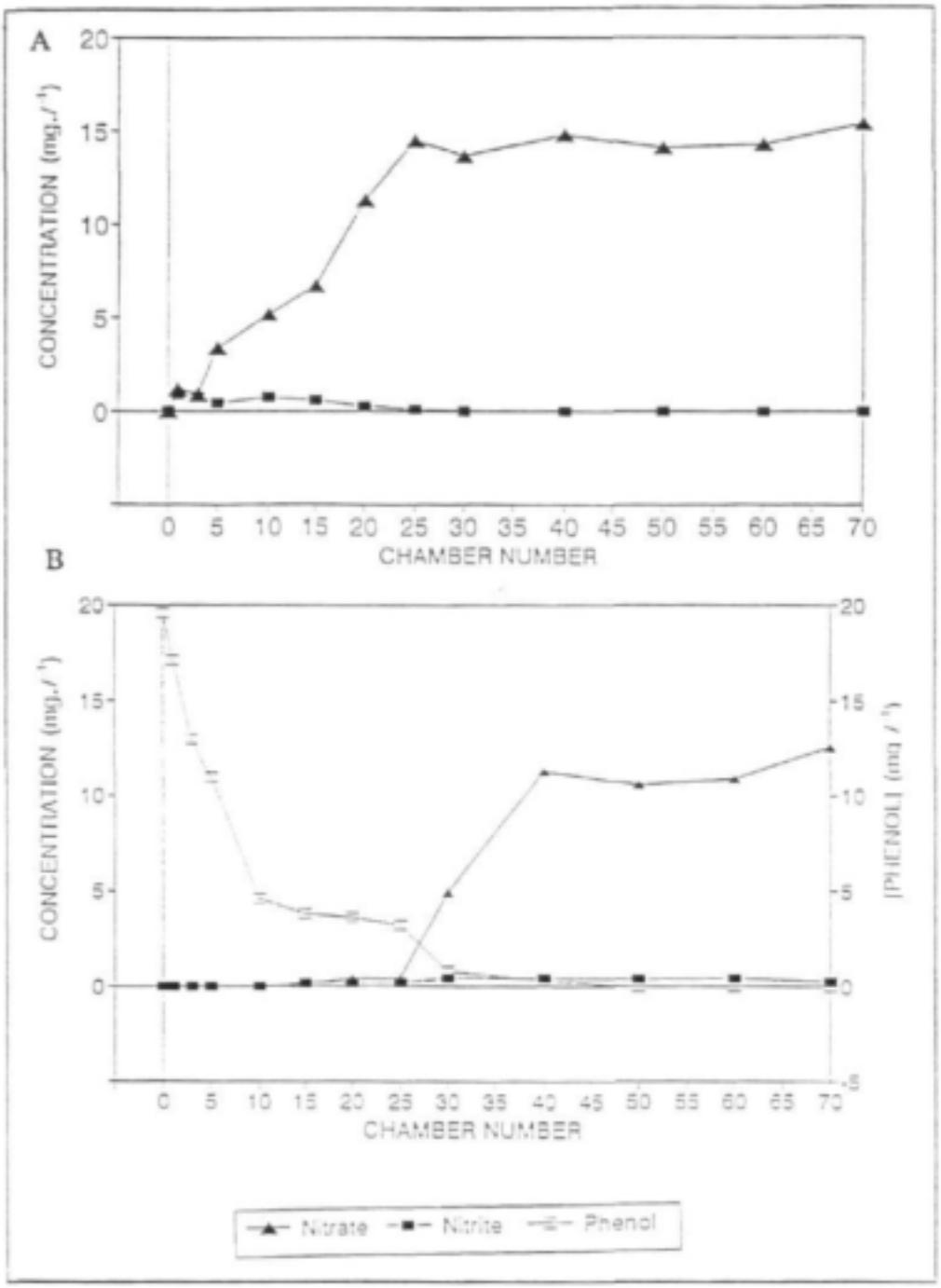


Figure 4.4 A Course of nitrification in Channel A prior to perturbation with phenol.

Figure 4.4 B 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.

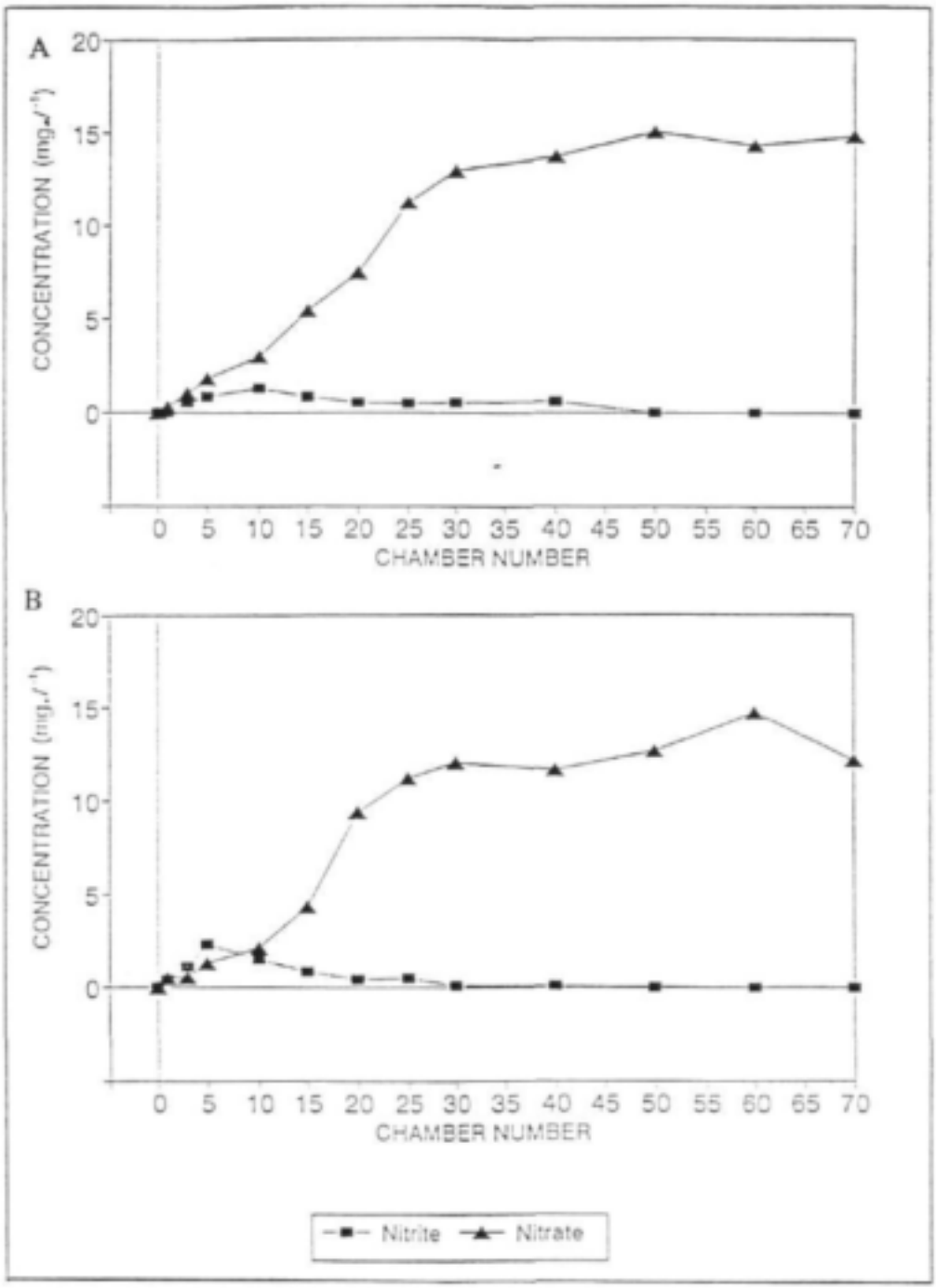


Figure 4.5 A and B Course of nitrification in Control Channel D prior to (A) and subsequent to (B) the 14-day perturbation study.

It was evident that nitrification was inhibited until the residual phenol concentration had been reduced to < 4 mg.l^{-1} (34 μM). 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 (Stafford, 1974; Hockenbury and Grady, 1977).

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

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, and 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.

2) Acute Impacts

To rationalize the screening procedure the duration of a perturbation study was reduced to 72 hours. Individual perturbation studies were made with phenol concentrations of 20 and 60 mg.l^{-1} and with 2,4-dichlorophenol concentrations of 10 and 20 mg.l^{-1}.

The fates of these perturbant molecules within the individual channels of the model are shown in Figures 4.6 (A and B) and 4.7 (A and B). The impacts of these perturbant compounds on nitrifying activity are presented in Figures 4.8 to 4.11. Inherent variability in the nitrite and nitrate concentrations of the control channel (Channel D) during a 216h experimental period are shown in Figures 4.12 (A and B).

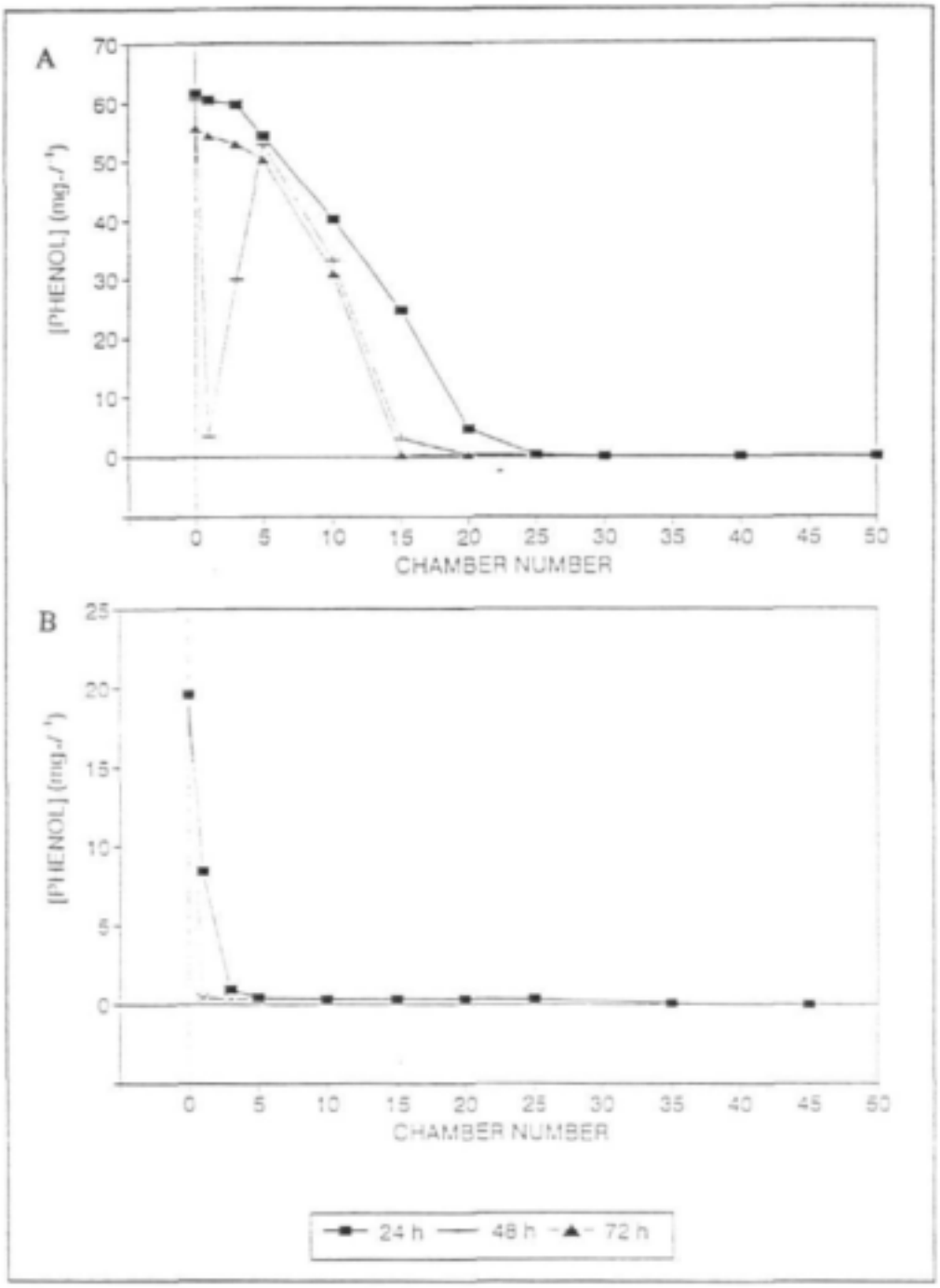


Figure 4.6 A and B Residual phenol concentrations after a continuous 72-hour perturbation of phenol[60 mg.ℓ^{-1} (A) and 20 mg.ℓ^{-1} (B)] to Chamber 1 of individual channels of the multi-stage model.

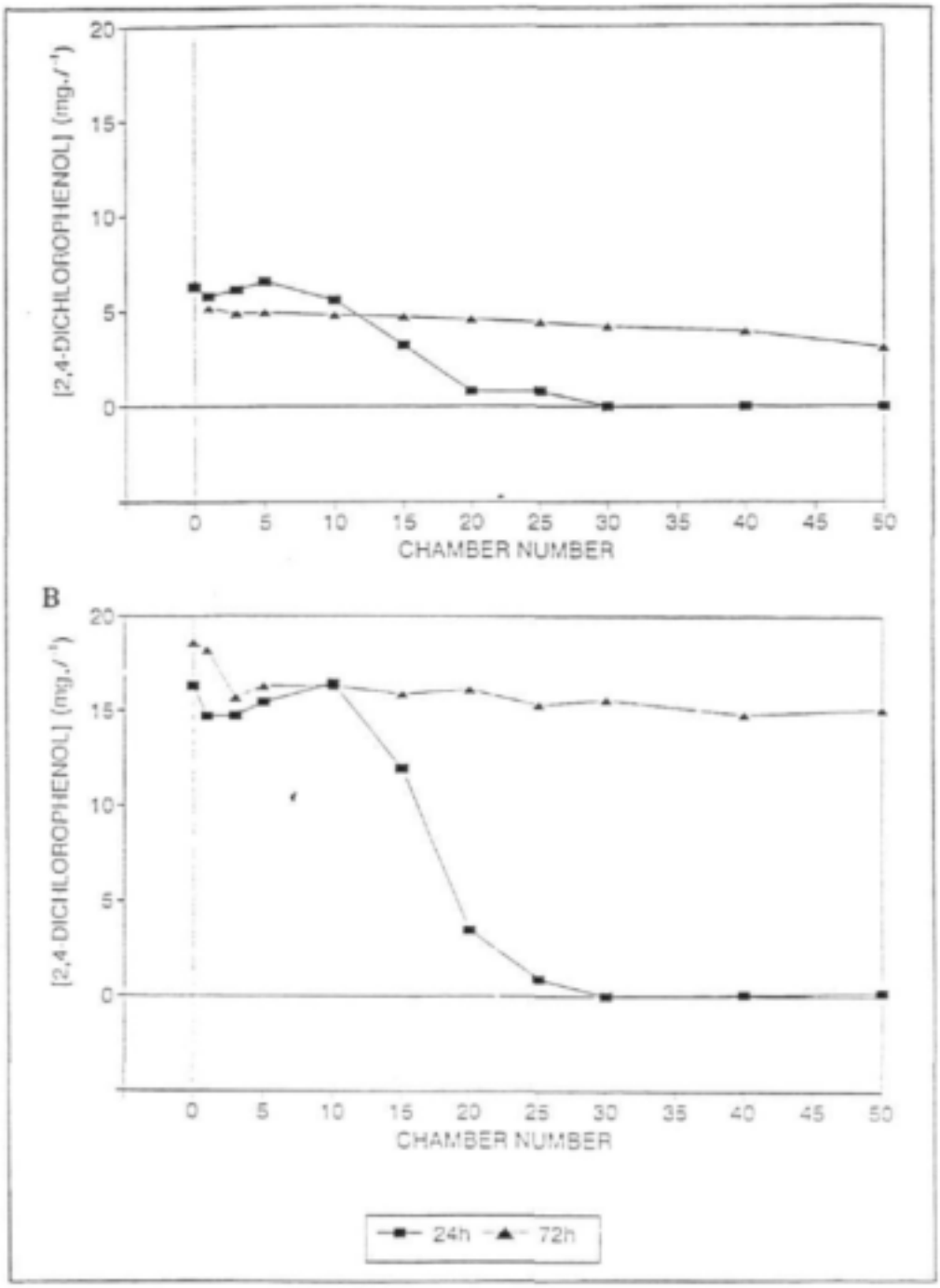


Figure 4.7 A and B Residual 2,4-dichlorophenol concentrations after a continuous 72-hour perturbation of 2,4-dichlorophenol [10 mg.l^{-1} (A) and 20 mg.l^{-1} (B)] to Chamber 1 of individual channels of the multi-stage model.

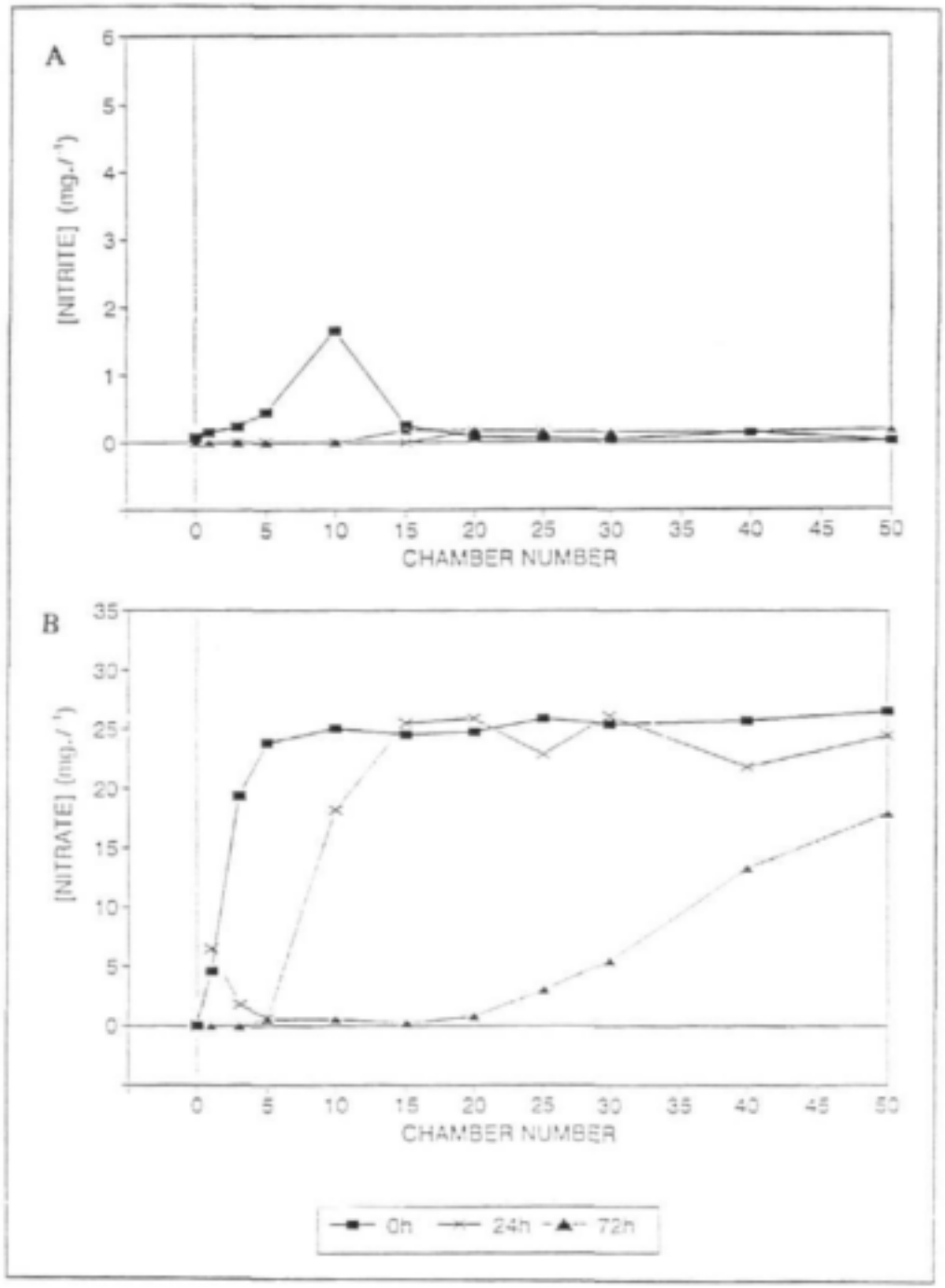


Figure 4.8 A and B

Changes in nitrite (A) and nitrate (b) concentrations in response to phenol (60 mg.l^{-1}) perturbation within an experimental channel of the multi-stage model.

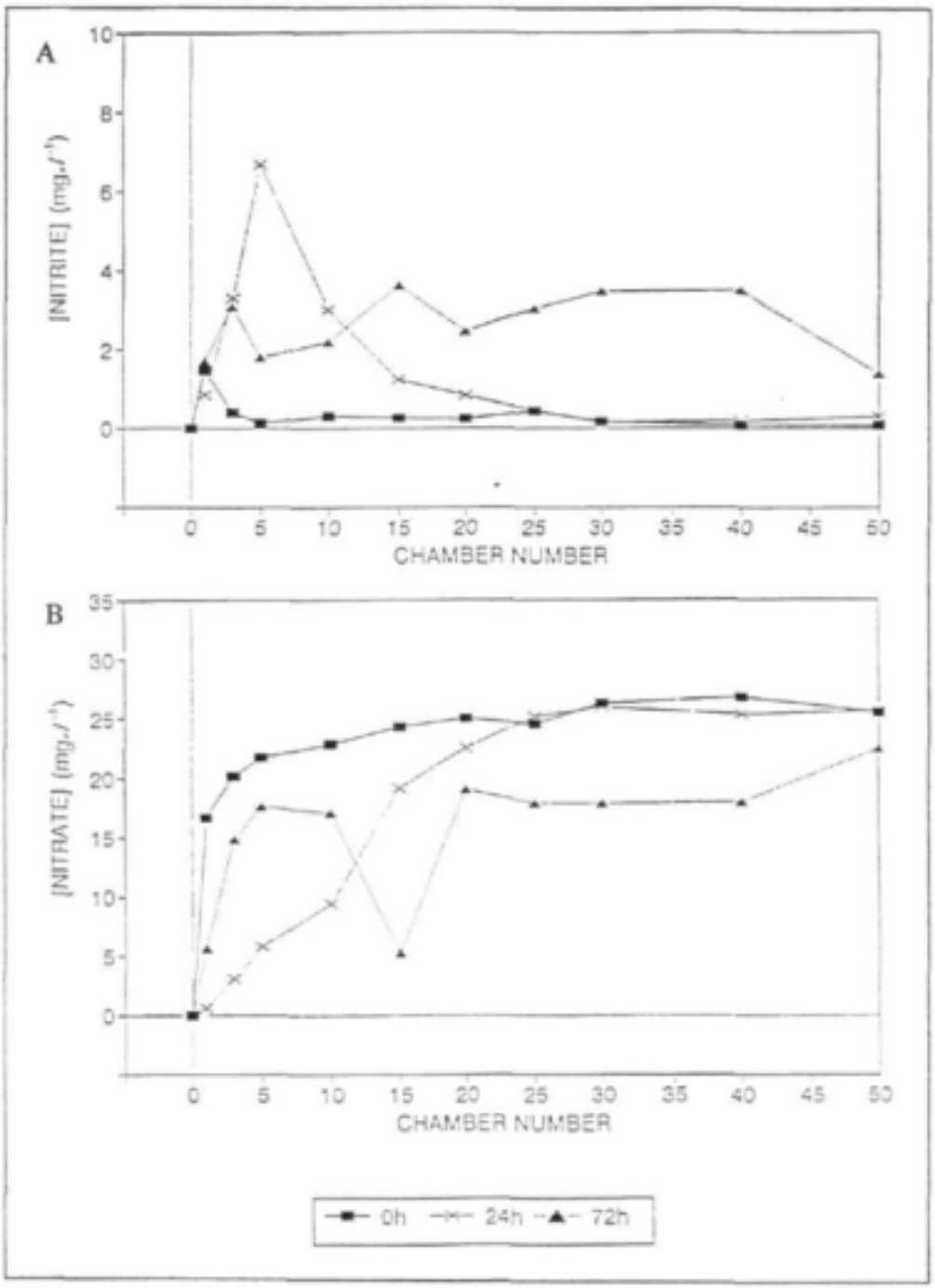


Figure 4.9 A and B Changes in nitrite (A) and nitrate (B) concentrations in response to phenol (20 mg.l^{-1}) perturbation within an experimental channel of the multi-stage model.

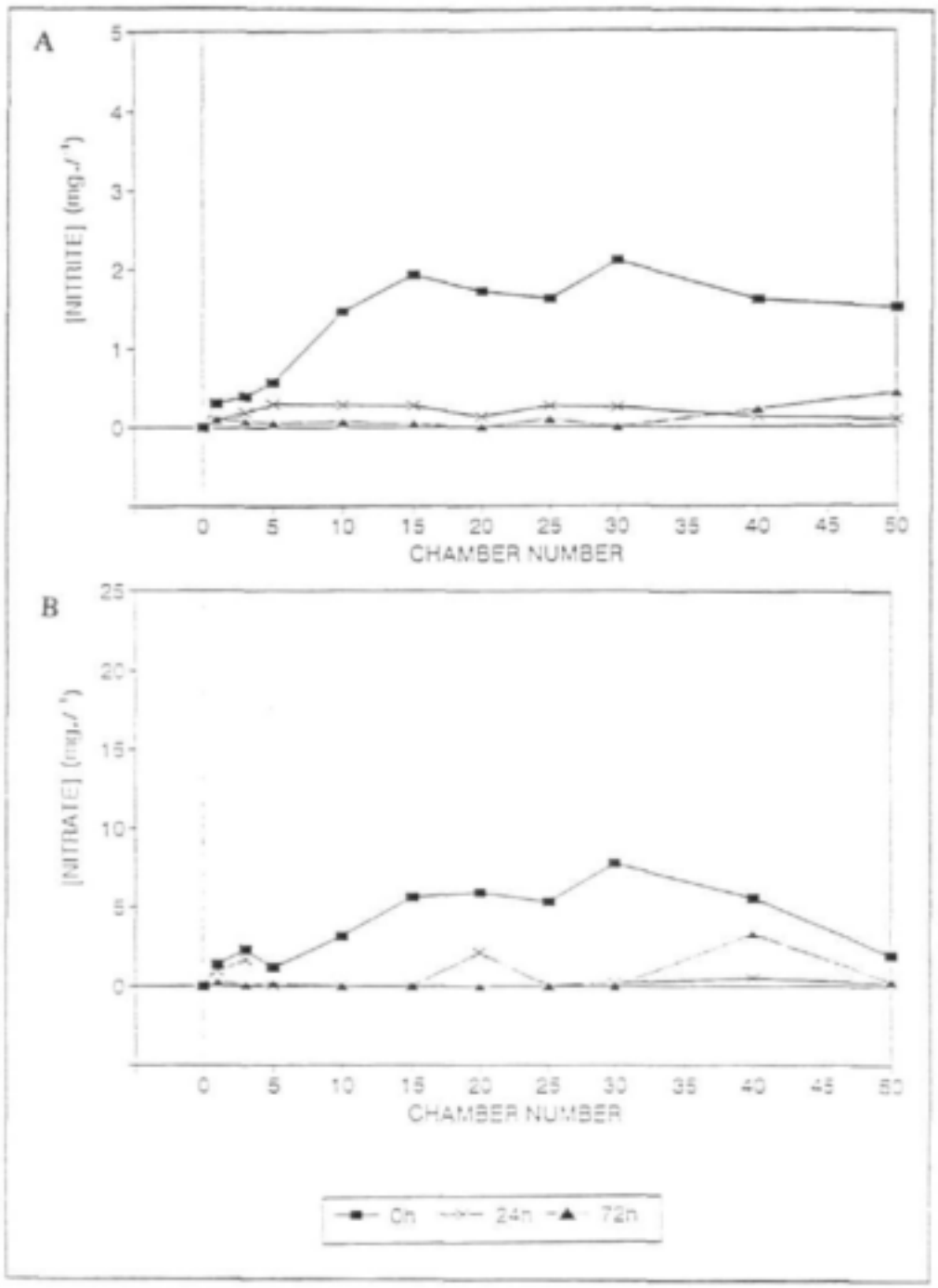


Figure 4.10 A and B Changes in nitrite (A) and nitrate (B) concentrations in response to 2,4-dichlorophenol (10 mg.l^{-1}) perturbation within an experimental channel of the multi-stage model.

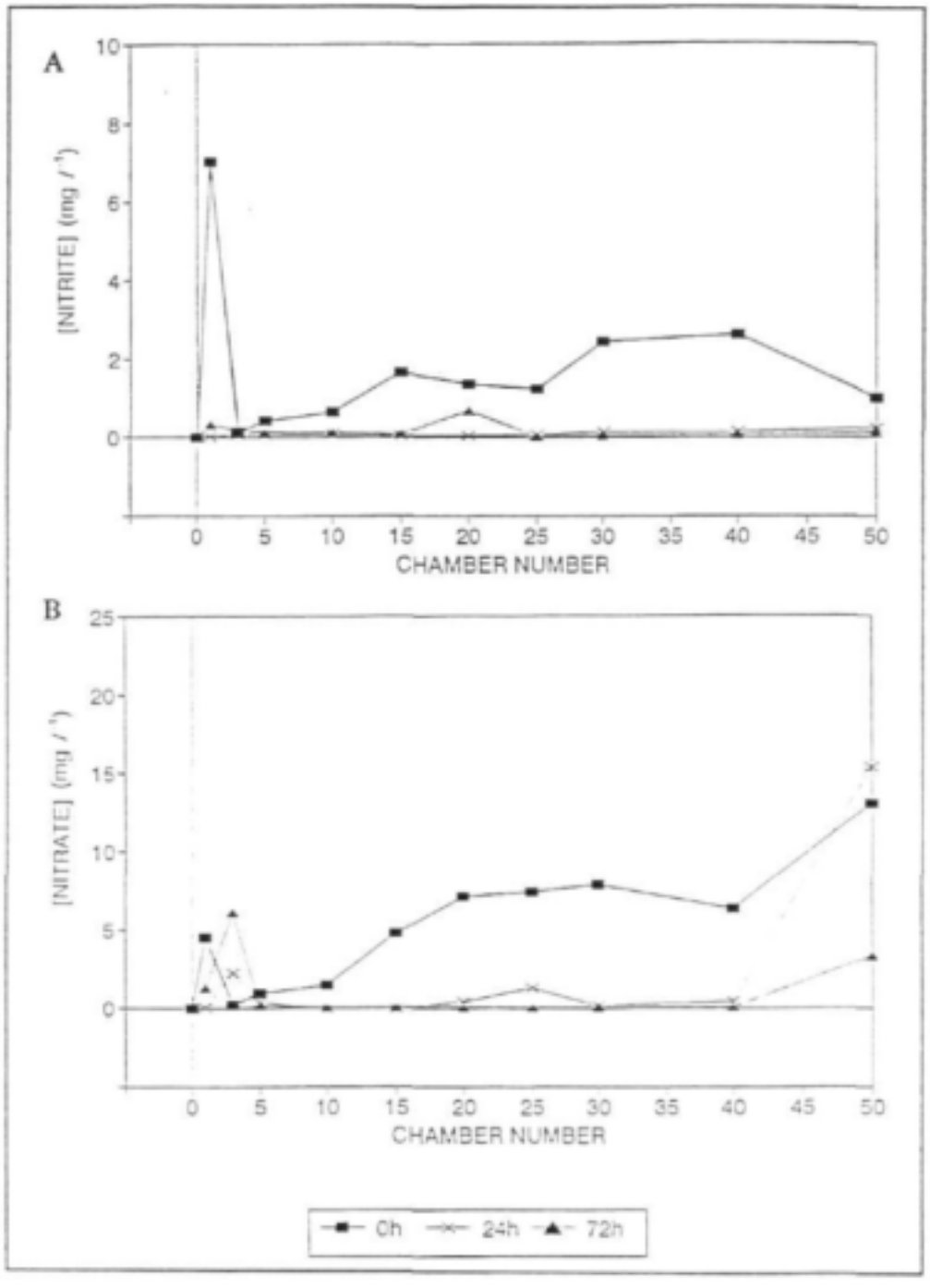


Figure 4.11 A and B Changes in nitrite (A) and nitrate (B) concentrations in response to 2,4-dichlorophenol (20 mg.l^{-1}) perturbation within an experimental channel of the multi-stage model.

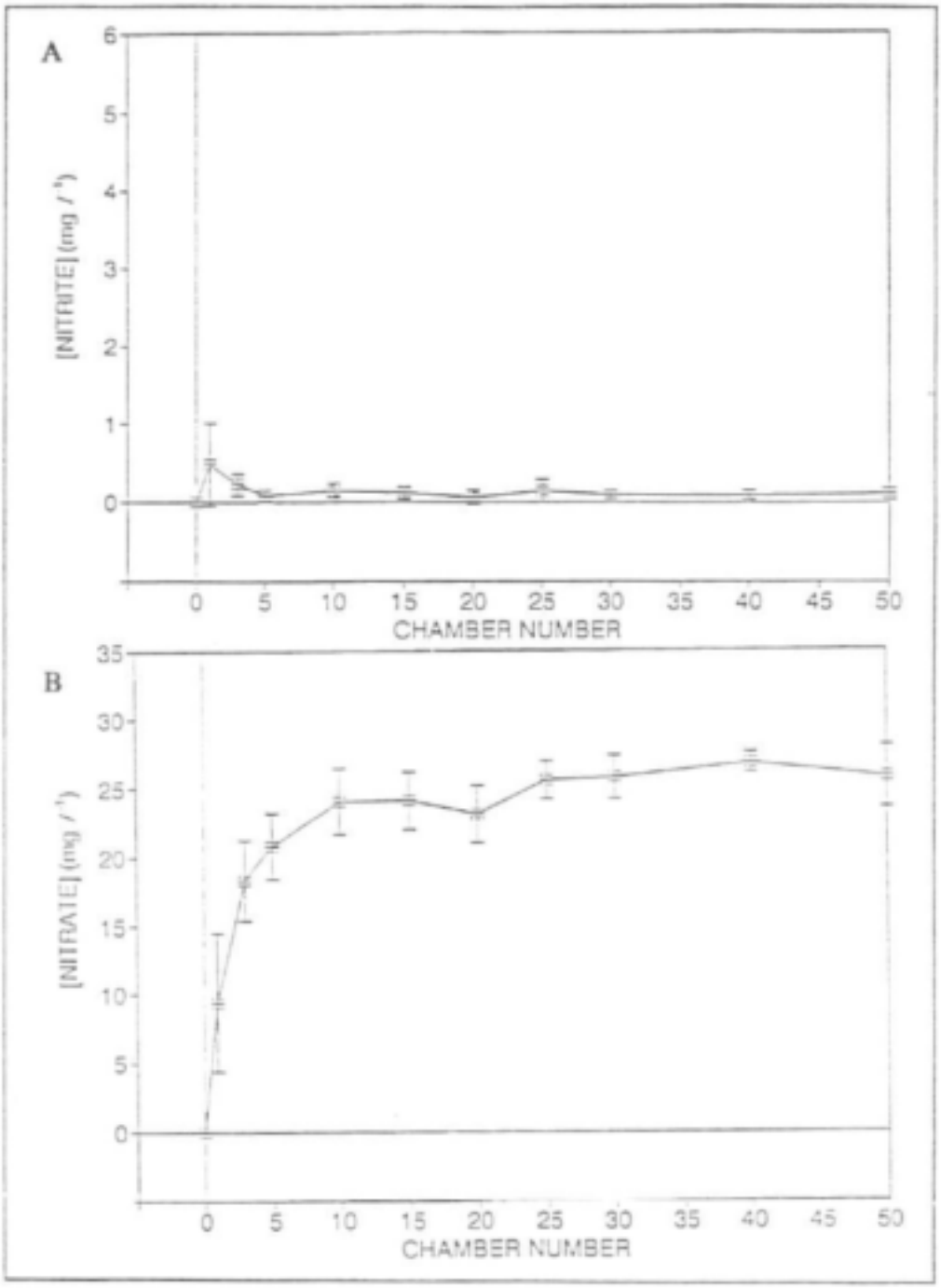


Figure 4.12 A and B

Discrete concentrations of nitrite (A) and nitrate (B) in Control Channel D at the end of the 216-hour experimental period. The mean values of each sampled chamber are shown together with vertical bars depicting 95% confidence intervals.

The results confirmed that a 72-hour perturbation period was sufficient to illicit an inhibitory response on nitrification with the concentrations of the perturbant molecules tested (Figures 4.8 - 4.11). It was evident that the toxicological impacts of a perturbant compound appeared to be a direct function of the removal or transformation of the molecule.

Phenol was found to readily attenuate within the model for each concentration tested whereas 2,4-dichlorophenol was found to persist (Figures 4.6 and 4.7). This illustrated the relevance of making biodegradation studies in conjunction with environmental impact assessments. Compounds which are labile will have less of an impact than compounds which are recalcitrant and may become biomagnified.

An important limitation of the perturbation assessment was the empirical nature of the protocol. To optimise time and resources there was, thus, a requirement for an initial screening protocol to determine an appropriate concentration range for testing in the model.

Several factors such as cell density, the presence of inert organic matter, nutrient availability, environmental conditions and previous exposure to the perturbant compound all contribute to the potential degradation of a particular compound and its overall toxicity. These factors are important considerations when screening test compounds in relatively low concentrations. Removal of the test compound from solution early in the test system can, potentially, lead to an underestimation of the toxicological impacts of the compound on nitrification. For example, the impacts of 20 mg.l^{-1} phenol on nitrification were minimised by the rapid attenuation of the molecule within the model (Figure 4.6 B). This rapid attenuation was thought to result from the possible effect of acclimation of the selected microbial association. This emphasised the importance of using unacclimated associations to assess potential impacts to aquatic environments.

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 flocculent biomass or 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. A consequence of this was that reproducibility in the experimental channels for subsequent perturbation studies was impaired and this impacted on the interpretation of the subsequent data. Cognisance of this variability was taken into consideration when interpreting the data.

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 the perturbant compound, and differing sensitivities of the nitrifying populations within the experimental channels. Other factors which could contribute to variability within the model were the discrepancies that arose in the aeration and mixing of the individual chambers which resulted from air flow impairment by aeration nozzle clogging.

Inherent variabilities in nitrite and nitrate concentrations within the control channel were found to stabilise with time and were thought to be directly related to the "age" of the nitrifying population (Figure 4.12).

Recovery of nitrifying activity was also investigated subsequent to the omission of the perturbant compound from the influent medium (Figures 4.13 - 4.15).

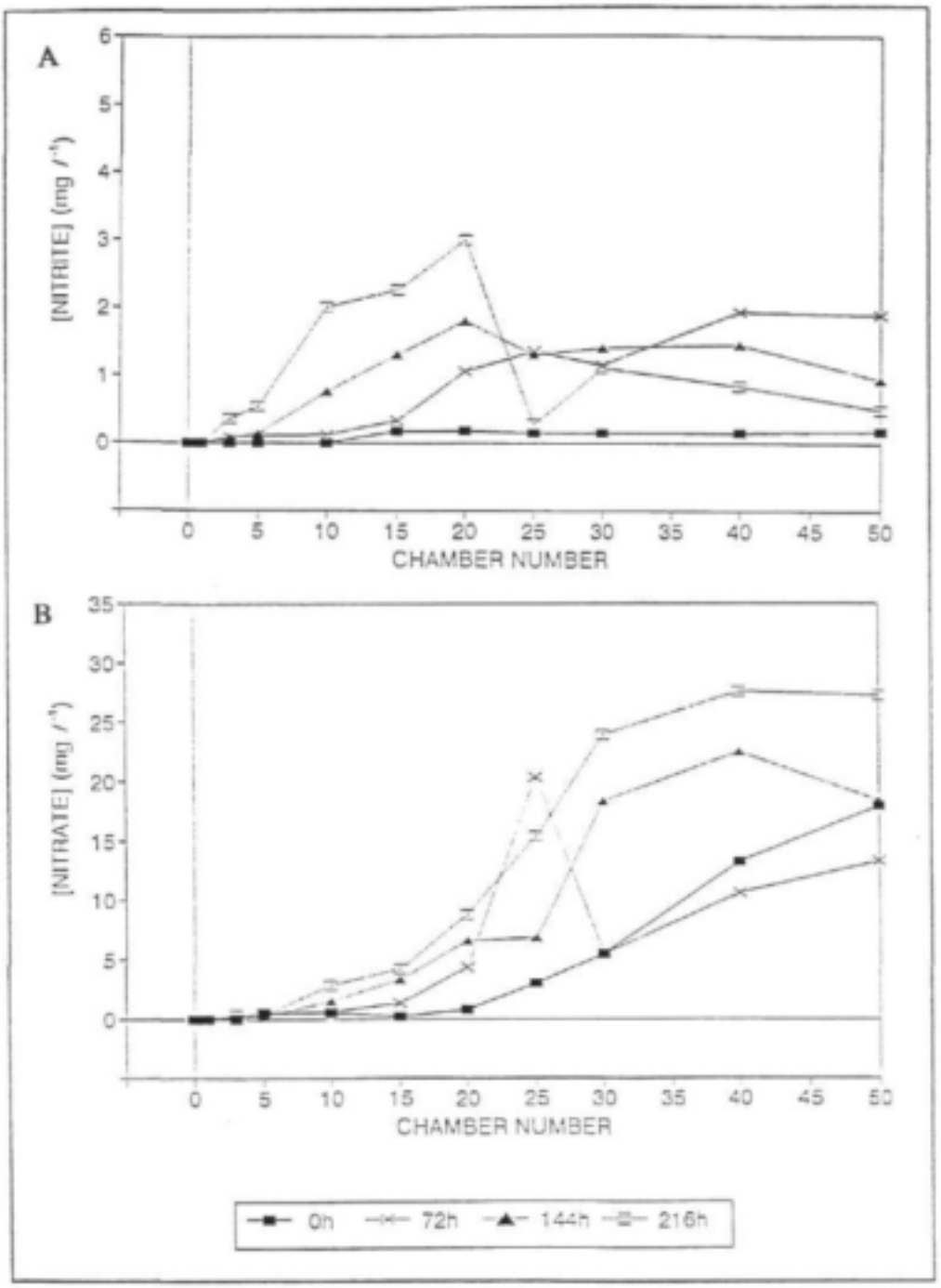


Figure 4.13 A and B Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 216-hour recovery period, subsequent to a 72-hour phenol perturbation (60 mg.l^{-1}).

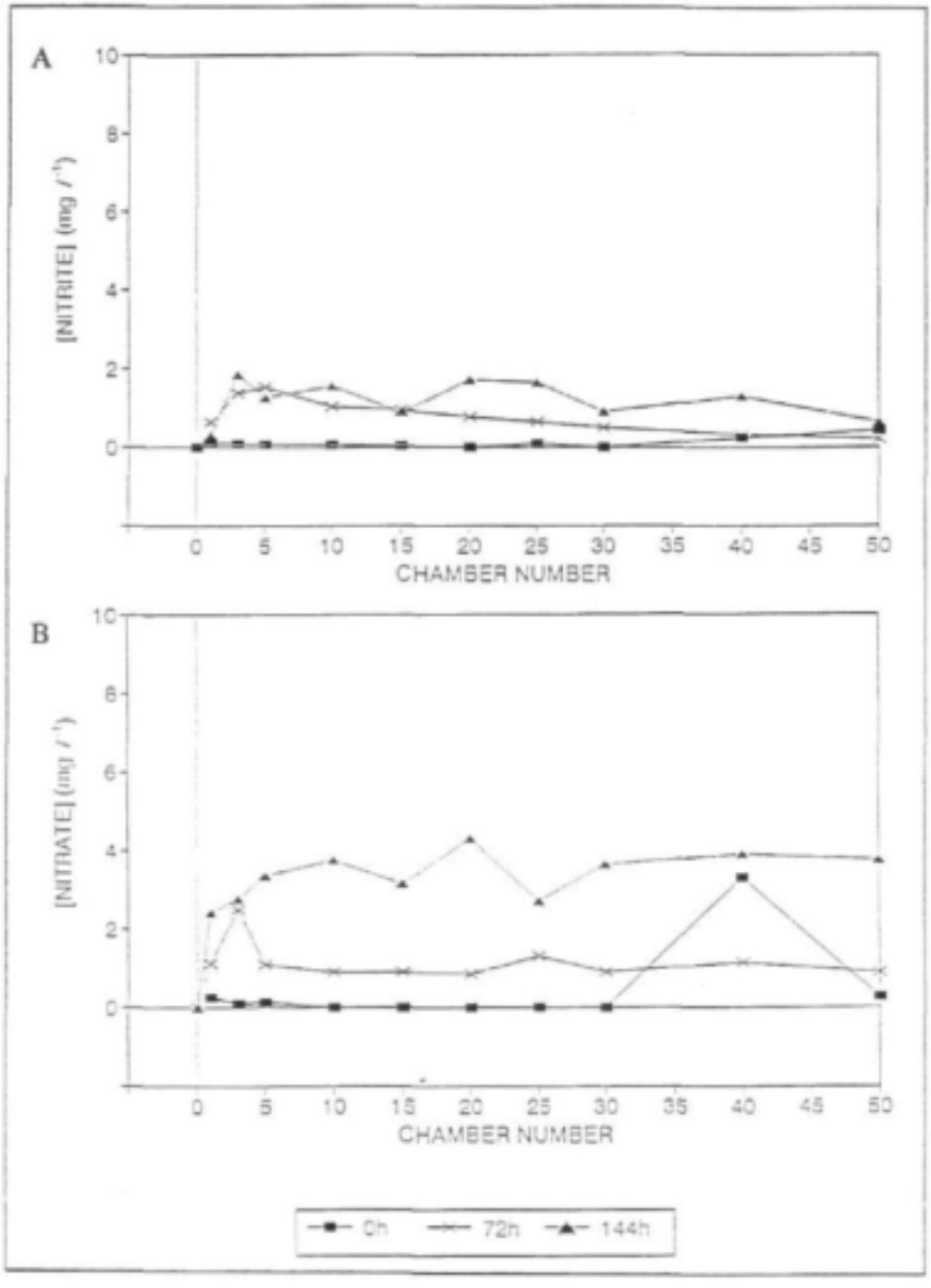


Figure 4.14 A and B Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 216-hour recovery period, subsequent to a 72-hour phenol perturbation (20 mg.l^{-1}).

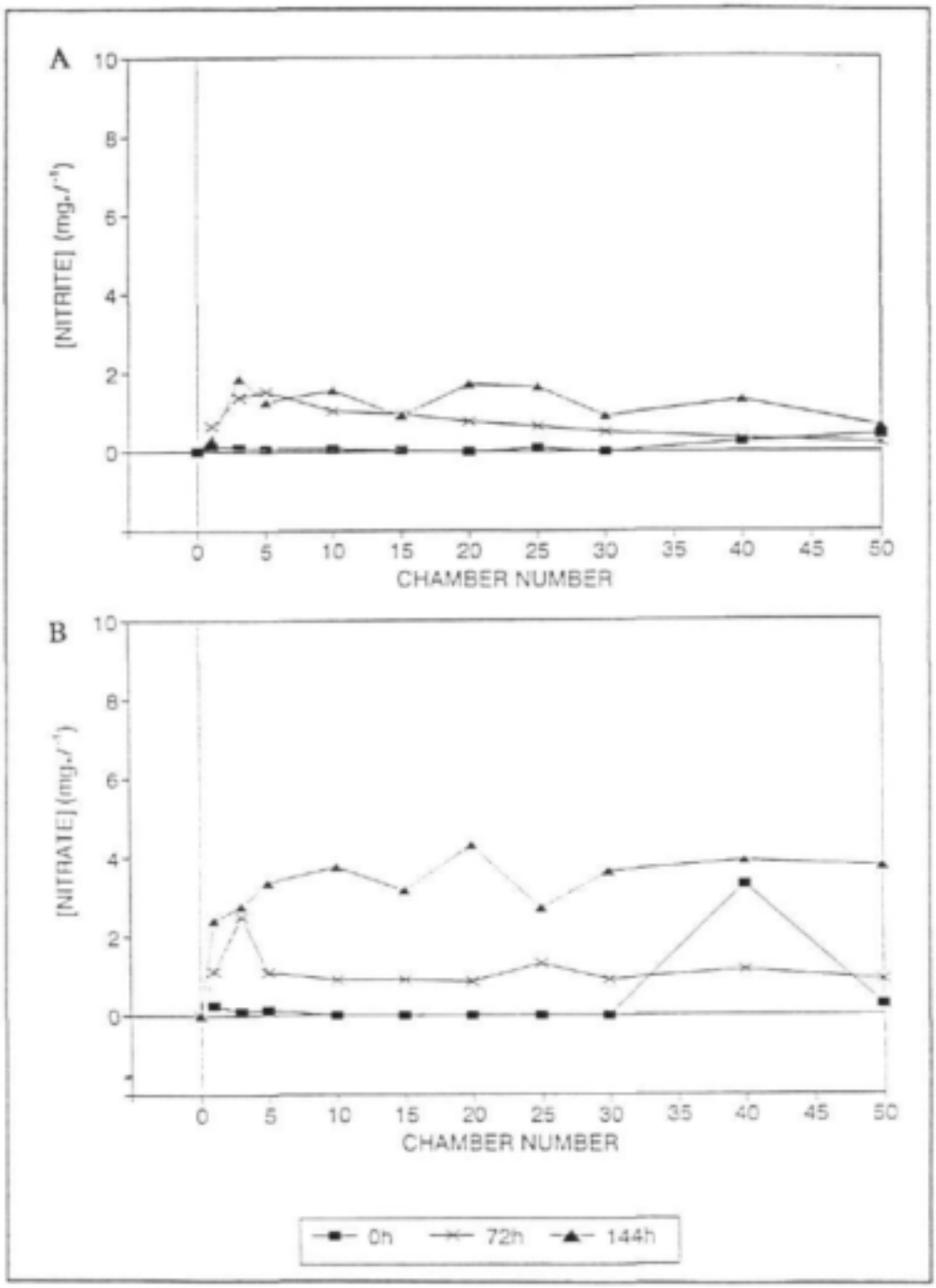


Figure 4.15 A and B Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 144-hour recovery period, subsequent to a 72-hour 2,4-dichlorophenol perturbation (10 mg.l^{-1}).

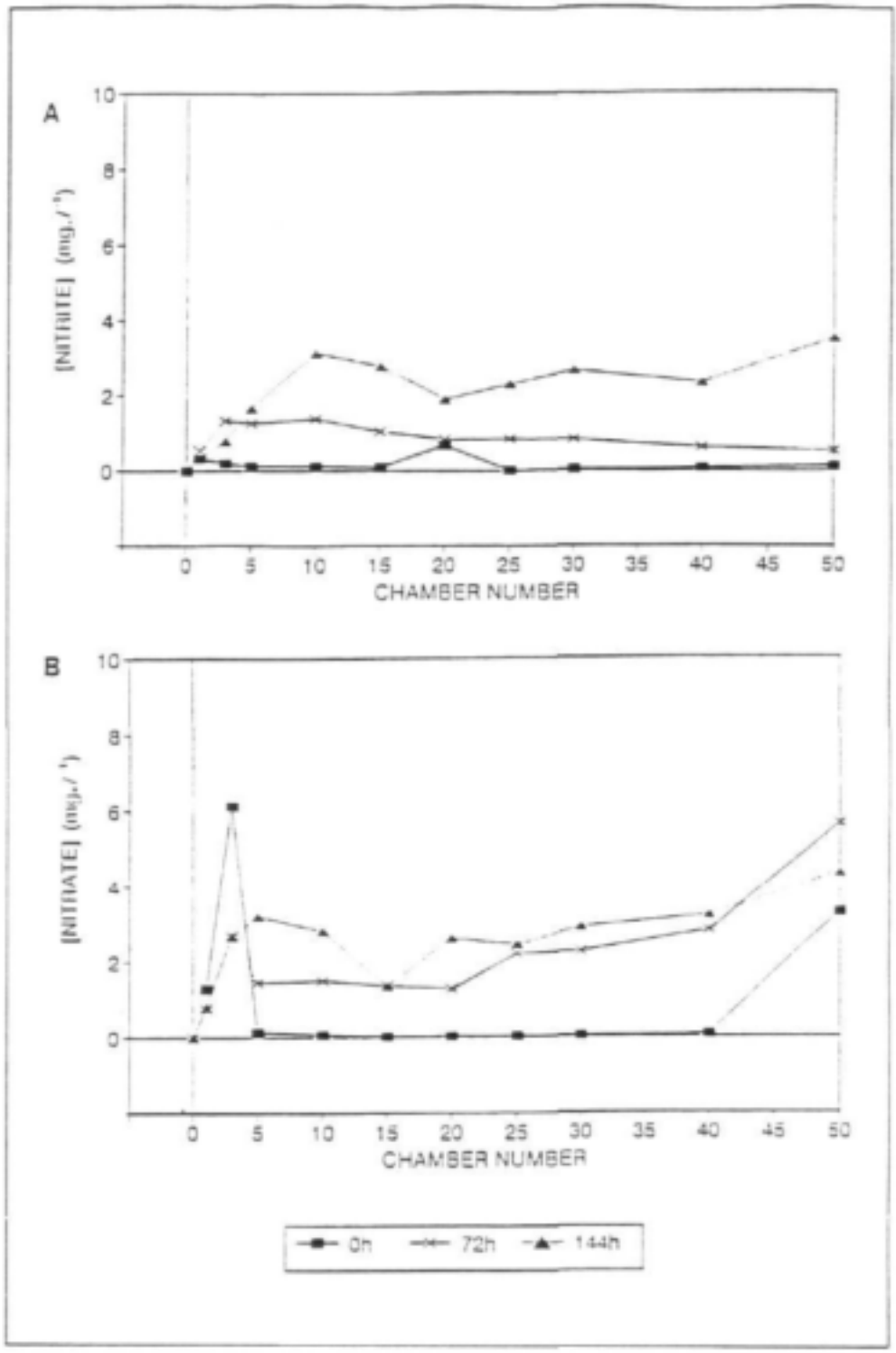


Figure 4.16 A and B Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 216-hour recovery period, subsequent to a 72-hour 2,4-dichlorophenol perturbation (20 mg./l).

It was found that phenol and 2,4-dichlorophenol both exhibited a temporary bacteriostatic effect and that nitrification activity recovered at a slow rate when these compounds were removed or diluted. This reinstatement of nitrification activity was consistent with the recovery of a small population of nitrifiers which were resistant or protected from the inhibitory phenol during the perturbation.

Impaired nitrification activity is of particular significance if one takes into consideration the impacts of a pollutant shock load on nutrient cycling processes within both wastewater treatment plants and aquatic environments. Inhibition of nitrifying activity can lead to prolonged recovery periods. With lowered nitrification rates this could have detrimental effects on nutrient removal processes and, thus, impair wastewater treatment efficiency. An important result of ammonia oxidizer inhibition is the expected increase in ammonia concentrations. Free ammonia is toxic to many aquatic organisms in relatively low concentrations (0.033 to 2.64 mg.l^{-1}) and can contribute to eutrophication of natural waters (Dallas and Day, 1993). Effluent standards for ammonia from the South African Department of Water Affairs and Forestry (DWAF)(1984) have been set at 10 mg.l^{-1}.

4.3 CONCLUSIONS

By use of the multi-stage laboratory model it was shown that inhibition of nitrification was a sensitive indicator of both phenol and 2,4-dichlorophenol toxicity. The fate of the perturbant compound within the multi-stage model was determined by direct analysis and the relationship between residual concentrations of the test compound and nitrifying activity could be assessed. Phenol, in concentrations of 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 10 and 20 mg.l^{-1} was found to persist within the model and inhibition of nitrification resulted. Biodegradation data with regards to a perturbant compound were, thus, considered an important requisite for assessing its potential impacts 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.

5. DEVELOPMENT AND TESTING OF A CHEMOAUTOTROPHIC NITRIFIER TOXICITY BIOASSAY

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. Inhibition of ammonia oxidation was chosen as the criterion for assessing toxicological impacts.

5.1 EXPERIMENTAL PROCEDURE

5.1.1 Enrichment/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. Ammonium sulphate was used as the sole energy source in the inorganic enrichment medium. 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 and was adjusted to the range 7-7.5 with sterile 5% (%) 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 (Spectroquant 14752, Merck).

5.1.2 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 perturbation experiments which were made over a 12-h period under batch culture conditions. 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. The overall phenol concentrations tested were 0.33, 3.33, 6.66 and 16.66 mg l^{-1}. The controls were diluted with 10 ml of distilled water. The ammonium concentrations were determined at times 0 and 12h. Each ammonium conversion was determined by calculating the difference in ammonium concentration at time 0 h and time 12 h and by expressing the value as a percentage of the ammonium concentration at time 0 h. The inhibitory effect of the perturbant molecule was then determined from the following equation:

$$\frac{(X_{CON}-X_{TEST}) \times 100}{X_{CON}} = \% \text{ inhibition} \qquad (5.1)$$

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.

5.2 RESULTS AND DISCUSSION

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

Figure 5.1 shows the course of ammonium conversion during the eight-week study period.

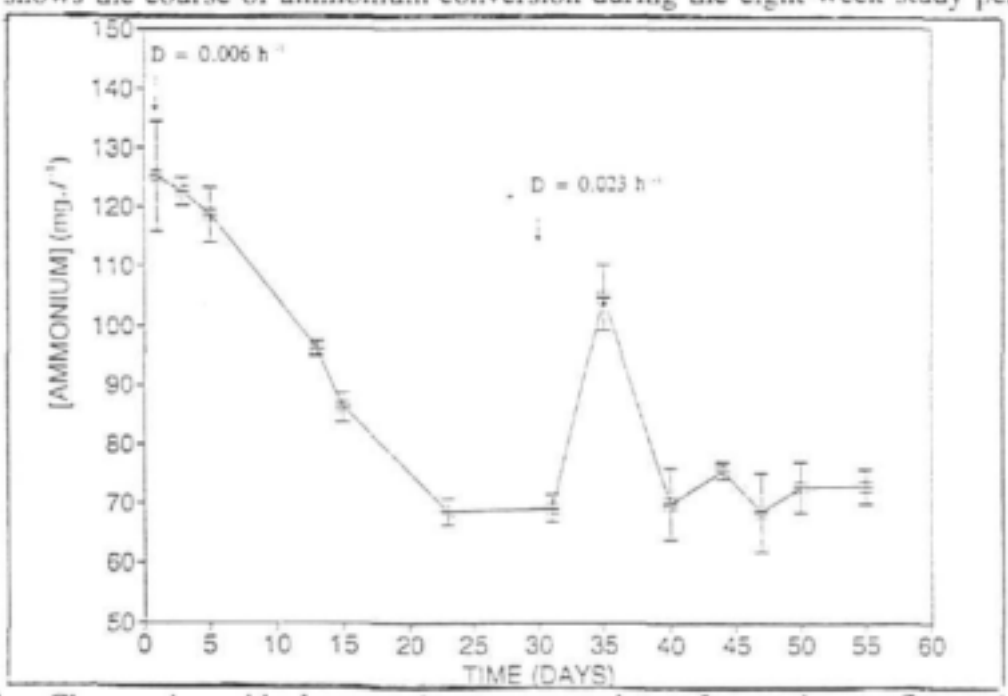


Figure 5.1 Changes in residual ammonium concentrations of a continuous-flow culture of nitrifying bacteria over an eight-week period. Data points are expressed as the means of triplicate samples. The vertical bars represent 95% confidence limits.

To facilitate nitrifier growth and prevent washout from the chemostat an overall dilution rate of 0.006 h^{-1} was initially chosen. 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.

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 because the substrate concentration increases. 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. 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 enables the test organisms to be available all year round 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 standardization of testing procedures.

5.2.2 Establishment of a 12-Hour 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 (Figure 5.2). The inhibitory effects of phenol on ammonia oxidation are presented graphically as percentage inhibitions (Figure 5.3).

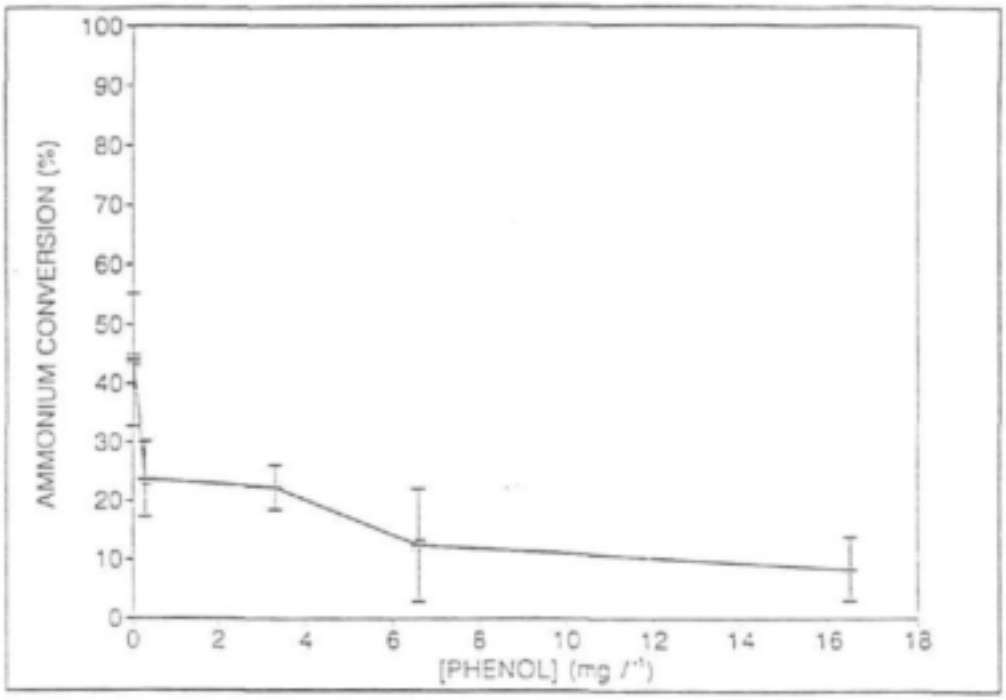


Figure 5.2 Ammonium conversions in the presence of increasing concentrations of perturbant phenol. Data points are expressed as the means of triplicate samples. The vertical bars represent 95% confidence limits.

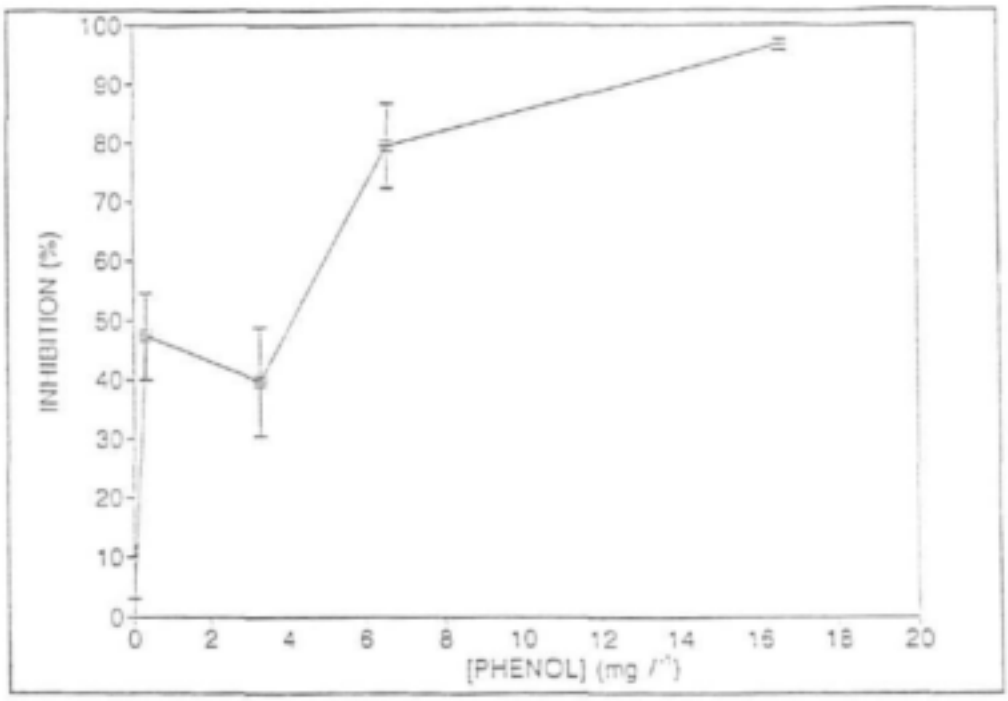


Figure 5.3 Percentage inhibition of ammonium conversion in response to increasing concentrations of phenol.

The intrinsic variabilities of replicates are represented in Figure 5.2 by the vertical bars which depict the 95% confidence levels. The results compared favourably with those derived from the multi-stage model where nitrification was found to be inhibited at phenol concentrations < 4 mg.l^{-1}.

Figure 5.3 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 mg.l^{-1} where a 47% inhibition was recorded. With 16.66 mg.l^{-1} phenol a 95% inhibition resulted and, thus, this concentration appeared to approach the threshold bactericidal concentration of ammonium oxidation. Taking into account the inherent variability of the results an EC_{50} in the range 0.33-4 mg.l^{-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, the replicates allow for statistical verification, and it is amenable to standardization.

5.3 CONCLUSIONS

With low overall dilution rates continuous culture can be used to enrich and isolate 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 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.

6. 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". The usefulness of a test system is usually based on its sensitivity and the representativeness of the ecosystem under consideration.

Microbial communities exhibit a structural and functional complexity which makes them useful in studying ecologically important elements such as species interactions, nutrient cycling and energy flow. Such properties have been considered as pertinent criteria for assessing ecotoxicological impacts. The aim of the research programme 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 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 will have 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. 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 but with the retention of microbial association integrity;

2) Selection pressures were kept constant which, theoretically, favoured the establishment of near steady-state conditions within the model. 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 and contributed 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 standardization 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 design and ultimately contributed to the impracticality of the model;

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 reductions in size and improved aeration delivery being important end criteria;

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 bioavailability 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 the 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 procedure;

8) Perturbation studies within the model were essentially found to be 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; and

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 South African Department of Water Affairs and Forestry (DWAF) recognises that biological monitoring (including ecotoxicological testing, bioassessment and bioaccumulation measurements) is needed in any comprehensive water quality monitoring programme (Roux *et al.*, 1993). Previously, national water quality monitoring in South Africa focused primarily on measuring physical and chemical variables and there has been a general lack of biological data in the water quality information systems. Developmental work in the field of biomonitoring is, therefore, encouraged by the DWAF and it has been suggested that such work should be consolidated from the collaborative efforts of resource mangers and researchers (Roux *et al.*, 1993).

Challenges that need to be addressed include:

1) Evaluating the feasibility of different biomonitoring approaches/techniques and their scope for potential application;
2) Developing the necessary infrastructure from which to implement these approaches/techniques; and
3) Integrating biological data with chemical and physical data in order to provide meaningful information to resource managers.

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 methodology for assessing ecotoxicological impacts will be used in preference to those that are structurally complex and require sophisticated and expensive equipment and skilled operators. 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.

6.1 FUTURE RESEARCH

1) Comparative studies have generally shown toxicity responses of bacterial bioassays are less sensitive than those of higher levels of biological organization (Yoshioka,1987; Arbuckle and Allemann, 1992). As a result, microbial bioassays have been regarded as useful tools for screening for toxicity rather than for establishing lower exposure limits for the environment. Further investigation is required to assess the sensitivity of the test protocol in relation to the toxicity responses of organisms from higher trophic levels.

2) There is relatively little information available on the toxic effects of perturbants on different groups of environmental bacteria (Dutka *et al.*, 1983; McFeters *et al.*, 1983; Bitton and Dutka, 1986; Blum and Speece, 1991). Nitrifiers have been identified as generally being 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 for monitoring effluent discharges. Extensive toxicity data exist with regards to nitrification inhibition in wastewater treatment processes (Tomlinson *et al.*, 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. 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.

3) *In situ* biomonitoring of nitrification has been suggested for routine monitoring of environmental impacts and 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 only appear to be suitable in environments which have high inputs of nitrogen/ammonium (e.g. wastewater treatment plants and polluted riverine systems).

4) The majority of data used to evaluate 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 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.

5) 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 use of these types of models, thus appears favourable for future ecotoxicological investigations.

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8. PUBLICATIONS

The following publications have resulted from the research project:-

1) HUNTER, CH. (1996) *Development of a Laboratory River Model to Determine the Environmental Impacts of Key Xenobiotic Compounds*. M.Sc. Thesis, University of Natal.

2) HUNTER, CH, SENIOR E, HOWARD, JR and BAILEY, IW. (1995) A multi-stage laboratory model for determining the impacts of anthropogenic substances on a microbial association found in aquatic ecosystems. *Water SA* 21 271-274.

3) LEHNERDT, RD, HUNTER, CH and SENIOR, E. (1995) Nitrification - an indicator/toxicity bioassay for determining the impacts of pollutant compounds in wastewater treatments and the aquatic environment. *South African Society for Microbiology (Natal), Eighth Annual Symposium, University of Zululand.*

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