The establishment and characterisation of a nitrifier population in a continuous-flow multi-stage model used to study microbial growth and interactions inherent in aquatic ecosystems

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Abstract

The enrichment, isolation and establishment of a population of nitrifiers within a continuous-flow multi-stage model is described. The establishment and, subsequent, spatial separation of the components of a nitrifier population within the model appeared to be dependent on: specific growth rate/overall dilution rate, as dictated by the cumulative chamber volume, at various points along the model; and the interactions between the micro-organisms and the prevailing environmental conditions. With time, nitrifying activity appeared to be growth-rate independent and true steady-state conditions were not achieved due to the presence of mixed microbial populations and the gradual accumulation of flocculant biomass and biofilms which facilitated increased nitrifying activity. These factors have important implications for subsequent perturbation studies which must be made before the various processes of nitrification become consolidated in the same space.

Introduction

Laboratory model micro-ecosystems have been developed to study microbial growth and interactions under *in situ* conditions (Wimpenny, 1988). They have been used to: provide simple analogues of the natural ecosystems in which characteristic structural and functional properties can be simulated and examined (Porcella et al., 1982); and determine the potential impacts of xenobiotic compounds under simulated *in situ* conditions (Porcella et al., 1982; Pratt et al., 1990; Freitsch, 1991; Scholz and Müller, 1992).

Cairns et al. (1992) proposed that microbial communities offer a number of advantages over using organisms from higher trophic levels for ecotoxicological testing. These include: the significant role they play in ecosystem dynamics; their ubiquitous nature; their sensitivity to anthropogenic stress; and a minimisation of scale effects. Various microbially-mediated processes such as organic matter decomposition, nitrogen transformations, sulphate reduction and methanogenesis have all been considered (Bitton and Dutka, 1986; Blum and Speece, 1992). Nitrification, incorporating specialised chemoautotrophic nitrifiers, has been shown to be particularly sensitive to xenobiotic perturbations and several bioassays have accordingly been developed (Williamson and Johnson, 1981; Powell, 1986; Blum and Speece, 1991).

The nitrogen cycle is pivotal in the regulation of water quality and usually maintains a balanced state in unperturbated aquatic environments (Welch, 1992). Disruption of this cycle can result from severe organic loading (substrate inhibition) and/or inputs of inimical pollutant compounds from agricultural, domestic or industrial sources (Dallas and Day, 1993). Inhibition of microbial transformation processes such as nitrification and denitrification can lead to accumulation of toxic intermediates such as ammonia, nitrite and nitrate (Welch, 1992). Elucidation of the factors controlling and inhibiting these regulatory processes is, therefore, extremely important in understanding and predicting the potential environmental impacts of perturbant compounds.

Nitrification has been defined as the biological conversion of reduced nitrogen (ammonia) to nitrite or nitrate (Alexander, 1977). Two distinct groups of chemolithotrophic bacteria, namely, the ammonia and nitrite oxidisers are thought to be primarily responsible for nitrification in the environment (Underhill, 1990). These bacteria have been grouped into the family Nitrobacteriaceae which comprise Gram-negative organisms categorised by their shape, size, arrangement of cytoplasmic membrane, DNA base ratios and metabolic capabilities (Underhill, 1990; Brock et al., 1991). *Nitrosomonas* (ammonia oxidisers) and *Nitrobacter* (nitrite oxidisers) are the two most commonly studied genera (Brock et al., 1991). Various heterotrophic micro-organisms, including bacteria (actinomycetes), algae and fungi have also been reported to have nitrifying activity (Focht and Verstraete, 1977). Since autotrophic nitrification usually occurs at higher rates than heterotrophic nitrification it is thought to play the more important role in nature (Kuenen and Robertson, 1988).

To facilitate examination of the environmental impacts of selected xenobiotic pollutants on fundamental cycling processes inherent to aquatic ecosystems, a multi-stage laboratory model was developed (Hunter, 1996). Particular emphasis was placed on establishing a population of nitrifying bacteria within the model with the subsequent aim of subjecting the resident microbial populations to perturbation testing with selected xenobiotic compounds.

This paper describes the enrichment, isolation and establishment of a population of nitrifiers within the multi-stage model. Qualitative and quantitative characterisations were made to determine the course of nitrification within the model. The significance of surface-attached (growth-rate independent) growth to the process of nitrification was assessed. Use of the multi-stage

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model as a sensitive tool for ecotoxicological investigations is proposed.

Materials and methods

Laboratory model configuration and operation

A full description of the model has been given previously (Hunter et al., 1995). The model had four identical channels (3 m in length, 36 mm wide and 95 mm deep) each consisting of 75 chambers. Each chamber had an operational volume of 122 ml (36 x 36 x 95 mm). Duplicate channels were constructed from Plexiglass and built-in unit blocks 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 facilitate mixing of nutrient medium within the individual chambers.

The model was operated as an open culture system with the influent medium pumped (Watson-Marlow 503U) into the first chamber of each channel. The medium flowed from one chamber to the next in weir fashion down the course of the array.

To limit variables, the model was operated under conditions of constant darkness and temperature (20°C ± 2°C). Air was bubbled continuously into each chamber via a system of interconnected aeration tubes (i.d. 3.5 mm) connected to an industrial blower (Regenair R2103, Gast Corp.).

Medium

A non-selective growth medium (pH 7.3) contained the following (mg.l^{-1} glass-distilled water): yeast extract, 10; proteose peptone, 10; casamino acid, 10; glucose, 10; soluble starch, 10; sodium pyruvate, 6; NaCl, 5.2; $CaCl_2$, 3; $MgSO_4$, 1.5; and $K_2HPO_4.3H_2O$, 21 and was sterilised by autoclaving (15 KPa, 121°C, 15 min).

Inoculum

The inoculum used was the same as that described previously (Hunter et al., 1995). Enrichment/isolation of the component heterotrophs and nitrifiers was achieved within the model after adding 1 l fibreglass wool-filtered inoculum to 9 l of growth medium prior to charging each channel via the first chamber at a dilution rate of 0.5 h^{-1}. The model was maintained as a batch culture overnight before initiating continuous flow.

A flow rate of 100 ml·h^{-1} was chosen to facilitate temporal and spatial separation of the component species of the inoculum. The dilution rate for each individual chamber was 0.82 h^{-1}.

Establishment of "steady-state" nitrification

Two channels (A and B) were operated as open cultures for 7 weeks during which samples were taken every week after an initial stabilisation period (from Week 2), to determine nitrifying activity. The first 45 chambers of each channel were used and the samples were taken from Chambers 1,3,5,10,15,25,35 and 45.

Characterisation of nitrification

Qualitative and quantitative characterisation of nitrification within the multi-stage model were determined by monitoring ammonia, nitrite and nitrate concentrations and pH.

Photometric analyses (Spectroquant Photometer, Merck), in conjunction with reagent kits, were used to quantify ammonium ion, nitrite and nitrate concentrations. Reagent kits (Spectroquant 14752, 14773 and 14776, Merck) were specific for ammonium (0.03 to 3.0 mg·l^{-1}), nitrite (0.03 to 3.0 mg·l^{-1}) and nitrate (1 to 90 mg·l^{-1}), respectively.

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

Nitrifying bacterial populations: free living or surface attached?

Following establishment and analysis of "steady-state" nitrification, Channel A was flushed with 12 l of sterile distilled water at an overall dilution rate of 0.5 h^{-1} to displace the free-living culture. The influent nutrient medium was then reinstated at the original flow rate. Samples for nitrification analysis were taken one week later, after the channel had undergone two complete culture volume changes, to determine if nitrifying activity had been reinstated.

Enrichment/isolation of autotrophic nitrifying bacteria

Batch cultures were used to isolate autotrophic nitrifying bacteria from the multi-stage model. Ammonium oxidisers were selected with ammonium sulphate as the sole energy source in a mineral salts medium (Soranio and Walker, 1968) while sodium nitrite was used to isolate the nitrite oxidisers (Smith and Hoare, 1968).

After the process had been operational for 7 weeks a 10 ml sample was taken from Chamber 10 of Channel A. The sample was vortexed in a test tube for 30 s to effect mixing, disperse cell aggregates and detach adhered micro-organisms. The sample was then serially diluted (10^{-2}, 10^{-4} and 10^{-5}) with quarter-strength Ringers solution. A 10% ($^{v}/_{v}$) inoculum from each dilution was then added to individual Erlenmeyer flasks (250 ml) which contained 100 ml of enrichment medium for isolating ammonium- or nitrite-oxidising autotrophs. The cultures were incubated aerobically at 27° C in the dark and shaken (150 r·min^{-1}) in a rotary shaker incubator (New Brunswick Scientific Co., Inc.). Samples (2 ml) were taken on a weekly basis for three weeks and analysed for the presence of nitrite or nitrate.

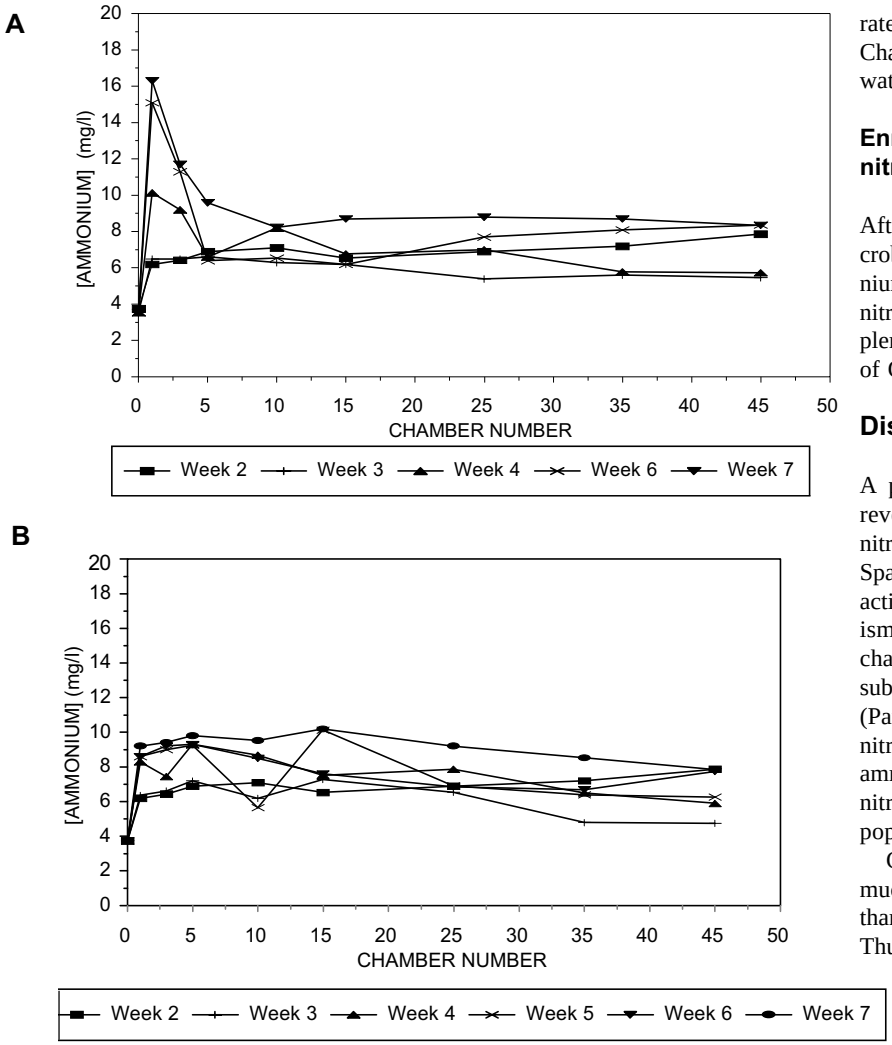
Results

Important environmental factors which affect the rate of nitrification are light intensity, dissolved oxygen (DO) concentration and temperature.

The multi-stage model was operated under constant dark conditions to facilitate nitrification, which has been shown to be photosensitive (Hooper and Terry, 1973; Yoshioka and Saijo, 1985), and to prevent algal growth. In aquatic environments, nitrification is associated with regions which are not readily exposed to light such as aerobic layers of sediment. Nitrate is readily assimilated by algae and thus poses a potential interference through nitrate assimilation.

Nitrifying micro-organisms have an obligate requirement for oxygen and inhibition occurs under anoxic conditions (Stensel and Barnard, 1992). The half-saturation constant (K_m) for oxygen has been found to range from 0.15 to 1 mg·l^{-1} (Goreau et al., 1980). To ensure that excess DO was present each chamber was aerated. Oxygen limitation was thought to be a potential problem only when the aeration tubes became blocked by biomass which effected localised microaerophilic regions.

The temperature limits for nitrification have been reported to



Figures 1 A and B

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

be between 5 and 40°C (Underhill, 1990) with optimal growth rates recorded between 25 and 30°C (Loveless and Painter, 1968). As a consequence, 25°C was chosen for this study.

Changes in ammonium, nitrite and nitrate concentrations, which were indicative of nitrifying activity in discrete chambers of Channels A and B, during the 7-week study period are shown in: Figs. 1 A and B (ammonium); Figs. 2 A and B (nitrite); and Figs. 3 A and B (nitrate). Figure 4 shows the changes in pH values recorded in selected chambers of Channel A after continuous cultivation of the microbial association for one month.

After 5 weeks it was found that the nozzle of the influent medium for Channel A had been displaced and was effectively feeding into Channel B. It was, however, estimated that Channel A had been in batch mode for < 48 h. Due to this problem, Channel A was not sampled (Week 5) until after two further complete culture volume changes had occurred.

Free-living/surface-attached nitrifiers

To resolve the question of whether the dominant nitrifiers were free-living (growth-rate dependent) or surface-attached (growth-rate independent), nitrification was monitored in Channel A before and after flushing with distilled water (Figs. 5 A and B).

Enrichment/isolation of autotrophic nitrifiers

After three weeks of batch culture incubation, microbial biomass was discernible within the ammonium- and nitrite-supplemented cultures. Assays for nitrite and nitrate confirmed oxidations of the supplements while Gram stains identified the presence of Gram-negative rods in both cultures.

Discussion

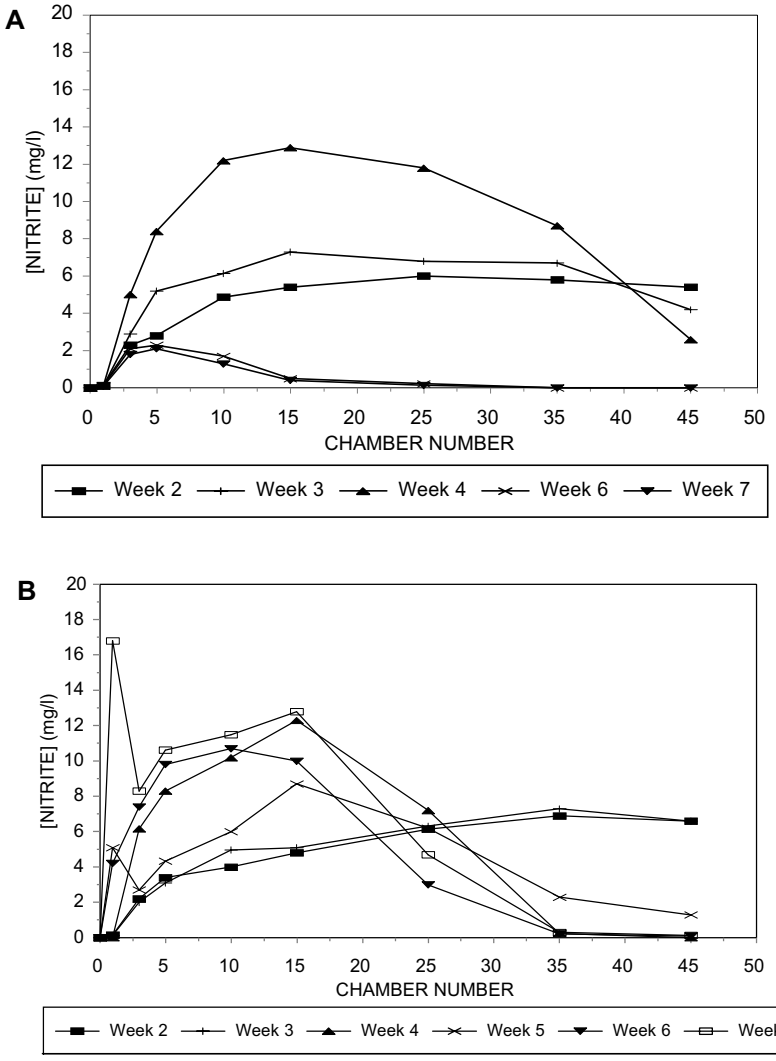
A preliminary investigation (Hunter et al., 1995) revealed that a microbial association containing nitrifiers established within the multi-stage model. Spatial separation of heterotrophic and nitrifying activity also resulted. Heterotrophic micro-organisms were expected to be selected in the early chambers of each channel on the basis of high substrate affinities and high specific growth rates (Parkes, 1982). The subsequent mineralisation of nitrogenous components of the medium released ammonia prior to biological oxidation to nitrate via nitrite and led to the establishment of nitrifying populations.

Characteristically, autotrophic nitrifiers have much lower maximum specific growth rates (μ_{max}) than mixed cultures of heterotrophs (Bitton, 1994). Thus, nitrification should have consolidated in regions of the model subjected to low overall dilution rates. To facilitate this, nitrogen concentrations of the influent medium must be in excess of the requirements of the heterotrophs (Gray, 1990). Only low concentrations of ammonium-nitrogen are assimilated into heterotrophic biomass. The catabolism of nitrogenous compounds determines the rate at which ammonium is made available for oxidation and is, thus, an important controlling factor in the establishment of an active nitrifying population.

Figures 1 A and B show the ammonium concentrations within Channels A and B during the study period. The general trend for both channels, at each sampling time, was that the first two to five chambers were characterised by elevated concentrations with the subsequent concentrations relatively unchanged. Possible explanations for this residual ammonium include: deficiencies of key nutrients; low K_N saturation constants for ammonium oxidation (K_N 0.5 to 2.0 mg·l^{-1}) (Bitton, 1994); and/or inhibition of ammonium oxidation due to a lowered pH.

In Channel A at Weeks 6 and 7 ammonium peaks were recorded in Chamber 1 which suggested that increased ammonification activity was operative. These coincided with the interrupted medium flow but were maintained even after continuous flow had been reinstated. This phenomenon could, possibly, be explained by the establishment of a larger population of heterotrophic ammonifiers during the batch culture mode.

Ammonium concentrations at discrete points along each channel are regulated by factors such as mineralisation of nitrogenous compounds, incorporation of ammonium-nitrogen into



Figures 2 A and B

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

heterotrophic biomass and the non-assimilative use of nitrogen as an energy source during nitrification. With a carbon to nitrogen loading ratio < 2:1 a small proportion of ammonium-nitrogen would be expected to be assimilated into heterotrophic biomass. Biological oxidation of ammonium to nitrate via nitrite was thus thought to be the main regulatory factor of ammonium concentrations within the model.

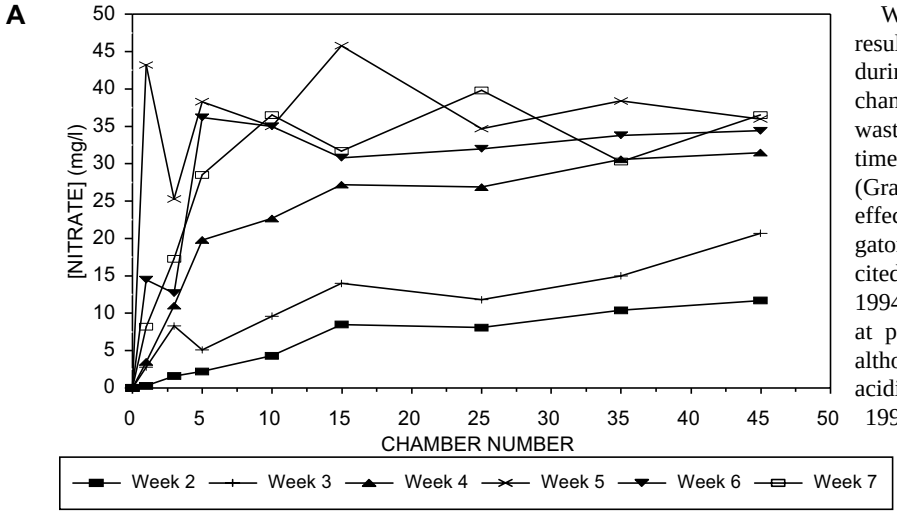
The course of nitrite metabolism is shown in Figs. 2 A and B. The results of Weeks 2 and 3 showed that initial increases in nitrite concentrations occurred in the first 5 to 10 chambers of each channel, thus indicating the establishment of ammonium oxidisers. At Week 4 increased concentrations of nitrite up to Chamber 15 were apparent which were followed by decreased nitrite concentrations indicative of nitrite oxidation. At Week 5, Channel B, effectively, was subjected to an increased flow rate due to the consolidation of the two influents. This appeared to effect lower concentrations of nitrite but these were reinstated during Weeks 6 and 7. In Week 7, the isolated increase in nitrite concentration in Chamber 1 of Channel B was thought to be related to flow impairment due to the development of microbial growth at the surface of the chamber. Channel A showed an interesting change with the reinstatement of flow after Week 5 since comparatively low concentrations of nitrite were recorded (Weeks 6 and 7), particularly from Chamber 15 onwards which suggested that concomitant oxidation of nitrite to nitrate had occurred (Figs. 3 A and B) due to the establishment of nitrite oxidisers.

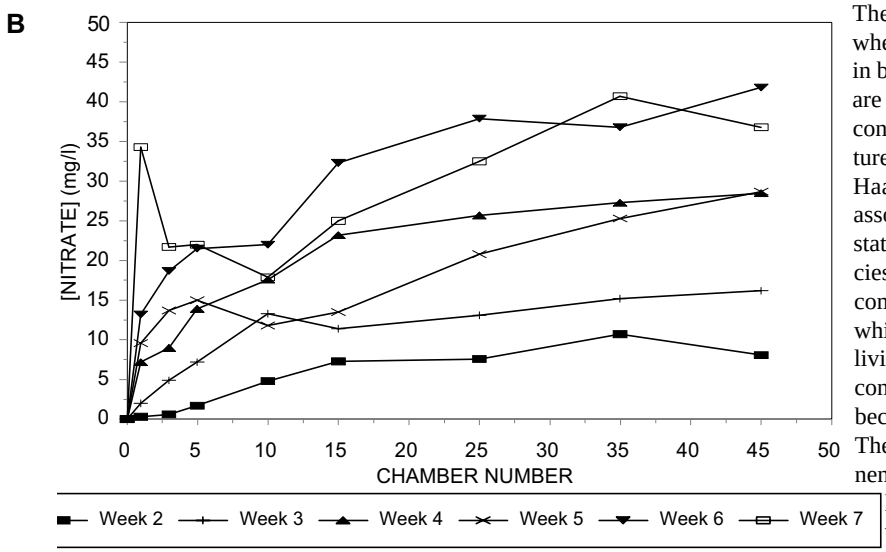
Figures 3 A and B show the course of nitrite oxidation within the same channels with increased nitrate concentrations indicative of the presence of nitrite oxidisers. Initially, substrate availability (nitrite) appeared to limit nitrite oxidiser establishment (Weeks 2 and 3). Up to Week 4 the duplicate channels gave comparable results. At Weeks 6 and 7, ammonium and nitrite oxidation in Channel A were differentiated temporally and spatially along the course of the channel probably due to the specific growth rates of the oxidative populations. In Channel A at Weeks 6 and 7, the nitrate concentrations stabilised in Chambers 5 to 10 onwards indicating the consolidation of nitrite oxidation activity.

Startup of nitrifying systems is often characterised by nitrite accumulation until nitrite oxidising populations reach equilibrium (Stensel and Barnard, 1992). This is attributed to ammonium oxidation generating more energy (66 to 84 kcal·mole^{-1} of ammonium) than nitrite oxidation (17.5 kcal·mole^{-1} of nitrite) (Painter, 1970). The ammonium oxidisers achieve higher cell yields and thus greater biomass production (Stensel and Barnard, 1992; Bitton, 1994). Thus, in nitrifying environments, ammonium oxidisers have been found in higher numbers than nitrite oxidisers (Gray, 1990). However, when populations of nitrifying bacteria establish under steady-state conditions it is often found that the nitrite concentrations are low (Gray, 1990) due to its oxidation to nitrate which is more rapid than the preceding oxidation (Stensel and Barnard, 1992). The oxidation of ammonium to nitrite is, thus, considered an important rate-limiting step in the process of nitrification (Gray, 1990).

This study demonstrated the slow rates at which the nitrifying populations established in the model. Maximum specific growth rates of nitrifiers from 0.023 to 0.057 h^{-1} have been reported in the literature (Underhill, 1990; Bitton, 1994) and have confirmed the protracted times ($\geq$6 weeks) required by nitrifiers to reach steady-state conditions in activated sludge (Gray, 1990).

Despite identical design and operation, the two channels differed considerably with regards to regions of nitrifying activity and nitrite accumulation. These discrepancies can be attributed to the interruption in flow to Channel B during Week 5. Under such enforced batch culture conditions, increased amounts of nitrifier biomass would be expected to develop in the early chambers, usually operated at dilution rates $>\mu_{max}$, and elevated nitrifying activity should result provided that biofilms at the weir overflows reinoculated the chambers. It is possible that with time progressive biofilm development could have effected consolidation of ammonium and nitrite oxidation in the same space thus eliminating spatial/temporal separation of the two oxidative steps. The efficacy of this model is, therefore, restricted to short-term studies.





Figures 3 A and B

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

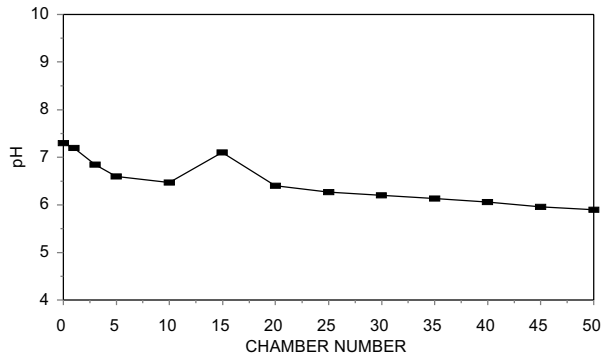


Figure 4

pH values recorded in selected chambers of Channel A of the multi-stage model after maintenance under continuous-flow conditions for one month

With the onset of nitrification a drop in pH resulted (Fig. 4) due to the release of protons (H^+) during the oxidation of nitrite to nitrate. Similar pH changes have been reported for closed systems, wastewater treatment processes with long retention times, and in waters with low buffering capacity (Gray, 1990; Underhill, 1990). The documented effects of pH on nitrification vary between investigators. pH ranges between 7.0 and 8.4 have been cited as optimal (Gray, 1990; Underhill, 1990; Bitton, 1994). In general, inhibition of nitrification occurs at pH values of below pH 6 (Underhill, 1990) although acclimation of nitrifying populations to acidic conditions has been reported (Antoniou et al., 1990; Underhill, 1990).

Nitrifying bacterial populations: Free-living or surface-attached?

The investigation was terminated after 7 weeks when "steady-state" conditions were approximated in both channels. In practice, steady-state conditions are reached when the biomass or residual substrate concentration remains constant for at least two culture volume changes (Middelbeek and Drijver-de Haas, 1992). A feature of interactive microbial associations is their inability to establish true steady states in continuous culture. Non-competitive species may be displaced, mutation may change the community structure, and wall growth may develop which may be physiologically dissimilar to the free-living community (Parkes, 1982). Near steady-state conditions are reached after a stable oscillating state becomes less pronounced with time (Senior, 1977). These oscillations have been attributed to component species competition for a common growth-limiting substrate with no species having a selective advantage. Small changes in culture conditions can cause considerable fluctuations in biomass and its activity (Parkes, 1982). Therefore, interactive microbial associations in continuous culture are in continuous transient growth phases.

Figures 5 A and B show the spatial separation of nitrifier activity which suggested that the populations were free-living. However, since nitrification occurred at discrete points along the channel (Fig. 5B) where the dilution rates were greater than the μ_{max} values cited in the literature, surface-attached populations were implicated and this was confirmed by flushing with sterile distilled water after which the oxidations were quickly reinstated. This observation was supported by reports that nitrifiers readily attach to surfaces (flocculent biomass aggregates) in activated sludge plants (Gray, 1990). Such attachment has also been reported to increase nitrifying activity and to promote the efficiency of a nitrifying system (Diab and Shilo, 1988).

Isolation of the component nitrifiers of the cultured association was undertaken and confirmed that autotrophic populations were responsible for the transformations recorded.

Since ecologically critical processes, namely, organic molecule catabolism, ammonification of organic nitrogen and nitrification, were differentiated, the following applications of the model are proposed:

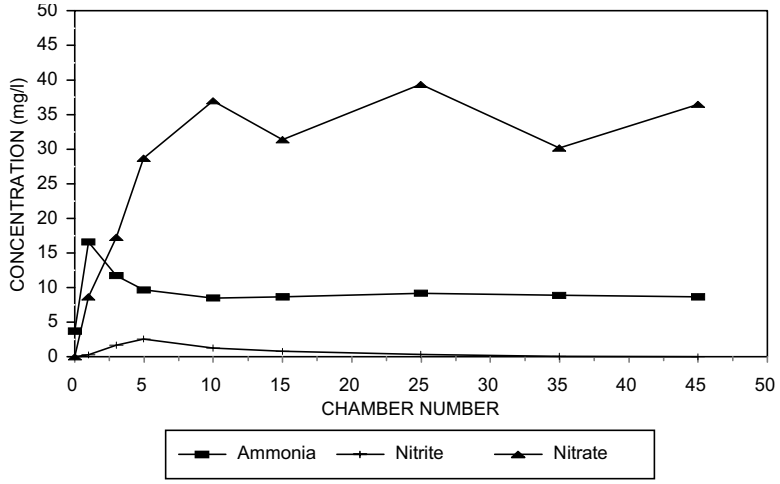


Figure 5A
Course of nitrification along the multi-stage laboratory model prior to flushing with sterile distilled water

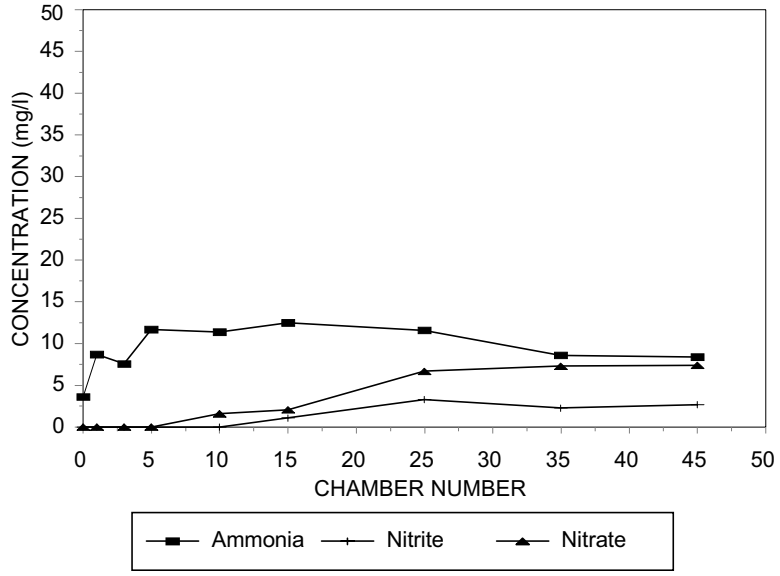


Figure 5B
Course of nitrification along the multi-stage laboratory model subsequent to flushing with sterile distilled water

- Evaluating environmental impacts (perturbation and recovery) of anthropogenic substances; and
- Determining the environmental fates of xenobiotic compounds and their susceptibility to biodegradation.

Conclusions

The establishment and, subsequent, spatial separation of the components of the microbial association within the model were, primarily, dependent on: the specific growth rate/overall dilution rate, as dictated by the cumulative chamber volume, at various points along the model; and the interactions between the microorganisms and the prevailing selection pressures.

With time, nitrifying activity appeared to be growth-rate independent and was identified as rate-limiting for the establishment of near steady-state conditions. True steady-state conditions were not achieved due to the presence of heterogeneous microbial populations and the gradual accumulation of flocculent biomass and biofilms which facilitated increased nitrifying activity.

These factors have important implications for subsequent perturbation studies which must be made before the various processes of nitrification become consolidated in the same space.

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References

ALEXANDER M (1977) *Introduction to Soil Microbiology*. Wiley, New York. 153-179.

ANTONIOU P, HAMILTON J, KOOPMAN B, JAIN B, HOLLOWAY B, LYBERATOS G and SVORONOS, SA (1990) Effect of temperature and pH on the effective maximum specific growth rate of nitrifying bacteria. *Water Res.* **24** 97-101.

BITTON G (1994) *Wastewater Microbiology*. Wiley-Liss, New York.

BITTON G and DUTKA BJ (1986) *Toxicity Testing Using Microorganisms, Vol.1* CRC Press, Boca Raton, Florida, 1-19.

BLUM DJ and SPEECE RE (1991) A database of chemical toxicity tests to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. Water Pollut. Control Fed*. **63** 198-207.

BLUM DJ and SPEECE RE (1992) The toxicity of organic chemicals to treatment processes. *Water Sci. Tech.* **25** 23-31.

BROCK E, KOOPS H-P, AHLERS B and HARMS H (1991) Oxidation of inorganic nitrogen compounds as an energy source. In: Balows A, Trüper HG, Dworkin M, Harder M and Schleifer K-H (eds.) *The Prokaryotes* (2nd edn.) Springer-Verlag, New York. 414-430.

CAIRNS J, MC CORMACK PV and NIEDERLEHNER BR (1992) Estimating ecotoxicological risk and impact using indigenous aquatic microbial communities. *Hydrobiologia* **237** 131-145.

DALLAS HF and DAY JA (1993) The Effect Of Water Quality Variables On A Riverine Ecosystem: A Review. Project Report 351. Water Research Commission, South Africa. 21-31.

DIAB S and SHILO M (1988) Effect of adhesion to particles on the survival and activity of *Nitrosomonas* sp. and *Nitrobacter* sp. *Arch. Microbiol.* **150** 387-393.

FOCHT DD and VERSTRAETE W (1977) Biochemical ecology of nitrification and denitrification. *Adv. Microbial Ecol.* **1** 135-214.

FREITSCH G (1991) Effects and degradation of 4-chloro-3-cresol in a system of natural self-purification. *Water Res.* **25** 717-727.

GOREAU TJ, KAPLAN WA, WOFSY SC, MCELROY MB, VALOIS FW and WATSON SW (1980) Production of nitrite and N_2O by nitrifying bacteria at reduced concentrations of oxygen. *Appl. Environ. Microbiol.* **40** 526-532.

GRAY NF (1990) *Activated Sludge: Theory and Practice*. Oxford University Press, Oxford. 153-176.

HOOPER AB and TERRY KR (1973) Specific inhibitors of ammonia in *Nitrosomonas. J. Bact.* **115** 480-485.

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

HUNTER CH, HOWARD JR, BAILEY IW and SENIOR E (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.

KUENEN JG and ROBERTSON LA (1988) Ecology of nitrification and denitrification. In: Cole JA and Ferguson SJ (eds.) *The Nitrogen and Sulphur Cycles.* 42nd Symp. of the Soc. for Gen. Microbiol., Cambridge University Press, Cambridge. 161-218.

LOVELESS JE and PAINTER HA (1968) The influence of metal ion concentration and pH value on the growth of a *Nitrosomonas* strain isolated from activated sludge. *J. Gen. Microbiol.* **52** 1-14.

MIDDELBEEK EJ and DRIJVER-DE HAAS JS (1992) Environmental factors influencing growth. In: Cartledge TG (ed.) *In Vitro Cultivation of Microorganisms.* Butterworth Heinemann, Oxford. 145-176.

PAINTER HA (1970) A review of literature on inorganic nitrogen micro-organisms. *Water Res.* **4** 393-402.

PARKES RJ (1982) Methods for enriching, isolating and analysing microbial communities in laboratory systems. In: Bull AT and Slater JH (eds.) *Microbial Interactions and Community Structure*. Academic Press, London. 45-102.

PORCELLA DB, ADAMS VD, MEDINE AJ and CONWAN PA (1982) Using three phase aquatic microcosms to assess fate and impacts of chemicals in microbial communities. *Water Res.* **16** 489-496.

POWELL SJ (1986) Studies of inhibition of nitrification. In: Prosser JI (ed.) *Nitrification: Special Publications of the Society for General Microbiology, Vol. 20* IRL Press, Oxford. 79-97.

PRATT JR, BOWERS NJ and CAIRNS J (1990) Effect of sediment on estimates of diquat toxicity in laboratory microcosms. *Water Res.* **24** 51-57.

SENIOR E (1977) Characterisation of a Microbial Association Growing on the Herbicide Dalapon. Ph.D. Thesis, University of Kent, United Kingdom.

SCHOLZ N and MÜLLER FJ (1992)The riverine biocoenosis model (aquatic stair case model) A test system for determining the ecotoxicity and biodegradation under reality-approximate riverine conditions. *Chemosphere* **25** 563-579.

SMITH AJ and HOARE DS (1968) Acetate assimilation by *Nitrobacter agilis* in relation to its "obligate autotrophy". *J. Bacteriol.* **95** 844-855.

SORANIO S and WALKER N (1968) Isolation of ammonia-oxidizing autotrophic bacteria. *J. Appl. Bact.* **31** 493-497.

STENSEL HD and BARNARD JL (1992) Principles of biological nutrient removal. In: Randal CW, Barnard JL and Stensel HD (eds.) *Design and Retrofit of Wastewater Treatment Plants for Biological Nutrient Removal.* Technomic Pub. Co. Inc., Lancaster. 25-45.

UNDERHILL SE (1990) Techniques for studying the microbial ecology of nitrification. In:Grigorova R and Norris JR (eds.) *Methods in Microbiology, Vol. 22*. Academic Press, London. 417-445.

WELCH EB (1992) *Ecological Effects of Wastewater*. Chapman and Hall, London. 54-76.

WILLIAMSON KJ and JOHNSON DG (1981) A bacterial bioassay for assessment of wastewater toxicity. *Water Res.* **15** 383-390.

WIMPENNY JWT (1988) *Handbook of Laboratory Model Systems for Microbial Ecosystems, Vol. 1*. CRC Press, Boca Raton, Florida. 1-18.

YOSHIOKA T and SAIJO Y (1985) Active nitrification in hypolimnion of Lake Kizaki in early summer: Effects of light on nitrification in water. *Arch. Hydrob.* **105** 1-9.