Assessment of phenolic compound perturbations of a nitrifier microbial association maintained within a continuous-flow multi-stage laboratory model

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Abstract

To facilitate rapid examination of the environmental impacts of selected molecules on cycling processes inherent to aquatic ecosystems, a multi-stage model ecosystem was developed.

Nitrification was chosen as the criterion for assessing the inhibitory effects of perturbant molecules on a microbial association maintained within the model. Inhibition of nitrification proved to be a sensitive indicator of both phenol and 2,4-dichlorophenol toxicity. Perturbant compound fate was determined by direct analysis and the relationship between residual concentration and nitrifying activity was assessed. Phenol, in concentrations of 20 and 60 mg·ℓ^{-1}, attenuated within the model and it was apparent that low concentrations (<4 mg·ℓ^{-1}) must be reached before nitrification proceeded. 2,4-dichlorophenol in concentrations of 10 and 20 mg·ℓ^{-1} was found to persist and inhibition of nitrification resulted. Biodegradation data of perturbant compounds were, thus, considered important requisites for assessing potential impacts on aquatic environments.

Introduction

Laboratory model micro-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; Hunter et al., 1995). Ranging in size, configuration and complexity, laboratory models have sought to provide simple analogues of natural ecosystems in which characteristic structural and functional properties can be simulated (Wimpenny, 1988).

Since micro-organisms play an integral role in ecosystem dynamics, evaluation of the effects of pollutants on mineral and nutrient cycles has been proposed as a means of assessing the impacts of compounds released into the environment (Bitton and Dutka, 1986; Cairns et al., 1992). Organic matter decomposition, nitrogen transformations, sulphate reduction and methanogenesis have all been considered (Blum and Speece, 1992). The premise is made that inhibition of micro-organism-mediated processes will have a direct bearing on the functioning of an ecosystem as a whole and will, therefore, reflect aspects of "ecosystem health".

To facilitate examination of environmental impacts of selected xenobiotic compounds on cycling processes inherent to aquatic ecosystems, a multi-stage model ecosystem was developed (Hunter, 1996). A primary aim was to establish a population of nitrifying bacteria within the model, as these species have been recognised as sensitive and rapid indicators of ecotoxicological perturbation (Williamson and Johnson, 1981; Blum and Speece, 1992).

In unperturbated aquatic environments the nitrogen cycle usually maintains a balanced state (Welch, 1992). Microbial transformations such as ammonification, nitrification and denitrification are the regulating mechanisms which contribute to the overall self-purification capacities of an aquatic environment. Disruption or inhibition of these transformations can result from severe organic loading (substrate inhibition) and/or toxic pollutant compounds and can lead to the accumulation of intermediates such as ammonia, nitrite and nitrate (Dallas and Day, 1993). All of these latter molecules have been found to be either toxic or to impair water quality when allowed to accumulate. Elucidation of the factors controlling and inhibiting these regulatory processes is thus important in understanding and predicting the potential impacts of perturbant compounds on aquatic environments.

To test the efficacy of the model ecosystem for rapid ecotoxicological assessments 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 model. Nitrification was chosen as the criterion for assessing the inhibitory effects of the selected molecules on the established microbial association and was monitored by nitrite and nitrate concentration analyses. Residual phenol and 2,4-dichlorophenol concentrations were assayed to determine their fates within the model.

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

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influent medium within individual chambers.

The model was operated as an open-flow system with the influent medium pumped (Watson-Marlow 503U) into the first chamber of each channel. The medium then 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

The non-selective growth medium (pH 7.3) contained the following ($mg \cdot \ell^{-1}$ 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; K_2HPO_4. $3H_2O$, 21 and was sterilised by autoclaving (15 KPa, 121°C, 15 min).

Inoculum

Water samples taken from various points along the Umzindusi River in the Pietermaritzburg, KwaZulu-Natal region were used as the inoculum (Hunter et al., 1995). Enrichment/isolation of the component microflora 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 (Hunter et al., 1998).

A flow rate of 100 $m\ell \cdot h^{-1}$ 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}.

Nitrification characterisation

The nitrification processes occurring within the model were characterised qualitatively and quantitatively by monitoring nitrite and nitrate concentrations (Hunter et al., 1998).

Phenol and 2,4-dichlorophenol determination

Phenol and 2,4-dichlorophenol analyses were done with a high pressure liquid chromatograph (Waters 600 E) fitted with a C_{18} Novapak column and a UV-absorbance detector (Waters 486) (Hunter et al., 1995).

Dispersion/dilution of phenol within the model

A 25-chamber uninoculated control channel was used to determine the dispersion/dilution of phenol following introduction into the first chamber of the model on a continuous basis. Distilled water was used as the diluent. In a series of separate experiments different concentrations of phenol (20, 60 and 100 $mg \cdot \ell^{-1}$) were continuously introduced for 48 h and phenol analysis was done 24 and 48 h thereafter.

Perturbation studies

After the establishment of near stable-state conditions within the model a series of experiments was performed to determine the perturbation effects of different concentrations of phenol (20 and 60 $mg \cdot \ell^{-1}$) and 2,4-dichlorophenol (10 and 20 $mg \cdot \ell^{-1}$), on the nutrient cycling processes operative in the laboratory model.

The perturbant molecules were introduced via the influent medium into Chamber 1 of each experimental channel and nitrite, nitrate and residual perturbant concentrations determined at regular intervals from selected chambers. Duplicate samples (5 $m\ell$) were taken from each sampling point. For each experiment a channel was maintained as an unperturbated control.

Recovery of nitrifying activity was also investigated subsequent to the omission of the perturbant from the influent medium.

Results

Residual concentrations of phenol in the controls after 24 and 48 h are shown in Fig. 1. Inherent variability within a control channel of nitrite (A) and nitrate (B) concentrations during the course of a 288 h experimental period is shown in Fig. 2. The impacts of 60 and 20 $mg \cdot \ell^{-1}$ phenol on nitrifying activity over a 72 h perturbation period are presented in Figs. 3 and 4, respectively, while the fates of phenol within the same channels are shown in Figs. 5 and 6 respectively. Similarly, the impacts of 20 and 10 $mg \cdot \ell^{-1}$ 2,4-dichlorophenol on nitrifying activity are presented in Figs. 7 and 8, whilst the fate of 2,4-dichlorophenol within each channel is shown in Figs. 9 A and 9 B. Recovery of nitrifying activity was also investigated subsequent to the omission of the perturbants and the results are shown in Figs. 10 to 13.

Discussion

It has previously been reported that nitrifier populations established within the multi-stage model over a 7 week period (Hunter et al., 1998). Although duplicate channels followed similar trends with regards nitrifying activity, temporal and spatial differences in nitrification were found between channels. These were attributed to the presence of mixed microbial populations and the gradual accumulation of flocculent biomass and biofilms which facilitated increased nitrifying activity. This emphasised the limitations of reproducibility within the model and, thus, the problems associated with replicating ecotoxicological impact studies. Cognizance of this was, therefore, taken when implementing perturbation studies and interpreting results.

The results obtained indicated that, for each molecule and concentration tested, microbial populations in duplicate channels exhibited similar responses to a perturbation. For the purpose of this paper the results of single experimental channels are, therefore, presented to illustrate the potential impacts of selected compounds on microbial populations established within the model.

Dispersion/dilution of phenol within the model

Phenol concentrations were found to equilibrate in the control channels within 24 h (Fig.1). 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 $m\ell \cdot h^{-1}$ a complete volume change was effected after 24 h.

The results confirmed that the flow characteristics of the model did not significantly affect the overall concentrations of the test compounds in the individual chambers. Fluctuations in phenol concentration (100 $mg \cdot \ell^{-1}$) in Channel A were attributed to artefacts arising during phenol analysis. Potential abiotic removal factors such as dilution and dispersion and volatilisation

Figure 1 (right)
Residual phenol concentrations of 25-chambered controls, 24 and 48 h after introduction in concentrations of 100 (A), 60 (B) and 20 (C) mg·ℓ⁻¹

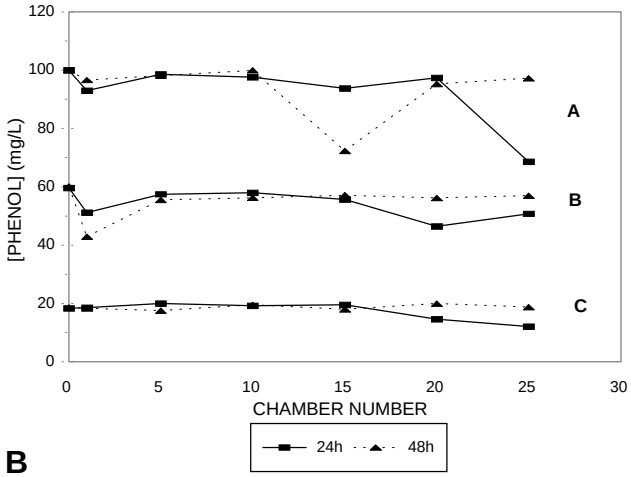
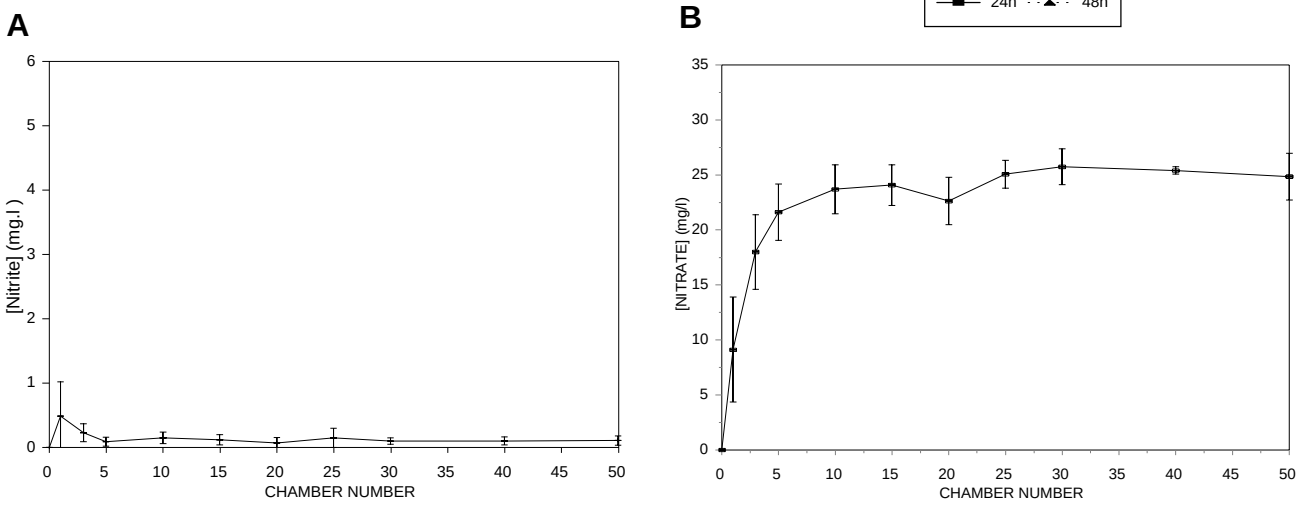


Figure 2 (bottom)
Discrete concentrations of nitrite (A) and nitrate (B) in a control channel during a 288h experimental period. The mean values of samples taken at 72 h intervals from selected chambers are shown together with vertical bars depicting 95% confidence intervals.



were not considered to be significant in the study. This was as expected since within aquatic environments phenol is readily transported in the aqueous phase due to its relatively high water solubility (6.7 g·ℓ⁻¹) and a low volatility (vapour pressure 0.8 mm at 20°C) (Weast, 1989).

Perturbation studies

Figure 2 illustrates the course of nitrification within a control channel during a 288h experimental period. Comparatively low concentrations of nitrite were recorded (Fig. 2A) which indicated that concomitant oxidation of nitrite to nitrate had occurred (Fig. 2B). 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.

In a preliminary perturbation study (Hunter et al., 1995), the addition of 20 mg·ℓ⁻¹ phenol via the influent effected a spatial shift in nitrification activity over a two-week period and indicated the sensitivity of the species to low phenol concentrations. However, in the context of the objectives of the study, it was felt that 2 weeks was too long to satisfy the criteria for a rapid ecotoxicological assessment protocol for determining impacts on aquatic ecosystems. To rationalise the screening procedure the duration of a perturbation study was, therefore, reduced to 72 h.

The addition of 60 mg·ℓ⁻¹ phenol to the experimental channel effected population shifts in nitrification activity during a 72 h perturbation period (Figs. 3 A and 3B). It was evident that nitrification was inhibited until the residual phenol concentration was reduced to < 4 mg·ℓ⁻¹ (Fig. 5). After the 48 h interval a temporary drop in phenol concentration occurred in Chambers 1 and 3 before increasing again. This was attributed to increased phenol degradation within these chambers resulting from a possible "damming effect" arising from a build-up of biomass between chambers. Nitrite did not accumulate (Fig. 3A) which indicated the sensitivity of the ammonium oxidisers to the perturbant. The inhibition of nitrate production was found to be more pronounced after 72 h than after 24 h (Fig. 3B) and was attributed to the protracted exposure time which effectively reduced the "masking effect" of associated biomass and organic matter. This finding illustrates one of the major limitations of short-term perturbation studies where long-term chronic effects are not considered.

Perturbation with 20 mg·ℓ⁻¹ phenol showed less of an impact on nitrifying activity within the model (Figs. 4 A and 4B). Increased nitrite concentrations arose after 24h, possibly indicating inhibition of nitrite oxidation activity. This was supported by lowered nitrate concentrations in Chambers 1 to 20. Subsequently, nitrifying activity appeared to recover after 48 h. The impacts of phenol on nitrification were minimised due to its attenuation within the model (Fig. 6). Phenol concentrations of < 1 mg·ℓ⁻¹ were recorded in Chamber 3 after 24 h.

These changes in nitrification activity indicated the sensitivity of the nitrifying populations to the lower concentration of phenol. Similar observations have been reported in the literature. For example, progressive inhibition of the respiration of nitrifiers was found to occur in the presence of concentrations of 4 to 10 mg·ℓ⁻¹ of phenol (Stafford, 1974). Hockenbury and Grady (1977)

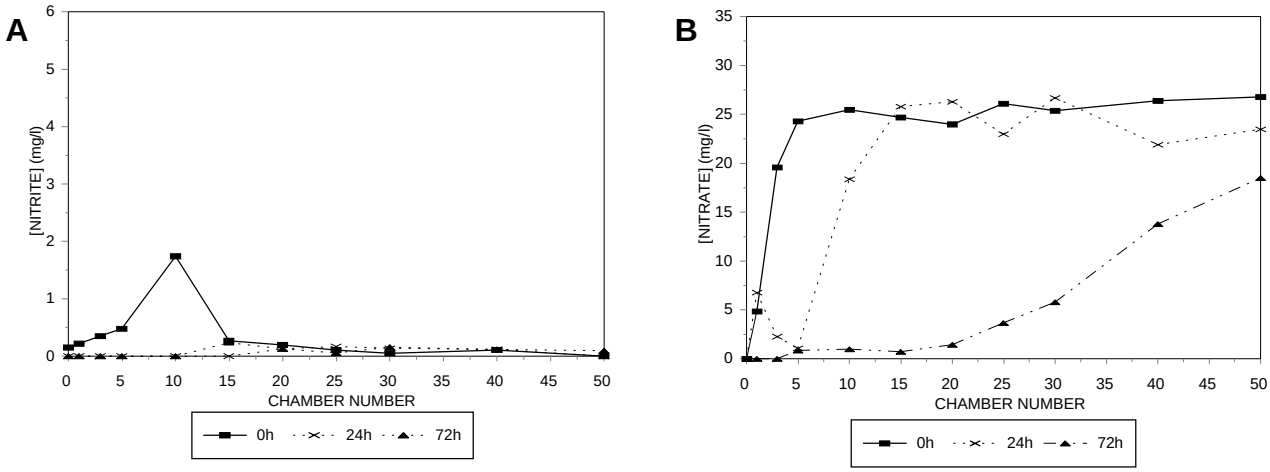


Figure 3
Changes in nitrite (A) and nitrate (B) concentrations in response to phenol (60 mg·ℓ¹) perturbation within an experimental channel of the multi-stage model

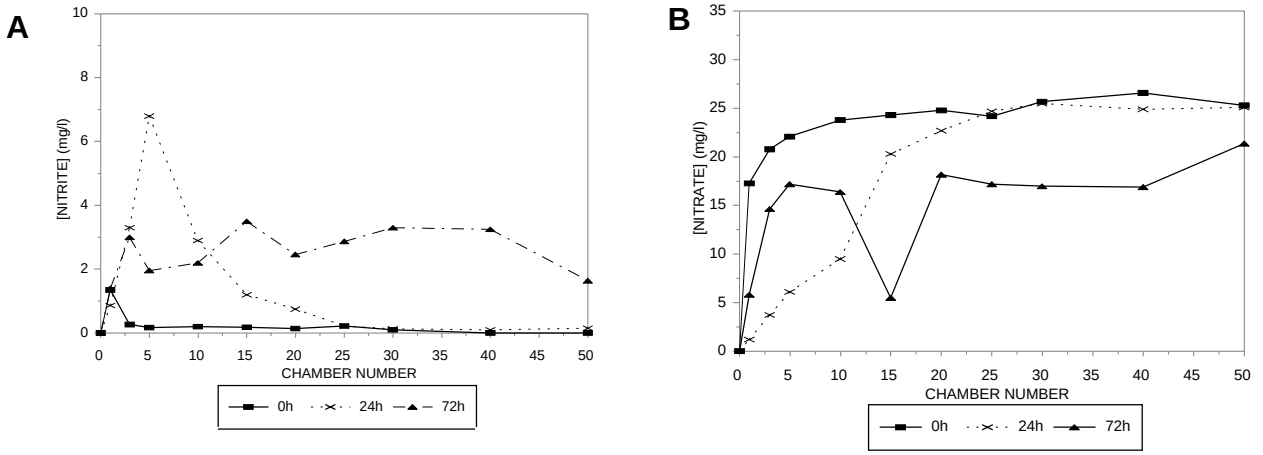


Figure 4
Changes in nitrite (A) and nitrate (B) concentrations in response to phenol (20 mg·ℓ¹) perturbation within an experimental channel of the multi-stage model

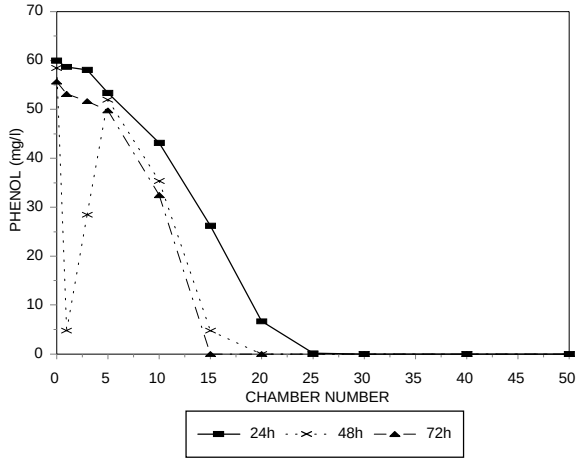


Figure 5
Residual phenol concentrations after a continuous 72 h perturbation of 60 mg·ℓ¹ phenol

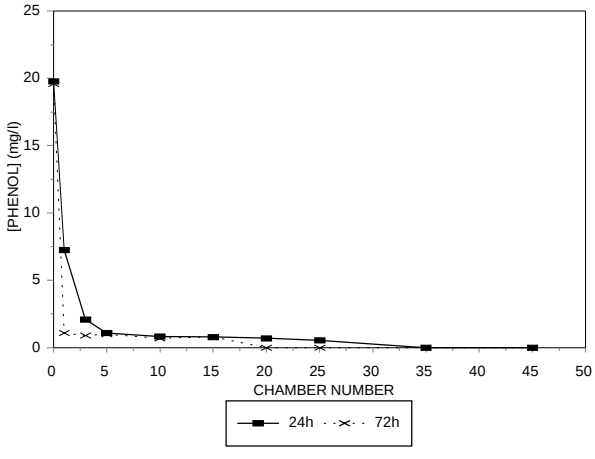


Figure 6
Residual phenol concentrations after a continuous 72 h perturbation of 20 mg·ℓ¹ phenol

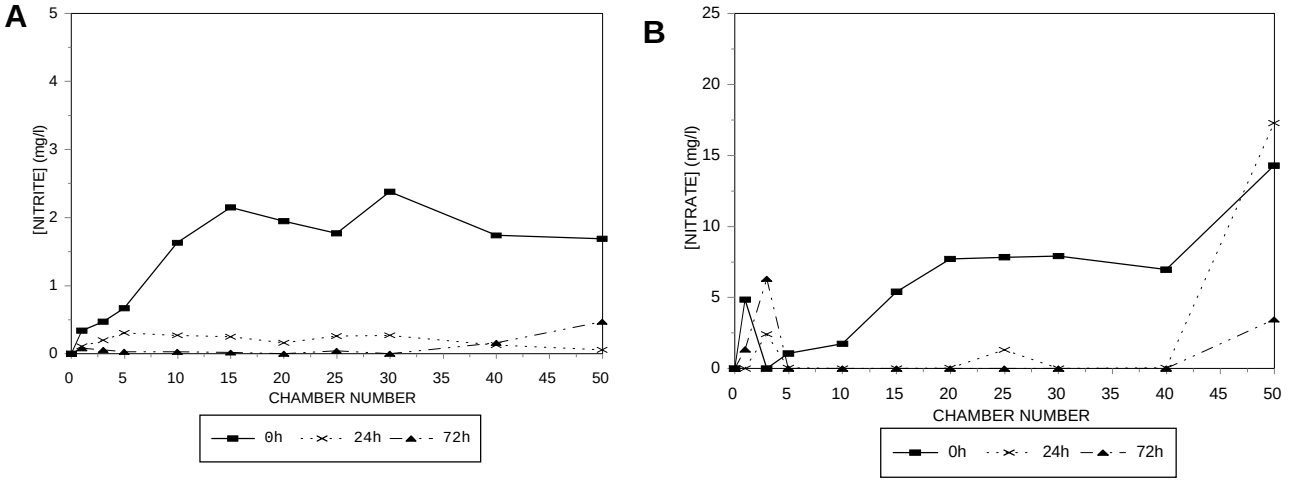


Figure 7

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

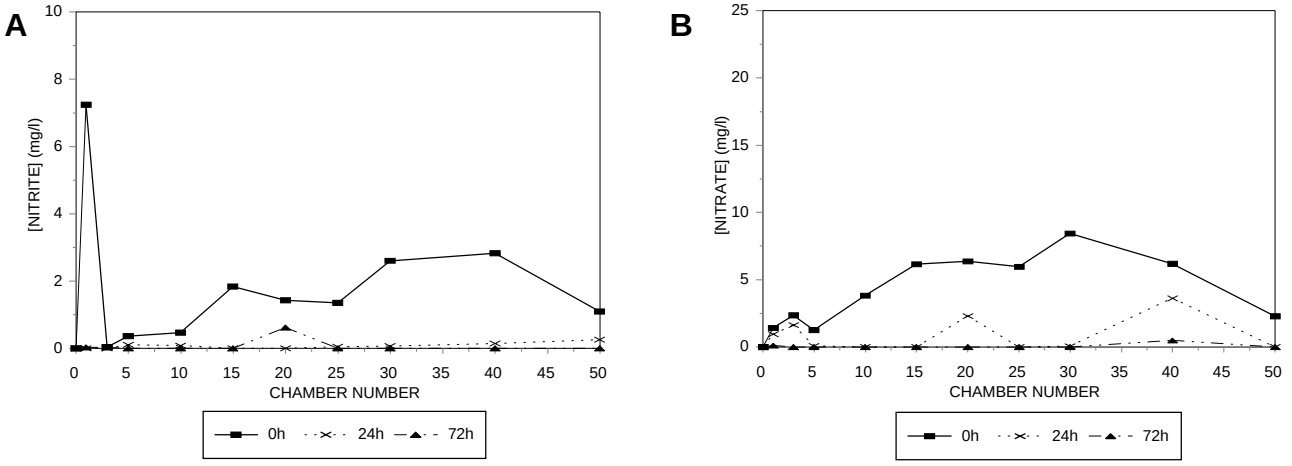


Figure 8

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

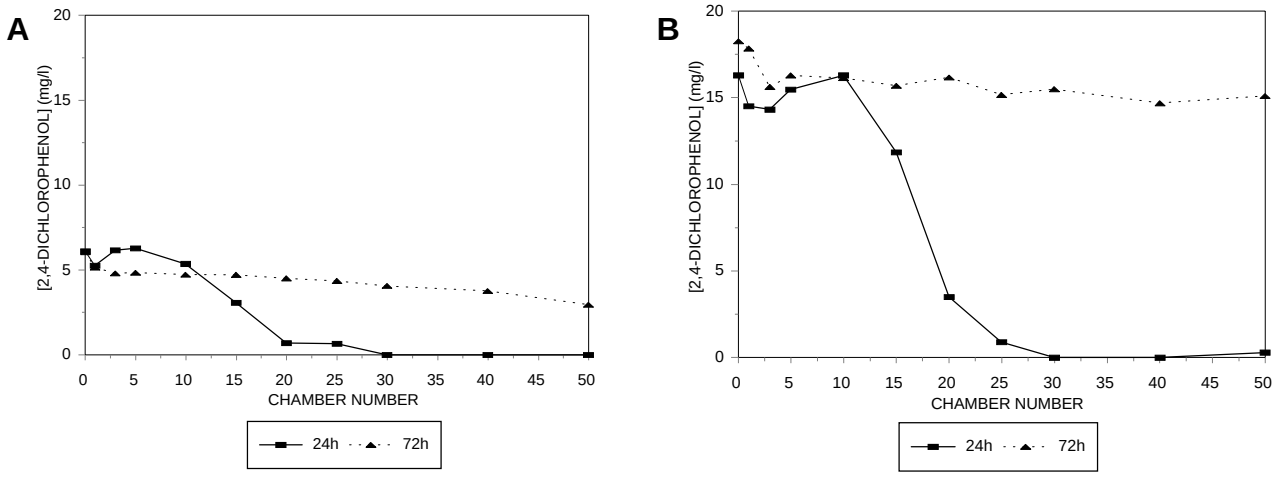


Figure 9

Residual 2,4-dichlorophenol concentrations after continuous 72 h perturbations of 10 mg·ℓ$^{-1}$ (A) and 20 mg·ℓ$^{-1}$ (B) 2,4-dichlorophenol

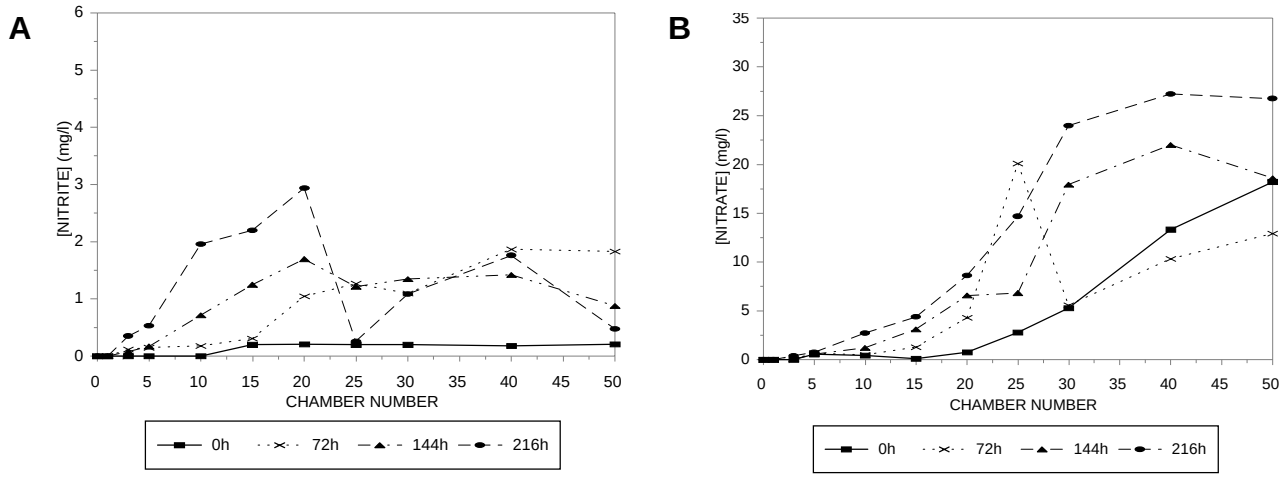


Figure 10

Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 216 h recovery period, following a 72 h phenol (60 mg·ℓ⁻¹) perturbation

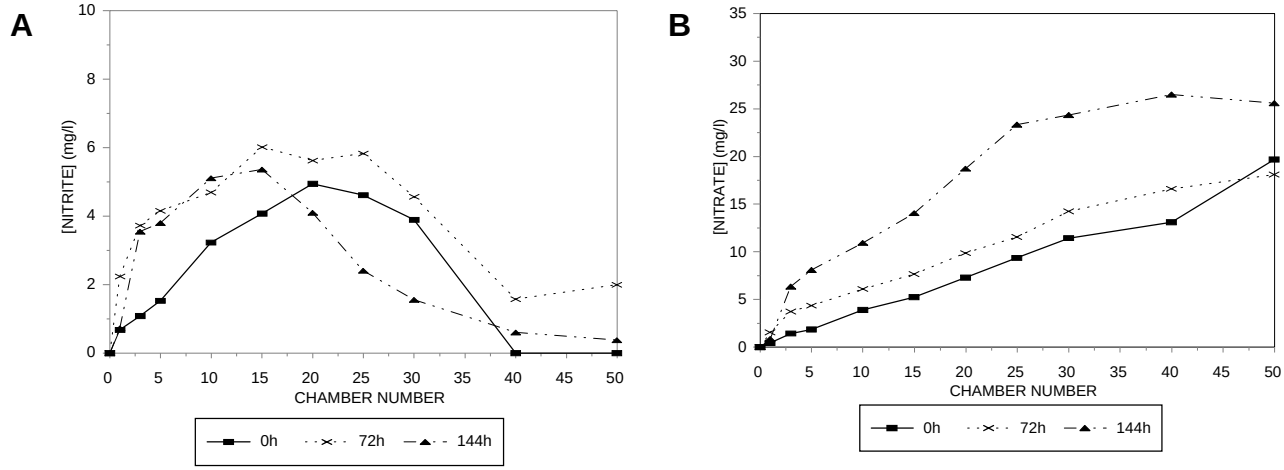


Figure 11

Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 144 h recovery period, following a 72 h phenol (20 mg·ℓ⁻¹) perturbation

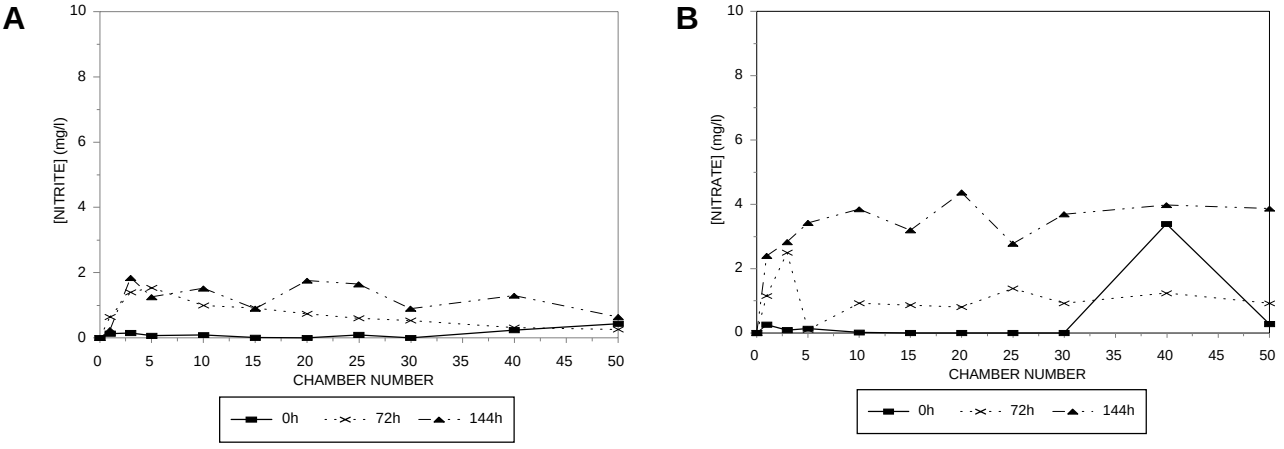


Figure 12

Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 144 h recovery period, following a 72 h 2,4-dichlorophenol (20 mg·ℓ⁻¹) perturbation

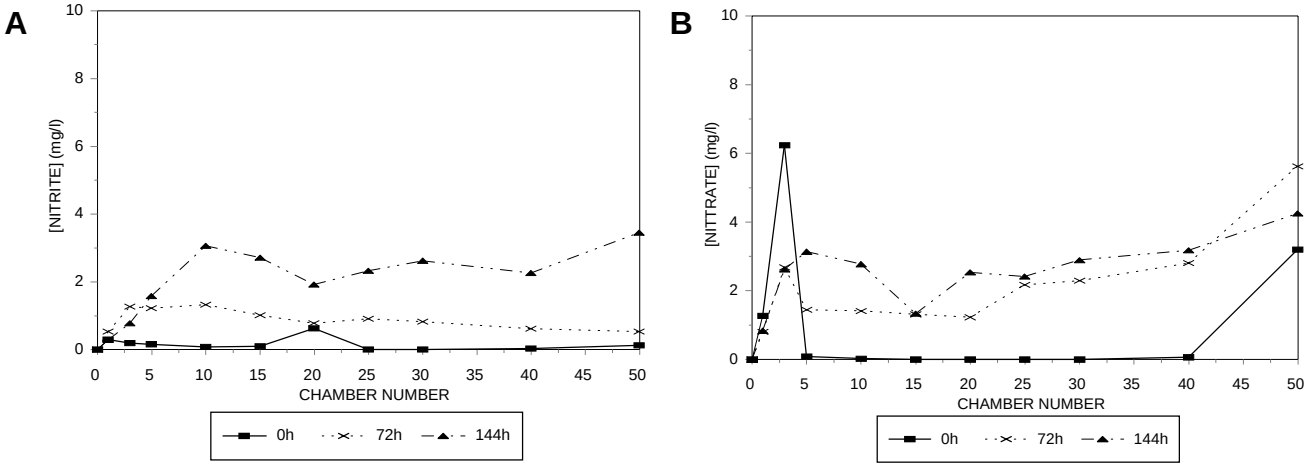


Figure 13

Changes in nitrite (A) and nitrate (B) concentrations in an experimental channel during a 144 h recovery period, following a 72 h 2,4-dichlorophenol (10 mg·ℓ$^{-1}$) perturbation

found that 5.6 mg·ℓ$^{-1}$ of phenol effected a 75% inhibition of nitrification in activated sludge. An LC_{50} for phenol of 21 mg·ℓ$^{-1}$ was indicated from a *Nitrosomonas* spp. bioassay (Blum and Speece, 1991).

Phenolic compounds were chosen for the perturbation study since nitrification has been found to be particularly sensitive to phenolic inhibition (Holladay, 1978). The sensitivities of nitrifiers to inhibitors are related, in part, to their relatively slow growth rates; thus, toxic effects at low concentrations are more marked since recovery through the growth of resistant or adapted species is slow (King and Dutka, 1986). Unsubstituted phenols display a high degree of surface activity and act by both disrupting cell membranes and inhibiting oxidase enzymes associated with the surface membranes (Hedgecock, 1967). Nitrifiers derive energy solely from the oxidation of reduced inorganic nitrogen mediated by such oxidase enzymes and, thus, exhibit a marked sensitivity to phenol (Brock et al., 1991).

Phenols are widely used as disinfectants and preservatives and have powerful antimicrobial activity with rapid bactericidal effects (Scott and Gorman, 1992), although their efficacies are markedly diminished by dilution and the presence of organic matter (Bitton and Dutka, 1986). Below bacteriostatic concentrations, phenol is readily degraded by a number of heterotrophic micro-organisms and has proved susceptible to catabolism via biological wastewater treatment processes (Rozich et al., 1983; Bitton, 1994). 2,4-dichlorophenol, in contrast, was chosen as an example of a semi-recalcitrant halogen-substituted phenol.

Marked inhibition of nitrifying activity resulted from the challenge of both concentrations of 2,4-dichlorophenol (Figs. 7 and 8), while concomitant 2,4-dichlorophenol attenuation did not result (Figs. 9 A and 9B). The actual test concentrations (6 mg·ℓ$^{-1}$ and 16 mg·ℓ$^{-1}$) introduced into each channel were found to be lower than anticipated due to possible adsorption to the filter membranes during sterilisation. Biodegradation was not thought to play a significant role in the attenuation. This emphasised one of the major limitations of perturbant assessments and illustrated the empirical nature of the assessment protocol. The initial choice of xenobiotic concentration is therefore, important if time and resources are to be optimised. Thus, there is a requirement for an initial screening protocol to determine an appropriate concentration range for testing in the model. For example, the LC_{50} of 2,4-dichlorophenol for the inhibition of nitrifying activity has been reported to be 0.74 mg·ℓ$^{-1}$ (Blum and Speece, 1991).

Recovery of nitrifying activity was also investigated subsequent to the removal of the perturbants from the influent medium (Figs. 10 to 13) for both molecules. Increasing concentrations of nitrite were recorded which indicated the re-establishment of ammonium-oxidising populations. Nitrate productions were, however, found to occur at lower overall rates than the pre-perturbation rates.

It was, thus, found that phenol and 2,4-dichlorophenol exhibited bacteriostatic effects and that the nitrification activities recovered at slow rates when these compounds were removed or diluted. These reinstatements were consistent with the recoveries of a small population of nitrifiers which were resistant to or protected from the inhibitory phenolics during the perturbations.

Impaired nitrification activity is of particular significance if one considers the impacts of pollutants on nutrient cycling processes in both wastewater treatment plants and aquatic environments. Inhibition of nitrifying activity can necessitate prolonged recovery periods. Thus, lowered nitrification rates could have detrimental effects on nutrient removal processes and, thus, impair wastewater treatment efficiency. An important consequence of ammonia oxidiser limitation is an 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).

The results confirmed that a 72 h study period was sufficient to illicit an inhibitory response on nitrification with the concentrations of the perturbant molecules tested. It was evident that the toxicological impacts of a perturbant compound were a direct function of the removal and/or transformation of the molecule.

Since nitrate is the end-product of nitrification its absence was considered a suitable indicator of nitrification inhibition. Several reports have confirmed that *Nitrosomonas* spp. are more sensitive to inhibitors than *Nitrobacter* spp. (Hockenbury and Grady, 1977; Blum and Speece, 1992). Ammonia oxidation is, thus, usually considered the principal step in nitrification inhibition. Blum and Speece (1991) reported that *Nitrobacter* spp. had similar sensitivities to toxic molecules as aerobic heterotrophs although they were more sensitive to phenols.

The rapid attenuation of phenol within each experimental channel illustrated the possible effect of acclimation of the selected microbial association. Factors such as cell density, nutrient availability, environmental conditions and previous exposure to the perturbant compound are contributory factors to phenol degradation and its overall toxicity. In relatively low concentrations, phenol attenuation may minimise the potential impacts on nitrification rendering it difficult to quantify the toxicological impacts. These findings emphasised the importance of using unacclimated associations to assess potential impacts to aquatic environments. They also illustrate the relevance of conducting biodegradation studies in conjunction with environmental impact assessments. Compounds which are labile will have lower impacts than compounds which are recalcitrant and may become biomagnified.

Conclusions

By use of the multi-stage laboratory model it was shown that inhibition of nitrification was a sensitive and rapid indicator of both phenol and 2,4-dichlorophenol toxicity. Through direct chemical analysis the relationship between the residual concentrations of the test compounds and nitrifying activity could be assessed. Phenol, in concentrations of 20 and 60 $mg \cdot \ell^{-1}$, was found to attenuate within the model and it was apparent that low concentrations (<4 $mg \cdot \ell^{-1}$) must be reached before nitrification would proceed. 2,4-dichlorophenol in concentrations of 10 and 20 $mg \cdot \ell^{-1}$ was found to persist within the model and inhibition of nitrification resulted. Biodegradation data with regards to a perturbant compound were, thus, considered important requisites for assessing its potential impacts on aquatic environments. Variability within the model was considered to be a major limitation for practical application as a rapid ecotoxicological assessment tool.

Acknowledgements

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