

# Ammonium-induced inhibition of ammonium-starved *Nitrosomonas europaea* cells in soil and sand slurries

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## Abstract

Ammonia-oxidising bacteria are poor competitors for limiting amounts of ammonium. Hence, starvation for ammonium seems to be the regular condition for these bacteria in natural environments. Long-term survival in the absence of ammonium will be dependent on the ability to maintain large population sizes at the expense of endogenous energy sources and on the preservation of a relatively large capacity for ammonium oxidation. The effect of freshly added ammonium on the performance of ammonia-oxidising bacteria was studied in ammonium-enriched slurries consisting of samples taken from non-water-saturated soil and sand columns inoculated with *Nitrosomonas europaea* and *Nitrobacter winogradskyi* and continuously percolated with mineral medium containing ammonium. Immediately after introduction of the nitrifying bacteria to the columns, ammonium oxidation started and nitrate leached from the columns. After 6 weeks of incubation of the columns, 94% of the ammonium supplied was recovered as nitrate in the effluent and net cell growth had ceased. In slurries with freshly added ammonium, ammonium oxidation decreased after an initial period of relatively high oxidation rates, which lasted 6 at the most. This indicated that the cells had been starved for ammonium in the columns. After 3 days of slurry incubation the ammonium-oxidising activity restarted, but not in the presence of chloramphenicol, indicating de novo synthesis of enzyme systems. Restart of activity after 3 days could not be attributed to the release of free-living cells from the sand particles or to the presence of organotrophic bacteria in the slurries. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Nitrification; Ammonium oxidation; Inhibition; *Nitrosomonas europaea*; *Nitrobacter winogradskyi*

## 1. Introduction

Inorganic nitrogen is an indispensable nutrient for plants as well as for micro-organisms. This means that under conditions of nitrogen limitation compe-

tition will occur, the outcome of which will determine whether an organism will grow or not. Chemolithotrophic nitrifying bacteria, and in particular the ammonia-oxidising bacteria, are not only dependent on ammonium for nitrogen assimilation, but also for generation of energy. Hence, competitive abilities with respect to the scavenging of limited amounts of ammonium seems to be of vital interest for these micro-organisms. However, ammonia-oxidising bac-

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teria compete poorly for ammonium with ammonium-assimilating, organotrophic bacteria and plants [1–8]. In the case of competition with plants, it was not always possible to conclude that the plant itself was the better competitor for ammonium or the organotrophic soil microbiota that grew at the expense of organic matter released from the plant roots. Experiments with  $^{15}\text{N}$ -labelled ammonium by Zak et al. [9] indicated that the organotrophic bacteria consumed more of the labelled inorganic nitrogen source than the plant and that nitrification was repressed.

For these ammonia-oxidising bacteria with highly unfavourable competitive abilities, it is very important to maintain the cell's homeostasis under conditions of ammonium limitation. Although the oxygen consumption rate is low under conditions of ammonium starvation, compared to situations of excess ammonium, the endogenous respiration is sufficient to maintain a considerable electrical potential across the cell membranes [10]. The existence of a relatively high potential in the absence of an external substrate such as ammonium might be of survival value. Not only the maintenance of the cell's homeostasis, but also the preservation of the ammonia-oxidising capacity might be of survival value as soon as ammonium becomes available again in the environment.

Although it has been mentioned recently that *Nitrosomonas eutropha* is able to grow on ammonium in the absence of oxygen [11], optimal growth of ammonia-oxidising bacteria is still thought to be aerobic. Hence, these chemolithotrophic bacteria do not only have to compete for ammonium in the natural environment, but also for oxygen. However on the basis of the respective kinetic abilities, it was concluded by Bodelier and Laanbroek [12] that in most natural ecosystems ammonia-oxidising bacteria are more likely to be limited by ammonium than by oxygen.

During competition experiments for limiting amounts of ammonium between the ammonia-oxidising *Nitrosomonas europaea* and the organotrophic *Arthrobacter globiformis* in non-saturated soil columns, cell numbers of *N. europaea* and the potential ammonia-oxidising activity per cell were repressed in the presence of glucose due to the better competitive abilities of the organotrophic cells for ammonium [6]. Irrespective of the presence of glucose-consuming *A. globiformis* cells, ammonium accumulated in the

columns, most notably in the upper layers, whereas the presence of oxygen could be demonstrated throughout the columns by the utilisation of oxygen micro-electrodes. Addition of the flagellate *Adriamonas peritocrescens* to soil columns with competing *N. europaea* and *A. globiformis* prevented largely the accumulation of ammonium in the columns and improved the performance of the ammonia-oxidising cells, although they still lost the competition for ammonium under ammonium-limiting conditions [7]. Even in the absence of glucose, when *A. globiformis* is not able to compete for ammonium, addition of *A. peritocrescens* diminished the accumulation of ammonium and enhanced the achievements of *N. europaea*. Hence, it was concluded by Verhagen and co-workers [6,7] that after 10–13 weeks of incubation, and irrespective of the presence of the competing organotrophic cells, the ammonia-oxidising bacteria were starved for ammonium by inaccessibility of their substrate. However, the onset of starvation for ammonium remained unknown due to the lack of more frequently harvesting of soil columns.

In this paper, we describe a series of experiments in non-saturated soil and sand columns. In contrast to the former experiments [6,7], the columns were only inoculated with nitrifying bacteria and triplicate columns were harvested at 14-day intervals for a period of 85 and 70 days for the soil and sand columns, respectively. In contrast to the usual analyses which last 5–6 h [13], ammonia-oxidising activities were measured in slurries with excess ammonium for 1 week to examine the time required by ammonium-starved cells to regain their maximum oxidising activities.

## 2. Materials and methods

### 2.1. Micro-organisms and culture conditions

*N. europaea* ATCC 19718 and *Nitrobacter winogradskyi* ATCC 25391 were used in the experiments. *N. winogradskyi* was added to the columns to prevent interference of accumulated nitrite formed by the ammonia-oxidising *N. europaea*. For inoculation of the soil or sand columns, 2-month-old pure cultures of nitrifying bacteria were used. These cultures had been grown in mineral medium containing 10

mM ammonium and 14.5 mM nitrite, respectively. Before inoculation, the pure cultures of ammonia- and nitrite-oxidising bacteria were diluted with mineral medium containing per litre 330 mg  $(\text{NH}_4)_2\text{SO}_4$ , 100 mg  $\text{K}_2\text{HPO}_4$ , 40 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 26.5 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 500 g NaCl and 1 ml of a trace elements solution [14] to obtain approximately equal numbers of *N. europaea* and *N. winogradskyi*. The pH of the mineral medium was 7.5. After dilution, the pure cultures were mixed. At the moment of inoculation, the mixed nitrifying cultures contained on average 4.54 mM ammonium, 0.87 mM nitrite and 1.58 mM nitrate.

## 2.2. Soil and sand columns

Growth and activity of the nitrifying bacteria were studied in continuously percolated, non-water-saturated soil and sand columns. For the soil columns, soil was collected from the top layer (0–30 cm) of an extensively used calcareous grassland in the forelands of the river IJssel near Brummen, the Netherlands. The sandy soil (21.3% coarse sand, 69.3% fine sand, 4.1% silt and 5.3% clay) contained 1.6% calcium carbonate and 4.2% organic matter, and the pH measured in water was 7.8. Moist, sieved (250 g, 2 mm mesh size) soil was added to a 30 cm long glass tube (internal diameter 40 mm) which was closed at the bottom by a hydrophobic nylon filter (pore size 0.2 mm) held in place by a hard plastic gauze and a stopper with a small outflow. This outflow was connected to an effluent bottle, which was kept under a suction of 1 m water to maintain a constant soil moisture content. Two openings in a rubber stopper at the upper end of the column enabled the introduction of sterile medium from a reservoir ( $9.6 \text{ ml day}^{-1}$ ) and of filter-sterilised air ( $25 \text{ ml min}^{-1}$ ), respectively, whereas a third opening connected to a conical flask served simultaneously as air outlet and for inoculation of the columns. Under these conditions, oxygen was available down to the bottom of the columns [6]. Total soil height in the columns was approximately 12.5 cm leaving a headspace of 17.5 cm. The columns filled with soil were sterilised by  $\gamma$ -irradiation (40 000 Gy) and then percolated with sterile demineralized water for 2 weeks to remove organic and inorganic soil compounds.

Subsequently, percolation with the mineral medium containing 5 mM ammonium was started. After 20 days of percolation with the mineral medium, each column was inoculated with 25 ml of a freshly mixed, nitrifying culture consisting of  $1.3 \times 10^8$  cells of *N. europaea* and  $1.2 \times 10^8$  cells of *N. winogradskyi*. At 14-day intervals, three columns were harvested and divided into three layers of 3, 3 and approximately 6.5 cm, respectively. The layers were subsequently analysed for numbers of nitrifying bacteria, potential ammonium- and nitrite-oxidising activities, the presence of unwanted organotrophic micro-organisms, mineral nitrogen concentrations and moisture content. Only the results of the upper 3 cm layer are presented as the two lower layers showed the same phenomena although at lower levels of activity and numbers.

After harvesting, the soil samples were checked for contamination by plating on nutrient rich agar medium. It was not possible to keep the glass soil columns free of contamination. For this reason the glass columns were replaced by Falcon bottle-top filters for the sand column experiments. No contamination was observed with these bottle-top filters. For the sand column experiments, calcareous river sand (pH in water of 7.8, 2.0% calcium carbonate and low in organic matter) from the river Rhine flood plains near Bommel was used. Sterile Falcon bottle-top filters (0.22 mm cellulose acetate membranes, Becton Dickinson) were filled with 100 g dry sterile sand obtained by sterilisation of moist sieved sand (2 mm mesh size) for 1 h on three subsequent days. Aeration was obtained by passive diffusion through cotton-wool. Mineral medium as described above was applied at a rate of  $4 \text{ ml day}^{-1}$ . The average residence time for the medium was comparable for the soil and sand column systems, i.e. 11.5 days. Non-water-saturated conditions in the sand columns were obtained by a suction of approximately 0.1 m water. The sterile sand columns were percolated with sterile demineralized water for 10 days followed by percolation with mineral medium. After 11 days of percolation with mineral medium, each column was inoculated with 25 ml of a freshly mixed, nitrifying culture consisting of  $9 \times 10^7$  cells of *N. europaea* and  $4.4 \times 10^7$  cells of *N. winogradskyi* and sampled as described above.

### 2.3. Determination of potential ammonium- and nitrite-oxidising activities

Potential ammonium- and nitrite-oxidising activities in the soil and sand column experiments were determined in slurries of 5 g moist soil or 15 g moist sand in 12.5 or 37.5 ml phosphate buffer (1 mM, pH 7.5), respectively. The slurries contained 2.5 mM ammonium sulfate and were incubated at 25°C and shaken at 150 rpm. Samples for determining the oxidation rates were taken at hourly intervals starting 30 min after addition of the substrate. In these slurries, nitrite always accumulated during the oxidation of ammonium indicating that the nitrite-oxidising step was rate-limiting. Accumulation rates of nitrite plus nitrate and of nitrate alone were used to calculate the potential ammonium- and nitrite-oxidising activities, respectively. Standard measurements lasted 6 h, but incubations were continued for 7 days with sampling after 24, 48, 72 and 168 h (soil experiment) and after 24, 48, 72, 96, 120, 144 and 168 h (sand experiments), respectively. Samples used for the determination of nitrite and nitrate were centrifuged at  $15000 \times g$  in a Biofuge A table centrifuge for 5 min. Subsequently, 0.75 ml of the supernatant was mixed with 0.75 ml of a 2 M KCl solution and stored at 4°C. Analyses of mineral nitrogen occurred within 3 days.

In the second sand column experiment, slurries were enriched with 1 g chloramphenicol  $l^{-1}$ . The preservation of activity of this inhibitor of de novo enzyme synthesis was checked by addition of 0.5 ml slurry to 0.13 ml of a nutrient rich medium inoculated with a well growing *Bacillus licheniformis* culture. In this way, chloramphenicol was proven to be inhibitory at 1 g  $l^{-1}$  for at least the duration of the whole chloramphenicol experiment, which was 168 h.

### 2.4. Determination of bacterial numbers

Ammonium- and nitrite-oxidising bacteria were enumerated by fluorescence-specific antibody (FA) microscopy and a most probable number (MPN) method, the latter as described before [6]. For the FA microscopy, a 1.0 g sample of moist soil or sand from the columns was mixed with 5.0 ml 10 mM tetrasodium pyrophosphate. The mixture was shaken for 15 min at 160 rpm and subsequently so-

nicated at 47 kHz for 2.5 min in a Branson 2000 sonification bath. Depending on the cell numbers present, the suspension was diluted 20–40 times and centrifuged at  $200 \times g$  for 1 min in a Biofuge A table centrifuge. Finally, 10 ml of the supernatant was transferred to a Nutricon microwell stock slide. After evaporation of the fluid, the cells were fixed on the glass surface by gentle heating. Fixed cells were treated with antisera prepared from blood from immunised rabbits as described before [15]. No cross reaction was observed between antibodies against *N. europaea* and *N. winogradskyi*. Numbers determined by FA microscopy were considered as total numbers, whereas the numbers estimated by the MPN method were considered as viable numbers.

### 2.5. Determination of mineral nitrogen concentrations, pH and moisture content

Mineral nitrogen concentrations in the soil or sand samples were determined by shaking 2.5 g of moist soil or sand in 25 ml of a 1 M KCl solution at 20°C. After 4 h of shaking, a slurry sample was taken and centrifuged at  $15000 \times g$  in a Biofuge A table centrifuge for 5 min. The supernatant was stored at 4°C and analysed within 3 days. Concentrations of ammonium, nitrite and nitrate in these supernatants as well as in the effluents of the columns were determined using a Traacs 800 autoanalyser (Technicon Instruments, Tarrytown, NY) with a detection limit of 0.01 mM N for all three compounds.

The pH of the slurries was determined by shaking 2.5 ml of moist soil or sand with 12.5 ml of demineralized water for 2 h. Moisture content was determined by analysis of weight loss after drying overnight at 110°C.

## 3. Results

### 3.1. Grassland soil columns

Twenty days before the nitrifying bacteria were introduced in the sterile soil columns, mineral medium containing 5 mM ammonium was continuously supplied to the top of the soil. Retention of ammonium by the soil is indicated by lack of recovery in the effluent (Fig. 1). As soon as the columns were

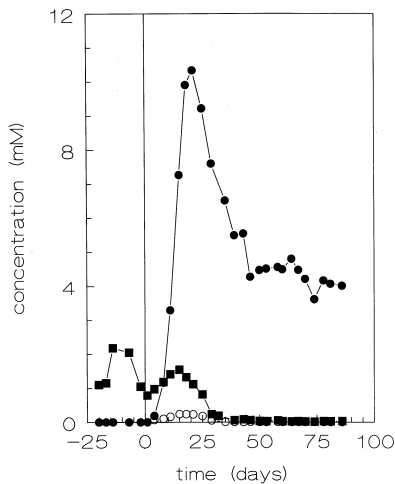


Fig. 1. Concentrations of ammonium (filled squares), nitrite (open circles) and nitrate (filled circles) in the effluent of columns filled with grassland soil. The non-water-saturated columns were continuously supplied with mineral medium containing 5 mM ammonium. The ammonia-oxidising *N. europaea* and the nitrite-oxidising *N. winogradskyi* were introduced at day 0.

inoculated with the ammonia-oxidising *N. europaea* and the nitrite-oxidising *N. winogradskyi*, nitrate and traces of nitrite leached from the columns. At 21 days, the nitrate concentration in the effluent reached a concentration of 10.3 mM and then declined, remaining constant after 43 days at approximately 4.3 mM. During the first weeks after inoculation nitrite accumulated slightly in the effluent up to a maximum of 0.2 mM. The pH of the effluent remained relatively constant during the experiment at an average value of 7.8 (range 7.5–8.1). After day 14, the concentrations of ammonium in the upper 3 cm of the

soil column steadily decreased until a sharp and significant ( $P < 0.05$ ) increase was observed at the end of the experiment (Table 1). The concentrations of nitrate in the upper layer remained almost constant throughout the experiment. Nitrite in the column was always below the detection limit, i.e. 0.14 mM  $\text{g}^{-1}$  dry soil. During the first 8 weeks of the experiment the soil moisture content remained relatively stable at 29% (w/w), but increased significantly ( $P < 0.05$ ) to 33% after 8 weeks of incubation.

Numbers of *N. europaea* and *N. winogradskyi* in the upper 3 cm of the soil column, determined by specific antibody fluorescence microscopy, increased significantly between weeks 2 and 4 and then remained approximately constant (Table 2). Only a minor, but significant, minimum was observed with both species during day 71. Potential ammonium- and nitrite-oxidising activities as indicators of actively nitrifying cells increased during the first 6 week after inoculation up to rates of 413 and 230  $\text{nM N g}^{-1}$  dry soil $^{-1}$ , respectively (Fig. 2). These activities declined between days 43 and 57, and became even significantly ( $P < 0.05$ ) lower at the end of the experiment when compared to day 43. Potential nitrite-oxidising activities were always lower than the ammonium-oxidising activities. Potential activities shown in Fig. 2, were calculated as usual from nitrite and nitrate production rates observed in soil slurries during 6 h. When the nitrite plus nitrate production measurements in the soil slurries were continued for 7 days, it was noticed that the production rate steadily decreased after the first 6 h of incubation (Fig. 3). Due to the sampling procedure, a possible decline of activity during these first 6 h remained unnoticed.

Table 1

Concentrations ( $\mu\text{M g}^{-1}$  dry soil or sand) of ammonium and nitrate in soil (upper 3 cm) and sand columns

Grassland soil columns			River sand columns		
Harvest day	Ammonium	Nitrate	Harvest day	Ammonium	Nitrate
–	–	–	0	0.96 b	0.15 b
14	4.10 cd	1.40 ab	14	1.72 c	1.21 ef
29	2.03 a	1.00 ab	28	0.73 a	1.23 f
43	3.20 abc	1.03 ab	42	0.68 a	1.06 e
57	2.97 b	0.93 a	56	0.53 ab	0.88 de
71	1.90 a	1.13 b	70	0.77 a	0.89 d
85	5.70 d	0.73 a	–	–	–

Concentrations of nitrite were always below detection limit, i.e. 0.14 mM  $\text{g}^{-1}$  dry soil. Concentrations within a column followed by different characters are significantly different ( $P < 0.05$ ).

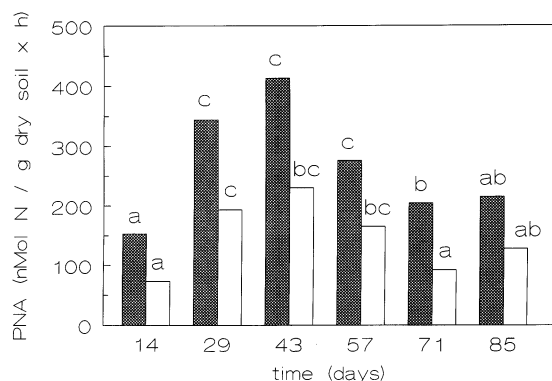


Fig. 2. Potential ammonium- (filled bars) and nitrite- (open bars) oxidising activities determined in ammonium amended slurries containing a soil suspension from the upper 3 cm of columns filled with grassland soil. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. Different characters indicate significant differences ( $P < 0.05$ ) between potential activities with respect to ammonium or nitrite oxidation, respectively.

The decline in activity after 6 h was most pronounced in the soil slurries from the columns that had been harvested after 57 days or more after inoculation of the nitrifying bacteria. Surprisingly, however, the nitrite plus nitrate production rates in these slurries increased again after 3 days of incubation.

After 6 h of incubation, nitrite concentrations in the soil slurries reached a maximum within a range of 0.1 and 0.2 mM and declined to low concentrations within the next 42 h (Fig. 4). Highest accumulation rates were observed in the most active slurries of days 29 and 43. In soil samples harvested at day

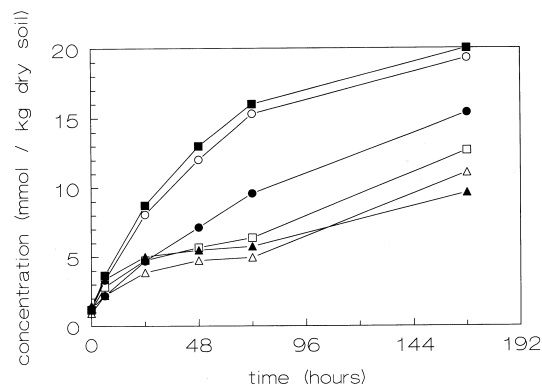


Fig. 3. Nitrite plus nitrate accumulation in ammonium amended slurries containing a soil suspension from the upper 3 cm of columns filled with grassland soil. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. The columns were harvested on days 14 (filled circles), 29 (open circles), 43 (filled squares), 57 (open squares), 71 (filled triangles) and 85 (open triangles, respectively).

85, a second significant ( $P < 0.05$ ) increase in nitrite concentration was observed after an incubation period of 168 h. Only the measurements of soil layers harvested at days 14 and 71 behaved differently as the nitrite continued to accumulate after 6 h. Nitrite accumulation in the samples of day 71 occurred more slowly and nitrite remained at 0.1 mM for several days.

### 3.2. River sand columns

As in the experiment with the grassland soil, ammonium supplied with the mineral medium accumu-

Table 2

Cell numbers ( $\text{g}^{-1}$  dry soil or sand) of ammonia-oxidising *Nitrosomonas europaea* and nitrite-oxidising *Nitrobacter winogradskyi* in soil (upper 3 cm) and sand columns enumerated by specific antibody fluorescence microscopy

Grassland soil columns			River sand columns		
Harvest day	Numbers of <i>N. europaea</i>	Numbers of <i>N. winogradskyi</i>	Harvest day	Numbers of <i>N. europaea</i>	Numbers of <i>N. winogradskyi</i>
–	–	–	0	$1.7 \times 10^5$ a	$1.6 \times 10^5$ a
14	$0.6 \times 10^8$ a	$0.3 \times 10^8$ a	14	$0.4 \times 10^7$ b	$0.4 \times 10^7$ b
29	$1.7 \times 10^8$ bc	$1.0 \times 10^8$ c	28	$2.6 \times 10^7$ c	$1.4 \times 10^7$ c
43	$1.8 \times 10^8$ c	$0.9 \times 10^8$ c	42	$4.1 \times 10^7$ d	$1.8 \times 10^7$ cd
57	$1.7 \times 10^8$ c	$1.0 \times 10^8$ c	56	$5.5 \times 10^7$ e	$2.3 \times 10^7$ de
71	$0.6 \times 10^8$ a	$0.6 \times 10^8$ b	70	$6.6 \times 10^7$ e	$2.2 \times 10^7$ d
85	$1.4 \times 10^8$ b	$0.9 \times 10^8$ c	–	–	–

Numbers within a column followed by different characters are significantly different ( $P < 0.05$ ).

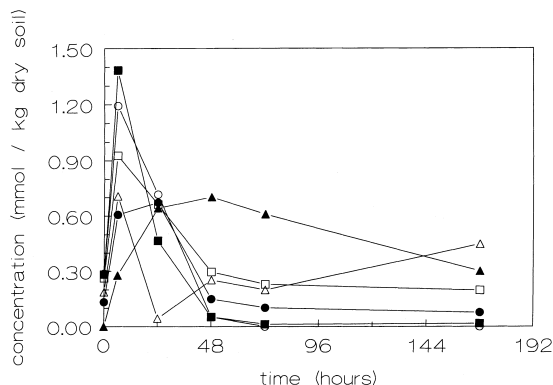


Fig. 4. Nitrite accumulation in ammonium amended slurries containing a soil suspension from the upper 3 cm of columns filled with grassland soil. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. The columns were harvested on days 14 (filled circles), 29 (open circles), 43 (filled squares), 57 (open squares), 71 (filled triangles) and 85 (open triangles), respectively.

lated in the river sand columns before these were inoculated with the ammonia- and nitrite-oxidising bacteria (not shown). As soon as the columns had been inoculated with *N. europaea* and *N. winogradskyi*, nitrite and nitrate appeared in the effluent indicating the oxidation of ammonia and nitrite by the bacteria. Nitrite reached a maximum of 0.3 mM after 9 days, whereas a maximum nitrate concentration of 6.8 mM was observed after 23 days. After day 23, the concentration of nitrate in the effluent decreased until it became approximately constant at 4.7 mM after 37 days. The average pH decreased from 7.6, after inoculation, to 6.8 at day 70.

Concentrations of ammonium and nitrate in the sand columns increased significantly ( $P < 0.05$ ) during the first weeks of the experiment and then declined (Table 1). Concentrations of ammonium were low compared to the experiment with grassland soil, whereas the concentrations of nitrate in both experiments were comparable. Moisture content was relatively constant at 21% (w/w), well below that of grassland soils, but no increase in moisture content was observed at the end of the incubation period.

Numbers of ammonium- and nitrite-oxidising bacteria increased during the first weeks of the experiment (Table 2) and then became approximately constant. Numbers of nitrifying bacteria determined by MPN varied irregularly during the whole experiment

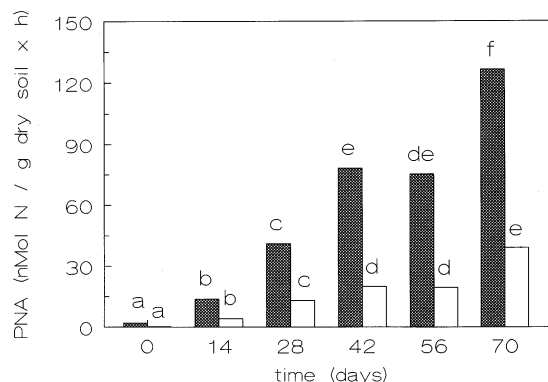


Fig. 5. Potential ammonium- (filled bars) and nitrite- (open bars) oxidising activities determined in ammonium amended slurries containing a sand suspension from columns filled with river sand. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. Different characters indicate significant differences ( $P < 0.05$ ) between potential activities with respect to ammonium or nitrite oxidation, respectively.

(not shown) and were on average four orders of magnitude lower than the numbers enumerated by specific antibody microscopy. Potential ammonium- and nitrite-oxidising activities determined in sand slurries increased significantly ( $P < 0.05$ ) up to rates of 126 and 39 nM g<sup>-1</sup> dry sand h<sup>-1</sup>, respectively (Fig. 5). During continued incubation of the sand slurries used for determining potential activities, the nitrite plus nitrate production rates decreased after

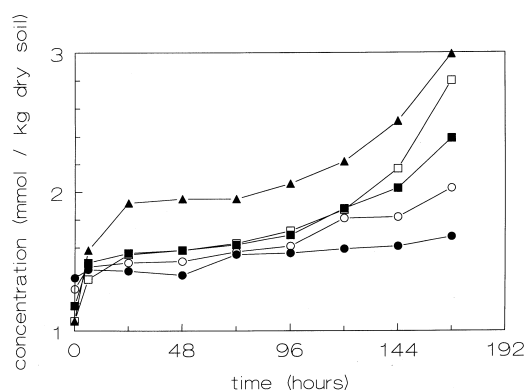


Fig. 6. Nitrite plus nitrate accumulation in ammonium amended slurries containing a sand suspension from columns filled with river sand. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. The columns were harvested on days 14 (filled circles), 28 (open circles), 42 (filled squares), 56 (open squares), 70 (filled triangles), respectively.

the first 6 h (Fig. 6), as with grassland soil. In this second experiment, the rates were approximately zero after 24 h, but increased again after 3 days of incubation. After 6 h of incubation, nitrite concentrations reached a maximum in the sand slurries within a range of 0.02–0.08 mM, generally well below the level observed during the soil slurries incubations (Fig. 7). In addition, the nitrite concentrations after 6 h in sand slurries from days 14 and 28 were significantly ( $P < 0.05$ ) lower than the maximum values measured in the other sand slurries. Nitrite concentrations declined within the next 3 days and then increased.

### 3.3. Additional experiments

To test if recovery of the nitrifying activity after 3 days observed in the experiments described above, was related to the release of free-living, nitrifying cells from the sand particles, well nitrifying, 1-week-old slurries from sand columns harvested at day 70 were subjected to filtration over 5- $\mu$ m mono-filament Nylon Heavy Duty sifting gauze (Merrem and LaPorte). The filtrate and the filters themselves resuspended in fresh mineral medium were incubated for another 4 days. Within these 4 days, the medium containing the sand particles oxidised 3.2 mM ammonium to nitrate (not shown). Although ammonium was available, no nitrite or nitrate production

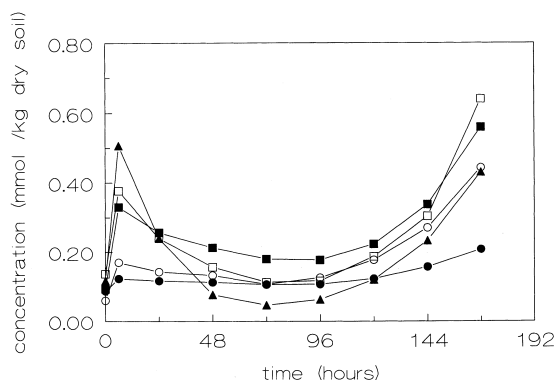


Fig. 7. Nitrite accumulation in ammonium amended slurries containing a soil suspension from the upper 3 cm of columns filled with grassland soil. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. The columns were harvested on days 14 (filled circles), 28 (open circles), 42 (filled squares), 56 (open squares), 70 (filled triangles), respectively.

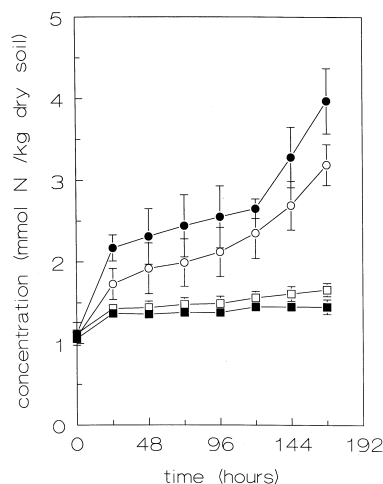


Fig. 8. Nitrite plus nitrate production by ammonium amended slurries containing a suspension of sand from columns filled with river sand that have been flushed with mineral medium supplemented with 5 mM ammonium for 44 days after inoculation with the ammonium-oxidising *N. europaea* and the nitrite-oxidising *N. winogradskyi*. Slurries were incubated at 25°C and shaken at 150 rpm in the dark. To half of the slurries, 1 g l<sup>-1</sup> chloramphenicol had been added (squares), the other half served as a control (circles). In addition, part of the slurries was handled aseptically (open symbols), whereas the other part was handled in the usual non-sterile manner (filled symbols).

was observed in the original filtrate during this incubation time indicating that the nitrifying activity was still associated with particles. To examine the possibility that the presence of organotrophic bacteria in the slurries unintentionally introduced by sampling of the sand slurries might have stimulated the nitrifying activity by release of growth-promoting factors, numbers of organotrophic bacteria in slurries made of sand columns harvested at day 70, were determined by plating on Tryptone Soy Broth agar (Oxoid). Numbers of colonies of organotrophic bacteria increased from  $4.0 \times 10^2$  to  $1.9 \times 10^6$  g<sup>-1</sup> dry sand during the first 48 h of the incubation of the sand slurries.

To test whether organotrophic bacteria could have been responsible for the recovery of the nitrifying activity in the sand slurries after 3 days, the sand column experiment was repeated and slurry incubations were performed under aseptic conditions. Slurries not aseptically handled served as a control. In addition, to half of the slurries, both aseptically treated and controls, chloramphenicol (1 g l<sup>-1</sup>) was

added to test if *de novo* synthesis of enzymes or even of cells was necessary for renewal of nitrifying activity. The general performance of the oxidation of ammonium to nitrite and nitrate by the inoculated nitrifying bacteria in the second river sand column experiment was similar to that observed in the first sand column experiment. In the effluents, maximum values of 1.0 mM nitrite and 7.0 mM nitrate were reached after 7 and 17 days, respectively. After day 17, the nitrate concentration in the effluent decreased slowly to 4.7 mM at day 32 and remained constant until 44 days after inoculation of the nitrifying bacteria. The pH values of effluents decreased slightly from 7.9 at the time of inoculation to 7.2 at day 44. At day 44, slurry experiments were performed. In the presence of chloramphenicol, no recovery of nitrifying activity was observed during the incubation period of 7 days (Fig. 8), whereas in its absence renewal of activity was observed. Although not significant at each sampling time, aseptic handling of the slurries had a slight negative effect on the production rate of nitrite plus nitrate in the absence of chloramphenicol. However, recovery of nitrifying activity itself could not be ascribed to the presence of organotrophic bacteria.

#### 4. Discussion

Although the nature of the grassland soil and river sand used in the columns was different, especially with respect to the amount of organic matter present, the patterns of nitrate leaching, and therefore of ammonium oxidation, were comparable. Maximum nitrate leaching was observed around day 22 after introduction of the nitrifying bacteria and the washout of nitrate reached a constant rate after approximately 40 days. From that time on, 94% of the ammonium supplied to both the soil and sand columns was recovered as nitrate. No nitrite was detected in the effluent of the columns, whereas 1 and 5% of the ammonium supplied leached from the soil and sand columns, respectively.

Although the pattern of leaching was not different, the peak in nitrate production during the first 40 days was much more pronounced in the grassland soil. During this initial period, the nitrogen balances amounted to 122 and 89% for the soil and sand

columns, respectively. Part of the additional ammonium in the grassland soil columns might have originated from mineralisation processes as it was not possible to keep those columns free of organotrophic bacteria. The additional ammonium available in the grassland soil was most likely responsible for the higher numbers of nitrifying bacteria observed in the soil columns. In addition, the presence of organic matter as particles for improved adherence of the nitrifying bacteria could have stimulated their activity and growth [1]. The higher potential activities of the nitrifying bacteria from the soil columns compared to those from the river sand columns can be attributed to the higher numbers as the potential activities of the bacteria on a cell basis were not different between both types of columns. In both column systems, a steady state, with respect to growth of the ammonium- and nitrite-oxidising populations, occurred after 40 days. At that moment the ammonium supplied to the columns was apparently just sufficient to satisfy the maintenance requirements of the cells or to balance growth and death in the population. When ignoring death of cells, the maintenance requirements of the ammonium- and nitrite-oxidising cells can be calculated for the sand columns assuming no spatial heterogeneity. During the period of constant nitrate production (i.e. after 40 days) the average oxidation rates in the sand columns were  $1.45 \times 10^{-16}$  and  $3.73 \times 10^{-16}$  M cell<sup>-1</sup> h<sup>-1</sup> for ammonium and nitrite, respectively. These rates amounted to 8 and 30% of the respective average potential oxidation activities per cell measured during this period. Hence, especially the ammonia-oxidising bacteria maintained a large overcapacity with respect to the oxidation of their major energy source. All numbers used for these calculations were based on FA-enumerations assuming the same physiological condition for each cell. For the soil columns, these calculations cannot be made, as only the upper layers of these columns were used for measurements, whereas part of the oxidation processes occurred in the deeper layers (not shown).

The reason for the ammonium-induced, temporary repression of nitrification in slurries is still obscure. The experiment with chloramphenicol convincingly proved that restart of nitrifying activity was dependent on *de novo* synthesis of enzymes, whatever the reason for ammonium-induced inhibition. Restart of

activity was not due to the presence of organotrophic bacteria or the formation of free-living nitrifying cells. After the restart of nitrifying activity, response was restricted to particle-bound bacteria as was demonstrated by the filtration experiment. Attachment to particles seems to be the preferred way of living for the ammonia-oxidising bacteria as demonstrated in water samples from the river Elbe [16].

The accumulation of nitrite during initial incubation of the slurries might have inhibited the ammonia-oxidising bacteria from the soil and sand columns. When accumulated nitrite was inhibitory to the ammonia-oxidising cells in the slurries, cells collected at the end of the incubation period of the soil columns were apparently more sensitive than the cells sampled at the start of the experiment, as the highest nitrite concentrations were encountered during the first weeks of the soil column experiment (Fig. 4). In the sand column experiments, the accumulation of nitrite (0.02–0.05 mM) during the first hours was still less severe, whereas the inhibition was even more pronounced (Fig. 7). Surprisingly, accumulation of nitrite to even higher levels during the second part of the incubation period of the sand-derived slurries had no inhibitory effect on the performance of the freshly started ammonia-oxidising bacteria. Hence, when nitrite was inhibitory to ammonia-oxidising bacteria it would only affect ammonium-starved cells.

In addition to nitrite, other inorganic nitrogen intermediate compounds may have been produced. For example, Stüven et al. [17] demonstrated the production of hydroxylamine by *N. europaea* in the presence of *N. winogradskyi* when pyruvate or formate was added as electron donor additional to ammonium. The production was explained by an imbalance between the electron-consuming steps in the metabolism of *N. europaea*, i.e. the hydroxylation of ammonium to hydroxylamine by ammonia mono-oxygenase and the reduction of oxygen by the electron transport system, and the electron-producing step, which is the oxidation of hydroxylamine to nitrite. Hydroxylamine temporarily inhibited the nitrite-oxidising bacteria, but had no effect on the ammonia-oxidising cells themselves. Growth of *N. europaea* on hydroxylamine seems to be impossible [18], although mixotrophic growth on ammonia and hydroxylamine has been demonstrated in continuous

culture [19]. So even when hydroxylamine would have been produced during the first hours of slurry incubation, it is not likely that this would have repressed the activity of the ammonia-oxidising bacteria in the presence of excess ammonium. The fact that the ammonia-oxidising bacteria in the experiments of Stüven et al. [17] were not repressed by the accumulation of nitrite stresses again that especially ammonium-starved cells are sensitive for nitrite. Also, in the experiment of Laanbroek and Gerards [20], ammonium-limited, but non-starved cells of *N. europaea* continued to oxidise ammonium when *N. winogradskyi* cells were repressed by oxygen limitation and nitrite accumulated. However, in this latter experiment, ammonium was not in excess, which might also have prevented substrate-induced inhibition of activity.

The inhibition of ammonium-starved cells of *N. europaea* by freshly supplied ammonium has been shown in soil and sand slurries, but does it also occur in the soils itself? Increased nitrite concentrations around active micro-colonies of ammonia-oxidising bacteria will attract nitrite-oxidising bacteria. In slurries from acid forest soils, mixed micro-colonies consisting of ammonia-oxidising bacteria in the centre and nitrite-oxidising bacteria in their periphery have been observed [21]. It is plausible that such mixed colonies also exist in more neutral soils and sediments. However, the fact that ammonia-oxidising bacteria seem to be firmly attached to sand particles, as was demonstrated in our experiments, does not necessarily mean that the nitrite-oxidising bacteria are also permanently adhered to these particles. It has been shown by Underhill and Prosser (cf. [22]) that ammonia- and nitrite-oxidising bacteria exhibit an opposite behaviour with respect to attachment to particles. Release of nitrite-oxidising bacteria from the sand particles can not be dismissed, as filtered free-living, nitrite-oxidising bacteria would not have been able to produce nitrate in the absence of nitrite-producing, ammonia-oxidising bacteria. Hence, the conditions in the slurries with respect to the respective (micro-)locations of the ammonia- and nitrite-oxidising bacteria may be different from the conditions in soils and sediments. Therefore accumulation of nitrite and a subsequent repression of the activity of the ammonia-oxidising bacteria as happened in the soil and sand slurries does not necessarily have

to occur in the soil itself. However, when mixed micro-colonies of ammonia- and nitrite-oxidising bacteria are stable in slurries, as was observed in slurries from acid forest soils [21], ammonium-induced inhibition of nitrifying activity of ammonium-starved cells would also be imaginable in soils.

In conclusion, the ammonia-oxidising bacteria, in general losers with respect to the competition for limiting amounts of ammonium, maintain a high ammonia-oxidising capacity during periods of starvation, but they can hardly profit from this, as the ammonium oxidation is repressed for a longer period shortly after the introduction of this substrate. Due to the relatively retarded activity of the nitrite-oxidising bacteria, nitrite accumulated in the system. The inhibiting compound being nitrite or an unknown intermediate metabolite destroys the enzyme system of the ammonia-oxidising cells as the re-establishment of activity is dependent on de novo synthesis of enzymes.

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