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Effect of salinity on temporal and spatial dynamics of ammonia-oxidising bacteria from intertidal freshwater sediment

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Abstract

Temporal and spatial dynamics within an ammonia-oxidising community from intertidal, freshwater sediments were studied in microcosms simulating flooding twice a day with fresh, brackish and marine waters. The microcosms had been filled with the upper 5 cm of intertidal freshwater sediment from the river Scheldt. Changes in community composition were examined by denaturing gradient gel electrophoresis of amplified DNA from the community. In the first week of incubation the initially present members of the *Nitrosomonas oligotropha* lineage were replaced by other members of the same lineage in the top layer of the sediment subjected to flooding with freshwater. Prolonged incubation extended niche differentiation to a depth of 5 cm. In the microcosms flooded with saline media, the initially present members of the *N. oligotropha* lineage were replaced by strains belonging to the *Nitrosomonas marina* lineage, but only in the top 1 cm. Shift in community composition occurred earlier in the marine microcosms than in the brackish microcosms and was slower than the change in the freshwater microcosms. Irrespective of the nature of the flooding medium, shifts in community composition were always consistent among replicate microcosms. We conclude that salinity is an important steering factor in niche differentiation among ammonia-oxidising bacteria and also that changes within the community of this functional group of bacteria may occur at different rates.

Keywords: Ammonia-oxidising bacteria; Freshwater sediment; Salt stress; Microcosms; Niche differentiation; Spatial and temporal community change

1. Introduction

Ammonia-oxidising bacteria (AOB) are chemolithoautotrophic microorganisms that generate their energy by oxidising ammonia to nitrite. Ammonia oxidation is the first and usually rate-limiting step in the nitrification and fulfils a critical role in global nitrogen cycle. Due to the vital role of AOB in elemental cycling and in environmental issues like acidification, eutrophication and greenhouse gas emission, a wealth of studies have been performed to elucidate ecological and physiological aspects of these bacteria in various environments [1–4]. Especially the use of cultivation-independent techniques has enhanced the knowledge on the biogeography of this very specialised group of bacteria [2,5,6]. AOB are among the rare cases in microbial ecology where pure cultures of nearly all recognised lineages are available. The latter makes it possible to connect ecophysiology with diversity and distribution of these

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bacteria [1,2]. Based on structural (16S rRNA) as well as functional (amoA) gene relationships, seven major lineages of AOB are recognised [6–8]: *Nitrosospira*, *Nitrosomonas europaea/Nitrosococcus mobilis*, *Nitrosomonas communis*, *Nitrosomonas marina*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans* and *Nitrosomonas* sp. Nm143. Despite the fact that all these lineages carry out the same basic energy-generating biochemical reaction, AOB seem to occupy clearly different niches and can therefore be classified into ecophysiological groups [1]. The former cluster 6, as defined by Stephen et al. [5] and comprising the *N. oligotropha* (6a) and the *N. marina* (6b) lineages, is predominantly found in aquatic environments. The *N. marina* lineage comprises obligate halophilic and salt-tolerant species, whereas members of the *N. oligotropha* lineage are found under oligotrophic freshwater conditions. The latter lineage seems to contain species growing relatively well at low ammonia concentrations [9,10] but also contains strains found in wastewater treatment plants [11]. Speksnijder and coworkers [12] detected a high micro-variation within the former cluster 6a, suggesting different eco-types and probably niche differentiation within this group. Niche differentiation within AOB communities has also been observed with respect to salinity [13–16].

In a survey of the diversity within the ammonia-oxidising *Betaproteobacteria* along a salinity gradient in the Scheldt estuary, a change in community composition has been observed at the transition zone between the freshwater and the brackish water parts of the estuary [13]. As indicated by enrichment experiments under different conditions of salinity, the freshwater part contained salt-sensitive as well as salt-tolerant ammonia-oxidising bacteria, whereas the brackish water samples contained only salt-tolerant ammonia-oxidising bacteria [9,14]. Due to the prevailing conditions in the freshwater part of the river, salt-sensitive bacteria were apparently dominant.

The salt-sensitive and the salt-tolerant ammonia-oxidising bacteria belonged to the *N. oligotropha* and the *N. marina* lineages, respectively [1,5]. In a study of ammonia-oxidising bacteria in intertidal freshwater sediments more upstream of the river, sequences related to *N. oligotropha* lineage were the most dominant among the sequences belonging to the betaproteobacterial ammonia-oxidisers ([12] and Speksnijder, personal communication). Studies on selection, adaptation or niche differentiation of AOB communities while environmentally perturbed are very scarce. Community changes or replacement of species may lead to changes in diversity, which may yield a community with different ecophysiological properties, certainly in the case of AOB, with possible consequences for the biogeochemical process of nitrification. However, it is not known how quick such changes occur, especially under natural conditions.

The aim of this study was to investigate how AOB communities change upon environmental perturbations. We focussed on the response of AOB communities from intertidal freshwater sediment to tidal floods with media of different salinities and on the possible niche differentiation among and within the lineages. Changes in community composition were followed in time and with depth in the sediment using PCR and denaturing gradient gel electrophoresis (DGGE) assays for ammonia oxidisers.

2. Material and methods

2.1. Description of the sampling site

The sampling site was a freshwater intertidal marshland near the village of Appels (51°01′N, 4°03′E, Belgium) that is located more than 100 km upstream of the Scheldt mouth. In June 2002, sediment from the upper 5 cm of the non-vegetated zone was collected during low tide and then stored in buckets at 4 °C until the filling of the microcosms. The mean tidal difference at Appels is 6 m and the average ammonia concentrations amount to 0.2 mM in the water and to 0.2–1.5 mM in the upper 10 cm of the sediment (Christelle Hyacinthe, personal communication). Salinity at Appels is 0.5 ppt and it does not change with the tides.

2.2. Description of the microcosms and sampling

Microcosms consisted of polymethylmethacrylate (PMMA) cylinders of 15 cm diameter and 25 cm height with a total volume of more than 4 l. The microcosms were filled with mixed sediment up to a height of 15 cm. In each microcosm four custom made rhizon soil solution samplers (Eijkelkamp, Giesbeek, The Netherlands), as described by Bodelier et al. [17], were mounted to enable sampling of the pore water at 1, 4, 8 and 12 cm depth, considering 0 cm at the interface with the flooding water. Each microcosm had also an inflow device (0.5 cm diameter) at 4 cm above the sediment surface, through which the flooding medium entered the system. The outflow device was located on the opposite side at the surface sediment level. The outflow device was protected by a metallic sieve to prevent sediment from leaving the microcosm system. Fig. 1 gives an overview of the microcosm system with the marine replicate microcosms in the foreground.

On every sampling day, a stainless steel cylinder (3 cm diameter and 20.5 cm height) was pushed into the sediment of the microcosms where it remained till the end of the experiment. Inside this stainless cylinder a second cylinder of PMMA (2.5 cm diameter and 23.5 cm height) was pushed and used to take a sediment core. In this way the sediment profile in the microcosm

Fig. 1. Overview of the microcosms: 12 microcosms in total with four replicates per treatment. The marine microcosms are in the foreground.

itself was not disturbed. Sediment cores were cut with a sharp tool in slices of 1 cm each; slices from different depths were used for analysis.

2.3. Microcosm experiment

The microcosms were subjected to a tidal regime using flooding media that differed mutually in salinity. The basic medium was a mineral salt medium containing 1 mM $(NH_4)_2SO_4$, 1 mM KCl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM KH_2PO_4 [18] and 1 ml SL8 trace element solution according to Verhagen and Laanbroek [19]. The basic medium was supplemented with 10, 260 or 520 mM NaCl. The microcosms exposed to these media will be referred to as freshwater microcosms (FM), brackish microcosms (BM) and marine microcosms (MM) (four replicates per treatment). The media were adjusted to pH 7.5–7.8 with 1 N NaOH before autoclaving for 25 min at 121 °C. Sterile KH_2PO_4 (0.4 mM) was added after separate autoclaving. Microcosms were incubated at 25 °C in the dark for 35 days.

The whole system was designed as a recycling system of 10 l flooding media that were replaced by fresh media once every week, after sampling of the microcosms. The flooding media covered the sediment surfaces for about 2 cm with a volume of approximately 350 ml. Tides were simulated according to the standard regime of 6 h high and 6 h low tide.

NH_4^+ and NO_2^- plus NO_3^- concentrations were daily measured in the three different 10 l reservoirs during one week and in the overlying water during one tidal cycle. NH_4^+ concentration in the media and overlying water was 1 mM in the whole experiment and this excludes ammonia to be a niche-differentiating factor.

2.4. Analysis of sediment water content and pore water analysis

Water content in percentage of weight was measured gravimetrically after drying 5 g sediment for 48 h at 60 °C. Measurements were done in sediment slices from 1, 2, 3, 4, 5 and 10 cm depth at day 7, day 24 and day 35 from four replicate microcosms per treatment.

Pore water samples were collected at day 0, 7, 14, 24, 35 from three replicate microcosms of each treatment type. Pore water samples were taken at 1, 4, 8, 12 cm depth in 3 ml Silicone Vacuum Venoject vials (Terumo) and immediately frozen at −20 °C. NH_3 and NO_2^- plus NO_3^- concentrations were measured with an Auto-Analyzer Traacs 800 (Technicon Instrument, Canada).

2.5. Potential nitrification activity

Potential nitrification activity was measured at day 0 and day 35 at different depths from two replicates per treatment. Measurements were done in slurries containing 2 g wet sediment in 20 ml mineral medium and incubated on a rotary shaker at 25 °C at 150 rpm. The sterilised medium contained 2.5 mM $(NH_4)_2SO_4$, 0.80 mM, K_2HPO_4, 0.20 mM KH_2PO_4, adjusted to pH 7.5 with 1 N NaOH [20]. Sub-samples from the slurries were taken after 1, 2, 4, 6, 12 and 24 h incubation. Nitrite and nitrate concentrations were measured colorimetrically with a Technicon Auto-Analyzer (Traacs 800). Potential nitrification activity in the intertidal freshwater sediment itself was measured as described above in slurries of sediment collected at the sampling site.

2.6. DNA extraction and purification

DNA was extracted from 0.5 g sediment with the mechanical disruption protocol described by Henckel [21]. Subsequently the extracted DNA was repurified with a commercial purification kit (Wizard® DNA Clean-up system, Promega Corporation, USA) according to the manufacturer's recommendations. Purified DNA was resuspended in deionised water. Extraction and purification were verified by electrophoresis of 5 μl of the DNA solution in 1.2% agarose gel and 0.5X TBE buffer (5X Tris-Borate: 54 g Tris base, 27.5 boric acid, 20 ml 0.5 M EDTA) and then visualised by ethidium bromide fluorescence.

2.7. Direct PCR amplification of ammonia-oxidising bacteria

Direct amplification of 16S rRNA gene fragments was performed for different replicates of each treatment

type. The AOB-specific primer set, CTO 189f-GC (an equimolar mixture of three forward primers) and CTO 654r, was used as described by Kowalchuk et al. [22]. The primer set amplified an approximately 465 bp fragment of the 16S rRNA gene of the betaproteobacteria. In this manuscript we refer to this fragment as the CTO fragment. The primers are degenerated with specificity and sensitivity clearly defined [2,22,28]. PCR amplification was performed in a total volume of 50 μl containing 1X PCR Mg-free buffer, 200 μM of each deoxynucleotide, 1.75 mM $MgCl_2$, two units of *Taq* DNA Polymerase (Invitrogen, Tech-Line USA), 400 ng μl^{-1} BSA (purified bovine serum albumin, New England BioLabs, Beverly, MA, USA), 0.5 μM of each primer, and 80–100 ng of purified DNA as template. Reactions were performed in a Multiblock Thermocycler System (Hybaid, USA) according to the following program: 3 min denaturation at 94 °C; 35 cycles of: 30 s denaturation at 92 °C, 30 s annealing at 57 °C and 45 s elongation at 72 °C; the last step was 5 min elongation at 72 °C. All the amplification reactions were verified loading 5 μl amplification products in 1.2% agarose gel, separated by electrophoresis in 0.5X TBE, stained in ethidium bromide solution and visualised with UV.

2.8. Nested PCR approach

As described by Bollmann and Laanbroek [14], 1:100 diluted CTO-PCR fragments were used as template for a nested PCR, using the eubacterial primers 357F with GC clamp and 518R [23]. CTO-PCR products (2.5 μl of 1:100 diluted) were used for 25 μl of master mix, whose components and concentrations were described above.

The thermocycling program has been described by Muyzer [23] as touchdown approach. In brief, the annealing temperature was set 10 °C above the expected annealing temperature and then lowered with 1 °C every second cycle until 55 °C.

2.9. DGGE analyses of ammonia-oxidising communities

Approximately 200–300 ng of PCR products were separated by DGGE with a Protean II system (Bio-Rad, USA) according to the protocol of Muyzer [23] as adapted by Kowalchuk et al. [22] for the study of ammonia-oxidising bacteria. The denaturant gradient to separate CTO fragments as well as PCR products resulting from the nested PCR approach were 30–55%, with a solution of 8% acrylamide, 7 M urea and 40% formamide defined as 100% denaturing. Gels were run for 16–17 h at 60 °C in 0.5X TAE buffer (50X Tris-acetate: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA per liter).

2.10. Sequencing of DNA fragments from DGGE and phylogenetic analysis

From the denaturing gradient gels, selected bands were cut out from the middle parts. The acrylamide pieces were stored in water overnight at 4 °C for the elution of PCR fragments [14]. Bands were used 1:10 diluted as template for a further 25 cycles PCR. Obtained PCR products were separated again with DGGE to confirm recovery of the desired single bands [24]. Single bands were sequenced with internal Cy 5 labelled primers, 357F and 518R, with a Thermo Sequenase Primer Cycle Sequencing Kit and ALF Express II Instrument (Amersham Biosciences, UK). Bands at the same height on the gel had the same nucleotide sequences and they were therefore submitted only once to the EMBL database.

Recovered sequences were aligned with the ARB program package (http://www.arb-home.de [25]). Alignments were refined by visual inspection and the Bioedit (Biological Sequence Alignment Editor) program. Phylogenetic trees were constructed in the Treecon program (version 1.3b, Van de Peer and de Watcher 1994) using the neighbour-joining method with the Jukes and Cantor correction. Gaps were not taken into account in the analysis. Bootstrap analyses were based upon 1000 replicates. For the phylogenetic analysis, we considered only the CTO fragments and not the nested amplicons of CTO fragments.

2.11. Nucleotide sequence accession numbers

Partial 16S rRNA gene sequences determined in this study have been deposited into the EMBL sequence database with the following accession numbers: AJ629220 (band N1), AJ628354 (band FM1), AJ628355 (band FM2), AJ628356 (band FM3), AJ628357 (band BM4), AJ628358 (band MM5), AJ628359 (band N3), AJ628360 (band N4), AJ628361 (band N5).

2.12. Statistical analysis

The effect of salinity and sediment depth on ammonium concentrations in pore water was tested per sampling day using a two-way ANOVA ($n = 3$). Salinity and depth were independent variables and ammonium concentration in pore water was the dependent variable. Potential nitrification activities of two replicates per treatment were compared between day 0 and day 35 with a *T*-test for dependent samples. One-way ANOVA analysis was used to test the effect of salinity on potential nitrification activities at the two different sampling days.

All data were analysed for normality (plots of SD vs. means) and were checked for homogeneity of variance (Levene's test). All the statistical analyses were made with the STATISTICA software package, version 6.0 for Windows (Tulsa, USA).

3. Results

3.1. Physical observations of the sediment

The sediment used to fill the microcosms contained many indigenous worms belonging to the class Oligochaetes, which constitute more than 95% of the total density of the benthic fauna of the river Scheldt [26]. Worms were active in the freshwater microcosms, bioturbating and thereby creating a oxidised sediment down to approximately 5 cm depth. In Fig. 2A, the burrows of the worms are clearly visible on a picture taken from a freshwater replicate microcosm. One cm sediment slides were washed with water on 210 μm diameter sieve and worms were counted by eyes; counting gave a density of 2–4 individuals per cm^3. In brackish and marine microcosms worms did not survive, probably due to osmotic stress. The absence of worm activity led to very different redox profiles in the brackish and marine treatments. In Fig. 2B, it is clearly visible that 1 cm below the sediment surface a layer of black deposit is formed. This putative iron sulphide layer marks the oxic–anoxic transition zone. Fig. 1 shows the blackish layer in the replicate marine microcosms.

Another indication of the physical effect of worm activity might be the water content of the sediment. At day 7, the average water content in percentage of dry weight in the freshwater microcosms was 60% at 1 cm depth and about 40% from 2 down to 10 cm depth. At day 24 and at day 35, we observed an increase in the water content within the first 5 cm of the sediment. In the brackish and marine microcosms, the average water content was maintained within a range of 38–45% along the whole depth profile without any significant change during the incubation period. Irrespective of sampling day, the water content decreased slightly with depth in the latter microcosms.

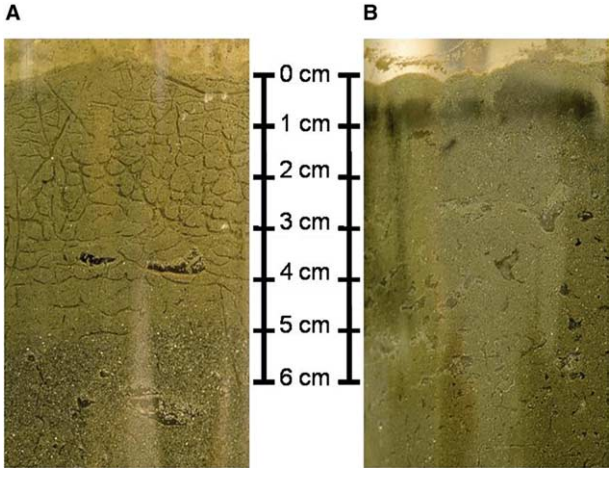

Fig. 2. Detail of sediment profiles. A: In the freshwater microcosm, the burrows of worms are visible down to approximately 5 cm. B: In the marine microcosm a black layer is visible at approximately 1 cm from the sediment surface.

3.2. Pore water characteristics

To reveal the effect of treatment on ammonium availability, pore water samples were retrieved from different depth in the sediment. At day 0, the average ammonium concentrations in the pore water of freshwater, brackish and marine microcosms were around 1 mM along the whole depth profile (Fig. 3). In the freshwater microcosms (Fig. 3A), ammonium concentrations increased during the incubation period to 2–2.5 mM at 1 and 4 cm depth and to 3–4 mM at 8 and 12 cm depth. However, in the brackish and marine microcosms (Fig. 3B and C) the steep increase in ammonium concentrations occurred already at 4 cm depth after 7 days and reached 5–6 mM at the end of the incubation. Nitrite and nitrate were not detected in the pore water samples. Statistical analysis showed that ammonium concentrations in the pore water depended significantly on salinity and depth. P values for salinity and depth were always below 0.05.

3.3. Potential nitrification activity

At day zero, potential nitrification activity was within the range of 2.0–3.0 μmol g^{-1} h^{-1} in the three treatments. At day 35, the potential nitrification activities decreased to a range of 0.5–1.5 μmol g^{-1} h^{-1}. This decrease was significant for all treatments (T-test for dependent samples, $P < 0.05$). However, salinity as such had no significant effect on potential nitrification activity (One way ANOVA). Measured PNA values of potential nitrification activity at the end of the incubation were in the range of 0.2–1.5 μmol g^{-1} h^{-1}, as were analysed at the sampling site.

3.4. Dynamics of ammonia-oxidising community revealed by nested PCR-DGGE

Temporal and spatial dynamics within the ammonia-oxidising community were investigated using denaturing gradient gel profiles of direct and nested PCR products.

DGGE profiles of nested PCR amplicons (see Section 2.8; Figs. 4 and 5) showed that changes in the ammonia-oxidising community occurred both in the freshwater microcosms and in the microcosms flooded with saline media. Moreover, the changes in the freshwater microcosms clearly differed from those in the brackish and marine microcosms. The initial ammonia-oxidising community was represented by band N1 at all depths (Fig. 4). In the freshwater microcosms the prominent band N1 had completely disappeared after 35 days from 1 to 3 cm depth and two new bands, N3 and N4, had appeared; band N1 persisted at 5 and 10 cm depth. After the same period of time, the intensity of band N1

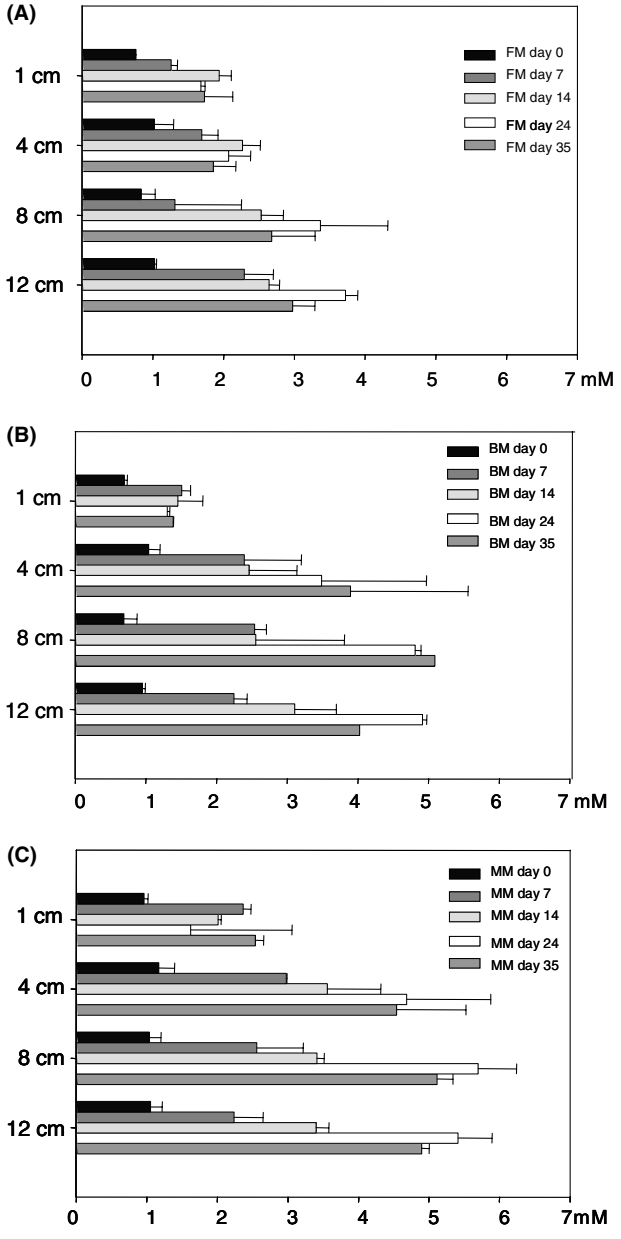

Fig. 3. Effect of treatment on the ammonium concentrations (mM) in the pore water at different sediment depths. Values are averages of three replicates per treatment. A: FM, freshwater microcosms; B: BM, brackish microcosms; C: MM, marine microcosms.

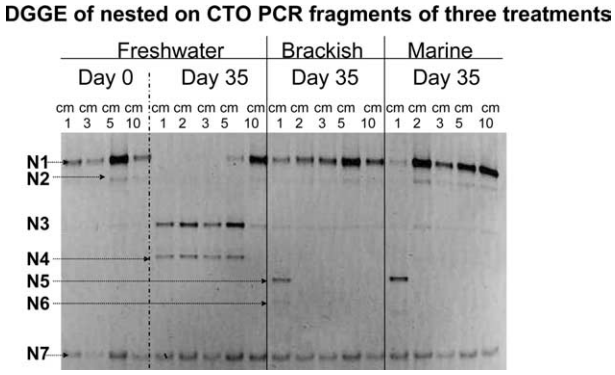

Fig. 4. DGGE of nested PCR of CTO fragments. One replicate per treatment (freshwater, brackish and marine) is shown. The spatial profile at day zero (1, 3, 5 and 10 cm) is only shown for the freshwater replicate. The spatial profile at day 35 is shown for the three treatments.

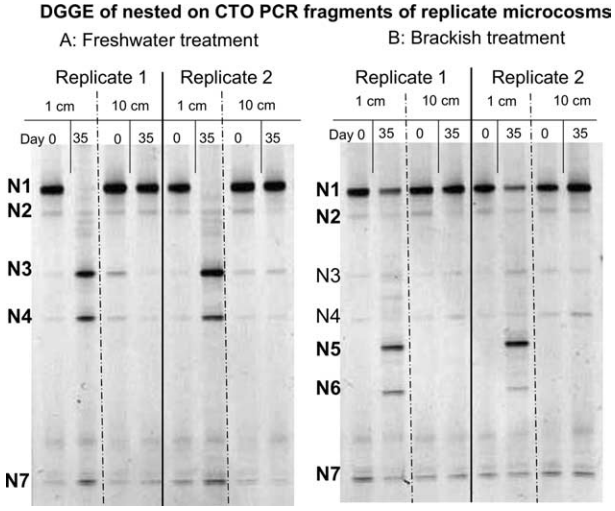

Fig. 5. DGGE of nested PCR of CTO fragments. A: Replicate microcosms 1 and 2 of the freshwater treatment. B: Replicate microcosms 1 and 2 of the brackish treatment. Per replicate, two different depths (1 and 10 cm) at two sampling days (day 0 and day 35) are displayed. Bands are numbered from N1 to N7.

decreased slightly in the brackish and in the marine microcosms at 1 cm depth and two new bands appeared at positions N5 and N6. In the brackish and marine microcosms no changes occurred at other depths. The changes occurring in replicate freshwater microcosms (Fig. 5A) were identical. Similarly, no differences were observed between replicates of the brackish treatment; in both treatments no changes occurred at 10 cm depth (Fig. 5B).

On the basis of BLAST analysis, band N1 belonged to the *N. oligotropha* lineage and showed 99% identity with clone 26Ft recovered from a wastewater treatment plant [27] and 97% identity with band FM1 recovered in our study with the direct PCR-DGGE approach (see below). Band N3 also belonged to the *N. oligotropha* lineage and showed 100% identity with *Nitrosomonas* strain Is 79 A3, which had been enriched with 0.25 mM NH_4^+ from freshwater lake sediment (A. Bollmann, personal communication). Band N5, recovered in the marine microcosm after 35 days belonged to the *N. marina* lineage. It had 99% identity with clone 11 Ft recovered from a wastewater treatment plant [26] and 99% identity with bands BM4 and MM5 recovered in this study with the direct PCR-DGGE approach (see below). Band N4 was not related to ammonia oxidisers' sequences and showed less then 95% identity with an environmental

clone of the *Methylophilus* spp. lineage. Recovery of non-ammonia oxidisers-related sequences has been reported in other studies and explained by degenerated positions in the CTO primer nucleotides [22,28]. Reamplification of bands N2, N6 and N7 after excision failed. The limited length of sequences obtained from bands N1, N3 and N5 prohibited direct inclusion into the phylogenetic tree (Fig. 8).

3.5. Ammonia-oxidising community using DGGE profiles of CTO amplicons

To obtain sufficient sequence information from DGGE bands for phylogenetic analysis, we also analysed the DGGE profiles of the samples through direct amplification from community DNA with the CTO primers (see Section 2.7; Figs. 6 and 7). The disadvantage of this direct procedure is that somewhat fuzzy bands are usually obtained. DGGE profile of CTO amplicons (Fig. 6) showed the changes in the initial freshwater ammonia-oxidising community over time at 1 cm depth. At day zero, band FM1 was retrieved in all microcosms. In the freshwater microcosm, band FM1 has disappeared after 7 days and two new bands, FM2 and FM3, appeared and persisted until day 35. A new band, BM4, appeared in the brackish microcosm after 35 days in addition to band FM1, while in the marine microcosm band MM5 had appeared already at day 24. The double-band pattern (BM4 and MM5 in Fig. 6) in the saline treatments is the result of the ambiguities in CTO oligonucleotides [22,28].

Because DGGE profile of nested PCR on CTO fragments (Fig. 4) showed no changes in the ammonia-oxidising community in the deeper layers of the brackish and marine microcosms, the complete spatial and temporal DGGE profile of CTO amplicons was analysed only for the freshwater microcosms (Fig. 7). Again, band FM1 was dominant at all depth at day zero. Band

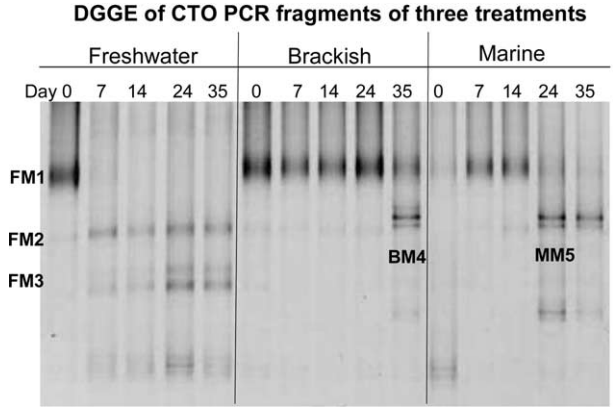

Fig. 6. Denaturing gradient gel of PCR amplified CTO fragments. Per treatment (freshwater, brackish and marine) samples at 1 cm depth are shown at five sampling days (day 0, 7, 14, 24 and 35).

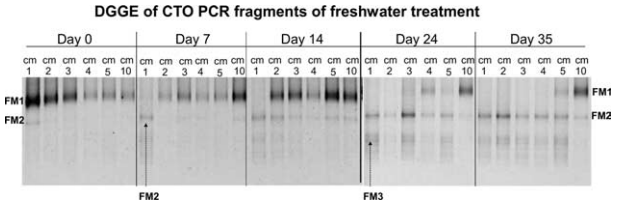

Fig. 7. Denaturing gradient gel of CTO-PCR fragments (direct amplification). A complete profile in a freshwater replicate microcosm over time (day 0, 7, 14, 24 and 35) at six different depths (1, 2, 3, 4, 5 and 10 cm) is shown.

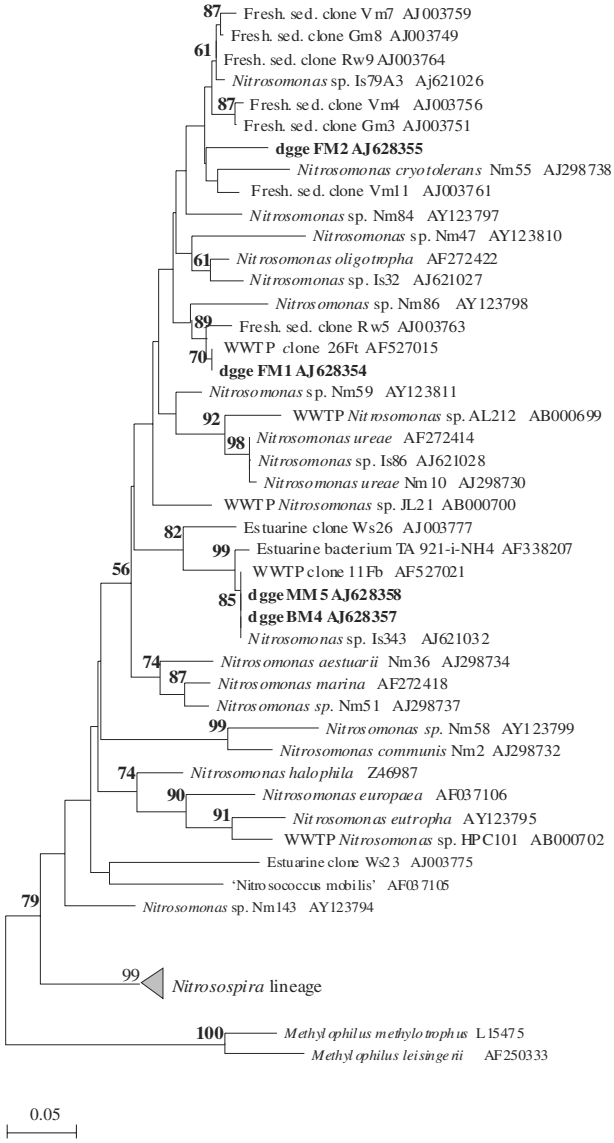

Fig. 8. Neighbour-joining tree of selected 16S rRNA genes of ammonia oxidisers from *Betaproteobacteria*. Sequences determined in this study are printed in bold and codes correspond to Figs. 6 and 7. The phylogenetic tree was based on 417 informative positions. The scale bar represents 0.05 fixed position mutations per nucleotide sequence position. Values at the node indicate bootstrap percentage over 1000 replicate trees. Trees were rooted using *E. coli* (U00006) and *Agrobacterium tumefaciens* (AJ389886) as outgroup.

FM2 had appeared at 1 cm depth after 7 days, at 2 cm after 14 days and at 5 cm depth after 24 days. The unclear band FM3 appeared at day 24 at all depths.

Band FM1 belonged to the *N. oligotropha* lineage with 97% and 95% identity to *N. oligotropha* and *Nitrosomonas ureae*, respectively. Band FM2 also belonged to the *N. oligotropha* lineage with 96% and 95% identity to *N. oligotropha* and *N. ureae*, respectively. Bands FM1 and FM2 both showed more than 97% identity to *Nitrosomonas* strain Is. 79 A3. Band FM3 was mostly related to a *Decholoromonas* species, which is not an ammonia oxidiser, and is thus not presented in the phylogenetic tree (Fig. 8). Band BM4 and MM5 were identical (100%) and belonged to the *N. marina* lineage; they showed 99% identity with *Nitrosomonas* strain Is. 343, which has been isolated from the brackish part of the Scheldt estuary (A. Bollmann, personal communication).

4. Discussion

The aim of this study was to assess spatial and temporal dynamics of AOB communities under environmental perturbations. The microcosms system designed for the experiment facilitated the assessment of the response of freshwater AOB communities to tidal flooding with saline water under nearly natural, but controlled conditions.

Bacteria from the *N. oligotropha* lineage of the beta-proteobacteria appeared to dominate the initial ammonia-oxidising community of the intertidal freshwater sediment. It is indeed known that in natural freshwater environments, members of the *N. oligotropha* lineage are generally the dominant representatives of AOB [2,12]. Our results match studies on other estuarine systems, such as the Seine [16] and the Elbe [15] estuaries, as well as with previous studies on the Scheldt estuary [13,14].

The initial salt-sensitive population was clearly replaced by salt-tolerant or halophilic ammonia oxidisers, when the sediments were flooded with saline water; their proliferation occurred after 14–24 days. Changes occurred earlier in the marine than in the brackish microcosms; apparently, higher salinity of the marine microcosms led to a faster shift from salt-sensitive members of the *N. oligotropha* lineage to salt-tolerant or even halophilic AOB members of the *N. marina* lineage. This difference indicates that AOB that are appearing are more likely halophilic than salt-tolerant. Enrichment of AOB from the brackish part of the Scheldt estuary yielded, on the contrary, salt-tolerant bacteria [14].

The *N. oligotropha* and the *N. marina* lineages of ammonia oxidisers are considered phylogenetically different [7,8] and have pronounced differences in distribution patterns and ecophysiological characteristics [1]. Obviously the conditions in the freshwater sediment of Appels are not such that species belonging to the *N. marina* lineage can become dominant members of the AOB community; these AOB remain in these sediments and wait for conditions that match their physiology such as higher salt concentration before emerging.

Unexpectedly, flooding with freshwater also induced changes in the ammonia-oxidising community. In the freshwater microcosms a shift occurred within the *N. oligotropha* lineage. This change occurred already in the first week of incubation at 1 cm depth.

In a study of ammonia-oxidising bacterial population of the Scheldt estuary, Bollmann and Laanbroek [14] showed that ammonia oxidisers grew better in their natural water supplemented with ammonia compared to mineral medium. They suggested that the presence of homoserine lactones or small organic compounds or ions, not provided in the mineral medium but present in the natural environment, could enhance the growth of strains different from those dominant under natural conditions. Another explanation for the observed community shift in the freshwater microcosms may be temperature. In addition to a partial effect of soil ammonia concentration, Avrahami and Conrad [29] demonstrated a direct effect of temperature on the composition of the ammonia-oxidising community in soils. Temperature at the sampling site was about 8–10 °C, while the experiment was conducted at 25 °C. The higher temperature could have selectively facilitated the growth of ammonia oxidisers that were negligible under the natural conditions. Although the data on the potential nitrification activities have to be considered with caution, because they were measured only at the beginning and at the end of the experiment in only two out of the four replicated treatments, we observed a clear decrease of activity with incubation time. Such a decrease in activity might be expected in the brackish and marine treatments due to osmotic stress and induced anoxicity, but it was quite surprising in the freshwater treatment. It might indicate that the original freshwater community had suffered from the mineral medium used for incubations and possibly also from higher temperature. However, at the end of the experiment, the potential nitrification activity was still within the range of values measured in sediment slurries collected at the sampling site.

We clearly observed that changes in the AOB community in the freshwater microcosms occurred down to a depth of 5 cm, while changes in the brackish and marine microcosms were detected only at 1 cm depth. This is interestingly linked with the presence of a blackish layer, hence of reduced and anoxic conditions, at the 1-cm depth in both brackish and marine treatments after 7 days of incubation. Since changes in the AOB community in the freshwater microcosms were observed down to 5 cm depth, it can be assumed that oxygen was not a limiting factor in this zone. It is significant to point

out that in this part of the sediment and down to 6–8 cm, the activity of worms took place. Worms persisted actively in the freshwater microcosms but not in the brackish and marine microcosms. The direct effect of the Oligochaetes on the microbial communities in our microcosms is not known. However, detailed studies demonstrate the strong impact that tubificids have on grain size distribution, erosion, water content, diffusion, permeability and oxygen demand of the sediments [26]. Data on the water content might be an indication of the impact of worms; in brackish and marine microcosms, water content was maintained constant during the time and along the depth. In contrast, the water content in the freshwater microcosms increased manifestly down to 6–8 cm during the incubation. This zone correlates strongly with the oxygenated layer we observed.

We are aware that our observations can not explain the shift that occurred in the freshwater microcosms because worms will also play a role in the natural environment, but the results open new questions about the discrete or combined role of various factors on niche differentiation of ammonia oxidisers even among closely related strains. In conclusion, our results confirmed that the composition of ammonia-oxidising community changes under environmental perturbations; further we showed that community changes or niche differentiation under natural conditions can take place within days and that salinity is a steering factor in selecting *Nitrosomonas* species in the sediment.

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