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Response of Induced Perturbation on Replicating β-Proteobacterial Ammonia-Oxidizing Populations in Soil

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Abstract The short-term response of induced perturbation by 4-ethylphenol on β-proteobacterial ammonia oxidizers (β-AOB) was investigated in two soils with initial differences in community structure. The hypotheses were that short-term effects of a disturbance of the AOB community is best monitored by specifically looking at the active populations and that soils with dissimilar active AOB populations would display different degree of resistance or resilience. Two soils from a previously characterized long-term field study fertilized with manure or sewage sludge was used. Soil microcosms were incubated in the laboratory over 15 days. The substrate-induced ammonia oxidation was measured, and the composition of β-AOB communities was determined by PCR–DGGE of specific β-AOB 16S rRNA gene fragments. Actively replicating members of the β-AOB were distinguished by the use of bromodeoxyuridine (BrdU) immunocapture. This approach demonstrated that only a minor fraction of the total AOB community was active. Exposure to 4-ethylphenol resulted in approximately 90% lowered substrate-induced ammonia oxidation rates in both soils. This activity inhibition was not accompanied by shifts in β-AOB community structure when total β-AOB DNA was studied. By contrast, changes were seen in the DGGE banding pattern of the BrdU-labeled community DNA after 4-ethylphenol addition in the manure-fertilized soil. In the sewage sludge fertilized soil, the banding pattern of the BrdU-labeled β-AOB remained unchanged, but bands were weaker after the disturbance. In conclusion, it was shown that BrdU immunocapture was applicable to detect shifts in community composition among replicating β-AOB populations in soil. However, this was not reflected by the soils' ammonia oxidation capacity to resist to or recover from the induced perturbation suggesting that rapid population shifts may not influence soil functioning in a short-term perspective.

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Introduction

Nucleic acid-based approaches have shown that microbial diversity is extremely high in soil ecosystems [38, 45]. The soil microbial communities provide important services and biodiversity is assumed to be an insurance for soils to resist to and recover from disturbances or cope with changing environmental conditions. For example, high bacterial diversity has been shown to increase the ability of a soil to withstand perturbations caused by pollutants [13]. Different microbial taxa are capable of performing the same functions and the hypothesis of functional redundancy claims that when many taxa have similar features, biodiversity loss would not have a major impact on ecosystem functioning [30, 50]. Redundancy has therefore been proposed as an indicator of soil quality [55]. However, recent studies suggest that the taxa in many microbial communities are not functionally redundant and that different communities are not functionally similar [1]. Genotypic dissimilarity is suggested as the major determinant of community performance [8, 17, 21] and low dissimilarity among bacteria has even been shown to have a negative effect on functioning due to increased competition even if richness is high [21].

As a model for studying functional redundancy, the autotrophic betaproteobacterial ammonia oxidizing bacteria (β-AOB) are good candidates [23]. In soil, both the β-AOB and the ammonia-oxidizing Thaumarcheota are responsible

for the oxidation of ammonia to nitrite, i.e., the first step in nitrification. The β-AOB are sensitive to a range of environmental disturbances and have therefore been used as indicator organisms for different kinds of soil perturbations using PCR-based approaches [9, 16, 19, 33, 35, 40]. However, with total DNA extraction it has proven difficult to link perturbations to rapid effects on the AOB community structure [3, 26, 27, 31, 35]. Total DNA extracts do not provide specific information about active taxa, which could be one explanation for the difficulty in identifying populations responding to certain short-term treatments. Targeting the actively growing populations under specific conditions instead of looking at the entire β-AOB community is one approach that can facilitate the study of perturbations on the soil β-AOB community, and estimate the degree of redundancy within this functional guild. By using bromodeoxyuridine (BrdU) immunocapture, in which the thymidine analogue BrdU is incorporated into cells during DNA synthesis, replicating bacterial cells can be labeled and studied separately [6, 48]. This method has been used in some studies to identify the active bacterial populations in soil that respond to different environmental factors, such as carbon substrate supply and presence of plants and mycorrhizal fungi [2, 6, 55], as well as in marine [15, 29, 34] and freshwater ecosystems [51]. However, the BrdU approach has not been used to detect specific functional communities and not for β-AOB.

Our aim was to explore the possibility of differential response of induced perturbation on the actively growing β-AOB populations in two soils with contrasting community composition. Our hypotheses were that short-term disturbances on the AOB community is best monitored by specifically looking at the active populations and that soils with dissimilar active AOB populations would respond differently to perturbations and display different degrees of resistance or resilience due to different genotypes being active. This would suggest that community composition matters for ecosystem functioning and that populations within the AOB community are not functionally redundant. As disturbance factor, 4-ethylphenol was chosen, since it is known to negatively affect the AOB activity [25]. Partial β-AOB-specific 16S rRNA genes were targeted to fingerprint the total and the replicating β-AOB communities, the latter analyzed through BrdU immunocapture, using denaturing gradient gel electrophoresis (DGGE) followed by sequencing of excised bands.

Material and Methods

Soil Sampling

Soil samples were collected in June 2005 from the Ultuna Long-term Soil Organic Matter Experiment, a field site located at Ultuna, Uppsala, Sweden [22]. The soil is a clay loam classified as a Eutric Cambisol according to the FAO system. The experimental site consists of three randomized blocks, each comprising 15 different fertilization treatments in plots of 2×2 m^2 separated by wooden frames, and has been under the same management practices since 1956. For this study, three replicate plots fertilized with manure (J) or sewage sludge (O) were sampled. The organic fertilizers are applied during fall every second year in amounts corresponding to 8 mt of ash-free organic matter per hectare. In addition, 22 kg phosphorus and 35–38 kg potassium chloride per hectare is applied yearly in spring. During the years 1956–1999, spring-sown cereals dominated and fodder rape and fodder beet were occasionally cultivated [22], but since 2000, the plots have been planted with corn (*Zea maize*). Composite samples of ten soil cores (2 cm diameter, 20 cm depth) were taken in between corn rows from each of the chosen plots. The soil was then sieved (4 mm mesh) and stored at −20°C. The soils differed in pH, total nitrogen content, and total organic content as reported by Enwall et al. [11], and summarized in Table 1. The most striking difference between the soil samples was the pH, which was 6.0 in the J-soil and 4.7 in the O-soil.

Experimental Setup and Soil Incubations

In the soil incubation experiment, soil from six field plots were used (the J and O field treatments sampled in three replicate plots). For the activity measurements, nine aliquots of 20 g soil from the composite samples of each plot were distributed to separate 250 ml Duran flasks. Three flasks with soil from each plot were supplemented with 1 ml of 80 mM 4-ethylphenol dissolved in water, added to a final concentration of 500 μg g^{-1} soil. The rest of the flasks were supplemented with 1 ml distilled water. The substrate-induced ammonia oxidation activity was measured in the soil without 4-ethylphenol at day 0 and after 15 days of incubation, and in the 4-ethylphenol amended soil after 15 days. The day-0 flasks were directly transferred to 25°C

Table 1 Soil pH, total nitrogen (Tot-N) and total organic carbon (Tot-C) for manure (J) and sewage sludge (O) fertilized soil in the Ultuna Long-term Soil Organic Matter Experiment

Soil	Fertilizer	pH[a]	Tot-N[a] (% of dw)	Tot-C[a] (% of dw)
J	Manure	6.02±0.09	0.20±0.01	2.20±0.05
O	Sludge	4.68±0.03	0.27±0.01	2.76±0.08

Mean values of the triplicate plots are shown with standard deviations (±S.D.)

[a] When the experimental site was established in 1956, the soil pH was 6.5 and the Tot-N and Tot-C were 0.17% and 1.5% of the soil dry weight (dw), respectively.

for activity measurement according to the method described below. The remaining flasks were incubated at 15°C to mimic normal climatic conditions for 15 days and aerated every second day.

In parallel, aliquots of 8 g soil for each field plot were transferred to separate 100 ml Duran flasks for analysis of the β-AOB community composition. In this way, the soil/flask volume/volume ratio was the same as for the activity measurements. In total, four flasks per plot were prepared. Two of the flasks from each plot were used as negative controls and two were supplemented with 4-ethylphenol (final concentration, 500 μg g^{-1} soil), as described for the activity measurements. To one control and one 4-ethylphenol amended soil from each plot, 0.8 ml of 200 mM bromodeoxyuridine (BrdU; Sigma Aldrich), dissolved in water was added. The BrdU was mixed into the soil with a sterile pipette tip. The flasks were incubated at 15°C for 15 days and treated in the same way as the 250-ml flasks. Total DNA was extracted from samples in these 100-ml flasks at the same time as the activity was measured in the 250-ml flasks described above, at day 0 and after 15 days.

Substrate-Induced Ammonia Oxidation Activity Measurements

The substrate-induced ammonia oxidation rate was assayed as accumulated nitrite after addition of ammonium according to a short incubation, chlorate inhibition technique [4, 44]. In brief, a potassium phosphate buffer containing 0.4 mM (di-) ammonium sulfate was added to 20 g soil and the soil slurries were incubated on a rotary shaker at 25°C for 6 h. Aliquots of 2 ml were taken at regular intervals and the nitrite concentration was determined spectrophotometrically by flow injection analysis (FIA, Tecator, Höganäs, Sweden). Potential ammonia oxidation rates were calculated by linear regression of the accumulated NO_2^-–N over time.

DNA Extraction and PCR Amplification

For each soil sample, three replicate DNA extractions were made using the FastDNA Spin Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's instructions. The three DNA extracts were pooled prior to further analysis.

Partial β-AOB 16S rRNA genes were amplified following a nested approach. First, a 1.4-kbp fragment of the 16S rRNA gene was amplified using the bacterial primers EC9-26 [52] and P13B [42]. In this first PCR, 20 ng soil DNA were used as template and the reaction contained 1.25 U Taq polymerase (Amersham Biosciences, Uppsala; Sweden), 10× reaction buffer with 15 mM $MgCl_2$ (Amersham Biosciences), 200 μM of each dNTP and 50 μM of each primer. An initial 2-min denaturing step at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C, with a final extension for 10 min at 72°C. The PCR products were diluted 50-fold and used as template in a second PCR targeting 16S rRNA genes from AOB of the *β*-subdivision of the *Proteobacteria* using primers CTO189fA/B-GC, CTO189fC-GC, and CTO654r [24]. Reactions were set up using the same concentrations as described above. An initial 2-min denaturing step at 94°C was followed by 34 cycles of 30 s at 94°C, 1 min at 57°C and 45 s at 72°C, and a final 10-min extension at 72°C. All PCRs were run in a PTC-100™ thermal cycler (MJ Research, Inc, Waltham, MA, USA).

Immunochemical Purification of BrdU-Containing DNA

Immunochemical purification of BrdU-labeled DNA was performed by the method of Urbach et al. [48], further modified by Artursson et al. [2]. For each extraction, 27 μl of herring sperm DNA (0.63 mg/ml in phosphate-buffered saline (PBS); Promega, Madison, WI, USA) was denatured at 100°C for 5 min and kept on ice for 5 min, and then mixed to a ratio of 9:1 with monoclonal anti-BrdU antibodies (3 μl; Roche, Basel, Switzerland). This mixture was then incubated on a shaker in the dark for 1 h at room temperature. A 25-μl portion of soil DNA, supplemented with 10 μl of PBS, was also denatured as described above, and then blended with 30 μl of the herring sperm–DNA–antibody mixture. This mixture was further incubated for 1 h in the dark at room temperature with constant agitation. Magnetic beads (Dynabeads), coated with goat anti-mouse immunoglobulin G (DYNAL, Oslo, Norway), were washed three times with 150 μl acetylated BSA in PBS buffer (PBS–BSA [1 mg ml^{-1}]) using a magnetic particle concentrator (DYNAL) and re-suspended in PBS–BSA to its initial concentration. The BrdU-DNA herring sperm antibody mixture was mixed with 10 μl portions of Dynabeads and the incubation was continued for an additional 1 h. After this incubation, the samples were washed eight times with 120 μl of PBS–BSA.

In order to separate the BrdU-containing DNA fraction from the beads, 30 μl of 1.7 mM BrdU (in PBS–BSA) were added to each sample, and a final incubation for 1 h in the dark at room temperature with constant agitation was performed. The DNA was then eluted with the magnetic particle concentrator, isolated by ethanol precipitation, and dissolved in 20 μl of sterile distilled water. After this purification, the BrdU-labeled β-AOB 16S rRNA genes were PCR-amplified by the same procedure as described above.

DGGE and Gel Analysis

DGGE was performed using the Biorad Dcode™ system (Bio-Rad Laboratories, Hercules, CA, USA). PCR products

(approximately 500 ng) were loaded onto 160×160 mm^2 polyacrylamide gels consisting of 7% (*v*/*v*) acrylamide–bisacrylamide (37.5:1), denaturant (urea and formamide) and run in 1.0× TAE–buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA, pH 8.3). The polyacrylamide gels were cast by a 170–9042 Model 475 Gradient Delivery System (Bio-Rad Laboratories), with a denaturing gradient ranging from 35% to 70% (100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60°C for 17 h at 130 V in 1× TAE buffer. The gels were stained for 30 min in 1:10,000 (*v*/*v*) SYBR Gold (Molecular probes, Eugene, Canada) in 1× TAE and visualized with UV-transillumination. Selected bands from the DGGE analysis were excised for sequencing, and were kept frozen in 160 µl sterile dH_2O at −70°C until further use.

Images were documented and analyzed with the Gel Doc 2000 System and Quantity One software (Bio-Rad Laboratories). The gels were analyzed using GelComparII (Version 4.50, Applied Maths). The DGGE band-matching matrices generated from GelComparII were further analyzed using the statistical program PC-ORD (Version 5.10, MjM Software). Cluster analysis was performed with unweighted pair group method with arithmetic mean (UPGMA) linkage using binary data and Bray–Curtis distance measures.

Cloning, Sequencing, and Phylogenetic Analysis

DNA was eluted from the excised DGGE bands by thawing for 1 h at room temperature and freezing for 1 h at −70°C, followed by thawing again at 8°C overnight. The eluted fragments were amplified as described above for the second PCR using CTO primers without GC-clamp in 50-µl reactions. The PCR products were purified with the MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA) after agarose gel (1%) electrophoresis and cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). The inserts were sequenced on one strand by Macrogen Inc. (Seoul, Korea) with an ABI3730 XL automatic DNA sequencer using the vector primers M13F.

The taxonomic affiliation of the sequences was confirmed using the RDPII classifier (http://rdp.cme.msu.edu). Nucleotide sequences were aligned with β-AOB 16S rRNA gene sequences of equivalent length from isolated strains deposited in the GenBank database (www.ncbi.nlm.nih.gov) using the CLUSTAL W software (http://www.ebi.ac.uk/clustalw/). A phylogenetic analysis was performed with the software TREECON [49], applying the Jukes and Cantor correction and the neighbor-joining method [39]. Tree topology was evaluated by bootstrap analysis based on 100 replicates.

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences obtained in this study has been deposited in the GenBank database under accession numbers FJ446558 to FJ446578.

Results

Ammonia Oxidation Activity

The soils from the two long-term fertilization treatments had different initial substrate-induced ammonia oxidation rates (Fig. 1). The three field replicate plots within each treatment did not differ greatly, and they responded in a similar fashion to incubation and perturbation by 4-ethylpehnol. The soil fertilized with manure (J) had the highest rate and the activity was maintained over the 15-day incubation time. The sewage sludge-fertilized soil (O) had a much lower activity. After 2 weeks incubation, it had increased slightly in the undisturbed soil. In both treatments, the activity was strongly inhibited by amendment with 4-ethylphenol (Fig. 1). The substrate-induced rates after perturbation were about 8% of the original activity in both treatments, resulting in very low potential ammonia oxidation rates in the sewage sludge-fertilized soil.

Total Versus Active β-AOB Community Fingerprints

The total β-AOB community structure that was fingerprinted by DGGE was different in the two differently fertilized soils (Fig. 2). The manure-fertilized soil (J) demonstrated a higher number of bands in the DGGE than

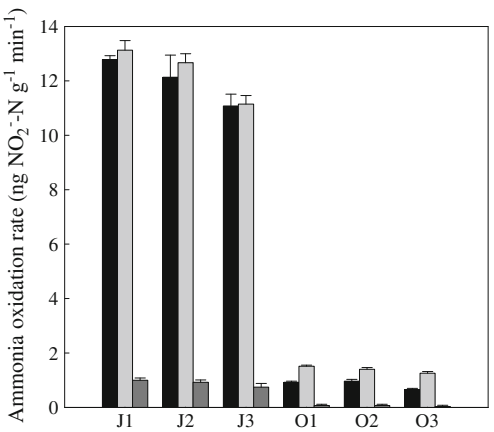

Figure 1 Substrate induced ammonia oxidizing activity in three field replicate plots (*1–3*) of soil fertilized with manure (*J*) or sewage sludge (*O*) in the initial soil samples (*black bars*) and after 15 days incubation at 15°C without (*light–gray bars*) and with stress (4-ethylphenol amendment; *dark–gray bars*). *Error bars* indicate standard deviation, $n=3$

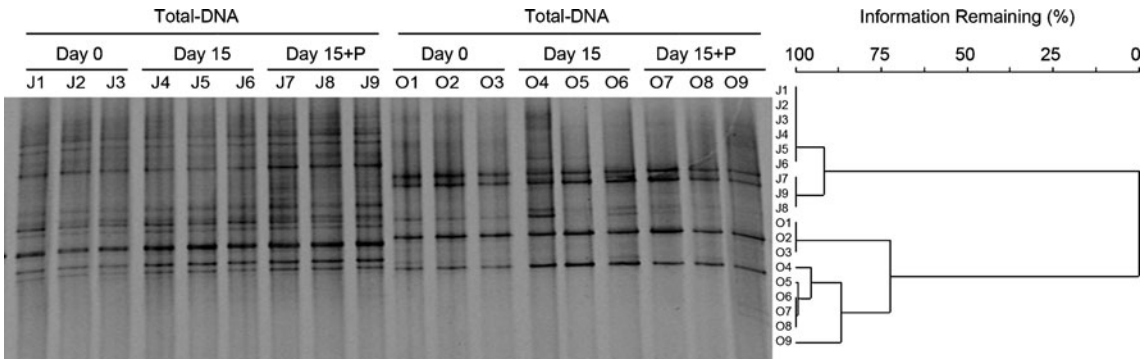

Figure 2 DGGE banding pattern of β-AOB 16S rRNA gene fragments accompanied by an UPGMA dendrogram based on presence–absence of bands from DNA extracted from the three field replicates of soils fertilized with manure (*J*) or sewage sludge (*O*) in the initial soil samples (*1–3*; day 0) and after 15 days incubation at 15°C without (*4–6*; day 15) and with 4-ethylphenol amendment (*7–9*; day 15+P). The *scale bar* over the dendrogram shows percent similarities

the sewage sludge-fertilized soil (O). Overall, the banding patterns in both soils remained unchanged over the incubation period in all field replicates.

PCR amplicons from the BrdU-labeled DNA were readily obtained using β-AOB specific primers. After immunocapture, no bands were detected on the agarose gel from PCR amplification of the negative controls to which no BrdU was added (data not shown). By using the BrdU immunocapture technique, it was shown that the number of active β-AOB populations, detected as DGGE bands, was lower compared to the total β-AOB community in both soils (Fig. 3). Similar to the results for the total DNA, the BrdU-DNA fingerprints of β-AOB differed between the two fertilizer treatments, whereas the field replicates were almost identical, which was confirmed by the cluster analysis (Fig. 3). The active β-AOB in the two soils responded somewhat differently to the addition of 4-ethylphenol, but for both soils, all bands detected in the active fractions were also seen in the total DNA fingerprints. In soil fertilized with manure (J), addition of phenol resulted in a shift in the active β-AOB community structure, with one band present in the undisturbed soil (lane J4-6) not detected in the phenol-amended soil (J7-9) and vice versa (bands J6:3 and J7:8). In the sewage sludge treatment (O), no change in banding pattern was observed after addition of 4-ethylphenol. However, all bands were weaker after incubation with phenol, possibly indicating a decreased abundance of replicating β-AOB populations (Fig. 3).

β-AOB Sequence Analysis

Sequencing of the excised DGGE bands revealed that all obtained sequences belonged to the *Nitrosospira* genus (Fig. 4). In total, 16 different sequences were obtained, as some bands, denoted J3:1, J3:2, and O3:11, harbored two or three sequences. Sequences from soil fertilized with manure (J) were related to *Nitrosospira* spp. belonging to clusters 0, 2, 3, and 4 (as defined by Purkhold et al. [36, 37]), whereas sequences from soil fertilized with sewage sludge (O)

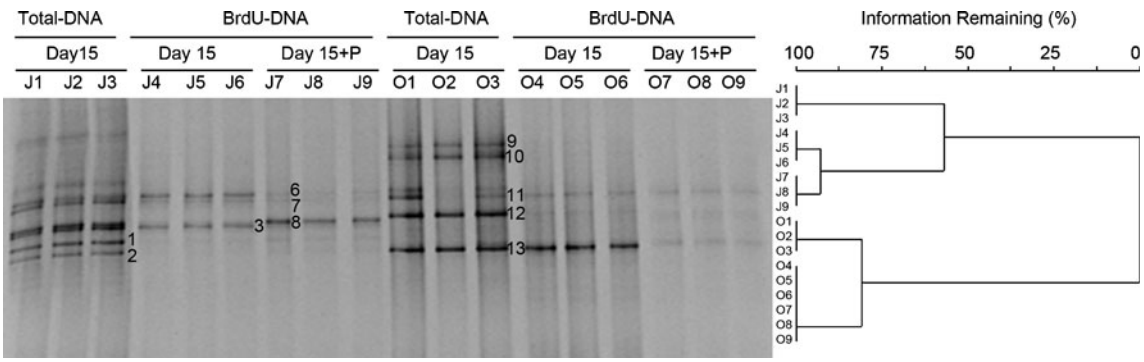

Figure 3 DGGE banding pattern of β-AOB 16S rRNA gene fragments accompanied by an UPGMA dendrogram based on presence–absence of bands from total and active βAOB communities in soils fertilized with manure (*J*) or sewage sludge (*O*). Total β-AOB fingerprints (*1–3*; total-DNA; day 15) originate from total DNA extracts after 15 days incubation at 15°C. The active β-AOB fingerprints were obtained from BrdU-labeled DNA after 15 days incubation at 15°C without (*4–6*; BrdU-DNA; day 15) and with 4-ethylphenol amendment (*7–9*; BrdU-DNA; day 15+P). Excised bands (*1–13*) are marked in the figure. The *scale bar* over the dendrogram shows percent similarities

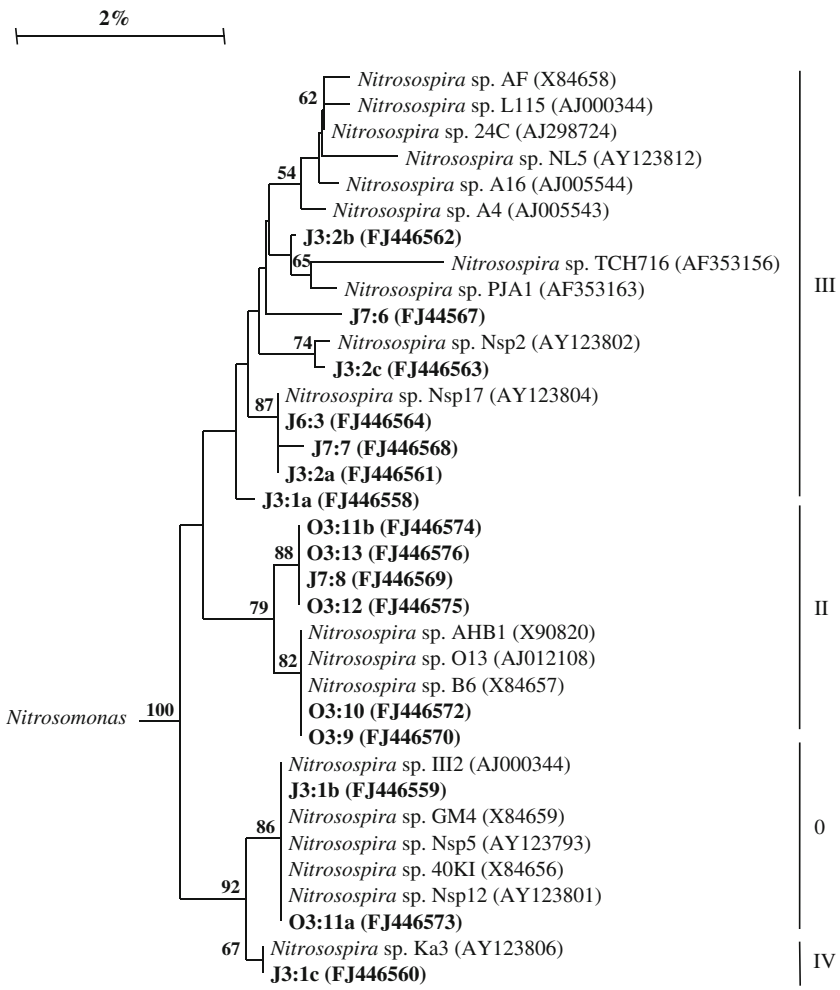

Figure 4 Neighbor-joining tree inferred from analysis of 424 bp 16S rRNA gene sequences from isolated β-AOB and environmental samples. The sequences obtained from the excised DGGE bands in the present study are in *bold*. Bootstrap values higher than 50% are shown

mainly related to sequences in cluster 2, with the exception of sequence O3:11a that related with *Nitrosospira* spp. in cluster 0. Sequencing confirmed that the bands J6:3 and J7:8, which are situated close in the gel, actually were two different sequences related to *Nitrosospira* spp. in clusters 3 and 2, respectively.

Discussion

In this study, BrdU immunocapture was used in combination with DGGE to monitor the short-term response of native β-AOB communities in arable soil to a model perturbation factor. We have demonstrated that the BrdU immunocapture technique is applicable to detect actively replicating β-AOB populations in the environment. Previous studies have argued that there may be differences in the capacity of bacteria to incorporate BrdU into their DNA [7, 48]. For instance, bacteria with a G+C rich genome were not detected in BrdU-labeled DNA in soil samples [2], although BrdU-positive cells from the G+C rich *Actinobacteria* were detected without problems in lakes [51]. Pernthaler et al. [34] found that most marine isolates tested in their study incorporated BrdU. It was also demonstrated recently that taxonomically diverse bacteria representing all the dominant phyla in soil incorporate BrdU, although BrdU uptake efficiencies varied and some bacteria were not capable of BrdU uptake at all [18]. It is important to be aware that BrdU uptake and incorporation may differ among bacteria when the technique is used to target active, uncultured populations in the environment. For comparison of bacterial response to perturbation in different soil samples, interpretation should be simpler using a certain sub-group of organisms, such as the β-AOB, rather than the total bacterial community. Because the AOB in the β-proteobacteria are phylogenetically close, it is more likely that the active β-AOB in our soil samples incorporated BrdU to a similar extent.

A prerequisite for the present study was that the soils would harbor different β-AOB communities prior to the imposed perturbation. Therefore, soils fertilized with manure (J) or sewage sludge (O) from a long-term field site was used and, as expected, the β-AOB fingerprints based on total DNA differed substantially between the two

soils, and they were nearly identical to those reported for soil sampled from the same plots 3 years earlier [11]. Amendment with 4-ethylphenol caused a substantial decrease in ammonia oxidation rates in both soils. However, this activity inhibition was not accompanied by any shift in DGGE banding pattern when the total β-AOB community was targeted. This is in agreement with findings in a previous study, in which a decrease in ammonia oxidizing activity caused by a mix of organic compounds, including different phenols, was not mirrored in a community shift of AOB [31]. Avrahami et al. [3] also reported changes in activity without modifications in the β-AOB community composition monitored by DGGE fingerprinting of *amoA* genes after 4 weeks of incubation when comparing effects of different concentrations of ammonium. Similarly, no effect on community structure of β-AOB was seen after nitrification inhibition with acetylene over a 30-day incubation period in microcosms [32]. Both these studies concluded that during the incubation period either no or slow growth of the β-AOB community occurred. It is possible that the majority of the targeted AOB were not active during these experiments and that small community changes may be more rapidly detected when only the active AOB populations are targeted. This was confirmed in the present study, in which BrdU immunocapture was used to exclude the inactive populations, demonstrating that only a fraction of the β-AOB present were active under the tested conditions.

Among the active β-AOB, only two major bands, and a few minor ones, were visible on the DGGE in both soils. In contrast to the total DNA fingerprints, 4-ethylphenol amendment had an effect on the active β-AOB populations. However, the communities in the two soils responded differently to the disturbance. In soil J, a shift in the community structure was detected, with one major population that was not present in the undisturbed soil appearing in the disturbed soil. In addition, two major populations either disappeared or became very faint. In agreement, Tourna et al. [46] detected minor changes in the AOB community structure as a response to a change in temperature by targeting the transcriptional activity of mRNA. In the present study, the DGGE band pattern in soil O remained unchanged, but the AOB populations most likely decreased in abundance after 4-ethylphenol disturbance as indicated by the faint bands. The fact that the two soils had different initial substrate-induced activity prior to the induced perturbation indicate differences in abundance or growth rates between the two AOB communities, which would support the observed population shift in soil J and the fading bands in soil O. We suggest that there were differences in growth rates rather than abundances since previous studies have shown that there is no difference in AOB community size between the two soils with both harboring about 7×10^6 *amoA* gene copies per gram dry weight soil [14]. The initial differences in activity might be an effect of the β-AOB populations in the sewage sludge-treated soil already being subjected to stress due to the combination of lower pH and the presence of heavy metals [5, 11, 12, 53, 54], which would make the AOB more predisposed to the imposed 4-ethylphenol stress. Tobor-Kaplon et al. [43] found lower resistance and/or resilience in soils with low pH and/or copper contamination, which supports the notion that environmental stress alters ecosystems in such a way that additional stress will have stronger impacts than in an unstressed system.

The soils did not display any functional responses supporting differences in resistance or resilience to the induced perturbation even though there were genotypic dissimilarities between the soils with respect to both the total and active dominant AOB populations, demonstrated by contrasted DGGE profiles and phylogenetic differences. Sequences from soil O mainly clustered within *Nitrosospira* spp. cluster 2, which is in agreement with previous findings of a tendency for acidic soils to be dominated by this group [24, 41]. Sequences from soil J clustered within *Nitrosospira* spp. clusters 0, 2, 3, and 4 suggesting greater phylogenetic divergence among the AOB 16S rRNA gene sequences in this soil, although this needs to be verified by sequencing a substantially larger fraction of the AOB communities. Even though genotypic dissimilarity is suggested as the major determinant of community performance [8, 17, 21], the shift in the active AOB community in soil J was not enough to ensure maintained ammonia oxidation potential at the initial level or a recovery of the activity in the present study. However, not only the β-AOB, but also the ammonia oxidizing archaea (AOA) could be important for ammonia oxidation in our two soils. In a previous study, it was shown that the AOB and AOA were equally abundant in soil J, but in the acidic soil O the AOB outnumbered the AOA with 2 logs [14]. Pure culture studies have demonstrated that the AOB prefer higher ammonia substrate concentrations than the AOA [28, 47]. It is therefore likely that the substrate-induced ammonia oxidation assay used in the present study favors the activity of the AOB due to the high concentration of ammonia used and the measured potential rates would reflect the activity of the AOB rather than that of the AOA. This assumption is further supported by other studies in which the AOB have been proposed to be functionally more important in nitrogen-rich environments compared with the AOA [10, 20, 56]. The incubation time was only 15 days and we cannot exclude that the community shift of AOB would result in recovery of the ammonia oxidation process in a long-term perspective. The time for recovery could also have been too short in relation to the severity of the perturbation and it is possible that a different result would

have been obtained if a lower concentration of 4-ethylphenol had been used. Mertens et al. [28] showed that soil nitrification was restored to pre-exposure levels within 3 years after Zn contamination in the field and accompanied by a distinct change in the AOB community structure. It was concluded that the Zn-contaminated soil harbored *Nitrosospira* populations that could be activated during a stress situation similar to what as observed for soil J in the present study, but that it can take substantial time before a recovery of soil nitrification is observed.

In conclusion, this study showed that the BrdU immunocapture technique is applicable to detect replicating β-AOB populations in soil samples. Only a minor fraction of the total AOB community was active and shifts in community structure after an induced perturbation were only observed among the active β-AOB. These results highlight a major drawback with analyzing only total DNA when studying short-term perturbation responses on microbial communities. Additionally, this study has shown that the resistance and resilience to the induced perturbation by 4-etylphenol was not different between soils harboring different AOB communities, despite the apparent shift in community composition among the replicating AOB. Our results suggest that rapid population shifts may not be relevant for soil functioning in a short-term perspective.

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