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Community Analysis of Ammonia Oxidizing Bacteria through Molecular Genetics in Activated Sludge of Effluent Treatment Plant

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Abstract

We investigated the communities of Ammonia-Oxidizing Bacteria (AOB) in activated sludge using Polymerase Chain Reaction (PCR) followed by Terminal Restriction Fragment Length Polymorphism (T-RFLP), cloning, and sequencing of the alpha-subunit of the ammonia monooxygenase gene (amoA). In this study the techniques of specific amplification of ammonia oxidiser 16S rDNA fragments by PCR, separation of mixed PCR samples by Denaturing Gradient Gel Electrophoresis (DGGE), and band identification by specific hybridization with oligonucleotide probes were combined to allow for the comparison of the community composition of multiple samples over space and time. DGGE bands of interest were also excised for DNA isolation, reamplification, sequence determination and phylogenetic analysis. We compared monthly samples by the emergent macrophyte *Glyceria maxima* to determine the seasonal effects that the plant roots and the oxygen availability might have on the β-subgroup ammonia-oxidiser populations present. Similarly, five soil or sediment samples, varying in oxygen availability, from different locations were compared. Although the presence of two previously defined Nitrosospira sequence clusters could be differentially detected in the samples examined, there was no evidence for a particular group which was specific to periodically anoxic environments.

Keywords: Nitrosospira; Nitrosomonas; Diversity; Nitrification; Oxygen limitation

Introduction

Rapid industrialization has necessitated the manufacture and use of different chemicals day to day life [1]. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide [2]. Pollution due to textile industry effluent has increased during recent years. Moreover, it is very difficult to treat textile industry effluents because of their high Biochemical Oxygen Demand, Chemical Oxygen Demand, heat, color, pH and the presence of metal ions [3]. Ammonia in aquatic environments can be toxic to fish and other aquatic life and contributes to eutrophication of water bodies [4]. Accordingly, removal of ammonia in wastewater is one of the primary tasks of the modern wastewater treatment process. Although activated sludge is a common process for wastewater treatment, nitrification failure unfortunately occurs frequently in many WWTPs [4,5], since nitrifiers, especially AOB, grow very slowly, and they are highly sensitive to several environmental and engineering factors, including temperature, pH, Dissolved Oxygen (DO), and a wide variety of chemical inhibitors [6,7]. Therefore, a better understanding of the microbial ecology of AOB in WWTPs could potentially improve the nitrification stability [8]. Culture-dependent methods are biased by the selection of species which obviously do not represent the real dominance structure, and hence give a poor understanding of AOB community structure [9]. To overcome these limitations, currently molecular biology techniques can be used to analyze sequences of the 16S rRNA and amoA genes to reveal AOB communities in various environments [10]. A number of studies have used molecular biology techniques to examine the influence of various factors on AOB community structure in WWTPs [4,11-14]. To date, however, the relative influence of specific deterministic environmental factors to AOB community dynamics in WWTP (with associated concurrent changes in a multitude of environmental parameters) is uncertain [5]. Also, the ecological principles underlying AOB community dynamics and nitrification stability and how they are related are poorly understood. Wittebolle et al. [15] have showed that in a laboratory-scale Sequential Batch Reactor (SBR), the AOB community had a weekly change rate of 1365% on 16S rRNA gene level despite the stable function of nitrification. This suggested that in laboratory-scale reactors, the functional stability of nitrification was not necessarily accompanied by AOB community stability. In the larger dimensional WWTPs, it remains unknown whether the frequent arrival of allochthonous organisms leads to a more stable or more dynamic community structure [11]. An equilibrium model based on island biogeography also predicts that the scale of the bioreactor will affect the microbial communities within it [16]. Wells et al. [5] observed the temporal oscillations of AOB populations within a full-scale WWTP while nitrification remained stable. However they did not evaluate the change rate, thus it is not clear whether the larger dimensional WWTP. The aim of this study was to investigate whether the seasonal dynamics and adaptations of the ammonia-oxidizing community in the root zone of *G. maxima*, as was found by Bodelier et al. [7], are reflected in the sequence cluster composition of these communities. Therefore, the same sediment and soil samples were analyzed using the PCR, DGGE and hybridization approach outlined above. Where hybridization results failed to fully resolve DGGE patterns or where DGGE patterns and hybridization results were in apparent conflict, DGGE bands were excised for subsequent sequence determination and phylogenetic analysis.

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Materials and Methods

Sample collection

Sediment samples were collected inside and outside the root zone of the emergent macrophyte *Glyceria maxima*. The influence of plant-derived oxygen on the soil environment in these locations shows seasonal fluctuations in the root zone, but not in the bare sediment. Five intact cores (25 cm in depth, 10 cm in diameter) were taken per sampling event, and the top 5 cm were excluded from all analyses. The sediment samples from the root zone of *G. maxima* collected were compared to soil samples from three other locations differing in their toxicity profiles: Five samples were collected per location, and each sample consisted of 32 cores (5 cm in depth, 2.5 cm in diameter).

DNA isolation

DNA from sediment and soil samples was isolated by a modified protocol of Stephen et al. [17]. Sediment or soil samples (0.5 g wet weight), 0.5 ml extraction buffer (120 mM K_2HPO_4 [pH 8]; 5% hexadecyl trimethyl ammonium bromide (CTAB), Sigma), 0.5 g glass beads and 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1 v/v, Sigma) were mixed in a 2-ml destruction tube. Samples were shaken three times at 5000 rpm for 30 s in a mini-bead beater (BioSpec). The tubes were cooled on ice between shaking periods. After centrifugation (5 min, 3000 Ug), 300 µL of the aqueous phase was removed and the rest of the tube's contents re-extracted with an additional 300 µL extraction buffer. The two resulting aqueous phases were pooled and twice extracted with 1 volume of chloroform/isoamyl alcohol (24:1 v/v). After centrifugation, the DNA was precipitated for at least 1 h at -20°C with 0.1 volume 3 M CH_3COONa [pH 5.2] and 1 volume of isopropanol. Centrifugation (15 min, 14000 Ug) resulted in a pellet which was subsequently washed with 70% ice-cold ethanol. Pellets were allowed to air dry, and DNA was resuspended in 40 µL TE buffer (10 mM Tris; 0.1 mM EDTA [pH 8.5]) and 10 µL loading dye. To remove humic compounds, DNA was loaded on a 1% agarose: 1% Polyvinyl Polypyrrol Idone (PVPP) composite gel (0.5UTBE; 1UTBE=90 mM Tris-borate, 2 mM EDTA, pH 8.3) as described by Kowalchuk et al. [17] and run at 100 V for 2 h. DNA longer than 10 kb was excised and isolated from the agarose using the QIA quick gel extraction kit (Qiagen, Chatsworth, CA, USA), and DNA was eluted with 50 µL 10 mM Tris (pH 8.5).

PCR conditions

PCR was first performed using 10-100 ng template DNA with the Eubacterial primers pA and pH [18] using Tbr polymerase according to the manufacturer's recommendations with the following thermocycling program: 1U (2 min, 94°C), 30U (30 s, 94°C; 60 s, 55°C and 75 s, +1 s/cycle, 72°C) and 1U (5 min, 72°C) with a reaction volume of 25 µL. PCR products (all 25 µL) were examined by electrophoresis in a 0.5UTBE 1% low-melting point agarose gel followed by ethidium bromide staining. For all samples, the product of the expected size (1.5 kb) was excised from the gel (total of 100 mg gel material). The gel fragment was melted by heating for 5 min at 65°C, and 1 µL was used as template in a second PCR using the CTO primers (CTOf189-GC, CTO654r), previously described to amplify specifically a 465-bp fragment of the 16S rRNA gene from L-Proteobacteria ammonia-oxidising bacteria [17] with the addition of a 5P GC-clamp [19]. These 50-µL reactions were performed using Expand High Fidelity polymerase (Boehringer, Mannheim) according to the manufacturer's specifications using the following thermo cycling program: 1U (1 min, 94°C), 25U (30 s, 92°C; 60 s, 57°C and 45 s, +1 s/cycle, 68°C) and 1U (5 min, 68°C). PCR amplification from plasmid controls was performed by direct use of the CTO primers using the conditions described above except that 5 ng of DNA was used as template and reaction volumes were 25 µL. All reactions were overlaid with an equal volume of mineral oil (Sigma, molecular biology grade) and run on Thermal Cycler. Final PCR products were examined by agarose gel electrophoresis (1.5% agarose, 0.5 UTBE) and stained with ethidium bromide for visualization upon UV illumination.

Oligonucleotide hybridization Degenerated Gradient Gel Electrophoresis and blotting

PCR products recovered with the CTO primers were subjected to DGGE according to the protocol of Muyzer et al. [20] as adapted by Kowalchuk et al. [17] for the study of ammonia-oxidising bacteria. Gels contained a 38-50% gradient of denaturing chemicals with 100% denaturant defined as 7 M urea and 40% formamide. DNA was visualized after ethidium bromide staining by UV transillumination, and gel images were stored using `The Imager' system. DNA in the polyacrylamide gels was blotted to Hybond-N. Nucleic Acid Transfer Membranes (Amersham, UK), using a Transblot SD (Bio-Rad) according to Muyzer et al. [20]. After completion of the transfer, the DNA was denatured (DNA-side down) on Whatman 3MM (Whatman) filter paper soaked with 0.4 M NaOH; 0.6 M NaCl and similarly neutralised with 1 M NaCl; 0.5 M Tris (pH 8). Membranes were sealed in plastic and stored at 4^3C until further use. Hybridization analyses were conducted using the oligonucleotide probes and hybridization conditions described by Stephen et al. [21]. Specifically, the probes L-Ammo 223r, Nsp436r, Nmonas244r, NspCl3-455r and NspCl4-446r were used to detect 16S rDNA fragments from all ammonia oxidisers, Nitrosospira, Nitrosomonas, cluster 3 Nitrosospira and cluster 4 Nitrosospira, respectively. No attempts to quantify the intensity of radioactive signals were made during the course of this study. 2.5. Sequence analysis of bands excised from DGGE gels Bands chosen for sequence analysis was carefully excised from the DGGE gel with a scalpel. Only the centre-most 50% of each band was excised in order to avoid the lane edges where smearing was observed. DNA extraction, reamplification and DNA sequencing were as described by Kowalchuk et al. [17]. DNA sequence manipulations were performed using the SeqApp program, version 1.9a169 [22], and phylogenetic analyses were implemented through PHYLIP 5.57 [23]. Distance matrix analyses were according to the method of Jukes and Cantor [24] with a masking function to exclude ambiguous data, and phylogenetic tree construction was by neighbour joining [25]. Phylogenetic analysis was performed for 287 positions which could be unambiguously aligned for all sequences used in the analysis. Bootstrapping was conducted with 100 replicates using the program SeqBoot [23]. Bootstrap supports for the sequence clusters were similar to those found previously [17]. Recovered sequences were also tested for homology to known sequences in the EMBL databank using the FastA program [26]. Bands whose nucleotide sequences were determined have been given labels in Figures 2 and 3 which correspond to the sequence names beginning with a `B' in Figure 1. The addition of an asterisk to a band label (Figures 2 and 3) indicates a difference of one base pair from the given numbered sequence. These differences have been shown to be introduced at the ambiguous position of the reverse primer by PCR [18] and have not been included in the phylogenetic analysis.

Results

Recovery of ammonia-oxidiser 16S rDNA

Fragments from sediment and soil samples Attempts to recover PCR products from direct amplification with the CTO primer pair, as

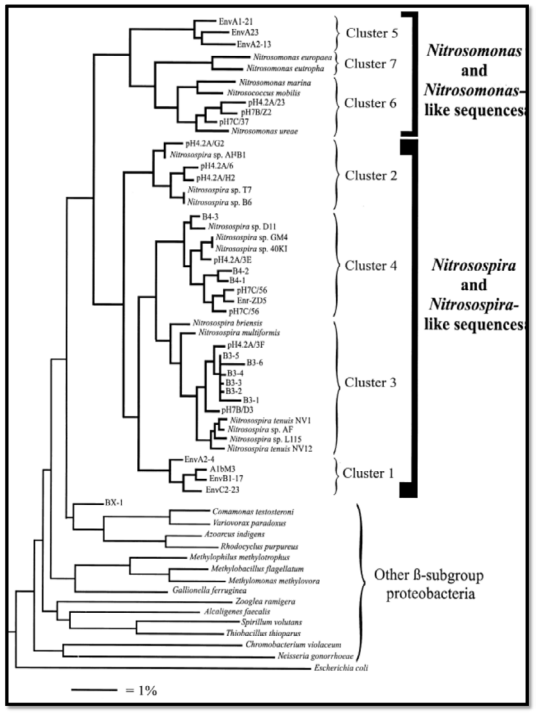

Figure 1: Neighbour-joining tree based upon partial 16S rDNA sequences from L-subgroup ammonia oxidisers.

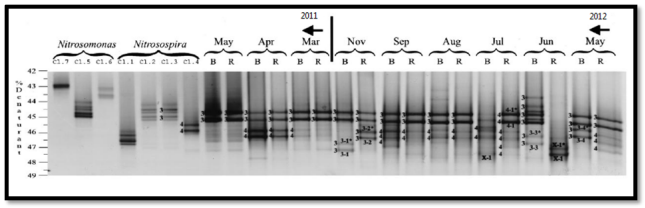

Figure 2: DGGE analysis is of PCR products.

described in Kowalchuk et al. [18], were not consistently successful for all samples, probably due to the inhibitory effect of some co-purified humic substances (results not shown). However, after employment of a nested PCR strategy in which DNA extracted from soil is first amplified with Eubacterial primers prior to specific amplification with the CTO primers, L-subgroup ammonia oxidisers could be detected from all sediment and soil samples analysed. For consistency, all DGGE and hybridization analyses were therefore conducted using the nested PCR protocol. The detection of L-subgroup ammonia oxidisers is in agreement with previous reports of detectable levels of chemo lithotrophic ammonia oxidation for all the sites, and all sampling dates examined [27]. Thus, the nested PCR strategy employed was able to detect less than 10^3 culturable cells g31 dry sediment, as determined previously by MPN analysis [27].

DGGE and hybridization analysis of Drontermeer sediment samples

Nested PCR products recovered from the monthly *G. maxima* root zone and bare sediment samples from Lake Drontermeer were subjected to DGGE analysis along with control fragments derived from cloned sequences of known cluster affinity (Figure 2) [17]. Fragment mobilities from environmental samples ranged from 44.5 to 47.2% denaturant, whereas control ammonia-oxidiser fragment mobilities ranged from 43.0 to 47.0% denaturant concentration. DGGE patterns from environmental samples were quite simple with usually between two and six detectable bands per sample. In both control and environmental DGGE patterns, bands often occurred in doublets, which is consistent with previous results which showed that a single template sequence can give rise to multiple DGGE bands due to an ambiguous position in the CTO reverse primer [18]. Most samples, except the root zone sample from June 1994 and the bare sediment sample from July 1994, displayed a clear double band at approximately 45% denaturant. Another doublet at approximately 46% denaturant could be observed in many samples although its relative intensity was quite variable. These two doublets correspond well to the band positions of Nitrosospira clusters 2 or 3 and cluster 4 controls, respectively. There were no apparent trends in banding patterns with respect to observed seasonal differences in ammonia oxidiser numbers and potential nitrification activities [27]. There were also no consistent differences detected between the DGGE patterns from root zone versus bare sediment samples. Both types of samples gave similar results, whereas other samples produced clear differences between the two zones. In order to determine the sequence cluster affinities of the detected DNA fragments, hybridization analysis was performed using both the genus- and cluster-specific probes described by Stephen et al. [22]. All bands, except the lower doublet root zone sample and the lowest band bare sediment sample, showed positive hybridization with the Nitrosospira-specific probe. The Nitrosospira-negative bands also failed to hybridise with the probe designed to detect all L-subgroup ammonia oxidisers. All Nitrosospira-like bands could be further classified by hybridization analysis into either Nitrosospira clusters 3 or 4, and labels to the left of the bands in Figure 2 indicate predicted cluster affinity. Neither DGGE banding pattern nor hybridization results indicated the recovery of detectable amounts of Nitrosomonas-like rDNA sequences. Hybridization results clearly demonstrated that similar DGGE mobilities were not predictive of common sequence cluster affinities, as was previously demonstrated with known control sequences by Kowalchuk et al. [18].

DGGE and hybridization analysis of soil and sediment samples with different oxicity profiles

PCR products recovered from five different soil/sediment types were also examined by DGGE (Figure 3). All samples gave very similar DGGE patterns with all bands within the range of 44.5-46.5% denaturant. The dominant feature of all DGGE patterns was a doublet

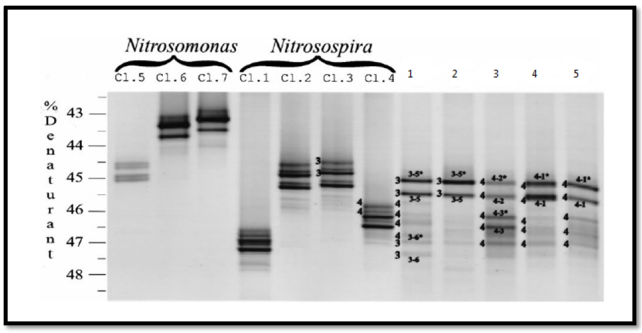

Figure 3: DGGE of soil and sediment samples di¡ering in oxicity. DGGE analysis of five environmental samples differing in their oxicity profiles, with the same clone references used in Figure 2.

at approximately 45% denaturant, although some samples contained other bands lower in the gel. All bands reacted positively with the Nitrosospira-specific probe, and there were no Nitrosomonas-like sequences detected. As with the Drontermeer samples, hybridization analysis revealed the presence of only Nitrosospira clusters 3 and 4 among the ammonia oxidiser sequences detected. Although DGGE banding patterns were quite similar for the five locations examined, the distribution of these two sequence clusters clearly differed, as denoted by the sequence cluster designations given in Figure 3. This is exemplified by the top doublets for each of the five samples which, although resolving to similar positions after DGGE, gave different cluster-specific hybridization results. Both Drontermeer samples (different cores were used as in the analysis of the seasonal dynamics) and the Brummen calcareous grassland soil revealed a predominance of Nitrosospira cluster 4 with no detectable signal from other cluster-specific probes.

Sequence analysis of excised bands

In cases where bands could not be classified by hybridization with cluster-specific probes and where hybridization results differed from those predicted by DGGE mobility, DGGE bands were excised for DNA isolation, reamplification and sequence analysis. The bands which were sequenced have been given names in Figures 2 and 3, and asterisks indicate a 1-bp deference introduced by the reverse primer during PCR. Identical numbers indicate identical nucleotide sequences and correspond to the numbers assigned to the `B' sequences shown in Figure 1. Phylogenetic analysis of the sequence derived from the DGGE doublet at 47.2% denaturant root zone sample revealed that it did not show direct affinity with the monophyletic group formed by all known L-subgroup ammonia-oxidiser 16S rDNA sequences (Figure 1). This sequence was also detected as a minor band bare sediment sample. Although this sequence shows the greatest similarity with 16S rDNA sequences from strains and sequences which fall within the L- subgroup ammonia-oxidiser clade (approximately 92% identity with a number of sequences), its phylogenetic position outside this group precludes the assumption that it was derived from an ammonia oxidiser. Based upon the limited phylogenetic information contained within this fragment, it is impossible to determine whether this sequence is derived from a novel group of ammonia-oxidising bacteria or if it comes from another L-subdivision Proteobacterium lacking this trait. Where DGGE mobility and hybridization analysis were in apparent conflict, DNA sequence analysis confirmed hybridization results for all cases (compare named bands from Figures 2 and 3 with phylogenetic placement in Figure 1). This result was not unexpected, as Kowalchuk et al. [18] demonstrated overlapping fragment mobilities between some sequence clusters.

Discussion

Recovery of ammonia-oxidiser 16S rDNA from natural samples

The nested PCR strategy employed was successful in detecting ammonia-oxidiser 16S rDNA from all soil and sediment samples tested. Previous MPN analyses of the same samples showed less than 103 culturable ammonia-oxidising bacteria g31 dry sediment for some samples [27]. Detection of this number of target cells in a background of at least 10^8 non-target cells compares favourably with previous attempts to detect known numbers of inoculated cells in soils [28,29]. However, as the MPN method detects only culturable cells, this may underestimate the actual number of target cells present. Unlike previous comparable studies [18,22], a nested PCR strategy was used here. Not all samples yielded PCR product after direct amplification with the CTO primers, probably due to a combination of inhibitory contaminants and low numbers of target cells. In samples for which both direct and nested PCR results could be compared, DGGE banding patterns showed only minor deference's in relative band intensities (results not shown), as might be caused by random events or small differences in amplification efficiencies [30]. Furthermore, multiple DNA extractions and PCR reactions from the same core yielded reproducible banding patterns (results not shown).

Fidelity of the CTO primers and cluster-specific oligonucleotide probes

All PCR products recovered by the CTO primers during this study were confirmed to have originated from ammonia oxidiser-like organisms by hybridization, save one double band found in root zone sample and the lowest band of bare sediment sample. As mentioned above, the phylogenetic placement of the sequence derived from these bands is basal to the established Nitrosomonas/Nitrosospira radiation and, in the absence of culture data, cannot be inferred to have originated from an autotrophic ammonia oxidiser. In all cases, phylogenetic analysis of nucleotide sequence data was in agreement with hybridization results. As in previous studies, migration of DGGE bands with control sequences was not an accurate predictor of cluster affinity [17,22] (Figure 1).

G. maxima root zone vs bare sediment samples

G. maxima root zone vs bare sediment samples, variation in space and time Only Nitrosospira clusters 3 and 4 were detected in Drontermeer sediment samples. However, Nitrosomonas urea-like sequences were recovered from MPN cultures from these samples (results not shown), suggesting bias in the MPN method. DGGE has revealed the presence of similar Nitrosomonas-like sequences in soils and sediments when they have comprised as little as 5% of the total recovered sequences [22] suggesting that Nitrosomonas-like strains comprised less than 5% of the ammonia-oxidiser population in these samples. Previous studies with specific antibodies had detected low numbers of Nitrosomonas cells in Drontermeer *G. maxima* root zone samples. No clear population trends corresponded to the seasonal fluctuations in ammonia oxidation [27]. Although month-to-month differences were seen in the distribution of Nitrosospira sequence clusters 3 and 4, such differences appeared random (Figures 2 and 3). Nor were consistent differences detected between root zone and bare sediment samples. The hypothesis that the balance of Nitrosospira clusters 3 and 4 is seasonally regulated in the root zone of *G. maxima* and the nearby bare sediment is not supported. Nitrifying bacteria are known to be able to survive long periods of dormancy [31,32], during which cells may show little or no activity and may exhibit a particular aversion to culturing. PCR-based techniques make no distinction between active and dormant cells, whereas MPN analyses select for easily activated cells, which may account for these differences. Detection of active ammonia-oxidiser populations might be addressed by use of reverse transcriptase PCR followed by the same DGGE and hybridization analyses [33-35]. The use of functionally relevant structural gene targets, such as the genes encoding ammonia monooxygenase [36], and studies related to their expression would also be of interest in this respect. Core-to-core variation might also complicate population distribution results. To address this issue, multiple cores (five) were examined for several sampling points. Slight differences were found between cores from a single sampling date and location (results not shown). Although the vernal peak in potential activity [27] was highly

significant, some months showed high standard deviations. The most variable months with respect to activity measurements did not show the greatest core-to-core variability as detected by DGGE. As fine-scale spatial heterogeneity is important with respect to overall microbial activity [37] and interactions with plants [38], further characterization of core-to-core variation, combining physiological and molecular techniques, is an important topic for future study.

Ammonia-oxidising bacteria from sediments and soils differing in oxicity profiles

No differences in community structure could be correlated with soil/sediment oxicity for the five different Dutch locations tested (Figure 3). Again, only Nitrosospira clusters 3 and 4 were detected. Although clear differences in the distribution of these two clusters were apparent between locations, such differences did not mirror either the oxygen kinetics of the resident ammonia-oxidising communities or oxygen availability [27]. These results do not support the hypothesis that specific sequence clusters of ammonia oxidizing bacteria are specially adapted to survival and growth under different oxygen tensions. Thus, it may be that the greater affinity for oxygen and the resistance to longer periods of anoxia displayed by sediment communities is a physiological adaptation of a generalist nitrifying community. Given that supported sequence clusters within the L-subgroup ammonia oxidisers exist, and that some of these groups correlate with specific environments, physiological differences may underlie phylogenetic groupings [18,22,27]. However, it is possible that very closely related organisms might also have different physiologies, for instance in their affinity for oxygen. This is plausible considering the known variation within cultured members of Nitrosospira cluster 3 [39,40]. The possibility remains that other organisms, such as nitrifiers from the Q-subgroup of the Proteobacteria or heterotrophic nitrifiers, are responsible for the seasonal fluctuations in potential ammonia-oxidising activities. Hybridization with specific probes has proved essential for the identification of recovered 16S rDNA fragments. The evidence presented demonstrates that Nitrosospira-like organisms related to sequence clusters 3 and 4 dominate the ammonia-oxidising community in the root zone of oxygen-releasing *G. maxima* stands. Low-oxygen, or periodically low-oxygen habitats, does not appear to select for a single phylogenetic cluster of ammonia-oxidising bacteria.

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