

Comparative analysis of ammonia monooxygenase (*amoA*) genes in the water column and sediment–water interface of two lakes and the Baltic Sea

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Abstract

The functional gene *amoA* was used to compare the diversity of ammonia-oxidizing bacteria (AOB) in the water column and sediment–water interface of the two freshwater lakes Plußsee and Schöhsee and the Baltic Sea. Nested amplifications were used to increase the sensitivity of *amoA* detection, and to amplify a 789-bp fragment from which clone libraries were prepared. The larger part of the sequences was only distantly related to any of the cultured AOB and is considered to represent new clusters of AOB within the *Nitrosomonas/Nitrosospira* group. Almost all sequences from the water column of the Baltic Sea and from 1-m depth of Schöhsee were related to different *Nitrosospira* clusters 0 and 2, respectively. The majority of sequences from Plußsee and Schöhsee were associated with sequences from Chesapeake Bay, from a previous study of Plußsee and from rice roots in *Nitrosospira*-like cluster A, which lacks sequences from Baltic Sea. Two groups of sequences from Baltic Sea sediment were related to clonal sequences from other brackish/marine habitats in the purely environmental *Nitrosospira*-like cluster B and the *Nitrosomonas*-like cluster. This confirms previous results from 16S rRNA gene libraries that indicated the existence of hitherto uncultivated AOB in lake and Baltic Sea samples, and showed a differential distribution of AOB along the water column and sediment of these environments.

Introduction

Aerobic nitrification is an important process in the cycling of nitrogen in terrestrial, marine, estuarine and freshwater environments. Until recently, only ammonia-oxidizing bacteria (AOB) were known to be responsible for the first, rate-limiting step of nitrification (Prosser, 1989; Head *et al.*, 1993; Kowalchuk & Stephen, 2001). However, meanwhile, the widespread existence of anaerobic ammonia oxidation (anammox) (Jetten *et al.*, 1997; Strous *et al.*, 1999) and the capability of some ammonia-oxidizing archaea (AOA) to oxidize ammonia to nitrite (Könneke *et al.*, 2005; Treusch *et al.*, 2005) became established. AOB use the conversion of ammonia to nitrite as their sole energy source. The oxidation of ammonia to nitrite by AOB is a two-step process: the oxidation of ammonia to hydroxylamine is catalyzed by the membrane-bound protein ammonia monooxygenase

(AMO) and the second step is catalyzed by hydroxylamine oxidoreductase (HAO) (Hooper *et al.*, 1997).

Since the publication of the first *amoA* sequence from *Nitrosomonas europaea* (McTavish *et al.*, 1993), more than 3000 partial *amoA* sequences from bacteria became available in GenBank. The majority originated from soil (40%), brackish or marine environments (37%) and wastewater treatment plants (21%). Only a minor fraction of 2% are from freshwater, illustrating that the investigation of *amoA* diversity in freshwater has been neglected so far. Previous work on communities of AOB in the freshwater lakes Plußsee and Schöhsee and of the Baltic Sea used 16S rRNA gene sequences (Kim *et al.*, 2006). Specific groups of freshwater AOB were observed in the two lakes, whereas in the Baltic Sea marine genotypes were detected, and in addition a differential distribution of *Nitrosomonas* and *Nitrosospira*-like sequences in the water column and sediment occurred.

Comparative phylogenetic analyses of 16S rRNA gene and *amoA* sequences have suggested similar but not identical phylogenetic relationships between these two molecular markers (Purkhold *et al.*, 2000). Relatively few informative sites have been found in the 16S rRNA gene due to the high overall similarity of the partial 16S rRNA gene sequences available from ammonia oxidizers, suggesting that *amoA* can be a more suitable marker for discriminating between closely related species or even ecotypes (Stephen *et al.*, 1996; Purkhold *et al.*, 2000). However, an important limitation of the *amoA*-based approach was the relatively short length and conservation of the *amoA* fragment analyzed in most of the studies carried out so far (Purkhold *et al.*, 2003). Therefore, in the present study a larger fragment representing the almost complete *amoA* gene was amplified to analyze the distribution and community composition of AOB. Owing to the low abundance of AOB in the habitats studied, a nested PCR approach was applied to increase the sensitivity. In many studies, nested amplifications have been applied successfully in order to increase the sensitivity of specific 16S rRNA gene PCR detection in environments with low abundances of AOB (Ward *et al.*, 1997, 2000; Kowalchuk *et al.*, 1998; Phillips *et al.*, 1999; Webster *et al.*, 2002, 2005; O'Mullan & Ward, 2005; Kim *et al.*, 2006).

The purpose of this study is to compare the AOB communities between the two freshwater lakes Plußsee and Schöhsee and the Baltic Sea using improved methods based on the functional marker *amoA*. In addition, considering that information on the composition of AOB communities from these habitats has been obtained previously based on the 16S rRNA gene using the same DNA extracts (Kim *et al.*, 2006), a comparison between both approaches, 16S rRNA gene and *amoA*, was performed as well. To our knowledge, this is the first detailed study that analyzes the diversity of *amoA* sequences in freshwater environments.

Materials and methods

Sampling and sampling sites

Samples from the lakes Plußsee and Schöhsee and the Baltic Sea were taken during thermal stratification in September 2004 (Kim *et al.*, 2006). The sampling depths and the environmental conditions are given in Table 1. In the eutrophic lake Plußsee, the pelagic zone was stratified into three distinct layers: (1) the oxic epilimnion roughly corresponding to the photic zone, (2) the metalimnion with steep gradients of oxygen, temperature and nutrients and (3) the anoxic hypolimnion with high concentrations of NH₃, H₂S and other reduced compounds. In the water–sediment interface high concentrations of nutrients were also observed (Table 2). Samples were taken from the epilimnion at 1-m depth (P1m), from the top of the metalimnion (4-m

Table 1. Environmental parameters for the samples

	Location*	Maximum depth (m)	Depth (m)	Temperature (°C)	DO (mg L ⁻¹)	Salinity (PSU)
Baltic Sea	54.5022 N,	27.5	2	18.0	10.3	13.8
	10.1067 E		10	16.5	9.1	16.5
			20	13.3	3.3	19.7
Plußsee	54.1722 N,	28	1	18.3	8.8	ND
	10.4444 E		4	18.3	8.6	ND
			7	8.2	0.1	ND
			Sediment	4.5	0	ND
Schöhsee	54.1625 N,	22	1	14.9	8.4	ND
	10.4367 E		11	13.7	6.3	ND
			12	9.8	2.0	ND
			Sediment	6.4	0	ND

*Location (latitude, longitude).

ND, not determined.

depth, P4m) and the top of the hypolimnion (7-m depth, P7m) and from the sediment–water interface (Psedi). Schöhsee is a mesotrophic, stratified lake with oxic conditions in the hypolimnion and an aerobic/anaerobic gradient at the sediment–water interface. Samples were taken from the epilimnion at 1-m depth (S1m), from the top of the metalimnion at 11 m (S11m), from the top of hypolimnion at 12-m depth (S12m) and from the sediment–water interface (Ssedi). The Baltic Sea out of Kiel Fjord is characterized as mesohaline with salinities ranging from 13.8 to 19.7 PSU through the stratified water column. Samples were taken from oxic surface water at 2-m depth (B2m), from chemocline at 10-m depth (B10m), from microoxic deep water at 20-m depth (B20m) and from the sediment–water interface at 27.5-m depth (Bsedi).

DNA extraction

For DNA extraction, water samples were filtered through 0.2-µm pore size filters (Supor-200, PALL Life Sciences). DNA was extracted using the UltraClean Soil DNA kit (MoBio), following the manufacturer's guidelines. The concentration and quality of the DNA was checked using electrophoresis on 0.8% agarose gels stained with ethidium bromide.

PCR amplification of *amoA* gene fragments

To increase the sensitivity of detection, nested PCR was performed. Six different primer combinations, including the primers *amoA34f/amoA-2R* used afterwards for nested

Table 2. The physicochemical data in Plußsee

Depth (m)	pH	PP ($\mu\text{g P L}^{-1}$)	TDP ($\mu\text{g P L}^{-1}$)	PO_4^- ($\mu\text{g P L}^{-1}$)	NH_4^+ ($\mu\text{g N L}^{-1}$)	NO_2^- ($\mu\text{g N L}^{-1}$)	NO_3^- ($\mu\text{g N L}^{-1}$)	PN ($\mu\text{g N L}^{-1}$)	TDN ($\mu\text{g N L}^{-1}$)	SiO_2 ($\mu\text{g Si L}^{-1}$)
1	8.4	18	14	1	23	1	1	143	891	138
4	8.4	19	14	1	23	1	0	142	858	136
7	6.8	23	51	19	25	1	0	171	885	146
25	6.5	17	475	435	1768	1	0	156	2381	938

PP, particulated phosphorus; TDP, total dissolved phosphorus; PN, particulated nitrogen; TDP, total dissolved nitrogen.

Table 3. Sequences and positions of the primers used for amplification of *amoCAB*

Gene	Primer	Primer sequence 5'–3'	Position*	References
<i>amoC</i>	amoC58f	CTAYGA CAT GTC RCT GTG G	58–76	Junier <i>et al.</i> (2008)
	305F	GTG GTT TGG AAC RGI CAR AGC AAA	763–786	Norton <i>et al.</i> (2002)
<i>amoA</i>	amoA34f	GCG GCR AAA ATG CCG CCG GAA GCG	34–57	Molina <i>et al.</i> (2007)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	802–822	Rotthauwe <i>et al.</i> (1997)
<i>amoB</i>	amoB1179r	CCA AAR CGR CTT TCC GG	1163–1179	Junier <i>et al.</i> (2008)

*Positions in the *amoCAB* gene of *Nitrosomonas europaea* ATCC 19718 (Acc. no. BX321859).

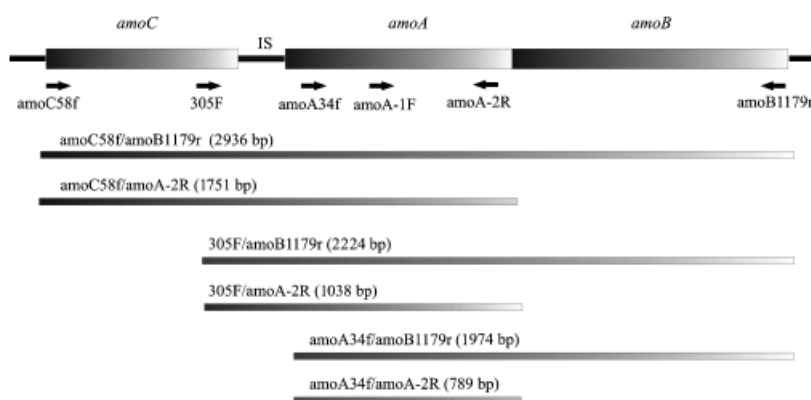


Fig. 1. Structure of the *amoCAB* operon and position of the primers used in this study. Initial PCR amplification with six different primer pairs was used to generate templates for nested *amoA* amplification. The predicted size of the PCR products is in parentheses.

PCR, were used for initial amplification of different regions of the *amoCAB* operon (Table 3 and Fig. 1). Because of the large size (*c.* 3000 bp) of the amoC58f/amoB1179r amplicon, this amplification was carried out using the Expand High Fidelity PCR system (Roche) in a total volume of 25 μL containing 1 \times PCR buffer, 200 μM of each dNTP, 200 nM of each primer, 2.5 mM MgCl_2 , 1% formamide, 0.1% bovine serum albumin (BSA) and 2.6 U of enzyme mix. The temperature program consisted of an initial denaturation at 94 $^\circ\text{C}$ for 2 min and 25 cycles of 94 $^\circ\text{C}$ for 15 s, 56 $^\circ\text{C}$ for 1 min and 68 $^\circ\text{C}$ for 5 min. For all other initial PCR reactions, amplification was carried out using 1 U of *Taq* DNA polymerase (Roche) and 1 \times PCR buffer for *Taq* polymerase. All other components and the concentrations in the master mix were the same as described above. The temperature program consisted of: initial denaturation at 94 $^\circ\text{C}$ for 5 min and 25 cycles of 94 $^\circ\text{C}$ for 1 min, 57 $^\circ\text{C}$ for 1 min and 72 $^\circ\text{C}$ for 1.5 min.

Each of the six initial *amoCAB* amplifications was used as a template for nested PCR or reamplification with amoA34f/amoA-2R using the proofreading *Pfu* polymerase. The amplification consisted of: initial denaturation at 95 $^\circ\text{C}$ for 2 min, hot start at 80 $^\circ\text{C}$ and 25 cycles of 95 $^\circ\text{C}$ for 30 s, 57 $^\circ\text{C}$ for 30 s and 73 $^\circ\text{C}$ for 3 min. Products were checked using electrophoresis in 1.0% agarose gels stained with ethidium bromide. With the sample from Baltic Sea 10 m, which produced a visible PCR product only with the primer combination amoA34f/amoB1179r, triplicates were performed. From Plußsee 7 m and Schöhsee 1 m only duplicates were performed.

Cloning and sequencing

For preparing clone libraries of each sample that was amplifiable, *amoA* products from the nested amplifications with the primers amoA34f/amoA-2R were pooled together,

concentrated in a multiscreen plate (Millipore Inc.) and agarose gel purified using the SNAP kit for gel DNA purification (Invitrogen). Although the use of pooled amplicons for the cloning does not allow estimation of PCR biases of each of the initial primer combinations, results from clone libraries prepared from products of each individual primer pair for the Baltic Sea sediment sample suggest that a similar spectrum of sequences is obtained for all primer combinations used (data not shown). Cloning and sequencing were performed as described previously (Kim *et al.*, 2006) but 48 colonies were picked from each sample. Sequencing of both strands was performed with the primers M13f and M13r. The amplified product of 789 bp (including the primer sequence) was checked using BLASTX (Altschul *et al.*, 1997) (<http://www.ncbi.nlm.nih.gov/blast/Blast>). These sequences were deposited in GenBank under accession numbers EF222028–EF222186 and EF615038–EF615208.

Phylogenetic analysis and rarefaction analysis

Phylogenetic analysis of *amoA* was performed using the software ARB (<http://magnum.mpi-bremen.de/molecol/arb/>). A database of 2152 *amoA* sequences published in GenBank was created. Sequences from this study were included in this database afterwards. An initial phylogenetic tree of *AmoA* was constructed with all sequences, using neighbor-joining clustering. The final phylogenetic tree was calculated with all clonal sequences from this study and the most closely related ones from the database. For tree calculation the sequence of the primers was removed and therefore from the initial amplified product of 789 bp, only 744 positions were considered. The tree was built by Fitch–Margoliash clustering with a substitution matrix of 248 amino acids, calculated using the Jones–Taylor Thornton method. Bootstrap analysis

was used to estimate the reliability of phylogenetic reconstructions (100 replicates).

To compare the diversity within each clone library, rarefaction analysis was performed using DOTUR (Schloss & Handelsman, 2005). Operational taxonomic units (OTUs) were defined at $\leq 5\%$ nucleotide sequence difference. This threshold was selected because it is adequate for the highly conserved *amoA*, to group closely related phylotypes without losing potentially valuable information by the inclusion of phylogenetically distinct sequences (Francis *et al.*, 2003).

Results

Increase of sensitivity for amplifying *amoA*

For *amoA* amplification six combinations of primer pairs were used (Fig. 1). Initial PCR reactions did not produce a visible band in agarose gels stained with ethidium bromide (data not shown). These PCR products were used as templates in a nested amplification with the primer pair *amoA34f/amoA-2R*. Although not all nested PCR resulted in *amoA* amplification, this strategy increased significantly the sensitivity of detection in most of the samples (Table 4). A band with the expected size was obtained with all initial templates from sediment samples of the three habitats and Baltic Sea 2 m. Nested amplification with the primers *amoA34f/amoB1179r* and *amoA34f/amoA2R* was more efficient in samples from the water column. With three samples from the water column of both lakes (1 and 4 m of Plußsee and 11 m of Schöhsee), it was not possible to obtain amplicons with any of the templates used.

The presence of *amoA* from AOA was also evaluated in all the samples. Three specific primer sets were tested (Francis *et al.*, 2005; Könneke *et al.*, 2005; Treusch *et al.*, 2005). A

Table 4. Results of nested amplifications of *amoA* with the primers *amoA34f/amoA-2R* using six different initial amplicons as templates

Site	Depth (m)	Primer combinations for initial amplification of the template					
		<i>amoC58f/amoB1179r</i>	<i>amoC58f/amoA-2R</i>	<i>305F/amoB1179r</i>	<i>305F/amoA-2R</i>	<i>amoA34f/amoB1179r</i>	<i>amoA34f/amoA-2R</i>
Baltic Sea	2	–	+	+	+	+	+
	10	–	–	–	–	+	–
	20	w	w	–	–	+	+
	Sediment	+	+	w	+	+	+
Plußsee	1	–	–	–	–	–	–
	4	–	–	–	–	–	–
	7	–	–	–	–	+	+
Schöhsee	Sediment	w	+	w	+	w	+
	1	–	–	–	–	+	+
	11	–	–	–	–	–	–
	12	–	+	–	–	+	+
	Sediment	w	+	+	+	w	+

+, visible product with predicted size; w, visible product with predicted size but weak band; –, no product.

Table 5. Number of clones carrying an insert amplified by nested PCR with the primers amoA34f/amoA-2R from the different samples, and assignment to 4 taxonomic groups after BLASTX search

Site	Depth (m)	No. of clones	No. of clones in the different groups after BLASTX search			
			<i>Nitrospira</i>	<i>Nitrospira</i> -like	<i>Nitrosomonas</i>	<i>Nitrosomonas</i> -like
Baltic Sea	2	41	40	0	1	0
	10	19	14	2	2	1
	20	33	33	0	0	0
Plußsee	Sediment	46	0	8	0	38
	7	45	3	42	0	0
Schöhsee	Sediment	48	10	38	0	0
	1	19	18	1	0	0
	12	36	0	35	1	0
	Sediment	43	1	31	11	0
All sites		330	119	157	15	39

Nitrospira, $\geq 95\%$ identity with *Nitrospira* spp.; *Nitrospira*-like, $< 95\%$ identity with *Nitrospira* spp.; *Nitrosomonas*, $\geq 95\%$ identity with *Nitrosomonas* spp.; *Nitrosomonas*-like, $< 95\%$ identity with *Nitrosomonas* spp.

band of the right size was obtained only with the primers ArchamoAF-R (Francis *et al.*, 2005) in Baltic Sea 20 m and sediment, Plußsee sediment and Schöhsee 11 m and sediment. Clone libraries were prepared with these PCR products. Although clones carrying inserts with the expected size were obtained, none of the sequences analyzed corresponded to *amoA* according to the results of BLASTX (data not shown). Although the results might suggest the absence of AOA in the samples, it cannot be ruled out that AOA are present in very low abundances and that nested amplification would possibly reveal the presence of archaeal *amoA*, as well.

Closest relatives of *amoA* clonal sequences identified using BLASTX

Clone libraries were made with all sediment and water samples from the Baltic Sea, 7 m from Plußsee and 1 and 12 m from Schöhsee. In total, 330 clones carrying an insert with the expected size (789 bp including the primer) were obtained. All of these clones were assigned to ammonia monooxygenase using BLASTX and no unspecific amplification was observed. The identity of the deduced amino acid sequences with the first hits of BLASTX varied from 88% to 100%. According to the percentage of identity with their closest hits, the sequences were classified into four groups (Table 5). The majority of the clones were assigned to the *Nitrospira*-like (157 clones) or *Nitrospira* (119 clones) groups, while only few clones were classified to the *Nitrosomonas*-like (39 clones) and *Nitrosomonas* (15 clones) groups.

Diversity of *amoA* sequences

Although the number of clones sequenced was not the same in all clone libraries, rarefaction analysis (Fig. 2) was performed using $\leq 5\%$ cutoff at the DNA level to define an OTU to compare the diversity of the *amoA* sequences.

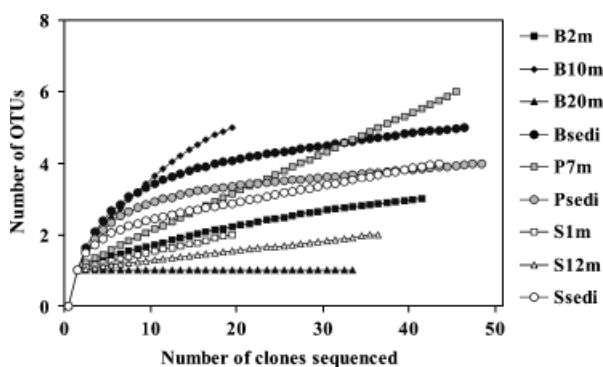


Fig. 2. Rarefaction curves of *amoA*-based OTUs at 5% cutoff at the DNA level. Coding of the samples with three different colors: black, Baltic Sea; gray, Plußsee; white, Schöhsee.

Rarefaction analysis showed that no saturation in the number of different OTUs recovered was obtained specially for Plußsee 7 m (6 OTUs) and Baltic Sea 10 m (5 OTUs recovered), suggesting a higher diversity in these samples. The rarefaction graphs showed saturation in Baltic Sea 20 m with only 1 OTU that corresponded to 16S rRNA gene cluster 0 (*Nitrospira*) in the phylogenetic analysis (Fig. 3), indicating a very low diversity in this sample. Intermediate levels of saturation were observed in the sediment samples from all three environments. The samples from Baltic Sea 2 m and Schöhsee 1 and 12 m showed relatively high saturation, indicating low diversity.

Phylogenetic analysis of *amoA* sequences

In the phylogenetic analysis, the sequences from this study fell into eight clusters. For five of these clusters (134 out of 330 sequences), which corresponded to the *Nitrosomonas*

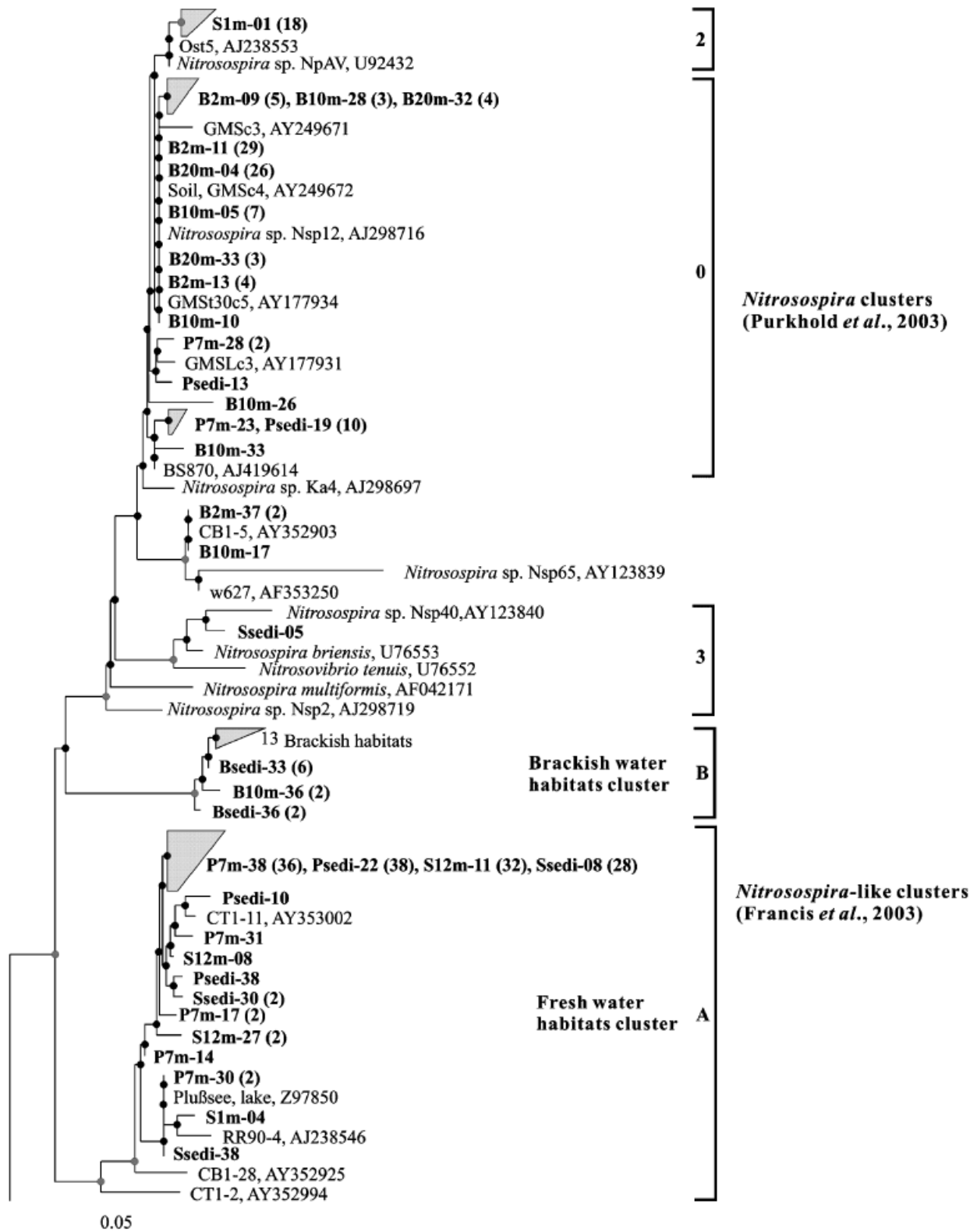


Fig. 3. Fitch–Margoliash phylogenetic tree calculated in ARB with 248 amino acids of AmoA using PmoA from *Crenothrix polyspora* (Acc. no. DQ295904) as an outgroup. Nomenclature of clusters according to Purkhold *et al.* (2003) and Francis *et al.* (2003). Clones from this study are in bold, with the number of identical clones in parentheses and their origin coded with three different letters: B for Baltic Sea, P for Plußsee and S for Schöhsee. Bootstrap values for ≥ 90 replicate trees are indicated at the nodes with three different colors: black (100%), gray (95–99%) and white (90–94%).

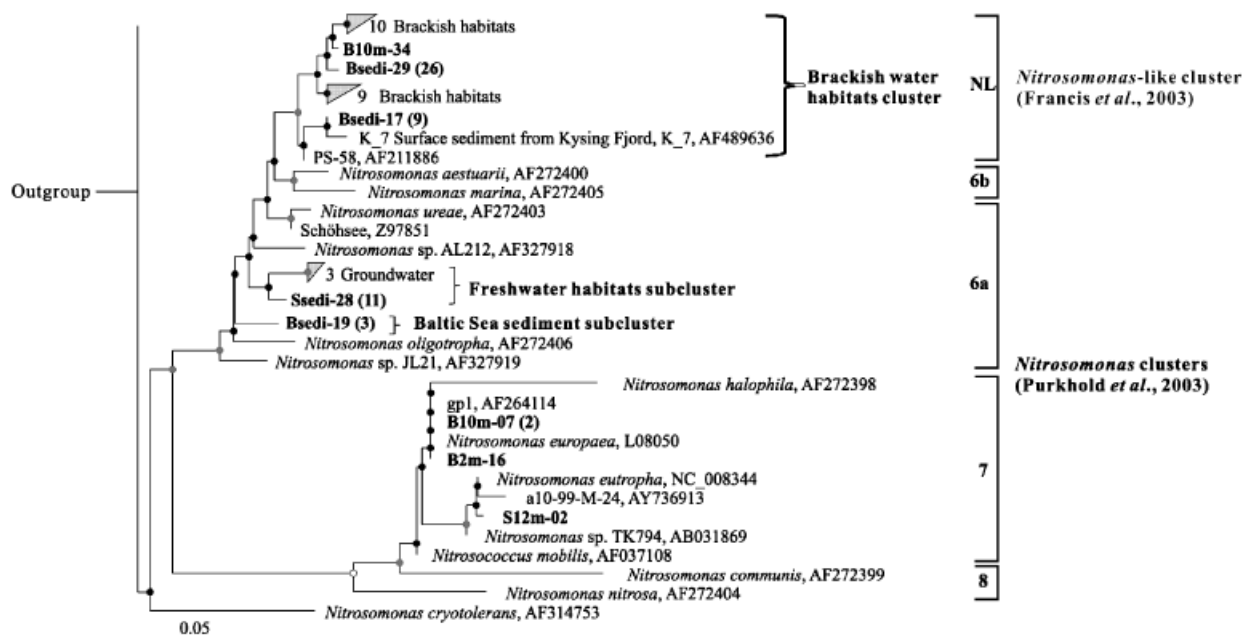


Fig. 3. Continued.

and *Nitrosospira* groups mentioned before, the 16S rRNA gene nomenclature based on sequences from cultured AOB (Purkhold *et al.*, 2003) was followed. For the other three clusters (196 out of 330 sequences), from which no cultured representatives are known yet and corresponded to the *Nitrosospira* and *Nitrosomonas*-like groups, the nomenclature from (Francis *et al.*, 2003) was used. *Nitrosospira* cluster 2 contained sequences from Schöhsee 1 m only. *Nitrosospira* cluster 0 was dominated by sequences from the Baltic Sea water column and included most of the sequences from the Baltic Sea water column and, in addition, a few from Plußsee 7 m and sediment. These were closely related to the *Nitrosospira* spp. Nsp5 and Nsp12, which had been isolated from a freshwater cave lake and soil, respectively (Koops & Harms, 1985), to *Nitrosospira* sp. 40KI isolated from soil (Jiang & Bakken, 1999), as well as to some environmental sequences from a meadow soil near Giessen (Avrahami & Conrad, 2003) and from the Baltic Sea (Tuomainen *et al.*, 2003). *Nitrosospira* cluster 3 had only one sequence from Schöhsee sediment.

Almost half of the sequences from this study (145 out of 330) fell into the *Nitrosospira*-like cluster A, which included sequences from both lakes, but not from the Baltic Sea. Up to now, this cluster contained only a few sequences retrieved from oligohaline habitats in Chesapeake Bay (Sites CT1 and CB1) (Francis *et al.*, 2003), from an enrichment culture prepared from Plußsee in a previous study (Rotthauwe *et al.*, 1997) and from roots of rice plants (Horz *et al.*, 2000). The *Nitrosospira*-like cluster B contained sequences from Baltic Sea 10 m and sediment only, which were related to sequences

from other brackish habitats (Nicolaisen & Ramsing, 2002; Francis *et al.*, 2003; Bernhard *et al.*, 2005; O'Mullan & Ward, 2005).

The *Nitrosomonas* clusters comprised 38 sequences from the Baltic Sea and 11 from Schöhsee, but none from Plußsee. Sequences from the Baltic Sea sediment (and one from 10 m) were grouped into the *Nitrosomonas*-like cluster, which is designated here as the NL cluster, that contained only clonal sequences from brackish and marine environments (Nold *et al.*, 2000; Nicolaisen & Ramsing, 2002; Francis *et al.*, 2003; Bernhard *et al.*, 2005; O'Mullan & Ward, 2005). Some sequences from sediments of Schöhsee and the Baltic Sea were included in the *Nitrosomonas* cluster 6a. Sequences from the Schöhsee sediment were associated with those from groundwater (Ivanova *et al.*, 2000). Sequences from the Baltic Sea sediment formed a cluster separate from those of the Schöhsee sediment and were only distantly related to any other environmental sequences or cultured AOB. Only four sequences from this study fell into cluster 7. Those from Baltic Sea were almost identical to *Nitrosomonas europaea* (over 99% identity of the deduced amino acid sequence) and the one from Schöhsee to *Nitrosomonas eutropha*.

Discussion

To the best of our knowledge, this is the first direct comparison of *amoA* gene diversity in natural freshwater lakes and a mesohaline marine environment. In previous

studies, *amoA* sequences from aquatic environments have been compared either in sediments from different habitats (Francis *et al.*, 2003; Bernhard *et al.*, 2005; Beman & Francis, 2006) or in different depths of the water column in the same habitat (Nold *et al.*, 2000; O'Mullan & Ward, 2005; Molina *et al.*, 2007). In the present study, *amoA* genes were detected in several – although not all – depths of the water column and in the sediment–water interface of all three environments. Therefore, this study provides a good opportunity to compare the diversity of ammonia-oxidizing bacterial communities in a wide range of environmental gradients.

Community differences in the three environments

AOB communities differ considerably between the two lakes and the Baltic Sea as can be expected considering the effect of different salinities (De Bie *et al.*, 2001; Francis *et al.*, 2003; Bernhard *et al.*, 2005; Kim *et al.*, 2006). Although some clusters contained clones from different habitats (*Nitrosospira* cluster 0, *Nitrosospira*-like cluster A and *Nitrosomonas* clusters 6a and 7), the marine and freshwater clones were separated into habitat-specific subclusters. Habitat specificity in the *Nitrosospira*-like subcluster A is well substantiated by the large number of sequences from both lakes and the absence of sequences from the Baltic Sea. This cluster also includes an ammonia-oxidizing enrichment culture from Plußsee (Acc. No. Z97850) that was described in an earlier study (Rotthauwe *et al.*, 1997). Interestingly enough, close relatives of this culture were detected now, about 10 years later, in the clone libraries from Plußsee and Schöhsee. In contrast, the cluster B exclusively contained sequences from the Baltic Sea, closely associated with *amoA* from several brackish environments (Nicolaisen & Ramsing, 2002; Francis *et al.*, 2003; Bernhard *et al.*, 2005; O'Mullan & Ward, 2005) and may be regarded, on the basis of our present knowledge, as specific for mesohaline environments.

Members of the *Nitrosomonas* clusters and the *Nitrosomonas*-like clusters, with a few exceptions, were found in sediment samples from the Baltic Sea and Schöhsee, but not in Plußsee. Representatives of cluster 6a have been found in soil, freshwater and freshwater sediment based on *amoA* (Ivanova *et al.*, 2000) and 16S rRNA gene (Stephen *et al.*, 1996, 1998; Speksnijder *et al.*, 1998; De Bie *et al.*, 2001). It has been suggested that organisms belonging to the *Nitrosomonas* cluster 6a may be restricted to nonsalty environments (De Bie *et al.*, 2001). However, in our study we found a separated subcluster containing sequences from the Baltic Sea sediment. The majority of *Nitrosomonas*-like sequences were retrieved from sediments of the Baltic Sea and clusters with a clonal group from brackish and salty habitats, so far not represented by any cultured representative.

Community differences within each environment

It is well known that AOB are not evenly distributed in stratified water bodies, but occur at certain times of the year and at specific locations within the system (Cavari, 1977; Garland, 1978; Christofi *et al.*, 1981; Robarts *et al.*, 1982). In general, AOB have to find their ecological niche in a counter gradient of oxygen and ammonia that occurs in most stratified lakes during summer immediately below the photic zone (usually the metalimnion), in areas with high nutrient concentrations, or in sediments, especially the sediment–water interface (Garland, 1978; Ward, 1986; Johnstone & Jones, 1988). In the two lakes studied, with the beginning of stratification in spring, different compartments are separated in depth providing niches for the development of specially adapted AOB communities.

In lake Plußsee, all clonal *amoA* sequences from 7 m were similar to those from the sediment. These sequences were grouped into the clusters *Nitrosospira* cluster 0 and *Nitrosospira*-like cluster A. Based on the distribution of the sequences found in lake Plußsee, it could be hypothesized that the members of the cluster A could be microaerophilic or tolerant to anoxic conditions. However, there are no clear evidences of a correlation with tolerance to low oxygen content for environmental sequences from other studies that are included in cluster A (Francis *et al.*, 2003). Whether the clear separation of the clones from Plußsee 7 m and sediment within the *Nitrosospira* cluster 0 indicates that part of these might also be adapted to low oxygen concentrations remains to be studied.

The distribution observed in clones from different depths of Schöhsee also seems to be correlated to oxygen content: almost all clones from the oxygen-rich water at 1-m depth formed a separate group within *Nitrosospira* cluster 2, while clones from sediment and the deeper water layers were grouped into *Nitrosospira*-like cluster A and *Nitrosomonas* clusters 6a and 7. Members of cluster 6a have also been detected by 16S rRNA gene sequences in the suboxic, ammonia-rich sediments of the Schelde estuary (Coci *et al.*, 2005), indicating some degree of tolerance to low-oxygen conditions (Speksnijder *et al.*, 1998; De Bie *et al.*, 2001).

In the Baltic Sea, AOB communities were also considerably different between the water column and the sediment. Most of the clones from the Baltic Sea water column were closely related to the *Nitrosospira* cluster 0. Cultured representatives of this cluster have been isolated mainly from sand, soil and freshwater (Kowalchuk & Stephen, 2001). Recently, a sequence belonging to this cluster was detected in the oxygen minimum zone (OMZ) off the Chilean Pacific coast (Molina *et al.*, 2007), suggesting that members of *Nitrosospira* cluster 0 can be relevant in brackish or marine environments as well. In contrast, sequences from the Baltic

Sea sediment clustered in the *Nitrosospira*-like cluster B and *Nitrosomonas*-like NL cluster, which both appear to be dominant in marine habitats of different salinities and wide geographic distribution (Nold *et al.*, 2000; Nicolaisen & Ramsing, 2002; Francis *et al.*, 2003; Bernhard *et al.*, 2005; O'Mullan & Ward, 2005; Beman & Francis, 2006; Molina *et al.*, 2007).

Comparison of AOB communities characterized by *amoA* and 16S rRNA gene

The *amoA* gene has been recommended as a better molecular marker to study AOB than the 16S rRNA gene (Rotthauwe *et al.*, 1997; Purkhold *et al.*, 2000). 16S rRNA gene clone libraries usually contain a significant proportion (up to 100%) of non-AOB (McCaig *et al.*, 1994; Kim *et al.*, 2006; Mahmood *et al.*, 2006), while clone libraries with *amoA* are usually 100% specific (O'Mullan & Ward, 2005; Molina *et al.*, 2007). This was confirmed when comparing the results of the present study with those from a previous one based on the 16S rRNA gene (Kim *et al.*, 2006). PCR with primers for *amoA* was more specific than with those for the 16S rRNA gene, and all clones with an insert of the expected size contained *amoA*, as confirmed using BLASTX.

The phylogenies of cultivated AOB obtained with the 16S rRNA gene or *amoA* are in general consistent (Purkhold *et al.*, 2000; Aakra *et al.*, 2001), although a few discrepancies have been observed (Juretschko *et al.*, 1998; Aakra *et al.*, 2001). However, this cannot be directly transferred to the comparison of clone libraries from environmental samples. Agreement between both markers has been found in some aquatic environments, such as the Shiprock groundwaters (Ivanova *et al.*, 2000), Monterey Bay (O'Mullan & Ward, 2005) and the OMZ off northern Chile (Molina *et al.*, 2007). Clone libraries of the 16S rRNA gene and *amoA* from the Baltic Sea were also in good agreement. In contrast, clone libraries of 16S rRNA gene and *amoA* from Plußsee did not coincide. In the 16S rRNA gene clone libraries, *Nitrosomonas* sequences were dominant (especially in the sediment sample), and only few *Nitrosospira* sequences were detected (Kim *et al.*, 2006). In the present study, all *amoA* sequences from Plußsee belonged to the *Nitrosospira* and *Nitrosospira*-like clusters, and *Nitrosomonas* spp. were not detected. Additionally, while 16S rRNA gene sequences related to *Nitrosomonas* were detected at 1 m, *amoA* could not be amplified from this sample with any of the primer combinations assayed here (Table 3).

Differences in the outcome of environmental studies using the 16S rRNA gene or the *amoA* approach might be due to: (1) different specificities of the PCR for the 16S rRNA gene and *amoA* or (2) differential sensitivity of the PCR, *amoA* PCR being more sensitive for *Nitrosospira* because of the higher number of *amoA* copies in *Nitrosospira*

(up to three copies) than in *Nitrosomonas* (up to two copies) (Norton *et al.*, 2002). One way to evaluate the effect of differences in the specificity of the primers is the analysis based on sequence comparison. Although the number of sequences encompassing the whole *amoA* is limited, an *in silico* characterization carried out previously. Junier *et al.* (2008) suggested that the primer amoA34f, which was used for generating the clone libraries, matched equally well all available sequences of *Nitrosospira* and *Nitrosomonas* in public databases. Similarly, the primer amoA2-R matches sequences of both genera of betaproteobacterial AOB. In the case of the primers used for the preamplification step of *amoC* and *amoB*, these primers were designed based on all sequences available of betaproteobacterial AOB, which included both *Nitrosomonas* and *Nitrosospira* sequences.

The suitability of the primers can be confirmed experimentally by results obtained from Baltic Sea and Schöhsee in which different groups of both, *Nitrosomonas* and *Nitrosospira* spp., are represented in the clone libraries. Thus, the differences in the results of the 16S rRNA gene and *amoA* for Plußsee could be associated with the complexity of the AOB communities in this particular environment in which a high micro-heterogeneity of the clonal sequences was found.

Comparison of *amoA* diversity in the clone libraries

In order to describe the fine-scale variability of clonal *amoA* sequences, OTUs can be defined at different cutoff values (Hughes *et al.*, 2001). For the 16S rRNA gene, cutoff values from 1% to 3% are most frequently used. However, for functional genes like *amoA*, cutoff values that are applicable for defining OTUs are still debatable (Ward, 2002). Based on rarefaction analysis at a 5% cutoff, the library from Plußsee 7 m was more diverse than that from Baltic Sea 10 m. However, comparing the number of clones sequenced between the two libraries, the library from Plußsee 7 m was less diverse after 20 clones were sequenced. Furthermore, in the phylogeny the clonal sequences from Plußsee 7 m were grouped into only two different clusters, *Nitrosospira* cluster 0 and *Nitrosospira*-like cluster A, whereas those from Baltic Sea 10 m were detected in four clusters in the phylogeny: *Nitrosospira* cluster 0, *Nitrosospira*-like cluster B, *Nitrosomonas*-like cluster and *Nitrosomonas* cluster 7 (Fig. 3). The number of OTUs in Plußsee 7 m changed at different cutoff values but in Baltic Sea 10 m it did not change after 1% cutoff values (data not shown), suggesting greater micro-heterogeneity of the sequences in the clone library of Plußsee 7 m. Similar to the clone libraries from Baltic Sea 10 m, the number of OTUs in the clone libraries from Baltic Sea 2 m and 20 m and Schöhsee 1 m remained almost constant after 1% cutoff values, indicating almost no variability at the DNA level.

The number of OTUs detected in this study was comparable to those of other studies, with the exception of the oligohaline station CB1 of Chesapeake Bay analyzed by Francis *et al.* (2003). In Chesapeake Bay, four OTUs were detected at two mesohaline stations (CB2 and CT2) and 11 at an oligohaline station (CB1) (Francis *et al.*, 2003). In Bahía del Tóbari, northwest coast of mainland Mexico, the clone libraries made with samples taken in January and October produced 6 and 7 OTUs (Beman & Francis, 2006).

To determine the influence that the inclusion of a longer fragment might have on the estimation of *amoA* diversity, rarefaction analyses were also carried out using sequences from clone libraries generated for the shorter region amplified with the primers *amoA1-F/amoA2-R* (Rotthauwe *et al.*, 1997). This was carried out only for the clone libraries from Baltic Sea sediment and Plußsee 7 m. In both cases, PCR products were only obtained after nested PCR amplification as mentioned for the longer *amoA* fragment (data not shown). In the case of the Baltic Sea sediment, the number of OTUs was the same for both fragments (5 OTUs detected). In the clone library of Plußsee 7 m, the number of OTUs decreased from seven for the longer piece (*amoA34f/amoA2-R*) to four for the short fragment (*amoA1-F/amoA2-R*), suggesting that the longer piece used in our study has a resolution at least equal to or even higher than the short piece used previously.

Definition of new clusters based on *amoA* sequence information

A sequence identity of < 80% for DNA and < 85% for protein sequences has been suggested as a threshold value for defining new AOB clusters [called lineages by Purkhold *et al.* (2000)] on the basis of partial *amoA* sequences of about 490 bp from the more variable 3' region (positions 332–822 of *amoA* from *Nitrosomonas europaea*). In the present study, we used the almost complete *amoA* sequence (positions 34–822), including the more conserved 5' part. We used the sequence identity between *amoA* from *Nitrosomonas cryotolerans* (Acc. no. AF314753) and *Nitrospira multififormis* (Acc. no. CP000103) as a reference, which was 88%/95% (DNA/protein) for the almost complete *amoA* gene targeted in this study compared with 84%/93% (DNA/protein) for the shorter region (Purkhold *et al.*, 2000). We suggest a threshold of sequence identity of $\leq 88\%$ for the DNA sequence and $\leq 95\%$ for the protein sequence to define new clusters based on the whole *amoA* gene sequences. Based on these thresholds *Nitrospira*-like clusters A and B, and the subclusters of the *Nitrosomonas* cluster 6a and of the *Nitrosomonas*-like cluster can be defined as potentially novel and habitat-specific representatives of AOB.

Conclusion

The present study showed clear differences between AOB communities not only among the three aquatic systems studied (Plußsee, Schöhsee and the Baltic Sea) but also within the different habitats generated due to the stratification of each aquatic body. Our results suggest that this differentiation is probably due to the chemical gradients, in particular oxygen, generated throughout the water column and sediments. The results also showed a good agreement between 16S rRNA gene and *amoA* studies, although this was not the case when high heterogeneity was found in the clone libraries. The results also showed that the application of nested amplification could enhance the sensitivity for *amoA* detection, and that the longer piece of *amoA* used here might have a higher resolution than the shorter *amoA* piece reported in most of the environmental studies.

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