Diversity of Functional Genes in the Aquatic Nitrogen Cycle

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Der Dekan

I dedicate this work to:

my family,

and my friends.

CONTENTS

SUMMARY

In this PhD thesis the diversity of functional bacterial genes in the nitrogen cycle was investigated with molecular methods in the lakes Plußsee and Schöhsee, and the Baltic Sea. The diversity of ammonia-oxidizing bacteria (AOB) was studied by diversity of specific 16S rDNA and *amoA* genes. Dominant sequences from Baltic Sea water column and Schöhsee 1 m were related to different *Nitrosospira* clusters. Sequences from Plußsee 7 m, Schöhsee 12 m and sediment of both lakes were clustered into a purely environmental cluster with no cultivated representatives. Two groups of *amoA* sequences from Baltic Sea sediment were related to environmental clusters from brackish and marine habitats.

The diversity of the evolutionarily related genes for ammonia monooxygenase (AMO) and particulate methane monooxygenase (pMMO) was analyzed. A higher frequency of *pmoA* sequences, mainly belonging to methane oxidizing bacteria of the gamma subgroup of proteobacteria (γ-MOB), was detected. Dominant *amoA* sequences were related to ammonia oxidizing bacteria of the beta subgroup of proteobacteria (β-AOB), no sequences related to *amoA* of the γ-AOB were detected. The deduced amino acid sequences of some clones from lake sediments were distantly related to PmoA from *Crenothrix polyspora*, a filamentous methane oxidizer with an unusual methane monooxygenase.

The distribution of denitrifying bacteria was studied by the nitrite reductase genes *nirK* and *nirS*. The dominant sequences of *nirK* from all clone libraries belonged to two distinct phylogenetic clusters, while *nirS* sequences from both lakes were scattered over several clusters throughout the complete phylogenetic tree, and only few sequences from Baltic Sea overlapped. In the Baltic Sea, *nirK*-denitrifiers were diverse throughout the water column, while *nirS*-denitrifiers were dominant in the sediment and almost absent in the water column. In Plußsee the community composition was inverted: *nirK*-denitrifiers were more diverse in the water column and *nirS*-denitrifiers in the sediment. In Schöhsee *nirS*-denitrifiers were highly diverse in water and sediment samples.

For *nirK* and *nirS* the sequences of the protein were less conserved than those of the gene while the *amoA* and *pmoA* protein were conserved, which might be an indication of a differential selection pressure.

ZUSAMMENFASSUNG

In der vorliegenden Dissertation wurde, unter Anwendung molekular-biologischer Methoden, die Diversität funktionaler bakterieller Gene im Stickstoffkreislauf im Plußsee, Schöhsee und einer küstennahen Station der Ostsee (Boknis Eck) untersucht.

Die Diversität Ammoniak-oxidierender Bakterien (AOB) wurde anhand von Klonbibliotheken aus PCR-Produkten des Gens für die Untereinheit A des Schlüsselenzyms Ammoniak-Monooxygenase (*amoA*) untersucht. Die häufigsten Sequenzen aus der Wassersäule bei Boknis Eck und 1m Wassertiefe Schöhsee waren mit verschiedenen Gruppen der *Nitrosospira* verwandt. Sequenzen aus dem Plußsee 7m, Schöhsee 12m und dem Sediment der beiden Seen wurden in einer Gruppe zusammengefasst, die nur Sequenzen aus verschiedenen Umweltproben enthält. Zwei Gruppen aus dem Ostsee Sediment waren verwandt mit einem bereits existierenden Cluster, das nur Sequenzen aus Brackwasser und marinen Habitaten enthält.

Es wurde die Diversität der beiden Gene mit gemeinsamem evolutionärem Ursprung Ammoniak-Monooxygenase (AMO) und partikuläre Methan-Monooxygenase (pMMO) analysiert. Klonbibliotheken enthielten vorwiegend Sequenzen der *pmoA* von Methan-oxidierenden gamma Proteobakterien (γ-MOB). Die *amoA* Sequenzen waren verwandt mit denen aus Ammoniak-oxidierenden beta Proteobakterien (β-AOB), es wurden keine *amoA* Sequenzen der gamma Proteobakterien gefunden. Bei einigen Klonen aus dem Sediment der beiden Seen hatte die aus der Basensequenz abgeleitete Aminosäuresequenz des Enzyms entfernte Ähnlichkeit mit PmoA aus *Crenothrix polyspora*, einem filamentösen, Methan-oxidierenden Bakterium mit ungewöhnlicher Methan-Monooxygenase.

Die Verbreitung denitrifizierender Bakterien wurde anhand der Gene *nirK* und *nirS* für Nitrit-Reduktase untersucht. Die meisten *nirK* Sequenzen aus den Klonbibliotheken von allen Standorten gehörten zu zwei phylogenetisch unterschiedlichen Gruppen. Die *nirS* Sequenzen aus beiden Seen waren auf verschiedene Gruppen des gesamten phylogenetischen Stammbaumes verteilt, die nur

3

wenige Sequenzen aus der Ostsee enthielten. Bei Boknis Eck waren die Sequenzen der *nirK*-Klone in den verschiedenen Proben aus der Wassersäule unterschiedlich, während *nirS*-Sequenzen im Sediment dominierten, aber in der Wassersäule fast vollständig fehlten. Im Plußsee waren diese Verhältnisse umgekehrt: Denitrifizierer mit *nirK* waren diverser in der Wassersäule, und solche mit *nirS* im Sediment.

1. INTRODUCTION

1.1 Nitrogen cycle

Nitrogen (N) is a major element in all organisms accounting for 6.25% of their dry mass on average. Nitrogen can be found in nature in both organic and inorganic forms. Earth's atmosphere contains about 78% nitrogen, the largest single component. However, most organisms cannot use nitrogen in this form. Plants must take up their nitrogen in "fixed" form, and animals as organic nitrogen either from plants or prey. Microorganisms connect the different organic and inorganic N-compounds, and they can use a great variety of them as N-source.

Redox reactions of the biological N-cycle (Figure 1-1) are fulfilled in different ways by bacteria, archaea and some fungi. Nitrification and denitrification are important processes in the global nitrogen cycle, and may counteract natural and manmade eutrophication by eliminating the nutrients NO_3^- and NH_3.
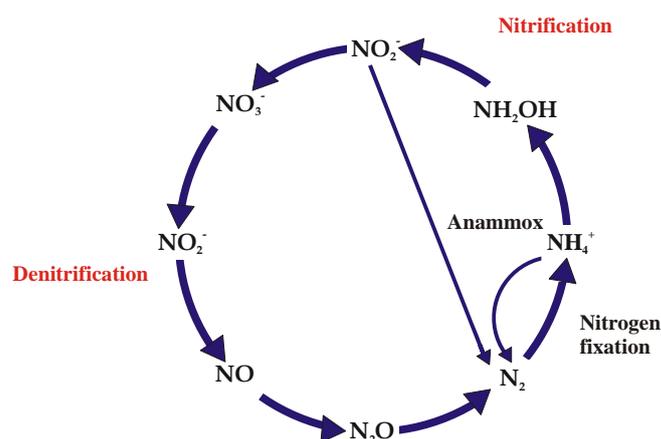
Figure 1- 1: Nitrogen cycle

Nitrification is the oxidation of reduced N in the form of NH_4^+ to NO_3^-. It plays a central role in the global N-cycle, providing a link between release of NH_4^+ by

decomposition of organic matter, and supply of the essential electron acceptor NO_3^- for denitrification. Denitrification as part of the N-cycle transforms nitrate (NO_3^-) into N_2 gas. This is a reductive process; which occurs in four stages starting from nitrate via nitrite, nitric oxide and nitrous oxide. It as three important ecological aspects (1) loss of fertilizer nitrogen may decrease the efficiency of fertilization in agriculture; (2) removal of nitrogen in wastewater treatment systems may reduce eutrophication; (3) release of nitrogen oxides may reduce ozone in the stratosphere and thus increase UV radiation on earth (Knowles, 1982).

1.2 Microorganisms and enzymes related to nitrification

Nitrification involves a relatively restricted number of microorganisms, mainly two distinct functional groups of the Proteobacteria, autotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Figure 1-2). However, increased understanding of the diversity of autotrophic nitrifiers, the discovery of the anammox process (ammonia oxidation under anaerobic conditions) (Op den Camp et al., 2007) and the more recent discovery of ammonia-oxidizing archaea (AOA) (Könneke et al., 2005; Treusch et al., 2005) have changed this relatively simple view of nitrification. As a result, the actual concept involves AOB, AOA, NOB and a number of heterotrophic bacteria and fungi for nitrification under aerobic conditions.

$$NH_4^+ \xrightarrow{\text{amo}} NH_2OH \xrightarrow{\text{hao}} NO_2^- \xrightarrow{\text{nor}} NO_3^-$$

ammonia   hydroxylamine   nitrite   nitrate

Ammonia-oxidizing bacteria (AOB)   **Nitrite-oxidizing bacteria (NOB)**
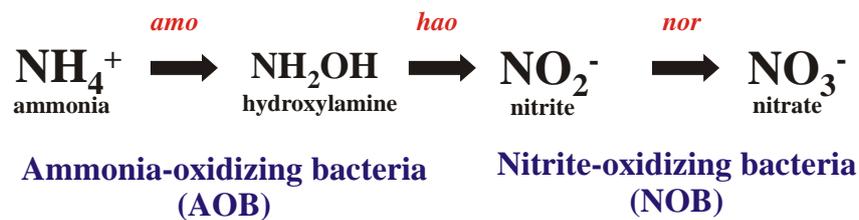
Figure 1- 2: Nitrification process

AOB are classified into the five different genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* (Koops and Möller, 1992), based on phenotypic characters such as cell morphology, intracellular membrane structure, motility, tolerance to high concentrations of NH_3 or salt and urease activity.

Phylogenetically, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* were combined into one common genus *Nitrosospira* (Head et al., 1993). *Nitrosomonas* and *Nitrosospira* belong to the beta-Proteobacteria (β-AOB) and *Nitrosococcus* to the gamma-Proteobacteria (γ-AOB). AOA were recently discovered in soil metagenomic libraries (Treusch et al., 2005). In addition, an ammonia-oxidizing, mesophilic crenarchaeota, *Nitrosopumilus maritimus*, belonging to the crenarchaeal group 1.1a lineage, has been isolated from a marine aquarium (Könneke et al., 2005) and grows chemolithoauto-trophically. NOB comprise four genera belonging to different subgroups of the Proteobacteria, *Nitrobacter* (alpha-Proteobacteria), *Nitrococcus* (gamma-Proteobacteria), *Nitrospina* (delta-Proteobacteria) and *Nitrospira* (beta-Proteobacteria), classified on the basis of phenotypic characters, mainly cell morphology and membrane ultrastructure (Prosser, 2007). Heterotrophic bacteria and fungi can also oxidize ammonia and organic nitrogen compounds (Focht and Verstraete, 1977) by different, xet not completely understood metabolic pathways. While N oxidation is the only energy-obtaining process in autotrophic nitrifiers, nitrification in heterotrophic organisms does not contribute significantly to their energy metabolism.

Cultured AOB and sequences from environments belonging to the beta-Proteobacteria were divided into 7 - 10 clusters based on 16S rDNA (Purkhold et al., 2000; Kowalchuk and Stephen, 2001). Cluster 0 - 4 belong to *Nitrosospira* spp., and cluster 5 - 8 to *Nitrosomonas* spp.. *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 5 contain environmental sequences, only, and no representative pure culture is known. *Nitrosospira* spp. were frequently observed in soil, whereas *Nitrosomonas* spp. dominantly detected in seawater, freshwater and activated sludge.

In soil, members of the *Nitrosospira* cluster 2 containing *Nitrosospira* strain AHB1 are dominant, particularly in acid soils (Stephen et al., 1996). Cluster 3 has been detected as the dominant AOB in a number of neutral pH arable fields receiving fertilizers, and these populations have been replaced by cluster 4 populations in response to prolonged periods without fertilizer use (Bruns et al., 1999; Kowalchuk et al., 2000).

1.INTRODUCTION

The γ-AOB, *Nitrosococcus oceani*, has been isolated from marine environments (Ward, 1986), but only few γ-AOB sequences are found in the ocean (McCaig et al., 1994; Nold et al., 2000). The *Nitrosomonas marina* lineage (*Nitrosomonas* cluster 6b) has been isolated from marine environments, and molecular techniques indicate widespread distribution of *Nitrosospira* and *Nitrosomonas* (McCaig et al., 1994). The sequences belonging to the *Nitrosomonas* Nm 143 lineage were usually found in marine environments or estuaries. Generally, these groups are phylogenetically distinct from those found among terrestrial and freshwater AOB. In addition, marine environments may contain sequences that fall into *Nitrosomonas* cluster 5 and *Nitrosospira* cluster 1 lineages, which do not contain cultivated representatives (McCaig et al., 1999; Bano and Hollibaugh, 2000).

In freshwater, AOB communities are dominated by members of the *Nitrosomonas* cluster 6a (*Nitrosomonas oligotropha* lineage) (Speksnijder et al., 1998; de Bie et al., 2001). Some sequences belonging to *Nitrosomonas* lineages, including cluster 5 and 6b (*N. marina* lineage) and 7 (*N. europaea*, *N. eutropha* lineage) (Speksnijder et al., 1998).

For the growth of AOB, namely for energy conservation during the oxidation process, the function of two key enzymes is necessary: ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). AMO catalyzes the oxidation of ammonia to hydroxylamine and HAO the oxidation of hydroxylamine to nitrite, which is the actual energy-generating step. AMO is a membrane-bound multiple subunit enzyme, consisting of AmoC, AmoA and AmoB, which are encoded by the genes *amoC*, *amoA* and *amoB* of the *amo* operon (Hommes et al., 1998; Sayavedra-Soto et al., 1998). HAO is an unusual enzyme with a highly complex structure, located as a soluble enzyme in the periplasmic space, but anchored in the cytoplasmic membrane (Arciero and Hooper, 1998).

From *Nitrosomonas europaea*, a 27-kDa membrane-associated protein AmoA, which contains the active site of AMO, (Hyman and Arp, 1992) and a second protein (AmoB) of 43 kDa have been isolated as probable AMO subunits (Hyman and Arp, 1992; Bergmann and Hopper, 1994). The full-length sequences of both genes, *amoA*

and *amoB*, coding for these subunit proteins have been determined from *Nitrosomonas europaea* (McTavish et al., 1993; Bergmann and Hopper, 1994), additional full-length *amoA* and *amoB* sequences from other AOB are available (Norton et al., 2002). An ORF upstream of the *amoA* gene in all AOB has been designated as the *amoC* gene (Klotz et al., 1997). This gene in *Nitrosospira* sp. NpAV, encodes an approximately 31-kDa membrane protein (Klotz et al., 1997). The *amo* operon occurs as multiple and almost identical copies in the β-AOB (Norton et al., 2002), while only single *amo* operon copies have been found in γ-AOB (Alzerreca et al., 1999). Intergenic space (IS) of strain-specific variability are located between the *amoC* and *amoA* genes (Alzerreca et al., 1999). In γ-AOB, an additional IR was observed between *amoA* and *amoB*.

The enzymes AMO in AOB and particulate methane monooxygenase (pMMO) in methane-oxidizing bacteria (MOB) are homologous and share many features (Bédard and Knowles, 1989; Ensign et al., 1993). The pMMO operon is comprised of three subunits encoded in the genes *pmoC, pmoA*, and *pmoB* (Zahn and Dispirito, Nguyen et al. 1998, Stolyar et al 1999, Gilbert et al 2000). The identical structures of their operon and high similarity of the nucleotide sequences (Figure 1-3) of the genes suggest a common evolutionary origin (Holmes et al., 1995; Semrau et al., 1995).
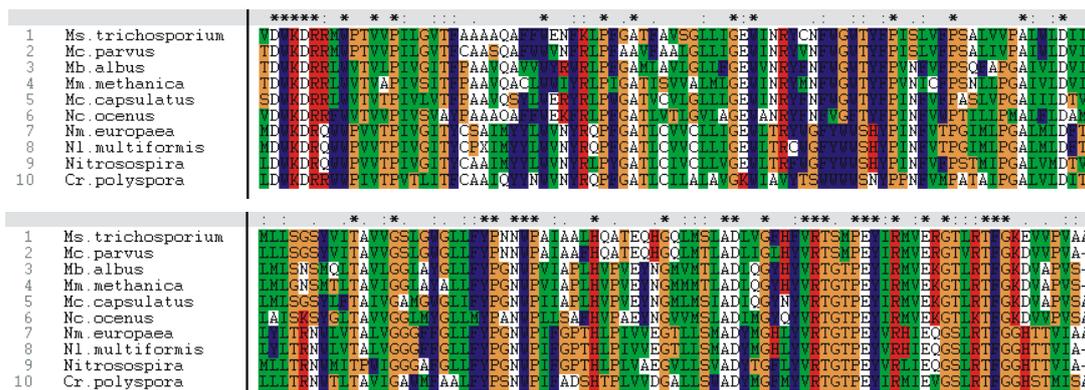
Figure 1- 3: Alignment of predicted amino acid sequences of *amoA* and *pmoA* genes from ammonia-oxidizing bacteria, methane-oxidizing bacteria and *Crenothrix polyspora*.

9

Genes homologous to *amoCAB* have also been described from archaea (Könneke et al., 2005; Treusch et al., 2005), but with relatively low sequence similarity to their bacterial counterparts (38 - 51%).

1.3 Microorganisms and enzymes related to denitrification

Four enzymes are involved in the denitrification process, nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) (Figure 1-4). The reduction of nitrate is associated with two homologous enzymes, membrane-bound (Nar) and periplasmic (Nap) nitrate reductases.

$$NO_3^- \xrightarrow{nar} NO_2^- \xrightarrow{nir} NO \xrightarrow{nor} N_2O \xrightarrow{nos} N_2$$

nitrate nitrite nitric oxide nitrous oxide denitrogen

Denitrifying bacteria

Figure 1- 4: Denitrification process

Nitrite reductase (Nir) is regarded as the key enzyme in the denitrification process producing the first gaseous product. Two different nitrite reductase catalyze the reduction of NO_2^- to NO (Zumft, 1997), Cu-type NO_2^--reductase is encoded by the *nirK* gene and cytochrome cd1-type NO_2^--reductase by *nirS*. The two Nir types are functionally and physiologically equivalent, as indicated by the expression of the Cu-Nir gene from *Pseudomonas aureofaciens* in a *Pseudomonas stutzeri* mutant lacking the gene encoding for cd_1-Nir (Glockner et al., 1993). These enzymes have never been observed to exist together in the same bacterial species (Coyne et al., 1989). While cd_1-Nir dominates in the different subgroups of the proteobacteria, Cu-containing Nir is spread more widely in the microbial world, including Archaea, and shows greater variation in molecular weight and immunological reactions (Coyne et al., 1989; Zumft, 1997; Philippot, 2002). It is still unknown why different classes of nitrite

reductases have evolved and what advantage the one may provide over the other. *nirS* is part of a *nir* gene cluster, in which the number of *nir* genes and the organization differ in different species. In some species, the *nirK* gene forms a cluster together with a *nirV* gene, located downstream and encoding a protein of unknown function (Philippot, 2002).

Denitrifiers are frequently isolated from soil, sediment and aquatic environments. In a remarkable study on denitrifier communities in various soil environments (Gamble et al., 1977) 1,500 bacteria were isolated, and 146 of these had the ability of denitrification. The major group isolated was *Pseudomonas fluorescens* and the second most prevalent group was *Alcaligens*. In this and some other cultivation-based studies of denitrifying bacteria, the genera *Alcaligenes*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Paracoccus*, *Ralstonia*, *Rhodobacter*, *Rubrivivax*, *Streptomyces*, and *Thauera* have been revealed as the dominant denitrifiers in diverse environments. Marine denitrifiers were dominated by *Shewanella baltica* (Brettar et al., 2001) and *Marinobacter* spp. (Goregues et al., 2005).

The capability of denitrification is widespread among microorganisms, e.g. Bacteria including Aquificae, Deinococcus-Thermus, Firmicutes, Actinobacteria, Bacteroides, and Proteobacteria lineages (Zumft, 1997), Archaea (Philippot, 2002) and some fungi (Shoun et al., 1992). Therefore, in order to study the diversity of denitrifying bacteria in the environment, it is necessary to focus on a gene directly involved in denitrification, and to link their distribution and activity to the processes (Bothe et al., 2000; Philippot, 2002).

The genes *nirK* and *nirS* coding for Nir were the first to be used for investigation of denitrifying bacterial diversity and have become the most widely used molecular markers (Braker et al., 1998). Marine habitats and soil showed a very high diversity of denitrifiers carrying *nir* genes grouped into novel clusters that are significantly distinct to the sequences obtained from isolated bacteria. These studies were carried out to correlate the diversity of *nir* genes with the abiotic/biotic factors that control the denitrifier communities.

1.INTRODUCTION

Marine denitrifier communities were studied in sediments (Braker et al., 2000; Braker et al., 2001; Liu et al., 2003) and the water column at oxic-anoxic interfaces (Jayakumar et al., 2004; Castro-González et al., 2005; Hannig et al., 2006). Amplification of *nirK* genes from marine habitats using published primer pairs seems to reveal more conserved sequences than of *nirS*. *nirS*-denitrifiers were diverse in the water column of the Arabian Sea with high concentrations of NO_2^- indicating high denitrification activity. In contrast, they were less efficiently detected from water column with low concentrations of H_2S or O_2 (Jayakumar et al., 2004). Communities of *nirS*-denitrifiers were inhabiting the oxic but not the sulfidic zone within the water column of the Baltic Sea (Hannig et al., 2006).

The effect of fertilization on the denitrifier community was analyzed in soil (Avrahami et al., 2002; Wolsing and Priemé, 2004). In a laboratory experiment, T-RFLP analysis of the *nirK* gene was used to detect the effect of ammonium addition at different concentrations on soil denitrifiers after one month (Avrahami et al., 2002). This resulted in a shift in the structure of the denitrifier community at medium and high ammonia concentration, which probably was indirectly caused by an increased supply of nitrate through nitrification of the added ammonium. In field studies using T-RFLP of *nirK* genes to compare the effect of mineral versus organic fertilizer (Wolsing and Priemé, 2004) a strong seasonal shift in the structure of the *nirK* communities was observed, whereas fertilizer type, rather than amount, appeared to affect the communities to a minor extent.

Sequencing of the dominant *nirK* and *nirS* genes in activated sludge from two different full-scale treatment plants revealed that some were distantly related to *Rhizobium* spp., *Azospirillum* spp., *Rhodobacter sphaeroides*, and *Paracoccus denitrificans*, although the majority clustered with other environmental clones (Throbäck et al., 2004). In a saline metallurgic wastewater treatment system, the diversity of *nirS* was greater than that of *nirK*, but both were much lower than in soil (Yoshie et al., 2004). In an aerated biofilm reactor, the highest numbers of *nirS* genes were observed in the outer region, while *nirK* was concentrated more to the inner part, demonstrating the possibility of a differential response of *nirK*- and *nirS*-denitrifiers to oxygen (Qiu et al., 2004).

Although strong gradients of nutrients are often present in sediments or biofilms of rivers, denitrifiers are obviously less diverse there than in soil and marine habitats (Nogales et al., 2002; Chenier et al., 2003). Denitrification activity and community composition of *nirK*-denitrifiers in river biofilms vary both with nutrient concentration and time (Chenier et al., 2003) and the diversity of expressed *nirS* genes was high in nitrogen-rich estuarine sediments (Nogales et al., 2002).

1.4 Molecular-based analysis of microbial diversity

Most microorganisms are difficult to isolate in pure cultures because of their special requirements of elements and sometimes low growth rates. Based on cultivation, our knowledge on the diversity of bacterial communities in natural environments is limited. Based on molecular methods the diversity of functional genes has been investigated in various environments.
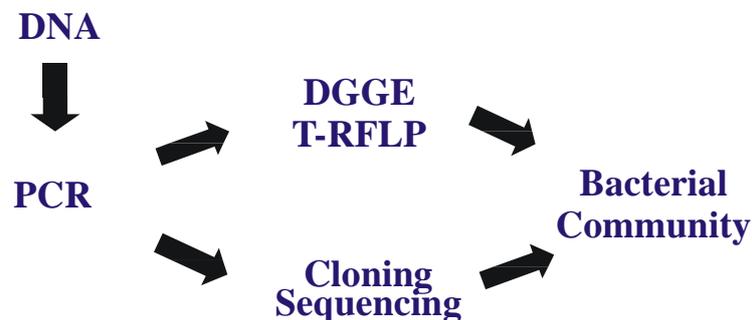
Figure 1- 5: Simplified scheme of molecular methods for studying bacterial communities.

Most of these approaches begin from amplification of the target functional genes from extracted DNA with the polymerase chain reaction (PCR) (Figure 1-5). PCR primers are designed to target the gene of interest. Following amplification of the gene, there are several methods for examining the diversity and community composition of that gene in a community. One efficient technique is terminal restriction fragment length polymorphism (T-RFLP), where the PCR-amplified gene fragments are cut with restriction enzymes, separated by gel electrophoresis, and the

length of the cut fragments measured quantitatively using a DNA sequencer (Liu et al., 1997). This technique quantifies the relative abundance of the dominant gene variants, presumably representing the dominant denitrifiers in the community. This is a useful technique for comparing communities, though it provides very little information about the identity of specific members of the community. Another widely used fingerprinting technique is denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). This technique separates the amplicons based on their G+C content, resulting in a series of bands on a gel, each band representing a unique variant of the gene, and therefore a unique member of the denitrifying community.

Other techniques, which involve gene sequencing, provide more specific information about variations of the gene between organisms and within communities. A common strategy is to develop a library of gene sequences from an environmental sample by cloning gene fragments that have been PCR amplified from mixed community nucleic acids. Randomly selected clones are sequenced and aligned, giving a census of the amplified gene fragments within a given sample. By collecting detailed sequence information for a large number of clones, the relative proportion of each gene variant can be assessed, providing a measure of overall gene diversity and specific community composition. This technique provides a very specific description of community structure.

1.5 Purpose of the thesis

Since the functional genes, *amoA*, *pmoA, nirK, nirS* from cultured organisms were published, microbial diversity based on these functional genes has been studied from several environments and consequently lots of sequences of functional genes were deposited in GenBank. However, most of these studies in natural environments have focused either on soils or marine habitats. The information of those sequences from freshwater lakes is scarce.

The purpose of this study was to fill this gap in part by investigating the diversity of functional genes related to the nitrogen cycle, in particular *amoA*, *pmoA*, *nirK* and *nirS* in two lakes and the Baltic Sea. At the time of sampling, all three

habitats showed a characteristic stratification (Fig. 2-2). The comparison was carried out as well between several depths in each environment, as indicated in Fig. 2-2.

1.6 Thesis outline

The results of this thesis are presented in six chapters in section 3, each structured with abstract, introduction, materials and methods, results, and discussion. This outline gives a short overview of each work.

3. I Comparative analysis and description of PCR primers for ammonia-monooxygenase genes of ammonia-oxidizing bacteria

The purpose of this work was to compare all primers that have been used to detect *amoCAB* genes with all available sequences in public databases and to design and test new primers to amplify the ammonia monooxygenase gene from known AOB and environmental samples.

3. II Application of novel PCR strategies and sequence analysis to study the *amoCAB* operon in beta ammonia-oxidizing bacteria

A new approach is described that uses nested amplification of *amo* with environmental samples. One major difficulty to study *amoA* genes in natural environments is the amplification because of the low abundance of AOB. Using initial amplicons of *amoCAB*, several nested amplifications of *amo* were performed and clone libraries were used to confirm the identity of the amplicons.

3. III Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea

In this chapter, I describe the community composition of ammonia oxidizing bacteria (AOB) in two lakes and the Baltic Sea. Based on 16S rDNA, the ammonia-oxidizing bacterial community was compared between environments, and within several depths in each of them.

3. IV Diversity of ammonia monooxxygenase (*amoA*) genes in the water column and sediment of two lakes and the Baltic Sea

Based on the new primers and strategy described in chapter I and II, the *amoA* gene was used to investigate the diversity of ammonia oxidizing bacterial communities in three contrasting environments. The good agreement of AOB phylogeny based on 16S rDNA and *amoA* was demonstrated.

3. V Genetic diversity of the evolutionarily related enzymes ammonia monooxygenase (AMO) and particulate methane monooxygenase (pMMO) in two lakes and the Baltic Sea

Unlike NirK and NirS in chapter VI, AMO and pMMO and their genes share several structural features, but their function is different. Therefore, this chapter analyses how *amoA* and *pmoA* genes evolved in closed environments (two lakes) and an open environment (Baltic Sea).

3. VI Distribution of nitrite reductase (*nirK* and *nirS*) genes in the water column and sediment-water interface of two lakes and the Baltic Sea

During denitrification, the enzymes NirK and NirS catalyze the reduction of nitrite to nitric oxide. Despite their similar function, these enzymes and their genes do not have any structural similarity. This work addresses how these genes are distributed in lakes and the Baltic Sea.

2. MATERIALS AND METHODS

2.1 Sampling sites

The two lakes Plußsee (54°10' N, 10°23' E) and Schöhsee (54°13' N, 10°26' E) are located in Schleswig-Holstein, northern Germany (Figure 6). Plußsee is a small eutrophic lake with funnel-shaped morphology and a diameter of 400 m, a surface area of 0.14 km^2, a maximum depth of 29 m and a mean depth of 9.4 m. Woodland encompass this lake and agricultural land lies on the higher surroundings. It has no direct inflow and only a minor outflow. During the summer, this eutrophic lake shows a stable thermal stratification, which is characterized by a thin (only a few meters deep) oxygen-rich epilimnion and a large anoxic hypolimnion beginning at a depth of 5 - 8 m, separated by the thermocline layer (metalimnion).

Figure 2-1: Map of the sampling sites of the two lakes Plußsee and Schöhsee (left) in northern Germany and the Baltic Sea (right) (© Google Earth)

Schöhsee is a mono-dimictic and mesotrophic lake with a surface area of 0.78 km^2, a maximum depth of 29.4 m and a mean depth of 10.9 m. This lake has a low productivity in the water column and sediment (Grey et al. 2004, Aberle and Wiltshire. 2006) at low total phosphorous (mean TP = 21 µg L^{-1}) and low chlorophyll-a contents (mean chlorophyll-a=21 µg L^{-1}) in the water column. Schöhsee has summer stratification as well, but with oxic hypolimnion.

The Baltic Sea is a brackish habitat with a surface area of 377,000 km^2, a mean depth of 53 mon the Swedish side of the center. The sampling site was the Boknis Eck out of the Kiel Fjord in the German coast (Figure 2-1) (54°30' N, 10°06' E), characterized as a eutrophic brackish region of the western Baltic Sea.

Details of chemical stratification at the time of sampling at the three sites are outlined in chapter III

2.2 Sample collection

The study sites included the lakes Plußsee and Schöhsee located in Schleswig-Holstein (North Germany) and the Boknis Eck out of the Kiel Fjord in the German coast of the Baltic Sea in September 2004. Water samples were collected using a Ruttner sampler at three different depths, which were determined in the field based on the temperature and oxygen profile (Fig. III-1). Additionally, samples from the overlaying sediment-water interface were also collected at 22 (Schöhsee), 28 (Plußsee) and 27.5 m (Baltic Sea). The designation of samples was as follows: Baltic Sea 2 m (B2m), 10 m (B10m), 20 m (B20m) and sediment-water interface (Bsedi); Plußsee 1 m (P1m), 4 m (P4m), 7 m (P7m) and sediment-water interface (Psedi); Schöhsee 1 m (S1m), 11 m (S11m), 12 m (S12m), 14 m (S14m) and sediment-water interface (Ssedi). Additional samples were obtained from rhizospheric soil from pea cultures in northern Germany and Jordan River from Israel for the experimental part of chapter I.

2.3 Strains

For this work the following strains were used: *Nitrosomonas europaea* ATCC 19718, *Nitrosomonas eutropha* C-31 ATCC 25978, *Nitrosospira briensis* C-128, *Nitrosospira tenuis* Nv1, *Nitrosomonas* sp. L13, *Nitrosococcus oceani* ATCC 19707, *Methylococcus capsulatus* Bath NCIMB 11853, *Methylocystis parvus* OBBP.

2.4 DNA extraction and PCR

Water samples were filtered through 0.2 μm pore size filters (Supor-200, PALL Life Sciences), and stored at −18°C until DNA extraction. Three independent filtrations and at least one DNA extraction per each of the filters was carried out,

producing not least than three replicates per sampling point. Differences between these replicates were not significant. DNA was extracted using the UltraClean Soil DNA kit (MoBio), following the manufacturer's guidelines. Concentration and quality of the DNA was checked by electrophoresis in 0.8% agarose gels stained with ethidium bromide.

2.4.1 16S rDNA of ammonia-oxidizing bacteria

Amplification of AOB-specific 16S rDNA was carried out using the nested PCR approach suggested by (Ward et al., 1997). Briefly, general 16S rDNA was amplified using the bacterial primers Eub9_27 and Eub1542 (Brosius et al., 1978). The PCR products with Eub9_27 and Eub1542 were cleaned and used as templates for amplification with the specific primers NitA and NitB (Voytek and Ward, 1995). The PCR conditions were described in previous study (Ward et al., 1997). For DGGE, these amplicons were diluted 100 times and used in a second nested PCR with the primers P3 (GC-clamped) and P2, using a touchdown temperature program (Muyzer et al., 1993).

2.4.2 *amo* genes

For amplification of the almost complete *amoCAB* operon with the primers amoC58f and amoB1179r, the Expand High Fidelity PCR system (Roche) was used. PCR was carried out a total volume of 25 μl containing 1× PCR buffer, 200 μM of each dNTP, 200 nM of each primer, 2.5 mM $MgCl_2$, 1% formamide, 0.1% BSA, and 2.6U of enzyme mix. Temperature program consisted of an initial denaturation at 94°C for 2 min, and 25 cycles of 94°C for 15s, 56°C for 1 min and 68°C for 5 min. For all other initial PCRs with 5 different primer pairs, amplification was carried out as described above, but using 1U of *Taq* DNA polymerase (Roche) and 1× buffer from the PCR Kit. The temperature program consisted of: initial denaturation at 94°C for 5 min; 25 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1.5 min.

For DGGE of *amoA* in chapter I, the PCR products were re-amplified with the primer pair amoA121f with a 40 bp GC-clamp attached (Muyzer et al., 1993) and amoA359rC in a touchdown temperature program from 65 to 55°C (Muyzer et al., 1993). For nested amplification, the PCR products were cleaned using a Multiscreen

Plate (Millipore Inc.) and diluted 100 times in HPLC water. Nested PCR was carried out as above except that 1U of *Taq* Polymerase (Roche) was used.

2.4.3 *amoA* and *pmoA* genes

The *amoA* and *pmoA* genes were amplified simultaneously with the primers A189 and A682 (Holmes et al 1995). PCR was performed with conditions described previously (Holmes et al 1995). However different annealing temperature was used at 53°C for samples from the Baltic Sea, or at 55°C for samples from Plußsee, or at 57°C for samples from Schöhsee. Different annealing temperatures were used because the most intensive bands were obtained using different temperature in each environment.

2.4.4 *nirK* and *nirS* genes

nirK and *niS* were amplified by PCR with the primer pairs F1aCu and R3Cu (Hallin and Lindgren, 1999) for *nirK* and cd3aF (Michotey et al., 2000) and R3cd (Throbäck et al., 2004) for *nirS*. Amplification was followed by (Throbäck et al., 2004).

2.5 Fingerprinting techniques

2.5.1 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) was carried out as described elsewhere (Kim et al., 2006) using the D-Gene System (BioRad) in polyacrylamide gels with a gradient of 35 to 75% of denaturants for chapter I in results and a gradient of 30 to 60% of denaturants for chapter III in results.

2.5.2 Terminal restriction fragment length polymorphism (T-RFLP)

Using the labeled primers A189-FAM (*amoA/pmoA*), F1aCu-FAM (*nirK*) and R3Cd-HEX (*nirS*), T-RFLP was carried out as described elsewhere (Junier et al., submitted) and principal component analysis (PCA) was carried out with Statistica 6 (Statsoft, USA) and the Primer 6 software (Primer E, Plymouth, UK)

2.6 Cloning and sequencing

The primers NitA and NitB were selected to prepare the products for cloning. PCR for cloning was carried out with the proof-reading *Pfu* DNA polymerase (Promega) according to the manufacturer's guidelines. In order to minimize PCR biases, three reactions were run in parallel and pooled using a Multiscreen plate (Millipore, Inc.). For cloning the Zero Blunt PCR cloning kit (Invitrogen) was used according to the manufacturer's guidelines. For 16S rDNA and *amoA*/*pmoA*, 96 clones were selected and for *amoA*, *nirK* and *nirS*, 48 clones were selected. Those clone wered checked for inserts of the expected size by PCR with plasmid-specific primers M13f and M13r and agarose electrophoresis. For screening of 16S rDNA, one-shot sequencing using M13f primer was performed using the BigDye terminator v3.1 cycle sequencing kit, and analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's recommendations. For functional genes, sequencing of both strands was performed with M13F and M13R primers.

The nucleotide sequences of 16S rDNA were compared with those in the GenBank using BLASTN (Altschul et al., 1997) on the NCBI's homepage (http://www.ncbi.nlm.nih.gov/blast/Blast) and classification of the clones was carried out using the hierarchy browser of the ribosomal database project (Cole et al., 2005). The sequences of functional genes were checked using BLASTX.

2.7 Diversity estimation of clone libraries

Rarefaction analysis with the DOTUR program (Schloss and Handelsman, 2005) was used to compare the functional genes diversity based on DNA within each clone library. Operational taxonomic units (OTUs) were defined as sequence groups in which sequences differed by 5%.

2.8 Phylogenetic analysis

Phylogenetic analysis of functional genes was performed with the software ARB (http://magnum.mpi-bremen.de/molecol/arb/). To build database, sequences of

each functional gene were collected from GenBank. Phylogenetic tree was constructed with the PHYLIP distance method by Neighbor-Joining and Fitch-Margoliash method.

2.9 Nucleotide sequence accession numbers

All sequences obtained in this study were deposited in GenBank. The accession numbers for different region of *amo* genes are EF204958-EF204996 and those for *amoA* genes are EF219168 - EF219191, EF222028 - EF222286 and EF615038 - EF615208. Using the primers A189/A682 for *amoA* and *pmoA*, accession numbers are EF623537 - EF623623 for *amoA* and EF623624 - EF623822 for *pmoA*. The accession numbers are DQ337713 - DQ337812 and EF615209 - EF615414 for *nirK* and DQ337813 - DQ337925 and EF615415 - EF615570 for *nirS*.

2.10 Bioinformatic analyses for chapter I in results

2.10.1 Sequences and alignments

For *in silico* analyses, the nucleotide sequences of *amo* genes were downloaded from GenBank using ENTREZ (http://www.ncbi.nlm.nih.gov/). Protein sequences were retrieved from Swissprot using Expasy (http://www.expasy.org). 16 sequences of *amoC* were analyzed, including beta- and gamma-AOB. Additionally eight sequences of the related subunit of the particulate methane monooxygenase (*pmoC*) and one sequence from the recently described ammonia-oxidizing archaea Candidatus *Nitrosopumilus maritimus* (Könneke et al., 2005) were retrieved for the analysis. 32 *amoB* sequences from both subclasses of proteobacteria and seven *pmoB* sequences were included in the analysis. Two *amoB* sequences from crenarchaeota were also included. A total of 2669 sequences consisting of cultured and uncultured *amoA* and the related alpha subunit of the particulate methane monooxygenase (*pmoA*) were retrieved. Although, *amoA* sequences from crenarchaeota were considered, they differed widely and were excluded from the analysis. *amoC, amoB* and *amoA* sequences were integrated in ARB (Ludwig et al., 2004). A database of complete and partial sequences of *amoA* from recognized AOB species was also

prepared in ARB. Before the analysis, all the sequences were verified manually and those including STOP codons or erroneous starting points were omitted. In order to simplify the presentation of the results, sequence similarity is shown for *amoA* sequences from cultured AOB (11 different phylogenetic clusters) and 10 *pmoA* sequences, only. Results from the complete database are available upon request. Sequences were aligned using ClustalW included in ARB.

2.10.2 Primer analyses

The primers analyzed in this study are summarized in Table I-1. Analysis of the primers was carried out using the software Oligo 6.0 (Table I-1). The position of each primer was determined after alignment of all the sequences in ARB. Specificity was evaluated using BLAST for short nearly exact matches (http://www.ncbi.nlm.nih.gov/BLAST/) and MATCH PROBE in ARB. The MATCH PROBE subroutine of ARB calculates two different parameters for specificity: number of mismatches and weight of the mismatches. The last parameter depends on the number, position and kind of mismatches. A maximum number of 5 mismatches were allowed in the analysis. New primers were designed by visual inspection of the multiple alignments or using the software GeneFisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). The new designed primers were also analyzed with Oligo v.6.0 (Table I-1). In order to simplify the comparison between primers that had been designed in different studies, we propose a standardized designation system according to the name of the targeted gene, followed by information on the position and orientation of the primers. Additional letters at the end of the designation indicate modifications such as shorter versions (s), wobble positions (IUPAC code), probe for FISH (p) or primer specific for AOB belonging to gammaproteobacteria (Gam).

2. MATERIALS AND METHODS

3. I   Comparative analysis and description of PCR primers for ammonia monooxygenase genes of ammonia-oxidizing bacteria
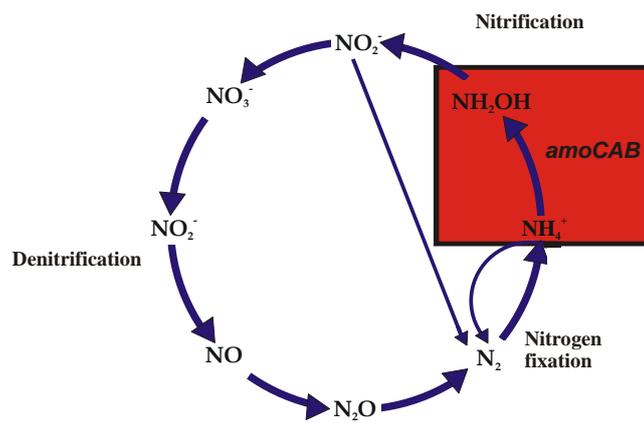
ABSTRACT

Over the past years several PCR primers have been described to amplify genes encoding the structural subunits of ammonia monooxygenase (AMO) from ammonia-oxidizing bacteria (AOB). Most of them target *amoA*, while *amoB* and *amoC* have been neglected so far. In this study we compared the nucleotide sequence of thirty-three primers that have been used to amplify different regions of the *amoCAB* operon with alignments of all available sequences in public databases. Advantages and disadvantages of these primers are discussed based on the original description and the spectrum of matching sequences obtained. Additionally, new primers are described to amplify the almost complete *amoCAB* operon of AOB belonging to betaproteobacteria (beta-AOB), generating products of about 3,000 bp that were useful for nested amplifications to increase the sensitivity of detection of these functional genes in environmental samples. A primer pair for DGGE analysis of *amoA* and specific primers for gamma-AOB are also described. With this strategy we detected differences in the composition of AOB communities in various environments including soil, the Jordan River, lakes and the Baltic Sea.

INTRODUCTION

Ammonia-oxidizing bacteria (AOB) are chemolithoautotrophic Gram-negative proteobacteria that fix CO_2 with the reducing power obtained from ammonia oxidation (Prosser, 1989). They belong to two monophyletic lineages: *Nitrosomonas* spp. (including *Nitrosococcus mobilis*) and *Nitrosospira* spp. (including *Nitrosolobus* and *Nitrosovibrio*) form a closely related clade within the beta subclass of proteobacteria (beta-AOB); whereas *Nitrosococcus oceani* is affiliated to the gamma subclass (gamma-AOB) of proteobacteria (Head et al., 1993; Purkhold et al., 2000; Purkhold et al., 2003).

Characterization of the species composition and diversity of AOB communities in nature has been hampered for a long time by the difficulties in

isolation and culture of these microorganisms. The analysis of AOB communities has become accomplishable by applying culture-independent molecular approaches, which are based on the amplification of 16S rRNA genes (16S rDNA) by PCR (Bothe et al., 2000; Kowalchuk and Stephen, 2001) or the detection of 16S rRNA by FISH (Wagner et al., 1993; Wagner et al., 1995; Mobarry et al., 1996). 16S rDNA is a good phylogenetic marker, but it is not necessarily related to the physiology of the target organisms (Kowalchuk and Stephen, 2001; Calvó and Garcia-Gil, 2004). Therefore, functional markers such as the genes encoding for key enzymes involved in their metabolism, provide an alternative in ecological studies (Rotthauwe et al., 1997). Diversity studies of AOB based on the sequence analysis of one of these genes, *amoA,* have shown a higher resolution to separate closely related strains (Rotthauwe et al., 1997; Alzerreca et al., 1999; Aakra et al., 2001a; Norton et al., 2002).

The ammonia monooxygenase (AMO) is a membrane-bound multiple subunit enzyme responsible for the conversion of ammonia to hydroxylamine (Hyman and Arp, 1992). The structural subunits of AMO in AOB are encoded by the genes *amoC*, *amoA* (containing the active site) and *amoB*, which are organized in one operon (Norton et al., 2002). The physical organization of the operon seems to be conserved in all AOB; multiple copies have been reported for beta-AOB (Norton et al., 2002), whereas so far it seems that a single copy is present in gamma-AOB (Alzerreca et al., 1999).

Since the publication of the first *amoA* sequence of *Nitrosomonas europaea* (McTavish et al., 1993), the number of partial and full-length sequences available in public databases has increased significantly. Several PCR primers to amplify *amoA* have been published (Holmes et al., 1995; Sinigalliano et al., 1995; Rotthauwe et al., 1997; Juretschko et al., 1998; Nold et al., 2000; Purkhold et al., 2000; Hoshino et al., 2001; Nicolaisen and Ramsing, 2002; Norton et al., 2002; Okano et al., 2004). The analysis of AMO-encoding genes has been extended to *amoC* and *amoB* (Purkhold et al., 2000; Norton et al., 2002; Calvó and Garcia-Gil, 2004), and more recently functional genes homologous to those in AOB have been described in archaea (Könneke et al., 2005; Treusch et al., 2005). Some of these primers were designed when only few sequences were available. Considering the new sequence information accumulated in recent years, including the complete genome of *Nitrosomonas*

europaea (Chain et al., 2003) and the draft sequences from the genomes of *Nitrosospira multiformis* and *Nitrosococcus oceani*, sequence analysis can contribute to estimate the advantages and failures of the available primers, and to assist the development of new strategies to study the structure of AOB communities. In this study we used all available *amoCAB* sequences from recognized AOB species, and whenever possible also the sequences from uncultured clones to characterize the published PCR primers and to propose new primers for the amplification of the *amoCAB* operon.

RESULTS AND DISCUSSION

Sequence analysis of *amoA* primers

Analysis of the primers in this study was carried out using the software Oligo 6.0 (Table I-1). Sequence matching of the *amoA* primers was analyzed in the ARB database prepared in this study. The complete alignment extended over 829 nucleotide positions numbered according to the sequence of *Nitrosomonas europaea* (L08050). The majority of the *amoA* sequences were concentrated in the region between positions 340 to 802. Therefore, the comparison of primers annealing outside of this region was limited to only a few sequences from the clusters: *Nitrosospira* cluster 3, *Nitrosomonas europaea*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans*, gamma-AOB, and *Methylococcus capsulatus* (Table I-2).

The primer pair AMO-F (amoA21f) and AMO-R (amoA686r) (Sinigalliano et al., 1995), had been derived from one sequence of *Nitrosomonas europaea* available at that time, and proved to be highly specific for the *Nitrosomonas europaea* cluster (Table I-2). The forward primer AMO-F (amoA21f) has three to five mismatches with some sequences of *Nitrosospira* cluster 3, and more than five mismatches with *Nitrosospira multiformis*, two sequences from *Nitrosomonas oligotropha* cluster, *Nitrosomonas cryotolerans*, gamma-AOB and MOB. In the GenBank search, the primer matched perfectly sequences from *Nitrosomonas europaea* and a probable

Table I-1: Primers analyzed in this study. New primer designations consider: target gene (amo followed by A, B or C), position in the alignment and orientation (forward f- or reverse r-). Modifications of the original primer sequence are shown in IUPAC code after the letter indicating the orientation of the primer. Other designations: p= probe for FISH; s= shorter version; Gam= specific for gamma-AOB. For amoA the positions were defined according to the sequence of *Nitrosomonas europaea* (L08050). For amoB and amoC the positions were defined according to the sequences of the *Nitrosomonas europaea* genome (BX321859). Melting temperature was calculated by nearest neighbor method. Deg.= number of different sequences due to wobble positions. mm= number of mismatched positions. N= no loops detected.

Gene	New designation	Original designation	Sequence 5'-3'	Position	length (bp)	Deg.	Tm (°C) oligo with different number of mm 0	1	2	3	4	Loop Tm (°C)	Reference
	amoA21f	AMO-F	AGA AAT CCT GAA AGC GGC	21-38	18	1	62.2	55.5	48.9	42.2	35.5	N	Sinigalliano et al., 1995
	amoA34f		GCG GCR AAA ATG CCG CCG GAA GCG	34-57	24	1	86.4	81.4	76.4	71.4	66.4	105	This study
	amoA40f	AMO-F2	AAG ATG CCG CCG GAA GC	40-56	17	1	68.7	61.6	54.6	47.5	40.4	N	Juretschko et al., 1998
	amoA49f		GAG GAA GCT GCT AAA GTC	49-66	18	1	53.6	46.9	40.2	33.6	26.9	N	This study
	amoA60r	304R	TAY CGC TTC CGG CGG CAT TTT CGC CGC	34-60	27	2	75.8	70.1	64.4	58.7	53.0	67.0	Norton et al., 2002
	amoA121f		ACC TAC CAC ATG CAC TT	121-137	17	1	51.0	44.0	36.9	29.9	22.8	N	This study
	amoA151f	A189	GGN GAC TGG GAC TTC TGG	151-168	18	4	59.0	52.4	45.7	39.0	32.4	N	Holmes et al., 1995
	amoA154f	301F	GAC TGG GAC TTC TGG CTG GAC TGG AA	154-179	26	1	67.9	62.2	56.5	50.8	45.1	N	Norton et al., 2002
	amoA154fs		GAC TGG GAC TTC TGG	154-168	15	1	46.3	38.3	30.3	22.3	14.3	N	This study
	amoA187f	amoA-1FF	CAA TGG TGG CCG GTT GT	187-203	17	1	64.4	57.3	50.2	43.2	36.1	16.0	Hoshino et al., 2001
	amoA310f	amoA-3F	GGT GAG TGG GYT AAC MG	310-326	17	4	51.1	44.0	36.9	29.9	22.8	N	Purkhold et al., 2000
amoA	amoA332f	amoA-1F	GGG GTT TCT ACT GGT GGT	332-349	18	1	58.3	51.6	45.0	38.3	31.6	N	Rotthauwe et al., 1997
	amoA332fHY	amoA1F mod	GGG GHT TYT ACT GGT GGT	332-349	18	6	58.8	52.1	45.4	38.8	32.1	N	Stephen et al., 1999
	amoA337p	A337	TTC TAC TGG TGG TCR CAC TAC CCC ATC AAC T	337-367	31	2	56.0	50.2	44.5	38.8	33.1	N	Okano et al., 2004
	amoA359rC		GGG TAG TGC GAC CAC CAG TA	340-359	20	1	65.2	59.2	53.2	47.2	41.2	30.0	This study
	amoA627r		CGT ACC TTT TTC AAC CAT CC	608-627	20	1	62.0	56.0	50.0	44.0	38.0	N	This study
	amoA665r	AMO-R2	GCT GCA ATA ACT GTG GTA	648-665	18	1	53.4	46.7	40.1	33.4	26.7	N	Juretschko et al., 1998
	amoA680r	A682 mod	AAV GCV GAG AAG AAW GC	664-680	17	18	51.5	44.4	37.4	30.3	23.3	N	Nold et al., 2000
	amoA681r	A682	GAA SGC NGA GAA GAA SGC	664-681	18	16	54.4	47.8	41.1	34.5	27.8	N	Holmes et al., 1995
	amoA686r	AMO-R	GAT ACG AAC GCA GAG AAG	669-686	18	4	54.9	48.3	41.6	34.9	28.3	N	Sinigalliano et al., 1995
	amoA820r	AMO-2R'	CCT CKG SAA AGC CTT CTT C	802-820	19	4	56.1	49.8	43.5	37.2	30.9	3.0	Okano et al., 2004
	amoA822r	AMO-2R	CCC CTC KGS AAA GCC TTC TTC	802-822	21	4	65.0	59.2	53.5	47.8	42.1	3.0	Rotthauwe et al., 1997
	amoA822rTC	amoA-2R-TC	CCC CTC TGC AAA GCC TTC TTC	802-822	21	1	70.2	64.5	58.7	47.3	41.6	3.0	Nicolaisen & Ramsing, 2002
	amoA822rTG	amoA-2R-TG	CCC CTC TGG AAA GCC TTC TTC	802-822	21	1	69.2	63.5	57.8	52.1	46.3	11.0	Okano et al., 2004
	amoA828r	302R	TTT GAT CCC CTC TGG AAA GCC TTC TTC	802-828	27	1	70.2	64.4	58.7	53.0	47.3	30.0	Norton et al., 2002
	amoB44r	amoB-4R	GCT AGC CAC TTT CTG G	29-44	16	1	51.9	44.4	36.9	29.4	21.9	41.0	Purkhold et al., 2000
	amoB160f	amoBMf	TGG TAY GAC ATK AWA TGG	160-177	18	8	47.0	40.3	33.6	27.0	20.3	N	Calvo & Garcia-Gil, 2004
amoB	amoB506r	308R	TCC CAG CTK CCG GTR ATG TTC ATC C	482-506	25	4	68.8	63.1	57.4	51.6	45.9	N	Norton et al., 2002
	amoB660r	amoBMr	RCG SGG CAR GAA CAT SGG	643-660	18	16	62.8	56.1	49.5	42.8	36.1	N	Calvo & Garcia-Gil, 2004
	amoB1179r		CCA AAR CGR CTT TCC GG	1164-1179	17	4	61.0	53.9	46.9	39.8	32.7	N	This study
	amoB1179rGam		GCA AAG CGG CTG TCT GG	1164-1179	17	1	64.8	57.8	50.7	43.7	36.6	N	This study
amoC	amoC58f		CTA YGA CAT GTC RCT GTG G	58-72	19	4	51.5	45.1	38.8	32.5	26.2	N	This study
	amoC763f	305F	GTG GTT TGG AAC RGI CAR AGC AAA	763-786	21	16	61.8	56.1	50.4	44.7	39.0	N	Norton et al., 2002

Table I-1: Comparison of the primer sequences with the ARB database. Sequences of beta-AOB were grouped in clusters according to their 16S rDNA phylogeny following the cluster designation of Purkhold et al., (2003). Sequences not grouped in any cluster are indicated as "no cluster". The number of mismatches is given in each box. Color coding: gray= no sequence in this area; black= more than 5 mismatches; blue gradient = increasing weight of the mismatches (see methods) starting in 0 (white) to more than 4 (dark blue). For the explanation of new primer designation see Table I-1 and text. Sequences from this study are indicated in bold.

Original designation (top) / New designation (bottom):

Original	AMO-F	AMO-F2	304R	A189	301F	amoA-1FF	amoA-3F	amoA-1F	amoA-1F	A337		AMO-R2	A682 mod	A682	AMO-R	amoA-2R'	amoA-2R	amoA-2RTG	amoA-2RTC	302R

Cluster	Strain	Accession	amoA21f	amoA34f	amoA40f	amoA49f	amoA60r	amoA121f	amoA151f	amoA154f	amoA154fs	amoA187f	amoA310f	amoA332f	amoA332fHY	amoA337p	amoA359rC	amoA627r	amoA665r	amoA680r	amoA681r	amoA686r	amoA820r	amoA822r	amoA822rTG	amoA822rTC	amoA828r
Cluster 0	Nitrosospira sp. III2	AJ298694																	4	3	0	2					
	Nitrosospira sp. Nsp5	AY123834																	4	3	0	2					
	Nitrosospira sp. Nsp12	AY123823																	4	3	0	2					
	Nitrosospira sp. 40KI	AJ298687																	4	3	0	2					
Cluster 2	Nitrosospira sp. B6	AJ298690																	4	3	0	2					
	Nitrosospira sp. III7	AJ298695																	4	3	0	2					
	Nitrosospira sp. O4	AJ298723																	4	3	0	2					
	Nitrosospira sp. O13	AJ298722																	4	3	0	2					
	Nitrosospira sp. L115	AJ298698																	4	3	0	3					
Cluster 3	Nitrosospira briensis	U76553	3	3	0	■	4	0	0	0	0	1		1	0	1	0					4	3	2	2	3	2
	Nitrosospira multiformis	AF042171	■	3	2	■	3	0	0	1	0	2	4	2	2	2	0		4	2	1	2	3	2	2	3	2
	Nitrosospira multiformis	AY177933						0	0	1	0	2	4	0	1	1	0			2	2	2					
	Nitrosospira tenuis	AY123824																	4	3	2	3					
	Nitrosovibrio tenuis	U76552	5	2	0	■	3	0	0	0	0	1	■	1	2	4	0		4	4	3		1	0	0	1	0
	Nitrosospira sp.	U31655										1	■	2	2	2	0										
	Nitrosospira sp. 24C	AJ298685																	4	2	2	3					
	Nitrosospira sp. A16	AJ298688																	4	2	2	3					
	Nitrosospira sp. AF	AJ298689																	4	2	2	3					
	Nitrosospira sp. L115	AY123817																	4	2	2	3					
	Nitrosospira sp. L13	AJ238542																		4	1	2					
	Nitrosospira sp. LT1FMf	AY189144														2	0				4		2	1	1	2	
	Nitrosospira sp. LT2MFa	AY189145														2	0				4		2	1	1	2	
	Nitrosospira sp. NI5	AY123832																	4	4	3	3					
	Nitrosospira sp. NpAV	AF032438	5	3	1	■	4	0	0	1	0	0	■	0	1	2	0		4	3	1	3	3	2	2	3	2
	Nitrosospira sp. Nsp1	AY123828																				4					
	Nitrosospira sp. Nsp2	AJ298719																	3	2	1	2					
	Nitrosospira sp. Nsp2	AY123822																		2	1	2					
	Nitrosospira sp. Nsp17	AY123825																	4	2	2	3					
	Nitrosospira sp. Nsp40	AY123840																				4					
	Nitrosospira sp. Nsp62	AY123837																				4					
	Nitrosospira sp. Nsp65	AY123839																		3	2	2					
	Nitrosospira sp. Nsp65	AY123838																	3	3	2	2					
	Nitrosospira sp. Np 39-19	AF042170						0	0	0	0	1	4	0	0	2	0		4	4	4		1	0	0	1	1
	Nitrosospira sp. Nv6	AY123826																	4	4	3	■					
Cluster 4	Nitrosospira sp. CT2F	AY189143														1	0		3	1	3		2	1	1	2	■
	Nitrosospira sp. Ka3	AJ298696																	3	1	3						
	Nitrosospira sp. Ka4	AJ298697																	3	1	3						
No cluster	Nitrosospira sp. Nsp41	AY123833																	4	2	1	3					
	Nitrosospira sp. Nsp58	AY123836																		2	1	3					
	Nitrosospira sp. Nsp57	AY123835																	4	4	4						
	Nitrosospira sp. NI20	AJ298703																	4	4	3	4					

Table I-2: *continued*.

Original designation (top): AMO-F | AMO-F2 | 304R | A189 | 301F | amoA-1FF | amoA-3F | amoA-1F | amoA-1F | A337 | | AMO-R2 | A682 mod | A682 | AMO-R | amoA-2R' | amoA-2R | amoA-2RTG | amoA-2RTC | 302R

	Strain	Accession	amoA21f	amoA34f	amoA40f	amoA49f	amoA60r	amoA121f	amoA151f	amoA154f	amoA154fs	amoA187f	amoA310f	amoA332f	amoA332fHY	amoA337p	amoA359rC	amoA627r	amoA665r	amoA680r	amoA681r	amoA686r	amoA820r	amoA822r	amoA822rTG	amoA822rTC	amoA828r
Nm. europaea	Nitrosomonas europaea	L08050	0	2	0		3	0	1	2	1	0	4	1	1	0	1		0	2	1	0	0	0	0	1	0
	Nitrosomonas eutropha	AY177932						2	1	2	1	2	4	0	1	4	2		1	3	3	2					
	Nitrosococcus mobilis	AF037108	0	2	0		3	0	1	2	1	0	4	1	1	0	1		0	2	1	0					
	Nitrosomonas sp. F3	AJ298691																	1	3	3	2					
	Nitrosomonas sp. F6	AJ298693																	1	3	3	2					
	Nitrosomonas sp. GH22	AF327917	1	2	0		4	2	1	2	1	2	4	0	1	4	2		2	3	3	2	2	1	1	2	1
	Nitrosomonas sp. Nm93	AF272401																	2								
	Nitrosomonas sp. Nm103	AF272411																	0	2	1	0					
	Nitrosomonas sp. Nm104	AF272409																	2								
	Nitrosomonas sp. Nm107	AF272407																	2								
	Nitrosomonas sp. TK794	AB031869	1	2	0		4	1	1	2	1	2	4	0	1	4	2		2	3	3	2	2	1	1	2	1
Nm. communis	Nitrosomonas communis	AF272399																	4	2		4					
	Nitrosomonas nitrosa	AF272404																5	2	4	4	4					
	Nitrosomonas sp. Nm33	AF272408																	2	3	3	3					
	Nitrosomonas sp. Nm41	AF272410																	1	2	3	4					
	Nitrosomonas sp. Nm58	AY123820																	2	4	4	4					
	Nitrosomonas sp. Nm148	AY123815																	2	4	4	4					
Nm. marina	Nitrosomonas aestuarii	AF272400																	2	1	3	3					
	Nitrosomonas marina	AF272405																	4	2	3	3					
	Nitrosomonas sp. C-113a	AF339042						0	1	0	2		1	2	2	0			4	3	2						
	Nitrosomonas sp. C-45	AF339041						0	1	0	1	3	1	0	2	0			3								
	Nitrosomonas sp. Nm51	AF272412																	4	3	3	2					
	Nitrosomonas sp. NO3W	AF339039						0	1	0	1	3	1	0	2	0			3	2	2						
	Nitrosomonas sp. TA-921-I-NH4	AF339043						0	2	0	1		1	2	2	1			2	2							
	Nitrosomonas sp. URW	AF339040						0	1	0	1	3	1	0	2	0			3								
Nm. oligotropha	Nitrosomonas oligotropha	AF272406																	2	4	2	2					
	Nitrosomonas ureae	AF272403																	4	4	4	4					
	Nitrosomonas sp. AL212	AF327918		3	0		5	4	1	2	1	2	4	1	1	4	2		4	2	3	3	2	3	3	2	3
	Nitrosomonas sp. JL21	AF327919		2	0		4	2	1	3	1	1	4	2	1	5	2		3	3	1	1	3	3	3	2	3
	Nitrosomonas sp. Nm143	AY123816																		2	2	2					
	Nitrosomonas sp. Nm47	AY123830																	3	4	3	3					
	Nitrosomonas sp. Nm59	AY123831																	2	4	3	4					
	Nitrosomonas sp. Nm84	AY123818																	2	3	1	1					
	Nitrosomonas sp. Nm86	AY123819																	2	3	2	2					
No cluster	Nitrosomonas cryotolerans	AF314753		4	3			0	0	0	0		4	0	1	2	2		4				2	2	2	2	4
	Nitrosomonas halophila	AF272398																	3	4	4	4					
	Nitrosomonas halophila	AY026907																	4	4	4	4					
	Nitrosomonas oligotropha	AJ298709																	3	4	4	4					
Nc. oceani	Nitrosococcus oceani	AF047705				0		0	3	0			1					0		2	3						
	Nitrosococcus halophilus	AF272521											0					3		4	4						
	Nitrosococcus sp. C-113	AF153344				0			1	4	1		1					0		2							
MOB	Methylocaldum gracile	U89301						0	2	0			2														
	Methylocaldum tepidum	U89304						0	2	0			3					1									
	Methylocapsa acidiphila	AJ278727											2														
	Methylococcus capsulatus	L40804						0	2	0			3											2	1	3		
	Methylohalobius crimeensis	AJ581836											3					2									
	Methylomicrobium album	U31654											3					1									
	Methylomonas methanica	U31653											2					3									
	Methylosarcina lacus	AY007286						0	3	0			3					2									
	Methylothermus thermalis	AY829010											3														
	Methylobacter sp. LW12	AY007285						0	3	0			2					2									

31

aminopeptidase of *Pirellula* sp. strain 1 (BX294141). Additionally, the comparison with clonal sequences from uncultured organisms showed that this primer has five mismatches to another region of *pmoA*. AMO-R (amoA686r) matched perfectly only three sequences of the *Nitrosomonas europaea* cluster, but possessed two to more than five mismatches with other sequences of this cluster. It has also two to four mismatches with almost all sequences from cultured beta-AOB, 1,190 sequences from uncultured beta-AOB, and *pmoA* from *Methylococcus capsulatus*. According to the sequence analysis, this primer pair may be suitable to amplify AOB closely related to *Nitrosomonas europaea* and exclude other AOB groups under stringent PCR conditions. The experimental evaluation (Sinigalliano et al., 1995) had shown that this primer pair can also amplify *amoA* from *Nitrosomonas cryotolerans* and *Nitrosococcus oceani*, but this should be possible only using PCR conditions favoring low specificity.
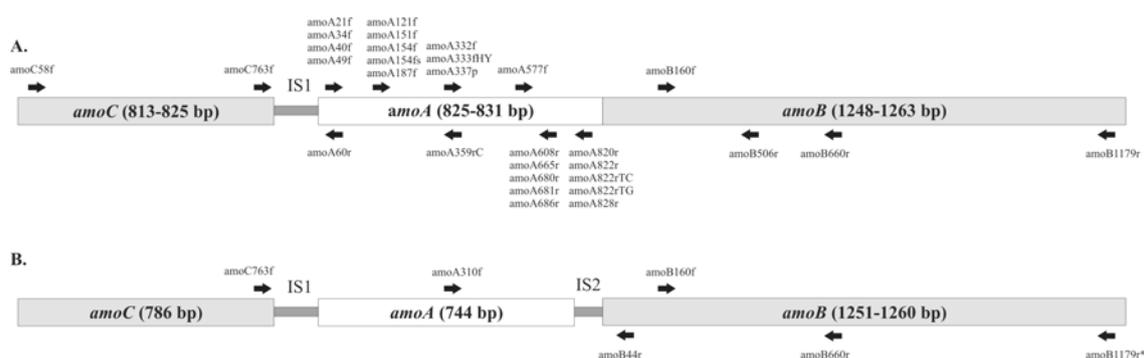
Figure I-1: Schematic diagram of the *amoCAB* operon in beta- (A) and gamma-AOB (B). IS= intergenic regions. The position and orientation of the different primers are shown by arrows. For primer designation see Tab. 1.

Consequently, only few primers have been described to amplify these genes. Primer 305F (amoC763f) (Norton et al., 2002) was designed to be used in combination with 304R (amoA60r) to generate a PCR product encompassing the 3' end of *amoC*, the intergenic region with *amoA* and the 5' part of *amoA* (see Fig. I-1). Alignment with *amoC* sequences showed that primer 305F (amoC763f) does not match perfectly any of the sequences analyzed (Fig. I-2) and it possesses a significant difference in the melting temperature (Table I-1) with 304R (amoA60r). It has between one to six mismatches with beta-AOB and more than 10 mismatches with

gamma-AOB and MOB. The two copies of *amoC* that are not located in the *amoCAB* operon of beta-AOB had more mismatches at different positions with the primers (Fig. I-2), suggesting that these genes might have evolved separately and that they could be targeted specifically.

Figure I-2: Alignment using CLUSTALW of *amoC* primers with all sequences available. Matches with the primer sequences are indicated by dots. Matches in wobble positions are shown shaded.

The primer amoB-4R (amoB44r) (Purkhold et al., 2000) which was designed to amplify *amoAB* from *Nitrosococcus halophilus* in combination with the primer amoA-3F (amoA 310f), does not match perfectly any sequence analyzed (Fig. I-3). This region is not highly conserved neither in gamma- nor in beta-AOB.

Figure I-3: Alignment using CLUSTALW of *amoB* primers with all sequences available. Matches with the primer sequences are indicated by dots. Matches in wobble positions are shown shaded. Dashes represent gaps in the alignment.

The primer pair amoBMf (amoB160f) and amoBMr (amoB660r) (Calvó and Garcia-Gil, 2004) has been published recently in order to use *amoB* as an alternative molecular marker for AOB. Both primers target regions relatively conserved in beta- and some gamma-AOB (Fig. I-3), but so far they have not been use extensively in environmental samples. The annealing temperature suggested for this primer pair (Calvó and Garcia-Gil, 2004) is significantly higher than the calculated values (Table I-1).

Strain	Copy	Accession	amoBMr (amoB660r) 5'RCGSGGCARGAACATSGG3'	amoB1179r 5'CCAAARCGRCTTTCCGG3'	amoB1179r* 5'GCAAAGCGGCTGTCTGG3'
Nitrosomonas europaea	1	BX321859	A . . T . . . . G . . . . . . C . .	. . . . . A . . G	C . . . . . A . . . . . T . . C . .
Nitrosomonas europaea		L08050	A . . T . . . . G . . . . . . C . .	. . . . . A . . G	C . . . . . A . . . . . T . . C . .
Nitrosomonas europaea	2	BX321863	A . . T . . . . G . . . . . . C . .	. . . . . A . . G	C . . . . . A . . . . . T . . C . .
Nitrosomonas europaea		AJ555508	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosomonas europaea		AJ555507	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosomonas eutropha		AJ555506	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosomonas aestuarii		AJ555504	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosomonas cryotolerans		AF314753	A . . T . . . . G . . . . . . C . .	. . . . . A . . A	C . . . . . A . . A . . . T . . C . .
Nitrosomonas sp. ENI-11		AB079054	A . . T . . . . G . . . . . . C . .	. . . . . G . . G	C . . . . T . . C . .
Nitrosomonas sp. ENI-11		AB079055	A . . T . . . . G . . . . . . C . .	. . . . . A . . G	C . . . . . A . . . . . T . . C . .
Nitrosomonas sp. TK794		AB031869	A . . T . . . . G . . . . . . C . .	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira briensis		AJ555495	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira multiformis		X90822	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira multiformis		AJ555501	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. 40KI		AJ555496	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. AF		AJ555502	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. AHB1		X90821	G . . C . . . . G . . . . . . G . .	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. B6		AJ555498	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. Ka4		AJ555497	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. NpAV	2	AF016003	G . . C . . . . G . . . . . . G . .	. . . . . G . . G	C . . . . T . . C . .
Nitrosospira sp. NpAV	3	U92432	G . . C . . . . G . . . . . . G . .	. . . . . G . . G	C . . . . T . . C . .
Nitrosospira sp. NpAV	1	AF032438	G . . C . . . . G . . . . . . G . .	. . . . . G . . G	C . . . . T . . C . .
Nitrosospira sp. Nsp1		AJ555500	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. Nsp17		AJ555503	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. Nsp2		AJ555494	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosospira sp. Nv6		AJ555499	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosococcus halophilus		AJ555509	- - - - - - - - - A - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Nitrosococcus oceani		CP000127	G . . G . . . . A . . . . . . G . .	G . . . . . G . . G . . G . . T . .	. . . .
Nitrosococcus oceani		AF047705	- . . T A . . A T G C C T T G G C C T	- - - - - - - - - - - - - - - - -	. . . .
Nitrosococcus sp. C-113		AF153344	G . . G . . . . A . . . . . . G . .	G . . . . . G . . G . . G . . T . .	. . . .
Uncultured ammonia-oxidizing		AJ555505	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Candidatus Nitrosopumilus		DQ085099	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Uncultured crenarchaeote		AJ627422	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - -
Methylocapsa acidiphila		CT005238	C . . G C C G . C . . . G C C G C .	G . G . . G G A G . . G . . G A C	. . G . . . . G A . . . . . . G A C
Methylococcus capsulatus		L40804	A . . G . . G . T . . . G . . C . .	G . G . . A . . G . . G	. . G . . A . . . . C . .
Methylococcus capsulatus		U94337	A . . G . . G . T . . . G . . C . .	G . G . . A . . G . . G	. . G . . A . . . . C . .
Methylocystis sp. M		U81596	C G A G . C G . T . . T G C C C T T	. . G . C C T . G . . G . . . . T	C . G . C C T . . . . C . T
Methylocystis sp. SC2		AJ584611	C G A A . C G . T . . T G C C C T T	. . G . T C T . G . . G . . G . T	C . G . T C T . . . . G . T
Methylocystis sp. SC2		BX649604	T . . G A C G . T . . T . C C C T T	. . . . . C T . G . . G . . G . T	C . . . . C T . . . . G . T
Methylosinus trichosporium		U31650	A G A A . C G . T . . T G C C C T T	. . G . T C T . G . . G . . . . T	C . G . T C T . . . . C . T
Uncultured bacterium		CT005232	C . . G C C G . T . . . G C C G C .	G . G . . C G A G . . A . . G A C	. . G . . C G A . . . A . . . G A C

Figure I-3. *continued*.

The primer 308R (amoB506r) (Norton et al., 2002) was proposed to be combined with 305F (amoC763f) as an alternative to obtain the full length of the *amoA* gene and its flanking regions. In the alignment with *amoB* sequences (Fig. I-3), this primer had 10 to 11 mismatches with sequences from gamma-AOB and therefore it is probably suitable only for beta-AOB.

Very recently the *amoB* sequences from two archaea have been deposited in GenBank (Könneke et al., 2005; Treusch et al., 2005). These partial sequences were too short for sequence comparison with the majority of primers analyzed here. The primers amoBMf (amoB160f) and 308R (amoB506r) presented more than 12 mismatches and are not expected to target these sequences.

Description of new primers for amplification of the *amoCAB* operon

Various motivations lead us to search for new primers for different pieces of the *amoCAB* operon. Conventional PCR approaches are often not sensitive enough to amplify functional genes from many natural habitats with low abundances of AOB. Nested PCR approaches for any of the *amo* genes could be useful to solve this problem, as has been demonstrated for 16S rDNA (Ward et al., 1997). However, adequate templates and internal primers need to be designed for this purpose.

In order to amplify the almost complete *amoCAB* operon, sequence conservation was inspected in the few sequences available for the flanking genes *amoC* and *amoB*. The primers amoC58f and amoB1179r (Table I-1) were designed to amplify the largest segment possible of the operon, which includes the three genes and the intergenic regions. The size of the PCR product is variable due to differences in the length of the genes and especially of the intergenic regions, but should be around 2,900 bp. Matching of the primer amoC58f with the *amoC* sequences available in GenBank is shown in Fig. I-2. A BLAST search retrieved only sequences from beta-AOB and did not have any unspecific match. This primer matched perfectly the sequences from beta-AOB, excepted for the *amoC* copies of *Nitrosomonas europaea* and *Nitrosospira* sp. NpAV that are not located in an operon. These extra copies of *amoC* are expected to be excluded from the amplification due not only to the difference in the sequence but also to the use of the reverse primer amoB1179r, which is located at the end of the *amoB* gene. The primer amoB1179r matches in a highly conserved region of *amoB* from beta-AOB and *Nitrosococcus halophilus* (Fig. I-3). In a BLAST search, it matched all *amoB* from beta-AOB and it had one mismatch with a hypothetical protein of *Geobacter metallireducens* (CP000148). Considering that a loop formation was detected with Oligo 6.0 (Table I-1), the experimental annealing temperature was higher than the melting temperature of the primer in order to avoid the formation of loops. In a modification of this primer (amoB1179rGam) the specificity is shifted to target only gamma-AOB.

The application of *amoA* for phylogenetic inference is partially limited due to short length and high conservation of the fragment analyzed (Purkhold et al., 2003). Therefore, one of the main challenges for applying this gene as a functional molecular marker is the search of primers that allow the amplification of a longer *amoA* piece.

Different conserved positions were detected in the *amoA* alignment. The primer amoA34f was designed to target positions close to 5' region of the gene that can be use in combination with primers for the 3' region of the gene such as amoA-2R (Rotthauwe et al., 1997) or 302R (Norton et al., 2002) to amplify almost the complete of *amoA*. This primer retrieved sequences from all beta AOB included in this study (Table I-2), and has been already used to characterized AOB communities in marine environments (Molina et al., 2007). The wider spectrum of beta AOB recognized by the primer, compared to the primer amoAF (Sinigalliano et al., 1995), makes them a better option for PCR in environments not dominated by *Nitrosomonas*-like AOB.

The primers amoA121f and amoA359rC were designed to amplify an internal fragment from beta-AOB suitable for DGGE. The primer amoA121f matches perfectly all *Nitrosospira* spp. and some *Nitrosomonas* spp. and with one to four mismatches *Nitrosomonas eutropha, Nitrosomonas* sp. GH22, *Nitrosomonas* sp. TK794, *Nitrosomonas* sp. AL212, *Nitrosomonas* sp. JL21. It has more than 5 mismatches with sequences from gamma-AOB (Table I-2). The reverse primer amoA359rC, having 9 bases overlap with amoA-1F (amoA332f) (Rotthauwe et al., 1997), matches perfectly the sequences from all *Nitrosospira* clusters and displays high similarity with the *Nitrosomonas* clusters. A former version of the primer combination amoA121fgc-amoA359r designed in our laboratory was used in the past by other research groups to analyze the impact of soil management on the diversity of AOB in soil (Webster et al., 2002). The primer amoA359rC reported in this manuscript is an improved variant of the originally primer designated amoA4-R, which was used in the DGGE without wobble positions to avoid artifacts. Besides the use of this primer combination for DGGE, the size of the expected PCR product makes it also potentially useful for quantification of AOB by real time PCR.

Although the number of *amoA* sequences from gamma-AOB is very limited (only two complete sequences), the primer pair amoA49f and amoA627r was designed to tentatively amplify a fragment of 559 bp exclusively from gamma-AOB. These primers, when checked in GenBank by BLAST, matched only the sequences used for primer design. Similarly, the evaluation in ARB showed that the forward primer matches only the two gamma-AOB while the reverse primer has three mismatches with *Nitrosococcus halophilus* but no mismatches with *Nitrosococcus*

oceani. Between one and three mismatches were recorded with some MOB and more than five with all beta-AOB (Table I-2). These primers have a similar melting temperature (Table I-1), desirable for specific amplification.
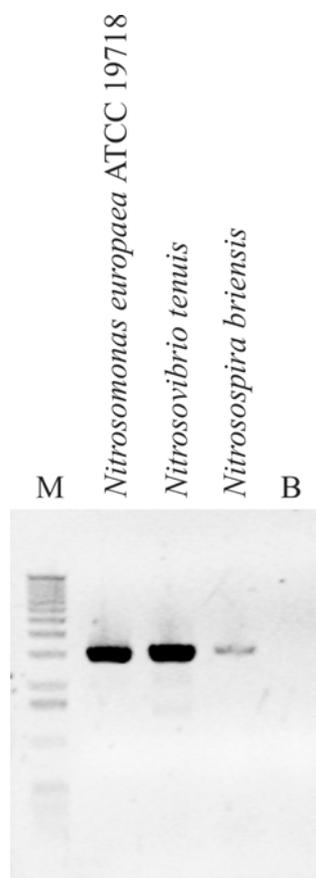
Figure I-4. Agarose gel electrophoresis of PCR products amplified with the primers amoC58f-amoB1179r from genomic DNA of recognized AOB species (A) and environmental samples (B). M= 1 Kb ladder; B=Negative control.

Experimental evaluation of the new PCR primers

The primers designed in this study were used to amplify almost the complete *amoCAB* operon from named species of AOB and environmental samples. A product of about 3,000 bp was obtained from genomic DNA of *Nitrosomonas europaea, Nitrosovibrio tenuis* and *Nitrosospira briensis* (Fig. I-4). Some environmental samples and a positive control were selected for testing a nested PCR approach (Fig. I-5). A PCR product was visible in the amplification with genomic DNA from *Nitrosomonas europaea* after 25 cycles with the most distant primers (amoC58f and amoB1179r), but not in the environmental samples (Fig. I-5A). Further nested amplification with the primer combination amoA121fgc-amoA359rC, using these

PCR products as templates, produced a fragment of the expected size (around 230 bp; Fig. I-5B).
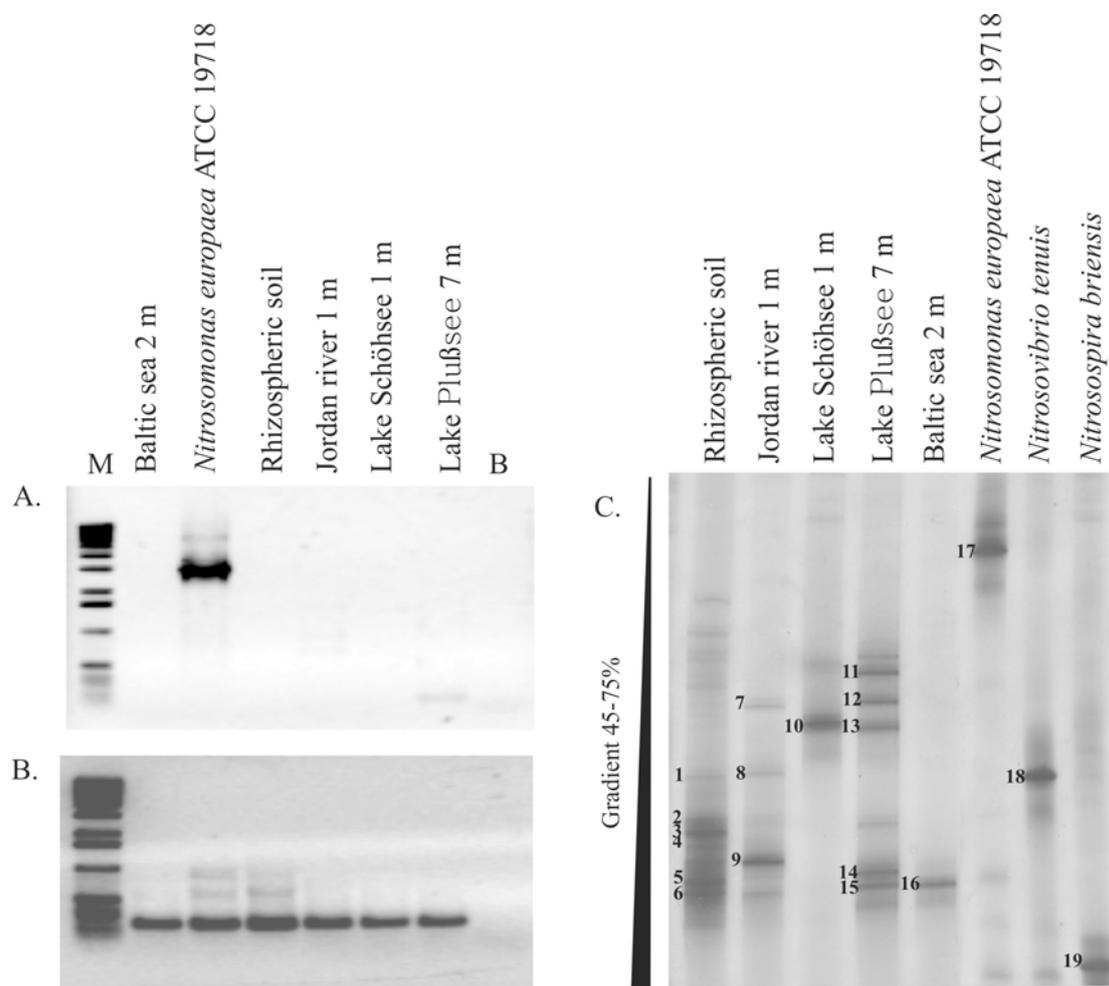
Figure I-5: Evaluation of the nested PCR approach and the DGGE for analyzing AOB in environmental samples. A. PCR with the primers amoC58f-amoB1179r directly from DNA. B. Nested PCR with the primers amoA121fgc-amoA359rC from the products shown in A. C. DGGE profiles of PCR amplicons obtained with the primers amoA121fgc-amoA359rC from environmental samples and _Nitrosomonas europaea_ shown in B. Amplicons from _Nitrosospira briensis_ and _Nitrosovibrio tenuis_ were included in the DGGE. M= 1 Kb ladder; B=Negative control.

Differences in the AOB community composition were evaluated by DGGE (Fig. I-5C). _Nitrosomonas europaea, Nitrosovibrio tenuis_ and _Nitrosospira briensis_ were used in the analysis. Although, more than one band was present on the DGGE gel, each culture produced a dominant band at different positions in the gradient: the band from _Nitrosomonas europaea_ was located at around 45%, the band from _Nitrosovibrio tenuis_ at 55%, and the one from _Nitrosospira briensis_ at around 65% of denaturant concentration. Each environmental sample was characterized by a specific

pattern of bands.

Table I-3: Identification of bands excised from the DGGE gel shown in Fig. I-5. The first BLAST hit of an identified species is given, as well.

Band no	Sample	lenght (bp)	First hit in BLAST (Accession)	Identity (%)	First identified hit in BLAST (Accession)	Identity (%)
1	Soil	218	Uncultured ammonia-oxidizing bacterium clone slEASLc44 (AY177930)	97	*Nitrosospira* sp. 40KI (AJ308612)	98
2	Soil	221	Uncultured ammonia-oxidizing bacterium clone slEASLc44 (AY177930)	97	*Nitrosospira* sp. NpAV (U92432)	94
3	Soil	215	Uncultured ammonia-oxidizing bacterium clone slEASLc44 (AY177930)	96	*Nitrosospira* sp. 40KI (AJ308612)	98
4	Soil	214	Uncultured ammonia-oxidizing bacterium clone slEASLc44 (AY177930)	97	*Nitrosospira* sp. 40KI (AJ308612)	98
5	Soil	211	Uncultured ammonia-oxidizing bacterium clone slGMSLc3 (AY177931)	96	*Nitrosospira* sp. 40KI (AJ308612)	99
6	Soil	212	Uncultured ammonia-oxidizing bacterium clone slGMSLc3 (AY177931)	96	*Nitrosospira* sp. 40KI (AJ308612)	99
7	Jordan river	218	Uncultured ammonia-oxidizing bacterium clone slEASLc41a (AY177929)	96	*Nitrosospira multiformis* (AY177933)	96
8	Jordan river	219	Nitrosospira sp. NpAV copy 3 (U92432)	91	*Nitrosospira* sp. NpAV copy 3 (U92432)	91
9	Jordan river	222	Uncultured ammonia-oxidizing bacterium clone slGMSLc3 (AY177931)	98	*Nitrosospira* sp. 40KI (AJ308612)	99
10	Lake Schöhsee	228	Nitrosospira sp. NpAV copy 2 (AF016003)	87	*Nitrosospira* sp. NpAV copy 2 (AF016003)	87
11	Lake Plußsee	133	Uncultured beta proteobacterium DGGE band U4a(3)B (AJ308620)	96	*Nitrosospira* sp. (X90821)	93
12	Lake Plußsee	220	Uncultured beta proteobacterium DGGE band U4a(3)B (AJ308620)	100	*Nitrosospira* sp. NpAV copy 3 (U92432)	95
13	Lake Plußsee	207	Nitrosospira sp. NpAV copy 3 (U92432)	94	*Nitrosospira* sp. NpAV copy 3 (U92432)	94
14	Lake Plußsee	202	Uncultured ammonia-oxidizing bacterium clone slGMSLc3 (AY177931)	96	*Nitrosospira* sp. 40KI (AJ308612)	99
15	Lake Plußsee	197	Uncultured ammonia-oxidizing bacterium clone slGMSLc3 (AY177931)	94	*Nitrosospira* sp. NpAV copy 3 (U92432)	92
16	Baltic Sea	220	Uncultured ammonia-oxidizing bacterium clone slGMSLc3 (AY177931)	96	*Nitrosospira* sp. 40KI (AJ308612)	99
17	Standard	226	*Nitrosomonas europaea* ATCC 19718 (BX321863)	98	*Nitrosomonas europaea* ATCC 19718 (BX321863)	98
18	Standard	221	*Nitrosovibrio tenuis* (U76552)	100	*Nitrosovibrio tenuis* (U76552)	100
19	Standard	228	*Nitrosospira briensis* (U76553)	99	*Nitrosospira briensis* (U76553)	99

The most prominent bands from each pattern were excised from the gel and sequenced. All the sequences matched *amoA* when compared to GenBank entries by BLAST (Table I-3). In all the cases, the first hit corresponding to an identified species was related to the *Nitrosospira* lineage. The most complex communities were observed in rhizospheric soil and Lake Plußsee, whereas the communities in Lake Schöhsee and the Baltic Sea were dominated by a single band. In some cases, the first hit in BLAST was the same for bands from different samples and in different positions in the gel, e.g. bands 5 and 6 (rhizospheric soil), 9 (Jordan River), 14 and 15 (Lake Plußsee) and 16 (Baltic Sea) matched the uncultured ammonia-oxidizing bacterium clone slGMSLc3. However, alignment of the sequences and the identity level obtained in BLAST (Table I-3) showed that the bands migrating in different positions in the gel corresponded to different sequences. The occurrence of the same BLAST hit in bands located at different positions in the gel can be explained by the low number of environmental sequences in the database for the region amplified with the primers amoA121fgc-amoA359rC.

3. II Application of novel PCR strategies and sequence analysis to study the *amoCAB* operon in ammonia-oxidizing betaproteobacteria
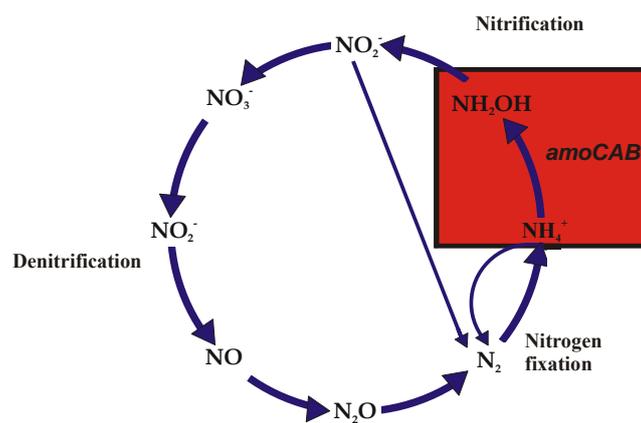
ABSTRACT

A novel PCR strategy involving nested amplification was assayed for the analysis of various regions of the complete *amoCAB* operon in ammonia-oxidizing bacteria belonging to the beta subclass of proteobacteria (beta-AOB). Primers to amplify the whole *amoCAB* and the different subunits and intergenic regions were applied. The fragments obtained were analyzed by cloning and sequencing and showed different grades of identity to *amoCAB* sequences in the database. Environmental sequences retrieved by this approach formed clusters separated from those of cultured ammonia-oxidizing bacterial species. By multiple alignments, one environmental sequence of *amoCAB* (2901 bp) could be reconstructed. This approach allows retrieving *amoCAB* sequences from environmental samples and open new insights on the study of the evolution and function of these coupled genes.

INTRODUCTION

Ammonia oxidation to nitrite, the first step in nitrification, is primarily mediated by chemolitoautotrophic microorganisms belonging to beta- and gammaproteobacteria (Prosser, 1989; Head et al., 1993; Teske et al., 1994; Kowalchuk and Stephen, 2001) and archaea (Könneke et al., 2005; Treusch et al., 2005). In order to examine the diversity of the ammonia-oxidizing bacteria (AOB) several molecular tools have been developed (Bothe et al., 2000; Kowalchuk and Stephen, 2001; Prosser and Embley, 2002). One of these techniques, targets the genes encoding the enzyme ammonia monooxygenase (AMO), which catalyzes the oxidation of ammonia to hydroxylamine (Hyman and Arp, 1992). The AMO is a membrane-bound multiple subunit enzyme encoded by the genes *amoC*, *amoA* (containing the active site) and *amoB*, which are organized into the *amoCAB* operon (Norton et al., 2002). Genes homologous to *amoCAB* have been also described in archaea (Könneke et al., 2005; Treusch et al., 2005), but with relatively low sequence similarity to their bacterial counterparts.

One of the limitations for the applicability of PCR detection of *amoCAB* genes is the relative low abundance of AOB in natural environments. Analysis based on 16S rRNA genes has shown that nested amplification can improve the detection of AOB (Ward et al., 1997). In this study we describe a novel PCR strategy that can be applied to amplify the different subunits of AMO in environmental samples using a nested PCR approach. This approach provides a more sensitive detection method and brings information on all the different subunits, which can be relevant to analyze the origin and evolution of this microbial group and its function in the environment.

RESULTS AND DISCUSSION

Nested PCR approach to amplify the *amoCAB* genes

Several combinations of published (Holmes et al., 1995; Rotthauwe et al., 1997; Norton et al., 2002; Calvó and Garcia-Gil, 2004) and newly designed PCR primers (Table II-1, Fig. II-1) were used to amplify different regions of the *amoCAB* operon in beta-AOB. Amplification of *amoCAB* with the primers amoC58f/amoB1179r (Fig. II-2A) produced a band of approximately 3,000 bp with beta-AOB (*Nitrosomonas europaea* ATCC 19718, *Nitrosomonas eutropha* C-31 ATCC 25978, *Nitrosospira briensis* C-128, *Nitrosospira tenuis* Nv1, *Nitrosomonas* sp. L13). The size of the *amoCAB* products in these beta-AOB was variable due to the variability of the intergenic spacer region between *amoC* and *amoA* in beta-AOB (Norton et al., 2002). This amplification did not produce any band with the gamma-AOB (*Nitrosococcus oceani* ATCC 19707) nor with the type I and type II methane oxidizing bacteria (MOB) (*Methylococcus capsulatus* Bath NCIMB 11853, *Methylocystis parvus* OBBP) (Fig. II-2A). In order to confirm the specificity of this amplification, *amoCAB* PCR products were used as template for *amoA* PCR with the primers amoA34f/amoA-2R, which produced one band of 789 bp in beta-AOB, but no products in gamma-AOB or MOB (Fig. II-2B).

3. RESULTS-II

Table II-1: Primers used to amplify fragments from *amoCAB* genes in betaproteobacterial ammonia-oxidizing bacteria.

Primer	Position[a]	Sequence 5'-3'	Reference
amoC58f	58-72	CTA YGA CAT GTC RCT GTG G	This study
305F	763-786	GTG GTT TGG AAC RGI CAR AGC AAA	(Norton et al., 2002)
amoA34f	34-57	GCG GCR AAA ATG CCG CCG GAA GCG	(Molina et al., 2007)
amoA-1F	332-349	GGG GTT TCT ACT GGT GGT	(Rotthauwe et al., 1997)
amoA349r	332-349	ACC ACC AGT AGA AAC CCC	This study
amoA664f	664-681	GCS TTC TTC TCN GCS TTC	This study
A682	664-681	GAA SGC NGA GAA GAA SGC	(Holmes et al., 1995)
amoA802f	802-822	GAA GAA GGC TTT SCM GAG GGG	This study
amoA-2R	802-822	CCC CTC KGS AAA GCC TTC TTC	(Rotthauwe et al., 1997)
amoBMf	160-177	TGG TAY GAC ATK AWA TGG	(Calvó and Garcia-Gil, 2004)
amoBMr	643-660	RCG SGG CAR GAA CAT SGG	(Calvó and Garcia-Gil, 2004)
amoB1179r	1164-1179	CCA AAR CGR CTT TCC GG	This study

[a] Positions in the amoCAB gene of *Nitrosomonas europaea* ATCC 19718 (Accession number, BX321859).
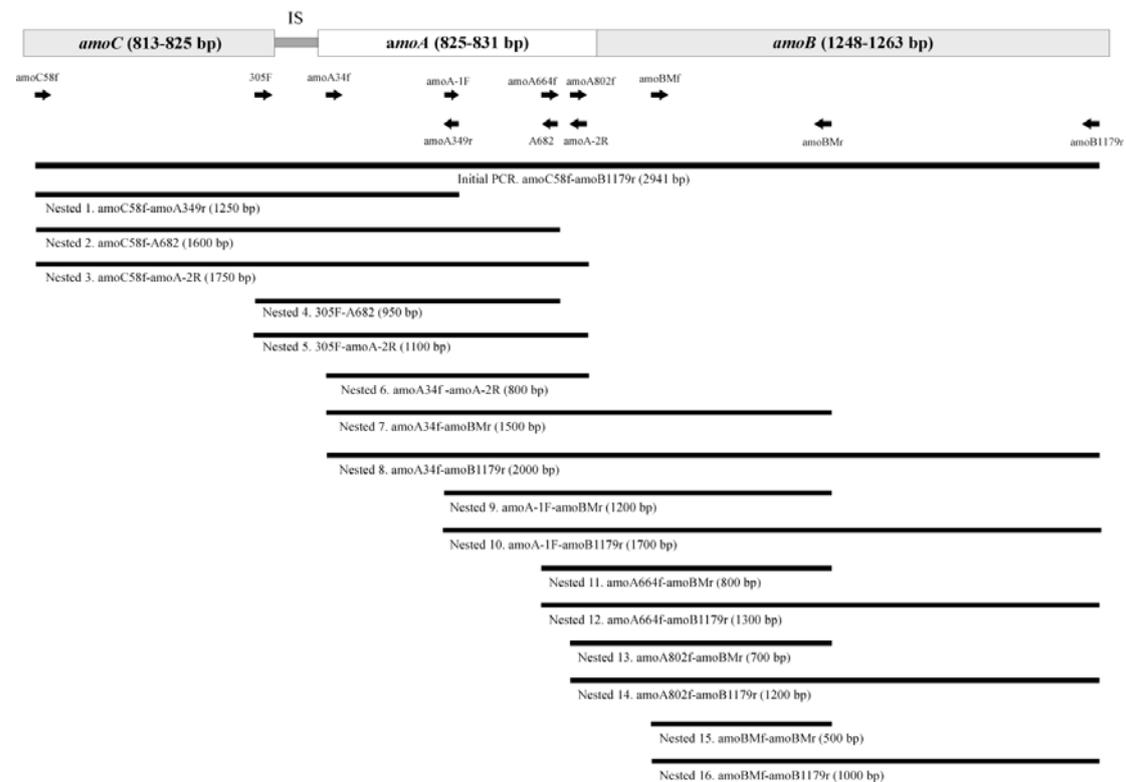
Figure II-1: Structure of *amoCAB* operon in β-AOB including intergenic space (IS) between *amoC* and *amoA* and designed PCR for this study. Initial PCR and 16 nested PCRs are described. The expected length of fragments is given in parenthesis.

44

Additional combinations of primers (see Table II-1, Fig. II-1) were used to amplify different regions of the *amoCAB* operon using the initial *amoCAB* template. These combinations were tested in genomic DNA from *Nitrosomonas europaea* ATCC 19718 and total DNA extracted from an environmental sample. A total of 16 nested amplifications were assayed, but only 10 of them produced a band of the expected size in both *Nitrosomonas europaea* and the environmental sample (primer combinations 1, 4, 5, 6, 7, 8, 9, 10, 13 and 14; Fig. II-3). In the primer combinations 2, 3, 11 and 12, a product with the expected size was obtained from *Nitrosomonas europaea*, but no-amplification or a wrong size product was obtained for the environmental sample. Although, a band with the expected size was also observed in PCR with the primer combinations 15 and 16, the amplification was very inefficient in both *Nitrosomonas europaea* and the environmental sample.
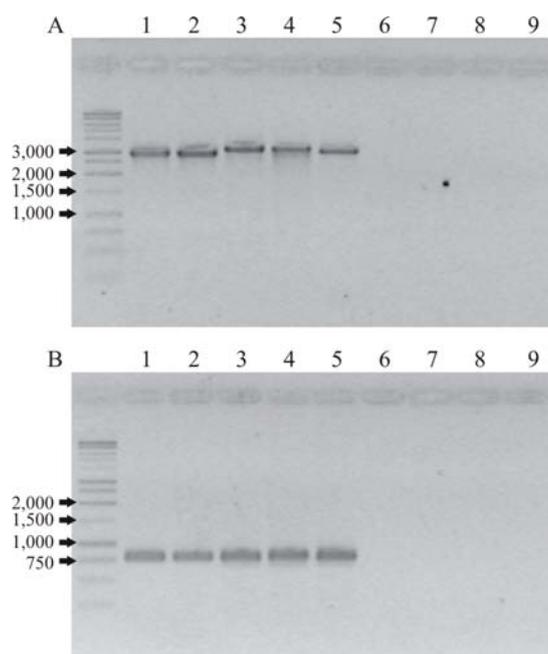
Figure II-2: Agarose gel electrophoresis of PCR products from *amoCAB* (approximately 3,000-bp) (A) and of 789-bp fragments of the *amoA* (789-bp) (B) from ammonia-oxidizing bacteria and methane-oxidizing bacteria. Lane 1: *Nitrosomonas europaea* ATCC 19718; lane 2: *Nitrosomonas eutropha*; lane 3: *Nitrosospira briensis*; lane 4: *Nitrosovibrio tenuis*; lane 5: *Nitrosomonas* sp. L13; lane 6: *Nitrosococcus oceani* ATCC 19707; lane 7: *Methylococcus capsulatus*; lane 8: *Methylocystis parvus*; lane 9: Blank.
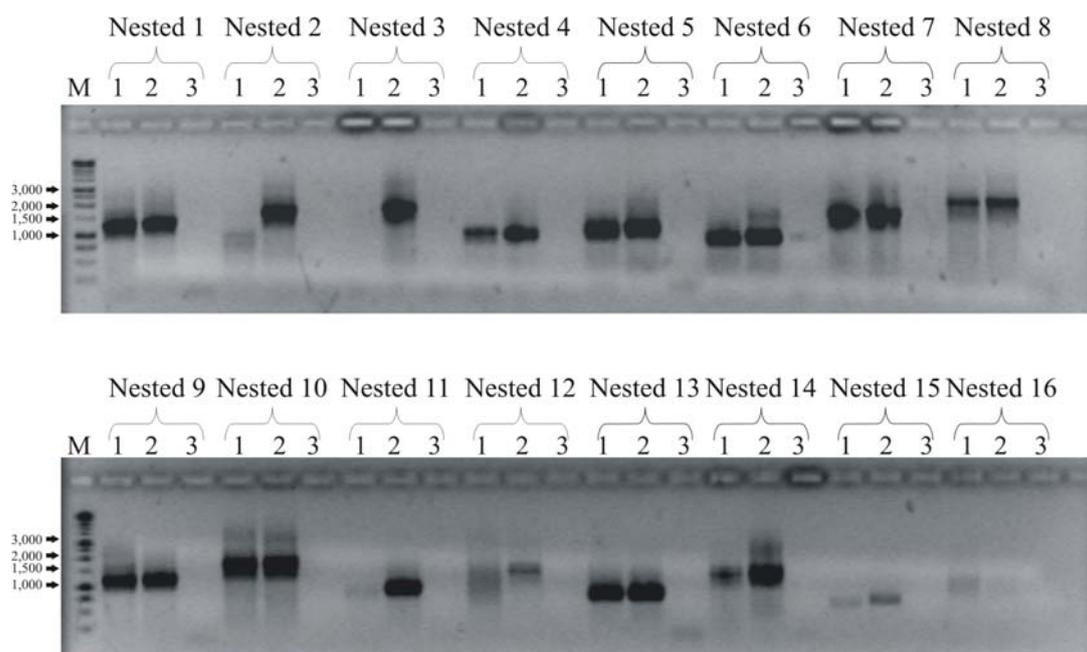
Figure II-3: *amoCAB* **nested PCR amplification of** *Nitrosomonas europaea* **ATCC 19718 and Baltic Sea sediment with different primer combinations using amoC58f-amoB1179r PCR fragment. M = 1 kb Ladder; 1 = Baltic Sea sediment; 2 =** *Nitrosomonas europaea* **ATCC 19718; 3 = Blank.**

Cloning and sequencing of *amoCAB* PCR products

In order to better characterize the *amoCAB* products obtained from the environmental sample, the following PCR products were selected for cloning and sequencing: amoC58f/amoA349r (nested 1), 305F/amoA-2R (nested 5), amoA34f /amoBMr (nested 7), amoA34f-amoB1179r (nested 8), amoA-1F/amoB1179r (nested 10) and amoA802f/amoBMr (nested 13). These combinations were selected because they represent different regions within the *amoCAB* operon, including *amoC* (nested 1), the intergenic space (IS) between *amoC* and *amoA* (nested 1 and 5), *amoA* (nested 1, 5, 7, 8 and 10) and *amoB* (nested 7, 8, 10 and 13). The cloning did not intended to thoroughly describe the diversity on the sample but rather to validate the methodological approach. Therefore, from each cloning only 12 clones were peaked randomly and checked for inserts. Finally, 47 clones containing a right-sized insert were obtained and the comparison of the sequences obtained with sequences deposited in the GenBank database showed that in all the cases the cloned products corresponded to the different subunits of AMO operon (see below).

Sequence analysis of clone libraries

In the phylogenetic analysis, all *amoC* clones (766 bp; from the nested 1) formed a single cluster (Cluster C-I) that was related to *Nitrosomonas* (Fig. II-4A). The corresponding *amoA* sequences from the same clones were also grouped into a single cluster (Cluster A-I, Fig. II-4B), that also contained clonal sequences from products obtained with the nested 5 (305F/amoA-2R; 1 clone), nested 7 (amoA34F/amoBMr; 3 clones), nested 8 (amoA34F/amoB1179R; 2 clones) and nested 10 (amoA-1F/amoB1179R; 5 clones). The cluster A-I was related to other sequences from *Nitrosomonas* spp., but formed an independent branch from cultured AOB species.

Since many of the *amoA* sequences analyzed here included the starting codon of the gene, the codon usage was analyzed in the clones. It has been noted before that the unusual start codon GTG is preferentially found in *Nitrosomonas amoA*, in contrast to the standard ATG start found in *Nitrosospira* strains (Norton et al., 2002). The analysis of the sequences included in the cluster A-I showed the triplet GTG as start codon, confirming their placement in the *Nitrosomonas* lineage.

Two additional *amoA* clusters related to *Nitrosospira* were observed (Fig. II-4B). This clusters included most of the clones from the nested 5 (9 clones in cluster A-II and 2 clones in cluster A-II) and some of the clones of the nested 7 (2 clones in cluster A-II). All this clones have the standard ATG start codon that has been observed in *Nitrosospira* strains (Norton et al., 2002).

The phylogenetic analysis of the *amoB* sequences was in agreement with the results of *amoA*. The clones in the cluster B-I in phylogeny of *amoB* (Fig. II-4C) corresponded to those in the *amoA* cluster A-I, whereas the clones in cluster B-II corresponded to those included in *amoA* cluster A-II. The cluster B-I that was related to the *Nitrosomonas* lineage, included clones from the nested 7 (3 clones), 8 (2 clones), 10 (5 clones) and 13 (amoA802F/amoBMr; 5 clones). The cluster B-II was related to the *Nitrosospira* lineage and contained sequences from nested 7 (2 clones) and nested 13 (5 clones).
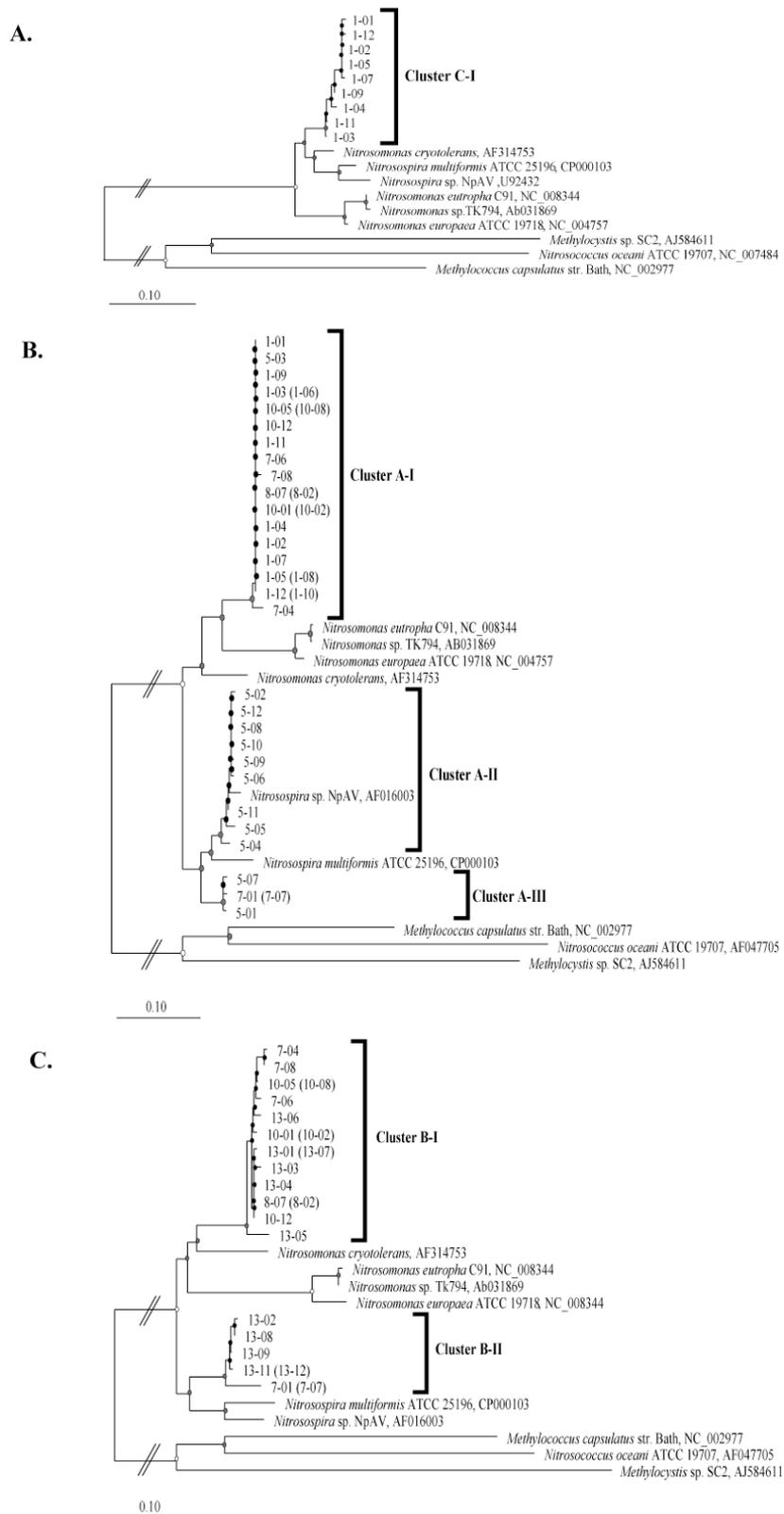
Figure II-4: Phylogenetic tree based on AmoC (A), AmoA (B) and AmoB (C) sequences. Phylogenies were constructed with the PHYLIP subroutine by Fitch-Margoliash in ARB using Amo from *Nitrosococcus occeani* and Pmo from *Methylococcus capsulatus* and *Methylocystis* as outgroups. Clones in this study are shown in bold. Bootstrap values ≥90% (100 replicates) are indicated at the branch points in two different colors: black (100%), gray (95 - 99%) and white (90 - 95%).

Analysis of the intergenic region *amoC-amoA* and *amoA-amoB*

Sequence analyses of cultured AOB species has indicated the existence of a IS between *amoC* and *amoA*. This region varies in size as well as in sequence, largely due to insertions and deletions of nucleotide blocks outside conserved regions (Norton et al., 2002). The phylogenetic relevance of the IS region between *amoC* and *amoA* has never been considered, only the intergenic space (IS) between the 16S rRNA and the 23S rRNA genes has been used in the past to study the phylogeny of AOB (Aakra et al., 2001b).

In the clones including *amoC* and *amoA*, an intergenic space between both genes was observed. For those clones obtained from the nested 1, the length of the IS varied between 136 to 138 bp, which is shorter than the IS reported for different species of identified ammonia-oxidizing bacteria (Norton et al., 2002). The length of the IS in some of the clones from the primer combination 305F/amoA-2R is similar to the IS of cultured AOB species (Norton et al., 2002). For example the clones 5-01, 5-02, 5-04, 5-06, 5-08, 5-09, 5-10 and 5-12 (233 bp) produced an IS similar to the one reported for *Nitrosospira* sp. NpAV (223 bp), while in the clones 5-05, 5-07 and 5-11 the length of the IS (426 or 428 bp) was similar to the one reported for *Nitrosospira tenuis* (427 bp).

Although for the majority of the clones the phylogenetic analysis and the IS length showed correspondence, in some others like 5-01, 5-07 and 5-11, it did not, suggesting that other evolutionary processes can be occurring in this non-coding region, that are not observed in the coding areas of the operon.

In all the clones related to *Nitrosospira* sp. the *amoA* was followed immediately by *amoB*. In contrast, a 1 bp overlap and shift in the open reading frame between *amoA* and *amoB* was observed in the clones related to *Nitrosomonas* sp.. This is in agreement with previous observations of the physical structure of the *amoCAB* operon indicating that in beta-AOB, *amoA* is either followed immediately by *amoB*, or there is a 1-bp overlap between both genes (Norton et al., 2002), whereas in gamma-AOB the genes *amoA* and *amoB* are separated by an intergenic space of approximately 65 bp (Alzerreca et al., 1999; Norton et al., 2002).
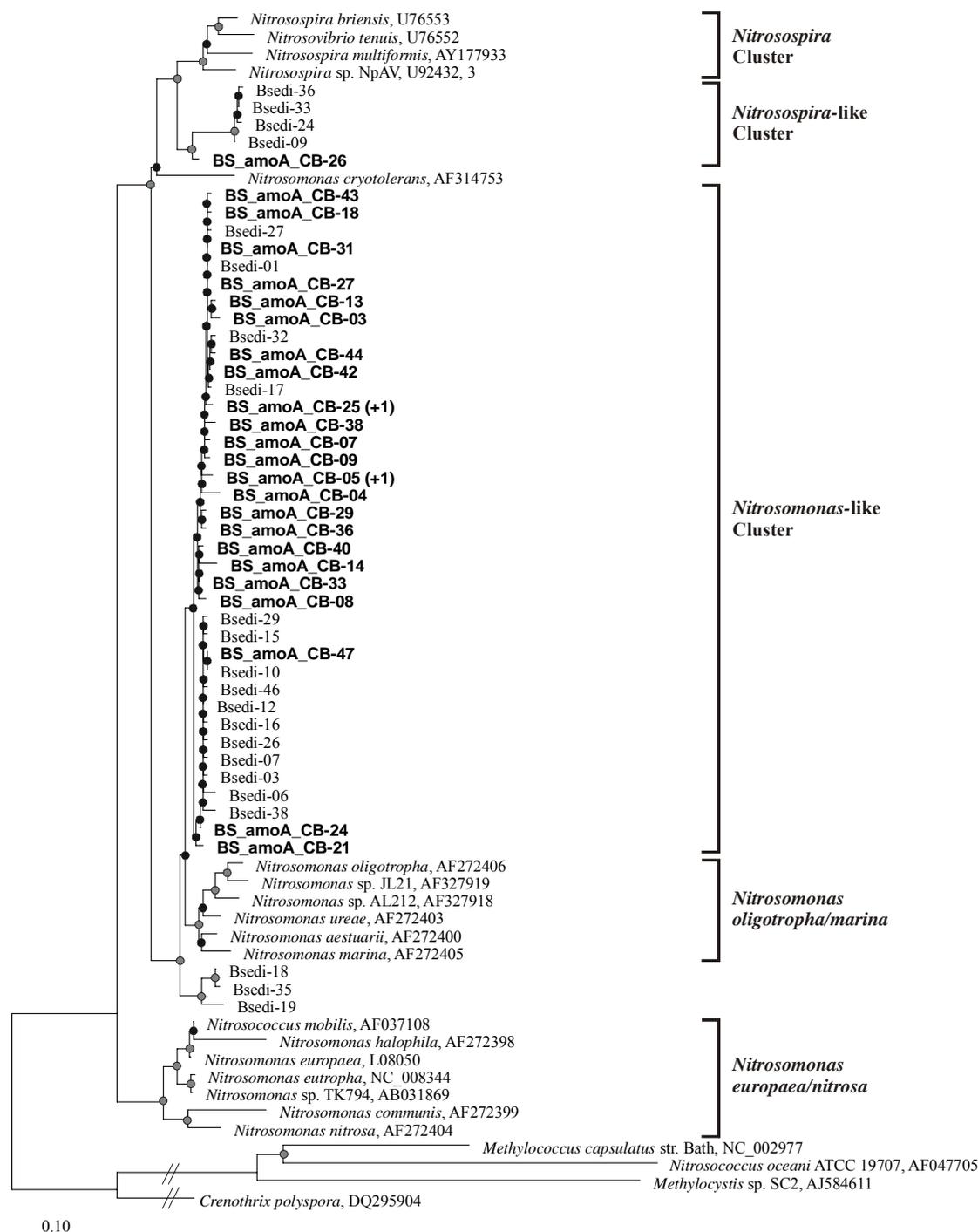
Figure II-5: Phylogenetic tree based on 263 amino acids of AmoA, constructed with the PHYLIP subroutine and Fitch-Margoliash method in ARB. Outgroups: AmoA from *Nitrosococcus oceani* and PmoA from *Crenothrix polyspora*, *Methylococcus capsulatus* and *Methylocystis parvus*. Clones from this study are coded as BS_amoA_CB and shown in bold; clones from our previous study are coded as Bsedi. Cluster designations are based on (Purkhold et al., 2000) and (Francis et al., 2003). Bootstrap values ≥95% (100 replicates) are indicated at the branch points in two different colors: black (100%) and gray (95 - 99%).

Alignment of an environmental *amoCAB* sequence

Using multiple alignments, the sequences from the six clone libraries were aligned in an attempt to reconstruct an environmental *amoCAB* sequence. One sequence of *amoCAB* (2901 bp) was assembled, with an overlapping approximately of 300 bp in *amoA*. The assembled sequence contains *amoC* (766 bp), IS (136 bp), *amoA* (825 bp) and *amoB* (1177 bp, 1 bp overlap with *amoA*). The comparison of this environmental sequence with complete sequences from the *amoCAB* operon reported in GenBank showed as closest relative *Nitrosomonas cryotolerance,* with identities in the DNA sequence of 82% for *amoC*, and 78% for both *amoA* and *amoB*. The identity at the level of the protein sequence was of 92, 89 and 86% for *amoC*, *amoA* and *amoB*, respectively.

Extended analysis of *amoA* sequences

After the publication of the first *amoA* sequence from *Nitrosomonas europaea* (McTavish et al., 1993), the number of sequences of this gene in public databases has increased enormously, including the complete genome of *Nitrosomonas europaea* (Chain et al., 2003), the genomes of *Nitrosospira multiformis* and *Nitrosococcus oceani*, and *amoA* sequences in metagenomic libraries (Venter et al., 2004; Treusch et al., 2005). Despite this, due to the limitations of the primers used, up to date, more than 90 % of the *amoA* sequences in GenBank ($\geq$ 2,500 sequences) are only partial sequences (about 500 bp from 800 bp possible). Although the potential of *amoA* as molecular markers has been widely recognized, the partial *amoA*/AmoA fragment analyzed in the vast majority of studies provides less resolution compared to 16S rDNA, since its relative small size and the high sequence conservation of the stretch analyzed (Purkhold et al., 2003).

Many of these limitations can be overcome by using primers that target a longer *amoA* fragment (Norton et al., 2002). The primer combination amoA34f/amoA-2R (nested 6) can be a very good alternative to target almost the whole *amoA*. In order to evaluate this, a clone library with PCR products using this primer pair was prepared. A total of 26 clones had an insert of the right size (789 bp). Although the efficiency of cloning was low, all the clones contained *amoA* sequences according to BLASTX (http://www.ncbi.nlm.nih.gov/blast/Blast) (Altschul et al.,

51

1997). The deduced amino acid sequence of 25 clones was 91~93% identical with *amoA* from *Nitrosomonas* sp. AL212 (Acc. No. AF327918), while the deduced amino acid of one clone (BS_amoA_CB-26) was 90% identical with *Nitrosospira* sp. NpAV (Acc. No. U92432).

A 789 bp region (corresponding to 263 amino acids) of the clonal *amoA* sequences was used to construct a phylogenetic tree. For comparative purposes this tree includes sequences of reference cultures of AOB and 22 clonal *amoA* sequences obtained in a previous, independent experiment from the same sample (Kim et al, in preparation).

The clonal sequences from the Baltic Sea sediment-water interface fell into three clusters, a *Nitrosospira*-like cluster and two *Nitrosomonas*-like clusters (Fig. II-5). The majority of clones was related to *Nitrosomonas*-like cluster A and B (cluster nomenclature according to (Purkhold et al., 2000) and (Francis et al., 2003)). One clone (BS_amoA_CB-26) was related to a *Nitrosospira*-like cluster. This distribution of the clones coincided with a previous study of the same sample based on 16S rDNA sequences (Kim et al., 2006). Also, the distribution of the clones obtained in this study coincided with *amoA* clones retrieved in a previous study from the same environment (clones coded by Bsedi in Fig. II-5). However, the Cluster C found in Bsedi was not observed in this clone library.

3. III Comparative Analysis
of ammonia-oxidizing bacterial communities
in two lakes in North Germany
and the Baltic Sea
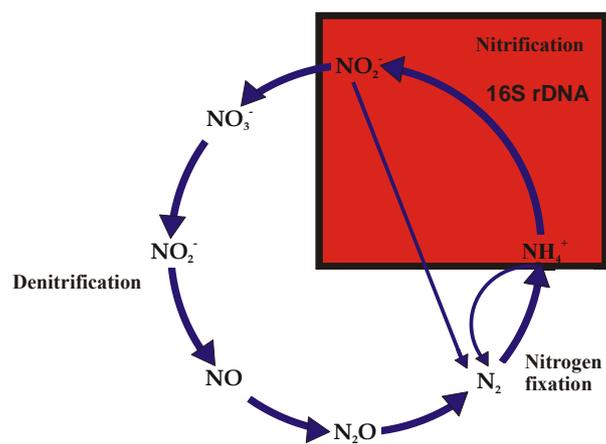
ABSTRACT

One important pathway of the nitrogen cycle in aquatic environments is the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB). In this study the composition of AOB communities was compared between fresh (lakes Plußsee and Schöhsee) and brackish (Baltic Sea) water at two different levels: i) between environments and ii) within different depths in each environment. Changes in the community structure were studied by denaturing gradient gel electrophoresis (DGGE) and clone libraries of PCR products of 16S rRNA genes (rDNA) from AOB of the beta subclass of proteobacteria. Each environment displayed a particular DGGE band pattern. In Plußsee and the Baltic Sea, the differentiation of communities in epi- and metalimnion from those in hypolimnion coincided with a distinct stratification of the water column. In Schöhsee with an aerobic hypolimnion, the communities at all depths were similar. AOB communities in sediments were different from those in the water column. The composition of clone libraries showed the presence of specific *Nitrosomonas* and *Nitrosospira*-like sequences in each environment and habitat.

INTRODUCTION

Nitrification, the microbiologically mediated two-step oxidation of ammonia to nitrite and then to nitrate, is an important process in aquatic environments. It links ammonia from different sources (organic matter mineralization or nitrogen fixation) to denitrification, which might result in the loss of nitrogen from the system in the form of gaseous dinitrogen (Ward, 2000). Nitrification is especially important in coastal and freshwater systems with elevated anthropogenic inputs of nitrogen, because it can lead ultimately to nitrate contamination, which produces eutrophication and ecosystem degradation (Seitzinger, 1988).

The microorganisms responsible for the first step of nitrification, autotrophic ammonia-oxidizing bacteria (AOB), are extremely refractory to traditional laboratory culture techniques, making the study of their ecological importance and *in situ* diversity difficult (Koops et al., 2003). AOB are restricted to two monophyletic

clusters within the beta and gamma subclasses of proteobacteria, also called beta- and gamma-AOB (Head et al., 1993; Teske et al., 1994). This has facilitated the use of molecular markers targeting the 16S rRNA gene (16S rDNA) to study the distribution and diversity of AOB in various environments (Bothe et al., 2000; Kowalchuk and Stephen, 2001).

Sequences from clone libraries recovered from different environments have shown that total diversity of AOB exceeds the diversity of culture collection isolates (Stephen et al., 1996). Available sequence information from cultured isolates identifies at least nine clusters within the beta-AOB (Purkhold et al., 2003). Two additional environmental clusters including 16S rDNA sequences from uncultured bacteria (*Nitrosospira* cluster 1 and *Nitrosomonas* cluster 5) that clustered in between the coherent group built by all known AOB have been proposed (Stephen et al., 1996; Kowalchuk et al., 1997). It has been shown that the occurrence of some of these clusters can be coupled with environmental gradients such as pH and ammonia concentration (Koops and Pommerening-Röser, 2001; Prosser and Embley, 2002).

In marine sediments and open waters, specific groups of 16S rDNA sequences of AOB have been recorded. Sequences similar to *Nitrosospira* cluster 1 (Bano and Hollibaugh, 2000; Hollibaugh et al., 2002) and *Nitrosomonas* cluster 5 and 7 (Phillips et al., 1999; Hollibaugh et al., 2002), have been found in clone libraries from Arctic, Antarctic and Mediterranean waters, as well as in sediments from the Pacific Northwest, Scotland, and Netherlands (Kowalchuk et al., 1997; McCaig et al., 1999; Freitag and Prosser, 2003, , 2004). Relatively little is known about the diversity and distribution of AOB in freshwater lakes. A previous study including lakes in North Germany found that AOB species composition differs among depths in vertical profiles, and among samples and enrichments (Ward et al., 1997). Additionally, an apparently dominant group of AOB belonging to *Nitrosomonas* cluster 6 (*Nitrosomonas marina* and *Nitrosomonas oligotropha*) has been detected in sediment and water samples from lakes in the Netherlands (Speksnijder et al., 1998).

The aim of this study was to compare the composition of AOB communities in two lakes in North Germany (Lakes Plußsee and Schöhsee) and the Baltic Sea. The comparison was carried out at two levels: between different layers of the water

column and sediment, and between the environments. Differences in the community composition were analyzed by denaturing gradient gel electrophoresis (DGGE) and clone libraries of PCR products amplified in a nested PCR approach with primers specific for 16S rDNA of beta-AOB.

RESULTS

Hydrographic data

The two lakes (Plußsee and Schöhsee) and the Baltic Sea were sampled in the period of thermal stratification. For the comparison of the AOB communities, four layers were established according to the temperature and oxygen profiles (Fig. III-1). These depths included epi-, meta- and hypolimnion in the three environments. Although in Schöhsee a thermal stratification was observed, this did not cause the formation of a strictly anaerobic hypolimnion. Additionally, in one sample taken at the water-sediment interface, the proportion of particulate material from sediment was very high and therefore they will be denominated sediment.

DGGE analysis of 16S rDNA

DGGE with PCR products of the hypervariable V3 region of the 16S rDNA (Muyzer et al., 1993) showed differences in the beta AOB communities in samples from different depths of the same location and between environments. Data from several DGGE gels obtained from different PCR reactions from various filters and DNA extractions were compared in order to evaluate whether the differences were due to variability within the samples. In all the cases the resulting patterns showed to be highly reproducible (data not shown). For the different samples, few very intense bands were observed in the patterns (Fig. III-2). Baltic Sea 1 and 10 m shared the same band pattern, which was clearly differentiated from the one observed at 20 m and sediment. Sediment produced the most complex pattern and none of the bands observed in this sample had the same positions as bands observed in the water column. In Lake Plußsee a similar situation was observed with the samples from 1 and 4 m sharing the same band patterns and with the differentiation of the samples at 7 m and sediment. In contrast, samples from 1, 11, 12 and 14 m in Lake Schöhsee

presented a very similar band pattern, and only the sediment sample was clearly different.
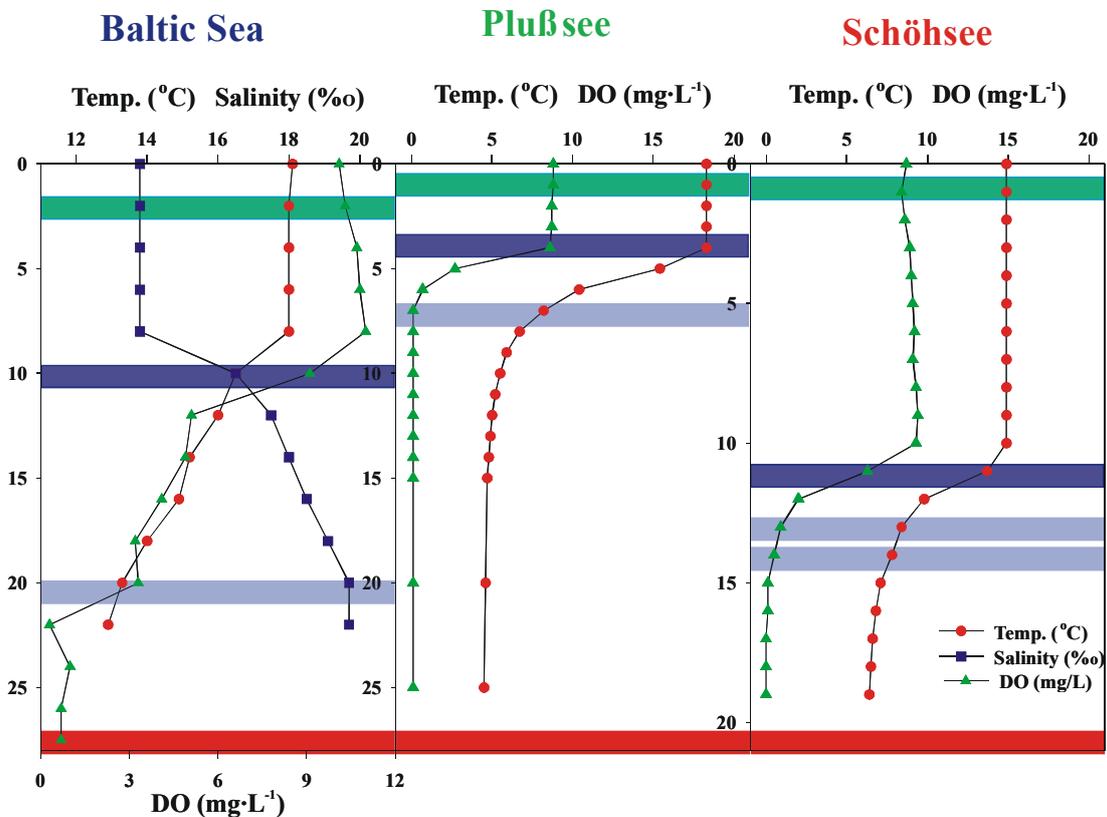
Figure III-1: Depth profiles taken at the Baltic Sea, Plußsee and Schöhsee in September 2004. Temperature (●), dissolved oxygen (▲), and salinity in the Baltic Sea (■). Samples for community analysis were taken from different depths: in the Baltic Sea from 2, 10 and 20 m; in Plußsee from 1, 4 and 7 m; in Schöhsee from 1, 11, 12, and 14 and. Samples from the sediment were taken from 27.5 (Baltic Sea), 28 (Plußsee) and 22 m (Schöhsee).

In order to compare different samples the bands in the DGGE gel were assigned to migration groups (see Fig. III-2A). Considering that DNA with different sequence can co-migrate to the same band position in the gel, these migration groups are not interpreted as a phylogenetic classification. For this comparison only very intense bands were taken into account, which produced 11 different migration groups. Some groups were commonly observed in samples from different locations. Migration group B was observed in all sediment samples, whereas group C was only detected in sediment samples from the lakes. Group H was detected with different intensities in the water column of both lakes and in sediment from Lake Plußsee.

A.

B.

Figure III-2: DGGE (A) and cluster (B) analysis of 16S rDNA AOB-specific PCR products from various depths of the Baltic Sea, Plusssee and Schöhsee. Cultures from AOB were used as standards. Arrows in A show the migration groups defined in the text.
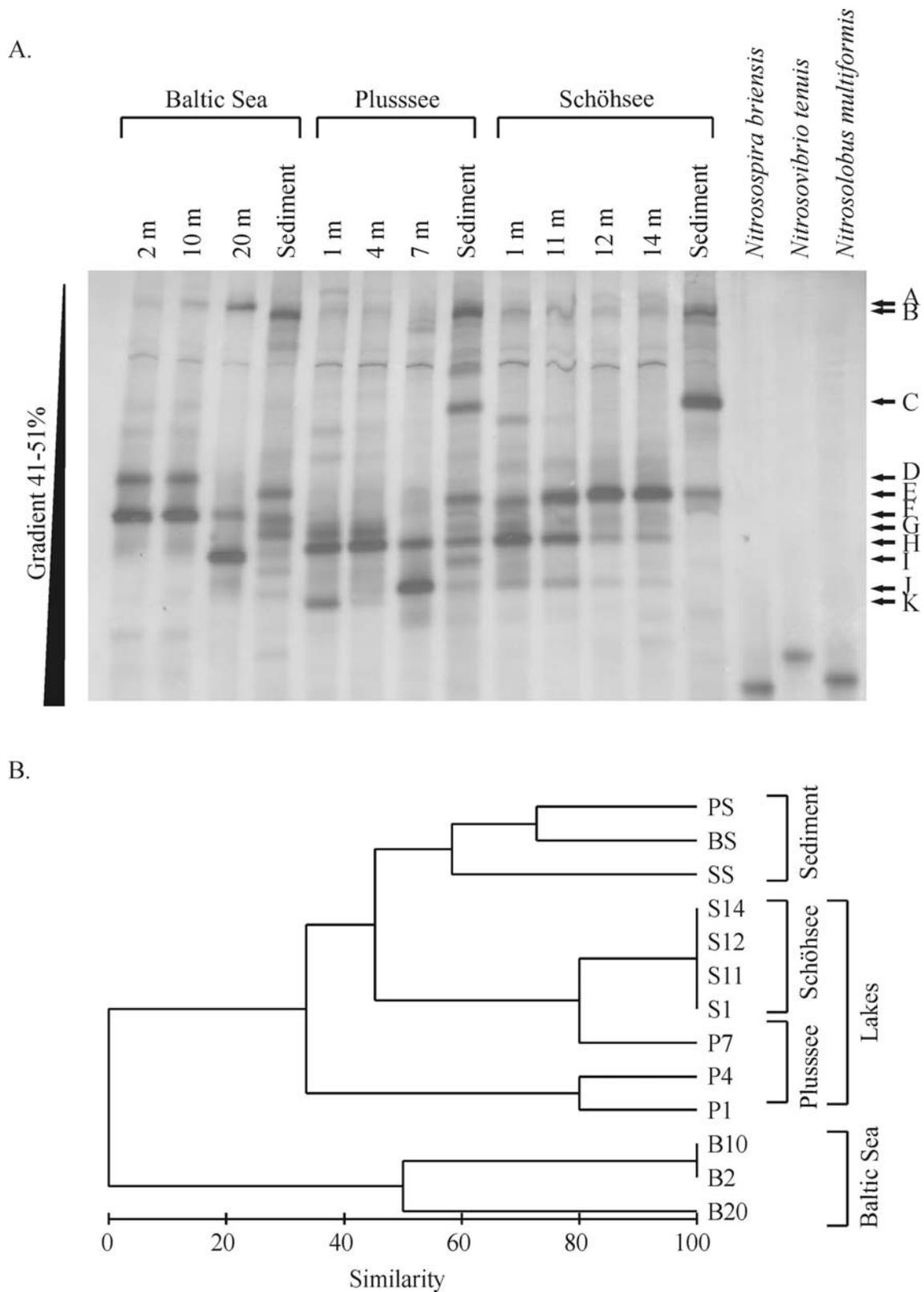
Cluster analysis, principal component analysis (PCA) and multidimensional scaling (MDS) were used to analyze the existence of common patterns between the samples. These analyses produced a similar ordination (data not shown) and therefore only data from the cluster analysis are shown. In a cluster analysis three clusters were observed: the first containing all sediment samples, the second the samples of water column from lakes and the last one with the samples from the Baltic Sea (Fig. III-2B). This analysis also showed that samples from the sediment of the three environments are more similar between them, than with samples from their respective water column. The formation of these clusters was confirmed independently by analysis of similarity (ANOSIM). This analysis showed that the formation of groups separating sediment samples, lake water column and the water column from the Baltic Sea was statistically significant.

The grouping of the samples according to the DGGE patterns was compared with environmental data in order to find which variable explained the separation of the samples best. We observed that oxygen and the combination of oxygen and temperature had the highest predictive capacity.

Recovery of AOB from clone libraries of 16S rDNA PCR products

Seven samples (Baltic Sea 2 and 20 m; Plußsee 1 and 7 m; Schöhsee 1 m; sediment from the Baltic Sea and Plusssee) were selected to construct clone libraries. These samples were selected because their DGGE band pattern contained all the bands observed in the different environments. From a total of 541 clones screened by one-shot sequencing, 538 clones (99.4%) were assigned to proteobacteria by comparison with the hierarchy browser of the ribosomal database project (RDP). The rest of the clones belonged to unclassified bacteria. Within the proteobacteria, 533 sequences were assigned to the beta subclass. Not all sequences from betaproteobacteria did belong to AOB. The percentage of AOB differed in each clone library (Table III-1). In the Baltic Sea the clone libraries from 20 m and sediment were dominated by AOB-like sequences, while at 2 m only three sequences were classified as such. Most of the sequences from 1 and 7 m of Plußsee were not related to AOB, which only dominated in the clone library from the sediment. All clones from Schöhsee 1 m belonged to betaproteobaceria, none was classified as AOB.

Table III-1: Summary of clone recovery in the different sampling sites including the number and percentage of clones assigned to ammonia-oxidizing bacteria (AOB).

Sample Site	Depth	Total No. of clones	Ammonia-oxidizing bacteria			
			Nitrosospira	*Nitrosomonas*	Total No. of AOB	%
	2 m	80	2	1	3	3.8
Baltic Sea	20 m	91	90	1	91	100.0
	Sediment	71	0	67	67	94.4
	1 m	72	1	15	16	22.2
Plußsee	7 m	78	0	1	1	1.3
	Sediment	65	10	31	41	63.1
Schöhsee	1 m	72	0	0	0	0.0

Identification of 16S rDNA sequences by BLAST

Sequences from the Baltic Sea were divided into eight groups according to the hits obtained in BLAST (Table III-2). The groups represented by the clones BS-54, BS-18, BS-71 and BS-91 were related to *Nitrosomonas* spp. Sequences represented by the clone BS-18 dominated the library from sediment. The clones BS-54 and BS-71 were only found in the sediment, representing a small proportion of the clones. The groups represented by the clones B20-68, BS-57 and B1-43 were related to *Nitrosospira* spp. The clone B20-68 (and 90 identical sequences) was only detected in the sample from 20 m. The clone BS-57 (and other four identical sequences) amplified from 20 m and sediment. Most of the sequences from 2 m (clone B1-83) did not belong to AOB and were related to sequences from *Hydrogenophaga*. In 2 m only two sequences represented by the clone B1-43 were related to *Nitrosospira* spp.

Sequences from Lake Plußsee were divided into 13 groups (Table III-3). Most sequences related to *Nitrosomonas*, represented by the clone PS-45, were observed in 1 m and the sediment. The other groups related to *Nitrosomonas* (P1-46, PS-35 and P1-56) included only few clones. Sequences related to *Nitrosospira* (PS-69 and P1-07) were also detected in low frequencies in sediment and 1 m (only one clone). The other clones were related to different groups of betaproteobacteria. Clones similar to *Polynucleobacter* (PS-74) dominated in the library from 7 m.

Table III-2. Identification and frequency of the 16S rDNA clonal sequences obtained in the Baltic Sea. The closest relative and the closest identified relative in BLAST are indicated.

Clone	Length bp	No. of identical clones			Closest relative	Accession	Identity %	Closest identified relative	Accession	Identity %
		1 m	20 m	Sed.						
BS-54	766	0	0	2	*Nitrosomonas* sp. BF16c57	AF386746	97	*Nitrosomonas* sp. BF16c57	AF386746	97
BS-18	737	0	0	58	Beta-proteobacterium clone EnvA2-13 from marine sediment beneath salmon fish cage close relationship to ammonia-oxidising bacteria	Z69097	99	*Nitrosomonas* sp. BF16c57	AF386746	96
BS-71	778	0	0	6	Uncultured ammonia-oxidizing bacterium clone TLBs234r from coastal marine sediment	AB239731	96	*Nitrosomonas* sp. Is79A3	AJ621026	96
BS-91	797	1	0	1	Uncultured ammonia-oxidizing bacterium clone B35s168r from eutrophic coastal marine sediment, Japan	AB212153	99	*Nitrosomonas* sp. NS20	AB212171	97
B20-68	677	0	90	0	Uncultured *Nitrosospira* sp. clone LD2-2 from coastal Scottish waters	AY461519	99	*Nitrosospira* sp. AHB1	X90820	95
B1-43	549	2	0	0	Uncultured *Nitrosospira* SM-42 associated to rhizoremediation based wastewater treatment	AJ275882	99	*Nitrosospira* sp. Nsp12	AY123801	99
BS-57	689	0	1	4	Uncultured *Nitrosospira* sp. clone 9NBGBact_38 basalt glass from 9N latitude East Pacific Rise	DQ070800	99	*Nitrosospira* sp. Nsp57	AY123791	97
B1-83	691	77	0	0	Uncultured Comamonadaceae bacterium clone DS152 from mangrove	DQ234234	98	*Hydrogenophaga* sp. R03-1	AY618576	95

Table III-3. Identification and frequency of the 16S rDNA clonal sequences obtained in Plußsee. The closest relative and the closest identified relative in BLAST are indicated.

| Clone | Length bp | No. of identical clones | | | Closest relative | Accession | Identity % | Closest identified relative | Accession | Identity % |
		1 m	7 m	Sed.						
P1-46	520	2	0	2	*Nitrosomonas* sp. R7c131	AF386752	98	*Nitrosomonas* sp. R7c131	AF386752	98
PS-45	767	10	0	24	Unidentified beta proteobacterium clone Rw9 recovered from freshwater environment; affinity with *Nitrosomonas* cluster 6	AJ003764	99	*Nitrosomonas* sp. Is79A3	AJ621026	99
PS-35	800	1	1	5	Uncultured bacterium clone RS 8-Bact29 melted red snow water; alpine lake Jöri XIII, Switzerland	AJ867669	94	*Nitrosomonas* sp. Nm86	AY123798	97
P1-56	630	2	0	0	Uncultured ammonia-oxidizing bacterium clone TLBs244r from coastal marine sediment	AB239735	98	*Nitrosomonas* sp. NS20	AB212171	96
PS-69	803	0	0	1	*Nitrosospira* sp. Nv6	AY123805	99	*Nitrosospira* sp. Nv6	AY123805	99
P1-07	600	1	0	9	Uncultured *Nitrosospira* sp. clone LD1-B10 anoxic marine sediment	AY114343	99	*Nitrosospira* sp. HB	X90820	96
PS-77	870	0	0	1	*Burkholderia cepacia* strain ATCC 25608	AY741339	92	*Burkholderia cepacia* strain ATCC 25608	AY741339	92
PS-74	740	7	72	1	*Polynucleobacter* sp. MWH-Mekk-C4	AM110093	99	*Polynucleobacter* sp. MWH-Mekk-C4	AM110093	99
P1-86	641	22	4	10	Uncultured bacterium clone Hot Creek 38 from arsenite-oxidizing biofilm	AY168745	97	*Leptothrix cholodnii*	X97070	97
P1-86	632	19	1	0	Uncultured bacterium clone J2_15 from a first order upland river, Germany	AJ508159	96	*Chitinimonas taiwanensis* strain cf	AY323827	95
P1-88	557	7	0	0	Uncultured beta proteobacterium NO27FW100501SAB29 from subsurface water, Kalahari Shield, South Africa	DQ230943	95	*Chromobacterium* sp. 71	AY117572	94
P1-38	603	1	0	4	Uncultured bacterium clone HTH4 16 from metal-rich particles in a freshwater reservoir	AF418964	99	Candidatus *Chlorothrix halophila*	AY395567	93
PS-32	681	0	0	8	Uncultured bacterium clone 1013-28-CG13 from an uranium-contaminated aquifer	AY532566	99	Uncultured beta proteobacterium clone BPM2_A03*)	AY689649	93

Table III-4. Identification and frequency of the 16S rDNA clonal sequences obtained in Lake Schöhsee. The closest relative and the closest identified relative in BLAST are indicated.

Clone	Length bp	No. of identical clones 1 m	Closest relative	Accession	Identity %	Closest identified relative	Accession	Identity %
S1-33	707	42	Uncultured bacterium clone 252ds10 from water 10 m downstream of manure	AY212701	99	*Polynucleobacter* sp. MWH-NZ7W17	AM110083	97
S1-75	683	14	Uncultured bacterium clone JFJ-ICE-Bact-06 from mountain snow Mönchsjoch, Switzerland	AJ867749	99	*Polaromonas ginsengisoli*	AB245355	98
S1-7	653	6	Uncultured bacterium clone SRRT18 from root-tip (0 to 40 mm) of Phragmites at Sosei River in Sapporo, Japan	AB240466	97	Ideonella sp. 0-0013	AB211233	97
S1-6	676	6	Uncultured bacterium clone J2_15 from a first order upland river, Germany	AJ508159	95	*Chitinimonas taiwanensis* strain cf	AY323827	94
S1-86	736	3	Uncultured betaproteobacterium clone A6 from alpine lake Jöri XIII, Switzerland	AJ867899	94	*Azospira* sp. R-25019	AM084030	91
S1-20	634	1	*Rubrivivax gelatinosus* strain OK3O3	AF487435	97	*Rubrivivax gelatinosus* strain OK3O3	AF487435	97

Sequences from Schöhsee 1 m were similar to six different groups of betaproteobacteria (Table III-4). Clones related to *Polynucleobacter*, represented by the clone S1-33, dominated in the library. The other clones were related to the genera *Polaromonas*, *Ideonella*, *Chitinimonas*, *Azospira* and *Rubrivivax*.

DISCUSSION

Community structure of AOB analyzed by DGGE

Amplification of 16S rDNA with specific primers and DGGE analysis is a good approach to characterize the community structure of AOB in different environments (Kowalchuk et al., 1997; Bano and Hollibaugh, 2000; Hollibaugh et al., 2002). Analysis of the DGGE patterns obtained from the samples of two lakes and the Baltic Sea showed that community composition of AOB in the water column is specific from each sampling site (Fig. III-2). This is not unexpected considering the variations in salinity and organic content between the lakes and the Baltic Sea (Ward et al., 1997).

Although community composition was different between the Baltic Sea and Plusssee, in both environments the stratification of the water column coincided with the separation of the communities from the surface and the anoxic deeper layers. In contrast, the existence of a more oxygenated deep layer in Schöhsee was reflected in the similarities of the AOB communities throughout the water column. These results were supported by the best correlation of the clustering analysis with oxygen content of the water as an environmental variable.

Differences between the DGGE band patterns derived from the water column and sediment, provided no evidence for links between benthic and pelagic AOB in any of the environments studied, a fact also reported before (Freitag and Prosser, 2004) for Loch Duich, thus showing the existence of specific pelagic and benthic ecotypes in AOB.

Composition of AOB communities analyzed by 16S rDNA clone libraries

Clone libraries from a subset of samples produced a different proportion of AOB-like sequences. The existence of a variable amount of non-AOB sequences in clone libraries prepared with specific 16S rDNA primers has been extensively documented in different studies (Speksnijder et al., 1998; Phillips et al., 1999; Bano and Hollibaugh, 2000; Hollibaugh et al., 2002). Several factors may influence the relative recovery of AOB sequences in the clone libraries, including the total counts of AOB and their abundance respect to the total bacterial population (Speksnijder et al., 1998). If the number of AOB-like clones is considered an indirect measure of the abundance of AOB in the samples, the comparison of the clone libraries from the lakes and the Baltic Sea suggested differential distribution of these microorganisms in different depths (Table III-1).

The frequency of AOB-like sequences in the surface (1 m in lake samples and 2 m in the Baltic Sea) was significantly lower than in the deeper layers and sediment, suggesting that this habitat is less suitable for AOB. In the Arctic, no amplification of AOB at the surface has been associated with photo-inhibition of the AOB cytochromes (Bano and Hollibaugh, 2000). It has been also suggested that nitrification generally is greatest immediately below the photic zone, where competition with phytoplankton for ammonia is reduced (Johnstone and Jones, 1988). Therefore, lower abundances of AOB in the superficial water might be a combined effect of lower NH_4^+ availability and light inhibition.

The recovery of AOB was lower in samples from the lakes compared to the Baltic Sea, with the only exception of Plußsee sediment. There are no previous studies comparing community composition of nitrifying bacteria in brackish and freshwater, however the results from this study suggested that during the stratification, brackish environments are a more suitable habitat for AOB.

Distribution of specific sequences of AOB in the samples

It has been observed that the distribution of distinct species of AOB depends on various environmental parameters (Koops and Pommerening-Röser, 2001).

Therefore, the detection of specific groups of AOB in a sample might be correlated with environmental variables.

Sequences from the Baltic Sea 20 m were related to clonal sequences from *Nitrosospira* isolated from other water samples (Phillips et al., 1999; Bano and Hollibaugh, 2000; Hollibaugh et al., 2002; Freitag and Prosser, 2004; O'Mullan and Ward, 2005). The detection of these sequences in several marine environments all over the world, has suggested a worldwide distribution throughout the ocean. The sequences found in the water column of the Baltic Sea support this cosmopolitan distribution.

Most of the sequences from the Baltic Sea sediment were related to sequences from *Nitrosomonas* that have been detected previously in sediments receiving high organic matter loads (McCaig et al., 1999), and in the Artic Ocean in samples from a region with higher productivity (Bano and Hollibaugh, 2000). In Plußsee sediment also a higher frequency of sequences related to *Nitrosomonas* was observed. However, these sequences were similar to *Nitrosomonas* species that have been recovered from other freshwater environments (Speksnijder et al., 1998).

3. IV Diversity of ammonia monooxygenase (*amoA*) genes in the water column and sediment-water interface of two lakes and the Baltic Sea
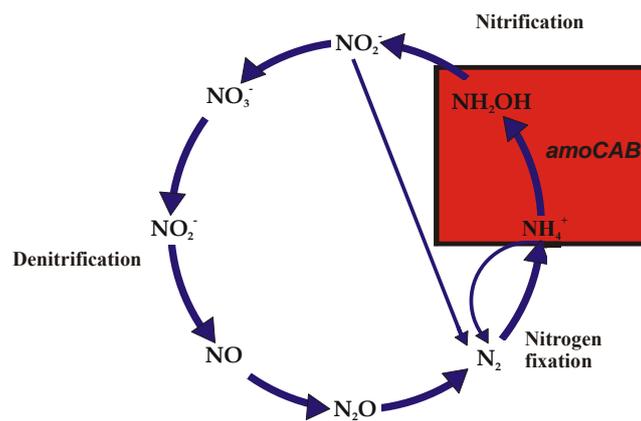
ABSTRACT

The functional gene *amoA* was used to investigate the diversity of ammonia-oxidizing bacteria (AOB) in the water column and sediment-water interface of Plußsee, Schöhsee and the Baltic Sea. This study represents the first extensive analysis of *amoA* diversity in freshwater lakes. Nested amplifications were used in order to increase the sensitivity of *amoA* detection, and to generate a 789 bp fragment from which clone libraries were prepared. In phylogenetic analyses, the larger part of sequences was not related to any of the cultured AOB. Almost all sequences from the water column of the Baltic Sea and 1 m of Schöhsee were related to different *Nitrosospira* clusters. Sequences from 7 m of Plußsee, 12 m of Schöhsee and the dominant ones from the lake sediment samples were clustered together with sequences from Chesapeake Bay, a previous study of Plußsee and rice root plants (*Nitrosospira*-like cluster A). The other sequences from Plußsee sediment fell into *Nitrosospira* cluster 0, while additional sequences of Schöhsee sediment clustered with *Nitrosomonas* cluster 6a (*Ns. oligotropha/Ns. ureae*). Two groups of sequences from Baltic Sea sediment were related to the purely environmental clusters from the brackish/marine habitats (*Nitrosomonas*-like cluster and the other with the *Nitrosospira*-like cluster B (Francis et al., 2003)). This confirms previous results from 16S rDNA libraries that indicated the existence of hitherto uncultivated AOB in lake and Baltic Sea samples, and show 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. It is a two-step process carried out by distinct functional groups of chemolithotrophic bacteria, the ammonia oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB). Until recently, only AOB were accepted to be responsible for the first, rate-limiting step of nitrification (Prosser, 1989; Head et al., 1993; Kowalchuk and Stephen, 2001). But meanwhile the widespread existence of anaerobic ammonia oxidation (anammox)

(Jetten et al., 1997; Strous et al., 1999) and the capability of some Crenarchaeota (AOA) to oxidize ammonia to nitrite (Könneke et al., 2005; Treusch et al., 2005) is well established.

AOB use the conversion of ammonia to nitrite as their sole energy source. The oxidation of ammonia to nitrite by AOB is also 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).

Based on 16S rDNA sequences, cultured AOB constitute two distinct monophyletic lineages within the Proteobacteria. Most strains belong to the beta-Proteobacteria, which include the genera *Nitrosomonas* and *Nitrosospira* (containing the former genera *Nitrosolobus* and *Nitrosovibrio*). But only two known species, *Nitrosococcus oceani* and *N. halophilus* belong to the gamma-Proteobacteria (Head et al., 1993; Purkhold et al., 2000). Cultured AOB and environmental sequences related to beta-Proteobacteria have been grouped into 8 clusters (*Nitrosospira* cluster 0 - 4 and *Nitrosomonas* cluster 5 - 8) based on 16S rDNA (Purkhold et al., 2000). *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 5 (Purkhold et al., 2000) contain environmental sequences, only. As they are defined by 16S rRNA, and no representative pure cultures of these two groups have been described, it is not clear yet if these bacteria are really able to oxidize ammonia.

Besides 16S rDNA, *amoA*, the gene encoding subunit A of ammonia monooxygenase, has been used as a molecular marker to study the diversity of the ammonia oxidizing bacteria in the environment (Rotthauwe et al., 1997). Using *amoA* has several advantages compared to 16S rDNA (Rotthauwe et al., 1997; Purkhold et al., 2000). First, this gene is related to the potential function of ammonia oxidation, thus giving relevant ecological information. Second, *amoA* sequences have a better phylogenetic resolution than 16S rDNA sequences.

Since the publication of the first *amoA* sequence from *Nitrosomonas europaea* (McTavish et al., 1993), more than 3,000 *amoA* sequences from bacteria, and a few from archaea, are available in GenBank, the majority from soil (40%), brackish and marine environments (37%) and wastewater treatment plants (21%). Only 2% of the

amoA sequences are from freshwater, illustrating that the investigation of *amoA* diversity in freshwater has been neglected so far.

In several aquatic environments, habitat specific clusters of *amoA* sequences have been reported. *Nitrosospira*-like and *Nitrosomonas*-like clusters distinctly related to known AOB have been observed in Washington continental margin (Pacific Ocean) and Puget Sound (Nold et al., 2000), Kysing Fjord (Nicolaisen and Ramsing, 2002), Chesapeake Bay (Francis et al., 2003), Monterey Bay (O'Mullan and Ward, 2005), Plum Island Sound (Bernhard et al., 2005), Bahío del Tóbari along Mexico coast (Beman and Francis, 2006) and OMZ off northern Chile (Molina et al., 2007). *amoA* sequences related to several *Nitrosomonas* clusters were dominant in the Lower Seine River and estuary (Cébron et al., 2003) and in groundwater (Ivanova et al., 2000). The information of the diversity and distribution of *amoA* in freshwater lake is almost absent, only few sequences are available in the database.

Distribution of ammonia oxidizing bacterial communities has been described previously (Kim et al., 2006) by 16S rDNA clone libraries from the same habitats as the present study. Baltic Sea 20 m was dominated by *Nitrosospira* cluster, Baltic Sea sediment by *Nitrosomonas* and Plußsee sediment by both of them. In the present study, community composition and distribution of AOB was analyzed using *amoA* gene. Nested amplification and new primer pairs were applied to increase the sensitivity and to analyze a larger fragment of 789 bp that covers the almost complete *amoA* gene.

RESULTS

Increase of sensitivity for amplifying *amoA*

In the past, nested amplification has been successfully applied in order to increase the sensitivity of specific 16S rDNA PCR detection in environments with low abundances of AOB (Ward et al., 1997; Kowalchuk et al., 1998; Phillips et al., 1999; Ward et al., 2000; Webster et al., 2002; O'Mullan and Ward, 2005; Webster et al., 2005; Kim et al., 2006). For *amoA* amplification, six combinations of primer pairs

were used for the initial amplification (Fig. IV-1 and Table IV-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 reactions resulted in *amoA* amplification, this strategy increased significantly the sensitivity of detection in most of the samples (Table IV-2). A band with the expected size was obtained with all initial templates from sediment samples of the three environments. With the exception of Baltic Sea 2 m, where most of the initial templates amplified in the nested PCR, the templates amoA34f/amoB1179r and amoA34f/amoA-2R produced more bands in the nested PCR with 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.
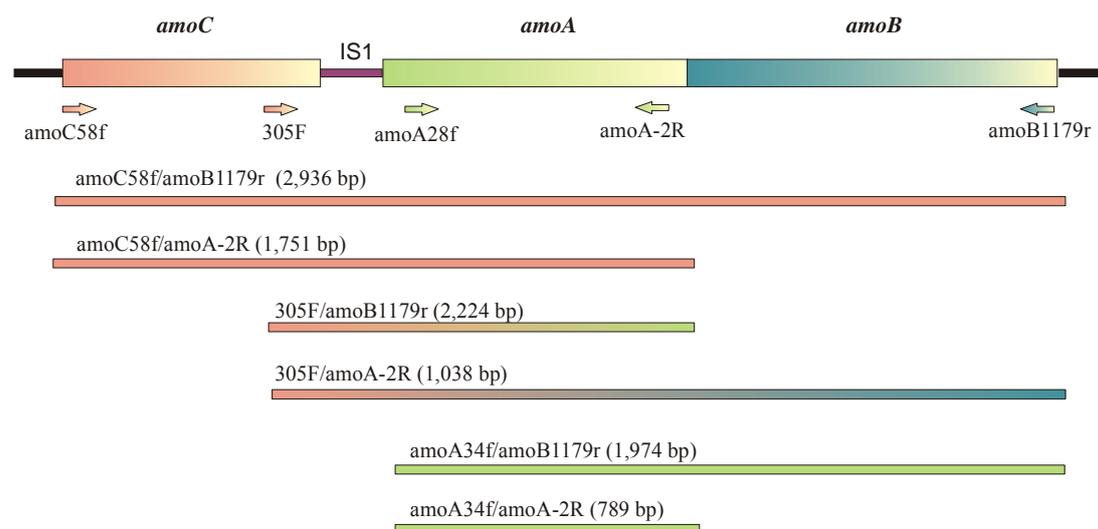
Figure IV-1: Structure of the *amoCAB* operon and position of the primers used in this study. Initial PCR amplification with 6 different primer pairs was used as templates for nested *amoA* amplification. Predicted size of the PCR-products in parenthesis.

Table IV-1: Sequences and positions of the primers used for amplification of *amoCAB*.

Gene	Primer	Primer sequence 5'-3'	Position[a]	Reference
amoC	amoC58f	CTA YGA CAT GTC RCT GTG G	58-76	In this study
	305F	GTG GTT TGG AAC RGI CAR AGC AAA	763-786	(Norton et al., 2002)
amoA	amoA34f	GCG GCR AAA ATG CCG CCG AAA GCG	34-57	(Molina et al., 2007)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	802-822	(Rotthauwe et al., 1997)
amoB	amoB1179r	CCA AAR CGR CTT TCC GG	1163-1179	In this study

[a] Positions in the amoCAB gene of *Nitrosomonas europaea* ATCC 19718 (Accession number, BX321859).

71

Table IV-2: Results of nested amplifications of *amoA* with the primers amoA34f/amoA-2R using 6 different initial amplicons as templates, coded by: +=visible product with predicted size; w=visible product with predicted size but weak band; -=no products.

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

Closest relatives of *amoA* clonal sequences identified by BLASTX

Clone libraries were made with pooled products of the nested amplification from all sediment samples, all 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) were obtained. No unspecific amplification was observed, all of these clones were assigned to ammonia monooxygenase by BLASTX. The identity of deduced amino acid sequences with the first hits of BLASTX varied from 89 to 100%. According to the percentage of identity, the sequences were classified into 4 groups (Table VI-3): *Nitrosospira* group (≥95% identity), *Nitrosospira*-like group (89 - 94 % identity) , *Nitrosomonas* group (≥95% identity), *Nitrosomonas*-like group (88 - 94% identity).

The majority of the clones were assigned to *Nitrosospira*-like (157 clones) or *Nitrosospira* group (119 clones), while only few clones were classified as *Nitrosomonas*-like group (39 clones) and *Nitrosomonas* (15 clones).

Clones belonging to either the *Nitrosospira* or *Nitrosospira*-like group, dominated in water column samples from the three environments. Only in Baltic Sea 10 m, clones belonging to all the groups were detected. Unlike the water column,

most of the clones from Baltic Sea sediment belonged to the *Nitrosomonas*-like group (38 clones out of 46) and few clones were affiliated to the *Nitrosospira*-like group. In the sediment samples from the two lakes, the majority of the clones belonged to *Nitrosospira*-like or *Nitrosospira* group (48 clones of Plußsee and 32 clones of Schöhsee). In Schöhsee sediment one quarter of the clones (11 out of 43) was affiliated to the *Nitrosomonas* group.

Table IV-3: Total number of clones carrying an insert with the primers amoA34f/amoA-2R obtained from the different samples. The frequency of clonal sequences checked by BLASTX and classified into 4 groups: *Nitrosospira* group ≥ 95% identity with *Nitrosospira* spp.; *Nitrosospira*-like group < 95% identity with *Nitrosospira* spp.; *Nitrosomonas* group ≥ 95% identity with *Nitrosomonas* spp.; *Nitrosomonas*-like group < 95% identity with *Nitrosomonas* spp.

Site	Depth (m)	No. of clones	No. of clones in the different groups after BLASTX search			
			Nitrosospira	*Nitrosospira* - like	*Nitrosomonas*	*Nitrosomonas* - like
Baltic Sea	2	41	40	0	1	0
	10	19	14	2	2	1
	20	33	33	0	0	0
	Sediment	46	0	8	0	38
Plußsee	7	45	3	42	0	0
	Sediment	48	10	38	0	0
Schöhsee	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

Diversity of *amoA* sequences

To compare the diversity of the *amoA* sequences in the different samples, rarefaction analysis (Fig. VI-2) was performed using ≤5% cutoff at DNA level to define an OTU (Francis et al., 2003). Rarefaction analysis showed that the highest *amoA* diversity was detected in Plußsee 7 m (6 OTUs), followed by Baltic Sea 10 m (5 OTUs). The lowest diversity was detected in Baltic Sea 20 m with only 1 OTU that corresponded to 16S rDNA cluster 0 (*Nitrosospira*) in the phylogenetic analysis (Fig. 3). Intermediate levels of diversity 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 low diversity.
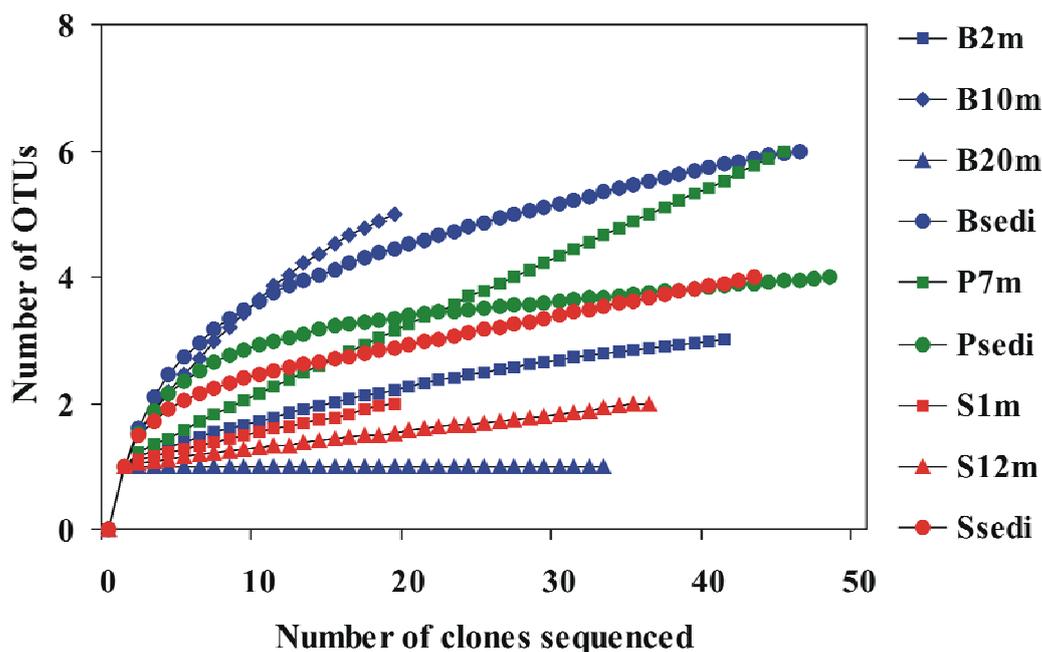
Figure IV-2: Rarefaction curves of *amoA*-based OTUs at 5% cutoff. Coding of the samples with three different colors: blue=Baltic Sea; green=Plußsee; red=Schöhsee.

Phylogenetic analysis of *amoA* sequences.

In the phylogenetic analysis, the sequences from this study fell into eight clusters. For five of these clusters (139/330 sequences) the 16S rDNA nomenclature based on (Purkhold et al., 2003) was followed, which contained the sequences closely related to cultured AOB. For the other three clusters (191/330 sequences) the nomenclature from (Francis et al., 2003) was used, because they included the pure environmental clusters.

Nitrosospira cluster 2 contained sequences only from Schöhsee 1 m, forming a separate cluster with cultured species. *Nitrosospira* cluster 0 included almost all sequences from Baltic Sea water column and some from Plußsee 7 m and sediment. These were closely related to the known AOB *Nitrosospira* sp. Nsp5 and Nsp12 isolated from a freshwater cave lake and soil, respectively (Koops and Harms, 1985) and *Nitrosospira* sp. 40KI isolated from soil (Jiang and Bakken, 1999). As well, some environmental sequences from a meadow soil in Giessen (Avrahami and Conrad, 2003) and from the Baltic Sea (Tuomainen et al., 2003) belong to this cluster. *Nitrosospira* cluster 3 had one sequence from Schöhsee sediment.
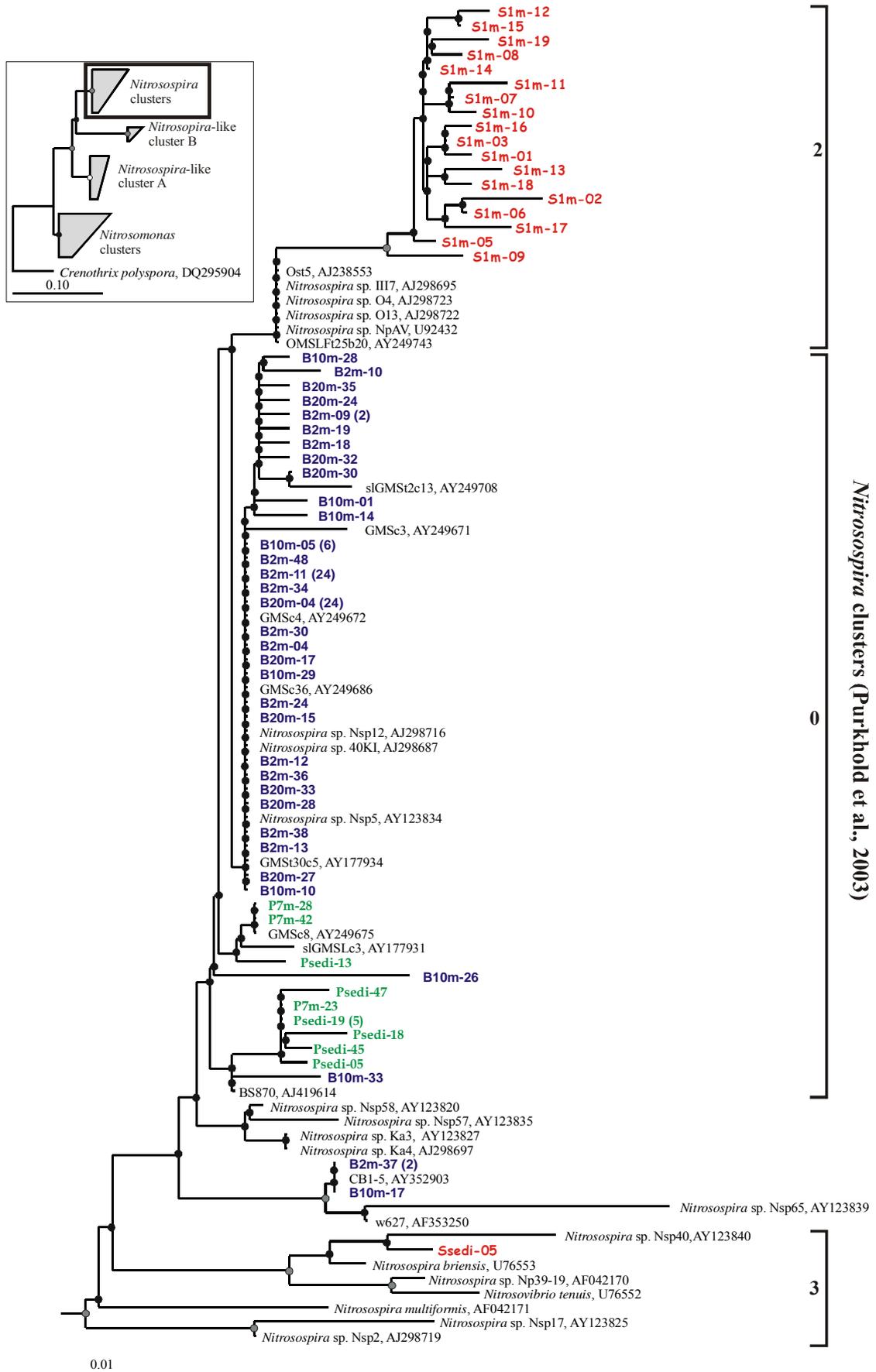
Figure IV-3
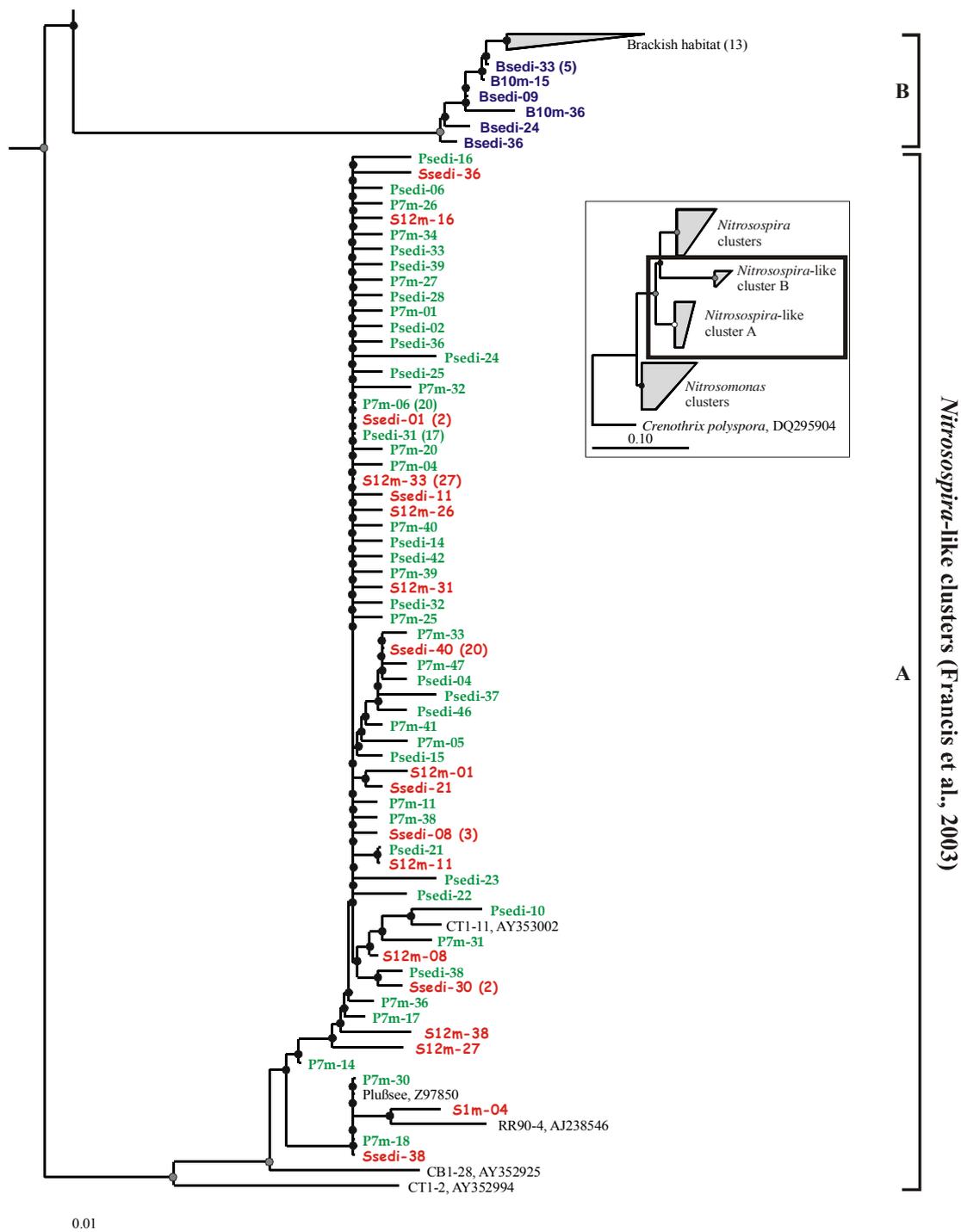
Figure IV-3: Fitch-Margoliash phylogenetic tree calculated in ARB with 263 amino acids of AmoA using PmoA from *Crenothrix polyspora* (Acc. No. DQ295904) as an outgroup. Nomenclature of clusters according to (Purkhold et al., 2003) and (Francis et al., 2003). Clones from this study are in bold, with the number of identical clones in brackets, and their origin coded with three different letter types: Arial for Baltic Sea with blue, Book Antiqua for Plußsee with green, and Comic Sans MS for Schöhsee with red. Bootstrap values for ≥90 replicate trees are indicated at the nodes with three different colors: black (100%), gray (95 - 99%) and white (90 - 94%).

76
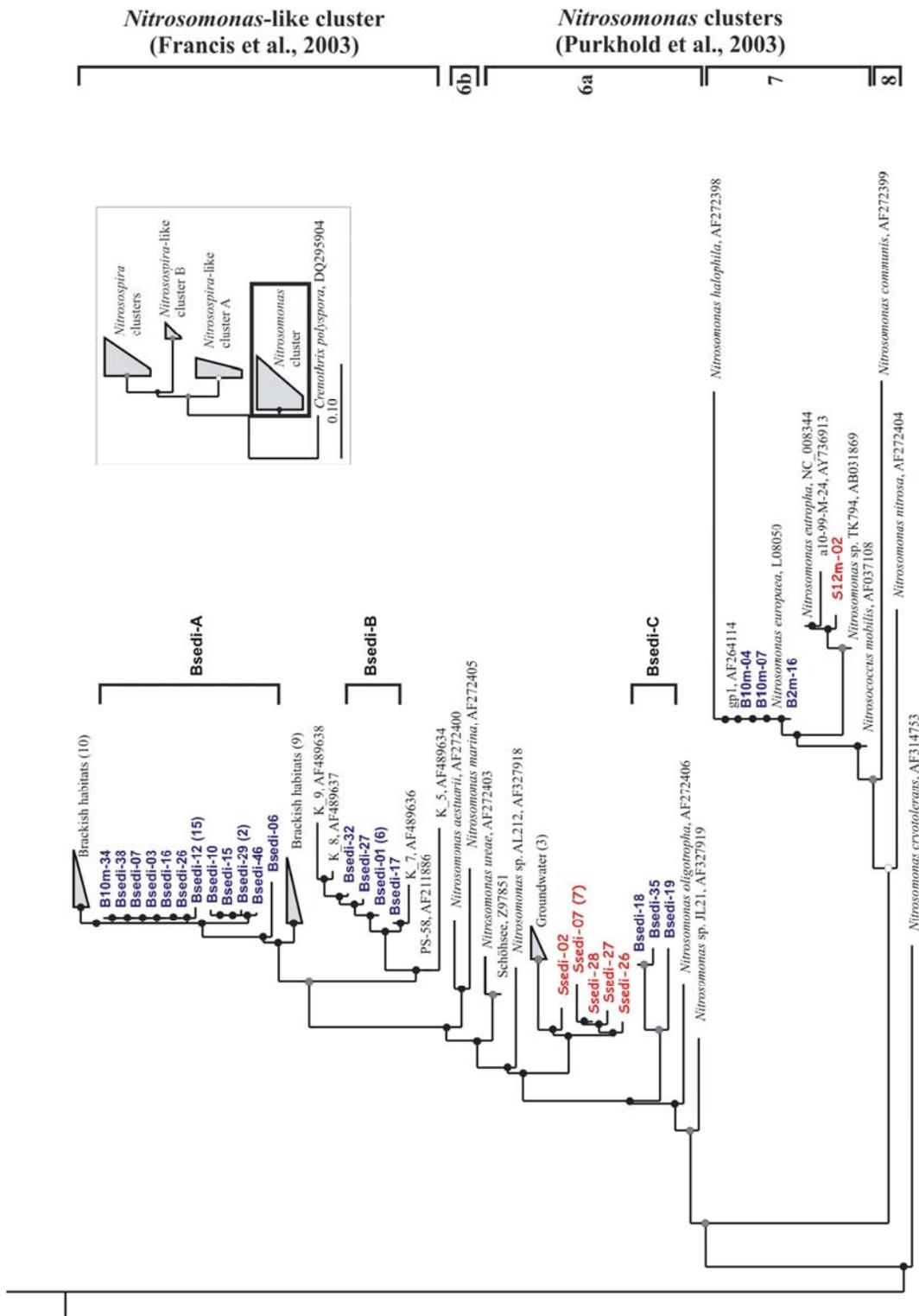
Figure IV-3: *continued.*

The *Nitrosospira*-like cluster contained almost half of the sequences from this study (155 out of 330), which so far includes environmental *amoA* sequences only, but none from any cultured AOB. In the characterization by BLASTX, all of these sequences showed 89% - 92% identity with AmoA from *Nitrosospira* sp. NpAV. 145 out of 330 sequences representing both lakes fell into the *Nitrosospira*-like cluster A. Up to now this cluster contained only few sequences retrieved from a few environments, like oligohaline habitats in Chesapeake Bay (Sites CT1 and CB1) (Francis et al., 2003), enrichment culture from Plußsee (Rotthauwe et al., 1997) and roots of rice plants (Horz et al., 2000). In contrast, the *Nitrosospira*-like cluster B contained sequences only from Baltic Sea 10 m and sediment, related to sequences from brackish habitats (Nicolaisen and Ramsing, 2002; Francis et al., 2003; Bernhard et al., 2005; O'Mullan and Ward, 2005).

The *Nitrosomonas* clusters comprised only 42 sequences from the Baltic Sea and 13 from Schöhsee that showed at least 91% identity with any of the *Nitrosomonas* spp. Sequences from Baltic Sea sediment (and one from 10 m) were grouped into the *Nitrosomonas*-like cluster that contained only environmental *amoA* sequences from brackish and marine environments (Nold et al., 2000; Nicolaisen and Ramsing, 2002; Francis et al., 2003; Bernhard et al., 2005; O'Mullan and Ward, 2005). These sequences were 91 - 93% identical with *Nitrosomonas* sp. AL212, the first hit in BLASTX,. Some sequences from sediment of Schöhsee and the Baltic Sea were included in the *Nitrosomonas* cluster 6b. Sequences from Schöhsee sediment were associated with those from groundwater (Ivanova et al., 2000) and showed 95 - 96% identity with *Nitrosomonas* sp. JL21, the first hit in BLASTX,. Sequences from the Baltic Sea sediment were 91 - 93% identical with *Nitrosomonas* sp. AL212, but they formed a cluster not related to any other environmental sequences nor cultured AOB. Only 4 sequences in this study fell into cluster 7 and were almost identical with *Nitrosomonas europaea* ($\geq$ 99% identity of deduced amino acid sequence).

DISSCUSSION

To our best 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 of different habitats (Francis et al., 2003; Bernhard et al., 2005; Beman and Francis, 2006) or in different depths of the water column in the same habitat (Nold et al., 2000; O'Mullan and Ward, 2005; Molina et al., 2007). In the present study, *amoA* genes were detected in several - though 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 over a wide environmental gradient.

Amplification efficiency

One of the major problems for the detection of AOB in many environments is that they represent only a small fraction of the total bacteria present. This has been overcome by the use of nested PCR with specific primer systems for 16S rDNA (Ward et al., 1997; Hastings et al., 1998; Ward et al., 2000; O'Mullan and Ward, 2005; Kim et al., 2006) and recently suggested as well for *amo* genes (Hastings et al., 1998)(Kim et al. submitted).

The low abundance of AOB in many natural environments poses a challenge when analyzing the diversity of *amoA* genes (Beman and Francis, 2006; Leininger et al., 2006). In the present study, the sensitivity of detection by PCR was increased considerably by using nested amplification. Though PCR products were detected in most of the samples analyzed here, with some samples from the water column of both lakes amplification products were not visible in agarose gels after ethidium bromide staining. This could be due to just the absence of significant numbers of AOB and the lack of sensitivity of detection. 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 niche in a countergradient of oxygen and ammonia which 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 and Jones, 1988). In the water column of the hypereutrophic lake Onondaga no apparent nitrification could be measured, but in

sediments the nitrification rates were quite high (Pauer and Auer, 2000). Likewise, numbers of AOB estimated by MPN, were small in the water column of the eutrophic Esthwaite Water (Hastings et al., 1998), but high in the sediment.

Community differences in the three environments

The samples were taken from three environments during thermal stratification in summer (Kim et al., 2006). In Plußsee, a eutrophic lake the pelagic zone was stratified into three distinct layers, the oxic epilimnion roughly corresponding to the photic zone, the metalimnion with steep gradients of oxygen, temperature and nutrients, and the anoxic hypolimnion with high concentrations of NH_3, H_2S and other reduced compounds. Schöhsee is a mesotrophic-stratified lake with oxic conditions in hypolimnion and an aerobic/anaerobic gradient at the sediment-water interface. Baltic Sea at Boknis Eck is characterized as mesohaline with salinities ranging from 13.8 to 19.7 PSU through the stratified water column.

AOB communities in these three environments differ significantly 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 share clones from different habitats, such as *Nitrosospira* cluster 0, *Nitrosospira*-like cluster and *Nitrosomonas* cluster 6a, the marine and freshwater clones are separated into habitat-specific subclusters. For some of these, e.g. subcluster A of the *Nitrosospira*-like clusters, habitat-specificity is quite well substantiated by the large number of sequences from both lakes. This group also includes an ammonia-oxidizing enrichment culture from Plußsee (Acc.No. Z97850) that has been described in an earlier study (Rotthauwe et al., 1997). Interestingly enough, close relatives of this culture have been detected now, about 10 years later, in the clone libraries from Plußsee and Schöhsee. In contrast, the well separated subcluster B contained some sequences from the Baltic Sea, closely associated with *amoA* from several brackish environments (Nicolaisen and Ramsing, 2002; Francis et al., 2003; Bernhard et al., 2005; O'Mullan and Ward, 2005) and may be regarded, on the basis of our present knowledge, as specific for mesohaline environments.

Members of the *Nitrosomonas* cluster 6a have been found in soil, freshwater, and freshwater sediment based on *amoA* (Ivanova et al., 2000) and 16S rDNA (Stephen et al., 1996; Speksnijder et al., 1998; Stephen et al., 1998; de Bie et al., 2001).This cluster also contains some sequences from this study, namely from Schöhsee sediment and, in a well separated subcluster, from Baltic Sea sediment. According to (de Bie et al., 2001) members of this specific *Nitrosomonas* lineage may be restricted to non-salty environments.

Community differences within each environment.

In the two lakes studied, upon overturn in autumn, ammonia and oxygen are mixed through the whole water column. During winter, at low temperatures, but high oxygen concentrations, the whole ammonia content of the lake is oxidized to nitrate. With the beginning of stratification in spring, different compartments are separated in depth providing niches for the development of specially adapted bacterial communities. The most important factors for the development of AOB communities are: light penetration, concentration of ammonia (or urea) and oxygen, and to some extent temperature.

In case of Plußsee 7 m and sediment which were under anoxic conditions, all clonal *amoA* sequences were similar to each other, and distributed into the two clusters *Nitrosospira* cluster 0 and *Nitrosospira*-like cluster A. It may be hypothesized that at least the members of the latter cluster A could be microaerophilic or tolerant to anoxic conditions. Whether the clear separation of the clones from Plußsee 7m and sediment within the *Nitrosospira* cluster 0 indicates that also these are somehow adapted to low oxygen concentrations, remains to be studied. This concept is also supported by the distribution of clones from different depths of Schöhsee: All clones from the oxygen-rich 1 m water depth formed a separate group within *Nitrosospira* cluster 2. The clones from sediment and the deeper water layers were grouped into *Nitrosospira*-like cluster A and *Nitrosomonas* cluster 6a. Members of the latter cluster 6a have been detected by 16S rDNA also in the suboxic, ammonia-rich sediments of the Schelde estuary (de Bie et al., 2001) indicating some degree of tolerance to low oxygen conditions (Speksnijder et al., 1998; de Bie et al., 2001). They are not included in the *amoA* based phylogenetic tree of Fig. 3.

Also in the Baltic Sea AOB communities were significantly different in the water column and sediment. It is well known from many studies that the yet uncultured *Nitrosospira*-like and *Nitrosomonas*-like *amoA* sequences were dominant in marine habitats of different salinities and wide geographic distribution (Nold et al., 2000; Nicolaisen and Ramsing, 2002; Francis et al., 2003; Bernhard et al., 2005; O'Mullan and Ward, 2005; Beman and Francis, 2006; Molina et al., 2007). While AOB communities in Baltic Sea sediment followed this pattern, all of the clones from Baltic Sea water column were closely related to *Nitrosospira* cluster 0, where they separated from the clones retrieved from the anoxic samples from Plußsee 7m and sediment. In the oxygen minimum zone off the Chilean Pacific coast sequences similar to *Nitrosospira* spp. were found while members of the *Nitrosospira*-like cluster dominated in the surface water and upper oxycline (Molina et al., 2007).

Definition of new species based on *amoA* sequence information.

Nitrosospira-like cluster B and *Nitrosomonas*-like cluster have been described as a novel group for marine habitats (Francis et al., 2003) whereas *Nitrosospira*-like cluster A contains many clones from both lakes, and clearly distinct from all other known *amoA* sequences.

Identity of 80% DNA or 85% protein has been suggested (Purkhold et al., 2000) as threshold value for defining new AOB species on the basis of partial *amoA* sequences of about 490 bp from the more variable 3' region (positions 332 to 822 of *amoA* from *Nitrosomonas europaea*). In the present study, we used the almost complete *amoA* (positions 34 to 822), including the more conserved 5' part. Consequently, sequence identity between *amoA* from *Nitrosomonas cryotolerans* (Acc. No. AF314753) and *Nitrosospira multiformis* (Acc. No. CP000103) was 88/95% (DNA/protein) for the almost complete *amoA* gene targeted in this study, and 84/93% (DNA/protein) for the shorter region from 332 to 822 used in the above mentioned study (Purkhold et al., 2000). Therefore, for whole *amoA* gene sequences we suggest a somewhat higher threshold of ≤88% for DNA and ≤95% for protein to define novel groups. Based on these thresholds *Nitrosospira*-like cluster A and B, and

the subgroups of *Nitrosomonas* cluster 6a and *Nitrosomonas*-like cluster are potentially novel and habitat-specific groups of AOB.

Comparison of the clone libraries diversity

In order to describe the fine-scale variability of clonal *amoA* sequences, usually operational taxonomic units (OTUs) are defined (Hughes et al., 2001) at different cutoff values. For 16S rDNA, cutoff values from 1 to 3% are most frequently used. However, for functional genes, like *amoA*, cutoffs that are applicable for defining OTUs are still up for discussion (Ward, 2002). Based on rarefaction analysis at 5% cutoff, the clonal sequences from Plußsee 7 m were most diverse, although in the phylogenetic analysis they were grouped into only two different clusters, *Nitrosospira* cluster 0 and *Nitrosospira*-like cluster A. In addition, the number of OTUs in these clusters changed at different cutoffs ranging from 2 to 5%, suggesting great micro-heterogeneity of the sequences in this clone library. In contrast, the number of OTUs in the clone libraries from Baltic Sea water column and Schöhsee 1 m remained constant at cutoffs from 1% to 5%, indicating almost no variability at the DNA level.

The number of OTUs detected in this study were lower than in comparative studies (Francis et al., 2003; Beman and Francis, 2006), which may at least in part be due to the inclusion of the more conserved part of the *amoA* gene. In Chesapeake Bay, four OTUs were detected at two mesohaline stations (CB2 and CT2) and 11 at an oligohaline station (CB1). In Bahía del Tóbari, northwest coast of mainland Mexico, the clone library in January and October produced 6 and 7 OTUs, respectively.

Comparison of AOB communities characterized by *amoA* and 16S rDNA

amoA gene is a better molecular marker to study AOB than 16S rDNA (Rotthauwe et al., 1997; Purkhold et al., 2000). This is confirmed in the present study, when comparing with previous results based on clone libraries of specific 16S rDNA PCR products of the same samples (Kim et al., 2006). PCR with primers for *amoA* are more specific than with those for 16S rDNA of β-AOB. The latter may contain up to

100% 16S rDNA from non-AOB (McCaig et al., 1994; Kim et al., 2006; Mahmood et al., 2006), while clone libraries with *amoA* usually are 100% specific (O'Mullan and Ward, 2005; Molina et al., 2007), as was the case in this study, where all clones with an insert of the expected size (789 bp) contained *amoA*, as confirmed by BLASTX. In former studies, the low sensitivity of PCR of *amoA* was disadvantageous for studies in environments with low abundance of AOB. However, this can be overcome with the nested approach described here.

Phylogeny based on 16S rDNA and *amoA* sequences from cultured AOB is highly congruent (Purkhold et al., 2000; Aakra et al., 2001a). This has been found as well in the studies of AOB community comparison between 16S rDNA and *amoA* with same samples from aquatic environments, such as Shiprock groundwaters (Ivanova et al., 2000), Monterey Bay (O'Mullan and Ward, 2005) and oxygen minimun zone (OMZ) off northern Chile (Molina et al., 2007). The clone libraries from the Baltic Sea showed a good agreement between 16S rDNA and *amoA* as well. Only in Plußsee clone libraries of 16S rDNA and *amoA* do not coincide at all. *Nitrosomonas* sequences were dominant in 16S rDNA, and only few *Nitrosospira* sequences were detected (Kim et al., 2006). In the present study, there was no single *amoA* sequence related to *Nitrosomonas* spp.

This discrepancy could be due to several reasons: (i) different specificities of the PCR for 16SrDNA and *amoA*; (ii) *amoA* PCR is more sensitive for *Nitrosospira* because of different numbers of gene copies in *Nitrosospira* (3) and *Nitrosomonas* (2) (Norton et al., 2002); (iii) horizontal gene transfer (Juretschko et al., 1998; Aakra et al., 2001a). Despite high consistency between the two phylogeny approaches, a few discrepancies have been also presented (Juretschko et al., 1998; Aakra et al., 2001a). Different clustering of some strains has been observed for 16S rDNA and *amoA* such as between *Nc. mobilis* and *Ns. europaea* (Juretschko et al., 1998) and between *Ns. cryotolerans* and *Ns. aestuarii* (Aakra et al., 2001a), and the possibility of horizontal gene transfer of *amoA* has been suggested as an explanation.

3. V Genetic diversity

of the evolutionarily related enzymes

ammonia monooxygenase (AMO)

and particulate methane monooxygenase (pMMO)

in two lakes and the Baltic Sea
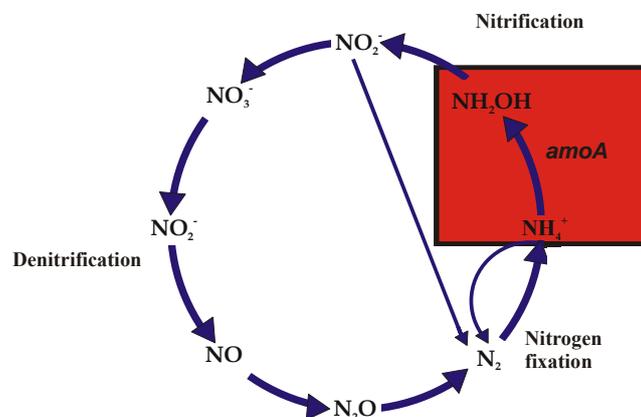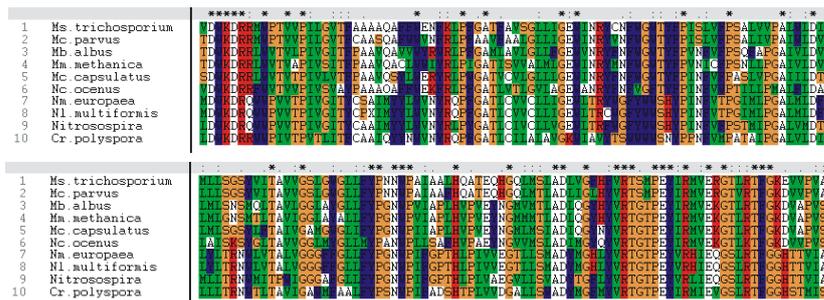
ABSTRACT

Evolutionary relationships between ammonia monooxygenase (AMO) and particulate methane monooxygenase (pMMO) lead us to analyze the diversity of their genes *amoA* and *pmoA* based on cloning, sequencing and T-RFLP in different habitats: Plußsee (eutrophic lake) and Schöhsee (mesotrophic lake) as closed environments and the Baltic Sea (brackish inland sea) as an open environment. A higher frequency of *pmoA* sequences, mainly belonging to methane oxidizing bacteria of the gamma subgroup of proteobacteria (γ-MOB), was detected. Dominant *amoA* sequences were related to ammonia oxidizing bacteria of the beta subgroup of proteobacteria (β-AOB), no sequences related to *amoA* of the γ-AOB were detected. The deduced amino acid sequences of some clones from lake sediments were distantly related to PmoA from *Crenothrix polyspora*, a filamentous methane oxidizer with an unusual methane monooxygenase. In the Baltic Sea all sequences from the water column were related to *amoA*, while almost all from sediment were related to *pmoA*. In Plußsee all sequences belonged to several *pmoA* clusters. In the water column of lake Schöhsee, *amoA* and *pmoA* sequences were detected simultaneously, whereas sequences related to *C. polyspora* dominated in sediment. Principal component analysis (PCA) of the T-RFLP data showed the separation of the communities from each environment. However, samples from the Baltic Sea grouped more closely together than those from the two lakes.

INTRODUCTION

Oxidation of ammonia to nitrite and methane to methanol are important processes in the biogeochemical cycles of nitrogen and carbon in terrestrial and aquatic ecosystems. Ammonia-oxidizing bacteria (AOB) and methane oxidizing bacteria (MOB) can grow utilizing ammonia or methane as the sole source of energy (Hanson and Hanson, 1996).

Oxidation of ammonia to nitrite, is catalyzed by two different enzymes, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Hooper et al., 1997). *amoA*, the gene encoding the catalytic subunit A of AMO has been used as a molecular marker to investigate the distribution and diversity of AOB in various environments (Avrahami and Conrad, 2003; Francis et al., 2003; Molina et al., 2007) and a relatively low sequence similarity with amino acid between AOB and AOA were described (38 - 51%) (Könneke et al., 2005).

Based on 16S rDNA sequences, AOB have been classified into two different subclasses of the Proteobacteria. The genera *Nitrosomonas* and *Nitrosospira* belong to the β-subclass of Proteobacteria (β-AOB) and the genus *Nitrosococcus* to the γ-subclass of Proteobacteria (γ-AOB) (Head et al., 1993). Recently, ammonia-oxidizing archaea (AOA) were discovered in a soil metagenomic library (Treusch et al., 2005), and a ammonia-oxidizing mesophilic crenarchaeote, *Nitrosopumilus maritimus*, has been isolated from a marine aquarium (Könneke et al., 2005).

Two distinct types of methane monooxygenases (MMO) catalyze the oxidation of methane to methanol in MOB: the membrane-bound particulate methane monooxygenase (pMMO) and the cytoplasmic soluble methane monooxygenase (sMMO) (Murrell et al., 2000). In spite of their similar function, these two enzymes do not share any similarity of genes nor structure.

Based on their phylogenetic relationship, MOB are affiliated to the α- and γ-subclasses of Proteobacteria (α-MOB type II and γ-MOB type I) (Hanson and Hanson, 1996; Murrell et al., 2000). γ-MOB type I include the genera *Methylococcus*, *Methylomicrobium*, *Methylobacter*, and *Methylomonas*, whereas α-MOB type II include the genera *Methylocystis* and *Methylosinus*.

It has been demonstrated that the genes encoding AMO in AOB and those encoding pMMO in MOB share high sequence similarity and gene structure (Holmes et al., 1995; Semrau et al., 1995). Furthermore, DNA and amino acid sequences of *amo* and *pmo* of the γ-subclass of the Proteobacteria are more similar with each other than with *amo* from β-AOB. It has been reported that *amo* from β- and γ-AOB and

pmo from γ-MOB might have separated into three lineages early in evolution (Alzerreca et al., 1999). It has also been proved that AMO and pMMO can both catalyze ammonia and methane oxidation, although displaying higher affinity for their respective substrate (Hanson and Hanson, 1996).

The purpose of this study was to compare the distribution and diversity of these two enzymes within each habitat and between habitats in diverse aquatic ecosystems, the eutrophic lake Plußsee and the mesotrophic lake Schöhsee as closed habitats and the Baltic Sea as open habitat.

RESULTS

1. Analysis of *amoA* and *pmoA* clonal sequences

1.1 Identification by BLASTX.

A total of 286 clones with an insert of 531 bp were obtained. Several clones carrying an unspecific product were also obtained. These "unspecific" amplifications were more common in water column samples except for 14 m of Schöhsee. More clones were collected from Schöhsee than from Baltic Sea and Plußsee (Table V-1), and from Baltic Sea and Plußsee more from sediment than from water column. No clones carrying *amoA* or *pmoA* were obtained from Baltic Sea 20 m and from Plußsee 1 and 4 m.

The clones were classified into 4 categories according to the first identified bacterium found by BLASTX search. Clones having 93 - 98% similarity with deduced amino acid from cultured β-AOB were assigned to β-AOB group (87 clones). Clones with an identity of 84 - 98% with PmoA from cultured γ-MOB were affiliated to *pmoA* γ-MOB type I (161 clones). One single *pmoA* clone was assigned to α-MOB type-II (98% identity with PmoA from *Methylocystis*). A group of 37 clones from both lakes showed 65 - 67% identity with the unusual PmoA from *Crenothrix polyspora*. These clones which showed higher similarity to AmoA (64%) than to

PmoA (51%) in the database.were assigned to the *Crenothrix* group, which has been described to be a methane oxidizer (Stoecker et al., 2006).

Table V-1: Number of clones carrying an insert of 531 bp belonging to the different taxonomic groups identified by BLASTX.

Site	Depth (m)	No. of clones	BLASTX results			
			β-AOB	*Crenothrix*	γ-MOB Type-I	α-MOB Type-II
Baltic Sea	2	16	16	0	0	0
	10	5	5	0	0	0
	Sediment	44	3	0	41	0
Plußsee	7	3	0	0	3	0
	Sediment	39	0	1	37	1
Schöhsee	1	34	18	0	16	0
	11	5	2	0	3	0
	12	9	3	0	6	0
	14	70	22	1	47	0
	Sediment	61	18	35	8	0
All sites		286	87	37	161	1

In Baltic Sea clones from the water column belonged only to the β-AOB group, while almost all clones from sediment corresponded to the γ-MOB type I group. Clones from Plußsee were affiliated to *pmoA* groups only, including dominant clones in the γ-MOB type I group, one clone in the *Crenothrix* group and a single clone in the α-MOB type II group. In Schöhsee, clones belonging to β-AOB, *Crenothrix* and γ-MOB type I group were detected. Here, the proportion of β-AOB and γ-MOB type I decreased in sediment, which was the only habitat that harbored a significant number of clones from the *Crenothrix* group.

1.2 Diversity estimation by rarefaction analysis

To estimate the diversity of *amoA* and *pmoA* sequences in the clone libraries, rarefaction analysis was performed using 5% cutoff to define OTUs (Fig. V-1). Rarefaction analysis showed that the diversity of sequences from lakes was higher than from the Baltic Sea. The sequences from Schöhsee 14 m were the most diverse with 14 OTUs detected without saturation of the rarefaction curve. Although from sediment of Plußsee fewer sequences were obtained than from 14 m of Schöhsee, the diversity was higher in Plußsee sediment (12 OTUs detected). While in the

phylogenetical analysis, the sequences from these samples belonged to several clusters, the lowest diversity was observed in 2 and 10 m of Baltic Sea with only 1 OTU that corresponded to *Nitrosospira* cluster in the phylogenetic tree. Sediment from the Baltic Sea and Schöhsee and the water column in all three environments (except for 14 m of Schöhsee) showed intermediate levels of diversity. Relatively higher diversity was observed in sediments (4 OTUs in Baltic Sea and 8 OTUs in Schöhsee), while lower diversity was observed in the water column (2 OTUs detected and 2 clusters found in the phylogenetic tree).
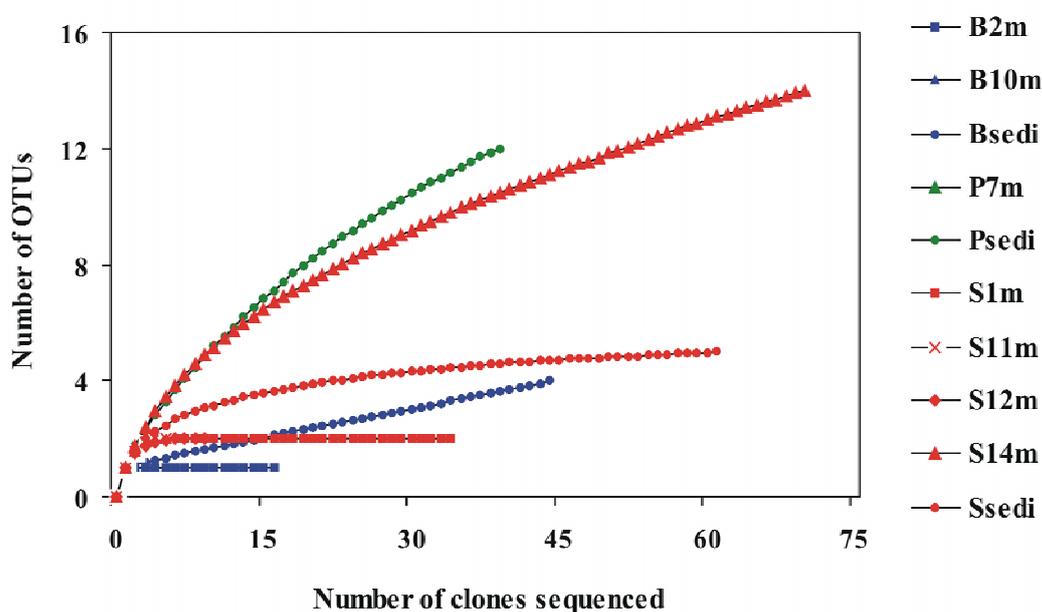
Figure V-1: Rarefaction curves showing the diversity of OTUs from 9 samples using 5% cutoff. Samples from the Baltic Sea are coded with blue from Plußsee with green and from Schöhsee with red.

1.3 Phylogenetic analysis

The sequences obtained in this study were compared to sequences from GenBank (Fig. V-2). The nomenclature of the clusters in the phylogenetic tree for *amoA* is used according to (Francis et al., 2003), and for *pmoA* according to (Pester et al., 2004) and (Bussmann et al., 2006). An additional cluster including the sequences related to *Crenothrix* group was also identified. The sequences from this study fell

Table V-2: Number of clones from each clone library in the different clusters of the phylogenetic tree (Fig. 3) and closest known bacteria in GenBank identified by BLASTX. Id=identity (%).

Cluster	Representative sequences	No. of clones from										Closest known bacteria		
		Baltic Sea			Plußsee		Schöhsee					Organism	Id	Accession
		2 m	10 m	Sediment	7 m	Sediment	1 m	11 m	12 m	14 m	Sediment			
Nitrosospira	S1m-11	16	5	1	0	0	18	2	0	12	0	*Nitrosospira* sp. NpAV	98	U92432
Nitrosospira-like	S14m-44	0	0	1	0	0	0	0	3	10	0	*Nitrosospira* sp. NpAV	93	U92432
Nitrosomonas	Ssedi-45	0	0	1	0	0	0	0	0	0	18	*Nitrosomonas* sp. JL21	96	AF327919
Crenothrix-like	Ssedi-10	0	0	0	0	1	0	0	0	1	35	*Crenothrix polyspora*	67	DQ295904
B11	S14m-08	0	0	0	0	0	0	0	0	7	0	*Methylococcaceae bacterium*	92	DQ119047
B8	Ssedi-08	0	0	0	0	1	0	0	0	0	3	*Methylobacter tundripaludum*	93	AJ414658
B1-a	Psedi-03	0	0	0	0	7	0	0	0	4	0	*Methylobacter tundripaludum*	96	AJ414658
B1-b	Psedi-11	0	0	0	0	8	0	0	0	0	0	*Methylobacter sp.* BB5.1	97	AF016982
B12	S14m-17	0	0	0	2	0	16	3	6	32	0	*Methylomicrobium pelagicum*	89	U31652
B9	P7m-03	0	0	0	1	0	0	0	0	0	0	*Methylomonas* sp. LW15	97	AF150795
B13	Bsedi-27	0	0	41	0	0	0	0	0	0	0	*Methylobacter sp.* BB5.1	89	AF016982
B3	Psedi-02	0	0	0	0	19	0	0	0	3	5	*Methylococcus capsulatus* str. Bath	90	NC_002977
B6	Psedi-35	0	0	0	0	2	0	0	0	1	0	*Methylococcus capsulatus* str. Bath	84	NC_002977
B7	Psedi-17	0	0	0	0	1	0	0	0	0	0	*Methylocystis* sp. SC2	98	AJ584611
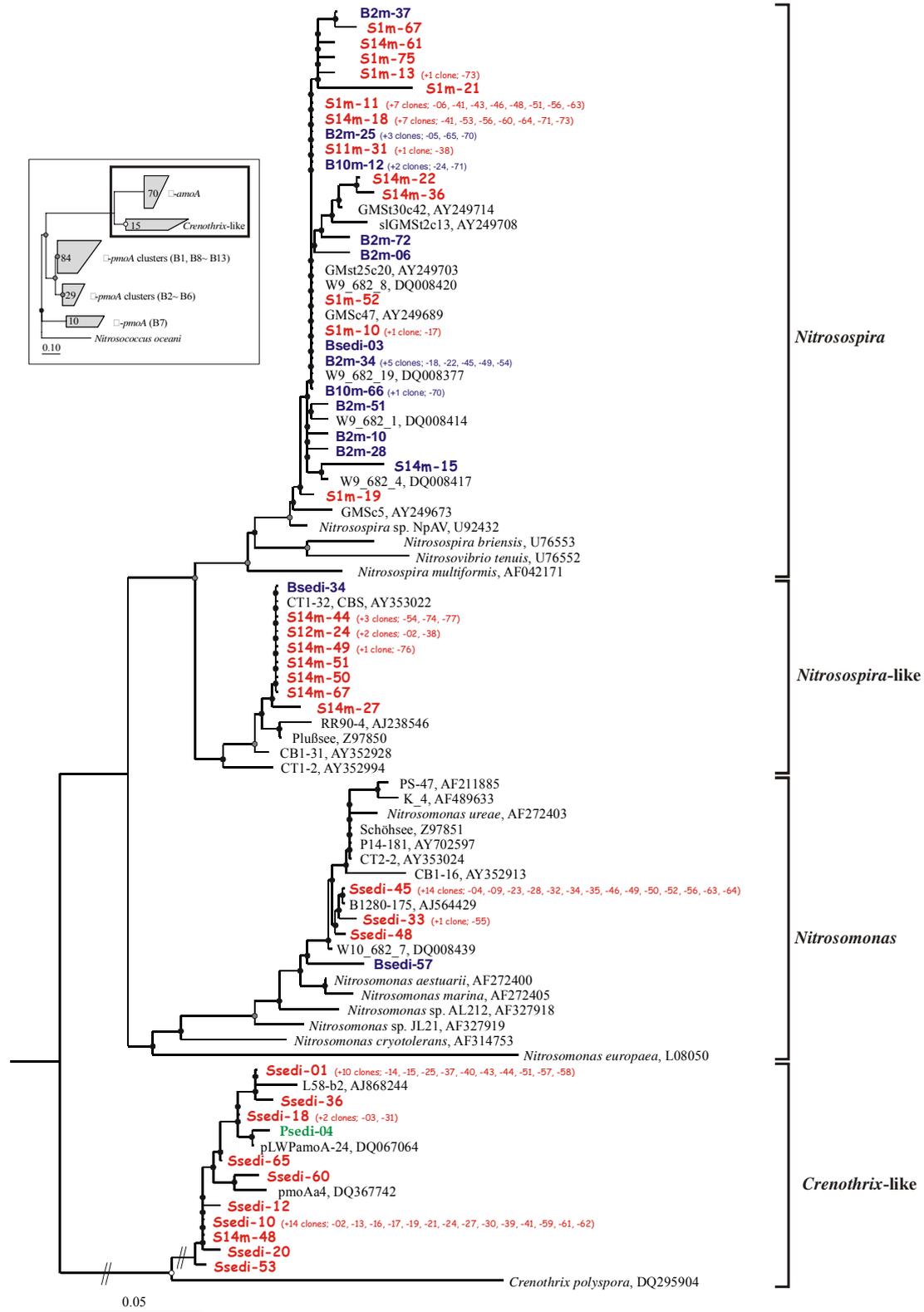
Figure V-2: Phylogenetic tree calculated with 177 amino acids of AmoA and PmoA using Fitch Margoliash method in ARB. Nomenclature of clusters follows (Francis et al., 2003) for *amoA* and (Pester et al., 2004) and (Bussmann et al., 2006) for *pmoA*. Clones from this study are in bold and typed with three different fonts: Arial for Baltic Sea with blue, Book Antiqua for Plußsee with green and Comic Sans MS for Schöhsee with red. Bootstrap values ≥ 90 replicate trees are indicated at the branch with three different colors: black (100%), gray (95 - 99%) and white (90 - 94%).

into 13 clusters: 3 *amoA* clusters, 1 *Crenothrix*-like cluster and 9 *pmoA* clusters. The number of sequences in each of these clusters is presented in Table V-2.

Two clusters of *amoA* were more than 96% similar to cultured AOB (*Nitrosospira* and *Nitrosomonas* clusters). The *Nitrosospira* cluster included sequences from the water column of Baltic Sea and Schöhsee that were closely related to those from Snake River (Erwin et al., 2005) and soil (Avrahami and Conrad, 2003). The *Nitrosomonas* cluster contained sequences from sediment of Schöhsee and Baltic Sea, only, associated with sequences from several aquatic environments (Nold et al., 2000; Nicolaisen and Ramsing, 2002; Francis et al., 2003; Lipponen et al., 2004; Erwin et al., 2005). The *Nitrosospira*-like cluster included sequences from Schöhsee 12 and 14 m and one from Baltic Sea sediment, associated with an enrichment culture from Plußsee and some environmental clones from soil and estuarine sediment (Rotthauwe et al., 1997; Horz et al., 2000; Francis et al., 2003). Sequences in this cluster were less than 94% similar with AmoA from known AOB.

The *Crenothrix*-like cluster contained sequences from both lakes that were closely related with the sequences from temporary flooded upland soil (Knief et al., 2003) and Lake Washington (Nercessian et al., 2005; Kalyuzhnaya et al., 2006) at 66% similarity with PmoA of *Crenothrix polyspora* (Stoecker et al., 2006).

The *pmoA* sequences from this study fell into nine different clusters. The clusters B1, B3, B6, B8, B9 (γ-MOB type I) and B7 (α-MOB type II) corresponded to groups previously described (Pester et al., 2004; Bussmann et al., 2006). The clusters B11, B12 and B13 (γ-MOB type I) were new groups identified in this study. Sequences in these newly described clusters showed ≤92% similarity with PmoA from known MOB.

Many sequences from sediment of both lakes and 14 m of Schöhsee were observed in clusters B1, B3, B6, and B8 that were closely associated with the sequences from sediment of different lakes: Lake Wintergreen (Nold et al., 2000), Lake Washington (Costello and Lidstrom, 1999; Nercessian et al., 2005; Kalyuzhnaya et al., 2006) and Lake Constance (Pester et al., 2004; Bussmann et al., 2006). Those in
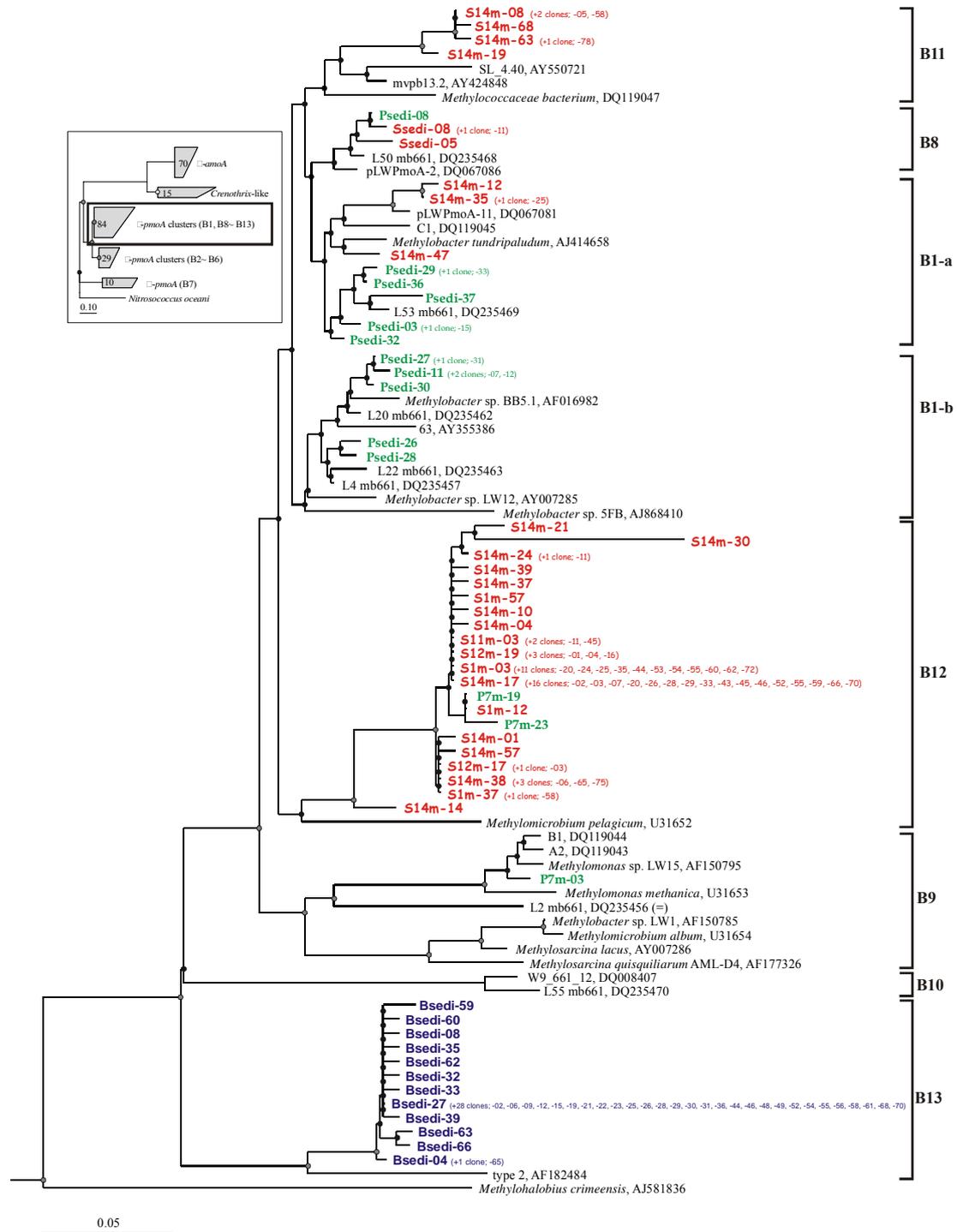
Figure V-2-*Continued*

94
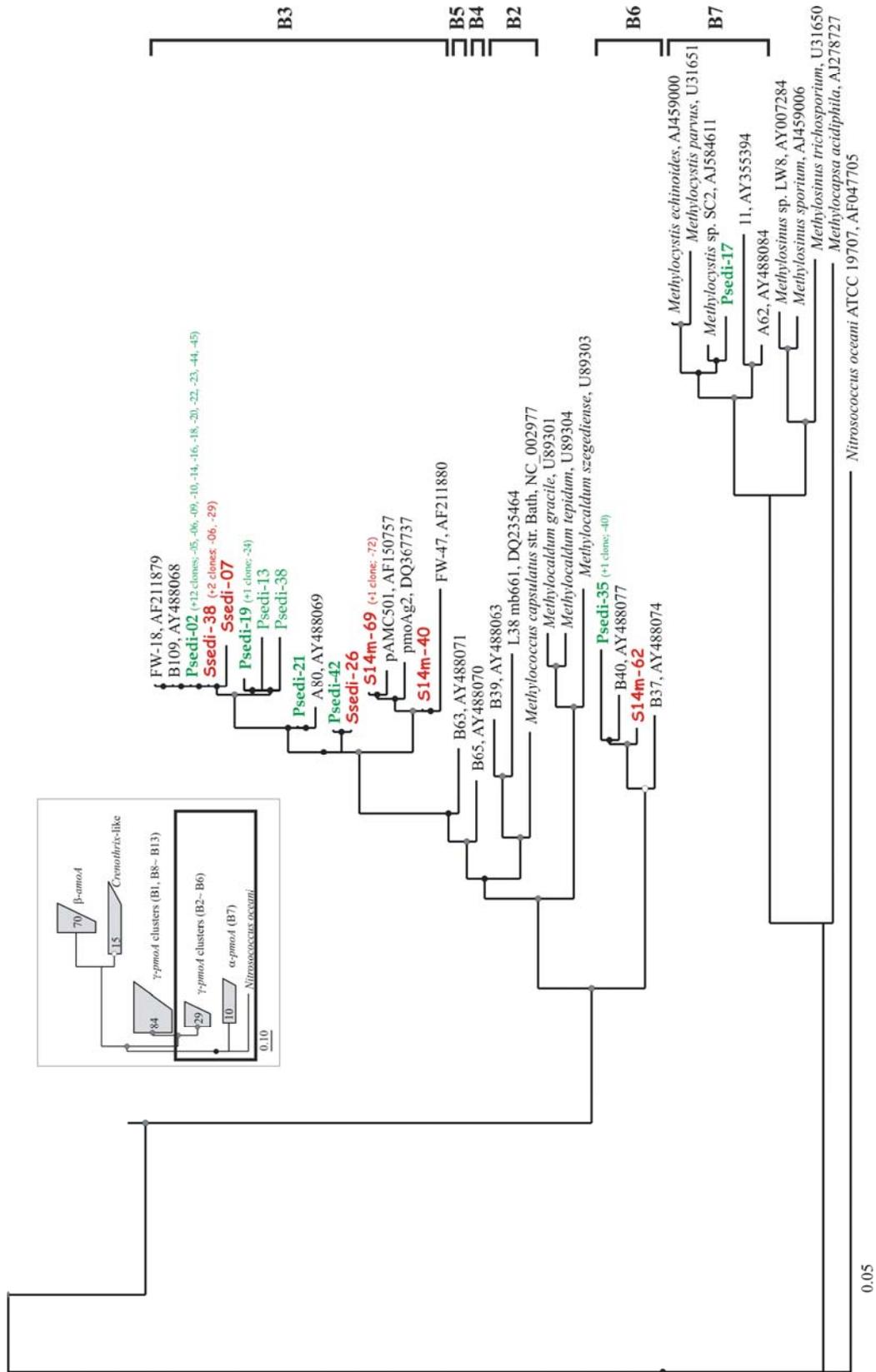
Fig. V-2-Continued

B8 and B1 showed 92 - 97% similarity with PmoA of *Methylobacter* spp., while those in B3 and B6 were 84 - 93% similar with the PmoA of *Methylococcus capsulatus* str. Bath. Cluster B9 and B7 contained a single sequence from 7 m and sediment of Plußsee respectively, which were closely related to PmoA from known MOB (*Methylomonas* sp. LW15 and *Methylocystis* sp. SC2). Sequences in the cluster B11 were associated with those from a Transbaikal soda lake (Lin et al., 2004) and Movile Cave (Hutchens et al., 2004). They were related to Methylococcaceae bacterium LC2 (DQ119047), which was isolated from Lake Constance (Bussmann et al., 2006). 21% of the sequences, all retrieved from the water column of both lakes, were found in cluster B12 and showed 85 - 89% similarity with PmoA from *Methylomicrobium pelagicum*. All *pmoA* sequences from Baltic Sea sediment were observed in cluster B13, related to a sequence from Newport Bay (McDonald et al., 2005).

2. T-RFLP analysis

2.1 Relative abundance

The relative abundance of T-RFs was analyzed after restriction with *Hae*III, which produced the highest number of different T-RFs (16 T-RFs) (Fig. V-3). Samples from each environment showed a distinct pattern of T-RFs. Within each environment T-RF patterns from the water column were more similar with each other than with those from the sediment. The most diverse T-RF pattern was observed in 14 m of Schöhsee (8 T-RFs), whereas the most uniform was observed in 1 m and 4 m of Plußsee (2 T-RFs).

Only few T-RFs were shared between samples from different environments. Two T-RFs (41 bp and 346 bp) were detected in sediment from the Baltic Sea and Plußsee and in all samples from Schöhsee. The T-RF 59 bp was common to samples from the water column in both lakes, and the T-RF 80 bp was detected in sediment of both lakes and Schöhsee 14 m. Specific T-RFs were found through the water column of each environment, such as 87 bp and 145 bp in the Baltic Sea, 94 bp in Plußsee, and 48 bp in Schöhsee. The T-RF 365 bp was dominant in Baltic Sea 20 m and T-RF 222 bp in sediment.

In Baltic Sea, the samples from 2 and 10 m displayed almost identical T-RF patterns. Some of the T-RFs from 2 and 10 m (87 and 145 bp) were also observed in 20 m. Similarly, the samples from Plußsee water column presented similar T-RF patterns dominated by T-RFs 59 and 94 bp. In the water column from Schöhsee, a shift in the communities was observed between 1 m and 14 m.
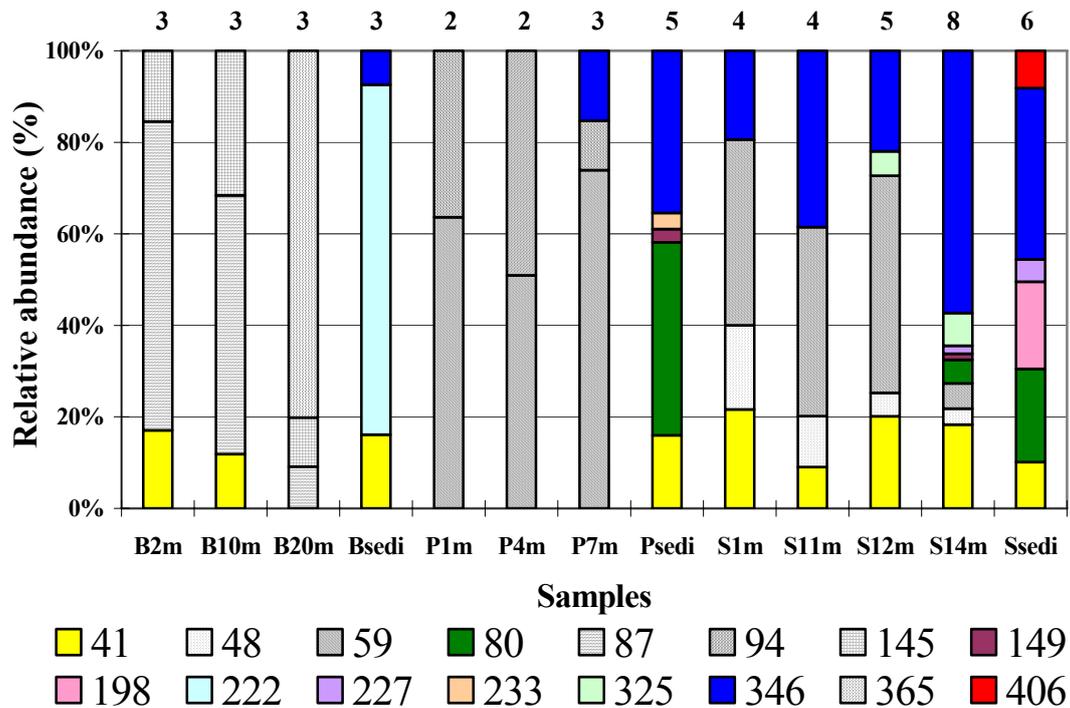
Figure V-3: Relative abundance of T-RFs after restriction with *Hae*III. Number in upper of the columns indicated the number of T-RFs detected in each sample. Length of the different fractions is coded according to the panel on the bottom. For the colored T-RFs a corresponding clones was identified in the clone libraries by simulated.

2.2 Principal component analysis (PCA)

PCA was performed with the combined results of T-RFLP from the five enzymes (Fig. V-4). The analysis was carried out independently for each environment and for all three environments combined. In the In the results from the digestion with *Hae*III, Baltic Sea 10 m was closer to 20 m and sediments than to 2 m. This correlation was due to the existence of common T-RFs in these samples that were detected with the other restriction enzymes (data not shown). In Plußsee PCA with the results from all digestions coincided with those observed for *Hae*III. Samples from the water column were separated in the PC1 from those of sediment. In Schöhsee,

according to the PC1, the samples could be divided into two groups: 1, 11 and 12 m as one group and 14 m and sediment as the other. However, 14 m and sediment were separated in the PC2.

In the PCA with all samples, Baltic Sea and water column of Plußsee formed two compact groups, which were segregated from all samples from Schöhsee and Plußsee sediment by PC2. Plußsee sediment was similar to 14 m and sediment of Schöhsee. Samples from the upper layer of Schöhsee (1, 11 and 12 m) were also grouped together.
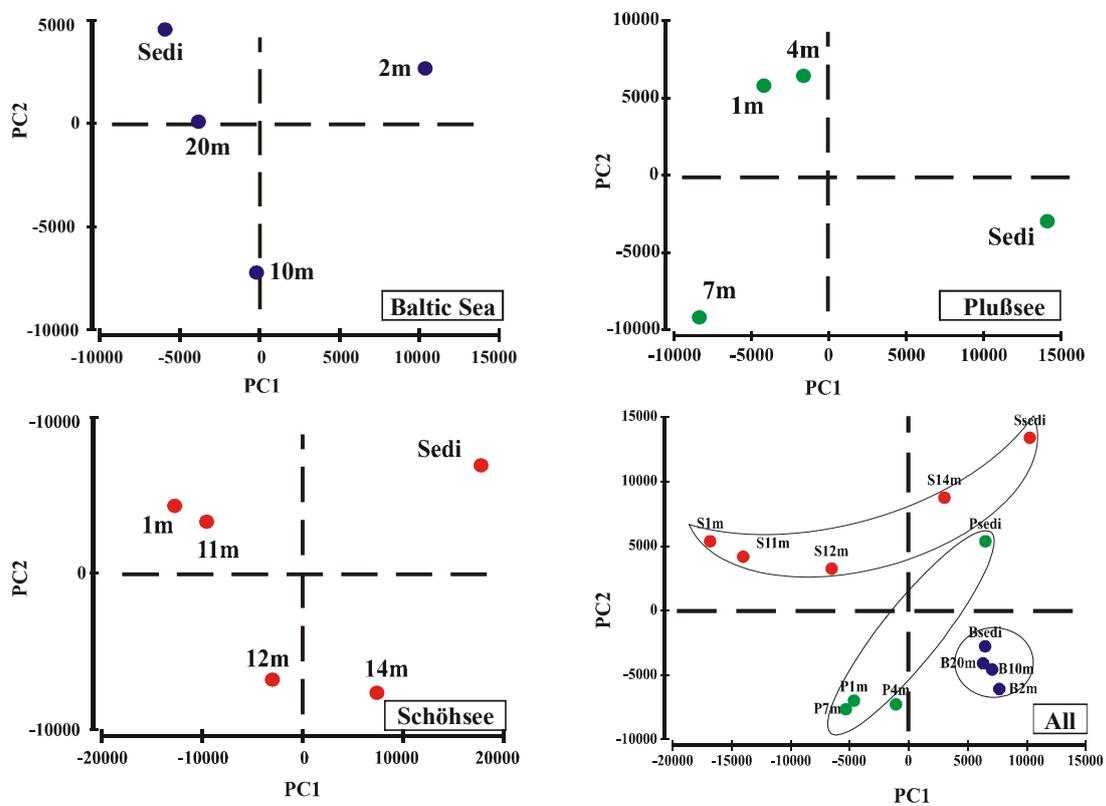
Figure V-4: Principal component analysis (PCA) of T-RFLP patterns after restriction with 5 enzymes. Samples are coded as follows: Baltic Sea, blue; Plußsee, green; Schöhsee, red.

2.3 Simulated T-RF from clonal sequences

To combine the results of T-RFLP and clone libraries, T-RFs were simulated from the clonal sequences (Table V-3). Because all sequences from the same cluster

usually produced the same T-RFs, only dominant T-RFs in each cluster were used for this analysis. Some clusters produced the same T-RFs indicating that T-RFLP has a limited applicability to characterize the whole diversity of the community. Simulated T-RFs were compared with the direct T-RFLP after *Hae*III digestion. T-RFs from the direct approach that corresponded to clones in the libraries are marked in colors in Figure 1. The actual T-RFs 41 bp and 346 bp corresponded to the simulated T-RFs 45 bp (three *amoA* clusters and *pmoA* cluster B7), and 348 bp (clusters B1, B8, B9 and B12) that were dominant T-RFs after simulation with the clones. T-RFs simulated with unspecific sequences from the library could be assigned to dominant T-RFs from the water column in all three environments, corresponding to the results from the clone libraries where a high proportion of unspecific clones were obtained.

Table V-3: Representative T-RFs simulated with the program TRiFLe with clonal sequences in different clusters of the phylogenetic tree (Fig. V-3).

Genes	Cluster	Restriction enzymes				
		*Alu*I	*Hae*III	*Mbo*I	*Msp*I	*Taq*I
amoA	*Nitrosospira*	531	45	59	46	464
	Nitrosospira-like	284	45	30	46	229
	Nitrosomonas	531	45	472	46	531
pmoA/*amoA*	*Crenothrix*-like	284	199	18	33	20
pmoA	B1-a	55	348	167	531	531
	B1-b	531	348	59	531	531
	B3	284	85	59	79	257
	B6	284	85	59	79	531
	B8	531	348	531	208	531
	B9	98	348	167	531	531
	B11	94	531	531	531	531
	B12	98	348	531	531	531
	B13	85	225	326	531	531
	B7	393	45	30	244	170

DISCUSSION

The evolutionary relationship between AMO and pMMO lead to design primers in conserved regions for simultaneous amplification of both genes (Holmes et al., 1995). Although many *amoA* and *pmoA* sequences have been reported from

aquatic environments and are available in the databases, several studies were focused on one of these enzymes independently and investigation of both genes are scarce (Nold et al., 2000; Erwin et al., 2005). Thus, the detection of both genes in this study gives a good chance to compare diversity and distribution of these genes in three different environments.

Distribution of both genes within/between environments

Different distribution of both genes in sediment of marine habitats and lakes has been studied with the same primers as used here (Nold et al., 2000). Clones of. β-*amoA* (75%) dominated in marine habitats, while in lake sediment only γ-*pmoA* clones were detected. From Eastern Snake River Plain aquifer, β-*amoA* was dominant with 80% and some α-*pmoA* (20%) were found (Erwin et al., 2005). However, applying the same primer pair, only *pmoA* genes were detected in some lakes. In Lake Constance γ-*pmoA* were dominant and some α-*pmoA* were detected (Bussmann et al., 2004; Pester et al., 2004). In sediment of Transbaikal soda lakes (Lin et al., 2004) and Mono Lake (Lin et al., 2005) only *pmoA* of the γ-MOB type I was found.

Although this primer set targets *amoA* gene of β- and γ-AOB and *pmoA* of α-MOB and γ-MOB, it has more mismatches with *pmoA* than with *amoA*. In the present study only β-AOB and dominant γ-MOB (only a single α-*pmoA* sequence in Plußsee sediment) were found. This finding is in good agreement with the results from a study of marine and freshwater sediments where β-*amoA* and γ-*pmoA* has been detected (Nold et al., 2000).

The investigated habitats in the present study showed significantly different diversity of both genes in each environment. The results of clone libraries from the Baltic Sea indicated that the habitats were partitioned with only β-*amoA* sequences related to *Nitrosospira* found in the water column and almost all γ-*pmoA* sequences detected in sediment. This finding in the water column coincides with results from the same samples studying amoA diversity with a different set of primers (Kim et al, submitted a). However, in this study only few *amoA* sequences could be detected in sediment although diverse *amoA* sequences were discovered in a previous study (Kim

et al., submitted a) with a different set of primers. Probably, *pmoA* is more frequent in Baltic Sea sediment than *amoA*. In Mono Lake (Lin et al., 2005) only *pmoA* was detected, though four different β-AOB had been found in another study based on 16S rDNA (Ward et al., 2000). This could be due to either low abundance of *amoA* sequences or lack of primer specificity. To detect *amoA*, nested amplification was carried out because of low abundance of AOB (Kim et al., submitted a). However, the intensive products using A189/A682 primers were obtained with direct amplification with genomic DNA.

In Plußsee, almost all sequences were γ-pmoA and only one α-*pmoA* and a *Crenothrix*-like *pmoA* were detected. Using 16S rRNA probe with FISH revealed that γ-MOB were dominant in Plußsee, and α-MOB were not found (Eller et al., 2005). As well in soda lakes, only γ-*pmoA* have been in clone libraries with the same primers (Lin et al., 2004; Carini et al., 2005; Lin et al., 2005). A previous study with the same samples but different primers specific for only *amoA* or 16S rDNA (Kim et al., 2006), (Kim et al., submitted a) revealed several different AOB communities.

In contrast to the results of Baltic Sea and Plußsee, the mesotrophic lake Schöhsee showed a coexistence of both genes through the water body. The shift of the distribution was significant and coincided with the concentration of O_2 (Kim et al., 2006). Coexistence of β-*amoA* and γ-*pmoA* was observed at O_2 concentrations of 8.4 – 2 mg L^{-1} in 1, 11 and 12 m. γ-*pmoA* dominated at 0.5 mg L^{-1} in 14 m, and *Crenothrix*-like *pmoA* sequences under anoxic conditions in sediment. *Nitrosospira* and *Nitrosospira*-like *amoA* sequences were detected through the water column, and *Nitrosomonas* sequences in sediment. This coincides with a previous study showing different adaptation to particle-associated habitats by *Nitrosomonas* and *Nitrosospira* (Phillips et al., 1999). More diverse γ-*pmoA* sequences were found in 14 m. It could be hypothesized that relatively high rates of methane oxidation occur at this depth. In stratified lakes, methane oxidation usually occurs near the deeper part of the chemocline (metalimnion), where dissolved-oxygen levels are relatively low compared with the epilimnion (Hanson and Hanson, 1996). (Lin et al., 2005) suggested a correlation of methane oxidation activity with a change of the community composition of methane-oxidizing bacteria based on *pmoA* genes. In DGGE profiles,

they detected an additional band in the depth, with the highest rates of aerobic methane oxidation.

The fact that sequences related to *Crenothrix polyspora* were found in sediment is very interesting, which was isolated from the backwash water of rapid sand filters for removal of iron, manganese, and ammonium of German drinking water treatment plant (Stoecker et al., 2006). *Crenothrix polyspora* is a filamentous methane oxidizing bacterium that can take up acetate and glucose when methane is absent, indicating that it could be a facultative methane oxidizer. Contrasting kinds of interaction have been reported between nitrification and methane oxidation, such as inhibition (King and Schnell, 1994) and stimulation (Bodelier et al., 2000) of methane oxidation by ammonia availability, and the possibility that nitrification either contributes to (Bodelier and Frenzel, 1999) or suppresses (Megraw and Knowles, 1987; Roy and Knowles, 1994) methane oxidation. However, the interaction can be more complicated since ammonia oxidizing archaea and methane oxidizing *C. polyspora* were discovered recently (Könneke et al., 2005; Treusch et al., 2005; Stoecker et al., 2006), and could be an important part of the community in specific habitats.

Habitat specific clusters in aquatic environments

Habitat specific clusters were found in this study. While the same clusters of *amoA* sequences were detected in both Baltic Sea and Schöhsee, the *pmoA* sequences observed in Baltic Sea did not appear in lakes or the other way around. In Baltic Sea *pmoA* sequences from cluster B13 are closely related to the sequences from Newport Bay, Southern California (McDonald et al., 2005), forming a cluster specific for brackish environments. The *pmoA* cluster B12 is a novel cluster dominated by sequences from the water column of Schöhsee (and 2 clones from Plußsee 7m) and has some similarity with *Methylomicrobium pelagicum*. On the other hand, *pmoA* sequences from sediment of Plußsee and 14 m and sediment of Schöhsee were closely related to the sequences from sediment in Lake Constance (Bussmann et al., 2004; Pester et al., 2004; Bussmann et al., 2006), Lake Washington (Costello and Lidstrom, 1999; Nercessian et al., 2005; Kalyuzhnaya et al., 2006) and Pacific Northwest (Nold et al., 2000), suggesting a more cosmopolitan distribution. Sequences related to

unusual *pmoA* of *Crenothrix polyspora,* which were found mainly in Schöhsee, showed 65% - 67% similarity of the deduced amino acid sequences. Similarity of DNA between those sequences and *C. polyspora*, however, was ≤53%. It could well be that this group of amoA/pmoA sequences represents a novel group of either methane- or ammonia-oxidizing bacteria.

Comparison of the clone libraries diversity

Using 5% cutoff, *pmoA* genes were much more diverse with 25 OTUs than *amoA* genes (6 OTUs). *pmoA* was more diverse and divergent in the samples from both lakes than in Baltic Sea. For *pmoA* genes, Plußsee sediment showed the highest diversity with 12 OTUs. Most of these sequences were grouped into cluster B3 that consisted of only 1 OTU, whereas cluster B1 contained 7 OTUs (5 OTUs in subcluster B1-a and 2 OTUs in subcluster B1-b) indicating more divergent sequences in B1 and conserved sequences in B3. Sequences in cluster B1 were 86% identical, while those in cluster B3 were 96% identical. *pmoA* sequences from Schöhsee 14 m were widely dispersed throughout the phylogenetic tree with 11 OTUs. 3 OTUs in cluster B12, 2 OTUs in clusters B11, B1 and B3 and one OTU in clusters *Crenothrix*-like and B6 indicated the huge diversity of *pmoA* genes in this habitat. *pmoA* sequences of Schöhsee sediment showed relatively low diversity with 4 OTUs, 2 OTUs in *Crenotrhix*-like cluster and 1 OTU in clusters B3 and B8. Diversity was reduced in the water column of Schöhsee (1 OUT) and Plußsee (2 OTUs). Although lots of *pmoA* sequences were observed in Baltic Sea sediment, they belonged to 1 OTU with 98% sequence identity, indicating the presence of conserved *pmoA* genes in this habitat. In contrast to this, amoA sequences from Baltic Sea sediment were distributed into 3 OTUs with 1 sequence each. 3 OTUs were found as well in Schöhsee 14 m, while sequences from Baltic Sea water column and Schöhsee 1, 11 and 12 m and sediment formed 1 OTU, each, indicating low diversity of *amoA* in these habitats.

Application of T-RFLP to study *amoA* and *pmoA* genes

Fingerprinting techniques such as DGGE (Henckel et al., 1999; Henckel et al., 2000a; Henckel et al., 2000b; Fjellbirkeland et al., 2001; Hoffman et al., 2002; Knief

et al., 2005) and T-RFLP (Horz et al., 2001; Hoffman et al., 2002; Pester et al., 2004) have been applied to investigate the communities in several environments. T-RFLP is regarded as a useful method to study diversity of complex bacterial communities and to rapidly compare community structure in different ecosystems (Liu et al., 1997). However, linking the T-RFLP results with the simulation of T-RFs in the clone libraries from this study indicated that this technique has only a limited resolution to separate different members of the community. Another problem is the unspecific PCR amplification that could misestimate the community composition by T-RFLP method. In the clone libraries of this study, unspecific PCR products were also observed. Other studies using the same primer set, also described unspecific PCR amplification (Nold et al., 2000; Bourne et al., 2001).

3. VI Distribution

of nitrite reductase (*nirK* and *nirS*) genes in the water column and sediment-water interface of two lakes and the Baltic Sea
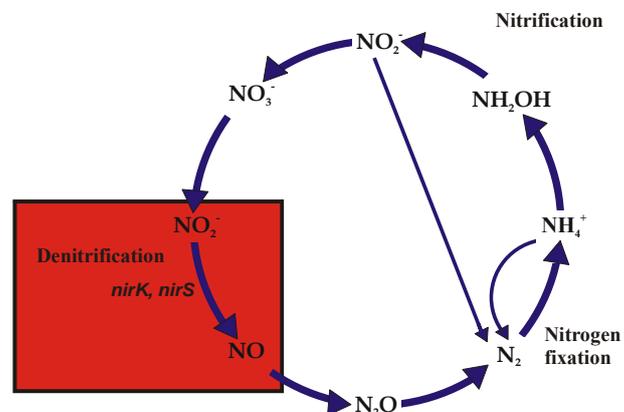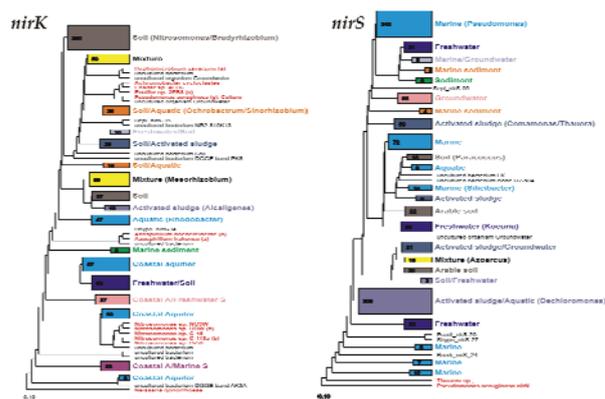
ABSTRACT

The distribution of denitrifying bacteria carrying *nirK* or *nirS* genes was studied in samples from the water column and sediment-water interface of lake Plußsee as a eutrophic, and lake Schöhsee as a mesotrophic lake in Schleswig-Holstein (Germany), and a near-shore station of the German Baltic Sea. Both, *nirK* and *nirS* genes were amplified by PCR and analyzed by terminal restriction fragment length polymorphism (T-RFLP), cloning and sequencing. Based on relative abundance, clone libraries and rarefaction curve, *nirS*-denitrifiers were more diverse than *nirK*-denitrifiers. Principal component analysis (PCA) of T-RFLP showed that in all environments sediment *nirK*-denitrifiers and *nirS*-denitrifiers were different from those in the water column. For *nirK*, dominant sequences from all clone libraries were detected in two distinct phylogenetic clusters, and for *nirS*, sequences from both lakes shared several clusters throughout the complete phylogenetic tree and only few sequences from Baltic Sea overlapped. Different distribution between *nirK* and *nirS* sequences was observed in three environments. In the Baltic Sea, *nirK*-denitrifiers were diverse throughout the water column, while *nirS*-denitrifiers were dominant in the sediment and almost absent in the water column. Lake Plußsee communities showed inverse composition: *nirK*-denitrifiers were more diverse in the water column and *nirS*-denitrifiers in the sediment. In Lake Schöhsee *nirS*-denitrifiers showed high diversity across the water body.

INTRODUCTION

Denitrification is a respiratory microbial process in which oxidized nitrogen is used as an alternative electron acceptor for energy production when oxygen is limiting. Ecologically, this process is important because it converts the nutrient nitrate (NO_3^-) into the more inert gaseous nitrogen (N_2), which is eventually equilibrated with the atmosphere and may thus cause a loss of nitrogen. Gaseous intermediates, like NO, NO_2 and N_2O may contribute to the depletion of ozone if they reach the atmosphere (Knowles, 1982). This process is catalyzed sequentially under anaerobic

or microaerobic conditions by four different enzymes: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), nitrous oxide reductase (Nos). Microorganisms possessing these enzymes are distributed over a wide range of taxonomically distinct groups of Bacteria and Archaea (Zumft, 1997; Philippot, 2002).

Nitrite reductase (Nir) is the key enzyme of denitrification, catalyzing the reduction of NO_2^- to NO, the first gaseous product (Zumft, 1997). There are two nitrite reductase enzymes that are evolutionarily distinct but have a similar function: the copper-containing NirK encoded by the gene *nirK*, and the cytochrome *cd1*-containing NirS encoded by *nirS* (Zumft, 1997; Philippot, 2002). These two enzymes have never been observed to coexist within the same bacterial species (Coyne et al., 1989). *nirS* is part of a *nir* gene cluster, in which the number of *nir* genes and the organization differ in different species. In some species, the *nirK* gene forms a cluster together with a *nirV* gene, located downstream and encoding a protein of unknown function (Philippot, 2002).

Recent studies using nitrite reductase genes as functional markers have explored their diversity, focused on diverse environments comprising soil (Avrahami et al., 2002; Priemé et al., 2002; Rösch et al., 2002; Throbäck et al., 2004; Wolsing and Priemé, 2004; Sharma et al., 2005), groundwater (Yan et al., 2003), estuarine sediment (Nogales et al., 2002; Santoro et al., 2006), marine sediment (Braker et al., 2000; Liu et al., 2003) and seawater (Tuomainen et al., 2003; Jayakumar et al., 2004; Castro-González et al., 2005; Hannig et al., 2006; Oakley et al., 2007). Up to date, most studies in environments have focused on either soils or marine environments with little attention given to freshwater (Junier et al. submitted).

In this study, we focus on the diversity and distribution of the nitrite reductase genes *nirK* and *nirS* in the water column and sediment-water interface of two lakes (Plußsee and Schöhsee) in northern Germany and the Baltic Sea.

RESULTS

1. T-RFLP analysis of *nirK* and *nirS*

1.1 Relative abundance of T-RFs

PCR products of *nirK* and *nirS* were obtained from all the samples. Analysis of T-RFLP showed that *Hae*III produced the highest number of T-RFs (19 T-RFs for *nirK* and and 20 for *nirS*), while *Alu*I and *Msp*I produced less T-RFs (13 and 16 for *nirK*; 15 and 17 for *nirS*). Therefore, the relative abundances of individual T-RFs after *Hae*III restriction were used for further analyses (Fig. VI-1 and VI-2).

In the Baltic Sea, Plußsee and Schöhsee, *nirK*-denitrifier communities showed a clearly distinguishable pattern of T-RFs (Fig. VI-1). Only a few T-RFs (174 bp and 279 bp) were shared between environments. The T-RFs 35 bp, 41 bp and 205 bp were detected in both lakes and the T-RF 240 bp was found in the Baltic Sea and Schöhsee. In all three environments, the T-RF pattern from sediment samples was different from those in the water column. Sediments from the Baltic Sea and Plußsee were not dominated by any particular T-RF, while in Schöhsee sediment the T-RF 143 bp showed a significantly higher frequency (more than 60% of the fluorescence).
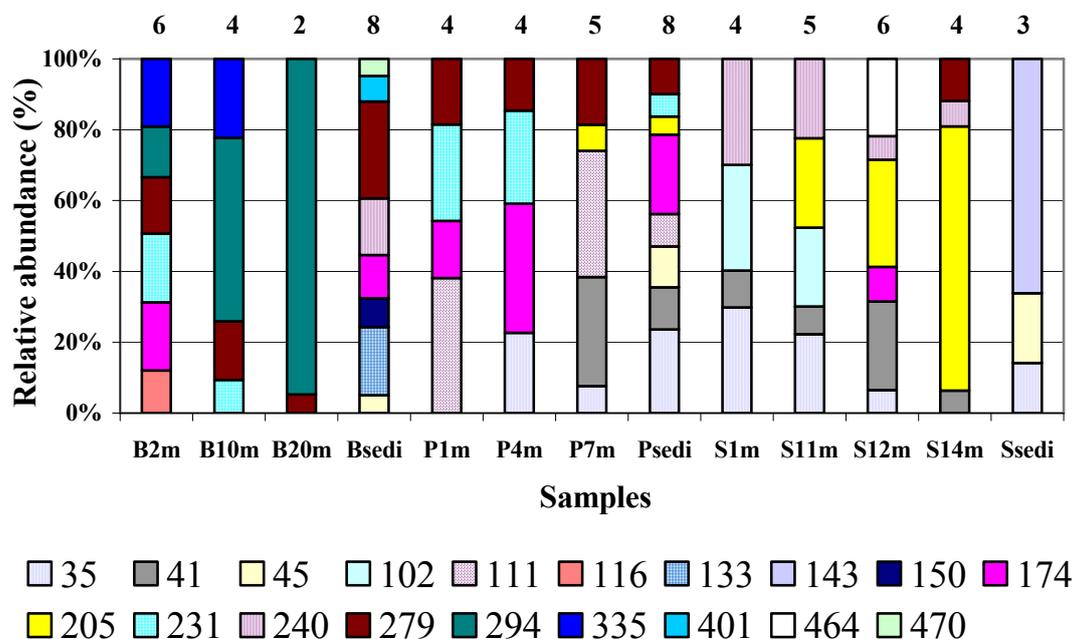
Figure VI-1: Relative abundance of T-RFs after restriction with *Hae*III of amplified *nirK* in samples from the Baltic Sea, Plußsee and Schöhsee. Number in upper of the columns indicated the number of T-RFs detected in each sample. Numbers in bottom indicate the lengths of T-RFs in base pair.

A higher proportion of T-RFs was shared between different samples from the same environment. In the Baltic Sea, the relative abundance of the T-RFs 231 bp, 279 bp, 294 bp and 335 bp changed with depth. Likewise, in Schöhsee a similar shift in the abundance of the T-RFs 41 bp, 205 bp and 240 bp was observed. The T-RF 111 bp was unique to Plußsee, and was detected there in 1, 4, 7m and sediment.

The T-RFLP patterns of *nirS* were more diverse than those of *nirK* (Fig. VI-2). The smallest T-RF 36 bp was common to all samples, except Plußsee 7 m and Schöhsee 14 m. Similarly, the T-RFs 66 bp, 136 bp and 147 bp appeared in all three environments. The T-RFs 130 bp, 222 bp, 271 bp and 407 bp appeared in the Baltic Sea and Plußsee, whereas the Baltic Sea and Schöhsee shared the T-RFs 53 bp, 72 bp, 91 bp, 176 bp, 301 bp and 372 bp. The T-RF 160 bp was detected only in the two lakes. T-RF 102 bp was observed in the Baltic Sea and T-RF 333 bp in Plußsee, only. There was no indigenous T-RF in Schöhsee. In the Baltic Sea, none of the samples was dominated by any particular T-RF, while one T-RF was dominant in Plußsee 7 m (60 bp) and Schöhsee 1 m and 12 m (36 bp).
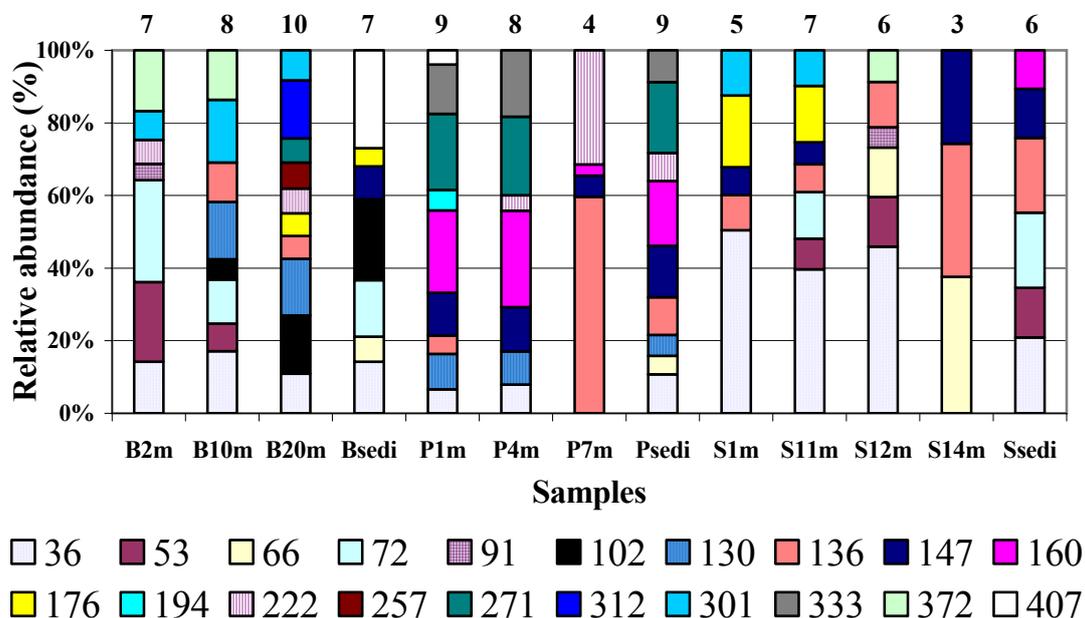
Figure VI-2: Relative abundance of T-RFs after restriction with *Hae*III of amplified *nirS* in samples from the Baltic Sea, Plußsee and Schöhsee. Number in upper of the columns indicated the number of T-RFs detected in each sample. Numbers in bottom indicate the lengths of T-RFs in base pair.

1.2 Principal component analysis (PCA) of T-RFLP of *nirK* and *nirS*

PCA of T-RFLP of *nirK* and *nirS* with the combined results of the three restriction enzymes is presented in Fig. VI-3. In all environments, *nirK* communities in sediment from three environments were separated from those in water column. Through the water column, in the Baltic Sea the composition of the *nirK* communities at 10 m depth was intermediated to those at 2 m and 20 m. In each lake, *nirK* communities from 1and 4 m of Plußsee and 11, 12, and 14 m of Schöhsee were grouped together. In Baltic Sea *nirS* communities of 2 and 10 m were similar and apart those from the other samples. *nirS*-communities from Baltic Sea 20 m, Plußsee 7 m and Schöhsee 14 m were separated from the other samples in each environment by the PC 1 (Fig. VI-3. A-C).

When all the samples were analyzed simultaneously (Fig. VI-3. D), the denitrifying communities with *nirK* were more related to each other in the two lakes and clearly separated from those in the Baltic Sea. Similar results were obtained for *nirS*, although in this case, the sediment communities of the Baltic Sea were grouped together with those in the lakes.

2. Clone libraries and sequence analysis of *nirK* and *nirS* PCR products

2.1 Clone libraries

Based on the PCA results of the T-RFLP, nine samples for *nirK* and ten samples for *nirS* were selected to generate clone libraries because they should cover the total diversity in the whole set of samples. These include for both genes all sediment samples from the three environments, 2 m and 20 m from Baltic Sea, 1 and 7 m from Plußsee and 1 and 12 m from Schöhsee. Additionally, *nirS* from 14 m in Schöhsee was chosen for cloning because it was separated from the other samples in the PCA.

A total of 305 *nirK* and 269 *nirS* clones with an insert of 470 and 473 bp for *nirK* and 410 - 425 bp for nirS were sequenced (Table VI-1). More clones from both genes were obtained with sediment samples from the Baltic Sea and Schöhsee than with water samples. Most *nirK* clones were recovered from Plußsee 1 m, while most
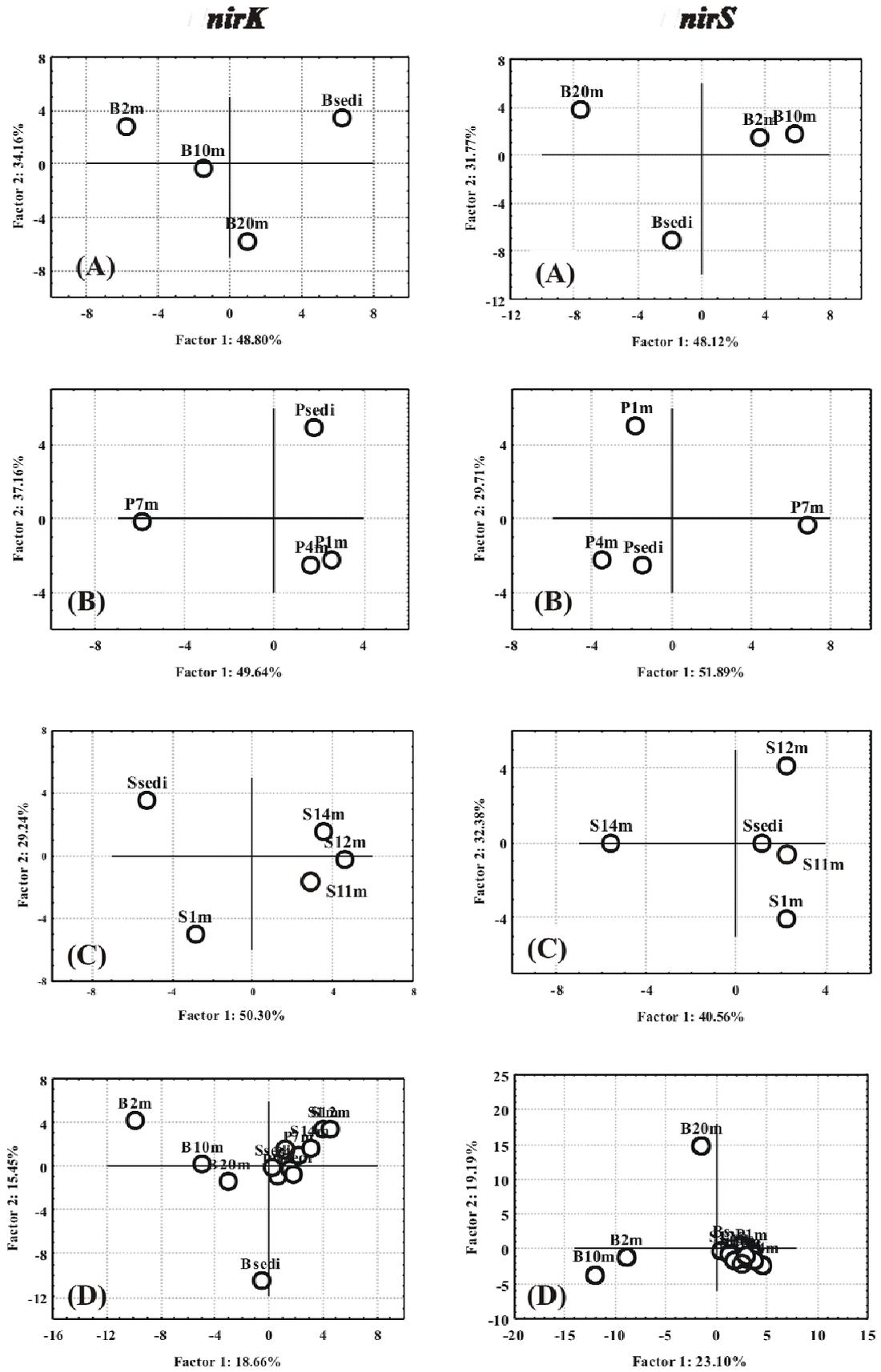
Figure VI-3: Principal component analysis of T-RFLP of *nirK* (left) and *nirS* (right) from samples of the Baltic Sea (A), Plußsee (B), Schöhsee (C) and all samples (D).

111

nirS clones originated from sediment. In the clone libraries from 2 and 20 m of the Baltic Sea, *nirS* clonal sequences were almost absent (only four and two clones with the right insert were detected).

Table VI-1: Number of sequences in clusters of the phylogenetic trees (Fig. VI-4 and VI-5)

Genes	Cluster	No. of clones sequenced										No. of sequences in each cluster
		Baltic Sea			Plußsee			Schöhsee				
		2 m	20 m	Sediment	1 m	7 m	Sediment	1 m	12 m	14 m	Sediment	
nirK	I	13	2	0	0	0	0	0	12	N.D.	2	29
	II	2	0	0	4	2	0	2	5	N.D.	0	15
	V	0	0	0	0	0	0	0	0	N.D.	6	6
	VI	15	33	31	18	7	12	2	2	N.D.	10	130
	VII	2	6	10	18	23	6	16	19	N.D.	22	122
	VIII	0	0	3	0	0	0	0	0	N.D.	0	3
	Total	32	41	44	40	32	18	20	38	N.D.	40	305
nirS	I	1	0	0	8	17	15	10	11	10	16	88
	II	0	0	0	0	0	2	1	0	0	4	7
	III	0	0	0	1	0	3	0	9	3	7	23
	IV	0	0	7	0	0	0	1	0	0	2	10
	V	0	1	0	0	16	4	0	2	34	7	64
	VI	0	0	13	0	0	0	0	0	0	0	13
	VII	0	0	0	2	0	12	1	21	0	7	43
	VIII	3	1	0	0	0	0	7	1	0	0	12
	IX	0	0	3	0	0	1	0	0	0	4	8
	X	0	0	1	0	0	0	0	0	0	0	1
	Total	4	2	24	11	33	37	20	44	47	47	269

Almost all clonal sequences were related to uncultured denitrifying bacteria as revealed by BLASTX at similarities from 74% to 100% for NirK and from 59% to 96% for NirS. Only two *nirK* sequences in cluster IIf were 100% identical with NirK from the cultured denitrifier *Ochrobactrum* sp. 2FB10 (AY078249).

2.2 *nirK* phylogeny

In *nirK* phylogeny (Fig. VI-4), the clusters I - VI correspond to those described by (Priemé et al., 2002) and Junier et al. (submitted). The clusters VII and VIII were identified in this study. All sequences from this study were grouped into six of the eight clusters. No clones were obtained from cluster III and IV, the majority fell
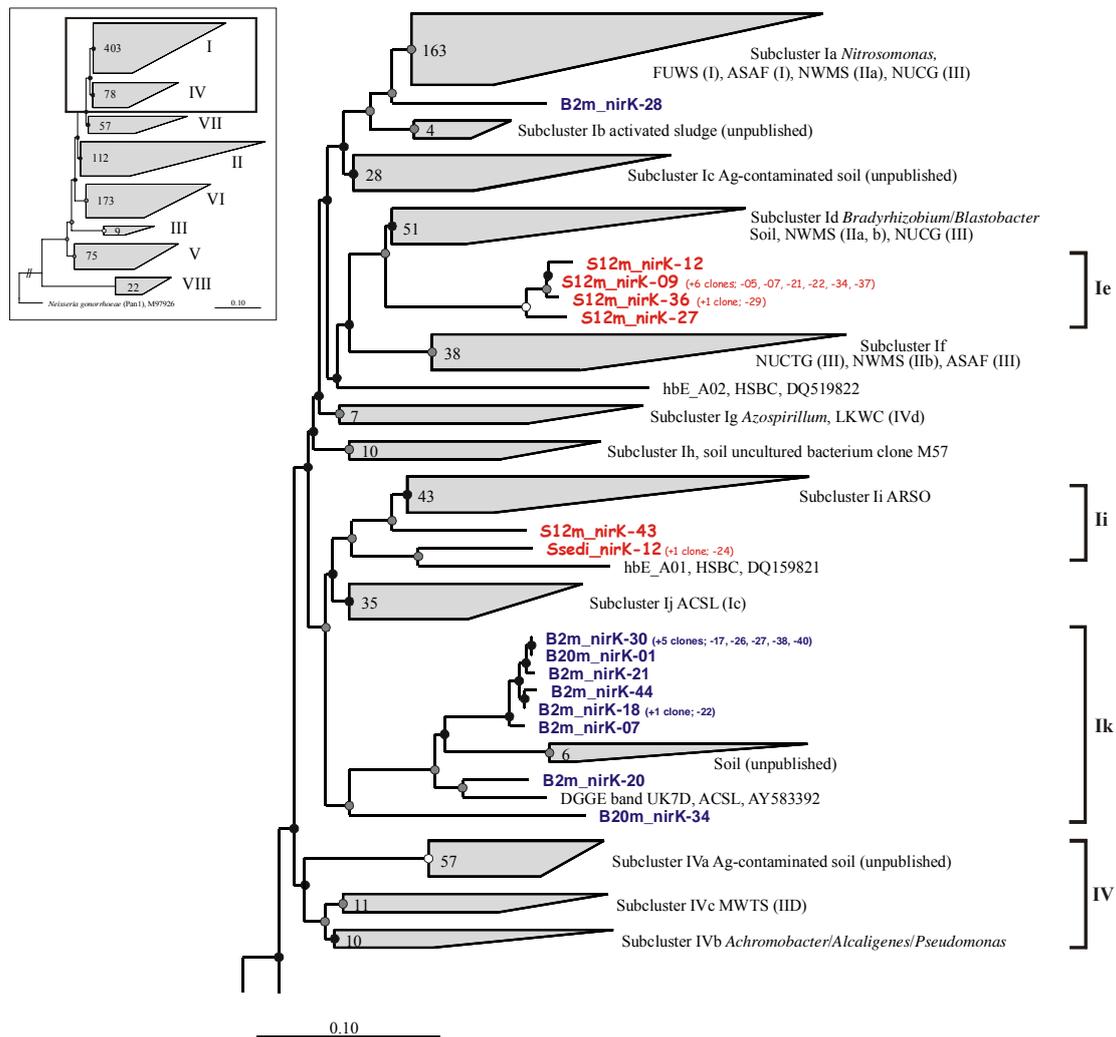
Figure VI-4: Phylogenetic tree of *nirK* gene, based on 158 deduced amino acids, constructed by neighbor joining method in ARB with aniA from *Neisseria gonorrhoeae* (M97926) as the outgroup. Clones from this study are shown in bold and designated as B, Baltic Sea in Arial with blue; P, Plußsee in Book Antiqua with green; S, Schöhsee in Comic Sans MS with red. and followed by depth. Sedi=sample from sediment-water interface. Bootstrap values for 1,000 replicates are indicated at the branch points by circles: black (100%), gray (95 - 99%) and white (90 - 94%). PNWS, Pacific Northwest sediment (Braker et al., 2000); RCES, River Colne estuary sediment (Nogales et al., 2002); FUWS, forested upland and wetland soil (Priemé et al., 2002); SDAC, incubated soil with different ammonia concentration (Avrahami et al., 2002); NWMS, northwestern Mexico sediment (Liu et al., 2003); NUCG, nitrate- and uranium-contaminated groundwater (Yan et al., 2003); BSCB, Baltic Sea cyanobacterial bloom (Tuomainen et al., 2003); MWTS, metallurgic wastewater treatment systems (Yoshie et al., 2004); ASAF, arable soil receiving artifical fertilizer (Wolsing and Priemé, 2004); ASWC, Arabian Sea water column (Jayakumar et al., 2004); ARSO (arable soil), ACSL (activated sludge) and PESO (peat soil) (Throbäck et al., 2004); OESP, OMZ in the eastern South Pacific (Castro-González et al., 2005); HSBC, Huntington State Beach, California (Santoro et al., 2006); BSWC, Baltic Sea water column (Hannig et al., 2006); AGCS, Ag-contaminated soil (unpublished); LKWC, Lake Kinneret water column (Junier et al. submitted)
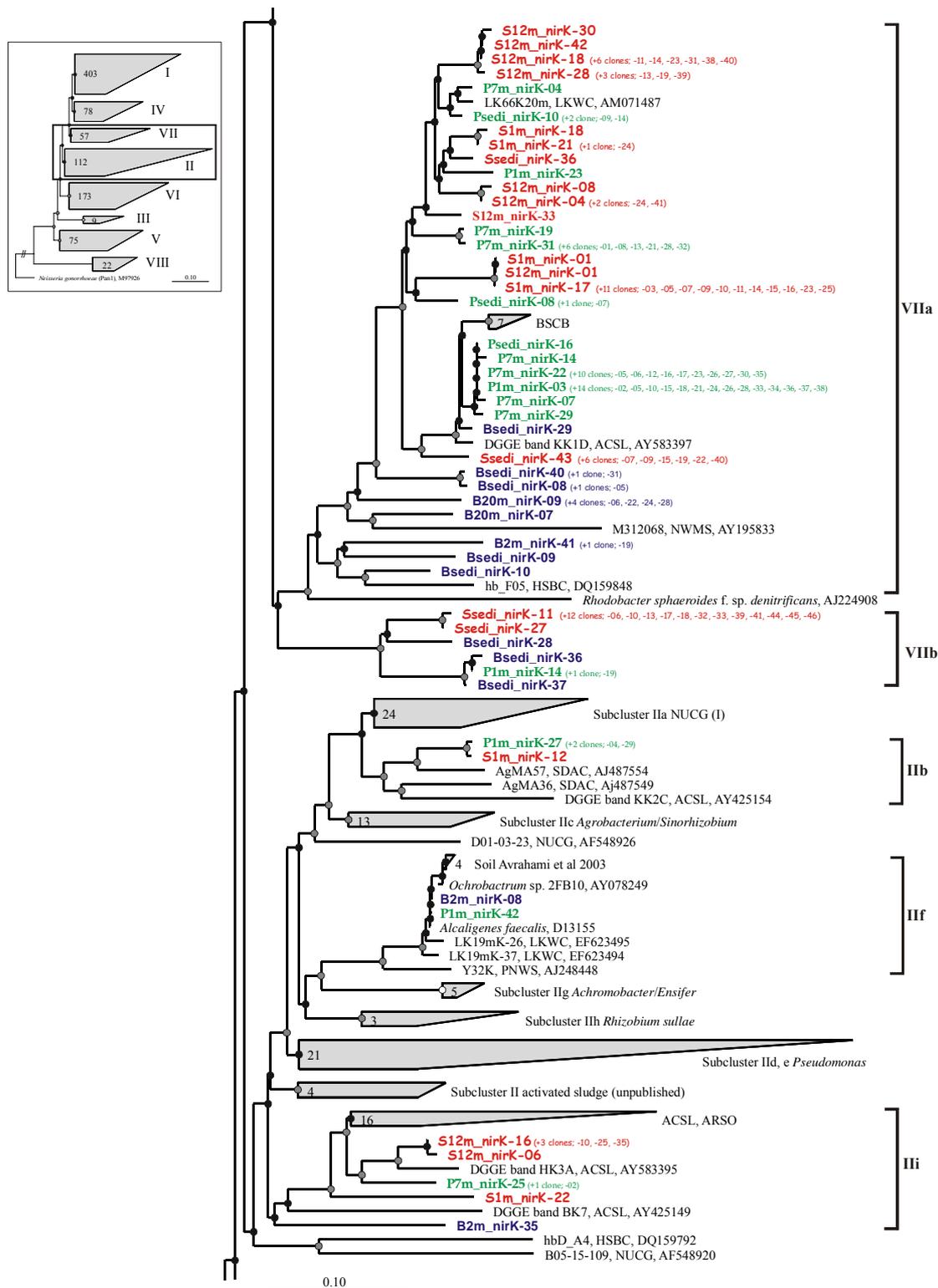
Figure V-4: *continued.*

Figure V-4: *continued.*

115
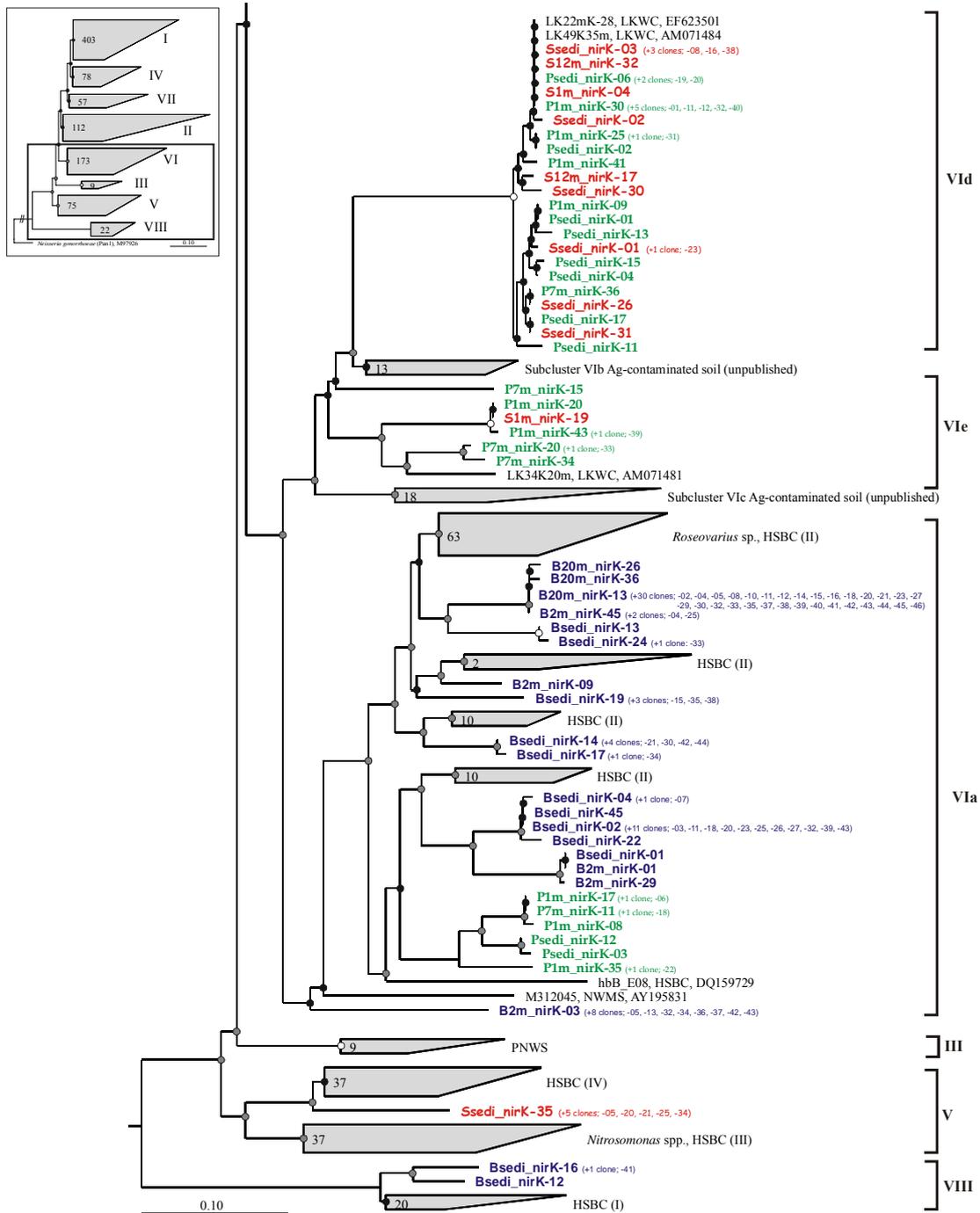
into cluster VI (130) and the new cluster VII (122). These two clusters contained clones from all samples. The sequences in cluster VII could be divided into at least two subclusters based on the identity with NirK from *Rhodobacter sphaeroides* f. sp. *denitrificans* which was isolated from polluted water of a lagoon pond in Japan (Satoh et al., 1976). Sequences in subcluster VIIa were 67 - 72% similar, those in subcluster VIIb 58 - 60%. The smaller subcluster VIIb contained only sequences from this study, while subcluster VIIa contained in addition a few sequences from a wide range of environments, such as Lake Kinneret (Junier et al., 2007 submitted), Baltic Sea (Tuomainen et al., 2003), northwestern Mexico sediment (Liu et al., 2003), Huntington State Beach, California (Santoro et al., 2006), and activated sludge (Throbäck et al., 2004). Cluster VI included NirK from *Roseovarius* sp. 217 (Acc. No. NZ_AAMV01000015) isolated from surface seawater collected near Plymouth, England (Schäfer et al., 2005), and environmental sequences that were 65 ~ 86% identical to NirK from this bacterium. This cluster VI was separated into three subclusters VId, VIe and VIa. Subcluster VIa included the sequences from Baltic Sea and Plußsee that were closely related with those from Huntington State Beach, California (Santoro et al., 2006). Sequences in subclusters VId and VIe were associated with those from Lake Kinneret (Junier et al. submitted) and from Ag-contaminated soil (Throbäck et al., unpublished).

Cluster I contained in the three subclusters Ie, Ii and Ik sequences from water column of the Baltic Sea as well as from 12m and sediment of Schöhsee, all closely related to sequences from soil (Priemé et al., 2002; Throbäck et al., 2004; Wolsing and Priemé, 2004). Similarly, cluster II included sequences from the water column of all three environments that were related to those from diverse environments. Cluster V contained sequences from Schöhsee sediment, and cluster VIII those from Baltic Sea sediment that were related to *nirK* from Huntington State Beach, California (Santoro et al., 2006).

2.3 *nirS* phylogeny

Ten clusters were identified in the phylogeny of *nirS* (Fig. VI-5). Clusters I - V are the same groups as previously identified (Yan et al., 2003), clusters VI - X are new from this study. Most of the *nirS* sequences (88) belonged to cluster I, which
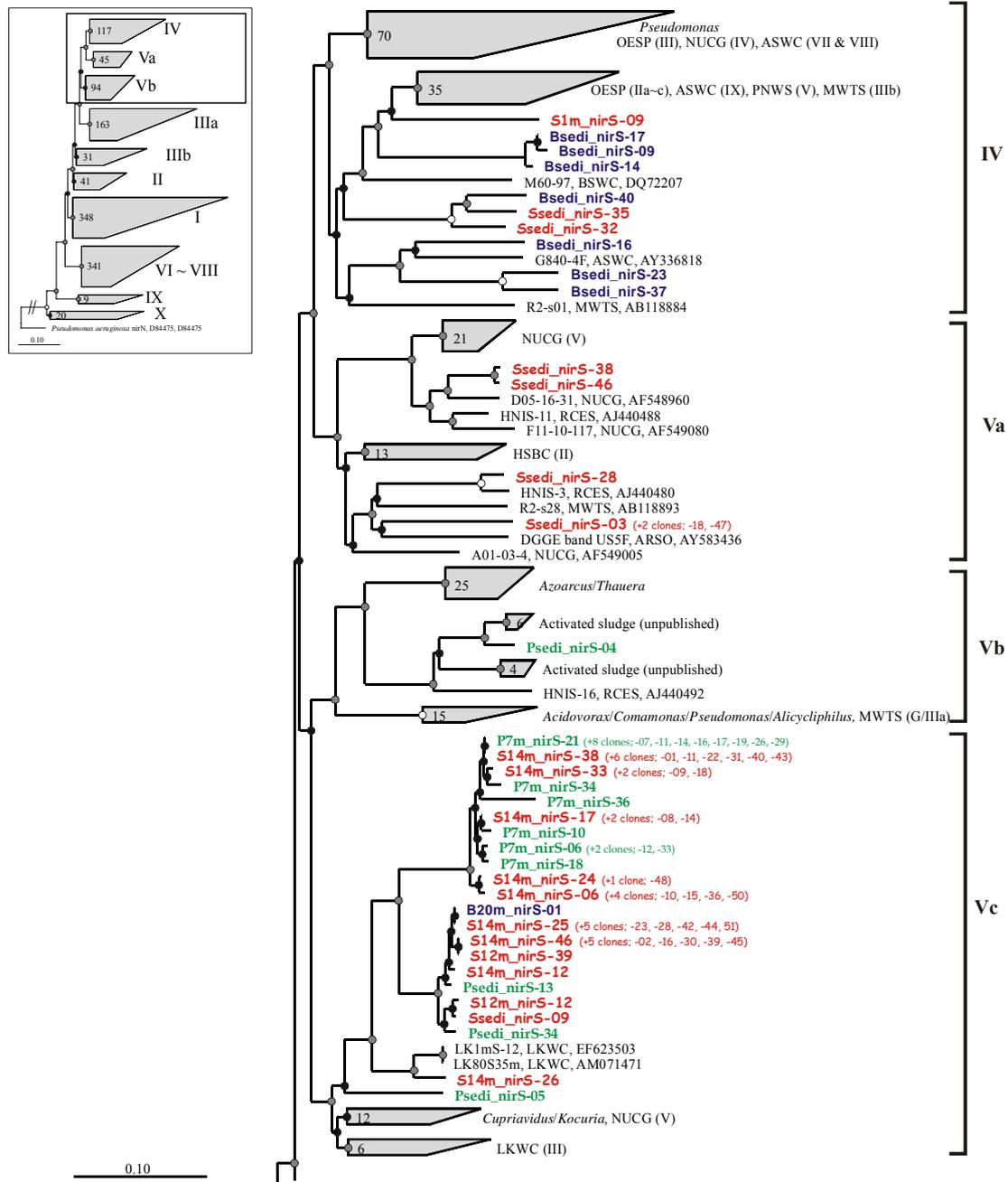
Figure V-5: Phylogenetic tree of *nirS* gene, based on 142 deduced amino acid sites, constructed by neighbor joining method in ARB with *nirN* from *Pseudomonas aeruginosa* (D84475) as the outgroup. For explanation see Fig. V-4.
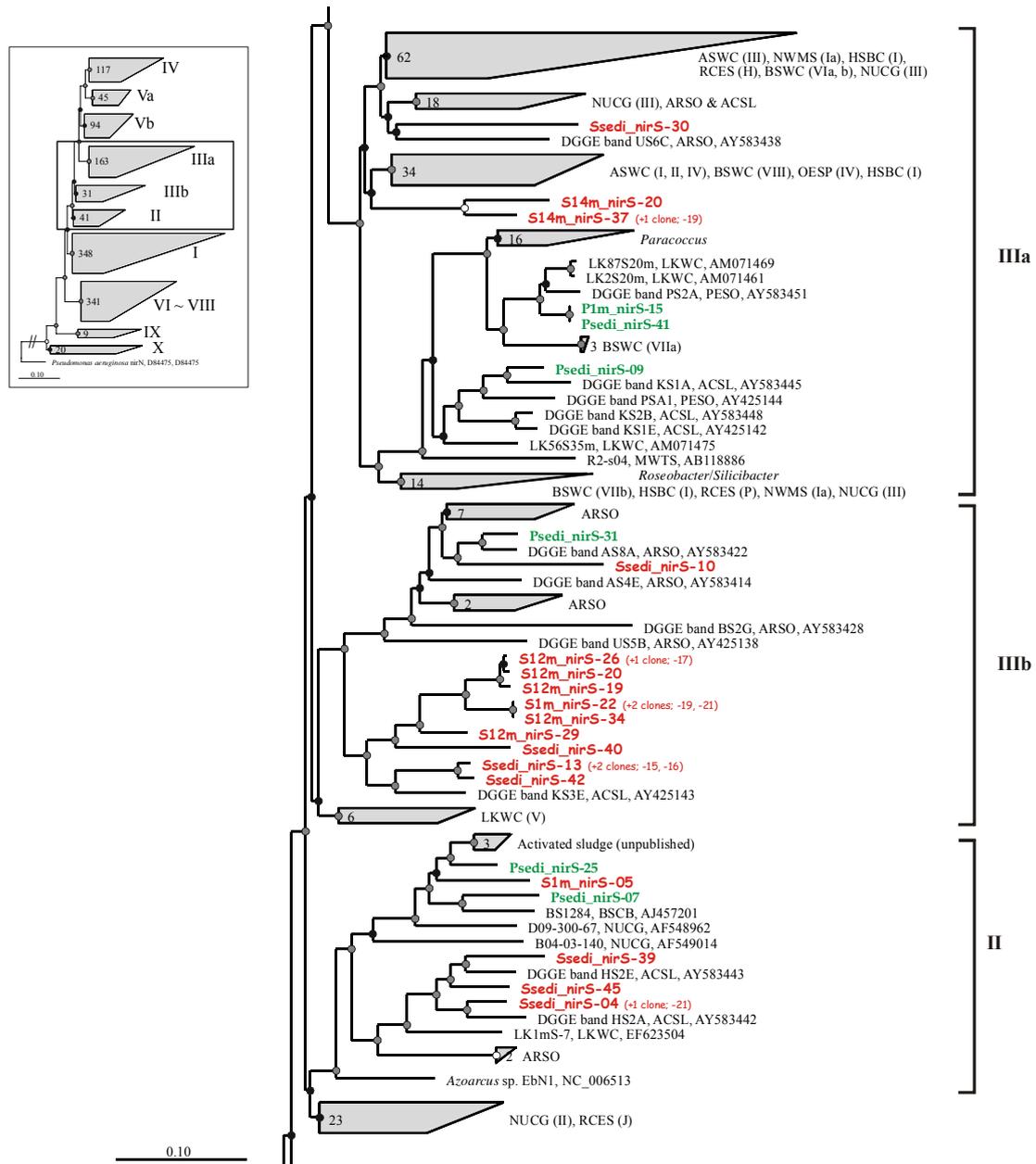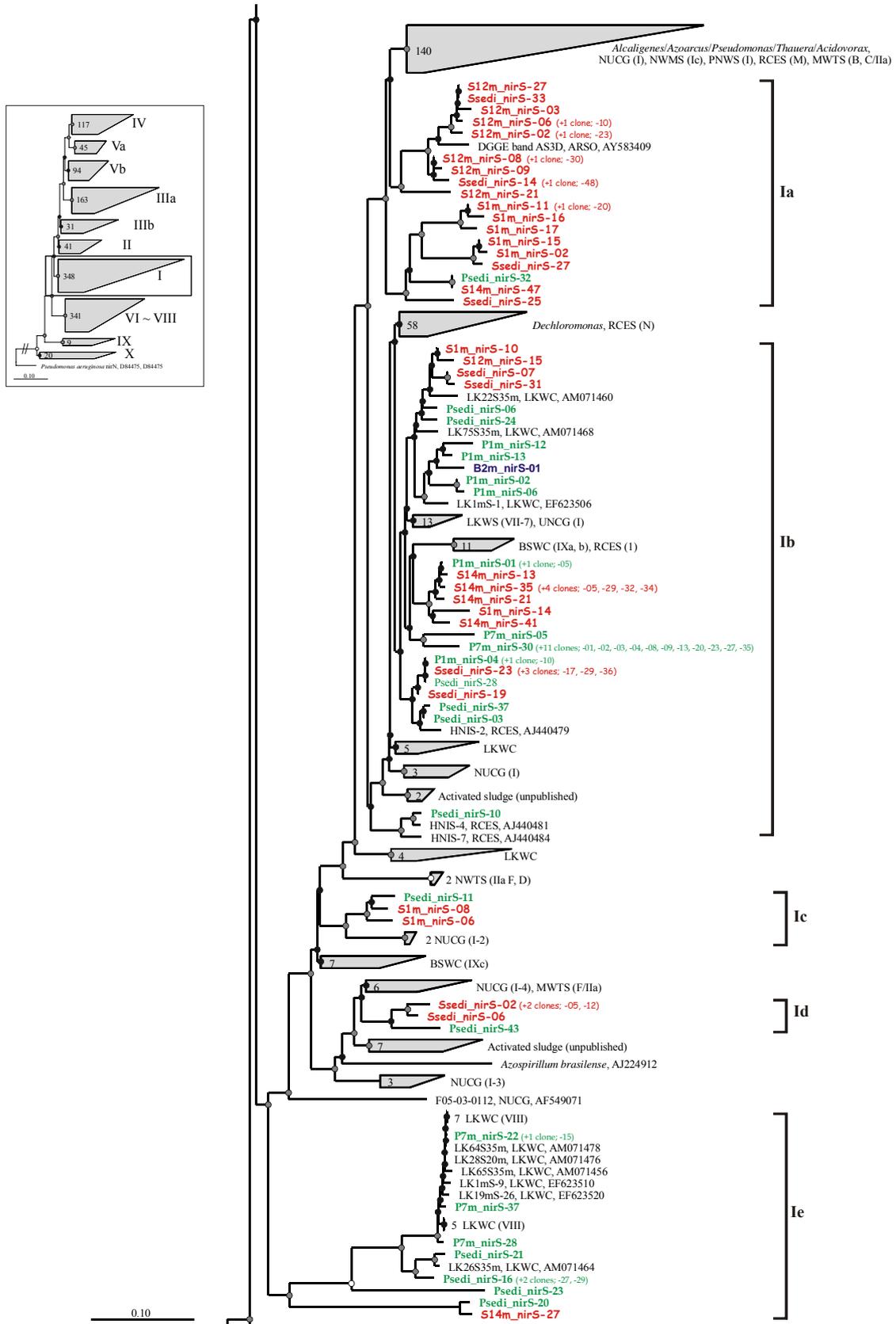
Figure V-5: *continued.*

Figure V-5: *continued.*
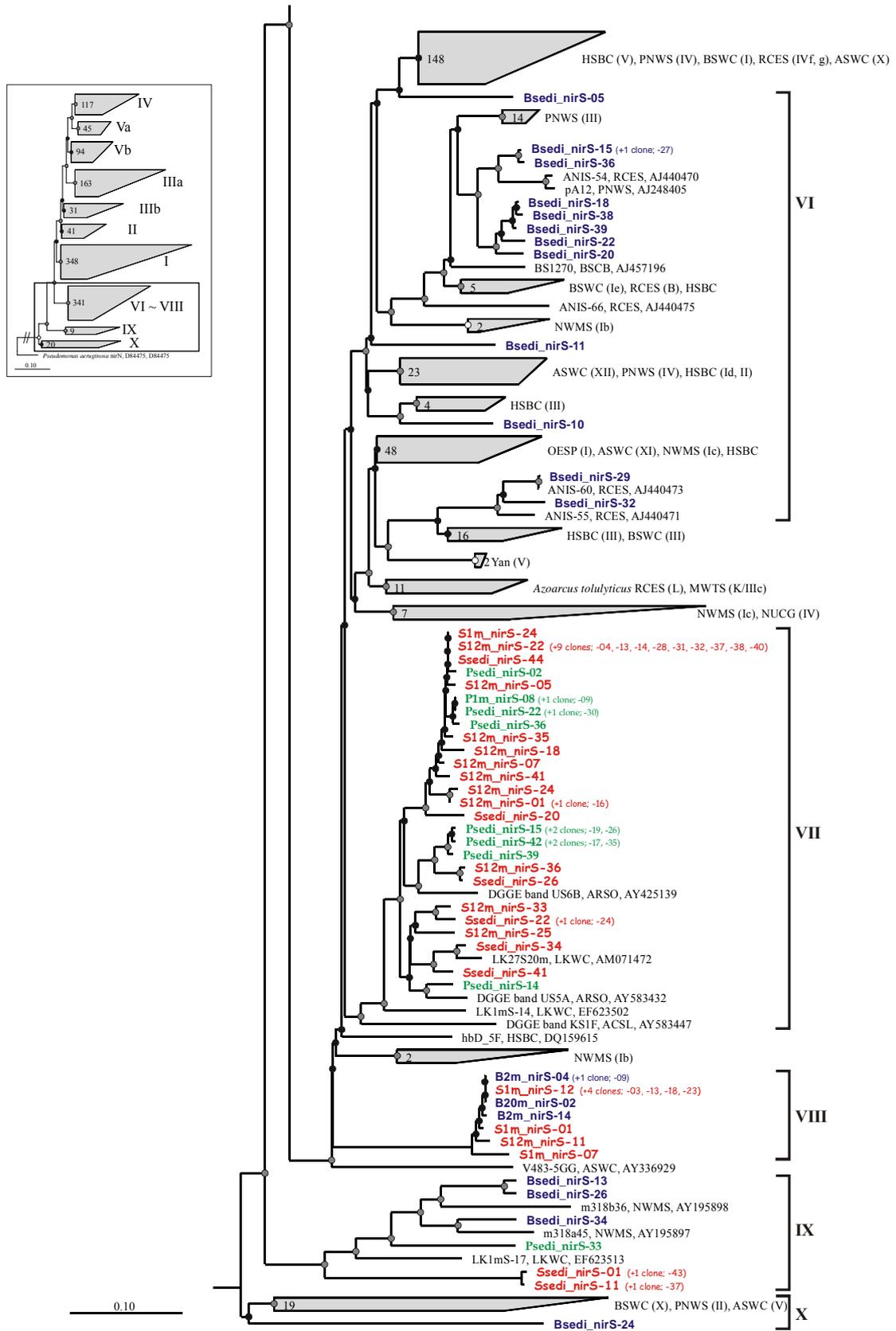
Figure V-5: *continued*.

includes sequences from all samples of both lakes, but nor from the Baltic Sea. These sequences were separated into five subclusters. Subcluster Ia contained sequences from Schöhsee and another one from arable soil (Acc. No. AY583409) (Throbäck et al., 2004). This purely environmental subcluster is only distantly related to known denitrifying bacteria. Subcluster Ib, containing NirS from *Dechloromonas* spp., also includes sequences from the water column of Lake Kinneret (Junier et al., 2007 submitted) and Baltic Sea (Hannig et al., 2006), and sediment of River Colne estuary (Nogales et al., 2002). Sequences in the smaller subclusters Ic and Id were related to those from nitrate- and uranium-contaminated groundwater(Yan et al., 2003). Subcluster Ie is a lake specific cluster with sequences from Plußsee and Lake Kinneret (Junier et al. submitted) and one sequence from Schöhsee.

Sequences from both lakes were also grouped into the clusters V and VII. Subcluster Vc was dominated by sequences from 7 m of Plußsee and 14 m of Schöhsee, closely related to some sequences from Lake Kinneret and at <85% identity with NirS from *Cupriavidus necator* (AM230890). Cluster VII comprised sequences from water and sediment of Plußsee and Schöhsee, from water of Lake Kinneret, arable soil and activated sludge, at ≤74% identity with the NirS from *Azoarcus tolulyticus* (AY078272).

The dominant sequences from Baltic Sea sediment and a few from lake Schöhsee (from 1 m depth and from the sediment were grouped into clusters IV and VI, together with sequences from brackish and marine habitats. Some sequences from both lakes were grouped into clusters II and III, together with sequences from arable soil, activated sludge, nitrate- and uranium-contaminated groundwater and Lake Kinneret. Cluster VIII, with sequences from the Baltic Sea and Schöhsee, was related at 68% identity to NirS from the Arabian Sea water column.

2.4 Diversity of *nirK* and *nirS* sequences

Rarefaction analysis comparing the diversity of both genes at 5% cutoff for OTU definition (Fig. VI-6) showed that the community with *nirS* was more diverse than the one with *nirK*. Saturation was not reached in both cases. The highest diversity of *nirK* was observed in the sample from Baltic Sea sediment with 14 OTUs

followed by Baltic Sea 2 m (12 OTUs). The lowest *nirK* diversity was detected in Baltic Sea 20 m with 5 OTUs. Diversity in both lakes was at an intermediate level.
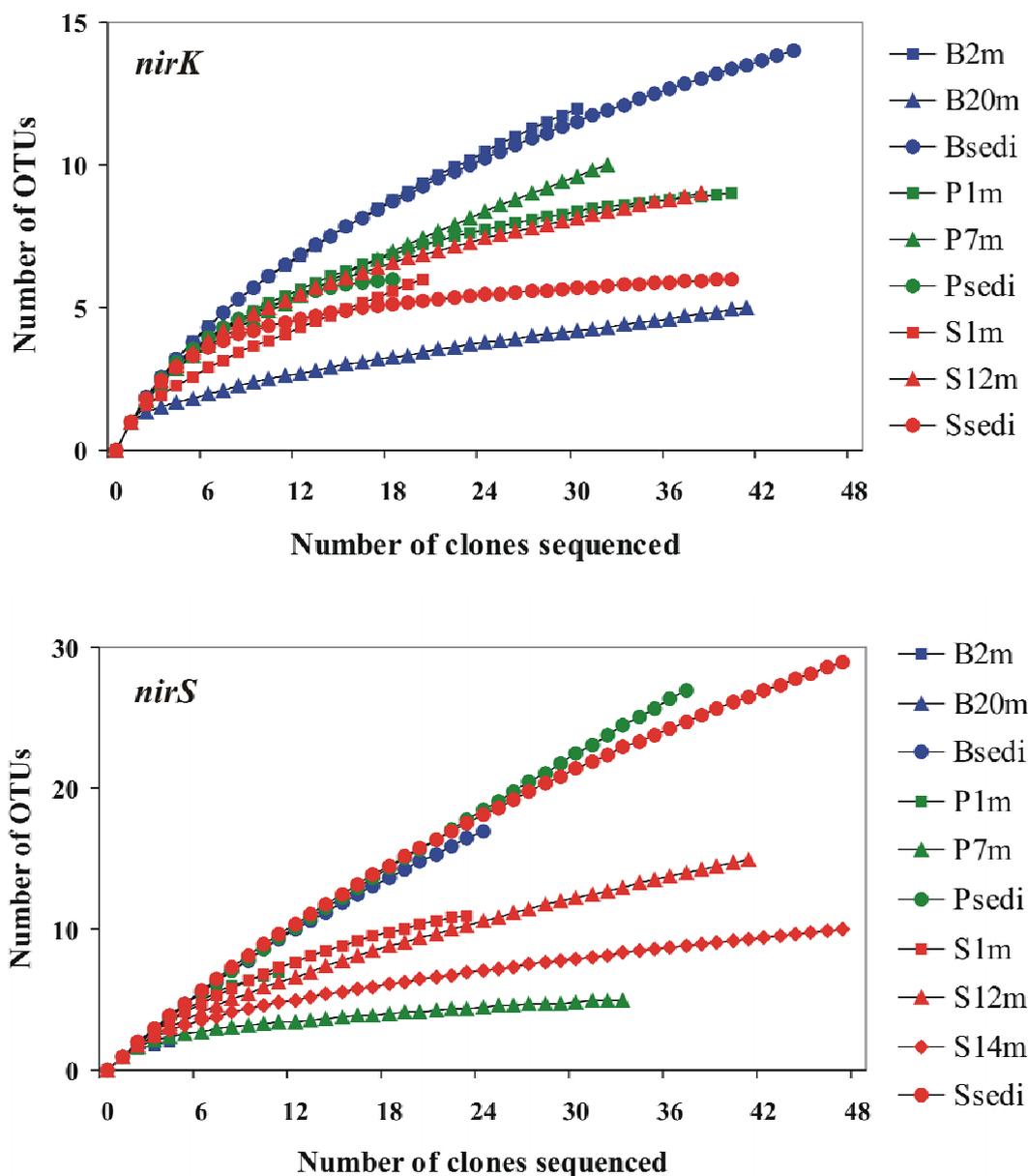
Figure V-6: Rarefaction curves indicating *nirK* and *nirS* diversity in clone libraries derived from each environment. OTUs were defined as groups of sequences differing by ≤5% at the DNA level. Coding of the samples with three different colors: blue=Baltic Sea; green=Plußsee; red=Schöhsee.

In the case of *nirS*, higher diversity was observed in samples from sediment than from the water column of all three environments. Among the sediment samples,

most of the OTUs were detected in Schöhsee (29), followed by Plußsee (27) and Baltic Sea (17).

In the Baltic Sea, the highest diversity for both genes was observed in the sediment. In the sample from Plußsee at 7 m depth *nirK* was the most diverse but *nirS* the least. In the sediment samples from both lakes, the highest diversity was observed for *nirS* and the lowest for *nirK*.

DISCUSSION

Up to date most studies in environments have paid attention to either soils or marine environments (Wallenstein et al., 2006), with less information provided from lakes (Junier et al., submitted). In this study, we combined T-RFLP and clone libraries to characterize the diversity and distribution of denitrifiers through the water column and sediment-water interface in two lakes and the Baltic Sea.

Comparison of *nirK* and *nirS* genes on the basis of T-RFLP

Recent studies have combined the amplification of *nirK* and *nirS* genes with the fingerprint techniques DGGE (Throbäck et al., 2004) and T-RFLP (Braker et al., 2001) to characterize the diversity and composition of denitrifiers. Applying T-RFLP analysis, the denitrifying bacterial community has been characterized in several environments, like soil (Avrahami et al., 2002; Priemé et al., 2002; Wolsing and Priemé, 2004) and marine habitats (Braker et al., 2001; Castro-González et al., 2005; Hannig et al., 2006). Generally low levels of *nirK* diversity (5 T-RFs) were found in soil (Avrahami et al., 2002; Wolsing and Priemé, 2004), and higher levels of *nirS* diversity (13 - 32 T-RFs) in marine habitats (Braker et al., 2001; Castro-González et al., 2005; Hannig et al., 2006).

In all three environments T-RFLP revealed higher diversity of *nirS* (11 - 17 T-RFs) than of *nirK* (10 to 12 T-RFs). This is in good agreement with the results from the clone libraries, based on the phylogeny, rarefaction analysis and the number of

OTUs detected. However, this finding is in contrast to the results obtained with water column samples from Lake Kinneret of *nirK* and *nirS* in which revealed that *nirK* was more diverse (20 T-RFs with *Hae*III) than *nirS* (13 T-RFs with *Alu*I) (Junier et al, submitted).

Distribution of denitrifiers within/between environments based on sequences

In general, *nirS*-denitrifiers are more frequent in estuarine and marine habitats (Braker et al., 2000; Liu et al., 2003; Jayakumar et al., 2004; Santoro et al., 2006), and *nirK*-denitrifiers in soil (Avrahami et al., 2002; Priemé et al., 2002; Wolsing and Priemé, 2004; Sharma et al., 2005). For freshwater there is only one study available that compared diversity of both genes in Lake Kinneret (Junier et al., submitted) and found that *nirS*-denitrifiers are most diverse in the epilimnion (1 m) and *nirK*-denitrifiers are most diverse in the hypolimnion (at 22 m). The differential distribution of both genes in the three environments suggests that diversity of these genes could result from a colonization of particular environments. The results from Baltic Sea in this study were exceptional in the general finding that *nirK*-denitrifiers are more diverse through the water body. *nirS*-denitrifiers are almost absent in the water column and detected in the sediment, only. This was similar in the Black Sea suboxic zone where *nirK*-denitrifiers showed much higher variation through the suboxic zone than with *nirS*-denitrifiers (Oakley et al., 2007).

Both denitrifiers are recovered in the clone libraries from all samples of both lakes as in Lake Kinneret (Junier et al. submitted). Similar distribution in both lakes showed that *nirS*-denitrifiers dominate in sediment. However, both lakes are distinguished by the distribution of *nirK*-denitrifiers. Plußsee showed inverse distribution between both denitrifiers that *nirK*-denitrifiers were dominant in the water column (at 7 m) and *nirS*-denitrifiers in sediment-water interface. Inverse distribution between both denitrifiers were presented from nitrate-contaminated groundwater (Yan et al., 2003) and Lake Kinneret (Junier et al., 2007). In contrast, both denitrifiers are more diverse in sediment than in water column of Schöhsee. Several studies have described factors influencing the distribution of denitrifying bacterial communities such as oxygen, nitrate (Liu et al., 2003), DOC, inorganic nitrogen and salinity gradients (Taroncher-Oldenburg et al., 2003), nitrite, oxygen, H_2S (Jayakumar et al.,

2004). We could conclude that dissolved oxygen concentration controls the distribution of both denitrifier groups in the two lakes. Many studies have been reported about the O_2 concentration, which is an important factor to influence the composition of denitrifiers (Liu et al., 2003; Jayakumar et al., 2004; Castro-González et al., 2005; Hannig et al., 2006). The Plußsee is an eutrophic freshwater lake, has a stable thermal stratification during summer with anoxia in the hypolimnion (Overbeck and Chróst, 1994). The Schöhsee is a mesotrophic freshwater lake with oxic conditions down to the sediment-water interface and low algal biomass (Rai et al., 1997; Aberle and Wiltshire, 2006). Therefore, oxygen in Schöhsee could be delivered to the sediment-water interface but in Plußsee oxygen was depleted below 7 m. It may be hypothesized that *nirK*-denitrifiers are more sensitively influenced by oxic condition, whereas *nirS*-denitrifiers are able to tolerate a broad range of oxygen conditions but more favorable to anoxic condition.

Habitat specific clusters based on phylogeny

nirK genes might be more specialized for particular habitats. The vast majority of *nirK* sequences was grouped into two distinct clusters VI and VII that were dominantly detected in this study indicating that these cluster are specific for freshwater to brackish habitats. Furthermore they are closely related to the sequences from Lake Kinneret (Junier et al., 2007 submitted) and a coastal aquifer at Huntington Beach (Santoro et al., 2006).

Unique *nirS* communities were found in the Baltic Sea with little overlap with the communities in the two lakes. They were more similar with *nirS* from brackish and marine habitats, such as from Pacific Northwest sediment (Braker et al., 2000), Baltic Sea cyanobacterial bloom (Tuomainen et al., 2003), northwestern Mexico sediment (Liu et al., 2003), Arabian Sea water column (Jayakumar et al., 2004), Huntington State Beach in California (Santoro et al., 2006), Baltic Sea water column (Hannig et al., 2006). However, *nirS* sequences, especially those from sediment, shared many clusters between both lakes and were dispersed throughout the complete phylogenetic system of this gene. These widely distributed *nirS*-denitrifier from lakes are related to those from a broad range of environments, like River Colne estuary sediment (Nogales et al., 2002), Baltic Sea cyanobacterial bloom (Tuomainen et al.,

2003), nitrate- and uranium-contaminated groundwater (Yan et al., 2003), arable soil, activated sludge and peat soil (Throbäck et al., 2004), metallurgic wastewater treatment systems (Yoshie et al., 2004), OMZ in the eastern South Pacific (Castro-González et al., 2005), Baltic Sea water column (Hannig et al., 2006), Huntington State Beach in California (Santoro et al., 2006), Lake Kinneret water column (Junier et al., 2007, submitted). It has been reported the worldwide distribution of *nirS*-denitrifiers in a wide range of habitats (Santoro et al., 2006) (Junier et al. submitted).

Comparison of the clone libraries diversity

As shown by the rarefaction curves, *nirS* was more diverse than *nirK* in sediment of all three environments. These findings are in agreement with those recently reported from the coastal aquifer at Huntington Beach, California where 13 - 19 OTUs were detected for *nirK* and 22 - 32 OTUs for *niS*. However, in the water column of the Baltic Sea and Plußsee *nirK* was more diverse (5 – 12 OTUs) than *nirS* (2 – 7 OTUs), similar to the OMZ of the Black Sea that *nirK* ranged 1 – 9 OTUs and *nirS* ranged 2 - 5 OTUs (Oakley et al., 2007).

Comparing *nirS* diversity in several marine OMZs (Oakley et al., 2007), the diversity of *nirS* sequences in both lake sediments is much higher than in the OMZ area; 23 OTUs in the Baltic Sea previously studied (Hannig et al., 2006), 20 OTUs and 22 OTUs in Arabian Sea (Jayakumar et al., 2004), 8 OTUs in Black Sea (Oakley et al., 2007) and 5 OTUs in Chilean OMZ (Castro-González et al., 2005).

The higher diversity does not necessarily mean that all these genes will be expressed and contribute to denitrification equivalently. Expression studies with five denitrifying genes in estuarine sediment showed that only *nirS* and *nosZ* were expressed (Nogales et al., 2002). This indicates that, despite the potential ability for denitrification is everywhere, expression of these genes is influenced by particular conditions. Nevertheless, the present study offers the first and necessary insight into the composition of bacterial communities with the potential for denitrification, and their interaction with the typical biotic and abiotic factors in stratified aquatic ecosystems, represented by two different lakes and the Baltic Sea, where investigation of denitrifying bacterial communities has been neglected so far.

4. DISCUSSION

Microbes are important components in the ecosystem, forming the basis of the food web and playing key roles in controlling the cycling of chemicals. One of the significant revolutions in microbial ecology is the application of rapid methods for sequencing the genes of microorganisms without cultivation from natural environments. At first, based on sequences from 16S rDNA, it is possible to identify and characterize microorganisms and to discover the previously unexpected huge diversity of the microbial communities. To focus on functional genes which encode enzymes catalyzing biogeochemical transformations, makes it possible to link microbial diversity to ecosystem function. This application has opened insights into the regulation of microbial activity and the microbial role in biogeochemical processes previously unknown.

In this present study, microbial diversity of functional genes related to the nitrogen cycle, in particular *amoA*, *pmoA*, *nirK*, and *nirS* has been investigated by molecular approaches in lakes Plußsee and Schöhsee and the Baltic Sea. Up to date, the information on functional genes related to the nitrogen cycle in freshwater lakes is scarce. Therefore, this work makes possible to compare freshwater communities with the diversity in soil and marine environments where the study of functional genes has been focused so far.

4.1 Diversity of ammonia-oxidizing bacteria (AOB)

4.1.1 Strategy to study AOB in environments

One of the problems faced when studying ammonia-oxidizing bacteria (AOB) is the low abundance in natural environments (Ward et al., 1997; Hastings et al., 1998; O'Mullan and Ward, 2005; Molina et al., 2007). Before the application of molecular methods in microbial ecology, ammonia oxidation was a difficult process to study, basically because of the slow growth in culture. Therefore cultivation, isolation and in vitro experiments with AOB have been a time-consuming process.

Based on sequence analysis, phylogeny of 16S rDNA in AOB are consistent with that derived from the functional gene, *amoA* (Aakra et al., 2001a; Purkhold et al., 2003).

Specific primers to amplify 16S rDNA and *amo* in AOB were compared (appendix A-1 and chapter I). For the detection of 16S rDNA (appendix A-1), the clones obtained with primers NitA/NitB recovered the highest frequency of AOB sequences, compared with primers βAMOf/βAMOr, βAMOf/Nso1255g and NitA/Nso1225g. For example, in the clone library of Plußsee sediment many sequences related to AOB were detected using the primer combination NitA/NitB, but not a single AOB sequence was detected using the primers βAMOf/βAMOr. The experimental examination of AOB-specific 16S rDNA primers showed that neither specificity nor sensitivity of the existing primer combinations can be evaluated reliably only by sequence analysis.

The re-examination of specific primers to amplify the *amoCAB* operon was carried out (chapter I) by sequence analyses. This underlines the possible strengths and weaknesses of primers to study community composition of AOB in environmental samples.

Hastings and coworker (Hastings et al., 1998) emphasized the necessity of nested amplification of *amo* gene. In this study, to overcome the problem of sensitivity for detection of *amo* due to low abundance in the environments studied, nested amplification using several primer pairs were compared (chapter II and IV). Nested amplification produced strong bands in agarose gels, although the initial amplification did not produce any visible band, indicating the advantages of nested amplification. Furthermore, this approach makes possible to detect the different regions of AMO genes (chapter II) and opens new possibilities to analyze *amoB* and *amoC* besides *amoA* or as coupled units of *amoCAB*.

4.1.2 Community comparison between 16S rDNA and *amoA*

When comparing 16S rDNA and the functional gene *amo* to study AOB community, in general *amoA* allows for more specific detection and better resolution of the diversity (Rotthauwe et al., 1997; Purkhold et al., 2000) because the rate of mutation and divergence of functional genes generally exceeds that of ribosomal genes (Ward, 2005). Therefore, the diversity of a functional group can be described

with greater resolution on the basis of functional genes than using 16S rDNA of the same group. Likewise, this study confirmed that *amoA* was better suited as a molecular marker for studying AOB communities than 16S rDNA (comparison of chapter III and IV).

The community composition of AOB by 16S rDNA varied between freshwater lakes and the Baltic Sea (chapter III). The Baltic Sea has a more diverse community and higher proportion of AOB sequences than the two freshwater lakes. Habitat-specific groups of marine or freshwater sequences were detected, suggesting the adaptation of AOB to particular environments. The comparison between the different layers of the two lakes and the Baltic Sea confirms earlier findings that *Nitrosomonas* spp. are more abundant in the sediment (Speksnijder et al., 1998; McCaig et al., 1999; Bano and Hollibaugh, 2000), whereas *Nitrosospira* spp. are preferentially found in the water column below the metalimnion (Hollibaugh et al., 2002; Freitag and Prosser, 2004; O'Mullan and Ward, 2005).

Dominant sequences of *amoA* genes in this study were distantly related to cultured organisms (chapter IV). Those from the Baltic Sea sediment belonged to clusters (*Nitrosospira*-like cluster B and *Nitrosomonas*-like cluster) which contained the sequences distributed worldwide in marine environments (Francis et al., 2003; Bernhard et al., 2005; O'Mullan and Ward, 2005). However, *amoA* genes from both lakes formed a lake-specific cluster (*Nitrosospira*-like cluster A) and were different from marine lineages (*Nitrosospira*-like cluster B).

4.1.3 Comparison of *amoA* and *pmoA* diversity

The genes encoding AMO are evolutionarily related to those encoding pMMO based on the similarity of nucleotide and amino acid sequences and protein structure (Holmes et al., 1995), although their function is different. In the three aquatic environments, γ-*pmoA* genes are most dominant (Chapter V). Similar findings were reported from several aquatic environments, such as freshwater lakes (Nold et al., 2000; Bussmann et al., 2004; Pester et al., 2004) and soda lakes (Lin et al., 2004; Lin et al., 2005). Plußsee and Schöhsee shared many phylogenetic clusters of γ-*pmoA* genes that were closely associated with sequences from Lake Constance (Bussmann et al., 2004; Pester et al., 2004) and Lake Washington (Nercessian et al., 2005; Kalyuzhnaya et al., 2006) in spite of distant geographic locations. While no overlap

between the two lakes and the Baltic Sea were found, *pmoA* genes from the Baltic Sea were clustered together with those from another brackish environment (McDonald et al., 2005). Many β-*amoA* from the Baltic Sea water column and Schöhsee were detected which were similar to those from the *Nitrosospira* cluster. Unusual *pmoA* genes from sediment of both lakes were detected and distinctly related to *Crenothrix polyspora* (Stoecker et al., 2006).

4.2 Diversity of denitrifying bacteria

4.2.1 Strategy to study denitrifying bacteria in environments

The difficulties to study denitrifying bacteria are quite different from those for ammonia-oxidizing bacteria. Because of the diverse affiliation of denitrifying bacteria in taxonomy, it is necessary to focus on a gene directly involved in denitrification to investigate their diversity in the environment, and to link their distribution and activity with the process itself.

4.2.2 Comparison of the diversity of *nirK* and *nirS*

Using PCR primers to detect *nirS* genes from different marine (Braker et al., 2000; Liu et al., 2003; Jayakumar et al., 2004; Castro-González et al., 2005; Hannig et al., 2006), terrestrial environments (Priemé et al., 2002; Throbäck et al., 2004) and lakes (Junier et al., submitted and chapter VI), it has been shown that *nirS* is widely distributed and that a remarkably high variety of *nirS* genes can be observed. In this study, all sequences of *nirS* are only distantly related to those from cultivated denitrifiers.

The PCR approach has been less successful for *nirK*, probably because this gene occurs more widely among different groups of Bacteria and Archaea, and is more diverse itself (Braker et al., 2000). Despite several sets of PCR primers for *nirK* have been reported and applied to both marine and terrestrial environments, fewer sequences have been collected in the database. This reflects the possibility of either the limited occurrence of the Cu variant of the enzyme or a limitation of the sequence database for primer development (Ward, 2005).

In overall, *nirS* genes were more diverse than *nirK* genes in the investigated environments, such as marine sediment (Braker et al., 2000), soil (Priemé et al., 2002), groundwater (Yan et al., 2003), activated sludge (Yoshie et al., 2004), Lake Kinneret, Israel (Appendix A), Huntington State Beach, California (Santoro et al., 2006) and both lakes and the Baltic Sea sediment of this study (chapter V). In the phylogeny of *nirS* genes several clusters contained sequences from different aquatic environments while freshwater and marine clusters were separated. However, in the phylogeny of *nirK* genes no separation of freshwater and marine environments was apparent. This could be because of the limited information on *nirK* genes from aquatic environments in the database or the *nirK* gene is not adequately amplified by any set of PCR primers. Only few studies were carried out about *nirK* genes in marine environments (Braker et al., 2000; Santoro et al., 2006; Oakley et al., 2007) and in lakes (Junier et al., submitted and chapter V) so far.

4.3 How many species are there?

The question of how to define a species and how many species exist in a particular environment are not yet clear (Hughes et al., 2001; Ward, 2002). Both *nirK* and *nirS* databases suggest that denitrifiers contain a large and highly diverse group of species. Despite substantial sequence diversity of *amoA* and *pmoA* genes has been observed, it appears that both genes are less diverse overall than *nirK* and *nirS*. This is obvious at the level of cultured strains and environmental clone libraries. Based on rarefaction analysis using 5% cutoff to define OTUs, logarithmic regression discovers that several hundreds of sequences are necessary to describe the complete diversity of *amoA* in Plußsee 7 m, where *amoA* genes were the most diverse (chapter III). In contrast, more than thousand sequences are necessary to describe the complete diversity of *nirS* from Schöhsee sediment, where *nirS* genes were most diverse. Nevertheless, clone libraries like those in the present study, are suitable to discover the most representative types of functional genes from the environments.

4.4 Selection pressure

For *nirK* and *nirS* the sequences of the protein were less conserved than those of the gene. This was the opposite way around for *amoA* and *pmoA* and might be an indication of a differential selection pressure. The optimal function of AMO, and to a certain degree also MMO, is mandatory for the survival of AOB or MOB, respectively. In contrast, bacteria with *nirK* or *nirS* are not obligate denitrifiers, and do not necessarily need this function for survival. In the case of *nirK*/*nirS* sequences, the exchange of one of the first two bases of a codon was frequently observed, while in *amoA*/*pmoA* sequences, mutations occurred in the neutral third position of the codons. Similar high rates of amino acid exchange in NirK and NirS, indicating low selection pressure have been shown (Braker et al., 2000) in denitrifying communities of marine sediments.

In this present study, the deduced amino acid sequences of all four enzymes were more conserved in both lakes than in Baltic Sea. In each of these environments, amino acid sequences were more conserved in sediment than in the water column. This indicates higher selection pressure in the lakes compared to Baltic Sea, and in sediment compared to water column. This interesting phenomenon has not gained adequate attention so far.

4.5 Gene expression

Huge amounts of information about the diversity and distribution of functional genes *amoA*, *pmoA*, *nirK* and *nirS* have been collected in molecular studies on various environments, apart from freshwater. These studies do not reveal if those genes have been active at the time sampled from the environments, but merely give an insight into the genetic capacity of the community. Gene expression is under the control of regulatory mechanisms in the cell that respond to environmental conditions. To understand the expression under the biotic and abiotic conditions in the environment would be helpful for understanding the factors that regulate these processes and decide which of the microorganisms are active at a certain time. Amplification of

DNA and mRNA by PCR and RT-PCR has been applied to detect differential expression of *amoA* (Ebie et al., 2004), *pmoA* (Nercessian et al., 2005), *nirK* (Sharma et al., 2005) and *nirS* (Nogales et al., 2002).

The data obtained in this study represent a genetic characterization of specific microorganisms present under different environmental conditions, and thus constitute a framework for more detailed studies of the function and activity of these bacterial communities involved in the nitrogen cycle of hitherto neglected aquatic ecosystems.

4. DISCUSSION

REFERENCES

Aakra Å, Utåker JB, Nes IF (2001a) Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammonia-oxidizing bacteria. FEMS Microbiol. Lett. 205:237–242

Aakra Å, Utåker JB, Pommerening-Roser A, Koops HP, Nes IF (2001b) Detailed phylogeny of ammonia-oxidizing bacteria determined by rDNA sequences and DNA homology values. Int. J. Syst. Evol. Microbiol. 51:2021-2030

Aberle N, Wiltshire KH (2006) Seasonality and diversity patterns of microphytobenthos in a mesotrophic lake. Arch. Hydrobiol. 167:447-465

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang ZWM, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402

Alzerreca JJ, Norton JM, Klotz MG (1999) The *amo* operon in marine, ammonia-oxidizing γ-proteobacteria. FEMS Microbiol. Lett. 180:21-29

Arciero DM, Hooper AB (1998) Consideration of a phlorin structure for haem P-460 of hydroxylamine oxidoreductase and its implications regarding reaction mechanism. Biochem. Soc. Trans. 26

Avrahami S, Conrad R (2003) Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures. Appl. Environ. Microbiol. 69:6152-6164

Avrahami S, Conrad R, Braker G (2002) Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. Appl. Environ. Microbiol. 68:5685-5692

Bano N, Hollibaugh JT (2000) Diversity and distribution of DNA sequences with affinity to ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in the arctic ocean. Appl. Environ. Microbiol. 66:1960-1969

Bédard C, Knowles R (1989) Physiology, Biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. Microbio. Rev. 53:68-84

Beman JM, Francis CA (2006) Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified subtropical estuary: Bahía del Tóbari, Mexico. Appl. Environ. Microbiol. 72:7767-7777

REFERENCES

Bergmann DJ, Hopper AB (1994) Sequence of the Gene, *amoB*, for the 43-kDa polypeptide of ammonia monooxygenase of *Nitrosomonas europaea*. Biochem. Biophys. Res. Communi. 204:759-762

Bernhard AE, Donn T, Giblin AE, Stahl DA (2005) Loss of diversity of ammonia-oxidizing bacteria correlates with increasing salinity in an estuary system. Environ. Microbiol. 7:1289-1297

Bodelier PLE, Frenzel P (1999) Contribution of methanotrophic and nitrifying bacteria to CH_4 and NH_4^+ oxidation in the rhizosphere of rice plants as determined by new methods of discrimination. Appl. Environ. Microbiol. 65:1826-1833

Bodelier PLE, Roslev P, Henckel T, Frenzel P (2000) Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. Nature 403:421-424

Bothe H, Jost G, Schloter M, Ward BB, Witzel K-P (2000) Molecular analysis of ammonia oxidation and denitrification in natural environments. FEMS Microbiol. Rev. 24:673-690

Bourne DG, McDonald IR, Murrell JC (2001) Comparison of *pmoA* PCR primer sets as tools for investigating methanotroph diversity in three danish soils. Appl. Environ. Microbiol. 67:3802-3809

Braker G, Ayala-DEL-Río EL, Devol AH, Fesefeldt A, Tiedje JM (2001) Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. Appl. Environ. Microbiol. 67:1893-1901

Braker G, Fesefeldt A, Witzel K-P (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 64:3769-3775

Braker G, Zhou J, Wu L, Devol AH, Tiedje JM (2000) Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment community. Appl. Environ. Microbiol. 66:2096-2104

Brettar I, Moore ERB, Höfle MG (2001) Phylogeny and abundance of novel denitrifying bacteria isolated from the water column of the central Baltic Sea. Microb. Ecol. 42:295-305

Brosius J, Palmer ML, Kennedy PJ, Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 75:4801-4805

Bruns MA, Stephen JR, Kowalchuk GA, Prosser JI, Paul EA (1999) Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl. Environ. Microbiol. 65:2994-3000

Bussmann I, Pester M, Brune A, Schink B (2004) Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance). FEMS Microbiol. Ecol. 47:179-189

Bussmann I, Rahalker M, Schink B (2006) Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. FEMS Microbiol. Ecol. 56:331-344

Calvó L, Garcia-Gil LJ (2004) Use of *amoB* as a new molecular marker for ammonia-oxidizing bacteria. J. Microbiol. Methods 57:69-78

Carini SA, Bano N, LeCleir G, Joye SB (2005) Aerobic methane oxidation and methanotroph community composition during seasonal stratificatioin in Mono Lake, California (USA). Environ. Microbiol 7:1127-1138

Castro-González M, Braker G, Farías L, Ulloa O (2005) Communities of *nirS*-type denitrifiers in the water column of the oxygen minimum zone in the eastern South Pacific. Environ. Microbiol 7:1298-1306

Cavari BZ (1977) Nitrification potential and factors governing the rate of nitrification in Lake Kinneret. Oikos 28:285-290

Cébron A, Berthe T, Garnier J (2003) Nitrification and nitrifying bacteria in the lower Seine River and estuary (France). Appl. Environ. Microbiol. 69:7091-7100

Chain P, Lamerdin J, Larimer F, Regala W, Lao V, Land M, Hauser L, Hooper A, Klotz MG, Norton JM, Sayavedra-Soto L, Arciero D, Hommes N, Whittaker M, Arp D (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. J. Bacteriol. 185:2759-2773

Chenier MR, Beaumier D, Roy R, Driscoll BT, Lawrence JR, Greer CW (2003) Impact of seasonal variations and nutrient inputs on nitrogen cycleing and degradation of hexadecane by replicated river biofilms. Appl. Environ. Microbiol. 69:5170-5177

REFERENCES

Christofi N, Preston T, Stewart WDP (1981) Endogenous nitrate production in an experimental enclosure during summer stratification. Wat. Res. 15:343-349

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM, Tiedje JM (2005) The ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 1:D294-D296

Costello AM, Lidstrom ME (1999) Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl. Environ. Microbiol. 65:5066-5074

Coyne MS, Arunakumari A, Averill BA, Tiedje JM (1989) Immunological identification and distribution of dissmiliratory heme *cd1* and non-heme copper nitrite reductases in denitrifying bacteria. Appl. Environ. Microbiol. 55:2924-2931

de Bie MJM, Speksnijder AGCL, Kowalchuk GA, Schuurman T, Zwart G, Stephen JR, Diekmann OE (2001) Shift in the dominant populations of ammonia-oxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat. Microb. Ecol. 23:225-236

Ebie Y, Noda N, Miura H, Matsumura M, Tsuneda S, Hirata A, Inamori Y (2004) Comparative analysis of genetic diversity and expression of *amoA* in wastewater treatment processes. Appl. Microbiol. Biotechnol. 64:740–744

Eller G, Känel L, Krüger M (2005) Cooccurrence of aerobic and anaerobic methane oxidation in the water column of Lake Plußsee. Appl. Environ. Microbiol. 71:8925-8928

Ensign SA, Hyman MR, Arp DJ (1993) In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. J. Bacteriol. 175:1971-1980

Erwin DP, Erickson IK, Delwiche ME, Colwell FS, Strap JL, Crawford RL (2005) Diversity of oxygenase genes from methane- and ammonia-oxidizing bacteria in the Eastern Snake River Plain Aquifer. Appl. Environ. Microbiol. 71:2016-2025

Fjellbirkeland A, Torsvik V, Øvreås L (2001) Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmoA*, *mxaF* and 16S rDNA sequences. Antonie van Leeuwenhoek 79:209–217

Focht DD, Verstraete W (1977) Biochemical ecology of nitrification and denitrification. In: Alexander M (ed) Advances in Microbial ecology, Vol 1. Plenum Press, New York

Francis CA, O'Mullan GD, Ward BB (2003) Diversity of ammonia monooxygenase (*amoA*) genes across environmental gradients in Chesapeake Bay sediments. Geobiology 1:129-140

Freitag TE, Prosser JI (2003) Community structure of ammonia-oxidizing bacteria within anoxic marine sediments. Appl. Environ. Microbiol. 69:1359-1371

Freitag TE, Prosser JI (2004) Differences between betaproteobacterial ammonia-oxidizing communities in marine sediments and those in overlying water. Appl. Environ. Microbiol. 70:3789-3793

Gamble TN, Betlach MR, Tiedje JM (1977) Numerically dominant denitrifying bacteria from world soils. Appl. Environ. Microbiol. 33:926-939

Garland JHN (1978) Nitrification in the River Trent. In: James A (ed) Mathematical models in water pollution control. John Wiley and Sons Ltd., Chichester

Glockner AB, Jünst A, G. ZW (1993) Copper containing nitrite reductases from Pseudomonas aureofaciens is functional in a mutationally cytochrome cd1-free background (NirS⁻) of Pseudomonas stutzeri. Arch. Microbiol. 160:2136-2141

Goregues CM, Michtey V, Bonin P (2005) Molecular, biochemical, and physiological approaches for understanding the ecology of denitrification. Microb. Ecol. 49:198-208

Hallin S, Lindgren PE (1999) PCR detection of genes encoding nitrite reductase in denitrifying bacteria. Appl. Environ. Microbiol. 65

Hannig M, Braker G, Dppner J, Jürgens K (2006) Linking denitrifier community sturucture and prevalent biogeochemical parameters in the pelagial of the central Baltic Sea Proper (Baltic Sea). FEMS Microbiol. Ecol. 57:260-271

Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol. Rev. 60:439-471

Hastings RC, Saunders JR, Hall GH, Pickup RW, McCarthy AJ (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl. Environ. Microbiol. 64:3674-3682

Head IM, Hiorns WD, Embley TM, McCarthy AJ, Saunders JR (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. 139:1147-1153

REFERENCES

Henckel T, Friedrich M, Conrad R (1999) Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. Appl. Environ. Microbiol. 65:1980-1990

Henckel T, Jäckel U, Schnell S, Conrad R (2000a) Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane. Appl. Environ. Microbiol. 66:1801-1808

Henckel T, Roslev P, Conrad R (2000b) Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. Environ. Microbiol 2:666-679

Hoffman T, Horz H-P, Kemnitz D, Conrad R (2002) Diversity of the particulate methane monooxygenase gene in methanotrophic samples from different rice field soils in China and the Philippines. Syst. Appl. Microbiol. 25:267-274

Hollibaugh JT, Bano N, Ducklow HW (2002) Widespread distribution in polar oceans of a 16S rRNA gene sequence with affinity to *Nitrosospira*-like ammonia-oxidizing bacteria. Appl. Environ. Microbiol. 68:1478-1484

Holmes AJ, Costello A, Lidstrom ME, Murrell JC (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionary related. FEMS Microbiol. Lett. 132:203-208

Hommes NG, Sayavedra-Soto L, Arp DJ (1998) Mutagenesis and expression of *amo*, which codes for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol 180:3353-3359

Hooper AB, Vannelli T, Bergmann DJ, Arciero D (1997) Enzymology of the oxidation of ammonia to nitrite by bacteria. Antonie van Leeuwenhoek 71:59–67

Horz H-P, Rotthauwe J-H, Lukow T, Liesack W (2000) Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. J. Microbiol. Methods 39:197-204

Horz H-P, Yimga M, Liesack W (2001) Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. Appl. Environ. Microbiol. 67:4177-4185

Hoshino T, Noda N, Tsuneda S, Hirata A, Inamori Y (2001) Direct detection by in situ PCR of the *amoA* gene in biofilm resulting from a nitrogen removal process. Appl. Environ. Microbiol. 67:5261-5266

Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJ (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. 67:4399-4406

Hutchens E, Radajewski S, Dumont MG, McDonald IR, Murrell JC (2004) Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ. Microbiol 6:111-120

Hyman MR, Arp DJ (1992) $^{14}C_2H_2$- and $^{14}CO_2$-labeling studies of the de novo synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. J. Biol. Chem. 267:1534-1545

Ivanova IA, Stephen JR, Chang Y-J, Brüggemann J, Long PE, McKinley JP, Kowalchuk GA, White DC, Macnaughton SJ (2000) A survey of 16S rRNA and *amoA* genes related to autotrophic ammonia-oxidizing bacteria of the β-subdivision of the class proteobacteria in contaminated groundwater. Can. J. Microbiol. 46:1012-1020

Jayakumar DA, Francis CA, Naqvi SWA, Ward BB (2004) Diversity ofnitrite reductase genes (*nirS*) in the denitrifying water column of the coastal Arabian Sea. Aquat. Microb. Ecol. 34:69-78

Jetten MSM, Logemann S, Muyzer G, Robertson LA, Devries S, Vanloosdrecht MCM, Kuenen JG (1997) Novel principles in the microbial conversion of nitrogen compounds. Antonie van Leeuwenhoek 71:75-93

Jiang QQ, Bakken LR (1999) Comparison of *Nitrosospira* strains isolated from terrestrial environments. FEMS Microbiol. Ecol. 30:171-186

Johnstone BH, Jones RD (1988) Effects of light and CO_2 on the survival of a marine ammonia-oxidizing bacterium during energy source deprivation. Appl. Environ. Microbiol. 54:2890-2893

Juretschko S, G. T, Schmid M, Schleifer KH, Pommerening-Röser A, Koops HP, Wagner M (1998) Combined molecular and conventional analysis of nitirfying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrosospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64:3042-3051

REFERENCES

Kalyuzhnaya MG, Zabinsky R, Bowerman S, Baker DR, Lidstrom ME, Chistoserdova L (2006) Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. Appl. Environ. Microbiol. 72:4293-4301

Kim O-S, Junier P, Imhoff JF, Witzel K-P (2006) Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea. Arch. Hydrobiol. 167:335-350

King GM, Schnell S (1994) Ammonium and nitrite inhibition of methane oxidation by *Methylobacter albus* BG8 and *Methylosinus trichosporium* OB3b at low methane concentrations. Appl. Environ. Microbiol. 60:3508-3513

Klotz MG, Alzerreca J, Norton JM (1997) A gene encoding a membrane protein exists upstream of the *amoA/amoB* genes in ammonia oxidizing bacteira: a third member of the *amo* operon? FEMS Microbiol. Lett. 150:65-73

Knief C, Lipski A, Dunfield PF (2003) Diversity and activity of methanotrophic bacteria in different upland soils. Appl. Environ. Microbiol. 69:6703-6714

Knief C, Vanitchung S, Harvey NWH, Conrad R, Dunfield PF, Chidthaisong A (2005) Diversity of methanotrophic bacteria in tropical upland soils under different land uses. Appl. Environ. Microbiol. 71:3826-3831

Knowles R (1982) Denitrification. Microbio. Rev. 46:43-70

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437:543-546

Koops H-P, Harms H (1985) Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. Arch. Microbiol. 141:214-218

Koops H-P, Pommerening-Röser A (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. 37:1-9

Koops H-P, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M (2003) The Lithoautotrophic Ammonia-Oxidizing Bacteria. In: Dworkin M (ed) The Prokaryotes. Springer-Verlag, New York

Koops HP, Möller UC (1992) The lithotrophic ammonia-oxidizing bacteria. In: Balows A, Tüper HG, Dworkin M, Harder W, Schleifer KH (eds) The Prokaryotes. Springer Verlag, New York

Kowalchuk GA, Bodelier PLE, Heilig GHJ, Stephen JR, Laanbroek HJ (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 27:339-350

Kowalchuk GA, Stephen JR (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol. 55:485-529

Kowalchuk GA, Stephen JR, De Boer W, Prosser JI, Embley TM, Boldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. 63:1489-1497

Kowalchuk GA, Stienstra AW, Heilig GHJ, Stephen JR, Woldendorp JW (2000) Changes in the community structure of ammonia-oxidising bacteria during secondary succession of calcareous grasslands. Environ. Microbiol 2:99-110

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806-809

Lin JL, Joye SB, Scholten CM, Schäffer H, McDonald IR, Murrell JC (2005) Analysis of methane monooxygenase genes in Mono Lake suggests that increased methane oxidation activity may correlate with a change in methanotroph community structure. Appl. Environ. Microbiol. 71:6458-6462

Lin JL, Radajewski S, Eshinimaev BT, Trotsenko YA, McDonald IR, Murrell JC (2004) Molecular diversity of methanotrophs in Transbaikal soda lake sediments and identification of potentially active populations by stable isotope probing. Environ. Microbiol. 6:1049-1060

Lipponen MT, Martikainen PJ, Vasara RE, Servomaa K, Zacheus O, Kontro MH (2004) Occurrence of nitrifiers and diversity of ammonia-oxidizing bacteria in developing drinking water biofilms. Water Res. 38:4424-4434

Liu W-T, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522

Liu X, Tiquia SM, Holguin G, Wu L, Nold SC, Devol AH, Luo K, Palumbo AV, Tiedje JM, Zhou J (2003) Molecular diversity of denitrifying genes in

continental margin sediments within the oxygen-deficient zone off the Pacific coast of Mexico. Appl. Environ. Microbiol. 69:3549-3560

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H (2004) ARB: a software environment for sequence data. Nucleic Acids Res. 32:1363-1371

Mahmood S, Freitag TE, Prosser JI (2006) Comparison of PCR primer-based strategies for characterization of ammonia oxidizer communities in environmental samples. FEMS Microbiol. Ecol. 56:482-493

McCaig AE, Embley TM, Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonium oxidisers. FEMS Microbiol. Lett. 120:363-368

McCaig AE, Glover LA, Prosser JI (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65:1721-1730

McDonald IR, Smith K, Lidstrom ME (2005) Methanotrophic populations in estuarine sediment from Newport Bay, California. FEMS Microbiol. Lett. 250:287-293

McTavish H, Fuchs JA, Hooper AB (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. 175:2436-2444

Megraw SR, Knowles R (1987) Active methanotrophs suppress nitrification in a humisol. Biol. Fertil. Soils 4:205-212

Michotey V, Mejean V, Bonin P (2000) Comparison of methods for quantification of cytochrome cd1-denitrifying bacteria in environmental marine samples. . Appl. Environ. Microbiol. 66:1564-1571

Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62:2156-2162

Molina V, Ulloa O, Farías L, Urrutia H, Ramírez S, Junier P, Witzel K-P (2007) Ammonia-oxidizing β-proteobacteria from the oxygen minimum zone off northern Chile. Appl. Environ. Microbiol. 73:3547-3555

Murrell JC, Gilbert B, McDonald IR (2000) Molecular biology and regulation of methane monooxygenase. Arch. Microbiol. 173:325-332

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59:695-700

Nercessian O, Noyes E, Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L (2005) Bacterial populations active in metabolism of C_1 compounds in the sediment of Lake Washington, a freshwater lake. Appl. Environ. Microbiol. 71:6885-6899

Nicolaisen MH, Ramsing NB (2002) Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J. Microbiol. Methods 50:189-203

Nogales B, Timmis KN, Nedwell DB, Osborn AM (2002) Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Appl. Environ. Microbiol. 68:5017-5025

Nold SC, Zhou J, Devol AH, Tiedje JM (2000) Pacific Northwest marine sediments contain ammonia-oxidizing bacteria in the β subdivision of the *Proteobacteria*. Appl. Environ. Microbiol. 66:4532-4535

Norton JM, Alzerreca JJ, Suwa Y, Klotz MG (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Arch. Microbiol. 177:139-149

O'Mullan GD, Ward BB (2005) Relationship of temporal and spatial variabilities of ammonia-oxidizing bacteria to nitrification rates in Monterey Bay, California. Appl. Environ. Microbiol. 71:697-705

Oakley BB, Francis CA, Roberts KJ, Fuchsman CA, Srinivasan S, Staley JT (2007) Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. Environ. Microbiol. 9:118-130

Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004) Application of real-time PCR to study effects of

ammonium on population size of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol. 70:1008-1016

Op den Camp HJM, Jetten MSM, Strous M (2007) Anammox. In: Bothe H, Ferguson SJ, Newton WE (eds) Biology of the nitrogen cycle. Elsevier, Amsterdam, the Netherland, p 245-262

Overbeck J, Chróst (1994) Microbial ecology of Lake Plußsee. Ecol. Studies 105:1-44

Pauer JJ, Auer M (2000) Nitrification in the water column and sediment of a hypereutrophic lake and adjoining river system. Wat. Res. 34:1247-1254

Pester M, Friedrich MW, Schink B, Brune A (2004) *pmoA*-based analysis of methanotrophs in a littoral lake sediment reveals a divers and stable community in a dynamic environment. Appl. Environ. Microbiol. 70:3138-3142

Philippot L (2002) Denitrifying genes in bacterial and archaeal genomes. Biochim. Biophys. Acta 1577:355-376

Phillips CJ, Smith Z, Embley TM, Prosser JI (1999) Phylogenetic differences between particle-associated and planktonic ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in the northwestern Mediterranean Sea. Appl. Environ. Microbiol. 65:779–786

Priemé A, Braker G, Tiedje JM (2002) Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. Appl. Environ. Microbiol. 68:1893-1900

Prosser JI (1989) Autotrophic nitrification in bacteria. Adv. Microbiol. Physiol. 30:125-181

Prosser JI (2007) The ecology of nitrifying bacteria. In: Bothe H, Ferguson SJ, Newton WE (eds) Biology of the nitrogen cycle. Elsevier, Amsterdam, The Netherlands, p 223-243

Prosser JI, Embley TM (2002) Cultivation-based and molecular approaches to characterization of terrestrial and aquatic nitrifiers. Antonie Van Leeuwenhoek 81:165-179

Purkhold U, Pommerening-Röser A, Juretschko S, Schmid MC, Koops HP, Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: Implications for molecular diversity surveys. Appl. Environ. Microbiol. 66:5368-5382

Purkhold U, Wagner M, Timmermann G, Pommerening-Röser A, Koops HP (2003) 16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. Int. J. Syst. Evol. Microbiol. 53:1485-1494

Qiu XY, Hurt RA, Wu LY, Chen CH, Tiedje JM, Zhou J (2004) Detection and quantification of copper-denitrifying bacteria by quantitative competitive PCR. J. Microbiol. Meth. 59:199-210

Rai H, Arts MT, Wainman BC, Dockal N, Krambeck HJ (1997) Lipid production in natural phytoplakton communities in a small freshwater Baltic lake, Lake Schöhsee, Germany. Freshwater Biology 38:581-590

Robarts RD, Ashton PJ, Thornton JA, Taussig HG, Sephton LM (1982) Overturn in a hypertrophic, warm, monomictic impoundment (Hartbeespoort Dam, South Africa). Hydrobiologica 97:209-224

Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol. 68:3818-3829

Rotthauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63:4704-4712

Roy R, Knowles R (1994) Effects of methane metabolism on nitrification and nitrous oxide production in polluted freshwater sediment. Appl. Environ. Microbiol. 60:3307-3314

Sanguinetti CJ, Neto ED, Simpson AJG (1994) Rapid silver staining and recovery of PCR products separated by polyacrylamide gels. Biotechniques 17:915-918

Santoro AE, Boehm AB, Francis CA (2006) Denitrifier community composition along a nitrate and salinity gradient in a coastal aquifer. Appl. Environ. Microbiol. 72:2102-2109

Satoh T, Hoshino Y, Kitamura H (1976) *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. Arch. Microbiol. 108:265-269

Sayavedra-Soto LA, Hommes NG, Alzerreca JJ, Arp DJ, Norton JM, Klotz MG (1998) Transcription of the *amoC*, *amoA* and *amoB* genes in *Nitrosomonas europaea* and *Nitrosospira* sp. NpAV. FEMS Microbiol. Lett. 167:81-88

REFERENCES

Schäfer H, McDonald IR, Nightingale PD, Murrell JC (2005) Evidence for the presence of a CmuA methyltransferase pathway in novel marine methyl halide-oxidizing bacteria. Environ. Microbiol 7:839-852

Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71:1501-1506

Seitzinger SP (1988) Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. Limnol. Oceanogr. 34:702-724

Semrau JD, Chistoserdova L, Lebron J, Costello A, Davagnino J, Kenna E, Holmes AJ, Finch R, Murrell JC, Lidstrom ME (1995) Particulate methane monooxygenase genes in methanotrophs. J. Bacteriol. 177:3071-3079

Sharma S, Aneja MK, Mayer J, Munch JC, Schloter M (2005) Diversity of transcripts of nitrite reductase genes (*nirK* and *nirS*) in rhizospheres of grain legumes. Appl. Environ. Microbiol. 71:2001-2007

Shoun H, Kim DH, Uchiyama H, Sugiyama J (1992) Denitrification by fungi. FEMS Microbiol. Lett. 94:277-281

Sinigalliano CD, Kuhn DN, Jones RD (1995) Amplification of the *amoA* gene from diverse species of ammonium-oxidizing bacteria and from an indigenous bacterial population from seawater. Appl. Environ. Microbiol. 61:2702-2706

Speksnijder AG, Kowalchuk GA, Roest K, Laanbroek HJ (1998) Recovery of a *Nitrosomonas*-like 16S rDNA sequence group from freshwater habitats. Syst. Appl. Microbiol. 21:321-330

Stephen JR, Chang YJ, Macnaughton SJ, Kowalchuk GA, Leung KT, Flemming CA, White DC (1999) Effect of toxic metals on indigenous soil beta-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Appl. Environ. Microbiol. 65:95-101

Stephen JR, Kowalchuk GA, Bruns MA, McCaig AE, Phillips CJ, Embley TM, Prosser JI (1998) Analysis of β-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. Appl. Environ. Microbiol. 64:2958-2965

Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. 62:4147-4154

Stoecker K, Bendinger B, Schöning B, Nielsen PH, Nealson JL, Baranyi C, Toenshoff ER, Daims H, Wagner M (2006) Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. Proc. Natl. Acad. Sci. USA 103:2363-2367

Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MSM (1999) Missing lithotroph identified as new planctomycete. Nature 400:446-449

Taroncher-Oldenburg G, Griner EM, Francis CA, Ward BB (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl. Environ. Microbiol. 69:1159-1171

Teske A, Alm E, Regan JM, Toze S, Rittmann BE, Stahl DA (1994) Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. 176:6623-6630

Throbäck IN, Enwall K, Jarvis Å, Hallin S (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49:401-417

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP, Schleper C (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environ. Microbiol. 7:1985-1995

Tuomainen JM, Hietanen S, Kuparinen J, Martikainen PJ, Servomaa K (2003) Baltic Sea cyanobacterial bloom contains denitrification and nitrification genes, but has negligible denitrification activity. FEMS Microbiol. Ecol. 45:83-96

Utaker JB, Nes IF (1998) A qualitative evaluation of the published oligonucleotides specific for the 16S rRNA gene sequences of the ammonia-oxidizing bacteria. Syst. Appl. Microbiol. 21:72-88

Venter JC, Remington K, Heidelberg JF, Halpernm AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkuch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66-74

REFERENCES

Voytek MA, Ward BB (1995) Detection of ammonium-oxidizing bacteria of the beta-subclass of the class Proteobacteria in aquatic samples with the PCR. Appl. Environ. Microbiol. 61:1444-1450

Wagner M, Amann R, Lemmer H, Schleifer KH (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. 59:1520-1525

Wagner M, Rath G, Amman R, Koops H-P, Schleifer KH (1995) In situ identification of ammonia-oxidizing bacteria. . Syst. Appl. Microbiol. 18:251-264

Wallenstein MD, Myrold DD, Fireston M, Voytek MA (2006) Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecol. Appl. 16:2143-2152

Ward BB (1986) Nitrification in marine environments. In: Prosser JI (ed) Nitrification. Oxford, Washington, D.C. , p 157–184

Ward BB (2000) Nitrification and the marine nitrogen cycle. In: Kirchman DL (ed) Microbial ecology of the oceans. John Wiley and Sons, New York, p 427-453

Ward BB (2002) How many species of prokaryotes are there? Proc. Natl. Acad. Sci. USA 99:10234-10236

Ward BB (2005) Molecular approaches to marine microbial ecology and the marine nitrogen cycle. Annu. Rev. Earth Planet. Sci. 33:301-333

Ward BB, Martino DP, Diaz MC, Joye SB (2000) Analysis of ammonia-oxidizing bacteria from hypersaline Mono Lake, California, on the basis of 16S rRNA sequences. Appl. Environ. Microbiol. 66:2873-2881

Ward BB, Voytek MA, Witzel K-P (1997) Phylogenetic diversity of natural populations of ammonia oxidizers investigated by specific PCR amplification. Microb. Ecol. 33:87-96

Webster G, Embley TM, Freitag TE, Smith Z, Prosser JI (2005) Links between ammonia-oxidizer species composition functional diversity and nitrification kinetics in grassland soils. Environ. Microbiol. 7:676-684

Webster G, Embley TM, Prosser JI (2002) Grassland management regimens reduce small-scale heterogeneity and species diversity of β-Proteobacterial ammonia oxidizer populations. Appl. Environ. Microbiol. 68:20-30

Wolsing M, Priemé A (2004) Observation of high seasonal variation in community sturucture of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. FEMS Microbiol. Ecol. 48:261-271

Yan T, Fields MW, Wu L, Zu Y, Tiedje JM, Zhou J (2003) Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. Environ. Microbiol 5:13-24

Yoshie S, Noda N, Tsuneda S, Hirata A, Inamori Y (2004) Salinity decreases nitrite reductase gene diversity in denitrifying bacteria of wastewater treatment systems. Appl. Environ. Microbiol. 70:3152-3157

Zumft WG (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61:533-616

REFERENCES

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Ahn, T.S., Hong, S.H., **Kim O.-S.,** You, J.J., Jeon, S.O., Choi, S.I. 2001. The changes of *Bacillus* spp. in municipal wastewater treatment plant with B3 process. Korean J. Microbiol. 37(3): 209-213.

Hong, S.H., **Kim, O.-S.,** Jeon, S.O., You, J.J., Ahn T.S. 2002. Bacterial community of free-living and aggregated bacteria at thawing period in Lake Baikal. Korean J. Microbiol. 38(3): 192-197.

Byeon, M.S., Yoo, J.J., **Kim, O.-S.,** Choi, S.I. and Ahn, T.S. 2002. Bacterial abundance and enzymatic activity under artificial floating island in Lake Paldang. Korean J. Limnol. 35(4): 266-272.

Song, H.G., **Kim, O.-S.**, You, J.J., Jeon, S.O., Hong, S.H., LEE, D.-H., Ahn, T.S. 2004. Monitoring of bacterial community in a coniferous forest soil after a wildfire. Korean J. Microbiol. 42(4) 285-291.

Ahn, T.S., **Kim, O.-S**., Joh, K.S., Spiglazov, L.P., Drucker, V.V., Hong, S.H. 2006. Community analysis of aggregated bacteria in southern Lake Baikal. Hydrobiol. 568:5-8.

CURRICULUM VITAE

Kim, O.-S., Junier, P., Imhoff, J.F., Witzel, K-P. 2006. Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea. Arch. Hydrobiol. 167:335-350

Submitted

Pilar Junier, **Ok-Sun Kim**, Karl-Paul Witzel, Johannes F. Imhoff and Ora Hadas. 2007. Habitat-partitioning of denitrifying bacterial communities carrying *nirS*/*nirK* genes in the stratified water column of Lake Kinneret, Israel. Aquat. Microb. Ecol. Submitted.

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren. Die Promotion soll im Fach Mikrobiologie erfolgen. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

_____

Ok-Sun Kim

APPENDIX A

List of related publications and individual scientific contributions to multiple

author publications

Part of this thesis has been published, submitted or prepared as manuscripts with multiple authors. This list serves as a clarification of personal contributions on each publication.

PUBLISHED

Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea. Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff, and Karl-Paul Witzel. 2006. Arch. Hydrobiol. 167:335-350.

Ok-Sun Kim and Karl-Paul Witzel took the samples. Ok-Sun Kim carried out the experimental work and Pilar Junier helped to analyze the data. Preparation of the manuscript was done by Pilar Junier. Johannes F. Imhoff contributed by critical revision of the manuscript.

SUBMITTED

Habitat-partitioning of denitrifying bacterial communities carrying *nirS*/*nirK* genes in the stratified water column of Lake Kinneret, Israel. Pilar Junier, Ok-Sun Kim, Karl-Paul Witzel, Johannes F. Imhoff and Ora Hadas. 2007. Aquat. Microb. Ecol. Submitted.

Pilar Junier carried out the experimental part and analyzed the data. Ok-Sun Kim constructed the databases of *nirK* and *nirS* with ARB software. Pilar Junier evaluated the data and prepared the manuscript under supervision of Karl-Paul Witzel. All co-authors contributed by critical revision of the manuscript.

PREPARED

Diversity of ammonia monooxygenase (*amoA*) genes in the water column and sediment-water interface of two lakes and the Baltic Sea. Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel.

Ok-Sun Kim carried out the experimental part and analyzed the data. Pilar Junier constructed the database of *amoA*. Ok-Sun Kim evaluated the data and prepared the manuscript under supervision of Pilar Junier and Karl-Paul Witzel. Johannes F. Imhoff contributed by critical revision of the manuscript.

Genetic diversity of the evolutionarily related enzymes ammonia monooxygenase (AMO) and particulate methane monooxygenase (pMMO) in two lakes and the Baltic Sea. Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel.

Ok-Sun Kim carried out the experimental part and analyzed the data. Pilar Junier constructed the database of *amoA* and *pmoA* with ARB software. Ok-Sun Kim evaluated the data and prepared the manuscript under supervision of Pilar Junier and Karl-Paul Witzel. Johannes F. Imhoff contributed by critical revision of the manuscript.

Distribution of nitrite reductase (*nirK* and *nirS*) genes in the water column and sediment-water interface of two lakes and the Baltic Sea. Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel.

Ok-Sun Kim carried out the experimental part and analyzed the data. Pilar Junier helped to analyze the data with statistic. Ok-Sun Kim evaluated the data and prepared the manuscript under supervision of Pilar Junier and Karl-Paul Witzel. Johannes F. Imhoff contributed by critical revision of the manuscript.

Comparative analysis and description of PCR primers for ammonia-monooxygenase genes of ammonia-oxidizing bacteria. Pilar Junier, Ok-Sun Kim, Verónica Molina, Petra Limburg, Thomas Junier, Johannes F. Imhoff and Karl-Paul Witzel.

Ok-Sun Kim and Pilar Junier contributed equally to analyze all primers and to carry out the experimental part. Pilar Junier evaluated the data and to prepare the manuscript under supervision of Karl-Paul Witzel. All co-workers contributed by critical revision of the manuscript.

Application of novel PCR strategies and sequence analysis to study the *amoCAB* operon in beta ammonia-oxidizing bacteria. Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel.

Ok-Sun Kim carried out the experimental part. Ok-Sun Kim and Pilar Junier contributed equally to evaluate the data and to prepare the manuscript under supervision of Karl-Paul Witzel. Professor Imhoff contributed by critical revision of the manuscript.

Experimental comparison of specific 16S rDNA primers for analyzing ammonia oxidizing bacteria (AOB) in environmental samples. Pilar Junier, Ok-Sun Kim, Johannes F. Imhoff and Karl-Paul Witzel.

Pilar Junier and Ok-Sun Kim carried out the experimental part. Pilar Junier analyzed the bioinformatics, evaluated data and prepared the manuscript under supervision of Karl-Paul Witzel. Johannes F. Imhoff contributed by critical revision of the manuscript.

Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea

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With 2 figures and 4 tables

Abstract: One important pathway of the nitrogen cycle in aquatic environments is the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB). In this study the composition of AOB communities was compared between fresh (lakes Plusssee and Schöhsee) and brackish (Baltic Sea) water at two different levels: i) between environments and ii) within different depths in each environment. Changes in the community structure were studied by denaturing gradient gel electrophoresis (DGGE) and clone libraries of PCR products of 16S rRNA genes (rDNA) from AOB of the beta subclass of proteobacteria. Each environment displayed a particular DGGE band pattern. In Plusssee and the Baltic Sea, the differentiation of communities in epi- and metalimnion from those in hypolimnion coincided with a distinct stratification of the water column. In Schöhsee with an aerobic hypolimnion, the communities at all depths were similar. AOB communities in sediments were different from those in the water column. The composition of clone libraries showed the presence of specific *Nitrosomonas* and *Nitrosospira*-like sequences in each environment and habitat.

Key words: Ammonia-oxidizing bacteria, *Nitrosospira* spp., *Nitrosomonas* spp., Baltic Sea, Lake Schöhsee, Lake Plusssee.

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Habitat-partitioning of denitrifying bacterial communities carrying *nirS*/*nirK* genes in the stratified water column of Lake Kinneret, Israel

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ABSTRACT

Community composition of denitrifying bacteria was studied in the stratified water column of Lake Kinneret. The nitrite reductase genes, *nirS* and *nirK*, were amplified by PCR from water samples taken at 1, 14, 19 and 22 m depth. The PCR products were analyzed by terminal restriction fragment length polymorphism (T-RFLP) and clone libraries. The highest diversity of *nirS* denitrifying communities was observed at 1 m depth. According to the T-RFLP profiles and clone libraries of *nirS* products, two groups of denitrifiers were common and dominant in all depths. Deduced protein sequences from one of these groups displayed 77% identity to sequences of *nirS* reported in GenBank, indicating that it corresponds to previously unidentified species. Denitrifying bacterial communities with *nirK* were most diversified at 22 m and showed highest similarity to those at 19 m depth. At 1 m depth sequences only distantly related to *nirK* dominated, suggesting that denitrifying bacteria with copper-containing nitrite reductase were practically absent in this depth. The results indicate habitat-partitioning of denitrifying communities with different nitrite reductases in the stratified water column of Lake Kinneret. We conclude that diversity of denitrifying bacterial communities in stratified environments results from adaptation to particular environmental conditions.

Experimental comparison of specific 16S rDNA primers for analyzing ammonia-oxidizing bacteria (AOB) in environmental samples

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Status: Prepared

ABSTRACT

The specificity of PCR primers that are currently used to amplify the 16S rRNA genes (rDNA) of betaproteobacterial ammonia-oxidizers (βAOB) was rechecked by sequence comparison with updated GenBank entries, experimentally by clone libraries from four aquatic samples, and by analyzing published clone libraries generated with different primer combinations from a wide spectrum of habitats. Clone libraries prepared from products with different combinations of the primers NitA, NitB, βAMOf, βAMOr and Nso1225g showed discrepancies in the recovery of AOB-like sequences with the different primer pairs. In all the cases, the primers NitA/NitB produced the highest frequency of AOB-like sequences, compared to βAMOf/βAMOr, βAMOf/Nso1255g and NitA/Nso1225g. The experimental examination showed that neither specificity nor sensitivity of the existing primer combinations can be evaluated reliably by sequence analysis, only.

APPENDIX B

List of presented posters

Part of this thesis has been presented as posters at following symposia:

➢ **Ok-Sun Kim, Pilar Corredor and Karl-Paul Witzel:** Denitrifying bacteria in lakes and the sea studied by T-RFLP and clone libraries of the nitrite reductase genes *nirK* and *nirS*. International Symposium "American Society of Limnology and Oceanogrophy", June 2005 in Santiago de Compostella, Spain

➢ **Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel:** Community composition of denitrifying bacteria along a redox gradient of the stratified Lake Plußsee, studied by T-RFLP, DGGE and clone libraries of *nirS* and *nirK*. Annual meeting of "The 9th COST 856 meeting", March 2006 in Nijmegen, the Netherlands

➢ **Pilar Junier, Ok-Sun Kim, and Karl-Paul Witzel:** Delineation of ecotypes of denitrifying bacteria in lakes and the ocean by sequence analysis of *nirS* and *nirK*. Oral presentation by Pilar Junier. Annual meeting of the "the 9th COST 856 meeting", March 2006 in Nijmegen, the Netherlands.

➢ **Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel:** Using the *amoCAB* operon to study ammonia oxidizing bacteria (AOB) in lakes and the Baltic Sea. "11th International Symposium on Microbial Ecology ISME-11", August 2006 in Vienna, Austria

➢ **Pilar Junier, Ok-Sun Kim, Cornelia Burghardt, Gazalah Sabehi, Oded Beja, Ora Hadas and Karl-Paul Witzel:** Detection and characterization of uncultured ammonia-oxidizing bacteria (AOB) in a metagenomic library from Lake Kinneret, Israel. "11th International Symposium on Microbial Ecology ISME-11", August 2006 in Vienna, Austria

➢ **Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel:** Diversity of ammonia-oxidizing bacteria in the water column and sediment of two lakes and the Baltic Sea. Annual conference "Vereinigung für Allgemeine und Angewandte Microbiologie VAAM", April 2007 in Osnabrück, Germany.

➢ **Ok-Sun Kim, Pilar Junier, Johannes F. Imhoff and Karl-Paul Witzel:**

Appendix B-1: Poster presented at the International Symposium 'American Society of Limnology and Oceanogrophy', June 2005 in Santiago de Compostella, Spain

Appendix B-2: Poster presented at "the 9th COST 856 meeting", March 2006 in Nijmegen, the Netherlands

Appendix B-3: Oral presentation by Pilar Junier at "the 9th COST 856 meeting", March 2006 in Nijmegen, the Netherlands. Part of the work was presented.

Appendix B-4: Poster presented at the 11th International Symposium on Microbial Ecology ISME-11". August 2006 in Vienna, Austria

Appendix B-5: Poster presented at the 11th International Symposium on Microbial Ecology ISME-11", August 2006 in Vienna, Austria. I participated in a part work for this poster.

Appendix B-6: Poster presented at the Annual conference for "Vereinigung für Allgemeine und Angewandte Microbiologie VAAM". April 2007 in Osnabrück, Germany