Diversity and Ecology

of Marine Planctomycetes

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Summary

The phylum *Planctomycetes* is comprised of bacteria with characteristic morphological properties. Members of this phylum are widely distributed in terrestrial and aquatic habitats. Except for a group of *Planctomycetes* which anaerobically oxidizes ammonium to dinitrogen gas - the anammox bacteria - little is known about their detailed physiology. During this PhD thesis, the diversity and ecology of marine *Planctomycetes* were investigated with a special focus on marine anammox bacteria.

The anammox process was shown to play an important role in the loss of fixed nitrogen in marine oxygen minimum zones (OMZs). Anammox was detected in ammonium depleted waters of the Namibian upwelling system with oxygen concentrations which are supposed to reversibly inhibit the anammox reaction. Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) of samples from the Namibian OMZ showed that a fraction of the anammox bacteria was associated with particles that can provide anoxic microniches. Additionally, the co-occurring microbial community was dominated by members of the *Cytophaga/Flavobacterium* cluster and the *Gammaproteobacteria*. Bacteria of both groups are known for their capability to degrade proteins, which could provide the anammox bacteria with ammonium.

Furthermore, anammox bacteria were detected in suboxic waters of the Peruvian upwelling system and in the Arabian and the Black Sea. A detailed comparative analysis of 16S rRNA gene sequences and 16S-23S rRNA intergenic spacer regions (ITS) revealed a limited diversity of anammox bacteria in these habitats. While sequences belonging to a potential novel group of anammox bacteria were only detected in samples from the Namibian OMZ, sequences closely related to the *Candidatus* Scalindua cluster were found in all samples. This study revealed distinct subclusters of these sequences which were unique to each OMZ. Apparently, anammox sequences from Namibia and Peru were more closely related to each other than either group to the anammox sequences from the Arabian Sea. Anammox sequences from the Arabian Sea had less than 97% 16S rRNA sequence identity with sequences of other cultured or uncultured microorganisms. Therefore, a novel anammox species *Candidatus* Scalindua arabica is proposed.

In a metagenomic approach, six fosmids generated from marine samples were sequenced and annotated. Five of them contained genomic fragments related to so far uncultured planctomycetes. A comparative sequence analysis of these fosmids with all five available planctomycete genomes revealed a high number of genes coding for sulfatases on all of the marine genomes and on one fosmid. Sulfatases might enable those

planctomycetes to degrade sulphated heteropolysaccharides and to use the carbon skeleton as a source of energy. Additionally, genes for the conversion of C1 carbon compounds were found in all planctomycete genomes - except for *Candidatus* Kuenenia stuttgartiensis - as well as in two fosmids. The C1 metabolism is well known from methanogenic *Archaea* or methylotrophic *Alphaproteobacteria*, but up to now nothing is known about the function and relevance of these genes in *Planctomycetes.* Comparison of the genomes and fosmids furthermore revealed genes, which were specific for *Planctomycetes*. These planctomycete-specific genes might be involved in the distinct features of *Planctomycetes*.

Zusammenfassung

Das Phylum *Planctomycetes* umfasst Bakterien mit charakteristischen morphologischen Eigenschaften. Planktomyceten sind weit verbreitet in terrestrischen wie auch aquatischen Habitaten. Mit Ausnahme der Planktomyceten, die Ammonium anaerob zu molekularem Stickstoff (N_2) oxidieren – den sogenannten Anammox-Bakterien – ist wenig über ihre genaue Physiologie bekannt. In dieser Doktorarbeit wurden die Diversität und Ökologie mariner Planktomyceten untersucht, wobei der Schwerpunkt auf den marinen Anammox-Bakterien lag.

Der Anammox-Prozess spielt eine wichtige Rolle für den Verlust von fixiertem Stickstoff in marinen „Sauerstoff-Minimum-Zonen“ (OMZ). Er wurde in Ammonium limitierten Gewässern des Namibischen Auftriebsgebietes nachgewiesen, in denen Sauerstoffkonzentrationen verherrschten, die den Anammox-Prozess reversibel inhibieren sollten. Fluoreszenz in situ Hybridisierung mit enzymmarkierten Oligonukleotidsonden und Tyramid-Signalverstärkung von Proben der Namibischen OMZ zeigten, dass ein Teil der Anammox-Bakterien mit Partikeln assoziiert war. Innerhalb der Partikel kann es zu für den Anammox-Prozess günstigen anoxischen Bedingungen kommen. Desweiteren wurde die Partikel besiedelnde mikrobielle Gemeinschaft von Mitgliedern des *Cytophaga/ Flavobacterium* Clusters und der Gammaproteobakterien dominiert. Diese sind für ihre Fähigkeit bekannt, Proteine abzubauen. Das dabei freigesetzte Ammonium könnte von den Anammox-Bakterien genutzt werden.

Zusätzlich konnten Anammox-Bakterien auch im Peruanischen Auftriebsgebiet und in der Arabischen See und im Schwarzen Meer in sub-oxischen Gewässern nachgewiesen werden. Eine detaillierte vergleichende Untersuchung der 16S rRNA Gensequenzen und der 16S-23S rRNA intergenen Regionen ließ eine reduzierte Diversität der Anammox-Bakterien in allen Standorten erkennen. Abgesehen von Sequenzen des Namibischen Auftriebsgebietes, die zu einer potentiell neuen Gruppe von Anammox-Bakterien gehörten, wurden in allen Genbanken Sequenzen gefunden, die mit dem *Candidatus* Scalindua Cluster nahe verwandt waren. Darüber hinaus konnten Sub-Cluster von diesen Sequenzen unterschieden werden, die für die einzelnen Habitate spezifisch waren. Die Anammox-Bakterien der Namibischen und Peruanischen Auftriebsgebiete waren näher miteinander verwandt als jede der beiden Gruppen mit Anammox-Bakterien des Arabischen Meeres. Die 16S rRNA Gensequenzen des Arabischen Meeres hatten weniger als 97% Übereinstimmung mit den bekannten Sequenzen von kultivierten und auch unkultivierten

Bakterien. Es handelt sich bei diesen Sequenzen des Arabischen Meeres womöglich um eine neue Anammox-Spezies, *Candidatus* Scalindua arabica.

In einer Metagenom-Studie wurden sechs Fosmide von Proben aus marinen Habitaten sequenziert und annotiert. Fünf von ihnen enthielten Genomfragmente, die mit nicht kultivierten Planktomyceten verwandt waren. Eine vergleichende Sequenzanalyse der Fosmide mit den fünf bisher verfügbaren Planktomyceten-Genomen ließ in allen marinen Planktomyceten eine hohe Anzahl von Genen erkennen, die für Sulfatasen kodieren. Sulfatasen ermöglichen einen Abbau von sulfatisierten Heteropolysacchariden. Das freiwerdende Kohlenstoffgerüst könnte von den Planktomyceten als Energiequelle genutzt werden. Zusätzlich wurden in allen Genomen, mit Ausnahme des Anammox-Bakteriums *Candidatus* Kuenenia stuttgartiensis, und in zwei Fosmiden Gene für die Umwandlung von C1-Kohlenstoffverbindungen gefunden. Der C1-Metabolismus ist in methanogenen Archaeen und methanotrophen Alphaproteobakterien von Bedeutung, doch bisher ist noch nichts über die Funktion und Relevanz dieser Gene in Planktomyceten bekannt. Weiterhin identifizierte die vergleichende Analyse Gene, welche für Planktomyceten spezifisch waren. Diese Gene könnten zum Beispiel für die charakteristischen Eigenschaften der Planktomyceten verantwortlich sein.

List of abbreviations

anammox	anaerobic oxidation of ammonium with nitrite
bp	base pairs
C1	compounds containing one carbon atom
CARD-FISH	catalyzed reporter deposition-FISH
DAPI	4’,6-diamidino-2-phenylindole
DIN	dissolved inorganic nitrogen
DNA	deoxyribonucleic acid
DUF	domain of unknown function
et al.	and co-worker
FISH	fluorescence in situ hybridization
G+C content	guanine/cytosine-content
h	hours
HRP	horseradish peroxidase
ITS	internal transcribed spacer
kb	kilo bases
Mb	mega bases
OMZ	oxygen minimum zone
ORF	open reading frame
PCR	polymerase chain reaction
rDNA	gene of ribosomal RNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
spp.	species
tRNA	transfer RNA

Part I:

Combined Presentation of Results

A. Introduction

1. The Phylum *Planctomycetes*

1.1. Phylogeny

The phylum *Planctomycetes* (Garrity and Holt 2001) within the domain *Bacteria* contains one family (*Planctomycetaceae*) comprised of six genera (*Rhodopirellula*, *Blastopirellula*, *Pirellula*, *Planctomyces*, *Isosphaera*, *Gemmata*) (Schlesner et al. 2004) and four *Candidatus* genera (Kuenenia, Brocadia, Scalindua, Anammoxoglobus). The diversity within the *Planctomycetes* is astonishingly high; the 16S rRNA sequence identity of genus-representatives reaches from 76% to a maximum of 93%.

The phylogeny of the *Planctomycetes* has been under debate for some time with conflicting views of them being either rapidly evolving (Woese 1987, Fuerst 1995), deep (Stackebrandt et al. 1984) or even deepest branching within the bacterial domain (Brochier and Philippe 2002), or being remotely related to the *Chlamydiae* (Teeling et al. 2004, Weisburg et al. 1986). Recent studies suggest that the *Planctomycetes* are part of the so called PVC superphylum, a monophyletic clade that in addition to the *Planctomycetes* includes the phyla *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae*, and the candidate phyla 'Poribacteria' and OP3 (which so far have no cultured representatives) (Fieseler et al. 2004, Wagner and Horn 2006) (Fig. 1). The combination of these groups into a superphylum was supported by common features like a complex life style in many members, the peptidoglycan-lacking cell wall within *Planctomycetes* and *Chlamydiae*, intracellular compartments shared by the *Planctomycetes* and '*Poribacteria*' and by genomic and metagenomic information (Wagner and Horn 2006, Fieseler et al. 2006).

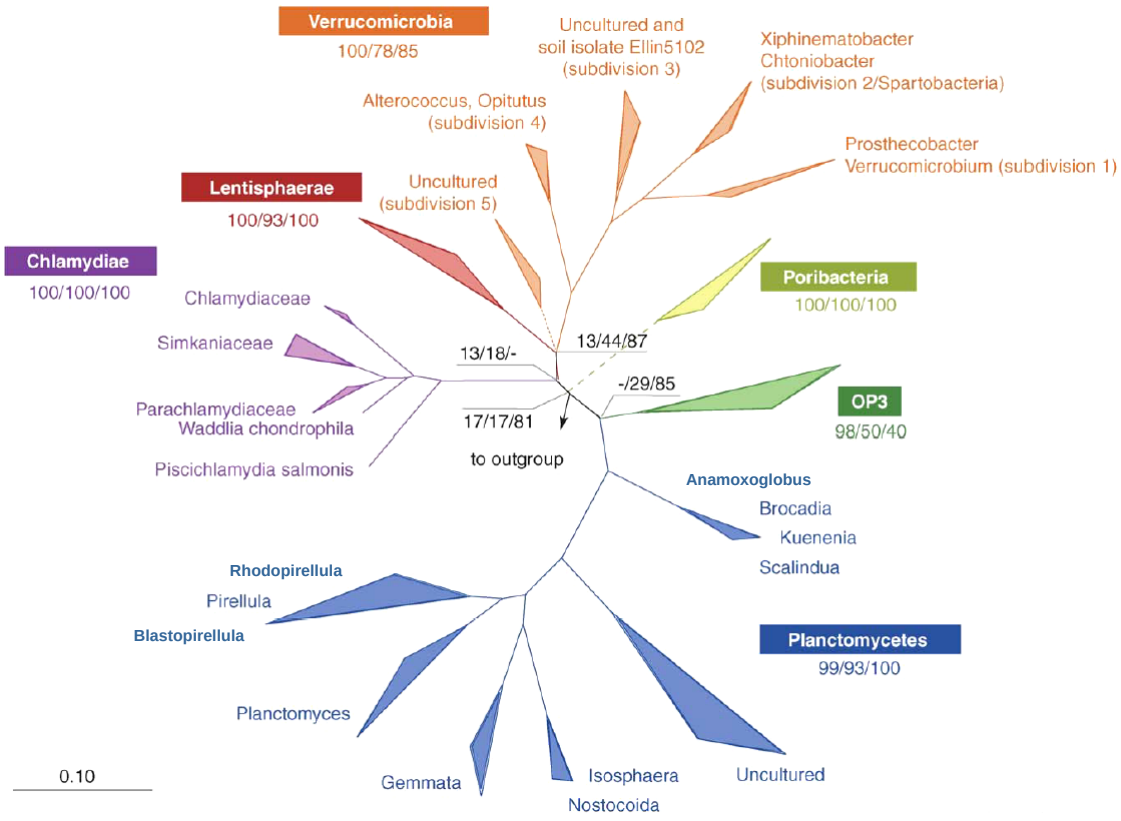


Fig.1: Phylogenetic tree based on 16S rRNA gene sequences showing the PVC superphylum, which is comprised of the phyla *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae*, and the *Candidatus* phyla 'Poribacteria' and OP3 (modified from Wagner and Horn 2006). Dotted lines indicate unresolved positioning of groups and numbers represent bootstrap values calculated with three different algorithms.

1.2. Morphology

All *Planctomycetes* known to date are characterized by a unique set of characteristic morphological features. Their cell wall lacks the common bacterial cell wall constituent peptidoglycan. Instead they possess a proteinaceous cell wall mainly consisting of proline and cystein, the latter stabilizing the protein envelope by disulfide bonds (König et al. 1984, Liesack et al. 1986). The cytoplasmic membranes of *Planctomycetes* often contain characteristic crateriform structures that are either evenly distributed throughout the whole cell surface (genus *Planctomyces*) or confined around the reproductive pole (genus *Pirellula*) (Liesack et al. 1986).

The most distinctive feature of the *Planctomycetes* is their internal compartmentalization (Lindsay et al. 1997, Lindsay et al. 2001, Fuerst 2005). All planctomycete cells exhibit a peripheral ribosome-free region, the paryphoplasm, and an interior region containing the DNA and ribosomes, bounded by a single intracytoplasmic membrane and called the riboplasm (or pirellulosome). The intracellular membrane in *Isosphaera pallida* and *Planctomyces maris* is invaginated, so that the paryphoplasm forms

the major portion of the cell interior. In other planctomycetes, the paryphoplasm remains as a peripheral zone (Fig. 2).

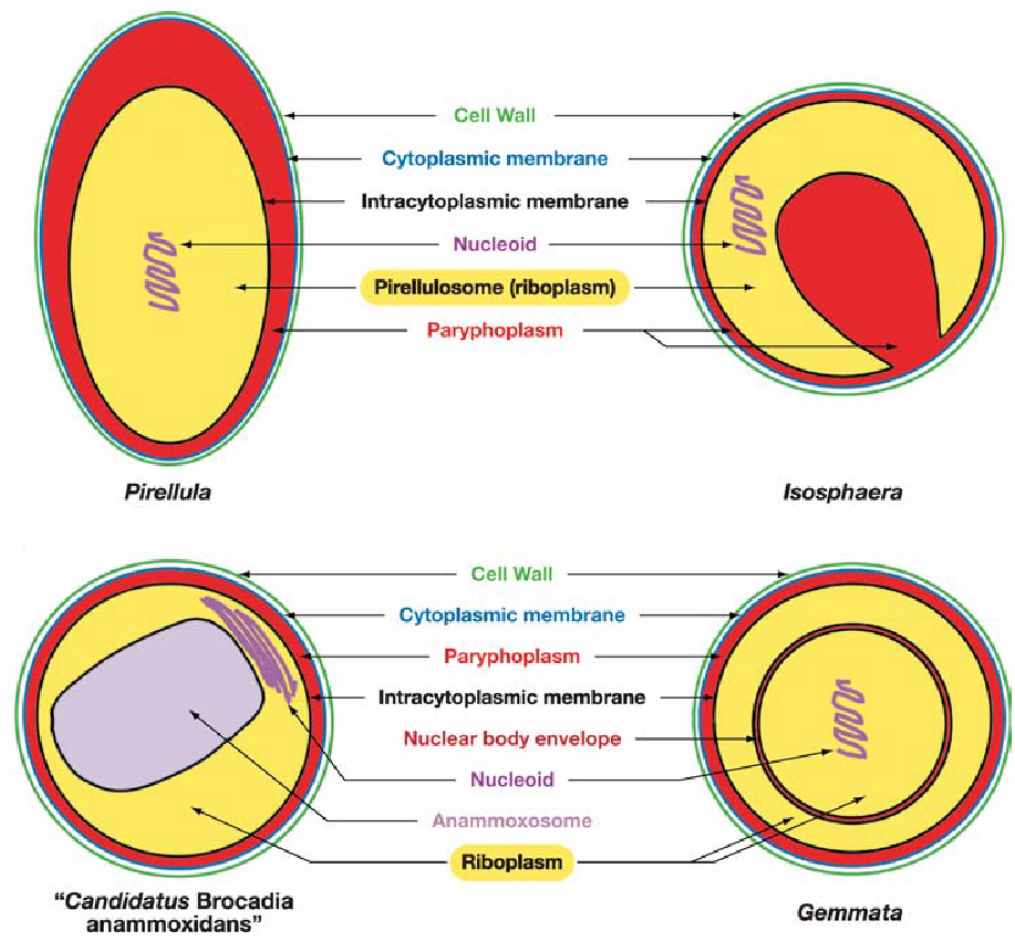


Fig. 2: Different modes of internal compartmentalization within the *Planctomycetes*, represented by the genera *Pirellula*, *Isosphaera*, *Gemmata* and *Candidatus* Brocadia anammoxidans as an example for anaerobic ammonium oxidizing (anammox) bacteria (Fuerst 2005).

Gemmata species in addition have a double-membrane around their nucleoid that, however, might be in contact with the intracytoplasmic membrane and thus is not fully closed (not visible in Fig. 2). Planctomycetes that anaerobically oxidize ammonium (anammox bacteria) possess the anammoxosome, a single-membrane-bound compartment that harbors enzymes and intermediates central to the anammox metabolism (see chapter A 2.1.). The diversity of subcellular organization is exceptional for prokaryotes.

Planctomycete cells are organized in a polar manner in some cases with nonprosthecate appendages (stalks) or polar holdfast structures. In addition, planctomycete cells have a generative pole from which daughter cells are produced in a yeast-like budding process. For some planctomycetes a life cycle has been described with flagellated, non-

reproductive swarmer cells and non-motile but reproductive adult cells (Franzmann and Skerman 1984, Fuerst 1995, Tekniepe et al. 1981). *Isosphaera pallida* is a multicellular filamentous bacterium that moves by gliding (Giovannoni et al. 1987).

1.3. "Classical" chemoorganoheterotrophic planctomycetes (all genera excluding the anammox bacteria)

1.3.1. Physiology

Knowledge about the physiology and metabolism of the genera *Rhodopirellula*, *Blastopirellula*, *Pirellula*, *Planctomyces*, *Isosphaera* and *Gemmata* is rather limited. Strains in pure culture are all chemoorganoheterotrophs and either obligate or facultative aerobes. Carbohydrates seem to be their major carbon source. *Planctomycetes* are slowly growing organisms, with the shortest generation time of 13 h in *Planctomyces maris* DSM 9787^T (Bauld and Staley 1976, Fuerst 1995) and 12 h in *Rhodopirellula baltica* SH 1^T (personal communication, P. Wecker).

Recently, the first planctomycete was investigated by a genomic approach. *Rhodopirellula baltica* SH 1^T, formerly known as *Pirellula* sp. strain 1, was isolated from brackish water at the Kiel Fjord in the Baltic Sea (Schlesner 1994) and is a pigmented, aerobic and heterotrophic representative of the *Planctomycetes*. Astonishingly, its genome contained 110 genes encoding for proteins with significant similarity to prokaryotic and eukaryotic sulfatases (Glöckner et al. 2003). It has been suggested that these sulfatases are metabolically important by efficient degradation of sulfated glycopolymers, which are found in marine snow aggregates, and allow the use of the derived carbon skeleton as an energy source. Another feature of *Rhodopirellula baltica* SH 1^T is the genetic potential for utilizing C1 compounds as well as growth on N-acetylglucosamine as sole source of carbon and nitrogen under aerobic conditions (Rabus et al. 2002).

In addition, the Gordon and Betty Moore foundation has funded draft sequencing of *Blastopirellula marina* DSM 3645^T and *Planctomyces maris* DSM 8797^T. Ward and co-workers have generated an early draft of the genome of *Gemmata obscuriglobus* UQM 2246^T. The unpigmented *Blastopirellula marina* DSM 3645^T was, like *Rhodopirellula baltica* SH 1^T, isolated from brackish surface waters at Kiel Fjord in the Baltic Sea (Schlesner 1986). Both are strict aerobes and can be considered as marine bacteria, since they do not grow in freshwater media (Schlesner et al. 2004). *Planctomyces maris* DSM 8797^T was isolated from surface waters at Pudget Sound, Washington, USA. It is

heterotrophic, obligately aerobic, mesophilic, has the absolute requirement for Na^+ and grows at a salinity of 1.5-4.0% (w/v) (Bauld and Staley 1980, Bauld and Staley 1976).

Gemmata obscuriglobus UQM 2246^T is a freshwater planctomycete, as well aerobic and mesophilic and was isolated in the early 1980s from the surface waters of a freshwater dam (Maroon Dam) in Queensland, Australia (Franzmann and Skerman 1984). Genomic information of these planctomycetes together with the genome of the planctomycete *Candidatus* Kuenenia stuttgartiensis (chapter A 2.2) and planctomycete fosmids were used in the comparative genomics study of this PhD thesis (chapter B 6).

1.3.2. Ecology

Most cultured and isolated planctomycetes originate from aquatic and aerobic habitats (Bauld and Staley 1976, Franzmann and Skerman 1984, Giovannoni et al. 1987, Schlesner 1994, Wang et al. 2002). However, molecular methods like fluorescence in situ hybridization (FISH) and 16S rRNA gene sequencing have revealed a much broader distribution of the *Planctomycetes* in the environment. They were detected in the water column and anoxic sediments of lakes (Miskin et al. 1999, Neef et al. 1998, Wang et al. 2002), in the water column (DeLong et al. 1993, Vergin et al. 1998) and in sediments of marine systems (Llobet-Brossa et al. 1998) and in oxic and anoxic soils (Wang et al. 2002). While they constitute only a few percent of the microbial community in coastal waters and even less in the marine pelagial (Rusch 2007), their abundances are much higher in marine sediments (Musat et al. 2006, Rusch et al. 2003). Furthermore, *Planctomycetes* have been detected in marine sponges (Pimentel-Elardo et al. 2003) or in the hepatopancreas of the crustacean *Panaeus monodon* (Fuerst et al. 1997, Fuerst et al. 1991). *Isosphaera pallida* commonly occurs in neutral and alkaline warm springs at temperatures from 35 to 55°C in Europe and North America (Giovannoni et al. 1987). *Planctomycetes* are often associated with surfaces, which is facilitated e.g. by the production of holdfast structures.

Planctomycetes also inhabit marine snow particles (Crump et al. 1999, DeLong et al. 1993) and can be involved in their mineralization and therefore might be of global significance for nutrient cycling processes in marine habitats. Marine snow is suggested to be important for the flux of biogenic carbon from the sea surface to the deep ocean. During sinking, biological and chemical transformations influence the quantity and quality of organic matter in the particles that reach the deep sea (Fuerst 1995) (see chapter A 4.1.1).

2. Planctomycetes mediating the anaerobic oxidation of ammonium (anammox)

2.1. Discovery of the anammox bacteria and their characteristics

For decades scientists believed that the anaerobic oxidation of ammonium should be thermodynamically possible with nitrate or nitrite as the electron acceptor, and the putatively responsible organisms were entitled "lithotrophs missing from nature" (Broda 1977, Richards 1965). However, it took until 1995 for the anammox process to be finally discovered in a denitrifying pilot plant for waste water treatment (Mulder et al. 1995), where ammonium disappeared under anaerobic conditions with production of dinitrogen gas. Further studies showed that the anaerobic oxidation of ammonium was mediated by microorganisms and that nitrite rather than nitrate was the electron acceptor (see equation 1) (van de Graaf et al. 1995).

(1) $NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O$ $\Delta G^{0f} = -335$ kJ mol^{-1} NH_4^+

Additionally it turned out that the bacteria carrying out anammox were carbon autotrophic, i.e. fixing CO_2 and incorporating it into biomass. The required electrons are derived from the anaerobic oxidation of nitrite to nitrate (van de Graaf et al. 1996). Treatment of anammox-mediating bioreactors with different oxygen concentrations showed that the anammox process was reversibly inhibited already by as little as 1 µM oxygen (0.5% of air saturation) (Strous et al. 1997) and irreversibly inhibited by high concentrations of nitrite (>10 mM) (Jetten et al. 1999).

The microorganisms carrying out anammox had a very long doubling time of about 10 days (Strous et al. 1998), which complicated their enrichment and isolation. Only one study reported doubling times of two days (Isaka et al. 2006). Their enrichment was facilitated by the use of very efficient biomass retention in continuous reactors. However, until now it was not possible to gain pure cultures of anammox bacteria, but their physical purification was accomplished by Percoll density-gradient centrifugation (Strous et al. 1999). This allowed the identification of the organism responsible for the anammox process. 16S rRNA gene sequence analysis showed that the anammox organism was a deep-branching planctomycete with only 74-77% sequence identity to the other *Planctomycetes* (Strous et al. 1999) (Fig. 3).

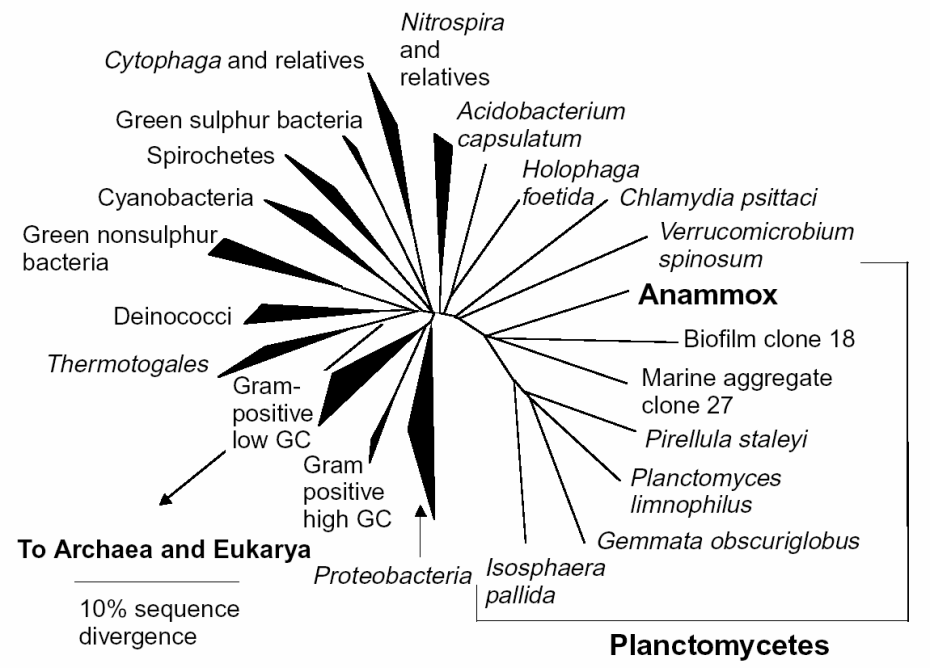


Fig. 3: Phylogenetic position of the first anammox bacterium, *Candidatus* Brocadia anammoxidans - labelled 'Anammox' in the tree - within the phylum *Planctomycetes* based on 16 rRNA phylogeny (Strous et al. 1999).

The first described anammox bacterium (Strous et al. 1999) was named provisionally *Candidatus* Brocadia anammoxidans (Fig. 4) to indicate that it is not in pure culture and therefore its uniqueness is defined only by 16S rRNA sequence (Murray and Schleifer 1994).

Furthermore, it was shown that anammox bacteria exhibited planctomycete-characteristic features like budding reproduction, crateriform structures and the anammoxosome (Fig. 4). This compartment was found to be surrounded by a membrane mostly composed of unique ladderane lipids (Sinninghe Damste et al. 2002) with three to five linearly concatenated cyclobutane rings. Ladderane lipids occurred both as ether and ester lipids in anammox bacteria, which is unusual since these bacteria are neither thermophiles nor related to *Archaea*.

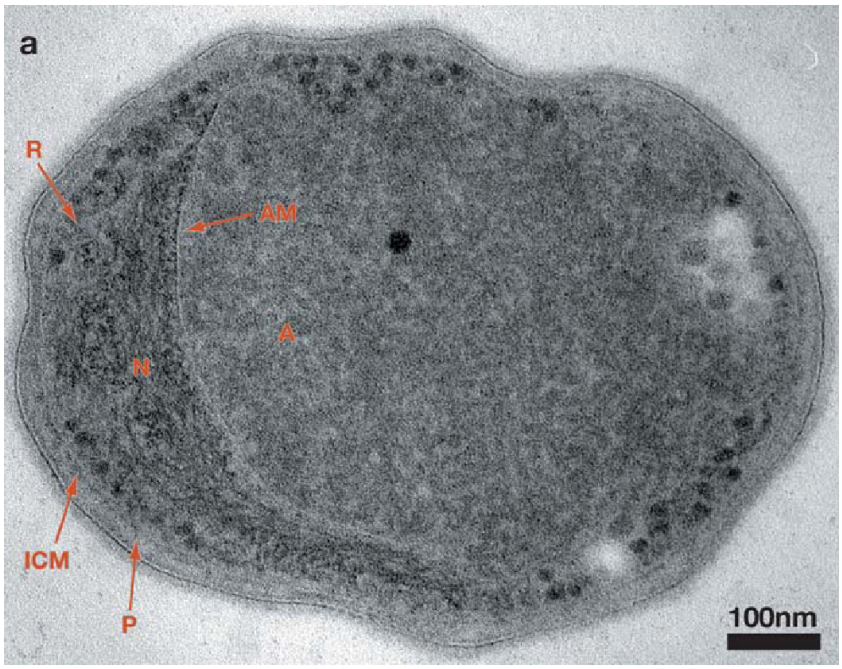


Fig. 4: Transmission electron micrograph of the anammox bacterium *Candidatus* Brocadia anammoxidans showing its anammoxosome (A), surrounded by a single membrane (AM). The cytoplasm external to the anammoxosome contains the nucleoid (N) and ribosomes (R) and is enclosed by the intracytoplasmic membrane (ICM). The paryphoplasm (P) lies at the rim of cell between the ICM and the cytoplasmic membrane (Fuerst 2005).

After the first discovery of the anammox process in 1995, it was found in several other waste water treatment plants or bioreactors (e.g. Egli et al. 2001, Pynaert et al. 2003, Schmid et al. 2000, Schmid et al. 2003). The investigation of the responsible microorganisms revealed the presence of new anammox bacteria, which gave evidence for a genus level diversity of anammox bacteria (Schmid et al. 2000). *Candidatus* Kuenenia stuttgartiensis was discovered in a trickling filter biofilm (Schmid et al. 2000), whereas *Candidatus* Scalindua brodae and *Candidatus* Scalindua wagneri were found in a waste water treatment plant in the UK (Schmid et al. 2003). *Candidatus* Brocadia fulgida originated from an anammox wastewater treatment plant and showed only 96% sequence identity to *Candidatus* Brocadia anammoxidans (Kartal et al. 2004).

Anammox bacteria were assumed to have a completely chemolithoautotrophic lifestyle. However, an anammox bacterium was recently shown to be capable of oxidizing propionate to CO_2. Additionally, it was shown for the first time that anammox bacteria were able to couple the oxidation of propionate to the reduction of nitrate to nitrite (Guven et al. 2005). This could imply that anammox bacteria can use nitrate in addition to nitrite for oxidation of ammonium in the anammox reaction via dissimilatory nitrate reduction, if a suitable electron donor (like propionate) is available.

Recently the anammox bacterium *Candidatus* Anammoxoglobus propionicus was enriched, which had at most 91% 16S rRNA sequence identity to the anammox species *Candidatus* Brocadia fulgida. It oxidized small organic acids like acetate and propionate at a high rate and out-competed other anammox bacteria and heterotrophic denitrifiers for the oxidation of propionate in the presence of ammonium, nitrite and nitrate (Kartal et al. 2007). *Candidatus* Anammoxoglobus propionicus did not incorporate propionate into biomass, indicating that the propionate oxidation occurs only for generation of energy. Additionally, a recent study has shown that *Candidatus* Kuenenia stuttgartiensis can perform dissimilatory nitrate reduction to ammonium (DNRA) (Kartal et al. 2007). It reduced nitrate to nitrite and in a second step to ammonium even in the presence of 10 mM NH_4^+. Subsequently, it converted nitrite and ammonium into N_2 by the usual anammox metabolism. This process further indicates that anammox bacteria might have a more versatile metabolism than previously assumed.

2.2. Insights into an anammox bacterial genome (*Cand*. Kuenenia stuttgartiensis)

Recently, the genome of *Candidatus* Kuenenia stuttgartiensis was assembled in an environmental genomics approach from a laboratory bioreactor comprising 29 different taxonomic units, with *Candidatus* Kuenenia stuttgartiensis constituting about 73% of the community (Strous et al. 2006). The genome analysis opened the possibility to discover the genes encoding the unique features of the anammox bacteria, such as the anaerobic oxidation of ammonium or ladderane lipid biosynthesis.

Strous and co-workers could deduce a new metabolic pathway for the anammox process with NO as intermediate but without hydroxylamine as it was proposed before (van de Graaf et al. 1997) (Fig. 5).

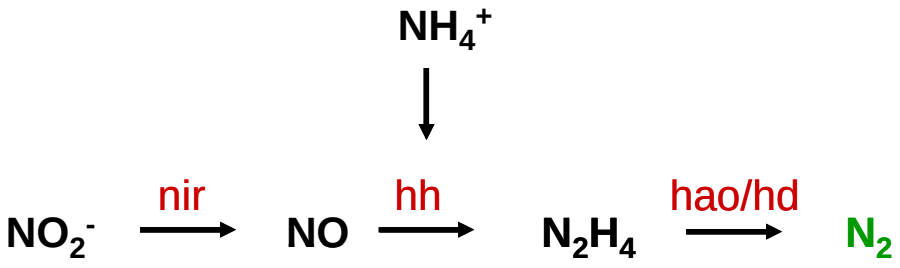


Fig. 5: Metabolic pathway of the anammox reaction: Nitrite is reduced to nitric oxide, which together with ammonium reacts to hydrazine (N_2H_4) and finally results in dinitrogen gas (nir= nitrite: nitric oxide oxidoreductase, hh= hydrazine hydrolase, hao= hydroxylamine oxidoreductase, hd= hydrazine oxidoreductase).

Besides, this study revealed a branched respiratory chain, which would enable the respiration of different energy sources with different electron acceptors. And indeed, iron and manganese oxides were experimentally shown to be respired by *Candidatus* Kuenenia stuttgartiensis. The anammox bacteria use the energy from redox reactions for autotrophic growth. The complete acetyl-CoA pathway was detected in the genome of *Candidatus* Kuenenia stuttgartiensis, and could be coupled to the use of nitrite as electron donor for the carbon fixation. The pathway of ladderane lipid biosynthesis was completely unknown and could not be immediately inferred, but two gene clusters on the genome were identified as promising candidates (Strous et al. 2006). The genome analysis underpins the possibility that anammox bacteria could have a less restricted life style than expected.

2.3. Anammox bacteria in the marine environment

After the identification of the bacteria responsible for the anammox process in waste water treatment plants and bioreactors, PCR-based methods and the design of planctomycete- and anammox bacteria-specific primers and probes allowed for the discovery and investigation of anammox bacteria in the marine environment. The first marine anammox species was identified in the Black Sea (Kuypers et al. 2003). It was most closely related to *Candidatus* Scalindua brodae originating from a wastewater treatment plant (98.1% sequence identity), but only distantly related to other anammox bacteria like *Candidatus* Kuenenia stuttgartiensis and *Candidatus* Brocadia anammoxidans (87.9% and 89.6% sequence identity, respectively). Consequently it was named *Candidatus* Scalindua sorokinii.

3. The marine nitrogen (N) cycle

The nitrogen cycle is composed of multiple transformations of nitrogen compounds, primarily catalyzed by microorganisms (Zehr and Ward 2002). It controls the availability of nitrogenous nutrients globally, and also has a profound impact on the biological productivity in marine systems (Ryther and Dunstan 1971). Figure 6 shows the complexity of the nitrogen cycle. Nitrogen undergoes a series of oxidation-reduction reactions in which it changes its valence state form from -III to +V.

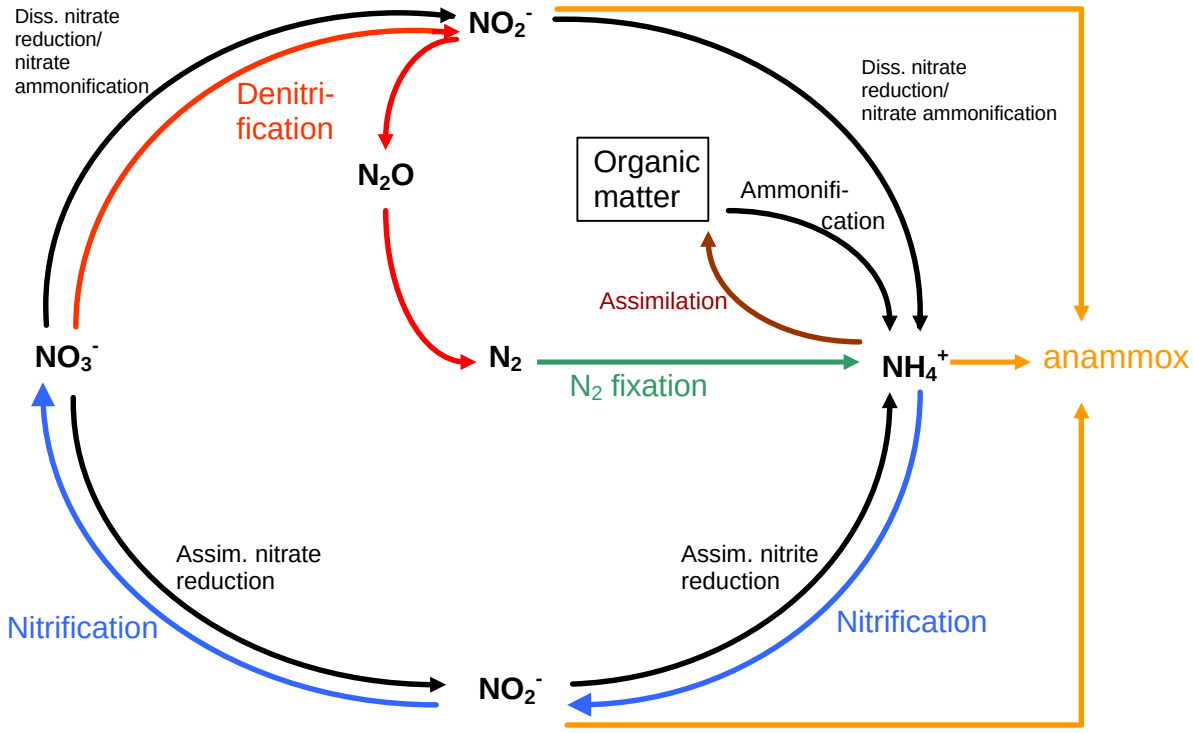


Fig. 6: The marine nitrogen cycle showing the chemical forms and key processes involved in the biogeochemical cycling of nitrogen (modified from Herbert 1999).

Inorganic nitrogen is often limiting in the oceans and most microorganisms can (in the absence of organic nitrogen) use only dissolved inorganic nitrogen (DIN) compounds in form of nitrate, nitrite and ammonium. Low concentrations of DIN limit productivity in the surface waters of oligotrophic open ocean gyres (Capone 2000) as well as in coastal upwelling regions (Kudela and Dugdale 2000). DIN can be liberated by death, lysis and decay of phytoplankton. A major source of nitrate in the ocean surface water is the upwelling of nitrate-rich deep ocean water (Zehr and Ward 2002). Nitrate is assimilated after sequential reduction to nitrite (assimilatory nitrate reduction) and ammonium (assimilatory nitrite reduction).

Heterotrophic bacteria release inorganic N (NH_4^+) during decomposition of organic matter and thereby recycle N to phytoplankton. Their role is emphasized in the microbial loop by transferring C, N and other nutrients from dissolved organic matter into higher trophic levels by being grazed from micrograzers (Azam 1998). However, heterotrophic bacteria also use DIN in addition to organic compounds, thereby competing with phytoplankton (Kirchman and Wheeler 1998).

Inorganic nitrogen compounds are limited in the oceans, and therefore it is important to study the processes responsible for the loss of fixed nitrogen. All living matter

contains nitrogenous macromolecules (nucleic acids, proteins, polyamino-sugars etc.) (Herbert 1999). Mineralization of biomass by hydrolytic reactions produces ammonium, a process which is called ammonification. In oxic waters, microorganisms oxidize ammonium via nitrite to nitrate in a process called nitrification. In suboxic and anoxic water layers, the produced nitrate gets reduced to N_2 during denitrification. A number of fermentative and strictly anaerobic (heterotrophic) bacteria (within the genera *Aeromonas*, *Clostridium*, *Vibrio* and *Desulfovibrio*) can reduce nitrate to ammonium, a process called nitrate ammonification or dissimilatory nitrate reduction (Herbert 1999). Besides denitrification the anaerobic oxidation of ammonium with nitrite or nitrate (anammox) transforms DIN into N_2. Both processes reduce the amount of biologically available nitrogen compounds in marine systems, but usually the loss is well balanced by nitrogen fixation.

In the following section, the main processes which govern the nitrogen budget in marine systems are described in more detail, i.e. nitrogen fixation, nitrification, denitrification and the anaerobic oxidation of ammonium (anammox).

3.1. Nitrogen fixation

79% of the earth's atmosphere is composed of molecular dinitrogen gas (N_2). This major N reservoir is unavailable to most organisms (Herbert 1999). The biological N_2 fixation - the reduction of atmospheric N_2 to the redox state of ammonia - is the major process supplying the oceans with dissolved inorganic nitrogen. It is confined to specialized microorganisms which possess the iron requiring enzyme nitrogenase. The filamentous cyanobacterium *Trichodesmium* (Capone et al. 1997, Orcutt et al. 2001) and cyanobacterial symbionts of diatoms (Carpenter et al. 1999, Villareal and Carpenter 1989) were believed to be the major N_2 fixers in the open ocean. However, there is recent evidence that some species of unicellular cyanobacteria and proteobacteria also fix N_2 (Zehr et al. 2000, Zehr et al. 1998, Zehr et al. 2001), although their distribution and N_2-fixation rates are not well known yet. It was estimated that 100-200 Tg N are fixed per year in the global oceans (Karl et al. 2002). In comparison, 175-450 Tg fixed N yr^{-1} are globally lost within oxygen depleted environments due to denitrification (Codispoti et al. 2001). A very recent study proposed that biological N_2 fixation, which channels N compounds into the sea, is intimately associated, both geographically and temporarily, with marine nitrogen removal (denitrification and anammox) and not with atmospheric iron deposition (Deutsch et al.

2007). This suggests that nitrogen fixation stabilizes the oceanic inventory of fixed nitrogen over time.

3.2. Nitrification

Nitrification is a two step process that involves two groups of bacteria: ammonia oxidizing bacteria (AOB), which oxidize ammonium to nitrite via hydroxylamine, and nitrite oxidizing bacteria (NOB), which oxidize nitrite to nitrate (Bothe et al. 2000). In general, AOBs are obligate chemolithoautotrophs and were long thought to be restricted to the *Beta-* and *Gammaproteobacteria*. *Nitrosomonas, Nitrosospira, Nitrosolobus* and *Nitrosovibrio* spp. and *Nitrosococcus mobilis* are grouped within the *Betaproteobacteria* (Bock and Wagner 2006), whereas *Nitrosococcus oceani* and *Nitrosococcus halophilus* belong to the *Gammaproteobacteria*. The enzyme ammonia monooxygenase (AMO) catalyses the oxidation of ammonium to hydroxylamine, and hydroxylamine oxidoreductase (HAO) the oxidation to nitrite (Bothe et al. 2000).

Nitrite oxidizers are found within the *Alpha-* (*Nitrobacter*), *Gamma-* (*Nitrococcus*) and *Deltaproteobacteria* (*Nitrospina*) and in the *Nitrospira* phylum (Bock and Wagner 2006, Teske et al. 1994). The enzyme responsible for the oxidation of nitrite to nitrate is nitrite oxidoreductase (NO_2-OR). Current studies show that the aerobic autotrophic nitrifiers occur in relatively low abundances in natural environments (Ward 2002).

Recently, the crenarchaeon '*Nitrosopumilus maritimus*' was shown to grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite (Könneke et al. 2005). Potential for ammonium oxidation in another crenarchaeon, *Cenarchaeum symbiosum,* was shown by a metagenome study (Hallam et al. 2006). In the meanwhile, genes encoding for the crenarchaeal ammonia monooxygenase a-subunit (*amoA*) were recovered from ocean waters around the world (Hallam et al. 2006, Wuchter et al. 2006) further suggesting an important role of crenarchaeota in nitrification.

Nitrification does not affect the budget of fixed nitrogen in the environment, but does change its form and supplies the substrate for denitrification. The linkage of both processes is responsible for the net loss of fixed N in aquatic environments (Ward 2005).

3.3. Heterotrophic denitrification

In the classical denitrification, oxides of nitrogen (including nitrate, nitrite and nitric and nitrous oxides) are used in respiration instead of oxygen by facultative anaerobic bacteria

(Ward 2005). The end product of complete denitrification is dinitrogen gas. In contrast to the very restricted phylogeny of nitrifiers, denitrification is performed by a diverse range of *Bacteria* (*Alpha-*, *Beta-* and *Gammaproteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*) and *Archaea* (Scala and Kerkhof 1998). Since denitrification is widespread among unrelated groups and therefore not a monophyletic property, the 16S rRNA approach is not suitable for investigation of denitrifying microorganisms (Braker et al. 2000). Instead, it is necessary to focus on a functional gene directly involved in denitrification to study the diversity of denitrifying microbes in the environment. However, phylogenies based on functional genes involved in dentrification do not always correlate well with phylogenies based on 16S rRNA genes. Therefore, identification of denitrifying organisms can be difficult.

3.4. Anaerobic oxidation of ammonium (anammox) in marine systems

Scientists have noticed for a long time that far less ammonium accumulates in marine oxygen-deficient waters and sediments than should be produced during anaerobic mineralization of organic matter from the stoichiometry of heterotrophic denitrification (Devol 2003).

Anammox activity in the oceans was first shown in anoxic sediments by the unique labeling pattern in anoxic ^{15}N-tracer experiments (so-called isotope pairing technique) (Thamdrup and Dalsgaard 2002). During the anammox process N_2 is formed through pairing of one N atom from both NO_2^- and NH_4^+ (Thamdrup and Dalsgaard 2002), whereas denitrification combines N from two NO_3^- molecules to form N_2. If anammox is the main process and ^{15}N-labeled NH_4^+, NO_2^- or NO_3^- is used, $^{29}N_2$ is formed. If denitrification is the main process $^{30}N_2$ can be detected. Thamdrup et al. found that anammox accounted for 24-67% of the total N_2 production in continental shelf sediments (Thamdrup and Dalsgaard 2002). Two studies conducted in suboxic to anoxic basins, the Golfo Dulce in Costa Rica and the Black Sea, provided first evidence for anammox occurring in the water column. In the suboxic waters of Golfo Dulce anammox accounted for 19-35% of the total N_2 production, at some depths even for 58% (Dalsgaard et al. 2003). The Black Sea is the world's largest anoxic basin and very low ammonium concentrations in suboxic waters suggested that ammonium was anaerobically oxidized to dinitrogen gas. Indeed, Kuypers and co-workers showed that anammox was an important process in suboxic waters of the Black Sea, possibly consuming more than 40% of the fixed nitrogen that gets transported into the suboxic zone (Kuypers et al. 2003). These

findings led to the assumption that globally anammox may be responsible for 30-50% of the N_2 production in the ocean (Devol 2003). To date, anammox has been detected in marine, coastal and estuarine sediments (Engstroem et al. 2005, Risgaard-Petersen et al. 2004, Rysgaard 2004, Thamdrup and Dalsgaard 2002, Trimmer et al. 2003), in anoxic basins (Dalsgaard et al. 2003, Kuypers et al. 2003), in mangroves (Meyer et al. 2005), sea-ice (Rysgaard and Glud 2004) and freshwater lakes (Schubert et al. 2006).

In the early years of anammox research, it was believed that the anammox bacteria are restricted to a chemolithoautotrophic life style. However, recent studies have changed our view of the metabolic capabilities of the anammox bacteria and we are beginning to understand that they are far more versatile than assumed. If anammox bacteria in the marine environments produce ammonium from nitrate or nitrite and use this ammonium in the production of N_2 (coupled DNRA-anammox), the whole process will be indistinguishable from denitrification in conventional isotopic tracer experiments. Consequently, the anammox process could be responsible for an even larger fraction of the N loss in suboxic to anoxic ecosystems as oxygen minimum zones (OMZs).

4. Marine oxygen minimum zones (OMZs)

Oxygen minimum zones are defined as oceanic regions with reduced oxygen concentrations below about 4.5 µM (Morrison et al. 1999) or 0.5 ml/l (Rogers 2000). OMZs are confined to a number of regions (Fig. 7) as the Arabian Sea, the Bay of Bengal, the Phillipine region, the northwest Pacific margin, the eastern Pacific and off southwestern Africa beneath the Benguela current (Kamykowski and Zentara 1990).

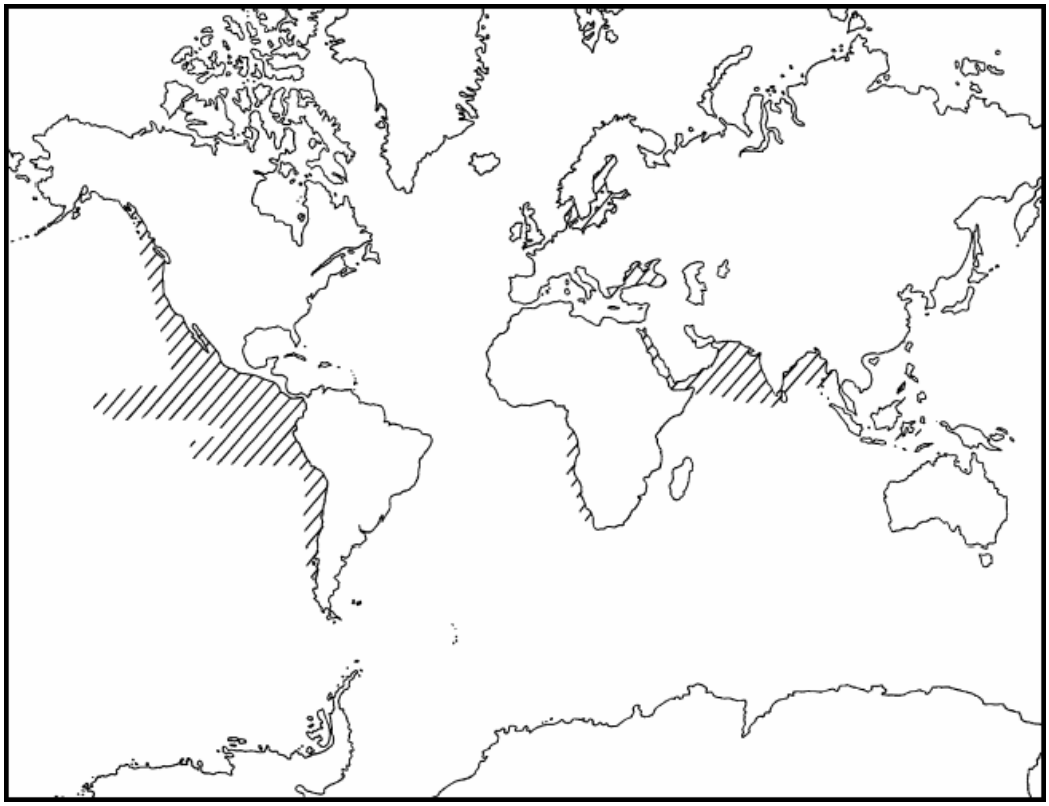


Fig. 7: Map showing the distribution of oxygen minimum zones in the world's oceans (from Rogers 2000 and Kamykowski and Zentara 1990).

In general, the OMZs are formed because of high primary productivity, followed by oxygen consumption due to the biological degradation of organic matter sinking from the surface into deeper waters and poor water circulation (Rogers 2000). Regions of high primary production are often areas of coastal upwelling, like the coast off Peru and Namibia (Mann and Lazier 1996). In the following paragraphs, the formation of the major OMZs is described from which samples were investigated during this thesis.

4.1. The Benguela upwelling system off Namibia

The Benguela current is a northward moving current, occupying a zone about 200 km wide off the west coast of southern Africa from Cape Town north to about latitude 15°S (Mann and Lazier 1996). The upwelling in the Benguela current system sustains some of the highest rates of primary production in the oceans (Carr 2002). It is caused by alongshore southerly winds, which transport surface water 45° to the right of the wind direction and Ekman transport 90° to the right of the wind direction (Colling 2002). The divergence of surface waters away from land leads to their replacement by upwelled subsurface South Atlantic water. The upwelling of deeper water masses in the euphotic zone is of high ecological importance, since they originate from 100-200 m depth and are colder and more nutrient-rich than surface waters. These water masses replace the nutrient-depleted surface waters and stimulate primary production (Colling 2002). Settling organic matter is then decomposed accompanied by oxygen consumption, which can lead to oxygen-depleted bottom waters (<10 µM O_2) (Chapman 1985).

4.1.1. Particles in marine systems

In the Namibian upwelling system, the concentrations of marine snow particles are among the highest reported for marine environments (Kiorboe et al. 1998, Simon et al. 2002). “Marine snow” is a collective term for a variety of fragile, amorphous, macroscopic particles ranging from 0.5 mm to many centimetres in longest dimension (Prezelin and Alldredge 1983). They consist of inorganic particles, detritus, phytoplankton, microorganisms etc.

Marine snow particles are believed to be the main vehicle for transporting organic matter from the upper ocean to the ocean floor (Fowler and Knauer 1986). However, marine snow particles are also subject to degradation most importantly through biological processes (Kiorboe 2001). Bacteria attracted to the organic particles intensively colonize marine snow, reaching densities of ~10^8 cells per millilitre (Smith et al. 1992) (Fig. 8).

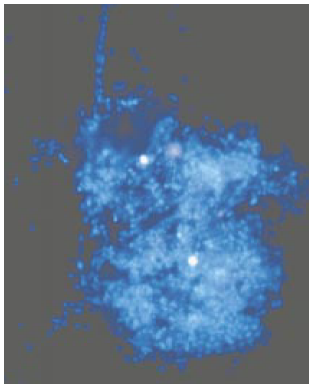


Fig. 8: 37 µm x 27 µm segment from a marine snow particle from Monterey Bay, California. The particle is intensively colonized by microorganisms (in blue) which were stained by the DNA-stain 4',6-diamidino-2-phenylindole (DAPI) (Azam and Long 2001).

Particle-inhabiting bacteria can produce a variety of extra-cellular enzymes, which break down components of the marine snow like proteins and polysaccharides and bring them into solution (Smith et al. 1992), resulting e.g. in higher ammonia concentrations compared to the ambient seawater (Shanks 1979). These remineralization processes convert the sinking organic matter into non-sinking DOM and keeps it in the upper ocean.

Aerobic respiration of microorganisms inhabiting marine snow particles reduces the oxygen concentration within particles and can lead to the formation of microaerophilic or even anaerobic microhabitats (Ploug 2001). These particles can thereby facilitate processes that do not take place at oxygen concentrations in the planctonic non-particulate seawater.

4.2. The Peruvian upwelling system

The Peruvian upwelling system in the eastern tropical South Pacific Ocean is another area of high primary production that creates one of the most important OMZs in today's oceans. Southerly alongshore winds cause, as in the Namibian upwelling system, the Ekman transport of surface waters offshore, which brings subsurface waters to the euphotic zone, driving primary production. The upwelled water originates from the poleward Equatorial Undercurrent and is cold, high in nutrient content and oxygen depleted (Copin-Montégut and Raimbault 1994, Friederich and Codispoti 1987). The nutrient-rich waters stimulate primary production in the euphotic zone and the decomposition of phytoplankton biomass further depletes dissolved oxygen, leading to the formation of the OMZ, which extends from the thermocline (~20 m water depth) to the shelf sediment and westward over the continental slope and abyssal plain.

4.3. The Arabian Sea

The Arabian Sea is located in the northern Indian Ocean and is enclosed to the north by the coast of Somalia, South-Yemen, Oman, Iran, Pakistan and India, which restricts circulation and exchange of water. The Arabian Sea contains a very stable low-oxygen layer, which is the thickest known in the present day's oceans, often ranging from the bottom of the euphotic layer (~100 m depth) down to ~ 1000 m (Sarma 2002).

Oxygen deficient conditions in the Arabian Sea are the consequence of its unusual geographic settings and a combination of physical and biological processes (Sarma 2002). Atmospheric and surface oceanic circulations are governed by two distinctly opposing monsoon cycles. During the southwest monsoon (June-August), strong winds blow from the southwest and produce offshore Ekman transport triggering intense coastal upwelling off Somalia, Yemen and Oman (Devol et al. 2006). Oxygen depleted, nutrient rich subsurface water is pumped into the surface layers by coastal upwelling and gets transported hundreds of kilometres offshore, enriching the euphotic zone with nutrients, which results in high primary production. Unlike in the Peruvian and Namibian upwelling systems, the coastal upwelling and the OMZ in the Arabian Sea are geographically separated (Morrison et al. 1999).

The northwest monsoon (December-February) drives cold, dry winds from the northeast and causes convective mixing over a large area, bringing oxygen-rich waters to the subsurface layers (Sarma 2002). Associated nutrient-pumping sustains primary production, which is comparable with that during the southwest monsoon (Barber et al. 2001). High primary production creates high oxygen demand and is one major factor for suboxic conditions in the Arabian Sea.

In summary, during the monsoon seasons the primary production increases and concomitantly the oxygen consumption in deeper water layers. Due to wind-driven circulations, oxygen is transported into the water, which prevents anoxic conditions in the Arabian Sea despite high oxygen demand (Sarma 2002). Suboxic conditions between the monsoon seasons when surface waters are oligotrophic are maintained by lower primary production and less oxygen transport in deeper water layers by lacking monsoon winds and physical transport. Another factor contributing to the suboxic conditions in the Arabian Sea is the restricted supply of oxygen by inflowing water from the Persian Golf, the Red Sea and the southern Indian Ocean.

5. Methodological aspects

Anammox bacteria have a doubling time of about 10 days (Strous et al. 1998). Therefore, enriching for them is a time-consuming procedure in which only a limited number of laboratories have so far succeeded. Until now, no one has achieved a pure culture of anammox bacteria, which is necessary for a valid taxonomical description. Therefore culture independent studies are of great importance to investigate the diversity, distribution, abundance and function of anammox bacteria in the environment.

5.1. The full cycle 16S rRNA approach

In 1977, the 16S rRNA proved to be a powerful molecule for the investigation of phylogenetic relationships, leading to the separation of prokaryotes into *Bacteria* and *Archaea* (Woese and Fox 1977). Reasons for its use as a "molecular clock" are its existence since the early stage of evolution, the presence in all organisms and its highly conserved function. It contains very conserved but also variable regions, which allows sequence comparison and phylogenetic analysis down to the genus and species level (Rossello-Mora and Amann 2001, Woese 1987).

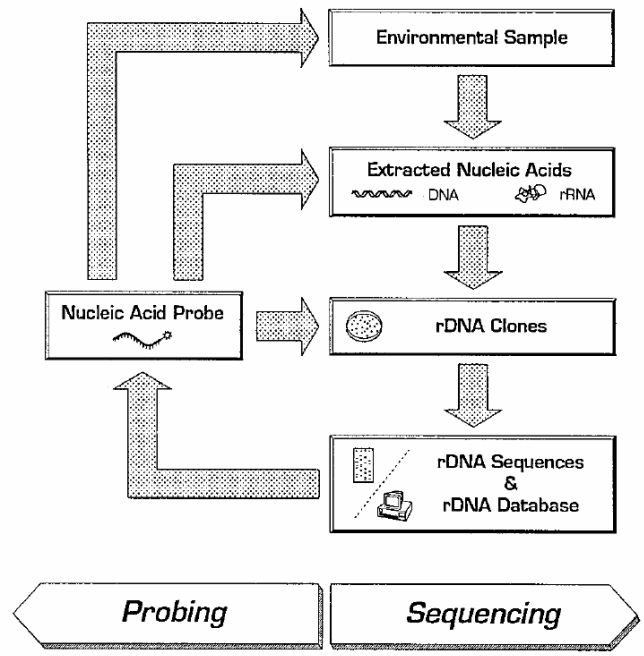


Fig. 9: Strategies of sequencing and probing within the 16S rRNA approach

After DNA extraction from environmental samples, rRNA genes can be amplified by polymerase chain reaction (PCR), cloned, sequenced and phylogenetically analyzed, which gives insights into the microbial diversity of the sample. Identification and quantification of the microorganisms is facilitated by hybridization (e.g. fluorescence in situ hybridization, FISH) using nucleic acid probes (Amann et al. 1995, Olsen et al. 1986).

Based on 16S rRNA gene sequencing novel microbial groups could be found, which were never observed in culture collections before, but seemed to represent an important fraction of environmental microbiota. Examples are the bacterial groups SAR11 (Giovannoni et al. 1990) and SAR86 (Mullins et al. 1995), as well as new groups of *Crenarchaeota* and *Euryarchaeota* (DeLong 1992, Fuhrman et al. 1992). The novel archaeal groups were not specialists or extremophiles, but represented "non-extreme" archaea, widespread in terrestrial and aquatic habitats (DeLong 1998). Up to now, the Ribosomal Database Project II (RDP II) contains 335,830 16S rRNA sequences (Release 9.48, status 1st March 2007) and the new SILVA database project even 353,366 sequences (www.arb-silva.de). These comprehensive databases allow the design of primers and probes with different specificity.

FISH is of importance since 16S rRNA sequence libraries do not contain information about the in situ abundance of microorganisms (Suzuki and Giovannoni 1996) and their ecological niche. This can be addressed by FISH. It is a technique which specifically stains the ribosomes of cells and allows cultivation-independent identification and quantification of microorganisms e.g. by epifluorescence microscopy (Amann et al. 1991, DeLong et al. 1989). The application of FISH with fluorochrome monolabeled probes on marine bacterioplankton was limited for a long time due to low signal intensity. Low signal intensity was most likely caused by small cell size or very low ribosome numbers in slow-growing cells. A very effective way to increase the signal to noise ratio and thereby the signal intensity is the catalyzed reporter deposition (CARD)-FISH method (Amann et al. 1992, Bobrow et al. 1989, Pernthaler et al. 2002, Schönhuber et al. 1997). Hybridization with horseradish peroxidase-labeled oligonucleotide probes is followed by an incubation with fluorochrome-labeled tyramides. Each horseradish peroxidase molecule can turn over several tyramide molecules, which leads to a strong signal amplification.

5.2. 16S-23S rRNA intergenic spacer region (ISR, ITS) as phylogenetic marker

Phylogeny based on 16S rRNA has restricted resolution power. Although the 16S rRNA contains variable and hypervariable regions (Stackebrandt and Rainey 1995), it is often not divergent enough to give good separation in close relationships, e.g. of all species in one genus (Normand et al. 1996). For studying the diversity and phylogeny of closely related strains, other phylogenetic marker genes should be used. The evolutionary rate of the 16S-23S rDNA intergenic spacer region, also known as internal transcribed spacer (ITS), is about 10 times greater than the evolutionary rate of the 16S rDNA (Leblond-Bourget et al. 1996). The ITS is extremely variable in size and sequence even within closely related

taxonomic groups (Gürtler and Stanisich 1996) permitting discrimination of species and even strain clusters.

Therefore, these two molecular markers provide different complementary information: The 16S rRNA gene is a good tool for inferring inter- and intrageneric relationships (Fox et al. 1992), comparison of the 16S-23S rDNA spacer sequence provides information concerning intra- and interspecific links (Leblond-Bourget et al. 1996) and allows the detection of recently diverged species.

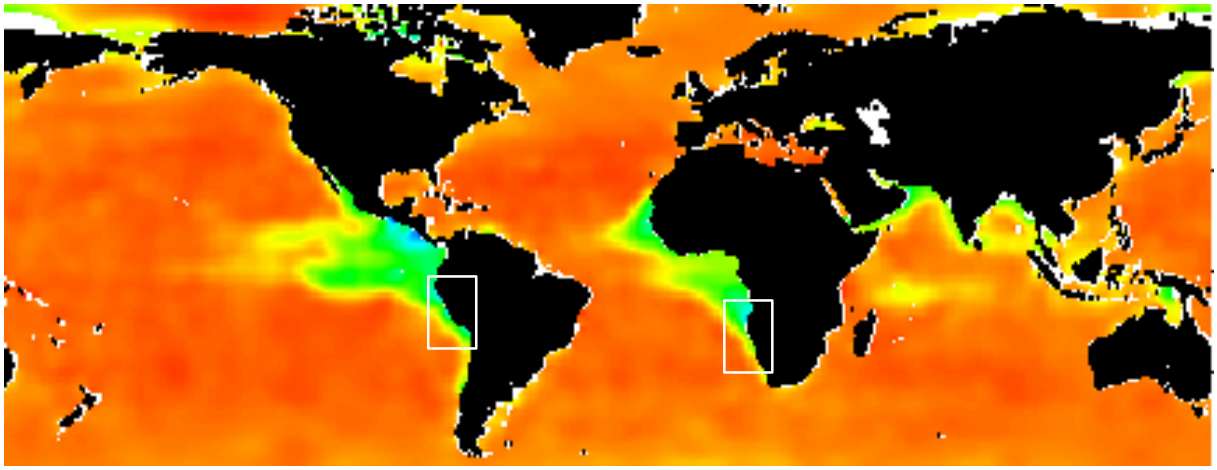
5.3. Genomics and Metagenomics

Improved sequencing techniques enabled the investigation of entire genomes from cultured microorganisms. The first sequenced marine microorganism was the archaeon *Methanocaldococcus jannaschii* isolated from deep-sea hydrothermal vents (Bult et al. 1996), but many followed. With the derived genomic information, it was possible to identify new genes, elucidate the enzymes involved in metabolic pathways or study the evolutionary relationships of organisms based on multiple genes or genome structure. However, the majority of microorganisms still resist cultivation. The genomic information of uncultured organisms can be addressed by the so-called metagenome approach, which comprises the genomes of all microorganisms in a certain habitat (Handelsman et al. 1998). Sequencing of uncultured organisms is facilitated by shotgun sequencing or by extracting high molecular weight DNA from the environment, cloning of large genomic fragments (>35 kb; e.g. into fosmids, cosmids and BACs), and screening for the clones containing the gene or expressing the enzyme of interest. With this approach, it was possible to gain insight into the metabolic potential of uncultured microorganisms. Another advantage of metagenomic libraries is that environmental DNA is cloned without a prior PCR step, which allows the investigation of microbial community diversity without PCR biases.

6. Aims

Within this PhD thesis the diversity and ecology of marine *Planctomycetes* were investigated. A special focus was drawn onto *Planctomycetes* which mediate the anaerobic oxidation of ammonium in marine OMZs, the anammox bacteria. This included their distribution, abundance and their ecological niche, as well as their identity and phylogenetic affiliation.

About 30-50% of the total nitrogen loss is currently estimated to take place in OMZs (Codispoti et al. 2001) and heterotrophic denitrification was assumed to be the major process responsible for this nitrogen-loss. However, very low concentrations of ammonium in suboxic waters indicated that the anammox process could be as well of importance in these ecosystems. But the anammox bacteria are known to be slowly growing organisms with a doubling time of about two weeks (Strous et al. 1998). Additionally, it was reported that anammox bacteria were very sensitive to oxygen and that oxygen concentrations as little as 1 µM already reversibly inhibited the anammox reaction (Strous et al. 1997). Thus, it was unknown, whether they would be able to thrive in more dynamic marine systems like the marine OMZs of coastal upwelling systems, where waters are more rapidly exchanged and concentrations of oxygen can vary considerably. The importance of the anammox process in the OMZs of the Namibian and Peruvian upwelling systems (Fig. 10) was studied with multiple approaches, of which the quantification of the anammox bacteria by fluorescence in situ hybridization (FISH) and their identification and phylogenetic affiliation was my contribution to these investigations.



Source: NOAA NODC World Ocean Atlas

Fig. 10: Map showing the location of the world's major oxygen minimum zones: the green-blue color at the eastern boundary of South and Central America and Africa represents regions with oxygen concentrations below ~ 4.5 µM and the orange color regions with higher oxygen concentrations.

The next goal was to answer the question, how the anammox bacteria can cope in these relatively dynamic systems with fluctuating oxygen and nutrient concentrations. Therefore it was planned to study their ecological niche in the water column. However, certain habitats in the water column like marine snow particles exhibit high autoflorescence which complicates fluorescence in situ hybridization with monolabeled oligonucleotide probes. To improve the signal to noise ratio of FISH for the confident detection of anammox bacteria, an improved protocol to stain the anammox bacteria had to be optimized. It was the goal to use the catalyzed reporter deposition (CARD)-FISH protocol for the visualization of the anammox bacteria to overcome autofluorescent-background problems.

Another objective of this thesis was the investigation of the diversity of anammox bacteria in marine OMZs. Since first results supposed a low diversity within the marine anammox bacteria, not only 16S rRNA sequences were used as phylogenetic marker genes, but also the 16S-23S rRNA intergenic spacer region, also known as internal transcribed spacer (ITS). 16S rRNA sequence analysis generally provides robust separation on the genus-level. The evolutionary rate of the ITS region is approximately 10 times higher than the evolutionary rate of the 16S rRNA gene (Leblond-Bourget et al. 1996). Therefore, the ITS permits a higher-resolution diversity analysis potentially down to the sub-species level (Gürtler and Stanisich 1996). During this study, samples from some of the major OMZs were analyzed: The Namibian and the Peruvian OMZ as well as the OMZ of the Arabian Sea and suboxic waters of the Black Sea.

Based on 16S rRNA sequences, the anammox bacteria are assumed to be deep-branching within the phylum *Planctomycetes* (Strous et al. 1999). The *Planctomycetes* are characterized by several unique features e.g. internal cell compartmentalization (Fuerst 2005) and a cell wall that lacks the typical bacterial cell wall component peptidoglycan, but instead is composed of proteins (König et al. 1984). While the knowledge about the chemolithoautotrophic anammox bacteria increases steadily, little is know about the chemoorganoheterotrophic planctomycetes, i.e. the genera *Pirellula*, *Rhodopirellula*, *Blastopirellula*, *Gemmata*, *Planctomyces* and *Isosphaera*. The third project within this PhD thesis was a comparative analysis of six planctomycete fosmids from the Namibian and Oregon upwelling with five planctomycete genomes including the waste water anammox bacterium *Candidatus* Kuenenia stuttgartiensis. The goal was to gain information about the physiological capabilities of marine planktonic planctomycetes with a focus on planctomycete-specific features and conspicuous metabolic pathways.

B. Results and Discussion

The Nutrient group headed by M. Kuypers has over the past years coordinated the interdepartmental anammox project of the MPI Bremen. OMZ waters were investigated by nutrient analyses, ^{15}N-tracer experiments, detection of ladderane lipids as biomarkers and by molecular techniques like 16S rRNA gene sequencing and fluorescence in situ hybridization (FISH). My contribution to the study of Kuypers et al, 2005 investigating the Namibian OMZ was the quantification of anammox bacteria by FISH. For the study from Hamersley et al, 2007, the investigation of the Peruvian OMZ, I provided 16S rRNA sequences of anammox bacteria in addition to their quantification by FISH. The objectives of my investigations were to quantify the anammox bacteria in the two OMZs to allow the calculation of anammox rates per cell in these habitats and their comparison with those in bioreactors, as well as the identification of the anammox bacteria and their phylogenetic affiliation.

1. The anammox process in the Namibian oxygen minimum zone

If heterotrophic denitrification was the only process responsible for the loss of fixed nitrogen in suboxic waters of the Namibian OMZ, ammonium should have accumulated in these waters. However, the ammonium concentrations were below the detection limit (Kuypers et al. 2005). In heterotrophic denitrification, two nitrate ions are combined to form N_2, whereas in the anammox process, one molecule of ammonium and one molecule of nitrite are used to form N_2. Thus, by ^{15}N-tracer experiments, anammox and denitrification can be distinguished, since denitrification produces $^{30}N_2$ and anammox $^{29}N_2$. ^{15}N incubations of Namibian OMZ waters indicated, that anammox rather than heterotrophic denitrification was responsible for the nitrogen loss (Fig. 11).

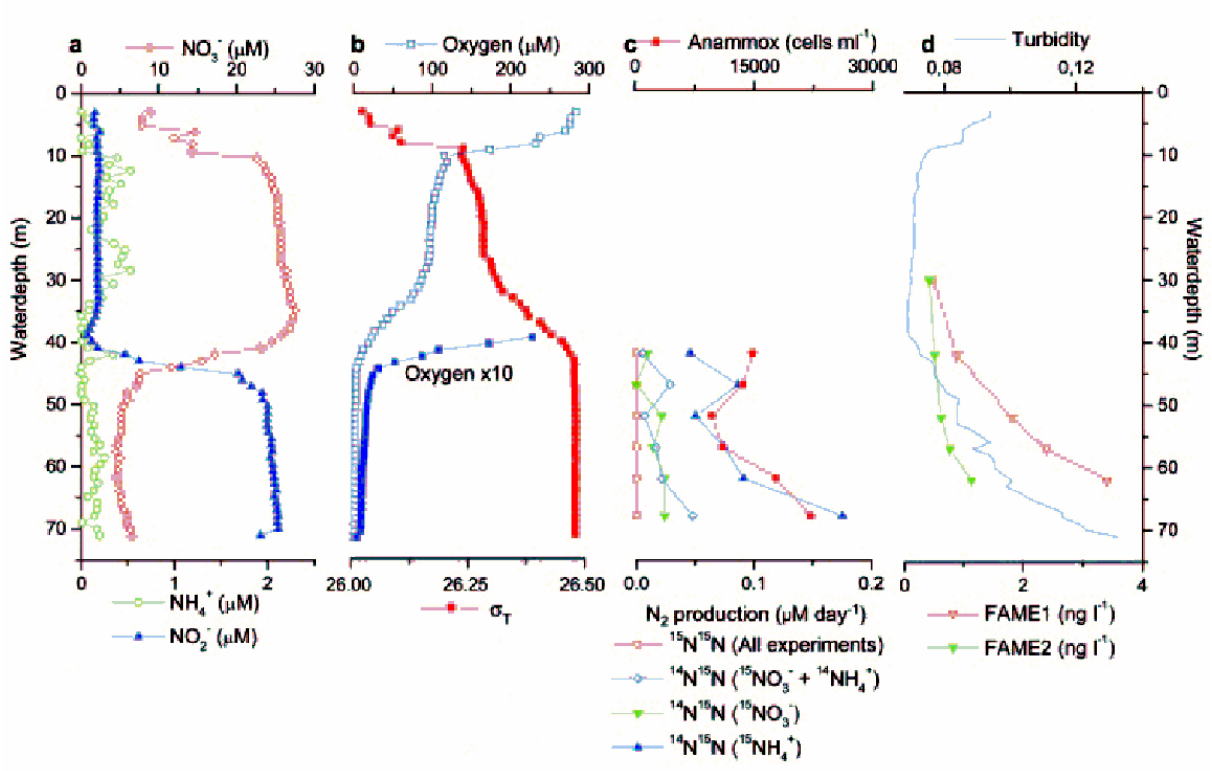


Fig. 11: Detection of the anammox process at station M202 in the Namibian upwelling system. (a) Nitrate, nitrite and ammonium concentrations over the water column. (b) Water density (s_T, the density of seawater in kg·m^{-3}- 1,000) and oxygen concentration (notice the 10-fold expanded O_2 gradient). (c) Anammox cell number per milliliter seawater and N_2 production. (d) Turbidity and depth distribution of ladderane lipids specific for anammox bacteria. FAME, fatty acid methyl ester. (Kuypers et al. 2005)

To link the anammox process to the responsible bacteria, firstly, the concentrations of anammox specific ladderane lipids were examined. Their distributions in the water column versus depth were similar to the anammox activity. Secondly, FISH with oligonucleotide probes specific for anammox bacteria was used for enumeration of the anammox bacteria. The relative abundance of anammox bacteria in the Namibian OMZ was quite low, ranging from 0.5% to 1.2%. With these low numbers, FISH of anammox bacteria reaches the detection limit of this method. However, a statistically relevant quantification is possible through the investigation of replicates and the use of multiple probes combined with the use of other anammox bacteria specific markers like ladderane lipids.

The total number of anammox cells in the Namibian OMZ ranged from 4000 to 22.000 cells per ml seawater. A strong linear correlation between anammox cell numbers and activity ($R^2 > 0.8$) indicated that anammox bacteria were responsible for the anaerobic oxidation of ammonium in the suboxic Namibian waters. The quantification allowed the calculation of cell-specific anammox activity (~ 4.5 fmol of ammonium per day). These rates were comparable with the rates estimated for the suboxic zone of the Black Sea (3-4

fmol of ammonium per day) and well within the range of rates measured for anammox bacteria in bioreactors (2-20 fmol of ammonium per day).

16S rRNA gene clone libraries showed that the anammox bacteria of the Namibian OMZ were closely related to *Candidatus* Scalindua sorokinii and *Candidatus* Scalindua wagneri (98% sequence identity).

Chemical profiles, ^{15}N-labeling experiments as well as ladderane lipid analyses and FISH counts indicated that active anammox bacteria were abundant at depths, where oxygen concentrations up to 9 µM were measured. Since it was known that anammox bacteria are reversibly inhibited by oxygen concentrations as low as 1 µM, we hypothesized the occurrence of anammox bacteria in anoxic microniches existing in macro-aggregates. During sampling in the Namibian OMZ, a high density of particles was observed. This is supported by studies reporting particle concentrations in this ecosystem which are among the highest known for marine systems (Kiorboe et al. 1998, Simon et al. 2002). The oxygen concentration in particles decreases due to aerobic respiration of particle inhabiting bacteria (Ploug 2001). This can lead to anaerobic microniches within a particle at low ambient oxygen concentrations, thus allowing the anammox reaction under otherwise unfavourable conditions. To investigate the occurrence of anammox bacteria in particles I used catalyzed reporter deposition (CARD)-FISH and indeed could observe anammox bacteria attached to particles (Fig. 12).

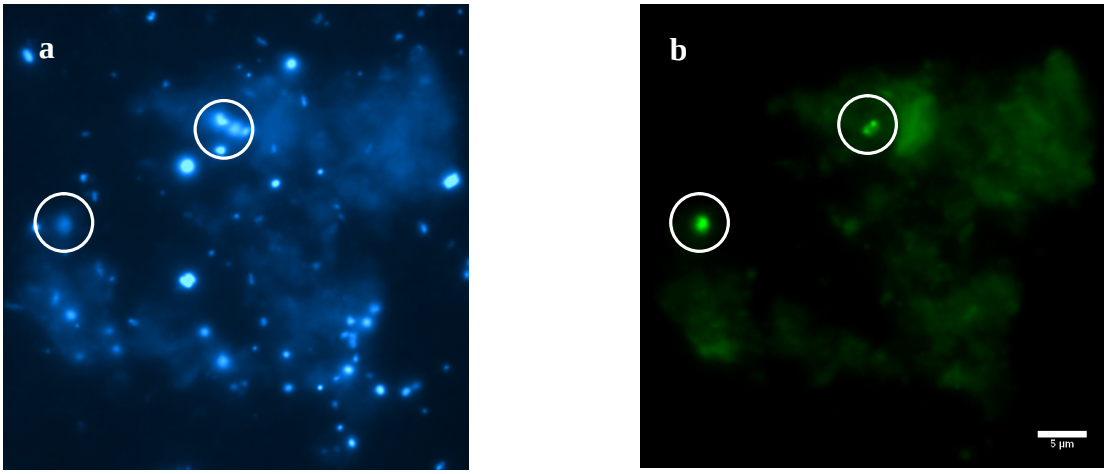


Fig. 12: Photomicrographs showing particle-associated microorganisms unspecifically stained with the DNA stain 4',6-diamidino-2-phenylindole (DAPI) (a) and anammox bacteria (encircled) stained specifically by CARD-FISH (b).

2. Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian OMZ

The major objective of this study was to further investigate the distribution and particle association of anammox bacteria in the Namibian OMZ. CARD-FISH was used to identify and localize anammox bacteria and the co-occurring microbiota. Special emphasis was drawn on comparing the relative abundances of co-occurring particle-attached groups versus their abundance in the free water phase and on their potential contribution to the anammox process.

The low signal intensity of anammox bacteria stained with standard FISH and on the other hand the high autofluorescence of particles complicated the reliable visualization and investigation of particle-attached anammox bacteria. Therefore, the CARD-FISH protocol was optimized for the in situ detection of particle-attached anammox bacteria. With this improved protocol it was observed that around 55% of the stained anammox cells were organized in tight clusters (Fig. 13a), ~25% were attached to particles (Fig. 13b) and ~20% were single cells not organized in clusters or attached to particles. We assume that the anammox clusters were originally attached to particles, but were separated from them during the sampling or fixation procedure. The same might also apply for the observed single cells.

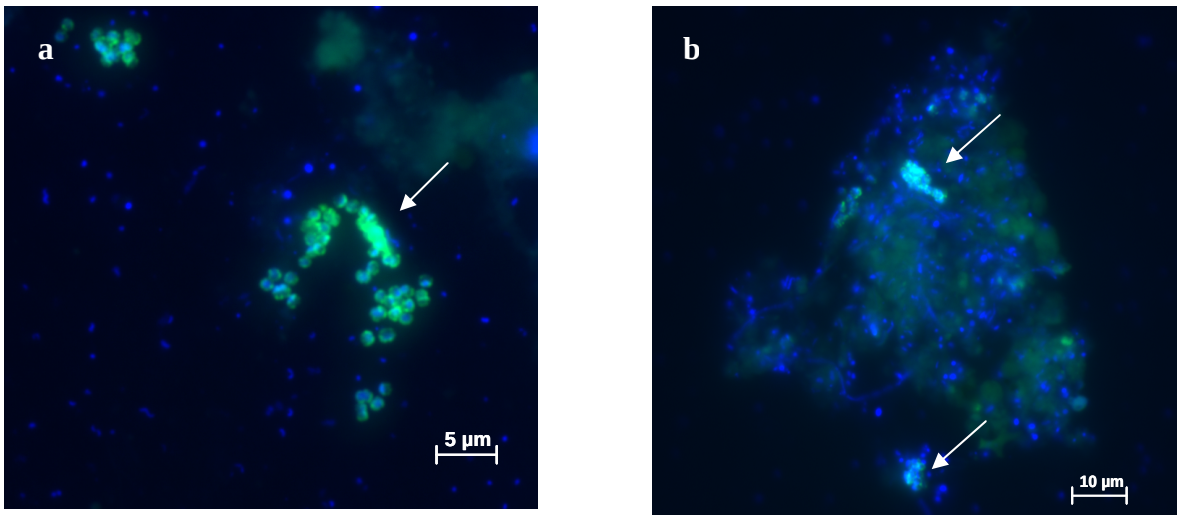


Fig. 13: Combined epifluorescence micrographs from samples of the Namibian OMZ. (a) anammox cells forming clusters, (b) anammox cells in association with particles. Anammox cells are stained both with DAPI (blue) and Fluorescein–labeled tyramide (green) after CARD-FISH with HRP-labeled probe BS-820 and appear turquoise. Non-anammox cells are stained only with DAPI and appear blue.

Methodological restrictions of catalyzed reporter deposition (CARD)-FISH

When comparing standard FISH counts of anammox cells with CARD-FISH counts an apparent discrepancy was observed. CARD-FISH detected at most 40% (27±13%) of the anammox cells detected by standard FISH (Fig. 14). However, both methods showed similar trends in depth distribution of anammox cells through the water column, which is supported by a strong linear correlation of both methods detecting anammox cells (R=0.91, P<0.005, n=12).

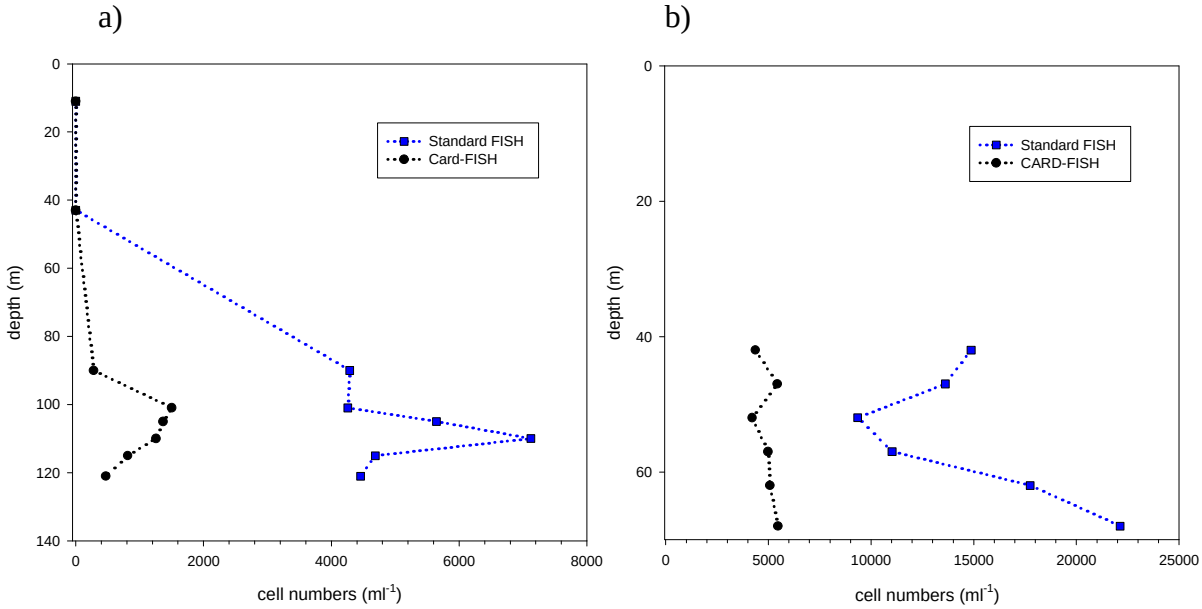


Fig. 14: Cell numbers of anammox bacteria stained by standard FISH (blue) or CARD-FISH (black) at station M182 (a) and M202 (b) in the Namibian OMZ.

It is an interesting methodological aspect that CARD-FISH counts of anammox bacteria were consistently lower than those of standard FISH. For activated sludge aggregates Schönhuber et al. had reported a similar phenomenon (Schönhuber et al. 1997). CARD-FISH stained only half of the cells which were stained with standard FISH. This is likely caused by the larger horseradish peroxidase label used for CARD-FISH (~44 kDa), compared to an oligonucleotide labeled with a fluorescent dye (~700 Da). During hybridization the enzyme-labeled probe has to penetrate the extracellular polymeric material in the bacterial cell wall. Depending on the exact composition of the cell wall, probe penetration might be inhibited, e.g., in gram-positive bacteria (Bidnenko et al. 1998). Therefore, a permeabilization step is performed prior to the hybridization. The cell wall of gram-negative bacteria is for example treated with lysozyme, whereas the archaeal cell wall can be easily digested by a short treatment with HCl (1 min, 0.1 M).

The planctomycete cell wall lacks the typical bacterial cell-wall component peptidoglycan. Studies in the 1980s have shown that they have a proteinaceous cell wall, mainly consisting of proline and cysteine (König et al. 1984, Liesack et al. 1986). The cell wall is stabilized by disulfide bonds formed by two cysteine residues. But the exact composition and structure of the anammox bacterial cell wall has not been studied in detail. Therefore, a variety of permeabilization methods were applied in an attempt to optimize the CARD-FISH protocol for anammox bacteria. Treatments with enzymes, detergents or a protocol which targeted the disulfide bonds of the proteinaceous cell wall were applied. However, even harsh treatments with proteinase K or achromopeptidase in high concentrations, which led to a complete digestion of the surrounding microbial community, did not result in staining of the entire anammox bacteria population.

Some planctomycetes like *Rhodopirellula baltica* SH1^T have different morphotypes in certain growth phases. Budding cells release swarmer cells which distribute, settle, become sessile cells and reproduce again (Gade et al. 2005). It is possible that also anammox bacteria exhibit different morphotypes and that some life cycle stages have an extreme stable, pronounced and thus impermeable cell-wall. It is also known from gram-positive bacteria that not all life cycle stages can by successfully hybridized (Chatzinotas et al. 1998, Hahn et al. 1993). Therefore we conclude that standard FISH is still the method of choice to quantify anammox cells in homogenized samples. However, CARD-FISH is the method of choice for monitoring and localizing anammox bacteria on particles with elevated autofluorescence.

Microbial community inhabiting particles

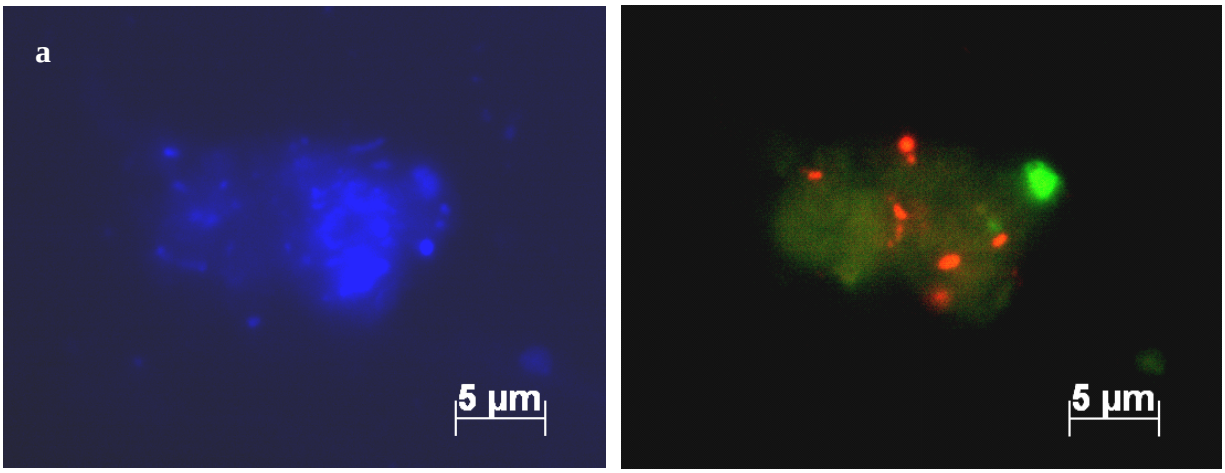
The microbial community inhabiting particles and the free water phase was investigated by CARD-FISH. We could identify microorganisms which clearly preferred the free water zone, while other groups preferred the particle as their habitat (Table 1). The abundance of *Gammaproteobacteria* was significantly higher in particles than in the free water phase. On the contrary, members of the SAR11 cluster as well as *Crenarchaeota* and *Euryarchaeota* preferred the free water phase. No significant differences in abundance were detected for the *Cytophaga*/*Flavobacterium* cluster, the SAR406 cluster or the *Alphaproteobacteria*. However, the species composition within the groups might differ.

Table 1: Overview of the relative abundances of different phylogenetic groups in the free water phase (suboxic to anoxic waters) and occurring in particles in the Namibian upwelling system.

Phylogenetic group	Relative abundance (%) Free water phase (suboxic to anoxic)	Particle associated	Significance
Alphaproteobacteria	17.1 (±7.3)	11.6 (±4.8)	P>0.05
SAR11-clade	16.5 (±3.5)	2.6 (±1.7)	**P<0.001**
Roseobacter-clade	3.1 (±2.2)	3.2 (±1.8)	P>0.05
Gammaproteobacteria	8.1 (±1.8)	11.8 (±5.0)	**P<0.01**
SAR86-clade	<1	<1	--
NOR5/OM60 clade	<1	1.6 (±1.6)	--
Pseudoalteromonas, Colwellia	<1	<1	--
Alteromonas/Colwellia	1.1 (±1.3)	<1	--
Nitrosococcus oceani, N. halophilus	ND	ND	--
Ammonia-oxidizing ***Betaproteobacteria***	ND	ND	--
***Cytophaga/Flavobacterium* cluster**	13.7 (±6.4)	12.4 (±3.5)	P>0.05
SAR406-clade	3.3 (±1.0)	4.0 (±2.2)	P>0.05
***Crenarchaeota* group I**	14.9 (±4.6)	2.2 (±1.8)	**P<0.001**
Euryarchaea	2.7 (±1.9)	<1	**P<0.001**

ND: not detectable; in bold: significant differences in abundance

The most abundant groups in particles were *Alphaproteobacteria*, *Gammaproteobacteria*, members of the *Cytophaga*/*Flavobacterium* cluster as well as the SAR406 cluster (Fig. 15). These results were supported by investigating aggregates from the Peruvian OMZ, where the same groups dominated the microbial community in aggregates. Similar to the Namibian OMZ, *Crenarchaeota*, *Euryarchaeota* and members of the SAR11 cluster were only rarely found in aggregates.



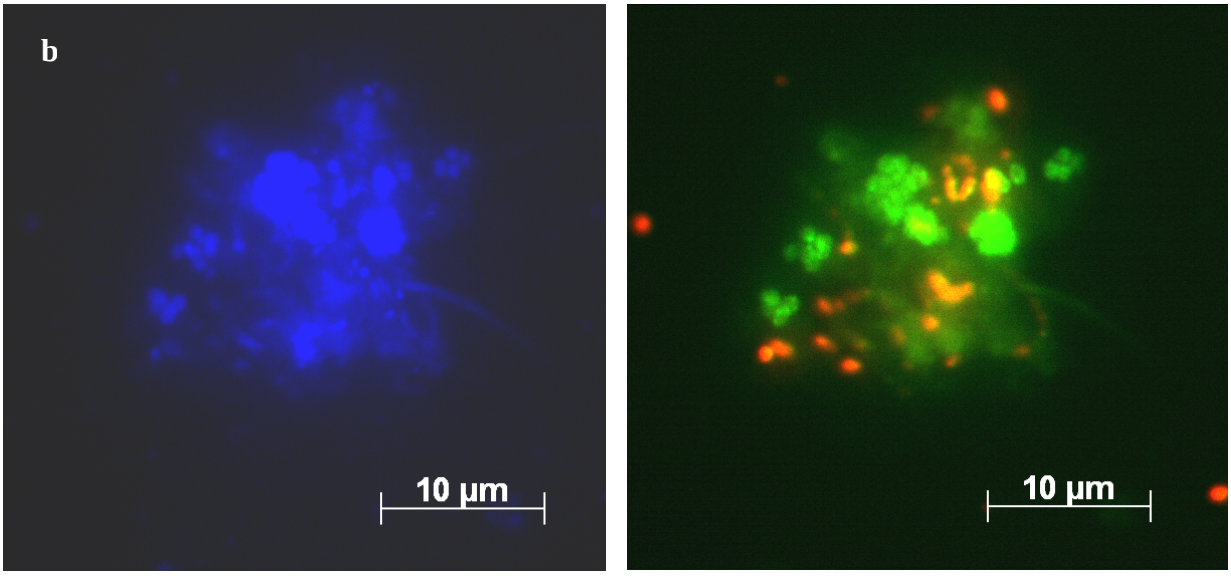


Fig. 15: Combined epifluorescence micrographs from particles in the Namibian OMZ stained with CARD-FISH. Double hybridizations were conducted with the anammox specific probe BS-820 and probes for other phylogenetic groups. (a) Probe CF319a targets members of the *Cytophaga/Flavobacterium* cluster, (b) Probe Gam42a targets *Gammaproteobacteria*. The left panel shows cells stained with DAPI in blue. In the right panel, anammox bacteria are shown in green; other groups of bacteria are stained in red.

This study provides the first quantification of major bacterial and archaeal groups in marine particles by fluorescence in situ hybridization. It also gives insight into potential interactions between anammox bacteria and other microbial groups, even though no specific group is tightly physically associated with anammox bacteria. We hypothesize that heterotrophic bacteria like *Alpha-* and *Gammaproteobacteria* and members of the *Cytophaga*/*Flavobacterium* cluster will reduce the oxygen concentration in particles by aerobic respiration (Fig. 16a), thereby reducing it to levels suitable for the anammox reaction (Fig. 16c). Furthermore, remineralization activity of members of the same groups could increase the ammonium concentration in particles (Fig. 16b). This would supply anammox bacteria with this nutrient, since the ambient water is depleted in ammonium. Aerobic ammonium oxidizing bacteria were not detected by FISH or CARD-FISH; however, nitrite could be supplied by *Crenarchaeota* (Fig. 16d). This group was highly abundant in the free water phase and closely related to '*Nitrosopumilus maritimus*', which is known to aerobically oxidize ammonium to nitrite (Könneke et al. 2005).

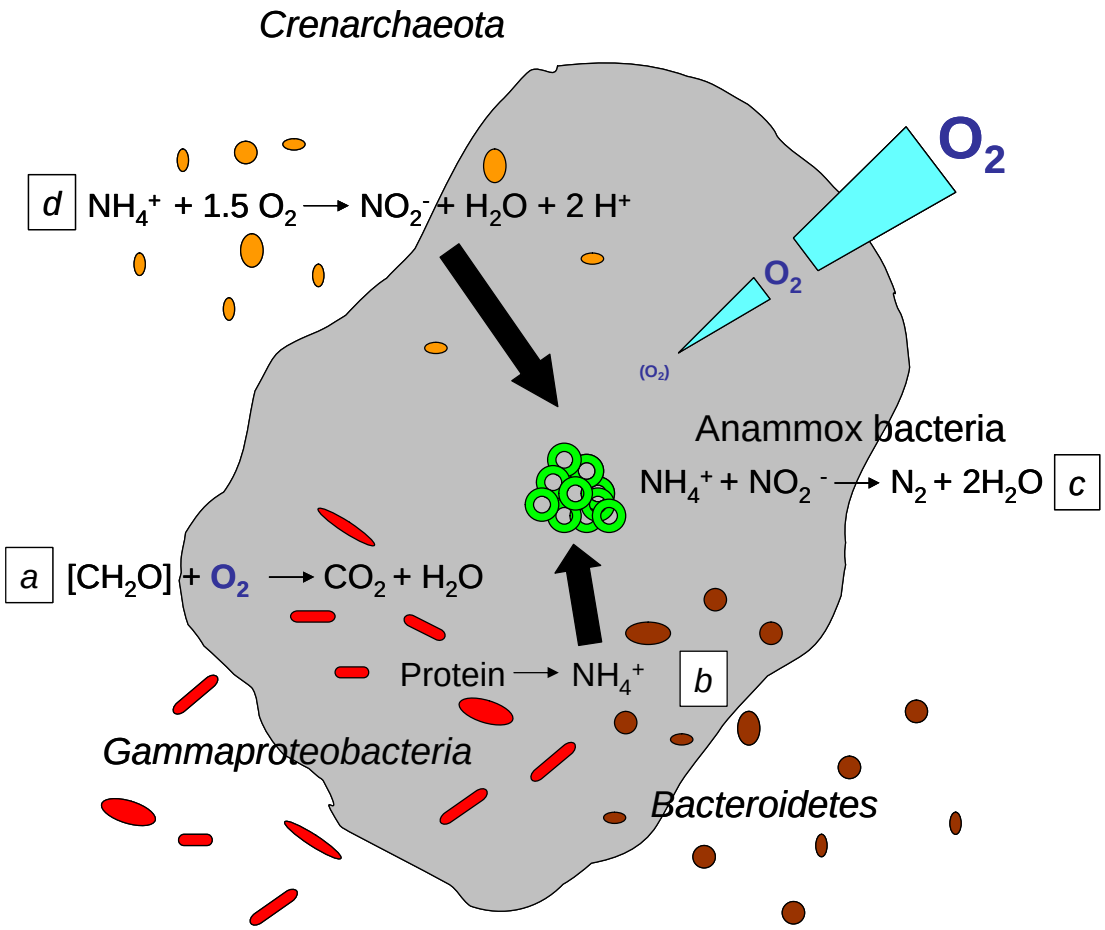


Fig. 16: Summary of potential microbial interactions on particles in the water column.

Only recently it was shown that anammox bacteria might be able to reduce nitrate to ammonium via dissimilatory nitrate and nitrite reduction (Kartal et al. 2007). In addition to the previously proposed scenario, this could constitute another possibility for the supply of the anammox bacteria with ammonium for the anammox reaction.

3. The anammox process in the Peruvian OMZ

The finding that anammox rather than denitrification was responsible for the loss of fixed nitrogen in the OMZ of Namibia led to the hypothesis, that anammox might be important in other OMZs such as the Peruvian. The role of anammox and denitrification in nitrogen loss from Peruvian OMZ waters was alike the previous Namibian study investigated by a multidisciplinary approach. My focus was the quantification of anammox bacteria by fluorescence in situ hybridization in addition to their identification through 16S rRNA gene clone libraries.

As in the Namibian OMZ, the predominant process responsible for the nitrogen loss in the Peruvian OMZ was shown to be the anaerobic oxidation of ammonium. Denitrification could not be measured in the samples studied (Fig. 17c).

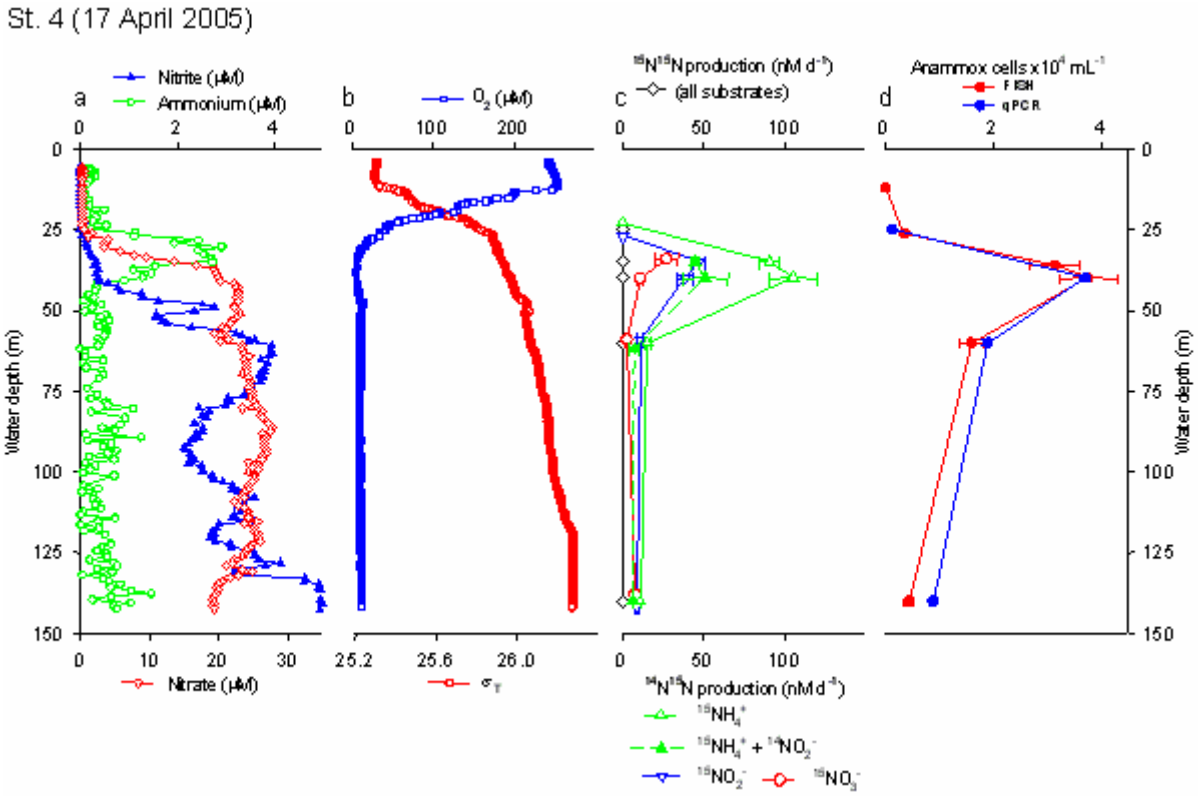


Fig. 17: Detection of the anammox process in the Peruvian upwelling system at station 4. (a) Concentration of nitrite, ammonium and nitrate. (b) Oxygen concentration and density. (c) N_2 production measured by ^{15}N-tracer experiments. Denitrification in black (not detected) and anammox rates in green, red and blue. (d) Anammox cell number determined by FISH (red) and quantative PCR (blue).

Additionally, dissimilatory nitrate reduction to nitrite coupled to anammox was detected, although the microorganisms responsible for this conversion could not be elucidated. Ladderane lipids were found at all depths where anammox activity was measured (not shown in Fig. 17). Likewise, anammox bacteria detected by FISH could be found at all depths where anammox took place, but not in the mixed water layer (Fig. 17d). In average, the relative abundance of anammox bacteria was higher in Peruvian OMZ (0.8-3.1%) than in the Namibian OMZ (0.6-1.2%). Their total cell numbers reached a maximum of $13x10^4$ to $15x10^4$ cells per ml seawater, which are so far the highest anammox numbers reported for the water column. The mean anammox cell number at each site was highly correlated with the mean ladderane concentration, when all depths were averaged over the whole water column. The depths distributions of anammox cell numbers were highly correlated with anammox rates within stations. Cell-specific anammox activities ranged

from 0.4 to 2.4 fmol per day and were comparable to rates from the Namibian OMZ and Black Sea suboxic waters.

^{15}N-tracer incubations as well as quantification of anammox cells and ladderane lipids showed that anammox bacteria were abundant in waters with in situ oxygen concentrations of up to 20 µM. After establishment of anoxic conditions, the bacteria immediately started with the anammox metabolism, as observed in the Namibian OMZ, suggesting that the cells in these waters were active in situ. In summary, studying the anammox process in the Peruvian OMZ has shown that anammox bacteria have the capability to persist in this dynamic environment. Alike the observed particle-association of anammox bacteria in the Namibian OMZ, preliminary results suggested that anammox bacteria in the Peruvian OMZ occur in aggregates. Decreased oxygen concentrations within an aggregate could facilitate the anammox reaction in ambient waters with oxygen concentrations up to 20 µM.

4. Microdiversity of anammox bacteria from major marine OMZs

In the second major part of this thesis I took a closer look at the diversity of anammox bacteria in different major OMZs. This included besides the Namibian and Peruvian OMZ the Black Sea and as a not yet studied OMZ the one in the Arabian Sea. For the latter it was unknown, whether anammox was of importance in this thickest low-oxygen layer in the present day's oceans.

The previous 16S rRNA gene clone libraries from the Namibian and Peruvian OMZs showed a rather reduced diversity. All anammox sequences were closely related to *Candidatus* Scalindua sorokinii. This finding was supported by other studies investigating marine and freshwater sediment samples as well as marine water samples by 16S rRNA gene sequences (Penton et al. 2006, Schmid et al. 2007). However, the study accomplished during this PhD thesis investigated the diversity of anammox bacteria of marine OMZs in greater detail since one of the most important OMZ, the Arabian Sea OMZ, was included. Furthermore, in addition to the 16S rRNA sequence, which normally gives robust separation on the genus-level, we used the 16S-23S rRNA intergenic spacer region (ITS), that has a greater evolutionary rate than the 16S rRNA gene (Leblond-Bourget et al. 1996). Thus, the ITS allows a higher-resolution diversity analysis potentially down to the sub-species level (Gürtler and Stanisich 1996) and enables the investigation of anammox bacterial microdiversity in marine OMZs.

Additionally, in previous studies very often at least one PCR primer was used that was specific for the known anammox bacteria. This will restrain the diversity analysis to the established anammox bacteria. In this study, the planctomycete-specific forward primer Pla46F (Neef et al. 1998) that targets the 5' end of the 16S rRNA gene and the universal reverse primer 1037R (targeting *E. coli* position 1930-1948 on the 23S rRNA gene) (Ludwig et al. 1992) were used for the amplification of the 16S rRNA-ITS-23S rRNA genes. The planctomycete-specific forward primer was used in order not to restrict the investigation to established anammox bacteria of the *Candidatus* genera Scalindua, Brocadia, Kuenenia and Anammoxoglobus, but to allow the discovery of potentially new clades of anammox bacteria. In previous studies it was observed that the clone libraries with primer Pla46F under-represented the abundance of anammox bacteria (Egli et al. 2001, Schmid et al. 2000, Schmid et al. 2003). However, it is known that the polymerase chain reaction is biased (Polz and Cavanaugh 1998, Suzuki and Giovannoni 1996) and that clone frequencies do not necessarily reflect the actual abundance of a specific group in the environment. The clone libraries from the four different suboxic water columns indeed resulted in apparent different frequencies of clones related to established anammox bacteria. Peruvian OMZ waters gave the highest percentage of anammox-like sequences, whereas the Black Sea sample gave the lowest. Another planctomycete-specific primer (58F, Liesack and Stackebrandt 1992) was tested, but yielded no detectable PCR amplicons.

Based on 16S rRNA genes, the Namibian OMZ clone library contained six sequences which were not closely related to the established anammox bacteria, but to sequences supposedly comprising a novel group of anammox bacteria (93% sequence identity; Fig. 18, group including Namibia_n173) (Tal et al. 2005). However, the16S rRNA sequence identity of this group to known anammox bacteria is quite low (79-84%) and the ability of this group of bacteria to anaerobically oxidize ammonium remains to be verified either by enrichment cultures or metagenomic studies.

In contrast and as already mentioned above, all four clone libraries contained clones with DNA fragments related to established anammox bacteria. The 16S rRNA sequences of the 87 clones were closely related to the *Candidatus* Scalindua clade (Fig. 18) and their 16S rRNA sequence identity to *Candidatus* Scalindua sorokinii ranged from 95.3% (Arabian Sea) to 98.3% (Black Sea, Namibia and Peru).

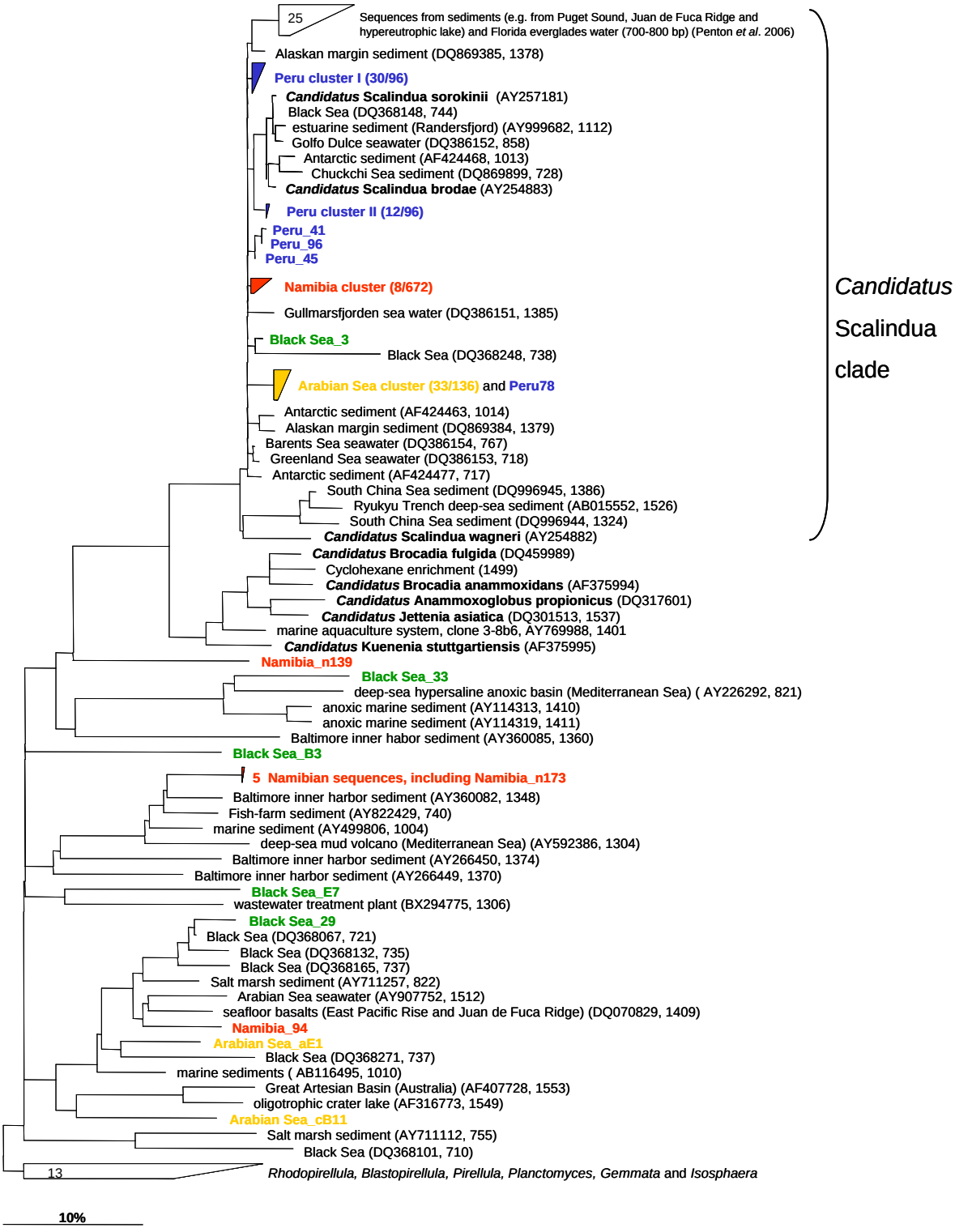


Fig. 18: Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic affiliations of anammox bacterial sequences from the Namibian, Peruvian and Arabian Sea OMZs, as well as the Black Sea suboxic waters with previously published anammox bacterial sequences. The consensus tree was built based on maximum likelihood, neighbour joining and maximum parsimony trees, calculated without and with 50% position variability filters. Sequences retrieved in this study are pointed out by a colour code (Peru: blue, Namibia: red, Arabian Sea: yellow and Black Sea: green). Accession numbers and sequence lengths are stated in parentheses.

Microdiversity studies of these clones based on 16S rRNA and ITS sequence showed distinct subclusters of the sequences which were unique to each OMZ (Fig. 19) and to a remarkable degree similar for 16S rRNA and ITS sequences. One Namibian OMZ specific cluster, one cluster specific for Arabian Sea sequences and two clusters specific for sequences from the Peruvian OMZ could be determined. Only three Peruvian sequences showed a slightly different phylogenetic affiliation based on 16S rRNA or ITS sequences (Peru41, Peru96 and Peru45). From the Black Sea, only a single anammox bacterial sequence could be retrieved. The high sequence identity of >98% within in each OMZ suggests that we see representatives of several strains of a singly species.

The ITS sequences from all anammox related sequences of all samples encoded the tRNAs for alanine and isoleucine. In comparison with wastewater anammox species (sequence identity from 63% to 82%, length from 376-529 nucleotides), the marine anammox ITS sequences were surprisingly similar to each other. They showed little sequence variation, both with respect to identity (minimum sequence identity of 80%) and length (403-462 nucleotides). The Arabian Sea sequences were distinctly longer (442-462 nucleotides) than ITS sequences from the Peruvian and Namibian OMZs (403-414 nucleotides).

The distinctiveness of anammox bacterial sequence from the Arabian Sea was supported by sequence identity values based on 16S rRNA and ITS sequences. The anammox bacterial 16S rRNA sequences from the Namibian and Peruvian OMZs showed ~98% sequence identity, whereas both sequence types had only 96-97% sequence identity to Arabian Sea sequences. The difference is even more obvious based on ITS sequences, since Peruvian and Namibian sequences shared 90-95% identity, but their sequence identities with Arabian Sea ITS sequences reached only 79-86%. Apparently, anammox sequences from Namibia and Peru were more closely related to each other than either group to the anammox sequences from the Arabian Sea. One could speculate that the closer relationship of the Namibian and Peruvian anammox bacteria reflects the more similar habitat they originate from.

Anammox sequences from the Arabian Sea had at maximum 96.6% sequence identity with another uncultured organism from the Alaskan margin sediment (DQ 869384) and at maximum 96.5% sequence identity with a cultured anammox bacterium (*Candidatus* Scalindua brodae). If the typical 97% sequence identity is used for species level cut-off (Rossello-Mora and Amann 2001), we speculate that the group of anammox bacteria from

the Arabian Sea comprise a novel species within the *Candidatus* genus Scalindua and propose the name *Candidatus* Scalindua arabica.

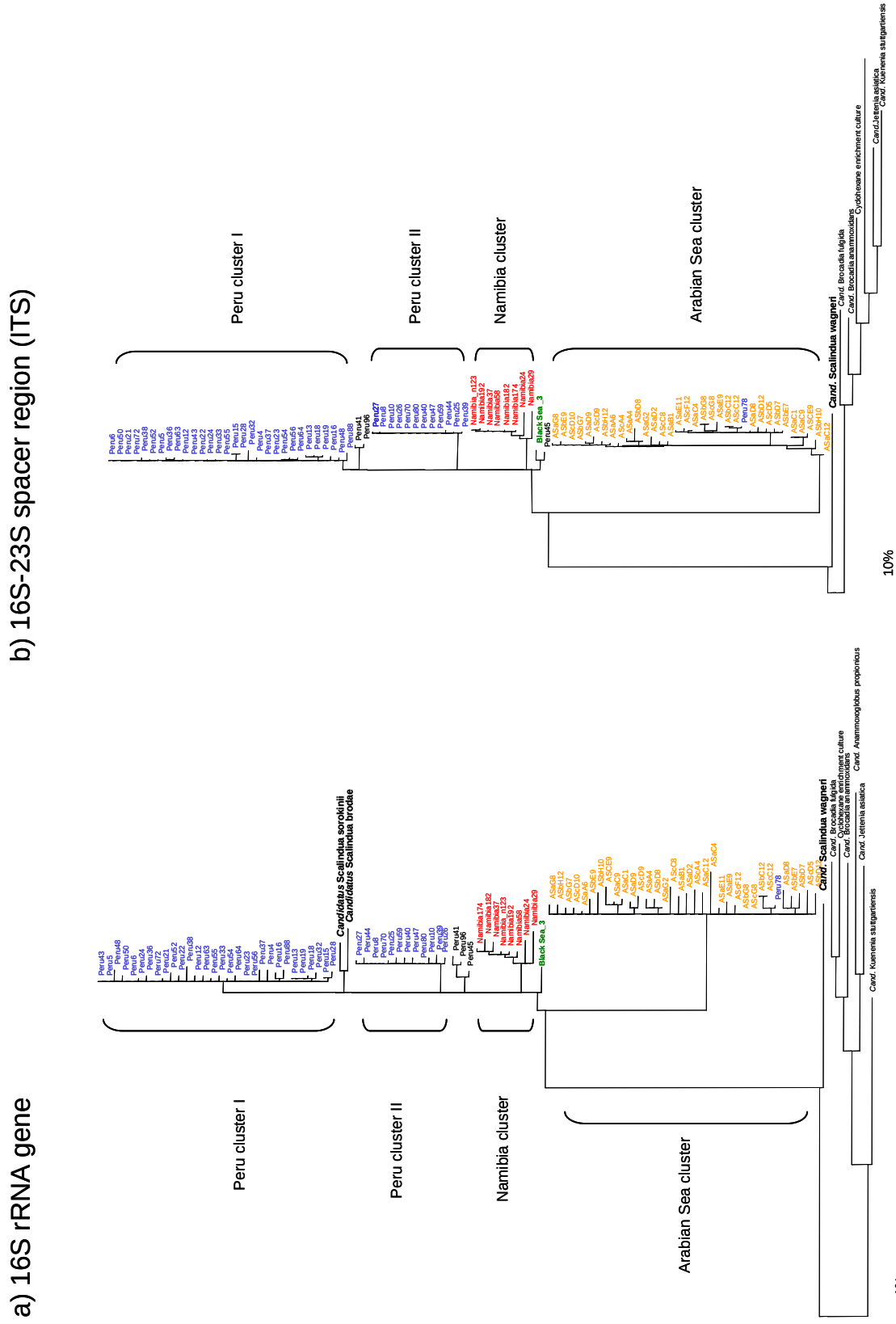


Fig. 19: (a) Consensus tree based on 16S rRNA sequences showing the grouping of the anammox bacterial sequences from the Arabian Sea, the Peruvian and Namibian OMZs, as well as the Black Sea in unique clusters. (b) Consensus tree based on ITS sequences from the Arabian Sea, the Peruvian and Namibian OMZs, as well as the Black Sea supporting the cluster pattern based on 16S rRNA sequences.

Conclusive remarks about anammox in marine ecosystems and outlook

The anammox process has been detected in diverse ecosystems, like marine, coastal and estuarine sediments (Engstroem et al. 2005, Risgaard-Petersen et al. 2004, Rysgaard 2004, Thamdrup and Dalsgaard 2002, Trimmer et al. 2003), anoxic basins (Dalsgaard et al. 2003, Kuypers et al. 2003), mangroves (Meyer et al. 2005), sea-ice (Rysgaard and Glud 2004) and freshwater lakes (Schubert et al. 2006). After the discovery of anammox in the Black Sea and Golfo Dulce it was questioned that the anammox process could be of importance in the marine OMZs. The reason for this skepticism was that anammox bacteria were shown to be active only under very restricted conditions, like lack of oxygen and sulphide and presence of nitrite. Thus it was assumed that the extent of oceanic OMZs in which anammox might occur would be rather limited (Ward 2003).

However, our studies together with findings of other groups to date provide strong evidence, that anammox is the major pathway for the loss of fixed nitrogen as N_2 from the Namibian and the Peruvian OMZs. A recent study reported the anammox process to take place in the Chilean OMZ (Thamdrup et al. 2006), whereas heterotrophic denitrification could not be measured. Very recently, anammox-specific ladderane lipids were detected in Arabian Sea OMZ waters (Jaeschke et al. 2007). This together with the anammox bacteria related sequences reported in chapter B 4 provides evidence for the existence of anammox bacteria in the Arabian Sea. However, the exact contribution of anammox to the fixed nitrogen-loss in the Arabian Sea remains to be resolved (Devol et al. 2006, Nicholls et al. 2007). All these studies indicate besides a world wide distribution of anammox that the anammox process might be of equal or perhaps even greater importance for the nitrogen-loss in marine systems than heterotrophic denitrification.

Additionally, we have to change our view of the metabolic capabilities of the anammox bacteria. We are beginning to understand that they are far more metabolic versatile than we thought (see chapter A 2). For example, if marine anammox bacteria can reduce nitrate to nitrite and in a second step to ammonium, they can produce the reactants necessary for the anammox reaction. Consequently, anammox will be indistinguishable from denitrification based on conventional isotopic tracer experiments. Thus, the nitrogen loss so far attributed to denitrification might arise from anammox, meaning that the anammox process could be responsible for an even larger fraction of the nitrogen loss in marine suboxic to anoxic ecosystems.

The microdiversity study presented in chapter B 4 supports recent studies, which indicate that most marine environments harbour anammox bacteria closely related to the

Candidatus Scalindua group (Hamersley et al. 2007, Kuypers et al. 2005, Penton et al. 2006, Schmid et al. 2007). However, it also suggests species-level endemism of e.g. *Candidatus* Scalindua arabica in the Arabian Sea. The investigation of other marine systems like the Chilean OMZ will shed further light on this hypothesis. The design and application of FISH probes specific for certain marine anammox bacterial clusters will in the future allow the investigation of their distribution and potential seasonal dynamics. Additionally, the ITS sequences of marine anammox bacteria now enable the design of FISH probes targeting the ITS. This allows, different from FISH of 16S rRNA, the detection of active anammox bacteria (Schmid et al. 2001). Furthermore, one could confirm that anammox bacteria are active in waters with 10-20 µM oxygen and investigate, whether they are active in the free water phase or only in particles.

The increasing number of anammox bacterial 16S rRNA sequences has shown that in addition to the sequences found in the Peruvian OMZ, also a substantial fraction of the Arabian Sea sequences showed a mismatch with the commonly used FISH probe BS-820, specific for *Candidatus* Scalindua wagneri and *Candidatus* Scalindua sorokinii. To detect the entire anammox population in these ecosystems, it is necessary to complement the probe with an additional probe (as described in Hamersley et al. 2007) or to design a new probe specific for marine anammox bacteria. Indeed, high priority should be given to the design and optimization of an updated probe set for marine anammox bacteria.

The discovery of other groups of bacteria mediating the anammox reaction solely based on 16S rRNA gene clone libraries from the environment is rather difficult if not impossible. It is necessary to couple the 16S rRNA gene sequences to the detection of anammox activity, which has traditionally been done in cultivation approaches. Alternatively, the potential ability of the anaerobic oxidation of ammonium could be studied by metagenome libraries. This needs to be done for the presumable novel group of anammox bacteria proposed by Tal and co-workers (Tal et al. 2005), of which related sequences were also found in the 16S rRNA gene clone libraries from the Namibian OMZ. One could screen a metagenomic library for the 16S rRNA of these planctomycetes and the genomic information adjacent to this marker gene could reveal the metabolic potential of this group of microorganisms. In another approach searching for novel anammox bacteria, more sequenced genomes of anammox bacteria could enable the design of primers specific for genes unique for the anammox reaction, like hydrazine hydrolase (hh) and hydrazine dehydrogenase (hd). Metagenome libraries could then be screened for these functional genes and together with neighbouring genomic information might reveal the identity of

new microorganisms mediating the anammox process. Detection of the mRNA of these functional genes could become a new screening tool for anammox activity in environmental samples.

6. Fosmids of novel marine *Planctomycetes* from the Namibian and Oregon coast upwelling systems and their cross-comparison with five planctomycete genomes

The above mentioned metagenomic approach was applied in a preliminary study investigating characteristics and metabolic features of chemoorganoheterophic planctomycetes. Molecular methods like FISH and 16S rRNA sequence analysis have shown that *Planctomycetes* are more widely distributed than assumed based on culture-depended studies. *Planctomycetes* are much more abundant in marine sediments than in marine water columns (Musat et al. 2006, Rusch et al. 2003); however, they seem to be of importance for the marine carbon cycling. In order to broaden our knowledge about marine *Planctomycetes*, six fosmids from two different marine upwelling systems were investigated. Two of the fosmids originate from a metagenome library generated from waters of the upwelling system off the coast of Oregon (Stein et al. 1996, Vergin et al. 1998). The other four sequenced fosmids came from a metagenome library produced from samples of the Namibian upwelling system during this thesis (Table 2).

Table 2: Characterization of the sequenced fomids: Insert size, G+C content, number of ORFs per fosmid and sampling location

	3FN	6FN	8FN	13FN	5H12	6N14
Fosmid insert size (kb)	37.0	40.7	34.6	36.7	41.0	42.5
G+C-content	59.7	47.8	50.8	55.0	51.0	52.4
Number of ORFs after manual annotation	28	28	33	31	31	32
Sampling location	Namibian upwelling system (Kuypers et al., 2005)				Oregon upwelling system (Stein et al., 1996)	

Five of the fosmids were most closely related to so far uncultured planctomycetes (Fig. 20). New draft genomes of *Blastopirellula marina* DSM 3645^T and *Planctomyces maris* DSM 8797^T were included in this investigation in addition to the sequenced genomes of *Rhodopirellula baltica* SH 1^T, *Candidatus* Kuenenia stuttgartiensis and *Gemmata obscuriglobus* DSM 2246^T. Thereby, genomic information from all cultured *Planctomycete* genera except from the *Isosphaera* and *Pirellula* lineage was used. This together with the

fosmids of uncultured planctomycete extended the study to a substantial fraction of all known planctomycete groups. The phylogenetic affiliation of the studied fosmids and the available planctomycete genomes are indicated in Fig. 20.

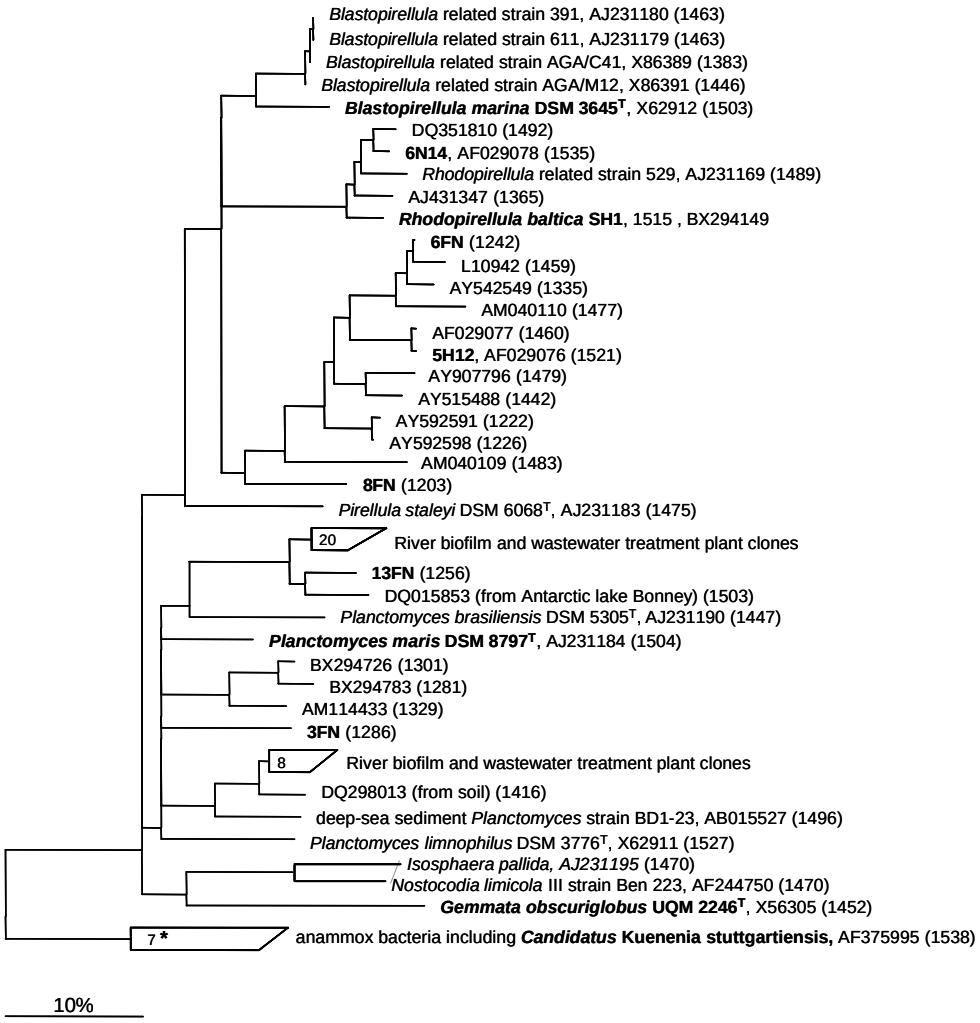


Fig. 20: Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic affiliation of fosmids and genomes investigated during this study. The consensus tree was constructed after tree calculation with neighbor joining, maximum parsimony and maximum likelihood algorithms without and with 50% position variability filters. Lengths of the sequences are indicated in parentheses. Anammox bacteria (group indicated by an asterisk comprising of *Cand.* K. stuttgartiensis, *Cand*. B. anammoxidans, *Cand*. B. fulgida, *Cand* A. propionicus, *Cand.* J. asiatica, *Cand.* S. sorokinii and *Cand*. S. brodae) were used as an outgroup. The bar repesents 10% estimated sequence divergence.

The comparative sequence analysis revealed a high number of genes coding for sulfatases in all genomes from marine *Planctomycetes*. *Rhodopirellula baltica* SH 1^T contained the highest number (109), while the freshwater planctomycetes *Gemmata obscuriglobus* and *Candidatus* Kuenenia stuttgartiensis only held 12 and 3 sulfatases, respectively. Additionally, fosmid 6FN from the Namibian upwelling system contained one sulfatase. These enzymes might enable the *Planctomycetes* to degrade sulfated

heteropolysaccharides and to use the derived carbon skeletons as energy source. Sulfated polysaccharides are produced in large quantities in marine environments and might be entrapped in marine snow particles which are known to be inhabited by *Planctomycetes*. The large chemical complexity of these compounds would explain the high number of genes encoding sulfatases on marine *Planctomycete* genomes.

Two fosmids from the Namibian upwelling and all *Planctomycete* genomes - except for *Candidatus* Kuenenia stuttgartiensis - contained genes involved in the conversion of C1-carbon compounds known from methanogenic *Archaea* or methylotrophic *Alphaproteobacteria* (Fig. 21) (Chistoserdova et al. 1998).

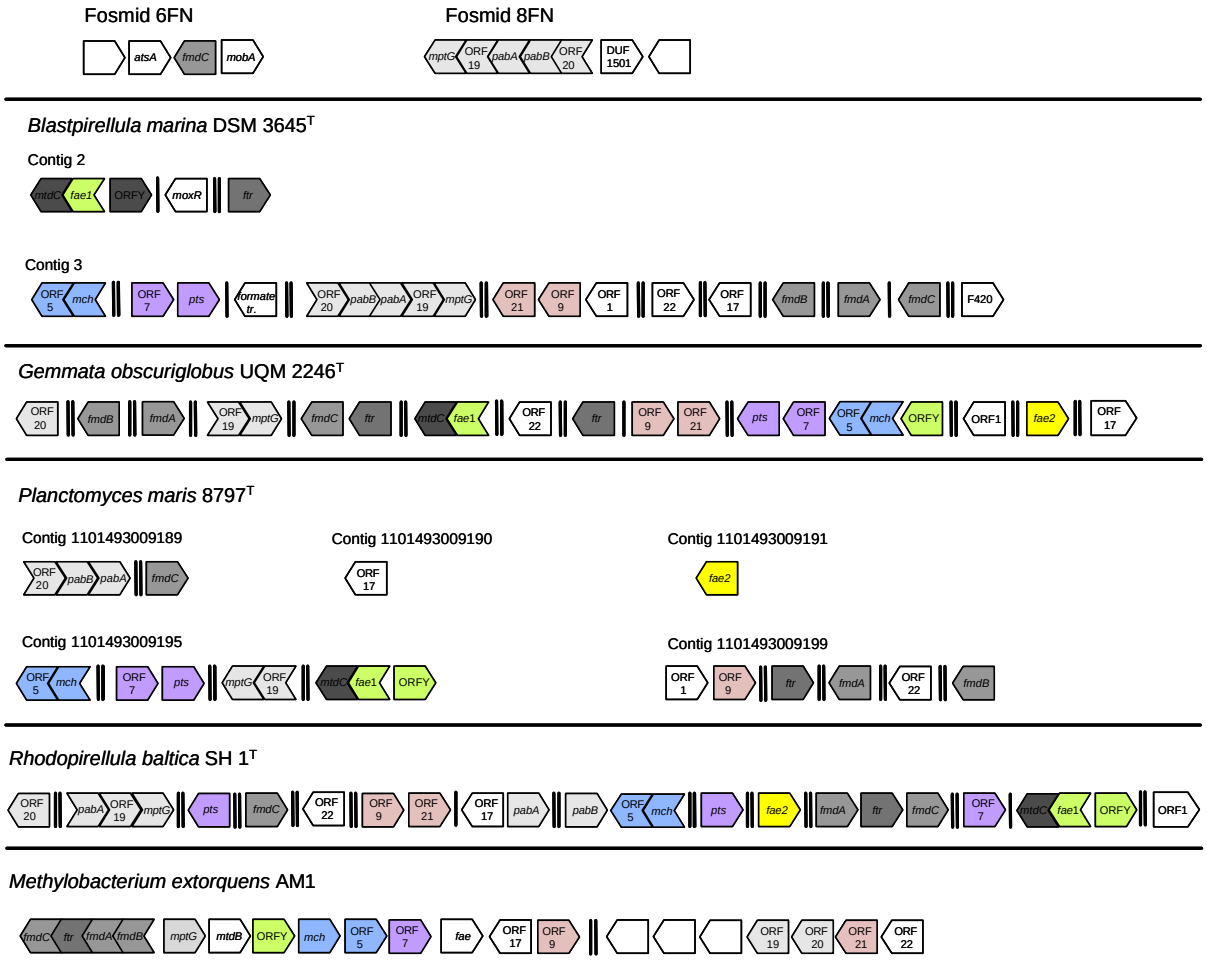


Fig. 21: Genomic arrangement of genes involved in H_4MPT-dependend C1- transfer. Comparison of fosmids 6FN and 8FN from the Namibian upwelling system, genomes of the planctomycetes *Blastopirellula marina* DSM 3645^T, *Gemmata obscuriglobus* UQM 2246^T, *Planctomyces maris* 8797^T, *Rhodopirellula baltica* SH 1^T and the methylotrophic *Alphaproteobacterium Methylobacterium extorquens* AM1. Inserted arrows state that ORFs are organized in operons. A single line separating arrows indicate that ORFs are separated by at most 50 ORFs; double lines represent a separation by > 50 ORFs.

Up to now it is not known whether these genes are functional in *Planctomycetes* and what role they play. So far, *Rhodopirellula baltica* SH 1^T could not be grown on C1 substrates (Bauer et al. 2004). On the other hand, the arrangement in conserved modules and the high expression levels as indicated by codon usage in some of the planctomycetes implied that these genes are of great importance to the *Planctomycetes*. It has been proposed that they are involved in the detoxification of formaldehyde (Chistoserdova et al. 2004). However, since other more energy-effective pathways for the detoxification of formaldehyde were found on the planctomycete genomes, it is very likely that these C1 metabolism genes play an important role in other, so far unknown pathways.

The investigation of all five sequenced planctomycete genomes and the six fosmids containing planctomycete DNA allowed the identification of genes, which were only present in planctomycete genomes and lacked similarity with other bacterial or archaeal genes - so called group-specific genes (GSG). These planctomycete-specific genes might include genes which are involved in their unique cellular characteristics. However, the anammox bacterium *Candidatus* Kuenenia stuttgartiensis only shared very few of these genes with the other planctomycete genomes, while the other planctomycetes had up to 700 genes in common (Fig. 22).

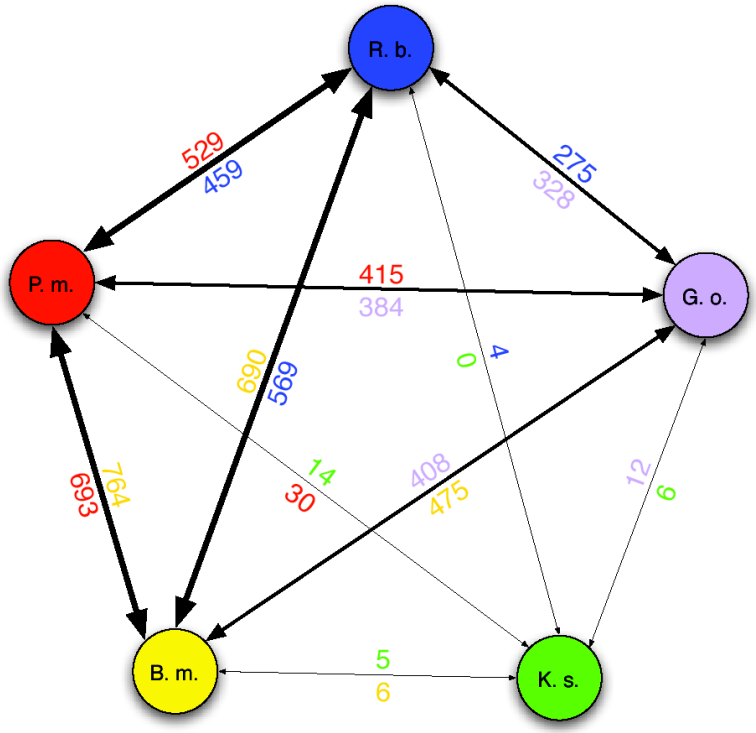


Fig. 22: Number of group specific genes shared between the five investigated genomes. R.b.: *Rhodopirellula baltica* $SH1^T$; G.o.: *Gemmata obscuriglobus* UQM 2246^T; P.m.: *Planctomyces maris* 8797^T; B.m.: *Blastopirellula marina* DSM 3645^T; K.s.: *Candidatus* Kuenenia stuttgartiensis. The arrow thickness indicates the amount of shared genes.

Apparently the anammox bacteria are very distinct from all other *Planctomycetes*, because they feature a unique metabolism and *Candidatus* Kuenenia stuttgartiensis lacks the C1 metabolism genes in addition to the planctomycete-specific genes on its genome. Further investigations like sequencing of additional anammox bacterial genomes will show, whether the placement of the anammox bacteria in a separate phylum within the PVC superphylum is more appropriate than their placement within the *Planctomycetes*.

This first comparison of six sequenced fosmids and five genomes allowed a comprehensive investigation of the genetic potential of the order *Planctomycetes*. One metabolic pathway and one gene family were found to be conserved and universally distributed, but their physiological role is so far not known. Additionally, this investigation shed light on the phylogenetic affiliation of the anammox bacterium *Candidatus* Kuenenia stuttgartiensis within the *Planctomycetes*.

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Part II:

Publications

A. List of publications presented in this thesis

Contributions to the manuscripts and publications presented in this thesis

1) **Marcel M. M. Kuypers, Gaute Lavik, Dagmar Woebken, Markus Schmid, Bernhard M. Fuchs, Rudolf I. Amann, Bo B. Jorgensen and Mike. S. M. Jetten.** 2005. Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proceedings of the National Academy of Sciences of the United States of America **102:**6478-6483.

D.W.: Quantification of anammox bacteria by FISH

2) **Dagmar Woebken**, **Bernhard M. Fuchs, Marcel M. M. Kuypers, Rudolf I. Amann.** 2007. Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Submitted.*

Idea M. K.; realization of experiments D.W.; development of the manuscript D.W. under editorial cooperation of M.K., B.F., and R. A.

3) **M. Robert Hamersley, Gaute Lavik, Dagmar Woebken, Jayne E. Rattray, Phyllis Lam, Ellen C. Hopmans, Jaap S. Sinninghe Damsté, Siegfried Krüger, Michelle Graco, Dimtri Gutiérrez, and Marcel M. M. Kuypers**. 2007. Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. Limnology and Oceanography. **52**: 923-933.

D.W.: Quantification of anammox bacteria by FISH and identification of anammox bacteria by 16S rRNA gene clone libraries.

4) **Dagmar Woebken, Phyllis Lam, Bernhard M. Fuchs, Marcel M. M. Kuypers, S. Wajih A. Naqvi, Boran Kartal, Marc Strous, Mike. S. M. Jetten and Rudolf I. Amann.** 2007. Microdiversity study of marine anammox bacteria reveals novel *Candidatus* Scalindua type in the Arabian Sea. *Manuscript in preparation.*

Idea and concept D.W.and P.L.; realization of experiments D.W. with support of P.L.; development of the manuscript D.W. under editorial cooperation of P.L., M.K., B. F., and R.A.

5) **Dagmar Woebken, Hanno Teeling, Alexandra Dumitriu, Ivaylo Kostadinov, Rudolf I. Amann and Frank Oliver Glöckner.** 2007. Fosmids of novel marine Planctomycetes from the Namibian and Oregon coast upwelling systems and their cross-comparison with one complete and four almost complete Planctomycete genomes. *Manuscript in preparation*.

Idea and concept D.W. and H.T.; realization of experiments D.W., A.D., I.K., and H.T.; development of the manuscript D.W. under editorial cooperation of H.T.

B. Publications

1

Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation

Marcel M.M. Kuypers, Gaute Lavik, Dagmar Woebken, Markus Schmid, Bernhard M. Fuchs, Rudolf Amann, Bo Barker Jørgenesen, and Mike S.M. Jetten

Proceedings of the National Academy of Sciences of the United States of America (PNAS). **102**: 6478-6483 (2005)

Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation

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In many oceanic regions, growth of phytoplankton is nitrogen-limited because fixation of N_2 cannot make up for the removal of fixed inorganic nitrogen (NH_4^+, NO_2^-, and NO_3^-) by anaerobic microbial processes. Globally, 30–50% of the total nitrogen loss occurs in oxygen-minimum zones (OMZs) and is commonly attributed to denitrification (reduction of nitrate to N_2 by heterotrophic bacteria). Here, we show that instead, the anammox process (the anaerobic oxidation of ammonium by nitrite to yield N_2) is mainly responsible for nitrogen loss in the OMZ waters of one of the most productive regions of the world ocean, the Benguela upwelling system. Our *in situ* experiments indicate that nitrate is not directly converted to N_2 by heterotrophic denitrification in the suboxic zone. In the Benguela system, nutrient profiles, anammox rates, abundances of anammox cells, and specific biomarker lipids indicate that anammox bacteria are responsible for massive losses of fixed nitrogen. We have identified and directly linked anammox bacteria to the removal of fixed inorganic nitrogen in the OMZ waters of an open-ocean setting. We hypothesize that anammox could also be responsible for substantial nitrogen loss from other OMZ waters of the ocean.

anammox | denitrification | oceanic nitrogen cycle | oxygen-minimum zone

Large amounts of dissolved inorganic carbon, fixed inorganic nitrogen (NH_4^+, NO_2^-, and NO_3^-), and phosphate in ocean-surface waters are consumed by algae and cyanobacteria during photosynthesis. The N:C and P:C ratios of the organic products are similar throughout the marine realm (1, 2). On average, the C:N:P molar ratio of this phytoplanktonic biomass is 106:16:1 (3). Carbon dioxide, ammonium, and phosphate are released in the same ratio, the so-called Redfield ratio, when this phytoplankton-derived organic matter is remineralized (3). Under oxic conditions, nitrifying organisms (mainly chemoautotrophic bacteria) will consume O_2 and oxidize ammonium through nitrite to nitrate. The nitrate can be reutilized to produce new phytoplanktonic biomass. Alternatively, in oxygen-deficient marine environments, predominately facultative anaerobic prokaryotes can use nitrate as an electron acceptor while oxidizing organic matter. During this process (i.e., heterotrophic denitrification) nitrate is reduced to N_2. Some ammonium is also released and, in the absence of oxygen, is expected to remain as ammonium (4).

For some time, however, oceanographers have known that far less ammonium accumulates in anoxic fjords and basins than would be expected from the stoichiometry of heterotrophic denitrification (5, 6). To explain this shortfall, it was suggested that microorganisms can combine ammonium and nitrate to yield N_2 (5). Direct evidence for the anaerobic oxidation of ammonium was found in wastewater bioreactors, where so-called "anammox" bacteria belonging to the order *Planctomycetales* directly oxidize ammonium to N_2 with nitrite as the electron acceptor (7–9). Nitrite is an intermediate in both heterotrophic denitrification (7) and aerobic ammonium oxidation (10, 11). Experiments with enrichment cultures and purified cells indicate that anammox bacteria can also reduce nitrate to nitrite while oxidizing short-chain fatty acids (e.g., acetate and propionate) (12).

The anaerobic oxidation of ammonium with nitrite to N_2 (anammox) was recently recognized as a major sink for fixed inorganic nitrogen in coastal sediments and the anoxic waters of basins isolated from oxygenated deep circulation (4, 13–19). Globally, 30–50% of the total nitrogen loss occurs in oxygen-minimum zones (OMZs) and is commonly attributed to heterotrophic denitrification (20, 21). There has been, until now, no published direct evidence for anammox in OMZs. However, the extremely low concentration of ammonium could indicate that anammox bacteria also play an important role in the nitrogen removal from OMZ waters (e.g., refs. 4, 16, and 18).

Upwelling of nutrient-rich South Atlantic midwaters in the Benguela current system along the southwest African continental margin sustains some of the highest rates of primary production in the ocean (Fig. 1*a*) (22, 23). Although the upwelling water is generally well oxygenated (>200 μM O_2), bottom waters become severely oxygen-depleted (<10 μM O_2; Fig. 1*b*) over large areas of the southwest African shelf. This condition results from the consumption of O_2 during the decomposition of settling algal biomass (23). A strong N deficit (i.e., a decrease in the ratio of fixed inorganic N to P) (24) in the bottom waters (Fig. 1*b*) has been attributed to denitrification (23, 24). Water in the Benguela OMZ is exchanged rapidly, and concentrations of oxygen vary significantly (23). One of the aims of this study was to investigate whether anammox bacteria, with their slow growth rate and anaerobic physiology, are able to thrive under such highly dynamic conditions.

Methods

Salinity, temperature, density, turbidity, and oxygen profiles were obtained by a conductivity–temperature–depth (CTD) system equipped with an oxygen sensor (Sea-Bird Electronics, Bellevue, WA). The oxygen sensor was calibrated by manual Winkler titration (in duplicate) of 100-ml water samples collected from four depths throughout the water column for each station.

Nutrient Analyses. Water samples for nutrient analyses were obtained by a pump-CTD system or go-flow bottles (Hydrobios, Loudeac, France). Nitrate, nitrite, ammonium, and phosphate concentrations (detection limits 0.1, 0.03, 0.3, and 0.1 μM, respectively) were determined on board with an autoanalyzer (TRAACS 800, Bran & Lubbe, Hamburg, Germany) immediately after sampling. The fixed-inorganic-nitrogen deficit was calculated from nutrient concentrations by using the equation N deficit = 16 × [phosphate] − ([nitrate] + [nitrite] + [ammonium]), in which 16 is the Redfield ratio of fixed inorganic nitrogen to phosphate (24).

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Abbreviations: CTD, conductivity–temperature–depth; OMZ, oxygen-minimum zone.

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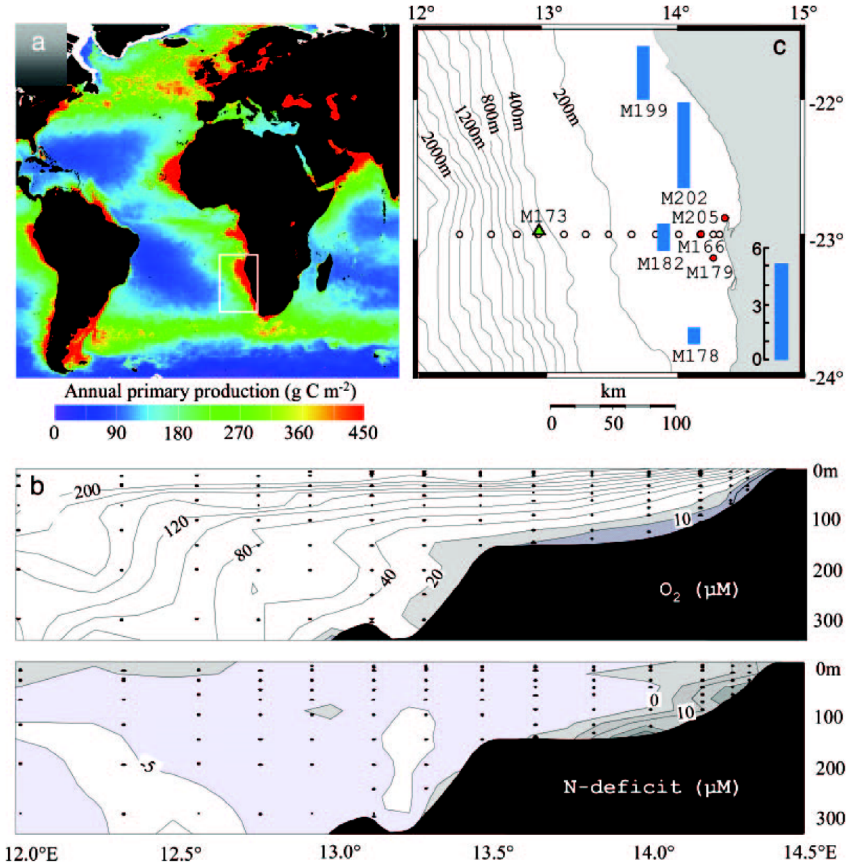

Fig. 1. The Benguela system. (*a*) Distribution of annual primary production (source: http://marine.rutgers.edu/opp/swf/Production/results/all2_swf.html). The white box indicates the extent of the Benguela upwelling system. (*b*) Vertical transect showing the lateral extension and fixed-inorganic-nitrogen deficit in the OMZ off Namibia at 23° south (see *Nutrient Analyses* in *Methods*). (*c*) Sites and nitrogen losses. The open circles represent sites used to construct the lateral transect in *b*. The blue bars represent the depth-integrated nitrogen loss (mmol·m^{-2}·d^{-1}) through anammox determined from anaerobic ^{15}N incubations of water collected from six or seven depths throughout the suboxic zone at sites M178, M182, M199, and M202. For sites M179 and M205 (red circles) anaerobic ^{15}N incubations indicate anammox activity, but rates were not measured. The integrated nitrogen loss for site M166 (red circle) is not shown because anaerobic ^{15}N incubations were performed for only one depth (107 m, Fig. 4). Anammox activity was undetectable at site M173 (green triangle), where oxygen concentrations in the bottom waters exceed 20 μM.

^{15}N Incubations and Analysis. For the ^{15}N incubations, we slightly modified the method previously published by Dalsgaard *et al.* (16). Briefly, 250 ml of Namibian shelf water collected from specific water depths with the pump-CTD were flushed with helium for 15 min after the addition of 5 μmol of $Na^{15}NO_3$, 2.5 μmol of $^{15}NH_4Cl$, or 5 μmol of $Na^{15}NO_3$ and 2.5 μmol of $^{14}NH_4Cl$ (reagents from Campro Scientific, Berlin). The ^{15}N incubations were started immediately after sampling. The water was transferred into 12-ml Exetainers (Labco, High Wycombe, Buckinghamshire, U.K.) and incubated for up to 48 h at *in situ* temperatures. Samples were taken after 0, 12, 24, and 48 h by removing 1 ml of water while replacing it with helium. Mercuric chloride was added to the samples to stop biological activity, and the samples were stored at 4°C until analysis. $^{15}N^{14}N$:$^{14}N^{14}N$ and $^{15}N^{15}N$:$^{14}N^{14}N$ ratios were determined by GC isotope ratio MS.

Particulate Organic Carbon and Lipid Analysis. Particulate matter was collected from specific water depths by using *in situ* pumps (Challenger Oceanic, Haslemere, Surrey, U.K.) to filter large volumes of water (≈500 liters per sample) through glass-fiber filters (GFF) (Whatman, Maidstone, U.K.) precombusted at 450°C (GFF nominal pore size, 0.7 μm). Contents of particulate organic carbon were determined by using a carbon and nitrogen (CN) analyzer (Carlo Erba, Milan). The GFF filters were Soxhlet-extracted for 24 h to obtain the total lipid extracts. Aliquots of the total extracts were saponified after the addition of an internal standard and separated into fatty-acid and neutral-lipid fractions. The fatty-acid fractions were methylated and analyzed by GC-MS for identification and quantification of ladderane lipids. Repeated concentration measurements agreed within ±10%. Because filters with a pore size of 0.7 μm may undersample anammox cells, the calculated ladderane-lipid concentrations represent minimum values.

Phylogenetic Analysis. Extraction, isolation, and cloning of DNA followed procedures described in ref. 25. The software package ARB (26) was used for phylogenetic analyses.

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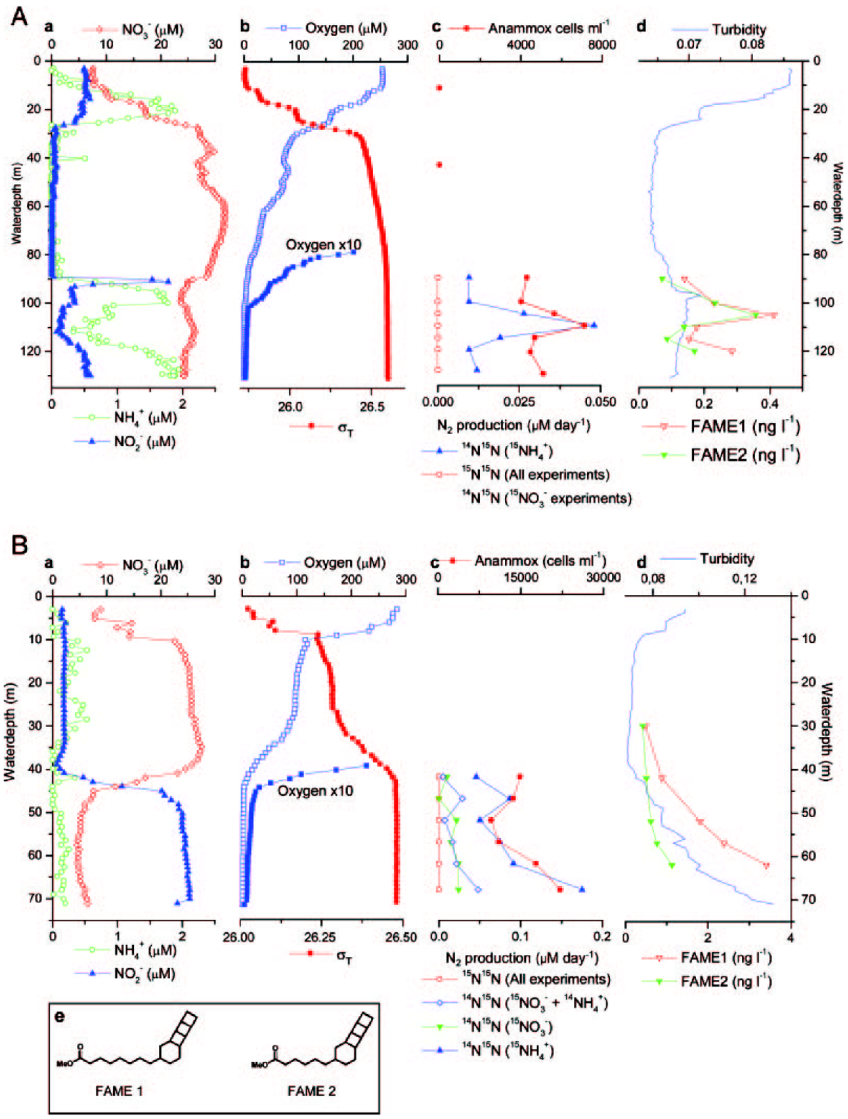

Fig. 2. Chemical zonation and distribution of anammox indicators at sites M182 on March 21, 2003 (*A*) and M202 on March 27, 2003 (*B*). (*a*) Concentrations of fixed inorganic nitrogen species. (*b*) Water density (σ_T, the density of seawater in kg·m^{-3} − 1,000) and oxygen concentrations (notice the 10-fold expanded O_2 gradient). (*c*) Anammox cells per milliliter and rates of production of N_2. The isotopic species pertain to the incubations indicated. At M182, production of $^{15}N^{15}N$ in all incubations and of $^{14}N^{15}N$ with added $^{15}NO_3^-$ and $^{14}NH_4^+$ or with added $^{15}NO_3^-$ was undetectable. Accordingly, all are represented by a single line. (*d*) Turbidity and depth distributions of membrane lipids specific for anammox bacteria. FAME, fatty acid methyl ester. (*e*) Molecular structures of the two ladderane fatty acid methyl esters represented in *d*.

FISH. Particulate organic matter for FISH analyses was collected from specific water depths by filtration of 30 ml of water through polycarbonate filters (0.2-μm pore size). The filter material was fixed with paraformaldehyde and hybridized with fluorescently labeled oligonucleotide probes specific to *Planctomycetes* (27) and to anammox bacteria (25, 28). Total cell counts were based on DAPI staining. The average number of anammox bacteria was determined by epifluorescence microscopy (28).

Results and Discussion

During an R/V *Meteor* cruise in March and April, 2003, we investigated the role of denitrification and anammox in Namibian shelf waters by combining microbiological and biogeochemical techniques. As observed in ref. 23, concentrations of nitrate maximized at 25–30 μM in the midwaters of the Namibian shelf (Fig. 2) and, because of uptake of nitrate during phytoplankton growth, were substantially lower in the surface

waters. Vertical distributions of nitrate, nitrite, and ammonium differ substantially between sites M182 and M202 (Fig. 2), reflecting the heterogeneous and dynamic nature of the Benguela upwelling system (23). However, at both sites, concentrations of nitrate drop at the base of the oxic zone (<10 μM O_2; Fig. 2). This decrease has been attributed to the conversion of nitrate to N_2 by denitrifying bacteria (23, 24). Nitrite is an intermediate in this process, and nitrite maxima were associated with the decrease in nitrate concentrations at both sites (Fig. 2). The N:P ratios for dissolved inorganic nutrients are well below Redfield values and indicate extensive loss of nitrogen from the oxygen-deficient waters at sites M182 and M202 (see Tables 1 and 2, which are published as supporting information on the PNAS web site). Ammonium should have accumulated in these waters if the nitrogen loss were due solely to heterotrophic denitrification (5, 6). The low ammonium concentrations (below detection limit) in the suboxic zone at site M202 (Fig. 2*B*) could indicate that anammox bacteria play an important role in nitrogen removal from the waters of the Benguela OMZ (4, 16, 18).

The N deficit (Fig. 1*b*) requires that large amounts of fixed inorganic nitrogen are being removed from the Namibian suboxic waters. To elucidate the microbial process responsible for this loss of N, we incubated water samples from various depths after the addition of [^{15}N]nitrate or [^{15}N]ammonium. Denitrification combines two nitrate ions to form one molecule of N_2. Because all water samples contained a natural abundance of [^{14}N]nitrate in addition to the added [^{15}N]nitrate, denitrification would produce $^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$ through random isotope pairing (13, 14, 29, 30). Even though the water samples were incubated with 45–80% ^{15}N-labeled nitrate, production of $^{15}N^{15}N$ was undetectable in all 0-, 12-, and 24-h incubations and in 96 of 100 48-h incubations (Figs. 2 and 3). The potential to respire nitrate is widespread among bacteria and archaea (31). The production of $^{15}N^{15}N$ in 4 of 100 48-h incubations (Fig. 4) indicates that facultatively anaerobic heterotrophs that can use nitrate in the absence of oxygen are present in the Namibian shelf waters but does not indicate that nitrate is directly converted to N_2 by denitrifying microorganisms in the suboxic shelf water.

Denitrification requires electron donors (e.g., organic matter, sulfide). No sulfide was detected in Namibian shelf waters during the 2003 R/V *Meteor* cruise. However, concentrations of particulate organic carbon were 30–95 μg·liter^{-1} at M182 and 70–300 μg·liter^{-1} at M202, indicating that organic matter is not limiting. Apparently, the expression or activity of denitrifying enzymes was suppressed in the shelf waters. We attribute this suppression to regular incursions of oxygen into the suboxic zone (23). Although some bacterial strains can denitrify under aerobic conditions in the laboratory (31), denitrifying enzymes are generally repressed or inhibited by free oxygen, and induction of full denitrifying capacity after reestablishment of anaerobic conditions can take at least 20 h (31, 32).

In contrast, anammox-enrichment cultures that have been exposed to oxygen resume activity immediately after the reestablishment of anaerobic conditions (33). Water samples from site M202 that were anaerobically incubated after the addition of [^{15}N]nitrate and [^{14}N]ammonium produced significant amounts of $^{14}N^{15}N$ (Figs. 2*B* and 3), indicating either that the reduction of nitrate to nitrite by heterotrophic denitrifiers was coupled to anammox (7, 14) or that anammox bacteria could be using nitrate as an electron acceptor concurrently with nitrite. The latter process, in which anammox bacteria reduce nitrate to nitrite while oxidizing short-chain fatty acids, was recently discovered in anammox bioreactors (12). The anaerobic incubation of water samples after the addition of [^{15}N]ammonium resulted in substantial $^{14}N^{15}N$ production in samples from the suboxic zone (Figs. 2 and 3), providing direct evidence for removal of fixed inorganic nitrogen by anammox

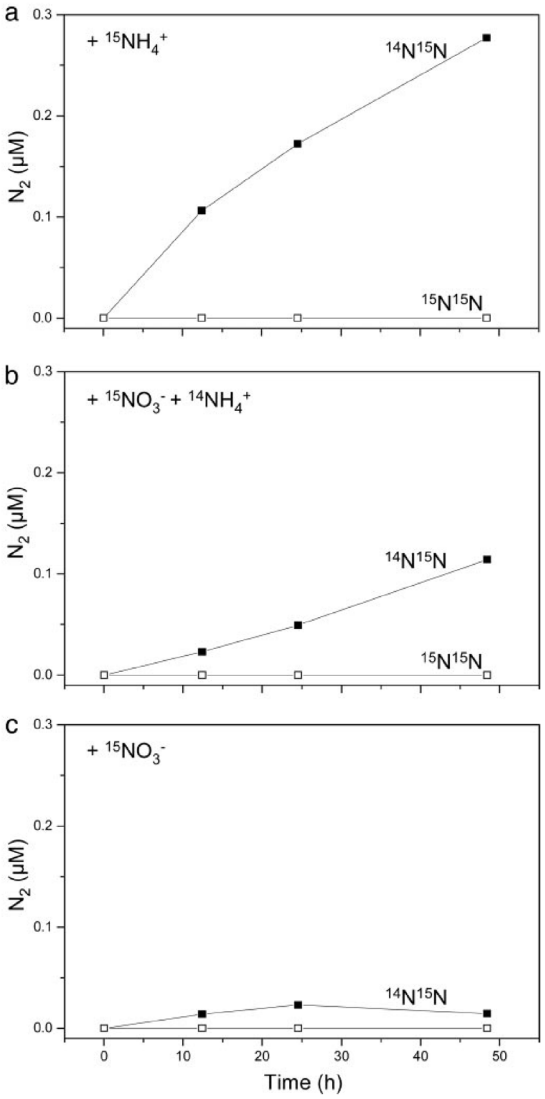

Fig. 3. Changes in concentrations of isotopically labeled N_2 species vs. time during incubations of samples from a depth of 68 m at site M202.

bacteria. The time-dependent data from M182 (data not shown) and M202 (Fig. 3) show rather linear accumulations of $^{14}N^{15}N$ for both the $^{15}NO_3^-$ and $^{15}NH_4^+$ additions. This lack of a lag time indicates that the anammox bacteria are active from time 0 and are likely active *in situ*.

Production of N_2 by anammox at site M182 maximizes in the suboxic zone between 100- and 115-m depth, where ammonium and nitrite disappear (Fig. 2*A*). Production of $^{14}N^{15}N$ was below the detection limit for sea water incubated for up to 48 h with [^{15}N]nitrate, indicating that aerobic oxidation of ammonium, rather than nitrate reduction, is the source of nitrite for anammox at site M182 (≈110-m depth; Fig. 2*A*). At site M202, the production of N_2 by anammox increases with depth, with

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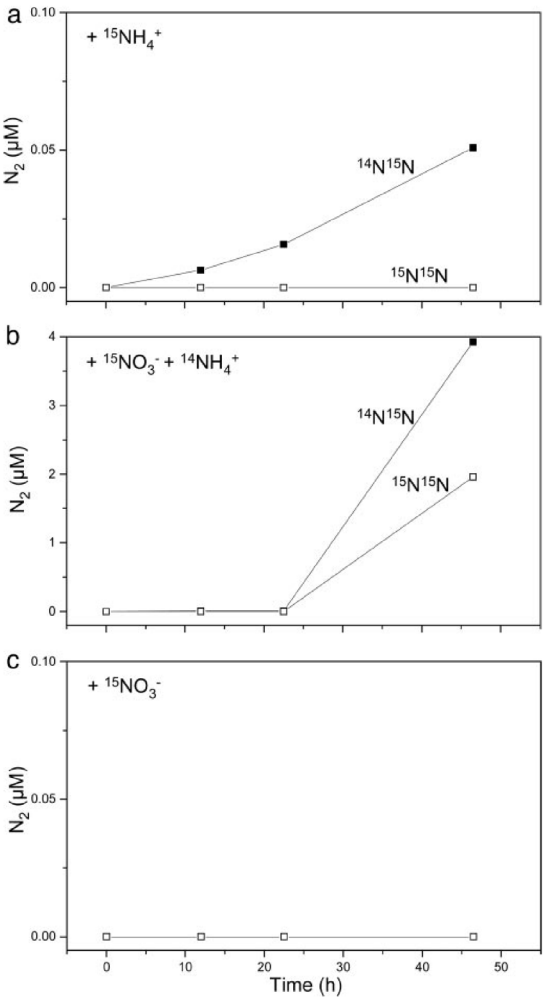

Fig. 4. Changes in concentrations of isotopically labeled N_2 species vs. time during incubations of samples from a depth of 107 m at site M166.

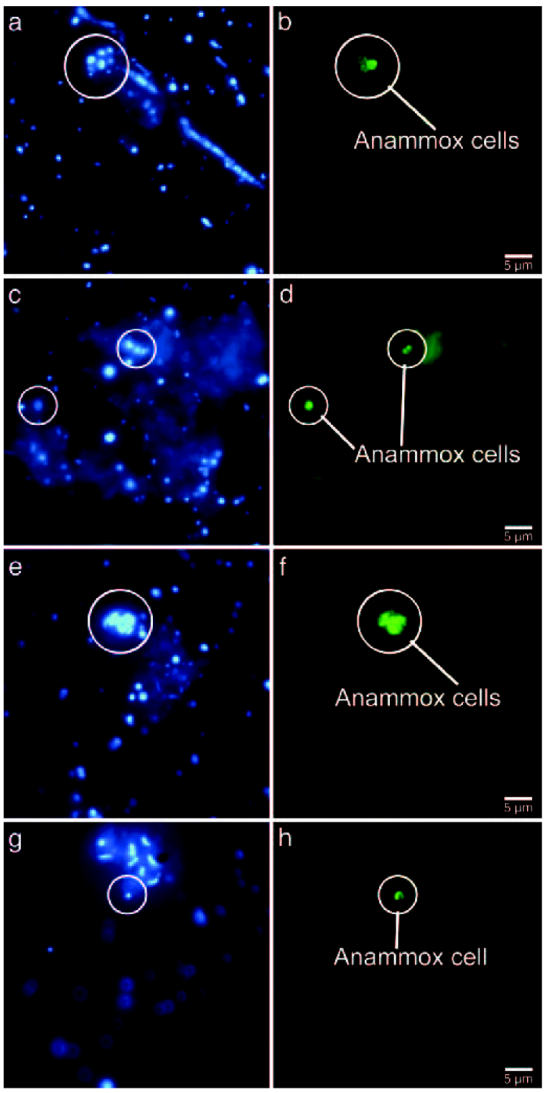

Fig. 5. *In situ* identification of aggregate-associated anammox cells (encircled) from station M202 (62 m). Single *xy* images of the same section were combined. (*a*, *c*, *e*, and *g*) Micrographs show aggregates stained with DAPI. (*b*, *d*, *f*, and *h*) Micrographs show results of hybridization with an oligonucleotide probe (AmxBS820) specific for anammox bacteria.

the highest rates near the sea floor. Concentrations of nitrite remain constant, indicating transient conditions (Fig. 2*B*).

Specific biomarkers, so-called ladderane lipids, were used to trace anammox bacteria in particulate organic matter collected from various depths across the suboxic zone. Ladderane lipids (17, 34, 35) are the main building blocks of a unique bacterial membrane that surrounds the "anammoxosome," a special compartment in the anammox cell where N_2 is produced. Two different ladderane lipids were found in the fatty-acid fraction obtained after saponification of the total lipid extract. Their distribution vs. depth was similar to that of anammox activity (Fig. 2).

In addition, DNA was isolated from various depths through the suboxic zone. A clone library was generated after the 16S ribosomal RNA gene was amplified with primers specific for *Planctomycetes* (25). Phylogenetic analysis of the resulting 16S rRNA sequences showed that *Planctomycetes* closely related (98% sequence similarity) to known anammox bacteria, *Candidatus* "*Scalindua sorokinii*" and *Candidatus* "*Scalindua brodae*" (17, 25), were present in the Namibian shelf waters.

FISH with oligonucleotide probes specific for anammox bacteria (25) was used to quantify anammox bacteria in the Namibian shelf waters. Up to 22,000 anammox cells per ml (≈1% of the total number of cells counted after DAPI staining) were detected in the suboxic zone. The depth distribution was similar to that of the anammox activity. No anammox cells were found in surface waters, where concentrations of O_2 exceed 20 μM (Fig. 2). A strong linear correlation between cell numbers and

activity ($R^2 > 0.8$) indicates that anammox bacteria are responsible for the anaerobic ammonium oxidation in the suboxic Namibian shelf waters. The cell-specific anammox activity calculated (≈4.5 fmol of ammonium per cell per day) for the Namibian shelf water is comparable with the rates estimated for the suboxic zone of the Black Sea (3–4 fmol of ammonium per cell per day) (17) and is well within the range found for anammox bacteria in laboratory bioreactors (2–20 fmol of ammonium per cell per day) (9).

Intriguingly, chemical profiles, ^{15}N-labeling experiments, ladderane-lipid distributions, and FISH analysis indicate that active anammox bacteria are abundant at oxygen concentrations up to 9 μM (Fig. 2). The abundance of anammox bacteria in the presence of free oxygen is surprising because the anammox metabolism is inhibited (33) by oxygen concentrations as low as 1 μM. The anammox bacteria present at higher oxygen concentrations could be dormant. Alternatively, marine snow aggregates are abundant in the Namibian shelf water and could provide the anammox bacteria with anaerobic microenvironments at low ambient-oxygen concentrations (<25 μM) (36). The abundance of aggregate-associated anammox cells visualized by FISH provides evidence for a particle association of the anammox bacteria at site M202 (Fig. 5). This finding is supported by the strong covariation among anammox rates, lipid profiles, anammox cell numbers, and turbidity as a measure of particle abundance (Fig. 2*B*). Whether anammox bacteria are particle-associated or not, our results clearly show that these bacteria are well adapted to the dynamic conditions encountered in a coastal upwelling region.

Labeling experiments with [^{15}N]ammonium at a number of sampling sites showed that anammox occurred throughout the lower 20–30 m of the water column over wide areas of the Namibian shelf (Fig. 1*c*). Labeling experiments with [^{15}N]nitrate at sites M178, M199 (data not shown), M182 (Fig. 2*A*), and M202 (Fig. 2*B*) did not provide evidence for significant production of N_2 by heterotrophic denitrifiers in Namibian shelf waters. Instead, our results indicate that fixed nitrogen is lost through anammox coupled to (*i*) reduction of nitrate to nitrite by heterotrophic denitrifiers or anammox bacteria and (*ii*) aerobic ammonium oxidation. Data from the Chilean OMZ show a similar dominance of anammox over heterotrophic denitrification (B. Thamdrup, personal communication).

Depth-integrated rates indicated that 1–5 mmol of fixed inorganic nitrogen per square meter per day was lost from the suboxic waters of the Benguela upwelling system due to anammox (Fig. 1*c*). If we assume that the main area (≈100,000 km^2) of suboxic shelf water extends from 28° to 18° south (23), 1.4 ± 1 Tg of fixed nitrogen per year might be lost through anammox from the Benguela system. The total estimated nitrogen loss from the OMZ waters of the world ocean (80–150 Tg·yr^{-1}) is based on nutrient measurements and has been fully attributed to heterotrophic denitrification (20, 21, 37) because, before the discovery of anammox bacteria, there was no other process known that could transform fixed inorganic nitrogen into N_2. In fact, to the best of our knowledge, there is, so far, no published evidence from ^{15}N-labeling experiments that nitrate is directly converted to N_2 by heterotrophic denitrifiers in the OMZ waters of the ocean. Our combined results show that anammox bacteria are responsible for massive losses of fixed nitrogen as gaseous N_2 from the Benguela OMZ water. The possibility that anammox is also a dominant process for nitrogen removal in other OMZ waters of the ocean should now be explored.

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Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system

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Abstract

Recent studies have shown that the anaerobic oxidation of ammonium by anammox bacteria plays an important role in catalyzing the loss of nitrogen from marine oxygen minimum zones (OMZ). However, in situ oxygen concentrations of up to 25 µM and ammonium concentrations close to or below the detection limit in the layer of anammox activity are hard to reconcile with the current knowledge of the physiology of anammox bacteria. We therefore investigated samples from the Namibian OMZ by comparative 16S rRNA gene analysis and fluorescence in situ hybridization. Our results showed that *Candidatus* Scalindua spp., the typical marine anammox bacteria, colonized microscopic particles that were likely the remains of either macroscopic marine snow particles or resuspended particles. These particles were specifically enriched in *Gammaproteobacteria* (11.8±5.0%) compared to the free water phase (8.1±1.8%). No preference for the attachment to particles could be observed for members of the *Alphaproteobacteria* and *Bacteroidetes*, which were abundant (12-17%) in both habitats. The alphaproteobacterial SAR11 clade, the *Euryarcheaota* and group I *Crenarchaeota* were all significantly depleted in particles compared to the free water phase (16.5±3.5% versus 2.6±1.7%, 2.7±1.9% versus <1%, and 14.9±4.6% versus 2.2±1.8%, respectively). Sequence analysis of the crenarchaeotal 16S rRNA genes showed a 99% sequence identity to the nitrifying '*Nitrosopumilus maritimus*'. Even though we could not observe conspicuous consortia – like structures of anammox bacteria with particle-enriched bacterioplankton groups, we hypothesize that members of *Gammaproteobacteria*, *Alphaproteobacteria* and *Bacteroidetes* play a critical role in extending the anammox reaction to nutrient depleted suboxic water layers in the Namibian upwelling system by creating anoxic, nutrient-enriched microniches.

Introduction

Fixed inorganic nitrogen compounds are important nutrients for the growth of phytoplankton in marine ecosystems and are often growth limiting. Up to 450 Tg of fixed nitrogen are yearly lost in the world's oceans (Codispoti et al. 2001). Until recently this loss was fully attributed to heterotrophic denitrification. Thirty to 50% of the nitrogen loss occurs in upwelling areas and associated oxygen minimum zones (OMZ), for instance in the Arabian Sea and the upwelling areas off the coasts of Namibia, Chile, and Peru.

Upwelling systems are ecosystems with intense primary production in the surface waters. Decomposition of settling biomass through aerobic respiration in the water column

(Carr 2002, Chapman 1985) leads to oxygen depletion in deeper water layers. In the Namibian upwelling system the oxygen concentration is often < 10 µM in deeper layers, even though surface waters are well oxygenated (> 200 µM O_2). These bottom waters are characterized by a strong N deficit, i.e. a decrease in the ratio of fixed inorganic N to P relative to the Redfield Ratio (16:1) (Redfield et al. 1963).

The observed N deficit could be partly explained by the anaerobic oxidation of ammonium ("anammox") with nitrite to N_2 in the OMZ water. The first bacteria able to anaerobically oxidize ammonium were discovered in a waste water treatment plant (Mulder et al. 1995, van de Graaf et al. 1995) and were affiliated to a separate cluster within the phylum *Planctomycetes* (Strous et al. 1999). Subsequently, the process was also detected in sediments (Dalsgaard and Thamdrup 2002, Engstroem et al. 2005, Rysgaard 2004, Thamdrup and Dalsgaard 2002, Trimmer et al. 2003) and in stratified water bodies like Golfo Dulce (a coastal bay in Costa Rica) and the Black Sea (Dalsgaard et al. 2003 and Kuypers et al. 2003, respectively). Recently it was shown that anammox is mainly responsible for the loss of fixed nitrogen as N_2 from the Namibian (Kuypers et al. 2005) and the Peruvian-Chilean upwelling systems (Hamersley et al. 2007, Thamdrup et al. 2006).

Most of what is known about the physiology of anammox bacteria is based on bioreactor experiments with anammox bacteria enriched at rather high ammonium concentrations from wastewater treatment plants. However, ammonium concentrations in the Namibian and Peruvian upwelling systems are often near or below the detection limit. Bioreactor studies have also shown that 1 µM O_2 reversibly inhibits the anammox metabolism (Strous et al. 1997). In the Namibian OMZ, anammox activity could be detected in samples from water depths with oxygen concentrations up to 9 µM O_2 (Kuypers et al. 2005). Preliminary results indicated particle association of anammox bacteria. These particles were believed to provide an anaerobic microenvironments for the anammox bacteria at low ambient-oxygen concentrations up to 25 µM oxygen (Kuypers et al. 2005).

The aims of this study were to further investigate the distribution and particle association of anammox bacteria in the Namibian OMZ. We used comparative 16S rRNA gene analysis and catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) to identify and localize anammox bacteria and the co-occurring microbiota. Special emphasis was drawn on comparing the relative abundances of co-occurring particle-attached groups versus their abundance in the free water phase and on their potential contribution to the anammox process.

Materials and Methods

Sample collection and preparation. Bacterioplankton samples were taken during an R/V Meteor cruise in March/April 2003 in Namibian shelf waters (station M182 at 23.17°S and 14.08°E, station M202 at 22.64°S and 14.30°E, station M199 at 22.0°S and 13.86°, transect along 23.0°S from 14.36°E to 12.0°E.). For DNA isolation, approximately 500 ml of water was filtered onto polycarbonate filters (type GTTP, pore size 0.22 µm, 47 mm diameter, Millipore, Eschborn, Germany) by a vacuum pump without prefiltration and stored at -20°C. For FISH, water samples were fixed with particle-free paraformaldehyde solution (final concentration, 1% volume/volume, v/v) for 1 hour at room temperature or overnight at 4°C. Afterwards, aliquots of 10 to 200 ml were filtered onto white polycarbonate membrane filters (type GTTP, pore size 0.22 µm, 47 mm diameter, Millipore) and stored at -20°C until further processing.

Turbidity, oxygen and ammonium profiles were obtained and particulate organic carbon and particulate organic nitrogen were analyzed as described before (Kuypers et al. 2005).

Construction of 16S rRNA gene clone libraries. DNA was isolated from the polycarbonate filters according to (Massana et al. 1997). The universal bacterial primers GM3F and GM4R (Muyzer et al. 1995), and universal archaeal primers 20F and 1392R (Massana et al. 1997 and Stahl et al. 1988, respectively) were used to amplify almost full length 16S rRNA genes from Namibian water samples. We used standard PCR conditions and reduced the cycle number as much as possible to minimize PCR bias. The PCR products were cleaned using the QIAquick PCR Purification Protocol (QIAGEN, Hilden, Germany) and their size was verified on agarose gels.

For the archaea clone library, a preparative gel was necessary because only one of two observed PCR products had the correct size of ~1300 bp. The cleaned PCR products were ligated into the vector pGEM-T Easy (Promega, Madison, USA) according to the manufacturer protocol and the vector was introduced into chemical competent *E.coli* strain TOP10. Clones were screened by PCR for inserts of correct size. Plasmids were isolated from positive clones with the Montage Plasmid Miniprep96 Kit (Millipore, Bedford, USA) and sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) with vector primers or 16S rRNA gene primers. The sequences were edited with the software Sequencing Analysis (Applied Biosystems),

imported into the ARB 16S rRNA gene database (Ludwig et al. 2004) and phylogenetically analyzed.

In addition, a *Planctomycetes*-enriched 16S rRNA gene clone library was constructed from station M202 (52 m depth) in the Namibian OMZ with the primers Pla46F (Neef et al. 1998) and the universal primer 1392R (Stahl et al. 1988). From the same sample we constructed two anammox bacteria-enriched clone libraries: one with the general anammox primer Amx368 (Schmid et al. 2003) and the universal primer 1392R, and another clone library with primer Pla46F and the *Candidatus* Scalindua-specific primer 1309R (Schmid et al. 2003). Nearly full length sequences are published in GenBank under accession numbers xxx-yyy.

FISH and CARD-FISH. Standard FISH identification of anammox bacteria with 16S rRNA-targeted oligonucleotide probes was done according to (Schmid et al. 2003).

For CARD-FISH we followed the protocol of (Pernthaler, et al. 2002) including the lysozyme permeabilization with the following modifications. Subsequent to cell immobilization with agarose and prior to hybridization the strongly active endogenous peroxidases inside anammox bacteria were destroyed by treatment with H_2O_2 solution (final concentration 3% in MilliQ water) for 10 min at room temperature. Anammox bacteria were permeabilized by Achromopeptidase (0.6 U ml^{-1}, final concentration; buffer contained 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0; incubation at 37°C for 30 min). To permeabilize Archaea, filters were incubated in 0.1 M HCl for 1 minute at room temperature.

A set of group- and species-specific probes (Table 1) was selected based on the 16S rRNA diversity indicated by the clone libraries constructed from Namibia.

Table 1: Overview of the probes and hybridization conditions applied for FISH and CARD-FISH.

Probe	Specificity	Sequence (5' → 3')	*Escherichia coli* position[a]	FA [%] [b]	Source
EUB338-I-III	*Bacteria*	GCWGCCWCCCGTAGGWGT	338-355	55	(Amann et al. 1990, Daims et al. 1999)
NON338	Negativ control	ACTCCTACGGGAGGCAGC	338-355	55	(Wallner et al. 1993)
CREN554	*Crenarchaeota* group I	TTAGGCCCAATAATCMTCCT	554-573	20	(Massan, et al. 1997)
EURY806	*Euryarchaea*	CACAGCGTTTACACCTAG	806-823	20	(Teira et al. 2004)
BS-820	*Candidatus* Scalindua sorokinii and Scalindua brodae	TAATTCCCTCTACTTAGTGCCC	820-841	40	(Kuypers et al. 2003)
BS-820-C		TAATCCCCTCTACTTAGTGCCC		40	(Hamersley et al.)
AMX-820 [e]	*Candidatus* Kuenenia stuttgardiensis	AAAACCCCTCTACTTAGTGCCC	820-841	40	(Schmid et al. 2000)
ALF968	Most *Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	968-986	55	(Neef 1997)
SAR11/441	SAR11-clade	TACAGTCATTTTCTTCCCCGAC	486-503	45	(Rappe et al. 2002)
ROS537	*Roseobacter*-clade	CAACGCTAACCCCCTCC	537-553	55	(Eilers et al. 2001)
GAM42a [c]	Most *Gammaproteobacteria*	GCCTTCCCACATCGTTT	1027-1043[d]	55	(Manz et al. 1992)
SAR 86/1245	SAR86-clade	TTAGCGTCCGTCTGTAT	1245-1262	55	(Zubkov et al. 2001)
NOR5/730	NOR5/OM60 clade	TCGAGCCAGGAGGCCGCC	730-747	50	(Eilers et al. 2001)
PSA184	*Pseudoalteromonas, Colwellia*	CCCCTTTGGTCCGTAGAC	184-210	50	(Eilers et al. 2000)
ALT1413	*Alteromonas/Colwellia*	TTTGCATCCCACTCCCAT	1413-1430	60	(Eilers et al. 2000)
Nscoc128	*Nitrosococcus oceani*, *N. halophilus (Gammaproteobacteria)*	CCC CTC TAG AGG CCA GAT	128-146	35[f]	(Juretschko 2000), (Loy et al. 2003)
NSO1225	Ammonia-oxidizing Betaproteobacteria	CGCGATTGTATTACGTGTGA	1225-1244	35[f]	(Mobarry et al. 1996)
NSO190	Ammonia-oxidizing Betaproteobacteria	CGATCCCCTGCTTTTCTCC	190-208	55[f]	(Mobarry et al. 1996)
CF319a	*Cytophaga/Flavobacteria*	TGGTCCGTGTCTCAGTAC	319-336	55	(Manz et al. 1996)
SAR406-97	SAR406-clade	CACCCGTTCGCCAGTTTA	97-114	65	(Fuchs et al. 2005)

a: Probe target position on *E. coli* 16S rRNA according to (Brosius et al. 1981)
b: Formamide concentration (v / v) in CARD-FISH hybridization buffer at 35°C
c: Including an unlabeled competitor probe BET42a (5'- GCCTTCCCACTTCGTTT-3'), for details see (Manz et al. 1992)
d: Probe target position on *E. coli* 23S rRNA according to (Brosius et al. 1981)
e: Probe AMX-820 used as competitor in hybridizations with probesBS-820 and BS-820-C.
f: FA concentration for hybridization at 46°C

All probes were purchased from biomers.net, Ulm (Germany). For standard FISH and CARD-FISH of anammox bacteria competitor AMX820 was used together with probe

BS820 (Table 1). Hybridization conditions were optimized for CARD-FISH applications by conducting a formamide series at 35°C hybridization temperature.

For CARD-FISH with single probes, carboxyfluorescein-labeled tyramide (Invitrogen, Karlsruhe, Germany) was used. For sequential hybridizations with two different probes we used tyramides labeled with $Alexa_{546}$ and with $Alexa_{488}$ (both from Invitrogen, Karlsruhe, Germany; used at a dilution of tyramide into amplification buffer of 1:500). After the first tyramide amplification, the filters were washed twice in 50 ml phosphate buffered saline (PBS; 145mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4, [pH 7.6], and once in MilliQ water. Then the filters were incubated in 3% H_2O_2 for 10 minutes to inactivate the HRP attached to the first probe. Subsequently, the filters were washed twice in MilliQ water, dehydrated in 96% ethanol and dried at room temperature before the second hybridization was done.

Microscopic evaluation. After FISH/CARD-FISH filter sections were treated with 4',6-diamidino-2-phenylindole (DAPI), and subsequently evaluated on a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) as described in (Pernthaler et al. 2002). Pictures were taken with a black and white camera (AxioCam MRm, Carl Zeiss, Jena, Germany).
The distribution of anammox clusters and anammox cells attached to particles on the filter were rather patchy. Since low abundance populations were targeted, we increased the number of counted fields of view from 10 to 20, and scored >2000 DAPI-stained cells per filter. For CARD-FISH of particles we evaluated a minimum of 20 particles per probe applied. Relative abundances are presented as mean values with standard deviations.

Results

In the water column of the Namibian upwelling system, we observed a high density of macroscopic particles during sampling by camera imaging from a remotely operated vehicle (Zabel et al. 2004). The sampling with a pump cast system or with go-flow bottles, fixation with paraformaldehyde, and filtration largely disintegrated the fragile aggregates resulting in a quite even distribution of single cells and particles of a size of up to 30 µm on the polycarbonate filters.

Particle-association of anammox bacteria. The vertical profiles of anammox bacteria at station M182 and M202 detected by standard FISH were published previously (Kuypers et al. 2005) and show a significant correlation with the anammox rates measured at both

stations (R=0.82, P<0.01). Additionally, the anammox cell numbers at station M182 and M202 are significantly correlated with the distribution of particulate organic nitrogen (R=0.84, P<0.01) and with particulate organic carbon (R=0.79, P=0.01), suggesting a preference for particle association of anammox cells in the Namibian OMZ.

Standard FISH allowed for accurate quantification of single anammox bacteria. However, the signal intensity of anammox bacteria stained with standard FISH was rather low, and the remaining particles had a high autofluorescence which made it impossible to reliably visualize anammox bacteria in particles. Therefore, we optimized CARD-FISH for the in situ detection of anammox bacteria in particles. With this protocol the particle associated anammox bacteria can be visualized based on a stronger signal and a much improved signal-to-noise ratio (Fig.1a). The CARD-FISH protocol is also superior in the visualization of anammox cell clusters which contained 2-20 and sometimes even more cells (Fig. 1 b).

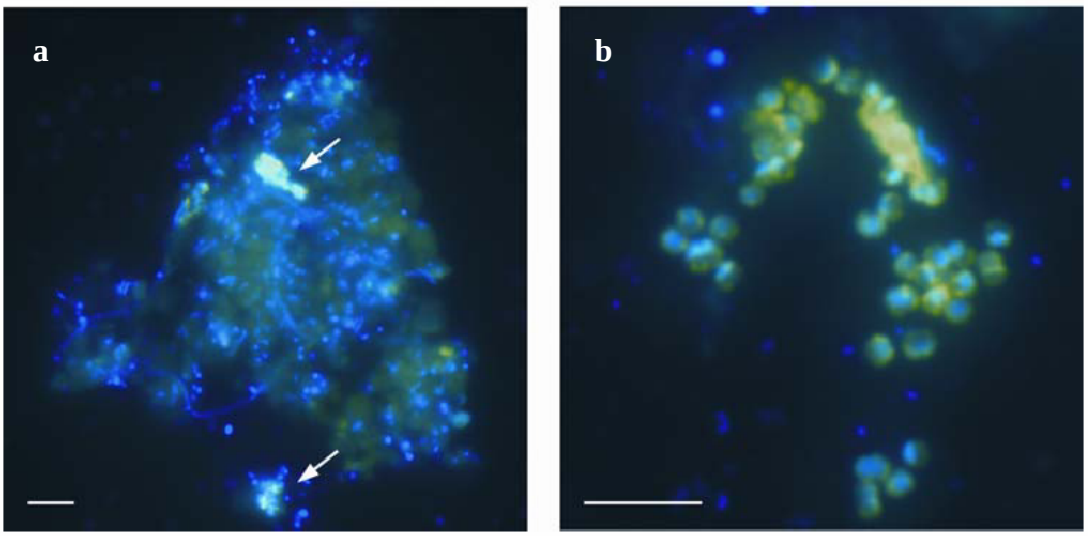


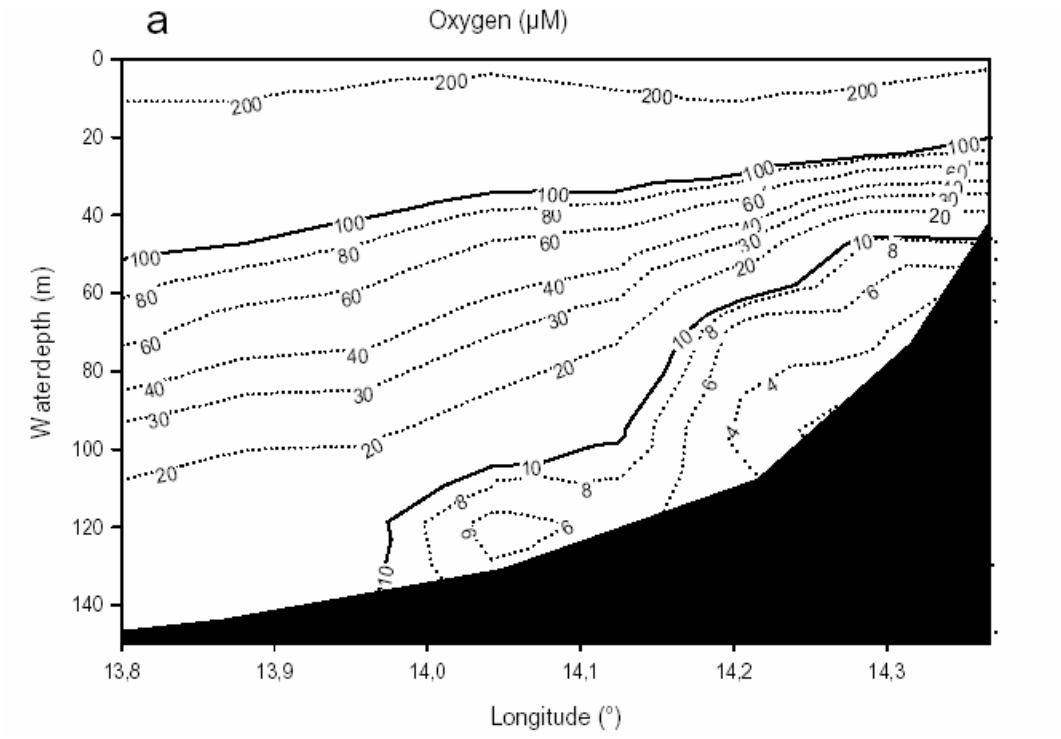
Fig. 1: Combined epifluorescence micrographs from samples of the Namibian OMZ (a) anammox cells in association with particles, (b) anammox cells forming clusters. Anammox cells are stained both with DAPI (blue) and Fluorescein–labeled tyramide (green) after CARD-FISH with HRP-labeled probe BS-820 and appear turquoise. Non-anammox cells are stained only with DAPI and appear blue. Anammox bacteria show the typical “doughnut-shaped” FISH signal caused by internal structures. Bar: 5 µm.

With this improved method we determined the fraction of anammox cells occurring in clusters, in particles or as single cells at stations M202 and M199 by hybridizing six different depths from each station (station M202: 42, 47, 52, 57, 62 and 68 m; station M199: 60, 65, 70, 75, 80 and 87 m) in triplicates. We found that 56.2 ±8.3% of the anammox cells stained with CARD-FISH were organized in clusters, 24.5±8.4% were

associated with particles (either as single cells or in clusters of anammox cells), and 19.3±10.8% were single cells not organized in clusters nor attached to particles.

Spatial distribution of anammox bacteria along the 23°S transect on the Namibian shelf. To further investigate the distribution of anammox cells in the Namibian upwelling system, samples were taken along a transect at 23°S latitude that extended from the OMZ into more oxygenated waters (Fig. 2).

The highest concentration of CARD-FISH stained anammox cells (~2,300 cells ml^{-1}) was found close to the coast at 14.32° E (Fig. 2b). Absolute anammox cell numbers are most likely under-estimated, because CARD-FISH does not detect all anammox cells (see discussion). In waters containing anammox bacteria the oxygen concentrations ranged from 30 µM oxygen in upper waters down to oxygen concentrations below detection limit above the sediment (Fig. 2a). Further offshore than 14.05°E the number of anammox cells was below the detection limit. In summary, our data show that in March/April 2003, the anammox populations were restricted to the Namibian shelf waters in a water layer between 30 m and the sediment surface.



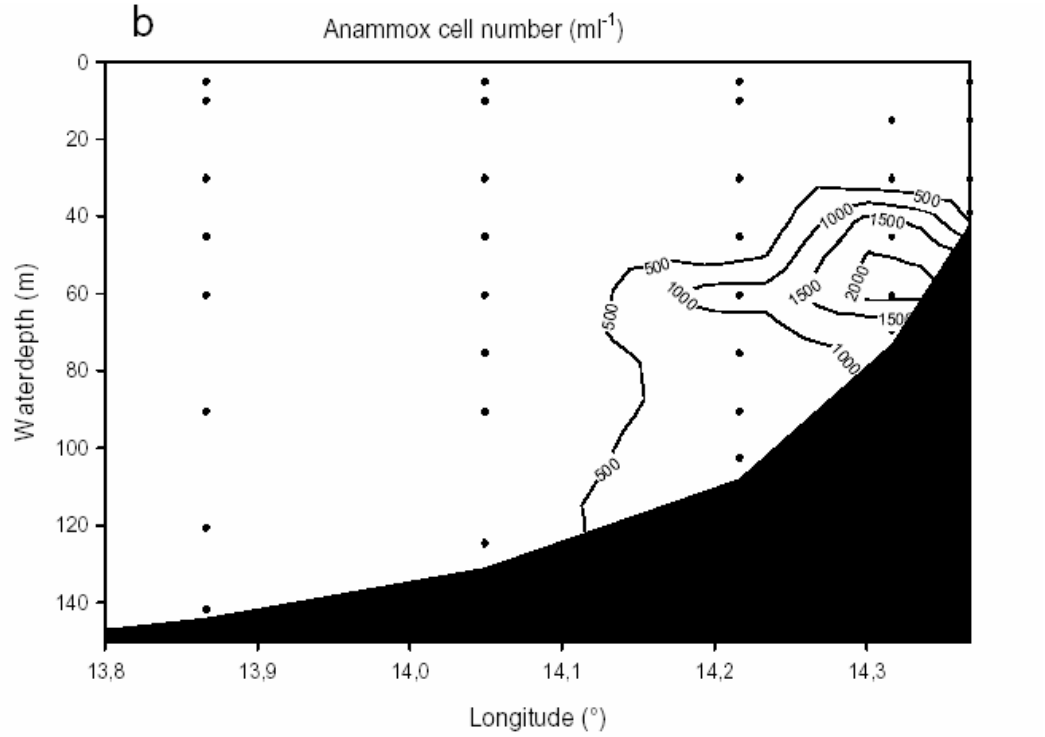


Fig. 2: (a) Oxygen concentration (µM) over the Namibian shelf along 23°S on the 22nd to the 23rd of March, 2003. Note that oxygen concentrations are < 10 µM above the sediment from approximately 13.95°E to 14.35°E.
(b) Distribution of CARD-FISH stained anammox cells per ml seawater along a transect on the 23°S off the coast of Namibia from 14.37°E to 13.85°E. The highest concentration of anammox cells was located close to the coast at 14.32° E. Further offshore than 14.05°E the number of anammox cells was below detection limit. The dots represent the sample depths. Note that by CARD-FISH only 40 (27±13%) of the anammox bacteria are detected.

Microbial diversity in the Namibian upwelling system. Particle association allows for specific interactions between anammox bacteria and other species. Therefore, the microbial diversity in the Namibian upwelling system was studied. We investigated six 16S rRNA gene clone libraries. This included two general bacterial libraries at station M182 from depths of 119 m and 130 m (suboxic to anoxic waters) where anammox occurred, one archaeal library from 130 m, a *Planctomycetes*-enriched library and two anammox bacteria-enriched libraries from station M202. The latter three were from 52 m depth where anammox activity was detected (Kuypers et al. 2005).

The two bacterial libraries from 119 m and 130 m depths at station 182 are in the following treated as one, because they originate from the same water mass according to physical and chemical parameters. A total of 231 clones were analyzed by partial sequencing of the approximately 400 nucleotides at the 5'-end of 16S rRNA. The libraries were dominated by sequences related to *Gammaproteobacteria* (94/231). Many of the sequences clustered into two groups of uncultured G*ammaproteobacteria*. The first cluster (19/231) showed up to 99% sequence identities with uncultured bacterial sequences from the Arctic Ocean (accession number AF354606), the Atlantic (AF382103), deep-sea

hydrothermal vent water (AB186990) and from the North Sea after an algal bloom (AJ400348). This cluster of sequences also showed 90% to 96% sequence identity with chemoautotrophic clam gill symbionts (*Calyptogena* spp. and *Vesicomya lepta*; AF035722, AF035723, AY310507 and AF035727). The second group of uncultured *Gammaproteobacteria* (49/231) had highest sequence identity to sequences from the Atlantic (AF382102), the Southern Ocean (AY135674), the Arctic Ocean (AF354595) and the Pacific (AY627383) indicating a wide distribution of this cluster. The other gammaproteobacterial sequences were related to the SAR92 cluster (6), to the genus *Vibrio* (5), to the *Alteromonadales* (4), the *Oceanospirillaceae* (3), the SAR86 cluster (3), and to the genera *Colwellia* (2), *Kangiella* (2) and *Psychromonas* (1).

The next dominant groups were sequences related to the *Alphaproteobacteria* (60/231) and the *Bacteroidetes* (43/231). Most of the sequences belonging to the *Alphaproteobacteria* were affiliated to the SAR11 cluster (46/231). About two thirds of the sequences within the *Bacteroidetes* showed highest sequence similarities to members of the genus *Cytophaga*, about one third to uncultured clades of *Bacteroidetes* and a single sequence to *Flavobacterium johnsoniae*. Some sequences had very high sequence identity (up to 100%) with sequences belonging to the AGG58 cluster (L10946; DeLong et al. 1993).

In addition, sequences were affiliated with the *Deltaproteobacteria* (11/231) including the SAR324 cluster (7/231), the *Betaproteobacteria* (6/231), with *Nitrospina* (6/231), *Actinobacteria* (4/238) and the SAR406 cluster (3/231). Furthermore, we retrieved two planctomycete related sequences having up to 88% sequence identity with a sequence from a particle-associated uncultured bacterium (L10943; DeLong et al. 1993). Single sequences grouped within the *Epsilonproteobacteria* and *Spirochaeta*. Due to potential biases during PCR amplification of the 16S rRNA genes (e.g. Suzuki and Giovannoni 1996), we did not use clone frequencies in our clone libraries to quantify microbial groups.

Since no anammox related sequences were retrieved with the general bacterial primer set, a PCR-amplification with a planctomycete-specific primer was performed. This library also failed to retrieve sequences related to anammox bacteria. Therefore, two clone libraries with anammox-specific primers were constructed. These resulted in 59 anammox related sequences of 700 to 900 nucleotides length. The majority (55/59) showed 97 to 99% sequence identity with *Candidatus* Scalindua brodae and *Candidatus* Scalindua sorokinii, the remaining four sequences had a lower sequence identity of about 96%.

The 16S rRNA gene clone library of *Archaea* at 130 m depth was strongly dominated by marine group I *Crenarchaeota* (18/23). Five sequences were affiliated with *Euryarchaeota*. Twelve of the 18 almost full length crenarchaeotal 16S rRNA sequences clustered tightly with an identity of 99% to that of '*Nitrosopumilus maritimus*' (Könneke et al. 2005).

Relative abundance of major microbial groups in the Namibian OMZ and characterization of a particle-specific microflora. We investigated the relative abundance of major groups of *Bacteria* and *Archaea* in the Namibian upwelling system by CARD-FISH using a set of group-specific probes chosen based on the results of the 16S rRNA gene clone libraries. For analysis of the free water phase, we hybridized samples from water masses containing less than 10 µM oxygen from 8 different stations along the Namibian coast. From similar water layers the results of up to 4 depths per station were combined. To determine the relative abundances of the microbial flora co-occurring with anammox bacteria on particles, we hybridized filters of station M199 from suboxic to anoxic waters.

Overall detection efficiency by CARD-FISH from the free water phase was high. With the *Bacteria*-specific probe mix EUB I-III, 72.7±14.3% of the cells in the Namibian OMZ were detected. The relative abundance of *Crenarchaeota* group I is significantly higher ($P<0.001$, Mann-Whitney-U-Test) in the free water phase (14.9±4.6%) than in particles (2.2±1.8%) (Table 2). The same applies for the *Euryarchaeota*, which account for 2.7±1.9% of all microorganisms in the free water phase, whereas their abundance in particles was below 1% ($P<0.001$, Mann-Whitney-U-Test).

Bacteria of the *Cytophaga/Flavobacterium* cluster, *Gammaproteobacteria* and *Alphaproteobacteria* inhabit both, the free water phase and particles, in high relative abundances. Statistical tests showed that the abundance of the *Cytophaga/Flavobacteria* was not significantly different in the free water phase and in the particles (13.7±6.4 % and 12.4±3.5%, respectively, $P>0.05$, Mann-Whitney-U-Test) (Fig. 3b).

Cells stained with probe ALF968 made up 17.1±7.3% of the cells in the free water phase and 11.6±4.8% of particle associated cells ($P>0.05$, Mann-Whitney-U-Test). Within the *Alphaproteobacteria* the counts with probe ROS537 targeting the *Roseobacter* cluster were 3.1±2.2% in free water phase and 3.2±1.8% attached to particles, and those with probe SAR11-441 were 16.5±3.5 % in free water phase and 2.6±1.7% in particles. The relative abundance of *Roseobacter* did not differ significantly in the free water phase and in

particles (P>0.05, T-test), in contrast to the significant higher relative abundance of the SAR11 group in the free water phase (P<0.001, T-test).

Table 2: Overview of the relative abundances of different phylogenetic groups in the free water phase (suboxic to anoxic waters) and occurring in particles in the Namibian upwelling system.

Phylogenetic group	Relative abundance (%) Free water phase (suboxic to anoxic)	Particle associated	Significance
Alphaproteobacteria	17.1 (±7.3)	11.6 (±4.8)	P>0.05
SAR11-clade	16.5 (±3.5)	2.6 (±1.7)	**P<0.001**
Roseobacter-clade	3.1 (±2.2)	3.2 (±1.8)	P>0.05
Gammaproteobacteria	8.1 (±1.8)	11.8 (±5.0)	**P<0.01**
SAR86-clade	<1	<1	--
NOR5/OM60 clade	<1	1.6 (±1.6)	--
Pseudoalteromonas, Colwellia	<1	<1	--
Alteromonas/Colwellia	1.1 (±1.3)	<1	--
Nitrosococcus oceani, N. halophilus	ND	ND	--
Ammonia-oxidizing ***Betaproteobacteria***	ND	ND	--
Cytophaga/Flavobacterium cluster	13.7 (±6.4)	12.4 (±3.5)	P>0.05
SAR406-clade	3.3 (±1.0)	4.0 (±2.2)	P>0.05
***Crenarchaeota* group I**	14.9 (±4.6)	2.2 (±1.8)	**P<0.001**
Euryarchaea	2.7 (±1.9)	<1	**P<0.001**

ND: not detectable; in bold: significant difference in abundance

The relative abundance of *Gammaproteobacteria* in particles is with 11.8±5.0% significantly higher than in the free water phase with 8.1±1.8% (P<0.01, T-test) (Fig. 3a). Probes specific for subgroups of the *Gammaproteobacteria* (NOR5/OM60, SAR86, *Alteromonas* and *Pseudoalteromonas*) only revealed minor abundances of about 1% or less of those groups in both habitats. Bacteria of the SAR406 cluster accounted for 3.3±1.0% in the free water phase and 4.0±2.2% in particles, showing no significant difference (P>0.05, T-test).

The ammonia-oxidizing *Beta-* and *Gammaproteobacteria* were quantified both, by standard FISH and CARD-FISH, at stations M182 (30, 42, 96 and 110 m) and M202 (42 and 68 m) with the probes NSO1225, NSO190 (specific for ammonia-oxidizing *Betaproteobacteria*) and Nscoc128 (*Nitrosococcus oceani* and *N. halophilus*) (Table 1). With both methods no ammonia-oxidizing bacteria could be detected.

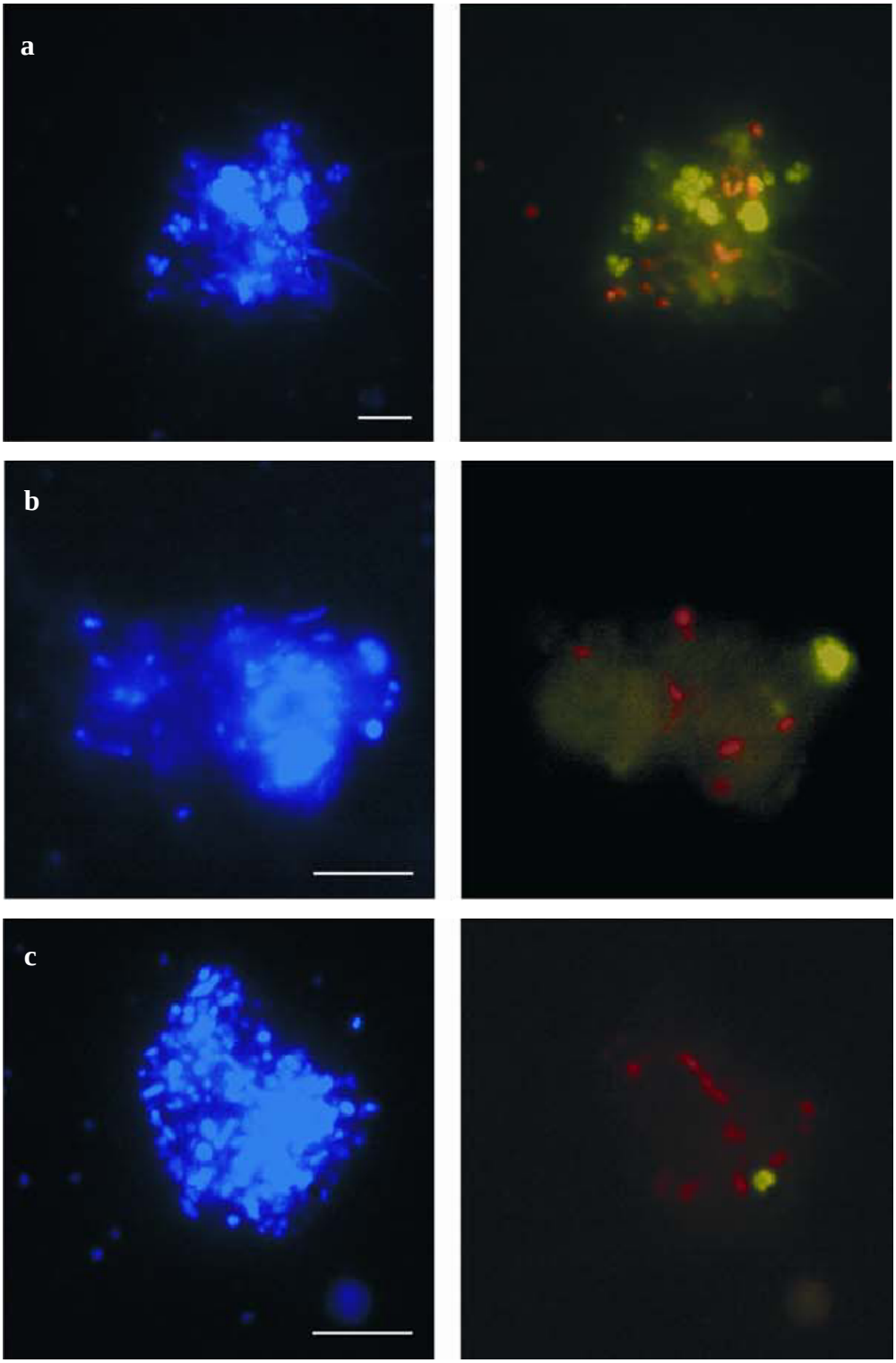


Fig. 3: Combined epifluorescence micrographs from particles in the Namibian and Peruvian OMZ stained with CARD-FISH. We conducted double hybridizations with the anammox specific probe BS-820 (and a mixture of BS-820 and BS-820-C for samples from the Peruvian OMZ) and probes for other phylogenetic groups (a, Gam42a; b and c, CF319a). The left panel shows cells stained with DAPI in blue. In the right panel anammox bacteria are shown in green, other groups of bacteria are stained in red. Fig. 3 a and b show samples from the Namibian OMZ, Fig. 3 c is from the Peruvian OMZ. Bar 5 µm.

Discussion

Particle association of anammox bacteria has been used to explain the discrepancy observed between the known oxygen-sensitivity of anammox bacteria and their occurrence in the Namibian OMZ, where anammox activity was detected in waters with oxygen concentrations up to 9 µM and ammonium concentrations below detection limit (Kuypers et al. 2005). This study focuses on the visualization of particle-associated anammox bacteria and their potential interactions with other microbial populations.

Methodological aspects. For the more reliable identification of anammox bacteria in the particles we developed an improved CARD-FISH protocol. This was necessary because the low signal intensity of standard FISH did not allow for the detection of anammox bacteria against the elevated autofluorescence of particles. When comparing standard FISH counts of anammox cells with CARD-FISH counts we discovered an apparent discrepancy. CARD-FISH detected at most 40% (27±13%) of the anammox cells detected by standard FISH. However, both methods showed similar trends in depth distribution of anammox cells through the water column, which is supported by a strong linear correlation of both methods detecting anammox cells ($R=0.91$, $P<0.005$, $n=12$).

It is an interesting methodological aspect that CARD-FISH counts of anammox bacteria were consistently lower than those of standard FISH. For activated sludge aggregates Schönhuber et al. had reported a similar phenomenon (Schönhuber et al. 1997). CARD-FISH stained only half of the cells which were stained with standard FISH (Schönhuber et al. 1997). This is likely caused by the larger horseradish peroxidase label used for CARD-FISH (~44 kDa), compared to an oligonucleotide labeled with a fluorescent dye (~700 Da). During hybridization the enzyme-labeled probe has to penetrate the extracellular polymeric material in the bacterial cell wall. Depending on the exact composition of the cell wall, probe penetration might be inhibited, e.g., in gram-positive bacteria (Bidnenko et al. 1998). Therefore we conclude that standard FISH is still the method of choice to quantify anammox cells in homogenized samples. However, CARD-FISH is the method of choice for monitoring and localizing anammox bacteria on particles with elevated autofluorescence.

Anammox in particles. During sampling in the Namibian upwelling system, a high abundance of macroscopic particles in the water column was detected by a submersible camera. The particles contributed to high turbidity in the Namibian upwelling (Kuypers et

al. 2005). In an earlier study, Kiørboe counted 85 to 640 macroaggregates per liter seawater further south in the Benguela upwelling off the coast of South Africa (Kiorboe et al. 1998). These numbers are among the highest concentrations reported for the marine environments (Kiorboe et al. 1998, Simon et al. 2002).

About 50% (56.2±8.3%) of the CARD-FISH stained anammox bacteria were organized in tight clusters (Fig. 1b). We also observed single anammox cells which could have been detached from particles during sampling. Considering the relatively high shear forces occurring during pump cast sampling as well as during sampling with go-flow bottles, we speculate that anammox bacteria formed clusters which are relatively stable compared to the other components of particles. Our data provide direct evidence for particle-association of anammox bacteria at least in the Namibian OMZ. With the samples available for this study, we can, however, only provide estimates of the exact fraction of anammox bacteria on particles and the in situ particle size. Exact numbers would require the retrieval of intact macroscopic particles which is currently only possible by SCUBA diving or specifically designed devices for catching particles.

Anammox bacteria distribution over the Namibian Shelf. Our study focused on the particle-association of anammox bacteria. To monitor their occurrence in a transect at 23°S from the coast to the open ocean (Fig. 2b), we used CARD-FISH because it provides a better reproducibility and allows analysis of numerous samples. There was a clear maximum in anammox bacterial abundance close to the coast between 14.37°E and 14.20°E which perfectly matches the zone of anammox activity (Kuypers et al. 2005). The oxygen concentrations in these waters range from ~30 µM at the upper boundary of the anammox zone to values below detection limit (<1 µM) close to the sediment (Fig. 2a). In the same waters, we found an increase in particle concentration as revealed by turbidity measurements (e.g. particle maximum at 14.31°E below 45 m and at 14.2°E at 60 m and close to the sediment; data not shown). This could indicate that anammox cells were resuspended together with particles from the sediment and that resuspension of suspended particles containing anammox bacteria might play a major role in this ecosystem. Only recently it has been shown that resuspension is an important process in the Benguela upwelling system of the coast off South Africa and that lateral particle transport is the primary mechanism controlling the supply and burial of organic carbon at the SW African shelf and slope (Inthorn et al. 2006). The significant correlations of anammox cell numbers and anammox activity, anammox cell numbers and particulate organic nitrogen and

anammox cell numbers and particulate organic carbon support our finding, that anammox bacteria are particle-associated. No experiments for anammox rate measurements were conducted at ambient oxygen concentrations higher then 9 µM. However, the association to oxygen-depleted microniches probably allows the anammox bacteria to maintain activity at oxygen concentrations as high as 25 µM (Ploug 2001), significantly expanding the zone of potential nitrogen loss due to anammox in the Namibian OMZ.

Microbial diversity in the OMZ. The apparent particle association of anammox bacteria opens the possibility for specific interactions of anammox bacteria with other microorgansims in the particles.

Macroscopic aggregates are known for their diverse microbiota, (e.g. DeLong et al. 1993, Fandino 2001, Gram et al. 2002, Rath et al. 1998). Also our general bacterial 16S rRNA gene clone libraries from the anammox zone (station M182, 119 m and 130 m) showed a high diversity with "standard" marine representatives of *Alphaproteobacteria*, *Gammaproteo-bacteria*, *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, and the SAR406 cluster.

Within the > 200 bacterial sequences there were, on the one hand, sequences of phylogenetic groups also found in fully oxygenated waters such as SAR11 (Giovannoni et al. 1990), SAR86 (Mullins et al. 1995), SAR92 (Britschgi and Giovannoni 1991) and members of the SAR406 cluster (Gordon and Giovannoni 1996). This could be due to sedimentation of biomass from surface waters. On the other hand, we found sequences related to those of the *Bacteroidetes* and *Planctomycetes* which are known for their ability to associate with particles. One example are the sequences which showed up to 100% sequence identity with members of the cluster AGG58. The principal member of this cluster (clone env.agg58) was amplified from particles in the Santa Barbara Channel in California in (DeLong et al. 1993).

Different composition of attached versus free-living microbial community. By CARD-FISH we could show that the relative abundance of *Gammaproteobacteria* in particles was significantly higher than in the free water phase. SAR11 as well as group I *Crenarchaeota* and *Euryarchaeota* clearly preferred the free water phase. On the group-level, we detected no differences in relative abundances of the *Cytophaga/Flavobacterium* cluster, the SAR406 cluster and *Alphaproteobacteria* (Table 2). This does not exclude that the species compositions differ within the groups.

Peruvian upwelling. The results from the Namibian OMZ led to the question, whether particle-associated anammox bacteria and their co-occurring microbiota can also be found in other OMZs. We studied samples from the Peruvian OMZ and found anammox organisms with 98% sequence identity to *Candidatus* Scalindua brodae and *Candidatus* Scalindua sorokinii (Hamersley et al. 2007). Double hybridizations showed that a fraction of anammox bacteria was attached to aggregates and like their relatives from the Namibian OMZ were in association with *Alphaproteobacteria*, *Gammaproteobacteria*, bacteria of the *Cytophaga/Flavobacterium* cluster (Fig. 3e) and the SAR406 cluster. Similar to the Namibian OMZ, *Crenarchaeota*, members of the SAR11 cluster and *Euryarchaeota* were only rarely found in aggregates. However, further investigations are needed to statistically confirm these preliminary results from the Peruvian OMZ.

Potential interactions in particles. Our study provides direct quantification of major bacterial and archaeal groups in marine particles using FISH. These results provide insight in the potential interactions between anammox bacteria and other microorganisms. Even though the observed aggregates are certainly not as tightly structured like e.g. the methane oxidizing consortia found in microbial mats on methane hydrates (Boetius et al. 2000), there may still exist discrete functional interactions. We hypothesize that heterotrophic bacteria like *Alpha-* and *Gammaproteobacteria* and members of the *Cytophaga/Flavobacterium* cluster will reduce the oxygen concentration in particles by respiration (Fig. 4a) to levels suitable for the anammox reaction (Fig. 4c). It as been shown, that microbial oxygen consumption can result in the development of anaerobic microenvironments in particles at ambient oxygen concentrations up to 25 µM (Ploug 2001).

Furthermore, remineralization activity of microorganisms in particles can result in significantly higher concentration of ammonium in macroscopic aggregates than in ambient seawater (Shanks 1979) (Fig. 4b). This elevated ammonium concentration is likely due to hydrolytic enzyme activity which is significantly higher in particles than in the ambient seawater (Smith et al. 1992). Heterotrophic bacteria on particles express a whole array of hydrolytic enzymes. Interestingly, the protease activity is 10-100 times higher than the activity of polysaccharidase, alpha- and beta-glucosidase (Smith et al. 1992). The ability to degrade a wide variety of high molecular weight compounds like proteins and the production of exoenzymes is known for members of the *Bacteroidetes* (Cottrell and Kirchman 2000, DeLong et al. 1993, Dworkin et al. 2005). Also *Alpha-* and

Gammaproteobacteria might be involved in degradation of complex compounds (Cottrell and Kirchman 2000). For some members of the latter group it is known that they regulate their hydrolytic enzyme activity by quorum sensing mechanisms (Christensen et al. 2003, Givskov et al. 1997).

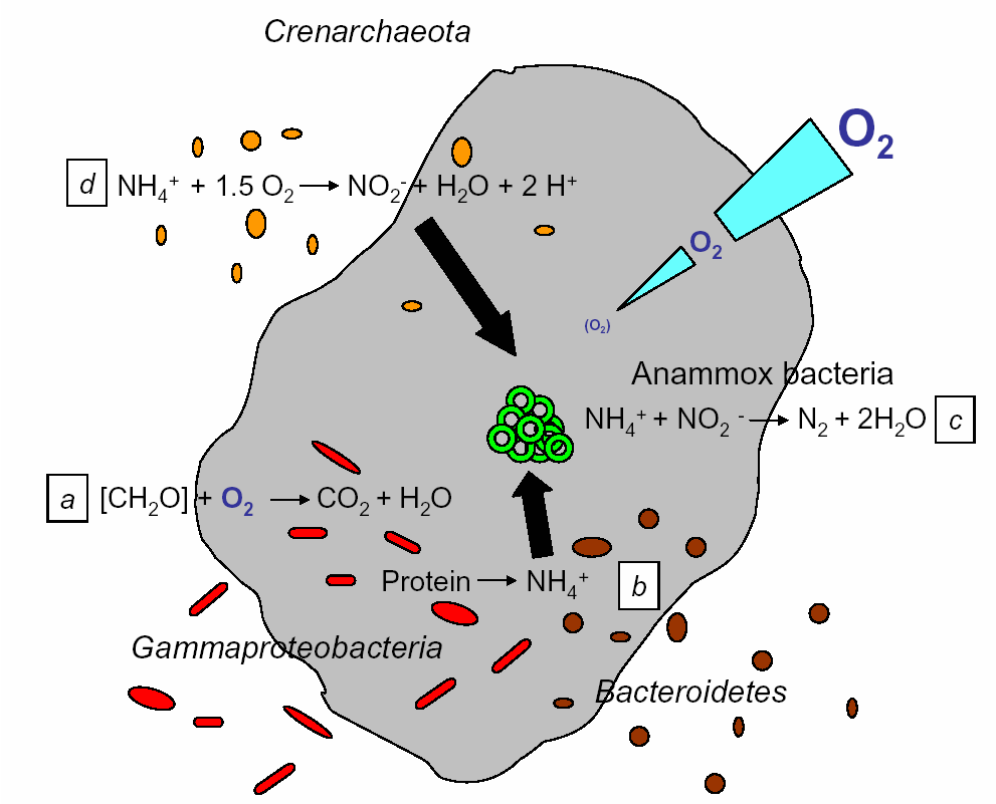


Fig. 4: Summary of potential microbial interactions on particles in the water column. We assume that the observed particles on filters were previously parts of larger particles in the water column. See text for details.

Previous results indicate that aerobic ammonium oxidation, rather than nitrate reduction, is the source of nitrite for anammox at site M182 in the Namibian OMZ (Kuypers et al. 2005). However, by standard FISH and CARD-FISH no ammonia-oxidizing bacteria were detected. This infers that the abundance of ammonia oxidizing bacteria stained with the probes used (Table 1) was below the detection limit. Recently, the crenarchaeon '*Nitrosopumilus maritimus*' was shown to grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite (Könneke et al. 2005). Fosmid sequences of *Cenarchaeum symbiosum* indicated genetic potential for ammonium oxidation (Hallam et al. 2006) in this crenarchaeon as well. Additionally, genes encoding for the creanarchaeal ammonia monooxygenase a-subunit (amoA) were recovered from ocean waters world wide (Hallam et al. 2006) and (Wuchter et al. 2006) further suggesting an important role of *Crenarchaeota* in oceanic nitrification. Most of the 16S rRNA sequences of the

Crenarchaeota in the Namibian Upwelling System show a sequence identity of 99% with '*Nitrosopumilus maritimus*'. Could these *Crenarchaeota* provide the anammox bacteria with nitrite in the Namibian OMZ?

The cell specific anammox activity calculated (~4.5 fmol of ammonium per cell per day) for the Namibian shelf water (Kuypers et al. 2005) is comparable to the cell specific aerobic ammonium oxidation rates (4 fmol of ammonium per cell per day) recently reported for marine group I *Crenarchaeota* (Wuchter et al. 2006). While *Crenarchaeota* account for ~15% of all microorganisms, anammox bacteria account for only ~1% of the microbial community in the Namibian OMZ waters (Kuypers et al. 2005). Hence only a small fraction of the *Crenarchaeota* at site M182 would need to be aerobic ammonium oxidizers if we assume a similar cell specific activity as for the North Atlantic (~4 fmol per cell per day) (Wuchter et al. 2006) and could provide the anammox bacteria with the necessary nitrite (Fig. 4c and 4d).

Future directions. Our hypothesis could be tested in future cruises by SCUBA diving based sampling of undisturbed macroscopic particles or by using particle collectors. The oxygen concentrations inside the particle could be measured by micro-sensors (Ploug 2001) to support our hypothesis of anoxic microniches. Preservation of the three-dimensional structure would allow CARD-FISH analysis of intact particles to prove the co-localization of anoxic niches and anammox bacteria. In addition, the exact local distribution of other bacteria in intact particles could be studied.

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Abstract

We investigated the microbial pathways of nitrogen (N) loss in an April 2005 transect through the Peruvian oxygen minimum zone (OMZ) at 12°S latitude using short anaerobic incubations with ^{15}N-labeled substrates and molecular–ecological and lipid–biomarker studies. In incubations with $^{15}NH_4^+$, immediate production of $^{14}N^{15}N$, but not $^{15}N^{15}N$, indicated that N_2 was produced by the pairing of labeled $^{15}NH_4^+$ with in situ $^{14}NO_2^-$ via anaerobic ammonium oxidation (anammox). Supporting this finding, we also found anammox-related 16S ribosomal ribonucleic acid gene sequences similar to those previously known from other marine water columns in which anammox activity was measured. We identified and enumerated anammox bacteria via fluorescence in situ hybridization and quantitative polymerase chain reaction and found ladderane membrane lipids specific to anammox bacteria wherever anammox activity was measured by our isotope tracer method. However, in incubations with $^{15}NO_3^-$ or $^{15}NO_2^-$, in which denitrification would have been expected to produce $^{15}N^{15}N$ by pairing of oxidized ^{15}N ions, $^{15}N^{15}N$ production was not detected before 24 h, showing that denitrification of fixed N to N_2 was not taking place in our samples. At the time and locality of our study, anammox, rather than denitrification, was responsible for N_2 production in the Peruvian OMZ waters.

The Peruvian upwelling system in the eastern tropical South Pacific Ocean (ETSP) is an area of high productivity that generates one of the ocean's largest masses of suboxic water. Longshore geostrophic winds cause the Ekman transport of the subtropical surface water (ca. >25 m deep)

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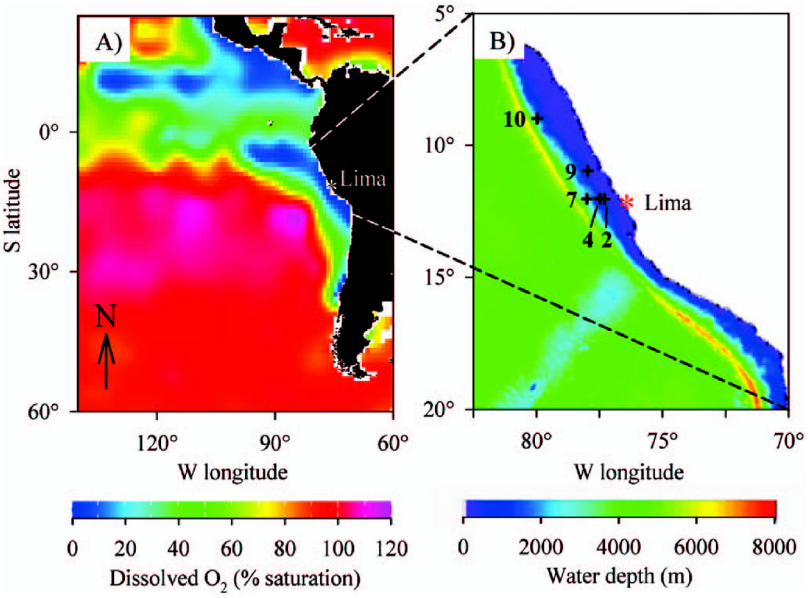

Fig. 1. (A) Dissolved oxygen concentrations (% saturation) in the Equatorial Pacific at 100 m in depth (image source: http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/.NODC/.WOA01/). (B). Bathymetry of Peruvian shelf and location of sampling stations. See Table 1 for station reference data. Sta. 2, 4, and 7 of transect at 12°S are shown; Sta. 1, 3, 5, and 6 (not sampled for anammox) are not shown (image source: http://topex.ucsd.edu/marine_topo/).

westward, bringing subsurface waters to the euphotic zone (Friederich and Codispoti 1987; Copin-Montégut and Raimbault 1994). The upwelled water has its source in the Equatorial Undercurrent, a poleward current of hypoxic, nutrient-enriched Equatorial subsurface water. The upwelling of this water into the euphotic zone supports high primary productivity, and decomposition of this phytoplankton biomass further depletes dissolved oxygen, resulting in the development of an oxygen minimum zone (OMZ) that extends from the thermocline—as little as 20 m below the surface—to the shelf sediments and westward over the continental slope and abyssal plain (Fig. 1A).

Decomposing organic matter should release dissolved nutrients in the same proportion as that of the originating material. A deficit in the ratio of regenerated inorganic nitrogen (NH_4^+, NO_3^-, and NO_2^-) to phosphate relative to the nitrogen : phosphorus (N : P) ratio of mixed-layer organic matter (the 'N-deficit') has been attributed to a loss of fixed nitrogen as N_2 within this and other oceanic OMZs (Codispoti and Packard 1980; Deutsch et al. 2001). Such calculations have indicated that 30% to 50% of the total oceanic nitrogen loss may occur in OMZs (Codispoti et al. 2001). Until recently, the only known process that could explain this loss was denitrification, the reduction of nitrate to N_2 gas via $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ by heterotrophic bacteria (Codispoti and Christensen 1985). However, direct measurements of the reduction of NO_3^- to N_2 in oceanic water columns have been lacking. Denitrification has been inferred from the distribution of water column nitrite, thought to accumulate as a result of incomplete nitrate reduction (Codispoti and Packard 1980; Codispoti and Christensen 1985), and from the ^{15}N enrichment of residual nitrate by the isotopic discrimination associated with nitrate reduction (Brandes et al. 1998). The activity of the electron transport system (ETS) in cell-free extracts, a measure of the reductive potential of the water column cellular machinery, has been assumed (in suboxic waters) to indicate denitrification potential, and denitrification rates have been estimated from ETS activity using theoretical and empirical correction factors (Codispoti and Packard 1980). Denitrification has also been inferred from the spatial gradients in N-deficits, with advection and diffusion calculated from reference and literature values (Codispoti and Packard 1980), or from chlorofluorocarbon-derived water-mass ages (Deutsch et al. 2001). Other incubation-based measures of nitrogen metabolism have not directly addressed N_2 production. Measurements of N_2O production under acetylene amendment have given contradictory results, and their interpretation may be uncertain because of the multiple pathways of N_2O production and consumption and the artifacts associated with the acetylene technique. Other measures have included nitrite production from nitrate in cell-free extracts (Packard et al. 1978) and isotopic tracer measurements of the reduction of nitrate to nitrite (Lipschultz et al. 1990). However, none of these approaches have directly

measured the loss of fixed inorganic nitrogen as N_2 gas, nor have they confirmed the mechanism of N_2 production with metabolic or microbiological evidence.

Recently, a novel microbial process known as anammox (anaerobic ammonium oxidation) has been show to be a significant contributor to nitrogen losses in marine and estuarine sediments (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003; Myer et al. 2005), in sea ice (Rysgaard and Glud 2004), and in the anoxic waters of basins isolated from oxygenated deep circulation (Dalsgaard et al. 2003; Kuypers et al. 2003). Anammox is a chemolithotrophic process whereby one mole of ammonium is oxidized with one mole of nitrite in the absence of oxygen to produce N_2 gas (Srous et al. 1999):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

The potential for such a pathway in the ocean was postulated by Richards et al. (1965) but was first observed in wastewater (Mulder et al. 1995) and was shown to be performed by hitherto-unknown members of the bacterial order *Planctomycetales* (Strous et al. 1999). N_2 generated through anammox can be distinguished from that generated by denitrification by the pattern of N_2 labeling observed in anaerobic incubations with $^{15}NH_4^+$, $^{15}NO_2^-$[15], or $^{15}NO_3^-$. The technique of isotope pairing can distinguish anammox from denitrification through the measurement of the relative production of the differently labeled N_2 isotopologues ($^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$) (Nielsen 1992; Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2003). Since this discovery, anammox has been shown to play a significant role in many marine environments (reviewed in Dalsgaard et al. 2005; Kuypers et al. 2006). Further, anammox has been shown to be responsible for large N losses from the OMZ waters of the dynamic Benguela upwelling system off the coast of Namibia, whereas no evidence for heterotrophic denitrification was found (Kuypers et al. 2005). This discovery led to the hypothesis that anammox might be important in other OMZs such as the Peruvian. The aim of this study was to investigate the role of these two microbial N_2 loss processes—denitrification and/or anammox—in nitrogen loss from Peruvian OMZ waters.

Methods

Study site—Water column profiles of physico–chemical parameters and water samples for nutrient analyses, ^{15}N incubations, and anammox indicators were collected during a cruise of the IMARPE R/V *José Olaya* off the coast of Peru in April 2005. Sampling stations were located along a transect at 12°S latitude near Callao, ranging from nearshore waters (47-m water depth) to the continental slope (ca. 2,400-m depth) (Table 1; Fig. 1B). In addition, two shelf stations were sampled farther north (Sta. 9 and 10 [respectively, 11°S and 9°S latitude: Ocean Drilling Project Sta. 1228 and 1229]; Fig. 1B). All the investigated stations are within the region of intense seasonal upwelling, primary production, and water column anoxia that form the extensive OMZ off the coast of Peru and northern Chile (Fig. 1A; Codispoti and Packard 1980).

Table 1. Characteristics of study stations within the Peruvian upwelling system (*see Fig. 1B*).

Station	Latitude (°S)	Longitude (°W)	Water depth (m)	Sampling date(s)
2	12.05	77.30	96	15 Apr 2005
				21 Apr 2005
4	12.03	77.49	141	17 Apr 2005
7	12.04	77.99	2,400	22 Apr 2005
9	10.96	77.96	152	20 Apr 2005
10	8.99	79.96	442	19 Apr 2005

Water column profiles—We obtained profiles of salinity, temperature, oxygen, and chlorophyll *a* (Chl *a*) fluorescence with a conductivity, temperature, and depth (CTD) system equipped with an oxygen electrode and a fluorometer (Sea-Bird Electronics). Water samples for high-resolution nutrient analyses and ^{15}N incubations were obtained by a pump-CTD (Kuypers et al. 2003) or by GoFlow bottles (Hydrobios). Nitrite was measured immediately on board (detection limit 0.03 μmol L^{-1}), and water samples were frozen (−20°C) for later analysis of nitrate, ammonium, and phosphate by autoanalysis (TRAACS 800, Bran & Lubbe; detection limits of 0.1, 0.3, and 0.1 μmol L^{-1}, respectively) (Grasshoff et al. 1999). The fixed N deficit was determined by the formula

$$\text{N deficit} = 12.6 \times (PO_4^{3-}) - ([NO_3^-] + [NO_2^-] + [NH_4^+]) \quad (2)$$

where the constant 12.6 is the empirically determined N : P ratio of organic matter produced in these waters (Codispoti and Packard 1980).

Long-term temperature and O_2 monitoring—A time series (1996–2005) of vertical profiles of temperature and oxygen at Sta. 2 has been constructed to show interannual variability (Gutiérrez et al. in press). Water samples were collected monthly with Niskin bottles. Temperature was measured by inversion thermometer through 2001 and by CTD from 2002 onwards. Dissolved oxygen was determined by the Winkler method (Grasshoff et al. 1999).

^{15}N incubations and analysis—^{15}N labeling experiments were performed at Sta. 2, 4, 7, 9, and 10 (Fig. 1B). Water was collected across the suboxic zone at four to seven depths per station, with one further sample collected from within the oxycline (ca. 25–50 μmol L^{-1} O_2) as a control. The ^{15}N incubations were begun immediately after sampling. The experimental method was slightly modified from that previously published by Dalsgaard et al. (2003). Briefly, 250 mL of Peruvian OMZ waters collected by pump-CTD were flushed with helium for 15 min and amended with $^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_2^-$, or $^{15}NO_2^-$ (to 5 μmol L^{-1} each) or with $^{15}NO_3^-$ (20 μmol L^{-1}) (isotopes: Campro Scientific). Water from above the suboxic zone was equilibrated with 20% v/v air to produce a final O_2 concentration of ca. 50 μmol L^{-1} (similar to in situ values).

Labeled seawater was transferred into 12-mL Exetainers (Labco) and incubated for up to 24 h in the dark at in situ temperatures. The Exetainers were destructively sampled at 0, 6, 12, and 24 h with $HgCl_2$ to stop biological activity. $^{14}N^{15}N:{}^{14}N^{14}N$ and $^{15}N^{15}N:{}^{14}N^{14}N$ ratios of He-equilibrated headspaces were determined by gas chromatography/isotope ratio mass spectrometry (Fisons VG Optima).

Lipid biomarkers—Evidence for the presence of anammox bacteria was sought using anammox-specific ladderane membrane lipids (Sinninghe Damsté et al. 2002). Particulate matter was collected using in situ pumps (McLane WTS-LV Sampler) to filter large volumes of water (ca. 500 liters per sample) through precombusted glass-fiber filters (GF/F, nominal pore size: 0.7 μm, Whatman). The filters were freeze-dried and extracted with 3× methanol, 3× methanol : dichloromethane (1 : 1 v/v), and 3× dichloromethane to obtain total lipid extracts. Aliquots of the lipid extracts were saponified and subsequently methylated with diazomethane. Samples were analyzed by high-performance liquid chromatography/mass spectrometry (HPLC/MS), as described elsewhere (Hopmans et al. 2006). Lipid quantification was performed using external authentic ladderane standards, isolated from anammox enrichment cultures. The reproducibility of the HPLC/MS ladderane concentration measurements was >95% (determined by triplicate analysis). Since GF/F filters, with a pore size of 0.7 μm, may undersample anammox cells, the calculated ladderane–lipid concentrations represent minimum values.

DNA extraction and phylogenetic analysis—For deoxyribonucleic acid (DNA) extraction, seawater samples (200 to 400 mL) were filtered onto polycarbonate GTTP membrane filters (47 mm in diameter, 0.22-μm pore size, Millipore) and stored at −20°C. DNA extraction and polymerase chain reaction (PCR) amplification targeting Planctomycete 16S ribosomal RNA (rRNA) genes were as described in Schmid et al. (2000), except that 1392R (Stahl et al. 1988) was used as the reverse primer. The anammox-related 16S rRNA sequences obtained in this study were deposited in GenBank (accession numbers DQ534720–DQ534744).

Fluorescence in situ hybridization (FISH) and total microbial abundance—Water samples were fixed with particle-free paraformaldehyde solution (final concentration, 1% v/v) for 1 h at room temperature or overnight at 4°C. Fixed samples were filtered onto white polycarbonate membrane filters (described above) and stored at −20°C until analysis. Filter material was hybridized (Glöckner et al. 1996) with fluorescently labeled oligonucleotide probes (biomers.net) specific for anammox bacteria. For the detection of the entire Candidatus *Scalindua wagneri* and *Scalindua sorokinii* clade a mixture of two probes were used: probe BS-820 (5′-TAA TTC CCT CTA CTT AGT GCC C-3′) (Kuypers et al. 2003) and a newly developed probe BS-820-C (5′-TAA TCC CCT CTA CTT AGT GCC C-3′). We used the unlabeled probe AMX-820 (5′-AAA ACC CCT CTA CTT AGT GCC C-3′) (Schmid et al. 2000) as a competitor. Filters were also stained nonspecifically for DNA with 4,6-diamidino-2-phenylindole (DAPI), and the relative abundance of anammox cells was determined by epifluorescence microscopy. Total microbial abundance was determined by flow cytometry calibrated with manual microscopic counting of DAPI-stained cells.

Quantitative PCR (qPCR)—Anammox bacteria were also quantified with the TaqMan fluorogenic PCR method (Livak et al. 1995) using DNA extracted from polycarbonate membrane filters (described above). A primer and fluorogenic probe set was designed for all known anammox or anammox-like bacteria using the Primer Express v.2.0 software (Applied Biosystems), based on sequences retrieved from the GenBank and from our own environmental sequence databases. The resulting primer set, AMX-808-F (5′-ARC YGT AAA CGA TGG GCA CTA A-3′) and AMX-1040-R (5′-CAG CCA TGC AAC ACC TGT RAT A-3′), and the probe AMX-931 (5′-TCG CAC AAG CGG TGG AGC ATG TGG CTT A-3′) had melting temperatures (58°C, 57°C, and 67°C, respectively) within the recommended range for the TaqMan system, and the specificity of their sequences was verified with the ARB software package BLAST (Altschul et al. 1990) and the Probe Match tool from the Ribosomal Database Project II (Cole et al. 2005). qPCR was performed on a iQ5 Real-Time PCR System (Bio-Rad Laboratories) for 2 min at 50°C and for 10 min at 95°C, followed by 50 cycles of 15 s each at 95°C and 2 min at 60°C. DNAs extracted from various anammox enrichment cultures were used as positive controls and standards. All samples and standards were analyzed in triplicate. The resulting anammox cell numbers were calibrated for absolute quantity against FISH counts (qPCR = 0.0307 [FISH] + 2,100; $r^2 = 0.7$, $p < 0.01$).

Results

Site characterization—The Peruvian shelf waters along our vertical transect at 12°S latitude were thermally stratified in mid-April 2005 with a pronounced OMZ extending from ca. 30-m water depth to the shelf sediments (Figs. 2A, 3). Within the OMZ, O_2 concentrations were typically less than 10 μmol L^{-1} (Fig. 2A). N deficits in the OMZ waters were largest near the coast and in shelf bottom waters, ranging from <5 μmol N L^{-1} over the slope to >15 μmol N L^{-1} near the shore (Fig. 2B), while Chl *a* concentrations in the mixed layer of the euphotic zone ranged up to 1.0 μg L^{-1} (Fig. 2C). Long-term (10-yr) temperature and O_2 monitoring of the water column at Sta. 2 shows that thermal stratification and subsurface hypoxia are common conditions, and the oxycline depth typically fluctuates between 10 and 50 m (Fig. 3). Instances of complete water column mixing and oxygenation were related to El Niño–Southern Oscillation (ENSO) temperature anomalies, as during the El Niño of 1997 to 1998 (red bars on Fig. 3 indicate ENSO warm periods), which otherwise were generally accompanied by a deepening of the oxycline. Cold-temperature anomalies, as during the La Niña of 1998 to 1999 (blue bars on Fig. 3 indicate ENSO

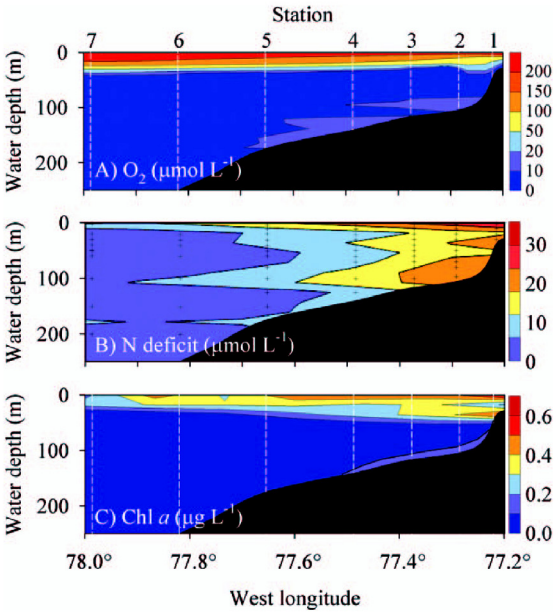

Fig. 2. Depth profiles along 12°S. Station numbers are those of Table 1 and Fig. 1B, with four additional stations interposed along the same latitude. Dashed lines in panels A and C represent locations of continuous profiles. Crosses in panel B represent locations of GoFlow bottle sample collection. (A) Dissolved O_2. (B) N deficit = 12.6 × ($^{15}NO_4^{3-}$) − ($NH_4^+ + NO_2^- + NO_3^-$). (C) Chlorophyll *a* (fluorescence).

cold periods), were generally associated with shoaling of the oxycline and a water column dominated by suboxic conditions.

Physico–chemical structure of the water column—During our cruise over the Peruvian shelf in April 2005, nitrate concentrations within the OMZ waters ranged from ca. 10 to 30 μmol L^{-1}, and nitrite concentrations ranged from ca. 0.2 to 5.0 μmol L^{-1} (Fig. 4, first column). Nitrate and nitrite concentrations were much lower in waters of the mixed layer. Two nitrite maxima were observed at all stations, one just below the oxycline and a second near the sediments, or centered at ca. 300 m over the slope at Sta. 7. Ammonium concentrations were typically <1 μmol L^{-1}, though maxima (up to 3 μmol L^{-1}) were found at the base of the oxycline at all stations. Nutrient-, oxygen-, and temperature-compensated density (σ_T) profiles taken 6 d apart at Sta. 2 showed the dynamic physico–chemical structure of the water column, which is also apparent from the long-term monitoring record (Fig. 3). On 15 April there was a poorly defined mixed layer at Sta. 2 with lenses of denser water present at 10 m depth, and high nitrate concentrations penetrated into surface waters, indicating a breakdown of stratification and upwelling of subsurface waters. By 21 April, Sta. 2 waters had stratified, forming a defined and deeper mixed layer nearly 30 m thick (Fig. 4B), possibly following reduced wind stress. The O_2 concentrations in the surface waters at this station on both dates were low (<50% saturation), indicating the upwelling of low-oxygen waters and high respiration rates. Although surface-water O_2 concentrations at Sta. 4 and 7—farther offshore—were near saturation, the oxycline depth was similar to that at Sta. 2, with steep oxygen gradients near the surface and extensive water-column suboxia. Steep density gradients of up to 0.033 kg m^{-4} maintained the oxyclines at Sta. 4 and 7 (Fig. 4, second column).

^{15}N-labeling incubations—Significant $^{14}N^{15}N$ production was detected in 48 out of 60 OMZ water-sample incubations (i.e., with $^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_2^-$, $^{15}NO_2^-$, or $^{15}NO_3^-$ amendments; average detection limit [including sample handling] for $^{14}N^{15}N$ = 1.1 nmol L^{-1}) (Fig. 4, third column). $^{14}N^{15}N$ was produced at every location and depth within the OMZ from at least one of the isotope treatments. The production of $^{14}N^{15}N$ in these incubations was linear with time ($r^2 \geq 0.7$; e.g., Fig. 5), although with $^{15}NO_3^-$ it was sometimes delayed (e.g., Fig. 5D). Of the incubations in which $^{14}N^{15}N$ production was not detected, half (6 out of 12) were incubations with $^{15}NO_3^-$. $^{14}N^{15}N$ production in anaerobic incubations with $^{15}NH_4^+$ ranged from 1.5 ± 0.1 nmol L^{-1} d^{-1} at 60(m at Sta. 2 (21 April; Fig. 4B) to a maximum of 384 ± 11 nmol(L(1(d(1 at Sta. 2 (15 April; Fig. 4A). At two stations (Sta. 2 [15 April] and Sta. 7), $^{14}N^{15}N$ production rates with $^{15}NH_4^+$ were significantly enhanced by the addition of $^{14}NO_2^-$. $^{14}N^{15}N$ production from $^{15}NO_2^-$ ranged from 0(nmol(L(1(d(1 at two depths at Sta. 7 to 48 ± 4 nmol L^{-1} d^{-1} at Sta. 4. Significantly lower rates of $^{14}N^{15}N$ production (maximum 27 nmol L^{-1} d^{-1}) were measured in incubations with $^{15}NO_3^-$. In contrast to $^{14}N^{15}N$, the production of $^{15}N^{15}N$ was undetectable in all 6-h and 12-h incubations (average detection limit [including sample handling] = 0.9 nmol L^{-1}). $^{15}N^{15}N$ was detected only in 6 out of 60 of the 24-h incubations and only from the $^{15}NO_2^-$ or $^{15}NO_3^-$ substrate. Both $^{14}N^{15}N$ and $^{15}N^{15}N$ production were undetectable in aerobic incubations with water from the mixed layer.

Abundance, phylogeny, and lipid biomarkers of anammox bacteria—Anammox bacteria were enumerated by FISH with anammox-specific 16S rRNA oligonucleotide probes and by qPCR. Anammox bacteria were found at all depths where $^{14}N^{15}N$ production was observed (Fig. 4). Their abundance in the OMZ ranged up to a maximum of 13 $\times 10^4$ to 15 $\times 10^4$ mL^{-1} at Sta. 2 (15 April). Anammox cells represented on average from 2.2% to 3.1% of the total microbial abundance at Sta. 2, but represented only 0.8% at Sta. 4 (Table 2). Phylogenetic analysis of the Planctomycete-specific 16S rDNA clone library detected sequences with 98% sequence identity as the known anammox bacteria Candidatus "*Scalindua sorokinii*" found in the Black Sea and the Benguela upwelling (Kuypers et al. 2003, 2005).

Particulate matter collected from the OMZ waters was also examined for the presence of ladderanes: membrane lipids specific to anammox bacteria (Table 2; Fig. 4, fourth

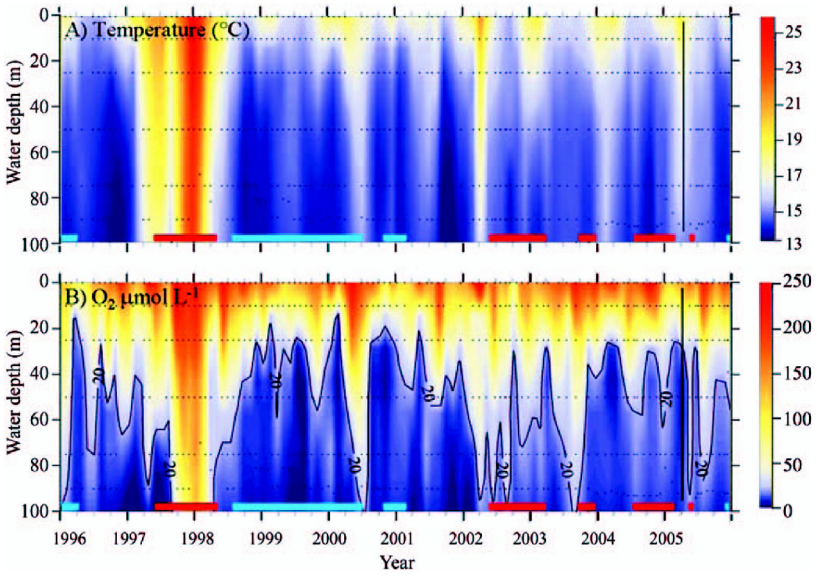

Fig. 3. Time-series of (A) temperature (°C) and (B) O_2 (μmol L^{-1}) profiles at Sta. 2 (Fig. 1B) (Gutiérrez et al. in press). Vertical line on A represents our study date (April 2005). Contour line on B indicates the 20 μmol O_2 L^{-1} isopleth. Temperature anomalies are the Oceanic Niño Index (http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ensoyears.shtml) and are shown as bars near the x-axis in red (>0.5°C: El Niño/Southern Oscillation [ENSO] warm periods) and light blue (<−0.5°C: ENSO cold periods). Note correspondence between ENSO events and water column oxygenation.

column). No ladderanes were found in the mixed layer, but they were found at all depths at which $^{14}N^{15}N$ production was observed, at concentrations ranging up to 58 ng L^{-1}. The differing depth distributions of anammox bacteria numbers and ladderane lipid concentrations at some stations may have resulted from the >12-h difference in sampling times, but when averaged over the whole water column, mean anammox bacteria numbers at each site were highly correlated with mean ladderane lipid concentrations ($r = 0.96$).

Discussion

Denitrification—We incubated water column samples from the Peruvian OMZ with $^{15}NO_2^-$ or $^{15}NO_3^-$ to elucidate the microbial process responsible for the loss of fixed nitrogen from the Peruvian OMZ. Since denitrification pairs two nitrite and/or nitrate ions to produce N_2, and since the pools of these N species in our incubations were labeled >50% with ^{15}N, denitrification would be expected to produce significant amounts of $^{15}N^{15}N$ through random pairing (Nielsen 1992). The theoretical ratio of the production of $^{15}N^{15}N$ to $^{14}N^{15}N$ by denitrification is

$$\frac{^{15}N^{15}N}{^{14}N^{15}N} = \frac{F}{2(1-F)} \quad (3)$$

where F is the fractional ^{15}N composition of the nitrate or nitrite pools (Nielsen 1992; Kuypers et al. 2006). Isotopic fractionation would not have an appreciable effect with this degree of labeling. Since the value of F in our experiments was no less than 0.5, N_2 production via denitrification would have yielded at least 0.5 mole of $^{15}N^{15}N$ for every mole of $^{14}N^{15}N$ produced. Although $^{14}N^{15}N$ was produced linearly beginning at 0–6 h in 20 of the 32 experiments with $^{15}NO_2^-$ or $^{15}NO_3^-$ ($r^2 \geq 0.7$), no $^{15}N^{15}N$ production was detected during any of the experiments before 24 h. Thus, our results did not provide evidence for in situ denitrification of nitrate or nitrite to N_2. However, $^{15}N^{15}N$

→

shows the abundance of anammox cells as determined by FISH and qPCR (top axis—note different y-axis scales) and the concentration of anammox-specific ladderane membrane lipid biomarkers (bottom axis).

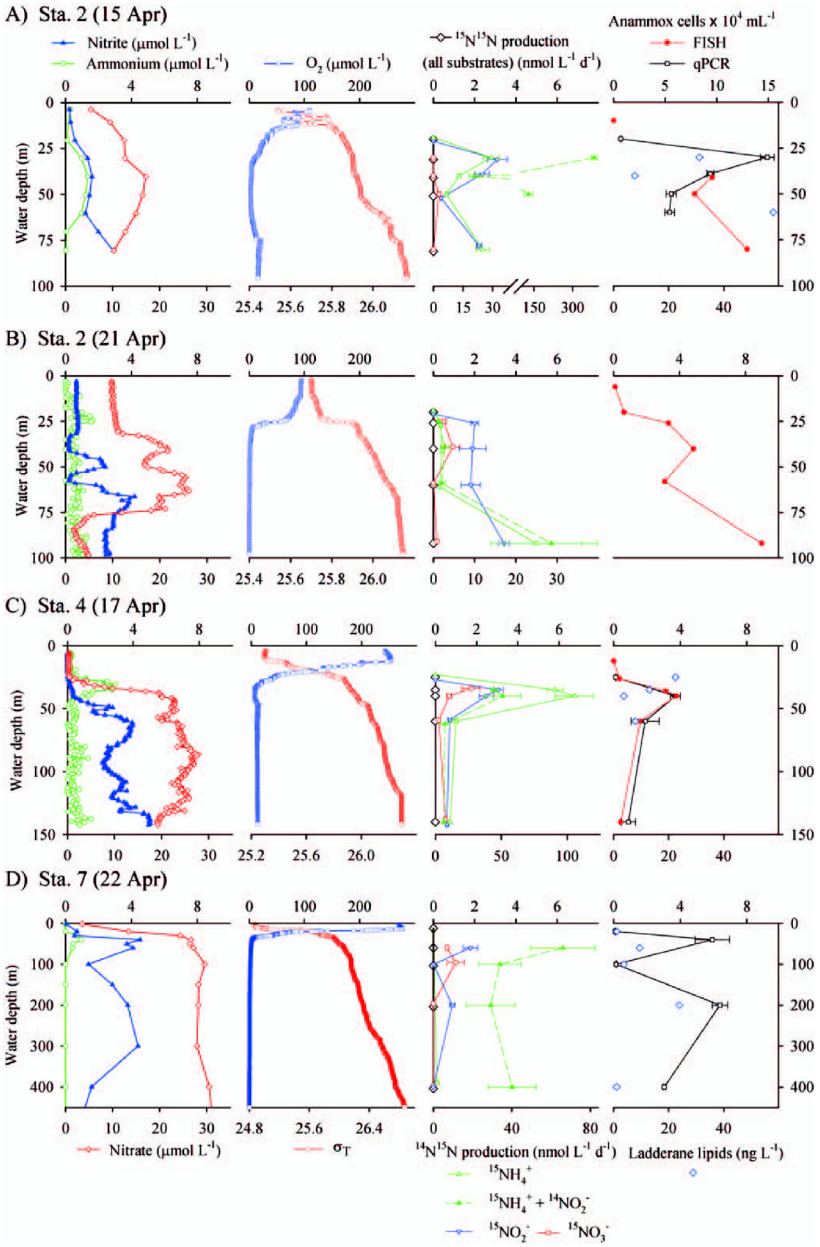

Fig. 4. Chemical zonation and distribution of anammox indicators (*see Fig. 1B* for station locations). (A) Sta. 2 (15 April 2005). (B) Sta. 2 (21 April 2005). (C) Sta. 4. (D) Sta. 7. The first column of figures shows the depth distribution of ammonium and nitrite (top axis) and nitrate (bottom nitrate), with water samples collected either by high-resolution pumpcast (B and C) or by GoFlow bottle water collection (A and D). The second column shows profiles of dissolved O_2 (top axis) and σ_T (bottom axis—note different y-axis scales). The third column shows $^{15}N^{15}N$ (top axis) and $^{14}N^{15}N$ (bottom axis—note different y-axis scales) production in incubations with four combinations of isotopically enriched dissolved inorganic nitrogen species. The fourth column

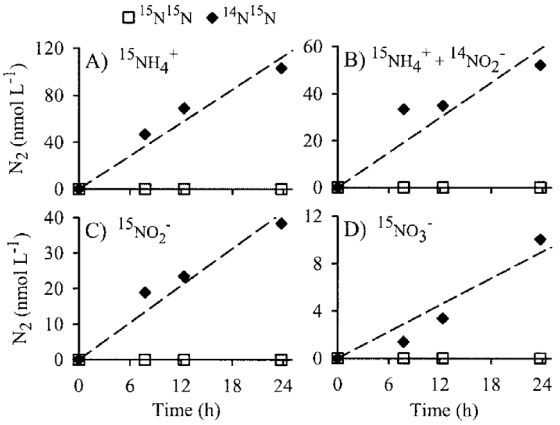

Fig. 5. Production of ^{15}N-labeled N_2 from ^{15}N-labeled substrates in anaerobic incubations of water from 40-m depth at Sta. 4. Dashed line is least-squares fit to $^{14}N^{15}N$ data. (A) Production of $^{14}N^{15}N$ from $^{15}NH_4^+$ indicates anammox activity. (Rate: 106 nmol $^{14}N^{15}N$ L^{-1} d^{-1}, $r^2 = 0.96$). (B) Unlabeled $^{14}NO_2^-$ added along with $^{15}NH_4^+$ to test for nitrite limitation of anammox. (Rate: 51 nmol $^{14}N^{15}N$ L^{-1} d^{-1}, $r^2 = 0.87$). (C) Production of $^{14}N^{15}N$ from $^{15}NO_2^-$ without $^{15}N^{15}N$ production indicates anammox, rather than denitrification, since the in situ NO_2^- pool was highly (>50%) ^{15}N labeled. (Rate: 39 nmol $^{14}N^{15}N$ L^{-1} d^{-1}, $r^2 = 0.96$). (D) Production of $^{14}N^{15}N$ from $^{15}NO_3^-$ without $^{15}N^{15}N$ production indicates nitrate reduction to nitrite coupled to anammox, rather than denitrification, since the situ NO_3^- pool was highly (>50%) ^{15}N labeled. (Rate: 11 nmol $^{14}N^{15}N$ L^{-1} d^{-1}, $r^2 = 0.94$).

was detected at 24 h in 6 of 32 experiments. Similar results were obtained in both the Benguela upwelling system and in the Golfo Dulce, where $^{15}N^{15}N$ production was not detected until 48 h (Dalsgaard et al. 2003; Kuypers et al. 2005). The potential to respire nitrate is widespread among bacteria and archaea (Zumft 1997). The production of $^{15}N^{15}N$ in six of the thirty-two 24-h incubations indicates that facultatively anaerobic heterotrophs that can use nitrate in the absence of oxygen are present in the Peruvian OMZ waters but does not indicate that nitrate was directly converted to N_2 by denitrifying microorganisms in situ in the Peruvian OMZ waters.

Anammox—We found ^{15}N tracer, molecular, and lipid biomarker evidence for anaerobic ammonium oxidation throughout the Peruvian upwelling OMZ in April 2005. Water samples incubated anaerobically with $^{15}NH_4^+$ produced significant amounts of $^{14}N^{15}N$ but no detectable $^{15}N^{15}N$. The exclusive production of $^{14}N^{15}N$ without delay indicated that $^{15}NH_4^+$ was being oxidized by in situ $^{14}NO_2^-$ to N_2 via the anammox reaction (Eq. 1) and excluded the suggested (Hulth et al. 1999) alternative pathways of anaerobic oxidation of NH_4^+ to NO_3^-, NO_2^- or of N_2 with MnO_2 (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003). $^{14}N^{15}N$ production in these incubations was linear ($r^2 \geq 0.7$), indicating that the anammox bacteria were active from time 0 and were therefore likely active in situ (Fig. 5A). Since $^{15}N^{15}N$ production was not detected in incubations with $^{15}NO_2^-$ or $^{15}NO_3^-$, even though the in situ NO_2^- and NO_3^- pools were highly labeled, all of the $^{14}N^{15}N$ produced must have been produced via the pairing of ^{15}N-labeled $^{15}NO_2^-$ with in situ $^{15}NH_4^+$ via the anammox reaction. The production of $^{14}N^{15}N$ in incubations with $^{15}NO_3^-$ therefore indicates that $^{15}NO_3^-$ was first converted to $^{15}NO_2^-$ by dissimilatory nitrate reduction and that this reaction was coupled to anammox. However, it is not yet known whether the reduction of nitrate to nitrite resulted from incomplete denitrification by classical heterotrophic denitrifiers or by other nitrate-respiring organisms. In contrast to these results from OMZ waters, no ^{15}N-labled N_2 was produced in any of the incubations with water from the oxic mixed layer (Fig. 4). Further evidence for the importance of anammox bacteria in the Peruvian OMZ waters came from molecular identification of the anammox bacteria via FISH and qPCR (Fig. 4). Ladderane lipids specific for anammox bacteria were also found within the Peruvian OMZ waters we investigated. These anammox-specific molecular and lipid biomarkers were found at all stations and depths in which anammox activity was measured with ^{15}N incubations but were not found

Table 2. Anammox indicators and activities in the Peruvian upwelling OMZ (station locations: Fig. 1B). All values are arithmetic means of OMZ measurements except the depth-integrated potential anammox rate.* The number of observations for each mean is as in Fig. 4. Errors are standard errors (nd, no data).

Station	Anammox cell numbers† ($\times 10^4$ mL^{-1})	Anammox cells (% of total cells)	Ladderane lipids (ng L^{-1})	Depth-integrated anammox rate* (mmol N m^{-2} d^{-1})	Cell-specific anammox rate (fmol $cell^{-1}$ d^{-1})
2 (15 Apr)	10.1±1.5	3.1±0.1	36±11	11.5	1.7
2 (21 Apr)	5.1±1.4	2.2±0.4	45 (at 92 m)	1.3	0.4
4	2.2±0.8	0.8±0.2	12±4	6.2	2.4
7	3.9±1.4	nd	8±4	34.0	1.9
9	nd	nd	16±14	8.8	nd
10	0.21±0.05	nd	4±2	1.5	1.9

* This is a potential rate calculated from the non–substrate-limited $^{15}NH_4^+ + {}^{14}NO_2^-$ treatment (Fig. 4). Rate measurements were depth-integrated by summing the product of the averages of two adjacent rate measurements and the depth interval between them. At Sta. 7, where anammox activity was only determined in the upper 400 m of the 2,400-m water column, potential anammox rates were arbitrarily set to decrease to 0 at 600 m.
† Anammox cell numbers for Sta. 2 and 4 determined by FISH; Sta. 7 and 10 determined by qPCR.

(lipids, FISH) or were found at greatly reduced concentrations (qPCR) above the oxycline.

Anammox activity measured by our ^{15}N incubations had maxima at the base of the oxycline at all stations and near the sediments at Sta. 2 (Fig. 4). The depth distributions of FISH and qPCR counts of anammox cells were highly correlated with $^{14}N^{15}N$ production (from $^{15}NH_4^+$ or $^{15}NO_3^-$) within stations (r = 0.77–0.95). Cell-specific potential anammox rates in the Peruvian OMZ (0.4–2.4 fmol cell^{-1} d^{-1}; Table 2) were comparable to rates from the Benguela upwelling system and the Black Sea (4.5 and 3–4 fmol cell^{-1} d^{-1}, respectively) (Kuypers et al. 2005). Although anammox metabolism in wastewater bioreactors is reversibly inhibited at O_2 concentrations as low as 1 μmol L^{-1} (Strous et al. 1997), ^{15}N-labeling experiments, FISH, qPCR, and ladderane biomarkers showed that anammox bacteria were abundant in waters with in situ oxygen concentrations of up to 20 μmol L^{-1} (Fig. 4), and these cells were able to begin anammox metabolism immediately following establishment of anoxic incubation conditions. Similar activity patterns were also observed in oxygen-containing samples collected from the Benguela upwelling system (Kuypers et al. 2005). The anammox bacteria at this high oxygen concentration may be dormant (although they were able to produce N_2 from NH_4^+ immediately upon reestablishment of anoxia during our labeling experiments), or they may be metabolizing in anoxic microniches within marine snow particles (Ploug 2001). Anammox bacteria in wastewater reactors may have doubling rates as short as 1.8 d, in contrast with early reports of an 11-d doubling period, indicating that they may have considerable flexibility in growing to meet changing environmental conditions (Isaka et al. 2006). The large temporal, spatial, and vertical heterogeneity in anammox activity, cell numbers and activities in the Peruvian OMZ (Fig. 4; Table 2) and the presence of active populations in oxygen-containing strata indicate that anammox bacteria have the physiological flexibility to persist in this dynamic environment.

Ammonium concentrations in OMZs are often very low, and it has been suggested that anammox could account for the "missing" ammonium that was predicted to have been regenerated by heterotrophic denitrification in the water column (Richards et al. 1965; Devol 2003). An initial appraisal of the significance of anammox in the ocean indicated a scenario in which anammox 'scavenging' of the ammonium regenerated during organic matter respiration by heterotrophic denitrification could at most be responsible for 28% to 48% of the total OMZ N_2 production, depending on the C : N ratio of the organic matter respired by denitrifiers (Devol 2003). However, if anammox can occur without denitrification in OMZ water columns, as indicated by our data and those of Kuypers et al. (2005), then another source of the ammonium to drive anammox must be found.

The peak in ammonium concentration and anammox activity that we observed just below the oxycline (also observed by Kuypers et al. [2005] in the Benguela upwelling) indicates that anammox may be driven in part by aerobically regenerated ammonium diffusing into the suboxic zone. Earlier measures of Peruvian OMZ community metabolism (ETS activity and nitrate reduction) also showed intense activity just below the oxycline (Codispoti and Packard 1980; Lipschultz et al. 1990). Heterotrophic dissimilatory reduction of nitrate, indicated by our $^{15}NO_3^-$ incubations, could provide an additional source of remineralized ammonium at these depths. A second maximum in anammox activity seen near the sediments at Sta. 2 (Fig. 4A,B) and at Sta. 9 and 10 (data not shown)—as well as in the Benguela upwelling (Kuypers et al. 2005)—might be driven by benthic ammonium fluxes (Dalsgaard et al. 2003). These observations indicate that maximum N_2 loss may not take place in the core of the deep nitrite maximum, as suggested earlier, but rather near the sediments and the oxycline, where ammonium is most available (Fig. 4).

Contribution of anammox and denitrification to N deficits in the Peruvian OMZ—Annual N losses in the Peruvian upwelling system previously have been estimated at 15–25 Tg N yr^{-1} based on measurements of ETS activity from the "inner main secondary nitrite maximum zone" within 175 km of the Peruvian coast (3.26×10^{11} m^2; Codispoti and Packard 1980). Lipschultz et al. (1990) estimated 8.9 Tg N yr^{-1} for the same area from the production of $^{15}NO_2^-$ in incubations with $^{15}NO_3^-$ tracer. The estimate of Codispoti and Packard (1980), derived from the diffusive and advective transport of N deficits through 16°S latitude and adjusted to the same area, was ca. 14 Tg N yr^{-1}, while the estimate of Deutsch et al. (2001), based on the change in N* (an N deficit measure) with chlorofluorocarbon–derived ETSP water-mass ages, when adjusted to the same area, yields ca. 19 Tg N yr^{-1}.

The rates of $^{14}N^{15}N$ production attributable to anammox that we derived from our ^{15}N incubation experiments were made under conditions of excess substrate availability, and we did not derive in situ anammox rates from them. Further, we recognize that the temporal and spatial scale of our sampling cannot be considered representative of the full range of variation within the Peruvian OMZ. However, our measurements represent the first direct measurements of N_2 production in the Peruvian OMZ waters, and we do not believe that they are necessarily less realistic than previous estimates from denitrification proxies and similarly restricted data sets. To compare the magnitude of our results to previously published areal N-loss estimates, we calculated a depth-integrated potential anammox rate (using results from the non–substrate-limited $^{15}NH_4^+ + {}^{15}NO_2^-$ incubations) at our five Peruvian OMZ stations to obtain an overall mean potential anammox rate of 11 mmol N m^{-2} d^{-1} (Table 2), equivalent to 18 Tg N yr^{-1} when scaled to the same area as the above estimates from previous studies (3.26×10^{11} m^2). This estimate falls well within the range estimated in the previous studies (9–25 Tg N yr^{-1}), indicating that the magnitude of the anammox N-loss rates we measured was not unreasonable and could potentially account for a significant proportion of the total N-loss from the Peruvian upwelling system.

N_2 losses via anammox have been shown to be significant in many anoxic marine sediments and water

columns investigated so far. However, the consequences of anammox activity for carbon and nitrogen cycling within the marine water column are still poorly understood. The significance of N_2 production via anammox or denitrification in OMZs lies not only in the identity of the responsible species but also in the metabolic differences between the two processes, which have differing consequences for the carbon cycle (chemoautotrophy vs. heterotrophy) and for the cycling of other inorganic N species in the N cycle, particularly ammonium and nitrous oxide. Our results showing that the N_2 production in the Peruvian upwelling OMZ in April 2005 was via anammox, rather than denitrification, are surprising, given the long-held expectation that OMZ N deficits would result from heterotrophic denitrification. However, earlier studies assumed that OMZ N deficits resulted from heterotrophic denitrification because no other N_2-generating process was known, and the assays used in these studies could not differentiate denitrification from anammox. Our results from ^{15}N-labeling experiments, supported by molecular and lipid biomarker data, are in agreement with prior results (Dalsgaard et al. 2003; Kuypers et al. 2005) and show that a hitherto unexpected process—anammox—can be the sole N-loss pathway in the OMZ waters of the ETSP.

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Microdiversity study of marine anammox bacteria reveals novel *Candidatus* Scalindua type in the Arabian Sea

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Microdiversity study of marine anammox bacteria reveals novel *Candidatus* Scalindua type in the Arabian Sea

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Key words: anammox, Planctomycetes, OMZ, ITS, diversity, *Candidatus* Scalindua arabica

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Abstract

The anaerobic oxidation of ammonium (anammox) is a globally significant nitrogen loss process catalyzed by a particular clade of bacteria deep branching within the phylum *Planctomycetes*. In marine oxygen minimum zones (OMZs), about 30-50% of the total nitrogen loss is currently estimated to take place and recent studies indicated that anammox might be the most important process responsible for this nitrogen loss.

Anammox bacteria were detected in three major marine oxygen minimum zones and in the Black Sea. Their diversity was investigated based on comparative sequence analysis of 16S rRNA and of the 16S-23S rRNA intergenic spacer regions, also known as internal transcribed spacer (ITS). In the Arabian Sea, the Namibian and Peruvian OMZ as well as the suboxic water column of the Black Sea, all anammox bacterial sequences were closely related to the *Candidatus* Scalindua genus. Both, 16S rRNA and ITS sequences, showed distinct and tight subclusters of *Candidatus* Scalindua which were unique to each OMZ. The anammox bacteria of the Namibian and Peruvian OMZs were more closely related to each other than to the anammox bacteria of the Arabian Sea OMZ. This study provides the first report on the identity and phylogenetic relationship of anammox bacteria in the Arabian Sea. Based on 16S rRNA and ITS sequences, the anammox bacteria in the Arabian Sea could represent a novel anammox species for which the name *Candidatus* Scalindua arabica is proposed.

Introduction

The Peruvian and Namibian upwelling systems along with the Arabian Sea are amongst the most productive regions of the world's oceans, as the upwelling of nitrate-rich deep water stimulates high biological production in surface waters (Carr 2002, Chapman and Shannon 1985, Copin-Montégut and Raimbault 1994, Friederich and Codispoti 1987). High primary productivity results in a large downward flux of particulate organic matter. In the water layers below, heterotrophic activities result in the formation of subsurface oxygen minima or so-called oxygen minimum zones (OMZs). About 30-50% of the total nitrogen loss is currently estimated to take place in these OMZs (Codispoti et al. 2001).

Heterotrophic denitrification has long been considered the only pathway for nitrogen loss. However, about a decade ago the anammox process has been detected in waste water treatment plants (Mulder et al. 1995, van de Graaf et al. 1995), in which ammonium is oxidized by nitrite to dinitrogen gas. This provides an alternative pathway for marine nitrogen loss. Since then anammox has indeed been detected in various marine and freshwater environments such as the suboxic water columns of the Black Sea (Kuypers et al. 2003) and Golfo Dulce (Dalsgaard et al. 2003), as well as in the Namibian (Kuypers et al. 2005) and Peruvian-Chilean OMZs (Hamersley et al. 2007, Thamdrup et al. 2006).

Kuypers et al. (Kuypers et al. 2003) showed that anammox bacteria constituted 0.75% of the total microbial community in the Black Sea, while their contribution to the total microbial community was slightly higher in the Namibian (~1%) (Kuypers et al. 2005) and the Peruvian OMZ (0.8-3%) (Hamersley et al. 2007). Recently, anammox specific ladderane lipids have been detected in the OMZ of the northwestern Arabian Sea (Jaeschke et al. 2007), even though the exact contribution of anammox to the fixed nitrogen-loss in the Arabian Sea remains to be resolved (Devol et al. 2006, Nicholls et al. 2007).

The taxonomy of anammox bacteria is complicated for the lack of pure cultures even after many years of cultivation. This is due to long doubling times in the range of weeks and probably the need for a microbial partner. The first marine anammox bacterium, *Candidatus* Scalindua sorokinii, was described for the Black Sea (Kuypers et al. 2003). On 16S rRNA level this strain had 98.1% sequence identity to *Candidatus* Scalindua brodae, originating from a wastewater treatment plant (Schmid et al. 2003). It was only distantly related to other known anammox strains *Candidatus* Kuenenia stuttgartiensis, *Candidatus* Brocadia anammoxidans and *Candidatus* Scalindua wagneri found in bioreactors and wastewater treatment plants (87.9, 89.6, and 93% 16S rRNA gene sequence identity, respectively) (Schmid et al. 2000, Schmid et al. 2003, Strous et al. 1999). Similarly, all

subsequent anammox bacterial 16S rRNA sequences retrieved from various natural habitats - e.g. marine and freshwater sediments (Penton et al. 2006, Schmid et al. 2007), lakes (Schubert et al. 2006), marine water columns like the Golfo Dulce, the Barents Sea and Greenland Sea (Schmid et al. 2007) as well as the Namibian (Kuypers et al. 2005) and Peruvian OMZ (Hamersley et al. 2007) - were closely related to the *Candidatus* Scalindua species (96-99% 16S rRNA sequence identity).

In the present study, we investigated whether anammox bacteria in three major marine OMZs and the Black Sea belong exclusively to the *Candidatus* Scalindua genus, or if bacteria related to the *Candidatus* genera Kuenenia, Brocadia or Anammoxoglobus, or if other undiscovered anammox-like bacteria might be present. We examined and compared the diversity of anammox bacteria in four different suboxic water columns: the Black Sea, the Arabian Sea OMZ as well as the Namibian and Peruvian OMZs. In addition to 16S rRNA sequence analyses, 16S-23S rRNA intergenic spacer regions, also known as internal transcribed spacer (ITS) were also examined. The evolutionary rate of the ITS region is approximately 10 times greater than the evolutionary rate of the 16S rDNA (Leblond-Bourget et al. 1996), thereby permitting a higher-resolution diversity analysis potentially down to the sub-species level (Gürtler and Stanisich 1996, Normand et al. 1996).

Methods and Materials

Sample collection and DNA extraction. Water samples investigated in this study were taken from the Namibian, the Peruvian and the Arabian Sea OMZs, in addition to the suboxic waters of the Black Sea. For details for sampling see Table 1. All filters were stored at -80°C until further processing. DNA extractions were done according to (Zhou et al. 1996) or (Somerville et al. 1989) (Table 1). To avoid DNA loss due to binding of the DNA to the glass fibres of the GF/F filter, cells were washed off from the filter with the extraction buffer without proteinase K and SDS prior to cell lyses.

PCR amplification of 16S rRNA gene and 16S-23S-rRNA intergenic spacer region (ITS). For the amplification of a ~ 4 kbp fragment encompassing the almost full length 16S rRNA gene, the ITS and a major part of the 23S rRNA genes, polymerase chain reactions were conducted according to (Schmid et al. 2001) using the primer Pla46F (targeting position 46-63 on 16S rRNA gene) specific for *Planctomycetes* and 1037R (targeting *E. coli* position 1930-48 on 23S rRNA gene) (Table 1 and 2).

Table 1: Overview of sampling, sample processing and construction of planctomyete-specific clone libraries from the Arabian Sea, Namibian and Peruvvian OMZs as well as from Black Sea suboxic waters

	Arabian Sea OMZ	**Black Sea suboxic waters**	**Namibian OMZ**		**Peruvian OMZ**
Cruise, Date	September 2004	R/V Professor Vodyanitskiy August 2005	R/V Meteor March/April 2003		IMARPE R/V José Olaya, April, 2005
Sampling position	Station 1 17.004°S, 68.0034°E	43.14°N, 34.00°E	Station 202 22.64°S, 14.30°E		Station 4 12.02°S, 77.29°W
Depth [m]	221	100	52	62	35
Chemistry: 1 = O_2 [µM] 2 = NO_2^- [µM] 3 = NO_3^- [µM] 4 = NH_4^+ [µM]	1 = n.d. 2 = 5 3 = n.d. 4 = n.d.	1= below d.l. 2 = close to d.l. 3 = 153 nM 4 = 383 nM	1= b.d.l. 2= 2 3= 4.6 4= b.d.l.	1= b.d.l. 2= 2.1 3= 5.6 4= b.d.l.	1= 6 2= 0.3 3= 13.5 4= 2.7
Sampled volume [L]	~ 200	9	~500		0.5
Sampling device	In situ pumps	Go-Flo bottle, CTD-rosette system	In situ pumps		CTD-pump cast system
Filter	GF/F (Whatman, precombusted, 450°C, nominal pore size 0.7 µm)	Sterivex filters (Millipore, GS 0.2 µm pore size)	GF/F (Whatman, precombusted, 450°C, nominal pore size, 0.7 µm)		Polycarbonate filter (Millipore, GTTP, 42 mm diameter, 0.2 µm pore size)
DNA extraction protocol	(Zhou et al. 1996)	(Somerville et al. 1989)	(Zhou et al. 1996)		(Zhou et al. 1996)
Frequency of established marine anammox clones related to *Cand.* Scalindua	33/136	1/480	7/384	1/288	46/96

n.d.: not determined
b.d.l.: below detection limit

PCR conditions were optimized to minimize PCR biases by reducing the cycle number as much as possible and by conducting 5-10 reactions in parallel (Polz and Cavanaugh 1998, Suzuki and Giovannoni 1996).

Cloning and sequencing. In order to avoid cloning of small and non-specific PCR products, fragments of the correct size (~ 4 kb) were excised from a preparative agarose gel (1.5%), and subsequently extracted and purified using the QIAquick Gel Extraction Kit Protocol (QIAGEN, Hilden, Germany), prior to cloning with TOPO TA Cloning kits with vector pCR4 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Clone screening was conducted with the Colony Fast-Screen™ Kit (Epicentre, Madison, USA). Plasmids were isolated from the positive clones with the Montage™ Plasmid Miniprep$_{96}$ Kit (Millipore, Eschborn, Germany) and were first sequenced with the primer 630R, targeting position 1529-1545 of the 16S rRNA gene (Juretschko et al. 1998). These sequences were checked for their closest relatives in BLAST (Altschul et al. 1997), and

clones with anammox bacteria related inserts were fully sequenced using primers 1035R, 907RM, GM1R in addition to 630R (Table 2).

Table 2. Primers used for PCR amplification and sequencing.

Name	Position	Sequence (5`? 3')	Reference
Pla46F[a1]	46-63	GACTTGCATGCCTAATCC	(Neef, et al. 1998)
Pla58F	42-59	GGCATGGATTAGGCATGC	(Liesack and Stackebrandt 1992)
An7F	52-70	GGCATGCAAGTCGAACGAGG	(Penton et al. 2006)
GM1R[a2]	518-534	ATTACCGCGGCTGCTGG	(Muyzer et al. 1993)
907RM[a2]	907-927	CCGTCAATTCMTTTGAGTTT	(Schäfer and Muyzer 2001)
630R[a2]	1529-1545	CAKAAAGGAGGTGATCC	(Juretschko et al. 1998)
1035R[b2]	242-256	TTCGCTCGCCRCTAC	(Ludwig et al. 1992)
1037R[b1]	1930-48	CGACAAGGAATTTCGCTAC	(Ludwig et al. 1992)

a: 16S rRNA position, E. coli numbering (Brosius et al. 1978)
b: 23S rRNA position, E. coli numbering (Brosius et al. 1978)
1: Primer was used for PCR
2: Primer was used for sequencing

Phylogenetic analysis. Contigs were assembled with the software SEQUENCHER Version 4.5 (Gen Codes Corporation, Ann Arbor, USA) resulting in sequences containing the almost complete 16S rRNA gene, the ITS and the beginning ~200 bp of the 23S rRNA gene. The 16S rRNA gene sequences were checked for the presence of chimeric sequences by using the CHIMERA_CHECK program from RDP (Cole et al. 2003) and for the ITS by visual inspection. Sequences were first aligned in Clustal X (Thompson et al. 1997). The 16S rRNA sequences were further aligned in Arb (Ludwig et al. 2004) and the overall alignment was further refined manually. Phylogenetic analyses of the 16S rRNA and ITS were performed separately in Arb using distance matrix, maximum parsimony and maximum likelihood algorithms with and without 50% position variability filters, and the consensus trees were derived thereafter. Sequence identities were calculated using the similarity function of the neighbour joining algorithm in the Arb program. The occurrence of tRNA genes within the ITS were predicted using tRNASCAN-SE, version 1.21 (Lowe and Eddy 1997).

Results

Phylogenetic analysis based on 16S rRNA sequences

We obtained a total of 1384 clones from the four libraries. Sequence-based screening of the 3' region of the 16S rRNA gene by BLAST indicated that besides planctomycetal fragments also fragments of *Verrucomicrobia* and unclassified bacteria were amplified. Within the true planctomycetal PCR fragments, 87 sequences were closely affiliated to established *Candidate* genera of anammox bacteria and were further analyzed.

Other planctomycetes possibly related to known anammox bacteria. In addition to the 87 sequences closely related to anammox bacteria, the clone libraries from the Black Sea suboxic waters as well as the Arabian Sea and Namibian OMZs contained rare planctomycete sequences only distantly related to the established anammox species (Black Sea: 4 clones out of 480, Arabian Sea: 2/136 and Namibian OMZ: 7/672) (Fig. 1).

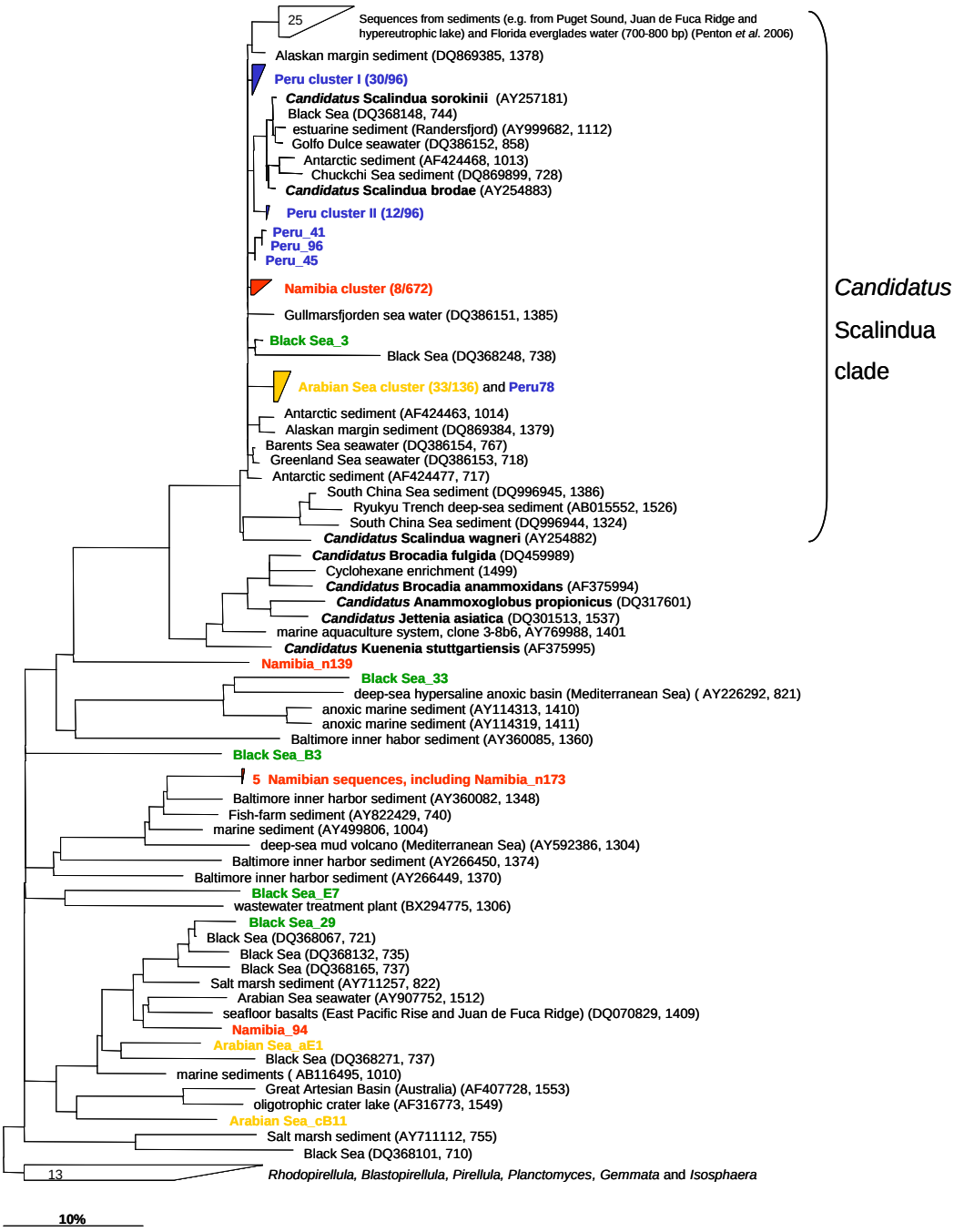

Fig. 1: Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic affiliations of anammox bacterial sequences from the Namibian, Peruvian and Arabian Sea OMZs as well as from the Black Sea suboxic waters with previously published anammox bacterial sequences. The consensus tree was built based on maximum likelihood, neighbour joining and maximum parsimony trees, calculated without and with 50% position variability filters using *Pirellula*, *Rhodopirellula*, *Blastopirellula*, *Planctomyces*, *Gemmata* and *Isoshpaera* sequences as outgroup. Sequences retrieved in this study are pointed out by a colour code (Peru: blue; Namibia: red; Arabian Sea: yellow and Black Sea: green) also used in Fig. 2. Accession numbers and sequence lengths are stated in parentheses. The bar represents 10% estimated sequence divergence.

Sequence Namibia_n139 did not group with any known anammox organism but was most closely related to a Baltimore Inner harbour sediment clone (AY266449, 82%, Fig. 1), which was presumed to belong to novel group of anammox bacteria (Tal et al. 2005). Five sequences from the Namibian OMZ had 99.8% sequence identity to each other and in the following, Namibia_n173 will be used as their representative sequence (Fig. 1). These sequences showed the closest affiliation to another sequence from the Baltimore Inner harbor sediment (AY360082, 93% sequence identity), also believed to originate from a novel anammox bacterium. Sequences BlackSea_B3 and BlackSea_29 and Namibia_94 had the highest sequence identity to a sequence retrieved from seafloor basalts (DQ070829, 81%, 91.3% and 92.6% sequence identity, respectively) and sequence BlackSea_E7 to a sequence published as a novel planctomycete found in a wastewater treatment plant (BX294775, 82% sequence identity) (Chouari et al. 2003). Sequence AS_aE1 from the Arabian Sea was closely related to a sequence previously retrieved from the Arabian Sea (AF907752, 89.4%; Fuchs et al. 2005) and AS_cB11 had highest sequence identity with a sequence from an ultra-oligotrophic crater lake (AF316773, 84.5% sequence identity). BlackSea_33 exhibited highest sequence identity with a sequence from ammonia-oxidizing, anoxic marine sediment (AY114319, 88.4%). The sequence identities of the above specified sequences derived from the three different OMZs to established anammox bacteria varied from 76% to 81%.

Sequences related to established anammox bacteria

Samples taken from the three OMZs and the Black Sea suboxic water column gave different frequencies of clones related to *Candidatus* Scalindua spp. The clone library of the Peruvian OMZ consisted of 46 anammox bacteria related sequences out of 96 screened clones (Table 1). The two clone libraries of the Namibian OMZ (from 52 m and 62 m depth) together contained 8 anammox bacteria related sequences out of 672 screened clones. Thirty-three clones out of 136 from the clone library of the Arabian Sea were positive, whereas only one out of 480 clones from the Black Sea clone library contained anammox bacteria related DNA.

All 87 sequences fell within a big cluster ("*Candidatus* Scalindua clade", Fig.1) and were related to *Candidatus* Scalindua spp. This cluster consisted of sequences from diverse marine sediments, such as the Puget Sound (Penton et al. 2006), Juan de Fuca Ridge (deep sea sediment) (Penton et al. 2006), Alaskan margin (Penton et al. 2006), Chuckchi Sea (Penton et al. 2006), the Antarctic continental shelf (Bowman and McCuaig 2003), South

China Sea, as well as subtropical wetlands in Florida (Penton et al. 2006) and a freshwater sediment sample from a hypereutrophic lake (Penton et al. 2006). Furthermore, the "*Candidatus* Scalindua clade" contained sequences from the water column of the Golfo Dulce (Schmid et al. 2007), the Gullmarsfjorden, the Barents Sea and Greenland Sea. The first described anammox species from the water column, *Candidatus* Scalindua sorokinii (Kuypers et al. 2003) from the Black Sea, also grouped within this cluster. Additionally, it contained sequences of *Candidatus* Scalindua brodae and *Candidatus* Scalindua wagneri, which were retrieved from a wastewater treatment plant (Schmid et al. 2003). Partial and nearly full-length sequences within the "*Candidatus* Scalindua clade" shared sequence identities of 91.2% to 99.9%. Similarity matrices calculated only with nearly full-length sequences result in 91.6% to 98.6% sequence identities.

The 16S rRNA sequences from each system formed one (Arabian Sea and Namibian OMZ, respectively) or two (Peruvian OMZ, named Peru I and II) monophyletic clusters of highly similar sequences (Fig. 2). All sequences from the Peruvian OMZ together with the sequences from the Namibian OMZ showed the highest sequence identities (98.2-98.5%) with sequence BlackSea_3 from the Black Sea clone library conducted in this study. BlackSea_3 itself had highest sequence identity (98.6%) with sequence DQ869385 from the Barrow Canyon sediment (Alaskan margin, Penton et al. 2006). The closest relative of the Arabian Sea cluster was sequence DQ869384 (96.6%) from the Alaskan margin sediment (Penton et al. 2006). Additionally, several sequences of the Peru I cluster, the Namibian and the Arabian Sea cluster showed high sequence identities with partial sequences (700-800 pb) from the Greenland and the Barents Sea (Peru I: up to 98.8% with DQ386154; Namibia: up to 98.4% with DQ386151 and up to 98.3% with DQ386153; Arabian Sea: up to 97.8% with DQ386154 and up to 97.6% with DQ386153).

All phylogenetic trees calculated by three different algorithms (maximum likelihood, neighbour joining and maximum parsimony) with different filters supported the grouping of anammox bacterial sequences into four dominant, coherent clusters (Fig. 2a), Peru I and II, one Namibia cluster and one Arabian Sea cluster. Within one cluster of anammox sequences, the Peruvian cluster I (consisting of 30 sequences) and cluster II (composed of 12 sequences) showed the highest 16S rRNA gene sequence identity of >99% (Table 3). Sequences within the Namibian (8 sequences) and Arabian Sea (33 sequences) clusters were slightly less similar (>98% sequence identity) to each other.

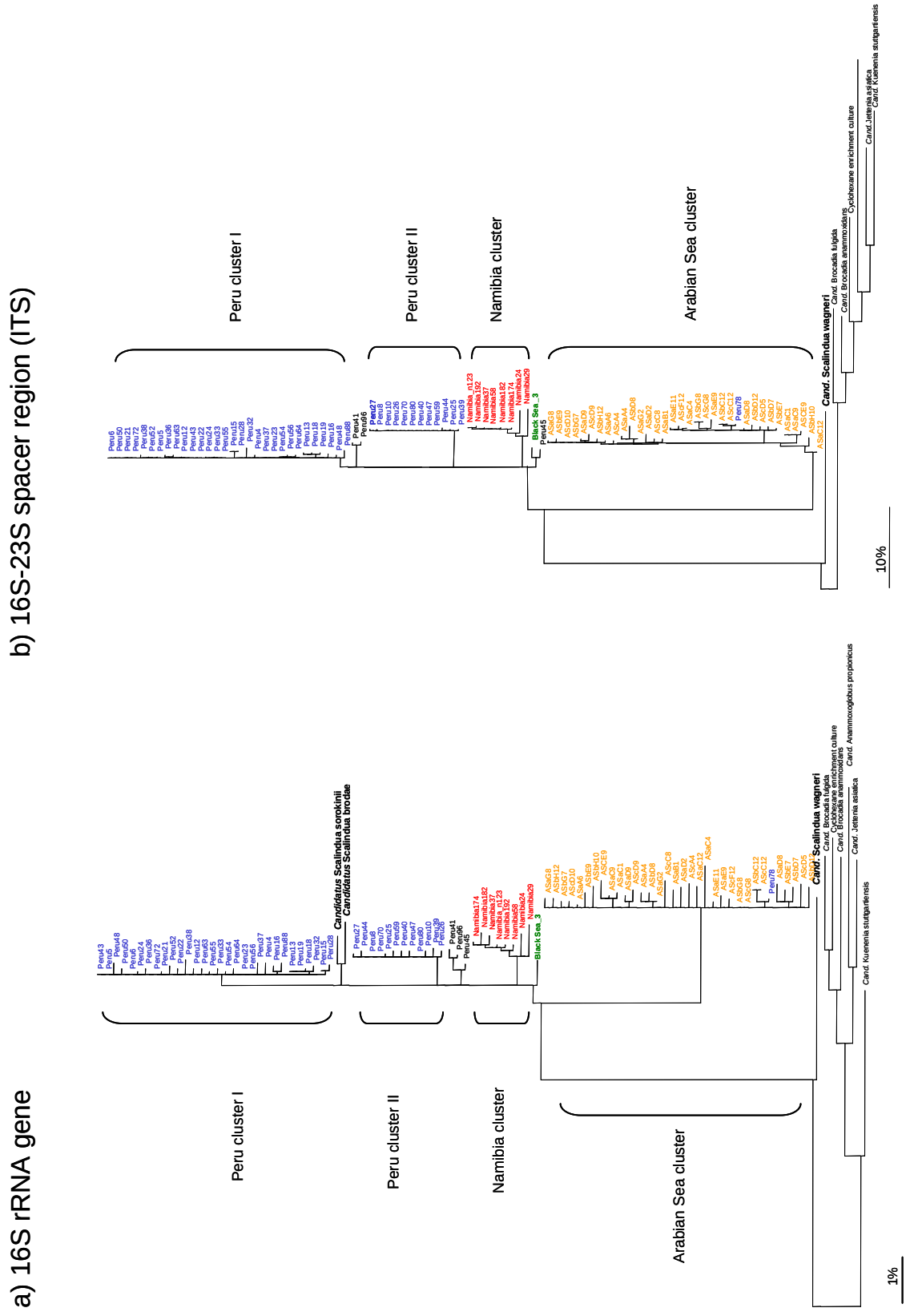

Fig. 2a: Consensus tree based on 16S rRNA sequences showing the grouping of the anammox bacterial sequences from the Arabian Sea, the Peruvian and Namibian OMZ as well as the Black Sea in unique clusters.
Fig. 2b: Consensus tree based on ITS sequences from the Arabian Sea, the Peruvian and Namibian OMZ as well as the Black Sea supporting the cluster pattern observed based on 16S rRNA sequences. Please note that the bar in the tree based on 16S rRNA sequences represents 1% sequence divergence instead of 10% for the tree based on ITS. ITS sequences for *Candidatus* Scalindua sorokinii and *Candidatus* S. brodae are missing due to lack of samples.

Table 3: 16S rRNA sequence identities (%) of the retrieved environmental anammox clusters with described *Candidatus* anammox species.

16S rRNA sequences	Peru cluster 1	Peru cluster 2	Namibia cluster	Arabian Sea cluster	BlackSea_3
Peru cluster 1	99.0 - 100				
Peru cluster 2	97.9 - 98.4	99.7 - 100			
Namibia cluster	97.9 - 98.8	97.8 - 98.4	98.4 - 99.9		
Arabian Sea cluster	96.1 - 97.4	95.4 - 96.4	95.5 – 97.0	98.2 - 100	
BlackSea_3	98.4 - 98.8	98.2 - 98.4	98.4 - 98.8	95.7 - 96.7	-
Cand. S. sorokinii	97.8 - 98.3	98.1 - 98.3	97.9 - 98.3	95.3 - 96.3	98.3
Cand. S. brodae	97.8 - 98.4	98.0 - 98.2	97.7 - 98.0	95.5 - 96.5	97.7
Cand. S. wagneri	93.8 - 94.3	93.9 - 94.1	93.6 - 93.9	92.2 - 93.1	93.7
Cand. B. anammoxidans	87.2 - 87.6	87.1 - 87.3	87.1 - 87.5	85.9 - 87.1	87.1
Cand. K. stuttgartiensis	88.1 - 88.6	88.0 - 88.2	87.8 - 88.2	87.2 - 88.1	87.9

Comparing 16S rRNA gene sequences of different clusters, Peru I and II and the Namibian cluster showed ~98% sequence identity with each other, but lower sequence identity towards the Arabian Sea cluster (96-97%). Sequence identities of cluster Peru I and II and Namibian cluster with *Candidatus* Scalindua sorokinii and *Candidatus* Scalindua brodae were around 98%, whereas the sequences of the Arabian Sea showed only 95-96% identity with both *Candidatus* Scalindua species (Table 3). Three Peruvian clones (Peru41, Peru45 and Peru96) did not group within cluster I or II (Fig. 1 and 2a), and one clone (Peru78) was not found in the Peruvian specific clusters, but grouped with the 33 sequences from the Arabian Sea (Fig. 2a).

Phylogenetic analysis based on the 16S-23S rRNA intergenic spacer region (ITS)

Similar to the ITS of waste water anammox species, the ITS of our marine anammox bacteria related to *Candidatus* Scalindua were encoding the tRNAs for alanine and isoleucine (Table 4). The length of the ITS of anammox bacteria varied strongly. Within the waste water anammox species, *Candidatus* Brocadia fulgida and *Candidatus* Scalindua wagneri contained the shortest ITS (376 and 381 nucleotides, respectively), while *Candidatus* Kuenenia stuttgartiensis (Schmid et al. 2001) and *Candidatus* Anammoxoglobus propionicus had the longest ITS (509 and 529 nucleotides, respectively) (Table 4). The sequence identities ranged from a maximum of 82% between *Candidatus* Brocadia fulgida and *Candidatus* B. anammoxidans down to 63% between *Candidatus* Anammoxoglobus and *Candidatus* Kuenenia stuttgartiensis.

Table 4. Characteristics of 16S-23S intergenic spacer regions (ITS) from the investigated OMZs and cultured anammox species

	ITS size (bp)	Predicted tRNA genes	Reference
Peru cluster 1	410-413	Ile, Ala	this study
Peru cluster 2	413	Ile, Ala	this study
Namibia cluster	403-414	Ile, Ala	this study
Arabian Sea cluster	442-462 (one exception: 434)	Ile, Ala	this study
BlackSea_3	409	Ile, Ala	this study
Cand. S. wagneri	381	Ile, Ala	this study
Cand. B. anammoxidans	442	Ile, Ala	(Schmid et al. 2001)
Cand. K. stuttgartiensis	509	Ile, Ala	(Schmid et al. 2001)
Cand. J. asiatica	464	Ile, Ala	DQ301513
Cyclohexane enrichment	423	Ile, Ala	this study
Cand. B. fulgida	376	Ile, Ala	this study
Cand. A. propionicus	529	Ile, Ala	this study

The anammox ITS from the Peruvian OMZ had a length of 410-413 nucleotides. ITS sequences from the Namibian OMZ (403-414 nucleotides) and the Black Sea (409 nucleotides) had a similar length. However, the ITS sequences from the Arabian Sea (442-462 nucleotides) were considerably longer. Tree calculations with distance matrix, maximum parsimony and maximum likelihood algorithms with and without filters showed that the clustering based on ITS (Figure 2b) was similar to that based on 16S rRNA sequences (Figure 2a). As expected, the sequence identities of ITS sequences within clusters were lower than those of the 16S rRNA sequences (Table 5). The within-cluster sequence identities of Peru I and II were higher (97-99% and 99-100%, respectively) than within the Namibian and Arabian Sea cluster (94-100%).

Table 5: ITS sequence identities (%) of the environmental anammox clusters with described *Candidatus* anammox species.

ITS sequences	Peru cluster 1	Peru cluster 2	Namibia cluster	Arabian Sea cluster	BlackSea_3
Peru cluster 1	97.1 - 100				
Peru cluster 2	91.4 - 93.1	99.3 - 100			
Namibia cluster	91.0 - 95.3	90.2 - 92.3	93.5 - 99.8		
Arabian Sea cluster	81.3 - 86.0	79.5 - 83.5	78.7 - 84.5	93.7 - 100	
BlackSea_3	94.8 - 96.6	92.1 - 92.6	91.3 - 94.8	81.4 - 84.7	-
Cand. S. wagneri	73.7 - 75.1	72.4 - 72.9	72.6 - 75.3	77.7 - 81.1	75.3
Cand. B. anammoxidans	67.3 - 68.4	68.0 - 68.3	67.9 - 68.9	68.8 - 71.1	68.6
Cand. K. stuttgartiensis	62.6 - 64.2	64.3 - 64.9	64.7 - 65.4	61.9 - 64.5	63.8

In contrast to the wastewater anammox species, the ITS sequences of the marine anammox bacteria were surprisingly similar to each other. As for 16S rRNA gene sequences, the sequence identities of Peruvian and Namibian ITS sequences towards each other (90-95%) were considerably higher then their sequence identity with Arabian Sea ITS

sequences (79-86%). One apparent difference between 16S rRNA and ITS sequences was the higher sequence identity of Arabian Sea ITS sequences with *Candidatus* Scalindua wagneri (78-81%) compared to sequences of Peru and Namibia (72-75%), which was not observed based on 16S rRNA sequences (Table 3 and 5).

Three Peruvian clones showed slightly different phylogenetic affiliations based on their 16S rRNA or ITS sequence. Based on their 16S rRNA gene sequence, all three clones group together (Fig. 2a). In comparison, ITS sequence analyses revealed that only clone Peru41 and Peru96 would fall into the same group yet clone Peru45 would group together with clone BlackSea_3.

Discussion

Potentially novel anammox bacteria in marine OMZs?

Very recently, Tal and co-workers postulated based on 16S rRNA sequences a novel group of anammox bacteria distantly related to known anammox bacteria (Baltimore Inner Harbor sediment) (Tal et al. 2005). Based on 16S rRNA sequences, 13 sequences of the present study did not group with any known anammox bacteria, but still within the phylum *Planctomycetes* but outside the known genera *Pirellula*, *Rhodopirellula*, *Blastopirellula*, *Planctomyces*, *Gemmata* and *Isosphaera* (Fig. 1). Six of them (Namibia_n139 and the group of five sequences including Namibia_n173) had as closest relatives clones from the supposedly novel group of anammox bacteria proposed by Tal and co-workers (AY266449 and AY360082, respectively, (Tal et al. 2005)). However, the low sequence identity (79-84%) to known anammox bacteria suggests a very distant affiliation. In addition, the ITS sequences of Namibia_n139 and Namibia_n173 were very distinct from the ITS of known anammox bacteria. The ability of this group of bacteria to anaerobically oxidize ammonia remains to be verified either by enrichment cultures or metagenome studies.

Besides, four uncultured planctomycete sequences from the Black Sea (BlackSea_29), Arabian Sea (AS_aE1 and AS_cB11) and the Namibian upwelling (Namibia_94) were affiliated with sequences derived from the Black Sea during an earlier study which were assigned to a group called “unknown planctomycetes/BO84” based on an uncharacterized enrichment culture (DQ368132, DQ368165, DQ368067 and DQ368271; Kirkpatrick et al. 2006). Like the previous group of unknown bacteria, the 16S rRNA sequence identities of the planctomycete sequences from Arabian Sea, Namibia and Peru to

known anammox bacteria were low (76-80%) and the physiological properties of this group of bacteria are still unknown.

Diversity of established anammox bacteria in marine OMZs

The 16S rRNA gene-ITS clone libraries constructed with primer Pla46F and 1037R resulted in apparent different frequencies of clones containing anammox bacterial DNA fragments in the four investigated samples (Table 1). Therefore, another planctomycete-specific primer (58F Liesack and Stackebrandt 1992, Table 2) was additionally tested in combination with the reverse primer 1037R to amplify the 16S rRNA–ITS region, but yielded no detectable amplicons. In principle, PCR primers specific for established anammox bacteria were avoided in our study in order not to discriminate against unknown anammox bacteria. However, we did test the recently published primer An7F (Penton et al. 2006) with the reverse primer 1037R to check for any anammox sequences not detected by Pla46F due to suggested PCR biases. But again, amplifications with An7F were unsuccessful for various environmental and bioreactor samples tested.

We used 16S rRNA and 16S-23S ITS sequences to study the microdiversity of planktonic anammox bacteria in three major marine OMZs. The ITS is extremely variable in size and sequence, permitting discrimination of species and even strain clusters (Gurtler and Stanisich 1996, Normand et al. 1996). Amongst the *Planctomycetes* only the genus *Isosphaera* and the anammox bacteria contain 16S rRNA and 23S rRNA genes that are linked by the ITS (Liesack and Stackebrandt 1989, Menke et al. 1991, Ward et al. 2000). The ITS of two wastewater anammox species (*Candidatus* B. anammoxidans and *Candidatus* K. stuttgartiensis) were studied previously and revealed spacer regions of about 450 nucleotides in length, containing tRNA genes for alanine and isoloeucine (Schmid et al. 2001). The same tRNA genes were found in all newly investigated waste water species presented in this study, as well as in the marine anammox bacteria, reflecting a high conservation of this feature.

The 16S rRNA gene sequences of marine planktonic anammox bacteria retrieved in this study are all closely related to *Candidatus* Scalindua sorokinii and *Candidatus* Scalindua brodae (Fig. 1). Within the "*Candidatus* Scalindua clade", a sequence identity of 92-99% was observed. Beside the sequences closely related to *Candidatus* Scalindua, no sequences closely related to other anammox bacteria such as *Candidatus* Kuenenia, Brocadia and Anammoxoglobus were found. This finding supports earlier studies reporting a low diversity of anammox bacteria in marine sediments (Penton et al. 2006, Schmid et al.

2007, Hamersley et al. 2007, Kuypers et al. 2005). Penton et al. provided evidence for a widespread distribution of closely related anammox bacteria in 11 different freshwater and marine sediments, with all anammox sequences closely related to the *Candidatus* Scalindua group (Penton et al. 2006). A recent study investigating diverse marine sediment and few water samples came to the same conclusion (Schmid et al. 2007).

Interestingly, each OMZ had its typical distinct and specific types of established anammox sequences. Two Peruvian specific clusters could be detected, one specific cluster for the Namibian OMZ and one for the Arabian Sea OMZ. In theory it is possible that the two Peruvian clusters represent merely two copies of the 16S-ITS-23S operon within the genome of one anammox bacterium and not two distinct strains. Earlier studies of *Blastopirellula marina* (IFAM 1313) (Liesack and Stackebrandt 1989), *Planctomyces limnophilus* (Menke et al. 1991) and *Gemmata obscuriglobus* (Menke et al. 1991) have revealed two copies of unlinked 16S rRNA genes in *B. marina* and *P. linmophilus* and five in *G. obscuriglobus.* However, *Rhodopirellula baltica* SH1^{T} (Glöckner et al. 2003) and more important, the anammox bacterium *Candidatus* Kuenenia stuttgartiensis (Strous et al. 2006) only possess one 16S rRNA gene and one 16S-ITS-23S operon, respectively, in their genomes. Additionally, it is very improbable that some very closely related anammox bacteria have two copies (Peru) and others only one (Namibia and Arabian Sea). Therefore, it is very likely that the two clusters in the Peruvian OMZ reflect the existence of two different anammox bacterial strains. Simultaneous hybridization with two probes specific for the two different Peruvian clusters would resolve the question, whether the two clusters correspond to two anammox bacterial strains or whether the Peruvian anammox bacteria possess two 16S-ITS-23S operons (Amann et al. 2000).

It is apparent that each of the clusters is currently only dominating in one OMZ. In other words, within one cluster specific for a certain OMZ, no sequences from other OMZs were found. The only exception was clone Peru78 which clustered with the Arabian Sea sequences. The distinct clustering of anammox bacteria in each OMZ is the prerequisite for the design of cluster-specific probes for fluorescence in situ hybridization (FISH). This would allow a more specific investigation and quantification of the anammox bacteria in each OMZ. Potentially, each OMZ is permanently dominated by one specific anammox bacteria population. Alternatively, the clustering of clone Peru78 with the Arabian Sea cluster suggests variability of different anammox types at least in the Peruvian OMZ. This can be investigated by FISH with cluster-specific probes.

The clustering of marine anammox 16S rRNA sequences into distinct groups was nicely confirmed by comparative sequence analysis of ITS sequences. The same was previously observed in a microdiversity study of the SAR11 cluster and marine crenarchaeota (Garcia-Martinez and Rodriguez-Valera 2000). Solely based on the minute differences in 16S rRNA sequences, the different clusters could have been artefacts. However, the ITS sequences clearly support genotypic differentiation between the OMZs.

The sequence idiosyncrasies within ITS would allow for the design of cluster-specific oligonucleotide probes which specifically target precursor rRNA. Since the primary transcript of the 16S-ITS-23S-5S operon is subsequently spliced into the different ribosomal RNAs and transfer RNAs, the concentration of precursor rRNA reflects the physiological activity of a microorganism better than the mature rRNAs (Cangelosi and Brabant 1997) which may be maintained during periods of starvation. FISH with probes targeting the ITS region (Oerther et al. 2000) has already been used for anammox bacteria (Schmid et al. 2001). Hence, in addition to anammox bacterial abundance, the activity of anammox bacteria in marine OMZs could be investigated by FISH and also by PCR of the ITS region.

Another interesting finding is that the anammox sequences from the Peruvian and Namibian OMZs are more closely related to each other than either group to the anammox sequences of the Arabian Sea OMZ. This is reflected by both, 16S rRNA sequences and ITS sequences. The closer relationship of Namibian and Peruvian anammox sequences in contrast to the Arabian Sea sequences is mirrored in their habitat. The Namibian and Peruvian OMZs are rather dynamic and result from southerly alongshore winds which lead to an upwelling of nutrient-rich subsurface waters and thus drives primary production. Settling organic matter is decomposed and followed by oxygen consumption which leads to oxygen depleted waters- the oxygen minimum zone (Chapman 1985, Copin-Montégut and Raimbault 1994, Friederich and Codispoti 1987). In contrast, the Arabian Sea contains a very stable and wide OMZ, often ranging from the bottom of the euphotic layer (~100 m) down to ~1000 m (Sarma 2002). The OMZ is the result of monsoon driven upwelling and restricted oxygen supply by inflowing waters from the Persian Golf, the Red Sea and the southern Indian Ocean. The upwelled water gets transported hundreds of kilometres offshore, where it results in high primary production and oxygen depleted waters. Therefore, in contrast to the Namibian and Peruvian upwelling, the coastal upwelling and the OMZ in the Arabian Sea are geographically separated (Morrison et al. 1999). However,

we can only speculate that this is among the reasons for the observed differences between anammox bacteria in the Namibian and Peruvian OMZs versus the Arabian Sea OMZ.

Anammox sequences of the Arabian Sea had at maximum 96.6% 16S rRNA sequence identity with another uncultured organism from the Alaskan margin sediment (DQ869384), 96.5% sequence identity with *Candidatus* Scalindua brodae and 96.3% with *Candidatus* Scalindua sorokinii (Table 3). A partial sequence of ~760 bases showed 97.8% sequence identity (DQ386154). However, since this is only half of the entire length of the 16S rRNA, the sequence was neglected in this discussion. Sequence identities of <97% usually indicate different species (Rossello-Mora and Amann 2001). Therefore we speculate that the Arabian Sea cluster comprises a novel species within the *Candidatus* genus Scalindua, for which we propose the name *Candidatus* Scalindua arabica. This is also consistent with the fact that Arabian Sea anammox ITS sequences have distinctly different lengths compared to other *Candidatus* Scalindua species. The diversity of anammox bacteria within each OMZ was quite low. The sequence identity of >98% suggests that within an OMZ we see representatives of several strains of a single species.

Conclusion. Our results showed that the anammox bacterial sequences detected in the Namibian, Peruvian and Arabian Sea OMZs, as well as in Black Sea suboxic waters, were closely related to the *Candidatus* Scalindua genus with low diversity. Both, 16S rRNA sequence and 16S-23S intergenic spacer (ITS) sequence analyses revealed distinct subclusters of *Candidatus* Scalindua that are unique to each OMZ examined, including the first report on a new *candidate* species in the Arabian Sea.

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5

Fosmids of novel marine *Planctomycetes* from the Namibian and Oregon coast upwelling systems and their cross-comparison with one complete and four almost complete planctomycete genomes

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Fosmids of novel marine *Planctomycetes* from the Namibian and Oregon coast upwelling systems and their cross-comparison with one complete and four almost complete planctomycete genomes

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Abstract

Molecular methods like fluorescence in situ hybridization (FISH) and 16S rRNA sequence analysis have shown that *Planctomycetes* are more widely distributed then assumed based on culture-depended studies. *Planctomycetes* are much more abundant in marine sediments than in marine water columns; however, they seem to be of importance for the marine carbon cycling. In order to broaden our knowledge about marine *Planctomycete*s, six fosmids from two different marine upwelling systems were investigated, some of which were closely related with so far uncultured *Planctomycete* groups. In a comparative sequence analysis with all available *Planctomycete* genomes, planctomycete-specific genes, as well as metabolic genes like sulfatases and those involved in C1 metabolism were investigated. This study is the first which includes the almost complete genomes of *Blastopirellula marina* DSM 3645^T and *Planctomyces maris* DSM 9787^T.

A high number of genes coding for sulfatases was found on all marine *Planctomycete* genomes and on one fosmid from the Namibian upwelling system. These enzymes might enable the *Planctomycetes* to degrade sulfated heteropolysaccharides and to use the derived carbon skeletons as energy source. Sulfated polysaccharides are produced in large quantities in marine environments and might be entrapped in marine snow particles which are known to be inhabited by *Planctomycetes*. The great chemical complexity of these compounds would explain the high number of genes encoding sulfatases on marine *Planctomycete* genomes. All *Planctomycete* genomes, but *Candidatus* Kuenenia stuttgartiensis, as well as two fosmids contained genes involved in the conversion of C1 carbon compounds known from methanogenic *Archaea* or methylotrophic *Alphaproteobacteria*. It is still not known what role these genes in *Planctomycetes* play, but codon usage analysis indicated high expression levels for some of these genes. Additionally, since easier pathways for the detoxification of formaldehyde are found on the genomes, it is very likely that these C1 metabolism genes in *Planctomycetes* play an important role in another as yet unknown pathway. The investigation of all five sequenced *Planctomycete* genomes and six fosmids containing planctomycete DNA allowed the identification of planctomycete-specific genes. These might include genes which are involved in their unique cellular characteristics. However, the anammox bacterium *Candidatus* Kuenenia stuttgartiensis was devoid of almost all genes that occur in other Planctomycetes. This together with the lack of C1 metabolism genes supports them being very distinct from all other *Planctomycetes*.

Introduction

Upwelling in marine coastal regions is a common phenomenon which can for example be caused by alongshore winds that drive surface water away from the shore and bring nutrient-rich deep sea water to the sun-lit surface. This results in an enhanced primary production that forms the basis for an elevated biomass on all subsequent trophic levels from plankton to fishes. It is in particular the high fish stock that constitutes the economic value of marine coastal upwelling regions. Hence, it is of crucial importance to understand the microbial communities underlying these ecosystems, even more so since they can trigger hazardous algal blooms, e.g. by toxic dinoflagellates.

The high levels of available nutrients in marine upwelling systems lead to a depletion of oxygen in deeper waters forming the so called oxygen-minimum zone (OMZ). Massive losses of nitrogen occur in the OMZ, mainly caused by planctomycetes living via anammox - the comproportionation of nitrite and ammonia to dinitrogen gas (Kuypers et al. 2005). Planctomycetes are also part of the microbial communities that are attached to macroscopic detrital aggregates (DeLong 1993, Crump et al. 1999), where they are likely involved in the breakdown of complex heteropolysaccharides (Glöckner et al. 2003). Such marine snow particles play a major role in highly productive marine ecosystems like upwelling regions.

These observations notwithstanding, the overall ecological functions of the *Planctomycetes* are not well-studied and hence not well understood. This might be attributed to the fact that *Planctomycetes* do not belong to the key players in marine surface waters in terms of abundance. Typically, they amount only for a few percent of the total microbial biomass in coastal waters, and even much less in the marine pelagial (Rusch et al. 2007), while abundances are much higher in marine sediments (Rusch et al. 2003, Inagaki et al. 2006, Musat et al. 2006).

In general, the *Planctomycetes* constitute an independent phylum within the domain *Bacteria* (Woese 1987), consisting of only one single family (*Planctomycetaceae*) with six accepted (*Rhodopirellula*, *Blastopirellula*, *Pirellula*, *Planctomyces*, *Isosphaera*, *Gemmata*) (Schlesner et al. 2004) and four candidate genera (Kuenenia, Brocadia, Scalindua, Anammoxoglobus). The phylogeny of the *Planctomycetes* has been under debate for some time with conflicting views of them as being either rapidly evolving (Woese 1987, Fuerst 1995), deep (Stackebrandt et al. 1984) or even deepest branching within the bacterial domain (Brochier and Philippe 2002) or being remotely related to the *Chlamydiae* (Weisburg et al. 1986, Liesack et al. 1992, Teeling et al. 2004). Recent studies suggest that the *Planctomycetes* are part of the so called PVC superphylum, a monophyletic clade that

besides the *Planctomycetes* is formed by the phyla *Chlamydiae*, *Verrucomicrobia*, *Lentisphaerae*, and the candidate phyla 'Poribacteria' and OP3 (which have no cultured representative so far) (Fieseler et al. 2004, Wagner and Horn 2006).

All *Planctomycetes* known to date are characterized by a unique set of characteristic morphological features. Their cells are organized in a polar manner with in some cases non-prosthecate appendages (stalks) or polar holdfast structures. In addition, planctomycete cells have a generative pole from which daughter cells are produced in a yeast-like budding process. A life cycle has been described for some planctomycetes that resembles that of *Caulobacter crescentus* with flagellated, non-reproductive swarmer cells and non-motile but reproductive adult cells (Tekniepe et al. 1981, Franzmann and Skerman 1984, Fuerst 1995, Glöckner et al. 2003). The cell walls of *Planctomycetes* lack the common bacterial cell wall constituent peptidoglycan, but instead consist out of proline and cystein-rich proteins that are stabilized by disulfide cross-links (König et al. 1984, Liesack et al. 1986). Likewise, S-layer-like arrayed proteins have been reported (Jetten et al. 2002). The cytoplasmic membranes of *Planctomycetes* contain characteristic pore-like crateriform structures that are either evenly distributed throughout the whole surface (genus *Planctomyces*) or confined around the reproductive cell pole (genus *Pirellula*) (Liesack et al. 1986). In addition, the DNA of *Planctomycetes* is highly compacted and hence often visible on electron micrographs as so called nucleoids.

The most distinctive feature of the *Planctomycetes*, however, is their internal compartmentalization (Lindsay et al. 1997, Lindsay et al. 2001, Fuerst, 2005). Members of the genera *Rhodopirellula*, *Blastopirellula*, *Pirellula*, *Planctomyces* and *Isosphera* have a single, large, membrane-bound compartment, the Pirellulosome. *Gemmata* species in addition have a double-membrane around their nucleoid that, however, might be in contact with the intracytoplasmic membrane and thus is not fully closed. Anammox planctomycetes finally have a special compartment, the anammoxosome that contains unique ladderane lipids (Sinninghe Damsté et al. 2002) and separates hazardous hydrazine from the cytoplasm in the course of the anammox process. The latter is carried out exclusively by a group of bacteria that branches deeply within the *Planctomycetes*.

Since these anammox planctomycetes are of industrial importance for wastewater treatment (Jetten et al. 1997) they are intensely studied. Apart from that, our knowledge on planctomycetal physiologies is quite limited. Most of the as yet cultured strains have been isolated from aquatic and aerobic habitats (Bauld J. and Staley 1976, Franzmann and Skerman 1984, Giovannoni et al. 1987b, Schlesner 1994) and they all are obligate or

facultative aerobic chemoheterotrophs that use carbohydrates as their major source of carbon. However, molecular methods like fluorescence *in situ* hybridization (FISH) and 16S rRNA gene sequencing have revealed a much broader distribution of the *Planctomycetes* in the environment. They were detected in the water column and sediments of fresh water lakes (Neef et al. 1998, Miskin et al. 1999, Wang et al. 2002, Kalyuzhnaya et al. 2004, Kalyuzhnaya et al. 2005), hot springs (Giovannoni et al. 1987a), in the water column (DeLong 1993, Vergin et al. 1998) as well as in shallow and deep sea sediments of marine systems (Llobet-Brossa et al. 1998, Rusch et al. 2003, Inagaki et al. 2006, Musat et al. 2006) and in oxic and anoxic soils (Wang et al. 2002). Furthermore, planctomycetes have been detected in marine sponges (Fuerst et al.1998, Fuerst et al. 1999, Pimentel-Elardo et al. 2003), the hepatopancreas of the crustacean *Panaeus monodon* (Fuerst et al. 1991, Fuerst et al. 1997) and in freshwater (Crump et al. 1999) and marine detritus particles (DeLong 1993, Fuerst 1995, Crump et al. 1999).

At present, only a few planctomycetes have been investigated by whole genome sequencing. The genome of the marine planctomycete *Rhodopirellula baltica* SH 1^T (formerly *Pirellula* sp. strain 1) is currently the only fully closed one (Glöckner et al. 2003). Besides that, four largely completed planctomycete genomes are available. The anammox planctomycete *Candidatus* Kuenenia stuttgartiensis was investigated by a metagenomic approach from a waste water treatment plant enrichment culture (Strous et al. 2006). In addition, the Gordon and Betty Moore foundation has funded draft sequencing of the two marine planctomycetes *Blastopirellula marina* DSM 3645^T (Schlesner et al. 2004) and *Planctomyces maris* DSM 9787^T (Bauld J. and Staley 1976) on our behalf, and finally Ward and co-workers at The Institute for Genomic Research (TIGR) have generated an early draft of the genome of the freshwater isolate of *Gemmata obscuriglobus* UQM 2246^T (Franzmann and Skerman 1984).

In order to broaden our understanding about marine *Planctomycetes*, we present here comparative sequence analysis of six novel *Planctomycete* fosmids from two different upwelling systems and their comparison with all of the available *Planctomycete* genomic sequences (see suppl. Fig. 1 for sampling sites). Four of the fosmids were retrieved from the OMZ of the Benguela upwelling system at the Namibian coast, and two originate from the coast of Oregon (Stein et al. 1996, Vergin et al. 1998) and were obtained by courtesy of Ed DeLong. At the same time, this is the first study to include results from our annotation of the almost complete genomes of *Blastopirellula marina* DSM 3645^T and *Planctomyces maris* DSM 9787^T. With these new draft genomes, we now have genomic sequences from

four of the six accepted planctomycete genera. In this study we investigated planctomycete-specific genes, metabolic genes like sulfatases and those involved in the C1 metabolism.

Materials and Methods

Metagenome sampling

In this study, two metagenome libraries were investigated: The first was constructed from picoplankton samples taken during a cruise in the eastern North Pacific at 200 m depth of the Oregon coast (44°02.72'N, 124°57.30'W) in August 1992 as described in (Stein et al. 1996). The second was constructed from Namibian shelf water that was sampled during an R/V *Meteor* cruise in March/April 2003 at station M202 (22.64°S and 14.30°E) (suppl. Fig. 1). This sample was taken from 52 m depth, where 2 µM nitrite, 4.6 µM nitrate and oxygen and ammonium concentration below the detection limit were measured (Kuypers et al. 2005). About 500 l seawater were brought onto pre-combusted (at 450 ºC) fibre glass filters (GFF; nominal pore size, 0.7 µm) and stored at -80 °C until further processing.

Fosmid library construction

The metagenome library from the North Pacific was constructed as described in (Stein et al. 1996). The library from the Namibian upwelling system was constructed as follows: High molecular weight DNA was extracted according to the protocol from Zhou et al. (1996), taking special care not to shear the DNA by using cell saver tips during the extraction. In order to avoid DNA loss due to DNA binding to the filter glass material, cells were washed off the filters with the extraction buffer without proteinase K and SDS prior to the extraction procedure.

After DNA extraction, the RNA was digested with RNAse in 0.5 x TE for one hour at room temperature. Subsequently, the RNAse was inactivated at 60 °C for 10 min, an equal amount of chloroform/isoamyl alcohol mixture was added and the supernatant was precipitated with 1/10 volume of NaoAc and 0.6 volume of isopropanol (incubation for 1 h at room temperature and centrifugation). The DNA was stored in 0.5x TE and the ends of the DNA were filled blunt ended according to the protocol of the Copy Control™ Fosmid Library Production Kit (EPICENTRE Biotechnologies, Madison, WI, USA).

By Pulsed Field Gelelectrophoresis (PFGE) we selected only DNA of 30-45 kb for subsequent ligation into the vector (1% LMT agarose; in 0.5x TBE; program of PFGE: run time 12 h at 14 °C; angle of 120 degrees; 6 V/cm; initial switch time 1 s, final switch time

10 s; afterwards for 1.3 h initial and final switch time 2 min). After cutting the band of the desired size (30-45 kb) we equilibrated the DNA three times in 1x TE (each 30 min) and digested the agarose with β-agarase (1,000 U/ml; 1 µl/100 mg agarose gel). The DNA was concentrated and washed with 1 x TE using a Microcon tube (Millipore, Billerica, MA, USA) and subsequently eluated in PCR-water.

The DNA was ligated into CopyControl pCC1FOS vectors (EPICENTRE Biotechnologies, Madison, WI, USA), but the ligation was conducted at 4 °C for two days. The vectors were packaged into the MaxPlax lambda phage (EPICENTRE Biotechnologies, Madison, WI, USA). *E. coli* cells of the strain EPI300-T1R (EPICENTRE Biotechnologies, Madison, WI, USA) were infected with the phages upon reaching an OD of 0.8 to 1.0 and plated onto LB-chloramphenicol plates (12.5 µg/ml). About 10,000 grown colonies were transferred in LB medium containing chloramphenicol (12.5 µg/ml), $MgSO_4$ (10 mM), and Glycerol (8%) and stored at -80 °C. Additionally, clones were pooled according to the row-column-plate scheme and incubated over night at 37 °C. The DNA was subsequently extracted using the 96 well Montage plasmid preparation kit (Millipore, Billerica, MA, USA) and stored at -80 °C.

Fosmid library screening

The metagenome library from the coast of Oregon was screened for fosmids containing planctomycete 16S rRNA genes as described in (Vergin et al. 1998). Four planctomycete fosmids were detected in 3552 screened clones.

The fosmid library from the Namibian upwelling system was screened by PCR with the planctomycete primer Pla46F (Neef et al. 1998) and the universal primer 1392R (Pace et al. 1986). Based on the pooling scheme, we could trace back the positive PCR products to single clones of the fosmid library. The 16S rRNA genes were sequenced and a their phylogeny was reconstructed using the Arb software (Ludwig et al. 2004). In total, 10,000 clones were screened and twelve clones with planctomycete DNA were obtained.

Insert size determination

The respective clones were induced for high copy number according to the manual of CopyControl™ Fosmid Library Production Kit (EPICENTRE Biotechnologies, Madison, WI, USA). Afterwards, fosmids were isolated following the QIAprep Spin Miniprep Protocol (QIAGEN, Hilden, Germany). Aliquots of 25 µl of fosmid DNA were digested with NotI (10,000 U/µl), the digestion was stopped by heating at 65 °C for 10 min and

quickly placed on ice. The fragment sizes were checked by PFGE (0.5x TBE, 1% pulsed field certified agarose (Bio-Rad, Hercules, CA, USA), initial switch time 5 s, final switch time 15 s, 6 V/cm, angle: 120 degrees, run time: 17 h, 14 °C).

Fosmid sequencing

Two of the four planctomycete fosmid inserts from the coast of Oregon were selected and sequenced at Integrated Genomics (Jena, Germany). From the twelve planctomycete fosmid from the Namibian metagenome library, four fosmids were chosen for sequencing based on the phylogenetic position of the planctomycete 16S rRNA gene and the determined insert size. For that purpose, large amounts of fosmid DNA were extracted after induction of the fosmids with the QIAGEN® Large-Construct Kit (QIAGEN, Hilden, Germany). Shotgun sequencing of the fosmids was conducted by AGOWA (Berlin, Germany) resulting in single contigs.

Gene prediction and annotation

Gene prediction was adapted for each sequence individually. For the rather short fosmid sequences, all open reading frames (ORFs) exceeding 90 nucleotides were taken into account. These were analyzed by the annotation system (see below). Over-predicted genes were sorted out during manual annotation. For the published genomes of *R. baltica* SH 1^T and *Candidatus* K. stuttgartiensis, the original gene prediction was retained. GLIMMER v2 (Delcher et al. 1999) was used for the gene prediction of the *G. obscuriglobus* UQM 2245^T draft genome. For the two planctomycete draft genomes sequenced by the Moore foundation, the supplied gene prediction was evaluated against an in-house gene prediction pipeline (mORFind, unpublished) that post-processes the outputs of CRITICA (Badger and Olsen, 1999), GLIMMER v2 (Delcher et al. 1999) and Zcurve (Guo et al. 2003). In case of *P. maris* DSM 8797^T the original gene prediction was kept, since it could not be further enhanced, but for *B. marina* DSM 3645^T, a completely new and much enhanced gene prediction was generated.

Annotation was accomplished with the GenDB v2 system (Meyer et al., 2003), using various bioinformatic tools for each predicted gene ranging from similarity searches against sequence databases (NCBI nr, NCBI nt, SwissProt) and protein family databases (Pfam, Prosite, InterPro, COG) to signal peptide- (SignalP v2.0, Nielson et al. 1997) and transmembrane helix predictions (TMHMM v2.0, Krogh et al. 2001). From these predictions, an annotation was generated in automated manner using a fuzzy logic-based

approach (Quast, in preparation). High-quality annotations were generated by manual revision of each gene's annotation for the fosmids and the draft genome of *B. marina* DSM 3645^T, because these sequences were of special interest.

Gene prediction and annotation of *B. marina* DSM 3645^T and *P. maris* DSM 8797^T is available on request.

Sequence availability

Fosmid sequences and genomes from *B. marina* DSM 3645^T and *P. maris* DSM 8797^T will be deposited at NCBI.

Comparative genomics

All comparative analyses was performed using a self-written JAVA-based framework that operates directly on GenDB MySQL databases and allows to work with multiple of these databases at once.

Planctomycete-specific genes were identified by searching all genes of the investigated planctomycete sequences with BLAST against a specific database (genomes DB). This database was constructed from the proteome FASTA files of all fully sequenced bacterial and archaeal genomes, in which each protein was tagged by a unique numerical identifier in the header that encoded the specie, the chromosome and the gene itself. Two approaches were taken in order to determine whether or not a gene was planctomycete-specific. In the first, two E-value thresholds separating planctomycetes and non-planctomycetes hits were used: A lower boundary (E-15) below which only hits to *Planctomycetes* were allowed and an upper boundary (E-6) below which no hits to any other genome were allowed. In the second approach, all BLAST hits were extracted for each genome, whose BLAST hits below E-10 exclusively targeted other *Planctomycetes*.

Besides planctomycete-specific genes, we also searched for orphaned, genome-specific genes. i.e. genes without known homologs in any other genome. A genes was regarded as orphaned, if it had no BLAST hit below E-7 in the non-redundant NCBI nr or the genomes DB.

Codon usage analysis

Codon usage analysis was carried out with CIAJava (Carbone et al. 2003) and codonw (Peden 1999). CAIJava was used for each genome with the full set of predicted genes and with the standard 15 iterations. Codonw was used for each of the genomes with a high-

quality training set of genes for the initial correspondence analysis that was generated by filtering all genes smaller than 300 bp as well as genes coding for hypothetical proteins, phage proteins, transposases and integrases. Thereafter, the extracted codon usage characteristics were used to analyze the codon usage of all genes.

Results

Characterization and phylogenetic assignment of the obtained fosmid sequences

The size of the fosmid inserts ranged from 34.6 kb (fosmid 8FN) to 42.5 kb (fosmid 6N14) (Table 1) and the G+C-content from ~48% to ~60% which is very similar to the G+C-content variation of the *Planctomycete* genomes (Table 3). The number of ORFs per fosmid insert ranged from 28 to 33.

Table 1: Characterization of fosmids: Insert size, G+C-content, number of ORFs and sampling location

	3FN	6FN	8FN	13FN	5H12	6N14
Fosmid insert size (kb)	37.0	40.7	34.6	36.7	41.0	42.5
G+C-content	59.7	47.8	50.8	55.0	51.0	52.4
Number of ORFs after manual annotation	28	28	33	31	31	32
Sampling location	Namibian upwelling system (Kuypers et al., 2005)				Oregon upwelling system (Stein et al., 1996)	

Based on 16S rRNA analysis, phylogentic assignments were made for the fosmid insert sequences obtained from the Namibian (3FN, 6FN, 8FN, 13FN) and Oregon (5H12, 6N14) coasts. A phylogenetic reconstruction based on tree calculation with neighbour joining, maximum likelihood and maximum parsimony algorithms (without and with 50% position variability filter) is shown in Fig. 1.

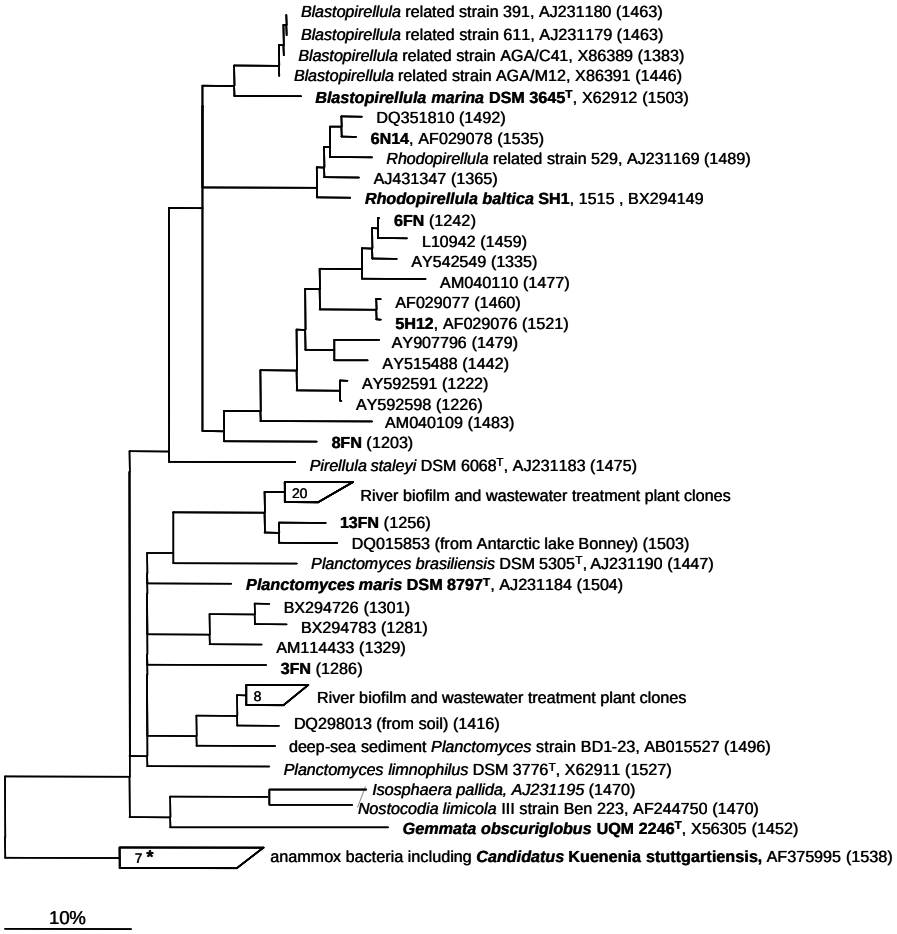

Fig. 1: Phylogenetic tree based on 16S rRNA sequences showing the phylogenetic affiliation of fosmids and genomes used in this study. The consensus tree was constructed after tree calculation with neighbor joining, maximum parsimony and maximum likelihood algorithms without and with 50% position variability filters. Lengths of the sequences are indicated in parentheses. Anammox bacteria (group indicated by an asterisk comprising of *Cand.* K. stuttgartiensis, *Cand.* B. anammoxidans, *Cand*. B. fulgida, *Cand* A. propionicus, *Cand*. J. asiatica, *Cand*. S. sorokinii and *Cand*. S. brodea) were used as outgroup. The bar repesents 10% estimated sequence divergence.

Fosmid insert 13FN exhibited highest 16S rRNA sequence similarity to an uncultured planctomycete sequence from Lake Bonney, a permanently ice-covered lake in Antarctica (DQ015853, 94.9% identity). Its closest cultivated planctomycete was *P. maris* DSM 8797^T (87.5% identity). Likewise, the 16S rRNA of *P. maris* DSM 8797^T was also the closest cultured relative to fosmid insert 3FN (88.7% identity), which however, was most similar to a planctomycete sequence from a hydrocarbon-contaminated soil (DQ298013, 89.2%). Fosmid insert 6FN had a very high similarity to the 16S rRNA sequence from an uncultured marine snow associated planctomycete (L10942, 99.0% identity). The sequence identity to the next cultured relatives, two *B. marina* DSM 3645^T related strains (AJ231179 and AJ231180), was considerably lower (87.5% identity). Clone 8FN exhibited highest sequence identity to a sequence originating from a deep-sea mud volcano in the eastern

Mediterranean (AY592598, 92% identity). The next cultured relative was a *Blastopirellula* related planctomycete isolated from the postlarvae of the giant tiger prawn (*Penaeus monodon*) (89.9% identity, X86391).

Fosmid 6N14 had highest sequence identity with an uncultured planctomycete from heavy metal contaminated surface sediments in the North Sea (98.2%, DQ351810) and is related to *Rhodopirellula baltica* SH 1^T (95.7% identity). Clone 5H12 grouped, together with 8FN and 6FN, within a cluster of uncultured planctomycetes and showed highest sequence identity to a fosmid from the same metagenome library (AF029077, 99.7%, identity). The closest cultured representatives were a group of isolates retrieved from the postlarvae of *Penaeus monodon* (e.g. X86389, 88.9% identity).

Genes involved in C1 metabolism

It was one of the most surprising findings in previous studies of the genomes of *R. baltica* SH 1^T and the draft genome of *G. obscuriglobus* UQM 2246^T that both code for proteins involved in tetrahydromethanopterin-(H_4MPT)-linked C1-compound conversions (supp. Fig. 2) and the biosynthesis of the associated essential cofactor methanopterin (Glöckner et al. 2003, Bauer et al. 2004, Chistoserdova et al. 2004). Before, these genes were believed to occur only in methanogenic *Archaea* and methylotrophic *Alphaproteobacteria*, where they play the central role in either the reductive or oxidative gain of energy from C1-substrates and the detoxification of the hazardous metabolic intermediate formaldehyde. Many of the participating genes have been identified by functional studies on the methylotroph *Methylobacterium extorquens* AM1 (Chistoserdova et al. 2005) with the notable exception of *ORF1*, that is absent in *M. extorquens* AM1 but present in various other methylotrophs (see suppl. Tab. 1 for an overview on the functions).

In addition to the already described C1 metabolism genes in *R. baltica* SH 1^T and *G. obscuriglobus* UQM 2246^T, we could identify homologs to all known C1 genes in the draft genome of *B. marina* DSM 3645^T and, with the exception of *ORF21*, also in the *P. maris* DSM 9787^T draft. In contrast, none of these genes were found in the draft genome of *K. stuttgartiensis* (Table 2 and Fig. 2).

Table 2: Presence and absence of archaea-like and bacteria-like H_4MPT-dependent genes in the studied *Planctomycete* genomes and fosmids

Gene	R.b.	G.o.	B.m.	P.m.	K.s.	3FN	6FN	8FN	13FN	5H12	6N14
ORF5	+	+	+	+							
ORF7	+	+	+	+							
ORF9	+	+	+	+							
ORF17	+	+	+	+							
ORF19	+	+	+	+				+			
ORF20	+	+	+	+				+			
ORF21	+	+	+								
ORF22	+	+	+	+							
ORFY	+	+	+	+							
fae [2]	+	+	+	+							
fae [2]	+	+	-	+							
ftr [1]	+	+	+	+							
mch	+	+	+	+							
mptG	+	+	+	+				+			
mtdC	+	+	+	+							
fmdABC [1]	+/-/+	+/+/+	+/+/+	+/+/+			-/-/+				
ORF1	+	+	+	+							
pabAB	+		+	+				+			
ptS		+	+	+							

[1] Ftr together with the three subunits of Fmd forms the formyltransferase/hydrolase complex (fhc)

R. baltica SH 1^T and *G. obscuriglobus* UQM 2246^T have two copies of the formaldehyde activating enzyme gene (*fae1* and *fae2* – 31% and 28% amino acid identity, respectively). We also found two *fae* copies in *P. maris* DSM 8797^T (38% aa identity) but only one in *B. marina* DSM 3645^T. Within *R. baltica* SH 1^T, *fae1* is characterized by a rigidly optimized codon usage indicating a high level of expression (Bauer et al., 2004). This is also the case within *B. marina* DSM 3645^T, whose *fae1* gene has the third highest codon adaptation index (CAI) of all of its genes, but not for the *P. maris* DSM 8797^T *fae* gene. In all four genomes, *fae1* is located upstream of a gene coding for the recently discovered methylene tetrahydromethanopterin gene *mtdC* (Vorholt et al., 2005). In *R. baltica* SH 1^T, *B. marina* DSM 3645^T and *P. maris* DSM 8797^T, *fae1* and *mtdC* are located adjacent to *ORFY*, a gene with as yet unknown function. Likewise, *mch* (encoding methenyl H_4MPT cyclohydrolase) is located directly upstream of *ORF5* (most likely involved in the biosynthesis of H_4MPT) in all four *Planctomycete* genomes.

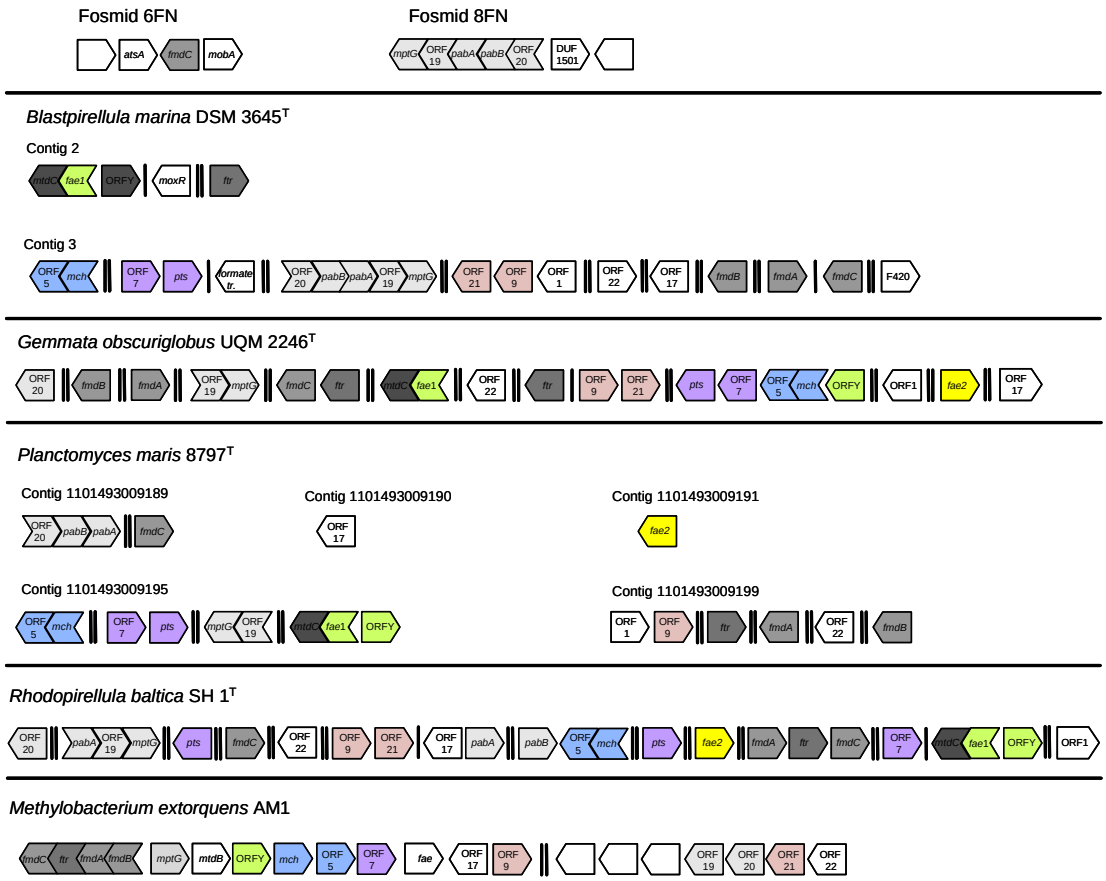

Fig. 2: Genomic arrangement of genes involved in H_4MPT-dependent C1-transfer. Comparison of fosmids 6FN and 8FN from the Namibian upwelling system, genomes of the planctomycetes *Blastopirellula marina* DSM 3645^T, *Gemmata obscuriglobus* UQM 2246^T, *Planctomyces maris* 8797^T, *Rhodopirellula baltica* SH 1^T and the methylotrophic *Alphaproteobacterium Methylobacterium extorquens* AM1. Inserted arrows state that ORFs are organized in operons. A single line separating arrows indicate that ORFs are separated by at most 50 ORFs; double lines represent a separation by > 50 ORFs.

Clustering of formyl-H_4MPT-dehydrogenase subunits A and C (*fm(w)dA/C*) with formylmethanofuran:H_4MPT-formyltransferase (*ftr*) could be observed in *R. baltica* SH 1^T, while in *G. obscuriglobus* UQM 2246^T the *fm(w)dC* and *ftr* genes are co-located. Within *B. marina* DSM 3645^T and *P. maris* DSM 8797^T, the *ftr* gene was not located adjacent to any of the *fm(w)d* genes. In all four *Planctomycete* genomes, *ORF19* was found to be linked to *mptG*. The first is involved in dH_4MPT biosynthesis (Chistoserdova et al. 2005), while the latter codes for beta-RFAP synthase, an enzyme that catalyzes the first reaction distinguishing the methanopterin biosynthesis pathway from that of folate biosynthesis (Scott and Rasche 2002, Chistoserdova et al. 2004). Clustering of *ORF9* with *ORF21* was observed in three genomes: *R. baltica* SH 1^T, *B. marina* DSM 3645^T and *G. obscuriglobus* UQM 2246^T. These two genes are also involved in dH_4MPT biosynthesis (Chistoserdova et al. 2005). Further co-localizations cover *ORF9* and *ORF1* in *B. marina* DSM3645^T and *P.*

maris DSM 8797^T, and *ORF7* and *pts* in *B. marina* DSM 3645^T, *P. maris* DSM 8797^T *and G. obscuriglobus* UQM 2246^T.

Genes involved in the H_4MPT-linked C1 metabolism were also found on two of the four fosmids from the Namibian OMZ (Tab. 2, Fig. 2). Fosmid 6FN harbors an instance of *fmdC* plus the gene *mobA* that encodes the molybdopterin-guanine dinucleotide biosynthesis protein A. Fosmid 8FN contains a larger C1 module comprising *ORF20-pabB-pabA-ORF19-mptG*. This complete module could also be identified in *B. marina* DSM 3645^T, while *P. maris* DSM 8797^T contained these genes in two separate clusters of *ORF20-pabB-pabA* and *ORF19-mptG*. *R. baltica* SH 1^T has the cluster *pabA-ORF19-mptG,* while ORF20 is isolated and pabB is co-localized with the C1 genes *ORF5* and *mch*. In *G. obsucriglobus* UQM 2246^T finally, only the genes *ORF19-mptG* from this cluster could be found. *ORF19* and *ORF20* are also co-localized in the methylotroph *M. extorquens* AM1 and have been shown to be essential for the biosynthesis of dH_4MPT (Chistoserdova et al. 2005). *MptG* encodes an GHMP family kinase, and *pabA* and *pabB* the para-aminobenzoate synthase glutamine amidotransferase component II and the para-aminobenzoate synthase component I, respectively (Kalyuzhnaya et al. 2005).

Planctomycete-specific genes

Having multiple *Planctomycete* genomes at hand opens the possibility for the identification of planctomycete-specific genes. Among these, the ones that are present in all five planctomycetes investigated are of particular interest, since they define a *Planctomycete* and hence must include the genes coding for their unique cellular characteristics.

Much to our surprise, only 2-3 genes were found when searching for such genes. A closer investigation revealed that *Candidatus* K. stuttgartiensis was almost devoid of genes that occur in all other planctomycetes. If left out, the remaining four planctomycetes had between 125 and 249 genes that were specific for them (Table 3).

A large proportion of these planctomycete-specific genes were made up by large paralogous gene families that were defined by the occurrence of one or more specific domains of as yet unknown function (DUF), in particular the DUF1501, PSD1 (DUF1553) and DUF1559 as well as the planctomycete-specific cytochrome c-like domains PSCyt1 and PSCyt2 (DUF1549).

Table 3: Characterization of the *Planctomycete* genomes: Genomes size, G+C-content, number of sulfatases, number of orphan genes and number of *Planctomycete* group-specific genes (GSG) and their domains of unknown functions

	R. baltica	*P. maris*	*B. marina*	*G. obscuriglobus*	*K. stuttgartiensis*
Genome size	7,145 kb	7,775 kb	6,655 kb	9,106 kb	4,218 kb
G+C content (%)	55.4	51.2	57.2	67.1	40.8
No. of predicted genes	7,325	6,531	5,600	(13,024)[1]	4,710
No. of orphan genes[2]	2897 (39.5%)	1559 (24.5%)	1088 (19.4%)	(5989)[1] (46.0%)[1]	1364 (29.0%)
No. of GSG w/o KS[3]	125	200	249	154	-
No. of GSG with KS[3]	3	3	3	3	2
No. of sulfatases[4]	109 (15.3/Mb)	83 (10.7/Mb)	41 (6.2/Mb)	12 (1.3/Mb)	3 (0.7/Mb)
No. of PFAM profile DUF1501[4]	41	94	31	61	0
No. of PFAM profile PSCyt2 (DUF1549)[4]	41	68	32	43	0
No. of PFAM profile PSD1 (DUF1553)[4]	41	68	32	43	0
No. of PFAM profile PSCyt1 (CytC)[4]	53	54	32	27	0
No. of PFAM profile SBP_bac_10 (DUF1559)[4]	74	134	197	125	0
No. of PFAM profile DUF1551[4]	6	2	1	7	0

[1] overprediction by Glimmer 2; [2] E value E-7 or better; [3] upper boundary: E-15, lower boundary: E-6; [4] E value E-5 or better

Oftentimes, these planctomycete-specific genes appeared in tandem. The most prominent of these tandems consisted of a gene carrying DUF1501 and a gene containing DUF1549 plus DUF1553 and sometimes an additional planctomycete-specific cytochrome c domain (Table 4). A remarkable high proportion of these genes had signal peptide predictions, especially the DUF1549/DUF1553 genes.

Table 4: Statistical analysis of DUFs in tandem structures on the Planctomycete genomes

	R. baltica	*P. maris*	*B.marina*	*G. obsuriglobus*	*K. stuttgartiensis*
Number of tandem occurrences (search started with DUF1501)	39	nd	30	22	0
Percentage of genes with DUF1501 that appear in tandem	95.1%	nd	96.8%	36.1%	0
Number of genes containing DUF1549 and DUF1553 from tandems having signal peptides	21	nd	21	12	0
Percentage of secreted genes with DUF1549 and DUF1553 from tandems	53.8%	nd	70.0%	54.5%	0
Number of genes with DUF1549 and DUF1553 from tandems which also contain CytC domain	28	nd	18	11	0
Percentage of genes with DUF1549, DUF1553 and CytC domain present in tandems	71.8%	nd	60.0%	50.0%	0

DUF1553 = PSD1
DUF1549 = PSCyt2

If the five planctomycete genomic sequences were searched for planctomycete-specific genes occuring in at least one other *Planctomycete* species, vastly diverging numbers were obtained (Fig. 3). The genomes of *R. baltica* SH 1^T, *P. maris* DSM 8797^T and *B. marina* DSM 3645^T were found to share 429 - 764 genes, while *G. obscuriglobus* UQM 2246^T was more distant to these three with 275 - 475 shared genes. In *Candidatus* K. stuttgartiensis finally, merely 0 - 30 planctomycete-specific genes were found that were shared with at least one of the other four planctomycetes.

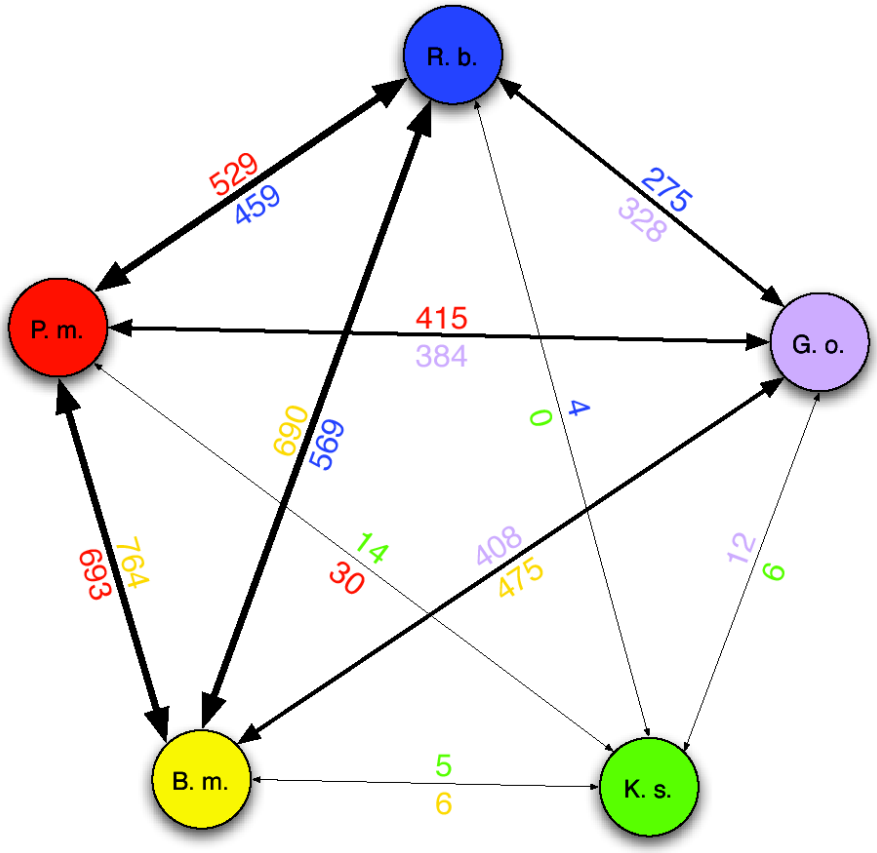

Fig. 3: Genes whose BLAST hits below E-10 exclusively targeted *Planctomycetes*. Colors indicate the respective reference genome for the BLAST searches. Corresponding colors represent the number of hits in the respective target genome, that is also expressed by thickness of the connection lines.

All five investigated genomes contained high proportions of hypothetical proteins of which a large proportional were found to be orphaned, i.e. present only in one particular planctomyete with no known homolog in any other species (Table 3).

One the six sequenced fosmids 28 genes with reliable BLAST hits exclusively to *Planctomycetes* were found (Table 5). It is noteworthy that from these 28 planctomycete-specific genes, only a single one exhibited high similarity to a gene from *Candidatus* K. stuttgartiensis. As within the genome sequences, many of the planctomycete-specific genes

on the fosmids contained the Pfam domains DUF1501, DUF1550 and DUF1559, and co-occurrences of the domains DUF1549 and DUF1533.

Table 5: Overview of Planctomycete-specific genes and their domain of unknown functions (DUFs) detected on the investigated fosmids

Fosmid	Gene	Function	Observations
3FN	3FN_13		
	3FN_15	Protein containing terpenoid cylases/protein prenyltransferase alpha-alpha toroid domain	
	3FN_24	Protein containing DUF1549, DUF1553	
	3FN_26	Protein containing DUF1501	
	3FN_27	Protein containing DUF1549, DUF1553	
6FN	6FN_5		
	6FN_20	Protein containing DUF1501	
	6FN_21	Protein containing planctomycete cytochrome c domain, DUF1549 and DUF1553	
	6FN_26	Protein containing DUF1550	Hit also for *Candidatus* K. stuttgartiensis
8FN	8FN_8	Protein containing DUF1501	
	8FN_9		
	8FN_23	Protein containing DUF1559, membrane	
	8FN_33	membrane	
13FN	13FN_31		
6N14	6N14_18		
	6N14_21		
	6N14_25		
5H12	5H12_7		
	5H12_8		
	5H12_9	DUF1501	
	5H12_10	DUF1553	
	5H12_11		
	5H12_17		
	5H12_25		
	5H12_26		
	5H12_29		

Sulfatases

The number of genes encoding sulfatases varied considerably among the different planctomcetes investigated (Table 3). While the marine planctomycetes *R. baltica* SH 1^T, *P. maris* DSM 8797^T and *B. marina* DSM 3645^T encode high numbers of sulfatases, only few sulfatases could be found in the non-marine planctomycetes *G. obscuriglobus* UQM2246^T and *Candidatus* K. stuttgartiensis.

R. baltica SH 1^T codes for no less than 109 sulfatases (15.3 per Mb), which to date is the highest number found in any bacterial genome. In the *P. maris* DSM 8797^T draft genome we found 87 sulfatases (10.7/Mb) and in the one of *B. marina* DSM 3645^T 41

(6.16/Mb), whereas in the *G. obscurilobus* UQM2246^T draft sequence only twelve sulfatases were found (1.3/Mb) and in *K. stuttgartiensis* only three (0.7/Mb). Additionally, one sulfatase could be found on fosmid 6FN from the Namibian upwelling system (suppl. Table 3).

Discussion

In the present study six *Planctomycete* fosmid insert sequences from two different marine upwelling systems were compared with all available complete or almost complete *Planctomycete* genomes. Taking the two new draft genomes of *B. marina* DSM 3645^T and *P. maris* DSM 8797^T into account, we now have genomic information from all *Planctomycete* genera at hand except but from the *Isosphaera* and *Pirellula* lineage.

R. baltica SH 1^T and *B. marina* DSM 3645^T were both isolated from the Kiel Fjord in the Baltic Sea (Schlesner 1994) and are aerobic heterotrophs. They are marine representatives of the *Planctomycete* phylum, since they do not at all grow in freshwater media (Schlesner et al. 2004). *P. maris* DSM 8797^T was isolated from shallow waters at Pudget Sound, Washington, USA (Bauld and Staley 1976; Bauld and Staley 1980) and is a heterotrophic, aerobic, marine planctomycete as well. *G. obscuriglobus* UQM 2246^T is a freshwater isolate from the Maroon Dam in Queensland, Australia (Franzmann and Skerman 1984) and *Candidatus* K. stuttgartiensis was enriched from a waste water treatment plant in Stuttgart, Germany (Schmid et al. 2000).

Environmental functioning of *Planctomycetes*

The planctomycetes from marine habitats contained considerably higher sulfatase copy numbers in their genomes than the planctomycetes from freshwater habitats (Table 3). Likewise, one of the four fosmid sequences from the Namibian upwelling system contained a sulfatase. Since those four fosmids together account for ~150 kb of sequence information, this amounts to 6.7 sulfatases per Mb. This is in line with what was observed for sulfatase-rich marine planctomycetes (Table 3). The presence of high sulfatase copy numbers might be attributed to a particular marine lifestyle. The most likely candidate substrates for these sulfatases are sulfated heteropolysaccharides which are produced in large quantities in marine environments, e.g. by fish (chondritin in cartilage), red algae (agars and carrageenans) and brown algae (sulfated fucans). These compounds are of a great chemical complexity, and hence require a versatile repertoire of specific sulfatases for successful biodegradation. We proposed earlier that sulfated polysaccharides are entrapped in marine

snow aggregates, which are known to be colonized by planctomycetes. Sulfatase-rich planctomycetes are then supposed to be able to degrade these polysaccharides and subsequently use their carbon skeletons as energy source (Glöckner et al. 2003). Sulfatase activity has meanwhile been proven for *R. baltica* SH 1^T, and not only for linear but also on sterically demanding sulfated compounds (Wallner et al. 2005). In addition, it has been demonstrated that *R. baltica* H 1^T, while apparently not capable of degrading agar, does degrade carrageenan (Gurvan Michel, pers. communication). This fits perfectly to the proposed lifestyle for these organisms. A more in-depth analysis of the sulfatases in *B. marina* DSM 3645^T revealed that 39 of its sulfatases carried the essential canonical motif [CS]-x-[PA]-x-R which in 37 cases could be extended to [CS]-x-[PA]-x-R-x(4)-[ST]-G. Thirty-four of these proteins had a proline at the third motif position, whereas four have a proline to alanine mutation. It is unclear, whether this has any impact on the functioning or specificity of the respective enzymes, but the presence of the canonical motif indicates that these sulfatases are active. Considering the sheer amount of sulfated heteropolysaccharides that are produced in marine environments and the ubiquity of lateral gene transfer (LGT), it is highly unlikely that only representatives of the marine *Plancomycetes* have adapted to exploit this resource. Hence it can be expected that high sulfatase copy numbers will be discovered in marine representatives from other lineages as well and likely play and important role in highly productive marine upwelling systems.

Investigation of the studied genomes and fosmids for genes involved in the conversion of C1 carbon compounds revealed that four of the genomes and two of the fosmids (8FN and 6FN) contained such genes (Tab. 2 and Fig. 2). The notable exception in the genomes is the deep-branching anammox bacterium *Candidatus* K. stuttgartiensis, which lacks those genes completely. Earlier studies discussed that the clustering of C1-transfer genes in *Planctomycetes* is much looser than in the genomes of methylotrophic proteobacteria where the genes are arranged in few main clusters (Bauer et al. 2004, Kalyuzhnaya et al. 2005). Our study with four planctomycete genomes containing these genes supported this finding. The genes were scattered widely over the genomes and at most five genes were arranged in one cluster (Fig. 2). This cluster of *Orf20-pabA-pabB-Orf19-mptG* was found on fosmid 8FN and in the genome of *B. marina* DSM 3645^T. Most likely, this cluster reflects the original organisation of these genes in the *Planctomycetes*, and in the course of evolution it was split into smaller modules, like for example in *P. maris* DSM 8797^T, where these genes are organized in the two distinct clusters *Orf20-pabA-pabB* and *Orf19-mptG*. With our study we could also support some previously

postulated clustering patterns of C1 genes in *Planctomycetes*, in particular clustering of *mptG* with *orf19* and of *mtdC* with *fae1* (Bauer et al. 2004, Kalyuzhnaya et al. 2005). However, the previous finding that the *fmdA*, *ftr* and *fmdC* are always co-located in all known bacterial genomes containing these *Archaea*-like genes (Bauer et al. 2004, Kalyuzhnaya et al. 2005) did not hold with the investigation of more *Planctomycete* genomes. The existence of H_4MPT-depended genes in representatives of *Proteobacteria* and *Planctomycetes* has let to numerous theories about the origin and distribution of these genes (Bauer et al. 2004). One scenario assumes two LGT events by which the genes were passed on from *Archaea* to *Proteobacteria* and from there to the *Planctomycetes*. Another scenario presumes that the last universal common ancestor was already equipped with the C1-transfer genes and that they were preserved only within few lineages. These questions are hard to answer, especially since the phylogenetic position of the entire PVC superphylum. The easiest explanation would be to question the monophyly of the eubacteria, which has been proposed by Cavalier-Smith (Cavalier-Smith 2006), but this discussion is clearly beyond the scope of this study.

Up to now it is not possible to answer the question whether these genes are functional in planctomycetes and what role they play. So far, *R. baltica* SH 1^T could not be shown to growth on C1 substrates (Bauer et al. 2004). On the other hand, codon usage analysis indicated high expression levels for *fae1* in *R. baltica* SH 1^T (Bauer et al. 2004) and in *B. marina* DSM 3645^T. Furthermore, proteome analysis proved that *fae1*, *mtdA* and *mch* are expressed in *R. baltica* SH 1^T (Bauer et al. 2004). Another indication that the genes involved in H_4MPT-dependent C1-compound conversions are active in the planctomycetes that possess them is their arrangement in conserved modules (Fig. 2). In summary, this suggests that the respective pathway is of great physiological and environmental importance to these planctomycetes. It has been proposed that the sole role of these genes within the *Planctomycetes* is the detoxification of formaldehyde (Chistoserdova et al. 2004). However, to have this pathway with its many steps solely as a means for formaldehyde detoxification seems to be rather complicated, especially since *R. baltica* SH 1^T, *B. marina* DSM 3645^T, *P. maris* DSM 8797^T and *G. obscuriglobus* UQM 2245^T contain glutathione-dependent formaldehyde dehydrogenases and *R. baltica* SH 1^T also contains a glutathione-independent formaldehyde dehydrogenase that can decompose formaldehyde in a much simpler pathway (Goenrich et al. 2002). Therefore, it seems likely that these C1 metabolism genes in *Planctomycetes* play an important role in an as yet unknown context. The presence of C1 metabolism genes in the fosmids from the Nambian upwelling region

indicates that this pathway is of importance in the suboxic waters of this highly productive region. The presence of non-anammox plancomycetes in this region further implies that these planctomycetes can cope with low oxygen concentrations. This is also backed up by the fact that all of the investigated planctomycete genomes with the exception of *Candidatus* K. stuttgartiensis harbour typical fermentation genes like acetate/butyrate kinase or phosphoketolase. Nonetheless, these planctomycetes are considered as obligate aerobes since they could not be cultivated under anoxic conditions so far.

All in all, the sulfatase-rich planctomycetes seem to be well-adapted to the nutrient-rich conditions in marine upwelling systems and are also capable to thrive under oxygen limiting conditions which can occur seasonally (Oregon upwelling system) or more permanently (OMZ of the Namibian upwelling system).

Features that set *Candidatus* K. stuttgartiensis and the other *Planctomycetes* apart

One striking result of our comparative genomics approach is the distinctness of *Candidatus* K. stuttgartiensis. It is almost completely devoid of genes that are shared with all other four investigated almost fully sequenced planctomycetes. In particular, the large paralogous gene families with otherwise planctomycete-specific domains that are even present on the small fosmids in this study are notably absent from *Candidatus* K. stuttgartiensis. Even when the E-value threshold is lowered to E-10, there are only eleven such genes. Likewise, when searched for planctomycete-specific genes that are not present in all but shared by at least two planctomycetes, there are only very few of these genes in *Candidatus* K. stuttgartiensis (Fig. 3). The same applies to the C1 carbon metabolism genes, that are absent from *Candidatus* K. stuttgartiensis but present in the four other almost fully sequenced planctomycetes and also in the fosmids. As a consequence, the anammox bacteria have likely diverged at a very early stage from the last common planctomycete ancestor. The most parsimonious assumption is, that his must have been before the radiation of planctomycete genera (*Pirellula*, *Blastopirellula*, *Rhodopirellula*, *Planctomyces*, *Gemmata* and *Isosphaera*), when the planctomycete-specific domains had not yet evolved and - if acquired by LGT - before the C1 metabolism genes were transferred. This is in agreement with phylogenetic studies based on 16S rRNA sequences, according to which anammox bacteria are deep branching within the *Planctomycetes* (Strous et al. 1999). However, the extent of the distinctness of *Candidatus* K. stuttgartiensis is surprising. If the distinctness of the anammox bacteria is confirmed in the future by other

anammox bacteria sequencing projects, one might consider whether a placement of the anammox bacteria within a separate phylum in the PVC superphylum is more appropriate.

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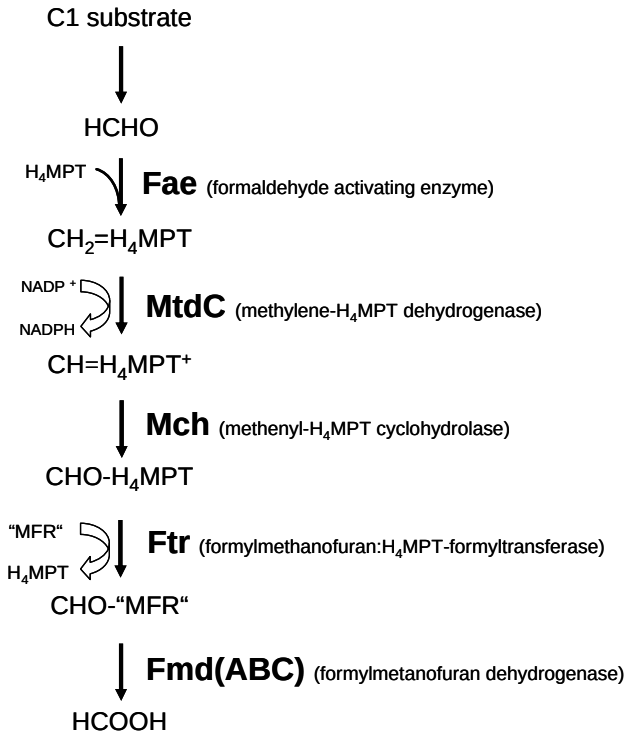
Supplemental Figures

Suppl. Fig. 1: World map showing the sampling sites for the planctomycete fosmids and cultured strains investigated in this study.



Suppl. Fig. 2: Scheme of H_4MPT-dependent C1 carbon compound conversion steps

Supplemental Table 1: Genes involved in H_4MPT – C1 pathway and the function of their encoded proteins

H_4MPT – pathway gene	**Function**
ORF5	Tetrahydromethanopterin:alpha-L-glutamate ligase (H_4MPT:alpha-L-glutamate ligase); Catalyzes the ATP or GTP-dependent addition of one L-glutamate molecule to tetrahydromethanopterin, producing tetrahydrosarcinapterin
ORF7	Kinase that participates in the biosynthesis of a prosthetic group of citrate lyase
ORF9	Protein belonging to a family that includes hydantoinase and oxoprolinase, both reactions involving the hydrolysis of five-membered rings via hydrolysis of their internal imide bonds
ORF17	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase related protein
ORF19	Protein containing DUF447, unknown function
ORF20	Dihydropterin synthesis protein
ORF21	Amino acid kinase
ORF22	Protein containing DUF556, unknown function
ORFY	ATP-dependent carboligase related to biotin carboxylase; may be involved in cofactor biosynthesis or regulation of the pathway
fae	Formaldehyde activating enzyme
ftr[1]	Tetrahydromethanopterin formyltransferase
mch	Methenyltetrahydromethanopterin cyclohydrolase
mptG	beta-ribofuranosylaminobenzene 5-prime-phosphate synthase
mtdC	Methylene tetrahydrofolate/methylene tetrahydromethanopterin dehydrogenase-like protein; ortholog of bacterial enzymes MtdA and MtdB with higher sequence similarity to MtdA enzymes, but homologous to the function of MtdB
fmdABC[1]	Formylmethanofuran dehydrogenase
ORF1	Conserved hypothetical protein containing DUF201, unknown function
pabAB within appropriate genomic context	Biosynthesis of para-aminobenzoic acid, a precursor of H_4MPT
ptpS within appropriate genomic context	6-pyruvoyl tetrahydrobiopterin synthase

[1] Ftr together with the three subunits of Fmd forms the formyltransferase/hydrolase complex (fhc)

Supplemental Table 2: Function of all ORFs on fosmid 3FN after manual annotation

orf	size in aa	annotated function
3FN_1	694	Hypothetical protein
3FN_2	466	Conserved hypothetical protein
3FN_3	389	Choloromuconate cycloisomerase
3FN_4	439	Oxidoreductase family protein
3FN_5	403	4-hydroxy-3-methylbut-2-3n-1-yl diphosphate synthase
3FN_6	584	Protein containing TPR repeats
3FN_7	269	Phosphinothricin N-acetyltransferase
3FN_8	408	Lipase/esterase
3FN_9	465	Serine/threonine protein kinase, secreted
3FN_10	510	Pectate lyase family protein
3FN_11	132	Hypothetical protein - remotely similar to RNA polymerase sigma factor Y, N-terminus
3FN_12	283	Secreted protein - remotely similar to RNA polymerase sigma factor Y, C-terminus
3FN_13	266	Protein containing DUF1080, possible secreted glycosyl hydrolase
3FN_14	294	Transciptional regulator, GntR family
3FN_15	834	Protein containing terpenoid cylases/protein prenyltransferase alpha-alpha toroid domain, membrane
3FN_16	150	Biopolymer transport protein ExbD/TolR
3FN_17	514	Imidazolonepropionase or related amidohydrolase
3FN_18	716	Sucrose-6-phosphate hydrolase, secreted
3FN_19	126	Hypothetical protein
3FN_20	124	ORF16-lacZ fusion protein
3FN_21	120	Hypothetical protein
3FN_22	416	Alanine racemase
3FN_23	399	DNA-directed DNA polymerase IV
3FN_24	746	Protein containing DUF1549 and DUF1553
3FN_25	232	RNA polymerase sigma-E factor
3FN_26	427	Protein containing DUF1501
3FN_27	593	Protein containing DUF1553 and DUF1549
3FN_28	230	Conserved hypothetical protein

Supplemental Table 3: Function of all ORFs on fosmid 6FN after manual annotation

orf	size in aa	predicted function
6FN_1	340	Tetracycline-efflux transporter/ tetracycline resistance protein
6FN_2	625	Transporter for Na-K-Cl or cationic amino acids
6FN_3	315	Auxin eflux carrier - putative malate permease
6FN_4	602	Quinonprotein alcohol dehydrogenase-like
6FN_5	1409	Conserved hypothetical protein
6FN_6	569	Arylsulfatase**,** secreted
6FN_7	282	Molybdenum or tungsten-containing formylmethanofuran dehydrogenase 1, subunit C
6FN_8	232	Molybdopterin-guanine dinucleotide biosynthesis protein A
6FN_9	737	Glycogen or alpha-glucan phosphorylase
6FN_10	363	Acyl-CoA dehydrogenase family protein
6FN_11	226	Adenylylsulfate kinase
6FN_12	447	Hypothetical protein
6FN_13	186	Hypothetical protein
6FN_14	93	Hypothetical protein
6FN_15	350	Lipase/esterase
6FN_16	126	Hypothetical protein
6FN_17	92	Hypothetical protein
6FN_18	218	Protein containing DUF84
6FN_19	130	Conserved hypothetical protein
6FN_20	462	Protein containing DUF1501
6FN_21	852	Protein containing planctomycete cytochrome c domain, DUF1549 and DUF1553
6FN_22	140	Hypothetical protein, secreted
6FN_23	274	Lipoate-protein ligase A
6FN_24	278	Hypothetical protein, membrane
6FN_25	787	Protein containing DUF1355, membrane
6FN_26	805	Conserved hypothetical protein, DUF1550
6FN_27	321	Protein containing DUF58
6FN_28	346	Methanol dehydrogenase regulator (MoxR) homolog

Supplemental Table 4: Function of all ORFs on fosmid 8FN after manual annotation

orf	size in aa	predicted function
8FN_1	122	Pyridoxal phosphate biosynthetic protein PdxJ (partial)
8FN_2	332	GHMP kinase (mptG)
8FN_3	225	Protein containing DUF447 (ORF 19)
8FN_4	238	Para-aminobenzoate synthase glutamine amidotransferase component II or Anthranilate synthase component II
8FN_5	504	Para-aminobenzoate synthase component I or anthranilate synthase component I
8FN_6	474	Dihydropteroate synthase (ORF 20)
8FN_7	400	N-acetylglucosamine-6-phosphate deacetylase
8FN_8	497	Protein containing DUF1501
8FN_9	461	Conserved hypothetical protein
8FN_10	263	Two-component system response regulator
8FN_11	171	Protein of unknown function UPF0079
8FN_12	121	Hypothetical protein
8FN_13	270	30S ribosomal protein S2
8FN_14	233	Elongation factor Ts
8FN_15	271	(Aspartate/glutamate/) uridylate kinase
8FN_16	201	Ribosome recycling factor
8FN_17	107	Hypothetical protein
8FN_18	142	Hypothetical protein, membrane
8FN_19	181	Hypothetical protein (secreted?)
8FN_20	375	Oxidoreductase, GFO/IDH/MocA family
8FN_21	254	DctM-like transporter
8FN_22	665	Dihydroxy-acid and 6-phosphogluconate dehydratase
8FN_23	387	Protein containing DUF1559, membrane
8FN_24	280	Haloacid dehalogenase-like hydrolase family protein
8FN_25	480	Conserved hypothetical protein
8FN_26	95	Hypothetical protein
8FN_27	278	Conserved hypothetical protein
8FN_28	110	Hypothetical protein
8FN_29	271	Conserved hypothetical protein containing DUF344
8FN_30	419	Protein containing prenyltransferase domains, secreted
8FN_31	259	GGDEF family protein, membrane
8FN_32	222	HAD-superfamily hydrolase
8FN_33	739	Protein kinase superfamily protein, membrane

Supplemental Table 5: Function of all ORFs on fosmid 13FN after manual annotation

orf	size in aa	predicted function
13FN_1	427	Molybdopterin oxidoreductase, membrane subunit
13FN_2	1082	Molybdopterin oxidoreductase, iron-sulfur binding subunit
13FN_3	236	Chaperone protein htpG
13FN_4	260	Succinate dehydrogenase iron-sulfur protein
13FN_5	642	Succinate dehydrogenase, flavoprotein subunit,
13FN_6	325	Succinate dehydrogenase cytochrome b558 subunit, membrane
13FN_7	392	RNA polymerase sigma factor (Major vegetative sigma factor, Sigma-A)
13FN_8	100	Hypothetical protein
13FN_9	129	Hypothetical protein
13FN_10	120	Hypothetical protein
13FN_11	337	Protein containing fasciclin domains, secreted
13FN_12	229	GTP cyclohydrolase I
13FN_13	188	6-pyruvoyl-tetrahydropterin synthase family protein
13FN_14	190	Cytidine and deoxycytidylate deaminase family protein
13FN_15	283	DNA glycosylase or AP lyase
13FN_16	1007	ATP-dependent DNA helicase, C-terminal part
13FN_17	522	ATP-dependent DNA helicase, N-terminal part
13FN_18	530	Deoxyribodipyrimidine photolyase-related family protein
13FN_19	305	Conserved hypothetical protein, membrane
13FN_20	490	Conserved hypothetical protein, membrane
13FN_21	127	Transposase
13FN_22	314	Transposase
13FN_23	359	Protein containing fasciclin domains
13FN_24	105	Hypothetical protein
13FN_25	222	SOUL heme-binding protein
13FN_26	340	UDP-glucuronic acid epimerase
13FN_27	140	Hypothetical protein, membrane
13FN_28	324	dTDP-glucose 4,6-dehydratase
13FN_29	624	Glutamyl-tRNA synthetase (Glutamate-tRNA ligase, GluRS)
13FN_30	267	Short-chain dehydrogenase/reductase family protein
13FN_31	293	Conserved hypothetical protein

Supplemental Table 6: Function of all ORFs on fosmid 5H12 after manual annotation

orf	size in aa	predicted function
5H12_1	174	Hypothetical protein
5H12_2	362	Conserved hypothetical protein, containing DUF1598
5H12_3	246	4-diphosphocytidyl-2C-methyl-D-erythritol synthase
5H12_4	636	Conserved hypothetical protein, membrane
5H12_5	78	Hypothetical protein
5H12_6	425	Hypothetical protein, secreted
5H12_7	914	Conserved hypothetical protein, secreted
5H12_8	465	Conserved hypothetical protein
5H12_9	441	Conserved hypothetical protein, containing DUF1501
5H12_10	578	Conserved hypothetical protein, containing DUF1553
5H12_11	464	Conserved hypothetical protein, membrane
5H12_12	434	Molybdopterin binding domain protein
5H12_13	608	Arylsulfatase precursor, secreted
5H12_14	955	DNA translocase ftsK (partial), membrane, secreted
5H12_15	403	Riboflavin biosynthesis protein ribAB (GTP cyclohydrolase-2 and 3,4-dihydroxy-2-butanone 4-phosphate synthase)
5H12_16	350	Lipase/esterase, secreted
5H12_17	282	Conserved hypothetical protein
5H12_18	234	Ribulose-phosphate 3-epimerase
5H12_19	203	Phosphoglycerate mutase family protein
5H12_20	366	Glycosyl transferase family 9 (heptosyltransferase) protein
5H12_21	78	Hypothetical protein
5H12_22	477	ATP-dependent hsl protease ATP-binding subunit hslU
5H12_23	206	ATP-dependent protease hslV
5H12_24	202	Isochorismatase hydrolase family protein
5H12_25	73	Conserved hypothetical protein, membrane
5H12_26	208	Conserved hypothetical protein, membrane
5H12_27	239	Conserved hypothetical protein, containing DUF558
5H12_28	194	Conserved hypothetical protein
5H12_29	227	Conserved hypothetical protein
5H12_30	531	Amino acid permease family protein, membrane
5H12_31	280	Ribonuclease D (partial)

Supplemental Table 7: Function of all ORFs on fosmid 6N14 after manual annotation

orf	size in aa	predicted function
6N14_1	308	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase
6N14_2	209	Protein containing a response regulator receiver domain
6N14_3	564	NAD-dependent malic enzyme
6N14_4	421	Conserved hypothetical protein
6N14_5	480	Cytochrome b/b6, membrane
6N14_6	174	Cytochrome b6-f ccomplex iron-sulfur protein
6N14_7	104	Hypothetical protein
6N14_8	300	Conserved hypothetical protein, membrane
6N14_9	754	Cytochrome c oxidase, subunit I, membrane
6N14_10	324	Cytochrome c oxidase, subunit II (precursor), membrane
6N14_11	576	Conserved hypothetical protein
6N14_12	100	Hypothetical protein
6N14_13	288	Conserved hypothetical protein, containing HD domain
6N14_14	88	Hypothetical protein
6N14_15	65	Hypothetical protein
6N14_16	229	Conserved hypothetical protein, containing DUF124
6N14_17	77	Hypothetical protein
6N14_18	658	Conserved hypothetical protein, membrane
6N14_19	229	Conserved hypothetical protein
6N14_20	373	3-dehydroquinate synthase
6N14_21	410	Conserved hypothetical protein, secreted
6N14_22	283	Phosphoadenosine phosphosulfate reductase
6N14_23	197	Transcriptional regulator family protein
6N14_24	818	60 kDa inner membrane insertion protein, membrane
6N14_25	1266	Soluble quinoprotein glucose dehydrogenase, secreted
6N14_26	84	Hypothetical protein
6N14_27	348	Conserved hypothetical protein, putative SAM-depedent methyltransferase
6N14_28	465	Amidohydrolase 2 family protein
6N14_29	801	Serine/threonine protein kinase family protein
6N14_30	352	D-isomer specific 2-hydroxyacid dehydrogenase family containing the catalytic and NAD binding domains
6N14_31	579	Altronate hydrolase or D-galactarate dehydratase
6N14_32	129	hypothetical protein

List of Publications

1. **Dagmar Woebken**, **Bernhard M. Fuchs, Marcel M. M. Kuypers, Rudolf Amann.** 2007. Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Submitted*.

2. **Dagmar Woebken, Phyllis Lam, Bernhard M. Fuchs, Marcel M. M. Kuypers, S. Wajih A. Naqvi, Boran Kartal, Marc Strous, Mike. S. M. Jetten and Rudolf Amann.** 2007. Microdiversity study of marine anammox bacteria reveals novel *Candidatus* Scalindua type in the Arabian Sea. *Manuscript in preparation*.

3. **Dagmar Woebken, Hanno Teeling, Alexandra Dumitriu, Ivaylo Kostadinov, Rudolf Amann and Frank Oliver Glöckner.** 2007. Fosmids of novel marine *Planctomycetes* from the Namibian and Oregon coast upwelling systems and their cross-comparison with one complete and four almost complete planctomycete genomes. *Manuscript in preparation*.

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