

Potential Interactions of Particle-Associated Anammox Bacteria with Bacterial and Archaeal Partners in the Namibian Upwelling System[∇]

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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 slightly but significantly ($P < 0.01$) enriched in *Gammaproteobacteria* (11.8% \pm 5.0%) compared to the free-water phase (8.1% \pm 1.8%). No preference for the attachment to particles could be observed for members of the *Alphaproteobacteria* and *Bacteroidetes*, which were abundant (12 to 17%) in both habitats. The alphaproteobacterial SAR11 clade, the *Euryarchaeota*, and group I *Crenarchaeota*, were all significantly depleted in particles compared to their presence in the free-water phase (16.5% \pm 3.5% versus 2.6% \pm 1.7%, 2.7% \pm 1.9% versus <1%, and 14.9% \pm 4.6% versus 2.2% \pm 1.8%, respectively, all $P < 0.001$). 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 consortium-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.

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 is lost yearly in the world’s oceans (9). 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 (31).

Upwelling systems are ecosystems with intense primary production in the surface waters. The decomposition of settling biomass through aerobic respiration in the water column (6, 7) leads to oxygen depletion in deeper water layers. In the Namibian upwelling system, the oxygen concentration is often less than 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) (49).

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 wastewater treatment plant (39, 65) and were affiliated with a separate cluster within

the order *Planctomycetales* (58). Subsequently, the process was also detected in sediments (13, 18, 50, 62, 64) and in stratified water bodies like Golfo Dulce (a coastal bay in Costa Rica) and the Black Sea (12, 32). Recently it was shown that anammox is mainly responsible for the loss of fixed nitrogen as N_2 from the Namibian (31) and Peruvian-Chilean upwelling systems (26) (63).

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 (59). In the Namibian OMZ, anammox activity could be detected in samples from water depths with oxygen concentrations up to 9 μM O_2 (31). Preliminary results indicated particle association of anammox bacteria. These particles were believed to provide anaerobic microenvironments for the anammox bacteria at low ambient oxygen concentrations of up to 25 μM oxygen (31).

The aims of this study were to further investigate the distribution and particle association of anammox bacteria in 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 cooccurring microbiota. Special emphasis was placed on comparing the relative abundances of cooccurring particle-associated groups and their abundance in the free-water phase

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and was placed 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 (stations M179 at 23.17°S, 14.32°E; M182 at 23.17°S, 14.08°E; M199 at 22.0°S, 13.86°E; and M202 at 22.64°S, 14.30°E and a transect along 23.0°S from 14.36°E to 12.0°E with stations M164/M184 at 14.36°E, M165/M185 at 14.32°E, and M166/M186 at 14.22°E) (68). For DNA isolation, approximately 50 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% [vol/vol]) for 1 h 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 (31).

Construction of 16S rRNA gene clone libraries. DNA was isolated from the polycarbonate filters according to the method of Massana et al. (37). We used standard PCR conditions and reduced the cycle number as much as possible to minimize PCR bias. The universal bacterial primers GM3F and GM4R (26 cycles) (41) and universal archaeal primers 20F and 1392R (34 cycles) (37, 57) were used to amplify almost full-length 16S rRNA genes from Namibian water samples. The PCR products were cleaned using the QIAquick PCR purification protocol (QIAGEN, Hilden, Germany), and their sizes were verified on agarose gels.

For the archaeal clone library, a preparative gel was necessary because only one of two observed PCR products had the correct size of $\sim 1,300$ bp. The gel-excised and cleaned PCR products were ligated into the vector pGEM-T Easy (Promega, Madison, WI) according to the manufacturer's protocol, and the vector was introduced into chemical competent *Escherichia 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, MA) and sequenced with the BigDye Terminator cycle sequencing kit, version 3.1 (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 (34), and phylogenetically analyzed.

In addition, a *Planctomycetales*-enriched 16S rRNA gene clone library was constructed from station M202 (52 m depth) in the Namibian OMZ with the primer Pla46F (43) and the universal primer 1392R (57). From the same sample, we constructed two anammox bacterium-enriched clone libraries, one with the general anammox primer Amx368F (52) and the universal primer 1392R and another clone library with primer Pla46F and the "*Candidatus* Scalindua"-specific primer 1309R (52).

FISH and CARD-FISH. Standard FISH identification of anammox bacteria with 16S rRNA-targeted oligonucleotide probes was carried out according to the method in reference 52.

For CARD-FISH, we followed the protocol of reference 44, including the lysozyme permeabilization with the following modifications. Because we detected strong endogenous peroxidase activity inside the anammox cells, the agarose-embedded cells were incubated with H_2O_2 solution (final concentration, 3% in Milli-Q 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 min 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. 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 Alexa₅₄₆ and with Alexa₄₈₈ (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 (145 mM NaCl, 1.4 mM NaH_2PO_4 , 8 mM Na_2HPO_4 , [pH 7.6]) and once in Milli-Q water. Then the filters were incubated in 3% H_2O_2 for 10 min to inactivate the horseradish peroxidase attached to the first probe. Subsequently, the filters were washed twice in Milli-Q water, dehydrated in 96% ethanol, and dried at room temperature before the second hybridization was performed.

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 previously by Pernthaler et al. (44), pictures were taken with a black and white camera (AxioCam MRm; Carl Zeiss, Jena, Germany).

For the free-water phase, we counted a minimum of 2,000 DAPI-stained cells per sample and per probe (equivalent to approximately 20 fields of view) (45). The relative abundance was determined as a percentage of DAPI-stained cells. Eight stations were investigated for the quantification of different phylogenetic groups in the Namibian upwelling system with up to 14 probes (M164, M165, M166, M179, M182, M184, M185, and M186). Per station, two to four depths representing the same body of water were counted with each probe. The mean value obtained from each of the examined stations was used for subsequent statistical analyses. Therein, the number of observations was equivalent with the examined number of stations. For particle-attached bacteria, we scored a minimum 20 particles per sample per probe from station M199, which equaled about 2,000 DAPI-stained cells. All values are given as means with standard deviations.

Statistical analysis. For statistical analyses, the normality of the data was checked. If data were normally distributed, the parametric *t* test was applied; if not, the nonparametric Mann-Whitney U test was used.

Nucleotide sequence accession numbers. Sequences are published in GenBank under accession numbers EF645838 to EF646149.

RESULTS AND DISCUSSION

Particle association of anammox bacteria. The particle association of anammox bacteria has been used to explain the discrepancy observed between the known oxygen sensitivity of anammox bacteria and the occurrence of these bacteria in the Namibian OMZ, where anammox activity was detected in waters with oxygen concentrations of up to 9 μM and ammonium concentrations below the detection limit (31). This study focuses on the visualization of particle-associated anammox bacteria and their potential interactions with other microbial populations.

We observed a high density of macroscopic particles in the water column of the Namibian upwelling system during sampling by camera imaging from a remotely operated vehicle (68). The particles contributed to high turbidity in the Namibian upwelling (31). In an earlier study, Kiørboe counted 100 to 640 macroaggregates (>0.45 mm) per liter of seawater further south in the Benguela upwelling off the coast of South Africa (29). These numbers are among the highest concentrations reported for the marine environments (29, 55). However, the sampling with a pumpcast system or with go-flow bottles, fixation with paraformaldehyde, and filtration largely disintegrated the fragile aggregates, resulting in quite even distribution of single cells and particles of a size of up to 30 μm on the polycarbonate filters.

The vertical profiles of anammox bacteria at stations M182 and M202 detected by standard FISH were published previously (31) and show significant correlations with the anammox rates measured at both stations ($R = 0.82$; $P < 0.01$). During the present study, we found that additionally, the anammox cell numbers at stations M182 and M202 significantly correlate 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.

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

Probe	Specificity	Sequence (5' → 3')	<i>Escherichia coli</i> position no. ^a	% FA ^b	Reference(s)
EUB338-I-III	<i>Bacteria</i>	GCWGCCWCCCGTAG GWGT	338–355	55	1, 11
NON338	Negative control	ACTCCTACGGGAGG CAGC	338–355	55	66
CREN554	<i>Crenarchaeota</i> group I	TTAGGCCCAATAATCM TCCT	554–573	20	37
EURY806	<i>Euryarchaeota</i>	CACAGCGTTTACACCTAG	806–823	20	61
BS-820/BS-820-C	“ <i>Candidatus</i> Scalindua sorokinii” and “ <i>Candidatus</i> Scalindua brodae”	TAATYCCCTCTACTTAGT GCCC	820–841	40	26, 32
AMX-820 ^c	“ <i>Candidatus</i> Kuenenia stuttgartiensis”	AAAACCCCTCTACTTAGT GCCC	820–841	40	51
ALF968	Most <i>Alphaproteobacteria</i>	GGTAAGGTTCTGCGCGTT	968–986	55	42
SAR11/441	SAR11 clade	TACAGTCATTTTCTTCCC CGAC	486–503	45	47
ROS537	<i>Roseobacter</i> clade	CAACGCTAACCCCTCC	537–553	55	17
GAM42a ^c	Most <i>Gammaproteobacteria</i>	GCCTTCCCACATCGTTT	1027–1043 ^d	55	36
SAR86/1245	SAR86 clade	TTAGCGTCCGTCTGTAT	1245–1262	55	69
NOR5/730	NOR5/OM60 clade	TCGAGCCAGGAGGC CGCC	730–747	50	17
PSA184	<i>Pseudoalteromonas</i> , <i>Colwellia</i>	CCCCTTTGGTCCGTAGAC	184–210	50	16
ALT1413	<i>Alteromonas/Colwellia</i>	TTTGCATCCCCTCCCAT	1413–1430	60	16
Nscoc128	<i>Nitrosococcus oceani</i> , <i>N. halophilus</i> (<i>Gammaproteobacteria</i>)	CCCCTCTAGAGGCCAGAT	128–146	35 ^f	28, 33
NSO1225	Ammonia-oxidizing <i>Betaproteobacteria</i>	CGCCATTGTATTACGT GTGA	1225–1244	35 ^f	38
NSO190	Ammonia-oxidizing <i>Betaproteobacteria</i>	CGATCCCCTGCTTTTCTCC	190–208	55 ^f	38
CF319a	<i>Cytophaga/Flavobacterium</i>	TGGTCCGTGTCTCAGTAC	319–336	55	35
SAR406-97	SAR406 clade	CACCCGTTCCGCCAGTTTA	97–114	65	20

^a Probe target position on *E. coli* 16S rRNA according to reference 5.

^b Formamide concentration (vol/vol) in CARD-FISH hybridization buffer at 35°C.

^c Includes an unlabeled competitor probe BET42a (5'-GCCTTCCCCTCGTTT-3'). For details, see reference 36.

^d Probe target position on *E. coli* 23S rRNA according to reference 5.

^e Probe AMX-820 used as competitor in hybridizations with probes BS-820 and BS-820-C.

^f Formamide (FA) concentration for hybridization at 46°C.

Standard FISH allowed accurate quantification of single anammox bacteria. However, the signal intensity of anammox bacteria stained with standard FISH was rather low and the 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 to 20 and sometimes even more cells (Fig. 1B).

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

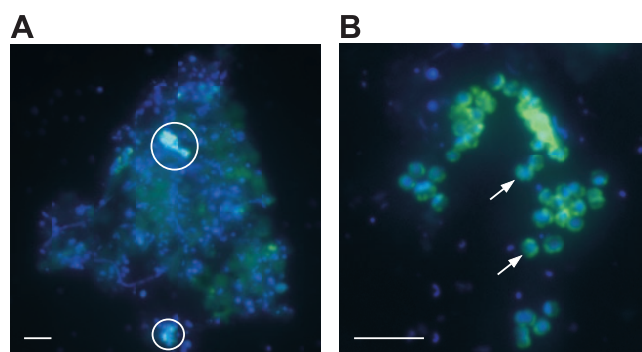


FIG. 1. Combined epifluorescence micrographs from samples of the Namibian OMZ. (A) Anammox cells (circles) in association with particles. The blue DAPI signals indicate DNA-containing cells. The green signals are derived from fluorescein-labeled tyramide (green) after CARD-FISH with the anammox-specific probe BS-820. Turquoise cells represent anammox bacteria stained by both dyes. (B) Individual anammox cells (arrows) forming clusters. Because of internal compartmentalization, single anammox cells at higher microscopic magnifications do not appear uniformly in turquoise. Instead, parts of the cells containing the nucleoid appear only in blue and those containing the riboplasm appear only in green. Nonanammox cells are stained with only DAPI and appear blue. Bar = 5 μm .

counts of anammox bacteria were consistently lower than those of standard FISH. For activated sludge aggregates, Schönhuber et al. previously reported a similar phenomenon (53). CARD-FISH stained only half of the cells which were stained with standard FISH (53). This is likely caused by the larger horseradish peroxidase label used for CARD-FISH (~44 kDa) compared to that for 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 (2). 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.

With the improved CARD-FISH 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 (for station M202, 42, 47, 52, 57, 62, and 68 m; for station M199, 60, 65, 70, 75, 80, and 87 m) in triplicate. We found that $56.2\% \pm 8.3\%$ of the anammox cells stained with CARD-FISH were organized in clusters but not attached to particles (Fig. 1B), $24.5\% \pm 8.4\%$ were associated with particles (either as single cells or as clusters of anammox cells; Fig. 1A), and $19.3\% \pm 10.8\%$ were single cells not organized in clusters or attached to particles. Single anammox cells could have been detached from particles during sampling. Considering the relatively high shear forces occurring during pumpcast sampling as well as during sampling with go-flow bottles, we speculate that anammox bacteria formed clusters that 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, provide only 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 possible only by scuba diving or with specifically designed devices for catching particles.

Spatial distribution of anammox bacteria along the 23°S transect on the Namibian shelf. Our study focused on the particle association of anammox bacteria in the Namibian upwelling system. As described above, CARD-FISH allows confident detection of probe-positive cells within particles and the analysis of numerous samples. 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 ($\sim 2,300$ cells ml^{-1}) was found close to the coast at 14.32° E (Fig. 2b), which perfectly matches the zone of anammox activity (31). Absolute anammox cell numbers are most likely underestimated, because CARD-FISH does not detect all anammox cells (see above). In waters containing anammox bacteria, the oxygen concentrations ranged from 30 μM oxygen in upper waters to oxygen concentrations below detection limit (<1 μM) above 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 result could indicate that anammox cells were resuspended, together with particles from the sediment, and that resuspension of particles containing anammox bacteria might play a major role in this ecosystem. 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.

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 southwestern African shelf and slope (27). 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 than 9 μM . However, the association with oxygen-depleted microniches probably allows the anammox bacteria to maintain activity at oxygen concentrations as high as 25 μM (46), significantly expanding the zone of potential nitrogen loss due to anammox in the Namibian OMZ.

Microbial diversity in the Namibian upwelling system. The presence of particles would allow 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 planctomycete-enriched library, and two anammox-enriched libraries from station M202. The latter three were from a 52-m depth where anammox activity was detected (31).

The two bacterial libraries from 119-m and 130-m depths at station 182 are treated as one below, because they originate from the same water mass according to physical and chemical parameters. A total of 235 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* (96/235). Many of the sequences clustered into two groups of uncultured *Gammaproteobacteria*. The first cluster (19/235) 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 the North Sea after an algal bloom (AJ400348). This cluster of sequences also showed 90 to 96% sequence identity with chemoautotrophic clam gill symbionts (*Calyptogenia* spp. and *Vesicomya lepta*; AF035722, AF035723, AY310507, and AF035727). The second group of uncultured *Gammaproteobacteria* (49/235) 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); the genus *Vibrio* (5); the *Alteromonadales*

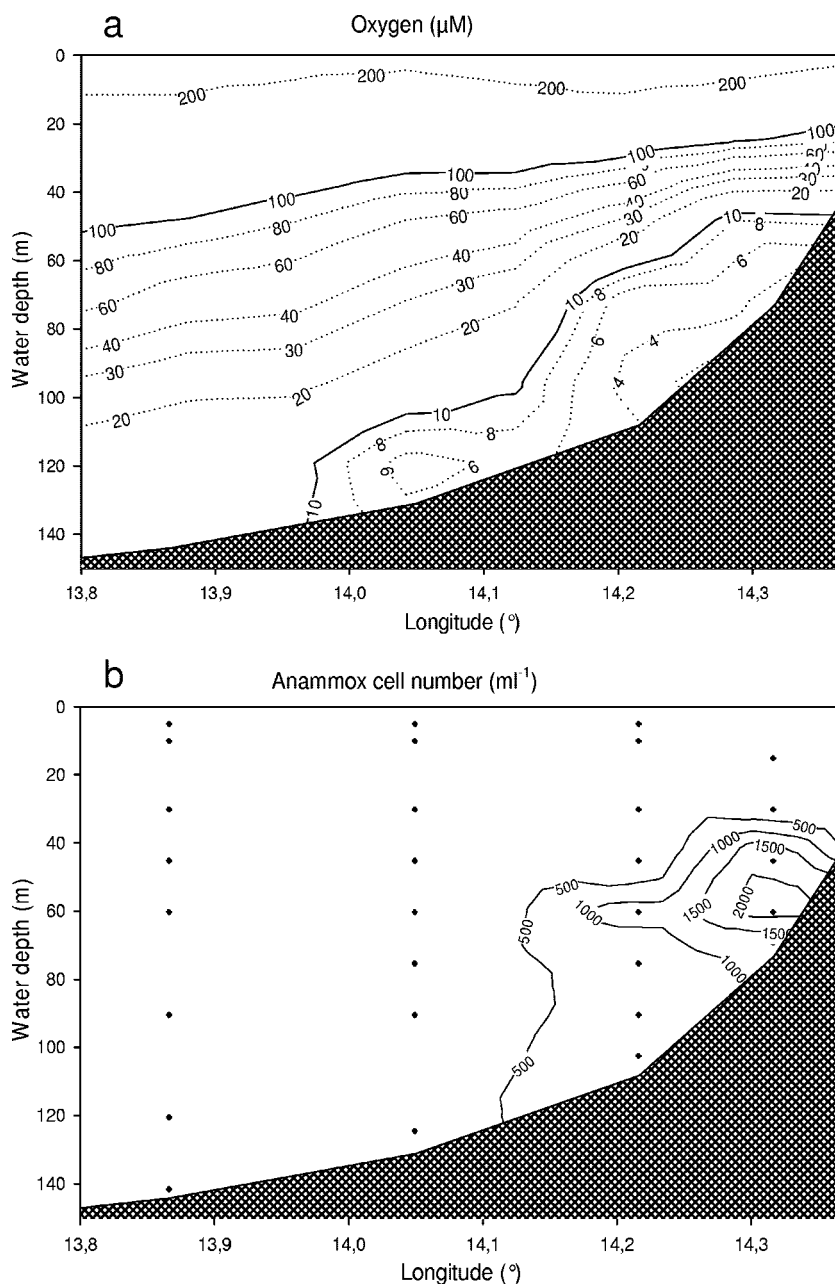


FIG. 2. (a) Oxygen concentration (μM) over the Namibian shelf along the 23°S transect on 22 to 23 March 2003. Note that oxygen concentrations are less than $10 \mu\text{M}$ above the sediment from approximately 13.95°E to 14.35°E . (b) Distribution of CARD-FISH-stained anammox cells per milliliter of seawater along the 23°S transect 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.

(4); the *Oceanospirillaceae* (3); the SAR86 cluster (3); and the genera *Cowellia* (2), *Kangiella* (2), and *Psychromonas* (1).

The next dominant groups were sequences related to the *Alphaproteobacteria* (57/235) and the *Bacteroidetes* (43/235). Most of the sequences belonging to the *Alphaproteobacteria* were affiliated with the SAR11 cluster (43/235). About two-thirds of the sequences within the *Bacteroidetes* showed highest sequence similarities to members of the class *Flavobacteria*, about one-third showed highest sequence similarities to uncultured clades of *Bacteroidetes*, and a single sequence

showed highest sequence similarity to *Flavobacterium johnsoniae*. Some sequences had very high sequence identity (up to 100%) with sequences belonging to the AGG58 cluster (L10946) (14).

In addition, sequences were affiliated with the *Deltaproteobacteria* (11/235), including the SAR324 cluster (7/235), *Betaproteobacteria* (6/235), *Nitrospina* (10/235), *Actinobacteria* (4/235), and the SAR406 cluster (3/235). Furthermore, we retrieved two planctomycete-related sequences having up to 88% sequence identity with a sequence from a particle-associ-

ated uncultured bacterium (L10943) (14). Single sequences grouped within the *Epsilonproteobacteria* and *Spirochaeta*.

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 a 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*" (30).

The apparent particle association of anammox bacteria opens the possibility for specific interactions of anammox bacteria with other microorganisms in the particles. Macroscopic aggregates are known for their diverse microbiota (e.g., see references 14, 19, 24, and 48). 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*, *Gamma-proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, and the SAR406 cluster.

Within the more than 200 bacterial sequences, there were, on the one hand, sequences of phylogenetic groups also found in fully oxygenated waters, such as SAR11 (21), SAR86 (40), SAR92 (4), and members of the SAR406 cluster (23). This could be due to the 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 abilities to associate with particles. The sequences which showed up to 100% sequence identity with members of the cluster AGG58 are an example. The principal member of this cluster (clone env.agg58) was amplified from particles in the Santa Barbara Channel in California (14). However, due to potential biases during PCR amplification of the 16S rRNA genes (e.g., see reference 60), clone frequencies in our libraries were not used to infer cellular abundances in the water column.

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 an analysis of the free-water phase, we hybridized samples from water masses containing less than 10 μM oxygen from eight different stations along the Namibian coast. From similar water layers, the results of up to four depths per station were combined. To determine the relative abundances of the microbial flora cooccurring with anammox bacteria on particles, we hybridized filters of station M199 from suboxic to anoxic waters. Representative photomicrographs of double hybridizations

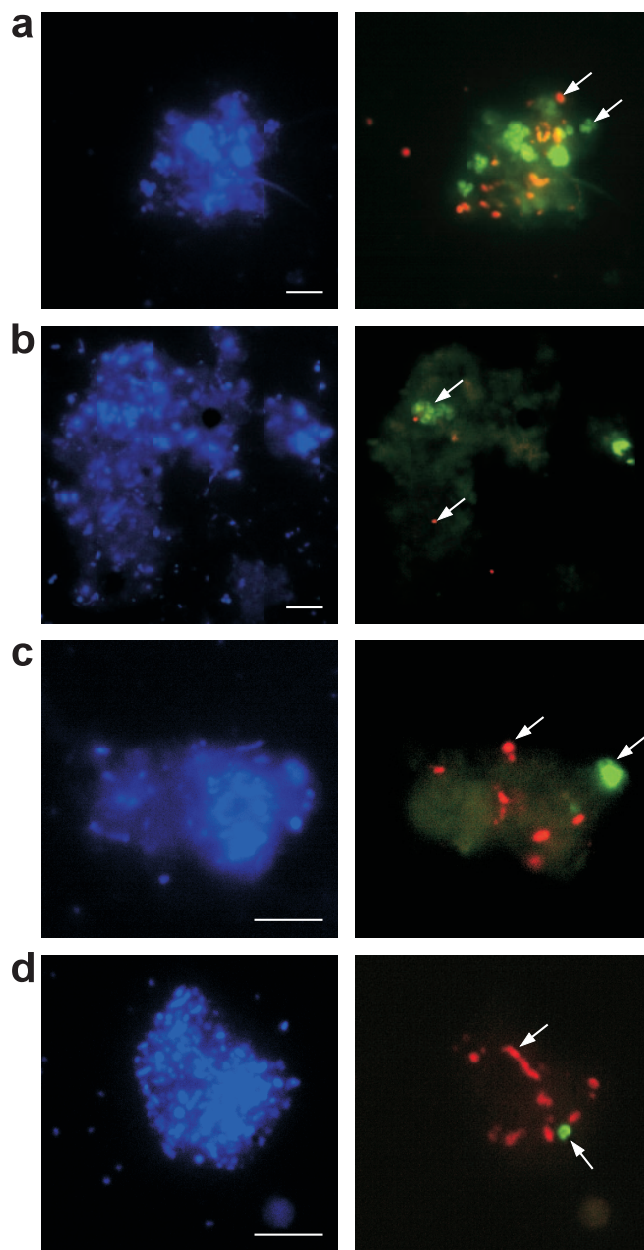


FIG. 3. Combined epifluorescence micrographs from particles in the Namibian and Peruvian OMZs stained with CARD-FISH. We conducted double hybridizations with the anammox-specific probe BS-820 (and a mixture of BS-820 and BS-820-C for samples from the Peruvian OMZ) and probes for other phylogenetic groups (panel a, Gam42a; panel b, Cren554; panels c and d, CF319a). The left panels show cells stained with DAPI in blue. In the right panels, anammox bacteria are shown in green, and other groups of bacteria are stained in red. Figure 3a to c show samples from the Namibian OMZ. Fig. 3d shows samples from the Peruvian OMZ. Bar = 5 μm . Some exemplary cells of both types are indicated by arrows.

with anammox-specific and other group-specific probes are shown in Fig. 3.

Overall detection efficiency by CARD-FISH from the free-water phase was high. With the *Bacteria*-specific probe mix EUB I-III, 72.7% \pm 14.3% of the cells in the Namibian OMZ were detected. The relative abundance of *Crenarchaeota* group

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^a

Phylogenetic group	Relative abundance (%)		<i>P</i>	No. of observations	
	Fwp	P		Fwp	P
<i>Alphaproteobacteria</i>	17.1 (±7.3)	11.6 (±4.8)	>0.05	4	28
SAR11 clade	16.5 (±3.5)	2.6 (±1.7)	<0.001	8	27
<i>Roseobacter</i> clade	3.1 (±2.2)	3.2 (±1.8)	>0.05	5	24
<i>Gammaproteobacteria</i>	8.1 (±1.8)	11.8 (±5.0)	<0.01	4	24
SAR86 clade	<1	<1			
NOR5/OM60 clade	<1	1.6 (±1.6)			
<i>Pseudoalteromonas</i> , <i>Colwellia</i>	<1	<1			
<i>Alteromonas/Colwellia</i>	1.1 (±1.3)	<1			
<i>Nitrosococcus oceani</i> , <i>N. halophilus</i>	ND	ND			
Ammonia-oxidizing <i>Betaproteobacteria</i>	ND	ND			
<i>Cytophaga/Flavobacterium</i>	13.7 (±6.4)	12.4 (±3.5)	>0.05	5	27
SAR406 clade	3.3 (±1.0)	4.0 (±2.2)	>0.05	4	21
<i>Crenarchaeota</i> group I	14.9 (±4.6)	2.2 (±1.8)	<0.001	7	23
<i>Euryarchaeota</i>	2.7 (±1.9)	<1	<0.001	7	20

^a Shown are relative abundances of different phylogenetic groups in the free-water phase (suboxic to anoxic waters) and occurring in particles in the Namibian upwelling system. ND, not detectable; Fwp, free-water phase; P, particles. Bold type indicates a significant difference in abundance. Please note that the number of observations in the free-water phase is equivalent to the number of stations investigated, where two to four depths per station were counted and the mean value was used for statistical analyses.

I is significantly higher ($P < 0.001$, Mann-Whitney U test) in the free-water phase ($14.9\% \pm 4.6\%$) than in particles ($2.2\% \pm 1.8\%$) (Table 2). The same applies for the *Euryarchaeota*, which account for $2.7\% \pm 1.9\%$ of all microorganisms in the free-water phase, whereas the abundance of *Euryarchaeota* 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 at high relative abundances. Statistical tests showed that the abundance of the *Cytophaga/Flavobacterium* was not significantly different in the free-water phase and in the particles ($13.7\% \pm 6.4\%$ and $12.4\% \pm 3.5\%$, respectively; $P > 0.05$, Mann-Whitney U test). Cells stained with probe ALF968 made up $17.1\% \pm 7.3\%$ of the cells in the free-water phase and $11.6\% \pm 4.8\%$ of particle-associated cells ($P > 0.05$, Mann-Whitney U test). Within the *Alphaproteobacteria*, the counts with probe ROS537 targeting the *Roseobacter* cluster were $3.1\% \pm 2.2\%$ in the free-water phase and $3.2\% \pm 1.8\%$ attached to particles, and those with probe SAR11-441 were $16.5\% \pm 3.5\%$ in the free-water phase and $2.6\% \pm 1.7\%$ 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).

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

The ammonia-oxidizing *Beta*- and *Gammaproteobacteria* were quantified by both 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 *Nitrosococcus halophilus*) (Table 1). No ammonia-oxidizing bacteria could be detected with either method.

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

Peruvian upwelling. The results from the Namibian OMZ led to the question of whether particle-associated anammox bacteria and their cooccurring 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*" (26). 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, and the SAR406 cluster. Similar to the Namibian OMZ, *Crenarchaeota*, members of the SAR11 cluster, and *Euryarchaeota* were found only rarely 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 give insight in the potential interactions between anammox bacteria and other microorganisms. Even though the observed aggregates are certainly not as tightly structured as, for example, the methane-oxidizing consortia found in microbial mats on methane hydrates (3), there may still exist discrete functional interactions.

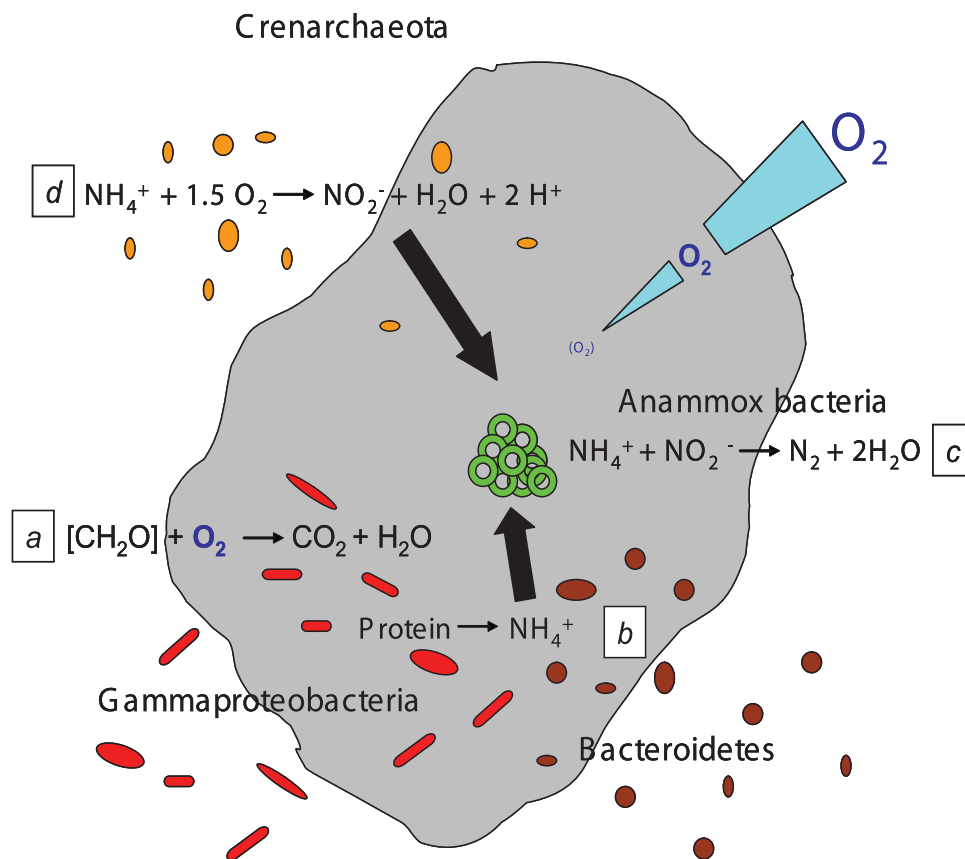


FIG. 4. A schematic view of a marine snow particle (gray) and associated microbial cells (brown, red, and orange shapes). Green circles indicate anammox bacteria. Potential microbial interactions on the particle in the water column are summarized (equations and arrows). Oxygen concentration (turquoise) decreases by respiration towards the center of the particle. We assume that the observed particles on filters were previously parts of larger particles in the water column. See text for details.

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 to levels suitable for the anammox reaction (Fig. 4a). It has been shown that microbial oxygen consumption can result in the development of anaerobic microenvironments in particles at ambient oxygen concentrations of up to 25 μM (46).

Furthermore, remineralization activity of microorganisms in particles can result in a significantly higher concentration of ammonium in macroscopic aggregates than in ambient seawater (54) (Fig. 4b). This elevated ammonium concentration is likely due to hydrolytic enzyme activity, which is significantly higher in particles than in the ambient seawater (56). Heterotrophic bacteria on particles express a whole array of hydrolytic enzymes. Interestingly, the protease activity is 10 to 100 times higher than the activity of polysaccharidase and alpha- and beta-glucosidase (56). 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* (10, 14, 15). Also, *Alpha*- and *Gammaproteobacteria* might be involved in the degradation of complex compounds (10). For some members of the latter group, it is known that they regulate their hydrolytic enzyme activity by quorum-sensing mechanisms (8, 22).

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 (31). However, by standard FISH and CARD-FISH, no ammonia-oxidizing bacteria were detected. This result 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 (30). Fosmid sequences of *Cenarchaeum symbiosum* showed potential for ammonium oxidation (25) in this crenarchaeon as well. Additionally, genes encoding the crenarchaeal ammonia monooxygenase α -subunit (*amoA*) were recovered from ocean waters worldwide (see references 25 and 67), 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 (31) 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* (67). While *Crenarchaeota*

account for ~15% of all microorganisms, anammox bacteria account for only ~1% of the microbial community in the Namibian OMZ waters (31). Hence, only a small fraction of the *Crenarchaeota* at site M182 would need to be aerobic ammonium oxidizers if we assume a cell-specific activity similar to that for the North Atlantic (~4 fmol per cell per day) (67) and provide the anammox bacteria with the necessary nitrite (Fig. 4c and d).

Future directions. Our hypothesis could be tested on 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 microsensors (46) to support our hypothesis of anoxic microniches. Preservation of the three-dimensional structure would allow CARD-FISH analysis of intact particles to prove the colocalization 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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