

Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean

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Nitrification, the microbial oxidation of ammonia to nitrite and nitrate, occurs in a wide variety of environments and plays a central role in the global nitrogen cycle. Catalyzed by the enzyme ammonia monooxygenase, the ability to oxidize ammonia was previously thought to be restricted to a few groups within the β - and γ -Proteobacteria. However, recent metagenomic studies have revealed the existence of unique ammonia monooxygenase α -subunit (*amoA*) genes derived from uncultivated, nonextremophilic Crenarchaeota. Here, we report molecular evidence for the widespread presence of ammonia-oxidizing archaea (AOA) in marine water columns and sediments. Using PCR primers designed to specifically target archaeal *amoA*, we find AOA to be pervasive in areas of the ocean that are critical for the global nitrogen cycle, including the base of the euphotic zone, suboxic water columns, and estuarine and coastal sediments. Diverse and distinct AOA communities are associated with each of these habitats, with little overlap between water columns and sediments. Within marine sediments, most AOA sequences are unique to individual sampling locations, whereas a small number of sequences are evidently cosmopolitan in distribution. Considering the abundance of non-extremophilic archaea in the ocean, our results suggest that AOA may play a significant, but previously unrecognized, role in the global nitrogen cycle.

Crenarchaeota | nitrification | ammonia monooxygenase

Nitrogen (N) is an essential nutrient in the ocean and limits biological productivity in most marine ecosystems. At the global scale, biological N fixation is the largest source of N to the ocean, whereas anaerobic microbial processes are responsible for N losses (1, 2). However, biological N fixation and anaerobic N losses are ultimately connected by nitrification, the microbially mediated, two-step conversion of ammonium (NH_4^+) to nitrate (NO_3^-) via nitrite (NO_2^-). As much as 4×10^{11} kg of N cycles through the ocean each year (1), and nearly all of this N must be nitrified at least once (3). Although nitrification is important throughout the ocean, it plays a critical role in the coastal ocean by linking the decomposition of nitrogenous organic matter to N loss via denitrification (microbial conversion of NO_3^- to N_2 gas). By removing a large percentage of anthropogenic N pollution from estuaries and continental shelf regions before it can reach the open ocean (4), coupled nitrification/denitrification effectively isolates the marine N cycle from the heavily altered terrestrial N cycle (1). In the open ocean, 30–50% of all N loss occurs in pelagic oxygen minimum zones (OMZs), where massive N losses have recently been attributed to anaerobic ammonium oxidation, or “anammox” (5). During anammox, oxidation of NH_4^+ occurs at the expense of NO_2^- produced by either heterotrophic NO_3^- reduction or aerobic ammonia oxidation, the first step of nitrification. Nitrification is therefore a particularly significant process in OMZs and at oxic/anoxic interfaces in coastal sediments, where the complex interplay between nitrification, denitrification, and anammox drives rapid N transformations and large N losses to the atmosphere.

Only a few phylogenetically restricted groups of microorganisms are known to perform either of the two steps of nitrification

(conversion of NH_4^+ to NO_2^- and NO_2^- to NO_3^-), all of which are members of the domain Bacteria. Because they catalyze the first and rate-limiting step of nitrification, ammonia-oxidizing bacteria (AOB) have received considerable attention in a wide variety of habitats, including soils, freshwater, marine water columns, estuaries, and sediments (6). Despite their critical biogeochemical role in both pelagic and benthic oceanic environments, AOB often comprise only 0.1% of bacterial assemblages (7). In contrast, microorganisms from the domain Archaea are ubiquitous and often abundant in the ocean (8–10), but their biogeochemical role remains unclear. Archaea represent the third domain of life, evolutionarily distinct from the Eukarya and Bacteria, and were previously thought to inhabit mostly extreme environments (11). Nonextremophilic archaea (Crenarchaeota and Euryarchaeota) are now recognized to be widespread in the ocean (12, 13), but our current understanding of their physiology and biogeochemical function remains largely speculative. Although some planktonic archaea may be heterotrophic because of their uptake of amino acids (14, 15), there is evidence that some marine archaea may be chemoautotrophs (16), capable of light-independent carbon (C) fixation (15, 17). Further insight into potential metabolic capabilities of planktonic archaea comes from recent work by Venter *et al.* (18), who investigated microbial genomic diversity in the Sargasso Sea by shotgun DNA sequencing. Based on their discovery of a unique ammonia monooxygenase gene on an archaeal-associated scaffold, Venter *et al.* suggest that some archaea may be capable of performing chemoautotrophic nitrification.

amoA encodes the catalytic α -subunit of ammonia monooxygenase, the enzyme responsible for catalyzing the rate-limiting step in bacterial ammonia oxidation. Because *amoA* is well conserved and required by all AOB, this gene has been used extensively as a molecular marker for cultivation-independent studies of AOB communities. The possibility that some archaea carry an *amoA* was confirmed by Schleper *et al.* (19), who found a Sargasso Sea-like *amoA* homolog on the same 43-kb metagenomic fragment as a 16S rRNA gene from soil Crenarchaeota, suggesting that archaea capable of ammonia oxidation may be present in soils. A definitive link between this novel *amoA* and archaeal ammonia oxidation was only recently established by cultivation of an ammonia-oxidizing member of the marine group 1 Crenarchaeota, the first cultivated representative from this dominant archaeal lineage (20). How widespread ammonia-oxidizing archaea (AOA) are in the marine environment, and their relative importance in the global N cycle, remain unknown.

In this study, we use unique PCR primers specifically targeting the archaeal *amoA* to document the distribution and diversity of AOA in water columns and sediments of the ocean.

Abbreviations: *amoA*, ammonia monooxygenase α -subunit; AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; ETNP, Eastern Tropical North Pacific; OMZ, oxygen minimum zone; OTU, operational taxonomic unit.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ14825–DQ14848 and DQ148573–DQ148905).

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Materials and Methods

Site Descriptions, Sample Collection, and DNA Extraction. Water samples were collected from three depths ($\sigma_T = 15.7, 15.8,$ and 15.9) bracketing the secondary nitrite maximum in the suboxic zone of the Black Sea (April 2003) and two stations (designated C1 and M2) in Monterey Bay (April 2005) by using Niskin bottles mounted on a hydrography wire. Water samples were either filtered directly onto Sterivex capsules ($0.2 \mu\text{m}$ filter, Millipore) with positive pressure (Black Sea) or a peristaltic pump was used (Monterey Bay). After filtration, capsules were capped, packed in polypropylene bags, frozen in liquid nitrogen, and stored at -80°C until DNA extraction. Briefly, DNA was extracted from Sterivex capsules by using 1.6 ml of lysis buffer (20 mM EDTA/400 mM NaCl/750 mM sucrose/50 mM Tris), 200 μl of 10% SDS, and 100 μl of proteinase K (10 mg/ml), incubated at 55°C for 2 h. After the addition of 1 ml of 100% ethanol, lysates were purified by using DNeasy columns (Qiagen, Valencia, CA).

Ten liters of water was collected in November 2003 from the secondary nitrite maximum (200-m depth) within the OMZ off the Pacific coast of Mexico, filtered onto a SpiralCap capsule, frozen, and stored below -70°C . For lysis, 12 ml of Tris-EDTA/1% SDS and 750 μl of proteinase K (10 mg/ml) was added to the capsules, followed by incubation at 55°C for 2 h. The lysate was removed, placed on ice for 90 min, and centrifuged for 40 min at $17,000 \times g$ to remove cell debris. DNA was ethanol-precipitated overnight, pelleted at $32,000 \times g$ for 30 min, resuspended in 300 μl of sterile water, and further purified by using Qiagen DNeasy columns.

Surface sediments were collected from San Francisco Bay and Bahía del Tóbari, Mexico (Gulf of California), using box cores deployed off small vessels in July 2004 and October 2004, respectively. Sediments were collected by hand from three sites in the Elkhorn Slough estuary (from head to mouth: Hudson's Landing, Hummingbird Island, and Vierra Marsh) in October 2004 and from the surf zone at the mouth of the unconfined Huntington Beach aquifer in July 2004. Soil aggregates were collected in June 2004 from the saturated C horizon at a noncontaminated "background" site near Oak Ridge, TN. Cores were collected from each environment by using cut-off 5-cc syringes, frozen on dry ice, and stored at -80°C . DNA was extracted from ≈ 0.25 g of sediment (0–0.5 cm depth interval) or soil by using the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA).

PCR Primer Design, Amplification, and Cloning of Archaeal *amoA* Fragments. PCR primers were designed based on alignments of archaeal *amoA* genes and deduced amino acid sequences from the Sargasso Sea (GenBank accession no. AACY01435967) and German soil (GenBank accession no. AJ627422). Gene fragments (635 bp) were amplified by using the PCR primers Arch-*amoA*F (5'-STAATGGTCTGGCTTAGACG-3') and Arch-*amoA*R (5'-GCGGCCATCCATCTGTATGT-3') with the following protocol: 95°C for 5 min; 30 cycles consisting of 94°C for 45 s, 53°C for 60 s, and 72°C for 60 s; and 72°C for 15 min. Triplicate PCRs were pooled (to minimize PCR bias), gel-purified, and cloned with the TOPO-TA cloning kit (Invitrogen). White colonies were transferred to 96-well plates containing LB broth (with 50 μ g/ml kanamycin), grown overnight at 37°C, and PCR-screened directly for the presence of inserts by using T7 and M13R vector primers.

Sequencing and Phylogenetic, Rarefaction, and Statistical Analyses. Sequencing of T7/M13 PCR products was performed by using vector primers on Applied Biosystems 3730xl capillary sequencers. Nucleotide sequences were assembled and edited by using SEQUENCHER, version 4.1 (Gene Codes, Ann Arbor, MI). A 606-bp region of 377 archaeal *amoA* sequences was chosen for

phylogenetic analysis. Nucleotide and amino acid alignments (based on 202 residues) were generated by using MACCLADE (<http://macclade.org>). Neighbor-joining phylogenetic trees were constructed based on alignments of DNA sequences (using Jukes–Cantor corrected distances) and amino acid sequences within PAUP*4.0b10. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1,000 replicates). To compare the archaeal *amoA*-based richness within each clone library, rarefaction analysis and Chao1 nonparametric richness estimations were performed by using DOTUR (21). Operational taxonomic units (OTUs) were defined as sequence groups in which sequences differed by $\leq 2\%$ or 5% .

Results and Discussion

Ubiquity of AOA. Nonextremophilic Crenarchaeota have been previously detected in a wide variety of environments and are widespread in the ocean (12, 13); however, their role in the marine C or N cycle has remained a mystery. Our results suggest that archaea capable of ammonia oxidation are ubiquitous in marine water columns and sediments. An extensive PCR survey of archaeal *amoA* genes using our primers resulted in amplification of 635-bp fragments (corresponding to essentially the entire gene) from multiple sites or depths within eight different geographic locations: the water columns of Monterey Bay (base of the euphotic zone), the Eastern Tropical North Pacific (ETNP; OMZ), and the Black Sea (suboxic zone); coastal and estuarine sediments from San Francisco Bay, Elkhorn Slough, and Huntington Beach, CA, and Bahía del Tóbari, Mexico, and for purposes of comparison, a saturated (suboxic) soil sample from Oak Ridge, TN. In total, we produced 15 clone libraries and a database of 377 archaeal *amoA* sequences from water columns, sediments, and soil (Fig. 1).

Primers were designed based on the novel archaeal *amoA* sequences from German soil and the Sargasso Sea water column (18, 19). These two genes shared 76% DNA identity, but 85% identity and 91% similarity at the amino acid level. Because these archaeal *amoA* genes are distant homologs of known bacterial *amoA* genes from β - and γ -*Proteobacteria* (19), it is not possible to recover them from environmental DNA samples with bacterial *amoA* (or *pmoA*) primers. Although designed based on a limited number of available sequences, our primers recovered 377 archaeal *amoA* sequences ranging from 69% to 96% identity to the German soil sequence and from 69% to 99% identity to the Sargasso Sea sequence. Based on this extensive database, it may be possible to design new, more degenerate primers that capture even greater archaeal *amoA* diversity.

AOA Diversity. Overall, we recovered a total of 138 unique OTUs (based on a 2% cutoff) from 377 individual archaeal *amoA* sequences. Both observed and extrapolated AOA richness varied widely within water column and sediment libraries (Fig. 2), with Chao1 richness estimates ranging from 5 to 37 OTUs for water column libraries and 11 to 48 OTUs for sediment libraries (see Table 1, which is published as supporting information on the PNAS web site). These richness levels are comparable to, or higher than, those observed for bacterial *amoA* clones obtained from water columns and sediments (22, 23). Based on phylogenetic analysis of PCR clone libraries, individual sample locations often had unique archaeal *amoA* sequences associated with them (Fig. 1); however, clades of these unique AOA sequences clustered according to habitat. For example, all sequences from North San Francisco Bay (NB-1), our only freshwater site, fell exclusively into one coherent cluster. Similarly, all *amoA* sequences recovered from Tennessee soil fell into one large, phylogenetically distinct cluster (see soil/sediment cluster in Fig. 1). This cluster also included the *amoA* sequence from soil metagenomic clone 54d9, which is derived from a member of the most widely distributed Crenarchaeota lineage (group 1.1b)

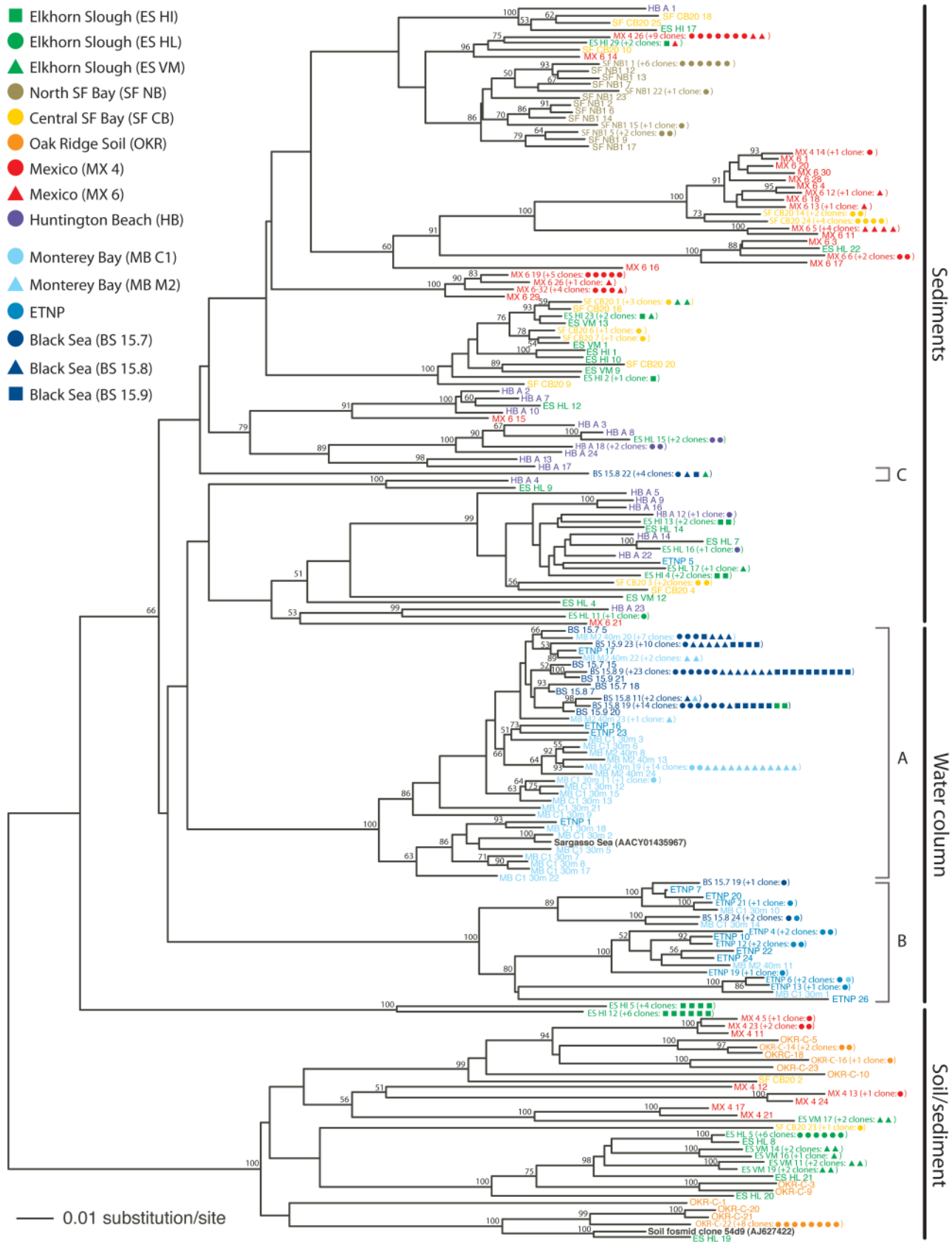


Fig. 1. Phylogenetic relationships among archaeal *amoA* sequences from water columns, sediments, and soil. Sequences are color-coded according to location; symbols are used to distinguish different sites or depths within a given location. One representative of sequence groups $\geq 99\%$ identical is shown; additional symbols show the total number of clones represented by a sequence. Database sequences are shown in black. Bootstrap values ($>50\%$) are indicated at branch points. Brackets highlight phylogenetic clusters referred to in the text. HI, Hummingbird Island; HL, Hudson's Landing; VM, Vierra Marsh.

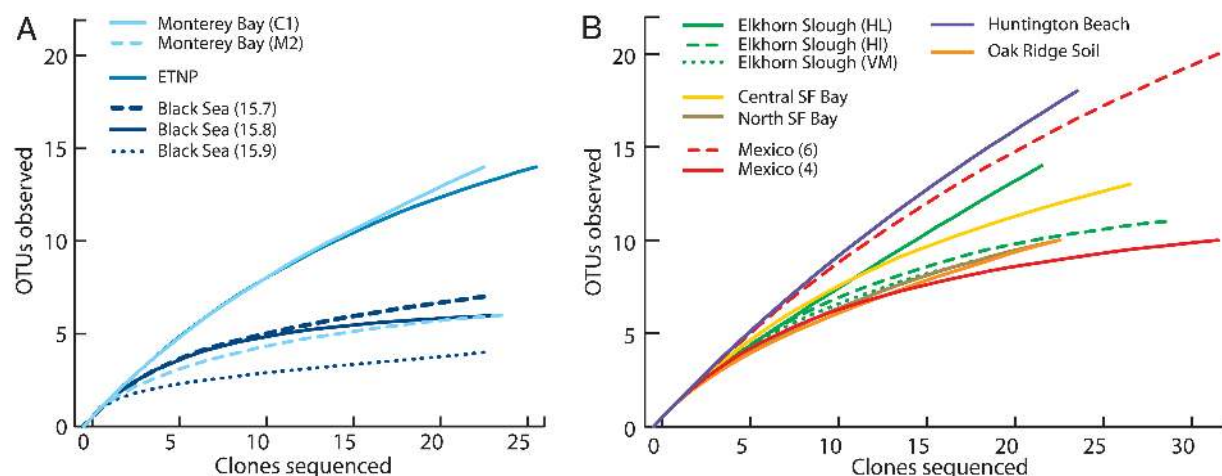


Fig. 2. Rarefaction curves indicating archaeal *amoA* richness within clone libraries derived from water columns (A) and sediments and soil (B). OTUs were defined as groups of sequences differing by $\leq 2\%$ at the DNA level. Curves are color-coded as in Fig. 1. HI, Hummingbird Island; HL, Hudson's Landing; VM, Vierra Marsh.

known in soils (19, 24). Interestingly, 141 of 142 water column sequences from the Black Sea, Monterey Bay, and the ETNP, despite widely different geographical locations and biogeochemical characteristics, fell into three distinct clusters (A, B, and C; Fig. 1). The largest of these clusters (cluster A) contains $>70\%$ of the water column sequences, including the Sargasso Sea sequence. It is tempting to speculate that these water column sequences correspond to strictly "planktonic" AOA; however, several sequences from Elkhorn Slough sediments also fell into these clades and were $>99\%$ identical to Black Sea and Monterey Bay water column sequences (Fig. 1). A number of sedimentary sequences also fell into the soil/sediment cluster. Based on Fig. 1, it is evident that while all but one of the water column sequences fell into the water column clusters, and all soil sequences fell into the large soil/sediment cluster, sequences within these clusters are not found exclusively in those environments. This finding in part reflects the wide range of archaeal *amoA* sequences recovered from sediments, with sequences falling in numerous clusters throughout the phylogenetic tree (Fig. 1).

AOA Diversity in the Water Column: The Black Sea, Monterey Bay, and ETNP. Based on the identification of an archaeal *amoA* gene in a Sargasso Sea surface water genomic library, we anticipated that similar genes would be present in other marine water column environments where nitrification plays a significant role. The Black Sea is the world's largest chemically stratified anoxic marine basin and encompasses a pronounced suboxic ($O_2 < 5 \mu M$) zone in which nitrogen cycling (e.g., denitrification, anammox) is extremely important (25, 26). Archaeal *amoA* was amplified from multiple depths within the suboxic zone of the Black Sea. Interestingly, this gene was present at densities (σ_T) similar to those where anaerobic ammonia oxidation has been reported (26). The majority ($>88\%$) of the archaeal *amoA* sequences obtained from the Black Sea suboxic zone fell into cluster A and showed 90–92% nucleotide identity to the Sargasso Sea gene sequence. Within this cluster, there were four predominant sequence types differing by $\approx 1\text{--}2\%$ at the DNA level (visible in an expanded tree, Fig. 3, which is published as supporting information on the PNAS web site), primarily at the third position. The remaining Black Sea sequences fell into clusters B and C. Overall, the Black Sea clone libraries were among the least diverse in this study (Fig. 2), and we appear to have recovered most or all of the OTUs predicted by the Chao1 richness estimator (Table 1).

The majority of sequences from the upper water column of Monterey Bay fell into cluster A along with the closely related Black Sea sequences (Fig. 1). Although archaeal *amoA* was successfully amplified from all depths analyzed between 30 and 80 m, for brevity we report here only sequence data derived from 30 m at a coastal site (C1) and 40 m at an offshore site (M2). The coastal station (C1) was used in part to determine the extent of overlap between planktonic AOA communities in Monterey Bay and benthic communities in Elkhorn Slough. (Because Elkhorn Slough is a small estuary that is tidally flushed by Monterey Bay seawater, Monterey Bay represents a potential source and sink for nutrients and microorganisms to the slough.) Near the offshore site (M2), nitrification rates (27) and AOB diversity (22) have recently been examined. Although there was some compositional overlap between the coastal (C1) and offshore (M2) archaeal *amoA* clone libraries, the coastal library was more diverse (Fig. 2). One subcluster within cluster A is comprised of seven C1 sequences (and one ETNP sequence) that share 93–99% identity with the Sargasso Sea sequence at the DNA level. Translation of these sequences revealed that all are identical to the Sargasso sequence at the amino acid level. These results indicate that the "microdiversity" within these coastal Monterey Bay sequences corresponds entirely to synonymous changes, with no effect on archaeal ammonia monooxygenase function. Additionally, most of the microdiverse (1–2% divergent) clusters of Black Sea and Monterey Bay sequences in cluster A are identical at the amino acid level. Given the differences between the suboxic zone of the Black Sea and the oxygenated euphotic zone of Monterey Bay, the compositional overlap between the archaeal *amoA* sequences from these two environments is surprising and suggests that planktonic AOA can tolerate a wide range of O_2 levels.

This conclusion is consistent with our results from the ETNP, one of the largest pelagic OMZs in the ocean, where archaeal *amoA* was amplified successfully from a 200-m depth under low oxygen conditions ($O_2 = 3.1 \mu M$) at the secondary nitrite maximum ($NO_2^- = 2.5 \mu M$). The ETNP and coastal Monterey Bay sequences were the most diverse of the six water column samples in the study (Fig. 2), with sequences from the ETNP falling into water column clusters A and B and one sediment cluster (see below). In contrast to the Black Sea and Monterey Bay libraries, the majority (21 of 26) of the ETNP sequences fell into cluster B, which included only nine sequences from the other water column libraries. Overall, we appear to have identified several key phylogenetic groups/clusters of planktonic AOA,

nitrogen by sedimentary denitrification. A common characteristic of all of the sediment samples analyzed in this study, as well as the suboxic water column and soil samples, is that denitrification functional genes (i.e., nitrite reductase) have also been successfully amplified from each of them. These results suggest that AOA may be intimately associated with denitrifiers and, like AOB, play an important role in coupled nitrification–denitrification. Interestingly, bacterial *amoA* could not be amplified from some of the samples analyzed in this study (e.g., Tóbari samples), which may indicate that archaeal *amoA* is more widespread than bacterial *amoA*, and that AOA are actually more widespread than AOB in sedimentary environments. Future studies focused on quantifying the relative abundance and expression of bacterial versus archaeal *amoA* genes under nitrifying conditions will be imperative for

understanding the relative contributions of what are apparently two functionally equivalent, but evolutionarily distinct, groups of microorganisms.

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