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Abundance and Diversity of Archaeal Ammonia Oxidizers in a Coastal Groundwater System▽†

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Nitrification, the microbially catalyzed oxidation of ammonia to nitrate, is a key process in the nitrogen cycle. Archaea have been implicated in the first part of the nitrification pathway (oxidation of ammonia to nitrite), but the ecology and physiology of these organisms remain largely unknown. This work describes two different populations of sediment-associated ammonia-oxidizing archaea (AOA) in a coastal groundwater system in Cape Cod, MA. Sequence analysis of the ammonia monooxygenase subunit A gene (*amoA*) shows that one population of putative AOA inhabits the upper meter of the sediment, where they may experience frequent ventilation, with tidally driven overtopping and infiltration of bay water supplying dissolved oxygen, ammonium, and perhaps organic carbon. A genetically distinct population occurs deeper in the sediment, in a mixing zone between a nitrate- and oxygen-rich freshwater zone and a reduced, ammonium-bearing saltwater wedge. Both of these AOA populations are coincident with increases in the abundance of group I crenarchaeota 16S rRNA gene copies.

Delivery of excess nutrients to coastal ecosystems can result in a myriad of deleterious effects, ranging from excessive or harmful algal blooms to loss of sea grass habitat, hypoxia, and loss of fish stocks (78). Terrestrially derived nitrogen in the form of ammonium (NH_4^+) or nitrate (NO_3^-) is transported to the coastal ocean through runoff and riverine inputs, but up to half of the nitrogen may travel through groundwater (36, 48, 57). While rivers amount to point sources of nitrogen and other solutes to the coastal ocean and are amenable to direct investigation, discharge of groundwater is a more diffuse source of nutrients to the coastal ocean, which is difficult to track. Importantly, groundwater systems can be chemically distinct from river systems and thus harbor different biological communities and biogeochemical processes (49). However, due to the large spatial and temporal variability as well the remote and difficult-to-sample nature of the groundwater/sediment matrix, the role of groundwater in coastal biogeochemistry has until recently been understudied and underappreciated. In an effort to better understand the initial stages of nitrogen transformation in a coastal groundwater system, we examined the microbial communities and more specifically the populations of ammonia-oxidizing microorganisms in an anthropogenically influenced coastal groundwater system in Cape Cod, MA.

Reduced nitrogen is often introduced to groundwater as NH_4^+ or organic nitrogen that is subsequently catabolized to NH_4^+. Nitrification is the microbially mediated oxidation of ammonia (NH_3) to nitrite (NO_2^-) and NO_3^-, which may ultimately be removed from the system by reduction to dinitrogen by denitrification or anaerobic ammonia oxidation (anammox). The rate-limiting step in the nitrification process is the oxidation of ammonia to nitrite. For many years, it was thought that ammonia oxidation was carried out exclusively by two groups of obligately aerobic autotrophs known as ammonia-oxidizing bacteria (AOB), which are found within the *Beta-* and *Gammaproteobacteria* (31, 58, 59). The populations of these organisms can be characterized by the genes that encode ammonia monooxygenase, an enzyme that catalyzes the oxidation of ammonia to hydroxylamine (32, 59, 69). The most often used genetic marker for this metabolism is the ammonia monooxygenase subunit A gene (*amoA*), which codes for the active site of the enzyme.

The discovery of an *amoA*-like gene sequence on a genomic fragment associated with a ubiquitous group of marine archaea (84) has prompted a reconsideration of the role of archaea in the nitrogen cycle and specifically the biogeochemistry of ammonia oxidation. Subsequently, markers for these organisms have been found in a variety of settings, including high-temperature vents (17, 87), terrestrial soils (30, 40, 66, 80), marine sediments (19, 23), open ocean waters (23, 64, 86), and oxygen minimum zones (15, 38) as well as a large number of coastal waters, including estuaries (3, 8, 50) and groundwater systems (65). Recent reports argue that these ubiquitous organisms may even represent a new phylum of *Archaea* known as the *Thaumarchaeota* (71). Understanding how these organisms are distributed in the environment, as well as what drives their distribution, i.e., their physiology and ecology, will lead to a better understanding of the controls on nitrogen cycling and nitrogen removal in the environment. The current study aims to address how ammonia oxidizers are distributed throughout a coastal groundwater system in relation to the gradients in geochemical variables.

MATERIALS AND METHODS

Study area and sample collection. The groundwater system at the Waquoit Bay National Estuarine Research Reserve (41°34′49″N, 70°31′27″W; Waquoit, MA) (Fig. 1) consists of outwash gravel, sand, and silt deposited during the retreat of the Wisconsin glaciation (53). The upper 10 m of sediment is homo-

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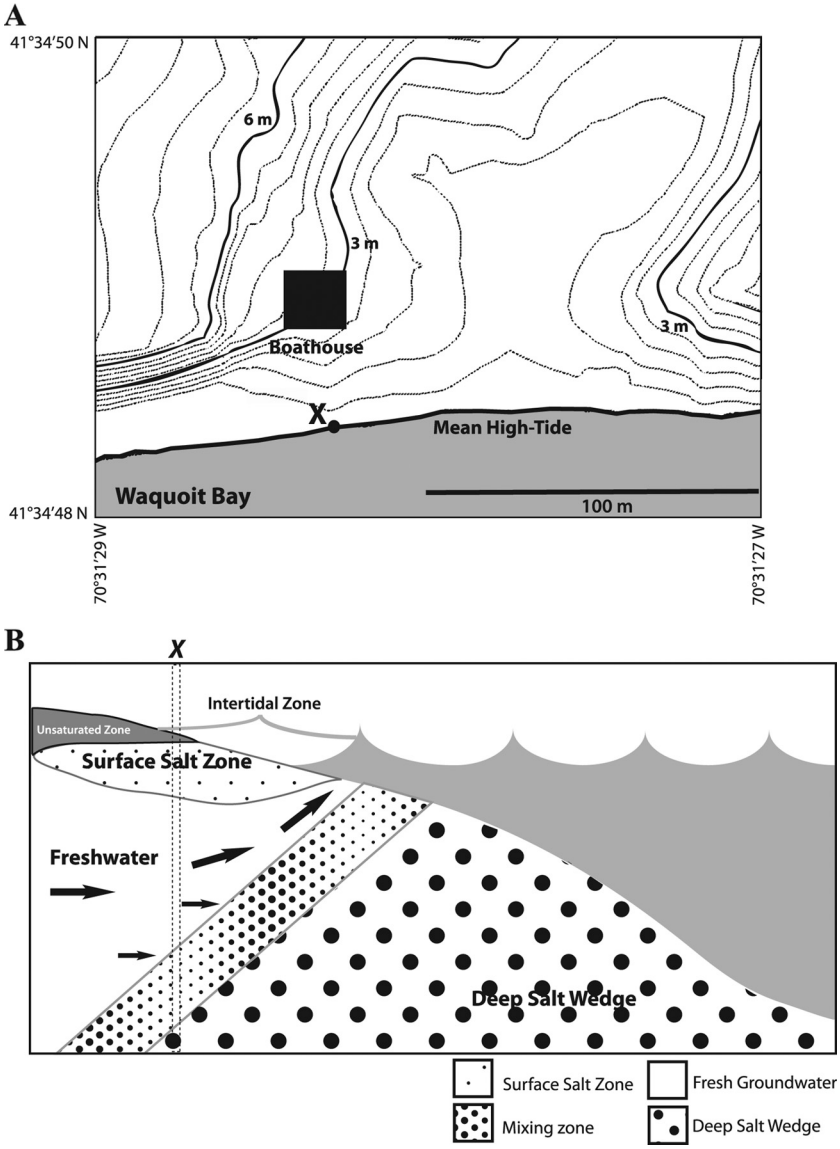

FIG. 1. (A) Map of Waquoit Bay (Waquoit, MA) with 0.6-m contours (including 3- and 6-m major contours) of the land surface. The location of the sediment and groundwater sampling, in relation to the boathouse (black box), at the mean high tide line, is denoted with "X." (B) Cartoon representing an idealized view of the groundwater system underlying the beach at Waquoit Bay. Three major water layers are depicted: the surface salt zone, the freshwater layer, and the deep salt wedge. "X" shows the location of sampling in the high intertidal zone.

geneous and highly permeable, providing conduits for transport of fresh groundwater toward the bay and tidally forced saltwater intrusions (13).

One-third of the fresh water entering Waquoit Bay comes from direct discharge of groundwater, while over 50% comes from ground water channeled into streams (9). Water recharges the groundwater quickly due to the high permeability of the surface soils, which are composed of coarse grain sands (95%, mostly 125 to 500 μm) and silts (5%) (13). Below the surface sand unit, a matrix of fine grain sands and silts occurs to a depth of 45 m (51). The groundwater is unconfined, resting on bedrock located 100 to 125 m below sea level (45, 54). Water flow through the surface region is predominantly horizontal, proceeding toward the bay with an average seepage rate at the head of the bay of 5 to 10 cm day^{-1} during high tide and increasing to 10 to 40 cm day^{-1} at low tide (51).

A long-term monitoring program for nutrient advection into the bay has been established at this site (43, 45, 81), and the Waquoit Bay groundwater system has been the focus of several studies addressing the importance of the groundwater discharge into the bay for trace metal, nutrient, and pollutant budgets (11, 68, 76, 77, 82).

Groundwater and sediment sampling. Groundwater was collected in June 2008 at the head of Waquoit Bay to a depth of 4 m (coinciding with the top of the salt wedge) using a piezometer with a stainless steel "Retract-a-tip" (AMS, ID). The piezometer was driven through the sediment using a hammer-drill with a vertical resolution of 15 to 10 cm. At each sampling depth, water was gently pumped to the surface using a Geopump Series I peristaltic pump (Geotech, Denver, CO) and nylon tubing. After the tubing was slowly flushed with at least 1 liter of groundwater (more than 6-fold flushing of tubing volume), pH, dissolved oxygen, salinity, and temperature were measured using a YSI 600R multiprobe calibrated in accordance with the manufacturer's protocols (10). Salinity is reported on the practical salinity scale with appropriate units (practical salinity unit [PSU]).

At each sampling depth, triplicate groundwater samples were filtered through a 0.2-μm-pore-size Sterivex filter, collected in 60-ml high-density polyethylene (HDPE) bottles, and stored on ice for subsequent analyses of NO_3^-, NO_2^-, and NH_4^+ concentrations. Samples were analyzed within 12 h of sampling upon return to the laboratory. Nitrite concentrations were measured colorimetrically

using sulfanilamide and *N*-(1-napthyl) ethylenediamine (56). Nitrate plus nitrite (NO_x) concentrations were measured by chemiluminescence following a hot vanadium reduction (16, 25, 26). Ammonium was measured using the phenol-hypochlorite method (70).

Guided by the field measurements of the groundwater composition, a sediment core was collected within <2 h (ca. slack low-tide) and 0.5 m of the piezometer site using a hand-operated bailer-boring auger (Eijkelkamp, Netherlands) to a depth of 4 meters. Sediment from the bailer was laid on sterilized aluminum foil, and 1 to 5 g of sediment was aseptically subsampled with a vertical resolution of 30 to 50 cm. Sediment samples were immediately frozen in liquid nitrogen and stored at −80°C in the laboratory for later DNA extraction and analysis.

DNA extraction. DNA extractions were performed in triplicate, each from 1 g of sediment using lysozyme (100 mg ml^{-1}) and proteinase K (20 mg ml^{-1}) digestions, followed by physical disruption by successive freeze-thaw cycles and phenol-chloroform-isoamyl extraction (20). Extracted DNA was precipitated using isopropyl alcohol and 3 M sodium acetate with final resuspension in 50 μl of nuclease-free water or Tris-EDTA buffer (TE). The extracts were quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific) and diluted using nuclease-free water to appropriate concentrations (see below) for amplification and quantification by PCR methods.

qPCR. Quantitative PCR (qPCR) was employed to determine the gene copy numbers of bacterial and archaeal 16S rRNA and *amoA* genes throughout the core. Triplicate DNA extractions of each sample were diluted to 10 ng μl^{-1} and amplified using either the iQ SYBR green supermix (Bio-Rad) or a cocktail consisting of 1× colorless master mix (Promega, Madison, WI), 10 nM deoxynucleoside triphosphates (dNTPs; Promega), 0.5× SBYR green I (Invitrogen, Carlsbad, CA), 20 nM fluorescein (Sigma), 1 mM $MgCl_2$ (Promega), forward and reverse primers (300 to 1,000 nM) (Table 1), and 1.25 units of GoTaq Flexi DNA polymerase (Promega). Four different sets of qPCR primers (23, 47, 80, 86) were used to analyze the abundance of archaeal *amoA* (Table 1; see also Fig. S1 in the supplemental material). All qPCR amplifications were repeated on three separate days with optimized thermal programs (Table 1) using an iCycler thermocycler (Bio-Rad, Hercules, CA). qPCR efficiencies ranged from 88 to 105% for all reactions, resulting in linear standard curves (r^2 = 0.90 to 0.99).

Standard curves were constructed from serial dilutions (10^7 to 10^1 copies μl^{-1} plus a water blank) of linearized p-Gem T-Easy plasmids (Promega) containing inserts of the gene of interest (Table 1). Plasmids (0.5 μg μl^{-1}) were linearized by digestion with the EcoRI (New England Biolabs) restriction enzyme in the supplied buffer (2 h at 37°C, with heat inactivation for 20 min at 65°C). Restriction digests were cleaned using an SV Wizard PCR purification kit (Promega), eluted in 30 μl, and quantified using a Nanodrop spectrophotometer (ThermoFisher). Melt curve analyses were performed on all reactions to confirm specificity.

PCR, cloning, and sequencing. Clone libraries were constructed for the archaeal 16S rRNA and *amoA* genes from 0.5 m, 1 m, 2 m, 2.5 m, 3 m, and 4 m. Briefly, targets were amplified using published protocols and primers (Table 1 and references therein), and the products of three separate amplifications were pooled for each gene at each depth. The pooled products were cleaned using the SV Wizard PCR cleanup kit (Promega), followed by ligation into the p-Gem T-Easy vector and transformation into the host *Escherichia coli* (JM109) in accordance with the manufacturer's protocols (Promega). Plasmid purification and sequencing were performed at the Keck Facility, Josephine Bay Paul Center at the Marine Biological Laboratories (JBPC-MBL; Woods Hole, MA). Sequencing chemistry was done in accordance with a modified BigDye reaction mixture containing 0.5 μl of BigDye Terminator, 0.4 μl of primer (M13R [5′-C AGGAAACAGCTATGAC]; 15 μM), 0.1 μl dimethyl sulfoxide, 1/3× reaction buffer, template (200 to 400 ng DNA), and water added to give a final volume of 6 μl. The recommended thermal protocol of 60 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min was used for sequencing.

Sequence analysis. Base calling and sequence quality checks were performed by the Phred and Phrap software packages (21, 22). The Lucy software program (14) was used for vector trimming. Processed sequences were searched against the nonredundant nucleotide (nr/nt) database using the BLASTn algorithm. 16S rRNA gene sequences were imported into ARB (41), aligned with the ARB aligner, manually checked and corrected, and then added by parsimony to an ARB alignment from Greengenes. *amoA* genes were also imported into ARB, aligned, and added by parsimony to an alignment of archaeal *amoA* gene sequences from the GenBank database (4). Conservative column filters were constructed, masking any sites missing data for any of the sequences. Trees were constructed on aligned sequences using the ARB neighbor-joining function.

Nucleotide sequence accession numbers. All sequences generated and used in this study have been reported to GenBank under the accession numbers HM160162 to HM160497.

RESULTS

Environmental context. Long-term chemical monitoring has helped to elucidate the biogeochemical setting within the Waquoit Bay subterranean estuary (11–13, 37, 51, 76). The most-prominent features observed during the current study include (i) a surface salt layer (salinity up to 4 PSU) with low dissolved oxygen (<50 μM), nitrate (<50 μM), and ammonium (<5 μM) concentrations, (ii) an intermediate oxic freshwater layer with high levels of both nitrate (up to 350 mM) and dissolved oxygen (up to 175 μM), and (iii) an underlying deep, anoxic reducing salt wedge that lacked nitrate but contained up to 53 μM ammonium (Fig. 2A). These zones provide a variety of niches that could harbor distinct microbial communities adapted to different salinities, oxygen levels, or nutrient concentrations. While dissolved organic carbon (DOC) was not measured in the current study, previous work at a nearby site within Waquoit Bay (approximately 600 m east of the current study site) showed low (less than 50 μM) DOC concentrations in the fresh ground water, with variable but generally higher concentrations in brackish surface sediments (50 to 750 μM) as well as in the deep salt wedge (100 to 200 μM) (12).

16S rRNA gene abundance. The abundance of bacterial 16S rRNA genes ranged from 3.9×10^8 to 1.3×10^9 copies per gram of sediment, with the maximum bacterial 16S rRNA gene abundance observed at a 2-meter depth (Fig. 2B). Archaeal 16S rRNA gene abundance was comparable with bacterial 16S rRNA gene abundance in the upper 1.7 m (4.8×10^8 to 7.3×10^8 copies per gram of sediment). Below 1.7 m, the archaeal 16S rRNA gene abundance increased gradually, reaching a maximum (6.3×10^9 copies per gram of sediment) at a depth of 3 m.

Taking a closer look at the archaea using qPCR primers specific to group 1 (also known as marine group I [MG1]) archaea (47), we found that the abundance of MG1 (4.5×10^4 to 9.8×10^6 copies g^{-1} sediment) was approximately 1 percent of the total archaeal 16S rRNA gene abundance determined by qPCR at all but the shallowest depths (Fig. 2C). The 16S rRNA gene abundance of MG1 crenarchaea showed two peaks in the sediment column: one within the surface salt layer and the second at a depth of 2.8 m at the deep salt transition zone.

Archaeal 16S rRNA gene diversity. The archaeal 16S rRNA genes recovered from the sediments belonged to both the *Euryarchaeota* and the *Crenarchaeota* subdivisions, with roughly equal contributions from the *Euryarchaeota* (36 out of 84, 43%) and the *Crenarchaeota* (48 out of 84, 57%), for which MG1 *Crenarchaeota* accounted for the majority of the sequences (Fig. 3). Therefore, in contrast to the qPCR results, which showed MG1 abundance to be approximately 1% of total archaea, the archaeal clone libraries were approximately 50% MG1. As discussed below, this discrepancy may be the result of primer biases in PCR amplification and/or qPCR protocols or sampling biases in the clone libraries.

Crenarchaeota sequences were recovered from all depths, and they were mostly (37 out of 48, or 77%) *Cenarchaeales*-like sequences (which includes *Nitrosopumilus maritimus*). Se-

TABLE 1. Primers and methods used in this study

Target gene	Primer name(s) and sequence(s)	Thermal program	qPCR efficiency (%)	Positive control	Reference
Bacterial 16S rRNA (sequencing only)	8F (400 nM), AGAGTTTGATCCTGGCTCAG; 1492R (400 nM), GGTTACCTTGTTACG ACTT	94°C for 120 s; 30 cycles of 94°C for 30 s, 47°C for 60 s, and 72°C for 60 s; and final extension of 72°C for 600 s		*Escherichia coli*, *Pseudomonas stutzeri*	28
Bacterial 16S rRNA	Bac1369 (1,000 nM), CGGTGAATACGTTCY CGG; Prok1541 (1,000 nM), AAGGAGGTG ATCCRGCCGCA	50°C for 120 s, 94°C for 120 s, and 40 cycles of 94°C for 15 s and 59°C for 60 s	88–90	*P. stutzeri*	74
Archaeal 16S rRNA (sequencing only)	21Fa (400 nM), TTCCGGTTGATCCYGCC GGA; 1492R (400 nM), GGTTACCTTGTTA CGACTT	94°C for 120 s; 30 cycles of 94°C for 30 s, 47°C for 60 s, and 72°C for 60 s; and final extension of 72°C for 600 s		*Ferroplama acidarmonas*	60
Archaeal 16S rRNA	Arcmix1369F (1:1 mixture) (500 nM), CGGTG AATACGTCCCTGC and CGGTGAATATG CCCCTGC; Prok1541 (1,000 nM), AAGGAG GTGATCCRGCCGCA	50°C for 120 s; 94°C for 120 s; and 40 cycles of 94°C for 15 s, 59°C for 60 s, and 80°C for 15 s (read)	98–105	*F. acidarmonas*, *N. maritimus*	74
MG1 *Crenarchaeota* 16S rRNA	GI-751F (500 nM), GTCTACCAGAACAYG TTC; GI_956R (500 nM), HGGCGTTGACT CCAATG	95°C for 900 s and 50 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 30 s, and 80°C for 15 s (read)	93–95	*N. maritimus*, environmental clone	47
Archaeal *amoA* from Francis et al. (sequencing and qPCR)	amoAF (500 nM), STAATGGTCTGGCTTAG ACG; amoAR (500 nM), GCGGCCATCCAT CTGTATGT	95°C for 300 s and 30 cycles of 94°C for 30 s, 53°C for 60 s, 72°C for 60 s, and 80°C for 15 s (read)	93–95	*N. maritimus*, environmental clone	23
Archaeal *amoA* from Wuchter et al.	archamoAF (500 nM), CTGAYTGGGCYTGG ACATC; archamoR (500 nM), TTCTTCTTT GTTGCCCAGTA	95°C for 300 s and 35 cycles of 94°C for 30 s, 61.5°C for 40 s, 72°C for 40 s, and 80°C for 15 s (read)	95–96	*N. maritimus*, environmental clone	86
Archaeal *amoA* from Mincer et al.	crenamoAmodF (500 nM), TGGCTAAGACG MTGTA; crenamoA-Q-F (500 nM), GCARG TMGGWAARTTCTAYAA; crenamoAmodR (500 nM), AAGCGGCCATCCATCTGTA	95°C for 900 s and 50 cycles of 94°C for 15 s, 52°C for 30 s, 72°C for 30 s, and 80°C for 15 s (read)	95–96	*N. maritimus*, environmental clone	47
Archaeal *amoA* from Treusch et al.	Amo196F (1,000 nM), GGWGTKCCRGGRAC WGCMAC; Amo277R (1,000 nM), CRATGA AGTCRTAHGGRTADC	95°C for 150 s and 45 cycles of 95°C for 15 s, 50°C for 40 s, and 80°C for 15 s (read)	90–91	*N. maritimus*, environmental clone	80
Bacterial *amoA*	amo189F (300 nM), GGHGACTGGGAYTTC TGG; amoA2, R′ (900 nM), CCTCKGSAAA GCCTTCTTC	50°C for 120 s; 95°C for 600 s; and 40 cycles of 95°C for 45 s, 55°C for 60 s, 72°C for 45 s, and 80°C for 15 s (read)	96–97	*Nitrosomonas* sp.	52

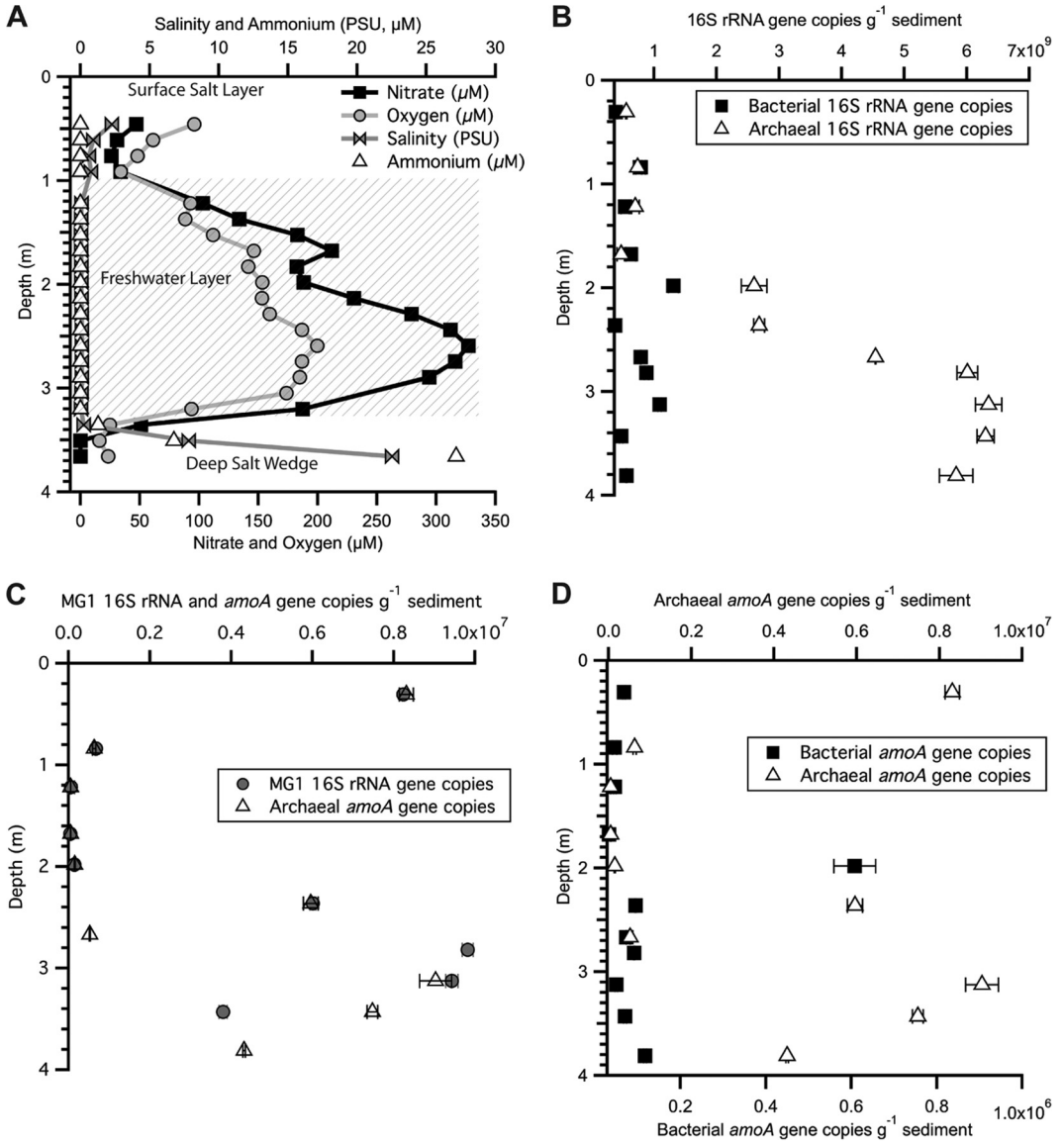

FIG. 2. Groundwater chemistry at study site. (A) Salinity, ammonium, nitrate, and oxygen concentrations in the groundwater at the time of coring. Three distinct parcels of water are shown: (i) a saltwater surface zone (to about 1 m), with low oxygen and nitrate levels; (ii) a persistent fresh groundwater that carries elevated oxygen and nitrate concentrations; and (iii) a deep anoxic salt wedge that introduces ammonium in to the system. (B) Depth distribution of total bacterial and archaeal 16S rRNA gene abundance. (C) Abundance of MG1 16S rRNA and archaeal *amoA* genes. (D) Depth distribution of bacterial and archaeal *amoA* genes. Error bars are calculated for panels as 1 standard deviation for all data points; some bars are hidden within the data symbol.

quences were also recovered from the pSL12 (4 out of 48, or 8%) and the SAGMA (South African gold mine archaea; 7 of 48, or 15%) subgroups of the *Crenarchaeota*. Both MG1 and pSL12 clades have been implicated in nitrification (29, 35, 47), but it is unknown whether SAGMA-like organisms harbor *amoA* genes. Related 16S rRNA sequences have been found in many environments, including surface marine waters, Antarctic bathypelagic and other deep marine sediments (27), uranium mine tailings (75), and agricultural soils (44).

Archaeal *amoA* gene distributions. The results from all four qPCR primer sets targeting archaeal *amoA* genes (Table 1; see also Fig. S1 in the supplemental material) provided reproducible (self-consistent) data under the recommended amplification conditions. Moreover, all four primer sets yielded the same range of abundances for the archaeal *amoA* gene abundance throughout the upper half of the sediment core, although differences between the primers from Treusch et al. (80) and the other primers deeper in the sediments were observed (Fig. S2). Given the general consistency between the primer sets from Wuchter et al. (86), Mincer et al. (47), and Francis et al. (23) throughout the sediment core, we have chosen to present our *amoA* gene abundance data in accordance with those obtained using the primer set from Francis et al. (23) for the remainder of the discussion.

As with MG1 abundance, archaeal *amoA* genes occurred in high abundance in the surface meter (8.3×10^6 copies per

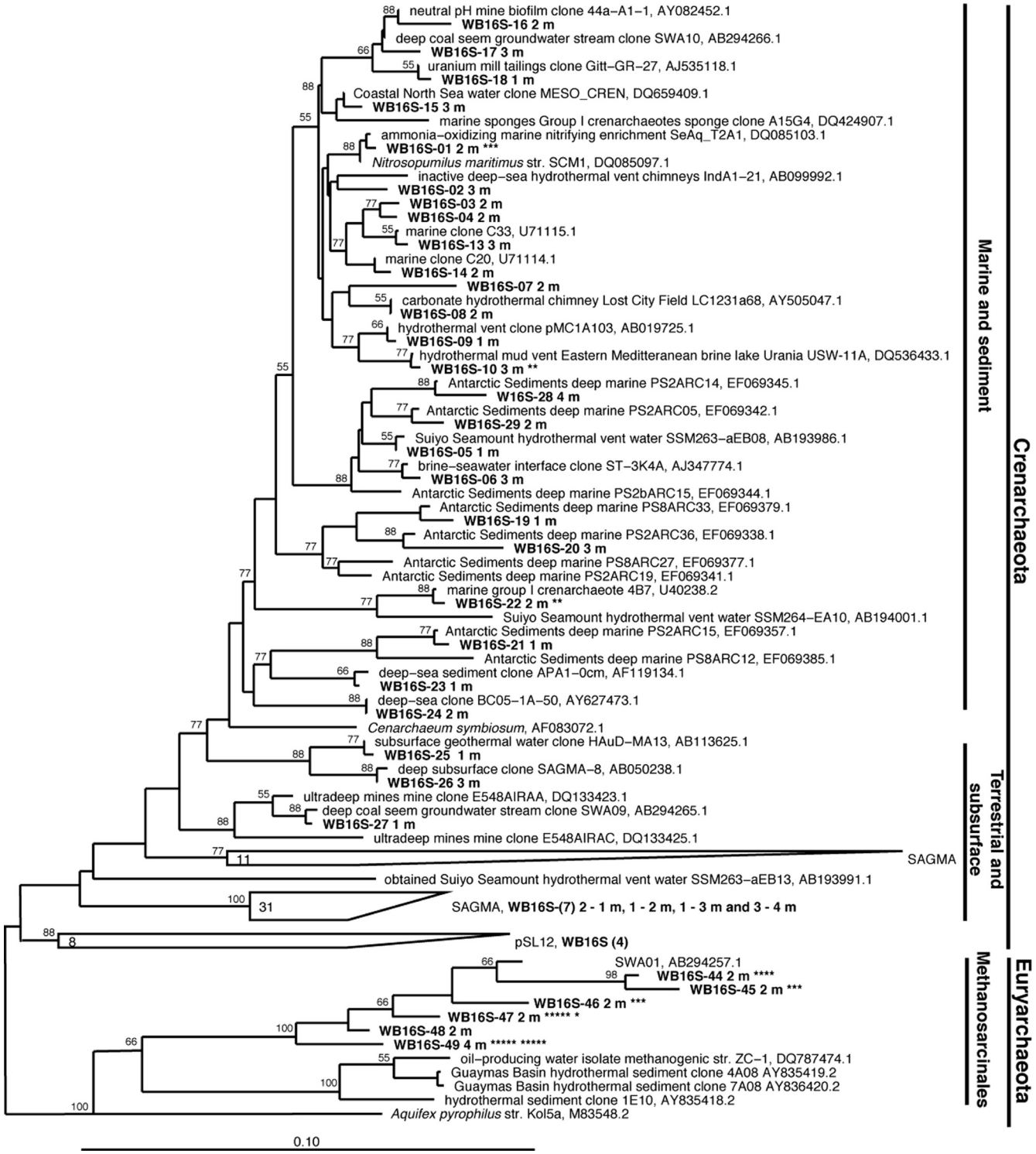

FIG. 3. Neighbor-joining tree, based on 1,309 bp of the archaeal 16S rRNA gene. Type strains are represented with italicized font. Sequences recovered from this study are in bold, with sequences that were repeatedly recovered marked with an asterisk(s); the number of asterisks corresponds to the number of times the sequence was recovered. ARB neighbor-joining methods, with Jukes-Cantor corrections and 1,000 iterations of bootstrapping, were used for tree construction. Bootstrap values greater than 50% are shown.

gram of sediment) and at 2.8 m (1.1×10^7 *amoA* copies per gram of sediment) in the core (Fig. 2D). Moreover, the ratio of archaeal *amoA* gene abundance to MG1 16S rRNA gene abundance was close to 1 throughout most of the sediment column (Fig. 2C), consistent with genomic evidence for 1 *amoA* gene copy per AOA genome (47, 85). Rather than corresponding directly to particular environmental parameters, these peaks in archaeal *amoA* gene abundance appeared in or near transi-

tions between freshwater and saline groundwater, which may correspond to regions of periodic availability of O_2, NH_3, and dissolved organic carbon (46).

Bacterial *amoA* gene distribution. Bacterial *amoA* abundance was low (1.3×10^3 to 3.3×10^4 copies per gram of sediment) throughout much of the sediment column, with a small peak (6.0×10^5 copies per gram of sediment) occurring at 2 m (Fig. 2D). In this peak within the core of the freshwater layer, bacterial *amoA* accounted for approximately 80% of the total *amoA* genes copies. Elsewhere, bacterial *amoA* was less than 1% of the total *amoA* gene abundance.

Archaeal *amoA* gene clone libraries. The archaeal *amoA* gene clone libraries (consisting of 343 clones in total: 92 from 1 m, 96 from 2 m, 86 from 3 m, and 69 from 4 m) from the sediments revealed two genetically distinct communities within the system. The first community was mostly found in the upper 2 meters of the sediments and a second community between 2 and 4 meters within the sediments.

The *amoA* genes recovered from the upper 2 meters clustered primarily with water column A and B sequences within the archaeal *amoA* phylogeny (Fig. 4) (3, 23). This group includes sequences from open ocean and coastal waters, as well as estuarine sediments (3, 50), and the sequences from Waquoit Bay were most similar to sequences from the low-salinity sediments of North San Francisco Bay (50). Below 2 meters, the recovered *amoA* genes were distributed within a cluster of archaeal *amoA* genes obtained from sediment, soil, and groundwater (30, 83).

DISCUSSION

Many studies concerning nitrogen biogeochemistry in coastal groundwater focus on the role of denitrification as a mechanism of fixed nitrogen loss (1, 39), with less consideration for how this process fits in the broader context of nitrogen transformations. Traditionally, nitrification and denitrification have been thought to occur in separate niches due to the incongruent environmental requirements of the processes. However, tightly coupled nitrification and denitrification are thought to be important for nitrogen removal where appropriate environmental conditions are separated by small spatial scales. Estuarine systems, where strong redox gradients compress the spatial separation of these two processes, represent one area where tightly coupled nitrification and denitrification may occur (34, 61). Groundwater systems where terrestrially derived freshwater and infiltrating saline waters mix represent another setting where nitrifying and denitrifying niches may overlap.

Near the head of Waquoit Bay, MA, lies a zone where fresh groundwater mixes with intruding saline bay water to form a subterranean estuary. Three distinct layers are present in the upper 4 m of the subterranean estuary where the sediments for the current study were obtained (Fig. 2A): (i) a surface salt zone within the upper meter of sediments overlying (ii) a freshwater layer that is underlain by (iii) a deep salt wedge. These layers are separated by transitions marked by gradients in salinity, oxygen, ammonium, nitrate, dissolved organic carbon, and other redox-sensitive constituents (12, 37, 76).

Previous studies have modeled and/or measured the fluxes of nitrogen at different points within the Waquoit Bay subterranean estuary (37, 72). These studies have suggested that coupled nitrification and denitrification may occur in both the surface and deep salt transition zones, where waters containing ammonium and oxygen mix. Here, we have used molecular techniques to describe the microbial communities residing on or within the sediments and examine whether these communities indeed contain the genetic potential for nitrification. We specifically examined the communities of AOA and AOB, focusing on the changes in their abundance and community structure in the different zones of the Waquoit Bay subterranean estuary.

Surface salt zone. The uppermost water layer within the Waquoit bay subterranean estuary is a mixture of groundwater and bay water that overtops and percolates through the coarse grain sands. This tidally induced mixing is thought to be rapid, given the estimated residence time of this water of hours to days based on the hydraulic gradient, offshore seepage meters, and reaction and transport modeling (46). Nitrate and oxygen are present in this layer, with concentrations decreasing between 0.5 m and 0.9 m; in contrast, ammonium is only occasionally observed in this layer. Nitrate and ammonium are likely a product of local organic matter degradation or sediment desorption rather than influx from the bay, given that the concentrations in the bay water of both solutes are low (<2 and <5 μM, respectively) (76). The wide range of DOC concentrations reported (7, 12) in the upper meter of the groundwater system also suggests that this is a dynamic region of the sediment and that the microbial community within this zone may be exposed to a broad range of DOC, O_2, and nutrient availability.

The microbial community within this depth interval is apparently composed of equal numbers of bacteria and archaea, and abundances for both groups are similar to those reported for marine surface sediments (67). However, the discrepancy in the proportion of total archaea that MG1 represents based on clone libraries versus qPCR suggests that total archaeal abundance measurements may be overestimated by qPCR. This could be a result of analytical biases (i.e., low primer specificity obtained using the mixture of archaeal qPCR forward primers, potentially resulting in nonspecific amplification) or isolation of extracellular DNA preserved by attachment to mineral surfaces or clays (18, 62). Because there is a good correlation between the abundance of MG1-like 16S rRNA genes and archaeal *amoA* genes, with a 1:1 ratio maintained throughout much of the sediment column (Fig. 2C), this gene ratio being consistent with genomic (17, 35) and environmental (2, 47, 86) observations of AOA, we believe that the MG1 16S rRNA gene abundances may be more reliable than the total archaeal 16S rRNA gene abundance estimates in this case. If so, the total abundance of archaea may be lower than that of bacteria throughout the core.

The relation between MG1 16S rRNA and archaeal *amoA* gene abundances indicates a potential population of crenarchaeal ammonia oxidizers. The peak in archaeal *amoA* gene abundance in the surface meter is consistent with results from a reactive transport model that predicted elevated nitrification rates in the upper meter of the sediment column due to the dispersive mixing of ammonium and oxygen between the overtopping bay water and advecting freshwater (73). Our data suggest that a potential nitrifying population resides in these

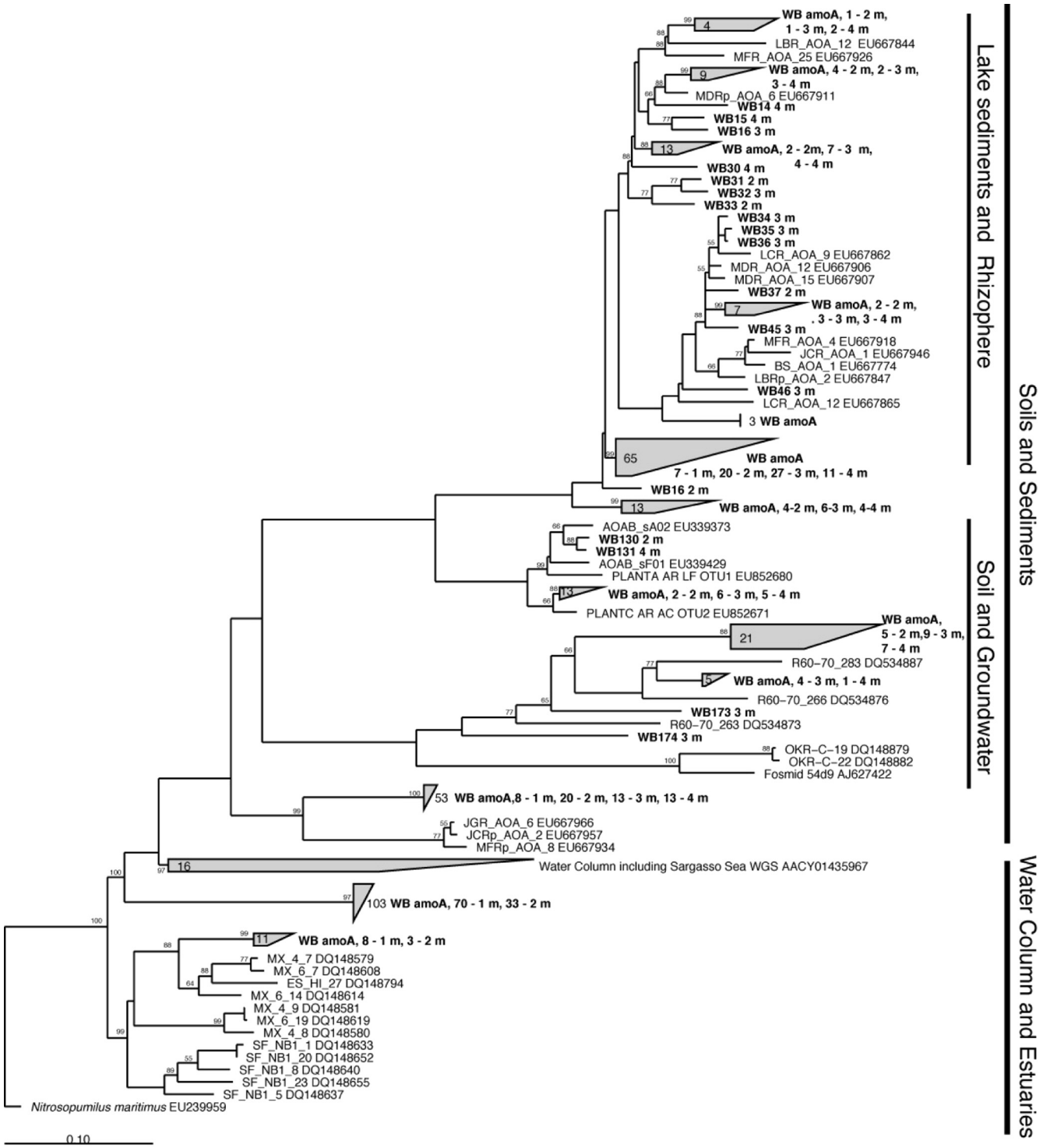

FIG. 4. Neighbor-joining tree of the archaeal *amoA* gene based on 529 bp (EU239959 positions 57 to 586) of unambiguous nucleotides. Clone libraries were constructed at 6 depths within the sediment column using the primer pair from Francis et al. (23). Numbers within the wedges represent the number of sequences included in that branch. Numbers located in the branch label indicate the quantity of sequences recovered at specific depths. ARB neighbor-joining methods, with Jukes-Cantor corrections and 1,000 iterations for bootstrapping, were used for tree construction. Bootstrap values greater than 50% are shown.

surface sediments and that the bulk of the population is archaeal rather than bacterial. It is possible that this population represents organisms that are washed into the sediments from the surface bay waters and that the diminishing abundance in the upper meter is a result of filtering through the sands. Activity studies, such as those involving the consumption of added ammonium or isolation of *amoA* mRNA, are needed to determine whether this is an active population of AOA.

Freshwater plume. The second layer of water encountered was a terrestrially derived fresh groundwater plume flowing laterally through the strata toward the bay. The AOA abundance declined within this layer, while AOB reached peak abundances, resulting in an ammonia-oxidizing population dominated by bacteria. This layer is replete in both nitrate and oxygen but low in ammonium content. In other regions of the bay and in more-landward sections of the groundwater system, a terrestrially derived plume carrying a large supply of ammonium (>100 μM) and reduced metals (Fe^{2+}, Mn^{2+}, etc.) has been observed within this freshwater layer, mainly during times of high groundwater recharge, such as the early spring (12, 63, 77). This reduced, ammonium-rich plume was not observed at our site in June 2008, although periodic influxes of ammonium derived from inland sources may fuel nitrification at this depth at other times of the year. The AOB population could also exploit a tightly controlled release of ammonium from carbon degradation or mobilization from the sediment matrix. However, DOC concentrations are routinely less than 50 μM in the fresh groundwater (12). Further investigation is required to determine if and when the AOB population is active at this depth and what factors control that activity.

Deep salt wedge transition. The transition zone between the freshwater layer and the deep salt wedge is the location of the second peak in archaeal *amoA* gene abundance (Fig. 2D). This transition represents a mixing zone between the freshwater layer flowing toward the bay containing oxygen and nitrate and the reduced intruding salt water containing ammonium and most likely DOC (12). Mixing between the two sources of water in this zone is therefore expected to supply ammonium, oxygen, and DOC to the microbial community. While the depth of the deep AOA peak is not perfectly coincident with the salinity gradient, fluctuations in the depth of the deep salt wedge due to changes in hydraulic head and tidal pumping have been observed or modeled at nearby sites (13, 37, 72). In this system, the distribution of sediment-associated microbes may reflect an average, rather than instantaneous, groundwater condition.

Based on previous measurements, DOC content is expected to increase as salinity increases into the deep salt wedge, approaching 100 to 150 μM in the upper part of the deep salt wedge (12). In the data set reported by Charette et al. in 2006 (12), there was a small deviation from the straight mixing curve for DOC (linear fit $r^2 = 0.72$) in the transition zone where salinity increases from ca. 0.5 to 20 PSU, indicating a potential loss of carbon in the deep salt transition. The 2006 data set also suggests a loss of oxygen over this same region (linear fit $r^2 = 0.58$). While heterotrophic organisms may contribute to these losses in DOC and dissolved oxygen, the presence of O_2, DOC, and NH_4^+ could produce a favorable environment for (potentially mixotrophic) ammonia-oxidizing archaea (29, 33, 55). Below, we examine the potential relationship between this chemical data and the ammonia-oxidizing populations.

AOA versus AOB. Three populations of ammonia oxidizers are present in the groundwater system: a shallow AOA population, an intermediate AOB population, and a deep AOA population that is genetically distinct from the shallow population. The AOA populations are observed in mixing zones where ammonium, oxygen, nitrate, and most likely DOC are dispersively mixed, whereas the AOB population is largely contained within the relatively stable core of the freshwater plume. Recent reports have examined spatial patterns in the distribution of the archaeal and bacterial *amoA* genes in relation to ammonium and organic carbon concentrations, salinity fluctuations, and other chemical loads (5, 6, 23, 24, 50). Caffrey et al. (8) reported a relationship of archaeal *amoA* gene abundance to salinity in several estuaries. Relative changes in abundance between AOB and AOA have also been found to correlate with C/N ratio (50), salinity and oxygen (3, 50, 65), and trace metal availability (lead and nickel) (8, 50). AOB abundance has been found to correlate positively with salinity (for example 50, 65). Based on these studies, AOB might be expected to be more abundant in the surface salt layer and the transition to the deep salt wedge due to the increase in salinity in these zones. However, this was not the case in our study. Of course, these environmental parameters do not vary in isolation; for instance, C/N ratios and salinity may covary (for example 50), obscuring any relationship between the environmental parameter and the microbiota. In the present study, abundance of sediment-associated AOA or AOB did not show a strong ($r^2 > 0.50$) correlation with any of the simultaneously measured groundwater parameters (O_2, salinity, NO_3^-, NO_2^-, NH_4^+, pH, or redox potential). Instead, peaks in AOA abundance occurred within the gradients of fresh/salt transition zones, which may shift relative to the depth in the sand depending on hydraulic gradients and tidal pumping (46).

AOA and AOB may have different abilities, as suggested by recent studies, to fill different niches. The reports of potential mixotrophy in *Cenarchaeum symbiosum* (29) and planktonic archaea (33, 55) hint at the potential plasticity of the metabolic capabilities, which may allow AOA to thrive in dynamic environments such as mixing zones, where DOC and mobilized ammonium may periodically be introduced (e.g., both the surface meter and the transition to the deep salt wedge). Mixotrophic AOA might be more competitive than the obligately autotrophic AOB in these zones. It is also possible that the AOA are better adapted to compete for ammonium in these dynamic regions of the groundwater system that more consistently see low oxygen levels. *Nitrosopumilus maritimus* SCM1 has been shown to have a high affinity for ammonium, as much as 200-fold higher than that of AOB, and a low substrate threshold of 10 nM NH_4^+ or less (42).

Interestingly, the *amoA* gene phylogenies from the surface meter and the deep salt wedge transition zone reveal that these populations represent two distinct AOA communities (Fig. 4). Sequences of *amoA* genes recovered from transition to the deep salt wedge suggest that this population closely resembles a soil/sediment community, while *amoA* gene sequences obtained from the upper meter are similar to sequences recovered from low-salinity, low-nutrient, estuarine sediments (23, 50). These results are consistent with the environments from which they were drawn and strengthen the idea that environmental niches within the AOA are reflected in the *amoA* phylogeny. Ecological niche separation for AOA, based on *amoA* gene phylogenies, has been suggested previously, especially for water column-derived sequences (2, 29, 47, 64). A recent report (64) supports this idea of niche separation by showing differences in the distributions of the "shallow-adapted" cluster A and "deep-adapted" cluster B *amoA* gene abundances and transcriptional activities in a transect through the California

Current. This niche separation is not limited to the two water column clusters. Soil-derived *amoA* gene sequences have been reported to occur in horizon-specific clusters, suggesting that phylogenic distinctions may be related to environmental conditions (pH, redox potential, etc.) (30).

It is important to note that while archaeal nitrification within the groundwater system may seem to be focused in specific mixing zones, these zones ought to converge as the water flows toward the bay and the layer of fresh groundwater thins (9). In fact, most of the discharge of groundwater to the bay is saline in composition, a result of the mixing of the terrestrially derived freshwater with bay water prior to discharge. It could be expected then that most fresh groundwater may be exposed to an AOA population prior to discharge, when it is recalled that each zone where bay water and freshwater mix, i.e., the surface salt zone and the transition to the deep salt wedge, harbor large populations of AOA. Future work will address the activity of these communities and examine the potential of denitrification in these zones to yield a better understanding of how nitrogen is cycled and removed from this anthropogenically influenced groundwater system.

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