Applied and Environmental Microbiology, Dec. 2006, p. 7767–7777 Vol. 72, No. 12
0099-2240/06/$08.00+0 doi:10.1128/AEM.00946-06

Diversity of Ammonia-Oxidizing Archaea and Bacteria in the Sediments of a Hypernutrified Subtropical Estuary: Bahía del Tóbari, Mexico[▽]

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Received 20 April 2006/Accepted 22 September 2006

Nitrification within estuarine sediments plays an important role in the nitrogen cycle, both at the global scale and in individual estuaries. Although bacteria were once thought to be solely responsible for catalyzing the first and rate-limiting step of this process, several recent studies have suggested that mesophilic *Crenarchaeota* are capable of performing ammonia oxidation. Here we examine the diversity (richness and community composition) of ammonia-oxidizing archaea (AOA) and bacteria (AOB) within sediments of Bahía del Tóbari, a hypernutrified estuary receiving substantial amounts of ammonium in agricultural runoff. Using PCR primers designed to specifically target the archaeal ammonia monooxygenase α-subunit (*amoA*) gene, we found AOA to be present at five sampling sites within this estuary and at two sampling time points (January and October 2004). In contrast, the bacterial *amoA* gene was PCR amplifiable from only 40% of samples. Bacterial *amoA* libraries were dominated by a few widely distributed *Nitrosomonas*-like sequence types, whereas AOA diversity showed significant variation in both richness and community composition. AOA communities nevertheless exhibited consistent spatial structuring, with two distinct end member assemblages recovered from the interior and the mouths of the estuary and a mixed assemblage from an intermediate site. These findings represent the first detailed examination of archaeal *amoA* diversity in estuarine sediments and demonstrate that diverse communities of *Crenarchaeota* capable of ammonia oxidation are present within estuaries, where they may be actively involved in nitrification.

Nitrification, the microbial oxidation of ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-), plays a critical biogeochemical role in both individual ecosystems and the global nitrogen (N) cycle. This process is of particular significance in estuarine sediments, where it is frequently linked to anaerobic N losses via denitrification and anaerobic ammonium oxidation. In individual estuaries, coupled nitrification/denitrification can remove a substantial percentage (10 to 80%) of anthropogenic N pollution (43), while globally, ca. 30% of all fixed-N loss occurs in estuarine and continental-shelf sediments (15). Despite the evident importance of nitrification, surprisingly little is known about the microorganisms that mediate this process in the natural environment.

Previous studies have focused on the ammonia-oxidizing bacteria (AOB), microorganisms that are known to perform the first and rate-limiting step of chemoautotrophic nitrification, ammonia oxidation. AOB molecular studies have targeted both the 16S rRNA gene—because known AOB are phylogenetically restricted to the beta- and gammaproteobacteria—and the *amoA* gene, which encodes the catalytic α subunit of the ammonia monooxygenase enzyme. In one of the first 16S rRNA-based estuarine studies, de Bie et al. (8) reported distinct geographical patterns in AOB sequence distributions linked to steep gradients of salinity, ammonium (NH_4^+), and oxygen concentrations in the Scheldt estuary. Similarly, studies targeting the bacterial *amoA* gene in the Chesapeake Bay and Plum Island Sound estuaries showed shifts in AOB diversity related to differences in salinity (3, 12). Although these data have contributed significantly to understanding AOB dynamics in estuarine sediments, what is currently known about the microbial ecology of ammonia oxidation and nitrification must be reassessed following the discovery of nitrification among microorganisms from the domain *Archaea*.

Archaea constitute a ubiquitous and exceptionally abundant component of microbial communities (9, 14, 21, 45), with mesophilic members of the kingdom *Crenarchaeota* alone numbering as many as 10^{28} cells in the ocean (21). Although the roles played by these organisms in marine biogeochemistry have remained elusive, multiple converging lines of evidence now suggest that many *Crenarchaeota* are capable of performing ammonia oxidation. These include metagenomic evidence for ammonia monooxygenase (*amo*) genes on an archaeon-associated scaffold from the Sargasso Sea (48) and on the same 43-kb metagenomic fragment as a 16S rRNA gene from group 1.1b soil *Crenarchaeota* (46), as well as cultivation of *Nitrosopumilus maritimus*, a member of the marine group 1.1a *Crenarchaeota* that grows chemoautotrophically, oxidizes ammonia to nitrite, and contains putative *amoA*, *amoB*, and *amoC* genes (22).

The widespread presence of archaeal *amoA* genes in marine water columns and sediments indicates that the ability to oxidize ammonia may be broadly distributed within the *Crenarchaeota* and biogeochemically important in the ocean (13). In fact, Ingalls et al. (20) demonstrated that ca. 83% of in situ archaeal production in mesopelagic waters of the North Pacific is chemoautotrophic—consistent with ammonia oxidation (20)—

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▽ Published ahead of print on 29 September 2006.

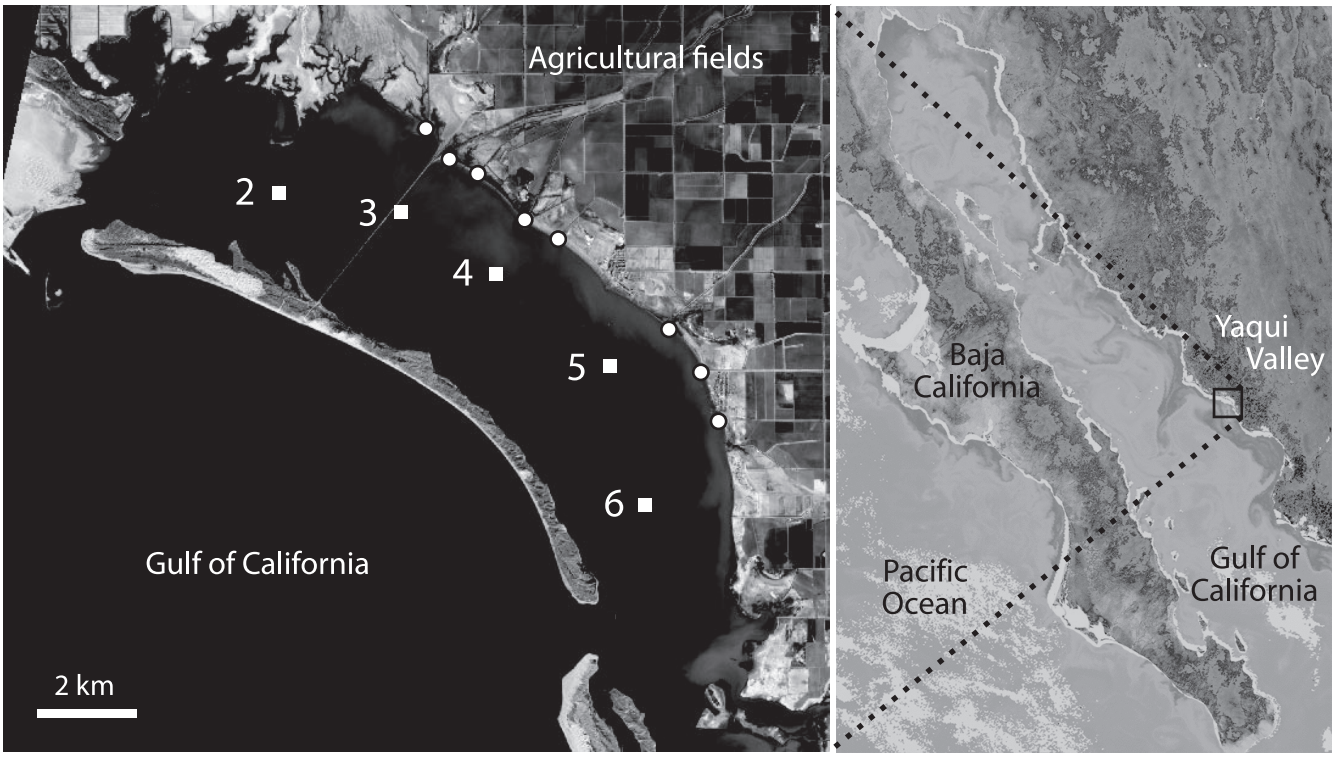


FIG. 1. Locations of Bahía del Tóbari and sampling transect (left) and Yaqui Valley-Gulf of California region of northwest Mexico (right). Locations of sampling points within Bahía del Tóbari plotted on 10-m resolution panchromatic data from the Advanced Land Imager (data provided by National Aeronautics and Space Administration and the Carnegie Institution, courtesy of G. P. Asner). The filled white circles show the locations of agricultural-drain inputs into Tóbari. The scale bar is at the lower left.

while the genome of the archaeal sponge symbiont *Cenarchaeum symbiosum* contains genes predicted to encode pathways involved in autotrophic carbon fixation, as well as *amoA*, *amoB*, and *amoC* genes similar to those found in *N. maritimus* (16). More recently, Park et al. (33) documented the occurrence of ammonia-oxidizing archaea (AOA) in nitrifying wastewater treatment plant bioreactors, while Wuchter et al. (50) found archaeal *amoA* gene copy numbers to be 10 to 1,000 times those of bacterial *amoA* in the North Sea and North Atlantic and comparable to cell counts of *Crenarchaeota*—findings that suggest most pelagic *Crenarchaeota* are AOA and that these organisms are the numerically dominant ammonia oxidizers in the ocean (50). Similar conclusions can be drawn from a study that analyzed a range of soils: archaeal *amoA* gene copy numbers were up to 3,000 times those of bacteria and correlated with *Crenarchaeota*-specific lipids, including crenarchaeol (24).

Taken together, these data demonstrate convincingly that many *Crenarchaeota* are capable of oxidizing ammonia to nitrite, yet no study has examined AOA diversity in detail within estuarine sediments, where this process is of particular significance. Based on archaeal and bacterial *amoA* genes, we analyzed AOA and AOB diversity in Bahía del Tóbari, a subtropical estuary located along the northwest coast of mainland Mexico. Nitrification is expected to be biogeochemically important in this estuary, because it receives substantial amounts of N in runoff from the adjacent Yaqui Valley agricultural system, and this N-rich runoff can fuel large phytoplankton blooms in the Gulf of California downstream (2). Initial characterization of two sites in Tóbari found diverse AOA communities within the sediments of the estuary (13); here, we greatly expand these analyses, examining AOA diversity across a five-site transect, at two points in time, and in comparison to AOB diversity.

MATERIALS AND METHODS

Site description. Bahía del Tóbari is an ~90-km^2 estuary located along the northwest coast of mainland Mexico at 27.08°N 109.96°W (Fig. 1) and is situated between the Yaqui Valley—a 225,000-ha region of intensive wheat-based agriculture (27)—and the Gulf of California—one of the most productive and biodiverse regions of the world's oceans (35, 52). The aridity of this broader region (the Sonoran Desert) has important implications for estuarine circulation within Bahía del Tóbari and the amount, source, and timing of freshwater runoff that it receives.

Circulation within many Gulf of California estuaries is characterized as "negative," driven by evaporative generation of warm, salty waters that flow out of the estuaries at depth, balanced by shallow inflow. Some are also "mixed" estuaries, exhibiting characteristics of both negative estuaries and "positive" estuaries in different seasons (47). Bahía del Tóbari is a mixed estuary in which physical circulation is driven primarily by wind and tides (M. E. Cruz-Colin, S. G. Monismith, A. Valle-Levinson, and J. A. Delgado-Contreras, unpublished data) and inhibited by a causeway and two small bridges that span the width of the estuary (Fig. 1). The Yaqui Valley and Bahía del Tóbari also receive very little precipitation, so that agriculture in the Yaqui Valley is irrigated, and what little freshwater reaches Tóbari arrives almost entirely in the form of agricultural runoff. Irrigation canals transport water to Yaqui Valley wheat fields from reservoirs located in the adjacent foothills of the Sierra Madre, and the fields are in turn drained by a separate system of drainage canals. In multiple cases, these drainage canals discharge into the interior of Bahía del Tóbari, transporting agricultural runoff directly to the estuary (Fig. 1). Of the 2.9×10^9 m^3 of

irrigation water that is typically applied to the Yaqui Valley as a whole, about half is lost via evapotranspiration, and only 15% flows out of the agricultural fields (42); however, fertilizer application rates are exceptionally high in the Yaqui Valley (27), and N is applied primarily as urea or anhydrous ammonia. As a result, Tóbari receives substantial amounts of N in runoff, and primarily in the form of dissolved NH_4^+ (17). Runoff occurs in four large, discrete pulses per year—in late January, early March, early April, and late November—following irrigation in the Yaqui Valley (2).

Sample collection and biogeochemical analyses. Surface sediments were collected from Bahía del Tóbari using box cores deployed off "pangas"—small, outboard motor-powered fishing vessels—in January and October 2004. Cores were collected using cutoff 5-cm^3 syringes, frozen immediately on dry ice, and stored at −80°C until the DNA was extracted. Salinity and temperature were measured using a handheld YSI-85 dissolved-oxygen, conductivity, salinity, and temperature instrument (YSI Incorporated, Yellow Springs, OH), and water samples were collected for nutrient analyses in 10-ml Lachat tubes, immediately frozen on dry ice, and stored at −20°C until analysis. An Alpkem Flow Solution IV autoanalyzer was used to measure NH_4^+ concentrations in water samples (32). The instrument was calibrated with five standards ranging from 2.22 to 139 μM, with an R^2 value of 0.997 over the linear range of the measurement.

DNA extraction and PCR amplification, cloning, and sequencing of *amoA* gene fragments. DNA was extracted from ca. 0.25 g of sediment (0- to 0.5-cm depth interval) using the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA). Archaeal *amoA* PCR primers were designed based on alignments and deduced amino acid sequences of *amoA* genes from the Sargasso Sea and German soil, as previously reported by Francis et al. (13). Archaeal *amoA* gene fragments (~635 bp) were amplified with the PCR primers Arch-amoAF (5′-STAATGGTCTGG CTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) using 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. Bacterial *amoA* gene fragments (~490 bp) were amplified from sediment DNA extracts using PCR primers (AmoA-1F* and AmoA-2R) and conditions described previously (36, 45a). Triplicate PCR products were pooled, gel purified, and cloned using the TOPO-TA cloning kit (Invitrogen). White transformants were transferred to 96-well plates containing LB broth (with 50 μg/ml kanamycin), grown overnight at 37°C, and PCR screened directly for the presence of inserts using T7 and M13R vector primers. Sequencing of T7/M13 PCR products was performed using vector primers on ABI 3730xl capillary sequencers (PE Applied Biosystems).

Richness, phylogenetic, and statistical analyses. Nucleotide sequences were assembled and edited using Sequencher v.4.2 (GeneCodes, Ann Arbor, MI). For archaeal *amoA*, phylogenetic analysis was conducted on a 594-bp region corresponding to most of the gene. A total of 694 archaeal *amoA* sequences were analyzed, including 218 new sequences from Tóbari, 370 environmental sequences from Francis et al. (13) (accession no. DQ14825 to DQ14848 and DQ148573 to DQ148905), three water column sequences from Monterey Bay and Antarctic surface waters reported by Hallam et al. (16) (accession no. DQ333419, DQ333421, and DQ433422), 24 sediment sequences from Park et al. (33) (accession no. DQ278569 to DQ278592), 74 soil sequences from Leininger et al. (24) (accession no. DQ534815 to DQ534888), metagenomic sequences from the Sargasso Sea (accession no. AACY01435967) and German soil (accession no. AJ627422), the *amoA* gene from *N. maritimus* (accession no. DQ085098), and two *amoA* genes from *C. symbiosum* (accession no. DQ397569 and DQ397580). Additional environmental sequences from marine water columns (13, 16, 50), sediments (13), and soils (24) less than 594-bp in length were excluded from this analysis.

For bacterial *amoA*, a 449-bp region was selected for phylogenetic analysis. For both genes, nucleotide and amino acid alignments were generated using MacClade (http://macclade.org). All bacterial *amoA* sequences were compared with GenBank database sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), and the three closest matches were included in the tree, along with various cultivated AOB sequences (see Fig. 3 for the accession numbers).

Operational taxonomic units (OTUs) were defined as sequence groups in which sequences differed by ≤5%, and all analyses of richness—including rarefaction analysis (18) and Chao1 nonparametric richness estimations (5)—were performed using the software program DOTUR (39). Neighbor-joining phylogenetic trees (based on Jukes-Cantor-corrected distances) and parsimony trees were constructed based on alignments of DNA sequences using ARB (http://www.arb-home.de) (25). We elected to analyze nucleic acid rather than predicted amino acid sequences to highlight the genetic (rather than enzyme level) heterogeneity among sites and time points. Distance-based and parsimony-based bootstrap analyses were conducted in PAUP *4.0b10 (Sinauer Associates) and used to estimate the reliability of phylogenetic reconstructions, with 1,000 replicates for both archaeal and bacterial *amoA*.

Observed differences in community composition between libraries were statistically compared using ∫-Libshuff (41), with 10,000 randomizations and a distance interval (D) of 0.01 on PAUP-generated Jukes-Cantor pairwise distance matrices. ∫-Libshuff uses Monte Carlo methods to calculate the Cramér-von Mise statistic by constructing random communities from the entire data set and comparing the coverage of random communities to the observed coverage of our libraries. Significant P values were evaluated after correcting for multiple pairwise comparisons using the Dunn-Sidák method (44), where $P_{\text{corrected}} = 1 - (1 - P_{\text{uncorrected}})^{1/k}$, and k is the total number of comparisons (for 10 archaeal *amoA* libraries, each compared with the corresponding 9 other libraries, $k = 10 \times 9 = 90$). In cases where coverage of the homologous library is significantly different from a randomly constructed heterologous library, we can be reasonably certain that the clone libraries represent distinct populations. To compare overlap between duplicate archaeal *amoA* clone libraries and original libraries, abundance-based Sørensen similarity indices (6) and the standard errors of these values were calculated using the program SONS (40).

Nucleotide sequence accession numbers. The *amoA* gene sequences reported in this study have been deposited in GenBank under accession numbers DQ500959 to DQ501176 (archaeal) and DQ501177 to DQ501238 (bacterial).

RESULTS AND DISCUSSION

Study site biogeochemistry. Ammonia oxidation is biogeochemically important in most estuaries, and this microbially mediated process is expected to be particularly significant in the sediments of Bahía del Tóbari, Mexico: extremely high rates of fertilizer application in adjacent agricultural fields of the Yaqui Valley result in large N losses (27), much of the N lost via runoff occurs in the form of NH_4^+ (17), and this N is known to fuel large phytoplankton blooms in the downstream Gulf of California (2). In this context, we examined ammonia-oxidizing microbial communities—as well as NH_4^+ concentrations, temperature, and salinity—at multiple sites along an ~10-km transect starting at the north mouth (site 2) and ending at the south mouth (site 6) of Bahía del Tóbari (Fig. 1). "Mouth" sites 2 and 6 were located in 4- to 8-m-deep channels near the mouths of the estuary, while "interior" sites 3, 4, and 5 were located in ~1.5 m of water, on average (Fig. 1).

Salinity data showed sites 2 and 6 to be essentially marine (34.6 to 36.6 practical salinity units [psu]), while sites 3 and 4 (and site 5 in January) showed slightly depressed salinities due to freshwater input (Table 1). Consistent with the aridity of the Gulf of California region, salinities and temperatures were higher at all sites in October 2004 than in January 2004 (Table 1). In January 2003, NH_4^+ concentrations as high as 115 μM were associated with lower salinities in the interior of the bay; however, when sediment samples were collected in January 2004, the NH_4^+ concentrations were much lower—most likely a result of reduced agricultural activity within the Yaqui Valley due to drought. Values in January 2004 were more comparable to those observed in October 2003 and October 2004 (Table 1). Given the similarities between NH_4^+ concentrations measured at these different time points, these may represent "baseline" NH_4^+ concentrations within Tóbari; however, these concentrations (22.2 to 45.0 μM) are quite high relative to many estuarine systems (e.g., the Chesapeake Bay and San Francisco Bay) (12, 49).

Amplification and cloning of bacterial and archaeal *amoA* genes. Bahía del Tóbari sediment samples were collected in January and October 2004 for DNA extraction and PCR amplification of archaeal and bacterial *amoA* genes. Interestingly, while archaeal *amoA* was successfully amplified from all five

TABLE 1. Physical and biogeochemical characteristics of sampling sites[a]

Site	2004						2003			
	Salinity (psu)		Temp (°C)		NH_4^+ (μM)		Salinity (psu)	Temp (°C)	NH_4^+ (μM)	
	January	October	January	October	January	October	January	January	January	October
2	35.2	36.6	18.1	25.2	22.1	45.0	35.2–35.7	17.8–19.5	60.8–71.5	26.2
3	34.2	34.9	18.7	23.8	80.4	22.3	33.4–34.6	17.8–18.9	69.9–115	29.7
4	34.0	35.3	19.0	24.9	22.9	40.1	33.5–34.8	18.1–19.6	61.1–91.7	36.7
5	33.9	36.0	18.6	25.5	27.1	26.5	34.1–35.3	18.5–19.3	66.5–89.1	24.1
6	34.6	36.1	18.4	26.9	34.2	28.9	34.8–35.4	18.3–20.5	65.0–70.5	22.7

[a] Data from 2004 were collected during sampling for molecular analyses. Data from January 2003 are presented as the range of values observed from 14 to 23 January 2003; each site was sampled every 1 to 3 days during this period (five times in total) and once in October 2003 for analysis of NH_4^+ concentrations only.

sites in both January and October, bacterial *amoA* could only be amplified—despite repeated screening—from 4 of 10 samples: site 2 in January, site 4 in January and October, and site 5 in October (Table 2). Clearly, these results should not be considered conclusive evidence for the absence of AOB in the other six samples; however, they are suggestive of differences between bacterial and archaeal ammonia oxidizer communities in Bahía del Tóbari, and in light of recent studies showing AOA to be more abundant than AOB in both the marine water column (50) and soils (24), they may represent evidence for numerical dominance of AOA in estuarine sediments, as well.

Diversity of bacterial *amoA* gene sequences. To assess AOB diversity within the sediments of Bahía del Tóbari, bacterial *amoA* gene libraries were generated for both site 4 samples (January and October 2004), where the widest range of sequence types was expected to be found based on results for the archaeal *amoA* gene (see below). We sequenced 24 clones from the January library and recovered six OTUs (based on a 5% cutoff) and sequenced 37 clones from the October library and recovered seven OTUs. These represent virtually all of the OTUs predicted by the Chao1 estimator, which yielded a value of 7 for both January and October (Table 2). These levels of richness are comparable to those found in other estuarine environments (e.g., the Chesapeake Bay) (12) but appear to be slightly lower than archaeal *amoA* richness within the same samples based on rarefaction analysis (Fig. 2).

Bacterial *amoA* sequences recovered from Tóbari were compared with the nearest database sequences, and although previous studies recovered both *Nitrosomonas*- and *Nitrosospira*-like *amoA* sequences from estuarine environments (3, 12), only *Nitrosomonas*-like sequences were recovered from Tóbari (Fig. 3). This is consistent with a study that used different PCR primers but recovered only *Nitrosomonas*-like sequences from marine sediments of the Pacific Northwest (30) yet inconsistent with a study in the water column of the Guaymas basin (located approximately 150 km north of Tóbari), which recovered both *Nitrosospira*- and *Nitrosomonas*-like sequences (23). It is therefore possible that *Nitrosospira*-like sequences are present within Tóbari yet extremely rare within our clone libraries from site 4 or that conditions at site 4 may simply favor *Nitrosomonas* over *Nitrosospira* species. The nearest cultivated organism to Tóbari sequences was the estuarine isolate *Nitrosomonas* sp. strain Nm143, which shared 86 to 89% nucleotide sequence identity and 93 to 95% amino acid identity with MX-JAN-1, MX-JAN-4, MX-OCT-4, and MX-OCT-7 (see the legend to Fig. 4 for an explanation of sequence names).

TABLE 2. Observed and estimated richnesses of archaeal and bacterial *amoA* gene libraries[a]

Site	Archaeal *amoA*			Bacterial *amoA*[b]		
	No. of clones sequenced	No. of OTUs (5%)	Chao1 (5%)	PCR amplification/ no. of clones sequenced	No. of OTUs (5%)	Chao1 (5%)
January						
2	32	4	5	+		
3	24	4	5	−		
4	30	9	12	24	6	7
5	23	6	12	−		
6	32	10	13	−		
October						
2	31	8	9	−		
3	24	14	21	−		
4	32	8	8	37	7	7
5	22	6	7	+		
6	32	10	15	−		
Total	282	42	71	61	9	9

[a] OTUs were defined as 5% difference in nucleic acid sequence alignment, and Chao1-estimated richness was calculated using DOTUR.

[b] For bacterial *amoA*, positive PCR amplification is indicated by a plus sign or the number of clones screened; unsuccessful amplification is indicated by a minus sign.

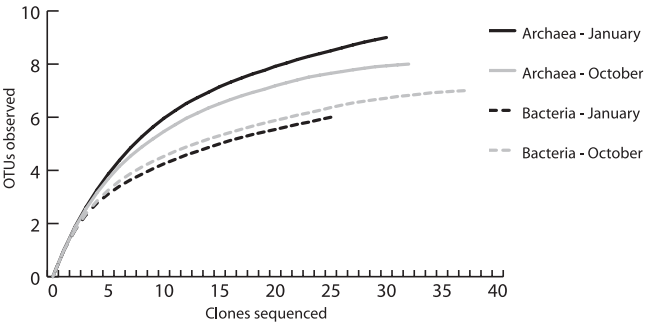


FIG. 2. Rarefaction curves showing relative richnesses of archaeal and bacterial *amoA* genes at site 4 within Tóbari. January libraries are shown in black and October libraries in gray, with archaeal *amoA* data shown as solid lines and bacterial data as dashed lines. OTUs were defined based on a 5% cutoff; 95% confidence intervals for the curves are not shown, as these values are identical to the observed richnesses at the end points of the curves.

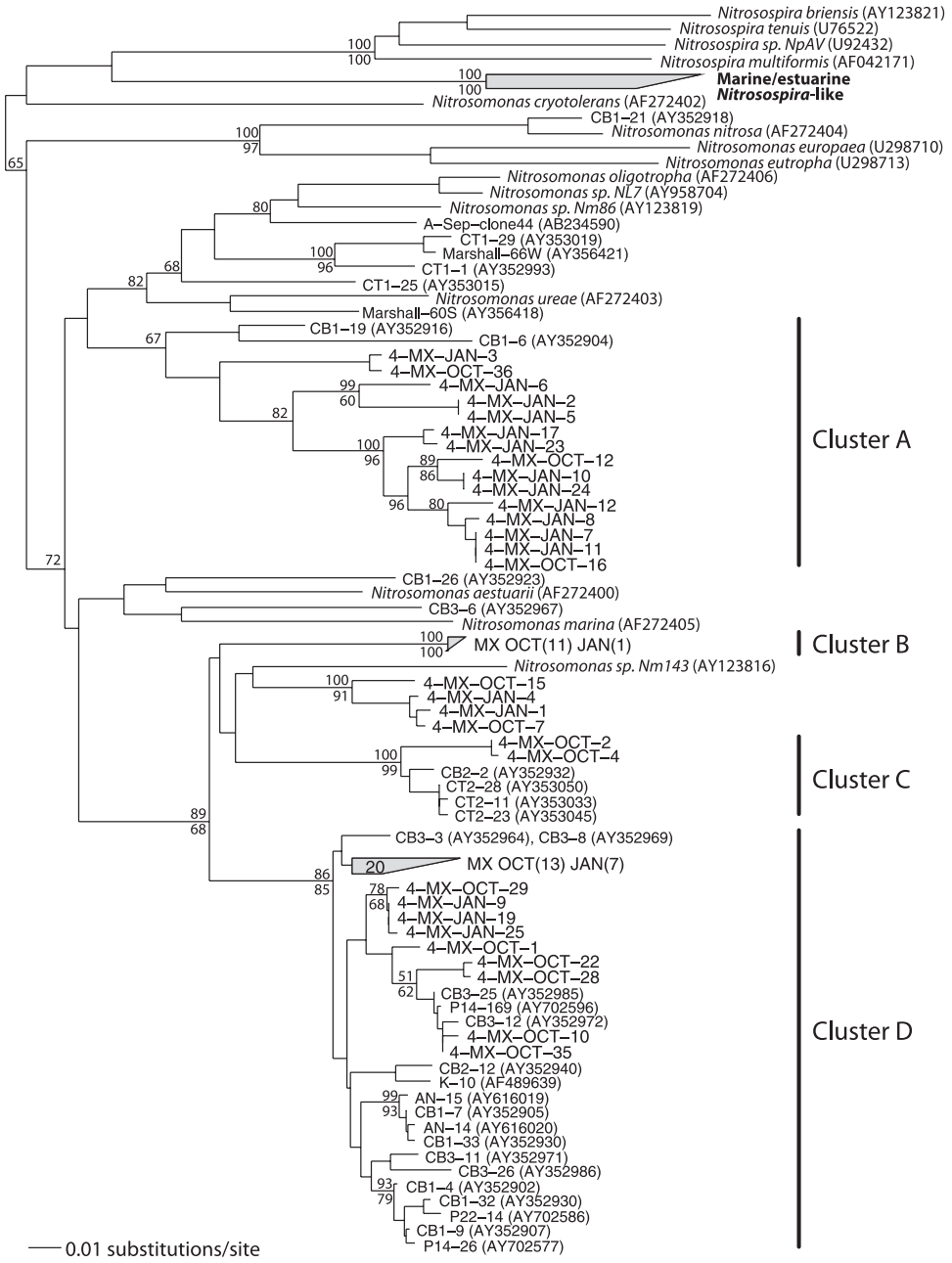
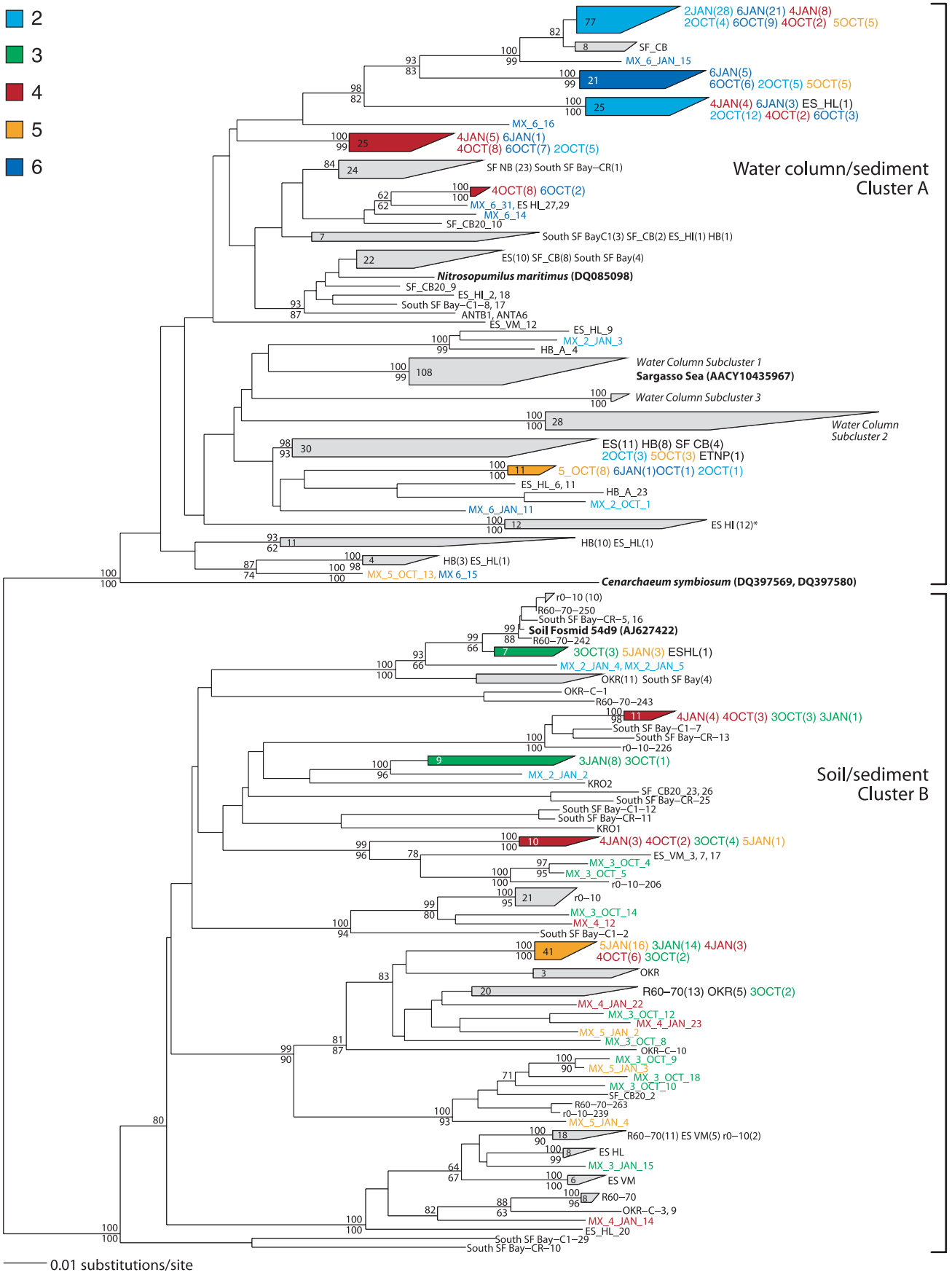


FIG. 3. Phylogenetic relationships among bacterial *amoA* sequences from Bahía del Tóbari, closely related database sequences, and cultivated AOB (accession numbers are in parentheses). Clusters containing Tóbari sequences referred to in the text are labeled on the right. Bootstrap values (>60%) are indicated at branch points, with distance bootstrap values above the line and parsimony values below. The scale bar is at the lower left. The tree is neighbor joining, based on Jukes-Cantor-corrected DNA distances, and rooted with *Nitrosospira* and *Nitrosospira*-like sequences and *Nitrosomonas cryotolerans*.

In a DNA-based phylogenetic tree, Tóbari bacterial *amoA* sequences fell into four main clusters (A, B, C, and D) (Fig. 3). Cluster A was dominated by 12 January sequences, contained 3 sequences from October, and included several distinct subclusters. In contrast, cluster B contained 11 October sequences and 1 January sequence, all of which were >99% identical at the DNA level. In addition, there is distance-based bootstrap support for the inclusion of database sequences from Chesapeake Bay (CB1-6 and CB1-19) in cluster A, whereas cluster B formed a distinct branch of the tree. Cluster C comprised two sequences from Tóbari and several Chesapeake Bay and Choptank River sequences, while nearly half of the bacterial *amoA* sequences recovered from Tóbari fell into cluster D. These 29 sequences were closely related to sequences from the Chesapeake Bay (12), Plum Island Sound (3), Kysing Fjord (29), and Norsminde Fjord (34). Upon translation, sequences in cluster D shared >98% amino acid identity, with many of the Tóbari sequences identical to database sequences at the


2
3
4
5
6
2JAN(28) 6JAN(21) 4JAN(8)
2OCT(4) 6OCT(9) 4OCT(2) 5OCT(5)
SF_CB
MX_6_JAN_15
6JAN(5)
6OCT(6) 2OCT(5) 5OCT(5)
4JAN(4) 6JAN(3) ES_HL(1)
2OCT(12) 4OCT(2) 6OCT(3)
MX_6_16
4JAN(5) 6JAN(1)
4OCT(8) 6OCT(7) 2OCT(5)
SF NB (23) South SF Bay–CR(1)
4OCT(8) 6OCT(2)
MX_6_31, ES HL_27,29
MX_6_14
SF_CB20_10
South SF BayC1(3) SF_CB(2) ES_HI(1) HB(1)
ES(10) SF_CB(8) South SF Bay(4)
Nitrosopumilus maritimus (DQ085098)
SF_CB20_9
ES_HI_2, 18
South SF Bay–C1–8, 17
ANTB1, ANTA6
ES_VM_12
ES_HL_9
MX_2_JAN_3
HB_A_4
Water Column Subcluster 1
Sargasso Sea (AACY10435967)
Water Column Subcluster 3
Water Column
Subcluster 2
ES(11) HB(8) SF CB(4)
2OCT(3) 5OCT(3) ETNP(1)
5_OCT(8) 6JAN(1)OCT(1) 2OCT(1)
ES_HL_6, 11
HB_A_23
MX_2_OCT_1
MX_6_JAN_11
ES HI (12)*
HB(10) ES_HL(1)
HB(3) ES_HL(1)
MX_5_OCT_13, MX 6_15
Cenarchaeum symbiosum (DQ397569, DQ397580)
Water column/sediment
Cluster A
r0–10 (10)
R60–70–250
South SF Bay–CR–5, 16
Soil Fosmid 54d9 (AJ627422)
R60–70–242
3OCT(3) 5JAN(3) ESHL(1)
MX_2_JAN_4, MX_2_JAN_5
OKR(11) South SF Bay(4)
OKR–C–1
R60–70–243
4JAN(4) 4OCT(3) 3OCT(3) 3JAN(1)
South SF Bay–C1–7
South SF Bay–CR–13
r0–10–226
3JAN(8) 3OCT(1)
MX_2_JAN_2
KRO2
SF_CB20_23, 26
South SF Bay–CR–25
South SF Bay–C1–12
South SF Bay–CR–11
KRO1
4JAN(3) 4OCT(2) 3OCT(4) 5JAN(1)
ES_VM_3, 7, 17
MX_3_OCT_4
MX_3_OCT_5
r0–10–206
r0–10
MX_3_OCT_14
MX_4_12
South SF Bay–C1–2
5JAN(16) 3JAN(14) 4JAN(3)
4OCT(6) 3OCT(2)
OKR
R60–70(13) OKR(5) 3OCT(2)
MX_4_JAN_22
MX_3_OCT_12
MX_4_JAN_23
MX_5_JAN_2
MX_3_OCT_8
OKR–C–10
MX_3_OCT_9
MX_5_JAN_3
MX_3_OCT_18
MX_3_OCT_10
SF_CB20_2
R60–70–263
r0–10–239
MX_5_JAN_4
R60–70(11) ES VM(5) r0–10(2)
ES HL
MX_3_JAN_15
ES VM
R60–70
OKR–C–3, 9
MX_4_JAN_14
ES_HL_20
South SF Bay–C1–29
South SF Bay–CR–10
Soil/sediment
Cluster B
0.01 substitutions/site

amino acid level. Given the variability in salinity, temperature, and N loads observed within Tóbari in relation to other estuarine and coastal environments, our results support the contention that this *Nitrosomonas*-like group represents AOB capable of withstanding an extensive range of environmental conditions (12). Likewise, previous authors have suggested that this group represents a widespread bacterial *amoA* sequence type, in light of its frequent recovery from marine and estuarine sediments and broad geographic distribution (3). Our results lend further support to this hypothesis, as 48% of the bacterial *amoA* sequences recovered from Tóbari fell into this group.

Diversity of archaeal *amoA* gene sequences. Archaeal *amoA* clone libraries were generated for all five sites within Bahía del Tóbari for both the January and October sampling time points. From these 10 libraries, a total of 282 clones were sequenced and 42 OTUs were recovered, based on a 5% cutoff. The numbers of OTUs recovered in individual libraries ranged from 4 OTUs at sites 2 and 3 in January to 14 OTUs at site 3 in October, and extrapolated richness values based on the Chao1 estimator—often considered a lower bound on total richness (4)—ranged from 5 to 21 OTUs (Table 2). For both observed richness and Chao1-estimated richness, the highest and lowest richnesses occurred in archaeal *amoA* libraries from site 3.

The structures of these AOA communities were examined via phylogenetic analysis of 694 archaeal *amoA* sequences. They included the 218 sequences from Bahía del Tóbari produced in this study, as well as 370 sequences from soils, marine water columns, and sediments (including 64 sequences from Tóbari) reported previously by Francis et al. (13); metagenomic sequences from Antarctic surface waters and Monterey Bay (16); sediment sequences from southern San Francisco Bay (33); soil sequences from Norway and Germany (24); metagenomic sequences from the Sargasso Sea (48) and German soil (46); and *amoA* genes from *N. maritimus* (22) and *C. symbiosum* (16). In a DNA-based phylogenetic tree, these sequences fell into two primary groups: 456 sequences fell into a large "water column/sediment" cluster (cluster A), and 238 sequences fell into a "soil/sediment" cluster (cluster B) (Fig. 4). The water column/sediment and soil/sediment clusters were distinct from each other, with any two sequences in these clusters sharing only 66 to 76% identity at the nucleotide level.

Sequences from Bahía del Tóbari fell into both cluster A and cluster B. Within cluster A, all but three of the water column sequences from Antarctic surface waters, the Black Sea, Monterey Bay, and the eastern tropical North Pacific fell into three subclusters ("water column subclusters" in Fig. 4). In contrast, none of the 282 sequences recovered from Tóbari fell into these subclusters, lending further support to the idea that archaeal *amoA* sequences recovered from sediments and from water columns are, for the most part, phylogenetically distinct (13). Tóbari sequences shared only 69 to 90% nucleotide sequence identity with the *N. maritimus* sequence (22), despite the fact that the *N. maritimus* sequence grouped closely with sequences from Elkhorn Slough and San Francisco Bay sediments.

Of the 182 sequences from Tóbari that fell in the water column/sediment cluster, 124 fell into a distinct region in the upper portion of the tree. This region of the tree was comprised of several subclades dominated by Tóbari sequences, with only nine sequences recovered from other environments (Fig. 4). Within these clusters, there was a significant amount of overlap between sequences from different sites; however, these clusters contained only sequences from sites 2, 4, and 6 (both January and October) and site 5 in October—no sequences from site 3 fell into water column/sediment cluster A, nor did any sequences from site 5 in January. Similarly, no sequences from site 6 and only three sequences from site 2 fell into soil/sediment cluster B. All site 3 sequences, all January site 5 sequences, and all archaeal *amoA* sequences from soils fell into cluster B (KRO, OKR, r0-10, and R60-70 sequences in Fig. 4).

Statistical analysis of AOA community structure. These observed variations in community composition were statistically compared using ∫-Libshuff (41), an approach that has been applied to 16S rRNA gene libraries (11) and was recently shown to be effective for comparison of N-cycling microbial communities based on functional genes, including nitrous oxide reductase (*nosZ*), nitrite reductase (*nirS* and *nirK*), and nitrogenase (*nifH*) gene libraries (19, 37, 51). ∫-Libshuff compares the coverage of clone libraries to a hypothetical coverage generated from multiple randomizations of sequences and calculates a population *P* value. If the comparisons between both library X and library Y and library Y and library X yield significant (<0.05) *P* values, the patterns displayed by the libraries are most likely representative of distinct populations (Table 3). In cases where Y versus X produces a significant *P* value but X versus Y does not, library X is considered a subset of library Y. The opposite is also true, where library Y is a subset of library X.

Based on this analysis, only at site 5 were the January and

FIG. 4. Phylogenetic relationships among archaeal *amoA* sequences from Bahía del Tóbari and previously reported environmental sequences. The sequences are color coded according to the sampling site within Tóbari. The sequence names denote the overall location (Mexico), the sampling site (2 to 6), the sampling time point (January or October), and the individual sequence number. For example, "MX-2-JAN-1" indicates Mexico site 2, January sampling, sequence 1. Previously reported site 4 and 6 October sequences do not contain "OCT" within the sequence names. Other environmental sequences are shown in black, and critical database sequences in boldface (see the text). The clusters are color coded by the most abundant sampling site represented in the cluster, and the sampling sites and times of the represented sequences are indicated, followed by the number of corresponding sequences in parentheses. These are ordered by abundance within the cluster. Next to large clusters, January sequences appear on the first line and October sequences on the lower line. Bootstrap values (>60%) are indicated at branch points, with distance bootstrap values above the line and parsimony values below. The scale bar is at the lower left. This tree is a neighbor joining tree based on Jukes-Cantor-corrected DNA distances and is midpoint rooted. Accession numbers corresponding to the 694 sequences represented in this tree are listed in Materials and Methods. The sequence group from Elkhorn Slough (Hummingbird Island) referred to in the text is indicated by an asterisk.

TABLE 3. *P* values from ∫-Libshuff corrected using the Dunn-Sidák method[a]

Homologous library X site	*P* value for heterologous library Y site:									
	January					October				
	2	3	4	5	6	2	3	4	5	6
January										
2		**0**	*0.784*	**0**	*0.692*	*0.194*	**0**	*0.216*	*0.971*	*0.102*
3	**0**		*0.102*	***0.044***	**0**	**0**	*0.759*	*0.094*	**0**	**0**
4	***0***	***0***		***0***	***0***	**0**	**0**	0.968	**0**	***0***
5	**0**	*0.165*	*0.748*		**0**	**0**	0.999	*1.000*	**0**	**0**
6	**0**	**0**	*0.488*	**0**		0.290	**0**	*0.187*	0.899	0.397
October										
2	***0***	**0**	**0.018**	**0**	0.061		**0**	**0.009**	**0**	1.000
3	**0**	***0***	**0.009**	0.478	**0**	**0**		*0.061*	**0**	**0**
4	***0***	***0***	0.413	***0***	***0***	**0**	**0**		**0**	**0**
5	***0***	**0**	**0**	**0**	0.925	*1.000*	**0**	**0**		1.000
6	***0***	**0**	*0.565*	**0**	0.815	1.000	**0**	0.613	0.150	

[a] In comparisons between the homologous library X and the heterologous library Y, boldface *P* values indicate that the libraries are drawn from significantly ($P < 0.05$) different communities, while italics indicate that library X is a subset of library Y, and bold italics indicate that library Y is a subset of X.

October archaeal *amoA* gene libraries significantly different from one another (Table 3). Sites 4 and 6 were statistically indistinguishable in January and October, whereas January libraries from sites 2 and 3 were subsets of the October libraries—most likely reflecting the greater OTU richness recovered in October libraries from sites 2 and 3 (Table 2). The general lack of statistically significant differences between January and October libraries suggests that AOA community compositions are fairly stable at sites 2, 3, 4, and 6, since there is evidently little variation between AOA communities sampled 9 months apart.

At site 5, both phylogenetic comparisons and those based on ∫-Libshuff show significant differences between the January and October clone libraries. The differences in these libraries may be indicative of a shift in AOA community structure with time; however, further study is needed to confirm this possibility, as well as the more general possibility that AOA communities exhibit such temporal variation. Nevertheless, the clear phylogenetic and statistical differences between the site 5 libraries mean that they are similar to libraries recovered from different sites at different times: that is, the site 5 January library is statistically indistinguishable from the site 3 October library, a subset of libraries drawn from sites 3 and 4 in January, and the site 4 October library; the site 5 October library is indistinguishable from the January and October libraries from site 6 and a subset of the site 2 October library. The site 5 January library is therefore statistically similar to that of interior sites (3 and 4) and the October library to that of mouth sites (2 and 6). In fact, consistent interrelationships among interior sites and among mouth sites are evident based on this analysis: site 3 is a subset of site 4 in both January and October, and sites 2 and 6 are in many cases statistically indistinguishable. These results are consistent with the phylogeny presented in Fig. 4, since sites 2 and 6 are found in many of the same subgroups within cluster A, whereas site 4 sequences fall into both clusters A and B—in the latter case, frequently overlapping with sequences from site 3.

Confirmation of AOA community structure via analysis of duplicate clone libraries. To more thoroughly explore these similarities and differences between and within AOA communities present at different sites, we generated duplicate clone libraries for all sites from an additional set of sediment cores collected in January 2004. All sample collection, extraction, amplification, purification, and cloning protocols were carried out identically for these duplicate "B" libraries, and for purposes of comparison, two libraries (6B and 6C) were generated from pooled PCRs for the January site 6 duplicate. Between 17 and 31 clones were sequenced at random from each of these duplicate libraries, and to avoid confusion in terms of distinguishing Tóbari archaeal *amoA* sequences from different dates and sites, the sequences obtained from the duplicate libraries were not included in Fig. 4.

With the exception of site 4, all of these duplicate libraries were highly similar to the original January libraries: duplicate libraries for sites 2, 3, and 6 were all statistically indistinguishable from the original libraries (∫-Libshuff *P* values ranged from 0.41 to 1.00), while the original library from site 5 was a subset of the 5B library. To compare the similarities of these three sets of libraries (January, October, and duplicates from January), the program SONS (40) was used to calculate abundance-based Sørensen's indices of similarity (L_{abd}) (6) using a 5% OTU definition. These values represent the probability that a randomly selected OTU in either of the two libraries will be found in both libraries (6, 40). Based on this analysis, sites 2 and 6 showed the highest similarity among original and duplicate libraries: similarity indices for the site 2 duplicate library compared with the January and October libraries were 0.86 (±0.24 standard error) and 0.87 ± 0.21, while the site 6 indices were 0.70 ± 0.19 and 0.86 ± 0.12 for the original January and October libraries compared with the 6B library and 0.98 ± 0.13 and 1.0 ± 0.16 for the 6C library. (For purposes of comparison, the 6B and 6C libraries generated from the same pooled PCRs shared an L_{abd} value of 0.93 ± 0.11.) Although the similarity index for the site 3B library and the original January library was only 0.56 ± 0.28, the 3B library shared an L_{abd} of 0.88 ± 0.21 with the October library. This likely reflects the OTU richness recovered in the 3B library relative to the January and October libraries—9 OTUs in the

duplicate library versus 4 OTUs in the January library and 14 in October (Table 2). It may also reflect the fact that the 3B library contained three sequences that fell into cluster A (data not shown), whereas none of the OTUs from the January and October libraries were represented by sequences in this cluster. Along these lines, the site 5 duplicate library (from January) showed no similarity to the October library, which is consistent with the phylogenetic differences between the original January library and the October library.

Surprisingly, neither of the original libraries from site 4 was similar to the duplicate library. Instead, all of the sequences in the 4B library were most closely related (83 to 85% identical) to a group of 12 sequences from a single sampling site (Hummingbird Island) in Elkhorn Slough, California (Fig. 4). This sequence group appears to be relatively rare based on the range of archaeal *amoA* sequences reported thus far and forms a distinct branch in Fig. 4, sharing less than 79% nucleotide identity with other sequences in cluster A. Although the presence of this sequence type in our site 4 duplicate library is both intriguing and difficult to explain, it seems plausible that slight differences in sampling depth between duplicate sediment cores could be a factor (see below). Disregarding the novelty of these sequences relative to the other 14 clone libraries analyzed, it is worth noting that the original site 4 January and October libraries shared an L_{abd} of 0.69 ± 0.15—only the site 6 January and October libraries were more similar (L_{abd} = 0.80 ± 0.18). This relatively high similarity is surprising given the wide phylogenetic distribution of sequences recovered in the site 4 libraries from January and October: sequences from the site 4 libraries fall in both cluster A and B in Fig. 4, and based on statistical analysis, only the October libraries from sites 2 and 5 are not subsets of the site 4 libraries (Table 3). These data suggest that although the original site 4 libraries are diverse, they are similar, since the probability that an OTU found in either library will be present in both is greater than two-thirds. While the 4B library does not show any similarity to the original libraries, the fact that it contains a seemingly "rare" archaeal *amoA* sequence type does support the idea that site 4 harbors a remarkably heterogeneous AOA population. Overall, the duplicate libraries from January corroborate our original findings for sites 2, 3, 5, and 6.

Structuring of AOA communities. Taken together, these clone libraries effectively represent triplicate samples for comparing the spatial variation of AOA communities within Bahía del Tóbari, as each site was sampled in duplicate in January and once in October. Based on these samples, mouth sites 2 and 6 show a great deal of similarity among archaeal *amoA* libraries and are for the most part statistically indistinguishable, and 210 of 213 sequences from these sites fall into the water column/sediment cluster A (Fig. 4). In contrast, site 3 sequences fall almost entirely into the soil/sediment cluster B, and in all cases, the site 3 libraries are statistically different from those of sites 2 and 6. In only two cases do site 3 libraries show any similarity to site 2 and 6 libraries; however, these similarity indices are only 0.05 ± 0.10 (site 3 October and site 2 January) and 0.14 ± 0.14 (site 3 January duplicate and site 6 January) and are not significantly different from zero. Overall, the similarities and differences among these libraries are strongly suggestive of spatial variability in AOA community composition across this estuarine transect: AOA communities within the interior of the bay (site 3) were distinct from those at the mouths of the estuary (sites 2 and 6), whereas site 4 was a mixed assemblage, with sequences falling in clusters A and B and nearly all libraries as a subset of site 4 libraries.

Previous studies using the 16S rRNA gene have found similar structuring of archaeal communities within an estuary. In the Columbia River, Columbia River estuary, and adjoining coastal ocean, Crump and Baross (7) found only "freshwater" sequences in the river to be most closely related to sequences recovered previously from freshwater lake sediments (26), while coastal ocean sequences were predominantly group 1.1a marine *Crenarchaeota* (7). Sequences from the Columbia River estuary were a mixture of freshwater and marine sequences, suggesting that the estuarine archaeal community is a mixture of end member assemblages from the Columbia River and the coastal ocean (7). However, results from other estuarine and sedimentary environments are more varied: despite low salinities of 0 to 4 psu, only 16S rRNA sequences that cluster with "marine" group 1.1a *Crenarchaeota* were recovered from the Duoro estuary sediments (1); no crenarchaeal sequences were recovered from salt marsh sediments of the River Colne (28); and a recent survey of archaeal 16S rRNA genes in soils, freshwater sediments, microbial mats, and a hypersaline pond found archaeal diversity within freshwater sediments to be high, with both group 1.1a and 1.1b *Crenarchaeota* present, in addition to other groups (31).

Although it now appears that many mesophilic *Crenarchaeota* may posses the *amoA* gene (13, 16, 22, 24, 50), which of these crenarchaeal groups are related to specific *amoA* sequence types has, for the most part, yet to be determined. However, given that the 16S rRNA genes from *N. maritimus* and *C. symbiosum* are associated with group 1.1a *Crenarchaeota* and that their *amoA* genes fall in the water column/sediment cluster A, it seems likely that archaeal *amoA* genes associated with *Crenarchaeota* group 1.1a fall predominantly into cluster A. In parallel, the 16S rRNA gene from soil fosmid 54d9 is associated with group 1.1b *Crenarchaeota*, and its *amoA* gene falls in the soil/sediment cluster B, suggesting that archaeal *amoA* genes associated with group 1.1b fall into cluster B. In further support of this idea, approximately equal numbers of archaeal 16S rRNA genes and *amoA* genes were recovered from the RUD soil cDNA library of Leininger et al. (24), and the archaeal 16S rRNA genes in this soil belonged exclusively to *Crenarchaeota* group 1.1b (31). All of the archaeal *amoA* sequences from RUD soil fell into cluster B ("r0-10" and "R60-70" sequences [Fig. 4]). More broadly, group 1.1b *Crenarchaeota* are nearly ubiquitous in soils (10, 31, 38), and all known soil *amoA* sequences fall in cluster B, while group 1.1a *Crenarchaeota* dominate the oceanic water column (10, 38) and all *amoA* sequences from the water column fall into cluster A.

These parallels between 16S rRNA phylogeny and archaeal *amoA* phylogeny are clearly intriguing, yet there are undoubtedly exceptions, and several unknowns remain. For example, other crenarchaeal groups may be associated with the *amoA* sequences in clusters A and B (e.g., marine benthic groups, SAGMCG-1, and groups 1.1c, 1.2, and 1.3) (38), and the factors that ultimately drive differences between crenarchaeal or AOA groups are poorly understood. Some authors have suggested that depth may play a role in structuring AOA commu-

nities in the marine environment (16) and in soils (24). While water column depth differences between mouth sites and interior sites may influence AOA communities within Tóbari, sediment depth is likely to be of greater importance for these sedimentary communities. In this study, we purposely focused on the 0- to 0.5-cm depth interval, where ammonia oxidation is expected to be most active within estuarine sediments. This depth interval may not be strictly comparable across different sites and time points, however, and even subcentimeter differences in the interval analyzed could explain some AOA community differences—for example, the novel sequences recovered in the site 4 duplicate library compared to the original. More simplistically, many of the sequences that fall into the soil/sediment cluster B may be soil-derived sequences transported into the bay via agricultural runoff, since Tóbari receives relatively high sediment loads from the Yaqui Valley drainage canals and flushing is inhibited within the estuary. Clearly, understanding factors, such as depth, that may favor particular AOA "ecotypes" should be the focus of future studies.

Our results demonstrate the presence of at least two broad AOA groups in the sediments of Bahía del Tóbari (clusters A and B) (Fig. 4). Based on phylogenetic and statistical analyses of three sets of archaeal *amoA* gene libraries (15 libraries in total), these groups appear to show spatial structuring across the estuary, and it seems likely that archaeal *amoA* sequences falling in cluster A are associated with group 1.1a *Crenarchaeota* and those in cluster B with 1.1b *Crenarchaeota*. Our findings also suggest that AOA may be more widespread and possibly more abundant than AOB in estuarine sediments; however, confirmation of this hypothesis—as well as the relative contributions of these different groups of organisms to estuarine ammonia oxidation and N biogeochemistry—merits further investigation.

ACKNOWLEDGMENTS

We thank Esther Cruz-Colin and Juan Delgado-Contreras for assistance with fieldwork and sampling, Manuel Nieblas-Lopez and the pescadores of Bahía del Tóbari for boat access, Peter Jewett for performing nutrient analyses, Greg Asner and David Lobell for providing satellite imagery, and Alyson Santoro and three anonymous reviewers for helpful comments on the manuscript.

We thank Pamela Matson for logistical and monetary support supplied to J.M.B. This work was supported in part by a National Science Foundation (NSF) Graduate Research Fellowship (to J.M.B.); by an NSF Office of International Science and Engineering Americas Program Dissertation Enhancement Grant (to J.M.B.); by NSF grant MCB-0433804 (to C.A.F.); and by support for the Yaqui Valley Sustainability Project provided by the David and Lucile Packard Foundation.

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