Aerobic nitrification is the microbially mediated two-step oxidation of ammonia to nitrite and then to nitrate. The two steps are performed by different groups of bacteria, the ammonia-oxidizing bacteria (AOB) and the nitrite oxidizers. The process of nitrification is important in aquatic environments, because it links N mineralization, the formation of ammonia from organic materials, to denitrification, which results in the loss of nitrogen from the system in the form of gaseous dinitrogen (43). This linkage is especially important in coastal aquatic systems where anthropogenic inputs of nitrogen can result in heavy nutrient loading, and denitrification can account for the loss of up to 50% of the inorganic N input (34). Disruption of nitrification, denitrification, or the linkage of these processes can lead to eutrophication and system degradation.

The evolutionary history and genetic diversity of AOB have been examined in numerous environments with AOB-specific 16S rRNA primers (for examples see references 2, 7, 14, 20, 36, and 39). Unlike most functional groups, AOB-specific 16S rRNA amplification is feasible because the AOB are restricted to two monophyletic clusters within the beta- and gamma-Proteobacteria (9, 28). A second gene that can be used to investigate AOB diversity is amoA, which encodes the first subunit of ammonia monooxygenase, the protein that catalyzes the oxidation of ammonia to hydroxylamine (21), which is then converted to nitrite (11). amoA has two major advantages over 16S rRNA for comparisons of genetic diversity. First, amoA encodes a protein involved directly in ammonia oxidation, therefore genetic differences are more likely to be of functional importance to the process of interest, nitrification. Second, the rate of molecular divergence in amoA is expected to exceed that of 16S rRNA (28), allowing for greater resolution of genetic differences in natural populations. Unlike the evolutionary history of many functional genes, horizontal transfer of amoA in AOB appears to be minimal or absent. This provides a parallel phylogenetic topology for amoA and the gene encoding 16S rRNA, which simplifies inference of the evolutionary history of the organism based upon these genes.

Although the number of studies is small, common insights are beginning to emerge about the diversity and composition of marine AOB assemblages. For example, the dominant betaproteobacterial AOB 16S rRNA gene sequence in clone libraries from the polar oceans (2, 10) has also been observed as a dominant sequence in clone libraries from the Mediterranean Sea (27). As sequence data sets represented as phylogenetic trees increase in size, visual interpretation of patterns is no longer adequate to examine divergence within and between samples. The quantitative genetic comparisons utilized in this study help to interpret patterns of diversity and allow quantitative comparisons to environmental variables and biogeochemical function, such as nitrification rate. This type of analysis will be vital in the interpretation of the large sequence data sets required for the analysis of system dynamics, an important step in the transition to true molecular ecology of microbial systems.

The present study of AOB at a mid-latitude Pacific site had three main goals: (i) to elucidate the dynamic diversity and structuring of AOB communities using both amoA and 16S
rRNA genes from one station at three depths and four dates in Monterey Bay (MB), Calif.; (ii) to compare AOB communities in MB and the adjacent estuarine system of Elkhorn Slough in an attempt to differentiate regional supply from biological selection in neighboring, but sharply contrasting, ecosystems; and (iii) to investigate potential links between environmental variation, AOB diversity, and biogeochemical function by utilizing pairwise comparisons of AOB diversity, nitrification rates, and measured hydrographic, nutrient, and biological variation.

### Materials and Methods

**Sampling.** Bimonthly sampling cruises began in February 1998 and ended in October 1999. Water samples were collected throughout the euphotic zone (defined as the 0.1% light level) at a mid-bay station (depth, 900 m; 36°45'N, 122°01'W) in Monterey Bay, Calif., using a 10-liter Niskin rosette mounted on a conductivity-temperature-depth (CTD) sensor. This sampling scheme was used to cover the range of nitrification rates in the water column, because the nitrification maximum typically occurs above the depth of 0.1% light but below the depth of 10% light (43). Temperature and salinity measurements (Table 1) were made using a Seabird CTD sensor, and subsamples were collected for nutrient analysis. A complete analysis of the temporal variability in hydrographic and biogeochemical parameters from the 2-year period is under way (44). Six samples were analyzed for molecular diversity of AOB communities: April 98-M (M, surface depth; 93% light), October 99-M, and October 99-D (D, deep; 0.1% light). Temperature and salinity measurements (Table 1) for the two samples were collected on October 14, 2017 by guest http://aem.asm.org/ Downloaded from...
TABLE 3. Diversity estimators for pairwise comparisons of 16S rRNA sequences

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<tbody>
<tr>
<td>4/98-M</td>
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<tr>
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<td>0.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10/99-D</td>
<td>2.78&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.36&lt;sup&gt;c&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup> Mean pairwise sequence differences within (π<sub>w</sub>) sites are on the diagonal in boldface.
<sup>b</sup> Mean pairwise sequence differences between (π<sub>W</sub>) sites are below the diagonal.
<sup>c</sup> Pairwise Fst values are above the diagonal. An asterisk indicates a non-significant (P > 0.05) observed Fst value compared to Fst values from 100 randomizations of the sequences; i.e., it indicates random structuring of sequences.

A similar range of variation was found among the four dates included in this study (Table 2). The nitrification rate maximum occurred at 40 to 60 m, and it ranged from 27 to 74 nmol per liter per day (Table 1). Surface samples (<20 m) had the lowest nitrification rates (21 to 28 nmol per liter per day).

**Betaproteobacterial AOB 16S rRNA diversity.** An analysis of molecular variance of the betaproteobacterial AOB 16S rRNA MB clones indicated that 59.2% of the total variation occurred within the samples, versus 40.8% among samples (data not shown). Thus, 40.8% of the observed variation depended on comparisons between clone libraries and was not observed within a single library. Diversity estimators (π<sub>w</sub> and π<sub>W</sub>) and pairwise dissimilarity indices (Fst) were used to examine the partitioning of this variation within and between the samples. All values for the mean number of pairwise sequence differences within a sample (π<sub>w</sub>) were similar and small (<4.0), i.e., sequences within a sample were similar, except for the October 99-M sample (π<sub>W</sub> = 13.94) (Table 3). This suggests that the 16S rRNA sequences in the October 99-M clone library were not very similar to each other compared to levels of similarity within the other libraries. A similar pattern of variation was also found in comparisons between samples. All values for the mean number of pairwise differences between samples (π<sub>W</sub>) were similar and small (<4.0), except for pairwise comparisons to the October 99-M sample (π<sub>W</sub> = 17.21) (Table 3). These π<sub>W</sub> and π<sub>W</sub> values indicate that, excluding the October 99-M sample, the gene sequences found within a clone library (from one sample) were similar, and when the sequences in two libraries (two samples) were compared, they were also very similar to one another. This is consistent with the relationship of samples in the 16S rRNA phylogenetic tree (Fig. 1A). Although levels of divergence in 16S rRNA were limited, Fst values, estimating the partitioning of variation within versus between samples, indicated a nonrandom structuring in all pairwise comparisons of samples except for the pairings of April 99-M with October 98-M (P = 0.06), October 98-M with October 99-D (P = 0.10), and October 99-S with October 99-D (P = 0.59). This indicates that although most libraries were similar, some variation was detected and stochastic sampling alone could not explain this variation (except in the pairs identified above); i.e., although most samples were similar, meaningful community structure was observed in time and space.

**Betaproteobacterial AOB amoA diversity.** AMOVA of the amoA MB clones indicated that 52.0% of the total variation occurred within the samples versus 48.0% between samples, suggesting that the sequences within individual clone libraries account for approximately half of the total observed variation. This is consistent with the patterns displayed on the phylogenetic tree shown in Fig. 2. The smallest π<sub>W</sub> for amoA was found in the sample from April 98-M (Table 4), while the largest π<sub>W</sub> was found within the October 99-M sample. The smallest pairwise π<sub>W</sub> indicates that samples October 99-D and April 98-M are most similar to each other. In contrast, the most divergent pair of samples is October 99-M and April 98-M. The largest pairwise differences (π<sub>W</sub>) in this study all involve comparisons to the October 99-M sample. Fst comparisons show a nonrandom structuring in all pairwise comparisons except for the pairing of April 99-M with October 98-M (P = 0.32). This indicates that both spatial and temporal structure was observed in the amoA sequence data set that was not due to stochastic sampling.
FIG. 1. Distance neighbor-joining trees of the betaproteobacterial AOB 16S rRNA based on (A) a subset of cloned sequences based on the 1,060-bp fragment or (B) the 295-bp fragment. Database sequences for cultured strains and environmental sequences are in boldface and are identified by GenBank accession numbers. Monterey Bay sequences are labeled as month-year-depth (surface [S], mid [M], or deep [D])-clone number; e.g., 10-99-S-18 represents October 1999 surface clone 18. Bootstrap values of >50 are displayed.
FIG. 2. Distance neighbor-joining tree of betaproteobacterial AOB AmoA sequences based on 149 amino acid residues. Database sequences for cultured strains and environmental sequences are in boldface and are identified by GenBank accession numbers. Monterey Bay sequences are labeled A (for AmoA)-month-year-depth (surface [S], mid [M])-clone number; e.g., A10-99-M-21 represents AmoA October 1999 mid-depth clone 21. Bootstrap values of >60 are displayed.
Nitrosococcus cococcus serotypes in marine environments (40), typically by two
methods.

diversity of gammaproteobacterial AOB in these environ-
ments. One of the sequences that have been examined and was composed primarily of October 99-S se-
quences plus three October 99-D sequences. The sequences in
October 99-M sample contained two sequences from the MB clone libraries, both from October 99-M, that cluster tightly with

Nitrosomonas eutropha. The divergence of the October 99-M sequences cannot be clearly attributed to any of the measured environmental
variables (Table 1) (discussed below).

Comparison of 16S and amoA gene diversity. Both 16S rRNA and amoA genes displayed similar patterns of partition-
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* Mean pairwise sequence distances within (\( \pi_m \)) sites are on the diagonal in boldface.
  
* Mean pairwise sequence distances between (\( \pi_{ab} \)) sites are below the diagonal.
  
* Pairwise population Fst values are above the diagonal. An asterisk indicates a nonsignificant (\( P > 0.05 \)) observed Fst value compared to Fst values from 100 randomizations of the sequences; i.e., it indicates random structuring of sequences.

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Gammaproteobacterial AOB 16S rRNA phylogenetic analysis. Using the NOC 1,2 primers, a 1,100-bp fragment of 16S rRNA was examined for gammaproteobacterial AOB diver-
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Mediterranean Sea (27). Only the October 99-M sample had
sequences that clustered into both MB groups 1 and 2. The MB
16S rRNA sequences reveal limited diversity but high similarity to assemblages of AOB sequences that have been found in
other marine environs. Hollibaugh et al. (10) hypothe-
sized that the 16S rRNA sequences retrieved from the polar
oceans (polar sequences in group 1; Fig. 1A) may correspond to
the dominant polar AOB and possibly a dominant global
marine AOB. The occurrence of nearly identical sequences in
high frequency in Monterey Bay as well as the Mediterranean
Sea (27) and Loch Duich (7) suggests that this AOB type may
indeed be a dominant marine AOB not limited to polar hab-

pairwise population Fst values are above the diagonal. An asterisk indicates a nonsignificant (\( P > 0.05 \)) observed Fst value compared to Fst values from 100 randomizations of the sequences; i.e., it indicates random structuring of sequences.

ns serotypes). In Pacific northwest marine and estuarine sed-
iments, gammaproteobacterial AOB contributed minimally to
amoA clone libraries compared to betaproteobacterial AOB
(25). Ward and O’Mullan (46) reported limited molecular diver-
sity but widespread distribution of genes encoding gamma-
 proteobacterial AOB 16S rRNA in marine samples. The rela-
tive importance of gammaproteobacterial subdivision AOB in
marine nitrification and the diversity of the betaproteobacte-
rial versus gammaproteobacterial AOBs remain unclear but
are important questions demanding further investigation.

Betaproteobacterial AOB AmoA phylogenetic analysis. A
distance-based neighbor-joining tree of 149 amino acid resi-
dues from AmoA was constructed (Fig. 2) with the 91 se-
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quences. AmoA displayed a more complex phylogenetic
topology than 16S rRNA sequences, comprising four major
groups (A, B, C, and D). Most sequences (58%) fell into group
A and clustered with sequences from mesohaline and polyhal-
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mental clones from the sediments of Kysing Fjord (24), but
they were distinct from any other published AOB sequences.
Within group A, amino acid distances of >6% occurred be-
tween sequences, but these sequences did not form strongly
supported subclusters, nor did they cluster with database se-
quences that could help to differentiate the subgroups. Group
A contained sequences from every sample in this study, al-
though the majority of October 99-M and October 99-S se-
quences were found outside of group A (in groups B and C,
respectively). Group B represented 20% of the sequences ex-
amined and was composed primarily of October 99-S se-
quences plus three October 99-D sequences. The sequences in
group B are <96% identical to group A sequences, but groups
A and B are joined by a bootstrap value of 68 and are more
closely related to each other than to any cultured strains.

Group C represents 20% of the sequences from the clone
libraries and is composed primarily of sequences from the
October 99-M sample. These sequences cluster with AmoA

TABLE 4. Diversity estimators for pairwise comparisons of amoA sequences

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closely related to each other than to any cultured strains.

Group C represents 20% of the sequences from the clone
libraries and is composed primarily of sequences from the
October 99-M sample. These sequences cluster with AmoA
sequences from cultivated Nitrosospira strains, brackish water
sediment samples from the northern Chesapeake Bay (6), and
sediment samples from Azvedo Pond, a site in Elkhorn
Slough, Calif. (3). Groups A, B, and C (Fig. 2) may corres-
pond to the 16S rRNA sequences in Nitrosospira-like group 1 (Fig.
1A), but this relationship of amoA and 16S rRNA environmen-
tal clones cannot be definitively tested with this data set.

Groups A, B, and C are joined to Nitrosospira-like AmoA
sequences with a bootstrap value of 60. The final group (D)
contained only two sequences from the MB clone libraries,
both from October 99-M, that cluster tightly with N. eutropha
(>98% identity). This group is particularly interesting, because
the only 16S rRNA Nitrosomonas-like sequences were also
from the October 99-M sample and grouped tightly with N.
eutropha. The divergence of the October 99-M sequences cannot
be clearly attributed to any of the measured environmental
variables (Table 1) (discussed below).

Comparison of 16S and amoA gene diversity. Both 16S rRNA and amoA genes displayed similar patterns of partition-
ing of variation within versus between samples (see AMOVA
results discussed above), indicating significant variation that is
only detected in comparisons between samples. The greatest
amount of variation for both genes was found in the October
99-M sample and similar patterns of variation between samples ($\pi_d$), although different levels were observed for both genes. Fst values for 16S rRNA sequences indicated a slightly larger number (3 of 15; Table 3) of sample pairs with randomly distributed sequences than amoA Fst values (1 of 15; Table 4), but the greater resolution (relative amount of sequence divergence) of amoA would allow samples to be more easily differentiated. This analysis also reveals that samples separated by $<15$ m at the same station can have higher levels of genetic differentiation than samples that are temporally separated in distinct seasons or years (Tables 3 and 4).

An analysis of $\pi_d$ values (normalized to sequence fragment length to allow for comparison to cultivated strains) for amoA and 16S rRNA genes found a strong positive correlation ($r^2 = 0.88$; F ratio $= 95.73$; $P < 0.0001$) (Fig. 3), although the data are bimodal and rely heavily on comparisons to the October 99-M sample. The absence of data points in the upper-left or lower-right portion of the plot suggests that the two sequence data sets were sampled from overlapping portions of the same AOB community. Although it is impossible to associate functional and ribosomal genes directly without cultured representatives, the diversity and similarity values from these environmental samples are consistent with genetic distances for the two genes that would be obtained from a well-sampled population with overlap of membership.

Five studies have made the direct comparison of amoA and 16S rRNA in the same environmental sample, but none provide the number of samples or sequences examined in the present study (3, 8, 15, 24, 47). For example, Caffrey et al. (3) examined betaproteobacterial AOB 16S rRNA and amoA in sediments of Azvedo Pond, a saline tidal pond located directly adjacent to MB in Elkhorn Slough. It is interesting that Azvedo Pond sediment sequences were dominated by N. marina-like sequences, while the adjacent MB water column habitat was dominated by Nitrosospira-like sequences. Most (18 of 21) 16S rRNA sequences from Azvedo Pond were N. marina-like, while the remaining sequences were Nitrosospira-like. In agreement with the 16S rRNA data, 8 of 11 amoA sequences examined from the same sample were N. marina-like, while the remaining sequences clustered with Nitrosospira-like sequences. Comparisons from the literature (3, 8, 15, 24, 47) show general phylogenetic agreement between 16S rRNA and amoA in environmental samples and support the more quantitative findings of this study, suggesting that 16S rRNA and amoA clones sample the same or similar AOB populations. Although amoA allows much greater resolution than 16S rRNA, the general trend of pairwise divergence for amoA and 16S rRNA between cultivated strains is also observed in pairwise comparisons of 16S rRNA and amoA quantitative diversity estimators from environmental samples.

**Genetic diversity and biogeochemical function.** To study the interaction of environmental variation, AOB diversity, and biogeochemical function (i.e., nitrification rate), five hypothetical associations were considered. First, the environment, independent of AOB diversity and dynamics, could be driving variation in nitrification rate. Second, the environment could cause changes in AOB that control variation in nitrification rate. Third, the environment could cause changes in the total number or relative activity of AOB that controls variation in nitrification rate. Fourth, AOB could be invariant over time, not responding to the changing environment, suggesting that environmental variation at this scale was not relevant to ecological or functional fluctuation. Fifth, variation could be detected in the environment, AOB diversity, and nitrification rates, but the measured variables would not be correlated. This could be due to a complex interaction of multiple parameters, lacking either a dominant cause-and-effect relationship or lacking adequate signal to resolve the dominant relationship.

Analysis of the 2-year data set of hydrographic and biogeochemical variation did not uncover correlations to nitrification rate, suggesting that physical and/or chemical forcing does not directly control nitrification rate on the measured time scale (44). We therefore rejected hypothesis one to explain the interactions in our data set. Ward also noted that nitrification rates had less variation than expected compared to variation in other coastal systems or to biological processes measured from the same samples (e.g., ammonium assimilation, chlorophyll, bacterial cell count). This may suggest that the AOB community does not respond to environmental variation or that changes in AOB diversity cause a population response that stabilizes function across a variable environment. AOB strains in culture demonstrate a kinetic response to the experimental addition of ammonia (4, 13, 42), but this type of response is typically not observed with natural AOB assemblages (26, 41, 45). AOB population dynamics may explain the lack of a kinetic response in natural assemblages to experimental ammonia increases, resulting in a stabilized nitrification rate over variable conditions.

Temporal stability of ecosystem function as a result of dynamic population structure or activity has been predicted by theory (16, 18, 22, 33), but it is difficult to study experimentally in systems that change population structure slowly (e.g., many eukaryotes). In populations with long generation times, it is more straightforward to measure the effects of total diversity, changes in activity, or spatial niche differentiation rather than dynamic structuring due to temporal variation. Stabilized function across a temporarily variable environment should be maximized by a population with high turnover or short generation time (33), such as bacteria. It is important to note that the dynamic component is not total species richness but instead...
population structure. Our present sampling of AOB populations measures changes in numerically dominant sequences rather than total species (or sequence) richness.

Small but significant genetic differentiation was detected by AMOVA in AOB populations from MB (Tables 3 and 4 and Fig. 1 and 2), with approximately half of the variation only occurring between samples. A Monte Carlo-based test for non-random sequence assemblages showed that in most cases (Tables 3 and 4) samples were significantly different from a random distribution of sequences. Significant variation was detected in both the depth profile and temporal samples. Therefore, we can reject hypothesis four, that no variation was present in AOB populations.

Hypotheses two, three, and five all expect dynamic AOB populations to be detected. A Mantel test (35) was used to examine the correlation of Fst values (for both 16S rRNA and AmoA genes) and pairwise differences in the rate of nitrification, but no correlation was found (data not shown). This finding does not indicate a lack of ecological interaction of AOB dynamics and biogeochemical function. It may be that the variation in AOB populations is responsible for maintaining the relatively stable nitrification rates, while the other biological processes examined were substantially more variable (44).

If AOB population dynamics do stabilize function, a correlation between environmental variation and AOB dynamics would be expected to exist (Hypotheses two and three). Hypothesis five expects no correlations among environment, AOB populations, or nitrification rates. Additional Mantel tests examining AOB Fst values together with temperature, salinity, bacterial abundance, nitrite, and nitrate (data not shown) did not yield a significant correlation between environmental variation and AOB population dynamics. Hypothesis three, which expects a correlation between environmental variation and changes in the abundance or activity of AOB, could not be evaluated due to the lack of data on total AOB abundance or differential expression patterns. We are unable to reject hypothesis three or five. Although significant variation was detected in AOB communities, none of the environmental parameters measured can be implicated in controlling the pattern of variation in AOB. It also remains unclear whether AOB population dynamics indicate that changes in the total number of AOB and changes in AOB expression dynamics may act to stabilize nitrification rates or whether no clear pattern links environmental variation, AOB dynamics, and nitrification rates. Additional complications are due to the incomplete information on gammaproteobacterial AOB, which may at times contribute significantly to ammonia oxidation in this environment. The solution to these problems is to increase the number of samples being examined, include mRNA-based assays, and examine samples which have a greater range in the rates of nitrification. We are presently investigating the use of mRNA microarrays and experimentally manipulated systems to further explore these interactions.

Conclusions. AOB populations from Monterey Bay were dominated by Nitrosospira-like sequences, while AOB populations in the adjacent Elkhorn Slough were dominated by N. marina-like sequences. The MB samples with the highest degree of genetic overlap were separated in time. In contrast, samples with no temporal separation but small vertical separation had comparatively less genetic overlap. AmoA cluster B (Fig. 2) represents novel gene sequences that are phylogenetically distinct from any previously identified AmoA sequences. This study is consistent with recent AOB environmental studies that show that the majority of marine AmoA or AOB 16S rRNA sequences are phylogenetically distinct from any cultured strain. AOB 16S rRNA types identified by Hollibaugh et al. (9) as dominant polar types may indeed represent dominant marine AOB. This reinforces the need for novel approaches and additional effort to culture AOB from various environments and provides a number of target groups for culture work.

No correlation between changes in AOB diversity and composition and changes in nitrification rates were found. It appears that, within the nutrient and hydrographic regimes of Monterey Bay, AOB population structure and function are either not tightly linked, are controlled by changes in AOB abundance and expression dynamics, or lack sufficient variation to discern their relationship. Correlations among AOB community structure and environmental variables and function would be expected to be stronger in environments with steeper gradients and greater temporal variability.

ACKNOWLEDGMENTS

We thank Mary Hogan, Neil Harrington, Jane Caflrey, and Darryl Martino for help in the collection of samples and Neil Harrington and Jane Caflrey for technical assistance with biogeochemical measurements.

This work was supported by the NSF (OCE-9896240). G.D.O. received stipend support from an NSF grant to B.B.W. (OCE-9981482).

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