Characterization of Nitrifying, Denitrifying, and Overall Bacterial Communities in Permeable Marine Sediments of the Northeastern Gulf of Mexico$^\dagger$

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Sandy or permeable sediment deposits cover the majority of the shallow ocean seafloor, and yet the associated bacterial communities remain poorly described. The objective of this study was to expand the characterization of bacterial community diversity in permeable sediment impacted by advective pore water exchange and to assess effects of spatial, temporal, hydrodynamic, and geochemical gradients. Terminal restriction fragment length polymorphism (TRFLP) was used to analyze nearly 100 sediment samples collected from two northeastern Gulf of Mexico subtidal sites that primarily differed in their hydrodynamic conditions. Communities were described across multiple taxonomic levels using universal bacterial small subunit (SSU) rRNA targets (RNA- and DNA-based) and functional markers for nitrification ($amoA$) and denitrification ($nosZ$). Clonal analysis of SSU rRNA targets identified several taxa not previously detected in sandy sediments (i.e., Acidobacteria, Actinobacteria, Chloroflexi, Cyanobacteria, and Firmicutes). Sequence diversity was high among the overall bacterial and denitrifying communities, with members of the Alphaproteobacteria predominant in both. Diversity of bacterial nitrifiers ($amoA$) remained comparatively low and did not covary with the other gene targets. TRFLP fingerprinting revealed changes in sequence diversity from the family to species level across sediment depth and study site. The high diversity of facultative denitrifiers was consistent with the high permeability, deeper oxygen penetration, and high rates of aerobic respiration determined in these sediments. The high relative abundance of Gammaproteobacteria in RNA clone libraries suggests that this group may be poised to respond to short-term periodic pulses of growth substrates, and this observation warrants further investigation.

Permeable marine sediments, and their associated bacterial communities, act as biocatalytic filters for the overlying water column in the coastal ocean. Nearly half of the biomass produced from primary production is thought to deposit onto the shallow continental shelf seafloor, where highly active bacterial communities mineralize the organic matter and release inorganic nutrients back into the water column (11, 32, 34, 38, 63). Thus, enhanced pore water exchange in permeable sediments or sands may stimulate bacterial metabolism through the delivery of substrates and the removal of metabolites (7, 11, 35). Due to the high permeability of these sediments, hydrodynamic forces likely affect the bacterial niches within the uppermost layer of the seabed and impact overall bacterial diversity (33). Although more information is available on bacterial communities present in less permeable, muddy sediments (37), relatively few studies have investigated the microbiology of marine sands, and thus little is known about the diversity of microorganisms inhabiting these environments.

Nitrogen often limits primary production in marine environments (30), and the predominant sink or loss of nitrogen in the coastal ocean is nitrification-denitrification. Nitrification and denitrification are bacterially mediated processes that, when coupled, link the mineralization of nitrogenous compounds to the liberation of gaseous nitrogen that can be released to the atmosphere (15, 42, 70). Understanding the diversity of the bacterial groups associated with nitrification and denitrification is critical to understanding the factors that may influence this important part of the nitrogen cycle in the marine environment. In order to elucidate the nitrifier communities, the functional gene, $amoA$, was assayed which encodes the first subunit of ammonia monoxygenase, a protein involved in the first step of nitrification. This gene has been used to identify nitrifiers, and a previous study by Hunter et al. (33) reported a low diversity of $amoA$ genes in sandy marine sediments. However, a limited number of samples was examined, and very little is known about the diversity of bacterial $amoA$ genes in permeable marine sediments across environmental gradients. For monitoring denitrifying bacteria in marine environments, three functional genes ($nirS$, $nirK$, and $nosZ$) have been used to (9, 10, 43, 66, 67) due to their broad phylogenetic diversity. While $nirS$ and $nirK$ encode enzymes active early in the denitrification pathway, $nosZ$ encodes the enzyme nitrous oxide reductase, which catalyzes the final step in denitrification, thus representing the process that leads to the loss of biologically available nitrogen from the sediment. Although reports of

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2† Supplemental material for this article may be found at http://aem.asm.org.
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denitrifier diversity in continental shelf sediments have been published (66–68), the database for marine sediments remains small, and past studies have often focused on methodological development.

The objective of the present study was to expand the characterization of bacterial community diversity in understudied permeable shelf sediments across spatial, temporal, and hydrodynamic gradients. Simultaneous purification and analysis of community DNA and RNA compared total and metabolically active bacterial populations. High-throughput techniques were developed and coupled with clonal analysis to facilitate the determination of overall, denitrifier, and nitrifier bacterial community composition at increased spatial resolution. Whereas clonal analysis revealed no substantial changes across environmental gradients, terminal restriction fragment length polymorphism (TRFLP) profiles showed distinct differences in bacterial community composition according to site, depth, and time period sampled.

### MATERIALS AND METHODS

**Site and sample description.** Permeable marine sediments were studied on St. George Island, FL, in the northeastern Gulf of Mexico (NEGOM). The continental shelf near St. George Island is considered to be relatively pristine and unaffected by heavy anthropogenic impact. Sediment samples were collected in March and May 2005 from the gulf (29°44.885N, 84°42.594W) and from Apalachicola Bay (29°45.034N, 84°42.719W) sides of St. George Island and were comprised predominantly of moderately well-sorted quartz sand with a relatively low organic matter content (<0.2%) and a porosity of 40 to 45% (Table 1). More information on these biogeochemical determinations can be found in the Table 1.

<table>
<thead>
<tr>
<th>Parameter†</th>
<th>March</th>
<th>Gulf</th>
<th>May</th>
<th>Bay</th>
<th>May</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median grain size (µm)‡</td>
<td>180.9</td>
<td>209.6</td>
<td>307.0</td>
<td>220.0</td>
<td></td>
</tr>
<tr>
<td>Sorting coefficient‡</td>
<td>1.5</td>
<td>1.8</td>
<td>1.6</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Porosity (%‡)</td>
<td>40.0 ± 0.4</td>
<td>40.1 ± 0.6</td>
<td>39.8 ± 1.3</td>
<td>42.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Mean permeability (m² day⁻¹)</td>
<td>(2.0 × 10⁻¹³) ± (1.7 × 10⁻¹³)</td>
<td>(2.8 × 10⁻¹³) ± (8.2 × 10⁻¹³)</td>
<td>(4.6 × 10⁻¹³) ± (1.2 × 10⁻¹³)</td>
<td>(3.4 × 10⁻¹³) ± (6.9 × 10⁻¹²)</td>
<td></td>
</tr>
<tr>
<td>Oxygen production (mmol m⁻² day⁻¹ù)</td>
<td>14.17</td>
<td>51.36</td>
<td>91.85</td>
<td>54.70</td>
<td></td>
</tr>
<tr>
<td>Oxygen consumption (mmol m⁻² day⁻¹ù)</td>
<td>-11.82</td>
<td>-19.54</td>
<td>-17.11</td>
<td>-16.56</td>
<td></td>
</tr>
<tr>
<td>Photosynthesis (mmol m⁻² day⁻¹ù)</td>
<td>25.98</td>
<td>70.90</td>
<td>98.96</td>
<td>51.26</td>
<td></td>
</tr>
<tr>
<td>Mean C content (%‡ SD±)</td>
<td>0.12 ± 0.11</td>
<td>0.11 ± 0.14</td>
<td></td>
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* Mean C content (%): Calculated according to the method of Blott and Pye (6); † calculated according to the method of Holme and McIntyre (28); ‡ calculated according to the method of Cook et al. (18); † calculated from oxygen production corrected for sediment oxygen consumption during daylight; **, calculated in a CarloErba Element CNS analyzer, with sulfuramide as a standard.

Slightly gravelly fine sand, moderately sorted.
Slightly gravelly fine sand, moderately well sorted.

### Bacterial communities in permeable marine sediments

**MATERIALS AND METHODS**

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TRFLP analysis. For TRFLP, the bacterial SSU rRNA and nosZ genes were PCR amplified from the same community DNA used for clone library construction. Similar PCR amplification conditions were used as described above, except the forward primers were labeled with 6-carboxyfluorescien (ABI). Fluorescent amplification of amoA was unsuccessful due to gene template concentrations being below our detection limit. TRFLP analysis of SSU rRNA and nosZ genes was performed according to the method of Kerkhof et al. (40) using MnlI as the restriction enzyme and the forward primers listed in Table 2. TRFLP fingerprinting was performed on an ABI 310 genetic analyzer (ABI) using Genescan software. To identify individual peaks, the 5' terminal restriction fragment (TRF) length for each sequenced clone was determined by an in silico digest using the MnlI recognition site and matched to the peak sizes from the TRFLP analysis. Ten phylotypes were chosen at random to have in silico digests verified by individual TRFLP analysis. For each phylotype, the in silico predictions were within one base of the size observed on TRFLP electropherograms with a majority being exact matches. In silico digests from Hunter et al. (33) were also included in the analysis of TRFLP profiles. Similar TRF lengths that were obtained from multiple unrelated clone sequences were discarded, and associated electropherogram peaks were not included in statistical calculations. TRFs from representative phylotype sequences were matched to peaks from each of the 28 sediment sample profiles (i.e., seven depths, two sites, and two months). Further, comparative analysis of the TRFLP profiles was performed based on the Sorensen's similarity index (47, 56) and UPGMA (for weighted pair-group method with arithmetic averages) analysis using the COMbinatorial Polythetic Agglomerative Hierarchical clustering package (COMPAH96; http://www.esumb.edu/edgwebp.htm). Dendrograms were constructed by inserting distance matrices into Clustering Calculator (www2.biology.ualberta.ca/bruzasto/cluster.php#ClusterCalc) using Canberra Distance for distance measure and Saitou and Nei neighbor-joining for the clustering method. Output trees were visualized on TreeView (taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Phylogenetic and statistical analyses. All clone sequences were checked for chimeras using Chimera Check from the Ribosomal Database Project II Release 9 (48). Sequences from the present study and reference sequences, as determined by highest sequence similarity during BLAST analysis, were subsequently aligned by using the Fast Aligner algorithm in the ARB package (71). All SSU rRNA alignments were visually verified and manually adjusted according to Escherichia coli SSU rRNA secondary structure. According to the method of Hunter et al. (33), neighbor-joining trees incorporating a Jukes-Cantor distance method with arithmetic averages) analysis using the COMbinatorial Polythetic Agglomerative Hierarchical clustering package (COMPAH96; http://www.esumb.edu/edgwebp.htm). Dendrograms were constructed by inserting distance matrices into Clustering Calculator (www2.biology.ualberta.ca/bruzasto/cluster.php#ClusterCalc) using Canberra Distance for distance measure and Saitou and Nei neighbor-joining for the clustering method. Output trees were visualized on TreeView (taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Nucleotide sequence accession numbers. The 52 total nucleotide SSU rRNA, nosZ, and amoA sequences reported here were submitted to the GenBank database under accession numbers DQ431855 to DQ431906.

RESULTS

Sediment characteristics and biogeochemical rate measurements. Bulk-phase sediment characteristics and organic matter content between the gulf and bay sites or the different sampling times were largely the same (Table 1). The observed production and consumption rates of oxygen are among the highest rates measured for shallow subtidal marine sediments and indicate that the turnover of organic matter in the marine sands of the NEGOM equals or exceeds that of muddy sediments in similar subtidal locations. Oxygen consumption rates, a proxy for organic matter degradation, were similar between the sites and times (Table 1). Photosynthetic production was about four times higher at the bay site in comparison to the gulf site during March, but lower in May. A distinct difference in the heights and spacing of sand ripples was observed that can be attributed to the fact that the gulf site is exposed to stronger waves and currents in comparison to the bay site. When the bottom currents generated by waves and tides are deflected by the ripple topography, pressure gradients develop that pump water through the upper layers of the permeable sediments. In situ tracer clearing rate measurements with benthic advection chambers (11, 32, 34, 38, 63) revealed that under moderate bottom currents of 10 cm s\(^{-1}\) approximately 461 ± 94 (standard deviation [SD]) liters m\(^{-2}\) day\(^{-1}\) are filtered through the upper 6 cm of the gulf sediment. In contrast on the bay side, filtration reached only 137 ± 13 (SD) liters m\(^{-2}\) day\(^{-1}\) with a pore water penetration of 3 cm. During stormy periods, these filtration rates and filtration depth could be doubled. The contrasting hydrodynamic conditions and ensuing flushing depths at the two study sites were also reflected in the stratification of iron minerals present in sediment cores, observed as a color change from brown Fe(III) oxides to black Fe(II) sulfides. A distinct redox transition was observed in bay site cores at a depth of approximately 4 cm, whereas no such stratification was observed in gulf site cores.
TABLE 3. Statistical analyses of SSU rRNA and nosZ gene clone libraries using standard ecological and molecular estimates of sequence diversity

<table>
<thead>
<tr>
<th>PCR target</th>
<th>Sample</th>
<th>No. of clones (no. of phylotypes)</th>
<th>% Coverage</th>
<th>Species richness (95% CI)*</th>
<th>Shannon-Weiner index</th>
<th>1/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSU rRNA gene (DNA)</td>
<td>3B02</td>
<td>70 (38)</td>
<td>42</td>
<td>64 (48–101)</td>
<td>3.44</td>
<td>40.3</td>
</tr>
<tr>
<td></td>
<td>3B1820</td>
<td>67 (44)</td>
<td>36</td>
<td>85 (63–132)</td>
<td>3.66</td>
<td>67.0</td>
</tr>
<tr>
<td></td>
<td>3G02</td>
<td>55 (34)</td>
<td>32</td>
<td>66 (47–110)</td>
<td>3.34</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>3G1820</td>
<td>57 (31)</td>
<td>42</td>
<td>50 (38–85)</td>
<td>3.25</td>
<td>34.0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>249 (71)</td>
<td>66</td>
<td>92 (80–122)</td>
<td>3.89</td>
<td>42.2</td>
</tr>
<tr>
<td>SSU rRNA (RNA)</td>
<td>3B02</td>
<td>26 (18)</td>
<td>54</td>
<td>29 (21–55)</td>
<td>2.81</td>
<td>37.5</td>
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<tr>
<td></td>
<td>3B1820</td>
<td>39 (20)</td>
<td>69</td>
<td>37 (25–74)</td>
<td>2.78</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>3G02</td>
<td>39 (26)</td>
<td>54</td>
<td>52 (36–94)</td>
<td>3.11</td>
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<td></td>
<td>3G1820</td>
<td>32 (15)</td>
<td>87</td>
<td>16 (15–22)</td>
<td>2.62</td>
<td>21.1</td>
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<tr>
<td></td>
<td>Total</td>
<td>136 (42)</td>
<td>88</td>
<td>57 (47–85)</td>
<td>3.41</td>
<td>28.5</td>
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<tr>
<td>nosZ</td>
<td>3B02N</td>
<td>27 (13)</td>
<td>54</td>
<td>18 (14–39)</td>
<td>2.42</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>3B1820N</td>
<td>33 (18)</td>
<td>39</td>
<td>38 (23–105)</td>
<td>2.68</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>3G02N</td>
<td>81 (27)</td>
<td>52</td>
<td>41 (31–78)</td>
<td>2.67</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>3G1820N</td>
<td>90 (23)</td>
<td>65</td>
<td>28 (24–48)</td>
<td>2.70</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>231 (38)</td>
<td>74</td>
<td>46 (40–66)</td>
<td>3.03</td>
<td>14.1</td>
</tr>
</tbody>
</table>

*a CI, confidence interval.

RFLP and statistical analyses of SSU rRNA, nosZ, and amoA clone libraries. Fifteen clone libraries constructed by using nucleic acids extracted from March bay 0- to 2-cm (3B02) and 18- to 20-cm (3B1820) and gulf 0- to 2-cm (3G02) and 18- to 20-cm (3G1820) sediment core sections resulted in a total of 249 Bacteria SSU rRNA gene clones (DNA-derived), 136 Bacteria SSU rRNA clones (RNA-derived), 231 nosZ clones, and 64 amoA clones. The sample abbreviations given in parentheses will henceforth be used to identify the clone sequences retrieved. Clone sequences with designations beginning with LC or SC are from Hunter et al. (33) and represent phylotypes detected in both studies. The percent coverage for all four SSU rRNA gene clone libraries was 66% (Table 3), with individual library coverage ranging from 32 to 42%. RNA-derived SSU rRNA clone libraries had higher percent coverages for the individual libraries (54 to 87%) and the combined, total library coverage ranging from 32 to 65% (Table 3). Statistical estimators suggested the percent coverage for all four SSU rRNA gene libraries was 66% (Table 3), with individual library coverage ranging from 32 to 42%. RNA-derived SSU rRNA clone libraries had higher percent coverages for the individual libraries (54 to 87%) and the combined, total library coverage (88%). All diversity indices suggested lower diversity in the RNA-derived libraries compared to the corresponding DNA-derived libraries (Table 3). The total nosZ clone library, integrating all samples, had a calculated total percent coverage of 74% with lower individual clone library percent coverages (39 to 65%) (Table 3). Statistical estimators suggested the nosZ gene diversity was similar to that of the SSU rRNA (Table 3), but much higher than that of the amoA gene. Two phylotypes were detected in the three amoA clone libraries (see Table S1 in the supplemental material) with a percent coverage of 100% for each (data not shown). Rarefaction curves for the total SSU rRNA (DNA- and RNA-derived) and nosZ libraries and the total and individual amoA gene libraries suggested a sufficient number of clones were sampled to represent library diversity (see Fig. S1 in the supplemental material). Although rarefaction curves for the remaining libraries did not indicate sample saturation, sufficient clones were screened to indicate trends within the bacterial communities.

SSU rRNA gene phylogenetic analysis. Sequence analysis of 46 of the 73 SSU rRNA gene phylotypes (DNA-derived) indicated nine distinct phyla with a majority of the sequences most closely related to sequences of uncultured organisms obtained from other marine environments (see Table S2 in the supplemental material). Two separate distance-based neighbor-joining trees were constructed with 20 Proteobacteria and 26 non-Proteobacteria-related sequences from the present study, with additional reference sequences from the GenBank database providing classification of most sequences to the family taxonomic level (Fig. 1 and 2). Additional sequences indicated in boldface type the figures indicate clones with matching TRF lengths used in TRFLP analysis. Nearly half of the total SSU rRNA gene clones (46%) grouped within the phylum Proteobacteria (see Table S2 in the supplemental material). Alphaproteobacteria-related clones accounted for 56% of the total Proteobacteria (see Table S2 in the supplemental material). Similar to a previous sandy-sediment study (33), no clone sequences within the classes Betaproteobacteria and Epsilonproteobacteria were detected.

Proteobacteria. The 20 DNA-derived Proteobacteria-related phylotypes grouped into three classes, i.e., Alpha-, Delta-, and Gammaproteobacteria. A majority of the DNA-derived phylotypes (n = 9) was most closely related to the Alphaproteobacteria (see Table S2 in the supplemental material). Phylotypes 3G02-39 and LC1-35 clustered within the family Hyphomicrobiaceae (92 and 94%, respectively; Fig. 1) and were found in all four libraries, representing 31% of the Alphaproteobacteria-related clones (see Table S2 in the supplemental material). The six DNA-derived Gammaproteobacteria-related phylotypes (Fig. 1; SC1-11 and SC1-44 were only detected in TRFLP analysis and not in the clone libraries) exhibited little site or depth specificity. DNA-derived phylotypes 3G02-43 and 3B1820-38 represented 58% of the total Delaprotobacteria-related clones and clustered within the group II sulfate reducer (46) family Desulfobacteraceae (see Table S2 in the supplemental material and Fig. 1), while phylotype SC3-7 clustered with the group III sulfate-reducing family Desulfobulbaceae (46) (Fig. 1). Representing 32% of the total Delaprotobacteria-related clones, 3G02-06 and LC1-13 formed their own clade and could not be further classified beyond the class level (Fig. 1).
Non-Proteobacteria lineages. Eight non-Proteobacteria phyla were identified by phylogenetic analysis. Of the 26 non-Proteobacteria-related phylotypes, 5 grouped within the phylum Planctomycetes, which contains the *Pirellula*-associated anammox functional group (Fig. 2). Although two phylotypes branch within the *Pirellula* clade, the other three phylotypes, represented by clones 3B1820-36, LCI-9, and LCI-1, diverged to form a second clade that was supported by strong bootstrap values (Fig. 2). The *Bacteroidetes/Chlorobi* superphylum contained six diverse phylotypes, accounting for 14% of the total SSU rRNA gene clones (Table 4), and was related to the orders Sphingobacteriales and Chlorobiales and the families Saprospiraceae, Flexibacteraceae, and Flavobacteriaceae. All *Acidobacteria* and *Actinobacteria*-related phylotypes had low sequence similarity to identified cultured isolates; thus, classification beyond the phyla level was not possible (Fig. 2). Five of the six phylotypes in the phylum *Actinobacteria*, clustered within their own clade and apart from any cultured isolates (Fig. 2), with only SC3-41 (33) being affiliated with the cultured genus “*Candidatus Microthrix parvicella*” (89%). A single phylotype, 3B1820-43, clustered within the phylum *Cyanobacteria* and was 99% similar to the chloroplast DNA sequence from *Altinuncana* (Fig. 2 and see Table S2 in the supplemental material). Three phylotypes—LC1-24, 3G02-01, and 3G1820-58—clustered within the family Anaerolinaceae in the phylum Chloroflexi (Fig. 2). Phylotypes 3G02-02 and 3G1820-01 clustered with the families *Paenibacillaceae* and *Turbibacteraceae*, respectively, within the phylum *Firmicutes* (see Table S2 in the supplemental material and Fig. 2). The final two phylotypes, 3G1820-03 and 3G1820-56, branched into the aerobic heterotrophic phylum *Gemmataimonadetes* (Fig. 2).

SSU rRNA (RNA-derived) phylogenetic analysis. Sequence analysis of 23 of the 42 SSU rRNA phylotypes (RNA-derived) indicated six distinct phyla. Although *Firmicutes*, *Bacteroidetes*/Chlorobi, and *Gemmataimonadetes* were detected in the DNA-derived SSU rRNA gene libraries, no RNA-derived clones grouped into these phyla. Similar to the DNA-derived clones, nearly half of the RNA-derived clone sequences (41%) grouped into 12 Proteobacteria-related phylotypes; however, *Gammaproteobacteria*-related clones represented 59% of the total Proteobacteria (see Table S3 in the supplemental material).

Twelve RNA-derived *Proteobacteria*-related phylotypes grouped into three classes: Alpha-, Delta-, and Gammaproteobacteria. A majority of the DNA-derived phylotypes (ν = 9) was most related to the *Alphaproteobacteria* (see Table S2 in the supplemental material), while only one RNA-derived phylotype grouped in this lineage. Six of the twelve *Proteobacteria*-related RNA-derived phylotypes grouped within the class *Gammaproteobacteria*. Although no *Pseudomonadaceae*-related clones were detected in the DNA-derived libraries, they were the most frequently detected *Gammaproteobacteria* lineage in the RNA-derived library (see Table S3 in the supplemental material) and were widely identified in the TRFLP profiles. *Delta*proteobacteria*-related phylotypes were more depth specific in RNA-derived libraries compared to the DNA-derived libraries. A total of 11 RNA-derived non-Proteobacteria-related phylotypes grouped into five different phyla, all represented in the DNA-derived libraries. *Actinobacteria*, *Planctomycetes*, and *Acidobacteria*-related phylotypes, incorporating 13, 12, and 5% of the total RNA-derived clones, respectively, were detected in all depths at each site (see Table S3 in the supplemental material). All three phylotypes related to phototrophic *Cyanobacteria* (11% of the total RNA-derived clones) were detected in the 18- to 20-cm libraries.

Phylogenetic analysis of the nosZ gene. Sequence analysis of the 42 nosZ gene phylotypes indicated that all sequences were most closely related to sequences of uncultured organisms that putatively clustered with *Alphaproteobacteria*-related nosZ genes (Fig. 3). However, due to low sequence similarity to cultured strains and the presence of nosZ on mobile genetic elements (69), characterization of these genes as being *Alphaproteobacteria*-related is reported with caution. The most frequently detected phylotype (17% of the total nosZ clones), 3G02N-02, was 81% similar to the environmental clone CSS bacterium 696M, retrieved from permeable sediments off the coast of New Jersey (66) (see Table S4 in the supplemental material). Several deep branching clades were comprised solely of clones from the South Atlantic Bight and NEGOM (Fig. 3) (2, 66).

TRFLP-based analysis of the bacterial community structure. Initially, triplicate samples from each site, depth, and time were analyzed by using TRFLP, resulting in 84 profiles (see representative profiles in Fig. 4). Comparative analysis, as described below, showed little difference between individual profiles and a sample formed by combining the triplicate extracts prior to fluorescent amplification. Thus, 28 profiles from the combined extracts were subjected to cluster analysis using pairwise Sorensen’s indices to construct dendrograms for the bacterial and the denitrifier communities with the SSU rRNA and nosZ gene targets (Fig. 5). In contrast to the clonal analysis, the SSU rRNA and nosZ gene TRFLP profiles from gulf and bay samples formed separate and distinct clusters with less than 50 and 38% sequence similarity, respectively. Furthermore, all SSU rRNA gene profiles from March and May clustered in separate clades (<63% similarity) and with the exception of the May 4-6 cm and 6-8 cm bay samples, all bay samples from March and May also formed distinct clusters (<52% similarity; Fig. 5A). The nosZ-derived TRFLP profiles...
from the bay March and May samples also showed a very distinct clustering (<52% similarity; Fig. 5B) with gulf March and May samples having less distinct clustering. These data are supported by the observation that 10 of the 23 TRFLP identified nosZ phylotypes were detected only in bay samples, while 6 were only found in gulf samples. Of note, for both gene targets, the 0- to 2-cm samples from the bay and gulf were more similar to the 18- to 20-cm samples from that same site compared to the other site’s 0- to 2-cm sample.

In each of the 28 SSU rRNA TRFLP composite profiles, ca. 50% of the SSU rRNA gene-related peaks were identified and matched to clone phylotypes using in silico-derived TRF sizes. The Alphaproteobacteria identified TRFLP peaks represented between 5.2 and 25.3% of the total peak area for each sample (Fig. 6). Abundance profiles for this class suggest similar distribution patterns for the two bay samples with more Alphaproteobacteria detected in the gulf sediments (Fig. 6). Supporting this range and abundance, TRFLP peaks matching in silico TRF sizes for phylotypes 3G02-39 and LC1-35, clustering within the family Hyphomicrobiaceae, were detected at almost every depth, site, and time point and had the largest percentage of total peak area for any sequence (see Fig. S2 in the supplemental material). Additional Alphaproteobacteria lineages were more depth specific (e.g., Rhodobacterales-related phylotype LC1-30 was detected in the shallow depths of all four samples) and site specific (e.g., Rhizobiales-related LC1-25 was observed almost exclusively in bay sediments profiles with relatively high peak areas [see Fig. S2 in the supplemental material]).

Classwide comparisons for both Gamma- and Deltaproteobacteria indicated larger peak areas below 8 cm in bay sediments and below 14 cm in gulf sediments (Fig. 6). For example, all three Deltaproteobacteria-related phylotypes were observed only in the deeper sediment depths. With the exception of phylotype 3B1820-38, bay Deltaproteobacteria-related phylotypes were detected higher in the sediment column, i.e., between 4 and 8 cm, compared to the gulf phylotypes (see Fig. S2 in the supplemental material). Deltaproteobacteria-related phylotypes were also detected higher in the column in May compared to March at both sites.

Multiple non-Proteobacteria-related phylotypes were detected and showed similar site, depth, and time specificity. Pirellula-related phylotypes were detected at mid-depths in bay and gulf samples (Fig. 6), whereas Actinobacteria-related phylotypes from bay sediments were observed higher in the sediment column compared to those detected in gulf sediments (Fig. 6). Firmicutes-related phylotypes 3G02-02 and 3B1820-01 clustered with the families Paenibacillaceae and Turicibacteraceae, respectively (see Table S2 in the supplemental material and Fig. 2) and were only detected in the shallow sediment depth TRFLP profiles (Fig. 6).

Similar to the community fingerprints of the SSU rRNA gene, approximately half of the TRFLP peaks from the nosZ fingerprints were identified and given a corresponding phylotype. Although numerous phylotypes were detected in both the bay and gulf samples including several that were detected at nearly all depths (i.e., SC3N-21/SC1N-18, SC3N-27, SC3N-19; see Fig. S3 in the supplemental material), some phylotypes were exclusive to one site or detected at limited depths within the cores. Peaks associated with phylotypes 3G1820N-06, 3G1820N-48, and SC3N-10 were detected on both sample dates but only in gulf sediments (see Fig. S3 in the supplemental material). A total of 87% of the clones detected in phylotype 3G02N-02 were isolated from gulf samples and 78% of these were in 3G02N (see Table S4 in the supplemental material). This is in agreement with TRFLP peak areas associated with this phylotype being much higher, with wider distribution through the gulf samples (see Fig. S3 in the supplemental material). More than 30% of the TRFLP peak area in the shallow bay sediment samples was associated with the phylotype 3G1820N-14 (10% of the total nosZ clones). Interestingly, this phylotype was not detected at any depth or time point in gulf sediments.

**DISCUSSION**

Our results from the NEGOM demonstrate that the diversity, as well as the metabolic activity, of bacterial communities is high in permeable sediment environments, likely due to increased transport of growth substrates and the removal of metabolites by advective exchange with the overlying water column (20). Using our expanded sequence database, the present study elucidated an unprecedented diversity of overall and denitrifying bacterial communities in marine sands (33, 55), and we expanded current knowledge to show the presence of numerous taxa that had not been previously recognized in marine sands. We further suggest that the Gammaproteobacteria are poised to respond rapidly to the input of growth substrates based on their relative abundance in rRNA clone libraries, although this observation should be tested by using more quantitative methods.

**Phylogenetic analysis of the total and metabolically active bacterial communities in marine sands.** Similar to previous studies of marine sediments (12, 33, 44, 45, 55), as well as of marine bacterioplanktonic communities (72), we showed that the Gammaproteobacteria, Planctomycetes, and Bacteroidetes groups predominate over the sequence diversity of marine sands. In contrast to previous work (45, 55), we observed that...
the Alphaproteobacteria showed the highest relative abundance in overall and denitrifying communities. The Deltaproteobacteria and gram-positive groups (Actinobacteria and Firmicutes) also comprised a substantial fraction of the overall diversity observed in permeable sediments, in agreement with previous work (12, 33, 45, 55). The relative abundance of Alphaproteobacteria phylotypes in particular suggests that this group is well adapted to sediments undergoing continuous and rapid pore water advection as indicated by the high permeability and rapid rates of organic matter turnover that we observed in the sands of the NEGOM. The fact that a predominance of Alphaproteobacteria sequences is also observed in marine bacterioplankton communities (72) may be explained by the recent observations showing enhanced exchange of particles between permeable sediments and the overlying water column (5, 18, 31, 36). In comparison to muddy marine sediments, bacteria could more readily switch between attached and planktonic lifestyles in marine sands.

A surprising observation of our study was that Gammaproteobacteria-related phylotypes were more frequently detected in RNA-derived clone libraries, suggesting high levels of metabolic activity within this group. Alternatively, Gammaproteobacteria found in the present study may maintain a higher ribosome content per cell under nongrowth conditions, as has been demonstrated in some pure cultures (22) and seawater enrichments (21). Regardless of the cause, increased rRNA levels may permit certain Gammaproteobacteria to respond better to the rapid changes in oxygen and organic substrates that occur in marine sands. One genus with the capacity for denitrification, i.e., Marinobacter (64), was detected within both gulf and bay sediment RNA-derived libraries, and previous cultivation work suggests that this group is among the most abundant of denitrifiers in marine sediments (4, 25). Specifically targeting this lineage with quantitative PCR methods would provide valuable insight into its ecological impact.

The results from the denitrifying communities are consistent with the rRNA findings with a large sequence diversity detected. However, nosZ-based phylogenetic classification beyond the class level, i.e., Alphaproteobacteria, is tentative at best. Linking nosZ diversity with SSU rRNA diversity is difficult due to primarily partial nosZ sequences from environmental samples being predominant in the database. Although additional marine denitrifiers have been cultured (62), gaps exist in the sequence database between SSU rRNA and nosZ phylogenies. For example, while Hyphomicrobiaceae, a family with known denitrifying lineages, was frequently detected in the DNA libraries, no nosZ genes and no full genome sequences for this group are available in public databases. Thus, many

FIG. 3. Neighbor-joining phylogenetic tree, incorporating a Jukes-Cantor distance correction, of the nosZ gene sequences from 3B02, 3B1820, 3G02, and 3G1820 samples. Sequences from the present study and close relatives were aligned by using the Fast Aligner algorithm and verified by hand by using the ARB software package. Sequence names in boldface type represent both cloned sequences and sequences from Hunter et al. (33) used in TRFLP peak analysis. Bootstrap analyses were conducted on 1,000 samples, and percentages greater than 50% are indicated at the nodes. Ralstonia eutropha strain H16 was used as the outgroup. Scale bar, 0.10 change per nucleotide position.

FIG. 4. Replicate TRFLP fingerprints of May bay and gulf 6- to 8-cm samples. Replicates from this depth demonstrate the reproducibility of TRFLP profiles from different cores collected at the same time and from the same site. Difference in peak presence and height can be observed between sites.
nosZ sequences from the present study remain tightly clustered with previously reported clone sequences from environmental samples (33, 66) and not to cultivated species. These results underscore the need for cultivation of marine denitrifying model microorganisms that better represent the indigenous populations.

Analysis of the amoA gene showed a low diversity \( (n = 2) \) in NEGOM sediments in comparison with previous studies of marine muds (3, 13, 23, 58, 60). Bacterial amoA sequences showed a high sequence identity (98\%) to sequences retrieved from the water column and muddy sediments of other coastal marine ecosystems. However, two ubiquitous phylotypes at both NEGOM sites represent a comparable level of diversity to a previous sandy sediment study (33). Therefore, while ammonia oxidation has been shown to play a critical role in the coastal ecosystem in other marine habitats (1, 8, 29, 49, 51, 57, 73), results presented here suggest that ammonia oxidizing-related taxa may be under-represented in these clone libraries, potentially due to targeting only the bacterial contribution to the ammonia oxidizing community (22a).

**Profiling of permeable marine sediment communities over expanded scales using DNA fingerprinting.** An understanding of bacterial community structure and function in marine sediments has been hampered by the excessive time and cost of sample processing. Previous studies of bacterial diversity in marine sediments have relied mainly on clonal analysis and have often lacked replication or based interpretation of bacterial community structure on relatively few samples. In the present study, DNA fingerprinting provided improved replication and spatial coverage that was not achievable with clonal analysis. Consistent with past work in marine sands (55), we observed community composition at the phylum/subphylum level to be relatively stable in clone libraries, whereas we observed substantial trends with depth and between sites at the

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**FIG. 5.** Sorensen’s index-based dendrograms of SSU rRNA gene (A) and nosZ gene (B). TRFLP profiles from each depth, site, and time were compared for peak absence or presence to produce a distance matrix pairwise comparison. Neighbor-joining trees were then constructed from the distance matrices.
family to species level using our community fingerprinting approach. Such differences in community interpretation highlight the need for more robust replication in sampling and characterization. The results presented here are indicative of in situ conditions, not total community potential as shown by the microcosm experiments of Hunter et al. (33).

Sorensen's index-based analysis of TRFLP profiles provided evidence for bacterial niche specialization and suggested that the bacterial community diversity is affected by advective pore water flow in permeable sands. The gulf site was exposed to higher flushing rates due to increased exposure to larger waves compared to bay site sediments. Lower sediment flushing rates and a well-defined redox gradient, as determined by a clearly visible zone of black iron sulfides at a depth of ~4 cm, were observed in the bay sediments. These differences may thus lead to at least some of the variations in the community structure that were observed. By correlating specific TRFLP peaks to cloned sequences, changes in community structure at each depth interval could be analyzed for potential ecological impacts. Two Alphaproteobacteria phylotypes were related to a known denitrifier, i.e., Hyphomicrobium denitrificans strain DSM 1869 (62), and were detected at all sites, depths, and time points, thus supporting the broad distribution observed in a previous lab-based sandy sediment community structure determination (33). Hyphomicrobiaceae lineages have been detected in a wide variety of environments (i.e., wastewater [19], deep-sea sediment [55, 74], and Antarctic sediment [52]) and linked not only to nitrogen cycling but also to growth on methylated sulfur compounds (52). Such metabolically diverse lineages with the capacity for denitrification may prove to be a significant contributor to geochemical processes in dynamic environments such as coastal sediments that are subjected to variable pulses of carbon and oxygen. At deeper depths, Deltaproteobacteria (i.e., Desulfobulbaceae and Desulfobacteraceae) became more relatively abundant, supporting the location of the redox boundary as observed by the presence of iron sulfides in the cores. Due to pore water flow within these sediments, it is interesting to hypothesize that the depth-specific taxa identified are surface adherent and thus less affected by water influx.

Large differences in community structure with depth were expected, and the differences in the distribution of bacterial taxa between sites at comparable depth are consistent with prior findings in coastal sands from the Mid-Atlantic Bight (67). The Sorensen's index-based analysis of the TRFLP profiles indicated as low as 50% similarity for SSU rRNA gene and 38% similarity for nosZ between the bay and gulf bacterial communities. Thus, differences in flushing rates or redox zone stability may allow different populations to become more numerous between sites. Free-living, non-surface-adherent populations (26) may be forced by pore water flow through the sediment and thus detected at multiple locations within the core. Such movement within the sediment may explain why some groups (i.e., denitrifiers and phototrophs) were detected throughout the core despite having limited geochemical ranges.

FIG. 6. Relative abundance of microbial populations across sediment depth, site and time. Phylogenetic groups were determined by comparing in silico digests of cloned sequences to electropherogram peak sizes during TRFLP analysis. The percentage of the total peak area was then reported for each clone and combined with similar lineages to produce abundance profiles. TRFLP peak area profiles for Gemmatimonadetes were omitted due to a single clone detected in March Gulf samples from 4 to 10 cm. Please note the differences in x-axis scales.
Vertical and lateral movement of sediment particles may also contribute to site-specific similarities in the bacterial community structure. Although wave action and bottom water currents can suspend and mix surface sediments (61, 65), horizontal transport of sediments (61, 65), homogeneity within the sediment were very different. While Rhodobacteraceae and Cyano bacteria are known phototrophs; however, their profiles within the sediment were very different. While Rhodobacteraceae and Cyano bacteria were detected in the shallow depths exclusively, Cyano bacteria-related lineages were observed at all depths.

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REFERENCES


concentrations using the pathline counting method. Ground Water 32:719–726.