

## Diversity and Distribution of *Planctomycetes* and Related Bacteria in the Suboxic Zone of the Black Sea

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Received 19 September 2005/Accepted 10 February 2006

**Samples from six depths of the Black Sea's suboxic zone were analyzed for 16S rRNA gene sequence information. A gradient in phylotype diversity was found. The distributions of known anaerobic ammonium oxidation (anammox) bacteria, many unknown *Planctomycetes*, and other phylotypes were examined in relation to the local nutrient and redox conditions.**

The Black Sea is the world's largest permanent anoxic basin and is an excellent analog for early-Earth oceans (1) and modern-day oxygen-limited systems. Bacteria belonging to the phylum *Planctomycetes* that can produce N<sub>2</sub> gas from NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, i.e., anaerobic ammonium oxidation (anammox) bacteria, were recently identified in the suboxic zone here (13). This recently discovered N cycle pathway has been estimated to account for between one-fifth (5) and essentially all (14) of the N<sub>2</sub> production in marine oxygen-minimal zones. All known anammox bacteria belong to the bacterial phylum *Planctomycetes* (3, 10); their activity has also been reported to occur in wastewater treatment facilities (30), marine sediments (31), and sea ice (22).

*Planctomycetes* are characterized by intracellular membranes (sometimes enclosing condensed nuclei [17]), reproductive budding, a lack of peptidoglycan, and a wide distribution (8, 9). Interestingly, ether- and ester-linked lipids (6), a sterol production pathway (21), and C<sub>1</sub> transfer genes phylogenetically intermediate between *Archaea* and *Proteobacteria* (3) have all been found in this phylum. The genera *Planctomyces*, *Gemmata*, *Isosphaera*, *Pirellula*, *Blastopirellula*, and *Rhodopirellula* are known from pure culture (28). Discovery and cultivation of anammox bacteria has resulted in the recognition of "Candidatus" genera "Brocadia," "Kuenenia," and "Scalindua" (25, 26, 27, 30).

The aim of this study was to elucidate the composition of *Planctomycetes* in the Black Sea in order to shed some light on this intriguing phylum, as the complete extent of its diversity, distribution, and metabolic potential remains unknown. The first task undertaken was to obtain and assess detailed 16S rRNA gene sequence information about the planctomycetes which reside in the suboxic zone. The second was to apply this information to concurrent chemical data to better understand how the community structure relates to natural abundances of different N species.

**Sample collection.** Samples were obtained in April 2003 onboard the R/V *Knorr* from the Black Sea's central gyre

(42°30.79' N, 30°59.60' E) by use of a conductivity-temperature-depth Rosette with SeaBird sensors. Two liters of seawater for DNA extraction was pressure filtered from Niskin bottles onto 0.2- $\mu$ m Millipore Sterivex filters and frozen.

**Chemical data.** Nutrient samples were analyzed onboard using a two-channel Technicon Autoanalyzer II. Nitrite, nitrate, and ammonia were measured as described elsewhere (2, 28). Oxygen and hydrogen sulfide samples were measured with wet chemical and polarographic techniques by S. Kononov and A. Romanov (Marine Hydrophysical Institute, Sevastopol, Ukraine). A complete chemical analysis is provided elsewhere (18a).

**DNA extraction and analysis.** DNA was extracted from filters as described elsewhere (34). Samples were PCR amplified for partial 16S rRNA gene sequencing by using *Planctomycetes*-specific primers 58f (5'-GGCATGGATTAGGCATGC-3') (16) and 926r (5'-CCACCGCTTGTGTGAGCCCC-3') (35) for 32 cycles with an annealing temperature of 60°C. PCR products were cloned and sequenced with standard methods (see, e.g., reference 23). Sequences were edited, aligned, and analyzed for tree construction and rarefaction analysis with the following programs: Sequencher (<http://www.genecodes.com>), ClustalX (32), Genedoc (20), Bellerophon (12), RDP's CHECK\_CHIMERA (4), TreeCon (33), Phylip (<http://evolution.genetics.washington.edu/phylip.html>), and DOTUR (24).

**Chemical profiles.** The suboxic zone of the Black Sea is defined by low oxygen (<10  $\mu$ M) and H<sub>2</sub>S (<10 nM) concentrations. At the time of sampling, suboxic conditions were found between  $\sigma_{\theta}$  of 15.59 (approximately 53 m) and  $\sigma_{\theta}$  of 16.01 (approximately 69 m), where sulfide was first detectable (Fig. 1a). Although the absolute depth may change (18), the density stratification creates a stable region where NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> are completely consumed (Fig. 1b), and thus depth is given in density units ( $\sigma_{\theta}$ ) rather than meters.

**Diversity of *Planctomycetes* and related organisms.** Three hundred thirty complete insert sequences from six depths defined 56 unique operational taxonomic units (OTUs) by use of the furthest-neighbor approach with a 97% sequence similarity cutoff (Table 1; Fig. 2). The primer set utilized proved effective in selecting for many unknown *Planctomycetes* sequences, although non-*Planctomycetes* phylotypes were also amplified. Eleven distinct groups were defined (Fig. 2 and 3). Four of

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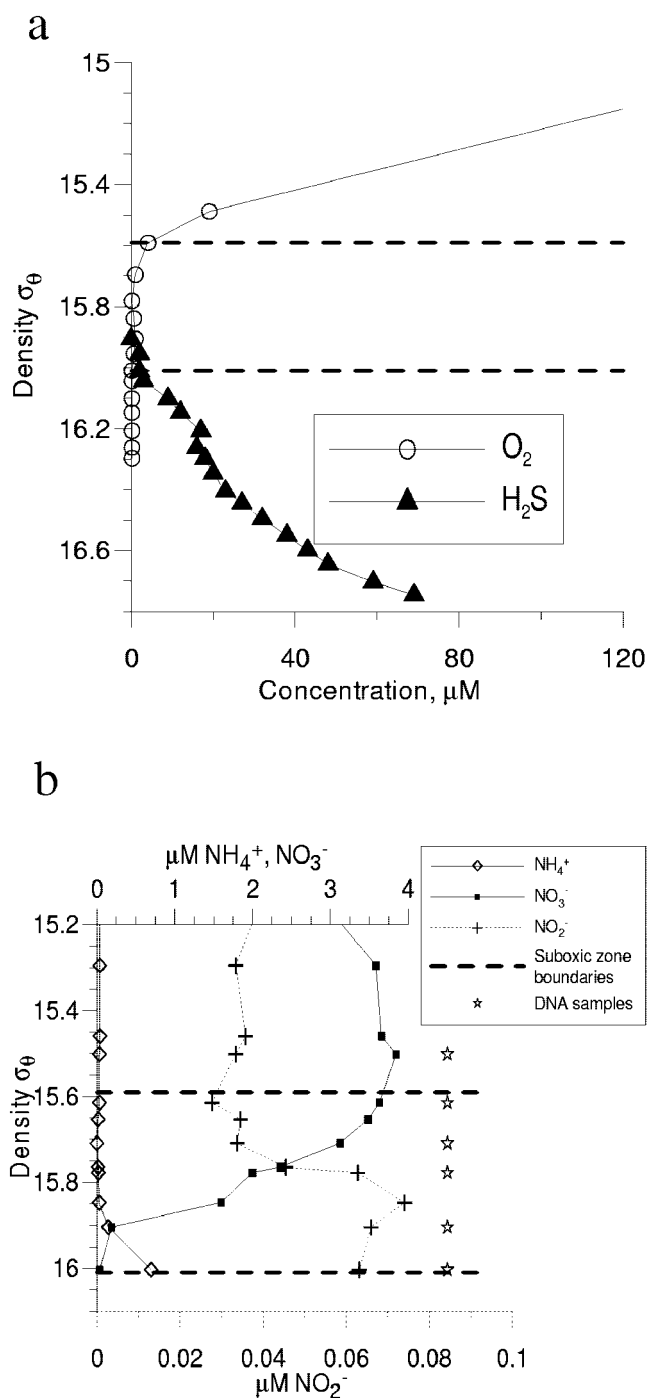


FIG. 1. Chemical data depicting the suboxic zone. (a) Dashed horizontal lines indicate the boundaries of the suboxic zone. They correspond to a depth of 53 m to 69 m. (b) N species and DNA sampling pattern. Note the different scale bars for nitrite.

these groups were clearly *Planctomycetes*: *Pirellula* (group A), *Planctomyces* spp. (group B), unknown *Planctomycetes*/BO84 sequence type (from enrichment culture [unpublished data]) (group C), and known anammox bacteria (group D). *Chlamydia* (group E) was represented by a single sequence but shared subdivision/division level affiliation with a larger un-

known group (group F), found throughout the suboxic zone but with some genus-level depth specificity.

Several phylotypes were found for *Verrucomicrobia* (group G) and a single sequence for *Lentisphaerae* (group H). Some other non-*Planctomycetes* sequences appear more derived, such as candidate division OD1 (group I), although they are not closely related ( $\sim 80\%$  similarity) to previously sampled organisms. The greatest diversity of this group is found deep in the suboxic zone, near the onset of sulfide. Despite their phylogenetic divergence, organisms in this group may be involved in sulfur cycling, consistent with previous observations of the candidate division OD1 (11). Note that the new OD1 sequences obtained have a 100% sequence identity with the probe PLA46 (19), originally designed for the *Planctomycetes*. Another uncultured group (group J) is apparently related to candidate division OP3. Group K appears deep in the suboxic zone and expands the known diversity of candidate division WS3. Representatives of this group, originally discovered in a methanogenic aquifer (7), are absent at shallower depths (Fig. 3). The most similar known sequences (similarity of 73 to 89%) are salt marsh sediment clones.

**Anammox bacteria.** "*Candidatus Scalindua sorokinii*" is the only known marine anammox bacterium and has been previously reported from the Black Sea (13, 27, 30). It was found at most depths, except  $\sigma_\theta$  of 15.5 and 15.7.  $\text{NO}_2^-$  and  $\text{NH}_4^+$  overlapped at the lowest depths; however, "*Candidatus Scalindua sorokinii*" sequences dominated clone libraries at the intermediate depth of  $\sigma_\theta$  of 15.8 (Fig. 3 and 4b). It is curious that bacteria at this depth were separated from the upward flux of  $\text{NH}_4^+$  from depth.  $\text{NH}_4^+$  was not measurable until multiple layers deeper in the suboxic zone ( $\sigma_\theta > 15.90$ ), where a different community structure is found. This can be explained several ways: (i) some proportion of anammox activity may rely on remineralization of  $\text{NH}_4^+$  from organic matter, which is consistent with natural nitrogen isotope ratios in the Black Sea (18a), (ii) interspecies  $\text{NH}_4^+$  transfer could be supplying this substrate for anammox bacteria, or (iii) the "*Candidatus Scalindua*"-type sequences found at middle and upper depths may represent inactive bacteria.

**Overall diversity.** Clone library diversity varied greatly between the different density layers (Fig. 3 and 4). Libraries from the shallow to intermediate depths returned fewer OTUs and tended to be dominated by a few sequences, such as "*Candidatus Scalindua*" or the BO84 type. Deeper areas of the suboxic zone ( $\sigma_\theta = 15.9$  and  $\sigma_\theta = 16.0$ ) exhibited a broad array of

TABLE 1. Sequence OTUs with depth, summarized from the clone libraries sequenced

Density ( $\sigma_\theta$ )	GenBank accession no.	No. of OTUs <sup>a</sup>	Most common group(s)
15.502	DQ368063–DQ368116	14	BO84 (C)
15.615	DQ368117–DQ368162	14	BO84 (C)
15.709	DQ368163–DQ368203	12	BO84 (C)
15.777	DQ368204–DQ368250	12	Known anammox (" <i>Ca. Scalindua sorokinii</i> ") (D)
15.904	DQ368251–DQ368290	27	<i>Pirellula</i> (A), unknown (F)
16.003	DQ368291–DQ368333	24	Candidate division WS3 (K)

<sup>a</sup> The number of OTUs for each depth was determined by use of the furthest-neighbor approach, with 97% similarity set as the threshold value.

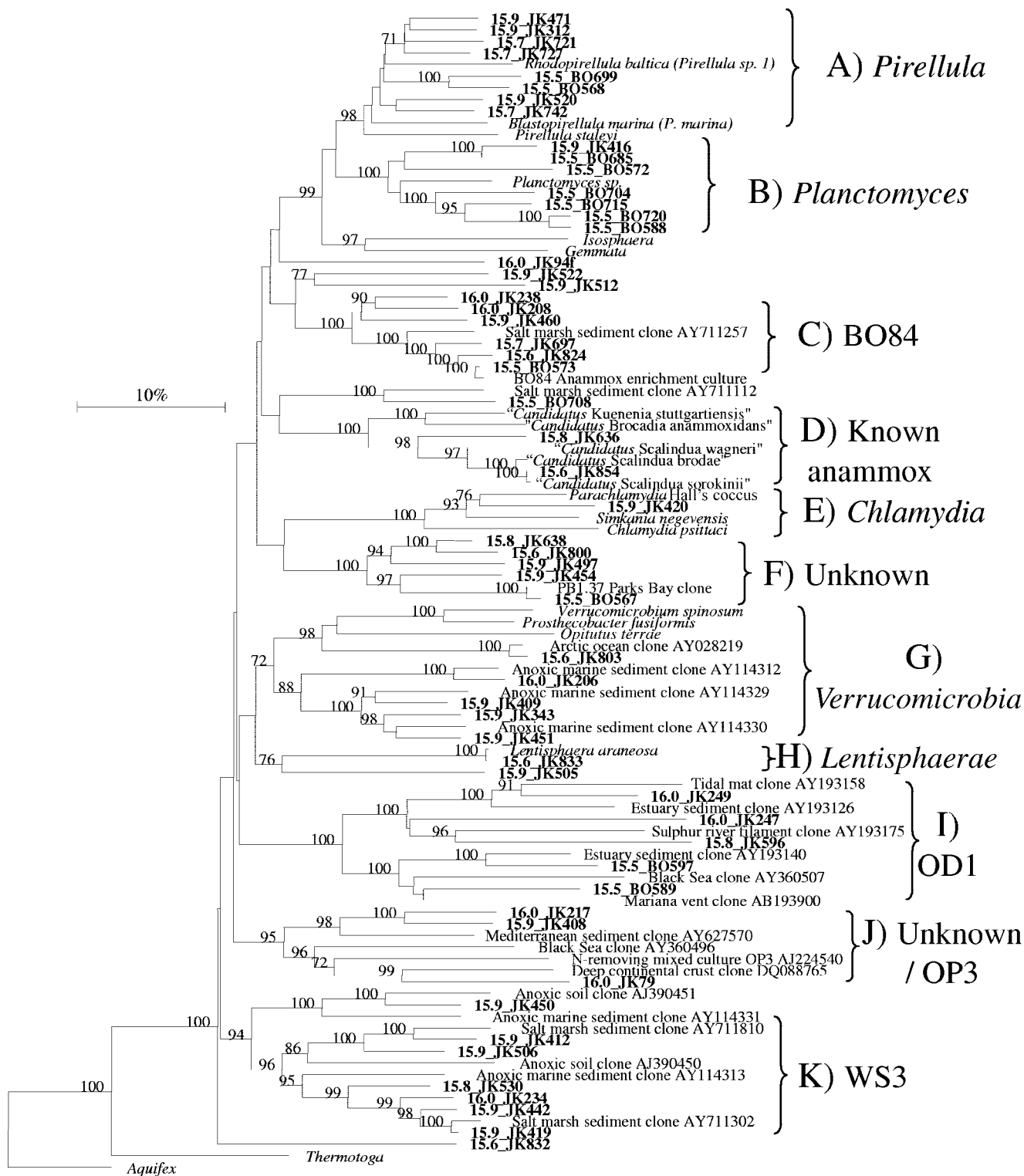


FIG. 2. Phylogenetic tree displaying representatives of all of the 56 unique OTUs found in the suboxic zone, with percentages of sequence similarity shown at left. Environmental sequences are displayed in bold; the leading number in each sequence ID corresponds to the shallowest depth where the OTU is present.

highly divergent sequences, as reflected in rarefaction curves; the six different densities sampled show two distinct patterns (Fig. 4). Clone libraries from the bottom two depths, where nitrate is absent and ammonium and sulfide begin to permeate

into the suboxic zone, had a steeper rarefaction curve. The relative taxonomic richness here is in part due to the presence of deeply branching groups such as OD1 (Fig. 2 and 3). Even when the data set is restricted to “true” *Planctomyces* se-

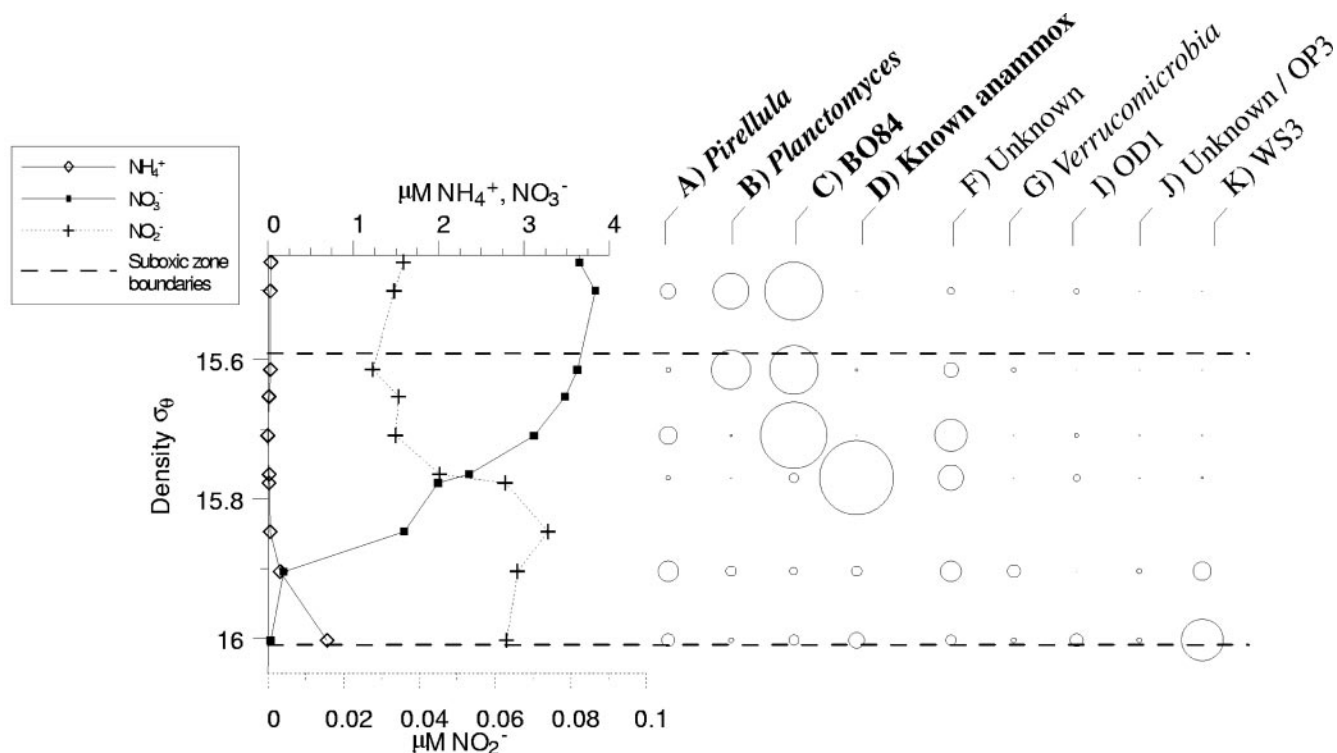


FIG. 3. Bubble graph depicting group depth profiles of clone libraries from each density layer, contrasted with nutrient data. Groups are based on the phylogenetic tree (Fig. 2), with true *Planctomyces* groups in bold.

quences (Fig. 4b), however, lower depths appear to harbor a greater diversity of bacteria than the more oxidized upper layers.

Overall, there were marked differences in community structure between as little as 0.1 density units (typically 2 or 3 m

here). Depths which had measurable levels of nitrate had lower diversity, both in the overall number of unique OTUs and in their relatedness to each other (Table 1; Fig. 3 and 4). When nitrate decreases and there is a transition to a more reducing environment ( $\sigma_\theta = 15.9$  and  $\sigma_\theta = 16.0$ ), there is a notable

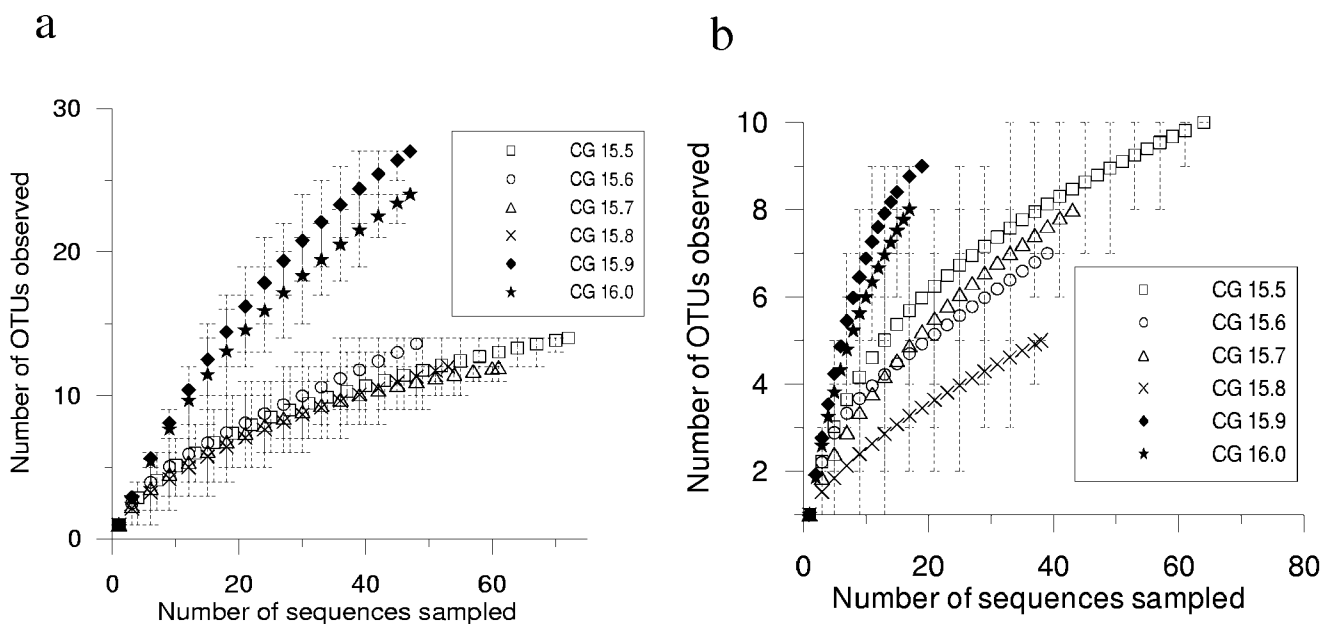


FIG. 4. Rarefaction curves with 95% confidence intervals for the clone libraries from each density sampled. (a) Summary for all OTUs found. (b) Same curves for the groups definitively identified as *Planctomyces* (groups A, B, C, and D). CG, Central Gyre.

increase in the overall number of OTUs and a decrease in their relatedness to each other. This was mainly due to the presence of uncultured organisms related to candidate divisions OD1 and WS3, but *Planctomycetes* sequences contribute to this effect. It has long been noted that transition zones or ecotones such as these can host high levels of diversity (see, e.g., references 15 and 29); the suboxic-zone sequences are consistent with these observations. Further work is needed, however, to characterize the unknown bacteria found here and to detail their significance to biogeochemical cycling.

**Nucleotide sequence accession numbers.** Nucleotide sequence accession numbers in the GenBank database are DQ368063 to DQ368333.

We are grateful to William Brazelton, David Stahl, Cheryl Jenkins, and the R/V *Knorr* crew for their help.

This work was funded by NSF Microbial Observatories grant MCB-0132101 and NSF-IGERT grant DGE-9870713 for astrobiology.

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