Free-living and aggregate-associated Planctomycetes in the Black Sea

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Abstract

We examined the distribution of uncultured Planctomycetes phylotypes along depth profiles spanning the redox gradient of the Black Sea suboxic zone to gain insight into their respective ecological niches. Planctomycetes phylogeny correlated with depth and chemical profiles, implying similar metabolisms within phylogenetic groups. A suboxic zone sample was split into >30 and <30 μm fractions to examine putative aggregate-attached and free-living Planctomycetes. All identified Planctomycetes were present in the >30 μm fraction except for members of the Scalindua genus, which were apparently free-living. Sequences from Candidatus Scalindua, known to carry out the anammox process, formed two distinct clusters with nonoverlapping depth ranges. One cluster, only 97.1% similar to the named species, was present at high nitrite/nitrate and low ammonium concentrations in the upper suboxic zone. We propose this sequence type be named 'Candidatus Scalindua richardsi'. A second cluster, containing sequences more similar to Candidatus Scalindua sorokini, was present at high ammonium and low nitrite conditions in the lower suboxic zone. Sequences obtained from the sulfidic zone (1000 m depth) yielded Planctomycetes from two uncharacterized Planctomycetacia clusters and three potentially new genera as well as sequences from the uncultured OP3 phylum.

Introduction

Two pathways are involved in the conversion of fixed nitrogen to N2: the anammox process where \( \text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 \) via hydroxylamine and hydrazine intermediates (van de Graaf et al., 1996, 1997) and denitrification where \( 2\text{NO}_3^- + \text{organic carbon}/\text{H}_2\text{S} \rightarrow \text{N}_2 + \text{CO}_2/\text{SO}_4^{2-} \) via a nitrous oxide intermediate (Zumft, 1997). Although a wide variety of bacteria can mediate denitrification (Zumft, 1997), only a subset of genera in the Planctomycetes phylum are known to mediate the anammox process. In the marine environment, members of the Candidatus Scalindua genus utilize the anammox process in both sediments and oxygen-deficient water columns, and several other Candidatus genera (Kuenenia, Brocadia, Anamnoxoglobus) mediate the process in freshwater and wastewater treatment plants (Schmid et al., 2007). Anammox bacteria have an unusual internal compartment, the anammoxosome, composed of tightly packed ladderane lipids, which is necessary to contain the volatile intermediate hydrazine (Sinninghe Damste et al., 2002). Internal cell compartments are a characteristic feature of the Planctomycetes phylum (Lindsay et al., 2001), as are their protein envelopes and lack of a peptidoglycan cell wall (Liesack et al., 1986; Hieu et al., 2008). Owing to their internal compartments and compartmentalization machinery, Planctomycetes have been proposed as the progenitor of eukaryotes (Forte & Gribaldo, 2010; Reynaud & Devos, 2011). However, it seems more likely that similarities between Planctomycetes and eukaryotes are a case of convergent evolution (Fuchsm & Rocap, 2006; Strous et al., 2006; McNerney et al., 2011).

Anammox bacteria are quite different from other characterized Planctomycetes both phylogenetically and physiologically. While anammox bacteria are primarily autotrophic, all of the other characterized Planctomycetes...
are heterotrophic. Furthermore, the anammox bacterium *Candidatus Kuenenia stuttgartiensis*’s genome shares 0–30% *Planctomycetes*-specific genes with other *Planctomycetes* genomes, as defined by reciprocal BLAST scores, compared to 275–764 between the other *Planctomycetes* genomes: *Rhodopirellula baltica*, *Blastopirellula marina*, *Gemmata obscuriglobus*, and *Planctomyces maris* (Woebken et al., 2007b). It should be noted, however, that these four organisms are all in the same class, *Planctomycetacia*, while the anammox genera form a separate class with, and presumably share more genes with, the uncultured members of the agg27 group (Elshahed et al., 2007).

Members of the Scalindua genus have a versatile metabolism. Two Scalindua species have been enriched from marine sediments and closely examined (van de Vossenberg et al., 2008). Both of these Scalindua enrichments and *Candidatus Kuenenia stuttgartiensis*, enriched from a wastewater treatment facility, have at least three metabolisms: the anammox process, iron and manganese reduction with formate, and nitrate reduction with formate (Strous et al., 2006; Kartal et al., 2007; van de Vossenberg et al., 2008). In the presence of nitrate, *Candidatus Kuenenia stuttgartiensis* uses formate as an energy source for dissipatory nitrate reduction to ammonium (DNRA); the ammonium is then converted to N2 using the anammox process (Kartal et al., 2007). Presumably, members of Scalindua use the same pathway.

Contrastingly, members of the class *Planctomycetacia*, including *Rhodopirellula*, *Blastopirellula*, *Pirellula*, and *Planctomyces*, serve an important ecological function by performing the initial aerobic breakdown of complex organic matter into simpler compounds (Glockner et al., 2003). In culture, *R. baltica* and *B. marina* can degrade sulfated heteropolysaccharides (Glockner et al., 2003; Schlesner et al., 2004) as well as N-acetylglucosamine (Rabus et al., 2002; Schlesner et al., 2004), a monomer of chitin and a subunit of bacterial peptidoglycan. Sulfated heteropolysaccharides are abundant in the marine environment (Woebken et al., 2007b and references therein). Sulfatases, enzymes used to degrade sulfated heteropolysaccharides, are abundant in the genome of marine bacteria *P. maris*, *R. baltica*, *B. marina* (Glockner et al., 2003; Woebken et al., 2007b), so this ability appears to be common in the *Planctomycetacia* class.

Some members of the *Planctomycetalia* class can also break down organic matter under both anaerobic and sulfidic conditions. *Blastopirellula* strain Zi62 was isolated from a sulfur- and sulfide-rich spring and was found to undergo fermentation and sulfur reduction (Elshahed et al., 2007). The genes for fermentation exist in the genomes of aerobic *Planctomyces* *R. baltica*, *B. marina*, and *P. maris* although these strains are not known to perform fermentation (Woebken et al., 2007b). In summary, the *Planctomycetalia* consume organic matter under both aerobic and anaerobic conditions.

One bacterial strain has been isolated from a third class of *Planctomycetes*, the WPS-1 class, now known as *Phyiciphiarae*. This recently cultured planctomyzete, *Phyiciphia mikurensis*, is a facultatively anaerobic heterotroph and performs fermentation as well as nitrate reduction to nitrite (Fukunaga et al., 2009). Fermentation may explain the ubiquity of *Phyiciphia* at the sulfate-methane transition zone in marine sediments (Harrison et al., 2009).

In aerobic environments, *Planctomyces* have been found attached to sinking marine aggregates (DeLong et al., 1993), which is consistent with their role of degrading complex organic matter. Stable isotope probing experiments indicate that members of *Rhodopirellula* metabolize phytodetritus (Ghiring et al., 2009). Attachment to surfaces is important in the cell cycle of many *Planctomyces*. In the cell cycle of *Rhodopirellula*, *Blastopirellula*, *Pirellula* and *Planctomyces*, a bud on the mother cell turns into a motile swarmer cell with flagella, which then differentiates into adult stalked nonmotile cells with a holdfast, allowing cells to remain attached to a surface (Baud & Staley, 1976; Schlesner et al., 2004; Gade et al., 2005). For example, members of *Pirellula* have been found attached to marine diatoms during a diatom bloom (Morris et al., 2006), *P. mikurensis* (WPS-1) was found attached to seaweed (Fukunaga et al., 2009), several *Planctomyces* lineages were found attached to kelp (Bengtson & Øvreås, 2010), and members of the agg27 group were found on sinking marine particles (DeLong et al., 1993). Owing to the presence of attached bacteria in all three classes of *Planctomycetes*, one might assume that anammox bacteria in the marine environment are also particle-attached. On the Namibia shelf, 24 ± 8% of anammox cells visualized by CARD-FISH were particle-associated, while the majority of anammox cells were not (Woebken et al., 2007a). However, the size of these particles were relatively small, and the particle maximum was close to the sediments, indicating that active anammox cells were likely resuspended with sediments (Woebken et al., 2007a). Thus, it is yet to be determined whether or not anammox bacteria are associated with sinking particles in open water low-oxygen systems.

*Planctomyces* living in suboxic and sulfidic water columns have not been as well characterized. Here, we identify *Planctomyces* of the Black Sea suboxic and sulfidic waters. The Black Sea is a brackish semi-enclosed permanently stratified basin, with an oxic zone where the cold intermediate layer (core density of σ0 ≈ 14.5) represents the lower boundary of direct communication with the surface. Below this is a ~50-m-thick suboxic zone, where oxygen is < 10 μM and hydrogen sulfide is undetectable.
(Murray et al., 1995). Below the suboxic zone is a sulfidic layer, which continues to the bottom. The diversity of Planctomycetes in the Black Sea suboxic zone was previously examined using 16S rRNA gene sequencing (Kirkpatrick et al., 2006). Bacteria were found related to the Rhodopirellula, Planctomycetes, and Candidatus Scalindua genera as well as to several uncultured groups including agg27 and a cluster of sequences labeled 'Unknown' by the authors (Kirkpatrick et al., 2006) and here labeled Black Sea I. Using a different 16S rRNA gene primer set, Physicisphaerae (formerly WPS-1) have also been found in the Black Sea suboxic zone, and two members of the WPS-1 group were categorized as aggregate-attached (Fuchsman et al., 2011).

By examining depth profiles of Planctomycetes across the redox gradient of the Black Sea suboxic zone, we aimed to gain insight into their metabolisms and ecological niches. With this goal, we examined Planctomycetes that are attached to sinking organic matter (> 30 μm fraction) and those that are potentially free-living (< 30 μm). Anammox bacteria are of particular interest for this study because of their known geochemical importance in the Black Sea (Kuypers et al., 2003; Jensen et al., 2008).

Materials and methods

Owing to the salinity stratification of the Black Sea, characteristic inflections in the water-column profiles (such as oxygen) are associated with specific density values regardless of when and where they were sampled (Murray et al., 1995). Therefore, results presented here are plotted against potential density (σ0) rather than depth (m). A comparison of depth and density for each cruise is shown in Supporting information, Fig. S1.

Sampling

DNA samples were collected in the western central gyre of the Black Sea on four separate cruises: (1) in September 2000 at station M10-L41 on the R/V Bilim (42°10′N, 29°41′E), (2) in early June 2001 at station 2 Voyage 162 leg 17 of the R/V Knorr (42°30′N, 30°46′E), (3) in April 2003 at station 19 on Voyage 172 leg 7 of the R/V Knorr (42°30′N, 31°00′E), and (4) in late March 2005 at station 2 on cruise 403 of the R/V Endeavor (42°30′N, 30°45′E).

For the Knorr and Endeavor cruises, samples were collected using a CTD–Rosette with 10-L Niskin bottles and Sea Bird sensors. Approximately 2 L of seawater was filtered onto 0.2-μm Millipore Sterivex filters. Samples were frozen immediately and later stored at −80 °C. For the R/V Bilim cruise, samples were obtained from 30-L Nansen bottles, and approximately 7 L of water were filtered onto a 0.2-μm Millipore Sterivex filters after passing through a 30-μm prefilter. Samples were frozen immediately and later stored at −22 °C. A set of DNA samples were also prefiltered with 30-μm prefilters on the R/V Endeavor cruise in 2005. On the Bilim and Knorr cruises, sampling focused in the suboxic region, but on the Endeavor cruise, a more complete depth profile of the oxic and sulfidic layers was obtained, including samples at 1000 m (σ0 = 17.19) and 2000 m (σ0 = 17.21). In addition, to look at the Planctomycetes community under fully oxygenated conditions, 2 L from one Niskin bottle triggered at 50 m (σ0 = 13.9) was filtered onto a Sterivex on the R/V AKBAHABT cruise 2900 in May 2007 in the Northeast Black Sea (44.5°N, 37.7°E), frozen immediately and stored at −80 °C.

DNA extraction

The DNA extraction protocol was adapted from the study of Vetriani et al. (2003) and included 8–10 freeze–thaw cycles between a dry ice/ethanol bath and a 55 °C water bath, followed by chemical lysis with lysozyme and proteinase K. DNA was then purified with phenol–chloroform followed by spin columns (Qiagen, Valencia, CA).

16S rRNA gene clones

Cloned 16S rRNA gene sequences were amplified from sulfidic water at 1000 m depth from the western central gyre in March 2005. Some partial length sequences obtained from suboxic water collected from the western central gyre in April 2003 in the study of Kirkpatrick et al. (2006) were further sequenced for this article [58F and 926R (Wang et al., 2002); complete insert equals 900 bp]: Genbank accession numbers: DQ368172, DQ368174, DQ368178, DQ368186, DQ368199, DQ3682178, DQ368229, DQ368248, DQ368251, DQ368256, DQ368260, DQ368262, DQ368269, DQ368274, DQ368283, DQ368292–3, DQ368295, DQ368299, DQ368300, DQ368302, DQ368307–9, DQ368311, DQ368313, DQ368316, DQ368318, DQ368320, DQ368325, DQ368330–2. Sequencing was performed at High-Throughput Sequencing Solutions (http://www.htseq.org) using primers T7 and M13R. Chromatograms were hand inspected, and contigs assembled using the Sequencher program (GeneCodes Corporation, Ann Arbor, MI). Resequenced suboxic zone sample sequences were then processed in the SEPIK program (Collins & Rocap, 2007) to choose which restriction enzymes should be used in terminal restriction fragment length polymorphism (TRFLP) analyses, in order to ensure that groups of interest could be distinguished with the restriction enzymes used. 16S rRNA gene from 1000 m depth was amplified (58F and 926R) and cloned as described in the study of Kirkpatrick.
et al. (2006) using Invitrogen (Carlsbad, CA) TOPO vectors. Sequencing was first performed using 58F. Some 1000-m clones were resequenced in full using primers T7 and M13R (fully sequenced clones identified by 1K: e.g. 1K11E). All clones were chimera checked with Bellerophon (Huber et al., 2004) and submitted to GenBank (accession numbers HQ883377-HQ883474).

**TRFLP**

TRFLP chromatograms were obtained using *Planctomycetes*-specific 16S rRNA gene primers (58F-FAM and 926 R; Wang et al., 2002). Five samples from 2001 and eight samples from 2003 were analyzed for TRFLP from the suboxic zone of the western gyre as well as eight prefiltered samples from 2000 and one whole sample from \( \sigma_\theta = 15.9 \). An additional sample from \( \sigma_\theta = 16.1 \) in 2001 could not be amplified well enough for TRFLP chromatograms to be obtained. In 2005, TRFLP chromatograms were obtained across the redox gradient: one oxic sample, one hypoxic zone (39 \( \mu \)M) sample, seven suboxic zone samples, and four sulfidic zone samples. The chromatogram from MspI at \( \sigma_\theta = 16.4 \) was rejected later in analysis because of insufficient total peak height. Five suboxic zone samples that were prefiltered with a 30-\( \mu \)m prefilter and one prefilter were also examined. PCR products were amplified for 30 cycles at 60 °C, purified (Qiagen columns; Qiagen, Valencia, CA) and digested separately overnight with restriction enzymes HaeIII, Hpy88I, MspI, and immediately precipitated with ethanol. Fragment analysis was performed on a MegaBACE 1000 apparatus (Molecular Dynamics) at the University of Washington Marine Molecular Biotechnology Laboratory. Electrophoretic profiles were visualized with DAX software (Van Mierlo Software Consultancy, the Netherlands).

**Identification of TRFLP peaks**

The ReFLP program (Collins & Rocap, 2007) produced a list of predicted fragment lengths for 16S rRNA gene clones. These fragment lengths were ground truthed by two clones, corresponding to *Planctomyces* JK211 and *Candidatus* Scalindua JK200, which were digested with all restriction enzymes individually. TRFLP peaks were identified using the criteria that an identified peak was \( \pm 1 \) base pair from the length of the predicted fragment length for all restriction enzymes [in two cases, the identified peak was 2 base pairs from the predicted fragment (shown in parentheses in Table 1)]. An identified peak was allowed a total range of 1 base pair between samples. Using a small bin size, the risk that an identified TRFLP peak actually represented more than one unrelated bacterial species was reduced. In most of the suboxic zone samples, there were 26–30 peaks in TRFLP chromatograms using MspI. Approximately 60% of peaks were identified in suboxic zone samples. In the lower suboxic zone (\( \sigma_\theta \geq 15.9 \)), there were between 30 and 40 peaks, and the percentage of identified peaks was lower. With HaeIII, there were also 25–40 peaks in TRFLP chromatograms, but Hpy88I only produced 20–25 peaks.

The primers used in this study were designed to amplify the *Planctomycetes* phylum. However, these primers have previously been found to also amplify sequences affiliated with other members of the *Planctomycetes*–*Verrucomicrobia*–*Chlamydia superphylum* (which include *Lentisphaerae*, candidate divisions Obsidian Pool 3 (OP3) and *Poribacteria* (Wagner & Horn, 2006) as well as the candidate division WS3 (Kirkpatrick et al., 2006). Although the database used to identify TRFLP peaks included clones representing *Lentisphaera*, WS3, *Verrucomicrobia*, and *Chlamydia*, previously found in clone libraries from the Black Sea, no actual peaks corresponding to these phyla were identified. Identified TRFLP peaks only corresponded to members of the *Planctomycetes* and OP3 phyla. This difference between the communities obtained by sequencing and TRFLP could be due to steric interactions of the fluorescent fluorescein amidite (FAM) molecule attached to the forward primer used in TRFLP or to the low abundance of the tangential phyla.

<table>
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Statistical analyses

TRFLP profiles were normalized downward by total peak height with a minimum cutoff of 0.3% of the total peak height. TRFLP peaks were binned using frame shifting (Hewson and Fuhrman, 2006) with four frames at 0.5 base pair intervals. For each enzyme, resemblance matrices were obtained using the Whittaker index, which takes abundance (peak height) into account, and the Jaccard index, which uses presence/absence. Trends were the same for both indices, so only the Whittaker index, which contains more information, is discussed. For each comparison between two samples, the maximum similarity of the four frames was used. Hierarchical cluster analyses (using the group average) and principal component analysis (PCA) were performed using the Primer 6 program. The restriction enzyme MspI was primarily used in these analyses because it distinguished the greatest number of phylotypes in the suboxic zone samples. Data from the HaeIII enzyme formed the same large clusters but with slightly higher percentage of similarities. Owing to prefiltration of most samples from 2000, they could not be included in these analyses. Error in the resemblance matrix and significance level of the cluster diagram were determined using a Monte-Carlo simulation of 50 replicates using the average error of both normalized peak height (+ 83 rfu where total peak height is 15 000 rfu) and base pairs (+ 0.06 bp) as determined by 14 sets of duplicate TRFLP profiles. All simulated replicates had at least 85% Whittaker similarity.

Phylogenetic trees

Trees were constructed in MEGA4 (Tamura et al., 2007) after aligning with CLUSTALX (Larkin et al., 2007). In the phylogenetic analyses where multiple sequence lengths were necessary, trees were constructed using the minimum evolution method (Rzhetsky & Nei, 1992) with 1000 bootstraps, and distances were computed using the maximum composite likelihood method (Tamura et al., 2004). These multiple sequence alignments contained complete sequences and were not trimmed down to partial Black Sea sequences to maintain previously published phylogenetic relationships. The neighbor-joining tree in Fig. 5 was created using 720-bp sequences with 100 bootstraps.

Nutrient concentrations

S. Konovalov and A. Romanov (MHI, Ukraine) measured oxygen with the Winkler method and assayed sulfide by iodometric titration (Cline, 1969). In both cases, reagents were bubbled with argon to avoid contamination by atmospheric oxygen. During Bilim 2000 and Knorr 2001, nutrients were analyzed on shipboard by S. Tugrul (METU, Turkey). For Knorr 2003 and Endeavor 2005, nutrients were analyzed shipboard using a Technicon Autoanalyzer II system. Nitrate was reduced to nitrite using a cadmium column, which was measured using sulphanilamide and N(1-naphthyl)-ethylenediamine (Armstrong et al., 1967). Ammonium was analyzed using the indophenol-blue procedure (Slayke & MacIsaac, 1972). Deep-water ammonium samples were diluted with nutrient-free Black Sea surface water to reduce sulfide content.

Results

Chemistry

Oxygen and sulfide data for each cruise are discussed in the study of Fuchsman et al. (2008). The boundaries of the suboxic zone for each year, < 10 μM O₂, for the upper boundary and first appearance of detectable sulfide for the lower boundary, are indicated by dashed lines in Fig. 1. These density surfaces were slightly shoaled in 2003 compared to 2000, 2001, and 2005 (Fig. S1).

Nitrite concentrations were low through the oxycline and peaked in the lower suboxic zone (Fig. 1). In 2000 and 2005, the nitrite maximum was at σθ = 15.85, while in 2003, it was at σθ = 15.8, and it was absent in 2001. Nitrite concentrations from 2000 were significantly larger than in other years, with a maximum at 0.28 μM. The maxima were < 0.1 μM in the other years (Fig. 1).

While the flux of ammonium across the sulfide boundary into the suboxic zone was similar between years (Fuchsman et al., 2008), measurable ammonium reached different depths in the suboxic zone (Fig. 1). In 2000, ammonium became undetectable at σθ = 16.05, in 2001 at σθ = 15.95, in 2003 at σθ = 15.8, and in 2005 at σθ = 15.85.

Molecular biology

Bacteria identified in TRFLP chromatograms and their respective fragment lengths are shown in Table 1. The phylogenetic relationships of these identified Planctomycetes are shown in Fig. 2. All the Planctomycetes identified in the suboxic zone in 2005 were found on particulate matter (> 30 μm) except for Scalindua, which were only found in the prefiltered sample (< 30 μm; Fig. 3).

A hierarchical cluster analysis of the similarity in TRFLP samples from the western central gyre suboxic zone were compared using Whittaker indices, based on restriction digests with enzyme MspI. The Planctomycetes/OP3 community separated into oxic (σθ = 14.9–15.3),
upper suboxic ($\sigma_0 = 15.6–15.85$), lower suboxic ($\sigma_0 = 15.9–16.0$), and sulfidic [$\sigma_0 = 16.1–17.21$ (2000 m)] clusters (Fig. S2). Although no sulfide was detected at $\sigma_0 = 16.1$ 2005, the $\sigma_0 = 16.1$ 2005 sample clustered with the sulfidic samples when either restriction enzymes MspI or HaeIII were examined. However, sulfide was detected 3 m below this sample, and the detection limit for sulfide with this technique is 3 $\mu$M (Konovalov et al., 2003), so sulfide may have been present at $\sigma_0 = 16.1$ at low concentrations. Although suboxic zone samples from 3 years were analyzed, they all separated into lower and upper suboxic zone clusters at $\sigma_0 = 15.9$ when either MspI or HaeIII were examined (Fig. S2). Each set of samples clustered most closely with the other samples from the same cruise, excepting samples in the $\sigma_0 = 15.75–15.85$ range from 2003 to 2005, which clustered together.

Hierarchical clusters indicated variability in the suboxic zone Planctomycetes community. A PCA was used to find which TRFLP peaks contributed the greatest microbial variability between depths and cruises (Fig. S3). For MspI, eigenvector PC1, representing 37% of the variability, was strongly correlated with peak 210 (BO823 BSI; linear coefficient of 0.783), which was particularly high in

![Fig. 1. Depth profiles of TRFLP peak height for Candidatus Scalindua (MspI) compared to reactants ($NO_2^-$ and $NH_4^+$) and product (biological $N_2$; Fuchsman et al., 2008) of the anammox process: (a) in 2000 (b) 2001 (c) 2003 (d) 2005. Note different scale for axes for 2000. Average error for peak height is ± 83 rfu. Dashed lines denote suboxic zone boundaries. Nutrients and DNA sequences were collected from the same cast where possible.](image-url)
suboxic zone samples from 2001 (Fig. 4), and also correlated with peak 259 (Scalindua; linear coefficient 0.3), which was abundant for lower suboxic zone samples. Eigenvector PC2, representing 16% of the variability, indicated opposite trends for peak 459 (BO832 agg27) and peak 127 (linear coefficients of −0.554 and 0.584, respectively); BO832 agg27 was abundant in upper suboxic zone samples from 2003 to 2005 and low in 2001 (Fig. 4), while peak 127 was absent in 2003 samples and abundant in 2001 samples. BO832, Scalindua, and peak 370 (which matches peak 127) also contributed the most to microbial variability when peaks cut with the HaeIII enzyme were examined (data not shown). BO832 is not cut by the HaeIII enzyme.
Some Planctomycetes are found both in oxic and suboxic zones. Comparing the various suboxic zone samples to a sample with 105 μM oxygen (σO₂ = 14.9), seven phylotypes were found in common: agg27 BO832, BSI BO813, BSI BO823, BSI JK389, Planctomyces JK211, Planctomyces BO821, Planctomyces JK221, Rhodopirellula JK742. Three of these phylotypes (BSI BO813, Planctomyces JK211, Planctomyces BO821) were also identified in a fully oxygenated sample (294 μM O₂ and 0.6 μM NO₃⁻; E. Yakushev, P.P. Shirshov Institute of Oceanology, Russia, unpublished data) from 50 m depth (σO₂ = 13.9) in the Northeast Black Sea in May 2007. These two oxygenated Planctomycetes communities shared only 22% similarity using the Whittaker Index, but as they also differed in location, time, and depth, we cannot pinpoint the cause of these community differences.

**Sulfidic zone**

Planctomycetes-specific 16S rRNA gene primers amplified Planctomycetes, Lentisphaera, and OP3 clones from 1000 m (Fig. 2 and Fig. S4). These primers are known to amplify some sequences from phyla in the Planctomycetes –Verrucomicrobia–Chlamydia superphylum (Kirkpatrick et al., 2006). A large number of OP3 sequences were obtained from 1000 m (Fig. S4). These sequences correspond to OP3 groups II, III, and IV as defined by Glockner et al. (2010). The most abundant sequence group (phylotypes 1K2C from OP3-III) was also identified in TRFLP profiles from the sulfidic zone with its highest normalized peak height at 1000 and 2000 m (Figs S4 and S5). OP3 phylotype JK79, also in OP3-III, had a contrasting depth profile where peak height was highest at σO₂ = 16.4 in the upper sulfidic zone (Fig. S5).

The Planctomycetes sequences from 1000 m in the sulfidic zone clustered into group A17 and in uncharacterized sulfidic group A in the class Planctomycetacia and in a series of small clusters in the deeply branching class of Planctomycetes (Fig. 2). Interestingly, sequences 1K8B and 1K11E from 1000 m, in the A17 group, are closely related to sequences found in a diatom bloom in oxic surface waters (Morris et al., 2006) and from the suboxic zone of Lake Tanganyika (Schubert et al., 2006), implying a wide range of ecological niches in the A17 group. However, Planctomycetes from 1000 m primarily are found in uncharacterized groups outside the Planctomycetacia class. These Planctomycetes imply that novel genera and perhaps novel metabolic groups of Planctomycetes reside in sulfidic environments.

**Discussion**

**Particle-attached and free-living bacteria**

As aggregates larger than 53 μm dominate the vertical mass flux in the ocean (Clegg & Whitfield, 1990; Amiel et al., 2002), bacteria caught on the 30-μm-pore-size filter collected from the center of the suboxic zone in 2005 (σO₂ = 15.8) were likely mainly attached to sinking aggregates or large suspended particles. These particles contained diatom chloroplasts (Fuchsman et al., 2011). Particulate organic carbon (determined by a 0.7-μm-pore-size filter) was around 4 μM in the suboxic zone at that time, and C/N ratios of these particles were about 9, indicating that the particulate organic matter was at least partially degraded (Fuchsman et al., 2011). Particulate manganese had a maximum at σO₂ = 15.85 and was detectable throughout the suboxic zone (Fuchsman et al., 2011).

Identified 16S rRNA gene phylotypes were categorized as particle-attached or free-living based on a comparison of TRFLP chromatograms between the prefiltered sample and the 30-μm-pore-size prefiltro from σO₂ = 15.8 2005 (Fig. 3). If > 75% of the combined normalized peak height for a phylotype was found on the prefiltro chromatogram, it is considered to be particle-attached. If > 75% of the combined peak height for a phylotype was found in the filtrate chromatogram, it is considered free-living. These findings were supported and extended by...
Fig. 4. Depth profiles of TRFLP peak height from 2001, 2003 and 2005, with restriction enzymes as per Table 1. Names are color-coded by aggregate affinity: aggregate-attached (green), free-living (red), found in both fractions (blue) or not determined (black), and presence in fully oxygenated sample (box). Average peak height error is ± 83 rfu.
comparing presence and absence in prefiltered and bulk water samples $\sigma_0 = 15.7 - 15.9$. Some *Planctomycetes* were identified as particle-attached including *Planctomyces* phylotype JK221; agg27 phylotypes BO825 and BO832; and BSI phylotypes BO823, JK782, JK80, JK589, and BO813. Other *Planctomycetes* were found equally in both fractions, including *Planctomyces* phylotype BO821, *Rhodopirellula* BO815, the unidentified peak pair 370/127 (HaeIII/MspI), and unidentified peak 166 (MspI). These organisms may be attached to both large and small ($< 30 \mu m$) particles or live some of their life cycle as free-living organisms (Bauld & Staley, 1976; Schlesner et al., 2004; Gade et al., 2005). In contrast, TRFLP peak 259 corresponding to the Scalindua phylotype was the only peak that was only present in the filtrate, and thus Scalindua are classified here as free-living. *Planctomyces* JK211, although present at low levels on the particulate material, was also classified as free-living. On the Namibian shelf, $24 \pm 8\%$ of anammox cells were associated with small particles, likely resuspended sediment (Woebken et al., 2007a). With our dataset, we cannot eliminate the possibility that anammox bacteria exclusively attach to $< 30 \mu m$ particulate material. However, they certainly do not attach to sinking particles (defined as $> 50 \mu m$ by Clegg & Whitfield, 1990).

Some *Planctomycetes* were found in a range of oxygen concentrations from fully oxygenated to suboxic waters (such as *Planctomyces* JK211, BO821 and BSI BO813), while others were found at 105 $\mu M$ oxygen and suboxic waters (such as agg27 BO832 and BO825 and *Planctomyces* JK221). As all these *Planctomycetes* were found on particles, they may have been attached to sinking particles in oxic waters and remained on the particles as they sank into the suboxic zone. Other *Planctomycetes* were present only under suboxic conditions (BSI JK782, JK80, BO823, and *Rhodopirellula* BO815; Fig. 4), implying a community

Fig. 5. A bootstrapped neighbor-joining phylogenetic tree of Black Sea anammox 720 bp sequences which shows a new Candidatus species, ‘Candidatus Scalindua richardsi.’ Sequences where 869 bp are available are underlined. Density level is noted by symbol.
specific to particles in low-oxygen waters. These Planctomy-
cetes either have a rapid chemotactic response to nutrient plumes generated by sinking particles as seen in some other marine bacteria (Stocker et al., 2008), or they create their own particles from processes such as manganese oxidation. Manganese- and iron-encrusted Planctomyces have been found in lakes, although these organisms have not yet been well characterized using molecular methods (Schmidt et al., 1981, 1982).

**Metabolic implications**

Genomic and cultivation studies in the open ocean imply that most Planctomyces, excluding anammox bacteria, are oxygenic heterotrophs able to degrade complex organic compounds (Glockner et al., 2003; Schlesner et al., 2004; Woebken et al., 2007b). Rhodopirellula-like JK742, which is only detectable in samples from 2005 with more than 5 μM oxygen (Fig. 4), may be such an organism. However, the suboxic and sulfidic zones of the Black Sea are an ideal place to find Planctomyces with alternative metabolisms. For example, the Rhodopirellula-like phylotype BO815 was not found in oxic samples but had a maximum in the σ0 = 15.7–15.95 region and was detectable throughout the sulfidic zone (Fig. 4). This organism, as well as the other Planctomycesetacetica sequenced from 1000 m (> 300 μM H2S; Fig. 2), may be carrying out fermentation in the sulfidic zone, as noted previously in a sulfur spring by isolate Zi62 (Elshahed et al., 2007). Organic acids, indicative of fermentation, have a maximum in the Black Sea’s upper sulfidic zone and are present throughout the lower sulfidic zone (Albert et al., 1995).

We used depth profiles across the redox gradient to investigate linkages to substrates available to these organisms. Consistent vertical profiles throughout the 3 years examined indicate robust trends in the distribution of Planctomyces in the suboxic zone (Fig. 4). The genera Planctomyces, agg27, and Black Sea I (BSI) had fairly consistent depth profiles between identified genotypes within the group (Fig. 4). Agg27 clones were generally present in the upper suboxic zone and the oxic zone (Fig. 4). A microaerophilic enrichment culture of an agg27 strain sampled in 2003 grew in Black Sea water enriched with ammonium and nitrite (Kirkpatrick et al., 2006). Although organic matter was present in the Black Sea water, no additional organic compounds were added. The enrichment in this medium implies, but does not guarantee, the use of nitrite or ammonium in its metabolism. The ability to utilize oxygen at a large range of concentrations or to switch to nitrate reduction under low-oxygen conditions may explain the existence of Planctomyces in both oxic and suboxic zones. Contrastingly, the depth profiles of BSI group phylotypes had maxima in the center of the suboxic zone but were not present in the sulfidic zone (Fig. 4) and were generally not found under higher-oxygen conditions. The similarities between BSI TRFLP depth profiles and nitrate and particulate manganese imply involvement in nitrate or metal cycling. The depth range of the BSI phylotype’s TRFLP peak height coincides with the presence of particulate manganese oxides; the maximum in particulate manganese was at σ0 = 16.05 in 2001 (Konovalov et al., 2003), σ0 = 15.8 in 2003 (Trouwborst et al., 2006), and σ0 = 15.85 in 2005 (Fuchsman et al., 2011).

**Anammox**

*Candidatus* Scalindua sorokinii has been implicated in the anammox process in the suboxic zone of the Black Sea (Kuypers et al., 2003). The substrates for the anammox process are readily available in the Black Sea. Nitrite is produced by nitrate reduction linked to organic matter degradation (Ward & Kilpatrick, 1991). There is a constant flux of ammonium from the sulfidic layer into the suboxic zone. This ammonium can be autotrophically oxidized to nitrite even at extremely low oxygen concentrations (Ward & Kilpatrick, 1991; Lam et al., 2007). Ammonium oxidation to nitrite can then be linked to the anammox process (Lam et al., 2007) allowing anammox to proceed under low-organic-matter conditions (Fuchsman et al., 2008). The reactants and products of the anammox process, along with the Scalindua normalized peak height, are seen in Fig. 1. We used normalized TRFLP peak height as a proxy for the relative abundance of Scalindua. Owing to the prefiltering of DNA samples from 2000, peak heights from this year cannot be compared to other years. Biological N2 (N2 excess) was calculated by subtracting the N2 present because of physical processes such as gas solubility and mixing in the study of Fuchsman et al. (2008). Scalindua peak height and concentration of biologically produced N2 generally correlate (R2 = 0.6).

In 2000 and 2003, two maxima were seen in the depth profile of Scalindua peak height: one at the nitrite maximum and the second at the bottom of the suboxic zone. In 2005, no DNA samples were collected at the nitrite maximum. These two TRFLP peak maxima may correspond to different ecotypes of Scalindua. All the Scalindua sequences obtained from the Black Sea have the same TRFLP cut pattern with the restriction enzymes used in this study. However, Scalindua clones sequenced from the 2003 cruise (Kirkpatrick et al., 2006; 720 bp) form three clusters (Fig. 5). For five select longer sequences, similarity was obtained using MatGat 2.0 over 869 base pairs. These longer sequences are used to represent each cluster.
JK215 was > 99% similar to Candidatus S. sorokinii, a named sequence originally recovered from the lower suboxic zone of the Black Sea (Kuypers et al., 2003), and they are in the same cluster (Fig. 5). Another cluster of sequences represented by phylotype JK410 was 2.2% different from Candidatus S. sorokinii. This group was only found in the lower suboxic zone (σ:h = 15.9 and 16.0) under conditions of high ammonium concentration and low nitrite concentration. However, bootstrap analysis of the neighbor-joining phylogenetic tree could not determine that the Candidatus S. sorokinii cluster and the JK410 cluster were significantly different (Fig. 5). A third cluster, represented by 869-bp sequence JK616, was 2.9% different from Candidatus S. sorokinii and 1.8% different from JK410. The mean difference between Candidatus S. sorokinii and all the shorter sequences in the JK616 cluster was 2.5%. Sequences in the JK616 cluster were only found at σ:h = 15.8, the nitrite maximum where nitrate was in high concentration and ammonium was in very low concentration. This group was statistically different from the JK410 and Candidatus S. sorokinii clusters (Fig. 5) as well as from Peru clusters I, II, and III and the Namibian cluster from the study of Woebken et al. (2008). Candidatus Scalindua arabica (Woebken et al., 2008) was 5% different from this cluster.

As JK616 is sufficiently divergent from the named species ‘Candidatus S. sorokinii’ to be named a new species (Stackebrandt & Goebel, 1994), we are proposing a new species ‘Candidatus Scalindua richardisi’, named in honor of Francis A. Richards, a chemical oceanographer who hypothesized the existence of anaerobic ammonium oxidation based on chemical fluxes (Richards, 1965). The depth distribution of ‘Candidatus S. richardisi’ suggests that it is adapted to extremely low ammonium concentrations. It is possible that it can perform DNRA, an ability found in other anammox bacteria (Kartal et al., 2007; van de Vossenberg et al., 2008). In contrast, the JK410/Candidatus S. sorokinii cluster appears to be adapted to extremely low nitrite concentrations, but where ammonium is available. They may be the anammox bacteria that were found to be closely linked to active gammaproteobacterial ammonium oxidizers in the lower suboxic zone (Lam et al., 2007).

In all four cruises, biological N2 profiles imply small amounts of N2 production may occur in the presence of low concentrations of sulfide (σ:h = 16.1–16.25; Fig. 1). Scalindua DNA was found in the upper sulfidic layer (9.5 μM sulfide at σ:h = 16.2 in 2000). At depths below this (20 μM H2S at σ:h = 16.4), Scalindua peak heights were undetectable. This finding is consistent with the presence of ladderane lipids associated with anammox bacteria at depths with detectable sulfide in 2003 (Wakeham et al., 2007). While 1–2 μM sulfide has been shown to inhibit anammox activity in the upper suboxic zone of the Black Sea (Jensen et al., 2008), some anammox bacteria can tolerate sulfide (Kalyuzhnyi et al., 2006). Our data suggest that different species of Scalindua may be found in the upper and lower suboxic zone, leaving the possibility of sulfide tolerant Scalindua mediating the anammox process in the lower suboxic zone. Scalindua may alternatively catalyze metal reduction (van de Vossenberg et al., 2008) in the upper sulfidic zone. At σ:h = 16.2 in 2000, particulate manganese was < 1 nM but particulate iron was 6 nM (Yigiterhan et al., 2011). The Black Sea Sulfurimonas, a bacterium closely related to autotrophic denitrifiers and found to be active in the upper sulfidic zone (Glaubitz et al., 2010), may also be responsible for N2 production below the suboxic zone. Sulfurimonas DNA was found from σ:h = 16–16.2 in 2000 using TRFLP with universal bacterial primer 27F (unpublished data, CAF). Thus, Scalindua and Sulfurimonas coexisted in this depth range. Future work should examine more closely N2 production pathways at the transition between suboxic and sulfidic zones.

Conclusions

In this study, we examined the spatial and temporal distribution of the many uncultured Planctomycetes in the Black Sea. The Planctomycetes in the same phylogenetic groups generally had similar depth profiles, implying similar metabolisms within phylogenetic groups. However, these potential metabolisms were clearly more diverse than those found in cultured Planctomycetes.

All cultured Planctomycetes have been found to attach to surfaces. Organic aggregates are sources of abundant but transitory nutrients. Theoretically, heterotrophic aggregate-attached bacteria should be adapted to consume substrates quickly and reproduce rapidly while adaptation to life at free-living energy-limited conditions includes efficient use of resources and high affinity transporters for acquiring nutrients. All identified Planctomycetes in this study were present on aggregates except for members of Scalindua known to facilitate the anammox reaction. Scalindua were only found in the < 30 μm fraction and existed in conditions that require the ability to acquire reactants at nanomolar concentrations: ‘Candidatus S. richardisi’ (phylotype JK616) was present at high nitrite and nitrate and low ammonium concentrations, while a ‘Candidatus S. sorokinii’ sequence cluster was present at high ammonium and low nitrite concentrations. The fact that Scalindua bacteria in the Black Sea are free-living and not linked to sinking organic matter is consistent with their activity under oligotrophic conditions (Fuchsman et al., 2008). This situation contrasts to that of heterotrophic denitrification, which is stimulated by high organic matter conditions (Ward et al., 2008).
Acknowledgements
We would like to thank B. Paul for collecting DNA samples from 2000 to 2001 and for nutrients data from 2005 and S. Konovalov (MH1, Ukraine) and S. Tugrul (METU, Turkey) for use of oxygen and nutrient data. We would like to thank an anonymous reviewer and G. Rocap for helpful comments on the manuscript. C.A.F. and J.B.K. were supported by IGERT trainships in Astrobiology under NSF grant 05-04219. This work was also supported by NSF OISE 0637866, OISE 0637845, and OCE 0649223. Sampling in May 2007 was supported by a Lewis and Clark Astrobiology Travel Grant and was accomplished with the help of Evgeniy Yakushev (CRDF Grant # CGP-15123). The authors have no conflict of interest to declare.

References


### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Density vs. depth for 2000 (blue triangles), 2001 (purple circles), 2003 (black squares) and 2005 (maroon stars).

**Fig. S2.** Hierarchical cluster of Whittaker similarity among *Planctomycetes*-specific TRFLP profiles in samples across the redox gradient of the Black Sea.

**Fig. S3.** A PCA indicating which TRFLP peaks contribute the greatest variation to differences among samples.

**Fig. S4.** Minimum evolution phylogenetic tree of OP3 and *Lentisphaera* sequences obtained from 1000 m (bold) in the sulfidic zone of the Black Sea in 2005.

**Fig. S5.** Depth profiles of sulfide (triangles) compared to TRFLP peak height of two identified OP3 clones from the OP3-III clade: 1k2C (red circles), JK79 (purple squares).

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