

Abundance and diversity of *Planctomycetes* in a Tyrrhenian coastal system of central Italy

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ABSTRACT: The phylum *Planctomycetes* is involved in important processes, such as the mineralization of algal biomass and the removal of nitrogen. Using a combination of 16S rRNA sequence analysis and *in situ* hybridization, we analyzed the diversity and dynamics of *Planctomycetes* in a shallow meso-eutrophic lake, Lago di Paola, Italy. *Planctomycetes* detected by the probe PLA46 accounted for 1 to 5% of prokaryotic picoplankton. Abundances were higher in the coastal lake than in the adjacent marine waters. In the surface waters of the lake, the numbers of *Planctomycetes* fluctuated greatly, reaching a maximum of 1.5×10^6 cells ml⁻¹ in July. The hypoxic bottom waters had less variable cell abundances. The *Planctomycetes* counts were positively correlated with chlorophyll *a* concentrations, confirming the role of this phylum in the degradation of algal biomass. We obtained 70 almost full-length 16S rRNA gene sequences of *Planctomycetes* from 2 libraries. Four distinct clades could be identified. The *Pirellula*-related group F and the uncultured *Planctomycetes* group B both had the highest identity with sequences retrieved from marine habitats, whereas the *Pirellula*-related group E was affiliated with sequences known from freshwater and brackish water environments. The *Planctomyces*-related group A seems to have a wide habitat range. Catalyzed reported deposition fluorescence *in situ* hybridization with newly developed probes revealed abundances of the 4 clades in surface and bottom waters ranging from 1×10^3 to 5×10^4 cells ml⁻¹. Together, the 4 clade-specific probes identified only about a quarter of the *Planctomycetes* detected by probe PLA46. This indicates that the diversity of *Planctomycetes* has not yet been fully explored.

KEY WORDS: Coastal lake ecology · *Planctomycetes* · Bacterial diversity · FISH · CARD-FISH · 16S rRNA gene sequencing

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INTRODUCTION

Planctomycetes are a phylum of budding bacteria with a peptidoglycan-free proteinaceous cell wall of increasing interest for ecology, genomics and, particularly, cell biology and microbial evolution (Fuerst 1995). Their most distinctive feature is an internal compartmentalization (Lindsay et al. 1997, 2001,

Fuerst 2005), which is reminiscent of the eukaryotic lineage (Fuerst 2005). They are part of the *Planctomycetes/Verrucomicrobia/Chlamydiae* superphylum, which, in addition to *Planctomycetes*, includes the phyla *Verrucomicrobia*, *Chlamydiae* and *Lentisphaerae*, and the candidate phyla Poribacteria and OP3 which do not yet have any culture representatives (Fieseler et al. 2004, Wagner & Horn 2006).

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Most cultured strains have been isolated from marine, freshwater and terrestrial habitats (Bauld & Staley 1976, Franzmann & Skerman 1984, Giovannoni et al. 1987, Schlesner 1994, Wang et al. 2002). Despite the recent success in isolating new members of the *Planctomycetes* from aquatic (Fukunaga et al. 2009, Winkelmann & Harder 2009) and terrestrial habitats (Kulichevskaya et al. 2007, 2008, 2009), the phylum continues to be one of the least represented in microbial culture collections. *Planctomycetes* are most commonly heterotrophs (Fuerst 1995), with the notable exception being the autotrophic anammox bacteria, which catalyze anaerobic ammonia oxidation with nitrite (Strous et al. 1999).

Studies based on molecular methods, such as 16S ribosomal RNA (rRNA) gene sequencing and fluorescence *in situ* hybridization (FISH), have revealed a broad distribution of *Planctomycetes* in aquatic environments. They have been detected in the marine water column (DeLong et al. 1993, Vergin et al. 1998, Shu & Jiao 2008a), where they often colonize marine snow particles (DeLong et al. 1993, Rath et al. 1998, Crump et al. 1999), in marine sediments (Llobet-Brossa et al. 1998, Rusch et al. 2003, Inagaki et al. 2006, Musat et al. 2006, Shu & Jiao 2008c) and in the water column and sediments of freshwater systems (Neef et al. 1998, Miskin et al. 1999, Kalyuzhnaya et al. 2004, 2005, Amalfitano et al. 2008). Many studies have reported high abundances of *Planctomycetes* following algal blooms (Janse et al. 2000, Brümmer et al. 2004, Eiler & Bertilsson 2004, Ward et al. 2006, Tadonlécé 2007, Pizzetti et al. 2011). Morris and co-workers (Morris et al. 2006) found that high abundances of *Pirellula*-related cells (up to $4 \times 10^4 \text{ ml}^{-1}$) occurred during a diatom bloom in Oregon coastal waters. Furthermore, genomic and metagenomic evidence indicates the specialization of marine *Planctomycetes* for algal polymer degradation (Glöckner et al. 2003, Woebken et al. 2007b). Recent studies have shown the presence of *Planctomycetes* in the Venice Lagoon (Simonato et al. 2010) and the Río de la Plata estuary (Alonso et al. 2010). An in-depth assessment of the diversity, abundance and distribution of coastal *Planctomycetes* has only recently been initiated for the North Sea (Pizzetti et al. 2011). Herein, we extend this study to a Mediterranean coastal lake. This is a meso-eutrophic aquatic environment representative of South European coastal systems for which no study focusing on the ecology of *Planctomycetes* has yet been performed.

The aim of this study was to analyze the abundance and diversity of *Planctomycetes* in a particular coastal system by using 16S rRNA-based methods. Based on

previous results obtained in the North Sea (Pizzetti et al. 2011), we hypothesized that Lago di Paola, with its meso-eutrophic character, would be particularly rich in *Planctomycetes* in terms of both abundance and diversity.

MATERIALS AND METHODS

Study area, sample collection and processing

Lago di Paola is a shallow, meso-eutrophic lake located on the Tyrrhenian coast of Central Italy (*Latium*). It is part of a cluster of 4 coastal lakes (Fogliano, Paola, Caprolace and Monaci) and is characterized by a water residence time of 8 to 12 mo. Two narrow artificial channels at the northwestern and southeastern ends of the lake allow for a limited water exchange with the sea, which is enough to sustain a high degree of salinity throughout the year. Freshwater only enters the lake because of the intrusion of groundwater, which, in summer, is drastically reduced because of its exploitation for agricultural purposes. The lake has a surface area of ~400 ha (main axis 7 km) and a mean depth of 4 m, except in its central area, where a small depression reaching a depth of 10 m is located. The lake has been exploited for fish farming since the time of the Roman Empire.

Between January and December 2003, surface water samples were collected monthly (fortnightly in May, June and July) using a Niskin bottle at a depth of ~50 cm from 3 stations along Lago di Paola's main axis. We have named these stations SAB1, SAB2 and SAB3 (Fig. 1). Samples were also taken from the marine coastal waters off the mouth of the lake outlet, ~100 m from the shore line (SAB4) (Fig. 1). From July onwards (monthly, except September), samples were also collected from the trench that represents the deepest point in the lake (~10 m; SAB2b).

The environmental parameters were ascertained as described by Manganelli (2005). Briefly, temperature, salinity, pH and dissolved oxygen (DO) were determined with probes (Wissenschaftlich-Technische Werkstätten; Kent Electronic Instrument). The analytical methods used for the determination of dissolved nutrients (phosphate, nitrate and nitrite) were those reported by Parsons et al. (1984) and Valderrama (1981). The dissolved organic carbon (DOC) concentration was analyzed by high temperature catalytic oxidation using a Shimadzu TOC-5000 analyzer (Pettine et al. 1999). Chlorophyll *a* (chl *a*) was extracted in 90% acetone and concentrations were measured by spectrophotometry according to Lorenzen (1967).

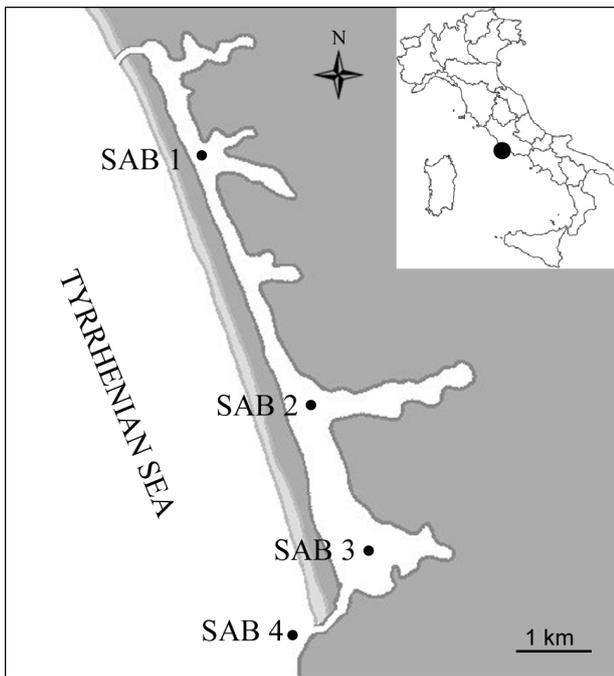


Fig. 1. Schematic map of the Lago di Paola indicating the 4 sampling sites. SAB1: 41° 18' 0.4" N, 13° 00' 47.6" E; SAB2: 41° 16' 12.3" N, 13° 02' 15.0" E; SAB3: 41° 15' 21.9" N, 13° 02' 37.0" E; SAB4: 41° 14' 48.0" N, 13° 02' 11.0" E

Starting in July, the abundance of the *Planctomycetes* was quantified by fluorescence *in situ* hybridization (FISH) and catalyzed reporter deposition (CARD)-FISH. Aliquots were fixed with formaldehyde (final concentration 2%) for 1 h at room temperature and for up to 24 h at 4°C. Thereafter, 4 to 10 ml aliquots were filtered at a low vacuum level (200 mbar) onto 0.2 µm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore). All of the filters were stored at -20°C until further processing.

To analyze *Planctomycetes* diversity, additional sampling was carried out on 15 September 2008 at stations SAB2 and SAB2b. Temperature, salinity and DO were recorded during this process. Overall, the same procedures were followed as in 2003, except that the bacterial cell fixation for the FISH and CARD-FISH was performed with a paraformaldehyde solution (20% w/v, Electron Microscopy Sciences; final concentration 1%), and the prefiltration of the water samples was through 10 µm pore-size polycarbonate filters (type TCTP; diameter, 47 mm; Millipore). Moreover, for the purposes of DNA isolation, aliquots of between 750 and 1250 ml were filtered onto 0.2 µm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore) after pre-

filtration through 10 µm pore-size polycarbonate filters (type TCTP; diameter, 47 mm; Millipore). The filters were frozen semi-dry and kept at -20°C until processed for nucleic acid extraction.

FISH and CARD-FISH

Planctomycetes abundance in both the 2003 and 2008 samples was quantified by FISH and CARD-FISH with probe PLA46 (Neef et al. 1998), and the cell numbers obtained were then compared. All of the samples were also inspected for false-positive signals using the NON338 probe (Wallner et al. 1993). In the 2003 formaldehyde fixed samples (2% final concentration), the numbers of *Planctomycetes* cells after FISH were higher (43% more) than the numbers of cells obtained by CARD-FISH (Fig. S1 in the supplement at www.int-res.com/articles/suppl/a065p129_supp.pdf). No false-positive signals were detected in any of the samples. Consequently, the *Planctomycetes* in the 2003 sample were further quantified by FISH. Conversely, the *Planctomycetes* in the 2008 samples fixed with paraformaldehyde solution (1% final concentration) were quantified by CARD-FISH, because this approach yielded higher probe-positive cell numbers than was the case for the FISH preparations (Fig. S1). No false-positive signals were detected in these samples by CARD-FISH.

FISH was performed according to the protocol of Glöckner et al. (1996). CARD-FISH was also performed using the same 2 probes according to the protocols established by Pernthaler et al. (2002, 2004) and slightly modified by Pizzetti et al. (2011) to improve the fluorescence signals of the *Planctomycetes*. The samples were permeabilized with lysozyme (10 mg ml⁻¹; Fluka; buffer contained 0.05 M EDTA, pH 8, 0.1 M Tris-HCl, pH 7.4) for 1 h at 37°C and then with achromopeptidase (60 U ml⁻¹; Sigma; buffer contained 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37°C. Additionally, hybridization was performed at 46°C for 2.5 h with a washing step at 48°C for 5 min. Filter sections, counterstained with 4',6-diamidino-2-phenylindole (DAPI; final concentration 1 µg ml⁻¹) and washed for 1 min in Milli-Q water and 96% ethanol, were subsequently arranged on microscope slides and embedded in a mixture of 2 anti-fading re-agents (VECTASHIELD® Mounting Medium H-1000, Vector Laboratories and Citifluor [1:5]). The slides were stored at -20°C until further analysis, and the cells were quantified on an Axioplan II Imaging epifluorescence microscope (Zeiss).

DNA extraction and PCR amplification

Total nucleic acids were extracted according to the protocol established by Zhou et al. (1996) and slightly modified by Pizzetti et al. (2011). Picoplankton samples were collected by filtration from the surface (SAB2) and the bottom waters of the lake (SAB2b) (Fig. 1).

Fragments of ~1.4 kb in size, encompassing almost full-length 16S rRNA genes, were amplified using both the primer PLA46F (5'-GGA TTA GGC ATG CAA GTC-3', targeting position 46–63 on the 16S rRNA gene; Neef et al. 1998), specific for *Planctomycetes*, and the general primer, 1392R (5'-ACG GGC GGT GTG TAC-3', targeting *Escherichia coli* position 1392–1406 on 16S rRNA gene; Xu et al. 1998). Each PCR reaction was carried out with a 50 µl master mix including: 0.3 mg ml⁻¹ BSA (final concentration; Sigma Chemie); 1× reaction buffer (final concentration; Eppendorf); 0.25 mM dNTPs (final concentration; Roche Diagnostics); 0.5 µM for each primer (final concentration; <http://Biomers.net>); 0.02 U µl⁻¹ *Taq* Polymerase (final concentration; Eppendorf); and 1 µl DNA. The PCR was run with the following cycling conditions: an initial denaturation step at 94°C for 4 min; 33 cycles of 1 min denaturation at 94°C; 2 min annealing at 59°C; 1.5 min elongation at 72°C; and a final extension step of 10 min at 72°C. The PCR cycle numbers were reduced as much as possible, while 10 replicates were processed in parallel to minimize PCR bias (Suzuki & Giovannoni 1996, Polz & Cavanaugh 1998). Finally, the PCR products were checked in a 1% agarose gel electrophoresis (Biozym LE agarose).

16S rRNA gene libraries, sequencing and phylogenetic reconstruction

The PCR product was purified by agarose gel electrophoresis, and the 1.4 kb band was excised and purified using a Qiaquick Gel extraction kit (QIAGEN). Purified amplicons were ligated in vector P-GEM T-Easy (Promega) according to the manufacturer's instructions. The recombinant plasmids were then transformed into chemically competent *Escherichia coli* cells (Invitrogen), plated and picked. Plasmid DNA was isolated with the Montage™ Plasmid Miniprep Kit (Millipore). It was then sequenced using the 2 vector primers, M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'), as well as the primer GM1F (5'-CCA GCA GCC GCG GTA AT-3'; Lane 1991), to obtain

almost full-length 16S rRNA gene sequences. Contigs were assembled using the SEQUENCHER software Version 4.6 (Gene Codes Corporation). The 16S rRNA gene sequences were subsequently deposited in the EMBL Nucleotide Sequence Database with the accession numbers FR714331 to FR714400.

The sequences were aligned in the ARB software package (www.arb-home.de; Ludwig et al. 2004) using the SINA_aligner (Pruesse et al. 2007). The alignments were further refined manually by conducting a comparison with their closest relatives. For the in-depth phylogenetic rRNA analysis, only sequences with >1200 nucleotides were included. The phylogenetic tree reconstruction was based on the distance matrix (e.g. neighbor-joining) and maximum likelihood methods with and without 30 and 50% *Planctomycetes* positional conservatory filters, respectively. The *Verrucomicrobia*, *Lentisphaerae*, *Chlamydiae* and *Candidatus* OP3 sequences were used as out-groups. A consensus tree was then constructed (Peplies et al. 2008).

The diversity coverage of the sequenced clones was analyzed using the computer program DOTUR (distance-based operational taxonomic unit [OTU] and richness; Schloss & Handelsman 2005). OTUs were calculated using the furthest neighbor algorithm for each distance level, from a distance matrix generated in ARB. To construct the rarefaction curves, we then selected 97% (species level) and 95% (genus level) sequence identities, as reported by Rosselló-Mora & Amann (2001). The coverage index (C) of the clone libraries was calculated with the formula $C = (1 - n/N)100$, where N is the number of all of the clones with a *Planctomycetes* insert and n the number of phylotypes at a 97 and 95% identity level appearing only once in the library (Good 1953, Kemp & Aller 2004).

Probe design

Using the ARB software's probe design tool, oligonucleotide probes targeting the 16S rRNA of the *Planctomycetes* clades were designed based on the newly created phylogenetic tree. Probe specificities were checked *in silico* against the SILVA 16S rRNA database (version 100; Pruesse et al. 2007) (Table S1 in the supplement at www.int-res.com/articles/suppl/a065p129_supp.pdf). To optimize the stringency conditions, a series of hybridizations at increasing formamide concentrations (10 to 60%) were evaluated in appropriate natural samples. The optimal formamide concentration was the highest concentration

before the signal intensity started to decrease (Table S2 in the supplement). Probes were applied for the CARD-FISH performed on the 2008 samples.

Statistics

Multiple linear correlations between *Planctomycetes* absolute abundance and environmental parameters (salinity, temperature, DO, pH, chl *a*, DOC, PO_4^{3-} , NO_2^- , and NO_3^-) were firstly calculated using the Spearman rank correlation test and the p-value was Bonferroni corrected to test for multiple comparisons (Legendre & Legendre 1998).

The partial least squares regression (PLSR) was then applied to model the relationship between *Planctomycetes* absolute abundances (*Y*-response variables) and physico-chemical parameters (*X*-descriptive variables; here: salinity, temperature, DO, pH, chl *a*, DOC, PO_4^{3-} , NO_2^- , and NO_3^-). Descriptive variables were log transformed and normalized before analysis (means were centered to 0 and the variables were standardized by dividing each of them by their standard deviation). This robust method generalizes multiple linear regression by analyzing data with strongly collinear, correlated, noisy and many *X*-descriptive variables, and can simultaneously model several *Y*-response variables, also in cases of incomplete data sets (Wold et al. 2001).

Statistical analyses were carried out using Sigma Stat 3.5 (Systat Software) and the R statistical environment (R version 2.10.0; R Development Core Team 2009), including the *pls* package (Mevik & Wehrens 2007) and custom R scripts.

RESULTS

Physico-chemical parameters

During 2003, the environmental parameters within the lake displayed remarkable seasonal variability in comparison to those registered in the neighboring marine coastal waters (Fig. 2). Indeed, the salinity values ranged between 29.0 (March) and 38.0 (August) in the lake, and between 37.5 (February) and 39.0 (September) in the marine waters (Fig. 2). Oxygen saturation in the lake fluctuated greatly throughout the year, with the highest value registered in May (162%) and the lowest in August (37%). In the coastal marine waters, these values ranged between 73% in September and 114% at the beginning of May (Fig. 2).

At the beginning of July, the water column was stratified, as indicated by a steep gradient in temperature (29.1°C surface; 24.8°C bottom). Indeed, the oxygen saturation values at that time were 131 and 27% in the surface and bottom waters, respectively (Fig. 3A). In August, the water masses started to re-mix, with homogeneous temperature values for surface and bottom waters (~28°C). Salinity was ~38 through the entire water column and the hypoxia extended from the bottom waters (13% oxygen) into the surface waters (35% oxygen) (Fig. 3A). At that time, low oxygen concentrations were observed along the entire lake (SAB1 = 38% and SAB3 = 39%), and the oxygen in the surface waters remained low, including in September. In the months that followed, however, oxygen concentrations increased in both the surface and the bottom waters.

Microbial and *Planctomycetes* abundance in 2003

In the lake, the absolute numbers of prokaryotic picoplankton in the surface waters ranged between $6.2 \times 10^6 \text{ ml}^{-1}$ in December at SAB3 and $3.4 \times 10^7 \text{ ml}^{-1}$ in July at SAB2 (Table S3 in the supplement). In the deeper layers, the cell numbers ranged between $8.9 \times 10^6 \text{ ml}^{-1}$ in December and $2.1 \times 10^7 \text{ ml}^{-1}$ in July (Table S3). Meanwhile, in the marine coastal waters, the absolute cell counts ranged between $9.1 \times 10^5 \text{ ml}^{-1}$ in September and $4.7 \times 10^6 \text{ ml}^{-1}$ in July (Table S3). Overall, the mean cell numbers of prokaryotic picoplankton in the lake ($1.8 \times 10^7 \text{ ml}^{-1}$) were 7.7-fold higher compared with what was found at a nearshore sampling site in the adjacent Tyrrhenian Sea ($2.3 \times 10^6 \text{ ml}^{-1}$).

The relative abundance of *Planctomycetes* at SAB1 was at its highest at the beginning of July, representing $4.7 \pm 0.4\%$ (mean \pm SD) of the prokaryotic picoplankton community. This decreased to $0.8 \pm 0.5\%$ in September (Fig. 4, Table S3). In October, the percentage increased to $1.9 \pm 0.2\%$ and remained at almost the same level in November ($1.4 \pm 0.6\%$) and December ($1.8 \pm 1.1\%$). At SAB2, *Planctomycetes* abundance increased between the beginning ($1.5 \pm 0.4\%$) and the end of July ($5.4 \pm 2.1\%$) (Figs. 3b & 4, Table S3). Indeed, the highest abundances of *Planctomycetes* were registered at the end of this month. *Planctomycetes* abundance then decreased to $0.8 \pm 0.2\%$ in September, increased to $2.8 \pm 0.7\%$ in October and then fell slightly to $1.5 \pm 0.7\%$ in December. Relative abundances at the bottom of SAB2 were more stable, ranging between $1.4 \pm 0.9\%$ in October and $2.1 \pm 0.4\%$ in August (Fig. 3B). *Planctomycetes* relative abundance at SAB3 increased from 1.8 ± 0.2

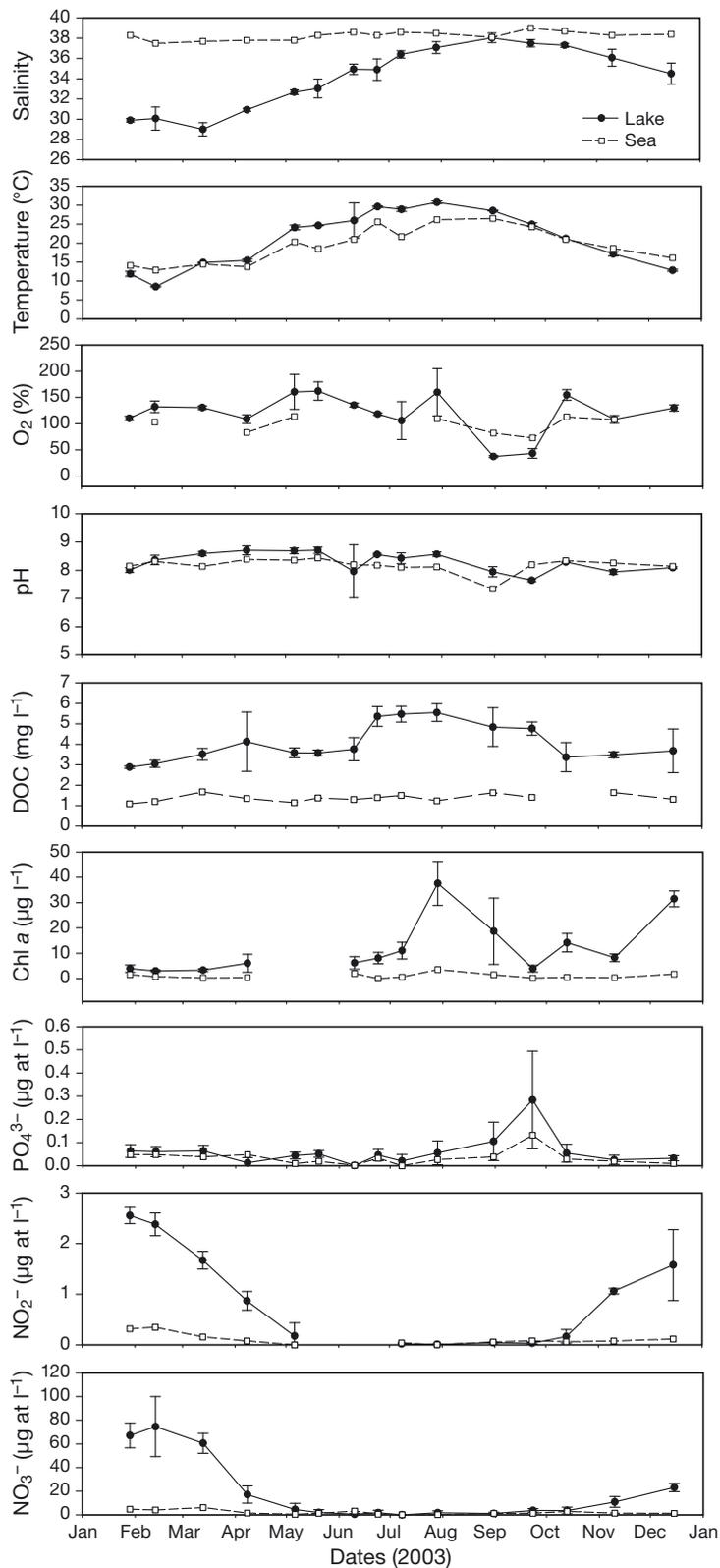


Fig. 2. Environmental parameters collected between January and December 2003. Modified from Manganello (2005). Means of SAB1, SAB2 and SAB3 (see Fig. 1) values (\pm SD) are shown. For a few time points no data were recorded (gaps). DOC: dissolved organic carbon

to $3.4 \pm 3.4\%$ between the beginning and the end of July and decreased to $0.9 \pm 0.6\%$ in August. There was then an increasing trend until October ($3.2 \pm 1.4\%$). Thereafter, the abundance decreased to $2.0 \pm 0.3\%$ in December. Generally, the highest abundances of *Planctomycetes* in the lake were registered in July and October (Fig. 4, Table S3). In the neighboring marine coastal waters (SAB4), relative abundance increased from $0.8 \pm 0.3\%$ in July to $2.0 \pm 0.8\%$ in September, before decreasing to $0.9 \pm 0.2\%$ in December (Fig. 4, Table S3). Overall, mean *Planctomycetes* relative abundance was 1.6-fold higher in the lake than in the sea.

Correlation analyses revealed that the absolute abundances of *Planctomycetes* were significantly correlated with chl *a* ($n = 28$, $r_s = 0.65$, $p \leq 0.001$), DOC ($r_s = 0.62$, $p \leq 0.001$), salinity ($r_s = -0.56$, $p \leq 0.01$), oxygen ($r_s = 0.48$, $p \leq 0.05$) and pH ($r_s = 0.38$, $p \leq 0.05$). No significant correlations with temperature, PO_4^{3-} , NO_3^- or NO_2^- were found (Table S4 in the supplement).

A PLSR was performed to elucidate the main relationships between the variance of *Planctomycetes* abundance (*Y* variable) and physico-chemical variables (*X* variables). The first 2 latent variables (LV 1 and 2) of the model explained 50 and 8% of the total variation in the *Y* variable, respectively (Fig. S2 in the supplement). The score plot indicates environmental conditions and seems to induce a grouping of samples according to sampling months or sampling sites (Fig. S2A). Indeed, July samples are separated from other samples along the LV 1 axis (dark blue circle, Fig. S2A), and August and September samples seem to be separated from other samples along the LV 2 axis (pink circle, Fig. S2A). Samples from site SAB4 also grouped together, validating conditions from this coastal offshore site, different from the other sites in the lake. LV 1 explained 23% of the variation in the environmental variables and was positively influenced mostly by pH, chl *a*, temperature and DOC and negatively influenced by salinity, nitrite and nitrate (Fig. S2B). Also, 11% of the variation in the environmental variables was explained by LV 2, which was mostly positively influ-

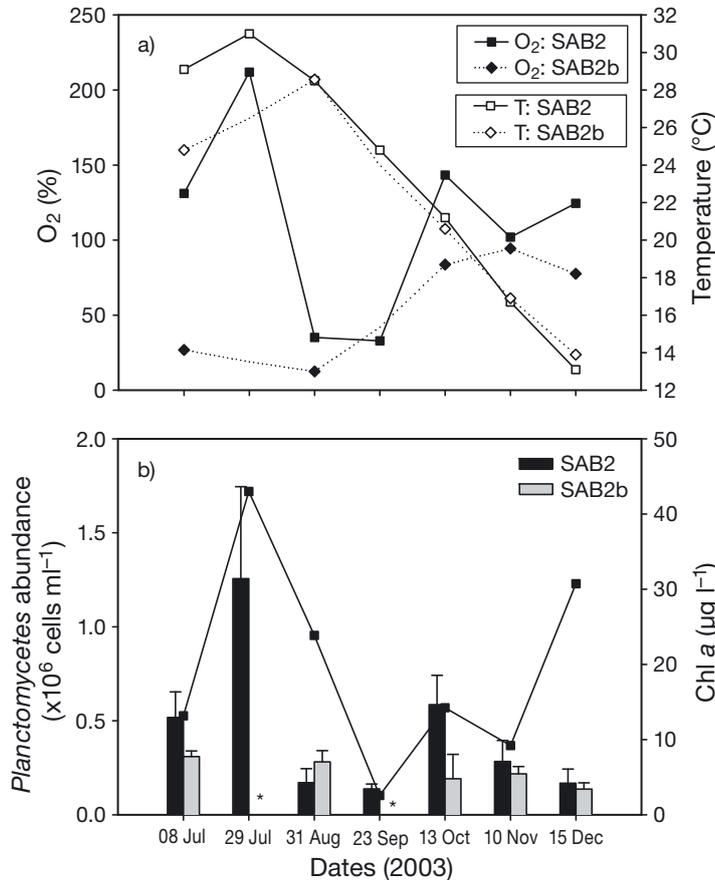


Fig. 3. (A) Oxygen and temperature and (B) *Planctomycetes* absolute counts at the surface (SAB2) and the bottom (SAB2b) of the Lago di Paola at the 7 sampling days between July and December 2003. Means \pm SD of 3 replicates are shown. Chl *a* values registered at SAB2 are shown. *Sampling not done

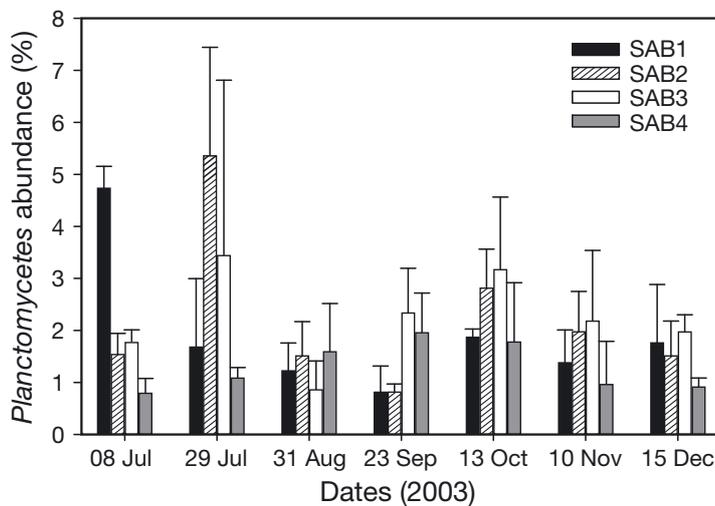


Fig. 4. *Planctomycetes* relative abundances at the 3 sampling sites along the lake (see Fig. 1) and at the adjacent marine waters between July and December 2003. Relative abundances were calculated by comparison to DAPI-stained cells. Means \pm SD of 3 replicates are shown

enced by DO and nitrite, and negatively influenced by temperature and DOC. *Planctomycetes* abundance was situated on the positive part of both latent variables, and thus seems to be mostly linked to chl *a*, DOC, pH, water column temperature, and DO and nitrite.

Diversity of *Planctomycetes*

Two clone libraries were constructed from the surface (SAB2) and bottom waters (SAB2b) sampled on 15 September 2008, using a primer pair targeting the 16S rRNA genes of *Planctomycetes* (PLA46F/1392R). PLA46 currently has a group coverage of 52%, and is fully complementary to 1903 of the 3645 *Planctomycetes* 16S rRNA sequences. It also has some out-group hits, most of which are within the Lentisphaerae. On 15 September, temperature (26.0°C) and salinity (37.1) did not vary over depth, and hypoxic conditions were observed in both the surface (21%) and the bottom waters (12%). The percentage of *Planctomycetes* determined by CARD-FISH was (mean \pm SD) 3.4 \pm 0.1% (2.8 \pm 0.09 $\times 10^5$ ml⁻¹) at the surface of Lago di Paola and 5.2 \pm 0.6% (3.6 \pm 0.4 $\times 10^5$ ml⁻¹) at the bottom. In total, we analyzed 161 sequences, of which 101 were affiliated with *Planctomycetes*. Forty of 83 sequences retrieved from SAB2 were affiliated to *Planctomycetes*, 21 to *Chlamydiae*, 16 to *Lentisphaerae*, 4 to *Verrucomicrobia* and 2 to the candidate division OP3. Of the 78 sequences, 61 retrieved from the SAB2b sequences belonged to the *Planctomycetes*, 11 to *Lentisphaerae*, 5 to *Chlamydiae* and 1 to the candidate division OP3. We were able to determine almost full-length sequences for 70 of the 101 *Planctomycetes* clones.

Comparative sequence analyses enabled us to distinguish 4 phylogenetic clades. These were named: *Pirellula*-related group F, *Pirellula*-related group E, uncultured *Planctomycetes* group B and *Planctomycetes*-related group A (Fig. 5). These clades remained stable in different types of tree reconstructions (neighbor-joining, maximum likelihood) with and without *Planctomycetes* positional conservative filters (50 and 30%, respectively).

The *Pirellula*-related group F sequences accounted for 3 of 31 of the almost full-length *Planctomycetes* sequences obtained in this

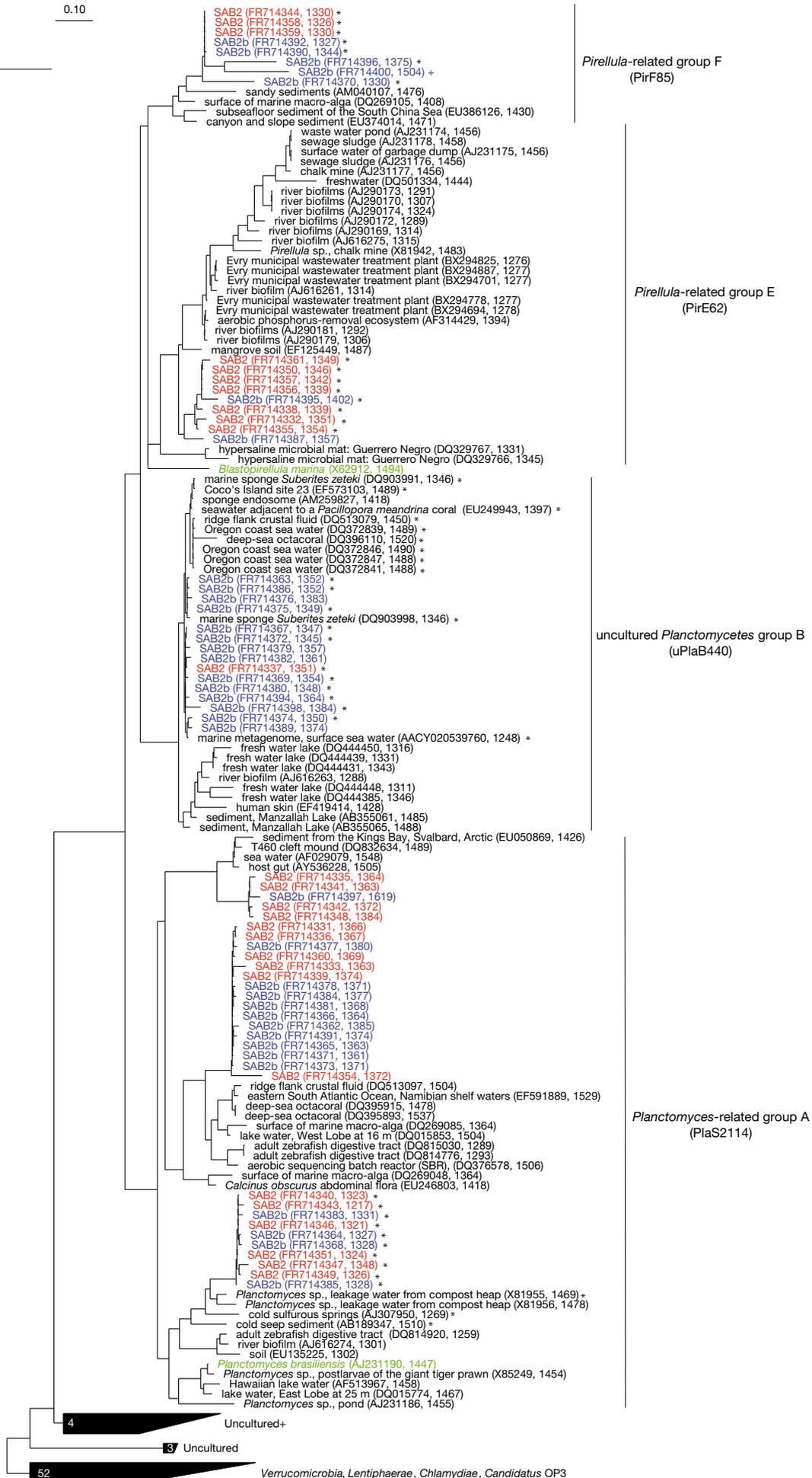


Fig. 5. Phylogenetic tree based on almost full-length 16S rRNA sequences (>1200 nucleotides) showing the affiliations of different clusters. Sequences belonging to the 4 clades are pointed out by a color code [SAB2]: red; top (surface [SAB2]: blue; type strains: green). Accession numbers and sequence length are stated in parentheses. *: sequences targeted by newly designed probes, stated in parentheses, at 0 weighted mismatches; +: a clone targeted by PirE62; +: a clone targeted by PlaS2114. The numbers in the boxes of the uncultured clades indicate the number of sequences retrieved in this study. The bar represents 10% sequence divergence.

52 Verrucomicrobia, Lentipharae, Chlamydiae, Candidatus OP3

study from the surface water (SAB2), and 5 of 39 found in the bottom water (SAB2b). The sequences shared ~86% sequence identity with *Blastopirellula marina* (Fig. 5). The frequencies of the *Pirellula*-related group E were 7/31 and 2/39 in SAB2 and SAB2b, respectively. The sequences in this clade shared ~89% sequence identity with *B. marina* and ~87% with a strain of the genus *Pirellula* (Fig. 5). The sequences from the uncultured *Planctomyces* group B were retrieved much less frequently from SAB2 (1/31) than from SAB2b (14/39) (Fig. 5), whereas the *Planctomyces*-related group A appeared frequently in both libraries (16/31 in SAB2 and 15/39 in SAB2b). The former group shared ~87% sequence identity with *Planctomyces brasiliensis*, whereas for the latter this was between 85 and 87% with strains of the genus *Planctomyces* (Fig. 5).

The coverage of *Planctomyces* diversity at SAB2 was 84% at the species level (97% sequences identity) and 87% at the genus level (95% sequence identity). The library coverage in SAB2b was lower, at 74 and 77% at the species and genus levels, respectively (Fig. S3 in the supplement).

Abundance of specific *Planctomyces* clades

Three specific oligonucleotide probes were designed for the newly defined *Planctomyces* clades. Along with a previously designed probe (Pizzetti et al. 2011), the 3 new probes targeted all 4 dominant clades identified in the Lago di Paola clone libraries (Table S2). Probe PirF85 is specific to the *Pirellula*-related group F, encompassing 7 of 8 sequences retrieved in the present study. Probe PirE62 is fully specific to 8 of 9 sequences of the *Pirellula*-related group E. Probe PlaS2114 targeted a monophyletic clade within the *Planctomyces*-related group A, which encompassed 10 of 31 sequences retrieved in the present study. Probe uPlaB440 was previously designed by Pizzetti et al. (2011) based on sequences retrieved from North Sea coastal waters. It targeted 11 of 15 sequences that belonged to the uncultured *Planctomyces* group B.

The counts with probe PirF85 in the bottom waters of Lago di Paola were (mean \pm SD) $1.1 \pm 0.4 \times 10^4$ ml⁻¹ ($0.2 \pm 0.1\%$ of prokaryotic picoplankton). In the surface water samples, only single PirF85-positive cells occurred, and the counts were below the detection limit of 0.1% (Fig. 6). The *Pirellula*-related group E, which was identified with probe PirE62, accounted for $1.2 \pm 0.5 \times 10^4$ ml⁻¹ ($0.2 \pm 0.1\%$) and $2.0 \pm 0.5 \times 10^4$ ml⁻¹ ($0.3 \pm 0.1\%$) in the surface and bottom waters,

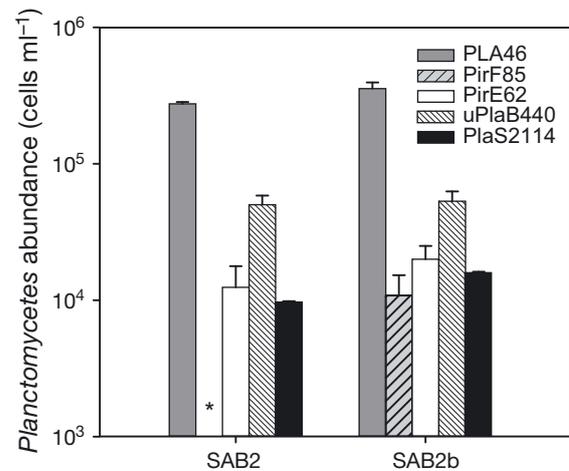


Fig. 6. Logarithmic scale (note that the y-axis does not start at zero) of absolute abundances of *Planctomyces* (PLA46), *Pirellula*-related group F (PirF85), *Pirellula*-related group E (PirE62), uncultured *Planctomyces* group B (uPlaB440) and *Planctomyces*-related group A (PlaS2114) on 15 September 2008. Means \pm SD of 3 replicates are shown. Data are from site SAB2 (see Fig. 1; surface [SAB2]; bottom [SAB2b]). *: below detection level

respectively (Fig. 6). The number of cells hybridized with probe uPlaB440 was $5.0 \pm 0.8 \times 10^4$ ml⁻¹ ($0.6 \pm 0.1\%$) at the surface and $5.3 \pm 1.0 \times 10^4$ ml⁻¹ ($0.8 \pm 0.1\%$) at the bottom (Fig. 6). Finally, probe PlaS2114 targeted $9.7 \pm 0.2 \times 10^3$ cells ml⁻¹ (0.1%) and $1.6 \pm 0.03 \times 10^4$ cells ml⁻¹ (0.2%) in the upper and lower water column of the lake (Fig. 6). Throughout the samples from 2008, the sum of the counts with the 4 clade-specific probes was 26 and 28% of the PLA46 counts at the surface and the bottom of the lake, respectively.

DISCUSSION

Before discussing the abundances of *Planctomyces* in Lago di Paola in detail, the methodological evolution from FISH to CARD-FISH, which occurred during the course of this study, needs to be considered. Initially, *Planctomyces* counts were higher with FISH than with CARD-FISH in the samples taken in 2003. Later, the opposite was true for the 2008 samples. We believe that this was probably due to a change in the sample fixation from a final concentration of 2% formaldehyde in 2003 to the lower concentration of 1% of paraformaldehyde that was used in 2008. Aldehydes are cross-linking amino groups. After fixation at 2%, the 16S rRNA of part of the *Planctomyces* may no longer have been accessible to the large HRP-labeled probes used in CARD-FISH, due to the larger size of the label (~44 kDa).

The smaller Cy3-labeled probes (the molecular weight of Cy3 is ~700 Da) probably penetrated the cells in the 2003 samples more efficiently. The samples from 2008, however, were less cross-linked by the 1% paraformaldehyde fixative, and here CARD-FISH was superior to FISH due to its greater sensitivity. We are confident that the counts obtained with the 2 methods for the 2 different sample types are reliable. With both methods we observed the doughnut-shaped hybridization pattern that is typical of *Planctomycetes* (Woebken et al. 2007a). If there was a counting bias, the *Planctomycetes* numbers were somewhat underestimated as opposed to overestimated.

Planctomycetes abundances in Lago di Paola were high. The abundance maximum of 1.5×10^6 cells ml⁻¹ at SAB1 at the beginning of July 2003 is, to our knowledge, the highest value reported for a coastal system, exceeding previous reports by one order of magnitude (Morris et al. 2006, Alonso et al. 2010, Simonato et al. 2010). The values found by these authors are very similar to the maximum of 5×10^4 ml⁻¹ that we counted at the adjacent nearshore marine sampling site, SAB4. The exceptionally high abundances of *Planctomycetes* in Lago di Paola are probably due to the meso-eutrophic character of the lake, which receives high amounts of organic carbon from autochthonous (internal primary production) and allochthonous sources, such as agriculture.

Planctomycetes abundance correlated positively with chl *a* and DOC, which are also positively correlated to each other (Table S4). A likely scenario is that a summer bloom of algae formed fresh substrate for the growth of the *Planctomycetes*. This further confirms earlier reports of high abundances of *Planctomycetes* being linked to algal blooms (Janse et al. 2000, Brümmer et al. 2004, Eiler & Bertilsson 2004, Ward et al. 2006, Tadolniké 2007, Pizzetti et al. 2011).

The variation in *Planctomycetes* abundance could also be explained by the variance of DO. Lago di Paola is stratified during the summer. However, at the end of this season, when the waters begin to mix, the oxygen concentration dramatically decreases at the surface, leading to hypoxia throughout the entire water column (Fig. 3). At that time, *Planctomycetes* abundance was higher at the lake bottom than at the surface, suggesting that the *Planctomycetes* were strongly affected by the change from oxic to hypoxic conditions occurring in the surface water. Indeed, when oxygen saturation was re-established, an increase in *Planctomycetes* was registered. This, along with a similar diversity of *Planctomycetes* in the surface and bottom water

libraries (SAB2 vs. SAB2b; Fig. 5), indicates that this phylum can deal with hypoxic conditions with an oxygen saturation of ~12%.

In accordance with previous studies (Chouari et al. 2003, Elshahed et al. 2007), we used primer Pla46F, which is specific to *Planctomycetes*, and the universal primer 1392R to analyze *Planctomycetes* 16S rRNA diversity in Lago di Paola. Recently, Shu & Jiao (2008b) suggested and tested a new primer set to amplify planctomycetal 16S rRNA genes from seawater, revealing that it retrieved a broader *Planctomycetes* diversity and less out-group sequences. Testing this primer set on our sequences demonstrated that it would have reduced the retrieval of non-*Planctomycetes*. However, it would have also underestimated the *Planctomycetes* diversity in our sample by not amplifying the 16S rRNA of the *Planctomycetes*-related group A. We conclude that the primer set we used was adequate, even if in the future it might be possible to design more specific primers that are based, for example, on PCR-independent metagenome libraries.

With our study, several new *Planctomycetes* phylogenotypes have been added to the database as almost full-length 16S rRNA gene sequences. We were able to define 4 stable coastal groups by conducting a comparative sequence analysis. Sequences of all of the clades were retrieved from the surface and bottom waters of Lago di Paola, suggesting a high connectivity, which reflects mixing. Clone frequencies in the surface and bottom water libraries were similar except for the uncultured *Planctomycetes* group B, of which more sequences were obtained from the bottom water. However, this finding is unlikely to be significant, as the CARD-FISH counts for this group obtained with probe uPlaB440 were $\sim 5 \times 10^4$ ml⁻¹ in the surface as well as the bottom water. The clade-specific CARD-FISH counts with probes PirF85, PirE62, and PlaS2114 confirmed the simultaneous presence of all clades in the surface and bottom waters in similar abundances. None of the clades accounted for more than 1% of the total picoplankton in the samples examined. We cannot exclude the possibility that the 4 *Planctomycetes* clades examined herein might be more abundant in terms of particle numbers because this research was conducted on the 0.2–10 µm fraction, which excluded aggregates.

The *Pirellula*-related group F clones obtained in this study were closely related to sequences retrieved from marine sediment and the surface of marine macroalgae. Most of the *Pirellula*-related group E sequences, however, were best affiliated with sequences

retrieved from wastewater treatment plants, river biofilms and mangrove soils (Fig. 5). Our sequences from the *Planctomyces*-related group A currently have the highest identity (~91% sequence identity) with sequences from compost leakage water, cold sulphurous springs and cold seep sediment (Fig. 5). *Planctomyces brasiliensis*, which also belongs to this clade, was isolated from a hypersaline lake in Brazil. The *Planctomycetes*-related group A clade seems to have a wide habitat range, whereas the uncultured *Planctomycetes* group B clade was first described by Pizzetti et al. (2011) in North Sea coastal waters. We have demonstrated that this group may be more widely distributed in coastal marine habitats. Interestingly, in addition to the common doughnut-shaped cells, probe uPlaB440 also visualized a second, rather rod-shaped morphotype, indicating the presence of distinct sub-populations (Fig. S4 in the supplement).

With this study we have not only increased our knowledge of *Planctomycetes* diversity, but have also produced 3 new clade-specific probes. Our data will facilitate future studies of the ecology and biogeography of the coastal marine clades of *Planctomycetes*.

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