Abundance, distribution and diversity of planktonic *Planctomycetes* in coastal zones

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A mio padre e a Claudio

Johannes Müller to the young Ernst Haeckel at Helgoland in 1854:

"As soon as you have entered into this pelagic wonderland you will see that you cannot leave it."

(Haeckel 1890, transl. G.W. Field 1893, p. 573; cited from Taylor 1980)

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Abstract

The phylum *Planctomycetes* is a group of budding, peptidoglycan-less bacteria of increasing interest for microbial evolution, ecology, cell biology and genomics. Although members of the *Planctomycetes* seem to have a cosmopolitan distribution, little is known about their spatial and temporal occurrence in coastal zones. In this thesis the abundance, distribution and phylogenetic diversity of *Planctomycetes* were investigated in different coastal systems, the German North Sea island Helgoland, the Tyrrhenian coastal lake Lago di Paola and the adjacent marine coastal waters.

Seawater was collected in different seasons at the station "Kabeltonne" near the island Helgoland. Starting from late spring to summer samples were size-fractionated. By catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) previous episodic observations could be corroborated that *Planctomycetes* prefer an attached lifestyle. An increase in *Planctomycetes* absolute numbers was registered in spring showing a positive correlation with centric diatoms (*Centrales*). The results suggested that *Planctomycetes* are most likely bottom-up controlled benefiting from algal production.

Similar to findings in North Sea coastal waters *Planctomycetes* increased in summer at Lago di Paola - during stratification - in surface waters and mainly responded to high dissolved organic carbon (DOC) and chlorophyll concentrations confirming their close link with the degradation of algal biomass. In bottom waters the conditions seemed to be more stable, e.g. during hypoxia in summer, and the abundances of *Planctomycetes* were less variable. At the end of the summer when the water column became completely hypoxic *Planctomycetes* abundance was higher at the lake bottom than at the surface suggesting that *Planctomycetes* are strongly influenced by the change from oxic to hypoxic conditions occurring in the surface water.

In the course of both studies 224 almost full length 16S rRNA gene sequences of *Planctomycetes* have been added to the database. By comparative sequence analysis of samples from the North Sea and Lago di Paola we could define six coastal clades. *Pirellula*-related group D and *Pirellula*-related group C were exclusively found in Helgoland samples while *Pirellula*-related group E and *Pirellula*-related group F occurred in Lago di Paola samples. Uncultured *Planctomycetes* group B and *Planctomyces*-related group A sequences were retrieved both from Helgoland and Lago di Paola samples.

Based on these results a new set of 16S rRNA-targeted oligonucleotide probes was generated. While nearly 100% of the *Planctomycetes* community at Helgoland could be

explained by the clade-specific probes, only about a quarter of the *Planctomycetes* in Lago di Paola could be classified indicating the presence of additional, yet unidentified clades of *Planctomycetes* in Lago di Paola. At Helgoland the clade-specific counts revealed a seasonal succession which might be linked to abundances of centric and pennate diatoms. At Lago di Paola, cell numbers indicated the simultaneous presence of all clades in the surface and bottom water in similar abundances.

In clone libraries from Lago di Paola, 20 almost full-length 16S rRNA gene sequences affiliated with *Chlamydiales* were retrieved. Two main clusters were identified and two oligonucleotide probes were designed, tested and optimized. By CARD-FISH it was possible to visualize high numbers of individual environmental chlamydiae as well as chlamydiae inside a host, presumably a protist. Our findings enlarge the knowledge of the biodiversity of environmental chlamydiae in aquatic habitats and deserve attention from a sanitary point of view.

Zusammenfassung

Das Phylum *Planctomycetes* umfasst Bakterien, die sich durch Knospung teilen und deren Zellwand nicht aus Peptidoglykan besteht. Bei der Erforschung der mikrobiellen Evolution, Ökologie, Zellbiologie und Genomik rücken sie immer mehr ins Zentrum des Interesses. Obwohl *Planctomycetes* weltweit verbreitet sind, ist wenig über ihre räumliche und zeitliche Verteilung in Küstenzonen bekannt. Diese Studie befasst sich mit der Abundanz, Verbreitung und Phylogenie der *Planctomycetes* in verschiedenen Küstengebieten – vor der deutschen Nordseeinsel Helgoland, im Küstensee ‚Lago di Paola' am Tyrrhenischen Meer und in den angrenzenden Küstengewässern.

Im Rahmen dieser Doktorarbeit wurden vom späten Frühjahr bis zum Sommer Wasserproben an der Station „Kabeltonne", Helgoland entnommen und das Plankton wurde größenfraktioniert. Mittels Fluoreszenz *in situ* Hybridisierungs-Techniken (CARD-FISH) konnten frühere episodische Beobachtungen bestätigt werden, dass *Planctomycetes* eine Anhaftung an Partikel bevorzugen. Einen Anstieg der Abundanz wurde im Frühjahr verzeichnet und es konnte eine positiven Korrelation mit der Häufigkeit von Diatomeen der *Centrales*-Gruppe gezeigt werden. Diese Ergebnisse deuten an, dass *Planctomycetes* höchstwahrscheinlich durch Substratverfügbarkeit kontrolliert sind und von der Algen-Primärproduktion profitieren.

Der Lago di Paola weist während des Sommer eine stabile Schichtung des Wasserkörpers auf. Parallel zum Konzentrationsanstieg an gelöstem organischem Kohlenstoff infolge des Abbaus von Algenbiomasse wurde dort ein Anstieg der *Planctomycetes* im Oberflächenwasser verzeichnet. Im Tiefenwasser scheinen die Bedingungen – zum Beispiel während des sommerlichen Sauerstoffmangels – stabiler zu sein, denn die Abundanz der *Planctomycetes* variierte nicht sehr. Zum Ende des Sommers, wenn die Wassersäule komplett hypoxisch ist, weist das Tiefenwasser eine wesentlich höhere Häufigkeit an *Planctomycetes* auf als das Oberflächenwasser. Vermutlich werden die *Planctomycetes* im Oberflächenwasser stärker von den wechselnden Bedingungen – oxisch zu hypoxisch – beeinflusst.

Im Zuge beider Studien konnten 224 qualitativ hochwertige 16S rRNA-Gensequenzen der *Planctomycetes* zur Datenbank hinzugefügt werden. Durch den Vergleich der Nordsee-Sequenzen mit denen von Lago di Paola konnten sechs Untergruppen der *Planctomycetes* definiert werden, die spezifisch für Küstengewässer sind. Die *Pirellula*-verwandten Gruppen D und C kamen ausschließlich in den Wasserproben von Helgoland vor, während die *Pirellula*-verwandten Gruppen E und F nur im Lago di Paola auftraten. Die nicht kultivierte *Planctomyctes* Gruppe B und die *Planctomycetes*-verwandte Gruppe A wurden an beiden Standorten gefunden.

Aufgrund dieser Ergebnisse wurde ein neuer Satz an 16S rRNA-gerichteter Oligonukleotidsonden entwickelt. Mit diesen gruppenspezifischen Sonden konnte für Helgoland fast 100% der *Planctomycetes* klassifiziert werden, während für den Lago di Paola nur etwa ein Viertel identifizierbar waren. Dies deutet auf weitere, bis jetzt noch unbekannte *Planctomycetes* Gruppen im Lago di Paola hin. Für Helgoland zeigten die gruppenspezifischen Zählungen eine saisonale Sukzession, welche mit dem Auftreten von Diatomeen zusammenhängen könnte. Für den Lago di Paola deuten die Zellzahlen eine Gleichverteilung aller Gruppen mit annähernd gleichen Abundanzen im Oberflächen- und Tiefenwasser an.

In Klonbanken vom Lago di Paola befanden sich 20 16S rRNA-Gensequenzen, die den *Chlamydiales* zugeordnet werden konnten. Zwei Hauptgruppen wurden identifiziert, für die zwei Oligonukleotidsonden entwickelt, erprobt und optimiert wurden. Mittels CARD-FISH war es möglich, viele einzelne Chlamydien, wie auch Chlamydien innerhalb eines Wirtes - vermutlich eines Protisten - in Proben von Lago di Paola zu visualisieren. Diese Ergebnisse erweitern das Wissen über die Biodiversität von Chlamydien in aquatischen Habitaten und verdienen Aufmerksamkeit hinsichtlich hygienischer Gesichtspunkte.

List of abbreviations

anammox	anaerobic oxidation of ammonium with nitrite
C1	compounds containing one carbon atom
CARD-FISH	catalyzed reporter deposition-FISH
CCW	Continental Coastal Water
Chl a	chlorophyll a
DAPI	4',6-diamidino-2-phenylindole
Da	dalton
DO	dissolved oxygen
DOC	dissolved organic carbon
DOM	dissolved organic matter
EB	elementary body
FISH	fluorescence *in situ* hybridization
HRP	horseradish peroxidase
Mb	mega bases
NSW	Central (Southern) North Sea Water
OTU	operational taxonomic unit
PCA	Principal Components Analysis
PCR	polymerase chain reaction
PVC	*Planctomycetes*, *Verrucomicrobia*,*Chlamydiae*
RB	reticulate body
rRNA	ribosomal RNA
SSU	small subunit
TSA	tyramide signal amplification

1. Introduction and Aims

Introduction

1.1. Coastal environments

The coastal zone is where land, ocean, and atmosphere interact. The world coastline, which extends over 350,000-1,000,000 km, displays a wide diversity of geomorphological types and ecosystems (Gattuso et al., 1998). The total surface area of the coastal zone occupies only about 7% (26 x 10^6 km^2) of the surface of the global ocean (360 x 10^6 km^2) (Gattuso et al., 1998). However, the high productivity of coastal seas equates to 14-30% of the oceanic primary production, 80% of organic matter burial, 90% of sedimentary mineralization, 75-90% of the oceanic sink of suspended river load, ca. 50% of the deposition of calcium carbonate (Mantoura et al., 1991; Pernetta and Milliman, 1995) and at least 90% of the global fish catch (Pauly and Christensen, 1995). Coastal zones plays an important role in biogeochemical cycles receiving high inputs of terrestrial organic matter and nutrient through run-off and groundwater discharge. Furthermore, the coastal ocean exchanges large amounts of matter and energy with the open ocean. The coastal ocean is also the area of greatest human impact on the marine environment since 91% of coastal region inhabitants reside within 50 km of the sea (eurostat, 2010). Therefore, residents of coastal regions exert greater pressure near the coastline. The coastal zone is expected to be home to nearly 75% of the world population by 2025. Coastal zone overall economic value has been recently estimated as 43% of the world's ecosystem services and natural capital (Costanza et al., 1997). Overall, the coastal ocean constitutes one of the most geochemically and biologically active areas of the biosphere and it is particularly vulnerable to climate variability and change. Key concerns include the responses to sea level rise and implications for water resources. Indeed, the International Panel on Climate Change (IPCC) estimates that the global average sea level will rise between 0.18 to 0.59 meters in the next century (IPCC, 2007). Potentially, sea level rise will exacerbate the severe socio-economic and environmental problems coastal zones are facing. It is therefore necessary to assess the impacts of climate change to better understand the vulnerability of coastal zones and to implement mitigation and adaptation strategies for meeting the climate change challenge.

1.1.1. The German Bight of the North Sea (Helgoland)

The North Sea is a typical semi-enclosed continental shelf sea with a wide range of tidal conditions, winds, circulation and effects of stratification (Howarth, 2001). In a simplified view, the North Sea is mainly characterized by the following water bodies: (1) Atlantic Ocean water entering in the north, characterized by high salinities (> 35) and high winter temperatures; (2) Channel current water coming through the English Channel from the southwest, also oceanic with high salinity and high winter temperatures; (3) varying amounts of coastal water characterized by low salinity because of river water influence, with low winter and high summer temperatures; (4) mixed water from the central North Sea, hydrographically in-between coastal and oceanic water; (5) Baltic Sea water of low salinity from the Skagerrak, flowing in western to north western direction (Hagmeier and Künne, 1951). The flow is broadly anticlockwise round the coasts of the North Sea, with weak and varied circulation in its center.

The German Bight, the south-eastern bight of the North Sea, is characterized by strong mesoscale variability in physical properties such as fronts, meanders and eddies. Generally, two main water masses are found in the German Bight: Continental Coastal Water (CCW) and Central (Southern) North Sea Water (NSW). The NSW can be divided into two slightly differing water masses, a surface layer and a bottom layer component. The CCW is a mixture of water from the Atlantic and from the English Channel, together with water from the rivers Rhine, Meuse, Ems, and Elbe (Becker et al., 1992).

In the German Bight, about 60 km off the estuaries of the rivers Elbe and Weser is placed the island of Helgoland (Fig. 1). Helgoland is located in the mixed water area of the North Sea (see above), close to the area of coastal water influence. Helgoland was extensively studied, indeed since 1962, the waters at Helgoland Roads (sampling station “Kabeltonne”) have been sampled daily to determine temperature, salinity, Secci disk depth, macronutrients and phytoplankton. Currently, chlorophyll, light penetration depths, zooplankton, intertidal macroalgae, macrozoobenthos and bacterioplankton are now sampled on a regular basis (Franke et al. 1999; Bartsch et al. 2004; Gerdts et al. 2004; Gutow et al. 2007; Reichert et al. 2008). Thus, the Helgoland roads pelagic time series is supported by additional data turning on one of the richest temporal marine data sets available. The salinity at Helgoland ranges between 28 and 33 and the water temperature can be between 22 °C in summer and below zero (-2 °C) in winter (Wiltshire and Manly, 2004). Salinity and annual mean temperature have increased since 1962 and the latter by

an average of 1.67 °C (Wiltshire at al., 2010). The mean flagellate biomass has changed only slightly (Radach, 1998). A steady decrease in all nutrients since the early 1980s has been observed. This is likely due to a reduction in nutrient input (Hickel et al. 1993; van Beusekom 2004; van Beusekom et al. 2008) and also to the fact that the current direction is less influenced by coastal waters, and more open North Sea water reaches the site than 45 years ago (Wiltshire et al., 2010). Ultimately, it has been observed that the bacterial community displays a pronounced seasonality (Gerdts et al., 2004) and it has a direct dependency on abiotic and biotic factors (Sapp et al., 2007).

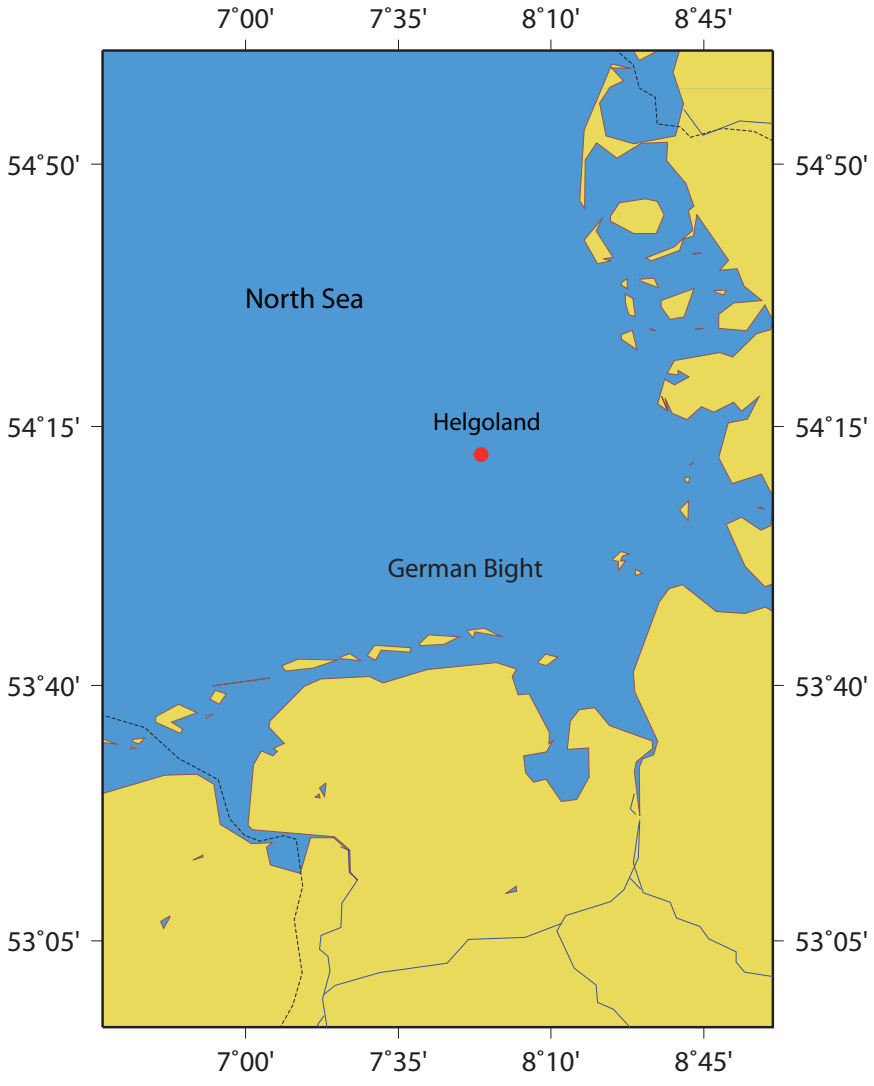

Fig. 1: Map of the German Bight of the North Sea. In red is highlighted the island of Helgoland.

1.1.2. Coastal lakes: Lago di Paola (*Latium*-Italy)

Coastal lakes are semi-enclosed environments generally separated from the sea by a barrier and sometimes connected to the sea by one or more inlets. Their hydrodynamism is not significantly influenced by tides (Brambati, 1988). Coastal lakes receive high nutrient inputs from allochthonous sources and sustain high level of primary production and biodiversity. Their environmental features result in a high variability of the physico-chemical characteristics and biological communities (Nuccio et al., 2003). These basins can act either as a sink for the organic matter (OM) or as a source of organic and inorganic nutrients for adjacent coastal areas. The balance between the OM export and its accumulation largely depends on the geomorphologic features and physical factors and it is affected by *in situ* heterotrophic utilization (Manini et al., 2003).

Lago di Paola that belongs to "Wetlands of International Importance" in accordance with Ramsar Convention (Iran, 1971) is a coastal lake inhabited and exploited for fish farming since the Roman Empire. It is located along the Tyrrhenian coast of central Italy (*Latium*) (Fig. 2) and it is characterized by a high water residence time. Two narrow artificial channels (Caterattino and Torre Paola) at the north-western and south-eastern ends of the lake allow for a limited water exchange with the sea; freshwater enters the lake only by groundwater intrusion that in summer is drastically reduced by exploitation for agricultural purposes. The lake has an extension of about 400 ha (main axis 7 km) and an average depth of 4 m, except in the central area, where a narrow depression reaching 10 m depth and representing the deepest point in the lake is located (fossa della Molella). Lago di Paola results to be a meso-eutrophic system which apparently does not influence the marine system that remains oligotrophic, a typical condition in Tyrrhenian coastal areas (Manganelli et al., 2005). Particularly, the highest dissolved organic carbon (DOC) concentration in the lake suggests it to act as a sink for autochthonous (biological production) and allochthonous sources (mainly agricultural areas). The high bacterial abundance, production and very high respiration rates of the bacterial community show how the lake functions in the mineralization of the organic matter (Manganelli et al., 2005). The high bacterial abundance in the lake could be mostly related to high DOC concentration and nutrient availability (bottom-up). Besides, the bacterial community seem to be also controlled by top-down factors (high predation) (Manganelli et al., 2005).

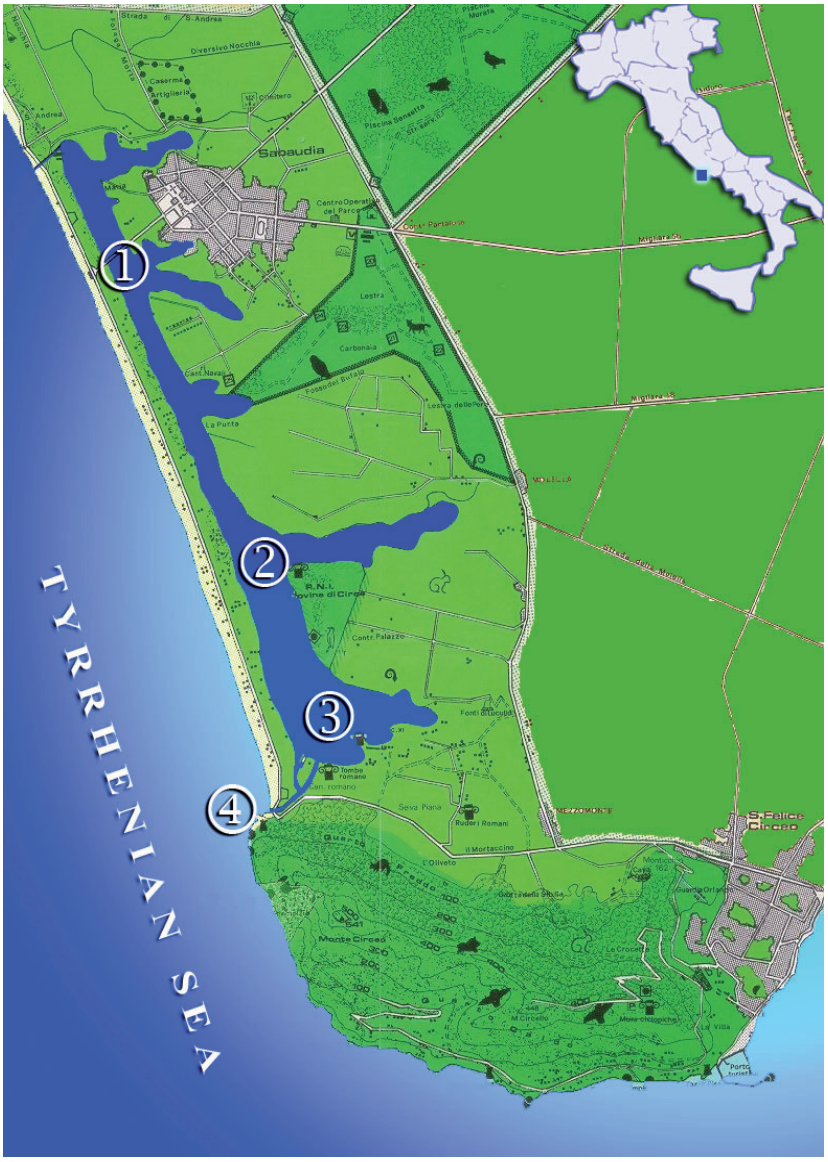

Fig. 2: Map of Lago di Paola indicating the four sampling sites analyzed within this thesis.

1.2. The phylum *Planctomycetes*

1.2.1. Phylogeny

On the basis of 16S ribosomal RNA (rRNA) gene sequence analysis the *Planctomycetes* were defined as an independent phylum of the domain *Bacteria* (Woese, 1987; Ward et al., 1995). Their phylogeny has been under debate for some time (Stackebrandt et al., 1984; Weisburg et al., 1986; Fuerst, 1995; Brochier and Philippe 2002; Teeling et al., 2004). Latest studies grouped them in the *Planctomycetes*, *Verrucomicrobia, Chlamydiae* - (PVC) superphylum, a monophyletic clade that in addition to *Planctomycetes* includes the phyla *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae* and the candidate phyla Poribacteria and OP3 that have no cultured representatives yet (Fieseler et al., 2004; Wagner and Horn, 2006). The grouping of these phyla into a superphylum was supported by common and unique features that distinguish them from other *Bacteria,* such as a peptidoglycan-free cell wall within *Planctomycetes* and *Chlamydiae* and the presence of a cellular compartmentalization in *Planctomycetes* and Poribacteria. Furthermore, the *Planctomycetes* and *Verrucomicrobia* have several phenotypic and molecular features considered to be typical for eukaryotes; for example *Verrucomicrobia* carry genes encoding tubulin-like proteins. Indeed, some researchers hypothesized that an ancestor of the *Planctomycetes* and *Verrucomucrobia* may have been a possible candidate as progenitor of eukaryotic lineage (Fuerst, 2005; Wagner and Horn, 2006).

Currently, the phylum *Planctomycetes* is comprised of the two classes *Planctomycetacia* and *Phycisphaerae.* The class *Planctomycetacia* is organized in one order (*Planctomycetales*) and one family (*Planctomycetaceae*) with nine accepted genera (*Planctomyces, Pirellula, Blastopirellula, Rhodopirellula, Gemmata, Isosphaera, Singulisphaera, Schlesneria, Zavarzinella)* (Schlesner et al., 2004; Ward et al., 2006; Kulichevskaya et al., 2007; Kulichevskaya et al., 2008; Kulichevskaya et al., 2009) (Fig. 3). The class *Planctomycetacia* also contains five candidate genera (*Candidatus* Kuenenia, *Candidatus* Brocadia, *Candidatus* Scalindua, *Candidatus* Anammoxoglobus, *Candidatus* Jettenia) (Fig. 3). *Phycisphaerae* is a recently accepted new class of *Planctomycetes* (Fukunaga et al., 2009) that is currently composed of only one species *Phycisphaera mikurensis* isolated from the marine alga *Porphyra* sp.

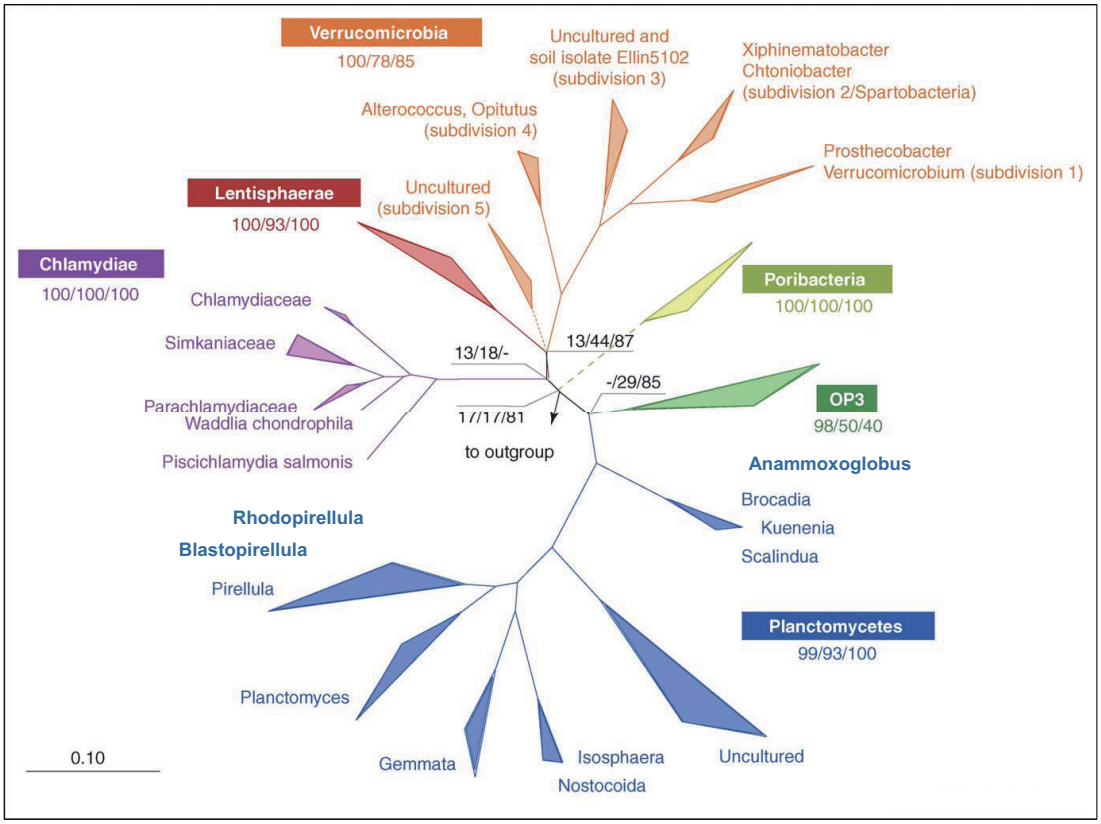

Fig. 3: The PVC superphylum is shown by a phylogenetic tree based on 16S rRNA gene sequences (modified by Wagner and Horn, 2006). Dotted lines show unresolved positioning of groups and numbers indicate bootstrap values calculated with three different algorithms. *Singulisphaera, Schlesneria, Zavarzinella, Candidatus* Jettenia and *Phycisphaera mikurensis* are not shown.

1.2.2. Morphological features

Planctomycetes are a group of budding, peptidoglycan-less bacteria of increasing interest for microbial evolution, ecology, cell biology and genomics (Fuerst, 2004). *Planctomycetes* cells are spherical, ovoid or pear-shaped in most genera (Starr et al., 1983) and a special rosette cell arrangement in pure culture is observed (Schmidt et al., 1981; Schmidt et al., 1982). One genus, *Isosphaera*, is a multicellular filamentous bacterium that moves by gliding.

The presence of a proteinaceous cell wall instead of the common bacterial cell wall constituent peptidoglycan was initially reported for several strains of *Planctomyces* and *Pirellula* (König et al., 1984). It explained the resistance of these strains to antibiotics known to target the synthesis of peptidoglycan. An in-depth analysis of the cell wall composition of members of *Planctomyces* and *Pirellula* has shown proteins rich in

glutamate and cystine/cysteine, the disulfide bonds of which might contribute to the sodium dodecyl sulfate (SDS)-resistance of the cell envelope (Liesack et al., 1986).

Planctomycetes cells are often organized in a polar manner including non-prosthecate appendages (stalks) or polar holdfast structures. Their cytoplasmic membranes contain characteristic pore-like crateriform structures either evenly distributed (genus *Planctomyces*) or just around the reproductive cell-pole (genus *Pirellula*) (Liesack et al., 1986). *Planctomycetes* cells show a generative pole from which daughter cells are formed in a yeast-like budding process. For some *Planctomycetes* the budding type of reprodution results in a cell cycle which is characterized by motile and sessile morphotypes (Tekniepe, 1981; Franzmann and Skerman, 1984; Fuerst 1995).

Moreover, the most distinctive feature of the *Planctomycetes* is their internal compartmentalization (Lindsay et al., 1997; Lindsay et al., 2001; Fuerst, 2005). All *Planctomycetes* cells have a single intracytoplasmic membrane that separates a peripheral ribosome-free paryphoplasm from fibrillar condensed nucleoid and ribosome-like particles (pirellulosome) (Fig. 4). The cell nucleoid is usually highly condensed. In *Pirellula* and *Isosphaera*, the intracytoplasmic membrane binds and defines a single interior cell compartment which holds the cell DNA and ribosome-like particles (Lindsay et al., 2001) (Fig. 4). In *Isosphaera* the intracytoplasmic membrane is invaginated, so the paryphoplasm represents the major portion of the cell interior while in other *Planctomycetes* occupies a peripheral zone (Fig. 4). In *Gemmata* and in *Planctomycetes* carrying out anaerobic ammonia oxidation [“anammox”; Strous et al., 1999; Schmid et al., 2000; Van Dongen et al., 2001)], the compartment bounded by the intracytoplasmic membrane contains a second membrane-bounded compartment (Strous et al., 1999; Lindsay et al., 2001). In *Gemmata* this compartment contains the nucleoid and is surrounded by a double membrane that may be in contact with the intracytoplasmic membrane and so is not fully closed (not visible in Fig. 4). In anammox bacteria the compartment, called anammoxosome, is bounded by a single bilayer membrane possessing unique ladderane lipids (Sinninghe Damstè et al., 2002) (Fig. 4). The anammoxosome is the site where catabolism takes place.

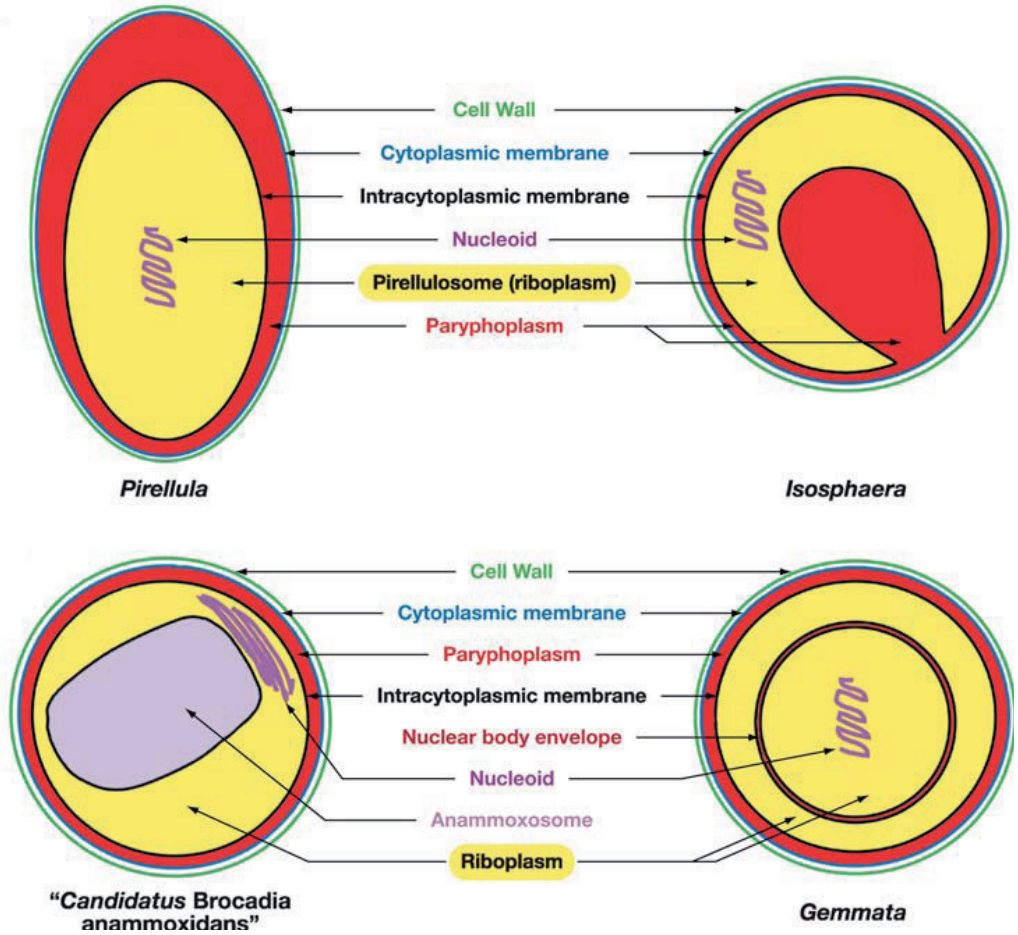

Fig. 4: Cell organization and internal compartmentalization in different *Planctomycetes* is shown (Fuerst, 2005).

1.2.3. Genomics and physiology

Planctomycetes possess one of the largest genomes known in Bacteria (e.g. 9 Mb for *Gemmata obscuriglobus*) (Fuerst, 2004). In recent years several planctomycete genome projects have started. Currently, four completed whole-genome sequences exist for the planctomycete *Rhodopirellula baltica* SH 1^T, *Pirellula staleyi* DSM 6068^T, *Planctomyces limnophilus* DSM 3776^T and *Candidatus* Kuenenia stuttgartiensis. Furthermore, other three draft genomes are publicly available for *Blastopirellula marina* DSM 3645^T, *Gemmata obscuriglobus* UQM 2246^T and *Planctomyces maris* DSM 8797^T. A further draft of a *Gemmata* sp. genome, strain Wa-1, is being sequenced commercially with some academic release. In progress are the genomes of *Isosphaera pallida* ATCC 43644^T, *Planctomyces brasiliensis* DSM 5305^T and the anammox planctomycete *Candidatus* Brocadia anammoxidans.

The first planctomycete investigated by genomic approach was *Rhodopirellula baltica* SH 1^T, formely known as *Pirellula* sp. strain 1 (Glöckner et al., 2003). It was isolated from brackish water at the Kiel Fjord in the Baltic Sea (Schlesner, 1994) and is a pigmented, marine, strictly aerobic and heterotrophic representative of the *Planctomycetes*. The presence of genes for interconversion of C1 compounds, all genes for heterolactic acid fermentation and 110 sulfatases were not expected for such an isolate (Glöckner et al., 2003). *Rhodopirellula baltica* SH 1^T shows a proteinaceous cell wall although remnants of genes for peptidoglycan synthesis were found. Gene sets for lipid A synthesis and homologues to the flagellar basal body L- and P- rings suggests a former Gram-negative type of the cell wall. Among the open reading frames identified, 83% have best BLAST homologous with Bacteria, 9% with Archaea and 8% with eukaryotic proteins. Another feature of *Rhodopirellula baltica* SH 1^T is the growth on N-acetylglucosamine as sole source of carbon and nitrogen under aerobic condition (Rabus et al., 2002). A recent transcriptome study showed that over 3000 of the 7325 genes of *Rhodopirellula baltica* SH 1^T were affected by temperature and/or salinity changes (Wecker et al., 2009). Recently, Woebken and co-workers (Woebken et al., 2007b) compared all planctomycete genomes available at that time with six planctomycete fosmids from two marine upwelling systems. Similar to what was shown before for *Rhodopirellula baltica* SH 1^T they found a high number of sulfatase genes known to be involved in the breakdown of sulfatated heteropolysaccharides and the presence of C1 metabolism genes (Glöckner et al., 2003).

Pirellula staleyi DSM 6068^T was isolated from the freshwater Lake Lansing, MI, USA (Schlesner and Hirsch, 1984; Schlesner and Hirsch, 1987). It is an aerobic, heterotrophic, pear- or teardrop-shaped bacterium with a clearly visible pointed attachment pole and the absence of true stalks. A unique feature observed in *Pirellula staleyi* is the presence of “hump” protrusions including both cell wall and cytoplasm and appear to conform to the definition of prosthecae. Functions proposed for the prosthecae include stalk function, reproduction and increasing surface area (Clum et al., 2009). Strains closely related to the species have been isolated from fresh, brackish water and hypersaline lakes. The genome comprises one main circular chromosome. The majority of the protein-coding genes (54.5%) were assigned with a putative function and the remaining were annotated as hypothetical proteins (Clum et al., 2009).

Planctomyces limnophilus DSM 3776^T is a stalked, budding bacterium isolated from the freshwater Lake Pluβsee in Germany (Hirsch and Müller, 1985). It shows red pigmented colonies, the formation of multicellular rosettes, a narrow temperature range for

growth and low salt tolerance. The majority of the protein-coding genes (53.9%) were assigned with a putative function and the remaining were annotated as hypothetical proteins (Labutti et al., 2010).

The anammox planctomycete *Candidatus* Kuenenia stuttgartiensis was investigated by metagenomic approach from a wastewater treatment plant enrichment culture (Strous et al., 2006). The present genomic data of *Candidatus* Kuenenia stuttgartiensis have revealed some unique pathways such as ladderane biosynthesis, biological hydrazine metabolism and carbon fixation (Strous et al., 2006).

Gemmata obscuriglobus UQM 2246^T was isolated from the surface waters of a freshwater dam (Maroon Dam) in Queensland, Australia (Franzmann and Skerman, 1984). It is a budding, aerobic, nonprosthecate bacteria with spherical to ovoid cells and taxonomically the deepest branching organism within the order *Planctomycetales. Gemmata obscuriglobus* is the first representative of bacteria that has been shown to possess a membrane-bound DNA-containing nucleoid resembling the eukaryotic nucleus. Thus, *Gemmata obscuriglobus* is the only known exception of prokaryote/eukaryote dichotomy and an interesting object for understanding the evolutionary implications of compartmentalization on major molecular processes in the cell. Its draft genome revealed the presence of sterol biosynthesis and C1 transfer metabolism. In addition a soil isolate Wa-1 of *Gemmata* is being sequenced commercially, which will complement the *Gemmata obscuriglobus* results providing data for intrageneric comparisons. *Gemmata* sp. strain Wa-1 has recently been investigated with respect to a possible role of the ancestor of *Planctomycetes* in the evolution of the *Eukarya* (Staley et al., 2005).

Blastopirellula marina DSM 3645^T is an unpigmented, aerobic, heterotrophic and strict marine bacterium as well as *Rhodopirellula baltica* SH 1^T; both they do not grow in freshwater media (Schlesner et al., 2004). Like *Rhodopirellula baltica* SH 1^T it was isolated from brackish water at the Kiel Fjord in the Baltic Sea (Schlesner, 1986). Cells are ovoid, ellipsoidal or pear-shaped, occurring singly or in rosettes (Schlesner et al., 2004).

Planctomyces maris DSM 8797^T was isolated from waters at Puget Sound, Washington, USA (Bauld and Staley 1976; Bauld and Staley, 1980). It is a heterotrophic, obligately aerobic, mesophilic bateria with the absolute requirement for Na^+ and grows at a salinity of 1.5-4.0% (w/v) and in the presence of at least 100 mM-NaCl suggesting that the bacterium is indigenous to the marine environment (Bauld and Staley 1976; Bauld and Staley, 1980).

Isosphaera pallida ATCC 43644^T is an unusual filamentous, budding bacterium isolated from an algal mat from Kah-nee-tah Hot Spring in Oregon (Giovannoni et al., 1987). It is an obligately aerobic chemoheterotroph, moderately thermophilic with a limited nutritional range and slow growth rate (Giovannoni et al., 1987). *Isosphaera pallida* is phototactic and motile by gliding.

Planctomyces brasiliensis DSM 5305^T was isolated from a water sample of Lagoa Vermelha, a salt pit near Rio de Janeiro in Brazil (Schlesner, 1989). It is a budding peptidoglycan-less, nonprosthecate bacterium. Compared to *Planctomyces maris* and *Planctomyces limnophilus* it shows a significantly higher tolerance to artificial sea water or to NaCl (Schlesner, 1989).

Candidatus Brocadia anammoxidans is able to anaerobically oxidize ammonium and nitrite to nitrogen gas. The ability to oxidize ammonium anaerobically makes *Candidatus* Brocadia anammoxidans potentially useful for reducing, or eliminating, ammonium from wastewater. This organism will be used in comparative genomic studies with other anaerobic ammonium oxidizers.

Overall, the whole-genome reciprocal BLAST-analysis of *Rhodopirellula baltica* SH 1^T, *Gemmata obscuriglobus* UQM 2246^T, *Blastopirellula marina* DSM 3645^T and *Candidatus* Kuenenia stuttgartiensis suggests that these *Planctomycetes* do not share an unusually large number of genes with the *Archea* and *Eukarya* (Fuchsman and Rocap, 2006) which is in contrast with previous reports (Glöckner et al., 2003; Teeling et al., 2004) letting to a further complicated debate of *Planctomycetes* evolution.

1.2.4. Diversity, abundance and function

Although the first microscopic observation of rosette-forming "planktonic-mycetes" was reported back in 1924, pure cultures were not obtained until 1973 (Staley, 1973). By now, strains have been mostly isolated from marine, freshwater and terrestrial habitats (Bauld and Staley, 1976; Franzmann and Skerman, 1984; Giovannoni et al., 1987; Schlesner, 1994; Wang et al., 2002; Kulichevskaya et al., 2007; Kulichevskaya *et al.,* 2008; Fukunaga et al., 2009; Kulichevskaya et al., 2009). Despite the recent success in isolating new members of the *Planctomycetaceae* (Winkelmann and Harder, 2009), the phylum remains one of the less represented in microbial culture collections.

Studies based on cultivation-independent molecular methods like 16S rRNA gene sequencing and fluorescence *in situ* hybridization (FISH) confirmed their occurrence in

aquatic and terrestrial habitats and revealed an even broader distribution of *Planctomycetes* in the environment. They were detected in the marine water column (DeLong et al., 1993; Vergin et al., 1998; Shu & Jiao, 2008c) often colonizing marine snow particles (DeLong et al., 1993; Rath et al., 1998; Crump et al., 1999), in shallow and deep sea sediments of marine systems (Llobet-Brossa et al., 1998; Rusch et al., 2003; Inagaki et al., 2006; Musat et al., 2006; Shu & Jiao, 2008b), in the water column and sediments of fresh water lakes (Neef et al., 1998; Miskin et al., 1999; Kalyuzhnaya et al., 2004; Kalyuzhnaya et al., 2005) and in river biofilms (Brümmer et al., 2004). Many studies have reported high abundances of *Planctomycetes* following algal blooms (Janse et al., 2000; Brümmer et al., 2004; Eiler and Bertilsson, 2004; Ward et al., 2006; Tadonleke, 2007). Morris and coworkers (Morris et al., 2006) have found high abundances of *Pirellula*-related cells (up to 4 x 10^7 l^{-1}) during a diatom bloom in Oregon coastal waters. Furthermore, *Planctomycetes* have been also detected in marine sponges (Fuerst et al., 1998; Friedrich et al., 1999; Fuerst et al., 1999; Friedrich et al., 2001; Webster et al., 2001; Pimentel-Elardo et al., 2003 ; Zhu et al., 2008), and in crustaceans (Staley, 1973; Fuerst et al., 1991; Fuerst et al., 1997).

Besides aquatic habitats *Planctomycetes* were also identified in soils (Buckley et al., 2006), in acidic *Sphagnum* wetlands (Dedysh et al., 2006) and recently, they have been detected in the intestinal tract of various animals (Frey et al., 2006), including humans (De Hertogh et al., 2006) and in the termite gut (Shinzato et al., 2005; Köhler et al., 2008). *Planctomycetes* seem to be nearly everywhere. This is supported by the finding that they represented 4.8% of the clones from a community library from the Paleolithic Altamira cave paintings in Spain (Schabereiter-Gurtner et al., 2002).

The application of FISH techniques revealed the ubiquitous distribution and the abundance of *Planctomycetes*. Neef and colleagues (Neef et al., 1998) designed two 16S rRNA-targeted oligonucleotide probes specific for *Planctomycetes* (PLA46 and PLA886) and Gade and coworkers (Gade et al., 2004) developed a 16S rRNA-targeted oligonucleotide probe for the genus *Pirellula* (PIR1223) and a species-specific probe for *Rhodopirellula baltica* SH 1^T (RB454) which have been successfully applied in many studies (Glöckner et al., 1999; Ivanova and Dedysh, 2006; Morris et al., 2006; Schattenhofer et al., 2009). The use of such probes showed that *Planctomycetes* represent a significant proportion of the microbial community in various environment occupying < 2 to 10% of the DAPI counts in two polluted rivers (Brümmer et al., 2000; Brümmer et al., 2004), 3 to 19% in North Sea sandy sediments (Musat et al., 2006) and 4 to 13% in anoxic layers of a *Sphagnum* peat bog (Kulichevskaya et al., 2006). Compared to that, they

represent only a few percent of the entire picoplankton community in coastal waters (Glöckner et al., 1999) and even less in the marine pelagial (Rusch et al., 2007; Schattenhofer et al., 2009). These observations can explain why the ecological functions of *Planctomycetes* in the marine system are not well studied and understood. However, the abundance is not necessarily reflecting the importance since key metabolisms can be performed by minor groups (e.g. anammox *Planctomycetes,* see below).

Due to their ability to grow on various sugars, it has been proposed that *Planctomycetes* are involved in the degradation of carbohydrates (e.g. N-acetylglucosamine) (Schlesner, 1994; Fuerst et al., 1995; Rabus et al., 2002). They can also be of global significance for nutrient cycling processes in marine habitats for the mineralization of marine snow particles (DeLong et al., 1993; Fuerst, 1995). Recently, Woebken and coworkers (Woebken et al., 2007b), to deepen our understanding about *Planctomycetes* ecology, compared all planctomycete genomes available at that time with six planctomycete fosmids from two marine upwelling systems. Similar to what was shown before for *Rhodopirellula baltica* SH 1^T (Glöckner et al., 2003) they found a high number of sulfatase genes in all marine planctomycete genomes and in two fosmids and the presence of C1 metabolism genes, only known for methanogenic *Archaea* and methylotrophic *Proteobacteria*, in two fosmids and all genomes except for *Candidatus* Kuenenia stuttgartiensis. The presence of sulfatases in marine *Planctomycetes* could be attribute to the marine lifestyle. Most probably the substrates for sulfatases are sulfated heteropolysaccharides, produced in the marine environment mainly by red algae (agars and carrageenans) and brown algae (sulfated fucans) and entrapped in macroaggregates known to be colonized by *Planctomycetes* that derive its energy from those compounds (Woebken et al., 2007b). Sulfated polymers are complex compounds, hence it requires a versatile repertoire of specific sulfatases for biodegradation and that could explain the high copy numbers in the marine *Planctomycetes* genomes. With respect to C1 compounds is not clear whether the genes are functional in *Planctomycetes* and which is their role. *Rhodopirellula baltica* SH 1^T does not grow on C1 substrates (Bauer et al., 2004). It has been suggested that the sole role of those genes within the *Planctomycetes* is the detoxification of formaldehyde (Chistoserdova et al., 2004) but *Rhodopirellula baltica* SH 1^T, *Blastopirellula marina* DSM 3645^T, *Planctomyces maris* DSM 8797^T and *Gemmata obscuriglobus* UQM 2246^T contain glutathione-dependent formaldehyde dehydrogenases and *Rhodopirellula baltica* SH 1^T contains a glutathione-independent formaldehyde dehydrogenase that can decompose formaldehyde in a simpler way (Goenrich et al., 2002;

Woebken et al., 2007b). Hence, it suggests a general importance of these genes for *Planctomycetes* other than formaldehyde detoxification that should be definitely investigated in the future.

Members of the candidate genera Kuenenia, Brocadia, Scalindua, Anammoxoglobus and Jettenia are responsible for the anaerobic oxidation of ammonia in wastewater treatment plants, in a granular sludge reactor, coastal marine sediments and oceanic and freshwater oxygen minimum zones (Strous et al., 1999; Schmid et al., 2000; Dalsgaard et al., 2003; Schmid et al., 2003; Kuypers et al., 2005; Kartal et al., 2007; Quan et al., 2008; Woebken et al., 2008). Anammox *Planctomycetes* are able to remove ammonia nitrogen from wastewater, combining ammonia and nitrite into dinitrogen gas. The anammox process is important for the reduction of ammonia emissions due to agricultural use of chemical fertilizers (Ward et al., 2006). Latest studies identified anammox *Planctomycetes* in marine sediments from the Baltic-North Sea (Dalsgaard and Thamdrup, 2002), in the Black Sea (Kuypers et al., 2003) and in an anoxic bay off the coast of Costa Rica (Dalsgaard et al., 2003) indicating an important role for annamox *Planctomycetes* in the global nitrogen cycle, for molecular nitrogen regeneration in anoxic conditions. The absence of C1 genes in *Candidatus* Kuenenia stuttgartiensis and the lack of almost any planctomycete-specific gene indicates that anammox bacteria are distinct from all other *Planctomycetes* (Woebken et al., 2007b).

1.3. The phylum *Chlamydiae*

The order *Chlamydiales*, until recently, comprised the single family *Chlamydiaceae* exclusively consisting of obligate intracellular bacteria. *Chlamydiae* represented a small group of closely related bacteria deeply branching within the domain Bacteria, with a unique developmental cycle, and thus being biologically and phylogenetically well separated from all other known bacteria. Everett and co-workers (Everett et al., 1999; Bush and Everett, 2001), based on comparative sequence analysis of different macromolecules, reclassified these pathogens into nine species belonging to two different genera (*Chlamydophila* and *Chlamydia*) within the family *Chlamydiaceae* (Horn and Wagner, 2001). *Chlamydiae* of the family *Chlamydiaceae* represent the world's major cause of preventable blindness and sexually transmitted disease [*Chlamydia trachomatis*, trachoma and urogenital infections in humans (WHO, 2001, 2008)].

In the 1990s, “novel chlamydiae” were discovered as symbionts of free-living amoebae or insects, as pathogens of crustaceans, as contaminants of a laboratory cell culture, and in an aborted bovine fetus (Kahane et al., 1995; Amann et al., 1997; Rurangirwa et al., 1999; Horn et al., 2000; Thao et al., 2003; Kostanjsek et al., 2004). Based on these findings three novel families within the phylum *Chlamydiae* were described: *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae* (Rurangirwa et al., 1999; Everett et al., 1999). Molecular data suggest an additional diversity of *Chlamydiae* in the environment (Ossewaarde and Meijer, 1999; Horn and Wagner, 2001; Corsaro et al., 2003; Corsaro and Venditti, 2009). These “novel chlamydiae” (all the *Chlamydiae* not belonging to the family *Chlamydiaceae*) are also called environmental chlamydiae. By now the diversity of environmental chlamydiae outgoes the diversity of previously recognized pathogenic chlamydiae. Water systems have been shown to be a potential source of *Chlamydiae*. However, their diversity is underestimated and their natural hosts are unknown in many cases (Corsaro and Venditti, 2009).

Recent studies suggest *Chlamydiae* as part of the *Planctomycetes*, *Verrucomicrobia, Chlamydiae* - (PVC) superphylum, a monophyletic clade that also includes the phyla *Planctomycetes*, *Verrucomicrobia*, *Lentisphaerae* and the candidate phyla Poribacteria and OP3 that have no cultured representatives yet (Fieseler et al., 2004; Wagner and Horn, 2006).

Twenty-nine genomes, including complete, draft and in progress, are currently available for *Chlamydiales* of which only four belongs to environmental chlamydiae:

Candidatus Protochlamydia amoebophila UWE25, *Parachlamydia acathamoebae, Simkania negevensis* and *Waddlia chondrophila*.

Environmental chlamydiae have phylogenetically different hosts than pathogenic chlamydiae (amoebae vs. humans) and show the same obligate intracellular life style and a similar morphology of their developmental stages. Particularly the developmental cycle of *Chlamydiae* involves a metabolically inactive infectious form called the elementary body (EB), that, after the entry in the target cell (e.g. free-living amoebae), differentiates into a larger, metabolically active reticulate body (RB) (Fig. 5). After multiple rounds of replication, RBs go through a secondary differentiation process back to infectious EBs (Fig. 5) (Subtil and Dautry-Varsat 2004).

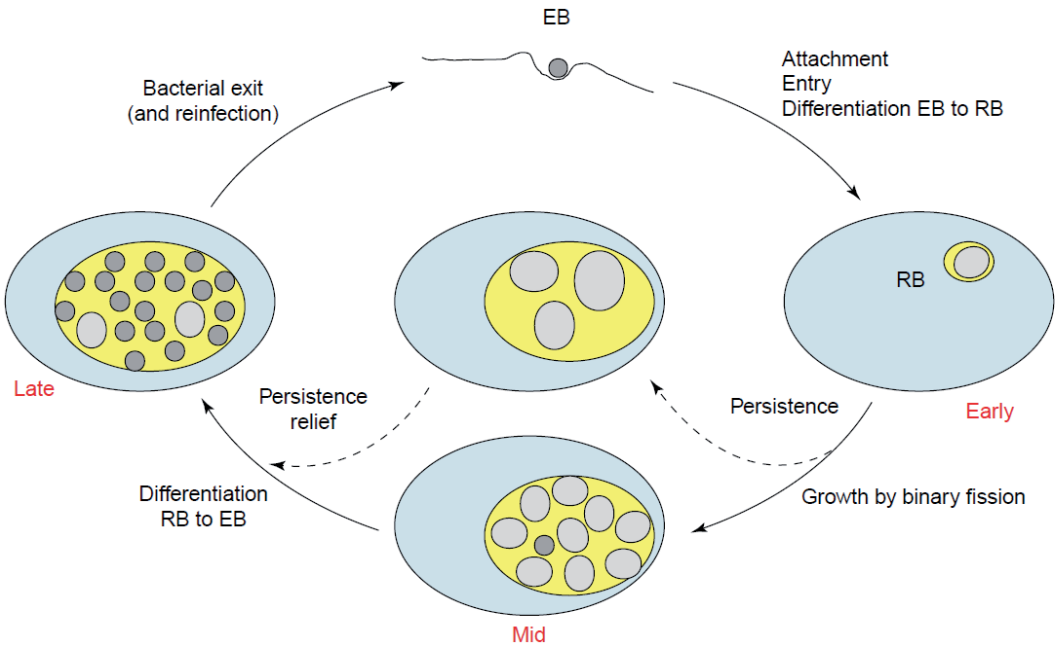

Fig. 5: Infectious cycle of *Chlamydiaceae* (Subtil and Dautry-Varsat 2004).

Environmental chlamydiae are more closely related to pathogenic chlamydiae than to any other bacteria. Most probably an in-depth analysis of those “novel chlamydiae” will show further similarities with pathogenic chlamydiae making environmental chlamydiae a suitable model system to study chlamydial biology. Recent studies suggest environmental chlamydiae as potential emerging pathogens. In fact, a possible association of environmental chlamydiae with respiratory disease of humans has been shown (Greub and Raoult, 2002). In patients with respiratory disease antibodies against *Parachlamydia* or

Simkania were identified and 16S rRNA genes more similar to rRNA genes of environmental chlamydiae than to rRNA genes of pathogenic chlamydiae were found. Ultimately, *Parachlamydiae* which naturally occur within amoebae, could enter and multiply within human cells (Greub et al., 2003; Collingro et al., 2005).

1.4. Methodological aspects

1.4.1. The 16S rRNA approach

While prokaryotes are invisible to the naked eye they are "the unseen majority". The oceans contain in total 3.6 x 10^{28} cells and the total number of cells in saline lakes and freshwaters is 2.3 x 10^{26} (Whitman et al., 1998). In the last thirty years the use of molecular techniques revealed informations regarding the biodiversity, abundance and role of microorganisms in the environment, three important aspects of the microbial ecology.

Studies of 16S rRNA partial sequences by Carl Woese (Woese and Fox, 1977) led to the three domains of life: *Archaea* (archaebacteria), *Bacteria* (eubacteria) and *Eukarya* (eukaryotes). Comparisons of phylogenetic sequences allowed to study yet uncultured microorganisms in their environment. The 16S rRNA molecule is ancient, functionally and evolutionarily homologous in all organisms and contains very conserved but also variable regions that allows sequence comparison and phylogenetic analysis to the genus and species levels (Rossello-Mora and Amann, 2001). Furthermore, 16S rRNA provides sufficient sequence information (~ 1,500 nucleotides) to permit statistically significant comparisons. For that reason, in 1986, Norman Pace and colleagues (Olsen et al., 1986) proposed the "ribosomal RNA approach" for studies in microbial ecology. After DNA extraction from environmental samples the 16S rRNA genes of microorganisms are amplified by polymerase chain reaction (PCR), cloned, sequenced and phylogenetically analyzed to elucidate the microbial diversity of the sample (Amann et al., 1995) (Fig. 6). The analysis of 16S rRNA sequences produces numerical values of 16S rRNA identity that can be used as circumscription limits for taxa (Rossello-Mora and Amann, 2001). A genus could be defined by species with 95% 16S rRNA sequence identity (Ludwig et al., 1998) and 97% 16S rRNA sequence identity can be used as a boundary for the species circumscription (Rossello-Mora and Amann, 2001). The ribosomal RNA approach has become an integral part of environmental microbiology and since its development, an immense diversity has been revealed. At the moment there are 25 phyla with cultured representatives and more than 50 others defined only by the relatedness of 16S rRNA gene sequences (Pace, 2009).

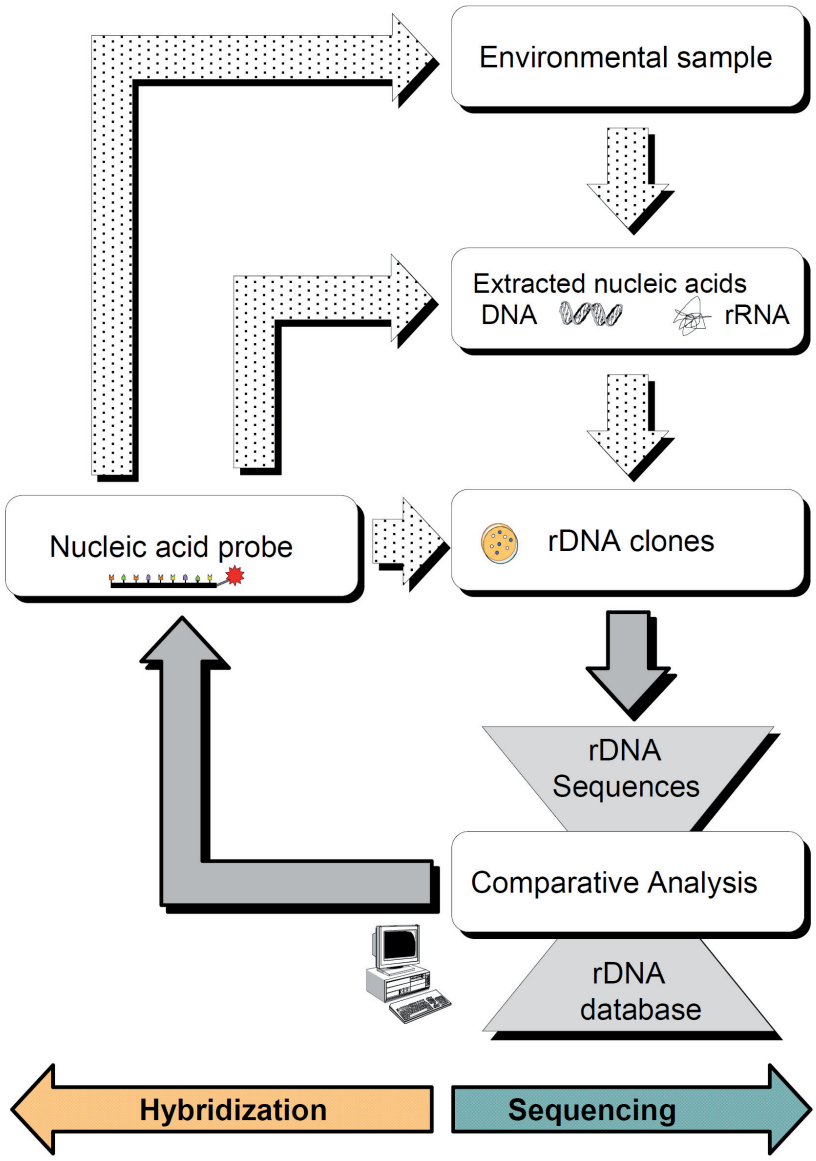

Fig. 6: The 16S rRNA approach (Amann et al., 1995).

The number of 16S rRNA sequences is still increasing. Currently, the SILVA database release 102 from February 2010 (Pruesse et al., 2007) contains 460,783 full sequences and 1,246,462 partial sequences (www.arb-silva.de). These can be handled by the software package ARB (Ludwig et al., 2004) and phylogenies can be calculated. Since 16S rRNA sequence libraries do not provide information about the *in situ* abundance of microorganisms (Suzuki and Giovannoni, 1996) and their ecological niche, in a second phase, nucleic acid probes are designed and hybridization (e.g. FISH) is carried out. FISH is a molecular technique that stains ribosomes of cells and allows the identification,

quantification and, in combination with other techniques, characterization of microbial populations in complex environments (Amann et al., 1991; Wagner et al. 2003). Theoretically, each ribosome within a bacterial cell, that contains one copy each of 5S, 16S, 23S rRNA, is stained by one probe molecule during the hybridization, so the high numbers of ribosomes per cell provide a natural signal amplification (Amann et al., 1995). However, most of bacteria in aquatic habitats are small, slow growing and starving thus with very low ribosome numbers, so the signal intensities of hybridized cells are really low and frequently below the detection limit (Pernthaler et al., 2001). In order to increase the signal to noise ratio the tyramide signal amplification (TSA), also known as catalyzed reporter deposition (CARD) was proposed (Amann et al., 1992; Pernthaler et al., 2002). CARD is based on the deposition of a large number of labeled tyramine molecules by peroxidase activity (Pernthaler et al., 2002). Hence, in contrast to FISH numerous fluorescent molecules can be introduced at the hybridization site *in situ* leading to a strong signal amplification. Using CARD-FISH the percentage of identified microorganisms in marine systems increased three times (Pernthaler et al., 2002) allowing a reliable quantification in the oligotrophic open ocean (Schattenhofer et al., 2009).

Regarding the probe design, probe specificities must be checked *in silico* against a database (e.g. SILVA database) and to optimize the stringency conditions, a series of hybridizations at increasing formamide concentrations (e.g. 10–70%) must be evaluated. The optimal formamide concentration is the highest concentration before signal intensity decreases.

1.4.2. Optimization of *Planctomycetes* cells permeabilization for CARD-FISH

It is well known that the critical step of the CARD-FISH method is the diffusion of large molecules such as enzymes into whole fixed cells (Amann et al., 1992). The larger horseradish peroxidase label (~ 44 kDa) is used for CARD-FISH compared to an oligonucleotide labeled with a fluorescent dye (~ 700 Da) used in the FISH method (Woebken et al., 2007a). Since the exact composition of the cell wall varies within prokaryotes, probe penetration might be inhibited (e.g. gram-positive) (Bidnenko et al., 1998). Hence, it is needed to include a controlled permeabilization step before the enzymatic signal amplification, balancing permeability with cellular integrity (Schönhuber et al., 1997; Pernthaler et al., 2002). Pernthaler and colleagues (Pernthaler et al., 2002) provided an improved protocol for CARD-FISH of marine planktonic and benthic microbial assemblages. Particularly, samples firstly concentrated onto membrane filters

and embedded in low-gelling-point agarose are subsequently incubated with lysozyme (10 mg ml^{-1} at 37°C) to allow the penetration of the enzyme labeled probe into the extracellular polymeric material in the gram-negative bacterial cell wall. Whereas the archaeal cell wall can be digested by a treatment with HCl (1 min, 0.1 M).

In the 80's it was shown that *Planctomycetes* have a proteinaceous cell wall mainly consisting of proline and cysteine (König et al., 1984; Liesack et al., 1986). The cell wall is stabilized by disulfide bonds formed by two cysteine residues. By contrast, in the genome of *Rhodopirellula baltica* remnants of genes for peptidoglycan synthesis were found (Glöckner et al., 2003).

Therefore, since the protocol of Pernthaler and colleagues (Pernthaler et al., 2002) was not specifically tested for *Planctomycete*s, here different permeabilization treatments were applied to optimize the CARD-FISH protocol for this phylum (Fig. 7).

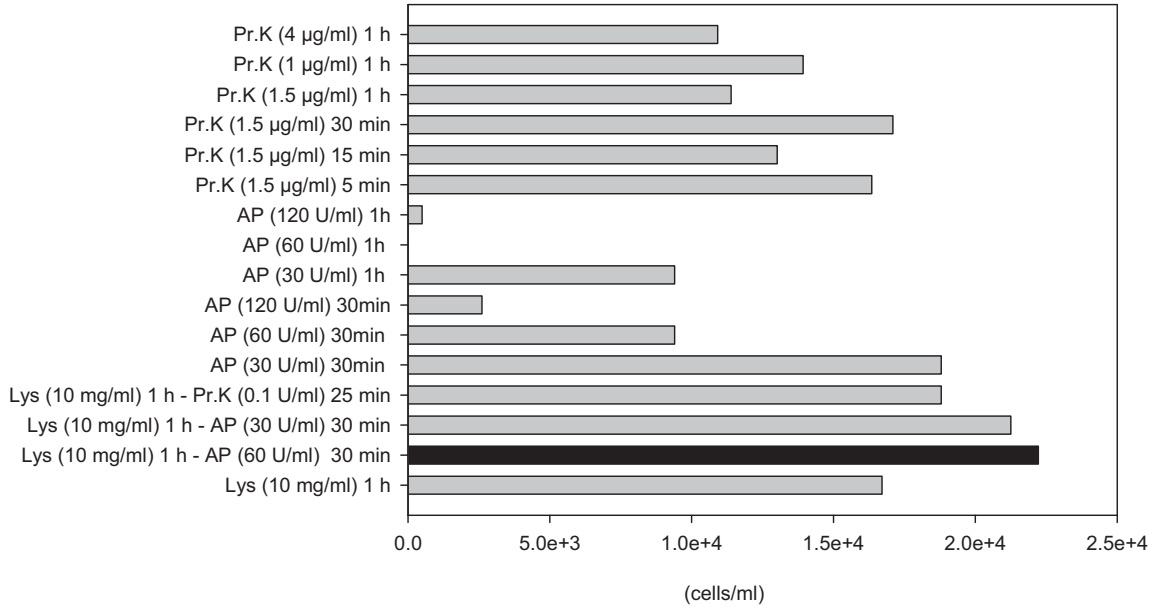

Fig. 7: Optimization of *Planctomycetes* cells permeabilization for CARD-FISH. The probe PLA46 (Neef et al., 1998) was used to assess *Planctomycetes* abundance. All incubations were performed at 37 °C. Lys: lysozyme; AP: achromopeptidase; Pr.K: proteinase K. Enzyme concentrations and times are displayed. In black the best permeabilization treatment is shown.

The best result, displayed in Fig. 7, was obtained using lysozyme (10 mg ml^{-1} buffer contained 0.05 M EDTA, pH 8, 0.1 M Tris-HCl, pH 7.4) for 1 h at 37 °C followed by achromopeptidase (60 U ml^{-1}; buffer contained 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37 °C. This permeabilization step was applied to all samples analyzed within

this thesis. Since *Planctomycetes* and *Chlamydiae* share a peptidoglycan-free cell wall the treatment was also used to detect *Chlamydiae.*

1.4.3. Optimization of DNA isolation

Zhou and colleagues developed a rapid method for bacterial lysis and extraction of DNA from soils with minimal shearing (Zhou et al., 1996). This method is based on lysis with a high-salt extraction buffer (1.5 M NaCl) and a prolonged heating (2 to 3 h) of the soil suspension with sodium dodecyl sulfate (SDS) to remove membrane lipids, hexadecyltrimethylammonium bromide and proteinase K to break cell walls and remove proteins. Although this protocol was already used for extracting total nucleic acids from water (Gómez-Pereira et al., 2010; Simonato et al., 2010) here it was slightly modified: (i) cut sections of polycarbonate filters were used as samples, (ii) all centrifugation steps were performed at 53,250 x g excepted for the recovery of the aqueous phase after the mixing with chloroform/isoamylalcohol which was performed at 10,000 x g, (iii) the precipitation of DNA with isopropanol was done overnight at room temperature, (iv) the pellet of crude nucleic acids was resuspended in 1:10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8). Overall, a longer time period of DNA precipitation using isopropanol and an increased speed of centrifugation steps compared to the original protocol were necessary to retrieve sufficient amounts of DNA.

1.5. Aims

The general aim of this PhD thesis was to define the abundance, distribution and phylogenetic diversity of *Planctomycetes* in different coastal systems under different environmental conditions trying to conclude on potential activities based on the correlation of *Planctomycetes* diversity and abundance data with environmental parameters. The main objectives of the thesis are listed below:

- To quantify *Planctomycetes* throughout the year in the seawater column off the German North Sea island Helgoland, focusing on the 0.2-3 µm, 3-10 µm, 0.2-10 µm and > 0.2 µm fractions, and in macroaggregates of mostly algal origin since indirect evidence for *Planctomycetes* attached life style was so far mostly coming from clone frequencies in SSU libraries (DeLong et al., 1993; Rath et al., 1998; Crump et al., 1999). In our study we aimed to corroborate this by *in situ* hybridization techniques such as CARD-FISH. Chapter 2.1

- To optimize the CARD-FISH protocol of Pernthaler and colleagues (Pernthaler et al., 2002) that was not specifically tested for *Planctomycete*s to improve their fluorescence signals since they are peptidoglycan-less bacteria showing a proteinaceous cell wall mainly consisting of proline and cysteine. Chapter 2.1

- To correlate *Planctomycetes* abundance with environmental parameters such as temperature, salinity, time, silicate, phosphate, nitrate, nitrite, ammonium, Chl a, DOC, dissolved oxygen, pH and centric and pennate diatoms trying to explain the potential activities of *Planctomycetes* in aquatic habitats. Chapters 2.1 and 2.2

- To define the diversity of *Planctomycetes* by comparative sequence analysis of almost full length 16S rRNA genes in winter and summer samples and in 3-10 µm and 0.2-3 µm fractions. Chapter 2.1

- Based on the created phylogenetic tree to design test and optimize oligonucleotide probes for clades of the *Planctomycetes* phylum in order to quantify specific marine subgroups and to determine how these groups are distributed in different years, seasons, fractions and samples. Chapter 2.1

- Based on a previous evidence of the presence of *Planctomycetes* at the coastal lake Lago di Paola, Italy (not published) to analyze the seasonal dynamics of *Planctomycetes* in the coastal lake and in the adjacent marine coastal waters. The coastal lake is a type of aquatic system for which to our knowledge no study focusing on ecology of *Planctomycetes* has been performed. Particularly, within the lake, to define the distribution of *Planctomycetes* on the transitional period from summer to winter seasons along the lake main axis and between its surface and bottom from stratified to mixed waters. Chapter 2.2

- To assess *Planctomycetes* diversity in surface and bottom waters in order to identify the most representative *Planctomycetes* clades within the lake. Design, test and optimize oligonucleotide probes for such clades to determine how they are distributed over depth. Chapter 2.2

- To define the biodiversity of environmental chlamydiae in Lago di Paola since monitoring *Planctomycetes*, 16S rRNA gene sequences affiliated with *Chlamydiales* were retrieved. Design, test and optimize oligonucleotide probes for the new identified clades. Chapter 2.3

2. Results and Discussion

List of manuscripts presented in this thesis

Contributions to the manuscripts presented in this thesis

2.1. Abundance and seasonality of three clades of *Planctomycetes* in the North Sea

Ilaria Pizzetti, Bernhard M. Fuchs, Gunnar Gerdts, Antje Wichels, Karen H. Wiltshire, Rudolf Amann

Submitted to *Environmental Microbiology*. Idea I.P., B.M.F. and R.A.; I.P., B.M.F. and R.A, designed research; I.P. realization of experiments; G.G., A.W., K.H.W. contributed experimental data; I.P. wrote the manuscript with editorial assistance of B.M.F., K.H.W. and R.A.

2.2. Abundance and diversity of *Planctomycetes* in a Tyrrhenian coastal lake (Lago di Paola, Italy)

Ilaria Pizzetti, Bernhard M. Fuchs, Rudolf Amann, Stefano Fazi

Submitted to *Aquatic Microbial Ecology*. Idea I.P. and S.F.; I.P., B.M.F., R.A. and S.F. designed research; I.P. realization of experiments; S.F. contributed experimental data; I.P. wrote the manuscript with editorial assistance of B.M.F., R.A and S.F.

2.3. *In situ* indentification of novel environmental chlamydiae in a Mediterranean coastal lake (Lago di Paola, Italy)

Ilaria Pizzetti, Stefano Fazi, Bernhard M. Fuchs, Rudolf Amann

Manuscript in preparation. Intended for *Environmental Microbiology Reports*. Idea I.P.; I.P., S.F., B.M.F., R.A. designed research; I.P. realization of experiments; I.P. wrote the manuscript with editorial assistance of S.F., B.M.F., and R.A.

2.1.

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Submitted to *Environmental Microbiology*.

Abundance and seasonality of three clades of *Planctomycetes* in the North Sea

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Running Title: Seasonality of marine planktonic *Planctomycetes*

Keywords: *Planctomycetes*; coastal marine system; picoplankton; CARD-FISH; 16S rRNA gene sequencing.

Summary

Members of the bacterial phylum *Planctomycetes* are widely distributed in terrestrial and aquatic habitats. However, little is known regarding their spatial and temporal distribution in the marine water column. Particularly, quantitative information on specific clades of *Planctomycetes* is scarce. Here we investigated the abundance, distribution and phylogenetic diversity of *Planctomycetes* in surface waters off the German North Sea island Helgoland in different seasons. Seawater was size-fractionated, and in addition marine macroaggregates were collected. A high seasonal diversity of *Planctomycetes* was revealed by comparative sequence analysis of almost full length 16S ribosomal RNA (rRNA) genes in winter and summer samples and in 3-10 µm and 0.2-3 µm fractions. A *Planctomyces*-related group A, an uncultured *Planctomycetes* group B and a *Pirellula*-related group D were identified as the three major clades. Relative abundance of *Planctomycetes* throughout the year as analyzed by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with probe PLA46 was between 0.5-5%. *Planctomycetes* were enriched on particles with counts of 6-10% on marine macroaggregates. A general increase of PLA46 counts after algal blooms suggested that *Planctomycetes* were bottom-up controlled and benefited from the increase in productivity. CARD-FISH with newly developed probes confirmed distinct seasonal variations of the three clades in 2007 and 2009, and provided first hints on a stable seasonal succession which might be linked to abundances of centric and pennate diatoms.

Introduction

On the basis of 16S ribosomal RNA (rRNA) gene sequence analysis the *Planctomycetes* were defined as an independent phylum of the domain *Bacteria* (Woese, 1987). Their phylogeny has been under debate for some time (Stackebrandt *et al.*, 1984; Fuerst, 1995; Brochier and Philippe, 2002; Teeling *et al*., 2004). There is currently a broad consensus that *Planctomycetes* belong to the so-called PVC superphylum, a monophyletic clade that also includes the phyla *Verrucomicrobia, Chlamydiae, Lentisphaerae* and the candidate phyla Poribacteria and OP3 which have no cultured representative so far (Fieseler *et al.*, 2004; Wagner and Horn, 2006). The grouping of these phyla into a superphylum was supported by common and unique features that distinguish them from other *Bacteria,* such as a peptidoglycan-free proteinaceous cell wall (König *et al.*, 1984; Liesack *et al.*, 1986), a cellular compartmentalization (Lindsay *et al.*, 1997; Lindsay *et al.*, 2001) and a budding type of reproduction (Fuerst, 1995). Furthermore, *Planctomycetes* cells are often organized in a polar manner including non-prosthecate appendages (stalks) or polar holdfast structures.

The phylum *Planctomycetes* is comprised of the two classes *Planctomycetacia* and *Phycisphaerae.* The class *Planctomycetacia* is currently organized in one order (*Planctomycetales*) and one family (*Planctomycetaceae*) with nine accepted genera (*Planctomyces, Pirellula, Blastopirellula, Rhodopirellula, Gemmata, Isosphaera, Singulisphaera, Schlesneria, Zavarzinella)* (Schlesner *et al.*, 2004; Ward *et al.*, 2006; Kulichevskaya *et al.*, 2007; Kulichevskaya *et al.*, 2008; Kulichevskaya *et al.*, 2009). The class *Planctomycetacia* also contains five candidate genera (*Candidatus* Kuenenia, *Candidatus* Brocadia, *Candidatus* Scalindua, *Candidatus* Anammoxoglobus, *Candidatus* Jettenia). *Phycisphaerae* is a recently accepted new class of *Planctomycetes* (Fukunaga *et*

69 *al.*, 2009) that is currently composed of only one species *Phycisphaera mikurensis* isolated
70 from the marine alga *Porphyra* sp.

71 Although the first microscopic observation of rosette-forming "planktonic-
72 mycetes" was reported back in 1924, pure cultures were not obtained until 1973 (Staley,
73 1973). By now strains have been mostly isolated from marine, freshwater and terrestrial
74 habitats (Bauld and Staley, 1976; Franzmann and Skerman, 1984; Giovannoni *et al.*, 1987;
75 Schlesner, 1994; Wang *et al*., 2002; Kulichevskaya *et al.*, 2007; Kulichevskaya *et al.*,
76 2008; Fukunaga *et al.*, 2009; Kulichevskaya *et al.*, 2009). Despite the recent success in
77 isolating new members of the *Planctomycetaceae* (Winkelmann and Harder, 2009), the
78 phylum remains one of the less represented in microbial culture collections.

79 Using methods like 16S rRNA gene sequencing and fluorescence *in situ*
80 hybridization (FISH) *Planctomycetes* were detected in the marine water column (DeLong
81 *et al.*, 1993; Vergin *et al.*, 1998) often colonizing marine snow particles (DeLong *et al.*,
82 1993; Crump *et al.*, 1999), in sediments of marine systems (Llobet-Brossa *et al.*, 1998;
83 Rusch *et al.*, 2003; Musat *et al.*, 2006) and in the water column and sediments of fresh
84 water lakes (Neef *et al.*, 1998; Miskin *et al.*, 1999; Kalyuzhnaya *et al.*, 2004; Kalyuzhnaya
85 *et al.*, 2005). Many studies have reported blooms of *Planctomycetes* following algal
86 blooms (Janse *et al.*, 2000; Brümmer *et al.*, 2004; Eiler and Bertilsson, 2004; Ward *et al.*,
87 2006; Tadonleke, 2007). Morris and coworkers (Morris *et al*., 2006) have found high
88 abundances of *Pirellula*-related cells (up to 4 x 10^7 l^{-1}) to occur during a diatom bloom in
89 Oregon coastal waters.

90 The complete genome sequence of the marine isolate *Rhodopirellula baltica* has
91 shown a high number of sulfatase genes known to be involved in the breakdown of
92 sulfated heteropolysaccharides mainly produced by algae and the presence of C1
93 metabolism genes (Glöckner *et al.*, 2003). Recently, a comparison of all *Planctomycetes*

genomes available with six *Planctomycetes* fosmids from two marine upwelling systems corroborated the specialization of marine *Planctomycetaceae* for algal polymer degradation (Woebken *et al.*, 2007b).

Although many data have been gathered for the entire *Planctomycetes* phylum, little is known about their spatial and temporal distribution in the marine water column and particularly, quantitative information on specific *Planctomycetes* clades is scarce. Furthermore, previous anecdotal observations of *Planctomycetes* colonization of marine snow particles and algae deserve a broader investigation on dedicated samples.

The aim of this study was an in-depth assessment of the abundance, distribution and diversity of planktonic *Planctomycetes* at the coastal marine station "Kabeltonne" off the island Helgoland in the German Bight of the North Sea. A special emphasis was put on the quantification of *Planctomycetes* in the 3-10 µm and 0.2-3 µm fractions in the water column and on marine aggregates.

Results

Environmental parameters.

Temperature, salinity and the concentration of silicate, phosphate, nitrate, nitrite and ammonium were determined daily at station "Kabeltonne" near the North Sea island Helgoland (Table S1). Water temperature was low and mostly constant between February and March (around 6 °C), increasing between March and July to a maximum of 18.0 °C, decreasing in October to 15.2 °C (Fig. 1, Table S1). The values of salinity ranged from 31.7 (May) to 33.6 (February). A general decreasing trend, between winter and spring, was registered for all nutrients excepted for the ammonium (Fig. 1, Table S1).

Picoplankton and *Planctomycetes* abundances.

Overall, 23 samples were analyzed in 2007 (Table 1). The abundances of total picoplankton, quantified by DAPI staining and microscopic counting, ranged between 6.3 x 10^5 cells ml^{-1} in February and 1.7 x 10^6 cells ml^{-1} in October (Fig. 1, Table 1). The abundance of *Planctomycetes* was quantified with probe PLA46 (Neef *et al.*, 1998) by an improved catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) protocol optimized for the phylum. The contribution of *Planctomycetes* to the entire picoplankton community throughout the year was quite stable at about 4-5% relative abundance (Fig. 1, Table 1). Absolute cell numbers of *Planctomycetes* varied throughout the year and increased from a minimum of 2.9 x 10^4 ml^{-1} (4.5%) in February to 6.4 x 10^4 ml^{-1} (4.2%) in March reaching a maximum in May with 8.6 x 10^4 ml^{-1} (5.5%). In summer cell numbers gradually decreased to 6.8 x 10^4 ml^{-1} (4.2%) in June and 5.7 x 10^4 ml^{-1} (4.1%) in July remaining on almost the same level in October [(6.1 x 10^4 ml^{-1} (3.7%)] (Fig. 1, Table 1).

In 2009 counts of *Planctomycetes* reached the highest values in autumn [2.1 x 10^4 cells ml^{-1} (1.4%) and 1.6 x 10^4 cells ml^{-1} (1.5%) in October, 1.5 x 10^4 cells ml^{-1} (1.6%) in November] (Fig. S1a).

Abundances of *Planctomycetes* in size-fractionated samples and macroaggregates.

On six sampling days in May (4), June (1) and July (1), water samples were size-fractionated. The filtrate of a 10 µm pore-size filtration was either directly collected on a 0.2 µm filter (we refer to these filters as the 0.2-10 µm fractions) or subsequently filtered through 3 µm pore-size filters before final collection of cells on 0.2 µm pore-size filters (0.2-3 µm fractions).

143 The average cell numbers of *Planctomycetes* in the 0.2-10 µm fraction (6.7 x 10^4
144 ml^{-1}) were one order of magnitude higher compared to the 0.2-3 µm fraction (6.5 x 10^3 ml^{-}
145 ^{1}) (Fig. 2). Total cell numbers in the 0.2-3 µm fraction ranged between 4.0 x 10^5 ml^{-1} (May)
146 and 7.9 x 10^5 ml^{-1} (July). *Planctomycetes* cells in the 0.2-3 µm fraction ranged between 5.7
147 x 10^2 ml^{-1} to 1.0 x 10^4 ml^{-1} in May and were 1.3 x 10^4 ml^{-1} and 5.1 x 10^3 ml^{-1} in June and
148 July, respectively (Fig. 2).

149 Total cell numbers in the 0.2-10 µm fraction ranged between 1.3 x 10^6 ml^{-1} (May)
150 and 1.6 x 10^6 ml^{-1} (May and June). *Planctomycetes* numbers in the 0.2-10 µm fraction, at
151 the same sampling times, ranged between 5.6 x 10^4 ml^{-1} and 8.6 x 10^4 ml^{-1} in May and
152 reached 6.8 x 10^4 ml^{-1} and 5.7 x 10^4 ml^{-1} in June and July, respectively (Fig. 2). Subtracting
153 total picoplankton cell numbers of the 0.2-3 µm fractions from those of the respective 0.2-
154 10 µm fractions we calculated that the picoplankton cell numbers in the 3-10 µm fraction
155 ranged between 5.9 x 10^5 ml^{-1} and 1.0 x 10^6 ml^{-1}, indicating that attached picoplankton
156 outnumbers free-living picoplankton by about 1.5-fold.

157 Further evidence for an attached lifestyle of *Planctomycetes* was obtained by
158 examining three macroaggregates of mostly algal origin (i.e. *Polysiphonia violacea,*
159 *Bryopsis* sp.) retrieved by SCUBA diving on June 5th, 2007. Relative *Planctomycetes*
160 abundances on those macroaggregates were 6 %, 8% and 10%.

161

162 **Correlation of *Planctomycetes* with diatom counts.**

163 Based on a previous finding in coastal waters off Oregon (Morris *et al.*, 2006) the
164 correlation of *Planctomycetes* and diatom cells was further investigated. Counts of centric
165 (*Centrales*) and pennate (*Pennales*) diatoms between February and December 2007 were
166 provided by the long term monitoring program of the Biological Station Helgoland.
167 *Centrales* showed the lowest value in February (3.6 x 10^2 L^{-1}) starting to bloom in the

middle of April (3.0 x 10^6 L^{-1}) and reaching a pronounced peak at the beginning of May (3.8 x 10^6 L^{-1}) (Fig. S2). With respect to *Centrales* our picoplankton sampling covered the pre-bloom situation and the end of the bloom (Fig. S2). On the other hand, *Pennales* began to bloom at the end of August (7.1 x 10^5 L^{-1}) reaching a peak at the beginning of September (8.5 x 10^5 L^{-1}) but also showed a second peak in November (8.2 x 10^5 L^{-1}) (Fig. S2). The minimum value was registered in November (2.0 x 10^1 L^{-1}). None of our picoplankton samples were from a period in which the *Pennales* bloomed (Figs 1 and S2).

Considering our picoplankton samples collected in 2007 (n = 23) absolute abundances of *Planctomycetes* were significantly correlated with counts of *Centrales* (r_s = 0.54; P≤0.01), temperature (r_s = 0.60; P ≤ 0.01), salinity (r_s = -0.52; P ≤ 0.05), time (r_s = 0.67; P ≤ 0.001), NO_2^- (r_s = -0.56; P ≤ 0.01), NO_3^- (r_s = -0.60; P ≤ 0.01), and NH_4^+ (r_s = 0.50; P ≤ 0.05). No correlation with SiO_2, PO_4^{3-} and *Pennales* was found (Fig. S3, Table S2).

Clone libraries and phylogenetic affiliation of marine *Planctomycetes*.

Three clone libraries were constructed with the *Planctomycetes*-specific primer PLA46F and the universal primer 1392R. A total of 216 sequences (partial and almost full-length) were analyzed of which 184 were affiliated to *Planctomycetes*. One clone library was constructed from a winter sample [February-T7 (Table 1)]. From one summer sample [July-T22 (Table 1)] we constructed a library of the 3-10 µm fraction and a second from the 0.2-3 µm fraction.

Fig. 3 shows an overview consensus tree that summarizes the 16S rRNA-based phylogeny of 352 almost full-length sequences (longer than 1200 nucleotides) of which 154 were obtained in this study. One hundred and fifty *Planctomycetes* sequences grouped into four distinct phylogenetic clusters. These clusters were termed: *Pirellula*-related group

193 D, *Pirellula*-related group C, uncultured *Planctomycetes* group B and *Planctomyces*-
194 related group A. The arrangement of groups A-D remained stable in different types of tree
195 reconstructions (neighbor joining, maximum likelihood) with and without *Planctomycetes*
196 positional conservatory filters (50% and 30%).

197 The sequences most frequently retrieved from the February 2007 sample (total
198 picoplankton collected on 0.2 μm) was the *Planctomyces*-related group A (62% of the
199 *Planctomycetes* almost full length sequences in the clone library) (Fig. S4c). Only three
200 clones of the group were closely related to *Planctomyces maris* (~ 97% sequence identity),
201 the rest shared < 90% sequence identity with *Planctomyces maris* and < 89% sequence
202 identity with *Planctomyces brasiliensis.*

203 The 16S rRNA gene library made from the 0.2-3 μm fraction of July 2007 was
204 dominated by uncultured *Planctomycetes* group B (54% of the almost full length sequences
205 of *Planctomycetes* in the clone library) (Fig. S4b), whereas the 3-10 μm fraction of the
206 same surface water sample was dominated by *Pirellula*-related group D (54% of the
207 *Planctomycetes* almost full length sequences in the clone library) (Fig. S4a). The later
208 sequences share ~ 86% identity with *Rhodopirellula baltica,* ~ 88% identity with
209 *Blastopirellula marina* and ~ 89% identity with strains of the genus *Pirellula*.

210 The coverage of *Planctomycetes* diversity in February was 84 and 89% at species
211 (97% sequences identity) and genus levels (95% sequence identity), respectively, and 85
212 and 92% at species and genus levels in the 3-10 μm July library, respectively. Rarefaction
213 analysis of the July 0.2-3 μm library indicated coverage of 92 and 96% at species and
214 genus levels, respectively (Fig. S5).

215

216

217

Quantification of marine *Planctomycetes* clades.

Based on these results, three new specific oligonucleotide probes were designed for the three major clades identified in the clone libraries (Table S3): (i) probe PirD1039 targeted all sequences in the *Pirellula*-related group D; (ii) probe uPlaB440 is fully complementary to 32 of 40 sequences retrieved from the uncultured *Planctomycetes* group B, and (iii) probe PlaA1228 targeted a monophyletic clade within the *Planctomyces*-related group A which encompassed 30 of 39 sequences retrieved in this study (Fig. S4, Table S3). Initially, seven sampling days were chosen in different seasons of 2007 to quantify each clade by CARD-FISH (Fig. 4). This quantification was performed on the 0.2-10 µm fractions for the May, June and July samples to remove resuspended particles.

The number of cells hybridized with PirD1039 ranged from 5.1 x 10^3 ml^{-1} (0.6% of total picoplankton) in March to 2.0 x 10^4 ml^{-1} (1.3%) in May showing highest values in the late spring and summer compared to winter and autumn (Fig. 4). No cells hybridizing with probe PirD1039 were detected in the 0.2-3 µm fraction from July. Cells identified as *Pirellula*-related group D were, however, detected on all three macroaggregates in abundances ranging between 0.6% and 0.8%.

With probe uPlaB440 we could detect between 3.3 x 10^3 cells ml^{-1} (0.4%) in February and 2.3 x 10^4 cells ml^{-1} (1.4%) in October (Fig. 4). Since the majority of the uncultured *Planctomycetes* group B sequences were retrieved from the 0.2-3 µm fraction we also quantified this population in the respective size fractions of May, June and July. While in July the abundance was 5.1 x 10^3 cells ml^{-1}, in May and June no cells could be detected. The abundance of uPlaB440 counts in the July 0.2-10 µm fraction was 1.7 x 10^4 cells ml^{-1} indicated that also this clade prefers an attached lifestyle. uPlaB440 positive cells were also detected on one of the algal macroaggregates, though in low abundance (0.2%).

PlaA1228, designed for a cluster of sequences mainly retrieved from February, showed higher numbers in February [1.3 x 10^4 cells ml^{-1} (1.5%)] and October [3.1 x 10^4 cells ml^{-1} (1.9%)] compared with July [9.0 x 10^2 cells ml^{-1} (0.1%)] (Fig. 4). In May and June no probe positive cells could be detected. Furthermore, the abundance of this clade on the three macroaggregates was 0.1%, 0.3% and 0.5%.

Throughout the year 2007 the sum of counts with the three newly designed probes PlaA1228, uPlaB440 and PirD1039 accounted for 32% to 103% of the PLA46 counts (Fig. S6a). The coverage of *Planctomycetes* was complete in October 2007, and lowest in May 2007.

Furthermore, thirteen sampling days were analyzed throughout 2009 to quantify each clade. Each of the three clades of marine *Planctomycetes* was also detected in 2009. The different clades had their maxima at different time points. With the probe PirD1039 highest counts were obtained in the beginning and the end of the summer (e.g. 2.4 x 10^3 cells ml^{-1} or 0.2% in June) (Fig. S1a). uPlaB440 showed the highest number in September [5.7 x 10^3 cells ml^{-1} (0.4%)], lower abundances were registered in the other seasons (Fig. S1a). The number of cells hybridized with PlaA1228 were 6.0 x 10^3 ml^{-1} (0.5%) and 7.7 x 10^3 ml^{-1} (0.9%) in October and 5.8 x 10^3 ml^{-1} (0.8%) in November, showing lower abundances in the other sampling days (Fig. S1a). Throughout the samples of 2009 the sum of counts with the three clade-specific probes ranged between 12% and 93% of the PLA46 counts (Fig. S6b).

Discussion

Abundance and distribution.

In comparison with data previously reported for the Helgoland sampling site (Glöckner *et al.*, 1999) and for other marine waters (Schattenhofer *et al.*, 2009), relative abundances of *Planctomycetes* showed higher values in this study. This is likely due to the use of the more sensitive CARD-FISH protocol that results in enhanced fluorescence intensities and signal-to-background ratios (Pernthaler *et al.*, 2002). Furthermore, we improved the CARD-FISH protocol for a better identification of *Planctomycetes*. As originally suggested by Woebken and colleagues (Woebken *et al.*, 2007a) for anammox bacteria, achromopeptidase was used to specifically permeabilize the proteinaceous cell wall of *Planctomycetes* (König *et al*., 1984; Liesack *et al*., 1986).

Currently, PLA46 and PLA886 are the probes available for the quantification of *Planctomycetes*. In this study, PLA46 was used because it is more specific than PLA886 in terms of outgroup hits (Amann and Fuchs, 2008). PLA46 does not cover all *Planctomycetes*, but we confirmed *in silico* by a cross-check with the curated high-quality SSU database SILVA (Pruesse *et al.*, 2007) that the marine genera were all detected. We therefore assumed that PLA46 provided reliable counts of total *Planctomycetes* in our coastal marine system.

Planctomycetes abundances increased in absolute numbers in spring suggesting that they were bottom-up controlled and benefited from algal production. With respect to inorganic nutrients, *Planctomycetes* showed a negative correlation with nitrate and nitrite and a positive correlation with ammonium. The abundance of *Planctomycetes* was positively correlated with centric diatoms (C*entrales*). However, these apparent correlations were also a consequence of dependent behavior of these properties, as reflected by co-variation of most environmental variables in the samples (Fig. S3). Overall,

292 the most likely scenario, in our system, was that a bloom of *Centrales* resulted in an
293 increase of *Planctomycetes.* At that time nitrate and nitrite were depleted by the growing
294 algae. Our results provide further evidence for a direct interaction and carbon flow between
295 algae and *Planctomycetes* as suggested previously by Morris and coworkers (Morris *et al.*,
296 2006). The genome annotation of *Rhodopirellula baltica* SH 1^T suggested that it derives its
297 energy from degradation of sulfated polymers produced mainly by algae (carrageenans,
298 agars, sulfated fucans) and entrapped in macroaggregates (Glöckner *et al.*, 2003; Schlesner
299 *et al.*, 2004). The main storage carbohydrate in marine diatoms is the polysaccharide
300 chrysolaminarin which can contribute up to 80% of the organic carbon (Alderkamp *et al*.,
301 2006). While it is located intracellularly in live algae (Chiovitti *et al.*, 2004) it may be
302 released by algal cell lysis (Brussaard *et al*., 1995). All marine cultured *Planctomycetes* so
303 far seem to be specialized in sugar metabolism (Ward *et al.*, 2006; Woebken *et al.*, 2007b).
304 Even if to date there is no direct proof that marine strains use chrysolaminarin as a carbon
305 source the increase of *Planctomycetes* absolute numbers coinciding with the end of a
306 *Centrales* bloom, when most probably there is a massive algal lysis, suggests this
307 compound as a possible link between *Planctomycetes* and diatoms. This hypothesis
308 requires further investigation.

309 While Morris and coworkers showed a general coupling between diatoms and
310 *Planctomycetes*, evidences of the specific correlation between *Centrales* and
311 *Planctomycetes* abundances have not yet been reported, although the diatom images
312 provided by Morris and coworkers showed centric-like diatom cells. Since we did not
313 study a bloom of pennate diatoms which occurred in the late summer of 2007, we can not
314 exclude that *Planctomycetes* might benefit from these also, which is quite likely as the
315 cellular composition is similar in centric and pennate diatoms.

316 Between late spring and summer we compared *Planctomycetes* abundances of 0.2-3
317 µm and 3-10 µm fractions in water samples with the abundances in marine aggregate
318 samples. Our results showed that most of *Planctomycetes* are attached-living bacteria.
319 Indirect evidence for that life style was so far mostly coming from clone frequencies in
320 SSU libraries (DeLong *et al.*, 1993; Rath *et al.*, 1998; Crump *et al.*, 1999). In our study we
321 corroborated this by *in situ* hybridization techniques (Fig. S7). *Planctomycetes* are well
322 known for their ability to attach to surfaces by the excretion of holdfast substances (Ward
323 *et al*., 2006).
324
325 ***Planctomycetes* diversity and clades quantification.**
326 This study almost doubled the database size on almost full length 16S rRNA gene
327 sequences of marine *Planctomycetes*. Many new phylotypes were added. It was possible to
328 identify three stable coastal groups. These clades were present in winter and summer but
329 the clone frequencies varied between seasons (winter vs. summer) and samples (3-10 µm
330 vs. 0.2-3 µm fractions) (Fig. S4) suggesting a high variability of clades of *Planctomycetes*
331 in different size fractions and seasons.
332 Based on CARD-FISH quantification the *Pirellula*-related group D (PirD1039) was
333 more abundant in summer than in winter 2007 and 2009 (Figs S6a and S6b). Most of the
334 retrieved clones belonged to the 3-10 µm fraction, and they were closest related to
335 published sequences retrieved from marine sponges and the surface of marine macro-alga
336 (Fig. S4a) suggesting that this clade prefers an attached life style.
337 In the 2007 samples CARD-FISH counts of uncultured *Planctomycetes* group B
338 (uPlaB440) peaked in October (Fig. 4). This clade seems to be more abundant in the 3-10
339 µm fraction although it could be detected also in the 0.2-3 µm fraction. In contrast with
340 this result this clade contains clones mostly retrieved from the 0.2-3 µm fraction and most

341 closely related to sequences retrieved from seawater but also from marine sponges (Fig.
342 S4b). In 2009 uPlaB440 counts were highest in September and October (Fig. S6b), with a
343 seasonal pattern similar of that of the year 2007.

344 On the other hand, *Planctomyces*-related group A (PlaA1228) was more abundant
345 in winter and autumn and almost not present in summer of 2007 (Fig. 4). The clade
346 comprised sequences mainly retrieved from winter and closest related to sea water,
347 sediment and host (marine bivalve) gut (Fig. S4c). Also in 2009 the *Planctomyces*-related
348 group A was most abundant in autumn (Fig. S1a). The more finely resolved clade-specific
349 counts from 2009 provide a first hint to a correlation of *Planctomyces*-related group A and
350 the autumn bloom of *Pennales* (Figs S1a and S1b).

351 Overall, with the clade-specific probes, we were able to detect between 32 and
352 103% of the *Planctomycetes* community in 2007 and between 12 and 93% of the
353 *Planctomycetes* community in 2009. We conclude that this new set of probes enables the
354 quantification of a major fraction of the marine coastal *Planctomycetes*. It would be
355 interesting to apply this set of probes to other coastal sites and to test our hypotheses on a
356 correlation of *Planctomycetes* with particular groups of algae (i.e. pennate and centric
357 diatoms). At the long-term ecological research site "Kabeltonne", Helgoland, it should be
358 specifically checked whether the 2009 succession of *Pirellula*-related group D at the
359 beginning of the summer, followed by uncultured *Planctomycetes* group B towards the end
360 of the summer and finally the dominance of *Planctomycetes*-related group A in autumn is a
361 stable seasonal pattern.
362
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364
365

Experimental procedures

Sample collection, abiotic and biotic parameters.

Water samples were collected every workday at a depth of 1 m at the station "Kabeltonne" (54° 11.3' N, 7° 54.0' E), Helgoland Roads, North Sea by the research boat Aade. Overall, 23 samples sampled between February and October 2007 were analyzed. Marine macroaggregates were collected by SCUBA diving using open-ended syringes on June 5th, 2007 from the same sampling site. Furthermore, 13 samples were analyzed between February and November 2009.

Determination of salinity was performed using an inductive salinometer (GDT Autosal8400 Salinometer, Guildline, Ontario, Canada) followed by conversion to a salinity value using UNESCO tables (Cox, 1966; Grasshoff *et al.*, 1999). The concentration of silicate, phosphate, nitrate, nitrite and ammonium were measured photometrically as described previously (Grasshoff and Johannsen, 1974; Grasshoff *et al.*, 1999). For the enumeration of diatoms samples were preserved with Lugols' solution and counted using the Uthermöhl method in an inverted microscope (Wiltshire *et al.*, 2008) (Table S1).

Sample processing and CARD-FISH.

For CARD-FISH water samples were collected and fixed for 1 hour at room temperature and up to 24 hours at 4 °C with paraformaldehyde solution (20% w/v, Electron Microscopy Sciences, Hatfield, PA; final concentration 1%). Afterwards, 15 ml aliquots were filtered at low pressure (200 mbar) onto 0.2 µm polycarbonate filters (type GTTP; diameter, 47 mm; Millipore, Eschborn, Germany). In May, June and July, water samples were first passed through 10 µm polycarbonate filters (type TCTP; diameter, 47 mm; Millipore); the filtrate was filtered onto 3 µm (type TSTP; diameter, 47 mm; Millipore) and 0.2 µm (type GTTP; diameter, 47 mm; Millipore) filters in succession to collect the

391 biomass of attached and free-living prokaryotes. In addition, 15 ml aliquots were directly
392 filtered onto 0.2 µm filters for comparison. All filters were stored at -20 °C until further
393 processing. For 2009 samples, 100 ml aliquots were directly filtered onto 0.2 µm
394 polycarbonate filters (type GTTP; diameter, 47 mm; Millipore).

395 Macroaggregates were resuspended in sterile seawater, fixed (see above) and
396 sonicated on ice (21 W for 60 s, Sonopuls HD 60, Bandelin, Berlin, Germany). Aliquots of
397 15 ml were first prefiltered in 10 µm polycarbonate filter (type TCTP; diameter, 47 mm;
398 Millipore) to get rid of the algae and subsequently filtered onto 0.2 µm polycarbonate
399 filters (type GTTP; diameter, 47 mm; Millipore).

400 Prior to CARD-FISH, filters were cut in sections and embedded in 0.2% (w/v)
401 agarose (gel strength ≥ 300 g cm^{-2}, Biozym, Oldendorf, Germany) (Pernthaler *et al.*, 2002).
402 The efficiency of the permeabilization treatments of the *Planctomycetes* cell wall for
403 CARD-FISH was checked using different enzymes such as lysozyme, achromopeptidase,
404 and proteinase K. The highest numbers of *Planctomycetes* hybridized by CARD-FISH
405 were obtained after incubation with lysozyme (10 mg ml^{-1}; Fluka, Steinheim, Germany;
406 buffer contained 0.05 M EDTA, pH 8, 0.1 M Tris-HCl, pH 7.4) for 1 h at 37 °C followed
407 by achromopeptidase (60 U ml^{-1}; Sigma, Steinheim, Germany; buffer contained 0.01 M
408 NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37 °C.

409 Endogenous peroxidases were inactivated by using 0.01 M HCl at room
410 temperature for 10 min. Hybridization with horseradish peroxidase (HRP)-labeled
411 oligonucleotide probes (Table S3; http://Biomers.net, Ulm, Germany) and tyramide signal
412 amplification was carried out according to the protocol by Pernthaler and colleagues
413 (Pernthaler *et al.*, 2004) with the following modifications: hybridization was performed at
414 46 °C for 2.5 h and the washing step at 48 °C for 5 min. The probe-delivered horseradish
415 peroxidase was detected with tyramides labeled with fluorescein (Pernthaler *et al*., 2004).

The probe NON338 (Wallner *et al*., 1993) was used as a negative control. No false-positive signals were detected. After amplification, filter sections were washed twice in 96% ethanol before drying. Filter sections were then counterstained with DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 1 µg ml^{-1} and washed for 1 min in Milli-Q water and 96% EtOH. Filter sections were arranged on microscope slides, and embedded in an antifading reagent (VECTASHIELD® Mounting Medium H-1000, Vector Laboratories, Inc., Burlingame, CA, USA and Citifluor Ltd., London, UK). Slides were stored at -20 °C until further analyses. Cells were quantified with an Axioplan II Imaging epifluorescence microscope (Zeiss, Jena, Germany).

Rational for size fractionation in 2007.

In order to remove resuspended material from sediment surfaces, we decided to first prefilter water samples through 10 µm filters. Then, the water samples were filtered onto 3 µm and finally onto 0.2 µm filters to quantify *Planctomycetes* as free-living and attached-living to small algae (e.g. small diatoms) and to microaggregates such as transparent exopolymer particles (TEP). Ultimately, to collect all *Planctomycetes* for comparison, after prefiltration through 10 µm filters, we also filtered the water directly onto 0.2 µm filters. *Planctomycetes* abundances were always quantified on the 0.2 µm filters. This sampling strategy was chosen because it allowed us to easily quantify *Planctomycetes* since an accurate cell count directly on particles is difficult. Uneven the distribution of particles on filters and difficult visualization of *Planctomycetes* cells could lead to an under or overestimation of the real number of *Planctomycetes*. The same strategy was adopted for water samples to construct 16S rRNA gene clone libraries and for CARD-FISH. However, with this approach we unfortunately excluded *Planctomycetes* which colonize macroaggregates, so we consequently collected three different

441 macroaggregates, mostly of algal origin for evaluation. Since the quantification of bacteria
442 on such samples is also problematic we included a sonication step which facilitated their
443 detachment from the aggregate and allowed counting. On the other hand, we might
444 underestimate the number of *Planctomycetes* since this sonication step, not optimized for
445 the sample, could either destroy cells but most probably do not detach them quantitatively
446 from the aggregate.
447
448 **DNA isolation and PCR amplification.**
449 For DNA isolation, approximately 1 L of water was filtered onto 0.2 µm
450 polycarbonate filters (type GTTP; diameter, 47 mm; Millipore), under low pressure (200
451 mbar - 1 bar), to collect the total microbial biomass. Particularly in May, June and July,
452 water samples were first prefiltered through 10 µm polycarbonate filters (type TCTP;
453 diameter, 47 mm; Millipore); the filtrate was filtered onto 3 µm (type TSTP; diameter, 47
454 mm; Millipore) and 0.2 µm (type GTTP; diameter, 47 mm; Millipore) filters in succession.
455 Total nucleic acids were isolated according to the protocol of Zhou and colleagues
456 (Zhou *et al.*, 1996). The following method modifications were carried out: (i) cut sections
457 of polycarbonate filters were used as samples, (ii) centrifugation steps were performed at
458 53,250 x g beside the one done to recover the aqueous phase after the mixing with
459 chloroform/isoamylalcohol that was performed at 10,000 x g, (iii) the precipitation of DNA
460 with isopropanol was done overnight at room temperature, (iv) the pellet of crude nucleic
461 acids was resuspended in 1:10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8). A long
462 time period of DNA precipitation using isopropanol and an increased speed of
463 centrifugation steps were necessary to retrieve sufficient amounts of DNA.
464 A fragment of about 1.4 kb in length -encompassing most of the 16S rRNA gene-
465 was amplified using 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 1392R [5'-ACG GGC GGT GTG TAC-3', targeting the *E. coli* position 1392-1406 on 16S rRNA gene; (Xu *et al.*, 1998)] a general primer for *Bacteria*. Each PCR reaction mixture with a volume of 50 µl contained: 0.3 mg ml^{-1} BSA (final concentration; Sigma Chemie, Taufkirchen, Germany), 1 x reaction buffer (final concentration; Eppendorf, Hamburg, Germany), 0.25 mM dNTPs (final concentration; Roche Diagnostics, Mannheim, Germany), 0.5 µM each primer (final concentration; http://Biomers.net, Ulm, Germany), 0.02 U $µl^{-1}$ Taq Polymerase (final concentration; Eppendorf, Hamburg, Germany) and 1 µl of DNA. PCR cycling conditions were as follows: 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, and 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 and 10 replicates were processed in parallel to minimize PCR biases (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). PCR products were determined with 1% agarose gel electrophoresis (Biozym LE agarose, Biozym, Oldendorf, Germany).

Cloning, sequencing and phylogenetic analysis.

To avoid cloning of non-specific and small PCR products, fragments (~ 1.4 kb) were excised from a 1.5% agarose gel and subsequently extracted and purified using Qiaquick Gel extraction kit (QIAGEN, Hilden, Germany) prior to cloning them in P-GEM T-Easy (Promega, Madison, USA) according to the manufacturer's instructions. The recombinant vectors were then transformed into chemically competent *E. coli* cells (Invitrogen, Gröningen, Netherlands), plated and picked. Plasmids DNA were isolated with the Montage™ Plasmid Miniprep Kit (Millipore, Eschborn, Germany) and then sequenced using the two vector primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-

491 CAG GAA ACA GCT ATG AC-3') together with GM1F [5′-CCA GCA GCC GCG GTA
492 AT-3'; (Lane, 1991)] to obtain almost full length sequences. The 16S rRNA gene
493 sequences of this study were deposited in the EMBL Nucleotide Sequence Database with
494 the accession numbers FN822075- FN822228.

495 Contigs were assembled with the software SEQUENCHER Version 4.6 (Gene
496 Codes Corporation, Ann Harbor, USA) resulting in sequences encompassing almost full
497 length 16S rRNA genes. The sequences were aligned in the ARB software package
498 [http://www.arb-home.de; (Ludwig *et al.*, 2004)] using the SINA_aligner (Pruesse *et al.*,
499 2007). Alignments were further refined manually by comparison of closest relatives. For
500 in-depth phylogenetic rRNA analysis only sequences with more than 1200 nucleotides
501 were included. Phylogenetic tree reconstruction was based on distance matrix (DM) (e.g.
502 neighbor-joining) and maximum likelihood (ML) methods with and without 30% and 50%
503 *Planctomycetes* positional conservatory filters. Subsequently, a consensus tree was
504 constructed (Peplies *et al.*, 2008).

505

506 **Rarefaction analysis.**

507 Diversity coverage by the sequenced clones was analyzed using the computer
508 program DOTUR [Distance-Based OTU and Richness (Schloss and Handelsman, 2005)
509 http://schloss.micro.umass.edu/software/dotur.html]. Operational taxonomic units (OTUs)
510 were calculated using the furthest neighbor algorithm for each distance level, from a
511 distance matrix generated in ARB. Then, 97% and 95% sequences identity were selected to
512 generate the rarefaction curves. Coverage index of the clone libraries was calculated with
513 the formula C = (1-*n*/N)*100, where N is the numbers of all clones with a *Planctomycetes*
514 insert and *n* the number of phylotypes at 97% and 95% identity which appeared only once
515 in the library (Good, 1953; Kemp and Aller, 2004).

Probe design.

Oligonucleotide probes specific for clusters of *Planctomycetes* were designed based on the newly created phylogenetic tree using the probe design tool of the ARB software. Probe specificities were checked *in silico* against the SILVA 16S rRNA database [version 100; (Pruesse *et al.*, 2007)] (Table S4). To optimize the stringency conditions, a series of hybridizations at increasing formamide concentrations (10–70%) were evaluated. The optimal formamide concentration was the highest concentration before signal intensity decreased (Table S3). Furthermore, upon CARD-FISH, *Planctomycetes* showed a peculiar doughnut-shaped morphology in fluorescent mode (Fig. S7) that allowed us to verify the specificity of the newly designed probes *in situ*.

Statistics.

Multiple linear correlations between *Planctomycetes* abundance and abiotic (temperature, salinity, time, dissolved silicate, phosphate, nitrate, nitrite, ammonium) and biotic (centric and pennate diatoms) parameters were calculated with Spearman rank correlation test using SigmaStat 3.5 (Systat software, Santa Clara, CA, USA). The significance level of the correlations was also adjusted by the Bonferroni correction for multiple comparisons, according to which the *P*-value is divided by the total number of simultaneous independent comparisons (Legendre and Legendre, 1998).

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Figure and Table legends

Fig. 1: Environmental parameters, picoplankton, *Planctomycetes* (PLA46) and diatom (*Centrales* and *Pennales*) absolute counts. For microbial and *Planctomycetes* abundances means ± SD of three replicates are shown. Twenty three sampling days analyzed through the year 2007.

Fig. 2: Absolute abundances of *Planctomycetes* in 0.2-10 µm and 0.2-3 µm fractions at the six sampling days between late spring and summer. Means ± SD of three replicates.

Fig. 3: Phylogenetic tree based on almost full length 16S rRNA sequences (> 1200 nucleotides) showing the affiliations of different clusters. The consensus tree was built based on maximum likelihood and neighbor joining trees calculated without and with 50% and 30% *Planctomycetes* positional conservatory filters using *Verrucomicrobia, Lentisphaerae, Chlamydiae* and *Candidatus* OP3 sequences as outgroup. Sequences retrieved in this study are pointed out by a color code (July_3-10 µm fraction: red; July_0.2-3 µm fraction: blue; Febr._0.2 µm: green). The numbers in the boxes indicate the number of sequences retrieved in this study for each sample. The bar represents 10% sequence divergence.

Fig. 4: Absolute abundances of *Planctomycetes* (PLA46), *Pirellula*-related group D (PirD1039), uncultured *Planctomycetes* group B (uPlaB440), *Planctomyces*-related group A (PlaA1228) in seven sampling dates through the year 2007. *: not detected. Means ± SD of three replicates.

Table 1: *Planctomycetes* relative abundances and total microbial abundances in twenty three sampling days through the year 2007. Means ± SD of three replicates.

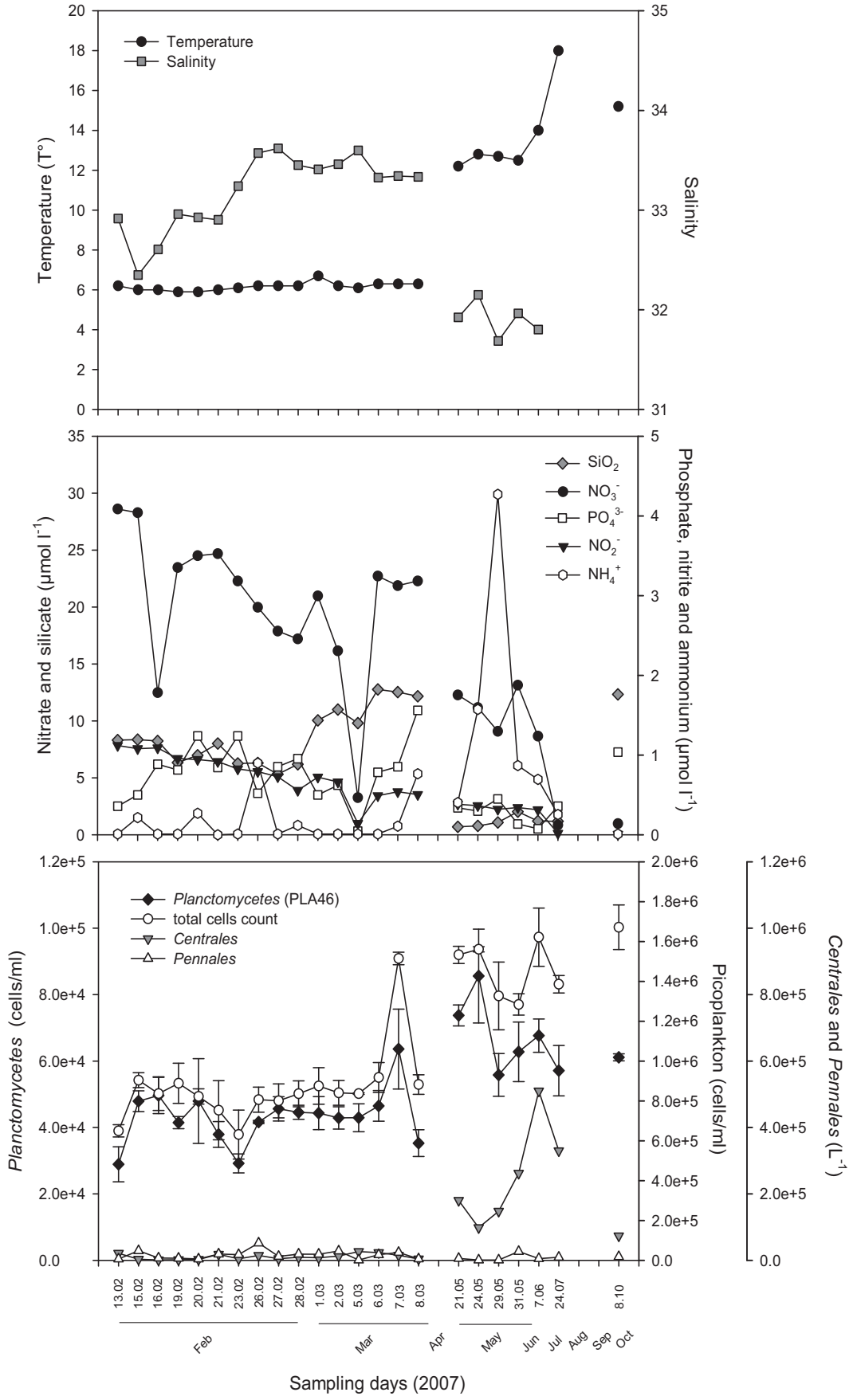
Temperature
Salinity
Temperature (T°)
Salinity
Nitrate and silicate (µmol l-1)
Phosphate, nitrite and ammonium (µmol l-1)
SiO2
NO3-
PO43-
NO2-
NH4+
Planctomycetes (PLA46)
total cells count
Centrales
Pennales
Planctomycetes (cells/ml)
Picoplankton (cells/ml)
Centrales and Pennales (L-1)
13.02
15.02
16.02
19.02
20.02
21.02
23.02
26.02
27.02
28.02
1.03
2.03
5.03
6.03
7.03
8.03
21.05
24.05
29.05
31.05
7.06
24.07
8.10
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Sampling days (2007)

Fig. 1

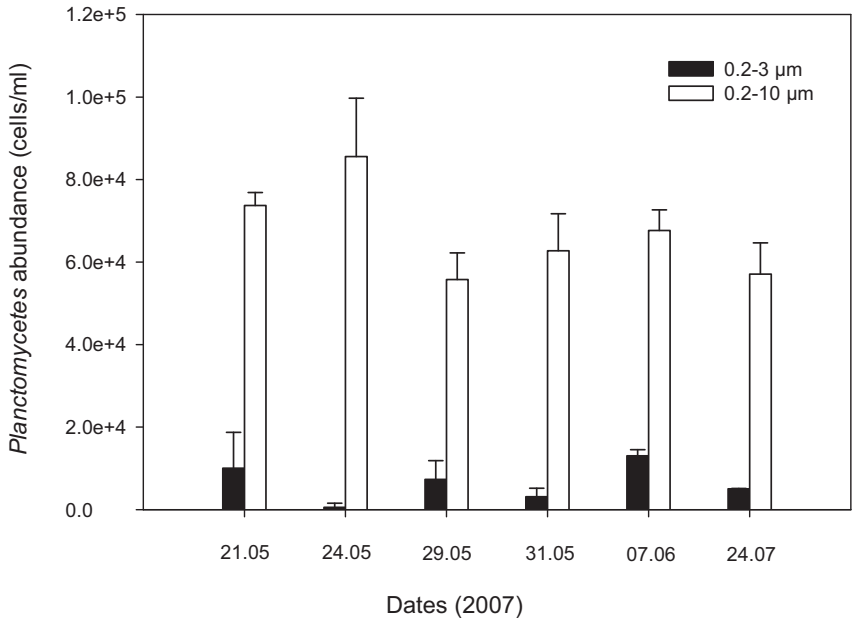
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Planctomycetes abundance (cells/ml)
0.2-3 µm
0.2-10 µm
21.05
24.05
29.05
31.05
07.06
24.07
Dates (2007)

Fig. 2

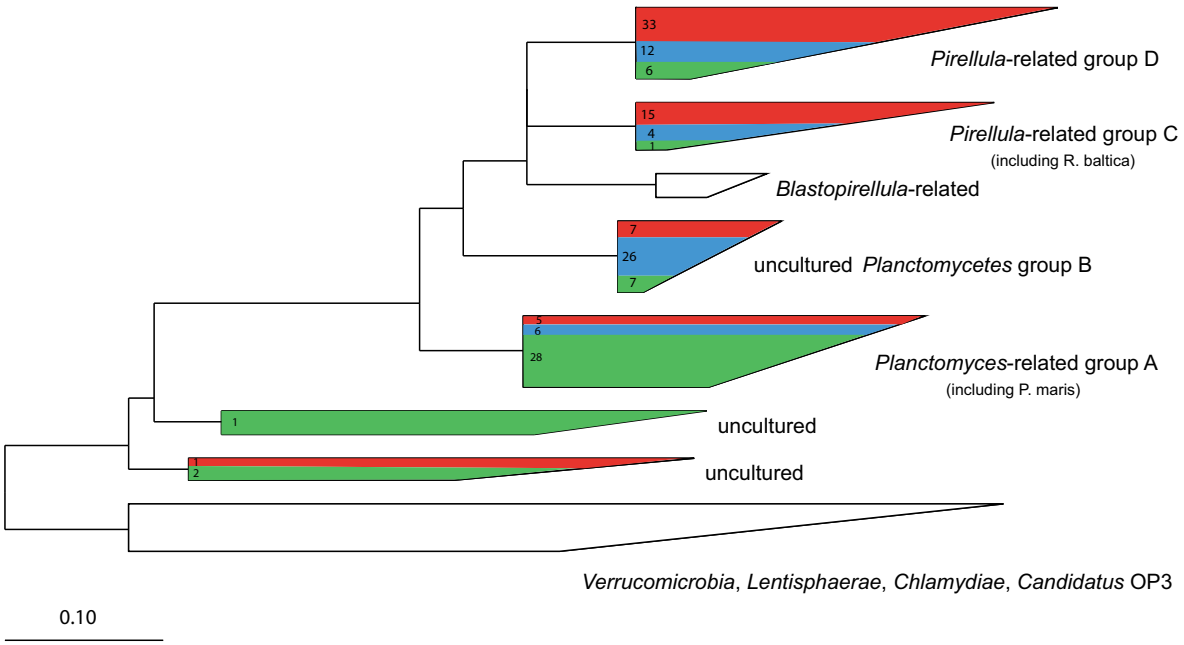
33
12
6
Pirellula-related group D
15
4
1
Pirellula-related group C
(including R. baltica)
Blastopirellula-related
7
26
7
uncultured *Planctomycetes* group B
5
6
28
Planctomyces-related group A
(including P. maris)
1
uncultured
1
2
uncultured
Verrucomicrobia, *Lentisphaerae*, *Chlamydiae*, *Candidatus* OP3
0.10

Fig. 3

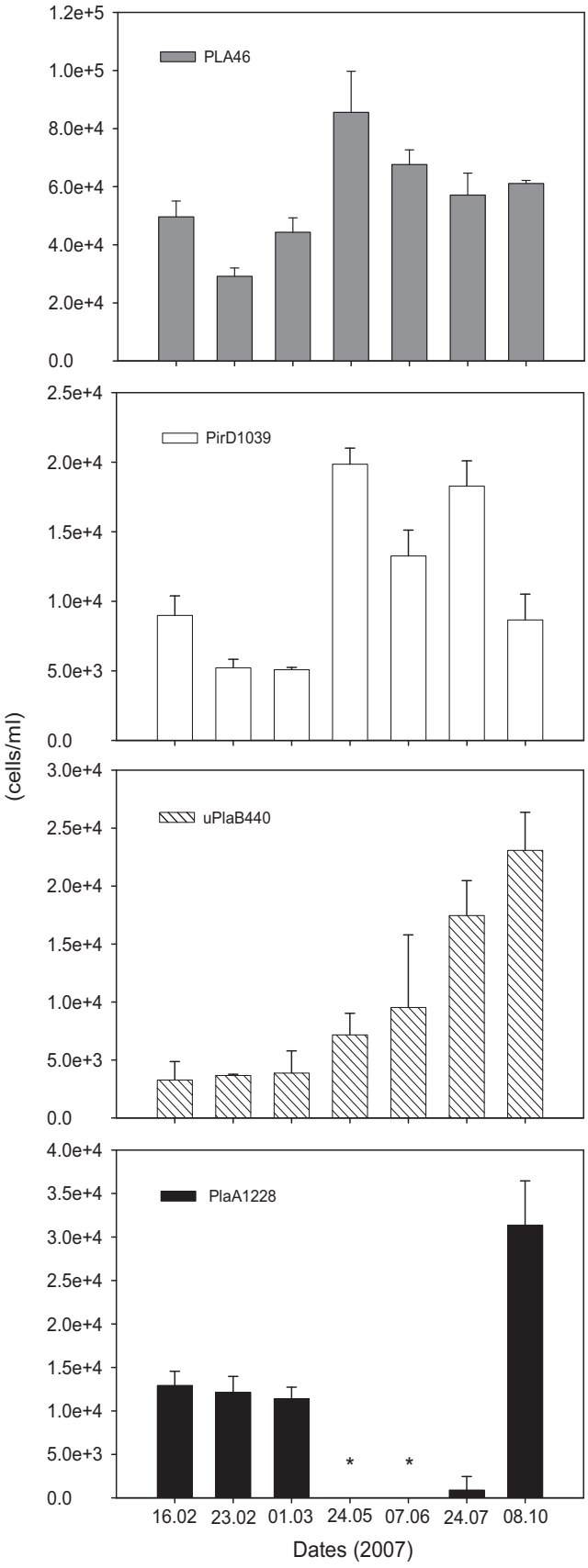
PLA46
PirD1039
uPlaB440
PlaA1228
(cells/ml)
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
2.5e+4
1.5e+4
1.0e+4
5.0e+3
3.0e+4
3.5e+4
*
16.02
23.02
01.03
24.05
07.06
24.07
08.10
Dates (2007)

Fig. 4

Table 1

Sample number	Date 2007	*Planctomycetes* relative abundance [%]	Total cell abundance [$x10^6$ cells ml^{-1}]
T1	13.2.2007	4.5 ± 0.8	0.65 ± 0.03
T2	15.2.2007	5.3 ± 0.3	0.90 ± 0.04
T3	16.2.2007	5.9 ± 0.7	0.84 ± 0.09
T4	19.2.2007	4.7 ± 0.2	0.89 ± 0.1
T5	20.2.2007	5.8 ± 1.6	0.82 ± 0.04
T6	21.2.2007	5.0 ± 0.5	0.75 ± 0.1
T7	23.2.2007	4.6 ± 0.5	0.63 ± 0.1
T8	26.2.2007	5.2 ± 0.1	0.81 ± 0.06
T9	27.2.2007	5.7 ± 0.5	0.80 ± 0.09
T10	28.2.2007	5.3 ± 0.3	0.84 ± 0.07
T11	1.3.2007	5.1 ± 0.6	0.88 ± 0.09
T12	2.3.2007	5.1 ± 0.4	0.84 ± 0.06
T13	5.3.2007	5.1 ± 0.5	0.84 ± 0.004
T14	6.3.2007	5.1 ± 0.5	0.92 ± 0.08
T15	7.3.2007	4.2 ± 0.8	1.5 ± 0.03
T16	8.3.2007	4.0 ± 0.5	0.88 ± 0.05
T17	21.5.2007	4.8 ± 0.2	1.5 ± 0.04
T18	24.5.2007	5.5 ± 0.9	1.6 ± 0.01
T19	29.5.2007	4.2 ± 0.5	1.3 ± 0.2
T20	31.5.2007	4.9 ± 0.7	1.3 ± 0.05
T21	7.6.2007	4.2 ± 0.3	1.6 ± 0.1
T22	24.7.2007	4.1 ± 0.5	1.4 ± 0.04
T23	8.10.2007	3.7 ± 0.1	1.7 ± 0.1

Supporting information

Fig. S1: (a) Counts of *Planctomycetes* (PLA46), *Pirellula*-related group D (PirD1039), uncultured *Planctomycetes* group B (uPlaB440), *Planctomyces*-related group A (PlaA1228) in thirteen sampling dates through the year 2009. (b) Chlorophyll a concentration, centric diatom (*Centrales*), pennate diatom (*Pennales*) and Flagellates counts between December 2008 and December 2009.

Fig. S2: Counts of *Centrales* and *Pennales* between February and December 2007. *: our samples.

Fig. S3: Correlations between *Planctomycetes* absolute abundance and abiotic [temperature (Temp.), salinity (Sal.), time, dissolved silicate, phosphate, nitrate, nitrite, ammonium] and biotic (*Centrales* and *Pennales*) parameters measured at all twenty three sampling dates between February and October 2007. Only significant Spearman's correlations at the level $P \leq 0.05$ (thin lines) and at the Bonferroni-corrected level $P \leq 0.0009$ [(0.05/55)-thick lines)] are shown. Thin and large connecting lines also represent significant correlations with coefficient < 0.66 and ≥ 0.66, respectively. Dashed and continuous lines represent negative and positive coefficient, respectively. The size of the dots is proportional to the number of significant correlations that a variable has with other parameters.

Fig. S4: Detailed consensus tree based on almost full length 16S rRNA sequences (> 1200 nucleotides) showing the grouping of *Planctomycetes* sequences retrieved in this study. Please note that the three trees are the same and correspond to the Fig. 3. Sequences are pointed out by a color code (July_3-10 µm fraction (AF): red; July_0.2-3 µm fraction (FLF): blue; Febr._0.2 µm (TF): green; type strains: violet). Accession numbers and sequence length are stated in parentheses. In grey boxes sequences targeted by newly designed probes at 0 weighted mismatches are

highlighted. (a) Sequences belonging to *Pirellula*-related group D (PirD1039), (b) sequences belonging to uncultured *Planctomycetes* group B (uPlaB440), (c) sequences belonging to *Planctomyces*-related group A (PlaA1228) are shown.

Fig. S5: Rarefaction curves of almost full length 16S rRNA sequences (> 1200 nucleotides) recovered from February_0.2 µm (T7) and July_0.2-3 µm and 3-10 µm fractions (T22), respectively. The expected numbers of OTUs was calculated using cut-off values of sequence identity for species (97%) and genus (95%) levels.

Fig. S6: Coverage of *Planctomycetes* by clade-specific probes in 2007 (a) and 2009 (b).

Fig. S7: Epifluorescence micrographs of bacteria from Helgoland water column samples, from March (c), October (a, e, f), and the 0.2-10 µm fraction of May (b) and July (d). Blue, DAPI signals of DNA containing cells; green, signals of catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)-positive cells using the probes: (a), (b), (c): PLA46, (d): PirD1039, (e): uPlaB440, (f): PlaA1228. (b) and (c): *Planctomycetes* and phytoplankton cells. Bar = 5µm.

Table S1: Abiotic and biotic parameters in twenty three sampling days through the year 2007.

Table S2: Spearman rank correlation between *Planctomycetes* absolute abundance and abiotic and biotic parameters measured at all twenty three sampling dates through the year 2007. *Pennales* are not shown since they are not correlated to any parameter. Temp: Temperature; Sal: Salinity. *: $P \leq 0.05$; **: $P \leq 0.01$. In bold statistically significant r_s at the Bonferroni-corrected level $P \leq 0.0009$. n=55 being the number of simultaneous independent comparisons.

Table S3: *Planctomycetes* specific oligonucleotide probes.

Table S4: Specificity of probes: PirD1039 (*Pirellula*-related group D), uPlaB440 (uncultured *Planctomycetes* group B) and PlaA1228 (*Planctomyces*-related group

A). Dots indicate full identity to the probe target sequence. Upper-case letters indicate nucleotide changes leading to strong mismatches; lower-case letters indicate changes leading to weak mismatches. The isolation source and the weighted mismatches of non-target organisms are represented. R (A/G), D (A/G/U), S (C/G), K (G/U), B (C/G/U), V (A/C/G), Y (C/U), W (A/U).

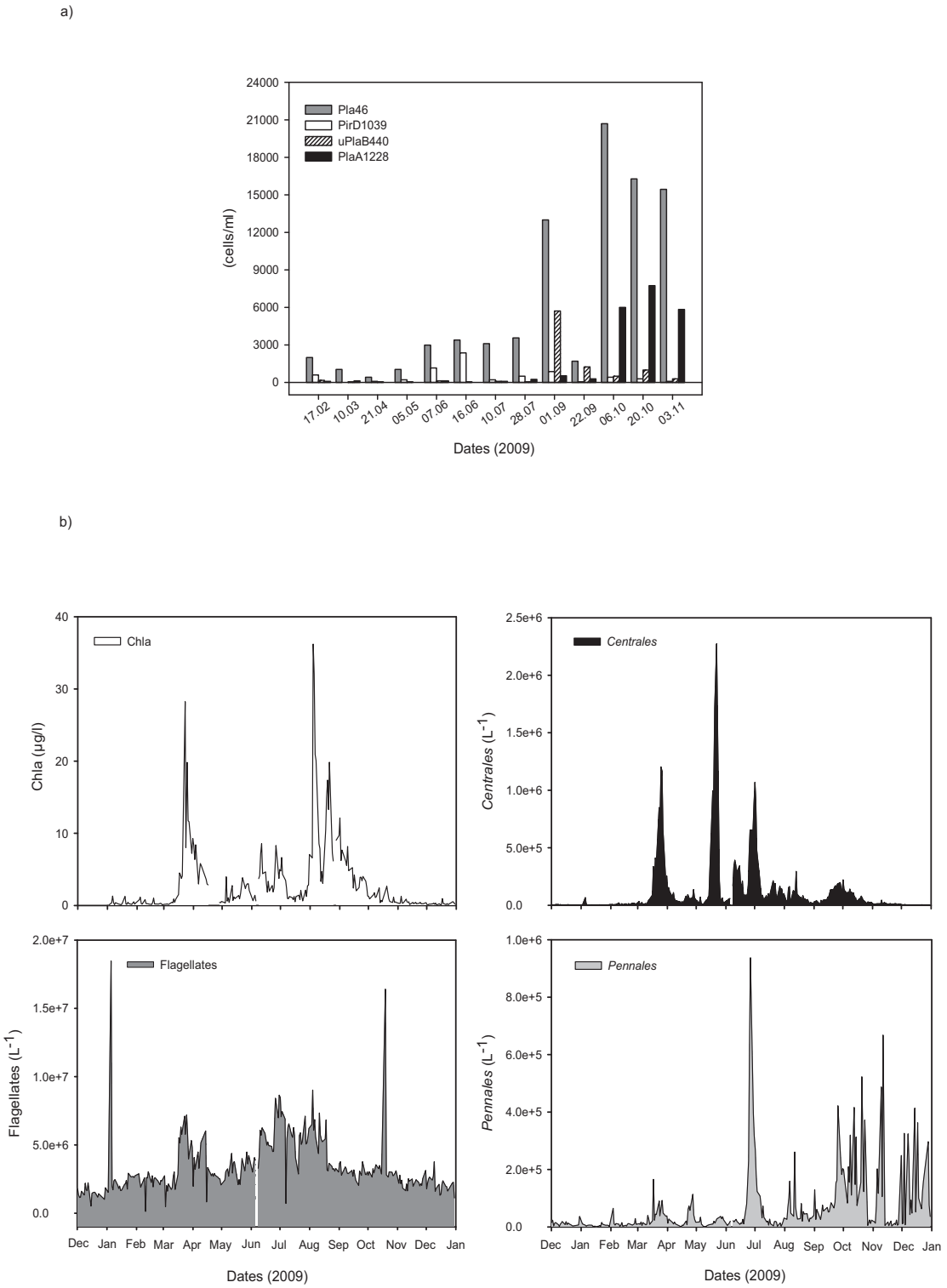
a)
Pla46
PirD1039
uPlaB440
PlaA1228
(cells/ml)
Dates (2009)
b)
Chla
Chla (µg/l)
Centrales
Centrales (L-1)
Flagellates
Flagellates (L-1)
Pennales
Pennales (L-1)
Dates (2009)

Fig. S1

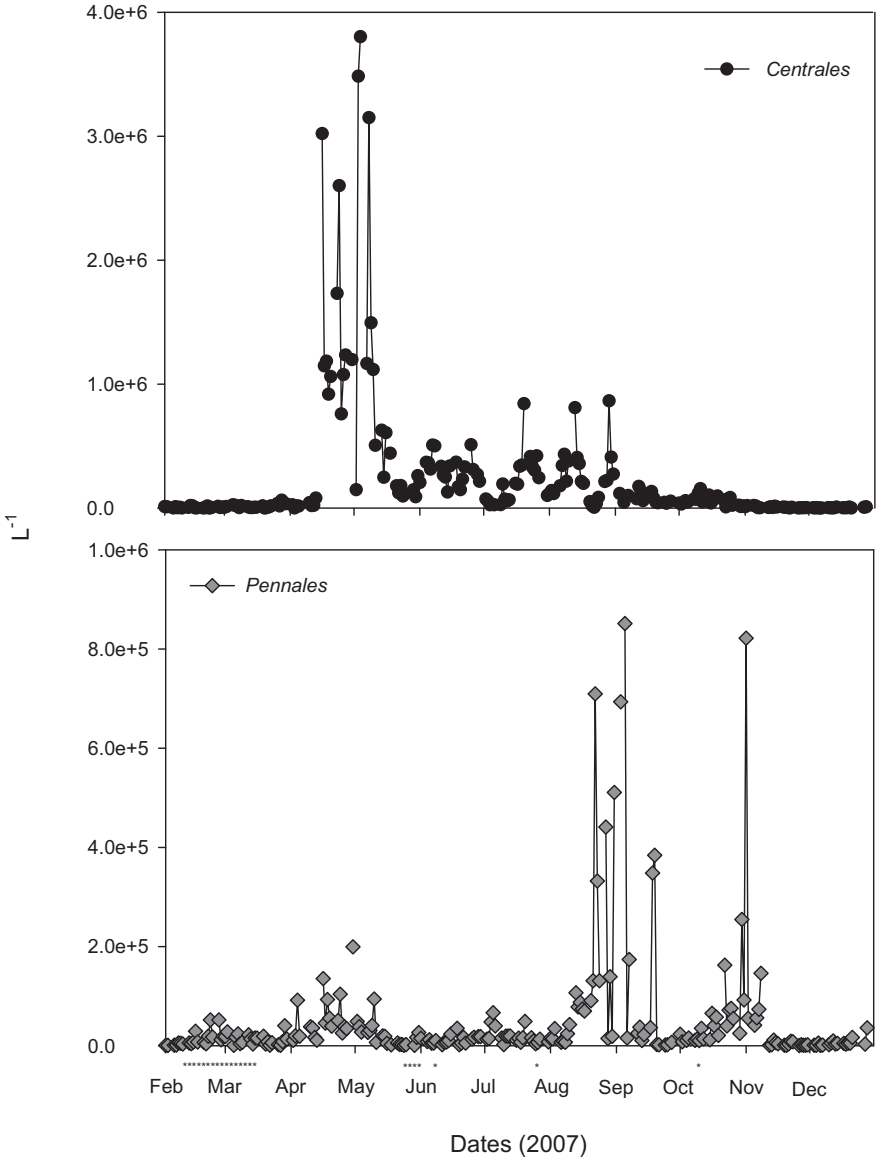
4.0e+6
3.0e+6
2.0e+6
1.0e+6
0.0
Centrales
L-1
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
Pennales
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Dates (2007)

Fig. S2

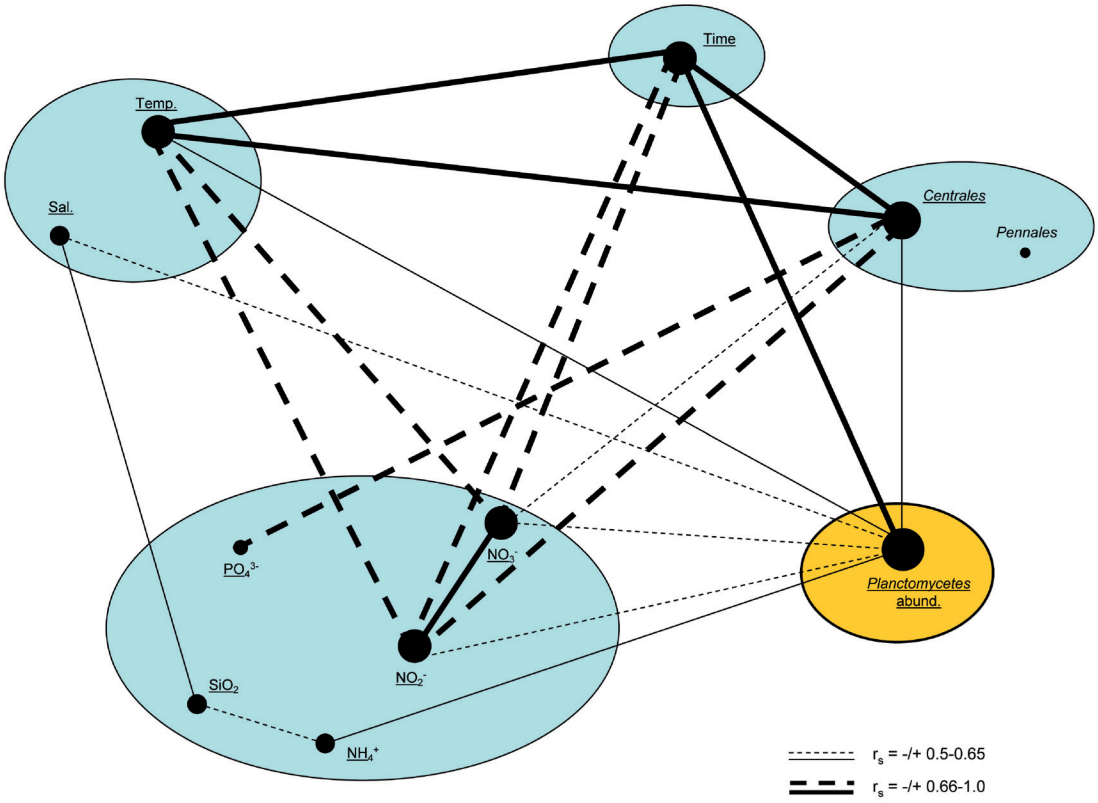
Time
Temp.
Sal.
Centrales
Pennales
PO_4^{3-}
NO_3^-
Planctomycetes abund.
NO_2^-
SiO_2
NH_4^+
r_s = -/+ 0.5-0.65
r_s = -/+ 0.66-1.0

Fig. S3

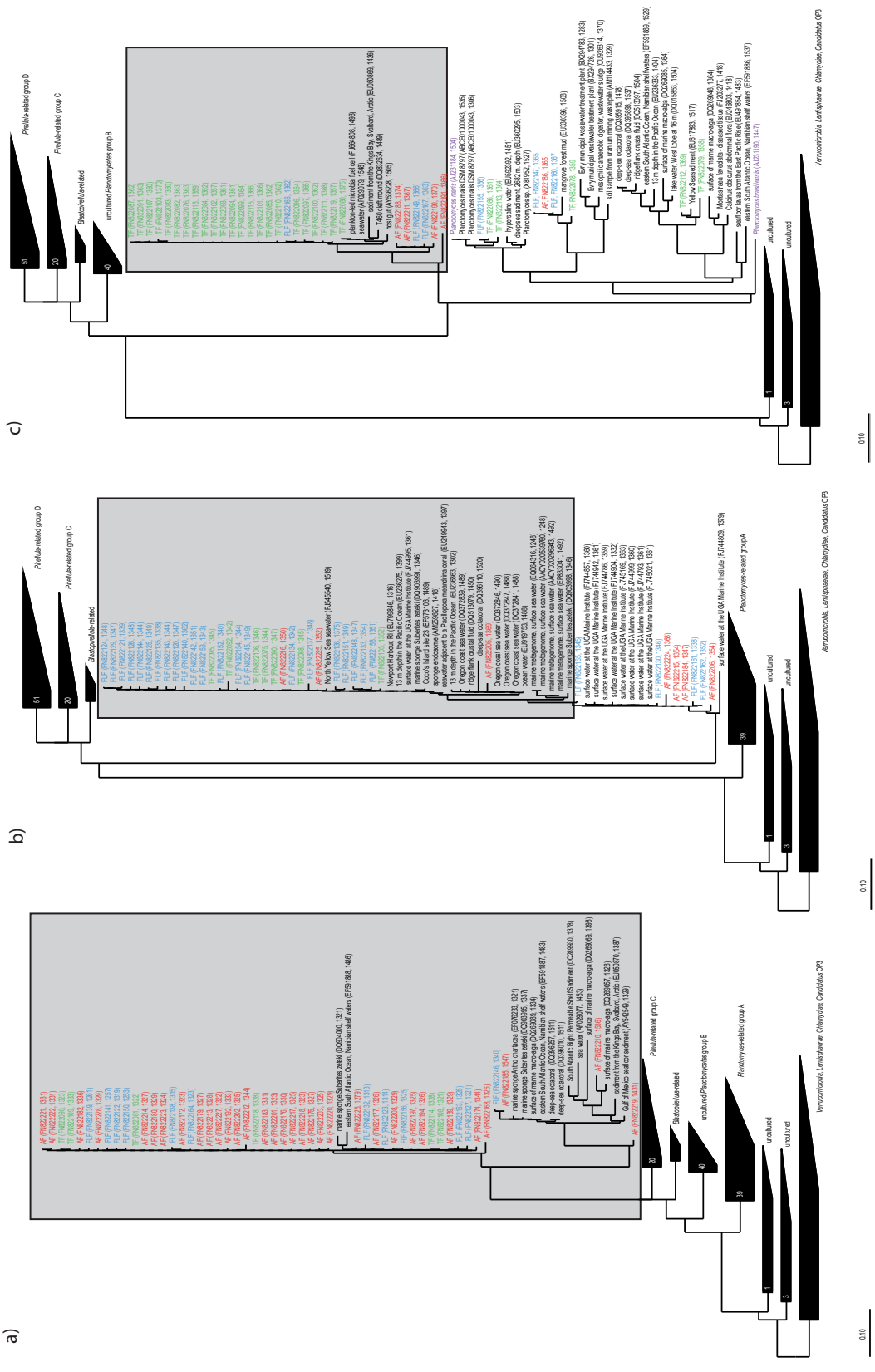
a)
b)
c)
Pirellula-related group D
Pirellula-related group C
Blastopirellula-related
uncultured Planctomycetes group B
Planctomycetes-related group A
uncultured Planctomycetes group B
Verrucomicrobia, Lentisphaerae, Chlamydiae, Candidatus OP3
0.10

Fig. S4

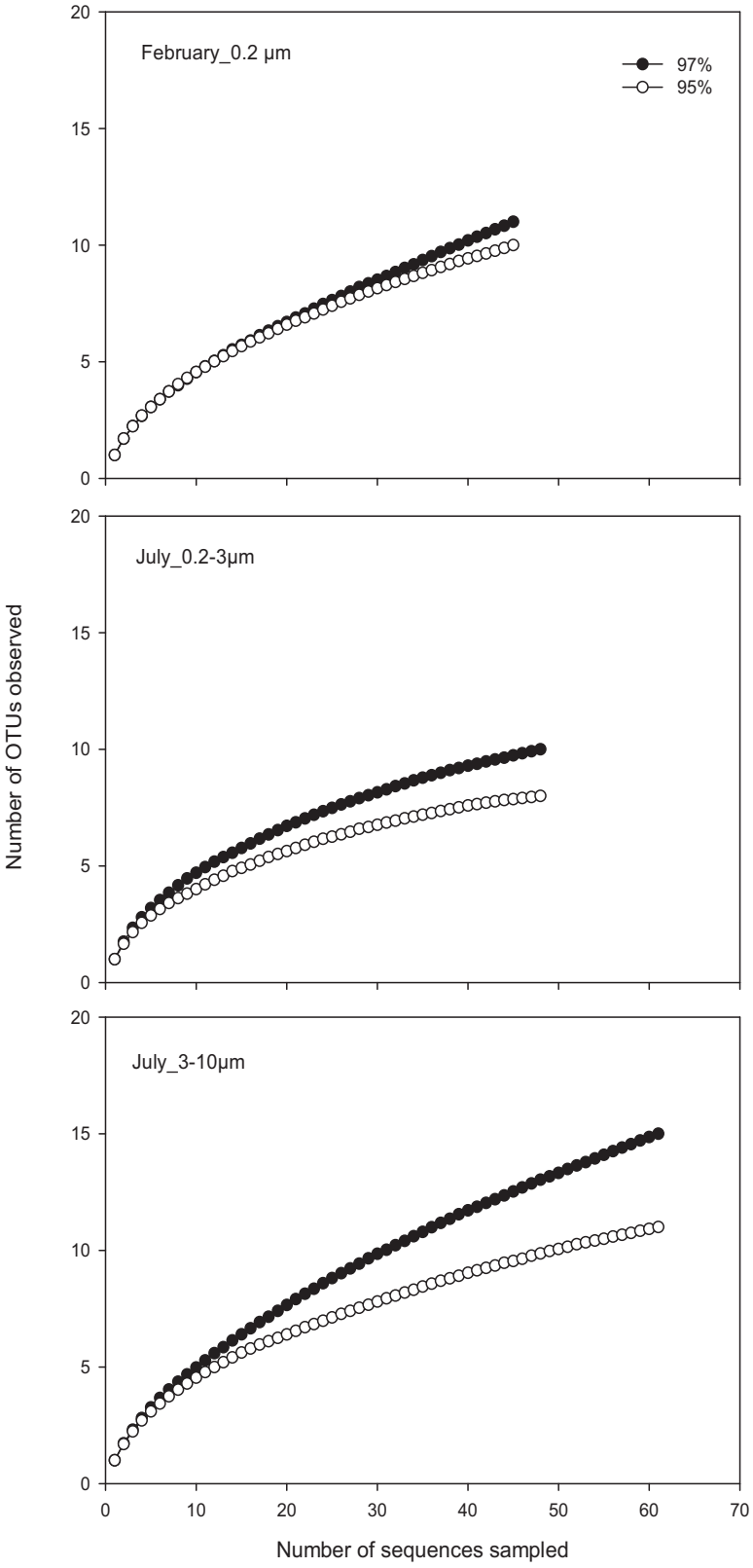
February_0.2 µm
97%
95%
July_0.2-3µm
July_3-10µm
Number of OTUs observed
Number of sequences sampled
0
5
10
15
20
0
10
20
30
40
50
60
70

Fig. S5

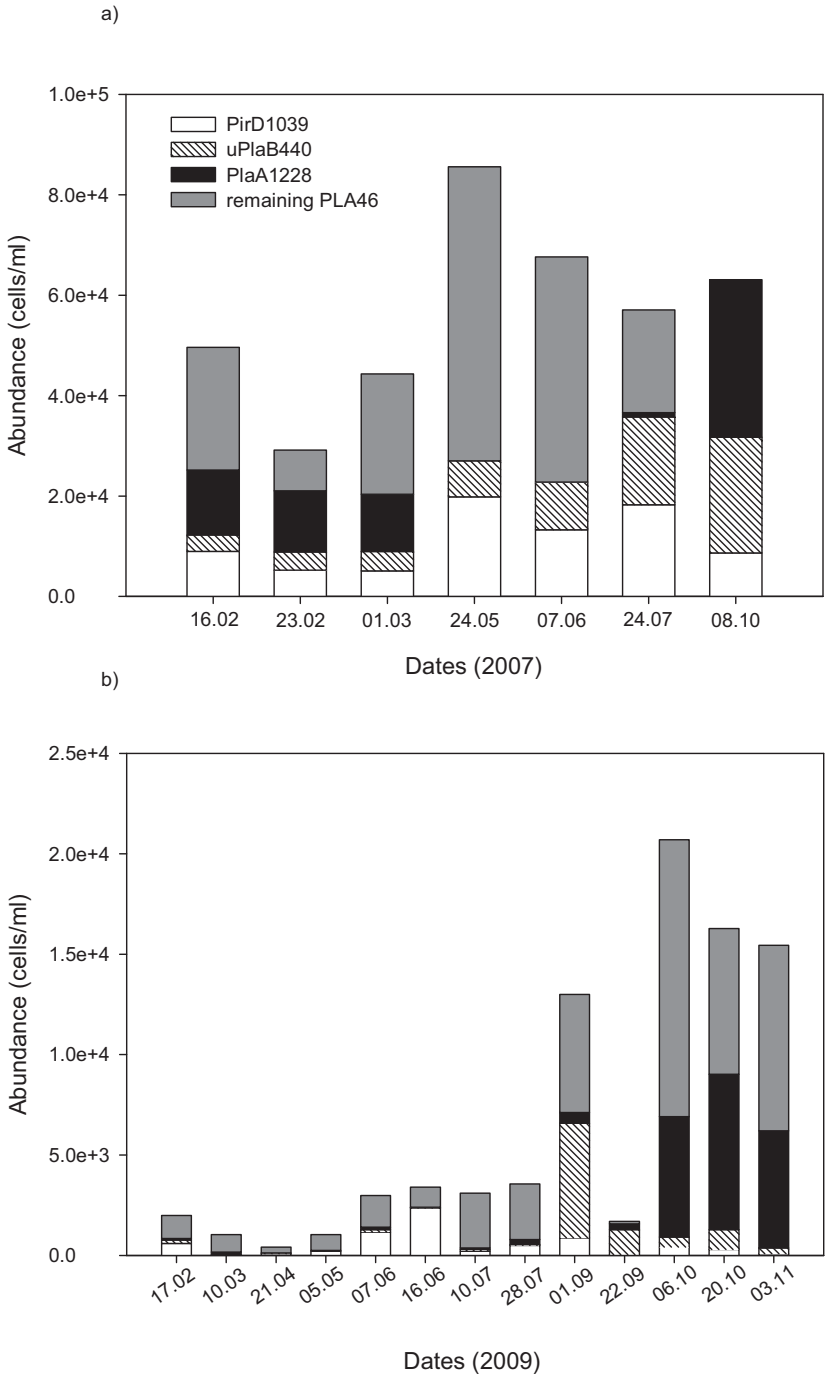
a)
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Abundance (cells/ml)
PirD1039
uPlaB440
PlaA1228
remaining PLA46
16.02
23.02
01.03
24.05
07.06
24.07
08.10
Dates (2007)
b)
2.5e+4
2.0e+4
1.5e+4
1.0e+4
5.0e+3
0.0
Abundance (cells/ml)
17.02
10.03
21.04
05.05
07.06
16.06
10.07
28.07
01.09
22.09
06.10
20.10
03.11
Dates (2009)

Fig. S6

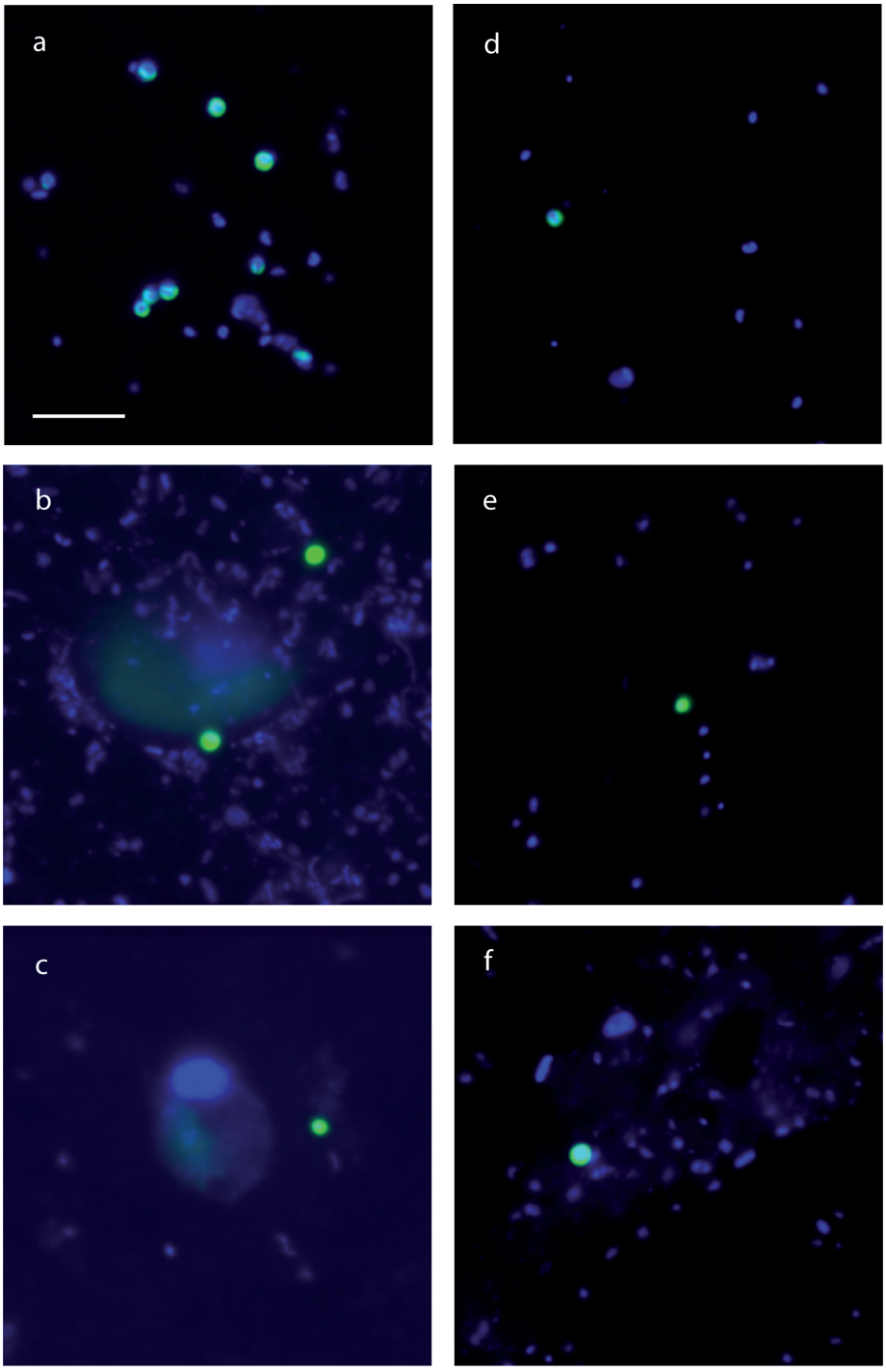

Fig. S7

Table S1

Date	Temp (°C)	Sal	SiO_2 (µmol l^{-1})	PO_4^{3-} (µmol l^{-1})	NO_2^- (µmol l^{-1})	NO_3^- (µmol l^{-1})	NH_4^+ (µmol l^{-1})	*Centrales* (x 10^4 L^{-1})	*Pennales* (x 10^4 L^{-1})
13.02.07	6.2	32.916	8.31	0.36	1.12	28.62	≤0.01	2.1	0.4
15.02.07	6.0	32.349	8.35	0.50	1.08	28.30	0.22	0.3	2.9
16.02.07	6.0	32.606	8.23	0.88	1.09	12.49	≤0.01	0.1	0.7
19.02.07	5.9	32.959	6.34	0.81	0.96	23.47	≤0.01	0.2	0.6
20.02.07	5.9	32.928	6.97	1.24	0.94	24.52	0.27	0.3	0.4
21.02.07	6.0	32.903	8.02	0.84	0.92	24.70	0.00	1.8	1.9
23.02.07	6.1	33.241	6.26	1.24	0.82	22.29	≤0.01	0.5	1.7
26.02.07	6.2	33.574	6.31	0.52	0.79	19.99	0.90	1.5	5.2
27.02.07	6.2	33.620	5.31	0.85	0.73	17.90	≤0.01	0.5	1.2
28.02.07	6.2	33.452	6.19	0.96	0.56	17.20	0.12	0.9	1.9
01.03.07	6.7	33.409	10.05	0.50	0.72	20.99	≤0.01	0.8	1.8
02.03.07	6.2	33.461	11.00	0.62	0.66	16.16	≤0.01	1.3	2.8
05.03.07	6.1	33.600	9.82	0.04	0.14	3.25	≤0.01	2.6	0.1
06.03.07	6.3	33.327	12.77	0.78	0.49	22.73	≤0.01	2.3	1.7
07.03.07	6.3	33.343	12.53	0.85	0.54	21.89	0.11	1.5	2.4
08.03.07	6.3	33.334	12.17	1.56	0.50	22.30	0.77	0.4	0.4
21.05.07	12.2	31.924	0.69	0.34	0.38	12.29	0.40	18.0	0.6
24.05.07	12.8	32.151	0.78	0.30	0.37	11.18	1.57	9.8	0.1
29.05.07	12.7	31.687	1.07	0.45	0.32	9.09	4.27	14.8	0.1
31.05.07	12.5	31.964	2.02	0.14	0.34	13.14	0.87	26.2	2.6
07.06.07	14.0	31.802	1.21	0.07	0.31	8.65	0.70	51.0	0.5
24.07.07	18.0		1.17	0.36	0.02	0.85	0.25	33.0	0.9
08.10.07	15.2		12.34	1.04	0.01	0.97	≤0.01	7.3	1.1

Table S2

	PLA46	SiO_2	PO_4^{3-}	NO_2^-	NO_3^-	NH_4^+	Temp	Sal	*Centrales*	Time
PLA46										
SiO_2										
PO_4^{3-}		0.41*								
NO_2^-	-0.56**									
NO_3^-	-0.60**			**0.80**						
NH_4^+	0.50*	-0.54**								
Temp	0.60**			**-0.81**	**-0.66**	0.47*				
Sal	-0.52*	0.51*				-0.43*				
Centrales	0.54**		**-0.70**	**-0.79**	-0.62**		**0.80**			
Time	**0.67**		-0.47*	**-0.90**	**-0.75**		**0.90**		**0.80**	

Table S3

Probe	Target organism	Sequence (5' 3')	FA[a] [%]	Reference
PLA46	*Planctomycetes*	GACTTGCATGCCTAATCC	30	(Neef *et al*., 1998)
PirD1039	*Pirellula*-related group D	ATGCAGCACCTACGTAAG	30	(This study)
uPlaB440	uncultured *Planctomycetes* group B	TGCATTTCCTCCCCAACG	30	(This study)
PlaA1228	*Planctomyces*-related group A	AATACCACGCAGACCGCT	30	(This study)

a. Formamide (FA) concentration (v/v) in CARD-FISH hybridization buffer.

Table S4

PirD1039	**isolation source**	**weighted mismatches**	Probe (3-'5')	GAATGCATCCACGACGTA
Pirellula-related group D			Target (5'-3')	CUUACGUAGGUGCUGCAU
uncultured planctomycetales (*Pirellula*)	marine aquaculture / sponge	0.2		...g..............
uncultured planctomycetes	soil / waste water treatment plant	1		.g.........u.....
uncultured planctomycetales	hypersaline microbial mat	1.5		...g..C...........
uncultured planctomycetales	soil / wastewater treatment plant	1.8		.g.g..C...........
uncultured bacteroidales (*Phorphiromonas*)	fungus growing termite	0.8		u...u.............
uncultured acidobacteria	soil	0.9		GC................
consensus uncultured *Planctomycetes* group B				.A.R.AC...........
consensus *Planctomyces*-related group A				.D.RSAC...........
uPlaB440			Probe (3-'5')	GCAACCCCTCCTTTACGT
uncultured *Planctomycetes* group B			Target (5'-3')	CGUUGGGGAGGAAAUGCA
FN822075 (+7)	our sample	0.2		............g....
uncultured planctomyceteales	termite gut	0.3		................u.
uncultured planctomycetales	waste water treatment plant	0.8		..A.............g
uncultured planctomycetales	freshwater/sediment lake	1		...A..............
uncultured planctomycetales	sea sediment / terrestrial	1.2		.Ag...............
uncultured planctomycetales	terrestrial / sludge	1.3		...A.............C
uncultured planctomycetales	sediment	1.4		..gA..............
uncultured gammaproteobacteria	subseafloor sediments	0.6		a................U
uncultured acidobacteria	terrestrial	0.8		..A...............
uncultured *Treponema* sp. (Spirochetes)	termite gut	0.9		a..........g.....C
consensus *Pirellula*-related group D				.R.A...........UUU
consensus *Planctomyces*-related group A				.AGA.....K.....BVD
PlaA1228			Probe (3-'5')	TCGCCACACGCACCATAA
Planctomyces-related group A			Target (5'-3')	AGCGGUCUGCGUGGUAUU
uncultured planctomyces	waste water treatment plant	1.2		.....C............
uncultured bacteroidetes (*Parabacteroides*)	TCE-dechlorinating microbial community	1.4		U....Nu.........g.
uncultured delta proteobacterium	soil	1.5		..G........g......
consensus *Pirellula*-related group D				...R..Y...K.WC....
consensus uncultured *Planctomycetes* group B				..G..C....A.CC....

2.2.

Abundance and diversity of *Planctomycetes* in a Tyrrhenian coastal lake (Lago di Paola, Italy)

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Abundance and diversity of *Planctomycetes* in a Tyrrhenian coastal lake (Lago di Paola, Italy)

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Running heading: *Planctomycetes* abundance and diversity in a Tyrrhenian coastal lake

Keywords: coastal lake; *Planctomycetes*; bacterial diversity; FISH; CARD-FISH; 16S rRNA gene sequencing.

Abstract

Although members of the bacterial phylum *Planctomycetes* seem to be cosmopolitan, little is known about their spatial and temporal distribution in coastal areas. Here we analyzed the dynamic of *Planctomycetes* in the Tyrrhenian coastal lake Lago di Paola and in the adjacent marine coastal waters. Lago di Paola is a highly variable meso-eutrophic environment compared to the more stable oligotrophic marine system and harboured a higher abundance of *Planctomycetes* compared to the marine waters. At the end of summer higher *Planctomycetes* abundances were registered in the hypoxic bottom waters compared to surface waters.

To assess *Planctomycetes* diversity comparative 16S ribosomal RNA (rRNA) sequence analysis was performed. In a surface water 16S rRNA gene library the coverage of *Planctomycetes* diversity was 84% and 87% at species and genus levels, respectively, whereas in a bottom water library these values were 74% and 77%, suggesting a higher *Planctomycetes* diversity closer to the sediment. Four clades were identified in Lago di Paola: *Pirellula*-related group F and uncultured *Planctomycetes* group B show both highest identity with sequences retrieved from marine habitats, whereas *Pirellula*-related group E was affiliated to sequences known from freshwater and brackish water environments. The *Planctomyces*-related group A seems to have a wide habitat range. Quantification by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with newly developed probes revealed similar abundances of the four clades in surface and bottom waters in the range of 10^3 to 10^4 cells ml^{-1}. The four clade-specific probes identified, however, only about one quarter of the *Planctomycetes* indicating the presence of additional, yet unidentified clades.

Introduction

Planctomycetes are a group of budding, peptidoglycan-less bacteria of increasing interest for microbial evolution, ecology, cell biology and genomics (Fuerst 1995). A distinctive feature of members of this bacterial phylum is the cellular compartmentalization (Lindsay et al. 1997, Lindsay et al. 2001, Fuerst 2005). They are part of the *Planctomycetes*/*Verrucomicrobia*/*Chlamydiae* superphylum which in addition to *Planctomycetes* includes the phyla *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae* and the candidate phyla Poribacteria and OP3 that have no cultured representatives yet (Fieseler et al. 2004, Wagner & Horn 2006).

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 *Planctomycetes* (Kulichevskaya et al. 2007, Kulichevskaya et al. 2008, Fukunaga et al. 2009, Kulichevskaya et al. 2009, Winkelmann & Harder 2009), the phylum remains one of the less represented in microbial culture collections. Studies based on molecular methods like 16S ribosomal RNA (rRNA) gene sequencing and fluorescence *in situ* hybridization (FISH) revealed a broad distribution in the environment. With cultivation-independent methods *Planctomycetes* were detected in the marine water column (DeLong et al. 1993, Vergin et al. 1998, Shu & Jiao 2008a) where they were often colonizing 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 2008b) and in the water column and sediments of fresh water systems (Neef et al. 1998, Miskin et al. 1999, Kalyuzhnaya et al. 2004, Kalyuzhnaya et al. 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,

Tadonleke 2007, Pizzetti et al. submitted). Morris and coworkers (Morris et al. 2006) have found high abundances of *Pirellula*-related cells (up to 4 x 10^4 ml^{-1}) to occur during a diatom bloom in Oregon coastal waters. Furthermore, genomic and metagenomic evidence indicates a close link of *Planctomycetes* with the degradation of algal biomass (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 at 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. submitted). Here, we extend this study to a Mediterranean coastal lake, a type of aquatic system for which to our knowledge no study focusing on ecology of *Planctomycetes* has been performed.

Coastal lakes are semi-enclosed environments characterized by a high variability of the physico-chemical parameters. They receive high nutrient inputs from allochthonous sources. A high level of primary production supports a large biodiversity (Nuccio et al. 2003). Coastal lakes can act both as a source or a sink for organic matter (OM) for adjacent coastal areas. The balance between the OM export and its accumulation largely depends on the geomorphologic features and physical factors and it is affected by *in situ* heterotrophic utilization (Manini et al. 2003).

Lago di Paola, a lake exploited for fish farming since the Roman Empire, is located along the Tyrrhenian coast of central Italy (*Latium*). It is characterized by a high water residence time. Two narrow artificial channels, at the north-western and south-eastern ends of the lake allow for a limited water exchange with the sea which is sufficient to support high salinity throughout the year. Freshwater enters the lake only by groundwater intrusion that in summer is drastically reduced due to exploitation for agricultural purposes. The lake

100 has an extension of about 400 ha (main axis 7 km) and an average depth of 4 m, except in
101 the central area, where a small depression reaching 10 m depth is located.

102 The aim of this study was to analyze the seasonal dynamics of *Planctomycetes* in
103 the context of a coastal lake and in the adjacent marine coastal waters. The main study was
104 carried out in 2003 when a severe water deficiency crisis in the period 2001-2002 in
105 Central Italy (Preziosi et al. 2008) resulted in a drastic reduction in freshwater inputs from
106 the aquifer with a consequent increase in water residence time. Recently, it has been
107 proposed that the assessment of microbial communities in harsh environmental conditions,
108 or along drastic environmental gradients, could allow improving knowledge on the
109 composition, functioning and dynamic of the microbial world (Schimel et al. 2007).
110 Therefore, in order to assess how *Planctomycetes* relate to environmental characteristics,
111 the study was focused on the transitional period from summer to winter seasons when
112 physico-chemical characteristics of the lake changed drastically. During this period the
113 summer stratification broke down and hypoxia extended from bottom waters into surface
114 waters. An additional sampling was performed in the central area of the lake, in order to
115 carry out an in-depth analysis of *Planctomycetes* diversity, including the specific
116 quantification of newly identified *Planctomycete*s clades. This second campaign was
117 carried out at the end of summer 2008 when, as a consequence of another water deficiency
118 crisis in the year 2007 (Preziosi et al. 2008), water physico-chemical characteristics of the
119 lake were comparable to those observed in 2003.
120
121 ## Materials and Methods
122 **Sample collection and processing.**
123 Surface water samples were collected monthly (fortnightly in May, June and July)
124 at three stations along the lake main axis named SAB1, SAB2, and SAB3 between January

125 and December 2003 (Fig. S1). Samples were also taken in marine coastal waters off the
126 mouth of the lake outlet (SAB4) (Fig. S1). From the month of July (monthly, except
127 September) samples were also collected at the trench that represents the deepest point in
128 the lake [about 10 m depth (SAB2b)].

129 Environmental parameters were determined as described by Manganelli
130 (Manganelli 2005). Briefly, temperature, salinity, pH and dissolved oxygen were
131 determined by probes [(Wissenschaftlich-Technische Werkstätten (WTW), Weilheim,
132 Germany; Kent Electronic Instrument Limited (EIL), Chertsey, Surrey, UK)]. The
133 analytical methods used for the determination of dissolved nutrients (phosphate, nitrate and
134 nitrite) were those reported by Parsons and colleagues (Parsons et al. 1984) and
135 Valderrama (Valderrama 1981). Dissolved organic carbon (DOC) concentration was
136 analyzed by high temperature catalytic oxidation using a Shimadzu TOC-5000 analyzer
137 (Pettine et al. 1999). Chlorophyll *a* (Chl a) was extracted in 90% acetone and
138 concentrations were measured by spectrophotometry according to Lorenzen (Lorenzen
139 1967).

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

146 For in-depth analysis of *Planctomycetes* diversity, an additional sampling was
147 carried out on September 15th, 2008 at stations SAB2 and SAB2b. During this sampling
148 temperature, salinity and dissolved oxygen were recorded. Overall the same procedures
149 were followed as in 2003, excepted for the bacterial cell fixation for FISH and CARD-

FISH that was performed with a paraformaldehyde solution (20% w/v, Electron Microscopy Sciences, Hatfield, PA; final concentration 1%) and a prefiltration of water samples through 10 μm pore-size polycarbonate filters (type TCTP; diameter, 47 mm; Millipore). Moreover, for DNA isolation, between 750 and 1250 ml aliquots were filtered onto 0.2 μm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore) after prefiltration through 10 μm pore-size polycarbonate filters (type TCTP; diameter, 47 mm; Millipore). Filters were frozen semidry and kept at -20 °C until processed for nucleic acid extraction.

FISH and CARD-FISH.

FISH was performed according to the protocol by Glöckner and coworkers (Glöckner et al. 1996). The probe PLA46 (Neef et al. 1998) was used to assess *Planctomycetes* abundance and the probe NON338 (Wallner et al. 1993) was used as a negative control. Since false-positive signals were detected by the NON338 probe cell counts from the negative control were subtracted from all counts with the probe PLA46.

CARD-FISH was performed, using the same two probes, according to protocols by Pernthaler and colleagues (Pernthaler et al. 2002, 2004) and slightly modified by Pizzetti and coworkers (Pizzetti et al. submitted) to improve the fluorescence signals of *Planctomycetes*. Shortly, samples were permeabilized with lysozyme (10 mg ml^{-1}; Fluka, Steinheim, Germany; buffer contained 0.05 M EDTA, pH 8, 0.1 M Tris-HCl, pH 7.4) for 1 h at 37 °C followed by achromopeptidase (60 U ml^{-1}; Sigma, Steinheim, Germany; buffer contained 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37 °C. Moreover, the hybridization was performed at 46 °C for 2.5 h and the washing step at 48 °C for 5 min. The probe NON338 was used as a negative control; no false-positive signals were detected in none of the samples. Filter sections, counterstained with DAPI (4',6-diamidino-2-

175 phenylindole) [final concentration 1 μg ml^{-1}] and washed for 1 min in Milli-Q water and
176 96% ethanol were subsequently arranged on microscope slides, and embedded in a mix of
177 two antifading reagents [VECTASHIELD® Mounting Medium H-1000, Vector
178 Laboratories, Inc., Burlingame, CA, USA and Citifluor Ltd., London, UK (1:5)]. Slides
179 were stored at -20 °C until further analyses. Cells were quantified on an Axioplan II
180 Imaging epifluorescence microscope (Zeiss, Jena, Germany).
181
182 **DNA extraction and PCR amplification.**
183 Total nucleic acids were extracted according to the protocol of Zhou and colleagues
184 (Zhou et al. 1996), modified by Pizzetti and coworkers (Pizzetti et al. submitted) from
185 picoplankton collected by filtration from surface (SAB2) and bottom water (SAB2b)
186 collected at site 2 (Fig. S1). Briefly, polycarbonate filters were cut in small pieces and
187 incubated in an extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0),
188 100 mM Na-phosphate (pH 8.0), 1.5 M NaCl, 1% CTAB] together with proteinase K (10
189 mg ml^{-1}) in order to break the cell wall and remove proteins. Cell lysis was completed by
190 adding 20% SDS to a final concentration of 2%: this also removed membrane lipids. All
191 centrifugation steps were performed at 53,250 x g excepted for the recovery of the aqueous
192 phase after the mixing with chloroform/isoamylalcohol which was performed at 10,000 x
193 g. The precipitation of DNA with isopropanol was done overnight at room temperature.
194 After washing in ethanol and drying, the nucleic acids pellet was resuspended in 1:10 TE
195 buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8). The high speed centrifugation steps and
196 the longer time period of DNA precipitation using isopropanol improved the yield of DNA.
197 Fragments of about 1.4 kb encompassing almost full length 16S rRNA genes were
198 amplified using the primer PLA46F [5'-GGA TTA GGC ATG CAA GTC-3', targeting
199 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 *E. coli* position 1392-1406 on 16S rRNA gene; (Xu et al. 1998)]. Each PCR reaction was carried out with 50 µl master mix including: 0.3 mg ml^{-1} BSA (final concentration; Sigma Chemie, Taufkirchen, Germany), 1 x reaction buffer (final concentration; Eppendorf, Hamburg, Germany), 0.25 mM dNTPs (final concentration; Roche Diagnostics, Mannheim, Germany), 0.5 µM each primer (final concentration; http://Biomers.net, Ulm, Germany), 0.02 U μl^{-1} Taq Polymerase (final concentration; Eppendorf, Hamburg, Germany) and 1 µl of DNA. The PCR was run at 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, and 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 and 10 replicates were processed in parallel to minimize PCR biases (Suzuki & Giovannoni 1996, Polz & Cavanaugh 1998). PCR products were checked in 1% agarose gel electrophoresis (Biozym LE agarose, Biozym, Oldendorf, Germany).

16S rRNA gene libraries, sequencing and phylogenetic reconstruction.

The PCR product was purified by agarose gel electrophoresis. The 1.4 kb band was excised from the gel and subsequently extracted and purified using Qiaquick Gel extraction kit (QIAGEN, Hilden, Germany) to avoid cloning of non-specific and small PCR products. Purified amplicons were ligated in vector P-GEM T-Easy (Promega, Madison, USA) according to the manufacturer's instructions. The recombinant plasmids were then transformed into chemically competent *E. coli* cells (Invitrogen, Gröningen, Netherlands), plated and picked. Plasmids DNA were isolated with the Montage™ Plasmid Miniprep Kit (Millipore, Eschborn, Germany) and then were sequenced using the two vector primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG

AC-3') together with GM1F [5′-CCA GCA GCC GCG GTA AT-3'; (Lane 1991)] to obtain almost full length 16S rRNA gene sequences. Contigs were assembled with the software SEQUENCHER Version 4.6 (Gene Codes Corporation, Ann Harbor, USA). The 16S rRNA gene sequences of this study were deposited in the EMBL Nucleotide Sequence Database with the accession numbers FR714331-FR714400.

The sequences were aligned in the ARB software package [http://www.arb-home.de; (Ludwig et al. 2004)] using the SINA_aligner (Pruesse et al. 2007). Alignments were further refined manually by comparison of closest relatives. For in-depth phylogenetic rRNA analysis only sequences with more than 1200 nucleotides were included. Phylogenetic tree reconstruction was based on distance matrix (DM) (e.g. neighbor-joining) and maximum likelihood (ML) methods with and without 30% and 50% *Planctomycetes* positional conservatory filters. A consensus tree was constructed thereafter (Peplies et al. 2008).

Diversity coverage by the sequenced clones was analyzed using the computer program DOTUR [Distance-Based OTU and Richness (Schloss & Handelsman 2005) http://schloss.micro.umass.edu/software/dotur.html]. Operational taxonomic units (OTUs) were calculated using the furthest neighbor algorithm for each distance level, from a distance matrix generated in ARB. Then, we selected 97% and 95% sequences identity to construct the rarefaction curves. Coverage index of the clone libraries was calculated with the formula C = (1-*n*/N)*100, where N is the numbers of all clones with a *Planctomycetes* insert and *n* the number of phylotypes at 97% and 95% identity appearing only once in the library (Good 1953, Kemp & Aller 2004).

Probe design.

Oligonucleotide probes targeting the 16S rRNA of *Planctomycetes* clades were designed based on the newly created phylogenetic tree using the probe design tool of the ARB software. Probe specificities were checked *in silico* against the SILVA 16S rRNA database [version 100; (Pruesse et al. 2007)]. For optimization of stringency conditions, a series of hybridizations at increasing formamide concentrations (10–60%) were evaluated. The optimal formamide concentration was the highest concentration before signal intensity started to decrease (Table 1). Probes were applied for CARD-FISH on 2008 samples as described below.

Statistics.

Linear correlations between *Planctomycetes* abundance and environmental parameters were calculated with the Spearman rank correlation test using SigmaStat 3.5 (Systat software, Santa Clara, CA, USA). For an ordination of sampling sites, Principal Components Analysis (PCA) was performed with the software PAST (PAlaeontological STatistics, ver. 2.01) [http://folk.uio.no/ohammer/past/; (Hammer et al. 2001)]. Component loadings visualized in a biplot were calculated using all physico-chemical parameters and *Planctomycetes* absolute abundances. All variables were normalized using division by their standard deviations, and loading values were computed by the data correlation matrix.

Results

Physico-chemical parameters.

During 2003 the environmental parameters showed remarkable seasonal variability within the lake in comparison to those registered in the neighboring marine coastal waters (Fig. S2). Indeed, the values of salinity ranged between 29.0 (March) and 38.0 (August) in

the lake and from 37.5 (February) to 39.0 (September) in marine waters (Fig. S2). Oxygen saturation in the lake fluctuated strongly throughout the year with the highest value registered in May (134%) and the lowest in August (24%). In coastal marine waters oxygen saturation ranged between 58% in September and 91% at the beginning of May (Fig. S2). Lake water temperature ranged between 8.5 °C in February and 30.8 °C in July; in marine waters the range was between 12.9 °C in February and 26.5 °C in August (Fig. S2). DOC concentrations ranged from 2.89 mg l^{-1} (January) to 5.55 mg l^{-1} (July) in the lake and between 1.09 mg l^{-1} (January) and 1.68 mg l^{-1} (March) in marine waters (Fig. S2). Chlorophyll *a* showed the highest value in July (37.6 µg l^{-1}) and the lowest in February (3.0 µg l^{-1}) in the lake. In coastal waters chlorophyll *a* ranged between the detection limit registered in June and 3.5 µg l^{-1} in July (Fig. S2). For phosphate, values ranged between the detection limit in June and the highest value in September (0.3 µg at l^{-1}). In marine waters the highest value was also registered in September (0.1 µg at l^{-1}) (Fig. S2). The highest value of nitrate was 74.6 µg at l^{-1} in February decreasing to 0.1 µg at l^{-1} in July. In the marine system nitrate showed the highest value in March (6.2 µg at l^{-1}) and the lowest in July (0.1 µg at l^{-1}) (Fig. S2). Nitrite ranged between the detection limit in July and 2.6 µg at l^{-1} in January. In coastal waters values ranged from the detection limit in July to 0.3 µg at l^{-1} in February (Fig. S2).

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 were 110% and 22% in surface and bottom waters, respectively (Fig. 1a). In August, water masses started to re-mix with homogeneous temperature values along the water column (~ 28 °C). Salinity was around 38 along the water column and the hypoxia extended from bottom waters (27% oxygen) into surface waters (10% oxygen) (Fig. 1a). At that time hypoxia was observed along the whole lake (including SAB1 and SAB3) and

300 the oxygen concentration in surface waters remained low also in September. In the
301 following months the oxygen concentration increased both in surface and bottom waters.
302
303 **Microbial and *Planctomycetes* abundance in 2003.**
304 In the lake surface waters absolute numbers of prokaryotic picoplankton ranged
305 between 6.2 x 10^6 ml^{-1} in December at SAB3 and 3.4 x 10^7 ml^{-1} in July at SAB2 (Table
306 S1). In the deeper layers cell numbers ranged between 8.9 x 10^6 ml^{-1} in December and 2.1
307 x 10^7 ml^{-1} in July (Table S1). In marine coastal waters absolute cell counts ranged between
308 9.1 x 10^5 ml^{-1} in September and 4.7 x 10^6 ml^{-1} in July (Table S1). Overall, the average cell
309 numbers of prokaryotic picoplankton in the lake (1.8 x 10^7 ml^{-1}) was 7.7-fold higher
310 compared to the sea (2.3 x 10^6 ml^{-1}).
311 *Planctomycetes* abundances were quantified by FISH and CARD-FISH with probe
312 PLA46 and the cells numbers obtained on the same samples by both techniques were
313 compared. In these formaldehyde fixed samples (2% final concentration), *Planctomycetes*
314 cell numbers after FISH were higher (43% more) compared to cell numbers obtained by
315 CARD-FISH (Fig. S3). Consequently, *Planctomycetes* were further quantified by FISH in
316 the 2003 sampling.
317 The relative abundance of *Planctomycetes* at SAB1 showed highest numbers at the
318 beginning of July with 4.7 ± 0.4% of the prokaryotic picoplankton community with a
319 decreasing trend to 0.8 ± 0.5% in September (Fig. 2, Table S1). In October the percentage
320 increased to 1.9 ± 0.2% remaining on almost the same level in November (1.4 ± 0.6%) and
321 December (1.8 ± 1.1%).
322 At SAB2 *Planctomycetes* abundance increased from the beginning of July (1.5 ±
323 0.4%) to the end of July (5.4 ± 2.1%) when highest abundances were registered throughout
324 the year (Fig. 1b & 2, Table S1). Then, *Planctomycetes* abundance decreased to 0.8 ± 0.2%

in September and increased to 2.8 ± 0.7% in October with a slight decrease to 1.5 ± 0.7% in December. At the bottom of SAB2 relative abundances were more stable ranging between 1.4 ± 0.9% in October and 2.1 ± 0.4% in August (Fig. 1b).

Planctomycetes relative abundance at SAB3 increased from 1.8 ± 0.2% to 3.4 ± 3.4% between the beginning and the end of July and decreased to 0.9 ± 0.6% in August. An increasing trend until October (3.2 ± 1.4%) was registered. Then, the abundance decreased to 2.0 ± 0.3% in December. Generally in the lake the highest *Planctomycetes* abundances were registered in July and in October (Fig. 2, Table S1).

In the neighboring marine coastal waters (SAB4) the relative abundance increased from 0.8 ± 0.3% in July to 2.0 ± 0.8% in September decreasing to 0.9 ± 0.2% in December (Fig. 2, Table S1). Overall the average of *Planctomycetes* relative abundance was 1.6-fold higher in the lake than in the sea.

Correlation analyses showed that absolute abundances of *Planctomycetes* were significantly correlated with chlorophyll *a* ($n = 28$, $r_s = 0.65$; $P \leq 0.001$), dissolved organic carbon ($r_s = 0.62$; $P \leq 0.001$), 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 correlation with temperature, PO_4^{3-}, NO_3^-, or NO_2^- was found.

A PCA of the whole data set of surface waters was performed in order to elucidate the main relationships between *Planctomycetes* and physico-chemical variables. Figure 3 shows the distribution of the variables in the space formed by the first two components, which explained 65% of the variance. Performing PCA, the component 1, explaining 40% of the total variance, clearly discriminates among summer and winter sampling points, with temperature and salinity having the highest positive and nitrite and nitrate the highest negative loading values (Temp = 0.441, Sal = 0.435, NO_2^- = -0.483, NO_3^- = -0.491). *Planctomycetes* showed low but positive loadings (0.046) (Fig. 3). The component 2,

350 explaining 25% of the total variance, is likely to discriminate lake from seawater
351 conditions with highest positive loadings for DOC (0.488) and Chl a (0.467) (Fig. 3).
352
353 **Phylogenetic affiliation of *Planctomycetes*.**
354 A phylogenetic analysis of *Planctomycetes* was done on samples from September
355 15th, 2008. At station SAB2, salinity, temperature and oxygen values were comparable
356 with those observed in August and September 2003. Particularly, temperature (26.0 °C)
357 and salinity (37.1) did not vary along the water column and hypoxic condition was
358 observed both at the surface (17.1%) and bottom water (8.1%).
359 *Planctomycetes* abundance was quantified by FISH and CARD-FISH using PLA46.
360 In these samples fixed with paraformaldehyde solution (1% final concentration) CARD-
361 FISH yielded higher probe-positive cell numbers than FISH preparations (Fig. S3). The
362 percentage of *Planctomycetes* determined by CARD-FISH was 3.4 ± 0.1% (2.8 ± 0.09 x
363 10^5 ml^{-1}) at the surface and 5.2 ± 0.6% (3.6 ± 0.4 x 10^5 ml^{-1}) at the bottom of Lago di
364 Paola.
365 One clone library each was constructed from surface (SAB2) and bottom water
366 (SAB2b) collected at site 2. In total, we analyzed 161 sequences (partial and almost full-
367 length) of which 101 were affiliated with *Planctomycetes*. Forty of 83 sequences retrieved
368 from SAB2 were affiliated to *Planctomycetes*, 61 of 78 SAB2b sequences belonged to
369 *Planctomycetes*.
370 By comparative sequence analyses four phylogenetic clades could be distinguished.
371 They were named *Pirellula*-related group F, *Pirellula*-related group E, uncultured
372 *Planctomycetes* group B and *Planctomyces*-related group A (Fig. 4). These clades
373 remained stable in different types of tree reconstructions (neighbour joining, maximum

likelihood) with and without *Planctomycetes* positional conservatory filters (50% and 30%).

Pirellula-related group F sequences accounted for 3 out of 31 almost full length *Planctomycetes* sequences obtained in this study from surface water (SAB2) and 5 out of 39 in bottom water (SAB2b). The sequences shared $\simeq$ 86% sequence identity with *Blastopirellula marina* (Fig. 4). The frequencies of *Pirellula*-related group E were 7/31 and 2/39 in SAB2 and SAB2b, respectively. Sequences in this clade shared $\simeq$ 89% sequence identity with *Blastopirellula marina* and $\simeq$ 87% with a strain of the genus *Pirellula* (Fig. 4). Sequences from uncultured *Planctomycetes* group B were retrieved much less frequently from SAB2 (1/31) than from SAB2b (14/39) (Fig. 4). *Planctomyces*-related group A was frequent in both libraries (16/31 in SAB2 and 15/39 in SAB2b). The former group shared $\simeq$ 87% sequence identity with *Planctomyces brasiliensis* and the later between 85% and 87% with strains of the genus *Planctomyces* (Fig. 4).

The coverage of the *Planctomycetes* diversity at SAB2 was 84% at species level (97% sequences identity) and 87% at the genus level (95% sequence identity). In SAB2b library coverage was lower with 74% and 77% at species and genus levels, respectively (Fig. S4).

Abundance of specific *Planctomycetes* clades.

Three specific oligonucleotide probes were designed for the newly defined *Planctomycetes* clades. Together with one previously designed probe (Pizzetti et al. submitted) the new probes were targeting all four dominant clades identified in the clone libraries of Lago di Paola (Table 1). Probe PirF85 is specific for *Pirellula*-related group F encompassing 7 of 8 sequences retrieved in this study. Probe PirE62 is fully complementary to 8 of 9 sequences of the *Pirellula*-related group E. Probe PlaS2114

399 targeted a monophyletic clade within the *Planctomyces*-related group A which
400 encompassed 10 of 31 sequences retrieved in this study. Probe uPlaB440 was previously
401 designed by Pizzetti and colleagues (Pizzetti et al. submitted) based on sequences retrieved
402 from North Sea coastal waters. It targeted 11 of 15 sequences that belonged to uncultured
403 *Planctomycetes* group B.

404 Counts with probe PirF85 in the bottom water were $1.1 \pm 0.4 \times 10^4$ ml^{-1} ($0.2 \pm 0.1\%$
405 of prokaryotic picoplankton). In surface water samples of Lago di Paola only single
406 PirF85-positive cells occurred and the counts were below the detection limit of 0.1% (Fig.
407 5). *Pirellula*-related group E identified with probe PirE62 accounted for $1.2 \pm 0.5 \times 10^4$ ml^{-1}
408 ($0.2 \pm 0.1\%$) and $2.0 \pm 0.5 \times 10^4$ ml^{-1} ($0.3 \pm 0.1\%$) in the surface and bottom water,
409 respectively (Fig. 5). The number of cells hybridized with probe uPlaB440 was $5.0 \pm 0.8 \times$
410 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
411 (Fig. 5). Finally, probe PlaS2114 targeted $9.7 \pm 0.2 \times 10^3$ cells ml^{-1} (0.1%) and $1.6 \pm 0.03 \times$
412 10^4 cells ml^{-1} (0.2%) in the upper and lower water column of Lago di Paola (Fig. 5).
413 Throughout the samples of 2008 the sum of counts with the four clade-specific probes was
414 26% and 28% of the PLA46 counts on the surface and bottom of the lake, respectively.
415

416 Discussion

417 Lago di Paola is a highly variable aquatic environment compared to the more stable
418 marine system. The physico-chemical data clearly identify this coastal lake as a meso-
419 eutrophic system which apparently did not directly influence the adjacent marine coastal
420 site investigated in this study (SAB4). The highest DOC concentration in the lake, in fact,
421 suggests it to act as a sink for autochthonous (biological production) and allochthonous
422 sources (mainly agricultural areas). Lago di Paola during summer is stratified and at the

423 end of the summer when waters started to mix, the oxygen concentration dramatically
424 decreases at the surface resulting in hypoxia in the entire water column.
425
426 **Seasonality of *Planctomycetes*.**
427 Before abundances of *Planctomycetes* in Lago di Paola are discussed in detail the
428 methodological evolution from FISH to CARD-FISH which occurred during the course of
429 this study needs to be considered. Initially, *Planctomycetes* counts were higher with FISH
430 than with CARD-FISH in the samples taken in 2003. Later, the opposite was true for the
431 2008 samples. We would like to argue that this was likely due to a change in sample
432 fixation from a final concentration of 2% formaldehyde in 2003 to the lower concentration
433 of 1% used in 2008. Formaldehyde is cross-linking amino-groups. After the fixation at 2%
434 the 16SrRNA of a part of the *Planctomycetes* might no longer be accessible to large HRP-
435 labeled probes used in CARD-FISH due to the larger size of the label (~ 44 kDa). The
436 smaller Cy3-labeled probes (MW of CY3 is ~ 700 Da) likely penetrated the cells in the
437 2003 samples more efficiently. The samples of 2008 were less cross-linked by the 1%
438 paraformaldehyde fixative, and here CARD-FISH was superior to FISH due to its higher
439 sensitivity. We are confident that the counts obtained with the two methods for the two
440 different sample types are reliable. With both methods we observed mainly the doughnut-
441 shaped hybridization pattern typical for *Planctomycetes* (Woebken et al. 2007a). If there
442 was a counting bias, the *Planctomycetes* numbers were rather under- than overestimated.
443 *Planctomycetes* abundances were assessed separately at three stations within the
444 lake. They showed a high seasonality with the highest values of 1.5×10^6 cells ml^{-1} at
445 SAB1 at the beginning of July. Particularly during stratification *Planctomycetes* increased
446 in surface waters in absolute numbers in response to high DOC, chlorophyll concentration,
447 temperature and oxygen saturation (Fig. 3). This scenario changed when a dystrophic crisis

448 occurred. At the end of the summer when waters started to mix, the oxygen concentration
449 dramatically decreased at the surface resulting in hypoxic conditions in the entire water
450 column. At that time, *Planctomycetes* abundances were rather low. This change was best
451 correlated with changes in physical parameters and in phosphate concentration (Fig. 3).
452 When oxygen saturation was re-established an increase of *Planctomycetes* was registered.
453 This upshift coincided with shifts in inorganic nutrients (Fig. 3). Overall, our results are
454 similar to findings in North Sea coastal waters (Pizzetti et al. submitted) where
455 *Planctomycetes* seemed to be bottom-up controlled and most likely benefited from algal
456 production.

457 In bottom waters the conditions seem to be more stable, e.g. during hypoxia in
458 summer, and the abundances of *Planctomycetes* were less variable. In August when the
459 water column became completely hypoxic *Planctomycetes* abundance was higher at the
460 lake bottom than at the surface suggesting that *Planctomycetes* were strongly influenced by
461 the change from oxic to hypoxic conditions occurring in the surface water. At the marine
462 station SAB4 the *Planctomycetes* had lower abundance values compared to the lake
463 stations SAB1-3 likely because of lower DOC. However, also phosphate could represent a
464 limiting factor for *Planctomycetes* in such a system. In fact, in many areas of the
465 Mediterranean Sea P limitation appears to influence primary productivity and bacterial
466 uptake of DOC, the latter as a result of a low assimilation of dissolved organic substrates
467 when P is not available for bacterial metabolism (Thingstad & Rassoulzadegan 1995,
468 Zoppini et al. 1995, Vaulot et al. 1996, Zohary & Robarts 1998, Puddu et al. 2003).
469
470 **Diversity and clades quantification.**
471 With this study several new phylotypes of *Planctomycetes* have been added to the
472 database as almost full length 16S rRNA gene sequences. By comparative sequence

analysis we could define four stable coastal groups. Sequences of all clades were retrieved from surface and bottom waters of Lago di Paola indicating a high connectivity which reflects mixing. Clone frequencies in surface and bottom water libraries were similar except for the uncultured *Planctomycetes* group B of which more sequences were obtained from bottom water. CARD-FISH counts for this group obtained with probe uPlaB440 were, however, 5 x 10^4 ml^{-1} in the surface water as well as in the bottom water. The clade-specific CARD-FISH counts with PirF85, PirE62, and PlaS2114 confirmed the simultaneous presence of all clades in the surface and bottom water in similar abundances. None of the clades accounted for more than 1% of the total picoplankton in the samples examined. We can, however, not exclude that the four *Planctomycetes* clades examined in this study might bloom in particular settings.

The *Pirellula*-related group F clones obtained in this study were closely related to published sequences retrieved from marine sediment and the surface of marine macro-alga, whereas most *Pirellula*-related group E sequences showed best affiliation with sequences retrieved from waste water treatment plant, river biofilms and mangrove soil (Fig. 4). Our sequences from *Planctomyces*-related group A currently show the highest identity (~ 91 % sequence identity) with sequences from compost leakage water, cold sulphurous springs and cold seep sediment (Fig. 4). *Planctomyces brasiliensis*, that 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. The clade of uncultured *Planctomycetes* group B has first been described by Pizzetti and coworkers (Pizzetti et al. submitted) in the North Sea coastal waters. We here demonstrate that this group might be more widely distributed in coastal marine habitats. Interestingly, probe uPlaB440 visualized in addition to the common doughnut-shaped cells also a second, rather rod-shaped morphotype indicating the presence of distinct subpopulations (Fig. S5). It is now possible to learn more about the

biogeography of coastal marine clades of *Planctomycetes* by applying the set of specific probes developed in this study.

Overall, the diversity of *Planctomycetes* in Lago di Paola was only partially covered in this study. The coverage of *Planctomycetes* diversity was 84% and 87% and 74% and 77% at species and genus levels at the surface and bottom, respectively (Fig. S4), suggesting higher *Planctomycetes* diversity particularly at the bottom water of the lake. With the four clade-specific probes used in this study we were able to detect between 26% and 28% of the PLA46 counts in the surface and bottom waters of the lake, respectively. This indicates the presence of additional, yet unidentified clades of *Planctomycetes* in Lago di Paola that should be definitely investigated in the future by further comparative sequence analysis and the development of additional clade-specific oligonucleotide probes.

Conclusions

This study yielded first data on the diversity, abundance and spatial distribution of *Planctomycetes* in a highly variable meso-eutrophic coastal lake system. The relative abundances of *Planctomycetes* varied between 1% and 5% with highest counts of 1.5×10^6 cells ml^{-1}. Similar to a study in the coastal North Sea (Pizzetti et al. submitted) in which *Planctomycetes* abundances were linked to numbers of centric and pennate diatoms we obtained first hints on a also seasonal succession in Lago di Paola that correlates with Chl a concentrations. It would be interesting to apply, in different seasons and years, the new set of probes specific for clades found in the Lago di Paola to check whether there is a seasonal succession and to test whether particular clades of *Planctomycetes* are indeed correlated to the occurrence of specific phytoplankton. Such studies must also consider that the diversity of *Planctomycetes* in Lago di Paola is high and has not yet been fully covered.

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831
832 Zoppini A, Pettine M, Totti C, Puddu A, Artegiani A, Pagnotta R (1995) Nutrients,
833 standing crop and primary production in western coastal waters of the Adriatic Sea.
834 Estuar Coast Shelf Sci 41:493-513
835
836
837
838
839
840
841
842
843
844

Figure legends

Fig. 1: (a) Oxygen and temperature at the surface and the bottom of the Lago di Paola at the seven sampling days between July and December 2003. (b) *Planctomycetes* absolute counts at the surface and the bottom of the Lago di Paola at the seven sampling days between July and December 2003. Means ± SD of three replicates are shown.

Fig. 2: *Planctomycetes* relative abundances at the three sampling sites along the lake and at the adjacent marine waters between July and December 2003. Means ± SD of three replicates are shown.

Fig. 3: Two-dimensional plot of the Principal Components Analysis **(**PCA**)** performed for the whole surface water data set from July to December 2003, including physico-chemical parameters and *Planctomycetes* absolute abundances. For seawater (SAB4) samples from the first sampling campaign in July, October and December were not included because the data set was incomplete. All variables were normalized using division by their standard deviations, and loading values were computed by the data correlation matrix. Symbols refers to the four sampling sites ♦: SAB1; ●: SAB2; ■: SAB3; ▲: SAB4; empty symbols refer to the second sampling campaign in July. Temp: Temperature; Sal: Salinity; DO: Dissolved oxygen; Chl a: Chlorophyll *a*; DOC: Dissolved organic carbon; PLA: *Planctomycetes*. Dotted circles separate data registered in the lake from the sea. Continuous circles surround samples from the same month within the lake. Black lines represent all variables used. The length of each line indicates the degree of correlation with the ordination axis. The percent variation explained by each axis is indicated in parentheses.

869 **Fig. 4:** In order to summarize the *Planctomycetes* 16S rRNA-based phylogeny in the lake a
870 consensus tree was constructed with 223 almost full-length sequences (longer than
871 1200 nucleotides) of which 27 from the surface and 36 from the bottom of the lake
872 were obtained in this study and grouped into four distinct clades. The consensus
873 tree was built based on maximum likelihood and neighbor joining trees calculated
874 without and with 50% and 30% *Planctomycetes* positional conservatory filters
875 using *Verrucomicrobia, Lentisphaerae, Chlamydiae* and *Candidatus* OP3
876 sequences as outgroup. Sequences belonged to the four clades are pointed out by a
877 color code [surface (SAB2): red; bottom (SAB2b): blue; type strains: green).
878 Accession numbers and sequence length are stated in parentheses. *: sequences
879 targeted by newly designed probes, stated in parentheses, at 0 weighted
880 mismatches. + blue: a clone targeted by PirE62. +: a clone targeted by PlaS2114.
881 The numbers in the boxes of the uncultured clades indicate the number of
882 sequences retrieved in this study. The bar represents 10% sequence divergence.

883 **Fig. 5:** Logarithmic scale of absolute abundances of *Planctomycetes* (PLA46), *Pirellula*-
884 related group F (PirF85), *Pirellula*-related group E (PirE62), uncultured
885 *Planctomycetes* group B (uPlaB440), *Planctomyces*-related group A (PlaS2114) on
886 September 15th, 2008. Means ± SD of three replicates.

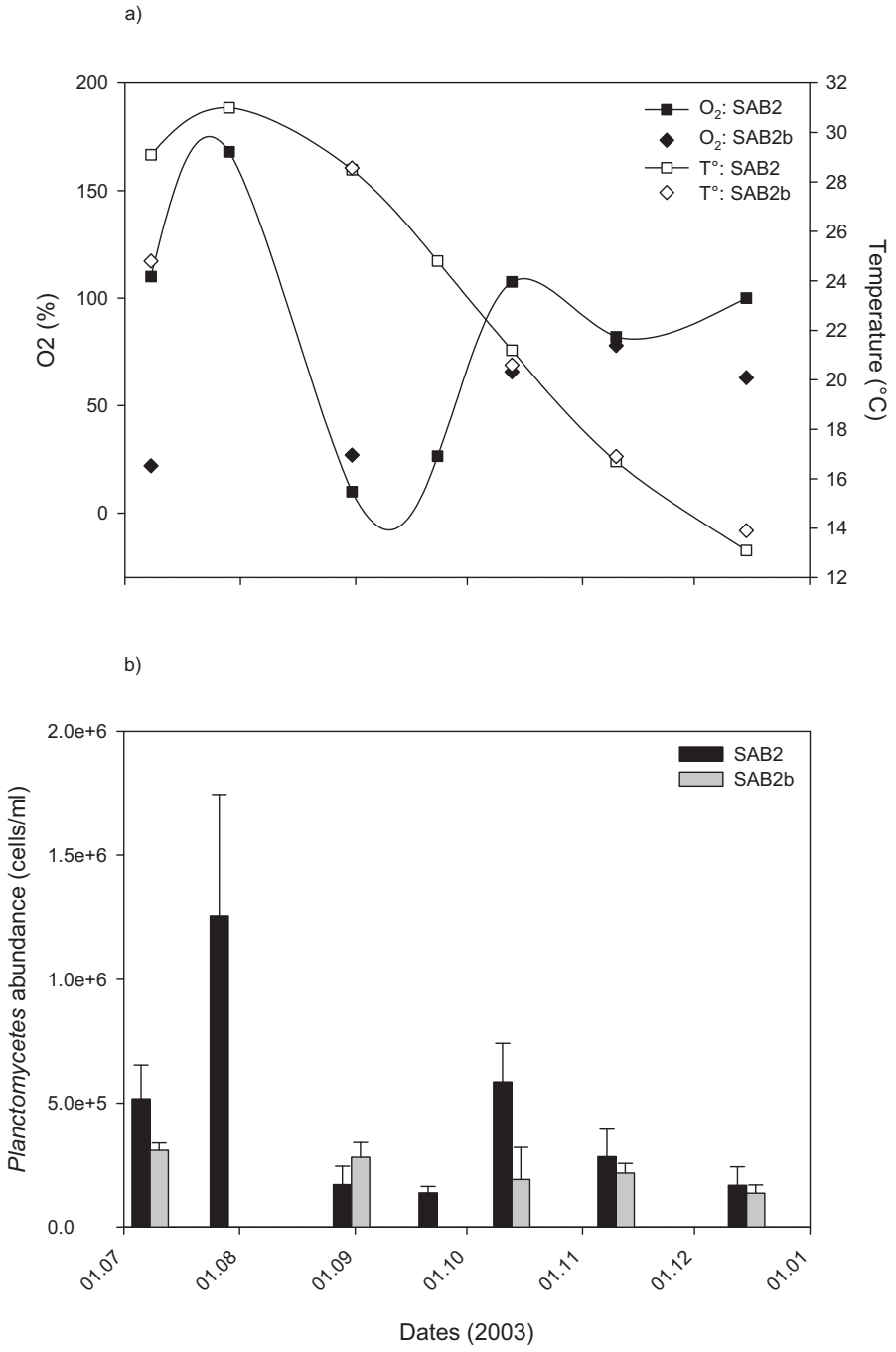
a)
O2 (%)
Temperature (°C)
O_2: SAB2
O_2: SAB2b
T°: SAB2
T°: SAB2b
b)
Planctomycetes abundance (cells/ml)
SAB2
SAB2b
Dates (2003)
01.07
01.08
01.09
01.10
01.11
01.12
01.01

Fig. 1

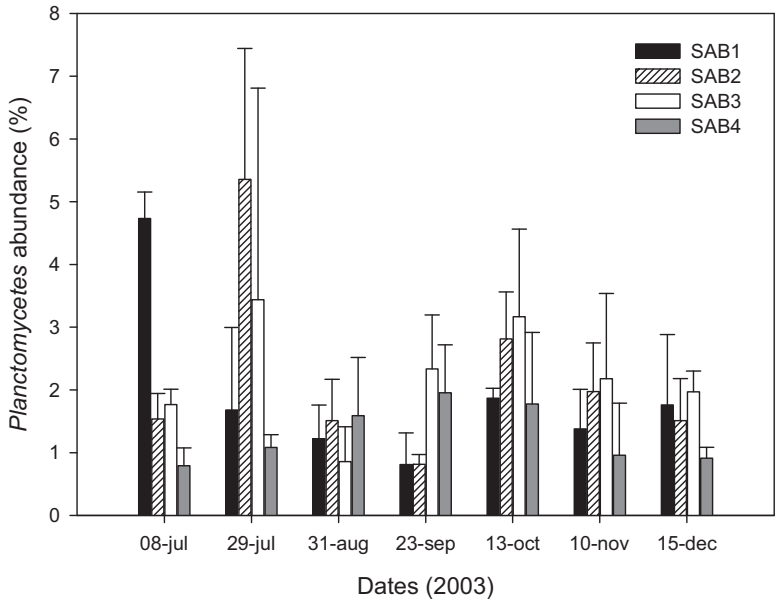
SAB1
SAB2
SAB3
SAB4
Planctomycetes abundance (%)
0
1
2
3
4
5
6
7
8
08-jul
29-jul
31-aug
23-sep
13-oct
10-nov
15-dec
Dates (2003)

Fig. 2

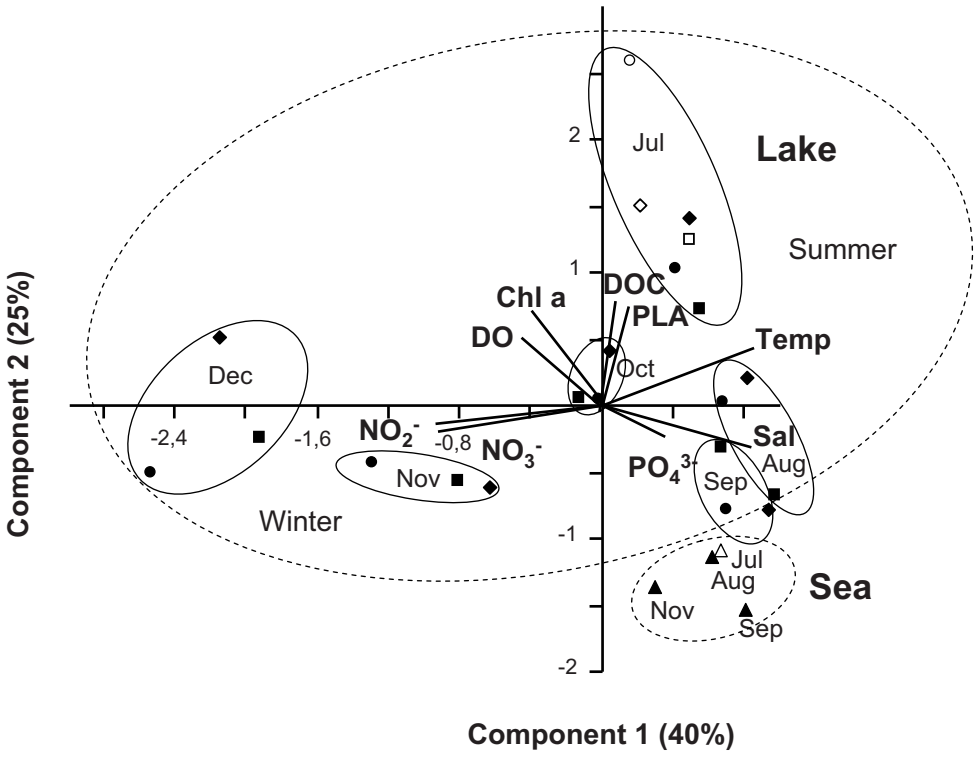
Component 2 (25%)
Component 1 (40%)
Lake
Summer
Sea
Winter
Jul
Aug
Sep
Oct
Nov
Dec
Chl a
DOC
PLA
DO
Temp
Sal
NO_2^-
NO_3^-
PO_4^{3-}
2
1
-1
-2
-2,4
-1,6
-0,8

Fig. 3

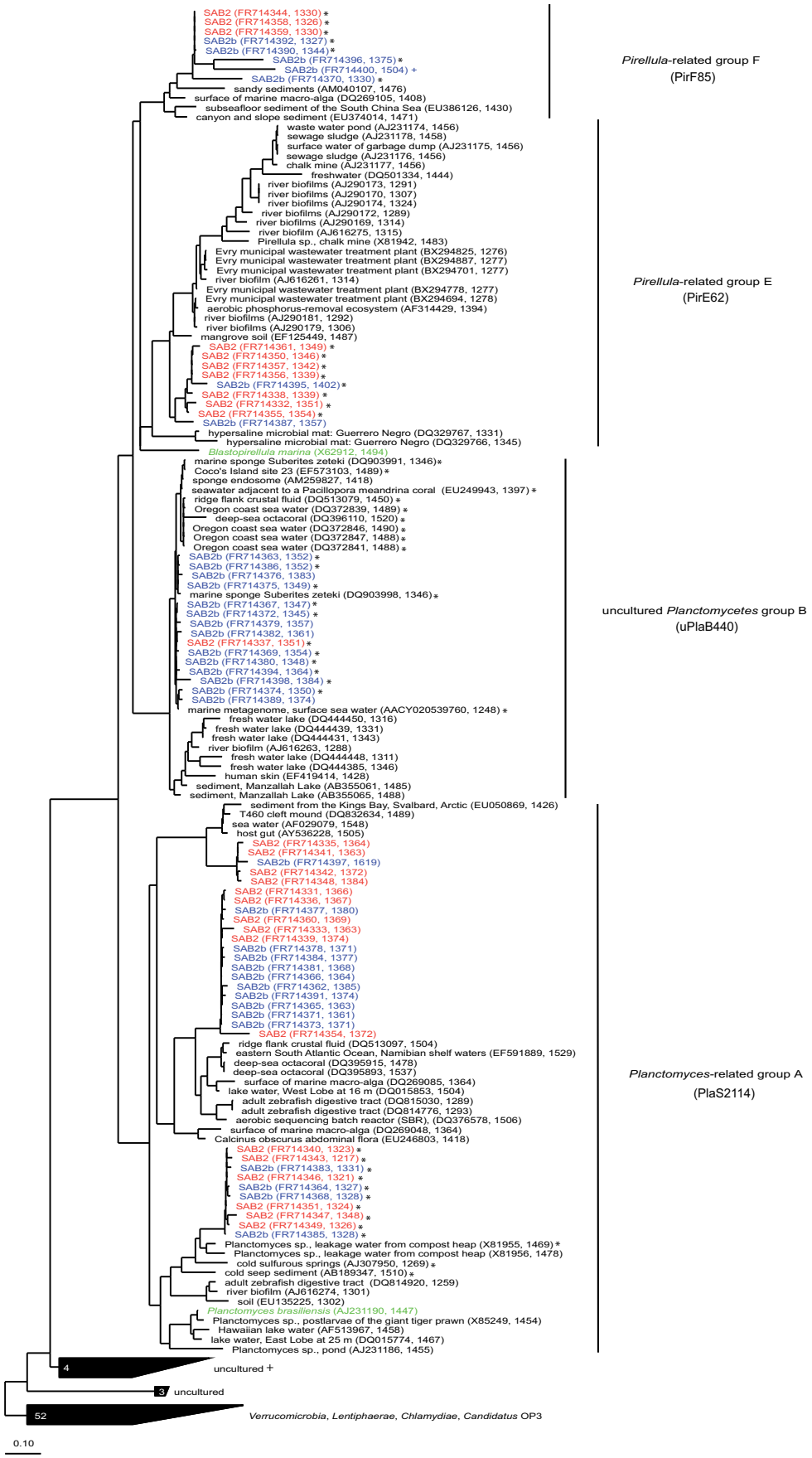
SAB2 (FR714344, 1330)
SAB2 (FR714358, 1326)
SAB2 (FR714359, 1330)
SAB2b (FR714392, 1327)
SAB2b (FR714390, 1344)
SAB2b (FR714396, 1375)
SAB2b (FR714400, 1504)
SAB2b (FR714370, 1330)
sandy sediments (AM040107, 1476)
surface of marine macro-alga (DQ269105, 1408)
subseafloor sediment of the South China Sea (EU386126, 1430)
canyon and slope sediment (EU374014, 1471)
waste water pond (AJ231174, 1456)
sewage sludge (AJ231178, 1458)
surface water of garbage dump (AJ231175, 1456)
sewage sludge (AJ231176, 1456)
chalk mine (AJ231177, 1456)
freshwater (DQ501334, 1444)
river biofilms (AJ290173, 1291)
river biofilms (AJ290170, 1307)
river biofilms (AJ290174, 1324)
river biofilms (AJ290172, 1289)
river biofilms (AJ290169, 1314)
river biofilm (AJ616275, 1315)
Pirellula sp., chalk mine (X81942, 1483)
Evry municipal wastewater treatment plant (BX294825, 1276)
Evry municipal wastewater treatment plant (BX294887, 1277)
Evry municipal wastewater treatment plant (BX294701, 1277)
river biofilm (AJ616261, 1314)
Evry municipal wastewater treatment plant (BX294778, 1277)
Evry municipal wastewater treatment plant (BX294694, 1278)
aerobic phosphorus-removal ecosystem (AF314429, 1394)
river biofilms (AJ290181, 1292)
river biofilms (AJ290179, 1306)
mangrove soil (EF125449, 1487)
SAB2 (FR714361, 1349)
SAB2 (FR714350, 1346)
SAB2 (FR714357, 1342)
SAB2 (FR714356, 1339)
SAB2b (FR714395, 1402)
SAB2 (FR714338, 1339)
SAB2 (FR714332, 1351)
SAB2 (FR714355, 1354)
SAB2b (FR714387, 1357)
hypersaline microbial mat: Guerrero Negro (DQ329767, 1331)
hypersaline microbial mat: Guerrero Negro (DQ329766, 1345)
Blastopirellula marina (X62912, 1494)
marine sponge Suberites zeteki (DQ903991, 1346)
Coco's Island site 23 (EF573103, 1489)
sponge endosome (AM259827, 1418)
seawater adjacent to a Pacillopora meandrina coral (EU249943, 1397)
ridge flank crustal fluid (DQ513079, 1450)
Oregon coast sea water (DQ372839, 1489)
deep-sea octacoral (DQ396110, 1520)
Oregon coast sea water (DQ372846, 1490)
Oregon coast sea water (DQ372847, 1488)
Oregon coast sea water (DQ372841, 1488)
SAB2b (FR714363, 1352)
SAB2b (FR714386, 1352)
SAB2b (FR714376, 1383)
SAB2b (FR714375, 1349)
marine sponge Suberites zeteki (DQ903998, 1346)
SAB2b (FR714367, 1347)
SAB2b (FR714372, 1345)
SAB2b (FR714379, 1357)
SAB2b (FR714382, 1361)
SAB2 (FR714337, 1351)
SAB2b (FR714369, 1354)
SAB2b (FR714380, 1348)
SAB2b (FR714394, 1364)
SAB2b (FR714398, 1384)
SAB2b (FR714374, 1350)
SAB2b (FR714389, 1374)
marine metagenome, surface sea water (AACY020539760, 1248)
fresh water lake (DQ444450, 1316)
fresh water lake (DQ444439, 1331)
fresh water lake (DQ444431, 1343)
river biofilm (AJ616263, 1288)
fresh water lake (DQ444448, 1311)
fresh water lake (DQ444385, 1346)
human skin (EF419414, 1428)
sediment, Manzallah Lake (AB355061, 1485)
sediment, Manzallah Lake (AB355065, 1488)
sediment from the Kings Bay, Svalbard, Arctic (EU050869, 1426)
T460 cleft mound (DQ832634, 1489)
sea water (AF029079, 1548)
host gut (AY536228, 1505)
SAB2 (FR714335, 1364)
SAB2 (FR714341, 1363)
SAB2b (FR714397, 1619)
SAB2 (FR714342, 1372)
SAB2 (FR714348, 1384)
SAB2 (FR714331, 1366)
SAB2 (FR714336, 1367)
SAB2b (FR714377, 1380)
SAB2 (FR714360, 1369)
SAB2 (FR714333, 1363)
SAB2 (FR714339, 1374)
SAB2b (FR714378, 1371)
SAB2b (FR714384, 1377)
SAB2b (FR714381, 1368)
SAB2b (FR714366, 1364)
SAB2b (FR714362, 1385)
SAB2b (FR714391, 1374)
SAB2b (FR714365, 1363)
SAB2b (FR714371, 1361)
SAB2b (FR714373, 1371)
SAB2 (FR714354, 1372)
ridge flank crustal fluid (DQ513097, 1504)
eastern South Atlantic Ocean, Namibian shelf waters (EF591889, 1529)
deep-sea octacoral (DQ395915, 1478)
deep-sea octacoral (DQ395893, 1537)
surface of marine macro-alga (DQ269085, 1364)
lake water, West Lobe at 16 m (DQ015853, 1504)
adult zebrafish digestive tract (DQ815030, 1289)
adult zebrafish digestive tract (DQ814776, 1293)
aerobic sequencing batch reactor (SBR), (DQ376578, 1506)
surface of marine macro-alga (DQ269048, 1364)
Calcinus obscurus abdominal flora (EU246803, 1418)
SAB2 (FR714340, 1323)
SAB2 (FR714343, 1217)
SAB2b (FR714383, 1331)
SAB2 (FR714346, 1321)
SAB2b (FR714364, 1327)
SAB2b (FR714368, 1328)
SAB2 (FR714351, 1324)
SAB2 (FR714347, 1348)
SAB2 (FR714349, 1326)
SAB2b (FR714385, 1328)
Planctomyces sp., leakage water from compost heap (X81955, 1469)
Planctomyces sp., leakage water from compost heap (X81956, 1478)
cold sulfurous springs (AJ307950, 1269)
cold seep sediment (AB189347, 1510)
adult zebrafish digestive tract (DQ814920, 1259)
river biofilm (AJ616274, 1301)
soil (EU135225, 1302)
Planctomyces brasiliensis (AJ231190, 1447)
Planctomyces sp., postlarvae of the giant tiger prawn (X85249, 1454)
Hawaiian lake water (AF513967, 1458)
lake water, East Lobe at 25 m (DQ015774, 1467)
Planctomyces sp., pond (AJ231186, 1455)
4
uncultured
3
uncultured
52
Verrucomicrobia, Lentiphaerae, Chlamydiae, Candidatus OP3
0.10
Pirellula-related group F
(PirF85)
Pirellula-related group E
(PirE62)
uncultured Planctomycetes group B
(uPlaB440)
Planctomyces-related group A
(PlaS2114)

Fig. 4

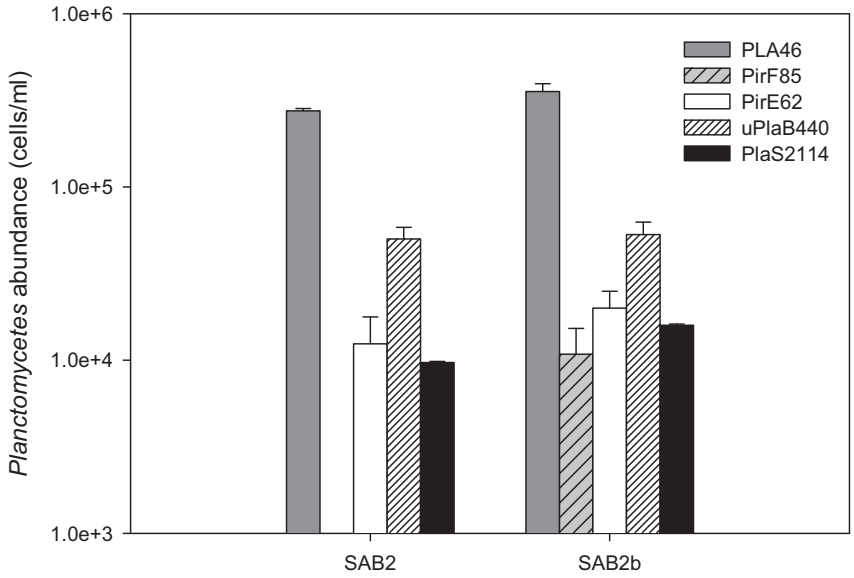
1.0e+6
1.0e+5
1.0e+4
1.0e+3
Planctomycetes abundance (cells/ml)
PLA46
PirF85
PirE62
uPlaB440
PlaS2114
SAB2
SAB2b

Fig. 5

Table 1: *Planctomycetes* specific oligonucleotide probes.

Probe	Target organism	Sequence (5' → 3')	FA[a] [%]	Reference
PLA46	*Planctomycetes*	GACTTGCATGCCTAATCC	30	(Neef et al. 1998)
uPlaB440	uncultured *Planctomycetes* group B	TGCATTTCCTCCCCAACG	30	(Pizzetti et al. submitted)
PirF85	*Pirellula*-related group F	GCTGTCCGTCTTAATGCA	45	(This study)
PirE62	*Pirellula*-related group E	AGGAAGCTTCTCGCACGA	20	(This study)
PlaS2114	*Plantomycetes*-related group A	GTTCCGATGCCTTACTAC	30	(This study)

a. Formamide (FA) concentration (v/v) in CARD-FISH hybridization buffer.

Supplementary material

Fig. S1: Schematic map of the Lago di Paola indicating the four sampling sites.

Fig. S2: Environmental parameters collected between January and December 2003. Data modified from Manganelli (Manganelli 2005).

Fig. S3: Comparison of samples fixed with formaldehyde (2% final concentration) in 2003 with samples from 2008 fixed with paraformaldehyde (1% final concentration). *Planctomycetes* absolute counts after FISH and CARD-FISH. Each bar represents the average of counts at site 2 in the surface and bottom of the lake.

Fig. S4: Rarefaction curves of almost full length 16S rRNA sequences (> 1200 nucleotides) recovered from the surface and bottom of the lake on September 15th, 2008. The expected numbers of OTUs was calculated using cut-off values of sequence identity for species (97%) and genus (95%) levels.

Fig. S5: Epifluorescence micrographs of bacteria from the surface of Lago di Paola. Blue, DAPI signals of DNA containing cells; green, signals of catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)-positive cells using the probes: (a), (b): PLA46 (10ml), (c): PirF85 (50 ml), (d) PlaS2114 (50ml), (e) PirE62 (50ml), (f), (g) uPlaB440 (50 ml). Bar = 5μm.

Table S1: *Planctomycetes* relative and absolute abundances (means ± SD of three replicates) and total microbial abundances between July and December 2003.

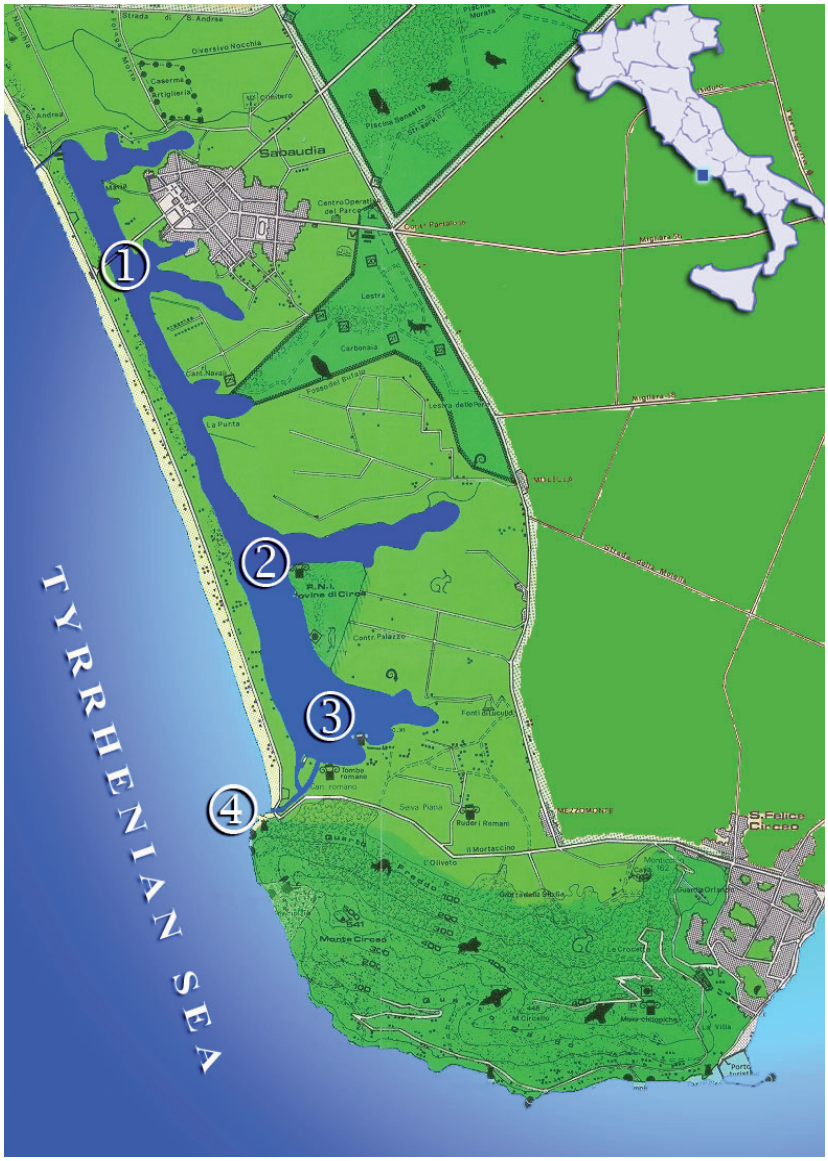

Fig. S1

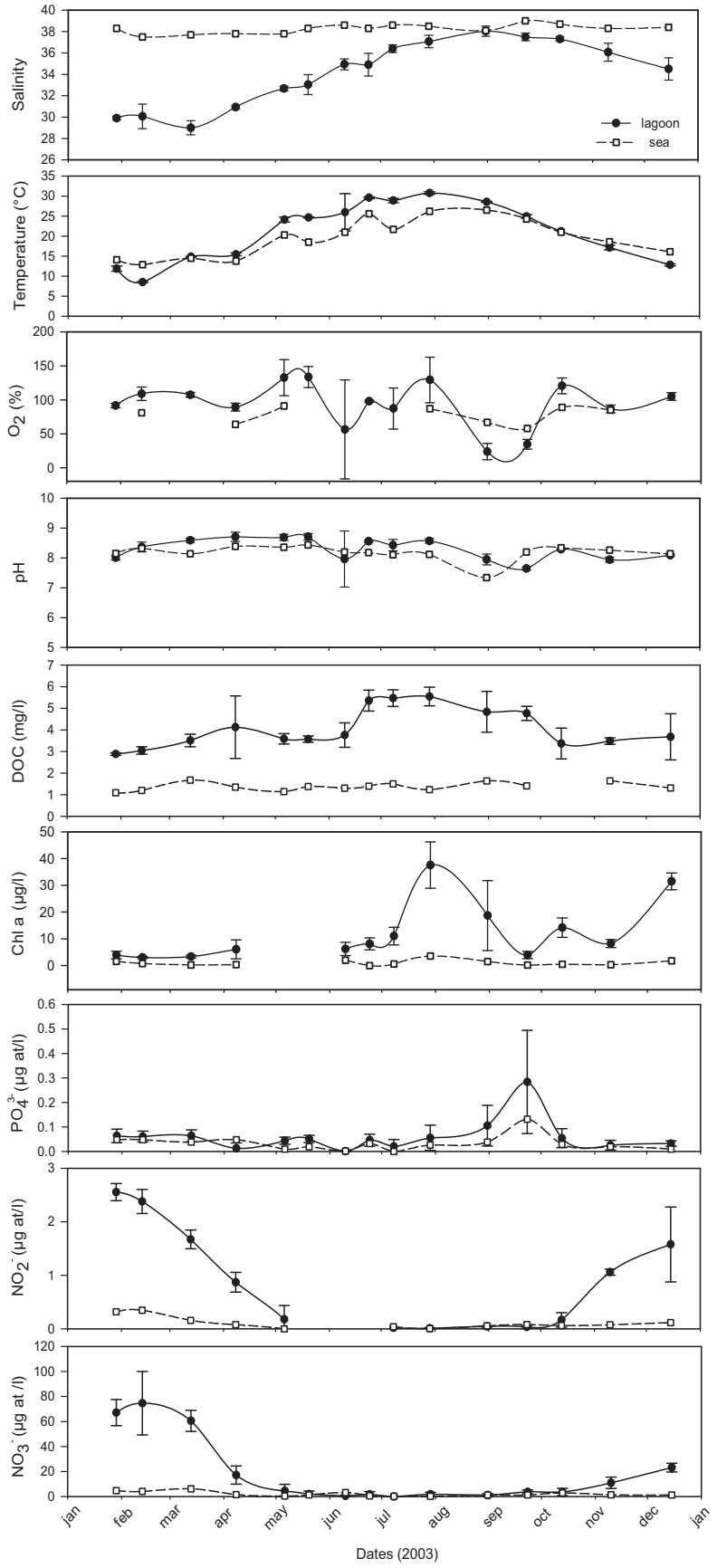
Salinity
lagoon
sea
Temperature (°C)
O2 (%)
pH
DOC (mg/l)
Chl a (µg/l)
PO4 3- (µg at/l)
NO2- (µg at/l)
NO3- (µg at /l)
jan
feb
mar
apr
may
jun
jul
aug
sep
oct
nov
dec
jan
Dates (2003)

Fig. S2

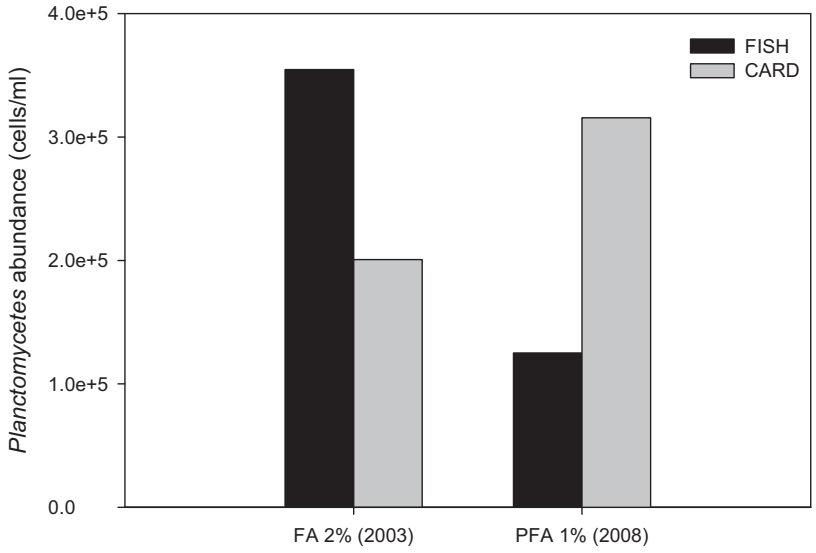
4.0e+5
3.0e+5
2.0e+5
1.0e+5
0.0
Planctomycetes abundance (cells/ml)
FISH
CARD
FA 2% (2003)
PFA 1% (2008)

Fig. S3

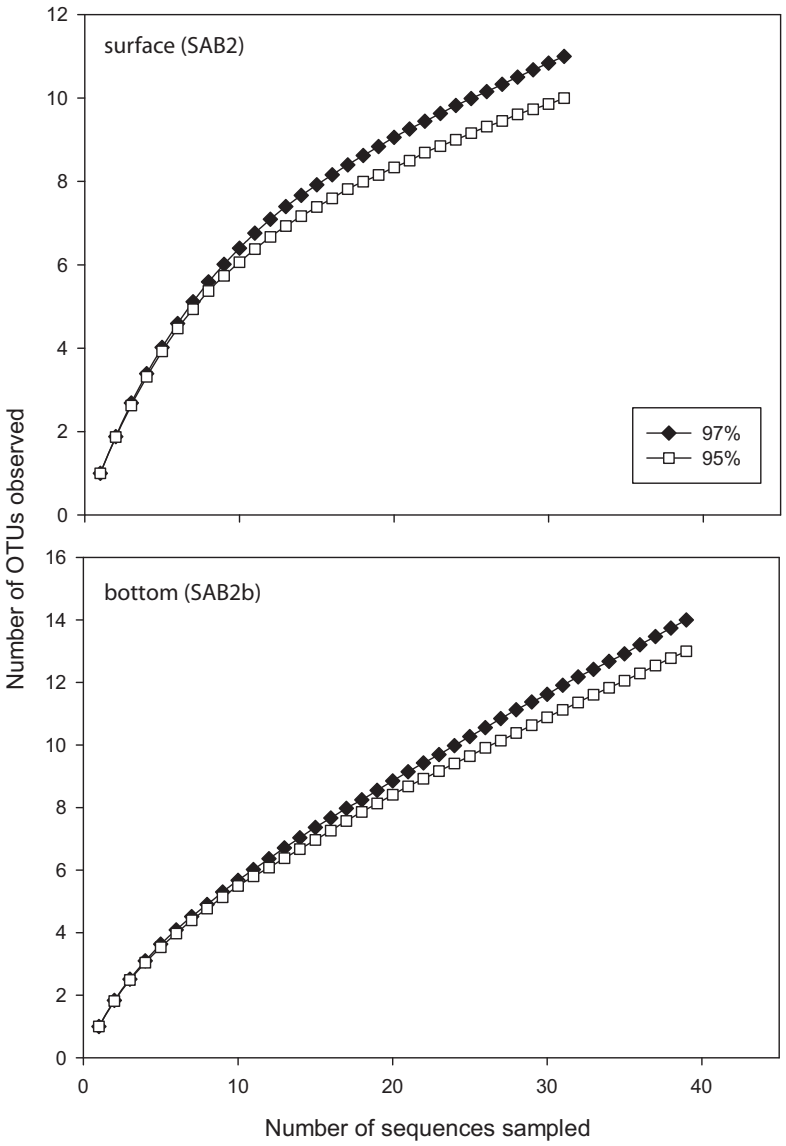
surface (SAB2)
bottom (SAB2b)
97%
95%
Number of OTUs observed
Number of sequences sampled
0
2
4
6
8
10
12
14
16
0
10
20
30
40

Fig. S4

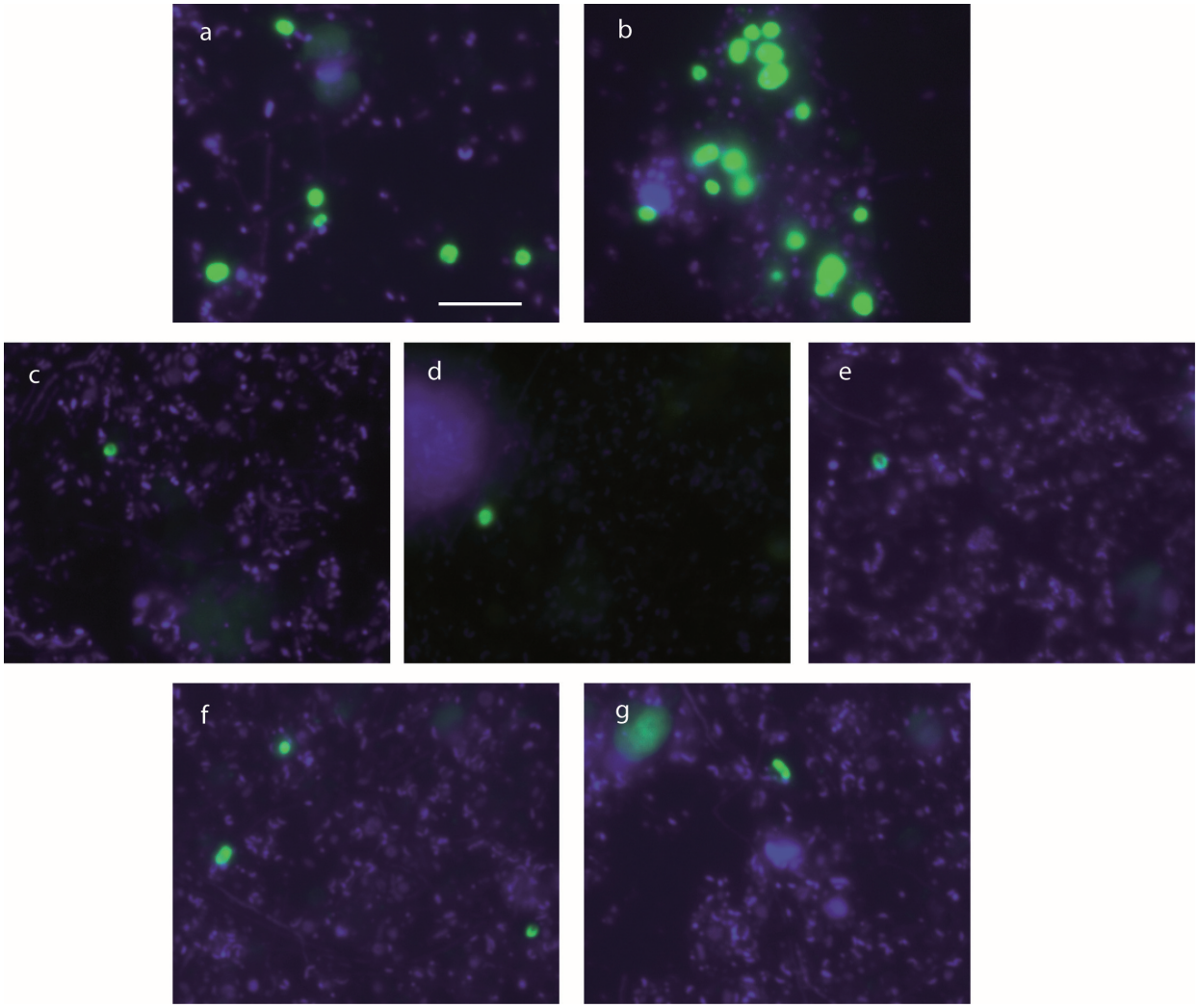

Fig. S5

Table S1

Dates	Stations	*Planctomycetes* relative abundance [%]	*Planctomycetes* absolute abundance [$x10^5$ cells ml^{-1}]	Total cell abundance [$x10^7$ cells ml^{-1}]
08/07/03	SAB1	4.7 ± 0.4	15. 4 ± 1.4	3.3
	SAB2	1.5 ± 0.4	5.2 ± 1.4	3.4
	SAB2b	1.5 ± 0.1	3.1 ± 0.3	2.1
	SAB3	1.8 ± 0.2	5.1 ± 0.7	2.9
	SAB4	0.8 ± 0.3	0.37 ± 0.1	0.47
29/07/03	SAB1	1.7 ± 1.3	2.9 ± 2.3	1.7
	SAB2	5.4 ± 2.1	12.6 ± 4.9	2.3
	SAB3	3.4 ± 3.4	5.9 ± 5.8	1.7
	SAB4	1.1 ± 0.2	0.35 ± 0.06	0.32
31/08/03	SAB1	1.2 ± 0.5	1.7 ± 0.7	1.4
	SAB2	1.5 ± 0.7	1.7 ± 0.7	1.1
	SAB2b	2.1 ± 0.4	2.8 ± 0.6	1.4
	SAB3	0.9 ± 0.6	1.1 ± 0.7	1.3
	SAB4	1.6 ± 0.9	0.56 ± 0.3	0.35
23/09/03	SAB1	0.8 ± 0.5	1.2 ± 0.8	1.5
	SAB2	0.8 ± 0.2	1.4 ± 0.3	1.7
	SAB3	2.3 ± 0.9	4.0 ± 1.5	1.7
	SAB4	2.0 ± 0.8	0.18 ± 0.07	0.091
13/10/03	SAB1	1.9 ± 0.2	3.3 ± 0.3	1.7
	SAB2	2.8 ± 0.7	5.9 ± 1.6	2.1
	SAB2b	1.4 ± 0.9	1.9 ± 1.3	1.4
	SAB3	3.2 ± 1.4	6.2 ± 2.7	2.0
	SAB4	1.8 ± 1.1	0.23 ± 0.1	0.13
10/11/03	SAB1	1.4 ± 0.6	1.9 ± 0.9	1.4
	SAB2	2.0 ± 0.8	2.8 ± 1.1	1.4
	SAB2b	1.7 ± 0.3	2.2 ± 0.4	1.3
	SAB3	2.2 ± 1.4	2.8 ± 1.8	1.3
	SAB4	1.0 ± 0.8	0.098 ± 0.09	0.10
15/12/03	SAB1	1.8 ± 1.1	2.1 ± 1.3	1.2
	SAB2	1.5 ± 0.7	1.7 ± 0.7	1.1
	SAB2b	1.5 ± 0.4	1.4 ± 0.3	0.89
	SAB3	2.0 ± 0.3	1.2 ± 0.2	0.62
	SAB4	0.9 ± 0.2	0.12 ± 0.02	0.13

2.3.

In situ identification of novel chlamydiae in a Mediterranean coastal lake (Lago di Paola, Italy)

Ilaria Pizzetti, Stefano Fazi, Bernhard M. Fuchs, Rudolf Amann

Manuscript in preparation. Intended for *Environmental Microbiology Reports*.

In situ indentification of novel environmental chlamydiae in a Mediterranean coastal lake (Lago di Paola, Italy)

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Running title: Chlamydial diversity in a Mediterranean coastal lake

Keywords: environmental chlamydiae; 16S rRNA gene sequencing; CARD-FISH; coastal lake.

Summary

Until recently the order *Chlamydiales* exclusively included one family of obligate intracellular bacteria, the *Chlamydiaceae*, which encompassed causative agents of severe diseases. In the 1990s, "novel chlamydiae" were discovered as symbionts of free-living amoebae and other eukaryotic hosts. These findings showed an additional diversity of *Chlamydiae* in the environment. Moreover, recent studies suggest "novel chlamydiae" as potential emerging pathogens.

While monitoring *Planctomycetes*, 20 almost full-length 16S ribosomal RNA (rRNA) gene sequences affiliated with *Chlamydiales* were retrieved from a coastal lake along the Tyrrhenian coast of central Italy (Lago di Paola, *Latium*). Two main clusters were identified and two oligonucleotide probes were designed, tested and optimized. Chl282 targets the cluster I and almost all other *Chlamydiales* thus most likely the probe allowed us to visualize and quantify the majority of *Chlamydiales* in such environment. With Chl282bis that is specific for the cluster II it was possible to visualize free-living environmental chlamydiae as well as chlamydiae inside a host, presumably a protist. The cluster I shares $\simeq$ 98% sequence identity within its sequences while the cluster II $\simeq$ 94% sequence identity. Our findings enlarge the knowledge of the biodiversity of environmental chlamydiae in aquatic habitats and deserve attention from a sanitary point of view.

Introduction

Until recently, the bacterial phylum *Chlamydiae* consisted of a single family *Chlamydiaceae*. It represented a small group of closely related obligate intracellular bacteria deeply branching within the domain Bacteria. *Chlamydiae* are characterized by a unique developmental cycle consisting of a metabolically inactive infectious form called the elementary body (EB), that, after the entry in the target cell differentiates into a larger, metabolically active reticulate body (RB). After various rounds of replication, RBs go through a secondary differentiation process back to infectious EBs (Subtil and Dautry-Varsat, 2004). *Chlamydiae* are biologically and phylogenetically well separated from all other bacteria. Everett and coworkers (Everett et al., 1999; Bush and Everett, 2001), based on comparative sequence analysis of different macromolecules, reclassified these pathogens into nine species belonging to two different genera (*Chlamydophila* and *Chlamydia*) within the family *Chlamydiaceae* representing the world's major causes of preventable blindness and sexually transmitted disease [*Chlamydia trachomatis*, trachoma and urogenital infections in humans (WHO, 2001, 2008)]. Recent phylogenetic studies showed that the phylum *Chlamydiae* forms part of a stable superphylum encompassing *Planctomycetes*, *Verrucomicrobia* and *Chlamydiae*, referred to as PVC superphylum. This monophyletic group also includes *Lentisphaerae*, and the candidate phyla Poribacteria and OP3 that have no cultured representatives, yet (Fieseler et al., 2004; Wagner and Horn, 2006).

In the 1990s, “novel chlamydiae” were discovered as symbionts of free-living amoebae or insects, as pathogens of crustaceans, as contaminants of a laboratory cell culture, and in an aborted bovine fetus (Kahane et al., 1995; Amann et al., 1997; Rurangirwa et al., 1999; Horn et al., 2000; Thao et al., 2003; Kostanjsek et al., 2004). Based on these findings three novel families within the phylum *Chlamydiae* were

described: *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae* (Everett et al., 1999; Rurangirwa et al., 1999). These "novel chlamydiae" (all the *Chlamydiae* not belonging to the family *Chlamydiaceae*) were also called environmental chlamydiae. Furthermore, molecular data suggested an additional diversity of *Chlamydiae* in the environment (Ossewaarde and Meijer, 1999; Horn and Wagner, 2001; Corsaro et al., 2003; Corsaro and Venditti, 2009).

Water systems have been shown to be a potential source of *Chlamydiae*. However, their diversity is underestimated and their natural hosts are unknown in many cases (Corsaro and Venditti, 2009). Environmental chlamydiae are more closely related to pathogenic chlamydiae than to any other bacteria. They have different hosts compared to pathogenic chlamydiae (amoebae vs. humans), but have the same obligate intracellular life style and a similar morphology of their developmental stages as pathogenic chlamydiae. There is evidence of a possible association of the environmental chlamydiae with respiratory disease of humans (Greub and Raoult, 2002).

Here we report evidence for the presence of two novel clusters of 16S ribosomal RNA (rRNA) gene sequences of *Chlamydiales* affiliated with the family *Simkaniaceae* in a coastal lake in central Italy (Lago di Paola, *Latium*). By catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with two newly developed probes we were able to quantify and localize these environmental chlamydiae in the lake water. Evidence was obtained for an abundance > 10^4 individual small coccoid cells per ml and for replication of larger cells of environmental chlamydiae most likely in protists.

Results and Discussion

At the time of sampling (September 15th, 2008) the water column of meso-eutrophic Lago di Paola had already been re-mixed after the summer stratification. Environmental

parameters such as temperature (26.0 °C) and salinity (37.1) were constant over depth and hypoxia was observed both in surface (17.1% of oxygen saturation) and bottom waters (8.1%).

For an analysis of the diversity of *Planctomyetes* two 16S rRNA gene libraries had been constructed based on the primer set PLA46-UNIV1392, one from the surface (SAB2) and one from the bottom (SAB2b) of the Lago di Paola (Pizzetti et al., submitted). Twenty-one sequences (20 almost full-length and 1 partial) related to the order *Chlamydiales* and affiliated with the family *Simkaniaceae* were exclusively retrieved from the surface of the lake (Fig. 1). The primer pair PLA46-UNIV1392 resulted to be not specific for *Planctomycetes* and it is not surprising since PLA46F the general primer used to amplify the 16S rRNA gene of *Planctomycetes* is also amplifying the 16S rRNA gene of some outgroups of which the phylum *Chlamydiae* (family *Simkaniaceae*). In the future, it might be possible, to design more specific primers based, for example, on PCR-independent metagenome libraries, in order to reduce the mismatches to non-planctomycetes.

Two main clusters were identified which consisted of 9 (cluster I) and 8 (cluster II) closely related sequences (Fig. 1). Those clusters remained stable in different types of tree reconstructions (neighbour joining, maximum likelihood) with and without chlamydial positional conservatory filters (50% and 30%). The cluster I shared $\simeq$ 98% sequence identity within its sequences, $\simeq$ 88% sequence identity with the cluster II, $\simeq$ 85% sequence identity with *Waddlia chondrophila* and *Simkania negevensis* (Fig. 1) and 87% sequence identity with *Neochlamydia hartmannellae* and *Parachlamydia acanthamoebae*. Furthermore, cluster I resulted to be closely related to uncultured *Chlamydia* sp. (AY114327) retrieved from anoxic marine sediment (Fig. 1) and *Candidatus* Protochlamydia naegleriophila (DQ632609) ($\simeq$ 88% sequence identity). A new developed oligonucleotide probe Chl282 targeted the cluster I but also almost all sequences belonging

126 to *Chlamydiales* and one sequence that belongs to Candidate division OP10. The number
127 of cells hybridized with probe Chl282 was $4.5 \pm 0.5 \times 10^4$ ml^{-1} ($0.6 \pm 0.1\%$) on the surface
128 and $5.0 \pm 0.8 \times 10^4$ ml^{-1} ($0.7 \pm 0.1\%$) at the bottom of the lake. Since Chl282 is not specific
129 for the cluster I, most likely the probe allowed us to quantify the majority of *Chlamydiales*
130 in such environment. Hence we suggest for the future to use Chl282 together with
131 Chl282bis to quantify the complete order. CARD-FISH showed a dot-shaped morphology
132 of those tiny cells and no host could be identified (Fig. 2d). That should be due to the
133 prefiltration treatment in 10 µm pore-size filters that most probably removes the host.
134 The cluster II shared $\simeq$ 94% sequence identity within its sequences, $\simeq$ 87%
135 sequence identity with *Simkania negevensis* and *Neochlamydia hartmannellae* and $\simeq$ 88%
136 sequence identity with *Parachlamydia acanthamoebae.* It was mostly closest related to the
137 uncultured bacterium (EU491566) from seafloor lavas from East Pacific Rise (Fig. 1),
138 Chlamydiales bacterium CRIB33 (EU683887) from raw surface water by Acanthamoeba
139 co-culture, *Candidatus* Protochlamydia amoebophila UWE25 (BX908798) and
140 *Parachlamydia acanthamoebae* from river water (DQ309029) ($\simeq$ 88% sequence identity).
141 The new oligonucleotide probe Chl282bis that differs from the probe Chl282 for just one
142 nucleotide (Table S1) is specific for the cluster II. CARD-FISH showed cells with dot-
143 shaped morphology and interestingly a host was visualized, thus chlamydial cells were
144 identified in different developmental stages, outside the host as EBs-like and inside the
145 host as RBs-like (Fig. 2c). Unfortunately it was not possible to classify the host that most
146 likely is a protist.
147 The multi-color CARD-FISH showed no overlap between the two probes
148 confirming that they target different sequences within *Chlamydiae*. Among available
149 oligonucleotide probes only Bn_9658 (Amann et al., 1997) is able to target almost all our
150 sequences but is not specific only for them. Furthermore, all sequences from this study

151 resulted to be not covered by available chlamydial primer sets. Since Chl282 could be
152 considered a general probe and Chl282bis is specific for the newly defined cluster II we
153 propose to use the probes together as a general probe for *Chlamydiae* since they target 251
154 out of 264 sequences currently published for *Chlamydiae.* It would be also possible to use
155 them as a new general chlamydial primer set. Ultimately, the result could deserve attention
156 not only from a phylogenetic but also from a sanitary point of view since there is already
157 evidence of pathogenicity of environmental chlamydiae.
158
159 **Experimental procedures**
160 Surface water samples were collected on September 15th, 2008 in the central area of
161 the Lago di Paola at the surface (SAB2) and bottom (SAB2b) of the lake, the latter
162 representing the deepest point in the lake (about 10 m depth). During this sampling
163 temperature, salinity and dissolved oxygen were recorded.
164 Samples for CARD-FISH were fixed either for 1 hour at room temperature and or
165 up to 24 hours at 4 °C with paraformaldehyde solution (20% w/v, Electron Microscopy
166 Sciences, Hatfield, PA; final concentration 1%). Afterwards, aliquots were filtered at low
167 vacuum (200 mbar) onto 0.2 μm pore-size polycarbonate filters (type GTTP; diameter, 47
168 mm; Millipore, Eschborn, Germany) after prefiltration through 10 μm pore-size
169 polycarbonate filters (type TCTP; diameter, 47 mm; Millipore). CARD-FISH was
170 performed according to protocols by Pernthaler and colleagues (Pernthaler et al., 2002,
171 2004) and slightly modified by Pizzetti and coworkers (Pizzetti et al., submitted). Multi-
172 color CARD-FISH was done according to the protocol by Pernthaler and colleagues
173 (Pernthaler et al., 2004) with the following modifications: (i) after the first hybridization
174 the probe delivered HRP was inactivated by 3% H_2O_2, (ii) Alexa 488 and 594 labeled
175 tyramides were used.

For DNA isolation, between 750 and 1250 ml aliquots were filtered onto 0.2 µm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore) after prefiltration through 10 µm pore-size polycarbonate filters (type TCTP; diameter, 47 mm; Millipore). Filters were frozen and kept at -20 °C until processed for nucleic acid extraction. Total nucleic acids were extracted according to the protocol of Zhou and colleagues (Zhou et al., 1996) modified by Pizzetti and coworkers (Pizzetti et al., submitted).

Almost full-length 16S rRNA genes were amplified by PCR using 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 universal reverse primer 1392R [5'-ACG GGC GGT GTG TAC-3', targeting *E. coli* position 1392-1406 on 16S rRNA gene; (Xu et al., 1998)]. PCR reaction, amplification, cloning and sequencing were carried out as previously described (Pizzetti et al., submitted). The 16S rRNA gene sequences of this study were deposited in the EMBL Nucleotide Sequence Database with the accession numbers FR714401- FR714420.

Contigs were assembled with the software SEQUENCHER Version 4.6 (Gene Codes Corporation, Ann Harbor, USA) resulting in sequences encompassing almost full length 16S rRNA genes. The sequences were aligned in the ARB software package [http://www.arb-home.de; (Ludwig et al., 2004)] using the SINA_aligner (Pruesse et al., 2007). Alignments were further refined manually by comparison of closest relatives. Only sequences with more than 1300 nucleotides were included. Phylogenetic tree reconstruction was based on distance matrix (DM) (e.g. neighbor-joining) and maximum likelihood (ML) methods with and without 30% and 50% chlamydial positional conservatory filters. A consensus tree was constructed thereafter (Peplies et al., 2008).

Two oligonucleotide probes targeting chlamydial 16S rRNA were designed based on the newly created phylogenetic tree using the probe design tool of the ARB software.

Probe specificities were checked *in silico* against the SILVA 16S rRNA database [version 100; (Pruesse et al., 2007)] (Table S1). For optimization of stringency conditions, a series of hybridizations at increasing formamide concentrations (10–40%) were evaluated. The optimal formamide concentration was the highest concentration before signal intensity decreased.

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371 **Figure legends**

372 **Fig. 1:** Consensus tree based on maximum likelihood and neighbor joining trees calculated
373 without and with 50% and 30% chlamydial positional conservatory filters using
374 *Planctomycetes*, *Verrucomicrobia* and *Lentisphaerae* as outgroup. Sequences
375 targeted by the two newly designed probes (0 weighted mismatches) retrieved from
376 this study together with closest relatives are pointed out by a color code (red:
377 CHL282; blue: CHL282bis). SAB2: surface sample. The red rectangle surrounds
378 sequences belonging to the cluster I and the blue rectangle sequences belonging to
379 the cluster II. Type strains are surrounded by a grey rectangle. The green rectangle
380 surrounds sequences belonging to the family *Simkaniaceae*. *: the sequence is not
381 belonging to the family *Simkaniaceae*. Accession numbers and sequence length are
382 displayed. The bar represents 10% sequence divergence.

383

384 **Fig. 2**: (A) and (B) reproduced from Amann et al., 1997: Trophozoite of *Acanthamoeba*
385 *mauritaniensis* $Berg_{17}$ with one large and several small vacuoles containing
386 different developmental stages of the obligate intracellular parasites. Arrows mark
387 the thin-walled gram-negative RBs, the gram-positive condensed infective stages
388 EBs are shown by arrowheads. Magnifications, ca. 7,400 (A) and 28,000 (B). N,
389 nucleus of the host amoeba; mi, mitochondria; um, unit membrane. (c) and (d):
390 Epifluorescence micrographs of bacteria from the surface of Lago di Paola. Blue,
391 DAPI signals of DNA containing cells; green, fluorescein (d) and Alexa 488 (c)
392 signals of catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-
393 FISH)-positive cells using the probes: (c) Chl282bis, (d) Chl282. In the picture (c):
394 Red, background fluorescence due to unspecific binding of Alexa 594. Bar = 5µm.

395

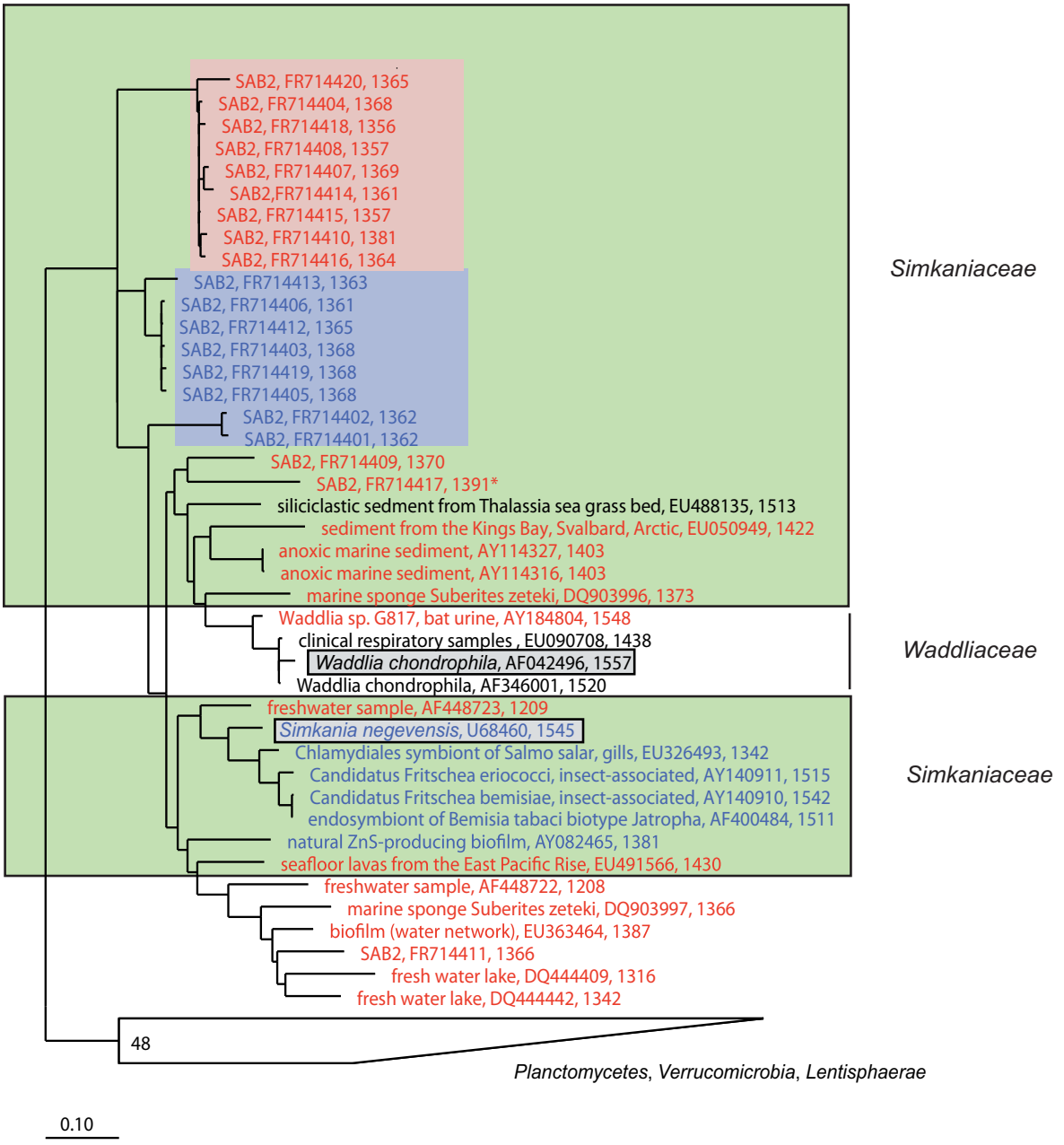
SAB2, FR714420, 1365
SAB2, FR714404, 1368
SAB2, FR714418, 1356
SAB2, FR714408, 1357
SAB2, FR714407, 1369
SAB2,FR714414, 1361
SAB2, FR714415, 1357
SAB2, FR714410, 1381
SAB2, FR714416, 1364
SAB2, FR714413, 1363
SAB2, FR714406, 1361
SAB2, FR714412, 1365
SAB2, FR714403, 1368
SAB2, FR714419, 1368
SAB2, FR714405, 1368
SAB2, FR714402, 1362
SAB2, FR714401, 1362
SAB2, FR714409, 1370
SAB2, FR714417, 1391*
siliciclastic sedment from Thalassia sea grass bed, EU488135, 1513
sediment from the Kings Bay, Svalbard, Arctic, EU050949, 1422
anoxic marine sediment, AY114327, 1403
anoxic marine sediment, AY114316, 1403
marine sponge Suberites zeteki, DQ903996, 1373
Waddlia sp. G817, bat urine, AY184804, 1548
clinical respiratory samples , EU090708, 1438
Waddlia chondrophila, AF042496, 1557
Waddlia chondrophila, AF346001, 1520
freshwater sample, AF448723, 1209
Simkania negevensis, U68460, 1545
Chlamydiales symbiont of Salmo salar, gills, EU326493, 1342
Candidatus Fritschea eriococci, insect-associated, AY140911, 1515
Candidatus Fritschea bemisiae, insect-associated, AY140910, 1542
endosymbiont of Bemisia tabaci biotype Jatropha, AF400484, 1511
natural ZnS-producing biofilm, AY082465, 1381
seafloor lavas from the East Pacific Rise, EU491566, 1430
freshwater sample, AF448722, 1208
marine sponge Suberites zeteki, DQ903997, 1366
biofilm (water network), EU363464, 1387
SAB2, FR714411, 1366
fresh water lake, DQ444409, 1316
fresh water lake, DQ444442, 1342
48
Planctomycetes, Verrucomicrobia, Lentisphaerae
Simkaniaceae
Waddliaceae
Simkaniaceae
0.10

Fig. 1

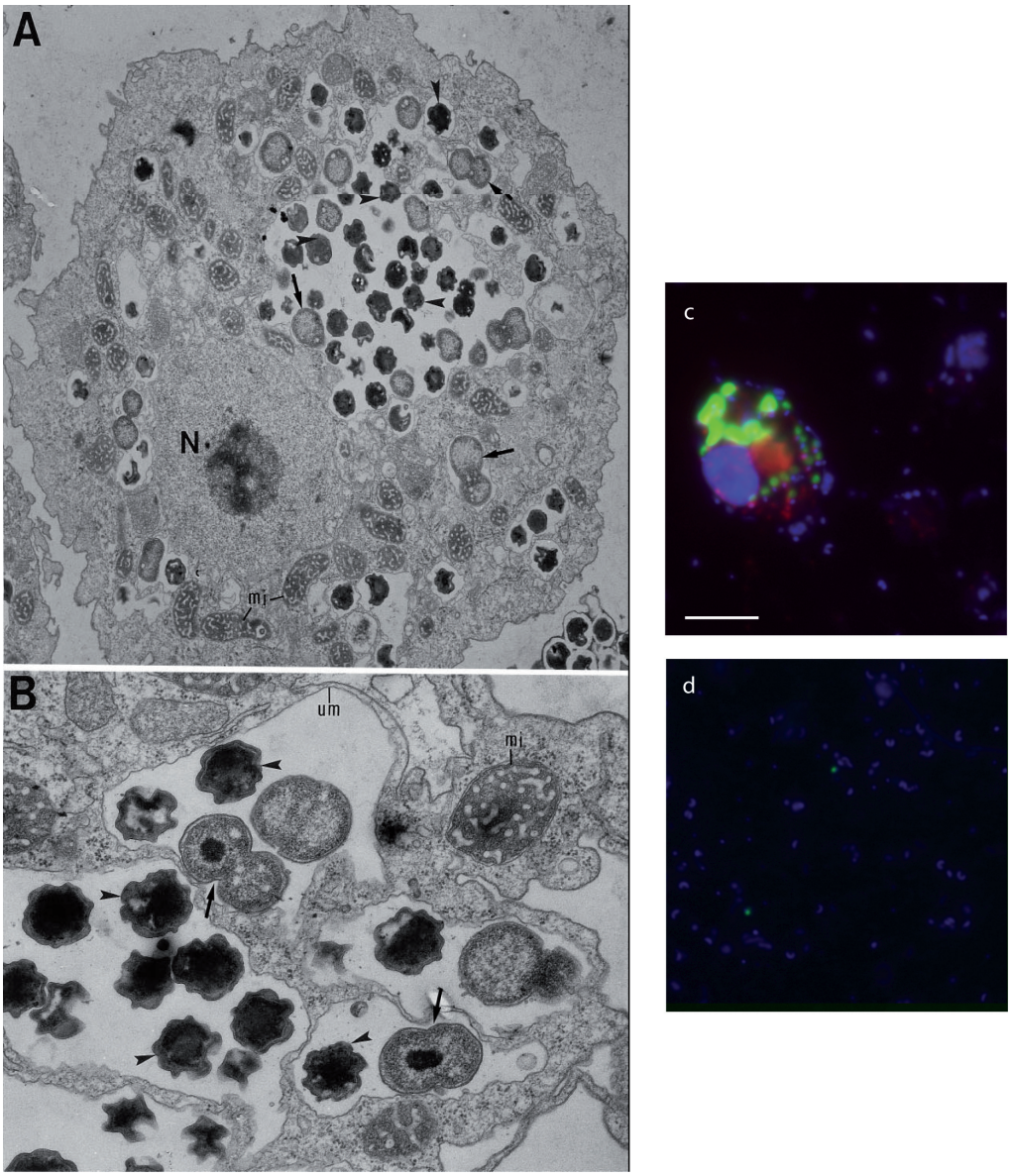

Fig. 2

Table S1: Specificity of probes: Chl282 (25% FA) and Chl282bis (30% FA). Dots indicate full identity to the probe target sequence. Upper-case letters indicatenucleotide changes leading to strong mismatches; lower-case letters indicate changes leading to weak mismatches. The isolation source and the weighted mismatches (w. m.) of non-target organisms are represented. Y (C/U). In red the nucleotide that differs between the two probes.

Chl282	**isolation source**	**w. m.**	Probe (3-'5')	TGCAGATCCGCCTAACTC
Cluster I			Target (5'-3')	ACGUCUAGGCGGAUUGAG
uncultured *Chlamydia* sp./*Waddlia* sp./clone from Candidate division OP10	anoxic marine sediment/bat urine/biofilm reactor	0.2		..........g.....
clones from Candidate division WS3	soil/hypersaline microbial mat	0.6		g.............gY
FR714413 (+6)/clones from Candidatus Fritschea	our sample/*Salmo salar*/insect-associated	1.1		..........C....
Simkania negevensis +FR714401	contamined Vero cells	1.3		..........gC....
Arcobacter butzleri (epsilonproteobacteria)	humans	1.6		g...G..........U
Chl282bis			Probe (3-'5')	TGCAGATCCGCCTGACTC
Cluster II			Target (5'-3')	ACGUCUAGGCGGACUGAG
Simkania negevensis + FR714401	contamined Vero cells	0.2		..........g.....
FR714420 (+10)/uncultured *Chlamydiales* bacteria/*C. suis*/*C. psittaci*/*C. pecorum*/*C. abortus*/*C. trachomatis*	our sample/fresh water/waste water treatment plants/animals	0.6		..........u....
uncultured *Chlamydia* sp./*Waddlia* sp./clone from Candidate division OP10	anoxic marine sediment/bat urine/biofilm reactor	0.8		..........gu....
Candidatus Pisciclamydia salmonis	salmon gill	1		...C..........
clones from Candidate division WS3	hypersaline microbial mat	1.2		g..........u..gC
uncultured *Lentisphaerae*/*Firmicutes*	wastewater sludge/rumen/sediment/colobus feces	1.3		...g.....u.....
uncultured *Firmicutes*/*Alphaproteobacteria*	sheep feces/maricultured scallop	1.6		g...G..........Y

3. General Conclusions and Outlook

3.1. Abundance of *Planctomycetes* in coastal zones

Relative abundances of *Planctomycetes* in seawater samples analyzed throughout the year were between 0.5-5% at station "Kabeltonne" near the North Sea island Helgoland (Germany). Comparing our results with data previously reported for the Helgoland sampling site (Glöckner et al., 1999) and for other marine waters (Schattenhofer et al., 2009), *Planctomycetes* abundances showed higher values in this study. *Planctomycetes* abundances analyzed from summer to winter seasons in the Tyrrhenian Sea varied between 0.8% and 2%. At the beginning of the previous century, it was realized at Helgoland that rapid changes, mostly anthropogenically induced, such as the effects of increased pollutants and nutrients, could affect the marine system (Wiltshire et al., 2010). Although since the early 1980's a decrease in all nutrients has been observed, nutrient inputs are still higher in the North Sea compared to the oligotrophic conditions typical of Tyrrhenian coastal areas, thus affecting the abundance of *Planctomycetes* in such environments. Nevertheless, the relative abundance of *Planctomycetes* in Lago di Paola, a coastal lake located along the Tyrrhenian coast of central Italy (*Latium*), varied between 1% and 5% on the transitional period from summer to winter seasons when physico-chemical characteristics of the lake changed drastically. Lago di Paola is a highly variable aquatic environment compared to the more stable marine system and *Planctomycetes* abundances at the marine station are lower because of lower DOC. The physico-chemical data clearly identify this coastal lake as a meso-eutrophic system which apparently does not directly influence the adjacent marine coastal site investigated in this study. Overall we can conclude that results obtained from the North Sea are comparable with values from the meso-eutrophic Lago di Paola.

Planctomycetes abundances analyzed from samples collected both at Helgoland and Lago di Paola showed a high seasonality. At Helgoland an increase in absolute numbers was registered in spring showing a positive correlation with centric diatoms (*Centrales*) and a negative correlation with nutrients depleted by the growing algae. Moreover, at Lago di Paola *Planctomycetes* increased in summer - during stratification - in surface waters and mainly in response to high DOC and chlorophyll concentrations. Overall, the similar results obtained by both studies suggested *Planctomycetes* are bottom-up controlled and most likely benefit by algal production. These results and particularly the correlation between *Planctomycetes* and diatoms provide further evidence for a direct interaction and carbon flow between algae and *Planctomycetes* as suggested previously by

Morris and coworkers (Morris et al., 2006). Furthermore, genomic and metagenomic evidence indicates a close link of *Planctomycetes* with the degradation of algal biomass (Glöckner et al., 2003; Woebken et al., 2007b). In detail, the genome annotation of *Rhodopirellula baltica* SH 1^T suggested that it derives its energy from degradation of sulfated polymers produced mainly by algae (carrageenans, agars, sulfated fucans) and entrapped in macroaggregates (Glöckner et al., 2003; Schlesner et al., 2004). Comparing all planctomycete genomes available with six planctomycete fosmids from two marine upwelling systems Woebken and coworkers (Woebken et al., 2007b), similar to what was shown for *Rhodopirellula baltica* SH 1^T, found a high number of sulfatase genes in all marine planctomycete genomes and in two fosmids. Sulfated polymers are complex compounds, hence it requires a versatile repertoire of specific sulfatases for biodegradation and that could explain the high copy numbers in the marine *Planctomycetes* genomes. Moreover, the main storage carbohydrate in marine diatoms is the polysaccharide chrysolaminarin which can contribute up to 80% of the organic carbon (Alderkamp et al., 2006). While it is located intracellularly in live algae (Chiovitti et al., 2004) it may be released by algal cell lysis (Brussaard et al., 1995). All marine cultured *Planctomycetes* so far seem to be specialized in sugar metabolism (Ward et al., 2006; Woebken et al., 2007b). Even if to date there is no direct proof that marine strains use chrysolaminarin as a carbon source the increase of *Planctomycetes* absolute numbers coinciding with the end of a *Centrales* bloom, when most probably there is a massive algal lysis, suggests this compound as a possible link between *Planctomycetes* and diatoms. This hypothesis requires further investigation. While Morris and coworkers showed a general coupling between diatoms and *Planctomycetes*, evidences of the specific correlation between *Centrales* and *Planctomycetes* abundances have not yet been reported, although the diatom images provided by Morris and coworkers showed centric-like diatom cells. Since we did not study a bloom of pennate diatoms we can not exclude that *Planctomycetes* might benefit from these also, which is quite likely as the cellular composition is similar in centric and pennate diatoms.

Only for samples of the North Sea, between late spring and summer we compared *Planctomycetes* abundances of 0.2-3 µm and 3-10 µm fractions in water samples with the abundances in marine aggregate samples. Our results showed that most of *Planctomycetes* are attached-living bacteria (4.8 - 8.5 x 10^4 cells ml^{-1}). Indirect evidence for that life style was so far mostly coming from clone frequencies in SSU libraries (DeLong et al., 1993;

Rath et al., 1998; Crump et al., 1999). In our study we corroborated this by *in situ* hybridization techniques (Fig. 8). In fact, *Planctomycetes* are well known for their ability to attach to surfaces by the excretion of holdfast substances (Ward et al., 2006).

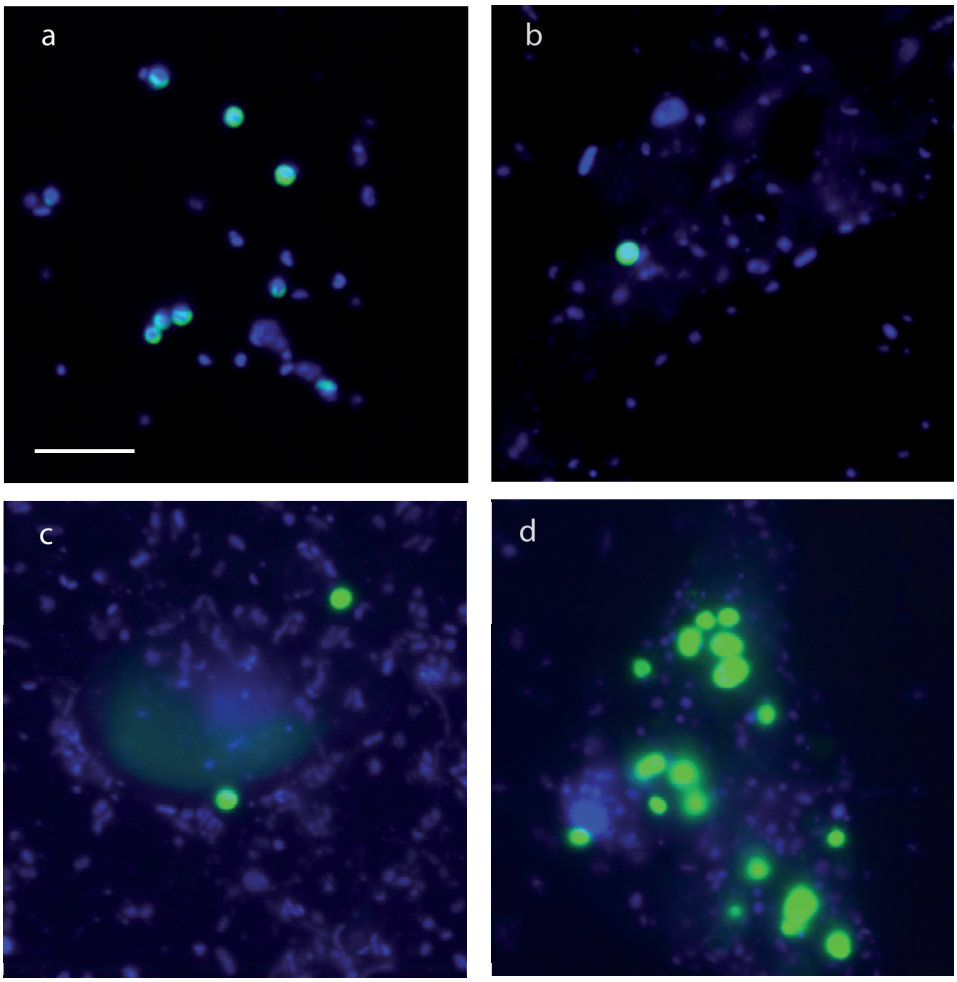

Fig. 8: Epifluorescence micrographs of *Planctomycetes* from Helgoland water column and Lago di Paola samples. Blue, DAPI signals of DNA containing cells; green, signals of catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)-positive cells using the probes: (a), (c): PLA46 (Helgoland), (b): PlaA1228 (Helgoland), (d) PLA46 (Lago di Paola). (c): *Planctomycetes* and phytoplankton cells. Bar = 5µm.

Beside surface water samples, at Lago di Paola we also analyzed samples collected at a small depression reaching 10 m depth and representing the deepest point in the lake. The conditions seem to be more stable, e.g. during hypoxia in summer, and the abundances of *Planctomycetes* were less variable compared to surface waters. At the end of the summer when the water column became completely hypoxic *Planctomycetes* abundance was higher at the lake bottom than at the surface suggesting that *Planctomycetes* are strongly influenced by the change from oxic to hypoxic conditions occurring in the surface water.

In these studies molecular techniques such as FISH and CARD-FISH were used to identify and quantify *Planctomycetes* in the environment. Since the CARD-FISH includes a permeabilization step before the enzymatic signal amplification and the general protocol of Pernthaler and colleagues (Pernthaler et al., 2002) was not specifically tested for *Planctomycete*s, here different permeabilization treatments were applied to optimize the CARD-FISH protocol for this phylum. As originally suggested by Woebken and colleagues (Woebken et al., 2007a) for anammox bacteria, achromopeptidase was used to specifically permeabilize the proteinaceous cell wall of Planctomycetes (König et al., 1984; Liesack et al., 1986). Currently, PLA46 and PLA886 (Neef et al., 1998) are the probes available for the quantification of *Planctomycetes*. In these studies, PLA46 was used because it is more specific than PLA886 in terms of outgroup hits (Amann and Fuchs, 2008). Furthermore, PLA886 hybridizes to the 18S rRNA of many eukaryotes, including diatoms. PLA46 does not cover all *Planctomycetes*, but we confirmed *in silico* by a cross-check with the curated high-quality SSU database SILVA (Pruesse et al., 2007) that the marine genera were all detected. We therefore assumed that PLA46 provided reliable counts of total *Planctomycetes* in our coastal marine systems, however it is possible that the probe does not target all *Planctomycetes* in Lago di Paola where the diversity is much higher.

3.2. *Planctomycetes* diversity and distribution

With this studies 224 almost full length 16S rRNA gene sequences of *Planctomycetes* have been added to the database. By comparative sequence analysis in winter and summer samples and in 3-10 µm and 0.2-3 µm fractions in the North Sea and in surface and bottom waters at the end of the summer at Lago di Paola we could define six stable coastal groups (Fig. 9). *Pirellula*-related group D and *Pirellula*-related group C were exclusively found in Helgoland samples while *Pirellula*-related group E and *Pirellula*-related group F belonged to Lago di Paola samples (Fig. 9). Uncultured *Planctomycetes* group B and *Planctomyces*-related group A comprised sequences retrieved both from Helgoland and Lago di Paola samples (Fig. 9).

Sequence identities of clades described in Helgoland and Lago di Paola samples were compared. *Pirellula*-related group D shared $\simeq$ 88% sequence identity with *Pirellula*-related group E and $\simeq$ 87% with *Pirellula*-related group F. It also shared $\simeq$ 88% sequence identity with *Blastopirellula marina*, the closest cultured relative, and $\simeq$ 89% with strains of the genus *Pirellula*. Based on CARD-FISH quantification the *Pirellula*-related group D, targeted by the newly developed probe PirD1039 (Table 1), was more abundant in the North Sea in summer than in winter 2007 and 2009. Most of the retrieved clones belonged to the 3-10 µm fraction and they were closest related to published sequences retrieved from marine sponges and the surface of marine macro-alga suggesting that this clade prefers an attached life style. Sequences from *Pirellula*-related group C shared $\simeq$ 87% and $\simeq$ 85% sequence identities with *Pirellula*-related group E and *Pirellula*-related group F, respectively, and $\simeq$ 93% sequence identity with *Rhodopirellula baltica* and $\simeq$ 92% with strains of the genus *Pirellula.*

On the other hand, *Pirellula*-related group E was closely related to *Blastopirellula marina* and strains of the genus *Pirellula* sharing with them $\simeq$ 89% sequence identity. Most clones showed best affiliation with sequences retrieved from waste water treatment plant, river biofilms and mangrove soil. Sequences of the *Pirellula*-related group F shared $\simeq$ 87% sequence identity with strains of the genus *Pirellula* and $\simeq$ 86% with *Blastopirellula marina*. The clones obtained in this study were closest related to published sequences retrieved from marine sediment and the surface of marine macro-alga. The clade-specific CARD-FISH counts with the newly designed probes PirE62 and PirF85 (Table 1) showed their presence in the surface and bottom water of the Lago di Paola in similar abundances.

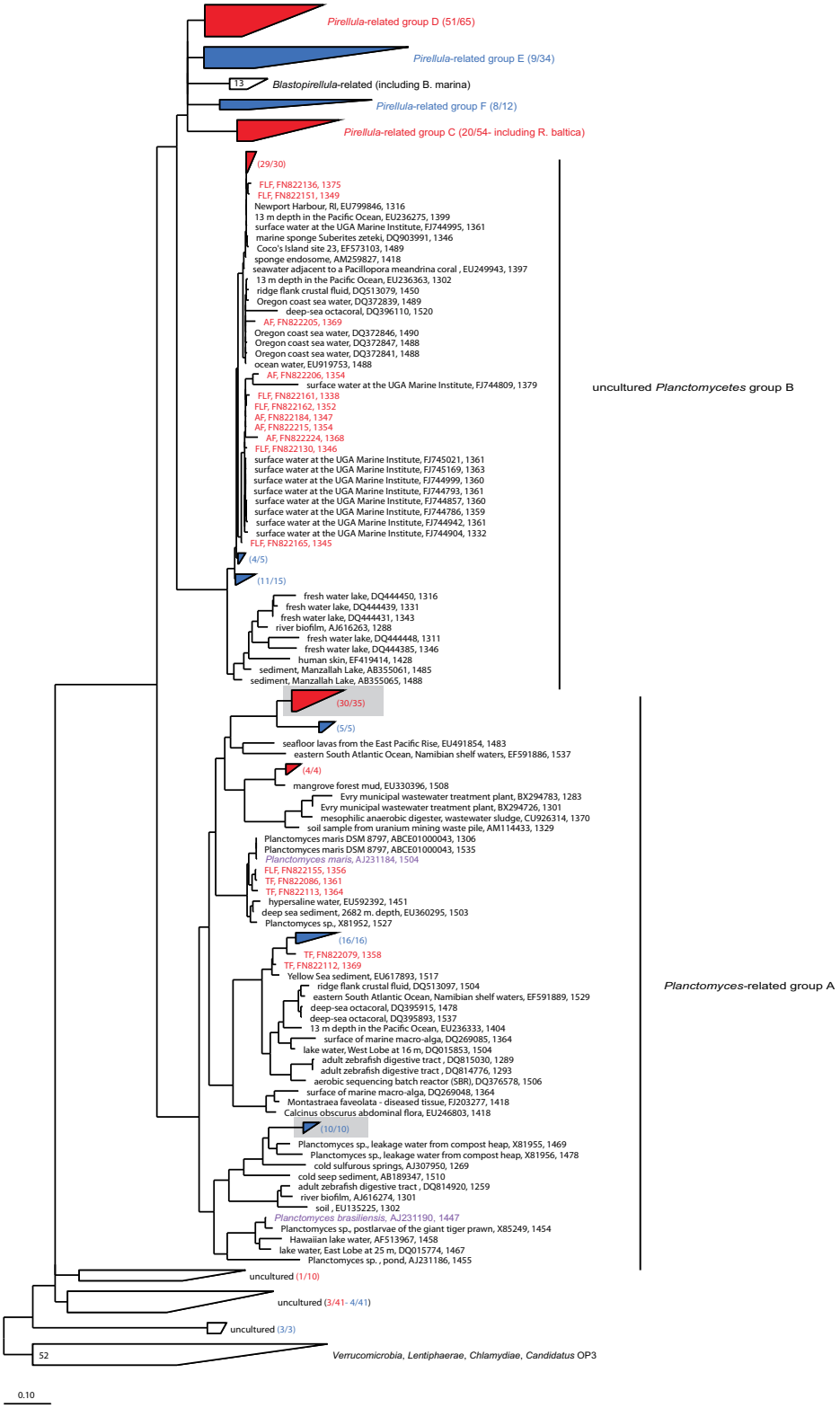

Fig. 9: Phylogenetic tree based on almost full length 16S rRNA sequences (> 1200 nucleotides) showing the affiliations of different clusters from Helgoland and Lago di Paola samples. The consensus tree was built based on maximum likelihood and neighbor joining trees calculated without and with 50% and 30% *Planctomycetes* positional conservatory filters using *Verrucomicrobia, Lentisphaerae, Chlamydiae* and *Candidatus* OP3 sequences as outgroup. Sequences retrieved in this study are pointed out by a color code (Helgoland samples: red; Lago di Paola samples: blue; type strains: violet). Total sequences available and total sequences retrieved in each study are stated in parentheses. Accession numbers and sequence length are shown. In grey boxes the monophyletic clades within *Planctomyces*-related group A targeted by two newly designed probes (PlaA1228, PlaS2114). The bar represents 10% sequence divergence.

The clade of uncultured *Planctomycetes* group B has been first described at Helgoland and subsequently at Lago di Paola. Sequences from Helgoland shared ≃ 97% sequence identity with sequences from Lago di Paola samples, thus it was possible to use the same newly designed probe uPlaB440 (Table 1) to quantify the clade in both samples. In the North Sea, in the 2007 samples, CARD-FISH counts of uncultured *Planctomycetes* group B peaked in October. This clade seems to be more abundant in the 3-10 µm fraction although it could be detected also in the 0.2-3 µm fraction. In contrast with this result this clade contains clones mostly retrieved from the 0.2-3 µm fraction and most closely related to sequences retrieved from seawater but also from marine sponges. In 2009 uPlaB440 counts were highest in September and October with a seasonal pattern similar of that of the year 2007. On the other hand, uncultured *Planctomycetes* group B showed the same cell numbers in the surface and bottom of Lago di Paola. New clones added from this study to the group also resulted closely related to seawater and marine sponges. We here demonstrated that this group might be more widely distributed in coastal marine habitats. Interestingly, probe uPlaB440 visualized in addition to the common doughnut-shaped cells also a second, rather rod-shaped morphotype indicating the presence of distinct subpopulations.

Since sequences belonging to *Planctomyces*-related group A retrieved from Helgoland samples shared only ≃ 87% with sequences from Lago di Paola two different probes were designed and used to quantify different monophyletic clades within *Planctomyces*-related group A. PlaA1228 targeted sequences retrieved from Helgoland samples while PlaS2114 was designed to target sequences obtained from Lago di Paola samples (Fig. 9). In the North Sea, only three clones of the group were closely related to *Planctomyces maris* (≃ 98% sequence identity), the rest shared ≃ 88% sequence identity with *Planctomyces maris* and ≃ 87% sequence identity with *Planctomyces brasiliensis*. *Planctomyces*-related group A (PlaA1228, Table 1) was more abundant in winter and autumn and almost not present in summer of 2007. The clade comprised sequences mainly retrieved from winter and closest related to sea water, sediment and host (marine bivalve) gut. Also in 2009 the *Planctomyces*-related group A was most abundant in autumn. Interestingly, the more finely resolved clade-specific counts from 2009 provide a first hint to a correlation of *Planctomyces*-related group A and the autumn bloom of pennate diatoms (*Pennales*). Sequences from *Planctomyces*-related group A (PlaS2114, Table 1) at Lago di Paola showed the highest identity (≃ 91 % sequence identity) with sequences from

compost leakage water, cold sulphurous springs and cold seep sediment and ≃ 88% sequence identity with *Planctomyces brasiliensis*, the closest cultured relative, that was isolated from a hypersaline lake in Brazil. The *Planctomycetes*-related group A clade seems to have a wide habitat range. The clade was present in the surface and bottom water of lake in similar abundances.

Overall at Helgoland the clade-specific CARD-FISH counts, ranging between 0.1% and 1.9% of the total picoplankton, showed a seasonal pattern and we were able to detect between 32 and 103% of the *Planctomycetes* community in 2007 and between 12 and 93% of the *Planctomycetes* community in 2009. We conclude that this new set of probes enables the quantification of a major fraction of the marine coastal *Planctomycetes*. It would be interesting to apply this set of probes to other coastal sites and to test our hypotheses on a correlation of *Planctomycetes* with particular groups of algae (i.e. pennate and centric diatoms). At the long-term ecological research site "Kabeltonne", Helgoland, it should be specifically checked whether the 2009 succession of *Pirellula*-related group D at the beginning of the summer, followed by uncultured *Planctomycetes* group B towards the end of the summer and finally the dominance of *Planctomycetes*-related group A in autumn is a stable seasonal pattern.

At Lago di Paola, cell numbers confirmed the simultaneous presence of all clades in the surface and bottom water in similar abundances. None of the clades accounted for more than 1% of the total picoplankton in the samples examined. We can, however, not exclude that the four *Planctomycetes* clades examined in this study might bloom in particular settings. The diversity of *Planctomycetes* in Lago di Paola was only partially covered in this study. The coverage of *Planctomycetes* diversity was 84% and 87% and 74% and 77% at species and genus levels at the surface and bottom, respectively, suggesting higher *Planctomycetes* diversity particularly at the bottom water of the lake. With the four clade-specific probes used in this study we were able to detect between 26% and 28% of the PLA46 counts in the surface and bottom waters of the lake, respectively. This indicates the presence of additional, yet unidentified clades of *Planctomycetes* in Lago di Paola that should be definitely investigated in the future by further comparative sequence analysis and the development of additional clade-specific oligonucleotide probes. It would be interesting to apply, in different seasons and years, the new set of probes specific for clades found in the Lago di Paola to check whether there is a seasonal succession and to test whether particular clades of *Planctomycetes* are indeed correlated to the occurrence of

specific phytoplankton. Such studies must also consider that the diversity of *Planctomycetes* in Lago di Paola is high and has not yet been fully covered.

The Pla46F primer, specific for *Planctomycetes,* together with the universal primer 1392R were chosen to determine *Planctomycetes* diversity in the Helgoland and in Lago di Paola water columns as previous studies already suggested (Chouari et al., 2003; Elshahed et al., 2007). However, recently, Shu and Jiao (Shu & Jiao, 2008a) suggested and tested a new primer set to amplify seawater DNA showing that, in comparison with the commonly used, it both reduced mismatches to non-planctomycetes and supplemented *Planctomycetes* diversity in their sample. Testing this primer set on our sequences it reveals that the mismatches to non-planctomycetes can be reduced but at the same time it underestimates the *Planctomycetes* diversity in our sample, not amplifying the 16S rRNA of *Planctomycetes*-related group A. This result confirms that the primer set we used is the best one available even if, in the future, it might be possible, to design more specific primers based, for example, on PCR-independent metagenome libraries, in order to reduce the mismatches to non-planctomycetes.

Table 1: *Planctomycetes* specific oligonucleotide probes.

Probe	Target organism	Sequence (5' → 3')	FA[a] [%]	Reference
PLA46	*Planctomycetes*	GACTTGCATGCCTAATCC	30	(Neef *et al*., 1998)
PirD1039	*Pirellula*-related group D	ATGCAGCACCTACGTAAG	30	(Helgoland study)
uPlaB440	uncultured *Planctomycetes* group B	TGCATTTCCTCCCCAACG	30	(Helgoland study)
PlaA1228	*Planctomyces*-related group A	AATACCACGCAGACCGCT	30	(Helgoland study)
PirF85	*Pirellula*-related group F	GCTGTCCGTCTTAATGCA	45	(Lago di Paola study)
PirE62	*Pirellula*-related group E	AGGAAGCTTCTCGCACGA	20	(Lago di Paola study)
PlaS2114	*Plantomycetes*-related group A	GTTCCGATGCCTTACTAC	30	(Lago di Paola study)

a. Formamide (FA) concentration (v/v) in CARD-FISH hybridization buffer.

3.3. Novel environmental chlamydiae

While monitoring *Planctomycetes*, 21 16S rRNA gene sequences related to the order *Chlamydiales* and affiliated with the family *Simkaniaceae* were exclusively retrieved from the surface of Lago di Paola. The primer pair PLA46F-1392R resulted to be not specific for *Planctomycetes* and it is not surprising since PLA46F is also amplifying the 16S rRNA gene of some outgroups including the phylum *Chlamydiae* (family *Simkaniaceae*).

Two clusters which consisted of 9 (cluster I) and 8 (cluster II) closely related sequences were identified (Fig. 10). Those clusters remained stable in different types of tree reconstructions (neighbour joining, maximum likelihood) with and without chlamydial positional conservatory filters (50% and 30%). The cluster I shared ≃ 98% sequence identity within its sequences, ≃ 88% sequence identity with the cluster II, ≃ 85% sequence identity with *Waddlia chondrophila* and *Simkania negevensis* (Fig. 10) and 87% sequence identity with *Neochlamydia hartmannellae* and *Parachlamydia acanthamoebae*. Furthermore, cluster I resulted to be closely related to uncultured *Chlamydia* sp. (AY114327) retrieved from anoxic marine sediment (Fig. 10) and *Candidatus* Protochlamydia naegleriophila (DQ632609) (≃ 88% sequence identity). A new developed oligonucleotide probe Chl282 targeted the cluster I but also almost all sequences belonging to *Chlamydiales* and one sequence that belongs to Candidate division OP10. Evidence was obtained for an abundance > 10^4 individual small coccoid cells per ml on the surface and at the bottom of the lake. Since Chl282 is not specific for the cluster I, most likely the probe allowed us to quantify the majority of *Chlamydiales* in such environment. Hence we suggest for the future to use Chl282 together with Chl282bis to quantify the complete order. CARD-FISH showed a dot-shaped morphology of those tiny cells and no host could be identified (Fig. 11d). That should be due to the prefiltration treatment in 10 µm pore-size filters that most probably removes the host.

The cluster II shared ≃ 94% sequence identity within its sequences, ≃ 87% sequence identity with *Simkania negevensis* and *Neochlamydia hartmannellae* and ≃ 88% sequence identity with *Parachlamydia acanthamoebae.* It was mostly closest related to the uncultured bacterium (EU491566) from seafloor lavas from East Pacific Rise (Fig. 10), Chlamydiales bacterium CRIB33 (EU683887) from raw surface water by Acanthamoeba co-culture, *Candidatus* Protochlamydia amoebophila UWE25 (BX908798) and *Parachlamydia acanthamoebae* from river water (DQ309029) (≃ 88% sequence identity).

The new oligonucleotide probe Chl282bis that differs from the probe Chl282 for just one nucleotide (Table 2) is specific for the cluster II. CARD-FISH showed cells with dot-shaped morphology and interestingly a host was visualized, thus chlamydial cells were identified in different developmental stages, outside the host as EBs-like and inside the host as RBs-like (Fig. 11c). Unfortunately it was not possible to classify the host that most likely is a protist.

The multi-color CARD-FISH showed no overlap between the two probes confirming that they target different sequences within *Chlamydiae*. Besides we suggest to use Chl282bis with Chl282 as a competitor to definitively discriminate between clusters. Among available oligonucleotide probes only Bn_9658 (Amann et al., 1997) is able to target almost all our sequences but is not specific only for them. Since Chl282 could be considered a general probe and Chl282bis is specific for the newly defined cluster II we propose to use the probes together as a general probe for *Chlamydiae* since they target 251 out of 264 sequences currently published for *Chlamydiae.* Furthermore, all sequences from this study resulted to be not covered by available chlamydial primer sets. It would be also possible to use Chl282 and Chl282bis together, for example, with 1392R as a new general chlamydial primer set. Ultimately, the result could deserve attention not only from a phylogenetic but also from a sanitary point of view since there is already evidence of pathogenicity of environmental chlamydiae.

Fig. 10: Consensus tree based on maximum likelihood and neighbor joining trees calculated without and with 50% and 30% chlamydial positional conservatory filters using *Planctomycetes*, *Verrucomicrobia* and *Lentisphaerae* as outgroup. Sequences targeted by the two newly designed probes (0 weighted mismatches) retrieved from this study together with closest relatives are pointed out by a color code (red: CHL282; blue: CHL282bis). SAB2: surface sample. The red rectangle surrounds sequences belonging to the cluster I and the blue rectangle sequences belonging to the cluster II. Type strains are surrounded by a grey rectangle. The green rectangle surrounds sequences belonging to the family Simkaniaceae. *: the sequence is not belonging to the family Simkaniaceae. Accession numbers and sequence length are displayed. The bar represents 10% sequence divergence.

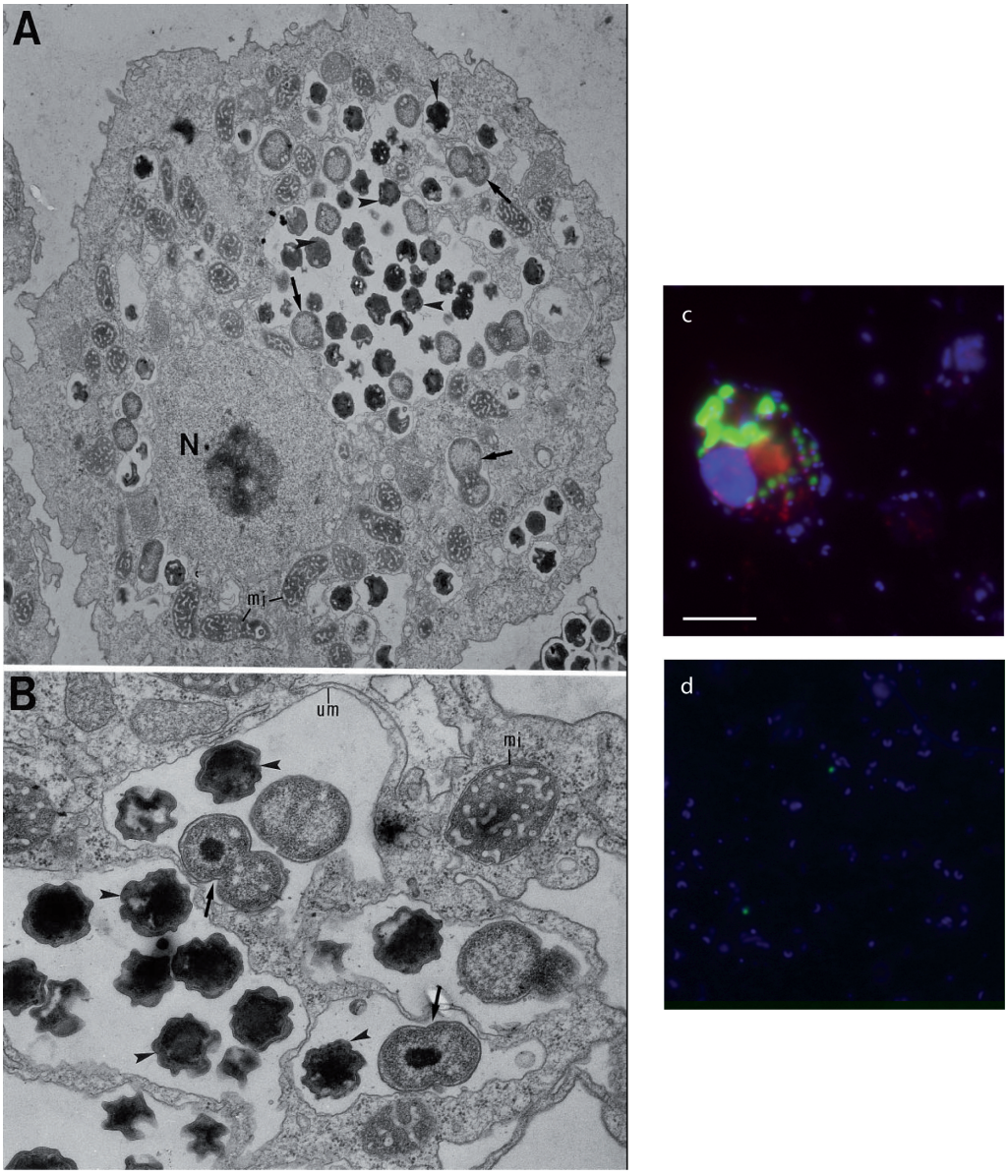

Fig. 11: (A) and (B) reproduced from Amann et al., 1997: Trophozoite of *Acanthamoeba mauritaniensis* $Berg_{17}$ with one large and several small vacuoles containing different developmental stages of the obligate intracellular parasites. Arrows mark the thin-walled gram-negative RBs, the gram-positive condensed infective stages EBs are shown by arrowheads. Magnifications, ca. 7,400 (A) and 28,000 (B). N, nucleus of the host amoeba; mi, mitochondria; um, unit membrane. (c) and (d): Epifluorescence micrographs of bacteria from the surface of Lago di Paola. Blue, DAPI signals of DNA containing cells; green, fluorescein (d) and Alexa 488 (c) signals of catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)-positive cells using the probes: (c) Chl282bis, (d) Chl282. In the picture (c): Red, background fluorescence due to unspecific binding of Alexa 594. Bar = 5µm.

Table 2: Specificity of probes: Chl282 (25% FA) and Chl282bis (30% FA). Dots indicate full identity to the probe target sequence. Upper-case letters indicate nucleotide changes leading to strong mismatches; lower-case letters indicate changes leading to weak mismatches. The isolation source and the weighted mismatches (w. m.) of non-target organisms are represented. Y (C/U). In red the nucleotide that differs between the two probes.

Chl282	**isolation source**	**w. m.**	Probe (3-'5')	TGCAGATCCGCCTAACTC
Cluster I			Target (5'-3')	ACGUCUAGGCGGAUUGAG
uncultured *Chlamydia* sp./*Waddlia* sp./clone from Candidate division OP10	anoxic marine sediment/bat urine/biofilm reactor	0.2		..........g.....
clones from Candidate division WS3	soil/hypersaline microbial mat	0.6		g..............gY
FR714413 (+6)/clones from Candidatus Fritschea	our sample/*Salmo salar*/insect-associated	1.1		..........C....
Simkania negevensis +FR714401	contamined Vero cells	1.3		..........gC....
Arcobacter butzleri (epsilonproteobacteria)	humans	1.6		g...G..........U
Chl282bis			Probe (3-'5')	TGCAGATCCGCCTGACTC
Cluster II			Target (5'-3')	ACGUCUAGGCGGACUGAG
Simkania negevensis + FR714401	contamined Vero cells	0.2		..........g.....
FR714420 (+10)/uncultured *Chlamydiales* bacteria/*C. suis*/*C. psittaci*/*C. pecorum*/*C. abortus*/*C. trachomatis*	our sample/fresh water/waste water treatment plants/animals	0.6		..........u....
uncultured *Chlamydia* sp./*Waddlia* sp./clone from Candidate division OP10	anoxic marine sediment/bat urine/biofilm reactor	0.8		..........gu....
Candidatus Pisciclamydia salmonis	salmon gill	1		...C..........
clones from Candidate division WS3	hypersaline microbial mat	1.2		g..........u..gC
uncultured *Lentisphaerae*/*Firmicutes*	wastewater sludge/rumen/sediment/colobus feces	1.3		...g.....u.....
uncultured *Firmicutes*/*Alphaproteobacteria*	sheep feces/maricultured scallop	1.6		g...G..........Y

3.4. Conclusive remarks

With a first study in the North Sea we showed by *in situ* hybridization techniques that most of *Planctomycetes* are particle inhabitants and we found a positive correlation between *Planctomycetes* and centric diatoms (*Centrales*) suggesting *Planctomycetes* may benefit from substances released from diatoms e.g. chrysolaminarin but also providing a further evidence for a possible direct interaction and carbon flow between algae and *Planctomycetes.* We also gave a first hint to a correlation of a newly defined *Planctomycetes* clade, *Planctomyces*-related group A, and a bloom of pennate diatoms (*Pennales*).

With a second study we obtained first hints on a *Planctomycetes* seasonal succession in Lago di Paola that correlates with chlorophyll a concentrations. The similar results obtained by both studies strongly suggested *Planctomycetes* are bottom-up controlled and most likely benefited by algal production. Since a new set of probes is now available for clades of *Planctomycetes* it would be interesting to apply these probes to other coastal sites, in different seasons and years, and to test our hypotheses on a correlations of *Planctomycetes* with the occurrence of specific phytoplankton (i.e. pennate and centric diatoms).

A metagenomic approach will be useful to obtain insights into the potential function of *Planctomycetes* in coastal systems and should complement direct *in situ* observations of *Planctomycetes* using CARD-FISH. It will therefore be used in order to explore the ecology of the *Planctomycetes* phylum and particularly their link with diatoms.

Further studies should define the structure of the *Planctomycetes*-phytoplankton micro-scale interaction and the identification of the partners. Atomic force microscopy (AFM) showed that a substantial fraction of bacteria, including *Synechococcus*, occur conjoint as bacteria-bacteria and bacteria-*Synechococcus* in the marine plankton (Malfatti and Azam, 2009). Since AFM can be used in combination with epifluorescence microscopy (EFM) (Mangold et al., 2008) it should enable the identification of partners by fluorescence *in situ* hybridization before AFM imaging.

Follow up studies should address the *in situ* activity of *Planctomycetes*. Single cell techniques like microautoradiography (MAR) and nanometer-scale secondary-ion mass spectrometry (NanoSIMS) in combination with FISH (Behrens et al., 2008; Musat et al., 2008) has enabled the phylogenetic identification of microbial partners and the monitoring of the transfer of metabolites between them (Behrens et al., 2008). It would be worthwhile

to perform incubation experiments in order to identify *Planctomycetes* and phytoplankton and look for a possible transfer of metabolites between them.

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Erklärung

Gemäß §6 (5) Nr. 1 - 3 Promotionsordnung erkläre ich hiermit, dass ich die Arbeit mit dem Titel:

Abundance, distribution and diversity of planktonic *Planctomycetes* in coastal zones

1. ohne unerlaubte fremde Hilfe angefertigt habe,
2. keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe,
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeit um drei identische Exemplare handelt.

Bremen, 18 November, 2010

Ilaria Pizzetti