

# Comparison of primer sets for the study of *Planctomycetes* communities in lentic freshwater ecosystems

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## Summary

In search of a primer set that could be used to study *Planctomycetes* dynamics in lakes and especially via fingerprinting methods, e.g. denaturing gradient gel electrophoresis (DGGE), three existing specific primer sets, developed for marine and soil systems, have been tested on water samples from four freshwater ecosystems. The first primer set (PLA46F/PLA886R) allowed PCR amplification of *Planctomycetes* sequences in only one of the four ecosystems, whereas the second primer set (PLA40F/P518R) amplified *Planctomycetes* sequences in all the studied ecosystems but with a low specificity, since sequences belonging to *Verrucomicrobiales* and *Chlamydiales* clades were also amplified. Finally, the third primer set (PLA352F/PLA920R) allowed amplification of *Planctomycetes* sequences in the four ecosystems with a very high specificity. It amplified all known *Planctomycetes* genera and yielded the highest Operational Taxonomic Unit (OTU) richness and diversity estimates. *In silico* analyses supported these results. Further experiments comparing PLA352F/PLA920R to PLA46F/P1390R (a primer set generating a longer PCR fragment, also used to study *Planctomycetes*) yielded very similar results. Our findings suggest that the primer set PLA352F/PLA920R provides good estimates of *Planctomycetes* richness and diversity compared with other, and can thus be used to study *Planctomycetes* dynamics in lentic freshwater ecosystems.

## Introduction

The order of *Planctomycetales* belongs to a separate phylum in the domain bacteria on the basis of their 16S rRNA gene sequences ([Schlesner and Stackebrandt, 1986](#); [Woese, 1987](#)). It is now known that these bacteria are ubiquitous and display several specificities in terms of cell structure (e.g. cell compartmentalization), genetics and physiology ([Fuerst, 1995](#); [Lindsay et al., 2001](#)). These bacteria have been isolated from soils, marine and freshwaters, hot springs, wastewater treatment plants and invertebrate animals (e.g. [Schlesner, 1986; 1994](#); [Giovannoni et al., 1987](#); [Bornemann and Triplett, 1997](#); [Fuerst et al., 1997](#); [Axelrood et al., 2002](#); [Pimentel-Elardo et al., 2003](#); [Gade et al., 2004](#); [Köhler et al., 2008](#)). Since the discovery of the implication of *Planctomycetes* in the anaerobic ammonia oxidation (anammox) process ([Strous et al., 1999](#); [Kuypers et al., 2003](#)), the number of studies dealing with these bacteria has considerably increased over years. However these studies generally concern marine ecosystems, soils, wastewater treatment plants and springs ([Strous et al., 1999](#); [Schmid et al., 2001](#); [Chouari et al., 2003](#); [Elshahed et al., 2007](#); [Rich et al., 2008](#); [Shu and Jiao, 2008](#); [Woebken et al., 2008](#)) and only a few studies have been carried out on these bacteria in lentic freshwater ecosystems. In the latter, *Planctomycetes* have been found to be involved in anammox ([Schubert et al., 2006](#)) and in change in the quality of dissolved organic matter ([Tadonlélé, 2007](#)), two key processes in the functioning of aquatic systems. However while the first cited study focused on particular *Planctomycetes*, the second was based on fluorescence *in situ* hybridization, with no information on *Planctomycetes* composition.

In order to increase our knowledge on the structural and functional diversity of the *Planctomycetes* communities in freshwater ecosystems, we need to have a set of primers allowing specific amplification of this group and an evaluation of its diversity. We wanted that this primer set generates a small size fragment, which can be used in a fingerprinting approach such as denaturing gradient gel electrophoresis (DGGE), although the latter is not restricted to short fragments. In this goal, we tested three existing primer sets developed in marine and soil systems PLA40F/P518R ([Derakshani et al., 2001](#)), PLA46/PLA886R ([Pyneart et al., 2003](#)) and PLA352F/PLA920R ([Mühling et al., 2008](#)), and described as *Planctomycetes*-specific ([Table 1](#)). Our study was performed on water samples from four contrasting freshwater ecosystems (two French peri-alpine lakes, Annecy and Bourget, and two reservoirs in Burkina Faso, Bagre and Pouytenga) and was complemented by *in silico* analyses, which compared the theoretical performance of these primers in the context of the current Ribosomal Database Project (RDP) database. Finally, we compared the results provided by the best primer set with those obtained with a primer set (PLA46F/P1390R) generating a longer fragment, and used by other authors to study *Planctomycetes* ([Chouari et al., 2003](#)).

Table 1. *Planctomycetes*-specific 16S rRNA gene PCR primer sets used in this study and conditions of each PCR reaction.

Primers	Target group	Sequences (5'-3')	No. of cycles	PCR conditions						References
				Denaturation Temperature (°C)	Denaturation Time (min)	Annealing Temperature (°C)	Annealing Time (min)	Elongation Temperature (°C)	Elongation Time (min)	
1.										For DNA extraction and PCR amplification, 250 ml of water sample from each lake was first filtered through a 2- $\mu$ m-pore-size polycarbonate membrane filter (Nucleopore) to eliminate larger eukaryotes. The bacterioplankton remaining in the filtrate was then collected, through a gentle filtration, on a 0.2 - $\mu$ m-pore-size polycarbonate membrane filters (Nucleopore). Nucleic acid extraction was carried out as described in <a href="#">Dorigo and colleagues (2006)</a> . PCR amplifications were performed with the PTC-100 <sup>TM</sup> Thermal Cycler (MJ Research) using three sets of primers (PLA46F/PLA886R, <a href="#">Pyneart et al., 2003</a> ; PLA40F/P518R, <a href="#">Derakshani et al., 2001</a> ; and PLA352F/PLA920R, <a href="#">Mühling et al., 2008</a> ). To compare the two sets of primers PLA352F/PLA920R and PLA46F/P1390R, PCR amplifications have been performed only on water samples from the Lake Bourget (referred to as 'Bourget 2'). For each primer set, a range of annealing temperatures was tested to determine empirically the temperature that resulted in the most specific PCR product.
PLA40F	<i>Planctomycetales</i>	CGG CRT GGA TTA GGC ATG	30	94	0,5	60	1	72	2	<a href="#">Boon et al. (2000)</a> ; <a href="#">Derakshani et al. (2001)</a>
P518R	<i>Eubacteria</i>	ATT ACC GCG GCT GCT GG								
PLA46F	<i>Planctomycetales</i>	GGA TTA GGC ATG CAA GTC GCC TTG	30	95	1	56	1	72	2	<a href="#">Pyneart et al. (2003)</a>
PLA886R	<i>Planctomycetales</i>	CGA CCA TAC TCC C GGC TGC								
PLA352F	<i>Planctomycetales</i>	AGT CGA GRA TCT TGT GTG	30	96	1	58	1	74	1	<a href="#">Mühling et al. (2008)</a>
PLA920R	<i>Planctomycetales</i>	AGC CCC CGT CAA								
PLA46F	<i>Planctomycetales</i>	GGA TTA GGC	30	94	1	57	1	72	2	<a href="#">Chouari et al. (2003)</a>

Table 1. *Planctomycetes*-specific 16S rRNA gene PCR primer sets used in this study and conditions of each PCR reaction.

Primers	Target group	Sequences (5'–3')	No. of cycles	PCR conditions			References	
				Denaturation Temperature (°C)	Time (min)	Annealing Temperature (°C)		Time (min)
P1390R	<i>Eubacteria</i>	ATG						
		CAA						
		GTC						
		GAC						
		GGG						
		CGG						
		TGT						
		GTA						
		CAA						

## Results and discussion

### Efficacy and ability of each primer set to amplify *Planctomycetes* sequences

The *in silico* analyses (theoretical approach) using the RDP database showed that the forward and the reverse of the primer set PLA352F/PLA920R matched with a higher number of *Planctomycetes* sequences (60% and 52% respectively), and also exhibited lower percentages of match outside this bacterial group than the forward and the reverse from the two others primer sets (Table 2). It is, however, important to note that in spite of a good specificity of primers obtained by theoretical analysis, experimental analyses are necessary and can reveal a high degree of non-specificity (Morales and Holben, 2009).

Table 2. *Planctomycetes*-specific 16S rRNA gene PCR primers used in this study and their specificity revealed by the *in silico* analysis.

Primer	Target group	Sequence (5'→3')	<i>Escherichia coli</i> position	Identical matches within the target group	Percentage matches within the target group compared with all <i>Planctomycetes</i> sequences <sup>a</sup>	Percentage matches within the target group compared with total hits
PLA46F	Planctomycetales	GGA TTA	46–63	2838	30	77
		GGC ATG				
		CAA GTC				
PLA886R	Planctomycetales	GCC TTG	886–904	4574	48	95
		CGA CCA				
		TAC TCC				
PLA40F	Planctomycetales	CGG CRT	40–57	2446	26	86
		GGA TTA				
		GGC ATG				
P518R	<i>Eubacteria</i>	ATT ACC	518–534	1784	18	0.24
		GCG GCT				
PLA352F	Planctomycetales	GCT GG	350–367	5684	60	91
		GGC TGC				
		AGT CGA				
PLA920R	Planctomycetales	GRA TCT	920–937	4973	52	99
		TGT GTG				
		AGC CCC				
		CGT CAA				

- a.

A total of 9488 *Planctomycetes* sequences were available in the RDP database.

- The theoretical performance of each PCR primer was tested (*in silico* analyses) using the 'Probe Match' function within the Ribosomal Database Project (RDP) (Cole *et al.*, 2005).

Very contrasting results were found in the PCR efficiency and specificity using the first three primer sets tested. The primer set PLA46F/PLA886R allowed amplification of *Planctomycetes* sequences (100% of sequences obtained belonged to *Planctomycetales* order) but only in Lake Bourget. No amplification was obtained with this primer set for samples from the three other ecosystems (Table 3). This primer set has previously been used to estimate the *Planctomycetes* diversity in marine environments and in digesters of wastewater treatment plants (Pyneart *et al.*, 2003; Shu and Jiao, 2008). Previous studies indicated that a low specificity of the primers and a poor efficacy of the PCR reaction can yield poor amplification (Becker *et al.*, 2000), as found with this primer set in our study. Moreover, recent studies (Bru *et al.*, 2008; Wu *et al.*, 2009) have shown that single mismatch in the regions where primers bind onto the template strands can also greatly affect the PCR efficacy. This set of primers might thus be able to detect and amplify only a restricted number of *Planctomycetes* sequences among those present in freshwater ecosystems. In a recent review, Amann and Fuchs (2008) reported that the coverage of the probe PLA46 was 44% (559 of 1271) of the *Planctomycetes* 16S rRNA sequences in the SILVA database. Our *in silico* analysis in the context of a larger database (RDP) indicates a lower coverage (30%) for the PLA46F as a primer for this bacterial group (Table 2).

Table 3. Analysis of the 16S rDNA diversity (Chao1 estimator, total number of sequences, number of *Planctomycetes* sequences and OTUs and percentage of *Planctomycetes* sequences) for each primer set and lake.

Primers	Lakes	Total number of sequences	Number of <i>Planctomycetes</i>		Chao1 estimates (±SD)
			Sequences	OTUs	
1. After amplification, fragments were cloned in the pGEM-T system II vector (Promega) and then transformed into JM109-competent cell (Promega) according to the manufacturer's instructions. Ninety-six positive clones (white colonies) obtained from each clone library from each lake and each primer set were randomly selected, verified by PCR using the commercial primers SP6/T7 and finally sequenced (GATC Biotech). Sequences were then edited, aligned with Genedoc (Nicholas and Nicholas, 1997) and finally checked for chimera using Bellerophon (Huber <i>et al.</i> , 2004) and the Ribosomal Database Project (RDP, Cole <i>et al.</i> , 2005). Operational Taxonomic Units (OTUs) were defined on the basis of a ≥ 98% sequence identity. The Chaos-1 and abundance-based coverage estimators of species richness were calculated using the software 'EstimateS' ( <a href="http://viceroy.eeb.uconn.edu/estimates">http://viceroy.eeb.uconn.edu/estimates</a> ). ND, not determined.					
PLA46F/PLA886R	Annecy	ND	ND	ND	ND
	Bourget 1	94	94	10	10.5 (1.29)
	Bagre	ND	ND	ND	ND
	Pouytenga	ND	ND	ND	ND
PLA40F/P518R	Annecy	26	5	5	ND
	Bourget 1	72	48	7	12 (7.04)
	Bagre	70	47	5	5 (0.01)
	Pouytenga	57	55	2	2 (0)
PLA352F/PLA920R	Annecy	86	85	9	9 (0.25)
	Bourget 1	94	93	9	12 (4.18)
	Bourget 2	90	89	5	6 (2.19)
	Bagre	88	84	21	26 (4.21)
PLA46F/P1390R	Pouytenga	88	86	5	5 (0)
	Bourget 2	78	76	6	7 (2.22)

The second primer set, PLA40F/P518R, allowed to amplify *Planctomycetes* sequences in the four lakes but the specificity of the amplification varied considerably (Table 3). The proportion of sequences belonging to *Planctomycetes* ranged from a value as low as 13% in Lake Annecy to 96% in the reservoir Pouytenga, intermediate values being found in Lake Bourget (51%) and in the reservoir Bagre (67%). This primer set amplified members of other bacterial orders, which are phylogenetically close to *Planctomycetes*, namely *Verrucomicrobiales* and *Parachlamydiales* (13% and 9% of the total of sequences respectively) in Lake Annecy, and *Chlamydiales* (6% of all sequences) in the reservoir Bagre. This lack of *Planctomycetes* specificity has previously been reported for the primer PLA40F in soils and rice roots (Derakshani *et al.*, 2001). These authors pointed out that PLA40F revealed no mismatch for almost all *Planctomycetes* and only a very few mismatches for members of the *Verrucomicrobia* and some *Chlamydia*. Our results suggest that the primer set PLA40F/P518R might not be suitable for study of freshwater planktonic *Planctomycetes*, especially in the framework of fingerprinting approach on these communities.

The third primer set tested (PLA352F/PLA920R) provided the best results in term of specificity and efficiency ([Table 3](#)). Indeed, a positive PCR amplification was obtained in all sampling sites and almost all the sequences (> 97%) belonged to the *Planctomycetes* phylum. The remaining sequences (< 3%) belonged to a group of 'unclassified bacteria'.

### **Operational Taxonomic Unit (OTU) richness found in the *Planctomycetes* communities using the three sets of primers**

The number of *Planctomycetes* Operational Taxonomic Units (OTUs) identified in the samples from the four ecosystems with the different primer sets did not differ significantly from the values of the Chao1 estimator (Sign test,  $P = 0.25$ , [Table 3](#)), showing that we obtained a sufficient number of sequences to evaluate the *Planctomycetes* OTU richness in our samples. This finding was confirmed by the rarefaction curves which were asymptotic in most cases (not shown). Finally it appeared for the same primer set that there were large variations in the richness index between the samples from the four ecosystems ([Table 3](#)). Compared with the two others, the primer set PLA352F/PLA920R allowed to obtain higher numbers of OTUs and higher values of the Chao1 index in almost all cases ([Table 3](#)). This suggests that the primer set PLA352F/PLA920R was able to amplify a larger set of *Planctomycetes* sequences than the two others, and that the latter underestimated the richness of the *Planctomycetes* community. These results were supported by the *in silico* analyses, which revealed only a few sequences detected by PLA352F/PLA920R outside the group of *Planctomycetes* in the context of RDP ([Table 2](#)). As pointed out in previous works ([Lueders and Friedrich, 2003](#)), our findings confirmed that it is necessary to perform a careful evaluation of the PCR primers used to assess the diversity in microbial communities. Hence, the primer set PLA352F/PLA920R seemed to provide the best compromise considering the ability to obtain a positive PCR amplification, the specificity of the amplification and the ability to detect a large number of OTUs within the *Planctomycetes* communities.

### ***Planctomycetes* diversity obtained using PLA352F/PLA920R versus PLA46F/P1390R**

Based on the above results, further experiments were performed to compare results obtained with PLA352F/PLA920R with those found with the primer set PLA46F/P1390R ([Chouari et al., 2003](#)). PLA352F/PLA920R generates a short enough PCR fragments (568 bp) to be used in DGGE ([Mühling et al., 2008](#)), one of the most used fingerprinting approach. PLA46F/P1390R allows amplification of a 1344 bp fragment, and has been used to study *Planctomycetes* communities in waste water treatment plants ([Chouari et al., 2003](#)). The comparison was performed on a sample from Lake Bourget (Bourget 2). Results showed that the two primer sets presented a high specificity to *Planctomycetes*, as 99% and 98% of sequences obtained with PLA352F/PLA920R and PLA46F/P1390R, respectively, belonged to *Planctomycetales*. The number of OTUs (5 and 6) and the composition of the *Planctomycetes* community amplified by these two primer sets were very similar ([Table 3](#)). In addition, these two primer sets yielded similar values for the Chao1 ([Table 3](#)). This indicated that, despite the smaller size of PCR fragments generated by PLA352F/PLA920R, the latter did not underestimate *Planctomycetes* diversity, in our samples, compared with PLA46F/P1390R.

### **Phylogenetic analysis of the sequences obtained with the primers PLA352F/PLA920R**

Based on the above results, a phylogenetic analysis was performed with data obtained with PLA352F/PLA920R. To date, seven cultured genera of *Planctomycetes* (*Planctomyces*, *Gemmata*, *Isosphaera*, *Pirellula*, *Schlesneria*, *Singulisphaera* and *Zavarzinella*) have been described ([Staley et al., 1992](#); [Kulichevskaya et al., 2007](#); [2008](#); [2009](#)) but several additional *Candidatus* genera (*Brocadia*, *Kuenenia*, *Scalindua*, *Annamoxoglobus* and *Jettenia*) playing a key role in the anammox process ([Strous et al., 1999](#); [Kuypers et al., 2003](#); [Kartal et al., 2007](#); [Schmid et al., 2007](#); [Quan et al., 2008](#)) have recently been proposed. Among the 437 *Planctomycetes* sequences obtained with the primer set PLA352F/PLA920R in our four sampling sites, 39 OTUs displaying a  $\geq 98\%$  sequence identity were identified. Thirty-eight of these OTUs were distributed in four clusters containing all *Planctomycetes* genera [cluster I (*Pirellula/Blastopirellula*), cluster II (*Planctomyces/Schlesneria*), cluster III (*Isosphaera/Singulisphaera*) and cluster IV (*Gemmata/Zavarzinella*)]. This indicated that the primers PLA352F/PLA920R were able to amplify members of all the *Planctomycetes* genera currently described ([Fig. 1](#)). Among these 38 OTUs, 20 (~53%) belonged to cluster I, which comprises *Pirellula/Blastopirellula* genera. A high diversity of the *Pirellula* genus and its dominance within *Planctomycetes* communities have been reported by previous studies in other freshwater environments, namely wastewater treatment plants and river biofilms ([Chouari et al., 2003](#); [Brümmer et al., 2004](#)). Our results, together with those from these previous studies, suggest that members of this genus are probably well adapted to freshwater ecosystems and might play a particular role in these environments.

One OTU (OTU 28), amplified in the reservoir Bagre, showed a high degree of similarity with sequences belonging to anammox *Planctomycetes* ([Fig. 1](#)). This suggests that the primer set PLA352F/PLA920R is potentially able to detect and amplify freshwater *Planctomycetes* involved in anaerobic ammonium oxidizing processes. This may be of interest even though obtaining of a better representation of anammox organisms when they are present might require primer specific to these organisms ([Schmid et al., 2005](#)). The existence of anoxic

microzones in detrital aggregates ([Shanks and Reeder, 1993](#)) might be a factor allowing the presence of anammox bacteria in oxygenated water columns, as found in this study.

All the sequences sharing  $\geq 98\%$  sequence identity with sequences from GenBank were closely related to environmental sequences and not to cultivated organisms. In addition, 46% of the OTUs displayed sequence similarity  $< 98\%$  with sequences from GenBank® database (Table S1), which confirmed that the *Planctomycetes* from freshwater ecosystems are still poorly known. As previously found for dominant phyla in freshwater communities ([Humbert et al., 2009](#)), the fact that best blast hits were all obtained with sequences from freshwater habitats (lakes, rivers and reservoirs) seems to support the conclusions of [Brümmer and colleagues \(2004\)](#) suggesting that *Planctomycetes* species from freshwaters are different from those from marine environments.

In conclusion, we propose that at the current state of knowledge, the primer set PLA352F/PLA920R could be used to study pelagic *Planctomycetes* in freshwater ecosystems specifically via fingerprinting methods.

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## References

- Amann, R., and Fuchs, B.M. (2008) Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nat Rev Microbiol* 6: 339–348.
- Axelrood, P.E., Chow, M.L., Radomski, C.C., McDermott, J.M., and Davies, J. (2002) Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Can J Microbiol* 48: 655–674.
- Becker, S., Böger, P., Oehlmann, R., and Ernst, A. (2000) PCR bias ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. *Appl Environ Microbiol* 66: 4945–4953.
- Boon, N., Goris, J., De Vos, P., Verstraete, W., and Top, E.M. (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. *Appl Environ Microbiol* 66: 2906–2913.
- Bornemann, J., and Triplett, E.W. (1997) Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and population shifts associated with deforestation. *Appl Environ Microbiol* 63: 2647–2653.
- Bru, D., Martin-Laurent, F., and Philippot, L. (2008) Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an example. *Appl Environ Microbiol* 74: 1660–1663.
- Brümmer, I.H.M., Felske, A.D.M., and Wagner-Döbler, I. (2004) Diversity and seasonal changes of uncultured *Planctomycetales* in river biofilms. *Appl Environ Microbiol* 70: 5094–5101.
- Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J., and Sghir, A. (2003) Molecular evidence for novel *Planctomycete* diversity in a municipal wastewater treatment plant. *Appl Environ Microbiol* 69: 7354–7363.
- Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acid Res* 33 (Database Issue): D294–D296. doi:10.1093/nar/gki038.
- Derakshani, M., Lukow, T., and Liesack, W. (2001) Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Appl Environ Microbiol* 67: 623–631.
- Dorigo, U., Fontvieille, D., and Humbert, J.F. (2006) Spatial variability in the abundance and composition of the free-living bacterioplankton community in the pelagic zone of Lake Bourget (France). *FEMS Microbiol Ecol* 58: 109–119.  
Direct Link:
- Elshahed, M.S., Youssef, N.H., Luo, Q., Najjar, F.Z., Roe, B.A., Sisk, T.M., et al. (2007) Phylogenetic and metabolic diversity of *Planctomycetes* from anaerobic sulphide- and sulphur-rich zodletone spring, Oklahoma. *Appl Environ Microbiol* 73: 4707–4716.
- Fuerst, J.A. (1995) The *Planctomycetes*: emerging models for microbial ecology, evolution and cell biology. *Microbiology* 141: 1493–1506.

- Fuerst, J.A., Gwilliam, H.G., Lindsay, M.A., Lichanska, A., Belcher, C., Vickers, J.E., and Hugenholtz, P. (1997) Isolation and molecular identification of Planctomycete bacteria from postlarvae of the giant tiger prawn, *Penaeus monodon*. *Appl Environ Microbiol* 63: 254–262.
- Gade, D., Schlesner, H., Glöckner, F.O., Amann, R., Pfeiffer, F., and Thomm, M. (2004) Identification of Planctomycetes with Order-, Genus-, and Strain-specific 16S rRNA-targeted probes. *Microb Ecol* 47: 243–251.
- Giovannoni, S.J., Schabtach, E., and Castenholz, R.W. (1987) *Isosphaera pallida* gen. and comb. nov., a gliding, budding eubacterium from hot springs. *Arch Microbiol* 147: 276–284.
- Huber, T., Faulkner, G., and Hugenholtz, P. (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics Application Note* 20: 2317–2319.
- Humbert, J.F., Dorigo, U., Cecchi, P., Le Berre, B., and Bouvy, M. (2009) Comparison of the structure and composition of bacterial communities from temperate and tropical freshwater ecosystems. *Environ Microbiol* 11: 2339–2350.  
Direct Link:
- Kartal, B., Rattray, J., van Niftrik, L.A., van de Vossenberg, J., Schmid, M.C., Webb, R.I., et al. (2007) *Candidatus 'Anammoxoglobus propionicus'* a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* 30: 39–49.
- Köhler, T., Stingl, U., Meuser, K., and Brune, A. (2008) Novel lineages of Planctomycetes densely colonize the alkaline gut of soil-feeding termites (*Cubitermes* spp). *Environ Microbiol* 10: 1260–1270.  
Direct Link
- Kulichevskaya, I.S., Ivanova, A.O., Belova, S.E., Baulina, O.I., Bodelier, P.L.E., Rijpstra, W.I.C., et al. (2007) *Schlesneria paludicola* gen. nov., sp. nov, the first acidophilic member of the order Planctomycetales from sphagnum-dominated boreal wetlands. *Int J Syst Evol Microbiol* 57: 2680–2687.
- ulichevskaya, I.S., Ivanova, A.O., Baulina, O.I., Bodelier, P.L.E., Damsté, J.S.S., and Dedysh, S.N. (2008) *Singulisphaera acidiphila* gen. nov., sp. nov., a non-filamentous, *Isosphaera*-like Planctomycete from acidic northern wetlands. *Int J Syst Evol Microbiol* 58: 1186–1193.
- Kulichevskaya, I.S., Baulina, O.I., Bodelier, P.L.E., Rijpstra, W.I.C., Damsté, J.S.S., and Dedysh, S.N. (2009) *Zavarzinella formosa* gen. nov., sp. nov., a novel stalked, *Gemmata*-like Planctomycete from a Siberian peat bog. *Int J Syst Evol Microbiol* 59: 357–364.
- Kuypers, M.M.M., Sliemers, A.O., Lavik, G., Schmid, M., Jørgensen, B.B., Kuenen, J.G., et al. (2003) Anaerobic ammonium oxidation by bacteria in the Black Sea. *Nature* 422: 608–611.
- Lindsay, M.R., Webb, R.I., Strous, M., Jetten, M.S., Butler, M.K., Forde, R.J., and Fuerst, J.A. (2001) Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. *Arch Microbiol* 175: 413–429.
- Lueders, T., and Friedrich, M.W. (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl Environ Microbiol* 69: 320–326.
- Morales, S.E., and Holben, W.E. (2009) Empirical testing of 16S rRNA gene PCR primer pairs reveals variance in target specificity and efficacy not suggested by in silico analysis. *Appl Environ Microbiol* 75: 2677–2683.
- Mühling, M., Woolven-Allen, J., Murrell, J.C., and Joint, I. (2008) Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME J* 2: 379–392.
- Nicholas, K.B., and Nicholas, H.B.J. (1997) *Genedoc: a tool for editing and annotating multiple sequence alignments*. Distributed by the author [WWW document]. URL
- Pimentel-Elardo, S., Wehrl, M., Friedrich, A.B., Jensen, P.R., and Hentschel, U. (2003) Isolation of Planctomycetes from *Aplysina* sponges. *Aquat Microb Ecol* 33: 239–245.
- Pyneart, K., Smets, B.F., Wyffels, S., Beheydt, D., Siciliano, S.D., and Verstraete, W. (2003) Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor. *Appl Environ Microbiol* 69: 3626–3635.
- Quan, Z.X., Rhee, Z.K., Zuo, J.E., Yang, Y., Bae, J.W., Park, J.R., et al. (2008) Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. *Environ Microbiol* 10: 3130–3139.  
Direct Link:
- Rich, J.J., Dale, O.R., Song, B., and Ward, B.B. (2008) Anaerobic ammonium oxidation (anammox) in Chesapeake Bay sediments. *Microb Ecol* 55: 311–320.

- Schlesner, H. (1986) *Pirella marina* sp. nov., a budding, peptidoglycan-less bacterium from brackish water. *Syst Appl Microbiol* 8: 177–180.
- Schlesner, H. (1994) The development of media suitable for microorganisms morphologically resembling *Planctomyces* spp., *Pirellula* spp., and other *Planctomycetales* from various aquatic habitats using dilute media. *Syst Appl Microbiol* 17: 135–145.
- Schlesner, H., and Stackebrandt, E. (1986) Assignment of the genera *Planctomyces* and *Pirella* to a new family *Planctomycetaceae* fam. nov. and description of the order *Planctomycetales* ord. nov. *Syst Appl Microbiol* 8: 174–176.
- Schmid, M., Schmitz-Esser, S., Jetten, M., and Wagner, M. (2001) 16S–23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environ Microbiol* 3: 450–459.  
Direct Link:
- Schmid, M.C., Mass, B., Dapena, A., van de Pas-Schoonen, K., van de Vossenberg, J., Kartal, B., et al. (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (*Anammox*) bacteria (Minireview). *Appl Environ Microbiol* 71: 1677–1684.
- Schmid, M.C., Risgaard-Petersen, N., van de Vossenberg, J., Kuypers, M.M.M., Lavik, G., Petersen, J., et al. (2007) Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. *Environ Microbiol* 9: 1476–1484.  
Direct Link:
- Schubert, C.J., Durisch-Kaiser, E., Wehrli, B., Thamdrup, B., Lam, P., and Kuypers, M.M.M. (2006) Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). *Environ Microbiol* 8: 1857–1863.  
Direct Link:
- Shanks, A.L., and Reeder, M.L. (1993) Reducing microzones and sulfide production in marine snow. *Mar Ecol Prog Ser* 96: 43–47.
- Shu, Q., and Jiao, N. (2008) Different *Planctomycetes* diversity patterns in latitudinal surface seawater of the open sea and in sediment. *J Microbiol* 46: 154–159.
- Staley, J.T., Fuerst, J.A., Giovannoni, S., and Schlesner, H. (1992) The order *Planctomycetales* and the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera*. In *The Prokaryotes: A Handbook of the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds). New York, NY, USA: Springer-Verlag, pp. 3710–3731.
- Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., Van de Pas-Schoonen, K.T., et al. (1999) Missing lithotroph identified as new *Planctomycete*. *Nature* 400: 446–449.
- Tadonlélé, D.R. (2007) Strong coupling between natural *Planctomycetes* and changes in the quality of dissolved organic matter in freshwater samples. *FEMS Microbiol Ecol* 59: 543–555.  
Direct Link:
- Woebken, D., Lam, P., Kuypers, M.M.M., Naqvi, S.W.A., Kartal, B., Jetten, M.S.M., et al. (2008) A microdiversity study of *anammox* bacteria reveals a novel *Candidatus Scalindula* phylotype in marine oxygen minimum zones. *Environ Microbiol* 10: 3106–3119.  
Direct Link:
- Woese, C.R. (1987) Bacterial evolution. *Microbiol Rev* 51: 221–271.
- Wu, J.H., Hong, P.Y., and Liu, W.T. (2009) Quantitative effects of position and type of single mismatch on single base primer extension. *J Microbiol Methods* 77: 267–275.