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Validation of a new catalysed reporter deposition–fluorescence *in situ* hybridization probe for the accurate quantification of marine *Bacteroidetes* populations

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Summary

Catalysed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) is a powerful approach to quantify bacterial taxa. In this study, we compare the performance of the widely used *Bacteroidetes* CF319a probe with the new CF968 probe. *In silico* analyses and tests with isolates demonstrate that CF319a hybridizes with non-*Bacteroidetes* sequences from the *Rhodobacteraceae* and *Alteromonadaceae* families. We test the probes' accuracy in 37 globally distributed marine samples and over two consecutive years at the Blanes Bay Microbial Observatory (NW Mediterranean). We also compared the CARD-FISH data with the *Bacteroidetes* 16S rRNA gene sequences retrieved from 27 marine metagenomes from the TARA Oceans expedition. We find no significant differences in abundances between both approaches, although CF319a targeted some unspecific sequences and both probes displayed different abundances of specific *Bacteroidetes* phylotypes. Our results demonstrate that quantitative estimations by using both probes are significantly different in certain oceanographic regions (Mediterranean Sea, Red Sea and Arabian Sea) and that CF968 shows seasonality within marine *Bacteroidetes*, notably large differences between summer and winter that is overlooked by CF319a. We propose CF968 as an alternative to CF319a for targeting the whole *Bacteroidetes* phylum since it has better coverage, greater specificity and overall better quantifies marine *Bacteroidetes*.

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Introduction

Marine *Bacteroidetes,* mostly belonging to the class *Flavobacteria,* represent one of the major groups of bacterioplankton in a variety of environments (Kirchman *et al*., 2003; Chen *et al*., 2006; Alonso *et al*., 2007; Teeling *et al*., 2012). Quantitative and genomic approaches have unveiled relevant ecological features of this group. For example, they are particularly successful in the degradation of organic matter in the ocean (Riemann *et al*., 2000; Pinhassi *et al*., 2004; Sarmento and Gasol, 2012) and have the capacity to consume proteins and other high-molecular-weight polymers (Cottrell and Kirchman, 2000; Bauer *et al*., 2006; Gómez-Pereira *et al*., 2012; Fernández-Gómez *et al*., 2013). In addition, the ability of some members of the *Bacteroidetes* to obtain additional energy from light by using proteorhodopsin (Gómez-Consarnau *et al*., 2007; González *et al*., 2008) makes them ecologically relevant marine taxa. Marine *Bacteroidetes* are typically associated with cold waters, upwelling systems and phytoplankton blooms (Simon *et al*., 1999; Pinhassi *et al*., 2004; Abell and Bowman, 2005; Schattenhofer *et al*., 2009; Gómez-Pereira *et al*., 2010) and are usually found enriched in the particle-attached fraction of the bacterioplankton (DeLong *et al*., 1993; Crespo *et al*., 2013). Although lower abundances of *Bacteroidetes* are reported in the oligotrophic open ocean and in deeper waters (Schattenhofer *et al*., 2009; Gómez-Pereira *et al*., 2010; Díez-Vives *et al*., 2014; Lefort and Gasol, 2013), this group represents a cosmopolitan phylum in marine environments.

Several quantification approaches have been used to evaluate the abundance of the group. In

marine environments, catalysed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) analyses have shown *Bacteroidetes* abundances to be 10–20% of the prokaryotes in surface waters (Glöckner *et al*., 1999; Cottrell and Kirchman, 2000; Alonso-Sáez *et al*., 2007; Ruiz-González *et al*., 2012a; Lefort and Gasol, 2013), while quantitative PCR (QPCR) has reported abundances ranging from less than 1% to 53%, with the highest numbers associated with phytoplankton blooms (Abell and Bowman, 2005; Fandino *et al*., 2005). Recently, the relative abundance of marine *Bacteroidetes* sequences has also been obtained by using high-throughput sequencing technologies such as 454 FLX Titanium and Illumina (HiSeq2000/GAIIx), providing ranges between 8% and 20% (e.g. Rusch *et al*., 2007; Kirchman *et al*., 2010; Campbell *et al*., 2011; Crespo *et al*., 2013).

Growing interest in *Bacteroidetes* over time has led to the design of several primers and oligonucleotide FISH probes owing to their accurate quantification (Manz *et al*., 1996; Weller *et al*., 2000; O'Sullivan *et al*., 2002; Kirchman *et al*., 2003; Chen *et al*., 2006; Blumel *et al*., 2007; Mühling *et al*., 2008). However, the probe CF319a, introduced 18 years ago by Manz and colleagues (1996) is still the most commonly used to quantify the abundance of marine *Bacteroidetes* despite being originally designed to target the classes of *Cytophaga-Flavobacteria* rather than the whole *Bacteroidetes* phylum. Because of the continuing expansion of sequence databases, the first evaluation of the *in silico* coverage of available probes for this taxon was performed in 2008, highlighting the flaws related to the high number of outgroup hits (Amann and Fuchs, 2008). Additionally, a recent review that considered not only the exponential growth of sequence databases but also used *in silico* and culture tests indicated that the PCR primer CF968 has the best *in silico* coverage for *Flavobacteria* (Díez-Vives *et al*., 2012). Yet, CF968 was not tested as a CARD-FISH probe and, therefore, a comparative quantitative study remains lacking.

In the present paper, we compare the abundance of marine *Bacteroidetes* populations by using CARD-FISH analyses based on two specific probes that target different V regions of the 16S rRNA gene (rDNA). We analyse their differences in isolates, *in silico* and *in situ*, by comparing the most commonly used marine *Bacteroidetes* probe (CF319a), which targets the V3 region, and the new CF968 probe binding to the V6 region. We then compare their abundance in environmental samples globally distributed across the world's oceans and for two consecutive years at the Blanes Bay Microbial Observatory (BBMO; NW Mediterranean) in order to assess their temporal dynamics. Additionally, owing to the potential of metagenomics as a PCR bias-free technique for quantitative diversity studies, we conduct a comparative analysis of CARD-FISH counts and 16S rDNA sequences of *Bacteroidetes* retrieved from a set of marine metagenomes from the TARA Oceans global expedition (Karsenti *et al*., 2011) and analyse the specific phylogenetic taxa retrieved by each of the evaluated probes.

Results and discussion

In silico *comparison of the CF319a and CF968* Bacteroidetes *probes*

We compared the usefulness of the most commonly used *Bacteroidetes* probe (CF319a) with a new probe (CF968). CF968 was previously designed as a PCR-DGGE (denaturing gradient gel electrophoresis) primer (Chen *et al*., 2006; Mühling *et al*., 2008; Díez-Vives *et al*., 2012) and has also been reported as a QPCR primer (De Gregoris *et al*., 2011). However, it has not been tested as a CARD-FISH probe. *In silico* testing by using the latest SILVA database (v. 115, 2013 August) clearly showed that probe CF319a does not fully detect all members of the phylum *Bacteroidetes*, as previously observed by Amann and Fuchs (2008). Our analyses showed a coverage of 42.6% of the phylum *Bacteroidetes* for CF319a. Among this coverage, 86.2% of the sequences belonged to the class *Flavobacteria*, 34.2% to *Sphingobacteria*, 8.7% to *Cytophaga* and 30.2% to *Bacteroidia* (Supporting Information Table S1). On the contrary, CF968 exhibited a better *in silico* performance with a recovery of 91.2% of the total *Bacteroidetes* phylum, showing an increase in coverage for *Flavobacteria* (up to 94.8%) and better coverage for the *Sphingobacteria*, *Cytophaga* and *Bacteroidia* classes (92%, 78.6% and 91.8% respectively) (Supporting Information Table S1).

Additionally, we compared the differences for both probes at the genus level (Supporting Information Table S2). For the class *Flavobacteria*, which contains most of the marine *Bacteroidetes* taxa, we found better coverage for CF968 with over a 10% difference to CF319a in several marine genera such as the uncultured group NS5 and the genera *Galbibacter*, *Aquamarina* and *Capnocytophaga* among others. However, in some cases, CF319a provided better coverage, such as in the genera *Gaetbulibacter* and *Sufflavibacter* (Supporting Information Table S2). For the *Sphingobacteria* and *Cytophaga* classes, the marine groups with clear differences between both probes were restricted to the *Chitinophaga* in the *Sphingobacteriales* and *Cytophaga* and *Microscilla* in the *Cytophagales*, all of them being only detected by CF968 (Supporting Information Table S1). Most of the taxa belonging to *Bacteroidia* were not marine but rather intestinal microbiota.

Furthermore, we confirmed that one of the main flaws of CF319a is its high number of outgroup hits distributed mainly within different families of the phyla *Thaumarchaeota* (0.1%) and *Proteobacteria* (0.5%), in particular the class *Epsilonproteobacteria* (14.7%) (Supporting Information Table S1). Instead, CF968 outgroup hits were mostly constrained within the *Spirochaeta* (54%) and the uncultivated TM6 phylum (64.8%), which are rare groups in marine bacterioplankton communities, as well as in some *Proteobacteria* such as certain members of the class *Deltaproteobacteria* (0.4%). Some outgroup hits were also recovered by both probes such as some members of *Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Verrucomicrobia* (Supporting Information Table S1). At lower taxonomic ranks (family level), some outgroup hits from typical marine taxa for both probes were related to alphaproteobacterial members within *Rhodobacteraceae* (0.1%) and gammaproteobacterial *Alteromonadaceae* (0.1%) (Supporting Information Table S1). Yet, CF319a presented hits within the common marine *Rhodobacter* genus, which were absent for CF968.

Although the *in silico* analyses of both probes showed better coverage of CF968 for the whole *Bacteroidetes* phylum (42.6% with CF319a versus 91.2% with CF968) and the *Flavobacteria* class (86.2% CF319a versus 94.8% CF968), the non-specific probe binding to other taxa and the low accessibility of the probe to the ribosomal gene could affect abundance estimations when tested in marine samples. However, the accessibility of both probes was similar (Class III) based on a previous study of relative fluorescence hybridization intensities expressed as different classes of brightness levels (from Class I to Class VI) (Behrens *et al*., 2003). Thus, hypothetical differences in abundance between these probes are not likely to be caused by the different accessibility levels of the probe.

Both probes were also evaluated with a mechanistic FISH model based on the thermodynamics of nucleic acid hybridization (Yilmaz and Noguera, 2004). In this model, the affinity of a probe to its folded nucleic acid target is defined as the overall Gibbs-free energy change ($\Delta G°_{overall}$). A lower (more negative) $\Delta G°_{overall}$ represents greater potential for the formation of probe–RNA duplexes and thus higher brightness. By using the mathFISH webtool (http://mathfish.cee.wisc.edu) (Yilmaz *et al*., 2011), we found that $\Delta G°_{overall}$ for CF319a is -15.2 kcal mol^{-1} compared with -16.1 kcal mol^{-1} for CF968 (this is an average of the $\Delta G°_{overall}$ for each probe with 10 different sequences). Considering that a threshold of $\Delta G°_{overall}$ of -13.0 kcal mol^{-1} was proposed in the design of FISH probes to maximize hybridization efficiency without compromising specificity, we concluded that both probes are equivalent from a thermodynamic point of view.

Unspecific hybridization of the CF319a probe

Some of the CF319a and CF968 marine outgroup hits found *in silico* were related to the *Rhodobacteraceae* and *Alteromonadaceae* families. Although the number of outgroup hits *in silico* were not that high (0.1% of total hits), these two groups of bacteria are relevant in many marine environments. *Rhodobacteraceae* can account for up to 20% of total bacterioplankton in coastal areas (Buchan *et al*., 2005), and they range between 6% and 17% of the total community in our study site (BBMO) (Alonso-Sáez *et al*., 2007; Ferrera *et al*., 2011). *Alteromonadaceae* taxa can bloom (> 20%), usually associated to organic matter aggregates or under specific conditions, such as post-algal bloom events (Acinas *et al*., 1999; Alonso-Sáez *et al*., 2007; Crespo *et al*., 2013). Furthermore, in a previous study that used DGGE, sequencing bands from the PCR amplicons of primer set CF319a-907RM resulted in the retrieval of sequences related to *Rhodobacteraceae* and *Alteromonadaceae*, whereas no unspecific amplification was observed when using primer set 357F-CF968R (Díez-Vives *et al*., 2012). The CF319a and CF968 PCR primers have the same position and sequence as their respective CARD-FISH probes. To further confirm these observations, we tested for the specificity of both probes in cultures and environmental samples. As suspected, the hybridizations on the *Rhodobacterales*-like strains ZOCON-B2 and ZOCON-B3 and the *Alteromonadales*-like isolates ZOCON-D11 and ZOCON-F9 from our BBMO culture collection (G. Salazar unpublished) were positive for CF319a and negative for CF968 (see the examples in Fig. 1A). Additionally, multicolor CARD-FISH showed that in seawater samples where *Rhodobacteraceae* and *Alteromonadaceae* accounted for a large proportion of the total prokaryotes, many of the positive cells for the specific probes of these groups (i.e. ROS537 for *Rhodobacteraceae* and ALT1413 for *Alteromonadaceae*) were also hybridized by CF319a (Fig. 1B). Contrarily, no dual hybridization was observed with CF968. These results confirm that CF319a hybridizes with the *Rhodobacteraceae* and *Alteromonadaceae* groups. The non-specific hybridization of CF319a may reduce the accuracy of quantification estimates, particularly when these other marine taxa are abundant.

Abundance of Bacteroidetes *in marine samples*

We compared the performance of both probes in 37 seawater samples from contrasting marine environments including the Arctic Ocean, Antarctic Ocean, North Indian Ocean, Red Sea, Arabian Sea and Mediterranean Sea (Fig. 2, Supporting Information Fig. S1, Supporting Information Table S3). The quantification of *Bacteroidetes* included samples from surface, deep-chlorophyll maximum (DCM), oxygen minimum zones (OMZ; 270 and

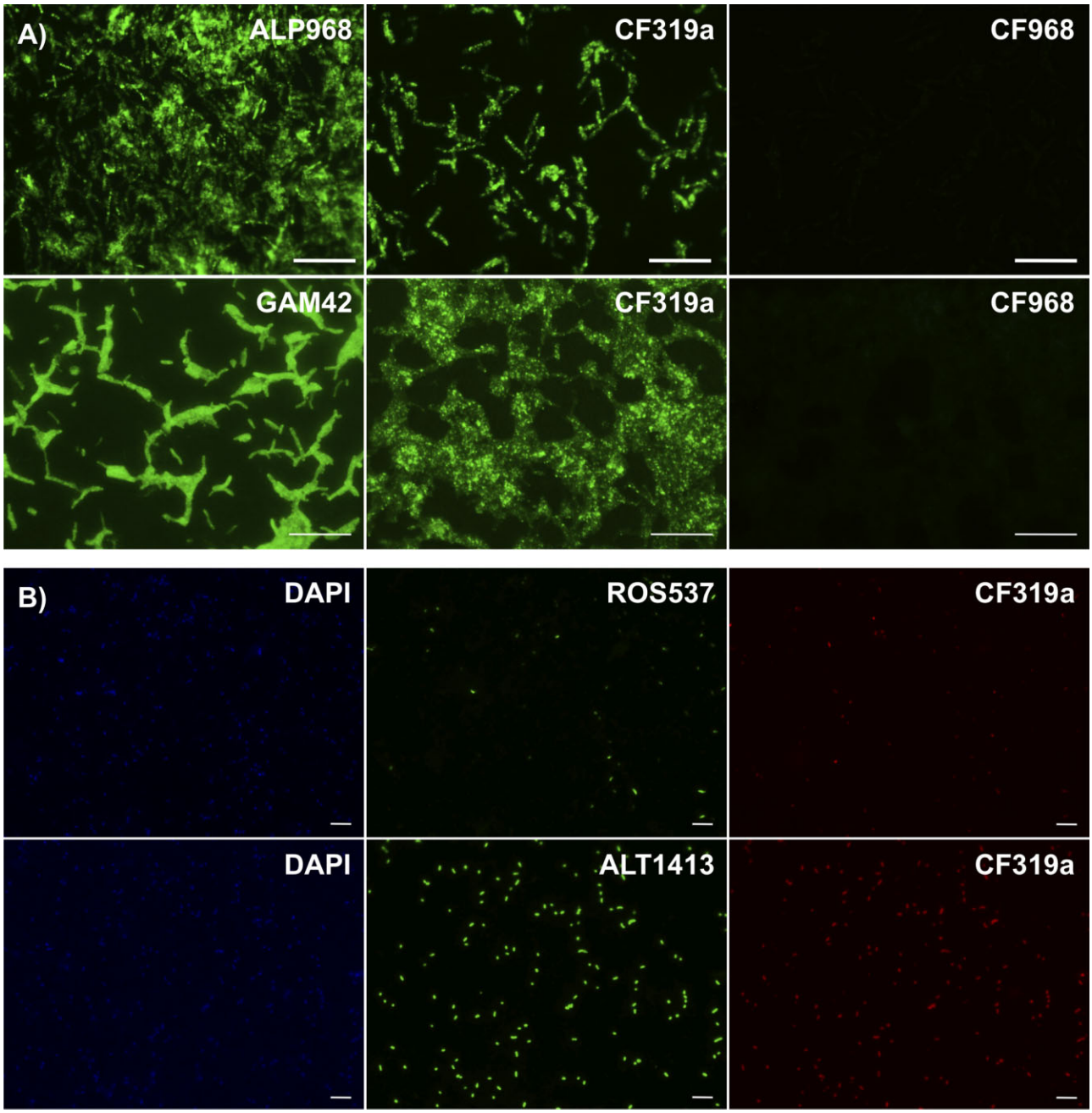

Fig. 1. A. CARD-FISH photomicrographs of the alphaproteobacterial isolate ZOCON-B3 (upper panel) and gammaproteobacterial isolate ZOCON-D11 (lower panel) hybridized with HRP-labelled oligonucleotide probes. For each panel, the left image shows the positive control hybridization, the central one the CF319a probe and the right panel the CF968 probe.
B. CARD-FISH photomicrographs of the dual hybridization of marine samples. The left panel shows total prokaryotes stained with DAPI, the central panel shows cells hybridized with the oligonucleotide probes for *Rhodobacteraceae* (ROS537) and *Alteromonadaceae* [Alexa 488 (green) HRP-labelled] and the right panel shows cells hybridized with the *Bacteroidetes* probe CF319a [Alexa 594 (red) HRP-labelled]. Scale bar = 5 μm.

350 m) and bathypelagic waters (2000 m) (Fig. 2). Positive cell hybridization was found in all samples for both probes; however, CF319a detected higher cell numbers in 70% of the marine samples tested (Figs 2 and 3, Supporting Information Fig. S2). Although the overall average differences between both probes were not significant, clear differences were observed in 35% of the total samples (13 out of 37) with differences in abundance values of > 20% (Fig. 2). A comparison by oceanographic regions showed statistically significant (paired *t*-tests or nonparametric

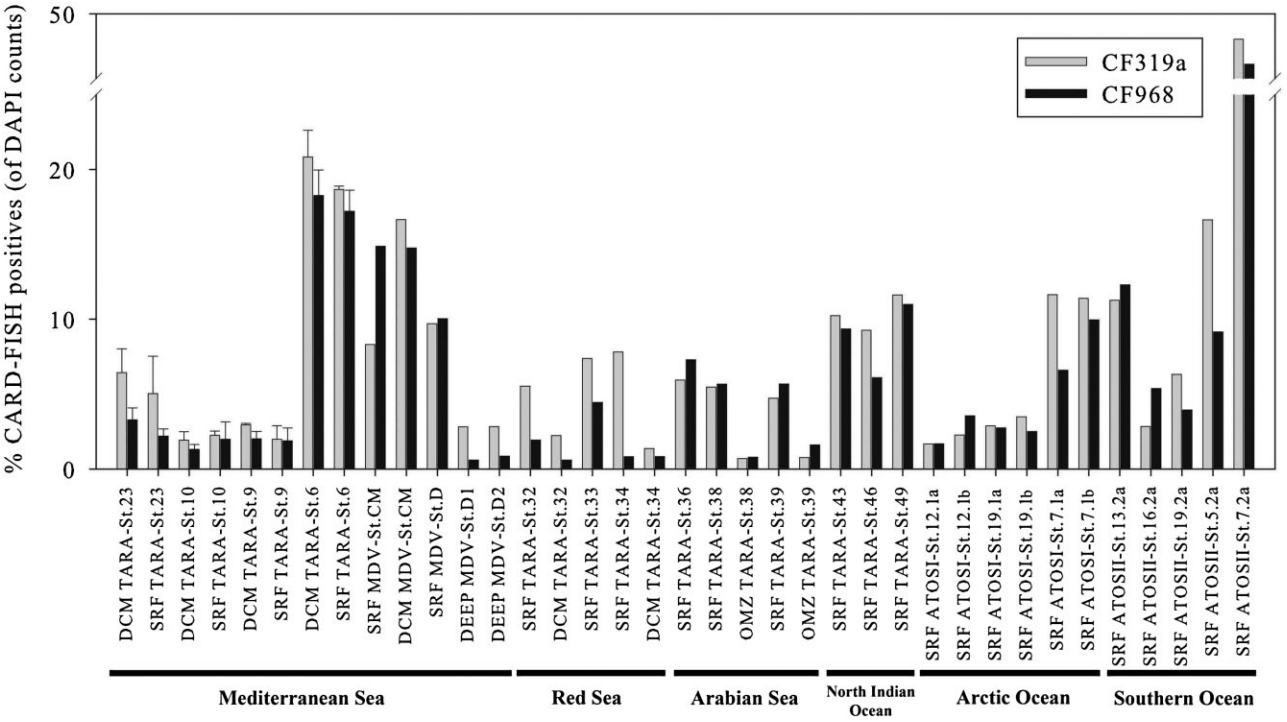

Fig. 2. Histogram showing the percentage of cells hybridized with the CF319a and CF968 probes in different oceanographic regions.

Wilcoxon signed rank tests, $P < 0.05$) differences between the hybridization values of the two probes in samples from the Mediterranean Sea (TARA-St.23, MDV-St.CM), Red Sea (TARA-St.32, TARA-St.33) and Arabian Sea (TARA-St.36, TARA-St.38, TARA-St.39). However, hybridization with the two probes was not significantly different in samples from the North Indian Ocean (TARA-St.46), Arctic Ocean (ATOSI-St.7.1a) and Antarctic Ocean (ATOSII-St.5.2a). Higher abundances with the new probe CF968 compared with CF319a were also detected in one surface Mediterranean Sea sample, MDV-St.CM, where 8% and 15% of cells were hybridized with CF319a and CF968 respectively, and in a few other stations with minor differences (Fig. 2).

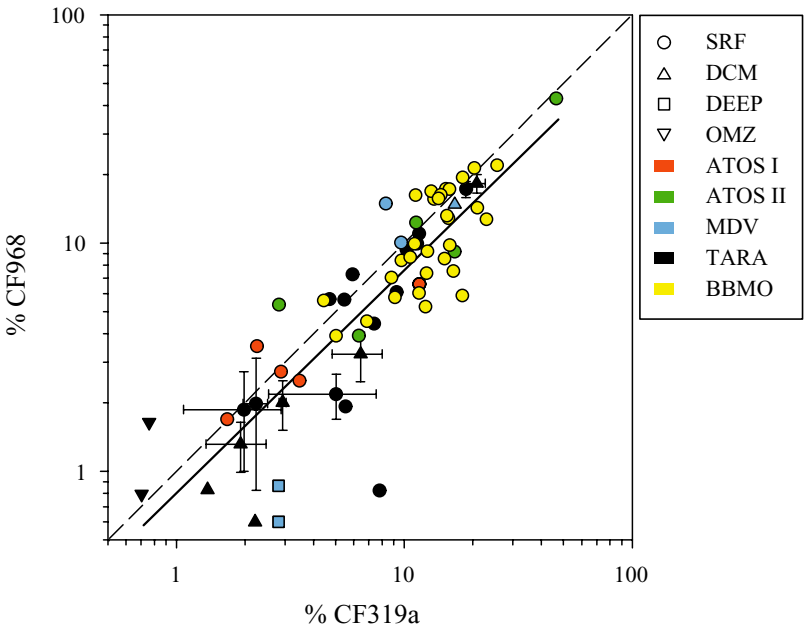

Fig. 3. Comparison of the counts with probes CF319a and CF968 in marine samples across different oceanographic regions and during the 2 years of temporal series at the BBMO.

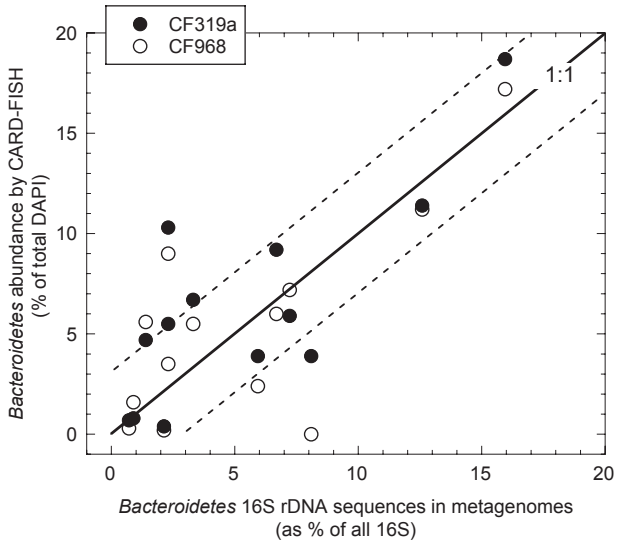

Fig. 4. Plot comparing the relative abundance of *Bacteroidetes* as observed with CARD-FISH and the 16S rDNA sequences extracted from the TARA Oceans metagenomes.

The highest abundances of *Bacteroidetes* were found in Antarctic waters, where 43% and 46% of cells were hybridized with CF968 and CF319a respectively (Fig. 2). These large numbers were associated with a diatom bloom of *Thalassiosira* (Ruiz-González *et al*., 2012b). A high abundance of marine *Bacteroidetes* in cold waters associated with algal blooms is recurrently reported in polar regions with values up to 72% during a *Phaeocystis* bloom as measured with FISH (Simon *et al*., 1999; Ruiz-González *et al*., 2012b) and 17–30% estimated with QPCR (Abell and Bowman, 2005). *Bacteroidetes* can also contribute up to 50% after algal blooms in temperate waters (Teeling *et al*., 2012).

We also compared *Bacteroidetes* abundances in the Mediterranean Sea from west to east in surface and DCM samples from a set of stations from the TARA Oceans global expedition (Fig. 2, Supporting Information Table S3). The main differences between the results obtained with both probes (CF319a versus CF968) were found in coastal stations: at the surface of St.10 (12% and 2% respectively) as well as St.6, St.9 and St.23 at the DCM (Fig. 2, Supporting Information Table S4). Furthermore, in the vertical gradient sampled during the transect MODIVUS (MDV) in the Mediterranean Sea from the surface down to 2000 m, *Bacteroidetes* had maximum abundances at the surface (5 m) and DCM (44 m) with average contributions of 12–14% of total prokaryotes with both probes. By contrast, much lower numbers were found in deeper waters (500 m and 2000 m), with between 1% and 3% of prokaryote abundances (Figs 2 and 3).

We further compared the abundance of *Bacteroidetes* obtained by CARD-FISH with the abundance of 16S rDNA sequences that belonged to *Bacteroidetes* from 27 metagenomes sequenced by the TARA Oceans expedition (Fig. 4). Most relative abundances retrieved by both probes were within 3% of all the 16S rDNA metagenomic sequences with no significant differences observed.

Temporal dynamics of marine Bacteroidetes *at the BBMO*

We also investigated the abundance of marine *Bacteroidetes* with both probes during a 2-year temporal series at the BBMO. Interestingly, at this temporal scale we noticed pronounced differences between the two probes (Fig. 5). *Bacteroidetes* abundance varied over time (2008–2009) from 4% to 26% with CF319a and from 4% to 22% with CF968. These results are comparable with the quantitative abundance found in a recent parallel study performed in the same coastal site (8–22%) by using CF968 between 2007 and 2008 (Díez-Vives *et al*., 2014). While the quantification of *Bacteroidetes* with the CF319a probe overlooked any seasonality, providing similar numbers through the year with an average of 16% for 2008 and 10% for 2009, the CF968 probe unveiled a clear seasonal pattern with a significant decrease in the number of hybridized cells for the samples between August to January in 2008 and from June to November in 2009 (Fig. 5). In fact, the hybridization values of the two probes were significantly different in this pool of samples (paired Student's *t*-test, $P < 0.001$). Thus, CF968 uncovered differences between winter and summer for *Bacteroidetes* assemblages, in agreement with previous results that have used DGGE community profiling, which have shown distinct patterns in *Bacteroidetes* assemblages in winter–spring and summer–fall in the same sampling station (Díez-Vives *et al*., 2014).

We postulated the hypothesis that the differences between both probes in these samples coincided with the high abundances of some members of *Rhodobacteraceae* and *Alteromonadaceae* because we had observed unspecific band sequences in DGGE when using the primer CF319a as well as the unspecific hybridizations of these taxa with CF319a in cultures and seawater samples that were not observed with CF968. During the 2-year survey at the BBMO, *Gammaproteobacteria* (which include *Alteromonadales*) ranged between 5% and 28% (GAM42a probe) and *Rhodobacteraceae* (ROS537 probe) comprised between 1% and 17% of total prokaryotes, and thus these groups were relevant components of the community in this coastal site (Ruiz-González *et al*., 2012a). However, we found no clear relationship between the abundances of these groups and those of *Bacteroidetes* in the BBMO

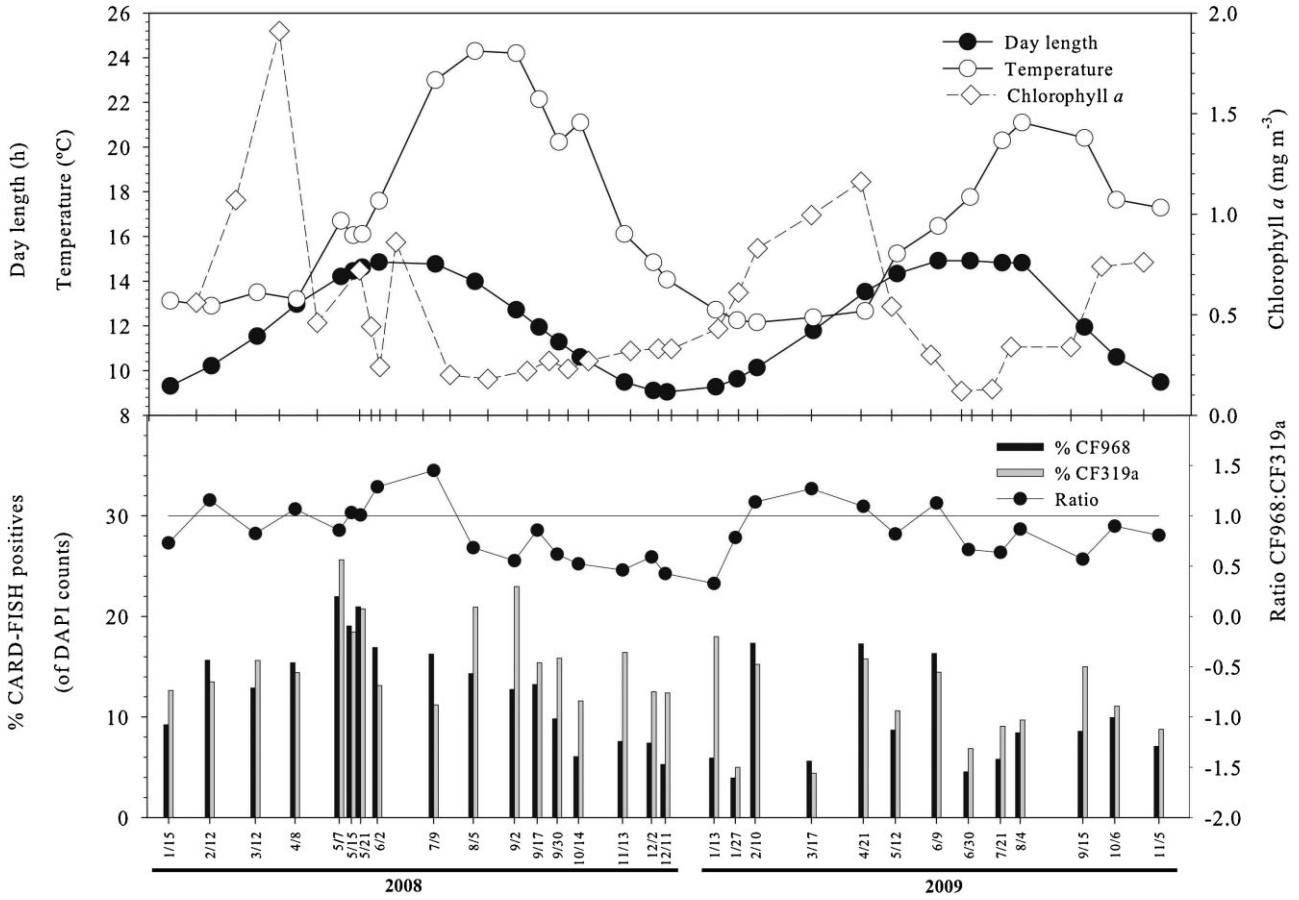

Fig. 5. Upper panel: seasonal evolution of temperature, day length and chlorophyll *a* at the BBMO between January 2008 and November 2009. Lower panels: Histograms showing the seasonal variation in the percentage of cells hybridized with the CF319a and CF968 probes over 2 years at the BBMO. At the top, the evolution of the ratio between counts with CF968 and those with CF319a. Labels on the x-axis indicate the month and day of sampling.

data. Nonetheless, the multicolor hybridization tests performed in marine samples in which the percentages of *Alteromonadales* and *Rhodobacteraceae* were high (22–37% and ~ 23% of total prokaryotes respectively) resulted in large differences in *Bacteroidetes* estimates between CF319a (25–29%) and CF968 (4–15%) (Fig. 1B). These dual hybridization experiments clearly showed that many positive cells for probes ALT1413 and ROS537 were also hybridized by using CF319a, confirming the *in silico* and in-culture observations of its unspecificity against these groups. Furthermore, the TARA-St.6 surface and DCM water samples in which we had observed large differences between the two probes (21.4–22% with CF319a, 16.1–17.4% with CF968) had relatively high contributions of *Rhodobacteraceae* (5.1% and 5.8%) (Supporting Information Table S4). These results suggest that the unspecific hybridization of CF319a may influence the enumeration of marine *Bacteroidetes*, particularly in samples where these groups are abundant.

Difference in the relative abundance of specific taxa retrieved by each probe

As shown above, a discrepancy between the CF319a and CF968 probes was observed when comparing CARD-FISH counts for a variety of marine samples in both scenarios, at different oceanographical regions and in our temporal dynamic study (Fig. 3). Our findings support the hypothesis that CF319a overestimates *Bacteroidetes* abundance owing to unspecific hybridization (Fig. 1, Supporting Information Fig. S3). Nevertheless, the differences observed between probes could be due to other reasons such as (i) differences in the specific *Bacteroidetes* taxa retrieved by each probe and/or (ii) differences in the relative abundance of the taxa retrieved by both probes.

To test this possibility, we first built a 16S rDNA dataset with 7185 sequences retrieved from the 27 TARA Oceans metagenomes (Supporting Information Table S5).

According to SILVA, 5.4% of the total sequences (390 out of 7185) corresponded to *Bacteroidetes* sequences. We then conducted an *in silico* test with these 16S rDNA metagenomic sequences by using the match probe tool of ARB to recruit sequences matching perfectly (zero mismatches) with either the CF319a or the CF968 probe sequences. We retrieved 111 and 112 matching sequences respectively. The Ribosomal Database Project (RDP) taxonomic assignment of those sequences targeting each probe confirmed that (i) CF319a hybridized non-specifically with members of *Alphaproteobacteria* (*Rhodospirillaceae*) and *Deltaproteobacteria* (*Desulfobacterales*, Supporting Information Fig. S3) and (ii) there were differences in the relative abundance of the taxa retrieved by both probes (Supporting Information Fig. S3). Although the RDP taxonomic assignation at the genus level must be taken with caution, it is noteworthy that only 38% of the *Bacteroidetes* genera were retrieved by both probes and that many of the shared genera were detected in different frequencies by each probe (Supporting Information Fig. S3).

To further investigate those differences at finer scale (phylotype level), we constructed a phylogenetic tree by mapping the specific 16S rDNA *Bacteroidetes* sequences (111 and 112 sequences retrieved by CF319a and CF968 respectively) onto a SILVA reference tree (Supporting Information Fig. S4). The *Bacteroidetes* sequences belonged to marine metagenomes globally distributed from three depths (surface, DCM and OMZ) and three distinct size fractions (0.22–1.6 μm, 5–20 μm and 180–2000 μm) of the five size fractions available. Although many of the monophyletic clusters were composed of phylotypes targeted by both probes, some clear differences emerged. Interestingly, there were more monophyletic clusters among the sequences detected only by CF968 (clusters A, C, D and E in Supporting Information Fig. S4) than among those retrieved only by probe CF319a (cluster B). Cluster A was found in surface waters from the Mediterranean Sea (St.7; close to the Gibraltar Strait) and within the size fraction 5–20 μm. However, clusters C, D and E were more cosmopolitan, being found in surface waters and DCM (cluster C) and widely distributed across several TARA stations (Supporting Information Fig. S4). Therefore, these data also confirm the differential target of each probe and the differences in the relative abundances of the targeted marine *Bacteroidetes*.

Conclusions

We tested the probe CF968, new for CARD-FISH, and the results indicate that it displays higher specificity for the *Bacteroidetes* phylum and performs better than probe CF319a with non-specific hybridizations in some of the marine taxa tested. We demonstrate the unspecific hybridization of probe CF319a in cultures of *Alphaproteobacteria* (*Rhodobacteraceae*) and *Gammaproteobacteria* (*Alteromonadaceae*) and in marine samples by using multicolor hybridizations performed with CF319a and specific probes for such groups. The comparative analyses of CARD-FISH and 16S rDNA *Bacteroidetes* sequences show no significant differences, although the analyses of the 16S rDNA metagenomic sequences targeted by probe CF319a show additional potential outgroup hits related to *Alphaproteobacteria* (*Rhodospirillaceae*) and *Deltaproteobacteria* (*Desulfobacterales*). We also demonstrate that CF968 unveils seasonal patterns that are not detected by CF319a and therefore this probe is recommended for studies that cover seasonality. We also observe, by analysing metagenomes, differences in the relative abundances of the targeted taxa by both probes. We finally conclude that using multiple probes is a recommended strategy for the accurate quantification of marine microbial populations.

Experimental procedures

Collection of environmental CARD-FISH samples

The environmental samples for the CARD-FISH counts were collected during four oceanographic expeditions (Supporting Information Fig. S1, Supporting Information Table S3): cruises ATOS I and II in the Arctic and Antarctic Oceans respectively; cruise MODIVUS, which covered both a horizontal transect and vertical profiles in the Northwestern Mediterranean Sea; and the TARA Oceans expedition, which includes a selection of stations from the Western to Eastern Mediterranean Sea, the Red Sea and the North Indian Ocean and Arabian Sea (Supporting Information Fig. S1). All seawater samples (200 μm pre-filtered) were fixed (1–12 h) with paraformaldehyde (1% final concentration) at 4°C in the dark and gently filtered through 0.2 μm polycarbonate filters, rinsed with milli-Q water and stored at –20°C until processing. For the BBMO samples, 25 ml was filtered onto 47 mm filters, whereas for the ATOS and MODIVUS samples, 10 to 15 ml was filtered through 25 mm filters. In TARA Oceans, 10 ml of surface and DCM water, and 90 ml of mesopelagic samples were filtered through 25 mm filters respectively.

CARD-FISH

CARD-FISH was carried out following the protocol described by Pernthaler and colleagues (2004). Two horseradish peroxidase (HRP)-labelled probes were used to determine the relative abundances of *Bacteroidetes* cells in the seawater samples: CF319a (Manz *et al.*, 1996) and CF968 (the new *Bacteroidetes* CARD-FISH probe). The eubacterial antisense probe Non338 (Wallner *et al.*, 1993) was used as a negative control. The hybridization results with Non338 never exceeded 0.5% of DAPI counts and these were not subtracted from CARD-FISH counts (Supporting Information

Fig. S5). The CF319a and Non338 probes were purchased from biomers.net (Ulm, Germany) and CF968 was synthesized with a 5′-aminolink (Thermo Fisher Scientific, Ulm, Germany) and subsequently labelled with HRP (Roche Diagnostic, Mannheim, Germany) according to Urdea and colleagues (1988) and Amann and colleagues (1992). The filters were first coated with a thin layer of agarose and permeabilized with lysozime (10 mg ml^{-1}, 37°C, 1 h) and achromopeptidase (60 U ml^{-1}, 37°C, 30 min). Hybridizations were carried out on filter sections overnight at 35°C and specific hybridization conditions were established by the addition of formamide to the hybridization buffer (55% formamide for both *Bacteroidetes* probes, 20% for Non338). After hybridization, filter sections were washed for 15 min at 37°C and the signal was amplified with Alexa 488-labelled tyramide for 15 min at 46°C.

The specificity of CF319a and CF968 was tested in the alphaproteobacterial isolates ZOCON-B2 and ZOCON-B3, and gammaproteobacterial isolates ZOCON-D11 and ZOCON-F9 available from the BBMO culture collection (GenBank Accession Numbers KJ700452-KJ700455). Control hybridizations were performed with probes ALF968 and GAM42a as described in Alonso-Sáez and colleagues' (2007). Moreover, multicolor CARD-FISH was performed with both *Bacteroidetes*-specific probes, the *Alteromonadales*-specific probe ALT1413 (Eilers *et al*., 2000) and the *Rhodobacterales*-specific probe ROS537 (Eilers *et al*., 2001) by using Alexa 488- and Alexa 594-labelled tyramides and using a step of peroxidase inactivation with 0.01 M HCl for 10 min at room temperature between hybridizations as described by Pernthaler and colleagues (2004). Dual hybridizations were performed in samples collected from the BBMO seawater incubation experiments in which *Alteromonadales* and *Rhodobacterales* represented a significant proportion of the bacterioplankton (F. Baltar, J. Palovaara, M. Vila-Costa, G. Salazar, E. Calvo, C. Pelejero, C. Marrasé, J.M. Gasol and J. Pinhassi, submitted).

CARD-FISH preparations were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1}) to enumerate total prokaryotes. Cell enumeration was carried out by using an Olympus BX61 epifluorescence microscope either manually or by applying a threshold-based macro (details in Massana *et al*., 1997) in the IMAGE PRO PLUS software adapted for CARD-FISH enumeration. The results of the manual and semi-automatic counts were calibrated successfully. Between 500 and 800 DAPI-positive cells were counted manually, with 1000–2000 in the semi-automatic protocol, in both cases in a minimum of 10 fields. The results are presented as the percentage of the positive hybridized cells to total prokaryotic cells.

CARD-FISH CF968 probe optimization

The CF968 (5′-GGTAAGGTTCCTCGCGTA-3′) probe was tested against two *Flavobacteriaceae* cultures, MED134 (*Dokdonia donghaensis*) and MED152 (*Polaribacter dokdonensis*) from our culture collection isolated from the BBMO (Lekunberri *et al*., 2014). During probe testing, the temperature was kept constant at 35°C and the stringency varied with different formamide concentrations (from 0 to 60%) in the hybridization buffer. The optimal formamide concentration for the CF968 probe was found to be 55%.

In silico *test of the CARD-FISH probes*

Probe evaluation was carried out by using the ARB software (Ludwig *et al*., 2004) with the curated SILVA (http://www.arb-silva.de) rDNA database (Pruesse *et al*., 2007; Quast *et al*., 2013). The release used was the 'non-redundant' SSU Ref NR 115 dataset, which is a modified version of the full reference release in which redundancies (> 99% identity) were filtered out (about 50% of the entries). The dataset contained 469,229 SSU rDNA sequences, including 40,931 of *Bacteroidetes*. Target and non-target matches were evaluated with the zero-weighted option in ARB's probe match tool.

TARA Oceans collection samples for metagenomes

Water samples were collected from two depths (surface and DCM) by using a large peristaltic pump (TECH-POMPES, A40) or a rosette sampler, and subsequently separated into five size fractions (0.2–1.6, 0.8–5, 5–20, 20–180 and 180–2000 μm). For fractions larger than 5 μm, samples were obtained after filtering the collected seawater by using the Gravity Plankton Sieving System, a series of nets of different-sized fractions (Karsenti *et al*., 2011). To obtain fractions smaller than 5 μm, 100 l of seawater was first filtered through 200 and 20 μm meshes to remove large plankton. Further filtering was carried out by pumping water serially through 142 mm filters of either a 1.6 and 0.2 μm or a 5 and 0.8 μm pore size with a peristaltic pump (Masterflex, EW-77410-10). After filtration, filters were flash frozen and kept for about 4 weeks at −20°C on the schooner and then at −80°C in the laboratory until the DNA extraction was performed.

Bacteroidetes *16S rDNA sequences extracted from TARA Oceans metagenomes*

Twenty-seven metagenomes from the TARA Oceans expedition were sequenced by 454 FLX-Ti pyrosequencing by the French National Genome Sequencing Center (Genoscope), France. All details on the DNA extraction, sequencing and accession numbers for these metagenomes can be found elsewhere (Hingamp *et al*., 2013). The selected marine metagenomes correspond to 13 distinct TARA Oceans stations, including samples from surface, DCM and OMZ. These TARA Oceans metagenomes belong to different plankton size fractions: 0.2–1.6, 0.8–5, 5–20, 20–180 and 180–2000 μm (details in Supporting Information Table S4).

The 16S rRNA gene sequences were extracted from these TARA Oceans metagenomes by using a customized version of rna_hmm.py, a python wrapper script that uses hidden Markov models (HMMs) to identify and annotate metagenomic reads that contain fragments of rRNA genes (Huang *et al*., 2009). Briefly, HMMs for 16S rRNA gene sequences were generated by using hmmbuild (version 3.0; Eddy, 2011) on aligned 16S rRNA gene sequences that were downloaded from the SILVA database (Quast *et al*., 2013) (http://www.arb-silva.de/projects/ssu-ref-nr/) (version SSU r104; alignment quality = 100; sequence length > =1200;

sequence quality > =75; restrict search to SILVA). Each candidate read was trimmed (and reverse-complemented if necessary) to segments that were predicted to contain 16S rRNA gene sequences, and a length filter of 250 bp for 454 reads was applied.

A total of 7185 16S rRNA reads were retrieved from the TARA Oceans metagenomes to build a database by using ARB software (version 5.1) (Ludwig *et al*., 2004). By using the match probe tool of ARB, only the 16S rRNA reads with perfect matches (zero mismatches) with either the CF319a or the CF968 probe sequences were enumerated and included into our analyses. First, the taxonomic assignment of the retrieved 16S rRNA reads was carried out by using the classifier tool of the RDP web (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Cole *et al*., 2009). Second, a phylogenetic tree was constructed. Sequences were first aligned by using the SINA multiple alignment algorithm (http://www.arb-silva.de/aligner/) (Pruesse *et al*., 2012) and imported into the ARB software (Ludwig *et al*., 2004). The phylogenetic tree was built by the insertion of sequences into the ARB-formatted SILVA 108 release database by using the parsimony algorithm.

The 16S rDNA sequences from the TARA Oceans metagenomes data used in this study are accessible from the Sequence Read Archive of the European Nucleotide Archive through Accession Numbers ERA155563, ERA155562 and ERA241291. Statistical analyses were carried out in the R environment (www.r-project.org) by using the Vegan package (Oksanen *et al*., 2009).

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References

Abell, G.C.J., and Bowman, J.P. (2005) Colonization and community dynamics of class *Flavobacteria* on diatom detritus in experimental mesocosms based on Southern Ocean seawater. *FEMS Microbiol Ecol* **53:** 379–391.

Acinas, S.G., Antón, J., and Rodríguez-Valera, F. (1999) Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl Environ Microbiol* **65:** 514–522.

Alonso, C., Warnecke, F., Amann, R., and Pernthaler, J. (2007) High local and global diversity of *Flavobacteria* in marine plankton. *Environ Microbiol* **9:** 1253–1266.

Alonso-Sáez, L., Balagué, V., Sà, E.L., Sánchez, O., González, J.M., Pinhassi, J., *et al*. (2007) Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiol Ecol* **60:** 98–112.

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.

Amann, R.I., Zarda, B., Stahl, D.A., and Schleifer, K.H. (1992) Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* **58:** 3007–3011.

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., *et al*. (2006) Whole genome analysis of the marine *Bacteroidetes* '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8:** 2201–2213.

Behrens, S., Fuchs, B.M., Mueller, F., and Amann, R. (2003) Is the *in situ* accessibility of the 16S rRNA of *Escherichia coli* for Cy3-labeled oligonucleotide probes predicted by a three-dimensional structure model of the 30S ribosomal subunit*? Appl Environ Microbiol* **69:** 4935–4941.

Blumel, M., Suling, J., and Imhoff, J. (2007) Depth-specific distribution of *Bacteroidetes* in the oligotrophic eastern Mediterranean Sea. *Aquat Microb Ecol* **46:** 209–224.

Buchan, A., González, J.M., and Moran, M.A. (2005) Overview of the marine *Roseobacter* lineage. *Appl Environ Microbiol* **71:** 5665–5677.

Campbell, B.J., Yu, L., Heidelberg, J.F., and Kirchman, D.L. (2011) Activity of abundant and rare bacteria in a coastal ocean. *Proc Natl Acad Sci USA* **108:** 12776–12781.

Chen, X., Zeng, Y., and Jiao, N. (2006) Development and evaluation of specific 16S rDNA primers for marine *Cytophaga-Flavobacteria* cluster. *Mol Ecol Notes* **6:** 1278–1281.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., *et al*. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37:** 141–145.

Cottrell, M.T., and Kirchman, D.L. (2000) Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66:** 1692–1697.

Crespo, B.G., Pommier, T., Fernández-Gómez, B., and Pedrós-Alió, C. (2013) Taxonomic composition of the particle-attached and free-living bacterial assemblages in the Northwest Mediterranean Sea analyzed by pyrosequencing of the 16S rRNA. *Microbiologyopen* **2:** 541–552.

De Gregoris, T.B., Aldred, N., Clare, A.S., and Burgess, J.G. (2011) Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J Microbiol Methods* **86:** 351–356.

DeLong, E.F., Franks, D.G., and Alldredge, A.L. (1993) Phylogenetic diversity of aggregate-attached vs. free-living

marine bacterial assemblages. *Limnol Oceanogr* **38:** 924–934.

Díez-Vives, C., Gasol, J.M., and Acinas, S.G. (2012) Evaluation of marine *Bacteroidetes*-specific primers for microbial diversity and dynamics studies. *Microb Ecol* **64:** 1047–1055.

Díez-Vives, C., Gasol, J.M., and Acinas, S.G. (2014) Spatial and temporal variability among *Bacteroidetes* populations in the NW Mediterranean Sea. *Syst Appl Microbiol* **37:** 68–78. doi:10.1016/j.syapm.2013.08.006; 2013.

Eddy, S.R. (2011) Accelerated Profile HMM Searches. *PLoS Comput Biol* **7:** e1002195.

Eilers, H., Pernthaler, J., Glöckner, F.O., and Amann, R. (2000) Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66:** 3044–3051.

Eilers, H., Pernthaler, J., Peplies, P., Glöckner, F.O., Gerdts, G., and Amann, R. (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. *Appl Environ Microbiol* **67:** 5134–5142.

Fandino, L.B., Riemann, L., Steward, G.F., and Azam, F. (2005) Population dynamics of *Cytophaga-Flavobacteria* during marine phytoplankton blooms analyzed by real-time quantitative PCR. *Aquat Microb Ecol* **40:** 251–257.

Fernández-Gómez, B., Richter, M., Schüler, M., Pinhassi, J., Acinas, S.G., González, J.M., and Pedrós-Alió, C. (2013) Ecology of marine *Bacteroidetes*: a comparative genomics approach. *ISME J* **7:** 1026–1037.

Ferrera, I., Gasol, J.M., Sebastian, M., Hojerova, E., and Koblizek, M. (2011) Growth rates of aerobic anoxygenic phototrophic bacteria as compared to other bacterioplankton groups in coastal Mediterranean waters. *Appl Environ Microbiol* **77:** 7451–7458.

Glöckner, F.O., Fuchs, B.M., and Amann, R. (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl Environ Microbiol* **65:** 3721–3726.

Gómez-Consarnau, L., González, J.M., Coll-Lladó, M., Gourdon, P., Pascher, T., Neutze, R., *et al*. (2007) Light stimulates growth of proteorhodopsin-containing marine *Flavobacteria*. *Nature* **445:** 210–213.

Gómez-Pereira, P.R., Fuchs, B.M., Alonso, C., Oliver, M.J., van Beusekom, J.E., and Amann, R. (2010) Distinct flavobacterial communities in contrasting water masses of the north Atlantic Ocean. *ISME J* **4:** 472–487.

Gómez-Pereira, P.R., Schüler, M., Fuchs, B.M., Bennke, C., Teeling, H., Waldmann, J., *et al*. (2012) Genomic content of uncultured *Bacteroidetes* from contrasting oceanic provinces in the North Atlantic Ocean. *Environ Microbiol* **14:** 52–66.

González, J.M., Fernandez-Gomez, B., Fernandez-Guerra, A., Gomez-Consarnau, L., Sanchez, O., Coll-Llado, M., *et al*. (2008) Genome analysis of the proteorhodopsin-containing marine bacterium *Polaribacter* sp MED152 (*Flavobacteria*). *Proc Natl Acad Sci USA* **105:** 8724–8729.

Hingamp, P., Grimsley, N., Acinas, S.G., Clerissi, C., Subirana, L., Poulain, J., *et al*. (2013) Exploring nucleocytoplasmic large DNA viruses in Tara Oceans microbial metagenomes. *ISME J* **7:** 1678–1695.

Huang, Y., Gilna, P., and Li, W. (2009) Identification of ribosomal RNA genes in metagenomic fragments. *Bioinformatics* **25:** 1338–1340.

Karsenti, E., Acinas, S.G., Bork, P., Bowler, C., De Vargas, C., Raes, J., *et al*. (2011) A holistic approach to marine eco-systems biology. *PLoS Biol* **9:** e1001177.

Kirchman, D.L., Yu, L., and Cottrell, M.T. (2003) Diversity and abundance of uncultured *Cytophaga*-like bacteria in the Delaware estuary. *Appl Environ Microbiol* **69:** 6587–6596.

Kirchman, D.L., Cottrell, M.T., and Lovejoy, C. (2010) The structure of bacterial communities in the western Arctic Ocean as revealed by pyrosequencing of 16S rRNA genes. *Environ Microbiol* **12:** 1132–1143.

Lefort, T., and Gasol, J.M. (2013) Global-scale distributions of marine surface bacterioplankton groups along gradients of salinity, temperature, and chlorophyll: a meta-analysis of fluorescent *in situ* hybridization studies. *Aquat Microb Ecol* **70:** 111–130. doi:10.3354/ame01643.

Lekunberri, I., Gasol, J.M., Acinas, S.G., Gómez-Consarnau, L., Crespo, B.G., Casamayor, E.O., *et al*. (2014) The phylogenetic and ecological context of cultured and whole-genome-sequenced marine bacteria in the coastal NW Mediterranean Sea. *Syst Appl Microbiol* **37:** 216–228. doi:10.1016/j.syapm.2013.11.005.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al*. (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.-H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142:** 1097–1106.

Massana, R., Gasol, J.M., Bjørnsen, P.K., Blackburn, N., Hagstrøm, A., Hietanen, S., *et al*. (1997) Measurement of bacterial size via image analysis of epifluorescence preparations: description of an inexpensive system and solutions to some of the most common problems. *Sci Mar* **61:** 397–407.

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.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G.L., Solymos, P., *et al*. (2009) Vegan: community ecology package. R package version 1.15-2. URL http://CRAN.R-project.org/package=vegan.

O'Sullivan, L.A., Weightman, A.J., and Fry, J.C. (2002) New degenerate *Cytophaga-Flexibacter-Bacteroides*-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. *Appl Environ Microbiol* **68:** 201–210.

Pernthaler, A., Pernthaler, J., and Amann, R. (2004) Sensitive multi-color fluorescence *in situ* hybridization for the identification of environmental microorganisms. In *Molecular Microbial Ecology Manual*. Kowalchuk, G.A., De Bruin, F.J., Head, I.M., Akkermans, A.D.L., and Van Elsas, J.D. (eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 711–726.

Pinhassi, J., Sala, M.M., Havskum, H., Peters, F., Guadayol, O., Malits, A., and Marrasé, C. (2004) Changes in bacterioplankton composition under different phytoplankton regimens. *Appl Environ Microbiol* **70:** 6753–6766.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., *et al*. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35:** 7188–7196.

Pruesse, E., Peplies, J., and Glöckner, F.O. (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* **28:** 1823–1829.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al*. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41:** 590–596.

Riemann, L., Steward, G.F., and Azam, F. (2000) Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* **66:** 578–587.

Ruiz-González, C., Galí, M., Lefort, T., Simó, R., and Gasol, J.M. (2012a) Annual variability in light modulation of bacterial heterotrophic activity in surface northwestern Mediterranean waters. *Limnol Oceanogr* **57:** 1376–1388.

Ruiz-González, C., Galí, M., Simó, R., and Gasol, J.M. (2012b) Sunlight effects on the DMSP-sulfur and leucine assimilation activities of polar heterotrophic bacterioplankton. *Biogeochemistry* **110:** 57–74.

Rusch, D.B., Halpern, A.L., Sutton, G., Heidelberg, K.B., Williamson, S., Yooseph, S., *et al*. (2007) The Sorcerer II Global Ocean Sampling Expedition: northwest Atlantic through eastern tropical Pacific. *PLoS Biol* **5:** e77.

Sarmento, H., and Gasol, J.M. (2012) Use of phytoplankton-derived dissolved organic carbon by different types of bacterioplankton. *Environ Microbiol* **14:** 2348–2360.

Schattenhofer, M., Fuchs, B.M., Amann, R., Zubkov, M.V., Tarran, G.A., and Pernthaler, J. (2009) Latitudinal distribution of prokaryotic picoplankton populations in the Atlantic Ocean. *Environ Microbiol* **11:** 2078–2093.

Simon, M., Glockner, F.O., and Amann, R. (1999) Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquat Microb Ecol* **18:** 275–284.

Teeling, H., Fuchs, B.M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C.M., *et al*. (2012) Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336:** 608–611.

Urdea, M.S., Warner, B.D., Running, J.A., Stempien, M., Clyne, J., and Horn, T. (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. *Nucleic Acids Res* **16:** 4937–4956.

Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14:** 136–143.

Weller, R., Glöckner, F.O., and Amann, R. (2000) 16S rRNA-targeted oligonucleotide probes for the *in situ* detection of members of the phylum *Cytophaga-Flavobacterium-Bacteroides*. *Syst Appl Microbiol* **23:** 107–114.

Yilmaz, L.S., and Noguera, D.R. (2004) Mechanistic approach to the problem of hybridization efficiency in fluorescent *in situ* hybridization. *Appl Environ Microbiol* **70:** 7126–7139.

Yilmaz, L.S., Parkernar, S., and Noguera, D.R. (2011) mathFISH, a web tool that uses thermodynamics-based mathematical models for *in silico* evalulation of oligonucleotide probes for fluorescence *in situ* hybridization. *Appl Environ Microbiol* **77:** 1118–1122.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Map showing the geographic location of the samples used in this study.

Fig. S2. CARD-FISH-composed photomicrographs of marine prokaryotes from a Red Sea environmental sample (St. 32-Surface, TARA Oceans), stained with DAPI and hybridized with:

A. HRP-labelled CF319a.

B. HRP-labelled CF968 oligonucleotide probes.

Scale bar = 20 μm.

Fig. S3. A and B. The upper pie chart represents the RDP taxonomy classification of the 16S rDNA sequences retrieved by the CF319a probe (111 sequences) and the CF968 probe (112 sequences) from 27 TARA Oceans metagenomes. The ring in the bottom shows the main genera of *Flavobacteria* (represented in green in the upper pie) captured by each probe. The percentage numbers in the ring represent the relative abundances, and only those with percentages > 3 are shown in the graphic.

Fig. S4. Phylogenetic tree of the *Bacteroidetes* using the 16S rDNA sequences from the TARA Oceans metagenomes that were targeted by each probe (light blue CF319 and light red CF968 inner ring). The second ring shows each TARA station analysed. The third ring represents the three depths sampled at TARA St (Surface, DCM and OMZ). Finally, the outer ring displays the three different size fractions with positive hits (0.22–1.6 μm, 5–20 μm, and 180–2000 μm).

A–E. Different clusters exclusively retrieved by one of the probes.

Fig. S5. Photomicrographs of marine prokaryotes:

A. Stained with DAPI for total prokaryotes

B. Hybridized with HRP-labelled Non338 probe showing a lack of signal for the non-sense probe (negative control).

Scale bar = 20 μm.

Table S1. Summary of the *in silico* analyses of the probes CF319a and CF968 based on the SILVA ref 115 dataset showing the target hits (% coverage) and outgroup hits at different taxonomic ranks (Phylum, Class, Family and Genus).

Table S2. Difference in the relative abundance of the *in silico* coverage analyses between genera within *Flavobacteria* for probes CF319a and CF968.

Table S3. Geographic location and depth of the samples used for CARD-FISH comparisons in this study obtained in four different oceanographic cruises.

Table S4. Comparative abundances of *Bacteroidetes* (using probes CF319a and CF968), *Gammaproteobacteria* (probe Gam42a), *Alteromonadaceae* (probe ALT1413) and *Rhodobacteraceae* (probe ROS537) in samples from the Mediterranean Sea collected by the TARA Oceans expedition. Numbers in bold represent the highest abundance of *Bacteroidetes* that coincided with the highest abundance of *Rhodobacteraceae* (shown in red).

Table S5. TARA Oceans metagenomes used in this study to extract the 16S rDNA *Bacteroidetes* sequences.