

ORIGINAL ARTICLE

Distinct flavobacterial communities in contrasting water masses of the North Atlantic Ocean

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Members of the class *Flavobacteria* in the phylum *Bacteroidetes* are among the most abundant picoplankton in coastal and polar oceans. Their diversity is high in marine waters. However, quantitative information about distribution patterns of flavobacterial clades is scarce. We analyzed the diversity and clade-specific abundances of individual *Flavobacteria* in different oceanic provinces in the North Atlantic Ocean. Samples were taken along the 30°W meridian between the East Greenland current and the North Atlantic subtropical gyre. Comparative sequence analysis of 16S ribosomal RNA (rRNA) gene libraries revealed high diversity and significant spatial variability within the class *Flavobacteria*. Published and newly designed oligonucleotide probes were used to enumerate eleven flavobacterial clades by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). We found that different provinces harbor distinct flavobacterial communities. Clade DE2 accounted for a substantial fraction of total *Flavobacteria* only in the Polar Biome (BPLR), whereas the VISION clades VIS1 and VIS4 significantly increased in the Arctic (ARCT) province. Members of the genus *Polaribacter* were the most abundant clade in all the water masses analyzed, with highest absolute numbers in BPLR and ARCT. We improved the CARD-FISH protocol to quantify the rare clades VIS2, VIS3, VIS5 and VIS6, which were present in abundances below 0.5%. They all showed pronounced regional distribution patterns. Microscopic analysis proved a specific enrichment of *Flavobacteria* in the phycosphere of nanophytoplankton of BPLR and ARCT. Our results suggest that different marine flavobacterial clades have distinct niches and different life strategies.

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Introduction

Members of the *Bacteroidetes* phylum, formerly also known as *Cytophaga-Flavobacteria-Bacteroides* cluster, constitute one of the major groups of picoplankton (Glöckner *et al.*, 1999; Kirchman, 2002). *Bacteroidetes* thrive in a variety of marine systems, including hydrothermal vents (Sievert *et al.*, 2000; Kormas *et al.*, 2006), coastal sediments (Llobet-Brossa *et al.*, 1998) and waters (Eilers *et al.*, 2001; O'Sullivan *et al.*, 2004) and in open ocean waters (Simon *et al.*, 1999; Abell and Bowman, 2005; Schattener *et al.*, 2009). Taxonomically, the phylum *Bacteroidetes* consists of the classes

Bacteroidia, *Flavobacteria*, *Sphingobacteria* and *Cytophagia*, the latter previously included in the class *Sphingobacteria* (Ludwig *et al.*, 2008). There is more and more evidence, for example, from comparative 16S ribosomal RNA (rRNA) sequence analysis, that *Flavobacteria* is the dominant class of *Bacteroidetes* in marine picoplankton (for example, Kirchman *et al.*, 2003; Alonso *et al.*, 2007; Chen *et al.*, 2008). High abundance has been linked to cold waters (Simon *et al.*, 1999; Abell and Bowman, 2005), phytoplankton blooms (Simon *et al.*, 1999), photic zone (Schattener *et al.*, 2009) and upwelling systems (Alonso-Saez *et al.*, 2007). This distribution suggests a preference for more productive conditions. However, *Flavobacteria* have also been detected in oligotrophic marine surface waters in significant numbers (Schattener *et al.*, 2009).

Sequence-based studies have shown the dominance of particular flavobacterial phylotypes during algal blooms, whereas other clades of *Flavobacteria*

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dominated before the blooms (Riemann *et al.*, 2000; O'Sullivan *et al.*, 2004; Pinhassi *et al.*, 2004). In addition, *Flavobacteria* could be localized in the phycosphere (Rooney-Varga *et al.*, 2005; Sapp *et al.*, 2007), which is the space immediately surrounding an algal cell (Bell and Mitchell, 1972). Moreover, this association might depend on the composition and dynamics of the phytoplankton and flavobacterial communities (Grossart *et al.*, 2005; Rooney-Varga *et al.*, 2005). However, most of these studies were not combined with a quantitative technique and, therefore, there is no information about the clade-specific population sizes.

Cell abundances and distribution patterns of members of the phylum *Bacteroidetes* in marine picoplankton have been obtained by fluorescence *in situ* hybridization (FISH) using probes CF319a (Manz *et al.*, 1996) and CFB560 (O'Sullivan *et al.*, 2002); the specificity of these two probes has recently been reviewed (Amann and Fuchs, 2008). Evidence for distribution patterns of distinct flavobacterial clades, however, is sparse. Examples include FISH-based quantifications of the clade *Cytophaga marinoflava-latercula*, the uncultured cluster DE2 and the genus *Polaribacter*. *Cytophaga marinoflava-latercula* was shown to constitute up to 6% of total picoplankton population during spring and summer, off the island of Helgoland, in the German Bight of the North Sea (Eilers *et al.*, 2001). Clade DE2, originally retrieved from the Delaware Estuary, was found to account for up to 10% of picoplankton population in this estuary as well as in the Chukchi Sea, Arctic Ocean (Kirchman *et al.*, 2003; Malmstrom *et al.*, 2007). Members of the genus *Polaribacter* have been shown to form prominent fractions in polar oceans (Brinkmeyer *et al.*, 2003; Malmstrom *et al.*, 2007).

This study was performed in the North Atlantic between the East Greenland current and the North Atlantic subtropical gyre (NAST), an area characterized by annual spring blooms known as one of the most conspicuous seasonal events in the world ocean (for example, Ducklow and Harris, 1993). The spring bloom starts north of about 40°N latitude and subsequently develops northwards (Longhurst, 1998). The concentration of chlorophyll *a* remains high during summer and it gradually returns to winter concentrations (Henson *et al.*, 2006). During summer, there is a shift from dominance by the >20 µm chlorophyll fraction to dominance by the <20 µm chlorophyll fraction (Gifford *et al.*, 1995). In contrast, the NAST is an oligotrophic area with low levels of primary production (Longhurst, 1995). Thus, the North Atlantic Ocean is characterized by strong gradients that could influence the distribution patterns of picoplankton groups.

The objective of this study is to quantify flavobacterial clades that inhabit these contrasting oceanic provinces in the northern North Atlantic Ocean and to identify environmental factors that control their abundance. We analyzed samples of four

oceanic provinces as defined by Longhurst (1995), which encompassed nine significantly different water masses according to the classification of Oliver and Irwin (2008). We hypothesized that: (i) members of the class *Flavobacteria* are present throughout the transect, (ii) general flavobacterial abundances are higher at the more northern stations where temperature is low and nutrient concentration high, and in the euphotic zone in which phytoplankton abundance is high, (iii) the flavobacterial diversity varies locally, and (iv) different flavobacterial clades prevail in different water masses and are correlated with distinct environmental parameters (for example, temperature, nutrient concentration and phytoplankton abundance).

Materials and methods

Samples were taken along a transect in the North Atlantic Ocean from 66°39.27'N; 29°36.65'W (station 2 = S2) to 34°24.87'N; 28°28.90'W (S19) during the VISION cruise (diVersItY, Structure and functiON) MSM03/01 on board the research vessel Maria S. Merian from September 21 to September 30, 2006 (Figure 1a, Table 1). Along this transect, each station was sampled at 12 depths, from 10 m down to 250 m or 500 m. Samples were collected with a rosette of 20-l Niskin bottles mounted on a conductivity–temperature–density profiler. Chlorophyll *a* fluorescence was measured on board by a flow through fluorometer (microFlu-Chl, TriOS Optical Sensors GmbH, Oldenburg, Germany). Total picoplankton and nanophytoplankton populations were enumerated after fixation with particle-free formaldehyde solution (37% w/v, Fluka, Taufkirchen, Germany; final concentration, 1% v/v) and staining with SYBR Green (1:10 000 dilution of stock; Sigma-Aldrich, Taufkirchen, Germany) (Marie *et al.*, 1997) using a FACScalibur flow cytometer equipped with an air-cooled laser providing blue light at 488 nm as described previously (Tarran *et al.*, 2006). Profiles were grouped according to oceanic provinces defined by two classification systems (Longhurst, 1998; Oliver and Irwin 2008). The Longhurstian provinces used in this study include the Boreal Polar (BPLR), the Arctic (ARCT), the North Atlantic Drift (NADR) and the North Atlantic Subtropical East (NAST) provinces. Oliver and Irwin (2008) delineated provinces by objectively clustering satellite observed thermal and optical properties. The provinces defined by Oliver and Irwin (2008) were computed on the basis of the September 2006 mean of the Advanced Very High Resolution Radiometer (AVHRR) sea surface temperature and the Sea-viewing Wide Field-of-view Sensor (SeaWiFS) water leaving radiance.

Nutrient analysis

Water samples for nutrients analysis were filtered directly after sampling through 0.45-µm in-line

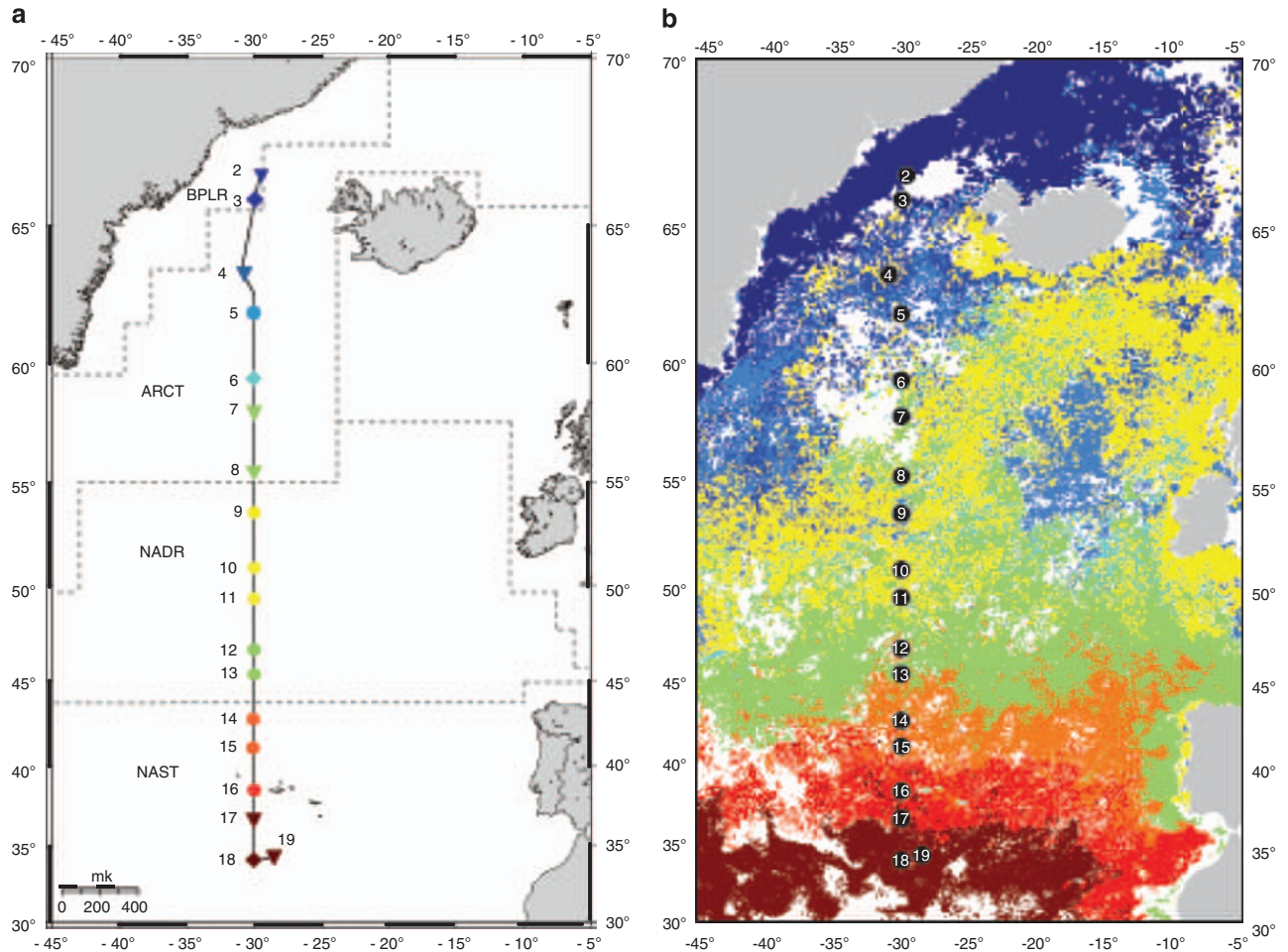


Figure 1 (a) Map indicating the VISION cruise track and sampled stations S2–S19 and boundaries of the oceanic provinces Boreal Polar (BPLR), Arctic (ARCT), North Atlantic Drift (NADR) and North Atlantic Subtropical (NAST) provinces. Color of symbols represents different water masses as showed in Figure 1b. Symbol types represent analyses done in the sampled stations: Spheres (○) indicate stations where only CF319a counts were performed; triangles (▼) indicate stations where flavobacterial clade-specific and CF319a counts were performed; and rhomboids (◆) indicate stations where 16S rRNA gene clone libraries and counts were performed. (b) Significant water masses determined by the September 2006 mean of AVHRR sea surface temperature and SeaWiFS water leaving radiance according to Oliver and Irwin (2008). The different colors represent different water mass types.

Table 1 Coordinates, date and time, temperature (temp), salinity (sal), dissolved oxygen (DO) in the 20-m samples of those stations (S) from which the 16S rRNA gene libraries were constructed

Province	S	Coordinates		Date; time (UTC)	Temp (°C)	Sal (PSU)	DO (ml l ⁻¹)
		Latitude	Longitude				
BPLR	3	65°52,64'N	29°56,54'W	22.09.06; 19:00	0.7	33.0	7.7
ARCT	6	59°20,87'N	29°59,89'W	24.09.06; 08:00	10.9	35.0	5.9
NAST	18	34°04,43'N	30°00,09'W	30.09.06; 08:00	23.5	36.4	4.7

Abbreviations: ARCT, Arctic; BPLR, Boreal Polar; NAST, North Atlantic subtropical province; UTC, Coordinated Universal Time; PSU, practical salinity units.

Time indicated is the time when sampling procedure started.

filters attached to a 60-ml pre-cleaned syringe into two 12-ml polystyrene tubes. Samples were stored at 4 °C (dissolved silicate) or –80 °C (NH₄⁺, PO₄³⁻, NO₃⁻ and NO₂⁻). The samples were spectrophotometrically measured with a continuous-flow analyzer

using standard AA3 methods (Seal Analytical, Norderstedt, Germany) using a variant of the method of Grasshoff *et al.* (1983). The s.d. values were calculated for repeated measurements of five surface samples (20 m) and five deep samples (200 m), and

were for PO_4^{3-} and NO_2^- $0.01\ \mu\text{M}$ at all levels, for NO_{2+3} $0.03\ \mu\text{M}$ at the $1\ \mu\text{M}$ level and $0.07\ \mu\text{M}$ at the $18\ \mu\text{M}$ level, and for NH_4^+ $0.07\text{--}0.08\ \mu\text{M}$ at all levels.

DNA extraction and 16S rRNA gene libraries

The 16S rRNA gene libraries were constructed from the 20-m samples at S3, S6 and S18 (Figure 1). Approximately 100-l samples were pooled from five Niskin bottles, pre-filtered through a $10\text{-}\mu\text{m}$ steel mesh cartridge (Wolftechnik, Weil der Stadt, Germany), and microorganisms were collected on cellulose acetate filters ($142\ \text{mm}$ diameter, $0.2\ \mu\text{m}$ pore size; Sartorius, Goettingen, Germany). Filters were stored at $-80\ ^\circ\text{C}$. DNA was extracted from the $0.2\text{-}\mu\text{m}$ filters according to procedure used by Zhou *et al.* (1996).

Almost complete bacterial 16S rRNA genes were amplified from the extracted DNA using the primer pairs GM3F, 5'-AGAGTTTGATCMTGGC-3', and GM4R, 5'-TACCTTGTACGACTT-3' (Muyzer *et al.*, 1995). The PCR was run at the following cycling conditions: initial denaturation at $96\ ^\circ\text{C}$ for 3 min, followed by 20 cycles consisting of denaturation ($96\ ^\circ\text{C}$ for 1 min), annealing ($48\ ^\circ\text{C}$ for 2 min) and extension ($72\ ^\circ\text{C}$ for 3 min) and a final step at $72\ ^\circ\text{C}$ for 15 min. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), ligated using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany) or pGEM-T-Easy (Promega, Mannheim, Germany) and cloned into high-efficiency competent cells of *Escherichia coli* (JM109). Libraries were screened for *Bacteroidetes* containing clones by PCR with the primer pair CF315-F, 5'-ACKGGYACTGAGAYACGG-3'; and CF967-R, 5'-GGTAAGGTTCTCGCGTA-3' (Chen *et al.*, 2006). The PCR reaction and cycling conditions were as described above but with 30 cycles. Annealing temperature was $61\ ^\circ\text{C}$ after optimization of the primers to avoid false positives. Plasmids of positive clones were isolated with Montage plasmid MiniPrep₉₆ kit (Millipore, Eschborn, Germany) and $50\text{--}100\ \text{ng}$ of template was used per sequencing reaction performed with ABI BigDye and ABI PRISM3100 Genetic Analyser (Applied Biosystems, Darmstadt, Germany). All sequences were checked for chimera origin using the online tool Bellerophon from Greengenes (Huber *et al.*, 2004; DeSantis *et al.*, 2006) and possible chimera sequences were excluded from further analysis. The 16S rRNA gene sequences of this study were deposited in GeneBank under the accession numbers FN433282–FN433465.

Phylogenetic reconstruction

Sequences were analyzed with the software package ARB (Ludwig *et al.*, 2004) using Silva database release 96, October 2008 (Pruesse *et al.*, 2007). Phylogenetic reconstruction was done following the 'standard operating procedure for phylogenetic

inference' (Peplies *et al.*, 2008). Operational taxonomic units were calculated with DOTUR (Distance-Based OTU and Richness; Schloss and Handelsman, 2005) using the furthest neighbor algorithm from a distance matrix generated in ARB. From the DOTUR results, we identified all the sequences that contributed to each operational taxonomic unit. These data were imported to EstimateS (Colwell, 2005) and similarity indexes calculated according to Chao *et al.* (2005). Coverage index of the clone libraries was calculated using the formula $C = (1 - n/N) * 100$, where N represents the number of all clones carrying a *Bacteroidetes* insert and n the number of phylotypes at 99% identity appearing only once in the library (Good 1953; Kemp and Aller 2004).

Catalyzed reporter deposition (CARD)-FISH

Samples for CARD-FISH were fixed with particle-free formaldehyde solution (37% w/v, Fluka; final concentration, 1% v/v) for 2 h at room temperature. Water samples were filtered with low pressure Hg onto polycarbonate filters (type GTTP; $0.2\ \mu\text{m}$ pore size; $47\ \text{mm}$ diameter; Millipore, Eschborn, Germany) in varying volumes. Typically $15\text{--}30\ \text{ml}$ was filtered from the surface water layer and $50\text{--}150\ \text{ml}$ from samples below $100\ \text{m}$ depth. In addition, 500-ml aliquots were filtered from the surface water layer. The filters were stored frozen at $-20\ ^\circ\text{C}$ for further analyses. The CARD-FISH procedure was performed according to Perenthaler *et al.* (2004) with modifications as published elsewhere (Schattenhofer *et al.*, 2009). Hybridizations were done with horseradish peroxidase-labeled oligonucleotides probes (Biomers, Ulm, Germany) at varying formamide concentrations depending on the probe used (Table 2). Working solutions of probes and competitors (both at $50\ \text{ng}\ \mu\text{l}^{-1}$) were mixed with hybridization buffer in a 1:1:300 proportion and hybridization was carried out overnight at $46\ ^\circ\text{C}$. The probe-delivered horseradish peroxidase was detected with tyramides that were custom labeled with fluorescein (Molecular Probes, Eugene, OR, USA). Subsequently, all cells were stained with 4,6-diamidino-2-phenylindole ($1\ \mu\text{g}\ \text{ml}^{-1}$). Hybridized and 4,6-diamidino-2-phenylindole-stained cells were quantified on an Axioplan II Imaging epifluorescence microscope (Zeiss, Jena, Germany).

The signals in the negative control (NON338) were low, accounting for less than one cell in 10 fields. Differences in clade abundance between provinces were tested by *t*-test on $\log(x+1)$ transformed data. If data did not pass normality and equal variance test, Mann–Whitney–Wilcoxon test was applied.

For the evaluation of *Bacteroidetes* associated with nanophytoplankton cells, hybridizations were performed on 500-ml samples from surface waters using probes, CF319a and SAR11-441, targeting the SAR11 clade (Morris *et al.*, 2002) at 25% formamide. Nanophytoplankton cells were identified by

Table 2 Flavobacterial clade-specific oligonucleotide probes used in this study

Probe	Target	Sequence (5' → 3')	FA (%)	Reference
CF319a	<i>Bacteroidetes</i>	TGGTCCGTGTCTCAGTAC	35	Manz <i>et al.</i> (1996)
POL740	<i>Polaribacter</i>	CCCTCAGCGTCAGTACATACGT	35	Malmstrom <i>et al.</i> (2007)
CF6-1267	DE2	GAAGATTCGCTCCTCCTC	35	Kirchman <i>et al.</i> (2003)
FL197	VIS4	TCATCTCATACCGTAACC	35	This study
Comp FL197_1		TCATCCCTACCGTAACC		
Comp FL197_2		YCATCTCKTACCGTAACC		
VIS6-814	VIS6	CAGCGAGTGATGATCGTT	15	This study
Comp VIS6-814		CAGCGAGTGATGATCGTT		
VIS5-586	VIS5	CCACTGACTTAGATTCCC	25	This study
Comp VIS5-586_1		CCACTGACTTAGAGGCC		
Comp VIS5-586_2		CCACTGACTTAAATTTCC		
FL845	VIS2	GCTTAGCCACTCATCTAA	15	This study
Comp FL845_1		GCTTAGCCACTCAACTAA		
Comp FL845_2		GCTTAGCCACTCAACCAA		
VIS3-183	VIS3	CTCTAGAAAGTGATGCCAC	25	This study
VIS1-575	VIS1	CTTAACAAACAGCCTGCGGACC	35	This study
Comp VIS1-575		CTTAAAAAACAGCCTGCGGACC		
FL443	<i>Gramella</i>	TAGATGTGTTCTTCCCG	65	Alonso unpub
LEE82	<i>Leeuwenhoekiella</i>	ACTCGTCAGCTTCCCGAA	25	This study
Comp LEE82_1		GCTCGCCAGCCTCCCGAA		
Comp LEE82_2		ACTCGTCAGCATCCCGAA		
DOK827	<i>Dokdonia</i>	GTCCGAAAACCAAACAAC TAG	20	This study
Comp DOK827_1		GTCCGAAAACCAACAGCTAG		
Comp DOK827_2		GUCCGAAAACCGAACAGCTAG		

Abbreviations: Comp, unlabeled competitor oligonucleotides; FA, formamide concentration (v/v) in the hybridization buffer.

epifluorescence microscopy from their pigment fluorescence and an approximate diameter of 5–10 μm . Pictures were taken with a black and white camera (AxioCam MRm; Carl Zeiss, Jena, Germany). The phycosphere, which is defined as the area immediately surrounding nanophytoplankton cells (Bell and Mitchell, 1972), was delimited and measured on the micrographs using the software AxioVision Rel. 4.7.2 (Carl Zeiss). More than 30 nanophytoplankton cells were evaluated in more than 10 images. The CF319a- and SAR11-positive signals were quantified inside the phycosphere, including the nanophytoplankton cell, and outside of it. Ratios of counts per area unit between inside and outside the phycosphere were calculated and used for statistical analysis with the *t*-test.

Testing a modified counting protocol for rare populations

For quantification of rare populations, we enumerated picoplankton populations from filters containing increasing volumes of water samples. Samples were taken at the German North Sea island Helgoland (54°11.3'N; 07°54.0'E) and were filtered on polycarbonate filters (0.2 μm pore size) in increasing amounts from 10, 25, 50, 100, 250 to 400 ml in triplicates for each volume. Hybridizations were performed as described above using probes ROS537, for members of the *Roseobacter* clade (Eilers *et al.*, 2001) at 35% formamide; NOR5-730, for members of the gammaproteobacterial NOR5/OM60 clade (Eilers *et al.*, 2001) at 50% formamide and PLA46, for *Plantomycetes* (Neef *et al.*, 1998) at 30%. The

NON338 probe was used as negative control at 35% formamide. Only the probe-conferred fluorescence was counted in 20 microscopic fields for each volume replicate and the absolute abundance of the clade was determined after subtraction of the negative control. To test whether significant differences exist among the different volumes, one-way analysis of variance was applied. When normality or equal variance test were not passed, analysis of variance on ranks was used.

Probe design

Oligonucleotide probes targeting the 16S rRNA of flavobacterial clades were designed with the probe design tool in ARB, as well as unlabeled competitor oligonucleotides (Manz *et al.*, 1992; Table 2; Supplementary Table 1). For optimization of stringency conditions, a series of hybridizations at increasing formamide concentrations (10–50%) were evaluated. The optimal formamide concentration was the highest concentration before signal intensity decreased (Supplementary Table 2). Unlabeled competitor oligonucleotides, which allow a single mismatch discrimination by blocking non-target probe-binding sites (Manz *et al.*, 1992), were used at all times to assure specificity (Supplementary Table 1).

Probe POL740 (Malmstrom *et al.*, 2007) was re-evaluated on the strain *Polaribacter filamentus* (DSMZ13964, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The LEE82 and DOK827 probes were tested on *Leeuwenhoekiella* sp. and *Dokdonia* sp. strains,

respectively. The strains were isolated during the cruise and kindly provided by J Harder (unpublished data).

Multi- and univariate statistics on Flavobacteria abundances

Multivariate statistical analyses were applied to test the differences in the flavobacterial community among the different water masses. A similarity matrix was calculated with Bray–Curtis coefficient on square root-transformed abundance data. The Bray–Curtis matrix was represented by hierarchical agglomerative cluster analysis using group average as the linkage algorithm. Significance of the grouping in the cluster analysis was tested using similarity profile permutation test at 99% significance level. The similarity profile permutation tests for statistically significant evidence of genuine clusters in an *a priori* unstructured set of samples (Clarke *et al.*, 2008). Multivariate statistics were performed with software Primer v6 (Version 6.1.5, Primer-E, Plymouth, UK; Clarke and Gorley, 2006).

Correlations between environmental variables and flavobacterial abundances were analyzed with Spearman rank correlation test. The test was performed with physicochemical parameters (temperature, salinity, dissolved silicate, NH_4^+ and PO_4^{3-} , which is correlated with NO_3^- and NO_2^-), chlorophyll *a* fluorescence and abundance of nanophytoplankton and of different microphytoplankton classes (dinoflagellates, diatoms and coccolithophores). Microphytoplankton data were kindly provided by J Ufkes and V Smetacek (personal communication). Correlations with counts obtained with probe CF319a were performed with data from surface to 50 m of all stations. The significance level of the correlations was 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). All univariate statistical analyses were performed using the software Sigma-Stat 3.5 (SYSTAT, Santa Clara, CA, USA).

Results

Characterization of study area

Boundaries of the oceanic provinces as defined by the Longhurstian classification were in good agreement with delimitations according to Oliver and Irwin (2008), based on the satellite-observed thermal and optical properties of the study area during September 2006 (Figure 1b). However, the four Longhurstian provinces could be further separated by the latter approach into nine distinct water masses (Figure 1). The northernmost stations (S2 and S3) were located at the boundary of BPLR and ARCT provinces and satellite-derived water masses confirmed that they represented a separate area. These stations had an average chlorophyll *a* con-

centration of $0.7 \mu\text{g l}^{-1}$, surface water temperature below 3°C and low salinity (<33 PSU, practical salinity units) (Supplementary Figures 1, 2a and b). Within ARCT province, four water masses (stations S4, S5, S6, S7 + S8) were differentiated with chlorophyll *a* concentrations ranging between 0.3 and $1 \mu\text{g l}^{-1}$, and temperature between 10 and 12°C . Along the NADR province (S9–S13), the temperature gradually increased up to 20°C . In the southernmost water body (S17–S19) of the NAST province (S14–S19), the temperature reached 24°C , and chlorophyll *a* had the lowest value of $<0.1 \mu\text{g l}^{-1}$. The concentrations of phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-) and dissolved silicate (Si) in the upper 50 m were higher in the BPLR and ARCT provinces, ranging between 0.2 and $0.6 \mu\text{M}$, 2.0 and $6.5 \mu\text{M}$, 0.1 and $0.2 \mu\text{M}$, and 0.6 and $3.2 \mu\text{M}$, respectively. The concentrations decreased along the NADR province to very low concentrations in the NAST with PO_4^{3-} , NO_3^- , $\text{NO}_2^- < 0.06 \mu\text{M}$, and Si $< 0.3 \mu\text{M}$. The NH_4^+ concentrations ranged between 0.2 and $0.6 \mu\text{M}$ in surface layers and decreased below detection limit in deeper waters.

The abundance of picoplankton in the surface mixed layer was higher in the BPLR, ARCT and NADR provinces ($0.4\text{--}1.1 \times 10^6$ cells per ml) than in the NAST province ($0.2\text{--}0.6 \times 10^6$ cells per ml; Supplementary Figure 2c). The abundance of nanophytoplankton was also higher in the upper mix layer of the BPLR, ARCT and NADR provinces ($4.9\text{--}36 \times 10^3$ cells per ml) than in the NAST province ($2.7\text{--}8.2 \times 10^3$ cells per ml; Supplementary Figure 2d).

Bacteroidetes diversity and distribution

The abundance of *Bacteroidetes* along the transect was quantified by CARD-FISH using the probe CF319a down to a depth of 500 m (Figure 2; Supplementary Table 3). Maximum counts of $18.2 \pm 2.8\%$ of total picoplankton were detected in surface waters of the BPLR province. *Bacteroidetes*

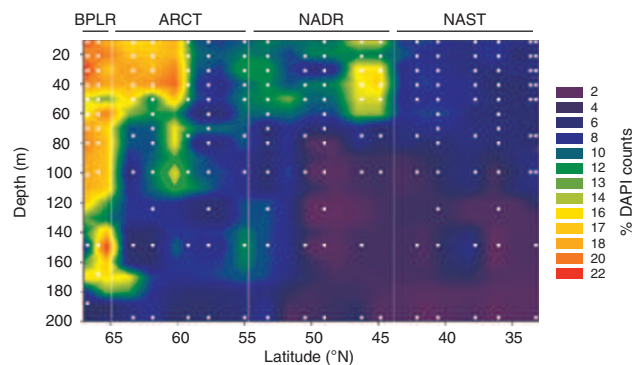


Figure 2 Latitudinal depth distribution of *Bacteroidetes* as detected by probe CF319a (relative abundance, % of 4,6-diamidino-2-phenylindole (DAPI) counts). Oceanic provinces as indicated in Figure 1a. Dots represent the sampling depths.

abundance decreased along ARCT and NADR provinces to a minimum of $7.0 \pm 1.1\%$ in the NAST province. Abundances decreased with depth and were between 0.9 and 8% at 200 m. As the abundance of *Bacteroidetes* was highest in the upper 50 m, we focused further analysis on the mixed surface water layer.

As a basis for the subsequent quantification of flavobacterial clades, three 16S rRNA gene libraries were constructed with general bacterial primers from surface water samples of S3 (BPLR), S6 (ARCT) and S18 (NAST) in which *Bacteroidetes* represented 19, 17 and 7% of picoplankton population, respectively. We screened a total of 1632 clones of which 190 carried a *Bacteroidetes* insert. After removal of six putative chimeric sequences, we further analyzed 71 clones from S3 (61 > 1400 nt, 58 unique), 55 from S6 (52 > 1400 nt, 43 unique), and 58 from S18 (55 > 1400 nt, 54 unique). We opted for almost full-length 16S rRNA sequences to facilitate subsequent probe design.

The coverage of the *Bacteroidetes* diversity at 99% sequence identity level was high at S3 and S6 with 79 and 92%, respectively. It was lower at S18 (63%). Moreover, the three libraries had little overlap in their diversity (Supplementary Table 4). The similarity between S3 and S6 was low with indices ranging between 0.1–0.3 and no operational taxonomic units were shared between S3/S6 and S18. Similarity indices at 97% identity confirmed that S3 and S6 were more similar to each other (0.2–0.4) than both were to S18 (0.02–0.05).

Comparative sequence analysis showed that sequences affiliated with the class *Flavobacteria* dominated all three libraries (Figure 3) despite the fact that the primer pair used for screening had a similar coverage of the two classes *Flavobacteria* and *Sphingobacteria* (Chen *et al.*, 2006), including the new class *Cytophagia*. A total of 180 sequences belonged to the class *Flavobacteria*, only three to the class *Sphingobacteria*, one to the class *Cytophagia* and none to the class *Bacteroidia*.

A detailed phylogenetic analysis placed 83% of the flavobacterial sequences (151/180) within the 'marine clade' that comprises genera that have been exclusively isolated from marine habitats (Bowman, 2006). A total of 43 sequences within the marine clade grouped closely with those of cultured genera. For example, S3 contained 27 sequences affiliated with the genus *Polaribacter*. They all had the target site of probe POL740 (Malmstrom *et al.*, 2007). From S18, we obtained eight sequences of *Leeuwenhoekella* spp. and four of *Dokdonia* spp. For these two genera, probes were designed in this study (LEE82, DOK827; Supplementary Table 5). Another 107 sequences clustered with yet uncultured clades mostly retrieved from various marine pelagic environments, both coastal and open ocean, as well as temperate and polar sites. Sequences related to clade DE2, originally described by sequences retrieved from the Delaware Estuary (Kirchman *et al.*, 2003), were present in S3 (11), S6 (9) and S18 (6).

The DE2 clade was subsequently quantified using the probe CF6-1267 (Kirchman *et al.*, 2003).

We also defined new clades on the basis of sequences from the VISION cruise. Clade VIS1 contained 9 and clade VIS4 contained 18 sequences that originated mainly from S6 and S18. The probes VIS1-575 and FL197 were designed to quantify VIS1 and VIS4, respectively. The VIS2 clade includes sequences from both coastal and open ocean environments; this clade was quantified by probe FL845. Clade VIS5, targeted by VIS5-586, contained sequences from S3 and S6, and also sequences from other diverse marine habitats. The VIS3 clade, targeted by VIS3-183, contained 10 sequences from S3 and 8 from S6 that were most closely related to sequences previously retrieved from the North Sea (Alonso *et al.*, 2007). A total of 19 sequences were related to the uncultured lineage 'AGG58' (DeLong *et al.*, 1993; O'Sullivan *et al.*, 2004). Moreover, three sequences from S6 clustered tightly with those of a natural iron fertilization event (West *et al.*, 2008). We named this clade VIS6 and targeted it with probe VIS6-814.

Quantifying rare flavobacterial clades

First FISH analyses using probes for the clades VIS2, VIS3, VIS5 and VIS6 resulted in counts of less than 1 cell per microscopic field, corresponding to relative abundances of 0.5% and lower. Similarly, in the more southern stations, the otherwise abundant clades VIS1, VIS4 and DE2 dropped below 0.5% of total bacteria in abundance. Such infrequent events were below the threshold for confident quantification by FISH. We therefore tested whether this limitation could be overcome by filtering larger volumes resulting in a higher cell density. Probe-specific counts proportionally increased with increasing amounts of filtered sample ($r^2 = 0.9$; Supplementary Figures 3a, c and e). Signals in controls for unspecific staining by CARD-FISH stayed low, accounting for only 1–2 signals in 10 microscopic fields, in both the filters through which high and low volumes of seawater were filtered (from here on named high- and low-volume filters). Although calculated clade abundances were not significantly different, the s.e. values were markedly lower for the high-volume filters (Supplementary Figures 3d and f). Moreover, when cells of clade DE2, detected by probe CF6-1267, were counted on low- and high-volume filters from S3, no statistical difference was detected in the counts ($P > 0.05$). On the basis of these results, except for *Polaribacter* spp. all flavobacterial clades were quantified on 500-ml filters.

On these filters, the average numbers of probe-positive cells per counting grid for the abundant clades were in the range of 20–226 for probe VIS1-575; 5–200 for probe FL197; and 1–78 for probe CF6-1267 along the transect. For the low-abundance clades, the numbers ranged from 1–55 for VIS3-183; 1–38 for VIS6-586; 5–10 for FL845; and 1–9 for VIS5-586. The lower limit for quantification with the

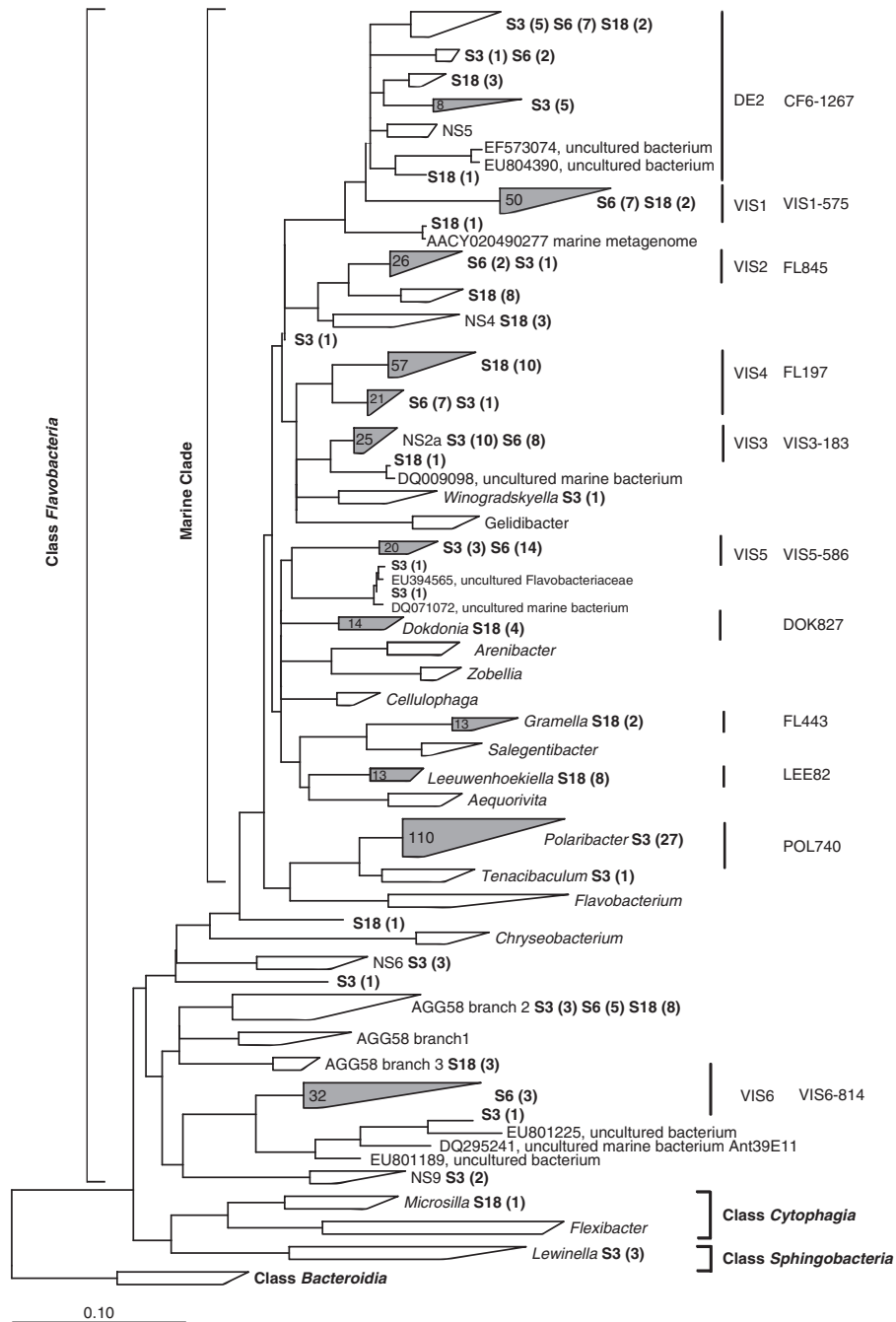


Figure 3 Phylogenetic association of sequences retrieved in this study from Boreal Polar (BPLR) (S3), Arctic (ARCT) (S6) and North Atlantic Subtropical (NAST) (S18), indicating the number of sequences of each station that contributes to the clades shown in wedges. Consensus tree was built with full-length sequences on the basis of neighbor joining and maximum likelihood methods, calculated without and with 50% bacteria positional conservatory and 30% *Bacteroidetes* positional conservatory filters. Gray shadows indicate target clades of flavobacterial clade-specific oligonucleotide probes indicating the number of targeted sequences in the database Silva 96 (Pruesse et al., 2007). Name of the probes are given to the right. VIS: VISION clades.

modified protocol was set to 1 cell per counting grid on average.

Abundance of flavobacterial clades

The genus *Polaribacter* and the clades VIS1, VIS4 and DE2 were detected throughout the transect (Figure 4a; Supplementary Table 6). *Polaribacter*

spp. was the most abundant clade at all the stations and depths analyzed, except for S8 in which clade VIS1 was slightly more abundant. Abundances were higher in BPLR and ARCT provinces, with a maximum detected at S4 with $72 \pm 45 \times 10^3$ *Polaribacter* spp. cells per ml that account for $6.7 \pm 3.5\%$ of picoplankton population. Counts decreased toward S7 ($P < 0.01$) and further south to

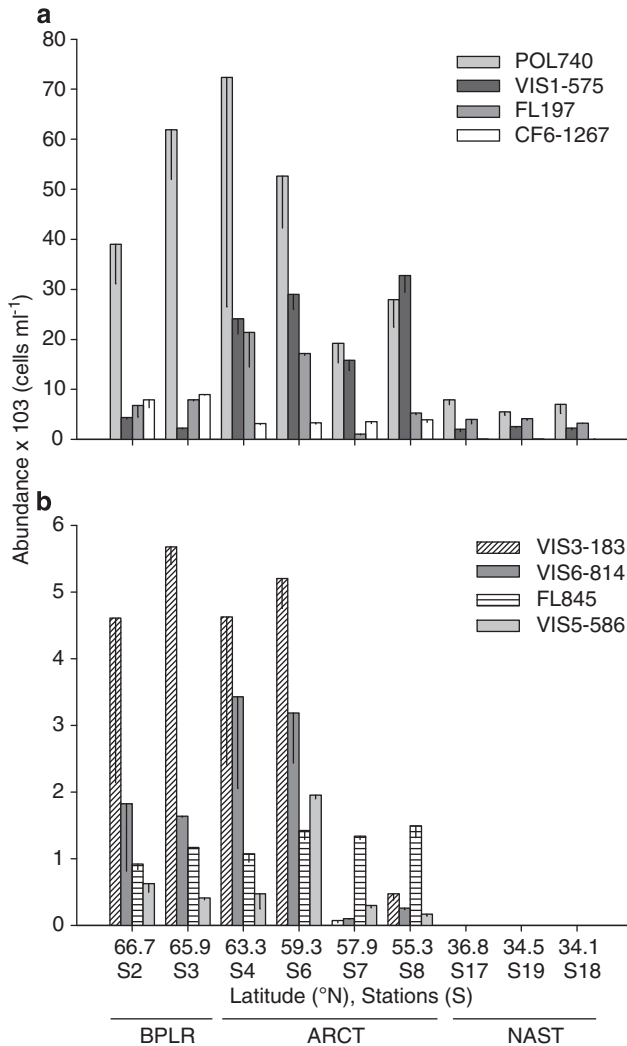


Figure 4 Total abundance of flavobacterial clade-specific probes in the Boreal Polar (BPLR), Arctic (ARCT) and North Atlantic Subtropical (NAST) provinces. (a) High-abundance clades and (b) low-abundance clades. Error bars represent s.d. values for duplicates (S2–S4; 20 and 40 m) or triplicates (S6–S19, 10, 20 and 30 m), shown as the negative s.d. value. All samples are from the upper mix layer and, therefore, considered as replicates.

S19 ($5.5 \pm 0.7 \times 10^3$ cells per ml; $1.0 \pm 0.1\%$). Members of the clade VIS1 significantly increased from BPLR to ARCT province ($P < 0.01$), reaching a peak of $29 \pm 3 \times 10^3$ cells per ml ($3.4 \pm 0.4\%$) at S8. The VIS1 abundance in surface water was again lower at S19 ($2.5 \pm 0.2 \times 10^3$ cells per ml; $0.5 \pm 0.05\%$). At S4–S6, there was a significant increase in clade VIS4 ($P < 0.001$), with a maximum of $21 \pm 7 \times 10^3$ cells per ml or $2.1 \pm 0.3\%$ at S4. This was followed by a steep decrease toward S7 ($1.0 \pm 0.2 \times 10^3$ cells per ml; $0.1 \pm 0.03\%$; $P < 0.001$). The DE2 clade was most abundant in BPLR at S3 ($8.9 \pm 0.1 \times 10^3$ cells per ml; $0.9 \pm 0.07\%$); it showed a step-like decrease in the ARCT province ($3.3 \pm 0.2 \times 10^3$ cells per ml; $0.4 \pm 0.03\%$; S4; $P < 0.001$) and a second step

in the NAST stations (105 ± 12 cells per ml; 0.02% ; S18; $P < 0.001$).

Clades VIS3, VIS6, VIS2 and VIS5 were detected in the BPLR and ARCT provinces, but not in the NAST province (Figure 4b; Supplementary Table 6). The VIS3 and VIS6 clades were more abundant between S2 and S6 with a maximum of VIS3 at S3 with $5.6 \pm 0.2 \times 10^3$ cells per ml ($0.6 \pm 0.02\%$), and one of VIS6 at S4 with $3.4 \pm 1.3 \times 10^3$ cells per ml ($0.3 \pm 0.08\%$). As for VIS4, both groups significantly decreased within the ARCT province from S6 to S7 ($P < 0.01$). The VIS2 clade had a relatively uniform distribution in BPLR and ARCT provinces with only a slight increase towards its maximum at S8 ($1.5 \pm 0.2 \times 10^3$ cells per ml; $0.1 \pm 0.03\%$). The group VIS5 represented a low fraction of the total bacterial community in both provinces (maximum at S6: $19.5 \pm 0.5 \times 10^2$ cells per ml; 0.2%).

Flavobacterial community composition in different provinces

Cluster analysis of absolute clade abundances showed that the flavobacterial communities were rather homogeneous within distinct water masses and different between them (Figure 5). Replicate samples of S2 and S3—representing BPLR—clustered with each other and were distinct from the adjacent ARCT stations S4 and S6 (Figure 5a). Within the ARCT province, the S4/S6 cluster was clearly separated from the flavobacterial communities at S7 and S8. The NAST stations S17–S19 were clustered tightly and were different from all other samples. The clustering was generally in good agreement with the water mass classification on the basis of its thermal and optical properties. There are only two minor differences between the satellite-derived classification of water masses and the clustering of flavobacterial communities. The first difference is that S7 and S8 occur in the same satellite-derived water mass (Figure 1), but are statistically distinct according to the cluster analysis (Figure 5a). The second is that S4 and S6 are in distinct water masses according to the satellite-based classification, but are not statistically different with respect to the clustering of flavobacterial clades.

A visualization of flavobacterial community composition in the different water bodies supported the clustering (Figure 5b). In the ARCT samples about 70% of the members of the class *Flavobacteria* could be assigned to specific clades, whereas in BPLR and NAST provinces the overall coverage with the set of clade-specific probes used in this study was $< 50\%$. Overall, the *Polaribacter* clade was the most important one accounting on average for about 30% of *Flavobacteria*, a fraction only equaled by VIS1 in S7 and S8. In the NAST province, the community was significantly different with *Polaribacter* and VIS4 accounting for 11 and 10% of *Flavobacteria*, respectively, followed by VIS1 (5.8%).

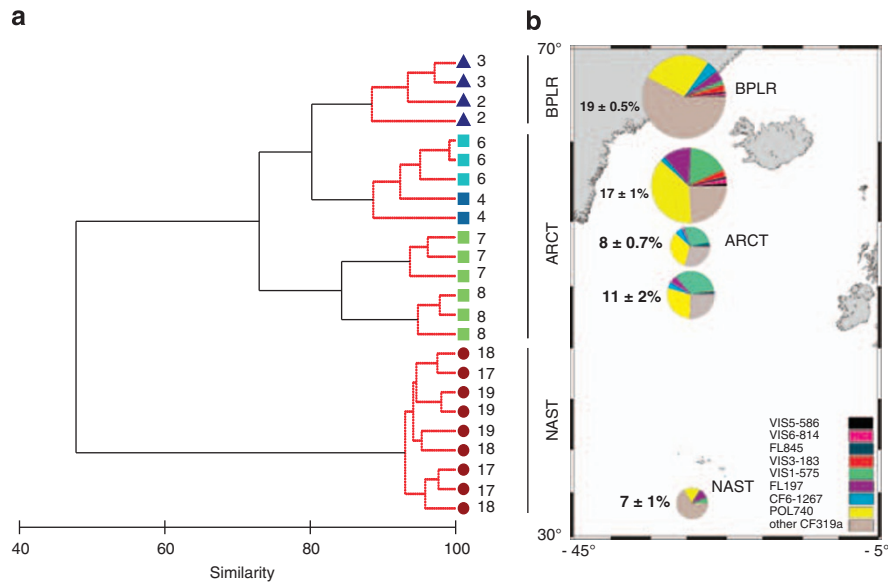


Figure 5 Flavobacterial community composition, as analyzed by fluorescence *in situ* hybridization (FISH) with clade specific-probes in the Boreal Polar (BPLR), Arctic (ARCT) and North Atlantic Subtropical (NAST) provinces. **(a)** Dendrogram representing the similarity of the flavobacterial community in the three provinces. Symbols are color coded as in Figure 1 and numbers indicate station replicates. Black lines in the dendrogram represent significant clusters at the 99% level using SIMPROF (similarity profile permutation test) test; red lines represent non-significant clusters at this level. **(b)** Relative contribution of each flavobacterial subgroup to the total CF319a. Pie chart represents average group sizes of clustering stations in **(a)**; area of pie represents the relative abundance of CF319a (percentage is given to the left).

Table 3 Spearman rank correlation between temperature (temp), salinity (sal), phosphate (PO_4^{3-}) and dissolved silicate concentration (Si), chlorophyll *a* fluorescence (Chl *a*), total abundance of microphytoplankton (microphyto), nanophytoplankton (nanophyto) and *Flavobacteria* clade abundances

	CF319a	POL740	CF6-1267	FL-845	VIS1-575
Temp	-0.87	-0.89	-0.79	0.73	
Sal	-0.86	-0.82	-0.87		
PO_4^{3-}	0.84	0.87			
Si	0.87	0.84	0.77	-0.78	
Chl <i>a</i>	0.66				0.85
Microphyto	0.63				
Nanophyto	0.76		0.57		0.74

r_s values indicated are statistically significant after Bonferroni correction, at the level $P < 0.05$. *n* being the number of simultaneous independent comparisons.

Further correlation analysis of single environmental variables revealed that counts with probe CF319a, as well as abundance of *Polaribacter* spp. and clade DE2 were significantly negatively correlated with temperature with r_s values of -0.87, -0.89 and -0.79, respectively (Table 3). The same applies for salinity with r_s value ranging between -0.82 and -0.87. The correlation with silicate concentration was significantly positive (r_s values: 0.77-0.87). Abundance of clade VIS2 was significantly positively correlated with temperature ($r_s = 0.73$) and negatively with silicate concentration ($r_s = -0.78$). Abundances of VIS1, VIS3, VIS4, VIS5 and VIS6 clades did not correlate with any physicochemical parameter.

Taking biological variables into consideration, counts with probe CF319a were significantly correlated with chlorophyll *a* fluorescence ($r_s = 0.66$), and with the total abundance of microphytoplankton ($r_s = 0.63$) and nanophytoplankton ($r_s = 0.76$). A stronger correlation was detected between the group VIS1 and chlorophyll *a* fluorescence ($r_s = 0.85$) and nanophytoplankton abundance ($r_s = 0.74$). Members of the DE2 clade were significantly correlated with the abundance of nanophytoplankton ($r_s = 0.57$). The high-density 500-ml filters of S3 were used to do a preliminary check regarding whether the correlation is caused by a direct attachment of *Flavobacteria* to the phycosphere of nanophytoplankton cells (Figure 6). We quantified the CF319a-positive cells inside and outside the phycosphere of nanophytoplankton (Figure 6a) and compared the ratio with the relative localization of the free-living SAR11 clade (Figure 6b). The SAR11 cells were more abundant (27% at S3, 20m; M Schattenhofer, unpublished results) than *Flavobacteria* (19% at S3, 20m) in the BPLR province. Assuming a random co-occurrence, more SAR11 cells would be expected per nanophytoplankton cell compared with flavobacterial cells. However, the ratio inside and outside the phycosphere was 2.5 ± 0.8 ($n = 13$) for CF319a and 0.8 ± 0.2 ($n = 10$) for SAR11-441. The difference was significant ($P < 0.01$), implying that *Flavobacteria* were specifically enriched in the phycosphere compared with SAR11 cells. The clades *Polaribacter*, VIS1, VIS4 and DE2 were identified in the phycosphere of nanophytoplankton (Figures 6c-f), but an exact

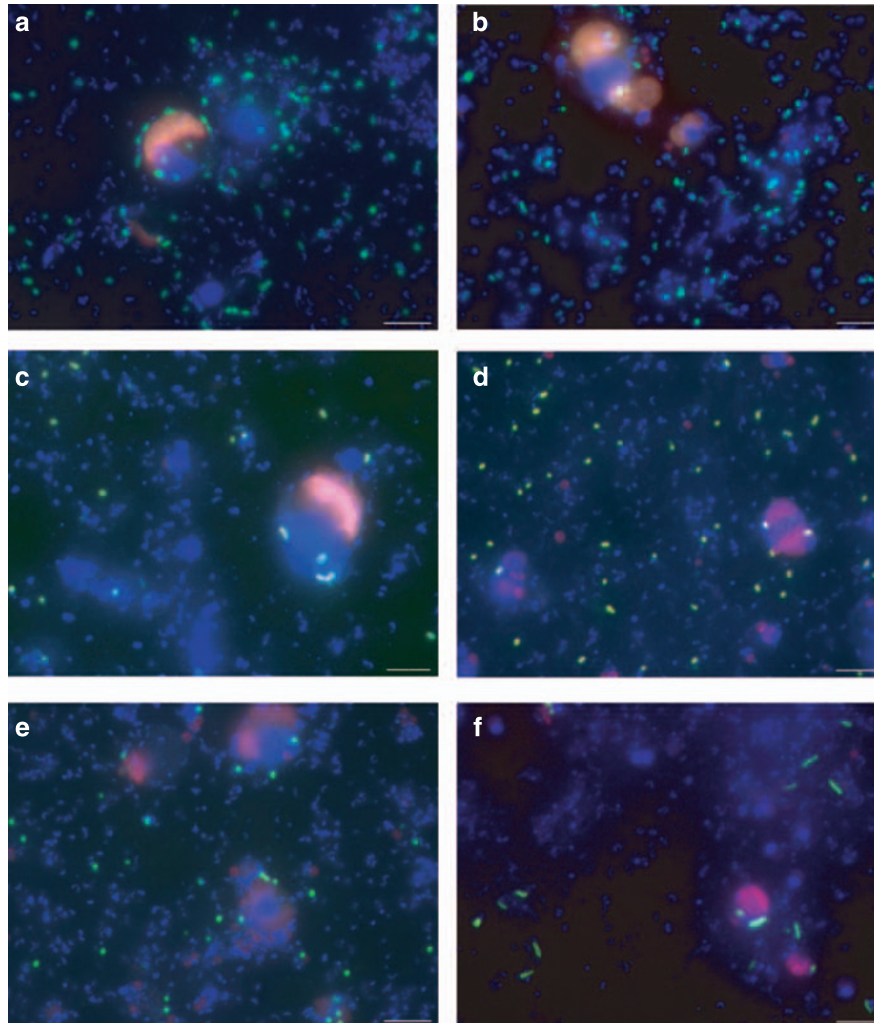


Figure 6 Epifluorescence micrographs of nanophytoplankton cells and bacteria in the phycosphere. Red, autofluorescence of nanophytoplankton cells; blue, DAPI signals of DNA containing cells; and green, signals of catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)-positive cells using the probes: (a) CF319a, (b) SAR11-441, (c) POL740, (d) VIS1-575, (e) FL197 and (f) CF6-1267. a–c and f: S3, 20 m; d and e: S4, 20 m. Bar = 5 μ m.

quantification was not possible. Filters obtained during the VISION cruise had too low numbers of algae with attached flavobacteria for a reliable quantification. Even higher volumes should have been filtered or the nanophytoplankton fraction should have been concentrated before filtration.

Discussion

High abundance of members of the class *Flavobacteria* in marine waters has already been reported by ZoBell (1946) in his famous monography 'Marine Microbiology'. He linked the occurrence of these aerobic heterotrophic bacteria that often form yellow-to-orange colonies on agar plates to the aerobic degradation of algal polymers. We here add molecular evidence supporting (i) the general importance of *Flavobacteria* in marine picoplankton and (ii) the occurrence of distinct flavobacterial

communities in different marine water bodies. Our data of a transect in the North Atlantic between 65°N and 33°N confirm the preference of certain flavobacterial clades for cold and nutrient-rich water masses (Figure 2), as it had been reported by others (Simon *et al.*, 1999; Brinkmeyer *et al.*, 2003; Kirchman *et al.*, 2003; Abell and Bowman, 2005). Extending data of an Atlantic meridional transect cruise between 40°N and 20°S (Schattenhofer *et al.*, 2009), we demonstrate that other flavobacterial clades thrive under oligotrophic conditions in warm waters, both in the NADR and the NAST provinces.

Comparative 16S rRNA sequence analysis and high-resolution quantitative CARD-FISH using a newly designed set of clade-specific oligonucleotide probes provide independent evidence for the existence of distinct flavobacterial communities in different water bodies. The coverage of flavobacterial diversity in three representative clone libraries (S3, S6 and S18) was not complete, but

with values between 63% (NAST) and 92% (ARCT) was sufficient to clearly prove that there was only little overlap between the different water bodies. S3 and S18 shared only 2–5% of its phylotypes (Supplementary Table 4), whereas S3 and S6, which were more similar in terms of environmental characteristics, shared 25–40% of flavobacterial phylotypes.

The CARD-FISH quantification added numbers in support of distinct flavobacterial populations along the transect (Figures 4 and 5). These shifts coincided with transitions from one water mass to another (Figure 5a). Within the ARCT province, for example, a sharp decrease in chlorophyll *a* fluorescence was evident from S6 to S7, which was mirrored by an abrupt reduction of VIS3, VIS4 and VIS6 populations as well as of total *Flavobacteria* (Figures 1,2 and 4).

Overall, we identified marked distribution patterns of flavobacterial clades in contrasting water bodies of the North Atlantic Ocean. Our results are in line with the increasing body of evidence that distinct picoplankton populations show pronounced seasonal (Fuhrman *et al.*, 2006), latitudinal (Fuhrman *et al.*, 2008) and depth (DeLong *et al.*, 2006) distribution patterns in marine systems. For example, SAR11 abundances follow distinct annual distribution patterns in the euphotic and upper mesopelagic zones of the Sargasso Sea (Carlson *et al.*, 2009). Different high light- and low light-adapted ecotypes of *Prochlorococcus* show marked depth distributions in the subtropical and tropical Atlantic Ocean (Johnson *et al.*, 2006). Similarly, characteristic latitudinal distribution patterns of *Synechococcus* clades have been identified, with particular clades dominating temperate mesotrophic waters (Zwirgmaier *et al.*, 2007).

Distinct distribution patterns of abundant flavobacterial clades

The CARD-FISH analysis proved that members of the genus *Polaribacter* were present throughout the transect, although related sequences were only retrieved from BPLR province. The absolute numbers in the surface waters were consistently high and ranged between 7×10^4 (S4) and 5×10^3 (S19) cells per ml. The abundance was negatively correlated with temperature indicating a preference for colder water masses of less than 10 °C, which is within the optimal growth temperature of several cultured strains (Gosink *et al.*, 1998). Our data further support the importance of *Polaribacter* spp. in polar environments (Bano and Hollibaugh, 2002; Brinkmeyer *et al.*, 2003). Yet, although abundances of *Polaribacter* spp. decreased more than one order of magnitude in NAST province, in which the temperature was more than 20 °C, they were still the largest of the flavobacterial clades in these blue waters. This could be the result of a more cosmopolitan distribution of a single *Polaribacter* lineage.

Alternatively, this could reflect a lack of discrimination, by probe POL740, of several mutually exclusive species in different water bodies. Recently, *P. dokdonensis* has been described to have a growth optimum of 25–28 °C (Yoon *et al.*, 2006). *P. dokdonensis* MED152 can grow in nutrient-poor environments and use proteorhodopsin for generating energy from light (Gonzalez *et al.*, 2008). Considering the high abundance of *Polaribacter* spp. and its significant intra-genus diversity both in its physiology and in 16S rRNA similarity, for example, *P. dokdonensis* shares only 96.2–96.8% with the other *Polaribacter* spp. type strains (Yoon *et al.*, 2006), we recommend that future studies should attempt to subdivide this important genus. We hypothesize that this will reveal uncoupled population dynamics of warm- and cold-water phylotypes with different life styles.

In contrast to *Polaribacter* spp., clades VIS1 and VIS4—both encompassing mostly 16S rRNA gene sequences from temperate and subtropical open ocean sites—had more narrow population maxima. The VIS1 clade was above 1×10^4 cells per ml throughout ARCT province only, a density reached by VIS4 at S4 and S6, but not at S7 and S8. Clade DE2 was again different, with a maximum slightly below 1×10^4 cells per ml in BPLR. The DE2 clade comprises sequences that have mostly been retrieved from coastal systems, such as the Delaware Estuary (Kirchman *et al.*, 2003), the North Sea (Eilers *et al.*, 2001, Alonso *et al.*, 2007) and the coast of Plymouth (O'Sullivan *et al.*, 2004). The abundance of DE2 in our open ocean samples was significantly lower than the 10% previously reported, for example, for the Delaware Estuary (Kirchman *et al.*, 2003). On our transect, the BPLR stations might have been the ones with the most significant coastal influence.

Toward a reliable quantification of rare flavobacterial populations

A major output of this study was a protocol for the identification and quantification of rare clades (Figure 4b). The VIS2, VIS3, VIS5 and VIS6 clades consistently formed groups of <0.5% relative abundance. In this study, we could quantify these populations down to absolute numbers of only 10^2 cells per ml, which was equivalent to detecting one target cell in 10^4 non-target cells. Interestingly, of the rare clades only the abundance of VIS2 was explained by an environmental variable. It was positively correlated with temperature ($r_s = 0.73$). This group consists of sequences retrieved from coastal environments and open ocean, but none from polar origin pointing at a preference for warmer waters.

As mentioned above, the rare clades, VIS3 and VIS6, significantly decreased from S6 to S7 (Figure 4b; Supplementary Table 6), mirroring a change in the optical properties of the water masses

(Figure 1b and Figure 5a). Clade VIS6 comprises also phylotypes previously retrieved during a phytoplankton bloom in naturally iron-fertilized waters (West *et al.*, 2008); West *et al.* (2008) had reported a dominance of these phylotypes during the bloom. In this study, we could not detect a correlation between abundance of VIS6 and micro- or nanophytoplankton abundance or chlorophyll *a* fluorescence.

Except for *Polaribacter* spp. readily culturable flavobacteria were rarely detected. Members of the genus *Gramella* could not be detected in any of the samples, whereas those of genera *Dokdonia* and *Leeuwenhoekella* were only observed in samples from the NAST province. Their abundance was too low to be quantified, but their conspicuously large morphology allowed for a confident identification in all three last stations (S17–S19). Genome analysis of *Dokdonia* spp. MED134 revealed that this strain contains a gene for proteorhodopsin and its growth is enhanced when exposed to light under low or intermediate labile organic matter concentrations (Gomez-Consarnau *et al.*, 2007), which could point to an adaptation of this group to oligotrophic environments.

In summary, the rare flavobacterial clades also showed differential distribution patterns. These clades, except for one case, did not correlate with other biological or environmental variables. With CARD-FISH of high volume filters, quantitative studies on 'the rare biosphere' (Sogin *et al.*, 2006) are now within reach. Moreover, rare clades can be responsible for most of the uptake of key nutrients (Musat *et al.*, 2008) and can carry out fundamental ecosystem processes (Kuypers *et al.*, 2005). Additional studies are now required to find out whether the rare flavobacterial clades are always present in low numbers, or if they bloom under particular conditions. Their exact ecosystem functions are yet to be elucidated.

Phytoplankton association of flavobacterial clades

Links between members of the class *Flavobacteria* and phytoplankton have been frequently reported (Simon *et al.*, 1999; O'Sullivan *et al.*, 2004; Pinhassi *et al.*, 2004; Grossart *et al.*, 2005; Sapp *et al.*, 2007). Phytoplankton cells release polymers, mainly polysaccharides, to the phycosphere during all the phases of their growth (Myklestad, 1995). Therefore, the association of *Flavobacteria* with algae has been related to their potential ability to degrade molecules released by phytoplankton (Kirchman, 2002). During phytoplankton blooms certain flavobacterial clades develop short-term maxima, whereas others dominate before the blooms (Riemann *et al.*, 2000; Pinhassi *et al.*, 2004; West *et al.*, 2008). Flavobacterial lineages might respond differentially to phytoplankton as a consequence of a physiological ability to take up different compounds released by algal cells. For example, the use of complex macromolecules varies among cultured genera of the class

Flavobacteria (Bernardet and Nakagawa, 2006). Furthermore, genomic data point to a specialization of distinct flavobacterial clades for degradation of polysaccharides and proteins (Bauer *et al.*, 2006; Woyke *et al.*, 2009).

In our study, we detected a significantly positive correlation between total *Flavobacteria*, as quantified by probe CF319a, and nanophytoplankton, as well as for the clades VIS1 and DE2 with nanophytoplankton (Table 3). Microscopy demonstrated that cells of four flavobacterial clades: VIS1, DE2, VIS4 and *Polaribacter*, did indeed reside in the phycosphere of nanophytoplankton cells (Figures 6c–f). As this observation could be a consequence of random agglomeration of cells during filtration, we compared the localization of CF319a-positive cells with the localization of the abundant alphaproteobacterial SAR11 clade. Our results clearly indicate a preference of *Flavobacteria* for attachment to nanophytoplankton at S3 in comparison with a typical free-living bacterium like SAR11 (Giovannoni *et al.*, 1990; Rappe *et al.*, 2002). Follow-up studies should simultaneously identify nanophytoplankton species and flavobacterial clades to investigate the presence of distinct and predictable species-specific interactions. We are confident that the combination of genomic data on metabolic potential, substrate incubation experiments and information on *in situ* co-localization will soon yield detailed insight in how exactly this group of 'old' marine bacteria is interacting with phytoplankton.

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