Bacterial community dynamics during the winter–spring transition in the North Sea

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Abstract

Bacterioplankton dynamics at Helgoland Roads (54°11.3′N, 7°54.0′E) in the North Sea over the winter–spring transition were investigated. The bacterial community was analyzed and correlated with phytoplankton community data and abiotic parameters. The community structure was analyzed by ribosomal intergenic spacer analysis (RISA) and by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes followed by DNA sequence analysis. The linkage of abiotic and biotic environmental factors and bacterial community as well as phylotypes (sequenced DGGE bands) was analyzed by the ordination technique of canonical correspondence analysis (CCA). Generally, an influence of temperature and phytoplankton on the bacterial community during the sampling period was observed. Additionally, multivariate analysis by factors revealed an influence on specific bacterial phylotypes of these factors. Overall, results indicate that changes in the bacterial community were caused not only by abiotic factors but also by the phytoplankton community.

Introduction

Bacterioplankton dynamics are governed by seasonal changes in abiotic and biotic factors, especially phytoplankton dynamics. Little is known about the controlling factors and their effects either on the bacterial community or on bacterial species. There are growing numbers of studies dealing with the seasonality of bacterioplankton community composition (Shiah & Ducklow, 1994; Pinhassi & Hagström, 2000; Gerdts *et al.*, 2004). One of the major controlling factors resulting in seasonality of the bacterial community composition was elucidated by Shiah & Ducklow (1994). Their investigation of the control of the whole bacterial community revealed temperature to be the major controlling factor in winter, autumn and spring, whereas limitation of inorganic nutrients and substrates was regarded to be the controlling factor in summer. Recent studies, including a mesocosm study, have supported this, indicating a general limitation of bacterioplankton in the summer due to organic carbon and inorganic nutrients in the natural environment (Rivkin & Anderson, 1997; Øvreås *et al.*, 2003). A direct relationship between temperature and bacterial production (Pinhassi & Hagström, 2000) as well as seasonal succession of the community structure (Gerdts *et al.*, 2004), was shown for marine bacterioplankton. Generally, seasonality of the bacterioplankton community structure has also been shown in freshwater ecosystems (Kent *et al.*, 2004). Furthermore, it has been observed that seasonal forces play a structuring role in bacterial communities in lakes, as determined by a study conducted over two consecutive years (Yannarell *et al.*, 2004). This was also shown for an Antarctic lake by Pearce (2005).

An influence of temperature on the community was particularly observed for lakes located in northern Sweden (Lindström, 2001). Additionally, several authors have observed changes in bacterial community composition during natural blooms or mesocosm phytoplankton experiments (Middelboe *et al.*, 1995; Riemann *et al.*, 2000; Fandino *et al.*, 2001; Arrieta & Herndl, 2002; Pinhassi *et al.*, 2004; Brussaard *et al.*, 2005; Rooney-Varga *et al.*, 2005), indicating close coupling of phytoplankton and bacterial community composition. A close link between bacterioplankton (especially attached bacteria) and phytoplankton dynamics has already been shown by Rooney-Varga *et al.* (2005).

Consequently, the phytoplankton community seems to have a direct effect on the bacterioplankton, especially attached bacteria, at the phylogenetic level.

Approximately 20% of marine bacteria can currently be cultivated by traditional techniques or by dilution culturing (Selje *et al.*, 2005). Therefore, the analysis of factors controlling the bacterioplankton must include culture-independent methods. Using this approach, Pinhassi *et al.* (2004) worked with mesocosms with different phytoplankton regimes and found that shifts in the bacterial community could be correlated with the phytoplankton composition. Brussaard *et al.* (2005) demonstrated that the breakdown of a *Phaeocystis globosa* bloom in a mesocosm study was accompanied by changes in bacterial community composition.

Here we try to elucidate the driving forces for shifts in bacterial community structure over the winter–spring transition in the North Sea. Additionally, the influence of specific abiotic and biotic factors on the phylotypes was studied. It was hypothesized that temperature as well as nutrients and phytoplankton dynamics account for the seasonality of bacterioplankton over the winter–spring transition. We investigated the changes in the free-living and attached bacterial community with ribosomal intergenic spacer analysis (RISA) and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes, followed by DNA sequence analysis. The linkage of abiotic and biotic environmental factors and community composition was analyzed by the multivariate ordination method of canonical correspondence analysis (CCA).

Materials and methods

Study site, sample collection, abiotic and biotic factors

Samples were collected twice weekly from a depth of 1 m from February to May 2004 at Helgoland Roads (54°11.3′N, 7°54.0′E), North Sea by the motor boat Aade. The sampling period covered the change of seasons from winter to spring, and included a phytoplankton bloom consisting mainly of *Phaeocystis* spp.

Water temperature was measured immediately after sampling. Determination of salinity was performed using an inductive salinometer (GDT Autosal8400B Salinometer, Guildline, Ontario, Canada) followed by conversion to a salinity value using UNESCO tables (Cox, 1966; Grasshoff *et al.*, 1999).

In order to monitor the concentration of nutrients, ammonium, nitrite, nitrate, silicate and phosphate were measured photometrically (Grasshoff & Johannsen, 1974; Grasshoff *et al.*, 1999).

The samples for the enumeration of phytoplankton cells were preserved with Lugols' solution before algal cell numbers were determined. Twenty-five-milliliter samples were counted using the Uthermöhl method and an inverted microscope (Wiltshire & Manly, 2004).

For the enumeration of bacteria, the samples were prefiltered through 10-μm gauze filters.

Direct counting was performed as described above, using the stain Acridine Orange (Gerdts *et al.*, 2004).

Sampling of biomass and extraction of nucleic acid

In order to collect the biomass of attached and free-living bacteria, 1 L of the seawater was filtered through 3-μm and 0.2-μm membrane filters (Millipore, Schwalbach, Germany) in succession. Filters were stored at −20 °C until DNA extraction.

DNA of attached and free-living bacteria was extracted from cut filters by a modified standard protocol of Anderson & McKay (1983), omitting the NaOH step. This method is comparable to the method of Somerville *et al.* (1989), who efficiently extracted DNA from the pelagic environment. Briefly, cell lysis was performed by adding lysozyme (1 mg mL^{-1}) and sodium dodecyl sulfate (1%). DNA extraction was carried out using phenol/chloroform/isoamyl alcohol (25:24:1). After precipitation of the DNA with isopropanol, all DNA extracts were eluted in sterile water and stored at −20 °C until further analyses.

Amplification of ribosomal intergenic spacer and RISA

For amplification of the intergenic spacers between the 16S and 23S subunits of ribosomal sequences, we used the primers S-D-Bact-1522-b-S-20 (5′-TGCGGCTGGATCC CCTCCTT-3′) and L-D-Bact-132-a-A-18 (5′-CCG GGT TTC CCC ATT CGG-3′) (Normand *et al.*, 1996; Ranjard *et al.*, 2000a, b). PCR reaction mixtures with a volume of 100 μL contained 10 μL of 10 × Taq buffer (Eppendorf, Hamburg, Germany), 20 μL of 5 × Master Enhancer (Eppendorf), 300 μM each dNTP (Perkin Elmer, Rodgau-Jügesheim, Germany), 0.5 μM each primer, 2 U of Taq DNA polymerase (Eppendorf), and 5 μL of DNA of 0.2 μm filters and 0.5 μL of 3-μm filters, respectively [Correction added after publication 8 January 2007: in the preceding sentence 0.5 μL of DNA of 0.2 μM filters and 0.5 μL of 3 μm filters was corrected to 5 μL of DNA of 0.2 μm filters and 0.5 μL of 3-μm filters]. The amplification protocol was as follows: a denaturing step at 95 °C for 3 min, 25 cycles at 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, and a final extension step of 72 °C for 5 min. PCR reactions were performed in an Eppendorf Mastercycler. Amplification of PCR products was confirmed by electrophoresis on a 1.4% (w/v) agarose gel. Fragments were resolved on 8% polyacrylamide gels (Qbiogene, Heidelberg, Germany) in 0.5 × (20 mM TrisHCL, 10 mM acetic acid, 0.5 mM EDTA) (TAE) buffer. Three lanes were used for 0.1 μg of a 100-bp ladder

(Invitrogen, Karlsruhe, Germany) in order to achieve comparability. Electrophoresis was performed at 20 °C and 50 V for 18 h, using a DCode system (BioRad, München, Germany). Gels were stained with SYBRGold as recommended by Molecular Probes (Invitrogen). Imaging was performed with the ChemiDoc XRS System (BioRad).

Amplification of 16S rRNA genes and DGGE

PCR amplification of 16S rRNA gene fragments was performed using the primers 341f with a 40-bp GC-rich sequence at the 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3′) and modified 907rm (5′-CCG TCA ATT CMT TTR AGT TT-3′) (Teske *et al.*, 1996). PCR reaction mixtures with a volume of 100 μL contained 10 μL of 10 × Taq buffer (Eppendorf), 20 μL of 5 × Master Enhancer (Eppendorf), 300 μM each dNTP (Perkin Elmer), 0.2 μM each primer, 2 U of Taq DNA polymerase (Eppendorf) and 5 μL of DNA of 0.2 μm filters and 0.5 μL of 3-μm filters, respectively [Correction added after publication 8 January 2007: in the preceding sentence 0.5 μL of DNA of 0.2 μM filters and 0.5 μL of 3 μm filters was corrected to 5 μL of DNA of 0.2 μm filters and 0.5 μL of 3-μm filters]. The 'touchdown' PCR started with a denaturing step at 94 °C for 5 min. Every cycle consisted of three steps, each for 1 min: 94 °C, annealing temperature, and 72 °C. The initial annealing temperature of 65 °C was decreased by 0.5 °C per cycle until a touchdown of 55 °C, at which temperature 12 additional cycles were carried out. Final primer extension was performed at 72 °C for 10 min, and this was followed by 22 cycles starting at 71 °C and decreasing by 1 °C per cycle. A slow decrease of the temperature leads to more accurate reannealing of homologous DNA strands and therefore prevents the formation of heteroduplexes. PCR reactions were performed in an Eppendorf Mastercycler. PCR products were inspected on 1.2% (w/v) agarose gels. These served for estimation of the amount of PCR product used for DGGE analyses, which were performed with a BioRad DCode system (see above). Fragments were resolved on 6% (w/v) polyacrylamide gels in 0.5% TAE buffer with denaturing gradients of 15–55% urea/formamide (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 60 °C and 150 V for 10 h (Sigler *et al.*, 2004). DGGE gels were stained with SYBRGold (see 'Amplification of ribosomal intergenic spacer and RISA'). Imaging was performed with a ChemiDoc XRS System (BioRad).

DNA sequencing

Prominent DGGE bands that connected or separated samples were excised, eluted (Sambrook *et al.*, 1989), repeatedly cleaned with additional DGGE gels, and reamplified using the primers 341f without GC-clamp and 907rm. DNA was purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Products were checked by electrophoresis in 1.2% (w/v) agarose gels. Sequencing was performed by Qiagen GmbH using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequencing primers were 907rm and 344f (5′-ACG GGA GGC AGC AG-3′). Nearest relatives were searched for using BLAST (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis

Sequence data were checked for the presence of PCR-amplified chimeric sequences with the check_chimera program (Cole *et al.*, 2003). The ARB software package (http://www.arb-home.de) was used for phylogenetic analysis (Ludwig *et al.*, 2004). After addition of sequences to the ARB 16S rRNA gene sequences database (released June 2002), alignment was carried out with the Fast Aligner integrated in the program and refined by comparison of closest relatives retrieved by BLAST. Sequences with more than 1300 nucleotides were used to calculate phylogenetic trees. The ARB 'parsimony interactive' tool was used to add partial sequences to respective trees. Phylogenetic relationships were deduced by the neighbor-joining method with the correction algorithm of Felsenstein (1993).

Nucleotide sequence accession numbers

The sequences obtained in this study are available from GenBank under accession numbers DQ289508–DQ289544.

Statistical analysis of RISA and DGGE profiles

We used two different fingerprinting methods to analyze the bacterial community during the sampling period. A general overview of bacterioplankton dynamics was achieved by RISA. Ordination techniques based on RISA fingerprints were performed to elucidate the factors affecting the whole bacterial community, whereas ordination techniques based on DGGE fingerprints were used to analyze the bacterial community at the phylotype level and the factors affecting specific bacterial phylotypes.

Analyses of RISA and DGGE fingerprints were carried out with the BIONUMERICS 4.0 software package (Applied Maths, BVBA, Belgium). Normalization of RISA gels was performed by BIONUMERICS software, using 100-bp ladders as references in every profile. Normalization of DGGE gels was performed using a specific sample including seven bands covering a broad area of positions as reference in addition to internal references in every profile. For sample comparison, band-matching analysis was performed. Bands were assigned to classes of common bands within all profiles. In the band-matching table based on DGGE fingerprints, sequenced DGGE bands were assigned to corresponding

band classes. We omitted DGGE fingerprints of attached bacteria from band-matching because of bias due to plastid rRNA gene (see Table 4). The procedure resulted in band-matching tables that included densitometric values of fingerprints for both community analyses (Muylaert *et al.*, 2002). These band-matching tables formed the basis for community ordination analysis.

To test whether weighted-averaging techniques or linear methods were appropriate, detrended correspondence analysis (DCA) was performed using CANOCO for Windows 4.53 (Biometris, the Netherlands). The longest gradients resulting from DCA were 2.694 for the analysis based on RISA profiles and 2.218 for the analysis based on DGGE profiles. Those values did not indicate a clear linear or unimodal relationship (Lepš & Šmilauer, 2003), so we performed redundancy analysis (RDA) as well as CCA to compare species–environment correlations.

The data were not transformed prior to RDA or CCA. Explanatory variables included temperature, salinity, concentrations of inorganic nutrients, namely ammonium, nitrate, nitrite, phosphate and silicate, as well as cell numbers of phytoplankton species. To differentiate the attached and the free-living community in the ordination analyses based on RISA profiles, a categorical variable was introduced (filter). This variable was set to 0 and 1 for the free-living and for the attached community respectively. Using this method, we were able to analyze the variation of the community also with respect to the influence of the fraction (Rooney-Varga *et al.*, 2005). Generally, RDA and CCA were performed as described by Lepš & Šmilauer (2003).

An automated forward selection was used to analyze intersample distances for both RISA and DGGE profiles. First, the variance inflation factor (VIF) of environmental variables was calculated. Variables displaying a value greater than 20 of this factor were excluded from RDA and CCA analyses, assuming collinearity of the respective variable with other variables included in the examined dataset.

The null hypothesis that species composition is independent of the measured variables was tested using constrained ordination with manual forward selection and a permutation test. The analysis was performed without transformation of data with focus scaling on intersample distances and manual selection of environmental variables, applying a partial Monte Carlo permutation test (499 permutations) including unrestricted permutation. The marginal effects of environmental variables were selected according to their significance level ($P < 0.05$) prior to permutation. The partial Monte Carlo permutation test provided the conditional effect of each variable. To estimate the influence of the measured variables on specific phylotypes, interspecies distances were calculated from the dataset derived from sequence data.

For all community ordination analyses, biplot scaling was used.

Results

Environmental parameters, and phytoplankton and bacterial cell counts

Temperature, salinity and the concentrations of phosphate, nitrate, nitrite, ammonium and silicate were determined as abiotic factors, whereas phytoplankton and bacterial cell counts were determined as biotic environmental parameters (Fig. 1). The water temperature increased constantly until the end of May, from 4.9 to 10.5 °C, despite slight variations. The values of salinity ranged from 29.33 to 33.51, and the values of the five major nutrients showed their lowest values in May (Fig. 1). Bacterial cell counts ranged from 3.69×10^6 to 0.84×10^6 cells mL^{-1}, with substantial variation during the sampling period. Generally, a decreasing trend could be observed from February to May (Fig. 1).

Phytoplankton counts revealed the appearance of six main diatom species, namely *Thalassionema nitzschioides* (Grunow) Grunow ex Hustedt, *Paralia sulcata* (Ehrenberg) Cleve, *Odontella aurita* (Lyngbye) C.A. Agardh, *Thalassiosira nordenskiöldii* Cleve, *Thalassiosira decipiens* (Grunow) Jørgensen, and *Guinardia delicatula* (Cleve) Hasle. Additionally, *Chattonella* spp., belonging to the family *Raphidophyceae*, *Phaeocystis* spp., belonging to the *Heterokontophyta*, and unclassified flagellates were counted (Fig. 1).

At the beginning of the sampling period, mainly *Thalassionema nitzschioides*, *Par. sulcata* and flagellates comprised the phytoplankton community. *Thalassionema nitzschioides* was observed at the beginning of March until the end of April, and *Par. sulcata* was observed until the end of March, whereas flagellates were apparent during the whole sampling period. *Odontella aurita* and *Thalassiosira nordenskiöldii* appeared in mid-April but disappeared at the end of that month. *Thalassiosira decipiens* could only be detected in mid-April for 2 weeks, whereas *G. delicatula* occurred first at the end of April, which was also true for *Chattonella* spp. *Guinardia delicatula* was observed until the end of the sampling period, whereas *Chattonella* spp. disappeared at the beginning of May. In addition, *Phaeocystis* spp. occurred in May and were observed until the end of the sampling period.

Community ordination analysis

Generally, we used two different fingerprinting methods to monitor changes in the bacterial community structure. RISA was performed to determine general differences between the attached and free-living bacterial communities during the sampling period (Fisher & Triplett, 1999; Ranjard *et al.*, 2000a, b, 2001), whereas DGGE of the free-living bacterial community was used to study the changes in the bacterial community based on specific phylotypes. As these fingerprinting methods have certain limitations, both were

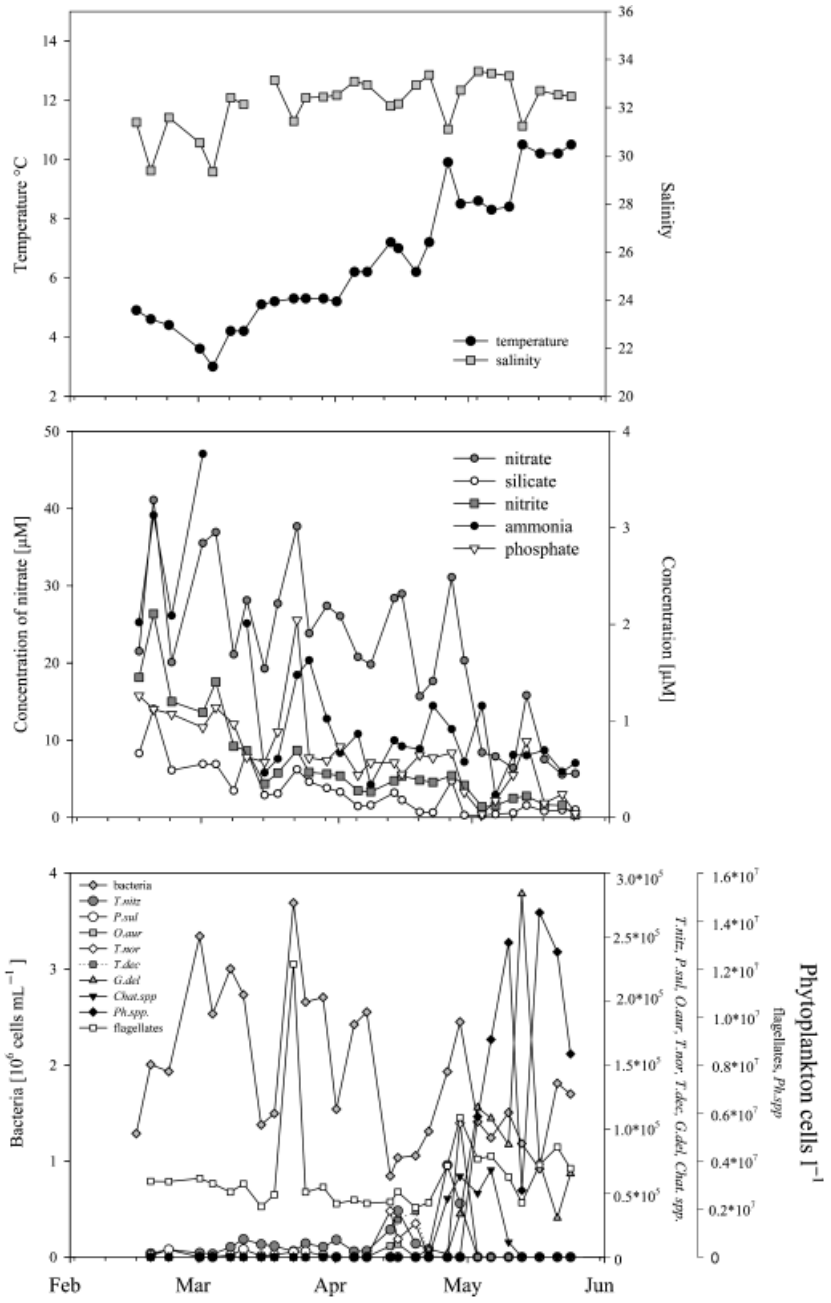

Fig. 1. Environmental parameters, phytoplankton and bacterial cell counts, including phytoplankton species. *T. nitz*, *Thalassionema nitzschioides*; *P. sul*, *Paralia sulcata*; *O. aur*, *Odontella aurita*; *T. nor*, *Thalassiosira nordenskiöldii*; *T. dec*, *Thalassiosira decipiens*; *G. del*, *Guinardia delicatula*; *Chat*. spp., *Chattonella* spp.; *Ph*. spp., *Phaeocystis* spp.

used to analyze the influence of environmental factors on the bacterioplankton.

Community ordination analysis based on RISA profiles

Generally, ordination analysis of the bacterial community was carried out using phytoplankton species cell counts, salinity, temperature, the nutrients ammonium, phosphate and silicate (Fig. 1), and the variable filter differentiating free-living and attached bacteria. Because of indistinct DCA results, both RDA and CCA were performed according to Lepš & Šmilauer (2003) to compare species–environment correlations. Lower values of species–environment correlations were obtained by RDA than by CCA (Table 1). Although the difference was not pronounced, it was assumed that unimodal methods would be more appropriate for analysis of the large dataset. Generally, nonlinear models are required for analysis of ecological data collected over a large range of habitat variation (Jongman *et al*., 1987). Hence, we decided that ordination techniques based on weighted averaging would be more suitable, assuming a

Table 1. Eigenvalues and variance decomposition for CCA and RDA

	Axes	Eigenvalues	Species–environment correlations	Cumulative percentage variance of species data	Cumulative percentage variance of species–environment correlation
Community analysis, CCA					
RISA intersample distances	Axis 1	0.424	0.944	20.3	44.5
	Axis 2	0.186	0.908	29.2	64.1
	Axis 3	0.107	0.791	34.3	75.4
	Axis 4	0.063	0.596	37.3	82.0
DGGE intersample and interspecies distances	Axis 1	0.420	0.981	47.0	58.2
	Axis 2	0.127	0.911	61.2	75.9
	Axis 3	0.057	0.860	67.6	83.9
	Axis 4	0.032	0.760	71.2	88.3
Community analysis, RDA					
RISA intersample distances	Axis 1	0.291	0.918	29.1	54.9
	Axis 2	0.099	0.871	39.0	73.6
	Axis 3	0.049	0.754	43.9	82.8
	Axis 4	0.024	0.707	46.3	87.2
DGGE intersample and interspecies distances	Axis 1	0.483	0.968	48.3	62.1
	Axis 2	0.103	0.829	58.6	75.4
	Axis 3	0.079	0.802	66.5	85.5
	Axis 4	0.042	0.848	70.7	90.9

CCA, canonical correspondence analysis; RDA, redundancy analysis; RISA, ribosomal intergenic spacer analysis; DGGE, denaturing gradient gel electrophoresis.

unimodal response of species to the environment, to elucidate the influence of the measured variables on the variation of the bacterial community.

The constrained ordination revealed high values of the VIF (> 20) for the variables nitrate and nitrite, which indicated collinearity with other variables. These factors were excluded from the final CCA.

The eigenvalues of the ordination analyses are presented in Table 1. The sum of all unconstrained eigenvalues indicated an overall variance in the dataset of 2.090. Total variation that could be explained by environmental variation accounted for 0.951, as indicated by the sum of all canonical eigenvalues. Concerning the variance of species data, the first axis explained 20.3% of the total variation, the first and the second axes explained 29.2%, and all four axes explained 37.3% (Table 1). Species–environment correlations were high, especially for axes 1 and 2 (0.944 and 0.908), indicating a relationship between species and environmental variables.

Biplot scaling of CCA is shown in Fig. 2. Canonical axes 1 and 2 are shown in Fig. 2a, demonstrating a strong influence of the nominal variable filter, which is equivalent to the respective fraction of the bacterial community, indicating a distinct differentiation of free-living and attached bacteria. This is supported by a high eigenvalue of canonical axis 1. Axis 1 represented a strong gradient caused by the nominal variable filter, indicated by the intraset correlation coefficient of 0.6052, which is also applicable for axis 2, which displayed an intraset correlation coefficient of – 0.6477 (Table 2). The influence of this variable was determined to be significant by a permutation test (Table 3). It became apparent that the variable filter had the highest value of lambda A of the conditional effects (Table 3), representing the highest additional variance explained by this variable at the time it was included in the permutation test (ter Braak & Šmilauer, 2002).

The influence of the factors *G. delicatula*, temperature, *Phaeocystis* spp., *Chattonella* spp., flagellates and salinity on the bacterial communities deriving from May samples is also shown (Fig. 2a). The variables *Phaeocystis* spp., *G. delicatula* and temperature contributed particularly to the gradient, as indicated by the intraset correlation coefficients (Table 2). Significance was retrieved for the factors *Phaeocystis* spp. and *G. delicatula* but not for the factor temperature, applying the 5% significance level ($P < 0.08$). The variable *Chattonella* spp. displayed minor influences on the gradient, as indicated by the intraset correlation coefficients shown in Table 2. The permutation test also showed no significance of this variable at the 5% level ($P < 0.08$).

However, temperature and the phytoplankton *Chattonella* spp. contributed to the environmental variables explaining variation, as shown by biplot scaling of canonical axes 2 and 3 (Fig. 2b), indicated by the lengths of the respective arrows.

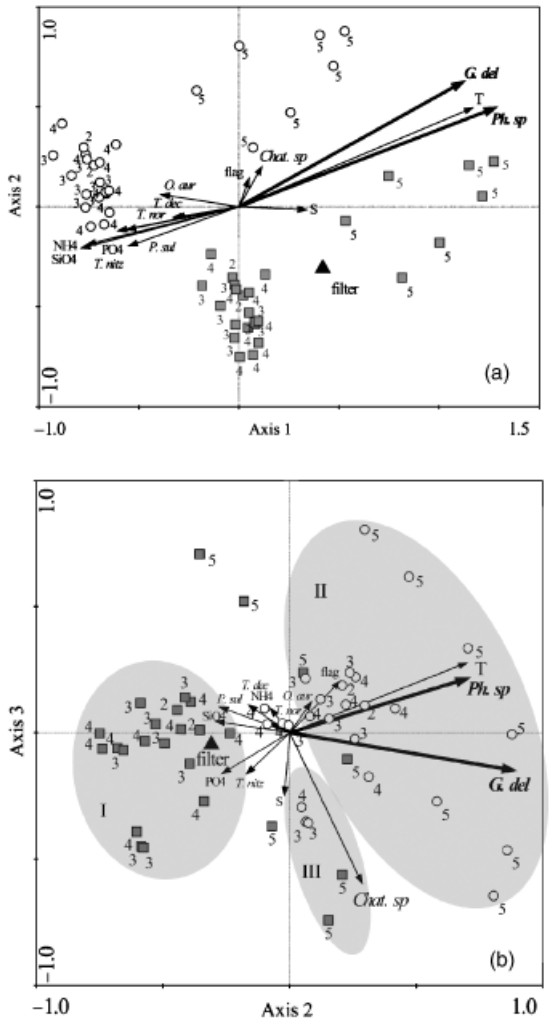

Fig. 2. CCA of RISA profiles showing attached and free-living bacterial communities, obtained using phytoplankton species cell counts of flagellates (flag, *Thalassionema nitzschioides* (*T. nitz*), *Paralia sulcata* (*P. sul*), *Odontella aurita* (*O. aur*), *Thalassiosira nordenskiöldii* (*T. nor*), *Thalassiosira decipiens* (*T. dec*), *Guinardia delicatula* (*G. del*), *Chattonella* spp. (*Chat*. sp.) and *Phaeocystis* spp. (*Ph*. sp.), salinity (S), temperature (T), the nutrients ammonium (NH_4), phosphate (PO_4) and silicate (SiO_4), as well as the variable 'filter' differentiating free-living and attached bacteria. Circles indicate free-living communities, and filled squares indicate attached communities. Numbers near the symbols indicate the month of sampling (2, February; 3, March; 4, April; 5, May). Arrows indicate the direction of increasing values of the respective variable, the length of arrows indicates the degree of correlation of the variable with community data, significant variables are indicated by bold arrows, and groups I, II and III of communities are indicated by a gray background. (a) Axes 1 and 2 of CCA biplot. (b) Axes 2 and 3 of CCA biplot.

Biplot scaling of canonical axes 2 and 3 (Fig. 2b) showed that the attached bacterial communities deriving from samples of February, March and April are grouped together (group I, Fig. 2b). This group showed little influence of nutrients and phytoplankton species. Furthermore, some free-living and attached communities were combined (group II, Fig. 2b). They were influenced by temperature, *Phaeocystis* spp., *G. delicatula* and *Chattonella* spp. In this group, some free-living communities from the May, April, March and February samples were mainly influenced by temperature, *Phaeocystis* spp. and *G. delicatula*, whereas *Chattonella* spp. influenced some free-living communities from the April and March samples and attached communities from the May samples (group III, Fig. 2b). It is obvious that communities from the May samples were particularly influenced by several factors. Summarizing the effects of environmental variables, it became apparent that the nominal variable filter had the strongest conditional effect, followed by *Phaeocystis* spp. and *G. delicatula*. Note that temperature had a strong marginal influence but a minor conditional influence compared with the variables filter, *Phaeocystis* spp., *G. delicatula* and *Chattonella* spp.

Generally, the first two axes together explained 64% of the variation that could be explained by the variables, whereas all four axes explained 82% of the variation (Table 1).

Phylogenetic analysis

The most prominent DGGE bands that separated or connected the bacterial community were sequenced from excised bands. Sequence data of 45 excised bands could be retrieved, representing 36 different phylotypes (Table 4). Sequence data revealed the presence of three phyla of the *Bacteria* (Fig. 3). Most sequences were related to members of the *Proteobacteria* and members of the *Bacteroidetes* phylum. Within the *Proteobacteria*, the *Alphaproteobacteria* and *Gammaproteobacteria* were most abundant. Additionally, we found one member of the *Betaproteobacteria* and members of the *Actinobacteria*. Furthermore, several chloroplast sequences were detected. The closest relatives of the sequenced bands derived from BLAST analyses are listed in Table 4. The results revealed many close matches with 98–100% similarity to bacterial 16S rRNA gene sequences in GenBank.

Comparison of sequence data of excised bands appearing at the same position in DGGE gels revealed identical closest relatives in most cases (e.g. bands F111 and F122, F120 and F200, and F196 and F296). Moreover, bands F067 and F132, as well as F074 and F306, did not result in the same sequence, although the respective bands were retrieved from the same positions in DGGE gels (Table 4).

Proteobacteria

A neighbor-joining tree of the *Alphaproteobacteria* revealed that the majority of sequences belonged to the *Roseobacter* clade (65%). Thirty-five percent belonged to clusters of the SAR116 and SAR1 phylotypes. Gene sequences of the *Gammaproteobacteria* were all assigned to the *Oceanospirillales*, whereas the phylotype classified as a member of the

Table 2. Intraset correlation coefficients of forwardly selected environmental variables with the four significant axes produced by CCA of RISA and DGGE fingerprints of the bacterial community

Community analysis, CCA	Environmental factors	Axis 1	Axis 2	Axis 3	Axis 4
RISA intersample distances	Filter	0.6052	−0.6477	−0.1121	0.1065
	Phaeocystis spp.	0.6717	0.3763	0.1322	0.0380
	Guinardia delicatula	0.5869	0.4754	−0.0911	−0.1107
	Chattonella spp.	0.0607	0.1546	−0.3704	0.2612
	Temperature	0.6164	0.3790	0.1727	−0.0561
DGGE intersample and interspecies distances	Temperature	0.9027	0.0169	0.0679	−0.0456
	Phaeocystis spp.	0.6730	−0.5602	−0.0414	0.0229
	Nitrite	−0.7178	−0.1004	−0.4813	0.0075

CCA, canonical correspondence analysis; RISA, ribosomal intergenic spacer analysis; DGGE, denaturing gradient gel electrophoresis.

Table 3. Marginal and conditional effects of forwardly selected environmental variables produced by CCA

		Marginal effects	Conditional effects		
Community analysis	Environmental variable	Lambda 1	Lambda A	*P*-value	*F*-factor
RISA intersample distances	Filter	0.27	0.27	0.002	7.53
	Phaeocystis spp.	0.26	0.24	0.002	7.31
	Guinardia delicatula	0.23	0.12	0.002	3.87
	Chattonella spp.	0.05	0.05	0.066	1.73
	Temperature	0.23	0.05	0.08	1.6
DGGE intersample distances and interspecies distances	Temperature	0.36	0.36	0.002	16.67
	Phaeocystis spp.	0.25	0.09	0.002	5.18
	Nitrite	0.25	0.05	0.020	2.43

CCA, canonical correspondence analysis; RISA, ribosomal intergenic spacer analysis; DGGE, denaturing gradient gel electrophoresis.

Betaproteobacteria belonged to the *Burkholderiales* (Fig. 3). Members of the *Alphaproteobacteria* contributed to both the attached and free-living bacterial fractions, whereas the members of the *Betaproteobacteria* and of the *Gammaproteobacteria* detected in this study belonged solely to the free-living bacteria (Table 4).

Bacteroidetes and *Actinobacteria*

Within the *Bacteroidetes* phylum, sequenced phylotypes clustered with the *Flavobacteria* (Fig. 3). These phylotypes formed part of both the attached and free-living bacterial communities.

Two phylotypes of the free-living fraction were classified as *Actinobacteria*, with substantial similarities to already described uncultured bacteria, namely 'uncultured bacterium ARKIA-43' and 'uncultured actinobacterium PI_RT340' (Fig. 3).

Succession of free-living bacterial species and community ordination analysis based on 16S rRNA gene sequence data

Generally, 28 phylotypes were identified at different positions on the DGGE gels of the free-living bacterial community. The succession of free-living bacterial phylotypes is shown in Table 5. At the beginning of the sampling period, the community was mainly composed of members of the *Alphaproteobacteria*. Additionally, members of the *Flavobacteria*, *Actinobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, as well as unknown phylotypes, were detected.

Some phylotypes were predominant during the whole sampling period, such as an alphaproteobacterium (F111), whereas some phylotypes disappeared in March or April. In April, additional members of the *Alphaproteobacteria* and *Flavobacteria* were detected, whereas a member of the *Gammaproteobacteria* (F069) and two unknown phylotypes [F070, F01ne (not excised)] were first detected in May samples. During this period, *Actinobacterium* F196, a specific *Flavobacterium* (F197) and *Betaproteobacterium* F200 were no longer found.

In order to achieve a detailed analysis of the free-living bacterial community and the factors influencing distinct phylotypes, RDA and CCA of bacterial phylotypes and environmental factors were performed. Bands F070, F01ne, F124 and F291 were omitted from ordination, as they appeared only once or twice in the dataset. Because of lower values of species–environment correlations in RDA (Table 1), we used weighted averaging to analyze the influence of environmental factors on the bacterial community and phylotypes.

The ordination analysis of the free-living bacterial community was performed using phytoplankton species cell

Table 4. Relatedness of bacteria to known organisms

DGGE band	Fraction	Phylogenetic group	Closest relative	Similarity (%)	Base positions compared	Accession number of closest relative
F067*	Free	*Alphaproteobacteria*	Uncultured marine bacterium, D015	98	530	AF177555
F068	Free	*Alphaproteobacteria*	*Sulfitobacter* sp., KMM 6006	81	518	AY682196
F105	Free	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, PI_RT284	100	530	AY580529
F111	Free	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, DC11-80-2	100	532	AY145625
F122	Free	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, DC11-80-2	100	531	AY145625
F125	Free	*Alphaproteobacteria*	Uncultured SAR116 *Alphaproteobacterium*, EF100-93A06	89	535	AY627368
F130	Free	*Alphaproteobacteria*	Uncultured *Rhodobacteraceae* bacterium, F4C74	82	517	AY697922
F132*	Free	*Alphaproteobacteria*	*Rhodobacteraceae* bacterium, AP-27	99	521	AY145564
F182	Free	*Alphaproteobacteria*	Uncultured SAR116 *Alphaproteobacterium*, EF100-93A06	98	528	AY627368
F198	Free	*Alphaproteobacteria*	*Rhodobacteraceae* bacterium, AP-27	100	530	AY145564
F202	Free	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, PI_4k2g	97	551	AY580512
F203	Free	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, PI_RT284	91	537	AY580529
F291	Free	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, PI_4z10f	100	534	AY580535
F077	Att	*Alphaproteobacteria*	*Rhodobacteraceae* bacterium, AP-27	99	536	AY145564
F089	Att	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, DC11-80-2	90	493	AY145625
F098	Att	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, DC11-80-2	99	562	AY145625
F160	Att	*Alphaproteobacteria*	Uncultured *Alphaproteobacterium*, DC11-80-2	99	551	AY145625
F310	Att	*Alphaproteobacteria*	*Rhodobacteraceae* bacterium, AP-27	100	563	AY145564
F120	Free	*Betaproteobacteria*	Uncultured bacterium, BN_32	98	528	AY550846
F200	Free	*Betaproteobacteria*	Uncultured bacterium, BN_32	95	534	AY550846
F069	Free	*Gammaproteobacteria*	Marine *Gammaproteobacterium*, HTCC2121	100	561	AY386341
F070	Free	*Gammaproteobacteria*	Marine *Gammaproteobacterium*, HTCC2121	81	541	AY386341
F141	Free	*Gammaproteobacteria*	Uncultured *Gammaproteobacterium*, PI_4r8d	100	560	AY580742
F309	Free	*Gammaproteobacteria*	Marine *Gammaproteobacterium*, HTCC2121	98	576	AY386341
F074†	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, PI_4j12f	99	550	AY580583
F114	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, PI_RT302	99	544	AY580649
F128	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, AB-4	88	551	AY353556
F137	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, PI_4d7b	94	569	AY580580
F188	Free	*Flavobacteria*	Uncultured bacterium, BN_34	94	517	AY550843
F197	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, GWS-c7-FL	89	525	DQ080954
F306†	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, B12	94	529	AF466917
F086	Att	*Flavobacteria*	Marine psychrophile *Bacteroidetes* bacterium, SW17	97	554	AF001368
F162	Att	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, AB-4	95	555	AY353556
F172	Att	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, PI_4d7b	99	546	AY580580
F270	Att	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, PI_RT302	99	530	AY580649
F289	Att	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, PI_4e10g	98	575	AY580688
F184	Free	*Actinobacteria*	Uncultured bacterium, ARKIA-43	99	538	AF468297
F196	Free	*Actinobacteria*	Uncultured *Actinobacterium*, PI_RT340	100	539	AY580357
F296	Free	*Actinobacteria*	Uncultured *Actinobacterium*, PI_RT340	98	474	AY580357
F075	Free		Unknown			
F124	Free		Unknown			
F187	Free		Unknown			
F192	Free		Unknown			
F01ne‡	Free		Not excised			
F02ne‡	Free		Not excised			
F097	Att	Plastid	*Teleaulax amphioxeia*	100	567	AY453067
F127	Free	Plastid	Uncultured *Prasinophyte* LUR7	98	526	AY960282
F146	Att	Plastid	Environmental clone OCS54	98	543	AF001657
F151a	Att	Plastid	*Teleaulax amphioxeia*	99	534	AY453067
F167	Att	Plastid	Uncultured phototrophic eukaryote, ANT18/2_25	99	532	AY135677
F169	Att	Plastid	Uncultured phototrophic eukaryote, JL-WNPG-T36	99	532	AY664132
F278	Att	Plastid	*Thalassiosira eccentrica*	98	542	AJ536458
F279	Att	Plastid	*Rhizosolenia setigera*, p112	98	555	AJ536461
F326	Att	Plastid	*Teleaulax amphioxeia*	100	566	AY453067

*Phylotypes with same position in DGGE profile but different sequence data within the *Alphaproteobacteria*.

†Phylotypes with same position in DGGE profile but different sequence data within the *Bacteroidetes*.

‡ne, nonexcised bands.

DGGE, denaturing gradient gel electrophoresis.

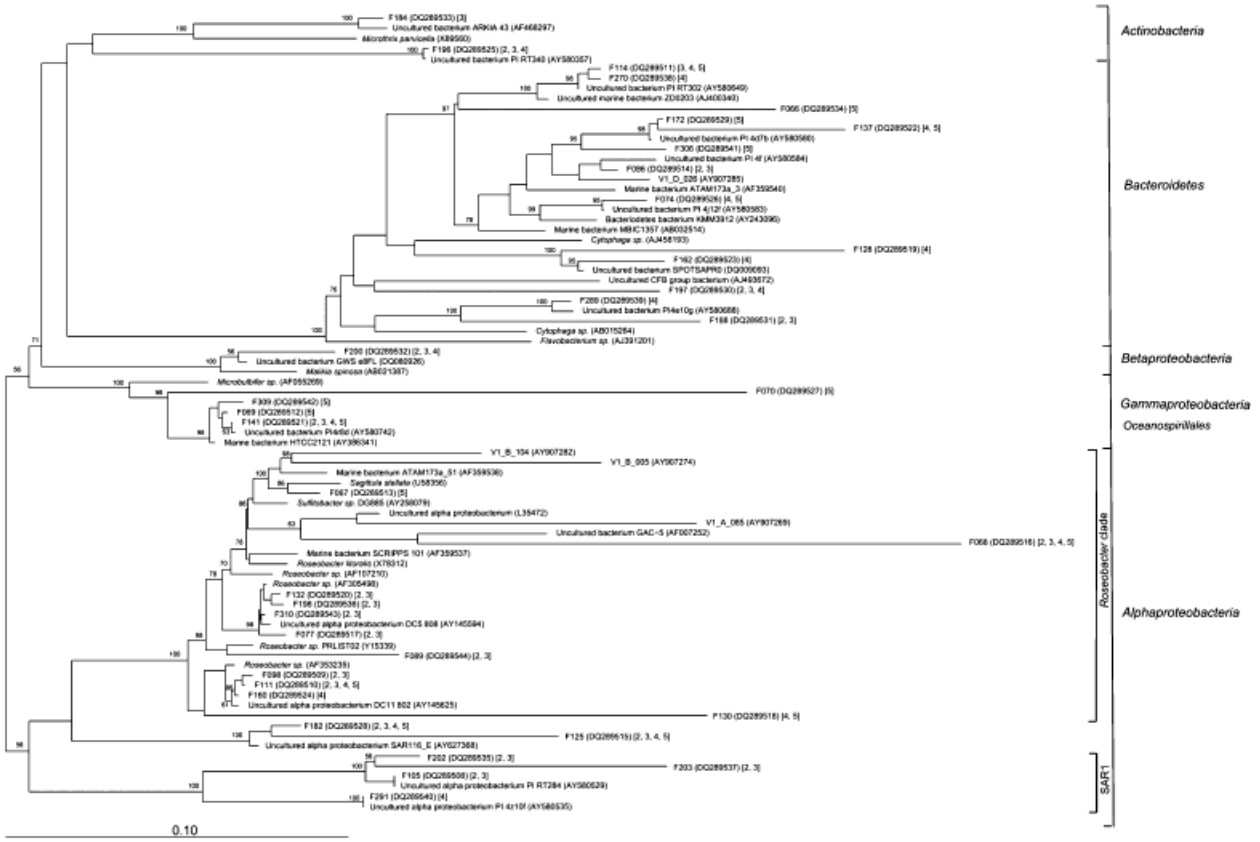

Fig. 3. Phylogenetic tree of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* and members of the *Bacteroidetes*. GenBank accession numbers are given in parentheses. Bootstrap values above 50% are displayed. Numbers in square brackets indicate the main appearance of the phylotype (2, February; 3, March; 4, April; 5, May).

counts, salinity, temperature and the nutrients ammonium, nitrate, nitrite and phosphate as explanatory variables (Fig. 1). CCA revealed high values of the variance inflation factor (> 20) for the variable silicate, indicating collinearity with other variables. Therefore, it was excluded from CCA.

The eigenvalue of axis 1 of CCA (0.420, Table 1) represents a gradient due to the environmental factors temperature, nitrite and *Phaeocystis* spp., as indicated by their intraset correlation coefficients (Table 2). The overall variance in the dataset accounted for 0.894, whereas the total variation explained by environmental variation accounted for 0.721. Additionally, the first axis explained 47.0% of the total variation, the first and second axes together explained 61.2%, and all four axes explained 71.2% (Table 1).

Phylotype–environment correlations were especially high for axes 1 and 2 (0.981 and 0.911), indicating a relationship between phylotype and environmental variables. The first two axes together explained 75.9% of the variation, whereas all four axes explained 88.3% of the variation (Table 1). Biplot scaling of CCA of DGGE fingerprints is shown in Fig. 4. The analysis based on intersample distances revealed a group of free-living communities in February and March that were mainly influenced by nutritional factors and the phytoplankton species *Par. sulcata* (group I, Fig. 4). The free-living communities of April and May appeared in separate groups. Salinity and *Chattonella* spp. displayed a minor correlation with the communities retrieved from April samples, whereas the phytoplankton species *O. aurita*, *Thalassiosira decipiens*, *Thalassiosira nordenskiöldii* and *Thalassionema nitzschioides* were correlated with this group to a greater extent (group II, Fig. 4). The communities retrieved from May samples were also grouped together and were generally related to *Phaeocystis* spp. and *G. delicatula* (group III, Fig. 4). A correlation with temperature was also observed.

Significant correlation with the sample variation was retrieved by three factors, namely temperature, nitrite and *Phaeocystis* spp., based on the 5% level in a partial Monte Carlo permutation test (Table 3). Both marginal and conditional effects are shown in Table 3. It is apparent that the factor temperature had the strongest influence, followed by *Phaeocystis* spp. and nitrite. CCA with interspecies distances was performed to calculate the influence of the environmental variables on specific phylotypes. Biplot scaling

Table 5. Succession of free-living bacterial species during the sampling period; filled boxes indicate appearance, and unfilled boxes indicate absence, of respective phylotype. The order is based on appearance in DGGE gels due to succession

Band	Phylogenetic group	16.02.	19.02.	23.02.	01.03.	04.03.	08.03.	11.03.	15.03.	18.03.	22.03.	25.03.	29.03.	01.04.	05.04.	08.04.	13.04.	15.04.	19.04.	22.04.	26.04.	29.04.	03.05.	06.05.	10.05.	13.05.	17.05.	21.05.	24.05.
F202	*Alphaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■															
F203	*Alphaproteobacteria*	■	■	■	■	■	■	■	■			■		■															
F105	*Alphaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■															
F187	unknown	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■
F196	*Actinobacteria*	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■							
F197	*Flavobacteria*	■	■	■	■	■	■	■	■			■		■	■	■	■	■	■	■	■								
F188	*Flavobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■															
F111	*Alphaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
F132	*Alphaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■															
F068	*Alphaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■
F200	*Betaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■						
F141	*Gammaproteobacteria*	■	■	■	■	■	■	■	■			■					■	■	■	■	■	■	■	■	■	■	■	■	■
F192	unknown	■	■	■	■	■	■										■	■	■	■	■	■	■	■	■	■	■	■	■
F125	*Alphaproteobacteria*	■	■	■	■	■	■	■	■						■		■	■	■	■	■	■	■	■	■	■	■	■	■
F02ne*	not excised	■	■	■	■	■	■	■	■			■					■	■	■	■	■	■	■						
F182	*Alphaproteobacteria*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
F114	*Flavobacteria*			■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
F184	*Actinobacteria*			■			■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
F127	plastid							■	■	■								■	■	■	■	■							
F291	*Alphaproteobacteria*														■														
F074	*Flavobacteria*														■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
F130	*Alphaproteobacteria*														■	■	■	■	■	■	■	■	■	■	■	■	■		
F137	*Flavobacteria*																		■	■		■	■	■	■		■		
F124	unknown																	■	■	■									
F128	*Flavobacteria*																	■	■	■									
F01ne*	not excised																						■						
F069	*Gammaproteobacteria*																						■	■	■	■	■	■	■
F070	*Gammaproteobacteria*																						■	■	■	■	■	■	■

*ne, nonexcised bands.

revealed four groups of phylotypes and two bands that could not be grouped with the other bands (Fig. 5). Group I was mainly influenced by nutrients (nitrite) and *Par. sulcata*. In this dataset, the influence of nutrients could be separated from the effect of temperature, as the analysis of the VIF revealed no collinearity for both factors. Group II did not show any correlation with a variable included in our dataset. Little correlation was displayed by salinity and the phytoplankton species *O. aurita* and *Chattonella* spp. in group III. Phylotypes positively correlating with the phytoplankton species *Phaeocystis* spp. and *G. delicatula* as well as temperature were grouped within group IV. The factors affecting bacterial species were those retrieved in CCA based on DGGE fingerprints of the free-living community (Fig. 4). However, the phylotype F069 (*Gammaproteobacteria*) appearing at the beginning of May (Table 5) was strongly correlated with the occurrence of *Phaeocystis* spp., as indicated by CCA (Fig. 5).

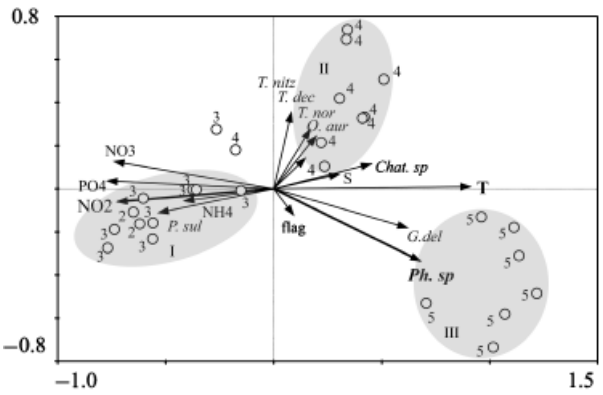

Fig. 4. CCA biplot of intersample distances of DGGE fingerprints of the free-living bacterial community using phytoplankton species cell counts of flagellates (flag, *Thalassionema nitzschioides* (*T. nitz*), *Paralia sulcata* (*P. sul*), *Odontella aurita* (*O. aur*), *Thalassiosira nordenskiöldii* (*T. nor*), *Thalassiosira decipiens* (*T. dec*), *Guinardia delicatula* (*G. del*), *Chattonella* spp. (*Chat*. sp.) and *Phaeocystis* spp. (*Ph*. sp.), salinity (S), temperature (T), and the nutrients nitrate (NO_3), nitrite (NO_2), ammonium (NH_4) and phosphate (PO_4). Circles indicate free-living communities, and numbers near the symbols indicate the month of sampling (2, February; 3, March; 4, April; 5, May). Arrows indicate the direction of increasing values of the respective variable, and the length of arrows indicates the degree of correlation of the variable with community data. Significant variables are indicated by bold arrows, and groups I, II and III of communities are indicated by a gray background.

Discussion

The linkage of phytoplankton and bacterioplankton dynamics demonstrated by several investigations (Brussaard *et al*., 2005; Rooney-Varga *et al*., 2005) is still not well understood. Until now, it has been assumed that there are specific factors and effects controlling specific bacterial populations. The findings of Shiah & Ducklow (1994), Pinhassi & Hagström (2000), Gerdts *et al*. (2004) and Kent *et al*. (2004) were supported by our study, as we also observed seasonal succession of bacterioplankton in the winter–spring transition. Like other investigators, we found temperature to be the most important factor influencing the bacterioplankton composition of Helgoland Roads over the winter–spring transition in 2004–2005. Additionally, the phytoplankton species *Phaeocystis* spp. and *G. delicatula* displayed strong effects on the bacterial community during the winter–spring transition, leading to a decrease in the influence of abiotic factors and an increase in the influence of biotic factors, as indicated by CCA (Fig. 2b).

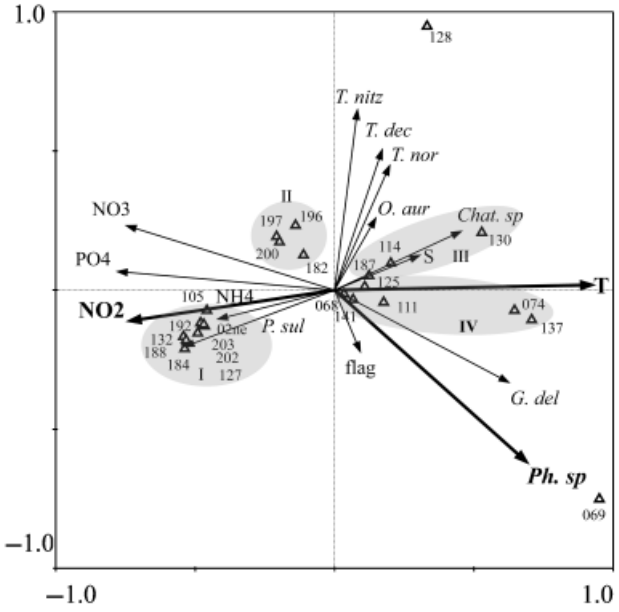

Fig. 5. Biplot of interspecies distances; CCA of DGGE fingerprints of the free-living bacterial community using phytoplankton species cell counts of flagellates (flag, *Thalassionema nitzschioides* (*T. nitz*), *Paralia sulcata* (*P. sul*), *Odontella aurita* (*O. aur*), *Thalassiosira nordenskiöldii* (*T. nor*), *Thalassiosira decipiens* (*T. dec*), *Guinardia delicatula* (*G. del*), *Chattonella* spp. (*Chat*. sp.) and *Phaeocystis* spp. (*Ph*. sp.), salinity (S), temperature (T), and the nutrients nitrate (NO_3), nitrite (NO_2), ammonium (NH_4) and phosphate (PO_4). Triangles with numbers indicate sequenced bands, and the suffix 'ne' indicates nonsequenced bands. Arrows indicate the direction of increasing values of the respective variable, and the length of arrows indicates the degree of correlation of the variable with community data. Significant variables are indicated by bold arrows, and groups I, II, III and IV of phylotypes are indicated by a gray background.

Community ordination analysis

Although the DCA results showed a linear or unimodal relationship, the results of the analysis are not clear. CCA was performed to analyze species–environment correlations, as the results of RDA showed lower correlation values. In general, the separation of the attached and free-living communities became evident as a strong effect on the analysis of RISA profiles. Distinct differences between attached and free-living bacteria have already been observed by DeLong *et al.* (1993) and Fandino *et al.* (2001), and in a multivariate analysis by Rooney-Varga *et al.* (2005).

Generally, the correlation of the measured environmental variables with the free-living fraction of the bacterial community appeared to be stronger than with the attached community, except for some attached communities in May that were strongly correlated with *G. delicatula* or *Chattonella* spp. (Fig. 2b). The correlation of *Phaeocystis* spp. with the bacterial community was determined to be significant for CCA of RISA and DGGE profiles (Table 3), indicating a structuring role for this phytoplankton species. This has already been described for culture experiments (Janse *et al.*, 2000). The authors stated that bacteria with specific enzyme activities might be favored by a bloom of this Heterokontophyte. Furthermore, Brussaard *et al.* (2005) observed changes in the bacterial community during the breakdown of a *Phaeocystis* bloom in a mesocosm study that might support the finding that *Phaeocystis* spp. has a structuring role. It has to be considered that probably not only the presence of *Phaeocystis* spp. but also the increase of algal-derived organic matter due to the high total abundance of phytoplankton in May had a strong effect on the bacterioplankton community.

Besides the occurrence of *Phaeocystis* spp., it can also be assumed that temperature contributed to the shifts in the bacterial community, although the significance test did not reach the 5% level ($P = 0.08$) in CCA of RISA profiles. It was correlated mainly with free-living bacterial communities in April and May, as determined by CCA of DGGE fingerprints. This aspect has already been described by Pinhassi & Hagström (2000), who observed a relationship between temperature and bacterial production. Furthermore, an influence of temperature on bacteria has been described by Shiah & Ducklow (1994) and Šestanović *et al.* (2004).

However, we could not ascribe a significant factor that shaped the attached communities in samples obtained in February to April.

Generally, a single band in a RISA profile might contain several species, and a species could result in several bands (Ranjard *et al.*, 2001), so we focused on differences between

the bacterial communities determined by RISA. In addition, the primer set used in this study might have resulted in amplification of plastid DNA. Nevertheless, it is concluded from our results that this bias did not harm our analyses, as the biotic factors did not correlate excessively with the attached bacterial communities (Fig. 2a and b).

In contrast to CCA of RISA profiles, CCA of DGGE fingerprints showed a significant conditional effect of nutrients (nitrite), indicating an important effect on the free-living community. This finding is supported by recent studies showing that limitation of the bacterial community due to inorganic nutrients occurs in periods when temperature is not limiting (Rivkin & Anderson, 1997; Øvreås *et al*., 2003).

Generally, succession of communities was obvious from both fingerprinting methods, RISA and DGGE.

In addition, the seasonal dynamics of phytoplankton are reflected in bacterioplankton dynamics based on the free-living bacterial community in general. In the first part of the sampling period, the phytoplankton community was stable, as was the bacterioplankton community, despite slight variations. In March, flagellate abundance increased, accompanied by changes in the bacterial community composition. In April, further changes were not directly linked with bacterioplankton dynamics. After this, a strong correlation of the bacterioplankton community with the appearance of *Phaeocystis* spp. was seen. This finding supports the hypothesis that, among other factors, phytoplankton accounts for seasonality of bacterioplankton dynamics.

The analysis based on interspecies distances revealed the influence of different factors on specific phylotypes. An influence of nutrients was observed for phylotypes detected in the first part of the sampling period. It is suggested that they contributed to a specific 'winter' community. Group II was not influenced by any of the variables. It can be assumed that other factors influencing this group were not included in our dataset. Additionally, it should be considered that the *Betaproteobacteria* (F200) are rare in marine pelagic environments and are found predominantly in freshwater and coastal areas (Rappé *et al*., 1997; Fuhrmann & Ouverney, 1998; Giovannoni & Stingl, 2005). It cannot be excluded that this phylotype was detected due to water mass displacement at the sampling station, as the Elbe river has a strong impact when there are eastern winds. Further studies are needed to determine whether such impacts can be correlated with detection of specific bacterial species.

The further development of the community resulted in a group separated from the earlier community, indicating a strong shift caused by the appearance of *Phaeocystis* spp. and the increase in temperature. In particular, a member of the *Gammaproteobacteria* belonging to the *Oceanospirillales* (F069) occurred at the beginning of May. Its appearance is directly correlated with the bloom of *Phaeocystis* spp., indicating a strong effect of this alga on this phylotype. Additionally, it became apparent that two members of the *Flavobacteria* (group IV, Fig. 5) were strongly correlated with temperature and the alga *Phaeocystis* spp.

It has to be taken into account that the fingerprinting method (DGGE) based on PCR amplification has potential biases, which have been discussed elsewhere (Suzuki & Giovannoni, 1996; von Wintzingerode *et al*., 1997; Bidle & Azam, 2001). The primer set used in this study resulted in amplification of plastid DNA, especially in the fraction $> 3\,\mu m$ (Table 4). These profiles of attached bacterial communities were not analyzed by ordination. Therefore, we could not consider the factors influencing those bacteria that appeared to be attached to the phytoplankton at the level of phylotypes. Factors influencing those bacteria, especially concerning their appearance as attached or free-living bacteria, remain unclear. Furthermore, one plastid phylotype was observed in the fraction of the free-living community ($< 3\,\mu m$ and $> 0.2\,\mu m$). Its influence on the ordination was estimated to be low (Fig. 5). The sequence was similar to that of the 16S rRNA gene of a plastid of the *Prasinophyceae* (similarity of 98%) and was grouped with those phylotypes that were correlated with nutrients (nitrite) and *Par. sulcata*. As already shown in other studies (Bonin *et al*., 2002) nearly all excised DGGE bands were sequenced without interference, suggesting that each band represented one unique 16S rRNA gene sequence. Furthermore, sequencing of the majority of the DGGE bands at the same position in the gels revealed identical sequences, except for bands F067/F132 and F074/F306. Accordingly, we presume that each band corresponded to one 'phylotype'.

The phylotypes retrieved in this study belonged mainly to the *Alphaproteobacteria*. Within this group, phylotype F068 clustered with sequence ATAM 173a_51, associated with the toxic dinoflagellate *Alexandrium* spp. (Hold *et al*., 2001), whereas bands F111 and F130 clustered with the sequence DC11-80-2 retrieved from the Weser estuary (Selje *et al*., 2004) and *Roseobacter* sp. (AF353235) retrieved from the Arctic Ocean (Bano & Hollibaugh, 2002). Members of the *Roseobacter* clade are thought to play an important role in the degradation of organic matter, colonizing a broad range of particles under algal bloom conditions (Pinhassi *et al*., 2004). Some members of the *Alphaproteobacteria* were detected, both free-living and attached. These were closely related to a member of the *Rhodobacteraceae*, namely AP-27 (AY145564), and the uncultured alphaproteobacterium DC11-80-2, both observed in the Weser estuary (Selje *et al*., 2004). In addition, a phylotype clustered with a sequence belonging to the SAR1 cluster (Fig. 3), which was retrieved in a study of the bacterial community of the Plum Island Sound estuary by Acinas *et al*. (2004). It might be that this phylotype is globally distributed. This can also be assumed for members of the *Gammaproteobacteria* clustering with

the sequence HTCC2121 retrieved from the Pacific Ocean (Cho & Giovannoni, 2004) or with members of the *Flavobacteria* clustering with a sequence found in the Plum Island Sound estuary (Acinas *et al.*, 2004). It has to be considered that the *Flavobacteria*, as particle colonizers, are thought to play an especially prominent role in the degradation of organic matter (Pinhassi *et al.*, 2004; Abell & Bowman, 2005).

The factors measured in our study were able to explain a large part of the variation occurring in the bacterial community. However, among others, the factors of grazing by ciliates or nanoflagellates as well as control by viruses could not be considered. The importance of these factors has been demonstrated in different studies. Šestanović *et al.* (2004) showed that heterotrophic nanoflagellates controlled planktonic bacteria of the Adriatic Sea in the period spring to summer, replacing the control by temperature, which was considered to be the controlling factor in the other seasons of the year in their study. The role of ciliates in the microbial food web has been investigated by Sherr & Sherr (1987), who stated that the observed clearance rates might be high enough to support the idea that ciliates contribute to a high extent to the microbial food web. Additionally, del Giorgio *et al.* (1996) demonstrated the control of total number of bacteria by heterotrophic nanoflagellates in dialysis experiments. A study of food selection showed that specific bacteria were selected by bacterivorous protists, indicating control by heterotrophic flagellates of the community composition of bacterioplankton (Jezbera *et al.*, 2005). The control of bacterial abundance by viruses has been shown by several studies (Weinbauer & Peduzzi, 1995; Winter *et al.*, 2004), and it has been suggested that viruses might have a stronger effect on bacterial abundance under certain conditions than grazing by heterotrophic nanoflagellates (Weinbauer & Peduzzi, 1995).

Conclusion

This study showed seasonal succession and dynamics of bacterioplankton in the winter–spring transition, supporting the findings of Gerdts *et al.* (2004). Additionally, relationships of several factors with the bacterioplankton were shown, especially for temperature, emphasizing its important role in the winter–spring transition. Also, the phytoplankton species *Phaeocystis* spp., *G. delicatula* and *Chattonella* spp., as well as nutrients (nitrite), contributed to shifts in the bacterial community. We also showed a correlation of factors with specific bacterial phylotypes. In particular, the strong relationship of *Phaeocystis* spp. with a member of the *Gammaproteobacteria* has been shown, indicating a strong influence of this alga on specific phylotypes of the bacterial community.

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