Interactions of marine bacteria

in the pelagic food web

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Der Dekan

Basic to the understanding of any ecosystem is knowledge of its food web, through which energy and materials flow.

Pomeroy, 1974

Preface

This study comprises a general introduction presented in Chapter I, four stand-alone publishable papers – Chapter II to V and an overall discussion – Chapter VI. Each of the Chapters II, III, IV and V contains a separate introduction, description of methods, presentation of data and discussion as well as a separate reference list. Chapter II has already been submitted for publication in *Microbial Ecology* and Chapter V has already been accepted by *FEMS Microbiology Ecology*. Chapters III and IV will be submitted for publication in *FEMS Microbiology Ecology*.

The titles and authors of the papers (Chapters II, III, IV and V) are briefly listed below:

Chapter II	Species-specific bacterial communities in the phycosphere of microalgae?
Authors	Sapp M, Schwaderer AS, Wiltshire KH, Hoppe HG, Wichels A, Gerdts G
Status	Submitted for publication in *Microbial Ecology*
	All analyses, the text writing and graphical presentation were done by Melanie Sapp under supervision of Dr. A. Wichels, Dr. G. Gerdts and Prof. Dr. K.H. Wiltshire. A.S. Schwaderer assisted during the experiment and provided phytoplankton data. Prof. Dr. H.-G. Hoppe provided fruitful discussion.

Chapter III	Bacteria in the phycosphere of marine diatoms: a closer look
Authors	Sapp M, Michel M, Wichels A, Gerdts G
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	The text writing and graphical presentation were done by Melanie Sapp under supervision of Dr. A. Wichels and Dr. G. Gerdts. The experiment and analyses were performed by M. Michel under supervision of Dr. A. Wichels, Dr. G. Gerdts and M. Sapp.

Chapter IV	Impacts of cultivation of marine diatoms on the associated bacterial community
Authors	Sapp M, Wichels A, Gerdts G
Status	To be submitted for publication in *FEMS Microbiology Ecology*
	All analyses, the text writing and graphical presentation were done by Melanie Sapp under supervision of Dr. A. Wichels and Dr. G. Gerdts.

Chapter V	Bacterial community dynamics during winter-spring transition in the North Sea
Authors	Sapp M, Gerdts G, Wiltshire KH, Wichels A
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	All analyses, the text writing and graphical presentation were done by Melanie Sapp under supervision of Dr. A. Wichels and Dr. G. Gerdts. Prof. Dr. K.H. Wiltshire provided oceanographic and phytoplankton data as well as fruitful discussion.

Contents

General Introduction

Bacteria in the pelagic ecosystem

As early as 1894, microbial oceanographers such as Bernhard Fischer (Institute of Hygiene in Kiel) considered bacteria to be a fundamental part in the marine environment (Fischer, 1894). Now, summarising the current knowledge on this topic, it has become obvious that marine prokaryotic microbes are an important unit in the marine ecosystem and an integral part of the microbial loop. Marine bacteria contribute to biogeochemical cycling by consumption of particulate and dissolved organic matter. In addition, they play an important role in nutrient cycling and serve as the main food source for nanoflagellates.

Marine bacteria are quite small compared to bacteria of other habitats; over 90 % of microscopically counted bacteria passed through 1 µm nucleopore filters (Cho & Azam, 1988). Generally, marine bacteria have a size of 1-2 µm with a few exceptions. The smallest bacterium displays a size of 0.3 µm (Sherr & Sherr, 2000). The physiological capacity of marine bacteria is manifold. Phototrophic cyanobacteria, heterotrophic bacteria, chemoautotrophic methanogens, sulfur-oxidising and nitrifying bacteria occur in the marine environment (Sherr & Sherr, 2000). The bacterioplankton is dominated by gram-negative heterotrophic bacteria which oxidise organic substrates. On the one hand, they consume low molecular weight organic compounds which can be transported directly across the cell membrane such as amino acids, acetate or sugars. On the other hand, extracellular hydrolysis or cleavage is needed for the consumption of polysaccharides or proteins. Ecto- and extracellular enzymes enable these reactions. The enzymes are either associated with the bacterial cell surface or they occur dissolved in the water (Hoppe, 1993). Apart from aerobic respiration, anaerobic processes can occur inside organic particles such as fecal pellets (Alldredge & Cohen, 1987) but these processes are usually considered to be of minor importance in marine pelagic food webs. However, suboxic conditions below the euphotic zones can also emerge in regions of upwelling-induced phytoplankton-blooms.

The pelagic environment provides microscale nutrient patches as a source of nutritional demands of bacteria. Therefore, one useful adaptation in the marine habitat is motility which enables chemotactic bacteria to reach nutrient patches, organic particles or algal cells (Blackburn *et al*., 1998; Grossart *et al.*, 2001).

Furthermore, another adaptation of bacteria in the pelagic environment might be the separation into particle-attached and free-living bacteria. Different phylogenetic and physiological characteristics of pelagic bacteria result in this discrimination (DeLong *et al.*, 1993; Middelboe *et al.*, 1995) which leads to the assumption that these bacteria play different roles in the pelagic ecosystem.

Prokaryotic diversity

In the history of marine microbial ecology, marine microbiologists first tried to study their objects of interest exclusively by cultivation and later by direct counting. It became evident that these methods would not increase the knowledge regarding the role of bacteria in the marine environment (ZoBell, 1946; Jannasch & Jones, 1959). The differences between these methods are known as "the great plate anomaly" (Staley & Konopka, 1985). The comparison of the results observed by these methods revealed that only few marine bacteria can be cultivated. Until now, methods were established which achieved successful cultivation of approximately 20 % only (Selje *et al.*, 2005). Therefore, analysis of the bacterial community in the marine habitat required new culture-independent methods which were established on the basis of the bacterial 16S rRNA genes. The analysis of the genes encoding for the small subunit of ribosomes has several advantages. First, the genes have very conserved and quite variable sites and second, the availability of rDNA databases allows comparative sequence analysis and phylogenetic classification (Amann & Ludwig, 2000).

Nowadays, a variety of different techniques is available to study the diversity of the marine bacterial community. Beside cloning and sequencing of the 16S rRNA genes, different community fingerprinting methods are most important. Beside these fluorescent *in situ* hybridisation provides information of the phylotype and the phenotype. Studies on microbial ecology often use community fingerprinting methods such as Amplified Ribosomal DNA Restriction Analysis (ARDRA), Ribosomal Intergenic Spacer Analysis (RISA), Single Strand Conformation Polymorphism (SSCP), Terminal Restriction Fragment Lenght Polymorphism (T-RFLP) or Denaturing Gradient Gel Electrophoresis (DGGE). Particularly DGGE is a widespread and often-used method to obtain genetic fingerprints of bacterial communities. It has the potential to provide information on the diversity and dynamics of bacterial communities. Additionally, the analysed 16S rRNA gene fragments may be used for identification of bacterial phylotypes via subsequent sequencing. Summarising the results of studies in molecular ecology, it can be stated that the most abundant rRNA genes do not

correspond to cultivated species (Giovannoni & Rappé, 2000). Therefore, molecular techniques are very important for the study of microbial ecology in the marine habitat.

The phylogenetic diversity of the marine bacterioplankton is dominated by gram-negative *Proteobacteria* in addition to members of the *Bacteroidetes* phylum. Furthermore, marine methylotrophic bacteria, *Planctomycetales*, *Cyanobacteria* and gram-positive bacteria are part of the bacterioplankton. Interestingly, the major prokaryotic groups appear to have cosmopolitan distributions. Of these, members of the *Alphaproteobacteria* are comprised in the *Roseobacter* clade. Members of this group are chemoorganotroph and occur exclusively in the marine environment. Some of these species are able to synthesise bacteriochlorophyll a (Allgaier *et al.*, 2003). Apart from the *Roseobacter* clade species belonging to *Sphingomonas*, SAR 11 and SAR 116 are important groups within the *Alphaproteobacteria* which can be highly specialised (Giovannoni & Rappé, 2000). Another very important group of the *Proteobacteria* is the group of *Gammaproteobacteria* which is the best cultivable group of the bacterioplankton. These chemoorganotrophic bacteria are often associated with surface. Species like *Alteromonas sp.* and *Pseudoalteromonas sp.* belong to this group as well as species like *Oceanospirillum sp.* or *Marinobacter sp.* which form a separate clade.

Within the *Bacteroidetes* phylum, a high variability in morphology and phenotype has been found. Members of this phylum displayed the ability for gliding motility as well as the ability to degrade biomacromolecules like chitin, agar, cellulose or DNA. They are widespread distributed in the marine environment. Typically, the *Bacteroidetes* are associated with surfaces such as algal cells or marine snow but they can also be found free-living. An association with marine snow was also found for members of the *Planctomycetales* (Giovannoni & Rappé, 2000). Identification of marine bacteria also revealed the presence of marine methylotrophs which can be divided into two groups: Type I methanotrophs contain species like *Methylomonas sp.* or *Methylobacter sp.* whereas the species *Methylosinus sp.* or *Methylocystis sp.* belong to Type II methanotrophs. Furthermore, gram-positive bacteria were detected as ubiquitous in the marine environment namely the *Actinobacteria.* But usually *Actinobacteria* are not abundant in the marine pelagic bacterial community. Additionally, oxygenic phototrophs which mostly possess chlorophyll a and phycobilisomes occur in the marine environment. Species like *Synechococcus sp.* belong to the *Cyanobacteria* and are included in the picophytoplankton. Culture-independent methods revealed that *Archaea* are also abundant in the marine habitat (DeLong *et al.*, 1999). Within this domain marine *Archaea* can be divided mainly into two groups. Members of the first group are peripherally related to the *Crenarcheota*. They can be found at dephts of 100 m whereas members of the

second group dominate the *Archaea* in surface water samples. Those *Archaea* have a common ancestry with the *Euryarchaeota*.

Microbial loop, marine food web

In the past, the role of bacteria in the pelagic ecosystem was exclusively ascribed to decomposition processes (Strickland, 1965). The classical marine food chain model consisted of primary production by diatoms and dinoflagellates consumed by copepods which in turn served as prey for larger consumers. Pomeroy (1974) hypothesised that this perspective might be inadequate. He considered photoautotrophic nanoplankton to be the major producer in the marine food web and stated that bacteria might be the largest consumers of the primary production. In his remarks he applied a concept which assumed uniform biomass distribution among different size classes and an inverse relation between size and metabolic rate. Additionally, he suggested that important food sources in marine food webs were particulate organic matter (POM) and dissolved organic matter (DOM) which would be primarily consumed by heterotrophic bacteria. He also postulated the universality of the detritus food web. Ten years later, the concept of the microbial loop was presented by Azam and coworkers (1983). They hypothesised that DOM deriving from the primary production would be consumed by bacteria which in turn would be consumed by flagellates. These would serve as prey for micro-zooplankton. Consequently, the microbial loop channels the energy within DOM and POM, which was assumed to be lost from the trophic system, back to the pelagic food chain. A simplified overview of the conceptual model of the microbial loop is given in Fig. 1 according a scheme published by Sherr & Sherr (2000). This figure illustrates major interactions of heterotrophic bacteria and it has to be considered that causal interactions in the microbial loop are circular and multibranched (Thingstad, 2000).

Major substrates for heterotrophic bacteria are labile DOM, dissolved inorganic nutrients and organic forms of mineral nutrients like amino acids. Control of heterotrophic bacteria within the microbial loop is ensured by bacterivory and viral lysis (Fig. 1). Bacterivorous protists are the main consumers of heterotrophic bacteria in the marine ecosystem; therefore it is the first important controlling factor. Del Giorgio *et al*. (1996) performed dialysis experiments to show this coherence. Additionally, they found that especially metabolically active bacteria were consumed by protists. Similar results have been observed for the middle Adriatic Sea, where heterotrophic nanoflagellates controlled bacteria in summer (Šestanović *et al.*, 2004). Beside nanoflagellates, it could be demonstrated that also pelagic ciliates are important consumers of bacteria (Sherr & Sherr, 1987). The second important factor controlling

heterotrophic bacteria is viral lysis. The mortality due to viral infection can exceed the mortality caused by grazing of nanoflagellates (Weinbauer & Peduzzi, 1995).

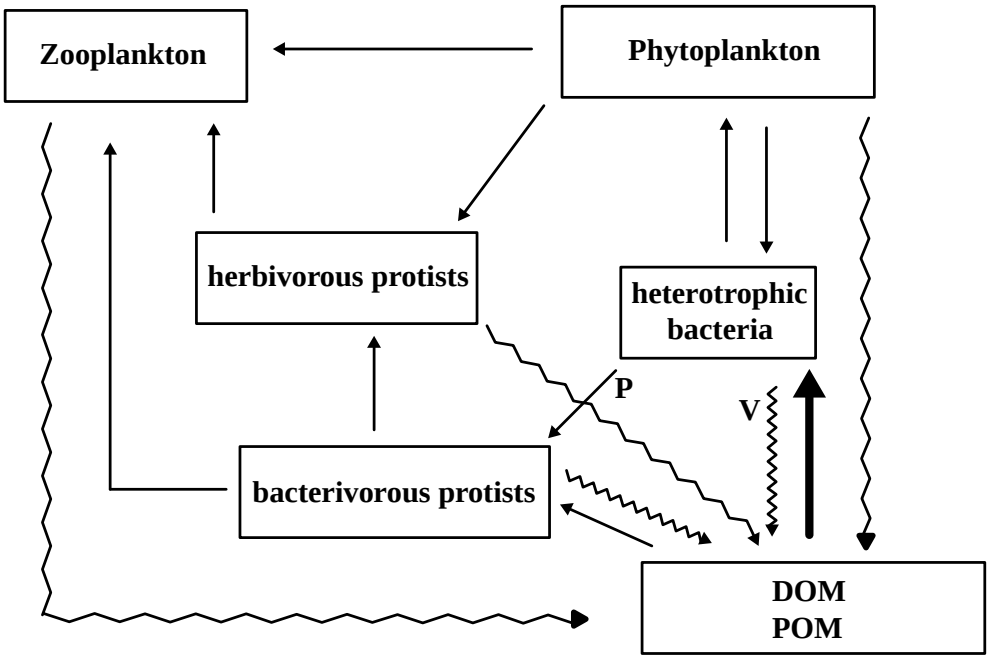

Figure 1: Simplified scheme of a pelagic microbial food web according to Sherr & Sherr (2000). P: Predation, V: Viral lysis

Generally, in surface waters, an overall percentage of 10-50 % of bacterial mortality is ascribed to viral lysis (Fuhrman, 1999). It is suggested that the infection by viruses is generally species specific and that it is density dependent (Fuhrman, 1999). Apart from the control by protists and viral lysis, heterotrophic bacteria have to compete with phytoplankton for mineral forms of nitrogen, phosphorus and iron in the surface layer of the marine environment. Where phosphorus is deficient relative to nitrogen, not only the phytoplankton but also the bacterial growth rate is phosphorus limited. It is likely that iron limitation also occurs (Kirchman, 2000) which could be confirmed by stimulation of bacterial growth after addition of iron in Antarctic waters (Pakulski *et al.*, 1996).

The phytoplankton-bacteria interaction was investigated in detail in this thesis. The habitat of phytoplankton-associated bacteria has been depicted by the "phycosphere" concept defining the area around algal cells where bacteria feed on extracellular products of the algae (Bell & Mitchell, 1972). The association of distinct bacterial communities and microalgae has already been shown in several studies (Schäfer *et al.*, 2002; Grossart *et al.*, 2005; Jasti *et al.*, 2005). Differences between bacterial phylotypes which live on the phytoplankton cells and those living in the surrounding medium were also shown (Kogure *et al.*, 1982; Vaqué *et al.*, 1990). Phytoplankton cells excrete organic compounds, including high proportions of carbohydrates

(Myklestad, 1995). Bacteria attached to algal surfaces consume these extracellular products. This process is an important part in the microbial loop (Azam, 1998). Hence, it is fundamental to elucidate the ecological role of bacteria interacting with phytoplankton. Several studies agreed that specific groups of bacteria are associated with different microalgae (Prokiç *et al.*, 1998; Hold *et al.*, 2001; Schäfer *et al.*, 2002; Green *et al*., 2004; Grossart *et al.*, 2005; Jasti *et al.*, 2005).

Apart from the interactions already described, bacteria and zooplankton interact via DOM and POM according to the concept of the microbial loop (Fig. 1). Bacterial cell counts are higher in fecal pellets than in the surrounding seawater (Delille & Razouls, 1994). Especially the species *Pseudomonas sp*. and *Vibrio sp*. could be identified in fecal pellets (Delille & Razouls, 1994). Recent investigations showed that copepods themselves are microbial hotspots in the ocean. Tang (2005) found a balance between bacterial growth stimulated by the host's feeding and bacteria loss through the host's defecation. He hypothesised that different types of diet might be selective forces for a diversification of bacterial communities inside copepods. According to these findings, the role of bacteria interacting with zooplankton should be investigated in detail in future.

Focussing on biogeochemical cycling, it should be mentioned that free-living bacteria are responsible for most of the bacterial production (inter alia in the central north Pacific gyre and the eutrophic Santa Monica basin (Cho & Azam, 1988)). This finding led to the hypothesis that free-living bacteria might be important for biogeochemical fluxes assuming that these bacteria might mediate the exchange equilibrium between dissolved and particulate phases (Azam, 1998). Further investigations are needed to test this hypothesis and to gain more insights of the bacterial role in biogeochemical fluxes.

Bacterial communities in the coastal environment

The coast is a highly dynamic environment and it is regarded as the boundary extending from the land into the shelf sea. It serves as interface between terrestrial and marine energy as well as matter fluxes. In shallow coastal seas, the marine productivity is very high in contrast to the open-ocean. It is likely that bacteria play an important role in this habitat especially with focus on biogeochemical cycling, supported by the fact that cell numbers and biomass of marine bacteria in coastal seas are higher than those of the open-ocean (Azam *et al.*, 1983). Therefore, it could be expected that bacterial species would be different in the coastal seas and the open-ocean (Giovannoni & Stingl, 2005). However, marine bacterioplankton species are generally similar, except for the group of *Betaproteobacteria* which could only be

identified in coastal areas. Still, the potential freshwater origin of these bacteria cannot be excluded.

The study site for all investigations presented in this thesis was Helgoland Roads (54°11.3' N and 7°54.0' E), North Sea. In a three year study of the bacterioplankton community the structure was analysed by DGGE. It could be shown that the bacterioplankton of Helgoland Roads varies with the season (Gerdts *et al.*, 2004). It was apparent that a stable community in winter was displaced by diverse communities in spring and strong shifts were displayed in summer. After these shifts the community again developed towards a winter community.

Generally, bacterioplankton dynamics are governed by seasonal changes of abiotic and biotic factors and are linked with phytoplankton dynamics. There is an increasing number of studies dealing with seasonality of bacterioplankton community composition (Shiah & Ducklow, 1994; Pinhassi & Hagström, 2000; Kent *et al.*, 2004). Temperature was considered to be the major controlling factor in winter, autumn and spring, whereas a limitation of inorganic nutrients and substrate was regarded to be the controlling factor in summer (Shiah & Ducklow, 1994). Thingstad (2000) stated that patterns in bacterioplankton succession can be ascribed to a delay between the peak in phytoplankton biomass and a subsequent response in bacterial biomass and production. He assumed that the delay might probably be caused by the need for bacteria to hydrolyse polymers to monomers. Otherwise, the production of organic substrates for bacterial growth might be mainly a result of activities in the predatory food chain, as labile material will then be produced only in the succession phase where grazers consume the phytoplankton. However, a study by Van Es & Meyer-Reil (Van Es & Meyer-Reil, 1982) showed a correlation of primary production and marine bacteria. Additionally, changes in bacterial community composition could be observed 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; Rooney-Varga *et al.*, 2005; Brussaard *et al.*, 2005). These studies indicated a close coupling of phytoplankton and bacterial community composition. However, further studies are needed to investigate seasonal succession of bacterioplankton due to phytoplankton dynamics.

Focus of the present study: bacteria-phytoplankton interaction

In this study, the bacteria-phytoplankton interaction in a coastal environment was investigated. In general, it was based on the fact that highly specific interactions of bacteria with phytoplankton like symbioses (Croft *et al.*, 2005) or parasitic relationships (Fukami *et*

al., 1997; Lovejoy *et al.*, 1998) can occur in addition to more unspecific interactions such as competition, commensalism or mutualism.

In this work, the interaction of microalgae and bacteria was studied with a focus on the association with microalgae considered to be key-species of Helgoland Roads. Bacterial communities of microalgal cultures were analysed concerning specificity of the association and related to algal growth phases. The results of this culture experiment are presented in Chapter II. The hypothesis that species-specific interactions occur between bacteria and different diatom species is tested in Chapter III by performance of resource competition experiments. In addition, it was investigated, if diatom exudates show a shaping influence on the structure of diatom associated bacterial communities. In Chapter IV shifts in the associated bacterial community structure resulting from isolation and cultivation of microalgae are displayed.

Additionally, Chapter V is based on the diversity and dynamics of the bacterioplankton of Helgoland Roads which was studied during winter-spring transition 2004 with regard to successional changes due to phytoplankton species dynamics as well as abiotic parameters. The linkage of the bacterial community and abiotic and biotic environmental factors was analysed by multivariate statistics.

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Species-specific bacterial communities in the phycosphere of microalgae?

Abstract

Specific associations of bacteria with phytoplankton have recently been reported in the literature. In our study, we analysed bacterial communities of microalgal cultures related to algal growth phases. Seven freshly isolated key diatom and dinoflagellate species from Helgoland Roads, North Sea, were investigated. The community composition of associated bacteria as well as the cell numbers, the photosynthetic efficiency of the algae and the depletion of anorganic nutrients in the medium were recorded over a period of eight weeks in batch cultures. Diversity and succession of bacterial communities was analysed by Ribosomal Intergenic Spacer Analysis (RISA). Phylogenetic analysis of bacterial populations was performed by Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA genes followed by DNA sequence analysis. Members of *Alpha* - and *Gammaproteobacteria* and the *Flavobacteria-Sphingobacteria* group within the *Bacteroidetes* phylum predominated in the cultures. Differences in free-living and attached bacterial populations were observed between the phylogenetic groups. Shifts in the bacterial communities could not be correlated to changes of nutrient levels or algal growth phases. Regarding our results, it should not be generalised, that the compositions of the bacterial communities are strictly species-specific for microalgae. The importance of factors like the composition of exudates is apparent.

Introduction

The linkage between bacterioplankton and phytoplankton dynamics was recently demonstrated by Rooney-Varga *et al*. (2005). Here it was shown that there was coherence in the dynamics of bacteria living attached to phytoplankton cells. In addition, the influence of phytoplankton blooms on bacterial communities has been shown by analysing the effects of phytoplankton blooms in a mesocosm study (Riemann *et al.*, 2000) and in the field (Fandino *et al.*, 2001). In the study by Riemann *et al*. (2000) major changes in the bacterial community composition were observed in the postbloom phase of a diatom bloom. Fandino *et al*. (2001) were able to detect shifts in the bacterial metabolism and in the community composition

during a dinoflagellate bloom. Thus, the topic of bacterial-algal association is of great ecological interest at present.

The habitat of phytoplankton-associated bacteria has been depicted by the "phycosphere" concept defining the area around algal cells where bacteria feed on extracellular products of the algae (Bell & Mitchell, 1972). An uptake of extracellular products of *Skeletonema costatum* by bacterial isolates could be demonstrated by Bell *et al*. (1974) indicating the role of exudates as a carbon source. Generally, phytoplankton cells excrete organic compounds, including high proportions of carbohydrates (Myklestad, 1995) contributing to the base of the microbial food web (Lancelot, 1983). Bacteria which live attached to algal surfaces and which consume extracellular products, consequently participate in biogeochemical cycling and play an important part in the microbial loop (Azam, 1998). Hence, it is important to elucidate the ecological role of bacteria interacting with phytoplankton. Beside highly specific interactions of bacteria with phytoplankton like symbioses (Croft *et al.*, 2005) or parasitic relationships (Fukami *et al.*, 1997; Lovejoy *et al.*, 1998), it is likely that interactions such as competition, commensalism or mutualism occur.

However, the factors controlling the process of colonisation might favour specific interactions. Recently, it has been reported that the influence might be due to environmental factors (Grossart, 1999). Furthermore, general specificity of bacteria associated with algae has been shown in several studies (Hold *et al.*, 2001; Schäfer *et al.*, 2002) leading to the assumption that the colonised cells have selecting influence to a certain extent. Studies on the process of colonisation showed that different bacterial phylotypes live on the phytoplankton cells and in the surrounding medium (Kogure *et al.*, 1982; Vaqué *et al.*, 1990). These findings support the idea of general specificity of attached versus free-living bacteria, but still it is not clear which factors control the interaction of bacteria and microalgae.

However, commonness can be found within groups of bacteria being associated with microalgae (Prokiç *et al.*, 1998; Hold *et al.*, 2001; Schäfer *et al.*, 2002; Green *et al*., 2004; Grossart *et al.*, 2005; Jasti *et al.*, 2005). Thus, the habitat "phycosphere" should be considered a niche which might provide a suitable microenvironment for a diverse subset of bacteria.

In this study, bacteria associated with key diatoms and dinoflagellates isolated from Helgoland Roads, North Sea, were identified and specificity was investigated concerning their "host". This was carried out by analysing the associated bacterial community of the respective algal species in clonal cultures obtained by micropipetting. Comparison of the associated communities was performed during different growth phases of the algae.

Methods

Algal cultures

In 2002, plankton samples from Helgoland Roads (54°11.3' N and 7°54.0' E) water were taken using a 20 µm and 80 µm net with the research vessel Aade. Single cells were isolated by micropipetting (Daste *et al.*, 1983) and washed sequentially in many steps before being cultivated. The process of repeated washing of single cells removed free-living bacteria from the sample and left only those bacteria strongly associated with the single algal cell at the time of isolation. Consequently, the free-living bacteria in the culture were originally most likely derived from attached bacteria. The culture medium was sterile.

Experimental design and sample collection

Seven key phytoplankton species were chosen for our experiments. These were a variety of microalgae which dominate in the water of Helgoland Roads during winter, spring, summer and autumn (Wiltshire & Dürselen, 2004). They included *Guinardia delicatula* (Cleve) Hasle, *Pseudonitzschia pungens* Grunow, *Thalassiosira rotula* Meunier isolated in April (spring) and August (summer), *Skeletonema costatum* (Greville) Cleve, *Ceratium horridum* (Cleve) Gran and *Akashiwo sanguinea* (Hirasaki) Hansen et Moestrup. The microalgae were incubated in batch culture in Guillard's f/2 medium (Guillard, 1975) in 2 l glass bottles at 16°C with 12:12 light-dark cycle at 40,0 µmol photons $m^{-2}sec^{-1}$. For the experiments, two litres of media were inoculated with 10 ml of the respective microalgal culture in the early stationary phase. Incubation of cultures was performed in three replicates over an eight week period. Samples were taken from the initial inoculum community and after one, two, three, four and eight weeks. The experiment was conducted from May to July 2003.

Sampling of bacterial biomass and extraction of nucleic acid

In order to collect the biomass of attached and free-living bacteria in the cultures, 50 ml of each culture were filtered through 3 µm and 0.2 µm membrane filters (Millipore, Germany) in succession. This was done with the initial inoculum and in weeks one to eight. Successive filtration separated the biomass of the attached bacteria, which were detained by a 3 µm filter, and free-living bacteria, which were detained by a 0.2 µm filter. Filters were stored at -20°C until DNA extraction.

Nucleic acid extraction was performed as described in detail by Wichels *et al*. (2004). Filters were cut into pieces and transferred to sterile 2 ml vials. Bacterial biomass was resuspended in STE buffer (6.7 % Saccharose, 50 mM Tris, 1 mM EDTA, pH 8) and cell lyses were facilitated by adding lysozyme (2 mg ml^{-1}) and SDS (1 %). DNA extraction was performed using phenol-chloroform-isoamylalkohol (25:24:1). After isopropanol precipitation of the DNA all DNA extracts were resuspended in sterile water and stored at -20°C until further analysis. These served as template DNA in the PCR. Prior to PCR amplification, the DNA extracts were analysed by agarose gel electrophoresis on 0.8 % agarose gels (45 minutes at 100 V in 0.5 x TBE (10 x TBE: 0.89 M Tris, 0.89 M boric acid, 0.025 M EDTA)). After electrophoresis, gels were stained with ethidium bromide (0.5 mg l^{-1}). Photographs were taken on a UV-transilluminator (302 nm) with Polaroid MP4 equipment (Cambridge, Mass., USA). The sample volume for subsequent PCR amplification was estimated from the photograph.

Community fingerprinting by Ribosomal Intergenic Spacer Analysis (RISA)

For amplification of the intergenic spacers (IGS) between the 16S and 23S subunits of ribosomal sequences we used the primers S-D-Bact-1522-b-S-20 (5'- TGC GGC TGG ATC CCC TCC TT -3') and L-D-Bact-132-a-A-18 (5'- CCG GGT TTC CCC ATT CGG -3') (Ranjard *et al.*, 2000a; Ranjard *et al.*, 2000b). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf, Germany), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer, Germany), 0.5 µM of each primer and 2 U of Taq DNA Polymerase (Eppendorf). We used 5 µl DNA of the 0.2 µm filters or 0.5 µl DNA of the 3 µm filters. The amplification started with a denaturing step at 95°C for three minutes and 25 cycles at 95°C for one minute, 53°C for one minute and 72°C for one minute followed by 72°C for five minutes for final extension. PCR reactions were done 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, Germany) in 0.5 x TAE buffer. Three lanes were used for 0.1 µg of a 100 bp ladder (invitrogen, Germany) in order to achieve comparability. Electrophoresis was run at 20°C for 18 h at 50 V using a DCode system (BioRad, Germany). Gels were stained with SYBRGold as recommended by Molecular Probes (Germany) and illuminated on a UV table (2011 Macrovue Transilluminator, LKB Bromma, Sweden), photographs were taken with Polaroid MP4 equipment.

RISA fingerprint analysis

Comparative analysis of RISA fingerprints was carried out with the BioNumerics 4.0 software (Applied Maths, Belgium). Multivariate analysis of fingerprints was performed using the subroutines MDS, ANOSIM, RELATE and BIO ENV of the PRIMER 5 software suite (PRIMER-E Ltd., UK; Clarke & Warwick, 2001). Normalisation of gels was carried out using 100 bp ladders as references in every profile. For sample comparison a band-matching analysis was carried out. Bands were assigned to classes of common bands within all profiles. This procedure included the densitometric values of bands as proposed by Muylaert *et al*. (2002). The resulting band matching table was imported into PRIMER and used to calculate Bray Curtis similarities of RISA profiles applying square root transformation (Clarke & Warwick, 2001) for each culture and for the complete dataset.

The Bray Curtis similarity between samples i and j is:

$$S_{il}^{'} = 100\left\{1 - \frac{\sum_{j=1}^{n}\left|y_{ij} - y_{lj}\right|}{\sum_{j=1}^{n}\left(y_{ij} + y_{lj}\right)}\right\}$$

The extreme values are (0,100) as:

S´= 0 if two samples have no bands in common

S´= 100 if two samples have all bands in common

Ordination of Bray Curtis similarities was performed by nonmetric multidimensional scaling (MDS) (Yannarell & Triplett, 2005). MDS was calculated using ten random starting configurations of sample points. It was assumed that the final configuration was optimal unless other configurations displayed lower stress levels. For clearness, all plots are presented two-dimensional although three-dimensional plots usually displayed lower stress levels.

Additionally, hierarchical agglomerative clustering of Bray Curtis similarities was performed using the complete linkage method of the PRIMER software.

To test the hypothesis that the within-group RISA profile similarity was greater than between groups, an analysis of similarity was conducted by the subroutine ANOSIM of the PRIMER software. ANOSIM is a nonparametric technique designed to allow statistical comparisons for multivariate data sets in a manner similar to univariate techniques (ANOVA). The null hypothesis is that no difference between associated bacterial communities of different microalgae exists. Testing the hypothesis results in a sample statistic R displaying the degree of separation between groups. Complete separation is indicated by R = 1, whereas R = 0 suggests no separation. Having determined R, ANOSIM randomly assigns samples to different groups to generate a null distribution for R (Monte Carlo test; Yannarell & Triplett,

2004) to test whether within-group samples were more closely related to each other than would be expected for a random distribution.

The influence of the measured biotic and abiotic variables on the structure of the associated communities was tested by the PRIMER 5 subroutines RELATE and BIO ENV (Gillanders, 2001). For these statistical analyses, diatom and dinoflagellate data were separated because the variable silicate was measured only in diatom cultures. In order to perform the routine RELATE, the variables such as nutrients, yield of photosystem II and algal cell counts of living, degrading and dead cells were used to calculate a similarity matrix using the normalised Euclidean distance coefficient and a square root transformation. The Bray Curtis similarity of samples and the normalised Euclidean distance of variables were compared by the RELATE routine (Clarke & Warwick, 2001) using the rank correlation by Spearman. This comparison provides a significance test with the matching coefficient ρ_m which is equivalent to the Mantel's test (Clarke & Warwick, 2001). The null hypothesis of the significance test implies that there is no relation between the two similarity matrices with $\rho_m = 0$. If there is a relation between sample and variable matrix, ρ_m will have values near one. If RELATE indicates a relation between the two matrices, the subroutine BIO ENV of the PRIMER software was used to search for a subset of individual variables that maximises the rank correlation value ρ. A rank correlation value of $\rho = 1$ indicates that the measured variables are able to explain the community data.

Statistical analysis was performed with all replicates of each culture except for the culture of *A. sanguinea* because of contamination of replicate three.

Phylogenetic analysis of communities

Amplification of 16S rRNA genes and DGGE. PCR amplification of 16S rDNA fragments was performed according to Wichels *et al*. (2004) 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 907rm modified (5'- CCG TCA ATT CMT TTR AGT TT -3'). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer), 0.2 µM of each primer, 2 U of Taq DNA Polymerase (Eppendorf) and 5 µl DNA of 0.2 µm filters or 0.5 µl of 3 µm filters. The "touchdown" PCR started with a denaturing step at 94°C for five minutes. Every cycle consisted of three steps of one minute, i.e., 94°C, annealing temperature and 72°C. The initial annealing temperature of 65°C 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 ten minutes followed by 22 cycles starting at 71°C decreasing by 1°C per cycle in order to avoid heteroduplices. PCR reactions were performed in an Eppendorf Mastercycler. PCR products were inspected on 1.2 % (w/v) agarose gels. DGGE analyses 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 - 70 % urea/formamide (100 % denaturant contains 7 M urea and 40 % formamide). Electrophoresis was run at 60 °C and 150 V for 10 hours (Sigler *et al.*, 2004). DGGE gels were stained with SYBRGold (see RISA) and illuminated on a UV table (see RISA).

DNA sequencing. Prominent DGGE bands which connected or separated samples were excised, eluted (Sambrook *et al.*, 1989) and reamplified using the primers 341f without GC-clamp and 907rm. DNA was purified via the Qiaquick PCR purification kit (QIAGEN, Germany) following the instructions of the manufacturers protocol. Products were checked by electrophoresis on 1.2 % (w/v) agarose gels. Sequencing was performed by using the SequiTherm Excel™ II long read sequencing Kit-LC (Biozym, Germany) following the manufacturer's instructions. Sequencing primers were 907rm-IRDye700 and 344f-IRDye800 (5'- ACG GGA GGC AGC AG -3'). Sequencing was carried out using a long range gel on a 4200 automated DNA sequencer (LI-COR Inc., Lincoln, Nebraska). 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 by 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 rDNA sequences database (release June 2002) alignment was carried out with the Fast Aligner integrated in the program and refined by comparison of closest relatives obtained 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 neighbour joining method including the correction algorithm of Felsenstein (1993).

Nucleotide sequence accession numbers. The sequences obtained in this study are available from GenBank under the accession numbers AY907263-AY907343.

Abiotic and biotic explanatory variables

Algal cell numbers. To determine algal cell numbers, three replicates were counted in Sedgewick-Rafter chambers using the method described by Lund *et al*. (1958). For each sample a minimum of 400 cells was counted with colonies being noted as single units. In the case of colony formation, the number of cells in a colony was recorded separately. Beside the living cells, degrading and dead cells were also counted to obtain a more detailed insight into the development of the cultures. The state of the chloroplasts and the cytoplasm was determined visually. Cells with few chloroplasts and significantly reduced cytoplasm were recorded as degrading and empty valves recorded as dead.

Photosynthetic efficiency and physiological fitness. The quantum yield during photosynthesis is a very sensitive indicator of a plant's physiological state and stress level (Durako & Kunzelmann, 2002; Juneau *et al.*, 2003). To determine the effective quantum yield of photochemical energy conversion the PAM (Pulse Amplitude Modulation) method of fluorescence measurement was applied. To acquire the quantum yield of Photosystem II, the first initial fluorescence F_0 and F'_0 for light exposed samples respectively is induced by low levels of actinic light after a period of dark adaptation. When the emission reaches a stable level, a strong pulse of light, the Saturation Pulse, is applied. This leads to a quick reduction of the electron transport chain between the two photosystems, forcing the cells to emit all energy as fluorescence. The maximum fluorescence value F_m and F'_m for light exposed samples respectively is recorded and the effective quantum yield calculated from $(F_m - F_0) / F_m$ for dark adapted and $(F'_m - F'_0)/ F'_m$ for previously light exposed samples (Genty *et al.*, 1989). This reflects directly the electron flow through Photosystem II at the time of the measurement and is regarded as an indicator for the fitness of the algal cells. The quantum yield was measured using a Xenon-PAM, Walz, Germany (1999). Samples were dark-adapted for five minutes, then transferred into a 10x10 mm quartz cuvette and the fluorescence yield measured.

Nutrients. In order to monitor the nutrient concentration and depletion, the five major inorganic nutrients in sea water: ammonium, nitrite, nitrate, silicate and phosphate were measured photometrically in triplicate (Grasshoff & Johannsen, 1974; Grasshoff *et al.*, 1999) and analysed in a Lambda 2S Perkin-Elmer UV/VIS Spectrometer.

Results

Community structure. Characterisation of the bacterial communities associated with the microalgae was performed by the fingerprinting method RISA. This was shown exemplarily for the associated bacterial community of *G. delicatula* (Fig. 2).

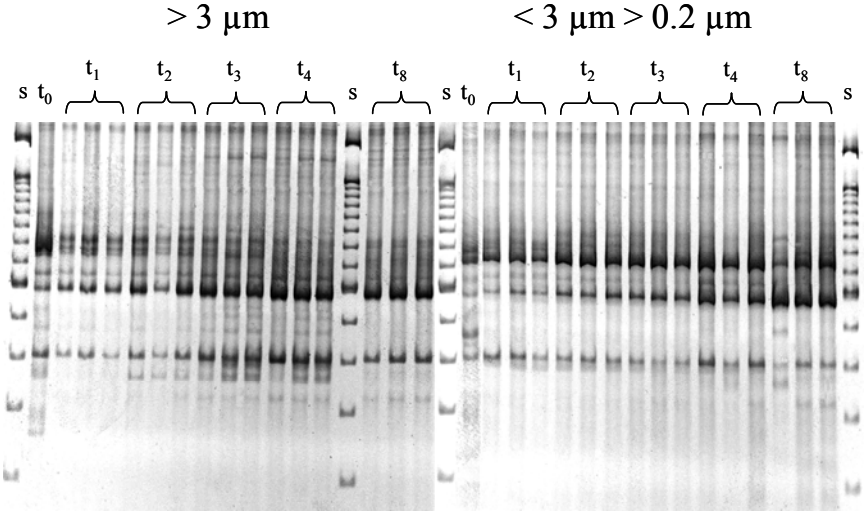

Figure 2: RISA fingerprint of bacterial communities of *G. delicatula* cultures (three replicates) resolved on 8 % polyacrylamide. t_0: initial inoculum community. $t_1 - t_8$: samples week 1 to week 8. s: 100 bp ladder.

To analyse the fingerprints, different multivariate analyses as well as hierarchical agglomerative clustering were applied. It is apparent that the bacterial communities from the examined microalgae as represented by the RISA fingerprints (Fig. 2 and Fig. 3), were separated to different degrees from each other. Regarding the complete MDS ordination and the cluster analysis, the communities of *T. rotula* (spring and summer) compared to *A. sanguinea* but also *G. delicatula* displayed clearly separated groups. From the cluster analysis it can also be seen, that only the community of *T. rotula* (spring and summer) forms a homogeneous cluster (Fig. 3).

The ANOSIM analysis revealed that regarding all cultures, the communities of *T. rotula* isolated in spring and *A. sanguinea* were most different from each other with R = 0.98 (ANOSIM; see Tab. 1) whereas the communities of *P. pungens* and *C. horridum* were most similar, indicated by a low value of R (R = 0.328, Tab. 1). It should be mentioned that the bacterial assemblages of the two cultures of *T. rotula* (spring and summer) were also different with R = 0.852 in the ANOSIM analysis, which could not be resolved by hierarchical agglomerative clustering (Fig. 3).

Table 1: ANOSIM (Analysis of similarity) statistics for tests involving comparisons of all seven cultures, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

sample statistic r	*G. delicatula*	*P. pungens*	*T. rotula* (spring)	*T. rotula* (summer)	*S. costatum*	*C. horridum*
G. delicatula						
P. pungens	0.617					
T. rotula (spring)	0.866	0.829				
T. rotula (summer)	0.841	0.715	0.852			
S. costatum	0.424	0.582	0.569	0.532		
C. horridum	0.697	0.328	0.836	0.785	0.589	
A. sanguinea	0.870	0.790	0.980	0.990	0.536	0.742

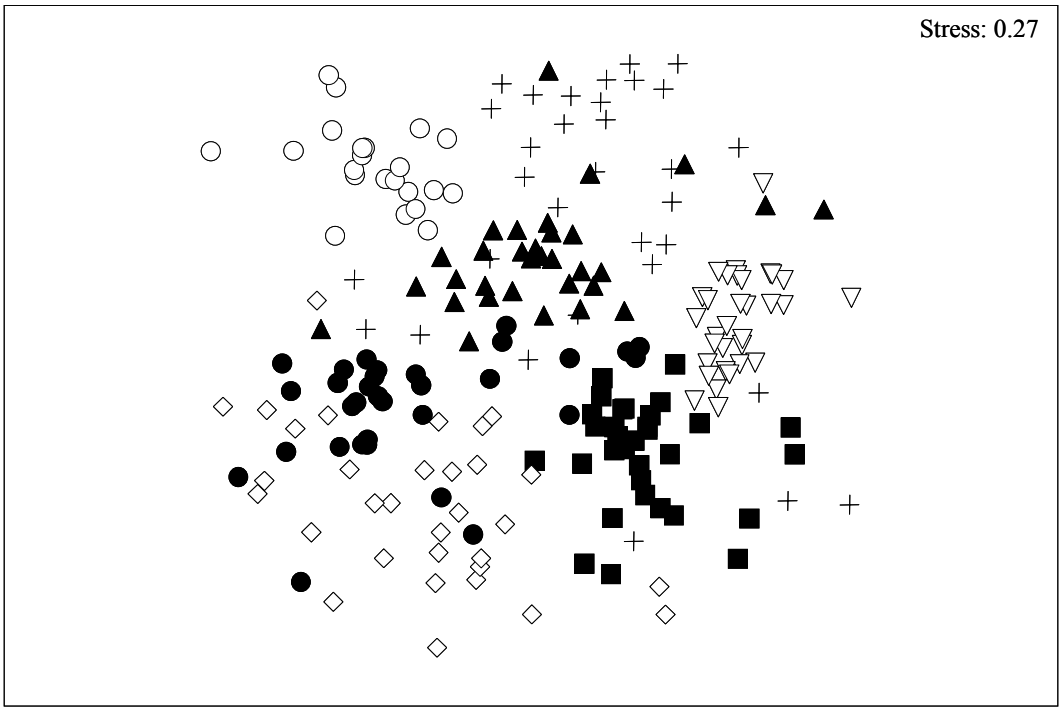

Figure 3: MDS plot based on Bray-Curtis similarities of RISA fingerprints of bacterial communities of different microalgal cultures (including three replicate batch cultures for each species, two sample fractions and five sampling dates) based on Bray-Curtis similarities. Microalgae: *G. delicatula* (▲), *T. rotula* spring (▽), *T. rotula* summer (■), *P. pungens* (◇), *A. sanguinea* (○), *C. horridum* (●), *S. costatum* (+). Stress level in 3D = 0.19.

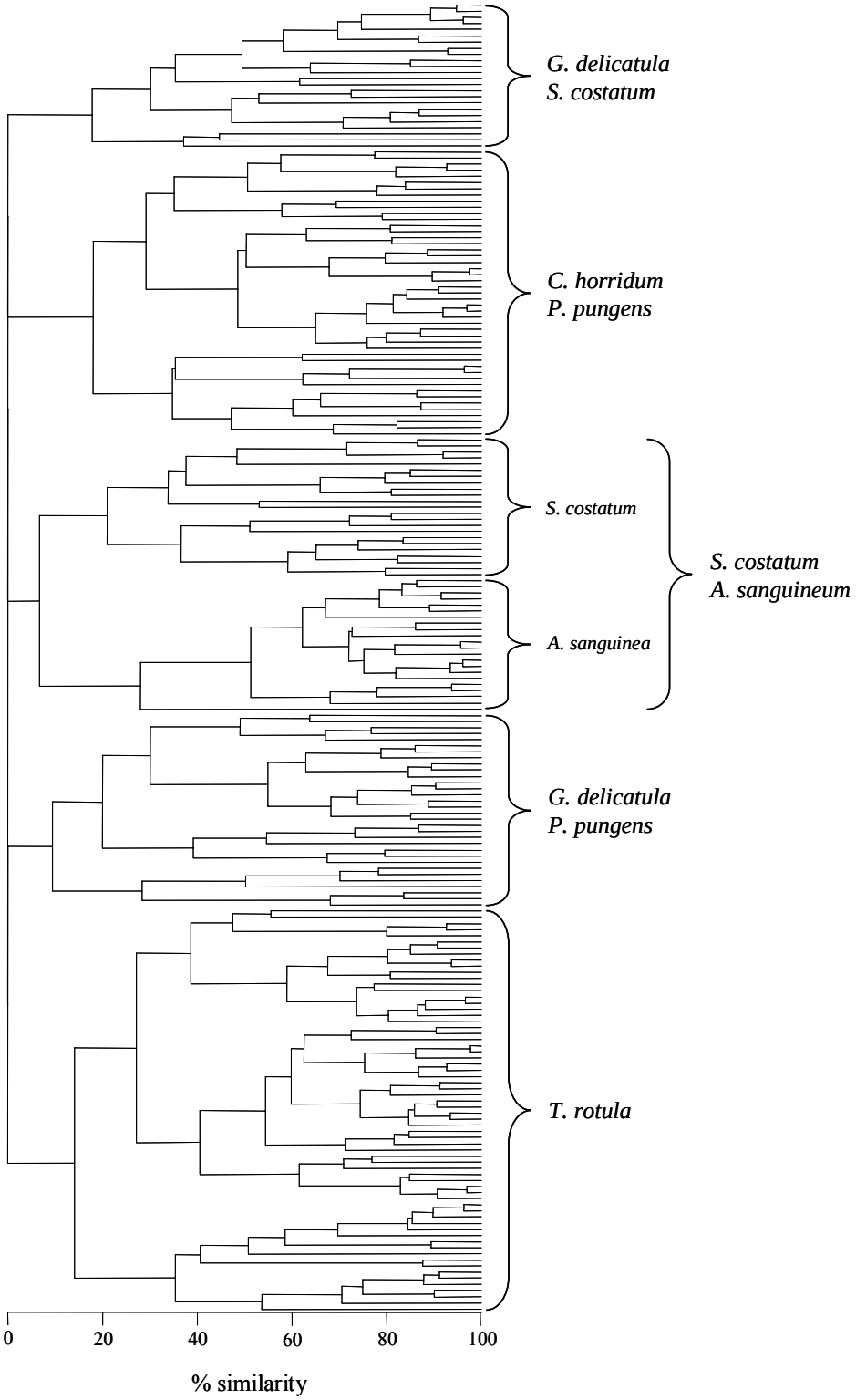

Figure 4: Cluster analysis (complete linkage) based on Bray-Curtis similarities of RISA fingerprints of bacterial communities of different microalgal cultures (including replicate batch cultures for each species, two sample fractions and five sampling dates).

MDS plots of single cultures revealed different patterns of bacterial communities over the course of the incubation (Fig. 5), although in most cases no clear trend or succession could be

observed. As an exception, *G. delicatula* displayed a pronounced succession in the total bacterial community with completely separate free-living and attached communities in week eight as indicated by the arrows in Fig. 5. In contrast the bacterial community of *A. sanguinea* displayed a single cluster with an outlier (week one) and generally no successional trends were visible (as indicated by the closed circle in Fig. 5).

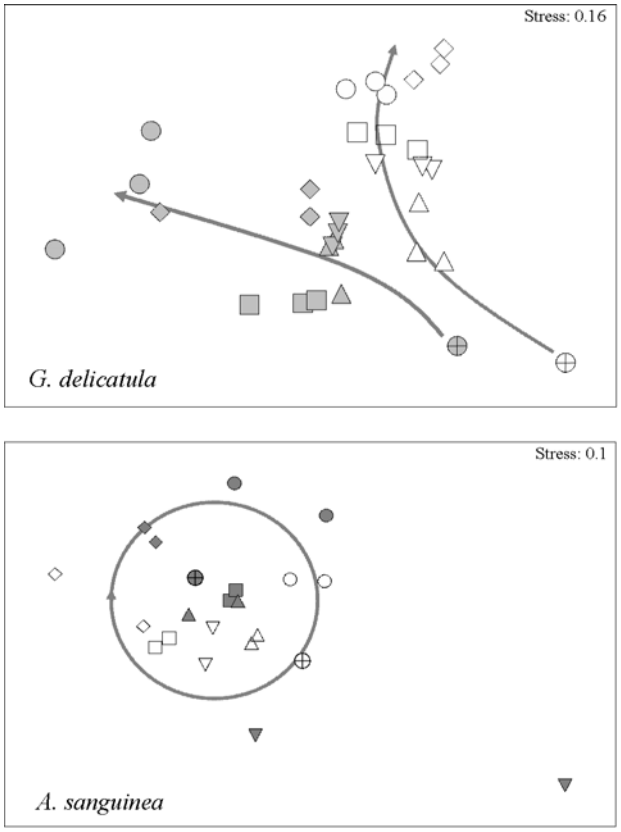

Figure 5: MDS plots based on Bray-Curtis similarities of RISA fingerprints of bacterial communities of *G. delicatula* and *A. sanguinea* cultures. Samples: Initial inoculum community (⊕), week 1 (▽), week 2 (△), week 3 (□), week 4 (◇), week 8 (○). Filled symbols indicate sample fraction < 3 µm > 0.2 µm (free-living bacteria), open symbols indicate sample fraction > 3 µm (attached bacteria).

Linking community structure to abiotic and biotic variables. Using the PRIMER subroutine RELATE, only weak relationships between the matrices of Bray Curtis similarities and the normalised Euclidean distance of abiotic and biotic variables were detected (Tab. 2). The analysis of the diatom and dinoflagellate associated communities related with the respective variables revealed a matching coefficient ρ_m of 0.172 for the diatom cultures and ρ_m of 0.281 for the dinoflagellate cultures indicating little relation. In spite of these low coefficients BIO ENV analyses were performed to indicate the influence of individual variables or a combination of variables. Highest rank correlations were found between diatom community data and the variables phosphate and living algal cells, with $\rho = 0.235$, whereas

algal fitness, nitrite, and phosphate related to dinoflagellate community data revealed highest rank correlations with $\rho = 0.323$ (Tab. 2).

Table 2: RELATE and BIO ENV statistics for relatedness of samples' and variables' similarity

COMPARISON	RELATE			BIOENV	
	statistic ρ_m	Permutations with scores $\geq R$	*P*-value	Maximum ρ	Combination of variables contributing to maximum ρ
Associated bacteria in diatom cultures and variables	0.172	0 out of 999	0.001	0.235	P, AL
Associated bacteria in dinoflagellate cultures and variables	0.281	0 out of 999	0.001	0.323	F, NO_2, P

NO_2 = nitrite, P = phosphate, AL = algal cells living, F = yield photosystem II

Phylogenetic analysis of communities. In order to identify the most prominent phylotypes in the different algal cultures, sequence analyses from excised DGGE bands were carried out.

Sequence data generally revealed the presence of two phyla of *Bacteria*. Most sequences were related to the *Alpha* - and *Gammaproteobacteria*. Additionally, we found members of the *Flavobacteria* -*Sphingobacteria* group within the *Bacteroidetes* phylum. Closest relatives of the sequenced phylotypes detected with a BLAST analysis are listed in Tab. 3.

The results revealed a few close matches with 98-99 % similarity to bacterial 16S rRNA gene sequences in the GenBank.

Overall, 43 % and 37 % of the phylotypes were assigned to the *Alpha* - and *Gammaproteobacteria*, respectively. 16 % of the phylotypes were assigned to the *Flavobacteria* group. We found one phylotype affiliated to Gram-positive bacteria in the culture of *S. costatum* which is closely related to *Bacillus* (G_245, AY907318). DNA of plastidal origin (chloroplasts) accounted for 2.5 % of the sequenced bands.

A neighbour joining tree of the *Alphaproteobacteria* revealed that the majority of sequences belonged to the *Roseobacter* clade (88.6 %, Fig. 6 A). Only 5.7 % were related to the order *Rhizobiales* or the family *Sphingomonadaceae,* respectively. Within the *Roseobacter* clade most sequences clustered with *Roseobacter sp*. or *Sulfitobacter sp*.. Some sequences assigned

to the *Alphaproteobacteria* were only distantly related to this group (A_071, A_073, A_085, B_007, D_043, E_069, G_221). Some phylotypes were related to the *Alphaproteobacteria* and appeared in more than one culture. The sequences were related to different species of *Roseobacter sp*. and were found in all cultures except for *T. rotula* (spring). Contrastingly, two sequences obtained from the cultures of *T. rotula* (summer) and *S. costatum* clustered with a sequence of a cultured bacterium associated with the toxic dinoflagellate *Prorocentrum lima,* namely *Roseobacter sp*. PRLIST02 (Fig. 6A). Several sequences obtained from the culture of *A. sanguinea* clustered with *Roseobacter sp*. RED-1 (AY136122), an isolate obtained from the Gulf of Eilat (Pinhassi & Berman, 2003). From all cultures sequences related to *Sulfitobacter sp*. were obtained whereas close matches were found especially with *Sulfitobacter pontiacus* in cultures of *S. costatum* and *P. pungens*. Most of the members of the *Alphaproteobacteria* belonged solely to the fraction of free-living bacteria (I_228, I_237, J_285, J_286, J_289, J_290). However, one phylotype assigned to the group of *Alphaproteobacteria* was found only as attached in the culture of *G. delicatula* (B_007).

Gene sequences of the *Gammaproteobacteria* were mainly assigned to two clusters, the *Alteromonadales* (53 %) and *Oceanospirillales* (47 %, Fig. 6 B).

In the family of *Alteromonadales* the phylotype associated with the culture of the dinoflagellate *A. sanguinea* was only detected in the fraction of the free-living bacteria. However, within the *Gammaproteobacteria* some phylotypes were also found exclusively within the fraction of attached bacteria (B_003, D_034, E_054, J_253).

Phylotypes related with *Oceanospirillales* were mainly derived from cultures of *C. horridum* and *P. pungens*. Sequences related to *Halomonas sp*. were obtained from the cultures of *T. rotula* (spring and summer) and *P. pungens*. Several sequences derived from the microalgal cultures of *G. delicatula*, *T. rotula* (summer), *S. costatum* and *A. sanguinea* clustered with *Alteromonas sp.* or *Glaciecola sp*.. Within the *Flavobacteria - Sphingobacteria* group all sequences belonged to the family of *Flavobacteriaceae* (Fig. 6 C). A cluster of sequences obtained from the cultures of *G. delicatula*, *P. pungens* and *S. costatum* was related to *Gelidibacter sp*. whereas several bacterial sequences from the culture of *C. horridum* clustered with an uncultured marine bacterium (AJ298376, Fig. 6 C). It should be pointed out that *T. rotula* (summer) and *A. sanguinea* harboured no members of the *Flavobacteria - Sphingobacteria* group. Some members of the family of *Flavobacteriaceae* belonged solely to the fraction of attached bacteria (A_016, G_158, I_183, I_189 and I_200) whereas no phylotype assigned to the *Flavobacteriaceae* was detected exclusively in the fraction of the free-living bacteria.

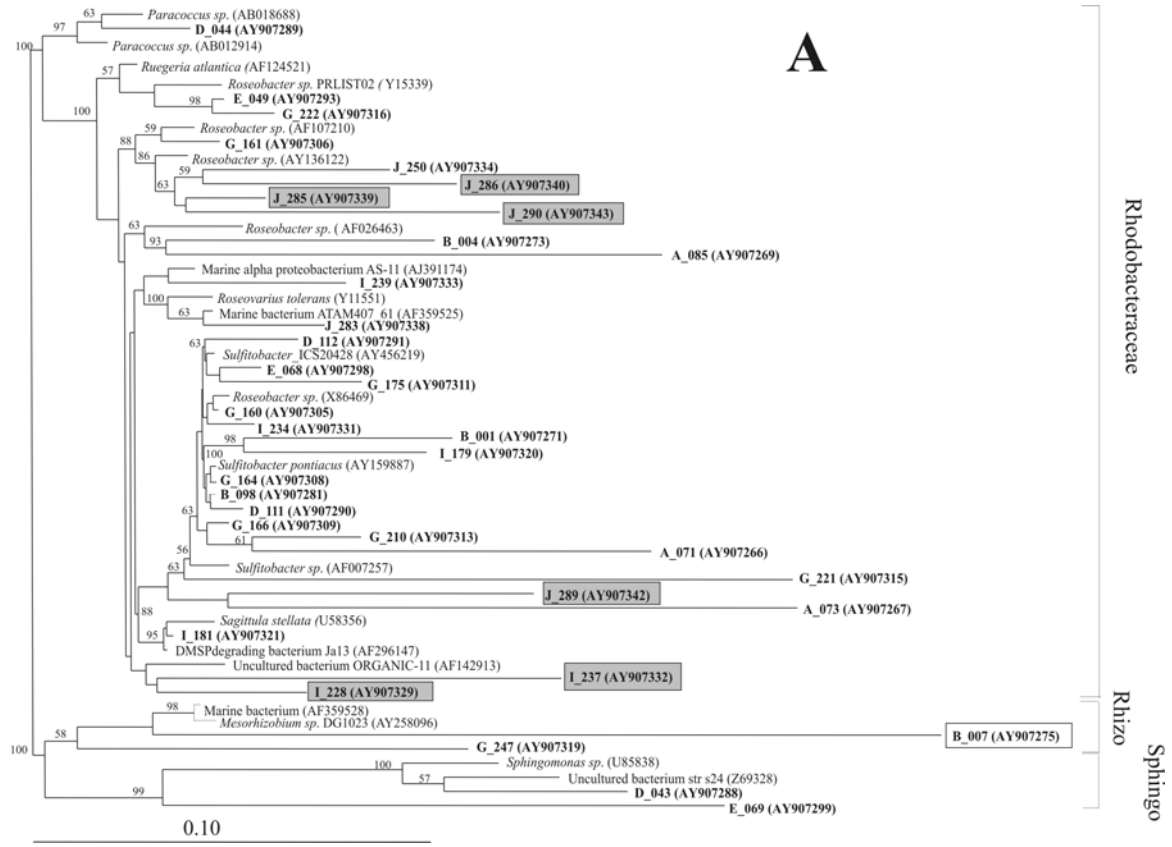

Figure 6: Phylogenetic trees of *Alpha* - and *Gammaproteobacteria* (A, B) and *Flavobacteriaceae* (C). Sequences obtained in this study are displayed in bold. Phylotypes appearing only in one fraction are shown in boxes: open boxes: attached, filled boxes: free-living bacteria; GenBank accession numbers are given in parentheses. Bootstrap values above 50 % are displayed. Rhizo = *Rhizobiales*; Sphingo = *Sphingomonadaceae*

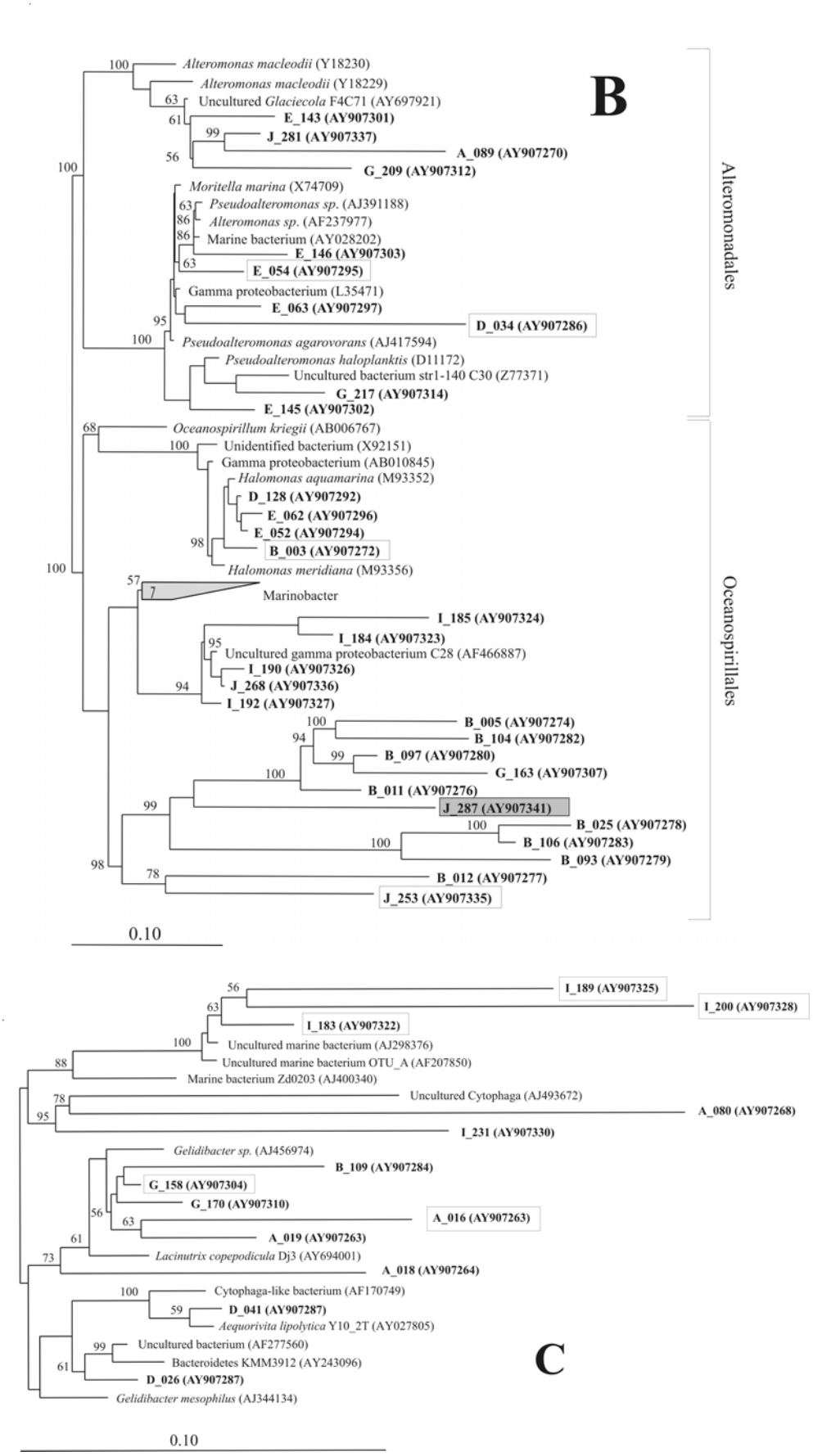

Figure 6 continued

Table 3: Relatedness of microalgae associated bacteria to known organisms

Alga	DGGE band	fraction	Phylogenetic group	Closest relative	Simi-larity [%]	Based positions compared	Accession number of closest relative
G. delicatula	A_071	Free*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	87	512	**AY159887**
	A_073	Free*	*Alphaproteobacteria*	Arctic sea ice bacterium ARK9994	83	483	**AF468380**
	A_085	Free*	*Alphaproteobacteria*	*Roseobacter sp.* PIC-68	90	455	**AJ534238**
	A_089	Free*	*Gammaproteobacteria*	Marine bacterium ATAM407_18	83	494	**AF359529**
	A_016	Att	*Bacteroidetes*	*Bacteroidetes* bacterium KMM 3906	89	469	**AY521224**
	A_018	Att*	*Bacteroidetes*	*Lacinutrix copepodicola*	87	516	**AY694001**
	A_019	Att*	*Bacteroidetes*	*Bacteroidetes* bacterium PI_4s2d	97	425	**AY580579**
	A_080	Free*	*Bacteroidetes*	*Gelidibacter mesophilus*	85	459	**AJ344134**
P. pungens	B_001	Att*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	89	487	**AY159887**
	B_004	Att*	*Alphaproteobacteria*	*Roseobacter sp.* RED 1	89	443	**AY136122**
	B_005	Att*	*Alphaproteobacteria*	*Roseobacter sp.* PIC-68	76	470	**AJ534238**
	B_007	Att*	*Alphaproteobacteria*	*Mesorhizobium sp.*	83	468	**AY258096**
	B_011	Att*	*Alphaproteobacteria*	*Roseobacter sp.* RED 1	94	470	**AY136122**
	B_097	Free*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	95	468	**AY159887**
	B_098	Free*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	99	463	**AY159887**
	B_104	Free*	*Alphaproteobacteria*	*Roseobacter sp.* KT0917	91	455	**AF173972**
	B_003	Att	*Gammaproteobacteria*	*Halomonas venusta*	91	456	**AJ306894**
	B_012	Att*	*Gammaproteobacteria*	*Pseudomonas sp.* NUSTO3	85	453	**AY293865**
	B_025	Att*	*Bacteroidetes*	Bacterium DG1025	94	472	**AY258129**
	B_093	Free*	*Bacteroidetes*	Bacteroidetes bacterium R43	93	452	**AF539755**
	B_106	Free*	*Bacteroidetes*	Bacterium DG1025	97	446	**AY258129**
	B_109	Free*	*Bacteroidetes*	Uncultured Bacterium SIC.B8236	94	509	**AF277562**
T. rotula spring	D_043	Att*	*Alphaproteobacteria*	Arctic sea ice bacterium ARK9996	94	532	**AF468381**
	D_044	Att*	*Alphaproteobacteria*	*Paracoccus sp.* MBIC4017	97	491	**AB025188**
	D_111	Free*	*Alphaproteobacteria*	*Sulfitobacter sp.* 4318-8/2	97	495	**AJ542658**
	D_112	Free*	*Alphaproteobacteria*	*Sulfitobacter sp.* ICS20428	96	455	**AY456219**
	D_034	Att	*Gammaproteobacteria*	*Pseudoalteromonas sp.* V4.BO.24	82	444	**AJ244742**
	D_128	Free*	*Gammaproteobacteria*	*Halomonas venusta*	98	485	**AJ306894**
	D_026	Att*	*Bacteroidetes*	*Bacteroidetes* bacterium KMM 3912	95	535	**AY243096**
	D_041	Att*	*Bacteroidetes*	*Aequorivita lipolytica*	97	530	**AY027805**
T. rotula summer	E_049	Att*	*Alphaproteobacteria*	*Ruegeria atlantica*	95	516	**AF124521**
	E_068	Att*	*Alphaproteobacteria*	*Sulfitobacter sp.* ICS20428	97	454	**AY456219**
	E_069	Att*	*Alphaproteobacteria*	Arctic sea ice bacterium ARK10031	87	481	**AF468357**
	E_052	Att*	*Gammaproteobacteria*	*Halomonas venusta*	98	442	**AJ306894**
	E_054	Att	*Gammaproteobacteria*	*Alteromonas sp.* MS23	94	525	**AF237977**
	E_062	Att*	*Gammaproteobacteria*	*Halomonas sp.*	97	491	**AB104435**
	E_063	Att*	*Gammaproteobacteria*	*Pseudoalteromonas sp.*	95	481	**AY305857**
	E_143	Free*	*Gammaproteobacteria*	*Glaciecola sp.* F4C71	94	457	**AY697921**
	E_145	Free*	*Gammaproteobacteria*	*Pseudoalteromonas sp.* ANT9388	95	502	**AY167333**
	E_146	Free*	*Gammaproteobacteria*	*Pseudoalteromonas sp.* BA1	91	485	**AF323549**
	E_138	Free*	chloroplast	*Thalassiosira plastid*	98	531	**AJ536458**

* OTU is also found in the other fraction

Table 3 continued: Relatedness of microalgae associated bacteria to known organisms

Alga	DGGE band	fraction	Phylogenetic group	Closest relative	Simi-larity [%]	Based positions compared	Accession number of closest relative
S. costatum	G_160	Att*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	99	517	**AY159887**
	G_161	Att*	*Alphaproteobacteria*	*Roseobacter sp.*	96	463	**AF107210**
	G_163	Att*	*Alphaproteobacteria*	Arctic sea ice bacterium ARK9994	92	460	**AF468380**
	G_164	Att*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	100	479	**AY159887**
	G_166	Att*	*Alphaproteobacteria*	*Sulfitobacter sp.* BIO-7	97	465	**AJ532580**
	G_175	Att*	*Alphaproteobacteria*	*Sulfitobacter sp.* ICS20428	95	434	**AY456219**
	G_210	Free*	*Alphaproteobacteria*	*Sulfitobacter sp.* BIO-7	94	496	**AJ532580**
	G_221	Free*	*Alphaproteobacteria*	*Sulfitobacter sp.* ICS20428	89	465	**AY456219**
	G_222	Free*	*Alphaproteobacteria*	Uncultured marine bacterium D009	95	463	**AF177551**
	G_247	Att*	*Alphaproteobacteria*	*Rhodovolum iodosum*	88	430	**Y15011**
	G_209	Free*	*Gammaproteobacteria*	*Glaciecola sp.* F4C71	91	475	**AY697921**
	G_217	Free*	*Gammaproteobacteria*	*Pseudoalteromonas sp.* SM9913	90	441	**AY305857**
	G_158	Att	*Bacteroidetes*	*Lacinutrix copepodicola*	96	517	**AY694001**
	G_170	Att*	*Bacteroidetes*	*Lacinutrix copepodicola*	93	470	**AY694001**
	G_241	Att	chloroplast	*Skeletonema plastid*	93	473	**X82154**
	G_245	Att	*Firmicutes*	*Bacillus sp.* LMG 20243	92	481	**AJ316317**
C. horridum	I_179	Att*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	95	433	**AY159887**
	I_181	Att	*Alphaproteobacteria*	DMSP-degrading marine bacterium JA13	98	459	**AF296147**
	I_228	Free	*Alphaproteobacteria*	DMSP-degrading marine bacterium JA13	91	470	**AF296147**
	I_234	Free*	*Alphaproteobacteria*	*Sulfitobacter pontiacus*	98	483	**AY159887**
	I_237	Free	*Alphaproteobacteria*	*Roseobacter sp.* TM1042	88	507	**AY332663**
	I_239	Free*	*Alphaproteobacteria*	*Roseobacter sp.* TM1042	94	520	**AY332663**
	I_184	Att*	*Gammaproteobacteria*	*Oceanospirillum sp.* MED92	93	455	**AY136116**
	I_185	Att*	*Gammaproteobacteria*	*Gammaproteobacteria* C28	90	450	**AF466887**
	I_190	Att*	*Gammaproteobacteria*	*Gammaproteobacteria* C28	97	454	**AF466887**
	I_192	Att*	*Gammaproteobacteria*	*Oceanospirillum sp.* MED92	97	440	**AY136116**
	I_183	Att	*Bacteroidetes*	Uncultured marine bacterium BY-65	97	496	**AJ298376**
	I_189	Att	*Bacteroidetes*	Uncultured marine bacterium BY-65	94	428	**AJ298376**
	I_200	Att	*Bacteroidetes*	*Tenacibaculum sp.* T-6	87	463	**AY573525**
	I_231	Free*	*Bacteroidetes*	*Gelidibacter mesophilus*	85	488	**AJ344134**
A. sanguinea	J_250	Att*	*Alphaproteobacteria*	*Roseobacter sp.* RED-1	92	408	**AY136122**
	J_283	Free*	*Alphaproteobacteria*	*Roseobacter sp.* TM1042	96	461	**AY332663**
	J_285	Free	*Alphaproteobacteria*	Uncultured bacterium JH10_C12	94	503	**AY568770**
	J_286	Free	*Alphaproteobacteria*	*Roseobacter sp.* RED-1	94	412	**AY136122**
	J_287	Free	*Alphaproteobacteria*	*Roseobacter sp.* RED-1	88	465	**AY136122**
	J_289	Free	*Alphaproteobacteria*	*Roseovarius sp.* DFL-35	89	448	**AJ534219**
	J_290	Free	*Alphaproteobacteria*	*Roseobacter sp.* RED-1	90	491	**AY136122**
	J_253	Att	*Gammaproteobacteria*	*Gammaproteobacteria* C28	83	468	**AF466887**
	J_268	Free*	*Gammaproteobacteria*	*Gammaproteobacteria* C28	99	466	**AF466887**
	J_281	Free*	*Gammaproteobacteria*	*Pseudoalteromonas sp.* SCc-7	95	449	**AJ456960**

* OTU is also found in the other fraction

Discussion

In this study, it was analysed whether freshly isolated microalgae harbour specific bacterial communities in their "phycosphere". The analysis of the bacterial communities by RISA and DGGE with subsequent phylogenetic analysis coupled with the assessment of several biotic and abiotic parameters describing the growth phases of microalgae or the depletion of anorganic nutrients was conducted in order to get more information on the interaction of bacteria and microalgae.

RISA was used as a fingerprinting method for the differentiation of the total bacterial community into different groups of populations (Ranjard *et al.*, 2000b). The specificity of associated bacterial communities differed depending on algal species as shown by ANOSIM (Tab. 1). Our results do not generally confirm the observations of Grossart *et al*. (2005) and Schäfer *et al*. (2002) who stated that microalgae harbour specific bacterial communities. The picture is more complex. In our study, where the similarities of the associated bacterial communities of *T. rotula, P. pungens*, *C. horridum*, *G. delicatula*, *S. costatum* and *A. sanguinea* were studied, it could be shown that specificity varied to different degrees. We showed that when comparing the microalgal species, *T. rotula* harbours a specific bacterial community in contrast to the communities of the other studied microalgal species which share bacterial similarities to different extents. Interestingly, according to the MDS ordination and the ANOSIM results, the bacterial communities of the two strains of *T. rotula* isolated in spring and summer 2002 were also separated. It must, however, be taken in consideration that the examined microalgae from different seasons might be genetically different (Orsini *et al.*, 2002; Rynearson & Armbrust, 2004). The morphology of cells of both *T. rotula* cultures matched those of known *Thalassiosira rotula* species (Savin *et al.*, 2004).

The question, as to whether the observed differences in the "phycosphere" bacterial communities of the respective algal cultures are more related to the environmental conditions at the time of isolation, or to genetic differences of *T. rotula* populations, needs to be addressed in future.

Generally, the composition of the associated bacterial communities could not be correlated with the growth or decline of the algae or the depletion of anorganic nutrients by using the RELATE and BIO ENV routines. This would indicate that environmental factors alone are not necessarily explanatory. None of the measured variables could explain the shifts in the associated communities satisfactorily. It is obvious that factors other than the anorganic nutrients were responsible for shaping the communities in our experiments. It can be assumed

that other factors are directly linked to the microalgae and could include the quality or quantity of exudates. Increasing similarities of attached communities during incubation of the cultures of *T. rotula* (spring and summer) and *A. sanguinea* might support this hypothesis. With inoculation, when microalgal cells including their associated bacteria are transferred to fresh media, thus the effect of algal metabolites might be reduced. This could explain the difference in community composition of the inoculum compared to the first sample after one week of incubation in fresh media. This effect might be comparable to the shifts occurring during confinement, which has already been described for natural bacterial communities (Ferguson *et al.*, 1984; Schäfer *et al.*, 2000).

Even though antibacterial metabolites are produced by some microalgae (Naviner *et al.*, 1999) which possibly inhibit certain bacterial species, the effect of algal exudates acting as organic nutrients is possibly much more pronounced in promoting bacterial populations and therefore "shaping" a community. This assumption is supported by a study of Myklestad (1995) analysing the composition of exudates, which differed in several microalgal cultures especially with regard to polysaccharides. Nevertheless, if microalgae share similarities in the composition of exudates they might also share similarities in the composition of associated bacterial communities. This question needs to be addressed in future.

In addition to the RISA fingerprinting followed by statistical analysis, DGGE and a phylogentic analysis of excised bands was performed in order to identify bacterial phylotypes and populations representing the bacterial communities of the different microalgae.

Sequencing of DGGE bands revealed that the *Alpha* - and *Gammaproteobacteria* as well as members of the *Flavobacteria-Sphingobacteria* group within the *Bacteroidetes* phylum were the predominant groups associated with the examined microalgal cultures. Bacteria of these groups have already been described in other investigations dealing with the interaction of bacteria and microalgae (Bidle & Azam, 2001; Hold *et al.*, 2001; Wichels *et al.*, 2004; Grossart *et al.*, 2005). Grossart *et al*. (2005) inoculated quasi axenic diatom cultures of *T. rotula* and *S. costatum* with a natural bacterial community and analysed the resulting shifts in bacterial community structure. They found an association of microalgae with *Alpha* - and *Gammaproteobacteria* as well as members of the *Flavo-Sphingobacteria* group and stated that the associated bacterial community of microalgae might be distinct depending on the microalgae. This might generally be true for certain microalgal species, we have found that even the same algal species can harbour different bacterial populations as shown for *T. rotula* isolated in spring and in summer (Fig. 3 and Tab. 3).

Some phylotypes occurring in several cultures have already been described in association with particles (Hold *et al.*, 2001; Schäfer *et al.*, 2002; Green *et al*., 2004; Grossart *et al.*, 2005) and it can be assumed that certain bacteria are able to live associated with microalgae but also on detrital particles. These bacteria might be adapted to opportunistic colonisation of any kind of particle. This might be true for the *Alphaproteobacteria* in particular, the most abundant group obtained in this study (47% of all phylotypes) with two phylotypes predominantly related to *Sulfitobacter sp*. and *Roseobacter sp*.. Within this group members related with *Roseobacter litoralis, Ruegeria algicola, Sulfitobacter pontiacus* and *Sagittula stellata* were identified which have previously been described for the bacterioplankton of Helgoland Roads (Eilers *et al*., 2001). Sequences mainly related to *Roseobacter sp*. and *Sulfitobacter sp*. were obtained from all cultures, except for the culture of *T. rotula* (spring) where no sequences related to *Roseobacter sp*. were present. In the literature, members of *Alphaproteobacteria* have been described as being associated with the dinoflagellate *Gymnodinium catenatum* (Green *et al*., 2004) or with *Alexandrium sp.* (Hold *et al.*, 2001). Moreover, species of *Roseobacter sp.* and *Sagittula stellata* are known to live as chemoheterotrophic organisms hydrolysing cellulose and lignin (Buchan *et al.*, 2000; Dang & Lovell, 2000). Species of *Sulfitobacter sp*. have also been found in association with diatom species (Schäfer *et al.*, 2002). Hence, our findings are confirmed by the study of Grossart *et al.* (2005). *Sulfitobacter pontiacus* is known to play a pivotal role in the sulfur cycle because of its ability to oxidise sulfite (Gonzáles & Moran, 1997) and thus the specific microenvironment provided by microalgae might be a suitable habitat for species like *Sulfitobacter pontiacus*.

37 % of the phylotypes we found belonged to the *Gammaproteobacteria*. Members of this group have also been found in several microalgal cultures (Prokiç *et al.*, 1998; Hold *et al.*, 2001). Other studies analysing the association of bacteria and microalgae also reported the occurrence of *Gammaproteobacteria.* It has been demonstrated that *Alteromonas macleodii* was associated with *Gymnodinium catenatum* (Green *et al*., 2004) while other studies described the association of *Alteromonas macleodii* and species of *Pseudoalteromonas* with *Noctiluca scintillans* (Seibold *et al.*, 2001) and also with the toxic dinoflagellate *Prorocentrum lima* (Prokiç *et al.*, 1998). In a study of Stewart *et al.* (1997) species like *Alteromonas sp*. were identified to stimulate algal growth and to be beneficial for microalgae such as *Pseudonitzschia multiseries*. Concerning the genus *Pseudoalteromonas*, the species *P. agarovorans* degrades algal polysaccharides such as agar, alginate and carrageenan. Further, it can produce lipase, caseinase and β-galactosidase (Romanenko *et al.*, 2003) and is

thought to be important for the production of POM (particulate organic matter). Species related to *Halomonas sp*. have also already been isolated from the bacterioplankton of Helgoland Roads (Eilers *et al.*, 2000). Hence, it is remarkable that one of the phylotypes (B_003) related to *Halomonas sp*. was only found in the fraction of attached bacteria.

Only a minor fraction of the sequences (16 %) were associated with members of the *Flavobacteria-Sphingobacteria* group within the *Bacteroidetes* phylum which have already been shown to be associated with phytoplankton (Bidle & Azam, 2001; Brown & Bowman, 2001; Schäfer *et al.*, 2002; Wichels *et al.*, 2004; Grossart *et al.*, 2005). In this study, only *Flavobacteriaceae* were identified. Other members of the *Bacteroidetes* phylum have been found in the study of Schäfer *et al*. (2002). A member of *Gelidibacter sp*., for instance, occurred in the cultures of *G. delicatula*, *P. pungens* and *S. costatum* while other sequences belonging to the family of *Flavobacteriaceae* could not be obtained in multiple cultures (Fig. 6 C). Nevertheless, several of these species potentially play an important role in the association of bacteria and microalgae. *Aequorivita lipolytica*, for example, has already been described as being associated with *Gymnodinium catenatum* (Green *et al*., 2004). This chemoheterotrophic bacterium produces lipase (Bowman & Nichols, 2002) which probably degrades fatty acids of the host cell.

With respect to the different fractions of attached and free-living bacteria, it has been observed that the fractions were clearly separated in some cultures. It is suggested that those bacteria termed free-living originally derived from the attached community and that these might be able to live in the ambient environment. Additionally, we found differences between the three phylogenetic groups due to the fractions. Most of the phylotypes found only in the fraction of attached bacteria belong to the group of *Flavobacteriaceae* (38.5 % of *Flavobacteriaceae*) whereas a proportion of 13.3 % of *Gammaproteobacteria* were ascribed only to this group. Within the *Alphaproteobacteria* only one phylotype was solely found in the fraction of attached bacteria (2.9 %). It is assumed that members of the *Flavobacteria* clade but also *Gammaproteobacteria* play an important part in the interaction of microalgae and bacteria and that they are possibly more specific regarding the choice of habitat than *Alphaproteobacteria*. Generally, the three phylogenetic groups found in this study, *Alpha* - and *Gammaproteobacteria* as well as *Flavobacteriaceae* are known to degrade dissolved and particulate organic matter (Cottrell & Kirchman, 2000). Coherence was also found for silicate dissolution by members of the *Gammaproteobacteria* and *Flavobacteria* - *Sphingobacteria* (Bidle *et al.*, 2002). Therefore, it is assumed that members of these groups participate in biogeochemical cycling and play an important part in the microbial loop. It can be suggested

that the physiological flexibility of the attached bacteria may support the colonisation of the microalgal environment. Further studies should take into account that associated bacteria might belong to certain ecological guilds or even might be functional redundant to sustain stability of specific ecosystem processes (Gaston, 1996).

Conclusion

Regarding our results, it should not be generalised, that the compositions of the bacterial communities are strictly species-specific for microalgae. Although the phylogenetic analysis revealed that most phylotypes were already described for the "phycosphere", the specific composition of bacterial communities can still not be exactly predicted for specific microalgal species. In future experiments, the fundamental niche(s) of those bacteria benefiting from phycosphere(s) should be defined to elucidate the structure of bacterial communities in the this niche(s). Here, the importance of factors like the composition of exudates is apparent. Further studies are necessary in order to examine the interaction of bacteria and microalgae and to specify parameters which favour the association of specific bacteria with specific algae.

We assume, that our experiments provide first information on the *in situ* bacterial community associated with the phytoplankton cells since algae were freshly isolated from the environment and not provided by culture collections.

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Bacteria in the phycosphere of marine diatoms: A closer look

Abstract

Different experiments were conducted to analyse the level of specificity of particular bacterial communities associated with diatoms. The design of treatments allowed the analysis of resource competition between bacterial communities and the effects of diatom exudates on the community structure. We hypothesised that species-specific associations of bacteria and the respective host alga would lead to communities similar to the originally-associated community and we hypothesised that bacteria associated with different diatom species would be suppressed. Interestingly, competition of associated bacterial populations could not be observed in any treatment. Furthermore, the influence of exudates on the composition of the associated bacterial communities was seen to be low. A separation according to attached and free-living bacteria could be observed in the treatments.

Summarising our results: we could not detect species-specific associations of bacteria and the studied diatom species. Bacteria associated with the investigated diatoms might be generally adapted to a "diatom" environment but not to a certain species. Hence, it could be hypothesised that associated bacterial species belong to ecological guilds.

Introduction

The association of distinct bacterial communities and microalgae has been shown in several studies (Schäfer *et al.*, 2002; Grossart *et al.*, 2005; Jasti *et al.*, 2005). Schäfer *et al*. (2002) found distinct "satellite bacteria" in microalgal cultures whereas Grossart *et al.* (2005) could demonstrate that specific bacterial groups from a natural community colonised the phycosphere of previously quasi axenic diatoms. Generally, studies on the process of colonisation revealed that different bacterial phylotypes live on the phytoplankton cells compared to the surrounding medium (Kogure *et al.*, 1982; Vaqué *et al.*, 1990). The findings of a general specificity of bacteria associated with algae (Hold *et al.*, 2001; Grossart *et al.*, 2005; Jasti *et al.*, 2005) led to the assumption that the microalgae might have a selecting influence. A close linkage of bacterioplankton and phytoplankton especially for bacteria living attached to phytoplankton cells could be demonstrated by Rooney-Varga *et al*. (2005). Additionally, a study conducted in the North Sea demonstrated a specific influence of

phytoplankton species on members of the free-living community (Chapter V, this volume). Although the results of culture experiments and *in situ* analyses led to the assumption of a strong link between the bacterioplankton and the phytoplankton, it still remains unclear which factors control the association. Generally, the habitat of phytoplankton associated bacteria has been depicted by the "phycosphere" concept defining the zone around algal cells where bacteria feed on extracellular products of the algae (Bell & Mitchell, 1972). An uptake of extracellular products of *Skeletonema costatum* by bacteria was already demonstrated by Bell *et al*. (1974). Phytoplankton cells excrete organic compounds including high proportions of carbohydrates (Myklestad, 1995) contributing to the base of the microbial food web (Lancelot, 1983). Therefore, bacteria living attached to algal surfaces consuming extracellular products participate in biogeochemical cycling and play an important part in the microbial loop (Azam, 1998).

Considering the phycosphere to be a habitat occupied by different bacterial populations it is assumed that the bacteria which have the ability to live in this habitat compete with other bacteria or they coexist in the phycosphere.

In this study we analysed to which extent the structure of a bacterial community associated with one diatom species is influenced by the phycosphere of a different diatom species. Due to the results of culture experiments conducted earlier (Chapter II, this volume), we assume that some of the associated bacterial species or populations might belong to ecological guilds or might be functionally redundant.

This hypothesis was tested by different experiments. On the one hand, resource competition experiments were conducted to analyse if diatom-associated bacterial communities compete for nutrients and if certain populations are eliminated by competitive exclusion. In addition, it was tested, if diatom exudates shape the bacterial community structure. Diatom exudates were obtained by filtration and diatom-associated bacterial communities as well as a seawater community were added to different exudates. It was suggested that the treatments would lead to communities dissimilar to the inoculum community if diatom exudates would shape the bacterial community structure.

Methods

Algal cultures

In 2004, plankton samples from Helgoland Roads (54°11.3' N and 7°54.0' E) were taken using a 20 µm and 80 µm net aboard the research vessel Aade. Single cells were isolated by

micropipetting (Daste, 1983) and passed through several washing steps before they were subsequently cultivated.

The experiments were conducted with cultures of three phytoplankton species isolated in April 2004. These were *Guinardia delicatula* (Cleve) Hasle, *Pseudonitzschia pungens* Grunow and *Thalassiosira rotula* Meunier. Microalgae were incubated as batch cultures (10 l) in Guillard's f/2 medium (Guillard, 1975) at 16°C with 12:12 light-dark cycle at 46,0 µmol photons $m^{-2}sec^{-1}$ until experimental setup.

Experimental design

Resource competition. Algal cultures and one seawater sample (Helgoland Roads) obtained in July 2005 were filtered through 10 µm membrane filters (Millipore, Germany) to remove algal cells and particles. This filtrate served as inoculum. 100 ml of each algal culture and 100 ml of the inoculum were added to 800 ml of Guillard's f/2 medium (Guillard, 1975). 200 ml of the tested algal culture were also added to 800 ml of Guillard's f/2 medium (Guillard, 1975) and served as control. In detail, the culture of *G. delicatula* was added to the filtrate of *T. rotula*, *P. pungens* or the filtrate of the seawater sample whereas 200 ml of this culture served as control. Experiments were also performed with *T. rotula* and *P. pungens* respectively. All experimental treatments were performed in duplicate. Incubation was performed as already described (see above).

Influence of exudates. The exudates of the studied algal cultures were obtained by successive filtration of the diatom cultures in the early stationary phase through 10 µm membrane filters (Millipore), GF/C filters (Whatman, Germany) and 0.2 µm membrane filters (Millipore). A volume of 990 ml of exudate was incubated with 10 ml of inoculum of the respective alga or the seawater sample (see above) obtained by filtration through 10 µm membrane filters (Millipore). The treatment including exudate and inoculum of the same culture served as control. In detail, the exudate of *G. delicatula* was incubated with the inoculum of *T. rotula*, *P. pungens*, the inoculum of the seawater sample as well as with the inoculum of the host alga which served as control. Experiments were also performed with *T. rotula* and *P. pungens* respectively. All experimental treatments were performed in duplicate. Incubation was performed as already described (see above). Sampling was carried out after 7, 14, 21 and 42 days of incubation.

Sampling of biomass and extraction of nucleic acid

A volume of 100 ml of each sample was filtered through 3 µm and 0.2 µm membrane filters (Millipore) in succession. The filtration separated the biomass of attached bacteria detained

by 3 µm filter and free-living bacteria detained by 0.2 µm filter. Filters were stored at -20°C until DNA extraction.

Nucleic acid extraction was performed as described by Wichels *et al*. (2004). Briefly, filters were cut into pieces and transferred to sterile 2 ml vials. Bacterial biomass was resuspended in STE buffer (6.7 % Saccharose, 50 mM Tris, 1 mM EDTA, pH 8) and cell lyses were facilitated by adding lysozyme (2 mg ml^{-1}) and SDS (1 %). DNA extraction was performed using phenol-chloroform-isoamylalkohol (25:24:1). After precipitation of the DNA with isopropanol all of the DNA extracts were finally kept in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored at -20°C until further analysis. These served as template DNA in the PCR. Prior to PCR amplification, the DNA extracts were analysed by agarose gel electrophoresis on 0.8 % agarose gels. After electrophoresis, gels were stained with ethidium bromide (0.5 mg l^{-1}). Imaging was performed with a ChemiDoc XRS System (BioRad, Germany).

Amplification of Ribosomal Intergenic Spacer and RISA

For amplification of the intergenic spacers (IGS) between the 16S and 23S subunits of ribosomal sequences we used the primers S-D-Bact-1522-b-S-20 (5'- TGC GGC TGG ATC CCC TCC TT -3') and L-D-Bact-132-a-A-18 (5'- CCG GGT TTC CCC ATT CGG -3') (Ranjard *et al.*, 2000a; Ranjard *et al.*, 2000b). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf, Germany), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer, Germany), 0.5 µM of each primer, 2 U of Taq DNA Polymerase (Eppendorf) and 5 µl DNA of 0.2 µm filters or 0.5 µl of 3 µm filters. The amplification started with a denaturing step at 95°C for three minutes and 25 cycles at 95°C for one minute, 53°C for one minute and 68°C for one minute followed by 68°C for five minutes for extension. 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, Germany) in 0.5 x TAE buffer. Three lanes were used for 0.1 µg of a 100 bp ladder (invitrogen, Germany) in order to achieve comparability. Electrophoresis was run at 20°C for 18 h at 50 V using a DCode system (BioRad). Gels were stained with SYBRGold as recommended by Molecular Probes (invitrogen). Imaging was performed with a ChemiDoc XRS System of BioRad.

Statistical analysis of RISA profiles

Analysis of RISA profiles was carried out with the BioNumerics 4.5 software package of Applied Maths BVBA (Belgium). Multivariate analysis of fingerprints was performed using

the subroutines ANOSIM and MDS of the PRIMER 5 software suite (PRIMER-E Ltd., UK; Clarke & Warwick, 2001). First, normalisation of gels was performed by the BioNumerics software using 100 bp ladders serving as references in every profile. For sample comparison a band-matching analysis was carried out. Bands were assigned to classes of common bands within all fingerprinting profiles. The procedure included densitometric values of the profiles (Muylaert *et al*., 2002). The resulting band-matching tables were imported into PRIMER. The Bray Curtis similarity of communities was calculated applying square root transformation (Clarke & Warwick, 2001). Statistical analysis was performed with all samples. Ordination of Bray Curtis similarities was carried out by nonmetric multidimensional scaling (MDS) (Yannarell *et al*., 2005). MDS was calculated using 10 random starting configurations of sample points. It was assumed that the final configuration was optimal unless other configurations displayed lower stress levels. To test the hypothesis that the within-group community similarity was greater than among-groups, an analysis of similarity was conducted by the subroutine ANOSIM of the PRIMER software. ANOSIM is a nonparametric technique designed to allow statistical comparison for multivariate data sets in a manner similar to univariate techniques (ANOVA). The null hypothesis applied here is, "that no differences between treatments exist". Testing the hypothesis resulted in a sample statistic R displaying the degree of separation between groups. Complete separation is indicated by R = 1, whereas R = 0 suggests no separation. Having determined R, ANOSIM randomly assigns samples to different groups to generate a null distribution for R (Monte Carlo test, 999 permutations) (Yannarell & Triplett, 2004) to test whether within-group samples were more closely related to each other than would be expected at random. The results of ANOSIM with a significance level of 0.1 % are displayed.

Results

Resource competition

Control experiments were conducted to investigate how the treatment alone affected the bacterial community associated with a specific diatom. Comparison of the controls revealed a general separation of the communities associated with *P. pungens* and *G. delicatula* which was also observed for the associated communities of *T. rotula* and *P. pungens* (Tab. 4). The communities associated with *T. rotula* and *G. delicatula* displayed no clear separation (Tab. 4). Analysing the resource competition of the associated communities of respective cultures, ANOSIM revealed no strong separation of the communities due to the value of the global R (Tab. 5).

Table 4: ANOSIM (Analysis of similarity) statistics for pairwise tests involving comparisons of all controls of competition treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic r	*G. delicatula*	*P. pungens*
P. pungens	0.500	
T. rotula	0.354	0.560

Table 5: ANOSIM (Analysis of similarity) statistics for global tests involving comparisons of all competition treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic r	Seawater sample	*G. delicatula*	*P. pungens*
G. delicatula Treatment Host algae	 0.358 -		
P. pungens Treatment Host algae	 0.115 -	 0.310 0.239	
T. rotula Treatment Host algae	 0.234 -	 0.325 0.249	 0.400 0.239

As shown by MDS, the competing communities associated with *P. pungens* and *G. delicatula* did not develop a community composition similar to the originally associated community or a composition similar to the added bacterial community (Fig. 7 A). This is supported by the R-values obtained by pairwise tests (Tab. 6). MDS displayed a slight separation of the communities associated with *G. delicatula* and *T. rotula.* The structure of the respective community developed in a direction originally associated with the respective alga (Fig. 7 B). A pairwise test revealed a difference between the treatments of *T. rotula* control and *T. rotula* inoculum in the culture of *G. delicatula* (R = 0.509, Tab. 6) indicating that the inoculum of *T. rotula* developed a community similar to that associated with *G. delicatula*. A shift in the community of the inoculum of *T. rotula* towards a community similar to the originally associated community was also observed for the treatment with communities associated with *T. rotula* and *P. pungens*. In this treatment MDS displayed a separation of the control of *T. rotula* but not of any other combination (Fig. 7 C). Additionally, R-values resulting from pairwise tests supported this finding with R = 0.619 (Tab. 6) for the treatment including the

inoculum of *T. rotula* in the culture of *P. pungens* tested against the control of *T. rotula* indicating that the inoculum community of *T. rotula* became dissimilar to the control community of *T. rotula*.

Table 6: ANOSIM (Analysis of similarity) statistics for pairwise tests involving comparisons of all competition treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic R	Competition
P. pungens C ↔ *P. pungens* I in *G. delicatula*	0.375
G. delicatula C ↔ *P. pungens* I in *G. delicatula*	0.238
G. delicatula C ↔ *G. delicatula* I in *P. pungens*	0.315
T. rotula C ↔ *T. rotula* I in *G. delicatula*	0.509
G. delicatula C ↔ *G. delicatula* I in *T. rotula*	0.222
G. delicatula I in *T. rotula* ↔ *T. rotula* I in *G. delicatula*	0.449
T. rotula C ↔ *P. pungens* I in *T. rotula*	0.384
T. rotula C ↔ *T. rotula* I in *P. pungens*	0.619
P. pungens C ↔ *T. rotula* I in *P. pungens*	0.32
P. pungens I in *T. rotula* ↔ *T. rotula* I in *P. pungens*	0.414
T. rotula C ↔ seawater I in *T. rotula*	0.264
G. delicatula C ↔ seawater I in *G. delicatula*	0.423

C: control, I: inoculum

No development towards a specific community composition could be observed in the treatment including the seawater community and the associated community of *P. pungens* as displayed by MDS (Fig. 7 D). This is supported by R-values obtained from pairwise tests (Tab. 6). Although pairwise tests could not show a clear separation of the seawater community and the bacterial communities associated with *T. rotula* or *G. delicatula* (Tab. 6) slight separation was displayed by MDS (Fig. 7 E and F) indicating that the seawater community did not develop a composition similar to the originally associated bacterial community in the cultures of *T. rotula* or *G. delicatula*.

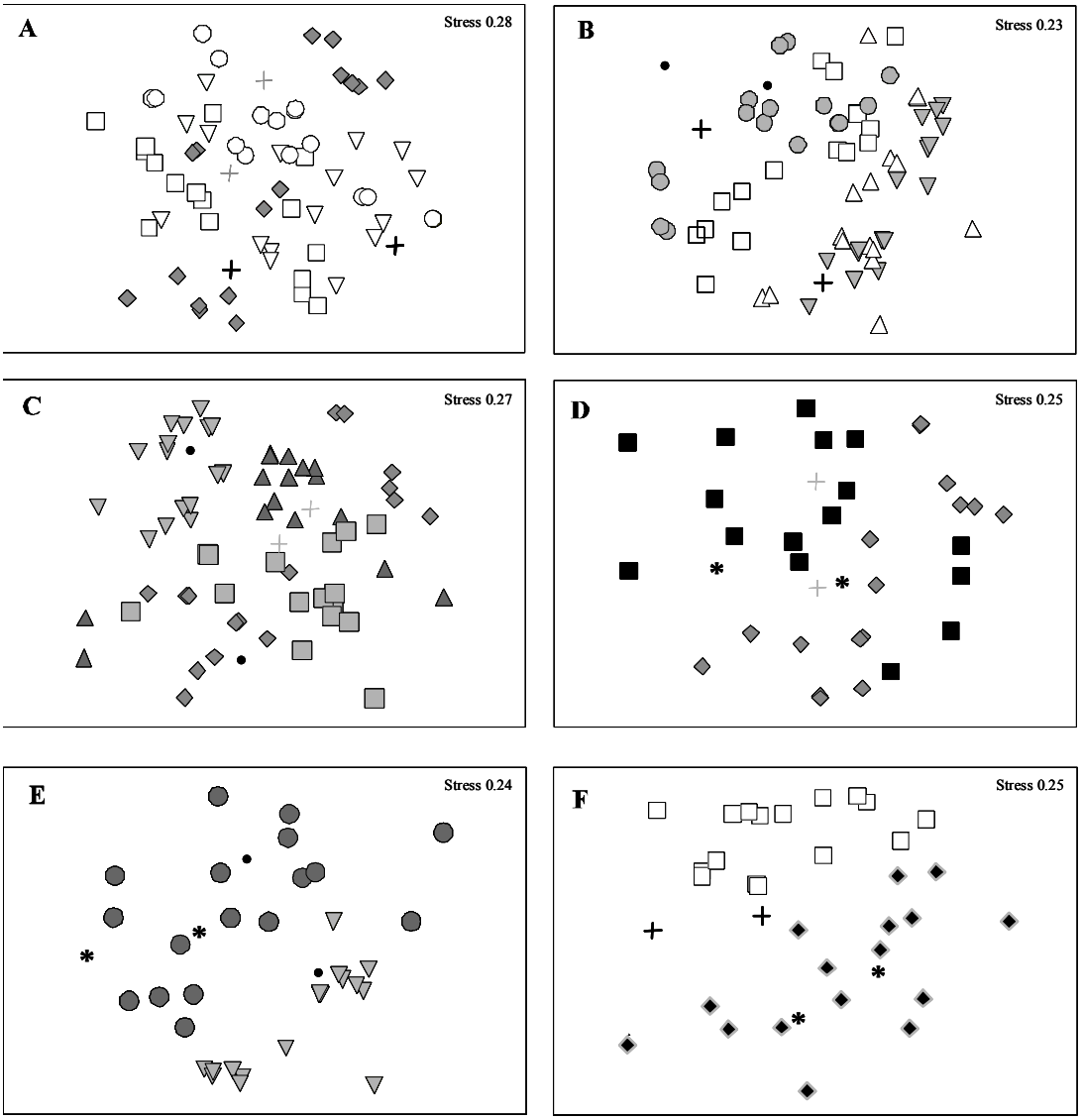

Figure 7: MDS plots based on Bray-Curtis similarities of RISA fingerprints of competing bacterial communities including two replicates, two sample fractions and four sampling dates. A: *P. pungens* and *G. delicatula*, B: *G. delicatula* and *T. rotula*, C: *T. rotula* and *P. pungens*, D: *P. pungens* and seawater community, E: *T. rotula* and seawater community, F: *G. delicatula* and seawater community.
P. pungens control (◆), *G. delicatula* control (□), *T. rotula* control (▼). Inocula of *P. pungens* (+), *G. delicatula* (+), seawater community (*) and *T. rotula* (•). *G. delicatula* inoculum in *P. pungens* culture (▽), *P. pungens* inoculum in *G. delicatula* culture (○), seawater community in *P. pungens* culture (■), seawater community in *T. rotula* culture (●), *P. pungens* inoculum in *T. rotula* culture (▲), *T. rotula* inoculum in *G. delicatula* culture (●), *G. delicatula* inoculum in *T. rotula* culture (△), *T. rotula* inoculum in P. *pungens* culture (■), seawater community in G. *delicatula* culture (◆).

Influence of exudates

Generally, a separation of the different controls was determined by ANOSIM (Tab. 7). Especially the controls of the *P. pungens* exudate or the seawater filtrate displayed strong differences from the other control treatments whereas the controls of the *G. delicatula* and the *T. rotula* exudate appeared to be similar.

Table 7: ANOSIM (Analysis of similarity) statistics for pairwise tests involving comparisons of all controls of exudate treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic r	Seawater sample	*G. delicatula*	*P. pungens*
G. delicatula	0.696		
P. pungens	0.546	0.72	
T. rotula	0.557	0.299	0.715

Furthermore, a distinct effect on the bacterial communities due to the respective exudate tested by ANOSIM was not found (Tab. 8). Instead of this, separation into attached and free-living bacteria was observed except for those experiments conducted with the seawater community serving as inoculum (Tab. 8). The influence of different inocula varied depending on the source of the inoculum. The treatment with *P. pungens*-associated bacteria serving as inoculum displayed the strongest separation of the respective communities (Fig. 8 A). A strong partition due to free-living and attached bacteria could also be observed for treatments including the inoculum of *T. rotula* (Fig. 8 B). In this MDS plot it is apparent that the community composition of the original inoculum of *T. rotula* changed during the incubation with exudates of the host alga *T. rotula*. The incubation of exudates with an inoculum of *G. delicatula* also resulted in a differentiation of free-living and attached bacteria (Fig. 8 C). However, some communities of the treatments including *T. rotula* and *P. pungens* serving as exudates were not separated which is also indicated by ANOSIM (global R = 0.416, Tab. 8). No separation of attached and free-living communities was observed in all treatments including the seawater sample serving as inoculum (Fig. 8 D).

Table 8: ANOSIM (Analysis of similarity) statistics for global tests involving comparisons of all exudate treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic r	**Inoculate**			
	G. delicatula	*P. pungens*	*T. rotula*	Seawater sample
Exudates all	0.016	0.123	0.010	0.047
Exudates fraction	0.416	0.494	0.507	0.151

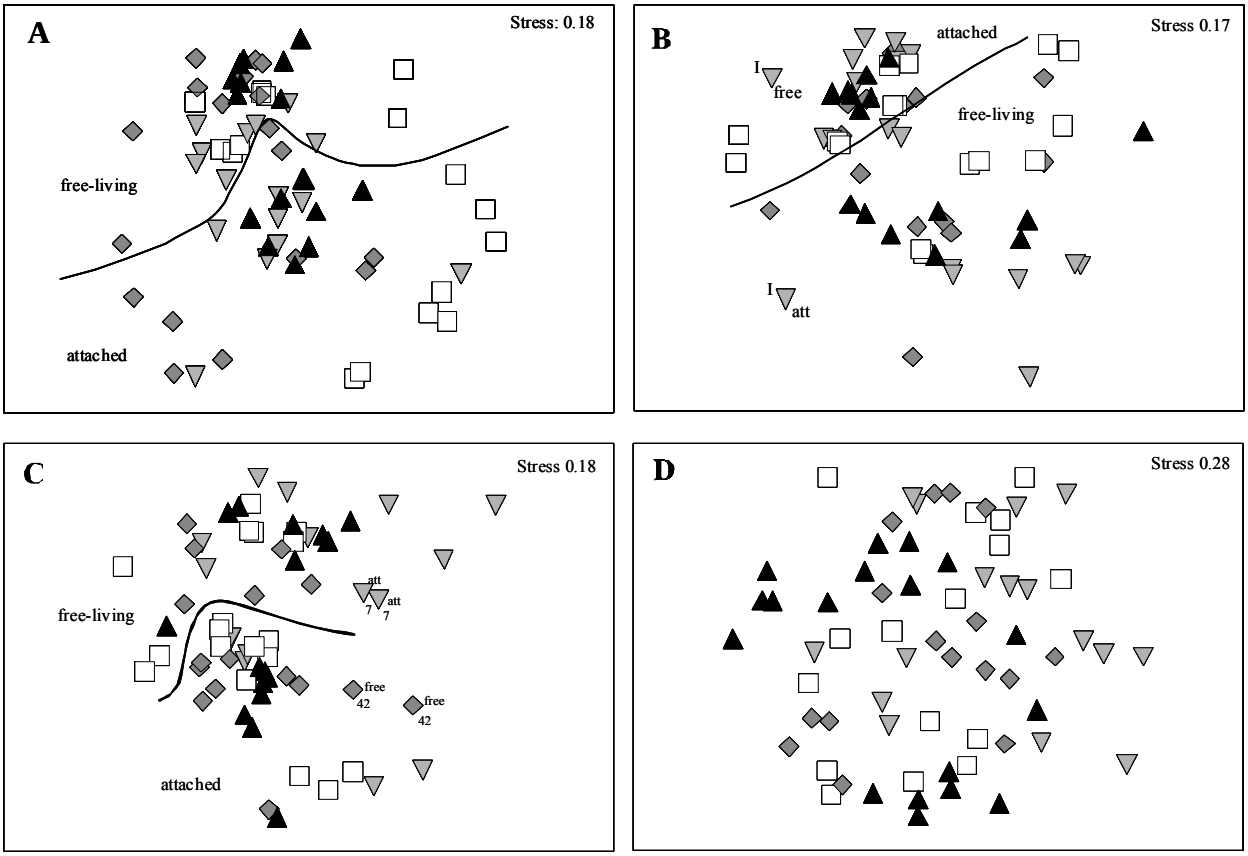

Figure 8: MDS plots based on Bray-Curtis similarities of RISA fingerprints of bacterial communities cultivated in algal exudates including two replicates, two sample fractions and four sampling dates. A: *P. pungens* inoculum in exudates of *P. pungens*, *G. delicatula*, *T. rotula* and the seawater sample, B: *T. rotula* inoculum in exudates of *P. pungens*, *G. delicatula*, *T. rotula* and the seawater sample, C: *G. delicatula* inoculum in exudates of *P. pungens*, *G. delicatula*, *T. rotula* and the seawater sample, D: seawater inoculum in exudates of *P. pungens*, *G. delicatula*, *T. rotula* and the seawater sample, *P. pungens* exudate (◆), *G. delicatula* exudate (□), *T. rotula* exudate (▼), seawater sample exudate (▲). Separation is indicated by black lines, I: inoculum, att: attached fraction, free: free-living fraction, numbers indicate sampling time.

Comparison of all different experimental treatments revealed that especially the inoculum of *P. pungens* favoured a separation into free-living and attached bacteria (Tab. 9). Similar effects were observed for the combinations of *G. delicatula* and the seawater community, *G. delicatula* and *P. pungens* as well as *P. pungens* and the seawater community (Tab. 9).
A specific influence of the exudate or the added inoculum was tested by pairwise tests. These revealed that the exudates of *P. pungens* or *G. delicatula* did not influence the community but the respective inocula (Tab. 10). This is also displayed in the MDS plot (Fig. 9 A). The treatments including the exudates of *T. rotula* and *G. delicatula* displayed no influence by inocula or exudates (Tab. 10, Fig. 9 B). However, the treatments including the exudates of *T. rotula* and *P. pungens* resulted in high R-values including those tested against the respective control (Tab. 10). The MDS plot supports this finding. Samples can be grouped with the inoculum community but not with the exudate (Fig. 9 C).

Table 9: ANOSIM (Analysis of similarity) statistics for specific global tests involving comparisons of all exudate treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic r	Seawater sample	*G. delicatula*	*P. pungens*
G. delicatula			
Treatment	0.417		
Exudate	-0.029		
Inoculate	0.615		
P. pungens			
Treatment	0.418	0.432	
Exudate	0.008	0.091	
Inoculate	0.575	0.522	
T. rotula			
Treatment	0.311	0.205	0.522
Exudate	-0.001	0.054	0.048
Inoculate	0.386	0.263	0.731

The investigation of an influence of the seawater community and the substances within the seawater filtrate on the bacterial communities associated with the studied diatoms revealed that the bacterial communities associated with *P. pungens* were not influenced by the seawater filtrate (Tab. 10). The inocula of *P. pungens* and the seawater sample had a strong influence on the community but the influence of the exudates was low (Fig. 9 D). This is also true for the combinations of *T. rotula* and the seawater community as well as for *G. delicatula* and the seawater community (Tab. 10, Fig. 9 E and F). It has to be considered that the seawater control and experimental treatments including the seawater community displayed

more dispersed plots in contrast to those treatments including inocula obtained from diatom cultures.

Table 10: ANOSIM (Analysis of similarity) statistics for pairwise tests involving comparisons of all exudate treatments, *P*-value = 0.001 with 0 out of 999 permutations with scores ≥ *R*

Sample statistic R	Exudates
P. pungens C ↔ *G. delicatula* C	0.720
G. delicatula C ↔ *P. pungens* I in *G. delicatula*	0.421
P. pungens C ↔ *G. delicatula* I in *P. pungens*	0.679
P. pungens I in *G. delicatula* ↔ *G. delicatula* I in *P. pungens*	0.451
G. delicatula I in *T. rotula* ↔ *T. rotula* I in *G. delicatula*	0.273
T. rotula C ↔ *G. delicatula* I in *T. rotula*	0.250
G. delicatula C ↔ *T. rotula* I in *G. delicatula*	0.261
T. rotula C ↔ *P. pungens* C	0.715
T. rotula C ↔ *P. pungens* I in *T. rotula*	0.652
P. pungens C ↔ *T. rotula* I in *P. pungens*	0.784
P. pungens I in *T. rotula* ↔ *T. rotula* I in *P. pungens*	0.768
P. pungens C ↔ seawater I in *P. pungens*	0.590
P. pungens C ↔ seawater C	0.634
seawater I in *P. pungens* ↔ *P. pungens* I in seawater sample	0.580
seawater C ↔ *P. pungens* I in seawater sample	0.635
T. rotula C ↔ seawater I in *T. rotula*	0.293
T. rotula C ↔ seawater C	0.557
Seawater C ↔ seawater I in *T. rotula*	0.223
seawater I in *T. rotula* ↔ *T. rotula* I in seawater sample	0.368
Seawater C ↔ *T. rotula* I in seawater sample	0.626
G. delicatula C ↔ seawater I in *G. delicatula*	0.618
G. delicatula C ↔ seawater C	0.732
seawater I in *G. delicatula* ↔ G. *delicatula* I in seawater sample	0.546
Seawater C ↔ *G. delicatula* I in seawater sample	0.712

C: control, I: inoculum

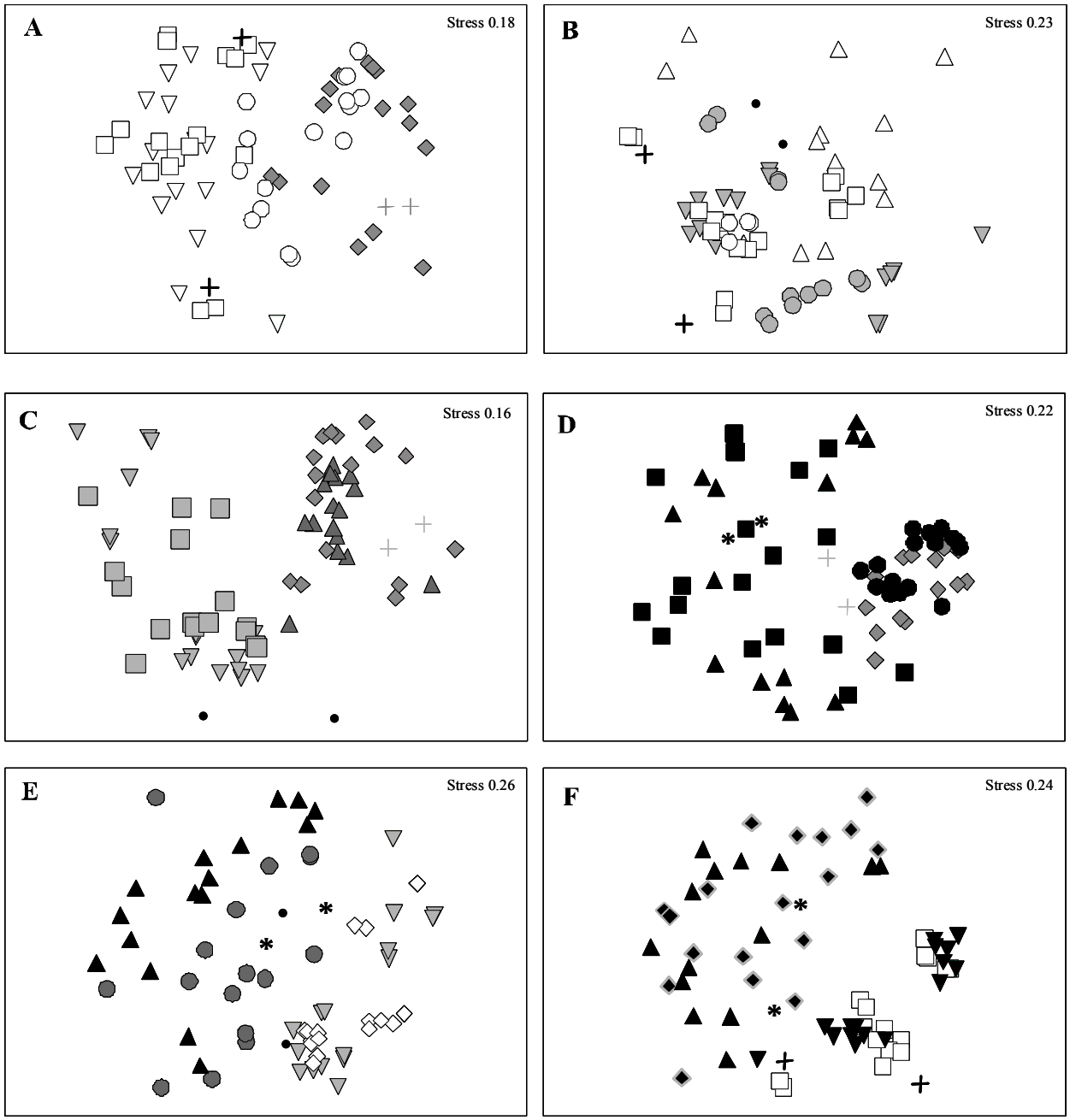

Figure 9: MDS plots based on Bray-Curtis similarities of RISA fingerprints of bacterial communities cultivated in algal exudates including two replicates, two sample fractions and four sampling dates. A: *P. pungens* and *G. delicatula*, B: *G. delicatula* and *T. rotula*, C: *T. rotula* and *P. pungens*, D: *P. pungens* and the seawater sample, E: *T. rotula* and the seawater sample, F: *G. delicatula* and the seawater sample.
P. pungens control (◆), *G. delicatula* control (□), *T. rotula* control (▼), seawater sample control (▲). Inocula of *P. pungens* (+), *G. delicatula* (+), seawater sample (*) and *T. rotula* (•). *G. delicatula* inoculum in *P. pungens* exudates (▽), *P. pungens* inoculum in *G. delicatula* exudates (○), seawater community in *P. pungens* exudates (■), seawater community in *T. rotula* exudates (●), *P. pungens* inoculum in *T. rotula* exudates (▲), *T. rotula* inoculum in *G. delicatula* exudates (●), *G. delicatula* inoculum in *T. rotula* exudates (△), *T. rotula* inoculum in P. *pungens* exudates (■), seawater community in G. *delicatula* exudates (◆), *G. delicatula* inoculum in seawater sample exudates (▼), P. *pungens* inoculum in seawater sample exudates (●), *T. rotula* inoculum in seawater sample exudates (◇).

Discussion

In this study, we investigated the specificity of the association of bacteria and diatoms by resource competition experiments of bacterial communities obtained from diatom cultures. In addition, the influence of exudates on the composition of the associated bacterial community was investigated.

Resource competition

MDS plots and ANOSIM of culture experiments displayed differences between the originally associated bacterial communities of the studied diatoms generally supporting the hypothesis of specificity of bacteria-phytoplankton interactions (Schäfer *et al.*, 2002; Grossart *et al.*, 2005; Jasti *et al.*, 2005). However, the separation was not complete and it is assumed that a part of the bacterial community might occur in several diatom cultures. This has already been found for microalgal cultures derived from Helgoland Roads (Chapter II, this volume). This is particularly the case for the slight differences between the bacterial communities associated with *G. delicatula* and *T. rotula*.

The experimental treatments dealing with competition between the associated bacterial communities revealed no influence on the community by the respective diatom except for the treatments including the inoculum of *T. rotula*. Regarding the treatments including inocula communities of *G. delicatula* and *P. pungens*, the community composition detected during the incubation was neither similar neither to the community of the inoculum nor to the community originally associated with the respective diatom. Therefore, it is suggested that in these treatments some species were eliminated by competitive exclusion and some were not. It is assumed that the eliminated bacteria were either not able to compete with the other bacteria or they were not able to live associated with the respective diatom. The latter might be caused by competition for nutrients or antibacterial substances produced by the diatom but this has to be addressed in future studies. Our results indicate that this might be particularly true for the community associated with *T. rotula.* The remaining bacteria which asserted themselves in the new environment might have outcompeted some originally associated bacteria. It is suggested that these bacteria might have overlapping ecological niches which might be true for the bacteria associated with *P. pungens* and *G. delicatula*. In an ecosystem species can live in the same or in overlapping niches. These species can have similar nutritional demands so that they can be grouped into ecological guilds (Odum, 1999). The ecological niche is usually defined in a spatial, trophical and multidimensional context in

ecology (Odum, 1999). Its definition comprises the place where a certain species lives, its nutritional demands and interactions with other species or the environment. Rosenfeld (2002) stated that a niche can be characterised by the function of a certain species in an ecosystem. Species which have the same function might be functionally redundant presupposed that they have the same physiological capacities according to the concept of the functional niche. If we apply this concept on diatom-associated bacterial species these might be functionally redundant meaning that they have functional overlap. Our results indicate that some diatoms provide a suitable habitat for a broad range of species indicated by the results of the treatments containing the seawater community and the cultures of *G. delicatula* and *T. rotula*. We presume that bacteria associated with diatoms might be generalists tolerating a wide range of resources.

Influence of exudates

In this study, it could be shown that the influence of exudates of the studied diatom species on the bacterial community structure was low. A shaping influence on the respective community by the exudates could not be observed in any treatment. Concerning multivariate statistics an effect of the inocula was shown comparing the treatments including the associated communities of *P. pungens/G. delicatula* or *P. pungens/T. rotula* displaying the strongest effect due to the inoculum of *P. pungens*. However, it is suggested that either the associated bacterial communities of *G. delicatula* or *T. rotula* or their exudates might be similar as they displayed neither an influence of exudates nor an influence by the respective inoculum. It has to be considered that phytoplankton cells excrete organic compounds which differ in their composition (Myklestad, 1995). We hypothesise that the exudates obtained from the studied diatoms also differ in the composition of extracellular substances but this needs further investigations with respect to the studied diatoms.

Assuming specialisation of bacteria for uptake and utilisation of compounds deriving from algal exudates it seems likely that we can observe an influence of exudates in our treatments. In contrast, Bell (1983) could demonstrate that bacteria adapted to exudates of a diatom or a flagellate were able to utilise the exudates of other microalgae. These findings indicate that not all bacteria are specialised on specific exudates and that they might adapt to different algal compounds. This is assumed for the associated bacterial populations studied in our treatments. Therefore, we hypothesise that associated bacteria are generally able to adapt to different exudate compounds.

In the exudate treatments a separation of the community into free-living and attached bacteria was observed except for the treatments including the seawater community. Regarding our

results it must be noted that the filtration of algal cultures retained dissolved organic material (DOM) or transparent exopolymer particles (TEP) (Verdugo *et al.*, 2004) in the medium. DOM is reported to assemble into polymers resulting in polymer networks (Verdugo *et al*., 2004). The so called "marine gels" provide microenvironments different from DOM alone (Verdugo *et al.*, 2004). It is thus likely that nets of polymers were formed in the treatments including algal exudates. This is the only manner in which particles < 10 µm could have been introduced by the inocula. Considering the separation of free-living and attached bacteria in those treatments it is assumed that bacteria of the respective inoculum favoured the inherent DOM as a nutrient source and had the capacity to contribute to the assemblage of marine snow and to attach to it. DeLong *et al*. (1993) have already observed a separation of attached and free-living bacterial assemblages. This separation leads to the assumption that specific bacteria might be responsible for particle decomposition.

The development of highly specialised attached bacterial populations has been observed during aggregate experiments with diatoms (Grossart *et al*., 2006). The authors elucidated that heterotrophic bacteria, especially those colonising phytoplankton cells, play an important role for the aggregation of phytoplankton. Processes leading to aggregation might have favoured a separation of attached and free-living bacteria in our treatments. Nevertheless, it is not clear why a separation into free-living and attached bacteria occurred in treaments with exudates but not in competition treatments. In both approaches the inocula included bacterial communities which were dominated by mainly free-living bacteria which derived from the associated bacterial community, but the competition treatments contained diatom cells. It is likely that the exudates in the competition treatments might also form polymers, therefore, we cannot explain the separation of free-living and attached bacteria satisfactorily.

Conclusion

From our results species-specific interactions between bacteria and the studied diatoms could not be shown. Our results indicate that associated bacteria are presumably adapted to a broad range of substrates. Additionally, we could not detect a community shaping influence of exudates. Apart from our results algae might promote specific bacterial communities with a specific composition of exudates and thereby shape these communities. Nevertheless, the bacterial communities might keep their ability to adapt to new conditions. Therefore, we assume that associated bacteria adapted to the diatom environment belong to ecological guilds.

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Impacts of cultivation of marine diatoms on the associated bacterial community

Abstract

Shifts in bacterial communities associated with four different diatom species were monitored during isolation and cultivation of microalgae. The bacterial community was analysed by the fingerprinting methods RISA (Ribosomal Intergenic Spacer Analysis) and DGGE (Denaturing Gradient Gel Electrophoresis) of 16S rRNA genes followed by DNA sequence analysis. Generally, members of *Alpha* - and *Gammaproteobacteria* as well as members of the *Bacteroidetes* predominated the cultures. Multidimensional scaling revealed strong shifts in the associated communities during cultivation. Especially the number of phylotypes belonging to the *Gammaproteobacteria* increased. It could be shown that the bacterial community associated with the diatoms at the time of isolation was completely different from the associated community after twelve months of cultivation.

Introduction

Specific associations of bacteria and phytoplankton cells *in situ* have recently been demonstrated by Kaczmarska *et al*. (2005). The authors could show a strong association of certain bacterial morphotypes and phytoplankton cells by scanning electron microscopy. Nevertheless, an identification of these bacteria was not achieved. However, comprehensive analysis of microalgae/bacteria consortia also requires the proper identification of the involved organisms. Whereas the identification of microalgae can usually be performed by microscopy, the bacterial community must be analysed using molecular methods.

Information on bacteria-phytoplankton interactions was obtained inter alia by experiments with algal batch cultures which provide well defined cultivation conditions and an easy access to microbial biomass for molecular analysis (Schäfer *et al*., 2002; Grossart *et al*., 2005; Jasti *et al*., 2005; Chapter II, this volume). Distinct “satellite bacteria” in microalgal cultures were found by Schäfer *et al*. (2002). Grossart *et al*. (2005) could demonstrate that specific bacterial groups deriving from a natural community colonised phytoplankton cells. Furthermore, specificity especially for *Roseobacter sp*. associated with *Alexandrium* species has been shown by Jasti *et al*. (2005).

Apart from this, shifts in marine bacterioplankton can result from confinement which has already been reported by Ferguson *et al*. (1984). The authors observed that bacteria of a seawater sample are subjected to changes when they are enclosed in a small volume. Shifts on the community level after confinement were observed by Schäfer *et al*. (2000) who noticed an increase of *Gammaproteobacteria* especially of *Alteromonas* like species.

Nevertheless, cultivation experiments with batch cultures provide the advantages of enclosed systems, hence they are useful in the analysis of specific interactions of bacteria and phytoplankton. First, an algal culture provides controlled conditions which might have an influence on the growth of algal cells and their associated bacterial community. Second, specific interactions of bacteria and phytoplankton cells can be investigated more easily in cultures than *in situ*.

However, it must be considered that isolation and cultivation of marine bacteria promotes certain bacterial species as shown by Eilers *et al*. (2000). High nutrient concentrations in the culture medium are mainly responsible for this observation. It has to be considered that the nutrient concentrations in most algal culture media are high (Guillard, 1975), what might also lead to shifts in the algal-associated bacterial community.

In this study, we investigated the shifts in the bacterial community during the isolation and subsequent cultivation of diatoms to assess if diatom-associated bacterial communities are influenced by confinement and cultivation.

Methods

Algal cultures

In 2004, plankton samples from Helgoland Roads (54°11.3’ N and 7°54.0’ E) were taken using a 20 µm and 80 µm net aboard the research vessel Aade. Single cells were isolated by micropipetting (Daste, 1983) and passed through several washing steps before they were subsequently cultivated.

To monitor shifts in the bacterial community during cultivation of diatoms we chose four diatom species isolated in April 2004. These were *Guinardia delicatula* (Cleve) Hasle, *Pseudonitzschia pungens* Grunow, *Thalassiosira rotula* Meunier and *Skeletonema costatum* (Greville) Cleve. Microalgae were incubated in batch culture in Guillard’s f/2 medium (Guillard, 1975) at 16°C with 12:12 light-dark cycle at 20.0 µmol photons $m^{-2}sec^{-1}$. After two months single cells were grown to clonal cultures. After this the cultures were recultivated every month. Samples were taken after two, four and twelve months (step 1, 3 and 11).

Additionally, samples of the *in situ* community obtained at the date of isolation were used to cover the original bacterial community (Chapter V, this volume).

Sampling of biomass and extraction of nucleic acid

A volume of 50 ml of each sample was filtered through 3 µm and 0.2 µm membrane filters (Millipore) in succession. This procedure separated biomass of attached bacteria detained by 3 µm filter and free-living bacteria detained by 0.2 µm filter. Filters were stored at -20°C until DNA extraction.

Nucleic acid extraction was performed as described by Wichels *et al.* (2004). Briefly, filters were cut into pieces and transferred to sterile 2 ml vials. Bacterial biomass was resuspended in STE buffer (6.7 % Saccharose, 50 mM Tris, 1 mM EDTA, pH 8) and cell lyses was performed by adding lysozyme (2 mg ml^{-1}) and SDS (1 %). DNA extraction was performed using phenol-chloroform-isoamylalkohol (25:24:1). After precipitation of the DNA with isopropanol all DNA extracts were finally kept in sterile water and stored at -20°C until further analysis. They served as template DNA in the PCR. Prior to PCR amplification, the DNA extracts were analysed by agarose gel electrophoresis on 0.8 % agarose gels (45 minutes at 100 V in 0.5 x TBE (10 x TBE: 0.89 M Tris, 0.89 M boric acid, 0.025 M EDTA)). After electrophoresis, gels were stained with ethidium bromide (0.5 mg l^{-1}). Imaging was performed with a ChemiDoc XRS System (BioRad, Germany).

Community Analyses

Amplification of Ribosomal Intergenic Spacer and RISA

For amplification of the intergenic spacers (IGS) between the 16S and 23S subunits of ribosomal sequences we used the primers S-D-Bact-1522-b-S-20 (5'- TGC GGC TGG ATC CCC TCC TT -3') and L-D-Bact-132-a-A-18 (5'- CCG GGT TTC CCC ATT CGG -3') (Ranjard *et al.*, 2000a; Ranjard *et al.*, 2000b). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf, Germany), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer, Germany), 0.5 µM of each primer, 2 U of Taq DNA Polymerase (Eppendorf) and 5 µl DNA of 0.2 µm filters or 0.5 µl of 3 µm filters. The amplification started with a denaturing step at 95°C for three minutes and 25 cycles at 95°C for one minute, 53°C for one minute and 72°C for one minute followed by 72°C for five minutes for extension. 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, Germany) in 0.5 x TAE buffer. Three lanes were used for 0.1 µg of a 100 bp ladder (invitrogen, Germany) in order to

achieve comparability. Electrophoresis was run at 20°C for 18 h at 50 V using a DCode system (BioRad). Gels were stained with SYBRGold as recommended by Molecular Probes (invitrogen). Imaging was performed with a ChemiDoc XRS System of BioRad.

Amplification of 16S rRNA genes and DGGE followed by DNA sequence analysis

To analyse which bacterial phylotypes caused the shifts in the bacterial community during the cultivation of algal cultures PCR amplification of 16S rDNA fragments was performed according to Wichels *et al*. (2004) 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 907rm modified (5'- CCG TCA ATT CMT TTR AGT TT -3'). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer), 0.2 µM of each primer, 2 U of Taq DNA Polymerase (Eppendorf) and 5 µl DNA of 0.2 µm filters or 0.5 µl of 3 µm filters. The "touchdown" PCR started with a denaturing step at 94°C for five minutes. Every cycle consisted of three steps each with one minute: 94°C, annealing temperature and 72°C. The initial annealing temperature of 65°C 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 minutes followed by 22 cycles of extension starting at 71°C decreasing by 1°C per cycle in order to avoid heteroduplices. PCR reactions were performed in an Eppendorf Mastercycler. PCR products were inspected on 1.2 % (w/v) agarose gels. DGGE analyses 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 run at 60°C and 150 V for 10 hours (Sigler *et al.*, 2004). DGGE gels were stained with SYBRGold (see RISA) and imaging was performed with a ChemiDoc XRS System of BioRad.

DNA sequencing. Prominent DGGE bands which connected or separated samples were excised, eluted (Sambrook *et al.*, 1989) and reamplified using the primers 341f without GC-clamp and 907rm. DNA was purified via the Qiaquick PCR purification kit (QIAGEN, Germany) following the instructions of the manufacturers protocol. Products were checked by electrophoresis on 1.2 % (w/v) agarose gels. Sequencing was performed by using the SequiTherm Excel™ II long read sequencing Kit-LC (Biozym) following the manufacturer's instructions. Sequencing primers were 907rm-IRDye700 and 344f-IRDye800 (5'- ACG GGA GGC AGC AG -3'). Sequencing was done by a long range gel on a 4200 automated DNA

sequencer (LI-COR Inc., Lincoln, Nebraska). Nearest relatives were searched by BLAST (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis. Sequence data were checked for the presence of PCR amplified chimeric sequences by 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 rDNA sequences database (release 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 neighbour joining method including the correction algorithm of Felsenstein (Felsenstein, 1993).

Nucleotide sequence accession numbers. The sequences obtained in this study are available from GenBank under accession numbers DQ376145-DQ376174.

Statistical Analysis of RISA and DGGE profiles

Analysis of RISA and DGGE profiles was carried out with the BioNumerics 4.5 software package of Applied Maths BVBA (Belgium). Multivariate analysis of fingerprints was performed using the subroutines ANOSIM and MDS of the PRIMER 5 software suite (PRIMER-E Ltd., UK; Clarke & Warwick, 2001). First, normalisation of gels was performed by BioNumerics software using 100 bp ladders in RISA profiles and a specific sample including seven bands in DGGE profiles as references in every profile. For sample comparison a band matching analysis was performed. Bands were assigned to classes of common bands within all profiles of a fingerprinting method. The procedure included densitometric values of the profiles (Muylaert *et al.*, 2002). The resulting band matching tables were imported into PRIMER. Bray Curtis similarity of bacterial communities was calculated applying square root transformation (Clarke & Warwick, 2001). Statistical analysis was performed with all samples. Ordination of Bray Curtis similarities was performed by nonmetric multidimensional scaling (MDS) (Yannarell *et al.*, 2005). MDS was calculated using 20 random starting configurations of sample points. It was assumed that the final configuration was optimal unless other configurations displayed lower stress levels.

To test the hypothesis that the within-group community similarity was greater than among-groups, an analysis of similarity was conducted by the subroutine ANOSIM of the PRIMER software. ANOSIM is a nonparametric technique designed to allow statistical comparisons for multivariate data sets in a manner similar to univariate techniques (ANOVA). The null

hypothesis is that no difference between associated bacterial communities of different cultivation steps exists. Testing the hypothesis results in a sample statistic R displaying the degree of separation between groups. Complete separation is indicated by R = 1, whereas R = 0 suggests no separation. Having determined R, ANOSIM randomly assigns samples to different groups to generate a null distribution for R (Monte Carlo test, 999 permutations) (Yannarell & Triplett, 2004) to test whether within-group samples were more closely related to each other than would be expected at random. The results of ANOSIM with a significance level of 0.1 % are displayed.

Results

Community shifts. It is apparent that the bacterial communities from the different cultivation steps are separated to different degrees from each other as represented by MDS of RISA and also DGGE fingerprints (Fig. 10). The MDS plot of RISA fingerprints (Fig. 10 A) displayed a development of bacterial communities associated with the studied microalgae within three steps which are indicated by circles. General separation of the communities during cultivation is also reflected by ANOSIM (global R = 0.636). The communities associated with freshly isolated microalgae could be grouped together (group I, step 1). Those communities were not similar to the community obtained from an *in situ* sample taken at the day of the initial isolation of the microalgae. Additionally, the communities displayed a shift after four months of cultivation of the clonal cultures (group II, step 3), which is supported by ANOSIM (pairwise test, R = 0.561). Differences were found between the algal species in step 3. The MDS plot also displayed a shift between cultivation after four and after twelve months (groups II and III, Fig. 10 A).

The communities included in group III (step 11) were more separated from the communities associated with freshly isolated microalgae than those obtained from cultures after four months of cultivation which is also shown by ANOSIM (pairwise test, R = 0.835). Generally, a similar development could be observed by MDS of DGGE fingerprints (Fig. 10 B). ANOSIM revealed an overall separation of the communities during cultivation (global R = 0.771). In this analysis the communities associated with freshly isolated microalgae can be grouped together (group I, step 1). A shift occurred after two to four months of cultivation which is also evident in the ANOSIM analysis (pairwise test, R = 0.787). It resulted in more similar communities associated with microalgal cultures after four to twelve months (group II, steps 3 and 11, pairwise test, R = 0.434). The associated communities in step 1 are completely

separated from those communities obtained after twelve months of cultivation which is shown by ANOSIM (pairwise test, R = 1.0).

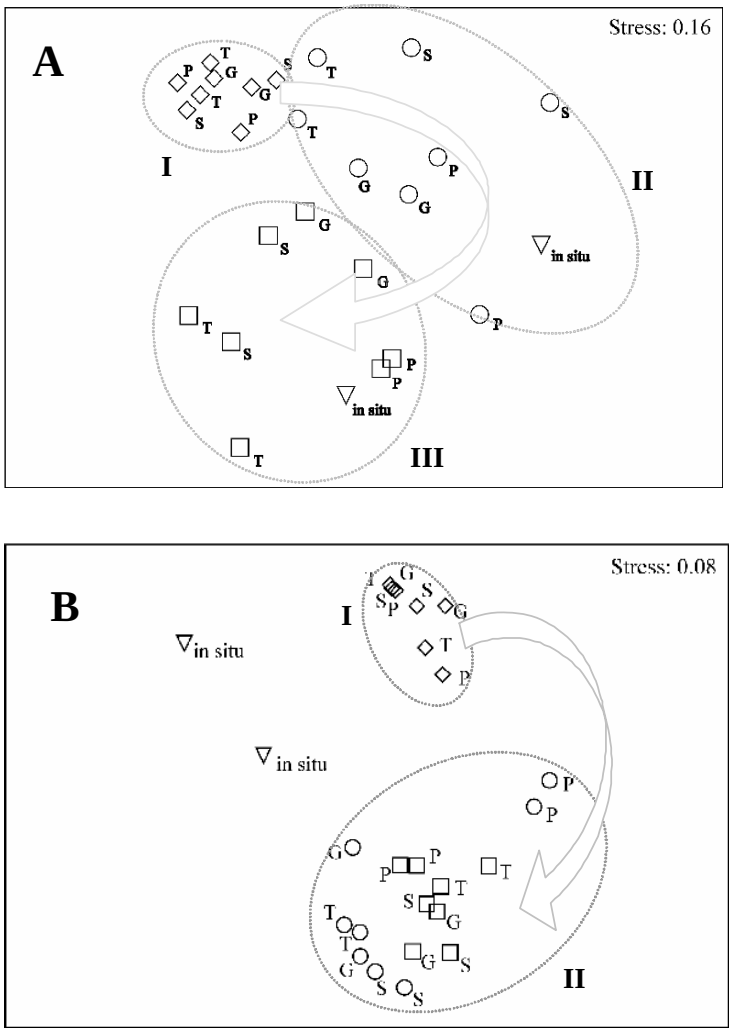

Figure 10: MDS plots based on Bray-Curtis similarities of community fingerprints of different microalgal cultures (G: *Guinardia delicatula*, T: *Thalassiosira rotula*, P: *Pseudonitzschia pungens*, S: *Skeletonema costatum*), two sample fractions and 4 sampling dates. Cultivation steps: *in situ* (▽), step 1 (◇), step 3 (○), step 11 (□). The arrows indicate the development of the communities. Circles display groups of samples. A: MDS plot of RISA fingerprints B: MDS plot of DGGE fingerprints

Phylogenetic analysis of communities. In order to identify the most prominent phylotypes in the different cultivation steps, sequence analysis from excised DGGE bands was performed. Sequence data generally revealed the presence of two phyla of *Bacteria* (Fig. 11). Most sequences were related to the *Alpha* - and *Gammaproteobacteria*. Additionally, we found members of the *Bacteroidetes* phylum. The results revealed close matches with 98-100 % similarity to bacterial 16S rRNA gene sequences in the GenBank. Overall 44 % and 31 % of the phylotypes were assigned to the *Alpha* - and *Gammaproteobacteria*, respectively. 19 % of the phylotypes belonged to the *Bacteroidetes* phylum. Additionally, we found two phylotypes affiliated to *Actinobacteria* (6 %). A neighbour joining tree revealed that sequences belonging to *Alphaproteobacteria* clustered with *Sulfitobacter sp.*, *Paracoccus marcusii* or an uncultured member of *Alphaproteobacteria* obtained from the Arctic Ocean (Fig. 11). Sequences related to the *Gammaproteobacteria* were mainly assigned to the

Alteromonadales. In detail, the phylotypes clustered with *Pseudoalteromonas sp*. or *Alteromonas sp*. (Fig. 11). Additionally, we found a phylotype which could be assigned to *Colwellia sp*.. In the order of *Pseudomonadales* a phylotype clustered with *Psychrobacter glacinocola*. Within the *Bacteroidetes* phylum the phylotypes clustered with *Cytophaga sp*., *Flavobacteria sp.* or uncultured members of the *Bacteroidetes*. Some phylotypes related with *Alpha-, Gammaproteobacteria* or *Bacteroidetes* were already found in microalgal cultures obtained from the phytoplankton of Helgoland Roads such as the phylotypes G009, G062 or G001 (Chapter II, this volume). Furthermore, the phylotypes G021 and G046 clustered with sequences obtained from an *in situ* sample collected at the date of isolation of actually studied microalgae (Chapter V, this volume).

Succession of phylotypes during cultivation. Generally, 18 phylotypes of the bacterial community associated with the studied microalgae were identified at different positions on the DGGE gels. The succession of phylotypes in the different cultures and different cultivation steps is summarised for the main phylotypes occurring in most cultures and cultivation steps in Tab. 11 and Fig. 12. Some phylotypes obtained from the *in situ* sample collected at the time of isolation could not be detected in the cultures after isolation. This is true for the phylotype F128 (Fig. 11 and 12 A, DQ289519; Chapter V, this volume) belonging to the *Bacteroidetes*. One phylotype occurred in the microalgal cultures after isolation and after four and twelve months of culturing (G003, Tab. 11, Fig. 12 B). Some phylotypes could be obtained in the cultures after isolation but did not occur during further cultivation. These phylotypes belonged to the *Gammaproteobacteria* (G048, G049, G061 and G062, Tab. 11, Fig 12 C, D, F) and the *Alphaproteobacteria* (G046, Tab. 11, Fig. 12 E). The phylotype G037 belonging to the *Gammaproteobacteria* was detected in all cultures after isolation (step 1) and in the culture of *G. delicatula* in step 3 (Tab. 11, Fig. 12 G). Furthermore, some phylotypes could be detected exclusively in steps 3 and 11 like G006 and G009 belonging to the *Alphaproteobacteria* (Tab. 11, Fig. 12 H + K) and one member of the *Gammaproteobacteria* (G010, Tab. 11, Fig. 12 J). The phylotype G020 belonging to the *Bacteroidetes* occurred *in situ* as well as in steps 3 and 11 (Tab. 11, Fig. 12 I), but it could not be detected in step 1. A member of the *Alphaproteobacteria* (G372) could solely be detected in some cultures in step 11 (Tab. 11, Fig. 12 L). Some phylotypes retrieved in this study were not displayed in the MDS plot or in the table as they occurred only in few samples: The phylotype G001 could be detected in the culture of *P. pungens* in step 3 whereas the phylotype G356 occurred in two samples in step 11. The DGGE band G021 could be found in one culture in step 3 as well as

the phylotype G012, whereas the DGGE band G024 could be detected in three samples in step 3.

Table 11: Appearance of phylotypes within cultivation steps

Phylotype	Identification		
	Cultivation step	Phylogenetic group	Microalga
F128	*in situ*	*Bacteroidetes*	
G046	*in situ* 1 1 1 1	*Alphaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G020	*in situ* 3 11 3	*Bacteroidetes*	*T. rotula* *P. pungens* *G. delicatula*
G048	1 1 1 1	*Gammaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G049	1 1 1 1	*Gammaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G061/G062	1 1 1 1	*Gammaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G037	1 1 1, 3 1	*Gammaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G003	1, 3, 11 1, 3, 11 11 11	*Alphaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G006	11 3, 11 11	*Alphaproteobacteria*	*T. rotula* *P. pungens* *S. costatum*
G009	3, 11 11 3, 11 3, 11	*Alphaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G010	3, 11 11 3, 11 3, 11	*Gammaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*
G372	11 11 11 11	*Alphaproteobacteria*	*T. rotula* *P. pungens* *G. delicatula* *S. costatum*

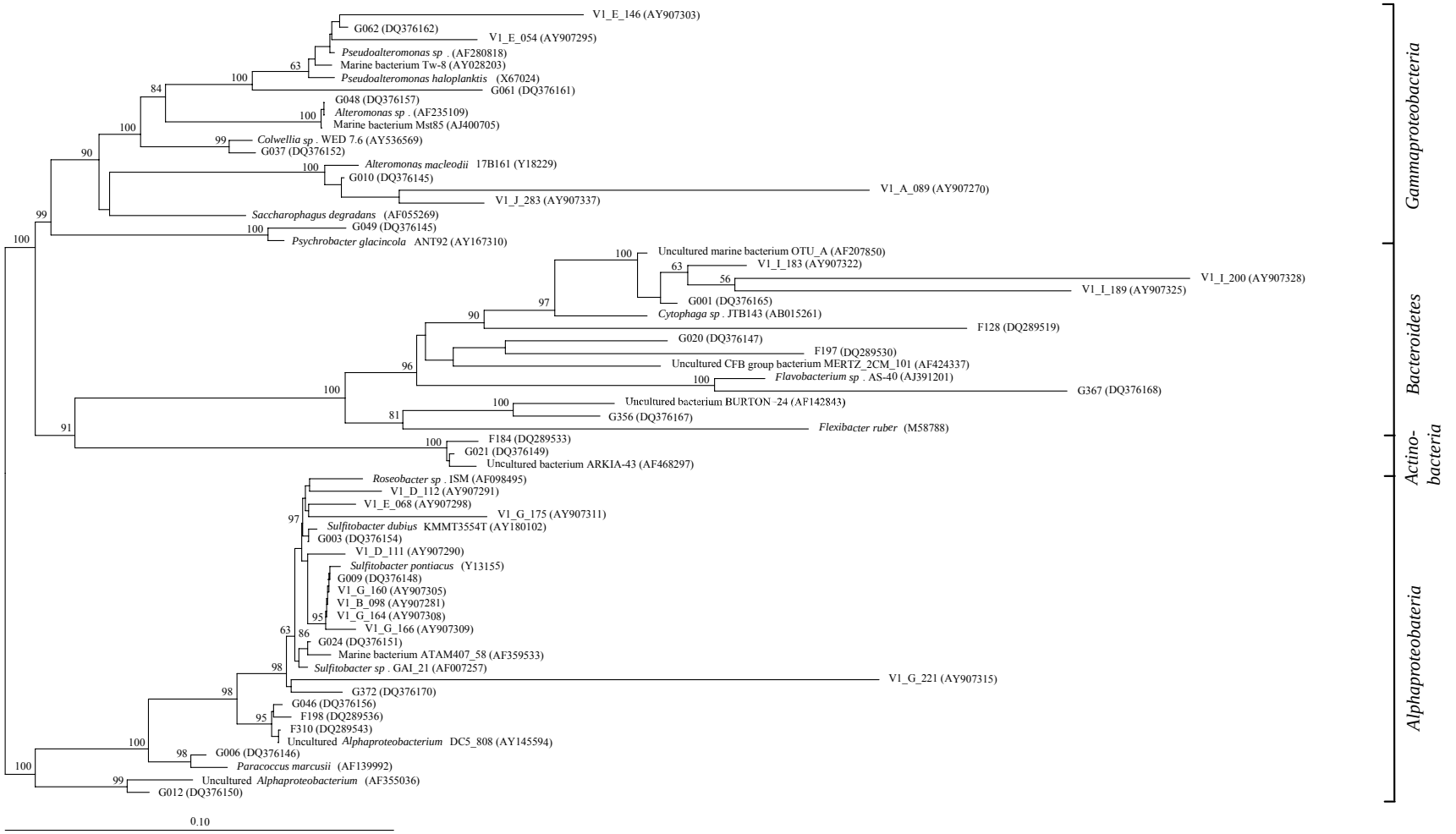

Figure 11: Phylogenetic tree of *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Actinobacteria* and members of *Bacteroidetes*. GenBank accession numbers are given in parentheses. Bootstrap values above 50 % are displayed.

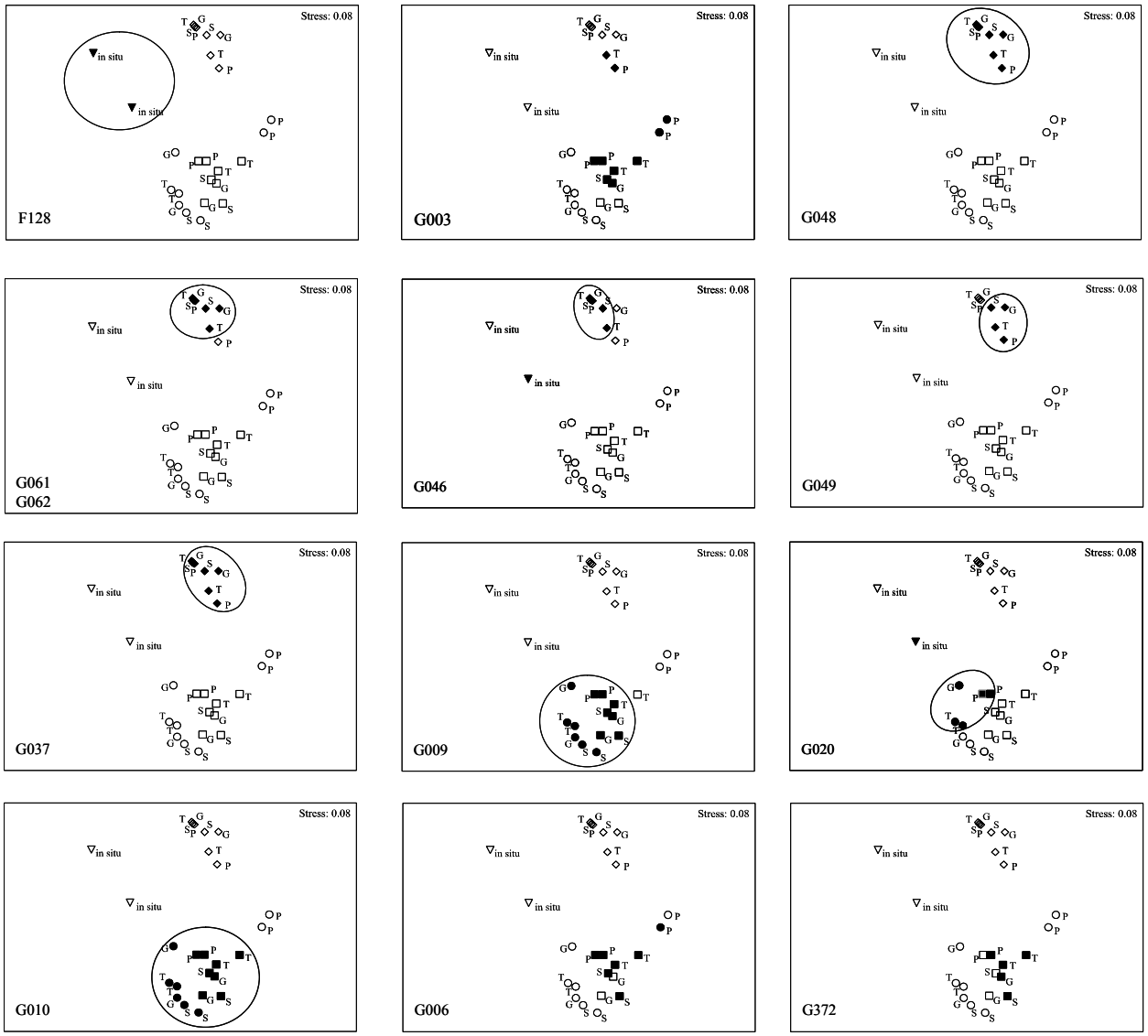

Figure 12: MDS plots based on Bray-Curtis similarities of DGGE fingerprints of bacterial communities of different microalgal cultures, two sample fractions and 4 sampling dates. Cultivation steps: *in situ* (▽), step 1 (◇), step 3 (○), step 11 (□). Identification of specific phylotypes is indicated by filled symbols.

Discussion

In this study, we investigated the development and succession of bacteria associated with diatoms in culture after consecutive cultivation steps. Generally, we detected strong shifts in the community structure resulting from isolation and cultivation of the microalgae which was shown by MDS based on the fingerprinting methods RISA and DGGE. The process of cultivation did not lead to species-specific bacterial communities associated with the studied diatom cultures. In detail, sequencing of DGGE bands revealed the predominance of *Alpha-* and *Gammaproteobacteria* in the studied cultures. Members of the *Bacteroidetes* also occurred during cultivation. In the literature, other investigations dealing with the interaction

of bacteria and microalgae have already identified *Alpha-* and *Gammaproteobacteria* as well as *Bacteroidetes* being associated with algal cells (Bidle & Azam, 2001; Hold *et al.*, 2001; Schäfer *et al.*, 2002; Green *et al*., 2004; Wichels *et al.*, 2004; Kaczmarska *et al*., 2005; Grossart *et al.*, 2005; Jasti *et al.*, 2005; Chapter II, this volume). In detail, the mentioned bacterial groups were detected in association with diatom detritus (Bidle & Azam, 2001) or diatom cells (Schäfer *et al.*, 2002; Kaczmarska *et al*., 2005; Grossart *et al.*, 2005; Chapter II, this volume). In addition, the association of *Alpha-*, *Gammaproteobacteria* and members of the *Bacteroidetes* with dinoflagellates was shown in culture (Green *et al*., 2004; Jasti *et al.*, 2005; Chapter II, this volume) or *in situ* within a bloom (Wichels *et al.*, 2004). Therefore, it is concluded that members of these bacterial groups might play an important part in the interaction of bacteria and microalgae explaining the overall appearance in algal cultures. Since the microalgae were isolated by the technique of micropipetting it is likely that phylotypes identified in the algal cultures originated *in situ*. Additionally, some identical phylotypes have already been isolated from the bacterioplankton of Helgoland Roads (Eilers *et al.*, 2000; Eilers *et al*., 2001), in particular *Sulfitobacter sp*., *Alteromonas sp*. and *Pseudoalteromonas sp*.. Referring to our study, the coherence of *in situ* detection and recovery during cultivation could be shown for two phylotypes belonging to the *Alphaproteobacteria* (G046) and the *Bacteroidetes* (G020). They clustered with phylotypes which were already obtained during the isolation period: G046 clustered with the phylotype F198 (DQ289536, *Alphaproteobacteria*) within the *Roseobacter* clade and G020 clustered with the phylotype F197 (DQ289530, *Bacteroidetes*) belonging to the *Flavobacteria*.

Presuming low abundance leads to non-detection of phylotypes, it can be hypothesised that the bacteria associated with microalgae which were not detected *in situ* but later on, must have grown rapidly during cultivation because of high substrate concentration. This has already been suggested for some *Gammaproteobacteria* obtained from cultivated samples of Helgoland Roads (Eilers *et al.*, 2000). In our study, this might also be the case for one member of the *Alphaproteobacteria* (G003) and some of the *Gammaproteobacteria* (G048, G061, G049, G037). Relatives of these phylotypes have already been found in other microalgal cultures obtained from Helgoland Roads (V1_E_146, V1_E_054, V1_A_089, V1_J_283, V1_D_112, V1_E_068, V1_G_175, V1_D_111, V1_G_160, V1_B_098, V1_G_164, V1_G_166, V1_G_221, Fig. 11, Chapter II, this volume). Therefore, it is assumed that those bacterial groups are important for the interaction of bacteria and phytoplankton. This assumption is supported by a study of Stewart *et al*. (1997) who demonstrated beneficial effects of *Alteromonas sp*. on microalgae like *Pseudonitzschia*

multiseries. However, due to methodological limitation it was not possible yet to successfully identify strongly attached bacterial species directly on the algal cells.

Although we detected a member of the *Actinobacteria* (G021) in several samples obtained also in the *in situ* community (F184, DQ289533, Fig. 11), it seems unlikely that this phylotype plays an important role in the interaction of bacteria and phytoplankton as in most cases *Actinobacteria* were not found to occur associated with algae before. Nevertheless, it could be obtained in some diatom cultures. The influence of the ambient community of the environment where the algal cells were isolated from has not been studied yet, but the identification of *Actinobacteria* in algal cultures might lead to the suggestion that the environment might play a certain role.

Summarising our results, it is assumed that the shifts occurring after isolation can be ascribed to high nutrient concentrations in the culture favouring specific bacterial species as described by Eilers *et al*. (2000). It has to be taken into account that these shifts might also result from confinement which was already described for natural bacterial communities which were enclosed in bottles or mesocosm tanks (Ferguson *et al.*, 1984; Schäfer *et al.*, 2000). Furthermore, during the process of cultivation the dilution after each recultivation step might have favoured additional shifts in the community composition.

In future the findings retrieved from culture experiments dealing with the interaction of bacteria and microalgae should be scrutinised regarding cultivation impacts. Still we do not know exactly which bacteria are important for the microalgae and if there are differences due to growth phases of the algae.

Bacterial dynamics should be studied *in situ* and related with phytoplankton dynamics. Several authors have already made approaches in this way (Middelboe *et al.*, 1995; Fandino *et al.*, 2001; Arrieta & Herndl, 2002; Wichels *et al.*, 2004; Kaczmarska *et al*., 2005; Rooney-Varga *et al.*, 2005 and Chapter V, this volume). Especially the study of Rooney-Varga *et al*. (2005) as well as the study of M. Sapp, G. Gerdts, K.H. Wiltshire, A. Wichels (Chapter V, this volume) demonstrated that a combination of fingerprinting methods and multivariate statistics provides a promising tool to analyse phytoplankton – bacterioplankton interactions.

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

Abstract

We investigated the bacterioplankton dynamics at Helgoland Roads in the North Sea over the winter-spring transition. The bacterial community was analysed and correlated with phytoplankton community data and abiotic parameters. The bacterial diversity was analysed by RISA (Ribosomal Intergenic Spacer Analysis) and by DGGE (Denaturing Gradient Gel Electrophoresis) of 16S rRNA genes followed by DNA sequence analysis. The linkage of abiotic and biotic environmental factors and bacterial community as well as phylotypes was analysed 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 by these factors. Overall, our 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 and linked with 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 seasonality of bacterioplankton community composition (Shiah & Ducklow, 1994; Pinhassi & Hagström, 2000; Gerdts *et al.*, 2004; Kent *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 major controlling factor in winter, autumn and spring, whereas a limitation of inorganic nutrients and substrate was regarded to be controlling factor in summer. Recent studies supported this coherence indicating a general limitation of bacterioplankton in the summer situation due to organic carbon and inorganic nutrients in the natural environment (Rivkin & Anderson, 1997) and in a mesocosm study (Øvreås *et al.*, 2003).

Additionally, several authors 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; Rooney-Varga *et al.*, 2005; Brussaard *et al.*, 2005) indicating close coupling of phytoplankton and bacterial community composition. A close link between bacterioplankton (especially of attached bacteria) with 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 on the phylogenetic level especially of attached bacteria.

Specific associations of microalgae and marine bacteria have already been described (Schäfer *et al.*, 2002; Grossart *et al.*, 2005), but in order to estimate the ecological relationship of bacterioplankton and phytoplankton, it must be elucidated which kind of interaction exists between both partners. Specific stimulative and inhibitory effects of isolated bacteria on algae have already been demonstrated by Fukami *et al*. (1997). Although the investigation of isolated bacteria offers new insights into their physiological capacities and symbiotic relationships it does not represent the whole spectrum of bacteria. Only 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. In detail, they found that specific *Roseobacter* and *Flexibacter* species disappeared during the breakdown of the bloom indicating a strong association with *P. globosa*.

Here we try to elucidate the driving forces of 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 should be clarified.

We investigated the changes in the free-living and attached bacterial community with RISA (Ribosomal Intergenic Spacer Analysis) and DGGE (Denaturing Gradient Gel Electrophoresis) of 16S rRNA genes followed by DNA sequence analysis. The linkage of abiotic and biotic environmental factors and community composition was analysed by the multivariate ordination method of Canonical Correspondence Analysis.

Methods

Study site, sample collection, abiotic and biotic factors

Samples were collected twice weekly from one meter depth 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 winter to spring situation 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. 25 ml of 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 one litre of the seawater was filtered through 3 µm and 0.2 µm membrane filters (Millipore, Germany) in succession. Filters were stored at -20°C until DNA extraction.

DNA was extracted from cut filters by a modified standard protocol of Anderson & McKay (1983) omitting the NAOH step. Briefly, the cell lyses was facilitated by adding lysozyme (1 mg ml^{-1}) and SDS (1 %). DNA extraction was carried out using phenol/chloroform/isoamylalcohol (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 (IGS) between the 16S and 23S subunits of ribosomal sequences we used the primers S-D-Bact-1522-b-S-20 (5'- TGC GGC TGG ATC

CCC TCC TT -3') and L-D-Bact-132-a-A-18 (5'- CCG GGT TTC CCC ATT CGG -3') (Ranjard *et al.*, 2000a; Ranjard *et al.*, 2000b). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf, Germany), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer, Germany), 0.5 µM of each primer, 2 U of Taq DNA Polymerase (Eppendorf) and 5 µl DNA of 0.2 µm filters or 0.5 µl of 3 µm filters. The amplification steps are displayed in Tab. 12. 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, Germany) in 0.5 x TAE buffer. Three lanes were used for 0.1 µg of a 100 bp ladder (invitrogen, Germany) in order to achieve comparability. Electrophoresis was run at 20°C for 18 h at 50 V using a DCode system (BioRad, Germany). Gels were stained with SYBRGold as recommended by Molecular Probes (invitrogen). Imaging was performed with the ChemiDoc XRS System of BioRad.

Table 12: Amplification of Ribosomal Intergenic Spacer

Step	Temperature [°C]	Time [minutes]	Cycles
1	95	3	1
2	95	1	25
3	53	1	
4	72	1	
5	72	5	1

Amplification of 16S rRNA genes and DGGE

PCR amplification of 16S rDNA 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 907rm modified (5'- CCG TCA ATT CMT TTR AGT TT -3'). PCR reaction mixtures with a volume of 100 µl contained 10 µl of 10 x Taq buffer (Eppendorf), 20 µl of 5 x Master Enhancer (Eppendorf), 300 µM of each dNTP (PerkinElmer), 0.2 µM of each primer, 2 U of Taq DNA Polymerase (Eppendorf) and 5 µl DNA of 0.2 µm filters or 0.5 µl of 3 µm filters. The "touchdown" PCR started with a denaturing step at 94°C for five minutes. Every cycle consisted of three steps each with one minute: 94°C, annealing temperature and 72°C. The initial annealing temperature of 65°C 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 minutes followed by 22 cycles starting at 71°C decreasing by 1°C per cycle in order to avoid heteroduplices. PCR reactions were performed in an Eppendorf Mastercycler. PCR products were inspected on 1.2 % (w/v) agarose gels. DGGE analyses 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 run at 60 °C and 150 V for 10 hours (Sigler *et al*. 2004). DGGE gels were stained with SYBRGold (see RISA). Imaging was performed with a ChemiDoc XRS System of BioRad.

DNA sequencing

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

Phylogenetic analysis

Sequence data were checked for the presence of PCR amplified chimeric sequences by 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 rDNA sequences database (release 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 neighbour joining method including 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 analyse the bacterial community during the sampling period. A general overview of bacterioplankton dynamics was achieved by the fingerprinting method 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 analyse the bacterial community on 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). Normalisation of RISA gels was performed by BioNumerics software using 100 bp ladders as references in every profile. Normalisation 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 correspondent band classes. We omitted DGGE fingerprints of attached bacteria from band matching because of bias due to plastid rDNA (see Tab. 16). The procedure resulted in band matching tables including densitometric values of fingerprints for both community analyses (Muylaert, 2002). These band matching tables were 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), therefore we performed Redundancy Analysis (RDA) as well as Canonical Correspondence Analysis (CCA) to compare species-environment correlations.

The data were not transformed prior to RDA or CCA. Explanatory variables included temperature, salinity, the concentration of anorganic 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 analyse 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 analyse 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 due 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 analysis with interspecies distances was calculated from the dataset derived from sequence data.

For all community ordination analyses biplot scaling was used.

Results

Environmental parameters, phytoplankton and bacterial cell counts

Temperature, salinity and the concentration of phosphate, nitrate, nitrite, ammonia and silicate were determined as abiotic factors whereas phytoplankton and bacterial cell counts covered biotic environmental parameters (Fig. 13). The water temperature increased constantly until the end of May from 4.9 °C to 10.5 °C despite slight variation. The values of salinity ranged from 29.33 to 33.51 and the values of the five major nutrients ranged from 41.10 to 5.54 µm for nitrate, from 2.11 to 0.02 µM for nitrite, from 3.77 to 0.23 µM for ammonia, from 2.04 to 0.03 µM for phosphate and from 14.03 to 0.28 µM for silicate. Lowest values of nutrients were obtained in May (Fig. 13).

Bacterial cell counts ranged from 3.69 10^6 cells ml^{-1} to 0.84 10^6 cells ml^{-1} with high variation during the sampling period. Generally, a decreasing trend could be observed from February to May (Fig. 13).

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, *Guinardia delicatula* (Cleve) Hasle. Additionally, the species *Chattonella spp*. belonging to the family of *Raphidophyceae*, the species *Phaeocystis spp*. belonging to the Heterokontophyta and not classified flagellates were counted (Fig. 13).

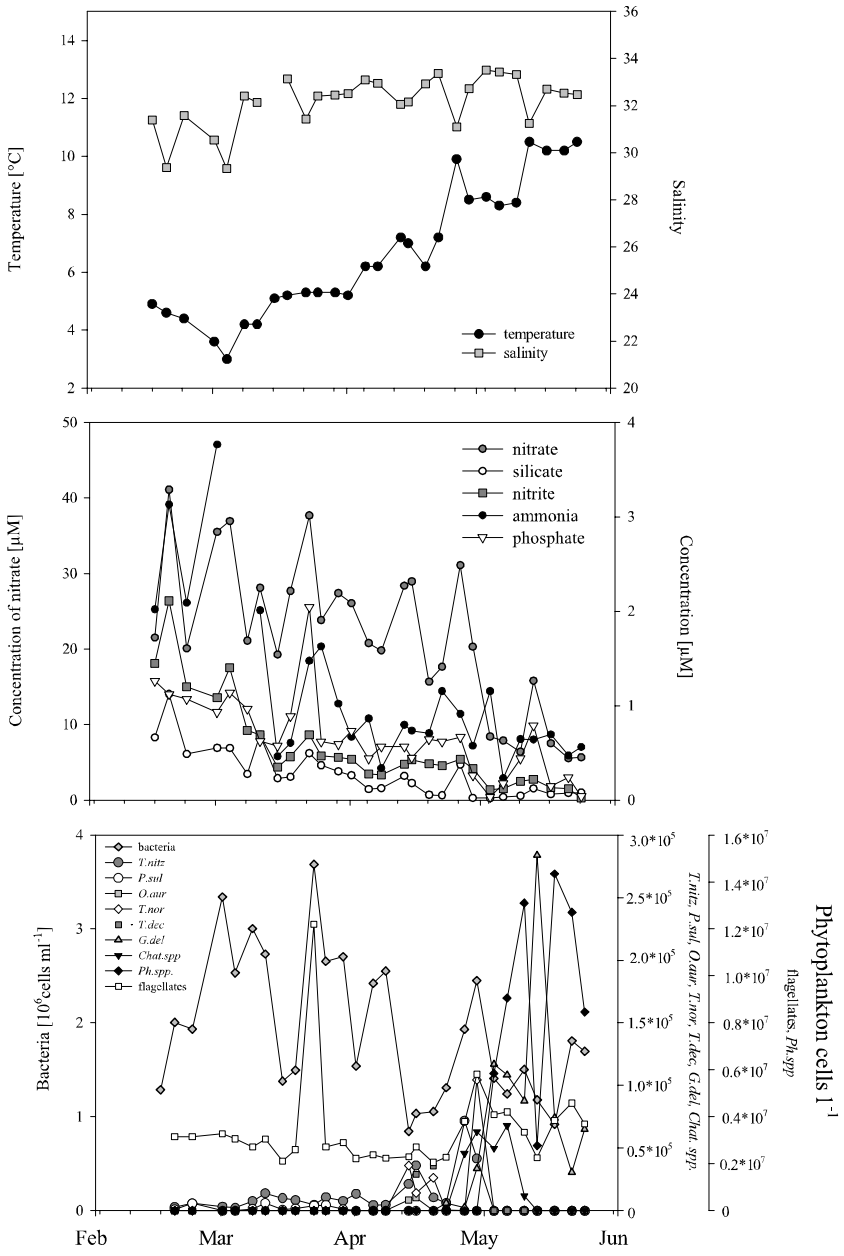

Figure 13: Environmental parameters, phytoplankton and bacterial cell counts including phytoplankton species. (*T. nitz: Thalassionema nitzschioides*, *P. sul: Paralia sulcata*, *O. aur: Odontella aurita*, *T. nor: Thalassiosira nordenskiöldii*, *T. dec: Thalassiosira decipiens*, *G. del: Guinardia delicatula*, *Chat. spp.: Chattonella spp*. and *Ph. spp.: Phaeocystis spp*.)

At the beginning of the sampling period mainly *T. nitzschioides*, *P. sulcata* and flagellates assembled the phytoplankton community. *T. nitzschioides* was observed until the end of April with the lowest value of 2.3 10^3 cells l^{-1} at the beginning of March and the highest value of 7.2 10^4 cells l^{-1} at the end of April. In May no cells of *T. nitzschioides* could be detected. *P. sulcata* was observed until the end of March with the lowest value of 800 cells l^{-1} and the highest value of 6.2 10^3 cells l^{-1} in March. Flagellates ranged from 3.0 10^6 cells l^{-1} with the highest value of 1.2 10^7 cells l^{-1} in March to 4.0 10^6 cells l^{-1} in May with the lowest value of 2.0 10^6 cells l^{-1} in April displaying high variation. *O. aurita* appeared in mid-April with 10^4 cells l^{-1}. At the end of April *O. aurita* reached 420 cells l^{-1} but disappeared afterwards. *T. nordenskiöldii* was also detected in mid-April in one week with a mean of 3.6 10^4 cells l^{-1}. It disappeared and was detected again at the end of April with 1.04 10^5 cells l^{-1}. *T. decipiens* could only be detected in mid-April within two weeks with cell counts of about 3.0 10^4 cells l^{-1}. *G. delicatula* occurred first at the end of April with the lowest value of 2.6 10^3 cells l^{-1} and the highest value in mid-May. *Chattonella spp*. was observed from the end of April to the beginning of May. This microalga reached the highest value of 6.8 10^4 cells l^{-1} and the lowest value of 1.2 10^4 cells l^{-1} in this period. *Phaeocystis spp*. occurred in May and was observed until the end of the sampling period. The numbers ranged between 2.7 10^6 cells l^{-1} and 1.4 10^7 cells l^{-1}.

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 ammonia, phosphate and silicate (Fig. 13) as well as 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 (Tab. 13). Although the difference was not pronounced it was assumed that unimodal methods would be more appropiate to analyse the large dataset. Generally, non-linear 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 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 variance inflation factor (> 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 Tab. 13. The sum of all unconstrained eigenvalues indicated an overall variance in the data set of 2.090. Total variation which could be explained by environmental variation accounted for 0.951 indicated by the sum of all canonical eigenvalues. Concerning the variance of species data the first axis explained 20.3 %, the first and the second axes explained 29.2 % and all four axes explained 37.3 % of the total variation (Tab. 13).

Table 13: Eigenvalues and variance decomposition for CCA and RDA

Community analysis CCA	**Axes**	**Eigenvalues**	**Species-environment correlations**	**Cumulative percentage variance of species data**	**Cumulative percentage variance of species-environment relation**
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	**Axes**	**Eigenvalues**	**Species-environment correlations**	**Cumulative percentage variance of species data**	**Cumulative percentage variance of species-environment relation**
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

Species-environment correlations were high, especially for axes 1 and 2 (0.944 and 0.908) indicating a relationship of species and environmental variables.

Biplot scaling of CCA is shown in Fig. 14. Canonical axes 1 and 2 are displayed in Fig. 14 A demonstrating a strong influence by 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 the canonical axis 1.

Axis 1 represented a strong gradient caused by the nominal variable filter indicated by the intraset correlation coefficient of 0.6052 what is also applicable for axis 2 which displayed an intraset correlation coefficient of -0.6477 (Tab. 14). The influence of this variable was determined as significant by a permutation test (Tab. 15). It became apparent that the variable filter had the highest value of lambda A of the conditional effects (Tab. 15) representing the highest additional variance what is 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 displayed. Especially the variables *Phaeocystis spp.*, *G. delicatula* and temperature contributed to the gradient indicated by the intraset correlation coefficients (Tab. 14). 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 indicated by the intraset correlation coefficients displayed in Tab. 14. The permutation test also showed no significance of this variable at the 5 % level ($p < 0.08$).

Table 14: 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

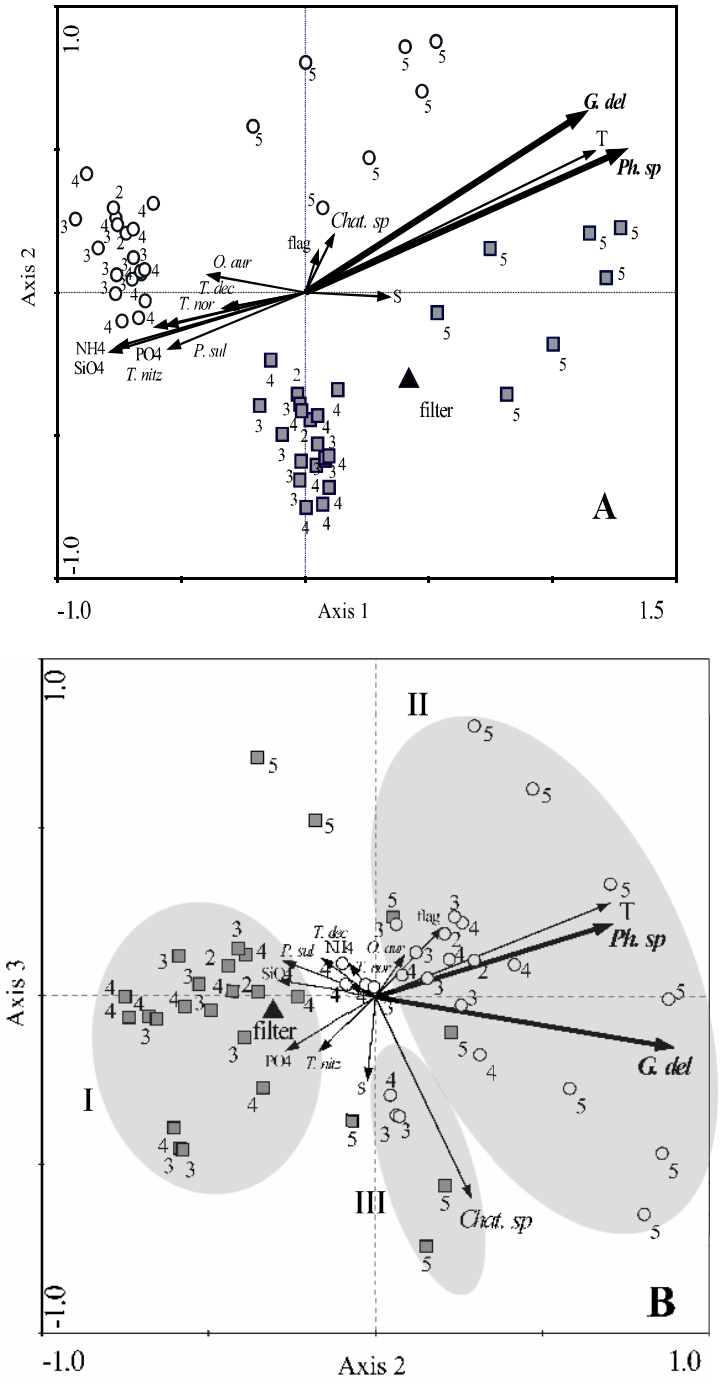

Figure 14: CCA of RISA profiles displaying attached and free-living bacterial communities using phytoplankton species cell counts of flagellates (flag), *Thalassionema nitzschioides* (*T. nitz*), *Paralia sulcata* (*P. sul*), *Odontella aurita* (*O. aur*), *Thalassiosira nordenskiöldii* (*T. nor*), *Thalassiosira decipiens* (*T. dec*), *Guinardia delicatula* (*G. del*), *Chattonella spp.* (*Chat. sp*) and *Phaeocystis spp.* (*Ph. sp*), salinity (S), temperature (T), the nutrients ammonia (NH4), phosphate (PO4) and silicate (SiO4) as well as the variable filter differentiating free-living and attached bacteria. Circles indicate free-living communities, filled squares indicate attached communities. Numbers near the symbols indicate the month of sampling (2: February, 3: March, 4: April, 5: May). Arrows reflect the direction of increasing values of the respective variable, the lenght of arrows indicates the degree of correlation of the variable with community data, significant variables are indicated by bold arrows, groups I, II, III of communities are indicated by grey background. A: Axes 1 and 2 of CCA biplot; B: Axes 2 and 3 of CCA biplot

However, temperature and the phytoplankton species *Chattonella spp*. contributed to the environmental variables explaining variation as shown by biplot scaling of canonical axes 2 and 3 (Fig. 14 B) indicated by the length of respective arrows.

Biplot scaling of canonical axes 2 and 3 (Fig. 14 B) showed that the attached bacterial communities deriving from samples of February, March and April are grouped together (group I, Fig. 14 B). This group displayed little influence by nutrients and phytoplankton species. Furthermore, some free-living and attached communities were combined (group II, Fig. 14 B). They were influenced by temperature, *Phaeocystis spp*., *G. delicatula* and *Chattonella spp*. In this group some free-living communities of May, April, March and February samples were mainly influenced by temperature, *Phaeocystis spp*. and *G. delicatula* whereas *Chattonella spp*. influenced some free-living communities of April and March samples and attached communities of May samples (group III, Fig. 14 B). It is obvious that especially communities of May samples were influenced by several factors. Summarising 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 which could be explained by the variables whereas all four axes explained 82 % of the variation (Tab. 13).

Table 15: Marginal and conditional effects of forwardly selected environmental variables produced by CCA

Community analysis	**Environmental variable**	**Marginal effects**	**Conditional effects**		
		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

Phylogenetic analysis

The most prominent DGGE bands which separated or connected the bacterial community were sequenced from excised bands. Sequence data of 45 excised bands could be obtained representing 36 different phylotypes (Tab. 16). Sequence data revealed the presence of three phyla of *Bacteria* (Fig. 15). Most sequences were related to members of *Proteobacteria* and members of the *Bacteroidetes* phylum. Within the *Proteobacteria, Alpha-* and *Gammaproteobacteria* were most abundant. Additionally, we found one member of the *Betaproteobacteria* and members of *Actinobacteria*. Furthermore, several chloroplast sequences were detected. Closest relatives of the sequenced bands derived from BLAST analyses are listed in Tab. 16. The results revealed many close matches with 98-100 % similarity to bacterial 16S rRNA gene sequences in the GenBank.

Comparison of sequence data of excised bands appearing at the same position in DGGE gel revealed identical closest relatives in most cases (e.g. bands F111 and F122, F120 and F200 as well as 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 obtained from the same positions in DGGE gels (Tab. 16). Generally, 44 % and 11 % of the sequenced bacterial phylotypes were assigned to the *Alpha-* and *Gammaproteobacteria*, respectively. 36 % of the phylotypes were assigned to the *Flavobacteria* whereas the *Actinobacteria* accounted for 5.5 % of sequenced bands. Additionally, the member of the *Betaproteobacteria* which was observed in several spring communities accounted for 2.8 % of sequenced bacterial phylotypes.

Proteobacteria

A neighbour joining tree of the *Alphaproteobacteria* revealed that the majority of sequences belonged to the *Roseobacter* clade (65 %). 35 % belonged to clusters of SAR116 and SAR1 phylotypes. Gene sequences of the *Gammaproteobacteria* were all assigned to the *Oceanospirillales* whereas the phylotype classified as a member of *Betaproteobacteria* belonged to the *Burkholderiales* (Fig. 15). Members of the *Alphaproteobacteria* contributed to both fractions of attached and free-living bacteria whereas the member of *Betaproteobacteria* as well as the *Gammaproteobacteria* detected in this study belonged solely to the free-living bacteria (Tab. 16).

Table 16: Relatedness of bacteria to known organisms

DGGE band	fraction	Phylogenetic group	Closest relative	Similarity [%]	Based positions compared	Accession number of closest relative
F067*[1]	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*[1]	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*[2]	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium, clone 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

*[1] phylotypes of same position in DGGE profile but different sequence data within *Alphaproteobacteria*
*[2] phylotypes of same position in DGGE profile but different sequence data within *Bacteroidetes*

Table 16 continued:

DGGE band	fraction	Phylogenetic group	Closest relative	Similarity [%]	Based positions compared	Accession number of closest relative
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*[2]	Free	*Flavobacteria*	Uncultured *Bacteroidetes* bacterium B12	94	529	AF466917
F086	Att	*Flavobacteria*	Marine psychrophile *Bacteroidetes* 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		unkown			
F124	Free		unkown			
F187	Free		unkown			
F192	Free		unkown			
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 eukariote ANT18/2_25	99	532	AY135677
F169	Att	plastid	Uncultured phototrophic eukariote 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

*[2] phylotypes of same position in DGGE profile but different sequence data within *Bacteroidetes*
ne*: not excised bands

Bacteroidetes and Actinobacteria

Within the *Bacteroidetes* phylum sequenced phylotypes clustered with the *Flavobacteria* (Fig. 15). Those phylotypes were part of both the attached and free-living bacterial community.

Two phylotypes of the free-living fraction were classified as *Actinobacteria* with high similarities to already described uncultured bacteria namely “uncultured bacterium ARKIA-43” and “uncultured Actinobacterium PI_RT340” (Fig. 15).

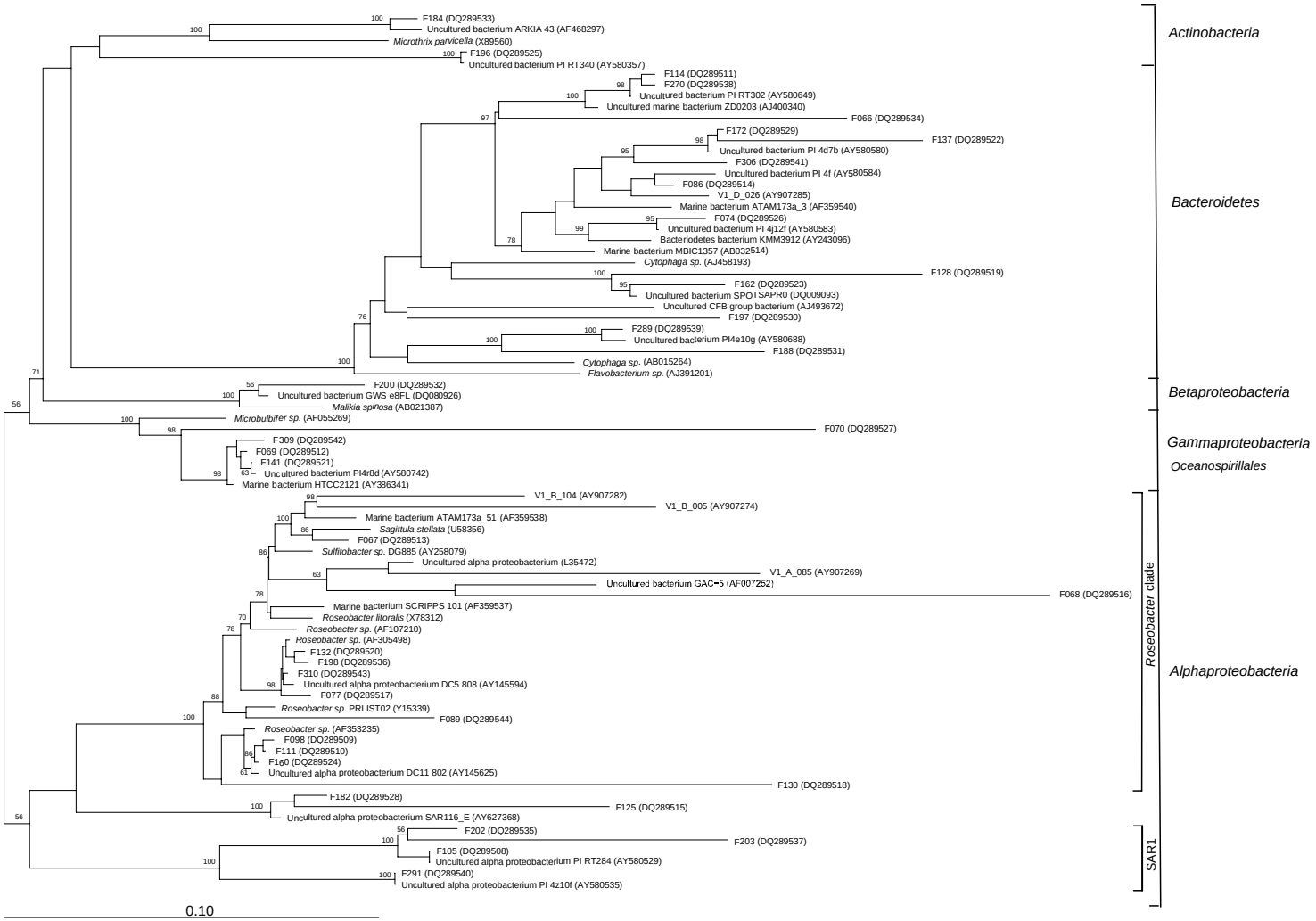

Figure 15: Phylogenetic tree of *Alpha-*, *Beta-* and *Gammaproteobacteria, Actinobacteria* and members of *Bacteroidetes*. GenBank accession numbers are given in parentheses. Bootstrap values above 50 % are displayed.

Succession of free-living bacterial species and Community Ordination Analysis based on 16S rDNA 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 displayed in Tab. 17. At the beginning of the sampling period the community was mainly composed of members of *Alphaproteobacteria* (F202, F203, F105, F111, F132, F068, F125, F182). Additionally, members of *Flavobacteria* (F114, F188, F197), members of *Actinobacteria* (F184, F196), members of *Beta-* and *Gammproteobacteria* (F200, F141) and unknown phylotypes (F187, F192, F02ne) were detected.

Some phylotypes were predominant during the whole sampling period (F111, F068, F187, F182, F141, F125, F114) whereas some phylotypes disappeared in March or April (F202, F203, F105, F188, F192, F02ne, F184). In April, additional members of the *Alphaproteobacteria* (F291, F130) and the *Flavobacteria* (F074, F137, F128) could be detected whereas a member of the *Gammproteobacteria* (F069) and two unknown phylotypes (F070, F01ne) were first detected in May samples. During this period *Actinobacteria* (F196), *Flavobacteria* (F197) and *Betaproteobacteria* (F200) were not found anymore.

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. The 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 (Tab. 13) we used weighted averaging to analyse 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 counts, salinity, temperature, the nutrients ammonia, nitrate, nitrite and phosphate as explanatory variables (Fig. 13). 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, Tab. 13) represents a gradient due to the environmental factors temperature, nitrite and *Phaeocystis spp*. as indicated by their intraset correlation coefficients (Tab. 14). The overall variance in the data set accounted for 0.894, whereas the total variation explained by environmental variation accounted for 0.721. Additionally, the first axis explained 47.0 %, the first and second axes together explained 61.2 % and all four axes explained 71.2 % of the total variation (Tab. 13).

Phylotype-environment correlations were high especially for axes 1 and 2 (0.981 and 0.911) indicating a relationship of phylotypes and environmental variables. 75.9 % of the variation could be explained by the first two axes together whereas all four axes explained 88.3 % of the variation (Tab. 13). Biplot scaling of CCA of DGGE fingerprints is shown in Fig. 16. The analysis based on intersample distances revealed a group of free-living communities in February and March mainly influenced by nutritional factors and the phytoplankton species *P. sulcata* (group I, Fig. 16). Free-living communities of April and May appeared in separate groups. Salinity and the species *Chattonella spp*. had a minor influence on the communities obtained from April samples whereas the phytoplankton species *O. aurita*, *T. decipiens*, *T. nordenskiöldii* and *T. nitzschioides* influenced this group to a greater extent (group II, Fig. 16). The communities obtained from May samples were also grouped together and were generally influenced by the factors *Phaeocystis spp*. and *G. delicatula* (group III, Fig. 16). A small influence could also be observed by the factor temperature.

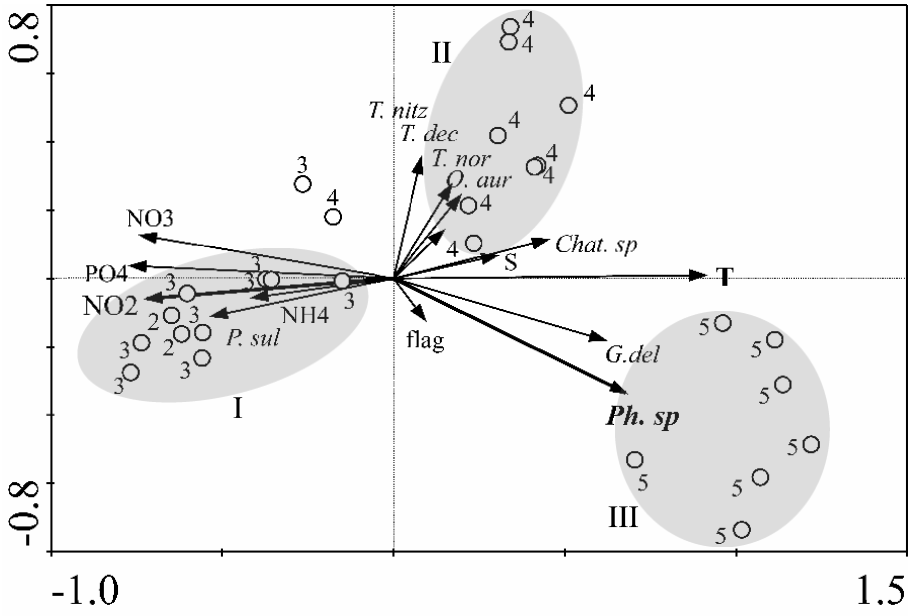

Figure 16: CCA biplot of intersample distances of DGGE fingerprints of the free-living bacterial community using phytoplankton species cell counts of flagellates (flag), *Thalassionema nitzschioides* (*T. nitz*), *Paralia sulcata* (*P. sul*), *Odontella aurita* (*O. aur*), *Thalassiosira nordenskiöldii* (*T. nor*), *Thalassiosira decipiens* (*T. dec*), *Guinardia delicatula* (*G. del*), *Chattonella spp*. (*Chat. sp*) and *Phaeocystis spp*. (*Ph. sp*), salinity (S), temperature (T), the nutrients nitrate (NO3), nitrite (NO2), ammonia (NH4) and phosphate (PO4). Circles indicate free-living communities, numbers near the symbols indicate the month of sampling (2: February, 3: March, 4: April, 5: May). Arrows reflect the direction of increasing values of the respective variable, the lenght of arrows indicates the degree of correlation of the variable with community data. Significant variables are indicated by bold arrows, groups I, II, III of communities are indicated by grey background.

Significant influence on the sample variation was retrieved by three factors namely temperature, nitrite and *Phaeocystis spp*. based on the 5 % level performing a partial Monte Carlo permutation test (Tab. 15). Both marginal and conditional effects are displayed in Tab. 15. 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 revealed four groups of phylotypes and two bands which could not be grouped with the other bands (Fig. 17).

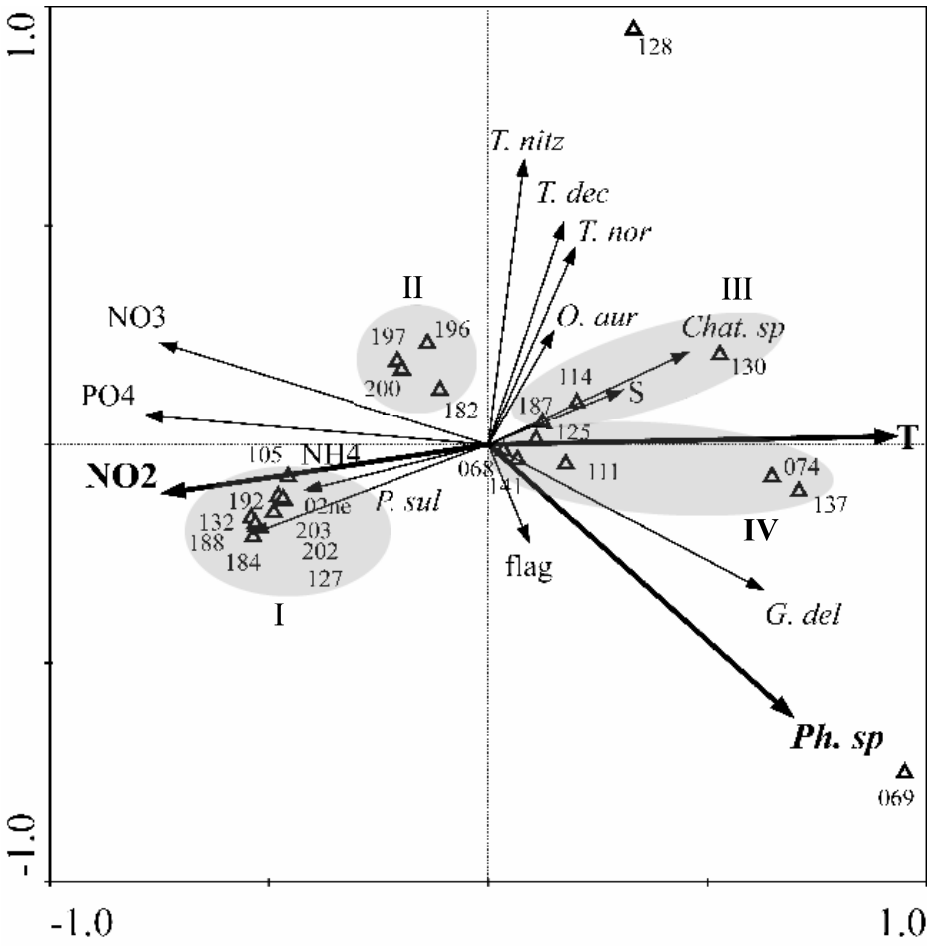

Figure 17: Biplot of interspecies distances, CCA of DGGE fingerprints of the free-living bacterial community using phytoplankton species cell counts of flagellates (flag), *Thalassionema nitzschioides* (*T. nitz*), *Paralia sulcata* (*P. sul*), *Odontella aurita* (*O. aur*), *Thalassiosira nordenskiöldii* (*T. nor*), *Thalassiosira decipiens* (*T. dec*), *Guinardia delicatula* (*G. del*), *Chattonella spp*. (*Chat. sp*) and *Phaeocystis spp*. (*Ph. sp*), salinity (S), temperature (T), the nutrients nitrate (NO3), nitrite (NO2), ammonia (NH4) and phosphate (PO4). Triangles with respective numbers indicate sequenced bands, the suffix "ne" not sequenced bands. Arrows reflect the direction of increasing values of the respective variable, the lenght 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 grey background.

Group I included the bands F202, F203, F132 and F105 (*Alphaproteobacteria*), F188 (*Flavobacteria*), F184 (*Actinobacteria*), F127 (plastid) and the unknown bands F192 and F02ne. This group was mainly influenced by nutrients (nitrite) and *P. sulcata*. Group II consisted of the bands F182 (*Alphaproteobacteria*), F197 (*Flavobacteria*), F200 (*Betaproteobacteria)* and F196 (*Actinobacteria*). This group did not show any influence due to a variable included in our dataset. Little influence was displayed by salinity and the phytoplankton species *O. aurita* and *Chattonella spp*. on group III including the bands F114, F128 (*Flavobacteria*), F130 (*Alphaproteobacteria*) and the unknown band F187. Phylotypes positively influenced by the phytoplankton species *Phaeocystis spp.* and *G. delicatula* as well as temperature were the bands of group IV consisting of the bands F141 (*Gammaproteobacteria)*, F068, F111 and F125 (*Alphaproteobacteria*) as well as the bands F074 and F137 (*Flavobacteria*). The factors affecting bacterial species were those obtained in CCA based on DGGE fingerprints of the free-living community (Fig. 16). However, the phylotype F069 (*Gammaproteobacteria*) appearing at the beginning of May (Tab. 17) was strongly affected by the occurrence of *Phaeocystis spp*. indicated by CCA (Fig. 17).

Table 17: Succession of free-living bacterial species during sampling period, filled boxes indicate appearance, unfilled boxes indicate absence of respective phylotype. Order 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	unknown																											■	■

*ne: not excised bands

Discussion

The linkage of phytoplankton and bacterioplankton dynamics demonstrated by several investigations (Rooney-Varga *et al.*, 2005; Brussaard *et al.*, 2005) is still not well understood. Until now, it is assumed that there are specific factors and effects controlling specific bacterial populations. The investigations 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 winter-spring transition. Like other studies, we found temperature to be the most important factor influencing bacterioplankton composition of Helgoland Roads over the winter-spring transition 2004. Additionally, the phytoplankton species *Phaeocystis spp*. and *G. delicatula* displayed strong effects on the bacterial community during winter-spring transition displacing the influence of abiotic towards an influence of biotic factors as indicated by CCA (Fig. 14 B).

Community Ordination Analysis

We used two different fingerprinting methods to analyse the bacterial community. The fingerprinting method RISA was performed to observe differences between the bacterial communities during the sampling period (Ranjard *et al.*, 2000; Fisher & Triplett, 1999; Ranjard *et al.*, 2001) and to correlate these with environmental variables whereas the fingerprinting method DGGE was performed to analyse changes of particular bacterial populations in relation to environmental variables.

Although results of DCA did not indicate clear unimodal analyses, CCA was performed to analyse species-environment correlations as results of RDA displayed lower correlation values. Therefore, CCA with RISA profiles and environmental data has been determined in order to link changes in the bacterial community to specific environmental factors.

In general, the separation of the attached and free-living community became evident as a strong effect on the analysis (Fig. 14 A). This is indicated by high intraset correlation coefficients (Tab. 14). Distinct differences between attached and free-living bacteria have already been observed by DeLong *et al*. (1993) and Fandino *et al*. (2001). Additionally, those differences were demonstrated in a multivariate analysis by Rooney-Varga *et al*. (2005).

Generally, the influence of the measured environmental variables appeared to be stronger on the free-living fraction of the bacterial community than on the attached community except for some attached communities in May influenced by *G. delicatula* or *Chattonella spp*. (Fig. 14 B). The influence of the environmental factors *Phaeocystis spp*. and *G. delicatula* on the

bacterial community was determined to be significant (Tab. 15) indicating a structuring role by these phytoplankton species. This has already been described for *Phaeocystis spp*. in culture experiments (Janse *et al.*, 2000). The authors stated that bacteria with specific enzyme capacity might be favoured 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 what might support the finding that *Phaeocystis spp.* probably has a structuring role.

Beside the occurrence of *Phaeocystis spp*. and *G. delicatula* it can also be assumed that temperature and *Chattonella spp*. contributed to the shifts in the bacterial community although the significance test reached not the 5 % level ($p < 0.08$). Especially temperature played a major role as it influenced mainly free-living bacterial communities in May (Fig. 14 B). This aspect has already been described by Pinhassi & Hagström (2000) who observed a relation of temperature and bacterial production. Furthermore, an influence on bacteria by temperature has been described by Shiah & Ducklow (1994) and Šestanović *et al*. (2004). However, we could not ascribe a significant factor shaping the attached communities in samples of 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 that we focussed on differences between the bacterial communities determined by RISA.

The influence of environmental factors on the free-living community was analysed in detail on the basis of DGGE fingerprints in contrast to RISA. The May communities (group III) appeared to be very different from those in winter as indicated by the distance in the CCA biplot (Fig. 16). Both temperature and nutrients (nitrite) displayed significant conditional effects in CCA of DGGE fingerprints indicating an important influence on the free-living community. This finding is supported by studies of Shiah & Ducklow (1994), Rivkin & Anderson (1997), Pinhassi & Hagström (2000), Øvreås *et al*. (2003) and Šestanović *et al*. (2004). In their studies, seasonal effects were apparent with temperature being the limiting factor in colder periods and inorganic nutrients limiting the community when temperature was no longer limiting. In this study, temperature affected both the groups of April and May samples.

The May sample group was strongly influenced by the appearance of *Phaeocystis spp*.. Its influence was calculated as a significant conditional effect on the free-living community. This finding supports the results of CCA based on RISA profiles emphasising the strong effect of *Phaeocystis spp*. as shown by Janse *et al*. (2000).

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

The analysis based on interspecies distances revealed the influence of different factors on specific phylotypes which were separated into four groups. An influence by nutrients was observed inter alia for three phylotypes of the *Alphaproteobacteria* (F105, F202, F203) in group I (Fig. 17) being closely related to a member of the *Alphaproteobacteria* belonging to the SAR1 cluster (Fig. 15), which was obtained 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. Additionally, the phylotypes F132 (*Alphaproteobacteria*), F188 (*Flavobacteria*), F184 (*Actinobacteria*), F127 (plastid) and the unknown bands F192 and F02ne were also influenced by nutrients in particular. These phylotypes could be detected in the first part of the sampling period and it is suggested that they contributed to a specific "winter" community (Tab. 17). Group II was not influenced by any of the variables (F200, F196, F197, F182). It can be assumed that other factors influencing this group were not included in our dataset. Additionally, it should be considered that *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).

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. However, *Actinobacteria* and *Betaproteobacteria* disappeared in May and were no longer detected in this group of phylotypes (Tab. 17). A member of the *Gammaproteobacteria* belonging to the *Oceanospirillales* (F069) occurred in the beginning of May. Especially the appearance of this *Gammaproteobacterium* might be directly linked to the bloom of *Phaeocystis spp*.. It can also be assumed that the occurrence of this alga influenced the member of *Betaproteobacteria* (F200) as its appearance and the absence of *Phaeocystis spp*. coincided (Fig. 17, Tab. 17). Additionally, phylotypes appearing in group IV (Fig. 17) might have been positively influenced by the algae *Phaeocystis spp*., *G. delicatula*, *Chattonella spp*. and the factor temperature.

The phylotypes retrieved in this study belonged mainly to the group of *Alphaproteobacteria.* Within this group the phylotype F068 clustered with the sequence ATAM 173a_51 associated with the toxic dinoflagellate *Alexandrium spp*. (Hold *et al.*, 2001), whereas the bands F111 and F130 clustered with the sequence DC11-80-2 obtained from the Weser estuary (Selje *et al.*, 2004) and *Roseobacter sp*. (AF353235) obtained from the Arctic Ocean (Bano & Hollibaugh, 2002). It is assumed that the bands F111 (*Roseobacter* clade) and F125

(SAR116) were mainly influenced by the increase of temperature whereas the phylotype F130 (*Roseobacter* clade) was mainly influenced by *Chattonella spp*. and salinity.

Members of *Flavobacteria* also contributed to the bacterioplankton community. They showed an influence due to *Phaeocystis spp*. and the increase of temperature (F114, F074, F137). Two of them displayed high similarity (99 %) to sequences obtained in the study of Acinas *et al*. (2004) indicating global distribution of those phylotypes. F074 and F137 (*Flavobacteria*) were mainly influenced by the increase of temperature. Furthermore, it is indicated that the phylotype F114 was mainly influenced by *Chattonella spp*. and salinity whereas the phylotype F128 was influenced by the phytoplankton species *T. decipiens*, *T. nordenskiöldii* and *T. nitzschioides.*

Within the group of *Gammaproteobacteria* globally distributed phylotypes were also detected. The phylotype F069 which was strongly influenced by *Phaeocystis spp*. clustered with the sequence HTCC2121 obtained from the Pacific Ocean (Cho & Giovannoni, 2004) and the phylotype F141 with a sequence found in the Plum Island Sound estuary (Acinas *et al*., 2004). This phylotype was not strongly influenced by *Phaeocystis spp*. or *G. delicatula* (Fig. 17).

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; v. 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 µm (Tab. 17). Those profiles of attached bacterial communities were not analysed by ordination. Therefore, we could not consider the factors influencing those bacteria which appeared to be attached to the phytoplankton on the level of phylotypes. This is true for the sequences F086 and F289 (*Flavobacteria*) obtained only from the fraction of attached bacteria but also for the sequences from both fractions (F077, F089, F098, F160, F310). The latter sequences were closely related to a member of *Rhodobacteraceae* namely AP-27 (AY145564) and the uncultured *Alphaproteobacterium* DC11-80-2 both observed in the Weser estuary (Selje *et al.*, 2004). It has to be considered that especially *Flavobacteria* as particle colonisers are thought to participate in degradation of organic matter (Pinhassi *et al.*, 2004; Abell & Bowman, 2005). Additionally, members of the *Roseobacter* clade of *Alphaproteobacteria* are thought to play a similar role colonising a broad range of particles under algal bloom conditions (Pinhassi *et al.*, 2004). 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 µm > 0.2 µm). This was intentionally included in the analysis in order to monitor its influence on the ordination which was estimated to be low (Fig. 17). The sequence was similar to the 16S rDNA gene of a plastid of *Prasinophyceae* (similarity of 98 %) and was grouped with those phylotypes which were influenced by nutrients (nitrite) and *P. sulcata*.
Generally, the total variation in bacterioplankton dynamics which could be explained by environmental variation accounted for 0.951 for CCA based on RISA fingerprints and for 0.721 for CCA based on DGGE fingerprints. Therefore, the factors which had been measured in our study were able to explain a large part of variation occurring in the bacterial community. However, the factors of grazing by ciliates or nanoflagellates as well as control by viruses could not be considered. The importance of those factors had been demonstrated in different studies. Šestanović *et al*. (2004) showed that heterotrophic nanoflagellates controlled planktonic bacteria of the Adratic Sea in the period of spring to summer replacing the control of 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 might contribute to a high extent to the microbial food web. Additionally, Del Giorgio *et al*. (1996) could demonstrate the control of total number of bacteria by heterotrophic nanoflagellates in dialysis experiments. The control of bacterial abundance by viruses has been shown by several studies (Weinbauer & Peduzzi, 1995; Winter *et al.*, 2004) and it is suggested that viruses might have a stronger effect on bacterial abundance under certain conditions than grazing of heterotrophic nanoflagellates (Weinbauer & Peduzzi, 1995).

Conclusion

This study showed seasonal succession and dynamics of bacterioplankton in winter-spring transition supporting the study of Gerdts *et al*. (2004). Additionally, an influence of several factors on the bacterioplankton could be shown especially for temperature emphasising its important role over 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 could also show an influence of factors affecting specific bacterial phylotypes. Particularly, the positive influence of *Phaeocystis spp*. on a member of the *Gammaproteobacteria* has been shown demonstrating the strong influence of this alga on specific phylotypes of the bacterial community.

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General Discussion

In this study, the specificity of the association between bacteria and phytoplankton was investigated in culture experiments. It was elucidated that *Proteobacteria* and members of the *Bacteroidetes* phylum are associated with microalgae considered to be key-species of Helgoland Roads, North Sea. Generally, species-specific associations could not be detected. The influence of diatoms and their exudates on the associated bacterial community was studied in detail and according to the results estimated to be low. It was further observed that the associated bacterial community is exposed to shifts due to isolation and cultivation of microalgae making it necessary to scrutinise results obtained from culture experiments investigating bacteria-microalgae associations.
The influence of phytoplankton on the bacterial community was also studied *in situ*. It was shown that the bacterioplankton community is influenced by seasonal effects during the winter-spring transition. Changes in inorganic nutrients, temperature and phytoplankton abundance governed a major part of bacterioplankton community shifts.

The association of bacteria and microalgae

Specificity

A culture experiment conducted with diatoms and dinoflagellates considered to be key species at Helgoland Roads showed that *Alpha-* and *Gammaproteobacteria* as well as *Flavobacteria* were associated with the key microalgae. This finding is supported by several studies as *Proteobacteria* and members of the *Bacteroidetes* phylum were detected in other microalgae cultures as well as phytoplankton blooms (Janse *et al.*, 2000; Riemann *et al.*, 2000; Hold *et al.*, 2001; Schäfer *et al.*, 2002; Pinhassi *et al.*, 2004; Green *et al*., 2004; Wichels *et al.*, 2004; Brussaard *et al.*, 2005; Jasti *et al.*, 2005). It is apparent that especially members of the *Roseobacter* clade are associated with phytoplankton (Riemann *et al.*, 2000; Hold *et al.*, 2001; Schäfer *et al.*, 2002; Green, 2004; Wichels *et al.*, 2004; Jasti *et al.*, 2005). Furthermore, the role of *Bacteroidetes* in the degradation of algal blooms can be emphasised (Riemann *et al.*, 2000; Pinhassi *et al.*, 2004). In addition, the inoculation of quasi axenic algal cultures with seawater resulted in a microalgal associated community composition comparable with the above mentioned bacterial groups (Grossart *et al.*, 2005). Therefore, it is concluded that

members of these bacterial groups generally play an important role in the interaction of bacteria and phytoplankton.

In contrast to experiments carried out with algal cultures from culture collections this study was conducted with freshly isolated algal cultures. Therefore these findings allow first insights into the *in situ* situation. Taking into account that *Alpha-* and *Gammaproteobacteria* as well as members of the *Bacteroidetes* phylum are the major groups in the marine environment (Giovannoni & Rappé, 2000) it is likely that species belonging to these groups made up the habitat known as the "phycosphere". This habitat can be defined as an area where bacteria and phytoplankton interact (Bell *et al.*, 1974). The "phycosphere" can be considered to be a microhabitat where algal cells defend themselves against colonisation and bacteria potentially utilise algal exudates (Cole, 1982). These interactions of bacteria and phytoplankton are of great importance especially for biogeochemical cycling (Azam, 1998). Recently, specificity of the association between bacteria and phytoplankton has been discussed (Grossart *et al.*, 2005; Jasti *et al.*, 2005). In contrast, in this study, species-specific associations of bacteria and microalgae could not be detected in general. Especially the concurrent identification of members of the *Roseobacter* clade in several diatom and dinoflagellate cultures contradicts the hypothesis of a species-specific association (Fig. 6). Additionally, the same algal species might harbour different bacterial populations as shown for *T. rotula* isolated in spring and in summer (Chapter II, this volume) supporting the idea that specificity might not occur at the species level.

Bacteria in the phycosphere

An adaptation of bacteria to different algal extracellular products has been hypothesised by Bell (1983). This suggestion could be confirmed for three diatom species by the resource competition experiments presented in Chapter III (this volume). Therefore, it is suggested that different bacterial populations are adapted to "phycospheres" of different diatoms. Further studies should investigate whether dinoflagellate-associated bacteria are also adapted to different "phycospheres". Furthermore, the shaping influence of diatom-derived exudates on diatom-associated bacterial communities could be neglected. If bacteria are highly specialised for the uptake and utilisation of compounds derived from algal exudates it seems likely that one could detect an influence of these different exudates on the associated bacterial community. Bell (1983) could demonstrate that bacteria adapted to exudates of a diatom or a flagellate were also able to utilise the exudates of other microalgae. These findings indicate that bacteria are not generally specialised to utilise specific exudates. It was therefore hypothesised that "phycosphere" associated bacteria are able to adapt to different exudate

compounds. Specific bacterial communities might be shaped by the "phycospheres" of the investigated diatoms with a specific composition of exudates, but at the same time, the bacterial communities might keep their ability to adapt to new conditions. Assuming that the "phycosphere" serves as ecological niche for associated bacteria these might have similar nutritional demands. Therefore, it is suggested that these species have overlapping niches. Generally, groups of species with similar nutritional demands belong to ecological guilds (Odum, 1999), if they live in the same or an overlapping niche. Those diatom-associated bacterial species which are able to adapt to different "phycospheres" might play similar roles in the community. The concept of the functional niche (Rosenfeld, 2002) emphasises that a niche can be defined by functional not only by spatial characteristics. If this concept is applied here, several bacterial species associated with diatoms might have functional overlap. These would be even functionally redundant, if the species have the same physiological capacities.

Concerning diatom-associated bacteria it is suggested that the diatom "phycosphere" is a suitable habitat for a broad range of species. These might tolerate a wide range of resources what would support the hypothesis that bacteria associated with diatoms might be generalists. Theoretically, these bacteria might be able to use POM when they are particle-associated and DOM when they are free-living as suggested by Miki & Yamamura (2005). This assumption would also explain the occurrence of most species as free-living and attached in the culture experiment (Tab. 3). These physiological adaptations need to be investigated in further studies.

Phytoplankton associated bacteria live in a microenvironment where the consumption of biomolecules has to be performed inter alia by extracellular hydrolysis. This implies certain adaptations of these bacteria. General differences were already observed for attached and free-living bacterial populations in the seawater (DeLong *et al.*, 1993). It is likely that physiological adaptations of attached bacteria are needed for the colonisation of particles and algal cells.

Apart from this, a correlation of extracellular enzyme activity combined with phylogenetic changes during the collapse of a bloom indicates specialisation of bacteria which utilise algal products (Arrieta & Herndl, 2002). A metabolic response to substrate enrichment has also been observed during a dinoflagellate bloom (Fandino *et al.*, 2001). The authors observed physiological and phylogenetical differences within attached and free-living bacteria supporting the statement of specialisation of attached bacteria. Similar results could be obtained by Middelboe *et al.* (1995) and Riemann *et al.* (2000). In this study no physiological characteristics of the bacterial communities were included. Nevertheless, assumptions

concerning physiological adaptations can be made from the results as the bacteria derived from diatom-attached bacteria. This is ensured by the technique of micropipetting (Daste *et al.*, 1983). The algal cells were washed sequentially in many steps before being cultivated. It is suggested that this process removed free-living bacteria from the algal cells and left only those bacteria which were strongly associated with the single algal cell at the time of isolation. Consequently, the free-living bacteria in the culture were originally most likely derived from attached bacteria. Therefore, it can be assumed that the identified bacteria are physiologically adapted to the habitat "phycosphere". Nevertheless, it was observed that attached and free-living bacteria were clearly separated in some cultures studied in the first culture experiment (Chapter II, this volume). Most of the phylotypes found only in the fraction of attached bacteria belonged to the group of *Flavobacteriaceae* whereas a smaller proportion of *Gammaproteobacteria* was ascribed only to this fraction. Within the *Alphaproteobacteria* only one phylotype was solely found in the fraction of attached bacteria. Therefore, it is concluded that members of the *Flavobacteria* and *Gammaproteobacteria* might be more specific regarding the choice of habitat than *Alphaproteobacteria*. Despite these differences it could be suggested that the attached bacteria might be physiologically flexible, and that this may support the colonisation of the microalgal environment. This hypothesis is also supported by the results of the first culture experiment (Chapter II, this volume) as no differences in the association of bacteria and microalgae occurred due to different growth phases of the algae.

Regarding the treatments where the influence of exudates was studied, the bacterial community derived from mainly free-living bacteria (Chapter III, this volume) and during cultivation, a separation of the community into free-living and attached bacteria could be observed except for the treatments including the seawater community. Assuming aggregation of DOM to polymers (Verdugo *et al.*, 2004) in these treatments it is suggested that bacteria of the respective inoculum favoured the provided DOM as nutrient source and had the capacity to contribute to the formation of marine gels from DOM polymers and to attach to it. Marine gels can be formed of colloidal DOM polymers and provide microenvironments with properties different from the polymers (Verdugo *et al*. 2004). Specific species might be attracted by these microenvironments, nevertheless, it is not clear why a separation into free-living and attached bacteria occurred in the treatments with exudates but not in competition treatments. In both approaches the inocula included mainly free-living bacteria derived from the associated bacterial community, but the competition treatments contained diatom cells. It

is likely that the exudates in the competition treatments also form polymers, therefore, the separation of free-living and attached bacteria cannot be explained satisfactorily.

Cultivation shifts

In literature, cultivation of marine bacteria from a seawater sample is often accompanied by shifts in the community (Eilers *et al*., 2000). Therefore, it was hypothesised that shifts in the bacterial community composition might occur within the community structure of phytoplankton-associated bacteria during cultivation of the microalgae. In Chapter IV (this volume) this hypothesis was confirmed. Considering the shifts in the bacterial community detected during isolation and cultivation of microalgae, the results obtained from culture experiments might be distorted. However, the association of diatoms with *Proteobacteria* and members of the *Bacteroidetes* phylum could be confirmed. Shifts were detected from a common diatom community after isolation to communities different from the original community structure. Particularly the increase of *Gammaproteobacteria* but also the occurrence of different *Alphaproteobacteria* during different cultivation steps indicate that cultivation serves for different conditions in the culture which might favour different bacterial phylotypes. In detail, recultivation shifts dilute the culture and therefore favour different conditions. Generally, *Gammaproteobacteria* especially *Alteromonas sp*., *Colwellia sp*. and *Vibrio sp*. are known to benefit from high nutrient concentration in the cultivation medium (Eilers *et al.*, 2000). These bacterial species are thought to live "feast and famine" as stated by Eilers *et al*. (2000). In the context of the results of the cultivation experiment it is assumed that *Gammaproteobacteria* are associated with algal cells but their abundance was too low to detect these phylotypes within the attached community *in situ* by the methods used in this study. During the isolation process these species were exposed to high nutrient concentrations which led to higher growth rates. It is concluded that these species are important for the association of bacteria and diatoms as members of the *Gammaproteobacteria* were detected in all studied cultures. This is supported by other studies on the association of bacteria and phytoplankton (Hold *et al.*, 2001; Pinhassi *et al.*, 2004; Wichels *et al.*, 2004; Jasti *et al.*, 2005; Grossart *et al.*, 2005). Furthermore, it has been demonstrated that especially *Alteromonas sp*. provided benefits for *Pseudonitzschia multiseries* cells in culture (Stewart *et al.*, 1997). In this study, the culture experiment and the monitoring of cultivation shifts revealed the presence of phylotypes clustering with *Sulfitobacter sp*. which have been found in association with microalgae supporting the findings of Schäfer *et al.* (2002) and Grossart *et al.* (2005). *Sulfitobacter pontiacus* is known to play a pivotal role in the sulfur cycle because of its ability to oxidise sulfite (Gonzáles & Moran, 1997) and thus the specific microenvironment provided

by microalgae might be a suitable habitat for species closely related to *Sulfitobacter pontiacus*.

Members of the *Actinobacteria* were identified in few samples during the cultivation experiment. The identified *Actinobacterium* appeared in the seawater sample from which the algal cells were isolated. Therefore, it is assumed that the ambient community might influence the composition of the associated bacterial community. As the association of *Actinobacteria* and phytoplankton has not been described before, it is suggested that these phylotypes do not play an important role in the interaction with diatoms. In future, the findings obtained from culture experiments dealing with the interaction of bacteria and microalgae should be scrutinised regarding cultivation impacts. It is concluded that it remains unknown which specific bacteria are important for the microalgae.

Seasonal dynamics

This study provided further insights into the control of seasonal succession and dynamics of bacterioplankton during the months February, March, April and May (winter-spring transition). Significant influence on the bacterial community could be shown for changes in temperature, nutrients and phytoplankton abundance. It is concluded that bacterioplankton dynamics are controlled by these factors and that the community structure is strongly characterised by seasonality what explains the strong shifts which occurred in the bacterioplankton of Helgoland Roads during this period. The latter is supported by the study of Gerdts *et al*. (2004) which already showed seasonality of bacterioplankton of Helgoland Roads.

The phylotypes obtained in this study belonged mainly to *Flavobacteria* and the group of *Alpha-* and *Gammaproteobacteria.* Moreover, gram-positive *Actinobacteria* were identified. These results support the finding that bacterioplankton species of coastal habitats and the open-ocean are similar (Giovannoni & Rappé, 2000). Furthermore, phylogenetic analyses revealed close matches with phylotypes identified in the Plum Island Sound estuary by Acinas *et al*. (2004) indicating cosmopolitan distribution of these phylotypes. Beside that, members of the *Betaproteobacteria* were identified, but it should be considered that *Betaproteobacteria* 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).

The linkage of phytoplankton and bacterioplankton dynamics has already been demonstrated by several investigations (Billen *et al.*, 1990; Rooney-Varga *et al.*, 2005; Brussaard *et al.*, 2005) but the control of these dynamics is poorly understood. Seasonal succession of

bacterioplankton has already been observed by Shiah & Ducklow (1994), Pinhassi & Hagström (2000), Gerdts *et al*. (2004) and Kent *et al*. (2004). As in other studies, temperature appeared to be the most important factor, apart from inorganic nutrients, influencing the bacterioplankton composition of Helgoland Roads over the winter-spring transition. Additionally, the phytoplankton species *Phaeocystis spp*. and *G. delicatula* displayed strong effects on the bacterial community displacing the influence of abiotic factors with biotic factors.

The measured environmental variables influenced mainly the free-living fraction of the bacterial community except for some attached communities in May. These latter were mainly affected by *G. delicatula* or *Chattonella spp*.. Distinct differences between attached and free-living bacteria have already been observed by DeLong *et al*. (1993) and Fandino *et al*. (2001) and were already supported in a multivariate analysis by Rooney-Varga *et al*. (2005). Therefore, it is likely that the factors influencing free-living and attached bacteria are different. Especially temperature and nutrients played a major role for free-living bacterial communities in the early period studied in this thesis. These findings are supported by several studies showing an influence of temperature or nutrients on bacteria (Shiah & Ducklow, 1994; Rivkin & Anderson, 1997; Pinhassi & Hagström, 2000; Øvreås *et al.*, 2003; Šestanović *et al.*, 2004). However, free-living bacteria were also strongly affected by the appearance of *Phaeocystis spp*. emphasising the strong effect of this phytoplankton species as shown by Janse *et al*. (2000). An influence of the phytoplankton species *G. delicatula* on the bacterial community was also shown. Especially the influence of *Phaeocystis spp*. is assumed to be strong as bacteria with specific enzyme capacity might be favoured by a bloom of this Heterokontophyte as it excretes complex mucopolysaccharides (Janse *et al.*, 2000). Furthermore, Brussaard *et al*. (2005) observed changes in the bacterial community structure and an increase in bacterial production during the breakdown of a *Phaeocystis* bloom in a mesocosm study which supports the finding that *Phaeocystis spp.* strongly influences bacterial populations.

In this study, it could be shown in detail, that specific phylotypes of free-living bacteria were influenced by different factors. An impact of nutrients was observed especially for *Alphaproteobacteria,* a *Flavobacterium* and an *Actinobacterium.* The respective phylotypes are assumed to contribute to a specific "winter" community. After a shift in the bacterial community the appearance of a *Gammaproteobacterium* might be directly linked to the bloom of *Phaeocystis spp*.. Additionally, phylotypes belonging to the May community might have been positively influenced by the algae *Phaeocystis spp*., *G. delicatula*, *Chattonella spp*. and

the factor temperature. Although the factors measured in this study explained the main variance in the dataset it could not be clarified which factors influenced a group of phylotypes consisting of an *Alphaproteobacterium*, a *Flavobacterium*, a *Betaproteobacterium* and an *Actinobacterium*. It is likely that the factors influencing these phylotypes were not included in the dataset. However, *Actinobacteria* and *Betaproteobacteria* disappeared in May and were no longer detected in the bacterioplankton. The factor which controlled this development could not be elucidated.

Due to methodical limitation, the factors influencing attached bacteria could not be considered on the level of specific phylotypes. Nevertheless, it is interesting that exclusively members of the *Flavobacteria* and the *Alphaproteobacteria* were found within the attached bacteria. It has to be considered that especially *Flavobacteria* as particle colonisers are thought to participate in degradation of organic matter (Pinhassi *et al.*, 2004; Abell & Bowman, 2005). Additionally, members of the *Roseobacter* clade of *Alphaproteobacteria* are thought to play a similar role colonising a broad range of particles under algal bloom conditions (Pinhassi *et al.*, 2004).

However, the factors of grazing by ciliates or nanoflagellates as well as the control by viruses could not be considered in this study although the importance of those factors had been demonstrated in different studies (Sherr & Sherr, 1987; Weinbauer & Peduzzi, 1995; Del Giorgio *et al.*, 1996; Šestanović *et al.*, 2004; Winter *et al.*, 2004).

Methodical approaches

Two different fingerprinting methods were used to analyse the bacterial community associated with microalgae and the bacterioplankton dynamics. The fingerprinting method RISA was performed to show differences between the bacterial communities (Fisher & Triplett, 1999; Ranjard *et al.*, 2000; Ranjard *et al.*, 2001) whereas the fingerprinting method DGGE with subsequent sequencing of 16S rRNA gene fragments was performed to analyse particular bacterial phylotypes. Beside the methodical limitation that amplification via polymerase chain reaction is the first step for both fingerprinting methods, the combination of these methods provides an excellent tool to study changes in bacterial communities in large datasets on different levels. The comparison of both methods within the analysis of bacterioplankton dynamics revealed good agreement concerning shifts in the bacterial community so that reliability of the data is provided by both methods. But each has potential advantages and disadvantages. Generally, a single band in a RISA profile might contain several species and a species could result in several bands (Ranjard *et al.*, 2001). Also a single

DGGE band might contain several species which has already been discussed elsewhere (Suzuki & Giovannoni, 1996; v. Wintzingerode *et al.*, 1997; Bidle & Azam, 2001). As RISA gels are easily compared by applying software for the analysis of fingerprints they provide a very good basis for statistical analyses of bacterial diversity. The information obtained from DGGE fingerprints with subsequent sequencing can also be used for statistical analysis but it is more time-consuming to get satisfactory results because of the missing linearity of the gradient in the gels.

However, the studies presented in this thesis combined fingerprinting methods and multivariate statistics to provide significant results. Especially the investigation of bacterial community dynamics related with phytoplankton dynamics is based on a new combination of methods. Several authors have already made approaches for analysing bacterioplankton and phytoplankton dynamics *in situ* (Middelboe *et al.*, 1995; Fandino *et al.*, 2001; Arrieta & Herndl, 2002; Wichels *et al.*, 2004) but a combination of different fingerprinting methods and multivariate statistics is promising. Especially the study of Rooney-Varga *et al*. (2005) as well as the present study demonstrated that a combination of fingerprinting methods and CCA provides an excellent tool for the analysis of phytoplankton – bacterioplankton interactions *in situ*.

The role of phytoplankton associated bacteria in the microbial loop

Within the pelagic food web the role of bacteria is not limited to a decomposer role. The concept of the microbial loop hypothesised that bacteria channel energy included within DOM and POM derived from primary production back to the food chain which consists mainly of phytoplankton and zooplankton as shown in a simplified scheme in Fig. 1 (Azam *et al.*, 1983). Thus, it is assumed that bacteria associated with microalgae participate in biogeochemical cycling and play an important part in the microbial loop by means of consumption of algal DOM and POM. In this context, the present study provides further insights into this relationship on a general level as well as for the food web of Helgoland Roads. *Alpha* - and *Gammaproteobacteria* as well as *Flavobacteriaceae* were primarily found as being associated with microalgae which are considered to be key species of Helgoland Roads. Therefore, it is suggested that members of these bacterial groups are important for the food web of the coastal area around Helgoland. Members of *Alpha* - and *Gammaproteobacteria* as well as *Flavobacteriaceae* are known to degrade dissolved and particulate organic matter (Cottrell & Kirchman, 2000) indicating that they generally play an important role in the microbial loop. A correlation was also found for silicate dissolution by

members of the *Gammaproteobacteria* and *Flavobacteria* - *Sphingobacteria* (Bidle *et al.*, 2002) supporting the importance of bacterial activity within biogeochemical cycling. Furthermore, it was elucidated that bacteria belonging to these phylogenetic groups are directly influenced by phytoplankton species indicating a close coupling of both trophic compartments which has already been indicated by high growth rates and enhanced hydrolytic ectoenzyme activities of bacteria in the presence of algae and polymer particles (Grossart, 1999). However, it could be suggested that the physiological flexibility of the attached bacteria may support the colonisation of the microalgal environment. Further studies should take into account that associated bacteria might live in overlapping niches and might have similar nutritional demands, which means they might belong to the same ecological guild (Odum, 1999). It is suggested that the stability of specific ecosystem processes might be sustained by these bacteria which in fact might have the same physiological capacities and might be functionally redundant (Gaston, 1996).

Outlook

In future studies methodical advances should be made with regard to specific interactions, including the analysis of the identity and the function of associated bacteria. Although numerous studies indicated specific interactions of bacteria and microalgae only few provided insights on a physiological level (Bidle *et al.*, 2002; Arrieta & Herndl, 2002). Generally, an uptake of extracellular products of *Skeletonema costatum* by bacterial isolates could be demonstrated by Bell *et al*. (1974) but new methods are required which facilitate a closer look at the bacterial community with regard to identity and specific function. Recently, a linkage of bacterial identity and physiological capacity was achieved by the combination of fluorescence *in situ* hybridisation and microautoradiography (FISH-MAR) or Stable Isotope Probing (SIP) (Wagner *et al.*, 2006). The majority of results recently published for both methods show single substrate utilisation (Wagner *et al.*, 2006). Collaborative approaches would probably enable microbial ecologists to study the utilisation of specific extracellular compounds obtained from microalgal exudates by applying FISH-MAR or SIP resulting in deeper insights into specific processes concerning the capacity of bacteria and their role for biogeochemical cycling.

In this study, it was shown that a combination of community fingerprinting methods and multivariate statistics is promising for the analysis of factors controlling specific phylotypes within bacterioplankton dynamics *in situ* (Chapter V). Extensive investigations based on this methodical approach should include the abundance and if possible the identity of

nanoflagellates and viruses in the analysis to study the influence of these factors on the structure of the bacterial community. Furthermore, it would be desirable to study bacterioplankton dynamics with regard to controlling factors within longer time-series. Such investigations could elucidate which factors influence the bacterial community e.g. of Helgoland Roads in autumn resulting in a stable "winter" community as shown by Gerdts *et al*. (2004). Additionally, time-series including bacterioplankton community structure, abiotic factors like temperature and nutrients as well as the abundance of phytoplankton, nanoflagellates and viruses would be a major step towards a holistic approach to elucidate those factors which control specific bacterial phylotypes throughout years.

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Abstract

A close link between bacteria and phytoplankton in the pelagic environment was observed in several studies. Generally, highly specific interactions of bacteria and phytoplankton (e.g. symbioses or parasitic relationships) can occur in addition to more unspecific interactions such as competition, commensalism or mutualism. The habitat of phytoplankton-associated bacteria has been depicted by the "phycosphere" concept defining the area around algal cells where bacteria feed on extracellular products of the algae. Since consumption of extracellular products by bacteria is an important process within the microbial loop, it is fundamental to elucidate the ecological role of bacteria interacting with phytoplankton.

In this thesis, bacteria-phytoplankton interactions were investigated with a focus on the association with microalgae considered to be key species at Helgoland Roads. Initially, bacterial communities of microalgal cultures were analysed concerning specificity of the association and related to algal growth phases. Seven freshly isolated key diatom and dinoflagellate species from Helgoland Roads, North Sea, were investigated. The community composition of associated bacteria as well as the cell numbers, the photosynthetic efficiency of the algae and the depletion of anorganic nutrients in the medium were recorded over a period of eight weeks in batch cultures. Members of *Alpha* - and *Gammaproteobacteria* and the *Flavobacteria-Sphingobacteria* group within the *Bacteroidetes* phylum predominated in the cultures. Differences in free-living and attached bacterial populations were observed between the phylogenetic groups. Shifts in the bacterial communities could not be correlated to changes of nutrient levels or algal growth phases. Regarding these results, it should not be generalised, that the compositions of the bacterial communities are strictly species-specific for microalgae.

In detail, the hypothesis regarding species-specific interactions between bacteria and different diatom species was tested by resource competition experiments. It was investigated, whether microalgae or their exudates have a shaping influence on the structure of diatom-associated bacterial communities. It was assumed that species-specific associations of bacteria and the respective host alga would lead to communities similar to the originally associated community. It was presumed that bacteria associated with different diatom species would be suppressed. Interestingly, competition of associated bacterial populations could not be observed in any treatment. Furthermore, the influence of exudates on the composition of the

associated bacterial communities was estimated to be low. Therefore, it could be shown that specificity at a species level did not occur among the studied diatom species. Bacteria associated with the investigated diatoms might be generally adapted to a diatom environment but not to a certain species. Hence, it can be hypothesised that associated bacterial species belong to ecological guilds.

Shifts in the associated bacterial community structure resulting from isolation and cultivation of microalgae were also investigated. Generally, members of *Alpha* - and *Gammaproteobacteria* as well as members of the *Bacteroidetes* predominated the cultures. Multidimensional scaling revealed strong shifts in the associated communities during cultivation. Especially the number of phylotypes belonging to the *Gammaproteobacteria* increased. It could be shown that the bacterial community associated with the diatoms at the time of isolation was completely different from the associated community after twelve months of cultivation. The findings observed in culture experiments dealing with the interaction of bacteria and microalgae should be scrutinised regarding cultivation impacts.

Additionally, the influence of phytoplankton on the bacterial community was studied *in situ* during winter-spring transition 2004. The diversity and dynamics of the bacterioplankton of Helgoland Roads were investigated with regard to successional changes due to phytoplankton dynamics as well as abiotic parameters. In detail, the bacterial community was analysed and correlated with phytoplankton community data and abiotic parameters by the ordination technique of Canonical Correspondence Analysis. Generally, an influence of temperature and phytoplankton on the bacterial community during the sampling period was observed. Additionally, multivariate analysis revealed an influence on specific bacterial phylotypes by these factors. In detail, free-living bacteria were strongly influenced by the appearance of *Phaeocystis spp*. and *Guinardia delicatula.* Overall, the results indicate that changes in the bacterial community were caused not only by abiotic factors but also by the phytoplankton community.

It could be suggested that the physiological flexibility of attached bacteria may support the colonisation of the microalgal environment by these bacteria. Further studies should take into account that associated bacteria might belong to certain ecological guilds or even might be functionally redundant to sustain stability of specific ecosystem processes.

Zusammenfassung

Eine enge Verflechtung von Bakterien und Phytoplankton im Pelagial konnte in mehreren Untersuchungen beobachtet werden. Allgemein können sowohl spezifische Interaktionen wie Symbiose oder Parasitismus als auch unspezifische Interaktionen wie Konkurrenz, Kommensalismus oder Mutualismus zwischen Bakterien und Phytoplankton vorkommen. Das Habitat von Phytoplankton-assoziierten Bakterien wurde durch das Phycosphären-Konzept beschrieben, welches den Bereich um Algenzellen beschreibt, in dem Bakterien sich von extrazellulären Produkten der Algen ernähren. Diese Interaktion ist ein wichtiger Teil des Nahrungsnetzes im Pelagial, der „Microbial Loop". Daher ist es wesentlich, die ökologische Rolle von Bakterien, die mit Phytoplankton interagieren, aufzuklären.

In dieser Doktorarbeit wurde die Interaktion von Bakterien und Phytoplankton in Hinblick auf eine Assoziation mit jenen Mikroalgen, die als Schlüsselarten im pelagischen Ökosystem vor Helgoland gelten, untersucht. Zunächst wurden bakterielle Gemeinschaften in Mikroalgenkulturen auf die Spezifität der Assoziation untersucht und mit den Wachstumsphasen der Algen in Beziehung gesetzt. Sieben frisch isolierte Diatomeen- und Dinoflagellatenarten, Schlüsselarten im Nahrungsnetz der Nordsee vor Helgoland wurden untersucht. Über einen Zeitraum von acht Wochen wurde die Zusammensetzung der Gemeinschaft assoziierter Bakterien, die Zellzahlen von Bakterien und Algen, die Leistungsfähigkeit der Photosynthese bei den untersuchten Algen und die Konzentrationsveränderung von anorganischen Nährstoffen im Medium in diskontinierlicher Kultur untersucht. In den Kulturen dominierten Bakterien, die zum einen den *Alpha-* oder *Gammaproteobakterien* sowie der Gruppe der *Flavobakterien-Sphingobakterien* im *Bacteriodetes* Phylum zugeordnet werden konnten. Innerhalb der phylogenetischen Gruppen konnten Unterschiede zwischen frei und angeheftet lebenden Bakterien festgestellt werden. Veränderungen der bakteriellen Gemeinschaften konnten nicht mit Änderungen der Nährstoffkonzentrationen oder Algenwachstumsphasen korreliert werden. Aufgrund dieser Ergebnisse wurde deutlich, dass eine Verallgemeinerung, die Zusammensetzung von assoziierten Bakterien sei strikt spezies-spezifisch bezogen auf die Mikroalgen, nicht getroffen werden sollte.

Im Einzelnen wurde die Hypothese getestet, ob spezies-spezifische Interaktionen zwischen Bakterien und verschiedenen Diatomeenarten auftreten. Dies wurde mittels Konkurrenz-

experimenten durchgeführt. Ferner wurde getestet, ob Exudate von Diatomeen einen Einfluss auf die Struktur ihrer assoziierten bakteriellen Gemeinschaft, ausüben. Die Annahme war, dass spezies-spezifische Assoziationen von Bakterien und der entsprechenden untersuchten Alge in den Konkurrenzversuchen zu Gemeinschaften führen würden, die ähnlich zu der ursprünglich assoziierten Gemeinschaft wären. Des Weiteren wurde vermutet, dass Bakterien, die mit anderen Diatomeen assoziiert sind, unterdrückt werden würden. Interessanterweise konnte Konkurrenz zwischen den bakteriellen Gemeinschaften in keinem Versuchsansatz nachgewiesen werden. Außerdem war der Einfluss von Exudaten auf die Zusammensetzung der assoziierten bakteriellen Gemeinschaft gerinfügig. Es konnten demnach keine spezies-spezifischen Assoziationen von Bakterien und den untersuchten Diatomeenarten nachgewiesen werden. Jene Bakterien, die mit den untersuchten Diatomeen assoziiert sind, könnten an eine Umgebung, die durch die Diatomeen geprägt ist, angepaßt sein aber nicht an spezielle Arten von Diatomeen. Dies führt zu der Hypothese, dass assoziierte Bakterien zu ökologischen Gilden gehören könnten.

Es wurden außerdem Veränderungen in der bakteriellen Gemeinschaft untersucht, die durch die Isolierung und Kultivierung von Mikroalgen hervorgerufen werden. In den Kulturen dominierten Bakterien, die den *Alpha-* und *Gammaproteobakterien* sowie zum *Bacteriodetes* Phylum zugeordnet werden konnten. Mittels multidimensionaler Skalierung konnten starke Veränderungen in der assoziierten Gemeinschaft während der Kultivierung statistisch nachgewiesen werden. Insbesondere die Zahl der Phylotypen, die zu den *Gammaproteobakterien* zugeordnet wurden, erhöhte sich. Es konnte in dieser Untersuchung gezeigt werden, dass die mit Diatomeen assoziierte bakterielle Gemeinschaft zum Zeitpunkt der Isolation vollständig anders war, als nach zwölf Monaten Kultivierung. Daher sollten die Ergebnisse aus Kulturexperimenten zur Interaktion von Bakterien und Mikroalgen eingehend auf Kultivierungseffekte hin geprüft werden.

Außerdem wurde der Einfluß von Phytoplankton auf die bakterielle Gemeinschaft *in situ* während des Übergangs von Winter zu Frühjahr untersucht. Dazu wurde die Diversität und Dynamik des Bakterioplanktons von Helgoland in Bezug auf sukzessionsbedingte Veränderungen des Phytoplanktons sowie abiotischer Parameter analysiert. Dabei wurde im speziellen die bakterielle Gemeinschaft mit Daten der Phytoplanktongemeinschaft und abiotischen Parametern mittels Kanonischer Korrespondenzanalyse korreliert. Es konnte während des Beprobungszeitraumes sowohl ein Einfluß von Temperatur als auch von Phytoplankton auf die bakterielle Gemeinschaft festgestellt werden. Außerdem konnte ein direkter Einfluß dieser Faktoren auf spezielle Bakterien mittels multivariater statistischer

Analyse nachgewiesen werden. Im Einzelnen wurden frei lebende Bakterien stark von *Phaeocystis spp*. und *Guinardia delicatula* beeinflußt. Insgesamt zeigen die Ergebnisse, dass Veränderungen innerhalb der bakteriellen Gemeinschaft nicht nur durch abiotische Faktoren, sondern auch durch die Phytoplanktongemeinschaft ausgelöst wurden.

Es ist anzunehmen, dass die physiologische Flexibilität von angeheftet lebenden Bakterien die Kolonisierung der Mikroalgenumgebung unterstützt. Weitere Studien sollten klären, ob assoziierte Bakterien zu ökologischen Gilden gehören oder eventuell sogar funktionell redundant sind, um zum Beispiel die Stabilität spezifischer Ökosystemprozesse zu gewährleisten.

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CURRICULUM VITAE

Melanie Sapp

geboren am 29. Januar 1977 in Remscheid

deutsch

ledig, keine Kinder

Promotion

seit	November ‘02	Tätigkeit als Doktorandin innerhalb des „FoodWeb Projektes“, Teilbereich Mikrobiologie am Alfred-Wegener Institut, Biologische Anstalt Helgoland
	Juni ‘04	Sapp, M., Schwaderer, A.S., Wiltshire, K.H., Hoppe, H.G., Wichels, A., Gerdts, G. (2004) Interaction of marine bacteria in the pelagic food web, Eingeladener Vortrag im mikrobiologischen Seminar, Kiel
	August ‘04	Sapp, M., Schwaderer, A.S., Wiltshire, K.H., Hoppe, H.G., Wichels, A., Gerdts, G. (2004) Diversity and succession of bacterial populations in microalgal cultures, Poster auf der 10. Internationalen ISME Tagung über Mikrobielle Ökologie, 22.-27.08. Cancun, Mexiko; Förderung durch die DFG
	April ‘05	Mitarbeit bei der Organisation des AWI-Doktorandentages auf Helgoland

Studium

	Okt. ‘96 - Aug. ‘02	Studium der Biologie an der RWTH Aachen Studienschwerpunkte: Ökochemie (Hauptfach), Mikrobiologie (Nebenfach), Neurobiologie (Nebenfach), Umwelthygiene (Nebenfach)
	Dez. ‘01 - Aug. ‘02	Diplomarbeit „Charakterisierung der gebundenen Rückstände des Fungizids Cyprodinil in Weizenzellkulturen“ Sapp, M., Ertunç, T., Bringmann, I., Schäffer, A., Schmidt, B. (2004) Characterization of the bound residues of the fungicide cyprodinil formed in plant cell suspension cultures of wheat. Pest management science 60:65-74.
	August ‘02	Diplom-Biologin

CURRICULUM VITAE

		Studentische Tätigkeiten
	Mai ‘98 - Nov. ‘01	Mitarbeit innerhalb der studentischen Selbstverwaltung des Wohnheims Halifaxstraße (Aachen) als Senatorin
	August ‘98	Teilnahme am meeresbiologischen Kurs der Meeresstation Helgoland
	April - Juni ‘99, April – Juni ‘00 April – Juni ‘01	Leitung des Organisationskomitees für das Sommerfest des Wohnheims Halifaxstraße
	Sept. ‘99 - Jan. ‘00	Studentische Hilfskraft am Institut für Ökologie / Ökotoxikologie / Ökochemie der RWTH Aachen (Prof. Schäffer)
	August ‘02	Teilnahme an der 10. Internationalen IUPAC-Tagung in Basel; Posterpreis innerhalb der Sektion „Disease Control“ ausgeschrieben durch Bayer CropScience
		Schulbildung
	‘83 - ‘87	Grundschule Wupper, Radevormwald
	‘87 - ‘96	Theodor-Heuss Gymnasium Radevormwald
	Juni ‘96	Abitur
	Mai ‘92, Sept. ‘94	Schüleraustausch mit Partnerschulen in Chateaubriant, Frankreich
		Kenntnisse
Sprachen		Englisch fließend in Wort und Schrift
		Französisch ausbaufähige Grundkenntnisse
EDV		fundierte Kenntnisse der Programme Word, Excel, PowerPoint, Corel Draw, Adobe Illustrator, LaTeX u.a.

Helgoland, 25. Mai 2006

Melanie Sapp