Response of Marine Bacterioplankton to Experimental Manipulations of Growth Conditions

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Das Meer ist mein
wahlverwandtes Element
und schon sein Anblick
ist mir heilsam.

Heinrich Heine, Helgoland 1899

Table of contents

Summary

Coastal prokaryotic picoplankton is exposed to a highly variable environment. In this thesis the full cycle 16S rRNA approach for a cultivation-independent analysis of prokaryotic communities was applied to monitor and investigate the fate of bacterial populations in experimental incubations in the context of changes of potentially causative ecological factors.

Observations at the level of the microbial food web revealed distinct successions of two bacterial populations. In mesocosms of Mediterranean Sea water *Alteromonadaceae*/*Colwelliaceae* (A/C) (*Gammaproteobacteria*) instantaneously responded to confinement-induced changes in the organic matter field. While these bacteria were rare at the beginning of the 8-day incubation period, they accounted for almost up to 60% of the prokaryotic community during the early stage (48 h) of the experiment. A/C were found to be extremely sensitive to grazing, as they were selectively reduced by heterotrophic nanoflagellates (HNF) in increasing densities. *Rhodobacteraceae*, which are members of the *Alphaproteobacteria*, were the dominant bacterial population during the second stage of the experiment. A tight relation to phytoplankton was suggested by a close correlation between the relative abundance of *Rhodobacteraceae* and the chlorophyll *a* concentration. This particular population therefore most likely benefited from algal-derived dissolved organic carbon (DOC).

A second investigation focused on the response of bacterial populations to a complex substrate source, the mucus released by a tropical coral species. Again, A/C were found to grow most rapidly upon addition of coral mucus. Specifically, 2 genotypes related to *Alteromonas macleodii* seemed to be the most competetive utilizers of this particular carbon source, and these bacteria accounted for >40% of the prokaryotic community after only 10 h of incubation. In view of its sensitivity to protistan grazing, it is likely that *A. macleodii* could transfer a considerable amount of the organic carbon derived from zooxanthellate photosynthesis to the higher levels of the pelagic food web of coral reefs.

The specific substrate and nutrient preferences of two closely related bacterial populations were addressed in a third incubation experiment. A/C and

Pseudoalteromonadaceae were enriched in dilution enrichments with various substrate and nutrient concentrations. However, high growth in unamended incubations impeded the identification of clear-cut preferences of either population. Both A/C and *Pseudoalteromonadaceae* responded within 48 h with *Pseudoalteromonadaceae* being the more abundant population irrespective of manipulation and the more competitive at elevated concentrations of amino acids.

Altogether, the results presented here allowed a description of distinct opportunistic growth patterns of bacterial populations as responses to changes of ecological factors. The abundance and composition of *Rhodobacteraceae* populations significantly depended on the presence of exudates of phytoplankton. A/C responded instantaneously with growth to disturbances of the organic matter field. However, their high sensitivity to grazing by HNF resulted only in short-lived phases of dominance. Thus, A/C potentially might play an important role in the channeling of organic substrates within the microbial loop.

Zusammenfassung

Das küstennahe, prokayrotische Picoplankton ist sehr wechselhaften Umweltbedingungen ausgesetzt. Ziel der vorliegenden Arbeit war es, die Reaktion dieser prokaryotischen Gemeinschaft auf ein sich änderndes Nährstoff- und Substratangebot mit Hilfe des kultivierungsunabhängigen 16SrRNA-Ansatzes näher zu analysieren.

Auf der Nahrungsnetzebene konnte eine deutliche Sukzession zweier bakterieller Populationen beobachtet werden. In Mesokosmen gefüllt mit Oberflächenwasser des Mittelmeeres reagierten *Alteromonadaceae*/*Colwelliaceae* (A/C) (*Gammaproteobacteria*) umgehend auf Veränderungen im Gefüge des organischen Materials, die durch die Eingrenzung des Wassers in Mesokosmen hervorgerufen worden war. Der Anteil der zu Beginn der Inkubationen selten auftretenden A/C stieg innerhalb von 2 Tagen auf 60% der prokaryotischen Gemeinschaft an, wurde aber im weiteren Verlauf durch zunehmenden Fraßdruck heranwachsender heterotropher Nanoflagellaten (HNF) äußerst selektiv reduziert. In der zweiten Phase des Experimentes dominierten *Rhodobacteraceae* (*Alphaproteobacteria*) die Gemeinschaft. Die gute Korrelation ihrer relativen Abundanzen mit der Konzentration an Chlorophyll *a* deutete auf eine enge Kopplung der *Rhodobacteraceae* an Phytoplanktondichten an. Diese bakterielle Population schien in der Lage zu sein, den organischen Kohlenstoff, den die Mikroalgen freisetzen, zu nutzen.

Eine zweite Untersuchung setzte den Schwerpunkt auf die Beschreibung der bakteriellen Reaktion auf Zugabe eines komplexen Substrates, wie es der Mucus tropischer Korallen darstellt. Die Gruppe der A/C waren auch hier die ersten, die auf Veränderungen des Kohlenstoffgefüges durch die Substratzugabe reagierten. Zwei mit *Alteromonas macleodii* verwandte Genotypen schienen die erfolgreichsten Nutzer dieser Kohlenstoffquelle gewesen zu sein. Nach nur 10-stündiger Inkubation stellten sie >40% der prokaryotischen Gemeinschaft. Im Hinblick auf die hohe Empfindlichkeit gegenüber dem Fraßdruck durch HNF, scheint es vorstellbar, dass *A. macleodii* beträchtliche Mengen des organischen Kohlenstoffs, den die Zooxanthellen als Symbionten der Koralle photosynthetisch produzieren, auf höhere Ebenen des pelagischen Nahrungsnetzes zu transportieren.

In einem dritten Experiment sollten bestimmte Substrat- und Nährstoffvorlieben zweier nahe verwandter bakterieller Populationen untersucht werden. A/C und *Pseudoalteromonadaceae* wurden in Verdünnungskultivierungen unter Zugabe verschiedener Substrate und Nährstoffe in unterschiedlichen Konzentrationen angereichert. Aufgrund der Tatsache, dass auch ohne diese Zugaben ausgeprägtes Wachstum zu beobachten war, konnten keine eindeutigen Schlüsse in Bezug auf eventuelle Präferenzen gezogen werden. Die Zellzahlen der A/C wie auch der *Pseudoalteromonadaceae* stiegen innerhalb von 48 h deutlich an. *Pseudoalteromonadaceae* stellten die größeren Populationen unabhängig von der Art der angewandten Manipulation. Zudem zeigten sie sich bei erhöhten Aminosäurekonzentrationen kompetitiver als A/C.

Alles in allem erlauben die hier präsentierten Ergebnisse die Beschreibung unterschiedlicher opportunistischer Wachstumsmuster. *Rhodobacteraceae* sind in Größe und Zusammensetzung ihrer Populationen insbesondere von Exudaten des Phytoplankton abhängig. A/C reagieren ungehend mit Wachstum auf Veränderungen im Gefüge des organischen Materials. Allerdings verhindert ihre enorme Empfindlichkeit gegenüber dem Fraßdruck durch HNF eine stabilere Dominanz in prokaryotischen Gemeinschaften. Dadurch könnten A/C eine wichtige Rolle dabei spielen, organische Substrate innerhalb der mikrobiellen Schleife auf die nächste trophische Ebene zu überführen.

List of abbreviations

A/C	*Alteromonadaceae* and *Colwelliaceae*
C	carbon
CARD	catalyzed reporter deposition
CFU	colony forming unit
Chl *a*	chlorophyll *a*
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DOM	dissolved organic matter
FISH	fluorescence *in situ* hybridization
HMW	high-molecular-weight
HNF	heterotrophic nanoflagellates
LMW	low-molcular-weight
Mbp	mega base pairs = 10^6 base pairs
N	nitrogen
P	phosphorus/phosphate
POM	particulate organic matter
PSU	practical salinity unit
RNA	ribonucleic acid
rRNA	ribosomal RNA
sp.	species
spp.	several species

Part I: Combined presentation of results

A Introduction

1 The marine pelagic zone

The oceans cover more than 70% of the surface of the Earth. Most of these marine waters belong to the oceanic zone, being off the continental shelfs and reaching depths below 1000 m. Only 8% belong to the neritic zone, which is closer to the continental shores and rather shallow in depth (Lalli and Parsons, 1997a). Nevertheless, this zone plays an important role in the cycling of elements, because primary productivity in the neritic environment is higher than in the oceanic (Lalli and Parsons, 1997b) (Fig. A-1). Consequently, the relatively small area of continental shelf seas is responsible for approximately 18% of the marine pelagic primary productivity (Smith and Hollibaugh, 1993).

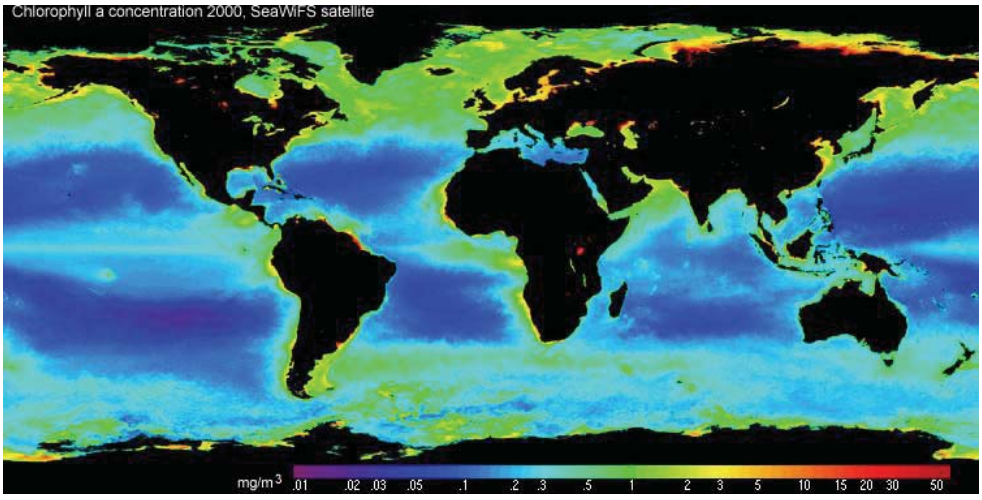

Fig. A-1: Chlororphyll *a* (Chl *a*) concentrations in the aquatic environment as observed from space. The colors yellow and red refer to areas of high primary productivity (max. 50 µg l^{-1}), whereas purple and blue refer to areas of low productivity (min. 0.1 µg l^{-1}) (www.deepocean.net).

The pelagic zone comprises all living space within the water column, from surface waters (epipelagic region) to the deepest zones of the deep sea (hadalpelagic region). The oceanic environment is generally characterized by lower nutrient levels as it is less influenced by terrestrial sources and rather relies on ocean circulation and atmospheric input. The neritic, coastal pelagic zone is a very dynamic ecosystem. Continental river run-off and tidal currents

are only two of the factors which impose great variability upon the system (Kennish, 2001). In temperate climatic zones, the different seasons add an extra component to the orchestra of factors.

The pelagic environment supports two basic types of marine organisms. One type comprises the nekton, or free-swimming animals, such as fish, squid and marine mammals, that are strong enough to swim against currents and are therefore independent of water movements. The remaining inhabitants of the pelagic environment form the plankton. These are organisms whose powers of locomotion are such that they are incapable of making their way against a current and thus are passively transported by currents in the sea (Lalli and Parsons, 1997a). The plankton is thus exposed to large scale as well as small scale changes of environmental parameters such as temperature, turbulence, and nutrient concentrations and needs to be able to cope with this heterogeneity.

There are two ways to distinguish between different classes within the plankton. One way is to divide it according to size, defining groups such as pico-, nano-, and microplankton. They range from 0.2–2.0 µm, 2.0–20 µm, and 20–200 µm, respectively, in size. Another approach is rather systematic and distinguishes between classes such as zooplankton, phytoplankton, and bacterioplankton according to their affiliation to animals, plants or bacteria (Lalli and Parsons, 1997a).

2 Prokaryotic picoplankton

Generally, marine planktonic prokaryotes have a size of <1-2 µm with a few exceptions. They attain peak numbers in estuarine waters (~10^6–10^8 cells ml^{-1}) (Kennish, 2001; Cottrell and Kirchman, 2003; Henriques *et al.*, 2004), and gradually decline in abundance from the coastal ocean (1 to 3 x 10^6 cells ml^{-1}) (Schauer *et al.*, 2003; Calvo-Díaz *et al.*, 2004; Pernthaler and Pernthaler, 2005) to oceanic regions (10^4–10^6 cells ml^{-1}) (Zubkov *et al.*, 2000; Morris *et al.*, 2002).

The physiological capacity of marine bacteria is manifold. Autotrophic bacteria derive energy through photosynthesis (phototroph) or through the oxidation of inorganic compounds (chemolithotroph). Heterotrophic bacteria obtain energy from organic compounds (chemoorganotroph) (Madigan and Martinko, 2006). Mixotrophy is yet another way to gain energy which combines phototrophic and heterotrophic features. *Dokdonia* sp. strain MED134, a member of the *Bacteroidetes*, and *Candidatus* Congregibacter litoralis, a member of the *Gammaproteobacteria*, can use proteorhodopsins to enhance growth yield (Fuchs *et al.*, 2007; Gómez-Consarnau *et al.*, 2007). On the other hand, the cyanobacteria *Prochlorococcus* spp. and *Synechococcus* spp. can take up amino acids at lower energetic costs as compared to uptake of inorganic nitrogen such as ammonium (Zubkov *et al.*, 2003). Heterotrophic bacteria will be focused on in this study.

2.1 The role of heterotrophic bacteria in pelagic food webs

Bacteria had long been regarded as remineralizers, responsible for converting organic to inorganic matter and recycling nutrients to primary producers. In 1983, however, Azam *et al.* (1983) and Ducklow (1983) conveyed the idea of a ‘microbial loop’, the cycling of organic matter through microbes before entering the classic food web. The observed tight coupling between phytoplankton and bacteria on the one hand, and between bacteria and zooflagellates on the other (e.g. Burney *et al.*, 1982) supported the idea of a trophically relevant role of

bacteria within the food web. In the microbial loop concept (Fig. A-2), pelagic bacteria generally utilize dissolved organic matter (DOM) as energy source. When DOM is supplied sufficiently, bacteria are primarily kept below a certain density by grazing of heterotrophic flagellates and virus-induced lysis (Weinbauer, 2004; Pernthaler, 2005). Both autotrophic and heterotrophic flagellates are in turn preyed upon by microzooplankton (Azam *et al.*, 1983). This indicates that bacteria play a role in marine food webs analog to phytoplankton: they are producers and are consumed by other organisms (Ducklow, 1983). Thus energy released as DOM by phytoplankton is returned to the main food chain via a microbial loop of bacteria-flagellates-microzooplankton (Azam *et al.*, 1983).

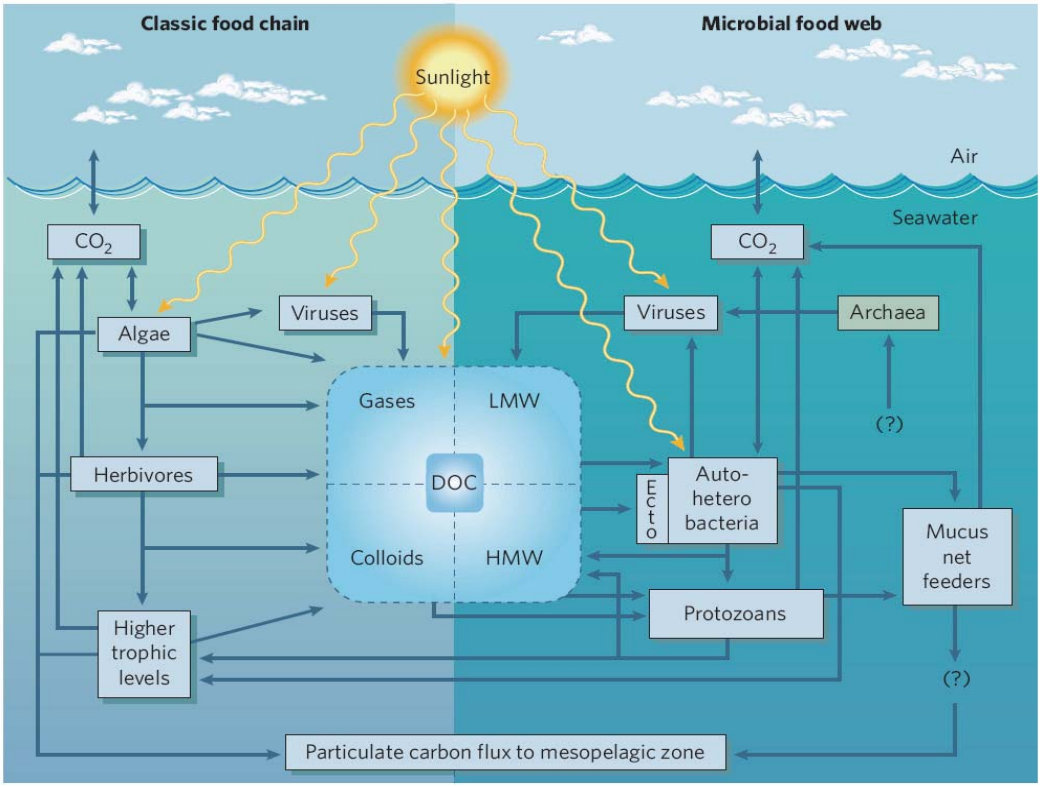

Fig. A-2. Classic food chain (left) and microbial loop/microbial food web (right) in the marine pelagic zone. Algae photosynthesize and the algal-derived energy is transported through herbivores to higher trophic levels. The microbial loop uses energy stored in the non-living carbon pool to produce microbial biomass that can re-enter the classic pathway of carbon and energy flow. Cell-associated ectoenzymes (Ecto) enable bacteria to use high-molecular-weight (HMW) dissolved organic carbon (DOC) in addition to low-molecular-weight (LMW) and gaseous carbon substances (from DeLong and Karl, 2005).

2.2 Environmental factors and community composition

Changes in environmental factors such as temperature and inorganic nutrients such as nitrogen (N) and phosphorus (P) are known to control the appearance of specific phytoplankton populations, whereas much less is known about their impact on the composition and abundance of bacterial communities. Temperature is considered to be the major controlling factor in winter, autumn and spring, whereas a limitation of nutrients and substrate is regarded to be the controlling factor in summer (Shiah and Ducklow, 1994). Since the environment displays variable conditions due to seasons and nutrient availability, it is not surprising that the associated bacterial community mirrors this variability to a certain extent.

Using whole-genome DNA hybridization in the Baltic Sea, Pinhassi and Hagström (2000) found a clear differentiation between bacterial assemblages during spring, dominated by *Bacteroidetes*, and summer, dominated by *Alphaproteobacteria*. Eilers *et al.* (2001) studied the seasonality of marine bacterial groups by fluorescence *in situ* hybridization (FISH) in the North Sea and found that *Bacteroidetes* dominated during spring and early summer, while a group of *Gammaproteobacteria* (i.e. NOR5) was abundant during summer (8%). A dominance of *Bacteroidetes* in spring and early summer and a dominance of *Alphaproteobacteria* from late summer to winter was also reported by Mary *et al.* (2006) for the English Channel. In the north-western (NW) Mediterranean Sea, year-round monitoring of the bacterial community with CARD-FISH revealed the dominance of *Alphaproteobacteria*, especially in spring and summer, when they accounted for ~40% of total cell counts (Alonso-Sáez *et al.*, 2007). So the coastal bacterioplankton displays a seasonality in community composition to a certain extent (Fuhrman *et al.*, 2006). Generally, *Alpha-* and *Gammaproteobacteria* play a significant role particularly in summer, whereas *Bacteroidetes* especially prosper in spring and early summer.

Furthermore, blooms of phytoplankton were observed to be accompanied by high abundances of *Rhodobacteraceae* (Eilers *et al.*, 2001). The bacterioplankton community structure can change rapidly in response to growth and decay of different phytoplankton in neritic as well as in oceanic areas, indicating that dissolved organic matter (DOM) from different algae select

for different bacteria (González *et al.*, 2000; Pinhassi *et al.*, 2004; Grossart *et al.*, 2005). Experiments with estuarine bacterioplankton showed that different complex dissolved organic carbon (DOC) sources selectively stimulated growth of specific bacterial genotypes (Covert and Moran, 2001).

In addition to organic substrates as e.g. provided by algal exudates, the availability of inorganic nutrients such as N and P seems to regulate the success of bacterial populations. In isolation attempts on media with reduced concentrations of N and P compared to common laboratory media, species that had not been isolated before were retrieved from the marine environment. Among those were members of the NOR5 (Eilers *et al.*, 2001) and SAR11 clades (Connon and Giovannoni, 2002), which were obviously adapted to low nutrient conditions.

Besides the availability of resources, mortality can influence a bacterial community tremendously. Selective grazing by predators may be detrimental for one but beneficial for the other population and thereby affects community composition (Pernthaler, 2005 and references therein). Additionally, viruses and their specificity potentially change community composition (Thingstad and Lignell, 1997; Schwalbach *et al.*, 2004). In general, bacteriophages may be responsible for 20-50% of bacterial mortality (Fuhrman, 1999).

On the whole, coastal bacterial communities are exposed to a wide variety of environmental conditions. In particular, temperature (as imposed by seasonality), substrate and nutrient availability and grazing seem to be critical parameters of the selection process for specific populations.

2.3 Cultured members of the bacterioplankton

Bacterial strains that can be grown under specific conditions in the laboratory can be subjected to comprehensive physiological analysis in order to understand their potential role in an ecosystem. Cultivation-independent studies based on comparative 16S rRNA gene sequence analysis have revealed that the discrepancy between bacterial diversity in the environment and in culture collections is tremendous (Fox, 2005; Alonso *et al.*, 2007). In 1987, all 12 known bacterial divisions had cultured representatives, whereas in 2004 only

one third of the 80 observed divisions had cultivated members (Fig. A-3). Figure A-3 illustrates the discrepancy between our knowledge of phylogenetic diversity and physiological needs. Some taxa have been easily and by now many times isolated from different environmental sites, e.g. members of the gammaproteobacterial genera *Vibrio* (Thompson *et al.*, 2004a and references therein). Others, as mentioned above, required special adjustments of the medium to become cultivated strains (Eilers *et al.*, 2001; Connon and Giovannoni, 2002). Yet other taxa have still not been obtained in pure culture, but only in enrichments such as the planctomycete *Candidatus* Brocadia anammoxidans (Strous and Jetten, 2004).

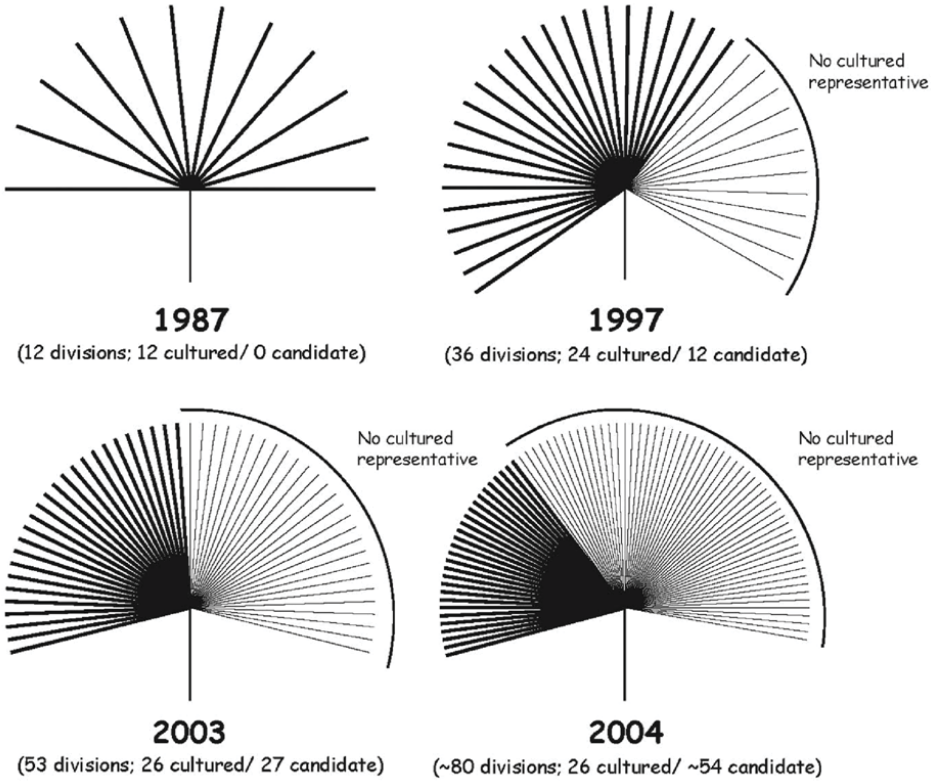

Fig. A-3. Diagram of the recent expansion in the number of known bacterial phylogenetic divisions. The extent of known diversity is expanding rapidly primarily due to culture-independent environmental surveys (Figure courtesy of Kirk Harris; from Fox, 2005).

The alphaproteobacterial *Candidatus* Pelagibacter ubique is a recent example of how the introduction of genome analysis changed the procedures in describing the potential role of a species in the environment (Giovannoni *et al.*, 2005b). *In situ* measurements demonstrated the relevance of the SAR11 clade, of which *Canditatus* P. ubique is a member (Morris *et al.*, 2002). For a long time, however, representatives of this clade seemed to be reluctant to grow at

laboratory conditions. Finally, scientists were successful to isolate and cultivate P. ubique (Connon and Giovannoni, 2002; Rappé *et al.*, 2002), and its genome was analyzed. From the small size of the genome (1.3 Mbp) and the genetic instrumentation regarding transport systems, Giovannoni and colleagues deduced that these organisms were well adapted to nutrient- and substrate-limited conditions (Giovannoni *et al.*, 2005b) at the expense of responsiveness to changes in nutrient concentration (Giovannoni *et al.*, 2005a). This preference of members of the SAR11 clade for low substrate concentrations has been shown to exist in coastal microbial communities (Alonso and Pernthaler, 2006).

Silicibacter pomeroyi, another representative of the *Alphaproteobacteria*, was observed to display a different strategy. The fact that this member of the *Rhodobacteraceae* can be grown in the laboratory quite easily provides an enormous advantage in efforts to understand their biology, as isolates can be probed for information on e.g. substrate utilization, metabolic pathways, physiological constraints, enzyme activity, nucleic acid sequence, and growth rates. *S. pomeroyi* belongs to the marine Roseobacter clade, the relatives of which comprise ~10-20% of coastal and oceanic mixed-layer bacterioplankton (González and Moran, 1997; Giovannoni and Rappé, 2000; González *et al.*, 2000; Eilers *et al.*, 2001). They are commonly found in association with microalgae (Riemann *et al.*, 2000; Zubkov *et al.*, 2001) and have a depth distribution that matches that of phytoplankton (González *et al.*, 2000). In addition, genes for the uptake of algal-derived compounds were found in the genome of *S. pomeroyi*, (Moran *et al.*, 2004). Thus genome analysis as well as environmental observations supported the idea of a tight association to phytoplankton blooms.

Another potential strategy to cope with changing nutrient concentrations in the environment became obvious when the genome of the gammaproteobacterial *Candidatus* Congregibacter litoralis KT71 was genome-analyzed. The strain had already been isolated from surface water of the German Bight in 1999 (Eilers *et al.*, 2001), but only with the genome in hands, scientists were able to derive and experimentally test hypotheses concerning the physiology and the ecological niche of *Candidatus* C. litoralis (Fuchs *et al.*, 2007). The complete genetic equipment for photosynthesis was found, and subsequent cultivation experiments under nutrient-limited condition

resulted in higher cell yields when light was available. Among other genomic and physiological features, these results led to the assumption that this strain seemed to be well adapted to a dynamic environment such as the German Bight, where conditions such as nutrient availability may change on a daily to seasonal scale (Fuchs *et al.*, 2007).

Candidatus Congregibacter litoralis KT71 had only been isolated in 1999, when Eilers and colleagues had reduced the concentration of N and P in the cultivation medium (Eilers *et al.*, 2001). In contrast, some genera had cultivated representatives already decades ago and these had been retrieved by rather conventional approaches. Strains of e.g. *Alteromonas*, *Pseudoalteromonas*, and *Vibrio* are easily enriched and kept in culture on/in standard medium such as Marine Agar and Zobell Medium (Baumann *et al.*, 1972; Pinhassi *et al.*, 1997; Thompson *et al.*, 2004a). In 1972, Baumann and colleagues isolated 218 strains from marine waters of Hawaii, and after thorough phenotypic characterization they created the new genus of *Alteromonas* and for 21 of the strains the new species of *Alteromonas macleodii* (Baumann *et al.*, 1972). More than 10 years later, after more physiological experiments, this species was finally entirely described (Baumann *et al.*, 1984) as motile, straight rod, 0.9–1.3 µm in width and 1.8–2.5 µm in length. Its representatives are able to produce extracellular enzymes such as amylase, gelatinase, and lipase and to use organic compounds, including hexoses, disaccharides, sugar acids, and amino acids (Baumann *et al.*, 1984). More than 20 years after isolation from the environment, the 16S rRNA gene sequence of *A. macleodii* was added to GenBank (Gauthier *et al.*, 1995). Comparative 16S rRNA gene sequence analysis led to the separation of the genus *Alteromonas* into two new genera, *Alteromonas* and *Pseudoalteromonas* (Gauthier *et al.*, 1995). Based on physiological characterization this distinction had not been possible before. *A. macleodii* was left within *Alteromonas* as the only species described, while all remaining species had been moved to the genus *Pseudoalteromonas*. With the additional application of cultivation-independent techniques Eilers and colleagues described the discrepancy between the culturability of members of the family of *Alteromonadaceae* - currently consisting of the genera *Alteromonas*, *Glaciecola* (Ivanova *et al.*, 2004), and *Aestuariibacter* (Yi *et al.*, 2004) - and their environmental abundance in the North Sea as detected by

FISH with the probe ALT1413, which targets members of the *Alteromonadaceae* and *Colwelliaceae* (A/C) (Eilers *et al.*, 2000b). Despite being frequently cultured, *Alteromonadaceae* (as well as *Pseudo-alteromonadaceae* and *Vibrionaceae*) typically constitute only a minor fraction of the bacterioplankton community in surface waters as detected by FISH (Eilers *et al.*, 2000b). In deep water samples (up to 200 m) they were observed via quantitative rRNA hybridization to dominate the bacterial community, especially the particle-attached community (>74%) (García-Martínez *et al.*, 2002). Altogether, as cultivation-independent approaches revealed, physiological characterization was neither sufficient to resolve phylogenetic affiliations between closely related genera nor to shed light on the ecological niche of *Alteromonas*.

The same discrepancy of being rare in the environment yet frequently isolated was observed for the genus *Pseudoalteromonas* (Eilers *et al.*, 2000b), as mentioned above. Many members of the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995) were isolated from marine eukaryotes (Holmström and Kjelleberg, 1999), such as mussels and scallops (Ivanova *et al.*, 2002b), sponges (Ivanova *et al.*, 2002c; Lau *et al.*, 2005), and tunicates (Holmström *et al.*, 1998; Egan *et al.*, 2001; Ivanova *et al.*, 2002a). Their existence in a variety of habitats and their world-wide spread suggest that the adaptive and survival strategies of *Pseudoalteromonas* species are diverse and efficient (Holmström and Kjelleberg, 1999). Many of the species produce biologically active metabolites such as agarases, toxins, bacteriolytic substances, and other enzymes which may assist them in their competition for nutrients and space as well as in their protection against grazing by predators (Holmström and Kjelleberg, 1999). Examples of violacein-producing bacteria have been isolated from freshwater, and their toxic pigment serves the purpose of grazing protection (Matz *et al.*, 2004). Currently, the description of the physiological traits of *Pseudoalteromonas* is based on 35 validly described species (Nam *et al.*, 2007), however, ecological implications have not yet been discussed thoroughly.

Another example of cultured and well-studied organisms are the members of the gammaproteobacterial genus V*ibrio*. They are widespread in coastal and estuarine habitats all over the world (Thompson *et al.*, 2004a and

references therein). The most infamous member of the genus is most likely the human-pathogen *Vibrio cholerae*, the causative agent of cholera. First isolates were obtained by Robert Koch in the 1880s (Thompson *et al.*, 2004a). In contrast to the majority of marine pelagic bacteria, also the ecological aspects of *V. cholerae* have been investigated in order to understand how this seasonally occurring disease is spread. Microbiological studies have shown that especially zooplankton play a significant role as a reservoir of *V. cholerae* (Colwell and Huq, 2001 and references therein). Additionally, rises in sea surface temperature have been found to be directly correlated with occurrence of cholera (Colwell, 1996; Jiang *et al.*, 2000a; Jiang *et al.*, 2000b).

Besides *V. cholerae* being a potential threat for human beings, animals can be affected by pathogenic *Vibrio*, such as *V. anguillarum*, *V. salmonicida*, and *V. vulnificus* for fish and *V. harveyi* for shrimp (Thompson *et al.*, 2004a and references therein), with sometimes devastating consequences especially when dense populations in aquaculture are affected. Corals are also damaged by diseases mediated by *Vibrio*. *V. shiloi* and *V. corallilyticus* were shown to be causative agents for coral bleaching and black band disease, respectively (Rosenberg and Ben-Haim, 2002; Ben-Haim *et al.*, 2003). Outbreaks of these pathogens were observed after environmental conditions had changed, such as a rise in water temperature or an increase in carbon and nutrient concentrations.

However, also non-pathogenic vibrios are commonly isolated from marine ecosystems (Thompson *et al.*, 2004a; Thompson *et al.*, 2005) and interactions between bacteria and host can as well be beneficial for both partners, e.g. the squid-vibrio association, which has been studied in detail on the symbiosis between the bobtail squid *Euprymna scolopes* and *Vibrio fischeri* (McFall-Ngai, 1999). The host regrows fresh pure cultures of *V. fischeri* in its light organs on a daily basis in order to ensure the emission of bioluminescence. The cell density of the symbionts regulates the activation of bacterial bioluminescence, which the host uses as counter-light to be protected against visual detection by potential predators. The fact of daily venting of 90% of the *V. fischeri* culture into the surrounding seawater (Boettcher *et al.*, 1996) points at high growth rates of these bacteria.

As indicated earlier, *Vibrionaceae*, and also *Alteromonadaceae* and *Pseudoalteromonadaceae*, have been observed to instantaneously respond to surplus carbon (Eilers *et al.*, 2000b; Alonso and Pernthaler, 2005; Kline *et al.*, 2006). In combination with short generation times these genera may manage to dominate bacterial enrichment cultures within a relatively short period of time (Eilers *et al.*, 2000a). Maintenance of high ribosomal contents in the cell even after long periods of starvation (Flärdh *et al.*, 1992; Eilers *et al.*, 2000a) might be another advantage. Irrespective of starvation duration cells remain highly responsive to nutrients and exhibit immediate several fold increases in the rates of protein synthesis and RNA synthesis when substrate is added (Flärdh *et al.*, 1992; Pernthaler *et al.*, 2001). On the other hand, only high grazing mortality caused by heterotrophic nanoflagellates (HNF) can counterbalance the rapid growth of these bacteria and might explain their low *in situ* abundances (Eilers *et al.*, 2000b; Beardsley *et al.*, 2003).

The here discussed examples of cultured species from the marine bacterioplankton illustrate 3 aspects: (i) the collection of cultivated species does not mirror the actual prokaryotic diversity in the environment; (ii) mainly for historical reasons, the knowledge about particular species differs in quality, i.e. it might be derived from physiological characterization (e.g. *Alteromonas*, Baumann *et al.*, 1972; Baumann *et al.*, 1984) or from genome analysis (e.g. *Candidatus* P. ubique; Giovannoni *et al.*, 2005b) and sometimes from overlapping sources (e.g. *S. pomeroyi*, Moran *et al.*, 2004; *Candidatus* C. litoralis KT71, Fuchs *et al.*, 2007); (iii) ecological implications of physiological differences have rarely been subject to speculations and experimentally verified in the environment.

3 Habitats of interest

Coastal seas offer a wide variety of different habitats. The climatic zone defines the impact of seasons on an ecosystem, and a seasonally variable temperature reigns in temperate and polar regions. The in- and outflow of matter into and from the particular sea play another key role. Salinity is often driven by a play of powers between fresh and salt water input and evaporation. Another important factor is the supply of nutrients, which in shallow seas often originate from terrestrial (river run-off) or atmospherical (precipitation) sources, whereas in upwelling systems nutrient-rich waters climb to the surface and allow high biological productivity.

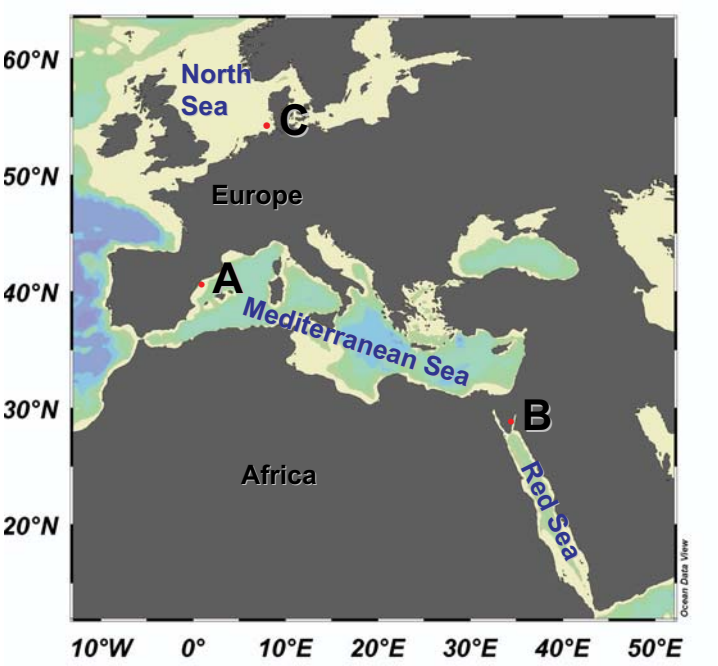

Fig. A-4. Map displaying the three locations from where samples of prokaryotic pico-plankton for the here presented studies were taken. **A**: Blanes Bay (Mediterranean Sea). **B**: Gulf of Aqaba (Red Sea). **C**: German Bight (North Sea). The map was plotted with Ocean Data View (Schlitzer, 2007).

For the presented study bacterial communities from three coastal sites were investigated: from the oligotrophic (low primary productivity) Gulf of Aqaba (Rasheed *et al.*, 2003), which is part of the Red Sea, from the Balearic Sea, which belongs to the oligotrophic (western) Mediterranean Sea (Thingstad and Rassoulzadegan, 1995; Pinhassi *et al.*, 2006), and from the German Bight as the south eastern part of the North Sea (Fig. A-4). The following paragraphs

give a short introduction to each of the habitats. Physico-chemical parameters are summarized in Table A-1. Generally, the habitats differ in characteristics such as the the trophic state (phytoplankton productivity), and the impact of oceanic or terrestrial input.

Tab. A-1. Average physico-chemical regimes of the seas where the microbial communities investigated in the presented studies originated from. Data was collected from several sources (see below).

	Gulf of Aqaba	Western Mediterranean Sea	German Bight
Temperature [°C]	23 – 26[b]	23 – 25[S]	10[a]
Salinity [PSU]	40	37	32
Chl *a* [µg l^{-1}]	< 0.8	0.2[S], > 1[W]	2 – 4[b]
NH_4 [µM]	0.1 - 0.3[b]	0.75[S]	4 – 5[W], 1 – 2[S]
NO_3 [µM]	0.1 - 0.5[b]	0.28[S],	20 – 30[W], < 5[S]
PO_4 [µM]	0.03 - 0.15[b]	0.05[S], 0.12[F]	1 -1.5[W], 0.3 – 0.5[S]
Tidal influence	scarce	scarce	pronounced
Max. depths [m]	~1800	5267	~ 40

Gulf of Aqaba: Data from Wolfvecht *et al.*, 1992; Rasheed *et al.*, 2003
Western Mediterranean Sea: Data from Sala *et al.*, 2002; Pinhassi *et al.*, 2006
German Bight: Brockmann and Topcu, 2001; Becker *et al.*, 1992; Becker, 2003; MURSYS: www.bsh.de/Meeresdaten
a – mean, b – range; S – summer; F – fall; W – winter

3.1 (Western) Mediterranean Sea

The Mediterranean Sea is – as the name suggests - almost completely enclosed by land: in the north by Europe, in the south by Africa, and in the east by Asia (Fig. A-4). In the west lies the only, 13 km wide, natural opening to the open ocean, namely the Strait of Gibraltar, which leads into the Atlantic Ocean. Mean salinity is at 37 PSU (practical salinity unit) slightly above oceanic levels due to the fact that evaporation exceeds precipitation and river run-off. The water which enters from the Atlantic Ocean travels along the coastline of the

African continent. Increasing salinity causes the descent of the water mass to the bottom, where it travels back along the European coastline. On its way it passes many distinct basins of the Mediterranean Sea, the deepest being the Ionean Sea with a depth of 5267 m.

The summer period (June to September) is characterized by high water temperatures (23-25°C) and low concentrations of Chl *a* (~0.2 µg l^{-1}) (Pinhassi *et al.*, 2006) (Tab. A-1).

Phosphorus (P) is usually the growth-limiting nutrient in summer, when concentration of total dissolved P decrease to 0.05 µM (Pinhassi *et al.*, 2006). It is generally assumed that growth of bacterioplankton in the Mediterranean Sea is limited by the availability of inorganic P (Zweifel *et al.*, 1993; Sala *et al.*, 2002). Schauer *et al.* (2003) provided a first analysis to the seasonality of bacterial assemblages of Blanes Bay (northwestern Mediterranean Sea, Fig. A-5) using the DGGE (Denaturing gradient gel electrophoresis) technique. Members of *Bacteroidetes* and *Alphaproteobacteria* were found to be the dominant groups all year round. Within the *Alphaproteobacteria* mostly representatives of the Roseobacter clade were detected. Interestingly, different genotypes within this particular clade seemed to be dominant in different seasons. In addition, members of the Roseobacter clade were observed to more abundant in winter, whereas another alphaproteobacterial clade, SAR11, was more abundant during the spring-summer period (Alonso-Sáez *et al.*, 2007). Members of the *Alteromonadaceae*/*Colwelliaceae* (A/C), which were usually rare, were detected to display a sudden increase in relative abundances to up to 30% once in summer (Alonso-Sáez *et al.*, 2007). Thus,

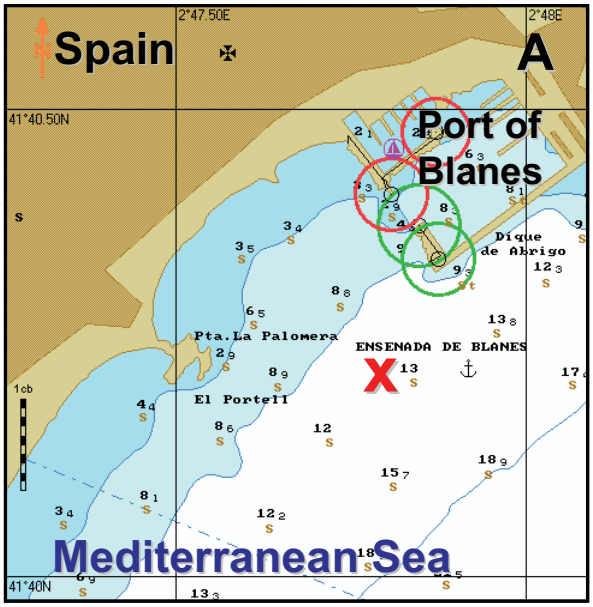

Fig. A-5. Sampling site in Blanes Bay close to Port of Blanes at the Spanish coast of the Mediterranean Sea. The blue lines depict isobaths. The site of sampling is between the 10- and 15-m isobaths indicated by the red cross. The map was plotted in NAVI 3000 (Transas Marine Ltd.).

seasonal variability obviously substantially contributes to the regulation of bacterioplankton growth and community composition.

Changes within the composition of the prokaryotic picoplankton of the coastal Mediterranean Sea may as well originate from natural disturbance events such as the flush floods of temporary rivers (McClain *et al.*, 2003; Tzoraki *et al.*, 2007) and the import of matter by Sahara dust storms (Goudie and Middleton, 2001). On the other hand anthropogenic sources such as fish-farming (Dalsgaard and Krause-Jensen, 2006) and a growing impact by residents and tourists on the Mediterranean watershed (Bethoux *et al.*, 2002) have brought increasing P and N loads into the sea.

3.2 Gulf of Aqaba (Red Sea)

The Gulf of Aqaba is the northernmost extension of the Red Sea, a narrow sea framed by the shores of the African continent and the Saudi Arabian Peninsula (Fig. A-4), and has a salinity of 40 PSU (Tab. A-1). Compared to the open ocean, this salt concentration is elevated; it results from oceanic water inflow from the Indian Ocean and high evaporation rates. Water temperatures are 23 - 26°C all year round and are another consequence of high solar irradiation, as large parts of the Red Sea are in the tropical/arid region. The Red Sea is permanently stratified, whereas the Gulf of Aqaba itself is characterized by deep winter mixing, which transports some inorganic nutrients into the otherwise nutrient-depleted euphotic zone (Wolfvecht *et al.*, 1992; Lindell and Post, 1995). In general, nutrient concentrations are

Fig. A-6. Sampling site Dahab Lagoon (indicated by the red cross), Gulf of Aqaba (Red Sea). The lagoon itself is shallow (reaching 20 m depth at its outer rims); right outside the lagoon depth reaches down several hundreds of meters. Corals were collected at 3-8 m depth. The map was plotted in NAVI 3000 (Transas Marine Ltd.).

rather low (Rasheed *et al.*, 2003), and thus low water turbidity and high solar irradiation allow the settlement of corals to build calcified reefs (*Scleractinia*). At shallow, sandy sites such as the Lagoon of Dahab (Fig. A-6) solitary corals like *Fungia* sp. can settle.

In nutrient-poor water, most of the primary production is sustained by recycled nutrients, which are utilized as soon as they are released (Eppley, 1980; Ducklow, 1983). The photosynthetic fixation of carbon by zooxanthellate symbionts of scleractinian corals is hence an important energy and carbon source for the whole ecosystem. The carbon is stored as mucous polysaccharides and is more or less continuously released into the free water by the coral (Crossland *et al.*, 1980), where it becomes available for microbes as well as higher animals (Johannes, 1967; Goreau *et al.*, 1970; Ducklow and Mitchell, 1979; Gottfried and Roman, 1983).

The bacterioplankton dynamics in the Red Sea are not yet studied in any detail. A positive correlation between the distribution of chlorophyll *a* (Chl *a*) and bacterioplankton biomass production has been observed (Grossart and Simon, 2002). Cultivation attempts have retrieved representatives of *Bacteroidetes*, *Alphaproteobacteria*, and *Gammaproteobacteria* (Pinhassi and Berman, 2003).

3.3 German Bight (North Sea)

The North Sea is a shallow shelf sea which opens northward into the northern Atlantic Ocean (Fig. A-4). Inflow of water with salinities above 35 PSU from the Atlantic and freshwater inflow from the Baltic Sea and rivers result in a decreasing salinity gradient running approximately from North West to South East. The salinity at Helgoland Station (Fig. A-7) in the German Bight is on average 32 PSU (Tab. A-1). Mean temperature is around 10°C (Becker, 2003).

The German Bight is vertically unstratified, and the water column is fully mixing throughout the year. The island of Helgoland is only 50 km off the mouth of rivers Elbe and Weser, and consequently nutrient levels are above mean values of the southern North Sea (Brockmann and Topcu, 2001). River run-off and atmospheric inputs are important sources for elevated nutrient levels in the consequently nutrient-rich North Sea (Brockmann *et al.*, 2003). In addition,

nutrient gradients are controlled by seasonal turnover. Nutrients such as nitrogen and phosphorus are in particular responsible for eutrophication as well as limitation events. On the whole, the German Bight is the most dynamic of the three habitats under investigation, as a pronounced seasonal impact and high terrestrial influence impose great variablity.

The bacterioplankton of the German Bight has been described as being comprised mainly of *Alpha-* and *Gamma-proteobacteria* and *Bacteroidetes* (Eilers *et al.*, 2001). Among and within these groups seasonal changes occur due to high variability within the North Sea system as a temperate sea (Gerdts *et al.*, 2004). Gerdts and colleagues (2004) showed by DGGE analysis that a stable community in winter was displaced by diverse communities in spring and strong shifts were displayed in summer. *Bacteroidetes* represented between 20 and 40% of the community in summer (Eilers *et al.*, 2001; Beardsley, 2003), when *Gammaproteobacteria* contributed between 10 and 14% on average. Approximately one third of all detected *Gamma-proteobacteria* could be identified by FISH with probes specific for only two distinct clades, namely SAR86 and NOR5. A more dynamic component of the gammaproteobacterial community seems to be the so-called opportunistic fraction of marine bacteria. In environmental microbiology, the term opportunistic is increasingly used for bacteria that respond rapidly to changes in environmental conditions. This is e.g. reflected in occasional peaks of abundances of A/C (Fig. A-8) in German Bight surface waters.

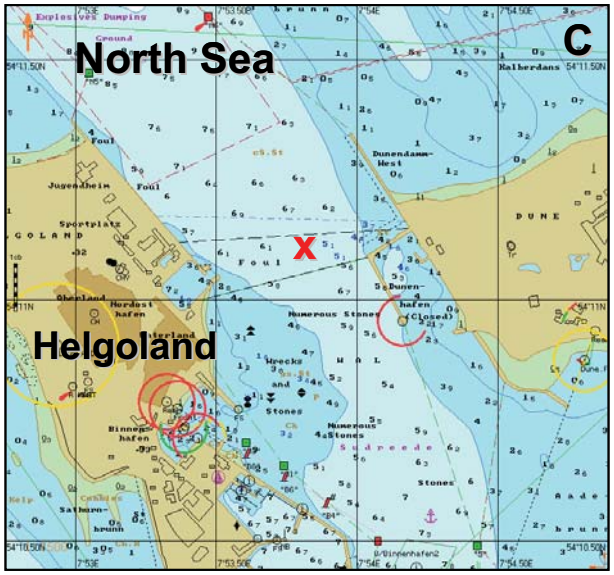

Fig. A-7. Sampling site Helgoland Roads (indicated by the red cross) in the German Bight, North Sea. The site is situated between the island of Helgoland and the Dune. Blue lines depict isobaths. At the sampling site water depth is 7 m. The map was plotted in NAVI 3000 (Transas Marine Ltd.).

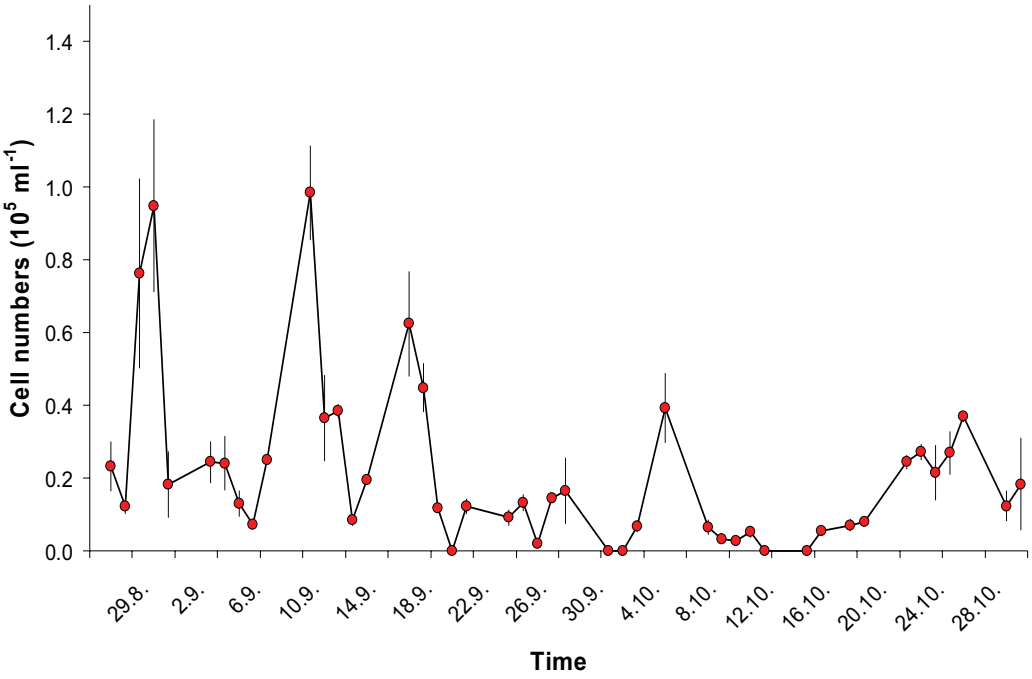

Fig. A-8. Total abundance of *Alteromonadaceae*/*Colwelliaceae* during 2 months of almost daily sampling of surface waters at Helgoland Roads, German Bight. Sampling began at end of August and continued until end of October 2002. Cells were detected by CARD-FISH with probe ALT1413 (Keller, 2003).

4 Methodological approaches

In order to describe bacterial populations and their response to environmental factors tools from different microbial disciplines need to be combined. The experimental work may be based on classic microbiological techniques such as enrichment, isolation and cultivation of microorganisms. The analytical approaches will often rely on molecular biological methods, in particular on the different components of the full cycle 16S rRNA approach (Olsen *et al.*, 1986; Pace *et al.*, 1986; Amann *et al.*, 1995) in order to identify and quantify bacterial genotypes and populations.

Enrichment cultivation allows exposing environmental microbial communities to a selected set of environmentally relevant factors. By the application of different manipulations mimicking changes in ecological factors, their effect on bacterial populations of interest can be studied. Examples for parameters which can be manipulated in laboratory experiments are temperature, food web structure, concentrations of macro- and micronutrients, and the type of carbon source.

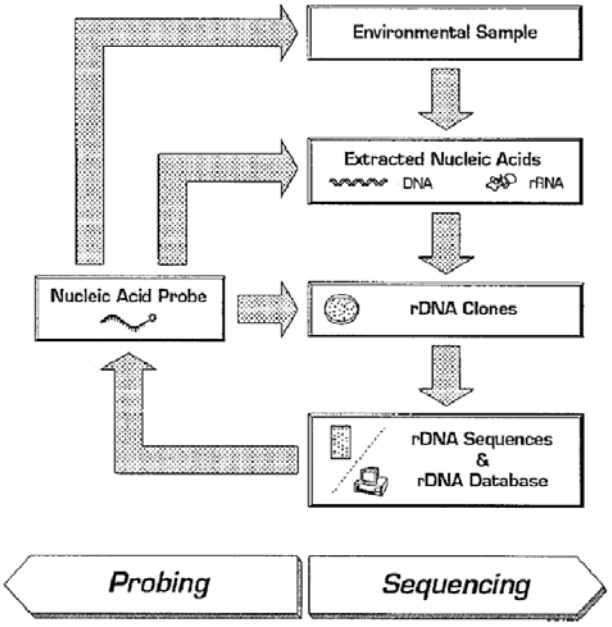

Fig. A-9. Principle phases of the full cycle 16S rRNA approach for cultivation-independent analysis of microbial communities. Nucleic acids are isolated directly from environmental (or experimental) samples to be amplified and cloned. The genes of the 16S rRNA are sequenced in order to compare the new genotypes against existing databases. A comprehensive dataset can be the basis to design specific probes for fluorescence *in situ* hybridization (FISH) in order to detect and quantify the microorganisms of interest in the environment (from Amann *et al.*, 1995).

Bacterial communities from environmental and experimental samples can be characterized by the application of the individual steps of the whole cycle 16S rRNA approach (Fig. A-9). The

16S rRNA gene as phylogenetic marker allows identification without cultivation and laborious characterization of isolates (Woese, 1987) of abundant bacterial taxa in the sea (Giovannoni *et al.*, 1990; Giovannoni and Rappé, 2000). A 16S rRNA gene sequence library serves as a first inventory of the members of prokaryotic communities. However, the resulting frequencies of different genotypes never represent the native community composition due to the so-called PCR bias which is introduced during analysis (Reysenbach *et al.*, 1992; Farrelly *et al.*, 1995; Suzuki and Giovannoni, 1996; v. Wintzingerode *et al.*, 1997). Based on comprehensive 16S rRNA gene sequences databases (such as RDP, Cole *et al.*, 2007), it is possible to design specific oligonucleotide probes in software environments such as ARB (Ludwig *et al.*, 2004). These probes are used for direct microscopic visualization and quantification of populations of interest, e.g. by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) (Pernthaler *et al.*, 2004). Total cell counts and relative abundances of different bacteria within aquatic samples can be evaluated in high-throughput, e.g. flow cytometry (Marie *et al.*, 1997) or automated microscopy (Pernthaler *et al.*, 2003).

Details on specific experimental procedures are presented in the corresponding manuscripts (Part II, B). Since the experiments were carried out in different seas, they are subsequently referred to as Blanes Enrichment (Mediterranean Sea, Publication I), Dahab Enrichment (Red Sea, Publication II), and Helgoland Enrichment (North Sea, Supplement).

5 Aims

The present PhD thesis aimed at the understanding of the ecological role of particular bacterial population in the environment. The main focus was put on marine bacteria that are known to rapidly respond to changes of environmental parameters, such as the substrate concentration or the grazing pressure. It was of interest (i) which populations are responsive to environmental changes, (ii) which trigger do they respond to, and (iii) how do they respond. Manipulative experiments with prokaryotic communities were conducted at three different ecological levels:

(a) At the level of the food web which included sources for bacterial growth as well as bacterial mortality. In order to detect effect-response-relations between the prokaryotic picoplankton and the environment, parameters such as abundances of bacterial populations, phytoplankton and predator densities were monitored in mesocosm. Incubations were set up for several days in order to detect patterns of succession, which elude themselves when taking one snap-shot only. Project details are presented in Publication I.

(b) At the level of the prokaryotic picoplankton community utilizing an undefined, environmental carbon source. Zooxanthellate symbionts of the reef-building corals produce mucus which is released into the water and represents a substrate that appears to be well suited for bacterial growth (Ducklow and Mitchell, 1979; Herndl and Velimirov, 1986). The goal of this project was the identification of microorganisms which would benefit from this type of carbon. The results of this investigation are presented and discussed in Publication II.

(c) At the level of the bacterial community and growth competition of particular populations under defined substrate and nutrient conditions. In isolation attempts and incubation experiments, *Gammaproteobacteria* were found to be overrepresented in resulting collections compared to their environmental abundances (Eilers *et al.*, 2000a). Particularly *Alteromonadaceae*, *Pseudoalteromonadaceae* and *Vibrionaceae* were readily isolated from coastal waters by plating (Eilers *et al.*, 2000a; Eilers *et al.*, 2000b;

Pinhassi and Berman, 2003). Not all of them appear always at the same time and place though (i.e. in the same experiments; e.g. Eilers *et al.*, 2000a; Schäfer *et al.*, 2000; Beardsley *et al.*, 2003; Massana and Jürgens, 2003). It was considered worthwhile investigating which preference determines the success of a microbial population. The findings of this project did not result in a completed publication. Details on methodological aspects and results are presented in the supplement section (Part III).

B Results & Discussion

1 Ecological factors affecting the abundance of studied bacteria

A bacterial community is always exposed to biotic and abiotic factors of the environment, while bacteria, in turn, have an effect on the chemical composition of ecosystems (Jannasch, 1955; Cho and Azam, 1988; Fenchel, 1988). Microbial community composition has often been considered as a result of water temperature, salinity, and/or availability of substrate and nutrients (Larson and Hagström, 1982; Huq *et al.*, 1984; Revilla *et al.*, 2000; Bouvier and del Giorgio, 2002; Cottrell and Kirchman, 2003; Eiler *et al.*, 2006). In the present investigation, the effect of substrate and nutrients was not only studied with respect to growth of one particular species, but also to population dynamics of co-occurring bacteria and their potential interactions, e.g. competition. Specifically, the impact of disturbances, i.e. sudden changes in the environment, on community composition was examined. Since bacteria are part of the food web (Azam *et al.*, 1983; Ducklow, 1983), not only the availability of resources (bottom-up) but also bacterial mortality determines the success of a population. Thus, the analysis of factors which influence mortality (top-down) were included in one of the experimental systems. These findings on population dynamics of the studied groups will subsequently be discussed in the context of their physiology, potential niches and role within the coastal ecosystem. In the following the three studies of this thesis will be discussed synoptically rather than separately in the context of bottom-up and top-down factors.

1.1 Bottom-up factors

Bottom-up controls comprise limiting factors for the production of microbial biomass. Factors which were investigated in this study were nutrient and substrate availability manipulated by amendment or dilution and poorly characterized disturbance by confinement. The effect of these manipulations

was studied on the time scale of hours to days. Changes of the organic matter field were produced by confinement or the induction of an artificial phytoplankton bloom (Blanes Enrichment, Publication I), but also by the addition of specific substrates (natural, complex substrates, Dahab Enrichment/Publication II or defined substrates, Helgoland Enrichment/Supplement).

Confinement

Disturbance by confinement was observed to affect bacterial community composition instantaneously (so-called bottle effect) in the Blanes Enrichment. The relevance of this phenomenon in marine, pelagic environments is not well understood. It is assumed that the confinement process itself somehow changes the structure of the organic matter continuum (Azam and Worden, 2004). We can only carefully speculate that comparable changes within the organic matter field of pelagic systems may occur, e.g. tidal rock pools may be habitats in which confinement may be found in the environment.

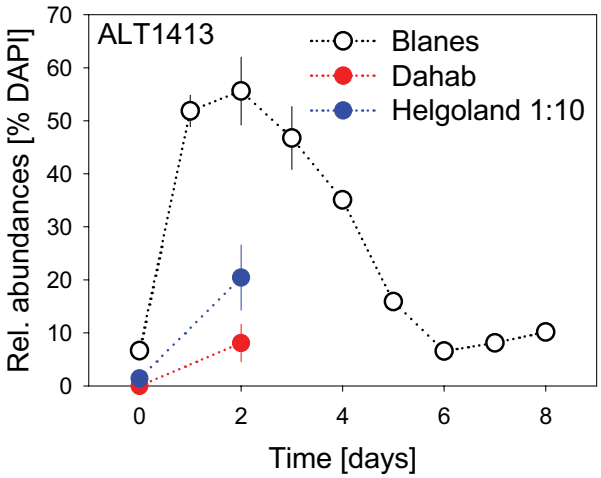

Fig. B-1. Relative abundances of *Alteromonadaceae*/*Colwelliaceae* (A/C) as targeted by probe ALT1413. The graph shows the response of A/C to confinement in incubations of Mediterranean Sea water (Blanes), of Gulf of Aqaba water (Dahab), and German Bight water (Helgoland) without addition of any allochthonous substrates. The Blanes Enrichment lasted for 8 days, whereas the Dahab and Helgoland Enrichments were stopped after 50 and 48 h, respectively.

In the Blanes Enrichment, one particular bacterial population was observed to be most responsive to confinement in mesocosms. Bacteria targeted by probe ALT1413 (group of *Alteromonadaceae* and *Colwelliaceae*; A/C) were rare at the beginning of the incubation (Fig. B-1). Within 24 - 48 h, their relative abundance increased by at least 5-fold in all mesocosms, regardless of any additional manipulation, and these bacteria accounted for up to 56% of total cell counts. A similar response of A/C was observed in bottle

incubations of North Sea water where this bacterial population enriched irrespective of substrate addition (Eilers *et al.*, 2000a). Bacterial growth patterns in the Dahab Enrichment differed in substrate amended and control incubations in contrast to these results (Fig. B-2), probably because they were carried out with comparatively nutrient-poor Red Sea water. While the relative abundances of A/C were 8 ± 4% after 50 h in the control, they reached 49 ± 1% in substrate amended incubations already after 10 h (see *Carbon availability and resources*). It is likely that the background substrate concentrations in the Red Sea waters were too low to fuel growth of the prokaryotic picoplankton despite advantageous conditions for specific bacterial populations due to confinement. In contrast, A/C might have benefited from the generally higher nutrient levels in the Mediterranean (Blanes Enrichment) and North Sea (Helgoland Enrichment) (Fig. B-1).

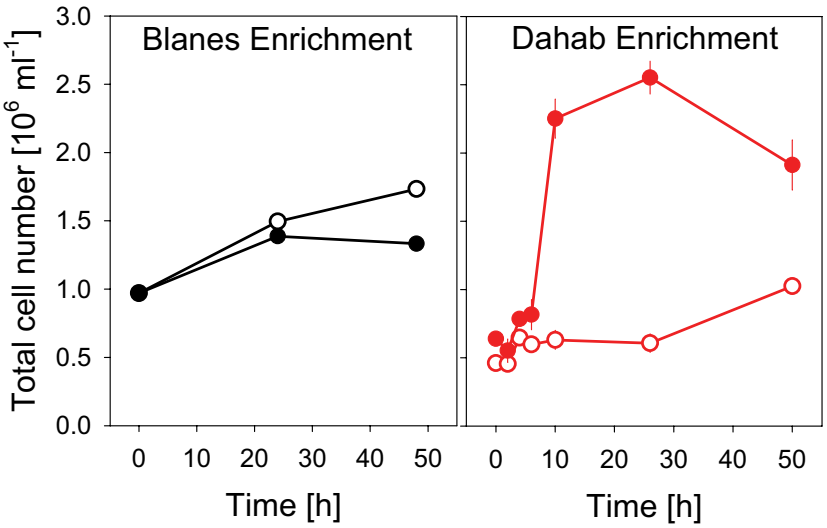

Fig. B-2. Development of total cell numbers during 48 to 50 h of incubation. The control is represented by open circles, the substrate amended incubation by solid circles. In the Blanes Enrichment (black) glucose was added at daily basis, whereas in the Dahab Enrichment (red) mucus of the coral *Fungia* sp. was added 1:10 (v/v) at set up.

Dilution

Another kind of disturbance in aquatic ecosystem is the mixing of water masses of different physico-chemical properties (e.g. estuaries; Revilla *et al.*, 2000; Kirchman *et al.*, 2005). Heavy precipitation periodically dilutes marine surface waters, thereby reducing salinity (Brown *et al.*, 2002) and affecting parameters

such as nutrient and substrate concentration and temperature (Longhurst, 1998).

In the Helgoland Enrichment, North Sea water was diluted 1:10 with aged seawater (7 years old, filtered North Sea water) prior to incubation. After 48 h of incubation at *in situ* temperature of 17°C, total bacterial cell numbers in the control (without substrate amendment) had increased by one order of magnitude to $3.0 \times 10^5 \pm 0.8 \times 10^5$ cells ml^{-1}. *Pseudoalteromonadaceae* and the group of A/C as targeted by oligonucleotide probes PSA184 and ALT1413, respectively, were observed as most responsive and most abundant (Fig. B-3). The absolute numbers of the population of A/C increased during 48 h

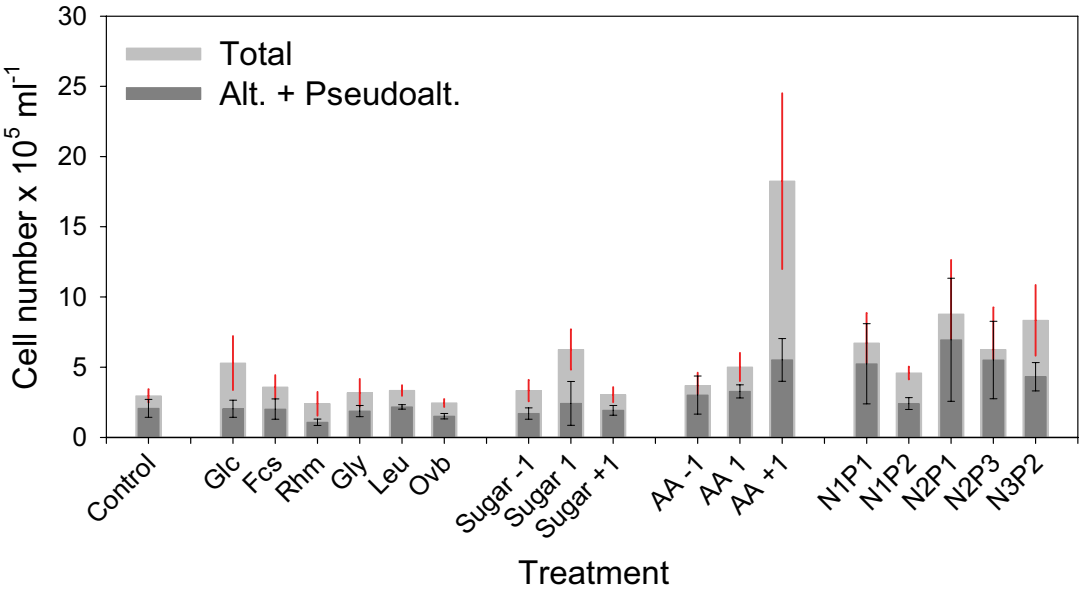

Fig. B-3. Absolut cell numbers in various treatments of the Helgoland Enrichment after 48h of incubation. Total cell counts are compared to the sum of ALT1413- and PSA184-positive cells. All designations are explained in more detail in the supplement section. Incubations were amended with simple sugars (Glucose Glc, Fucose Fcs, Rhamnose Rhm), amino acids (Glycin Gly, Leucin Leu), or protein (Ovalbumin Ovb), or with different concentrations of a mix of sugars (Sugar) or amino acids (AA). '- 1' designates the addition of substrate in lowest concentration, whereas '+ 1' indicates the highest. In addition NH_4 and PO_4 were supplied in different concentrations which are all described in Tab. S1 (Supplements). '1' means no addition of the respective nutrient, '3' indicates the addition in highest concentrations. All carbon treatments were amended with nutrients at medium concentration ('N2P2').Error bars indicate the standard error.

by >2 orders of magnitude to $6.4 \times 10^4 \pm 2.3 \times 10^4$ cells ml^{-1} and reached relative abundances of 20 ± 11% (Fig. B-4). *Pseudoalteromonadaceae* accounted for 46 ± 14%, which corresponded to a rise in total abundance by ~5 orders of magnitude to $1.4 \times 10^5 \pm 4.6 \times 10^4$ cells ml^{-1} within 48 h. In contrast to

the effect of confinement from which bacteria of the A/C group seemed to benefit most pronouncedly (Blanes Enrichment; Fig. B-1) (Eilers *et al.*, 2000a), dilution added another component to the experimental set up and obviously favored the enrichment of *Pseudoalteromonadaceae* over A/C instead

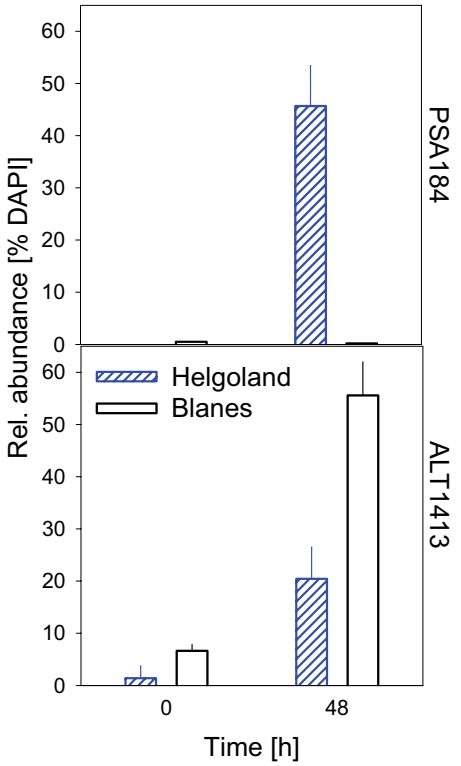

Fig. B-4. Relative abundances of *Alteromonadaceae*/*Colwelliaceae* (A/C; probe ALT1413) and *Pseudoalteromonadaceae* (probe PSA184) in two different enrichment approaches. In the control of Helgoland Enrichment (blue), the environmental microbial community was diluted 1:10 with aged seawater, and after 48 h of incubation it was dominated by *Pseudoalteromonadaceae* (top panel). In the control of the Blanes Enrichment (white), the environmental microbial community was transferred to confinement without any additional treatment or amendment. Members of A/C enriched more pronouncedly in this experiment than in the dilution experiment with water from the German Bight (Helgoland).

(Fig. B-4). The aged seawater was obviously not free of carbon to prevent growth of bacteria. In an incubation experiment with North Sea water which compared enrichment conditions such as dilution or prefiltration (0.8 µm pore size), only dilution resulted in a transient rise of relative abundance of *Pseudoalteromonadaceae* (Beardsley *et al.*, 2003). Dilution cultivation experiments with waters from the Red Sea (Pinhassi and Berman, 2003) resulted in bacterial taxa different from the ones observed in the Helgoland Enrichment. In those incubations mainly *Alphaproteobacteria* and members of the gammaproteobacterial clade *Oceanospirillum* enriched, but no *Alteromonadaceae* nor *Pseudoalteromonadacae*. These results of Pinhassi and Berman (2003) were based on identification after isolation on Zobell Agar only (as opposed to direct identification by FISH). Thus, they represented only 20% of enriched cells as the remaining 80% did not grow as colony forming units (CFU) on plates.

In conclusion, the success of specific populations in dilution cultivation probably does not depend so much on the initial bacterial community, but rather on the dilution medium.

Inorganic nutrients and limitation

Another factor that might influence the performance of bacterial populations in the environment is the availability of nutrients such as nitrogen (N) and phosphorus (P). For example, bacteria may exhibit different preferences in terms of optimum nutrient concentrations and responsiveness. High nutrient concentrations can inhibit some bacteria from forming colonies (Eilers *et al.*, 2001). In comparison to phytoplankton, bacteria are considered to be superior competitors for nutrients (Bratbak and Thingstad, 1985).

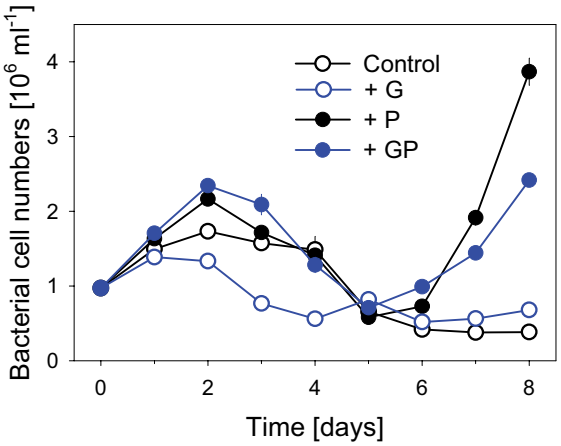

Fig. B-5. Development of total bacterial cell numbers in the different experimental mesocosms of the Blanes Enrichment. Control: unamended, + G: +Glucose, + P: +Phosphate; + GP: +Glucose and Phosphate.

Total cell numbers of the Blanes Enrichment after 2 days of incubation were almost 1.5 times and almost up to twice as high in the P- amended (phosphate only: +P, phosphate + glucose: +GP) mesocosms as compared to the non-P-amended (control, +G) (Fig. B-5). This hints at a P-limitation of bacterial production. In dilution enrichments carried out with water from the Mediterranean Sea and the Gulf of Aqaba, additions of phosphate and carbon had a pronounced effect on total bacterial cell numbers (Pinhassi and Berman, 2003). Another dilution cultivation experiment with Mediterranean Sea water from different seasons compared bacterial activity and growth response under nutrient-amended and unamended conditions (Pinhassi *et al.*, 2006). As medium for dilution, environmental water was filtered (0.2 µm pore size). When there was little or no P-limitation detected in the environment, similar community compositions were observed in nutrient-

amended and control incubations. However, when the environmental bacterioplankton was strongly P-limited, large differences between nutrient-amended and unamended dilution cultivations were found.

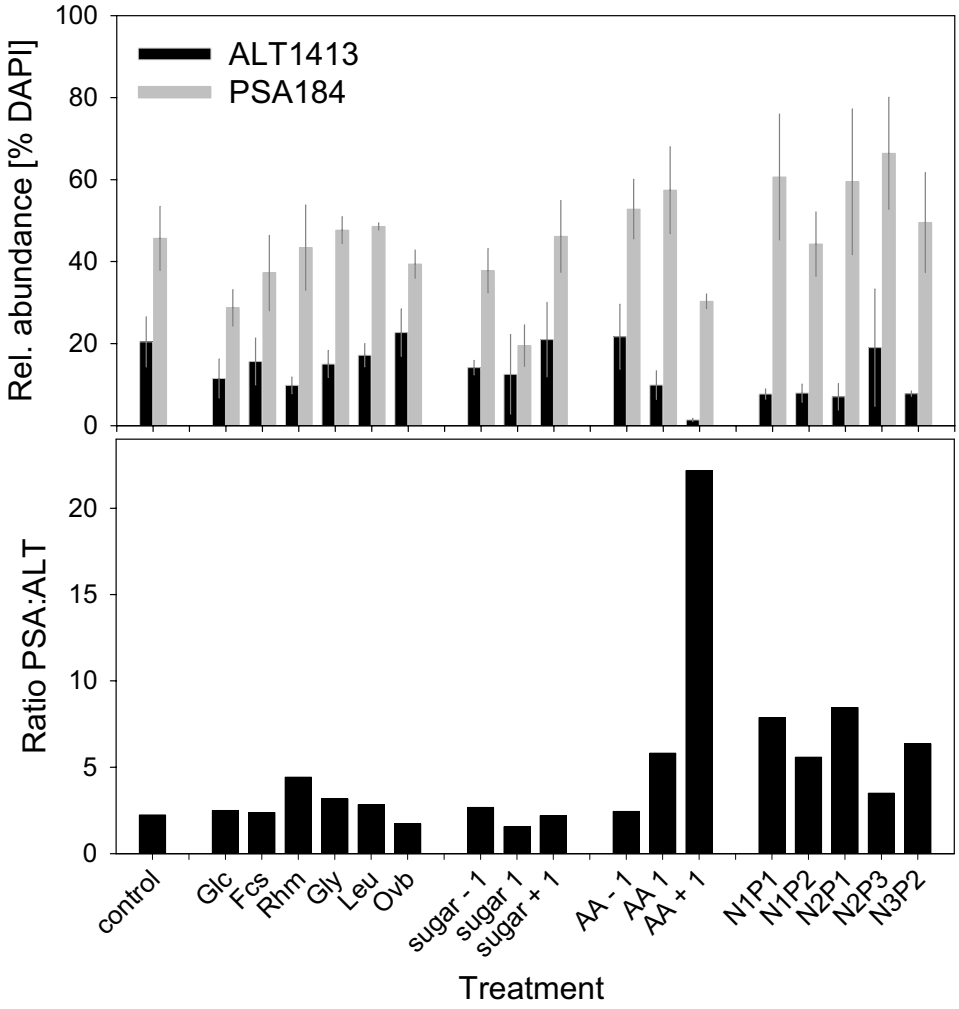

Fig. B-6. Top-panel: Relative abundances of *Alteromonadaceae*/*Colwelliaceae* (A/C; probe ALT1413) and *Pseudoalteromonadaceae* (probe PSA184) in the Helgoland Enrichment after 48 h of incubation with various combinations of substrate and nutrient concentrations. Bottom-panel: Corresponding ratio of the relative abundances of *Pseudoalteromonadaceae* to A/C. All treatment designations are explained in more detail in the supplement section. Incubations were amended with simple sugars (Glucose Glc, Fucose Fcs, Rhamnose Rhm), amino acids (Glycin Gly, Leucin Leu), or protein (Ovalbumin Ovb), or with different concentrations of a sugar mix (Sugar) or amino acids (AA). '- 1' designates the addition of substrate at the lowest concentration, whereas '+ 1' indicates the highest. In addition, NH_4 and PO_4 were supplied in different concentrations which are all described in Tab. S1 (Supplements). '1' means no addition of the respective nutrient, '3' indicates the addition in highest concentrations. All carbon treatments were amended with nutrients at medium concentration ('N2P2'). Error bars indicate the standard error.

In the Helgoland Enrichment, a manipulation of nutrients in addition to various substrate manipulations did scarcely result in significantly different bacterial growth patterns (Figs. B-3, B-6). This indicates that bacteria were rather limited by carbon than by inorganic nutrients. However, analysis of the microbial community revealed nevertheless an effect on the ratio of the relative abundances of A/C and *Pseudoalteromonadaceae*. Whenever nutrients were lacking or added in 'non-normal' ratios (Fig. B-6; treatments N1P1, N1P2, N2P1, N2P3, N3P2; for more details see Tab. S-1), *Pseudoalteromonadaceae* formed populations significantly larger than in a nutrient ratio as found in the environment (treatment Sugar 1).

Carbon availability and resources

Organic carbon, both in dissolved and particulate form (Azam *et al.*, 1983; Azam, 1998; Simon *et al.*, 2002), is a key resource for bacterial growth. Studies from various pelagic environments have shown that dissolved amino acids and neutral monosaccharides are the most important substrates for bacterioplankton growth (e.g. Kirchman, 1990; Rich *et al.*, 1996; Weiss and Simon, 1999). To study the effect of organic carbon addition on bacterial community composition in different habitats and under various conditions, enrichments with addition of simple (Publication I and Supplement I, SI) or complex (Publication II) carbon substrates were performed. In the Helgoland Enrichment (SI), increasing amino acid concentrations shifted the ratio of the relative abundances of *Pseudoalteromonadaceae* to A/C in favor of *Pseudoalteromonadaceae*

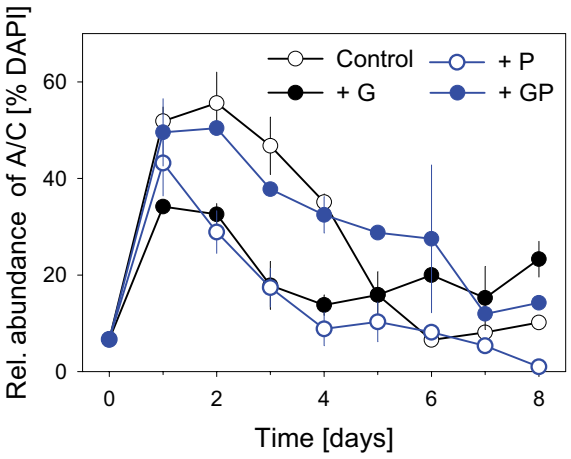

Fig. B-7. Changes in the relative abundance of *Alteromonadaceae*/*Colwelliaceae* in the control and amended mesocosms in the Blanes Enrichment. Treatment designations are: + G: +Glucose, + P: +Phosphate, + GP: +Glucose and Phosphate.

(Fig. B-6, bottom panel). Two different possibilities can explain these findings: (i) *Pseudoalteromonadaceae* were more competitive for amino acids, or (ii) members of the A/C cluster were inhibited by high amino acid concentrations. The low abundance of A/C (2 ± 1%; Fig. B-6, top panel; $2.0 \times 10^4 \pm 3.1 \times 10^3$ cells ml^{-1}, data set not shown) in the presence of the highest applied amino acid concentration rather supports the latter assumption. Kirchman (1994) suggested that uptake of dissolved free amino acids (DFAA) might be a feature of nutrient-rich coastal rather than nutrient-poor oceanic zones. The findings of the Helgoland enrichment hint, in addition, at populations within the coastal bacterioplankton that are more competitive for DFAA than others, i.e. *Pseudoalteromonadaceae*.

In the Blanes Enrichment, the addition of simple glucose (+G) had two clear-cut consequences: (i) The population of A/C persisted over time more successfully in incubations with glucose-amendment as compared to the control (C) and the P-amended mesocosm (+P) (Fig. B-7). The additional glucose might have fueled growth of the population against the grazing pressure by HNF, so that they were not suppressed as much as in the absence of glucose. Alternatively, some bacteria might have used the extra carbon to increase in cell size in order to obtain full or partial resistance against HNF predation (Thingstad *et al.*, 2005). (ii) The development of community composition of *Rhodobacteraceae* in the glucose-amended incubations differed

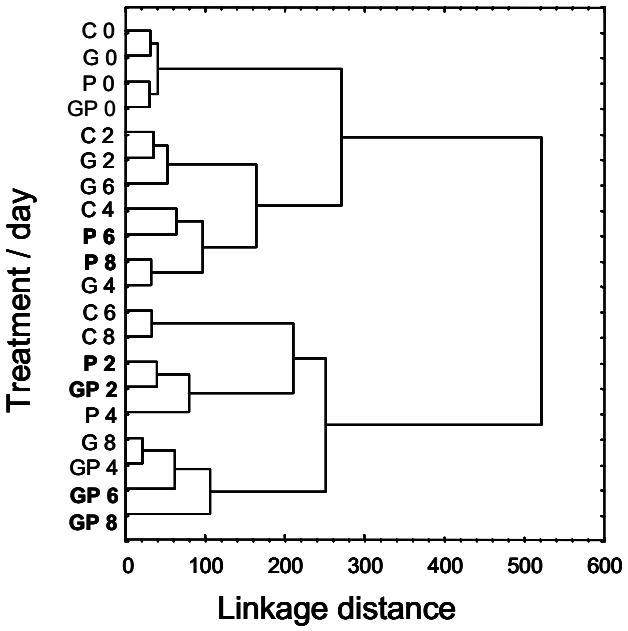

Fig. B-8. Similarities of the *Rhodobacteraceae* communities in the different treatments of the Blanes Enrichment at the beginning and at days 2, 4, 6, and 8 of the experiment. The dendrogram depicts the results of cluster analysis (Ward's method) of those bands in DGGE gels that were affiliated to *Rhodobacteraceae* according to sequence analysis. Treatment designations are: C: Control, G: +Glucose, P: +Phosphate, GP: +Glucose and Phosphate. Highlighted in bold: similarity of the *Rhodobacteraceae* community in +P and +GP at day 2 and their contrasting development (days 6-8).

from the one without daily glucose dosis. This resulted in 2 distinct bacterial communities for treatment +P and for treatment +GP (Fig. B-7; Fig. B-8). The additional glucose might have been directly taken up by these bacteria (Alonso and Pernthaler, 2006). Alternatively, it might have influenced algal growth which in turn might have resulted in a selection for different bacterial genotypes due to varying substrate sources (Pinhassi *et al.*, 2004).

In general, the phytoplankton represent another source of carbon in this experiment. Blooms of phytoplankton were most pronounced in mesocoms which were amended with phosphorus (+ P and + GP), as reflected by Chl *a* concentrations (Fig. B-9). Those phytoplankton blooms were accompanied by rising abundances of bacteria affiliated to the *Rhodobacteraceae* (Fig. B-9). At its maximum, this population accounted for approximately 50% of the bacterial community. From day 0 to day 5 (+P) and from day 0 to day 7 (+GP) absolute

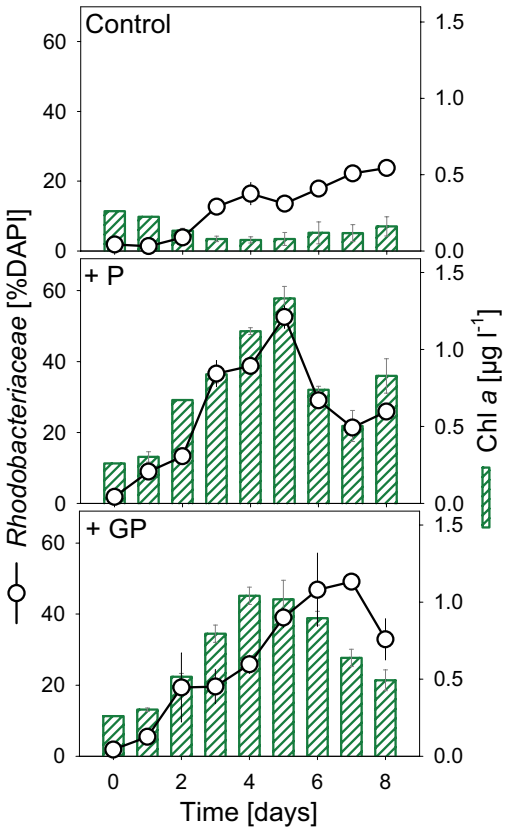

Fig. B-9. Relative abundances of *Rhodobacteraceae* and Chl *a* concentrations in the control and the phosphate amended treatments of the Blanes Enrichment. Designations are: + P: + phosphate, + GP: + glucose and phosphate.

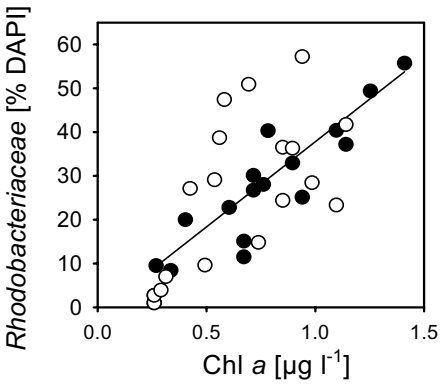

Fig. B-10. Correlation between the community contribution of *Rhodobacteraceae* and the concentration of Chl *a* in the +P (solid circle) and +GP (open circle) treatments of the Blanes Enrichment. The relationship of *Rhodobacteraceae* and Chl *a* is linear (r^2=0.58).

numbers of *Rhodobacteraceae* increased by a factor of 4 to 5 thousand, reaching densities of $5.7 \times 10^5 \pm 4.0 \times 10^4$ cells ml^{-1} (+P) and $7.1 \times 10^5 \pm 1.1 \times 10^4$ cells ml^{-1} (+GP) (data not shown). *Alphaproteobacteria* as well as *Bacteroidetes* were observed to co-occur with blooms of phytoplankton in natural and experimental systems before (Riemann *et al.*, 2000; Eilers *et al.*, 2001; Zubkov *et al.*, 2001; Pinhassi *et al.*, 2004). However, the present Blanes Enrichment provides strong statistical evidence for a tight relation between the occurrence and abundance of algae and members of the Roseobacter clade (Fig. B-10).

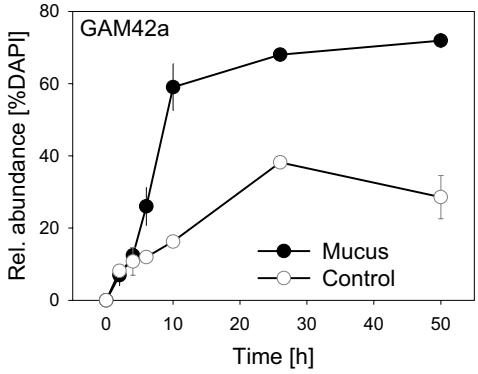

Fig. B-11. Relative abundance of *Gammaproteobacteria* as detected by FISH with probe GAM42a in control and mucus-amended incubations of the Dahab Enrichment.

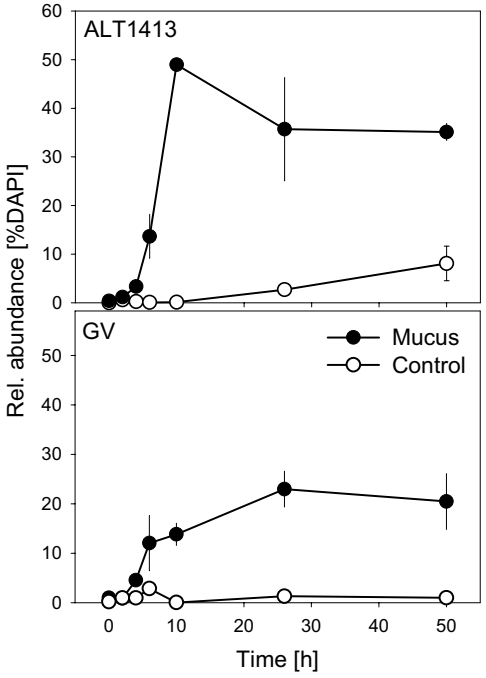

Fig. B-12. Changes of the relative abundances of *Alteromonadaceae*/*Colwelliaceae* (probe ALT1413) and *Vibrionaceae* (probe GV) of the Dahab Enrichment in mucus-amended and control incubations.

In the Dahab Enrichment, *Gammaproteobacteria* dominated a community grown in seawater amended with mucus from the coral *Fungia* sp. (Fig. B-11). The addition of a complex, natural source of organic carbon, i.e. coral mucus, to Red Sea water resulted in an almost instantaneous enrichment of A/C followed by *Vibrionaceae* (Fig. B-12). The probe AMAC83 was specifically designed for 2 genotypes within the gene sequences library from this experiment. These genotypes are closely related to *Alteromonas macleodii* (> 99% sequence identity) (Publication II, Fig. 3, p. 119) and accounted for 44% of the total bacterial community and for 90% of all detected members of the A/C

group after 10 h of incubation, whereas they were rare at the beginning and in the control incubation (Fig. B-13). These results indicate that some genotypes related to *A. macleodii* may be specialized to prosper on the dissolved fraction of coral mucus (which may be 56–80% of total mucus; Wild *et al.*, 2004). It has been shown in waters immediately surrounding coral reefs that there are elevated bacterial numbers compared to the remaining reef waters (Seymour *et al.*, 2005). In view of our results it is likely that *A. macleodii* may form substantial populations in the direct vicinity of corals. Therefore these bacteria may play an important role in channeling coral-released DOC to higher trophic levels of the pelagic zone of coral reefs.

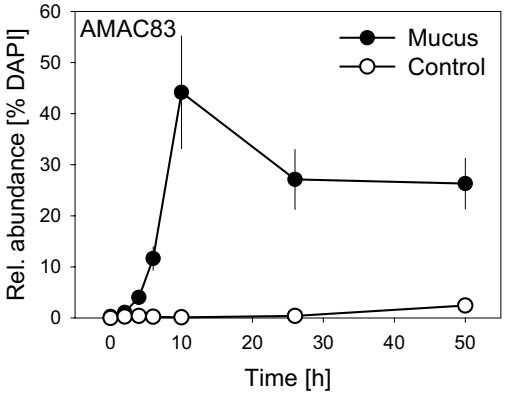

Fig. B-13. Changes of the relative abundances of cells hybridized by the *Alteromonas macleodii* specific probe AMAC83 (targeted to genotypes M10-016 and M26-001, Publication II, Tab. 2, p. 116) in mucus-amended and control incubations.

1.2 Top-down factors

Predation

In the Blanes Enrichment, the effect of grazing by heterotrophic nanoflagellates (HNF) on different bacterial populations was monitored for 8 days. During the first 48 h of the incubation, populations of A/C enriched quite rapidly in all mesocosms and accounted for 34 to 56% of the total bacterial community (Fig. B-14). When HNF numbers increased above densities of ~10^3 ml^{-1}, numbers of A/C started to decrease (control, +G, +P) or the increase was slowed down and eventually ceased at higher HNF densities (+GP), respectively, indicating that the consumption of that particular bacterial population fueled HNF growth (Fig. B-14). During the second half of the incubation, a population of *Rhodobacteraceae* dominated the communities in most mesocosms. In

contrast to A/C, these populations were less sensitive to grazing by HNFs as illustrated by the non-linear relationship between the abundance of grazers and the relative abundance of *Rhodobacteraceae* (Fig. B-15). At low HNF densities (i.e., before and after the HNF bloom), *Rhodobacteraceae* increased with HNF numbers, whereas the opposite was true at higher HNF densities of 1.5×10^4 ml^{-1} or above. A similar pattern, i.e. a smaller effect of HNF on *Rhodobacteraceae* than on *Alteromonadaceae* (at protistan densities $<10^4$ ml^{-1}) has also been observed during short-term bottle incubations (Beardsley *et al.*, 2003). Such high experimental HNF densities, however, are above those densities observed in coastal waters (Tanaka *et al.*, 1997; Beardsley *et al.*, 2003). There are substantial implications of our findings with respect to the occurrence of *Alteromonadaceae* in coastal waters. Specifically, it is likely that short-lived blooms of these bacteria are terminated by the activity of bacterivorous protists.

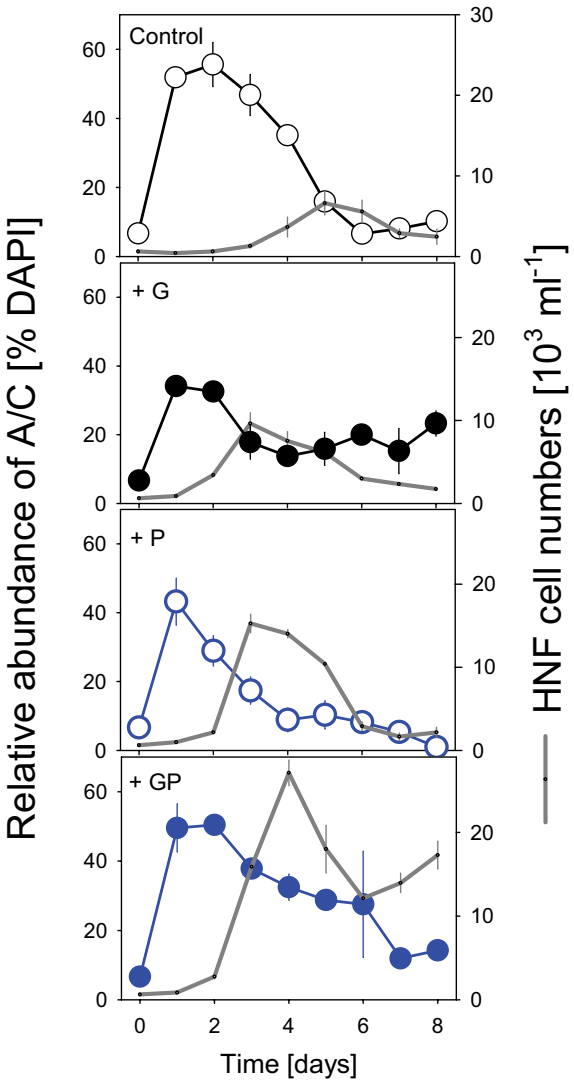

Fig. B-14. Development of the relative abundances of *Alteromonas*/*Colwellia* (A/C; circles) and the total numbers of heterotrophic nanoflagellates (HNF; line) in the Blanes Enrichment. Treatment designations are: + G: +Glucose, + P: +Phosphate, + GP: +Glucose and Phosphate.

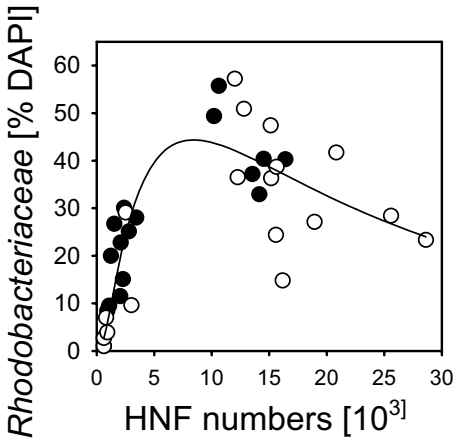

Fig. B-15. Correlation between the community contribution of *Rhodobacteraceae* and HNF numbers in the +P (solid circle) and +GP (open circle) treatments. The relationship of *Rhodobacteraceae* and HNF in both P-amended treatments is best described by a non-linear pattern (regression type: lognormal, r^2= 0.74). Treatment designations are: + P: +Phosphate, + GP: +Glucose and Phosphate.

Viral lysis

Viral lysis of bacterial cells may explain in parts the reduction of abundances of the group of A/C in the Blanes Enrichment. Since such a viral 'killing the winner' scenario (Thingstad and Lignell, 1997) is based on host-specificity, it would require low species diversity within the A/C. The results of strain isolation support this assumption: Only 4 different genotypes affiliated with this group could be retrieved at various time points, and 2 of those isolates were phylogenetically very closely related (sequence identity > 99%). Since *Alteromondaceae* are known to be readily cultivable (Eilers *et al.*, 2000b; Pinhassi and Berman, 2003) it is indeed likely that the diversity of these bacteria was low. If more genotypes had been present, they should have occurred in the collection of retrieved isolates. However, the total abundances of viral particles changed little in mesocosms without P addition (M. Weinbauer, unpublished data), suggesting that protistan predation probably represented the dominant cause of mortality for A/C in these treatments.

2 Bacteria with opportunistic growth strategy in coastal waters

In order to describe the potential ecological role of microorganisms, neither phylogenetic position nor morphological and physiological traits in pure culture nor stand-alone genome analysis will be sufficient. Additionally, it also appears to be essential to identify and monitor ecological factors which affect the performance of bacterial populations in the context of microbial competitors and the food web.

The microbial key players in the studies conducted here were *Gamma*- and *Alphaproteobacteria* with an apparently opportunistic growth strategy. Thus, it seems that 'opportunism' is no trait affiliated with a particular phylognetic group. However, both the *Gamma*- as well as the *Alphaproteobacteria* displayed distinct differences in their respective niches.

2.1 Members of *Rhodobacteraceae* – opportunistic companions of phytoplankton

The marine Roseobacter clade comprises phylogenetically diverse and physiologically versatile bacterial species (Buchan *et al.*, 2005). Members of this clade have been found frequently to be abundant in communities associated with phytoplankton blooms – in the environment as well as experimentally induced in the laboratory (González *et al.*, 2000; Eilers *et al.*, 2001; Zubkov *et al.*, 2001; Pinhassi *et al.*, 2004; Pinhassi *et al.*, 2005).

In the Blanes Enrichment, *Rhodobacteraceae* benefited directly from the contribution of the phytoplankton to the pool of DOC. Even though these bacteria responded readily to manipulations, they were outcompeted by A/C. There are two possible explanation for this: (i) Since *Rhodobacteraceae* benefit from algal exudates, their abundance did not increase before a certain density of phytoplankton was reached and a corresponding concentration of exudates

was present (Fig. B-10, p. 41), (ii) in general, *Rhodobacteraceae* respond more slowly to environmental changes than *Alteromonadaceae* (Publication I, Fig. 3). One reason for this might be a different number of rRNA operons (Klappenbach *et al.*, 2000). A high operon number is believed to be a prerequisite for an immediate and fast response to nutritional changes (Klappenbach *et al.*, 2000). Three rRNA operons were found in the genome of *Silicibacter pomeroyii* (*Rhodobacteraceae*), a species that responds to algal exudates (Moran *et al.*, 2004). Rapidly responding bacteria from soil have 5 rRNA operons (Klappenbach *et al.*, 2000). Unfortunately, a published copy number of *Alteromonas* is not yet available. A count of the complete rRNA operons in the draft genome of *Alteromonas macleodii* 'Deep ecotype' (Accession number: NZ_AAOD00000000) resulted in 5 copies. Other opportunistically growing bacteria (*Vibrio*) feature 8 and 14 copies of the rRNA operon (Heidelberg *et al.*, 2000; Thompson *et al.*, 2004b).

According to correlation analysis, predation was only harmful to the population of *Rhodobacteraceae* when grazer densities were extremely high (Fig. B-15, p. 45). Thus, they seem to exhibit some resistance against grazing, e.g. a smaller cell size or motility (Pernthaler, 2005). Alternatively, HNF might prefer to graze upon other populations which potentially better suit their nutritional requirements.

In summary, *Rhodobacteraceae* will prosper in coastal waters that are characterized by high phytoplankton and low to medium predator (e.g. HNF as part of the zooplankton) densities. The high diversity of *Rhodobacteraceae* that is even observed in enrichment cultivation (Fig. B-8, p. 40) furthermore hints at a versatile utilization of the organic material provided by the phytoplankton.

2.2 Members of *Alteromonadaceae* – opportunistic exploiters of rapid changes

Members of A/C were observed to thrive on simple as well as complex organic substrates (Fig. B-6, p. 38; Fig. B-7, p. 39; Fig. B-12, p. 42). They were competitive for glucose in the Blanes Enrichment (Publication I) even in comparison with *Rhodobacteraceae*. A/C were the first group to utilize the

organic carbon from coral mucus as substrate for growth in the Dahab Enrichment (Publication II), even outcompeting closely related gammaproteobacterial genera, e.g. *Vibrio* (Fig. B-12, p.42). Thus, they could be described as competition-specialists and 'early stage' opportunistic species; not only do they respond to various environmental changes, they are also the first to do so. Obviously, cells from the A/C group easily switch from 'standby' into 'growth' mode, since almost no lag phase and short doubling times were observed, e.g. in the Dahab Enrichment (Fig. B-12, p. 42). Genotypes of *Alteromonas macleodii* only required one hour for doubling in Red Sea water freshly amended with coral mucus. Thus these bacteria seemed to possess the necessary enzymatic 'equipment' for the degradation of coral mucus or consumption of dissolved organic material. This observation suggests that a versatile metabolism is absolutely advantageous for ubiquitous organisms with an opportunistic life style, which are probably substrate generalists.

Pronounced grazing-sensitivity seems to accompany these high growth capacities, probably due to an increased cell size (Publication I, Fig. 6) (Beardsley *et al.*, 2003). HNF were observed to prey upon A/C extremely selectively. It is possible that a few members of the A/C population might have managed to survive the HNF attack in the Blanes Enrichment by increasing their cell size beyond the optimal uptake size of their predators (Jürgens and Matz, 2002). Such a combination of traits of competition-specialists (for glucose) and defense-specialists (against HNF) has been described as 'Winnie-the-Pooh strategy' at the example of *Vibrio splendidus* (Thingstad *et al.*, 2005).

From our results it can be deduced that *Alteromonadaceae* will prosper in sites with a rather dynamic organic matter field. In particular, the periodic availability of DOC will be beneficial, as they are the first to respond to changes in growth condition. However, these populations will be kept in check by top-down control by predators and potentially by viral lysis. It is conceivable that in some ecological setups (e.g. in the vicinity of corals) considerable amounts of dissolved organic carbon are channeled to higher trophic levels through such opportunistically growing bacterial populations.

3 Outlook

Diversity and occurence of A. macleodii

Alteromonadaceae are found in the free water column as well as attached to particles and surfaces, and are usually observed in low abundances in coastal surface waters (Acinas *et al.*, 1999; Dang and Lovell, 2000; Eilers *et al.*, 2000b; Beardsley *et al.*, 2003). In the Dahab Enrichment particularly two closely related genotypes of *Alteromonas macleodii* rapidly increased in abundance in incubations amended with mucus of the coral *Fungia* sp. as a resource. This hints at a high specialization for particular habitats. A depth dependent distribution of ecotypes of *A. macleodii* in marine waters has been described. Comparative sequence analysis of ITS (Intergenic transcribed spacer), *gyrB* (encoding the beta subunit of DNA gyrase), and *rpoA* (encoding the alpha subunit of RNA polymerase) genes revealed a separation of *A. macleodii* into a deep-sea and a surface ecotype (López-López *et al.*, 2005). Since these bacteria displayed short generation times in the Blanes as well as in the Dahab Enrichment, any adaptation to distinct niches could rapidly be manifested in the genome (Cooper and Lenski, 2000). Since the coral mucus composition differs between coral species (Meikle *et al.*, 1988), and various *A. macleodii* have been found associated with different corals in locally separated seas (e.g. Bourne and Munn, 2005; Lampert *et al.*, 2006), different genotypes might be specialized to e.g. the utilization of particular polysaccharides as substrates. Since *A. macleodii* are culturable on agar plates, their microdiversity could be investigated by extended isolation and subsequent genotypic and phenotypic analyses. The resolution of the microdiversity within *A. macleodii* might reveal new insights about ecological adaptations within this species (Thompson *et al.*, 2005). Certainly, the appropriate set of genetic markers for such a distinction between ‘ecotypes’ needs to be established.

Identification of functional genes and comparative gene expression analysis

Opportunistic bacterial species share the traits of being responsive to environmental changes and of responding quickly. However, currently it is poorly understood how this is reflected on a genomic level (apart from the above discussed rRNA operon numbers). *In silico* (comparative genomics) analysis and *in vitro* (physiological testing and gene expression profiling) analysis should identify the involved gene apparatus which allows for the success of bacteria such as *Alteromonadaceae* and *Rhodobacteraceae* in their respective niches.

Monitoring of opportunistic bacterial species in the environment

Periodically occurring substrate and nutrients input results in the rapid growth of so called 'opportunitrophs' (Moran *et al.*, 2004), especially in oligotrophic environments. Such arbitrary availability of resources is therefore thought to select for species which are competition-specialists. However, to date environmental evidence for the occurrence of such bacteria is still scarce, because their short-lived blooms (e.g. *Alteromonadaceae*/*Colwelliaceae*; Fig. A-8, p. 26) are often overlooked at the typical sampling intervals of weeks to months. Therefore data such as bacterial population dynamics, nutrient concentrations, temperature, salinity, and density of predators needs to be collected simultaneously at a high resolution (on scale of days) in order to determine the exact combination of parameters that favor blooms of opportunistic bacteria. Thereby it may be possible to identify bacterial groups that could serve as indicators for particular disturbance events (e.g. input of allochthonous material).

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Part II: Publications

A List of publications

List of publications and my contributions the manuscripts

I Allers, E., Gómez-Consarnau, L., Pinhassi, J., Gasol, J.M., Simek, K., and Pernthaler, J. (2007) Response of *Alteromonadaceae* and *Rhodobacteriaceae* to glucose and phosphorus manipulation in marine mesocosms. *Environmental Microbiology (Online Early)*: doi: 10.1111/j.1462-2920.2007.01360.x.

Sampling for FISH during the experiment, sample analysis and data evaluation; writing of a first draft of the manuscript and of the first subsequent update.

II Allers, E., Niesner, C., Wild, C., and Pernthaler, J. (in prep.) Primary microbial colonizers of mucus from the scleractinian coral *Fungia* sp.

Contribution to experimental design; realization of the experiment and sampling; in parts sample analysis and FISH data evaluation; probe design; writing of a first draft of the manuscript and of the first subsequent update.

Not presented in this thesis:

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C.A., Quast, C., Kuhl, H., Knaust, F., Woebken, D., Bischof, K., Mussmann, M., Choudhuri, J.V., Meyer, F., Reinhardt, R., Amann, R.I., and Glöckner, F.O. (2006) Whole genome analysis of the marine *Bacteroidetes* '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ. Microbiol.* 8: 2201-2213.

Member of the annotation team; subsequent investigation of the genetic information with respect to pathways of the carbohydrate and amino acid metabolism

B Publications

Publication I

Response of *Alteromonadaceae* and *Rhodobacteriaceae* to glucose and phosphorus manipulation in marine mesocosms

Elke Allers, Laura Gómez-Consarnau, Jarone Pinhassi,
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Response of *Alteromonadaceae* and *Rhodobacteriaceae* to glucose and phosphorus manipulation in marine mesocosms

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Summary

Microbial successions were studied in experimental mesocosms of marine water in the presence of additional organic carbon (glucose), phosphorus (P) or both. P addition lead to pronounced blooms of phytoplankton and to significantly enhanced bacterial production. Characteristic succession patterns were observed for two phylogenetic groups of bacteria that both transiently formed > 50% of total cells. An initial bloom of bacteria affiliated to the *Alteromonadaceae* could not be assigned to any specific treatment and was interpreted as a response to the manipulations during mesocosm set-up. These bacteria rapidly declined with the appearance of heterotrophic nanoflagellates, suggesting a negative effect of selective grazing. The persistence of *Alteromonadaceae* in the microbial assemblages was significantly favored by the presence of additional glucose. During the second half of the experiment, bacteria affiliated to *Rhodobacteriaceae* formed a dominant component of the experimental assemblages in treatments with addition of P. The community contribution of *Rhodobacteriaceae* was significantly correlated with chlorophyll *a* concentrations only in the P-amended mesocosms ($r^2 = 0.58$). This was more pronounced in the absence of glucose ($r^2 = 0.85$). The phylogenetic and morphological diversity among *Rhodobacteriaceae* was high, and treatment-specific temporal successions of genotypes related to *Rhodobacteriaceae* were observed. We suggest that the observed succession patterns reflect different niche preferences: *Alteromonadaceae* rapidly responded to disturbance and profited from allochthonous glucose input, whereas *Rhodobacteriaceae* benefited from the phytoplankton bloom.

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Introduction

Recurring seasonal phenomena, such as phytoplankton spring blooms, are an important feature of coastal ecosystem dynamics that lead to predictable patterns of bacterial successions (Schauer *et al.*, 2003; Fuhrman *et al.*, 2006). In addition, some coastal environments such as lagoons and estuaries exhibit high and stochastic variability of, for example, salinity, nutrients or concentrations of dissolved organic carbon (DOC) (Kirchman *et al.*, 2005), potentially causing short-lived blooms of particular bacterial species (Piccini *et al.*, 2006). Succession within the bacterioplankton in coastal marine systems may thus be triggered by autochthonous processes, but also by other input of labile organic substrates, for example, the nutrient-rich run-off from rivers or organic contamination (Revilla *et al.*, 2000; Schendel *et al.*, 2004). Succession patterns are moreover shaped by selective mortality, as, for example, the grazing preferences of heterotrophic nanoflagellates (HNF) or selective viral lysis can affect the relative proportions of different microbial populations (Thingstad, 2000; Pernthaler, 2005).

Experimental manipulations of marine bacterioplankton assemblages suggest that there are at least two groups of planktonic bacteria that can rapidly respond to environmental changes, but that nevertheless appear to occupy different niches. On the one hand, the growth of bacteria from particular phylogenetic lineages is apparently stimulated by the input of certain labile substrates (e.g. glucose; Pinhassi and Berman, 2003) that might also be released during experimental manipulations due to disturbance of the autochthonous organic matter field (Kepkay, 1994; Azam and Worden, 2004). For example,

Gammaproteobacteria affiliated to the genera *Alteromonas*, *Pseudoalteromonas* and *Vibrio* spp. are typically rare *in situ* (Eilers *et al.*, 2000a), yet these bacteria are readily enriched during incubation of seawater in bottles or mesocosms with or without addition of substrates or after food web manipulation (Lebaron *et al.*, 1999; Pukall *et al.*, 1999; Eilers *et al.*, 2000b). At the same time, these bacteria appear to be particularly sensitive to selective HNF grazing (Beardsley *et al.*, 2003; Worden *et al.*, 2006).

On the other hand, some bacteria appear to be more tightly associated with blooms of phytoplankton: typically, the abundances of some *Bacteroidetes* and *Alphaproteobacteria* increase in the presence of natural and experimentally induced algal blooms (Riemann *et al.*, 2000; Zubkov *et al.*, 2001; Pinhassi *et al.*, 2004). In particular, bacteria from the physiologically diverse family of marine *Rhodobacteriaceae* specifically accompany artificially generated blooms of, for example, diatoms (Schäfer *et al.*, 2002; Grossart *et al.*, 2005). Such bacteria have been found to closely follow the seasonal development of primary producers in coastal North Sea waters (Eilers *et al.*, 2001). In contrast, no stimulation of *Rhodobacteriaceae* was observed during manipulations that did not trigger phytoplankton growth (Eilers *et al.*, 2000b).

Growth of bacteria from these two lineages in meso- or microcosms has been reported previously (Pukall *et al.*, 1999; Schäfer *et al.*, 2000; 2002; Pinhassi *et al.*, 2004). Currently it is unclear, however, how populations of *Alteromonadaceae* and *Rhodobacteriaceae* might interact in such systems. Specifically, it is not known if these groups form simultaneous blooms in experimental assemblages, or if there are distinct succession patterns that can be assigned to differences in their respective niche preferences as outlined above. Mesocosm set-ups seem to be particularly useful to study the relationship between these microbes in detail, because (i) the initial confinement in itself represents a disturbance of the original environment, (ii) mesocosms can be spiked with organic carbon (e.g. glucose), and (iii) sufficiently sized mesocosms allow the induction of phytoplankton blooms (by addition of nutrients).

We monitored the diversity and short-term temporal dynamics of bacteria related to *Alteromonadaceae* and *Rhodobacteriaceae* in mesocosms of coastal Mediterranean waters that were either amended with glucose (+G), phosphorus (+P), both (+GP) or left unmanipulated (control). This was studied in the context of changes in phytoplankton density and of the development of the HNF community.

Results

Dynamics of chlorophyll a *concentrations and HNF densities.*

Both P-amended mesocosms displayed pronounced maxima of phytoplankton biomass – as reflected by chlorophyll *a* (Chl *a*) concentrations – between days 4 and 5 (1.3 $\mu g\ l^{-1}$ and 1.0 $\mu g\ l^{-1}$ in +P and +GP respectively) (Fig. 1). The average Chl *a* concentrations in these treatments were significantly higher than in the other mesocosms (Fig. 1, Table 1). Interestingly, the addition of glucose also resulted in significantly elevated Chl *a* concentrations as compared with the control treatment (Table 1). A second maximum of Chl *a* was observed on day 8 only in +P.

The cell numbers of HNF during the first 6 days of the experiment generally followed the development of the bacterial assemblage with a time lag, and HNF reached their highest densities always at least 1 day after the first peak of bacterial abundances (Fig. 1). This maximum formed latest in the control mesocosms (day 5), when HNF densities in the other treatments had already started to decrease. HNF reached significantly higher average (Table 1) and maximal densities (day 4, 2.7×10^4 cells ml^{-1}) in +GP than in the control and +G. A second bloom of the flagellate population was observed in this treatment at the end of the experiment (Fig. 1). In all but the control mesocosms there was a highly significant positive correlation ($P < 0.001$) between HNF numbers and Chl *a* concentration (Spearman rank correlations, +G: $r_S = 0.78$; +P: $r_S = 0.88$, +GP: $r_S = 0.75$).

Table 1. Treatment-specific differences of Chl *a* concentrations, bacterial and HNF counts, bacterial production and of the community contributions of *Rhodobacteriaceae* and *Alteromonadaceae* in the experimental mesocosms as established by ANOVA and *post hoc* tests.

		Counts			Relative abundance	
Treatments	Chl *a* concentration	HNF	Bacteria	Bacterial production	*Rhodobacteriaceae*	*Alteromonadaceae*
Control	a	a	a	a	a	ab
+G	b	a	a	a	a	ab
+P	c	ab	b	b	b	a
+GP	c	b	b	b	b	b

Treatments denoted with 'a' are significantly different at $P < 0.05$ from 'b' and 'c', but not from 'ab', and treatments denoted with 'b' are significantly different from 'a' and 'c', but not from 'ab'.

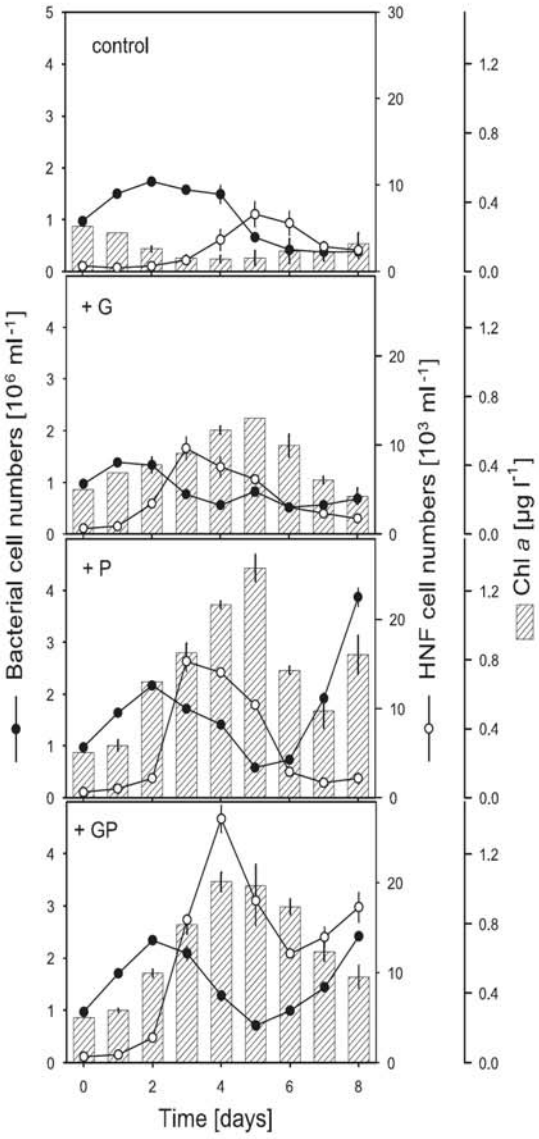

Fig. 1. Development of total cell numbers (solid circle), numbers of heterotrophic nanoflagellates (HNF; open circle), and of chlorophyll *a* (Chl *a*; columns) in the different experimental mesocosms (means and ranges of replicates). Control, unamended; +G, +glucose; +P, +phosphate; +GP, +glucose and phosphate.

Bacterial abundance and production

Initial bacterial cell numbers ranged around 1×10^6 cells ml^{-1} (Fig. 1). There was a significant difference both in cell numbers and in bacterial production between treatments with and without P addition (Table 1). Cell numbers in the control and the +G mesocosms less than doubled during the first 2 days of the experiment, whereas they rose to $> 2 \times 10^6$ cells ml^{-1} in +P and +GP. A second maximum of total abundances was observed towards the end of the experiment only in +P and +GP (Fig. 1). This increase of cell numbers was mirrored by higher bacterial production only in the +GP, but not in the +P variant (Fig. 2). Bacterial production in the P-amended treatments increased by more than two orders of magnitude within 24 h (Fig. 2). This initial peak was less pronounced in the control and +G variants, and production in these treatments subsequently decreased to < 80 µg C l^{-1} day^{-1} (Fig. 2).

Temporal dynamics of Alteromonadaceae *and* Rhodobacteriaceae

Cell detection rates by fluorescence *in situ* hybridization and catalyzed reporter deposition (CARD-FISH) were 58 ± 6% (mean ± range) in the original water sample and 88 ± 7% in the mesocosms (data not shown). In both P-amended treatments there was a clear succession pattern of bacteria affiliated with *Alteromonadaceae* and *Rhodobacteriaceae* over the course of the experiment, which was less obvious in the control mesocosms and absent in +G (Fig. 3). In all mesocosms *Alteromonadaceae* accounted for a substantial fraction of the rapid initial change in cell numbers (Figs 1 and 3) (and thus probably also of bacterial production, Fig. 2). Within 48 h of incubation, bacteria targeted by probe ALT1413 multiplied from 7×10^4 cells ml^{-1} in the original water sample to $0.5–1.0 \times 10^6$ cells ml^{-1} in the various treatments. During that phase the relative contribution of *Alteromonadaceae* to the respective bacterial assemblages increased by five- to sixfold in +G and +P, and by eightfold in +GP and the control mesocosms (Fig. 3). A subsequent disproportional decline of these bacteria to 1% (+P) to 12% (+GP) of total cells was observed in all mesocosms except for +G. In that treatment *Alteromonadaceae* maintained a community contribution of approximately 20% between days 3 and 8, reflecting the stability of total cell numbers during that period (Fig. 1).

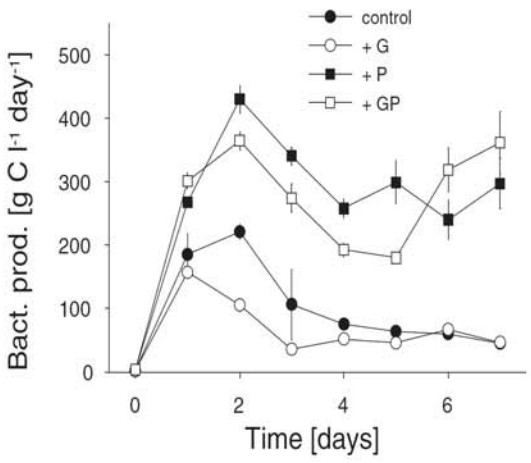

Fig. 2. Mean and range (error bars) of bacterial production in the different treatments. Control (solid circles), +G (+glucose; open circles), +P (+phosphate; solid squares) and +GP (+glucose and phosphate; open squares).

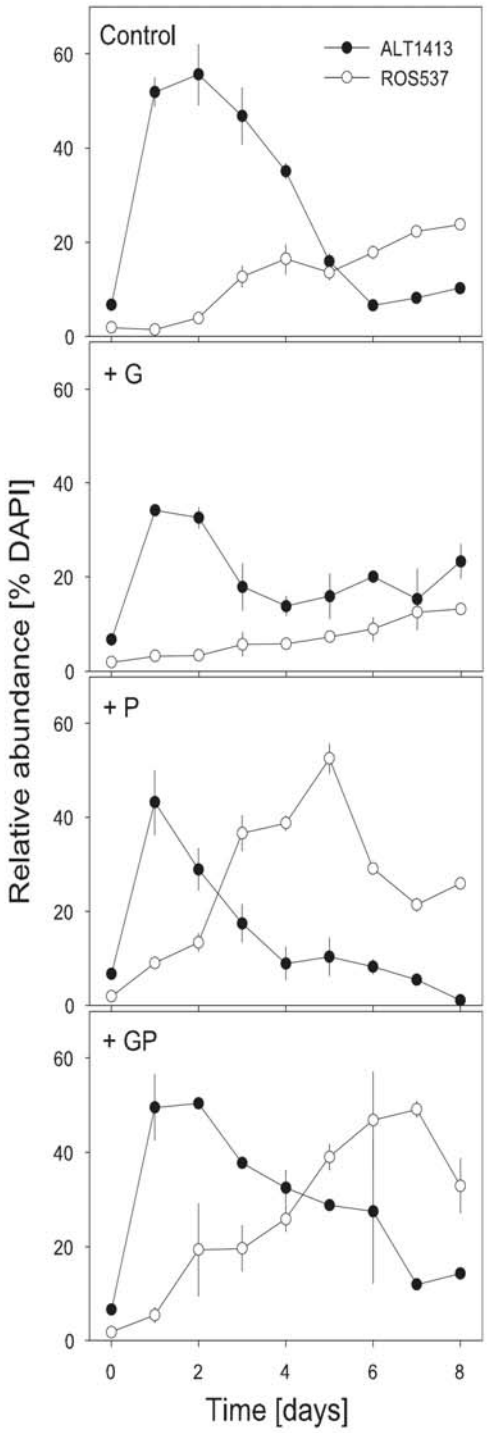

Fig. 3. Development of the relative abundances of *Alteromonadaceae* (ALT1413; solid circles) and of *Rhodobacteriaceae* (ROS537; open circles) in the different experimental mesocosms (means and ranges of replicates). Treatment designations are described in the legend to Fig. 1.

There was no significant relationship between the relative abundances of *Alteromonadaceae* and HNF numbers or Chl *a* concentration (data not shown). However, *Alteromonadaceae* and HNF abundances showed highly correlated patterns of population dynamics with a time delay of 1, 2 and 3 days in the +G, the +P and the control mesocosms respectively (Spearman's rank correlation, data not shown).

Members of the *Rhodobacteriaceae* were rare in the original water (day 0, Fig. 3). Their community contribution increased most steeply in treatments amended with P, where they accounted for approximately half of total cells between days 5 (+P) and 7 (+GP) and subsequently declined. The +G treatment, which sustained a stable population of *Alteromonadaceae*, in turn, triggered no substantial bloom of *Rhodobacteriaceae*. In terms of absolute cell numbers (details not shown), a first maximum of these bacteria was observed after 48–72 h of incubation, which ranged between 4 and 6×10^5 cells ml^{-1} in +P and +GP, and approximately half as much in the control treatment. Cell numbers of *Rhodobacteriaceae* subsequently declined, but a second, even denser bloom ($8–10 \times 10^5$ cells ml^{-1}) formed in the P-amended mesocosms on the last two sampling dates.

In the P-amended mesocosms, there was a significantly positive correlation between the relative abundances of *Rhodobacteriaceae* and Chl *a* concentrations, which was more tight in the +P ($r_S = 0.93$, $P < 0.001$) than in the +GP ($r_S = 0.62$, $P = 0.006$) mesocosms. While this correlation was most likely linear (Fig. 4, linear correlation coefficient, $r^2 = 0.58$), the relationship between *Rhodobacteriaceae* and HNF in these treatments was best modelled by a lognormal regression ($r^2 = 0.74$): the relative abundances of *Rhodobacteriaceae* reached a maximum at HNF densities > 10^4 ml^{-1} and declined thereafter (Fig. 4), reflecting the observation that the highest community contribution of *Rhodobacteriaceae* was found after the HNF bloom (Fig. 1 and Fig. 3).

Genotypic and phenotypic diversity of Rhodobacteriaceae *and* Alteromonadaceae

The diversity of bacteria affiliated with the *Rhodobacteriaceae* was clearly reflected in denaturing gradient gel electrophoresis (DGGE) and partial sequence analyses of 16S rRNA genes. Altogether, 21 different genotypes related to this lineage could be obtained from various treatments and sampling dates either from excised DGGE bands or from isolates obtained on agar plates (Table 2). In contrast, only four genotypes affiliated with *Alteromonadaceae* (including one *Glaciecola*) were obtained by cultivation, two of which were closely related (sequence identity > 99%). No sequence related to *Alteromonadaceae* was obtained by DGGE. This is surprising, because the here used primer pair should theoretically also match to the vast majority of bacteria from this lineage, including our isolates (probe match against all 16S rRNA gene sequences with the primer target site in

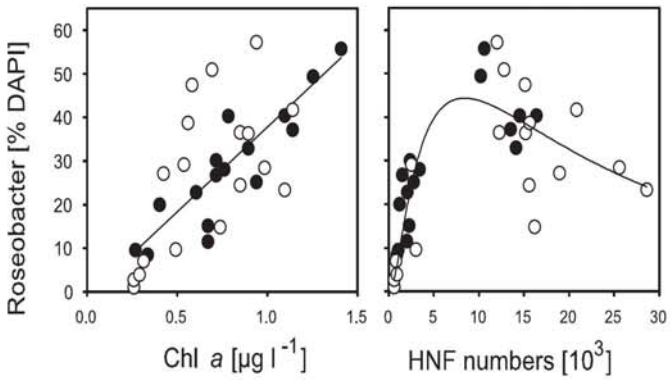

Fig. 4. Correlation between the community contribution of *Rhodobacteriaceae* and the concentration of Chl *a* (left) and HNF numbers (right) in the +P (solid circle) and +GP (open circle) treatments. The relationship of *Rhodobacteriaceae* and HNF in both P-amended treatments is best described by a non-linear pattern (regression type: lognormal, $r^2 = 0.74$), whereas it is a linear one for *Rhodobacteriaceae* and Chl *a* ($r^2 = 0.58$).

the database of the Ribosomal Database Project). DGGE bands that were related to genotypes targeted by probe ROS537 according to sequence analysis were submitted to cluster analysis (Fig. 5). In both P-amended treatments there was a clear distinction between the composition of sequence types related to *Rhodobacteriaceae* from the early and late sampling dates. During the initial bloom, very similar genotypes of *Rhodobacteriaceae* were present in +P and +GP (+P, days 2 and 4; +GP: day 2, Fig. 3), yet these bacteria were clearly distinct from the *Rhodobacteriaceae* in the original sample (Fig. 5). In contrast, during the second bloom (days 6–8), the assemblages of *Rhodobacteriaceae* in +P and +GP were clearly separated, both from the assemblages at the earlier dates and from each other.

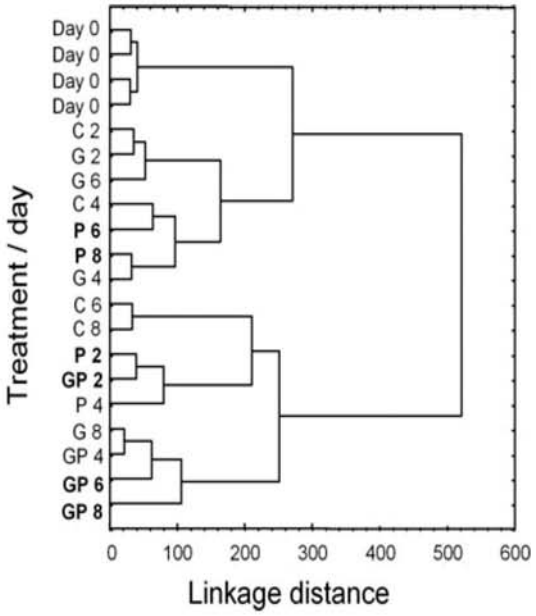

Fig. 5. Similarities of the *Rhodobacteriaceae* communities in the different treatments at the beginning and at days 2, 4, 6 and 8 of the experiment in the different treatments. The dendrogram depicts the results of cluster analysis (Ward's method) of those bands in DGGE gels that were related to *Rhodobacteriaceae* according to sequence analysis. Treatment designations are: C, control; G, +glucose; P, +phosphate; GP, +glucose and phosphate. Highlighted in bold: similarity of the *Roseobacter* community in +P and +GP at day 2 and their contrasting development (days 6–8).

Both *Rhodobacteriaceae* and *Alteromonadaceae* increased in cell size during the experiment in +GP. On day 8, cells of both populations were on average almost twice as long as in the beginning (Fig. 6). Additionally, a parameter for cell curvature (the ratio between the cell width and the width of a box enclosing the cell, i.e. the minimal Feret dimension) indicated the presence of at least one additional morphotype affiliate with *Rhodobacteriaceae* at the end of the experiment that was not present or rare during the initial bloom. In contrast, such morphological diversification was not observed for *Alteromonadaceae* (Fig. 6).

Discussion

Population dynamics of Alteromonadaceae

Our results suggest that the initial rise of *Alteromonadaceae* (Fig. 3) was a treatment-independent response to the disturbance caused by the transfer of seawater into the mesocosms. A rapid enrichment of these bacteria upon manipulation has been observed before during bottle incubations of North Sea water (Eilers *et al.*, 2000b; Beardsley *et al.*, 2003). *Alteromonadaceae* have also been detected in mesocosms of Mediterranean Sea water irrespective of nutrient addition, but only during the initial successional stages of the experimental assemblages (Schäfer *et al.*, 2001).

It is unclear what might have caused this conspicuous reaction to confinement. The mere handling of seawater might cause a disruption of the natural continuum of dissolved to particulate organic matter (Azam and Worden, 2004), e.g. the regular stirring of the mesocosms might cause a flocculation of colloidal material due to turbulence (Kepkay, 1994). This would favour bacterial taxa such as the *Alteromonadaceae* that can thrive on solid surfaces (Dang and Lovell, 2000) or particles (Acinas *et al.*, 1999) as well as in the water phase (Eilers *et al.*, 2000b; Beardsley *et al.*, 2003). Moreover, some *Alteromonadaceae*

Table 2. Genotypes and isolates related to *Alteromonadaceae* (in bold) and *Rhodobacteriaceae* lineages that were retrieved from different mesocosms and different times.

	Retrieved from	Designation	Closest genotype/ closest isolated relative	Identity (%)
Control	C 8 (C 6, P 2, GP 2)	Mes36	Uncultured alphaproteobacterium clone PI_4a9f (AY580451)	98
			Roseovarius sp. DFL-35 (AJ534219)	98
	C 8 (C 6, P 2, GP 2)	Mes15	Uncultured alphaproteobacterium clone PI_4a9f (AY580451)	98
			Roseobacter gallaeciensis (AY136134)	96
	C 8 (C 6)	Mes37	Uncultured marine bacterium D015 (AF177555)	95
			Roseobacter algocolus (X78315)	94
	C 8 (C 6)	Mes38	Bacterium K2-53B (AY345413)	97
			Phycococcus omphalius (AB193438)	97
	C 8	M625*	*Nereida ignava* (AJ748748)	99
			Thalassobacter oligotrophus (AJ631302)	95
	C 8	**M620***	**Uncultured marine eubacterium HstpL30 (AF159672)**	**94**
			***Glaciecola punicea strain* ANT9087 (AY167279)**	**92**
+Glucose	G 8	Mes47	*Roseobacter* sp. HYL-SA-18 (DQ008594)	97
			Agrobacterium gelatinovorum (D88523)	97
	G 8 (GP 6)	Mes26	*Agrobacterium gelatinovorum* (D88523)	98
			Thalassobacter oligotrophus (AJ631302)	98
	G 8 (P 2, GP 2, GP 8)	Mes49	*Rhodobacteraceae* bacterium CL-TA03 (AY962292)	97
			Jannaschia cystaugens (AB121782)	96
+Phosphate	P 4 (GP 4)	Mes8	Uncultured alphaproteobacterium clone JL-ECS-X8 (AY663968)	99
			Jannaschia cystaugens (AB121782)	96
	P 6	Mes9	Uncultured *Roseobacter* sp. clone (AY573530)	100
			Roseobacter gallaeciensis (AY136134)	97
	P 6	**M604***	**Uncultured *Alteromonas* sp. clone JL-ESNP-I29 (AY664213)**	**99**
			***Alteromonas macleodii* (AMY18231)**	**98**
	P 6	**M609***	**Uncultured gammaproteobacterium clone JL-ETNP-R11 (AY726784)**	**99**
			***Alteromonas alvinellae* (AF288360)**	**99**
	P 6	Mes11	Uncultured bacterium clone J-154 (AY600952)	96
			Agrobacterium gelatinovorum (D88523)	96
	P 8	M612*	Roseobacter sp. DSS-8 (AF098493)	98
			Agrobacterium gelatinovorum (D88523)	96
	P 8	M613*	Uncultured alphaproteobacterium clone JL-ECS-X8 (AY663968)	99
			Jannaschia cystaugens (AB121782)	96
	P 8	**M614***	**Uncultured gammaproteobacterium clone JL-ETNP-R11 (AY726784)**	**99**
			***Alteromonas alvinellae* (AB121782)**	**99**
+Glucose and phosphate	GP 2 (C 0, P 0, P 2)	Mes16	Marine arctic deep-sea bacterium HD9 (AJ557871)	98
			Agrobacterium gelatinovorum (D88523)	96
	GP 4	Mes23	CVSP bacterium CV919-312 (AF114484)	98
			Phycococcus omphalius (AB193438)	97
	GP 6	M602*	Uncultured alphaproteobacterium (AJ633943)	99
			Roseobacter gallaeciensis (RG16SRR)	98
	GP 6	M605*	*Thalassobius mediterraneus* (AJ878874)	99
			Agrobacterium gelatinovorum (D88523)	97
	GP 8	Mes28	*Roseobacter* sp. JL-126 (AY745859)	98
			Alphaproteobacterium MBIC1887 (AB026492)	98
	GP 8	Mes48	*Roseobacter* sp. HYL-SA-18 (DQ008594)	98
			Agrobacterium gelatinovorum (D88523)	97
	GP 8	M606*	*Roseobacter* sp. JL-126 (AY745859)	99
			Ruegeria atlantica (AF124521)	99
	GP 8	M618*	Uncultured *Roseobacter* sp. clone JL-ECS-X3 (AY663966)	97
			Roseobacter gallaeciensis (AY136134)	97

Genotypes were identified either by sequencing or by comparison with position of sequenced bands in the DGGE (reported in brackets). Isolates are indicated by asterisks. C, control; G, +glucose; P, +phosphate; GP, +glucose and phosphate.

apparently maintain high levels of ribosomes during extended periods of non-growth, which would allow these bacteria to rapidly initiate growth (Fig. 2) at changing environmental conditions (Eilers *et al.*, 2000b; Pernthaler *et al.*, 2001). This would also match the short estimated doubling times of this particular population during the first 24 h of the incubations, which varied between 7 (+P and +GP) and 12 h (control). Alternatively, the addition of ammonium to all treatments (to avoid N limitation, see *Experimental procedures*) might have also influenced community composition. High ammonium concentrations significantly favored the growth of colony-forming *Gammaproteobacteria* from North Sea waters (Eilers *et al.*, 2001).

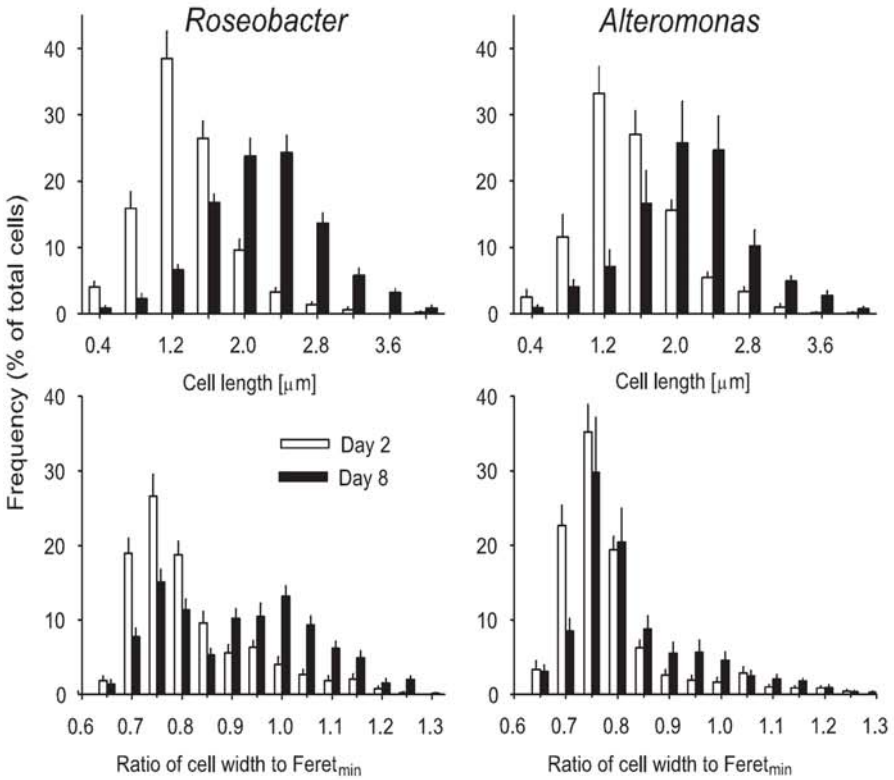

Fig. 6. Distributions of cell size and shape of *Rhodobacteriaceae* (left) and *Alteromonadaceae* (right) in the glucose- and phosphate-amended (+GP) treatment on days 2 (open bars) and 8 (closed bars). Top panels: cell length. Bottom panels: cell shape, expressed as the ratios of cell width to the minimum Feret dimension. Note the presence of two distinct morphotypes in *Rhodobacteriaceae* on day 8.

During the second half of the experiment the relative contribution of *Alteromonadaceae* to the microbial assemblages disproportionally declined in all treatments, concomitant with a rise of HNF numbers (Figs 1 and 3). The time delay between the two blooms suggests that the bacterial population was negatively affected by selective flagellate grazing (Pernthaler, 2005). A similar succession of *Alteromonadaceae* and HNF was observed during bottle incubations of North Sea water (Beardsley *et al.*, 2003). Our results thus support the conclusion that *Alteromonadaceae* are rare in coastal surface waters because their rapid growth response to changing environmental conditions is counterbalanced by high predation mortality. In addition, *Alteromonadaceae* might have been selectively reduced by viruses. As such a viral 'killing the winner' scenario (Thingstad and Lignell, 1997) is based on host specificity, it would require a low genotypic diversity within the *Alteromonadaceae*. The results of cultivation support this assumption: only four different genotypes affiliated with this lineage could be retrieved at various time points, and three of those isolates were phylogenetically closely related (identity, 93–99%, Table 2). As *Alteromonadaceae* are known to be readily culturable (Pinhassi and Berman, 2003) it is likely that the diversity of these bacteria was indeed low. On the other hand, the total abundances of viral particles changed little in mesocosms without P addition (M. Weinbauer, unpubl. data), suggesting that protistan predation probably represented the dominant mortality source for *Alteromonadaceae* in these treatments.

An initial stimulation by confinement followed by top-down control alone cannot provide an exhaustive explanation for the observed dynamics of the *Alteromonadaceae*. For example, the abundances of HNF declined in all treatments towards the end of the experiment (Fig. 1), probably due to predation by omnivorous, oligotrichous ciliates (J. Masmitjà and D. Vaqué, unpubl. data). However, there was no corresponding second bloom of *Alteromonadaceae* (Fig. 3), hinting at the presence of other competitors during the decline of the phytoplankton bloom, e.g. members of the *Bacteroidetes* (Riemann *et al.*, 2000; Pinhassi *et al.*, 2004). Moreover, the *Alteromonadaceae* maintained relative abundances of approximately 20% of total bacterial counts in the +G and the +GP treatments during the second half of the study, whereas they decreased to 10% or less in +P and in the controls (Fig. 3). This significant difference between treatments with and without glucose (days 5–8: Mann–Whitney *U*-test, $n = 16$, $P < 0.001$) indicates that the presence of an additional source of simple carbon positively influenced the persistence of *Alteromonadaceae* in

the respective microbial assemblages. Thingstad and colleagues (2005) suggested that some bacteria might utilize excess carbon to increase cell size in order to obtain full or partial resistance against HNF predation. Members of the *Alteromonadaceae* indeed formed significantly larger cells at the end of the experiment (Fig. 6). It is, however, unclear if a mean cell length of 2–3 μm (Fig. 6) would be sufficient to substantially reduce grazing mortality. Microscopic inspection showed that some of the dominant flagellate predators themselves were between 1.5 and 3 μm in size (K. Simek, unpubl. obs.), and thus were probably not able to feed on such large cells. Alternatively, glucose might have provided additional metabolic energy to compensate for high losses by higher growth rates than other bacterial groups. *Alteromonas* and related genera represented a large fraction of colony-forming units in glucose-amended dilution cultures of Mediterranean Sea water (Pinhassi and Berman, 2003).

The ubiquity of *Alteromonadaceae* and closely related genera in coastal marine habitats (Acinas *et al*., 1999; Dang and Lovell, 2000; Garcia-Martinez *et al*., 2002; Yoon *et al*., 2003), their typical rarity in bacterioplankton communities (Eilers *et al*., 2000b; Beardsley *et al*., 2003; Alonso-Sáez *et al*., 2007) and their characteristic response upon changes in growth conditions (Fig. 3) raises the question whether these bacteria might qualify as indicators of disturbance events in coastal surface waters. It is, for example, conceivable that blooms of these bacteria might be related to point sources of allochthonous organic carbon. In this context, it is intriguing that a pronounced bloom of bacteria related to *Alteromonadaceae* (> 30% of total cells) was observed in July 2003 at the site of our sampling (Alonso-Sáez *et al*., 2007).

Temporal dynamics of Rhodobacteriaceae

The physiologically highly diverse *Rhodobacteriaceae* are one of the major groups of marine bacteria, comprising up to 20% of coastal and 15% of mixed-layer ocean bacterioplankton communities (Buchan *et al*., 2005 and references therein). Between late autumn and spring (November to April) these bacteria constituted approximately 5% of total cells at our sampling site, whereas they were relatively rare during summer (Alonso-Sáez *et al*., 2007).

In contrast to the development of the *Alteromonadaceae* the relative contribution of *Rhodobacteriaceae* rose slowly, but these bacteria continuously gained importance over a period of several days (Fig. 3). During the second half of the investigation period, *Rhodobacteriaceae* transiently became the dominant bacteria in treatments amended with P. On the one hand, this conspicuous proliferation of *Rhodobacteriaceae* after P addition might have been induced directly by the nutrient. Low P concentrations can limit bacterioplankton growth in the Northwestern Mediterranean Sea during large parts of the year (Pinhassi *et al*., 2006). Moreover, some *Rhodobacteriaceae* contain bacteriochlorophyll *a* (Shiba, 1991; Buchan *et al*., 2005). It is thus conceivable that these bacteria might be able to increase growth at sufficient concentrations of P irrespective of DOC levels.

However, entirely phototrophic growth of these bacteria so far has not been shown, and is unlikely in view of recent genomic information (Swingley *et al*., 2007). Therefore, it is likely that *Rhodobacteriaceae* in the +P and +GP mesocosms benefited from the more pronounced blooms of phytoplankton (Fig. 1) rather than directly from the nutrient. This is suggested by the positive correlation between Chl *a* concentrations and the community contribution of *Rhodobacteriaceae* in these treatments (Fig. 4), which was moreover much tighter in the absence of an extra carbon source. *Rhodobacteriaceae* are typically abundant in bacterial communities that are associated with algae, including both natural (Eilers *et al*., 2001; Zubkov *et al*., 2001) and induced phytoplankton blooms (Pinhassi *et al*., 2004). During such periods these bacteria may increase in abundance by up to 2.5 times (Moran *et al*., 2003 and references therein). Some *Rhodobacteriaceae* can moreover prosper on phytoplankton-derived DOC, e.g. glucose (Alonso and Pernthaler, 2006) or the algal osmolyte dimethylsulfoniopropionate (Zubkov *et al*., 2001; Vila *et al*., 2004).

The high (morphological and genotypic) diversity of *Rhodobacteriaceae* in the experimental mesocosms might be another indication of the dependence of these bacteria on the phytoplankton (Table 2; Figs 5 and 6). Algal-derived DOM from exudates and cell lysis products consists of a variety of readily degradable substrates, such as mono- and polysaccharides, amino acids and proteins, etc. (Myklestad, 2000), potentially providing different niches for coexisting strains. In addition, the taxonomic composition of the phytoplankton assemblages differed in the various experimental treatments (P. Gasol, unpubl. obs.), and various algal species might have favoured specific bacterial genotypes (Schäfer *et al*., 2002; Pinhassi *et al*., 2004). A succession of ecophysiologically specialized genotypes of *Rhodobacteriaceae* is suggested by community fingerprinting (Fig. 5): during the first 48 h of the experiment the communities of *Rhodobacteriaceae* were highly similar in the +P and +GP treatments (Fig. 5), whereas they significantly differed at the end of the incubations. This implies that there were one or more genotypes from this family that acted as primary colonizers in both treatment types (Mes15, Mes16 and Mes49, Table 2). In contrast, the genotypes that dominated the respective assemblages during the later phase of the study apparently differed, for example, in their preference for high glucose concentrations.

A second aspect that might have led to the apparent success of *Rhodobacteriaceae* in the P-amended mesocosms could be a lower than average grazing rate on these bacteria at low to medium densities of the predators. This is suggested by the non-linear relationship between the community contribution of *Rhodobacteriaceae* and HNF abundances in the +P and +GP treatments (Fig. 4): at low HNF densities (i.e. before and after the HNF bloom) the fraction of *Rhodobacteriaceae* tended to increase with flagellate numbers, whereas the opposite was true at HNF densities of 1.5×10^4 ml^{-1} or above. A smaller effect of HNF on *Rhodobacteriaceae* than on *Alteromonadaceae* (at protistan densities $< 10^4$ ml^{-1}) has also been observed during short-term bottle incubations (Beardsley *et al*., 2003).

Conclusions

One might envisage heterotrophic picoplankton communities in coastal waters as being composed of rather stable populations and others with rapid temporal fluctuations. The former would include bacterial taxa that are adapted to constantly low substrate concentrations, e.g. members of the SAR 11 clade (Morris *et al*., 2002; Alonso and Pernthaler, 2006). The more variable components appear to fall into different categories, two of which were analysed in our study: (i) phylogenetic lineages such as the *Alteromonadaceae* harbour bacteria that respond to irregular disturbances by external events and to allochthonous DOC input, but do not maintain large population sizes in the plankton, and (ii) bacteria from lineages such as the *Rhodobacteriaceae* are related to more predictable autochthonous events, specifically phytoplankton blooms (Eilers *et al*., 2001; Zubkov *et al*., 2001). Obviously, mesocosms are extremely simplified systems that moreover greatly exaggerate the instable aspects of picoplankton assemblages. Nevertheless, our experimental incubations allowed distinguishing between the specific niches of two groups of planktonic marine bacteria that both can rapidly respond to a changing environment.

Experimental procedures

Experimental set-up and sampling

Water was collected from 1 m depth at the Blanes Bay Microbial Observatory, approximately 1 km off the Port of Blanes, Catalunya (Western Mediterranean Sea 41°40′N, 2°48′E) on 19 October 2004. Eight transparent rectangular polyethylene tanks were each filled with 200 l. These mesocosms were maintained for a period of 8 days at *in situ* temperature and in a 12:12 h light : dark cycle. All mesocosms were amended daily with 2 μM NH_4 (NH_4Cl) in order to prevent N limitation. In addition, two mesocosms were amended with 50 nM of PO_4 (KH_2PO_4) (treatment designation: +P), two mesocosms with 13.25 μM of glucose (treatment designation: +G), and two with both glucose and PO_4 at the same concentrations as in the separate additions (treatment designation: +GP). One set of two mesocosms was left untreated (treatment designation: control). All mesocosms were mixed twice a day by hand-held stirring, and samples were taken once every day.

Chlorophyll a concentrations

Chlorophyll *a* concentrations were determined after Parsons and colleagues (1984). Subsamples of 150 ml were filtered through glass fibre filters (GF/F, Whatman) and subsequently extracted in 90% acetone overnight at 4°C in the dark. Fluorescence was measured with a Turner Designs fluorometer.

Bacterial production, bacterial and flagellate cell numbers

Bacterial bulk growth activity during the enrichments was estimated from the incorporation rate of tritiated (^{3}H) leucine (Leu) as described by Kirchman and Ducklow (1993). Samples were incubated with 40 nM of ^{3}H Leu in microcentrifuge tubes in the dark for 1 h at ambient temperatures (Smith and Azam, 1992; Gasol and Morán, 1999). Trichloroacetic acid-killed samples were used as controls. Bacterial heterotrophic production was calculated as Leu incorporation rate times the standard 3.1 kg C mol Leucine^{-1} conversion factor (Gasol *et al*., 2002).

Total bacterial cell numbers were counted with a Becton Dickinson FACScalibur benchtop flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Daily samples from each mesocosm were processed as previously described (Gasol and del Giorgio, 2000). Portions of 1 ml were fixed with 1% buffered paraformaldehyde solution (PFA, pH 7.0) plus 0.05% glutaraldehyde, incubated for 10 min at room temperature and then stored in liquid nitrogen. For total bacterial cell counts, 200 μl of these subsamples were stained with a DMSO-diluted SybrGreen I stock solution (10:1; Molecular Probes, Eugene, OR, USA) at a final concentration of 2.5 μM. The staining was carried out for 10 min in the dark. For flow cytometric analysis 10 μl of a solution of yellow-green latex beads (size, 1 μm; final concentration, 10^6 ml^{-1}; Polyscience, Washington, PA, USA) was added to each sample as an internal standard. Bacterial cell numbers were determined from the ratios of cells to beads after Gasol and del Giorgio (2000).

For the enumeration of non-pigmented HNF, subsamples (100 ml) were preserved according to Simek and colleagues (1995). Portions of 5 ml from these samples were stained with 4′,6-diamidino-2′-phenylindol (DAPI, final concentration, 1 μg ml^{-1}) and passed through 0.8 μm Poretics polycarbonate filters (GE Osmonics, Minnetonka, MN, USA) by gentle vacuum filtration. Non-pigmented nanoflagellates were enumerated via epifluorescence microscopy. All samples were analysed within 24 h after preservation.

Temporal dynamics of Alteromonadaceae *and* Rhodobacteriaceae

Subsamples (50 ml) were fixed with 1% PFA for 24 h at 4°C for analysis of the bacterial community by CARD-FISH

(Pernthaler *et al.*, 2004). Subsamples were filtered onto white membrane filters (GTTP, 0.2 μm pore size, 47 mm in diameter, Millipore, Eschborn, Germany), and bacterial cells were fixed onto the filter by embedding the surface into 0.2% agarose (MetaPhor, Cambrex Bio Science, Rockland, USA). Before hybridization, cells were permeabilized by treatment with lysozyme and proteinase K as described previously (Teira *et al.*, 2004). Proteinase K concentration was adjusted to 0.075 μl ml^{-1} (2129 U mg^{-1}, 9.5 mg ml^{-1}, Fluka) and samples were incubated for 15 min. Filter sections were hybridized with horseradish peroxidase (HRP)-labelled oligonucleotide probes (Biomers, Ulm, Germany) as previously described (Pernthaler *et al.*, 2004). Probes were targeting members of the *Bacteria* (EUB I–III; Daims *et al.*, 1999), several genera within the *Rhodobacteriaceae* (ROS537; Eilers *et al.*, 2001), and bacteria affiliated to *Alteromonadaceae* and *Colwelliaceae* (ALT1413, genera *Alteromonas*, *Colwellia*, *Glaciecola*; Eilers *et al.*, 2000a). Stringent hybridization conditions were achieved by 55% Formamide (FA) in the hybridization buffer for EUB I–III and ROS537, and 60% FA for ALT1413 respectively. After 2.5 h of hybridization the probe-delivered HRP was detected with tyramides (diluted 1:700 in amplification buffer) that were custom labelled with fluorescein (Molecular Probes, Eugene, USA). The preparations were subsequently embedded onto glass slides in a previously described mountant mix containing 1 μg ml^{-1} DAPI (Pernthaler *et al.*, 2004). The fractions of CARD-FISH-stained cells of all DAPI-stained objects were determined by epifluorescence microscopy and semi-automated image analysis (Pernthaler *et al.*, 2003). Negative controls were routinely determined with the EUB I–III antisense probe NON338 (Wallner *et al.*, 1993).

Determination of cell morphology

Images of cells hybridized with probes ROS537 and ALT1413 in the +GP treatment at days 2 and 8 were acquired at blue excitation (CARD-FISH staining) from randomly selected filter areas with a ZEISS Axio Imager 1.1 equipped with a VDS COOL-1300Q digital camera (Vosskühler, Osnabrück, Germany) and image analysis software (LUCIA, G, Laboratory Imaging, Prague, Czech Republic). Morphological parameters (area, perimeter, length, width, Feret dimensions) of > 500 cells were determined in 10–25 images per preparation by previously described procedures (Posch *et al.*, 1997).

Extraction of microbial community DNA

Samples of microbial community DNA for subsequent analyses by DGGE, cloning and 16S rRNA gene sequencing were collected at the beginning of the experiment (day 0) and on every second day until day 8. Microbial biomass from approximately 700 ml of sample was collected onto 0.2 μm-pore-size polycarbonate filters (diameter, 47 mm, Durapore, Millipore). Filters were stored frozen at −70°C in sucrose buffer (0.75 M sucrose, 40 mM EDTA, 50 mM Tris pH = 8.3). DNA was extracted using a combined treatment with enzymes (lysozyme, proteinase K) and phenol-chloroform as described by Riemann and colleagues (2000). DNA was re-suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and quantified fluorometrically (PicoGreen; Molecular Probes).

Diversity analysis

Bacterial 16S rDNA was amplified by PCR using a bacterial primer complementary to position 341–358 with a 40 bp GC-clamp (GC341F; Muyzer *et al.*, 1993) and a universal primer complementary to position 907–927 (907RM; Muyzer *et al.*, 1998). Initial denaturation was at 95°C for 2 min followed by a thermal cycling programme as follows: denaturation for 30 s at 94°C; annealing for 30 s at an initial 63°C, decreasing 1°C every two cycles to a final of 53°C; extension for 90 s at 72°C. Ten cycles were run at 53°C for a total of 30 cycles followed by final 7 min of incubation at 72°C. The quality and size of PCR products were verified by agarose gel electrophoresis.

Sixty nanograms of PCR product was analysed by DGGE using the D Gene System (Bio-Rad) at 60°C for 6 h at 150 V. DGGE bands were excised using a sterile razor blade and eluted in 20 μl of MilliQ water overnight at 4°C, followed by a freeze–thaw cycle. A total of 5 μl of the eluate was used for re-amplification with the original primer set. A part of the PCR product was analysed by DGGE together with the original sample to verify the correct position of the band, and in cases where more than one band was present the target band was processed again as described above. PCR products were purified with the QIAquick PCR-Purification Kit (Qiagen) and quantified fluorometrically (PicoGreen; Molecular Probes).

Sequencing reactions were carried out using the DYEnamic™ ET terminator cycle sequencing kit (Amersham Biosciences) and primer 907RM as described by the manufacturer. The obtained partial 16S rRNA gene sequences were compared with existing prokaryotic sequences in GenBank (NCBI) using BLAST (Altschul *et al.*, 1990). Isolates were obtained by plating samples from dilution to extinction cultures (most probable number approach) or by direct plating from the mesocosms onto Zobell agar plates. The isolates were pre-screened according to colony morphology, and representative strains were identified by 16S rRNA gene sequencing as previously described (Pinhassi and Berman, 2003). All sequences were submitted to GenBank and their accession numbers are provided in Table S1.

Statistical analyses

Digitized DGGE images were analysed with the Quantity-one software (Bio-Rad). The software allows identification of different bands and calculating the contribution of each band to total intensity in each lane. Cluster analysis of banding patterns based on Ward's method was used to obtain a dendrogram using Statistica 6.0 (StatSoft, Tulsa, OK, USA).

The relationship between the relative abundances of *Alteromonadaceae* and *Rhodobacteriaceae* with HNF abundances, Chl *a* concentration and bacterial production were tested separately for each treatment type by pairwise correlations (Spearman's rank sum correlations, r_S and/or Pearson product moment correlations, r^2). Data from the two replicates

were pooled. In order to compensate for the chance of producing spurious significance by multiple correlations the critical alpha level required for significance was adjusted to $P < 0.01$.

Treatment-specific differences in bacterial abundance and relative abundances of *Alteromonadaceae* and *Rhodobacteriaceae,* HNF abundances, Chl *a* concentration and bacterial production were established by one-way ANOVA. In order to account for the different data distributions the relative abundances of *Alteromonadaceae* and *Rhodobacteriaceae* were arcsine transformed prior to analysis, whereas the other variables were log transformed. Subsets of treatments that were statistically indistinguishable for a particular parameter were determined by *post hoc* pairwise comparisons (Scheffé method). Analyses were performed using the software SPSS (SPSS, Chicago, IL, USA)

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Genotypes and isolates and their corresponding GenBank accession number.

This material is available as part of the online article from http://www.blackwell-synergy.com

Publication II

Primary microbial colonizers of mucus released from the scleractinian coral *Fungia* sp.

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Manuscript in preparation

Primary microbial colonizers of mucus released from the scleractinian coral *Fungia* sp.

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Running title: Enrichment of *Alteromonas macleodii* on coral mucus

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Abstract

Tropical coral reefs are highly productive ecosystems in an oligotrophic environment. A large fraction of the photosynthetically fixed carbon by corals is released as mucus into the ambient water, where it provides an important substrate source for microbial growth. We investigated which microbial taxa in coastal sea water (Southern Gulf of Aquaba) would profit most rapidly from the addition of freshly released mucus from the coral *Fungia* sp.. Microbial diversity of mucus-amended sea water enrichment cultures was studied by 16S rRNA gene sequence analysis, and changes in community composition were monitored by whole-cell fluorescence in situ hybridization over a period of 50 h. A decrease in the concentration and C:N ratio of particulate organic material during the first 26 h of incubation was accompanied by a steep rise of bacterial abundances, in particular of *Gammaproteobacteria*. More than 90% of the 16S rRNA gene sequences obtained from the enriched samples were affiliated to this lineage, specifically to *Alteromonas spp.* and *Vibrio spp.* Within 10 h of incubation *Alteromonadaceae* formed >50% of total cells, followed by *Vibrionaceae* (>20% after 26 h). Two almost identical genotypes closely related to *A. macleodii* on average represented >85% of all *Alteromonadaceae* (up to 45% of total cells) in the mucus-amended enrichments, but were rare in unamended control incubations and the original sea water. Thus, *A. macleodii* might represent an important link for the transfer of organic carbon from coral mucus to the pelagic microbial food webs of coral reefs.

Introduction

Coral reefs are located in some of the most nutrient depleted marine areas but nevertheless belong to the earth's ecosystems with the highest gross primary production rates (31, 43). Thus, there must be mechanisms in the reef ecosystem which conserve and rapidly recycle essential substrates and nutrients. Reef corals can secrete mucopolysaccharide material in such quantities that it can dominate the suspended matter in reefs (29, 38, 57, 59, 60). This mucus seems to play an important role as carrier of energy and nutrients to a range of planktonic and benthic consumers. Detached coral mucus can be consumed by a variety of reef organisms (26, 29), and it also represents an excellent growth substrate for some marine bacteria (15, 28). Thus, the surface coral mucus layer and its immediate vicinity are hotspots of microbial abundances and activity (45, 52). Moreover, incubations of bacterioplankton with freshly collected coral mucus have shown that the added organic carbon can be rapidly consumed, with C turnover rates of more than 7% h^{-1} (59). This is substantially higher than typical degradation rates of other types of suspended organic matter in coral reefs (58).

In general, corals may provide three different habitats for microorganisms: (i) the coral tissue itself, (ii) the surface mucus layer, which is only indirectly influenced by the host, e.g., by release of growth-inhibiting factors (50), and (iii) the waters in the direct vicinity, where coral exudates might affect bacterial densities and community composition (49, 50, 52). The overlap between the bacterial assemblages that are present on coral mucus and in the ambient water is poorly understood. Molecular fingerprinting of bacterial 16S rRNA genes suggested that the composition of the microbial community in the surface mucopolysaccharide layer of some reef corals may be similar to that of the surrounding waters (9). Other reports point at distinct differences between the two habitats (20, 27), e.g. the occurrence of gammaproteobacterial lineages in coral mucus that are considered rare in the bacterioplankton (33, 51).

As outlined above, a large amount of coral mucus is continuously released from the coral and floats in or dissolves into the ambient water. It is

conceivable that microbial species which rapidly incorporate the most easily metabolized coral exudates into their biomass potentially also play an important role in the remineralisation of this organic material and in reef trophodynamics in general. Therefore, we investigated which bacteria are the primary colonizers of freshly detached mucous material in sea water. For this purpose, the short-term changes of microbial community composition were analyzed in enrichment cultures of coastal sea water with mucus from one of the most common Red Sea scleractinian corals (*Fungia* sp.) by comparative 16S rRNA gene sequence analysis and fluorescence in situ hybridization (FISH) with oligonucleotide probes.

Materials & Methods

Experimental setup. Coral mucus and seawater were collected from the Lagoon of Dahab in the southern part of the Gulf of Aquaba (28°29'N, 34°30'E), Northern Red Sea, in May 2004. Ten polyps of the hermatypic coral *Fungia* sp. (5 - 10 cm in diameter) were collected from water depths of 3 - 8 m using SCUBA and immediately brought to the lagoon beach. There, the polyps were exposed to air for 3 min to trigger mucus production. Mucus released during the first min was discarded in order to avoid bacterial contamination from the coral surface and the covering water layer. The mucus produced during the following 2 min was collected in sterile glass dishes and subsequently filled into sterile glass bottles. Seawater was obtained from the same site in sterile bottles and immediately taken to the laboratory. Within 60 min after collection, coral mucus was homogenized and mixed 1:10 (v/v) with the collected fresh seawater. This mixture was filled in sterile 1-l glass bottles in triplicates. Triplicate bottles of untreated seawater served as control. All bottles were incubated at *in situ* temperature (24°C) and light conditions for a period of 50 h.

Sample fixation, development of total cell numbers. Samples were taken after 0, 2, 4, 6, 10, 26, and 50 h of incubation. Portions of 15 ml were fixed with 1% PFA for 24 h at 4°C for total bacterial counts and the analysis of microbial community structure by fluorescence *in situ* hybridization and catalyzed reporter deposition (CARD-FISH) (46). Sub-samples (1-10 ml) were filtered onto polycarbonate membrane filters (GTTP, pore size, 0.2 µm; diameter, 25mm for cell enumeration or 47mm for community analysis, Millipore, Eschborn, Germany). Total cell numbers were determined from 1-2 ml of sub-sample by staining with 4',6-diamino-2-phenylindole (DAPI, final concentration 1 µg ml^{-1}) and epifluorescence microscopy.

Particulate organic matter (POM). Samples for the measurement of particulate organic C (POC) and N (PN) concentrations and their stable isotope signatures were prepared by filtering portions of 50 ml from all 6 incubation bottles onto precombusted GF/F filters (Whatman, 25 mm in

diameter). This was carried out three times over the duration of the incubation at 0 h, 26 h and 50 h. The filters were dried for 48 hours at 40° C and kept dry until analysis at GeoBio-Center in Munich, Germany. Particulate organic carbon and nitrogen concentration measurements and stable isotope analyses were performed with a THERMO NA 2500 elemental analyzer coupled with a THERMO/Finnigan Conflo II- interface to a THERMO/Finnigan MAT Delta plus isotope ratio mass spectrometer. Standard deviations of C and N concentration measurements of replicates of the lab standard (peptone) were < 3 %. Stable isotope ratios are given in the conventional delta notation ($\delta^{13}C$ / $\delta^{15}N$) relative to PeeDee Belemnite standard (PDB) and atmospheric nitrogen (37). Standard deviation for repeated measurements of lab standard material was better than 0.15 ‰ for nitrogen and carbon.

Analysis of bacterial 16S rRNA genes. Mucus-amended incubations were sampled after 10 and 26 h for the construction of two bacterial 16S rRNA gene clone libraries (termed M10 and M26), and a third library (termed C50) was produced from the seawater control incubations after 50 h. Ten ml of unfixed sample were filtered onto white membrane filters (GTTP, 0.2 µm pore size, 25 mm in diameter, Millipore, Eschborn, Germany) and stored frozen until further analysis. DNA-free purified water (68 µl) was added to small pieces of filter (ca. 4 mm^2) from each sample in 0.5 ml PCR reaction tubes, and the tubes were treated with freeze-and-thaw-cycles ranging from -80°C to room temperature (RT). Afterwards DNA was directly amplified from the filter pieces by PCR (32) using the primer pair of GM3F and GM4R (41) under condition described before (21). *Taq* polymerase and PCR buffer were from Eppendorf (Hamburg, Germany).

The amplified 16S rRNA gene fragments were purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany), inserted into the TOPO vector (TOPO TA cloning kit; Invitrogen, Karlsruhe, Germany), and cloned into competent cells of *Escherichia coli* as described by the manufacturer. Insert-bearing clones were screened for right insert size by PCR using vector primer pair M13F (5'- GTT GTA AAA CGA CGG CCA GT -3') and M13R (5'- GGA AAC AGC TAT GAC CAT G-3'). For partial sequences, PCR products of the

screening PCR were used as templates in the sequencing reaction with primer GM1F (5'-CCA GCA GCC GCG GTA AT-3', modified after (40). Sequence analysis was carried out on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). For nearly full sequences, plasmids of interest were extracted with a QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's specifications and sequenced using primers GM1F, GM3 and GM4 (41). Sequences were assembled from partial sequences using the software Sequencher (Gene Codes Corp., Ann Arbor, Mich.). Partial and nearly full sequences were deposited in GenBank under accession numbers XX to YY.

The relative distribution of phylotypes in each library was used to calculate coverage values (25) and the nonparametric S_{Chao1} estimator (12) using a software tool provided by (30). Good's coverage estimates the proportion of phylotypes of a library of infinite size which would be represented in a smaller library. The S_{Chao1} parameter allows estimating the probable total number of phylotypes present in the source assemblage. Phylogenetic analysis of the obtained sequences was carried out using the ARB software package (36). Additional sequences were downloaded from RDP (13) to update the dataset. A maximum parsimony tree of *Alteromonas* spp. phylotypes was calculated using sequences >1200 nucleotides. Shorter sequences were subsequently added for reference by the ARB QUICK_ADD parsimony module without changing tree topology.

Relative abundances of different taxa. Changes of the bacterial community structure were analyzed by CARD-FISH (46). Before hybridization, bacterial cells from 5-10 ml of fixed subsample were fixed onto the filter by embedding the surface into 0.2% agarose (MetaPhor, Cambrex Bio Science, Rockland, USA). Cells were subsequently permeabilized by treatment with lysozyme and proteinase K (53). Proteinase K concentration was adjusted to 0.15 µl/ml (2129 U/mg, 9.5 mg/ml, Fluka) and samples were incubated for 15 min. Filter sections were hybridized with horseradish peroxidase (HRP)-labeled oligonucleotide probes (Biomers, Ulm, Germany) as described previously (46). Probes targeted members of the *Bacteria* (EUB I-III) (14), *Alteromonas* spp. + *Collwellia* spp. (ALT1413), (18) *Pseudoalteromonas* spp. (PSA184),

(18) and *Vibrio* spp. (GV) (24). Stringent hybridization conditions were established by adding 55% of formamide (FA) to the hybridization buffer for probes EUB I-III, PSA184 and GV, and 60% for probe ALT1413, respectively. After 2.5 h of hybridization at 35°C the probe-delivered HRP was detected with tyramides (diluted 1:700 in amplification buffer) that were custom labeled with fluorescein or Alexa488 (Molecular Probes, Eugene, USA). The preparations were subsequently embedded onto glass slides in a previously described mountant mix containing 1 µg ml^{-1} of DAPI (46). DAPI stained cells and the fractions of CARD-FISH stained cells of all DAPI stained objects were determined by epifluorescence microscopy and automated image analysis as described before (47). Negative controls were routinely determined with the EUB I-III antisense probe NON338 (56).

A highly specific probe was designed by means of the ARB PROBE_DESIGN tool that is targeted to two of the obtained sequence types affiliated to *Alteromonas macleodii* (clones M10-16 and M26-001). Probe AMAC83 (5' CGT AAC GCC ACT CGT CAT CTT 3'; *E. coli* position 83-104 (10)) was used in combination with unlabeled helper probes h-AMAC60 (5' CTA GCA AGC TAG AAA TGT TAC CG 3') and h-AMAC104 (5' CAA GTT CCC AAG CAT TAC TCA CC 3') in order to increase the accessibility of the target site (7). The theoretical specificity of the probe was verified *in silico* by performing a probe match against the ARB and RDP (release 51) databases. Since appropriate target organisms have not yet been isolated, stringent hybridization conditions for the new probe were determined in environmental samples by FISH at increasing concentrations of FA in the hybridization buffer and comparative cell counting. Optimal hybridization conditions were achieved at 55% of FA (see supplemental material).

Results

Concentration and isotopic signatures of POM. POC and PN concentrations in the control incubations at t = 0 were 0.71 ± 0.23 mg C l^{-1} and 0.07 ± 0.03 mg N l^{-1}, whereas POC and PN concentrations in the mucus incubations were increased by 3 fold with values of 2.34 ± 1.01 mg C l^{-1} and 0.17 ± 0.02 mg N l^{-1} (Tab. 1). Over the incubation time of 50 h, POC

concentrations decreased by 12% and 3% in mucus and control incubations, respectively. The average C:N ratio at t = 0 was 13.7 for the controls and 15.8 for the mucus incubations with increasing C:N ratio in the controls (17.4) and decreasing C:N ratio in the mucus incubations (10.0) over the experimental duration. The δ ^{13}C values of POM in mucus incubations (-19.97 ± 1.09‰) were less negative by almost 5‰ at t = 0 than in the controls (-24.75 ± 1.48 ‰) with no significant changes over the entire incubation time (Tab. 1). The average δ ^{15}N values of POM in the mucus incubations were always higher compared to the control treatments and reached positive values at t = 24 h (+2.16 ± 1.60 ‰) and t = 50 h (+1.82 ± 0.96 ‰).

Changes of total bacterial cell numbers. Initial cell numbers of the mucus-seawater-mixture with 6.4×10^5 cells ml^{-1} were slightly higher than cell numbers of the control with 4.6×10^5 cells ml^{-1} (Fig. 1). Mucus amendment lead to an almost 4-fold increase in cell numbers within 10 h of incubation, which corresponded to an estimated community doubling time of 5 h. By contrast, the total cell numbers of the seawater control approximately doubled within 50 h. Exponential growth of the bacterial community in the mucus-amended treatments terminated within the first 24 h of incubation. Microscopic inspection did not indicate a substantial growth of bacterivorous protists (heterotrophic nanoflagellates) over the incubation period (data not shown).

Diversity and phylogenetic analysis of 16S rRNA genes. Altogether, 95 distinct 16S rRNA genotypes were obtained from the three samples. In the C50 library 59% of all retrieved sequences affiliated to the *Gammaproteobacteria*, 22% to the *Alphaproteobacteria,* 11% to *Betaproteobacteria* and 7% to *Bacteroidetes*. By contrast, >90% of the sequences retrieved from both the M10 and the M26 library were affiliated to *Gammaproteobacteria*. Calculation of Good's coverage (M10: 0.8, M26:0.7, C50: 0.7) and of S_{Chao1} (Fig. 2) indicated that a large proportion of the expected gammaproteobacterial diversity was revealed by the number of sequenced clones in the individual libraries. Moreover, the asymptotic behaviour of the number of predicted phylotypes indicated that the relatively

small libraries M10 and M26 were nevertheless large enough to yield stable estimates of gammaproteobacterial phylotype richness (Fig. 2) (30). If data from the two libraries from the mucus-amended incubations were combined, approximately 90% (Good's coverage) of the total predicted gammaproteobacterial diversity was retrieved by our sequencing effort.

All gammaproteobacterial sequences were affiliated to *Alteromonadaceae*, *Colwelliaceae*, *Pseudoalteromonas* spp., and *Vibrio* spp.. Therefore, twenty-one distinct genotypes from these groups were selected for full sequencing (Tab. 2, Fig. 3). Sequence types of the *Alteromonadaceae* were affiliated to the species *A. macleodii* and *A.marina* as well as to uncultured *Alteromonas* spp. (Fig. 3).

Dynamics of bacteria targeted by FISH

Detection rates by CARD-FISH (probe EUB I-III) were 68% of total DAPI counts in the original water sample and 83 ± 5% in the incubations (data not shown). *Gammaproteobacteria,* as targeted by probe GAM42a, accounted for most bacteria in the mucus incubations at the end of the incubation period. Until 4 h of incubation, the relative abundances of these bacteria developed similarly in both mucus and control incubations, rising from close to 0% to 12 ± 2% and 11 ± 4% of total cell counts, respectively. In the mucus incubations the abundances continued doubling until the 10 h sampling, reaching abundances of 60 ± 6%. Towards the end of the experiment, *Gammaproteobacteria* constituted 72 ± 2% of the total bacterial community in these treatments. In contrast, the abundances of these bacteria in the seawater control started decreasing after 26 h of incubation and never exceeded 38 ± 2% of total counts (26 h) during the experiment.

A prominent fraction of the *Gammaproteobacteria* was affiliated to *Alteromonadaceae / Colwelliaceae* (A/C) as targeted by probe ALT1413 (Fig. 4). Their relative abundances increased from <1% to almost 50 ± 1% of total DAPI counts within 10 h. During the second half of the experiment, the relative abundances of A/C decreased to 35 ± 2%. The relative abundances of these bacteria in the controls rose steadily but more slowly to a maximum of 8 ± 4% at the end of the incubation period. Besides A/C, populations of bacteria affiliated to *Vibrionaceae* (GV) increased in the mucus-amended

treatments, to 21 ± 6% of total DAPI counts (Fig. 4). The community contribution of *Vibrionaceae* was substantially lower (<2%) in the seawater controls. Bacteria affiliated to *Pseudoalteromonadaceae* (probe PSA184) only enriched to a small extent in mucus-amended incubations, as compared to the untreated controls (Fig. 4).

The vast majority of cells that were detected by ALT1413 were hybridized by the newly designed specific probe AMAC-83 (Fig. 6). Cells targeted by AMAC-83 accounted for up to 44 ± 11% (10 h) of the bacterial community –indicating that a close relative of *Alteromonas macleodii* (Fig. 5) was the dominating genotype in the mucus incubations. The development of the total cell counts during the first hours of the incubations (0 h: 1.8×10^3 cell ml^{-1}; 10 h: 9.9×10^5 cells ml^{-1}) suggested an extremely low doubling time of these bacteria of approximately 1 h.

Discussion

Bacterial growth and POC consumption: Oligotrophic tropical seas are typically depleted in organic matter and nutrients, and coral mucus represents an important resource for microbial growth in reef ecosystems (11) and references therein). Already in 1979 Ducklow and Mitchell suggested that coral mucus could form the basis of a microbial food chain and that microorganisms might play a role in the initial degradation and further consumption of mucus by other organisms (15). This has subsequently been shown by various approaches (e.g. 44, 50). The rapid enrichment of bacteria (Fig. 2) and the 4 times higher reduction of POC concentrations (Table 1) in our mucus-amended incubations confirm earlier observations of enhanced bacterial growth in similar experimental setups (55) and in coral-containing mesocosms (19). Moreover, the decreasing C:N ratio of the particulate organic matter fraction in these treatments indicate an efficient microbial carbon mineralization, probably due to degradation of the complex polysaccharides which form a major fraction of the particulate mucous material (11). It should be noted that the POM collected in the mucus-amended incubations at the end of the experiment most probably also included the newly formed bacterial biomass. Filters of type GF/F typically do not retain small cells from oligotrophic waters, but larger bacteria in nutrient-rich samples or enrichments are quantitatively collected (22). However, even if the cell volumes of bacteria in the enrichments were 10 times larger than of typical planktonic cells, their total biomass would contribute <10% to total measured POC, as estimated by standard conversion factors (42).

Coral mucus may contain substances that inhibit the growth of some bacteria (50) and a considerable part of organic carbon from coral mucus (mainly lipids and polysaccharides, 11) may be in the dissolved fraction (57). Bacteria might, therefore, profit most substantially from mixtures of mucus and seawater (55). The diffusion of dissolved organic carbon (DOC) from the mucus layer of corals into the surrounding waters has been suggested as a possible cause for enhanced bacterial abundances in the immediate vicinity (1-3 cm) above coral surfaces (52). Unfortunately, changes of DOC concentration in our experiments were not analyzed due to technical reasons.

In view of the rapid response of the bacterial assemblage to mucus addition (Fig. 1), it is nevertheless likely that microbial growth was also fuelled by the fraction of dissolved organic material from the added mucous material.

Succession of *Gammaproteobacteria* in coral mucus-amended treatments. Research about the microbiology of the coral surface mucus layer itself has mainly focussed on its role in the maintenance of coral health and the transformation of microbial assemblages during disease (4, 49-51). So far, there is no direct information about the abundances of different bacterial taxa in coral mucus, e.g. by microscopic analysis. Cultivation-based studies have shown that particular *Gammaproteobacteria* (*Halomonas* spp., *Vibrio* spp., *Pseudoalteromonas* spp. and *Alteromonas*) can be 'residents' and/or 'visitors' of the surface mucus of *Fungia* and *Acropora* (34, 50). A high fraction (>50%) of gammaproteobacterial 16S rRNA genes were retrieved by PCR and sequence analysis from the surface mucus of healthy *Favia* (4), and the majority of sequence types associated with the mucus of *Oculina* were closely affiliated to *Vibrio splendidus* (33). By contrast, cultivation-independent analysis of bacterial diversity in the surface mucus of *Pocillopora* reported a dominance of *Alphaproteobacteria* (9). In that study *Alteromonas* sp. genotypes were nevertheless detected in slurries of coral tissue.

In the present study we did not aim at describing the community composition of coral mucus. Instead, we wanted to assess which bacterial taxa would be primarily enriched on mucus material freshly released into ambient water irrespective of their origin (i.e, the mucus itself or sea water). The addition of mucus to sea water clearly favored the growth of *Gammaproteobacteria*. More than 90 % of 16S rRNA gene sequences retrieved from coral mucus incubations at two timepoints were affiliated to this lineage, but only 52 % of sequence types in the control library. Analysis by CARD-FISH revealed that *Gammaproteobacteria* reached almost twice as high fractions of total cells and more than 5 times higher cell numbers in the mucus-amended incubation than in the control treatments. Within this lineage, the *Alteromonadaceae* were the most successful of the studied bacterial populations, in particular during the initial phase of the incubation (Fig. 4). Starting at $<5 \times 10^3$ cells ml^{-1} (in both the mucus amended treatments and the

controls) these bacteria increased to >10^6 cells ml^{-1} in the presence of mucus within 10 h. This was approximately ten times higher than their maximal cell numbers in the controls. Such rapid growth of *Alteromonadaceae* upon confinement has been observed in incubation of marine waters from habitats as different as the North Sea, the Mediterranean Sea, and the Red Sea (2, 17, 48).

Vibrionaceae represented the second most successful bacterial group in mucus-amended enrichments, but the population maximum of these bacteria (0.6 × 10^6 cells ml^{-1}) only formed after 26 h (Fig. 4). This delayed growth response might indicate that these bacteria were not able to consume organic material in mucus directly, but profited from the exoenzymatic activity of *Alteromonadaceae*. Alternatively, antagonistic effects between the two populations are conceivable. Representatives of both *Alteromonas* spp. and *Vibrio* spp. have been described that can inhibit the growth of strains from their own as well as from the other genus (35). Interestingly, one 16S rRNA gene sequence type of *Vibrionaceae* (M26-030) was most similar to *V. coralliilyticus* (Tab 2), a known coral pathogen (8). *Pseudoalteromonadaece*, which may also be stimulated by substrate addition (17) and which can be present in the mucus layer of healthy corals (51), did not show pronounced growth during our experiments (Fig. 4).

***Alteromonas macleodii*, a primary colonizer of detached coral mucus.**

Two highly similar (>99.9% sequence similarity) genotypes of *Alteromonadaceae* related to *A. macleodii*, ML10-016 and ML26-001 (Fig. 3) rapidly outcompeted all other bacterioplankton species in the primary utilization of mucus material, as monitored by FISH with the newly designed probe AMAC-83 (Fig. 6). By contrast, these bacteria formed no significant populations in the control treatment. The type species of the genus *Alteromonas*, *A. macleodii* IAM 12920 (sequence identity 99.8% and 99.9%) (23) is the closest cultivated relative to genotypes ML10-016 and ML26-001. It is, however, not targeted by probe AMAC-83 due to a single base deletion at *E. coli* position 100, which should allow reliable discrimination at the here chosen stringent hybridization conditions (see supplementary figure 1).

A. macleodii exhibits hydrolytic exoenzymatic activity of, e.g., amylases, gelatinases, and lipases (5). Thus, they appear to be well equipped to degrade particular components of coral mucus (11). *Alteromonas* spp. can moreover utilize the resulting monomers such as hexoses, disaccharides, sugar acids, amino acids, and ethanol (5). The versatile metabolism of these microorganisms may help to successfully exploit rapid changes in the supply of a complex substrate source such as coral mucus. In addition, the microscopic inspection of mucus-enriched samples revealed that *A. macleodii* were present as apparently free-living single cells, but also formed aggregates on particles, most likely originating from condensed coral mucus. A transition from dissolved to particulate matter is a typical aspect of the coral mucus cycle in coral reefs (57). *A. macleodii*, which are able to thrive both on particles (1) and in the water phase (17), are thus probably favored in an organic matter field that features a pronounced gradient of DOM to POM.

Trophic link to pelagic food webs? The sea water at distances of a few cm from corals has been shown to harbor elevated concentrations of bacteria (52). In view of our results it is, therefore, likely that *A. macleodii* is common in the immediate vicinity of *Fungia* sp. Bacteria from culturable gamma-proteobacterial lineages such as *Alteromonadaceae* and *Vibrionaceae* are known to exhibit a high sensitivity to grazing by heterotrophic nanoflagellates (HNF) than other members of marine bacterioplankton (6). For example, *Alteromonadaceae* were the first bacteria to respond to substrate and nutrient addition during a mesocosm experiment with Mediterranean water, but they were rapidly removed during a subsequent bloom of HNF (2). It is thus conceivable that a large fraction of the photosynthetically fixed carbon in coral reef systems, which is released as mucus into the ecosystem, is channeled to higher trophic levels via such tight predator-prey interactions. Moreover, selective protistan grazing might also explain why bacteria that are apparently able to exploit a major source of organic material in the pelagic zone of coral reefs are not found in high abundances in the bacterioplankton (see timepoint 0 in Fig.3).

On the other hand, elevated viral concentrations have also been detected in the close vicinity of corals (52). Viral lysis is known as an effective

mechanism of "killing the winner" (54), i.e. to selectively eliminate particularly successful or fast growing bacterial species. A rapid enrichment of only a limited number of genotypes as observed in our experiments (Fig. 6) would thus provide ideal conditions for such population control by specific viral lysis.

Conclusions: Our study illustrates that mucus from the coral *Fungia* sp. is efficiently utilized by bacterial groups that have been detected in coral tissue (9), mucus (33, 50), and that typically occur in low numbers in the plankton of coastal surface waters (3, 16, 18). Using rRNA gene sequence analysis and FISH we could identify bacterial genotypes closely related to *Alteromonas macleodii* as the most successful initial microbial colonizers of mucus released by the coral *Fungia* sp. This might help to provide an ecological framework for the interpretation of the diversification and the physiological potential of *Alteromonas macleodii,* e.g. as revealed by whole genome analysis. It should be noted that the composition of mucous material may vary with environmental factors and in different coral species (11, 39). Thus it is likely that different bacteria, either related to *A. macleodii* or other phylogenetic lineages, might be favored by the dissolution of mucus from various coral species into sea water. Future studies should thus aim at resolving to which extent the here described enrichment pattern can be generalized for different corals and/or geographic locations.

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TAB. 1: Changes in the concentrations and isotopic signatures of particulate nitrogen (N) and phosphorus (P) in enrichments with coral mucus and unamended controls during 50 h of incubation (means and standard deviations of three replicates).

Sample	Time [h]	N [mg l^{-1}]	C [mg l^{-1}]	C:N ratio (molar)	$\delta^{15}N$ [permil]	$\delta^{13}C$ [permil]
Control	0	0.07 ± 0.03	0.71 ± 0.23	13.68	-0.81 ± 5.96	-24.75 ± 1.48
	26	0.06 ± 0.04	1.02 ± 0.37	21.95	-0.56 ± 1.97	-25.29 ± 1.02
	50	0.05 ± 0.02	0.69 ± 0.06	17.24	-2.05 ± 6.84	-24.79 ± 0.74
Mucus	0	0.17 ± 0.02	2.34 ± 1.01	15.82	-0.45 ± 1.94	-19.97 ± 1.09
	26	0.24 ± 0.04	2.13 ± 0.19	10.30	2.16 ± 1.60	-19.59 ± 0.36
	50	0.24 ± 0.03	2.05 ± 0.15	9.99	1.82 ± 0.96	-19.45 ± 0.12

TAB. 2: 16S rRNA gene sequence types affiliated to *Pseudoalteromonas* spp. and *Vibrio* spp. from enrichments with coral mucus (M10, M26: 10 h and 26 h of incubation).

Designation	No. of nucleotides	Identity [%]	Closest relative / Closest cultivated relative	Accession no.
M10-004	1399	99.9	coral mucus clone M26-003	this study
		99.9	*Pseudoalteromonas* sp.	AJ874345
M10-022	1386			
		99.8	*Pseudoalteromonas* sp. EBD	DQ218321
M26-003	1402	99.9	coral mucus clone M10-004	this study
		99.8	*Pseudoalteromonas* sp. A28	AF227238
M26-004	1383			
		96.8	*Thalassomonas ganghwensis* JC2041	AY194066
M26-031	1382			
		99.8	*Pseudoalteromonas* sp. 03/034	AJ874351
M26-041	1387			
		95.5	marine bacterium Tw-2	AY028197
		95.1	*Alteromonas* sp. KT0232	AF235125
M26-005	1418			
		99.4	*Vibrio fortis* LMG 21562	AJ514915
M26-029	1408			
		98.6	*Vibrio brasiliensis* LMG 20546	AJ316172
M26-030	1404			
		99.3	bacterium CWISO20	DQ334358
		99.2	*Vibrio coralliilyticus* LMG 21349	AJ440004
M26-032	1404			
		98.9	*Vibrio* sp. LMG 20548	AJ316170
M26-036	1402	98.7	coral mucus clone ML26-032	this study
		98.6	*Vibrio* sp. LMG 19270	AJ316169
M26-037	1411			
		97.3	*Vibrio chagasii* LMG 13237	AJ490157

Legends to figures:

Figure 1: Changes of the total abundances of prokaryotic cells in mucus-amended and control incubations

Figure 2: Coverage of the diversity of *Gammaproteobacteria* (left panel) and of all bacteria (right panel) in 16S gene clone libraries from mucus-amended (M10, M26) and unamended (C50) enrichments as estimated by the parameter S_{Chao1}

Figure 3: Phylogenetic relationship of genotypes affiliated with *Alteromonas* from mucus-amended (M10, M26) and control (C50) incubations. Depicted in bold: Sequence types targeted by the oligonucleotide probe AMAC83

Figure 4: Relative abundances of *Alteromonadaceae* (probe ALT1413), *Vibrionaceae* (probe GV), *Pseudoalteromonadaceae* (probe PSA184), and in mucus-amended and control incubations.

Figure 5: Relative abundances of cells hybridized by probe AMAC83 (targeted to genotypes M10-016 and M26-001) in mucus-amended and control incubations.

Supplementary material:

Suppl. Figure A: Changes of the numbers of hybridized cells by probe AMAC83 at increasing levels formamide (FA) in the presence (black) and absence (white) of helper oligonucleotides. Stringent hybridization conditions are indicated by the dashed box.

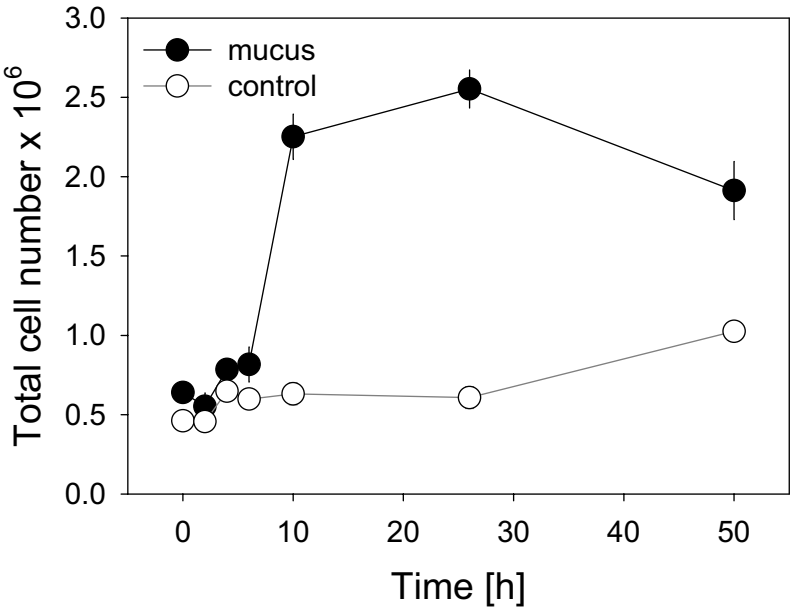


Figure 1

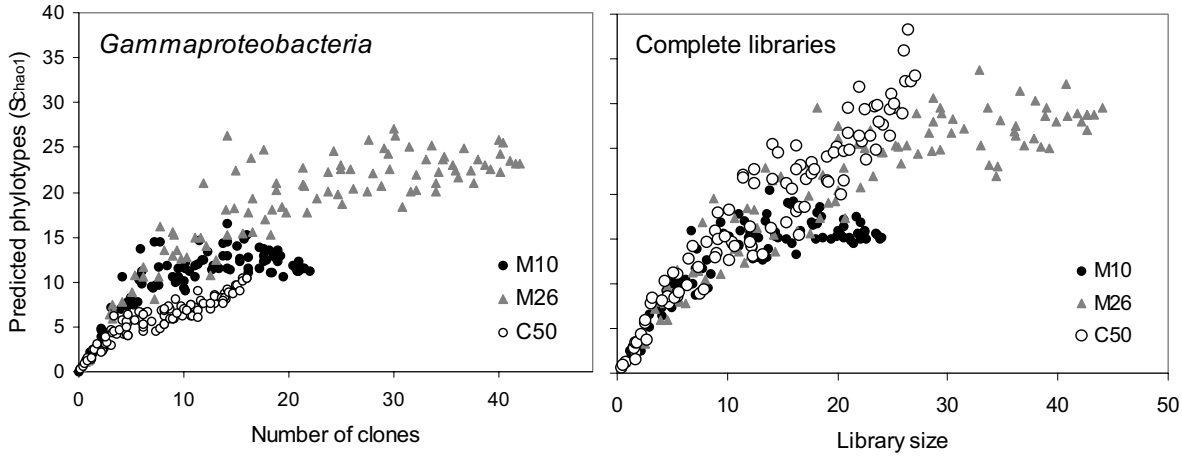


Figure 2

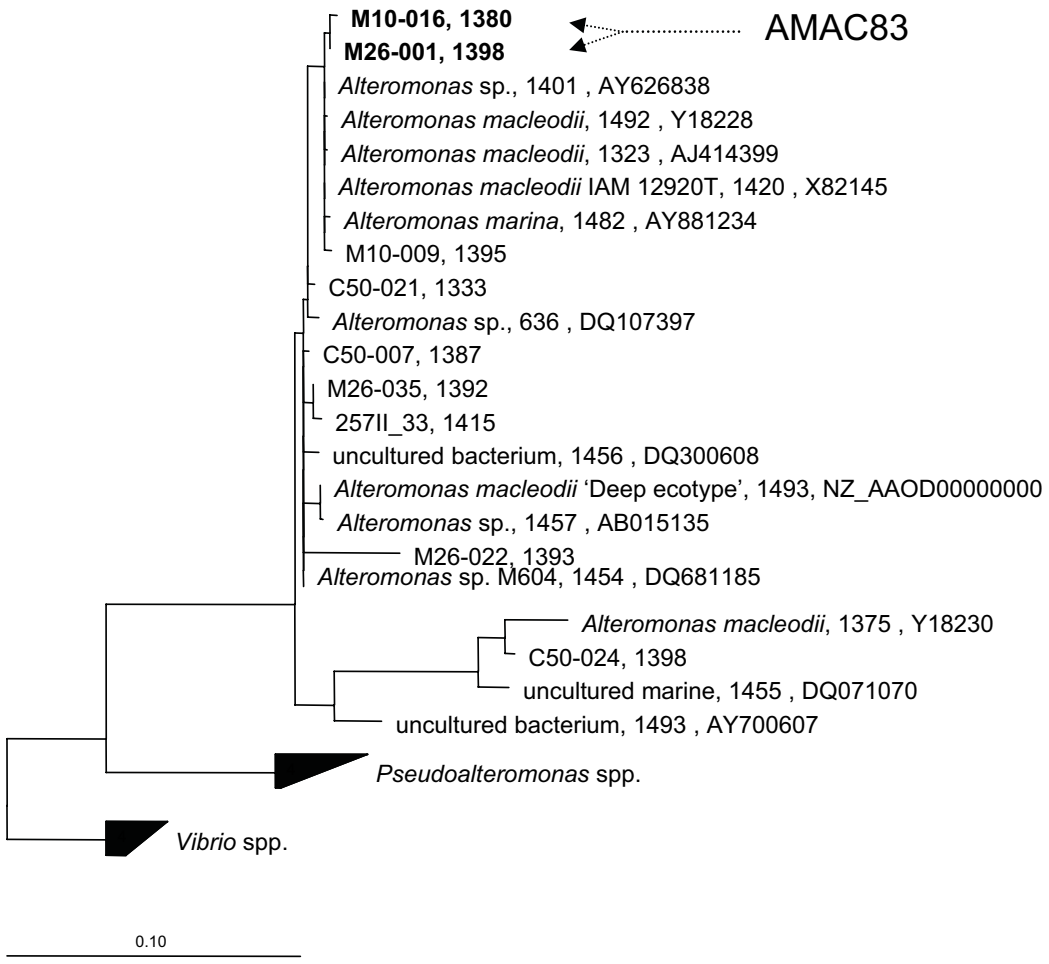

M10-016, 1380
M26-001, 1398
AMAC83
Alteromonas sp., 1401 , AY626838
Alteromonas macleodii, 1492 , Y18228
Alteromonas macleodii, 1323 , AJ414399
Alteromonas macleodii IAM 12920T, 1420 , X82145
Alteromonas marina, 1482 , AY881234
M10-009, 1395
C50-021, 1333
Alteromonas sp., 636 , DQ107397
C50-007, 1387
M26-035, 1392
257II_33, 1415
uncultured bacterium, 1456 , DQ300608
Alteromonas macleodii 'Deep ecotype', 1493, NZ_AAOD00000000
Alteromonas sp., 1457 , AB015135
M26-022, 1393
Alteromonas sp. M604, 1454 , DQ681185
Alteromonas macleodii, 1375 , Y18230
C50-024, 1398
uncultured marine, 1455 , DQ071070
uncultured bacterium, 1493 , AY700607
Pseudoalteromonas spp.
Vibrio spp.
0.10

Figure 3

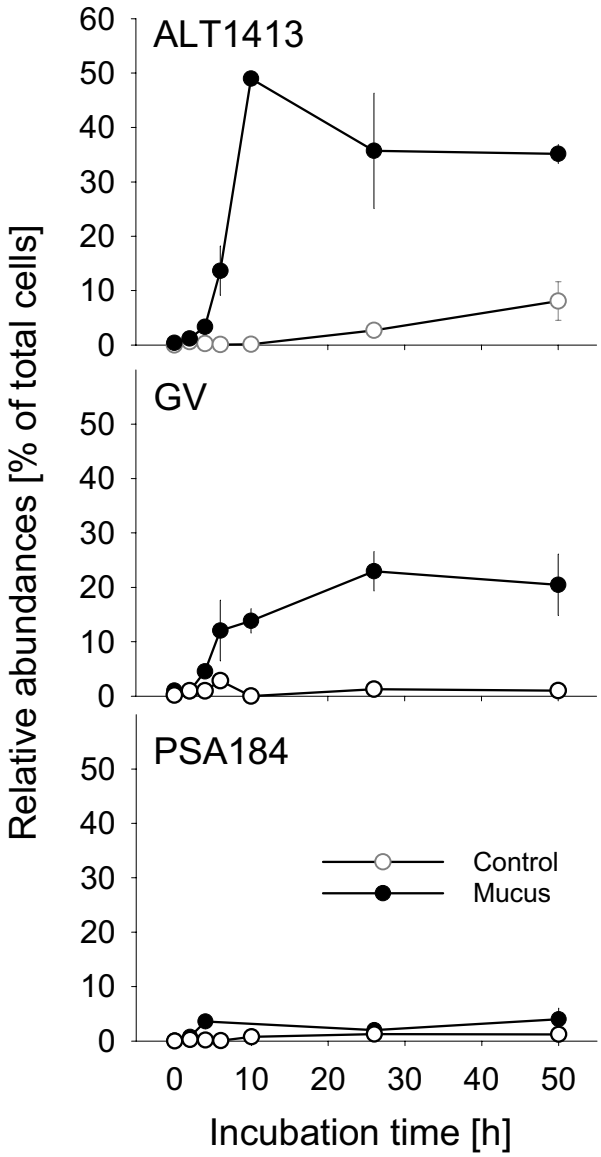

ALT1413
GV
PSA184
Relative abundances [% of total cells]
Incubation time [h]
Control
Mucus
0
10
20
30
40
50
60

Figure 4

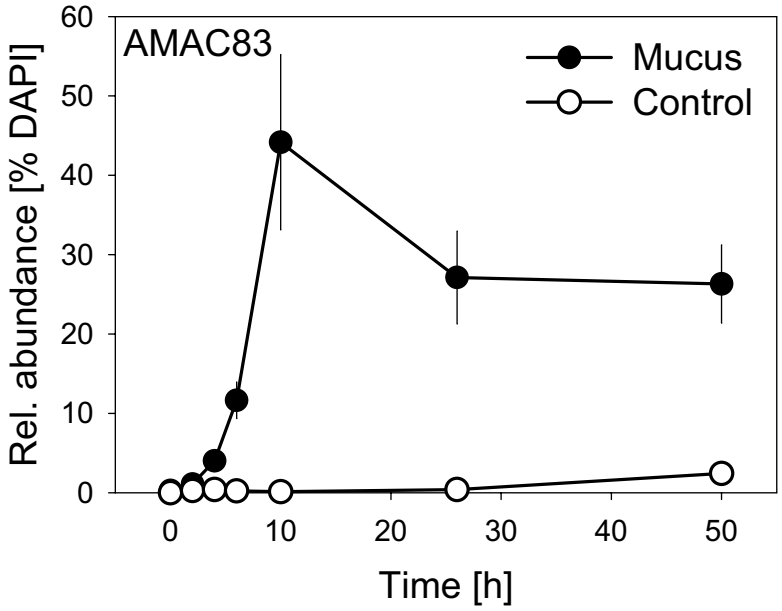
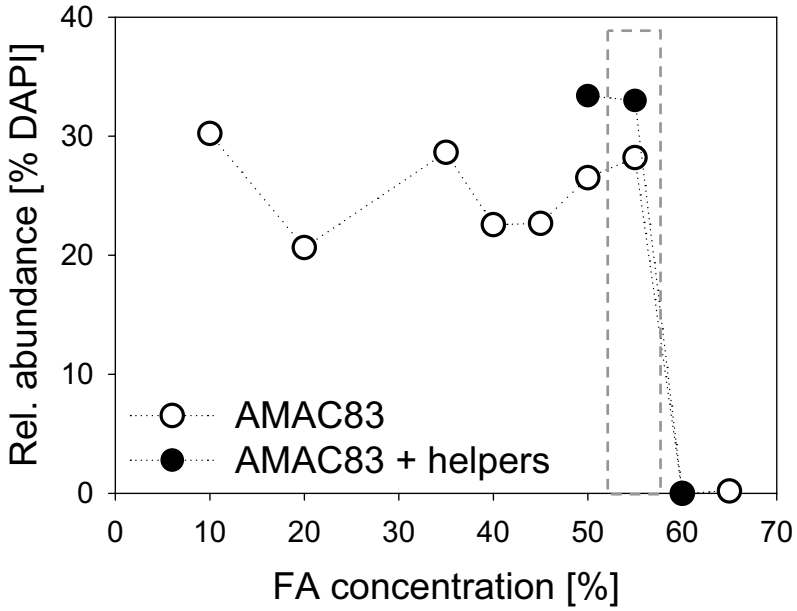


Figure 5



Suppl. Figure A

Part III: Supplements

SI Helgoland Enrichment

1 Material & Methods

Sampling site and substrate incubation setup. In August 2005, surface water was collected from 1 m depth at Helgoland Roads (54°11' N, 7°54' E; salinity: 32 PSU) near the island of Helgoland, which is situated approximately in the German Bight of the North Sea. Pre-filtered (1.2 µm) North Sea water was diluted 1:10 with 0.2-µm-filtered aged seawater. Dilution subsamples of 10 ml were amended with several C sources, such as sugars, amino acids and protein, and nutrients phosphate (NaH_2PO_4) and ammonium (NH_4Cl) in different combinations and concentrations (Tab. 1) and set up in 50 ml tubes in triplicates. Incubations were kept at *in situ* temperature (17°C) and in the dark for 48 h. The experiment was stopped by adding the fixative PFA (1% final concentration).

Total bacterial cell numbers. Cell numbers were counted with a Becton Dickinson FACScalibur benchtop flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Fixed samples were kept at 4°C until analysis. Following (Marie *et al.*, 1997), the nucleic acid stain SYBR Green I (Molecular Probes Inc., Eugene, OR, USA) and potassium citrate (Sigma, Steinheim, Germany) were added to subsamples of 500 µl to a final concentration of 26 mM and 29 mM, respectively. The staining was carried out for 30 min at 4°C in the dark directly prior to analysis. For flow cytometric analysis 50 µl of a solution of latex beads (size, 1 µm; concentration, 4×10^6 ml^{-1}; Polyscience, Inc., Washington, PA, USA) were added to each sample as internal standard. Bacterial cell numbers were determined from the ratios of cells to beads.

Bacterial abundances. Samples were fixed with 1% PFA for 1 h at RT for analysis of the bacterial community by fluorescence *in situ* hybridization and catalyzed reporter deposition (CARD-FISH) (Pernthaler *et al.*, 2004). Sub-samples were filtered onto white membrane filters (GTTP, 0.2 µm pore size, 47 mm in diameter, Millipore, Eschborn, Germany), and bacterial cells were fixed onto the filter by embedding the surface into 0.2% agarose (MetaPhor, Cambrex Bio Science, Rockland, USA). Before hybridization, cells were permeabilized by treatment with lysozyme. Filter sections were hybridized with horseradish peroxidase (HRP)-labelled oligonucleotide probes (Biomers, Ulm, Germany) as previously described (Pernthaler *et al.*, 2004). Probes were targeting members of *Pseudoalteromonadaceae*. (PSA184; Eilers *et al.*, 2000), *Alteromonadaceae*./*Colwelliaceae* (ALT1413; Eilers *et al.*, 2000), and *Vibrionaceae* (GV; Giuliano *et al.*, 1999). Stringent hybridization conditions were achieved by 55% Formamide (FA) in the hybridization buffer for PSA184 and GV, and 60% FA for ALT1413, respectively. After 2.5 h of hybridization the probe-delivered HRP was detected with tyramides (diluted 1:700 in amplification buffer) that were custom labeled with fluorescein (Molecular Probes, Eugene, USA). The preparations were subsequently embedded onto glass slides in a previously described mountant mix containing 1 µg ml^{-1} of DAPI (Pernthaler *et al.*, 2004). The fractions of CARD-FISH stained cells of all DAPI stained objects were determined by epifluorescence microscopy and semi-automated image analysis (Pernthaler *et al.*, 2003). Negative controls were routinely determined with the EUB I-III antisense probe NON338 (Wallner *et al.*, 1993).

Tab. S-1. Treatment designation for the incubations of the Helgoland Enrichment. With nutrients and substrates presented 1:10 diluted water of the German Bight was incubated for 48 h at 17°C.

Designation	Substrate	Substrate Concentration	NH_4 concentration	PO_4 concentration
Control	none	-	5 µM	1 µM
Glc	Glucose	10 µM		
Fcs	Fucose			
Rhm	Rhamnose			
Gly	Glycine			
Leu	Leucine			
Ovb	Ovalbumine			
Sugar -1	Glc + Fcs + Rhm	100 nM	5 µM	1 µM
Sugar 1	Glc + Fcs + Rhm	10 µM		
Sugar +1	Glc + Fcs + Rhm	100µM		
AA -1	Gly + Leu	100 nM		
AA 1	Gly + Leu	10 µM		
AA +1	Gly + Leu	100µM		
N1P1	Glc + Fcs + Rhm	10 µM	-	-
N1P2	Glc + Fcs + Rhm		-	1 µM
N2P1	Glc + Fcs + Rhm		5 µM	-
N2P3	Glc + Fcs + Rhm		5 µM	100 µM
N3P2	Glc + Fcs + Rhm		500 µM	1 µM

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