# Dynamics of the Genetic Diversity of Marine Bacterial Assemblages

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# Chapter 1

# Synopsis

# Introduction

## The biogeochemical importance of heterotrophic, pelagic, marine bacteria

Classical concepts of trophic interaction in the ocean never ascribed an important role to marine bacteria. These implied that most of the primary production produced by phytoplankton is channelled into higher trophic levels, thus to larger organisms through microzooplankton. Even after estimates of the actual numbers of bacteria present in seawater had been corrected (Hobbie *et al*., 1977; Jannasch & Jones, 1959) some doubt remained about the activity of the cells. The ocean was then regarded as just too dilute in organic carbon concentrations to support growth and activity of such a large number of cells. However, the application of radiotracer experiments suggested that the organic matter pool in seawater had short turn-over times (see references in Azam, 2001). Incorporation of tritiated thymidine was used to estimate bacterial production (Fuhrman & Azam, 1982) showing that bacteria are an important component of marine food webs (Azam *et al*., 1983). There is still considerable uncertainty about the actual amounts, but accepted estimates attribute 15 to 50% of the organic carbon produced by phototrophic primary producers to be channelled through bacteria (see Williams, 2000). However, to date it is still not clear what fraction of bacterial cells can be regarded as active, dormant or dead, as different methods to quantify the categories yield different numbers (Choi, 1999; Gasol *et al*., 1995; Karner & Fuhrman, 1997; Zweifel & Hagström, 1995). Another matter of debate are bacterial growth efficiencies. There are indications that these have usually been overestimated significantly (see review of Del Giorgio & Cole, 1998) and that bacterial growth efficiency may be as low as 15 to 20%. This finding has important consequences for estimating what fraction of organic matter oxidation is eventually represented by a bacterial cell. Variations in growth efficiency estimates lead to great uncertainties in the general balancing of marine production and consumption of organic matter (Williams, 2000), and make assessment of whole ocean regions as net sources or sinks of $CO_2$ extremely difficult (Del Giorgio *et al*., 1997; Williams, 1998). The importance of bacteria in cycling of organic matter is further underlined by their ability to act as competitors to phytoplankton in the uptake of inorganic nutrients (Caron, 1994). Thus bacterial nutrient uptake can affect nutrient pools available for primary production, and bacterioplankton growth may be limited by the availability of inorganic nutrients itself (Cotner *et al*., 1997).

## Diversity of marine microbial communities

It is clear that bacteria are important, but why should one study bacterial diversity? Studies of marine bacterioplankton have often applied a so-called “black-box” approach, meaning that bacteria have mainly been investigated according to the extent of fluxes or transformations that are mediated by them, without taking into account that only a subset of the community might actually be contributing to the measured rate. Thus, without knowledge which bacteria are responsible for the activity and hence relating the performance of the active fraction to the community as a whole, bacterioplankton remains a black-box. While this may seem to be ignorant at a first glance, the objective to quantify a bacterially mediated transformation and at the same time determine the identity of the responsible population(s) is far from trivial. Microbial ecological studies have only recently begun to explore the links between structure and function or identity and activity of microbial assemblages by applying new tools. Especially molecular biological techniques using small subunit ribosomal RNA sequences or the encoding genes as a molecular marker have been used for assessing the taxonomic composition of microbial communities (Amann *et al*., 1995; Giovannoni *et al*., 1990; Muyzer, 1998; Ward *et al*., 1990). The application of molecular biological techniques in biological oceanography has resulted in a revised conception of marine microbial community composition (see Giovannoni & Rappé, 2000 for a review). The conventional method of

plating bacteria on more or less diverse agar based media typically results in certain culturable groups of bacteria being detected, while the larger portion of bacteria can not be readily cultured using this strategy. An inventory made by molecular biological techniques commonly displays a greater diversity than culture-dependent methods, and moreover gives a different display of taxonomic composition, with most types retrieved not related to cultured bacterial isolates. Fluorescent in situ hybridisation (FISH) with taxon-specific gene probes has confirmed the general problems associated with the culture-dependent methods (Amann, *et al*., 1995; Glöckner *et al*., 1999). The incongruent nature of displays of community composition given by culture-dependent and independent approaches has also been verified in studies using the two approaches simultaneously (Eilers *et al*., 2000; Suzuki *et al*., 1997).

The consequence of the strong biases associated with the culture-dependent approach to analysing community composition has been a trend in microbial ecology to replace cultivation based methods with the new molecular tools. However, up to now, most studies using molecular biological techniques have only provided snap-shots of community composition only. Cloning and sequencing of complete 16S rRNA genes to analyse community composition with subsequent in situ identification of defined populations by FISH are powerful tools in microbial ecology, but there is always a trade-off between the amount of sequence information that can be gathered by the cloning approach and the number of samples that can be analysed. Genetic fingerprinting techniques, e.g. denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999; Muyzer & Smalla, 1998), are excellent tools for studying the changes in bacterial genetic diversity over both, temporal and spatial scales, because they facilitate the analysis of numerous samples.

**Temporal changes in marine bacterial community composition**

Few studies have applied molecular methods to study microbial community composition over longer time-spans, for instance to study seasonal and annual patterns of microbial community composition. Therefore, studies using cloning and sequencing to address dynamics in bacterial community composition have usually been limited to few samples resulting in a low temporal resolution (Kelly & Chistoserdov, 2001; Kerkhof *et al*., 1999). Using gene probes and hybridisation techniques a few autecological studies have provided higher temporal resolution of the distribution of specific bacterial and archaeal phylotypes in the marine environment (Field *et al*., 1997; Gordon & Giovannoni, 1996; Massana *et al*., 2000). Furthermore, a more extensive application of FISH detection of bacterial populations in combination with cloning and culturing was done by Eilers and colleagues (Eilers, *et al*., 2000). FISH of bacterial populations showed that culturable bacteria form the North Sea made up only a minor portion of the bacterioplankton assemblage, and at the same time demonstrated that abundance of probe-defined populations may undergo seasonal fluctuation. The first report on seasonal changes in marine microbial communities using a genetic fingerprinting approach, was the study by Murray et al. (Murray *et al*., 1998) who used PCR-DGGE to analyse fluctuation in the composition of bacterial assemblages in the waters around Anvers Island (Antarctica) along period of a nine months. They noted that the genetic richness of the assemblage increased at the beginning of the productive summer season and decreased again thereafter.

**Bacteria underlie various control mechanisms within the microbial food web.**

The goal of microbial ecology is to identify the factors determining the composition of microbial assemblages and their performance in driving the global biogeochemical cycles. Only a thorough understanding of how individual populations contribute to community performance and how they are regulated in their abundance and activity will ultimately add predictive power to microbial ecology, which is lacking at present. An answer to these questions can often only be given by correlation of measurements of abiotic and biotic

parameters with fluctuation in microbial community composition. Mechanisms controlling pelagic bacterial communities are classified as bottom-up and top-down regulation. Bottom-up regulation refers to the control conferred by availability of nutrients and carbon sources on the growth of microorganisms. Mortality conferred through grazing by microzooplankton (heterotrophic nanoflagellates, and ciliates) and viral infection is important in regulating the abundance of pelagic bacteria (Sanders, 1992; Sherr & Sherr, 1994; Weinbauer & Höfle, 1998) and is referred to as top-down control, and side-in control respectively (Suttle, 1994). Populations of picoplanktonic microorganisms may vary greatly in their response to these differing controlling factors. Reaction to bottom-up mechanisms will be determined by physiological characteristics, such as the substrate spectrum, substrate uptake affinity and kinetics. Likewise, there is variation in the susceptibility to different mortality mechanisms. It has been shown, for instance, that the chance of a bacterium being ingested by a grazing heterotroph is correlated to its size (Monger & Landry, 1991), which in turn is correlated to its activity (Del Giorgio *et al*., 1996; Gasol, *et al*., 1995). Some bacteria have evolved defence mechanisms that can reduce the mortality of the population, e.g. by developing an inedible morphotype (Hahn *et al*., 1999; Jürgens & Güde, 1994; Pernthaler *et al*., 1997) or outgrowing predation pressure (Pernthaler, *et al*., 1997). Thus, it is conceivable that variations in bottom-up and mortality mechanisms should have the potential to affect the composition of pelagic bacterial assemblages. Other studies that have addressed temporal changes of bacterial community composition recently, have provided indications that the development of phytoplankton blooms may affect bacterial community composition (Kelly & Chistoserdov, 2001; Kerkhof, *et al*., 1999). Thus, to understand bacterial community composition and its regulation a variety of factors has to be regarded. In this context it is of interest how grazing and eutrophication on the one hand and the predominant type of phytoplankton on the other hand may be able to affect the diversity and activity of bacterial assemblages.

Eutrophication of coastal waters is a problem with world-wide distribution and is of global importance. Eutrophication not only has economical impact, but also is a risk for the species diversity of natural ecosystems. Harmful algal blooms have been considered one of the effects of eutrophication in coastal environments (Paerl, 1998; Vollenweider, 1992). Eutrophication may disrupt natural pelagic food webs, and might therefore entail potential consequences for marine bacterial assemblages (Paerl, 1998).

**Outline of the thesis**

The aim of this thesis was to characterise fluctuations in the genetic diversity of bacterial communities occurring at a variety of temporal scales (hours to years) and to try to identify factors that influence the observed dynamics. Special attention was given to the influence of eutrophication on bacterial diversity.

A good part of the work presented in this dissertation has been carried out in the framework of the European-Union-funded project CHABADA (‘Changes in bacterial activity and diversity in Mediterranean coastal waters as effected by eutrophication’, project number MAS3-CT96-0047), which focussed on the effect of nutrient addition on bacterial activity and diversity. Analyses of microbial community composition reported on in the different chapters of this Thesis were mainly performed by denaturing gradient gel electrophoresis. Chapter 2 is an overview of the application of DGGE genetic fingerprinting in marine microbial ecology and also provides detailed protocols covering the practical aspects of the technique.

The CHABADA-project started out with mesocosm experiments that were used as model systems to evaluate short-term changes in Mediterranean bacterial communities as a consequence of nutrient addition. Generally, marked fluctuation occurred in the genetic diversity during the incubation, even in control mesocosms. Grazing of heterotrophic microzooplankton on bacteria was tentatively identified as an important factor affecting the genetic diversity of bacterial assemblages. The results of the mesocosm experiments are

reported in Chapter 3 and Chapter 4.

Originally, it was planned to complement the insights gained in the mesocosm experiments with observations from a natural eutrophication gradient such as in the Northern Adriatic Sea. Unfortunately, it was not possible to go there, so as an alternative the outflow plume of the Rhone river was studied, where strong gradients in both nutrient and salt concentrations occur. The results of that study are reported in Chapter 5.

Eventually, the chance to study the potential effects of eutrophication on bacterial diversity in a natural system arose in a collaboration with Olav Vadstein and Yngvar Olsen, from the Trondhjem Biological Station of the Norwegian University of Science and Technology, Trondheim, who were involved in the EU-funded project COMWEB ('Comparative Analysis of Food Webs Based on Flow Networks: Effects of Nutrient Supply on Structure and Function of Coastal Plankton Communities'; project number MAS3-CT96-0052). Within the COMWEB-project a eutrophication experiment in the landlocked bay Hopavågen on the coast of central Norway had been performed. Samples from the euphotic zone of the bay were analysed which were taken during a period of 40 months from 1996 until 1999. Two years, 1996 and 1997, served as control years, and during the summer seasons of 1998 and 1999 nutrients were added artificially. The samples were analysed for seasonal distribution patterns in the bacterial community composition and it was assessed whether emerging patterns were affected by eutrophication. The results are reported in Chapter 6.

Part of the rationale behind the final study, a survey of the diversity of so-called 'satellite' bacteria from diatom cultures (Chapter 7), was that eutrophication effects on bacterial diversity might only be indirect, and brought about through interactions that bacteria have with other components of pelagic food webs. One of the suggested effects of eutrophication on coastal zones has been the increased incidence of nuisance algal blooms, which might entail subsequent changes in the bacterial community, if algal-bacterial interactions (mutualisms and/or antagonisms) exist. Thus, the motivation to gather basic information by studying the bacterial diversity at the example of diatom laboratory cultures.

## Results

### Mesocosm studies

The results of the first mesocosm experiment showed that successional changes in the composition were occurring even in the untreated control mesocosm. Bacterial and protozoan counts revealed a typical mesocosm succession consisting of a growth phase of bacteria in the beginning, which was followed by a peak in protozoan counts (mainly heterotrophic nanoflagellates) and a concomitant reduction of bacterial numbers to initial levels. Estimations of bacterial mortality due to grazing indicated that grazing was the main factor responsible for bacterial mortality. Important changes in the composition of the bacterial community were suggested by marked changes in DGGE fingerprints of the bacterial assemblage during the incubation. Cloning and sequencing of 16S rRNA genes confirmed that a major shift in species composition was occurring during incubation and clone libraries of samples from after the peak in grazing activity were dominated by 16S rRNA genes related to those of the genus *Alteromonas*.

In a second mesocosm experiment, replicate tanks were used to evaluate the reproducibility of treatments (control and nutrient-enrichment with inorganic N and P). Additionally, samples from the original coastal sampling station were also analysed before and after the experiment to isolate the effect of manipulation in mesocosms. PCR-DGGE was also performed on reverse transcribed rRNA, which favours amplification of the most active bacterial populations due to their high rRNA content. Marked fluctuations in community

composition were evident from DGGE analyses and nutrient enrichment also entailed some differences in the community composition, but there were also similarities between the development of tanks from the two treatments, and nutrient addition seemed to affect mainly the speed and extent of the changes. Interestingly, the phase of intense grazing again marked important shifts in community fingerprints, and numbers of rRNA-derived DGGE bands (indicating active populations) that had increased during the initial growth phase, were reduced during the phase of protozoan grazing. Microbial populations were identified by sequencing of DGGE bands and contrary to the first mesocosm experiments the post grazing phase seemed to be dominated by α-Proteobacteria and members of the *Cytophaga-Flavobacterium-Bacteroides* group (CFB). Strong changes in community fingerprints of the bacterial assemblages in mesocosms were not mirrored at the natural site, where only little change was observed in community fingerprints during two weeks indicating a significant effect of confinement as suggested in the first mesocosm study.

**Field studies**

While the mesocosm experiments had lasted for about two weeks, the temporal scale of the samplings in the Rhone river plume was much shorter, extending just over a couple of hours. Riverine and marine bacterial assemblages behaved differently in the mixing zone. Bacterial abundances and activities showed a more drastic decrease in the low salinity range of the gradient than expected from dilution models, indicating that an important fraction of freshwater bacteria disappeared in the mixing area. The plume zone had a high total bacterial genetic richness (estimated by the number of DNA-derived DGGE bands), 13-55 bands compared to that reported in other aquatic ecosystems, which was the consequence of the mixing of riverine and marine assemblages. The proportion of active populations was estimated using the ratio of DGGE bands derived from RNA and DNA. This ratio was lower in Rhone water than in marine water indicating that only a part of the constitutive populations were active, while the activity was distributed within a larger fraction of populations in the marine assemblage. This fitted well with the observation of higher specific leucine incorporation rates in the marine assemblages. The marine community appeared to be strongly affected by decreasing salinity, which probably was a consequence of the mixing of marine assemblage with a much more abundant riverine community. No marked modification of the marine community by inflowing nutrient rich river water was observed. This lack of a response was probably the consequence of a very short residence time of water in the studied mixing area which did not allow for a growth response of marine populations to display in genetic fingerprints.

Seasonal and annual patterns in the bacterial community composition of the pristine, landlocked bay Hopavågen on the Norwegian coast were analysed in years with and without sustained experimental nutrient addition. Hopavågen was used as an experimental field for testing the consequences of nutrient addition in the summer seasons of 1998 and 1999, while 1996 and 1997 were investigated as control years without artificial eutrophication. Denaturing gradient gel electrophoresis analysis of bacterial 16S rRNA gene fragments showed seasonal variations in community composition that seemed to be recurrent annually with some phylotypes appearing at similar times during the years. *Synechococcus*–related cyanobacteria dominated the late summer community, α-Proteobacteria of the *Roseobacter* group seemed permanently present during the phase of phytoplankton production. The study showed that recurrent seasonal patterns exist in bacterial assemblages, with especially marked changes during the transition from winter to the productive seasons. Doubled amount of nutrient addition in 1999 as compared to 1998 had an effect on phytoplankton primary production and also some effect on phytoplankton community structure. An effect of eutrophication on the genetic diversity of the bacterial assemblage, was, however, not obvious, suggesting that the

level of artificial eutrophication, was not yet sufficient to alter the microbial food web structure and bacterial diversity of Hopavågen.

**'Satellite' bacteria in cultures of marine diatoms**

The motivation to analyse the genetic diversity of 'satellite-bacteria', bacteria accompanying uni-algal cultures of marine phytoplankton was to assess whether the different algal cultures may constitute niches for specific bacterial species. The six diatom cultures that were analysed were accompanied by distinct satellite assemblages, as the majority of the phylotypes detected in the six cultures was unique, only some phylotypes were common to more than one culture. Only minor variations of satellite assemblage genetic fingerprints was observed suggesting that the bacterial-algal associations were stable. An experimental approach to find evidence for specific algae-bacteria interactions by challenging algae cultures with heterologous satellite assemblages was unsuccessful. It was not possible to avoid carry-over of algae. Most satellite populations were identified by sequencing of DGGE bands as typical marine phylotypes of the α-Proteobacteria (related to the genera *Ruegeria*, *Sulfitobacter*, *Roseobacter*, and *Erythrobacter*), or of different genera of the CFB phylum. Surprisingly, β-Proteobacteria were also found in two of the cultures. A unifying theme in satellite bacterial assemblage composition was the presence of at least one representative of the α-Proteobacteria and of the CFB phylum, both of which have been identified as important representatives of the marine picoplankton. The results indicate that algae diversity may be an important factor for explaining some of the enormous bacterial diversity in marine assemblages, and vice versa.

## Discussion

The present study was successful in analysing dynamics of marine bacterioplankton assemblages in artificial as well natural systems at a variety of temporal scales. Simultaneous measurements of a variety of other biological parameters related to the microbial food web in the context of the European projects allowed to correlate fluctuations in bacterial diversity with co-varying parameters such as bacterial production, grazing conferred mortality, and phytoplankton production and composition.

Results presented in this dissertation have for instance demonstrated that the typical tri-phasic course often observed in incubation experiments (i.e. a growth-, a grazing, and a post-grazing phase; (Jürgens & Güde, 1994)) is accompanied by strong fluctuations in bacterial community composition. It was suggested that grazing by heterotrophic microzooplankton was an important factor in such incubations and was likely to be responsible for shifts in bacterial community composition between peak in bacterial biomass and subsequent reduction of bacterial numbers by grazing. Grazing has also been identified in other studies as a structuring force of bacterioplankton diversity (Jürgens *et al*., 1999; Pernthaler, *et al*., 1997; Suzuki, 1999; van Hannen *et al*., 1999) and it is now widely accepted that grazing by bacterivorous microzooplankton may affect community composition. Besides confirming that grazing can affect community composition in mesocosm experiments, DGGE-fingerprinting performed on DNA as well as on RNA (after reverse transcription) in the second mesocosm experiment also suggested that it most strongly affected active bacterial populations, matching previous suggestions and observations (Del Giorgio, *et al*., 1996; Gasol, *et al*., 1995). There was circumstantial evidence for removal of active bacterial populations in the fluctuation of the numbers of rRNA-derived bands representing active bacterial populations, which decreased after the grazing phase in mesocosm experiments. It has to be pointed out, however, that an increase in activity and growth rate may also be a bacterial strategy to compensate for grazing inflicted losses and hence to allow to co-exist with their predators (Pernthaler, *et al*., 1997).

Another important aspect of the present study is related to the question how mesocosm experiments, or incubation experiments in general, can help to answer questions in aquatic microbial ecology. Despite their wide application in the field (Duarte *et al*., 1997) only recently we and other authors have begun to analyse community composition of incubated bacterial assemblages by molecular methods, e.g. (Eilers *et al*., 2000; Jürgens, *et al*., 1999; Riemann *et al*., 2000). The present study extends previous observations on the effects of confinement (Ferguson *et al*., 1984) by giving examples of the way community composition can change significantly as analysed by molecular biological methods. Furthermore, the results presented here contrast sharply to results of previous studies suggesting only limited shifts in confined samples (Lee & Fuhrman, 1991), possibly because of different handling and incubation times and conditions. Additionally, the different methods to evaluate shifts in community composition, DGGE and cloning of 16S rRNA genes on the one hand, and community DNA hybridisation on the other hand, may have contributed to drawing the contrasting conclusions.

On the one hand the mesocosm experiments were useful to map changes in community composition in phases of varying gross activity, and hence to tentatively identify factors that structure the community, such as grazing by heterotrophic protozoa. Thus, mesocosm experiments may be of use for gaining information about potential short-term microbial community dynamics in marine environments, such as exemplified by episodic up-welling events and build-up and decay of phytoplankton blooms (Kerkhof, *et al*., 1999). On the other hand, the results show that mesocosm experiments may not be useful for experimental perturbation studies, despite the interesting successions of bacterial populations that are usually occurring. Although artificial nutrient addition to Mediterranean coastal water had a strong effect on global parameters such as bacterial production and biomass (Lebaron *et al*., 2001; Lebaron *et al*., 1999), the impact on bacterial diversity was less pronounced than might be expected. The aspect of food web manipulation in combination with confinement had much greater impact on bacterial diversity than had nutrient addition. This was evident from the marked deviation of DGGE patterns of microbial assemblages in control and nutrient enriched mesocosms from those in the natural situation (see Chapter 4). Apparently, a true negative control can hardly be achieved due to effects of sample handling (e.g. enrichment with organic carbon from damaged cells) and it seems obvious, therefore, that untreated mesocosms cannot be considered real controls against which to test the effect of experimental nutrient addition. Paerl noted (Paerl, 1998) that “microbial taxa (and their interactions) may respond opportunistically to anthropogenic alterations”, a description that also fits which the microbial assemblages of the mesocosms. In the control mesocosm of the first experiment the development of *Alteromonas* related bacteria was observed, which are often referred to as typical opportunistic bacteria. Thus, to some extent all mesocosms seemed to behave as if they were eutrophied, regarding their deviation from the natural state as even in control tanks strong fluctuations in parameters related to microbial activity and diversity were recorded. Eilers and colleagues (Eilers, *et al*., 2000) found that strong shifts in community composition such as those observed in our mesocosm experiments were due to selective activation of culturable groups of bacterioplankton, and, similar to the first mesocosm experiment (see Chapter 3), they observed an increase in the abundance of bacteria affiliated with *Alteromonas* during confinement without substrate addition, confirming our observations.

Studies in the field were conducted in the Rhone outflow plume, and in a landlocked bay on the coast of Norway. The short residence time of water in the Rhone outflow plume however, did not allow to follow the true response of bacterial populations to the changing environmental conditions in terms of changes in genetic diversity. The situation was further complicated by the significant differences in bacterial abundance in river and seawater, which biased the detection on DGGE of bacterial populations in favour of freshwater populations. Nevertheless, there were interesting differences regarding the fraction of active bacterial

populations as estimated by specific leucine incorporation rates on the one hand and the ratio of DNA and RNA derived DGGE bands on the other hand. Interestingly, both indicated that the activity was distributed amongst a larger fraction in the marine end part of the gradient.

To date there have been only few reports on seasonal changes in marine microbial assemblages. The study of the landlocked bay Hopavågen over a period of 40 month therefore certainly provides new information in showing that there were seasonal and annually recurring distribution of specific phylotypes. A marked change in community composition was concomitant with the beginning of the period of phytoplankton production in spring. At that time α-proteobacterial phylotypes related to *Roseobacter* became predominant in DGGE banding patterns and persisted throughout summer, emphasising that these bacteria are tightly coupled to phytoplankton production as has been suggested previously (González *et al*., 2000). Although, eutrophication affected primary production and algal biomass in 1999 (when nutrient loads were doubled with respect to those in 1998) and some marked peaks in diatom biomass during summer, there was no evidence of marked changes in the seasonal distribution of dominant bacterial phylotypes. However, this observation does not exclude a potential of eutrophication to affect bacterial community composition and dynamics, but may be a consequence of a still too low level of artificial eutrophication.

Eutrophication has also been implied in changing patterns of phytoplankton community composition (Paerl, 1998). While the attempt to experimentally check specificity of bacterial-algal associations in laboratory cultures was not completely successful, the results of the study demonstrate that there is potential for co-occurrence of algae and certain groups of bacteria. Especially, bacteria from the CFB and the α-Proteobacteria formed mini-consortia with the algae that may resemble the functional roles of the constituents in natural ecosystems. Furthermore, the observation that β-proteobacterial phylotypes are propagating in seawater media brought indirect evidence for existence of salt-water tolerant β-Proteobacteria.

**Influence of eutrophication?**

In principle a number of environmental conditions is potentially determining the composition of bacterial assemblages. The factors emphasised above, i.e. grazing, phytoplankton activity and production and composition, are those that have been implicated a pivotal role in shaping bacterial community composition in the present study, but what about the influence of eutrophication?

A direct influence of nutrient concentrations on physiological state of bacteria may be possible as bacteria differ in their uptake kinetics of inorganic nutrients. Furthermore, gross differences in the quality and availability of nitrogen in either inorganic or and organic form might potentially favour growth of adapted bacterial populations. In the mesocosms, however, it seemed that increases in nutrient concentrations only had limited effect on the composition of the community, although some predominant phylotypes in mesocosms (i.e. the dominant bands), were similar in controls and nutrient-enriched mesocosms. Eutrophication mainly affected the speed and magnitude of changes in biomass and thymidine incorporation (compare Lebaron, *et al*., 2001). Divergence between control and nutrient-enriched mesocosms was more profound at the end of the experiment when many other processes had had the chance to modify the composition of the bacterial community.

In the landlocked bay experiment an influence on bacterial diversity could neither be seen. However, this does not exclude the possibility that eutrophication affected some community members. On the one hand such populations might have been below the detection threshold of the DGGE method, on the other hand similar bands between years might represent physiologically distinct populations with regards to nutrient acquisition. Additionally, the level of nutrient addition may still have been to low in order to induce changes in food web structure sufficient to affect bacterial community dynamics and composition.

**Methodology applied and its constraints**

Molecular biological techniques have provided new insights in aquatic microbial ecology. They have helped substantially to change our view of the organisation and complexity of natural microbial assemblages. All molecular biological techniques have their strong and weak points, and therefore not a single technique is ideal for every purpose. Cloning and sequencing of complete 16S rRNA genes certainly allows for phylogenetic inferences with a superior confidence than is offered by partial 16S rRNA genes, such as those recovered from DGGE bands. However, DGGE allows processing and analysis of a greater number of samples than the cloning approach and hence, for good reason, was the preferred method in the work described here.

While 16S rRNA based techniques are still (and will be for some time) widely used tools for analysing microbial community composition, or fluctuation of composition, they do not yield information about the function of individual community members. Combination of molecular biological methods with microsensor measurements have resolved some of these problems in studies of biofilms, aggregates and sediments, by making it possible to sample the chemical microenvironment of the bacterial populations, and thus allowing to study fluxes and quantify substrate turnover *in situ* (Amann & Kühl, 1998; Santegoeds *et al*., 1998; Schramm *et al*., 1998). Unfortunately, no comparable tool is available currently for studying pelagic bacterial populations. Non-destructive sampling of the presumably gel-like matrix of the ocean (Azam, 1998) is not possible. Substrate addition experiments for probing the physiological status of pelagic bacterial communities rely on incubation for periods of a few hours and may fail to provide information on the populations predominating in situ, due to growth of opportunistic bacterial populations (Eilers, *et al*., 2000; Sherr *et al*., 1999). Combination of microautoradiography with FISH is one possibility to study uptake of defined substrates by natural prokaryotic populations that yields new information on potential activities of microbes (Cottrell & Kirchman, 2000; Lee *et al*., 1999; Ouverney & Fuhrman, 1999), yet the method does not discriminate between mere uptake and ability to oxidise a compound.

Another new technique holding promise for linking activity and identity of microbial populations is stable isotope labelling. Boschker and colleagues used stable isotope probing to label microbial populations involved in acetate and methane metabolism in estuary sediments. Subsequently the isotopic composition of lipid biomarkers was compared to known phospholipid fatty acid (PLFA) profiles as a means of identification of microbial populations (Boschker *et al*., 1998). A limitation for reliable identification may reside in a relatively small PLFA database, and the possibility that populations lack diagnostic biomarkers molecules.

A variation of stable isotope probing allows identification of microorganisms involved in processing of specific substrates on the basis of $^{13}$C-enriched genomic DNA (Radajewski *et al*., 2000). Radajewski and colleagues applied this technique to study methanol-consuming methylotroph populations in forest soil microcosms. DNA of populations assimilated $^{13}$C-labelled methanol became relatively heavy and could be separated by density gradient centrifugation from the $^{12}$C-DNA of microorganisms that had not incorporated the heavy carbon. Analysis of diversity in the heavy DNA-fraction literally identifies organisms that are processing the added substrate. These may also be involved in substrate turnover in situ, but the importance of the identified organisms in the environment should subsequently be verified by hybridisation of RNA with taxon-specific oligonucleotide probes. A drawback of the method is that it requires relatively long incubation times. Furthermore, dilution of substrates and/or crossfeeding of metabolites between different populations may render the isotopic enrichment of DNA ineffective (Radajewski, *et al*., 2000).

Application of environmental genomics as well as proteomics are exciting new possibilities to learn more about uncultured microbial taxa, and begin to make their mark in marine microbial ecology (DeLong, 2001). More genome sequences have become available

recently, and those of strains of the same species may differ markedly in gene content (Boucher *et al*., 2001). That adds to the concerns about a priori linking of 16S phylotype to phenotype and suggests a severe limit of 16S rRNA as a marker in ecological studies to discriminate between potentially phenotypic diverse members of clades such as the *Roseobacter* group.

Thus, special emphasis should therefore be put on the functional aspects of bacterial populations in future studies. This can, for instance, be achieved by targeting of functional genes which are associated with biogeochemical cycling of specific compounds. Sequencing of the so-called 'metagenome' and transcriptome of environmental samples may be an additional possibility to identify functional roles of microbes in the environment. Recently, Beja and colleagues identified by cloning and sequencing of genomic environmental DNA that uncultivated bacteria from the SAR86 group contain genes encoding bacteriorhodopsin, so far only been known to be expressed by halophilic archaea. Subsequently, they were able to show the presence of rhodopsin in membrane preparations from native marine picoplankton and also found evidence for widespread distribution of related proteorhodopsin genes in the surface ocean (Beja *et al*., 2001). Such genome sequencing studies may help to elucidate metabolic features of as yet uncultivated groups of bacterioplankton and might prove helpful in formulation of more promising culture conditions for retrieval of these groups as cellular clones.

On the other hand, the technological advance especially in genomic sequencing technology has been immense over the past few years, and only few research centres can keep up with the costs imposed by instrumentation and consumables required for genomic studies. Furthermore, it is debatable whether the possibilities for obtaining new cultures representative of dominant bacterioplankton have really been exhaustively exploited. A more extensive and maybe automated approach to finding more suitable culturing conditions for as yet uncultivated bacterioplankton might also have potential to contribute to progress in the field. At present and probably for some time in the future, isolated strains will be required for an understanding of the biochemical pathways that drive the biogeochemical cycling. Suggestions that microbial ecology is a field that no longer requires input from cultivation attempts but that can be brought forward simply by sequencing (of 16S rRNA genes) (Pace, 1996) are therefore clearly inappropriate.

16S rRNA based molecular ecological techniques have given microbial ecology a new direction and have yielded invaluable information on the composition and dynamics of microbial communities. Further progress in the field will rely on a combination of methods, such as the cloning techniques with stable isotope probing, or the use of functional genes as molecular markers. Genomic studies will probably provide more exciting findings in the future, but culturing of environmentally important bacteria will be as important for furthering our understanding of how bacterial populations are involved in biogeochemical cycling.

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# Chapter 2

# Denaturing Gradient Gel Electrophoresis in Marine Microbial Ecology

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## Introduction

During the past decade the approach of microbial community composition analysis has changed considerably. Classical techniques such as cultivation and microscopic identification are not sufficient to assess the diversity of bacteria in natural samples. On the one hand, lack of conspicuous morphology and small cell size do not allow microscopic identification of the majority of naturally occurring bacteria. On the other hand, media used for the cultivation of microbial strains are selective and hence give a biased view of the community composition. Furthermore, the isolation of the vast majority of naturally occurring bacteria in pure culture is hindered by our lack of knowledge of the specific culture conditions they need and by the potential for synergy between different organisms. Comparisons of culturable and total microscopic cell counts from diverse habitats have demonstrated the inadequacy of the culture-dependent approach to analyse microbial community composition (summarised in Amann et al., 1995). Therefore, other tools are required to supplement the conventional microbiological techniques. The introduction of molecular techniques in microbial ecology including those that use the gene sequences of the small subunit ribosomal RNA as a molecular marker for identification of microorganisms has changed our perception of the diversity of microbial communities. The genes encoding small subunit ribosomal RNAs reflect the evolutionary relationship of microorganisms (Woese, 1987) and the sequences of these genes allow to group and identify microorganisms. Despite some uncertainties about the phylogeny inferred from rRNA (e.g. the rooting of the different domains) which have emerged as a result of whole-genome sequencing studies and the use of alternative molecular markers (see e.g., Pennisi, 1998; Doolittle and Logsdon, 1998), the 16S rRNA approach remains the standard marker (see Ludwig and Schleifer, 1999). Giovannoni and co-workers (1990) for instance used a cultivation-independent approach consisting of PCR amplification, cloning and sequencing of 16S rRNA gene fragments to characterise the composition of Sargasso Sea bacterioplankton. The sequences obtained represented unknown 16S rRNA genes of heretofore uncultivated bacteria, and confirmed the limitations of cultivation-dependent approaches. Similar differences between culture-dependent and molecular approaches were observed by Ward and colleagues for a hot spring cyanobacterial mat community (Ward et al., 1990) and have been reported from microbial ecology studies repeatedly (for a review see Muyzer, 1998).

To get a better insight into the temporal dynamics or spatial variation of microbial communities, microbial ecosystems need to be studied over longer periods of time (e.g., days to years) or samples from many different locations have to be analysed. Although successful, the application of cloning and sequencing of 16S rRNA genes is too laborious and time consuming to analyse a large number of samples, even with the recent progress in sequencing technology. Genetic fingerprinting techniques, however, are excellently suited for comparison of large numbers of samples. Genetic fingerprinting of microbial communities provides banding patterns or profiles that reflect the genetic diversity of the community. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified gene fragments is one of the genetic fingerprinting techniques used in microbial ecology (Muyzer, 2000). In DGGE similar-sized DNA fragments are separated in a gradient of DNA denaturants according to differences in sequence. A variant of DGGE, temperature gradient gel electrophoresis (TGGE) makes use of a temperature gradient to separate gene fragments. DGGE is relatively easy to perform and is especially suited for the analysis of multiple samples. Since its introduction into microbial ecology by Muyzer et al. (1993) it has been adapted in many laboratories as a convenient tool for assessment of microbial diversity in natural samples. A general overview of PCR-DGGE fingerprinting of microbial communities is shown in Figure 1.

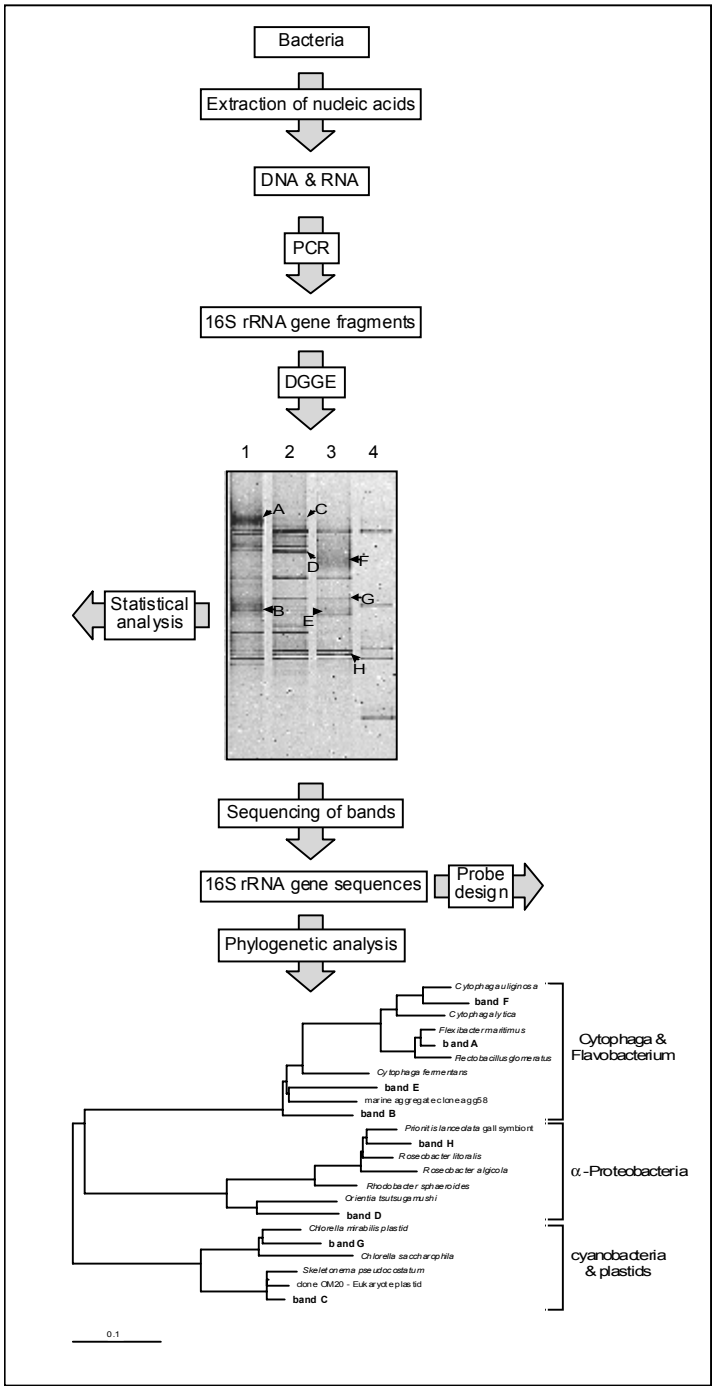



**Figure 1**

Flow diagram of PCR-DGGE analysis of microbial communities. The different steps are discussed in detail in this chapter. Briefly, bacteria are collected on filters, their nucleic acids are extracted and used as template in the PCR. The mixture of PCR products is analysed by DGGE. Community profiles can be further analysed with statistical methods, such as UPGMA and MDS (see Figure 3 for an example). To identify the community members, bands are excised from the denaturing gradient gels, re-amplified and sequenced. The sequence data are used for phylogenetic analysis, or can be used for the design of specific probes to detect bacterial cells in situ (see chapter by Amann). The gel shows temporal shifts in the bacterial diversity of mesocosm samples which are reflected in different community profiles. The time interval between the samplings were: 2 days between samples run on lane 1 and 2, and 3 days between samples of lane 2 and 3 (total time between sample 1 to 3: 5 days). Lane M shows a marker composed of PCR-products from 5 different DNAs (see section on DGGE standards). Sequences determined from the DGGE bands are shown in bold-type in the tree. The phylogenetic tree has been created with the special parsimony tool implemented in the software program ARB (Ludwig et al., 1998, Strunk and Ludwig, 1998), which allows the reliable positioning of partial sequence data in a tree derived from complete sequences, without affecting the topology of the tree.

## Principle of DGGE separation

Amplification of DNA extracted from mixed microbial communities with primers specific for 16S rRNA gene fragments of bacteria result in mixtures of PCR products. Because these products all have the same size, they can not be separated from each other by agarose gel electrophoresis. However, sequence variations between different bacterial rRNAs bring about different melting properties of these DNA molecules, and separation can be achieved in polyacrylamide gels containing a gradient of DNA denaturants, such as a mixture of urea and formamide. PCR products enter the gel as double-stranded molecules; as they proceed through the gel, the denaturing conditions gradually become stronger. PCR products with different sequences therefore start melting at different positions (i.e. at different denaturant concentrations) in the gel. Melting proceeds in so-called 'melting domains'. Once a domain with the lowest melting temperature reaches its melting temperature at a particular position in the denaturant gradient, a transition from a double stranded to a partially melted molecule occurs. The protruding single strands practically cause a halt of the molecule at that position. To prevent the complete dissociation of the two DNA strands, a 40-nucleotide GC-rich sequence ('GC-clamp') is attached at the 5'-end of one of the PCR primers.

## Applications of PCR-DGGE in marine microbial ecology

PCR-DGGE fingerprinting is a tool for monitoring variations in microbial genetic diversity, providing a minimum estimate of the richness of predominant community members. Furthermore, DGGE facilitates the identification of individual populations by hybridisation analysis of patterns with specific probes, or by sequence analysis of individual bands. PCR-DGGE has been used to investigate the diversity of microbial communities, to determine the spatial and temporal variability of bacterial populations, and to monitor community behaviour after natural or induced environmental perturbations. It has been used to study communities in various habitats, such as soil, sediments, water column, hydrothermal vents, microbial mats, mesocosms, or sewage treatment plants. Here we will only give some examples of the application of PCR-DGGE in marine ecosystems. For a more comprehensive overview of the use of PCR-DGGE in microbial ecology the reader is referred to Muyzer (1998, 1999) and Muyzer and Smalla (1998).

### DGGE to study spatial and temporal variability of bacterial populations

The distribution of microbial populations in the marine water column depends on numerous factors and variables. Especially in stratified systems exhibiting strong physicochemical gradients, DGGE fingerprinting can reveal a concomitant stratification of resident microbial assemblages. Teske et al. (1996) used PCR-DGGE to study the distribution of sulphate-reducing bacteria (SRB) in a stratified Danish fjord. PCR-DGGE combined with hybridisation analysis showed that the presence of SRB increased at and below the chemocline. Most-probable number (MPN) counts of SRB were done in parallel and showed a similar trend for the distribution of SRB. Interestingly, DGGE patterns of PCR-products obtained from cDNA after reverse transcription of RNA, representing the active populations, were different from those obtained after amplification of genomic DNA. Despite the agreement between MPN and DGGE, the hybridisation of DGGE patterns with oligonucleotide probes and sequencing analysis of DGGE bands revealed that the SRB enriched in the MPN-tubes had a different phylogenetic affiliation than the SRB detected in the natural samples. The finding that SRB obtained from the MPN cultures belonged to the genera *Desulfovibrio*, *Desulfobulbus*, and *Desulfobacter*, but those in the DGGE patterns of natural samples represented an independent

lineage of the δ-Proteobacteria, verified the potential disagreement between culture-dependent and molecular methods due to selection of culturable types of SRB.

The potential of PCR-DGGE for the analysis of large sets of samples was recognised by Ferrari and Hollibaugh (1999). They processed 100 samples from different stations in the Arctic Ocean to analyse the spatial variation in the diversity of bacterioplankton assemblages. DGGE fingerprints of the samples were subjected to image analysis and the spatial variation of the bacterioplankton assemblage was inferred by regression analysis of the similarity of densitometric curves derived from the DGGE patterns. The resulting dendrogram separated all DGGE patterns into five major clusters with minimally 80% similarity. While clustering of some samples corresponded to samples taken in a specific region of the Arctic Ocean, there was no correlation of geography and clustering of other samples. The authors noted that clustering of the majority of samples rather seemed to reflect different phases of the cruise and might therefore be confounded with temporal variation over the 44 day period of the cruise (Ferrari and Hollibaugh, 1999).

The bacterioplankton assemblages of two estuaries in California, San Francisco Bay and Tomales Bay, differing markedly in a number of physical and biological factors, had been shown to differ in metabolic properties. The analysis of samples from both estuaries by PCR-DGGE supported the hypothesis that metabolic differences were reflected in a different bacterioplankton composition (Murray et al., 1996). Yet, a few bands were common in all samples, and a number of bands were detectable at different times in both estuaries, raising the question as to what extent factors, such as the relative activity of the detected populations or metabolic plasticity, might influence the differences in metabolic profiles (Murray et al., 1996).

In another study, Murray and colleagues (1998) addressed spatial as well as temporal variations in bacterial community composition in the waters around Anvers Island (Antarctica). No obvious variation was detected between samples taken within one month from different points in an area of about three square nautical miles (3 and 50 m depth). However, samples retrieved from several depths up to 1,200 m on two occasions within seven weeks showed variations in DGGE-patterns especially at depths of 500 and 1,200 m indicating compositional changes of the bacterial community. The authors argued that due to the low bacterial activity (estimated by leucine incorporation) advective mixing processes rather than bacterial growth might have caused most of the variation. Seasonal variation in bacterial community composition of the surface waters was inferred from changes in DGGE-fingerprints over a period of almost 9 months at one station. Interestingly, the number of phylotypes decreased during the transition from spring to summer and increased from summer to autumn.

Riemann et al. (1999) used PCR-DGGE to map the genetic diversity of bacterioplankton in the surface-, mid- and deep water of the Arabian Sea during two consecutive monsoon periods and concluded that there was a high horizontal homogeneity of the microbial assemblages. Moreover, the dominant bands in DGGE profiles of the bacterial communities sampled eleven months apart, were remarkably similar, suggesting that if there was a seasonal variation in the bacterioplankton assemblage, it might be a predictable one. Predominant phylotypes were identified by cloning and sequencing of DGGE bands and were members of groups common in oceanic waters, e.g. members of the SAR11-cluster and the cyanobacteria. However, it was remarkable that none of the bands corresponded to γ-Proteobacteria or to members of the *Cytophaga-Flavobacterium-Bacteroides* phylum (CFB), and that 16S rRNA gene fragments similar to those of magnetotactic bacteria were retrieved.

West and Scanlan (1999) investigated the genetic structure of *Prochlorococcus* communities by molecular methods in two depth profiles from the surface to around 100 m

water depth in the Eastern North Atlantic, to assess the distribution of high-light (HL) and low-light (LL) adapted populations. Cloning and sequencing, as well as hybridisation with HL and LL specific gene probes of DNA amplified from different depths were performed. PCR products amplified with a cyanobacteria specific primer (Nübel et al., 1997) and a *Prochlorococcus* specific primer were separated on DGGE. All three methods indicated a niche-partitioning of *Prochlorococcus* genotypes HL and LL in the water column and provide a genetic support for flow cytometric observations of dim and bright *Prochlorococcus* populations.

**DGGE to monitor population shifts after environmental perturbation**

As pointed out above, PCR-DGGE analyses can be performed with DNA as well as with RNA. While DNA-derived PCR amplified 16S rRNA gene fragments are related to the presence of different bacterial populations, analyses of rRNA-derived PCR products can give an indication of which bacterial populations contribute to the RNA pool. As the cellular concentration of ribosomal RNA is related to the (recent) activity of cells it helps in surveying changes in the activity of bacterial populations. An example of potential differences between DNA- and RNA-derived DGGE fingerprints is shown in Figure 2. Similarly, the analysis of the genetic diversity and expression of functional genes can be performed using either DNA or mRNA. Here, PCR-DGGE analysis of DNA-derived PCR products show the genetic diversity (presence) of certain functional genes, while PCR-products obtained after DNase digest and reverse transcription of mRNA show the diversity of expressed genes (Wawer et al., 1997)

Rossello-Mora et al. (1999) investigated the response of the microbial community of marine sediments to amendment with cyanobacterial biomass under anaerobic conditions. Fluorescence in situ hybridisation (FISH), DGGE of PCR products obtained from DNA as well as from cDNA after reverse transcription of RNA, and sequencing of 16S rDNA PCR products were used to assess changes in the microbial community composition. Concomitant changes in the activity of the community were followed by measurements of carbon mineralisation, sulphate reduction, and ammonium production rates. Addition of cyanobacterial biomass resulted in marked changes in the composition. Dominant bands from RNA-derived banding patterns were affiliated with members of the CFB. FISH with probes specific for these CFB-populations showed that, although sulphate reduction was the main mineralisation process, members of the CFB, but not SRB showed the highest increase in abundance as detected by FISH. The authors concluded that these CFB played an important role in the anaerobic decomposition of complex organic matter and suggested that CFB might be responsible for hydrolysis of macromolecules and fermentation.

Mesocosm experiments were performed by Lebaron et al. (2001) and Schäfer et al. (2001) to study changes in the activity and diversity of bacterial assemblages from the Mediterranean Sea after addition of nutrients. Fluctuations in activity were recorded in parallel to variation in community composition, which was assessed by PCR-DGGE. Different phases were observed during the incubation corresponding to an initial increase of bacterial numbers, followed by an increase of heterotrophic protozoa cropping the bacterial production and a new increase of bacterial production after the peak in grazing activity (growth-, grazing, and post-grazing phase, respectively). These phases were reflected by concomitant changes in DGGE-fingerprints of the bacterial assemblage. Both, nutrient addition as well as grazing of protozoa seemed to effect changes in the bacterial genetic diversity. Multidimensional scaling analysis of DGGE patterns showed that differences in the development of the bacterial communities occurred between nutrient-enriched and control mesocosms and indicated that duplicate treatments were reproducible. Sequencing of DGGE

bands was used to identify several microbial populations. DGGE-bands of some populations disappeared from the DGGE patterns during the grazing phase, while members of the *Cytophaga-Flavobacterium-Bacteroides* phylum and *Ruegeria*-like bacteria became especially important after the peak in grazing activity. The latter populations also dominated the RNA-derived DGGE-fingerprints and hence it was suggested that these populations escaped the grazing pressure and were important contributors to bacterial production and activity in the post-grazing phase of the experiment.

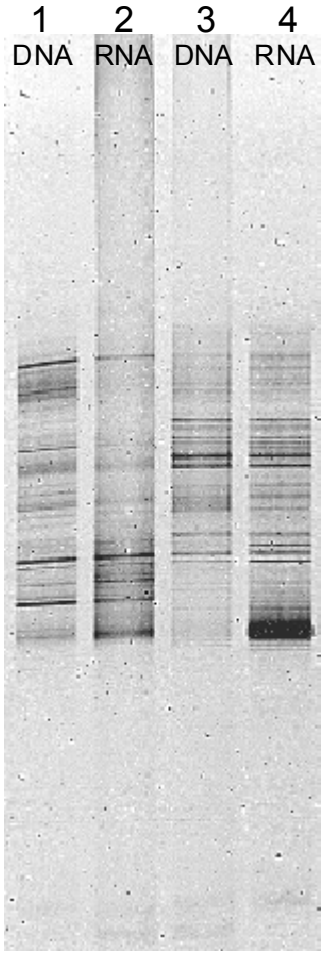



**Figure 2.**
DGGE patterns of PCR-amplified 16S rRNA gene fragments obtained from bacterial DNA, indicating the presence of bacteria, or from bacterial ribosomal RNA, showing the most active populations within the assemblage. Water samples from two different locations were analysed: Sample A (lanes 1 and 2) is from surface water taken off the coast of Banyuls-sur-mer (France) in May 1997; Sample B (lanes 3 and 4) is from coastal water taken near the mouth of the river Rhône (France) in April 1998. Fingerprints of the natural bacterial assemblages were obtained from DNA (lanes 1 and 3) or after DNA digestion and reverse transcription of RNA (lanes 2 and 4). Note the differences in DNA and RNA derived patterns in the upper part of lane 1 and 2, where some of the bands seen in the DNA derived pattern are weaker or not represented at all in the RNA derived patterns. This indicates that corresponding populations have a relatively low RNA content, and hence are probably less active than others. The marked difference in intensity of the band at the bottom of the profiles shown in lanes 3 and 4 indicates that a population contributes relatively less to the DNA pool, but relatively much to the RNA pool indicator of recent cellular activity. Hence, cells of this populations probably have a high rRNA content, which might indicate that they are active.

In a multidisciplinary approach McCaig et al. (1999) studied the impact of fish farming in cages on N-cycling and community structure of the underlying sediment. Organic carbon content and ammonium concentration of the sediment measured along a transect from the fish cage to a distance of 40 m from the cage. Carbon content and ammonium concentration were much higher under the fish cage than at the other sampling sites along the transect. Furthermore, nitrification and denitrification were strongly inhibited beneath the fish cage. DGGE was used to profile the diversity of beta-ammonium oxidising bacteria (β-AOB), along a transect from underneath the fish cages to 40 m from the cage, after PCR amplification of 16S rRNA gene fragments with primers specific for β-AOB (Kowalchuk et al., 1997). DGGE-profiles were blotted onto a membrane and hybridised with probes specific for subclusters of the β-AOB; to reveal the identity of β-AOB populations. DGGE-profiles of highly polluted sediments under the fish cage showed two prominent bands that were only faintly visible in DGGE profiles of samples from 20 and 40 m from the cage.

## DGGE to study archaea, eukaryotes, and viruses

The examples described above show that PCR-DGGE is increasingly being used in ecological contexts to get a better understanding of the factors regulating bacterial community composition. Bacterial community composition, however, is not only influenced by physicochemical factors, but may be effected by biotic factors such as primary production, grazing (Jürgens et al., 1999; Pernthaler et al., 1997; Schäfer et al., 2000; Suzuki, 1999; van Hannen et al., 1999a) and viral infection (Fuhrman 1999; van Hannen et al., 1999b). Therefore, it is of great interest to extend the molecular approach to study the genetic diversity of primary producers, grazers and viruses. Furthermore, *Archaea* might also play an important role in the marine system (DeLong 1992). Other than analysing bacterial communities by PCR-DGGE, specific PCR-DGGE assays allow to study eukaryotic microorganisms (van Hannen et al., 1998) and *Archaea*. Furthermore, PCR-DGGE applications for viral communities have been introduced (Scanlan and Wilson, 1999, Short and Suttle, 1999). These assays will be valuable for a more integrated study of the microbial communities.

Reports of unusual crenarchaeal 16S rRNA gene sequences retrieved from marine waters (DeLong, 1992, Fuhrman et al., 1992) have triggered further studies into the importance of *Archaea* in the marine environment. It has been shown by oligonucleotide hybridisation that archaeal rRNA may amount to a high percentage of total rRNA extracted from concentrated marine picoplankton (Murray et al., 1998), suggesting that *Archaea* have been largely ignored as potentially important members of marine microbial communities. Different PCR-DGGE assays have been used for the analysis of archaeal 16S rRNA gene fragments (Casamayor et al., 2000; Øvreås et al., 1997; Rölleke et al., 1998; Vetriani et al., 1999). PCR-DGGE analyses of microbial communities from meromictic lakes by Øvreås and colleagues (1997) and by Casamayor et al. (2000) have also reported crenarchaeal sequence types related to those reported from coastal surface waters (DeLong, 1992), marine archaeoplankton communities however, have not yet been analysed by PCR-DGGE.

DGGE-fingerprinting is not limited to the use of 16S rRNA gene fragments, but can also be performed with functional genes (e.g. Fesefeldt and Gliesche, 1997; Wawer and Muyzer, 1995). Using primers targeting the gene encoding [NiFe] hydrogenase of *Desulfovibrio* species Wawer et al. (1997) were able to analyse expression of these genes in complex microbial communities by DGGE.

## Eukaryotic microbial communities

Although small eukaryotes such as protozoa can be identified much easier by microscopy than bacteria because of their discriminative morphological features, their identification is time-consuming and can often be done by experts only. Identification of eukaryotic microbes by molecular methods can be achieved with primers developed by van Hannen et al. (1998), which amplify a 210 bp 18S rRNA gene fragment that can be separated by DGGE. Due to the limited size of the fragment, sequencing of gel bands may make identification possible at the phylum level only (van Hannen et al., 1998). However, the authors demonstrate, that using species specific oligonucleotide probes for hybridisation analysis of DGGE gels, identification at the species level is possible. Van Hannen et al. (1998) used the eukaryote-specific PCR-DGGE assay to compare the diversity of five Dutch lakes of a lagoon system. Analysis of DGGE-fingerprints and environmental variables of these lakes by UPGMA resulted in similar clustering of lakes and the respective genetic fingerprints of their eukaryotic diversity.

**Viral communities**

Viruses are a numerically important part of the microbial food web. Virus-induced mortality may contribute significantly to overall mortality of natural microbial populations (Suttle, 1994) and has therefore the potential to affect the diversity of bacterioplankton and primary producers (van Hannen et al., 1999b). Unfortunately, viruses do not contain ribosomal RNA, hence, for the study of natural virus communities other molecular markers are needed. Essential virus genes present in a large number of certain virus groups are candidates for molecular assays, and applications of PCR-DGGE assays to study diversity in virus communities have been described by Short and Suttle (1999) and Scanlan and Wilson (1999).

The assay by Short and Suttle is based on primers that specifically amplify gene fragments of the DNA polymerase genes (*pol*) of viruses infecting microalgae (*Phycodnaviridae*) (Chen and Suttle, 1995; Short and Suttle, 1999). Short and Suttle suggested that similarly, the development of primers specific for DNA polymerases of cyanophages and bacteriophages should be possible. Separation of virus PCR-amplified *pol*-gene fragments derived from cultures was accomplished by DGGE. A preliminary analysis of natural marine *Phycodnaviridae* communities demonstrated that they may undergo seasonal changes, and that community composition may vary over relatively small spatial scales (Short and Suttle, 1999).

Scanlan and Wilson (1999) have applied a cyanophage-specific PCR-DGGE assay based on primers described by Fuller et al. (1998) which target genes encoding capsid-proteins. DGGE was used to separate fragments of PCR-amplified virus-capsid protein genes obtained from a variety of virus strains. Application of cyanophage DGGE fingerprinting holds promise for gaining more insight into the influence of cyanophages on the diversity of cyanobacterial populations. Furthermore, they might facilitate elucidation of environmental stimuli, e.g. phosphate-limitation (P-limitation) that might decide whether a lytic, pseudolysogenic or lysogenic infection is established by cyanophages. Wilson et al. (1998) induced P-limitation in a mesocosm experiment by addition of excess nitrogen (N) at an N:P ratio of 75:1. Nutrient addition lead to a large *Synechococcus* bloom, which was shown to become P-limited by using a immunological marker for P-limitation in *Synechococcus*. Virus concentrations increased at the same time, just before the bloom collapsed after re-addition of P. The authors suggested that the P status of the *Synechococcus* population had important implications for the result of the host/phage interaction. They pointed out, however, that there was not sufficient data and too much variation in virus abundance estimates to establish whether or not the collapse of the *Synechococcus* bloom was due to lysogenic or pseudolysogenic host/phage interaction.

In the above section we have shown that PCR-DGGE is a useful tool for the analysis of complex microbial communities, and is contributing to change our perception of how the diversity of microbial communities is controlled. The examples discussed above emphasise that DGGE is not a stand-alone technique, but should rather be used in combination with other measurements related to the physicochemical and biotic factors that regulate the activity and diversity of microbial communities.

The different steps of the PCR-DGGE approach are described below. The method can also be used to study microbial communities from other ecosystems, yet some modifications in sample preparation and processing may be necessary.

# Practical aspects of PCR-DGGE

### Equipment

Apart from standard laboratory facilities, such as a refrigerator, freezer (-20°C and –80°C), and fume hood, the basic equipment needed for the analysis of bacterial communities by PCR-DGGE consists of:

- bench-top centrifuge with refrigeration for 15 ml tubes
- bench-top centrifuge for 0.5 and 1.5 ml tubes
- thermocycler
- water bath
- ice-bath
- vortex
- power supplies for electrophoresis systems
- agarose gel electrophoresis system
- denaturing gradient gel electrophoresis system, e.g., DCode system (Bio-Rad 170-9080; includes gradient former) or PhorU system (Ingeny)
- UV-transilluminator and Polaroid camera or a fluorescence imaging system, e.g., Fluor-S Multiimager (Bio-Rad 170-7701, Macintosh version)

### Optional equipment

- peristaltic pump (Model EP-1, Bio-Rad 731-8142) and gradient former (Model 385, Bio-Rad 165-2000) for casting gradient gels
- automatic DNA sequencer, e.g. ABI 310 Genetic Analyzer (Perkin Elmer)

## Sampling of bacteria

About $10^9$ bacterial cells are collected from water samples by gentle filtration on hydrophilic Durapore filters (Millipore, GVWP04700, polyvinylidene fluoride membrane, 0.22 µm, 47 mm diameter). After filtration, the filters are transferred into cryovials and immediately frozen in liquid nitrogen or otherwise. Upon return to the laboratory the filters can be stored at –80°C.

## Extraction of nucleic acids

Several protocols have been published in the literature for extraction of nucleic acids from marine microorganisms. We routinely use the protocol described here; it represents a combined extraction of DNA and RNA from bacteria collected on Millipore GVWP filters. The protocol is also suited for extraction of DNA and RNA from Gram-positive bacteria (Rossello-Mora et al., 1999), and Archaea (Casamayor et al., 2000).

### Reagents and disposables

- safety glasses, lab coat and gloves
- 15 ml sterile disposable centrifuge tubes
- 1.5 ml sterile disposable microcentrifuge tubes
- tube racks

- AE-buffer (20 mM sodium acetate, 1 mM EDTA, pH 5.5)
- phenol:chloroform:isoamylalcohol (25:24:1), pH 7 (PCI)
- 10% (w/v) sodium dodecyl sulphate (SDS)
- 3 M sodium acetate, pH 5.2
- 100% (v/v) ethanol
- 70% (v/v) ethanol
- water (Sigma W4502) or TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0)

**Method**

1. Pre-warm the PCI to 60°C in a water bath. Pre-cool the AE-buffer on ice. Set the temperature of the centrifuge at 4°C.
2. Transfer the filter from the cryovial to a 15 ml tube with clean forceps. Keep the tube on ice.
3. Rinse the filter with 2 ml of ice-cold AE-buffer. Vortex briefly, and put the tube back on ice.
4. Add 5 ml of hot PCI and 150 µl of SDS. Incubate for 5 minutes at 60°C. Vortex briefly every minute.
5. Cool the tube on ice.
6. Centrifuge at 4,000*g* for 5 minutes at 4°C to separate the aqueous and organic phase.
7. Transfer the aqueous phase to a clean 15 ml tube, and add 1/10 of a volume (ca. 200 µl) of sodium acetate.
8. Add 5 ml of PCI. Vortex briefly, and separate the two phases as described in step 6.
9. If necessary, repeat steps 7 and 8, until no proteineous material is visible at the interface between the aqueous and organic phase.
10. Transfer the aqueous phase to a clean 15 ml tube. Add 2.5 volumes of ice-cold 100% ethanol, and incubate for at least 3 hours at -20°C.
11. Centrifuge at 4,000*g* for 60 minutes at 4°C to pellet the precipitated nucleic acids. Remove the supernatant by gentle aspiration (use a fresh sterile pipette tip for each sample).
12. Rinse the DNA pellet with 1 ml of ice-cold 70% ethanol.
13. Centrifuge at 4,000*g* for 5 minutes at 4°C. Remove the supernatant.
14. Dry the pellet under vacuum.
15. Add 100 µl water (or TE-buffer) and incubate overnight at 4°C.
16. Redissolve the pellet by gentle pipetting. Aliquot the nucleic acid solution into sterile reaction tubes and store at –80°C. Inspect 5 µl of the solution by electrophoresis in a 1% (w/v) agarose gel together with an appropriate molecular weight standard, e.g., lambda *Hin*d III-digest (Stratagene 201109).

**Notes**

- All steps involving the handling of phenol or phenol-containing solutions should be performed in a fume hood, wearing safety glasses, gloves, and a lab coat.

# Purification of RNA

### Reagents and disposables

- 10x DNase buffer (400 mM Tris, 60 mM $MgCl_2$, pH 7.5)
- DNase I, RNase-free (10 u/µl, Pharmacia 27-0514-01)
- 3M sodium acetate pH 5.2
- phenol:chloroform:isoamylalcohol (25:24:1), pH 7 (PCI)
- 1.5 ml sterile disposable microcentrifuge tubes
- tube racks
- safety glasses, lab coat and gloves

### Method

1. Add the following reagents to a 1.5 ml tube:
   7 µl water
   2 µl 10x DNase buffer
   15 µl nucleic acid extract
2. Add 1 µl DNase, and incubate for 30 minutes at 37°C in a water bath or thermocycler.
3. Add 280 µl water and 30 µl sodium acetate. Vortex briefly. Remove the DNase enzyme by extracting with 300 µl of PCI.
4. Centrifuge at 4,000*g* for 15 minutes at 4°C to separate the aqueous and organic phase.
5. Transfer the aqueous phase to a clean 1.5 ml tube
6. Add 2.5 volumes 100% ethanol and incubate 2 hours at -20°C.
7. Centrifuge at 4,000*g* for 1 hour at 4°C.
8. Remove the supernatant by gentle aspiration. Rinse the RNA pellet with ice-cold 70% ethanol.
9. Dry the pellet under vacuum and redissolve in 15 µl water.
10. Use the solution directly for first strand synthesis and store the remainder at –80°C.

### Notes

- All steps involving the handling of phenol or phenol-containing solutions should be performed in a fume hood, wearing safety glasses, gloves and a lab coat.
- To avoid contamination with RNase enzymes all solutions should be prepared with RNase free water and chemicals should be molecular biology reagent grade ("RNase – none detected"). All steps should be done wearing gloves, additionally all lab bench surfaces and pipettes should be wiped with 70% ethanol.
- Using the above pipetting scheme the maximal amount of nucleic acids solution that can be used for preparation of RNA is 17 µl. In our experience 10-15 µl of nucleic acid extract usually is a sufficient amount to perform RT-PCR analyses. In case of nucleic acid extracts that are low in RNA content the above scheme must be scaled up (e.g. to 100 µl volumes or more).

## Preparation of 1st strand cDNA

### Reagents and disposables

- sterile PCR reaction tubes (0.2 or 0.5 ml)
- random hexanucleotides (1:50 [v/v] dilution of Boehringer 1277081 in water (Sigma W4502) ca. 40 ng/µl)
- dNTP solution (2.5 mM each dNTP; prepared from ultrapure dNTP-set [100 mM each dNTP; Pharmacia 27-2035-01] and PCR water [Sigma W4502]
- 5x RT buffer (250 mM Tris-HCl, pH 8.3 at 25°C, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol)
- MMLV-reverse-transcriptase (200 u/µl; Promega M1701)

### Method

1. Add 1 µl hexanucleotides (ca. 40 ng/µl) to 10 µl of RNA preparation in a microcentrifuge tube. Incubate for 10 minutes at 70°C in a water bath or thermocycler to denature the RNA. Cool on ice.
2. Centrifuge briefly to collect the liquid in the bottom of the tube and add 4 µl of 5x RT-buffer and 4 µl of dNTP-solution. Incubate for 2 minutes at 37°C.
3. Add 1 µl (200 u) of MMLV reverse transcriptase and incubate for 1 hour at 37°C
4. Incubate the tube at 95°C for 5 minutes. Cool on ice.
5. Use 1 to 5 µl as of the solution as template in the PCR (or make dilutions if too much PCR product is obtained). Store the remainder at –20°C.

### Notes:

- We do not determine the RNA concentration, but by following this protocol 10 µl of RNA preparation is usually sufficient for obtaining PCR products.
- It is important to save some of the RNA preparation for doing PCR controls to check the completeness of the DNA digestion.
- According to the above scheme, 1 µl of cDNA preparation corresponds to an initial input of 0.5 µl nucleic acid extract containing DNA and RNA. This will be important to consider for PCR controls.

## PCR and RT-PCR for DGGE

### Reagents and disposables

- sterile PCR reaction tubes
- 10x PCR reaction buffer (100 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$, 500 mM KCl)
- Taq DNA-polymerase (5 u/µl; Pharmacia 27-0799-02)
- dNTP solution (2.5 mM each dNTP; prepared as above)
- primers (50 µM) (see Table 1)
- water (Sigma W4502)

## Method

Make a 10-fold serial dilution of the extracted DNA and test several dilutions in the PCR to find the best concentration of template DNA that gives a good specific product. Note that at very high dilutions, some less abundant templates in the mixture may be lost and hence not be amplified sufficiently to form a band in the DGGE analysis.

1. Prepare a master-mix for the PCR reactions by adding for each reaction the following reagents:

| | |
|---|---|
| 10x PCR reaction buffer | 10 µl |
| dNTPs (2.5 mM each) | 10 µl |
| forward primer (50 µM) | 1 µl |
| reverse primer (50 µM) | 1 µl |
| water | 76.8 µl |
| Taq DNA polymerase (5 u/µl) | 0.2 µl |

2. Vortex, and spin briefly to collect the reagents in the bottom of the tube. Dispense 99 µl to each of the PCR-reaction tubes.
3. Add 1 µl of DNA (or cDNA) solution to each tube. Close the lid, and mark the tubes. Note which template and dilution was added to which tube.
4. Spin briefly to collect all the fluid in the bottom of the tube. If your thermocycler is not equipped with a heated bonnet, overlay the reactions with a drop of mineral oil (e.g. Sigma M8862)
5. Insert the tubes into the thermocycler and start the appropriate PCR-program (see Table 2).
6. When the run has completed store the reactions at 4°C or at –20°C until further use
7. Inspect 5 µl of the PCR products by electrophoresis on 1.5% (w/v) agarose gels together with appropriate size and mass standards.

## Notes

- Always perform the following PCR controls: (i) without addition of DNA template (negative control), and (ii) with addition of known DNA (positive control).
- We prefer to use single PCR-tubes with attached lids instead of strips of tubes with strips of caps. To avoid the risk of cross-contamination of samples during pipetting of template DNAs, the tubes are opened one at a time to add template DNA and immediately closed before proceeding to the next tube.
- For RT-PCR, an extra control reaction is necessary to check for the completeness of DNA digestion. A volume of RNA preparation corresponding to the amount of cDNA used as template in the PCR is added to a separate PCR reaction. If this control gives a product, then the DNA digestion was not complete, and traces of genomic DNA were present.

- Addition of bovine serum albumin (BSA; Sigma B6917) to the PCR reactions may help to overcome the effects of inhibitory substances present in the nucleic acid preparations, such as humic acids. Use a final concentration of up to 3 mg/ml in PCR reactions.

### Quantification of PCR products

An important point to consider in comparative DGGE analysis of multiple samples is that similar amounts of PCR products should be loaded onto the denaturing gels. Faint bands visible in one lane, might not be detectable in another lane. If the total amount of PCR product applied in these two lanes differ markedly, a fair comparison between samples is not possible. We use a mass standard (Precision Molecular Mass Standard; Bio-Rad 170-8207) for quantification of PCR products by agarose gel electrophoresis. At least three lanes are loaded with undiluted, 2-fold diluted and 4-fold diluted mass standard. Using the software MULTIANALYST (Bio-Rad) the pixel density values of marker bands with varying DNA-amounts (100, 70, 50, 35, 25, 20, 17.5, 12.5, 10 and 5 ng) can be used for regression analysis. Pixel densities are also read of unknown PCR-product bands, as well as of the background staining which is determined at least at one representative point of the gel. The regression curve and formula derived from the marker fragments is then used to estimate the DNA-concentration of the unknown samples.

### Notes

- Samples that are out of the range of the standard curve should be avoided. Instead, these samples should be analysed again with more or less sample applied
- Avoid oversaturation of portions of the gel-pictures (by overexposure)
- Thoroughly rinse the ethidium bromide stained gels in Milli-Q water to increase the signal-to-noise ratio.

### Troubleshooting – PCR

| | |
|---|---|
| No PCR product | If no product was obtained for positive control either, probably due to accidental omission of a vital ingredient, try again.<br>If positive control worked well, this may be related to presence of substances inhibiting Taq polymerase in nucleic acid extracts |
| Poor product yield | Use more template, or pool replicate reactions and concentrate by precipitation |
| Too much product or by-products | Use less template |
| Product in negative control | Contamination of solutions and/or plastic ware with DNA. UV-resistant plastics can be decontaminated by exposure to UV-light source (e.g. cross linker, clean bench). Use fresh aliquots of reagents, if problems persist. Prepare new stock solutions with nucleic acid-free water |
| Product from RNA preparation | DNA digestion was not complete. Repeat DNA digestion with more DNase and/or longer incubation time and/or less nucleic acid extract |
| Inhibition of Taq polymerase | Substances, such as humic acids are co-extracted with the nucleic acids and inhibit Taq-polymerase To overcome this problem (i) further purify the nucleic acid extracts or (ii) dilute nucleic acids which will also dilute inhibitory compounds and/or (iii) add BSA to PCR reactions (see notes above) |

Table 1. Target sites, sequences and specificity of primers targeting small subunit ribosomal RNA used for DGGE analysis

| Primer[a] | Target site[b] | Sequence (5' to 3') | Specificity[c] | Reference |
|---|---|---|---|---|
| 341F-GC[d] | 341-357 | CCT ACG GGA GGC AGC AG | *Bacteria* | Muyzer et al., 1993 |
| 907R[e] | 907-926 | CCG TCA ATT CMT TTG AGT TT | *Bacteria* | Muyzer et al., 1998 |
| 518R[f] | 518-534 | ATT ACC GCG GCT GCT GG | universal | Muyzer et al., 1993 |
| 1055F | 1055-1070 | ATG GCT GTC GTC AGC T | *Bacteria* | Ferris et al., 1996 |
| 1392R-GC | 1392-1406 | ACG GGC GGT GTG TAC | universal | Ferris et al., 1996 |
| 968F-GC | 968-984 | AAC GCG AAG AAC CTT AC | *Bacteria* | Nübel et al., 1996 |
| 1330R | 1330-1346 | TAG CGA TTC CGA CTT CA | *Bacteria* | Nübel et al., 1996 |
| 1385R | 1385-1401 | CGG TGT GTA CAA GAC CC | *Bacteria* | Nübel et al., 1996 |
| PRBA338F-GC | 338-357 | ACT CCT ACG GGA GGC AGC AG | *Bacteria* | Øvreås et al., 1997 |
| PARCH340FGC | 340-357 | CCC TAC GGG CYG CAS CAG | *Archaea* | Øvreås et al., 1997 |
| PARCH519R | 519-533 | TTA CCG CGG CKG CTG | *Archaea* | Øvreås et al., 1997 |
| ARC344 | 344-363 | ACG GGG AGC AGC AGG CGC GA | *Archaea* | Rölleke et al., 1998 |
| 907R-GC | 906-928 | CCC CGT CAA TTC ATT TGA GTT TT[g] | universal | Rölleke et al., 1998 |
| 344F-GC[h] | 344-363 | ACG GGG CGC AGC AGG CGC GA | *Archaea* | Vetriani et al., 1999 |
| ARC344F-GC | 344-363 | ACG GGG YGC AGC AGG CGC GA | *Archaea* | Casamayor et al., 2000 |
| 915R | 915-934 | GTG CTC CCC CGC CAA TTC CT | *Archaea* | Casamayor et al., 2000 |
| CYA359F-GC | 359-378 | GGG GAA TYT TCC GCA ATG GG | Cyanobacteria | Nübel et al., 1997 |
| CYA781R[h] | 781-805 | GAC TAC WGG GGT ATC TAA TCC CWT T | Cyanobacteria | Nübel et al., 1997 |
| PRO1017R | 1017-1035 | TCC CGA AGG CAC CCT CWA A | most *Prochlorococcus* | West and Scanlan, 1999 |
| CTO189F-GC[i] | 190-208 | GGA GRA AAG YAG GGG ATC G | β-AOB | Kowalchuk et al., 1997 |
| CTO654R | 633-654 | CTA GCY TTG TAG TTT CAA ACG C | β-AOB | Kowalchuk et al., 1997 |
| Eukaryotic forward | 1427-1453 | TCT GTG ATG CCC TTA GAT GTT CTG GG | Eukarya | van Hannen et al., 1998 |
| Eukaryotic reverse | 1616-1637 | GCG GTG TGT ACA AAG GGC AGG G | Eukarya | van Hannen et al., 1998 |

[a] F (forward) and R (reverse) indicate the orientation of the primers in relation to the rRNA sequence. Primers with identical designation but different sequence are reported individually.

[b] *E. coli* numbering according to Brosius et al. (1981) for primer sequences targeting 16S rRNA of Prokaryotes, and according to the numbering of *Saccharomyces cerevisiae* 18S rRNA for eukaryote specific primers (van Hannen et al., 1998)

[c] The specificity denoted is quoted from the original citation and may in some cases be applicable to the PCR assay (Table 2) with the appropriate second primer and cycling conditions. Furthermore, due to the increasing amount of sequence information in public databases, the stated specificities of primer sequences may not hold true upon re-assessment against the most recent database. It is therefore strongly recommended to re-assess the applicability of a certain primer sequence for specific applications.

[d] GC denotes that a GC-rich sequence (GC-clamp) is attached to the 5'-end of a PCR primer. For primers 341F-GC, ARC344F-GC used by Casamayor et al. (2000) and CYA359F-GC it comprises 40 nucleotides (sequence: 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3'). See other references for particular GC-clamp sequence used. For a compilation of different GC-clamps and calculated free energies of hairpin and primer-dimer formation see Muyzer et al. (1998).

[e] Component primers of 907R, i.e. 907RC (5'- CCG TCA ATT CCT TTG AGT TT-3') and 907RA (5'-CCG TCA ATT CAT TTG AGT TT-3') can be used individually [Schäfer, 2001). Component primer 907RC may exhibit a one nucleotide mismatch with certain bacterial 16S rRNA genes (e.g. members of γ-proteobacterial lineages *Acinetobacter*, *Pseudomonas*, *Beggiatoa*, *Enterobacteriacaea*, *Shewanella*, *Pseudoalteromonas*, *Alteromonas*, and many ε-proteobacterial 16S rRNA genes, as well as in members of *Gemmata*, *Verrucomicrobiales*, *Chlamydia*. This mismatch is avoided in most cases with primer 907RA.

[f] Primer 518R can be used in combination with 341F-GC for amplification of bacterial 16S rRNA genes (Muyzer et al., 1993). Furthermore, Vetriani et al. (1999) used it in combination with primer 344F-GC for amplification of archaeal 16S rRNA gene fragments.

[g] The primers has up to 4 mismatches with most *Archaea*.

[h] The reverse primer CYA781R used in PCR is an equimolar mixture of CYA781R(a) (5'-GAC TAC TGG GGT ATC TAA TCC CAT T-3') and CYA781R(b) (5'-GAC TAC AGG GGT ATC TAA TCC CTT T-3') (Nübel et al., 1997)

[i] See Kowalchuk et al. (1997) for details of primer synthesis. Note that in original paper component primers CTO189A-GC and CTO189B-GC had a typing error in the GC-clamp sequence (first 3 bases missing). The GC-clamp sequence should be the same as for component primer CTO189C-GC (Kowalchuk, personal communication). The same primers have been used by McCaig et al. (1999) with designations CTO178fGC and CTO637r.

Table 2. PCR-cycling conditions for various PCR-DGGE assays

| Primer combination | Specificity | PCR program[a] | Reference |
|---|---|---|---|
| 341F-GC/907R, and 341F-GC/518R | *Bacteria* | 5 min at 94°C, followed by 20 cycles of 1 min at 94°C, 1 min at 65°..55°C (touchdown –0.5°C cycle$^{-1}$), and 3 min at 72°C, followed by 15 cycles of 1 min at 94°C, 1 min at 55°C and 3 min at 72°C, followed by 7 min final extension at 72° | Muyzer et al., 1993, 1996 |
| 338F-GC/518R | *Bacteria* | 2 min 92°C, followed by 30 cycles of 1 min at 92°C, 30 s at 55°C, 1 min at 72°C, followed by 6 min final extension at 72°C | Øvreås et al., 1997 |
| 1055F/1392R-GC | *Bacteria* | 5 min 94°C, followed by 11 cycles 1 min at 94°C, 1 min at 53..43 (touchdown –1°C cycle$^{-1}$), and 3 min at 72°C, followed by 20 cycles of 1 min at 94°C, 1 min at 43°C, and 3 min at 72°C, final extension phase of 10 min | Ferris et al., 1996 |
| 968F-GC/1330R, and 968F-GC/1385R | *Bacteria* | 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C, followed by 5 min final extension at 72°C | Nübel et al., 1996 |
| CYA359F-GC/CYA781R | Cyanobacteria & chloroplasts | 5 min at 94°C, followed by 35 cycles of 1 min at 94°, 1 min at 60°C, and 1 min at 72° | Nübel et al., 1997 |
| CYA359F-GC/PRO1017R | most Prochlorococcus | 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C | West and Scanlan, 1999 |
| CTO189F-GC/CTO654R | β-ammonium oxidising bacteria | 1 min at 93°C, followed by 35 cycles of 30 s at 92°C, 60 s at 57°C, and 45 s at 68°C (+1 s cycle$^{-1}$), followed by 5 min final extension at 68°C | Kowalchuk et al., 1997 |
| PARCH340F-GC/ PARCH519R[b] | *Archaea* | 2 min at 92°C, followed by 30 cycles of 1 min at 92°C, 30 s at 53.5°C, 1 min at 72°C, followed by 6 min final extension at 72°C | Øvreås et al., 1997 |

| | | | |
|---|---|---|---|
| ARC344/907R-GC | *Archaea* | 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 65°C, 3 min at 72°C, then 1 µl of the PCR reaction is transferred to fresh reaction mixture and another 30 cycles are done[c]. | Rölleke et al., 1998 |
| ARC344F-GC/915R | *Archaea* | 5 min at 94°C, followed by 20 cycles of 1 min at 94°C, 1 min 71-61°C (touchdown: -0.5°C cycle$^{-1}$), and 3 min at 72°C, followed by 15 cycles of 1 min at 94°C, 1 min at 61°C, and 3 min at 72°C, followed by 10 min final extension at 72°C | Casamayor et al., 2000 |
| 344FGC/518R[b] | *Archaea* | 40 cycles of 30 s at 94°, 30 s at 48°C, and 30 s at 72°C | Vetriani et al., 1999 |
| EukaryoticF/EukaryoticR | *Eukarya* | 5 min at 94°C, followed by 25 cycles of 0.5 min at 94°C, 1 min at 52°C, and 1.5 min at 68°C, followed by 10 min final extension at 68°C | van Hannen et al., 1998 |

[a] potential hot start phases at 80°C have been omitted; touchdown denotes that annealing temperature is decreased in consecutive cycles

[b] second PCR of a nested PCR approach, see details in original study

[c] the two-step PCR was performed since only very limited amounts of original sample (Medieval wall paintings) were at disposition. Sometimes PCR products of the first round were gel-purified first, to avoid carry-over of by-products.

## Casting and running of denaturing gradient gels

To achieve the maximum resolution in DGGE patterns of unknown samples it is recommended to find out the best gradient conditions. This requires running perpendicular denaturing gels with the unknown sample to define the range of denaturant concentrations that allow the best separation possible. In our experience gradients ranging from ca. 10-20% to 70-80% denaturant concentration (urea and formamide; UF) result in a good separation of fragments obtained by PCR with primers 341f-GC and 907R and provide a security margin for fragments melting at unexpectedly high denaturant concentrations at the same time. It is strongly recommended to run time-travel experiments when starting DGGE analysis to check for optimal separation. For a description of casting and running perpendicular denaturing gradient gels and time travel experiments the reader is referred to Muyzer et al.(1996), or to the manual coming with the DGGE system.

### Preparation of reagents

*Formamide (de-ionised)*

Add 10 g of mixed bed resin (e.g. Sigma M8032) to 100 ml formamide in an Erlenmeyer and stir for 30-60 minutes. Decant or filter (e.g., Schleicher & Schuell folded filter 595 1/2, order no. 311647) the formamide to separate it from the resin beads. Store the de-ionised formamide in volumes of 35 ml at –20°C for the preparation of the 80% denaturing gel solution.

*Acrylamide/bis-acrylamide stock solution (37.5:1; 40% w/v)*

Acrylamide is a powerful neurotoxin and should be handled with extreme care. To avoid exposure to acrylamide dust, we recommend to buy ready-made acrylamide/bis-acrylamide stock solution (e.g., Bio-Rad 161-0149). If you prepare the solution from acrylamide powder, wear safety glasses, gloves, a lab coat, and a dust mask.

| | |
|---|---|
| Acrylamide | 38.93 g |
| Bis-acrylamide | 1.07 g |
| water | to 100 ml |

Filter the solution (e.g., through a e.g., Schleicher & Schuell folded filter 595 1/2) and store at 4°C in a dark bottle.

*DGGE acrylamide/bis-acrylamide solutions*

Prepare 6% (w/v) acrylamide/bis-acrylamide gradient solutions according to the amounts of reagents shown below. We use 6% acrylamide/bis-acrylamide solutions for PCR products obtained with primers 341F-GC / 907R as well as for CYA359F-GC / CYA781R (see Table 1). Higher concentrations of acrylamide/bis-acrylamide may be necessary for DGGE analysis of other 16S rRNA gene fragments (check original citations for details). The use of an 80% denaturing gel solution as high denaturing solution is usually sufficient for preparation of denaturing gradient gels. However, care has to be taken that bands are not lost from the analysis due to migration to higher denaturant concentrations than 80%. In this case a 100% denaturant acrylamide solution should be used.

| | 0% UF | 80% UF |
|---|---|---|
| acrylamide/bis-acrylamide (37.5:1, 40% w/v) | 15 ml | 15 ml |
| 50x TAE (pH 8.3) | 2 ml | 2 ml |
| Urea (U) | - | 33.6 g |
| formamide (deionised) (F) | - | 32 ml |
| Milli-Q water | to 100 ml | to 100 ml |

Filter through 0.45 µm filter or a Schleicher & Schuell folded filter 595 1/2 (Schleicher & Schuell 311647). Degas the acrylamide/bis-acrylamide solution for 15 minutes under vacuum, and store at 4°C in a dark bottle.

*10% Ammonium persulphate solution*

| | |
|---|---|
| ammonium persulphate | 1.0 g |
| water | to 10 ml |

Aliquot into single use portions and store at –20°C.

*TEMED*

TEMED is bought as a ready-to-use solution (e.g., from Fluka or Bio-Rad)

*50x TAE buffer (2M Tris, 2M Acetic acid, 50 mM EDTA; pH 8.3)*

| | |
|---|---|
| Tris base | 242.0 g |
| 0.5 M EDTA, pH 8.0 | 100.0 ml |
| Acetic acid (glacial) | 57.1 ml |
| water | to 1000 ml |

Autoclave the buffer solution for 20 minutes and store at room temperature.

*1X TAE running buffer*

Dilute 1 volume of 50x TAE-buffer with 49 volumes of Milli-Q water.

*Gradient-dye-solution*

To visually inspect proper gradient formation after casting, a dye solution can be added to the high denaturant solution.

| | |
|---|---|
| Bromophenolblue (0.5% w/v final) | 0.05 g |
| Xylenecyanole (0.5% w/v final) | 0.05 g |
| 1X TAE buffer | 10.0 ml |

*10x gel loading solution*

| | |
|---|---|
| Glycerol (100% v/v) | 5.0 ml |
| Bromophenolblue (0.25% w/v final) | 0.025 g |
| Xylenecyanole (0.25% w/v final) | 0.025 g |
| water | 5.0 ml |

Mix and store in small aliquots at room temperature.

**Assembly and casting of parallel denaturing gradient gels**

1. Clean the glass plates and spacers with water and soap. Rinse them with de-mineralised water.
2. Wipe the glass plates first with 70% ethanol and then with acetone. Use a dust-free cloth (e.g. Kimwipes). Do not wipe any plastics (e.g., spacers, combs, etc) with acetone.
3. Wipe the spacers (1 mm thickness) with ethanol and let them dry, then sparingly smear grease (High vacuum grease; Dow Corning, Auburn, MI, USA) along one of the long edges, such that around 2 mm are covered with a thin grease-film on each face of the spacer.
4. Place the large glass plate on a clean surface, and put the spacers onto the left and right margins, such that the greased edges face the outside edge of the glass plate.
5. Put the small glass plate on top of the spacers, to form a ‘sandwich’.
6. Align the spacers and the glass plates in such a way that they are flush at the bottom of the sandwich (this can be done on an even surface, or in the aligning slot of the casting stand).
7. Attach the clamps to the sandwich, tighten the clamp screws (finger-tight) and put the sandwich in the casting stand, fix in casting slot by turning the levers.
8. Before proceeding to the next step make sure that the device used for casting the gradient gel is ready installed and you are familiar with the procedure described below. The work has to proceed quickly otherwise you run the risk that gel solutions will polymerise before casting is finished. A gradient former comes with the DCode system from Bio-Rad. We use a combination of peristaltic pump (Model EP-1, Bio-Rad 731-8142) and gradient former (Model 385, Bio-Rad 165-2000) to cast gels. For detailed instruction on set-up and operation of these refer to the technical instructions of Bio-Rad. Connect the tubing of the pump with the outflow chamber of the gradient chamber. Attach an injection needle to the Luer-lock of the outlet tubing of the pump and insert the needle between the glass plates in the middle of the gel sandwich.
9. Prepare the high and low denaturant solutions for the gradient as required in disposable plastic tubes. Using 1 mm thick spacers, 12 ml each are recommended. The gradient gel will finally be overlaid with a 0% denaturant acrylamide solution (prepare 5 ml), as otherwise the presence of the denaturants hinders the formation of good sample wells.
10. Add ammonium persulphate and TEMED to the gradient solutions. We add 60 µl of ammonium persulphate and 8 µl of TEMED to each solution, directly pipett these into the solutions. Close tubes and mix thoroughly by inverting several times.
11. To inspect the gradient, add 120 µl of gradient-dye-solution to the high denaturant solution. Close the tube and mix by inverting several times.

12. Close the connection pipe between the two chambers of the gradient chamber, make sure pump is not running. Pour the high denaturant gel solution into the outflow chamber of the gradient chamber.
13. To remove air bubbles in the connection pipe, slowly open the pipe by turning the lever aside until the air has been expelled from the pipe and a drop of high denaturant gel solution is visible on the bottom of the second chamber. Then close connection pipe and pipette back all high denaturant gel solution back to the outflow chamber using a clean pipette tip.
14. Carefully add the low denaturant gel solution into the second chamber.
15. Turn on the magnetic stirrer at 250 rpm, then turn on the pump and slowly open the connection pipe, such that no extra high denaturant gel solution enters the second chamber. Cast the gel with ca. 4 ml/min. The last ca. 1 ml of the gradient gel will not be mixed properly (due to remains of high denaturant gel solution in the connecting pipe of the gradient chamber), hence avoid delivery of that last bit of gradient solution, as it will disturb the top of the gradient gel.
16. Remove needle from gel sandwich. Rinse gradient chamber and pump tubing with water to remove residual gel solution.
17. Clean a comb (1 mm thick) with ethanol and let it dry.
18. Add 25 µl of ammonium persulphate and 5 µl of TEMED to 5 ml of 0% denaturant gel solution. Close the tube and mix.
19. Carefully overlay the gradient gel with about half of the 0% denaturant gel solution using a 1000 µl pipette.
20. Insert the comb at an angle to avoid the formation of air-bubbles. Completely fill the gel sandwich with the remainder of the 0% denaturant gel solution.
21. Let the solution polymerise for at least 2 hours.

**Notes**

- There are two different kinds of spacers, those for casting perpendicular gels, which have grooves on the inside side of the gel sandwich and normal spacers without grooves. For normal parallel DGGE analysis we recommend to use spacers without grooves, as they are easier to grease and better safeguard against current leakage, which may cause considerable smiling of the gel.
- To facilitate mixing of the gradient solutions, the gradient chamber should be placed on a magnetic stirrer and small magnetic stirring bars should be added to each chamber. Furthermore the gradient chamber should be placed at a higher level than the peristaltic pump to improve gradient formation.
- To avoid too fast polymerisation of acrylamide solutions these can be kept on ice before casting. This may be especially important when temperatures in the lab rise to high levels during summer months.
- Polymerised gels can be stored overnight. To avoid drying out, the comb is removed, the wells filled with water and the gel covered with cellophane.

### Troubleshooting – DGGE gel casting

| | |
|---|---|
| Acrylamide solution leaves outflow chamber of gradient former, but gel solution from second chamber is not flowing into and mixing with solution in outflow chamber | Mostly due to air bubbles in the connection pipe between the two chambers of the gradient former |
| During casting, bubbles appear in the tubing between pump and needle | Mostly due to defect tubing, replace tubing |
| During casting many air bubbles are formed at the needle that get into the gradient gel between the glass plates | Mostly due to old needle, replace needle |
| Acrylamide solution leaks at the bottom of the glass plate assembly | Spacers and glass plates are not flush at the bottom |
| Gel does not polymerise | No or not enough TEMED and / or APS added |
| Gel polymerises, but remains viscous | Make sure that proper mixture or percentage of acrylamide and bis-acrylamide was used |

## Running parallel DGGE gels

### Sample preparation

After quantification of PCR-products, the samples are mixed with 10x gel loading solution. The total volume of PCR product to be loaded may vary between 15 µl and 60 µl. Using a 1 mm-thick, 16-well comb of the DCode system it is possible to load volumes up to approximately 70 µl. Apply the sample very slowly into the sample wells to avoid mixing with the electrophoresis buffer and to avoid overflow into the neighbouring wells. As bands tend to focus in DGGE there is no need to apply equal sample volumes. Alternatively, PCR products of low concentration can be precipitated and be re-dissolved in smaller volumes.

### DGGE standards

Sometimes more than 20 samples are to be compared on denaturing gradient gels, exceeding the number of wells formed with the 20-well comb, hence multiple gels are needed. Denaturing gradient gels, however, show some degree of gel-to-gel variation, caused by differences in the gradient. Therefore, it is recommended to use a marker standard on the gels that is composed of fragments halting at a range of denaturant concentrations. Such a marker facilitates gel-to-gel comparison, the marker we use routinely (see lane M of gel shown in Figure 1) is composed of five different fragments derived from chloroplast 16S rDNA of a *Nitzschia* sp., two cloned 16S rRNA genes obtained from an earlier study (Schäfer et al., 2000), and two commercially available genomic DNAs of *Clostridium perfringens* (Sigma D1760) and *Micrococcus lysodeikticus* (Sigma D8259). Bands halting at high denaturant concentrations can be used to normalise the migration length of individual bands which may vary between gels (Ferrari and Hollibaugh, 1999).

### DNA amounts to load

There is no general rule for the amount of PCR product to apply on denaturing gels, since the optimal amount will depend on the number of different sequence types (i.e. bands) in a given sample, as well as the relative contribution of the bands to the total PCR-product (i.e. the relative intensity of particular bands). For instance, loading 500 ng of PCR product in a

situation where the fluorescence intensity is equally distributed over 5 different bands will be different from samples showing 30 to 40 different bands. The absolute DNA amount to be loaded should therefore be tested empirically. Typically, we use about 500 ng (range 300-600 ng) PCR product for the analysis of marine bacterioplankton communities obtained by amplification with primers 341F-GC/907R. In our experience, using around 1 µg often leads to high background and overloading of individual dominant bands, potentially obscuring some other, fainter bands. Ferrari and Hollibaugh (1999) reported that about 1 µg was the optimal amount to use, however they often observed multiple bands for single organism templates, which may have been an effect of overloading DGGE lanes rather than representing sequence heterogeneity of multiple *rrn* operons. For analysis of oxygenic phototrophic communities Nübel and colleagues (1999) used around 500 ng.

1. Fill the electrophoresis tank with approximately 7 litres of 1x TAE buffer.
2. Insert the core. Two gels can be attached to the core and run at the same time. If only one gel is run, attach a buffer dam at the other site. The buffer dam can be made of a large and small glass plate without spacers and held together by the sandwich clamps.
3. Carefully place the lid (i.e. the electrophoresis/temperature control module) on the electrophoresis tank. Take care that the end of the stirring bar comes in its proper position.
4. Switch on the DCode system with the on/off button on the electrophoresis / temperature control module. Switch on the buffer recirculation pump and the heating element. Set the temperature to 60°C and set the ramp rate to 0. The buffer will reach the temperature in about 1 hour.
5. Prepare the samples by adding between 5 and 10 µl of gel loading solution. Mix the samples and spin briefly.
6. Remove the comb slowly, when the acrylamide gel is polymerised.
7. When the buffer has reached 60°C, switch off the electrophoresis unit, wait at least 15 seconds before removing the lid, and place the lid on the lid stand.
8. Take out the core, pre-wet the sandwich clamps of the gel sandwich and attach to core. Replace the core in the electrophoresis tank.
9. Take a 25 ml syringe, pull up the buffer from the electrophoresis tank, attach a needle and rinse the wells of the denaturing gel to remove traces of non-polymerised acrylamide.
10. Load the samples into the wells with a 50 µl Hamilton syringe. Thoroughly rinse the syringe with electrophoresis buffer between the different samples.
11. Put the lid on the buffer tank turn on electrophoresis unit and connect the cords to the power supply.
12. Run the gel at constant voltage of 10 volts for 10 minutes while the temperature is brought back to 60°C.
13. If some samples could not be loaded completely due to a too large sample volume, switch off power unit and electrophoresis unit, and repeat steps 10 and 11.
14. Run the gel at a constant voltage of 100 V for 18 hours. The amperage should be around 35 mA.
15. After 18 hours, turn off the power supply and the electrophoresis unit. Wait at least 15 sec before removing the lid. Take out the core and detach the gel sandwich.
16. Remove carefully one of the glass plates as well as the spacers. Stain the gel on the glass plate with ethidium bromide solution for 30 minutes (ethidium bromide 0.5 µg/ml in distilled water).

17. Rinse the gel for 20 to 30 minutes in distilled water.
18. Transfer the gel to an UV-transilluminator and photograph with a Polaroid camera or preferably use a gel documentation system equipped with a CCD camera and coupled to a computer (e.g. Fluor-S Multiimager, Bio-Rad). Take several photos of the gel with varying exposure times (optimal, underexposed, overexposed). Underexposed photographs may help to define very intense bands, while overexposed photographs may help to identify additional faint bands.

**Notes**

- Avoid powdered gloves as they may leave a background on the gel.
- DGGE gels can also be stained with Sybr Green (Muyzer et al., 1998) or Gelstar (Moeseneder et al., 1999). Specific filters might be necessary to optimise the acquisition of gel images. Denaturing gels can also be stained with silver. However, this might be disadvantageous for further re-amplification and sequencing of excised bands.
- Gels can be easily transferred into the Fluor-S Multiimager (Bio-Rad) using a large gels-coop (Sigma G7152). Avoid scratches in the scoop as this will show in gel images.
- In most cases, DGGE gels are 1 mm thin and therefore difficult to handle. However, gels can be transferred easily from UV-tables back to glass plates or moved to a blotting device or another UV-table using Whatman filter paper. Cut a piece to match the size of the gel and carefully put it on top of the gel, avoiding bubbles. Carefully lift the filter paper, make sure the gel remains attached, and put down on a glass plate/UV-table/blotting stack. Soak the filter paper completely with water (or buffer when moving to a blotting stack) and the filter paper will come off easily.

# Analysis of DGGE patterns

DGGE patterns from mixed microbial communities may be very complex. Different kinds of information can be extracted from DGGE patterns, i.e. the number, position (absence or presence of particular bands) and relative intensity of bands. Furthermore, the nucleotide sequence of bands can be determined. Information extracted from DGGE-patterns can be subject to numerical analysis to determine the extent of variation between DGGE patterns of different samples and thus help in the interpretation of DGGE analyses. A prerequisite for comparative analysis of DGGE patterns is that similar amounts of PCR-products were applied on the gel. Figure 3 schematically shows the steps in numerical analysis of DGGE patterns.

Deciding which features of gels represent bands and which do not is of pivotal importance. DGGE patterns can be analysed with band-finding algorithms after digitisation of gel photographs. Ferrari and Hollibaugh (1999), however, noted that visual inspection of gel patterns provides the most sensitive way. This agrees with our experience, although subjective assessment can not be ruled out with visual inspection, and analysis may vary between persons. Fragments of the 16S rDNA from different microorganisms may show varying degrees of sharpness as DGGE bands, some may focus very well, whereas others remain somewhat fuzzy. These are probably intrinsic features of the melting behaviour of different nucleic acid sequences. To remain as objective as possible, all features that look like a band should be scored as such. The basic assumption in DGGE analysis is that each band in a DGGE fingerprint corresponds to a unique type of 16S rRNA gene. Yet, there are some circumstances that prompt to think of this in relative terms (see section, Limitations of PCR-DGGE).

**Binary matrices**

A first step in the analysis of DGGE patterns by statistical methods, such as unweighted pair-wise grouping with mathematical averages (UPGMA) and multidimensional scaling (MDS) is to set up a binary matrix that is representative of the bands occurring in a set of DGGE patterns. The presence or absence of DGGE bands in a sample are scored as present (1) or absent (0), relative to the DGGE bands detectable in all samples of a set of DGGE patterns.

**Unweighted pair-wise grouping with mathematical averages (UPGMA)**

UPGMA is a clustering method for binary data whereby pair-wise similarities of DGGE patterns are used to infer a dendrogram that depicts these distances in graphical form. For UPGMA analysis of DGGE patterns a binary matrix is translated into a distance matrix representing the similarities of the DGGE-patterns using a similarity coefficient. Different similarity coefficients have been used by several authors. The Dice coefficient used for cluster analysis of data from restriction fragment length polymorphism (RFLP) of 16S rRNA genes (Heyndrickx et al., 1996) and ribopatterns of bacterial strains (Vachee et al., 1997) is identical to the Sorensen coefficients used by Murray et al. (1998) for calculation of pair-wise similarities and the Nei & Li coefficient used by van Hannen et al. (1998) and Lebaron et al. (1999) for cluster analysis of DGGE patterns. Other authors (Curtis and Craine, 1998; Ferrari and Hollibaugh, 1999; Liu et al., 1997) have used the Jaccard coefficient (Jaccard, 1908) for clustering of fingerprint patterns (T-RFLP and DGGE). This coefficient has also been used in the schematic example depicted in Figure 3. Both, the Jaccard and the Dice coefficients seem to be appropriate since they do not consider double absence of bands in the calculation of the pairwise similarity, and thereby avoid spuriously high similarity values in pairwise similarities of samples (i.e. DGGE patterns of two lanes in a DGGE gel) with high numbers of double-absent bands.

The Jaccard-similarity is calculated according to the formula:

$$S_{Jaccard} = N_{AB} / ( N_A + N_B - N_{AB})$$

Where $N_{AB}$ is the number of bands common in both samples (patterns), $N_A$ and $N_B$ represent the total number of bands in sample A and B, respectively.

The formula for the Dice coefficient as shown in (Heyndrickx et al., 1996) is:

$$S_{Dice} = 2 N_{AB} / ( N_A + N_B)$$

the designations of the terms are the same as given for Jaccard coefficient.

The distance matrix is further analysed by UPGMA (for examples see Lebaron et al., 1999; van Hannen et al., 1998).

**Multidimensional scaling analysis (MDS)**

MDS is a powerful data-reduction technique that may aid in the interpretation of large sets of complex DGGE patterns. Van Hannen et al. (1999a) were the first to use this statistical method in conjunction with DGGE fingerprinting in their study on the influence of predation on the genetic diversity of a microbial community. Schäfer and colleagues (2001) analysed by DGGE the development of Mediterranean bacterioplankton in nutrient-enriched mesocosms. Here, MDS not only served to show deviations between control and treatment mesocosms, but also confirmed the reproducibility of duplicate mesocosms. For MDS analyses the information of the DGGE patterns is again represented as a 0/1 binary matrix, which is used to derive a distance matrix, using the Dice or Jaccard coefficient (the Jaccard coefficient is for instance implemented in the statistics software SYSTAT 7.0).

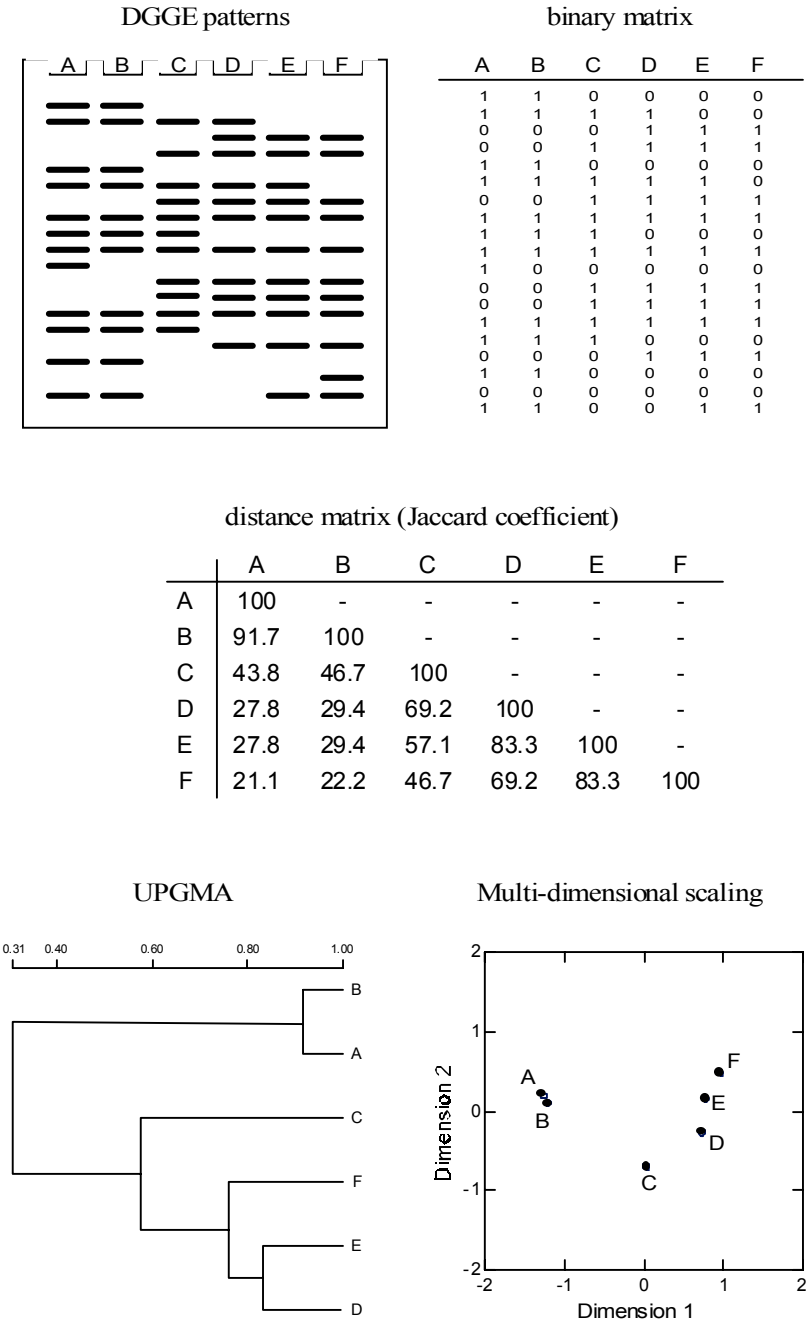



**Figure 3**.

Schematic example of statistical analysis of DGGE patterns. Briefly, the presence (1) and absence (0) of DGGE bands in different samples are scored in a binary matrix. The binary matrix is translated into a distance matrix using a similarity coefficient (e.g. Jaccard coefficient) that is used for UPGMA or MDS.

MDS reduces a complex DGGE pattern to a point in a two-dimensional space (when restricted to two dimensions). When, for instance, the development of a microbial community is studied during time by DGGE, the patterns can be analysed by MDS. Connecting the dots representing consecutive samples by lines, the development of the banding patterns can be visualised (for an example see van Hannen et al., 1999a).

### Densitometric analysis – relative fluorescence of DGGE bands

DGGE data may also be amenable to quantitative analysis. For this, the relative fluorescence (staining intensity) of DGGE bands has to be measured. This can usually be achieved using software such as NIH-image (available at http://rsb.info.nih.gov/nih-image/) by plotting the pixel density along the DGGE profile. This results in a peak pattern of which individual peaks and the baseline have to be defined. Subsequently relative fluorescence values can be obtained for individual bands.

### Diversity indices

DGGE derived values of genetic richness and abundance (defined as relative fluorescence of DGGE bands) can be used to calculate diversity indices. In a study of hyper-saline microbial mat communities Nübel and colleagues compared the diversity of oxygenic phototrophic microorganisms in mat samples from different sites (Nübel et al., 1999). Using a specific PCR (Nübel et al., 1997) they amplified 16S rRNA gene fragments of oxygenic phototrophs and separated them by DGGE. Different samples were compared according to the number of DGGE bands detectable (i.e. genetic richness), and their relative staining intensity (i.e. evenness). Using these PCR-DGGE-defined richness and evenness values, a Shannon-Weaver diversity index could be calculated which was compared to two other cultivation independently derived diversity estimates. It is important to note that the PCR conditions have to be adjusted such that the PCR does not reach the plateau-phase. Furthermore, using bacterial/universal primers with complex communities might not result in valid diversity estimates due to complex DGGE patterns.

## Identification of community members

Apart from facilitating the comparison of larger numbers of samples, DGGE-fingerprinting also makes possible the identification of predominant community members. Two approaches have been applied successfully. The first is hybridisation analysis of blotted denaturing gradient gels with oligonucleotide (e.g., Brinkhoff and Muyzer, 1997, Muyzer et al., 1998) or polynucleotide probes (Heuer et al., 1999). The second is sequencing of excised denaturing bands. The latter approach is, however, more straightforward than hybridisation analysis and also more universal, because only few of the "group-specific" target sites (Snaidr et al., 1997) lie within the fragment of the 16S rRNA encoding gene used for DGGE analysis of mixed bacterial communities.

### Excision of bands and re-amplification

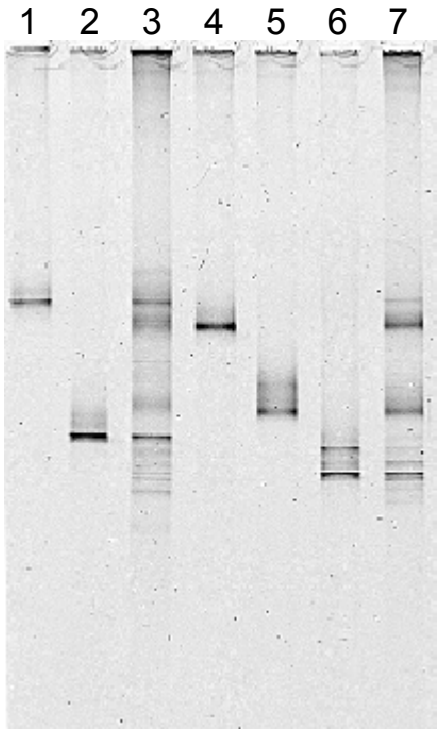
After documentation of the denaturing gel, make a printout of the gel and mark all bands that are to be excised and sequenced. Assign each band a number and label a corresponding number of 0.5 ml reaction tubes, accordingly.

1. Transfer the gel to a UV-table and set the UV-table to "preparative" (or "low") instead of "analytical" (or "high") mode.

2. Wipe a scalpel blade with ethanol and switch on UV-table, cut out band of interest and pick it up with the blade or with forceps.
3. Immediately switch off the UV-source to minimise the damage to the DNA bands in the gel.
4. Transfer the gel piece to the labelled tube.
5. Continue excising bands as described in step 2-4, until all bands have been excised.
6. Rinse the bands by adding 200 µl of nucleic acid free water, spin down contents of tubes and incubate at room temperature for 1-2 hours.
7. Remove the water by gentle aspiration (use a clean sterile tip for each band).
8. Add 25-50 µl of nucleic acid free water, spin down, and incubate at 4°C overnight.
9. Use water from the supernatant as template for re-amplification with the same primers as for the PCR for DGGE, store the remainder at –20 °C
10. Check the PCR product from the re-amplification alongside the original DGGE pattern to make sure it is the proper band and to see if it is a single band (see Figure 4)

**Figure 4.**

DGGE of re-amplified bands that were excised from a denaturing gel for sequence analysis (lanes 1 and 2, and lanes 4 to 6). The re-amps were run side by side with PCR products of the original samples (lanes 3 and 7) to verify that (i) the re-amplified products were single bands, and (ii) correspond to the excised band in the original pattern. Sometimes re-amplified products might consist of more than one band (e.g. lanes 5 and 6). In such cases the band should be excised and re-amplified again. Alternatively, such PCR products can be cloned to isolate the band of interest.




## Notes

- Ethidium bromide is a powerful mutagen. Wear always at least one pair of protective gloves.
- Protect yourself against exposure of UV radiation by wearing a UV-filtering face-shield. Shield your arm wrists by taping the ends of your lab coat sleeves tight around the wrists with tape.
- UV-light will also damage the DNA that you want to re-amplify. Therefore, excision should proceed as quickly as possible and UV-exposure has to be kept as short as possible. This can be achieved by switching off the UV-source as soon as a band has been excised and only turning it on when you are ready for excision of the next band

- Avoid scratching the surface of the UV-table, excise bands by rather pressing scalpel blade carefully through the gel than cutting with it.

## Cycle-sequencing of PCR-products

### Reagents and disposables

- PCR reaction tubes (single tubes not strips)
- ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Perkin Elmer 4303149)
- 5x BDT-buffer (400 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$)
- sequencing primer (5 µM) (primers used for PCR, without GC-clamp)

### Method

After inspection of re-amplified DGGE bands by DGGE the PCR products to be sequenced are purified using the Qiaquick PCR-purification kit (Qiagen 28106) according to the manufacturer's instructions and the concentration of the purified products is estimated as described above.

The pipetting scheme given below is based on the BigDye cycle sequencing ready reaction kit of Perkin Elmer (Perkin Elmer 4303149). It deviates from the recommendations of Perkin Elmer in that just a quarter of the recommended amount of ready reaction mix and extra buffer (BDT-buffer) is used (ready reaction mix includes buffer, dNTPs, fluorescently labelled ddNTPs, Amplitaq).

1. For each reaction prepare in a 0.2 ml PCR tube

| | |
|---|---|
| BDT-5x-buffer | 4 µl |
| Ready reaction mix | 2 µl |
| DNA | ca. 50 ng |
| Primer (5µM) | 0.8 µl |
| Water | to final volume of 20 µl |

   For multiple reactions with the same sequencing primer prepare a master mix consisting of ready reaction mix, 5x BDT-buffer, water and primer, dispense into 0.2 ml tubes and add water and template accordingly. Note that this is the recipe for a 1/4 reaction.
2. Briefly spin to collect the reagents in the bottom of the tube. Perform cycle sequencing: 25 cycles, each consisting of a rapid thermal ramp to 96 °C - hold for 10 sec, rapid thermal ramp to 50°C - hold for 5 sec, rapid thermal ramp to 60 °C – hold for 4 min. After the final cycle include a rapid thermal ramp to 4°C and hold temperature. (it might be necessary to adjust annealing temperature for particular primer sequences).

3. Store the reactions at 4 °C, until purification by isopropanol-precipitation (as recommended by Perkin Elmer)
4. Purify cycle sequencing products (e.g., by isopropanol precipitation, as recommended by Perkin Elmer) and finally resuspend the pellets in 13 µl of Template-suppression-reagent (Perkin Elmer).
5. Denature samples for 3 min at 95 °C in the thermocycler, then place on ice
6. Spin down briefly, transfer to ABI sample tubes and load on sequencer

**Comparative sequence analysis**

Analysis of sequences can be carried out using a number of different software programs and database services on the Internet, such as the Ribosomal Database Project (RDP; Maidak et al., 1999). We routinely use ARB, which is a comprehensive tool for comparative analysis of sequence data (Ludwig et al., 1998; Strunk and Ludwig, 1998).

## Reproducibility and sensitivity of PCR-DGGE

The PCR-DGGE approach to study microbial communities is a highly reproducible and a consistently performing fingerprinting technique (Ferrari and Hollibaugh, 1999, Murray et al., 1996, Muyzer, 1999, Øvreås et al., 1997, Riemann et al., 1999) and it has been suggested that even if biases affect the fingerprints generated by PCR-DGGE these biases are of a constant nature and thus changes in banding patterns do reflect variations in microbial community composition (Øvreås et al., 1997, Riemann et al., 1999).

Different studies have demonstrated the sensitivity of the PCR DGGE approach. Muyzer et al. (1993) found that in a mixture of different template DNAs the detection threshold of dilute templates by PCR-DGGE was around 1% of the total DNA. Similar values have been observed by others. Murray noted that in artificial mixtures template concentrations of 1.6% could be detected easily (Murray et al., 1996). In a study of the bacterioplankton of two Spanish lakes (Lakes Ciso and Vilar), Casamayor and colleagues (2000), could test the sensitivity of the method by comparing the identity of detected bands to counts of microscopically identifiable populations and also detected populations present at a density of around 1% of total cell counts (Casamayor et al., 2000). A new fingerprinting method introduced recently by Liu et al. (1997) into microbial ecology is terminal restriction fragment length polymorphism (T-RFLP). Marsh et al. (1998) analysed the eukaryotic microbial community of activated sludge samples and found that T-RFLP detected about twice as much phylotypes than DGGE. However, neither the primer sequences and specificity were given, nor did they describe their DGGE assay. A comparison of DGGE and T-RFLP was also made by Moeseneder et al., (1999) who analysed complex marine bacterial communities with optimised protocols for both methods. Although, T-RFLP detected a higher number of phylotypes (range 33 to 44, depending on restriction enzyme used) than did DGGE (range 28 to 35) the differences were not large.

# Limitations of the PCR-DGGE approach

As all other molecular biological applications that rely on PCR as an initial step, DGGE fingerprinting is potentially afflicted with PCR-inherent biases. These PCR-biases have been described in detail elsewhere (von Wintzingerode et al., 1997) and are discussed here only

briefly. The same is true for biases afflicted with unequal lysis of bacterial cells and efficiency to obtain a nucleic acid preparation representative of the community composition. Specific limitations of DGGE are that only short sequence fragments can be used (up to ca. 500 bp), thereby limiting the amount of sequence information for subsequent identification by comparative sequence analysis. Furthermore, the resolution of different sequences is not always accomplished and assignment of features as bands or not bands can also be difficult in some cases. Other biases also lead to artefacts with other molecular approaches. Bacteria may harbour more than one copy of the 16S rRNA encoding gene, with heterogeneous sequences, giving rise to more than one band on DGGE (Nübel et al., 1996). Furthermore, dissimilar sequences may co-migrate to the same position in a DGGE gradient (Buchholz Cleven et al., 1997; Fesefeldt and Gliesche, 1997; Kowalchuk et al., 1997; Rossello-Mora et al., 1999), causing a band to be a mixture of more than one sequence and preventing recovery of a clean sequence after reamplification. These problems potentially concern the reliable estimation of the number of different phylotypes (i.e. richness) by all genetic fingerprinting methods. Furthermore, artificial bands may be due to heteroduplex molecules (Ferris and Ward, 1997), which may form between single strands of two similar, but not identical DNA molecules. However, potential extra bands should not interfere with conclusions based on the comparison of patterns from different samples. Murray et al. (1996) noted that heteroduplex formation did not significantly interfere with DGGE-analysis of complex communities.

Molecular approaches to investigate microbial community composition are not free of biases either and attempts to cultivate bacterial strains representative of populations important in natural communities should not be neglected, since such strains have to be investigated to get new insights into the activities of marine microbes. To reduce misinterpretation of results due to biases or limitations of the techniques used it may be of advantage if other molecular, microbiological and geochemical measurements are made at the same time.

## Conclusions

Numerous studies in microbial ecology have used PCR-DGGE fingerprinting for the analysis of microbial community composition up to now. It has been shown by several studies that the approach is reproducible and sensitive. These aspects as well as the straightforwardness of DGGE will probably attract even more scientists to adapt this relatively inexpensive technique as a new tool in their study of microbial ecology in the future. While other new fingerprinting techniques, such as automated T-RFLP might be more sensitive, identification of predominant community members still requires cloning and sequencing of PCR-products. A potential future development in PCR-DGGE fingerprinting might be to use fluorescently labelled PCR primers which might (i) make staining of gels unnecessary and (ii) make possible to add intra-lane standards with a different fluorochrome, facilitating gel-to-gel comparisons.

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# Chapter 3

# Successional changes in the genetic diversity of a marine bacterial assemblage during confinement

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## Abstract

The successional changes in the genetic diversity of Mediterranean bacterioplankton subjected to confinement were studied in an experimental 300 liter seawater enclosure. Five samples were taken at different times and analyzed by PCR-DGGE fingerprinting to monitor changes in the bacterial genetic diversity rapidly. DGGE analysis clearly showed variations between the samples. Three of the five samples, with different DGGE banding patterns, were further analyzed by cloning and sequencing of 16S rRNA genes. Comparative sequence analysis indicated a shift from a mixed bacterial assemblage to a community dominated by bacteria closely affiliated to one single genus, *Alteromonas*. Sequences obtained at the start of the experiment were affiliated to two α–proteobacterial and three γ–proteobacterial lineages known from other studies of marine picoplankton. One sequence was affiliated to the *Verrucomicrobiales*. After 161 h of incubation two sequences represented a γ-proteobacterial lineage also present at 0 h, but the majority of sequences clustered around the sequence of *Alteromonas macleodii*. After 281 h only the dominant *Alteromonas*-like bacteria and bacteria distantly related to *Legionella* were found by cloning and sequencing. Mortality rates of bacteria indicated that grazing was the dominant mortality process when heterotrophic protozoa were abundant. Hence, changes in the genetic diversity of bacteria were partly influenced by differential mortality of bacterial populations during the course of incubation.

## Introduction

A considerable number of studies in marine microbial ecology have made use of enclosures or 'mesocosms', since they allow the control of experimental parameters (Duarte et al. 1997). However, enclosure experiments can introduce biases that stem from the lack of exchange of solutes and organisms with adjacent water masses or from adsorptive wall effects. One of the complications is the apparent increase in the number of bacterial cells that can be cultured on standard media (Ferguson et al. 1984; Lee and Fuhrman, 1991). This phenomenon has been recognized for a long time and represents one of many aspects of the so-called ‘bottle effect’. Ferguson and co-workers (1984) found that the bacterioplankton community changed significantly within 16 h of confinement from a community dominated by non-cultivable to a community dominated by cultivable bacteria. Lee and Fuhrman (1991) also noted increases in colony-forming-units, but according to the level of community DNA hybridization they estimated that changes in confined bacterioplankton were only minor, amounting to maximal 15% difference in DNA hybridization between original water sample and sample kept in 20 l containers for 2 days. Despite these biases, microcosm and mesocosm studies are still used in the investigation of aquatic bacteria (see, e.g. Duarte et al. 1997; Painting et al. 1989; Shiah and Ducklow 1995; Lebaron et al. 1999).

However, to date there have been no studies reporting on the identities of populations and their dynamics during confinement. Molecular methods using the small subunit ribosomal RNA and its encoding gene as molecular marker are excellent tools for this purpose, as they circumvent the recognized limitations of cultivation-based methods (for an overview, see Amann et al., 1995). Here we report on the changes in the bacterial diversity of a microbial assemblage during batch incubation of Mediterranean seawater in a mesocosm investigated by denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments (for a review, see Muyzer and Smalla, 1998) and by cloning and sequencing of amplified bacterial 16S rRNA genes.

## Materials and Methods

### Description of the mesocosm

A plastic tank ('mesocosm') was filled with 300 liter of seawater retrieved from the Gulf of Lyons (Mediterranean Sea) on 28 May 1996 offshore Banyuls-sur-mer (42°31'00" N, 3°11'83" E) in France. The water was filtered through a 200 µm plankton mesh to retain large organisms and was kept at ambient temperature (17.7 - 19.6°C) by immersion in a basin through which natural seawater from the nearby shore was pumped continuously. Light was provided for 12 h (five fluorescent tubes, 36 W, 120 cm). The water inside the mesocosm was circulated by a pump to assure efficient mixing (for more details see, Lebaron et al. 1999). Water samples were taken from the mesocosm after 0, 65, 113, 161 and 281 h. Bacterial cells (approximately 2.5 x $10^8$) were collected from the water samples by filtration on hydrophilic Durapore filters (Millipore GVWP 02500, polyvinylidene fluoride membrane, 0.22 µm, 25 mm diameter). After filtration, the filters were put into cryo-vials and frozen in liquid nitrogen immediately.

### Nucleic acid extraction

DNA was extracted and purified from the collected bacteria using the protocol described by Teske et al. (1996). Briefly, filters were thawed, transferred into sterile 15 ml centrifuge tubes and washed twice with 1 ml of ice-cold acetate-EDTA-buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA). 6 ml of hot phenol-chloroform-isoamylalcohol (25:24:1 v/v) and 60 µl of 25% (w/v) sodium-dodecyl-sulfate (SDS) were added, the samples were incubated for 5 min at 60°C in a water bath and vortexed briefly every minute. The aqueous phase was further purified by extracting with phenol-chloroform-isoamylalcohol (25:24:1 v/v) followed by ethanol precipitation to collect total nucleic acids. Precipitated nucleic acids were pelleted by 60 min centrifugation at approximately 4000 g and a temperature of 4°C (Heraeus, Labofuge 400R with rotor #8179 and buckets #8172; Heraeus, Hanau, Germany). Pellets were dried under vacuum and resuspended in 100 µl of sterile water (Sigma, Deisenhofen, Germany). 5 µl aliquots of the nucleic acid preparations were inspected on 1% (w/v) agarose gels.

### PCR amplification of 16S rRNA genes

The extracted genomic DNA was used as target DNA in the polymerase chain reaction (PCR; Saiki et al. 1988) to amplify the 16S rRNA genes. Two different primer sets were used, i.e., primer set 341F-GC/907RC which amplifies a ca. 550 bp long 16S rRNA gene fragment of Bacteria suitable for DGGE analysis (Muyzer et al. 1995) and a second primer pair, primers 8F and 1528R, which amplifies the nearly complete bacterial 16S rRNA gene (Garcia-Pichel et al. 1998). PCR with primer pair 341F-GC/907RC was performed as described previously (Muyzer et al. 1995). For the amplification of nearly complete 16S rRNA genes, total reaction volumes of 100 µl contained 10 µl of 10x PCR reaction buffer (100 mM Tris-HCl pH 9.0; 15 mM $MgCl_2$; 500 mM KCl; 1% (v/v) Triton X-100; 1% (v/v) gelatin), 10 µl of dNTPs (2.5 mM each), 5 µl of bovine serum albumin (BSA; 30 mg/ml), 50 pmol of each primer, ca. 10 ng of template DNA and water (Sigma) to adjust the volume to 99 µl. 'Hot-start' PCR (Chou 1992) was carried out on a Techne Cyclogene thermocycler by adding 1 µl of Super-Taq-polymerase (1 unit/µl; HT Biotechnology, Cambridge, UK) at a temperature of 80°C after the initial denaturation step (5 min at 94°C). Then 35 cycles of 1 min 94°C (except initial denaturation), 1 min primer annealing at 45°C and 3 min polymerization at 72°C were carried out. The last polymerization step at 72°C was extended for 30 min. PCR products were analyzed for quality and concentration by electrophoresis in 1% (w/v) agarose gels (1x TBE, Tris [ pH 8.3], boric-acid, EDTA), stained with ethidium bromide (0.5 mg/l) and photographed with a Polaroid system.

**DGGE analysis of PCR products**

DGGE was performed as described by Muyzer et al. (1998) to analyze PCR products obtained with the primer combination 341F-GC/907RC, using linear denaturing gradients of 10 to 70% urea and formamide (a 100% solution consists of 7M urea and 40% [v/v] formamide). About 500 ng of PCR product were applied to each lane. Gels were electrophoresed for 18 h at a constant voltage of 100V. After electrophoresis, the gels were incubated for 30 min in Milli-Q water containing ethidium bromide (0.5 mg/l), rinsed for 20 min in Milli-Q water and documented with a Fluor-S system at UV transillumination (Biorad, Hercules, CA). Gel scans were inverted and sharpness was enhanced with the filter option „sharpen in place“ as implemented in the software Multi-imager (Biorad).

**Cloning of PCR products**

The nearly complete 16S rRNA gene fragments were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The purified PCR products were then cloned using the pGEM-T (easy) Vector System II (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. The fragment of the 16S rRNA gene suitable for DGGE analysis was amplified from clones and screened by DGGE in order to identify as many inserts with different melting behavior as possible. Unique clones (i.e., a clone representing a group of clones with identical melting behavior) and replicates for some unique clones were further characterized by sequencing.

**Sequencing of cloned 16S rRNA genes**

10 ml of Luria-Broth containing ampicillin (100 µg/ml) was inoculated with a clone colony and grown overnight at 37°C. Plasmids were isolated using the Wizard Plus Minipreps DNA purification system (Promega GmbH., Mannheim, Germany) according to the manufacturer's instruction. The Taq Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, USA) was used to sequence the 16S rDNA fragments. 5'- and 3'- end partial sequences were obtained using M13 priming sites located on the plasmid upstream and downstream of the polylinker. Nearly complete sequences of some selected clones were obtained using primers 518F (Buchholz-Cleven et al. 1997), and in some cases 341F (without GC-clamp) and 907F (sequence: 5'-AAACTCAAAGGAATTGACGG-3'). Products of the sequencing reactions were electrophoresed on an Applied Biosystems 373S DNA sequencer.

**Comparative 16S rRNA sequence analysis and construction of phylogenetic trees**

New sequences were fitted into an alignment of about 5400 homologous full prokaryotic 16S rRNA primary structures (Maidak et al. 1997) using the automated aligning tool of the 'ARB' program package (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/). The sequence alignments were checked and misaligned positions were corrected manually. For complete clone sequences, trees were calculated by applying different algorithms (neighbor joining, maximum likelihood and maximum parsimony) using either a mask corresponding to the nucleotide positions of the 16S rRNA of *Escherichia coli* or nucleotide positions that were conserved to at least 50% within the different phyla (α-Proteobacteria, γ-Proteobacteria, and the group comprised of *Planctomyces*, *Pirellula*, *Gemmata*, *Isophaera* and *Verrucomicrobium*). Partial sequence data were inserted into the optimized tree, which was derived from full sequence data, according to the maximum parsimony criteria using a special ARB parsimony tool; the initial tree topology was not affected. Uncorrected pair wise 16S rRNA similarity values were calculated by taking into account all overlapping alignment positions between two sequences. Bootstrap analyses were carried out with almost complete sequences only, using the program DNAPARS from the PHYLIP program package as implemented in ARB, 1000 replications were performed.

### Nucleotide sequence accession numbers

The nucleotide sequence data reported here were deposited in the EMBL nucleotide sequence databases under the accession numbers AJ240909 to AJ240965.

### Enumeration of bacteria and protozoa

The abundance of bacteria and protozoa was determined by epifluorescence microscopy (Leitz, Laborlux D) after 4,6-diamidino-2-phenylindole (DAPI) staining. Subsamples for DAPI counts of bacteria were fixed with formaldehyde (2% final concentration), stained with DAPI for 30 min (2.5 µg/ml wt/vol) and collected by filtration on a 0.2 µm pore size black polycarbonate filter. For each sample a minimum of 20 fields (600 cells) were counted and averaged. Twenty ml of glutaraldehyde-preserved (0.5% v/v, final concentration) water samples were stained with DAPI (0.1 µg/l, final concentration) for 15 min. Stained protists were collected by filtration on a 0.8 µm pore size black filter (Nuclepore). Filters were mounted on microscopic slides and stored at 4°C until examination. Protozoa smaller than 20 µm were enumerated at a magnification of 1250 or 625 while larger micro-organisms (20-200 µm) were analyzed at a magnification of 125. A minimum of 100 organisms per filter was counted. Autotrophic species were distinguished from heterotrophs by the red autofluorescence of chlorophyll *a* observed under blue light excitation. Data presented in this paper concerns only the heterotrophic protozoa.

### Mortality and grazing rates estimation

Measurement of bacterial mortality and contribution of protozoa grazing in the overall mortality process was measured by following the decrease of radioactivity ($^{3}H$) associated with the bacterial genetic material, after labeling the bacterial assemblage with $^{3}H$-thymidine (Servais et al. 1985, 1989). 200 ml was sampled in the mesocosm and the bacteria were labeled with tritiated thymidine (50 $Ci.mmol^{-1}$, Amersham Life Science, England) at a final concentration of 4 nM for 10 to 12 hours in the dark at the mesocosm temperature. If the tritiated thymidine was not exhausted after this period, dialysis of the sample in Visking bags against circulating seawater for four hours was used to eliminate the unincorporated thymidine. After all the thymidine had been exhausted or eliminated, the sample was divided into two subsamples. The first one was not modified and the second one was filtered through a 2 µm pore size Nuclepore membrane to remove most of the eukaryotic micro-organisms from the sample. A mixture of cycloheximide-colchicine (200 mg/l cycloheximide and 100 mg/l colchicine) was added to the latter subsample to inhibit the possible grazing of the small fraction of protozoa that would have passed through the 2 µm filtration. This mixture was reported to be an inhibitor of protozoan grazing (Sherr et al. 1986). Ten ml aliquots were taken twice a day for 2 to 3 days from each subsample and were precipitated with cold trichloroacetic acid (5% w/v). The decrease of radioactivity associated with precipitated cellular materials was used to estimate the mortality. A linear decrease was usually observed in semi-log plot for each subsample. The slope for the unmodified subsample gave the first order rate of overall mortality while the slope in the other subsample corresponded to the first order rate of mortality not attributable to grazing. The difference between the two rates was considered to be the consequence of grazing by protozoa.

## Results

### PCR-DGGE analysis of mesocosm samples

Figure 1 shows the DGGE patterns of 16S rRNA gene fragments obtained after PCR-amplification of water samples taken from the mesocosm after 0, 65, 113, 161 and 281 h of

incubation. The number of apparent bands progressively decreased from 29 in the sample taken at t = 0 h (Fig. 1, lane 1) to 19 bands in the sample taken at t = 113 h (Fig. 1, lane 3). The number of bands increased and stabilized to 21 in the DGGE profiles of samples taken after 161 and 281 h (Fig. 1, lanes 4 and 5, respectively). Guided by these DGGE results, the samples from t = 0, 161 and 281 h were chosen for further analysis by cloning and sequencing of 16S rRNA genes.

**Comparative analysis of 16S rRNA gene sequences from sample t = 0 h.**

The phylogenetic affiliation of sequences obtained after cloning of 16S rRNA genes amplified from mesocosm water samples is shown in Figure 2 (A to C). Three out of 8 selected sequences from the clone library corresponding to sampling time 0 h belonged to the α subdivision of the Proteobacteria (Fig. 2A). Two sequences, CHAB-I-5 and CHAB-III-5 (similarity of 99.5%), were related to *Roseobacter denitrificans* (similarity of 95.3% and 96.4%, respectively) and to the sequence of clone SAR83 obtained from the Sargasso Sea (Giovannoni et al. 1990) (similarity of 93.3% and 96.7%, respectively). Another sequence of α–proteobacterial origin, CHAB-III-8, was related to clone OM25 of the SAR116 cluster (95.2%similarity) (Rappé et al. 1997) Distant cultured relatives are *Azospirillum lipoferum* and *Rhodospirillum salinarum* (87.8% and 85.9% similarity, respectively).

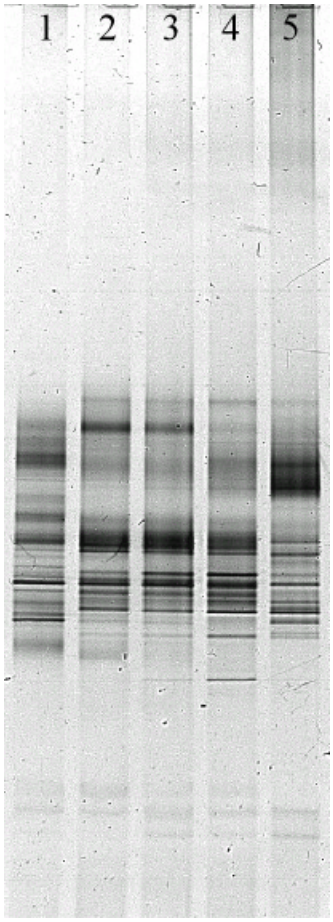


**Figure 1**

Negative image of an ethidium bromide-stained DGGE gel showing the banding patterns of PCR-amplified bacterial 16S rDNA fragments. The banding patterns represent fingerprints of the bacterial community present at sampling times 0 h (lane 1), 65 h (lane 2), 113 h (lane 3), 161 h (lane 4) and 281 h (lane 5) of the batch incubation.

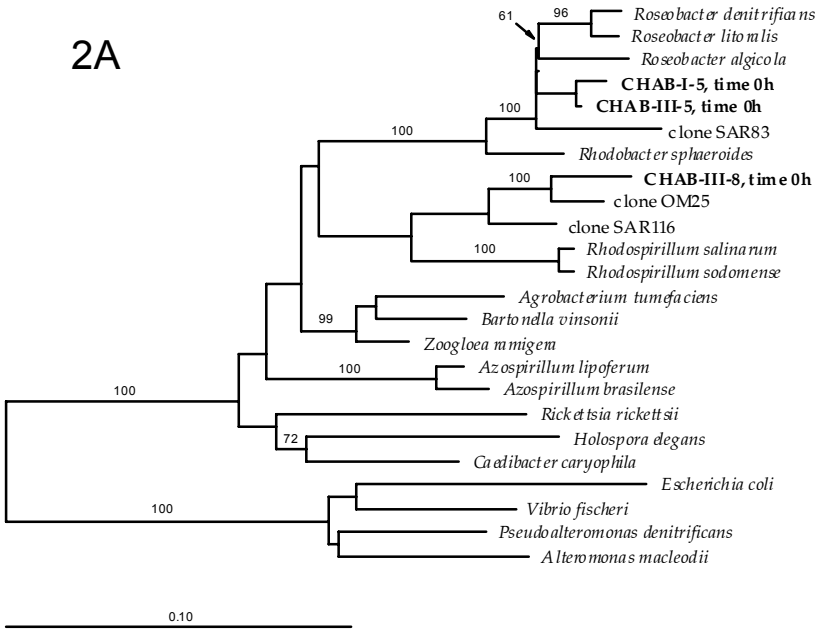


**Fig. 2A–C**

Parsimony trees show-ing the relationship of 16S rRNA gene sequen-ces from this study to 16S rRNA sequences of known taxa and sequences retrieved from environmental samples by others A: α subdivision of Proteo-bacteria, B: γ subdivision of Proteobacteria, C: *Planctomyces* group. The sequences determined in this study are in bold. The sampling time from which sequences were cloned is indicated together with the clone designation.

Four sequences belonged to the γ subdivision of the Proteobacteria, yet all of them were only distantly related to cultivated species (Fig. 2B). Two of these sequences, CHAB-III-1 and CHAB-II-39 (99.3% similarity), were 99% and 98.7% similar to the sequence of clone OM23, respectively (Rappé et al. 1997) and thus members of the SAR86 cluster (Giovannoni et al. 1990). Another sequence, CHAB-I-7, was monophyletic with the SAR86 cluster regardless of the treeing algorithm and masks used (bootstrap 100%). Yet, the low similarity of 87% to the closest relative, clone OM10, would mean a higher sequence variability than the 8% within this cluster noted by Rappé and colleagues (1997). Sequence CHAB-III-7 belonged to another group within the γ Proteobacteria (Fig. 2B). It was 96.2% similar to the sequence of clone OM252. Among their nearest cultivated relatives is *Oceanospirillum commune* (88.6% similarity to CHAB-III-7).

The sequence of clone CHAB-II-49 was affiliated to the recently proposed division of *Verrucomicrobiales* (Hedlund et al. 1997)(Fig. 2C).

**Comparative analysis of 16S rRNA gene sequences from sample t = 161 h.**

Sequence types found in the clone library corresponding to sampling time 161 h were different from that of 0 h. All were affiliated to the γ subdivision of Proteobacteria (Fig. 2B). Two out of 15 selected sequences, CHAB-VI-14 and CHAB-V-35 (99.3% similarity), had the highest sequence similarity with sequence CHAB-III-7 obtained from sampling time 0 h (similarities of 88.9% and 89.7% respectively). Among the closest cultivated relatives were *Oceanospirillum commune* and *Pseudomonas stutzeri* (similarities of ca 92.7 and 92.8% to CHAB-V-35, and 92.5 and 91.3% to CHAB-VI-14, respectively). The remaining 13 sequences were all affiliated to *Alteromonas macleodii*, the only species in the genus *Alteromonas* (Gauthier et al. 1995), with a similarity range of 92.6% to 98.4%.

**Comparative analysis of 16S rRNA gene sequences from sample t = 281 h.**

Sequences affiliated to the genus *Alteromonas* (11 sequences out of 14) also dominated the clone library corresponding to time 281 h (Fig. 2B), the similarity with the 16S rRNA gene sequence of *A. macleodii* ranged from 91.7% to 98.6%. Sequences related to CHAB-V-35 from sampling time 161 h were not detected. A new sequence-type appeared, CHAB-XI-27

(identical sequences of CHAB XI-13 and CHAB XII-17 are neither shown in tree nor deposited in database) which had as nearest cultivated relatives different *Legionella* species, e.g. *Legionella cincinnattiensis* (Fig. 2B). However, its phylogenetic affiliation could not be determined confidently with strong bootstrap values.


**Figure 2B.**

Parsimony tree showing the relationship of 16S rRNA gene sequences affiliated with the γ-Proteo-bacteria. The sequences determined in this study are in bold. The sampling time from which sequences were cloned is indicated together with the clone designation

Fig. 2C

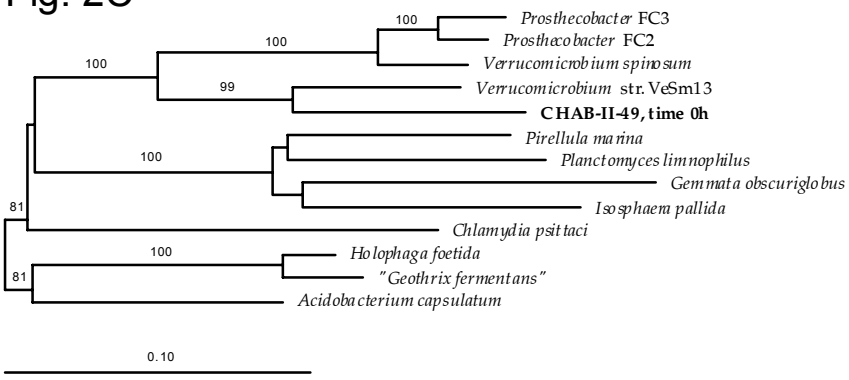


**Figure 2C.**

Parsimony tree showing the relationship of 16S rRNA gene sequences from the *Planctomyces* group. The sequence determined in this study is in bold, the sampling time from it was cloned is indicated.

## Bacterial and protozoan enumeration and estimation of mortality and grazing rates

In order to understand the control of bacterial abundance during the mesocosm experiment, the abundance of bacteria and protozoa, the bacterial mortality and the contribution of grazing by protozoa were investigated. Figure 3 shows the fluctuation of the abundance of bacteria and heterotrophic protozoa during the mesocosm experiment. During the first 120 hours the abundance of protozoa was quite stable. Then, between 120 and 150 hours a marked increase was observed. This maximum of the abundance of protozoa followed the maximum of bacterial abundance which occurred in the mesocosm after about 70 hours of incubation (Lebaron et al. 1999). This is a classical observation in batch incubation or mesocosm experiments in which the development of predators follows the development of prey (Jürgens and Güde 1994). After 150 hours the number of protozoa decreased reaching a value after 250 hours which was close to that observed at the beginning of the experiment.

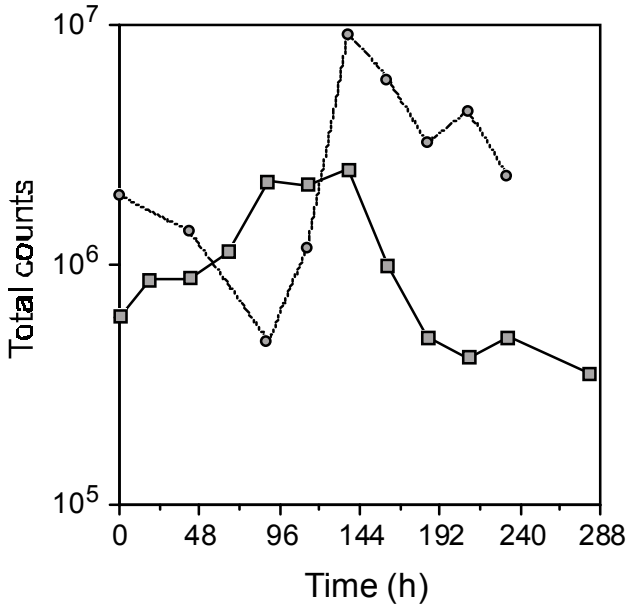


**Figure 3**

Fluctuation of the abundance of bacteria (per ml; squares and solid line) and protozoa (per liter; circles and dotted line) during the mesocosm experiment.

Results of overall mortality and first order grazing rates are presented in Table 1. The data show that for all the sampled situations, grazing by protozoa was the dominant process in the overall bacterial mortality (68 to 92 % of the overall mortality). The rates of mortality not due to grazing, such as bacteriophage-induced lysis or autolysis, were in the range of 0.02 to 0.07 $d^{-1}$ while the rates of mortality due to grazing by protozoa were between 0.13 to 0.29 $d^{-1}$. These values of mortality and grazing rates were in the same range of magnitude like values previously measured in various natural aquatic environments (Servais et al. 1989, 1992). The grazing rates confirmed the data of protozoa abundance indicating an important grazing pressure on the bacterial population especially between time 90 and 210 h.

TABLE 1. First order bacterial mortality and grazing rates during the mesocosm experiment[1]

| time interval | overall mortality rate | grazing mortality rate | % of grazing in the mortality |
|---|---|---|---|
| 90-130 h | 0.26 | 0.24 | 92 |
| 160-210 h | 0.36 | 0.29 | 81 |
| 210-230 h | 0.19 | 0.13 | 68 |

[1](expressed as $d^{-1}$)

## Discussion

Both approaches, DGGE as well as cloning and sequencing of PCR-amplified 16S rRNA genes indicated changes in the bacterial community composition over time. Cloning and sequencing of nearly complete 16S rRNA genes after PCR amplification from 3 of the 5 samples suggested a shift from a mixed bacterial community composed of sequences affiliated to different lines of descent from the α and γ Proteobacteria to a bacterial assemblage dominated by members closely related to *Alteromonas macleodii*.

In their study on the effect of confinement on changes in a bacterial community Lee and Fuhrman (1991) found a maximum of 15% difference at the level of community DNA of confined samples relative to the initial sample by DNA hybridization. Hence, they suggested that confined samples (20 l) did not undergo drastic changes in species composition within 48 h. We however, have found major differences in the composition of clone libraries from different time points and think that several factors, e.g. the longer duration of confinement, the larger volume of water and the prefiltration over a 200 µm mesh used in this study may explain different observations and conclusions regarding the extent of the effect of confinement.

Except for one sequence, sequences of clones determined in this study were affiliated to the α and γ Proteobacteria and similar to sequences belonging to the SAR83, SAR86 and SAR116 clusters or to other 16S rRNA sequences representing not-yet-cultivated marine bacteria (Giovannoni et al., 1990; Mullins et al. 1995; González and Moran 1997). A number of sequences were affiliated to the cultivable marine bacterium *Alteromonas macleodii*. One sequence represented a member of the *Verrucomicrobiales*, which have mainly been found in soil habitats (Liesack and Stackebrandt 1992). Although recently, Rath and colleagues (1998) reported the finding of sequences affiliated to members of this division in marine snow samples.

Sequence types that had been found in the t = 0 h sample were not detected in the sample corresponding to sampling time 161 h, except for sequences CHAB-V-35 and CHAB-VI-14 which clustered with the sequence of clone CHAB-III-7. However, these were only about 89% similar to sequence CHAB-III-7. Instead, the clone libraries prepared from DNA of water samples taken at 161 h and 281 h were dominated by sequences affiliated to members of the genus *Alteromonas*, suggesting a marked shift in the composition of the

bacterial community. Acinas et al. (1999) also reported on a variety of *A. macleodii* related sequences originating from different depths in offshore Mediterranean waters. Recent investigations by Pukall et al. (1999) indicated the presence of different *rrn* operons in *Alteromonas* strains isolated from Mediterranean coastal water. Therefore, the microdiversity in the *Alteromonas* cluster might overestimate the importance of *Alteromonas*-like bacteria. However, assuming the same bias at all sampling times investigated, there clearly is a shift in the genetic diversity during the incubation.

Typical mesocosm incubations proceed through distinct phases, whereby phase I is characterized by a stimulation of bacterial growth, phase II shows the development of heterotrophic nanoflagellates (HNF) feeding on the initial bacterial production, and phase III is characterized by an enrichment of 'grazing-resistant' bacterial morphotypes after the peak in grazing activity (Jürgens and Güde 1994). Mechanisms controlling bacterial abundance, such as grazing or viral lysis, should therefore also have impact on the genetic diversity of the bacterial assemblage.

The typical initial increase in culturability, growth rate and bacterial production observed in experiments using microcosms and mesocosms (Ferguson et al. 1984, Lee and Fuhrman 1991) is believed to stem partly from the release of nutrients during sample handling. Such an increase in culturability was also observed during the start of this incubation (see Lebaron et al. 1999). Comparison of the DGGE profiles of 16S rRNA gene fragments obtained from samples taken after 0, 65 and 113 h of incubation revealed a decrease in the number of apparent bands together with a shift in the relative band intensities. Differential growth of responsive bacterial populations is one way to explain these shifts. Furthermore, the disappearance of populations as revealed by DGGE analysis might be caused by unfavorable conditions (e.g. caused by initial sample handling and by confinement) for the corresponding populations or by lysis due to viral infection, rather than being an effect of protistan grazing, as numbers of protozoa increased only later during the course of incubation.

It has been shown that heterotrophic nanoflagellates exhibit prey-size-dependent feeding behavior (Monger and Landry, 1991; González et al. 1990) and have higher grazing rates on active than on inactive bacteria (Del Giorgio et al., 1996). Bacteria have developed strategies to escape grazing, e.g. by developing inedible morphologies (Jürgens and Güde, 1994) or by increasing their growth rate (Pernthaler et al., 1997). As grazing did control bacterial abundance in 'phase II', it might have changed the composition of the bacterial assemblage as well. Hence, dominance of *Alteromonas*-like 16S rRNA gene sequences in the clone libraries might indicate that these populations had an advantage during the phase with high grazing pressure.

The close similarity of many cloned sequences to the sequence of *Alteromonas macleodii*, a bacterium that is often recovered as part of the cultivable fraction of marine bacterioplankton, could suggest that these bacteria had high growth rates. This might be one way to alleviate the grazing pressure on the population and could help to explain the dominance of *Alteromonas*-like sequences in clone libraries prepared from samples taken after 161 and 281 h. Painting et al. (1989) found that a marked increase in colony-forming units during the first 5 days of a mesocosm experiment corresponded mainly to the development of *Pseudomonadaceae.* The classification criteria applied for *Pseudomonadaceae* would have included bacteria of the genus *Alteromonas* according to the present definition (Gauthier et al. 1995).

On the other hand flow cytometric analysis of water samples from the same mesocosm by Lebaron et al. (1999) showed an increase in the relative numbers of large bacterial cells (cell length >2.5 μm) that represented more than 80% of the total bacterial biovolume at time 209 h. Cells of *Alteromonas macleodii* have been described as 0.7 to 1 μm in diameter and 2-

3 µm in length (Gauthier et al. 1995). Thus, *Alteromonas*-related sequences most probably represent these large cells which might be too big to allow efficient ingestion by small HNF.

We have observed changes in the predominant 16S rRNA gene sequences during incubation of Mediterranean seawater during which grazing by protozoa controlled bacterial abundance. It appears that grazing also induced changes in the bacterial genetic diversity; while several different bacterial 16S rRNA gene sequences were found at the beginning of the incubation, *Alteromonas*-like sequences were predominant after the peak in grazing activity. Viral lysis can not be completely excluded as another source of bacterial mortality (for an overview see Suttle, 1994), but mortality data show that it was of minor importance. Therefore, the occurrence of *Alteromonas*-like bacteria after the peak in grazing activity was probably due to some advantage these bacteria had under grazing pressure, perhaps an "inedible" morphotype, high growth rates or a combination of both.

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# Chapter 4

# Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: Changes in the genetic diversity of bacterial populations

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## Abstract

A mesocosm experiment was performed to study the influence of nutrients on activity and diversity of bacterial assemblages from the Mediterranean Sea. Changes in the diversity of the predominant bacterial populations were monitored by DGGE fingerprinting of PCR products derived from 16S rRNA encoding genes. Fluctuations in the diversity of the most active populations was inferred by performing the DGGE fingerprinting on the basis of the cellular rRNA after reverse transcription and PCR amplification. DNA-derived DGGE patterns obtained from duplicate control and nutrient-enriched mesocosms showed differences in the development of the bacterial communities between control and nutrient-enriched experimental mesocosms. Multidimensional scaling analysis of the DNA-derived DGGE fingerprints indicated that duplicate treatments were reproducible. DNA- and RNA-derived DGGE fingerprints of bacterial assemblages changed over time, showing that the composition of the bacterial assemblages, as well as the most active bacterial populations changed during different phases of the incubation. Sequences of predominant DGGE bands in RNA-derived patterns were similar to 16S rRNA gene sequences of members of the α–, γ– and δ–*Proteobacteria* and of the *Cytophaga-Flavobacterium-Bacteroides* phylum (CFB). Bands corresponding to *Ruegeria*-like bacteria and members of the CFB became especially dominant during the course of incubation, suggesting that these populations were important contributors to bacterial production and activity in the post-grazing phase of the experiment.

## 1. Introduction

A major question in microbial ecology is to identify which members contribute to the activity and/or productivity of the whole bacterial community. During the last decade, the application of cultivation–independent molecular techniques using 16S rRNA or its encoding gene as molecular marker have provided new insights into microbial diversity and in the structure and dynamics of microbial communities from different habitats (for reviews see:[1,2]). Results of such studies indicate for instance that marine microbial communities are composed of ubiquitous members that can be found throughout the world's ocean [3-5]. Yet, differences have been recorded temporally and spatially [6,7], as well as between free-living and aggregate-attached communities [8-10]. However, most of these studies describe the composition of bacterial communities, but do not address bacterial activity.

So far, bacterial activity was mostly studied in bulk. A variety of techniques have been developed to quantify the productivity of natural communities and to characterise the metabolic activity of individual cells. Nevertheless, none of these methods can identify which microbial taxa contribute to community productivity.

Recent studies have addressed the question of relationships between structure and function of bacterial communities. A combination of methods was used to identify bacterial populations which are active within natural communities [11]. Similarly, the question of how these relationships can be affected by grazing was addressed [12,13]. By using a combination of community respiration measurements, and by fluorescent in situ hybridisation (FISH) and denaturing gradient gel electrophoresis analysis (DGGE) of PCR products after reverse transcription of RNA, Rossello-Mora et al. [14] were able to identify bacterial populations that were activated in marine sediment samples after amendment with cyanobacterial biomass. Several authors have applied a combination of microautoradiography and fluorescent in situ hybridisation (FISH), allowing to specifically detect cells and get information about their substrate uptake and hence potential in situ activity [15-17]. The specific nature of substrates however, and the laboriousness of FISH for a high number of samples do not make it an ideal tool to study global metabolic activity of bacterial populations when many samples are to be analysed.

In other studies [13,18], we have shown that bacterial community dynamics from the Mediterranean Sea was affected by inorganic nutrient addition. Observations at the community level (bacterial and protistan abundance, bacterial production) as well as changes in culturable populations and morphological changes in the bacterial assemblage are further explored in the paper by Lebaron et al. [18]. The first response was an increase in bacterial abundance. This growth phase was followed by an increase in the grazing pressure by nanoflagellates and ciliates (grazing phase), which caused a decrease in bacterial abundance (post-grazing phase). Morphological changes as well as marked changes in bacterial activity (thymidine incorporation rates) were observed during these different phases of the experiment.

The aim of this study was to monitor changes in the structure and composition of the bacterial community through DGGE analysis of PCR-amplified fragments of the 16S rRNA-encoding gene, and to investigate whether or not fluctuations in bulk bacterial activity during the mesocosm experiment were accompanied by a succession of dominant DGGE-defined populations. In order to identify the most active bacterial populations DGGE was used to analyse PCR-products derived from 16S rRNA after reverse transcription, which may favour the amplification of active populations with a high ribosome content [14,19]. The identity of the predominant community members was revealed after comparative analysis of sequences from DGGE-separated DNA fragments with sequences stored in databases.

## 2. Materials and methods

### 2.1. Mesocosm set-up and sampling

The source of seawater used in the experiment, the set-up of the mesocosms and the sampling schedule, as well as global parameters, such as total bacterial counts, chlorophyll *a* concentrations, etc., are described in the accompanying paper [18]. Briefly, 300 litres of Mediterranean coastal water retrieved from the Gulf of Lyons off the coast of Banyuls-sur-mer (France) were filtered through a 200 μm plankton net into each of four mesocosms. Two mesocosms were supplemented with inorganic nutrients (phosphate, nitrate, and ammonium) to reach concentrations 10-times higher than those in the natural water (hereafter referred to as 'experimental' mesocosms). Two mesocosms were not amended with nutrients (hereafter referred to as 'control'). Some of the analyses have been carried out with samples of one of the two replicate mesocosm of a treatment only. To clearly distinguish these replicates, and to make clear from which replicate specific results have been obtained, this has been indicated as replicate-1 or replicate-2 wherever necessary. The succession of bacterial assemblages in the four mesocosms was followed over a total time period of 312 h. Samples for analysis by molecular biological techniques were taken at the beginning of the incubation (t = 0 h) and after 48, 96, 144, 216 and 312 h. Water samples from the natural site were collected one day before the start (t = -24 h) as well as one day after the end of the mesocosm experiment (t = 336 h). A volume of water containing ca. $7.5 \times 10^8$ cells (range ca. 120 to 1800 ml) was filtered onto Durapore polyvinylidene fluoride filters with a pore size of 0.22 μm (Millipore, GVWP, 47 mm diameter). Subsequently, the filters were put in vials and immediately frozen in liquid nitrogen for transport to the lab, where the samples were stored at –80°C until further analysis.

### 2.2. Nucleic acid extraction, PCR-DGGE, and DNA sequencing

Nucleic acid extraction was performed following the hot phenol protocol described by Sahm and Berninger [20], however, without the bead beating and DNase step. Recent studies have shown that this protocol is also suitable to obtain DNA from Gram-positive bacteria, *e.g.* [14].

PCR amplification of 16S rRNA gene fragments from genomic DNA or from reverse transcribed RNA (cDNA) was carried out as described before [21] using primers 341F-GC and 907R except that two versions of the reverse primer, differing in one nucleotide position, were used (primer 907RC: 5'-CCGTCAATTCCTTTGAGT-TT-3'; primer 907RA: 5'-CCGTCAATTCATTTGAGTTT-3') since primer 907RC may not result in efficient amplification of 16S rRNA gene fragments of some marine representatives of the γ–*Proteobacteria* (*e.g.* the genera *Alteromonas*, *Pseudoalteromonas*, *Aeromonas*, and *Shewanella*) due to a mismatch in the target site. The concentration of PCR products was estimated by comparison with a molecular mass standard (Precision molecular mass standard; Biorad, Hercules, Ca.) by electrophoresis in 1% (w/v) agarose gels.

About 400 ng of PCR product from each sample were analysed on 6% polyacrylamide gels containing gradients of 20 to 80% denaturants (*i.e.*, urea and formamide). Gels were run at a constant voltage of 100V for 18 h at 60°C in 1x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA; pH 8.3). Following electrophoresis, the gels were incubated for 30 min in an ethidium bromide solution (0.5 μg $ml^{-1}$), then rinsed for 30 min in MilliQ water, before they were photographed using a Fluor-S Multi-Imager system (Bio-Rad Laboratories, Hercules, USA). Scans of DGGE gels were analysed for the numbers of bands per profile.

Individual bands were excised and re-amplified, inspected by DGGE alongside the original sample. PCR-products for sequencing were purified using the Qiaquick PCR purification system (Qiagen, Hilden, Germany) and sequenced with the BigDye cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA). Sequencing reactions were analysed on an Applied Biosystems 310 Genetic Analyzer.

### 2.3. Multidimensional scaling analysis of DGGE fingerprints

To assess changes in the genetic diversity of the bacterial communities over time, the complex DGGE banding profiles were analysed by multidimensional scaling (MDS) as described by van Hannen et al. [22]. For this purpose the presence and absence of DGGE bands over all profiles was recorded in a binary (1/0) matrix, and transformed into a distance matrix using Jaccard's similarity coefficient [23] as implemented in SYSTAT 7 for Windows. The distance matrix was further analysed by MDS with the software program SYSTAT 7 for Windows (analysis-settings: dimensions, 2; R-metric, 2; Iterations, 200; converge, 0.0001; loss function, Kruskal; regression, linear). MDS is a powerful data reducing method whereby a complex DGGE pattern is reduced to one point in a two dimensional space. By connecting the consecutive points the relative changes in the community structure can be visualised. The closer the points are to each other, the more similar are the DGGE banding patterns.

### 2.4. Comparative sequence analysis

New partial sequences (range: 338 - 549 bp) were analysed using BLAST [24] at the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and added together with the most important BLAST hits, to an alignment of about 5300 homologous bacterial 16S rRNA primary structures [25] by using the aligning tool of the ARB software package [26]. The tree was generated by Neighbour-joining with the correction method of Felsenstein as implemented in ARB, taking into account all unambiguous nucleotides between E. coli positions 392 and 774, negative branch lengths were collapsed to zero. The topology of the Neighbour-joining tree was in good agreement with (i) BLAST analyses and (ii) a tree derived by inserting partial sequences into a tree derived from almost complete sequences by using the ARB parsimony tool that enables a reliable positioning of new sequences without allowing changes of the overall tree topology [27]. Sequences obtained in this study have been deposited in the EMBL sequence database under accession numbers AJ298349 to AJ298386. Names of sequences consist of the prefix AY- or BY-, denoting the mesocosm of origin (AY for replicate-2 of the

control; BY for replicate-2 of experimental mesocosms) and a number. Numbers are identical to those given next to particular bands in Figures 1 and 3.

# 3. Results

## 3.1. DGGE analysis

DGGE analysis of PCR-amplified 16S rRNA gene fragments obtained after amplification of DNA from all sampling times of the four mesocosms were compared in order to visualise changes in the genetic diversity of the bacterial assemblages under the different conditions and to check the reproducibility of duplicate mesocosms. DNA-derived DGGE banding patterns for corresponding sampling times of the replicate mesocosms of both control and experimental mesocosms were very similar (result not shown). DGGE profiles of experimental and control mesocosms developed differently over the course of the experiment (Fig. 1). Visual inspection of DGGE fingerprints suggested a bi-phasic succession in the genetic diversity of bacteria in control tanks, with most changes occurring between sampling times 144 and 216 h. Patterns of the experimental mesocosms rather appeared three-phasic. A specific band (band AY-52) that became dominant in the pattern of the control after 216 h of incubation was also visible as a dominant band (band BY-70) in patterns of the experimental mesocosms, however, in the experimental mesocosms it already appeared after 144 h (see Fig. 1).

Since DNA-derived fingerprints of duplicate mesocosms were reproducible, analysis of RNA-derived DGGE-fingerprints was carried out only with samples of one replicate (replicates-2) of control and experimental mesocosms (Fig. 1). RNA-derived patterns showed some interesting differences to DNA-derived patterns. For instance, the RNA derived pattern of the t = 144 h sample of the control showed bands that were not present in the corresponding DNA-derived pattern. One of these RNA-derived bands corresponding to band AY-59 (see Fig. 1) would eventually become detectable in the DNA-derived pattern of the 216 h sample of the same mesocosm. The same band was also detectable in the experimental mesocosms at sampling time 312 h. Here it was present as an intense band (BY-75) in the RNA-derived DGGE profile, but only as a very faint band in the DNA-derived pattern. Another interesting difference between DNA- and RNA-derived fingerprints was that the fluctuations in the number of detectable bands, i.e., increases and decreases during the incubation, showed different trends (see below).

PCR-DGGE analysis was also carried out with a modified reverse primer, i.e. primer 907RA. Fingerprints of PCR products obtained using the two different reverse primers were dissimilar. Figure 3 shows a comparison of three samples each from replicates-2 of control and experimental mesocosms analysed using either 907RC or 907RA as reverse primer. Common bands between the fingerprints derived with these alternative reverse primers mostly corresponded to bands that were dominant in fingerprints obtained with the other primer, e.g. bands that were dominant in DGGE profiles of PCR products obtained with 907RC were mostly weak bands in DGGE profiles of PCR products obtained with primer 907RA. DGGE analysis of PCR products obtained with 341F-GC/907RA showed changes in the banding patterns of samples of the control mesocosm (replicate-2), which manifested after the peak in nanoprotozoan grazing activity, when some new bands appeared and relative intensities of bands shifted. Similar observations were made for the experimental mesocosm (replicate-2).

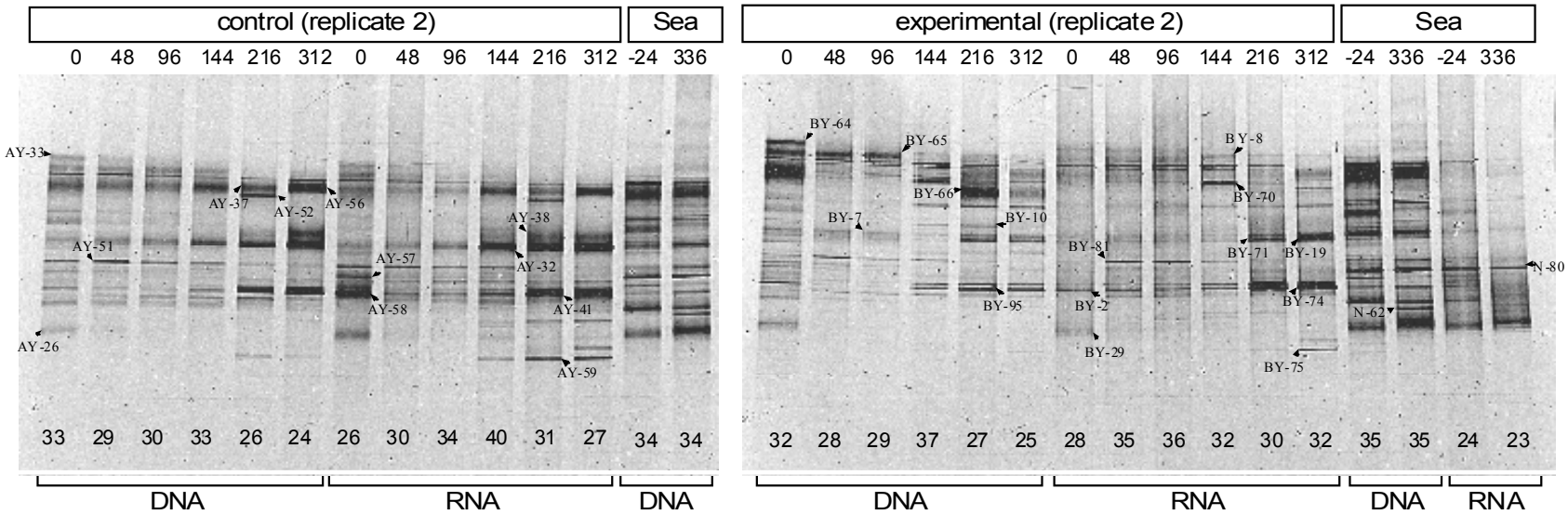


Fig. 1. DGGE analysis of PCR-amplified bacterial 16S rRNA gene fragments derived from DNA and RNA of samples taken from replicate-2 of control and replicate-2 of the experimental mesocosms as well as samples which were taken from the Mediterranean Sea before and after the mesocosm experiment (shown as Sea –24h and Sea 336 h, respectively). Time of sampling in hours (h) is indicated on top of the lanes. Reverse primers used in PCR was 907RC. Bands that were excised and sequenced are indicated (see also Fig. 4 and Table 1). The number of bands for each sample is indicated at the bottom of the lanes and represents the higher number of two separate runs.

Water samples from the original sampling site taken one day before the start of the mesocosm experiments (t = -24 h) and one day after the experiments had been terminated (t = 336 h) were also analysed by PCR-DGGE. There was less overall change in the banding patterns of the bacterial assemblage at the original sampling station (see Fig. 1, SEA) as compared to the changes occurring in the mesocosms.

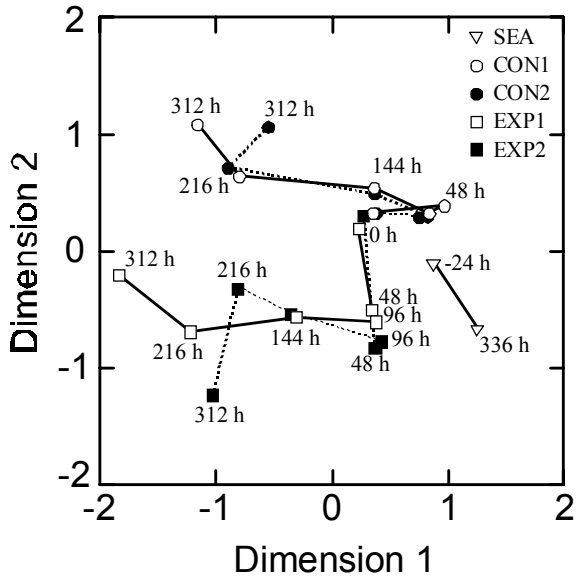


Fig. 2. Multidimensional scaling of DNA-derived DGGE banding patterns obtained with primers 341F-GC and 907RC illustrating changes in bacterial community structure in the four mesocosms and at the coastal sampling station (Sea). Consecutive time points (i.e., 0, 48, 96, 144, 216, and 312 h) have been connected by lines to depict the development of the banding patterns in each mesocosm during the incubation. The stress value of the final configuration was 0.16941, the proportion of variance 0.86642.

Multidimensional scaling (MDS) of the DNA-derived DGGE profiles obtained with the primers 341F-GC and 907RC confirmed that bacterial assemblages in replicate mesocosms developed reproducibly. The resulting graph (Fig. 2) shows a cluster of points for all mesocosm samples taken at t = 0 h, *i.e.*, at the start of the incubation, indicating nearly identical DGGE profiles or communities. Thereafter the DGGE fingerprints of the bacterial communities of nutrient-amended experimental mesocosm developed differently from those of the control mesocosms (Fig. 2). Samples from the control mesocosms taken after 48 and 96 h during the initial growth phase clustered very closely and were not very distant from the t = 0 h samples. Samples taken from the experimental mesocosms after 48 and 96 h also clustered closely together, however, they were further separated from the t = 0 h samples than their counterparts from the control. In addition, MDS-analysis showed that each sample taken later than 96 h from the experimental mesocosms (i.e. after 144, 216, and 312 h) was well separated from its preceding sample. In the control mesocosms, however, the greatest difference between fingerprints was evident between sampling times 144 h and 216 h, and minor differences were evident between 96 h and 144 h, as well as between samples taken after 216 h and 312 h. In contrast to fingerprints of samples taken from the mesocosms, those from the coastal sampling site showed only minor changes over a period of 15 days.

Numbers of DNA-derived bands in the DGGE profiles decreased in all mesocosms during the initial growth phase. (see Fig. 1). Subsequently, when bacterial abundance reached minimal values accompanied by maximal protist abundance, the number of DNA-derived DGGE bands increased again in samples taken from all mesocosms after 144 h, except for replicate-1 of the control where an increase was evident in the sample taken after 216 h. In the post-grazing phase the number of DNA derived DGGE bands decreased again.

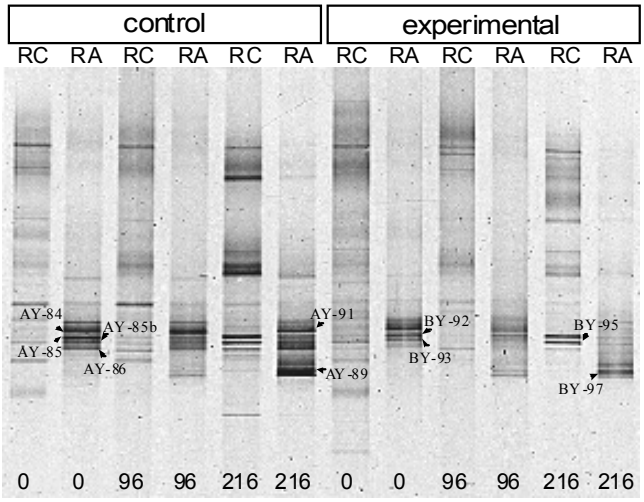


Fig. 3. Comparison of DNA-derived PCR-amplified bacterial 16S rRNA gene fragments obtained with alternative reverse primers 907RC and 907RA from each three samples of replicates-2 of control and experimental mesocosms. The primer used in PCR is denoted as RC or RA at the top of the lane for 907RC and 907RA, respectively, times of sampling in hours (h) are indicated at the bottom of the lanes. Bands that were excised and sequenced are indicated by an arrow and a number.

As the number of DNA-derived bands is rather related to the number of bacterial populations above the detection threshold of DGGE, the number of RNA-derived bands should reflect the predominant active populations, as it is rather related to the number of different sequence types in the RNA-pool, which is influenced by cellular bacterial activity [14,20]. DGGE analysis of RNA-derived PCR products showed a different trend from that of DNA-derived bands. In the phase of bacterial growth at the beginning of the incubation, the number of RNA-derived bands increased during the growth phase in both treatments and decreased again after the grazing phase. In the nutrient treatment (replicate-2) this corresponded to a maximum of RNA-derived bands for the sample taken after 96 h, in the control (replicate-2) the maximum number of RNA-derived DGGE bands was observed after 144 h.

### 3.2. Comparative sequence analysis

Sequencing of DGGE bands showed that bacterial populations belonged to various phylogenetic groups, representing the α–, γ– and δ–*Proteobacteria*, as well as the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum and oxygenic phototrophic micro-organisms from the phylum comprising cyanobacteria and algae chloroplasts (Fig. 4).

Different trends were observed during the incubation (compare the results presented in Fig. 4 and Table 1) for DGGE bands representing distinct bacterial populations. Some were detected in the natural seawater sample, but their DGGE bands became weaker or disappeared during the initial growth phase (i.e. incubation time 0 h until 96 h), *e.g.* *Synechococcus*-related cyanobacteria, *Rhodobacter* group bacteria AY-57 and N-80). The intensity of RNA-derived DGGE bands of these populations decreased sharply from the sample taken at the coastal sampling station (SEA) to time 48 h. A population related to *C. lytica* ('type 1' in Table 1) rapidly formed a dominant DGGE band at time 0 h, but disappeared again at time 48 h.

Bacteria related to *Alteromonas macleodii* were represented by dominant DGGE bands from the beginning of the incubation, but became weaker after the peak in grazing activity in DNA-derived PCR products, yet they still had dominant bands in the RNA-derived patterns (result now shown). These *Alteromonas*-related bacteria also dominated the DNA and RNA-derived DGGE patterns of the natural sample taken 24 h before the experiment. However, they were much less important in DNA and RNA-derived DGGE patterns of the natural samples taken after the experiment had been terminated as only one of the four bands (corresponding to AY-86) remained weakly visible (result not shown).

Other DNA-derived DGGE bands also detected in the natural seawater sample became more intense during the growth phase as well as their corresponding RNA-derived counterparts

were becoming more intense from natural to mesocosm conditions (*e.g.* bacteria related to *Flexibacter maritimus* and *Sulfitobacter*). Bands representing a population of the SAR86 cluster from the γ–*Proteobacteria* appeared in DGGE patterns of phase one of the experiment and became more dominant throughout the experiment in both treatments (result not shown). While some DGGE-defined populations were maintained throughout the incubation period (*e.g. Ruegeria* and Clone agg58 'type 1' in both treatments, *C. lytica* 'type 2' in the control), the DGGE bands of other populations became less dominant or disappeared (*e.g. Sulfitobacter and Flexibacter maritimus* related populations). Some DGGE bands were especially dominating the RNA-derived fingerprints during the grazing phase (*e.g. C. lytica* 'type 2', clone agg58 'type 1' in the control; *Orientia*-related bacteria in both treatments) or in the phase after the grazing with increasing bacterial production in replicate-2 of the experimental mesocosms (corresponding to *Ruegeria* and Clone agg58 'type 1' in both treatments, and additionally *C. lytica* 'type 2' in the control). After grazing chlorophyll *a* concentrations increased temporarily in nutrient-enriched mesocosms (result not shown) and newly appearing bands were affiliated to *Skeletonema* and *Chlorella* plastids. DGGE patterns from the last phase of the incubation of both mesocosms were dominated by a few bands, (*C. uliginosa* in experimental mesocosm replicate-2; *Lewinella*-like bacteria replicate-2 of control; as well as a sequence affiliated with the δ–*Proteobacteria* in both treatments).

# 4. Discussion

## 4.1. Genetic diversity of the bacterial assemblages

The changes in community structure observed in duplicate mesocosms were reproducible within each of both treatments, i.e. nutrient addition and control. Multidimensional scaling of the DNA-derived DGGE patterns confirmed this reproducibility of replicates and differences between treatments, indicating that the addition of nutrients not only had a quantitative effect on the bacterial community dynamics (i.e. markedly higher increases in bacterial cell counts and production; compare [18]) but also had a qualitative effect on the development of the bacterial assemblages. In contrast to the changes observed in genetic fingerprints of the bacterial assemblages in the mesocosms, the genetic fingerprints of the assemblage at the natural sampling site underwent only minor changes, which indicated a relative stability of the community composition in the natural environment over a period of 15 days. Hence, even the control mesocosms deviated from the natural situation. This was probably a consequence of the confinement and sample handling, e.g., the prefiltration over a 200 µm mesh-size net may have lead to trophic cascade effects. Although, mesocosm experiments introduce some bias in the evolution of bacterial communities compared to those naturally occurring in the field due to confinement and handling effect, these experimental tools are of great interest to investigate how some environmental factors and processes such as nutrient addition and grazing induce temporal variations of specific populations among bacterioplankton [28,29]. Furthermore, coastal areas may also be subject to rapid environmental changes due to pulsed nutrient input from river discharges and phytoplankton blooms, but variations in community structure are more complex to study and to understand since they occur at both spatial and temporal scales.

The changes in the genetic fingerprints of the bacterial communities in the mesocosms reflected the different phases during batch incubation observed with respect to bacterial and protozoan counts and bacterial productivity (compare [18]). Initial changes in banding patterns from sampling time 0 h to 96 h coincided with the cellular production in all mesocosms. These initial changes in the community were rather bottom-up regulated, as a

Table 1
Distribution of dominant DGGE-defined populations detected from DNA and RNA during different phases of the incubation in control (CON) and experimental (EXP) mesocosms (replicates-2 only), and in natural seawater samples (SEA) taken from the coastal sampling site one day before (-24 h) and one day after (336 h) the mesocosm experiments as identified by partial 16S rRNA gene sequencing (range: 338-549 bp).

| Phylogenetic group | DGGE-bands[a] | | | | GROWTH[b] | | | | GRAZING[c] | | | | POST-GRAZING[d] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Simil-arity (%) | Sea –24 h | | CON | | EXP | | CON | | EXP | | CON | | EXP | | Sea 336 h | |
| | | | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA |
| **α–Proteobacteria** | | | | | | | | | | | | | | | | | | |
| clone chab-I-5 | AY-57, N-80 | >97.6 | ++[e] | ++ | +– | +– | + | + | + | + | – | – | – | – | – | – | ++ | ++ |
| *Sulfitobacter* | AY-51, BY-81 | >99.8 | + | + | ++ | ++ | ++ | ++ | + | + | + | + | + | + | + | +– | + | – |
| *Ruegeria* (*C. virginica* 'symbiont') | AY-58, AY-41, BY-95, BY-2, BY-74 | >96.9 | + | + | + | ++ | + | + | + | + | + | + | ++ | ++ | ++ | ++ | + | – |
| *Paracoccus* | N-62 | 91.6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + |
| *Orientia* | AY-52, BY-70 | 89.4 | – | – | – | – | – | – | – | + | ++ | ++ | + | + | + | + | + | – |
| **γ–Proteobacteria** | | | | | | | | | | | | | | | | | | |
| *Alteromonas* | AY-84, AY-85, AY-85b, AY-86, AY-91, BY-92, BY-93 | 98.2-100.0 | ++ | ++ | ++ | + | ++ | + | ++ | + | ++ | + | + | – | + | – | + | – |
| clone OM60 (SAR86 cluster) | AY-89, BY-97 | 87.5 | + | – | + | – | + | – | + | – | + | + | + | ++ | + | ++ | + | – |
| **δ–Proteobacteria** | | | | | | | | | | | | | | | | | | |
| clone OM27 | AY-59, BY-75 | >92.0 | – | – | – | – | – | – | – | + | – | + | + | ++ | + | + | – | – |

Table 1 (continued)

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Cytophaga-Flavobacterium*** | | | | | | | | | | | | | | | | | | |
| *Cytophaga lytica* ('type 1') | AY-33, BY-64 | >91.2 | + | – | ++ | + | ++ | + | + | + | – | – | – | – | – | – | + | – |
| *Cytophaga lytica* ('type 2') | AY-37, AY-56 | >89.9 | ++ | – | ++ | + | + | + | ++ | ++ | + | + | ++ | ++ | + | + | ++ | + |
| *Flexibacter maritimus* | BY-65 | 97.3 | – | – | + | + | ++ | + | + | + | – | – | – | – | – | – | – | – |
| *Cytophaga uliginosa* | BY-66 | 92.6 | – | – | – | – | – | – | – | – | – | – | – | – | ++ | + | – | – |
| *Lewinella* / clone agg32 | AY-38 | 86.6 | – | – | – | – | – | – | – | + | – | – | + | + | – | +– | – | – |
| clone agg58 ('type 1') | AY-32, BY-19, BY-71 | >84.9-86.6 | + | +— | + | + | + | + | ++ | ++ | + | + | ++ | ++ | ++ | ++ | + | +— |
| clone agg58 ('type 2') | BY-7 | 87.5 | ++ | + | + | + | + | + | + | ++ | + | + | ++ | ++ | + | ++ | ++ | + |
| ***Cyanobacteria* & chloroplasts** | | | | | | | | | | | | | | | | | | |
| *Synechococcus* | AY-26, AY-29 | 99.0 | + | + | + | + | + | + | – | – | – | – | – | – | – | – | + | + |
| *Chlorella* plastid | BY-10 | 91.7 | + | — | + | – | +– | +— | – | – | + | + | – | – | + | – | + | – |
| Diatom plastid *(Skeletonema)* | BY-8 | 94.8 | – | – | – | – | – | – | – | – | + | + | – | – | + | + | – | – |

a: designation of band sequences: AY- marks sequences from DGGE analysis of the control (replicate-2), BY- those obtained from the experimental mesocosm (replicate-2), and N- marks sequences obtained from DGGE fingerprints of the natural samples from the coastal sampling site (compare Fig. 1 and Fig. 3 for positions of bands)
b: 'growth' phase comprises data of sampling times 0, 48 and 96 h of mesocosms;
c: 'grazing' phase refers to sampling time 144 h in both mesocosms;
d: 'post-grazing' phase comprises sampling times 216 and 312 h;
e: ++, band among dominant bands; +, band well visible; + —, band hardly visible; –, band not detectable

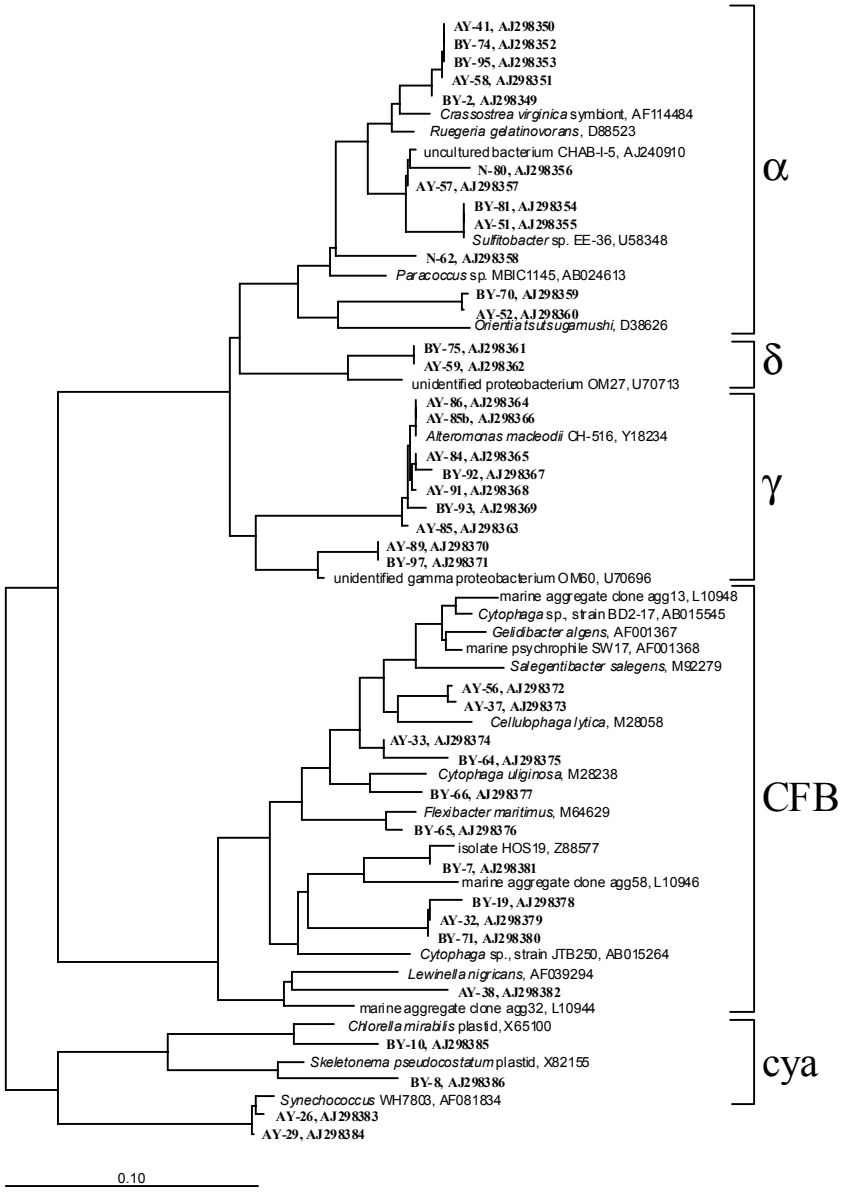


Fig. 4. Phylogenetic affiliation of predominant community members in mesocosms as revealed by comparative analysis of 16S rRNA sequences from DGGE bands and those stored in public nucleotide databases. Sequences determined in this study are shown in bold (for location of bands compare Fig. 1 and Fig. 3). Accession numbers of sequences are noted behind the taxon names, α, δ, γ, CFB and cya indicate the phylogenetic groups α–, δ– and γ–*Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* phylum and the phylum of cyanobacteria and algae chloroplasts, respectively. The scale-bar bar represents 10% estimated sequence divergence.

consequence of availability of substrates (which may be liberated during sample handling) and nutrients and subsequent cellular production. DGGE gels may show some gel-to-gel variation in resolution, and furthermore, despite careful quantification of PCR products, slight differences in the amounts of PCR products applied to individual lanes may occur. Therefore, the band numbers reported here are the higher number of two runs on replicate denaturing

gradient gels for replicates-2 of control and experimental mesocosms. The decrease of the number of DNA-derived DGGE bands might indicate changes in the evenness (i.e. a lower evenness) of bacterial populations, which would be plausible if a limited number of populations was responsible for the bulk of cellular production. A reduction in the number of bands in DNA-derived DGGE profiles may indicate a shift in the relative importance of r-selected versus K-selected microorganisms within the community (for the concept of r- and K-selection in microorganisms consult [30]). As the threshold for detection of a population by PCR-DGGE is around 1% [31,32], K-selected bacterial populations with a slow growth rate near this detection threshold could rapidly be eliminated from DGGE patterns during phases of cellular production with population bursts of r-strategic microorganisms. That such shifts in evenness occurred is very likely given the marked fluctuations in bacterial counts [18]. Furthermore, the disappearance of some bands during the start of the batch incubation may also have been due to increased mortality of specific populations, *e.g.*, by viral lysis.

In contrast to observations made on DNA-derived DGGE bands, in the experimental mesocosms (replicate-2) the number of RNA-derived DGGE bands increased during the growth phase, suggesting that RNA levels increased in a number of bacterial populations as a consequence of metabolic activation. However, after the peak in protistan biomass, which would have been the time of strongest grazing pressure, numbers of RNA-derived bands in the experimental mesocosms decreased again. This observation might be the consequence of preferential removal by grazing of active cells. In the control mesocosm the number of RNA-derived bands reached a maximum later (around sampling time 216 h), yet in the control the grazing phase was also less clearly identifiable than in the experimental mesocosms.

### 4.2. Identity of dominant bacterial populations

Sequencing showed that during the growth phase specific bacterial populations responded to the environmental changes. A very rapid response was that of the population of *Cytophaga lytica*-like bacteria ('type 1'), which only had a weak band in profiles of natural samples, but which had strong bands in time 0 h samples, suggesting a high growth rate for this population. In contrast to this another population distantly related to *C. lytica* ('type 2') seemed to be persistent throughout the experiment in control mesocosms, and also under natural conditions. Interestingly, the DGGE band of the latter population rapidly became less dominant during the growth phase in the nutrient treatment, and remained there only as weak bands throughout the experiment, possibly indicating that it was overgrown by bacterial populations with higher growth rates that better responded to the nutrient addition. A similar behaviour was noted for the α–proteobacterial populations represented by sequences AY-57 and N-80 (bacteria from the *Rhodobacter* group). The corresponding DGGE band was present in both natural samples, with strong RNA-derived bands, suggesting these bacteria were active in the natural situation. The corresponding DGGE bands became weaker and finally disappeared from the DGGE fingerprints of both treatments, suggesting they were not competitive in the new situation. Other bacterial populations from the *Rhodobacter* group developed in a different way. Bands representing *Sulfitobacter* species (AY-51 and BY-81) appeared to be activated, as the RNA-derived band of the natural sample only showed a weak band which became more dominant during the growth phase. This might suggest that bacteria related to *Sulfitobacter* may develop an r-strategy as proposed by Lebaron et al. [18].

As already discussed above and in the accompanying paper [18] nanoprotozoan grazing also appeared to affect bacterial community structure, since genetic fingerprints of bacterial communities remained almost identical during the growth phase in control mesocosms and changed only little in nutrient amended mesocosms (compare Fig. 1) until grazing of protists reduced bacterial abundance. Van Hannen et al. [22] also reported on the influence of protistan grazing on the genetic diversity of freshwater bacterial assemblages in a

continuous flow system investigated by DGGE. They, however, found a decrease in the number of detectable sequence types (*i.e.* DGGE bands) associated with high nanoflagellate grazing pressure. In this study and in a previous mesocosm experiment that showed a similar predator-prey succession [13,33], we found that changes in DGGE patterns associated with grazing rather consisted in an increase of the number of bands, detection of new band types, as well as in shifts of relative band intensities. These contrasting observations might be attributable to the different experimental systems used (continuous flow versus batch incubation) or point to principal differences between freshwater and marine bacterioplankton food webs.

Grazing associated changes in the genetic diversity manifested in nutrient-amended mesocosms in the banding pattern of sampling time 144 h, while in the control, largest changes were seen from 144 h to 216 h (see Fig. 1 and 2). If we accept that grazing eliminated the bulk of active cells, *i.e.* the predominant populations of the growth phase, then the increase in the number of apparent bands might be a consequence of higher evenness of bacterial populations as compared to the phase of cellular production. Bacterial populations that were detectable by DGGE after the phase of high grazing pressure, included a *C. lytica*-related population ('type 2'), the CFB related to a clone obtained from marine aggregates (clone agg58; [10]) and bacteria related to *Ruegeria gelatinovorans*. DGGE bands of these populations were very dominant in RNA-derived DGGE patterns of the post-grazing phase, suggesting that these bacteria were very active at the time of high grazing pressure.

Overall, sequencing of DGGE bands suggested that the taxonomic identity of active populations shifted during the experiment. As two PCR reactions with alternate reverse primers were performed on each sample, assignment of tentative dominant populations as concluded from band intensities would only be possible within the range of sequences amplified well by each primer set. However, whole cell hybridisation with fluorescently-labelled oligonucleotides suggested that α–*Proteobacteria* populations consisted of cells with a high ribosomal RNA content that became more abundant after the peak of grazing, while the fraction of γ–*Proteobacteria* declined during the incubation [18].

Members of the CFB phylum were only rarely encountered by fluorescent in situ hybridisation with a slight increase after the grazing peak [18], yet by DGGE they were detected throughout the incubation. Unfortunately, the DNA fragment used for DGGE analysis does not contain the target site of probe CF319a [34]. Weller et al. [35] have shown recently that only a part of the members of this phylum contain the proper target site. Inspection of the sequences to which the DGGE-band-sequences are most closely related, showed that almost all of them contained the proper target site of probe CF319a. Therefore, it seems unlikely that the low fluorescent in situ hybridisation counts were a consequence of bad probe coverage, at least in those subgroups of the CFB phylum that were detected here. Hence, the bands rather seem to represent populations with a high biomass and nucleic acid content. The strong bands of CFB in the DGGE patterns obtained from RNA after the grazing peak suggests these bacteria were active and thus the slight increase of CFB counts may have been due to a better detectability due to a higher ribosome content. Consequently, the strong RNA-derived DGGE bands of CFB in the post-grazing phase were probably reflecting an activation of the corresponding populations.

Bands representing γ–*Proteobacteria* related to *Alteromonas macleodii* and members of the SAR86-cluster were obtained with reverse primer 907RA. While *Alteromonas*-related bacteria were already found in the samples from the Mediterranean Sea at time –24 h until after the peak in grazing activity, representatives of the SAR86 cluster became more important during the incubation. The fraction of isolates affiliated to *Alteromonas* similarly decreased during the course of the experiment [18]. Recently, we have shown that *Alteromonas*-related bacteria dominated clone libraries of the post-grazing phase of a similar

batch incubation and suggested they had an advantage during high grazing pressure [33]. The observation that DGGE bands representing *Alteromonas* became less important after the peak in grazing activity in this experiment might therefore question our earlier hypothesis. Yet, we also found that these *Alteromonas* populations were already active in the beginning of the incubation and in the natural situation as suggested by their dominant rRNA-derived DGGE bands. Most of the DGGE-bands representing *Alteromonas* were also not detected in the natural samples taken after the mesocosm experiment. Hence, their potential to sustain an active and abundant population throughout the whole incubation period might have been limited. This finding indicates that mesocosm experiments may yield similar observations regarding global parameters (*e.g.* bacterial and protozoan counts, bacterial production), yet changes in bacterial community composition may be rather case-specific observations, that depend on the initial state and the history of the water taken for experiments.

**Conclusions**

Nutrient addition triggered qualitative differences in the bacterial assemblages of control and nutrient-amended mesocosms, and also in some differences regarding taxonomic identity of important community members. Nutrient addition as well as subsequent grazing processes affected bacterial genetic diversity and activity. The data presented here suggest that DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments as well as reverse transcribed RNA is a useful approach for monitoring changes in the identity of predominant and active populations within marine bacterial assemblages. While it is not possible to quantify the activity of particular populations via DGGE patterns, DGGE fingerprinting may be a first step of quantitative analyses providing an overview over a number of samples and aiding in the development of 16S rRNA targeted probes for subsequent slot blot hybridisation of rRNA [20,36] or whole cell hybridisation [2,14].

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# Chapter 5

# Bacterial activity and genetic richness along an estuarine gradient (Rhone river plume, France).

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## Abstract

Bacterial diversity and activity, were simultaneously investigated by microbial ecological and molecular biological methods along an estuarine gradient from the Rhone river to the Mediterranean Sea). Following a Lagrangian strategy, we have sampled plume, frontal and marine layers. The sampled estuarine gradient exhibited large changes both in physico-chemical and microbiological characteristics. Bacterial abundances and activities showed a more drastic decrease in the low salinity range of the gradient than expected from simple dilution models, indicating that an important fraction of freshwater bacteria disappeared in the mixing area. High specific activities, in particular for leucine, in the marine end-part of the gradient, suggested important bacterial protein synthesis, which may be the sign of active survival strategy for bacterial communities submitted to oligotrophic conditions. Bacterial genetic diversity of the sampled estuarine area, as estimated by the number of DNA-derived DGGE bands, was high (13-55 bands) compared to that reported in other aquatic ecosystems. This high diversity may be the consequence of the interface position of estuaries. The proportion of active populations was estimated using the ratio of DGGE bands derived from RNA and DNA. This ratio was lower in Rhone water than in marine water indicating that only a part of the constitutive populations were active, while the activity was distributed within a larger fraction of populations in the marine assemblage. Very few DGGE-bands detected from freshwater samples were also detected in the marine end-part of the gradient suggesting that a very limited number of freshwater bacteria could survive under marine conditions. Detection of these freshwater populations from RNA might indicate that these bacteria were able to synthesize different stress proteins as the result of a survival strategy or that these bacteria were able to maintain a metabolic activity under marine condition. The structure of marine communities was strongly affected by decreasing salinity. However, it seems that the decrease of DNA-derived bands may simply have been the consequence of the mixing of marine and freshwater.
No obvious relationship between genetic richness and activity changes was observed. This lack of relationship may be the consequence of a very short residence time of water in the studied mixing area.

## Introduction

Today, especially in the context of growing anthropogenic pressure on aquatic ecosystems, a large attention is given to prediction of the impact of anthropogenic perturbations on diversity of plants and animals and subsequent consequences on the functioning of these ecosystems (Proulx et al. 1996). In aquatic systems, bacterioplankton plays a main role in the biogeochemical cycles of major elements such as C, O, N, P and bacterial activity is an important process in controlling water quality. However, despite an increasing number of publications on bacterial diversity, little is known about (i) the effect of changes in environmental conditions on the composition and structure of bacterial communities, and about (ii) the relationships between bacterial diversity and productivity or activity (Schäfer et al. 2001). This can be explained by the inadequacy of culture-based methods used to record microbial diversity. The application of molecular biological methods, theoretically allows a more objective view on microbial diversity. However, so far only a few studies have tried to study bacterial diversity and activity in parallel. To our knowledge, such studies have been limited to very specific environments (e.g., biofilms, Santegoeds et al. 1998) or conduced in mesocosms (Lebaron et al. 1999, Schäfer et al. 2001), mainly because of difficulties in analyzing many samples simultaneously.

In this paper, we performed a joint study of bacterial diversity and productivity along the estuary of a large river flowing into the Mediterranean Sea (the Rhone river). Estuaries are coastal ecosystems where large changes in environmental conditions occur due to the mixture of fresh with marine waters. Inorganic nutrients and organic matter from watersheds are transported through estuaries, and can be processed by different freshwater, brackish and marine populations of microorganisms. Among marine ecosystems, estuaries appear to support high mean bacterial production values (Ducklow and Carlson 1992). Another striking aspect of estuarine bacterial production and biomass values is their large variations both from a spatial or temporal point of view (Ducklow and Shiah 1993, Painchaud et al. 1995, Goosen et al. 1999). Such ecosystems thus appeared as good systems to try to use in parallel classical microbial ecological methodologies and molecular biological methods (i.e. PCR -DGGE) for exploring possible links between bacterial diversity and activity.

## Material and methods

### Sampling strategy

The Rhone river plume (Gulf of Lion – northwestern Mediterranean Sea) was investigated following a Lagrangian strategy on April 20, 1998 (Fig. 1). For this experiment, a drifter (Naudin et al. 1997) was launched one mile south of the Rhone river mouth (Roustan buoy) to follow the mixing of the river plume with the marine underlying water. The drifter was tracked by the oceanographic research vessel Téthys II, on which continuous conductivity-temperature-depth (CTD) measurements were performed to select stations for sampling of water with different salinity. Five depths (0.5, 1.1, 2.2, 3.4, 10 m) were sampled along the halocline at stations B, C, D, and E see Fig. 1). Due to the limited water depth at the mouth of the river and the high-speed of the drifter only one surface sample was obtained from this area (A). Samples were collected outside the influence of the ship using a weighted polystyrene floating plate (1 m long, 0.6 m large) connected to the research vessel with 7 meters long Teflon tubes; the system allowed a very precise sampling at the desired depth (Naudin et al. 1997). Freshwater samples were collected in the Rhone river upstream the river mouth at three locations (R1, R2 and R3) across the river. For all measured variables, the reported results are mean values determined at the three stations. The marine reference station (S) (43°15’ N, 4° 44’ E; see Fig.1) (S) was also sampled at three depths (5, 30, 60 m). Samples were processed on board, either stored without any treatments or fixed in sterile bottles in the dark at 4°C.To avoid too long storage, samples were transported from the research vessel to a land-based laboratory (Salins de Giraud) by a high-speed shuttle.

Rough salinities were measured on board with a portable thermo-salinometer for the determination of the sampling stations. More accurate salinity measurements (using the Practical Salinity Scale) were performed at the land-based laboratory using a Guildline 8410 salinometer with an accuracy of ± 0.01.

### Ammonium concentration

Sub-samples for ammonium analysis were directly collected on board in 100-ml glass bottles. According to the Koroleff’s manual method (1976), the reagents were added immediately and the measurements were performed 24 hours after collection by spectrophotometry. The optical density was measured on each original sample to account for turbidity and salt effects in the final concentration value. The accuracy of the ammonium measurements was ± 0.2 µM.

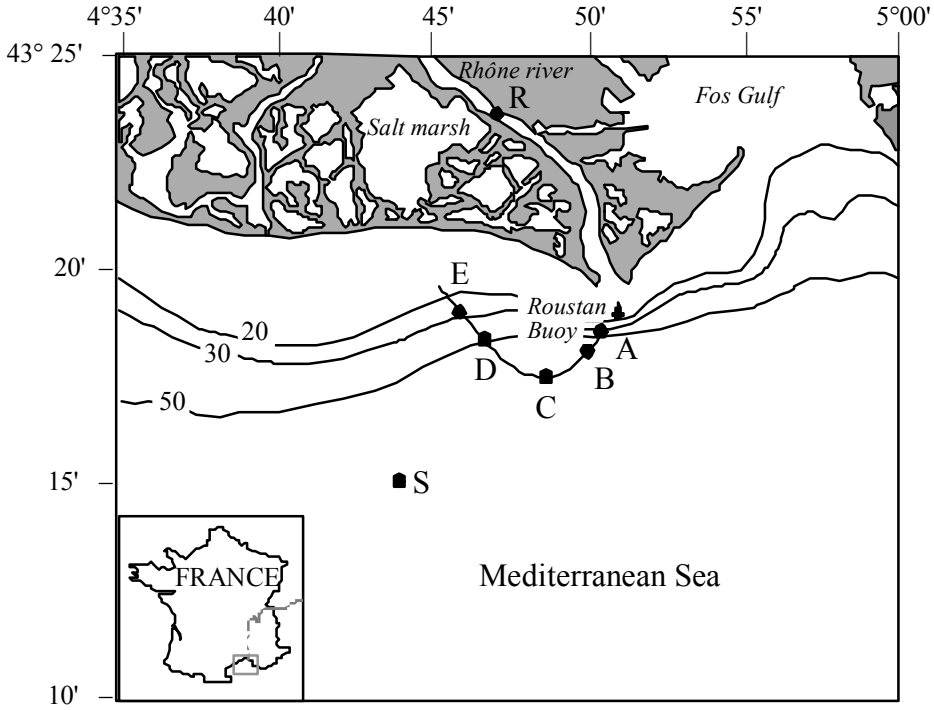



**Figure 1.**

Sampling locations in the Rhone river plume.

## Phytoplanktonic abundance

Phytoplankton counts were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Ca) equipped with an air-cooled argon laser (488 nm, 15 mW). Cells excited at 488 nm, were detected and enumerated according to their right angle light scattering properties (RALS) and their orange (585/42 nm) and red fluorescence (> 650 nm) emissions related to phycoerythrin and chlorophyll pigments, respectively. Fluorescent beads (0.94-µm) (Polysciences Inc., Warrington, PA) were systematically added to each sample. The ratio of mean fluorescence or RALS of a cellular population to that of 0.94-µm beads was used to normalize cell fluorescence emission and scatter values among samples. The precise volume analyzed and subsequent estimations of cell concentrations were calculated by measuring the remaining volume and subtracting it from the initial sub-sample volume (1-ml). Since part of the measured volume was used for flushing the sample line, the outer sleeve of the sample injection port of the flow cytometer was removed.

Different phytoplanktonic groups were discriminated according to their fluorescence and scatter characteristics as described previously (Troussellier et al. 1993, Campbell et al. 1994). For this paper, we have only considered two phytoplanktonic groups: prokaryotic (*Synechococcus*-like and *Prochlorococcus* cells) and eukaryotic (picoeukaryotic and larger cells) cells.

## Protozoa abundance

The abundance of protozoa was determined by epifluorescence microscopy (Leitz, Laborlux D) after DAPI staining. Water samples (20 ml) were preserved with glutaraldehyde (0.5 % (v/v) final concentration) and stained with DAPI (10 µg ml-1, final concentration) for 15 min. Stained protists were collected by filtration on 0.8 µm Nuclepore black filters. The filters were mounted on microscope slides and stored at +4°C until examination. Pico-sized (< 2 µm in diameter) and nano-sized (2-20 µm in diameter) micro-organisms were identified, counted and measured at a magnification of 1,250x and 625x, respectively, while micro-sized (20-200

µm in diameter) micro-organisms were analyzed at a magnification of 125x. A minimum of 100 organisms per filter was counted. Autotrophic species were distinguished from heterotrophs by the red autofluorescence of chlorophyll a observed under blue light excitation. Data presented in this paper only concern heterotrophic protozoa.

**Bacterial abundance and cellular characteristics**

Bacterial cells were enumerated using SYTO-13 staining and flow cytometry following Troussellier et al. (1999). Briefly, 1 -ml formaldehyde-fixed sub-samples were directly incubated with SYTO- 13 at a final concentration of 5 µM for 10 to 15 min. at room temperature in the dark. For each sub-sample, three replicate counts were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Ca) equipped with an air-cooled argon laser (488 nm, 15 mW). Stained bacterial cells, excited at 488 nm, were enumerated according to their right-angle light scatter (RALS) and green fluorescence (FL1) collected at 530/30 nm. These cell parameters were recorded on a four-decade logarithmic scale mapped onto 1024 channels. Standardized RALS values (cell RALS divided by 0.94-µm beads RALS) were used as an estimation of the relative size of bacterial cells (Troussellier et al. 1999). Cell concentrations were calculated as for phytoplankton (see above).

**Bacterial activity**

Two methods based on radioactive incorporation assays were used to estimate bacterial activity: measurement of 3H-thymidine incorporation rate into DNA (Fuhrman and Azam 1982) and 3H-leucine incorporation rate into bacterial proteins (Kirchman et al. 1985). Incorporation of 3H-thymidine (Amersham 84 Ci/mmol) was measured on each sample at four thymidine concentrations (6 to 32 nM). Four 10-ml subsamples were incubated in the presence of the four different concentrations of tritiated thymidine for one to two hours in the dark at in situ temperature. After incubation, cold trichloroacetic acid (TCA) (final concentration 5% [w/v]) was added and the samples were filtered over 0.2 -µm-pore size cellulose acetate membrane. Radioactivity associated with the filters was estimated by liquid scintillation. Incorporation rates expressed in pmol $l^{-1}$ $h^{-1}$ at the different thymidine concentrations were calculated and plotted against the added thymidine concentrations. The maximum incorporation rates were estimated by best fitting a hyperbolic function to the experimental data using a software based on the least squares criterion (Servais 1995). Data presented in the paper are maximum incorporation rates. Incorporation of 3H-leucine (Amersham 151 Ci/mmol) was measured at four leucine concentrations which ranged from 2 to 77 nM (2 nM of tritiated leucine in each case with 0-75 nM non-radioactive leucine) (Servais 1990). Four 10 -ml sub-samples were incubated in the presence of the four different concentrations of leucine for one to two hours in the dark at in situ temperature. After incubation, TCA was added and the samples were filtered over 0.2 µm pore size cellulose acetate membrane. Radioactivity associated with the filters was estimated by liquid scintillation. Maximum incorporation rates were calculated as for thymidine incorporation.

**Nucleic acid extraction, PCR and DGGE**

Nucleic acid preparation was performed following the hot phenol protocol of Sahm and Berninger (1998). Bead-beating and DNase steps were omitted. Recovery of sequences of Gram-positive bacteria in previous studies has shown that the modified protocol is also suitable to obtain DNA from Gram-positive bacteria (Rossello-Mora et al. 1999). PCR amplification of 16S rDNA fragments between E. coli positions 341 and 926 (Brosius et al. 1981) was carried out as previously described (Muyzer et al. 1998) but reactions contained 1 unit of Taq-polymerase and the buffering conditions recommended by the manufacturer (Pharmacia, Uppsala, Sweden). Reverse transcription of RNA and amplification of the 16S

crDNA was performed as previously described (Schäfer and Muyzer 2001). Denaturing gradient gel electrophoresis (DGGE) was done according to Muyzer et al. (1998) using denaturing gradients from 20 to 80% denaturants (100 % denaturants is a mixture of 7M urea and 40% [v/v] deionized formamide). Electrophoresis was performed at a constant voltage of 200 V for 3.5 h. Gels were stained in ethidiumbromide (0.5 µg/ml), rinsed in MilliQ-water and scanned under UV-illumination using the Fluor-S system (Biorad, Hercules, Ca.). The acquisition software Multi-analyst (Biorad, Hercules, Ca.) was used to invert images and for enhancing the sharpness using the "sharpen in place" filter.

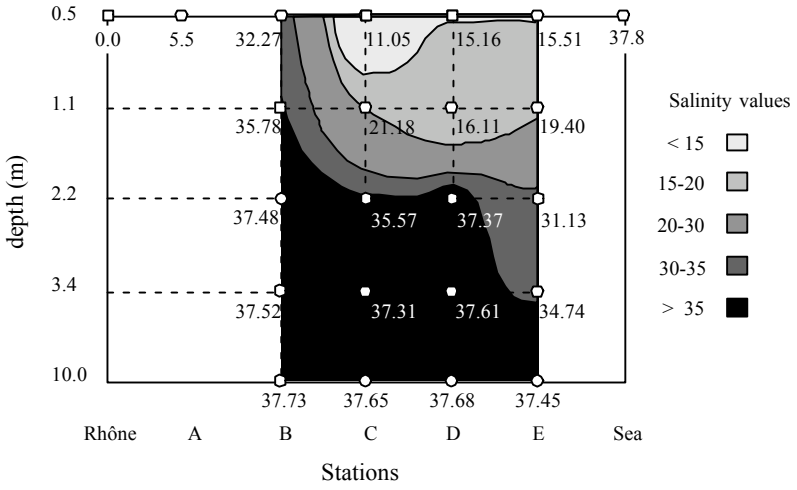



**Figure 2.**

Salinity distribution during the sampling campaign.

# Results

### 1. The environmental gradient

During the sampling cruise the Rhone river flow was 2500 m3 s-1 (river discharge measured at Beaucaire, 65 km upstream from the river mouth). The wind velocity during the preceding days was high (10 m s-1). As a consequence, the plume was not very well established. The time taken by the drifter to go from the beginning (Roustan buoy, Fig. 1) to the end of the sampling transect (station D, Fig. 1) was 5 hours and 30 min. Nevertheless, the estuarine area showed a 3-layer system as depicted by the salinity pattern (Fig. 2). A hydrological discontinuity could be seen in the plume at station B which may have been due to upwelling of deep water.

Plots of $NH_4$ concentration against salinity did not show significant deviations from the straight line linking river and seawater reference stations (Fig. 3a). This result indicates that the concentration of $NH_4$ followed the linear mixing model: When phytoplanktonic variables were plotted against salinity data, a significant increase of prokaryotic and eukaryotic cell numbers was observed (Fig. 3b). Regression analysis indicated that this increase was more important for prokaryotic than for eukaryotic cells, leading to an increase of the ratio prokaryotes:eukaryotes. Heterotrophic protozoa counts also increased along the salinity gradient (Fig. 3c); a 2.7 fold increase was observed between the river and the first station along the gradient.

### 2. Bacterial abundance and activity

Contrary to concentrations of $NH_4$, bacterial abundance showed a non-conservative pattern along the salinity gradient resulting in a dramatic decrease from the river to the mixing area (Fig. 4). Thymidine and leucine incorporation rates showed a similar decreasing pattern.

Abundance, leucine, and thymidine incorporation rates decreased by a factor of 1.6, 3.0 and 4.5, respectively, between the first two stations of the gradient.

However, when thymidine and leucine specific activities were computed, a strong decrease in thymidine specific activity was observed in the first part of the salinity gradient followed by an increase at the end part of the gradient (Fig. 5a). Leucine specific activity also decreased from the Rhone river water to the beginning of the mixture area, but then showed a strong increase appeared to increase from the beginning to the end of the salinity gradient (Fig. 5b). Apparent mean sizes of bacterial cells decreased all along the salinity gradient (Fig. 5c).

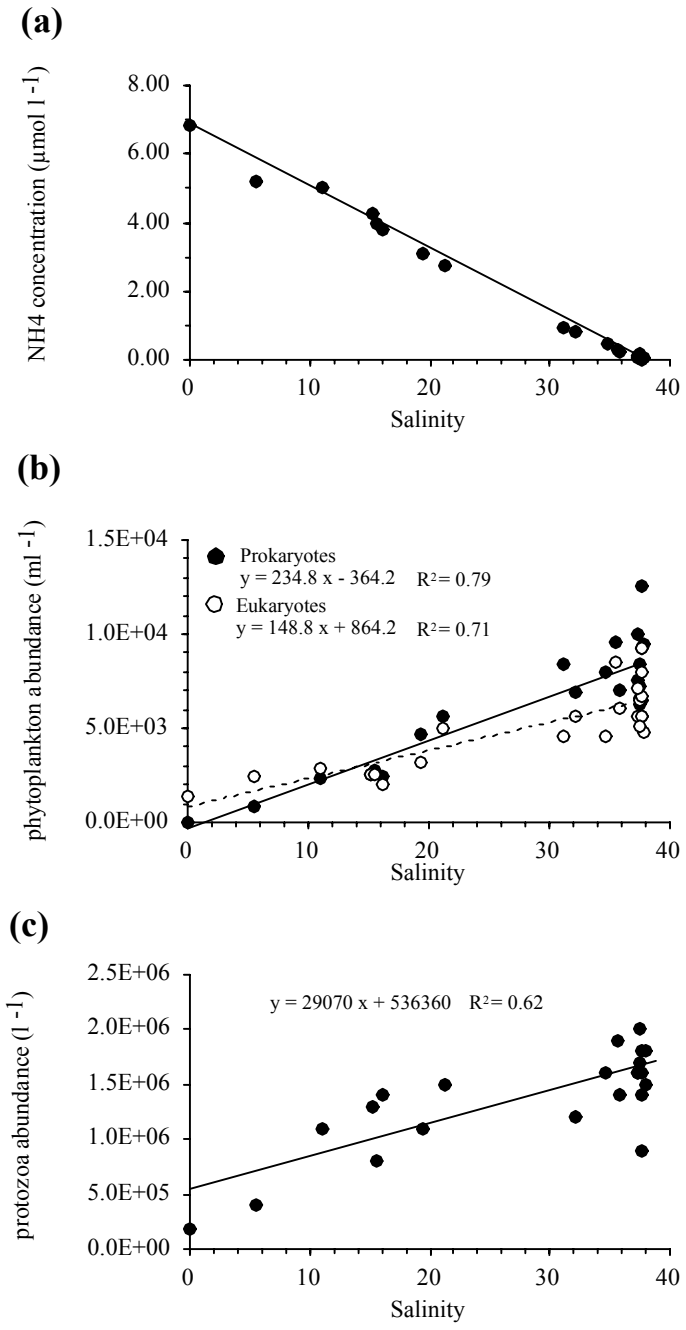



**Figure 3.**

$NH_4$ concentration (a), phytoplankton (b) and protozoa (c) abundances versus salinity values. Line in (a) corresponds to theoretical values for $NH_4$ if only dilution process occurred; Lines in (b) and (c) are regression lines obtained from linear regression models computed between abundance and salinity values.

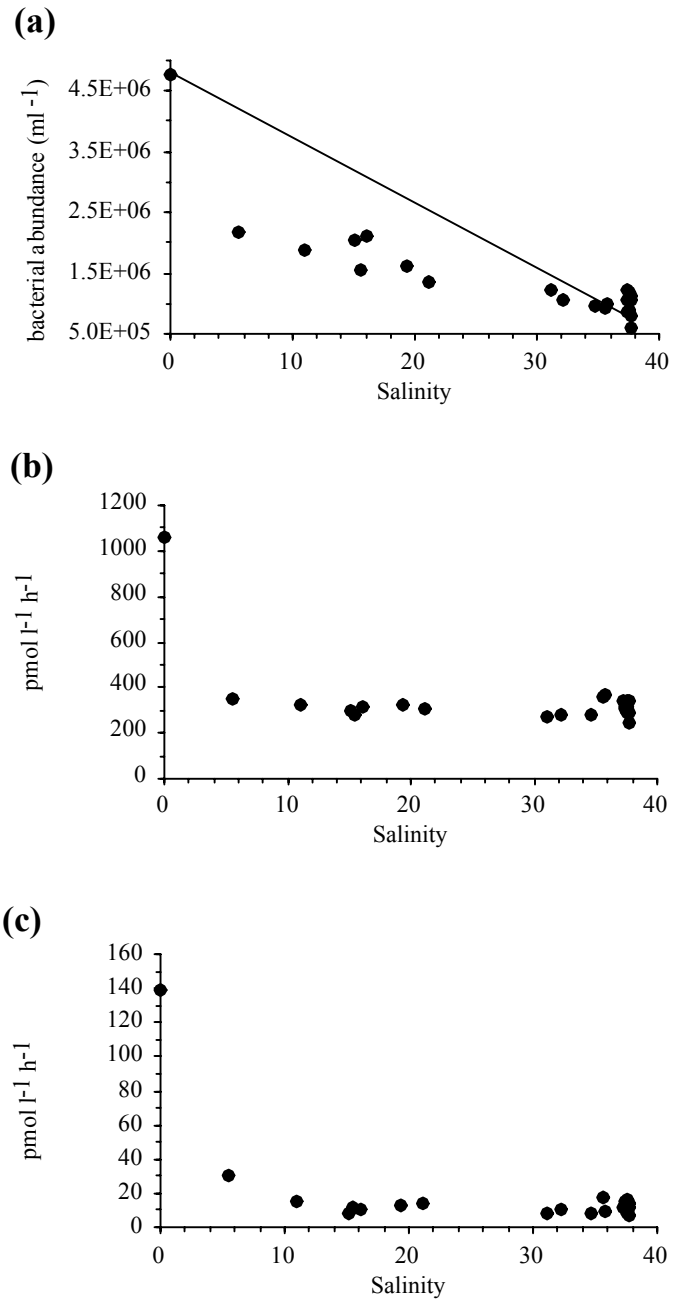



**Figure 4.**

Bacterial total counts (a), leucine (b) and thymidine (c) incorporation rates versus salinity values. Line in (a) corresponds to theoretical values for bacterial abundance if only dilution process occurred.

## 3. Genetic richness

The DNA-derived DGGE fingerprints of the bacterial communities from the river reference (station R) and from two depths (Sea 1: 5m; Sea 2: 30 m) of the marine reference station were obviously different (Fig. 6). The community fingerprint of the river sample showed a total of 47 discernible bands, while the fingerprint of the marine reference station displayed 35 and 34 bands for the water samples from 5 m and 30 m depth, respectively. These estimates, however, have to be regarded as minimum values, as dissimilar sequence types may co-migrate to the same position in the gel, thus leading to a potential underestimation of the number of different sequence types.

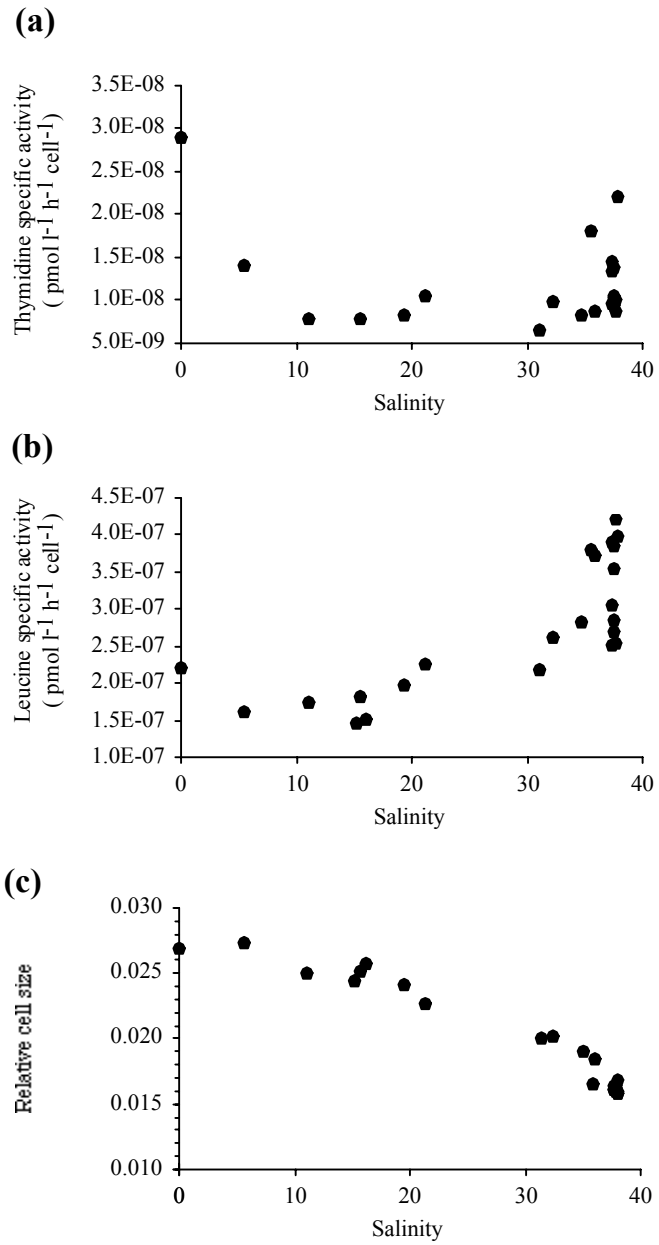



**Figure 5.**

Thymidine (a), leucine (b) specific activities and relative cell size (c) versus salinity values.

Assuming that the truly Mediterranean and Rhone bacterial communities shared no common bacterial populations, a total genetic richness, defined as the number of apparent DGGE bands across systems, accumulated to 82 different 16S rRNA sequence types. However, in a number of cases, bands from river and seawater samples were often at a similar position in the gel, and could not always be unambiguously identified as river or marine bands in the patterns obtained from samples of the zone where Mediterranean and Rhone water mass were mixing. Therefore, the number of discernible DGGE bands across systems (i.e. Rhone and Mediterranean) that could unambiguously be identified as originating from marine or river communities are 21 and 28 respectively

When the number of DNA-derived DGGE bands of the Rhone river (i.e., D-F bands, whereby D stands for DNA, and F for freshwater) were plotted against the salinity values, they exhibited a linear decrease (Fig. 7a) suggesting the disappearance of freshwater species.

D-M bands (DNA-derived DGGE bands from marine (M) and estuarine samples) showed the opposite pattern to the D-F profile (Fig. 7b), although in the marine part of the gradient (salinity between 40 and 30) there was an exponential decrease of the number of bands with salinity decrease.

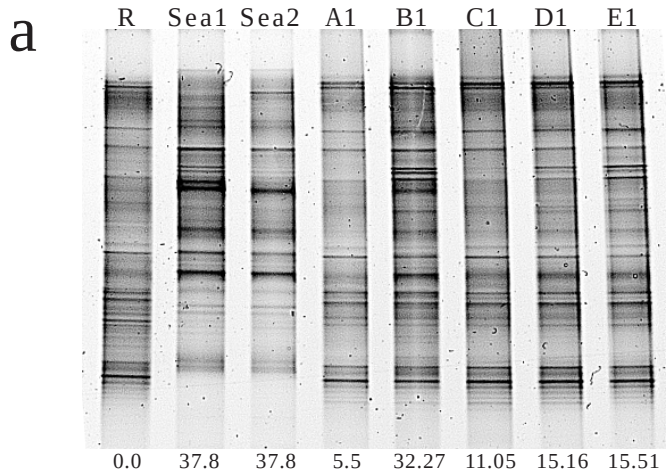
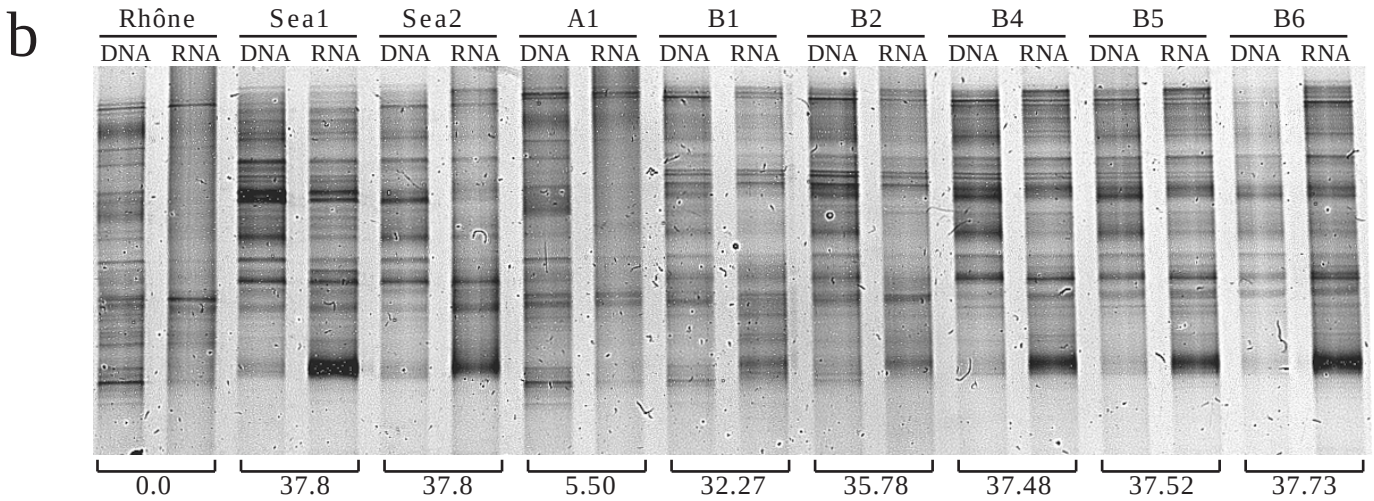



Figure 6.

(a) DNA-derived DGGE fingerprints obtained from the different sampling stations. Stations are: middle of the Rhone (R1), marine reference station 60 m and 30 m depth (S1 and S2, respectively), and surface samples of the stations in the river plume (A1, B1, C1, D1 and E1). The salinity of the water samples is indicated at the bottom of the lanes. (b) Example of comparison of DNA and RNA-derived DGGE-fingerprints from stations with different salinities. Samples B2, B4 and B6 are from depths of 1.1, 2.2, 3.4 and 10 m of station B, respectively.



Schäfer et al. (2001) have shown recently that changes in the number of detectable DGGE bands in a mesocosm experiment reflected trends in the activity of the bacterioplankton assemblage as estimated by leucine incorporation by Lebaron et al. (2001). Increases in the leucine incorporation rates were concomitant with increases in the number of rRNA-derived DGGE-bands, and vice versa. Furthermore, during phases of increased bacterial growth and production (as estimated from total counts and leucine incorporation), the number of rDNA-derived DGGE-bands declined, probably due to changes in the evenness of bacterial populations caused by differential growth. Therefore, in this study, the ratio between the number of DGGE bands obtained from RNA (R) and from DNA (D)-derived PCR products was used as an estimator of the proportion of active sequence-types in the bacterial community.

In the Rhone waters this ratio was 0.45, while in seawater it was 0.75. This ratio was computed for marine (M, Fig. 8a) and for freshwater (F, Fig. 8b) communities at each sampling station. M and F communities showed quite different patterns. For the bands corresponding to the freshwater pattern (Fig. 8a), the proportion of RNA-derived bands remained low in the surface layer where river and marine waters were progressively mixed (0-20) and then increased at higher salinity (>20). However, the highest values of the ratio (1.00) corresponded to a very limited number of DGGE bands (<5) suggesting that only a few populations from the freshwater community remained detectable from both DNA and RNA patterns. The proportion of RNA-derived bands from the marine community was high and less variable in the marine part of the salinity gradient but decreased strongly at salinity below 15 (Fig. 8b).

No statistical significant relationship was observed among genetic richness (i.e., the number of DGGE bands obtained either from DNA or RNA) and bacterial activity expressed by thymidine or leucine incorporation rates values.

## Discussion

The main objective of this study was to determine if a relationship exists between activity and diversity of bacterial communities in the estuarine area of the Rhone river in the South of France.

As expected, important changes in some environmental variables, which may affect directly or indirectly the bacterial assemblages, occurred along the Rhone estuary. According to the salinity variations, the sampled area was characterized by a 3-layer system (i.e., plume, frontal and marine layers) as described by Soto et al. (1993). Changes in $NH_4^+$ concentrations followed a simple dilution process of Rhone waters in Mediterranean seawater without significant gain or loss from biogeochemical processes. A similar trend was reported by Lochet and Leveau (1990).

The increase in abundance of both phytoplanktonic cells and heterotrophic protozoa along the salinity gradient has been already reported at least for the first part (i.e., the low salinity range) of the estuarine gradient (Soto et al. 1993) The ratio prokaryotes to eukaryotes increased from the freshwater to the seawater indicating a decrease in the water trophy, because prokaryotes predominate in oligotrophic waters as a consequence of their better efficiency to use scarce nutrients than eukaryotes (Weisse 1993).

Observed values of bacterial abundance and incorporation rates of tritiated thymidine and leucine in water samples taken along the transect were in the same range as those previously reported in the same area (Kirchman et al. 1989; Martinez et al. 1991; Bianchi et al. 1994). The drastic decrease of bacterial abundance observed in the low salinity range of the gradient indicated a net loss of freshwater bacterial cells when they encountered marine water. This trend is commonly found in different estuarine systems (see Painchaud et al.

[1995] and references herein). This decrease of bacterial counts was concomitant with a more important loss of activity as determined from thymidine and leucine incorporation rates. We can thus hypothesize that the activity of freshwater cells decreased strongly in the first part of the gradient and/or that an important fraction of active cells disappeared.

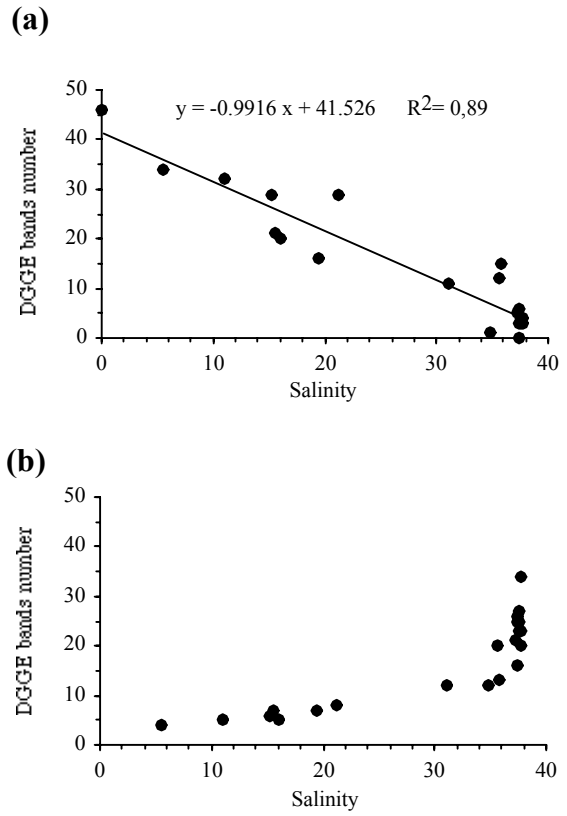



**Figure 7.**

Number of freshwater (a) and seawater (b) DNA-derived DGGE bands.

The decrease of activity for individual cells can be the consequence of environmental changes, such as the decrease of nutrient concentration and/or the increasing salinity. Disappearance of active cells can be the result of a lysis process due to an osmotic stress. However, at the beginning of the gradient only a limited change in salinity was observed. From the literature dedicated to the survival of allochthonous bacteria in seawater (e.g., Gauthier 2000) it seems that such a limited salinity change does not allow a significant decrease in total bacterial counts. Another explanation may be that active cells were preferentially grazed by protozoa (Del Giorgio et al. 1996; Bernard et al. 2000). This is congruent with the increasing number of heterotrophic protozoa from the beginning to the end of the salinity gradient. Furthermore, the decreasing cell size of bacteria from the river to the sea could be a consequence of this increasing grazing pressure, because heterotrophic protozoa more intensively grazed large cells than small cells.

At the marine end-part of the gradient, where more oligotrophic conditions predominate, overall thymidine and leucine incorporation rates were lower than in freshwater. However, thymidine and leucine specific activities showed that cells in the marine part of the gradient exhibited a relatively high specific activity. Leucine specific activities increased more than thymidine specific activities suggesting an important bacterial protein synthesis, which, in temperate estuaries, has been considered as the sign of a survival strategy of bacterial communities when they are submitted to oligotrophic conditions (Shiah and Ducklow 1997).

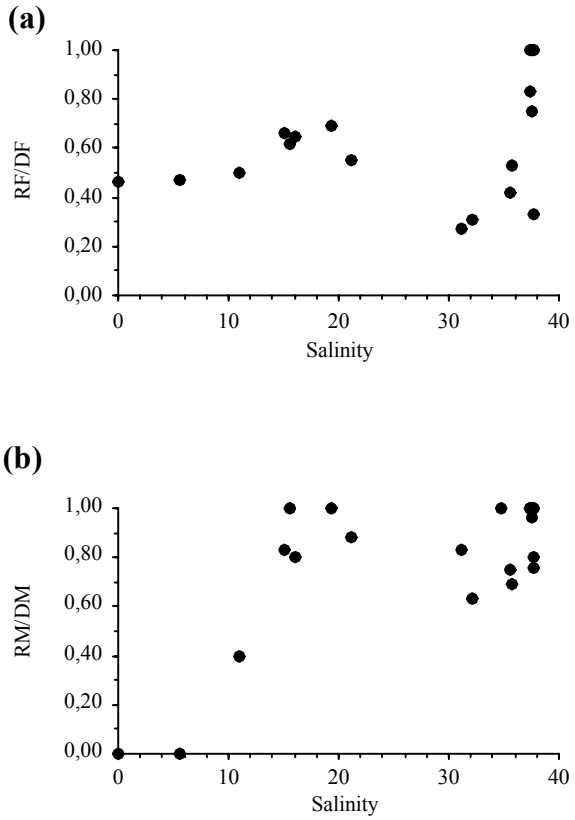



**Figure 8.**

Ratios between the number of RNA (R) and DNA (D) derived bands for marine (a) and freshwater communities versus salinity values.

The genetic richness of the Rhone river and Mediterranean Sea bacterial assemblages expressed by the number of bands in a DGGE profile was high compared to that reported in other aquatic ecosystems (Table 1). Diversity estimates from our study based on the analysis of 24 samples ranged from 13 to 55 DGGE bands for DNA-derived PCR products. It has to be kept in mind, though, that very few studies reported in table 1 have applied an appropriate sampling strategy to estimate genetic richness at the ecosystem level. Such a high genetic diversity may be the result of the interface position of estuaries where continental and marine bacterial communities are mixed. A link between diversity and activity was further investigated by comparing both DNA- and RNA-derived DGGE profiles

For freshwater samples, the number of bands found in DNA and RNA profiles was different and the ratio RNA/DNA was only 0.45. Assuming that bands are representative of different species, this suggest that only a part of the constitutive populations were active at the time of sampling. In contrast, the RNA/DNA ratio was 0.75 for marine communities showing that activity was distributed within a large diversity of populations. The linear decrease in the number of DNA-derived bands found in the profile of freshwater communities along the salinity gradient, indicated that continental bacterial populations were not able to significantly affect the structure and composition of marine communities. However, a few bands initially found in the freshwater profile ($n<5$) were detected in both RNA and DNA-derived DGGE patterns determined of samples from the end of the gradient. The corresponding increase of the RNA/DNA number of bands may be considered as the result of adaptive processes to survive under adverse conditions, which may necessitate the synthesis of different stress proteins, and thus previous expression of different genes. If so, this process seems to be operated only by a limited number of species because there was an overall decrease in the total number of cells. The fact that these freshwater populations were also detected in the RNA profile may also suggest that they are able not only to survive, but also to maintain a metabolic activity under marine conditions. Unfortunately, our results do not allow to discriminate between the two explanations.

Table 1. Number of DGGE bands (bacterial genetic richness) reported for different ecosystems (studies were ranked following decreasing maximum genetic richness).

| Reference | Ecosystem | Number of bands |
|---|---|---|
| van Hannen et al. 1999 | Algae-detritus | 54 |
| this study | Rhone estuary | 47 |
| Schäfer et al. 2001 | Mediterranean water mesocosms | 24-40 |
| Santegoeds et al. 1998 | Activated-sludge biofilm | 15-37 |
| This study | Mediterranean water | 34-35 |
| Moeseneder et al. 1999 | Aegean Sea | 28-36 |
| Murray et al. 1996 | San Francisco Bay | 22-31 |
| Schäfer et al. 2000 | Mediterranean water mesocosm | 19-29 |
| Murray et al. 1998 | Antarctic coastal waters | 8-29 |
| Murray et al. 1996 | Tomales Bay | 15-24 |
| Konopka et al. 1999 | Lakes | 8-23 |
| Gillan et al. 1998 | Biofilm of bivalve shell | 9-21 |
| Øvreas et al. 1997 | Meromictic lake (oxic zone) | 15-20 |
| Lindström 1998 | Boreal forest lake | 6-15 |
| Santegoeds et al. 1996 | Hot spring cyanobacterial mats | 14 |
| Jasper and Overmann 1997 | Water from shallow eutrophic lake | 6-10 |

The high ratio found between the number of RNA and DNA-derived bands in the marine community profiles suggests that most populations were able to grow in oligotrophic waters. To be able to survive and eventually to grow (e.g. to be able of an efficient uptake of scarce substrates) it may be important that these bacteria are able to maintain elevated levels of mRNA and tRNA. This hypothesis has early been suggested by Koch (1971) and verified in vitro by Morita (1982).

The structure of marine communities was strongly affected by decreasing salinity. One might hypothesize that the decrease of DNA-derived bands found in the seawater profile with decreasing salinity originated in a high sensitivity of some marine species to small changes in salinity. However, most probably, this pattern was a consequence of the different bacterial abundance in river versus marine samples. If the mean cell counts obtained from marine waters (6.15 105 ml-1) versus freshwater (4.6 106 ml-1) are considered, it can be easily demonstrated that mixing marine with river waters would establish an exponential decrease of the ratio between theoretical abundance of marine versus freshwater bacteria. Consequently, marine bacteria are quickly outnumbered and might not be detectable as distinct bands in DGGE patterns.

In the case of this study, the lack of relationship between bacterial diversity and activity measurements can be at least partly explained by hydrodynamic conditions. In fact, along the salinity gradient, we have sampled bacterial assemblages that either appeared as very different (such as the true Rhone and Mediterranean Sea communities) and adapted to their specific environment, or were extracted from a very dynamic area (plume) in which bacterial assemblages that were formed by the mixing of river and seawater bacteria have probably not enough time to adapt (the residence time of the plume was estimated less than 6 hours). Crump et al. (1999) found that a residence time of 1 to 2 days in case of the free-living bacterial communities in the Columbia River Estuary to be insufficient for the development of an adapted estuarine assemblage. On the other hand the aggregate-attached bacterial community of the Columbia River Estuary seemed to be adapted to the distinct estuarine conditions. Adaptation of the community is possible due to the prolonged residence time of particle-attached bacterial communities in the estuary, which was estimated as two to four weeks. We hypothesize that only in estuarine systems where the residence time of water from the mixing area is long enough, we can observed an adapted bacterial community which can support a relationship between activity and diversity.

To our knowledge, this is the first study that combine bacterial diversity and activity parameters in an attempt to get insight in the behavior of bacterial populations along a salinity gradient. Further understanding the role of bacterial populations in biogeochemical processes occurring along environmental gradients should require to provide information on the structure of bacterial communities (i.e., relative abundance of populations) and not only on richness. This is essential to really investigate the behavior of targeted species in these environments.

**Acknowledgements**

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# Chapter 6

# Does eutrophication alter bacterioplankton diversity? A four year experimental study in the Norwegian landlocked bay Hopavågen

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Manuscript in preparation

## Summary

Hopavågen, a small landlocked bay on the coast of Norway near Trondheim, was used as an experimental field for testing the consequences of experimental, sustained, enhanced nutrient addition in the summer seasons of 1998 and 1999 (with double amount of nutrients as compared to 1998), while 1996 and 1997 were investigated as control years without artificial eutrophication. Otherwise, Hopavågen is hardly impacted by anthropogenic eutrophication. This paper focuses on seasonal and annual patterns in bacterial community composition and on the question whether emerging patterns in the diversity of the bacterial community are impacted by eutrophication. Denaturing gradient gel electrophoresis analysis of bacterial 16S rRNA gene fragments showed seasonal variations in community composition that were annually recurrent. Largest changes were seen in the transition from winter/spring to the productive season. Some phylotypes appeared at similar times during the years, suggesting relatively stable seasonal distribution patterns of these phylotypes. *Synechococcus*–related cyanobacteria were predominant during summer until into autumn. α-Proteobacteria of the *Roseobacter* group seemed permanently present from spring through summer into autumn, suggesting coupling to the period of phytoplankton production or light availability. Other phylotypes also seemed to recur during discrete periods of the year. Primary production increased on an average by 16 and 75% in 1998 and 1999, respectively, compared to the average of the control years. Whereas no increase in chlorophyll *a* was observed in 1998 (104%), the higher dose of nutrients used in 1999 entailed significantly higher concentrations (164%). Nutrient addition created a clear response in the algal community, but an effect on the genetic diversity of the bacterial assemblage was not obvious.

## 1. Introduction

Eutrophication has become an apparent problem in the European coastal zone and is publicly associated mainly with the increased incidence of nuisance algal blooms Additionally to its economical impact, changes in species composition of natural communities and loss of diversity are among eutrophication effects that have received considerable scientific attention. Although hardly studied, changes in species composition and loss of diversity of the bacterial community might strongly affect flow of energy and material.

Recent mesocosm studies have demonstrated that the amount of eutrophication (thus, the nutrient dose) necessary to induce marked effects on pelagic food web structure may differ substantially between different types of European coastal waters (Olsen *et al*., 2001), and that it is for instance higher for Mediterranean than for Baltic waters (Olsen *et al*. a, in preparation; Olsen *et al*. b, in preparation). Mesocosm experiments with disturbed food web structure (organisms larger than 200 µm removed) and artificial nutrient addition to Mediterranean coastal water performed by Lebaron and co-workers found that nutrient addition had a strong effect on global parameters such as bacterial production and biomass (Lebaron *et al*., 2001). Analyses of bacterial diversity by PCR-DGGE and RT-PCR-DGGE of the same mesocosms revealed that changes in bacterial diversity in nutrient enriched and control mesocosms proceeded at a different pace and that the predominant and most active bacterial populations changed during the course of the incubation (Schäfer *et al*., 2001). In control and experimental mesocosms a few different phylotypes developed as judged from analysis of DGGE fingerprints. At the same time only little change in DGGE fingerprints occurred in field samples from the original sampling station, which strongly suggested that the incubation conditions and altered food web structure had severe impact on the community composition (Schäfer, *et al*., 2001). Thus, despite being widely used tools in microbial ecology (Duarte *et al*., 1997), mesocosms may be insufficient means to assess effect of eutrophication.

Seasonal distribution of bacteria in planktonic assemblages has been reported from limnic and marine systems (Höfle *et al*., 1999; Murray *et al*., 1998; Pernthaler *et al*., 1998; Pinhassi & Hagström, 2000). Pernthaler and colleagues (Pernthaler, *et al*., 1998) showed for a high mountain lake, that marked seasonal changes in community structure were occurring even during the phase of ice cover. Gordon and Giovannoni (Gordon & Giovannoni, 1996) found seasonal variation of the contribution of SAR402 phylotype to genomic DNA extracted from samples at a station in the Atlantic. Seasonal succession in bacterioplankton has also been assessed by whole genome probing. Data of Pinhassi and Hagstöm (Pinhassi and Hagström, 2000) indicated that a *Cytophaga*-dominated spring community formed after the collapse of the spring phytoplankton bloom, while the summer community was dominated by α-Proteobacteria related to *Sphingomonas* and *Caulobacter*. Murray et al. (1998) reported significant seasonal differences in bacterioplankton DGGE fingerprints from waters near Anvers Island (Antarctica) which were sampled over a period of nine months. The genetic richness as indicated by numbers of bands in DGGE patterns decreased from spring to summer, and increased again in autumn. Thus, there is increasing evidence that seasonal distribution patterns exist in marine bacterial assemblages.

The source of seasonality resides in strong changes of physical factors during the annual cycle, whereby the day-length and incident light angle ultimately influence the surface temperature, and hence stratification of pelagic systems. Especially, the light availability and stratification are important factors in promoting phytoplankton production.

In this study the microbial community of a landlocked bay in Norway, Hopavågen, was sampled over a period of almost 40 month from June 1996 until November 1999 and analysed by PCR-DGGE fingerprinting to assess the importance of seasonal changes during the annual cycle. Additionally, Hopavågen was eutrophied experimentally during the summer months of the years 1998 and 1999. Our interest was therefore two-fold: (a) to search for annual distribution patterns of specific phylotypes within the microbial assemblage, and (b) to assess whether experimental, sustained nutrient addition to a coastal ecosystem might alter the diversity of the bacterial assemblage.

## 2. Materials and Methods

### 2.1. Study area, experimental nutrient addition and sampling

The effect of experimental, sustained, enhanced nutrient supply to coastal waters on bacterioplankton diversity was examined in a landlocked bay, named Hopavågen, located in central Norway (Figure 1). Hopavågen, described in detail elsewhere (Reinertsen *et al*., in preparation; Vadstein *et al*., in preparation), is situated in a sparsely populated area close to the field station of the Norwegian University of Science and Technology (NTNU) on the West Coast of Norway. Hopavågen has a surface area of 275.000 $m^2$, a total volume of $5.4\times10^6$ $m^3$, and the volume of euphotic waters is $3.7\times10^6$ $m^3$ which corresponds to 68% of the total volume. The depth of the main basin is 22-32 m, and the bottom is relatively flat. The average depth is 20 m. Daily exchange of water with the coastal waters averages a volume corresponding to 19% of the productive water layer of Hopavågen, but it is variable and controlled mainly by tidal forces (tidal range 75-225 cm). Hopavågen has a narrow inlet which made the addition of nutrients well controllable. Experimental nutrient addition was done with each tidal cycle (usually two times per day). Artificial eutrophication was performed between 5 May 1998 and 12. October 1998, and from 25 May 1999 until 19 October 1999. The dose of nutrients corresponded to 0.4 µg P $litre^{-1}$ $day^{-1}$, and with a 14:5.4:1 molar ratio for N:Si:P (planned molar ratio was Redfield ratio, 16:8:1, but was not achieved due to technical problems) in 1998 and the double amount in 1999.

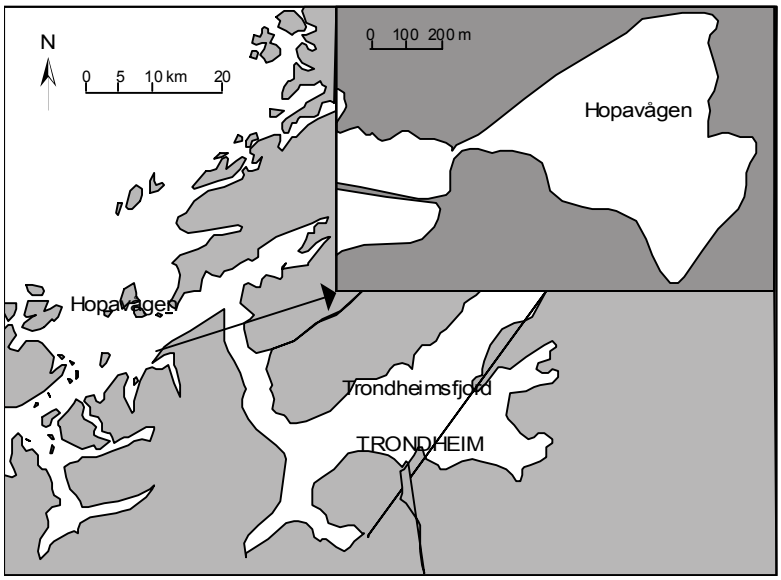



**Figure 1.**

Map showing the location of Hopavågen on the coast of Norway.

Integrated samples of productive (euphotic) waters were taken at a representative station with a 2 m long Ramberg sampler. The depth of the euphotic zone was based on profiles of salinity, temperature and *in vivo* fluorescence. In cases of a deep chlorophyll maximum, the composite euphotic sample was taken above this depth. In cases where no stratification was recorded, the mixed euphotic sample was made up of water from the 0-10m column. The sampling frequency was every week during summer/autumn (June-September) and less frequent in other periods (once or twice per month). A total of 22, 26, 27, and 26 samplings were done for 1996, 1997, 1998, and 1999 respectively, of which 11, 23, 27, and 26 (thus a total of 87) were available for molecular analyses. Samples were pre-screened through a 200 µm plankton net and collected on ignited Whatman GF/F glass fibre filters with a 47 mm diameter (Whatman, Maidstone, UK). Under the conditions used the GF/F filter retained >95% of the heterotrophic bacteria (Bratbak, 1985) Olav Vadstein, unpublished results). Sub-samples for different analysis were taken from the GF/F filter by cutting out circles with a sterilised cork borer.

### 2.2. Determination of chlorophyll *a* concentration

Samples for analysis of chlorophyll *a* were collected on Whatman GF/F glass fibre filters, extracted in methanol, and quantified by fluorometry using a Turner Designs fluorometer as described by (Strickland & Parsons, 1972). Two or three replicates were analysed per sample.

### 2.3. Nucleic acid extraction

Samples for molecular biological analyses consisted of entire GF/F filters (Whatman, diameter of 25 mm) up to 12 August 1997. Thereafter, small round portions of GF/F filters with an approximate diameter of 5 mm that were punched out of 47 mm GF/F filters with a cork-borer were used. A modification of the protocol described by Somerville et al. (1989) was used to extract DNA from glass fibre filters. Briefly, 1.5 ml of SET-buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-Cl [pH 7.6]) were added to a filterpiece in a 15 ml conical centrifuge tube (Greiner, Frickenhausen, Germany). The tubes were vortexed vigorously, then briefly spun down to collect liquid at the bottom of tube. 200 µl of a freshly prepared lysozyme solution (2% [w/v]; final concentration 0.2% [w/v]; article L7651, Sigma, St. Louis, Missouri) was added, gently mixed with the buffer and incubated at 37°C for 60 min. Sodium-dodecyl-sulphate was added to a final concentration of 0.5 % [w/v], then tubes were subjected to three freeze-thaw cycles in liquid $N_2$ and a waterbath at 55°C. 80 µl of Proteinase

K solution (final conc. 0.5 mg/ml, article no. 82456, Fluka, Buchs, Switzerland) was added and mixed with the buffer, then incubated for 30 min with brief vortexing pulses every 10 min. The aqueous solution was transferred into a 15 ml tube and kept on ice, while the filter was washed twice with 0.5 ml water (article no. W4502, Sigma) at 55°C in a waterbath to increase the yield of DNA which tends to bind to glass fibre at elevated salt concentrations. Washings were pooled with the aqueous buffer-phase and further purified by extracting once with phenol and in a second step with phenol:chloroform:isoamylalcohol (25:24:1 vol). Nucleic acids were precipitated for at least three hours at –27°C by adding NaCl (final concentration 0.1 M) and two volumes of ice-cold ethanol (100% v/v). Precipitated nucleic acids were pelleted by centrifugation for 60 minutes at 4,000 ×*g* and 4°C. Pellets were washed with 70% (v/v) ethanol and resuspended in 50 µl PCR-water overnight at 4°C. Aliquots were stored at –80°C. 5 µl of the extracted nucleic acid solution were loaded onto 0.7% (w/v) agarose gels to inspect quantity and quality of the DNA.

### 2.4. PCR-DGGE and DNA sequencing

Extraction of nucleic acids, amplification of 16S rRNA gene fragments and their analysis by DGGE as well as sequencing of excised DGGE bands were essentially done as described by Schäfer and Muyzer (Schäfer & Muyzer, 2001), except that 25-well combs were used to form slots, and consequently the amount of DNA-sample loaded onto the lanes was reduced to ca. 250 ng per sample. Bands were recovered from DGGE gels using a modification of the procedure of Mike Ferris (personal communication), which consists in pinching into the gels with sterile disposable pipette tips. Then pipette tips were transferred to 1.5 ml microcentrifuge tubes containing 10-30 µl of water. After overnight incubation at 4°C 1 to 5 µl of the water were taken as template for PCR reactions. The primers used for PCR-amplification of 16S rRNA gene fragments were 341F-GC (containing a 40-bp GC-rich sequence at its 5'end) and 907RM which is an equimolar mixture of the primers 907RC (5'-CCGTCAATTCCTTTGAGTTT-3') and 907RA (5'-CCGTCAATTCATTTGAGTTT-3'). Bovine serum albumin, fraction V (BSA; article no. 735086; Boehringer, Mannheim, Germany) was added to the amplification reactions to a final concentration of 3 µg/µl.

### 2.5. Comparative sequence analysis

New partial sequences were analysed using BLAST (Altschul *et al*., 1990) at the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and together with the highest scoring BLAST hits, added to an alignment of homologous bacterial 16S rRNA primary structures (Maidak *et al*., 1997) by using the aligning tool of the ARB software package (Strunk & Ludwig, 1998). A Neighbour-joining tree was generated using NEIGHBOUR (Felsenstein, 1993) with the Kimura-2 parameter model taking into account all overlapping unambiguous nucleotides of the set of sequences. Very short partial sequences were inserted into the tree using the special ARB parsimony tool (Ludwig *et al*., 1998). The names of sequences consist of the prefix HB (for Hopavågen Bacteria) and a DGGE-band number.

## 3. Results

### 3.1. Primary Production and chlorophyll *a* concentration during the four-year period

Primary production increased to significant levels in late March or early April dependent on the year (data not shown). During summer considerable fluctuations were observed, with coefficients of variation ranging between 0.35 and 0.67 (Table 1). In the two control years, 1996 and 1997, the average production rate was different, although not statistically significant. Whereas 1996 seemed to be dominated by a P-limited regime, N was limiting in the others years, and this may have caused the difference between 1996 and 1997 (Vadstein, *et al*., in preparation). In 1998 and 1999 the average primary production increased by 16 and

75%, respectively. If comparisons are made between the last three years of the study, the increase in 1998 and 1999 was 32 and 99%, respectively.

**Table 1.**

Average primary production (mg C $m^{-2}$ $d^{-1}$) during the period June-September for Hopavågen and grouped according to year.

| | 1996 | 1997 | 1998 | 1999 |
|---|---|---|---|---|
| Mean | 407 | 322 | 422 | 637 |
| +/-SD | 142 | 214 | 192 | 300 |
| Median | 405 | 256 | 393 | 694 |
| CV[a] | 0.35 | 0.67 | 0.45 | 0.47 |
| Average %[b] | 112 | 88 | 116 | 175 |

[a]Coefficient of variation
[b]Percent calculated using the average of 1996 and 1997 as 100%

**Table 2.**

Average chlorophyll *a* concentrations (µg $litre^{-1}$) during the period June-September for the euphotic zone in Hopavågen

| Year | N | Mean±SD | Percent[a] | CV[b] |
|---|---|---|---|---|
| 1996 | 16 | 2.05±0.82 | 106 | 0.40 |
| 1997 | 17 | 1.81±1.24 | 94 | 0.69 |
| 1998 | 18 | 2.01±1.08 | 104 | 0.54 |
| 1999 | 18 | 3.16±1.38 | 164 | 0.42 |

[a]Percent calculated using the average of 1996 and 1997 as 100%
[b]Coefficient of variation

The fluctuation in chl *a* concentration during the four-year period is shown in Figure 2. The concentrations varied along the seasons as a recurrent cycle with low chl *a* concentrations in the autumn and winter months and rising concentrations in March. No clear spring bloom was observed. Throughout the summer season, considerable fluctuation was observed, with coefficients of variation between 0.40 and 0.69 (Table 2). Whereas summer chl *a* concentrations did not increase due to the addition of nutrients in 1998, the doubling in nutrient addition in 1999 resulted in increased chl *a* concentrations. On average, the increase was 64% compared to the two control years (Table 2), the increase being statistically significant.

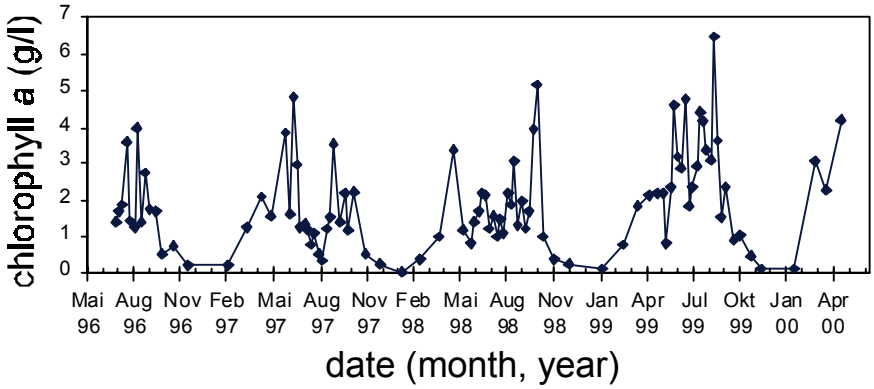



**Figure 2.**

Fluctuation of the chlorophyll *a* concentration during the study period.

### 3.2. Extraction and amplification of nucleic acid preparations.

DNA preparations for a total of 87 samples from the euphotic zone were performed. Inspection of preparations on agarose gels revealed distinct genomic DNA bands (approximate size of >20 kb) in most cases and besides variable bands and smears presumably corresponding to rRNA and tRNA (result not shown). In some cases nucleic acids were

hardly or not at all detectable on agarose gels. Still, bacterial 16S rRNA gene fragments could be amplified specifically from most of these preparations. For a number of samples multiple PCR reactions had to be pooled and precipitated in order to load comparable amounts of DNA onto the gel lanes. Sometimes, slightly brownish colour of dried nucleic acid pellets suggested the presence of co-extracted humic substances. Initial amplifications without the addition of BSA did not result in detectable amounts of PCR products on agarose gels (results not shown). Upon addition of BSA to subsequent reactions the inhibition of *Taq*-polymerase could be alleviated, and PCR products showing as discrete bands of the expected molecular size (i.e. ca. 550 bp) were obtained.

### 3.3. DGGE analyses

In general, DGGE patterns of all samples analysed showed seasonal changes but otherwise shared many similarities between years (Figure 3). The largest seasonal differences were evident between winter and the rest of the year. Gradual changes in DGGE fingerprints of consecutive samples from the productive seasons illustrated a certain degree of continuity in the development and succession of the bacterial assemblage over the summer period.

In 1996 only the second half of the year was analysed. The identity of the dominant bands remained the same throughout the summer and early autumn, yet some additional bands appeared in September and succeeding samples. In 1997 the DGGE fingerprints could be tentatively divided into four phases. Samples from April and May were quite similar and a transition to a stable composition was evident during June and early July (early summer community). The sample from 15-July-1997 formed a discontinuity before another stable composition of the patterns was evident from end of July until early September. This late summer community was dominated by a band identified as *Synechococcus*-related cyanobacteria (see below). Thereafter, fingerprints were quite variable, to a much higher degree than was the case in the 1996 autumn samples. Similarly, a transition from spring to early and late summer communities was evident in 1998 and 1999. In 1998, the genetic fingerprints of the bacterial community became more variable in autumn, like in 1997, while the dominant bands in autumn profiles of 1999 seemed comparable in their stability to those of 1996.

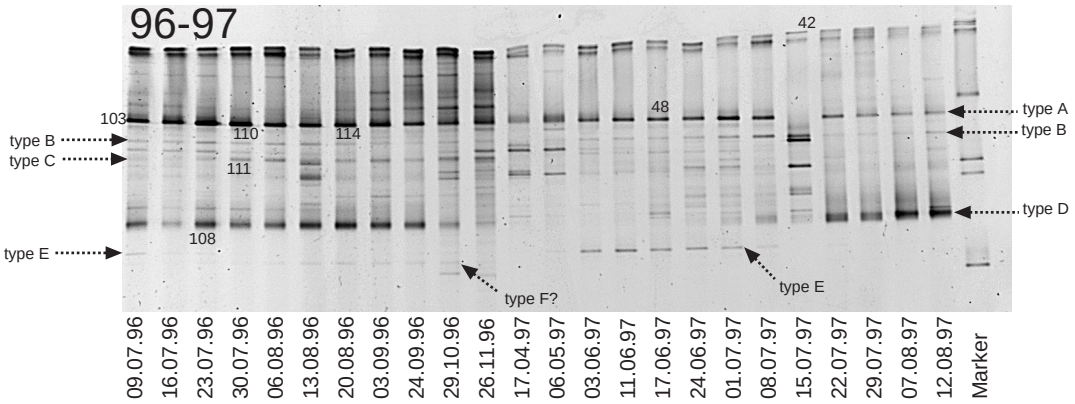
Richness of phylotypes seemed also fairly stable over the summer as there were no considerable changes in the number of bands in DGGE patterns, except for the sample taken on 1-September-1998 that deviated from this general trend since there was a marked increase in the number of bands as compared to neighbouring samples.

### 3.4. Phylotypes identified by sequencing

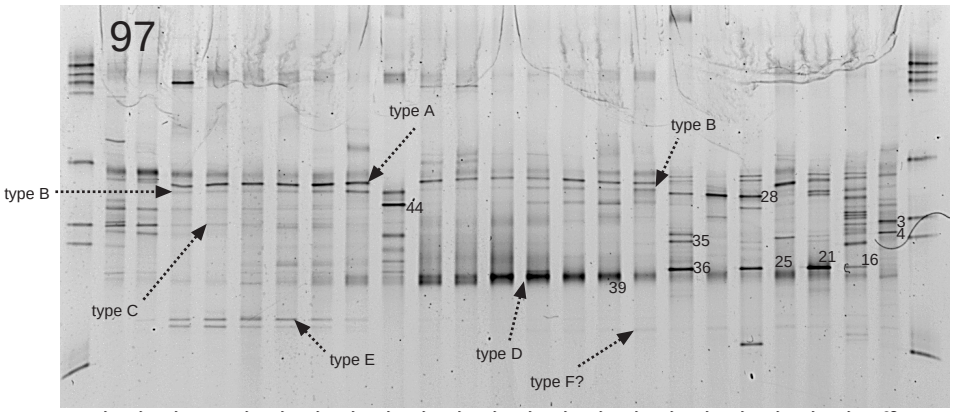
A number of phylotypes could be identified by sequencing of the respective bands. The results of the comparative sequence analysis are shown in Figure 4. Most sequences represented typical members of marine communities. Several sequences were similar to those of members of the *Roseobacter*-group in the α-Proteobacteria, whereby they were distributed in three clusters, corresponding to the different mobilities on DGGE. One sequence type, represented by bands HB-103, HB-110, HB-114, HB-62, and HB-48) was a dominant phylotype detected in the majority of samples. Whether or not HB-63 and HB-111 were migrating to different positions in the denaturing gradient gel could not be verified. These sequence types were also affiliated with the *Roseobacter* clade.

**Figure 3 (next page)**

DGGE analyses of PCR products obtained from samples of the GF/F filterable fraction of the microbial community of Hopavågen. The sampling date of each sample is indicated at the bottom of the lanes, "Marker" indicates lanes where a marker was applied. Numbers indicate the bands that were sequenced, the affiliation of the sequences is shown in Figure 4.




09.07.96 16.07.96 23.07.96 30.07.96 06.08.96 13.08.96 20.08.96 03.09.96 24.09.96 29.10.96 26.11.96 17.04.97 06.05.97 03.06.97 11.06.97 17.06.97 24.06.97 01.07.97 08.07.97 15.07.97 22.07.97 29.07.97 07.08.97 12.08.97 Marker

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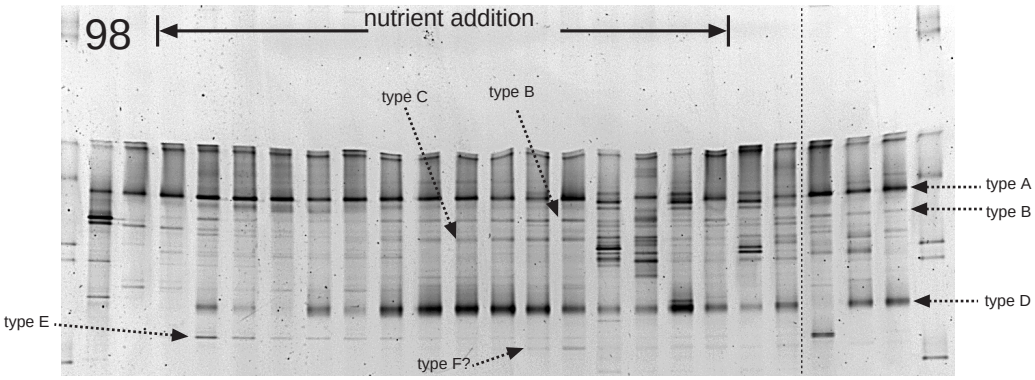


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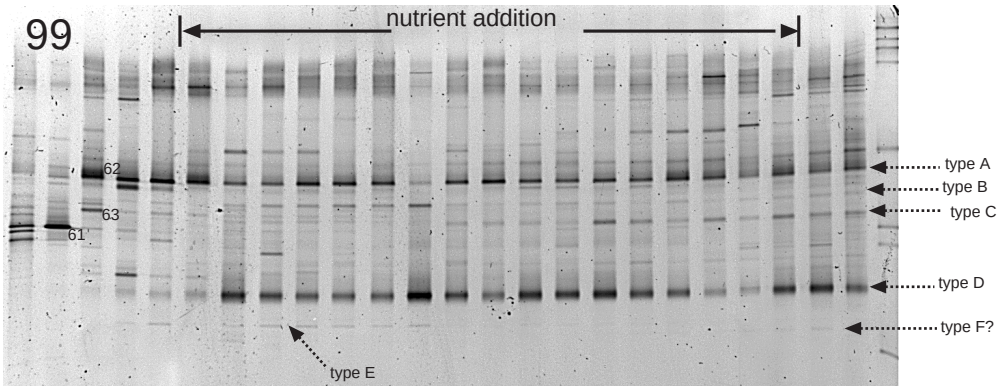


02.02.99 16.03.99 13.04.99 05.05.99 18.05.99 01.06.99 08.06.99 15.06.99 22.06.99 29.06.99 06.07.99 13.07.99 20.07.99 27.07.99 03.08.99 10.08.99 17.08.99 24.08.99 07.09.99 14.09.99 21.09.99 28.09.99 13.10.99 26.10.99 Marker

Sequences of γ-Proteobacteria, affiliated with members of the genus *Pseudoalteromonas*, were recovered from dominant bands (HB-61 and HB-3) in patterns of winter / early spring samples. Except for co-migrating (less dominant) bands in DGGE patterns of temporally neighbouring samples, no corresponding DGGE bands were detectable during the rest of the year. *Psychrobacter*-related γ-Proteobacteria were detected on two occasions in 1997 as dominant bands of samples that were discontinuous with respect to preceding and succeeding samples.

A population related to β-Proteobacteria was detected as a dominant band in a winter sample from January 1998. Co-migrating bands were not obvious in other DGGE fingerprints, except maybe for the sample of 2-February-99, where one of the dominant bands may have been similar. It formed part of a cluster uniting an uncultured bacterium detected in the rhizosphere of rape plants (unpublished, Kaiser, O. et al., Genbank entry AJ295478) with satellite bacterial populations from two marine diatoms (Schäfer *et al*., in preparation). Despite their different origin these three sequences were >99% similar.

The identity of 'type D' was verified from three different bands and sequences that were consistently affiliated with *Synechococcus*-related cyanobacteria.

A band, HB-42, from the top of the denaturing gradient which was present in many samples was related to a *Cytophaga* strain isolated from the North Sea (Eilers *et al*., 2000), and thus a member of the *Cytophaga-Flavobacterium-Bacteroides* phylum (CFB). Another representative of the CFB was band HB-36, which did not have close relatives (closest relative uncultured bacterium GKS2-21, 83% similarity) and might represent a distinct lineage in the CFB.

Two phylotypes from the gram positive bacteria were identified. One of these phylotypes was related to *Bacillus* sp. OS-5 (partial 16S rRNA gene similarity >98%) isolated from a coastal marsh in Galicia, Spain (unpublished, Genbank accession AJ296095, by Osorio C.R.) It was a dominant band (HB-21) in the DGGE pattern of the sample taken on 14-October-97, and a weaker band (HB-16) in the succeeding sample. The other gram positive phylotype was related to *Bacillus* sp. AS-38, a strain that was isolated from the Northern Adriatic Sea and expressed α-glucosidase (Fajon *et al*., submitted)

### 3.5. Seasonal distribution patterns of specific phylotypes

Seasonal distribution patterns of some phylotypes could be discerned, these are indicated as type A to type F in Figure 3. Most DGGE fingerprints showed two intense DGGE bands that represented two predominant phylotypes. One of these, 'type A', identified as *Roseobacter*-related α-Proteobacteria, was present in all but a few of the samples analysed by DGGE. With one exception (on 15-July-97) type A was present in all summer samples and it usually appeared as a dominant band in the DGGE patterns of samples taken in May or June. Thereafter, it was present as a dominant band until September, or even into October.

Type B and type C, which were not identified by sequencing, were also summer populations. While type B seemed relatively widely distributed over the productive season from early spring through to October, type C rather seemed to appear towards the mid to end of summer and remain detectable in autumn.

In all years there was the recurrent appearance of type D, identified as *Synechococcus*-related cyanobacteria, which formed a dominant band in the genetic fingerprints during most of the second half of the summer season and well into autumn. Yet, the time of onset and the duration of presence of the corresponding band in DGGE fingerprints was variable between years. In 1996 type D bands were already detectable as dominant bands when the survey began in the beginning of July. In 1997 they appeared at the end of June, but in 1998 and 1999 they appeared as a dominant band in the beginning of June. They were always present until at least into September or sometimes even until November.

Types E and F were most distinct with regards to their seasonal distribution. The time of presence of type E seemed well defined and stretched over four to six weeks from mid May, beginning of June to end of June/mid July. It was thus a typical member marking the early summer community. Type F was visible as very faint bands only at the end of summer and later on in autumn. Due to the weak signal, the definition of this type is uncertain to some extent.

Sequencing indicated that in the winter and spring samples γ-Proteobacteria related to the genus *Pseudoalteromonas* were detectable as dominant bands in DGGE fingerprints. Whether this constitutes a distinct distribution pattern for these populations (DGGE bands HB-3 and HB61) is uncertain, as only few winter and spring samples were analysed. However, the example of band HB-63 showed only predominance in patterns of February and March 1999, in April and May the band was only weakly visible, thereafter it disappeared.

## 4. Discussion

### 4.1. Seasonality in diversity of bacterioplankton communities

The existence of seasonal fluctuations in marine bacterioplankton communities has been reported earlier (Kelly & Chistoserdov, 2001; Murray, *et al*., 1998; Pinhassi & Hagström, 2000). This study confirms that seasonality observed in physical and biological factors also forces a seasonality in the genetic diversity of bacterioplankton communities. However, our study extends the observations made in earlier studies by demonstrating that not only seasonal changes exist, but that in addition there can be recurrent annual patterns in the bacterial community composition. Seasonal changes were obvious in all the analysed years. While relatively slow changes seemed to operate in the bacterial community during summer, and most samples were similar, the DGGE analysis of samples obtained from Hopavågen during late autumn, larger changes between consecutive samples were observed in winter and early spring samples. One reason may be that the sampling intervals were less frequent, but there was also a marked shift in the dominance of bands, namely those dominant during summer were not present or not dominant in non-summer samples.

It seems obvious and conceivable that much of the variation must be accounted for by the primary production that drives the system, which again is strongly coupled to the seasons as a consequence of daylight availability at this latitude and to stratification. This may explain why the strongest changes in DGGE fingerprints always occurred in the transition from winter/spring to summer from March to May, after which a relatively stable composition was established. In all years studied, the primary producers increased in biomass and activity in late March/early April (Figure 2, Tables 1 and 2). The community seemed fairly stable over the summer, with increasing dominance of cyanobacteria marking the late summer community, but the development of fingerprints in autumn months (September to November) seemed unsteady between years. While in 1996 and 1999 the fingerprints showed only little variation between summer and even late autumn samples, with a few additional sequence types appearing towards autumn, the situation in the years 1998 and especially in 1997 seemed much more unsteady. One possible explanation might be that in those autumn seasons physical factors such as storms caused higher instability and mixing of the water column, maybe also due to increased water exchange with the coastal waters. In general, the summer season of 1997 seemed to be quite unsteady, with a few discontinuous samples interspersed.

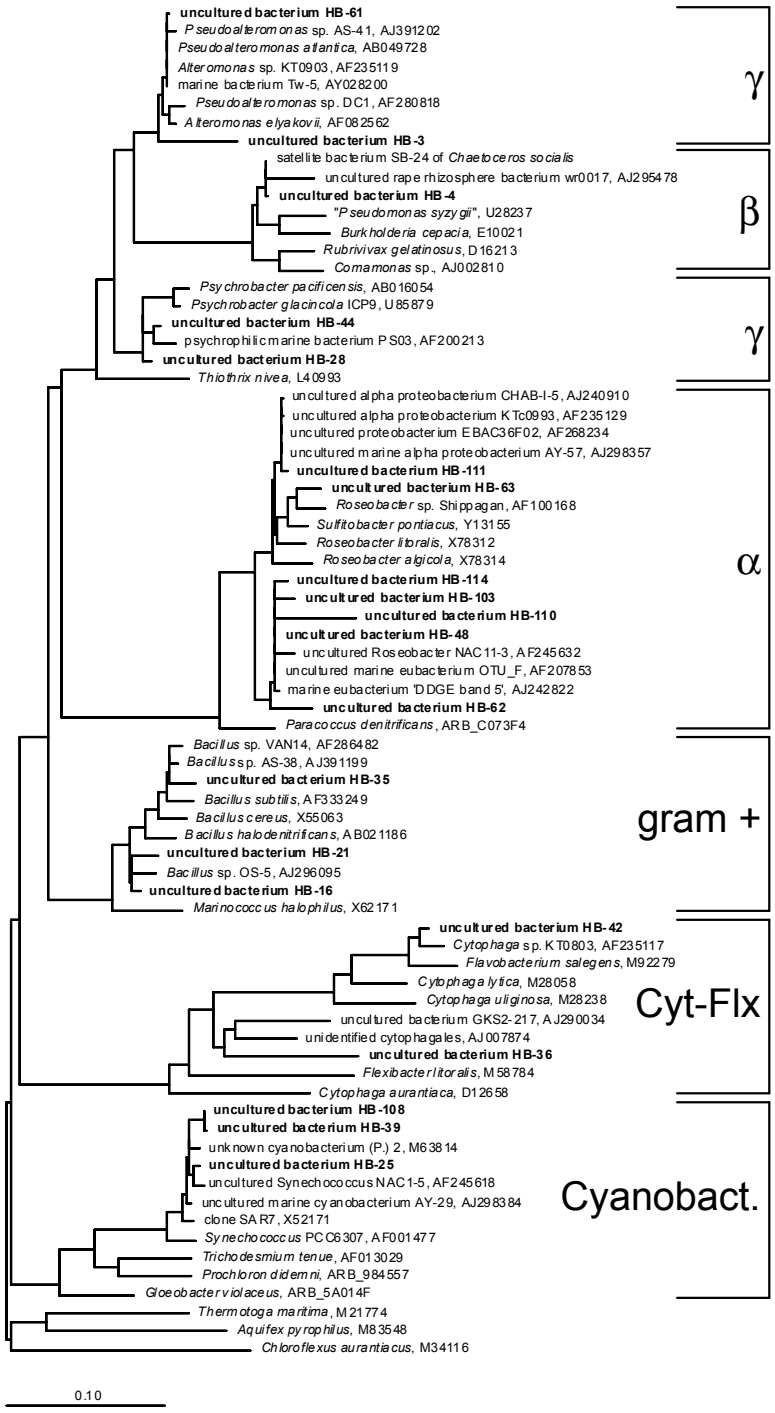


**Figure 4.**

Phylogram showing the relationships of sequences retrieved from DGGE bands from Hopavågen samples to reference sequences obtained from Genbank (accession given in parentheses). Names of sequences determined in this study are in bold and consist of the prefix HB- (for Hopavågen Bacteria and a band number as indicated in Figure 3). The tree was derived from analysis of the overlapping *E. coli* nucleotide positions using neighbour joining option of the program Neighbour from the Phylip-package (Felsenstein, 1993) as implemented in ARB, with the Kimura 2-parameter model and a transition-transversion-ratio of 2. Sequences marked with three asterisks (***) had only little overlap with the rest of the alignment and were therefore inserted into the Neighbour-Joining tree using the special ARB parsimony tool (Ludwig, et al., 1998). The sequences of *Thermotoga maritima*, *Aquifex pyrophilus* and *Chloroflexus aurantiacus* were defined as outgroup.

During summer, abrupt changes were only rarely seen in the DGGE fingerprints of consecutive samples. For instance samples of 8, 15 and 22 July 1997 seemed discontinuous with respect to the absence of a dominant band visible in patterns of 8 and 22 July but not on 15 July. Other bands did have counterparts in the neighbouring samples suggesting that the discontinuity was not a sampling artefact, but rather that a special event may have contributed to quicker and larger changes in the taxonomic composition of the microbial community than those that occurred gradually during summer.

### 4.2. Recovered sequence types

All sequences recovered from the gels had marine relatives, with the exception of HB-16 and 21, whose closest relative was isolated from a salt marsh. Surprisingly, only few members of the typical marine *Cytophaga-Flavobacterium-Bacteroides* phylum were amongst the sequenced bands. In other studies (Schäfer, *et al*., in preparation; Schäfer, *et al*., 2001) CFB populations have been detected more frequently. Most CFB populations are commonly distributed in the upper portion of the denaturing gradient, which may reflect their rather low genomic DNA GC-content. Some CFB populations may have gone undetected due to a low sequencing coverage of the DGGE bands in this study. Additionally, a possible complication for the identification of presumably heterotrophic phylotypes by DGGE and sequencing in this system may be that the microbial biomass was dominated by cyanobacteria during a long period in the summer. This might preclude efficient amplification of non-cyanobacterial sequence types by PCR, and hence limit the detection power of the method, for 'heterotrophic' populations. Still, CFBs were detected as a band permanently present at the top of the gradient (HB-42) but the resolution of this region of the gradient seemed variable to some extent between gels.

Surprisingly, a population of β-Proteobacteria was detected by sequencing. While β-Proteobacteria have been reported in marine systems in earlier studies (e.g. (Kelly & Chistoserdov, 2001; Rappé *et al*., 2000), they have commonly been considered allochthonous and of terrestrial or freshwater origin due to the frequent dominance of β-Proteobacteria in freshwater (Glöckner *et al*., 1999; Nold & Zwart, 1998). This β-proteobacterial phylotype, however, did not match any of those previously detected in marine samples, but had 'satellite-bacteria' growing in marine diatom cultures (Schäfer, *et al*., in preparation) as closest relatives, besides a population detected in the rape rhizosphere. The area is influenced by land, both directly and indirectly by in-flowing land-influenced water. However, in winter the salinity was normally >32 ppt which makes it appear unlikely that the phylotype was washed into Hopavågen. Also, the presence of similar phylotypes in marine diatom cultures strongly suggests physiological fitness of these bacteria at marine salt concentration.

The dominance of α-Proteobacteria from the *Roseobacter* clade emphasises the importance of members of this group in coastal surface waters (González & Moran, 1997), and adds to the clues that have been gathered by recent studies regarding the regulation of their activity and abundance. González and colleagues have reported that a major contribution of this clade may reside in the metabolism of organic sulphur compounds, such as algal derived DMSP (González *et al*., 1999). Considering the linkage with the productive season, the presence of *Roseobacter*-related populations strongly suggests that there is an important interaction of these bacteria with phytoplankton. This is also supported by analysis of so-called 'satellite' bacterial assemblages thriving in cultures of marine diatoms where members of the *Roseobacter*-lineage were detected in each of six diatom cultures analysed (Schäfer, *et al*., in preparation).

Pinhassi and Hagström (Pinhassi & Hagström, 2000) did not find any evidence of importance of *Roseobacter* species in the Gulf of Bothnia at any time of the year. While it has been suggested that the predominance of bacteria of this group may be overestimated by molecular methods (Cottrell & Kirchman, 2000), but the main reason for absence of

*Roseobacter* species in the Gulf of Bothnia is probably the very low salinity, which is generally below 6‰. Gonzales and Moran (González & Moran, 1997) noted that members of the *Roseobacter* lineage were not detectable at salinities of 5‰ or below in an estuarine gradient.

### 4.3. Seasonal distribution patterns

The single most important parameter for the food web in Hopavågen is probably phytoplankton production. Changes in bacterial community composition might be linked to predominance of different types of phytoplankton. The summer of 1997 might be a good example to illustrate this, as early and late summer communities were well defined due to a discontinuity (15-July-1997) in between. For instance, the early summer community, with the type E populations present, accompanied the development and decline of a strong fluctuation in chl *a* concentration. The late summer community, however, which was dominated by picocyanobacteria, completely lacked this phylotype. Also, the bands of type B populations were weaker in the time of cyanobacterial dominance, suggesting that some of the variation in bacterial diversity along the year may be caused by species oscillations in the autotrophic fraction of the microbial community. The possibility of such co-variance between algal and bacterial diversity has been addressed recently by several authors (González *et al*., 2000; Kelly & Chistoserdov, 2001; Kerkhof *et al*., 1999; Riemann *et al*., 2000). It seems clear that build-up and decay of blooms may trigger the development of modified bacterial assemblages (Kelly & Chistoserdov, 2001; Riemann, *et al*., 2000) and that bacterial community composition may vary along periods with increased primary production during phases of upwelling (Kerkhof, *et al*., 1999). A survey of the bacterial phylotypes growing in laboratory diatom cultures showed that the six diatom cultures that were analysed each had characteristic co-occurring bacterial assemblages that were stable over several subcultures (Schäfer, *et al.*, in preparation). However, no conclusive evidence has been found so far that the type of algae predominating in the water column may also modify the bacterial community composition. González et al. (2000) noted that the distribution of dominant phylotypes as assessed by specific hybridisation to extracted DNA did not change in relation to the variable phytoplankton community composition.

### 4.4. Effect of eutrophication?

Vadstein et al. (in preparation) noted that sustained experimental nutrient addition did not have dramatic effects on the phytoplankton biomass (cf. Figure 2 and Table 2). Yet, on average the chl *a* concentration increased by 64% in 1999 compared to control years, whereas no effect was detected in 1998 (Figure 2, Table 2). Neither were there clear differences in composition of phytoplankton between the years, except a somewhat higher share of dinoflagellates in 1996 (Sommer *et al*., in preparation). There was also no clear trend in phytoplankton succession, except an increase in cyanobacterial biomass in June, and dominance of dinoflagellates and diatoms in algal biomass. Picocyanobacteria normally increased in biomass in June, and constituted 40% of the phytoplankton biomass, but with oscillations in their contribution in 1998 and 1999.

However, the nutrient addition caused oscillations in the diatom biomass during summer (Sommer, *et al*., in preparation). Whereas diatoms contributed with <25% on most occasions in 1996 and 1997, they peaked 4 to 5 times in 1998 and 1998, and then comprised >70% of the phytoplankton biomass on several samplings. One could have expected that such oscillations in species composition could change the selective pressure on the heterotrophic bacterial community, but other biotic components may also affect the selective pressure on the individual bacterial populations, as both, bottom-up effects caused by changes in the nutrient supply, and top-down effects caused by grazing and virus infections. It is possible that eutrophication had an effect, but that it affected phenotypic rather than genotypic

properties of bacterial populations and that some variation might therefore go unnoticed in 16S rRNA based analyses.

**Conclusions**

Hopavågen exhibits strong seasonal differences in the composition of the bacterioplankton community. Annually recurring features in DGGE patterns suggest that the bacterial community composition as well as the succession within the community are non-random and do not appear to be modified markedly by eutrophication. The transition from winter/spring to summer triggers the largest changes in community composition, an indication for the importance of phytoplankton in fuelling the whole microbial food web of Hopavågen. Populations from the *Roseobacter* group are predominant throughout the productive seasons supporting recent studies implying these bacteria in cycling of phytoplankton derived dissolved organic matter.

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# Chapter 7

# Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms

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Manuscript in preparation

## Summary

Uni-algal, non-axenic cultures of six marine diatoms were screened by PCR-DGGE for the diversity of the accompanying bacterial communities ('satellite'-bacteria) in order to test the hypothesis that algal cells constitute niches for specific bacterial species. The complexity of the satellite assemblages, as judged from the number of detected phylotypes, was low when compared to the complexity of bacterial assemblages in nature. Generally, the six algae cultures were accompanied by distinct satellite assemblages, as the majority of the phylotypes detected in the six cultures was unique, only some phylotypes were common to more than one culture. Analysis of replicate incubations and repeated passage of cultures in most cases showed only minor variations of satellite assemblage genetic fingerprints, suggesting that the bacterial-algal associations were stable. An experimental approach to find evidence for specific algae-bacteria interactions by challenging algae cultures with heterologueous satellite assemblages was unsuccessful as it was not possible to avoid carry over of algae. Satellite populations were identified by sequencing of DGGE bands. Most of the populations represented typical marine phylotypes, such as members of the α–Proteobacteria (related to the genera *Ruegeria*, *Sulfitobacter*, *Roseobacter*, and *Erythrobacter*), or members of different genera of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. Surprisingly, β–Proteobacteria were also found in two of the cultures. A common point for all cultures was the presence of at least one representative of the α–Proteobacteria and of the CFB phylum, both of which have been reported as important representatives of the marine picoplankton. Their ubiquity in the sea and in the phytoplankton cultures analysed points to a specific role of these bacteria in the marine food web. The results indicate that algae diversity might be an important factor for explaining the enormous bacterial diversity in marine assemblages, and vice versa. Specific substances in the photosynthetic extracellular release and in the organic carbon produced by different phytoplankton species may require a variety of bacterial populations for the processing of this algae-derived organic matter.

## Introduction

So far most studies on algae-bacteria interactions have focused on the functional roles of phytoplankton and bacteria in the production and utilisation of phytoplankton derived dissolved organic matter (DOM). Interactions of algae and bacteria have been described in detail elsewhere (Cole, 1982) and range from symbiotic, via commensalic to parasitic interactions. Stimulation of bacterial growth by extracellular release of organic carbon by photosynthetic algae has led to the formulation of the 'phycosphere' as an important view of interaction of algae and bacteria. The carbon demand of heterotrophic bacteria is ultimately met by photosynthetically (algae-) derived carbon sources, except in situations where large inputs of allochthonous (e.g. terrestrial) organic matter is present (Bell, 1984). Thus, especially DOM, which becomes liberated upon senescence and after lysis of algal cells (e.g. through autolysis or 'sloppy-feeding' of microzooplankton) may be an important carbon source for heterotrophic marine bacteria.

The question of specificity of algal-bacterial interactions in the so-called 'phycosphere' is difficult to address due to the absence of adequate sampling methods. Studies in the field suffer from the inherent complexity of the pelagic food web and the general inability to rule out alternative mechanisms for explaining shifts in bacterial assemblage composition, yet they provide some circumstantial evidence that algal blooms may be able to affect the community composition of pelagic bacteria (González *et al*., 2000; Kerkhof *et al*., 1999; Riemann *et al*., 2000). González et al. (González, et al., 2000) reported that estimated abundance of *Roseobacter*-related bacteria was positively correlated with the concentration of dimethylsulfoniopropionate (DMSP) in samples from a DMSP-producing

algal bloom in the North Atlantic, while Riemann and colleagues found that marine α-Proteobacteria as well as members of the CFB-phylum were responding to the decay of a phytoplankton bloom dominated by *Thalassiosira* sp. (Riemann *et al*., 1999).

The aim of this study was to give complementary information on algal-bacterial interaction in systems where the complexity of the microbial community is reduced as compared to the natural situation, namely in diatom cultures. Uni-algal phytoplankton strains as they exist in algal culture collections may harbour a substantial number of commensalic bacteria, which have been termed 'satellite' bacteria, (e.g. Borisova, 1986). Given that these phytoplankton cultures are maintained by repeated passage, often over time spans of many years, it is conceivable that particular bacterial populations would be selected to live together with their algal hosts. While growth of bacteria in such phytoplankton cultures may in principle be photo-autotrophic or photoheterotrophic, it should mainly be heterotrophic as otherwise bacteria would be in direct competition for nutrients and light with the algal cells. Despite the DOM already present in the seawater, labile DOM provided by photosynthetic extracellular release by the algae is the main source of organic carbon for the bacteria. Another carbon source for the bacteria in phytoplankton cultures is particulate organic matter (POM) and DOM after senescence and lysis of the algae. On the one hand compounds in algae-cellular material may constitute a more or less stringent selection pressure as growth substrates and might invoke development of adapted bacteria (Janse *et al*., 2000). On the other hand, it has been hypothesised that in natural, oligotrophic environments bacteria possess uptake systems for organic carbon that have a high affinity, but a low specificity (Pointdexter, 1981). Bell stated that the extension of the 'high-affinity low-specificity' hypothesis predicts that bacteria should not specialise on extracellular organic carbon pools of individual species and found that bacteria are not specific in their ability to assimilate phytoplankton extracellular organic carbon (EOC), but could utilise EOC from other sources even after adjustment to specific EOC-pools (Bell, 1984).

Apart from reports on specific bacterial symbionts of marine macroalgae (Ashen & Goff, 2000) association of microalgae and bacteria in phytoplankton cultures has been demonstrated by isolation of bacterial strains from the cultures. Culturable bacteria isolated from diverse microalgal cultures were of similarly diverse affiliation (Borisova, 1996). Culturable bacteria from phytoplankton cultures also encompass toxin-producing bacteria, e.g. tetrodotoxin-producing bacteria from cultures of *Alexandrium tamarense* (Simidu *et al*., 1990; Simidu *et al*., 1987), or *Ruegeria algicola*, an okadeic acid producing bacterium from a culture of *Prorocentrum lima* (Lafay *et al*., 1995).

The objectives of this study were to analyse the genetic diversity of bacterial assemblages accompanying uni-algal, marine diatom strains, and to identify the dominant bacterial groups in these cultures by sequencing of 16S rRNA gene fragments. The composition of the bacterial satellite assemblage was observed over the course of repeated subculture and during different growth phases of the phytoplankton batch culture. Finally, an attempt was made to test the specificity of the association of algae and bacteria experimentally by reciprocally challenging two algae cultures with their respective satellite assemblages.

## Materials and Methods

### Algal cultures and sampling of satellite bacterial assemblages

The diatom strains used in this study are reported in Table 1. Diatoms were cultured at 15°C in seawater base F/2-medium as described by previously (Riegman *et al*., 2000), without soil extract in the medium, at a light intensity of 80 µE $m^{-2}$ $s^{-1}$ to 170 µE $m^{-2}$ $s^{-1}$ (8 h dark / 16 h light). Measurement of the absorbance at 435 nm was used to monitor growth of the algae. At

different times samples of the cultures were taken and filtered over 0.2 µm pore-size GVWP or GTTP filters (Millipore). Filters were stored at -80°C until further analysis. The kind of filter used (GVWP or GTTP) did not affect the results obtained by PCR-DGGE analysis.

**Table 1.**
Diatom strains used in this study.

| Species | Strain | Source |
|---|---|---|
| Dytilum brightwellii | CCMP358 | Gulf of Mexico |
| *Thalassiosira weissflogii* | CCMP1049 | Amityville, NY, USA |
| *Asterionella glacialis* | Oldenburg | - |
| Chaetoceros socialis | - | Roel Riegman |
| Leptocylindrus danicus | CCMP469 | - |
| *Coscinodiscus* sp. | - | Jörg Dutz |

## Reciprocal challenging experiment

To test the specificity of the algal-bacterial association a reciprocal challenging experiment was carried out as described below. All experimental manipulation was carried out in a laminar flow hood. All filtrations were performed with sterilised filtration equipment (i.e. vacuum flask, glass funnel and filter holder), filter discs were stored in cryovials and were transferred to –80°C until use. One-hundred ml cultures of *Leptocylindrus danicus* and *Chaetoceros socialis* were grown in triplicate into log-phase for 13 days, then 75 ml of each culture was aseptically removed. Of these 75 ml, 50 ml were aseptically filtered over GF/C filter discs (47 mm diameter; Whatman, Maidstone, UK). One half of the filtrate, 25 ml, of the GF/C filtrate was used as a challenger and was added to 25 ml of the algal culture to be challenged. The volume of the so-challenged culture was brought back to 100 ml using fresh sterile medium. The other half of the GF/C filtrate was filtered onto a 0.2 µm pore-size filter (GVWP) and was later used for analysis of the composition of the challenger assemblage by PCR-DGGE. 25 ml of the remaining unmodified algal culture were aseptically filtered with gentle pressure (max 100 mm Hg vacuum) over a 0.2µm filter (47 mm GVWP) and until they were used to analyse the genetic diversity of the whole cultures on the day of mixing. Two of the triplicate flasks of each algal culture were challenged, the third served as an unmodified control. For controls the challenger fraction was added back to the original culture. After 14 days of incubation of all cultures 25 ml were taken and filtered onto 0.2 µm pore-size GVWP filters, then the cultures were serially transferred into fresh medium and another sampling (25 ml onto 0.2 µm GVWP filters) was done after another 21 days of incubation.

## Extraction of nucleic acids, PCR-DGGE, and DNA sequencing

Extraction of nucleic acids, amplification of 16S rRNA gene fragments and their analysis by DGGE, as well as sequencing of excised DGGE bands were essentially as described by Schäfer and Muyzer (Schäfer & Muyzer, 2001). The primers used for PCR-amplification of 16S rRNA gene fragments were 341F-GC (containing a 40-bp GC-rich sequence at the 5'-end) and 907RM, which is an equimolar mixture of the primers 907RC (5'-CCGTCAATTCCTTTGAGTTT-3') and primer 907RA (5'-CCGTCAATTCATTTGAGTTT-3'). PCR products were inspected on 2% (w/v) agarose gels and quantified by comparison of band intensities with a molecular weight standard (Precision Molecular Mass Standard; Biorad, Hercules, Ca) using the software MULTIANALYST (Biorad) after scanning gels with a Fluor-S imaging system (Biorad). DGGE analyses were carried out with a DCode system (Biorad) using denaturing gradients of 20-80% denaturants (Schäfer and Muyzer, 2001) for further details). DGGE bands were sampled for re-amplification and sequencing by punching into individual bands with

disposable pipette tips (Mike Ferris, personal communication). It was not necessarily the case that gel-material was visibly carried over onto the pipette tip, rather the tips became 'contaminated' with DNA. The particular pipette tips used (2.5 µl tips, Eppendorf, Hamburg, Germany) were transferred to disposable 1.5 ml microcentrifuge tubes that contained 10 µl of PCR-buffer such that the tip would be immersed in the buffer. After overnight incubation at 4°C 1 to 5 µl of the buffer was taken as template for re-amplification of DGGE bands. Re-amplified PCR products were inspected alongside the original sample on DGGE to verify identity and purity of the bands. PCR products were purified using the QIAquick PCR purification kit. Sequencing was carried out using the ABI Prism® BigDye Terminator™ Cycle Sequencing Kit (PE Biosystems, Foster city, Ca) as described in (Schäfer and Muyzer, 2001) using the PCR primers (without GC-clamp).

## Comparative sequence analysis

The new partial sequences were analysed using BLAST (Altschul *et al*., 1990) at the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and aligned together with the highest scoring BLAST hits, to an alignment of homologous bacterial 16S rRNA sequences (Maidak *et al*., 1997) by using the aligning tool included in the ARB software package (Strunk & Ludwig, 1998). Neighbour-joining trees were generated using NEIGHBOUR (Felsenstein, 1993) with the Kimura-2 parameter model taking into account all overlapping unambiguous nucleotides within a given set of sequences. Sequences obtained in this study have been deposited in the sequence databases at the European Molecular Biology Laboratory under accession numbers AJ319823 to AJ319866. The names of sequences consist of the prefix SB (for satellite bacteria), a DGGE band number, and an abbreviation of the diatom culture in which the satellite bacterium was detected (see Table 2).

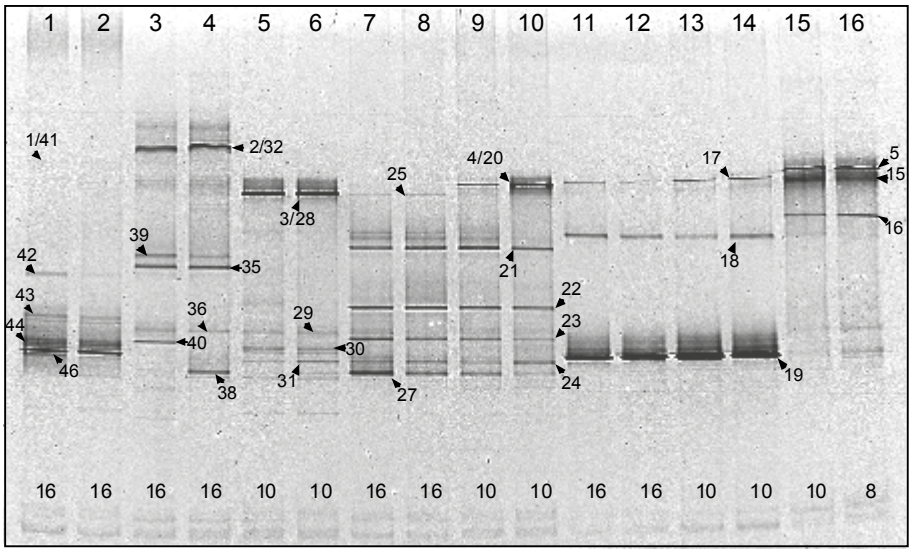



**Figure 1.**
DGGE analysis of PCR-amplified 16S rRNA gene fragments obtained from six uni-algal, non-axenic diatom cultures. Lanes: 1 and 2 *Dytilum brightwellii*; 3 and 4, *Thalassiosira weissflogii*; lanes 5 and 6, *Asterionella glacialis*; lanes 7 to 10, *Chaetoceros socialis*; lanes 11 and 12, *Leptocylindrus danicus*; lanes 13 and 14, *Leptocylindrus danicus*; lanes 15 and 16, *Coscinodiscus* sp. The age of the culture (in days) is indicated at the bottom of the lanes.

## Results

### Genetic diversity of satellite bacterial populations

DGGE showed that each culture had a unique composition of its satellite bacterial assemblage (Figure 1). There was hardly any overlap of band positions observed between cultures. There were also marked differences in richness over all cultures, the least in *Leptocylindrus danicus* (4 to 5 bands) and most in *Thalassiosira* and *Chaetoceros* with 20 and 16 bands maximum, respectively.

### Is the composition of satellite bacterial assemblages reproducible?

Analysis of samples from duplicates (Fig. 1) showed that the genetic diversity of the algal-bacterial cultures was generally reproducible. Some variation was recorded in the case of the cultures of *Thalassiosira weissflogii*, however, but except for two bands (band SB-40-TW, SB-38-TW) the differences were mainly a matter of shifted band intensities (note that bands SB-33 and 34 seemed to be heteroduplex bands). Figure 1 further shows that variation was also recorded when algae-bacteria cultures of *Chaetoceros socialis* were sampled at different times. While the band corresponding to the plastid of the algae (band 4/20) was visible in samples taken after 10 days of incubation, it could not be detected after 16 days of incubation. This finding might indicate that either the algae were not detectable due to altered extractability of the plastid DNA or - more probable - due to massive growth of bacteria.

### Does the genetic diversity of satellite bacterial assemblages vary in different phases of growth and senescence?

Further to the samples taken after 10 or 16 days for *C. socialis* and *L. danicus*, a more extensive survey of the genetic diversity of all six cultures was done spanning a period of almost two months with samples taken after 10, 16, 26 (*L. danicus* and *C. socialis* only), 37 and 56 days. In most cases DGGE profiles were very similar even between log-phase (10 days) and senescent phase cultures. Although, DGGE data does not strictly allow quantitative estimates of community composition, marked changes in the intensity of bands in different samples of all cultures strongly suggests that the relative abundance of different satellite populations changed over the course of batch incubation.

The culture of *Dytilum brightwellii* seemed very stable in composition, even over a time span of 56 days, a minor variation concerned the band 42 (sequence SB-42-DB) which was lacking in the genetic fingerprint obtained after 37 days of incubation. The only change in the culture of *Leptocylindrus danicus* consisted in the disappearance of the plastid band (band 17), probably due to senescence of the algae, in all other cultures variations in the banding patterns of the bacterial populations occurred.

The variability in the cultures of *Chaetoceros socialis* mostly concerned the detectability of the chloroplast 16S rRNA gene (see above). The *Asterionella glacialis* chloroplast sequence was detectable in each sample, only it was strongest (in comparison with bands of bacterial populations) in the day 10 sample, indicating that bacterial populations were less represented in the fingerprints, probably as a consequence of relatively lower bacterial total biomass.

In *Thalassiosira* cultures most variation was recorded between the samples obtained from duplicate cultures after 16 days. All other samples corresponded very well with the pattern of one of the duplicate 16 days old cultures.

The culture of *Coscinodiscus* was the only one in which a succession during prolonged incubation was recorded (Fig. 2, top panel). Generally, the richness of the satellite assemblage of *Coscinodiscus* increased as judged from the increase in the number of DGGE bands. Two additional populations detected in 37-days old cultures were identified as members of the CFB. Unfortunately, attempts to sequence further newly appearing DGGE bands were not

successful. It remains unpredictable whether freshly inoculated cultures would show similar successions, thus, the reproducibility after repeated passage is not certain.

**Identity of bacterial satellite populations**

The identification of satellite bacterial populations obtained by sequencing of DGGE bands is reported in Table 2 and Figure 3. The overall diversity was high, additionally to plastids of the diatoms, members of the α-, β-, and δ-subdivisions of the Proteobacteria, and members of the CFB group were identified by sequencing of DGGE bands.

There was a unifying theme in assemblage composition across all cultures, consisting in the presence of at least one type each of α-Proteobacteria and CFB-group members in each culture. Among the phylotypes detected were typical marine representatives, e.g. the phylotypes from the *Rhodobacter* group of the α-Proteobacteria (related to the genera *Roseobacter*, *Sulfitobacter* and *Ruegeria*), as well as other marine α–Proteobacteria (related to the genus *Erythrobacter*), a marine lineage formed by as yet uncultured members of the δ–Proteobacteria, as well as some of the CFB detected. Some sequences were atypical or hitherto not detected in marine samples. These encompassed the sequence distantly related to the Myxobacteria (SB-53-TW) and the β–proteobacterial phylotype almost identical to a 16S rRNA gene sequence obtained from the rhizosphere of rape (unpublished, Kaiser, O. et al., Genbank entry AJ295478). An interesting observation was that δ–Proteobacteria were only detected in the cultures of *Thalassiosira weissflogii*.

**Stability of algal-bacterial co-culture: reciprocal challenging of *L. danicus* and *C. socialis* cultures.**

The results of the challenging experiment are shown in Figure 4. The DGGE analysis shows that in the challenged cultures of *Leptocylindrus danicus* the band corresponding to the plastid of *Chaetoceros socialis* appeared upon subculture together with the complete set of bands from the *Chaetoceros* culture, indicating that the filtration over a GF-C filter did not retain all cells of *Chaetoceros* and that the challenged cultures of *Leptocylindrus* were quickly taken over and dominated by *Chaetoceros* and its satellite community. The control culture of *Leptocylindrus* remained stable, also after another subculture. On the contrary, the *Leptocylindrus* satellite community was not able to modify the composition of the *Chaetoceros* culture.

## Discussion

**Uniqueness and reproducibility of satellite assemblages**

All six cultures had distinct satellite assemblages whose compositions seemed to be stable, except for the culture of *Coscinodiscus* this could not be substantiated due to the too limited number of samples analysed.

**Satellite assemblages in different growth phases**

Limited changes in banding patterns from samples of different age indicated that satellite populations hardly changed between different growth phases, and can thus be considered stable assemblages. Minor variation in relative intensities and appearance of single extra DGGE bands in some samples as observed in cultures of *Asterionella* and *Thalassiosira* suggested that changes in evenness occurred without rendering the composition of the satellite assemblages unstable. The exception to this were cultures of *Coscinodiscus*, where marked changes were observed between growth phases. What might be the reason for these different patterns? Antibiotic substances released by the algae or little extracellular organic release? While additional infection of the cultures during the incubation period can not be excluded, it seems unlikely as this should potentially have occurred for all other cultures as well.

**Table 2.**
Satellite bacterial populations and plastids from diatom cultures identified by partial 16S rRNA gene analysis

| sequence name[1] | closest relative in database | accession no. | % similarity | phytoplankton culture |
|---|---|---|---|---|
| alpha Proteobacteria | | | | |
| SB-12-Cd | *Roseobacter gallaeciensis* | Y13244 | 90 | *Coscinodiscus sp.* |
| SB-13-CS | bacterium P91650 | AF214120 | 93 | *Chaetoceros socialis* |
| SB-14/19-LD | uncultured marine alpha proteobacterium BY-74 | AJ298352 | 99 | *Leptocylindrus danicus* |
| SB-23-CS | *Roseobacter* sp. DSS-8 | AF098493 | 98 | *Chaetoceros socialis* |
| SB-27-CS | *Crassostrea virginica* symbiont strain CV1 | AF114485 | 98 | *Chaetoceros socialis* |
| SB-38-TW | *Crassostrea virginica* symbiont strain CV1 | AF114485 | 98 | *Thalassiosira weissflogii* |
| SB-40-TW | *Sulfitobacter* sp. GAI-21 | AF007257 | 98 | *Thalassiosira weissflogii* |
| SB-43-DB | *Erythrobacter* sp. MBIC3019 | AB012062 | 97 | *Dytilum brightwellii* |
| SB-44-DB | uncultured alpha proteobacterium HetTri4-79 | AF216503 | 98 | *Dytilum brightwellii* |
| SB-46-DB | alpha proteobacterium SOGA34 | AJ244810 | 99 | *Dytilum brightwellii* |
| SB-58-AG | alpha proteobacterium Y3F | AF253467 | 92 | *Asterionella glacialis* |
| SB-59-AG | *Roseobacter* sp. (*Prionitis decipiens* symbiont) | AF107210 | 96 | *Asterionella glacialis* |
| SB-62-AG | *Tetracoccus cechii* | Y09609 | 92 | *Asterionella glacialis* |
| SB-8/30-AG | unknown alpha proteobacterium JP88 | AY007684 | 97 | *Asterionella glacialis* |
| SB-82-CS | unidentified alpha proteobacterium OM75 | U70683 | 91 | *Chaetoceros socialis* |
| beta-Proteobacteria | | | | |
| SB-24-CS | uncultured rape rhizosphere bacterium wr0017 | AJ295478 | 99 | *Chaetoceros socialis* |
| SB-31-AG | uncultured rape rhizosphere bacterium wr0017 | AJ295478 | 100 | *Asterionella glacialis* |
| delta-Proteobacteria | | | | |
| SB-52-TW | *Desulfobacterium indolicum* | AJ237607 | 83 | *Thalassiosira weissflogii* |
| SB-53-TW | *Cystobacter ferrugineus* (strain Cb fe27) | AJ233902 | 90 | *Thalassiosira weissflogii* |
| SB-56-TW | uncultured marine proteobacterium BY-75 | AJ298361 | 92 | *Thalassiosira weissflogii* |

(Table 2 continued)

| | | | | |
|---|---|---|---|---|
| CFB group | | | | |
| SB-10/22-CS | uncultured Cytophagales ESR 4 | AF268288 | 90 | *Chaetoceros socialis* |
| SB-11/16-Cd | uncultured Cytophagales ESR 4 | AF268288 | 87 | *Coscinodiscus sp.* |
| SB-15-Cd | uncultured marine eubacterium OTU_C | AF207850 | 97 | *Coscinodiscus sp.* |
| SB-18-LD | Flavobacteriaceae str. 2 | AB024308 | 98 | *Leptocylindrus danicus* |
| SB-25-CS | benzene mineralizing consortium clone SB-5 | AF029041 | 88 | *Chaetoceros socialis* |
| SB-29-AG | uncultured CFB group bacterium kpc103f | AF195431 | 99 | *Asterionella glacialis* |
| SB-35-TW | benzene mineralizing consortium clone SB-5 | AF029041 | 90 | *Thalassiosira weissflogii* |
| SB-36-TW | uncultured CFB group bacterium kpc103f | AF195431 | 99 | *Thalassiosira weissflogii* |
| SB-39 -TW | uncultured marine eubacterium OTU_B | AF207849 | 90 | *Thalassiosira weissflogii* |
| SB-42-DB | uncultured bacterium BA2 | AF087043 | 93 | *Dytilum brightwellii* |
| SB-51-TW | uncultured CFB group bacterium kpc103f | AF195431 | 99 | *Thalassiosira weissflogii* |
| SB-54-AG | uncultured eubacterium KEppib22 | AF188173 | 95 | *Asterionella glacialis* |
| SB-6/55-AG | uncultured *Microscilla* kpc117f | AF195442 | 99 | *Asterionella glacialis* |
| SB-63-AG | *Cytophaga* sp. clone NB1-m | AB013834 | 89 | *Asterionella glacialis* |
| SB-66-Cd | humic substances enrichment clone D47 | AF231443 | 88 | *Coscinodiscus sp.* |
| SB-68-Cd | *Cytophaga* sp. strain BD7-10 | AB015585 | 89 | *Coscinodiscus sp.* |
| SB-7/64-AG | uncultured CFB group bacterium NAC60-3 | AF245645 | 87 | *Asterionella glacialis* |
| SB-83-CS | uncultured CFB group bacterium kpc103f | AF195431 | 96 | *Chaetoceros socialis* |
| SB-9/21-CS | uncultured marine eubacterium OTU_B | AF207849 | 90 | *Chaetoceros socialis* |
| | | | | |
| Diatom chloroplasts | | | | |
| SB-1/41-DB | *Skeletonema pseudocostatum* plastid | X82155 | 98 | *Dytilum brightwellii* |
| SB-2/32-TW | *Skeletonema pseudocostatum* plastid | X82155 | 99 | *Thalassiosira weissflogii* |
| SB-3/28-AG | *Odontella sinensis* plastid | Z67753 | 97 | *Asterionella glacialis* |
| SB-4/20-CS | *Skeletonema pseudocostatum* plastid | X82155 | 97 | *Chaetoceros socialis* |
| SB-17-LD | uncultured vent bacterium ML-2e | AF208995 | 97 | *Leptocylindrus danicus* |
| SB-5-Cd | uncultured marine eubacterium HstpL35 | AF159636 | 96 | *Coscinodiscus sp.* |

[1]AG, *Asterionella glacialis*; CS, *Chaetoceros socialis*; Cd, *Coscinodiscus* sp.; DB, *Dytillum brightwellii*; LD, *Leptocylindrus danicus*; SB, satellite bacterium; TW, *Thalassiosira weissflogii*

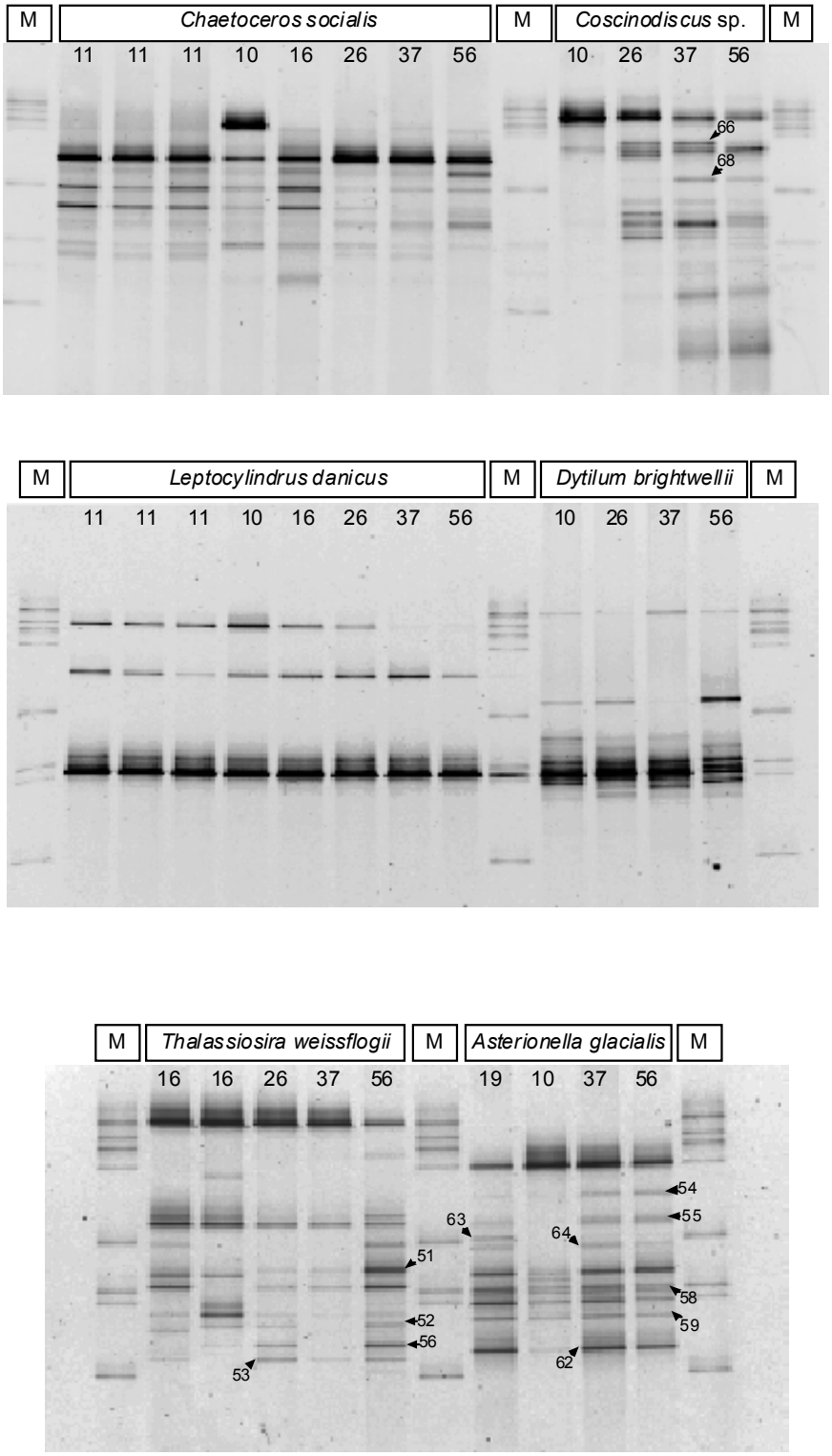



**Figure 2.**
DGGE analysis of diatom cultures from in different growth phases. The name of the culture is indicated above the respective lanes, M, indicates a marker lane. The age of the cultures on the day of sampling (in days) is indicated at the top of the lanes. The bands at the bottom of the gel are single stranded DNA.

**Coincidental selection or specific co-occurrence of algae and bacterial satellites**

For a number of reasons it is suggested that the observed patterns in the composition of the satellite assemblages are not coincidental, but to some extent the result of a selection process that enriches some well adapted bacterial populations. These may not be obligate but rather facultative satellites of their algal hosts. While Bell has suggested that native marine bacteria feed on a small set of low molecular weight compounds that may be present in PER originating from diverse algae (Bell, 1984), he also pointed out that long term selection should favour populations that are well adapted to the spectrum of organic carbon offered by the algae. The bacterial seeding stock at the time of isolation of algae (i.e. the species composition of the inoculate) might also expected to influence the outcome of the satellite assemblage to some degree, yet it should not preclude selection of suitable satellite bacteria., because culture-dependent approaches in marine microbial ecology suggest that at any one time a number of species is present that is not detectable by molecular methods (Bernard *et al*., 2000; Suzuki *et al*., 1997). Even if gross differences existed between inoculates at the time of isolation of algae, it may be expected that most samples share a set of taxa that are viable and present in low abundance. Numbers of genomes estimated to be present in environmental samples by DNA reassociation kinetics have shown presence of thousands of genomes in soil samples. Although, bacterioplankton samples show much lower complexity - on the order of around 165 genomes in freshwater (Ritz *et al.*, 1997) - there should be a sufficient diversity of bacterial populations to allow for selection of well adapted types, and make predominance of any kind of population that eventually is selected non-random. Therefore, obtaining different and unique genetic fingerprints from these cultures strongly suggests that there may be true co-occurrences between algae and bacteria in these diatom-bacteria cultures, formed by pairs of algae and bacteria that can co-exist efficiently.

**Phylotypes identified in diatom cultures**

To date their are no descriptions of satellite bacterial assemblages in the literature, thus the discussion of phylotypes found in diatom cultures refers to marine bacterial diversity in general. Additionally, there exist a number of Genbank sequence database entries that suggest that microalgae cultures have also been studied by others. In fact numerous of these sequences turned out to be close relatives of sequences determined in this study. Although algae cultures from strain collections represent artificial environments for both algae and bacteria, they harboured a range of phylotypes representative of several typical lineages of marine picoplankton. While β-Proteobacteria are often very abundant in freshwater habitats, in most cases they are present in low abundance or are not detectable at all in marine samples and therefore have not been considered typical members of marine bacterioplankton (Glöckner *et al*., 1999; Nold & Zwart, 1998). The exception to this are populations of ammonia-oxidising bacteria from the β-subdivision of the Proteobacteria which have been detected by molecular methods (using specific primers) in marine sediments (McCaig *et al*., 1999). Although detection of other β-Proteobacteria in coastal marine environments by molecular cloning techniques (Kelly & Chistoserdov, 2001; Kerkhof, *et al*., 1999; Rappé *et al*., 1997; Rappé *et al*., 2000; Suzuki, *et al*., 1997), fluorescent in situ hybridisation (Cottrell & Kirchman, 2000a; Glöckner, *et al*., 1999) and whole genome probing (Pinhassi & Hagström, 2000) has been reported, it has never been clear whether these constituted indigenous marine populations or rather were of terrestrial or freshwater origin. Therefore, an inability to grow at ionic strength of seawater which might explain the absence of β-Proteobacteria in marine samples physiologically has been suggested (Glöckner *et al*., 1996; Nold and Zwart, 1998), but there have also been some examples of isolation of β-Proteobacteria from marine samples (Hagström *et al*., 2000; Pinhassi and Hagström, 2000)

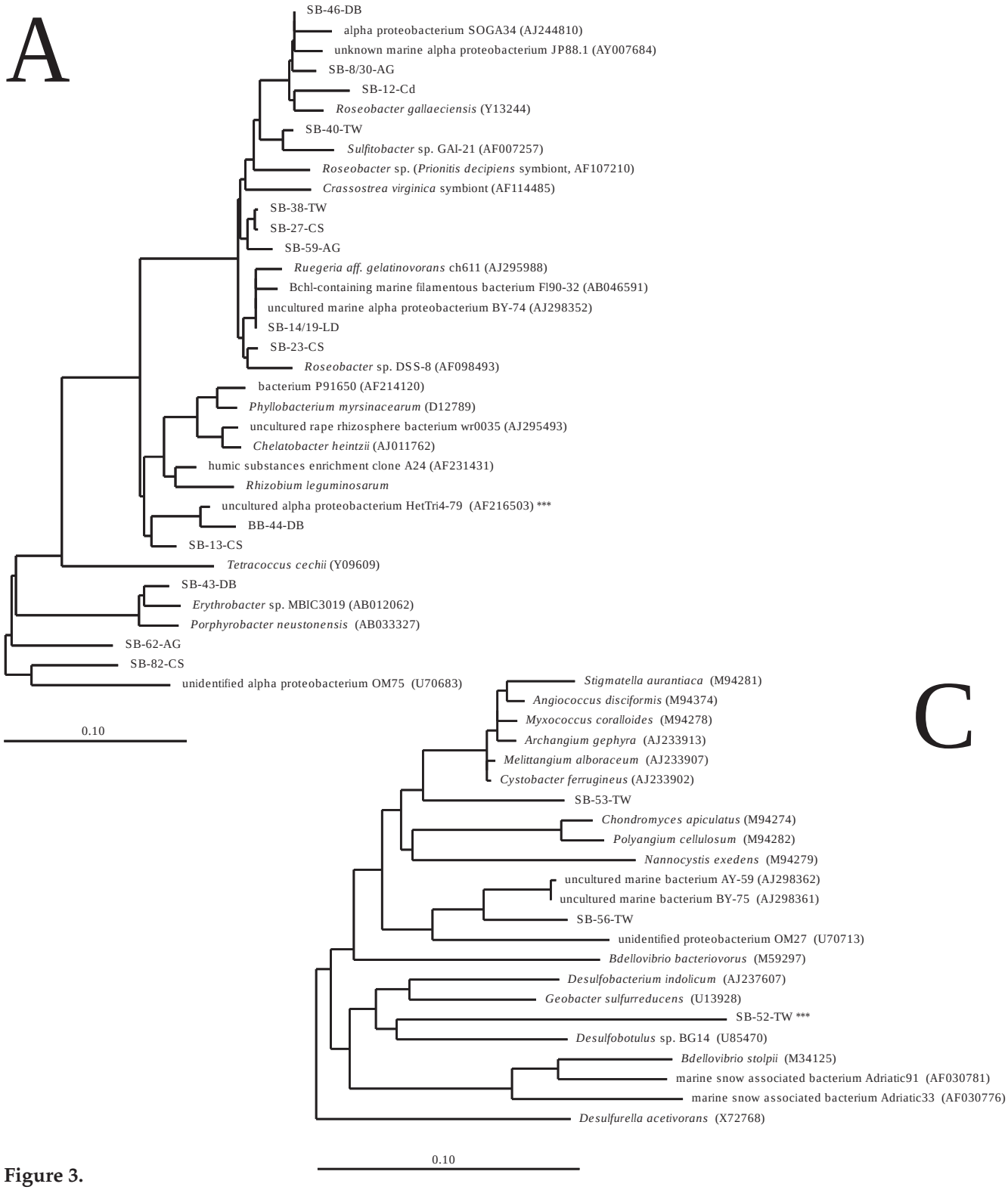
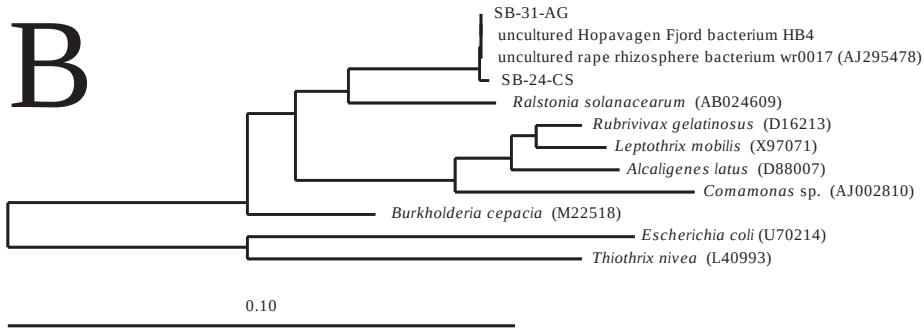
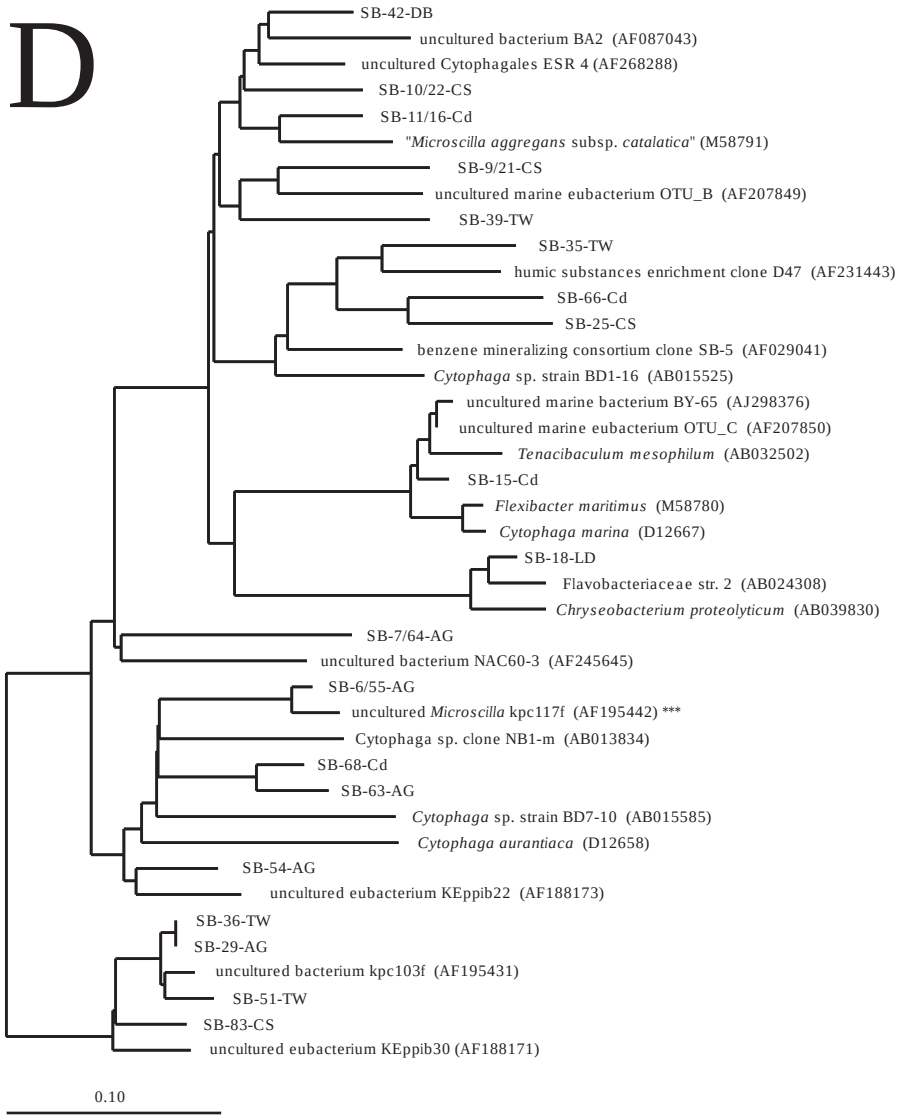



**Figure 3.**
Phylogenetic trees showing the relationships of sequences retrieved from DGGE bands from algae-bacteria cultures to reference sequences obtained from Genbank (accession numbers are given in parentheses). Names of sequences determined in this study are in bold and consist of the prefix SB-, a number referring to individual DGGE bands indicated in Fig. 1 and 2, and an abbreviation of the algal host species as indicated in Table 2. Some sequences have two numbers which indicates that the same band was retrieved and sequenced from two separate PCR products and DGGE gels (the DGGE gel of bands 1-14 is not shown), all duplicates had identical sequences. An example: sequence "SB-10/22-CS" was analysed from DGGE-bands 10 and 22, and was retrieved from the banding pattern of a *Chaetoceros socialis* culture. A, α-Proteobacteria; B, β-Proteobacteria; C, δ-Proteobacteria; D, Cytophaga-Flavobacterium-Bacteroides phylum; E, algae plastids. The root of the trees shown in Fig. 4A and 4D were determined with outgroups composed of various sequences from phyla spanning the bacterial domain. All neighbour joining trees were derived from the overlapping *E.coli* nucleotide positions using the Kimura 2-parameter model and a transition-transversion-ratio of 2 in Neighbor from the Phylip-package as implemented in ARB. Sequences marked with three asterisks (***) had only little overlap with the rest of the alignment and were therefore inserted into the neighbour-joining tree using the special ARB parsimony tool.


B
SB-31-AG
uncultured Hopavagen Fjord bacterium HB4
uncultured rape rhizosphere bacterium wr0017 (AJ295478)
SB-24-CS
Ralstonia solanacearum (AB024609)
Rubrivivax gelatinosus (D16213)
Leptothrix mobilis (X97071)
Alcaligenes latus (D88007)
Comamonas sp. (AJ002810)
Burkholderia cepacia (M22518)
Escherichia coli (U70214)
Thiothrix nivea (L40993)
0.10


D
SB-42-DB
uncultured bacterium BA2 (AF087043)
uncultured Cytophagales ESR 4 (AF268288)
SB-10/22-CS
SB-11/16-Cd
"Microscilla aggregans subsp. catalatica" (M58791)
SB-9/21-CS
uncultured marine eubacterium OTU_B (AF207849)
SB-39-TW
SB-35-TW
humic substances enrichment clone D47 (AF231443)
SB-66-Cd
SB-25-CS
benzene mineralizing consortium clone SB-5 (AF029041)
Cytophaga sp. strain BD1-16 (AB015525)
uncultured marine bacterium BY-65 (AJ298376)
uncultured marine eubacterium OTU_C (AF207850)
Tenacibaculum mesophilum (AB032502)
SB-15-Cd
Flexibacter maritimus (M58780)
Cytophaga marina (D12667)
SB-18-LD
Flavobacteriaceae str. 2 (AB024308)
Chryseobacterium proteolyticum (AB039830)
SB-7/64-AG
uncultured bacterium NAC60-3 (AF245645)
SB-6/55-AG
uncultured Microscilla kpc117f (AF195442) ***
Cytophaga sp. clone NB1-m (AB013834)
SB-68-Cd
SB-63-AG
Cytophaga sp. strain BD7-10 (AB015585)
Cytophaga aurantiaca (D12658)
SB-54-AG
uncultured eubacterium KEppib22 (AF188173)
SB-36-TW
SB-29-AG
uncultured bacterium kpc103f (AF195431)
SB-51-TW
SB-83-CS
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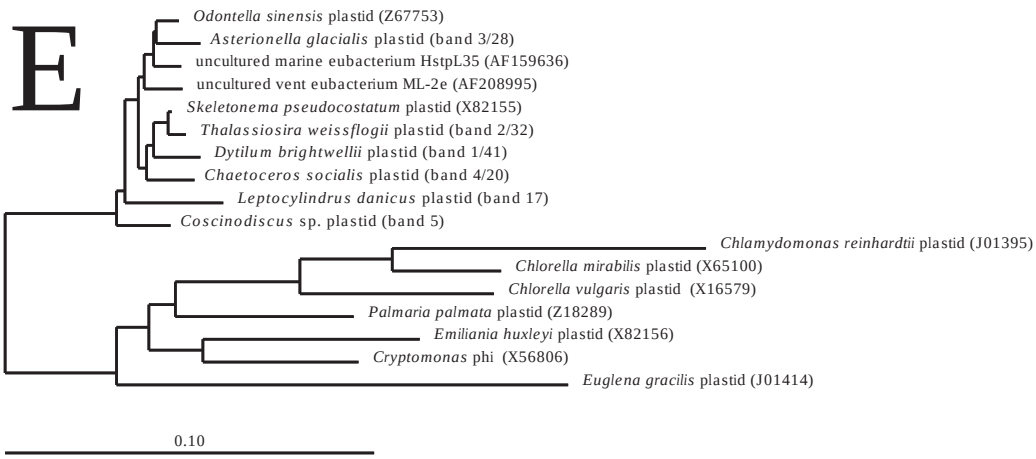
0.10


E
Odontella sinensis plastid (Z67753)
Asterionella glacialis plastid (band 3/28)
uncultured marine eubacterium HstpL35 (AF159636)
uncultured vent eubacterium ML-2e (AF208995)
Skeletonema pseudocostatum plastid (X82155)
Thalassiosira weissflogii plastid (band 2/32)
Dytilum brightwellii plastid (band 1/41)
Chaetoceros socialis plastid (band 4/20)
Leptocylindrus danicus plastid (band 17)
Coscinodiscus sp. plastid (band 5)
Chlamydomonas reinhardtii plastid (J01395)
Chlorella mirabilis plastid (X65100)
Chlorella vulgaris plastid (X16579)
Palmaria palmata plastid (Z18289)
Emiliania huxleyi plastid (X82156)
Cryptomonas phi (X56806)
Euglena gracilis plastid (J01414)
0.10

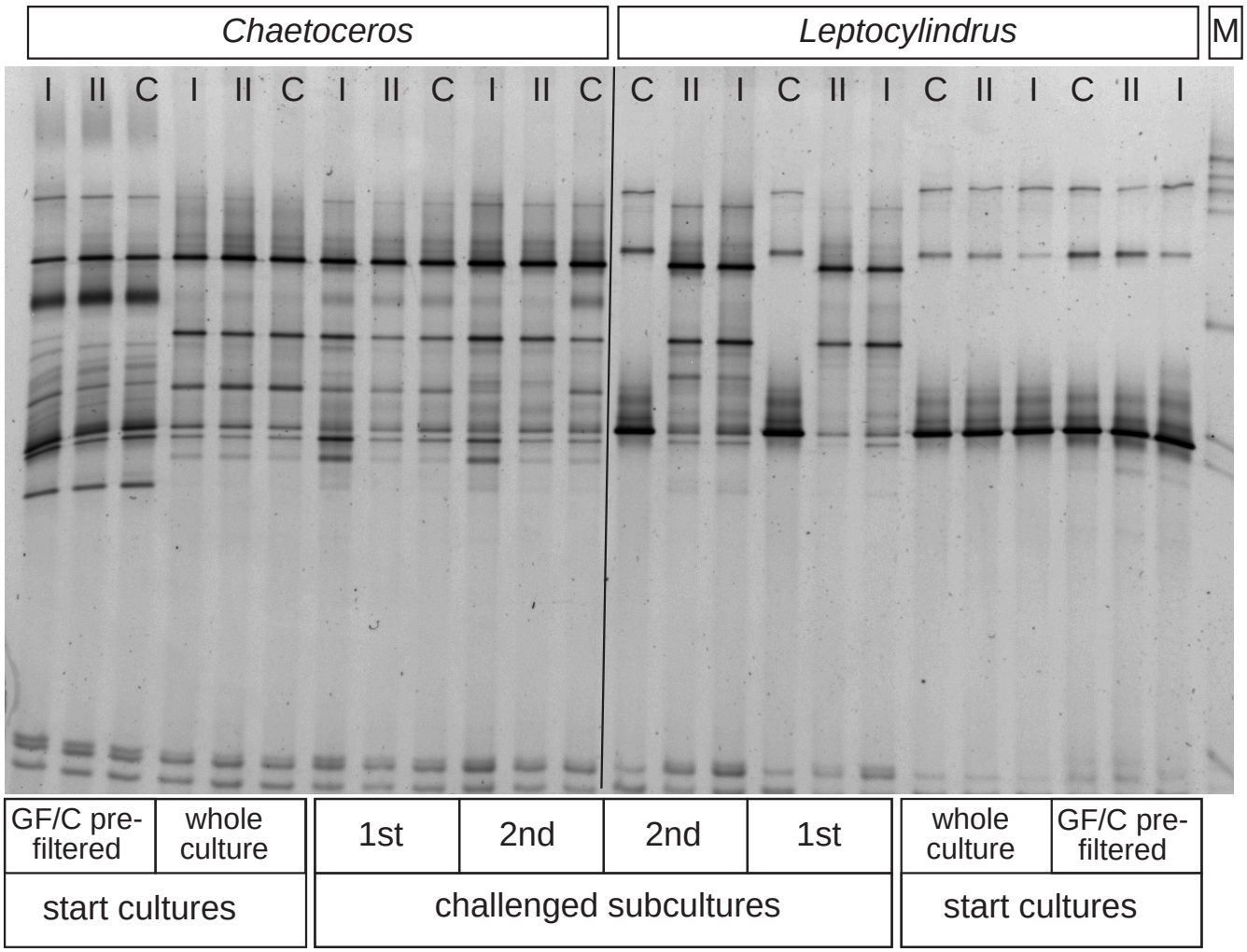



**Figure 4.**
DGGE analysis of the reciprocal challenging experiment to test the specificity of the association of algal hosts and satellite assemblages. The purpose of each sample is summarised at the bottom of the figure. The bands at the bottom of the gel are single stranded DNA. I, II and C indicate that the sample was from challenged culture-1, culture-2 or the unmodified control-culture of a series. M, indicates a marker lane.

The finding of β-proteobacterial phylotypes in these marine diatom cultures is interesting, as it further corroborates that at least some β-Proteobacteria are able to grow at marine salt concentration. However, the β-Proteobacteria found in this study were not closely related with any other β-proteobacterial phylotype retrieved from marine samples, but they formed a distinct cluster with a cloned 16S rRNA gene obtained from rhizosphere samples of rape. The only common denominator of these very contrasting environments seems to be the association or proximity to 'plant-tissue' probably forming microzones that receive plant derived organic molecules. Finding the same phylotype in DGGE patterns of samples from the Norwegian landlocked bay Hopavågen suggests that it does not represent an artefact of the culture conditions, but that these bacteria may at times be predominant members of marine microbial communities (Schäfer *et al*., in preparation). In contrast to finding unusual β-Proteobacteria, not a single band was affiliated with γ-Proteobacteria, who are rather typical members of marine bacterioplankton. This might suggest that there is no important role for γ-Proteobacteria in algae (diatom)-bacteria interaction. Yet, γ-Proteobacteria have been found for instance as tetrodotoxin producers in cultures of toxic dinoflagellates (Simidu, et al., 1990), thus they might for instance not be important in associations with diatoms, but instead link to other types of algae, e.g. dinoflagellates.

As expected there was a predominance of typical marine α-Proteobacteria and members of the CFB-phylum. Considering the findings of Cottrell and Kirchman (2000b) the hypothesised main roles of CFB and α-Proteobacteria are enzymatic breakdown of macromolecular DOM and utilisation of small molecular weight carbon units, respectively. Although this does not preclude involvement of α-Proteobacteria in degradation of complex organic matter by extracellular enzymatic activities as has been suggested by Riemann et al. (2000), it has also been shown by Cottrell and Kirchman (2000b) that no single phylogenetic group (i.e. CFB or α-Proteobacteria) dominated the consumption of all high- or low-molecular weight carbon.

**Conclusions**

Due to methodological constraints, direct proof for specific co-occurrence could not be gathered, yet the results presented here strongly suggest that laboratory diatom in cultures harbour distinct bacterial satellite assemblages. A common pattern of community composition was found constituting a elementary microbial community that is composed of a photosynthetic alga and members of the CFB and α-Proteobacteria. The common theme of predominance of α-Proteobacteria and members of the CFB group in all cultures may reflect specialisation in DOM and POM utilisation, respectively, whereby POM breakdown might be carried out especially (but not necessarily exclusively) by the CFB. α-Proteobacteria who have mainly been implicated in uptake of small organic molecules might co-feed on the organic matter made available by extracellular enzymic activity of CFB.

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# Appendix

## Summary

In the present study the diversity of coastal marine microbial communities was investigated. Emphasis was placed on the dynamic behaviour of the genetic diversity of the bacterial assemblages and on identification of factors that may exert structuring influence on composition of microbial communities. The influence of nutrient enrichment on microbial community composition was given special attention.

- The present study has demonstrated that the microbial communities in incubation experiments conducted in mesocosms may develop very differently from those in the field, and further stresses that the results obtained in mesocosm experiments must be interpreted with caution in relation to processes operating in the field.

- In mesocosm experiments marked changes in bacterial community composition were observed during the incubation. Decreasing numbers of detectable DGGE bands derived from DNA reflected growth of opportunistic bacterial population in the beginning of the incubation. Subsequent grazing by bacterivorous protozoa were concomitant with marked changes in genetic fingerprints of bacterial assemblages, suggesting an important influence of grazing on bacterial diversity. Grazing increased the number of DNA-derived bands, but reduced the number of DGGE bands derived from RNA after reverse-transcription PCR, suggesting that grazing restored the genetic evenness of the population by preferentially eliminating active cells.

- Nutrient addition to marine microbial communities in mesocosm experiments triggered some qualitative differences in community composition between unamended and nutrient-enriched bacterial communities, but primarily affected the speed at which fluctuations occurred in nutrient enriched versus unamended microbial assemblages.

- Strong seasonal fluctuation in bacterial community composition was recorded in samples from the landlocked bay Hopavågen (Norway). Some phylotypes exhibited annually recurring seasonal distribution patterns. Major changes were triggered by the beginning of the phytoplankton production period and a relative stable community persisted throughout the summer.

- Bacterial assemblages accompanying six different marine diatoms in laboratory culture (so-called satellite bacterial assemblages) were stable and of distinct composition, suggesting that bacteria-algae interaction in these cultures are non-random and hence algae and bacterial diversity may be linked. Genetic fingerprints of all six diatom cultures were predominated by bands representing members of the *Cytophaga-Flavobacterium-Bacteroides* group and α-proteobacterial phylotypes forming simple consortia that may be counterparts of interactions of algae and similar bacteria predominant in natural marine environments. Stable propagation of β-proteobacterial phylotypes in two cultures indicated physiological fitness of these populations in seawater based media, and hence demonstrates that β-Proteobacteria may be endemic in marine habitats.

## Zusammenfassung

Die vorliegende Arbeit befaßt sich mit der Diversität mariner mikrobieller Lebensgemeinschaften. Ein besonderer Schwerpunkt wurde auf die Dynamik der genetischen Diversität bakterieller Gemeinschaften gelegt und auf die Identifizierung solcher Faktoren, die strukturierenden Einfluß auf die Zusammensetzung mikrobieller Gemeinschaften ausüben. Dem Einfluß von Nährstoffanreicherung auf eine solche Zusammensetzung wurde besondere Aufmerksamkeit gewidmet.

- Die vorliegende Stude hat gezeigt, daß sich die Entwicklung mikrobieller Lebensgemeinschaften in in Mesokosmen durchgeführten Inkubationen sehr stark von der zeitgleichen Entwicklung im natürlichen Habitat unterscheiden kann und bekräftigt daher, daß Resultate aus solchen Experimenten Rückschlüsse auf die Entwicklung im Feld lediglich unter großem Vorbehalt zulassen.
- Während der Inkubation in Mesokosmen wurden starke Veränderungen in der Zusammensetzung bakterieller Lebensgemeinschaften beobachtet. Eine Abnahme der Anzahl von DNA abgeleiteter DGGE-Banden zu Beginn der Inkubation spiegelte das Wachstum opportunistischer bakterieller Populationen wider. Nachfolgende Beweidung durch bakterivore Protozoen ging mit deutlichen Veränderungen der genetischen Fingerabdrücke der bakteriellen Gemeinschaften einher. Diese Beobachtung läßt auf einen bedeutsamen Einfluß von Beweidung auf die bakterielle Diversität schließen. Die Beweidung erhöhte die Anzahl von DNA abgeleiteter DGGE Banden, aber reduzierte die Anzahl von RNA abgeleiteter Banden. Dies weist darauf hin, daß die Protozoen durch präferentielle Beweidung aktiver bakterieller Zellen (in diesem Zusammenhang insbesondere opportunistischer Populationen) das Gleichgewicht innerhalb der Gemeinschaft wieder herstellten.
- Nährstoffzugaben in Mesokosmos-Experimenten verursachten begrenzt qualitative Unterschiede in der Zusammensetzung der Gemeinschaften in mit Nährstoffen angereicherten und Kontrollansätzen. Jedoch schien die Anreicherung mit Nährstoffen vor allem die Geschwindigkeit der Veränderungen zu erhöhen.
- Erhebliche saisonale Fluktuationen in der Artenzusammensetzung bakterieller Lebensgemeinschaften konnten in Proben aus der Bucht Hopavågen (Norwegen) aufgezeigt werden. Einzelne Phylotypen zeigten jährlich wiederkehrende saisonale Verbreitungsmuster. Die größten Veränderungen wurden durch den Beginn der Phytoplankton Produktion eingeleitet. Während der Sommermonate war die Gemeinschaft verhältnismäßig stabil.
- Bakterielle Gemeinschaften, die sechs verschiedene marine Diatomeen in Labor-Kulturen begleiteten (sogenannte bakterielle Satelliten-Gemeinschaften), waren stabil und von charakteristischer Artenzusammensetzung. Diese Tatsache läßt darauf schließen, daß die Selektion von Satelliten-Bakterien in solchen Kulturen nicht dem Zufall überlassen ist, und daß die Diversität von Algen und Bakterien gekoppelt sein könnte. Genetische Fingerabdrücke aller sechs Diatomeen-Kulturen wurden durch DGGE-Banden dominiert, die Mitglieder der *Cytophaga-Flavobacterium-Bacteroides* Gruppe und α-Proteobakterien repräsentierten. Bakterien und Algen bildeten Konsortien, die den natürlichen Wechselwirkungen zwischen Algen und Mitgliedern dieser Bakteriengruppen im natürlichen Lebensraum entsprechen könnten. Die Anwesenheit β-proteobakterieller Phylotypen in zwei der Kulturen ließ auf die Lebensfähigkeit dieser Bakterien im marinen Milieu schließen.

## Samenvatting

Onderwerp van dit onderzoek was de bestudering van de diversiteit van marine bacteriële gemeenschappen, en in het bijzonder de bestudering van factoren die een rol zouden kunnen spelen bij de dynamiek van deze diversiteit, waarbij de nadruk werd gelegd op de invloed van nutriënten.

- Het onderzoek heeft aangetoont dat de dynamiek van bacteriële gemeenschappen in incubatie-experimenten sterk kan afwijken van die in het natuurlijke milieu. Het is daarom belangrijk zich te realiseren dat conclusies die uit deze experimenten worden getrokken niet dezelfde hoeven te zijn als die die uit een natuurlijke situatie getrokken zouden kunnen worden.
- Sterke veranderingen in de samenstelling van bacteriële gemeenschappen werden gezien tijdens de incubatie van zeewater in mesocosmen. Een vermindering van het aantal DGGE banden, een maat voor de genetisch diversiteit, werd aangetoont wat erop kan duiden dat er een sterke groei plaats vond van sommige opportunistische bacteriën in het begin van de incubatieperiode. De daarop volgende begrazing van de bacteriën door protozoën gaf eveneens een sterke verandering in het DGGE profiel. Na de begrazing was het aantal van DNA-afkomstige DGGE banden hoger dan voorheen, terwijl het aantal van RNA-afkomstige DGGE banden juist lager werd. Dit zou erop kunnen duiden dat protozoën de genetische diversiteit kunnen herstellen door preferentiele verwijdering van actieve bacteriën.
- Toevoeging van nutriënten had slechts een geringe invloed op de samenstelling van de bacteriële gemeenschappen in de verschillende mesocosmen, maar een grotere invloed op de snelheid en de mate waarmee deze veranderingen plaats vonden.
- In de kleine baai Hopavågen in Noorwegen manifesteerden zich sterke seizoenale veranderingen in de samenstelling van bacteriële gemeenschappen. Sommige fylotypen volgden een bepaalde seizoenale dynamiek. Bijzonder sterke veranderingen in de samenstelling vonden plaats tijdens de overgang van winter en lente naar die phase van phytoplankton productie. Daarna, tijdens de zomerperiode, was de samenzetting van de gemeenschap relatief stabiel.
- Bacteriële gemeenschappen die aanwezig waren in zes verschillende mariene diatomeën culturen (zogenaamde 'satellite bacteriën') waren stabiel en duidelijk verschillend in samenstelling wat er op kan duiden dat de interactie van bacteriën en algen in deze culturen niet toevallig was, en de diversiteit van deze twee groepen van elkaar afhankelijk zouden kunnen zijn. Bacteriën uit de *Cytophaga-Flavobacterium-Bacteroides* groep en uit de α-proteobacteriën waren dominant in de DGGE profielen van de 6 culturen. De bacteriën vormden consortia met de algen, die vergelijkbaar zouden kunnen zijn met de interacties tussen bacteriën en algen in het natuurlijke mariene milieu. Stabiele propagatie van β-proteobacteriën in twee van de culturen toonde aan dat deze bacteriën kunnen groeien in op zeewater gebaseerde media wat erop duidt dat deze populaties mariene vertegenwoordigers van de β-proteobacteriën zouden kunnen zijn.

## List of abbreviations

| | |
|---|---|
| APS | ammonium persulphate |
| BDT | BigDye™ Terminator |
| β-AOB | beta ammonium oxidising bacteria |
| bp | base pairs |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| CFB | *Cytophaga-Flavobacterium-Bacteroides* |
| chl *a* | chlorophyll *a* |
| CTD | conductivity temperature depth |
| DAPI | 4,6-diamidino-2-phenylindole |
| ddNTP | didesoxynucleotide-triphosphate |
| DGGE | denaturing gradient gel electrophoresis |
| DMSP | dimethylsulfoniopropionate |
| DNA | desoxyribonucleic acid |
| dNTP | desoxynucleotide-triphosphate |
| DOM | dissolved organic matter |
| EDTA | ethylendiamine tetra-acetic acid |
| EOC | extracellular organic carbon |
| FISH | fluorescent in situ hybridisation |
| HL | high-light adapted |
| HNF | heterotrophic nanoflagellates |
| LL | low-light adapted |
| MDS | multidimensional scaling |
| MMLV | Moloney Murine Leukemia Virus |
| MPN | most probable number |
| mRNA | messenger RNA |
| NCBI | National Center for Biotechnology Information |
| PCR | polymerase chain reaction |
| PER | photosynthetic extracellular release |
| PLFA | phospholipid fatty acid |
| POM | particulate organic matter |
| RALS | right angle light scatter |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcriptase-PCR |
| SDS | sodium-dodecyl-sulphate |
| SRB | sulphate reducing bacteria |
| TCA | trichloroacetic acid |
| TEMED | N,N,N',N'-tetramethylethylendiamin |
| TGGE | temperature gradient gel electrophoresis |
| T-RFLP | terminal restriction fragment length polymorphism |
| tRNA | transfer RNA |
| UF | urea formamide |
| UPGMA | unweighted pairwise grouping with mathematical averages |
| UV | ultraviolet |

# Contribution to the scientific publications presented in this dissertation

This dissertation is based on the publications listed below. These added explanations are to give an indication of the work that I, Hendrik Schäfer, have contributed to the individual chapters.

1. **Denaturing gradient gel electrophoresis in marine microbial ecology.**

   H. Schäfer and G. Muyzer

Gerard Muyzer was invited by Prof. John Paul to write this paper for a book dedicated to methods in marine microbiology. All practical work for examples of the application of DGGE in marine microbial ecology presented in the paper has been performed by Hendrik Schäfer. The manuscript has been written by Hendrik Schäfer with editorial help of Gerard Muyzer.

2. **Successional changes in the genetic diversity of a marine bacterial assemblage during confinement.**

   H. Schäfer , P. Servais, and G. Muyzer

Conception and execution of mesocosm experiments by complete CHABADA project-team. All molecular biological and phylogenetic analyses by Hendrik Schäfer. Data on protist abundance from Pierre Servais, writing of the manuscript by Hendrik Schäfer with editorial help of the co-authors.

3. **Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in the genetic diversity of bacterial populations.**

   H. Schäfer, L. Bernard, C. Courties, P. Lebaron, P. Servais, R. Pukall, E. Stackebrandt, M. Troussellier, T. Guindulain, J. Vives-Rego, and G. Muyzer

Conception and execution of mesocosm experiments by complete CHABADA project-team. All molecular biological and phylogenetic analyses by Hendrik Schäfer. Writing of the manuscript by Hendrik Schäfer with editorial help of the co-authors.

4. **Bacterial activity and genetic richness along an estuarine gradient (Rhône river plume, France)**

   M. Troussellier, H. Schäfer, N. Batailler, L. Bernard, T. Guindulain, M. Petit, R. Pukall, C. Courties, P. Lebaron, G. Muyzer, P. Servais, E. Stackebrandt, and J. Vives-Rego.

Conception and execution of field work by complete CHABADA project-team. Molecular biological analyses by Hendrik Schäfer. Writing of the manuscript by Marc Troussellier with editorial help of the co-authors.

**5. Does eutrophication alter bacterioplankton diversity? A four-year experimental study in the Norwegian landlocked bay Hopavågen.**

H. Schäfer, O. Vadstein, Y. Olsen, and G. Muyzer

Conception and execution of field experiments by Yngvar Olsen and Olav Vadstein. Molecular biological and phylogenetic analyses by Hendrik Schäfer. Data on chlorophyll *a* concentration and primary production by Olav Vadstein and Yngvar Olsen. Writing of the manuscript by Hendrik Schäfer with editorial help of the co-authors.

**6. Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms.**

H. Schäfer, B. Abbas, H. Witte, and G. Muyzer

Conception and execution of all experiments by Hendrik Schäfer, with technical assistance of Ben Abbas and Harry Witte. Phylogenetic analyses by Hendrik Schäfer. Writing of the manuscript by Hendrik Schäfer with editorial help of Gerard Muyzer.

## Further publications

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**Fajon C., Schäfer H. Cauwet G., Godon J.-J., Muyzer G., Stackebrandt E., Wassman P., and P. Lebaron. (submitted).** High phylogenetic diversity among bacterial strains with glucosidase and aminopeptidase activity from the Adriatic Sea.

**Schäfer. H., Ferdelman, T. G., Fossing, H., and G. Muyzer. (in preparation)**. Microbial diversity in sediments of the Benguela Upwelling System showing anaerobic methane oxidation.

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