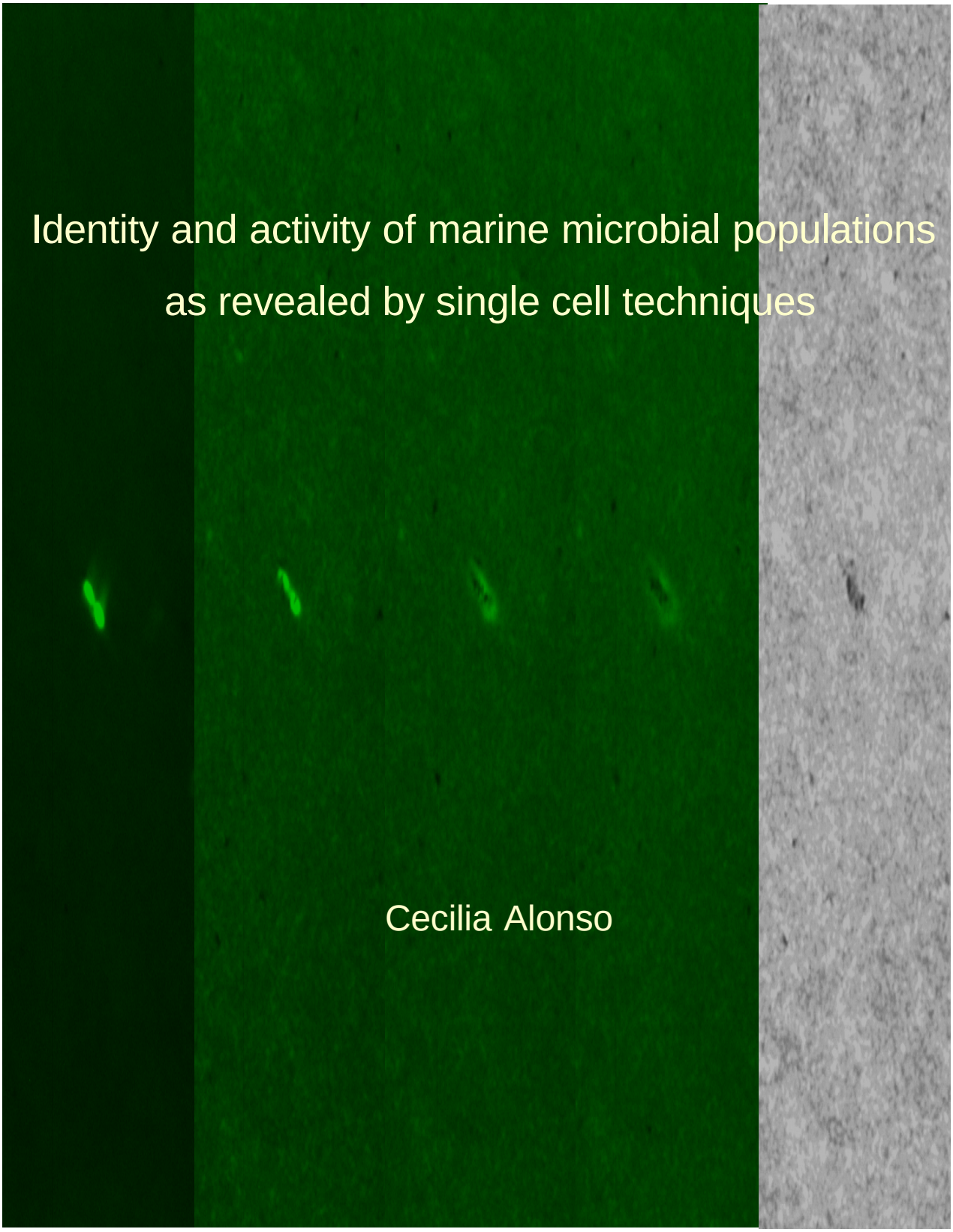

Identity and activity of marine microbial populations as revealed by single cell techniques

Cecilia Alonso

Este trabajo está dedicado a mis padres que hicieron todo lo posible -a veces también lo imposible- para ayudarme a encontrar mi camino.

Identity and activity of marine microbial populations as revealed by single cell techniques

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Cecilia Alonso

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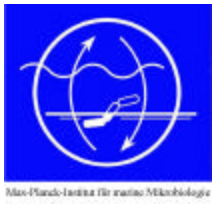

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"Tengo una banda amiga que me aguanta el corazón"

La Vela Puerca

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Summary

Summary

In most aquatic habitats the mere quantification of bacterial taxa does not appear to provide sufficient information about their ecological role. Consequently, there is a need for *in situ* approaches that allow simultaneous microbial identification and an estimate of microbial activity. These approaches should optimally provide a resolution at the level of single populations or even cells as bulk activity measurements seldom correlate with total abundances of bacteria and specific microbial populations may mediate central biogeochemical processes. At the end of the 1990's, a methodological approach was developed to track substrate uptake by specific prokaryotic groups. This was achieved by the combination of microautoradiography and fluorescence *in situ* hybridization (MARFISH). However, the original MARFISH method had several drawbacks for its application in marine samples. The first aim of this study was to overcome these limitations by introducing three major modifications that rendered the method more sensitive, accurate, and suitable for high-throughput sample processing. In the second half of this work this improved protocol was employed for two studies on the ecology of particular picoplankton populations in the coastal North Sea. In the first application the potential for anaerobic metabolism of pelagic bacteria was investigated. It has been suggested that in coastal environments the potential for anaerobic metabolism might be a common feature of bacterioplankton, but no direct evidence had been provided to support this hypothesis. Incorporation of glucose under anoxic conditions was found in *Alphaproteobacteria, Gammaproteobacteria* and the *Cytophaga-Flavobacteria*. Moreover, specific populations of copiotrophic bacteria (*Alteromonas, Pseudoalteromonas*) showed preferential glucose incorporation under anoxic conditions. In a second application, concentration-dependent uptake of glucose and leucine was assayed before and during a spring phytoplankton bloom. Coastal pelagic environments are characterized by concentration gradients of dissolved organic carbon, and by pronounced seasonal differences in substrate availability for the picoplankton. Microbial taxa that co-exist in such habitats might thus differ in their ability to incorporate substrates at various concentrations. Our results supported this hypothesis. Three patterns were observed for monomer uptake: high numbers of active cells regardless the substrate concentration (*Roseobacter*), preference for a specific concentration (SAR11 bacteria), and increasing numbers of active cells with increasing substrate concentration (SAR86, DE2 cluster of *Bacteroidetes,* and *Euryarchaeota*).

Zusammenfasung

Um die ökologische Rolle bestimmter bakterieller Taxa in aquatischen Lebensräumen zu verstehen, scheint es zumeist nicht ausreichend, nur ihre Populationsgrössen *in situ* zu quantifizieren. Vielmehr ist es notwendig, Ansätze zu entwickeln, welche die Identifizierung von Mikroorganismen mit einer Bestimmung ihrer Aktivität kombinieren. Solche Methoden sollten idealerweise auf dem Niveau der einzelnen Population oder sogar Zelle ansetzen, denn eine Aktivitätsbestimmung auf der Ebene der gesamten mikrobiellen Gemeinschaft korreliert selten mit den Gesamtzellzahlen, und verschiedene mikrobielle Populationen können für bestimmte biogeochemische Prozesse verantwortlich sein. Ende der 1990er Jahre wurden Ansätze entwickelt, um durch die Kombination von Mikroautoradiographie und Fluoreszenz *in situ* Hybridisierung (MARFISH) die Substrataufnahme in einzelnen Prokaryotengruppen zu untersuchen. Allerdings hatten die ersten MARFISH-Protokolle große Nachteile für eine Anwendung im marinen Bereich. Das erste Ziel dieser Arbeit war daher ein Umgehen dieser Nachteile mithilfe dreier Änderungen, welche die Empfindlichkeit und Genauigkeit der Methode verbesserten und einen größeren Probendurchsatz ermöglichten. Im zweiten Teil der Arbeit wurde dieses verbesserte Protokoll in zwei Untersuchungen über die ökologische Rolle bestimmter Picoplankton-Gruppen in der küstennahen Nordsee eingesetzt. In der ersten Studie wurde das Auftreten eines anaeroben Stoffwechsels bei pelagischen Bakterien untersucht. Es wurde zwar bereits die Ansicht geäußert, dass die Fähigkeit für anaeroben Stoffwechsel weit verbreitet im küstennahen Bakterioplankton sei, allerdings fehlte dafür bisher ein direkter Beweis. In dieser Arbeit wurde Glucoseeinbau bei *Alphaproteobakterien, Gammaproteobakterien* und Bakterien aus der *Cytophaga-Flavobacteria*-Gruppe nachgewiesen. Darüber hinaus zeigten bestimmte Gruppen copiotropher Bakterien (*Alteromonas, Pseudoalteromonas*) eine Präferenz für Glukoseeinbau unter anoxischen Bedingungen. In einer zweiten Studie wurde die konzentrationsabhängige Aufnahme von Glucose und Leucin vor und während einer Phytoplankton-Frühjahrsblüte untersucht. Küstennahe pelagische Habitate zeichnen sich aus durch Konzentrationsgradienten des gelösten organischen Kohlenstoffs sowie durch deutliche saisonale Unterschiede in der Substratverfügbarkeit für das Picoplankton. Mikrobielle Gruppen, welche in derartigen Habitaten zusammenleben, könnten sich daher in ihrer Fähigkeit unterscheiden, Substrate bei verschiedenen Konzentrationen aufzunehmen. Unsere Ergebnisse bestätigten diese Hypothese: Drei verschiedene Aufnahmemuster der Monomere wurden beobachtet: eine hohe Anzahl aktiver Zellen unabhängig von der Substratkonzentration („*Roseobacter*“ spp.), Vorliebe für eine bestimmte Konzentration (SAR11), und ein Ansteigen der Zahl aktiver Zellen mit steigender Substratkonzentration (SAR86, DE cluster 2 der *Bacteroidetes*, *Euryarchäen*).

Summary

Resumen

En la mayoría de los hábitats acuáticos la mera detección de un grupo bacteriano no brinda suficiente información sobre su rol ecológico. Para ello se necesitan técnicas que permitan simultáneamente determinar la identidad y la actividad de los microorganismos en su ambiente. Dichas técnicas óptimamente deberían ser de resolución unicelular dado que las medidas de actividad global raramente se correlacionan con la abundancia total de las bacterias y grupos altamente específicos de microorganismos pueden mediar procesos biogeoquímicos fundamentales. A fines de los '90 se desarrolló un método para estudiar la incorporación de sustratos por grupos específicos de procariotas a través de la combinación de microautoradiografía e hibridación *in situ* con sondas fluorescentes (MARFISH). Sin embargo, el método original tenía varias limitaciones para ser aplicado en muestras marinas. El primer objetivo de este estudio fue superarlas introduciendo tres modificaciones principales que tornaron el método más sensible, preciso y adecuado para la evaluación de muestras a gran escala. En la segunda parte de este trabajo el protocolo modificado fue empleado en dos estudios sobre la ecología de poblaciones picoplanctónicas en el Mar del Norte. En la primer aplicación se investigó el potencial de metabolismo anaeróbico de las bacterias pelágicas. Se ha sugerido que en ambientes costeros el metabolismo anaerobio puede ser una característica común en el bacterioplancton pero no se ha dado ninguna evidencia directa para apoyar esta hipótesis. En este estudio se encontró incorporación de glucosa en condiciones anóxicas en *Alfaproteobacteria, Gammaproteobacteria* y *Cytofaga-Flavobacteria*. Más aún, ciertas poblaciones de bacterias copiotróficas incorporaron glucosa preferentemente en condiciones anóxicas (*Alteromonas, Pseudoalteromonas*). En una segunda aplicación se estudió la incorporación de glucosa y leucina en función de su concentración antes y durante una floración algal. Los ambientes marinos costeros se caracterizan por gradientes de concentración de carbono disuelto y por pronunciadas diferencias estacionales en la disponibilidad de sustratos bacterianos. Los grupos microbianos que coexisten en estos habitats deberían diferir en su habilidad para incorporar sustratos a diferentes concentraciones. Nuestros resultados apoyan esta hipótesis. Tres patrones de incorporación de monómeros fueron observados: incoporación elevada independientemente de la concentración del sustrato (*Roseobacter*), preferencia por una concentración específica (SAR11) e incorporación incremental con la concentración de sustrato (SAR86, cluster DE2 de *Bacteroidetes y Euryarchaeota)*

Introduction

Identity and activity of marine prokaryotes

During the past decades the perception of bacteria as key components of the aquatic ecosystems has undergone an exciting revolution. The application of epifluorescence microscopy to enumerate bacteria in marine samples (Zimmermann R, 1974; Hobbie et al., 1977) has changed the paradigm derived from cultivation approaches that prokaryotes were typically rare and therefore unimportant in the water column (ZoBell, 1946). In parallel, methods for tracking the microbial uptake of radiolabeled substrates showed that the *in situ* substrates turnover rates were much faster than expected (Parsons and Strickland, 1961; Wright, 1965). By simultaneously estimating bacterial production and respiration it was shown that the prokaryotic fraction could be responsible for the utilization of as much as half of the material derived from primary producers (Fuhrman and Azam, 1980) and for a high proportion of the respiration of the whole planktonic assemblage (Pomeroy, 1974; Williams, 1981). These bulk measurements of microbial activity inspired the concept of "microbial loop" i.e. the flux of organic matter through prokaryotes and their grazers (Pomeroy, 1974; Azam et al., 1983).

However, the identity of the aquatic prokaryotes was still largely unknown. Microbial ecology was limited to the identification of the isolates that could be recovered on solid media, which represented as little as 0,1% of the total counts by epifluorescence microscopy (Kogure et al., 1978; Ferguson et al., 1984; Amann et al., 1995).

The first cultivation independent insight into marine prokaryotic diversity originated from the application of molecular tools that rely on the use of 16S ribosomal RNA as phylogenetic marker. Divergence in 16S sequences among different organisms has been used to define the primary lines of microbial evolution and it provided a tool for a natural classification of prokaryotes (Woese et al., 1985; 1987). In the early 1990's Giovanonni and co-workers revealed an unexpected diversity of the marine bacterioplankton through rRNA cloning and sequencing. They provided evidence for the presence of many novel bacterial groups, some of which still remain uncultured (e.g. SAR86 clade of *Gammaproteobacteria*) (Giovannoni et al., 1990; Britschgi and Giovannoni, 1991). The same approach lead, for instance, to the discovery of *Archaea* in the sea, organisms formerly believed to be restricted to extreme environments (DeLong, 1992; Fuhrman et al., 1992).

The introduction of rRNA hybridization techniques (Giovannoni et al., 1988; DeLong et al., 1989; Amann et al., 1990) allowed for quantification of different prokaryotic groups in their environment. Hence, microbial ecologists could not only gain insight into bacterial diversity in terms of richness, but also with respect to evenness. For instance, the application of fluorescence *in situ* hybridization (FISH) revealed that there are substantial differences in the composition of bacterial assemblages between freshwater and marine systems (Glöckner et al., 1999).

The combined approach of cloning, sequencing, probe design and quantification by FISH, termed the "full-cycle rRNA approach" (Amann et al., 1995), has been extensively applied to study prokaryotes in different natural environments, and during experimental manipulations (Pernthaler and Amann, 2005). However, it has also been observed that changes of microbial assemblages are often hard to interpret and do not always reflect the different ecological conditions. As a consequence, the mere *in situ* quantification of bacterial taxa usually does not provide enough information about their ecological role. Thus, there is a need for combined approaches that allow for simultaneous microbial identification and assessment of their activity.

Single cell approaches to address cellular activities

Activity is not homogeneously distributed within microbial assemblages. For example, only a fraction of bacterioplankton cells is responsible for the bacterial biomass production, measured as bulk incorporation of leucine or thymidine (Cottrell and Kirchman, 2003; Smith and del Giorgio, 2003). Furthermore, some central biogeochemical processes in the sea, as anaerobic methane oxidation or anaerobic ammonium oxidation, are mediated by very specific microbial populations (Boetius et al., 2000; Schmid et al., 2005). Therefore, activity measurements of whole microbial assemblages seldom correlate to total microbial abundances (e.g. del Giorgio et al., 1997) and methods with single cell resolution are needed for the study of the ecological role of single populations.

Several cellular criteria have been proposed as targets for specific stains and probes as indicators of cellular activity. Examples of properties that have been used to assess the metabolic status of individual cells include membrane integrity (Lopez-Amoros et al., 1995; Mason et al., 1998; Lopez-Amoros et al., 1998), respiratory activity (Zimmermann et al., 1978; Rodriguez et al., 1992; del Giorgio and Scarborough, 1995), DNA condensed in nucleoids (Zweifel and Hagström, 1995), cell

growth (Kogure et al., 1978; Urbach et al., 1999; Pernthaler et al., 2002b), rRNA content (Kemp et al., 1993; Amann, 1995), detection of specific mRNA (Hahn et al., 1993; Pernthaler and Amann, 2004), and substrate uptake (Brock, 1967; Hoppe, 1976). Most methods for determination of single cell activities were first developed independently from phylogenetic cell identification, but rather referred to total bacterial abundances as enumerated with general nucleic acids stains. Some of them have so far never been applied in combination with cell identification, whereas others have been combined with FISH.

FISH as activity indicator

The capacity to detect bacterial cells using oligonucleotide probes is in parts related to the cell specific rRNA content (Amann, 1995) (Figure 1, page 19). In turn, the ratio rRNA/rDNA has been correlated with the growth rate of cultured marine bacteria (Kerkhof and Ward, 1993; Kemp et al., 1993). Thus, rRNA content has been utilized as an index of cell activity (e.g. Karner and Fuhrman, 1997). Detection with oligonucleotide probes requires cells with a ribosomal content of 1000 to 10.000 copies per cell (Giovannoni et al., 1988; Lee and Kemp, 1994). However, there is evidence that total RNA content of cells in situ relates only inconsistently with metabolic activity (Jeffrey et al., 1996). For example, there are some bacterial taxa that keep a high ribosomal content during starvation (Flärdh et al., 1992; Eilers et al., 2000a).

In a recent publication Bouvier and del Giorgio (2003) reviewed the results so far obtained with FISH in natural bacterial assemblages. They showed that the proportion of cells that could be hybridized varied in a wide range from 1 to 100% of total counts. On average 56% of bacterioplankton cells were hybridized with the general bacterial probe EUB338 (Amann, 1995). The problem of detection due to low ribosome content have been solved with the use of more sensitive protocols, like hybridization with multiple labeled probes (DeLong et al., 1999; Pernthaler et al., 2002c; Church et al., 2003) and specially with probes labeled with horseradish peroxidase (CARDFISH) (Schönhuber et al., 1997; Pernthaler et al., 2002a). As a result of this increased sensitivity the FISH counts are much less dependent on variations of the physiological state of the cells (Bouvier and del Giorgio, 2003; Zwirglmaier, 2005). Moreover, the linearity of fluorescence intensity with ribosome

content may be lost during the signal amplification. These characteristics of the improved method preclude its utilization as a descriptor of single cell activity.

Vital stains for assessing membrane integrity

A bacterial cell is assumed to be viable if the membrane is not damaged, whereas it is probably dead if the membrane is compromised (Decamp and Rajendran, 1998a, b). The analysis of membrane integrity is based on the capacity of the cells to exclude specific compounds, such as fluorescent intercalating dyes which normally do not cross intact membranes. Such stains include propidium iodide, ethidium bromide, ethidium monazide and calcafluor white (Williams et al., 1998). For example, propidium iodide (PI) features a double positive charge thus it is assumed to be excluded by cells with intact cytoplasmatic membranes (Shapiro, 2000). This molecule accumulates in cells that have compromised membranes staining them with red color when visualized with wide green excitation. Thus, cells which take up PI are usually considered to be non viable (Jernaes and Steen, 1994; Lopez-Amoros et al., 1995; Williams et al., 1998). On the other hand, there are several fluorescent stains that are able to readily penetrate both living and dead cells (e.g. the SYTO series of stains). The LIVE/DEAD BacLight* bacterial viability kit by Molecular Probes provides a two-color fluorescent assay of bacterial viability using these two dyes: Propidium iodide and SYTO 9. When combined, the two stains are supposed to distinguish between live (fluorescent green) and dead cells (fluorescent red-yellowish) (Molecular Probes, 2003) (Figure 1, page 19). The evaluation can be performed either via epifluorescence microscopy or flow cytometry (Decamp and Rajendran, 1998a, b; Gasol et al., 1999). Critics to this method include the possibility of transient permeability to normally impermeant dyes. This can be induced by certain chemical and physical treatments (e.g. electroporation) with subsequent recovery of membrane integrity and viability (Shapiro, 2000; Molecular-Probes, 2001). Furthermore, the reliability of PI as an indicator of compromised cell membranes has been frequently tested on cultured bacteria using heat-killed bacteria (Lopez-Amoros et al., 1995; Williams et al., 1998). Recently, an *in situ* study showed that bacteria in natural environments are more permeable to PI-based stains as previously assumed (Pirker et al., 2005).

The propidium iodide staining has been combined with FISH in a technique termed "Vital Stain and Probe" (VSP) (Williams et al., 1998). This method

simultaneously assesses two independent criteria of cells that can be related to cell physiological status: i) membrane integrity and ii) ribosomal content. The combined staining with DAPI, propidium iodide and probes theoretically distinguishes between four categories of cells: alive and active, dead, dead but recently active and alive but relatively inactive, according to the combination shown in Table 1. So far, this method has not been applied in natural environments. A controversial aspect of this approach is the lack of a negative control. Moreover no explanation is offered of why active bacteria should suddenly die and maintain their cellular rRNA ("Dead but recently active").

Table 1. Classification of cells based on the combined staining with DAPI, PI and oligonucleotide probes (modified from Howard-Jones et al., 2001)

Cell status	DAPI	PI	Probe
Alive and active	+	-	+
Dead	+	+	-
Dead but recently active	+	+	+
Alive but with low or no activity	+	-	-

Redox dyes for detection of respiratory activity

The functioning of the respiratory chain can be visualized by the use of redox dyes e.g. 2-(p-iodophenyl)-3 (phenyl)-5-phenyl tetrazolium chloride (INT) or 5-cyano-2,3 ditolyl tetrazolium chloride (CTC) (Zimmermann et al., 1978; Rodriguez et al., 1992). Both INT and CTC are water soluble in their oxidized state and precipitate when reduced. In theory, bacterial cells that incorporate and reduce INT or CTC to visible formazan deposits should possess an active electron transport system (ETS). As activity within ETS is essential to cellular respiration (Packard, 1985) these cells are thought to be “actively respiring” (Rodriguez et al., 1992), (Figure 1, page 19). The enumeration of CTC+ cells has been used as a tool to detect active bacteria in freshwaters and marine systems (Rodriguez et al., 1992; del Giorgio and Scarborough, 1995; del Giorgio et al., 1997; Sherr et al., 1999a; Sherr et al., 1999b). Originally this technique was assumed to be applicable only to aerobic organisms but there is experimental evidence that tetrazolium salts could be also reduced under anaerobic conditions, particularly during glucose fermentation (Smith and McFeters,

1997). However, it is not suitable for application in reducing environments (e.g. sediments) where the *in situ* redox potentials are lower than the $E_{1/2}$ values of the tetrazolium salts, because non-biological reduction will occur (Smith and McFeters, 1997).

The determination of respiring bacteria has shown that the number of CTC+ bacteria was at least 10 fold more variable than total cell numbers across freshwater systems of different trophic states (del Giorgio and Scarborough, 1995; del Giorgio et al., 1997). Rates of bacterial production and respiration were more closely related to the abundance of CTC+ cells than to total counts in estuaries and lakes (Lovejoy et al., 1996; del Giorgio et al., 1997; Smith, 1998; Sherr et al., 1999a). Although in some studies close to 50% of bacteria can be CTC+ (del Giorgio and Scarborough, 1995), in general, the proportion of total cells scored as CTC+ tends to be low, less than 20% of total cells (del Giorgio et al., 1997; Sherr et al., 1999a; Smith and del Giorgio, 2003). In marine samples the fraction of CTC+ cells usually lies between 5 and 10% of all cells (Rodriguez et al., 1992; Gasol et al., 1995; Karner and Fuhrman, 1997; Berman et al., 2001). These low numbers have been interpreted as a limitation of the technique. It has been suggested that due to diversity in prokaryotic respiratory chains not all aerobic bacteria can reduce the particular tetrazolium salt or at least not in sufficient quantity to produce visible formazan grains (Smith and McFeters, 1997). Also toxicity of CTC or formazan precipitate has been shown (Ullrich et al., 1996). Currently, the method is regarded useful to enumerate the relative abundance of bacterioplankton cells with the highest respiration rates (Sherr et al., 2001).

Presence of a nucleoid body

From cultured bacteria it is known that DNA is organized in a condensed structure called a nucleoid. The nucleoid can be observed with epifluorescence microscopy after staining with a DNA specific fluorochrome [e.g. 4,6-diamidino-2-phenylindole (DAPI)]. As DAPI can bind non-specifically to other cell components, it has been suggested that bacterial counts using this technique overestimate the number of cells that actually contain DNA (Zweifel and Hagström, 1995). The NuCC technique (Nucleoid Containing Cells) attempts to enumerate only cells that contain nucleoids and to exclude bacterium like particles or dead cells. This is achieved by a destaining step with isopropanol (Figure 1, page 19).

The NuCC counts theoretically should represent the maximum estimate of the number of active bacteria since the presence of nucleoid does not ensure that a bacterium is growing or even capable of growth (Zweifel and Hagström, 1995). As initially proposed by Zweifel & Hagstrom (1995) the NuCC would be the living prokaryotes, whereas the rest of DAPI stained particles would be bacterial remainings, termed "ghosts". However, the proportion of NuCC found in marine plankton was unrealistically low, 2-32% of all DAPI counts (Zweifel and Hagström, 1995). In addition, it has not been possible to correlate this parameter to other methods for assessing activity of individual cells (Karner and Fuhrman, 1997). Subsequent studies showed that cells without nucleoids could regained them when supplied with a nutrient source (Choi et al., 1996).

A modified version of the protocol yielded higher values of NuCC (29-64% of DAPI counts) that were above the estimates of active cells as determined by two other methods (CTC precipitation and LIVE/DEAD kit). Currently, it is still unresolved what it is actually quantified by the NuCC method σ if all living marine bacteria should be assumed to contain a nucleoid.

Detection of growing cells

Inhibitors of cell division

During the late 1970s Kogure et al. (1978) developed an approach to identify and enumerate growing bacterioplankton cells. The method involves incubation of a water sample amended with a carbon source in the presence of nalidixic acid, a specific DNA gyrase inhibitor that interferes with cell division in many gram negative bacteria (King and Parker, 1988). Growing cells that respond to the antibiotic become elongated or swollen and can be directly enumerated microscopically to determine the actively growing fraction. A drawback from this method is the presence of bacteria that are resistant to nalidixic acid, and therefore able to grow and divide normally in the presence of this antibiotic. This limitation has led to modifications of the initial procedure and recent improvements have utilized multiple antibiotic cocktails (piromidic acid, pipemidic acid, ciproflaxin and cephalexin) that act similarly to nalidixic acid (Kogure et al., 1984; Servis et al., 1993; Joux and LeBaron, 1997). However, the presence of filamentous or pleomorphic cells in certain water samples may still interfere with the evaluation. Unfortunately, the recommended substrate addition (yeast extract) and prolonged incubation times (6-8 hours) may cause

activation of particular groups that normally were not proliferating in the natural sample. The growth of other bacterial groups may even been inhibited by the high substrates concentrations e.g. SAR11, (Rappe et al., 2002). Finally, some bacteria may have a long growth delay upon substrate addition (Pernthaler et al., 2001).

BrdU incorporation

5-bromo-2'-deoxyuridine (BrdU) is a thymidine analog. The bromodeoxyuridine-enriched DNA from the DNA synthesizing members of a microbial community can then be visualized either by immuno-fluorescence using anti bromodeoxyuridine monoclonal antibodies or isolated by immunochemical capture using antibody–coated paramagnetic beads (Urbach et al., 1999; Borneman, 1999). In 2002, a protocol combining detection of single cell BrdU incorporation and CARDFISH was published (Pernthaler et al., 2002b) (Figure 1, page 19). In this study only 3% of the bacterial cells showed positive BrdU incorporation, indicating a very small fraction of dividing cells in early autumn in the North Sea. The dividing cells were identified as belonging to three main populations: SAR86, *Roseobacter* and *Alteromonas*. SAR86 cells accounted for more than half of all BrdU positive *Bacteria* (Pernthaler et al., 2002b). Recently, the fluorescent signal intensity of BrdU incorporated by marine isolates has been correlated to their growth rates, indicating a potential for quantitative measurement of growth rates in natural bacterial assemblages (Hamasaki et al., 2004).

The main limitation of this technique is the inability of certain bacteria to incorporate this thymidine analog. This inability has been observed during uptake studies with isolates (Binnie and Coote, 1986; Urbach et al., 1999) and also in coastal seawater and lake samples where the BrdU:TdR incorporation ratio was significantly less than 1, indicating preferential incorporation of TdR (Steward and Azam, 1999).

Microautoradiography for tracking substrate uptake

Autoradiography is known from more than one century. It has, in fact, contributed to the discovery of radioactivity: Niepce in 1867 and later Becquerel in 1896 observed that uranium caused blackening of photographic emulsions (Rogers, 1979). Use of autoradiography to microbial ecology was first reported in the 1960's (Brock and Brock, 1968).

With this method it is possible to visualize bacteria that are engaged in substrate uptake. Microbial assemblages are supplied with a radiolabeled substrate, then the cells are put in contact with an autoradiographic emulsion. After exposure of the emulsion to their radioactive emissions, silver grains deposit around the cells (Figure 1, page 19).

Radioisotopes are greatly preferable to other ways of labeling such as staining or use of fluorescence elements because their chemical and physical properties largely coincide with those of the natural isotopes of the same elements and molecules they compose. In addition, the rate of radioactive disintegration is a purely statistical process and does not depend on physical or chemical conditions such as temperature, pressure, or pH. Moreover, radioisotopes can be detected at quantities less than 10^{-10}- 10^{-15} milligrams by counting radioactive disintegrations (Sorokin, 1999). The detection threshold of cell activity is so low that radiotracers are widely held as being the most sensitive technique for enumerating metabolically active bacteria in natural environments (Smith and del Giorgio, 2003).

Drawbacks of this technique include the potentially hazardous work with radioactivity, and the limited range of available radiolabeled substrates. Moreover it is possible that the added substrates are degraded during the incubation, leading to labeling of organisms that did not take up the original molecule but metabolites derived from it.

In a pioneer study, Brock (1967) used Microautoradiography with tritiated thymidine to calculate *in situ* growth rates of *Leucothrix mucor*, a conspicuous filamentous marine bacteria.

Microautoradiography + FISH

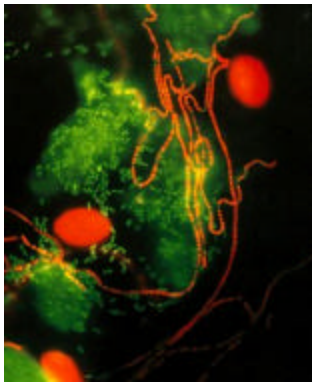
Recently, microautoradiography and FISH have been combined to profit from the complementary advantages of the two approaches. A number of acronyms have been proposed for a range of slightly different approaches, e.g. MAR-FISH (Micro-auto radiography-fluorescence *in situ* hybridization (Lee et al., 1999), STAR-FISH (Substrate tracking autoradiography- fluorescence *in situ* hybridization (Ouverney and Fuhrman, 1999), and MICRO-FISH (Microautoradiography -fluorescence *in situ* hybridization (Cottrell and Kirchman, 2000). In the following the approach will be referred to as MARFISH. To date, MARFISH has been applied in a small but increasing number of studies of natural and engineered environments. The method

was first used to determine microbial utilization of organic and inorganic substrates in activated sludge (Lee et al., 1999). Almost at the same time, Ouverney and Fuhrman (1999) demonstrated that most bacterial cells in the marine picoplankton were able to take up tritiated amino acids, in particular *Alphaproteobacteria* and members of the *Cytophaga-Flavobacterium* group. Subsequently, the same authors revealed that the planktonic marine *Archaea* were also capable of assimilating dissolved amino acids at nanomolar concentrations (Ouverney and Fuhrman, 2000). Thus, it was concluded that at least some of the *Archaea* were heterotrophic. Moreover, *Archaea* and *Bacteria* apparently successfully competed for the same pool of dissolved amino acid pool as no qualitative difference between the labeling of both prokaryotic groups was found.

MARFISH has also been used to determine the relative contribution of different prokaryotic groups to the utilization of marine dissolved organic matter (DOM) (Cottrell and Kirchman, 2000). The predominant groups present in coastal and estuarine waters showed very distinct patterns of DOM uptake and no single group dominated in the consumption of all tested substrates (chitin, N-acetyl glucosamine (NAG), protein, and amino acids). For instance, a high proportion of the *Cytophaga-Flavobacteria* group was consuming chitin, NAG and protein, even though they constituted a small fraction of the total population. In contrast, only a small proportion of this group was involved in the assimilation of dissolved amino acids, which were preferably consumed by *Alphaproteobacteria*. The complex patterns of DOM fraction utilization did not correlate with the relative abundance of the bacterial groups. This prompted the authors to suggest that it would be important to consider more than a single compartment for modeling the role of heterotrophic bacteria in carbon cycle (Cottrell and Kirchman, 2000). In a subsequent study, it was analyzed which bacteria assimilated ^{3}H-thymidine and ^{3}H-leucine in an estuary, where the community composition varied along the salinity gradient (Cottrell and Kirchman, 2003). Members of all majors groups of bacteria (*Proteobacteria* divisions and *Cytophaga*) were able to take up both tracers. About 50% of the variation in the assimilation by bacterial groups was explained by community composition, suggesting that abundance and activity are at least partially controlled by the same factors. The dominant phylogenetic groups were usually also responsible for the highest contribution to bacterial production. However, some results indicated that other factors than abundance must also play a role in defining the contribution of

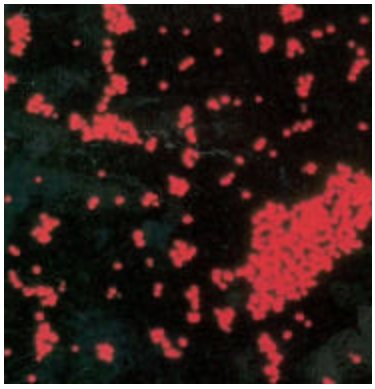
bacterial groups to total production. For example, *Alphaproteobacteria* appeared to account for 3 fold more assimilation of leucine and thymidine than *Cytophaga-Flavobacteria,* although both groups occurred at similar densities (Cottrell and Kirchman, 2003).

Examples of the use of MARFISH to elucidate physiological characteristics of more specific groups in the marine bacterioplankton are given by the work of Malmstrom and co-workers (2004 and 2005). These authors focused on the SAR11 cluster which often accounts for a large fraction of pelagic prokaryotes (Morris et al., 2002). Malmstrom et al. (2004), first studied the incorporation of the algal osmolite dimethyl sulfopropionate and of amino acids by SAR11 bacteria in coastal and open ocean waters. The fraction of SAR11 bacteria incorporating both substrates was equal to or greater than the average of the total bacterial community, being responsible for 50% of amino acid assimilation and 30% of DMSP assimilation in surface waters. The difference between the active fractions of SAR11 and other bacteria was greatest at the open ocean site, suggesting that SAR11 bacteria may be well adapted to oligotrophic conditions. In a second study (Malmstrom et al., 2005), the contribution of SAR11 bacteria to bacterial ^{3}H-leucine incorporation was investigated, as well as the assimilation of ^{3}H-glucose, amino acids and protein. SAR11 cells were one third to one half of bacterial cells that incorporated leucine; this represented a higher fraction than expected from their abundance (25% of total prokaryotes). Moreover, they disproportionately contributed to the uptake of glucose and amino acids. By contrast, SAR11 bacteria assimilated only as much or considerable less protein as predicted by their abundance. From these results it was argued that SAR11 bacteria would be more important for the biogeochemical flux of low molecular weight monomers, particularly amino acids, than for high molecular weight polymers.

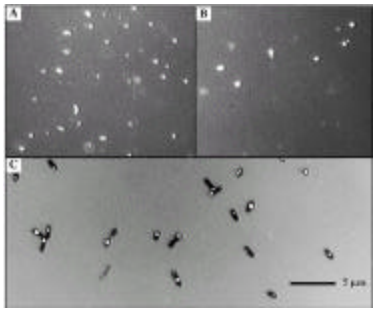


Live and dead Bacteria on top of epithelial cells

(Molecular Probes)

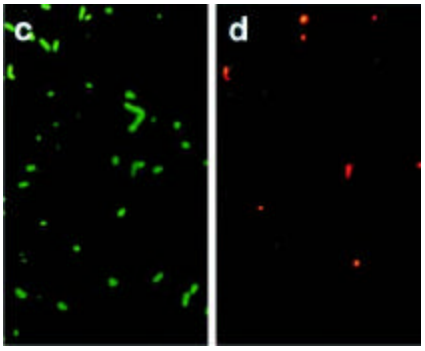


CTC positive cells from pure culture *Pseudomonas putida* (Rodriguez *et al*., 1992)



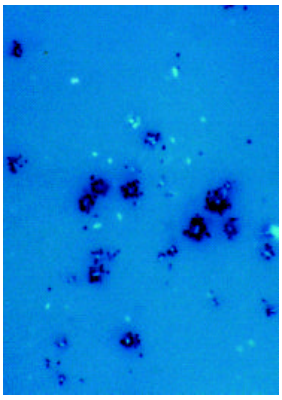
A: DAPI stain B: NUCC
C: superimposed image

(Zweifel & Hagström 1995)



Hybridized and Brdu positive Bacteria

(Pernthaler *et al*., 2002)



MAR positive Bacteria (DAPI stained)

(Cottrell & Kirchman 2000)

Fig. 1 Single cell methods for studying bacterial identity and activity.

What are active bacterioplankton cells?

Often a dominant fraction of the bacterial assemblage is typically unresponsive to at least some of the above discussed assays across a range of aquatic ecosystems (del Giorgio and Bouvier, 2002; Smith and del Giorgio, 2003). Gasol et al. (1999) proposed that the microbial abundances estimated by DAPI counts would be composed of 5 different types of particles: 1) large viruses and cell fragments, 2) dead cells but intact in shape, 3) inactive because the proper conditions for their development are not present, 4) growing at a very low rate, and 5) rapidly growing. Our current tools correctly identify the first and second group as dead and inactive and the fifth as alive and probably highly active with either method. The 3rd and 4th groups are probably the most abundant ones. For those cells each technique indicates different quantities, thus assigning them to the active or inactive pool depending on the methodology. The apparent lack of agreement between different approaches is the consequence of the diversity of physiological states in bacterioplankton and points to the large range of criteria for describing metabolic activity in bacterial communities. For example, Karner and Fuhrman (1997) observed that generally the percentage of cells incorporating amino acids determined by microautoradiography (MAR) was very similar to the percentage of cells hybridized by FISH, and that only a small fraction of cells was CTC positive.

Until very recently, many authors have applied one of these methods with the assumption that cells are either active or inactive in absolute terms depending on how they score with the technique in question (see review by Smith and del Giorgio, 2003). This has lead to high and likely pointless controversy as to which is “the best” method to study bacterial activity at the single cell level. However, evidence has accumulated that it is unlikely that a single technique can cover all the physiological diversity present in bacterioplankton assemblages. Moreover, irrespective of the method used, the physiological state of a cell is operationally defined and it is inferred from the general properties of a particular technique. Therefore, the results obtained by any single method may provide only partial information about bacterioplankton activity.

Currently, there is increasing agreement on that only using a suite of techniques would be possible to obtain an adequate picture of the complex phenotypes that bacterioplankton cells express in the environment.

Results and Discussion

1. Development of a MARFISH protocol

Microautoradiography

The autoradiographic process

The nuclear emulsions used in autoradiography are suspensions of crystals of silver bromide in gelatin (Kodak; Rogers, 1979). When these crystals are hit by electrons from the radioactive source a “latent image” is formed, due to the presence of a metallic silver nucleus inside the exposed crystals. This latent image is converted into a true image through photographic development. In the presence of the developing agent the nucleus of metallic silver catalyzes the conversion of the entire crystal into metallic silver. The bromide crystals that have not been reduced to silver are subsequently dissolved out of the emulsion by the fixative, leaving on the emulsion a pattern of silver grains reproducing the pattern of radioactivity location (Rogers, 1979).

Outline of the technique and practical considerations

1. Incubation with radiolabeled substrates.
2. Fixation of cells to stop substrate incorporation.
3. Filtration, bacteria with incorporated radioactive substrate are retained in the filter.
4. Exposure of the nuclear autoradiographic emulsion by bringing it in contact with the radioactive sample.
5. Chemical developing of the autoradiograms.

Incubation: Choice of Isotopes

Incubations with tritiated substrates are preferable because of its low radiation energy that would cause silver grain deposition sufficiently close to the cells that incorporated the substrate (Sorokin, 1999; Brock and Brock, 1968). ^{14}C and other ß emitters as ^{35}S and ^{125}I can also be suitable. In contrast, autoradiograms with ^{32}P may be difficult to interpret as the high-energy electrons can expose the emulsion distant from the source (Brock and Brock, 1968; Rogers, 1979).

Sufficient radioactivity must be present within the cell in order for adjacent emulsion grains to be exposed, ideally after being in contact with the emulsion for

only a few hours or days (Brock and Brock, 1968). It is preferable to use substrates with higher activities than to perform longer incubations. Specific radioactivity should not be less than 1mCi/mmol, it frequently ranges between 5 and 15 mCi/mmol (Brock and Brock, 1968; Sorokin, 1999). The tracers should be added in the highest specific radioactivity available to avoid increasing the organic concentration, and thus altering the system (Brock and Brock, 1968).

Fixation

Aldehydes used for fixation may react with autoradiographic emulsions by producing background grains and desensitizing the emulsion. Therefore, fixatives need to be removed before the sample comes in contact with the emulsion (Brock and Brock, 1968).

Emulsion handling

The emulsion (Eastman Kodak NTB-2 liquid nuclear track) must be handled only in the dark room illuminated with a safelight filter (Wratten #2, company) (Kodak). The darkroom must be absolutely light tight and the temperature should be kept preferably under 23°C (Brock and Brock, 1968; Rogers, 1979). Care is needed to block all light that may leak from the all luminous surfaces (e.g. indicator bulbs). Undiluted emulsion as delivered forms a too thick layer, it is better to dilute it to half concentration (Brock and Brock, 1968). It is highly recommendable to aliquot the fresh emulsion to avoid re-melting that can lead to spoiling. The emulsion must not be shaken but mixed through gentle inversion as shaking introduces air bubbles that distortion the autoradiograms. The emulsion should be kept refrigerated and protected from any form of radiation and freezing (Brock and Brock, 1968; Kodak).

As the nuclear emulsion dries, it becomes more sensitive to exposure by light, radiation and physical damage. It is recommended not to work closer than a meter to the safelight and to use the safelight only when is absolutely necessary (Brock and Brock, 1968; Rogers, 1979).

Exposure conditions

In order to reduce fading of the latent image it is important to reduce the humidity of the environment where the slides will be exposed. Exposure at low temperatures (typically 4°C) is preferable as it reduces the rate of chemical processes which may

increase background, but it also decreases the sensitivity of the emulsion (Brock and Brock, 1968).

If the exposure time is too long there may be a high density of silver grains obscuring the underlying material. Also, since the latent image may fade, prolonging the exposure time will not necessarily intensify a weak image when insufficient radioactivity is present. It is preferably to use short exposure times (maximally up to 1 week) (Brock and Brock, 1968).

Chemical developing of the autoradiograms.

Developing is a process of amplification, increasing the size of the deposit of metallic silver in a crystal until it reaches a threshold at which it can be recognized. This threshold is determined exclusively by the conditions of viewing (Rogers, 1979). With increasing contact time with the chemical developer, more silver is added to the silver deposit. The process ends when all the silver available in the crystal has been converted to metallic silver. At this point the developed grain occupies a volume of up to 3 times that of the original crystal (Rogers, 1979). Because the emulsion is extremely light sensitive when it is in contact with the developer, developing must be done in total darkness (Brock and Brock, 1968; Kodak).

Some of the factors affecting photographic development are i) the nature of developing agent, as some developers are more powerful reducing agents than others, ii) the developing time, as ultimately every crystal in the emulsion would be developed and iii) indirectly the temperature, as it controls the rate of the process. Therefore it is critical importance to work under 23°C (Rogers, 1979). Developing conditions might need to be optimized for all these factors to achieve the best signal-to-noise ratio.

After the chemical development, the fixation step dissolves the undeveloped silver bromide crystals that remain in the emulsion, this is usually carried out in a solution of thiosulphate. The thiosulphate ion forms a series of soluble complexes with ionic silver, without affecting the developed grains. The end point of fixation is generally taken as twice the period required for the emulsion to become transparent. As nuclear emulsions contain a higher percentage of silver bromide than do photographic emulsions, and the speed of fixation drops off rapidly as the products of fixation accumulate in the solution, it is important not to exceed the capacity of the fixer solution by over use (Rogers, 1979).

Background signals

In every autoradiogram, silver grains appear that are not due to radiation from the experimental source, but to other causes. One of them is over development. Even in absence of metallic silver the probability of development is finite and some crystals that were not exposed will nevertheless become developed grains. The aim of development is to produce the optimal ratio between grains at the source of radioactivity and unspecifically formed background grains.

Other causes for background include i) exposure to light (safelight filters only ensures that the wavelength of the light falling on the emulsion is the less harmful), ii) background due to pressure by accidentally touching the emulsion or due to too fast drying (because the shrinking of gelatin in the emulsion exerts pressure on the silver bromide crystals), iii) chemography, as some reactive groups, mainly from reducing agents can produce a latent image, iv) contamination of the emulsion due to improper cleaning, and v) environmental radiation (Rogers, 1979).

Fluorescence in situ hybridization and catalyzed reporter deposition

The FISH protocol consists of a hybridization step where the whole cells are in contact with a buffer containing the probe, and a subsequent washing step which removes the unbound probe (DeLong et al., 1989; Amann et al., 1990). The improved CARDFISH method was initially proposed by Amann and co-workers (1992), and is currently applied as the modified protocol by Pernthaler and co-workers (2002). Due to the use of horseradish peroxidase labeled probes, further steps for cell permeabilization and signal amplification are needed.

Outline of the technique and practical considerations

1. Fixation of the sample.
2. Filtration onto Nucleopore filters.
3. Cell immobilization via embedding of the filters in 0.2% low-gelling point agarose.
4. Cell permeabilization with 10 mg/ml Lysozyme solution for 1 hour at 37°C.
5. Hybridization at 35°C for 2 hours.
6. Washing of the hybridization for 15 minutes at 37°C.
7. Signal amplification: 15 minutes incubation in PBS at room temperature
   15 minutes incubation with fluorescent tyramide
   15 minutes washing in PBS at room temperature
8. Final washing with MilliQ water.

Compared to the original FISH protocol, the CARDFISH technique is longer and includes several incubation and washing steps that represent a further "aggression" to the sample. This needs to be taken into account for its combination with autoradiography.

MARFISH

The original MARFISH protocol for marine bacteria by Ouverney and Fuhrman (1999) has several drawbacks.

1. It is based on FISH rather than CARDFISH. FISH may yield lower detection rates in some marine environments (see Bouvier and del Giorgio (2003) for a review).
2. The step of transferring of cells from the filter to emulsion may cause extensive cell loss. Although there is no reference to cell loss in the original description, Cottrell and Kirchman (2000) later estimated that it could be up to 50% of all cells. In our own experience, this percentage is highly variable and depends on several handling factors.
3. The protocol is quite difficult and time consuming. Moreover, the risk of background generation is increased due to i) the obligatory use of light at distances shorter than the manufacturer's indications, and ii) accidental pressure during placing the filters on top of the emulsion.
4. A high fraction of preparations (up to 30%) is lost due to the detachment of filters during transfer and developing.
5. Often, a low reproducibility of replicates is achieved due to the complicated handling.
6. As a consequence of the above points the protocol provides a limited capacity for an accurate evaluation of numerous samples.

The aim of this study was to overcome these limitations by introducing three major modifications: 1) Combination of MAR with CARDFISH, 2) Avoidance of the cell transfer step, and 3) Development of an automated evaluation routine.

Protocol development

Samples used during the first phase of method development consisted in either water from the pond of the Max Planck Institute for Marine Microbiology, or from the Coastal North Sea. North Sea water samples were amended with 0.01% (w/v) Spirulina (Sigma), a cyanobacteria with very high protein content (Ciferri, 1983). All samples were incubated with 10 nM [methyl ^{3}H] thymidine for 4 hours.

Combination of autoradiography with CARDFISH

The first step of our modifications was combination of autoradiography with CARDFISH. This could be realized without problems if CARDFISH was performed before the MAR procedure (Figure 2).

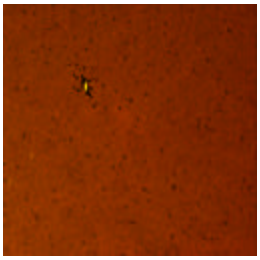


Fig. 2. First experience of combination of CARDFISH and Autoradiography

When CARDFISH was applied after the samples were already covered with the nuclear emulsion, a disruption of the thin coating layer was observed, leading to loss of the autoradiogram. To address this issue, the emulsion was not diluted with gelatin solution according to the standard procedure. Instead, agarose was tested as possible enhancement agent of the nuclear emulsion. Seven different brands of agarose with different melting and gelling points and gel strengths were tested at different concentrations to achieve melting at the same temperature as the nuclear emulsion (43°C, Table 2). The best results were achieved with Seakem LE agarose (final concentration 0.1% w/v). In a subsequent experiment, Seakem LE agarose was introduced as replacement of the Low-gelling point agarose that is used in the standard CARDFISH protocol (Pernthaler et al., 2002a). The nuclear emulsion diluted at 50:50 ratio (v/v) with Seakem LE agarose (concentration 0.2 %) was no longer disrupted when CARDFISH was performed after the MAR procedure. This modification is crucial as it allows to process samples that could loose rapidly their radioactive signal when using short-lived isotopes (like ^{35}S). It is also useful to re-

hybridize a sample with a second probe. However, when possible, it is recommended to perform CARDFISH before MAR as this is more suitable for the processing of large numbers of samples.

Table 2. Properties of the tested agarose brands for dilution of the nuclear emulsion

Agarose type	Melting point (°C)	Gelling point (°C)	Gel strength (g/cm^2)	Melting Performance
Low melting point	<65 (at 2%)	26-30 (at 2%)	300 (at 1%)	Good, but low strength
Ultra low gelling point	50	8-17	75 (at 2%)	Good, but low strength
Merck 12 239	<65 (at 4%)	<35 (at 4%)	Data not provided	Bad
Seakem LE	90	34.5-37.5	>1200 (at 1%)	Good, high strength
NuSieve	<35 (at 4%)	<35 (at 4%)	500 (at 4%)	Good, but low strength
Gibco 15517-014	<65 (at 1.5%)	24-28 (at 1.5%)	>250 (at 1%)	Bad
Metaphor	<75 (at 3%)	<35 (at 3%)	>300 (at 3%)	Bad

Avoidance of the cell transfer step

The original MARFISH protocol for marine bacteria includes a step where the cells are transferred from the filter to the emulsion causing significant cell loss. To avoid this step, a set of different filter types was first tested to act as support both for MAR and CARDFISH. From the suite of filters assayed (Polycarbonate track-etched, Cellulose nitrate and Anodisc), only the Polycarbonate track-etched filters that are routinely used for FISH were adequate.

However, autoradiography requires a rigid support surface. Therefore, it was necessary to test a series of glues to attach the polycarbonate filters to a glass slide. Several glues were tested: Ullrich's adhesive (Brock and Brock, 1968), Gelatin, domestic adhesive, Acrifix™, PAP gluing pen, and the UHU two-component epoxy adhesive. Only the last one was i) able to resist the whole procedure (including performance of CARDFISH after MAR) and ii) did not cause background for any of the two techniques.

Microautoradiography on polycarbonate filters was already introduced by Meyer-Reil in 1978, but Tabor and Neihof (1982) subsequently questioned it. These authors argued that the method was not suitable due to high background fluorescence for Acridine orange direct counts (Hobbie et al., 1977), and also because the filter pores would interfere with the recognition of cells and silver grains. Instead, they proposed the cell transfer from the filter to a glass slide coated with emulsion. As a consequence, this approach has become the standard for MAR of marine microbes. To examine whether this criticism would apply to the here developed protocol, the polycarbonate filters were glued to Cytoclear™ slides (Osmonics). These slides have a Teflon coating that renders the filter structure invisible under transmitted and fluorescent illumination (Osmonics). No improvement in the image quality was observed with respect to the use of normal glass slides, neither for the cell detection nor for the observation of silver grains. We concluded that probably the combined application of a different general nucleic acid stain (DAPI), a particular setting of illumination and the easy visualization of cells after CARDFISH allowed us to overcome the problems reported by Tabor and Neihof (1982).

Final protocol for MARFISH performance after incubation with radiolabeled substrates.

Reference: Alonso, C and J., Pernthaler. 2005. Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Appl. Env.Microbiol*.: **71**:1709-1716

1. Stop incubation by adding PFA 1% final concentration. Fix 1 hour at room temperature.
2. Filter sub-samples onto polycarbonate filters (diameter: 25 mm, pore size 0.2µ).
3. Rinse the filters 2 times with 5 ml of 1x PBS.
4. Cut the filters into 8 or more pieces
5. (Perform CARDFISH).
6. Glue the filter pieces onto the slides with epoxy glue.
7. In the dark room, melt the photographic emulsion 1/2 h at 43°C using a water bath.
8. Add an equal volume of agarose solution (0.2% w/v) to the emulsion to obtain a 1:1 dilution.
9. Coat the glass slides (with the glued filters) with the diluted emulsion and put them on a metal bar, allow drying for some minutes.
10. Place the slides in a lightproof box wrapped in aluminum foil and expose them at 4°C (exposure times need to be optimized for every experiment).
11. Develop the slides by using Kodak specifications:
12. Allow to air dry.
13. (Perform CARDFISH).
14. Stain with DAPI (final concentration: 1 µg/ml) at 4°C for 10 minutes, rinse for 1 minute with deionized water and then for 30 seconds with ethanol (80%).
15. Mount in a mix of Citifluor/Vectashield.

First field tests

In August 2002 an experiment was conducted for testing the method under development. Seawater was collected at the island of Helgoland and incubated for 4 hours after addition of 10 nM [methyl ^{3}H]-Thymidine. Samples for analyzing community composition were manually evaluated in triplicates. An average of 68% of DAPI counts were hybridized with the probe EUB I-III (*Bacteria,* Daims et al., 1999). More than 60 percent of *Bacteria* could be identified with the probes ROS537 (*Roseobacter* cluster, Eilers et al., 2001) and CF319a (*Cytophaga –Flavobacteria*, Manz et al., 1996).

For the autoradiograms, a series of exposure times was tested. The optimal exposure time was 18 hours. Triplicates preparations were manually counted. Ten fields were evaluated for each replicate. In every field, the total number of hybridized cells and the number of hybridized cells with silver grains deposits were counted. The counts were highly reproducible and the coefficients of variation of MAR+ hybridized cells (standard deviation as percentage of the mean) were between 6 and 12% (Figure 3).

On average, a quarter of all bacterial cells showed thymidine incorporation, indicating that this fraction of the community was synthesizing DNA at this summer sampling time (Figure 3). Members of *Roseobacter* cluster and *Cytophaga-Flavobacteria* showed a very different pattern of thymidine incorporation. On average only 8.5% of all *Cytophaga-Flavobacteria* cells incorporated the tracer, in contrast, almost all *Roseobacter* cells (91%) were MAR positive (Figure 3). Together, these two groups constituted 85% of all DNA synthesizing bacterial cells (Figure 4).

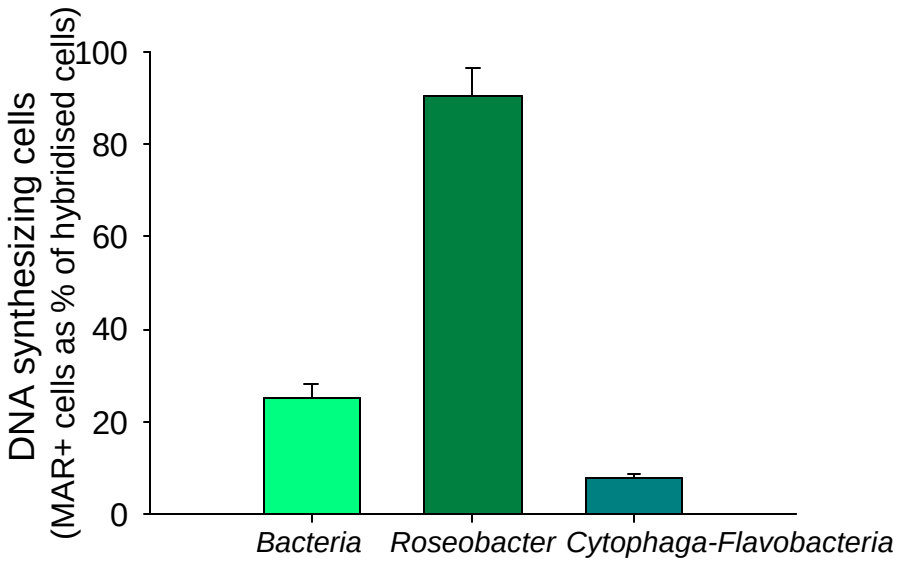


Fig. 3. Proportion of MAR positive cells within each bacterial group

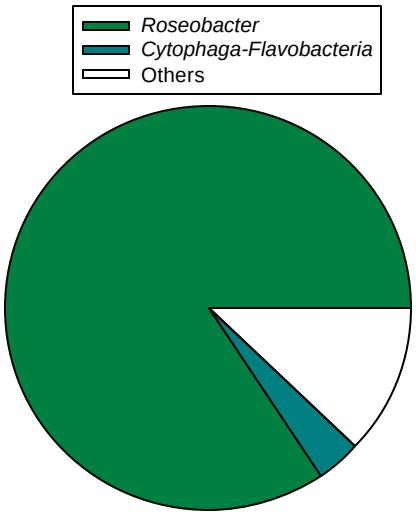


Fig. 4. Contribution of specific populations to total fraction of DNA synthetisizing *Bacteria*

Incubations with 10 nM [methyl ^{3}H]-Thymidine were also performed in samples from various depths (subphotic, meso- and bathypelagic zones) of the Mid Atlantic Ocean. The MARFISH protocol developed for North Sea samples proved to be equally suited to detection hybridization and autoradiographic signals from bacteria in the open and deep ocean.

Comparison with a standard protocol

Recently, a modification of the original protocol by Ouverney and Fuhrman (1999) was published by Teira et al. (2004). It substitutes FISH with CARDFISH, but it maintains the cell transfer. With the aim to compare both approaches, a series of parallel evaluations were carried out. This was done in co-operation with Karel Hornak from the Hydrobiological Institute of the Czech Academy of Sciences, Ceské Budejovice), who has experience with the Teira et al. protocol. The results of the comparison are presented as a list of advantages and disadvantages of each approach, and possible solutions are proposed to overcome the major problems.

Transferring cells from filters to emulsion

Advantages:

- The visualization of hybridized cells is less dependent on hybridization quality and on the number of grains in the vicinity of cells.
- The quality of DAPI staining is often better.

Disadvantages and possible solutions:

- The cell loss during transferring cannot be accurately quantified and varies in each assay. As a possible solution it was suggested to transfer the cells before the CARDFISH procedure since cells are much more difficult to remove after embedded and hybridization (see Sekar et al. (2004).
- Lower efficiency in processing of samples due to loss of filter pieces during development.
- Difficulties in removing the filters from the slides without damaging emulsion. A moistening with ethanol is a possible solution but it leads to increased cell loss.
- Often variability is observed between replicates in a set of preparations.
- Difficulties in evaluation of a single preparation are encountered due to very heterogeneous numbers of cells per microscopic field.

Performing MAR directly on filters

Advantages:

- The sample is maintained as it was filtered after fixation, there is no cell loss.
- The procedure is easier, minimizes dark room handling and has a higher success rate.
- Good reproducibility is obtained.
- Single preparations are homogeneous, allowing for a more rapid evaluation.

Disadvantages and possible solutions:

- It is more difficult to visualize the hybridized cells due to dim hybridizations or heavy grain coating. The solution to this problem is to adjust the brightness of hybridization (probe and tyramide concentration, hybridization time) and to carefully optimize exposure times.
- The quality of the DAPI staining is more variable. One possible solution is to perform staining at 4°C for a longer period of time.

<u>The automation of counting</u>

The automation of the simultaneous evaluation of autoradiograms and fluorescent hybridizations was mainly realized by the supervisor of this work: Dr. Jakob Pernthaler.

Evaluations of MAR-FISH preparations were carried out on an Axioplan II imaging fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a motorized stage, z-axis drive and fluorescent filter wheel, and with a digital camera (Orca I, Hamamatsu, Herrsching, Germany) linked to a personal computer. The image analysis software KS400 (Carl Zeiss Vision, Hallbergmoos, Germany) was used to develop a semi-automated image acquisition and evaluation strategy.

Image acquisition

As the filters are glued on the slide at variable positions it was not possible to develop a completely automated protocol for image acquisition as it was previously achieved for evaluation of CARDFISH preparations (Pernthaler et al., 2003).

The acquisition of images is achieved in three steps. First, the operator focuses on hybridized cells at UV excitation and 63x magnification. An image pair is acquired at UV (for DAPI staining) and blue excitation (for probe staining). Next, a stack of 5 bright field images is acquired automatically in a region of 1-5 µm above the filter containing the cells. The bright field images are combined into a single image and grains from different layers are detected by selecting the minimum brightness of each pixel from the image stack. This stack of images is taken to adequately record MAR grains in an emulsion of potentially variable thickness. During manual evaluation this is achieved by changing the focus depth.

Image evaluation

The detection of DAPI and FISH double-stained objects in the respective images is performed as described for the automation of CARDFISH evaluations (Pernthaler et al., 2003).

The MAR grains in the combined bright field image are detected by the following strategy: First the image is inverted and the average gray value is subtracted for background correction. Next, contrast is rescaled to cover 255 gray levels and the image is binarized at a fixed threshold (gray value 150). Objects below a custom size threshold are removed, and the binary image is further processed by two rounds of sequential object dilation and erosion by 1 pixel (morphological closing). Binary images from each image triplet are combined for the automated colocalization of DAPI-stained and hybridized cells, and of MAR grains. The binary images can be depicted on the screen as a three-color overlay which can be interactively combined with the original images for a manual elimination of artifacts before the counting procedure (Figure 5). This protocol of image acquisition and evaluation was eventually simplified to acquire only probe and MAR images. In the current procedure, the fraction of hybridized cells is determined separately before the autoradiographic process.

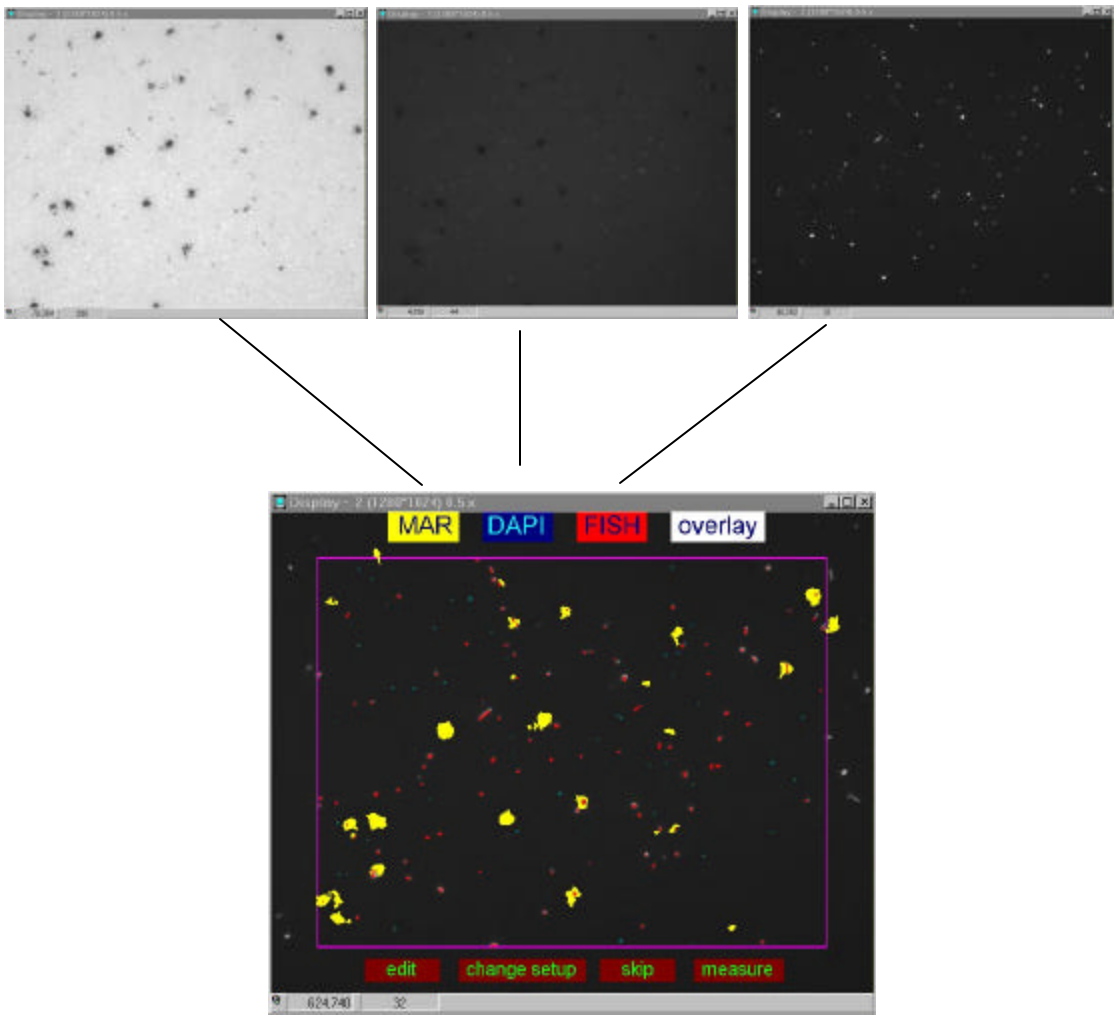


Fig. 5. Example of software assisted image analysis of MARFISH preparatio ns.

Validation of the image evaluation routine

The routine for semi-automated evaluation was tested for reproducibility at different levels: repeated evaluation of same sample, replicate evaluation of independent samples and comparison of results obtained by different users. Based on these data, the program was adapted to allow the user to define separate thresholds for probe intensity, cell size, size and connectivity of silver grains, providing a highly flexible and accurate system of evaluation.

Evaluation of reproducibility of the technique

The first application of the newly developed method provided the opportunity of examining the reproducibility of the approach, i.e. starting from separate triplicate incubations, until the image analysis evaluation. (The ecological question that was addressed in this context is discussed in the next section).

Samples from coastal North Sea water were evaluated with a hierarchical set of probes, targeted to bacterial taxa that formed between <1% to 68% of all DAPI counts. These bacterial groups also exhibited the whole range of possible MAR fractions of MAR+ cells (from 1 to 100%). The coefficients of variation (CV) were calculated (standard deviation/average) for every set of triplicates. The average of all CVs was 0.2, the median value was 0.15 and the modal (most frequent) values of were 0.11 and 0.12 (Table 3).

Table 3. Coefficients of variation of triplicate MARFISH evaluations

Set of triplicates	Average MAR+ (%)	Average FISH (n)	CV
1	14	14	0.31
2	20	14	0.51
3	30	14	0.26
4	14	33	0.20
5	11	41	0.18
6	20	27	0.38
7	33	18	0.64
8	13	62	0.25
9	13	61	0.31
10	26	32	0.43
11	3	281	0.25
12	86	12	0.07
13	31	54	0.16
14	94	24	0.06
15	8	330	0.33
16	21	209	0.11
17	16	335	0.21
18	11	528	0.08
19	12	497	0.12
20	12	519	0.05
21	32	222	0.07
22	84	104	0.05
23	59	157	0.15
24	11	864	0.12
25	71	162	0.03
26	19	714	0.12
27	37	367	0.16
28	12	1204	0.41
29	27	538	0.10
30	30	582	0.11
31	29	653	0.12
32	17	1554	0.11
33	30	1319	0.11

The CVs were plotted as a function of the number of hybridized cells and of the percentages of MAR positive cells. The higher CVs (>0.3) were found at combinations of low number of hybridized cells (<60) and low proportions of MAR positives cells (<33%). Altogether, the number of examined cells was the most decisive factor for precision (Figure 6). Based on these results it was concluded that to obtain CVs below 0.2, at least 50 MAR-positive hybridized cells should be counted for each triplicate.

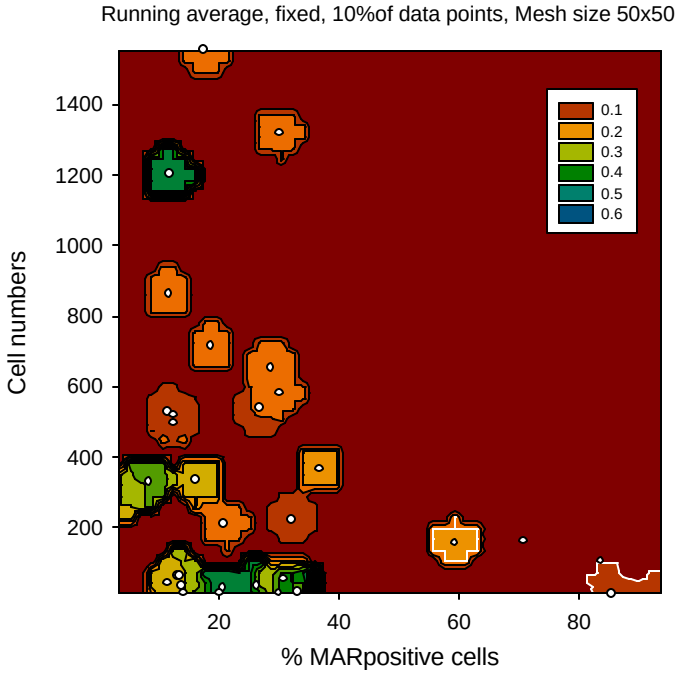


Fig. 6. Coefficients of variation among triplicate MARFISH evaluations as function of FISH counts and proportion of MAR positive cells

Further applications

Apart from the applications described in this section and the following ones, the modified MARFISH technique so far has been successfully applied in a humic lake (Sekar, unpublished results.), and oligotrophic Mediterranean waters (Vila, unpublished results.).

Conclusions

The modified MAR and CARDFISH staining and quantification protocol represents an improvement of the original technique in several respects:

- By combination with CARDFISH it allows a more quantitative detection of the prokaryotic community in marine waters. It avoids visualization problems due to low ribosome content and also to the attenuation of the fluorescent signal by the autoradiographic process.
- It avoids cell loss, permitting an accurate quantification of the proportion of cells responsible of tracer incorporation in a hybridized population
- It specifically circumvents the accidental loss of preparations due to unsuccessful cell transfer, which is a common problem of other MAR protocols.
- As the processing of preparations is substantially faster, it allows the design of complex investigations where many samples need to be evaluated. Presently, the typical number of data points in publications using MARFISH is between 10 and 30. In contrast, the average number of evaluated samples in the studies presented here is >100.

2. Facultative anaerobic metabolism among bacteria in the water column

Introduction

Anoxic microenvironments can be found in the fully oxygenated marine world (Alldredge and Cohen, 1987). In the coastal regions a significant fraction of microbial production is attributed to attached bacteria, that rapidly colonize and degrade particles of organic origin (Simon, 1987; Middelboe et al., 1995; Simon et al., 2002). Such zones of high turnover potentially represent a niche that might favor anaerobic metabolism. Moreover, in shallow habitats, such as the coastal southern North Sea, the frequent mixing between sediment and water column may contribute to the generation of at least transiently anoxic particles (Wainright, 1987; Tengberg et al., 2003). These resuspension-deposition events may also promote the exchange between bacteria from both habitats that would be potentially facing alternating oxic-anoxic phases (Wainright and Hopkinson, 1997).

Although it has been speculated that the ability of anaerobic metabolism might be a common feature of bacteria in coastal marine waters (Riemann and Azam, 2002), no direct evidence has been provided to support this hypothesis. Riemann and Azam (2002) described a significant decrease of [^{3}H]-Thymidine incorporation into marine bacteria after inhibition of the membrane transfer system responsible for the uptake of N-acetyglucosamine (NAG). The authors argued that this phospho-transferase system (PTS) is a typical feature of facultative anaerobic bacteria. They speculated that anaerobic metabolism could thus be a widespread feature of bacteria in the marine water column. However, in that study 22 of the 60 tested marine isolates that incorporated NAG via PTS were not capable of a facultative anaerobic metabolism. Moreover, the NAG uptake measured in marine water samples was probably not exclusively mediated via the PTS, as indicated by inhibition experiments with other substrates.

In the present work, MARFISH was employed to investigate *in situ* whether marine picoplankton populations were able of incorporating glucose under anoxic conditions. Glucose incorporation under oxic and anoxic conditions was traced by adding 10 nM tritiated glucose to freshly collected seawater in July and August 2003. The incubations were carried on for different periods of time (4 or 24 hours) in order to establish how rapid the microbial shift from aerobic to anaerobic metabolism could be.

Methodology

For every treatment type triplicate 10 ml sub-samples were incubated. Oxic incubations were performed in sterile 70 ml serum vials with cotton plugs. Anoxic incubation conditions were established in the same type of serum vials by flushing with nitrogen. The nitrogen flushing time required to produce anoxic conditions was verified by Winkler titration and with an oxygen microsensor.

Results

Incorporation of glucose under oxic and anoxic conditions was found in several major lineages of pelagic marine bacteria (*Alphaproteobacteria, Gammaproteobacteria* and the *Cytophaga-Flavobacteria* cluster of the *Bacteroidetes*) but not in marine *Euryarchaeaota,* at both sampling times and incubation periods (Figure 7).

In July, a very high proportion of the *Roseobacter* cells (~85%) were incorporating glucose in oxic conditions after 4 hours of incubations. This group represented more than a quarter of MAR positive *Bacteria*, despite being less than 10% of all bacterial cells (Figure 7).

In contrast, in August the *Gammaproteobacteria* exhibited the highest fraction of glucose incorporating cells, particularly under anoxic conditions. Members of this group represented a quarter of all *Bacteria* incorporating glucose under oxic conditions and more than half in the anoxic incubations (Figure 7). *Vibrio* spp. was the sub-population with higher fraction of MAR positive cells. Approximately 80-95% of all *Vibrio* spp. cells exhibited glucose incorporation under both conditions.

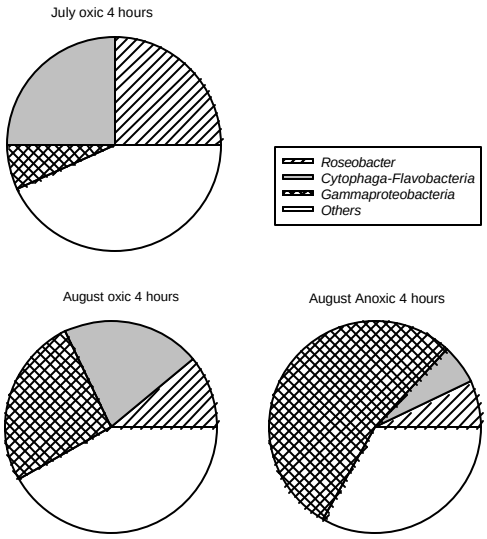


Fig.7. Contribution of specific groups to total Bacterial cells incorporating glucose (July and August, 4 hours incubations).

Members of *Cytophaga-Flavobacteria* cluster contributed similarly to the oxic glucose incorporation in both months, representing 25% of all MAR positive Bacteria in July and 21% in August (Figure 7). Their contribution was lower than expected from their relative abundances, which were 45% and 30% of all *Bacteria* in July and August, respectively. Their contribution to anoxic glucose uptake was even lower.

During the 24 hours of incubation a shift in the community composition was observed in both months, resulting in the enrichment in *Gammaproteobacteria.* In July these *Gammaproteobacteria* were almost exclusively *Alteromonas* whereas in August members of *Pseudoalteromonas* and *Vibrio* taxa were also enriched (Figure 8).

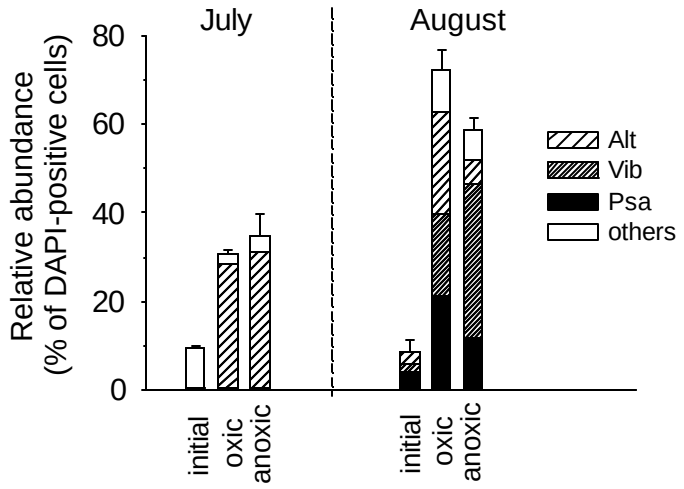


Fig.8. Proportion of *Gammaproteobacteria* in the initial sample and after 24 hours incubations
ALT: *Alteromonas*, PSA: *Pseudoalteromonas-Colwellia,* VIB: *Vibrio*

Members of *Cytophaga-Flavobacteria* were the main contributors to glucose uptake in July under oxic conditions when they represented more than half of all bacteria incorporating glucose (Figure 9). The proportion of MAR positives cells of the *Cytophaga-Flavobacteria* group was significantly lower under anoxic conditions although no differences were found in cell abundance. In contrast, both the abundance and proportion of MAR positive cells of *Gammaprotebacteria* increased under anoxic conditions, and this group became the main responsible of glucose uptake (Figure 9).

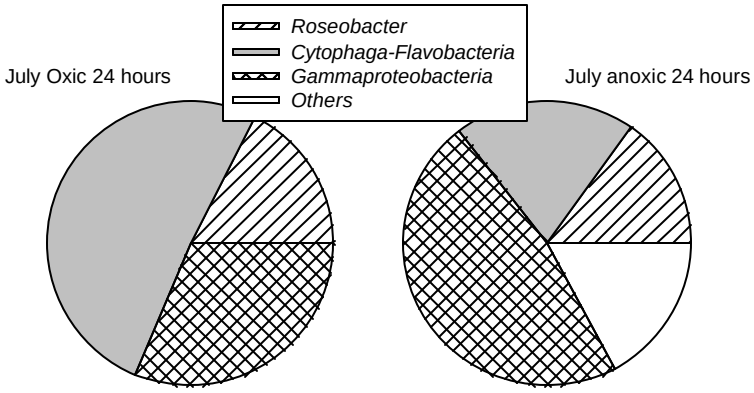


Fig.9. Contribution of specific groups to total Bacterial cells incorporating glucose (July, 24 hours incubations).

Although all bacterial groups analyzed in this study exhibited glucose uptake under both oxic and anoxic incubations, most of the taxa showed clear preferential incorporation at one condition. The proportion of MAR positive cells of *Roseobacter* and *Cytophaga-Flavobacteria* was typically higher under oxic conditions. In contrast, a preference for glucose uptake under anoxic conditions was observed for bacteria related to *Alteromonas* and *Pseudoalteromonas/Colwellia*. The fraction of active cells within a phylogenetic group usually differed between the sampling dates (e.g *Vibrio* spp. Figure 10).

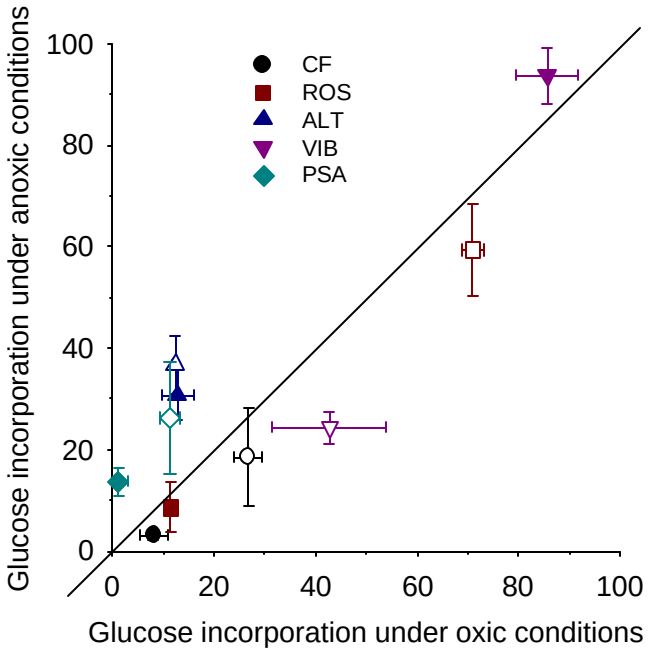


Fig.10. Glucose incorporation preferences for different bacterial groups: *Cytophaga-Flavobacteria* (CF), *Roseobacter* (ROS), *Alteromonas* (ALT), *Vibrio* (VIB) and *Pseudoalteromonas-Colwellia* (PSA).

Discussion

Our results showed that a substantial fraction of microbes in the coastal North Sea surface waters are capable of substrate uptake in the absence of oxygen, supporting the hypothesis that facultative anaerobic metabolism is widespread among bacteria in the water column.

The potential for anoxic glucose uptake may throw a new light on the ecology of members of the *Roseobacter* clade in coastal surface waters. The clade comprises bacteria with very different physiological capacities, e.g. hetero-organotrophs, anoxygenic phototrophs, sulfite oxidizers, and denitrifiers (Shiba, 1991; Sorokin, 1995; Wagner-Döbler et al., 2003). So far, no facultative fermentative strains have been described. Although the total abundances of *Roseobacter* cells were similar in July and August, a very different fraction of these cells was able to take up glucose at both sampling dates. This suggests that the activities of pelagic microbes are more dynamic than their population sizes, and that a small number of active cells can be responsible for a significant fraction of the turnover of particular substrates.

The observed enrichment and high levels of tracer incorporation in *Vibrio* spp. under anoxic conditions supported the interpretation that our experimental setup tested for a facultative anaerobic metabolism. Some marine *Vibrio* spp. are known for their ability to ferment sugars in the absence of oxygen (Macian et al., 2001).

Surprisingly, a higher fraction of *Alteromonas and Pseudoalteromonas* cells exhibited glucose incorporation under anoxic than under oxic conditions. The genus *Alteromonas* has been originally defined from a collection of predominatly aerobic gram-negative isolates (Baumann et al., 1972), and the described isolates from the genera *Pseudoalteromonas* and *Colwellia* are also known as obligate aerobic bacteria (Sawabe et al., 1998; Yumoto et al., 1998). However, recently Riemann and Azam (2002) reported a potential for anaerobic metabolism in several strains that were affiliated with the *Alteromonadaceae*, including a close relative of *Alteromonas macleodii*. 16S rRNA sequence types related to *Pseudoalteromonas* spp. have been obtained from the anoxic zone of the Cariaco Basin (Madrid et al., 2001), and from the suboxic layers overlying the sulfide-rich deeper waters of the Black Sea (Vetriani et al., 2003). In the context of our findings this suggests that the well-studied, strictly aerobic isolates from this family might not be ecophysiologically representative for those *Alteromonas* and *Pseudoalteromonas* spp. which are common in the marine water column.

Altogether, the observed anaerobic uptake of glucose by different pelagic bacteria suggests that there might be a substantial overlap between the microbial assemblages that inhabit the water column, organic particles and the sediment surfaces in shallow coastal systems. In future studies, it would be interesting to explore which metabolic pathways are responsible for the observed glucose utilization by marine bacteria (anoxic respiration, fermentation). A potential experimental design would include the study of anoxic glucose uptake in the presence of inhibitors of the respiratory chain (e.g. rotenone, (Cockerill et al., 1995; Chen and Suzuki, 2004) and under addition of alternative electron acceptors (e.g. nitrate salts). A complementary approach could focus on the analysis of surface sediments, and phyto- and zooplankton derived suspended particles as possible niches for the taxa that are suspected to be preferentially anaerobic.

3. Concentration dependent substrate uptake

Introduction

Differences in the usage of individual components of dissolved organic matter (DOM) may help explaining the distribution of the major bacterial groups among soil, freshwater and marine ecosystems (Cottrell and Kirchman, 2000). In order to improve models for carbon cycling in aquatic habitats it may also be important to know the minimum number of bacterial phylogenetic groups necessary to describe and explain DOM uptake. Currently, these models implicitly assume that all heterotrophic bacteria are the same and consist of a single phylogenetic type (Cottrell and Kirchman, 2000).

Organic matter in the photic zone in seawater is a complex and largely uncharacterized mixture of monomers and polymers. It has been proposed that it consists of a gel-like polymeric matrix with colloids and particles embedded (Azam, 1998). Thus, pelagic bacteria experience organic matter gradients, both in quality and in concentration of their substrates.

As most utilizable organic matter in the marine environment is polymeric (e.g. polysaccharides, proteins, nucleic acids), hydrolysis is required before it can be taken up by the bacteria. The monomers of these polymers are apparently the main conduit for organic matter flux into bacteria. Their pools turn over rapidly (generally 10-100 hours) and their concentrations are kept low by the bacterial uptake (generally 0.1nM-10nM for individual amino acids and 0.1nM-100nM for individual sugars) (Azam, 2001; Unanue et al., 1999; Kirchman et al., 2001).

Marine bacteria have been shown to possess multiple transport systems for monomers with different affinity constants (K_m) and velocities (V_{max}) (Azam and Hodson, 1981; Nissen et al., 1984; Unanue et al., 1999). The simultaneous possession of low flow and high flow systems would enable bacteria to take advantage of high substrate concentrations in microzones yet efficiently take up substrates at the low bulk-phase concentrations (Azam, 2001).

Presently, there is a gap in our understanding of microbial incorporation of monomers as function of their concentration. Current knowledge is derived from approaches that focus on two extremes: pure cultures studies (e.g. Nissen et al., 1984), and bulk uptake measurements by the whole microbial community (e.g. Azam and Hodson, 1981; Unanue et al., 1999). The application of MARFISH can help to fill

this gap, as monomer uptake of specific populations at different concentrations can be studied *in situ* at least semi-quantitatively.

In this work we investigated the incorporation of glucose and leucine at various concentrations by particular populations of *Bacteria* and *Archaea* in late winter and in a spring bloom dominated by the algae *Phaeocystis* sp. (Figure 11).

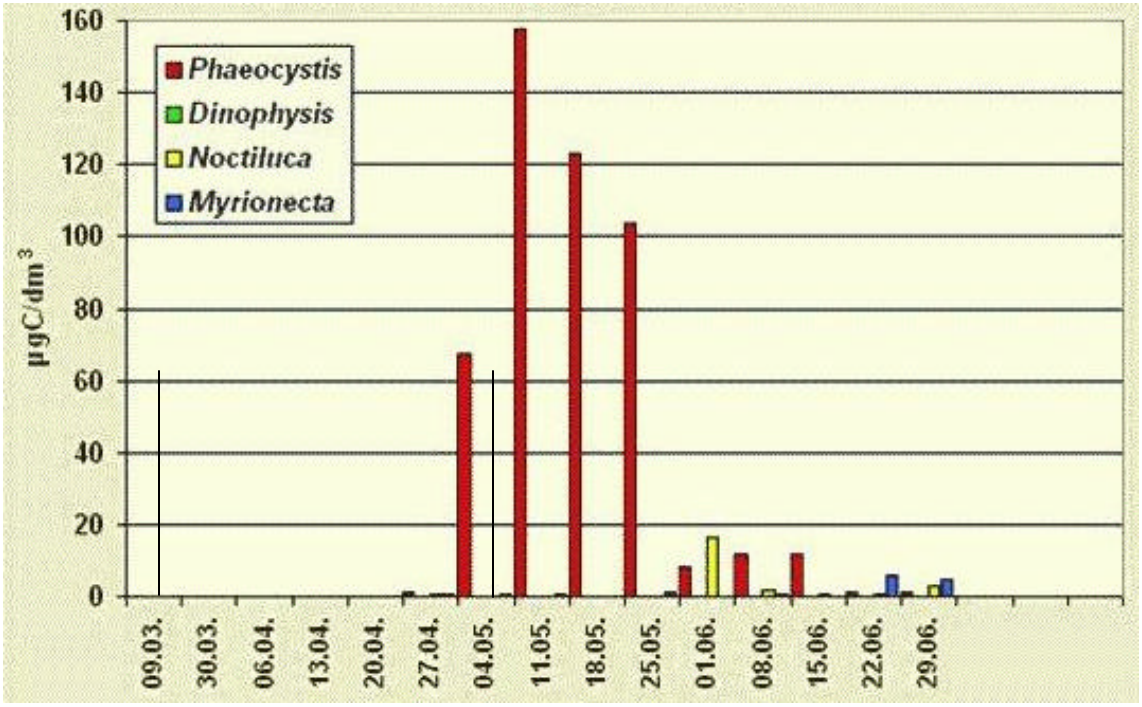


Fig.11. Development of the Phaeocystis dominated spring bloom. Arrows indicate sampling points. (source: Mursys Umweltreport, www.bsh.de).

Methodology

Two sets of samples of North Sea coastal water were incubated with either tritiated glucose or leucine in a concentration series (0.1 nM, 1 nM, 10 nM and 100 nM). One set of samples was used to measure the substrate incorporation rate of the whole community at the different concentrations by liquid scintillation counting. The other was employed to directly track monomers incorporation by single populations using MARFISH. For every treatment type triplicate 10 ml sub-samples were incubated plus one control sample consisting in seawater fixed with paraformaldehyde. The studied bacterioplankton groups were selected for their abundance and positive substrate incorporation. The following FISH probes were used for studying leucine uptake: EUB I-III (most *Bacteria*) (Daims et al., 1999), EURY806 (marine group II *Euryarchaeota*) (Teira et al., 2004), ROS537 (members of the alphaproteobacterial *Roseobacter*

clade) (Eilers et al., 2001), SAR86-1245 (members of the *Gammaproteobacteria* SAR86 clade) (Eilers et al., 2000b) and CF1267 -members of the DE2 clade of the *Bacteroidetes*- (Kirchman et al., 2003). The following FISH probes were used for studying glucose uptake: EUB I-III (most *Bacteria*) (Daims et al., 1999), ROS537 (members of the *Alphaproteobacteria Roseobacter* clade) (Eilers et al., 2001), SAR11-441 (members of the *Alphaproteobacteria* SAR11 clade) (Morris et al., 2002), CF319a (many groups from the *Cytophaga-Flavobacteria* cluster of the *Bacteroidetes*) (Manz et al., 1996), GAM42a (most *Gammaproteobacteria*) (Manz et al., 1992).

Results

The incorporation rates of both glucose and leucine were higher in May at all concentrations, in coincidence with the *Phaeocystis* bloom (Table 4). This was most pronounced for the uptake at the higher concentrations (10 and 100 nM) of both substrates, in particular for glucose. In March there was a slight preference for leucine, whereas in May both substrates were incorporated in similar quantities. Incorporation did not increase significantly beyond concentration of 10 nM in all cases, except for leucine which was highest at 100 nM in May.

Table 4 . Substrate incorporation rates by the whole microbial community

	Incorporated substrate (pmoles/l) average +/- stdv		Ratio May to March
	March	May	
Glucose 0.1 nM	1.4 +/- 0.2	6.7 +/- 0.7	5
Glucose 1 nM	11.7 +/-7.3	75.1 +/- 13.4	6
Glucose 10 nM	34.0 +/- 13.0	390.3 +/- 60.6	12
Glucose 100 nM	22.4 +/- 8.6	433.0 +/- 16.5	19
Leucine 0.1 nM	3.1 +/- 0.2	9.0 +/- 1.9	3
Leucine 1 nM	17.6 +/- 3.9	73.3 +/- 22.9	4
Leucine 10 nM	29.7 +/- 10.0	303.1 +/- 51.3	10
Leucine 100 nM	34.6 +/- 5.9	434.3 +/- 63.7	13

More bacterial cells were found to incorporate leucine at a given concentration (Figure 12). More cells were able to incorporate both substrates in May than in March, especially at the lower substrate concentrations (Figure 12). The percentage of bacterial cells able to incorporate both tracers did not significantly increase beyond the 10 nM concentration (Figure 12).

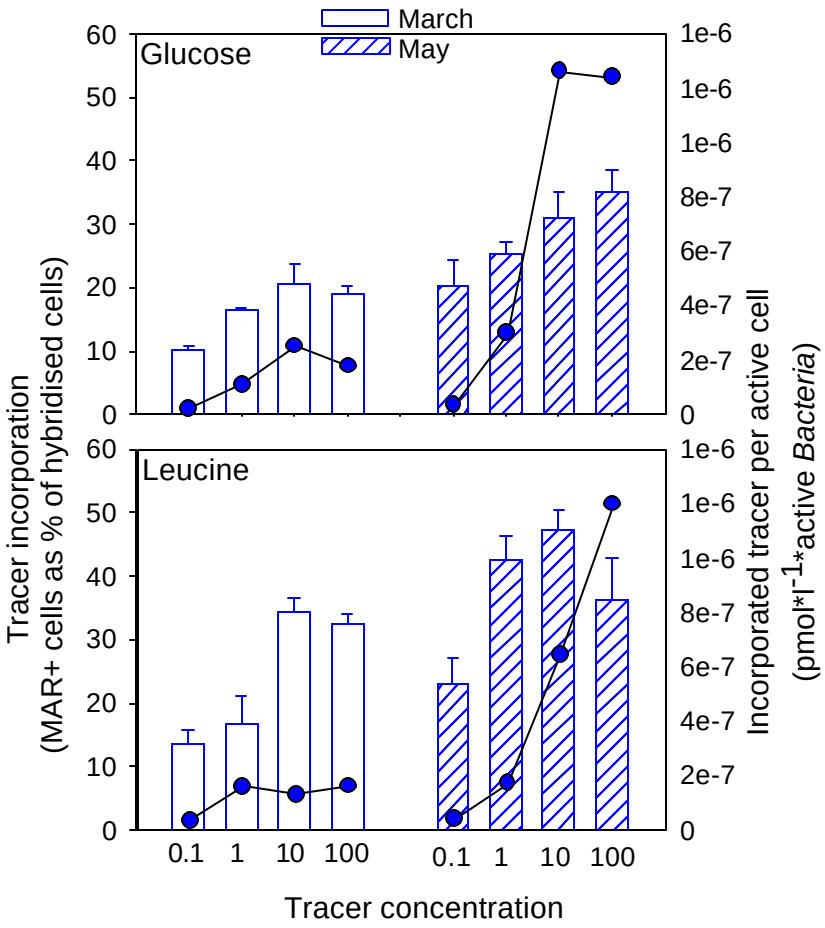


Fig. 12. Proportion of MAR positive cells of hybridized *Bacteria.*

While the incorporation rate particularly increased in May at the higher concentrations (10 and 100 nM) (Table 4), the increase in the proportion of MAR positive cells was more pronounced at the lower concentrations (0,1 and 1 nM) (Figure 12). This apparent discrepancy between the two methods indicates that i) more cells were able to incorporate the substrates even at lower concentrations in May and ii) the maximum uptake rate per active cell was higher in May. This goes in agreement with the fact that much shorter exposure times were needed for the May than the March samples for the same substrate concentration: e.g. for Glucose 0,1nM in March 6 days were required and 72 hours in May.

The increased leucine uptake rate and proportion of MAR positive cells in May could be due to an increase in the proportion of active cells within a defined population (e.g. *Roseobacter, Euryarchaeota*) but also due to the activity of other populations, either absent or inactive in the pre-bloom situation (e.g. DE2 cluster) (Figures 13 and 14).

Members of the *Roseobacter* clade were found to be very active in the incorporation of both offered substrates. A higher proportion of this population incorporated the monomers in May, as compared to March. However, in contrast with what was observed for *Bacteria*, there was no clear preference for leucine at any concentration, and the percentages of *Roseobacter* cells incorporating both substrates were very similar at both sampling times (Figure 13). In both months there was no or only little increase in the fraction of *Roseobacter* cells with visibly incorporated tracer with increased substrate concentration (Figure 13). The proportion of *Roseobacter* cells that incorporated both substrates was always above the community average, particularly at the lower concentrations (Table 5).

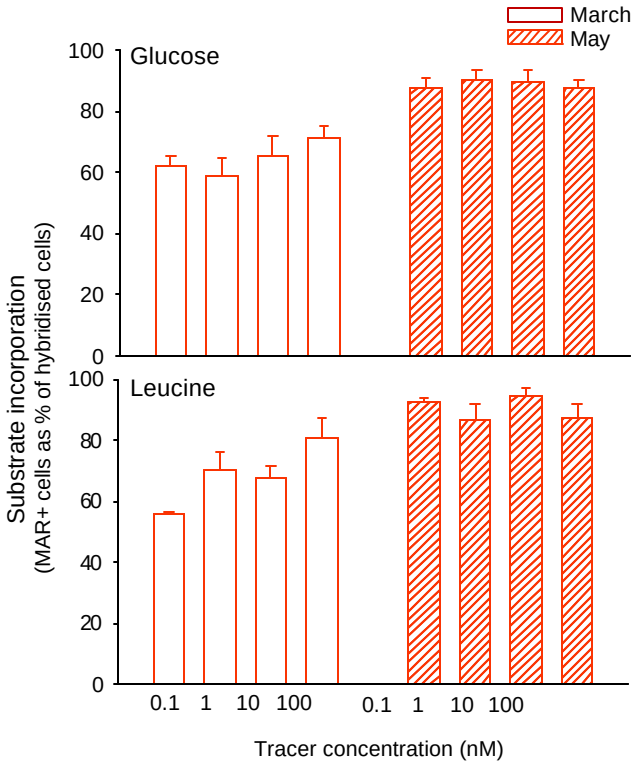


Fig.13. Proportion of MAR positive cells of hybridized *Roseobacter.*

SAR86, DE2 cluster of *Bacteroidetes* and *Euryarchaeota* only incorporated leucine and exhibited increasing uptake in response to increasing concentrations (Figure 14). In contrast to the whole community and other specific populations (*Roseobacter* and *Euryarchaeota*), the proportion of cells from the SAR86 clade incorporating leucine was almost invariant at a given tracer concentration between the two months (Figure 14). The proportion of cells from the SAR86 and DE2 clusters that took up leucine was always below the community average. Only at the highest

tracer concentration they formed a similarly high proportion of active cells as the whole bacterial assemblage (Table 5). The fraction of cells affiliated with *Euryarchaeota* that incorporated leucine was at least equal but typically higher than the bacterial fraction (Figure 14, Table 5).

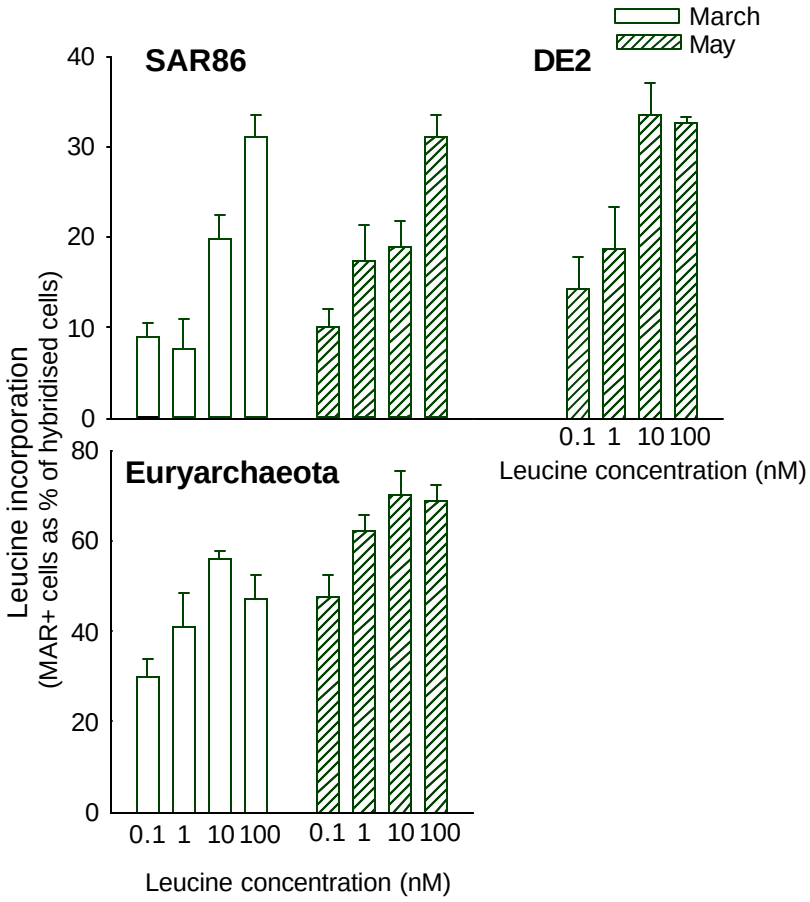


Fig.14. Proportion of MAR positive cells of hybridized SAR86, DE2 Bacteroidetes and *Euryarchaeota.*

Glucose uptake was observed in all major phylogenetic groups at both sampling time points (*Alphaproteobacteria*, *Gammaproteobacteria* and *Cytophaga-Flavobacteria*) (Figures 13 and 15). However, with the exception of *Roseobacter* and members of the SAR11 clade, none of the other specific populations tested (SAR86, NOR5, DE2) incorporated this substrate at any concentration.

The fraction of SAR11 bacteria showing glucose incorporation was highest at a concentration of 1 nM in both months (Figure 15). The proportion of MAR positive SAR11 cells at a given concentration was higher in May (Figure 15). Excepting the

incubation at 1 nM, the percentage of SAR11 cells that took up glucose was equal or below the MAR positive *Bacteria* average (Table 5).

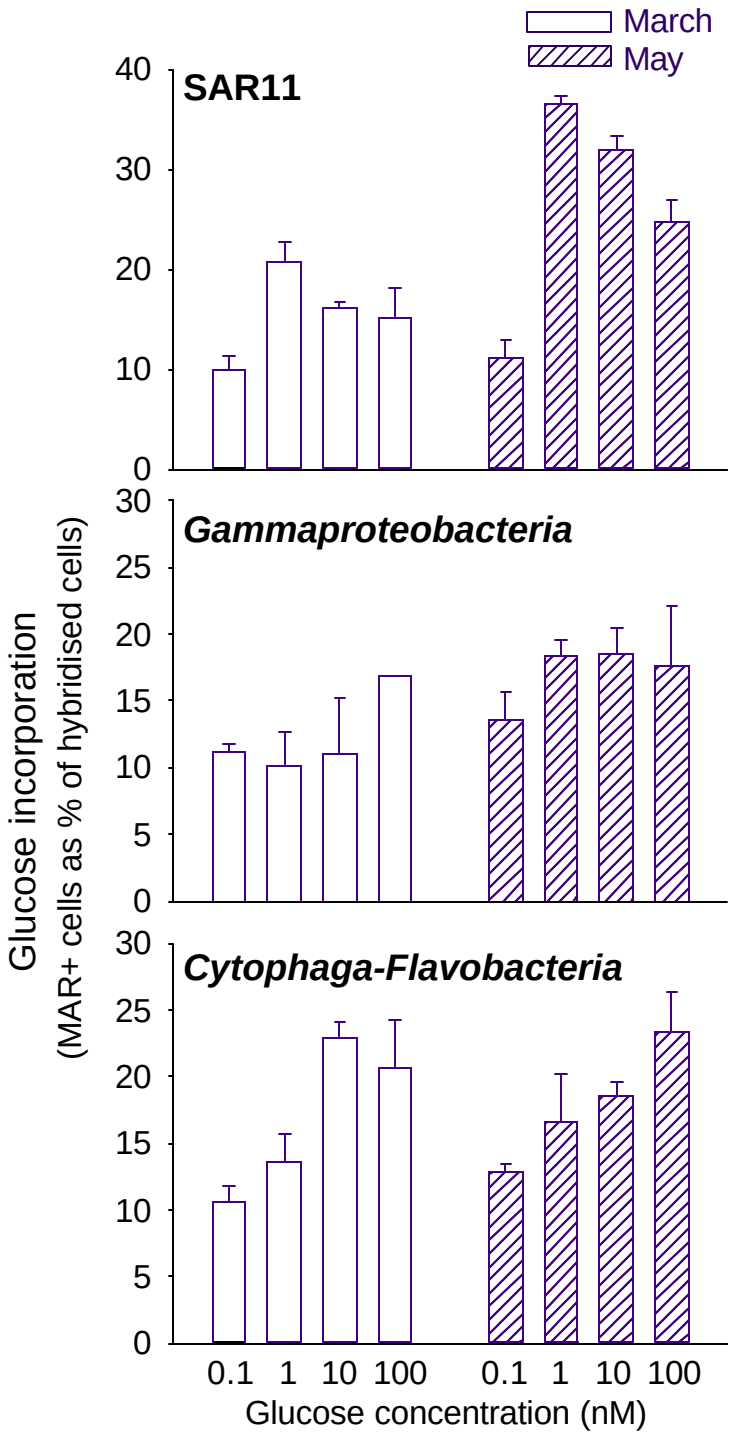


Fig. 15 Proportion of MAR positive cells in the hybridized cells of SAR11 clade, *Gamma-proteobacteria* and *Cytophaga-Flavobacteria*. .

Table 5. Ratio of fraction of MAR+ cells of single populations and fraction of MAR positive *Bacteria*

	Roseobacter		SAR11		SAR86		DE2		Euryarchaeota	
Glucose	March	May	March	May	March	May	March	May	March	May
0.1 nM	6.1	4.3	1.0	0.5	--	--	--	–	--	--
1 nM	3.6	3.6	1.3	1.5	--	--	--	–	--	--
10 nM	3.2	2.9	0.8	1.0	--	--	--	–	--	--
100 nM	3.7	2.5	0.8	0.7	--	--	--	–	--	--
Leucine										
0.1 nM	4.1	4.0	–	--	0.7	0.4	--	0.6	2.2	2.1
1 nM	4.2	2.0	–	--	0.5	0.4	--	0.4	2.4	1.5
10 nM	1.9	2.0	–	--	0.6	0.4	--	0.7	1.6	1.5
100 nM	2.5	2.4	–	--	1.0	0.9	--	0.9	1.0	1.9

For each single population there was a specific upper concentration where the number of MAR positive cells did not significantly increase (optimal substrate concentration). However, for *Euryarchaeota* and *Roseobacter* this concentration shifted between the two months (Figure 16).

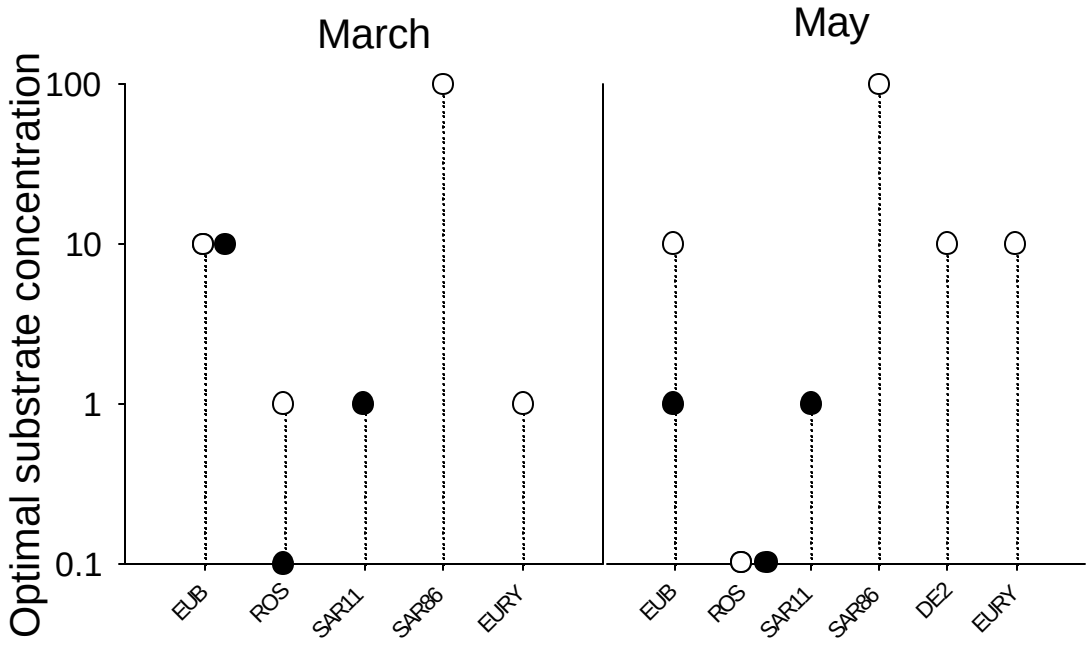


Fig.16. Optimal substrate concentration per prokariotic population and month. Optimal concentrations for total *Bacteria* are also depicted to compare glucose and leucine incorporation.

Discussion

Our results support the hypothesis that some heterotrophic microbial populations in marine picoplankton may harbor several uptake systems operating at different ranges of substrate concentration (Azam and Hodson, 1981; Nissen et al., 1984; Fuhrman and Ferguson, 1986). We provide evidence for the presence of taxa that can efficiently incorporate monomers across a wide gradient of concentrations e.g. *Roseobacter*. Alternatively, the pattern of multiphasic substrate uptake as observed for whole assemblages (Vaccaro and Jannasch, 1967; Azam and Hodson, 1981; Unanue et al., 1999) could also result from the coexistence of populations with different concentration optima for substrate uptake such as SAR86 and *Euryarchaeota*.

Members of *Roseobacter* accounted for approximately 20 to 50 percent of all bacterial cells showing leucine uptake. Their highest contribution to total MAR positives cells were found at the lower concentrations, indicating that the higher uptake at increasing concentrations was due to other populations with lower substrate affinity. Similarly, *Roseobacter* and SAR11 were the main groups responsible for glucose incorporation, particularly in the pre-bloom situation and at the lower concentrations (0.1 nM and 1 nM). These findings are consistent with the work from (Cottrell and Kirchman, 2000) where they proposed that *Alphaproteobacteria* would be specialized on simple susbtrates (amino acids, N-acetyl glucosamine) whereas *Cytophaga-Flavobacteria* would rather utilize polymers (proteins, chitin). However, no evident correlation with community composition at the level of major phylogenetic groups (i.e. *Alphaproteobacteria, Cytophaga-Flavobacteria)* was found to explain the very different proportions of *Bacteria* that incorporated glucose at different sampling times. The contribution to total substrate uptake of such broad phylogenetic groups results from the composite substrate incorporation of their constituent populations. As an example, *Roseobacter* and SAR11 appear to have different strategies of substrate incorporation although their contribution to glucose uptake was very similar. While a high fraction of *Roseobacter* cells were able to take up glucose at all concentrations, SAR11 cells exhibited a specific optimum concentration (1 nM) at which the higher proportion of MAR positive cells was found.

Our findings suggest that at low environmental concentrations substrate processing might be mainly carried out by a few specialized populations (e.g.

Roseobacter for monomers) whereas other populations participate in substrate utilization during more rich conditions (e.g. phytoplankton blooms).

It has been hypothesized that the increase in bulk incorporation rates during phytoplankton blooms could be due to a community replacement of oligotrophic bacteria by copiotrophic populations (Yager et al., 2001). However, we observed no replacement of the populations able to incorporate very low monomer concentrations (e.g. *Roseobacter* and SAR11). We conclude that it is possible that bacteria with both strategies coexist. Potentially, this coexistence could be related to the patchy nature of substrate distribution. During phytoplankton bloom situations, gradients of substrate concentration are expected to occur (Blackburn et al., 1997). Bacterial groups with different substrate affinity and maybe also different motility patterns (Blackburn et al., 1998; Grossart et al., 2001; Barbara and Mitchell, 2003) would profit at diverse points of this gradient. In contrast, during the pre-bloom a more uniform distribution of low concentrations of substrates is expected. This may explain why a low proportion of active cells and a relatively low and constant amount of substrate incorporated per cell were found at the lowest tracer concentration (Figure 12).

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Conclusions and Outlook

Ecology of prokaryotic populations in the North Sea

In every application of MARFISH to study prokaryotic activity in the North Sea only a fraction of prokaryotes was found to incorporate a particular substrate. This fraction significantly changed between close sampling timepoints without equivalent changes in the total number of cells. Such variation in the proportions of active cells between seasons without changes in cell numbers was also observed at the level of single populations. This indicates that the activity of pelagic microbes is substantially more dynamic than their population sizes. The causes for this variability of prokaryotic activity still need to be unveiled. One possible explanation could be a concerted utilization of multiple substrates according to their availability (Egli, 1993). Given the complexity of natural occurring bacterial substrates it would be interesting to perform simultaneous and separate uptake experiments with several substrates that differ, e.g., in elemental composition, concentration, or with respect to their specific cellular incorporation mechanisms.

The observed phenotypic diversity of substrate incorporation within prokaryotic communities reflects the complexity of the pelagic zone of the North Sea as a microbial habitat. For one, the observed anaerobic uptake of glucose in different bacterial taxa suggests that there might be a substantial exchange between the microbial assemblages that inhabit the water column, organic particles and the sediment surfaces in this shallow coastal system. Secondly, the coexistence of prokaryotic groups displaying different concentration-dependent patterns of substrate uptake is an indication for substrate patchiness. Likely, such heterogeneity is particularly relevant in the studied environment due to the terrestrial influence and the pronounced exchange processes between the sediment and the water column (Wainright and Hopkinson, 1997; Tengberg et al., 2003).

The number of microbial taxa or the phylogenetic resolution required to adequately describe the relationship between bacterial community composition and carbon flux patterns is still unclear. Therefore, operational taxonomic categories should be defined as coherent units in the context of particular studies (Pernthaler and Amann, 2005). It has been argued that consumption of DOM could be explained using a relatively small number of large phylogenetic groups (Cottrell and Kirchman, 2000). However, in the present work it is shown that substrate processing can be very heterogeneous within such major phylogenetic clades, and that a very small sized population may be responsible for a high proportion of uptake of a particular

substrate. A more realistic picture of an heterogeneous bacterial community, not only in terms of phylotypes but also in metabolic activity needs to be incorporated into our perception of aquatic ecosystems. An analysis of specific microbial activities at various levels of phylogenetic resolution will be required before it eventually might be possible to pool different genotypes into more coherent ecophysiological units. It is, moreover, likely that these units will not match the large phylogenetic groups (e.g., subclasses of *Proteobacteria*). "It now seems that things will get even more complicated before they get simpler" (Azam, 1998).

Perspectives for the application of MARFISH

MARFISH has emerged as a powerful technique to identify microbial phylogenetic lineages *in situ* that are responsible for specific processes in aquatic systems. However, there are also clear limits to its application. As a combination of two methods it suffers from the restrictions inherent to both. The main drawback of autoradiography is the availability of only a limited set of radiolabeled substrates which, moreover, were developed mainly for clinical applications and for work on eukaryotic cells. One strategy to overcome this limitation could be the custom production of radiolabeled substrates (e.g. Cottrell and Kirchman, 2000). However, this is a very demanding approach, and the majority of research groups in microbial ecology probably do not have the adequate human and technical resources to synthesize their own substrates.

The main restriction of FISH is set by the phylogenetic resolution of the available probes. Moreover, inconsistencies of substrate uptake within a probe-defined microbial population could be due to functional diversity below the discriminatory power of the 16S rRNA (Jaspers and Overmann, 2004). Apart from further probe design, e.g. targeted to 23S rRNA, other potentially useful techniques to distinguish physiology at the species or subspecies levels could be the coupling of autoradiography with *in situ* PCR methods (Hodson et al., 1995; Chen et al., 1999), or bacterial chromosome painting (Lanoil and Giovannoni, 1997; Lanoil et al., 2000). This would allow the visualization of cells based on more variable regions of the genome and therefore link the uptake of a specific substrate with the presence of a gene or genes that are known to be involved in its metabolism, potentially across phylogenetic diverse microbial groups. In addition, a combination of MARFISH and

mRNAFISH (Hahn et al., 1993; Bakermans and Madsen, 2002; Pernthaler and Amann, 2004) might allow to study the expression of specific genes in the context of substrate uptake.

Another logical extension of the MARFISH technique is to apply quantitative methods to evaluate relative or absolute substrate uptake rates within mixed communities. The measurement of radioactivity in an autoradiogram in terms of number of disintegrations that occur within a source is possible but technically difficult e. g., by track autoradiography or grain size area measurement (Rogers, 1979; Carney and Fahnenstiel, 1987; Davenport and Maguire, 1984). Carefully controlled experiments are needed to relate number of grains or tracks with absolute amounts of radioactivity and absolute measurements require the utilization of appropriate radioactive standards (Brock and Brock, 1968; Gray and Head, 2001; Nielsen et al., 2003).

A promising alternative to these tedious approaches for quantification of incorporated label would be the flow cytometric sorting of cells and the subsequent measurement of radioactivity by liquid scintillation counting (Lebaron et al., 2001; Zubkov et al., 2004). Sorting of hybridized cells from marine samples is already feasible (Sekar et al., 2004). Another interesting perspective for a quantification of incorporated radioactivity at the single cell level might eventually be the use of a ß-Imager, provided an increase of spatial resolution (Laniece et al., 1994; Barthe et al., 2004).

In summary, MARFISH was successfully applied to study coastal North Sea bacterioplankton, and to contribute to our understanding of the ecology of the microbial populations and communities in this habitat. The improved method is sensitive, accurate, and suitable for high throughput sample processing. This provides a perspective for its application in oligotrophic environments (e.g. open oceans, alpine lakes), and it allows for the design of more complex experiments, which require the evaluation of high number of samples.

References

References

Alldredge, A.L., and Cohen, Y. (1987) Can microscale chemical patches persist in the sea - microelectrode study of marine snow, fecal pellets. *Science* 235: 689-691.

Amann, R.I. (1995) *In situ* identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. *Molecular Microbial Ecology Manual* 3.3.6.: 1-15.

Amann, R.I., Krumholz, L., and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology* 172: 762-770.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* 59: 143-169.

Amann, R.I., Zarda, B., Stahl, D.A., and Schleifer, K.H. (1992) Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology* 58: 3007-3011.

Azam, F. (1998) Microbial control of oceanic carbon flux: the plot thickens. *Science* 280: 694-696.

Azam, F. (2001) Introduction, history, and overview: The 'methods' to our madness. In *Methods in Microbiology, Vol 30*, pp. 1-12.

Azam, F., and Hodson, R.E. (1981) Multiphasic Kinetics for D-Glucose Uptake by Assemblages of Natural Marine-Bacteria. *Marine Ecology-Progress Series* 6: 213-222.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A., and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series* 10: 257-263.

Bakermans, C., and Madsen, E.L. (2002) Detection in coal tar waste-contaminated groundwater of mRNA transcripts related to naphthalene dioxygenase by fluorescent *in situ* hybridization with tyramide signal amplification. *Journal of Microbiological Methods* 50: 75-84.

Barbara, G.M., and Mitchell, J.G. (2003) Bacterial tracking of motile algae. *FEMS Microbiology Ecology* 44: 79-87.

Barthe, N., Chatti, K., Coulon, P., Maitrejean, S., and Basse-Cathalinat, B. (2004) Recent technologic developments on high-resolution beta imaging systems for quantitative autoradiography and double labeling applications. *Nuclear Instruments & Methods in Physics Research Section a-Accelerators Spectrometers Detectors and Associated Equipment* 527: 41-45.

Baumann, L., Baumann, P., Mandel, M., and Allen, R.D. (1972) Taxonomy of aerobic marine eubacteria. *Journal of Bacteriology* 110: 402-429.

Berman, T., Kaplan, B., Chava, S., Viner, Y., Sherr, B.F., and Sherr, E.B. (2001) Metabolically active bacteria in Lake Kinneret. *Aquatic Microbial Ecology* 23: 213-224.

Binnie, C., and Coote, J.G. (1986) Inhibition of Sporulation in Bacillus-Subtilis by Bromodeoxyuridine and the Effect on DNA-Replication. *Journal of General Microbiology* 132: 493-502.

Blackburn, N., Azam, F., and Hagstrom, A. (1997) Spatially explicit simulations of a microbial food web. *Limnology and Oceanography* 42: 613-622.

Blackburn, N., Fenchel, T., and Mitchell, J. (1998) Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. *Science.* 282: 2254-2256.

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A. et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 407: 623-626.

Borneman, J. (1999) Culture-independent identification of microorganisms that respond to specified stimuli. *Applied and Environmental Microbiology* 65: 3398-3400.

Bouvier, T., and del Giorgio, P.A. (2003) Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): A quantitative review of published reports. *FEMS Microbiology Ecology* 44: 3-15.

Britschgi, T.B., and Giovannoni, S.J. (1991) Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. *Applied and Environmental Microbiology* 57: 1707-1713.

Brock, M.L., and Brock, T.D. (1968) The application of micro-autoradiographic techniques to ecological studies. *Mitt. Int. Verein. Limnol.*: 1-29.

Brock, T.D. (1967) Bacterial growth rate in the sea - direct analysis by thymidine autoradiography. *Science* 155: 81-83.

Carney, H.J., and Fahnenstiel, G.L. (1987) Quantification of Track and Grain Density Autoradiography and Evaluation of C-14 Loss on Preservation. *Journal of Plankton Research* 9: 41-50.

Chen, F., Dustman, W.A., and Hodson, R.E. (1999) Microscopic detection of the toluene dioxygenase gene and its expression inside bacterial cells in seawater using prokaryotic *in situ* PCR. *Hydrobiologia* 401: 131-138.

Chen, Y.Q., and Suzuki, I. (2004) Effect of uncouplers on endogenous respiration and ferrous iron oxidation in a chemolithoautotrophic bacterium Acidithiobacillus (Thiobacillus) ferrooxidans. *FEMS Microbiology Letters* 237: 139-145.

Choi, J.W., Sherr, E.B., and B.F., S. (1996) Relation between presence-absence of a visible nucleoid and metabolic activity in bacterioplankton cells. *Limnology and Oceanography* 41: 1161-1168.

Church, M.J., DeLong, E.F., Ducklow, H.W., Karner, M.B., Preston, C.M., and Karl, D.M. (2003) Abundance and distribution of planktonic Archaea and Bacteria in the waters west of the Antarctic Peninsula. *Limnology and Oceanography* 48: 1893-1902.

Ciferri, O. (1983) Spirulina, the Edible Microorganism. *Microbiological Reviews* 47: 551-578.

References

Cockerill, G.S., Levett, P.C., and Whiting, D.A. (1995) Synthesis of Novel Inhibitors of Electron-Transport. *Journal of the Chemical Society-Perkin Transactions 1* : 1103-1113.

Cottrell, M.T., and Kirchman, D.L. (2000) Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Applied and Environmental Microbiology* 66: 1692-1697.

Cottrell, M.T., and Kirchman, D.L. (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnology and Oceanography* 48: 168-178.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology.* 22: 434-444.

Davenport, J.B., and Maguire, B. (1984) Quantitative Grain Density Autoradiography and the Intraspecific Distribution of Primary Productivity in Phytoplankton. *Limnology and Oceanography* 29: 410-416.

Decamp, O., and Rajendran, N. (1998a) Bacterial loss and degradation of bacterial membrane in preserved seawater samples. *Marine Pollution Bulletin* 36: 856-859.

Decamp, O., and Rajendran, N. (1998b) Assessment of bacterioplankton viability by membrane integrity. *Marine Pollution Bulletin* 36: 739-741.

del Giorgio, P.A., and Bouvier, T.C. (2002) Linking the physiologic and phylogenetic successions in free- living bacterial communities along an estuarine salinity gradient. *Limnology and Oceanography* 47: 471-486.

del Giorgio, P.A., and Scarborough, G. (1995) Increase in the Proportion of Metabolically Active Bacteria Along Gradients of Enrichment in Fresh-Water and Marine Plankton - Implications for Estimates of Bacterial-Growth and Production-Rates. *Journal of Plankton Research* 17: 1905-1924.

del Giorgio, P.A., Prairie, Y.T., and Bird, D.F. (1997) Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. *Microbial Ecology* 34: 144-154.

DeLong, E.F. (1992) *Archaea* in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America* 89: 5685-5689.

DeLong, E.F., Wickham, G.S., and Pace, N.R. (1989) Phylogenetic strains: Ribosomal RNA-based probes for the identification of single cells. *Science* 243: 1360-1363.

DeLong, E.F., Taylor, L.T., Marsh, T.L., and Preston, C.M. (1999) Visualization and enumeration of marine planktonic *Archaea* and *Bacteria* by using polyribonucleotide probes and fluorescent *in situ* hybridization. *Applied and Environmental Microbiology* 65: 5554-5563.

Egli, T. (1993) The ecological and physiological significance of the growth of heterotrophic microorganisms with mixes of substrates. In *Advances in Microbial Ecology*. Jones, J.G. (ed). New York: Plenum Press, pp. 305-385.

Eilers, H., Pernthaler, J., and Amann, R. (2000a) Succession of pelagic marine bacteria during enrichment: A close look on cultivation-induced shifts. *Applied and Environmental Microbiology* 66: 4634-4640.

Eilers, H., Pernthaler, J., Glöckner, F.O., and Amann, R. (2000b) Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology* 66: 3044-3051.

Eilers, H., Pernthaler, J., Peplies, J., Glöckner, F.O., Gerdts, G., and Amann, R. (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. *Applied and Environmental Microbiology* 67: 5134-5142.

Ferguson, R.L., Buckley, E.N., and Palumbo, A.V. (1984) Response of marine bacterioplankton to differential filtration and confinement. *Applied and Environmental Microbiology* 47: 49-55.

Flärdh, K., Cohen, P.S., and Kjelleberg, S. (1992) Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. *Journal of Bacteriology* 174: 6780-6788.

Fuhrman, J.A., and Azam, F. (1980) Bacterioplankton Secondary Production Estimates for Coastal Waters of British-Columbia, Antarctica, and California. *Applied and Environmental Microbiology* 39: 1085-1095.

Fuhrman, J.A., and Ferguson, R.L. (1986) Nanomolar Concentrations and Rapid Turnover of Dissolved Free Amino-Acids in Seawater - Agreement between Chemical and Microbiological Measurements. *Marine Ecology-Progress Series* 33: 237-242.

Fuhrman, J.A., McCallum, K., and Davis, A.A. (1992) Novel major archaebacterial group from marine plankton. *Nature* 356: 148-149.

Gasol, J.M., delGiorgio, P.A., Massana, R., and Duarte, C.M. (1995) Active versus inactive bacteria: Size-dependence in a coastal marine plankton community. *Marine Ecology-Progress Series* 128: 91-97.

Gasol, J.M., Zweifel, U.L., Peters, F., Fuhrman, J.A., and Hagström (1999) Significance of Size and Nucleic Acid Content Heterogeneity as Measured by Flow Cytometry in Natural Planktonic Bacteria. *Appl Environ Microbiol* 65: 4475-4483.

Giovannoni, S.J., DeLong, E.F., Olsen, G.J., and Pace, N.R. (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *Journal of Bacteriology* 170: 720-726.

Giovannoni, S.J., Britschgi, T.B., Moyer, C.L., and Field, K.G. (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* 345: 60-63.

Glöckner, F.O., Fuchs, B.M., and Amann, R. (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence *in situ* hybridization. *Applied and Environmental Microbiology* 65: 3721-3726.

References

Gray, N.D., and Head, I.M. (2001) Linking genetic identity and function in communities of uncultured bacteria. *Environmental Microbiology* 3: 481-492.

Grossart, H.P., Riemann, L., and Azam, F. (2001) Bacterial motility in the sea and its ecological implications. *Aquatic Microbial Ecology* 25: 247-258.

Hahn, D., Amann, R.I., and Zeyer, J. (1993) Detection of Messenger-RNA in Streptomyces Cells by Whole-Cell Hybridization with Digoxigenin-Labeled Probes. *Applied and Environmental Microbiology* 59: 2753-2757.

Hamasaki, K., Long, R.A., and Azam, F. (2004) Individual cell growth rates of marine bacteria, measured by bromodeoxyuridine incorporation. *Aquatic Microbial Ecology* 35: 217-227.

Hobbie, J.E., Daley, R.J., and Jasper, S. (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology* 33: 1225-1228.

Hodson, R.E., Dustman, W.A., Garg, R.P., and Moran, M.A. (1995) *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Applied and Environmental Microbiology* 61: 4074-4082.

Hoppe, H.G. (1976) Determination and Properties of Actively Metabolizing Heterotrophic Bacteria in Sea, Investigated by Means of Micro-Autoradiography. *Marine Biology* 36: 291-302.

Howard-Jones, M.H., Frischer, M.E., and Verity, P.G. (2001) Determining the physiological status of individual bacterial cells. In *Methods in Microbiology, Vol 30*, pp. 175-205.

Jaspers, E., and Overmann, J. (2004) Ecological significance of microdiversity: Identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. *Applied and Environmental Microbiology* 70: 4831-4839.

Jeffrey, W.H., VonHaven, R., Hoch, M.P., and Coffin, R.B. (1996) Bacterioplankton RNA, DNA, protein content and relationships to rates of thymidine and leucine incorporation. *Aquatic Microbial Ecology* 10: 87-95.

Jernaes, M.W., and Steen, H.B. (1994) Staining of Escherichia-Coli for Flow-Cytometry - Influx and Efflux of Ethidium-Bromide. *Cytometry* 17: 302-309.

Joux, F., and LeBaron, P. (1997) Ecological implications of an improved direct viable count method for aquatic bacteria. *Applied and Environmental Microbiology* 63: 3643-3647.

Karner, M., and Fuhrman, J.A. (1997) Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. *Applied and Environmental Microbiology* 63: 1208-1213.

Kemp, P.F., Lee, S., and Laroche, J. (1993) Estimating the Growth-Rate of Slowly Growing Marine-Bacteria from RNA-Content. *Applied and Environmental Microbiology* 59: 2594-2601.

Kerkhof, L., and Ward, B.B. (1993) Comparison of Nucleic-Acid Hybridization and Fluorometry for Measurement of the Relationship between RNA/DNA Ratio and Growth-Rate in a Marine Bacterium. *Applied and Environmental Microbiology* 59: 1303-1309.

King, L.K., and Parker, B.C. (1988) A Simple, Rapid Method for Enumerating Total Viable and Metabolically Active Bacteria in Groundwater. *Applied and Environmental Microbiology* 54: 1630-1631.

Kirchman, D.L., Yu, L.Y., and Cottrell, M.T. (2003) Diversity and abundance of uncultured Cytophaga-like bacteria in the Delaware Estuary. *Applied and Environmental Microbiology* 69: 6587-6596.

Kirchman, D.L., Meon, B., Ducklow, H.W., Carlson, C.A., Hansell, D.A., and Steward, G.F. (2001) Glucose fluxes and concentrations of dissolved combined neutral sugars (polysaccharides) in the Ross Sea and Polar Front Zone, Antarctica. *Deep-Sea Research Part Ii-Topical Studies in Oceanography* 48: 4179-4197.

Kodak Product information.

Kogure, K., Simidu, U., and Taga, N. (1978) A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology* 25: 415-420.

Kogure, K., Simidu, U., and Taga, N. (1984) An Improved Direct Viable Count Method for Aquatic Bacteria. *Archiv Fur Hydrobiologie* 102: 117-122.

Laniece, P., Charon, Y., Dumas, S., Mastrippolito, R., Pinot, L., Tricoire, H., and Valentin, L. (1994) Hrri - a High-Resolution Radioimager for Fast, Direct Quantification in in-Situ Hybridization Experiments. *Biotechniques* 17: 338-345.

Lanoil, B.D., and Giovannoni, S.J. (1997) Identification of bacterial cells by chromosomal painting. *Applied and Environmental Microbiology* 63: 1118-1123.

Lanoil, B.D., Carlson, C.A., and Giovannoni, S.J. (2000) Bacterial chromosomal painting for *in situ* monitoring of cultured marine bacteria. *Environmental Microbiology* 2: 654-665.

Lebaron, P., Servais, P., Agogue, H., Courties, C., and Joux, F. (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Applied & Environmental Microbiology* 67: 1775-1782.

Lee, N., Nielsen, P., Andreasen, K., Juretschko, S., Nielsen, J., Schleifer, K., and Wagner, M. (1999) Combination of fluorescent *in situ* hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology. *Applied and Environmental Microbiology* 65: 1289-1297.

Lee, S.H., and Kemp, P.F. (1994) Single-Cell RNA-Content of Natural Marine Planktonic Bacteria Measured By Hybridization With Multiple 16s Ribosomal-RNA-Targeted Fluorescent-Probes. *Limnology and Oceanography* 39: 869-879.

Lopez-Amoros, R., Comas, J., and Vives-Rego, J. (1995) Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol. *Applied and Environmental Microbiology* 61: 2521-2526.

References

Lopez-Amoros, R., Comas, J., Garcia, M., and Vivesrego, J. (1998) Use of the 5-cyano-2,3-ditolyl tetrazolium chloride reduction test to assess respiring marine-bacteria and grazing effects by flow cytometry during linear alkylbenzene sulfonate degradation. *FEMS Microbiology Ecology* 27: 33-42.

Lovejoy, C., Legendre, L., Klein, B., Tremblay, J.E., Ingram, R.G., and Therriault, J.C. (1996) Bacterial activity during early winter mixing - (Gulf of St Lawrence, Canada). *Aquatic Microbial Ecology* 10: 1-13.

Macian, M.C., Ludwig, W., Schleifer, K.H., Pujalte, M.J., and Garay, E. (2001) Vibrio agarivorans sp nov., a novel agarolytic marine bacterium. *International Journal of Systematic and Evolutionary Microbiology* 51: 2031-2036.

Madrid, V.M., Taylor, G.T., Scranton, M.I., and Chistoserdov, A.Y. (2001) Phylogenetic diversity of bacterial and archaeal communities in the anoxic zone of the Cariaco Basin. *Applied and Environmental Microbiology* 67: 1663-1674.

Malmstrom, R.R., Kiene, R.P., Cottrell, M.T., and Kirchman, D.L. (2004) Contribution of SAR11 bacteria to dissolved dimethylsulfoniopropionate and amino acid uptake in the North Atlantic ocean. *Applied and Environmental Microbiology* 70: 4129-4135.

Malmstrom, R.R., Cottrell, M.T., Elifantz, H., and Kirchman, D.L. (2005) Biomass production and assimilation of dissolved organic matter by SAR11 bacteria in the Northwest Atlantic Ocean. *Applied and Environmental Microbiology* 71: 2979-2986.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: Problems and solutions. *Systematic and Applied Microbiology* 15: 593-600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.-H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* 142: 1097-1106.

Mason, D.J., Shanmuganathan, S., Mortimer, F.C., and Gant, V.A. (1998) A Fluorescent Gram Stain for Flow Cytometry and Epifluorescence Microscopy. *Applied and Environmental Microbiology* 64: 2681-2685.

Meyer-Reil, L.A. (1978) Autoradiography and epifluorescence microscopy combined for determination of number and spectrum of actively metabolizing bacteria in natural waters. *Applied and Environmental Microbiology* 36: 506-512.

Middelboe, M., Sondergaard, M., Letarte, Y., and Borch, N.H. (1995) Attached and free-living bacteria: Production and polymer hydrolysis during a diatom bloom. *Microbial Ecology* 29: 231-248.

Molecular-Probes (2001) LIVE BacLightTM Bacterial Gram Stain Kit (L-7005). Product information. www.invitrogen.com.

Morris, R.M., Rappe, M.S., Connon, S.A., Vergin, K.L., Siebold, W.A., Carlson, C.A., and Giovannoni, S.J. (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* 420: 806-810.

Nielsen, J.L., Christensen, D., Kloppenborg, M., and Nielsen, P.H. (2003) Quantification of cell-specific substrate uptake by probe- defined bacteria under *in situ* conditions by microautoradiography and fluorescence *in situ* hybridization. *Environmental Microbiology* 5: 202-211.

Nissen, H., Nissen, P., and Azam, F. (1984) Multiphasic Uptake of D-Glucose by an Oligotrophic Marine Bacterium. *Marine Ecology -Progress Series* 16: 155-160.

Osmonics Product information. www.osmolabstore.com.

Ouverney, C.C., and Fuhrman, J.A. (1999) Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. *Applied and Environmental Microbiology* 65: 1746-1752.

Ouverney, C.C., and Fuhrman, J.A. (2000) Marine planktonic Archaea take up amino acids. *Applied & Environmental Microbiology* 66: 4829-4833.

Packard, T.T. (1985) Measurement of electron transport activity of microplankton. In *Advances in Aquatic Microbiology*. Jannash, H., Leb Williams, P. J (ed). London: Academic Press, pp. 207-261.

Parsons, T.R., and Strickland, J.D.H. (1961) On the Production of Particulate Organic Carbon by Heterotrophic Processes in Sea Water. *Deep-Sea Research* 8: 211-222.

Pernthaler, A., and Amann, R. (2004) Simultaneous fluorescence *in situ* hybridization of mRNA and rRNA in environmental bacteria. *Applied and Environmental Microbiology* 70: 5526-5533.

Pernthaler, A., Pernthaler, J., and Amann, R. (2002a) Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Applied and Environmental Microbiology* 68: 3094-3101.

Pernthaler, A., Pernthaler, J., Eilers, H., and Amann, R. (2001) Growth patterns of two marine isolates: Adaptations to substrate patchiness? *Applied & Environmental Microbiology* 67: 4077-4083.

Pernthaler, A., Pernthaler, J., Schattenhofer, M., and Amann, R. (2002b) Identification of DNA-synthesizing bacterial cells in coastal North Sea plankton. *Applied and Environmental Microbiology* 68: 5728-5736.

Pernthaler, A., Preston, C.M., Pernthaler, J., DeLong, E.F., and Amann, R. (2002c) A comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. *Applied and Environmental Microbiology*: 661-668.

Pernthaler, J., and Amann, R. (2005) Fate of Heterotrophic Microbes in Pelagic Habitats:Focus on Populations. *Microbiology and molecular biology reviews* 69: 440–461.

Pernthaler, J., Pernthaler, A., and Amann, R. (2003) Automated enumeration of groups of marine picoplankton after fluorescence *in situ* hybridization. *Applied and Environmental Microbiology* 69: 2631-2637.

Pirker, H., Pausz, C., Stoderegger, K.E., and Herndl, G.J. (2005) Simultaneous measurement of metabolic activity and membrane integrity in marine bacterioplankton determined by confocal laser-scanning microscopy. *Aquatic Microbial Ecology* 39: 225-233.

Pomeroy, L.R. (1974) The ocean's food web, a changing paradigm. *Bioscience* 24: 499-504.

Rappe, M.S., Connon, S.A., Vergin, K.L., and Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* 418: 630-633.

Riemann, L., and Azam, F. (2002) Widespread N-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. *Applied and Environmental Microbiology* 68: 5554-5562.

Rodriguez, G., Phipps, D., Ishiguro, K., and Ridgway, H. (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied and Environmental Microbiology* 58: 1801-1808.

Rogers, A.W. (1979) *Techniques of Autoradiography*. Amsterdam: Elsevier.

Sawabe, T., Makino, H., Tatsumi, M., Nakano, K., Tajima, K., Iqbal, M.M. et al. (1998) Pseudoalteromonas bacteriolytica sp. nov., a marine bacterium that is the causative agent of red spot disease of Laminaria japonica. *International Journal of Systematic Bacteriology* 48: 769-774.

Schmid, M.C., Maas, B., Dapena, A., de Pas-Schoonen, K.V., de Vossenberg, J.V., Kartal, B. et al. (2005) Biomarkers for *in situ* detection of anaerobic ammonium-oxidizing (anammox) bacteria. *Applied and Environmental Microbiology* 71: 1677-1684.

Schönhuber, W., Fuchs, B., Juretschko, S., and Amann, R. (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Applied and Environmental Microbiology* 63: 3268-3273.

Sekar, R., Fuchs, B., Amann, R., and Pernthaler, J. (2004) Flow Sorting of marine bacterioplankton after fluorescence *in situ* hybridization. *Applied and Environmental Microbiology* 70: 6210-6219.

Servis, N.A., Lytle, M.S., Midthun, D.B., Leake, R.A., and Adams, J.C. (1993) Comparison of Isopropyl Cinodine with Nalidixic-Acid in the Direct Viable Count. *Journal of Applied Bacteriology* 75: 583-587.

Shapiro, H.M. (2000) Microbial analysis at the single-cell level: tasks and techniques. *J Microbiol Methods* 42: 3-16.

Sherr, B., Sherr, E., and del Giorgio, P. (2001) Enumeration of total and highly active bacteria. In *Methods in Microbiology, Vol 30*, pp. 129-159.

Sherr, B.F., Del Giorgio, P., and Sherr Evelyn, B. (1999a) Estimating abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. *Aquatic Microbial Ecology* 18: 117-131.

Sherr, E.B., Sherr, B.F., and Sigmon, C.T. (1999b) Activity of marine bacteria under incubated and *in situ* conditions. *Aquatic Microbial Ecology* 20: 213-223.

Shiba, T. (1991) *Roseobacter litoralis* gen-nov, sp-nov, and *Roseobacter denitrificans* sp-nov, aerobic pink pigmented bacteria which contain bacteriochlorophyll a. *Systematic and Applied Microbiology* 14: 140-145.

Simon, M. (1987) Biomass and Production of Small and Large Free-Living and Attached Bacteria in Lake Constance. *Limnology and Oceanography* 32: 591-607.

Simon, M., Grossart, H.P., Schweitzer, B., and Ploug, H. (2002) Microbial ecology of organic aggregates in aquatic ecosystems. *Aquatic Microbial Ecology* 28: 175-211.

Smith, E.M. (1998) Coherence of microbial respiration rate and cell-specific bacterial activity in a coastal planktonic community. *Aquatic Microbial Ecology* 16: 27-35.

Smith, E.M., and del Giorgio, P.A. (2003) Low fractions of active bacteria in natural aquatic communities? *Aquatic Microbial Ecology* 31: 203-208.

Smith, J.J., and McFeters, G.A. (1997) Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in Escherichia coli K-12. *Journal of Microbiological Methods* 29: 161-175.

Sorokin, D.Y. (1995) *Sulfitobacter pontiacus* gen-nov, sp-nov - a new heterotrophic bacterium from the black sea, specialized on sulfite oxidation. *Microbiology* 64: 295-305.

Sorokin, Y.I. (1999) *Radioisotopic Methods in Hydrobiology*. Heidelberg: Springer.

Steward, G.F., and Azam, F. (1999) Bromodeoxyuridine as an alternative to H-3-thymidine for measuring bacterial productivity in aquatic samples. *Aquatic Microbial Ecology* 19: 57-66.

Tabor, P.S., and Neihof, R.A. (1982) Improved micro-auto-radiographic method to determine individual microorganisms active in substrate uptake in natural waters. *Applied and Environmental Microbiology* 44: 945-953.

Teira, E., Reinthaler, T., Pernthaler, A., Pernthaler, J., and Herndl, G.J. (2004) Combining catalyzed reporter deposition-fluorescence *in situ* hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. *Appl. Environ. Microbiol.* 70: 4411-4414.

Tengberg, A., Almroth, E., and Hall, P. (2003) Resuspension and its effects on organic carbon recycling and nutrient exchange in coastal sediments: *in situ* measurements using new experimental technology. *Journal of Experimental Marine Biology and Ecology* 285: 119-142.

Ullrich, S., Karrasch, B., Hoppe, H.-G., Jeskulke, K., and Mehrens, M. (1996) Toxic effects of bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. *Applied and Environmental Microbiology* 62: 4587-4593.

Unanue, M., Ayo, B., Agis, M., Slezak, D., Herndl, G.J., and Iriberri, J. (1999) Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. *Microbial Ecology* 37: 36-48.

Urbach, E., Vergin, K.L., and Giovannoni, S.J. (1999) Immunochemical detection and isolation of DNA from metabolically active bacteria. *Applied & Environmental Microbiology* 65: 1207-1213.

Vaccaro, R.F., and Jannasch, H.W. (1967) Variations in Uptake Kinetics for Glucose by Natural Populations in Seawater. *Limnology and Oceanography* 12: 540-542.

Vetriani, C., Tran, H.V., and Kerkhof, L.J. (2003) Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. *Applied and Environmental Microbiology* 69: 6481-6488.

Wagner-Döbler, I., Rheims, H., Felske, A., Pukall, R., and Tindall, B.J. (2003) Jannaschia helgolandensis gen. nov., sp nov., a novel abundant member of the marine Roseobacter clade from the North Sea. *International Journal of Systematic and Evolutionary Microbiology* 53: 731-738.

Wainright, S., and Hopkinson, C. (1997) Effects of sediment resuspension on organic matter processing in coastal environments:asimulation model. *Journal of Marine Systems* 11: 353-368.

Wainright, S.C. (1987) Stimulation of heterotrophic microplankton production by resuspended marine sediments. *Science* 238: 1710-1712.

Williams, P.J.L. (1981) Microbial Contribution to Overall Marine Plankton Metabolism - Direct Measurements of Respiration. *Oceanologica Acta* 4: 359-364.

Williams, S.C., Hong, Y., Danavall, D.C.A., Howard-Jones, M.H., Gibson, D., Frischer, M.E., and Verity, P.G. (1998) Distinguishing between living and nonliving bacteria: Evaluation of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. *Journal of Microbiological Methods* 32: 225-236.

Woese, C.R. (1987) Bacterial evolution. *Microbiological Reviews* 51: 221-271.

Woese, C.R., Stackebrandt, E., Macke, T.J., and Fox, G.E. (1985) A Phylogenetic Definition of the Major Eubacterial Taxa. *Systematic and Applied Microbiology* 6: 143-151.

Wright, R.T.H., John E. (1965) The Uptake of Organic Solutes in Lake Water. *Limnology and Oceanography* 10: 22-28.

Yager, P.L., Connelly, T.L., Mortazavi, B., Wommack, K.E., Bano, N., Bauer, J.E. et al. (2001) Dynamic bacterial and viral response to an algal bloom at subzero temperatures. *Limnology and Oceanography* 46: 790-801.

Yumoto, I., Kawasaki, K., Iwata, H., Matsuyama, H., and Okuyama, H. (1998) Assignment of *Vibrio* sp. strain ABE-1 to *Colwellia maris* sp. nov., a new psychrophilic bacterium. *International Journal of Systematic Bacteriology* 48: 1357-1362.

Zimmermann, R., Iturriaga, R., and Beckerbirck, J. (1978) Simultaneous determination of total number of aquatic bacteria and number thereof involved in respiration. *Applied and Environmental Microbiology* 36: 926-935.

Zimmermann R, M.-R.L.-A. (1974) A new method for fluorescence staining of bacterial populations on membrane filters. *Kieler Meeresforsch* 30: 24–27.

ZoBell, C.E. (1946) *Marine microbiology. A monograph on hydrobacteriology*. Waltham, Mass, U.S.A.: Chronica Botanica Company.

Zubkov, M.V., Tarran, G.A., and Fuchs, B.M. (2004) Depth related amino acid uptake by Prochlorococcus cyanobacteria in the Southern Atlantic tropical gyre. *FEMS Microbiology Ecology* 50: 153-161.

Zweifel, U.L., and Hagström, A. (1995) Total counts of marine *Bacteria* include a large fraction of non-nucleoid-containing *Bacteria* (Ghosts). *Applied and Environmental Microbiology* 61: 2180-2185.

Zwirglmaier, K. (2005) Fluorescence *in situ* hybridisation (FISH) - the next generation. *FEMS Microbiology Letters* 246: 151-158.

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Incorporation of Glucose under Anoxic Conditions by Bacterioplankton from Coastal North Sea Surface Waters

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It has been hypothesized that the potential for anaerobic metabolism might be a common feature of bacteria in coastal marine waters (L. Riemann and F. Azam, Appl. Environ. Microbiol. 68: 5554-5562, 2002). Therefore, we investigated whether different phylogenetic groups of heterotrophic picoplankton from the coastal North Sea were able to take up a simple carbon source under anoxic conditions. Oxic and anoxic incubations (4 h) or enrichments (24 h) of seawater with radiolabeled glucose were performed in July and August 2003. Bacteria with incorporated substrate were identified by using a novel protocol in which we combined fluorescence in situ hybridization and microautoradiography of cells on membrane filters. Incorporation of glucose under oxic and anoxic conditions was found in α-*Proteobacteria*, γ-*Proteobacteria*, and the *Cytophaga-Flavobacterium* cluster of the *Bacteroidetes* at both times, but not in marine *Euryarchaeota*. In July, the majority of cells belonging to the α-proteobacterial *Roseobacter* clade showed tracer incorporation both in oxic incubations and in oxic and anoxic enrichments. In August, only a minority of the *Roseobacter* cells, but most bacteria affiliated with *Vibrio* spp., were able to incorporate the tracer under either condition. A preference for glucose uptake under anoxic conditions was observed for bacteria related to *Alteromonas* and the *Pseudoalteromonas-Colwellia* group. These genera are commonly considered to be strictly aerobic, but facultatively fermentative strains have been described. Our findings suggest that the ability to incorporate substrates anaerobically is widespread in pelagic marine bacteria belonging to different phylogenetic groups. Such bacteria may be abundant in fully aerated coastal marine surface waters.

Is facultatively anaerobic metabolism an exotic or widespread feature of marine bacterioplankton? The majority of heterotrophic microbes in the oceans live in a permanently aerobic environment (47). In the coastal regions, a significant fraction of microbial production is found within hot spots of particulate organic matter (marine snow) that is, e.g., composed of dead or senescent phytoplankton cells (3, 32). Such zones in which there is high turnover potentially represent a niche that might favor anaerobic metabolism (1, 6). In freely suspended or sinking aggregates, the rapid flux of oxygen largely compensates for microbial oxygen consumption, and anoxic microzones within marine snow particles are probably rather short lived (33). However, in shallow habitats, such as the coastal southern North Sea, the sporadic resuspension of detrital material from sediment surfaces (25) might provide a inoculum of bacteria that also thrive in the absence of oxygen.

Currently there is no direct field evidence that supports the hypothesis that facultatively anaerobic metabolism is a common feature of pelagic microbes. Although some bacteria that are readily isolated from the marine water column are capable of fermentative growth (e.g., the γ-proteobacterial genera *Moritella* and *Vibrio* [23, 49]), members of these phylogenetic lineages are usually rare in coastal surface waters, as determined by molecular techniques (13). Recently, Riemann and Azam (37) described a significant decrease in [^{3}H]thymidine incorporation into marine bacteria after inhibition of the membrane transfer system responsible for the uptake of *N*-acetylglucosamine (NAG). These authors argued that this phosphotransferase system (PTS) is a typical feature of facultatively anaerobic bacteria. They speculated that anaerobic metabolism could thus be a widespread feature of bacteria in the marine water column. However, in that study 22 of the 60 marine isolates that incorporated NAG via a PTS were not capable of a facultatively anaerobic metabolism (37). Moreover, the (aerobic) NAG uptake measured in marine water samples was probably not exclusively mediated via the PTS, as indicated by inhibition experiments with other substrates. Thus, it is still not known if pelagic bacteria are capable of substrate incorporation under anoxic conditions in coastal marine waters and, if they are, which bacteria are involved.

Microautoradiography (MAR) is an approach to track the uptake of radiolabeled tracers in single microbial cells (7). It provides a means of studying the facultatively anaerobic metabolic capacities of water column bacteria in situ via short-term incorporation of glucose under experimentally induced anoxic conditions. In order to assign physiological functions to particular bacterioplankton groups, MAR can be combined with single-cell identification by fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes (10, 22, 29, 45). Recently, the FISH technique has been substantially improved for better visualization of small planktonic cells by means of enzymatic signal amplification (catalyzed reported deposition [CARD]) (30). This modified CARD-FISH protocol has been successfully combined with MAR to measure activity in open-ocean microbes (45). However, in current pro-

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TABLE 1. Overview of protocol for MAR and CARD-FISH of marine bacterioplankton on membrane filters

Stage	Description
Incubation and preparation of filters	Add radiolabeled substrates at the desired concentration; end incubation by fixation with freshly prepared buffered paraformaldehyde fixative; filter onto polycarbonate filters.
CARD-FISH (can alternatively be performed after MAR)	See reference 30.
Microautoradiographic procedure	Glue the filter pieces onto glass slides; coat the slides with molten emulsion (diluted 1:1 with 0.2% agarose); let coated slides dry on top of an ice-cold metal surface; expose slides inside cardboard boxes at 4°C; develop photographic emulsion as described by the manufacturer.
DAPI counterstaining	Stain with a 1-μg/ml DAPI solution for 10 min in a refrigerator; rinse for 1 min with distilled water, followed by 1 min with 80% ethanol; air dry inside the refrigerator; mount in a 4:1 mixture of Citifluor and Vectashield.
Evaluation	Image capture for DAPI-, FISH-, and MAR-positive cells; offline image evaluation by semiautomated image analysis.

tocols, evaluation of MAR-FISH-stained bacterioplankton samples is still very time-consuming. Moreover, approximately 50% of the cells are lost during preparation (9), which potentially reduces the accuracy and reproducibility of the MAR technique. Therefore, there is a need for a simplified MAR-FISH approach that profits from the superior signal intensities of CARD-FISH staining and at the same time does not cause cell loss during the MAR procedure.

We tested the hypothesis that incorporation of glucose under anoxic conditions may be a widespread ability of bacteria that live in oxic coastal marine surface waters. This was done by performing short-term incubation and enrichment experiments at two times for samples from the German Bight of the North Sea. FISH with group-specific probes was used to assign aerobic and anaerobic glucose incorporation to individual bacterial populations. For this purpose, a modified protocol that combined MAR and CARD-FISH without the critical cell transfer step was developed and combined with a rapid evaluation system in which semiautomated image analysis was used.

MATERIALS AND METHODS

Sample acquisition, incubation, and fixation. Surface water samples (depth, 1 m) were collected in July and August 2003 at the Helgoland Roads sampling station (54°11′N, 7°54′E; water depth, 8 m), which is 50 km offshore in the German Bay of the North Sea. The sampling dates represented the onset and decline of a phytoplankton bloom that was mainly composed of *Thalassiosira* spp. and *Ceratium* spp. The median surface water temperatures were 18°C in July and 19°C in August, and the median salinities were 34.9 and 32.3 ppt, respectively (Mursys Umweltreport [www.bsh.de]). Incubations with radioactively labeled glucose under oxic and anoxic conditions were performed within 2 h after sample collection. Triplicate 10-ml subsamples were incubated for every treatment type. Oxic incubations were performed in sterile 70-ml serum vials with cotton plugs. Anoxic incubation conditions were established in the same type of serum vials by flushing the vials with nitrogen (purity, 4.0; Air Liquide, Stelle, Germany). The nitrogen flushing times required to produce anoxic conditions in the 10-ml subsamples in the serum vials were verified by Winkler titration and by using an oxygen microsensor (8, 36). The procedure used for anoxic incubations was as follows. First, the empty serum vials were preflushed for 2 min with nitrogen. Next, seawater was added without interrupting the flushing, and the water was bubbled with nitrogen for 15 min. Subsequently D-[6-^{3}H]glucose (specific activity, 1,29 TBq/mmol; Amersham) was added to a final concentration of 10 nM. Before the vials were closed with air-tight butyl rubber stoppers, the headspace in the bottles was flushed for 5 min. The bubbling procedure caused a small but significant increase in the pH, from pH 8.05 ± 0.01 to pH 8.28 ± 0.02, likely because of the removal of dissolved CO_2.

The preparations were incubated for either 4 or 24 h in the dark at the ambient water temperature (19 to 21°C). For each treatment one additional control sample was fixed at the beginning of the incubation, prior to the addition of radiolabeled substrate. Subsequently, freshly prepared buffered paraformaldehyde fixative (pH 7.0) was added to the samples to a final concentration of 1%. After fixation the samples for MAR were filtered through polycarbonate filters (type GTTP; pore size, 0.2 μm; diameter, 25 mm; Millipore, Eschborn, Germany). The filters were rinsed twice with sterile phosphate-buffered saline and stored at −20°C until they were analyzed.

Population analysis by FISH. The percentages of different microbial taxa were determined by FISH with horseradish peroxidase-labeled oligonucleotide probes and catalyzed reporter deposition (30). The following probes were used to characterize the microbial community in the original water samples and after incubation with radiolabeled glucose: EUB I-III (most *Bacteria*) (11), EURY806 (marine *Euryarchaeota*) (45), ALF968 (most α-*Proteobacteria*) (16), GAM42a (most γ-*Proteobacteria*), CF319a (many groups belonging to the *Cytophaga-Flavobacterium* cluster of the *Bacteroidetes*) (2), ROS537 (members of the *Roseobacter-Sulfitobacter-Silicibacter* clade [referred to as the *Roseobacter* clade below]), NOR5-730 (NOR5 subcluster of the γ-proteobacterial OM60 clade) (13), SAR86-1245 (members of the γ-proteobacterial SAR86 clade), ALT1413 (*Alteromonas* spp.), PSA184 (*Pseudoalteromonas* spp. and *Colwellia* spp.) (12), and GV822 (*Vibrio* spp.) (15). The EUB antisense probe NON338 (2) was used as a negative control. All probes were purchased from Biomers.net (Ulm, Germany). Hybridization of polycarbonate filter sections and signal amplification with $ALEXA_{488}$ (Molecular Probes, Eugene, Oreg.)-custom-labeled tyramides were performed as previously described (30). Specific hybridization conditions were established by addition of formamide to the hybridization buffers. Since the hybridizations were carried out at a lower temperature (35°C), the formamide concentrations in the hybridization buffers were increased by 20% compared to the concentrations reported previously for the directly fluorescently labeled probes (e.g., 55% instead of 35% for probe GAM42a). Counterstaining of CARD-FISH preparations with 4,6-diamidino-2-phenylindole (DAPI) (1 μg ml^{-1}) and mounting on microscopic slides were carried out as described previously (30). DAPI- and FISH-stained cells were counted by automated image analysis (31).

Development of a protocol for CARD-FISH and MAR on membrane filters. Since all previously described protocols for MAR-FISH of marine bacteria (10, 29, 45) include steps that may cause high cell losses (9), we developed a strategy to combine CARD-FISH and MAR staining of microbes on membrane filters without prior transfer of cells to glass slides (Table 1). For this purpose, we first tested different types of membrane filters (polycarbonate, cellulose nitrate, aluminum oxide). Polycarbonate membrane filters were most appropriate, but they were not rigid enough for the MAR procedure. Therefore, filter sections (one-quarter of a 25-mm-diameter filter) were glued onto glass slides prior to processing. Several glues were tested to determine whether they could resist the handling during the MAR-FISH procedure and cause no increase in the fluorescent background. The optimal glue for fixing the membrane filters onto the slides was a two-component epoxy adhesive (UHU plus sofortfest; UHU GmbH, Bühl, Germany).

We subsequently determined whether there were differences between performing the CARD-FISH staining before the MAR procedure and performing the CARD-FISH staining after the MAR procedure. No difficulties were encountered when we performed CARD-FISH of filter sections before the MAR procedure. In contrast, performing MAR and then CARD-FISH resulted in disruption of the photographic emulsion. To avoid this, the photographic emulsion was diluted with agarose instead of gelatin. Different types of agarose with different gel strengths and melting and gelling points were tested. The best results were achieved with Seakem LE agarose (gel strength, 1%; >1,200 g/cm^2;

gelling temperature at a concentration of 1.5%, 36 ± 1.5°C; Biozym, Oldenburg, Germany). In the final protocol 1 part of agarose was added to 1 part of molten photographic emulsion at a final agarose concentration of 0.1%. This allowed CARD-FISH to be carried out also after the MAR procedure.

Sample preparation for CARD-FISH and MAR. For evaluation of our samples, CARD-FISH was usually performed before the MAR analysis, because simultaneous hybridization of numerous filter sections was easier before they were glued onto microscopic slides. The standard CARD-FISH procedure was used (30), including prior embedding of filter sections in agarose and permeabilization with lysozyme. After the FISH-stained cells were counted, the filter pieces were glued onto slides, and MAR was performed within 24 h after hybridization. For the MAR procedure we essentially used the protocol described for cells that were transferred to slides (44). The photochemicals employed were purchased from Eastman Kodak (Rochester, N.Y,) and included autoradiography emulsion (NTB-2), developer (Dektol), and fixer. Different MAR exposure times were tested to obtain the maximum number of cells with silver grains while minimizing the number of false-positive cells, as judged from MAR of the prefixed controls. The optimal exposure times were 8 h for the samples obtained in July and 18 to 24 h for the samples obtained in August. For development of the exposed slides we used the instructions of the manufacturer (2 min of development, 10 s of rinsing with distilled water, and 5 min in fixer, followed by 5 min of washing with distilled water).

Evaluation by image analysis. Evaluations of MAR-FISH preparations were carried out by using an Axioplan II imaging fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a motorized stage, a *z*-axis drive, a fluorescent filter wheel, and a digital camera (Orca I; Hamamatsu, Herrsching, Germany) linked to a personal computer. The KS400 image analysis software (Carl Zeiss Vision, Hallbergmoos, Germany) was used to develop a semiautomated image acquisition and evaluation strategy. First, the operator focused on cells with UV excitation at a magnification of ×63. An image pair was acquired with UV and blue excitation. Next, a stack of five bright-field images was acquired automatically in a region that was 1 to 5 μm above the filtered cells, in order to adequately record MAR grains in an emulsion whose thickness was potentially variable. The bright-field images were combined to obtain a single image, and grains from different layers were detected by selecting the minimum brightness of each pixel from the image stack. For each sample, image triplets from at least 10 microscopic fields were acquired and evaluated.

Detection of DAPI- and FISH-double-stained objects in the respective images was performed as previously described (31). The MAR grains in the combined bright-field image were detected by using the following strategy. First, the image was inverted, and the average gray value was subtracted for background correction. Next, the contrast was rescaled to cover 255 grey levels, and the image was binarized with a fixed threshold (gray value, 150). Objects below a custom threshold were removed, and the binary image was further processed by two rounds of sequential object dilation and erosion by 1 pixel (morphological closing). Binary images from each image triplet were combined for automated colocalization of DAPI-stained and hybridized cells and of MAR grains. The binary images were depicted on the screen as a three-color overlay which could be interactively combined with the original images for manual elimination of artifacts before the object-counting procedure.

RESULTS

Quality of the novel MAR-FISH protocol. The modified MAR and CARD-FISH staining and quantification protocol allowed substantially more rapid processing of preparations than approaches that require transfer of cells to microscopic slides. It specifically eliminated the accidental loss of preparations due to unsuccessful cell transfer, which is a common problem of the MAR technique. Altogether, 99 individual MAR-FISH preparations were evaluated. Since the bacterial cells were embedded in agarose prior to treatment, no significant cell loss was caused by the procedure. On average, 350 FISH-stained cells were counted per sample. The average coefficient of variation of the fraction of cells with visible tracer uptake in triplicate incubations was 0.2 (i.e., 20% of the mean value). The number of MAR-positive cells counted and the variance of triplicate incubations were used to explore the relationship between evaluation effort and experimental precision (Fig. 1). The observed coefficients of variation were usually less than 0.2 if on average more than 50 MAR-positive cells were counted for each replicate (Fig. 1). Negative controls yielded similar percentages of false-positive MAR-active cells irrespective of the sampling time, incubation time, or incubation conditions (<2% of all FISH-stained cells).

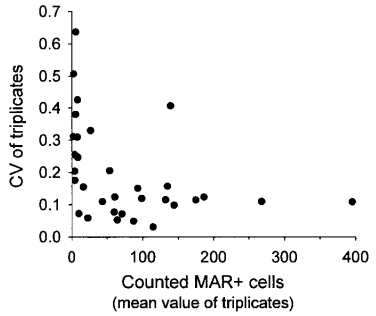

FIG. 1. Relationship between the mean number of counted cells with visible tracer uptake (MAR+) and the coefficient of variation (CV) of the fraction of these cells (percentage of all FISH-stained cells) in triplicate incubations.

Microbial community composition. In July, 87% of all DAPI-stained objects could be detected by FISH, but only 60% could be detected in August. *Euryarchaeota* accounted for 19 and 12% of the total counts in July and August, respectively. Bacteria that hybridized with probes ALF968, GAM42a, and CF319a accounted for around 90% of the cells targeted by the general bacteria probe EUB I-III at both times. Most α-*Proteobacteria* (as detected with probe ALF968) could be assigned to the *Roseobacter* clade in both July and August (data not shown). Therefore, in our subsequent evaluations we focused on this group rather than on α-*Proteobacteria* in general. In July, *Alteromonas* spp., *Pseudoalteromonas* spp., and *Vibrio* spp. were rare in the water column, whereas these groups accounted for the majority of γ-*Proteobacteria* in the August samples (Fig. 2). Members of the *Cytophaga-Flavobacterium* cluster of the *Bacteroidetes* were substantially more abundant in July than in August (Fig. 2).

Short-term (4-h) incorporation of radiolabeled glucose. In July, short-term glucose uptake experiments were carried out only under oxic conditions, whereas in August, both oxic and anoxic incubations were performed. Under oxic conditions, aerobic glucose incorporation was found in 29% ± 2% and 12% ± 5% of the bacteria in July and August, respectively (Fig. 3). *Euryarchaeota* did not show glucose incorporation under either oxic or anoxic conditions (data not shown). In July 84% ± 4% of *Roseobacter* spp. cells took up the tracer under oxic conditions, but in August the percentage was only 11% ± 1% (Fig. 3). The frequency of MAR-positive γ-*Proteobacteria* and members of the *Cytophaga-Flavobacterium* cluster ranged from <10% to approximately 20%, and there were no clear differences between the two times.

In August, the fractions of bacteria and of *Roseobacter* spp. that took up glucose were similar in oxic and anoxic incubations (Fig. 3). For the γ-*Proteobacteria* the tracer uptake was significantly higher under anoxic conditions than under oxic conditions. In contrast, fewer members of the *Cytophaga-Fla-*

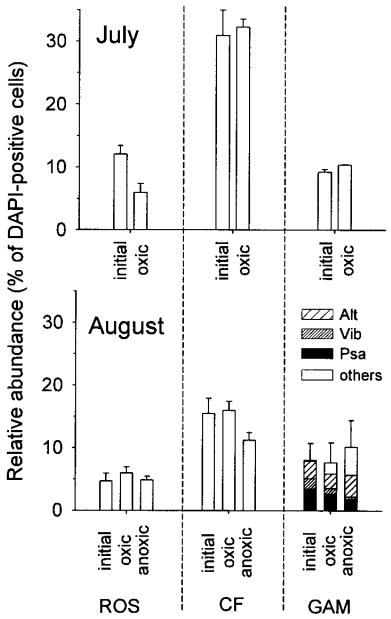

FIG. 2. Relative amounts of members of the *Roseobacter* clade (ROS), the *Cytophaga-Flavobacterium* cluster (CF), and the γ-*Proteobacteria* (GAM) in oxic and anoxic incubations (4 h) of coastal North Sea surface water. Alt, *Alteromonas*; Psa, *Pseudoalteromonas-Colwellia*; Vib, *Vibrio*; others, other γ-*Proteobacteria*. The term initial refers to a sample prior to incubation.

vobacterium cluster were found to incorporate radiolabeled glucose after anoxic incubation (Fig. 3).

Enrichment under oxic and anoxic conditions. During 24 h of incubation under oxic conditions, the total cell concentration increased from $0.6 \times 10^6 \pm 0.2 \times 10^6$ to $1.3 \times 10^6 \pm 0.3 \times 10^6$ cells ml^{-1} in July and from $1.2 \times 10^6 \pm 0.2 \times 10^6$ to $2.9 \times 10^6 \pm 0.6 \times 10^6$ cells ml^{-1} in August. Under anoxic conditions, the increase was less pronounced; in July the concentration increased to $1.0 \times 10^6 \pm 0.2 \times 10^6$ cells ml^{-1}, and in August the concentration increased to $1.7 \times 10^6 \pm 0.3 \times 10^6$ cells ml^{-1}. Under both incubation conditions, mainly γ-*Proteobacteria* were enriched (Fig. 4). In July, *Alteromonas* spp. constituted >90% of the newly grown γ-*Proteobacteria*, whereas in August the numbers of *Pseudoalteromonas* spp. and *Vibrio* spp. cells also significantly increased. In August the relative contribution of *Alteromonas* spp. to the γ-*Proteobacteria* was substantially smaller under anoxic conditions, whereas bacteria affiliated with *Vibrio* spp. were more numerous under these conditions (Fig. 4).

Glucose incorporation during the 24-h enrichments. Tracer uptake into microbial cells over a 24-h period was only studied with the July samples. The frequency of MAR-positive bacteria was approximately one-third higher under anoxic incubation conditions than under oxic incubation conditions, which mirrored the higher numbers of glucose-incorporating γ-*Proteobacteria* in the former treatments (Fig. 5). *Roseobacter* spp. showed the highest fraction of MAR-positive cells irrespective

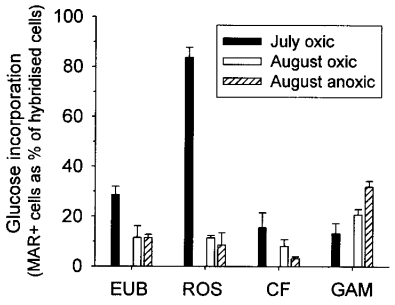

FIG. 3. Fractions of cells with visible tracer uptake (MAR+) affiliated with the *Bacteria* (EUB), the *Roseobacter* clade (ROS), the *Cytophaga-Flavobacterium* cluster (CF), and the γ-*Proteobacteria* (GAM) in oxic and anoxic incubations (4 h).

of the incubation conditions (under oxic conditions, 71% ± 2%; under anoxic conditions, 59% ± 9% [mean ± standard deviation]). Approximately 20% of the cells belonging to the *Cytophaga-Flavobacterium* cluster were capable of taking up glucose under anoxic enrichment conditions. In August, after 24 h of incubation a substantial fraction (>50%) of active cells was found in small aggregates that were composed of different γ-*Proteobacteria*. While the numbers of cells belonging to these bacterial groups could still be determined by FISH (Fig. 4), it was not possible to assign the MAR signals from such aggregated cells to individual populations.

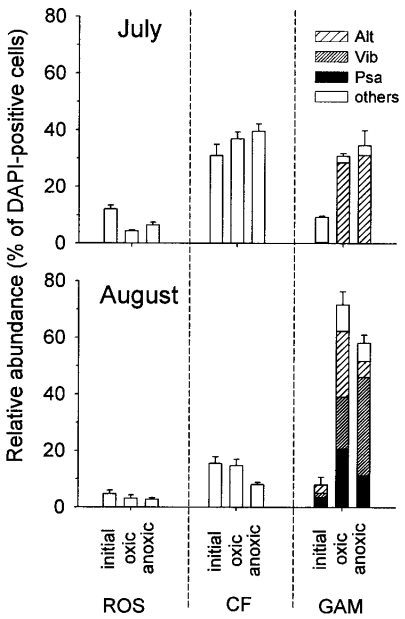

FIG. 4. Relative amounts of members of the *Roseobacter* clade (ROS), the *Cytophaga-Flavobacterium* cluster (CF), and the γ-*Proteobacteria* (GAM) in oxic and anoxic enrichments (24 h). Alt, *Alteromonas*; Psa, *Pseudoalteromonas-Colwellia*; Vib, *Vibrio*; others, other γ-*Proteobacteria*.

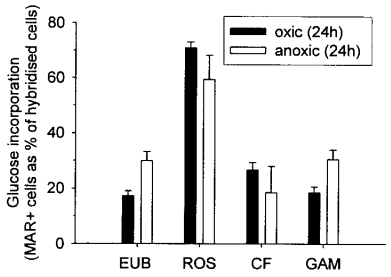

FIG. 5. Fractions of cells with visible tracer uptake (MAR+) affiliated with the *Bacteria* (EUB), the *Roseobacter* clade (ROS), the *Cytophaga-Flavobacterium* cluster (CF), and the γ-*Proteobacteria* (GAM) in oxic and anoxic enrichments (24 h) in July 2003.

Glucose incorporation for specific genera of the γ-*Proteobacteria*. Tracer uptake was specifically investigated for the three genera of γ-*Proteobacteria* that were found to be enriched over a 24-h period (Fig. 4). At both sampling times, significantly larger fractions of MAR-positive *Alteromonas* spp. and *Pseudoalteromonas* spp. cells were present after anoxic incubation for 24 h (July) and 4 h (August), respectively (Fig. 6). In July, nearly twice as many *Vibrio* spp. took up glucose under oxic conditions as under anoxic conditions. In contrast, little difference between oxic glucose uptake and anoxic glucose uptake by *Vibrio* spp. was observed in August. At that sampling time, approximately 80 to 95% of all *Vibrio* spp. cells were MAR positive. No visible glucose incorporation could be detected in cells belonging to other γ-proteobacterial groups (related to the NOR5 and SAR86 clades) under any conditions or at any time.

DISCUSSION

Method development. We successfully combined CARD-FISH and MAR to detect glucose uptake in coastal bacterioplankton populations under oxic and anoxic conditions. A satisfactory level of reproducibility was obtained for counts of active cells from triplicate incubations (Fig. 1). We addressed one of the most problematic issues of current MAR approaches by directly performing FISH and MAR with microbes concentrated on membrane filters without prior transfer of cells to glass slides. This strategy was originally introduced by Meyer-Reil (26), but it was later dismissed. Tabor and Neihof (44) stated that such an approach would result in background fluorescence that was too high to perform total cell counting by acridine orange staining and that the filter pores would interfere with the recognition of bacterial cells and silver grains. In our MAR preparations the background fluorescence with blue excitation was minimal and did not interfere with the CARD-FISH staining (Fig. 7). The filter pores did not present an obstacle to the visualization of MAR grains by bright-field illumination (Fig. 7) if (i) the aperture and field stops were sufficiently opened and (ii) the condenser was slightly misaligned from Köhler illumination to decrease contrast. Such empirical optimization of bright-field illumination is comparable to adjusting optimal Köhler illumination.

In this context we also compared MAR preparations on normal glass slides and on Cyto-Clear slides (GE Osmonics, Minnetonka, Minn.). These slides make polycarbonate filters nearly optically transparent with bright-field illumination (34). No substantial enhancement in the quality of the microscopic images was observed when Cyto-Clear slides were used. However, the quality of DAPI staining was indeed lower after MAR than after CARD-FISH alone. This was not a critical problem, since the fractions of hybridized cells could be readily determined before the actual MAR procedure was performed. Other DNA stains, such as SYBR Green I (28), might result in reduced background values for counts of total MAR-active cells.

In addition, our method also allowed us to perform the MAR procedure before the actual FISH staining (Table 1). While this is not important if substrates are labeled with ^{14}C or ^{3}H, it may be a great advantage for preserving the activity signal when isotopes with relatively short half-lives are used (e.g., in studies of the incorporation of dimethylsulfoniopropionate labeled with ^{35}S) (52).

Anoxic glucose incorporation as an indication of facultatively anaerobic metabolism. Our results suggest that a substantial fraction of microbes in coastal North Sea surface waters are capable of substrate uptake in the absence of oxygen. During 4-h incubations in August, equal fractions of bacterial cells incorporated glucose under anoxic and oxic conditions (Fig. 3). Thus, it seems likely that the slight increase in the ambient pH due to the oxygen removal procedure did not negatively affect microbial viability.

The ability to incorporate glucose in the absence of oxygen was present in bacterioplankton populations belonging to different phylogenetic lineages (Fig. 3 and 6). At our level of analysis, strictly oxic uptake was not observed in any of the groups studied, and some bacterial lineages preferentially incorporated the tracer under anoxic conditions (Fig. 8). Glucose uptake is an active transport mechanism, and there must be a simultaneous process to provide the required energy (e.g., fer-

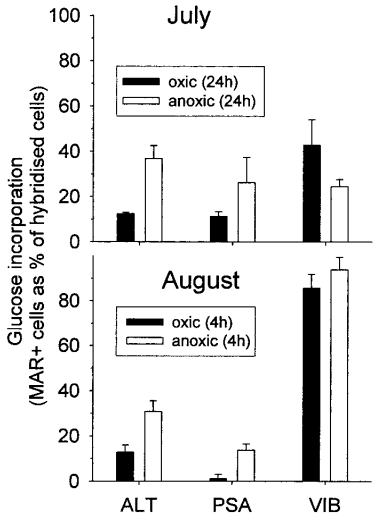

FIG. 6. Fractions of cells with visible tracer uptake (MAR+) affiliated with the γ-proteobacterial genera *Alteromonas* (ALT), *Pseudoalteromonas* and *Colwellia* (PSA), and *Vibrio* (VIB) in oxic and anoxic incubations (4 h) and enrichments (24 h).

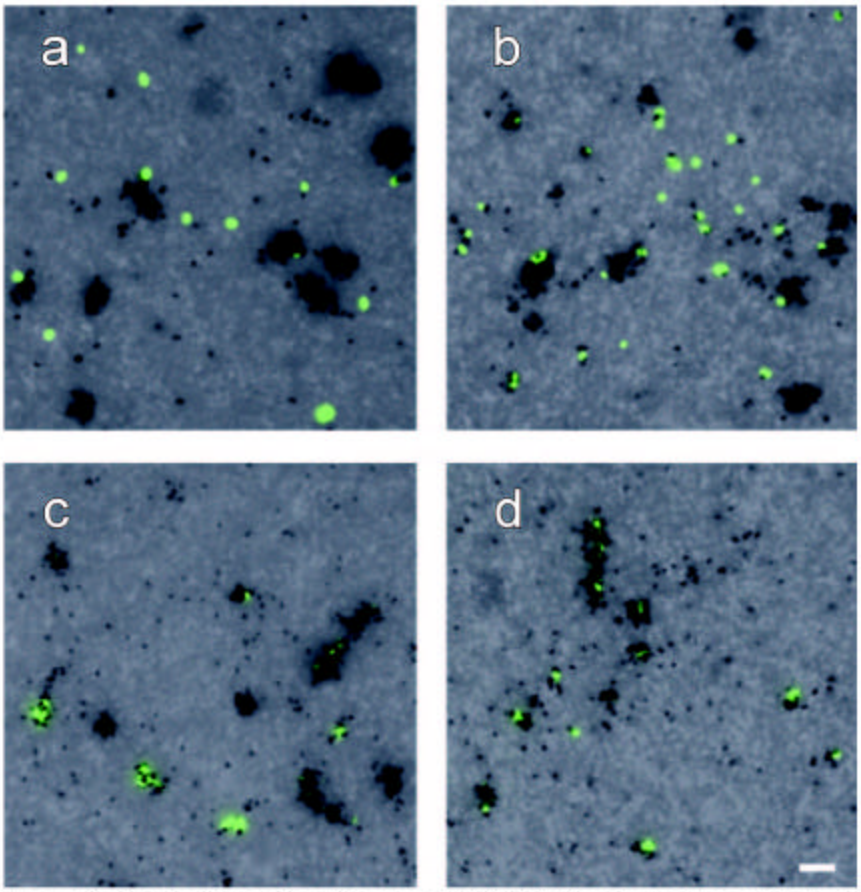

FIG. 7. Photomicrographs of hybridized bacteria from 24-h enrichments (July) with different levels of tracer uptake after MAR and CARD-FISH. (a and b) Probe ALT1413 (*Alteromonas* spp.) under oxic (a) and anoxic (b) conditions. (c and d) Probe ROS537 (members of the *Roseobacter* clade) under oxic (c) and anoxic (d) conditions.

mentation). In addition, some planktonic bacteria might be able to use electron acceptors other than oxygen for respiration. Isolates related to the *Alteromonadaceae*, *Vibrionaceae*, and *Roseobacter* spp. are capable of denitrification (14, 23, 41). However, the median monthly nitrate concentrations at the Helgoland Roads sampling station in July and August 2003 were <5 μmol liter^{-1} (MURSYS-report [www.bsh.de]). These concentrations were probably too low to explain the growth of these groups during the 24-h enrichments (Fig. 2 and 4).

Facultatively anaerobic marine γ-proteobacteria. The observed enrichment and high levels of tracer incorporation in *Vibrio* spp. under anoxic conditions (Fig. 6) support the interpretation that our experimental setup tested for facultatively anaerobic metabolism. Marine *Vibrio* spp. are known for their ability to ferment sugars in the absence of oxygen (23). Surprisingly, a higher fraction of cells that hybridized with probes ALT1412 and PSA184 exhibited glucose incorporation under anoxic conditions than under oxic conditions (Fig. 6). The genus *Alteromonas* was originally defined on the basis of a col-

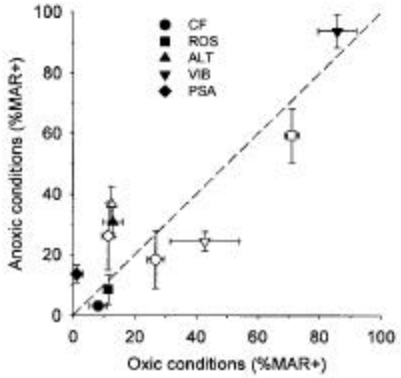

FIG. 8. Comparison of glucose incorporation under oxic and anoxic conditions. Open symbols, July, 4-h incubations; solid symbols, August, 24-h enrichments. ROS, *Roseobacter* clade; CF, *Cytophaga-Flavobacterium* cluster; ALT, *Alteromonas*; VIB, *Vibrio*; PSA, *Pseudoalteromonas-Colwellia*; MAR+, fraction of cells with visible tracer uptake.

lection of predominantly aerobic gram-negative isolates (5), and the described isolates belonging to the genera *Pseudoalteromonas* and *Colwellia* are also known as obligately aerobic bacteria (38, 54). However, recently Riemann and Azam (37) reported a potential for anaerobic metabolism in several strains that were affiliated with the *Alteromonadaceae*, including a close relative of *Alteromonas macleodii*. 16S rRNA sequence types related to *Pseudoalteromonas* spp. have been obtained from the anoxic zone of the Cariaco Basin (24) and from the suboxic layers overlying the sulfide-rich deeper waters of the Black Sea (51). This suggests that the well-studied, strictly aerobic isolates from this family might not be ecophysiologically representative of *Alteromonas* and *Pseudoalteromonas* spp. in the marine water column.

Glucose uptake by members of the *Roseobacter* clade. In July, a high fraction of microbes affiliated with the *Roseobacter* clade showed incorporation of radiolabeled glucose. They accounted for <10% of the total picoplankton but >25% of all bacteria that took up detectable amounts of tracer within 4 h during aerobic incubation. In contrast, only a minor fraction of cells belonging to the *Roseobacter* clade was able to incorporate glucose in August, although the total amounts of such bacteria were similar on the two sampling dates (Fig. 2). This suggests that the activities of pelagic microbes are substantially more dynamic than their population sizes and that a small number of active cells can be responsible for a significant fraction of the turnover of particular substrates. The *Roseobacter* clade as defined by the probe ROS537 harbors a number of subgroups with strikingly different physiological properties (41, 43, 53). Thus, the observed changes in activity might also reflect a succession of species between the sampling dates.

Bacteria belonging to the *Roseobacter* clade are widely distributed in the temperate oceans (17, 40), and some microbes related to the *Roseobacter* group are readily enriched and isolated from marine waters (13, 18). Planktonic members of this group are known to be major consumers of the algal osmolyte dimethylsulfoniopropionate, as observed, e.g., during a coccolithophore bloom in the North Sea (55). Substantial fractions of 16S rRNA gene sequence types and isolates from nonaxenic cultures of marine algae are affiliated with this lineage (35, 39). This close association of bacteria belonging to the *Roseobacter* lineage with primary producers agrees with the seasonally high numbers of tracer-incorporating cells (Fig. 3). In marine waters glucose originates mainly from the degradation of phytoplankton-derived exudates (20).

A large fraction of cells related to the *Roseobacter* group incorporated the tracer under anoxic conditions during the July 24-h enrichments (Fig. 5 and 7). The potential for anoxic glucose uptake may shed new light on the ecology of members of the *Roseobacter* clade in coastal surface waters. Although this clade comprises bacteria with very different physiological capacities (e.g., heteroorganotrophs, anoxygenic phototrophs, sulfite oxidizers, and denitrifiers) (41, 43, 53), no facultatively fermentative strains have been described. Recently, representatives of a new genus belonging to this group have been isolated from the German Bight (53), but these strains are also strictly aerobic heterotrophs.

Ecological role of facultatively anaerobic water column bacteria. The apparently widespread ability to take up glucose under anoxic conditions (Fig. 8) raises the question of why this is so common in bacteria from fully aerated marine surface waters. We support the argument of Riemann and Azam that this feature likely is an adaptation to growth in nutrient-rich microenvironments in which oxygen is periodically depleted (37). Zooplankton fecal pellets seasonally constitute the most common type of particulate organic matter in the coastal North Sea (50), and carbon from chitinous particles may account for a substantial fraction of bacterial production in coastal marine habitats (21). In contrast to entirely alga-derived marine snow, such compact particles might represent a habitat in which oxygen fluxes are sufficiently low to allow temporary anoxia (1), although this is disputed by other authors (42). *Vibrio* spp. are commonly found in fecal pellets of copepods (19), and some strains produce specific proteins to adhere to chitin (27). Isolates related to *Alteromonas* and *Pseudoalteromonas* readily colonize and degrade chitinous particles (4, 46), and a large fraction of the cells of members of the *Cytophaga-Flavobacterium* cluster from a coastal pelagic assemblage were able to incorporate chitin degradation products (10).

Even if marine snow aggregates do not become permanently anoxic while they are freely suspended, their sedimentation times are probably rather short. The average depth of the German Bight is only 20 m, and suspended particles and fecal pellets may sink through the water column at speeds of 100 m or more per day (48). Thus, bacteria associated with aggregated senescent algae (e.g., bacteria belonging to the *Roseobacter* lineage) might experience temporary anoxia while they are on the sediment surface. Subsequent reintroduction of such bacteria into the water column could be induced by periodic vertical mixing and resuspension of particulate organic matter (25). Altogether, the observed anaerobic uptake of glucose by different pelagic bacteria (Fig. 8) suggests that there might be a substantial overlap among the microbial assemblages that inhabit the water column, organic particles, and the sediment surfaces in shallow coastal systems.

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REFERENCES

1. **Alldredge, A. L., and Y. Cohen.** 1987. Can microscale chemical patches persist in the sea—microelectrode study of marine snow, fecal pellets. Science **235**:689–691.
2. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143–169.
3. **Azam, F.** 1998. Microbial control of oceanic carbon flux: the plot thickens. Science **280**:694–696.
4. **Baty, A. M., C. C. Eastburn, Z. Diwu, S. Techkarnjanaruk, A. E. Goodman, and G. G. Geesey.** 2000. Differentiation of chitinase-active and non-chitinase-active subpopulations of a marine bacterium during chitin degradation. Appl. Environ. Microbiol. **66**:3566–3573.
5. **Baumann, L., P. Baumann, M. Mandel, and R. D. Allen.** 1972. Taxonomy of aerobic marine eubacteria. J. Bacteriol. **110**:402–429.
6. **Bianchi, M., D. Marty, J. L. Teyssie, and S. W. Fowler.** 1992. Strictly aerobic and anaerobic bacteria associated with sinking particulate matter and zooplankton fecal pellets. Mar. Ecol. Prog. Ser. **88**:55–60.
7. **Brock, T. D.** 1967. Bacterial growth rate in the sea—analysis by thymidine autoradiography. Science **155**:81–83.

8. **Carpenter, J. H.** 1965. The Chesapeake Bay Institute technique for the Winkler dissolved oxygen method. Limnol. Oceanogr. **10:**141–143.
9. **Cottrell, M. T., and D. L. Kirchman.** 2003. Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. Limnol. Oceanogr. **48:**168–178.
10. **Cottrell, M. T., and D. L. Kirchman.** 2000. Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. **66:**1692–1697.
11. **Daims, H., A. Bruhl, R. Amann, K. H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434–444.
12. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.
13. **Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts, and R. Amann.** 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. Appl. Environ. Microbiol. **67:**5134–5142.
14. **Gauthier, G., M. Gauthier, and R. Christen.** 1995. Phylogenetic analysis of the genera *Alteromonas, Shewanella,* and *Moritella* using genes coding for small-subunit ribosomal RNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of 12 new species combinations. Int. J. Syst. Bacteriol. **45:** 755–761.
15. **Giuliano, L., E. De Domenico, M. G. Höfle, and M. M. Yakimov.** 1999. Identification of culturable oligotrophic bacteria within naturally occurring bacterioplankton communities of the Ligurian Sea by 16S rRNA sequencing and probing. Microb. Ecol. **37:**77–85.
16. **Glöckner, F. O., B. M. Fuchs, and R. Amann.** 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. **65:**3721–3726.
17. **Gonzalez, J. M., and M. A. Moran.** 1997. Numerical dominance of a group of marine bacteria in the alpha-subclass of the class *Proteobacteria* in coastal seawater. Appl. Environ. Microbiol. **63:**4237–4242.
18. **Gonzalez, J. M., W. B. Whitman, R. E. Hodson, and M. A. Moran.** 1996. Identifying numerically abundant culturable bacteria from complex communities: an example from a lignin enrichment culture. Appl. Environ. Microbiol. **62:**4433–4440.
19. **Hansen, B., and G. Bech.** 1996. Bacteria associated with a marine planktonic copepod in culture. 1. Bacterial genera in seawater, body surface, intestines and fecal pellets and succession during fecal pellet degradation. J. Plankton Res. **18:**257–273.
20. **Ittekkot, V., U. Brockmann, W. Michaelis, and E. T. Degens.** 1981. Dissolved free and combined carbohydrates during a phytoplankton bloom in the northern North Sea. Mar. Ecol. Prog. Ser. **4:**299–305.
21. **Kirchman, D. L., and J. White.** 1999. Hydrolysis and mineralization of chitin in the Delaware Estuary. Aquat. Microb. Ecol. **18:**187–196.
22. **Lee, N., P. Nielsen, K. Andreasen, S. Juretschko, J. Nielsen, K. Schleifer, and M. Wagner.** 1999. Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. **65:**1289–1297.
23. **Macian, M. C., W. Ludwig, K. H. Schleifer, M. J. Pujalte, and E. Garay.** 2001. *Vibrio agarivorans* sp. nov., a novel agarolytic marine bacterium. Int. J. Syst. Evol. Bacteriol. **51:**2031–2036.
24. **Madrid, V. M., G. T. Taylor, M. I. Scranton, and A. Y. Chistoserdov.** 2001. Phylogenetic diversity of bacterial and archaeal communities in the anoxic zone of the Cariaco Basin. Appl. Environ. Microbiol. **67:**1663–1674.
25. **McCandliss, R. R., S. E. Jones, M. Hearn, R. Latter, and C. F. Jago.** 2002. Dynamics of suspended particles in coastal waters (southern North Sea) during a spring bloom. J. Sea Res. **47:**285–302.
26. **Meyer-Reil, L. A.** 1978. Autoradiography and epifluorescence microscopy combined for determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. **36:**506–512.
27. **Montgomery, M. T., and D. L. Kirchman.** 1993. Role of chitin-binding proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. Appl. Environ. Microbiol. **59:**373–379.
28. **Noble, R. T., and J. A. Fuhrman.** 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat. Microb. Ecol. **14:**113–118.
29. **Ouverney, C. C., and J. A. Fuhrman.** 1999. Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. **65:**1746–1752.
30. **Pernthaler, A., J. Pernthaler, and R. Amann.** 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. **68:**3094–3101.
31. **Pernthaler, J., A. Pernthaler, and R. Amann.** 2003. Automated enumeration of groups of marine picoplankton after fluorescence in situ hybridization. Appl. Environ. Microbiol. **69:**2631–2637.
32. **Ploug, H., H. P. Grossart, F. Azam, and B. B. Jorgensen.** 1999. Photosynthesis, respiration, and carbon turnover in sinking marine snow from surface waters of Southern California Bight: implications for the carbon cycle in the ocean. Mar. Ecol. Prog. Ser. **179:**1–11.
33. **Ploug, H., M. Kühl, B. Buchholz Cleven, and B. B. Jörgensen.** 1997. Anoxic aggregates: an ephemeral phenomenon in the pelagic environment? Aquat. Microb. Ecol. **13:**285–294.
34. **Posch, T., J. Pernthaler, A. Alfreider, and R. Psenner.** 1997. Cell-specific respiratory activity of aquatic bacteria studied with the tetrazolium reduction method, cyto-clear slides, and image analysis. Appl. Environ. Microbiol. **63:** 867–873.
35. **Prokic, I., F. Brümmer, T. Brigge, G. H. D., G. Gerdts, C. Schütt, M. Elbrächter, and M. E. G. Müller.** 1998. Bacteria of the genus *Roseobacter* associated with the toxic dinoflagellate *Prorocentrum lima*. Protist **149:**347–357.
36. **Revsbech, N. P.** 1989. An oxygen microsensor with a guard cathode. Limnol. Oceanogr. **34:**474–478.
37. **Riemann, L., and F. Azam.** 2002. Widespread N-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. Appl. Environ. Microbiol. **68:**5554–5562.
38. **Sawabe, T., H. Makino, M. Tatsumi, K. Nakano, K. Tajima, M. M. Iqbal, I. Yumoto, Y. Ezura, and R. Christen.** 1998. *Pseudoalteromonas bacteriolytica* sp. nov., a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica*. Int. J. Syst. Bacteriol. **48:**769–774.
39. **Schäfer, H., B. Abbas, H. Witte, and G. Muyzer.** 2002. Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiol. Ecol. **42:**25–35.
40. **Selje, N., M. Simon, and T. Brinkhoff.** 2004. A newly discovered *Roseobacter* cluster in temperate and polar oceans. Nature **427:**445–448.
41. **Shiba, T.** 1991. *Roseobacter litoralis* gen. nov., sp. nov., and *Roseobacter denitrificans* sp. nov., aerobic pink pigmented bacteria which contain bacteriochlorophyll a. Syst. Appl. Microbiol. **14:**140–145.
42. **Simon, M., H. P. Grossart, B. Schweitzer, and H. Ploug.** 2002. Microbial ecology of organic aggregates in aquatic ecosystems. Aquat. Microb. Ecol. **28:**175–211.
43. **Sorokin, D. Y.** 1995. *Sulfitobacter pontiacus* gen. nov., sp. nov.—a new heterotrophic bacterium from the Black Sea, specialized on sulfite oxidation. Microbiology **64:**295–305.
44. **Tabor, P. S., and R. A. Neihof.** 1982. Improved micro-auto-radiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl. Environ. Microbiol. **44:**945–953.
45. **Teira, E., T. Reinthaler, A. Pernthaler, J. Pernthaler, and G. J. Herndl.** 2004. Combining catalyzed reporter deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. Appl. Environ. Microbiol. **70:**4411–4414.
46. **Tsujibo, H., H. Orikoshi, N. Baba, M. Miyahara, K. Miyamoto, M. Yasuda, and Y. Inamori.** 2002. Identification and characterization of the gene cluster involved in chitin degradation in a marine bacterium, *Alteromonas* sp. strain O-7. Appl. Environ. Microbiol. **68:**263–270.
47. **Turley, C. M., and P. J. Mackie.** 1994. Biogeochemical significance of attached and free-living bacteria and the flux of particles in the NE Atlantic Ocean. Mar. Ecol. Prog. Ser. **115:**191–203.
48. **Turner, J. T.** 2002. Zooplankton fecal pellets, marine snow and sinking phytoplankton blooms. Aquat. Microb. Ecol. **27:**57–102.
49. **Urakawa, H., K. Kita-Tsukamoto, S. E. Steven, K. Ohwada, and R. R. Colwell.** 1998. A proposal to transfer *Vibrio marinus* (Russell 1891) to a new genus, *Moritella* gen. nov., as *Moritella marina* comb. nov. FEMS Microbiol. Lett. **165:**373–378.
50. **Urban-Rich, J., E. Nordby, I. J. Andreassen, and P. Wassmann.** 1999. Contribution by mesozooplankton fecal pellets to the carbon flux on Nordvestbanken, north Norwegian shelf in 1994. Sarsia **84:**253–264.
51. **Vetriani, C., H. V. Tran, and L. J. Kerkhof.** 2003. Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. Appl. Environ. Microbiol. **69:**6481–6488.
52. **Vila, M., R. Simo, R. P. Kiene, J. Pinhassi, J. A. Gonzalez, M. A. Moran, and C. Pedros-Alio.** 2004. Use of microautoradiography combined with fluorescence in situ hybridization to determine dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa. Appl. Environ. Microbiol. **70:** 4648–4657.
53. **Wagner-Döbler, I., H. Rheims, A. Felske, R. Pukall, and B. J. Tindall.** 2003. *Jannaschia helgolandensis* gen. nov., sp nov., a novel abundant member of the marine *Roseobacter* clade from the North Sea. Int. J. Syst. Evol. Bacteriol. **53:** 731–738.
54. **Yumoto, I., K. Kawasaki, H. Iwata, H. Matsuyama, and H. Okuyama.** 1998. Assignment of *Vibrio* sp. strain ABE-1 to *Colwellia maris* sp. nov., a new psychrophilic bacterium. Int. J. Syst. Bacteriol. **48:**1357–1362.
55. **Zubkov, M. V., B. M. Fuchs, S. D. Archer, R. P. Kiene, R. Amann, and P. A. Burkill.** 2001. Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulfoniopropionate in an algal bloom in the North Sea. Environ. Microbiol. **3:**304–311.

Publications

Concentration-dependent patterns of leucine incorporation in coastal picoplankton

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Summary

Coastal pelagic environments are characterized by concentration gradients of dissolved organic carbon at a micro scale and by pronounced seasonal differences in substrate availability for the heterotrophic picoplankton. Microbial taxa that co-exist in such habitats might thus differ in their ability to incorporate substrates at various concentrations. We investigated uptake patterns of leucine in four microbial lineages from the coastal North Sea at concentrations between 0.1 and 100 nM before and during a spring phytoplankton bloom. Community bulk uptake rates and the fraction of leucine-incorporating cells in the different populations were analyzed. Significantly less bacterial cells incorporated the amino acid before (13-35%) than during (23-47%) the bloom at all but the highest concentration. In the pre-bloom situation the incorporation rate per active cell was constant above 0.1 nM of added leucine, whereas it steeply increased with substrate concentration during the bloom. At both timepoints, a high proportion of members of the *Roseobacter* clade incorporated leucine at all concentrations (55-80% and 86-94%, respectively). In contrast, the fractions of leucine-incorporating cells substantially increased with substrate availability in bacteria from the SAR86 clade (8-31%) and from the DE cluster 2 of the *Flavobacteria-Sphingobacteria* (14-33%). Uptake patterns of marine *Euryarchaeota* were in between these extremes (30-56% and 48-70%). Our results suggest that the contribution of microbial taxa to the turnover of particular substrates may be concentration-dependent. This may help to understand the specific niches of co-existing populations that appear to compete for the same resources.

Introduction

The pelagic environment is heterogeneous with respect to the small-scale distribution of dissolved organic molecules. Short-lived point sources of dissolved organic matter (DOM) may arise directly, e.g., from phytoplankton release (7), viral lysis of algal cells (9) and zooplankton feeding (32), or indirectly from the ecto-enzymatic hydrolytic activity of particle-associated microbes (23). In particular, the dissolved free amino acids (DFAA) may account for a substantial fraction of microbial carbon demand in aquatic systems (19, 20), and they also represent an energetically favorable source of nitrogen (59).

Bulk DFAA concentrations in seawater are typically low, as set by the high uptake affinities of pelagic microorganisms [(54) and references therein]. Moreover, their concentrations in coastal temperate waters are maintained within a narrow range regardless of high variability of primary production, likely due to a tight coupling between DFAA release and microbial consumption (20, 27). *In situ* measurements of amino acids uptake (54) as well as studies on isolates (50) indicate that marine bacteria may harbor several uptake systems for DFAA with different kinetic parameters that provide a means of enhanced incorporation over a broad range of substrate concentration. In addition, the assimilation rates of amino acids by microbial communities can rapidly change, e.g., during the course of a phytoplankton bloom (56).

Although the importance of DOM mineralization by picoplankton has been recognized for decades (5), the relative contributions of the various phylogenetic groups to the consumption of particular DOM fractions in the sea are currently subject of intense investigation (13, 34, 58, 59). It is theoretically conceivable that some microbial species in pelagic habitats may not be capable of incorporating monomeric substrates such as DFAA at all. Alternatively, it is also possible that the

relative contributions of different picoplankton taxa to the total turnover of these substrates are governed by their specific uptake patterns (i.e., affinity, velocity, saturation). Such differences moreover might have pronounced effects on interspecific competition (10). Currently, it is unknown whether the above discussed shifts in community uptake kinetics with changing substrate availability reflect the physiological plasticity of the dominant community members, a succession of specialized genotypes, or both. In the past years a promising combination of two single-cell methods has been established (microautoradiography and fluorescence in situ hybridization, MAR-FISH) that allows for a direct, semi-quantitative tracking of substrate incorporation by specific microbial populations in marine picoplankton (13, 38). Recent modifications of the approach have rendered it more suitable for work with aquatic microbes with low ribosome content (53), and for an accurate high-throughput sample processing (2).

We compared the *in situ* substrate uptake patterns of the pelagic microbial community in coastal North Sea surface waters in a late winter phytoplankton pre-bloom situation and during a spring bloom dominated by *Phaeocystis* sp. Specifically, we applied the MAR-FISH technique in combination with bulk uptake rates to investigate seasonal differences in the uptake patterns of leucine at various substrate concentrations by the whole assemblage and by different genotypic populations of free-living pelagic microbes.

Materials and Methods

Sampling site. Surface water samples (1 m depth) were collected on March 11 and May 6, 2004 at the sampling station Helgoland Roads (54°11'N, 7°54'E, water depth, 8 m), 50 km offshore in the German Bay of the North Sea. In March the surface water temperature was 4.2 °C and the density of phytoplankton was low. The algal community was dominated by diatoms, in particular Thalassiosira nitzschoides. In May the water temperature was 8.3°C. Phytoplankton biomass at the second sampling timepoint was more than 10 times higher. Although diatoms were still present, the phytoplankton community in May was dominated by colonies of the haptophyte Phaeocystis sp. (source: Mursys Umweltreport, www.bsh.de).

Incubations with [3H] leucine. Two sets of incubations were perfomed for the analysis of concentration-dependent leucine incorporation by the picoplankton assemblage. One set of samples -subsequently referred as "bulk incorporation"- was used to determine the rate of substrate incorporation by the total microbial community at different tracer concentrations. The other set served to track the tracer incorporation patterns of different populations by MAR-FISH. Both sets of samples were incubated with [3H] leucine (specific activity 2.26 TBq / mmol, Amersham Bioscience, Freiburg, Germany) in a concentration series (0.1 nM, 1 nM, 10 nM and 100 nM) within 1 h after sample collection. For every treatment type triplicate 10 ml subsamples plus one control (sea water fixed with paraformaldehyde) were incubated. The incubations were run for 4 hours in the dark at ambient water temperatures. Subsequently, freshly prepared buffered paraformaldehyde fixative was added to the incubations to a final concentration of 1%.

After 1 h of fixation at room temperature the samples were frozen for transport and stored at -20°C. The samples for bulk incorporation were filtered onto cellulose mixed

esters filters (type GSWP, pore size, 0.2 µm, diameter, 25 mm, Millipore, Eschborn, Germany) and rinsed twice with ice cold trichloroacetic acid and ethanol, (29). The samples for MAR-FISH analysis were filtered onto polycarbonate filters (type GTTP, pore size, 0.2 µm, diameter, 25 mm, Millipore, Eschborn, Germany) and rinsed twice with sterile phosphate buffered saline (PBS). All filters were stored at -20°C until further analysis.

Bulk measurements of substrate incorporation. The cellulose filters were placed in 6 ml scintillation vials and dissolved with 0.5 ml of ethyl acetate. Subsequently, 5 ml of Lumasafe plus scintillation cocktail (Lumac LSC BV, Groningen, The Netherlands) was added to each vial. The amount of radiolabeled substrate assimilated by microbial cells was measured in a scintillation counter (Packard TriCarb 2900 TR, Perkin Elmer, Wellesley, MA). Measurements were corrected for quench (external standard method), and by subtraction of counts from the prefixed controls.

Quantification of populations by FISH. Samples for the determination of the in situ abundance of the different bacterial populations were immediately fixed after collection with freshly prepared buffered paraformaldehyde at a final concentration of 1%. The abundances of different microbial taxa was determined by FISH with horseradish peroxidase labeled oligonucleotide probes and catalyzed reporter deposition (CARD-FISH) (40). In addition to probe EUBI-III (most Bacteria) (14), 4 specific probes were selected that targeted substantial populations (>1% of total counts) which also scored positively for substrate incorporation: EURY806 (marine group II Euryarchaeota) (53), ROS537 (members of the Roseobacter clade of?-Proteobacteria) (17), SAR86-1245 (members of the SAR86 clade?f?-Proteobacteria)

(16) and CF6-1267 (members of the DE cluster 2 of the Flavobacteria-
124 Sphingobacteria group of the phylum Bacteroidetes (31)). The EUB antisense probe NON338 (3) was used as negative control. All probes were purchased from
126 Biomers.net (Ulm, Germany). Counter-staining of CARD-FISH preparations was done with 4,6-diamidino-2-phenylindole (DAPI; final conc., 1 µg ml-1). Evaluation of DAPI
128 and FISH-stained cells was carried out manually, counting a minimum of 1000 DAPI cells per sample.

130

MAR-FISH analysis. A recently developed MAR-FISH protocol was applied to study
132 substrate uptake by specific picoplankton groups (2). The key features of this new protocol are the combination of CARD-FISH staining and microautoradiography of
134 microbes on membrane filters without a prior cell transfer onto glass slides and evaluation by semi-automated image acquisition and analysis. Triplicate samples
136 from every treatment type were analyzed by MAR-FISH with the different probes as described previously (2), and controls were evaluated with probe EUB I_III.
138 Altogether, 108 MAR-FISH stained preparations were evaluated. The photochemicals for MAR (autoradiography emulsion type NTB-2, developer type Dektol, fixer) were
140 purchased from Kodak (Eastman Kodak, Rochester, NY). Development of the exposed slides was performed according to the manufacturer's instructions (2 min of
142 development, 10 s of rinsing with deionized particle-free water [dH2O], 5 min exposure in fixer, washing for 5 min in dH2O). Different MAR exposure times were
144 used for the evaluation of different treatment types (Table 1). This strategy was chosen because we wanted to obtain the maximal number of MAR-positive cells at
146 the different tracer concentrations rather than to quantitatively compare tracer uptake at one exposure condition [e.g., by determination of grain size area (12)]. A fixed
148 exposure time in our experimental setup would have either resulted in the

underestimation of MAR-positive cells at low tracer concentration, or in the complete blackening of preparations at high tracer concentrations. Images of MAR-FISH preparations were captured with a digital camera (Orca I, Hamamatsu, Herrsching, Germany) mounted on a motorized epifluorescence microscope (Axioplan II Imaging, Carl Zeiss, Jena, Germany). Evaluation was performed as previously described (2) using the image analysis software KS400 (Carl Zeiss).

Statistical analysis. The following hypotheses were tested: (i) The fractions of MAR-positive Bacteria (as detected by probe EUB I-III) and MAR-positive cells affiliated with the SAR86 and Roseobacter clades and with marine Euryarchaeota were different between the two sampling times. (ii) The fractions of all MAR-positive Bacteria and of members of the SAR86 and Roseobacter clades, of the DE cluster 2 and of marine Euryarchaeota were different at the different tracer concentrations. This was tested separately for each probe-defined cell population (dependent variable) by two way linear analysis of variance of the sets of triplicate determinations (ANOVA, independent variables: concentrations, sampling timepoints), and by post-hoc pair wise multiple comparisons with Bonferroni corrections. For DE cluster 2, which was present only in May, hypothesis (ii) was tested by one way ANOVA (concentration) and post-hoc comparisons (Bonferroni's method). Calculations were performed using the software SigmaStat (SPSS Inc, Chicago, Ill.).

Results

Community contribution of the studied populations. Total cell numbers did not change substantially between both sampling dates: 0.8 (± 0.2) $*10^6$ cells ml^{-1} in March and 1.2 (± 0.4) $*10^6$ cells ml^{-1} in May. At both timepoints 84% of all DAPI-stained objects were detected by CARD-FISH (Table 2). *Euryarchaeota* constituted a constant fraction of total counts at both sampling dates. In May, the contribution of *Roseobacter* to all hybridized *Bacteria* was slightly higher. Members of the SAR86 clade formed a similar fraction of hybridized *Bacteria* and of total cell numbers at both months. Members of the DE cluster 2 of the *Flavobacteria-Sphingobacteria,* as targeted by the probe CF6-1267, were rare in March, and they represented 5% of hybridized *Bacteria* in May.

Incorporation of labeled substrate. Bulk incorporation rates of leucine were higher in May at tracer concentrations >0.1 nM (Fig. 1). In March the amount of incorporated leucine was similar at all concentrations whereas in May it steeply increased with increasing substrate concentration.

A significantly higher proportion of bacterial cells incorporated tritiated leucine in May than in March ($F=72.9$, $P<0.0001$), in particular at the lower substrate concentrations (interaction of variables date and concentration, $F=9.5$, $P<0.001$) (Fig. 2). The proportion of bacterial cells with visible tracer incorporation did not significantly increase at concentrations >10 nM in March and >1 nM in May (Bonferroni pair wise comparisons, $P>0.05$). Negative controls from pre-fixed samples yielded similar percentages of false-positive MAR-active cells irrespective of sampling timepoint, incubation and exposure conditions, approximately 1% of cells hybridized with probe EUB I-III (data not shown). In both months, the leucine incorporation rate per MAR-positive bacterial cell was five fold higher at 1 nM than at

196 0.1 nM of added tracer. In March this parameter did not further increase with concentration, whereas in May it increased by a factor of 30 across the total range of
198 concentrations (Fig.2).

The majority of members of the *Roseobacter* clade showed visible substrate
200 incorporation at all substrate concentrations (Fig. 3A). A higher fraction of cells from this population incorporated leucine in May than in March, as was observed for the
202 whole bacterial assemblage (Fig. 2). However, in contrast to *Bacteria* the proportion of MAR-positive cells affiliated to *Roseobacter* was already high at the lowest tracer
204 concentration and there was no (May) or only little (March) increase in the fraction of *Roseobacter* with visibly incorporated tracer at increasing substrate concentration
206 (Fig 3A). Although members of the *Roseobacter* clade only constituted 8% and 12% of total hybridized cells in March and May, respectively (Table 2), these bacteria
208 represented >30% and almost 50% of all MAR-positive *Bacteria* at the lowest tracer concentration (Fig. 3B).

210 Members of the DE cluster 2 of *Flavobacteria-Sphingobacteria* were only present in May. The number of cells with visible tracer incorporation increased with
212 substrate concentration (Fig. 4). The percentage of MAR-positive cells approximately doubled between concentrations of 1 and 10 nM, and their respective proportions
214 were very similar at the lower two and at the higher two tracer concentrations (Fig. 4). The fraction of cells from the SAR 86 clade that incorporated the radiolabeled
216 substrate also increased with increasing tracer concentration (Fig 4). In contrast to *Bacteria* and to the other studied populations, the proportion of MAR-positive SAR 86
218 cells at a given tracer concentration was very similar at the two sampling timepoints. The only exception to this was at 1 nM of tracer concentration, where the percentage
220 of MAR positive cells was significantly higher in May (Fig 4) (Bonferroni pair wise comparisons, $P>0.05$). A high proportion of cells from the marine group II

222 *Euryarchaeota* was able to incorporate leucine at all tracer concentrations (Fig 5). As observed for *Bacteria*, this fraction was significantly higher in May, when up to 70% of
224 all *Euryarchaeota* were also MAR positive (Fig 5). The percentage of cells incorporating leucine significantly increased at substrate concentrations >1 nM in
226 March and >0.1 nM in May.

The proportion of cells with visible leucine incorporation was two- to fourfold
228 higher in members of the *Roseobacter* clade than in hybridized *Bacteria* (Fig 6), and it was on average twice as high in *Euryarchaeota* (Fig. 6). This was particularly
230 pronounced in March, and at the lowest substrate concentrations. By contrast, the fraction of leucine-incorporating cells in bacteria affiliated with the SAR 86 and DE
232 cluster 2 was significantly below the community average at the three lower substrate concentrations. It matched the average fraction of all MAR-positive *Bacteria* at
234 leucine concentrations of 100 nM (Fig. 6).

236 Discussion

Substrate incorporation by the whole microbial assemblage. On average,
238 approximately 30% of all bacterial cells in our samples were able to incorporate leucine. This has been suggested to be a typical value for aquatic systems [e.g., (12),
240 for a review on the "active" microbial fraction see (52)]. Moreover, we also demonstrate that the fraction of cells that are "active" with respect to uptake of a
242 radiolabeled amino acid may be related to the substrate concentration (Fig. 2), in particular if specific populations are investigated. If we had used only one tracer
244 concentration, we would have concluded that 10% (1nM), 20% (10nM), or 30% (100nM) of bacteria from the SAR86 clade are "active" in March (Fig. 4).

246 Our results agree with earlier findings that blooms of *Phaeocystis* in the North Sea may stimulate microbial utilization of monomeric substrates (33). At the

248 concentrations of radiotracer typically used in investigations of microbial productivity
(1-10 nM) both the proportion of MAR-positive prokaryotic cells (Fig. 2, 5) and the
250 rate of leucine incorporation (Fig. 1) were significantly higher during the bloom than
before. This stimulation of activity has been assigned to phytoplankton excretion of
252 small organic molecules that are directly usable by the heterotrophic picoplankton
(33). Specifically, the release of amino acids may be enhanced during spring
254 phytoplankton blooms (11). In addition, higher water temperatures in May might also
have stimulated leucine uptake rates (11, 27).

256 In the pre-bloom situation the leucine incorporation rate per MAR-positive
microbial cell remained constant at tracer concentrations of 1 nM and above,
258 whereas it steeply increased over the whole range of offered substrate
concentrations in May (Fig. 2). This suggests a transition from an "oligotrophic" (high
260 affinity) to a more "copiotrophic" (high velocity) substrate uptake pattern of the
microbial assemblage, as previously observed during a spring bloom in the arctic
262 Chukchi Sea (56). The uptake rates of leucine per MAR-positive cell were very similar
at the lower two concentrations (0.1-1 nM) in both months (Fig. 2). However, in May
264 substantially more cells could incorporate the substrate at 1 nM, indicating a more
widespread ability of leucine incorporation during the Phaeocystis bloom, even at low
266 concentrations. Moreover, in May these "active" bacteria (and possibly also
Euryarchaeota) were able to incorporate higher amounts of this substrate as
268 indicated by the bulk uptake per active cell and the MAR-FISH shorter exposure
times (Fig 2, Table 1). Our observations might help to explain how the tight coupling
270 between the production and consumption of dissolved free amino acids in marine
waters (20, 27) is realized at the level of microbial ecophysiology.

272

Substrate uptake patters of single picoplankton groups. Incorporation of tritiated leucine was observed in all studied prokaryotic populations at all substrate concentrations (Figs. 3-5). However, there were clear differences in the concentrations beyond which the fraction of MAR positive cells within a group did not significantly increase. At least two distinct patterns of tracer incorporation were present. For one, a high fraction of cells from the *Roseobacter* clade were involved in leucine uptake in May irrespective of the substrate concentration (Fig. 3A). In contrast, an increasing number of MAR-positive cells were observed in bacteria affiliated to the SAR86 clade and DE cluster 2 with increasing tracer concentration (Fig. 4). *Euryarchaeota* (and *Roseobacter* during the pre-bloom situation) ranged in between these extremes, i.e. the relationship between the proportion of MAR-positive cells and substrate concentration was weak but significant (Figs. 3A, 5). Interestingly, none of the studied bacterial populations displayed the pattern of leucine-incorporating cells that was characteristic for the bacterial assemblage as a whole (Figs. 2, 3A, 4). This may indicate that the pattern observed at the community level was the result of summing up the contrasting patterns of different microbial species. However, the three bacterial groups examined here only accounted for 25 to 50 percent of all MAR-positive bacterial cells. Therefore, it cannot be excluded that other populations more closely resembled the "average" uptake pattern.

Members of the *Roseobacter* clade are often associated with natural or induced phytoplankton blooms (43, 47, 57). These bacteria can be colonizers of diatoms (24) and of algal-derived detrital particles (46, 47). They were found in the phycosphere of dinoflagellates (44) and as "satellite" bacteria in diatom cultures (48). Thus, some members of the *Roseobacter* lineage are probably dependent on organic compounds released by the phytoplankton. However, it has also been suggested that bacteria from this clade might be highly competitive at low nutrient conditions (42),

and *Roseobacter* spp. have been isolated on oligotrophic media and from filtered seawater (17, 21, 42). Our data might help to reconcile these contrasting observations. Free-living members of the *Roseobacter* lineage in coastal North Sea waters appeared to be good competitors for monomers such as leucine across a range of concentrations (Fig. 6). In May, an equal fraction of cells from this group incorporated the tracer at 0.1 nM and 100 nM (Fig. 3A), indicating that these bacteria are well adapted to thrive in habitats with high microscale variability of substrate concentrations (4, 28). Alternatively, it is conceivable that physiologically distinct subpopulations could have been responsible for tracer uptake in our samples at different substrate concentrations. The phylogenetic and metabolic variability inside this group (as defined by probe ROS537) is high, e.g., some genotypes can be free-living and others are preferentially attached to particles (18, 24). However, the constantly high percentage of MAR-positive cells at all substrate concentrations (Fig. 3A) indicates that the studied *Roseobacter* population behaved as a single ecophysiological unit at least with respect to leucine assimilation.

SAR86 is a phylogenetic lineage of free-living bacteria that are present at various depths and trophic conditions both in coastal and offshore waters (1, 16, 36). These bacteria have not been found in association with algae cultures (24, 43, 48), but they were present during a bloom of *Emiliania huxleyi* in the North Atlantic (22). In the German Bight, SAR86 bacteria grew more rapidly in late summer than during spring (39). The percentage of leucine-assimilating cells from the SAR86 group was below the community average at all but the highest concentration (Fig. 6). This suggests that SAR86-related bacteria are competitive for leucine only if its supply is high, and that they might not be specialized for uptake of low concentrations of amino acids and other monomers, e.g., glucose (2). This conclusion is also supported by the similar leucine uptake patterns of members of the SAR86 clade during the pre-

bloom and bloom situation (Fig. 4). We thus hypothesize that the activity and
326 abundance of this bacterial group is not primarily governed by the availability of algae derived monomeric substrates.

328 The marine DE cluster 2 is a monophyletic group of highly similar (>99%) 16S rRNA sequence types closely related to genera such as *Geldibacter* and
330 *Salgentibacter*. It was first described from the Delaware estuary (31). Bacteria from this lineage represented 20 to 50% of all *Flavobacteria-Sphingobacteria* the estuary
332 and in the Chukchi Sea. Members of the DE cluster 2 also occurred at considerable densities in the coastal North Sea (Table 2). While the three habitats arguably differ
334 in many respects, they also have some common characteristics, e.g., seasonally high primary production, influence of river discharge and a high exchange between the
336 sediment and the water column (31, 45, 49, 55). Members of the DE cluster 2 were present only during the *Phaeocystis* bloom, whereas they were too rare for FISH
338 quantification in the pre-bloom situation (Table 2). This suggests that their occurrence may be related to the development of the phytoplankton assemblage. Other
340 members of the highly diverse *Flavobacteria-Sphingobacteria* group of the *Bacteroidetes* have been linked to natural (18) and artificial (24, 43, 47)
342 phytoplankton blooms, including *Phaeocystis*-dominated blooms (51). Bacteria are frequently encountered in nutrient-rich microenvironments, e.g., organic aggregates
344 (15, 46), attached or in close vicinity to algae (24, 47, 48). Numerous representatives from this lineage have been isolated on rich solid media [e.g., (8, 25)]. The
346 significantly higher number of MAR-positive cells from the DE cluster 2 at higher tracer concentrations (Fig. 4) might indicate that these bacteria require the rich
348 conditions provided by the phytoplankton bloom, but also that amino acids are not the most favored substrate source of these bacteria (as was argued above for SAR86).
350 Some free-living marine representatives of the *Flavobacteria-Sphingobacteria* group

preferably incorporated high molecular weight dissolved organic matter such as chitin and protein (13), and isolates from this group were able to degrade polymers (30).

The planktonic marine group II *Euryarchaeota* are a common element of microbial assemblages in coastal waters (35, 37, 41) and the open ocean (26, 53). In the upper layers of the central North Atlantic, 20 and 30% of *Euryarchaeota* cells assimilated aspartate and leucine, respectively (26, 53). In our samples, the relative abundance of MAR-positive *Euryarchaeota* was always higher than of *Bacteria*, and twice as high at the lowest substrate concentration (Fig. 6). Their activity moreover increased at higher substrate concentrations and during the phytoplankton bloom (Fig. 5). Thus, these microbes appear to be highly competitive for DFAA across a wide range of concentrations. Interestingly, marine *Euryarchaeota* have recently been shown to also incorporate radiolabeled bicarbonate (26). In fact, an increasing body of evidence suggests that mixotrophy may be a widespread feature of planktonic marine microorganisms, e.g. it is also found in *Prochlorococcus* (59) or in members of the SAR86 clade (6).

Conclusions. We show that several microbial populations that compete for the same substrate differed in their respective concentration-dependent uptake patterns. Thus, their coexistence might be favored by substrate patchiness at a microscale. A large fraction of substrate turnover might be carried out by few specialized bacteria (e.g., *Roseobacter*) at low substrate concentrations, whereas more taxa might participate in substrate utilization during phytoplankton blooms. This is deduced from the presence of a bacterial population (DE cluster 2) during the *Phaeocystis* bloom that was absent in the pre-bloom situation and that exhibited a lower apparent affinity for leucine than *Roseobacter* (i.e., a lower fraction of MAR-positive cells at low substrate concentrations). Our results moreover support the hypothesis that some

heterotrophic microbial populations in marine picoplankton may harbor several uptake systems that operate at different ranges of substrate concentration.

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References:

1. **Acinas, S. G., J. Antón, and F. Rodríguez-Valera.** 1999. Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Appl. Environ. Microbiol. **65:**514-522.
2. **Alonso, C., and J. Pernthaler.** 2005. Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. Appl. Environ. Microbiol. **71:**1709-1716.
3. **Amann, R. I.** 1995. *In situ* identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Molecular Microbial Ecology Manual **3.3.6.:**1-15.
4. **Azam, F.** 1998. Microbial control of oceanic carbon flux: the plot thickens. Science **280:**694-696.
5. **Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad.** 1983. The ecological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. **10:**257-263.
6. **Beja, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich, and E. F. DeLong.** 2000. Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. Science **289:**1902-1906.
7. **Bell, W. H., and E. Sakshaug.** 1980. Bacterial utilization of algal extracellular products .2. A kinetic study of natural populations. Limnol. Oceanogr. **25:**1021-1033.
8. **Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin.** 1997. Diversity and association of psychrophilic bacteria in Antarctic Sea ice. Appl. Environ. Microbiol. **63:**3068-3078.
9. **Brussaard, C. P. D., R. Riegman, A. A. M. Noordeloos, G. C. Cadee, H. Witte, A. J. Kop, G. Nieuwland, F. C. Vanduyl, and R. P. M. Bak.** 1995. Effects of grazing, sedimentation and phytoplankton cell lysis on the structure of a coastal pelagic food web. Mar. Ecol. Prog. Ser. **123:**259-271.
10. **Button, D. K.** 1991. Biochemical basis for whole-cell uptake kinetics - Specific affinity, oligotrophic capacity, and themeaning of the Michaelis constant. Appl. Environ. Microbiol. **57:**2033-2038.
11. **Coffin, R. B.** 1989. Bacterial uptake of dissolved free and combined amino acids in estuarine waters. Limnol. Oceanogr. **34:**531-542.
12. **Cottrell, M. T., and D. L. Kirchman.** 2003. Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. Limnol. Oceanogr. **48:**168-178.
13. **Cottrell, M. T., and D. L. Kirchman.** 2000. Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. **66:**1692-1697.

14. **Daims, H., A. Bruhl, R. Amann, K. H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434-444.
15. **DeLong, E. F., D. G. Franks, and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. **38:**924-934.
16. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044-3051.
17. **Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts, and R. Amann.** 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. Appl. Environ. Microbiol. **67:**5134-5142.
18. **Fandino, L. B., L. Riemann, G. F. Steward, R. A. Long, and F. Azam.** 2001. Variations in bacterial community structure during a dinoflagellate bloom analyzed by DGGE and 16S rDNA sequencing. Aquat. Microb. Ecol. **23:**119-130.
19. **Ferguson, R. L., and W. G. Sunda.** 1984. Utilization of amino acids by planktonic marine bacteria - Importance of clean technique and low substrate additions. Limnol. Oceanogr. **29:**258-274.
20. **Fuhrman, J. A.** 1987. Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach. Mar. Ecol. Prog. Ser **37:**45-52.
21. **González, J. M., and M. A. Moran.** 1997. Numerical dominance of a group of marine bacteria in the alpha-subclass of the class *Proteobacteria* in coastal seawater. Appl. Environ. Microbiol. **63:**4237-4242.
22. **González, J. M., R. Simo, R. Massana, J. S. Covert, E. O. Casamayor, C. Pedros-Alio, and M. A. Moran.** 2000. Bacterial community structure associated with a dimethylsulfoniopropionate-producing North Atlantic algal bloom. Appl. Environ. Microbiol. **66:**4237-4246.
23. **Grossart, H. P., S. Hietanen, and H. Ploug.** 2003. Microbial dynamics on diatom aggregates in Oresund, Denmark. Mar. Ecol. Prog. Ser. **249:**69-78.
24. **Grossart, H. P., F. Levold, M. Allgaier, M. Simon, and T. Brinkhoff.** 2005. Marine diatom species harbour distinct bacterial communities. Environ. Microbiol. **7:**860-873.
25. **Hagström, A., J. Pinhassi, and U. L. Zweifel.** 2000. Biogeographical diversity among marine bacterioplankton. Aquat. Microb. Ecol. **21:**231-244.
26. **Herndl, G. J., T. Reinthaler, E. Teira, H. van Aken, C. Veth, A. Pernthaler, and J. Pernthaler.** 2005. Contribution of *Archaea* to total prokaryotic production in the deep Atlantic Ocean. Appl. Environ. Microbiol. **71:***in press.*
27. **Hoppe, H. G., S. J. Kim, and K. Gocke.** 1988. Microbial decomposition in aquatic environments - Combined process of extracellular enzyme activity and substrate uptake. Appl. Environ. Microbiol. **54:**784-790.

28. **Kiorboe, T., and G. A. Jackson.** 2001. Marine snow, organic solute plumes, and optimal chemosensory behavior of bacteria. Limnol. Oceanogr. **46:**1309-1318.
29. **Kirchman, D.** 2001. Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments, p. 227-237, Methods in Microbiology, Vol 30, vol. 30.
30. **Kirchman, D. L.** 2002. The ecology of Cytophaga-Flavobacteria in aquatic environments. FEMS Microbiol. Ecol. **39:**91-100.
31. **Kirchman, D. L., L. Y. Yu, and M. T. Cottrell.** 2003. Diversity and abundance of uncultured Cytophaga-like bacteria in the Delaware Estuary. Appl. Environ. Microbiol. **69:**6587-6596.
32. **Lampert, W.** 1978. Release of Dissolved Organic-Carbon By Grazing Zooplankton. Limnol. Oceanogr. **23:**831-834.
33. **Lancelot, C., and G. Billen.** 1984. Activity of heterotrophic bacteria and its coupling to primary production during the spring phytoplankton bloom in the southern bight of the North Sea. Limnol. Oceanogr. **29:**721-730.
34. **Malmstrom, R. R., M. T. Cottrell, H. Elifantz, and D. L. Kirchman.** 2005. Biomass production and assimilation of dissolved organic matter by SAR11 bacteria in the Northwest Atlantic Ocean. Appl. Environ. Microbiol. **71:**2979-2986.
35. **Massana, R., A. E. Murray, C. M. Preston, and E. F. Delong.** 1997. Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara channel. Appl. Environ. Microbiol. **63:**50-56.
36. **Mullins, T. D., T. B. Britschgi, R. L. Krest, and S. J. Giovannoni.** 1995. Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol. Oceanogr. **40:**148-158.
37. **Murray, A. E., A. Blakis, R. Massana, S. Strawzewski, U. Passow, A. Alldredge, and E. F. DeLong.** 1999. A time series assessment of planktonic archaeal variability in the Santa Barbara Channel. Aquat. Microb. Ecol. **20:**129-145.
38. **Ouverney, C. C., and J. A. Fuhrman.** 1999. Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. **65:**1746-1752.
39. **Pernthaler, A., and J. Pernthaler.** 2005. Diurnal variation of cell proliferation in three bacterial taxa from coastal North Sea waters. Appl. Environ. Microbiol. **71:**4638-4644.
40. **Pernthaler, A., J. Pernthaler, and R. Amann.** 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. **68:**3094-3101.
41. **Pernthaler, A., C. M. Preston, J. Pernthaler, E. F. DeLong, and R. Amann.** 2002. A comparison of fluorescently labeled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. Appl. Environ. Microbiol.**:**661-668.

42. **Pinhassi, J., and T. Berman.** 2003. Differential growth response of colony-forming alpha- and gamma-proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. Appl. Environ. Microbiol. **69:**199-211.
43. **Pinhassi, J., M. M. Sala, H. Havskum, F. Peters, O. Guadayol, A. Malits, and C. L. Marrase.** 2004. Changes in bacterioplankton composition under different phytoplankton regimens. Appl. Environ. Microbiol. **70:**6753-6766.
44. **Prokic, I., F. Brummer, T. Brigge, H. D. Gortz, G. Gerdts, C. Schutt, M. Elbrachter, and W. E. G. Muller.** 1998. Bacteria of the genus Roseobacter associated with the toxic dinoflagellate Prorocentrum lima. Protist **149:**347-357.
45. **Radach, G.** 1992. Ecosystem functioning in the German Bight under continental nutrient inputs by rivers. Estuaries **15:**477-496.
46. **Rath, J., K. Y. Wu, G. J. Herndl, and E. F. DeLong.** 1998. High phylogenetic diversity in a marine-snow associated bacterial assemblage. Aquat. Microb. Ecol. **14:**261-269.
47. **Riemann, L., G. F. Steward, and F. Azam.** 2000. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. Appl. Environ. Microbiol. **66:**578-587.
48. **Schäfer, H., B. Abbas, H. Witte, and G. Muyzer.** 2002. Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiol. Ecol. **42:**25-35.
49. **Scheurle, C., D. Hebbeln, and P. Jones.** 2005. An 800-year reconstruction of Elbe River discharge and German Bight sea-surface salinity. Holocene **15:**429-434.
50. **Schut, F., E. J. De Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture growth maintenance and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. **59:**2150-2160.
51. **Simon, M., F. O. Glöckner, and R. Amann.** 1999. Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. Aquat. Microb. Ecol. **18:**275-284.
52. **Smith, E. M., and P. A. del Giorgio.** 2003. Low fractions of active bacteria in natural aquatic communities? Aquat. Microb. Ecol. **31:**203-208.
53. **Teira, E., T. Reinthaler, A. Pernthaler, J. Pernthaler, and G. J. Herndl.** 2004. Combining catalyzed reporter deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. Appl. Environ. Microbiol. **70:**4411-4414.
54. **Unanue, M., B. Ayo, M. Agis, D. Slezak, G. J. Herndl, and J. Iriberri.** 1999. Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. Microb. Ecol. **37:**36-48.

55. **Wild-Allen, K., A. Lane, and P. Tett.** 2002. Phytoplankton, sediment and optical observations in Netherlands coastal water in spring. Journal of Sea Research **47:**303-315.

56. **Yager, P. L., T. L. Connelly, B. Mortazavi, K. E. Wommack, N. Bano, J. E. Bauer, S. Opsahl, and J. T. Hollibaugh.** 2001. Dynamic bacterial and viral response to an algal bloom at subzero temperatures. Limnol. Oceanogr. **46:**790-801.

57. **Zubkov, M. V., B. M. Fuchs, S. D. Archer, R. P. Kiene, R. Amann, and P. A. Burkill.** 2001. Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulfoniopropionate in an algal bloom in the North Sea. Environ. Microbiol. **3:**304-311.

58. **Zubkov, M. V., B. M. Fuchs, P. H. Burkill, and R. Amann.** 2001. Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. Appl. Environ. Microbiol. **67:**5210-5218.

59. **Zubkov, M. V., B. M. Fuchs, G. A. Tarran, P. H. Burkill, and R. Amann.** 2003. High rate of uptake of organic nitrogen compounds by Prochlorococcus cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Appl. Environ. Microbiol. **69:**1299-1304.

Table 1: Optimal autoradiographic exposure times for the different sampling timepoints and treatment types

Treatment type	March	May
0.1nM	72 h	72 h
1nM	48 h	24 h
10nM	48 h	24 h
100nM	24 h	12 h

Table 2: Cell numbers of the different microbial groups and fractions of total (DAPI stained) cells and of hybridized *Bacteria* (bacterial taxa only) .

	March			May		
Group	% total cells (% DAPI)	% *Bacteria* (% EUB)	Abundance (10^5 cells ml^{-1})	% total cells (% DAPI)	% *Bacteria* (% EUB)	Abundance (10^5 cells ml^{-1})
Bacteria	82		6.20	82		8.40
Euryarchaeota	2		0.15	2		0.22
Roseobacter	7	8	0.50	8	10	0.97
SAR86	4	5	0.32	3	4	0.37
DE cluster 2	b.d. [a]	b.d. [a]	b.d. [a]	3	4	0.39

[a] b.d.: below detection limit (1% of total cell counts)

Figure legends:

Figure 1: Incorporation rates of [^{3}H] leucine by coastal North Sea picoplankton in a pre-bloom situation (March) and during spring phytoplankton bloom (May) at different concentrations of offered substrate (mean ± 1 standard deviation).

Figure 2: *Bars*: Fractions of bacterial cells with visible uptake of leucine at different concentrations of offered substrate (mean ± 1 standard deviation). *Symbols and lines*: average per cell leucine incorporation rate normalized to cells with visible substrate uptake.

Figure 3: A: Fractions of cells from the *Roseobacter* clade with visible incorporation of leucine at different concentrations of offered substrate. B: Contribution of bacteria from this lineage to all leucine-assimilating bacterial cells.

Figure 4: Fractions of cells from the DE cluster 2 (upper panel) and SAR86 clade (lower panel) with visible uptake of leucine at different concentrations of offered substrate.

Figure 5: Fractions of marine group II *Euryarchaeota* showing incorporation of leucine at different concentrations of offered substrate.

Figure 6: Ratio of the fraction of leucine-assimilating cells in the studied phylogenetic groups to the fraction of leucine-assimilating *Bacteria*. Errors were estimated assuming additive error propagation.

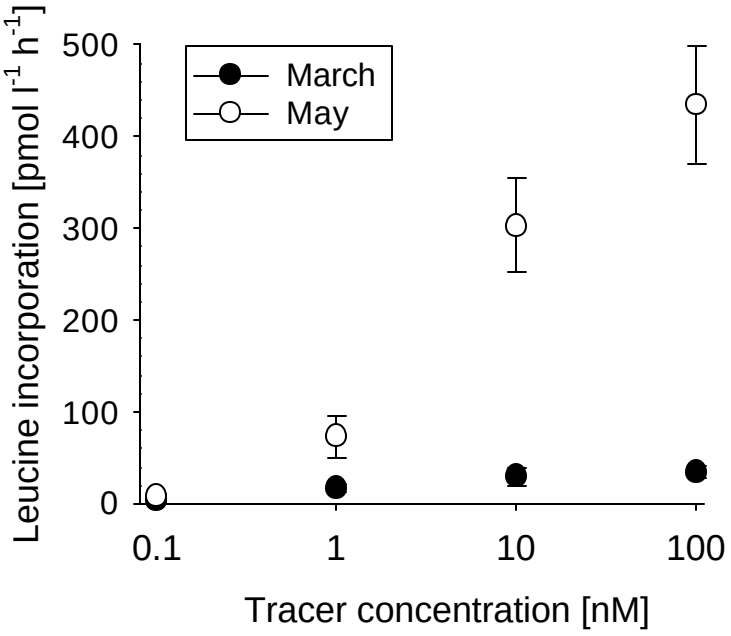
Leucine incorporation [pmol l-1 h-1]
500
400
300
200
100
0
March
May
0.1
1
10
100
Tracer concentration [nM]

Figure 1

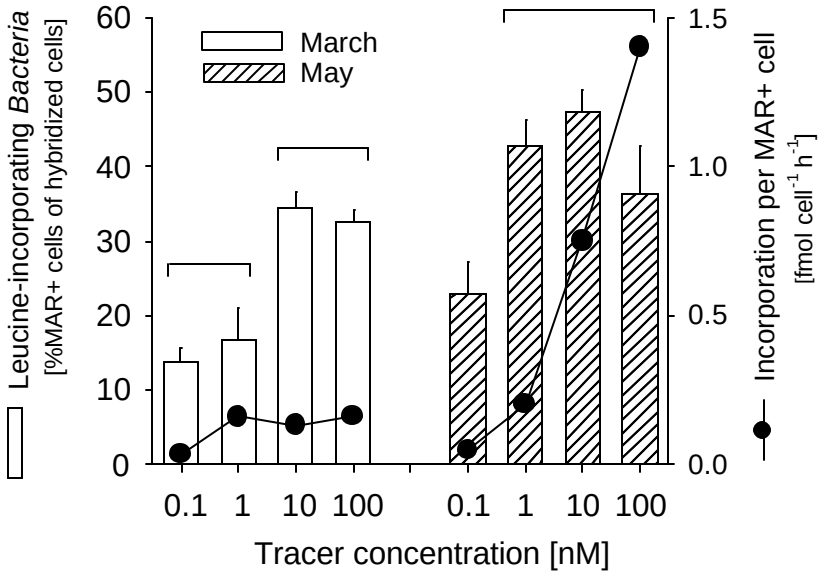
Leucine-incorporating *Bacteria*
[%MAR+ cells of hybridized cells]
Incorporation per MAR+ cell
[fmol cell^{-1} h^{-1}]
March
May
0
10
20
30
40
50
60
0.0
0.5
1.0
1.5
0.1
1
10
100
0.1
1
10
100
Tracer concentration [nM]

Figure 2

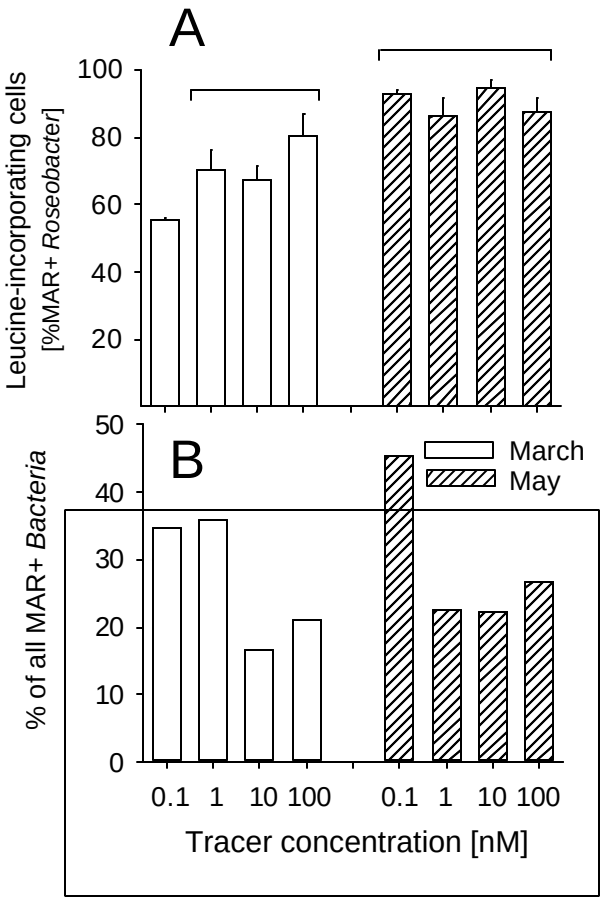
A
Leucine-incorporating cells
[%MAR+ Roseobacter]
100
80
60
40
20
B
% of all MAR+ Bacteria
50
40
30
20
10
0
March
May
0.1
1
10
100
0.1
1
10
100
Tracer concentration [nM]

Figure 3

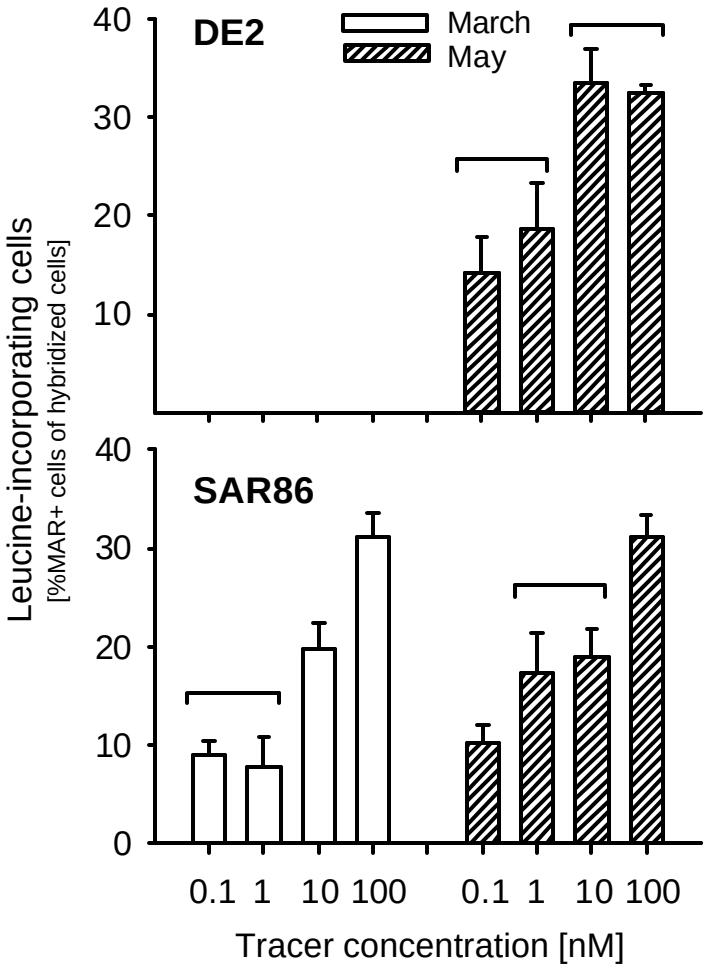
DE2
March
May
SAR86
Leucine-incorporating cells
[%MAR+ cells of hybridized cells]
40
30
20
10
0
0.1 1 10 100 0.1 1 10 100
Tracer concentration [nM]

Figure 4

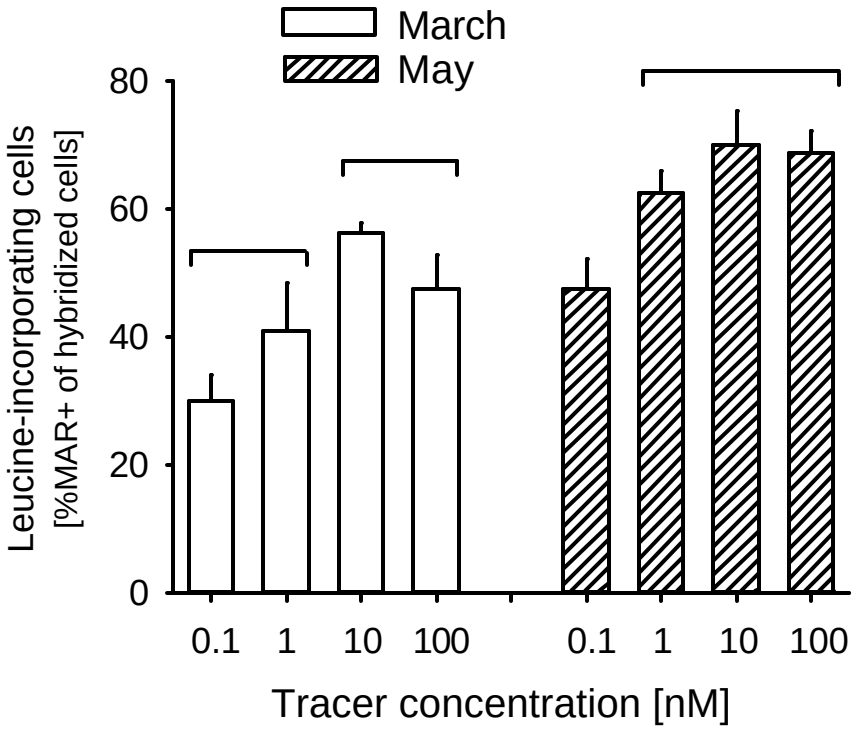
March
May
Leucine-incorporating cells
[%MAR+ of hybridized cells]
80
60
40
20
0
0.1
1
10
100
0.1
1
10
100
Tracer concentration [nM]

Figure 5

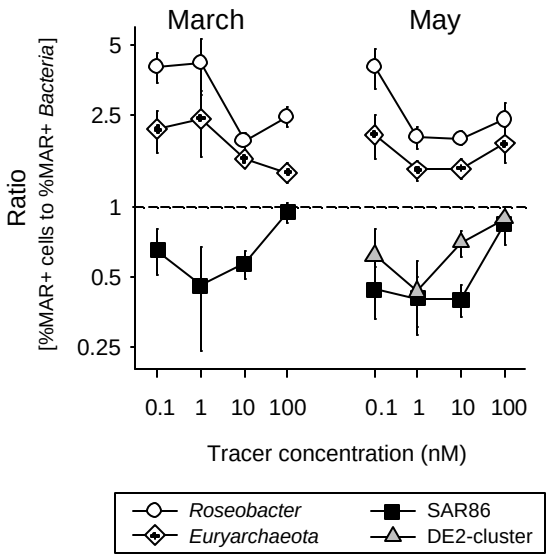
March
May
Ratio
[%MAR+ cells to %MAR+ Bacteria]
5
2.5
1
0.5
0.25
0.1
1
10
100
Tracer concentration (nM)
Roseobacter
Euryarchaeota
SAR86
DE2-cluster

Figure 6

Roseobacter and SAR11 dominate glucose uptake in coastal North Sea

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Appropriate section: Microbial Ecology

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Summary

Microbial taxa in pelagic marine habitats may differ in their respective substrate preferences. In addition, bacteria in coastal environments can respond to fluctuations in the availability of a single substrate, e.g. during spring phytoplankton blooms and in the vicinity of organic particles. We, therefore, studied the incorporation of glucose at various concentrations (0.1-100nM) by bacteria related to *Roseobacter*, SAR11, *Gammaproteobacteria* and *Cytophaga-Flavobacteria* in coastal North Sea waters in late winter and during a spring phytoplankton bloom dominated by *Phaeocystis* sp. (March and May 2004, respectively). Both, the fraction of glucose-assimilating bacterioplankton cells and the rate of substrate incorporation per active cell were higher in May. All studied bacterial groups exhibited a stimulation of glucose uptake during the phytoplankton bloom, and their respective contributions to all glucose-assimilating *Bacteria* were related to substrate concentration. The majority of glucose-incorporating bacterial cells at the lower concentrations were members of the *Roseobacter* and SAR11 lineages, whereas the proportion of active cells within *Cytophaga-Flavobacteria* significantly increased at higher levels of available substrate. The approximately equal contributions of the two alphaproteobacterial taxa was due to a low fraction of highly active *Roseobacter* cells and to a high number of SAR11 bacteria with a relative small proportion of glucose-assimilating cells. Our results point to the importance of concentration-dependent substrate incorporation patterns for a better understanding of the ecophysiological niches of different bacterioplankton populations.

Introduction

Glucose is regarded as one of the most universally utilized substrates for heterotrophic microbes, and it is typically the most abundant free neutral aldose in seawater (Rich et al., 1996), (Skoog et al., 1999). In marine surface waters it mainly originates from autotrophic sources, i.e. phytoplankton photosynthesis (Ittekkot et al., 1981), (Mopper et al., 1995), (Biddanda and Benner, 1997). Current estimates of glucose concentrations range from undetectably low (<1 nM) to approximately 100 nM (Rich et al., 1996), (Skoog et al., 1999), (Kirchman et al., 2001). The direct determination of glucose concentrations usually matches calculations based on the uptake kinetics of heterotrophic bacteria (Skoog et al., 1999), indicating a close coupling between release and uptake.

Glucose forms a prominent fraction of the storage polymers of some algal groups e. g Phaeocystis sp. (Janse et al., 1996b), (van Rijssel et al., 2000). A rapid release of glucose from polysaccharides by the activities of free-living and particle-bound (Janse et al., 1996a), (Becquevort et al., 1998), (Agis et al., 1998) microbes is suggested by the ubiquity of a range of dissolved beta-glucosidases with contrasting kinetic properties (Christian and Karl, 1995), (Arrieta and Herndl, 2002). The turnover of the (monomeric and polymeric) glucose pool in different oceanic regions ranges between days to months, and glucose assimilation in marine surface waters may represent from <1% to 40% of bacterial carbon production (Rich et al., 1996), (Skoog et al., 1999), (Kirchman et al., 2001).

The availability of glucose (and other dissolved free carbohydrates) to aquatic microbial assemblages is assumed to be related to changes of primary

production, e.g., during spring blooms of Phaeocystis in the North Sea (Ittekkot et al., 1981). In addition, there may be considerable short-term temporal fluctuations due to diurnal patterns of photosynthesis (Burney et al., 1982), (Skoog et al., 1999), as well as microscale patchiness (Blackburn et al., 1997) e.g., in the immediate vicinity of senescent or lysed algal cells. Pelagic bacteria in coastal habitats are thus probably exposed to glucose concentrations that range over several orders of magnitude. This is reflected by the presence of multiphasic uptake systems for the substrate both in single species of aquatic microbes (Nissen et al., 1984) and in whole bacterioplankton assemblages (Vaccaro and Jannasch, 1967), (Azam and Hodson, 1981), (Unanue et al., 1999).

Evidence is accumulating that the different phylogenetic groups of bacteria and archaea in marine waters also differ in their respective contributions to the flux of individual DOC components (Cottrell and Kirchman, 2000). For example, Malmstrom and co-workers have recently demonstrated that bacteria affiliated to one widespread lineage of alpha-Proteobacteria, SAR11, were responsible for a disproportionately high fraction of glucose assimilation in surface waters of the Northwest Atlantic Ocean (Malmstrom et al., 2005). In these experiments, glucose was offered at concentrations of 0.5 nM. While this approach reflects the ambient bulk concentrations in the open ocean, it may not adequately cover the potentially wide range of glucose available in coastal habitats, e.g. during bloom situations (Ittekkot et al., 1981), (Kirchman et al., 2001). It is, therefore, conceivable that other abundant groups of bacteria in coastal waters may be more competitive for glucose than SAR 11 bacteria at higher concentrations, e.g., alpha-Proteobacteria of the OM83

("Roseobacter") clade (González and Moran, 1997) or bacteria related to Cytophaga-Flavobacteria (Cottrell and Kirchman, 2000).

The concentration-dependent assimilation of glucose by different phylogenetic lineages of coastal North Sea bacterioplankton was studied at contrasting environmental settings. In particular, we compared the incorporation patterns of two groups of alpha-Proteobacteria, of gamma-Proteobacteria and of Cytophaga-Flavobacteria during a late winter pre-bloom situation and in a phytoplankton spring bloom dominated by Phaeocystis sp.. Microbial substrate uptake was investigated both by bulk incubations and by microautoradiography combined with fluorescence in situ hybridization (MAR-FISH)

Results

Community composition. The probes chosen to describe composition of the Bacterial assemblages accounted for 65% and 92% of cells hybridized with the EUBI-III probe in March and May, respectively (Fig. 1). In both months SAR11 cells constituted a high fraction of all Bacteria (Fig. 1). At both sampling dates, Gammaproteobacteria and Cytophaga-Flavobacteria constituted equivalent fractions of the Bacterial cells (Fig. 1). SAR11, Gammaproteobacteria and Cytophaga-Flavobacteria groups represented a higher proportion of the Bacteria in May. In contrast, the proportion of Roseobacter cells was similar in both months (Fig. 1). Total cell numbers did not change significantly between both months: 0,8 (± 0,2) *106 cells ml-1 in March and 1,2 (± 0,4) *106 cells ml-1 in May.

Glucose incorporation. More cells were able to incorporate glucose in May than in March at a given tracer concentration (Fig. 2A). Similarly, increased amounts of substrate were incorporated in May (Table 2). The percentage of MAR positive cells, as well as the amount of incorporated tracer, increased with increasing glucose concentration in both months until the 10 nM concentration (Fig. 2A, table 2). The incorporation rate per active cell was higher in May, particularly at the higher glucose concentrations (Fig. 2B).

Members of Roseobacter clade were found to be very active in the assimilation of glucose. A high fraction of this population (60% to 90%) showed glucose uptake. As for the whole bacterial assemblage, a higher proportion of this population incorporated the monomer in May, as compared to March. In each month the fraction of MAR positive Roseobacter cells was similarly high at all tracer concentrations (Fig. 3).

The fraction of SAR11 bacteria that showed glucose incorporation was maximal at 1nM concentration in both months (Fig. 3). The proportion of MAR positive SAR11 cells at a given concentration was higher in May (Fig. 3).

Gammaproteobacteria and Cytophaga-Flavobacteria exhibited increasing number of MAR positive cells with increasing glucose concentration (Fig. 4). The difference between the percentage of MAR positive cells at the highest and lowest concentrations was more pronounced for the Cytophaga-Flavobacteria cells (Fig. 4). Similar proportions of MAR positive cells were found within the Gammaproteobacteria at both sampling times, with the exception of the highest concentration where a significant higher fraction of cells showed glucose uptake in May (Fig. 4).

Negative controls yielded similar percentages of false-positive MAR-active cells irrespective of sampling time or incubation conditions, being on average less than1% of EUB I_III-stained cells (data not shown).

Contribution of specific groups to total uptake. The totality of MAR positive bacterial cells were belonging to the four groups analyzed, within the error of the method: these groups summed 74% to 119% of EUB I_III MAR positive cells. Roseobacter and SAR11 were the main glucose consumers, particularly at the lower concentrations (Fig. 5). Gammaproteobacteria and Cytophaga-Flavobacteria contributed similarly to glucose incorporating cells, increasing in importance with increasing glucose concentration (Fig. 5).

The relative contribution of these groups to MAR positive bacterial cells was compared to their relative contribution to all bacterial cells. This comparison shows whether the activity of a specific group is higher, lower or merely the one expected from their abundance. In this analysis, the contribution to total active cells by the Roseobacter group was always above that expected from their abundance by a minimum 3-fold factor (Table 3). This was specially marked for March and for lower substrate concentrations (Table 3). The relative contribution of SAR 11 cells to bacterial glucose uptake at 1nM concentration was higher than what expected from their abundance. Otherwise, these cells contributed to glucose uptake either accordingly to their relative abundance or below it (Table 3). The contribution to MAR positive cells of the Gammaproteobacteria and Cytophaga-Flavobacteria groups was different in both months. Whereas in March their participation as glucose

consuming cells was generally matching their relative abundance, in May they were underrepresented in the MAR positive cells (Table 3).

Discussion

Uptake of substrates by the whole microbial community. During both months, the amount of incorporated glucose per active cell increased with increasing substrate concentrations (Table 2, Fig. 2B). However, the rate of substrate incorporation per MAR positive cell in May was only about twice as high as in March at the lowest tracer concentration, whereas it was approximately 7 times as high at 100 nM (Fig. 2B). This stimulation of uptake at higher concentrations was not reflected in an equivalent concentration-dependent increase in the ratios of the numbers of MAR positive cells, which approximately doubled between the two timepoints across the range of tested concentrations (Fig. 2). Therefore, bacterial cells during the bloom situation were able to rapidly respond to higher amounts of substrate by increasing their incorporation rates. This suggests a transition of the whole bacterial assemblage from exhibiting an oligotrophic to a copiotrophic modality of substrate uptake, as previously observed for glucose by Yager and coworkers during a spring phytoplankton bloom in the coastal Alaskan Arctic Sea (Yager et al., 2001) and for leucine uptake in the German Bight (Alonso and Pernthaler, submitted).

Phytoplankton bloom events are typically accompanied by a rise in the fraction of particulate organic matter (Riemann et al., 2000). Laboratory studies show that there is a dynamic balance between the colonization and detachment of bacteria from such aggregates (Kiorboe et al., 2003). Thus, some apparently free-living bacteria might be particle-attached during other

phases of their life cycle. Microbes that thrive in pelagic habitats at times when such point sources of dissolved organic carbon are common (i.e., during the Phaeocystis bloom) would therefore profit from the ability to consume more rapidly substrates that are available at higher concentrations. By contrast, such a feature would be of little use if substrates are evenly distributed, as is likely during a late winter phytoplankton pre-bloom situation. This view is also supported by the results of Unanue et al (1999) who found a significantly higher Vmax for glucose uptake in bacteria associated with particles than in free-living bacteria in 5 out of 7 cases.

The discrepancy between the constant ratio of active cells and the increasing ratio of incorporation rate per cell (Fig. 2B) indicates that MARFISH can only provide a semi-quantitative appreciation of changes in bacterioplankton per cell activity. Thus, a logical extension of the MARFISH technique would be to apply quantitative methods to determine substrate uptake rates per cell within mixed communities. The measurement of radioactivity in an autoradiogram in terms of number of disintegrations that occur within a source is possible but technically difficult, e g., by track autoradiography or grain size area measurement (Rogers, 1979), (Carney and Fahnenstiel, 1987), (Davenport and Maguire, 1984). Carefully controlled experiments and appropriate standards are needed to reliably relate the number of grains or tracks to absolute amounts of radioactivity. These requirements render this approach inadequate for its use in natural samples where highly diverse cells are potentially responsible of substrate uptake at different rates (Brock and Brock, 1968; Gray and Head, 2001),(Nielsen et al., 2003). A promising alternative to these tedious approaches for the quantification of incorporated label is the

flow cytometric sorting of cells and the subsequent measurement of radioactivity by liquid scintillation counting (Lebaron et al., 2001; Zubkov et al., 2004). The sorting of hybridized cells from marine samples is already feasible (Sekar et al., 2004).

Contribution of specific groups to total uptake. Most glucose consuming bacterial cells were members of the Roseobacter and SAR11 lineages, particularly at the lower substrate concentrations (Fig. 5, Table 3). Their relative importance diminished at increasing concentration, partly due to a decrease in the proportion of MAR positive cells within the SAR11 clade (Fig. 3), but also due to the rising contribution of the other bacterial groups (Fig. 4). These findings suggest that Alphaproteobacteria are the main lineage responsible for glucose uptake at low ambient concentrations in coastal North Sea spring picoplankton. This agrees with previous studies where alphaproteobacteria where suggested as monomers specialists (Cottrell & Kirchman 2000). In crontrast, other groups, in particular Cytophaga-Flavobacteria participate more substantially in substrate turnover with its increasing availability. Representatives of the highly diverse Cytophaga-Flavobacteria group have been previously found to appear during Phaeocystis-dominated phytoplankton blooms (Simon et al., 1999) and bacteria from this lineage are frequently encountered in nutrient-rich microenvironments and on organic aggregates (Rath et al., 1998). This agrees with our observation that approximately twice as many bacteria from this lineage exhibited glucose assimilation at the highest substrate concentrations (Fig. 4).

224 At the level of larger subgroups (i.e., summing up Roseobacter and SAR11) the relative contributions of *Alphaproteobacteria, Gamma-proteobacteria* and 226 *Cytophaga-Flavobacteria* to the total numbers of glucose-assimilating cells typically corresponded well to what would be expected from their cell 228 numbers. This has been interpreted as indication that the same ("bottom-up") factors control the abundances and activity of these groups (Cottrell and 230 Kirchman, 2003). However, our results call for a more cautious appreciation of ecologically different microbial populations that may be hidden beneath such 232 large categories. Specifically, the two studied alphaproteobacterial populations appeared to be strikingly different in terms of ecophysiology. 234 Thus, the "average" *Alphaproteobacteria* did not seem to exist (Fig. 3), and the same might hold true for *Gammaproteobacteria* and *Cytophaga-* 236 *Flavobacteria.*

238 **Substrate uptake patterns of Roseobacter and SAR11.** Considering the constantly high contributions of Roseobacter and SAR11 bacteria to all 240 glucose incorporating Bacteria at both sampling timepoints (Fig. 5), it is likely that the concentration-dependent differences of uptake rates in March and 242 May (Table 2, Fig. 2B) were partially reflecting the physiological properties of cells from one or both of these lineages. The contrasting behavior of these 244 bacteria at the two sampling timepoints might reflect phenotypic plasticity, i.e., that identical genotypes were present at both sampling timepoints that differed 246 in their substrate uptake patterns due to the increase in temperature and substrate availability. Alternatively, a change in the dominant genotypes is

also conceivable that was masked by the use of FISH probes for rather large phylogenetic groups.

There is evidence that bacteria from the Roseobacter and SAR11 lineages might occupy comparable niches in different marine environments. For example, Roseobacter can be the main mediator of the flux of dimethyl sulfonopropionate (DMSP) (Malmstrom et al., 2004), (Zubkov et al., 2002), (Vila et al., 2004) in coastal waters or during blooms whereas DMSP was mainly consumed by SAR11 in open ocean samples (Malmstrom 2004). To our knowledge, this is the first study that simultaneously compares the substrate uptake patterns of both populations co-occurring in the same habitat. Roseobacter and SAR11 contributed approximately equally to the fraction of cells with visible glucose uptake (Fig. 5). However, the comparatively small Roseobacter population (Fig. 1) exhibited a very high proportion of active cells at all concentrations (Fig. 3). By contrast, the high contribution of the SAR11 bacteria to glucose uptake was due to their extremely high abundances (Fig. 1) that compensated for the relatively low fractions of active cells (Fig. 3).

Interestingly, these potentially different strategies for substrate incorporation appear to be partially reflected in the genomes of two isolates from the two clades, Silicibacter pomeroyi (Roseobacter) and Pelagibacter ubique (SAR11). A similar percentage of the genome codes for transporters and binding proteins in both isolates (around 12%) (Moran et al., 2004), (Giovannoni et al., 2005). However, the proportion of ABC transporters - known for high substrate affinity- is distinctly higher in Pelagibacter ubique (50% vs. 36%). S. pomeroyi features three rRNA operons, whereas SAR11

possesses only one. The number of rRNA operons has been related to the potential to respond rapidly to changes in resource availability (Klappenbach et al., 2000). This hints at a higher potential of S. pomeroyi to adjust to changes in growth conditions and to exploit pulses of substrate concentrations, whereas SAR11 might be better suited for slow but efficient growth in the ambient DOC field (Giovannoni et al., 2005). In addition, S. pomeroyi and other Roseobacter-related isolates possess quorum-sensing systems (Moran et al., 2004), (Gram et al., 2002), whereas Pelagibacter ubique does not (Giovannoni et al., 2005). Quorum sensing in S. pomeroyi has been interpreted to provide regulatory flexibility associated with the potential alternations between a particle-associated and planktonic life style (Moran et al., 2004). Roseobacter cells have been found both free-living and attached, e.g., during phytoplankton blooms (Riemann et al., 2000), or on marine snow (Rath et al., 1998). By contrast, SAR11 has only been observed in the free-living fraction of pelagic bacteria (Morris et al., 2002), and bacteria from this lineage do not grow at elevated substrate concentrations or on solid media (Rappe et al., 2002), (Simu and Hagström, 2004).

In a previous study by Malmstrom and co-workers (2005) the fraction of glucose-incorporating SAR11 cells clearly exceeded the community average, which was not generally the case in our samples (Table 3). However, the tracer concentrations used by Malmstrom et al (0.5nM) likely represented a competitive advantage for SAR11 bacteria at the low ambient substrate levels in the Northwest Atlantic Ocean. Thus, their results agree with the disproportionately high contribution of SAR11 bacteria to total glucose uptake in our samples at 1nM concentration (Fig. 3, Table 3). Nevertheless, it should

also be considered that SAR11 (as targeted by here applied FISH probe) represents a phylogenetically highly diversified lineage that should appropriately be regarded as a genus (Acinas et al., 2004). It is therefore conceivable that different ecotypes with a different affinity for glucose might occur in coastal and open ocean habitats.

Conclusions. In summary, we show that the two main representatives of Alpha-proteobacteria in coastal marine waters exhibit distinct concentration-dependent patterns of glucose assimilation. However, it should be noted that distinctly higher abundance of bacteria from the SAR11 clade than of the Roseobacter lineage in coastal North Sea waters during spring cannot be explained by their respective affinities to glucose. Other factors, such as top-down control by protistan grazers (Pernthaler, 2005) or viral lysis (Weinbauer and Rassoulzadegan, 2004), need to be taken into account. In our samples Roseobacter cells were substantially bigger and more active than cells from the SAR11 clade (Fig. 6), inviting speculations about food-web related population control of Roseobacter, e.g., in the context of the "killing-the-winner" concept (Thingstad, 2000), (Beardsley et al., 2003).

Experimental Procedures

Sampling site. Surface water samples (1 m depth) were collected at the sampling station Helgoland Roads (54°11’N, 7°54’E), in the German Bay of the North Sea. The sampling dates were 11th March 2004 (late winter) and 6th May 2004 (spring). The water temperature in March was 4.2°C and the algae cells were very scarce and mainly constituted by the diatom

Thalassionema nitzschioides that reached a biomass of 17 µg C/dm3. In May the water temperature was 8.3°C, and a spring bloom of Phaeocystis sp. -a typical phenomenon in coastal North Sea waters (Cadee and Hegeman, 2002)- was in its growing phase. It reached maximal biomasses (157 µg C/dm3) 5 days after the sampling date (source: Mursys Umweltreport, www.bsh.de).

Glucose incorporation. Triplicate 10 ml water sub samples incubations plus one control sample consisting in sea water fixed with paraformaldehyde were amended with tritiated glucose (Amersham, specific activity 1,29 TBq/mmol) in a concentration series (0.1 nM, 1 nM, 10 nM and 100nM) within 1 hour after sample collection. The incubations were run for 4 hours in the dark, at 4°C (March) and 10°C (May). Subsequently, freshly prepared buffered paraformaldehyde fixative was added to the samples to a final concentration of 1%. After fixation, the samples were frozen for transport, and then filtered through polycarbonate filters (type GTTP, pore size, 0.2 µm, diameter 25 mm, Millipore, Eschborn, Germany). The filters were rinsed twice with sterile phosphate buffered saline (PBS) and stored at -20°C until further MARFISH analysis. In parallel, identical incubations were run to measure the amount of substrate incorporated by the cells at the different concentrations. These samples were filtered onto cellulose mixed esters filters (type GSWP, pore size, 0.2 µm, diameter 25 mm, Millipore, Eschborn, Germany) and rinsed twice with ice cold trichloroacetic acid and ethanol, as described by (Kirchman, 2001). Filters were stored at –20°C until further processing.

Population analysis by FISH. Samples for determination of in situ abundance of specific bacterial populations were immediately fixed after collection with freshly prepared buffered paraformaldehyde at a final concentration of 1% (v/v). The abundances of different microbial taxa were determined by fluorescence in situ hybridization with horseradish peroxidase labeled oligonucleotide probes and catalyzed reporter deposition (CARD-FISH) (Pernthaler et al., 2002). Probes were selected which targeted all major bacterial groups: EUB I-III (most Bacteria) (Daims et al., 1999), SAR11 441 (members of the SAR11 clade of alphaproteobacteria) (Morris et al., 2002), ROS537 (members of the alphaproteobacterial SAR83 or "Roseobacter" clade) (Eilers et al., 2001), GAM42a (most gammaproteobacteria) (Manz et al., 1992) and CF319a (many groups from the Cytophaga-Flavobacteria lineage of the Bacteroidetes) (Manz et al., 1996). Probes were purchased from Biomers.net (Ulm, Germany). CARD-FISH preparations were counter-stained with 4,6-diamidino-2-phenylindole (DAPI; 1 µg ml-1). Evaluation of DAPI and FISH-stained cells was carried out manually, counting a minimum of 1000 DAPI cells per sample.

MARFISH analysis. To study the substrate uptake by specific bacterioplankton groups we applied a recently developed MARFISH protocol (Alonso and Pernthaler, 2005). The photochemicals employed (autoradiography emulsion type NTB-2, Dektol developer and fixer) were purchased from Kodak (Eastman Kodak, Rochester, NY). Different MAR exposure times were tested in order to obtain a maximum number of cells hybridized by probe EUB I-III with a halo of silver grains. The exposure time required to achieve the maximum number of MAR positive cells was particular

374 for each treatment type, as they differed in substrate concentration and in sampling dates (Table 1). Triplicates of every treatment type were evaluated 376 for every FISH probe. The controls were evaluated with the EUB I_III probe. The development of the exposed slides followed the instructions of the 378 manufacturer (2 min of development, 10 seconds rinsing with distilled water, 5 minutes in fixer, followed by 5 minutes washing with distilled water). 380 Evaluations of MAR-FISH preparations were carried out on an Axioplan II imaging fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with 382 a motorized stage, z-axis drive and fluorescence filter wheel, and with a digital camera (Orca I, Hamamatsu, Herrsching, Germany) linked to a personal 384 computer. Altogether 120 MARFISH preparations were evaluated.

386 **Bulk measurements of glucose incorporation.** The measurements of radiolabeled glucose incorporated by the cells were performed in a 388 scintillation counter (Packard TriCarb 2900 TR, Perkin Elmer, Wellesley, MA). The cellulose filters were placed on 6ml scintillation vials and dissolved with 390 0.5 ml of ethyl acetate. After dissolution was complete, 5 ml of Lumasafe plus scintillation cocktail was added to each vial (Lumac LSC BV, Groningen, The 392 Netherlands). Measurements were corrected for quench (external standard method), and by subtraction of counts from the formaldehyde fixed controls.

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References:

Acinas, S.G., Klepac-Ceraj, V., Hunt, D.E., Pharino, C., Ceraj, I., Distel, D.L., and Polz, M.F. (2004) Fine-scale phylogenetic architecture of a complex bacterial community. *Nature* **430**: 551-554.

Agis, M., Unanue, M., Iriberri, J., and Herndl, G.J. (1998) Bacterial colonization and ectoenzymatic activity in phytoplankton-derived model particles. Part II. Cleavage and uptake of carbohydrates. *Microbial Ecology* **36**: 66-74.

Alonso, C., and Pernthaler, J. (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Applied and Environmental Microbiology* **71**: 1709-1716.

Alonso, C., and Pernthaler, J. (submitted) Concentration-dependent patterns of leucine incorporation in coastal picoplankton. *Applied and Environmental Microbiology*.

Arrieta, J.M., and Herndl, G.I. (2002) Changes in bacterial beta-glucosidase diversity during a coastal phytoplankton bloom. *Limnology and Oceanography* **47**: 594-599.

Azam, F., and Hodson, R.E. (1981) Multiphasic Kinetics for D-Glucose Uptake by Assemblages of Natural Marine-Bacteria. *Marine Ecology-Progress Series* **6**: 213-222.

Beardsley, C., Pernthaler, J., Wosniok, W., and Amann, R. (2003) Are readily cultured bacteria in coastal North Sea waters suppressed by selective grazing mortality? *Applied and Environmental Microbiology* **69**: 2624-2630.

Becquevort, S., Rousseau, V., and Lancelot, C. (1998) Major and comparable roles for free-living and attached bacteria in the degradation of Phaeocystis-derived organic matter in Belgian coastal waters of the North Sea. *Aquatic Microbial Ecology* **14**: 39-48.

Biddanda, B., and Benner, R. (1997) Carbon, nitrogen, and carbohydrate fluxes during the production of particulate and dissolved organic matter by marine phytoplankton. *Limnology and Oceanography* **42**: 506-518.

Blackburn, N., Azam, F., and Hagstrom, A. (1997) Spatially explicit simulations of a microbial food web. *Limnology and Oceanography* **42**: 613-622.

Brock, M.L., and Brock, T.D. (1968) The application of micro-autoradiographic techniques to ecological studies. *Mitt. Int. Verein. Limnol.*: 1-29.

Burney, C.M., Davis, P.G., Johnson, K.M., and Sieburth, J.M. (1982) Diel Relationships of Microbial Trophic Groups and Insitu Dissolved Carbohydrate Dynamics in the Caribbean Sea. *Marine Biology* **67**: 311-322.

Cadee, G.C., and Hegeman, J. (2002) Phytoplankton in the Marsdiep at the end of the 20th century; 30 years monitoring biomass, primary production, and Phaeocystis blooms. *Journal of Sea Research* **48**: 97-110.

Carney, H.J., and Fahnenstiel, G.L. (1987) Quantification of Track and Grain Density Autoradiography and Evaluation of C-14 Loss on Preservation. *Journal of Plankton Research* **9**: 41-50.

Christian, J.R., and Karl, D.M. (1995) Bacterial Ectoenzymes in Marine Waters - Activity Ratios and Temperature Responses in 3 Oceanographic Provinces. *Limnology and Oceanography* **40**: 1042-1049.

Cottrell, M.T., and Kirchman, D.L. (2000) Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Applied and Environmental Microbiology* **66**: 1692-1697.

Cottrell, M.T., and Kirchman, D.L. (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnology and Oceanography* **48**: 168-178.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology.* **22**: 434-444.

Davenport, J.B., and Maguire, B. (1984) Quantitative Grain Density Autoradiography and the Intraspecific Distribution of Primary Productivity in Phytoplankton. *Limnology and Oceanography* **29**: 410-416.

Eilers, H., Pernthaler, J., Peplies, J., Glöckner, F.O., Gerdts, G., and Amann, R. (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. *Applied and Environmental Microbiology* **67**: 5134-5142.

Giovannoni, S.J., Tripp, H.J., Givan, S., Podar, M., Vergin, K.L., Baptista, D. et al. (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**: 1242-1245.

González, J.M., and Moran, M.A. (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class *Proteobacteria* in coastal seawater. *Applied and Environmental Microbiology* **63**: 4237-4242.

Gram, L., Grossart, H.P., Schlingloff, A., and Kiorboe, T. (2002) Possible quorum sensing in marine snow bacteria: Production of acylated homoserine lactones by Roseobacter strains isolated from marine snow. *Applied and Environmental Microbiology* **68**: 4111-4116.

Gray, N.D., and Head, I.M. (2001) Linking genetic identity and function in communities of uncultured bacteria. *Environmental Microbiology* **3**: 481-492.

Ittekkot, V., Brockmann, U., Michaelis, W., and Degens, E.T. (1981) Dissolved Free and Combined Carbohydrates During a Phytoplankton Bloom in the Northern North-Sea. *Marine Ecology-Progress Series* **4**: 299-305.

Janse, I., vanRijssel, M., Gottschal, J.C., Lancelot, C., and Gieskes, W.W.C. (1996a) Carbohydrates in the North Sea during spring blooms of Phaeocystis: A specific fingerprint. *Aquatic Microbial Ecology* **10**: 97-103.

Janse, I., vanRijssel, M., vanHall, P.J., Gerwig, G.J., Gottschal, J.C., and Prins, R.A. (1996b) The storage glucan of Phaeocystis globosa (prymnesiophyceae) cells. *Journal of Phycology* **32**: 382-387.

Kiorboe, T., Tang, K., Grossart, H.P., and Ploug, H. (2003) Dynamics of microbial communities on marine snow aggregates: Colonization, growth, detachment, and grazing mortality of attached bacteria. *Applied and Environmental Microbiology* **69**: 3036-3047.

Kirchman, D. (2001) Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments. In *Methods in Microbiology*. Paul, J.H. (ed). London: Academic Press, pp. 227-237.

Kirchman, D.L., Meon, B., Ducklow, H.W., Carlson, C.A., Hansell, D.A., and Steward, G.F. (2001) Glucose fluxes and concentrations of dissolved combined neutral sugars (polysaccharides) in the Ross Sea and Polar Front Zone, Antarctica. *Deep-Sea Research Part Ii-Topical Studies in Oceanography* **48**: 4179-4197.

Klappenbach, J.A., Dunbar, J.M., and Schmidt, T.M. (2000) RRNA operon copy number reflects ecological strategies of bacteria. *Applied and Environmental Microbiology* **66**: 1328-1333.

Lebaron, P., Servais, P., Agogue, H., Courties, C., and Joux, F. (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Applied & Environmental Microbiology* **67**: 1775-1782.

Malmstrom, R.R., Kiene, R.P., and Kirchman, D.L. (2004) Identification and enumeration of bacteria assimilating dimethylsulfoniopropionate (DMSP) in the North Atlantic and Gulf of Mexico. *Limnology and Oceanography*: 597-606.

Malmstrom, R.R., Cottrell, M.T., Elifantz, H., and Kirchman, D.L. (2005) Biomass production and assimilation of dissolved organic matter by SAR11 bacteria in the Northwest Atlantic Ocean. *Applied and Environmental Microbiology* **71**: 2979-2986.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: Problems and solutions. *Systematic and Applied Microbiology* **15**: 593-600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.-H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142**: 1097-1106.

Mopper, K., Zhou, J.A., Ramana, K.S., Passow, U., Dam, H.G., and Drapeau, D.T. (1995) The Role of Surface-Active Carbohydrates in the Flocculation of a Diatom Bloom in a Mesocosm. *Deep-Sea Research Part Ii-Topical Studies in Oceanography* **42**: 47-73.

Moran, M.A., Buchan, A., Gonzalez, J.M., Heidelberg, J.F., Whitman, W.B., Kiene, R.P. et al. (2004) Genome sequence of Silicibacter pomeroyi reveals adaptations to the marine environment. *Nature* **432**: 910-913.

Morris, R.M., Rappe, M.S., Connon, S.A., Vergin, K.L., Siebold, W.A., Carlson, C.A., and Giovannoni, S.J. (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420**: 806-810.

Nielsen, J.L., Christensen, D., Kloppenborg, M., and Nielsen, P.H. (2003) Quantification of cell-specific substrate uptake by probe- defined bacteria under in situ conditions by microautoradiography and fluorescence in situ hybridization. *Environmental Microbiology* **5**: 202-211.

Nissen, H., Nissen, P., and Azam, F. (1984) Multiphasic Uptake of D-Glucose by an Oligotrophic Marine Bacterium. *Marine Ecology-Progress Series* **16**: 155-160.

Pernthaler, A., Pernthaler, J., and Amann, R. (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Applied and Environmental Microbiology* **68**: 3094-3101.

Pernthaler, J. (2005) Predation on procaryotes in the water column and its ecological implications. *Nature Reviews Microbiology* **3**: 537-546.

Rappe, M.S., Connon, S.A., Vergin, K.L., and Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**: 630-633.

Rath, J., Wu, K.Y., Herndl, G.J., and DeLong, E.F. (1998) High phylogenetic diversity in a marine-snow associated bacterial assemblage. *Aquatic Microbial Ecology* **14**: 261-269.

Rich, J.H., Ducklow, H.W., and Kirchman, D.L. (1996) Concentrations and uptake of neutral monosaccharides along 140 degrees W in the equatorial Pacific: Contribution of glucose to heterotrophic bacterial activity and the DOM flux. *Limnology and Oceanography* **41**: 595-604.

Riemann, L., Steward, G.F., and Azam, F. (2000) Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Applied and Environmental Microbiology* **66**: 578-587.

Rogers, A.W. (1979) *Techniques of Autoradiography*. Amsterdam: Elsevier.
Sekar, R., Fuchs, B., Amann, R., and Pernthaler, J. (2004) Flow Sorting of marine bacterioplankton after fluorescence in situ hybridization. *Applied and Environmental Microbiology* **70**: 6210-6219.

Simon, M., Glöckner, F.O., and Amann, R. (1999) Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquatic Microbial Ecology* **18**: 275-284.

Simu, K., and Hagström, A. (2004) Oligotrophic bacterioplankton with a novel single-cell life strategy. *Applied and Environmental Microbiology* **70**: 2445-2451.

Skoog, A., Biddanda, B., and Benner, R. (1999) Bacterial utilization of dissolved glucose in the upper water column of the Gulf of Mexico. *Limnology and Oceanography* **44**: 1625-1633.

Thingstad, T.F. (2000) Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnology and Oceanography* **45**: 1320-1328.

Unanue, M., Ayo, B., Agis, M., Slezak, D., Herndl, G.J., and Iriberri, J. (1999) Ectoenzymatic activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. *Microbial Ecology* **37**: 36-48.

Vaccaro, R.F., and Jannasch, H.W. (1967) Variations in Uptake Kinetics for Glucose by Natural Populations in Seawater. *Limnology and Oceanography* **12**: 540-542.

van Rijssel, M., Janse, I., Noordkamp, D.J.B., and Gieskes, W.W.C. (2000) An inventory of factors that affect polysaccharide production by Phaeocystis globosa. *Journal of Sea Research* **43**: 297-306.

Vila, M., Simo, R., Kiene, R.P., Pinhassi, J., González, J.A., Moran, M.A., and Pedros-Alio, C. (2004) Use of microautoradiography combined with fluorescence in situ hybridization to determine dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa. *Applied and Environmental Microbiology* **70**: 4648-4657.

Weinbauer, M.G., and Rassoulzadegan, F. (2004) Are viruses driving microbial diversification and diversity? *Environmental Microbiology* **6**: 1-11.

Yager, P.L., Connelly, T.L., Mortazavi, B., Wommack, K.E., Bano, N., Bauer, J.E. et al. (2001) Dynamic bacterial and viral response to an algal bloom at subzero temperatures. *Limnology and Oceanography* **46**: 790-801.

Zubkov, M.V., Tarran, G.A., and Fuchs, B.M. (2004) Depth related amino acid uptake by Prochlorococcus cyanobacteria in the Southern Atlantic tropical gyre. *Fems Microbiology Ecology* **50**: 153-161.

Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R., and Burkill, P.H. (2002) Rapid turnover of dissolved DMS and DMSP by defined bacterioplankton communities in the stratified euphotic zone of the North Sea. *Deep-Sea Research Part Ii-Topical Studies in Oceanography* **49**: 3017-3038.

Table 1: Optimal autoradiographic exposure times for the different sampling timepoints and treatment types

Treatment type	**March**	**May**
0.1 nM	6 days	72 h
1 nM	72 h	48 h
10 nM	48 h	18 h
100 nM	18 h	12 h

Table 2. Glucose incorporation rates at different concentrations by the whole microbial community in March and May 2004

Treatment type	**Incorporated substrate (pmoles l^{-1} h^{-1})**		***Ratio May to March***
	March	May	
0.1 nM	1.4 +/- 0.2	6.7 +/- 0.7	5
1 nM	11.7 +/-7.3	75.1 +/- 13.4	6
10n M	34.0 +/- 13.0	390.3 +/- 60.6	12
100 nM	22.4 +/- 8.6	433.0 +/- 16.5	19

Table 3: Ratio of the fractions of glucose-assimilating cells in the studied phylogenetic groups to the fraction of glucose-assimilating *Bacteria*.

Treatment type	***Roseobacter***		**SAR11**		***Gamma-proteobacteria***		***Cytophaga-Flavobacteria***	
	March	May	March	May	March	May	March	May
0.1 nM	6.1	4.3	1.0	0.5	1.1	0.7	1.0	0.6
1 nM	3.6	3.6	1.3	1.5	0.6	0.7	0.8	0.6
10 nM	3.2	2.9	0.8	1.0	0.6	0.6	1.1	0.6
100 nM	3.7	2.5	0.8	0.7	0.9	0.5	1.0	0.7

Figure legends:

Figure 1: Contributions of bacteria affiliated to the *Roseobacter* and SAR11 clades, to *Gammaproteobacteria*, and to *Cytophaga-Flavobacteria* to all hybridized bacterial cells in coastal North Sea surface waters during a late winter situation (March 2004) and during a phytoplankton bloom dominated by Phaeocystis sp. (May 2004).

Figure 2: A) Fractions of bacterial cells with visible uptake of glucose (i.e., MAR+ cells) at different concentrations of offered substrate (mean ± 1 standard deviation). B) Ratios of glucose-incorporating cells (closed symbols) and of incorporation rates per MAR+ cell (open symbols) between the pre-bloom (March) and the phytoplankton bloom situation (May).

Figure 3: Fractions of cells from the *Roseobacter* and SAR11 clades with visible incorporation of glucose at different concentrations of offered substrate in March (upper panel) and May (lower panel).

Figure 4: Fractions of cells affiliated to *Gammaproteobacteria* and *Cytophaga-Flavobacteria* with visible incorporation of glucose at different concentrations of offered substrate in March (upper panel) and May (lower panel).

Figure 5: Relative contributions of glucose-assimilating cells from the studied phylogenetic groups to all glucose-assimilating *Bacteria*.

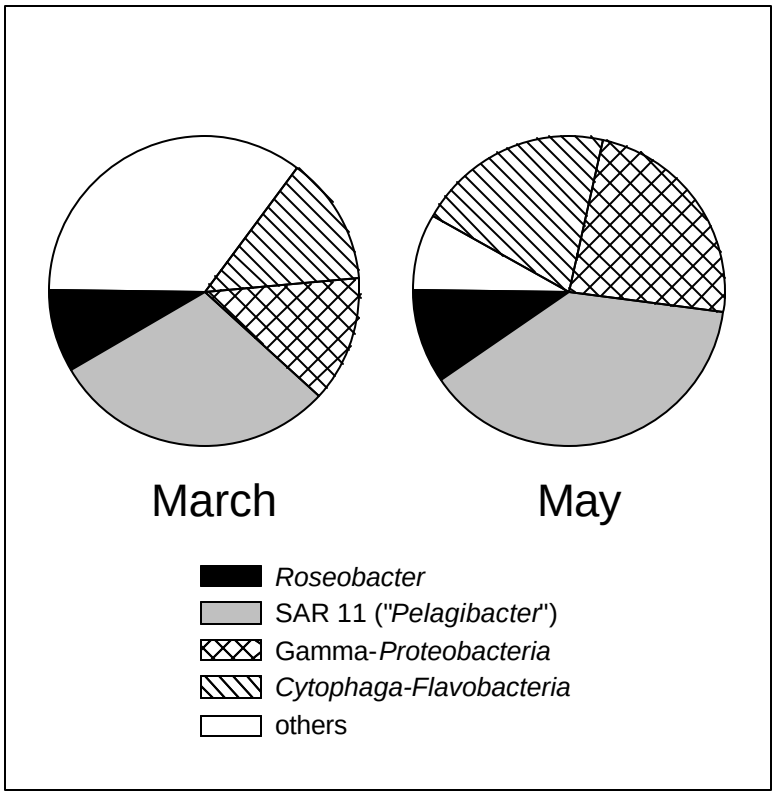
March
May
Roseobacter
SAR 11 ("*Pelagibacter*")
Gamma-*Proteobacteria*
Cytophaga-Flavobacteria
others

Fig. 1

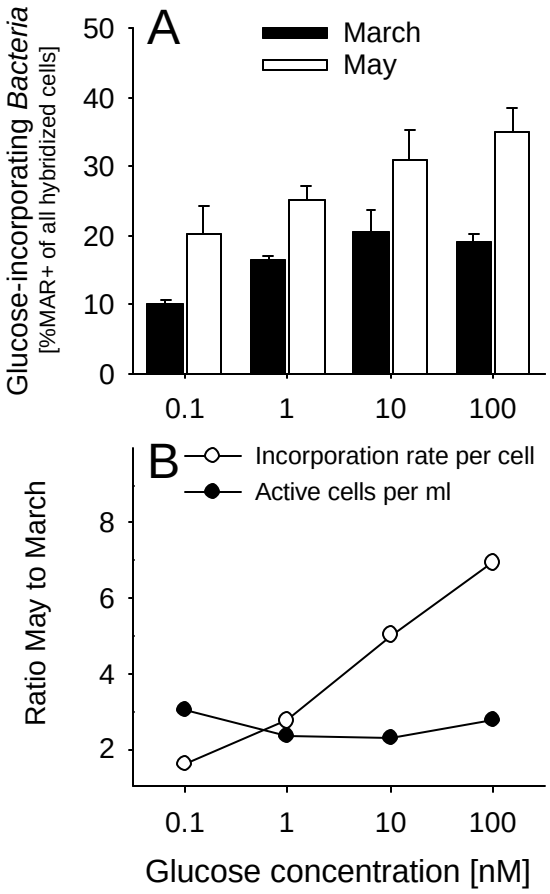
A
March
May
Glucose-incorporating Bacteria
[%MAR+ of all hybridized cells]
50
40
30
20
10
0
0.1
1
10
100
B
Incorporation rate per cell
Active cells per ml
Ratio May to March
8
6
4
2
0.1
1
10
100
Glucose concentration [nM]

Fig. 2

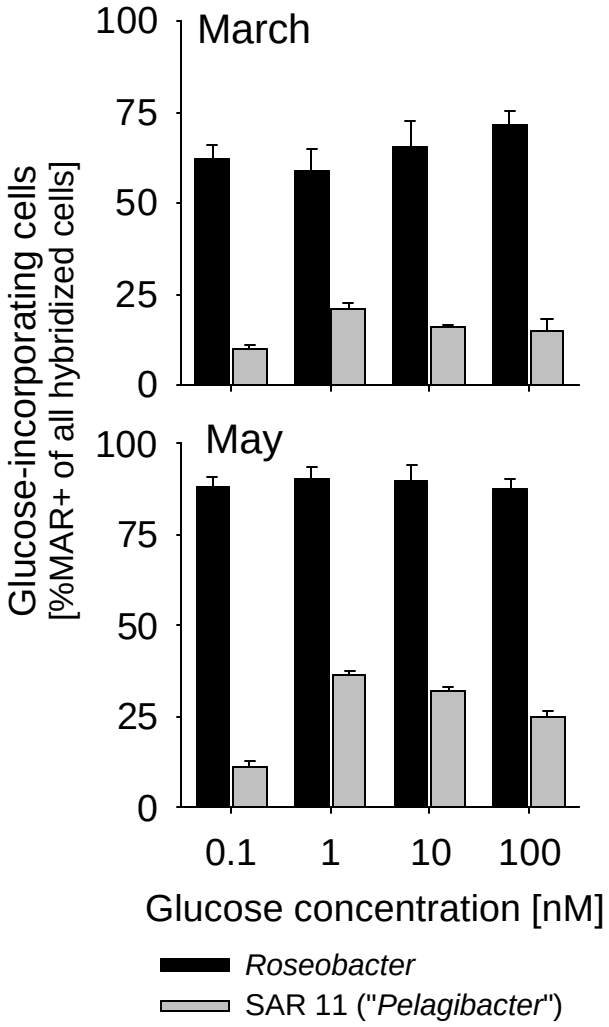
March
May
100
75
50
25
0
Glucose-incorporating cells
[%MAR+ of all hybridized cells]
0.1
1
10
100
Glucose concentration [nM]
Roseobacter
SAR 11 ("Pelagibacter")

Fig. 3

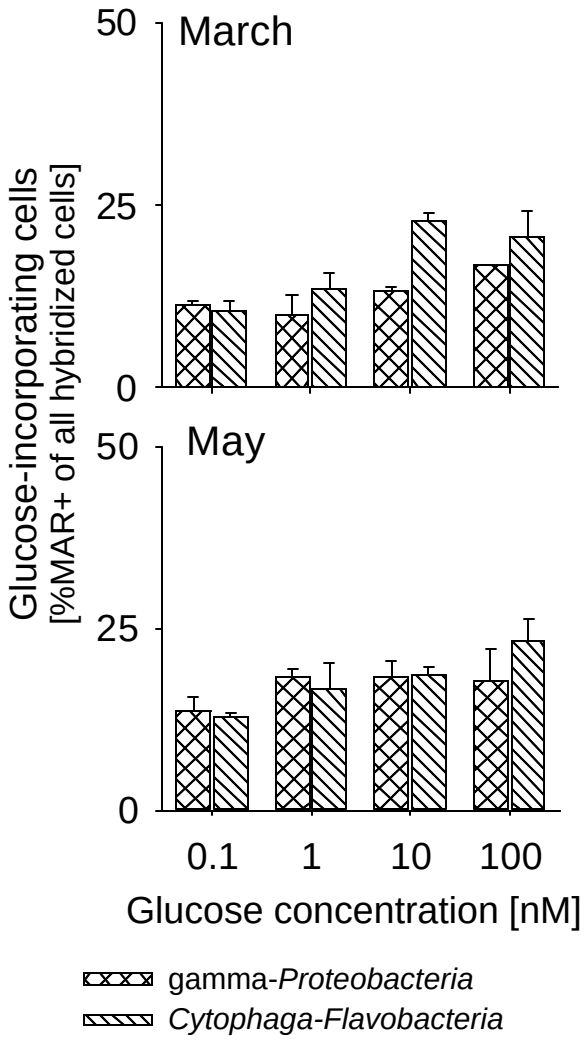
March
May
50
25
0
Glucose-incorporating cells
[%MAR+ of all hybridized cells]
0.1
1
10
100
Glucose concentration [nM]
gamma-Proteobacteria
Cytophaga-Flavobacteria

Fig. 4

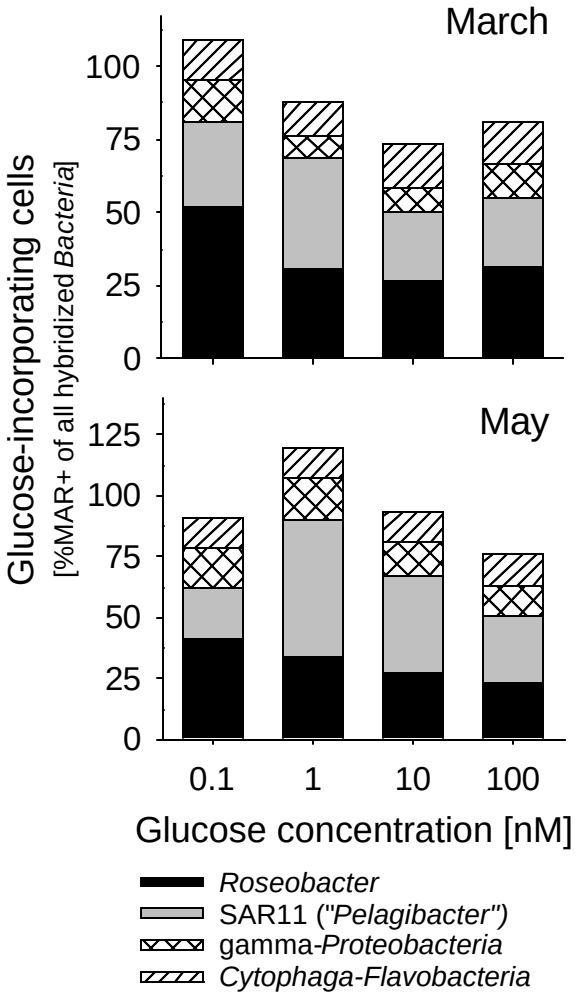
March
May
Glucose-incorporating cells
[%MAR+ of all hybridized Bacteria]
0
25
50
75
100
125
0.1
1
10
100
Glucose concentration [nM]
Roseobacter
SAR11 ("Pelagibacter")
gamma-Proteobacteria
Cytophaga-Flavobacteria

Fig. 5