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Abundance and heterotrophic activity of *Bacteria* and *Archaea* and bacterial community structure in the water column of the eastern Atlantic

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Einleitung

Die Bedeutung von Prokaryoten in den biogeochemischen Zyklen der Meere. In der ersten Hälfte des 20. Jahrhunderts wurden Mikrobiologen darauf aufmerksam, dass Bakterien mit einer Abundanz von 100 – 1000 Zellen ml^{-1} (Waksman and Renn 1934; ZoBell 1946) in den offenen Ozeanen vorkommen. Diese Abundanzen basierten auf der Plattenzähltechnik, also auf Bakterien die auf marinen Agarnährboden, dem sogenannten ZoBell-Agar aufwachsen können. Erst in den 70ern des letzen Jahrhunderts war man in der Lage marine Prokaryoten durch Farbmarkierungen der Nukleinsäuren (Hobbie, Daley et al. 1977) direkt unter dem Epifluoreszenzmikroskop zu zählen. Mit dieser neuen Methode werden wesentlich mehr Prokaryoten gezählt als mit der Plattentechnik, nicht 10^3 Zellen ml^{-1}, wie mit der „plate counting" Technik, sondern 10^6 Zellen ml^{-1}. Die um drei Größenordnungen höhere Abundanz von Prokaryoten in den Ozeanen führte zu einer Reihe von Fragen bezüglich des Wachstums und der Funktion von marinen Prokaryoten. Marine Mikroben, bis etwa 1975 ausschliesslich als Destruenten im marinen Nahrungsnetz angesehen, rückten somit mehr ins Zentrum der Nahrungsnetze (Williams 1972). Basierend auf den Ideen von Pomeroy (1974) und Williams (1972) führte diese geänderte Sichtweise von Nahrungsnetzen zu der Formulierung der Hypothese der „Mikrobiellen Schleife" (Microbial loop) von Azam et al. (1983). Die ursprüngliche Idee der mikrobiellen Schleife war, dass heterotrophe Bakterien gelöstes organisches Material (dissolved organic matter-DOM) über die Produktion von bakterieller Biomasse in partikuläres organisches Material (POM) überführen. Nur Bakterien sind in der Lage DOM aufzunehmen, alle anderen heterotrophen Organismen des Nahrungsnetzes benötigen POM als Nahrung. Diese heterotrophe bakterielle Biomasse wird dann von Flagellaten und Ciliaten konsumiert und dadurch für höhere trophische Niveaus verfügbar. Zahlreiche spätere Studien zeigten jedoch, dass nur ein kleiner Teil des von heterotrophen Prokaryoten aufgenommenen organischen Kohlenstoffs wirklich in die Metazoen-Nahrungskette gelangt. Der Großteil des durch die Mikroben aufgenommenen organischen Kohlenstoffs verbleibt innerhalb der mikrobiellen Schleife (Azam and Malfatti 2007). Die gegenwärtige Sichtweise ist, dass die Mikroben und Metazoen um den organischen Kohlenstoff, der hauptsächlich vom Phytoplankton produziert wird, konkurrieren (Herndl, Agogué et al. 2008).

Archaea und ihre Verteilung. Die Ansicht, dass Bakterien die einzig bedeutsamen Vertreter der Prokaryoten im Meer sind, wurde durch die Erkenntnis, dass auch Archaea in der marinen Wassersäule sehr abundant sind in den letzten beiden Jahrzehnten widerlegt (DeLong 1992; Fuhrman, McCallum et al. 1992). Rezente Studien bestätigten die allgegenwärtige Präsenz von mesophilen Archaea in allen ozeanischen Regionen (Karner et al., 2001; Teira et al., 2004). Im Wesentlichen bestehen sie aus zwei großen phylogenetischen Gruppen: den Crenarchaeota und den Euryarchaeota, wobei die marine Crenarchaeota Gruppe I [MCGI, nun auch Thaumarchaeota genannt (Brochier-Armanet, Boussau et al. 2008)] die häufigste planktonische Archaea-Gruppe ist und bis zu einem Drittel der Prokaryoten-Gemeinschaft unterhalb der euphotischen Zone in den Ozeanen stellt (Karner et al., 2001; Herndl et al. 2005; Varela et al., 2008). Im Pazifik erreicht der Beitrag der Crenarchaeota gemessen an der Gesamtzahl der Prokaryoten unter 150 m Tiefe ein Maximum von 40 % (Karner et al., 2001) und im nördlichen Nord-Atlantik übersteigt im Bathypelagial die Abundanz der Archaea (Euryarchaeota und Crenarchaeota) sogar die der Bakterien (Teira et al., 2004).

Metabolische Aktivität und die biogeochemische Rolle der Archaea. Obwohl einige Studien gezeigt haben, dass Archaea dominant in tiefen Wasserschichten sind, ist dennoch sehr wenig über ihre metabolische Aktivität und ihre biogeochemische Rolle im Ozean bekannt. Kürzlich durchgeführte Forschungen deuten, durch den Einbau von anorganischem Kohlenstoff (Wuchter et al., 2003) und dem Gebrauch von Ammonium als Energiequelle (Könneke et al. 2005; Wuchter et al., 2006, Ingalls et al. 2006, Hansman et al. 2009), auf eine chemoautotrophe Ernährung der MCGI hin. Eine Studie von Herndl et al. (2005) hat gezeigt, dass eine erhebliche Menge an gelöstem anorganischen Kohlenstoff, die ca. 84 % der heterotrophen prokaryoten Produktion entsprechen, von Archaea aufgenommen wird. Zudem wurde unterhalb der euphotischen Zone das Schlüsselgen *amo*A in den Archaea nachgewiesen. Dieses Gen kodiert das Enzym Ammonium-Monooxygenase, das die Oxidation von Ammonium zu Hydroxylamin katalysiert und somit den ersten Schritt der Nitrifikation darstellt. Analysen mit quantitativer PCR zeigten, dass das *amo*A Gen der Archaea um Grössenordnungen häufiger ist als das der Bakterien (Agogue et al. 2008, De Corte et al. 2009, Mincer et al. 2007). Das könnte bedeuten, dass MCGI eine wichtigere Rolle bei der ozeanischen Nitrifikation spielen als Bakterien. Diese Folgerung basiert allerdings nur

auf der Quantifizierung des *amo*A Gens im Vergleich zu der Häufigkeit des bakteriellen *amo*A Gens. Nitrifikationsraten wurden bisher von Archaea im Freiwasser nicht gemessen.

Obwohl Chemoautotrophie von MCGI oft dokumentiert wurde, wurde auch die Aufnahme von Aminosäuren nachgewiesen, was auf einen heterotrophen oder zumindest mixotrophen Stoffwechsel einiger MCGI Arten hinweist. Die Kombination von CARD-FISH (catalyzed reporter deposition-fluorescence in situ hybridization) mit Mikroautoradiographie (MICRO-CARD-FISH) ermöglicht es, die Aufnahme von spezifischen organischen und anorganischen Substanzen auf Einzelzellniveau nachzuweisen. Mit dieser Methode konnten Teira et al. (2006) und Varela et al. (2008) zeigen, dass MCGI bevorzugt D- anstelle von L-Asparagin im Bathypelagial des Atlantiks aufnehmen und Kirchman et al. (2007) demonstrierte die Aufnahme von verschiedenen organischen Substanzen (Leucin, Aminosäure-Mischung, Proteine, Glukose und Polysaccharide) durch Archaea in der Wassersäule (0 – 500 m) im westlichen Teil der Arktik. Obwohl diese Ergebnisse auf dem Einzelzellniveau aufdeckten, dass ein gewisser Anteil der MCGI heterotroph ist, muss der relative Beitrag der Archaea beim Fixieren des gelösten anorganischen Kohlenstoffs und deren Beitrag an der Umsetzung von organischem Material zur gesamten Prokaryoten-Aktivität erst noch geklärt werden.

Unterschiede und Veränderungen in den Strukturen von aktiven und metabolisch inaktiven Bakterien über die Tiefe. Aufgrund des sinkenden Substratangebots, welches überwiegend in Form von sinkenden parkikulären Material aus der euphotischen Zone kommt, sinkt auch in der Wassersäule mit zunehmender Tiefe die Abundanz und Aktivität der heterotrophen Prokaryoten (Byung and Azam 1988; Ducklow, Steinberg et al. 2001). Ähnlich dazu ändert sich, als Folge des geringeren Nährstoffangebotes und der niedrigeren Temperaturen, auch die Zusammensetzung der Prokaryoten-Gemeinschaft mit der Tiefe (Moeseneder er al. 2001). Es wurde gezeigt, dass nicht alle Zellen einer bakteriellen Gemeinschaft metabolisch aktiv sind, sondern dass sich ein Teil in einem Ruhestadium befindet (de Giorgio and Scarborough 1995; Gasol, de Giorgio et al. 1995). Analysiert man die 16S rRNA Genfragmente (16SrDNA) einer bakteriellen Gemeinschaft, detektiert man beide, die aktiven und inaktiven bakteriellen Gemeinschaften. Analysiert man jedoch die 16S rRNA Sequenzen, wird hauptsächlich der aktive Teil der Gemeinschaft detektiert (Moyer, Dobbs et al. 1994).

Eine direktere Methode, um die Unterschiede in der Zusammensetzung der gesamten und der aktiven Bakteriengemeinschaft zu erhalten, basiert auf den Einbau eines Tracers in die DNA der aktiven Bakterien. Bromodeoxyuridine (BrdU) dient als Thymidinanalog und wird in die neu synthetisierte DNA von aktiv wachsenden Bakterien eingebaut (Borneman 1999). DNA, die BrdU eingebaut hat, kann man mittels Antikörper binden und dann von der übrigen DNA mechanisch trennen. Anschließend kann man mit dieser Technik, die ihre Wurzeln in der Immunohistochemie hat (Morstyn, Hsu et al. 1983; Gomez, Ardakani et al. 1995), die erhaltene DNA verwenden, um alle gängigen molekularen Analysen durchzuführen.

Übersicht über die Inhalte der vorliegenden Arbeit. Ziel dieser Studie war es die Beiträge von Archaea und Bakterien an der Leucin-Aufnahme (als Indikator der Heterotrophie) der gesamten Prokaryotengemeinschaft in der Wassersäule des östlichen Atlantiks zu quantifizieren. Zusätzlich wurde die Zusammensetzung der Gemeinschaften in verschiedenen Tiefen analysiert, um die Unterschiede der Gemeinschaften aktiver und inaktiver Bakterien zu beschreiben.

Kapitel 1 vergleicht die Leucin-Aufnahmeraten von Bakterien mit jenen von Archaea. Spezielle Inhibitoren, die die Proteinsynthese hemmen, wurden verwendet, um selektiv Bakterien oder Archaea zu hemmen. Als Bakterien-Hemmer wurde Erythromycin (Kohanski et al. 2010) zugefügt und Diphtheria Toxin, um die Aktivität der Archaea (Stein et al. 1996) zu hemmen. Die zwei Inhibitoren wurden getrennt zu den Wasserproben zugefügt, wodurch es möglich wurde zwischen der Leucin-Aufnahmerate von Bakterien und Archaea zu unterscheiden. Die Spezifität von Erythormycin wurde durch die Kombination von Mikroautoradiographie mit Catalyzed Reporter Deposition-Fluorscence In Situ Hybridization (MICRO-CARD-FISH) getestet.

Kapitel 2 beschreibt die Zusammensetzung der aktiven und der gesamten bakteriellen Gemeinschaft, ermittelt durch die fingerprinting-Technik ARISA (Automated Ribosomal Intergenic Spacer Analysis). Mit ARISA wird die ITS1 Region zwischen dem 16S und dem 23S rRNA Gen auf dem rRNA Operon untersucht, weil diese bei den verschiedenen bakteriellen Genotypen sehr variabel ist bezüglich der Länge und Sequenz der Nukleotide (Fisher and Triplett 1999). Nachdem die mit BrdU markierte DNA vom restlichen DNA-Pool mittels Immunocapturing getrennt wurde, konnte die Analyse mit ARISA die

Zusammensetzung der metabolisch aktiven Bakteriengemeinschaft mit jener der Gesamtbakterien-gemeinschaft verglichen werden.

Introduction

Importance of prokaryotes for marine biogeochemical cycles. In the first half of the 20th century microbiologists became aware that Bacteria are abundant in the open ocean with abundances, based on plate counting techniques, ranging between 100-1000 ml^{-1} (Waksman and Renn 1934; ZoBell 1946). Only in the 70ies of the last century, however, marine prokaryotes were enumerated directly under the epifluorescence microscope using nucleic acid stains (Hobbie, Daley et al. 1977) resulting in prokaryotic abundances of 10^6 ml^{-1} rather than about 10^3 ml^{-1} as obtained by plate-counting techniques. These three orders of magnitude higher abundances of prokaryotes in the ocean than previously reported led to a number of questions concerning the growth and function of marine prokaryotes. Consequently, the marine microbes, traditionally seen as the terminal end of marine food webs, were placed more centrally into the context of food webs (Williams 1972). This paradigm shift culminated in the formulation of the 'microbial loop' hypothesis by Azam et al. (1983) building on the ideas of Pomeroy (1974) and Williams (1972). The main idea of the microbial loop hypothesis was originally that heterotrophic bacteria are converting dissolved organic matter (DOM) into particulate organic matter (POM) via bacterial biomass production. Only bacteria can take up DOM, all the other heterotrophs in the food web require POM. This heterotrophic bacterial biomass is grazed by flagellates and ciliates and hence channeled to the higher trophic levels of the marine food web. Numerous later studies, however, showed that only a small fraction of the organic carbon taken up by heterotrophic prokaryotes is really transferred to the marine metazoan food web. The majority of the organic carbon taken up by the microbes remains within the microbial food web (Azam and Malfatti 2007). The current view is that the microbial and the metazoan food web are competing for the phytoplankton derived organic carbon (Herndl, Agogué et al. 2008).

Archaea and their distribution. During the past decade, however, the general view that Bacteria are the main prokaryotes in the global ocean was challenged as evidence has been accumulating that Archaea are abundant in the oceanic water column as well (DeLong 1992; Fuhrman, McCallum et al. 1992). Subsequent studies revealed the ubiquitous presence of mesophilic Archaea in all major oceanic basins (Karner et al., 2001; Teira et al., 2004). The main groups of Archaea in the oxygenated water column of the ocean are Crenarchaeota and Euryarchaeota. Marine Crenarchaeota Group I [MCGI, now also coined Thaumarchaeota

(Brochier-Armanet et al., 2008)] are by far more abundant than Euryarchaeota and comprise about one third of the total prokaryotic abundance in the waters below the euphotic zone in the global ocean (Karner et al., 2001; Herndl et al., 2005; Varela et al., 2008). In the Pacific, the contribution of Crenarchaeota increased significantly below 150 m depth and reached a maximum of 40 % of the total prokaryotic abundance (Karner et al., 2001). In the northern North Atlantic, archaeal abundance (Crenarchaeota and Euryarchaeota) even exceeded the bacterial abundance in the bathypelagic water masses (Teira et al., 2004).

Archaeal metabolic activity and their biogeochemical roles. Although several studies showed that Archaea are dominant in deep waters, little is known about their metabolical activity and their biogeochemical roles. Recent studies showed that a large part of MCGI incorporate dissolved inorganic carbon (DIC) (Wuchter et al., 2003) and use ammonia as an energy source (Könneke et al., 2005; Wuchter et al., 2006 Ingalls et al. 2006, Hansman et al. 2009) suggesting a chemoautotrophic life style. Herndl et al. (2005) found that a substantial amount of dissolved inorganic carbon equivalent to up to 84 % of the heterotrophic prokaryotic production was incorporated by Archaea. The notion of archaeal chemoautotrophy is also supported by the high abundance of the archaeal *amo*A genes detected below the euphotic zone. This gene encodes the alpha subunit of the enzyme ammonia monooxygenase which catalyzes the first step in the nitrification from ammonia to hydroxylamine. Quantitative PCR analysis of this gene indicates that the distribution of the archaeal *amo*A gene is orders of magnitudes higher than the bacterial *amo*A (Agogue et al., 2008; De Corte et al., 2009; Mincer et al., 2007) suggesting that MCGI might be more important for oceanic nitrification than Bacteria. The assumption that MCGI are important nitrifiers in oceanic waters is based, however, entirely on the quantification of this a*mo*A gene in comparison to bacterial *amo*A gene abundance.

Although there are numerous reports that MCGI are chemoautotrophs, it was also documented that MCGI are taking up amino acids as well. The MICRO-CARD-FISH approach allows the determination of the distribution of activity on a single cell-level. With this method, Teira et al. (2006) and Varela et al. (2008) were able to demonstrate that MCGI prefer to take up D- over L-aspartic acid in the bathypelagic Atlantic. Kirchman et al. (2007) showed that several organic compounds (leucine, a mixture of amino acids, protein, glucose, and extracellular polysaccharides) were taken up by MCGI in the water column (0-500 m) of

the western Arctic. Although these results indicate that a certain fraction of the MCGI is, at least partially, living a heterotrophic life mode, the relative contribution of Archaea to total prokaryotic DIC fixation and heterotrophy remains to be shown.

Differences and changes over depths in the community structure of active and dormant bacteria. Heterotrophic prokaryotic abundance and activity decline with depth in the water column due to the decreasing substrate supply largely in the form of sinking particulate organic matter originating from the euphotic layer (Byung and Azam 1988; Ducklow, Steinberg et al. 2001). Similarly, prokaryotic community compositions are changing with depth in response to altered substrate supply and to changing temperature regimes (Moeseneder et al, 2001). However, it has been shown that not all of the cells in a given bacterial community are metabolically active but rather, a certain fraction is in a dormant stage (de Giorgio and Scarborough 1995; Gasol, de Giorgio et al. 1995). Performing bacterial community analysis using the 16S rRNA gene fragments (16S rDNA) is covering both the active and the dormant fraction of the bacterial community, while the 16S rRNA analysis reveals the more active bacterial community (Moyer, Dobbs et al. 1994). A more direct method to differentiate the composition of the total and the active bacterial community is based on the incorporation of a tracer in the DNA of growing and hence, active bacteria. Bromodeoxyuridine (BrdU) serves as analog to thymidine and consequently, is incorporated into the DNA of actively growing cells (Borneman 1999). The DNA resulting from the incorporation of BrdU can be separated using immunocapturing techniques which have their roots in immunohistochemistry (Morstyn, Hsu et al. 1983; Gomez, Ardakani et al. 1995). Subsequently, this BrdU-labeled DNA can be analyzed using the diverse range of available molecular techniques.

Thesis outline: The aim of this study was to specifically estimate the contribution of Archaea versus Bacteria to total leucine incorporation (as an indicator of heterotrophy) throughout the water column of the eastern Atlantic. Additionally, the community structure was analyzed to describe the differences in the composition of the community between the active and total fraction of the bacterial community at different depths.

Chapter 1 compares the leucine incorporation rates of Bacteria with that of the Archaea. Specific protein synthesis inhibitors were used to selectively inhibit either Bacteria or Archaea. As bacterial inhibitor, erythromycin (Kohanski et al. 2010) was applied and to inhibit archaeal activity (Stein et al. 1996), diphtheria toxin was used. With these two inhibitors applied separately to water samples, it was possible to distinguish between bacterial and archaeal leucine incorporation rates. The specificity of the erythromycin was tested using microautoradiography in combination with catalyzed reporter deposition-fluorescence in situ hybridization (MICRO-CARD-FISH).

Chapter 2 describes the composition of the active and the total bacterial community using ARISA (automated ribosomal intergenic spacer analysis) as a fingerprinting method. ARISA targets the ITS1 region between the 16S and the 23S rRNA genes in the rRNA operon which is highly variable (Fisher and Triplett 1999). After separation of the BrdU-labeled DNA from the total pool of DNA by immunocapturing, this DNA was also used for ARISA to determine the community composition of the active fraction of the bacterioplankton.

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Chapter 1: Heterotrophic activity of Archaea and Bacteria throughout water column of the eastern Atlantic

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Abstract

The abundance and leucine incorporation rate of Archaea and Bacteria was determined throughout the water column along a transect in the eastern Atlantic. Archaeal and bacterial abundances were determined by Catalyzed Reporter Deposition - Fluorescence In Situ Hybridization (CARD-FISH). Bacteria dominated throughout the water column, although their contribution to total prokaryotic abundance in the bathypelagic layer (1000 - 4000 m) was lower than in the surface and mesopelagic layers (0 - 1000 m). Crenarchaeota contributed about 30 ± 12% to the total prokaryotic abundance with a generally higher contribution in the bathypelagic layer than in the surface and mesopelagic layers. Euryarcheota contributed less than 5% to the total prokaryotic abundance throughout the water column. Leucine incorporation rates were determined for the total prokaryotic community as well as for Bacteria and Archaea separately using selective inhibitors. The bacterial inhibitor erythromycin and the archaeal inhibitor diphtheria toxin were used to determine the contribution of Archaea and Bacteria, respectively, to total heterotrophic activity. Using microautoradiography in combination with CARD-FISH, the specificity of erythromycin was tested. Erythromycin was found to selectively inhibit bacterial activity. The contribution of Bacteria to total leucine incorporation amounted to 65 ± 15% of total prokaryotic leucine incorporation decreasing in their contribution with depth. The mean cell-specific leucine incorporation rate of Crenarchaeota ($3.7 \pm 2.0 \times 10^{-5}$ fmol cell^{-1} d^{-1}) was 5 times lower than that of Bacteria ($18.3 \pm 17.6 \times 10^{-5}$ fmol cell^{-1} d^{-1}) in the surface and mesopelagic layer. In the bathypelagic layer, however, cell-specific leucine incorporation rates of Crenarchaeota were similar to those of Bacteria (1.8×10^{-5} fmol cell^{-1} d^{-1} for Crenarchaeota, 2.3×10^{-5} fmol cell^{-1} d^{-1} for Bacteria). Taken together, our results indicate that Crenarchaeota and Bacteria exhibit similar heterotrophic activity on a per-cell level in the bathypelagic waters of the Atlantic, while in the surface and upper mesopelagic waters, cell-specific heterotrophic archaeal activity is about one order of magnitude lower than that of Bacteria.

Introduction

Over the past decade, the notion emerged that mesophilic Archaea are ubiquitously present in all the major oceanic basins (Karner et al. 2001, Teira et al. 2006a, Kirchman et al. 2007). Among, the mesophilic marine Archaea, the marine Crenarchaeota Group I (MCGI) are by far the most abundant archaeal group contributing about one third to the total prokaryotes in the waters below the euphotic zone (Karner et al. 2001, Herndl et al. 2005, Varela et al. 2008).

Although MCGI are abundant in deep waters, little is known about their metabolic rates and consequently, their biogeochemical role in the ocean. Recent studies suggest that a substantial fraction of the MCGI is chemoautotrophic, incorporating dissolved inorganic carbon (DIC) (Wuchter et al. 2003) and using ammonia as an energy source (Könneke et al. 2005, Wuchter et al. 2006, Ingalls et al. 2006, Hansman et al. 2009). Quantitative PCR of the archaeal gene encoding the ammonia monooxygenase subunit A (amoA) indicates that archaeal *amo*A gene abundance is orders of magnitude higher than bacterial *amo*A in oceanic waters (Agogue et al. 2008, De Corte et al. 2009, Mincer et al. 2007) suggesting that MCGI might be more important for oceanic nitrification than Bacteria, although group or even domain-specific nitrification measurements have not been reported thus far.

Not all MCGI are autotrophs, however, but apparently heterotrophs or, at least mixotrophs, taking up amino acids as revealed by microautoradiography in combination with fluorescence in situ hybridization (MICRO-CARD-FISH) (Ouverney and Fuhrman 1999). MCGI have been shown to take up preferentially D-aspartic acid over L-aspartic acid in the bathypelagic waters of the Atlantic (Teira et al. 2006b, Varela et al. 2008). In the Arctic, several organic compounds (leucine, mixture of amino acids, proteins, glucose, and extracellular polysaccharides) are taken up by MCGI in the water column (0-500 m) of the western Arctic Ocean (Kirchman et al. 2007). Although these results obtained on a single-cell level indicate that a certain fraction of the MCGI is also heterotrophic, the relative contribution of Archaea to DIC fixation and/or heterotrophy of the bulk prokaryotic activity remains to be shown.

The aim of this study was to estimate the contribution of Archaea and Bacteria to total prokaryotic leucine incorporation (as an indicator of heterotrophy) throughout the water

column of the eastern Atlantic. To distinguish archaeal from bacterial leucine incorporation, specific inhibitors were used (Yokokawa et al. in prep). Erythromycin specifically affects protein synthesis in Bacteria (Kohanski et al. 2010) but does not affect archaeal protein synthesis, whereas diphtheria toxin inhibits protein synthesis in Archaea but not in Bacteria (Madigan and Martinko 2006). Archaea and eukaryotes have homologous amino acid sequences of the EF-2 protein, which is diphtheria toxin-sensitive (Stein et al. 1996). Using this dual inhibitor approach, we found that the contribution of archaeal leucine incorporation contributes about 25% to the total prokaryotic leucine incorporation throughout the water column.

Material and Methods

Sampling was carried out aboard the R/V Sarmiento de Gamboa (Consejo Superior de Investigaciones Cientificas, Spain) in the eastern Atlantic during the CAIBEX-2 and CAIBEX-3 cruises occupying six stations in July and August 2009 (Fig. 1). Water samples were collected with clean 12 L Niskin-bottles (General Oceanics) attached to a CTD (conductivity, temperature, depth) system (Seabird Model 9 plus) and tapped into acid-washed polycarbonate bottles (1 L) for measuring several microbial parameters as described below.

*Testing the specificity of the bacterial inhibitors erythromycin and streptomycin and the archaeal inhibitor diphtheria toxin***.** Several inhibitors were used to test their specificity to inhibit protein synthesis of either Bacteria or Archaea. Erythromycin (product #: 45673, Sigma-Aldrich) and streptomycin (product #: 85886, Sigma-Aldrich) was tested as bacterial inhibitors and diphtheria toxin (product #: D0564, Sigma-Aldrich) as inhibitor of archaeal protein synthesis. Initially, leucine incorporation rates with and without the bacterial inhibitors streptomycin (10 µg mL^{-1} final concentration) and erythromycin (10 µg mL^{-1} final conc.) and the archaeal inhibitor diphtheria toxin (10 µg mL^{-1} final conc.) were compared on individual samples. Summing up the percentages of inhibition of leucine incorporation in the erythromycin- or streptomycin-treated samples with that of the diphtheria toxin-treated samples revealed that streptomycin was not effectively inhibiting bacterial leucine

incorporation as the sum of the percentage of inhibition in streptomycin- and diphtheria toxin-treated samples were significantly lower than 100%. In contrast, the sum of inhibition in erythromycin- and diphtheria toxin-treated samples averaged 104 ± 26% compared to the untreated samples, tentatively indicating high specificity of erythromycin as bacterial inhibitor (Table 1). The specificity of erythromycin in inhibiting bacterial protein synthesis was also tested using microautoradiography with [^{3}H]-leucine as substrate in combination with CARD-FISH (described below). Consequently, erythromycin was used in the subsequent measurements as bacterial protein synthesis inhibitor and diphtheria toxin to inhibit archaeal activity.

Measuring leucine incorporation rates. Triplicate subsamples (1.5 mL) were dispensed into screw-capped centrifuge tubes (Scientific System Inc., 2.0 mL Screw Tube) and amended with 5 nmol L^{-1} (final conc.) of [^{3}H]-leucine (product #: NET460A080MC, PerkinElmer) and incubated at in situ temperature (± 2ºC) in the dark for 1h (for samples from 0-200m depth) or for 24 h (for samples from 250m to bottom) following the protocol of Kirchman (2001). One trichloroacetic acid (TCA) killed blank was used per sample. The incubation was stopped by adding TCA (final conc. 5%) and the samples centrifuged at 18,000 x *g* for 10 min, followed by an ethanol rinse (80%, ice-cold). The pelleted sample was radio-assayed with a liquid scintillation counter (Tri-Carb 3100TR, PerkinElmer) using Ultima-GOLD (Packard) scintillation cocktail. Quenching was corrected by external standard channel ratio. The disintegrations per minute (DPM) of the TCA-killed blank were subtracted from the average DPM of the samples, and the resulting DPM converted into leucine incorporation rates.

The relative contribution of Archaea and Bacteria to total prokaryotic leucine incorporation was determined using erythromycin and diphtheria toxin as bacterial and archaeal inhibitors of protein synthesis, respectively, and compared with the leucine incorporation in the untreated sample. The inhibitors were added to the respective treatments concurrently with the [^{3}H]-leucine. The contribution of Bacteria and Archaea to total prokaryotic leucine incorporation is calculated as follows: (1- leucine incorporation measured in the erythromycin- or diphtheria toxin-treatment/ leucine incorporporation in the untreated sample) x 100.

CARD-FISH. The abundance of Bacteria, Crenarchaeota and Euryarchaeota was determined by CARD-FISH using specific oligonucleotide probes (Teira et al. 2004). Water samples of 20 to 40 mL were fixed with paraformaldehyde (2% final conc.) and incubated in the dark overnight. Thereafter, samples were filtered through 0.2 µm polycarbonate filters (Millipore, GTTP) with 0.45 µm cellulose nitrate support filters (Millipore, HAWP), washed with Milli-Q water, air dried and stored at -20°C until further processing.

Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated either with lysozyme for the Bacteria probe-mix (Eub338, Eub338II and Eub338III) (Daims et al. 1999) or with proteinase-K for marine Euryarchaeota group II probe Eury806 (Teira et al. 2006a) and for the MCGI probe-mix [Cren537 (Teira et al. 2006a) and GI-554 (Massana et al. 1997)]. Hybridization buffer with 55% formamide was used for the Bacteria probe-mix and 20% formamide for Eury806 and the MCGI probe-mix.

The probes were added at a final concentration of 0.28 ng μL^{-1} (0.05µM) and hybridization was performed at 35°C for 12 to 15 h. Thereafter, the fluorescence signal was amplified by incubating the filters with H_2O_2 (0.0015%) and tyramide-Alexa488 at 37°C for 30 min. Filters were stained and mounted on slides with a DAPI (4,6-diamidino-2-phenylindole) mix (5.5 parts Citifluor, 1 part Vectashield, 0.5 parts PBS with DAPI at a final concentration of 1 µg mL^{-1}). Cells were detected under a Zeiss Axioplan-2 epifluorescence microscope with an Hg-lamp and corresponding filters for Alexia488 and DAPI.

MICRO-CARD-FISH. Since the bulk leucine incorporation measurements with erythromycin in combination with diphtheria toxin indicated that erythromycin might more effectively inhibit bacterial leucine incorporation than streptomycin, the specificity of erythromycin in inhibiting bacterial leucine incorporation was tested. For this specificity test, water from 300m depth of St. 3-23 (Fig. 1) was collected. To 40 mL subsample spiked with erythromycin, 5nM [^{3}H]-leucine (final conc.) was added and incubated in the dark at *in situ* temperature for 24 h. Controls without the addition of erythromycin were also run. Incubations were terminated by adding paraformaldehyde (2% final conc.) and subsequently, the samples were stored at 4°C in the dark for 12 h. Thereafter, the samples were filtered onto 0.2 µm pore-size polycarbonate filters (Millipore, GTTP, 25 mm filter diameter) supported by cellulose nitrate filters (Millipore, HAWP, 0.45 µm pore-size), rinsed twice with Milli-Q

water, dried, and stored in a microcentrifuge vial at -20°C until further processing in the home laboratory.

The FISH protocol for Bacteria was carried out as described above. Microautoradiographic development was performed as outlined in Teira et al. (2006b) by transferring previously hybridized filter sections onto slides coated with photographic emulsion (type NTB-2 melted at 43°C for 1 h). The slides were then placed in a light-tight box containing a drying agent and incubated for exposure at 4°C for 4 d. Finally, the slides were developed and fixed following Kodak's specifications [in Dektol developer (1:1 dilution with Milli-Q water) for 2 min, in Milli-Q water for 10 s, in fixer for 5 min and subsequently in Milli-Q water for 2 min]. Before completely dried, filter sections were removed and cells counterstained with a DAPI-mix and examined under a Zeiss Axioplan-2 epifluorescence microscope. The presence of silver grains surrounding the cells was checked in the transmission mode of the microscope. For each microscope field, we enumerated the relative abundance of probe-positive cells compared to DAPI-stained cells and the number of cells surrounded with a silver grain halo.

Results

Prokaryotic, bacterial and archaeal abundance throughout the water column. Prokaryotic abundance ranged from 5.0×10^4 to 7.2×10^5 cells mL^{-1} in surface and mesopelagic layers (88 - 700 m depth), and decreased exponentially with depth [range: 4.4 to 9.5×10^4 cells mL^{-1} in the bathypelagic layer (1100 - 3000 m depth), Fig. 2].

The recovery efficiency of prokaryotes using CARD-FISH (sum of Bacteria, Euryarchaeota and MCGI) was on average 86 ± 19% (± S.D., n = 22). The contribution of Bacteria to total prokaryotic abundance ranged from 45% to 78% and that of Archaea from 10% to 54% (Fig. 2). Bacteria dominated over Archaea throughout the water column (paired t-test; $p < 0.001$, Table 2). On average, Bacteria contributed 58 ± 9% to the total prokaryotic abundance with a higher contribution in surface and mesopelagic layers than in the bathypelagic layer (t-test; $p = 0.002$, Table 2). In contrast, the mean contribution of MCGI was 30 ± 12% with a higher contribution in the bathypelagic layer than in the surface and mesopelagic layers (t-test; $p = 0.011$, Table 2). Euryarchaeota always contributed less than 5% to total prokaryotic abundance (data not shown).

Testing the specificity of the inhibitors using MICRO-CARD-FISH. MICRO-CARD-FISH on Bacteria (collected at St. 3-23, 300 m depth) was used to test the specificity of erythromycin. At this stations, Bacteria comprised 62% of the total prokaryotes and 80% of the Bacteria took up leucine (cell with silver grain halo) in the absence of erythromycin (untreated sample). In the erythromycin-treated samples, only 3% of the Bacteria took up leucine indicating a high specificity of erythromycin to inhibit bacterial leucine uptake. No MCGI cells with silver grain halos were detected neither in untreated nor in erythromycin-treated samples. The specificity of diphtheria toxin was not determined because of logistic problems.

Prokaryotic leucine incorporation in untreated and erythromycin- and diphtheria toxin-treated samples. At all the six stations, prokaryotic leucine incorporation rates in untreated samples decreased by 2-3 orders of magnitude from the surface layer to the bathypelagic layer ranging from 205.56 - 0.26 pmol L^{-1} d^{-1} (Fig. 3). In the erythromycin-treated samples, leucine incorporation rates ranged from 47.15 - 0.13 pmol L^{-1} d^{-1} while in the diphtheria toxin-treated samples leucine incorporation ranged from 145.59 - 0.23 pmol L^{-1} d^{-1} (Fig. 3). The percentage of inhibition of leucine incorporation by erythromycin was always higher than the inhibition by diphtheria toxin except at St. 2-34 at 3000 m depth (Table 1). The sum of the percentage of inhibition of leucine incorporation by erythromycin and diphtheria toxin was on average 104 ± 26% (range: 62 - 153%, n = 28, Table 1). In 8 out of the 28 measurements, the sum of inhibition was higher than 110% (Table 1), indicating either unspecific inhibition or larger than expected variability in the determination of the leucine incorporation rates in one of the three treatments per sample used to calculate the percentage of inhibition. Therefore, these 8 measurements with an inhibition >110% were excluded from the below calculations. Excluding these 8 measurements, the mean percentage of inhibition of all the data shown in Table 1 is 91±17%.

The average percentage of inhibition by erythromycin and hence, the putative contribution of Bacteria to total prokaryotic leucine incorporation was 65 ± 15% (n = 22), Table 2). The average percentage of inhibition by diphtheria toxin, i.e., the putative contribution of Archaea to prokaryotic leucine incorporation was 26 ± 12% (n = 20, Table 2). The percentage of the putative contribution of Bacteria to leucine incorporation decreased significantly from the surface and mesopelagic waters (72 ± 13%) to the bathypelagic layer

(56 ± 14%, t-test, $p < 0.01$, Table 2, Fig. 4). There was no significant depth-related trend in the putative contribution of Archaea to the prokaryotic leucine corporation from the surface and mesopelagic layer to the bathypelagic layer (t-test, $p = 0.262$) (Fig. 4).

Single-cell activity of Bacteria and MCGI. Cell-specific leucine incorporation rates of Bacteria were calculated from the difference in leucine incorporation in the absence and presence of erythromycin and the fraction of bacterial cells identified by CARD-FISH relative to DAPI-stained cells. Cell-specific leucine incorporation rates of Bacteria were up to 2 orders of magnitude higher in the surface and mesopelagic layers than in bathypelagic layers (Mann-Whitney, $p=0.004$, range: 51.9 to 0.6 x 10^{-5} fmol leucine $cell^{-1}$ d^{-1}, Fig. 5, Table 2).

In MCGI, cell-specific leucine incorporation rates did not exhibit depth-related trends (Fig. 5). The average cell-specific leucine incorporation rate of MCGI in mesopelagic layers was not significantly different from that of the bathypelagic layers (Mann-Whitney, $p = 0.056$, Table 1). In the bathypelagic layers, there was no significant difference in cell-specific leucine incorporation rates between Bacteria and MCGI (t-test, $p = 0.499$, Table 2).

Discussion

Using erythromycin and diphtheria toxin to inhibit bacterial and archaeal protein synthesis, respectively, we found that Archaea contribute about 25% to the bulk leucine incorporation of prokaryotes in the water column of the eastern Atlantic. Euryarchaeota were present in abundances of only less than 5% of DAPI-stained cells throughout the different layers of the water column. This indicates that the detected archaeal leucine incorporation is most likely due to MCGI confirming earlier findings obtained by MICRO-CARD-FISH that some MCGI might be heterotrophic (Ouverney and Fuhrman 2000, Teira et al. 2006a, b, Kirchman et al. 2007, Varela et al. 2008).

The contribution of MCGI to prokaryotic leucine incorporation increases with depth (Table 2) in a similar way as MCGI abundance increases in the Pacific (Karner et al. 2001), the Atlantic (Herndl et al. 2005, Teira et al. 2006a, Varela et al. 2008), the Mediterranean Sea (De Corte et al. 2009) and the Arctic (Kirchman et al. 2007, Alonso-Saez et al. 2007).

Although there are a number of studies reporting the contribution of MCGI to total prokaryotic abundance, there are only a few studies on the contribution of MCGI to prokaryotic activity. Archaeal chemoautotrophy, based on nitrification as energy source has been shown to be widespread (Herndl et al. 2005, Wuchter et al. 2006). In the mesopelagic waters of the Atlantic, high DIC fixation rates by the prokaryotic community were found (Reinthaler et al. 2010), on the one hand, and on the other hand, several studies have shown heterotrophic activity of MCGI (Kirchman et al. 2007, Varela et al. 2008). These studies, however, did not determine the contribution of Archaea or MCGI to the leucine incorporation of the prokaryotic community.

MCGI contribute about 25% to the prokaryotic leucine incorporation in the lower latitudes of the North Atlantic (Table 1, 2) increasing with depth while the bacterial contribution slightly decreases with depth (Fig. 4). Yokokawa et al. (in prep.) found that diphtheria toxin inhibited up to 37% of the leucine incorporation in both the upper and deeper layers of the Pacific. Hence, the results obtained for the lower North Atlantic are in the same range as in the Pacific. Recently, the bacterial inhibitors streptomycin and ampicillin were used to estimate archaeal activity at a single station in the Red Sea (Ionescu et al. 2009). These authors found a high contribution of Archaea to DIC fixation at around 350m and 550m depth and a generally low archaeal contribution to prokaryotic leucine incorporation (Ionescu et al. 2009). Thus, the contribution of Archaea to heterotrophy in the ocean might be variable and depth dependent with generally relatively higher contributions to heterotrophy in the deeper (bathypelagic) waters than in surface and upper mesopelagic layers.

Cell-specific leucine incorporation rates of MCGI were similar to those of Bacteria in the bathypelagic layer, however, five times lower in the top 1000m layer (Table 2, Fig. 5). These cell-specific leucine incorporation rates are most likely conservative estimates, as they are based on the assumption that all MCGI are heterotrophs, which is most likely not the case. Likewise, we assumed that all Bacteria are heterotrophs, which is unlikely as well given the fairly high contribution of DIC-fixing Bacteria in the deep waters of the Arctic and Atlantic (Alonso-Saez et al 2010, Reinthaler et al, 2010, Varela et al. in press).

Taken together, this study showed the potential of erythromycin and diphtheria toxin as protein synthesis inhibitors in Bacteria and Archaea, respectively, to estimate the relative contribution of these two prokaryotic domains to the overall activity of the prokaryotic community. The efficiency of erythromycin as inhibitor of bacterial activity was shown by

MICRO-CARD-FISH, indicating that 97% of the bacterial cells were inhibited in their leucine uptake. Based on our data, we conclude that a substantial part (average: 25% over the entire water column) of the heterotrophic prokaryotic activity in the North Atlantic is due to MCGI. The cell-specific heterotrophic activity of Bacteria and MCGI appears to be similar in the bathypelagic waters. Given the generally high abundance of MCGI in bathypelagic waters of the global ocean, there is evidence now that deep-water heterotrophic prokaryotic activity is mediated not only by Bacteria but to a considerable extent also by mesophilic Crenarchaeota.

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Tables and Figures

Table 1. Percentage of inhibition of leucine incorporation in erythromycin- and diphtheria-treated samples relative to bulk leucine incorporation rates, and the sum of the percentage of inhibition in the erythromycin- and diphtheria toxin-treated samples at different stations and depths in the North Atlantic (for locations of the stations see Fig. 1). ND indicates not determined.

Station	Depth (m)	% inhibition to leucine incorporation rate			
		By Streptomycin	By Erythromycin	By Diphtheria toxin	Sum
2-34	100	-	78	26	104
	300	4	85	26	111
	700	13	67	35	103
	1100	3	66	42	109
	3000	27	30	37	67
2-56	120	-	81	11	92
	300	-	53	20	73
	700	-	87	66	153
	1200	-	66	44	109
	3000	-	64	56	120
2-65	80	-	77	29	106
	300	-	64	27	91
	700	-	66	38	104
	1100	-	71	8	79
	1500	-	49	13	62
3-1	100	-	91	6	97
	300	-	97	28	124
	700	-	82	21	102
	1100	-	72	30	103
	1800	-	85	56	142
3-23	100	-	78	ND	ND
	300	-	63	ND	ND
	700	-	47	21	68
	1100	-	54	17	71
	1800	-	55	46	101
3-41	100	-	84	25	109
	300	-	91	61	152
	700	-	77	53	130
	1100	-	84	63	147
	2000	-	41	28	70
Average ± SD		-	70 ± 16	33 ± 17	104 ± 26

Table 2. Mean ± SD contribution (in %) of Bacteria and marine Crenarchaeota Group I (MCGI) to total abundance, the percent contribution to leucine incorporation, and cell-specific leucine incorporation rate throughout the water column (0 - 3000 m), in the surface and mesopelagic layer (10 - 1000m depth) and in the bathypelagic layer (1000 - 3000m depth). The number of samples is given in parentheses.

Depth layer (m)	% contribution to total abundance		% contribution to leucine incorporation rates		Specific Leu incorporation rate ($x10^{-5}$ fmol $cell^{-1}$ d^{-1})	
	Bacteria	MCGI	Bacteria	MCGI	Bacteria	MCGI
0 – 3000	58 ± 9 (21)	30 ± 12 (15)	65 ± 15 (22)	26 ± 12 (20)	11.5 ± 15.4 (21)	2.4 ± 1.7 (13)
0 – 1000	63 ± 8 (12)	21 ± 9 (6)	72 ± 13 (13)	23 ± 9 (11)	18.3 ± 17.6 (12)	3.7 ± 2.0 (4)
1000 - 3000	51 ± 6 (9)	36 ± 9 (9)	56 ± 14 (9)	29 ± 14 (9)	2.3 ± 1.4 (9)	1.8 ± 1.2 (9)

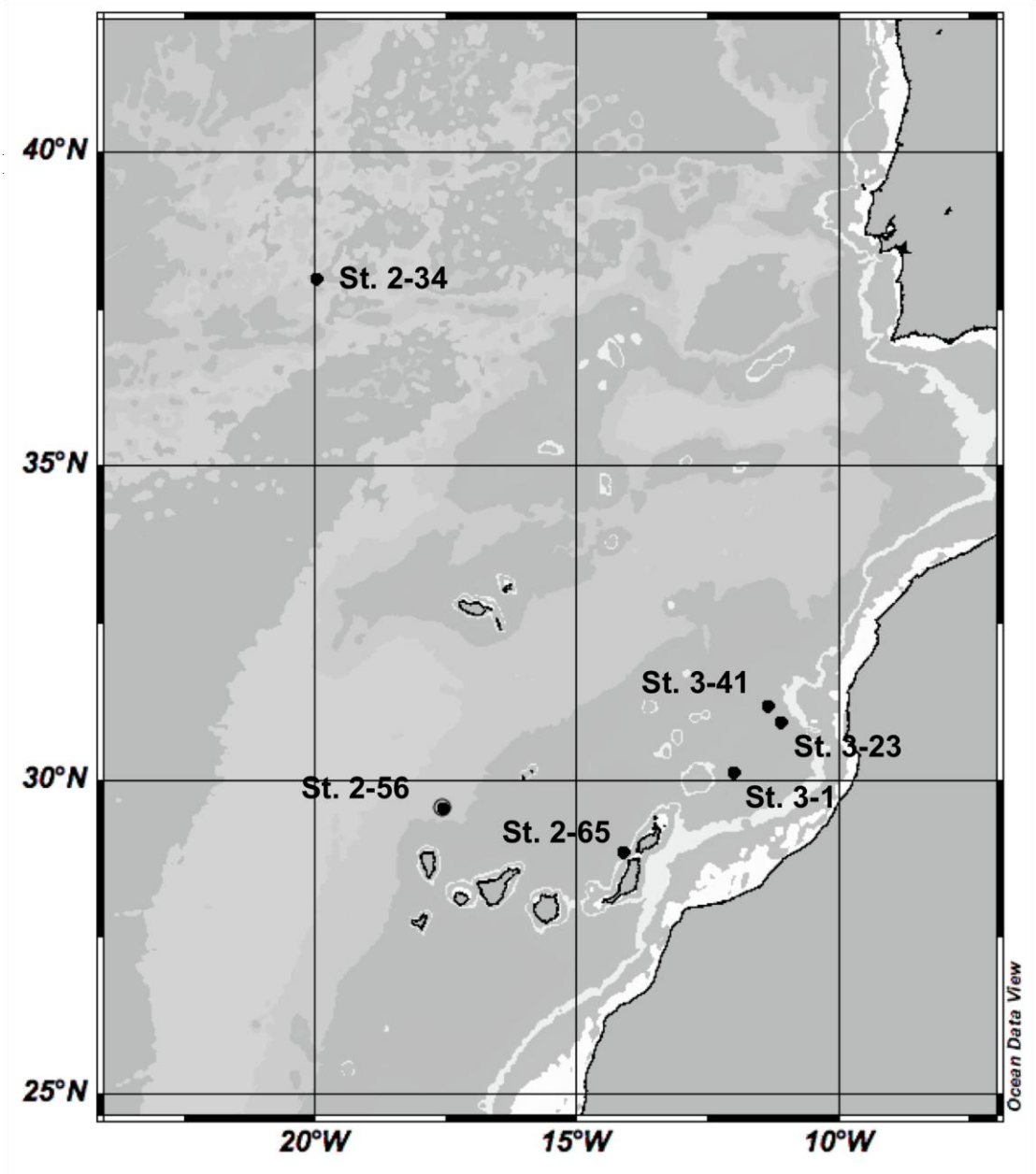

Fig. 1. Sampling sites in the eastern Atlantic occupied during the CAIBEX cruises in July and August 2009

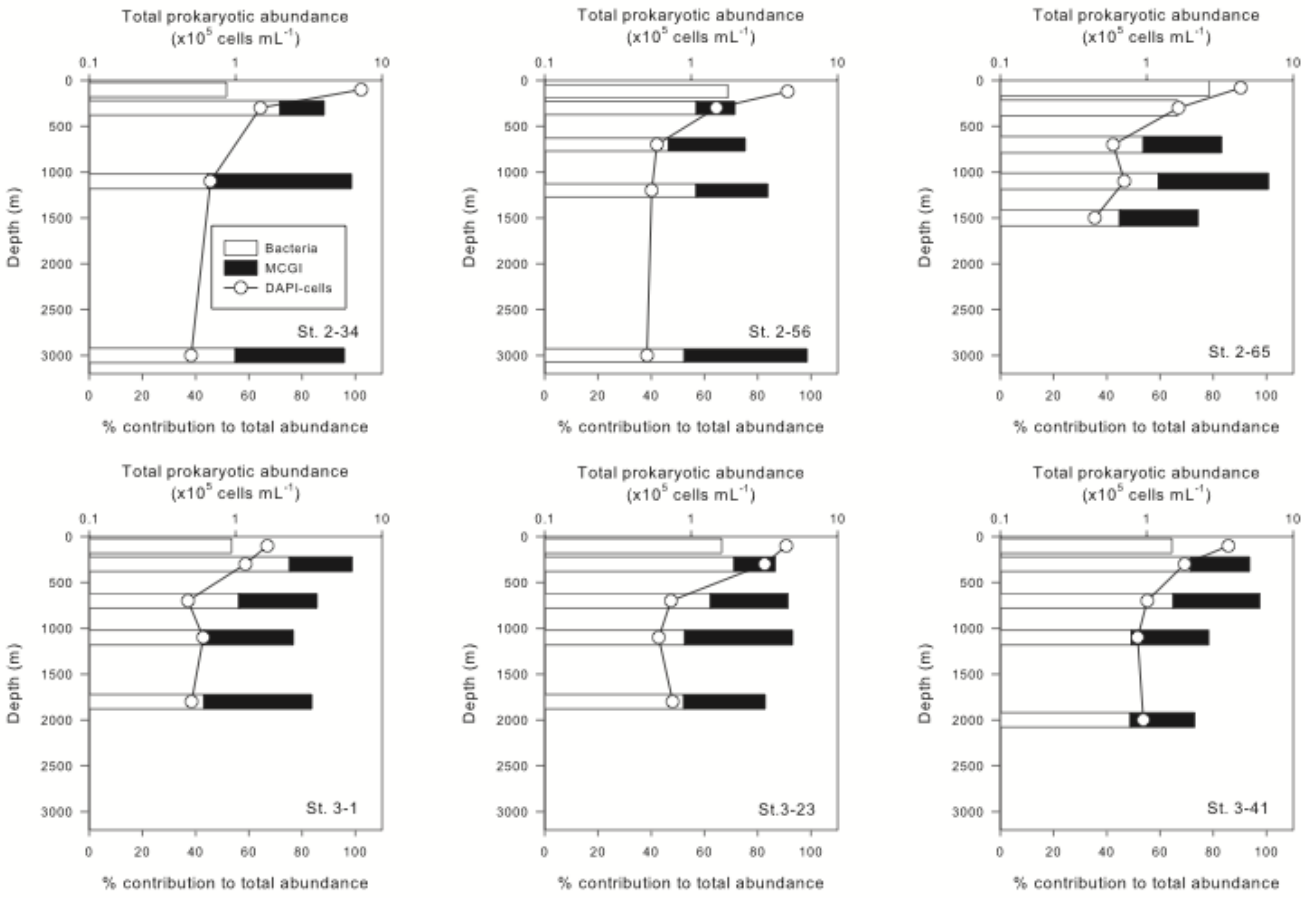

Fig. 2. Depth profiles of prokaryotic abundance (DAPI-stained cells), and % contribution of Bacteria and marine Crenarchaeota Group I (MCGI) to prokaryotic abundance at the sampling sites in the North Atlantic.

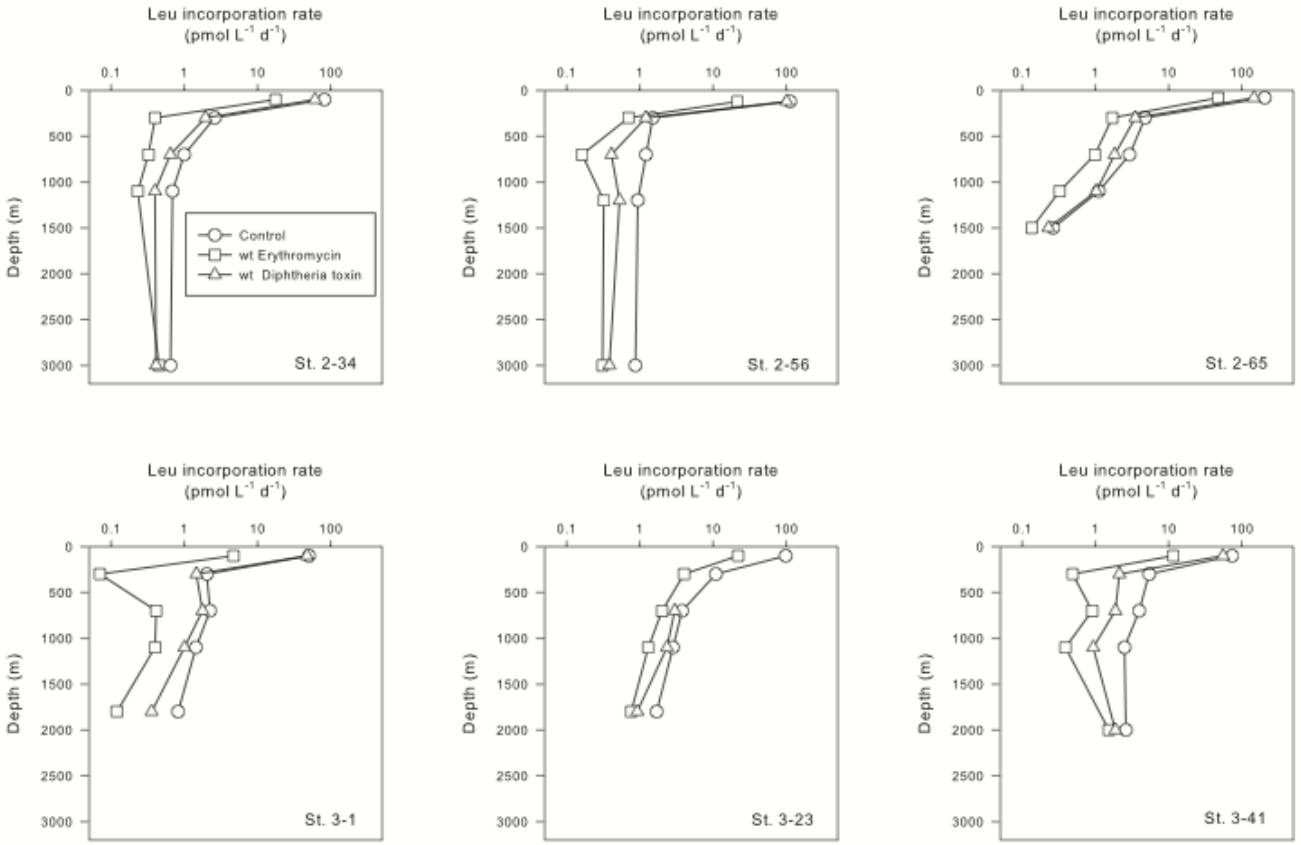

Fig. 3. Depth profiles of leucine incorporation rates without inhibitors (control) and in erythromycin- and diphtheria toxin-treated samples at the sampling sites.

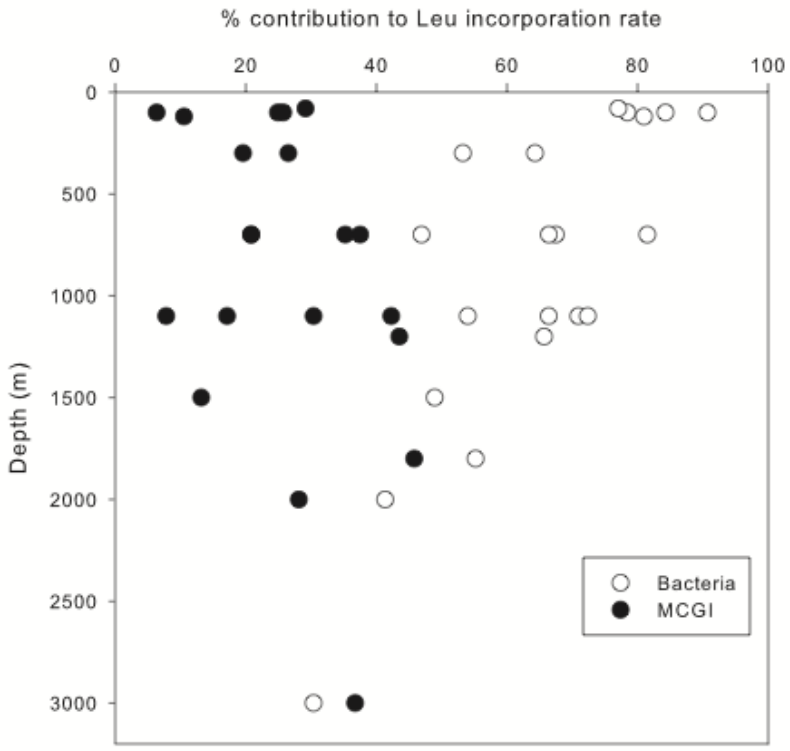

Fig. 4. Percentage of leucine incorporation rates in Bacteria and MCGI relative to total leucine incorporation throughout the water column of the stations sampled in the North Atlantic.

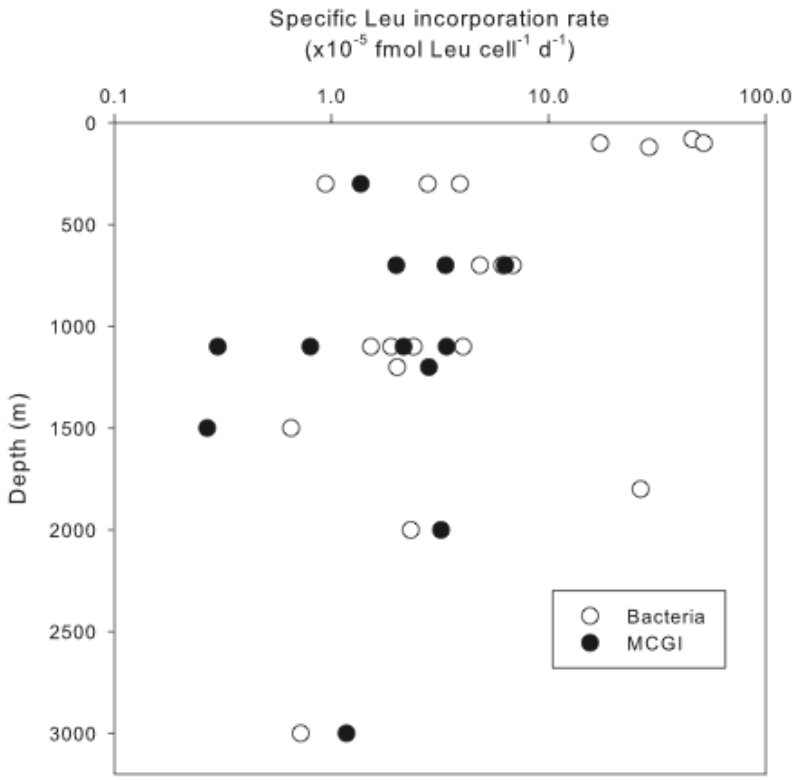

Fig. 5. Cell-specific leucine incorporations rate of Bacteria and MCGI throughout the water column.

Chapter 2: Composition of active and total bacterial communities separated by incorporating Bromodeoxyuridine (BrdU) throughout the water column of the eastern Atlantic

Abstract

To differentiate the community composition of metabolically active Bacteria from that of the total Bacteria, bromodeoxyuridine (BrdU) incorporation into the active bacterial community was used in combination with immunocapturing and automated ribosomal intergenic spacer analysis (ARISA) as a molecular fingerprinting approach. In total, 655 different operational taxonomic units (OTUs) were found of which 573 OTUs were also present in the active fraction. There was no significant difference (paired t-test) of the amount of OTUs over depth, neither in the community of total Bacteria nor in the community of active Bacteria. 44.6 % of the total and 39.1 % of the active OTUs were found to be unique. Results of the similarity analysis (ANOSIM, $r = 0.361$, $p = 0.001$) of the cluster showed a significant difference in the community composition throughout the water column between the active (Bacteria incorporating BrdU) and the total bacterial community. The similarity between surface and mesopelagic communities (100 – 1000 m depth) and bathypelagic communities (1000 – 3000 m depth) was higher for the active fraction ($r = 0.181$, $p = 0.007$) than for the total ($r = 0.332$, $p = 0.003$) bacterial community. As expected, the Shannon-Wiener diversity index (H') was significant higher for the total (paired t-test; $p = 0.002$) than for the active bacterial community. These findings suggest that the diversity of communities is mainly driven by the dormant fraction of the bacterial community, potentially forming a 'seed bank' in changing environmental conditions.

Introduction

Bacteria play an important role in the biogeochemical cycles of the oceans. Bacterial abundance and activity decline with depth in the water column due to the decreasing substrate supply largely in the form of sinking particulate organic matter (POM) from the euphotic layer (Byung and Azam 1988; Ducklow, Steinberg et al. 2001). Similarly, bacterial community composition is changing with depth in response to altered substrate supply and changing temperature (Moeseneder et al, 2001). However, it has been shown that not all members of a given bacterial community are metabolically active but a substantial, albeit variable fraction is in a dormant stage (de Giorgio and Scarborough 1995; Gasol, de Giorgio et al. 1995).

Numerous studies were carried out either to measure bacterial production or determine the community composition in a large range of marine environments. Recently, efforts were made to link the composition of communities to the function and productivity of specific species (Wagner et al. 2006, Fuhrman 2009). Performing bacterial community analysis using the 16S rRNA gene fragments (16S rDNA) is covers both the active and the dormant bacterial community, while the 16S rRNA analysis is assumed to reveal the more active bacterial community (Moyer, Dobbs et al. 1994). A direct method to differentiate the composition of the active from the total bacterial community is based on the incorporation of a tracer in the DNA of dividing bacteria. Bromodeoxyuridine (BrdU) serves as analog to thymidine and consequently, is incorporated into the DNA of actively growing cells (Borneman 1999). The DNA in which BrdU is incorporated can be separated using immunocapturing techniques which have their roots in immunohistochemistry (Morstyn, Hsu et al. 1983; Gomez, Ardakani et al. 1995). Subsequently, the BrdU-labeled DNA can be analyzed using the entire range of available molecular techniques. The aim of this study was to apply this method on bacterioplankton of the open North Atlantic to compare the composition of the actively growing fraction of the bacterial community with that of the bulk bacterial community and to determine potential shifts in the active community throughout the water column.

Material & Methods

Study site and sampling. Sampling was carried out aboard the R/V Sarmiento de Gamboa (Consejo Superior de Investigaciones Cientificas, Spain) during the CAIBEX2 and CAIBEX3 research cruises. Six stations located in the eastern Atlantic were sampled between July and August 2009 (Fig 1): during CAIBEX 2 St. 2-52 (29.9° N, 19.2° W); during CAIBEX3 St 3-1(30.1° N,12° W), St 3-18 (30.8°N, 10.8° W), St 3-23 (30.9° N, 11.1° W), St 3-41 (31.2° N, 11.4° W) and St 3-49 (31.1° N, 11.6° W). Water samples were collected with clean sampling bottles (12 liter, Niskin-X, General Oceanics) attached to a CTD (Seabird Model 9 plus) frame. Samples were stored in 1-L acid washed polycarbonate bottles until processing (described below).

For the molecular analysis of the active and the total bacterial community, between 7 and 10L of seawater were collected at different depths in acid-rinsed polycarbonate carboys. Bromodeoxyuridine (BrdU) was added to the samples at a final concentration of 20nM and incubated in the dark at *in situ* water temperature for 24 h. Subsequently, the samples were filtered through a 0.22 μm Sterivex filter cartridge (Millipore). The filters were stored at -80°C until further processing in the lab.

DNA extraction and separation of BrdU-labeled DNA. DNA extraction was performed with the Ultraclean Mega soil DNA isolation kit (Mobio) following the protocol of the manufacturer. The DNA extract was further concentrated using Centricon units (Millipore) and the DNA concentration was measured with a Nanodrop® spectrophotometer.

The BrdU-labeled DNA (representing the active Bacteria) was separated from the non-labeled DNA (corresponding to the inactive Bacteria) by immunocapturing following the protocol of Hamasaki et al. (2007). All the incubation steps were performed at room temperature. Herring sperm DNA was washed and incubated with anti-BrdU monoclonal antibody (Mouse IgG, Invitrogen, diluted 1:10 in PBS) for 30 min. The extracted DNA was boiled for 1 min, immediately frozen in dry-ice ethanol and subsequently, thawed. Each DNA sample was mixed with 10μL of the antibody mixture and incubated for 30 min. Paramagnetic beads coated with Goat anti-Mouse IgG (Dynabeads ® , Invitrogen) were washed with PBS containing BSA (1 mg ml^{-1}) by using a magnetic concentrator and subsequently, resuspended

with PBS-BSA to their original concentration. Thereafter, the coated beads were mixed with the DNA samples and incubated under gentle agitation for 30 min. Subsequently, the beads were washed seven times with 0.5 mL PBS-BSA. The captured DNA was eluted from the beads by adding 100 µL of 1.7 mM BrdU and incubating under constant agitation for 30 min. Subsequently, 2µL of glycogen (20mg ml^{-1}) was added to the supernatant and the DNA was collected by ethanol precipitation and resuspended in 10mM TRIS.

PCR and ARISA. One to 5µL of total DNA extract and 2µL of BrdU-labeled DNA extract was added to the ARISA Master Mix, consisting of 0.2µL of both primers (ITSF Fam and ITSR Reub, 0.2µL of Taq polymerase (Biotherm D, 5U μL^{-1} Gene Craft Germany), 4µL 10x PCR-buffer, 0.5µL 20x dNTP, 1µL BSA, filled up to 40µL with Sigma water. Each amplification consisted of: an initial denaturation step at 94°C (2 min), 30 or 36 cycles for the total or BrdU-labeled DNA, respectively, of denaturation at 94°C (15 sec), annealing at 55°C (30 sec), extension at 72°C (3 min) and a final extension step at 72°C (9 min). Subsequently a quality check of the PCR product was run on a 2% agarose gel. The remaining product was purified with the Quickclean PCR purification kit (Genscript) and the DNA concentration was quantified with a Nanodrop® spectrophotometer.

Twenty ng of DNA from the cleaned PCR product was denaturated at 95°C for 3 min, together with 10µL Hi-Di Formamide and 0.5µL of DNA size standard (GenScan 12000 LIZ Size Standard). FAM-labeled fragments were separated and detected with an ABI Prism capillary sequencer (Applied Biosystems). Subsequently, the electropherograms were analyzed with Peak Scanner Software (Applied Biosystems).

Statistical analysis. To determine the similarity between the composition of bacterial communities, the obtained matrix was analyzed with Primer software v6 (Primer-E). The communities were compared by calculating the Bray-Curtis similarity index from the square root transformed dataset using the peak-height (corresponding to the abundance of basepare fragments) of different size DNA bands.

Results

In total, 655 different operational taxonomic units (OTUs) were detected with ARISA ranging from 52 to 1200 bp fragments (Fig. 2). From these 655 OTUs, 573 (87.5 % of total OTUs) OTUs were also found in the BrdU-labeled DNA with 65 OTUs ubiquitously present in both, the BrdU-labeled Bacteria and total Bacteria. 44.6 % of total OTUs and 39.1 % of the BrdU-labelded OTUs occur unique in all samples. The amount of OTUs was not significantly different between the surface and mesopelagic bacterial communities (100 – 1000 m depth) and the bathypelagic communities (1000 – 3000 m depth) (paired t-test), neither for the BrdU-labeled bacteria nor for the total bacteria.

The difference in the community composition between the total and active bacterial community (BrdU-incorporating Bacteria) was significant (ANOSIM, $r = 0.361$, $p = 0.001$) throughout the entire water column. Analysis of similarities revealed significantly larger differences between surface and mesopelagic communities (100 – 1000 m) vs. bathypelagic bacterial communities (1000 – 3000 m) for total ($r = 0.332$, $p = 0.003$) than for active Bacteria ($r = 0.181$, $p = 0.007$). The diversity index Shannon-Wiener (H') was higher for the total Bacteria (4.8 ± 0.2) than for the active Bacteria (4.7 ± 0.2) (Table 1) (paired t-test; $p = 0.002$).

Discussion

In this study, the incorporation of BrdU was used to differentiate between the active and total bacterial communities throughout the water column. With this approach, it is possible to separate the BrdU-labeled DNA, thus the DNA representing the metabolically active bacteria, by immunocapturing and further process it by any desired fingerprinting technique. In this particular study, we used ARISA which gives a high resolution as it targets the highly variable intergenic spacer region between the 16S rRNA and the 23S rRNA on the rRNA operon.

Results from the cluster analysis showed that the composition of the total bacterial community was distinctly different from the BrdU-incorporating community (Fig. 3). Furthermore, the communities of total and active bacteria clustered with depth, indicating

stratification of both the total and the active bacterial community as shown previously for the Mediterranean sea using T-RFLP (Moeseneder, Winter et al. 2001). The largest shift in the community composition was found between the surface and mesopelagic (100 – 1000 m) versus the bathypelagic total bacterial community (1000 – 3000 m), whereas the between the active bacterial communities were more similar throughout the water column pointing to the presence of a less diverse and more widespread active bacterial community.

The ARISA OTUs obtained in the total communities reflect bacterial phylotypes with abundances above a certain threshold value commonly assumed to be about 0.01% of the total community DNA (Ruan Q et al. 2006). In contrast, the OTUs resulting from the BrdU-labeling approach comprise members of the actively growing bacterial communities with an abundance above this threshold. The observed differences between both fractions of the bacterial community is likely due to the fact that from the total pool of bacteria present in a given depth layer only a certain fraction of bacteria can utilize the available substrate in terms of quality and quantity (Taniguchi and Hamasaki 2008). On the other hand, some bacterial phylotypes might have high growth rates but low abundance due to high mortality rates associated to preferential grazing (Hahn and Höfle 2001) or virus infection (Fuhrman 1999) and thus, not be detected in the total community. In this study 117 OTUs were found which were present in the active community but absent in the total bacterial community.

The presence of dormant Bacteria in the total community agrees with the seed bank theory of bacterial communities (Pedrós-Alió 2006). In this scenario, a high number of species at low abundance in a particular system might be better adapted to environmental conditions occurring in other systems or in other seasons, and might become active when environmental conditions change.

Future work needs to focus in characterizing the main groups of active and non-active Bacteria of the community, using cloning and sequencing, and to investigate which are the environmental factors initiating changes in the bacterial community structure.

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Tables and Figures

Table 1. Shannon diversity index obtained for the total and active bacterial community at different stations and depths in the Atlantic. For location of the Stations – see Fig. 1.

Station	Depth (m)	H' $_{\text{total Bacteria}}$	H' $_{\text{active Bacteria}}$
52	100	4.8	
	300	5.1	5.0
	400	4.6	4.5
	1100	4.6	4.8
	3000	5.0	4.5
1	100	5.2	4.9
	300	4.7	4.8
	700	4.5	4.5
	1100	4.8	4.8
18	100	4.9	
	300	4.4	4.5
	700	4.5	4.6
	2000	4.7	4.8
23	100	5.1	4.4
	1100	4.9	4.7
	1900	4.8	4.6
41	100	5.0	4.6
	300	4.9	4.9
	1100	4.9	4.7
49	300		4.4
	700	5.1	4.5
	1100	4.9	4.8
	2850	5.0	
Average:		4.8	4.7
SD:		0.2	0.2

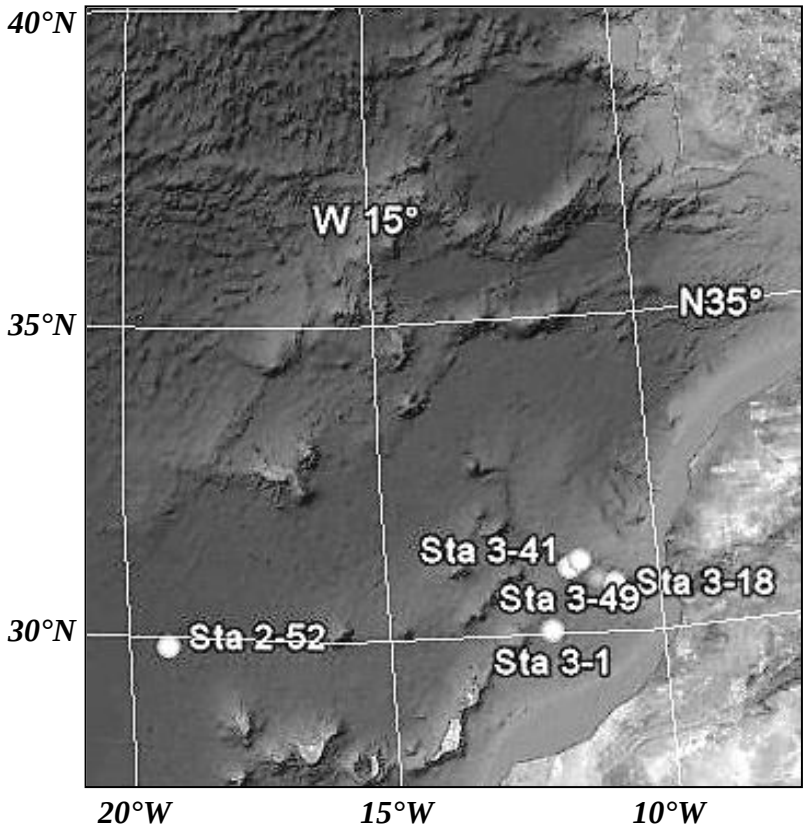

Fig. 1. Sampling sites in the eastern Atlantic occupied during the CAIBEX cruises in July and August 2009.

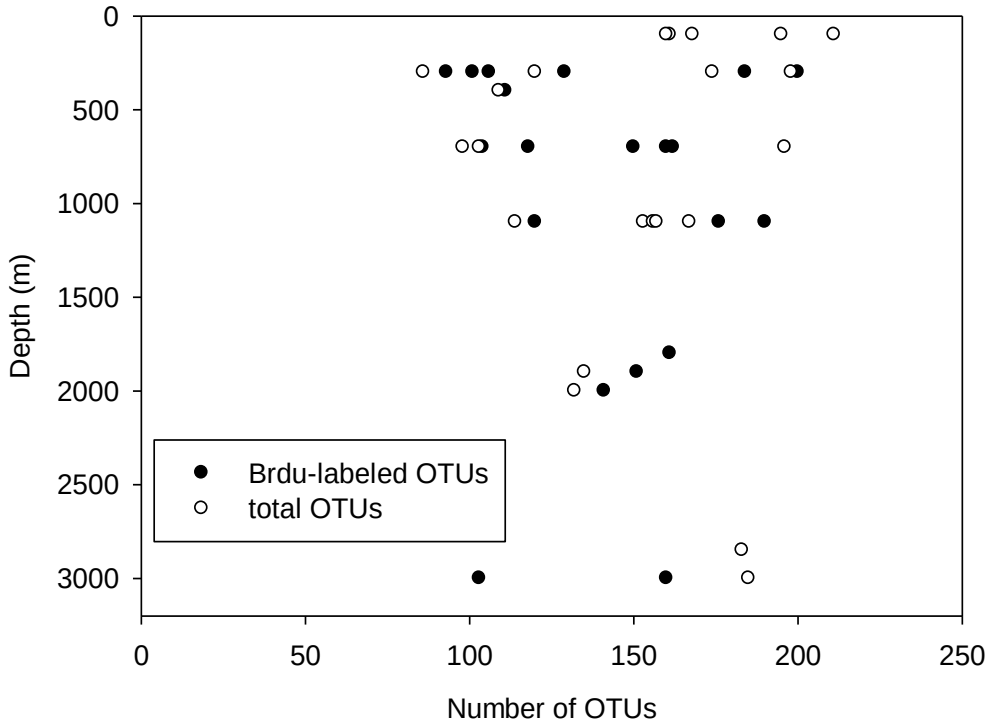

Fig. 2. Distribution of the number of operational taxonomic units (OTUs) with depth of all stations in the eastern Atlantic.

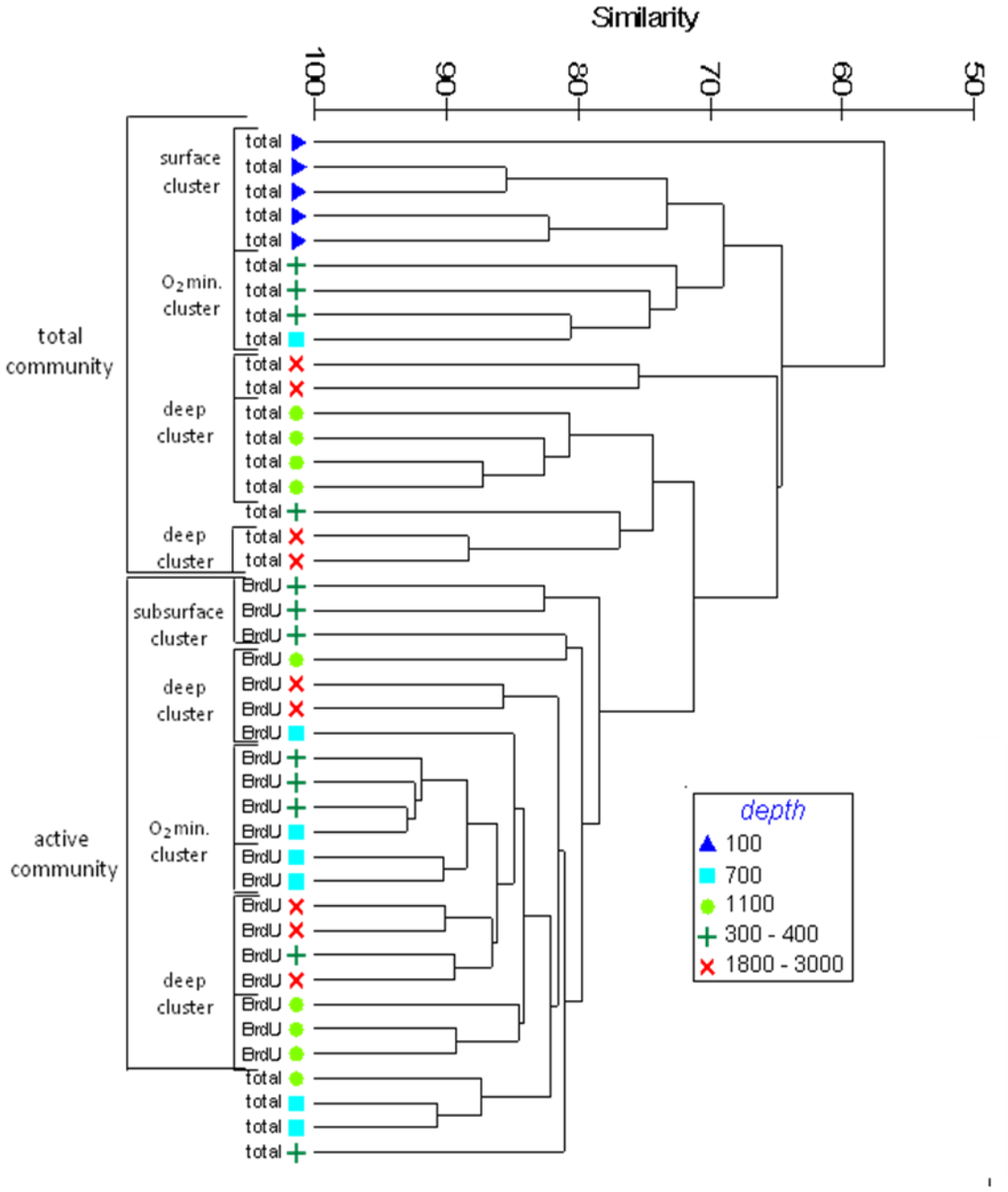

Fig. 3. Cluster analysis of the ARISA fingerprints based on Bray-Curtis analysis over different depths (indicated by the different symbols) for the total and the active (BrdU-incorporating) bacterial communities in the eastern Atlantic.

Zusammenfassung

Im Osten des Atlantiks wurden entlang eines Transekts die Abundanzen und Leucin-Aufnahmeraten von Bakterien und Archaea in der Wassersäule ermittelt. Außerdem wurde die Zusammensetzung von aktiven und gesamten Bakteriengemeinschaften durch molekulare Fingerprinting-Techniken analysiert.

Die Häufigkeiten von Bakterien, Crenarchaeota und Euryarchaeota wurden mittels dem Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (CARD-FISH) Verfahren ermittelt. Obwohl der Anteil der Bakterien an der gesamten Prokaryoten-Abundanz im Bathypelagial (1000 – 4000 m) geringer war als an der Oberfläche und dem Mesopelagial (100 – 1000 m), dominierten sie dennoch in der gesamten Wassersäule. Crenarchaota trugen 30 ± 12% zu der gesamten Prokaryoten-Abundanz bei, wobei sie eine generell höhere Verbreitung im Bathypelagial hatten als in der Oberflächenschicht und dem Mesopelagial. Euryarchaeota überstiegen in keiner Probe 5 % der gesamten Prokaryoten-Abundanz.

Zusätzlich zu den Leucin-Aufnahmeraten der Gesamtprokaryotengemeinschaft wurden auch die Leucin-Aufnahmeraten für Archaea und Bakterien ermittelt. Um die verschiedenen Aktivitätsraten von Bakterien und Archaea in Bezug zu der gesamten Prokaryoten-Produktivität zu bestimmen, wurde der bakterielle Hemmstoff Erythromycin und der Archaea Hemmstoff Diphtheria Toxin eingesetzt. Die Spezifität des Hemmstoffs Erythromycin wurde durch die Kombination von Microautoradiographie mit CARD-FISH getestet. Dabei wurde festgestellt, dass Erythromycin selektiv die bakterielle Produktivität unterdrückt. In der euphotischen Zone und dem Mesopelagial war der Beitrag der Bakterien an der gesamten Leucin-Aufnahmerate 65 ± 15% der gesamten Prokaryoten Leucin-Aufnahmerate und nahm mit zunehmender Tiefe ab. Die durchschnittliche Zellen-spezifische Aktivitätsrate der Crenarchaeota ($3.7 \pm 2.0 \times 10^{-5}$ fmol $cell^{-1}$ d^{-1}) war 5-mal geringer als die der Bakterien ($18.3 \pm 17.6 \times 10^{-5}$ fmol $cell^{-1}$ d^{-1}). Im Bathypelagial, jedoch, war die Zell-spezifischen Leucin-Aufnahmerate der Archaea vergleichbar mit der der Bakterien (1.8×10^{-5} fmol $cell^{-1}$ d^{-1} for Crenarchaeota, 2.3×10^{-5} fmol $cell^{-1}$ d^{-1} for Bacteria). Diese Ergebnisse deuten darauf hin, dass im Bathypelagial des Atlantiks Crenarchaeota und Bakterien bezogen auf Zellniveau vergleichbare heterotrophe Aktivitäten haben, in den Oberflächengewässern

und dem oberen Mesopelagial jedoch die heterotrophe Aktivität der Archaea um eine Größenordnung geringer als die der Bakterien ist.

Um zwischen der Zusammensetzung der Gemeinschaft von metabolisch aktiven Bakterien und der Gesamtbakteriengemeinschaft zu unterscheiden, wurde der Einbau von Bromodeoxyuridine (BrdU) in die aktive Gemeinschaft mit einer Kombination von immunocapturing der DNA in die BrdU eingebaut wurde und Automated Ribosomal Intergenic Spacer Analysis (ARISA), als molekulare Fingerprinting-Technik, verwendet. Insgesamt wurden dabei 655 verschiedene operational taxonomic units (OTUs) detektiert. Die Ähnlichkeitsanalyse der bakteriellen Gemeinschaften ergab zwischen den aktiven (BrdU-markierte Bakterien) und gesamten Bakterien einen signifikanten (ANOSIM, $r = 0.361$, $p = 0.001$) Unterschied in der Zusammensetzung der Gemeinschaften in der gesamten Wassersäule. Die Ähnlichkeiten zwischen Oberflächengewässer und Mesopelagial (100 – 1000 m) und Bathypelagial (1000 – 3000 m) war für die BrdU-markierte Bakteriengemeinschaft größer ($r = 0.181$, $p = 0.007$) als für die Gesamtbakteriengemeinschaft ($r = 0.332$, $p = 0.003$). Der Shannon-Wiener index (H’), als Index für die Diversität einer Gemeinschaft, war für die Gemeinschaft der Gesamtbakteriengemeinschaft größer als für die aktive (paired t-test; $p = 0.002$).

Diese Ergebnisse deuten darauf hin, dass die Diversität von Gemeinschaften im Wesentlichen durch die sich im Ruhestand befindenden Bakterien gebildet wird, die in einer sich verändernden Umwelt eine Art „Samenbank“ bilden könnte.

Summary

The abundance and leucine incorporation rate of Archaea and Bacteria were determined throughout the water column along a transect in the eastern Atlantic. Additionally, the composition of the active and total bacterial community was analyzed using molecular fingerprinting techniques.

The abundance of Bacteria, Crenarchaeota and Euryarchaeota was determined by Catalyzed Reporter Deposition - Fluorescence In Situ Hybridization (CARD-FISH). Bacteria dominated throughout the water column, although their contribution to total prokaryotic abundance in the bathypelagic layer (1000 - 4000 m) was lower than in the surface and mesopelagic layers (0 - 1000 m). Crenarchaeota contributed about 30 ± 12% to the total prokaryotic abundance with a generally higher contribution in the bathypelagic layer than in the surface and mesopelagic layers. Euryarcheota contributed less than 5% to the total prokaryotic abundance throughout the water column.

Leucine incorporation rates were determined for the total prokaryotic community as well as for Bacteria and Archaea separately using selective inhibitors. The bacterial inhibitor erythromycin and the archaeal inhibitor diphtheria toxin were used to determine the contribution of Archaea and Bacteria, respectively, to total heterotrophic activity. Using microautoradiography in combination with CARD-FISH, the specificity of erythromycin was tested. Erythromycin was found to selectively inhibit bacterial activity. The contribution of Bacteria to total leucine incorporation amounted to 65 ± 15% of total prokaryotic leucine incorporation decreasing in their contribution with depth. The mean cell-specific leucine incorporation rate of Crenarchaeota ($3.7 \pm 2.0 \times 10^{-5}$ fmol cell^{-1} d^{-1}) was 5 times lower than that of Bacteria ($18.3 \pm 17.6 \times 10^{-5}$ fmol cell^{-1} d^{-1}) in the surface and mesopelagic layer. In the bathypelagic layer, however, cell-specific leucine incorporation rates of Crenarchaeota were similar to those of Bacteria (1.8×10^{-5} fmol cell^{-1} d^{-1} for Crenarchaeota, 2.3×10^{-5} fmol cell^{-1} d^{-1} for Bacteria). These results indicate that Crenarchaeota and Bacteria exhibit similar heterotrophic activity on a per-cell level in the bathypelagic waters of the Atlantic, while in the surface and upper mesopelagic waters, cell-specific heterotrophic archaeal activity is about one order of magnitude lower than that of Bacteria.

To differentiate the community composition of metabolically active Bacteria from the total bacterial community, bromodeoxyuridine (BrdU) incorporation into the active bacterial community was used in combination with immunocapturing and automated ribosomal intergenic spacer analysis (ARISA) as a molecular fingerprinting approach. In total, 655 operational taxonomic units (OTUs) were found. Results of the similarity analysis (ANOSIM, $r = 0.361$, $p = 0.001$) of the obtained clusters showed a significant difference in the community composition throughout the water column between active (Bacteria incorporating BrdU) and the total bacterial community. The similarity between surface and mesopelagic communities (100 – 1000 m) and bathypelagic communities (1000 – 3000 m) was higher for the active ($r = 0.181$, $p = 0.007$) than for the total ($r = 0.332$, $p = 0.003$) bacterial community. The Shannon-Wiener diversity index (H') was significant higher for the total Bacteria (paired t-test; $p = 0.002$) than for the active ones.

These findings suggest that the diversity of communities is mainly driven by the dormant fraction of the bacterial community, potentially forming a 'seed bank' in changing environmental conditions.

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2009	Diploma thesis at the Department of Marine Biology, University of Vienna; Diploma title: Abundance and heterotrophic activity of *Bacteria* and *Archaea* and bacterial community structure in the water column of the eastern Atlantic
2004 - 2010	Diploma study of Biology/Ecology with specification in marine biology at the University of Vienna
1998 - 2003	College for Tourism, Catering and Hotel Management
1994 - 1998	Secondary school Guntramsdorf
1990 - 1990	Primary school Guntramsdorf

Marine and biological field courses

2009	Advanced marine ecology course with underwater field work including S.C.U.B.A (in STARESO, Calvi, Corsica, France)
2008	Ecology of coral reefs including S.C.U.B.A (Dahab, Egypt)
2008	Practical course for protection of sea turtles (*Caretta caretta*) in the Mediterranean sea (Fethiye, Turkey)
2008	Marine biological field course on the Mediterranean fauna and flora; Centre for Marine Research (Rovinj, Croatia)
2007	Tropical excursion at the field station La Gamba (Costa Rica)

Personal skills

Languages	German (first language), Spanish (fluent), English (fluent), French (only reading)
Computer skills	MS-Office, Sigma Plot, image processing (Adobe Photoshop, Adobe Illustrater), statistics (SPSS, Primer),
Additional qualifications	Electron microscopy (TEM, SEM) Confocal microscopy Light microscopy Analogue and digital photography S.C.U.B.A (PADI Divemaster)

Oceanographic cruises

2010	Geotraces cruise, Leg 2 on board of the RV Pelagia (Bermuda – Fortaleza/Brazil)
2011	Malaspina cruise, Leg 3 and 4 on board of the BIO Hespérides (Cape town/South Africa – Perth/Australia; Perth/Australia – Sydney/Australia)

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Youth hostel “Hostal Clan”(Argentina) - Receptionist
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Bank “Bank Austria Creditanstalt” (Austria) - Trainee

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