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Differentiating leucine incorporation of *Archaea* and *Bacteria* throughout the water column of the eastern Atlantic using metabolic inhibitors

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ABSTRACT: The abundance (based on catalyzed reporter deposition-fluorescence *in situ* hybridization, CARD-FISH) and leucine incorporation rates of *Archaea* and *Bacteria* were determined throughout the water column in the eastern Atlantic. *Bacteria* dominated throughout the water column, although their contribution to total prokaryotic abundance in the bathypelagic layer (1000 to 4000 m depth) was lower than in the surface and mesopelagic layers (0 to 1000 m depth). While marine *Crenarchaeota* Group I (MCG I) contributed 28 ± 12% to the total prokaryotic abundance, with a generally higher contribution in the bathypelagic layer than in the surface and mesopelagic layers, marine *Euryarchaeota* Group II contributed <5% throughout the water column. Using microautoradiography in combination with CARD-FISH, we tested the specificity of erythromycin and diphtheria toxin and found them to selectively inhibit bacterial and archaeal activity, respectively. These inhibitors were thus used to determine the contribution of *Bacteria* and *Archaea* to total leucine incorporation: *Bacteria* contributed 69 ± 15%, and this value decreased with depth; *Archaea* contributed 32 ± 16% over the entire water column, with no significant difference between surface and mesopelagic waters and the bathypelagic realm. The mean cell-specific leucine incorporation rate of MCG I ($5.3 \pm 3.0 \times 10^{-20}$ mol cell^{-1} d^{-1}) was 3.5-fold lower than that of *Bacteria* ($18.6 \pm 18.2 \times 10^{-20}$ mol cell^{-1} d^{-1}) in the surface and mesopelagic layer. In the bathypelagic layer, cell-specific leucine incorporation rates of *Crenarchaeota* were similar to those of *Bacteria* (2.3×10^{-20} mol cell^{-1} d^{-1} for MCG I, 2.9×10^{-20} mol cell^{-1} d^{-1} for *Bacteria*). In the surface and mesopelagic waters of the subtropical eastern North Atlantic, MCG I exhibited a lower heterotrophic activity on a per-cell level than *Bacteria*. In the bathypelagic zone, cell-specific heterotrophic activities of *Bacteria* and MCG I were similar.

KEY WORDS: *Archaea* · *Bacteria* · Leucine incorporation · Heterotrophic activity · Deep sea · Metabolic inhibitor

INTRODUCTION

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 (MCG I, recently coined *Thaumarchaeota*; Brochier-Armanet et al. 2008) are by far the most abundant archaeal group, accounting for about one-third of the total prokaryotic abundance in the waters

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below the euphotic zone (Karner et al. 2001, Herndl et al. 2005, Varela et al. 2008).

Although MCG I 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 MCG I is chemoautotrophic, incorporating dissolved inorganic carbon (DIC) (Wuchter et al. 2003) and using ammonia as an energy source (Könneke et al. 2005, Ingalls et al. 2006, Wuchter et al. 2006, Hansman et al. 2009). Quantitative PCR of the archaeal gene encoding the ammonia monooxygenase subunit A (*amo*A) indicates that the archaeal *amo*A gene abundance is orders of magnitude more abundant than bacterial *amo*A in oceanic waters (Mincer et al. 2007, Agogué et al. 2008, De Corte et al. 2009), suggesting that MCG I might be more important for oceanic ammonia oxidation than *Bacteria*. However, group- or even domain-specific ammonia oxidation measurements have not been reported to date.

Not all MCG I are autotrophs, but apparently, a fraction of MCG I is heterotrophic or at least mixotrophic, taking up amino acids as revealed by microautoradiography in combination with fluorescence *in situ* hybridization (MICRO-CARD-FISH) (Ouverney & Fuhrman 2000). Also, MCG I have been shown to preferentially take up D-aspartic acid over L-aspartic acid in the bathypelagic waters of the Atlantic (Teira et al. 2006b, Varela et al. 2008). Several organic compounds (leucine, a mixture of amino acids, proteins, glucose, and extracellular polysaccharides) were taken up by MCG I in the water column (0 to 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 MCG I 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 the present 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. Erythromycin specifically inhibits protein synthesis in *Bacteria* (Kohanski et al. 2010) but should not affect archaeal protein synthesis, whereas diphtheria toxin inhibits protein synthesis in *Archaea* but does not affect *Bacteria* (Kessel & Klink 1980, Rohwer & Azam 2000). Using this dual inhibitor approach, we found that *Archaea* contribute on average 32% to the total prokaryotic leucine incorporation throughout the water column.

MATERIALS AND METHODS

In the eastern Atlantic, sampling was carried out aboard the RV 'Sarmiento de Gamboa' (Consejo Superior de Investigaciones Cientificas, Spain) during the CAIBEX-2 and CAIBEX-3 cruises occupying 6 stations in July and August 2009 (Table 1). Water samples were collected throughout the water column with clean 12 l Niskin-bottles (General Oceanics) attached to a conductivity, temperature and depth (CTD) system (Seabird Model 9 plus). The seawater was placed into acid-washed polycarbonate bottles (1 l) for measuring several microbial parameters as described below.

Testing the specificity of the bacterial and the archaeal inhibitors

Several inhibitors were examined for their specificity to inhibit protein synthesis of either *Bacteria* or *Archaea*. Erythromycin (Sigma-Aldrich, product #45673) and streptomycin (Sigma-Aldrich, product #85886) were tested as bacterial inhibitors, and diphtheria toxin (Sigma-Aldrich, product #D0564) was tested as an inhibitor of archaeal protein synthesis, all provided at a final concentration of 10 µg ml^{-1}. Initially, leucine incorporation rates with and without the bacterial inhibitors streptomycin and erythromycin and the archaeal inhibitor diphtheria toxin were compared on individual samples collected during the CAIBEX-2 and CAIBEX-3 cruises. 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 apparently did not effectively inhibit bacterial leucine incorporation as the sum of the percentage of inhibition in streptomycin- and diphtheria toxin-treated samples were substantially lower than 100%. In contrast, the sum of inhibition in erythromycin- and diphtheria toxin-treated samples averaged 101 ± 23% compared to the untreated samples, tentatively indicating high specificity of erythromycin and diphtheria toxin as bacterial and archaeal inhibitors, respectively (Table 1). Consequently, the specificities of both erythromycin and diphtheria toxin for inhibiting bacterial and archaeal protein synthesis, respectively, were further tested using microautoradiography with [^{3}H]-leucine as a substrate in combination with CARD-FISH.

During the CAIBEX-3 cruise, Atlantic water from 300 m depth at Stn 3-23 (Table 1) was collected. To

Table 1. Location, mean percentage ± standard deviation (SD) of inhibition of leucine incorporation in erythromycin- and diphtheria toxin-treated samples relative to bulk (untreated control) 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 subtropical eastern North Atlantic. SD is the propagation error calculated from triplicate subsamples of the leucine incorporation rate with and without inhibitor. Values in parentheses indicate statistically not significant inhibition, indicated by a high SD ($p > 0.05$). nd: not determined. Means (±SD) of each column were calculated excluding the values in parentheses. The sum of the inhibition in a given sample was calculated only when both inhibitions were significant

Station (Location)	Depth (m)	% inhibition of leucine incorporation rate by streptomycin	by erythromycin	by diphtheria toxin	Sum
2-34	100	nd	78 ± 22	26 ± 5.1	104
(37.981° N, 19.988° W)	300	4.0 ± 0.3	(85 ± 82)	26 ± 2.5	nd
	700	13 ± 2.7	67 ± 20	35 ± 7.0	103
	1100	3.0 ± 1.1	(66 ± 57)	42 ± 17	nd
	3000	(27 ± 36)	30 ± 9.6	37 ± 8.8	67
2-56	120	nd	81 ± 7.0	11 ± 0.8	92
(29.548° N, 17.558° W)	300	nd	53 ± 22	20 ± 6.5	73
	700	nd	(87 ± 80)	(66 ± 63)	nd
	1200	nd	66 ± 12	44 ± 9.1	109
	3000	nd	64 ± 17	56 ± 12	120
2-65	80	nd	77 ± 18	29 ± 3.6	106
(28.853° N, 14.115° W)	300	nd	64 ± 13	27 ± 1.2	91
	700	nd	66 ± 24	38 ± 14	104
	1100	nd	71 ± 13	8.0 ± 0.8	79
	1500	nd	(49 ± 88)	13 ± 4.6	nd
3-1	100	nd	91 ± 27	6.0 ± 1.1	97
(30.118° N, 10.754° W)	300	nd	(96 ± 173)	28 ± 5.0	nd
	700	nd	82 ± 18	21 ± 3.1	102
	1100	nd	72 ± 19	30 ± 2.3	103
	1800	nd	85 ± 26	56 ± 8.2	142
3-23	100	nd	78 ± 7.9	nd	nd
(30.924° N, 11.095° W)	300	nd	63 ± 21	nd	nd
	700	nd	(47 ± 44)	21 ± 4.5	nd
	1100	nd	54 ± 12	17 ± 1.2	71
	1800	nd	55 ± 20	46 ± 19	101
3-41	100	nd	84 ± 19	25 ± 1.8	109
(31.178° N, 11.360° W)	300	nd	91 ± 46	61 ± 11	152
	700	nd	77 ± 33	53 ± 11	130
	1100	nd	(84 ± 59)	63 ± 29	nd
	2000	nd	41 ± 13	28 ± 9.2	70
Mean ± SD		nd	69 ± 15	32 ± 16	101 ± 23

40 ml subsamples spiked with erythromycin, 5 nM [^{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. The specificity of diphtheria toxin and erythromycin as archaeal and bacterial inhibitors, respectively, was evaluated on 2 samples collected in the North Atlantic at 200 m (2.5° N, 41.7° W) and 300 m (46.3° N, 39.7° W) depth during the Dutch GEOTRACES Cruises 1 and 2 (April to July 2011). Subsamples of 40 ml were spiked with either diphtheria toxin or erythromycin (final conc. as above) and kept at *in situ* temperature for 30 min. Subsequently, 10 nM [^{3}H]-leucine (final conc.) was added, and the samples were incubated at *in situ* temperature in the dark for 12 h. Controls without the addition of erythromycin or diphtheria toxin 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 laboratory.

Measuring leucine incorporation rates

During the CAIBEX-2 and CAIBEX-3 cruises, triplicate subsamples (1.5 ml) were dispensed into screw-capped centrifuge tubes (VWR International, 2.0 ml screw tube), amended with 5 nmol l^{-1} (final conc.) of [^{3}H]-leucine (PerkinElmer, product #NET460A080MC) and incubated at *in situ* temperature (±2°C) in the dark for 1 h (for samples from 0 to 200 m depth) or for 24 h (for samples from 250 m 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 were centrifuged at 18 000 × *g* for 10 min, followed by a TCA rinse (5%) and an ethanol rinse (80%, ice-cold). The pelleted sample was radio-assayed with a liquid scintillation counter (PerkinElmer, Tri-Carb 3100TR) using Ultima-GOLD (PerkinElmer) scintillation cocktail. Quenching was corrected based on an 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 was converted into leucine incorporation rates. Leucine additions of 5 nM were found to be sufficient to obtain maximum incorporation of [^{3}H]-leucine even in surface water prokaryotic communities.

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 was calculated as follows: (1 – [leucine incorporation measured in the erythromycin- or diphtheria toxin-treatment] / leucine incorporation in the untreated sample) × 100. The propagation error (standard deviation) of the percent inhibition was calculated according to Bevington & Robinson (2002).

Cell-specific leucine uptake was calculated from the leucine incorporation obtained in the erythromycin and diphtheria toxin-treated samples divided by the abundance of *Bacteria* and MCG I, respectively, determined using CARD-FISH as described below.

CARD-FISH

The abundance of *Bacteria*, MCG I, and marine *Euryarchaeota* Group II (MEG II) was determined using CARD-FISH with 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, the 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 the MEG II probe Eury806 (Teira et al. 2006a) and 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 MCG I probe mix.

The probes were added at a final concentration of 0.28 ng μl^{-1} (0.05 µM), 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. The filters were stained and mounted on slides with a 4′,6-diamidino-2-phenylindole (DAPI) mix (5.5 parts Citifluor, 1 part Vectashield, and 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 a 100 W Hg-lamp and corresponding filters for Alexa488 and DAPI.

MICRO-CARD-FISH

The CARD-FISH protocol for *Bacteria* was carried out as described above. Microautoradiographic development was performed as outlined by Teira et al. (2006b). Hybridized filter sections were transferred 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 for the CAIBEX samples and 2 d for the GEOTRACES samples. 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 the fixer for 5 min, and subsequently in Milli-Q water for 2 min). Before they were completely dried, the filter sections were removed, and the cells were counterstained with the DAPI mix and examined under a Zeiss Axioplan-2 epifluorescence microscope. The presence of silver grains surrounding the cells was checked in the bright-field mode of

the microscope at 1250× magnification. For each microscope field, we enumerated the relative abundance of probe-positive cells compared to DAPI-stained cells and the number of cells surrounded by a silver grain halo. In the GEOTRACES samples, the leucine uptake per cell was quantified using the quantitative MICRO-CARD-FISH approach as outlined by Sintes & Herndl (2006). Briefly, the area of the silver grain halo was measured using an image analysis system, and the total silver grain area per sample related to the bulk leucine uptake was calculated as described by Sintes & Herndl (2006). A total of >200 leucine-positive cells or >800 DAPI-stained cells were enumerated for each sample. The total leucine uptake associated to *Bacteria* or *Archaea* in the different treatments was determined as the abundance of bacterial or archaeal cells taking up leucine (cell with silver grain halo) multiplied by the average silver grain area per active cell. The reduction in leucine uptake due to the addition of erythromycin or diphtheria toxin was calculated as a percentage of the leucine uptake in the untreated controls.

RESULTS

Testing the specificity of the inhibitors using MICRO-CARD-FISH

MICRO-CARD-FISH was used to test the specificity of both erythromycin and diphtheria toxin. In the subtropical Atlantic (during the CAIBEX-3 cruise), water collected at Stn 3-23 at 300 m depth (Table 1) was used to check the specificity of erythromycin. On this sample, the specificity of diphtheria toxin was not determined for logistic reasons. At Stn 3-23, *Bacteria* comprised 62% of the total prokaryotic abundance, and 80% of the *Bacteria* took up leucine (based on the cells with a 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 in the untreated or in the erythromycin-treated sample.

Samples taken during the GEOTRACES Cruises 1 and 2 from the western North Atlantic at 200 m and 300 m depth were used to test the specificity of the inhibitors erythromycin and diphtheria toxin on *Bacteria* and *Archaea*. The contribution of *Bacteria* to total prokaryotic abundance was 80% at 200 m depth and 93% at 300 m depth, and MCGI contributed 20 and 7% at 200 m and 300 m depth, respectively, to the total prokaryotic abundance. The addition of erythromycin reduced the percentage of bacterial cells taking up leucine by 35% at 200 m and by 73% at 300 m depth compared to the abundance of leucine-positive bacterial cells in the untreated control. Using diphtheria toxin, 86% and 72% of the *Archaea* taking up leucine were inhibited at 200 m and 300 m depth, respectively, compared to the abundance of leucine-positive archaeal cells. These inhibition percentages are based on enumerating cells with at least 3 silver grains attached to the cell for classification as leucine-positive cells. Applying the quantitative MICRO-CARD-FISH approach and quantifying the silver grain halo area around leucine-positive cells, however, provided a more realistic assessment of the inhibition pattern of the 2 inhibitors used in the present study. The addition of erythromycin reduced the halo area of the *Bacteria* taking up leucine by 81% at 200 m and by 91% at 300 m depth compared to the halo area of leucine-positive bacterial cells in the untreated control. Conversely, the total silver grain halo area of MCGI cells taking up leucine was reduced by 88% and 74% at 200 m and 300 m depth, respectively, upon addition of diphtheria toxin. Diphtheria toxin decreased the total halo area of leucine-positive *Bacteria* by <5% as compared to the control, while erythromycin did not affect the halo area of MCGI taking up leucine, as judged by the essentially identical halo area around *Archaea* in the erythromycin treated and the untreated samples.

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 to 700 m depth) and decreased exponentially with depth (ranging from 4.4×10^4 to 9.5×10^4 cells ml^{-1} in the bathypelagic layer [1100 to 3000 m]; Fig. 1).

The recovery efficiency of prokaryotes using CARD-FISH (sum of *Bacteria*, MEGII, and MCGI) was on average 84 ± 9% (±1 SD, n = 25). The contribution of *Bacteria* to total prokaryotic abundance ranged from 43 to 78% and that of *Archaea* ranged from 10 to 54% (Fig. 1). *Bacteria* dominated over *Archaea* throughout the water column (paired *t*-test, $p < 0.001$; Table 2). On average, *Bacteria* contributed 57 ± 9% to the total prokaryotic abundance, with a higher contribution in surface and mesopelagic layers than in the bathypelagic layer (*t*-test, $p < 0.001$; Table 2). In contrast, the mean contribution of MCGI

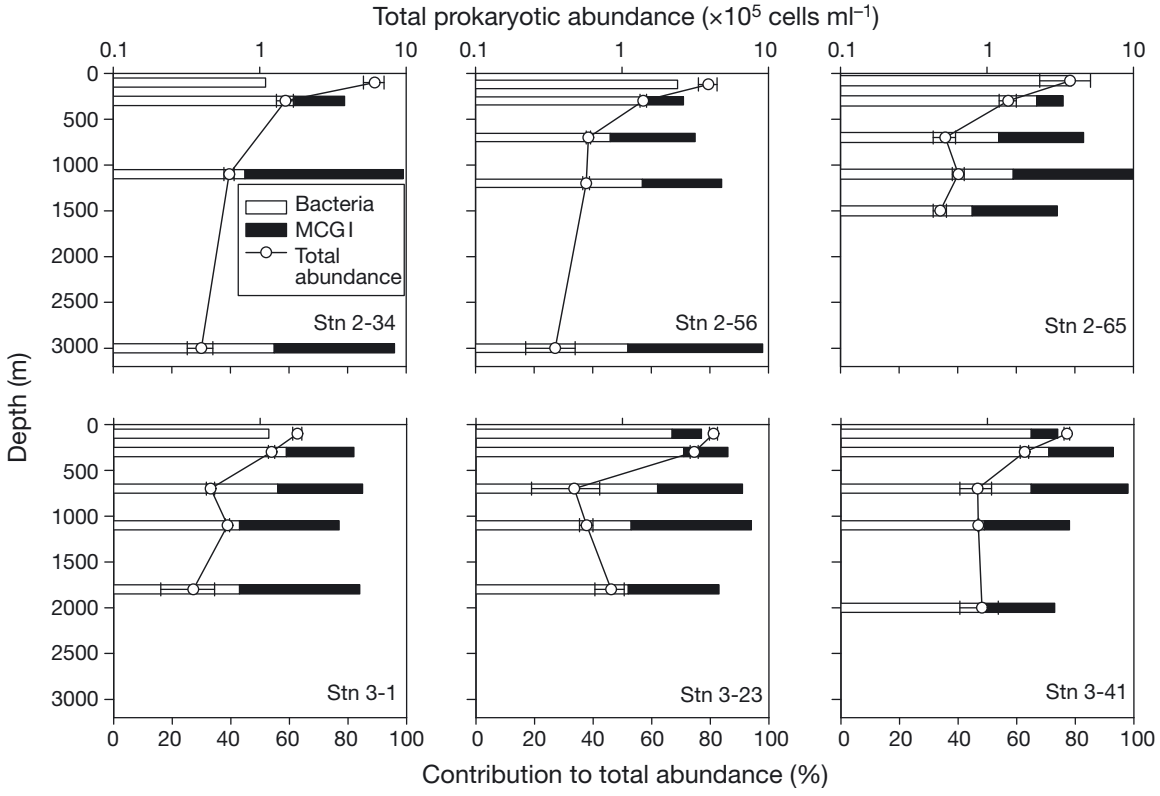

Fig. 1. Depth profiles of prokaryotic abundance (DAPI-stained cells) and the percentage of *Bacteria* and marine *Crenarchaeota* Group I (MCG I) determined using CARD-FISH relative to prokaryotic abundance at the sampling sites in the subtropical eastern North Atlantic occupied during the CAIBEX-2 and CAIBEX-3 cruises

was 28 ± 12%, with a higher contribution in the bathypelagic layer than in the surface and mesopelagic layers (*t*-test, $p < 0.001$; Table 2). MEG II always contributed <5% to total prokaryotic abundance (data not shown).

Prokaryotic leucine incorporation

At all 6 stations, prokaryotic leucine incorporation rates decreased in the untreated samples by 2 to 3 orders of magnitude from the surface layer to the bathypelagic layer, ranging from 0.26 to 205.56 pmol l^{-1} d^{-1} (Fig. 2). In the erythromycin-treated samples, leucine incorporation rates ranged from 0.13 to 47.15 pmol l^{-1} d^{-1}, while in the diphtheria toxin-treated samples, leucine incorporation ranged from 0.23 to 145.59 pmol l^{-1} d^{-1} (Fig. 2). The percentage of inhibition of leucine incorporation by erythromycin was always higher than the inhibition by diphtheria toxin, except at Stn 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 101 ± 23% (range: 67 to 152%, n = 21; Table 1).

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

Depth layer (m)	% contribution to total prokaryotic abundance		% contribution to leucine incorporation rates		Specific leucine incorporation rate ($\times 10^{-20}$ mol cell^{-1} d^{-1})	
	Bacteria	MCG I	*Bacteria*	MCG I	*Bacteria*	MCG I
10–3000	57 ± 9 (29)	28 ± 12 (25)	69 ± 15 (23)	32 ± 16 (27)	12.2 ± 15.9 (22)	3.7 ± 2.7 (21)
10–1000	62 ± 8 (17)	21 ± 9 (13)	75 ± 11 (14)	29 ± 14 (15)	18.6 ± 18.2 (13)	5.3 ± 3.0 (9)
1000–3000	50 ± 6 (12)	36 ± 9 (12)	60 ± 17 (9)	37 ± 18 (12)	2.9 ± 1.4 (9)	2.3 ± 1.6 (12)

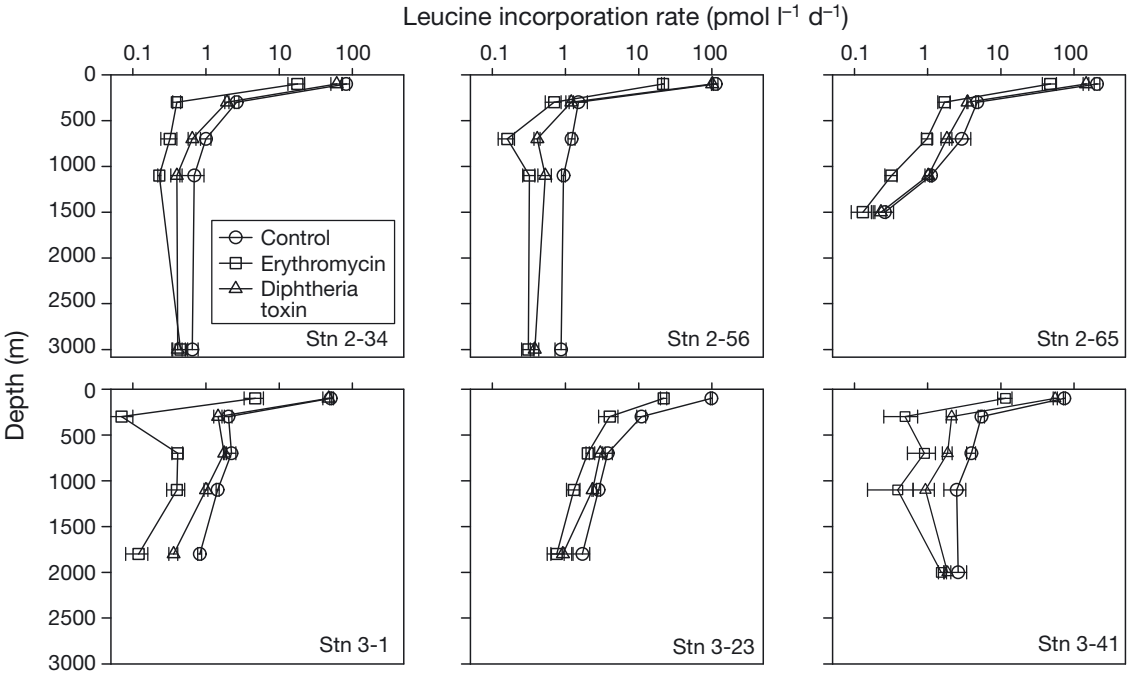

Fig. 2. Depth profiles of leucine incorporation rates by the prokaryotic community without inhibitors (untreated control) and in erythromycin- and diphtheria toxin-treated samples. Symbols represent the mean ± SD of 3 replicate measurements

The average percentage of inhibition by erythromycin and, hence, the putative contribution of *Bacteria* to total prokaryotic leucine incorporation was 69 ± 15% (n = 23; Table 2). The average percentage of inhibition by diphtheria toxin, i.e. the putative contribution of *Archaea* to prokaryotic leucine incorporation, was 32 ± 16% (n = 27; Table 2). The percentage of the putative contribution of *Bacteria* to leucine incorporation decreased significantly from the surface and mesopelagic waters (75 ± 11%) to the bathypelagic layer (60 ± 17%, *t*-test, p = 0.017; Table 2, Fig. 3). The percentage of leucine incorporation by *Archaea* did not significantly vary with depth (comparing surface and mesopelagic layers with the bathypelagic layer: *t*-test, p = 0.20; Table 2, Fig. 3).

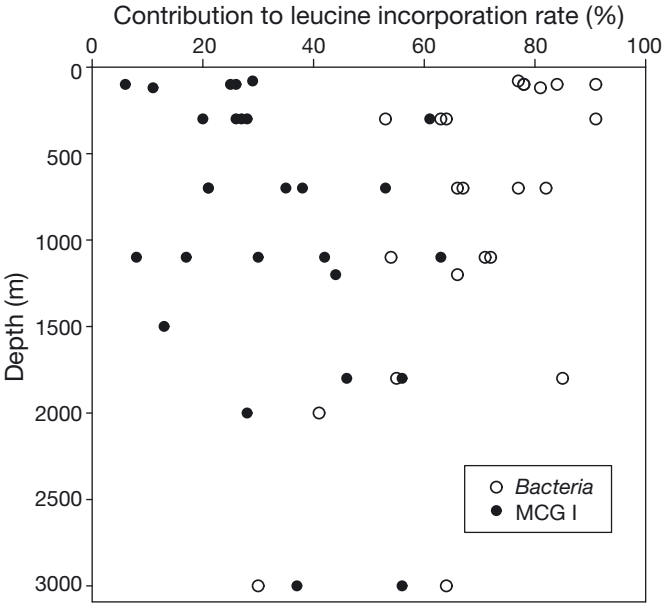

Fig. 3. Percentage of leucine incorporation rates in *Bacteria* and marine *Crenarchaeota* Group I (MCG I) relative to total leucine incorporation throughout the water column

Single-cell activity of *Bacteria* and MCG I

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 (*t*-test, p = 0.019, range: 0.9 × 10^{-20} to 54.5 × 10^{-20} mol leucine cell^{-1} d^{-1}; Table 2).

In MCG I, cell-specific leucine incorporation rates were slightly higher in the surface and mesopelagic layers than in bathypelagic layers (*t*-test, p = 0.008, range: 0.3 × 10^{-20} to 10.0 × 10^{-20} mol leucine cell^{-1} d^{-1}; Table 2). In the bathypelagic layers, there was no significant difference in cell-specific leucine incorporation rates between *Bacteria* and MCG I (*t*-test, p = 0.382; Table 2).

DISCUSSION

Using erythromycin and diphtheria toxin to inhibit bacterial and archaeal protein synthesis, respectively, we found that *Archaea* contribute on average 32% to the bulk leucine incorporation of prokaryotes in the water column of the eastern North Atlantic. *Euryarchaeota* were present in abundances of only <5% of DAPI-stained cells throughout the different layers of the water column. This indicates that the detected archaeal leucine incorporation is probably mainly due to MCG I, confirming earlier findings obtained using MICRO-CARD-FISH that a certain fraction of MCG I might be heterotrophic (Ouverney & Fuhrman 2000, Teira et al. 2006a,b, Kirchman et al. 2007, Varela et al. 2008). Also, radiocarbon analyses of mesopelagic MCG I collected at 2 depths in the North Pacific Gyre indicated that a fraction of MCG I is heterotrophic rather than chemoautotrophic (Hansman et al. 2009).

The contribution of total MCG I to prokaryotic leucine incorporation increased only insignificantly with depth (Table 2) in contrast to MCG I abundance, which has been shown to increase with depth 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 (Alonso-Sáez et al. 2007, Kirchman et al. 2007).

While a number of studies provide information on the contribution of MCG I to total prokaryotic abundance, only a few studies have explicitly measured MCG I activity. Archaeal chemoautotrophy, based on ammonia oxidation as energy source, has been shown to be widespread (Herndl et al. 2005, Wuchter et al. 2006). In the mesopelagic waters of the North Atlantic and in the Arctic Ocean, high DIC fixation rates by the prokaryotic community were found (Reinthaler et al. 2010, Alonso-Sáez et al. 2010, Varela et al. 2011), while other studies have shown heterotrophic activity of MCG I in the same regions (Kirchman et al. 2007, Varela et al. 2008). None of these studies, however, specifically determined the contribution of *Archaea* or MCG I to the leucine incorporation of the prokaryotic community.

Averaged over the entire water column, MCG I contributed 32% to the prokaryotic leucine incorporation in the subtropical North Atlantic (Tables 1 & 2) without any significant trend with depth, while the bacterial contribution decreases with depth (Fig. 3). T. Yokokawa and T. Nagata (unpubl. data) 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 subtropical North Atlantic are in the same range as those 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 350 and 550 m depth and a generally low archaeal contribution to prokaryotic leucine incorporation (Ionescu et al. 2009). Because these measurements were performed at a single station in the Red Sea, we do not know whether this is a general feature of the Red Sea. It might well be, however, that the contribution of *Archaea* to heterotrophy in the ocean is variable, particularly in regional seas like the Red Sea. Also, in the present study, the archaeal contribution to prokaryotic leucine incorporation was variable, as indicated by the coefficient of variation being about twice as high (50%, contribution mean ± SD: 32 ± 16%) as that of *Bacteria* (22%, mean ± SD: 69 ± 15%).

The substantial deviations from 100% of the total percentages of inhibition of leucine incorporation by erythromycin and diphtheria toxin for some samples (Table 1) might be caused by incomplete inhibition of the target group or by unspecific inhibition of the non-targeted group by one or both inhibitors. Depending on the prokaryotic community composition, the efficiency of the inhibitors against the target group and their effect on the non-target group might vary. Values of inhibition substantially higher than 100% could be related to some degree of inhibition of the non-target group by erythromycin or diphtheria toxin. In contrast, resistance to antibiotics (Baya et al. 1986, Levy & Marshall 2004) might explain inhibition values <100%. Moreover, the inhibitors might potentially serve as a substrate for the non-inhibited prokaryotic fraction (Dantas et al. 2008), thus inducing elevated activity in specific cells and consequently lowering the fraction of inhibition. Thus, the impact of the inhibitors on both the targeted and the non-targeted prokaryotic group needs to be thoroughly evaluated. However, taken together, the results of our specificity tests using a quantitative MICRO-CARD-FISH approach (Sintes & Herndl 2006) and the average inhibition obtained for *Archaea* and *Bacteria* in the Atlantic of 81% and 86%, respectively, validate our choice of inhibitors to target *Archaea* and *Bacteria*.

To date, the energy and carbon sources used by *Archaea* and the distribution patterns of heterotrophic and autotrophic prokaryotes are still not well understood in the ocean, particularly in the lower

meso- and bathypelagic realm. It has been suggested that *Archaea* are mainly chemoautotrophs, based on different approaches (Hallam et al. 2006, Wuchter et al. 2006, Hansman et al. 2009), down to bathy- and abyssopelagic waters (Agogué et al. 2008, Konstantinidis et al. 2009, Church et al. 2010). Nevertheless, the heterotrophic lifestyle of MCG I has also been shown (Teira et al. 2006b, Martin-Cuadrado et al. 2008). Our results further support the occurrence of some extent of heterotrophic activity in MCG I in the open ocean and might indicate either a mixotrophic lifestyle of *Archaea* or the presence of autotrophic and heterotrophic lifestyles in different taxa of MCG I.

The cell-specific leucine incorporation rates of MCG I were similar to those of *Bacteria* in the bathypelagic layer but were, however, 3.5-fold lower than those of *Bacteria* in the top 1000 m layer (Table 2). Because only leucine-positive MCGI and *Bacteria* were included in these calculations, the lower leucine uptake rate of MCG I than *Bacteria* in the surface and mesopelagic waters represents actual differences in the leucine uptake on a single-cell level rather than simply differences in the fraction of MCG I or bacterial cells capable of taking up leucine or being metabolically active. Likewise, the similar leucine uptake rates of MCG I and *Bacteria* in the bathypelagic waters indicate similar activity levels on a single-cell basis rather than shifts in the metabolically active fraction of the MCG I or bacterial community. Using the quantitative MICRO-CARD-FISH approach (Sintes & Herndl 2006), Varela et al. (2011) found that MCG I are fixing DIC at significantly lower rates than *Bacteria* throughout the water column of the subtropical North Atlantic. This might indicate that MCG I exhibit, on a per-cell basis, lower or similar activity levels as *Bacteria*, in both the auto- and heterotrophic life mode. More detailed studies are required, however, to verify this conclusion. The use of specific inhibitors for activity measurements in combination with single-cell analyses, such as MICRO-CARD-FISH, might be a valuable tool to differentiate the role of *Archaea* and *Bacteria* in the biogeochemical cycles of the ocean.

The present study demonstrates the potential of erythromycin and diphtheria toxin as protein synthesis inhibitors in *Bacteria* and *Archaea*, respectively. The fairly high specificity of the 2 inhibitors allows for estimations of the relative contribution of these 2 prokaryotic domains to the overall activity of the prokaryotic community. Our measurements throughout the water column of the eastern subtropical North Atlantic indicate that a substantial part (on average, 32% over the entire water column) of the heterotrophic prokaryotic activity is due to MCG I. The cell-specific leucine uptake of *Bacteria* and MCG I appears to be similar in the bathypelagic Atlantic waters. Given the generally high abundance of MCG I in bathypelagic waters of the global ocean, there is evidence now that deep-water prokaryotic heterotrophic activity is mediated not only by *Bacteria* but to a considerable extent also by marine *Crenarchaeota* Group I.

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