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ORIGINAL ARTICLE

Spatial distribution of *Bacteria* and *Archaea* and *amo*A gene copy numbers throughout the water column of the Eastern Mediterranean Sea

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Until recently, ammonia oxidation, a key process in the global nitrogen cycle, was thought to be mediated exclusively by a few bacterial groups. It has been shown now, that also *Crenarchaeota* are capable to perform this initial nitrification step. The abundance of ammonia oxidizing *Bacteria* and *Archaea* was determined using the bacterial and archaeal ammonia monooxygenase-α subunit (*amo*A) gene as functional markers in a quantitative PCR approach and related to the abundance of *Bacteria* and *Archaea* in the Eastern Mediterranean Sea. Archaeal *amo*A copy numbers decreased from 4000–5000 copies ml^{-1} seawater from the 200–500 m depth layer to 20 copies ml^{-1} at 1000 m depth. β-Proteobacterial *amo*A genes were below the detection limit in all the samples. The archaeal *amo*A copy numbers were correlated with NO_2^- concentrations, suggesting that ammonia-oxidizing *Archaea* may play a significant role in the nitrification in the mesopelagic waters of the Eastern Mediterranean Sea. In the bathypelagic waters, however, archaeal *amo*A gene abundance was rather low although *Crenarchaeota* were abundant, indicating that *Crenarchaeota* might largely lack the *amo*A gene in these deep waters. Terminal restriction fragment length polymorphism analysis of the archaeal community revealed a distinct clustering with the mesopelagic community distinctly different from the archaeal communities of both, the surface waters and the 3000–4000 m layers. Hence, the archaeal community in the Eastern Mediterranean Sea appears to be highly stratified despite the absence of major temperature and density gradients between the meso- and bathypelagic waters of the Mediterranean Sea.

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Introduction

In the oceanic water column, the archaeal contribution to prokaryotic abundance varies among different regions, water masses and depths (Karner *et al.*, 2001; Herndl *et al.*, 2005). *Crenarchaeota* appear to be more variable in abundance than *Euryarchaeota* as revealed by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) (Teira *et al.*, 2006a; Varela *et al.*, 2008). Using genomic approaches, light has been shed onto the potential role of archaeal communities in the oceanic carbon and nitrogen cycle (Hallam *et al.*, 2006; Lam *et al.*, 2007). It has been firmly established now that at least some *Crenarchaeota* are chemoauotrophs fixing CO_2 (Herndl *et al.*, 2005; Kirchman *et al.*, 2007) and using ammonia as an electron donor and energy source (Francis *et al.*, 2005; Könneke *et al.*, 2005). *Crenarchaeota* might be more important than *Bacteria* in oxidizing ammonia as a higher abundance of the archaeal gene encoding for a subunit of the enzyme ammonia monooxygenase A, *amo*A, was detectable than bacterial *amo*A gene abundance in the North Sea and the mesopelagic waters of the North Atlantic (Wuchter *et al.*, 2006).

There are only a few studies on the prokaryotic community composition of the water column of the

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Eastern Mediterranean Sea (Moeseneder *et al.*, 2001a, b, 2005). One of the most peculiar features of the Eastern Mediterranean Sea is its highly oligotrophic nature, its extremely low concentration of inorganic phosphorus (Thingstad *et al.*, 2005) and the warm deep waters (about 14 °C). The latter is a general characteristic of the bathypelagic realm of the Mediterranean. The composition of the water masses in the individual basins of the Eastern Mediterranean Sea differs remarkably (Zervakis *et al.*, 2004). In the northern Aegean Sea, outflow of Black Sea water introduces inorganic nutrients with decreasing influence towards the south. Several deep-water basins of the Eastern Mediterranean Sea harbor fairly isolated deep-water bodies (Zervakis *et al.*, 2004). The differences in the physical and chemical characteristics of the water bodies from north to south in the Eastern Mediterranean Sea are also reflected by considerable changes in the bacterial community composition as revealed by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting (Moeseneder *et al.*, 2001a).

The aim of this study was to determine the spatial abundance of *Crenarchaeota*, *Euryarchaeota* and *Bacteria* and the distribution of archaeal and bacterial *amo*A genes throughout the water column of the Eastern Mediterranean Sea along the north to south gradient, using CARD-FISH and quantitative-PCR (Q-PCR), respectively. T-RFLP was also used to compare the archaeal community composition among the stations and depth horizons. Our results suggest that ammonia-oxidizing *Archaea* may play a significant role in the nitrification in the mesopelagic realm of the Eastern Mediterranean Sea, while in the bathypelagic waters, *Archaea* are apparently lacking the *amo*A gene.

Materials and methods

Study site and sampling

The cruise in the Eastern Mediterranean Sea was conducted with the RV AEGAEO (Hellenic Center for Marine Research, Greece) occupying five stations in May 2007 (Figure 1). The stations were located in the North Aegean (St. 1; 48° 7.0′N, 24° 32.5′E), Mid Aegean (St. 2; 37° 42.1′N, 25° 26.0′E), Western Cretan Sea (St. 3; 36° 13.2′N, 23° 18.3′E), Ionian Sea (St. 4; 36° 15.9′N, 21° 30.1′E) and South Aegean (St. 5; 35° 47.2′N, 24° 54.7′E). Water samples were obtained from the mixed surface waters (10 m depth) and the meso- and bathypelagic layers (down to a maximum depth of 4350 m) with 5L-Niskin bottles mounted on a conductivity-temperature-depth rosette sampler. Dissolved oxygen concentrations were determined with a SBE oxygen sensor mounted on the conductivity-temperature-depth and nutrient concentrations (that is, phosphate, nitrate and nitrite) with a nutrient auto-analyzer (Bran & Luebbe Autoanalyzer II, Norderstedt, Germany).

For later molecular analyses of the prokaryotic community, 10 l of seawater was filtered through a 0.22 μm Sterivex filter cartridge (Millipore, Millford, MA, USA) to collect prokaryotes for molecular analyses. Thereafter, 1.8 ml of lysis buffer (40 mM EDTA, 50 mM Tris-HCL, 0.75 M sucrose) was added to the filter cartridge and the filter cartridges were stored at −80 °C.

Composition of specific phylogenetic groups

The composition of *Bacteria*, *Crenarchaeota* and *Euryarchaeota* was determined by CARD-FISH (Teira *et al.*, 2004). Water samples of 10–80 ml were collected from the Niskin bottles and preserved in paraformaldehyde (2% final concentration) at 4 °C in the dark for 18 h. Thereafter, the samples were filtered onto 0.2 μm polycarbonate filters (Millipore, GTTP) supported by 0.45 μm cellulose nitrate filters (Millipore, HAWP), rinsed with Milli-Q water, dried and stored in microfuge vials at −20 °C until further processing in the laboratory.

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) or with proteinase-K for marine *Euryarchaeota* Group II probe Eury806 and for the marine *Crenarchaeota* Group I probes Cren537 (Teira *et al.*, 2006a) and GI-554 (Massana *et al.*, 1997) following the method described by Teira *et al.* (2004). Filters were cut in sections and hybridized with horseradish peroxidase-labeled oligonucleotide probes and subsequently incubated with tyramide-Alexa488 for signal amplification. Thereafter, the filter sections were stained with a DAPI (4,6-diamidino-2-phenylindole) mix (5.5 parts of Citifluor (Citifluor, London, UK), 1 part of Vectashield (Vector Laboratories, Burlingame, CA, USA) and 0.5 parts of phosphate-buffered saline with DAPI (final concentration 5 μg ml^{-1})).

Hybridized and DAPI-stained cells were detected under a Zeiss Axioplan 2 epifluorescence microscope equipped with a 100 W Hg-lamp and appropriate filter sets for Alexa488 and DAPI. More than 600 DAPI-stained cells were counted per sample. For each microscope field, two categories were determined: total DAPI-stained cells and cells stained with the specific probe.

DNA extraction

The cartridges of the Sterivex filters were cracked open and the filters and the lysis buffer transferred into 50 ml sterile centrifuge tubes. DNA extraction was performed using the Mega Kit extraction (MoBIO laboratories, Carlsbad, CA, USA) and the protocol of the manufacturer. DNA extracts were concentrated (approximately 10 times) with a Centricon device (Millipore).

Q-PCR analysis

We conducted Q-PCR targeting 16S rRNA gene fragments of Marine *Crenarchaeota* Group I (MCGI)

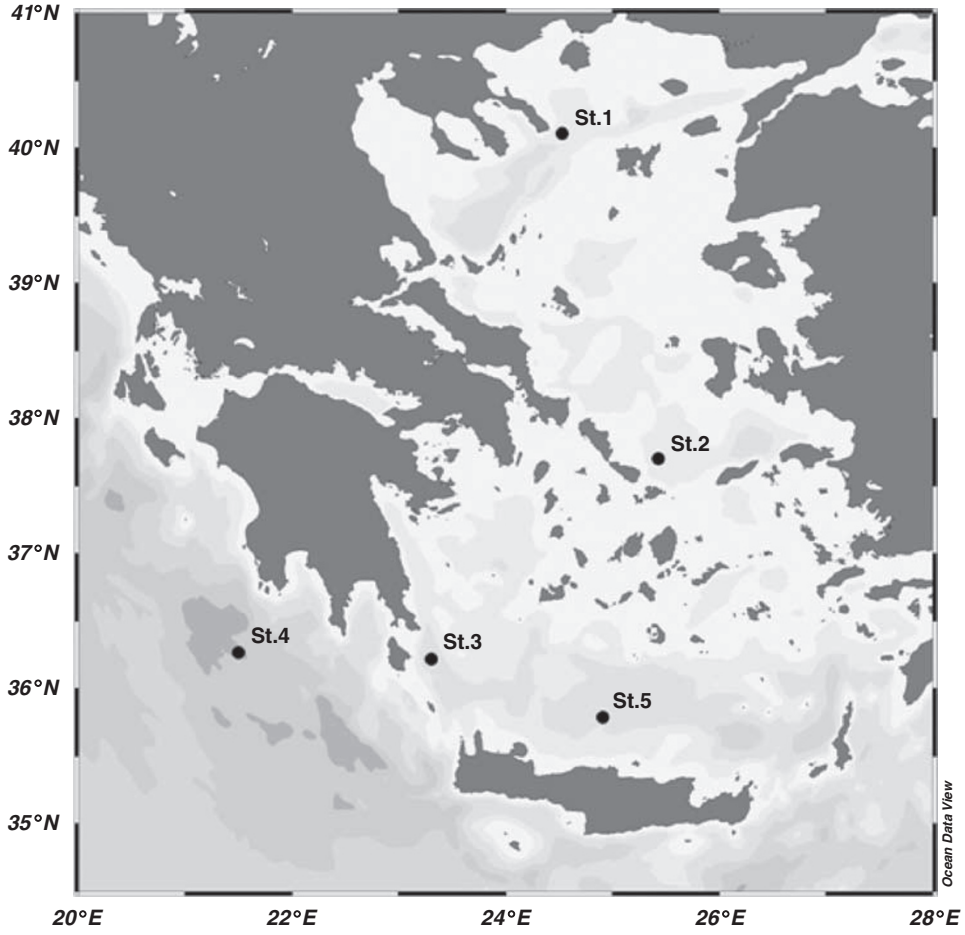

Figure 1 Sampling sites (Sts.1–5) in the Eastern Mediterranean Sea occupied during the POSEIDON cruise in May 2007.

and the pSL12 cluster and archaeal *amo*A genes for the samples collected at Sts. 1, 2 and 4 (St. 1: 100, 200 and 950 m; St. 2: 300, 400 and 750 m; St. 4: 1000, 3000 and 4350 m). β-Proteobacterial *amo*A genes were targeted using *amo*A-1F (5′-GGGGTTTC TACTGGTGGT)/*amo*Ar-new (5′-CCCCTCBGSAAAV CCTTCTTC) (Hornek *et al*., 2006). A mix of 25% of *Nitrosomonas europaea*, 25% of *N. eutropha*, 25% of *N. marina* and 25% of *Nitrosospira briensis* was used as a standard. β-Proteobacterial *amo*A genes, however, were not detected at any of the stations, although the same primer set readily detected β-Proteobacterial *amo*A in the coastal North Sea and the open North Atlantic (Wuchter *et al*., 2006). All Q-PCR experiments were performed on an iCycler iQ 5 thermocycler (Bio-Rad, Philadelphia, PA, USA) equipped with iCycler iQ software (version 3.1, Bio-Rad). The *amo*A copy numbers in the standards and environmental samples were determined in triplicate with three different dilutions. The reaction mixture (20 μl) contained 1 unit of Pico Maxx polymerase, 2 ml of 10 × Pico Maxx PCR buffer (Stratagene, La Jolla, CA, USA), 0.25 mM of each dNTP, 8 μg of bovine serum albumin, 0.2 mM of primers, 50 000 times diluted SYBR Green I (Molecular Probes), a final concentration of 10 nM fluorescein, 3 mM $MgCl_2$ and ultra-pure sterile water (Wuchter *et al*., 2006). The efficiency of the quantification reaction ranged between 74.5 and 111.5% with an r^2 ranging from 0.983 to 1.000. Marine *Crenarchaeota* Group I (MCGI) 16S rRNA gene fragments were detected using the primer set MCGI-391f (5′-AAGGTTARTCCGAGTGRTTTC) and MCGI-554r (5′-TGACCACTTGAGGTGCTG) with plasmid 88exp4 as a standard and primer annealing at 61 °C for 40 s (Wuchter *et al*., 2006). The pSL12 16S rRNA gene fragments were detected using the primer set pSL 12-750F (5′-GGTCCRCCAGAAC GCGC) and pSL12-876R (5′-GTACTCCCCAGGCGGC AA) with fosmid HF770_041/11 as a standard and primer annealing at 65 °C for 40 s (Mincer *et al*., 2007). Archaeal *amo*A genes were detected using the specific archaeal *amo*A primer set arch-amoA-for (5′-CTGAYTGGGCYTGGACATC) and arch-amoA-rev (5′-TTCTTCTTTGTTGCCCAGTA) with *Nitrosopumilus maritimus* as a standard and primer annealing at 58.5 °C for 40 s (Wuchter *et al*., 2006). The PCR efficiencies and correlation coefficients for the standard curves were as follows: for the MCGI 16S rDNA assay, 87.9–105.8% and $r^2 = 0.991$–0.999,

for the pSL12-like 16S rDNA assay, 94.7–110.5% and $r^2 = 0.949–0.999$, for the archaeal *amo*A assay, 86.5–97% and $r^2 = 0.983–0.999$ and for the β-proteobacterial *amo*A assay, 77.8–88.3% and $r^2 = 0.989–0.999$.

PCR and T-RFLP

PCR conditions and chemicals were applied as described by Moeseneder *et al*. (2001a). One μl of the DNA extract was used as a template in a 50 μl PCR mixture. For PCR, the universal primer 1492R-JOE (Lane, 1991) and the *Archaea*-specific primers 21F-FAM and 958R-JOE were used (Moeseneder *et al*., 2001a). Samples were amplified by an initial denaturation step at 94 °C (for 3 min), followed by 35 cycles of denaturation at 94 °C (1 min), annealing at 55 °C (1 min), and an extension at 72 °C (1 min). Cycling was completed by a final extension at 72 °C for 7 min. The PCR products were run on 1.0% agarose gel. The gel was stained with a working solution of SYBR Gold and the obtained bands were excised, purified with the Quick gel extraction kit (Genscript, Piscataway, NJ, USA), and quantified using a Nanodrop spectrophotometer. Fluorescently labeled PCR products were digested at 37 °C overnight. Each reaction contained 30 ng of cleaned PCR product, 5 U of tetrameric restriction enzyme (*Hha*I) and the respective buffer filled up to a final volume of 50 ml with ultra-pure water (Sigma, St Louis, MO, USA). The restriction enzyme was heat inactivated and precipitated by adding 4.5 μl LPA solution and 100 μl of 100% isopropanol. The samples were kept at room temperature for 15 min followed by centrifugation at 15 000 *g* for 15 min. Thereafter, the supernatant was discarded and the pellet rinsed with 100 μl 70% isopropanol and precipitated again by centrifugation (15 000 *g* for 5 min). Subsequently, the supernatant was removed again and the sample dried in the cycler at 94 °C for 1 min and stored at −20 °C until further analysis.

The pellet was resuspended in 2 ml of ultra-pure water and the product denatured in 7.8 ml of Hi-Di formamide at 94 °C for 3 min. Each sample contained 0.2 ml GeneTace 1000 (ROX) marker (Applied Biosystems, Foster City, CA, USA). Fluorescently labeled fragments were separated and detected with an ABI Prism 310 capillary sequencer (Applied Biosystem) run under GeneScan mode (van der Maarel *et al*., 1998; Moeseneder *et al*., 1999). The size of the fluorescently labeled fragment was determined by comparison with the internal GeneTace 1000 (ROX) size standard. Injection was performed electrokinetically at 15 kV for 15 s (adjustable) at 60 °C.

The output from the ABI Genescan software was transferred to the Fingerprinting II (Bio-Rad) to determine the peak area and for standardization using size markers. The obtained matrix was further analyzed with Primer software (Primer-E) to determine similarities of the T-RFLP fingerprints between samples.

Results

There was little variability in temperature and salinity among the five stations (Table 1). Phosphate and nitrate concentrations exhibited the common depth-related trend with low concentrations in the top 100 m layer and increasing with depth (Table 1). Oxygen concentrations remained rather constant with depth exhibiting no pronounced oxygen minimum layer.

Prokaryotic community composition determined by CARD-FISH

The mean contribution of *Bacteria* to total prokaryotic abundance (that is, DAPI-stained cell) was 45 ± 10% ($n = 29$) averaged over all the stations and depth layers (Figure 2). Overall, the variability in the relative abundance of *Crenarchaeota* and

Table 1 Sampling station, region name, layer, sample amount, physical parameters (temperature and salinity) and chemical parameters (phosphate, nitrate and nitrite)

Station	*Region*	*Bottom depth (m)*	*Layer (m)*	*Number of samples*	*Temperature (°C)*	*Salinity*	*Dissolved oxygen (μmol l⁻¹)*	*PO_4 (μmol l⁻¹)*	*NO_3 (μmol l⁻¹)*	*NO_2 (μmol l⁻¹)*
1	North Aegean	1010	>100	2	14.3–16.2	36.7–38.4	129.8–133.6	0.04–0.05	0.36–0.68	0.05–0.10
			100–950	6	13.8 ± 0.4	39.0 ± 0.0	124.2 ± 4.6	0.12 ± 0.04	3.10 ± 1.29	0.05 ± 0.01
2	Mid Aegean	800	>100	2	15.5–16.6	39.2–40.0	109.6–141.9	0.02	0.07–0.13	0.05
			100–750	7	14.4 ± 0.3	39.0 ± 0.1	132.6 ± 3.2	0.07 ± 0.02	1.76 ± 0.53	0.05 ± 0.01
3	Western Cretan	1245	>100	2	15.0 17.8	38.8	137.5	0.03	0.12–0.41	0.04–0.08
			100–1200	7	14.4 ± 0.1	38.9 ± 0.0	130.1 ± 2.3	0.10 ± 0.04	2.56 ± 1.06	0.05 ± 0.01
4	Ionian	4322	>100	2	16.8–19.9	38.7	137.3–147.0	0.02–0.03	0.03–0.06	0.04–0.05
			100–1000	7	14.9 ± 0.8	38.8 ± 0.1	129.9 ± 11.0	0.09 ± 0.07	1.76 ± 1.54	0.04 ± 0.01
			2000–4350	3	14.6 ± 0.8	38.8 ± 0.0	123.0 ± 1.3	0.15 ± 0.01	3.57 ± 0.45	0.04 ± 0.01
5	South Aegean	1420	>100	2	15.6–20.4	36.0–39.0	117.6–152.3	0.03	0.07–0.33	0.05–0.08
			100–1350	7	14.7 ± 0.5	39.0 ± 0.1	137.2 ± 4.7	0.10 ± 0.04	2.52 ± 1.26	0.07 ± 0.06

Parameters of the top 100 m layer are given as range, all other data represent mean ± s.d.

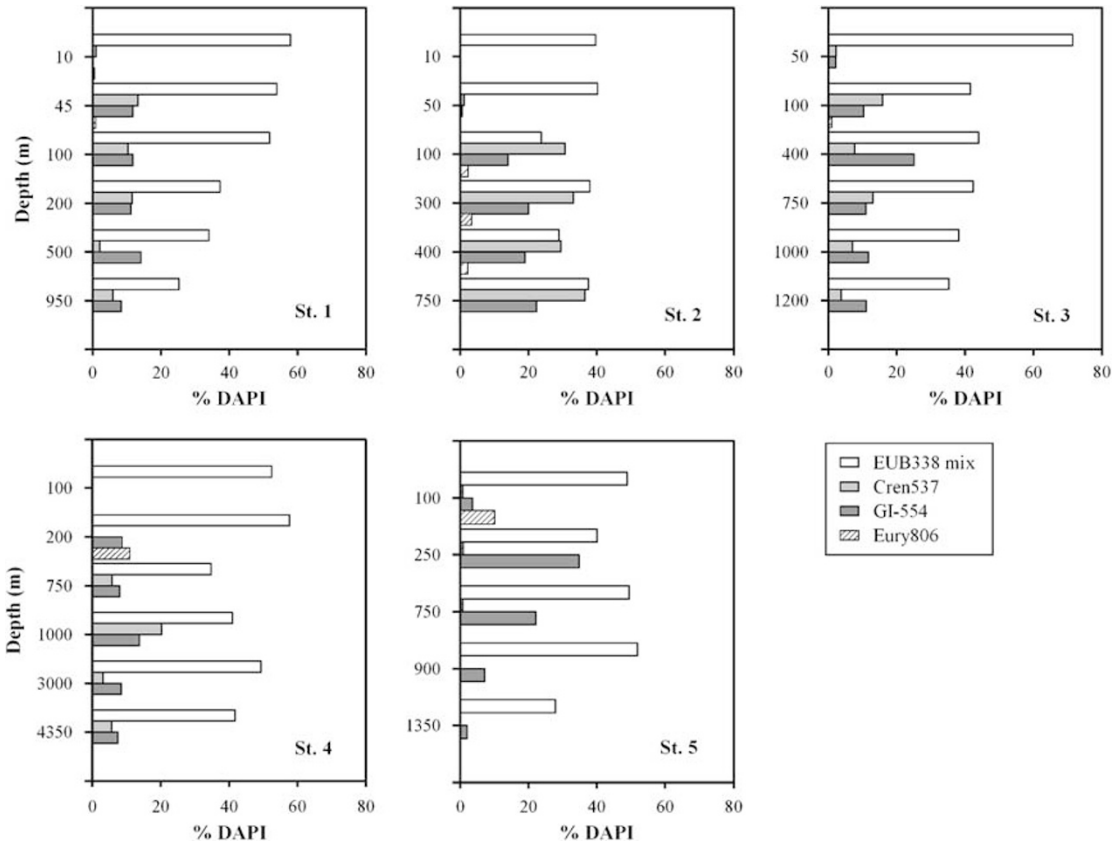

Figure 2 Contribution of *Bacteria* (EUB338 mix), Cren537-positive cells, GI-554-positive cells and Eury806-positive cells to total prokaryotic abundance (% of DAPI-stained cells) at the individual stations determined by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH).

Euryarchaeota was higher than that for *Bacteria*. Both, the Cren537 and the GI-554 probe, target the same crenarchaeal phylotypes with an additional 5 and 14% of phylotypes covered by the probe Cren537 and GI-554, respectively (Figure 3). The relative contribution of Cren537- and GI-554-positive cells to total prokaryotic abundance was $7 \pm 9\%$ (average $\pm$ s.d., $n = 29$), and $12 \pm 9\%$ ($n = 29$), respectively (Figure 2). The ratio of GI-554- to Cren537-positive cells ranged from 0.12 to 39.70. At St. 2, the relative abundance of Cren537-positive cells was higher than of GI-554-positive cells throughout the water column, whereas GI-554-positive cells dominated at St. 5 (Figure 2). At St. 5, the maximum percentage of GI-554-positive cells was 35% at 250 m decreasing with depth. Eury806-positive cells represented only a minor fraction of total prokaryotic abundance ($1 \pm 3\%$, $n = 29$) at all the stations and were detected only in the top 400 m of the water column (Figure 2).

Abundance of specific phylogenetic groups determined by CARD-FISH and Q-PCR

In 14 out of 29 samples, the difference in abundance between Cren537- and GI-554-positive cells was larger than a factor of 2 (Figure 4). These differences in abundance between *Crenarchaeota* enumerated by the Cren537 and GI-554 probe were highest in the upper mesopelagic realm.

Copy numbers of the 16S rRNA gene of the crenarchaeal MCGI and the pSL12 cluster were determined by Q-PCR from several depths at Sts. 1, 2 and 4. The average copy numbers of the 16S rRNA gene of MCGI was $10.9 \pm 11.1 \times 10^3$ copies ml^{-1} seawater ($n = 9$), while the corresponding copy numbers of the pSL12 cluster were about three times lower ($3.4 \pm 6.5 \times 10^3$ copies ml^{-1}, $n = 9$) (Figure 4). There was a distinct difference in the spatial distribution pattern between MCGI and the pSL12 cluster. Higher copy numbers of the 16S rRNA gene of MCGI were obtained for the mesopelagic layer (200–1000 m) than for the surface and bathypelagic waters (>1000 m), while copy numbers of the pSL12 cluster did not exhibit major fluctuations with depth (Figure 4).

Archaeal community composition based on T-RFLP fingerprinting

The T-RFLP pattern of the archaeal community revealed in total 27 operational taxonomic units (OTUs) on the 16S rDNA level ranging from 13.2 to 1016 bp fragments (Figure 5a). Only one of the 27

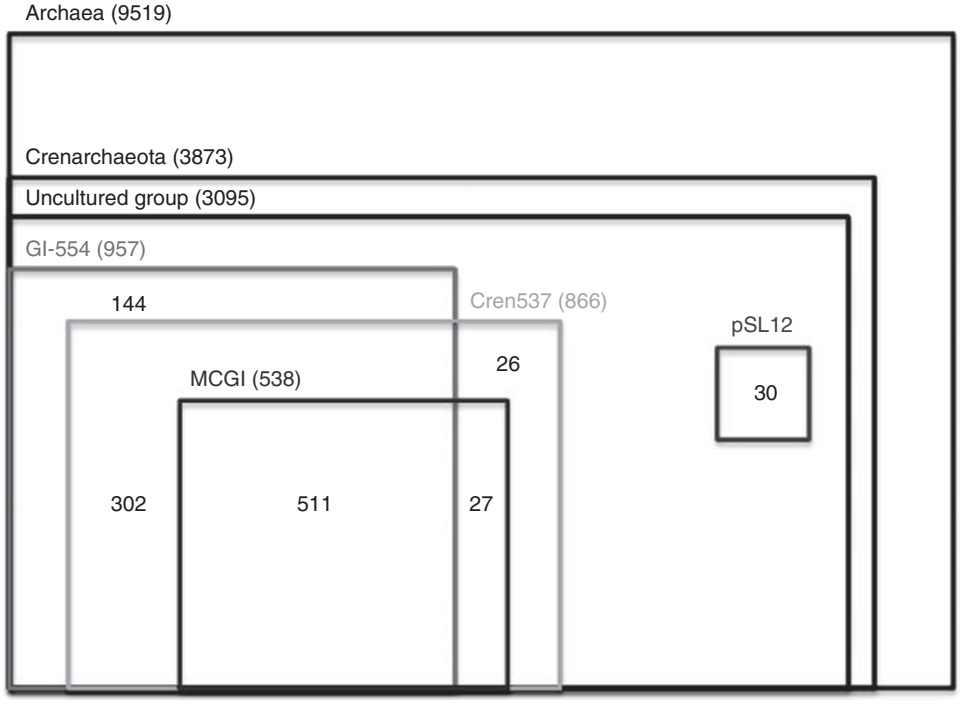

Figure 3 Scheme of the coverage of 16rRNA sequences deposited in SILVA database of the crenarchaeal FISH probes Cren537 and GI-554 and the PCR primers as of 4 April 2008. Numbers in parentheses are number of phylotypes of the specific target group in the database. Numbers in the boxes represent the number of phylotypes covered by the probe or primer.

OTUs (248.2 bp fragment) was present at all stations and depths and the OTUs of 580.99 and 591.29 pb were presented in almost all the samples without a clear vertical pattern. The OTU of 129.79 bp was detectable in all the samples until 1400 m depth but not at greater depth. Among all the stations, St. 4 exhibited the highest and lowest number of OTUs detectable in a specific sample, 13 OTUs at 750 m depth and 2 OTUs at 3000 m depth (Figure 5a). The Jaccard similarity cluster analysis indicated three distinct clusters, a subsurface water, a meso- and a bathypelagic cluster (Figure 5b). Analysis of similarities (ANOSIM) revealed significant differences in archaeal community composition between the surface layer (45–100 m depth) and the mesopelagic layer (200–1000 m depth, $r=0.69$, significant level: 0.1) and between the mesopelagic layer and the bathypelagic layer (>1400 m, $r=0.60$, significant level: 5.5).

Depth profile of archaeal amo*A copy numbers*
Copy numbers of the archaeal *amo*A gene were readily detectable (Figure 6a), while β-proteobacterial *amo*A genes were undetectable at all the stations. Copy numbers of the archaeal *amo*A gene were, on average, $3.9\pm1.2\times10^3$ copies ml^{-1} (mean ± s.d., $n=4$) in the 200–500 m layer and 1.6×10^3 copies ml^{-1} at 750 m depth. Below 950 m depth, archaeal *amo*A copy numbers never exceeded 10 copies ml^{-1} ($n=4$).

Ratios of archaeal *amo*A gene copy numbers to 16S rRNA gene copy numbers (sum of MCGI and pSL12) decreased drastically with depth (Figure 6b). The ratios decreased from 0.49 at 100 m to 0.12 at 400 m depth. Below 750 m depth, the ratios were below 0.05 (0.01 ± 0.02, $n=5$). The ratios of archaeal *amo*A gene copy numbers to crenarchaeal abundance, determined by CARD-FISH with the probes Cren537 and GI-554 (Figure 6c), were not significantly different from those obtained using the sum of the 16S rRNA copy numbers of MCGI and pSL12 (ANOVA on ranks, $\chi^2=0.222$, $P=0.895$). Copy numbers of the archaeal *amo*A gene correlated with nitrite concentrations ($r=0.79$, $P<0.01$, $n=9$), while no relation was found with nitrate ($P>0.05$, $n=9$); unfortunately no data are available for ammonium.

Discussion

Prokaryotic community composition
Bacteria contributed between 24 and 72% to total prokaryotic abundance at the sampling stations in the Eastern Mediterranean Sea. Although the contribution of *Bacteria* to total prokaryotes did not differ significantly among the stations and did not show any depth-related pattern, the bacterial contribution to total prokaryotic abundance is more variable in the Eastern Mediterranean Sea than in the North Atlantic determined by essentially the

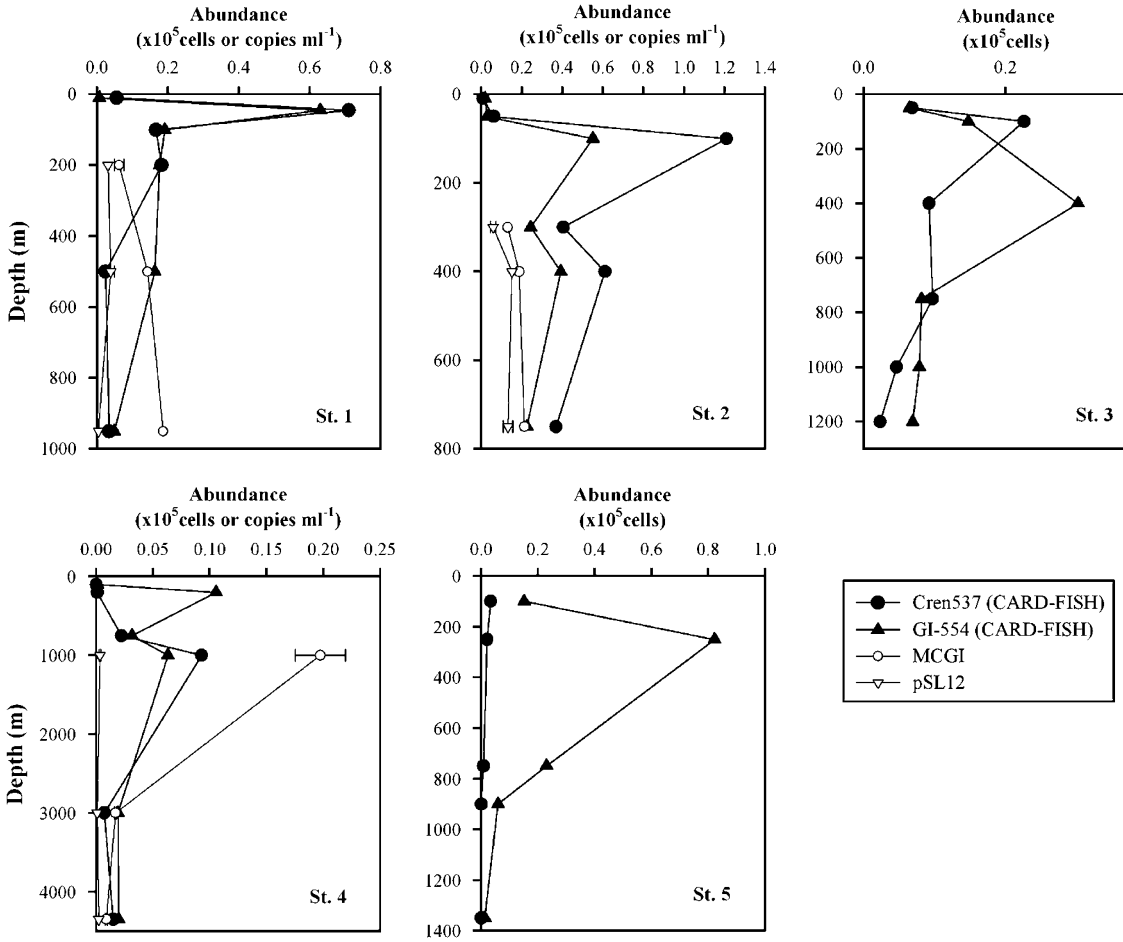

Figure 4 Depth profiles of Cren537- and GI-554-positive cells determined by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FSIH) (at Sts. 1–5), and MCGI and pSL12 gene copy numbers determined by quantitative-PCR (Q-PCR) (at Sts. 1, 2 and 4). For Q-PCR, the mean ± s.d. of determination is given for samples where triplicate determination has been performed, while the average of determination is given for samples where duplicate determination has been carried out.

same approach as used here (Teira *et al.*, 2006a; Varela *et al.*, 2008).

The mean contribution of the *Euryarchaeota* to total prokaryotic abundance averaged only 1.4% and they are confined to the subsurface and mesopelagic layers (Figure 2). Other studies also report only a minor contribution of *Euryarchaeota* to the prokaryotic community such as for the western Arctic Ocean (average: 3.5% in Table 1 of Kirchman *et al.* (2007)), the north-west Mediterranean coastal waters (average: 2.5% in Table 2 of Alonso-Saez *et al.* (2007)) and the (sub)tropical North Atlantic region (<5%, (Varela *et al.*, 2008)). Thus, the minor contribution of *Euryarchaeota* to prokaryotic abundance appears to be a common feature for this group, however, higher contributions than that reported by the above studies have been found in some deep waters of the North Atlantic (Teira *et al.*, 2006a) and by shotgun sequencing of fosmid clone libraries in the North Pacific (DeLong *et al.*, 2006). *Euryarchaeota* inhabiting the surface waters have been shown to harbor the proteorhodopsin gene allowing them to use light to drive the membrane proton pump, while deep-water *Euryarchaeota* lacked the proteorhodopsin gene (Frigaard *et al.*, 2006).

The relative contribution of Cren537- and GI-554-positive cells to total prokaryotic abundance was 7 ± 9 and 12 ± 9%, respectively (Figure 2). Cren537 covers 866 out of 3873 phylotypes of *Crenarchaeota* and all the MCGI present in the SILVA database as of 4 April 2008, while GI-554 covers 957 crenarchaeal phylotypes resulting in an overlapping coverage of 813 phylotypes (Figure 3). Applying both probes separately using CARD-FISH on the same sample, we detected a variable number of *Crenarchaeota*. At St. 5, GI-554-positive cells dominated throughout the water column, while at St. 2, Cren537-positive cells dominated (Figure 2). These large variations in the relative abundance of Cren537 (range: 0–37%) and GI-554 (range: 0–35%) might suggest compositional differences in the crenarchaeotal community among stations. Recent studies showed that the relative abundance of marine *Crenarchaeota* Group I increases with depth in the North Atlantic and

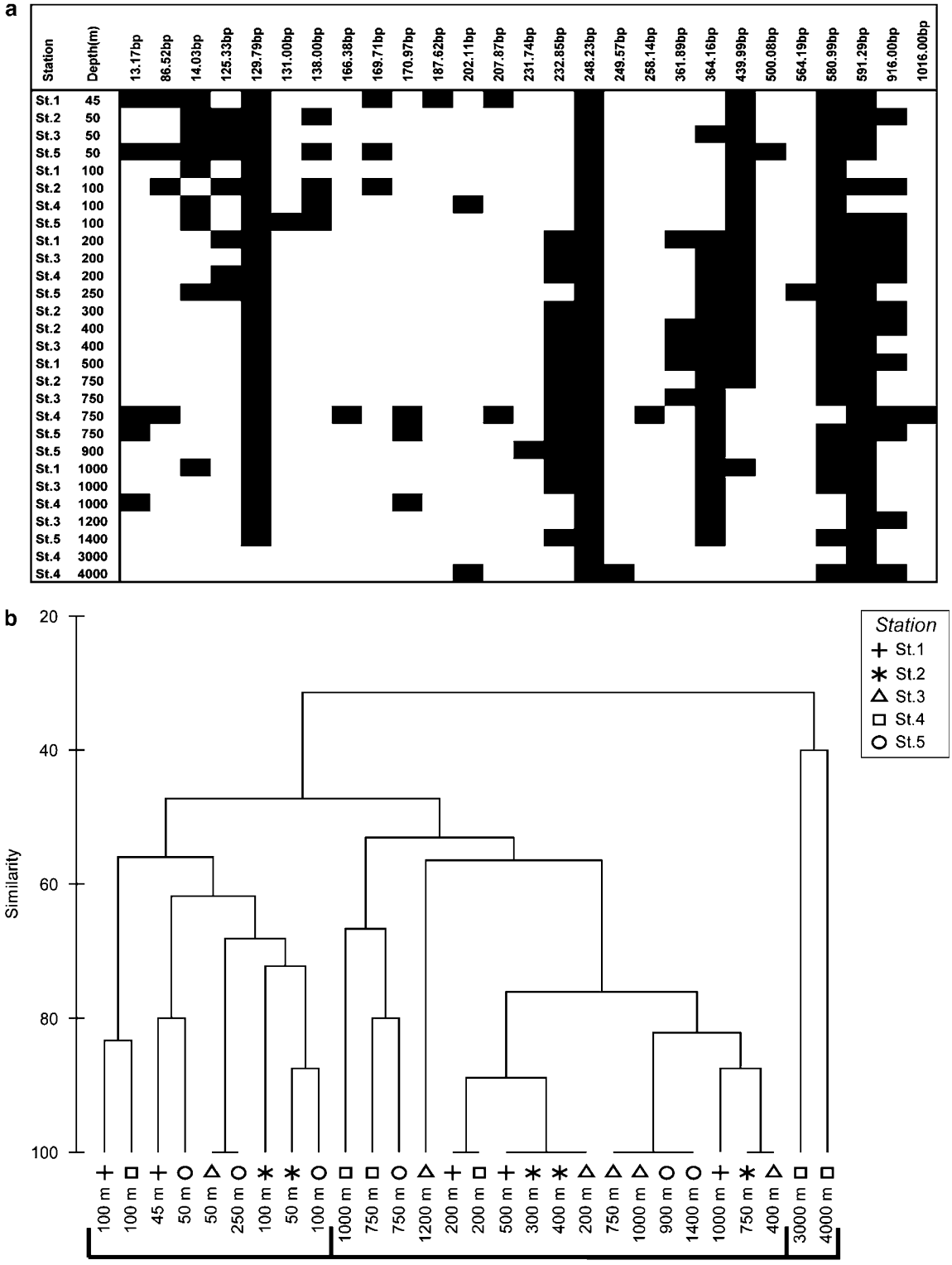

Figure 5 Archaeal community composition as revealed by terminal restriction fragment length polymorphism analysis. (**a**) Presence/absence distribution of all the individual archaeal OTUs detected at the individual stations and depth layers. Rows are aligned from surface to deep waters. (**b**) Similarity matrix for the individual samples obtained in the Eastern Mediterranean Sea.

the Arctic Ocean (Herndl *et al.*, 2005; Kirchman *et al.*, 2007). However, no consistent depth-related trends in the relative abundance of both Cren537-positive and GI-554-positive cells are detectable in the Eastern Mediterranean Sea (Figure 2). The lack of a depth-related trend in the distribution of *Crenarchaeota*

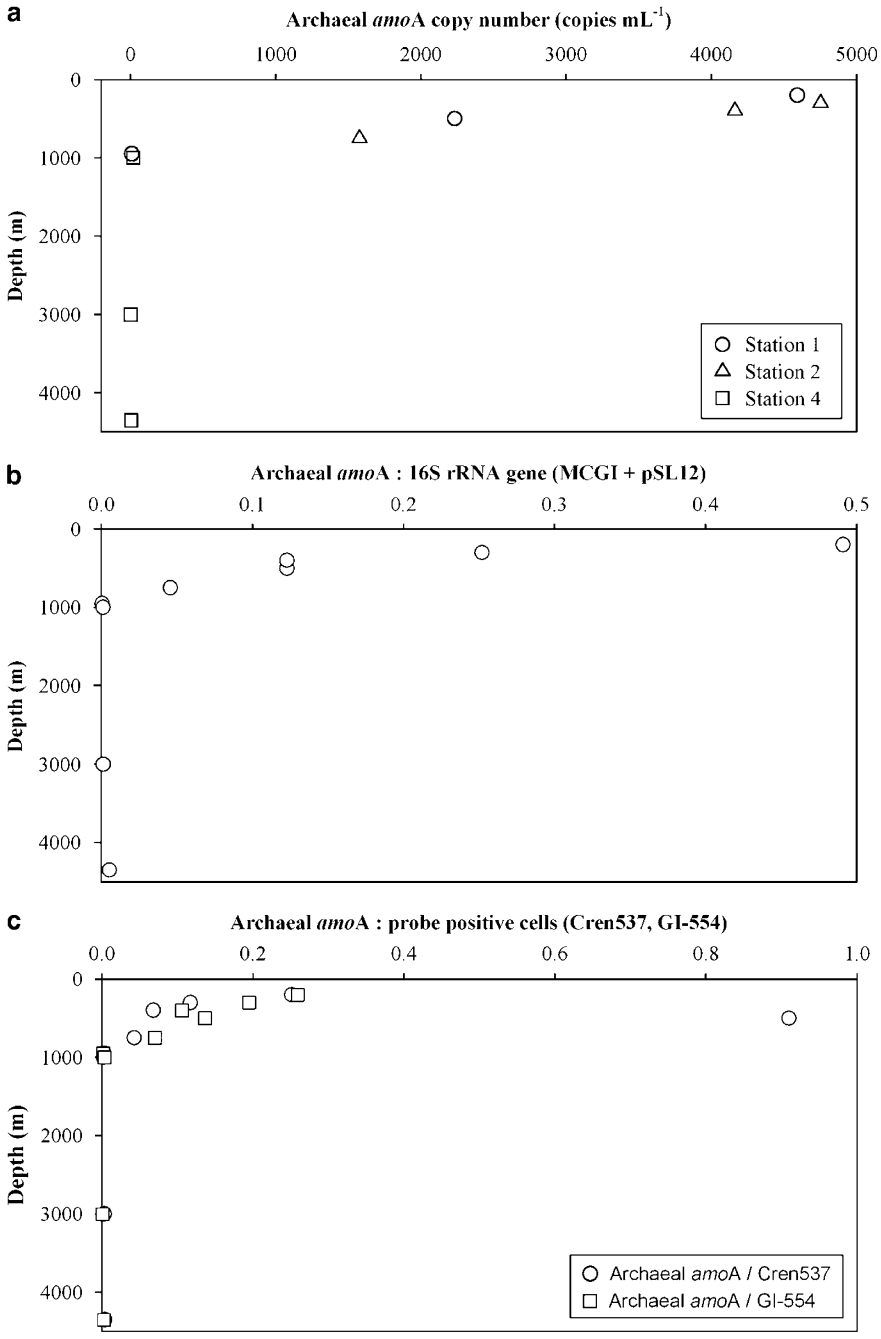

Figure 6 Depth distributions of archaeal *amo*A gene copy numbers at the Sts. 1, 2 and 4 (**a**); ratios of archaeal *amo*A to archaeal 16S rRNA gene (that is, sum of MCGI and pSL12 cluster) (**b**); ratios of archaeal *amo*A to Cren537 or GI-554 (**c**).

contrasts the pronounced decrease in the copy numbers of archaeal *amo*A genes with depth and the compositional differences in the archaeal community structure revealed by T-RFLP, discussed in more detail below.

The recovery efficiency with our CARD-FISH approach, that is, the sum of *Bacteria* determined by the EUB338 probe mix (average recovery efficiency: $44.7 \pm 10.0\%$, $n = 29$), *Euryarchaeota* (detected by Eury806, $1.4 \pm 3.6\%$, $n = 19$) and *Crenarchaeota* (detected either by Cren537 ($6.8 \pm 9.5\%$, $n = 29$) or GI-554 ($11.7 \pm 9.1\%$, $n = 29$)), ranged from 28 to 75%. Considering the almost complete coverage of all the bacterial groups with the EUB338 probe mix (Amann and Fuchs, 2008), the occasionally low recovery efficiency might be caused by a low coverage of the crenarchaeal community with the probes Cren537 and GI-554, by inefficient permeabilization of the cell membranes of certain prokaryotic cells or a variable contribution of dead or decaying cells still stainable with DAPI but lacking sufficient RNA (Del Giorgio and Gasol, 2008).

Abundance of specific phylogenetic groups determined by CARD-FISH and Q-PCR

Both, the abundance of Cren537- and GI-554-positive cells were within the range previously reported for *Crenarchaeota* (using the probe Cren537) in the North Atlantic and the Arctic Ocean (Herndl *et al.*, 2005; Kirchman *et al.*, 2007). Commonly, the crenarchaeal abundance determined by the Cren537 probe closely matched to that obtained by the GI-554 probe (Figure 4). Remarkable differences were noticed at specific stations and depths such as at St. 3 at 400 m depth, where the abundance of GI-554-positive *Crenarchaeota* was more than twice as high as the abundance of Cren537-positive cells. At St. 5, a pronounced peak in abundance of GI-554-positive *Crenarchaeota* was detected at around 200 m depth, while Cren537-positive *Crenarchaeota* were very low in abundance throughout the water column of this station (Figure 4).

The two crenarchaeal groups, MCGI and pSL12, have recently also been detected in the mespelagic zone at St. ALOHA in the subtropical gyre of the Pacific (Mincer *et al.*, 2007). Using Q-PCR, the abundance of MCGI was always higher than the abundance of the pSL12 cluster (Figure 4). Generally, the abundance of MCGI determined by Q-PCR should not be higher than that obtained by the Cren537 probe using CARD-FISH, because the latter targets all the known partial sequences of the MCGI (Figure 3). However, in two out of nine samples, MCGI copy numbers were higher than the crenarchaeal abundance determined by either the Cren537 or GI-554 oligonucleotide probe. This might be due to PCR bias (Becker *et al.*, 2000) or variable DNA extraction efficiency. Besides these two major discrepancies between the Q-PCR approach and CARD-FISH, both methods were in good agreement in seven out of the nine samples analyzed.

Archaeal community composition

Generally, the number of archaeal OTUs was almost twice as high in the top 100 m layer (8.9 ± 2.2 OTUs) than in the layers below 1000 m depth (4.8 ± 1.9 OTUs) (Figure 5a). The lowest number of OTUs was found at St. 4 at 3000 m with only 2 OTUs, while the highest number of OTUs was found at St.4 at 750 m with 13 OTUs. Only one OTU (248.23 bp) out of a total of 27 OTUs was ubiquitously present and 3 OTUs were present in almost every sample (Figure 5a). The rest of the OTUs are responsible for the observed distinct surface water and mesopelagic clusters of archaeal communities (Figure 5b). Despite the rather homogenous water column structure in the Eastern Mediterranean Sea with only small variations in salinity and temperature with depth, the surface water archaeal community is distinctly different from that in the other water layers reflecting the depth-related distribution pattern of archaeal *amo*A gene copy numbers discussed below. Overall, considerable spatial heterogeneity in the archaeal community composition between the individual depth layers of the different stations is apparent.

Depth profiles of archaeal amo*A gene*

Although bacterial *amo*A genes were not detected, archaeal *amo*A genes were readily detectable in the surface and mesopelagic waters (Figure 6a). A dominance of archaeal *amo*A over bacterial amoA genes was also found in the coastal North Sea and the mesopelagic waters of the North Atlantic (Wuchter *et al.*, 2006), as well as in soils (Leininger *et al.*, 2006). Our results support previous studies suggesting that marine *Crenarchaeota* are, at least partly, ammonia oxidizers (Francis *et al.*, 2005; Könneke *et al.*, 2005). Copy numbers of the archaeal *amo*A gene decreased with the depth and were essentially absent below 1000 m depth (Figure 6a). Thus, the copy numbers of archaeal *amo*A decreased more rapidly with depth than the abundance of *Crenarchaeota* determined by CARD-FISH and of MCGI and pSL12 determined by Q-PCR (Figure 4). This becomes evident, if the ratio of archaeal *amo*A gene copy numbers to crenarchaeal abundance (16S rRNA copy numbers, Cren537- and GI-554-positive cells) is calculated (Figures 6b and c). While in subsurface waters the ratio of *amo*A gene copy numbers: (MCGI + pSL12) is about 0.5, the corresponding ratios using crenarchaeal abundance determined by probes Cren537 and GI-554 range between 0.2 and 0.3 in subsurface waters. Genomic studies on *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus* revealed that both isolates contain one *amo*A copy per cell (Hallam *et al.*, 2006; D Stahl, personal communication). The ratios we obtained indicate that even in the surface waters and at St. 1 at 500 m depth not all of the detected *Crenarchaeota* harbor an *amo*A gene (Figures 6b and c). This ratio decreases rapidly with depth down to 1000 m implying that other energy sources than ammonia might be utilized to sustain the observed crenarchaeotal abundance. It has been shown that *Crenarchaeota* are also capable of utilizing organic substrates (Ouverney and Fuhrman, 2000) and that crenarchaeotal heterotrophy increases with depth (Teira *et al.*, 2006b).

For surface and mesopelagic waters, a positive relation between *Crenarchaeota* abundance and the concentration of ammonia was reported (Wuchter *et al.*, 2006; Kirchman *et al.*, 2007; Varela *et al.*, 2008), as well as with nitrite (Teira *et al.*, 2006a; Lam *et al.*, 2007). In this study, nitrite concentration explains about 60% of the variation in archaeal *amo*A copy numbers in the water column of the Eastern Mediterranean Sea (data not shown). This suggests that *Crenarchaeota* in the surface waters and in the mesopelagic realm are most likely oxidizing ammonia as an energy source (Lam *et al.*, 2007), while in the bathypelagic waters,

crenarchaeal abundance is most likely sustained by other energy sources, probably by organic compounds.

In conclusion, we have shown that in the Eastern Mediterranean Sea, crenarchaeal abundance remains fairly constant with depth and that the number of archaeal phylotypes identified by T-RFLP decreases with depth. The ratio archaeal *amo*A copy numbers/crenarchaeal abundance rapidly declines with depth indicating that putatively ammonia oxidizing *Crenarchaeota* are largely confined to the surface and mesopelagic waters, while in bathypelagic waters of the Eastern Mediterranean Sea, *Crenarchaeota* are likely utilizing energy sources other than ammonia, presumably organic sources as recently reported for the crenarchaeal community in the North Atlantic.

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References

Alonso-Saez L, Balague V, Sa EL, Sanchez O, Gonzalez JM, Pinhassi J *et al.* (2007). Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. *FEMS Microbiol Ecol* **60**: 98–112.

Amann R, Fuchs BM. (2008). Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nature Rev Microbiol* **6**: 339–348.

Becker S, Boger P, Oehlmann R, Ernst A. (2000). PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. *Appl Environ Microbiol* **66**: 4945–4953.

Del Giorgio PA, Gasol JM. (2008). Physiological structure and single-cell activity in marine bacterioplankton. In: Kirchman DL (ed). *Microbial Ecology of the Oceans*, 2nd edn. John Wiley & Sons: New York, pp 243–298.

DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, Frigaard NU *et al.* (2006). Community genomics among stratified microbial assemblages in the ocean's interior. *Science* **311**: 496–503.

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB. (2005). Ubiquity and diversity of ammonia-oxidizing *Archaea* in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* **102**: 14683–14688.

Frigaard NU, Martinez A, Mincer TJ, DeLong EF. (2006). Proteorhodopsin lateral gene transfer between marine planktonic *bacteria* and *archaea*. *Nature* **439**: 847–850.

Hallam SJ, Mincer TJ, Schleper C, Preston CM, Roberts K, Richardson PM *et al.* (2006). Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*. *PLoS Biol* **4**: 520–536.

Herndl GJ, Reinthaler T, Teira E, van Aken H, Veth C, Pernthaler A *et al.* (2005). Contribution of *Archaea* to total prokaryotic production in the deep Atlantic Ocean. *Appl Environ Microbiol* **71**: 2303–2309.

Hornek R, Pommerening-Roser A, Koops HP, Farnleitner AH, Kreuzinger N, Kirschner A *et al.* (2006). Primers containing universal bases reduce multiple amoA gene specific DGGE band patterns when analysing the diversity of beta-ammonia oxidizers in the environment. *J Microbiol Meth* **66**: 147–155.

Karner MB, DeLong EF, Karl DM. (2001). Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**: 507–510.

Kirchman DL, Elifantz H, Dittel AI, Malmstrom RR, Cottrell MT. (2007). Standing stocks and activity of *archaea* and bacteria in the western Arctic Ocean. *Limnol Oceanogr* **52**: 495–507.

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543–546.

Lam P, Jensen MM, Lavik G, McGinnis DF, Muller B, Schubert CJ *et al.* (2007). Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad Sci USA* **104**: 7104–7109.

Lane DJ. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E and Goodfellow M (eds). *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons: New York, pp 115–176.

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW *et al.* (2006). *Archaea* predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442**: 806–809.

Massana R, Murray AE, Preston CM, DeLong EF. (1997). Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara Channel. *Appl Environ Microbiol* **63**: 50–56.

Mincer TJ, Church MJ, Taylor LT, Preston C, Kar DM, DeLong EF. (2007). Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environ Microbiol* **9**: 1162–1175.

Moeseneder MM, Arrieta JM, Herndl GJ. (2005). A comparison of DNA- and RNA-based clone libraries from the same marine bacterioplankton community. *FEMS Microbiol Ecol* **51**: 341–352.

Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ. (1999). Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **65**: 3518–3525.

Moeseneder MM, Winter C, Arrieta JM, Herndl GJ. (2001a). Terminal-restriction fragment length polymorphism (T-RFLP) screening of a marine archaeal clone library to determine the different phylotypes. *J Microbiol Meth* **44**: 159–172.

Moeseneder MM, Winter C, Herndl GJ. (2001b). Horizontal and vertical complexity of attached and free-living bacteria of the Eastern Mediterranean Sea, determined by 16S rDNA and 16S rRNA fingerprints. *Limnol Oceanogr* **46**: 95–107.

Ouverney CC, Fuhrman JA. (2000). Marine planktonic *Archaea* take up amino acids. *Appl Environ Microbiol* **66**: 4829–4833.

Teira E, Lebaron P, van Aken H, Herndl GJ. (2006a). Distribution and activity of *Bacteria* and *Archaea* in the deep water masses of the North Atlantic. *Limnol Oceanogr* **51**: 2131–2144.

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

Teira E, van Aken H, Veth C, Herndl GJ. (2006b). Archaeal uptake of enantiomeric amino acids in the meso- and bathypelagic waters of the North Atlantic. *Limnol Oceanogr* **51**: 60–69.

Thingstad TF, Krom MD, Mantoura RFC, Flaten GAF, Groom S, Herut B *et al.* (2005). Nature of phosphorus limitation in the ultraoligotrophic Eastern Mediterranean. *Science* **309**: 1068–1071.

van der Maarel MJEC, Artz RRE, Haanstra R, Forney LJ. (1998). Association of marine *Archaea* with the digestive tracts of two marine fish species. *Appl Environ Microbiol* **64**: 2894–2898.

Varela MM, van Aken HM, Sintes E, Herndl GJ. (2008). Latitudinal trends of *Crenarchaeota* and *Bacteria* in the meso- and bathypelagic water masses of the Eastern North Atlantic. *Environ Microbiol* **10**: 110–124.

Wuchter C, Abbas B, Coolen MJL, Herfort L, van Bleijswijk J, Timmers P *et al.* (2006). Archaeal nitrification in the ocean. *Proc Natl Acad Sci USA* **103**: 12317–12322.

Zervakis V, Georgopoulos D, Karageorgis AP, Theocharis A. (2004). On the response of the Aegean sea to climatic variability: a review. *Internat J Climatol* **24**: 1845–1858.