

ORIGINAL ARTICLE

Contribution of crenarchaeal autotrophic ammonia oxidizers to the dark primary production in Tyrrhenian deep waters (Central Mediterranean Sea)

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Mesophilic *Crenarchaeota* have recently been thought to be significant contributors to nitrogen (N) and carbon (C) cycling. In this study, we examined the vertical distribution of ammonia-oxidizing *Crenarchaeota* at offshore site in Southern Tyrrhenian Sea. The median value of the crenarchaeal cell to *amoA* gene ratio was close to one suggesting that virtually all deep-sea *Crenarchaeota* possess the capacity to oxidize ammonia. Crenarchaea-specific genes, *nirK* and *ureC*, for nitrite reductase and urease were identified and their affiliation demonstrated the presence of ‘deep-sea’ clades distinct from ‘shallow’ representatives. Measured deep-sea dark CO₂ fixation estimates were comparable to the median value of photosynthetic biomass production calculated for this area of Tyrrhenian Sea, pointing to the significance of this process in the C cycle of aphotic marine ecosystems. To elucidate the pivotal organisms in this process, we targeted known marine crenarchaeal autotrophy-related genes, coding for acetyl-CoA carboxylase (*accA*) and 4-hydroxybutyryl-CoA dehydratase (*4-hbd*). As in case of *nirK* and *ureC*, these genes are grouped with deep-sea sequences being distantly related to those retrieved from the epipelagic zone. To pair the molecular data with specific functional attributes we performed [¹⁴C]HCO₃ incorporation experiments followed by analyses of radiolabeled proteins using shotgun proteomics approach. More than 100 oligopeptides were attributed to 40 marine crenarchaeal-specific proteins that are involved in 10 different metabolic processes, including autotrophy. Obtained results provided a clear proof of chemolithoautotrophic physiology of bathypelagic crenarchaeota and indicated that this numerically predominant group of microorganisms facilitate a hitherto unrecognized sink for inorganic C of a global importance.

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Introduction

Until recently, autotrophic ammonia oxidation was assumed to be performed exclusively by aerobic and anaerobic ammonia-oxidizing bacteria. Analyzing the Global Ocean Survey (GOS) metagenomic data

obtained from the photic waters of the Sargasso Sea, Venter *et al.* (2004) identified a putative ammonia monooxygenase (*amoA*)-like gene in an apparent archaeon-derived scaffold, and thus suggested ammonia (NH₃) oxidation to be attributed to crenarchaea. More direct indication of archaeal origin of *amoA*-like genes was obtained by analyzing the soil metagenome, when a similar gene was found on a 43-kb soil DNA fragment harboring the 16S rRNA gene of the group 1.1b *Crenarchaeota* (Treusch *et al.*, 2005). The definitive link between these novel *amoA*-like genes and archaeal NH₃ oxidation was convincingly established recently by cultivation of free-living ammonia-oxidizing crenarchaea (Könneke *et al.*, 2005; Hatzenpichler *et al.*, 2008; de la Torre *et al.*, 2008; Park *et al.*, 2010).

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The 16S rRNA gene phylogeny analysis placed them into the Marine Group 1.1a of *Crenarchaeota* to become the first cultivated representative of this exceptionally abundant archaeal group. The putative ammonia monooxygenase genes *amoA*, *amoB* and *amoC* organized in one operon were revealed in *Nitrosopumilus maritimus* SCM1 isolate (Walker *et al.*, 2010). The energy gained from the conversion of NH₃ to nitrite allows this crenarchaeon to use bicarbonate chemoautotrophically, as a principal carbon (C) source (Könneke *et al.*, 2005).

As it has been indicated in the review of Erguder *et al.* (2009), numerous studies have indicated that the archaeal *amoA* gene is very ubiquitous in the biosphere: it was found in coastal and marine waters, in subterranean estuary, in coastal, estuarine and cold seep sediments, in freshwater sediments, in a subsurface of radioactive thermal spring and neighboring biofilms, in the sediments and microbial mats/mud of hot springs and geothermal biofabrics and in coral reefs. Further, this gene was widely detected in terrestrial systems, including agricultural, forest and grassland soils, in sandy upland soils and saturated rice paddy soils, and in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. Finally, the *amoA* gene has been detected in man-made systems, such as aquarium biofilm systems and groundwater filter, and in activated sludge bioreactors (see Erguder *et al.*, 2009 for further references). Remarkably, the majority of studies quantifying archaeal *amoA* gene abundance in the marine pelagic ecosystems focused on the euphotic and mesopelagic subsystems, in which NH₃ is generated by remineralization processes, for example, in the oxygen minimum zone (OMZ) (Francis *et al.*, 2005; Wuchter *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008). However, *Crenarchaeota* are abundant in bathypelagic (1000–4000-m depth) NH₃-depleted waters as well (Karner *et al.*, 2001; Delong *et al.*, 2006; Teira *et al.*, 2006; Martin-Cuadrado *et al.*, 2007; Agogué *et al.*, 2008; Varela *et al.*, 2008) and, despite of their potential importance to the marine nitrogen (N) cycle, there is only limited information available on the distribution and physiological activities of these deep-sea organisms. While studying the archaeal *amoA* gene distribution in the bathypelagic waters, the question arose whether all deep-sea *Crenarchaeota* contribute to NH₃ oxidation (Mincer *et al.*, 2007; Agogué *et al.*, 2008; De Corte *et al.*, 2009; Church *et al.*, 2010). Studies performed in deep North Atlantic and Eastern Mediterranean Sea revealed that archaeal *amoA* gene abundance was rather low even though 16S rRNA genes of MGI of *Crenarchaeota* were abundant, suggesting that *Crenarchaeota* might largely lack the *amoA* gene in these deep-water environs (Agogué *et al.*, 2008; De Corte *et al.*, 2009). It was also shown that the general decrease in *amoA* abundance mirrored the patterns in C fixation, a phenomenon the authors attributed to a depth-dependent increase in archaeal

reliance on chemoorganoheterotrophy rather than autotrophy (Ouverney and Fuhrman, 2000; Pérez *et al.*, 2003; Kirchman *et al.*, 2007). Thus, researchers have postulated the existence of a specific heterotrophic *amoA* gene-lacking *Crenarchaeota*, that prefer deep ocean waters (Agogué *et al.*, 2008). However, no clear evidence of depth-dependent changes in the crenarchaeal *amoA*:16S rRNA gene ratio was found between the near-surface ocean and mesopelagic (down to 1000 m) waters of the central Pacific Ocean (Church *et al.*, 2010). Moreover, as we showed earlier (Yakimov *et al.*, 2009), above ratio approaches 1.0 in bathypelagic waters of Central Mediterranean, which contrasts with the data from previous studies (Agogué *et al.*, 2008; De Corte *et al.*, 2009). It is possible that these differences stem, in part, from the selection of quantitative (Q)-PCR primers (Schleper, 2008; Church *et al.*, 2010).

The purpose of the work reported was to closely examine Mediterranean bathypelagic ecosystems for the presence, abundance and ecological significance of autotrophic crenarchaeal organisms. Specifically, we collected samples from a depth of 3480 m in the Tyrrhenian Sea (Central Mediterranean) and examined the resident bathypelagic community for abundance of crenarchaeal *amoA* genes in relation to corresponding transcripts and cell numbers. Further, we analyzed the phylogenetic affiliation of recovered crenarchaeal *amoA* transcripts and genes putatively encoding for the α -subunit of urease, nitrite reductase (NirK), acetyl-CoA carboxylase (AccA) and 4-hydroxybutyryl-CoA dehydratase (4-HBD). To our knowledge, these are the first publicly available gene sequences for deep-sea crenarchaea of Mediterranean Sea. Deep-sea primary production rates were studied in on-deck experiments under the influence of various chemical and physical factors. Finally, in an attempt to definitely link autotrophy with marine *Crenarchaeota*, we performed the [¹⁴C]HCO₃ incorporation experiments followed by the analyses of radiolabeled proteins using shotgun proteomics approach.

Materials and methods

Study site and sample collection

Seawater samples were obtained from the Southern Tyrrhenian area of Mediterranean Sea (monitoring station 17 or V2 (39°32.050'N and 13°22.280'E)) from onboard the RV *Urania* during the CIESM-SUB2 (December 2005) and VECTOR-TM2 (February 2007) cruises. Samples from the different depths (200, 400 (OMZ), 2000 and 3480 m) were collected using 12-l Niskin bottles housed on a rosette (General Oceanics, Miami, FL, USA) equipped with SBE-911plus conductivity–temperature–depth sensors (Sea-Bird Electronics, Bellevue, WA, USA). In addition to the oxygen profile derived from the CTD sensor, parallel determination of oxygen concentration at chosen depths were carried out using the

Winkler method (Carpenter, 1965) with an automatic endpoint detection burette 716 DNS Titrino (Metrohm AG, Herisau, Switzerland). Samples for determining nutrient concentrations were collected in 20 ml polyethylene vials, quickly frozen in liquid N and then stored at -20°C . Nutrient concentrations were determined within a few weeks of the end of each cruise using SEAL QuAAtro Microflow Analyzer (SEAL Analytical, Hampshir, UK). All running standards were prepared with Low Nutrient Seawater and calibrated against Ocean Scientific standards (OSIL, Hampshir, UK). Sample analyses were performed at least twice using the same set of equipment.

Bacterioplankton sampling and enumeration

Hybridization was carried out using 4', 6'-diamidino-2-phenylindol (DAPI) staining and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). Seawater samples (1000 ml) collected at different depths were directly transferred to glass bottles, fixed with particle-free formaldehyde (2% final concentration) and stored at 4°C for 12 h. Microbial cells were collected by gentle filtration of 40–120 ml seawater portions onto 0.2- μm pore size, 47-mm diameter white polycarbonate filters GTTP (Millipore, Molsheim, France), which were then rinsed with sterile seawater, air-dried and stored at -20°C until further processing as described by Tamburini *et al.* (2009). Specifically, filters for CARD-FISH counts were embedded in low-gelling point agarose (0.1% final concentration, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), dried at 37°C for 10 min and dehydrated with 95% ethanol. For detection of bacteria, embedded cells were permeabilized by subsequent treatments with lysozyme (10 mg ml $^{-1}$, Sigma) in 0.5 M EDTA, 0.1 M Tris-HCl (pH 8.0) for 60 min at 37°C (Amann *et al.*, 1995) and with achromopeptidase (60 U in 0.01 M NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37°C according to Sekar *et al.* (2003). The archaeotal CARD-FISH enumeration was carried out according to the proteinase K protocol of Teira *et al.* (2004). Afterwards, the filters were rinsed three times with 0.2- μm filtered MilliQ water and placed in 0.01 M HCl at room temperature to denature endogenous peroxidase. Following acid treatment, filter sections were further hybridized with a solution of 5'-horseradish peroxidase (HRP)-labelled oligonucleotide probes (Pernthaler *et al.*, 2002) using a final concentration of 1 ng μl^{-1} . Probes used in this work are listed in the Supplementary Table S1. After an amplification step with Thyramide-Alexa, pieces of filter were mounted on slides, which were examined under an Olympus BX61 microscope equipped with a 100-W Hg-lamp and appropriate filter sets for 4', 6'-diamidino-2-phenylindol and Alexa488. The fraction of CARD-FISH-stained cells in at least 1000 4', 6'-diamidino-2-phenylindol-stained cells per sample was quantified. Negative control counts (hybridization with HRP-Non338;

Karner and Fuhrman, 1997) were always below 5% (1% average) of 4', 6'-diamidino-2-phenylindol-stained cells.

Nucleic acids extraction and cDNA synthesis

For nucleic acid extraction, three Niskin bottles were casted at each depth and seawater was immediately filtered onboard with Sterivex capsules (0.2 μm pore size, Millipore), using a peristaltic pump. After filtration, the collected material was resuspended by vortexing for 5 s in 400 μl of TE buffer (pH 8.0) containing lysozyme (5 mg ml $^{-1}$) and incubated 10 min at room temperature. Lysis was performed by the addition of 1600 μl of lysis buffer QRL1 (Qiagen, Milan, Italy) containing guanidine isothiocyanate and β -mercaptoethanol and, after vigorous vortexing for 30 s, filters were stored at -20°C until processing in laboratory. Keeping in mind that the short lifetime of mRNA molecules is critical for environmental transcriptomic studies, all operations were performed as fast as possible and with a consistent (60–90 min) time intervals between cast recovery and sample freezing. Nucleic acids extraction was continued in the laboratory and after thawing, the cartridges of the Sterivex filters were opened and the filters and the lysis buffer transferred into 50-ml sterile centrifuge tubes. Total DNA and RNA were further extracted using Qiagen RNA/DNA Mini Kit (Qiagen). The extraction was carried out according to the manufacturer's instructions. Before cDNA synthesis, RNA-containing extracts were purified from DNA by incubation with DNase I (DNA-free-Kit, Ambion, Austin, TX, USA) at 37°C for 30 min and the RNA concentrations determined using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Each RNA sample was immediately converted into cDNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers as described elsewhere (Yakimov *et al.*, 2007). The reaction was carried out in a MasterCycler 5331 Gradient (Eppendorf, Hamburg, Germany). The cDNA was used as the template for a further PCR amplification. Possible DNA contamination of RNA templates was routinely monitored by PCR amplification of RNA aliquots that were not reverse transcribed. No contaminating DNA was detected in any of these reactions.

PCR-amplification, gene cloning and sequencing

Archaeal 16S rRNA, putative archaeal *amoA*, *nirK* and *ureC* and key C fixation genes (*accA*, *4-bdh* and *cbbL*) of 3-hydroxypropionate/4-hydroxybutyrate and reductive pentose phosphate pathways, respectively, were amplified by PCR using primers listed in the Supplementary Table S2. The PCR reaction was carried out in a MasterCycler 5331 Gradient PCR (Eppendorf). The conditions of the PCR for all the genes were: initial denaturation at 94°C for

5 min; 35 cycles each of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C; final extension step of 10 min at 72 °C. PCR products were visualized on a 1% agarose gel, specific bands were cut out and purified using QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were ligated into pGEM-T-Easy Vector plasmid vector (Promega, Madison, WI, USA) and transformed by electroporation into the *E. coli* DH10B cells (Invitrogen). Transformants were selected on Luria-Bertani agar containing 100 µg ml⁻¹ of ampicillin under blue–white selection. Putative-positive clones of each library were transferred to a 96-well plate that contained Luria-Bertani broth supplemented with ampicillin (100 µg ml⁻¹). The clones were grown overnight at 37 °C and then stored in Luria-Bertani broth with 15% (v/v) glycerol at –70 °C before PCR screening and sequencing. Library clones were screened directly by PCR for the presence of inserts using two M13 universal primers, M13F (5'-GTTTCCCAGTCACGAC-3') and M13R (5'-TCACACAGGAAACAGCTATGAC-3'). The conditions for PCR screening were same as described above. Positive clones from each library were randomly selected and after PCR amplification, the DyeEx 96 Kit (Qiagen) was used for removal of unincorporated dye terminators. The purified products were further sequenced at Macrogen (Seoul, South Korea).

Phylogenetic analysis

We checked all sequences to remove chimeric sequences using the Chimera check program at Ribosomal Database Project (Cole *et al.*, 2005) and Basic Local Alignment Search Tool (BLAST) analysis of sequence segments (Altschul *et al.*, 1997). Phylogenetic analysis was performed as previously described (Yakimov *et al.*, 2007, 2009). Briefly, all sequences were edited and aligned with MacVector Software version 11.0.2 (Accelrys, San Diego, CA, USA) (Rajagopal, 2000). On the basis of distance matrix, clones having 97% sequence similarity or higher were treated as the same phylotype (Schloss and Handelsman, 2005). The sequences of phylotypes were realigned with other published sequences, including the closest one determined by BLAST. Phylogenetic trees were constructed using the Neighbour-Joining method and Jukes–Cantor distance matrix; 1000 bootstrap re-samplings were performed to estimate the reproducibility of the tree. Random order input of sequences, single jumbling and the global rearrangement option were used. A multiple sequence alignment and the phylogeny of protein homologs were constructed using MacVector 11.0.2 software with BLOSUM matrix and open and extend gap penalties of 10.0 and 0.05, respectively. The alignment file was then analyzed with the same software for calculation of the distance matrix. Neighbor-Joining algorithm and Poisson correction of distances were used to construct the phylogenetic tree; 1000 bootstrap replicates were performed to estimate the reproducibility of the tree.

Quantification of *amoA* transcripts and gene copies

Q-PCR analysis of environmental DNA templates was performed and the abundance of crenarchaeal *amoA*-like was evaluated by TaqMan gene expression assay. The reaction was performed in an ABI 7500 Fast Real-Time PCR System thermocycler. The primers and the TaqMan probe used are listed in Supplementary Table S2. 5'-6-FAM and 3'-TAMRA-labeled TaqMan probe was obtained from PE Applied Biosystems (Carlsbad, CA, USA). The experiment was performed using DNA templates originating from all depths sampled in triplicates along with a 'no template control'. Mixtures for Q-PCRs and the reaction conditions were as previously described (Yakimov *et al.*, 2007). Q-PCR amplification was analyzed using an automatic setting of the baseline and threshold values. The standard curve method was applied to generate the absolute quantification of *amoA* gene copies using a serial dilution ranging from 10⁷ to 10 copies of plasmid containing *amoA* gene (626-bp long fragment). The plasmid was the pGEM T-Easy Vector II harboring the crenarchaeal *amoA*-like gene (AM747269), extracted using PureLink HiPure Plasmid (Invitrogen S.R.L., San Giuliano Milanese, Italy) and quantified using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA).

Measurements of CO₂ fixation

Rates of CO₂ fixation were determined by the incorporation of NaH¹⁴CO₃ into particulate organic matter during radiotracing of water samples incubated at the *in situ* temperature (13 °C) immediately after cast retrieval on board of the research vessel. Three replicate gas-tight 60-ml serum vials were filled with 40 ml of 1 µm-prefiltered seawater collected at 200, 400 (OMZ), 2000 and 3480 m and 10 µCi of [¹⁴C]HCO₃ (52 mCi mM⁻¹, Amersham Italia, Milan, Italy) was added to each tube. The experiments included the following controls: experiment in the dark without any amendments and one fixed blank (addition of formaldehyde (2% final concentration) before the experiment) supplemented with [¹⁴C]HCO₃ (final concentration 0.25 µCi ml⁻¹). The effect of different additives and antibiotics was evaluated in combination with the [¹⁴C]HCO₃ incorporation. The additives tested were as follows: ammonium chloride (5.0, 50 µmol l⁻¹ and 0.6 mmol l⁻¹, final concentration), nitrite (1 mmol l⁻¹, final concentration), nitrate (1 mmol l⁻¹, final concentration), allylthiourea (86 µmol l⁻¹, final concentration), azide (25 µmol l⁻¹, final concentration), cysteine (1 mmol l⁻¹ final concentration) and urea (500 µmol l⁻¹, final concentration); the antibiotics tested were rifampicin (100 µg ml⁻¹), radicicol (20 µg ml⁻¹) and streptomycin (25 µg ml⁻¹). Additionally, the effect of light was also evaluated by exposition of the samples to led daylight lamp of 12 000 lux, corresponding to daylight intensity. Each experiment was performed in triplicate for 7 days and, at the end of this period

of incubations, was stopped by the addition of formaldehyde (2% final concentration). Before filtration, all samples were alkalified by the addition of 50 μ l of 10 mol NaOH and filtered through 0.1 μ m polycarbonate filters (Millipore). Filters were washed three times with 10 ml of filtered (0.1 μ m) seawater, acidified in an HCl fume hood for 12 h (to remove inorganic carbon) and air-dried before addition of scintillation cocktail for 14 C counting in a Wallac 1414 analyzer (PerkinElmer, Monza, Italy). The dpm values were corrected by subtracting the values detected in the controls from the absolute dpm obtained in the samples. Remarkably, the dpm values obtained in non-treated controls (radioactivity background) were comparable with those of formaldehyde-fixed, thus, unambiguously indicating the absence of any abiotic binding of 14 C-labeled bicarbonate. The ratio of 14 C-labelled bicarbonate to total inorganic C in the sample was determined using an average concentration of total TCO₂ of 2.29 ± 0.01 mM, measured in seawater column in the Southern Tyrrhenian Sea at the time of sampling (Rivaro *et al.*, 2010). Following the equation that 10 μ Ci of [14 C]HCO₃ (52 mCi mmol⁻¹, Amersham Italia) added to the sample corresponds to 2.31 μ g¹⁴C, one incorporated dpm means 177 pgC l⁻¹ per day and according to Herndl *et al.* (2005) was used for estimation of C uptake rates or values of dark ocean primary production. Differences in dark primary production rates measured in treated and untreated samples were analyzed with unpaired two-tailed Student's *t*-tests or one-way analysis of variance followed by Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA, USA). Data were expressed as mean \pm s.d. derived from at least three independent experiments. Differences were considered significant at $P < 0.05$.

[14 C]HCO₃ incorporation analysis by shotgun metaproteomics using reverse phase-high-performance liquid chromatography followed by tandem mass spectrometry (MALDI-TOF/TOF) for peptide sequencing and protein identification

In order to determine the eventual assimilation of labeled inorganic C into biomarkers and identify the organisms important in deep-sea primary production, 200 μ Ci of [14 C]HCO₃ was added to 200 ml of 1 μ m-filtered seawater collected from the depth of 3480 m and incubated in the gas-tight 500 ml flask at *in situ* water temperature (13 °C) in the dark for 7 days. A control culture was incubated under the same conditions without addition of 14 C-labeled bicarbonate. Total cells were harvested by filtration onto 0.22 μ m pore size, 47-mm diameter white polycarbonate filters GTTP (Millipore). Filters were cut in pieces, dipped in 2 ml BugBuster protein extraction reagent (Novagen Srl, Podenzano, Italy) and put on ice for 30 min with gentle agitation (\sim 100 r.p.m.) in an orbital shaker. After incubation, the samples were sonicated for a total of 1.2 min and

further spun at 15 000 g for 15 min and the supernatant retained. The protein mixture was then freeze-dried and stored at -86 °C until use. Total protein samples were solubilized in 1 ml solvent A (1 ml of trifluoroacetic acid in 1000 ml of water) and 14 C-labeled proteins were separated from the starting and non-labeled material by high-performance liquid chromatography using a ultraviolet detection system coupled with a flow-scintillation counter. The Vydac 218TP54 column (C18, 5 μ m, 4.6 mm i.d. \times 250 mm) was equilibrated with 70% solvent A (1 ml of trifluoroacetic acid in 1000 ml of water) and 30% solvent B (700 ml of acetonitrile, 300 ml of water and 1 ml of trifluoroacetic acid) to give a final concentration of 21% acetonitrile. A flow rate of 1.5 ml min⁻¹ with 100 min linear gradient was used to achieve 70% of buffer B (49% acetonitrile). The protein mixture was injected onto the column and the fraction collector was programmed to collect 0.1-min fractions. Finally, the 14 C-labelled protein fractions were pooled together and evaporated to dryness in a vacuum concentrator (Model 5301, Eppendorf AG). The pool of labeled proteins obtained using this protocol was further used for shotgun proteomic analysis: complete details describing trypsin digestion of 14 C-labeled proteins and following RP-LC-MALDI TOF/TOF MS analysis of obtained peptide are found in SI Materials and methods section. Raw shotgun-MS files containing a peak list of the precursors and fragment ions were filtered and exported with the Peak to Mascot tool (4000 Series Explorer Software, ABI). The filtered files were submitted for database search using MASCOT search engine version 2.3.01 (Matrix Science, London, UK). The search was performed in NCBI non-redundant 20100903 database (11 744 690 sequences). The following search parameters were used: enzyme, trypsin; allowed missed cleavages, 1; fixed modifications, β -methylthiolation of cysteine; variable modifications, oxidation of methionine and acetylation (N-term); mass tolerance for precursors was set to \pm 100 p.p.m. and for MS/MS fragment ions to \pm 0.8 Da. The confidence interval for protein identification was set to \geq 95% ($P < 0.05$) and only peptides with an individual ion score above the identity threshold were considered correctly identified.

Analysis of marine metagenomic data sets

Sequences of *N. maritimus* SCM1 and *C. symbiosum* A genes were queried against the NCBI non-redundant protein database using both BLASTP and BLASTX search with a cut-off value of $< 1e-50$. Only top BLAST high-scoring pairs derived from marine metagenomic libraries were considered for further analyses and tabulated according to the NCBI taxonomic identifier for each query. For comparative analysis, reciprocal BLASTN and TBLASTX searches between the different fosmids, scaffolds and genomic fragments were carried out, leading to the identification of regions of similarity,

insertions and rearrangements. To allow the interactive visualization of genomic fragment comparisons, MacVector (version 11.0.2) was used.

Nucleotide accession numbers

The nucleotide sequences reported in this paper have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers: HM241829 to HM241835 for the archaeal 16S rRNA gene sequences, HM355483 to HM355500 for the crenarchaeal *accA* gene sequences, HM355502 to HM355518 for the crenarchaeal *nirK* gene sequences, HM355519 to HM355532 for the crenarchaeal *ureC* gene sequences, HM355501 for the bacterial *cbbL* gene sequence, HM369442 to HM369457 for the crenarchaeal *amoA* gene sequences and HQ339937 to HQ339951 for crenarchaeal *4hbd* gene sequences.

Results and discussion

Description of the Tyrrhenian Deep Water (TDW) archaeal community composition

The Tyrrhenia Sea is a three-layer system in which the top layer (0–200 m) is occupied by the so-called Modified Atlantic Water (0–250 m), originated from the water entering at Gibraltar from the Atlantic Ocean. The intermediate layer (250–700 m) is dominated by the Levantine Intermediate Water, whereas the deeper compartments are occupied by TDW formed by the mixing of highly oligotrophic Levantine Intermediate Water with dense waters resident in the Western basin (Millot *et al.*, 2006; Ribera d'Alcalà *et al.*, 2009). At the monitoring Station 17 we mapped nutrient concentration on four depth horizons: 200, 400 m (corresponding to a somewhat pronounced OMZ with an oxygen concentration of $191 \pm 4 \mu\text{mol l}^{-1}$), 2000 and 3480 m. The deeper layers, encompassing the TDW, are characterized by minimal differences in salinity (0.01 unit), temperature (0.08 °C), oxygen (13 μmol) and nutrient (0.3–0.5 μmol) concentration. Temperature at the 3480 m depth at the time of sampling was 14.09 °C, salinity 38.48 practical salinity units (PSU) and oxygen concentration $233 \pm 4 \mu\text{mol dm}^{-3}$. Nutrients concentrations exhibited the common depth-increasing trend reaching at the depth of 3480 m the concentration of total inorganic N, phosphates and silicates were 7.94 ± 0.45 , 0.31 ± 0.03 and $9.32 \pm 0.51 \mu\text{mol l}^{-1}$, respectively. Dominant patterns for total inorganic N observed in all layers demonstrated an absolute dominance of NO_3^- at the expenses of NO_2^- and NH_4^+ , which is typical feature for highly oligotrophic TDW (Ribera d'Alcalà *et al.*, 2009).

According to results of archaeal 16S rRNA clone library analysis (88 clones sequenced in total), the bathypelagic zone of Station 17 was significantly enriched by *Crenarchaeota* belonging to four distinct subclusters within Marine Group I.1a (95.4% of all clones sequenced). Equally presented members of *Crenarchaeota* pSL12-like Clade and

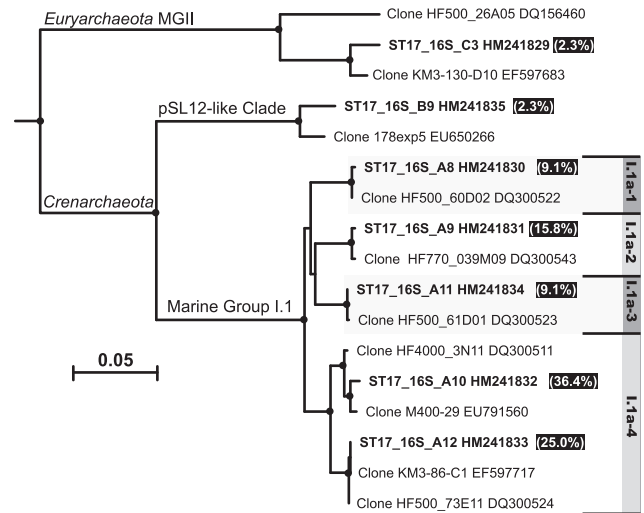


Figure 1 Phylogenetic tree of the archaeal 16S rRNA gene sequences retrieved from deep Tyrrhenian Sea (3480 m). The tree was constructed by Neighbour-Joining method and Jukes–Cantor distance matrix using MacVector 11.0.2 and a total of 550 non-ambiguously aligned positions. Non-parametric bootstrapping was performed upon 1000 iterations. Bootstrap values > 70 are shown with filled circles. The tree was rooted with *Alcanivorax borkumensis* strain SK2 16S ribosomal RNA gene (Y12579). The frequencies of all archaeal (sub)groups present in ST17 clone library are evidenced at the end of each sequence. The scale bar represents the number of fixed mutations per nucleotide position.

euryarchaeal Marine Group II encountered for remaining clones (Figure 1). The observed differences between eury- and crenarchaeotal presence in deep Tyrrhenian Sea might be a common phenomenon and is consistent with the findings of other studies (DeLong *et al.*, 2006; Martin-Cuadrado *et al.*, 2007, 2008; Mincer *et al.*, 2007; De Corte *et al.*, 2009; Yakimov *et al.*, 2009).

AmoA mRNA and gene-based diversity analyses and real-time PCR quantification

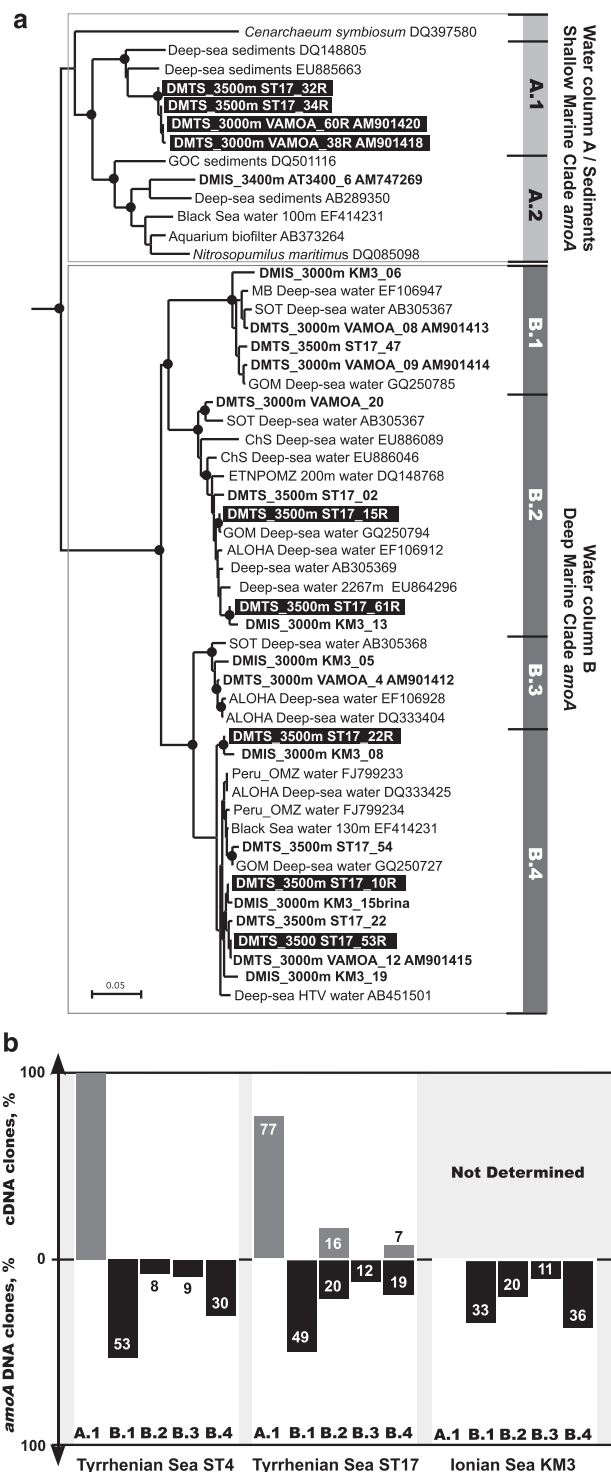
The abundance of *Crenarchaeota* MGI.1a in the deep Tyrrhenian Sea corroborated with significant abundance of crenarchaeal *amoA* gene as revealed by QPCR: the number of *amoA* gene copies found at the depth of 3480 m in Tyrrhenian Sea (7000 ± 475 copies per ml) was essentially the same as the number of crenarchaeal cells (5950 ± 360 cells ml^{-1}) counted at this site with CARD-FISH, resulting in *amoA* to crenarchaeal cells ratio of 1.18. A similar ratio (1.31) was obtained previously at another deep-sea site in Tyrrhenian Sea (monitoring Station 4, $39^{\circ}32.050'N$, $13^{\circ}22.280'E$) (Yakimov *et al.*, 2009). Estimates of *amoA* to total crenarchaeal 16S rRNA gene ratio (median value 1.3) observed in deep-sea environments in other parts of the world, did not vary significantly and have been used to indicate that the marine crenarchaea all possess the ability to perform nitrification (Wuchter *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008; Church *et al.*, 2010; Park *et al.*, 2010). In contrast, Agogué *et al.* (2008)

and De Corte *et al.* (2009) found ratios of archaeal *amoA*/16S rRNA gene copy number were never exceeded 0.05 (0.01 ± 0.02 , $n = 5$) in the bathypelagic waters of the Mediterranean Sea and Atlantic ocean, suggesting that a large part of the deep water crenarchaea do not have the ability to perform nitrification. It should be noted, however, that a recent metagenomic study of deep sea microbial communities indicated that the reported low archaeal *amoA*/16S rRNA gene copy number for deep sea crenarchaea may be erroneous due to mismatches in the primers (Konstantinidis *et al.*, 2009; Church *et al.*, 2010). Aligning these primers with all available deep Mediterranean *amoA* genes sequences (Yakimov *et al.*, 2007, 2009), we have found up to six mismatching nucleotides (Supplementary Figure S1).

On the basis of the RT-QPCR analysis of *amoA* mRNA transcripts, we examined the expression of this gene in bathypelagic waters of Tyrrhenian Sea. At the depth of 3480m the *amoA* transcript abundances (13.2 ± 0.06 copies per ml) were approximately 500 times lower than the gene numbers resulting in ratio of 1.86×10^{-3} . Similar values were observed in the mesopelagic waters of the Pacific Ocean (Church *et al.*, 2010), reflecting similarity in corresponding metabolic activity of crenarchaeal deep-sea communities worldwide. Besides RT-QPCR-based expression analysis of *amoA* transcripts, corresponding cDNA was used for clone library construction followed by sequencing of 96 clones and phylogenetic analysis. Almost three quarters of sequenced transcripts belonged to Shallow Marine *amoA* Clade (*amoA* SMC), a large cluster of sequences from shallow waters, marine sediments, corals, sponges and aquariums (Francis *et al.*, 2005; Mincer *et al.*, 2007; Beman *et al.*, 2008).

None of *amoA* SMC gene sequences were observed in DNA-derived library in which sequences formed four separated subgroups (>98% similarity) within Water column B/Deep Marine *amoA* Clade (*amoA* DMC). Only two subgroups, namely B.2 (16% of clones) and B.4 (7%), were represented in the *amoA* transcripts library (Figure 2). Thus, as previously observed (Yakimov *et al.*, 2009), the sampled TDW

Figure 2 Phylogenetic relationships of crenarchaeal *amoA*-like sequences retrieved from deep Mediterranean Sea: (a) Stations 4 (DMTS_3000 m (Yakimov *et al.*, 2009) and 17 (DMTS_3500 m) in Tyrrhenian Sea and Stations KM3 (DTIS_3000 m) and l'Atalante (DMIS_3400 m) in Ionian Sea. The abbreviations DMTS and DMIS stand for Deep Mediterranean Tyrrhenian and Ionian Sea, respectively. All Mediterranean Sea *amoA* clones are shown in bold. The clones obtained by RT-PCR of *amoA* derived from mRNA have the letter R at the end of the legend and black-shaded. The tree was constructed using sequences of comparable regions of the *amoA* gene sequences available in public databases. The tree is rooted and out-grouped with *amoA* genes of terrestrial origin (DQ148872 and AJ627422). Neighbor-Joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 75% is indicated by filled circles. The scale bar represents 5% sequence divergence. Abbreviation used: ALOHA, North Pacific Subtropical Gyre ALOHA station; ChS, China Sea; ETNPOMZ, Eastern Tropical North Pacific Oxygen Minimum Zone; GOC, Gulf of California; GOM, Gulf of Mexico; HTV, hydrothermal vent; MB, Monterey Bay; OMZ, Oxygen Minimum Zone; SOT, Southern Okinawa Trough. (b) Relative attribution (in percentage) of deep Mediterranean crenarchaeal *amoA* gene and gene transcripts to Marine Water Column Clades A and B. Four subgroup (B.1-B.4) are defined within Marine Water Column B or Deep Marine *amoA* Clade as it is shown in phylogenetic tree.



possessed remarkable differences between crenarchaeal *amoA* transcript and gene pools, that is, mRNA-based libraries were significantly enriched by SMC-like sequences, which were not found in DNA-derived libraries. To date, there is only limited information available on factors regulating transcription of *amoA* in of ammonia-oxidizing *Crenarchaeota* in the deep sea. A recent study found that numbers of crenarchaeal *amoA* gene copies normalized vs their transcripts were greatest in the epipelagic waters of South Pacific and decreased several orders of magnitude with depth (Church *et al.*, 2010). This finding was attributed to the reduced concentration of NH_4^+ in meso- and bathypelagic zones (Mincer *et al.*, 2007). The sampling biases, such as duration of deep-sea sampling cast, time intervals between cast recovery and sample freezing and fixation and the effect of decompression, could also affect the measurement of actual *amoA* expression in deep sea (Yakimov *et al.*, 2009).

Deep-sea Crenarchaeota-specific genes encoding NirK
The three genes (Nmar_1500, _1503 and _1502) annotated as *amoA*, *amoB* and *amoC* and coding for a putative ammonia monooxygenase complex are the only recognizable genetic hallmarks of NH_4^+ oxidation in recently published genome sequence of *N. maritimus* SCM1 (Walker *et al.*, 2010). Using available public databases of marine (meta) genomes, we attempted to compare the genomic context of *N. maritimus* SCM1 *amo* gene cluster with two bathypelagic microbial communities sampled out at Station ALOHA in the North-Pacific Subtropical Gyre (4000 m) and at the Eastern Mediterranean Station KM3 (3000 m) (Martin-Cuadrado *et al.*, 2007, 2008; Konstantinidis *et al.*, 2009). Two deep-sea ALOHA fosmids, HF4000_APKG4H17 and HF4000_APKG3E18 contained an incomplete *amoAB* operon along with the stretch of several predicted open reading frames highly syntenic at the amino-acid level to a 11-Kb genomic fragment situated downstream of the *N. maritimus* SCM1 *amoACB* operon (Supplementary Figure S2). The psi-BLAST and tBLASTX searches for *N. maritimus* SCM1 *amoC* homologs, revealed the presence of a relevant sequence in HF4000_ANIW97M7 fosmid recovered from the ALOHA deep-sea database. Downstream of *amoC*-like sequence, this fosmid predicted to encode a cupredoxin-containing oxidase related to the *N. maritimus* SCM1 nitrite reductases (NirKs) (Nmar_1259 and Nmar_1667, Walker *et al.*, 2010) (Supplementary Figure S2).

As downstream components of a potential NH_4^+ oxidation pathway, copper containing NirK could have a function in either determining tolerance to nitrite or under limiting oxygen concentrations, allow to use nitrite reduction as an alternative respiratory electron sink (Bartossek *et al.*, 2010; Walker *et al.*, 2010). All sequences available from the databases and potentially attributed to marine

Crenarchaeota possess high level of intra-cluster identity both at the nucleotide and amino-acid levels. On the basis of this finding, the pair of degenerated primers specific for marine crenarchaeal *nirK* sequences was designed (Supplementary Table S2) and *nirK* amplicon was obtained from 3480 m environmental DNA. Phylogenetic analyses of 40 clones demonstrated that all sequences but one were associated with the 'Deep Marine *nirK* Clade' originally formed by putative NirK genes found in three ALOHA fosmids. The remaining *nirK* clone was affiliated with *N. maritimus* genes and GOS sequences retrieved from superficial seawater. Both marine *nirK* clades were represented by the sequences retrieved exclusively from epi and bathypelagic seawater, and exhibited high divergence with 'soil' and 'thermophilic' crenarchaeal and bacterial *nirK* clades (Figure 3).

According to the predictions of MacPherson and Murphy (2007), the absence of four histidine residues at positions His⁴³, His⁸⁷, His²⁷⁰ and His³²¹ (*Achromobacter cycloclastes* NirK numbering), required for trinuclear copper coordination, strongly suggests that NirK-like proteins of 'marine' lineages are NirKs and not multicopper oxidases. Such His-containing multicopper oxidase (CENSYa_1582) was initially annotated as NirK in the genome of *C. symbiosum* (Hallam *et al.*, 2006a,b), the marine sponge symbiont, which is actually lacking NirK (Bartossek *et al.*, 2010). Although the role of NirK still has to be elucidated for both NH_4^+ -oxidizing bacteria and archaea, the NirK protein likely comprises a part of a defense mechanism against nitrosative intoxication similar to that of its bacterial counterpart (Cantera and Stein, 2007).

Molecular signatures of urea uptake system in Mediterranean bathypelagic crenarchaea

The deep Mediterranean Sea is permanently nutrient-depleted and one of the most oligotrophic marine ecosystems with ammonium concentrations significantly below 10 nM (Woodward, 1994). However, marine *Crenarchaeota* seem to be adapted to thrive under such extreme nutrient limitation via possessing the remarkably high affinity towards NH_4^+ (Martens-Habbena *et al.*, 2009) and the metabolic capacity to use as electron donors the reduced N, other than NH_4^+ , for example, urea and amino acids (Hallam *et al.*, 2006b). Two adjacent operons encoding three core urease subunits *ureCBA* and five urease accessory proteins *ureDEFGH* were identified in genome of *C. symbiosum* A along with urea transporter (CENSYa_0457) (Hallam *et al.*, 2006b). *N. maritimus* SCM1 lacks these genes, whereas urease operons with similar arrangements of open reading frames coding for urease subunits were found in two highly syntenic fragments in fosmids, HF4000_ANIW133O4 and HF4000_APKG7F11, in the ALOHA data set (North-Pacific Subtropical Gyre, 4000 m depth). The GOS database

also contains several genomic fragments for urease subunits, but none was found to contain integral *ure* operon (Supplementary Figure S2).

The *ureC* gene encodes the α -subunit of urease containing an active site and conserved nickel-binding ligands; these conserved regions were suitable targets for primer design to obtain *ureC*-like sequences from bathypelagic waters from

Station 17 (Supplementary Table S2). Recovered gene sequences and *ureC*-like sequences available from the public databases were used to make phylogenetic inferences. In total, 84 of 93 sequenced clones were found to be affiliated with four related sub-clades and were similar to *ureC* homologs in two deep-sea fosmids from ALOHA Station, namely HOTS_contig35539 and HF4000ANIW13304 (Figure 4). One numerically minor group of clones (6% of all clones sequenced) was clustered with sequences from GOS shallow water data set. Both clades formed distinct monophyletic groups distantly related to the two *ureC* genes found in *C. symbiosum* A genome. As it can be seen in Figures 2–4, the tree topology of TDW crenarchaeal *ureC* gene sequences was strikingly similar to those of *amoA* and *nirK* genes pointing to the occurrence of at least two vertically segregated groups of phylogenetically distinct crenarchaeal Shallow and Deep Marine clades.

Estimation of dark ocean primary production rates

The rates of prokaryotic dark primary production in seawater column at Station 17 were estimated by incorporation of ^{14}C -labeled bicarbonate into the microbial biomass. As described previously (Herndl *et al.*, 2005; Yakimov *et al.*, 2007, 2009), it was assumed that inorganic C is the only form of C available to autotrophic prokaryotes and that $[^{14}\text{C}]\text{HCO}_3^-$ would be taken up by these organisms at the same rates as natural bicarbonates. As shown in Figure 5, dark primary production strongly depends on the type of water masses encompassing the column. Superficial Modified Atlantic Water is characterized by the lower carbon fixation activity compared with Levantine Intermediate Water intermediate layer. The Levantine Intermediate Water masses are formed in Levantine Sea and, in contrast to their high oligotrophy, seemed to be among the most primary productive water masses of Mediterranean Sea (Yakimov *et al.*, 2007). Downward 2000 m, within the layer of TDW, autotrophic

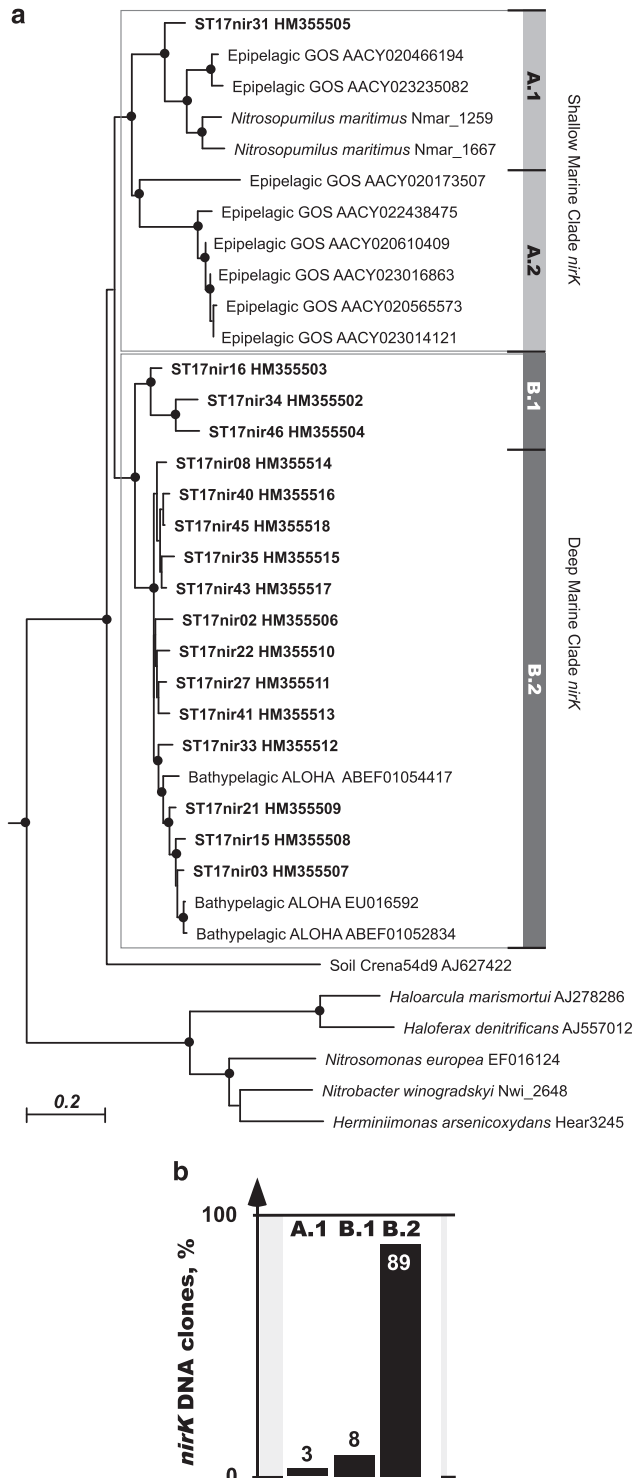


Figure 3 Phylogenetic relationships of crenarchaeal *nirK*-like sequences retrieved from the depth of 3480 m at Station 17 in Tyrrhenian Sea: (a) The tree was constructed using both archaeal and bacterial *nirK* gene sequences of comparable regions available in public databases. The tree is rooted and out-grouped with multicopper oxidases CENSYa_1582 and Nmar_1663 (DP000238.1 and CP000866.1), respectively. Neighbor-Joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 75% is indicated by filled circles. Phylogenetic trees constructed by other algorithms (maximum parsimony and maximum likelihood) resulted in identical topologies for principal clade grouping. The scale bar represents 20% nucleotide divergence. Abbreviation used: ALOHA, North Pacific Subtropical Gyre ALOHA station; GOS, Global Ocean Sampling. (b) Percentage of deep Mediterranean crenarchaeal *nirK* genes to cluster within two Marine Clades originally formed by the sequences retrieved from epi- (GOS) and bathypelagic (ALOHA) zones. Two subgroups are defined within each of these Clades.

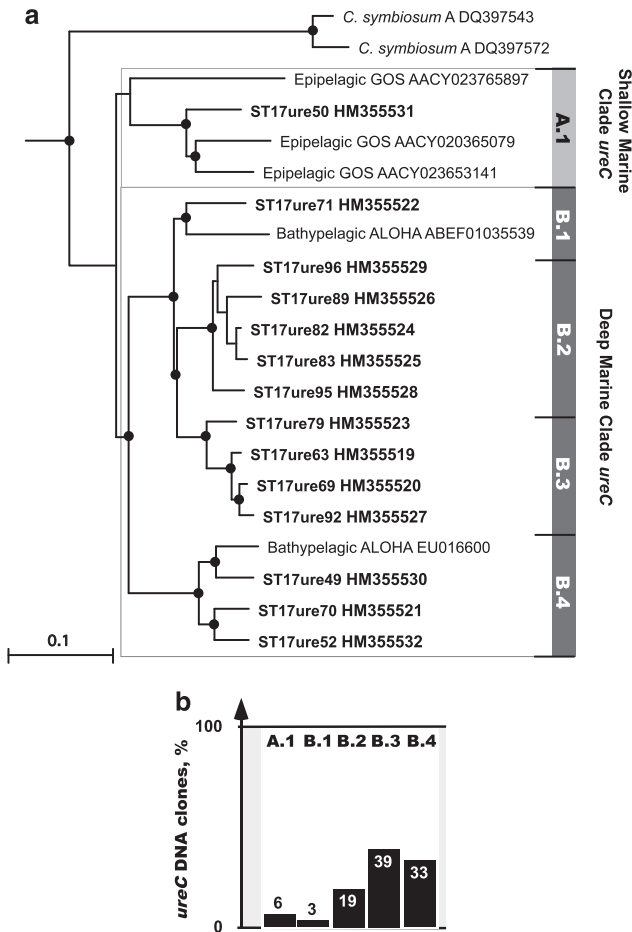


Figure 4 Phylogenetic relationships of crenarchaeal *ureC*-like sequences retrieved from the depth of 3480 m at Station 17 in Tyrrhenian Sea: (a) The tree was constructed using marine crenarchaeal sequences of comparable regions available in public databases. The tree is rooted and out-grouped with *ureC* sequences of *Alteromonas* sp. Deep and *E. coli* (CP001103 and AE005174), respectively. Neighbor-Joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 75% is indicated by filled circles. Phylogenetic trees constructed by maximum parsimony algorithm resulted in identical topologies for principal clade grouping. The scale bar represents 10% nucleotide sequence divergence. Abbreviation used: ALOHA, North Pacific Subtropical Gyre ALOHA station; GOS, Global Ocean Sampling. B. Percentage of deep Mediterranean crenarchaeal *nirK* genes affiliated to two Marine Clades originally formed by the sequences retrieved from epi- (GOS) and bathypelagic (ALOHA) zones.

activity declined to the values of 50–60 $\mu\text{gC m}^{-3}$ per day and remained constant down to the sea bed (Figure 5). The deep-sea dark CO_2 fixation values measured in our study corroborated with earlier estimates ($72.0 \pm 8.9 \mu\text{gC m}^{-3}$ per day) determined in this area at the depth of 3000 m (Tamburini *et al.*, 2009) and seem to be a characteristic feature of TDW, representing the densest, oldest and highly oligotrophic deep water masses of Mediterranean Sea (Millot *et al.*, 2006). By applying of obtained values to whole aphotic water column (from 200 m to the seafloor), we estimated the mean dark ocean

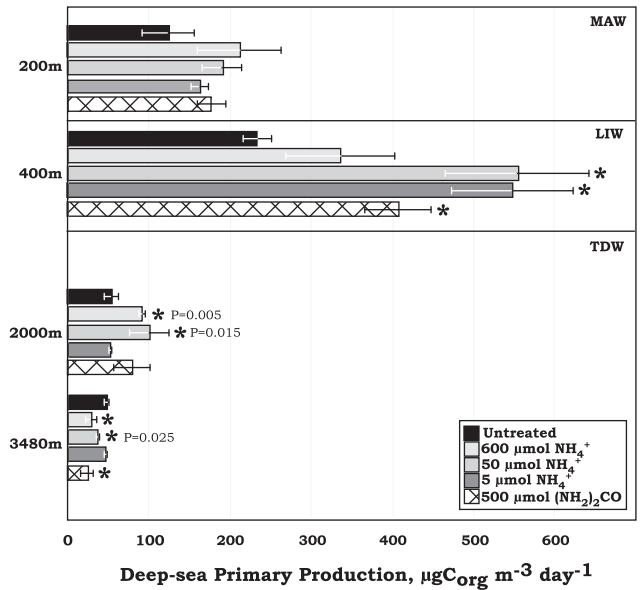


Figure 5 Prokaryotic primary production rates measured in samples taken at Station 17 from different depths and influence of different concentration of added ammonium (5, 50 and 600 μmol) and urea (500 μmol) on bicarbonate fixation activities. All error bars indicate one standard error from a mean of three replicate measurements. One-way analysis of variance (ANOVA test) was applied to analyse the obtained values. The values, significantly different from those of untreated samples, are indicated by asterisks. Resulting *P*-values different from $P < 0.001$ are indicated.

production at Station 17 as 257 mgC m^{-2} per day, which is comparable to the median value of photosynthetic biomass production ($273\text{--}429 \text{ mgC m}^{-2}$ per day) calculated for this area of Tyrrhenian Sea (Decembrini *et al.*, 2009). This observation pointed at both, the significance of the dark ocean primary production in the carbon cycle in aphotic marine ecosystems and at the nature of potential source of energy fueling this process.

Addition of various concentrations of NH_4^+ had similar positive effect on $[^{14}\text{C}]\text{HCO}_3^-$ fixation rates in epipelagic water masses, independently on amount of NH_4^+ applied (Figure 5). The autotrophic prokaryotes inhabiting the OMZ layer were strongly stimulated by low concentrations of ammonium. The increase of CO_2 fixation was also observed with 600 $\mu\text{mol l}^{-1}$, although in a lesser extent. In contrast, the autotrophy of mesopelagic community was insignificantly activated by higher concentration of NH_4^+ and no influence on CO_2 fixation was detected after addition of 5 $\mu\text{mol l}^{-1}$. Same indifferent response to addition of 5 $\mu\text{mol l}^{-1}$ ammonia was obtained for bathypelagic lithoautotrophic community. This community seemed to possess high sensitivity to ammonia: statistically significant inhibition of CO_2 fixation was detected at concentrations of NH_4^+ higher than 5 μmol (Figure 5).

The finding of marine crenarchaeal-specific *ureC* genes encoding urease α -subunit in deep Mediterranean Sea is consistent with the hypothesis that

marine *Crenarchaeota* are capable of using urea as a reduced N form for amino-acid biosynthesis, energy production or both (Hallam *et al.*, 2006b; Church *et al.*, 2010). We tested this hypothesis by addition of $500 \mu\text{mol l}^{-1}$ of urea to the seawater samples and measured $[^{14}\text{C}]\text{HCO}_3$ fixation rates were compared with those of untreated and NH_4^+ -supplemented experiments. As it can be seen in Figure 5, urea has influenced the primary production in a manner, absolutely identical to addition of comparable amount of NH_4^+ ($600 \mu\text{mol l}^{-1}$). The stimulating effect of urea on dark primary production has not been demonstrated so far. Even though there is a further need in elucidation of the mechanism of this phenomenon, our observation indicates that autotrophic members of deep-sea microbial community can catabolize urea to fuel their metabolism.

Influence of various factors and additives on dark primary production rates

The effect of various additives on autotrophic activity of bathypelagic microbial community collected at depth of 3480 m was examined in more detail (Figure 6). Similarly to other members of *Eury-* and *Crenarchaeota* (Jolivet *et al.*, 2003; Simon *et al.*, 2005; Pikuta *et al.*, 2007), the addition of rifampicin ($100 \mu\text{g ml}^{-1}$), an antibiotic agent inactivating prokaryotic DNA-dependent RNA polymerase, completely inhibited CO_2 fixation, whereas there was no toxic effect of radicicol ($20 \mu\text{g ml}^{-1}$), a natural product that inhibits DNA topoisomerase IV

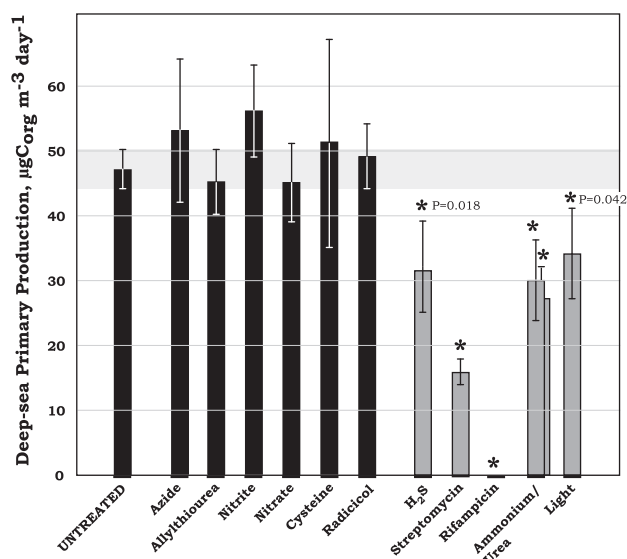


Figure 6 Influence of various additives and physical factors on primary production rates measured in seawater samples collected at 3480 m. The concentrations of all additives are given in corresponding part of Materials and methods section. All error bars indicate one standard error from a mean of three replicate measurements. Student's *t*-test was applied to analyze the obtained values. The values, significantly different from those of untreated samples, are indicated by asterisks. Resulting *P*-values different from ($P < 0.001$) are indicated.

of some thermophilic crenarchaea (Gadelle *et al.*, 2005). Depletion of oxygen in the deep seawater samples by addition of sulfide caused a slight decrease in primary production rates compared with untreated control ($P = 0.018$). This negative effect should be ascribed to the toxic effect of sulfide (Erguder *et al.*, 2009) rather than to oxygen depletion. Indeed, an addition of DL-cysteine could potentially have lead to the establishment of a micro-aerophilic environment ($45 \mu\text{mol O}_2 \text{l}^{-1}$), but it had no significant influence on bicarbonate fixation (Figure 6). The exposure of the deep seawater samples to full daylight (12 000 lux) also had a slightly negative effect on primary production rates ($72 \pm 15\%$ productivity of the control, $P = 0.042$). As was recently proposed (Mincer *et al.*, 2007; Church *et al.*, 2010), this effect could be explained by partial photo-inhibition of deep-sea *AmoA* proteins.

Addition of either nitrate or nitrite appeared to have no effect on bicarbonate fixation rates. As it was already demonstrated for the ammonia-oxidizing thermophilic crenarchaea (Hatzenpichler *et al.*, 2008), an addition of $86 \mu\text{M}$ allylthiourea, known inhibitor of bacterial ammonium mono-oxygenase, did not cause significant decline in autotrophic activity of deep-sea *Crenarchaeota*. Albeit the sodium azide has been also shown to inhibit bacterial ammonia and nitrite oxidation (Ginestet *et al.*, 1998), its addition had no significant influence on bicarbonate fixation at least given experimental conditions, thus suggesting the minor role of bacteria in observed autotrophy.

Molecular signatures of autotrophic pathways in Mediterranean bathypelagic crenarchaea

To analyse the distribution of CO_2 -fixing prokaryotes, the presence of crucial genes involved in autotrophic pathways was monitored across the water column. Six mechanisms are currently known by which autotrophic organisms can fix the inorganic carbon (Huber *et al.*, 2008; Berg *et al.*, 2010a,b). Because of the general sensitivity of the corresponding enzymes to the oxygen, only two pathways, namely Calvin–Benson–Bassham and 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycles, could occur in oxygenated seawater (Thauer, 2007; Berg *et al.*, 2007, 2010a,b). Specially designed and previously tested primer pairs (Elsaied and Naganuma, 2001; Yakimov *et al.*, 2007) were used to amplify the fragment of bacterial genes encoding the large subunit of red and green-like form I RuBisCO (CbbL), the key enzyme of Calvin–Benson–Bassham cycle (See Supplementary Table S2). Except OMZ (400 m depth), no amplification products were observed at any depth, indicating the presence of Calvin–Benson–Bassham cycle-operating prokaryotes only within oxygen-depleted layer. All 20 *cbbL* clones sequenced were found to be identical and deeply branched within cluster of autotrophic β -*Proteobacteria* (Supplementary Figure S3). The occurrence

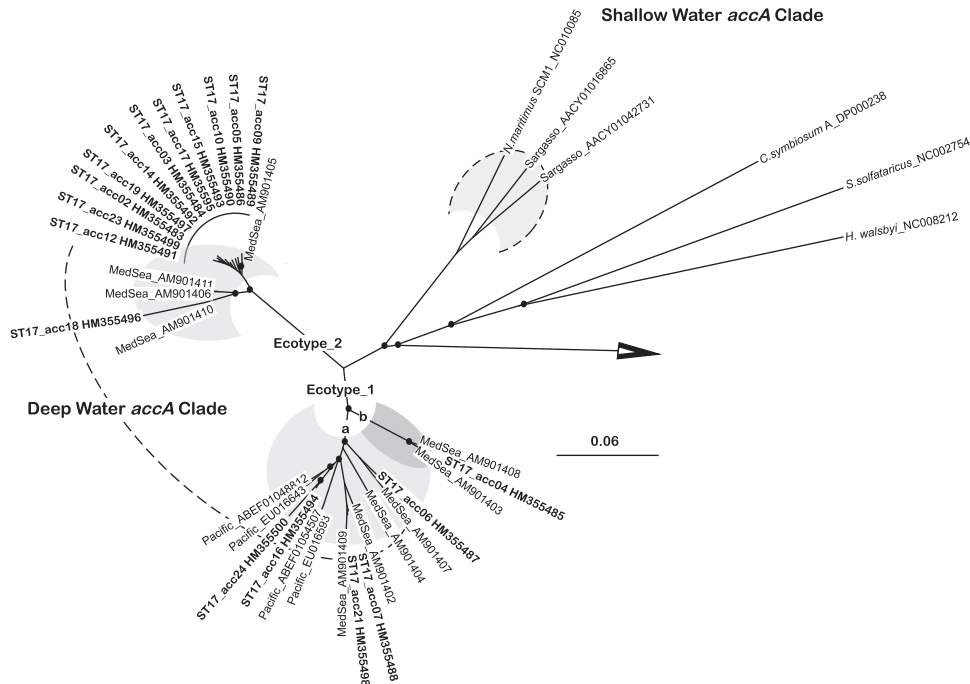


Figure 7 Phylogenetic relationships of crenarchaeal *accA*-like sequences retrieved from 3480 m depth at Station 17 in Tyrrhenian Sea. The tree was constructed using archaeal sequences of comparable regions available in public databases. The tree is rooted and outgrouped with *accA* sequence of *Caldicellulosiruptor saccharolyticus* (CP000679). Neighbor-Joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 70% is indicated by filled circles. The scale bar represents 6% nucleotide sequence divergence.

of NH_4^+ -oxidizing betaproteobacteria belonging to *Nitrosospira* and *Nitrosococcus* was already demonstrated in the oceanic OMZs worldwide (Lam *et al.*, 2007, 2009). Unfortunately, we were not able to affiliate our *cbbL*-like sequences to either sulphur-, metal- or NH_4^+ -respiring bacteria, thus, the type of energy-gaining pathway, utilized by autotrophic betaproteobacteria in the OMZ of Station 17, remained unclear.

In contrast to RuBisCO, the genes coding for crenarchaeal AccA, the key enzyme in autotrophic 3-HP/4-HB pathway, were retrieved from all sampled layers (data not shown). The pool of *accA*-like genes amplified from 3480 m is represented by two different ecotypes previously detected in this area (Yakimov *et al.*, 2009). The majority of *accA*-like clones (39 of 57 clones analyzed) were affiliated with a uniquely Mediterranean 'Ecotype_2' (Yakimov *et al.*, 2009) with no similar sequences known (Figure 7). The rest of *accA*-like clones formed the 'Ecotype_1', which also includes the sequences from bathypelagic seawater (4000 m depth) from North Pacific Subtropical Gyre, Hawaii Ocean Time Series Station ALOHA (DeLong *et al.*, 2006; Mincer *et al.*, 2007). As it is the case with the meso- and bathypelagic crenarchaeal *amoA*, *nirK* and *ureC* genes, both *accA* clades are separated from *N. maritimus* SCM1, *C. symbiosum* A and from the cluster of sequences found in superficial waters of Sargasso Sea (Figure 7), indicating the possible occurrence of at least two

vertically separated groups of phylogenetically distinct shallow and deep planktonic *Crenarchaeota*.

As stated by Berg *et al.* (2010a, b) the second key enzyme unambiguously indicating the presence of autotrophic 3-HP/4-HB pathway is the 4-HBD. Originally this enzyme was described in a few strict fermentative anaerobes, but recently 4-HBD has been postulated to be a pivotal enzyme in CO_2 -fixing 3H-P/4-HB cycle in autotrophic thermophilic and, possibly, also in mesophilic *Crenarchaeota* (Huber *et al.*, 2008; Berg *et al.*, 2007, 2010a, b). On the basis of the sequence similarities of these genes in *N. maritimus* SCM1 and *C. symbiosum* A genomes, we have found the 4-HBD homologs encoded in fosmids of bathypelagic ALOHA Station and in GOS contigs (La Cono *et al.*, 2010). Using the primer pair specifically targeting all marine crenarchaeal 4-*hbd* sequences, we were able to amplify this gene from the environmental DNA collected from 3480 m. All 20 clones analyzed harbored 4-*hbd* genes very similar each to another and to that coding for the putative 4-hydroxyphenylacetate 3-hydroxylase protein of uncultured marine crenarchaeote HF4000_APKG3H9. As in the case with *accA*, retrieved 4-*hbd* genes formed a tight cluster with the bathypelagic sequences, whereas a very distant relation was observed with those of 'shallow' representatives of marine crenarchaea exhibiting only 82 and 73% protein sequence identity with *N. maritimus* SCM1, *C. symbiosum*, respectively (Figure 8). Remarkably,

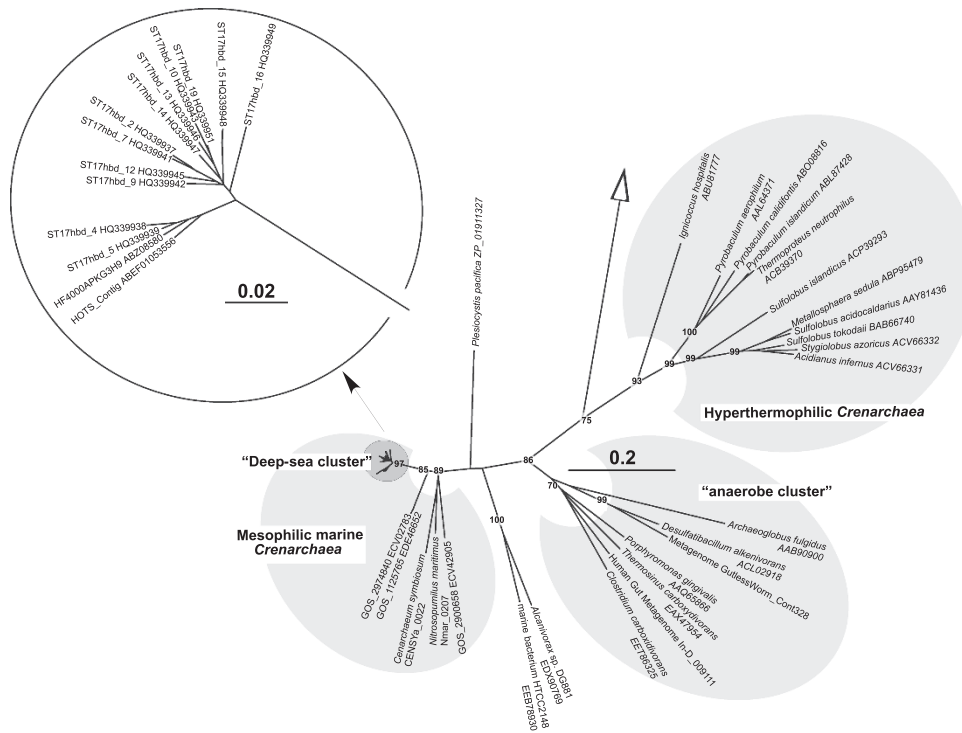


Figure 8 Phylogenetic affiliation of putative 4-hydroxybutyryl-CoA dehydratases 4-HBD recovered from deep Tyrrhenian Sea (3480 m) as determined by Neighbor-Joining analysis and Poisson correction of distances. The clones attributed to deep-sea *Crenarchaeota* lineage are shown in details in the insert. The tree is out-grouped with 3-hydroxybutyryl-CoA dehydrogenase of *N. maritimus* SCM1 (Nmar_1028, ABX12924). Neighbor-Joining analysis using 1000 bootstrap replicates was used to infer tree topology and the nodes with the percentage of bootstrap re-sampling above 70% are indicated. The scale bar represents percentage of amino-acid sequence divergence.

the ‘mesophilic’ marine crenarchaeal sequences are no more similar considering either their sequences or chromosomal arrangements, to ‘thermophilic’ crenarchaeal 4-*hbd* genes than they are to the homologs genes encoding bacterial dehydratases, suggesting functional and evolutionary differences between the ‘mesophilic’ and ‘thermophilic’ versions of crenarchaeal 4-HBD. This observation is coincident with a hypothesis that marine autotrophic archaea are likely the representatives of an evolutionary lineage different from *Crenarchaea* (Brochier-Armanet *et al.*, 2008; Gupta and Shami, 2010; Spang *et al.*, 2010).

Study of [¹⁴C]HCO₃ assimilation using shotgun metaproteomic analysis

Carbon assimilation mechanisms predominant in the bathypelagic zone and the exact role of marine crenarchaeota in this process have yet to be elucidated. The possibility of heterotrophic growth of marine crenarchaea is supported by studies that demonstrating the incorporation of ¹⁴C-labeled amino acids into the cells of marine crenarchaea (Ouverney and Fuhrman, 2000; Herndl *et al.*, 2005). In contrast, incorporation of ¹³C-labeled bicarbonate into the archaeal membrane lipids (Wuchter *et al.*, 2003) and archaeal cells (Herndl *et al.*, 2005) and the natural radiocarbon content of archaeal lipids from

the marine water column (Pearson *et al.*, 2001; Ingalls *et al.*, 2006) support the view of a predominantly autotrophic metabolism of marine crenarchaea. Recent isolation and genome analysis of *N. maritimus* SCM1 (Könneke *et al.*, 2005; Walker *et al.*, 2010) support the latter view. In the present study, we attempted to pair the molecular data with functional studies of microbial processes and with ¹⁴C-labeled bicarbonate incorporation experiments by using a shotgun proteomic approach. High-pressure liquid chromatography with combined ultraviolet and on-line flow-through radioactivity detection system was used for monitoring and separation of ¹⁴C-labeled proteins from not labeled material. After pooling, the radioactive proteins were subjected to trypsin digestion and the obtained peptides were analyzed using reverse phase liquid chromatography-tandem mass spectrometry. Compared with traditional proteomic methods, the application of this combined technique provides a powerful tool for proteome profiling and biomarker discovery. About 40 different proteins were properly attributed to marine *Crenarchaeota*, including hits from both *N. maritimus* SCM1, *C. symbiosum* A genomes and from the fosmid of bathypelagic waters of Pacific Ocean and Mediterranean Sea. The number of peptides per identified protein ranged from one to seven and the percentage of protein

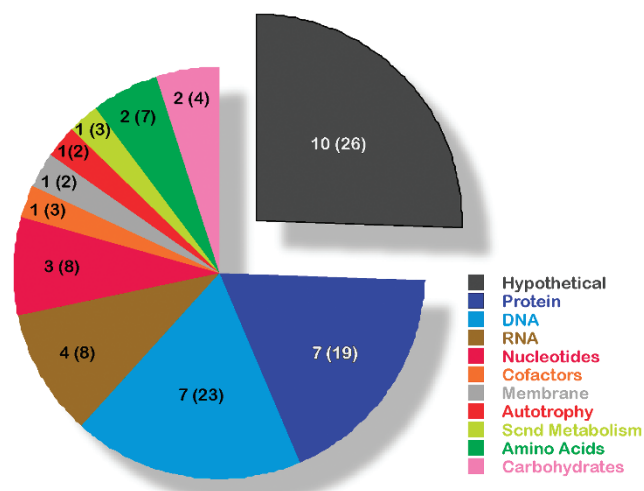


Figure 9 Number of identified crenarchaeal-specific ^{14}C labeled proteins and oligopeptide density grouped as clusters of orthologues genes (COGs). Abbreviations used for COGs: protein, posttranslational modifications and translation, ribosomal structure and biogenesis; DNA/RNA, replication, recombination and repair, transcription, genetic information processing, RNA degradation; nucleotide, nucleotide transport and metabolism; cofactors, coenzyme transport and metabolism; membrane, intracellular trafficking and secretion; inorganic ion transport and metabolism; scnd metabolism, secondary metabolites biosynthesis and transport, amino acids, amino-acid transport and metabolism; carbohydrate, carbohydrate transport and metabolism. Autotrophy stands for 4-hydroxybutyryl-CoA dehydrase, a key enzyme of 3-HP/4-HB autotrophic pathway.

amino-acid sequence coverage by the identified peptide(s) ranged from 2.2 to 65.1%, approximately (see Supplementary Table S3).

From the standpoint of energy metabolism, aerobic NH_4^+ oxidation is a relatively low energy-gaining process, thus, the energy limitation exerts a strong selective pressure in favor of energy-saving mechanisms and the energy costs are primarily spent for the synthesis of enzymes taking part of the basal cell's machinery and autotrophy (Berg *et al.*, 2010a). At least some part of bathypelagic *Crenarchaeota* possess an NH_4^+ -based chemolithoautotrophic energy metabolism, and thus, the biosynthesis of these enzymes, especially the auxiliary CO_2 fixation-related ones, should be considered vital for these organisms. As it is shown in Figure 9, the identified crenarchaeal-specific proteins are involved in at least 10 different biological processes with the absolute prevalence of DNA/RNA and protein metabolism-related enzymes (45%). Besides DNA and RNA polymerases, two helicases similar to those of *C. symbiosum* A (CENSYa_0180 and CENSYa_1010) were identified (Supplementary Table S3). At least 15 oligopeptides were attributed to four proteins of different chaperone systems indicating the importance of the folding pathways in crenarchaeal metabolism. Remarkably, of all the peptides subjected to shotgun proteomic we were able to identify two 9 and 19 amino-acid-long oligopeptides, unambiguously attributed to 4-HBD,

a key enzyme involved in autotrophic carbon fixation via 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg *et al.*, 2010a,b). It should be noted that significant amount of resolved oligopeptides (25%) were assigned to 10 hypothetical marine crenarchaeal-specific proteins of unknown function, thus precluding any speculation about their role in the cellular processes.

Conclusion

In the present work, we studied a number of issues related to dark ocean primary production driven, at least partially, by marine *Crenarchaeota*. Marine mesophilic *Crenarchaeota* are actively growing in the oxygenated water column of Tyrrhenian Sea using bicarbonate as a C source. The estimated mean dark ocean production at Station 17 was shown to be comparable to the median value of photosynthetic biomass production calculated for this area of Tyrrhenian Sea. Therefore, this group of chemoautotrophic prokaryotes, numerically dominating deep-sea microbial communities worldwide, might represent an important and hitherto unrecognized driver for sink of inorganic carbon in the largest oceanic subsystem embracing mesopelagic and bathypelagic zone. The resistance of marine *Crenarchaeota* to cultivation limits our capacities to unravel and define specific processes controlling their physiology and abundance. Therefore, further studies, including metagenomic and proteomic approaches and cultivation under *in situ* conditions, are necessary to overcome this circumstance and to provide important new insights into the mechanisms of element cycling in the deep-sea environment.

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