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Abstract

The dark ocean is a harsh environment where available energy and organic carbon sources for microorganisms are scarce. However, a large pool of dissolved inorganic carbon (DIC) is present there that can be potentially fixed through chemosynthesis by bacteria leaving the question on the available energy sources. Several reduced inorganic substrates are potentially available as energy source to fuel dark bacterial autotrophic carbon fixation such as ammonia, hydrogen and sulfur. The *apr*A gene encodes for the alpha subunit of the adenosine-5′-phosphosulfate (APS) reductase, responsible for either oxidizing or reducing sulfur. The oxidizing pathway might represent an energy source for the Calvin-Benson-Basham (CBB)-cycle in the dark ocean. In this study, *cbb*M, encoding RubisCO form II in Bacteria, and *apr*A gene abundance were determined along a transect in the Atlantic from 64°N to 50°S to quantify the potential for bacterial chemoautotrophy throughout the water column from the epi- to the bathypelagic realm. Both *cbb*M and *apr*A gene abundance were highest in the mesopelagic zone (200 - 1000 m), coinciding with the oxygen minimum layer, and upper bathypelagic (1000 - 2000 m) throughout the Atlantic. Moreover, bacterial autotrophic genes were relatively more abundant in oligotrophic regions, such as the North and South Atlantic Gyre and the Western Tropical Atlantic, as compared to high latitude regions. The co-occurrence of RubisCO and *apr*A genes in the same bacterial lineages affiliated to Gammaproteobacteria and Deltaproteobacteria subphyla indicates the potential of using sulfur oxidation as an energy source for CO_2 fixation through the CBB cycle in these widespread groups. Taken together, our results provide evidence that DIC fixation by chemoautotrophic microbes represents a potential alternative source of organic carbon for the deep ocean heterotrophs, especially in the oligotrophic gyral and tropical regions.

Key words: sulfur oxidation, adenosine-5′-phosphosulfate (APS) reductase, RubisCO, chemoautotrophy, Bacteria

1 Introduction

The ocean is a heterogeneous environment, with microbial hotspots of activity and abundance throughout the water column (Reinthaler *et al*., 2010), associated to local inputs of biologically available organic matter, such as copepods' fecal pellets (Tang et al., 2005) or particles (Baltar *et al*., 2009). Organic carbon is a limiting resource for heterotrophic prokaryotic growth in the dark ocean (>200 m deep) since only 1-40% of the epipelagic primary production is exported into the dark ocean below 200 m depth (Ducklow *et al*., 2001). Indeed, the measured heterotrophic bacterial carbon demand of the deep ocean is larger than the estimated input from epipelagic particulate organic carbon (POC) (Banse, 1990; Reinthaler *et al*., 2006). Consequently, there must be other sources of energy and carbon available to sustain bacterial metabolism in the ocean's interior. Recent studies indicate that dissolved inorganic carbon (DIC) fixation driven by chemical energy might represent an alternative, non-sinking source of organic carbon in the deep ocean (Reinthaler *et al*., 2010; Varela *et al*., 2011; Sintes *et al*., 2013).

Chemolithoautotrophic Bacteria are ubiquitously present in the dark ocean (Aristegui *et al*., 2009; Swan *et al*., 2011). The main pathway of chemoautotrophic carbon fixation is the Calvin-Benson-Basham (CBB) cycle (Tabita, 1988; 1999; Atomi, 2002). The key enzyme in this pathway is ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) forms I and II encoded by the *cbb*L and *cbb*M gene, respectively (Tabita, 1988; Cleland *et al*.,1998; Swan *et al*., 2011). The RubisCO form II is used in environments with high CO_2 and low O_2 levels, while RubisCO form I works better in low CO_2 and high O_2 concentration environments (Tabita, 1988; Badger and Bek, 2008).

Microbial chemosynthesis can potentially use a variety of energy sources, i.e., electron donors, such as ammonium, methane, reduced sulfur and hydrogen (Aristegui *et al*., 2009; Swan *et al*., 2011; Walsh *et al*., 2009; Reinthaler *et al.*, 2010; Anantharaman *et al*., 2013; Sintes *et al.*, 2013). Particularly, chemosynthesis based on the oxidation of reduced sulfur compounds is widely distributed among diverse marine environments such as hydrothermal vent plumes and oxygen minimum zones (OMZs) (Walsh *et al*., 2009; Anantharaman *et al*., 2013). In particular, the OMZs are widespread in the oceans, frequently expanding vertically for a few hundred meters in the mesopelagic zone (Ulloa *et al*., 2012). However, the extent to which reduced sulfur is used as an electron donor or energy source in the oxygenated oceanic water column remains poorly investigated. Reduced sulfur is low in concentration to

undetectable outside upwelling regions and the vicinity of hydrothermal vents (Walsh *et al*., 2009; Radford-Knoery *et al*., 2001). A potential source for reduced sulfur in the otherwise oxygenated water column might be marine snow where sulfide can be generated in oxygen depleted or anoxic microzones (Shanks and Reeder, 1993). Different forms of reduced sulfur compounds might be available as energy source for chemoautotrophic prokaryotes in the ocean (Ghosh and Dam, 2009).

The adenosine-5'-phosphosulfate pathway involves the enzymes adenosine-5′-phosphosulfate (APS) reductase and adenosine triphosphate (ATP) sulfurylase (Kappler and Dahl, 2011). APS reductase, comprising an alpha- and a beta-subunit (Lampreia *et al*., 1994; Hipp *et al*., 1997; Fritz *et al*., 2000; Meyer and Kuever, 2007), is the key enzyme in the sulfate reduction pathway of sulfate-reducing prokaryotes. However, this enzyme is also found in sulfur-oxidizing prokaryotes, where it catalyzes the transformation of sulfite to sulfate (Hipp *et al*., 1997; Meyer and Kuever, 2007).

The aim of this study was to determine the distribution and phylogenetic affiliation of DIC-fixing Bacteria in the Atlantic Ocean from 64°N to 50°S throughout the water column and hence covering several Atlantic oceanographic provinces. We also explored the potential of these chemoautotrophic Bacteria to use reduced inorganic sulfur as an energy source. Consequently, the study focused on the functional genes *cbb*M and *apr*A (APS reductase alpha subunit) using quantitative polymerase chain reaction (q-PCR). Also, the phylogeny of DIC-fixing sulfate reducing or oxidizing Bacteria in the water column was analyzed by cloning and sequencing.

2 Materials and Methods

Characteristics of the sampling area and sample collection – Water samples were collected on three research cruises (GEOTRACES 1 - 3) along a latitudinal transect in the Atlantic Ocean from 64°N to 50°S (Fig. 1). Water samples were taken using R/V *Pelagia* (GEOTRACES 1 - 2) during April and July 2010 and RSS *James Cook* (GEOTRACES 3) during February - March 2011 and a CTD (conductivity-temperature-depth;

Seabird, Bellevue, WA, USA) rosette sampler equipped with 25 L Niskin bottles and sensors for chlorophyll fluorescence, turbidity and oxygen.

Water samples for microbial DNA analysis (2 - 10 L) were collected at 6 to 8 depths per station, filtered onto 0.2 µm pore-size polycarbonate filters, immediately frozen in liquid N_2 and stored at -80°C. Samples for contextual abiotic and biotic parameters were taken at 24 depths per station. Inorganic nutrient concentrations and prokaryotic cell abundance were determined as described previously (De Corte *et al*., 2012; Sintes *et al*., 2013).

Four pelagic zones were distinguished throughout the depth profiles: epipelagic (50 - 200 m) (E), mesopelagic (200 - 1000 m) (M), upper bathypelagic (1000 - 2000 m) (UB) and lower bathypelagic (2000 - 4000 m) (LB). Moreover, six oceanic provinces were encountered and discriminated along the latitudinal transect following Longhurst (1998): North Atlantic Arctic (ARCT; 70°N - 55°N), North Atlantic Drift (NADR; 55°N - 40°N), North Atlantic Gyre (NAG; 40°N - 12°N), Western Tropical Atlantic province (WTRA; 12°N - 6°S), South Atlantic Gyre (SATL; 6°S - 40°S), and Subantarctic (SANT; 40°S - 55°S) (Fig.1).

DNA extraction and amplification – DNA was extracted from the filters using the Mobio UltraClean Soil DNA Isolation Kit following the manufacturer's protocol.

Two *cbb*M genes encoding for RubisCO form II named *cbb*M65 and *cbb*M68 (Swan *et al*., 2011), and one *apr*A gene encoding for the alpha subunit of APS reductase were amplified using polymerase chain reaction (PCR). The primer set used to amplify the *apr*A gene targeted both sulfate-reducing as well as sulfur-oxidizing prokaryotes (Blazejak *et al*., 2006; Meyer and Kuever, 2007).

The PCR amplification mix consisted of the specific primer pair (Table 1) for each gene (1 mM), 1 µL of DNA extract (9 - 25 ng of DNA), MgCl (2.5 mM), PicoMax Polymerase (1 U), 1x PicoMax polymerase buffer, bovine serum albumin (BSA) (0.2 µM), deoxyribonucleoside triphosphate (dNTPs) (0.2 mM) and sterile water filled up to a total volume of 25 µl. The PCR cycles consisted of an initial denaturation step (94°C for 5 min) followed by 30 cycles of denaturation at 94°C for 1 min, annealing at the specific primer temperature (Table 1) for 1 min, and extension at 72°C for 1 min. The PCR was finalized with a final extension at 72°C for 10 min and cooling down at 4°C.

***Cloning and sequencing of cbb*M65*, cbb*M68*, apr*A *gene* –** The microbial DNA from three different depth layers (mesopelagic, upper bathypelagic and lower bathypelagic) of station 33 (Fig. 1) was used to characterize the RubisCO- and *apr*A-harboring bacterial community. The amplified PCR products obtained as described above were checked on a 2% agarose gel. Thereafter, they were purified using 5 Prime PCRExtract Mini Kit, and the purified DNA concentration was determined on a Nanodrop 2000 spectrophotometer.

Subsequently, an adenine-tail extension was conducted on the purified DNA at 72°C for 15 min using Taq Polymerase (Fermentas). The amplified DNA was inserted in a vector and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Approximately 50 colonies were picked randomly after blue-white screening. These colonies were checked by gel electrophoresis in order to ensure the proper size of insert after PCR amplification using the vector primers *M*13F/*M*13R (Table 1). Subsequently, the clones containing the inserts of *cbb*M and *apr*A genes were submitted to GATC Biotech for Sanger sequencing.

***Phylogenetic analysis* –** All phylogenetic and evolutionary analyses were conducted using MEGA5 software (Tamura *et al*., 2011). The PCR amplified sequences were uploaded to ExPASy (Expert Protein Analysis System, Swiss Institute of Bioinformatics (SIB)) to trim and decrease redundancy. Gene sequences with ≥ 97% similarity were clustered into operational taxonomic units (OTUs) (Bairoch and Apweiler, 2000). The representative sequences of the OTUs were aligned together with known published sequences from NCBI using ClustalW. The evolutionary distances and history were computed using Maximum Composite Likelihood and the neighbor-joining method, respectively (Tamura *et al.*, 2011). The bootstrap consensus tree was inferred from 100 replicates (Felsenstein, 1985). The resulting phylogenetic tree was generated with iTOL (Leutnic and Bork, 2006 & 2011).

Quantitative-PCR (q-PCR) – Q-PCR was conducted in triplicate for all functional genes on a LightCycler 480 SW 1.5 (Roche). Internal standards for the different genes were prepared using plasmids from the clone libraries containing the target gene fragments using the primers and PCR conditions as given in Table 1. A serial dilution from 10^7 to 10^2 copies of the purified PCR product was prepared for the different genes based on the DNA concentration and the fragment size. One µL of standards, blanks and DNA extract were added to a 96-well q-PCR plate (Bio-Rad) containing 9 µL of reaction mix per well. The well was covered with an optical tape (Bio-Rad). The reaction mix for each well contained: 1x Mastermix (LighCycler 480 SYBR Green I Master, Roche), the specific primers (Table 1) (0.5 µM) and enough sterile DNAse-free water to give a total volume of 9 µl. The q-PCR for *cbb*M was composed of an initial denaturation step at 95°C for 10 min and 50 amplification cycles consisting of denaturation at 95°C for 5 s, annealing at the specific primer temperature (Table 1) for 5s, extension at 72°C for 15 s and plate reading at 76°C for 3 s. The average efficiency of the q-PCR for the *cbb*M65 gene was 73% and for *cbb*M68 gene 91%. The q-PCR cycling for *apr*A consisted of an initial denaturation step at 95°C held for 10 min, followed by 50 amplification cycles consisting of denaturation at 95°C for 5 s, annealing at 60°C for 15s, extension at 72°C for 30 s and plate read at 80°C for 3 s. The average efficiency of the q-PCR for *apr*A gene was 81%. The specificity of the q-PCR reaction for the different genes was checked with melting curve analyses (65 - 95°C).

The contribution of chemoautotrophic Bacteria containing *cbb*M or *apr*A genes to the bulk bacterial community was assessed by determining the ratio between the abundance of these functional genes and the abundance of the gene *rec*A. The *rec*A gene is encoding for *recombinase* A, an enzyme involved in DNA repair, and it is present in all known prokaryotic cells in one single copy (Miller and Kokjohn, 1990; Lin *et al*., 2006). The *rec*A gene abundance was measured on a different aliquot of extracted DNA from the same samples used for quantifying *cbb*M and *apr*A (Steiner, 2013). Due to the possibility that the extraction efficiency between replicate DNA extracts vary, the results presented here should be taken with caution. The *rec*A gene abundance will be determined on the same DNA extracts as used for functional gene abundance in the near future.

Statistical analysis – A one-way ANOVA followed by a *post hoc* Dunn's test on ranks was conducted to elucidate the differences between the different biogeographic provinces and depth layers for the two genes (*cbb*M and *apr*A). The results were considered

to be statistically significant at $p<0.05$. The Spearman Rank Order correlation was carried out using the SigmaPlot11 software to examine the influence of the different biotic and abiotic factors on the distribution of the *cbb*M and *apr*A genes. The distance-based multivariate analysis for a linear model using forward selection (DISTML forward) was applied to test the relationship of the genes *cbb*M and *apr*A with the biotic and abiotic factors.

3 Results

*cbbM*65, *cbbM*68 and *apr*A gene abundance determined by q-PCR

In the epipelagic waters, maximum gene abundance of *cbb*M65 was found in the SANT with an average of $5.97x10^{2} \pm 1.01x10^{3}$ mL^{-1} (Fig. 2A, Fig. 6A). The gene abundance of *cbb*M65 in the NADR province ($6.20x10^{1} \pm 5.29x10^{1}$ mL^{-1}) was not significantly different from that in the SATL (4.82 ± 5.50 mL^{-1}) (one way ANOVA on rank, $p>0.05$). The lowest abundance of *cbb*M65 genes was detected in the NAG province ($1.06\pm7.70x10^{-1}$ mL^{-1}) (Fig. 2A, Fig. 6A).

In the mesopelagic layer, *cbb*M65 gene abundance in the WTRA ($2.18x10^{3} \pm 1.27x10^{3}$ mL^{-1}) was similar to that in the SANT province ($7.76x10^{2} \pm 9.16x10^{2}$ mL^{-1}) (one way ANOVA on rank, $p>0.05$) as shown in Fig. 2B-D and Fig. 6A. The *cbb*M65 gene abundance was significantly higher in the mesopelagic of WTRA and SANT provinces than in the ARCT, NADR, NAG and SATL provinces (one way ANOVA on ranks, $p<0.05$) (Fig. 2B, Fig. 6A). In the bathypelagic layers, lower *cbb*M65 gene abundances were found than in the mesopelagic realm in all regions, except in the upper bathypelagic layers of SANT and SATL (Fig. 2B-D, Fig. 6A).

The *cbb*M68 gene abundance was low in the epipelagic layer of all six provinces (< 30 mL^{-1}, Fig. 3A). In the mesopelagic layer, the *cbb*M68 gene abundance was significantly higher in the WTRA province ($1.5x10^{2} \pm 7.99x10^{1}$ mL^{-1}) than in the ARCT, NADR, NAG, SATL and SANT province (one way ANOVA on rank, $p<0.05$) (Fig. 3B, 6B). Similar *cbb*M68 gene abundances were found in the mesopelagic layers of the NAG, SATL and SANT province (one way ANOVA on rank, $p>0.05$) (Fig. 3B). The same latitudinal distribution pattern of the *cbb*M68 gene abundance was observed for the mesopelagic and the upper bathypelagic

layer, albeit generally lower in the bathypelagic than in the mesopelagic waters (Figs. 3C and 6B). No significant latitudinal differences in the *cbb*M68 gene abundance were apparent in the lower bathypelagic communities (one way ANOVA on rank, $p>0.05$, Fig. 3D, 6B).

The ratio of *cbb*M65:*cbb*M68 generally decreased significantly from high to low latitudinal provinces (Spearman Rank Order correlation, $p<0.05$) (Table 2; Fig. 4) and was significantly higher in the mesopelagic than into epipelagic, upper bathypelagic and lower bathypelagic layers (one way ANOVA, $p>0.05$) (Fig. 5).

The *apr*A gene abundance in the epipelagic realm was similar throughout all the oceanic provinces (one way ANOVA, $p>0.05$) except the ARCT province (Figs. 6C and 7A). The abundance of the *apr*A gene increased towards the mesopelagic layer, except in the NADR (Fig. 6B). There was a significant difference in *apr*A gene abundance between the mesopelagic communities of the NAG ($6.20x10^2 \pm 4.29x10^2$ mL^{-1}), WTRA ($6.00x10^2 \pm 2.96x10^2$ mL^{-1}) and SATL province ($5.95x10^2 \pm 3.17x10^2$ mL^{-1}) as compared to the ARCT ($8.28x10^1 \pm 5.48x10^1$ mL^{-1}) and SANT ($1.41x10^2 \pm 1.59x10^2$ mL^{-1}) based on one-way ANOVA on ranks $p<0.05$. In the NADR province, the *apr*A gene abundance ($2.45x10^2 \pm 1.14x10^2$ mL^{-1}) decreased from the epipelagic to the mesopelagic layer. A general decrease in the *apr*A gene abundance was determined towards the bathypelagic as compared to the mesopelagic realm in all provinces, except the ARCT (Figs. 6C and 7).

The distribution of the *rec*A gene abundance along this longitudinal transect has been previously described (Steiner, 2013). The relative contribution of bacterial cells harboring the gene for RubisCO (*cbb*M65 and *cbb*M68) was high in the mesopelagic realm of the WTRA and SANT province. The proportion of Bacteria harboring the *cbb*M65 gene decreased towards the bathypelagic realm (Fig. 8A). Bacterial cells containing *cbb*M68 were relatively abundant in meso- to bathypelagic waters throughout the Atlantic (Fig. 8B). The *apr*A:*rec*A ratio showed a similar distribution to that of *cbb*M68:*rec*A (Fig. 8B, C).

*Phylogenetic affiliation of apr*A-, *cbb*M65- *and cbb*M68-*harboring Bacteria*

The phylogenetic affiliation of *apr*A and *cbb*M harboring Bacteria was determined in three depth layers at Station 33 located in the WTRA province (Fig.1). In total, 148 clones of *cbb*M65 were sequenced. All *cbb*M65 sequences obtained were putatively associated to the Gammaproteobacteria subphylum. One OTU (D1) was abundant in all pelagic layers

(represented by 29, 38 and 50 clones from the mesopelagic, upper bathypelagic and lower bathypelagic, respectively) (Fig. 9).

Only 35 clones of *cbb*M68 were effectively sequenced, due to the low efficiency of the cloning reaction with this specific gene. All the sequences of *cbb*M68 corresponded to the same OTU, putatively assigned to the Deltaproteobacteria subphylum.

A total of 151 clones containing the *apr*A gene fragment were successfully sequenced. The *apr*A gene primer pair used in this study is targeting either sulfate reducing bacteria (SRB) or sulfur oxidizing bacteria (SOB) (Meyer and Kuever, 2007). The most abundant OTU (A3) was present in the upper and lower bathypelagic (Fig. 10), and was putatively assigned to SOB Gammaproteobacteria. The next most abundant OTU (B3, Fig. 10) was obtained from the upper bathypelagic, and was presumably related to a SRB Deltaproteobacteria. Some OTUs were putatively associated to Alphaproteobacteria and Betaproteobacteria SOB (Fig. 10).

*Distribution of Bacteria harboring cbb***M** *and apr***A** *and the influence of abiotic and biotic factors*

Some background abiotic and biotic factors have already been analyzed including leucine incorporation as a proxy for prokaryotic heterotrophic production (De Corte *et al.,* 2012). In the present work, we specifically explored the relationships between these abiotic and biotic parameters and the functional genes *apr*A and *cbb*M related to chemoautotrophy.

The ratio of *cbb*M65:*cbb*M68, indicative of the dominance of different *cbb*M containing Bacteria, was positively correlated with the prokaryotic heterotrophic production, temperature and bacterial abundance (Table 2). A negative correlation was obtained between the ratio *cbb*M65:*cbb*M68 and salinity and depth (Table 2).

The relative contribution of cells harboring chemoautotrophic genes to the total bacterial abundance, represented by the ratios of *cbb*M65:*rec*A, *cbb*M68:*rec*A and *apr*A:*rec*A (Table 2), was negatively correlated with latitude, temperature, heterotrophic production, bacterial abundance and salinity. All these parameters were positively correlated with depth (Table 2).

Additionally, we explored the explanatory value of different environmental factors on the variability of genes indicative of chemoautotrophy (Table 3). The variability of the *cbb*M65:*cbb*M68 ratio was mainly explained by depth, temperature, salinity, bacterial abundance and latitude, together explaining 45.8% of the variation in this gene ratio (Table 3). The variability of *cbb*M65:*rec*A ratio was mainly explained by salinity (31.8%), depth (12.5%), bacterial abundance (6.3%) and temperature (1.8%), together explaing or 52.4% of the variation. The variability of *cbb*M68:*rec*A ratio was mainly explained by bacterial abundance (42.9%), latitude (3.9%), salinity (3.6%), bacterial activity (2.3%) and temperature (1.9%). The variability of *apr*A:*rec*A ratio was mainly explained by bacterial abundance and latitude, explaining 47.0% of the variation (Table 3).

4 Discussion

Latitudinal and depth related trends in Bacteria harboring cbbM or aprA

The total prokaryotic abundance in the ocean decreases with depth (Patching and Eardly, 1997; Nagata *et al*., 2000; Reinthaler *et al*., 2006; De Corte *et al*., 2012). The *rec*A gene encodes the recombinase A protein, responsible for DNA repair mechanisms; this gene is present in all prokaryotes in one copy per cell (Miller and Kokjohn, 1990; Lin *et al*., 2006). Thus, it can be used as a proxy for bacterial abundance (Steiner, 2013). Indeed, the *rec*A gene abundance decreases from epipelagic to lower bathypelagic waters in all Atlantic Provinces (Steiner, 2013) and correlated with the prokaryotic abundance measured by flow cytometry (Steiner, 2013). Thus, the ratio of a functional gene, such as *cbb*M or *apr*A, to the *rec*A gene is an indicator of the relative abundance of the Bacteria harboring a specific gene compared to the bulk bacterial community.

Prokaryotic abundance and heterotrophic production was highest in the ARCT and decreased towards the NAG and WTRA (De Corte *et al*., 2012), related to the input of biologically available organic matter from phytoplankton production in these oceanic provinces (Sathyendranath *et al*., 1995). The DIC fixation in the dark ocean by chemolithoautotrophic Bacteria and Achaea is of the same order of magnitude as heterotrophic biomass production (Herndl and Reinthaler, 2013). Thus, the activity of

chemolithoautotrophic Bacteria and Archaea represents another potential source of organic carbon for the meso- and bathypelagic ocean microbes (Alonso-Saez *et al*. 2010; Herndl and Reinthaler, 2013). Bacteria harboring either *cbb*M65, *cbb*M68 or *apr*A were more abundant in meso- to bathypelagic waters than in epipelagic layers throughout the Atlantic indicating that the potential for chemosynthesis and sulfur oxidation is widespread among bacteria in the dark ocean, even in the well-oxygenated layers of the mesopelagic realm. Furthermore, the higher contribution of bacteria harboring chemoautotrophy-related genes in regions with lower primary production (NAG, SATL, WTRA) supports the idea that, in these environments, chemoautotrophy can provide a complementary source of organic carbon for heterotrophic organisms.

The bacterial heterotrophic activity explains most of the variability of the genes *cbb*M68 and *apr*A, 42.87% and 41.31%, respectively (Table 3). Together with the negative correlation obtained between the leucine incorporation and the contribution of these two genes to the bulk bacterial community, this finding further supports the potential importance of the chemoautotrophic pathways in low productivity regions, such as the oligotrophic gyres and particularly the dark ocean.

*Phylogenetic community composition of cbb*M- *and apr*A-harboring Bacteria

The RubisCO genes *cbb*M65 and *cbb*M68 were found in several members of the phylum Bacteria, at least at the sequencing level reached in this study. Deltaproteobacteria were harboring the *cbb*M68 gene and Gammaproteobacteria possessed the *cbb*M65 gene (Fig 9). The different phylogenetic assignment of these two *cbb*M genes can help to explain the large difference in abundance of the genes (*cbb*M65>>*cbb*M68) in general with a distinct geographic distribution pattern of the two *cbb*M harboring groups. According to previous studies members of the Deltaproteobacteria have been reported to be more abundant in oxygen minimum zones (Wright *et al*., 1997; 2012), while Gammaproteobacteria have been reported to be more abundant in deep waters (Lopez-Garcia *et al*., 2001a).

The SAR324 clade of the class Deltaproteobacteria is ubiquitously present in the dark ocean (Brown and Donachi, 2007) and harbors *cbb*M and *apr*A genes (Swan *et al*., 2011). Although we could not find members of this subphylum with our Sanger sequencing approach, the

limited sequencing effort does not preclude that this group can be found by a more in-depth sequencing approach.

Based on the topology of the phylogenetic tree, the *apr*A gene of sulfur-oxidizing and sulfate-reducing bacteria form two phylogenetic lineages (Meyer and Kuever, 2007). The majority of sulfate-reducing bacterial sequences were related to Deltaproteobacteria sequences, while the majority of sulfur-oxidizing bacterial sequences were affiliated to Gammaproteobacteria, in agreement with previous studies (Hügler *et al.,* 2010). Members of the Gammaproteobacteria subphylum have been shown to increase in relative abundance with depth in the ocean (Lopez-Garcia *et al*., 2001a). As shown in this study, most of the *apr*A harboring bacteria were affiliated to Gammaproteobacteria. Taken together, these findings might indicate an adaptation of some members of this group to dark ocean conditions (Swan *et al*., 2011), and reflect metabolic versatility (Walsh *et al*., 2009). The co-occurrence of RubisCO and *apr*A genes in the same bacterial lineages of Gammaproteobacteria supports the possibility of using sulfur oxidation as an energy source for CO_2 fixation through the CBB cycle in this group of organisms, in agreement with previous studies (Swan *et al*., 2011; Hügler *et al*., 2010).

Overall, this study showed that dark carbon fixation is widespread among bacteria in the oceanic water column although oxygen minimum zones are not pronounced in the Atlantic.

5 Conclusion

The biomass and activity of dark ocean prokaryotic communities is constrained by the amount of organic carbon availability. However, the ocean contains a large pool of DIC that can potentially be fixed by Bacteria and Archaea through chemosynthesis, sulfur being one of the most probable energy sources (Swan *et al*., 2011). In this study, we found genes related to carbon fixation (*cbb*M) and sulfur oxidation (*apr*A) down to bathypelagic waters, and extending from the polar to the tropical and gyral systems. More importantly, the relative contribution of bacteria harboring these genes increases with depth, and towards the low productive low latitude regions. The most abundant microbe with co-occurrence of *apr*A and *cbb*M genes was putatively affiliated to Gammaproteobacteria. Taken together, these results indicate that the potential to carry out chemosynthesis is widespread, not only in oxygen minimum zones and hydrothermal vents, but throughout the oxygenated water column and under diverse environmental conditions.

6 Graphs and Figures

Table 1. Primers used to amplify the two *cbb*M genes, subsequently named *cbb*M65 and *cbb*M68, and *apr*A gene fragments for q-PCR, cloning and sequencing and annealing temperature.

Primer	Sequence	Fragment length	Target	Annealing temp.	Ref.
Plank1 *cbbM*65f Plank1 *cbbM*269r	5'-CACAAGGAAATCAAGTATATGCAAG-3' 5'-TCAACTAGKAAGGCAACATGGTG-3'	~204 bp	*cbb*M	59°C	Swan *et al*., 2011
Plank2 *cbbM*68f Plank2 *cbbM*270r	5'-AAGGTAATCAAATTTGGGGTCC-3' 5'-ATCAACAAGAAATGCAATGTTTTC-3'	~202 bp	*cbb*M	57°C	Swan *et al*., 2011
*M*13F *M*13R	5'-GTAAAACGACGGCCAG-3' 5'-CAGGAAACAGCNATGGG-3'	~243 bp	Cloning Seq.	60°C	
*apr*A-1F *apr*A-5R	5'-TGGCAGATCATGATYMAYGG-3' 5'-GCGCCAACYGGRCCRTA-3'	~412 bp	*apr*A	60°C	Meyer and Kuever, 2007

Table 2. Results of a Spearman Rank Order Correlation between the contribution of functional genes (*cbb*M and *apr*A) to the bulk community (as indicated by *rec*A) or relative *cbb*M contribution (*cbb*M65:*cbb*M68) and environmental parameters (BA-bacterial abundance determined by *rec*A, leucine-leucine incorporation into bacterial biomass as a proxy for heterotrophic bacterial production).

	Latitude	Depth	Temp.	Salinity	BA	Leucine
*cbb*M65:*cbb*M68	r=0.282 p<0.001	r=-0.405 p<0.001	r=0.238 p<0.001	r=-0.299 p<0.001	r=0.608 p<0.001	r=0.472 p<0.001
*cbb*M65:*rec*A	r=-0141 p<0.05	r=0.346 p<0.001	r=-0.353 p<0.001	r=-0.492 p<0.001	r=-0.322 p<0.001	r=-0.448 p<0.001
*cbb*M68:*rec*A	r=-0.421 p<0.001	r=0.490 p<0.001	r=-0.432 p<0.001	r=-0.327 p<0.001	r=-0.613 p<0.001	r=-0.647 p<0.001
*apr*A:*rec*A	r=-0.440 p<0.001	r=0.464 p<0.001	r=-0.313 p<0.001	r=-0.158 p<0.01	r=-0.651 p<0.001	r=-0.659 p<0.001

Table 3. Results of the multivariate multiple regression analysis with forward selection (DISTML forward) to explain the variability of the contribution of the functional genes to the bacterial community. The response variable was log transformed and the resulting data converted in Euclidean distance similarity matrices. The Pseudo-F and the p-value were obtained by permutation (*n*=999). For abbreviations see Table 2.

		Latitude	Depth	Temp	Salinity	BA	Leucine
*cbb*M65:*cbb*M68	prop	0.0806	0.1717	0.0588	0.1104	0.0366	0.0009
	p	0.001	0.001	0.002	0.001	0.004	0.634
*cbbb*M65:*rec*A	prop	0.0031	0.1248	0.0176	0.3184	0.0634	0.0095
	p	0.316	0.001	0.018	0.001	0.001	0.084
*cbbb*M68:*rec*A	prop	0.0391	0.0072	0.0193	0.0363	0.4287	0.023
	p	0.001	0.151	0.016	0.001	0.001	0.016
*apr*A:*rec*A	prop	0.0572	0.0005	0.0024	0.0017	0.4131	0.0047
	p	0.002	0.725	0.389	0.483	0.001	0.24

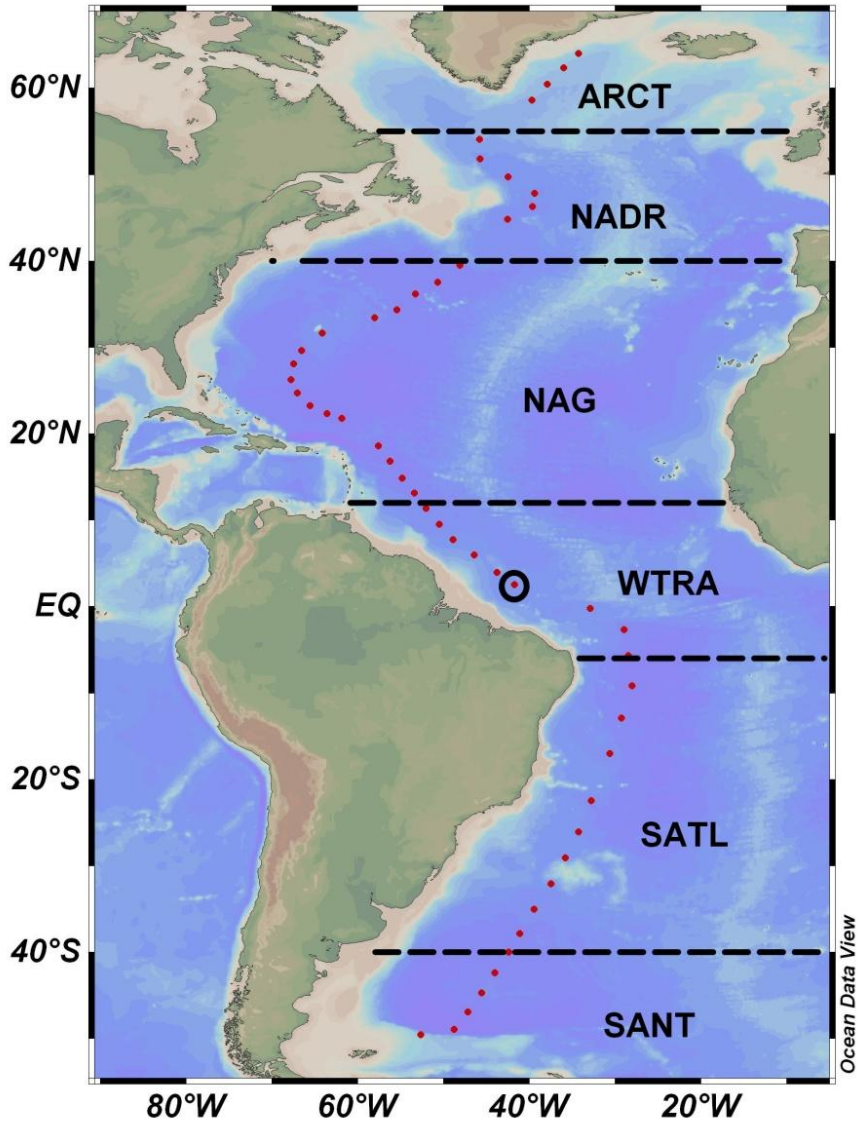

Figure 1. Sampling stations (red dots) along the latitudinal transect in the Atlantic. Dashed horizontal lines denote the borders between the 6 oceanic provinces (Longhurst, 1998). The encircled dot indicates the station where microbial communities were collected for cloning and sequencing of *cbb*M and *apr*A genes. ARCT - 70°N - 55°N, NADR - 55°N - 40°N, NAG - 40°N - 12°N, WTRA - 12°N - 6°S, SATL - 6°S - 40°S, SANT - 40°S - 55°S.

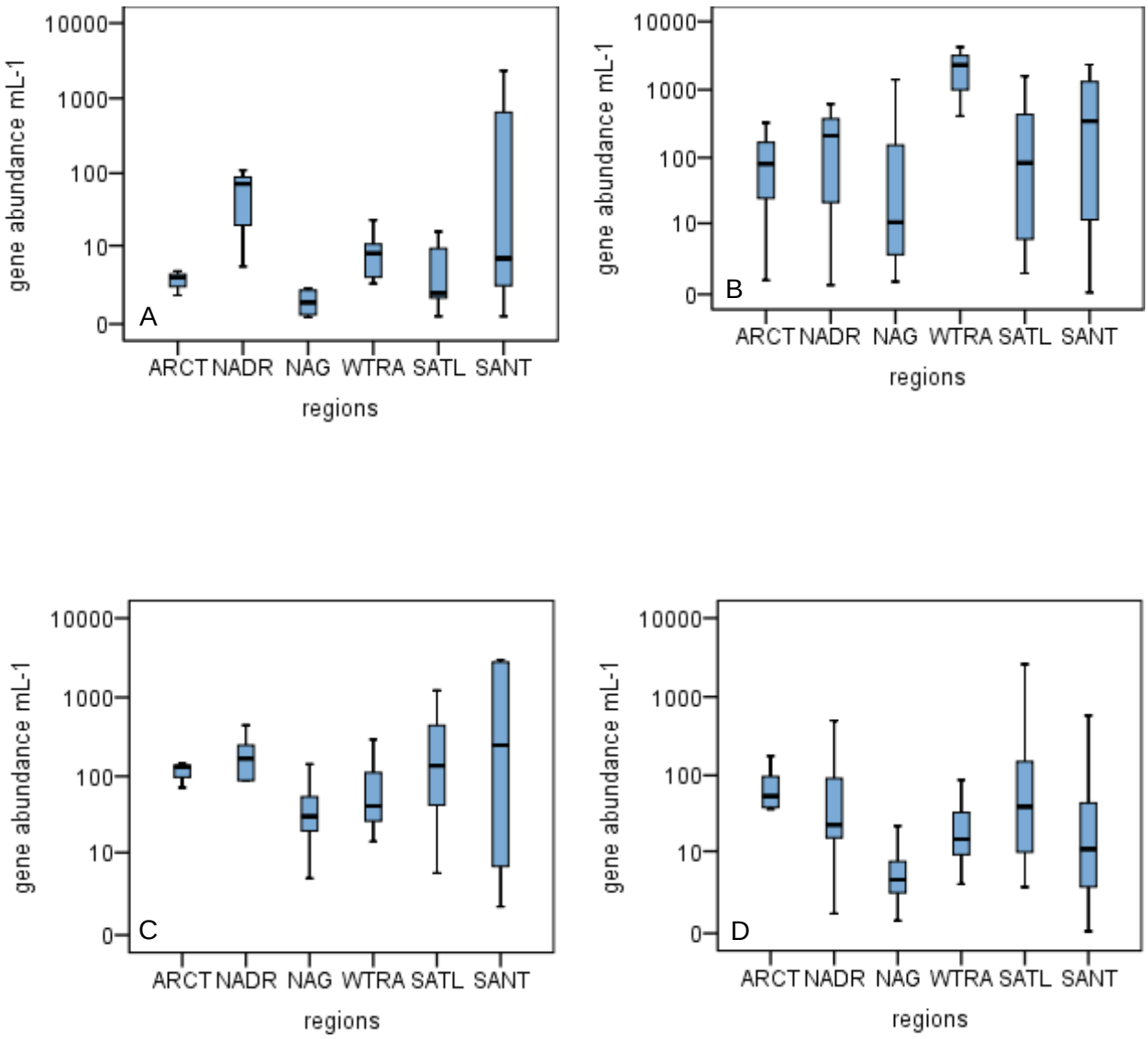

Figure 2. Distribution of the *cbb*M65 gene abundance [mL^{-1}] throughout the different provinces in the Atlantic for A) epipelagic, (B) mesopelagic, (C) upper bathypelagic and (D) lower bathypelagic realm. Abbreviations of the oceanic provinces are given in Fig. 1.

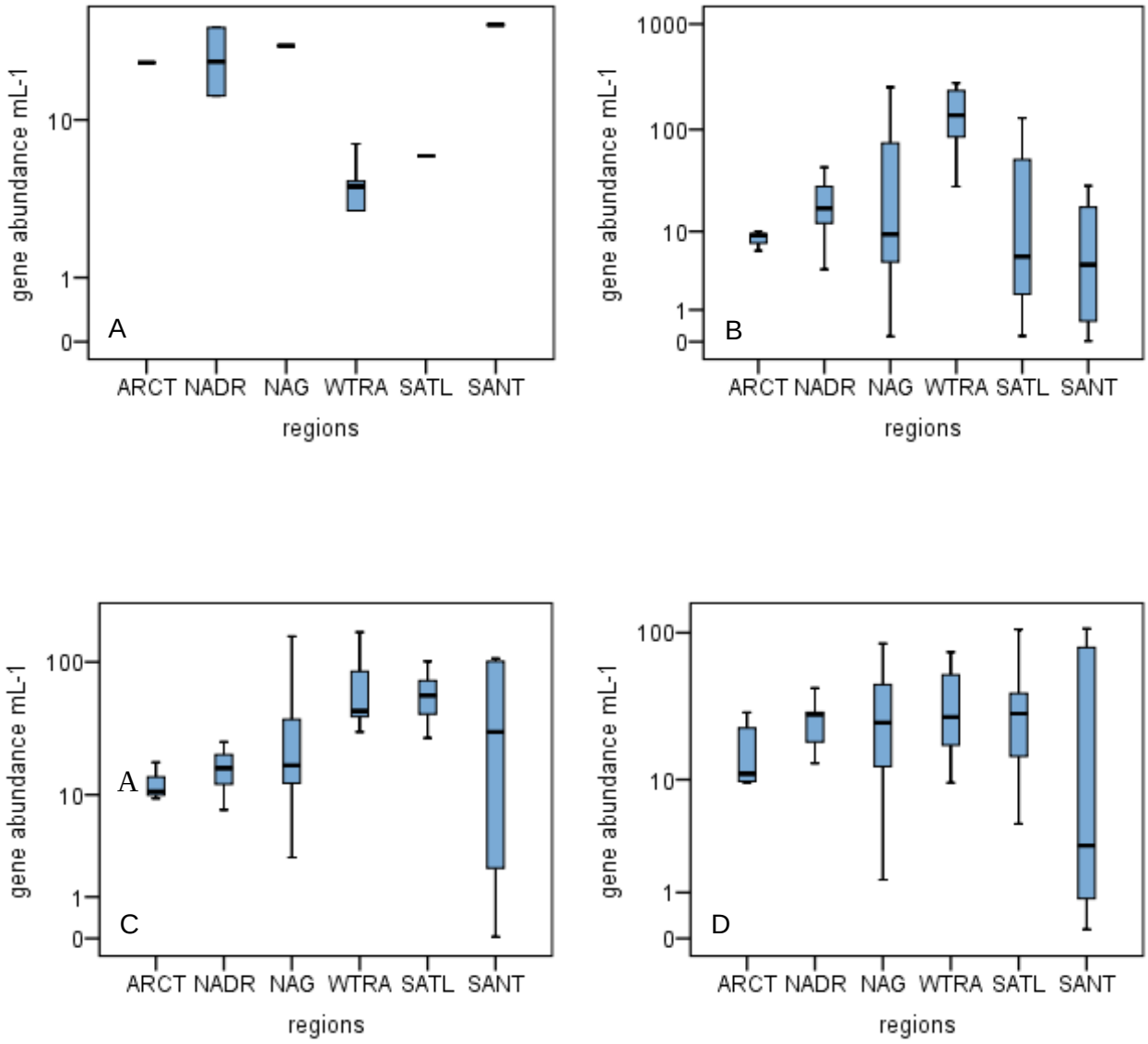

Figure 3. Distribution of the *cbb*M68 gene abundance [mL^{-1}] throughout the different provinces of the Atlantic in the (A) epipelagic, (B) mesopelagic, (C) upper bathypelagic, (D) lower bathypelagic realm. Abbreviation of oceanic provinces is given in Fig. 1.

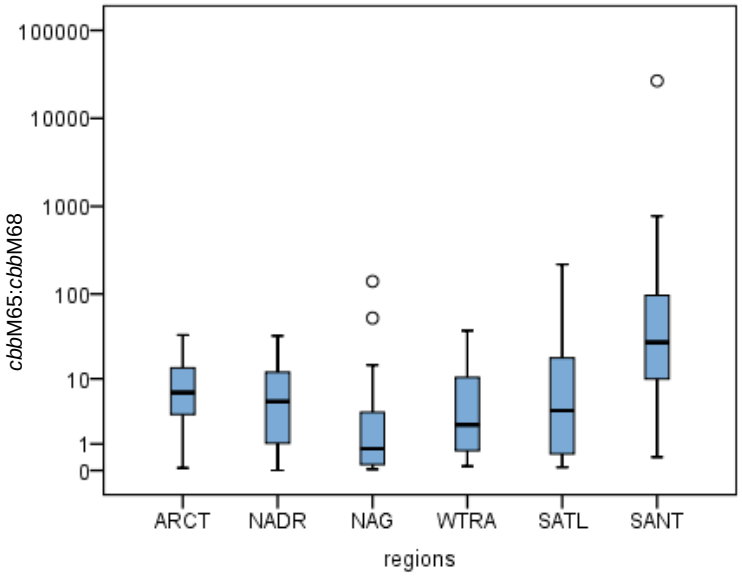

Figure 4. Ratio of *cbb*M65:*cbb*M68 gene abundance in the different oceanic provinces of the Atlantic. Abbreviation of oceanic provinces is given in Fig. 1.

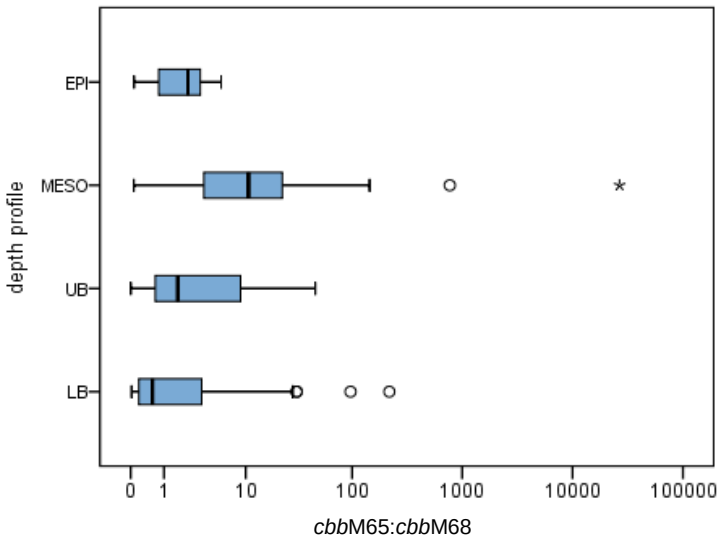

Figure 5. Ratio of *cbb*M65:*cbb*M68 gene abundance in different depth layers throughout the Atlantic. EPI-epipelagic (0-100 m), MESO-mesopelagic (100-1000 m), UB-upper bathypelagic (1000-2000 m) and LB-lower bathypelagic (2000-4000 m) realm.

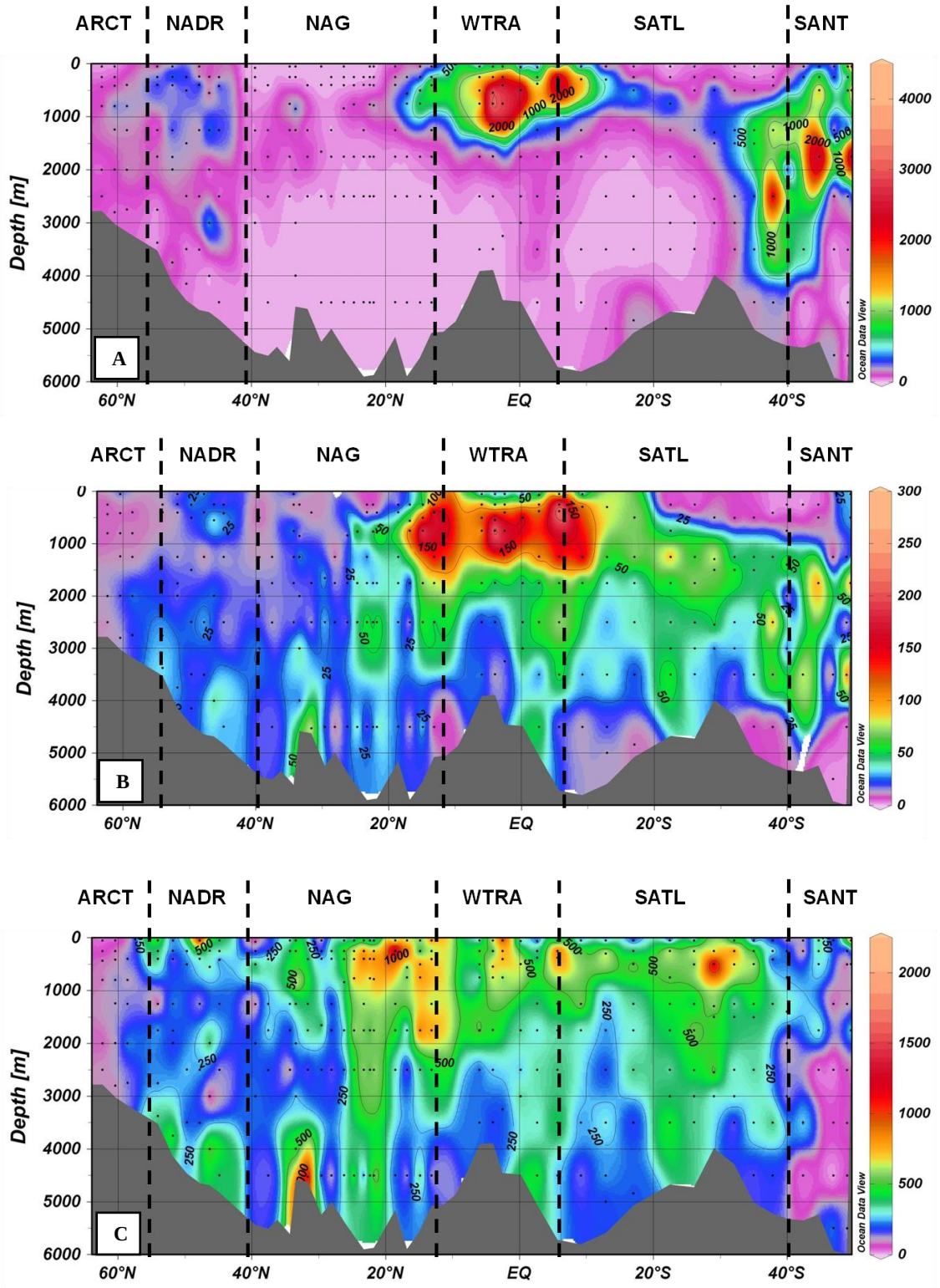

Figure 6. Distribution of the gene abundance [mL^{-1}] along the latitudinal transect and oceanic provinces versus depth: *cbb*M65 gene abundance mL^{-1} (A), *cbb*M68 gene abundance mL^{-1} (B) and *apr*A gene abundance mL^{-1} (C). Note the different scales for the different genes. Vertical dashed lines denote the limits between oceanographic regions. Abbreviation of oceanic provinces is given in Fig. 1.

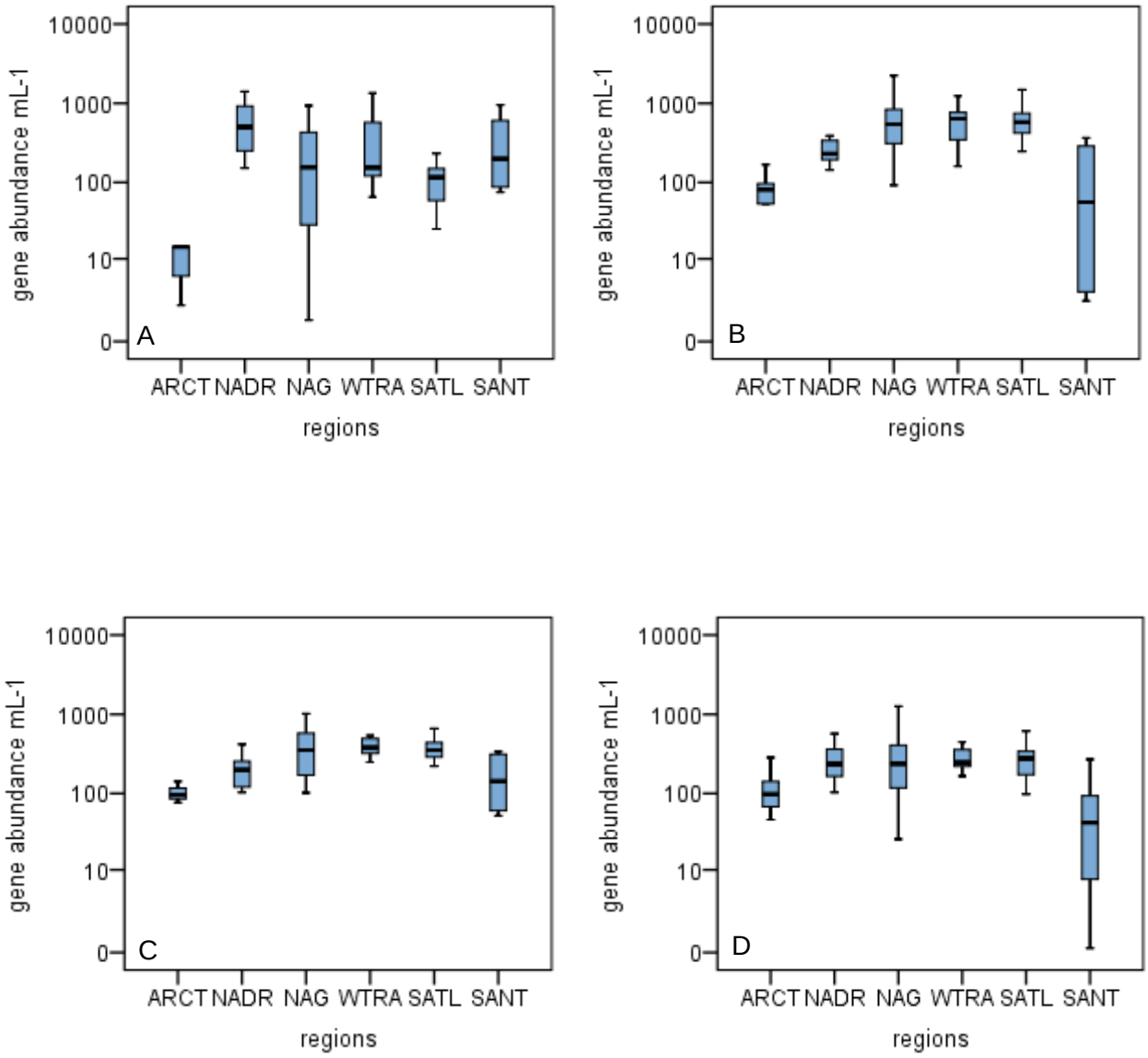

Figure 7. Distribution of the *apr*A gene abundance [mL^{-1}] throughout the different oceanographic regions in the Atlantic for (A) epipelagic, (B) mesopelagic, (C) upper bathypelagic, (D) lower bathypelagic.

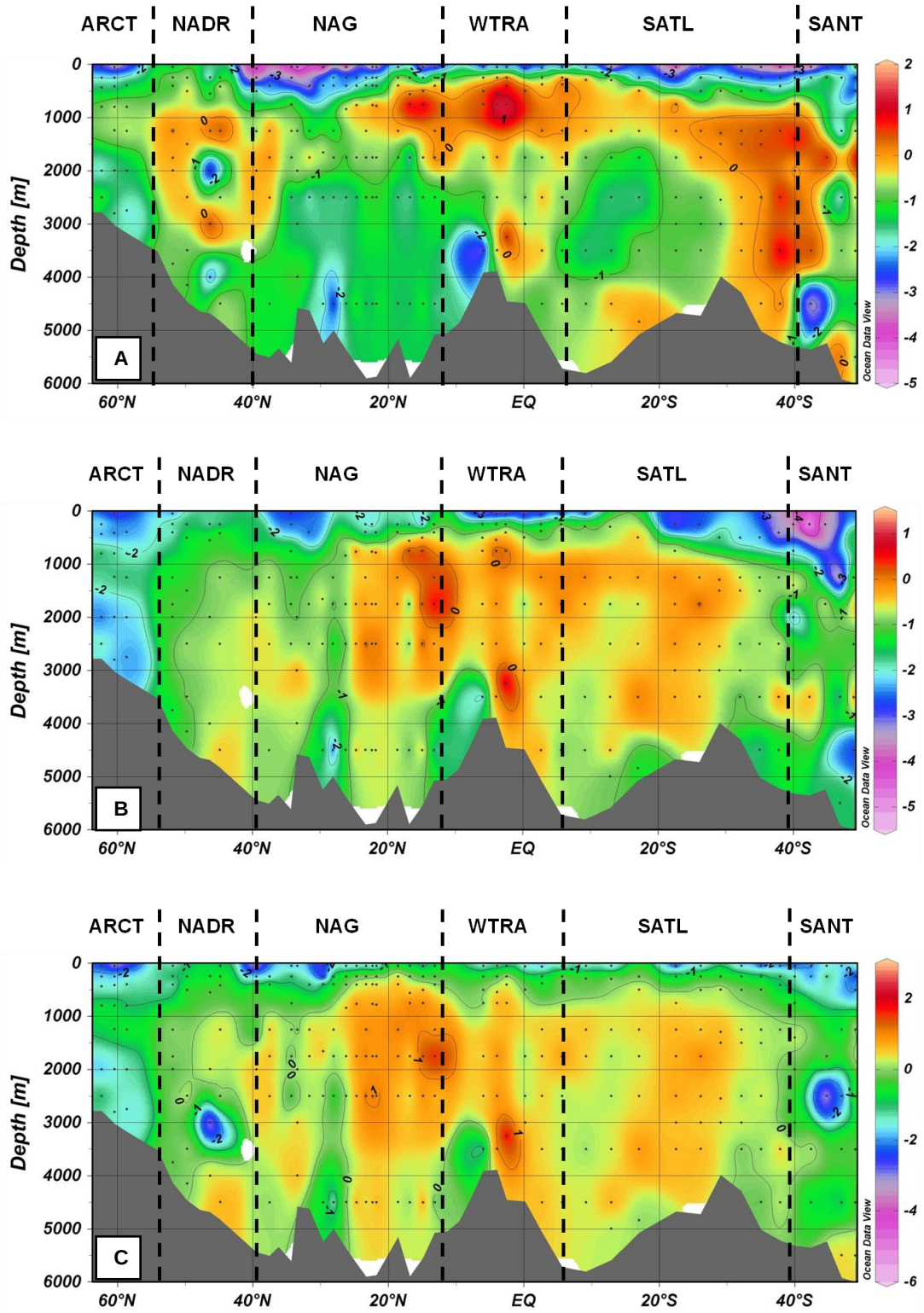

Figure 8. Proportion of bacterial cells harboring *cbb*M65 (A), *cbb*M68 (B) and *apr*A (C) along the latitudinal transect in the Atlantic and through the depth profile. The relative contribution of the cells containing the specific genes is given as the ratio between the abundance of the respective functional gene and *rec*A gene abundance.

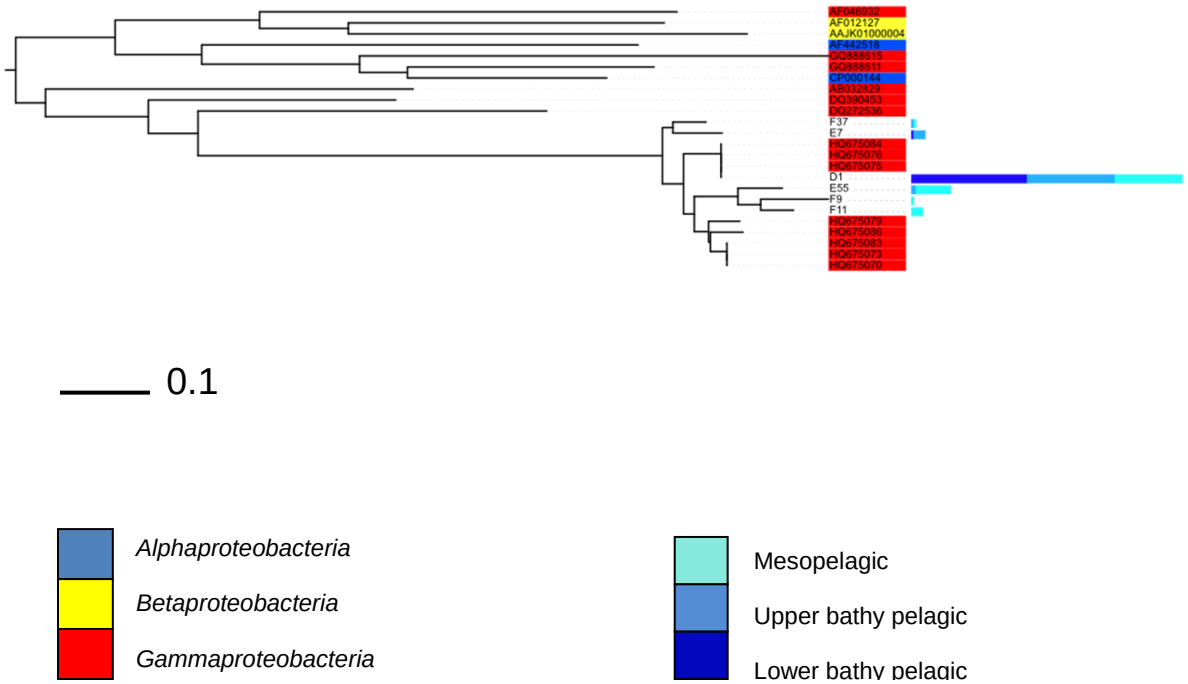

Figure 9. Neighbor–joining phylogenetic tree of the *cbb*M65 gene sequences at 97% similarity. The bar indicates the number of clones obtained from different depth layers (color code) represented by one sequence.

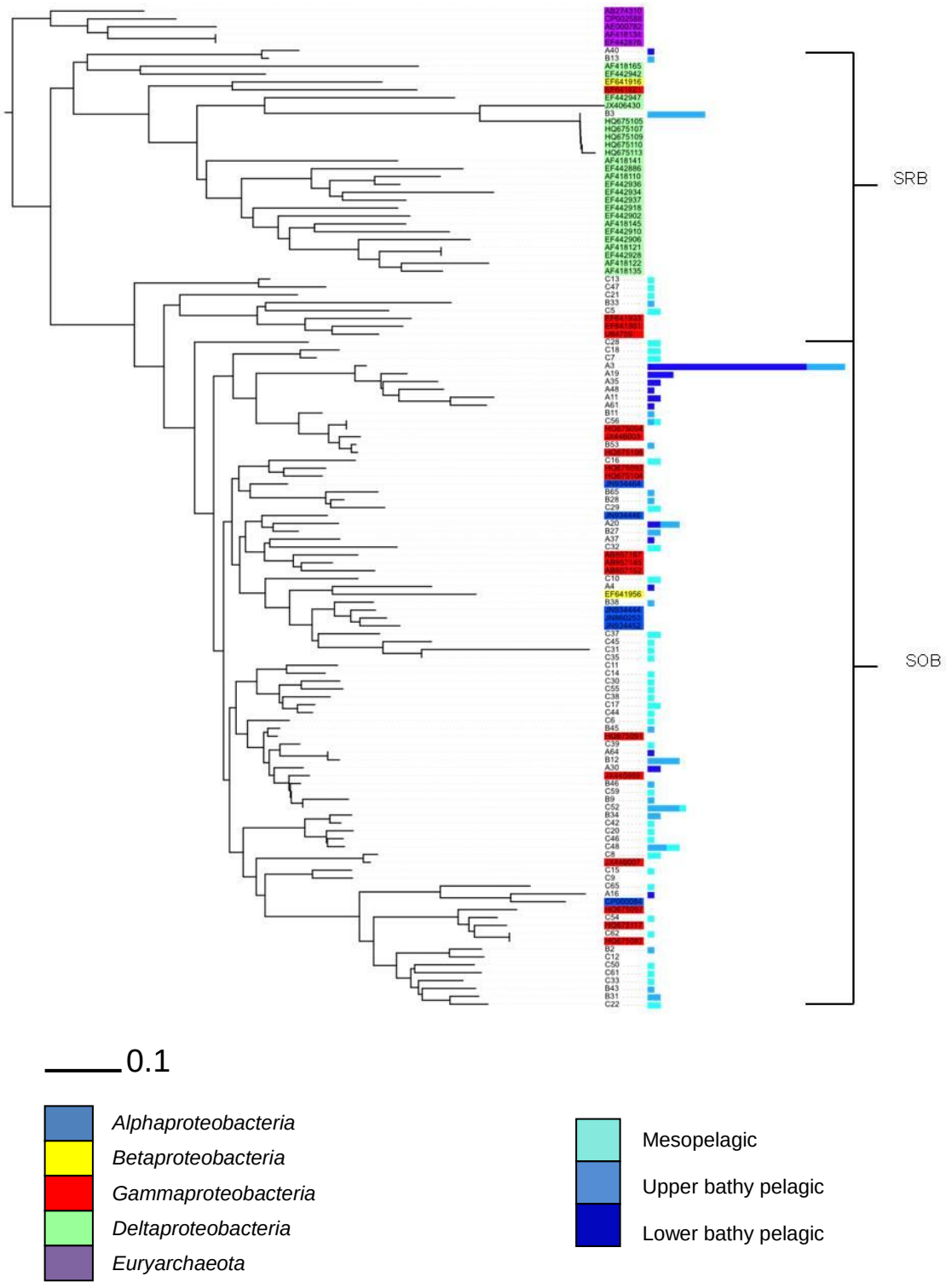

Figure 10 Neighbor–joining phylogenetic tree obtained by the *apr*A gene sequences at 97% similarity. The bar indicates the number of clones obtained from different depth layers (colour code) represented by one sequence. *Euryarchaeota* sequences obtained from NCBI were used to root the tree. Putative SRB (sulfate reducing bacteria) and SOB (sulfate oxidizing bacteria) are also indicated in brackets.

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Appendix

8 Abstract

The dark ocean is a harsh environment where available energy and organic carbon sources for microorganisms are scarce. However, a large pool of dissolved inorganic carbon (DIC) is present there that can be potentially fixed through chemosynthesis by bacteria leaving the question on the available energy sources. Several reduced inorganic substrates are potentially available as energy source to fuel dark bacterial autotrophic carbon fixation such as ammonia, hydrogen and sulfur. The *apr*A gene encodes for the alpha subunit of the adenosine-5′-phosphosulfate (APS) reductase, responsible for either oxidizing or reducing sulfur. The oxidizing pathway might represent an energy source for the Calvin-Benson-Basham (CBB)-cycle in the dark ocean. In this study, *cbb*M, encoding RubisCO form II in Bacteria, and *apr*A gene abundance were determined along a transect in the Atlantic from 64°N to 50°S to quantify the potential for bacterial chemoautotrophy throughout the water column from the epi- to the bathypelagic realm. Both *cbb*M and *apr*A gene abundance were highest in the mesopelagic zone (200 - 1000 m), coinciding with the oxygen minimum layer, and upper bathypelagic (1000 - 2000 m) throughout the Atlantic. Moreover, bacterial autotrophic genes were relatively more abundant in oligotrophic regions, such as the North and South Atlantic Gyre and the Western Tropical Atlantic, as compared to high latitude regions. The co-occurrence of RubisCO and *apr*A genes in the same bacterial lineages affiliated to Gammaproteobacteria and Deltaproteobacteria subphyla indicates the potential of using sulfur oxidation as an energy source for CO_2 fixation through the CBB cycle in these widespread groups. Taken together, our results provide evidence that DIC fixation by chemoautotrophic microbes represents a potential alternative source of organic carbon for the deep ocean heterotrophs, especially in the oligotrophic gyral and tropical regions.

9 Zusammenfassung

Für Mikroorganismen ist die Tiefsee ein spezielles Habitat, in dem sie sich adaptieren und auf verschiedene Nischen spezialisieren müssen. Bei Prokaryoten ist die Nutzung chemosynthetischer Prozesse weit verbreitet und kann in der gesamten Wassersäule nachgewiesen werden, insbesondere auch in sauerstoffarmen Zonen, hydrothermalen Tiefseespalten und bei unterschiedlichen Umweltbedingungen.

In der vorliegenden Arbeit wird untersucht, auf welche Art und Weise Bakterien in der Lage sind, das reiche Vorkommen von „dissolved inorganic carbon“ (DIC) als Energielieferant zu nutzen. Neben Ammonium und Wasserstoff ist Schwefel eine mögliche Energieressource für die lichtunabhängige bakterielle Kohlenstofffixierung. Die Forschungsfrage beruht darauf, zu klären, ob Schwefel während des Prozesses der Kohlenstofffixierung über den Calvin-Bassham-Bason (CBB)-Kreislauf eine mögliche Energiequelle ist, und zwar insbesondere während des lichtunabhängigen Schrittes der Fixierung.

Das Schlüsselenzym bei diesem Vorgang ist die Ribulose-1,5-Bisphosphat-Carboxylase / -Oxygenase (RubisCO) in Form I und II, kodiert von *cbb*L- beziehungsweise *cbb*M-Genen. Das Gen *apr*A, die Alpha-Einheit der Adenosine 5’-Phosphosulfat Reduktase, ist in der Lage Schwefel zu reduzieren oder zu oxidieren. Untersucht wird der Anteil der autotrophen Gene *apr*A und *cbb*M in der gesamten Wassersäule.

Im Rahmen der Arbeit wurden Wasserproben analysiert, die zwischen dem 64. nördlichen und dem 50. südlichen Breitengrad vom Epipelagial bis zum Bathypelagial im offenen Atlantik genommen wurden. Untersucht wurde, ob die oben genannte Energiegewinnung für die Chemosynthese im Fall der vorliegenden Wasserproben zutrifft. Die Proben wurden mithilfe eines CTD - („conductivity-temperature-depth“) Rosetten-Sammlers eingeholt, der mit 25 Liter Niskinflaschen, sowie mit Sensoren für Chlorophyllfluoreszenz, Strömung und Sauerstoff ausgestattet ist. Die Proben (2 - 10 Liter) für die DNA-Analysen wurden an 51 Stationen gezogen. Dabei wurden jeweils Meerwasserproben aus sechs bis acht unterschiedlichen Tiefen entnommen, die durch Polykarbonatfilter mit einer Porengröße von 0,2 µm gefiltert und anschließend sofort in flüssigem molekularen Stickstoff (N_2) eingefroren und bei -80°C bis zur weiteren Untersuchung gelagert wurden.

Methodisch stützt sich die Arbeit auf Polymerasekettenreaktionen (PCR) und quantitative Polymerasekettenreaktionen (q-PCR). Letztere ist eine Hochdurchsatzmethode, die dazu

dient, quantitative Aussagen über die Häufigkeit der Gene, in unserem Fall *cbb*M und *apr*A, zu erstellen. Klonieren und anschließende Sanger-Sequenzierung dienen der Feststellung der Phylogenie und Biodiversität. Mithilfe von „ocean data view“ werden die Daten in ihrer Häufigkeit und Tiefenverteilung graphisch dargestellt.

Die Ergebnisse zeigen, dass im gesamten Atlantik die beiden Gene *cbb*M und *apr*A ihr Maximum im Mesopelagial, welches sich mit den sauerstoffarmen Schichten deckt, und dem oberen Bathypelagial aufweisen. Außerdem ist der Anteil der Gene für Autotrophie in oligotrophen Regionen (nord- und südatlantischer „Gyre“ und westtropischer Atlantik) höher im Vergleich zu den weiter nördlich und südlich gelegenen Breitengraden.

Ein gemeinsames Vorkommen der *cbb*M- und *apr*A-Gene in denselben Bakteriengruppen, der Gammaproteobakterien und Deltaproteobakterien unterstützt die Annahme, dass Bakterien das Potential besitzen, Schwefeloxidation als Energieressource zu nutzen, um Kohlenstoffdioxid über den CBB-Kreislauf zu fixieren. Somit wird die Annahme unterstützt, dass die DIC-Fixierung durch chemoautotrophe Mikroorganismen in der Tiefsee eine mögliche alternative Ressource von organischem Kohlenstoff darstellt, speziell in oligotrophen „gyral“ und tropischen Regionen.

Curriculum vitae

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Education

2011-2014	MSc curriculum Ecology with specialization in “Marine Biology“ at the University of Vienna
2007-2011	Bachelor study “Biology” at the University of Vienna
1999-2007	Secondary School in Neunkirchen/Lower Austria

Marine courses

2012	Marine biological field course on the Mediterranean fauna and flora; Centre for Marine Research (Rovinj, Croatia)
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