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Abstract

Hydrostatic pressure is one of the important physical parameters affecting microbial life in the deep ocean. However, most studies measuring deep-sea microbial biomass synthesis are performed under atmospheric pressure conditions. In order to measure microbial activity *in situ* under hydrostatic pressure, an *in situ* microbial incubator was used during several research cruises in the Atlantic and the Indian sector of the Southern Ocean. Samples were taken from the mesopelagic (400m, 750m) and bathypelagic (1500m, 3500m, 4002m) layers of the water column. Catalyzed reporter deposition fluorescence *in situ* hybridization combined with microautoradiography was used to determine the microbial abundance and prokaryotic activity. Higher metabolic activities at atmospheric pressure conditions than under *in situ* pressure conditions were measured. SAR406 and *Alteromonas* showed a piezosensitive response. In contrast, the SAR202 cluster showed a piezotolerant response at all depths. The SAR11 cluster exhibited a piezotolerant response in mesopelagic layers while Thaumarchaeota are piezotolerant. Overall, piezophilic, piezotolerant and piezosensitive responses were determined at different depths for different taxonomic groups. The majority of the microbial community, however, appears to be piezotolerant.

Abstrakt

Der hydrostatische Druck ist einer der wichtigsten physikalischen Parameter, die das mikrobielle Leben in der Tiefsee beeinflussen. Die meisten Studien zur Messung der mikrobiellen Biomassenproduktion in der Tiefsee werden jedoch unter Atmosphärendruckbedingungen durchgeführt. Um die mikrobielle Aktivität an Ort und Stelle unter dem jeweiligen hydrostatischen Druck zu messen, wurde während mehrerer Forschungsfahrten im Atlantik und im indischen Sektor des Südlichen Ozeans ein mikrobieller In-situ-Inkubator verwendet. Die Proben wurden aus verschiedenen Tiefen entnommen, z.B. mesopelagisch (400m, 750m), bathypelagisch (1500m, 3500m, 4002m). Zur Bestimmung der Abundanz und der heterotrophen Aktivität der Prokaryoten wurde die Fluoreszenz-in-situ-Hybridisierung mit katalysierter Reporterdeposition in Kombination mit Mikroautoradiographie eingesetzt. Bei der Inkubation unter Atmosphärendruck wurden höhere Stoffwechselaktivitäten beobachtet als unter In-situ-Druckbedingungen. Wir konnten feststellen, dass SAR406 und einige der *Alteromonas* Taxa piezosensitive Eigenschaften aufwiesen. Die Gruppe SAR202 zeigte piezotolerante Eigenschaften in allen Tiefen und unter allen Versuchsbedingungen, während die Gruppe SAR11 piezotolerante Eigenschaften in mesopelagischen Schichten zeigte. Thaumarchaeota könnte piezotolerant sein. Insgesamt wurden die piezophilen, piezotoleranten und piezosensitiven Eigenschaften in verschiedenen Tiefen für verschiedene taxonomische Gruppen bestimmt. Die Mehrheit der mikrobiellen Gemeinschaft scheint jedoch piezotolerant zu sein.

INTRODUCTION

Characteristics of the deep sea

The dark ocean (>200 m depth) is subdivided into the mesopelagic (200–1000 m), bathypelagic (1000–4000 m), abyssopelagic (4000–6000 m) and the hadal zone (>6000 m) (Lauro Bartlett, 2008). This dark ocean is remarkably different from the epipelagic (0–200 m) ocean because of its low temperature and high concentration of inorganic nutrients (Aristegui *et al.*, 2009; Lauro & Bartlett, 2008). The waters of the meso- and bathypelagic realm comprising 82% of the total volume of the ocean (ETOPO1 Global Relief Model) are the largest but least studied realms of the ocean (Aristegui *et al.*, 2009; Nagata *et al.*, 2010; Teira *et al.*, 2004). The mesopelagic zone is confined between the seasonal and permanent thermocline with a residence time of decades (Aristegui *et al.*, 2009). In the bathypelagic and the abyssal zone, water mass residence time reaches up to thousands of years (Aristegui *et al.*, 2009). Sixty-two percent of all the water mass in the ocean has a temperature of about 2°C and faces a pressure of > 10 MPa (Jannasch and Taylor, 1984). In this dark environment, the most unique physical feature besides the low temperature is the high hydrostatic pressure caused by the weight of the overlying water column (Picard and Daniel, 2013).

Prokaryotic community composition in the dark ocean

Studies have shown that the prokaryotic community composition is depth stratified in the ocean water column (DeLong *et al.*, 2006). Marine prokaryotes are mainly composed of bacteria and the relative abundance of archaea increases with depth (Teira *et al.*, 2006; Dobal-Amador *et al.*, 2016; Lekunberri *et al.*, 2013; Nagata *et al.*, 2010). The archaeal abundance in the bathypelagic waters varies between 10–50 % of total prokaryotic abundance in the North Atlantic (Herndl *et al.*, 2005). Alphaproteobacteria, Gammaproteobacteria, Actinobacteria and Thaumarchaeota (Marine Group I) are typically the most abundant prokaryotic groups in the deep sea (Aristegui *et al.*, 2009). Herndl *et al.* (2005) found that Euryarchaeota were less abundant than Thaumarchaeota from the meso- to bathypelagic layers in the North Atlantic Ocean. Some lineages of Gammaproteobacteria (*Alteromonas*, *Pseudoalteromonas*, *Colwellia*) increase with depth in the North Atlantic (Agogu   *et al.*, 2011). SAR202 showed high relative abundance in the dark ocean which can be up to 66 % more abundant than in surface waters

(Morris *et al.*, 2004; Yilmaz *et al.*, 2016). The SAR11 clade, a member of Alphaproteobacteria, can reach up to 25 % of total prokaryotic abundance in the mesopelagic layers (Morris *et al.*, 2002). SAR406 clade, termed also as phylum Marinimicrobia (Rinke *et al.*, 2013), is more abundant in mesopelagic than epipelagic layers, apparently thriving in oxygen minimum zones (Yilmaz *et al.*, 2016) and playing an important role in sulfur cycling in the marine environment (Wright *et al.*, 2014).

Role of hydrostatic pressure on marine prokaryotes

ZoBell and Johnson (1949) were the pioneers of studying of the effect of hydrostatic pressure on microbial activity using pure cultures. They used the term “barophilic” to define the optimal growth higher than 0.1 MPa. However, this term has been replaced by Yayanos (1995) to “piezophilic” (“piezo” means pressure in Greek) (Tamburini *et al.*, 2013). Piezophiles are defined as pressure loving organisms which have an optimal growth rate and activity under hydrostatic pressure higher than atmospheric pressure conditions (Bartlett, 2008; Kato *et al.*, 2008). Microbes growing at similar rates under high hydrostatic and atmospheric pressure conditions are coined “piezotolerant”, and those growing best under atmospheric pressure condition are “piezosensitive” (Fang *et al.*, 2010; Kato, 2011; Tamburini *et al.*, 2009). Culture-based studies revealed microbial adaptations to high hydrostatic pressure such as membrane lipids (Yayanos, 1995), membrane proteins (Bartlett, 1999), and enzymes (Kato *et al.*, 2008). Hydrostatic pressure is considered an important parameter controlling microbial distribution and activity in the dark ocean (Bartlett, 2008; Aristegui *et al.*, 2009; Fang *et al.*, 2010; Jannasch and Wirsen, 1982; Lauro and Bartlett, 2008; Picard and Daniel, 2013; Tamburini *et al.*, 2009).

Effects of hydrostatic pressure on the activity of deep-sea natural prokaryotic communities have been studied for more than half a century (Tamburini *et al.*, 2013). In contrast to the early work by Jannasch and Wirsen (1973), recent studies indicate higher growth rates and metabolic activities of deep-sea microbes under *in situ* hydrostatic pressure than atmospheric pressure conditions (Tamburini, 2006). However, because of methodological challenges, the available data from the typically cold deep-sea environment (~2°C) are still scarce. Consequently, a firm conclusion of the effect of hydrostatic pressure on deep-sea prokaryotes in the global ocean has not been reached yet. Nevertheless, with a

combination of different and innovative approaches it is possible to arrive at a better understanding of the metabolic activity of the deep-sea microbial community under *in situ* hydrostatic pressure conditions now (Tamburini, 2006).

Previously, deep-sea prokaryotic communities have been considered as mostly inactive. In fact, prokaryotic activity measured under atmospheric pressure conditions generally decreases by two orders of magnitude from the euphotic to the bathypelagic zone (Jannasch and Wirsen, 1982). In the oligotrophic tropical Atlantic, prokaryotic heterotrophic activity decreased even by three orders of magnitude from the subsurface to the layers below 6000 m depth (Reinthal *et al.*, 2006; Lekunberri *et al.*, 2013). However, recent studies indicated that deep-sea heterotrophic microbes on a per-cell level are as active as epipelagic microbes (Teira *et al.*, 2006; Gasol *et al.*, 2009). Taken together, the vast majority of the early studies on deep sea microbial activity has been performed under atmospheric pressure conditions (Jannasch and Wirsen, 1982; Yayanos, 1986). Conflicting results have been reported on the influence of hydrostatic pressure on deep-sea microbes.

Thus, the aim of this study is to resolve the current enigma on the response of the deep-sea microbial community to hydrostatic pressure, hence resolving the conflicting results reported in the literature. Specifically, the following research questions are addressed: 1) How active are dark ocean heterotrophic prokaryotes at *in situ* hydrostatic pressure conditions as compared to atmospheric pressure conditions? 2) What is the fraction of piezophilic, piezotolerant and piezosensitive prokaryotes in the deep sea prokaryotic communities?

To answer these questions, we conducted incubations under *in situ* and atmospheric pressure conditions and analyzed prokaryotic activity on a single-cell level using catalyzed reporter deposition fluorescence *in situ* hybridization combined with microautoradiography (MICRO-CARD-FISH). CARD-FISH is a method to detect a specific taxonomic group of microbes (Pernthaler *et al.*, 2002). A combination of microautoradiography with CARD-FISH provides quantitative information on the substrate utilization in natural communities (Cottrell and Kirchman, 2003; Sintes and Herndl, 2006). Quantifying the halo area of the silver grains around a cell allows determining the single-cell activity levels (Sintes and Herndl, 2006).

MATERIALS AND METHODS

In situ incubations were performed with an *In situ* Microbial Incubator (ISMI). The ISMI is a device collecting seawater from defined depths in the water column and incubate and fix microbes under *in situ* conditions. Therefore, it allows measuring *in situ* microbial activity. As shown in Fig. 1–4, the ISMI consists of two major parts. The first part includes the incubation bottles which contains the radiolabeled substrate and the second part comprises the fixing bottles containing the fixative to stop the metabolic activity of the microbes at the end of the incubation. Transfer of sample seawater is carried out by peristaltic pumps via a controller system powered by a battery unit. Additionally, a rosette is responsible for the distribution of the water into the sampling bottles and also for the rinsing of the bottles between the samplings. These units are attached to the frame of the ISMI device. There are two sizes of the cylindrical bottles, 250 mL and 500 mL (Stefanschitz, 2018).

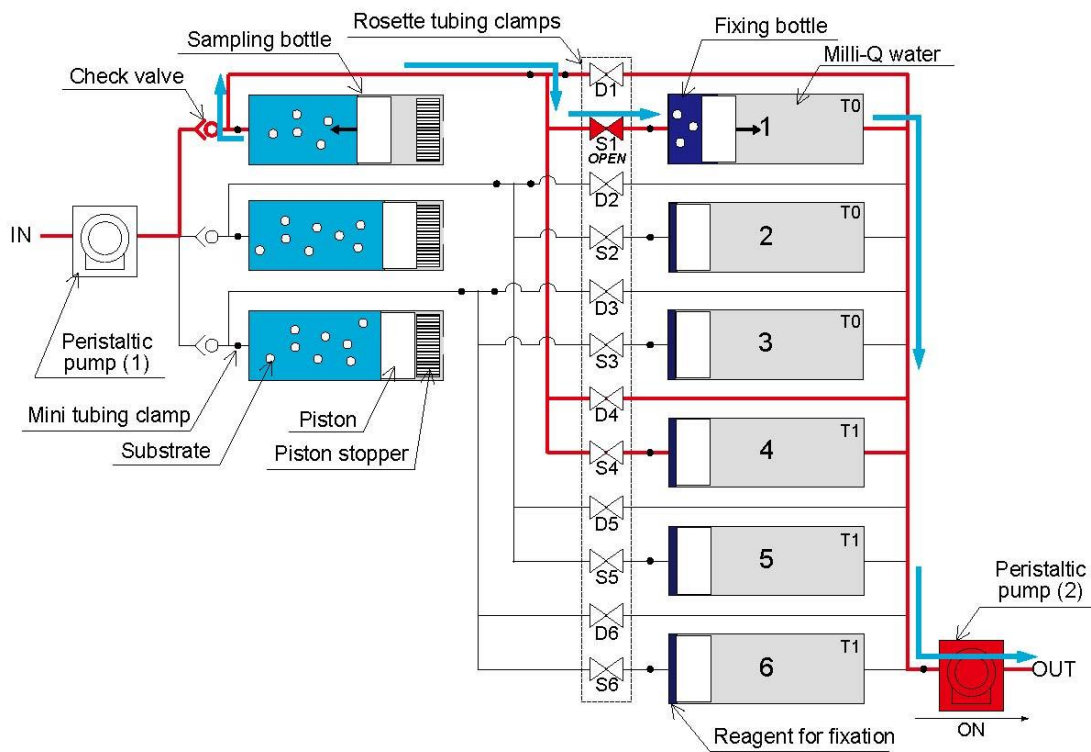


Fig 1. Scheme of the ISMI. After sampling, the seawater mixes with the H^3 -labelled leucine in the incubation flasks. The control incubation flasks are then transferred to the fixation flasks. T_0 indicates the beginning of the incubations and T_1 indicates the end of the incubations. The valves provide a one-way flow of the samples, tubings were filled with Milli-Q water to compensate the hydrostatic pressure.

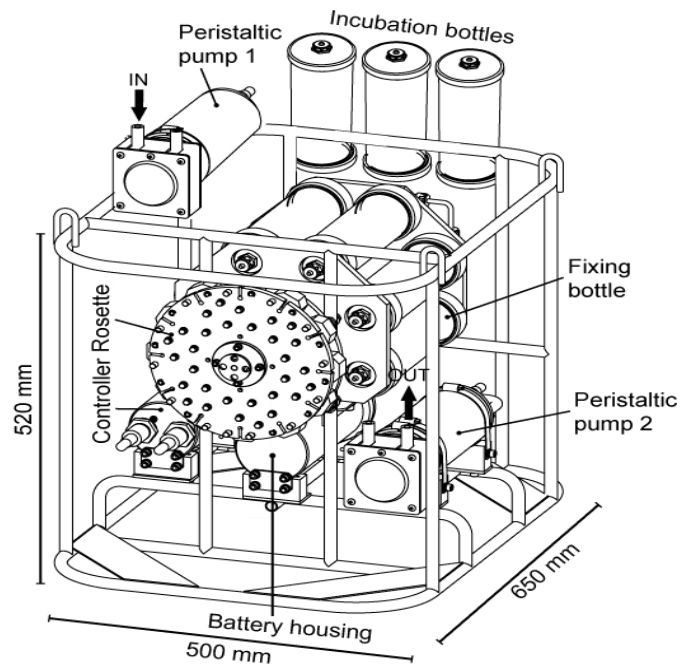


Fig 2. ISMI setup. Essential parts of the ISMI device are the peristaltic pumps, fixing flasks, incubation flasks, controlling unit, rosette and battery compartments.



Fig 3. ISMI actual setup used during the POSEIDON research cruise.

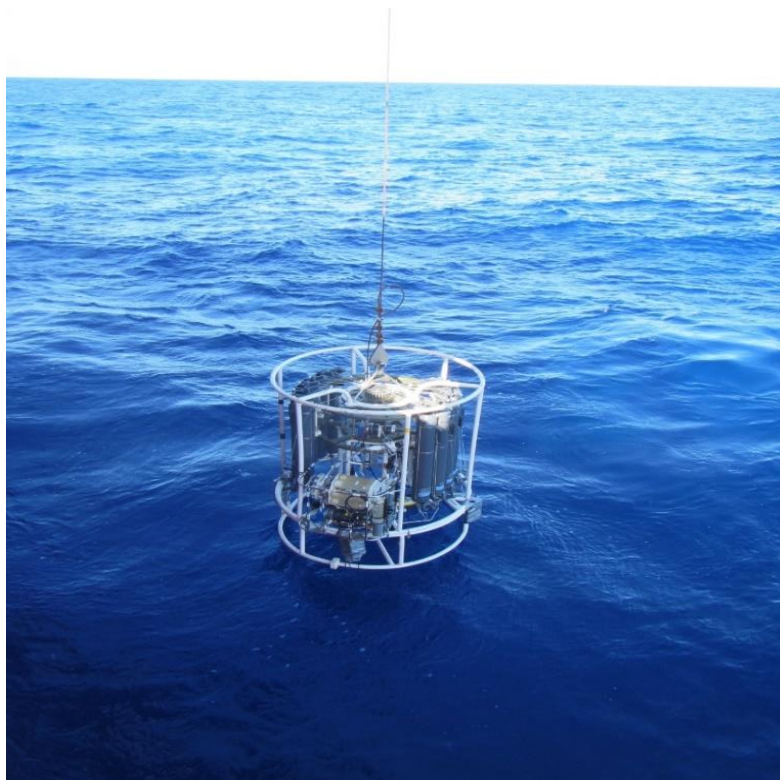


Fig. 4 Deployment of the CTD (Conductivity, temperature, depth rosette sampler with the Niskin bottles and the ISMI.

Cruise	Area	Station	Date	Latitude (°)	Longitude (°)
MOBYDICK	Southern Ocean	M2_2	6-Mar-2018	50,62514 S	72,0137 E
MOBYDICK	Southern Ocean	M3_3	18-Mar-2018	50,68773 S	68,0664 E
RadProf18	North Atlantic Ocean	C3	27-Aug-2018	43,7668 N	6,1672 W
RadProf18	North Atlantic Ocean	111	24-Aug-2018	42,9998 N	14,0350 W
POSEIDON	Atlantic Ocean	27	31-Mar-2019	23,79352 N	23,9188 W

Table 1. Names of the research cruises, geographic region, station numbers, dates, and exact positions where samples were taken for this study.

The samples analyzed in this study were taken from the research cruises MobyDick (stations M2_2 and M3_3), RadProf18 (Stations 111 and C3) and POSEIDON (St 27). Details on stations sampled are given in Table 1, Fig. 5 and 6.

The incubation flasks were washed with 0.5 N HCl and Milli-Q water and rinsed with 0.2 μm filtered seawater collected at a previous station at a similar depth prior to deploying

the ISMI device to the pre-determined depth. The incubation flasks contained ^3H -leucine with a final concentration of 5 nM and the fixing bottle formaldehyde with a final concentration of 2%. Sampling was done at specific depths (400 m, 750 m, 1500 m, 3500 m and 4002 m) with the specific volume of water (approximately 300–500 ml) in duplicate or triplicate. First, the water taken as sample was allowed to mix with the ^3H -leucine. Incubation time ranged between 7.2 to 8 h. Thereafter, the incubated sample was pumped into the fixation bottles where the samples were fixed with formaldehyde (2 % final conc.).

To compare samples incubated under *in situ* pressure conditions with that incubated under atmospheric pressure conditions on deck of the respective research vessel, samples were collected from the same station and depth as the ISMI was deployed by Niskin bottles mounted in a CTD frame. The atmospheric pressure samples were preserved in the dark and cold while ISMI samples were kept at the sampling depth for the entire incubation period.

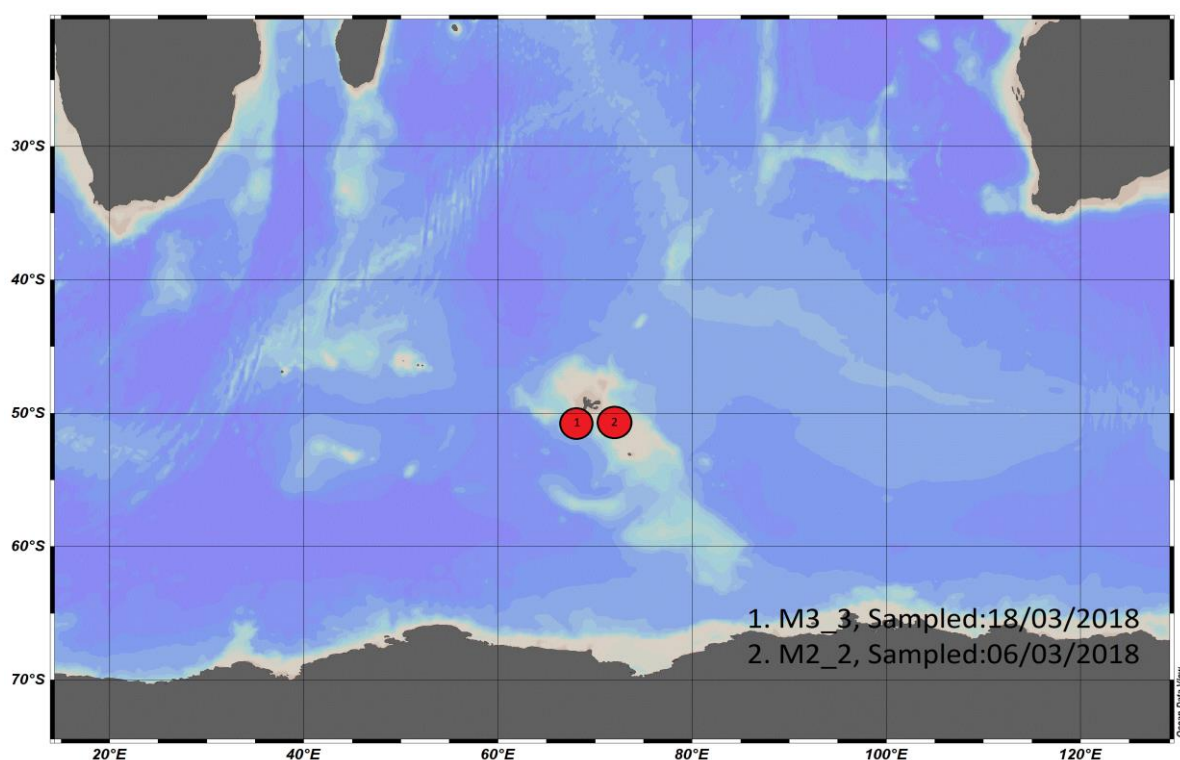


Fig 5. Sampling stations in the Southern Ocean. Samples were collected at Station M2_2 and M3_3 (in red) during MOBYDICK cruise in March, 2018.

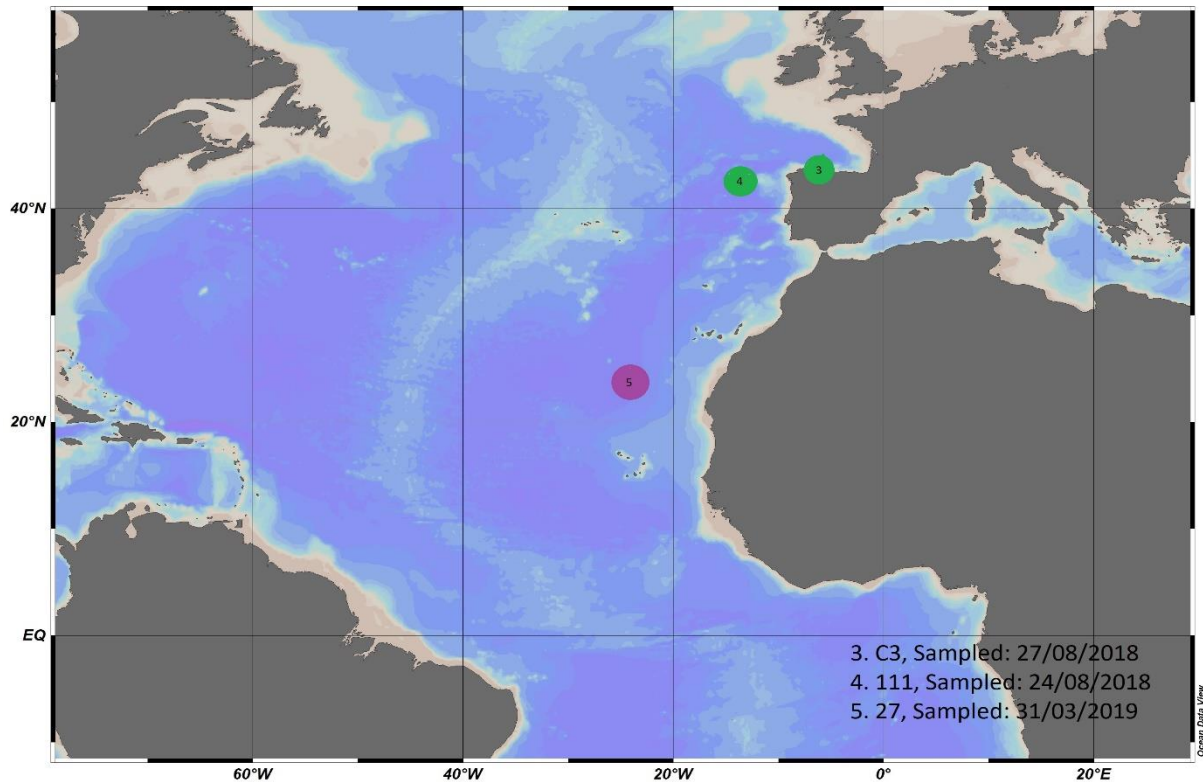


Fig 6. Sampling stations in the Atlantic Ocean. Samples were collected at Station C3 and 111 (in green) during the RadProf18 cruise in August, 2018. Samples taken from Station 27 (in purple) during the POSEIDON cruise in March, 2019.

Sample processing

The samples collected by the ISMI were filtered onto polycarbonate filters (0.2 μm pore size) for MICRO-CARD-FISH and for bulk leucine incorporation measurements on board. MICRO-CARD-FISH filters were stored at -20°C for later process. Incubations of samples under atmospheric pressure were placed in the same type of incubation bottles as used for the ISMI with seawater collected by Niskin bottles. These incubations were done in the dark at the same temperature as measured at the depth of ISMI deployment. To determine the abundance and single-cell activity of bacteria and archaea, MICRO-CARD-FISH was performed according to the protocol of the Microbial Oceanography lab (CARD-FISH and Microautoradiography Protocol by Gerhard J. Herndl Lab, 2018). A brief description is provided below.

CARD-FISH followed by microautoradiography (MICRO-CARD-FISH)

Generally, a modified procedure described in detail in Varela et al. (2008) was followed. For the CARD-FISH approach, low melting point agarose (0.1%) was heated up and 50–55 µl were transferred to a petri dish. The sample filter was placed sample-side down on the agarose and left in the hybridization oven at 37°C for 10-15 min. After taking out the sample from the hybridization oven, 96% ethanol was added to the petri dish to remobilize the filter under sterile conditions. Then, the filter was air dried. The whole filter was cut into 12 pie-shaped sections.

For the permeabilization of bacteria, the filter sections were placed sample-side down into a permeabilization mix (100 mg of lysosome, 1 ml of 1 M Tris-HCl, 1 ml of 0.5 M EDTA, 8 ml Milli-Q water) in the petri dish and placed in the hybridization oven at 37°C for 1 h. For permeabilization of archaea, the filters were placed in 0.1 M HCl at room temperature for 1 min. After the permeabilization, filters were washed once with Milli-Q water when bacteria were the target group and 3 times for archaea. After the washing step, the filter sections were placed into 0.01 M HCl at room temperature in the dark for 20-25 min, washed with Milli-Q water again, dipped into ethanol and then air-dried.

Filters were hybridized in 300 µl of hybridization buffer with the appropriate amount of oligonucleotide probes (1 µl of 50 ng/µL DNA stock solution). Formaldehyde concentrations in the hybridization buffer were specific for the target microbial groups (see Table 2). Hybridization took place in 0.6 ml vials, wrapped with an aluminum foil to shield off light and subsequently, the vials were placed on the rotor rod of the hybridization oven with a slow turning speed at 35°C for 12–15 h.

Thereafter, the filters were taken out of the vials under the fume hood and transferred into the Greiner tube filled with the washing buffer with a specific NaCl concentration depending on the target group (CARD-FISH and Microautoradiography Protocol by Gerhard J. Herndl Lab, 2018). The Greiner tube was left in the water bath at 37°C for 15 min.

After the washing step, 50 ml of PBS-T mix (50 ml of 1×PBS, 25 µl of 100% Triton X100) was prepared and half of it was poured into a petri dish. The filter sections were left in the petri dish with the PBS-T mix in the dark for 10–15 min. Excess amount of PBS-T was removed by blotting paper and then, the filter sections were placed into 1.6 ml vials filled with the

amplification buffer (20 ml of amplification buffer: 2 g of dextran sulfate, 8 ml of 5 M NaCl, 200 µl of 10% blocking reagent, 11.8 ml of 1×PBS) and placed in the hybridization oven at 46°C for 15 min. Thereafter, the filter sections were placed on a blotting paper to remove excess amplification buffer and then into the remaining PBS-T mix in the dark for 15 min. Finally, the filter sections were washed three times in Milli-Q water, dipped into 96 % ethyl alcohol and air-dried.

For microautoradiography following CARD-FISH, the filter sections were placed gently onto the foam wrapped slides. Meanwhile, ILFORD emulsion (Nuclear Emulsion Type K5, 0.2 µm crystal) was placed into the water bath at 43°C for 1 h. Since the autoradiography approach needs to be performed in total darkness, all the necessary equipment was prepared in advance. Microscope slides were dipped into the melted emulsion and placed on top of a metal sheet which had been placed on ice. The emulsion gets a sticky-solid texture after about 5 min of heating. The slides were taken from the top of the cold metal plate and placed carefully on the filter sections. The emulsion-covered slides on which the filter sections adhere were placed into a light-proof box filled with silica-gel beads in the lid, wrapped with aluminum foil and stored at 4°C for 2 weeks of exposure time.

One day prior to terminating the incubation, the developer and the fixer were prepared in 250 ml glass bottles and stored at 17°C. Developing and fixing was performed in the dark room at 17°C. The slides were taken out from the light proof box in dark, placed into the ILFORD developer solution for 4 min. Then, slides were placed in Milli-Q water for 10 sec and thereafter, into the ILFORD fixer solution for 7 min. Finally, the slides were dipped into Milli-Q water for 3 min. The wet slides were gently wiped with paper tissues and allowed to dry for 20 min. Contours of the filter sections were marked at the backside of the slides and the filter sections were gently peeled off. The filter section was stained with DAPI (4', 6-diamidino-2-phenylindole) mix with 2 µg ml⁻¹ final concentration (40 µl of DAPI 50 µg ml⁻¹, 70µl of 1 x PBS, 140 µl of Vectashield, 750 µl of Citifluor).

The slides were examined under a Carl Zeiss Axio Imager M2 microscope and images of the cells are made and quantified by using Axio Vision software. At least 1000 DAPI-positive cells per filter section were counted from a minimum of ten fields of views until 1000 cells were reached. Furthermore, probe-positive cells were counted and the halo area sizes measured via the Axio Vision software.

Table 2. Oligonucleotide probes used for specific target organisms, the sequence and the formamide concentration used.

Probe	Organisms	Sequence (5' - 3')	FA (%)	Reference
NON338	Negative control Bacteria	ACTCCTACGGGAGGCAGC	55	Wallner <i>et al.</i> (1993)
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	55	Amann <i>et al.</i> (1990)
EUB338-II	Bacteria	GCAGCCACCCGTAGGTGT	55	Daims <i>et al.</i> (1999)
EUB338-III	Bacteria	GCTGCCACCCGTAGGTGT	55	Daims <i>et al.</i> (1999)
SAR11-152	SAR11	ATTAGCACAAGTTTCCTCGTGT	45	Morris <i>et al.</i> (2002)
SAR11-441	SAR11	TACAGTCATTTTCTTCCCCGAC	45	Morris <i>et al.</i> (2002)
SAR11-542	SAR11	TCCGAACTACGCTAGGTC	45	Morris <i>et al.</i> (2002)
SAR11-732	SAR11	GTCAGTAATGATCCAGAAAGYTG	45	Morris <i>et al.</i> (2002)
SAR202-312	SAR202 clade	TGTCTCAGTCCCCCTCTG	55	Morris <i>et al.</i> (2004)
SAR406-97	SAR406 clade	CACCCGTTCCGCCAGTTTA	65	Fuchs <i>et al.</i> (2005)
Alt1413	Alteromonas / Colwellia	TTTGCATCCCACTCCCAT	55	Eilers <i>et al.</i> (2000)
Cren537	Thaumarchaeota Marine Group I	TGACCACTTGAGGTGCTG	20	Teira <i>et al.</i> (2004)
Cren554	Thaumarchaeota Marine Group I	TTAGGCCCAATAATCMTCTT	20	Massana <i>et al.</i> (1997)
Eury806	Euryarchaeota Marine Group II	CACAGCGTTTACACCTAG	20	Teira <i>et al.</i> (2004)
-(FA) :Formamide concentration in CARD-FISH hybridization buffer				
-Hybridization temperature 35°C; washing temperature 37°C				

RESULTS AND DISCUSSION

Abundance of DAPI-stained cells under *in situ* and atmospheric pressure conditions

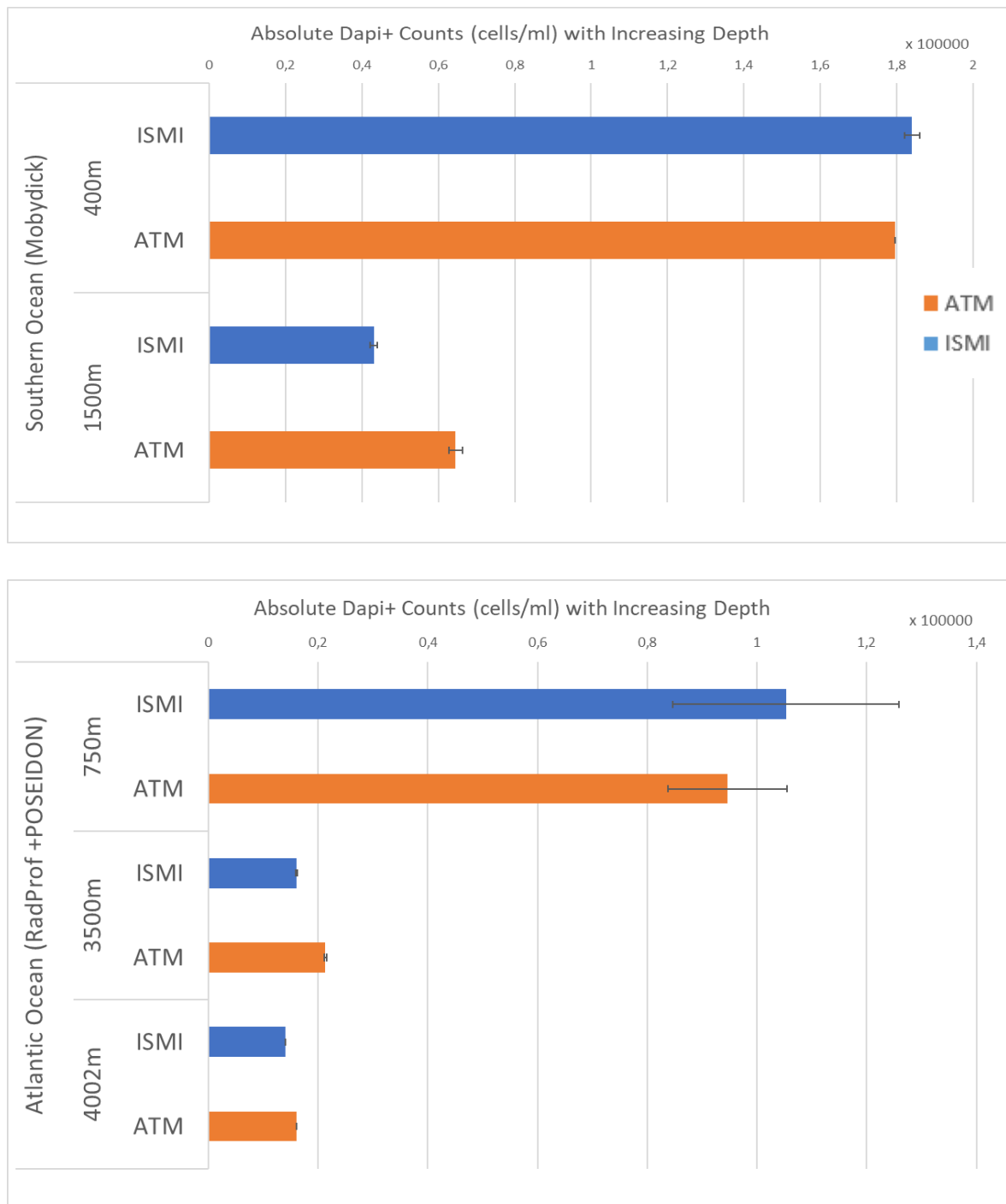


Fig. 7 Total DAPI-stained cell counts (10^5 cells ml^{-1}) at the end of the incubation under either *in situ* (ISMI, pressurized) or atmospheric pressure (ATM, depressurized) conditions. (upper panel) Samples collected from stations M2_2 (400 m) and M3_3 (1500 m) in the Southern

Ocean. (lower panel) Samples collected from stations C3 (750 m), 111 (3500 m), 27 (4002 m) in the Atlantic Ocean.

The abundance of cells was not significantly different in the depressurized and pressurized samples (Fig. 7). Only the prokaryotic abundance at station M3_3 (1500 m) incubated under atmospheric pressure was significantly higher than under in situ pressure conditions (Fig. 7, upper panel). Generally, the prokaryotic abundance decreased with increasing depth in the different oceanic regions as shown previously (Aristegui *et al.*, 2009).

Contribution of selected prokaryotic taxa to total prokaryotic abundance in pressurized and depressurized samples as determined by CARD-FISH

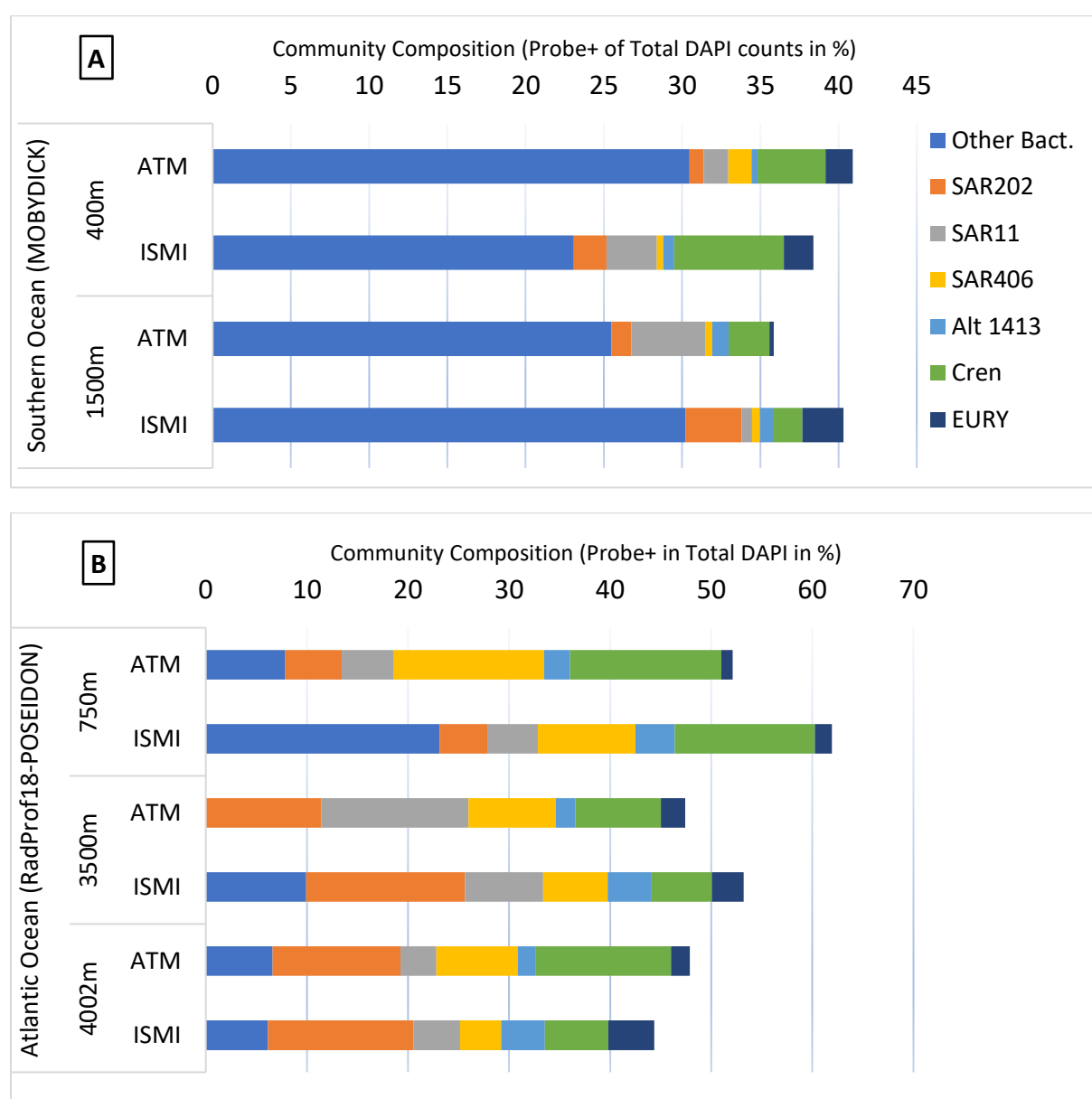


Fig 8. Relative abundance of different prokaryotic taxa at the end of the incubation under *in situ* and depressurized conditions. **(A)** M2_2 and M3_3 stations at 400 m and 1500m, respectively, in the Southern Ocean, **(B)** C3, 111 and 27 stations at 750m, 3500m and 4002 m, respectively, in the Atlantic Ocean. ATM: atmospheric pressure condition (depressurized); ISMI: *in situ* condition (pressurized). Bacteria include SAR202, SAR11, SAR406, *Alteromonas* (Alt1413). Cren: Marine Group I *Thaumarchaeota*; EURY: Marine group II *Euryarchaeota*.

Generally, the prokaryotic community was dominated by bacteria throughout the water column (Fig. 8). However, the relative contribution of bacteria to total prokaryotic abundance was lower than in coastal seas or surface waters (typically 60–90% of DAPI positive cells, De Corte *et al.*, 2013) and the epipelagic waters (70–90%, Schattenhofer *et al.*, 2009). However, previous CARD-FISH based studies in the dark ocean showed that bacteria comprised only $46 \pm 8\%$ of total DAPI counts of the mesopelagic depth of Atlantic (Teira *et al.*, 2005 ; Varela *et al.*, 2008).

The relative abundance of SAR202 ranged between 0.9% to 15.7% increasing with depth (Fig. 8). In the Southern Ocean, the relative abundance of SAR202 was lower than in the Atlantic in both, the meso- and bathypelagic zone. The highest proportion of SAR202 was observed under *in situ* hydrostatic pressure conditions with 15.7% and 14.4% of total DAPI counts at 3500 m and 4002 m, respectively, in the Atlantic (Fig. 8B). Previous studies found that SAR202 contribute about 10 % of total prokaryotic cells in the mesopelagic zone at the stations HOT, BATS and eastern coastal Pacific (Morris *et al.*, 2004) and approximately 6% was observed in the deep of South Atlantic Gyral Provinces (Varela *et al.*, 2008; Schattenhofer *et al.*, 2009). Hence, our results agree with previous studies indicating that SAR202 abundance increases with depth in the water column of the global ocean.

The relative abundance of SAR406 (Marinimicrobia) was higher under atmospheric than at *in situ* pressure conditions, except one station in the Southern Ocean (Fig. 8A). In contrast, the relative abundance of *Alteromonas* was slightly higher at *in situ* than atmospheric pressure conditions except at station M3_3 at 1500 m depth in the Southern Ocean (Fig. 8A). SAR11 is highly abundant in different depth layers of the ocean (Morris *et al.*, 2002). No difference was found between the relative abundance under *in situ* and atmospheric pressure conditions (Fig. 8).

Two probes (Cren537 and Cren554) were used to detect Marine group I Thaumarchaeota. For Marine group II Euryarchaeota, the Eury806 probe was used. In our study, the relative abundance of Thaumarchaeota was 4.3% to 15.6% of total DAPI positive cells. Previous studies showed that thaumarchaeal abundance can reach up to 10–50 % of total prokaryotic abundance in bathypelagic layers (Herndl *et al.*, 2005; Karner *et al.*, 2008). Seasonal dynamics and biogeographic differences might be the reason for the reported variability in the relative abundance of archaea in the oceanic water column (Varela *et al.*, 2008). At our sampling sites, Euryarchaeota contributed 0.3 to 4.6 % to total prokaryotic abundance (Fig. 8). The relative abundance of Thaumarchaeota was substantially higher than that of Euryarchaeota in both, under *in situ* and atmospheric pressure conditions (Fig. 8).

Relative abundance of cells taking up leucine

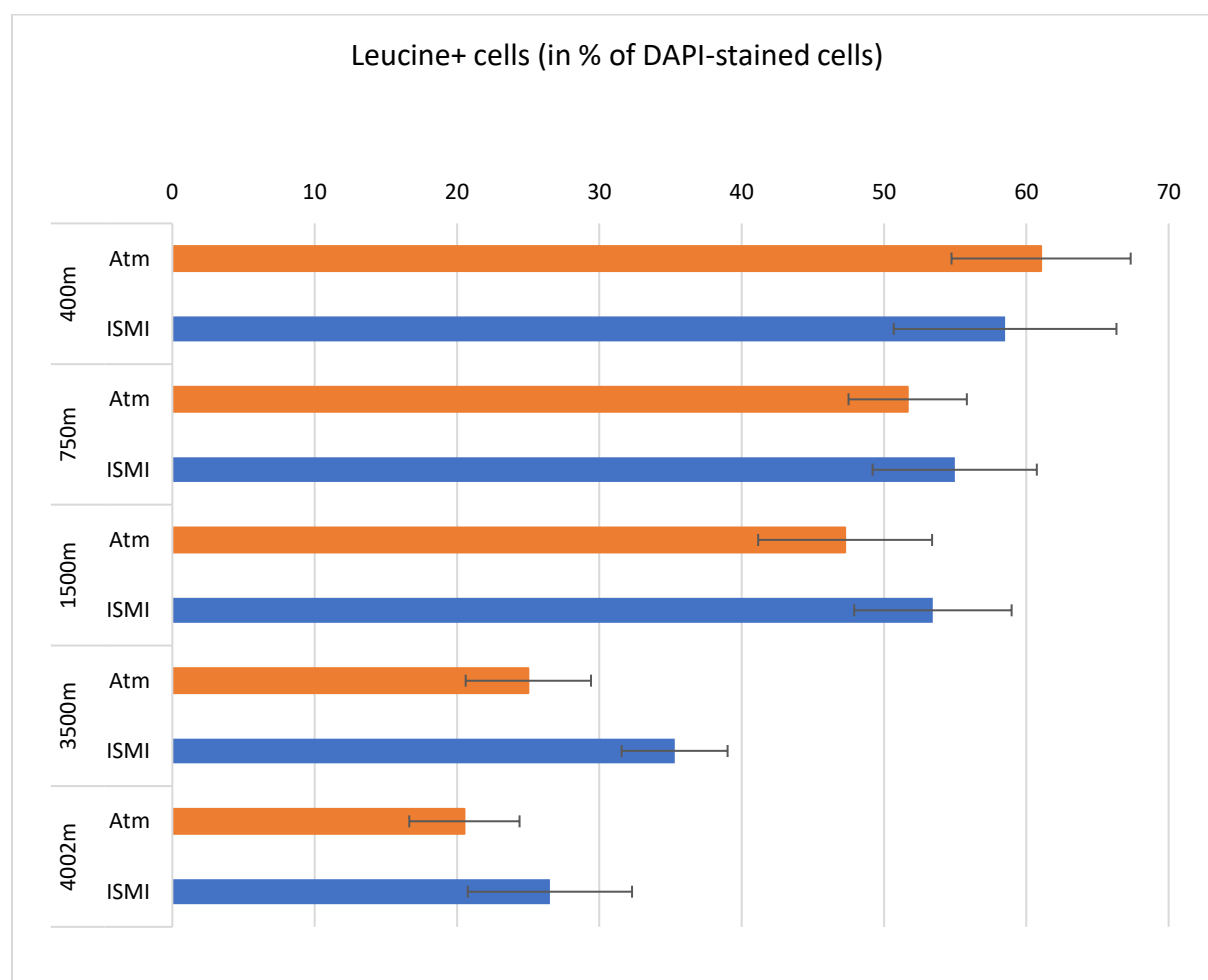


Fig. 9 Percentage of cells taking up leucine of total DAPI-stained cells at the two study sites (Atlantic and Southern Ocean) combined. Orange bars indicate atmospheric pressure conditions (Atm), blue bars *in situ* pressure conditions (ISMI).

Previous studies have shown that the abundance of active heterotrophic prokaryotes decreases with depth (Alderkamp, Sintes and Herndl, 2006; Lauro and Bartlett, 2008; Varela *et al.*, 2008; Tamburini *et al.*, 2013). Our measurements also revealed a decreased contribution of leucine-positive cells with increasing depth (Fig. 9). In the lower bathypelagic layers, a slightly higher contribution of leucine-positive cells was detected under *in situ* pressure than under atmospheric pressure conditions (Fig. 9). At 3500 m depth, the contribution of cells taking up leucine at *in situ* pressure conditions ($35.2 \pm 3.7\%$) was higher than under atmospheric pressure conditions ($25 \pm 4.4\%$), but otherwise, no significant difference in the contribution of leucine-positive cells to the total prokaryotic abundance was observed between *in situ* and atmospheric pressure conditions (Fig. 9).

Cell-specific activity estimated via the halo area size around cells

Cell-specific activities were measured based on the halo area around individual cells and related to the bulk leucine uptake measured in the same sample. The most important step during microautoradiography is the exposure of the cells to the photographic emulsion which causes the formation of a silver grain halo around cells taking up ^3H -leucine. The silver grain area surrounding the cell indicates microbial activity and, by combining with CARD-FISH, the activity of different microbial groups can be calculated on a single-cell level (Sintes and Herndl, 2006). Halo area sizes are expressed as $\mu\text{m}^2 \text{h}^{-1} \text{cell}^{-1}$ and presented as histograms according to the bin sizes (Fig. 10–16). A larger halo area size indicates higher leucine uptake. Bin sizes were defined as $0.2 \mu\text{m}^2 \text{cell}^{-1} \text{h}^{-1}$ and $0.1 \mu\text{m}^2 \text{cell}^{-1} \text{h}^{-1}$ for mesopelagic and for bathypelagic samples, respectively (Fig. 10-16).

Bacteria:

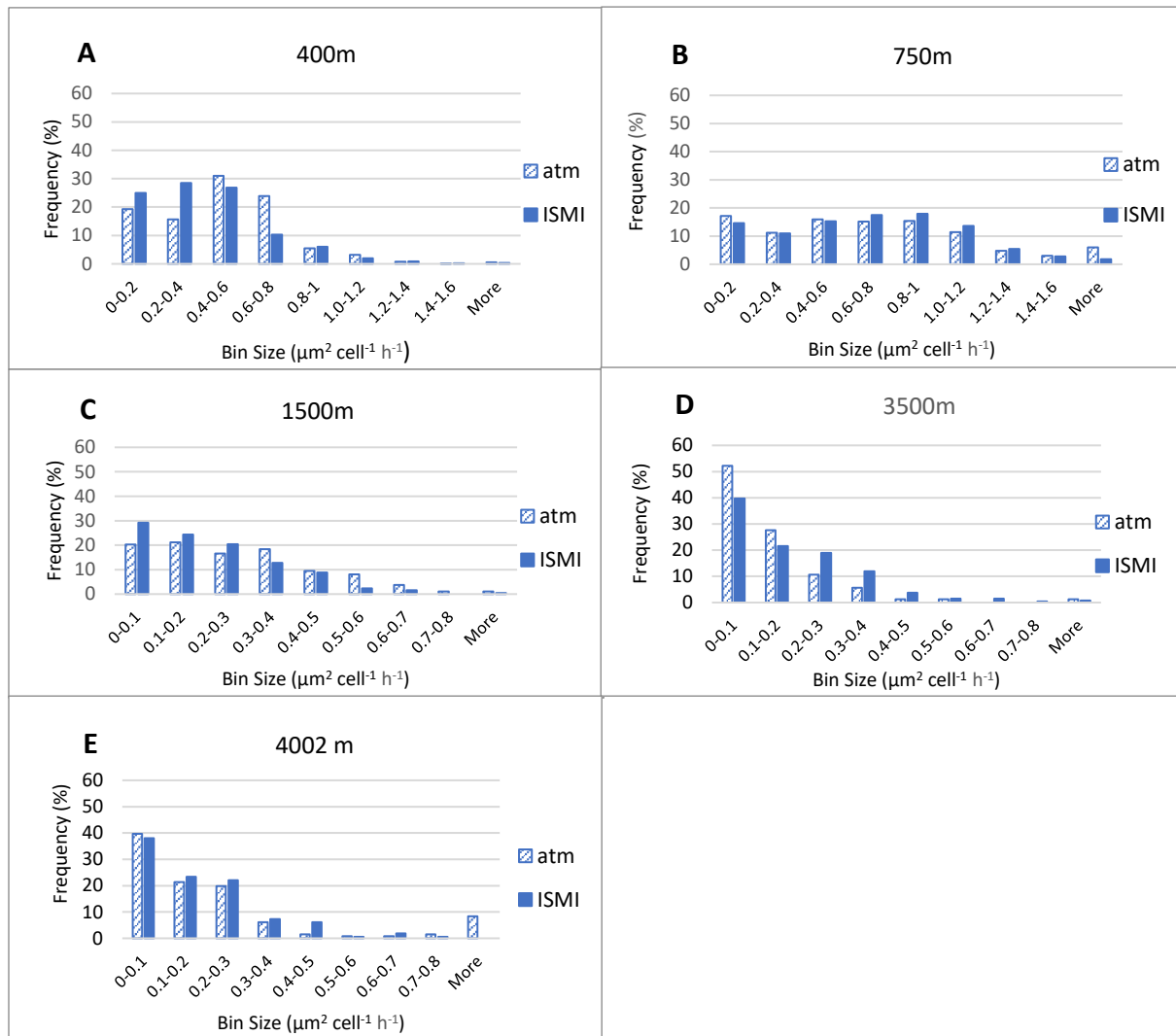


Fig 10. Distribution of the halo area around single bacterial cells taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions determined at station M2_2 and M3_3 of the Southern Ocean (400m and 1500m) and C3, 111, and St.27 (750m, 3500m and 4002m) of the Atlantic Ocean. Solid filled bars indicate single-cell activity under *in situ* pressure and hatched bars indicate single-cell activity under atmospheric pressure conditions.

Bacteria represented 30–45% of the total prokaryotic community (Fig. 8). While the distribution of halo area sizes was generally similar between *in situ* and atmospheric pressure conditions (Fig. 10 A–E), there was an increase in the frequency of active cells under atmospheric pressure conditions. To determine whether different prokaryotic taxa respond differently in their activity to depressurization, we further focused on specific bacterial and archaeal groups.

Marine Group I Thaumarchaeota

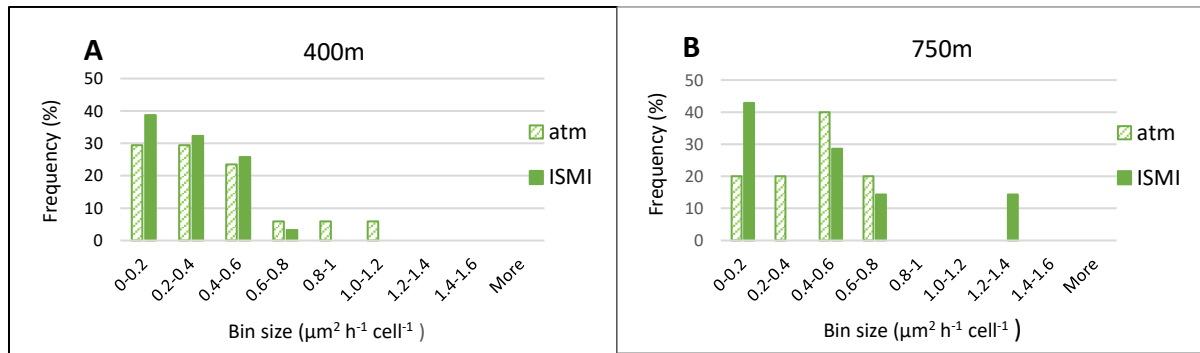


Fig. 11 Distribution of the halo area of Thaumarchaeota taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions determined at (A) station M2_2 of the Southern Ocean (400m) and (B) C3 (750m) of the Atlantic Ocean.. Solid bars indicate *in situ* and hatched bars atmospheric pressure conditions.

In Thaumarchaeota, cells with a high activity level were only found in the incubations under atmospheric pressure conditions at 400 m depth (Fig. 11A). In contrast, thaumarchaeal cells with high activity levels were found in under *in situ* pressure conditions at 750 m in the Atlantic (Fig. 11B). For the other samples, there were not enough leucine-positive thaumarchaeal cells (<10 cells per sample), even though Thaumarchaeota reached up to 15.6% of total prokaryotic abundance (Fig. 8B). Therefore, at the study sites, Thaumarchaeota are not utilizing leucine efficiently. It is well documented that the vast majority of Thaumarchaeota are chemolithoautotrophs (Elling *et al.*, 2017). From the few data we obtained for Thaumarchaeota it would appear that they are piezotolerant, however, definitely more data are required before a firm conclusion can be drawn.

Marine Group II Euryarchaeota

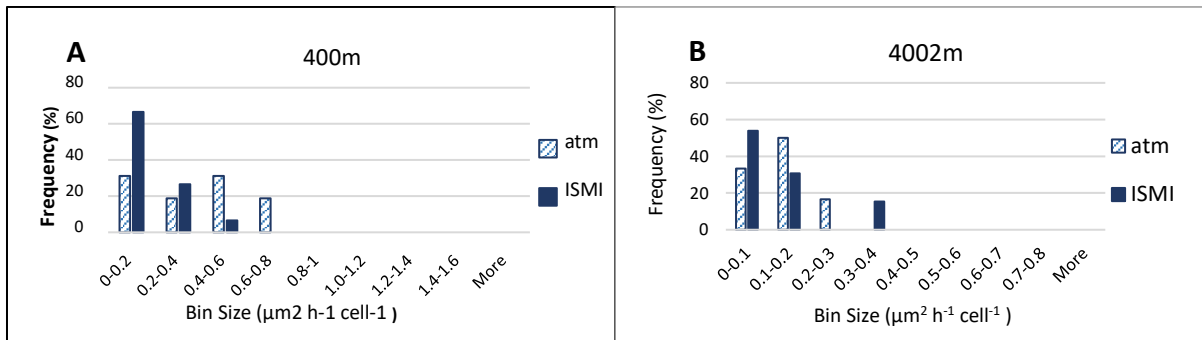


Fig. 12 Distribution of the halo area of Euryarchaeota taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions at (A) station M2_2 of the Southern Ocean (400m) and (B) St.27 (4002m) of the Atlantic Ocean. Solid bars indicate *in situ* and hatched bars atmospheric pressure conditions.

In addition to the low relative abundance of Euryarchaeota (Fig. 8), euryarchaeal cells taking up leucine were low in abundance. The distribution of halo area classes of Euryarchaeota was assessed at two different depths (Fig. 12). In the mesopelagic samples, the frequency of cells taking up leucine was rather high (>60%) in the lower area classes in under *in situ* pressure conditions (Fig. 12A). In the bathypelagic zone, there were more highly active cells under *in situ* pressure conditions (Fig. 12B). In any case, because of the small sample size, we cannot draw firm conclusions on the response of Euryarchaeota to hydrostatic pressure.

Alteromonas

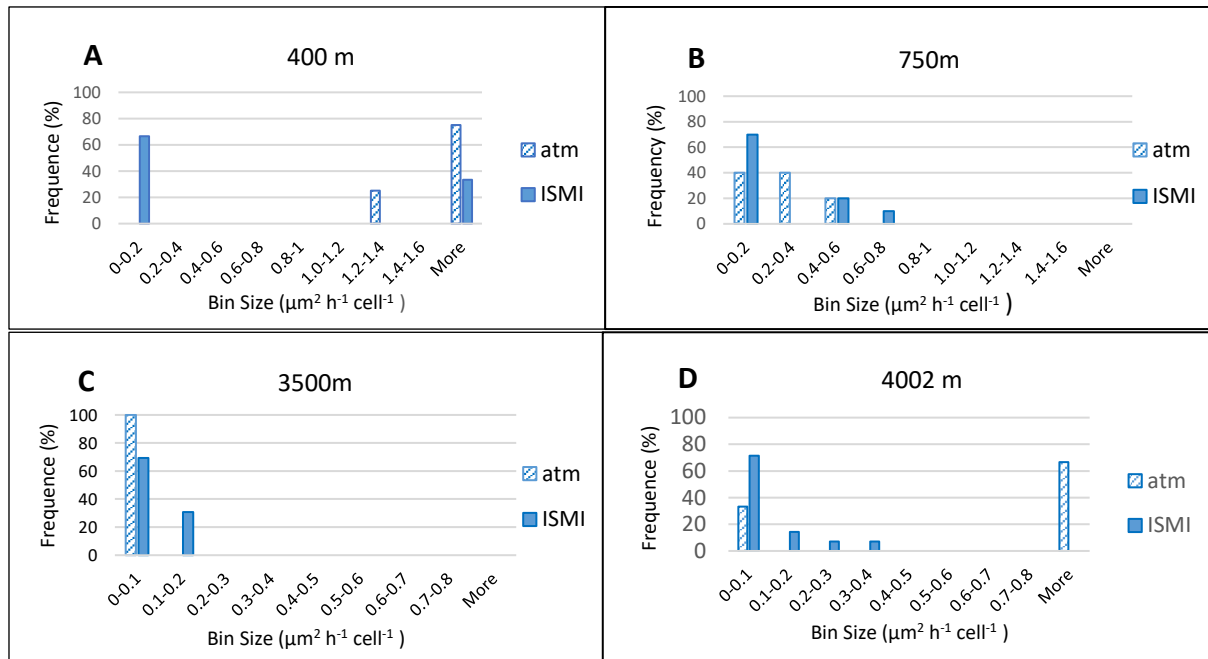


Fig. 13 Distribution of the halo area of *Alteromonas* taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions at (A) M2_2 of the Southern Ocean (400m) and (B) C3, (C) 111, and (D) St.27 of the Atlantic Ocean. Solid filled bars indicate single-cell activity under *in situ* pressure and hatched bars indicate single-cell activity under atmospheric pressure conditions.

At 750 m and 3500 m depth, the distribution of halo area classes was fairly similar between *in situ* and atmospheric pressure conditions (Fig. 13). However, at 400 m and 4002 m, *Alteromonas* cells showed either low ($< 0.4 \mu\text{m}^2 \text{ cell}^{-1} \text{ h}^{-1}$) or high ($> 0.8 \mu\text{m}^2 \text{ cell}^{-1} \text{ h}^{-1}$) activity levels (Fig. 13A, D) and highly active cells were found under atmospheric pressure conditions in *Alteromonas* collected at 400 m and 4002 m depth (Fig. 13A, D). Therefore, it is likely that some members of the *Alteromonas* group are piezosensitive.

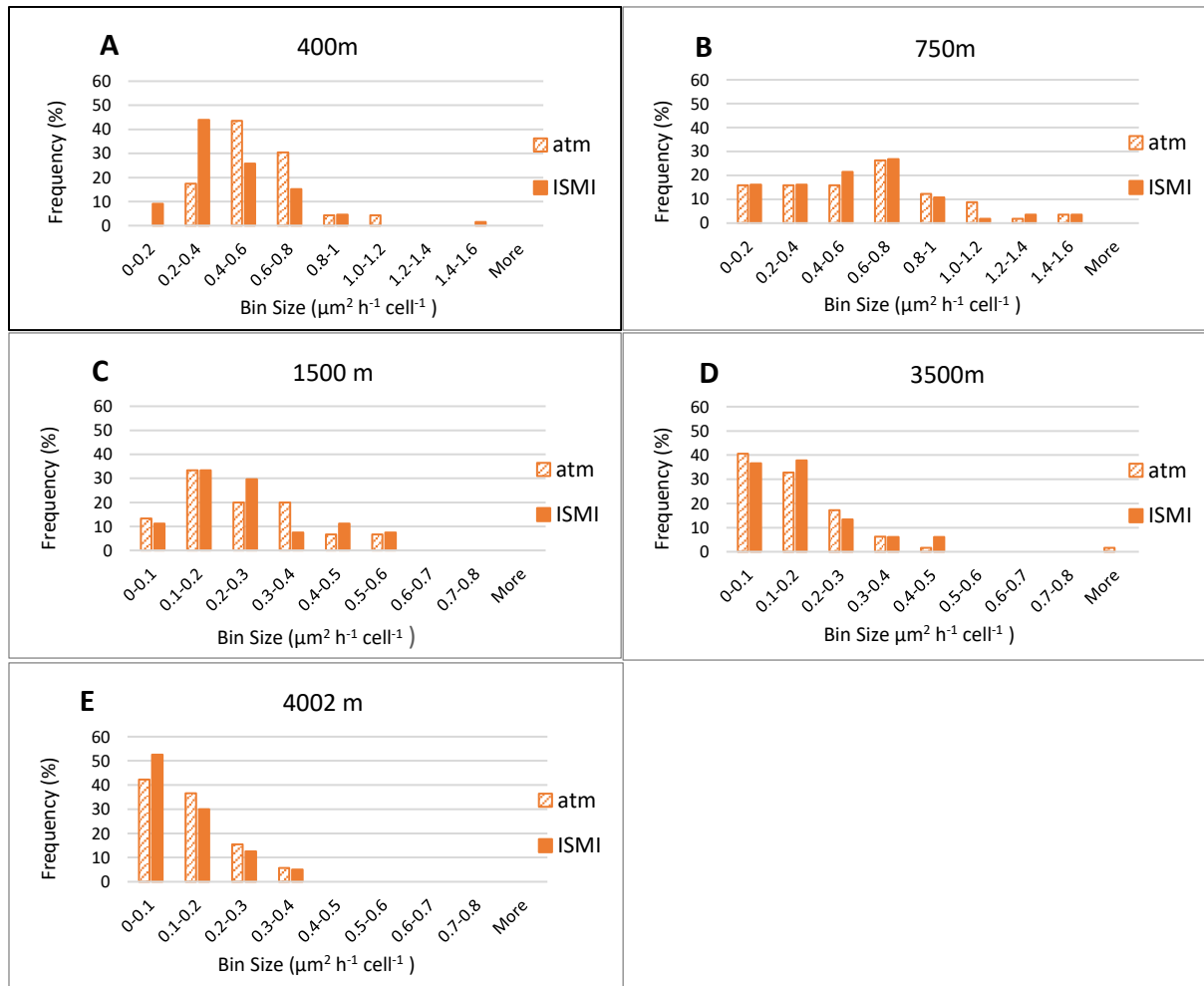


Fig. 14. Distribution of the halo area of SAR202 taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions at station M2_2 and M3_3 of the Southern Ocean (400m and 1500m) and C3, 111, and St.27 (750m, 3500m and 4002m) of the Atlantic Ocean .Solid bars indicate *in situ* and hatched bars atmospheric pressure conditions.

The distribution of the halo area of the SAR202 clade was similar between *in situ* and atmospheric pressure conditions (Fig. 14). Based on this, we conclude that the SAR202 clade is piezotolerant. Previous study suggested SAR202 plays an important role in the DOM oxidization in the deep sea (Landry *et al.*, 2017).

SAR11

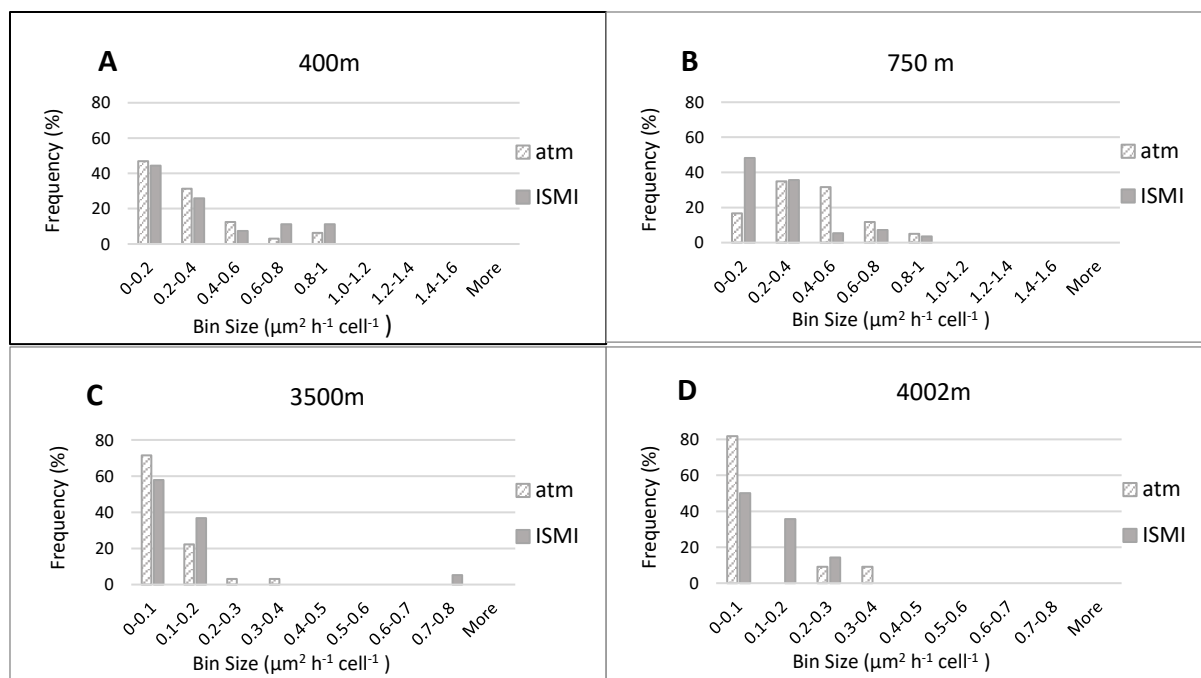


Fig. 15. Distribution of the halo area of SAR11 taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions determined at station M2_2 of the Southern Ocean (400m) and C3, 111, and St.27 (750m, 3500m and 4002m) of the Atlantic Ocean. Solid bars indicate *in situ* and hatched bars atmospheric pressure conditions.

The distribution of the activity levels of SAR11 was similar under *in situ* and atmospheric pressure conditions in both, the meso- and bathypelagic zone (Fig. 15). Relatively high activity levels under atmospheric pressure conditions were found for SAR11 collected at 3500 m and 4002 m depth (Fig. 15C, D). Overall, SAR11 showed piezotolerant characteristics.

SAR406 (*Marinimicrobia*)

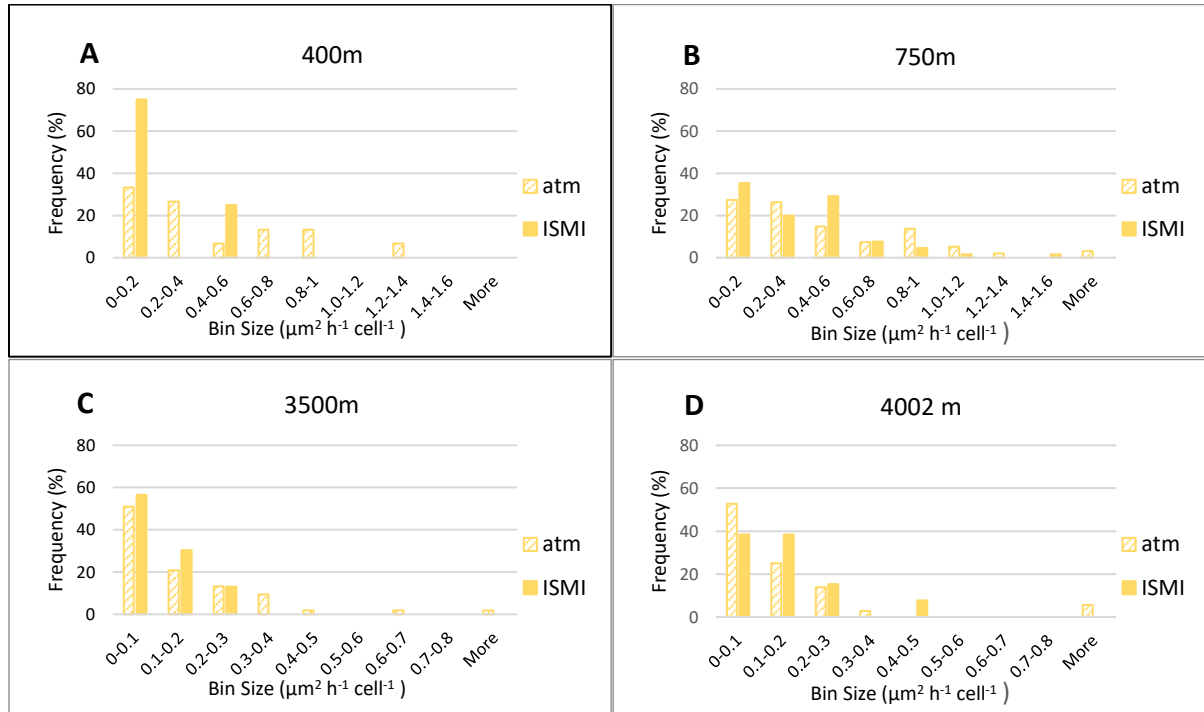


Fig. 16. Distribution of the halo area of SAR406 taking up ^3H -leucine at different depths under *in situ* and atmospheric pressure conditions at stations M2_2 of the Southern Ocean (400m) and C3, 111, and St.27 (750m, 3500m and 4002m) of the Atlantic Ocean. Solid bars indicate *in situ* and hatched bars atmospheric pressure conditions.

The distribution pattern of the single-cell activity indicated that SAR406 exhibits high cell-specific activity particularly under atmospheric pressure conditions (Fig. 16). Samples from 400 m depth at atmospheric pressure conditions exhibited a wide distribution of halo area classes as compared to *in situ* pressure conditions. High single-cell activity was observed under atmospheric pressure conditions on samples collected at 750, 3500 and 4002 m depth (Fig. 16B–D). Thus, we conclude that the SAR406 cluster has a tendency to be piezosensitive. A recent study suggests, however, that the metabolic activity varies among different members of *Marinimicrobia* (Bertagnolli *et al.*, 2017). This might explain also the wide distribution over all activity classes as shown in Fig. 16.

CONCLUSION

Our main goal of this study was to arrive at a better understanding of heterotrophic activity of different deep-sea microbial communities under *in situ* pressure conditions. We conducted *in situ* incubations and compared it to atmospheric pressure incubations. By targeting specific microbial groups representative of deep-sea prokaryotic communities, we observed different pressure responses by different taxonomic groups. SAR406 and some of *Alteromonas* showed piezosensitive characteristics. The SAR202 cluster is considered to be piezotolerant. The SAR11 cluster was piezotolerant in the mesopelagic layers. Heterotrophic Thaumarchaeota may be piezotolerant. Taken together, it appears that the majority of the examined deep-sea microbial taxa are not or only marginally affected by hydrostatic pressure.

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