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Hydrostatic pressure affects physiology and community structure of marine bacteria during settling to 4000 m: an experimental approach

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ABSTRACT: The response of 5 strains of shallow-water microbes to changing hydrostatic pressure was explored in a pressurized microcosm setup, simulating a sinking at 1000 m d^{-1} from surface waters to 4000 m depth in an isothermal ocean. Technology and methods combined a new, computer-controlled pressure laboratory with classical and new genetic tools to evaluate pressure responses of selected bacterial strains. Size, number, growth and species composition were obtained in a time series pattern under regular, non-saturating feeding. Planned as an initial step in a quest for quantifying the role of hydrostatic pressure in observed oceanic microbial distributions, results from this study indicate that selected bacterial strains from the surface respond individually to pressure exposure. A strong physiological response led to reduced bacterial numbers of all strains at 4000 m depth, a result corroborated elsewhere in the literature. Unexpectedly, pressure changes (during sinking) generated depth-specific maxima and minima in number of the selected strains, not seen in the ambient pressure control. A reshuffling of species and changes in sizes of all strains tested occurred in the pressurized treatment. Therefore, time series experiments with non-intrusive sampling features rather than end-member experiments become mandatory to document physiological responses to pressure. Our results, together with recent findings on pressure-related effects on microbial growth and community structure in the deep-sea, indicate that estimates of microbial organic matter turnover in the deep-sea—not taking pressure-related effects into account—need to be revisited.

KEY WORDS: Hydrostatic pressure · Pressure laboratory · Bacteria · Aggregates · Growth · Species composition · CARD-FISH

INTRODUCTION

In the ocean, horizontal and vertical physical processes coupled to variables such as flow, temperature and buoyancy, continuously redistribute dissolved and particulate organic matter. In particular, the biogeochemical significance of macroscopic organic aggregates (marine snow) as vehicles for vertical matter flux is widely acknowledged (Asper et al. 1992, Azam 1998). Particle aggregation and sedimentation remove photosynthetically fixed carbon from the ocean's surface and export it to the deep sea, thus sequestering atmospheric CO_2 on long time scales (Siegenthaler & Sarmiento 1993). On the other hand, aggregates provide important habitats for heterotrophic bacteria and other microorganisms. Grossart et al. (2007) showed that protease activity of attached bacteria was 10 to 20 times higher than that of free-living bacteria and that many bacteria could increase their protease activity within a short time (2 h) upon attachment to surfaces. Hence organic aggregates form hot-spots of microbial activity, leaving an extended plume of DOM behind, large enough to satisfy up to half of the bacterial carbon demand in the open ocean (Kiørboe & Jackson 2001). Thus activities of bacteria on sinking aggregates can play a crucial role in oceanic organic matter

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cycling. Ecosystems respond correspondingly to the ensuing spatial and temporal supply of nutrients (Simon et al. 2002). A key process responsible for ~75% of the surface to deep-sea gradient in dissolved inorganic carbon (DIC) is the biological carbon pump (Volk & Hoffert 1985). The efficiency of the biological pump largely depends on microbial organic matter mineralization and aggregation (as well as sinking). During sinking, organic matter leaves the euphotic zone and enters new water layers, where characterizing parameters also include hydrostatic pressure. While biomass models recognize a vertically distinct pattern and a variety of studies show differing species distributions with depth (e.g. Herndl et al. 2005, Winter et al. 2009), to date it has not been possible to clearly separate pressure effects from other environmental variables.

Although hydrostatic pressure has a major influence on the distribution and activities of bacteria in the marine environment (Marquis & Keller 1975, Tamburini et al. 2002), surprisingly few studies have been performed to identify which biochemical or physiological mechanisms play major roles in determining barotolerance. There exists a wide range of barotolerance from complete inability to grow at pressures as low as 20 MPa to a capacity for slow growth at pressures of about 100 MPa (Oppenheimer & Zobell 1952). Growth rates of deep-sea bacteria vary with pressure and different bacterial species exhibit maximal growth at different pressures (Yayanos 1986). Although Yayanos (1986) has shown that genuine deep-sea bacteria at different depths have several characteristics, presumably evolutionarily derived, distinguishing them from each other, this study has been greatly neglected in the past. Yayanos (1986) even demonstrates that pressure plays a significant role in determining the distribution of marine life and concludes that the role of organisms in biogeochemical cycles needs to be studied under *in situ* conditions, i.e. under ambient pressure. Recently, Tamburini et al. (2002, 2003) have shown that decompression of deep-sea bacteria results in reduced activities and, hence, great underestimations of *in situ* mineralization rates of organic matter. These results are in accordance with findings by Bianchi & Garcin (1994) and Baltar et al. (2009), which suggest that the role of deep-sea bacteria in the carbon cycling through the global ocean is far from negligible.

A dearth of data exists similarly for the fate and transformation of particle-attached, surface-generated bacteria sinking to the sea floor, as well as their role as one of the conduits of the biological pump. Tamburini et al. (2006) present an important example for this process. In contrast to previous studies, we fed the bacteria daily with fresh medium to avoid growth limitation by quantitative and qualitative changes of the growth substrate and to solely focus on pressure-related effects. Our new experimental setup allowed the withdrawal of samples from the pressurized inner chamber without depressurization, which otherwise may affect bacterial growth. In addition to a pure technical test of our experimental setup, we wanted to know if pressure-induced effects on microbial growth and consequences for species distribution for surface-originating bacteria are discernable. We gradually increased the pressure from 0.01 to 40 MPa to mimic natural sinking of microbes attached to particles (1000 m d^{-1}) from surface to depth at 4000 m. This settling speed, while on the high end of the sinking speed spectrum, was measured by various flux studies in sediment traps operating with novel collection cup sampling electronics with a triggering scheme assigning different settling velocities to different collection cups (Trull et al. 2008, Peterson et al. 2005). Our results show that bacteria collected from the ocean's surface are indeed affected by increasing pressure, indicating that pressure is in more than one way an important environmental variable which cannot be neglected when studying organic matter remineralization by marine bacteria attached to sinking aggregates.

MATERIALS AND METHODS

Experimental setup. To test for pressure effects, an experimental approach is needed that separates any pressure-associated effects from those of other environmental variables. Recently, such an approach became available by combining new pressure technology and sample evaluation in a new experimental design. We utilized a pressure laboratory of adequate size (24.5 l volume chamber, Fig. 1), filled with freshwater drawn from an outside water source via a high-pressure pump, permitting computer-controlled steady and unsteady pressure time series (de Jesus Mendes et al. 2007). An internal microcosm of 4 l volume (Gust 1987, Tengberg et al. 2004) was mounted within this pressurized experimental volume, which allowed for gentle agitation to maintain suspension of the bacteria. Inside this microcosm, by turning a stirrer disk in connection with metered central fluid recirculation, calibrated bottom stresses are exerted on the bottom. The ensuing fluid flow and turbulence inside the microcosm water volume (bacterial suspension) at the selected bottom stress setting (expressed here as friction velocity, u^*, 0.2 cm s^{-1}) kept the microbes suspended. A sterile food supply was stored in a compensation bladder with a volume of 2.0 l, connected via tubing to the bottom of the microcosm. The bladder carried sterile seawater spiked with nutrients for the microbes, which entered the experimental microcosm

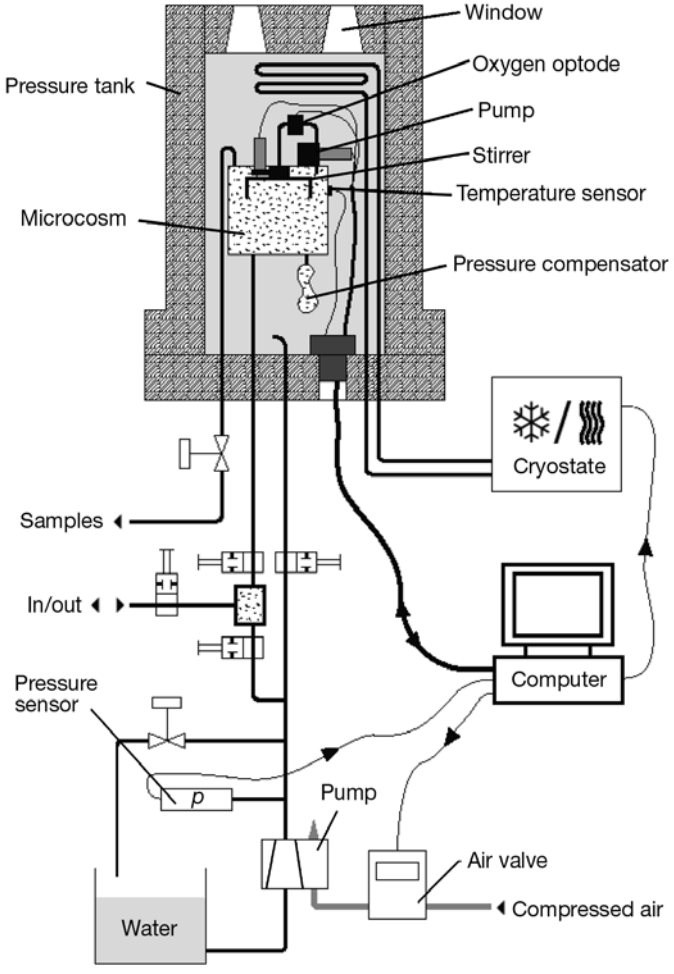

Fig. 1. Schematic view of the pressure setup. The 24.5 l pressure chamber carries an internal microcosm volume of 4 l which allowed for gentle agitation to maintain bacterial suspension. A sterile food supply was stored in a compensation bladder with a volume of 2.0 l. The microcosm and compensation bladder are embedded via rigid and flexible walls in ~18.5 l of freshwater where the pressure is adjusted via the control electronics of the high-pressure pump when samples are taken. This design prevents limitation of bacterial food supply, and tested microbes can be sampled at user-selected time increments without affecting the pressure in the chamber (for further details see 'Materials and methods')

space at the same volume rate only when samples were drawn. This design prevented limitation of bacterial food supply during the experiment (Fig. 1). Tested microbes were sampled at user-selected time increments without affecting the pressure in the 24.5 l chamber and thus the microcosm, since both fluid types, fresh- and saltwater, filled compartments that were separated only by flexible walls (decompression-free sample transfer and activity measurements are under development). The time duration of microbe sampling was approximately 10 s. Fifty ml were drawn to flush the tubing, and a second 50 ml sample was processed. Thus with each sampling, the original experimental volume was reduced by 100 ml and the water surrounding the microcosm and the compensation bladder (see Fig. 1) increased by 100 ml; this volume was pumped into the interior fluid surrounding the microcosm and compensation bladder to keep the total fluid volume of 24.5 l and thus the pressure constant inside the pressure chamber. The laboratory apparatus can be pressurized up to 50 MPa. The experiment was run at room temperature (20.5°C) in 2 microcosm setups: one pressurized (see above) and one kept at atmospheric pressure (control). Hence all settings and parameters in both microcosms were identical, except for the pressure.

The total experiment lasted for 7 d, where during the first 4 d the pressure increased linearly by 10 MPa, simulating a sinking of the microbes at 1000 m d^{-1}. After having reached 40 MPa, the pressure was held constant for 52.5 h. Except for the first day, samples of the seawater–microbe suspension were collected on a daily basis at 12:00 h. Fifty ml samples were processed for number and size of bacteria and for species composition for both control and pressurized microcosms.

Bacterial strains. The microbial population selected consisted of 5 strains which belong to different phylogenetic groups: HP2 (*Bacteroidetes* AY241555), HP4 (*Gammaproteobacterium* AY241549), HP10 (*Bacillus* AY167886), HP18 (*Alphaproteobacterium* [non-*Roseobacter*] AY239005) and Ros6996 (*Alphaproteobacterium*, *Roseobacter litoralis* Shiba 1991, DSMZ). Except for Ros6996—which was collected from seaweed in Japan (Shiba 1991), although close relatives also occur on diatom aggregates inoculated with North Sea water (Grossart et al. 2005)—all bacteria were isolated from aggregates collected in the German Wadden Sea (Grossart et al. 2004). Strains were selected based on their ability to grow free-living in seawater at 33.6 PSU and on their phylogenetic affiliation, which enables an easy distinction by fluorescence *in situ* hybridisation (FISH). All isolates were grown at 20°C; however, in the natural environment these isolates also grow well at temperatures ranging between10 and 25°C. So far, nothing is known about their pressure dependence. All isolates were grown in natural seawater (33.6 PSU) enriched with Marine Broth (0.01 g l^{-1}, MB2216, Difco) in the dark. The same nutrient solution was initially used in the experiments and, at each sampling, 100 ml of the removed liquid was replaced once per day at 12:00 h with 100 ml of fresh medium. Initial bacterial numbers were identical in both microcosms, but due to variances in growth rates, differed among strains (0.08, 0.11, 0.15, 0.32 and 0.35 × 10^6 ml^{-1} for strains HP2, HP4, HP18, HP10 and Ros6996, respectively).

Bacterial numbers and size. For enumeration of bacteria, 1 ml water samples were taken at 41, 64, 88, 109, 144 and 162 h from both the control and pressurized microcosms and fixed with formaldehyde (3% v/v). The samples were filtered through black 0.2 µm pore-size Nuclepore membranes and stained with DAPI using the protocol of Porter & Feig (1980). Bacteria were directly counted on filters by epifluorescence microscopy (Axioplan, Zeiss) at 1000× magnification. Cell length was determined by using a high resolution digital video camera and the software SIS Analysis Pro (both from Olympus).

FISH. Five ml subsamples from each assay (see above) were filtered onto 0.2 µm Nuclepore polycarbonate membranes. The filters were immediately fixed with fresh paraformaldehyde (4% v/v) for 4 h at 4°C and stored frozen at –20°C until further analysis. By using fluorescent oligonucleotide probes we were able to determine the abundance of each strain used in the experiment, i.e. cell numbers of *Bacteria* (EUB338: Amann et al. 1995), *Alpha-* and *Gammaproteobacteria* (ALF968 and GAM42a, respectively; Amann et al. 1995), *Bacteroidetes* (CF319a; Amann et al. 1995) and the *Roseobacter* group (ROS536r; Brinkmeyer et al. 2000). Abundance of *Bacillus*, however, was calculated as the difference between eubacterial counts minus those of all other probe-specific counts. Thus numbers of HP10 (*Bacillus*) are biased by methodological errors of all other strains. However, cells of HP10 were easily distinguished by cell morphology from all other cells and hence we are certain that the general temporal course throughout the experiment, particularly the absence of HP10 at 40 MPa pressure, are realistic. The protocol given by Glöckner et al. (1996) was used for hybridization. For all probes, 35% (v/v) formamide in hybridization buffer was used. Samples were counted within 24 h after hybridization at 1000× magnification using epifluorescence microscopy (Axioplan, Zeiss). Group-specific counts are given as percentage of total cell counts (DAPI).

Statistics. We compared measured bacterial parameters in the control and pressurized microcosms to search for pressure effects between the 2 treatments, which are otherwise identical. Statistically, such a comparison can be readily evaluated for hypothesis testing by non-parametric tests of paired observations such as the Wilcoxon signed rank test. The first hypothesis was that under pressure, fewer cell numbers would be found than in the control (1-tailed asymptotic Wilcoxon signed rank test, SPSS v.14.0). Additionally, we tested for differences in total number and relative proportion of each bacterial strain (2-tailed asymptotic Wilcoxon signed rank test, SPSS v.14.0).

RESULTS

The pressure time series of the microbiological suspension followed the computer-controlled path, representing settling over a 4000 m depth interval at 1000 m d^{-1} and a final 52.5 h exposure within the 4000 m deep near-bottom fluid layer.

Temperature, oxygen, bacterial numbers and sizes

Temperature remained constant throughout the experiment at 20.5 ± 0.5°C. Fig. 2 shows pressure and oxygen throughout the experiment. Although oxygen saturation rapidly dropped due to bacterial respiration from 85% to a low of ca. 10% at the end of the experiment in both control and pressurized treatments, no anaerobic conditions known to limit bacterial growth occurred (see bacterial numbers in the control, Fig. 3A). Whereas bacterial numbers in the control exponentially increased from 1×10^6 to 40×10^6 cells ml^{-1}, they remained significantly lower in the pressurized treatment ($Z = -2.201$, $p = 0.014$; Fig. 3a), indicating that pressure has a severe effect on growth of all selected shallow-water strains. Fig. 3B shows that in the pressurized treatment bacterial numbers increased throughout the first 3 d (although less than in the control) until a pressure maximum of ca. 25 MPa had been reached. Thereafter, bacterial numbers dramatically decreased to a low of 0.55×10^6 cells ml^{-1} on Day 4. Interestingly, bacterial numbers recovered on Day 5 and remained at ca. 1×10^6 cells ml^{-1} until the end of the experiment. Whereas cell length of single strains (detected by FISH) remained similar in the control from the start until the end of the experiment, indicat-

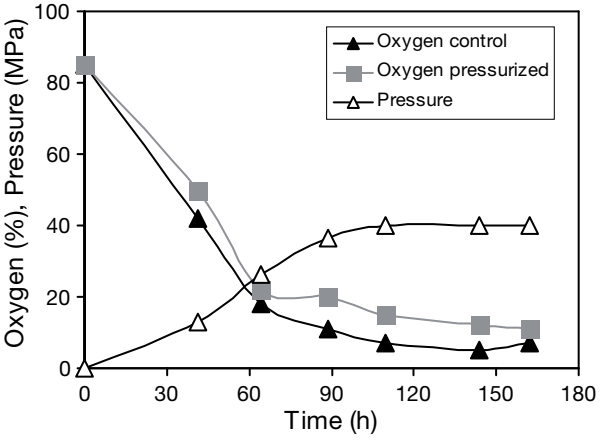

Fig. 2. Oxygen saturation (%) throughout the course of the experiment in both control and pressurized treatments. Changes in pressure (pressurized treatment only) throughout the experiment are also given

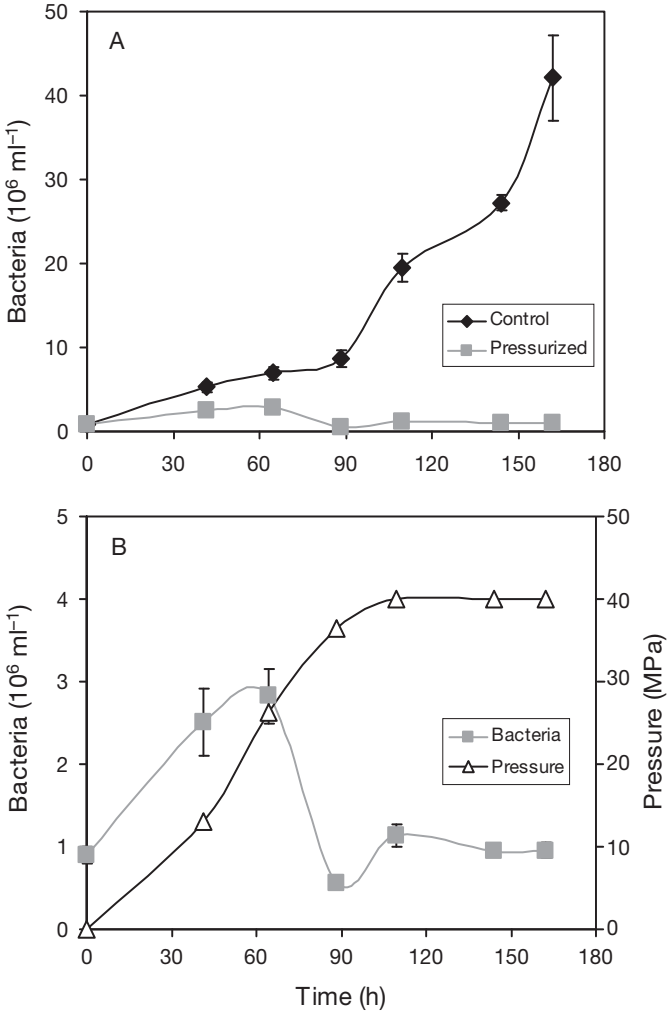

Fig. 3. (A) Time series of bacterial numbers in the control versus the pressurized treatment during the experiment. (B) To better compare temporal development of bacteria numbers in the pressurized treatment, the time series of pressure is also shown

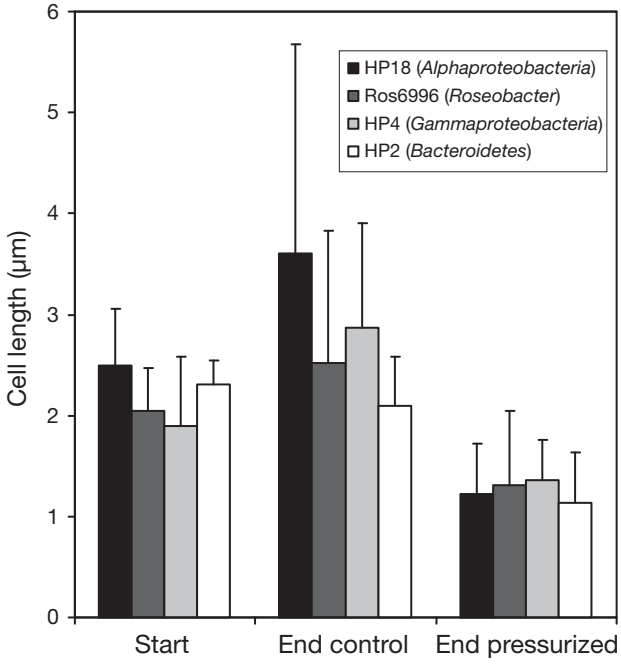

Fig. 4. Comparison of cell length at the beginning and the end of the incubation in the control versus the pressurized treatment

ing good growth conditions, it was lower in the pressurized treatment (Fig. 4), suggesting that pressure leads to morphological changes in bacteria.

Changes in bacterial community composition

FISH analysis (Fig. 5) revealed that absolute numbers of all strains, except HP2 (*Bacteroidetes*), significantly differed between control and pressurized treatments (Table 1). However, relative proportions of the tested strains in the control versus the pressurized treatment significantly differed only for strain HP4 (Table 1). In the control, strain HP4 contributed a relatively low fraction, but reached its highest numbers and a fraction of up to 60% in the pressurized treatment. In contrast, strain HP2 reached its highest numbers in the control and almost completely vanished in the pressurized treatment. These results show that pressure seems to select for survival and decline of specific surface bacteria that are well-adapted to pressure. Increases in numbers of strain HP4 between 88.5 and 144 h of incubation and of strain HP18 (*Alphaproteobacteria*, non-*Roseobacter*) between 109.5 and 162 h of incubation indicate that these bacterial isolates were able to adapt and to grow at 40 MPa pressure, although both strains have been isolated from the surface ocean. In contrast, strains HP2 and HP10 (*Bacillus*) had high doubling times (<0.5 d) during the first 41.5 h of the incubation, indicating substantial growth even at pressures up to 15 MPa. However, these strains were not able to actively grow at pressures >20 MPa, indicating that pressure potentially leads to changes in bacterial community composition during sinking of particulate organic matter.

DISCUSSION

A major advantage of our experimental approach is that it exceeds experimental resolution of similar techniques utilized to date; thus time series experiments of pressurized simulations of aggregates moving verti-

cally in the ocean (representing increasing or decreasing local hydrostatic pressure) are now feasible. This allows for realistic measurements of growth and species shifts on both sinking and rising aggregates. The results obtained from the present study support and extend the conclusions of earlier research pointing towards a strong role of pressure in microbial organic matter remineralization and species composition (Tamburini et al. 2003, 2006). Comparison between the control and the pressurized microcosm revealed a strong pressure response, not only in numbers, as reported earlier (Herndl et al. 2005, Winter et al. 2009), but also by different physiological effects, e.g. cell sizes. We have simulated fast sinking through different water regions, with aggregates finally residing in a benthic boundary layer (here at 4000 m depth), typical for marine aggregates. The applied pressure acted on the bacteria via 2 regimes, suggested by physiological reaction of the bacteria: pressure gradient and steady-state responses. A pressure gradient response depicts changes in bacterial physiology during the increase in pressure. Changes in bacterial parameters after this pressure increase (aggregates have been kept for 3 d at 40 MPa pressure) have been described as a steady-state response (Fig. 2). However, we do not know whether this pattern remains the same for slower settling speeds, allowing for prolonged times of bacterial adaptation to increasing ambient pressure. Our simulated sinking from surface waters appears to rapidly reduce cell division as indicated in the decline in cell numbers. The time series of pressure application with steady and unsteady components elicits reactions which can be ascribed to different physiological responses: (1) sensing and responding to the sinking process (pressure increase), (2) generation of distinct local maxima and minima in cell numbers at certain pressure thresholds and (3) adaptation and reshuffling of species at high steady-state pressure (40 MPa).

We chose to simulate organic matter sinking in an isothermal ocean by 5 selected, relatively well-characterized bacteria frequently found to be attached to organic matter in the upper 100 m of the water column. Of these, our data indicate selective growth and adaptation to the pressure changes by

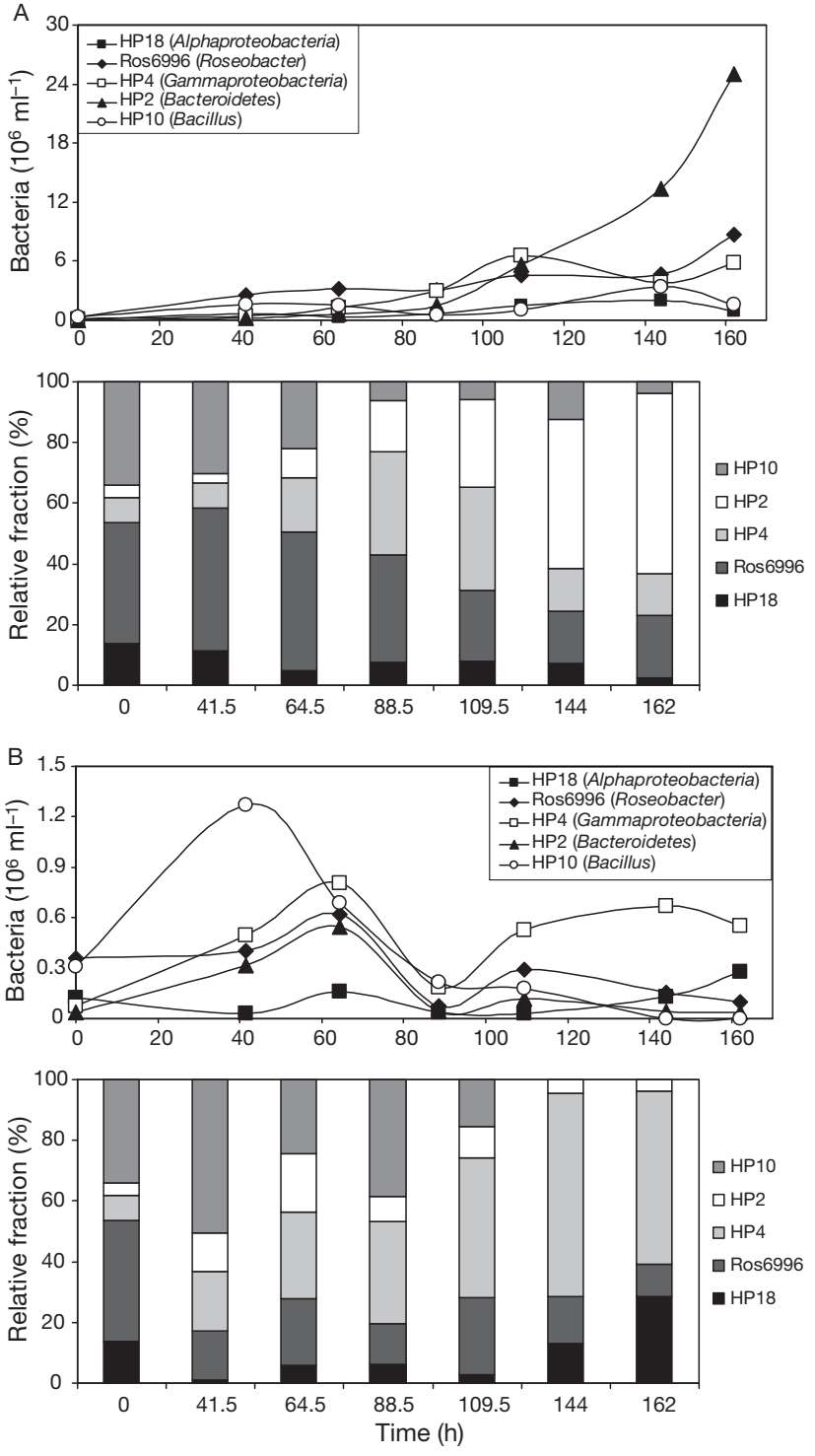

Fig. 5. Time series of the tested strains in the (A) control and (B) pressurized treatments. In both (A) and (B), the upper panel depicts cell numbers and the lower panel the relative fraction (%) of each strain

Table 1. Results of the 2-tailed asymptotic Wilcoxon signed rank test testing the hypothesis that relative proportion (%) and total number (cells) of each bacterial strain differ under pressure. (a) Based on positive ranks; (b) based on negative ranks. Significant values are marked in **bold**

Strain	*Z* (%)	p	*Z* (cells)	p
HP18 (*Alphaproteobacteria*)	–0.105(a)	0.917	–2.201(a)	**0.028**
Ros6996 (*Roseobacter*)	–1.782(b)	0.075	–2.201(a)	**0.028**
HP4 (*Gammaproteobacteria*)	–1.992(a)	**0.046**	–1.992(a)	**0.046**
HP2 (*Bacteroidetes*)	–1.153(b)	0.249	–1.782(a)	0.075
HP10 (*Bacillus*)	–0.943(a)	0.345	–2.201(a)	**0.028**

specific bacterial strains; e.g. increased percentage of strain HP4 (*Gammaproteobacteria*) but reduced fractions of strain HP10 (*Bacillus*) which becomes completely extinct under high pressure. The smaller cell size of all bacterial strains under pressure (significant at the 90% level) suggests a specific physiological response (Jannasch & Wirsen 1984). This holds true for both field- and laboratory-generated populations. Changes in growth in particular of strain HP4 at prolonged periods of high pressure demonstrate specific physiological pressure adaptations (e.g. Tamburini et al. 2003). Most of the available studies on deep-sea bacteria have been performed with samples at atmospheric pressure and hence fully neglect pressure-induced changes in physiology (e.g. Kamimura et al. 1992). As a consequence of our results, to obtain more realistic organic matter remineralization rates of deep-sea bacteria, pressure-related effects on microbial physiology and species distribution need to be taken into account.

We have to point out that our experimental setup does not reflect true *in situ* conditions: (1) temperature was kept constant at 20.5°C and (2) nutrients were daily added in a pulsed manner which is different from changes in temperature and nutrients during vertical sinking of aggregates. Although not representative of most *in situ* conditions, our results show that pressure is a major environmental variable for bacterial dynamics in the ocean. Furthermore, the present study suggests that, for selected species, transfer from shallow-water to deep-sea populations may be possible. We even hypothesize that the biological pump may consist of several (presently unknown) depth-sensitive and depth-specific transformation steps controlled by microbiological–hydrodynamical interactions.

The efficiency of the biological pump (Volk & Hoffert 1985) is affected by aggregation and sinking of organic matter combined with prevailing microbial mineralization rates. Our experiment suggests that not all surface-originating species will disappear during the settling process, and they may well add to the deep-sea population (Fig. 5B). Recently, Baltar et al. (2009) provided evidence that in the deep-sea most of the organic matter remineralization and hence CO_2 release is related to microbial activities on low-buoyancy and slow-sinking particulate organic matter which allow for better bacterial adaptation to pressure changes. Thus knowing the residence time of organic matter in certain water layers and the local microbial species pool is crucial for understanding carbon cycling in the ocean. Consequently, the suite of parameters controlling microbial organic matter remineralization in the ocean needs to be identified beyond the currently employed protocols, particularly in relation to hydrostatic pressure (Tamburini et al. 2002, 2003, 2006). The redistribution of particulate organic matter in the open ocean is not solely based on the 'sinking leg' but also on the 'rising leg', i.e. upward fluxes of organic particles due to water movements or inclusions of gas bubbles (Yayanos 1986, Riebesell 1992). In addition, bacteria may be transported through different water layers when attached to zooplankton (Grossart et al. 2009). As a result, bacteria attached to particles and zooplankton may experience frequent changes in ambient pressure. Tamburini et al. (2003) demonstrated that metabolic rates of deep-sea bacteria incubated under *in situ* pressure conditions are ca. 4.5 times higher than those measured under decompressed conditions. Our results on species reshuffling, cell sizes and growth responses obtained through our experimental approach (previously described by de Jesus Mendes et al. 2007) suggest that after the isothermal-ocean experiment (present study), full-scale environmental simulations are called for. This entails both simultaneously controlling the ambient pressure and temperature and sampling the tested microbes, not only in decompression mode at user-selected time increments without disturbing the experiment as we did here, but also in decompression-free mode for *in situ* activity measurements. While in our present experimental setup, sinking and ascending of organic matter can be simulated as desired, while keeping all other environmental variables (e.g. temperature, fluid flow, nutrient supply) constant, this constraint can be lifted in future experiments. The addition of a thermal control to the pressure control, along with decompression-free sampling and incubation, will result in an experimental design which tests for the (hitherto untestable) effects of hydrostatic pressure on species reshuffling, physiological responses of marine bacteria during continuous settling to a selected depth and identification of depth-specific process parameters of the biological pump. The data set presented here encourages us to continue such efforts.

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