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Abstract

The deep-sea mussel *Bathymodiolus azoricus* dominates hydrothermal vent fauna in the the Azores region. The gills of this species house methane- and sulfur-oxidizing bacteria that fulfill most of the mussel's nutritional requirements. Previous studies suggested that the ratio between methane- and sulfur-oxidizers could vary in response to the availability of electron donors in their environment, and this flexibility is considered a key factor in explaining the ecological success of the species. However, previous studies were based on non-isobaric recovery of specimens, with experiments at atmospheric pressure which may have induced artifacts. This study investigates the effect of pressure-related stress during recovery and experimentation on the relative abundances of bacterial symbionts. Mussel specimens were recovered for the first time using the pressure-maintaining device PERISCOP. Specimens were subsequently transferred into pressurized vessels and exposed to various chemical conditions. Using optimized fluorescence *in situ* hybridization-based approaches, relative abundance of symbionts were measured. Our results show that the recovery method (isobaric versus non-isobaric) does not influence the abundances of bacterial symbionts. Significant differences occur among specimens sampled from two contrasting sites. Exposure of mussels from the deeper site to sulfide and bicarbonate, and to bicarbonate alone, both resulted in a rapid and significant increase in the relative abundance of sulfur-oxidizers. Results reported herein are congruent with those from previous reports investigating mussels originating from shallow sites and kept at ambient pressure. Isobaric recovery and maintenance allowed us to perform *in vivo* experiments in specimens from a deeper site that could not be maintained alive at ambient pressure, and will greatly improve the chances of identifying the molecular mechanisms underlying the dialogue between bathymodioline hosts and symbionts.

Introduction

Bathymodiolinae mussels (family Mytilidae) are part of the remarkable fauna colonizing ecosystems such as hydrothermal vents and cold seeps in the deep-sea (Desbruyères et al., 2000; Duperron, 2010; Duperron et al., 2009; von Cosel et al., 1999, 2001). These mussels rely upon sulfur- or methane-oxidizing (SOX and MOX) bacteria occurring in their gill epithelial cells for all or part of their nutrition (Cavanaugh et al., 1981; Felbeck, 1981). The symbionts of mussels exploit compounds present in vent or seep fluids for their metabolism (Van Dover, 2000; Van Dover et al., 2002). Although sulfide is toxic to animals, symbiotic sulfur-oxidizers use hydrogen sulfide from the fluids as the source of energy for their metabolism and to fix inorganic carbon (Cavanaugh et al., 1988). Methane-oxidizing bacteria use methane both as a carbon and an energy source (Cavanaugh et al., 1992; Childress et al., 1986). Organic carbon compounds are subsequently transferred to their animal host and ultimately contribute to ecosystem productivity in habitats where only a small fraction of the photosynthetic primary production from upper layers of the oceans is brought in by sedimentation or advective transport (Cavanaugh, 1983; Corliss et al., 1979; Karl et al., 1980).

Bathymodiolus azoricus and its sister species *B. puteoserpentis* dominate several vent sites on the Mid-Atlantic Ridge (MAR). They possess both sulfur- and methane-oxidizing symbionts in their gill bacteriocytes, as demonstrated through ultrastructural studies, 16S rRNA-encoding gene sequence analyses, and enzyme assays (Cavanaugh et al., 1992; Distel et al., 1995; Duperron et al., 2006; Fiala-Medioni et al., 2002; Fisher et al., 1993). Dual symbiosis is thought to increase the environmental tolerance of hosts because the distinct metabolism of the sulfur- and methane-oxidizing symbionts may help the holobiont adapt to varying availability of reduced sulfur and methane (Distel et al., 1995; Fiala-Medioni et al., 2002). Several studies point to a high flexibility of the symbiont populations (Kádár et al., 2005; Riou et al., 2008). Bacteria indeed disappear from *B. azoricus* gill bacteriocytes when subjected to starvation in sulfide- and methane-free sea-water, but can be recovered when mussels return to sulfide-enriched aquaria (Kádár et al., 2005). The relative volume occupied by each symbiont type in bacteriocytes of *B. azoricus* varies within vent sites, and between sites displaying different chemical signatures (Halary et al., 2008). Experiments using mussels maintained in controlled conditions at atmospheric pressure with one, both or none of the electron donors necessary for endosymbiont metabolism confirm that symbiont relative abundances can change rapidly in response to changes in the availability of their respective substrates (Halary et al., 2008; Riou et al., 2010, 2008).

However, the previously mentioned results suffer numerous potential biases. First, specimen recovery from the MAR vent sites, which are located at depths between 800 m (Menez Gwen) and

3500 m (Logatchev), involved rapid (usually a few hours) and large de-pressurization of specimens (8 to 35 MPa). This results in high levels of stress, ultimately resulting in the death of specimens from the deepest sites (Halary et al., 2008). Second, specimens used in *in vivo* experiments are usually from shallower vent sites (Menez Gwen) and maintained in the laboratory at atmospheric pressure, *i. e.* ~80-fold lower than *in situ* conditions (Kádár et al., 2005; Riou et al., 2008). Results from these studies are thus potentially affected by artifacts associated with depressurization, and it remains to be confirmed whether observed symbiont dynamics were the consequence of these stresses or true biological responses. Another issue is with the quantification of symbionts itself. Several studies are based on a 3D fluorescence *in situ* hybridization (FISH) approach, which measures the fraction of the total volume occupied by each type of symbiont within bacteriocytes (Halary et al., 2008; Riou et al, 2008; Duperron et al, 2011). Although reliable, this approach is time consuming because it involves the acquisition of 3D images of gill sections, manual cropping of individual bacteriocytes, and computing volumes using a dedicated ImageJ plugin (Halary et al., 2008). This has limited the number of specimens that could be analyzed, reducing the statistical power of comparisons (Prosser, 2010).

Several pressurized vessels for live maintenance and pressurized recovery that prevent or reduce pressure-related stress have become available in recent years (Boutet et al., 2009). The first aim of the present study is to investigate relative symbiont abundances in *B. azoricus* mussels recovered from the Menez Gwen (800 m depth) and Rainbow (2300 m depth) vent sites using the pressure-maintaining PERISCOP sampling cell (Shillito et al., 2008). The second aim is to investigate these abundances in Rainbow specimens exposed to substrates used by the sulfide-oxidizing bacteria at their native pressure in the IPOCAMP vessel (Shillito et al., 2014). The percentage of total bacterial volume corresponding to methane- plus sulfur-oxidizing symbionts is measured by means of FISH and image analysis. In order to optimize the method, we compare results from three FISH-based approaches, and images acquired from the anterior and posterior regions of the gills. We then compare isobaric *vs.* non-isobaric recovery, Menez Gwen and Rainbow specimens, and the effect of treatments applied to live specimens from Rainbow. Results are discussed in relation to improvements of the methods used to investigate symbiont dynamics in animal tissue in light of previous reports, based on specimens from non-isobaric recoveries and exposed to various experimental conditions at atmospheric pressure.

Material and methods

Sampling sites

Bathymodiolus azoricus mussels (Bivalvia, Mytilidae) (von Cosel et al., 2001) were collected from two hydrothermal vent sites during the 2013 BioBaz cruise to the Mid-Atlantic Ridge aboard RV

Pourquoi Pas? using the ROV *Victor 6000*. Specimens were sampled from the vicinity of active smokers at Menez Gwen (MG2 marker, 37°50.669' N 31°31.156' W, 830 m depth) and Rainbow (France5 marker, 36°13.766' N 33°54.117' W, 2270 m depth) (Figure 1). Active vents were colonized by fauna typical for MAR vents, including one bivalve (*Bathymodiolus azoricus*) and three shrimp species (*Rimicaris exoculata, Mirocaris fortunata* and *Chorocaris chacei*) (Desbruyères et al., 2001).

Isobaric and non-isobaric recovery

At each sampling site, specimens were recovered in clean watertight BioBoxes, which were brought to the surface by the ROV or the shuttle within a few hours (non-isobaric sampling). Others were placed inside a 'CROCO' sampling cell that was fit into the pressure-keeping vessel PERISCOP (isobaric recovery, Shillito et al., 2008). PERISCOP was then closed and the shuttle to which it was attached was released within a few minutes, and surfaced within 45 minutes. Pressure was monitored during surfacing with autonomous pressure sensor (SP2T4000, NKE Instruments, France). Once onboard, PERISCOP was opened and mussels were recovered and either dissected and fixed, or transferred to the IPOCAMP pressure vessel within 10 minutes.

Exposure to bicarbonate and sulfide in pressurized vessels

Six mussels from Rainbow, recovered using the PERISCOP, were taken out and used in incubation experiments (shell length: 44.4-100.5 mm). Due to the limited volume of the IPOCAMP chamber and the size of specimens, only two specimens were transferred in each of three 1 liter Nalgene™ bottles filled with 11.8 µM $NaHCO_3$ and 36.4 µM Na_2S; with 11.8 µM $NaHCO_3$; or with filtered sea-water alone (control). These concentrations were within the range of values recorded in mussel aggregates on the site, and similar to values used in previous studies (Halary et al., 2008; Riou et al, 2008). Bottles were tightly closed and re-pressurized in IPOCAMP aquariums within 5 minutes. Mussels were incubated at 23MPa and 8°C for 5 h, then dissected immediately and fixed.

Sample fixation onboard

Anterior and posterior parts of mussel gill tissue were dissected at 4°C and fixed for fluorescence *in situ* hybridization (FISH). Gill fragments were transferred to 4% formaldehyde in twice-filtered sea-water (TFSW) (4°C, 2–4 h), rinsed, and dehydrated in increasing ethanol (50, 70 80, and 96%, 15 min each). The density of gill filaments per millimeter was measured under a dissecting microscope.

Fluorescence in situ hybridization (FISH)

Fragments of gills were embedded in polyethylene glycol distearate (PEG):1-hexadecanol (9:1), cut into 8 µm-thick sections and deposited on SuperFrost Plus slides (VWR International, USA). Wax

was removed and tissue rehydrated in decreasing ethanol series (96% to 70%). Sections were hybridized using 40% formamide for 1–3 h at 46°C as previously described (Duperron et al., 2008), rinsed, and mounted in DAPI-containing "Slow Fade" (Life Technologies) under a coverslip. Three 16S rRNA-specific probes were applied simultaneously on every section. FISH probes used are summarized in Table 1.

Image acquisition and analysis

Slides were observed under a BX61 epifluorescence (Olympus, Japan) or a SP5 confocal (Leica, Germany) microscope, and images were acquired at 400x magnification using ImagePro 6.0 (Olympus, Japan). On each section, a 2D image was first acquired by overlaying signals from the three probe-associated fluorochromes (2D acquisition). Second, an image stack was built by acquiring images every 0.3 µm over the thickness of the section (3D acquisition). Images were analyzed using ImageJ (Abramoff et al., 2004) and the total volume occupied by bacteria and respective proportions of the sulfur- and methane-oxidizing symbionts were computed for 2D and 3D images using the SymbiontJ plug-in as previously described, but applying the procedure to the whole field of view without isolating individual bacteriocytes (Halary et al., 2008). SymbiontJ applies filters and thresholds to the different color channels and computes the number of voxels corresponding to each symbiont type. Finally, ten bacteriocytes were manually cropped from each 3D acquisition, analyzed using SymbiontJ, and the mean percentage of volume occupied by each bacterial type was computed (bacteriocyte acquisition).

Statistical analyses

The percentage of bacterial volume occupied by methane-oxidizing bacteria was used for all analyses, after an Arcsine transformation (Halary et al., 2008). Values were then used for transformation-based redundancy analyses (tb-RDA). Sampling site (Menez Gwen versus Rainbow), recovery mode (isobaric versus non-isobaric), acquisition method (2D, 3D or bacteriocyte), gill region (anterior versus posterior), and specimen were used as factors into the constrained redundancy analysis (RDA), in order to estimate their contribution to the global variance. Significance was assessed using permutation tests (n=9999) using a full model, with separate test for each term (constraining variable). Shapiro-Wilk tests revealed non-normal distribution of the data, and non-parametric tests were thus applied for inter-groups comparisons. The Mann-Whitney-Wilcoxon (MWW, for 2 class factors) and Kruskal-Wallis (KW, for 3 or more class factors) tests were used to compare percentages of methanotrophs. All statistical analyses were performed using R (R Development Core Team, 2013); 'vegan' package was used for the constrained redundancy analysis.

Results

In order to optimize the 3D FISH method (Halary et al., 2008), three types of images were acquired and compared. For each mussel specimen, 10 acquisitions in 2D and 10 in 3D were obtained. From each of the later 3D acquisitions, 10 randomly chosen bacteriocytes were analyzed, resulting in 100 bacteriocytes per specimen. These acquisitions were obtained from at least 5 different sections, each containing 10-20 filaments. Results are summarized in Table 2, and a micrograph showing gill tissue with bacteriocytes containing the two symbionts is presented on Figure 2. The analyses of 2D, 3D, and bacteriocytes did not yield significantly different results (KW test on all measurements including anterior and posterior gill regions, p-value = 0.09).

The filament density per mm gill length along the anterior-posterior axis decreased significantly with increasing shell length (R^2=65%, Pearson's correlation test: p-value < 0.001, Figure 3). Gills form and grow from the posterior end. Percentages of methanotrophs (MOX) in the anterior and posterior regions of gill from 3 specimens per treatment (12 specimens) were computed using 2D and 3D methods, yielding in total 480 measurements (Table 2, Table S1). The percentage of volume occupied by methanotrophs was not significantly different between anterior and posterior regions of the gills (MWW test, p-value = 0.64). Subsequent analyses were thus made only on the anterior part of gills, for which more specimens were available.

When recovered in BioBoxes, mussels from Menez Gwen and Rainbow experienced pressure loss of around 8.3 and 23 MPa, respectively. The use of PERISCOP prevented that, and mussels arrived onboard at pressures close to those of the deep waters (Table 2). Percentages of volume occupied by methanotrophs were 46.5% ± 5.8 and 56.3% ± 6.4 in Menez Gwen and Rainbow mussels recovered in BioBoxes, and 44.3% ± 5.7 and 56.3% ± 8.3 in mussels recovered using PERISCOP (Figure 4, Table 2). A MWW test based on 1140 values from the anterior and posterior regions of the gills indicated no significant difference between the two types of recovery (p-value = 0.12).

Mussels from Menez Gwen and Rainbow sites displayed significantly different percentages of methanotrophs in their gills (MWW test, W = 36185.5, p-value < 2.2e-16). A unilateral test confirmed that methanotrophs occupied a higher fraction of the overall volume in bacteriocytes of *B. azoricus* from the deeper Rainbow site (Figure 4, Table 2, Table S1).

The influence of each aforementioned factor was estimated by constrained redundancy analysis (RDA) and ANOVA permutation tests. Overall, our model could explain 56% of the total variance. Sampling site was the most influential factor (41%; df = 1; F = 1026.1), followed by inter-individual differences, which accounted for more than 14% (df = 27; F = 13.3) of the total variance. Other factors explained much lower fractions of the variance: recovery mode (0.3%; df = 1; F = 7.7), acquisition method (0.3%; df = 2; F = 3.8) and gill region (0.04%; df = 1; F = 0.1) (Table S2).

Each experimental condition could be applied to 2 specimens only from Rainbow because of the

limited volume of bottles and the limited number of bottles that could be incubated at once in pressurized vessels. Nevertheless, 2 different treatments ($NaHCO_3$ + Na_2S or $NaHCO_3$ alone) and 2 control experiments (10 mussels fixed immediately after recovery using PERISCOP and 2 incubated in filtered sea-water in the same conditions as above) were applied, and 320 measurements were made using 2D and 3D methodologies (Figure 5, Table S3). There was no significant difference between 2D and 3D FISH acquisition methods in this dataset (MWW test, p-value = 0.54). The different treatments under pressure resulted in significant differences in percentages of methanotrophs (KW test, p-values < 0.011). The percentage of sulfur-oxidizers was twice higher in specimens exposed to sulfide and bicarbonate than in the sea-water treatment, with 90.1% ± 17.6 versus 38.5% ± 4.9 (Figure 5, Table S3). Specimens exposed to bicarbonate alone also displayed a markedly higher percentage of sulfur-oxidizers (SOX) than in the sea-water treatment. SOX in the bicarbonate treatment occupied 76.0% ± 23.0 of the bacterial volume. The percentage of SOX was slightly lower in mussels incubated in filtered seawater than prior to treatments (38.5 ± 4.9 versus 42.5% ± 7.6). Constrained RDA indicated that the overall variance was mainly explained by the 'treatment' factor (68.5%; df = 3; F = 506.7) and by inter-individual variability (18%; df = 12; F = 32.8), while 0.4% (df = 1; F = 0.9) was due to the method of image acquisition (Table S4).

Discussion

Image based quantification of volumes occupied by symbionts

Three FISH approaches were applied in this study, based on acquisition of 2D (Figure 2A) and 3D images, and of individual bacteriocytes (Figure 2B). They did not yield significantly different results. Although the results are similar, the bacteriocyte-based method used in previous studies (Riou et al., 2010) is by far the most time-consuming, because it involves manually cropping and computing percentages of methanotrophs in at least 10 bacteriocytes from each 3D image. If the aim is to maximize the number of acquisitions, the 2D or 3D approaches are equally suitable and far less time-consuming. In this study we managed to analyze a total of 36 specimens with two to three of these methods (Table 2), while previous studies using 3D-FISH were based 20 or fewer individuals (Duperron et al., 2011, 2007; Halary et al., 2008; Lorion et al., 2012; Riou et al., 2010, 2008).

Sampling methodology

The density of gill filaments was negatively related to shell length, *i.e.* the longer the shell was, the less dense gill filaments were (Figure 3). Similar filament densities and size-related trend are reported for the mussel *Mytilus edulis* (Jones et al., 1992). The gill forms and grows from the posterior end in bivalves (Cannuel et al., 2009; Wentrup et al., 2014); this process could result in

differences in symbiont relative abundances between the ‘young' posterior part and 'old' anterior one, but this was not confirmed by our data. It is likely that the absolute number of symbionts in each filament, which was not computed here, is different in the respective gill regions, in particular in the posterior-most budding zone (Wentrup et al., 2014). Measuring absolute numbers of symbionts would be necessary, but a reliable method still remains to be implemented (Boutet et al., 2011).

The two sites analyzed differed in depth (850 m at Menez Gwen and 2300 m at Rainbow) but non-isobaric recovery in BioBoxes involved significant pressure loss and potential physiological stress in both cases. Recovery stress is a major criticism often addressed to deep-sea biologists and physiologists by many colleagues who are dubious about reports from *ex situ* experiments. In the present study, mussels were for the first time recovered under their natural pressure until opening of the PERISCOP vessel on board, and compared with specimens recovered using classical, non-isobaric BioBoxes. Results indicate that, as far as symbiont relative abundances in gills are concerned, the recovery mode did not have any significant influence, not even for the deeper Rainbow site (Figure 4). This major finding validates *a posteriori* previous studies dealing with symbiont relative abundances that did use classical non-isobaric recovery methods involving pressure loss (Duperron et al., 2011, 2006; Halary et al., 2008; Lorion et al., 2012; Riou et al., 2010, 2008). However, although non-isobaric sampling is appropriate for studies focusing on symbiont relative abundances, isobaric recovery probably makes a difference when dealing with physiological parameters such as host and symbiont activities and gene expression. FISH signal intensities (but not areas and volumes) were indeed usually higher in specimens recovered using PERISCOP, suggesting that ribosomes to which FISH probes attach were less abundant in the latter (unpublished data).

Site-related differences in symbiont abundances

Besides depth, the Menez Gwen and Rainbow sites differ by several characteristics, including the chemical composition of end-member fluids. Fluids at Menez Gwen indeed display slightly lower concentrations of methane compared to Rainbow (Charlou et al., 2002). Sulfide concentrations are similar, but sulfide interacts with the high concentrations of iron at Rainbow, and is thus not readily available to organisms (Desbruyères et al., 2000; Le Bris and Duperron, 2010). Geochemical modeling applied to the mixing zone where mussels actually live indicate that more energy can be gained from sulfide at Menez Gwen compared to methane, while the opposite situation is encountered at Rainbow (Le Bris and Duperron, 2010). The result at Rainbow is however less clear-cut when including hydrogen, an alternative energy source for sulfur-oxidizing symbionts (Petersen et al., 2011). The ratio between sulfide and methane has been shown to influence the relative

abundance of sulfur- versus methane-oxidizers in mussel gills, and methanotrophs are more abundant than thiotrophs at Rainbow (Duperron et al., 2006). Previous estimations of symbiont relative abundances indicated that sulfur-oxidizers represented 53.1% ± 10.3 of the total volume of symbionts at Menez Gwen, compared to 39.4% at Rainbow (Halary et al., 2008; Le Bris and Duperron, 2010). Our results from PERISCOP-recovered specimens were remarkably close (52.6% ± 4.9 and 43.7% ± 6.4 respectively; Table 2). This is in line with the hypothesis that symbiont relative abundances depend on the availability of their respective substrates (Fiala-Medioni et al., 2002; Riou et al., 2008; Trask and Van Dover, 1999). Furthermore, the unexpected similarity between measurements made on specimens sampled in 2006 and 2013 suggests a certain level of long term stability in site-related differences, although short-term fluctuations certainly have occurred (Duperron et al., 2006; Halary et al., 2008).

Symbiont response to bicarbonate and sulfide pulses under pressure

Starvation experiments and exposure to increased concentrations of different chemosynthesis substrates have already been performed by several groups (Halary et al., 2008; Kádár et al., 2005; Riou et al., 2008), but most often on *B. azoricus* from the shallower Menez Gwen site and at ambient pressure. Here, we tested the effect of sulfide and bicarbonate exposure on pressurized *B. azoricus* from the deeper Rainbow site (2300 m depth). Mussels from this site are not easily maintained in the lab at ambient pressure (authors' personal observation). PERISCOP-recovered mussels were transferred to pressure vessels and maintained at 8°C and 23 MPa. A 5h incubation period with bicarbonate and sulfide resulted in doubling of the relative abundance of thiotrophs in the gills (Figure 5). Exposure to bicarbonate alone also significantly increased the relative abundance of thiotrophs (Figure 5). These rapid changes confirm that observations reported in Halary et al. 2008 and other works on mussels from Menez Gwen were not artifacts, and not linked to recovery stress or experimentation at atmospheric pressure. The increase in thiotroph relative volume observed in the present study is even more spectacular (65.0% in Halary et al., 2008). In another study, mussels subjected to a one-month starvation followed by 4 days of constant sulfide supply showed a 96% bacterial volume occupation by thiotrophs (Riou et al., 2008). Here, we show that non-starved *B. azoricus* can shift to an almost thiotrophic symbiosis within 5 hours of exposure to sulfide and bicarbonate (Figure 5). Unfortunately, only two specimens could be subjected to each treatment because of space limitations in IPOCAMP. So although the trend is clear, data from more specimens is needed to reliably estimate the true amplitude of this effect. Thiotrophic symbionts fix inorganic carbon using ribulose-1,5-bisphosphate Carboxylase Oxygenase (RubisCO) in the Calvin cycle (Cavanaugh et al., 1988). This process requires energy from the oxidation of reduced sulfur compounds (Cavanaugh et al., 1988). The less spectacular increase in relative abundance of

thiotrophs when only bicarbonate was available also suggests that another source of energy may have been used for chemosynthesis. Over a short period, bacteria might have used sulfur stored in compounds such as taurine or thiotaurine (Pruski and Fiala-Médioni, 2003). Alternatively, hydrogen, not measured in our study, has been demonstrated to be an alternative energy source for hydrothermal vent symbioses (Amend and Shock, 2001; Petersen et al., 2011). Although the increase in thiotrophs relative abundance could result directly from an actual increase in their number, it could alternatively result from a decrease in methanotrophs, whose substrate was absent and which may have been digested by host bacteriocytes before thiotrophs. Methanotrophs are indeed located more basally within bacteriocytes, closer to phagolysosome-like bodies (Distel et al., 1995; Duperron et al., 2005). It is not known which mechanisms on the host and symbionts sides control the dynamics of bacterial populations within bacteriocytes. Mussel symbionts have been shown to divide and to be digested within bacteriocytes, but additional mechanisms may explain their variations, including symbiont uptake from the environment by adults, as suggested in *B. brevior*, and symbiont release as documented in corals (Dubilier et al., 1998; Lesser, 2011; Thurber et al., 2009). Whatever the underlying mechanisms, the symbiont population as a whole seems to be rapidly and significantly affected by changes in the availability of energy and carbon sources, although results from more specimens are necessary.

Conclusions

In this study, a FISH-based method was used to quantify relative abundances of endosymbionts in gills of *Bathymodiolus azoricus*. Abundances in the anterior and posterior regions of the gill were similar. Mussels sampled in isobaric and non-isobaric recovery devices displayed similar relative volumes of the two types of symbionts indicating that non-isobaric recovery does not induce a major bias in measured volume occupation. However, the physiological status of mussels and their endosymbionts is probably not the same and isobaric recovery is certainly to be recommended for physiological or expression studies of specimens from the deepest sites. Symbiont relative volumes were different between the Menez Gwen and Rainbow sites, and similar to those previously measured on specimens sampled 7 years ago. On the other hand, symbiont populations displayed high flexibility in exposure experiments performed in pressurized aquaria. As postulated previously, this flexibility is certainly an advantage because physico-chemical micro-environments at hydrothermal vents are highly variable in time and space (Chevaldonné et al., 1991; Johnson et al., 1994, 1986; Le Bris et al., 2005), and maybe the key for the domination of *Bathymodiolus azoricus* at various hydrothermal vent sites of the Mid-Atlantic Ridge (Desbruyères et al., 2001, 2000; Von Cosel et al., 1999). More experiments involving methane, hydrogen, toxic compounds, but also thermal stress and symbiont release experiments will be necessary to further explore this flexibility.

The next step will then be to unravel the mechanisms that control this flexibility by exploring cell division patterns, genes and protein expressions of host and symbionts in various conditions.

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Table legends

Table 1: Probes, target groups, and labels used in FISH experiments.

Table 2: Sites, number of individuals, and percentages of methane- (MOX), and sulfur-oxidizers (SOX), standard deviation (SD) measured using the 2D, 3D, and bacteriocyte-based FISH methods in the anterior region of the gill. Maintained pressure during recovery has been calculated as follow: $P_{min}/P_{max}\times100\%$, where P_{max} is the pressure at sampling site, P_{min} – the minimal pressure during recovery.

Table S1: Sites, number of individuals, and percentages of methane- (MOX), and sulfur-oxidizers (SOX), standard deviation (SD) measured using the 2D and 3D -based FISH methods in the posterior region of the gill.

Table S2: Redundancy analysis estimating the contribution of factors site (SITE), recovery method (REC), FISH method (METH), localization in the gill (GILL), and inter-individual variability (IND) to the variability observed in percentages of methane-oxidizers in gills among Menez Gwen and Rainbow specimens.

Table S3: Percentages of methane- (MOX), and sulfur-oxidizers (SOX) and standard deviation (SD) measured using the 2D- and 3D -based FISH methods in the anterior region of the gill of Rainbow specimens upon recovery using PERISCOP (T0) and those exposed to 5-hours experimental treatments in pressurized vessels at 23 MPa.

Table S4: Redundancy analysis estimating the contribution of factors treatment (TREAT), FISH method (METH), and inter-individual variability (IND) in the variance observed in percentages of methane-oxidizers in gills among Rainbow specimens exposed to chemical treatments in pressurized vessels.

Figure legends

Figure 1: Localization of sampling sites on the Mid-Atlantic Ridge. MG – Menez Gwen site (830 m depth); Rb – Rainbow site (2270 m depth).

Figure 2: Overview (A) and detail (B) of transverse sections through *B. azoricus* gill filaments observed at 400× magnification. Filaments were cut parallel to the plane defined by the animals anterior-posterior and left-right axes, and perpendicular to its dorso-ventral axis. Nuclei from host tissue are labeled with DAPI (blue). FISH-labeled bacterial symbionts appear in pink (sulfur-oxidizers) and green (methanotrophs).

Figure 3: Density of gill filaments versus antero-posterior length of the shell. Fitted line is a linear regression (R^2=65%, Pearson's correlation test: p-value < 0.001).

Figure 4: Mean percentage and standard deviation of total bacterial volume occupied by methane-oxidizers in gill tissue of *B. azoricus* specimens from Menez Gwen and Rainbow recovered in BioBoxes (non-isobaric) and PERISCOP vessels (isobaric). Shades of grey correspond to the three FISH methods as indicated (see text for details).

Figure 5: Mean percentage and standard deviation of total bacterial volume occupied by sulfur-oxidizers in gill tissue of *B. azoricus* specimens from Rainbow upon recovery using PERISCOP (T0) and exposed to experimental treatments in pressurized vessels ($NaHCO_3$ + Na_2S, $NaHCO_3$, filtered sea-water). Shades of grey correspond to the two FISH methods as indicated (see text for details).

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Table 1

Probe	Target	Sequence [5' → 3']	Cy3	Cy5	FITC	Channel colour	Reference
EUB_338	Eubacteria	GCTGCCTCCCGTAGGAGT	+	+	+	red	Amann *et al.* , 1990
Imed_M-138	MOX	ACCATGTTGTCCCCCACTAA	+	-	-	green	Duperron *et al.* , 2008
Bang_T-642	SOX	CCTATACTCTAGCTTGCCAG	-	+	-	blue	Duperron *et al.* , 2005
NON_338	negative control	ACTCCTACGGGAGGCAGC	+	+	-	-	Wallner *et al.* , 1993

Table 2

Site		Menez Gwen		Rainbow	
Point		MG2		France 5	
Depth		830 m		2270 m	
Position		37°50.669 N 31°31.156 W		36°13.766 N 33°54.117 W	
Recovery mode		BioBox	PERISCOP	BioBox	PERISCOP
Maintained pressure		0%	83.60%	0%	76.50%
Number of individuals		7	10	3	16
Shell length mm (min-		32-47	26.3-39	88-97	67-88.5
2D FISH	% MOX	47.2	44.6	56.3	57.5
	% SOX	52.8	55.4	43.7	42.5
	S.D.	5.4	5.1	6.6	7.6
3D FISH	% MOX	46.5	44.3	56.3	56.3
	% SOX	53.5	55.7	43.7	43.7
	S.D.	5.8	5.7	6.4	8.3
10 bacteriocyte	% MOX	48.1	47.5	56.3	55.6
	% SOX	51.9	52.5	43.7	44.4
	S.D.	3.8	4.2	4.6	6.3

Figure 1

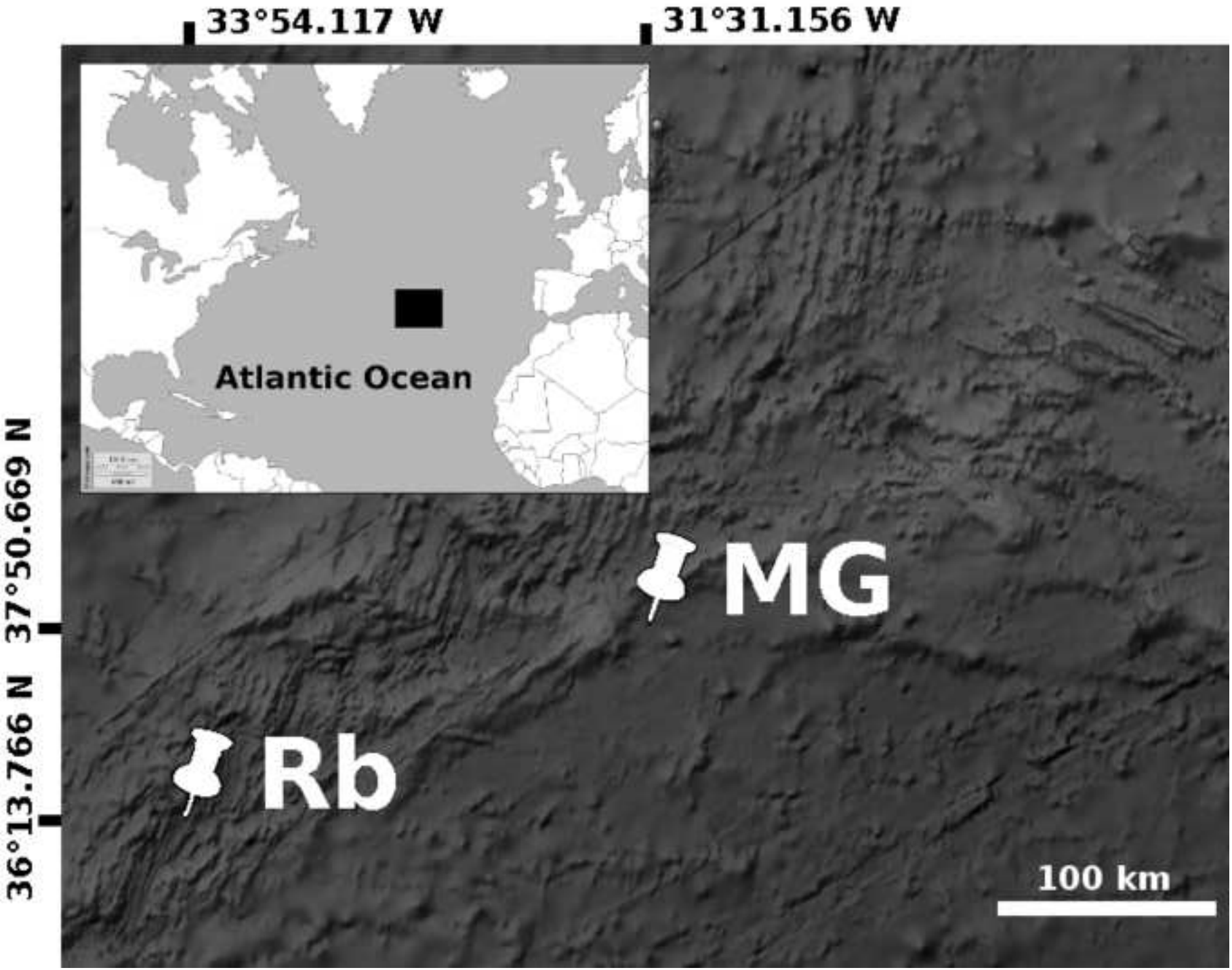

33°54.117 W
31°31.156 W
37°50.669 N
36°13.766 N
Atlantic Ocean
MG
Rb
100 km

Figure 2A

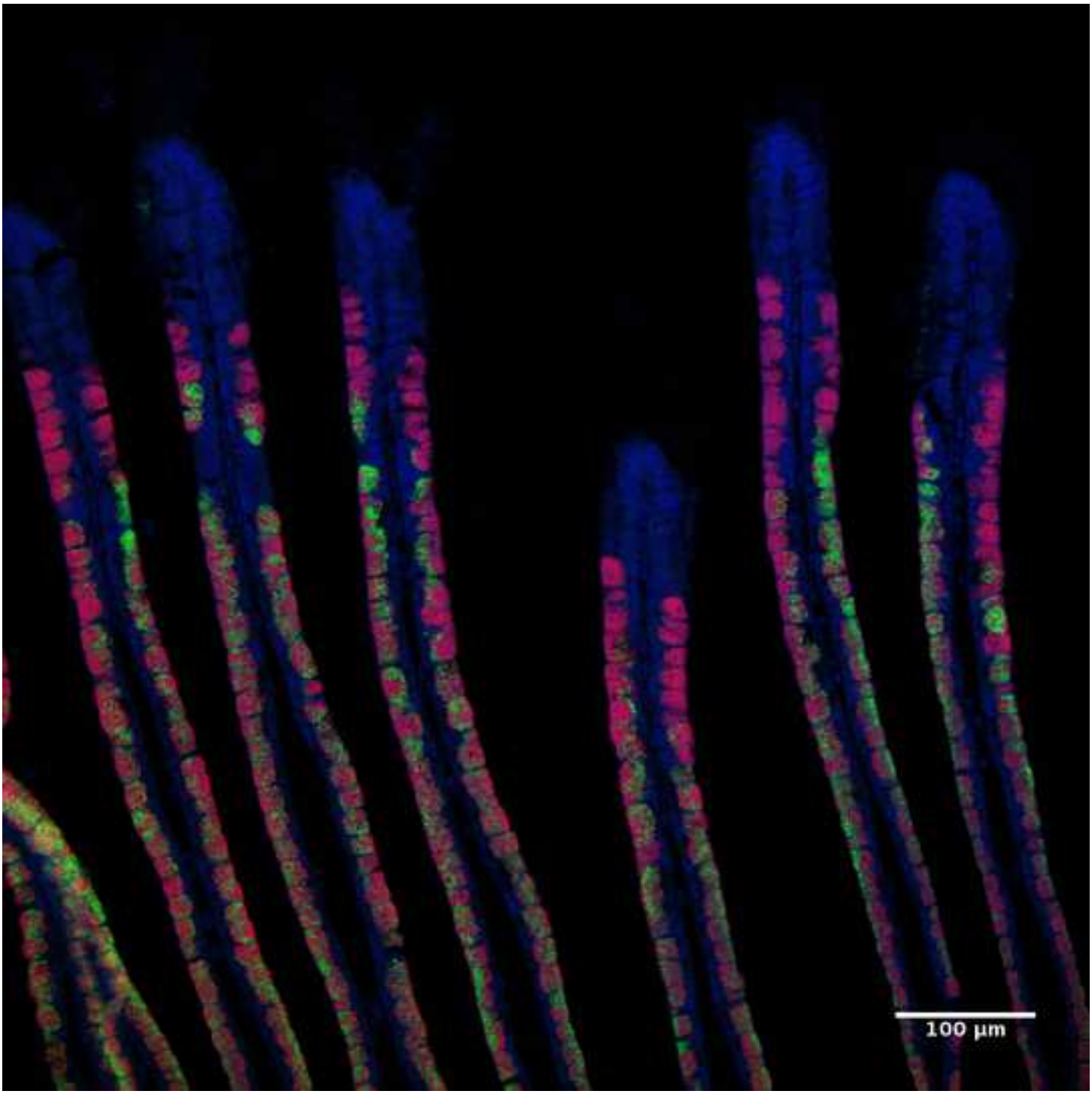


Figure 2B

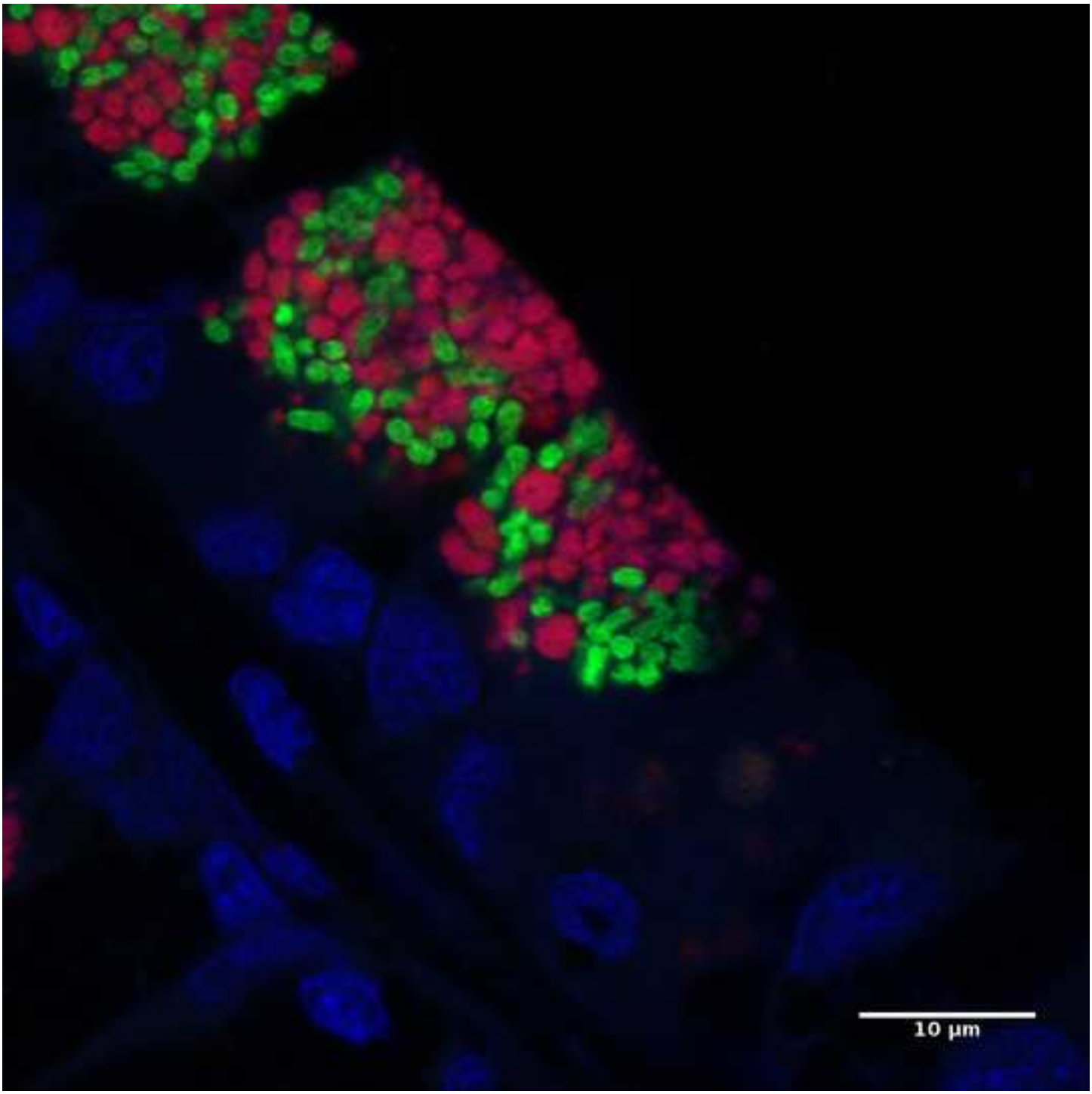


Figure 3

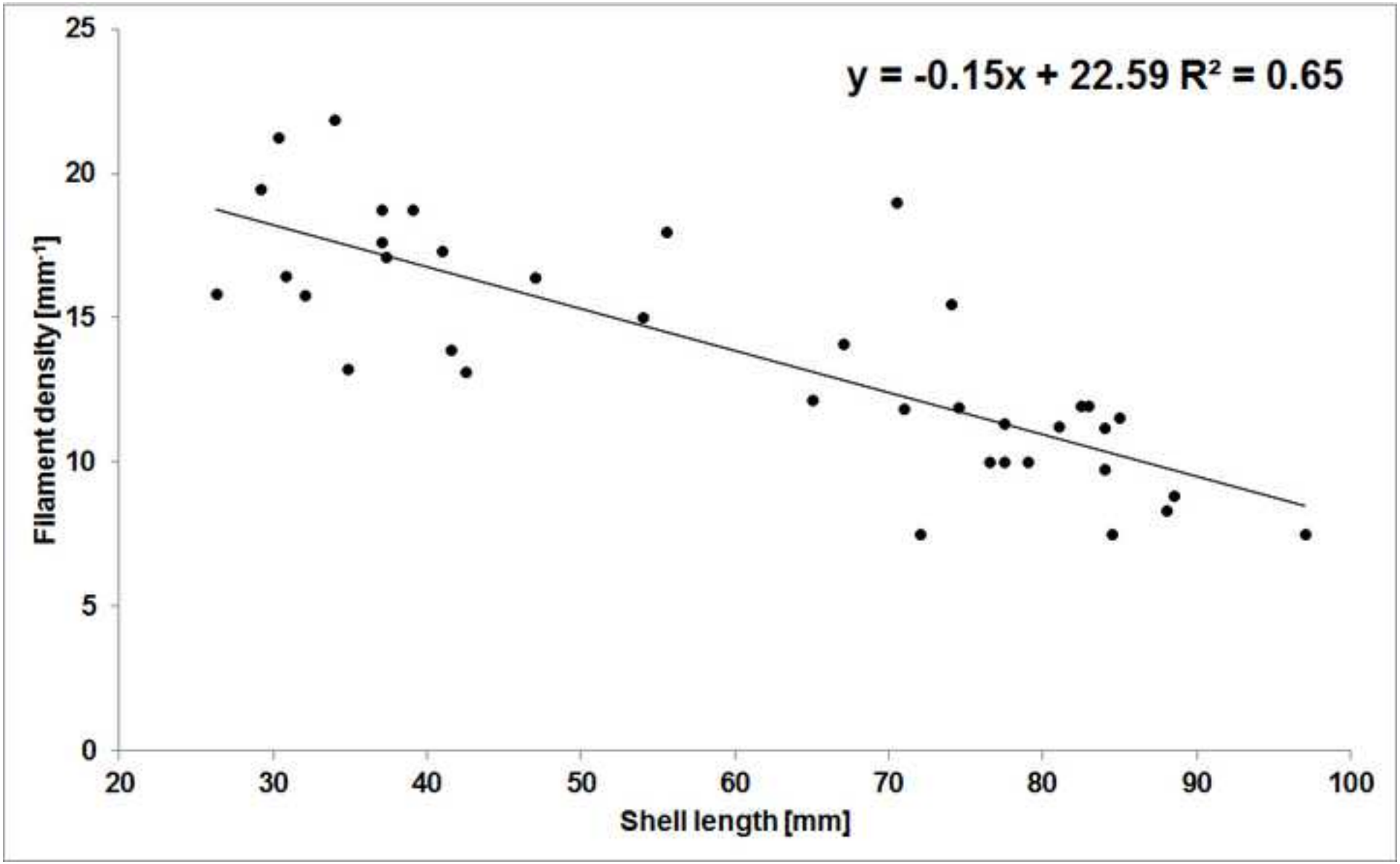
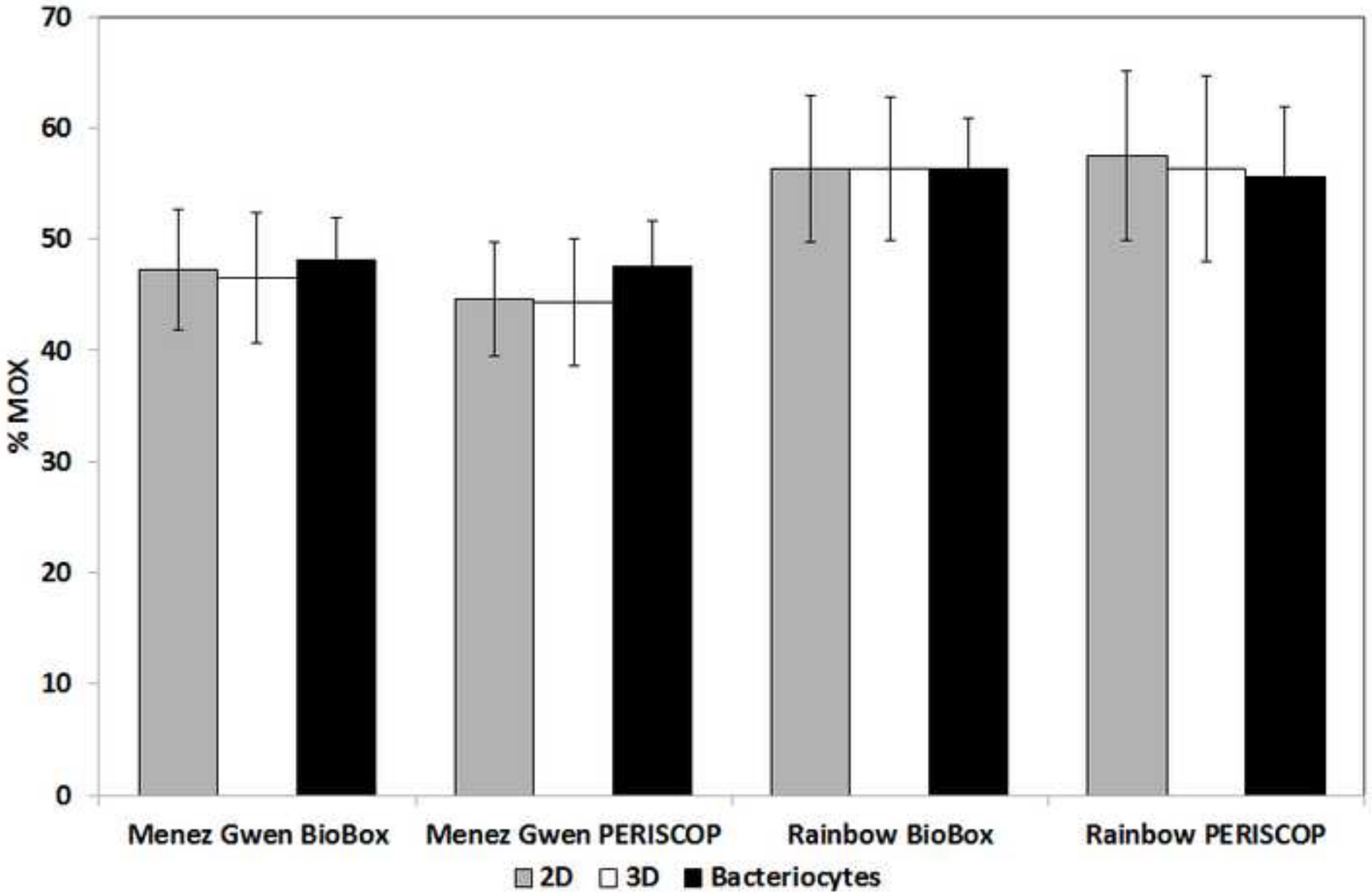

y = -0.15x + 22.59 R² = 0.65
Fllament density [mm⁻¹]
Shell length [mm]
0
5
10
15
20
25
20
30
40
50
60
70
80
90
100

Figure 4


70
60
50
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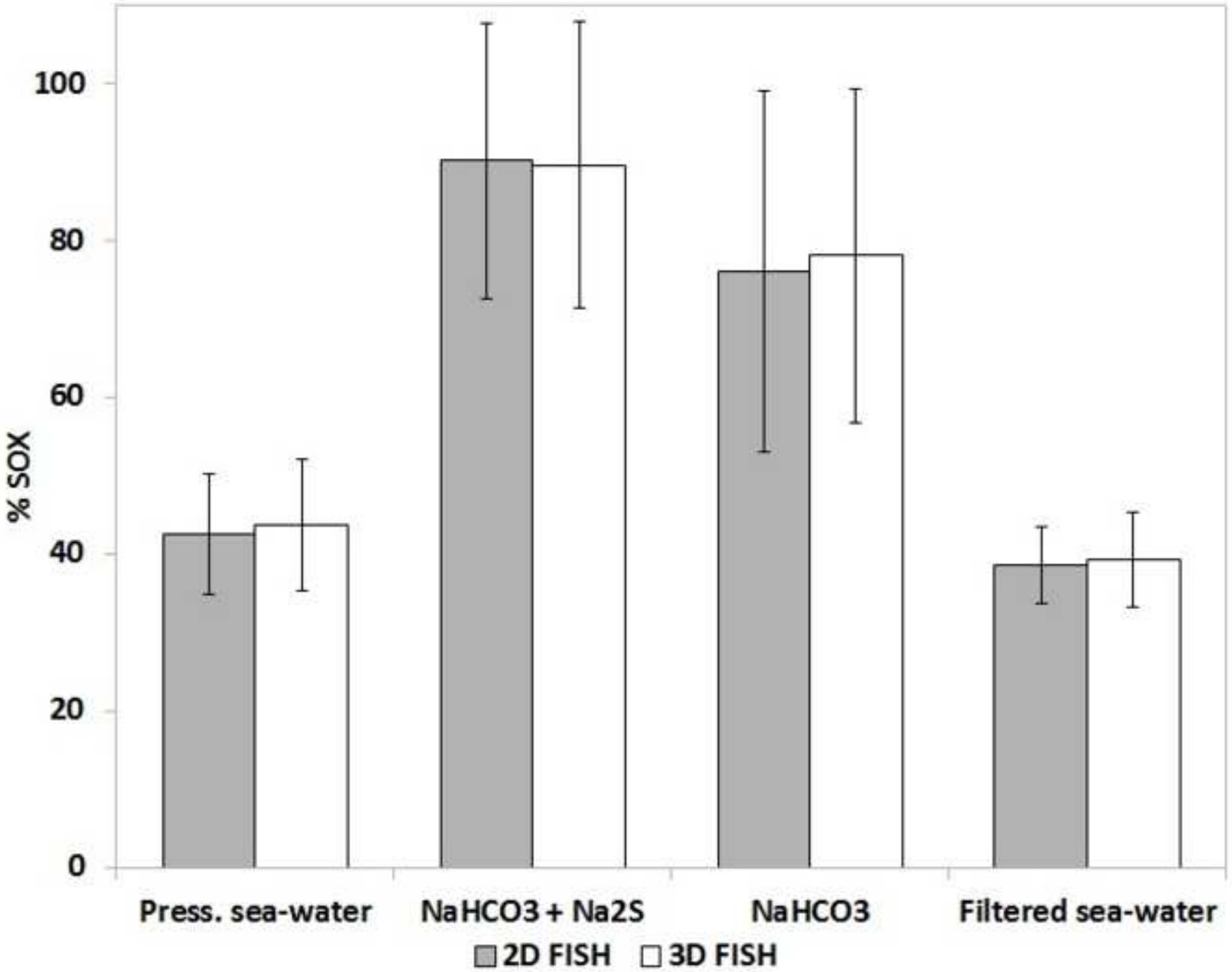
0
% MOX
Menez Gwen BioBox
Menez Gwen PERISCOP
Rainbow BioBox
Rainbow PERISCOP
2D
3D
Bacteriocytes

Figure 5


100
80
60
40
20
0
% SOX
Press. sea-water
NaHCO3 + Na2S
NaHCO3
Filtered sea-water
2D FISH
3D FISH