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Biogeosciences Discuss., 5, 2279–2304, 2008
www.biogeosciences-discuss.net/5/2279/2008/

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Influence of chemosynthetic substrates availability on symbiont densities, carbon assimilation and transfer in the dual symbiotic vent mussel *Bathymodiolus azoricus*

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Received: 7 April 2008 – Accepted: 22 April 2008 – Published: 29 May 2008

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Abstract

High densities of mussels of the genus *Bathymodiolus* are present at hydrothermal vents of the Mid-Atlantic Ridge. It was already proposed that the chemistry at vent sites would affect their sulphide- and methane-oxidizing endosymbionts' abundance. In this study, we confirmed the latter assumption using fluorescence in situ hybridization on *Bathymodiolus azoricus* specimens maintained in a controlled laboratory environment at atmospheric pressure with one, both or none of the chemical substrates. A high level of symbiosis plasticity was observed, methane-oxidizers occupying between 4 and 39% of total bacterial area and both symbionts developing accordingly to the presence or absence of their substrates. Using $H^{13}CO_3^-$ in the presence of sulphide, $^{13}CH_4$ or $^{13}CH_3OH$, we monitored carbon assimilation by the endosymbionts and its translocation to symbiont-free mussel tissues. Although no significant carbon assimilation could be evidenced with methanol, carbon was incorporated from methane and sulphide-oxidized inorganic carbon at rates 3 to 10 times slower in the host muscle tissue than in the symbiont-containing gill tissue. Both symbionts thus contribute actively to *B. azoricus* nutrition and adapt to the availability of their substrates. Further experiments with varying substrate concentrations using the same set-up should provide useful tools to study and even model the effects of changes in hydrothermal fluids on *B. azoricus*' chemosynthetic nutrition.

1 Introduction

Mytilids of the genus *Bathymodiolus* are among the dominant fauna inhabiting sulphide-hydrocarbon cold seeps and hydrothermal vents worldwide (Sibuet and Olu, 1998; Van Dover, 2000). Their nutrition seems to be mainly supported by sulphide- and/or methane-oxidizing bacterial (SOB, MOB) endosymbionts, located in specialized gill epithelial cells (Fisher, 1990). In fact, the coexistence of two distinct bacterial symbionts within a single cell of a multi-cellular eukaryote was demonstrated for the first

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time ever in the gills of *Bathymodiolus* spp. (Cavanaugh et al., 1987, 1992; Fisher et al., 1993). *B. puteoserpentis* and *B. azoricus*, the two mussel species present at Mid-Atlantic Ridge (MAR) vent sites, display the same general characteristics, with two distinct morphotypes of Gram-negative endosymbionts in gill bacteriocytes, and the presence of enzymes specific for sulphide and methane oxidizing metabolisms (type I ribulose 1,5-bisphosphate carboxylase/oxygenase, ATP sulfurylase, adenylyl sulfate reductase, and methanol dehydrogenase) (Robinson et al., 1998; Fiala-Médioni et al., 2002). Phylogenetic analyses show that the two Mytilid species share the same 16S rRNA phylotype of sulphide-oxidizing Gamma-proteobacterium, but harbour two distinct strains (Won et al., 2003). Duperron et al. (2006) found that the MOB 16S rRNA phylotype was also shared between the two mussel species, and that vent chemistry could affect the relative abundance of SOB and MOB. The volume occupied by each type of symbiont present within a bacteriocyte, quantified using a new 3-D fluorescence in situ hybridization (FISH) technique, varies from vent site to vent site between *B. azoricus* specimens (Halary et al., 2008). However, although physiological activity of the symbionts was observed in gill preparations of *B. thermophilus* (Nelson et al., 1995), *B. puteoserpentis* (Robinson et al., 1998) and in live specimens of the seep Bathymodiolid from the Gulf of Mexico (Childress et al., 1986; Fisher and Childress, 1992; Kochevar et al., 1992; Lee and Childress, 1995), it has to date not been demonstrated in live specimens of *B. azoricus*.

Stable isotope studies on Bathymodiolids have indicated the presence of two isotopically (and hence, nutritionally) distinct groups of mussels at the MAR (Trask and Van Dover, 1999; Fiala-Médioni et al., 2002; Colaço et al., 2002a). A first group (δ^{13}C~-30‰) would depend mostly on SOB, while the heavier isotopic values of a -20‰ group could be attributed to a larger reliance on MOB. *B. azoricus* is a complex organism which can derive its carbon and nitrogen from two different symbionts, particulate organic matter and possibly dissolved organic matter. In such a case, the analysis of stable isotopes at natural abundance in bulk tissue, giving information on the "average" diet over a period of time, is insufficient to accurately delineate the con-

tributions from the different food sources. Incubations with ^{13}C-enriched substrates are commonly used to study the activity of pure bacterial cultures as well as different complex samples taken from natural and artificial ecosystems and soils. Autotrophic SOB were already described performing such tracer experiments with ^{13}C-labelled bicarbonate (Knief et al., 2003a), as were communities of physiologically active MOB with $^{13}CH_4$ (Knief et al., 2003b). In the present study, we investigated symbiont populations and mussel nutrition in specimens of *B. azoricus* collected from the MAR and kept in aquaria under various conditions in a controlled laboratory environment (LabHorta facility in the Azores, Colaço and Santos, 2002b). Stable isotope tracer experiments were performed with ^{13}C-enriched bicarbonate, methane or methanol, followed by analysis of ^{13}C incorporation in the gill and muscle tissues. Densities of symbionts were estimated from FISH images using symbiont-specific probes. Results provide evidence that the symbionts develop according to the presence or absence of their substrates, and that inorganic carbon and C_1 substrates are assimilated and further translocated into symbiont-free mussel tissues.

2 Material and methods

2.1 Study site and animal collection

Acoustically retrievable cages were moored in August 2006 during the MOMARETO cruise (R/V "Pourquoi Pas?" IFREMER). They were positioned on diffuse venting areas at the Mid-Atlantic Ridge (MAR) hydrothermal vent field *Menez Gwen* (MG: 37°51′ N, 32°31′ W, 817 m) where mussel populations are very dense and cover virtually all available rock surfaces. Cages were filled with approximately 400 mussels using the French ROV VICTOR 6000. Our experiments were performed on adult mussels (53.6–74.1 mm shell length, mean 65.5±3.7 mm) from two cages recovered in January and May 2007. After a 20 min surfacing time, mussels were transferred to fresh cooled seawater for a 14 h transit to Faial Island (Azores) aboard the Portuguese vessel R/V Arquipélago.

10 specimens of each cage were dissected fresh (wild) on-board and remaining specimens transferred to the Azorean land-based hydrothermal vent laboratory set up, LabHorta. Before the transfer, the mussels' valves were scrubbed clean of visible material and rinsed in chilled seawater.

2.2 Laboratory maintenance

Tracer experiments were preceded by an acclimatization period of 4 days in LabHorta in January and by a maintenance period of 38 days before the start of tracer experiments with May mussels. Mussels were placed in 40 L tanks containing 7.5–10°C seawater (warmer water was found to inactivate methane oxidizers in *B. childressi*; Kochevar et al., 1992). Seawater was replaced every second day (pH 7.5–9) and oxygenated using compressed air to 12.5–67.5% oxygen saturation. A sulphide solution (20 mmol L^{-1} Na_2S in filtered seawater, pH 8.6–9.2) was dispensed discontinuously for 15 min every hour, using a peristaltic pump injecting 2 mL min^{-1}. Concentrations of 1 to 30 μmol L^{-1} were measured using the colorimetric diamine test, in the range of the 0 to 62 μmol L^{-1} measured at Northern MAR vent sites on mussel beds (Sarradin et al., 1998). Methane was bubbled continuously in seawater and dissolved methane monitored by sampling 60 mL seawater in a 300 mL sampling bottle rolled for 20 min before measuring the headspace concentration with a Gasurveyor 524. High methane concentrations above 300 μmol L^{-1} were found inhibitory for symbionts of *B. childressi* exhibiting high methane consumption rates (Kochevar et al., 1992), we thus tried to maintain its concentration below this level (8–91 μmol L^{-1}). Mussels were sampled before the January experiments (n=3), and after 22 (n=3), 32 (n=3), 38 (n=1), 42 (n=3) and 52 (n=3) days maintenance of May mussels. The effect of maintenance was assessed statistically through Kruskal-Wallis non-parametric analyses.

2.3 Stable isotope tracer experiments

All stable isotope enriched chemicals were purchased from Campro Scientific (The Netherlands). January tracer experiments were performed for 15 days on 3 specimens per treatment in artificial seawater (425 mmol L^{-1} NaCl, 9 mmol L^{-1} KCl, 9.3 mmol L^{-1} $CaCl_2$, 25.5 mmol L^{-1} $MgSO_4$, 23 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} total $NaHCO_3$; adjusted to pH 8) filtered on a 0.2 μm membrane and replaced every second day. Seawater was prepared with 10% $NaH^{13}CO_3$ (99% ^{13}C) and supplemented with sulphide (2–14 μmol L^{-1}). Two control experiments were run in parallel with either no addition of sulphide or the addition of sulphide with un-enriched bicarbonate.

May experiments were performed on 14 mussels per condition for up to 20 days. 0.2 μm-filtered natural seawater was supplemented with either methane (14–200 μmol L^{-1}, 25% ^{13}C), or hydrogen sulphide with bicarbonate (H_2S 0–32 μmol L^{-1} supplemented with $NaH^{13}CO_3$ 99% to obtain 2.85 mmol L^{-1}, 16.3% ^{13}C), or methanol (34 μmol L^{-1}, 22.2% ^{13}C). Two control experiments were run in parallel with either no addition of sulphide and methane, or with both sulphide and un-enriched methane, both in the presence of natural seawater bicarbonate (not supplemented with $NaH^{13}CO_3$). Temperature, pH and oxygen levels were monitored. One specimen of each experiment was sampled after 1 day enrichment, 3 specimens after 4 days (5 days in the case of the methane experiment) and 10 after 20 days (15 days with methane). At each sampling, mussels were opened and rinsed in distilled water to discard unincorporated tracers. The most anterior part of the left gill was removed from the specimens collected after 4 or 5 days experiment and fixed for FISH analysis in 2% formaldehyde.

All specimens (wild and from the maintenance and tracer experiments) were dissected into gill, mantle, muscle and remaining tissues which were frozen and lyophilized. Each tissue's dry weight was determined and a gill index (GI) was calculated from the gill tissue dry weight and shell length according to the following formula:

$$GI = [\text{gill tissue dry weight (g)/shell length (mm)}] \times 100 \quad (1)$$

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2.4 Fatty acids extraction

Lyophilized tissues were ground to a fine powder using a mortar and pestle. Lipids were extracted using a modified version of the Bligh and Dyer method (White et al., 1979) using chloroform/methanol/phosphate buffer pH 7.4 in 1:1:0.9 volume proportions. Total lipids retrieved in the chloroform phase were partitioned on silicic acid columns by sequential elution with chloroform, acetone, and methanol into apolar, neutral and polar lipids. Portions of the lipid extracts were transferred into 10 mL screw-top test tubes, previously decontaminated for 4 h at 450°C, to which a fresh solution of methanol/hydrochloric acid/chloroform (10:1:1 volume, 3 mL) was added for a transmethylation reaction run at 90°C for 60 min to form fatty acid methyl esters (FAMEs).

2.5 Stable isotope analyses

FAMEs chloroform extracts were transferred and dried under mild conditions (37°C, to avoid the evaporation of the most volatile FAMEs) into tin capsules (for liquids) previously decontaminated with acetone. Aliquots of lyophilized tissue powder were weighed into silver cups, previously decontaminated for 4 h at 450°C, and acidified with a few drops of dilute HCl (5%) before analysis, to remove any possible trace of carbonates. Tissues were re-dried overnight at 60°C. δ^{13}C analyses were performed on a Flash1112 elemental analyzer, coupled to a Delta V via a Conflo III interface (Thermo Finnigan). All carbon stable isotope ratios are expressed relative to the conventional standards (VPDB limestone) as δ values, defined as:

$$\delta^{13}\mathrm{C}=[(R_{\mathrm{sample}}-R_{\mathrm{standard}})/R_{\mathrm{standard}}] \times 10^3\ [‰] \text{ where } R={}^{13}\mathrm{C}/{}^{12}\mathrm{C} \quad (2)$$

Or as Atom percent values, defined as:

$$A = [{}^{13}\mathrm{C}/({}^{13}\mathrm{C} + {}^{12}\mathrm{C})] \times 100[\%] \quad (3)$$

The reference material used as standard for carbon isotopic ratio measurement was sucrose (IAEA-CH-6). The standard followed the same analytical processes as the unknown samples.

Net carbon incorporation in the mussel tissues (C_{inc}, in μmol C g tissue^{-1}) was calculated using the following formula:

$$C_{inc}=[(A_{exp}-A_{control}) \times C_{tissue}]/(A_{substrate}-A_{control}) \quad (4)$$

where A_{exp} is the ^{13}C atom percent measured in the mussel after the tracer experiment, $A_{control}$ is the ^{13}C atom percent measured in the control mussel, C_{tissue} is the carbon content of the tissue analyzed (μmol C g tissue^{-1}) and $A_{substrate}$ the ^{13}C atom percent of the substrate used in the experiment (i.e. 25% for CH_4, as given by the supplier, or 16.3% for $NaHCO_3$ and 22.2% for CH_3OH, as calculated from the preparation).

2.6 Fluorescence In Situ Hybridization (FISH)

FISH was performed according to the protocol used by Halary et al. (2008) with a few modifications. Upon recovery of mussel specimens, the most anterior part of their left gill was fixed in 2% formaldehyde (2–4 h, 4°C), rinsed twice with filtered seawater, and stored at −20°C in 1:1 ethanol/filtered seawater. In the lab, gill tissue was embedded in polyester wax and 10 μm-thick sections were cut using a microtome (JUNG, Heidelberg, Germany), collected on Superfrost® Plus slides (Roth, Germany), and hybridized as previously described (Halary et al., 2008) using the ATTO488-labelled ImedM probe (5′-ACCAGGTTGTCCCCCACTAA-3′, Duperron et al., 2008) specific for the methane-oxidizing symbiont and the Cy5-labelled BangT-642 probe (5′-CCTATACTCTAGCTTGCCAG-3′, Duperron et al., 2005) specific for the sulphide-oxidizing symbiont. Images were acquired using a BX61 microscope (Olympus Optical Co., Tokyo, Japan) under a 10× objective (NA 1.30) and analyzed using the ImageJ software (Abramoff, 2001). The green channel corresponding to the ImedM probe (methane-oxidizers) and the blue channel corresponding to the BangT probe (sulphide-oxidizers) were binarized by applying a luminosity threshold to distinguish between background fluorescence and probe signal, and numbers of pixels belonging to each type of symbiont were computed.

Total area occupied by bacteria and respective proportions of each phylotype were computed for the whole image. For an inter-comparison between images, bacterial areas were normalized by dividing areas by the total length of filaments visible and measured on each picture.

3 Results

3.1 Maintenance conditions effects

Before the tracer experiments, mussels were maintained in aquarium at atmospheric pressure in the presence of 30±16 μmol L^{-1} CH_4 (8–92 μmol L^{-1}) and 12±9 μmol L^{-1} H_2S to support the presence of endosymbionts. Mussels collected in January were allowed to recover from the decompression stress during 5 days at atmospheric pressure (transport followed by LabHorta). A quick drop of their gill index (GI) could be noticed over this short period (Fig. 1). This drop was also observed in May mussels maintained with H_2S and CH_4 up to 38 days before the tracer experiments (aquarium mean GI=0.46, n=11; wild animals mean GI=0.76, n=10, Kruskal-Wallis p=0.001, Fig. 1). Following this rapid loss in gill weight, no significant difference could further be evidenced between individuals collected at different time points of the maintenance period (Kruskal-Wallis p=0.475). GIs showed a significant decrease after 20 days tracer experiment (symbolized by an arrow on Fig. 1) with H_2S (Kruskal-Wallis p=0.016, n=10), a trend also observed for mussels kept in filtered seawater with dissolved enriched CH_4.

3.2 Tracer repartition in tissues and specific compound classes

Assimilation of ^{13}C from HCO_3^- was observed in the presence of H_2S (Fig. 2). Highest incorporation was found in the gill tissue, followed by other tissues such as the digestive tract and vital organs which displayed much lower incorporations. Slight assimilation of

inorganic carbon was also observed in the absence of H_2S, although to a much lesser extent. This coupling between HCO_3^- assimilation and the presence of H_2S was also observed in analyses of fatty acid methyl esters (FAMEs, including a small contribution from unlabeled C from the trans-methylation) from the polar lipid extracts of the different control experiments (Fig. 3). $\delta^{13}C$ analysis of FAMEs from lipid fractions of mussel A (the mussel specimen displaying the highest $\delta^{13}C$ values after January experiments) showed highest enrichment in polar fatty acids containing the phospholipid fatty acids (PLFA), followed by the neutral fatty acids found in part in glycolipids or as free fatty acids. A significantly lower increase in $\delta^{13}C$ was found in fatty acids associated with the apolar lipid fraction among which can be found di- and tri-acylglycerols and cholesterol derivatives.

3.3 Fixation rates

After 4 and 5 days of exposure to ^{13}C-labelled HCO_3^- (in the presence of H_2S) or CH_4, *B. azoricus* gill tissue already showed ^{13}C abundances significantly higher than background levels (Kruskal-Wallis p=0.049 for both treatments). At each sampling time, $\delta^{13}C$ values tended to be higher in gill than in muscle tissue. Labeling of the gill tissue appeared uniform between the different mussel specimens after 4 days in H_2S. However, strong differences in $\delta^{13}C$ between individuals were observed after 15 days with enriched CH_4 and 20 days with H_2S and enriched $NaHCO_3$. In the gill tissue, carbon incorporation rates varied from 151 to 323 nmol C g dry tissue^{-1} h^{-1} in the H_2S + labeled $NaHCO_3$ experiment, and from 56 to 228 nmol C g dry tissue^{-1} h^{-1} in the experiment with labeled CH_4 (Fig. 4, left panel). Although mussels seemed to incorporate efficiently enriched carbon from CH_3OH during the first days of the experiment, no further enrichment could be observed after the last 16 days. No incorporation rate was thus calculated for the experiment with methanol.

Under the hypothesis that carbon is assimilated by endosymbionts located in the gill tissue and then transferred to other tissues (see discussion) rates of carbon transfer

can be calculated. Transfer between gill and muscle tissues ranged from 52 to 104 nmol C g dry tissue^{-1} h^{-1} in the H_2S + labeled $NaHCO_3$ experiment, and from 11 to 21 nmol C g dry tissue^{-1} h^{-1} in the CH_4 experiment (Fig. 4, right panel).

3.4 Endosymbiont populations' response to experimental treatments

Gill filaments from May mussels were observed using FISH to assess the effects of the maintenance period in LabHorta and of the tracer experiments on symbiont populations (Fig. 5). Symbiont quantification results for a wild specimen, a specimen collected at the start of the experiments (after the acclimatization period) and others after 4 to 5 days in the absence or presence of either or both CH_4 and H_2S are displayed in Table 1. Gill filaments of aquarium specimens appeared narrower than the ones from a wild mussel dissected immediately after the cage recovery, and displayed much less bacteria (Fig. 5a–b). Quantifications confirmed a decrease in area covered by bacteria by a factor of 4 (from 16.7 to 3.7 $\mu m^2/\mu m$ filament), and indicated a shift in symbiont relative areas, with methane-oxidizing bacteria (MOB) representing 14% of the total area occupied by bacteria after acclimatization, versus 24% before (Table 1). At the beginning of the tracer experiments, bacterial densities remained low. A mussel kept in CH_4+H_2S, but at a different ratio with decreasing H_2S and 1/3rd lower CH_4 concentrations than during acclimatization, presented an overall lower bacterial area (1.7 $\mu m^2/\mu m$ filaments versus 3.7 before the tracer experiments, Fig. 5c). In these conditions, sulphide-oxidizing bacteria (SOB) area appeared to decrease slower than the MOB area, when compared with the mussel collected at the end of the acclimatization period. A mussel kept four days in filtered seawater devoid of CH_4 and H_2S also displayed a decrease in total area occupied by bacteria down to 2.1 $\mu m^2/\mu m$ filaments (Fig. 5d). The exclusive presence of 86.9±98.0 $\mu mol\,L^{-1}$ CH_4 favored an increase in the relative abundance of MOB to 39% of total bacterial area (Fig. 5e). Four days enrichment with exclusively 7.7±1.6 $\mu mol\,L^{-1}$ H_2S resulted in an increase in the overall endosymbiotic population and in the proportion of SOB representing 96% of total

bacterial area (Fig. 5f).

4 Discussion

Because of its capacity to recover and survive in aquaria for several months after the post-retrieval decompression stress, increasing data is being obtained from live *B. azoricus* maintained in aquaria at atmospheric pressure in the presence or absence of chemosynthetic substrates for the endosymbionts. Immune responses (Bettencourt et al., 2007), biochemical responses to recovery stress (Dixon et al., 2004) and to metal exposure (Company et al., 2008) have been investigated using such settings. Dixon et al. (2004) saw higher levels of DNA damage in mussels kept in the presence of methane and sulphide than in some kept without any "food supplement". In a recent study Bettencourt et al. (2008) investigated the physiological state of mussels during acclimatization to atmospheric pressure in the absence of dissolved gases over the course of several months post-capture and concluded that our laboratory set up was a suitable system to study physiological reactions. Response of symbionts to the experimental conditions was barely examined. A preliminary study done on sulphide-oxidizing bacterial symbionts (SOB) showed, on transmission electron micrographs, the loss and potential re-acquisition of SOB (Kadar et al., 2005).

In this study we tried to gain insight on the symbiont densities, physiology and impact on the nutrition of *B. azoricus* nutrition. Results from FISH observations and GIs indicate that despite a strong post-collection initial stress, with a marked decrease in total symbiont abundances and gill dry weight, both bacterial populations can be maintained in the gill tissue of mussels kept in aquaria. Furthermore, incubation experiments indicate that the presence of either sulphide or methane alone favors the increase in absolute and relative abundance of SOB and methane-oxidizing bacterial symbionts (MOB), respectively. This supports the hypothesis that symbiont populations quickly react to changes in environmental parameters, in particular to the balance between sulphide and methane, and thus optimize the use of available compounds as suggested

by previous authors (Trask and Van Dover, 1999; Colaço et al., 2002; Salerno et al., 2005; Duperron et al., 2006). The range of variation observed in symbiont relative abundances, with MOB occupying between 4 and 39% of total bacterial area, confirms the high level of plasticity of the dual symbiosis. In the absence of both sulphide and methane, MOB seem less affected than SOB, which could be due to their ability to use alternate carbon sources, for example by expressing ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) as demonstrated in a vent Bathymodiolid from the Mid-Okinawa Trough (Elsaied et al., 2006). The rate of digestion of each symbiont by the mussel, combined with their respective size (1.25 μm mean diameter for MOB, 0.35 μm for SOB; Fiala-Médioni et al., 2002) could also have a considerable impact on MOB/SOB surface ratios evolution with time. Although data must be taken with caution as only one individual per experimental condition was analyzed, the fact that observations qualitatively agree with expected results is a strong indication that our set-up is appropriate for the enrichment of each endosymbiont. Recently, a 3-D-FISH technique was employed to quantify the effect of a sulphide pulse in a similar experimental set-up, on the volume and relative abundance of symbionts in mussel gills (Halary et al., 2008). We herein provide the first indication that MOB also respond to a methane pulse.

Stable isotope tracer experiments evidenced that H_2S is needed for the assimilation of inorganic carbon and that, within the gill lipid fractions, this carbon is first incorporated into phospholipids which are major components of Gram-negative bacterial membranes (Kates, 1964). This confirms the chemoautotrophic sulphide-oxidizing activity of one of the endosymbionts in our experimental setting. We could also see a slight assimilation of $H^{13}CO_3^-$ in the absence of H_2S. This could be explained by the hypotheses that i) Sulphur or thiosulfate reserves are present in the gill tissue, although granules of elemental sulphur were never observed in *B. azoricus*; ii) MOB would harbor a RubisCO activity as observed in type X-methane oxidizers (Baxter et al., 2002) and in *Bathymodiolus sp.* (Elsaied et al., 2006); or iii) SOB is capable of using ATP from the host mussel to assimilate inorganic carbon. However, this carbon incorporation could be considered negligible when the $\delta^{13}C$ variation was converted to moles of

carbon incorporated, and more data is needed to confirm that it is significant.

Distribution of labeled carbon in tissues suggests that in the case of carbon uptake by SOB, inorganic carbon is first incorporated by bacteria within the gill tissue, then transferred to the rest of the tissues including the digestive system and vital organs, muscle and mantle tissues being the last ones to get this carbon. Although enrichment of the different tissues is only displayed for a single mussel here, we could observe the same pattern in 2 additional mussels from other enrichment attempts with H_2S and $NaH^{13}CO_3$ (data not shown). This hypothesis of carbon transfer from the gills to symbiont-free tissue was suggested for a seep *Bathymodiolus* sp. by Fisher and Childress (1992) who could observe an increase of radiolabel coming from $^{14}CH_4$ in symbiont-free tissues during a chase period. The latter authors suggested that the transfer might occur by intracellular digestion of the symbionts, from the observation of partially degraded bacteria in bacteriocyte bodies with lysosomal activity (also observed in *B. azoricus* by Fiala-Médioni et al., 2002). This could explain the fact that tissues in direct connection with the gill would benefit first from the carbon assimilated by SOB. Alternatively, the symbionts, grown into the mussel cell, could have been released in the food grove of the gills and passed through the digestive system. Slow transfer to muscle tissue, also observed with carbon incorporated by MOB, may also reflect differences in turnover: the gill tissue harbors bacteria that are continuously doubling and being digested, and hence show a ^{13}C abundance more like that of the symbionts; muscle tissue, in contrast, contains exclusively C accumulated through the entire life of the mussel, and its ^{13}C abundance, therefore, is less responsive to recently ingested food. Rough calculations gave carbon incorporation rates 3 times lower in muscle tissue than in symbiont-containing gill tissue for SOB and 5 to 10 times lower for MOB. Kochevar et al. (1992) described in more details the characteristics inherent to MOB from a seep Bathymodiolid in a study using $^{14}CH_4$. They showed that about 70% of the consumed methane was being incorporated as organic compounds and the highest net carbon incorporation rate was obtained at 250 μmol L^{-1} dissolved methane, with 5 μmoles carbon incorporated per gram whole mussel soft tissue wet weight per hour.

Our experiments were performed at 5 times lower methane concentration, and as rates depend upon concentration of substrates and are probably influenced by the physiological state of the animals and bacteria, we can not extrapolate our results to mussels in their home environment. However, these rates estimates, reported as amounts of carbon incorporated per dry specific tissue weight per hour, appear realistic in view of the results reported for *B. childressi* (Kochevar et al., 1992).

In an attempt to describe the physiology of MOB symbionts in more detail, we tested the possibility of C incorporation from methanol. Indeed, methanol dehydrogenase, necessary for growth on either methane or methanol, was found to be active in the gills of *B. azoricus* (Fiala-Médioni et al., 2002), and putative methanol-oxidizing symbionts were identified in the seep mussel *B. heckerae* by Duperron et al. (2007). Methanol, if available, would seem a better carbon and energy source compared to methane because the first step of methanotrophy, producing methanol, does not yield energy. However, no significant carbon uptake from methanol was observed, questioning whether symbionts can even get access to this potential resource.

Although working at atmospheric pressure, the LabHorta aquarium setting can thus be employed to study the dynamics of symbiont populations. In the future, pulses of varying intensities and durations or parallel measurements of rates of symbiont digestion by the host should be foreseen. Together with the identification of biomarkers for the endosymbionts following tracer experiments, such experiments should provide useful tools to study the effects of changes in hydrothermal fluids on the chemosynthetic nutrition of the Mid-Atlantic Ridge mussel and gain an insight on the extent to which the mussel beds interact with and impact their surroundings.

Acknowledgements. We thank C. Leal, V. Costa, M. Laranjo, H. Lopez and L. Pires for the experimental support, and P. Bodelier for helpful advice. Our acknowledgements also go to the Victor 6000 team, crews of R/V “Arquipélago” and “Pourquoi Pas?” and chief scientists of the MoMARETO cruise J. Sarrazin and P. M. Sarradin. Financial support for this research was provided by the EU Framework Contract No EVK3-CT1999-00003 (VENTOX, cage system), the Azorean DRCT (Portugal) and the Portuguese FCT (LabHorta and IMAR-DOP/UAç Research Unit #531 and Associate Laboratory #9 lab work and SFRH/BPD/35808/2007, grant RB), and

by the EU research training network MoMARnet-FP6-RTN/2003/505026 (grant VR), the EU network of excellence REX-3DEM (CT 502828, grant SH) and FWO-Vlaanderen (G.0632.06).

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Table 1. Results from rough endosymbiont quantifications obtained by FISH image processing. CH_4/ H_2S ratios are indicative, and in the case of fresh mussels, the value of 1.2 reported is a mean from end-member fluids at Menez Gwen (0.67–1.75, Desbruyères et al., 2000), which might not be representative of the situation in the immediate environment of mussels.

	CH_4/ H_2S	Surf Symb μm^2/ Fil length μm	% MOB	% SOB	MOB/SOB
Fresh	1.2	16.7	24	76	0.32
38 days H_2S+CH_4	2.4	3.7	14	86	0.17
+4 days H_2S+CH_4	1.8	1.7	8	92	0.09
+4 days Nothing	(–)	2.1	29	71	0.41
+4 days H_2S	100% H_2S	5.1	4	96	0.04
+5 days CH_4	100% CH_4	3.1	39	61	0.64

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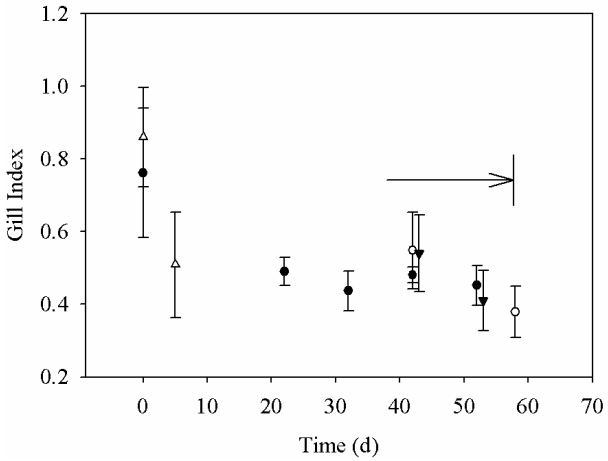

Fig. 1. Variations in gill index with time spent in aquarium and with the presence of methane and/or sulphide (mean±SD). Empty triangles: January fresh and control mussels exposed to methane and sulphide, filled circles: May fresh and control exposed to methane and sulphide, empty circles: May sulphide + bicarbonate tracer mussels, filled triangles: May methane tracer mussels.

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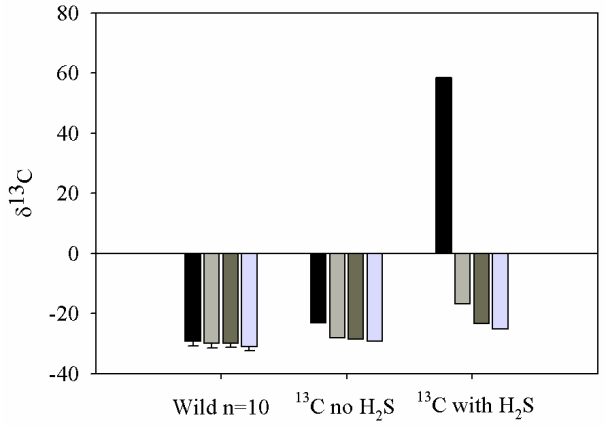

Fig. 2. $\delta^{13}C$ values measured in tissues of mussels collected in January (from left to right bar: gill, rest, mantle, muscle). Wild (mean±SD, n=10) or after 5 days acclimatization at atmospheric pressure followed by 15 days in the presence of 10% ^{13}C enriched bicarbonate with (^{13}C with H_2S, n=1) or without (^{13}C no H_2S, mussel A) hydrogen sulphide.

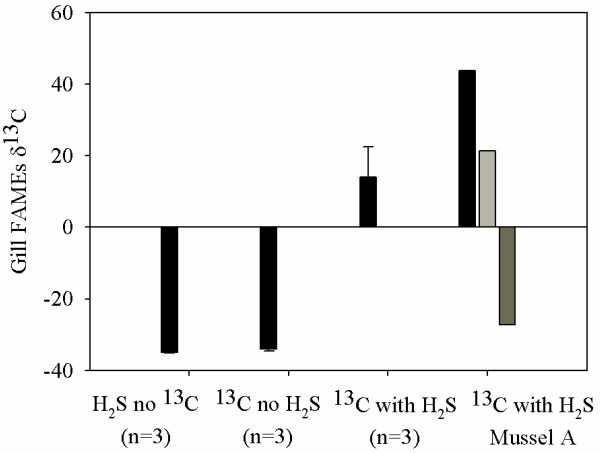

Fig. 3. $\delta^{13}C$ values measured in gill tissue PLFA (black bars) and in fatty acids of the different lipid classes extracted from the specimen displaying the highest bulk tissue $\delta^{13}C$ values after the $^{13}C+H_2S$ experiment (Mussel A. Black bars: polar, mean±SD, light grey bar: neutral, dark grey bar: apolar).

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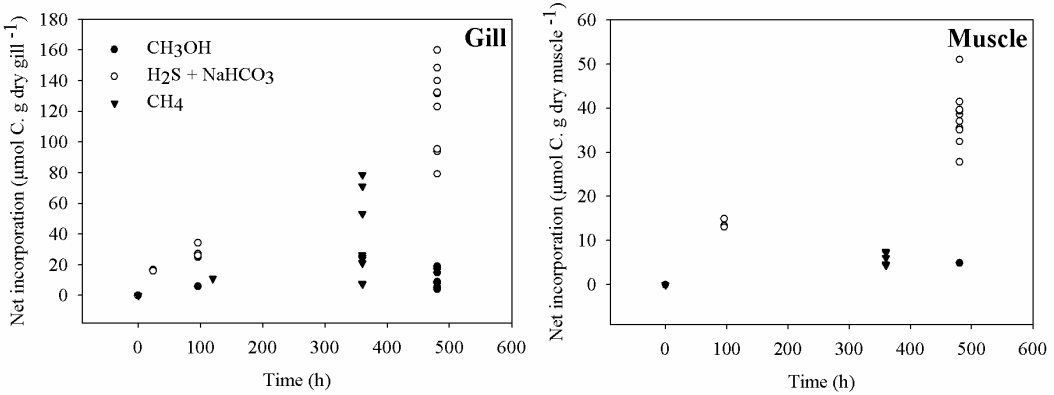

Fig. 4. Carbon net incorporation through time in gill (left panel) and muscle (right panel) tissues from the different experiments (Empty circles: sulphide plus bicarbonate, filled triangles: methane, filled circles: methanol).

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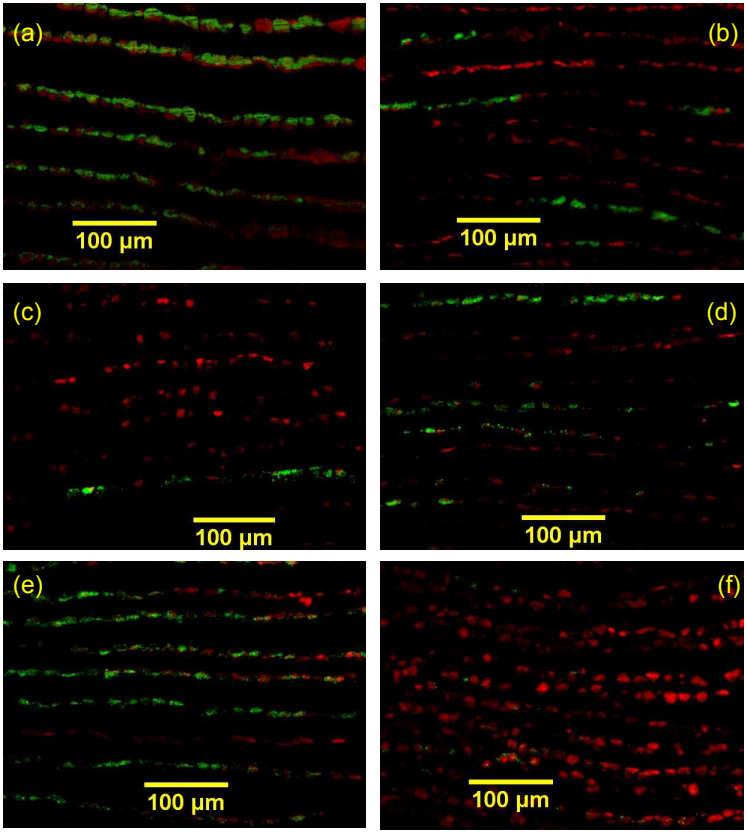

Fig. 5. FISH observations of transverse sections of gill filaments, displaying methane- (green) and sulphide-oxidisers (red). (a–b) gills from a fresh specimen **(a)** and a specimen maintained 38 days **(b)** in LabHorta with H_2S+CH_4. (d–f) Specimens observed 4-5 days after the beginning of the tracer experiments: 4 days with H_2S+CH_4 **(c)**; 4 days neither H_2S nor CH_4 **(d)**; 5 days with CH_4 **(e)**; 4 days with H_2S **(f)**.