

The description of the labeling experiments is a bit too condensed. They are fully described in previous publications, but it would help the reader to provide a general description of the experiments (experimental set up, actual amounts of label added, conditions during the experiment etc).

Referring to Reviewer 2's suggestions, paragraph 2.2 was changed for:

“All stable isotope enriched chemicals were purchased from Campro Scientific (The Netherlands). For the experiments, 42 adult mussels (55.6 to 72.4 mm Shell Length) were dispatched into 4 separate refrigerated 4-L aquaria with aerated 0.2  $\mu\text{m}$ -filtered natural seawater replaced every second day and monitored daily for temperature (7.8–9.8°C), pH (7.1–8.6) and O<sub>2</sub> saturation (median: 45%). The available quantity of labelled methane (25% <sup>13</sup>C) allowed running the tracer experiment with <sup>13</sup>CH<sub>4</sub> for a period of 15 days with a final dissolved concentration ranging between 14-200  $\mu\text{mol L}^{-1}$  (Riou et al., 2008). The control experiment (with filtered seawater only) and the experiments with NaH<sup>13</sup>CO<sub>3</sub> (99% added to the filtered seawater to obtain a concentration of 2.85 mmol L<sup>-1</sup>, 16.3% <sup>13</sup>C) + H<sub>2</sub>S (0-32  $\mu\text{mol L}^{-1}$ , Riou et al., 2008) or with 9 mg L<sup>-1</sup> of a <sup>13</sup>C-labeled amino acid mixture (98% <sup>13</sup>C, Riou et al., 2010) were continued over a period of 20 days. The amino acid mixture consisted of Gly 20-25%, Ala 15-20%, Tyr 10-15%, Leu 5-10%, Lys 5-10%, Ser 5-10%, Thr 2-5%, Phe 1-5%, Pro 1-5%, Val 1-5%, Met < 3%, Trp < 1%, Ile < 1%, His < 1%.

Three mussels from each experiment were selected for fatty acid analysis, and dissected into gill, mantle, muscle, and remaining tissues. Mussel tissues were immediately stored at -20°C till they were freeze-dried, a few days after dissection.”

It is not clear why PLFA were analyzed for the CO<sub>2</sub> and CH<sub>4</sub> incubations and total fatty acids (tFA) for the labeling experiment with amino acids. This should be explained in the manuscript.

Unlike plant and animal cells, bacteria do not contain *acyl lipid* stores (lipids containing O- or N-ester or ether linked fatty acids), and it is believed that their acyl lipids are confined to membranes. In addition, phospholipids represent 90 to 98% of bacterial lipids (King et al. 1977, White et al. 1979) and around 50% of eukaryotic lipids (Vestal & White 1989). Studying the PLFA somehow “concentrates” the signal of the bacteria, since the bacterial symbionts are hosted inside mussel cells, and in a tissue total lipid extract, the symbionts' fatty acid molecules are “diluted” by the fatty acids of the eukaryotic cells. In the perspective of identifying the fatty acids that are produced by the symbionts from the assimilation of CH<sub>4</sub> and CO<sub>2</sub> (in the presence of sulfide), it is thus preferred to examine the PLFA.

On the opposite, the fatty acid analysis in specimens incubated with enriched amino acids was designed both i) to observe the occurrence of lipogenesis from the assimilation of amino acids by the Mytilid cell machinery (which could also use free amino acids as osmotic regulators of the cells, metabolic fuel, protein synthesis or in the glucogenesis) AND ii) to try to answer the question of the autotrophy/mixotrophy of the symbionts. Thus, to retain most of the information relative to both the symbionts and the host's fatty acids, we preferred to use a technique with a high fatty acid yield and to analyse total fatty acids.

To clarify this, the end of the introduction modified to:

“In order to understand the modes of matter and energy transfer to the host, the phenotypes of *B. azoricus* symbionts' need to be characterized. Classification of phospholipid ester-linked fatty acids (PLFA) profiles has proven useful to clarify bacterial genus and species interrelationships established by DNA-based phylogeny (Bodelier et al., 2009). PLFA profiles thus allow to distinguish between different methane-oxidising bacteria (MOB) species as evidenced for example by the fact that methane-oxidising gamma-proteobacteria (formerly named after “Type I MOB”) mainly contain fatty acids with 14 and 16 carbon atoms, while

methane-oxidising alpha-proteobacteria (formerly “Type II MOB”) PLFA are mainly composed of 18 carbon atoms (Nichols et al., 1985). In addition, MOB bacteria possess fatty acids that are not found in any other known microorganism (methane-oxidising gamma-proteobacteria: 16:1(n-8) and 16:1(n-5); alpha-proteobacteria: 18:1(n-8)) and these compounds therefore represent valuable biomarkers (e.g. Nichols et al., 1985).

The main difficulty consists in the impossibility to grow *B. azoricus*’ symbionts in pure cultures. Tracer experiments represent powerful tools enabling the detection of the bacterial fatty acid signal inside the host. However, in a tissue total lipid extract, the symbionts’ fatty acid molecules are “diluted” by the fatty acids of the eukaryotic cells since the bacterial symbionts are hosted inside mussel cells. Unlike plant and animal cells, bacteria do not contain acyl lipid stores (lipids containing O- or N-ester or ether linked fatty acids), and it is believed that their acyl lipids are confined to membranes. Phospholipids represent 90 to 98% of bacterial lipids (King et al. 1977, White et al. 1979) and around 50% of eukaryotic lipids (Vestal & White 1989). Studying PLFA thus somehow “concentrates” the signal of the bacteria, and is more adapted than a total fatty acid study in the perspective of identifying the fatty acids that are produced by the symbionts from the assimilation of CH<sub>4</sub> and CO<sub>2</sub> (in the presence of sulfide).

In the present study, we thus investigated the incorporation of <sup>13</sup>C-labeled HCO<sub>3</sub><sup>-</sup> (in the presence of H<sub>2</sub>S) or CH<sub>4</sub> into gill-extracted PLFA from *B. azoricus* in order to assess the metabolic activity of the endosymbionts. This enabled us to establish specific fatty acid (FA) patterns which provide insights into the symbiont phenotypes. On the contrary, a technique with a high yield of tissue total fatty acid (tFA) recovery was preferred to retain most of the information relative to both the symbionts and the host’s fatty acids when analysing the assimilation of <sup>13</sup>C-labeled amino acids (dissolved into seawater) into *B. azoricus* gill fatty acids. This last experiment was indeed designed to observe i) the potential for heterotrophic growth by the symbionts, and/or ii) the occurrence of lipogenesis from the assimilation of amino acids by the Mytilid cell machinery (which could also use free amino acids as osmotic regulators of the cells, metabolic fuel, protein synthesis or in the glucogenesis). Close examination of the labeled FA patterns helped interpreting physiological processes occurring in *B. azoricus*.”

The data in Fig. 2 are mixed up: black bars present <sup>13</sup>CO<sub>2</sub>+H<sub>2</sub>S data and grey bars <sup>13</sup>CH<sub>4</sub> data. In addition, I do not see why the data for some of the highest labeled PLFA (Fig 2) and total FA (Fig 3) are cut off. Labeling levels are apparently higher (by how much?) than the y-axis range, but it seems to me that the highest labeled compounds are the most interesting ones. One may lose some resolution on the lower labeled compounds if the whole range is shown, but these are not that important anyway.

Figure 2 was modified according to Reviewer 1’s comments, and in view of Reviewer 2’s concerns the bar colors were changed and the whole range of values is now shown on a modified scale for Figs 2 and 3.

Finally, in the heading to Fig 1, NMID is explained but this is not seen in the figure.

The part “NMID: non-methylene interrupted di-unsaturated,” was removed from the caption of Fig. 1.

I would also add a figure showing the PLFA and tFA concentrations in gill tissue. These are now mentioned in the discussion in several places, but it would help to see the data.

In this sense, Figure 4 was added to the manuscript:

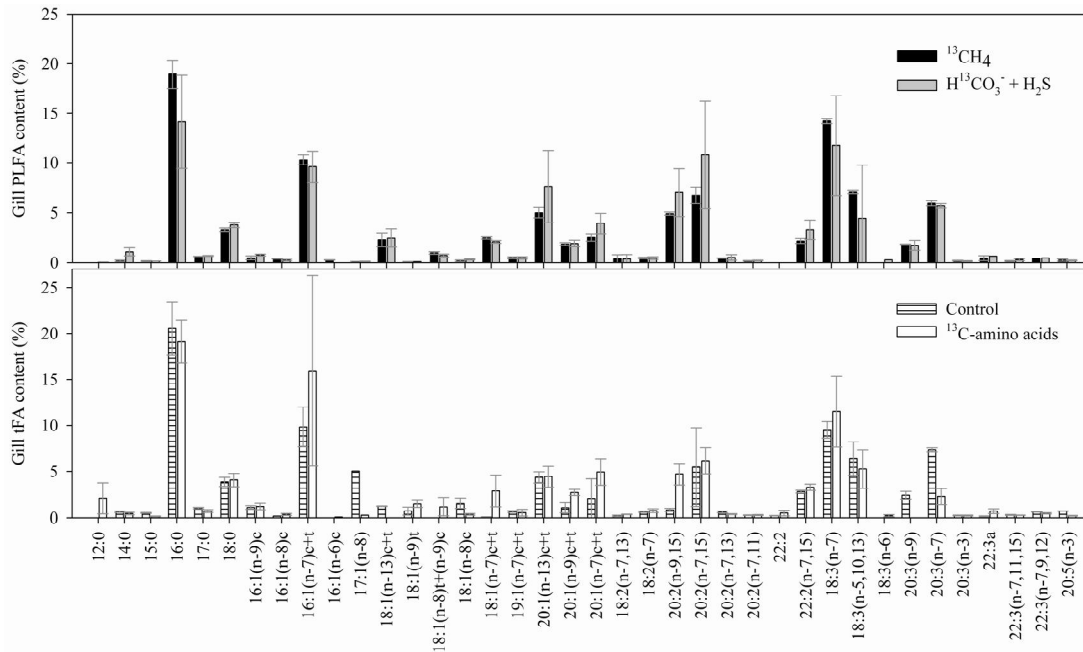


Figure 4: *Bathymodiolus azoricus* gill tissue PLFA or tFA content (n=3, average  $\pm$  standard deviation) after a 15 day supply with  $^{13}\text{CH}_4$  (black bars), or a 20 day supply with  $\text{H}^{13}\text{CO}_3^-$  in the presence of  $\text{H}_2\text{S}$  (grey bars), or with  $^{13}\text{C}$ -amino acids (white bars); striped bars represent control mussels.

In the amino-acid experiment, labeling was highest in 12:0 and 16:1(n-8)c, and label was also recovered in 16:1(n-6)c. These compounds are also highly labeled in the  $\text{CH}_4$  experiment and the two mono-unsaturated FA are used as biomarkers for methanotrophs. Doesn't this indicate that methanotrophic symbionts were important in adsorption of amino acids? This is not widely discussed as far as I can tell, and most of the discussion deals with the direct utilization of amino acids by the mussel..... Finally, given this finding the authors may reconsider some of the discussion on page 3465 about the substrates that can be used by symbiotic bacteria.

With respect to that comment, we modified the last paragraph of the discussion: "The incorporation of amino acid  $^{13}\text{C}$  into SOX and MOB FA biomarkers (12:0 and 16:1(n-8) and (n-6), respectively) might indicate that the symbionts could also be assimilating the amino acids directly. Bacteria indeed possess effective metabolic mechanisms..." Which was emphasized at the end of the paragraph: "However, our finding that some of the MOB (16:1(n-8) and (n-6)) and SOX (12:0) biomarkers were labeled after incubation with  $^{13}\text{C}$ -amino acids indicates that the symbionts had at least access to acetyl coA (or acetate) produced during the degradation of the amino acids by the host, and/or that they have the capacity to absorb and metabolise external amino acids. Further experiments are needed to ascertain these hypotheses."

Also, the sentence starting on page 3464 line 14, is basically incorrect as it doesn't acknowledge the high labeling in these two methanotrophic marker FA.

This sentence was mainly directed at drawing the attention to the (n-7) series. We might have expressed ourselves incorrectly, and for more clarity, the sentence was changed to:

“As can be noticed from Fig. 3, FA belonging to the (n-7) series were particularly enriched, with 16:1(n-7) showing the highest  $^{13}\text{C}$  incorporation within this series, followed by 18:1(n-7), 20:1(n-7), 18:3(n-7) and 18:2(n-7).”

Minor comments. P3458L11. “ The isotopic composition of individual FAME was : : .”

Corrected in the revised version that will be uploaded.

P3458L24. “ The d13C ratios of FAMES were corrected for the addition of one : : .”

The sentence was changed for: “The  $\delta^{13}\text{C}$  ratio of each FAME was corrected for the addition of one methanol carbon per molecule to obtain the isotopic signature of the fatty acid as in Abrajano et al. (1994) using the mass balance equation taking a measured  $\delta^{13}\text{C}_{\text{CH}_3\text{OH}}$  value of -40.3‰.”

P3459L27. “ to discuss fatty acid synthesis. “

Corrected in the revised version that will be uploaded.

P3463L21. NMID P3465L1. delete “switch on/switch off”

Deleted in the revised version that will be uploaded.