Use of carbon monoxide and hydrogen by a bacteria–animal symbiosis from seagrass sediments

Manuel Kleiner,1,2++ Cecilia Wentrup,1,3+++ Thomas Holler,1 Gaute Lavik,1 Jens Harder,1 Christian Lott,1,4 Sten Littmann,1 Marcel M. M. Kuypers1 and Nicole Dubilier1
1Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, Bremen 28359, Germany.
2Department of Geoscience, University of Calgary, 2500 University Drive, Calgary, AB T2N 1N4, Canada.
3Department of Microbiology and Ecosystem Science, Division of Microbial Ecology, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria.
4Elba Field Station, HYDRA Institute for Marine Sciences, Via del Forno 80, Campo nell’Elba, LI 57034, Italy.

Summary
The gutless marine worm Olavius algarvensis lives in symbiosis with chemosynthetic bacteria that provide nutrition by fixing carbon dioxide (CO2) into biomass using reduced sulfur compounds as energy sources. A recent metaproteomic analysis of the O. algarvensis symbiosis indicated that carbon monoxide (CO) and hydrogen (H2) might also be used as energy sources.

We provide direct evidence that the O. algarvensis symbiosis consumes CO and H2. Single cell imaging using nanoscale secondary ion mass spectrometry revealed that one of the symbionts, the γ3-symbiont, uses the energy from CO oxidation to fix CO2. Pore water analysis revealed considerable in-situ concentrations of CO and H2 in the O. algarvensis environment, Mediterranean seagrass sediments. Pore water H2 concentrations (89–2147 nM) were up to two orders of magnitude higher than in seawater, and up to 36-fold higher than previously known from shallow-water marine sediments. Pore water CO concentrations (17–51 nM) were twice as high as in the overlying seawater (no literature data from other shallow-water sediments are available for comparison).

Ex-situ incubation experiments showed that dead seagrass rhizomes produced large amounts of CO. CO production from decaying plant material could thus be a significant energy source for microbial primary production in seagrass sediments.

Introduction
Mutualistic symbioses between bacteria and animals are widespread, occur in almost all animal phyla and play major roles in the development, health and evolution of their hosts (McFall-Ngai, 2002; Walker and Crossman, 2007; Moya et al., 2008; Fraune and Bosch, 2010; McFall-Ngai et al., 2013). In many mutualistic symbioses, the function of the bacterial symbionts is to provide essential nutrients to their hosts (Moran, 2007; Moya et al., 2008). In chemosynthetic symbioses, the bacteria provide all or most of their host’s nutrition using inorganic compounds such as sulfide or hydrogen (H2) as energy sources to fix carbon dioxide (CO2) into biomass (Stewart et al., 2005; DeChaine and Cavanaugh, 2006; Dubilier et al., 2008; Petersen et al., 2011; Kleiner et al., 2012a).

The marine oligochaete Olavius algarvensis does not have a digestive or excretory system and relies on its bacterial symbionts for nutrition and waste recycling (Dubilier et al., 2001; Giere and Erseus, 2002; Woyke et al., 2006; Ruehlend et al., 2008; Kleiner et al., 2011; 2012b). It harbours two chemosynthetic sulfur-oxidizing gammaproteobacterial symbionts (γ1 and γ3), two sulfate-reducing deltaproteobacterial symbionts (δ1 and δ4) and a spirochaete between its cuticle and epidermal cells (Giere and Erseus, 2002; Ruehlend et al., 2008). The energy sources that fuel the O. algarvensis symbiosis are still not well understood. The collection site for O. algarvensis in this study and previous studies from the same site, a shallow bay off the coast of the Island of Elba (Italy) in the Mediterranean Sea (Dubilier et al., 2001; Giere and Erseus, 2002; Woyke et al., 2006; Ruehlend et al., 2008; Kleiner et al., 2011; 2012b), is characterized by Posidonia oceanica seagrass meadows and medium- to coarse-grained sandy sediments that cover a thick, peat-like structure consisting of dead seagrass rhizomes (Fig. 1). Concentrations of reduced sulfur compounds at this site are in the low nanomolar range, much lower than the micromolar concentrations that are usually present at sites with chemosynthetic symbioses (Dubilier et al., 2001; Kleiner et al., 2012b). In the O. algarvensis symbiosis, the reduced sulfur compounds required by the
sulfur-oxidizing $\gamma$-symbionts are provided internally by the sulfate-reducing $\delta$-symbionts (Dubilier et al., 2001). However, the external energy sources that power the symbiosis have remained enigmatic.

Metaproteomic analyses of the *O. algarvensis* association showed that three of its symbionts may use carbon monoxide (CO) and H$_2$ as energy sources (Kleiner et al., 2012b). Both sulfate-reducing symbionts abundantly expressed anaerobic carbon monoxide dehydrogenases (CODHs), which enable the use of CO as an energy source, as well as hydrogenases for the use of H$_2$ as an energy source (Kleiner et al., 2012b). The third symbiont, the sulfur-oxidizing $\gamma\delta$-symbiont, abundantly expressed an aerobic CODH (Kleiner et al., 2012b), an enzyme used by bacteria to oxidize CO with oxygen or nitrate (King and Weber, 2007). Because the symbionts are only separated from the environment by a thin cuticle, which is highly permeable for small molecules, they are unlikely to be limited in their access to dissolved gases in the worms’ environment (Dubilier et al., 2006).

The goal of the current study was to test if the metaproteomic predictions by Kleiner and colleagues (2012b) that CO and H$_2$ are used as energy sources by the *O. algarvensis* symbiosis are correct by examining the following questions: (1) Are CO and H$_2$ consumed by the *O. algarvensis* symbiosis? (2) If so, is the energy gained...
from CO and H2 oxidation used for CO2 fixation? (3) Are CO and H2 present in the *O. algarvensis* habitat, and if so what is their source and distribution?

**Results**

The *O. algarvensis* symbiosis oxidizes CO to CO2

In incubation experiments, CO consumption by live *O. algarvensis* worms began after 20–40 h and CO concentrations in the headspace of incubation bottles decreased from 3040 ± 30 ppm to 790 ± 680 ppm over 141 h (Fig. 2). No notable consumption of CO was observed in controls [dead worms, water that worms were washed in and pure artificial seawater (ASW) medium] (Fig. 2). The CO consumption rate of *O. algarvensis* was 2 ± 0.5 μmol g⁻¹ (wet weight) h⁻¹. In incubation experiments with ¹³C-labelled CO, *O. algarvensis* worms almost completely oxidized ¹³CO to ¹³CO₂ within 62 h, whereas no notable production of ¹³CO₂ occurred in the controls (dead worms) (Fig. 3). The average blank-corrected end-point CO concentration in the incubations with ¹³CO was 6.5 ± 35.8 nM (equivalent to 9 ± 48 ppm headspace concentration).

The *O. algarvensis* symbiosis consumes H2

H2 consumption by live *O. algarvensis* worms did not begin until after 40 h of incubation (Fig. 4A). After this lag phase, H2 consumption rates were high and H2 was nearly completely consumed after 86 h (from 2500 ± 320 ppm to 30 ± 20 ppm; Fig. 4A). A second injection of H2 into these incubations (t = 95.5 h) allowed us to better resolve H2 consumption over time. H2 decreased from 2630 ± 170 ppm to 270 ± 380 ppm within 17.5 h (Fig. 4B). The H2 consumption rate of the *O. algarvensis* symbiosis was 11 ± 1 μmol g⁻¹ (wet weight) h⁻¹. No notable consumption of H2 occurred in the controls (dead worms, water that worms were washed in and pure ASW medium).

The γ3-symbiont uses CO as an energy source to fix CO2 into biomass

Our bulk analyses of ¹³CO₂-incorporation in whole *O. algarvensis* worms showed that live worms always incorporated significant amounts of ¹³CO₂ compared with dead worms (Table 1). However, no significant differences in ¹³C-content were detectable between live worms incubated with CO and H2 compared with control incubations with no experimentally added energy source (Table 1). Nanoscale secondary ion mass spectrometry (nanoSIMS) analyses of the symbionts at the single cell level revealed that in live worms all symbionts, except the δ4-symbiont, had a higher ¹³C-content compared with whole dead worms and the carbon signal from polycarbonate filter background (Table 1 and Table S1; Figs 5 and 6 and Fig. S1). This suggests that all symbionts except the δ4-symbiont incorporated ¹³C under all three incubation conditions. However, because we could not measure ¹³C-content of single symbiont cells from dead worm controls, the exact amount of ¹³C-incorporation into individual symbionts could not be determined.

Analyses of single-cell ¹³CO₂-incorporation in each symbiont species for significant differences between the CO and H2 incubations compared with control incubations with no experimentally added energy source revealed: (1) The γ3-symbiont incorporated significantly more ¹³C-labelled CO₂ in the presence of CO compared with
incubations without an added energy source (Kruskal–Wallis, \(P = 7.7e-08\), Fig. 5). (2) The \(\delta\)-symbiont incorporated less \(^{13}\text{C}\)-labelled CO\(_2\) in the presence of CO or H\(_2\) compared with incubations without an added energy source (Kruskal–Wallis, \(P = 4.2e-08\) with CO and \(1.8e-04\) with H\(_2\)). (3) In the y\(_1\) and \(\delta\)-symbionts, no significant differences in \(^{13}\text{C}\)-content in the CO and H\(_2\) incubations compared with control incubations with no experimentally added energy source were observed. It is important to note that the \(^{13}\text{C}\)-content of the symbionts analysed with nanoSIMS was likely diluted because of the deposition of unlabelled carbon during the catalyzed reporter deposition fluorescence \textit{in situ} hybridization (CARD-FISH) treatment (Musat \textit{et al.}, 2014; Woebken \textit{et al.}, 2015) potentially obscuring additional significant differences. It is also noteworthy that \(^{13}\text{C}\)-incorporation by the symbionts can only explain part of \(^{13}\text{C}\)-incorporation into whole worms because \(^{13}\text{C}\)-incorporation in individual symbionts was in a similar range as in whole worms and the symbionts only make up a small fraction of the total worm biomass (Fig. 5, Table 1). The additional \(^{13}\text{C}\)-incorporation in whole worm bulk measurements is likely due to heterotrophic CO\(_2\) fixation by host tissues.

### Elevated CO and H\(_2\) concentrations in the \(O.\ algarvensis\) habitat

CO and H\(_2\) concentrations in sediment pore water where the worms were collected were much higher than in the seawater above the sediment (Fig. 7). Pore water CO concentrations (17–51 nM) were approximately twice as high as in seawater (8–16 nM), with the highest CO concentrations detected in pore water from within the dead rhizome mats (Figs 1 and 7). Pore water H\(_2\) concentrations (89–2147 nM) were up to two orders of magnitude higher than in the seawater (0–23 nM). In contrast to CO, the highest H\(_2\) concentrations were measured at 25 cm sediment depth and not in the deeper dead rhizome mats.

### Dead seagrass rhizomes release large amounts of CO

In incubations with dead seagrass rhizomes from the \(O.\ algarvensis\) habitat we observed high production rates of CO, whereas CO production rates in incubations with unfiltered seawater, and sediment with 0.2 \(\mu\text{m}\)-filtered water were much lower (Fig. 4). H\(_2\) consumption by the \(O.\ algarvensis\) symbiosis.

#### Table 1. \(^{13}\text{C}\)-content of whole worms based on bulk measurements in AT%.

|           | Mean AT%\(^a\) | SD AT% | Min. AT% | Max. AT% | n    | P-value t-test versus dead\(^a\) | P-value t-test w/o e-\text{donor}\(^b\) |
|-----------|----------------|--------|----------|----------|------|-------------------------------|---------------------------------------|
| \(^{13}\text{CO}_2 + \text{CO}\) | 1.284          | 0.013  | 1.274    | 1.299    | 3    | 5e-10                         | 0.56                                  |
| \(^{13}\text{CO}_2 + \text{H}_2\) | 1.295          | 0.038  | 1.257    | 1.332    | 3    | 5.2e-07                       | 0.83                                  |
| \(^{13}\text{CO}_2\) w/o e-\text{donor} | 1.303          | 0.048  | 1.26     | 1.355    | 3    | 2.3e-06                       | –                                     |
| Dead worms | 1.072          | 0.001  | 1.07     | 1.073    | 6    | –                             | –                                     |

\(\text{AT\%: atom percent }[^{13}\text{C} / (^{13}\text{C} + ^{12}\text{C}) \times 100]\).

\(^{a}\) P-values for comparisons of \(^{13}\text{C}\)-content in worms from incubations versus dead worms (t-test, one-tailed, \(H_0 = ^{13}\text{C}\)-content of live worms is not higher than that of dead worms). After Bonferroni correction for three comparisons the significance threshold \(P < 0.01\) corresponds to \(P < 0.003\).

\(^{b}\) P-values for comparisons of \(^{13}\text{C}\)-content in worms incubated without additional energy source versus incubations with CO or H\(_2\) added (t-test, two-tailed, \(H_0 = \text{means are equal}\)).

\(^{c}\) The detailed data including the measurements on the standard caffeine can be found in Table S3.
seawater were in the same range as in controls with double distilled water (Table 2). We measured the highest CO production rates in incubations with dead rhizomes to which ZnCl₂ was added to stop biological activity (Table 2). Dead rhizomes produced up to two orders of magnitude more CO in incubations with ZnCl₂ than in incubations without (3.64–55.60 versus 0.33–17.46 μmol CO kg⁻¹ (dry weight rhizome material) day⁻¹) (Table 2).

We did not detect H₂ production in any of the incubations with natural substrates from the *O. algarvensis* habitat.

**Discussion**

*Is CO an energy source for autotrophic carbon fixation in the *O. algarvensis* symbiosis?*

Our results indicate that part of the CO₂ fixation in the *O. algarvensis* symbiosis is powered by the oxidation of CO by at least the γ3-symbiont. This observation is in agreement with the abundant expression of an aerobic CODH and enzymes of the Calvin–Benson–Bassham (CBB) cycle under natural conditions in the γ3-symbiont (Kleiner *et al*., 2012b). It suggests that the γ3-symbiont uses its CODH (Fig. S2) to oxidize CO to CO₂ and that it uses a part of the energy from CO oxidation to fix CO₂ via the CBB cycle as described for other CO oxidizers (reviewed in King and Weber, 2007).

In contrast to the γ3-symbiont, the sulfate-reducing δ1- and δ4-symbionts did not incorporate significantly more ¹³CO₂ in the presence of CO compared with the control without CO (Fig. 5, Table S1), despite the fact that these symbionts express anaerobic CODHs under environmental conditions (Kleiner *et al*., 2012b). It is possible that the sulfate-reducing symbionts use CO as an energy source for other metabolic functions, as known from ‘carboxydovores’, microorganisms that oxidize CO.

---

Fig. 5. ¹³C-content of single symbiont cells based on nanoSIMS analysis. For all symbionts and treatments cells from three worms were analysed except for the γ3-symbiont in the H₂ treatment, for which only cells from two worms were analysed. Horizontal bars with P-values indicate significant differences based on a Kruskal–Wallis test. Due to the different ¹³C-contents of the four symbionts we used different scales for the y-axis for optimal visualization of the data. AT%: atom percent [¹³C / (¹²C + ¹³C) × 100]; n: total number of symbiont cells analysed. ¹³C isotope content values for all individual cells can be found in Table S1.
without coupling the energy gain to autotrophic growth (reviewed in King and Weber, 2007). The sulfate-reducing symbionts could use the energy released by the oxidation of CO for lithoheterotrophic growth on organic substrates such as fatty acids that are abundantly produced by the host under anoxic conditions (Kleiner et al., 2012b).

Unexpectedly, the δ4-symbiont incorporated significantly less labelled CO\(_2\) in the presence of CO or H\(_2\) compared with controls without an added energy source (Fig. 5). The δ4-symbiont might have been inhibited by the oxygen concentrations used in this study, or by end products of the other co-occurring symbionts. However, incubation conditions were similar in all three treatments, and \(^{13}\text{CO}_2\)-incorporation was clearly not inhibited in this symbiont in the control incubations without an external energy source (Fig. 5). We therefore do not have a satisfactory explanation for these results.

CO oxidation started after a lag phase of about 20 h (Fig. 2). This lag phase could be due to the CO-free pre-incubations of the worms (see Experimental Procedures). In aerobic CO-oxidizing microorganisms the oxidation of CO is catalysed by an inducible CODH, which was shown to only be induced in the presence of CO (Meyer and Schlegel, 1983). It is thus likely that the symbionts did not have substantial amounts of CODH at the beginning of the incubation and had to produce it after exposure to CO.

CO oxidation rates have, to our knowledge, not been previously measured in an animal–bacterial symbiosis. In the \textit{O. algarvensis} symbiosis, CO oxidation rates (2 \(\pm\) 0.5 \(\mu\)mol g\(^{-1}\) (wet weight) h\(^{-1}\)) were 10–100 times higher than those of bacterial CO oxidizers from coastal seaways (0.02–0.23 \(\mu\)mol g\(^{-1}\) (wet weight) h\(^{-1}\)) (Tolli et al., 2006), but comparable with rates of cultured carboxydotrophic microorganisms that can live on CO as their sole energy source (0.02 \(\mu\)mol to 18 mmol g\(^{-1}\) (wet weight) h\(^{-1}\)) (Diekert and Thauer, 1978; Cypionka et al., 1980; Tolli et al., 2006). This comparison of CO oxidation rates has several limitations. These include the fact that CO oxidation rates for cultured CO oxidizers were determined at a range of different CO concentrations and that the mixing environment within the worm will greatly differ from a well-shaken liquid culture.

It is likely that CO oxidation rates of the carboxydotrophic \textit{O. algarvensis} symbionts are even higher than 2 \(\pm\) 0.5 \(\mu\)mol g\(^{-1}\) (wet weight) h\(^{-1}\), because we estimated rates based on the biomass of the entire worm. If we assume that only the γ3-symbiont oxidized CO, based on the observation that it was the only symbiont that incorporated significantly more \(^{13}\text{CO}_2\) in the presence of CO (Fig. 5), and estimate its abundance at 5% of the total biomass of whole worms (Giere and Erseus, 2002; Ruehland et al., 2008), CO oxidation rates would be as high as 40 \(\mu\)mol g\(^{-1}\) (wet weight) h\(^{-1}\). However, \textit{in-situ} CO oxidation rates have, to our knowledge, not been previously measured in an animal–bacterial symbiosis. In the \textit{O. algarvensis} symbiosis, CO oxidation rates (2 \(\pm\) 0.5 \(\mu\)mol g\(^{-1}\) (wet weight) h\(^{-1}\)) were 10–100 times higher than those of bacterial CO oxidizers from coastal seaways (0.02–0.23 \(\mu\)mol g\(^{-1}\) (wet weight) h\(^{-1}\)) (Tolli et al., 2006), but comparable with rates of cultured carboxydotrophic microorganisms that can live on CO as their sole energy source (0.02 \(\mu\)mol to 18 mmol g\(^{-1}\) (wet weight) h\(^{-1}\)) (Diekert and Thauer, 1978; Cypionka et al., 1980; Tolli et al., 2006). This comparison of CO oxidation rates has several limitations. These include the fact that CO oxidation rates for cultured CO oxidizers were determined at a range of different CO concentrations and that the mixing environment within the worm will greatly differ from a well-shaken liquid culture.
oxidation rates of the *O. algarvensis* symbiosis are likely to be lower because of the lower in-situ concentrations of CO compared with the concentrations used in our incubations.

*Is H₂ also an energy source for the *O. algarvensis* symbiosis?*

H₂ consumption rates of the *O. algarvensis* symbiosis (11 ± 1 μmol g⁻¹ (wet weight) h⁻¹) were higher than those measured in the deep-sea hydrothermal vent mussel *Bathymodiolus* symbiosis (~3 μmol g⁻¹ (wet weight of gill tissue) h⁻¹) despite similar incubation H₂ concentrations of 1800 ppm (Petersen et al., 2011). H₂ consumption rates of free-living and cultivated microorganisms are higher than the *O. algarvensis* symbiosis and range from ∼100 μmol g⁻¹ (wet weight) h⁻¹ to several mmol g⁻¹ (wet weight) h⁻¹ (Vmax) at H₂ concentrations between 1 ppm and 20 000 ppm (Häring and Conrad, 1991; Klüber and Conrad, 1993; Perner et al., 2010). To adequately compare these rates to the H₂ consumption rate of the *O. algarvensis* symbiosis the reaction kinetics of H₂ oxidation would need to be characterized for the *O. algarvensis* symbiosis in future experiments. As discussed for CO, it is likely that the H₂ oxidation rates of the *O. algarvensis* symbionts are considerably higher, because we calculated these rates based on whole worm wet weight, instead of the biomass of the two sulfate-reducing symbionts predicted to be able to oxidize H₂ (Kleiner et al., 2012b). If we assume that only both deltaproteobacterial symbionts oxidized H₂ based on the observation that they possess hydrogenases (Kleiner et al., 2012b), and estimate their abundance at 10% of the total biomass of whole worms (Giere and Erseus, 2002; Ruehland et al., 2008), H₂ oxidation rates would be 110 μmol g⁻¹ (wet weight) h⁻¹, which is in the range of those of free-living and cultivated bacteria (Häring and Conrad, 1991; Klüber and Conrad, 1993; Perner et al., 2010).

The lag phase in H₂ consumption during the first 40 h of incubation (Fig. 4) could be due to the oxic pre-incubations of the worms and the relatively high oxygen concentrations at the beginning of the experiments (see Experimental Procedures). The expression of hydrogenases in anaerobic H₂-oxidizing microorganisms is generally only induced in the presence of H₂ and repressed by oxygen (reviewed in Vignais and Billoud, 2007). It is thus likely that the symbionts did not have substantial amounts of hydrogenases at the beginning of the incubation and had to produce these after exposure to H₂ and suboxic conditions in the incubation vessels. Once H₂ consumption began, H₂ was consumed down to 9 ppm in one incubation bottle, which corresponds to 5.4 nM H₂ in solution (Fig. 4A), indicating that the...
O. algarvensis symbionts have hydrogenases that can take up H₂ down to very low concentrations. This is in agreement with the metaproteome study of Kleiner and colleagues (2012b), which showed that the expressed uptake hydrogenases of the deltaproteobacterial symbionts are closely related to hydrogenases characterized as having a high-affinity for H₂ (Kleiner et al., 2012b).

Despite high H₂ consumption rates of live O. algarvensis worms, none of the symbionts showed increased CO₂ incorporation in the presence of H₂ in the nanoSIMS analyses (Fig. 5). This suggests that H₂ was not used as an energy source for autotrophic CO₂ fixation by the hydrogenase-possessing δ-symbionts under the applied incubation conditions. The δ-symbionts may have instead used the energy released by the oxidation of H₂ for lithoheterotrophic growth as discussed for CO above.

What are the sources of CO and H₂ in the habitat of O. algarvensis?

In the photic zone of the ocean, CO is produced through non-biological processes (abiotically) during the photochemical lysis of organic material. CO concentrations in the seawater above our study site were in the same low nanomolar range as those measured in other studies, and was most likely produced through photolytic processes (summarized and discussed in Tolli et al., 2006 and Moran and Miller, 2007). Surprisingly, there is currently no data on CO concentrations in marine sediments. In this study, we found CO concentrations in sediment pore waters that were twice as high as those in the overlying seawater, with the highest concentrations measured in the dead rhizome mats at sediment depths of 25 cm and more where photolysis of organic material is not possible (Figs 1 and 7). Accordingly, our results from incubations with different components from the O. algarvensis habitat showed that dead seagrass rhizomes incubated in the dark released considerable amounts of CO (Table 2). We therefore hypothesize that the large mats of dead seagrass rhizomes in the O. algarvensis habitat are a source of aphotically produced CO. Based on our observation that up to two orders of magnitude more CO was produced in the rhizome incubations in which biological activity was stopped with ZnCl₂ than in the rhizome incubations without ZnCl₂, we hypothesize that (1) CO production occurred abiotically, i.e. in the absence of live organisms, and (2) in the rhizome incubations without ZnCl₂, CO was not only produced, but also consumed by microorganisms associated with the dead rhizomes. This hypothesis is supported by earlier studies that found abiotic production of CO from humic acids, phenolic compounds and decaying plant material in soils in the absence of light and the presence of an oxidant (Conrad and Seiler, 1980; 1982; 1985). These authors observed that CO production increased with increasing temperature, moisture content and alkaline conditions (higher pH), indicating that a thermochemical process is involved in the production of CO from decaying plant material in soils. The exact reaction mechanism behind this process remains unknown.

CO production from organic material could also explain the widespread presence of microorganisms with the genetic potential to oxidize CO in the aphotic zones of the ocean. Several metagenomic and one metatranscriptomic study found high frequencies of CODH genes used for CO oxidation in the Mediterranean, Pacific and Atlantic Oceans at water depths between 200 and 6000 m, but the CO source remained elusive (Martin-Cuadrado et al., 2009; Quaiser et al., 2011; Smedile et al., 2013). We hypothesize that CO production from decaying organic matter is not limited to soils as described by Conrad and Seiler (1980; 1982; 1985) but could also explain CO production in the bathypelagic. This hypothesis is supported by studies showing ‘dark production’ of CO (i.e. not from photolytic processes) in coastal surface waters that can make up as much as 25% of the total CO production budget (Zhang et al., 2008; Day and Faloona, 2009).

In contrast to CO, none of the components from the O. algarvensis environment produced H₂ under the aerobic conditions used in this study. Potential sources for the high H₂ concentrations in the sediment pore waters at our collection site are the anaerobic oxidation of CO by carboxydotrophs that use protons as electron acceptors and thus release H₂ (Kerby et al., 1995; Maness et al., 2005; Oelgeschlager and Rother, 2008), and microbial fermentation that would produce H₂ as a by-product (Schwartz and Friedrich, 2006). Difficult to explain are the unusually high H₂ concentrations in the O. algarvensis sediments of 89–2147 nM because H₂ concentrations in aquatic sediments are usually very low (<60 nM) because of rapid oxidation by free-living H₂ oxidizers (Goodwin et al., 1988; Novelli et al., 1988).

Could CO production by dead seagrass rhizomes in the Mediterranean sediments support the O. algarvensis symbiosis?

CO concentrations in the pore waters of the O. algarvensis collection site (17–51 nM) correspond to atmospheric mixing ratios of 23–68 ppm. Such low concentrations (10–100 ppm) have been successfully used to incubate and isolate CO oxidizers from soils and seawater (Hendrickson and Kubisesske, 1991; Hardy and King, 2001; King, 2007; Weber and King, 2010).

In our incubations, O. algarvensis was also able to oxidize CO down to concentrations as low as 16 ppm (concentration at the end of one of the CO incubations) and down to 6.5 ± 35.8 nM in the incubations with ¹³COCO₂. We hypothesize that CO production in the Mediterranean, Pacific and Atlantic Oceans at water depths between 200 and 6000 m, but the CO source remained elusive (Martin-Cuadrado et al., 2009; Quaiser et al., 2011; Smedile et al., 2013). We hypothesize that CO production from decaying organic matter is not limited to soils as described by Conrad and Seiler (1980; 1982; 1985) but could also explain CO production in the bathypelagic. This hypothesis is supported by studies showing ‘dark production’ of CO (i.e. not from photolytic processes) in coastal surface waters that can make up as much as 25% of the total CO production budget (Zhang et al., 2008; Day and Faloona, 2009).

In contrast to CO, none of the components from the O. algarvensis environment produced H₂ under the aerobic conditions used in this study. Potential sources for the high H₂ concentrations in the sediment pore waters at our collection site are the anaerobic oxidation of CO by carboxydotrophs that use protons as electron acceptors and thus release H₂ (Kerby et al., 1995; Maness et al., 2005; Oelgeschlager and Rother, 2008), and microbial fermentation that would produce H₂ as a by-product (Schwartz and Friedrich, 2006). Difficult to explain are the unusually high H₂ concentrations in the O. algarvensis sediments of 89–2147 nM because H₂ concentrations in aquatic sediments are usually very low (<60 nM) because of rapid oxidation by free-living H₂ oxidizers (Goodwin et al., 1988; Novelli et al., 1988).
(average and standard deviation for four parallel $^{13}$CO incubations), indicating that the symbionts would be able to take up CO at the concentrations measured in the worm’s environment. Additionally, we consider it likely that the symbionts experience fluctuating conditions of CO and H$_2$ supply and that CO concentrations may often be higher than those that we measured in the pore waters for two reasons. First, based on our data we hypothesize that CO flux in the sediments close to the dead rhizomes is very high despite the low concentrations measured in pore waters. We base this assumption on the fact that on average five times more CO was produced in dead rhizome incubations with ZnCl$_2$ compared with without ZnCl$_2$ (Table 2), suggesting that an active CO-oxidizing microbial community rapidly oxidized the CO produced by the dead rhizomes. Second, the high variability in the measured pore water CO (and H$_2$) concentrations indicate a high spatial variation and suggest that small pockets with high concentrations may exist in the sediment. However, our measuring method, which required large sample volumes, would not have allowed the detection of such fine spatial differences.

To estimate how many *O. algarvensis* worms could be sustained by using CO as sole energy source for growth, we calculated the amount of carbon that could be fixed with the CO produced in the *O. algarvensis* habitat assuming that (1) between 0.02 and 0.164 mol CO$_2$ can be fixed autotrophically per mole CO oxidized using oxygen as terminal electron acceptor (Moersdorf et al., 1992); (2) an average *O. algarvensis* individual has a carbon content of 5 μmol; (3) 20 kg (dry weight) dead seagrass rhizome are buried in 1 m$^2$ of sediment (rough estimate based on samples taken for incubations) which produce 36.5 mmol CO per year (using a conservative mean CO production value of 5 μmol kg$^{-1}$ (dry weight) per day by dead rhizomes in their native state, Table 2); and (4) CO production by dead rhizomes is constant. We calculated that up to 6 mmol carbon could be fixed per year and square meter using CO as an energy source, which is equal to the carbon content of 1200 worms. These estimates indicate that CO production from decaying plant material could be a significant energy source for microbial primary production in marine seagrass sediments in the Mediterranean Sea and possibly in other coastal regions with large amounts of decaying plant material.

**Experimental procedures**

**Specimen collection and preparation for incubations**

Worms were collected by scuba diving in October 2009 and October 2011 off Capo di Sant’ Andrea, Elba in Italy (geographic position: 42°48’29.38”N, 10°8’31.57”E; 6–8 m of water depth). Only intact specimens were used in incubation experiments. Sexually mature worms that were identified as *O. ilvae*, a co-occurring less abundant gutless oligochaete species, were sorted out and not used in the experiments (Giere and Erseus, 2002).

Internally stored sulfur in the γ1-symbionts (Giere and Erseus, 2002) was removed by pre-incubating all worms in large glass bowls containing 0.2 μM-filtered oxic seawater and a thin (3 mm) layer of glass beads for a week. This pre-treatment was necessary because the γ1-symbionts use their stored sulfur for CO$_2$ fixation, and this would have masked differences in CO$_2$ fixation between treatments. After this pre-incubation, the symbionts had lost most of their stored sulfur as determined by the change of worm colour from bright white to transparent and decreased CO$_2$ fixation rates in test incubations without an external energy source (Fig. S3).

**Incubation experiments with $^{13}$C-labelled bicarbonate and CO, H$_2$ or no external energy source**

We compared uptake rates of $^{13}$C-labelled bicarbonate in *O. algarvensis* worms under three conditions: (1) in the presence of CO, (2) in the presence of H$_2$ and (3) in the absence of an externally added energy source. ASW with a salinity of 39‰ was prepared as previously described (Widdel and Bak, 1992) (Supporting Information). The pH of the ASW was adjusted to 7.5 corresponding to the conditions in the *O. algarvensis* habitat. $^{13}$C-labelled NaHCO$_3$ was added to detect CO$_2$ fixation in the symbionts.

Incubation bottles (serum bottles) were flushed with N$_2$ gas prior to filling with 20 ml of ASW to create microaerobic conditions. Oxygen concentrations were measured at the end of the incubations with an amperometric microelectrode (Revsbech, 1989) and were 0.18 mM in the control bottles and 0.11 mM in the bottles containing live worms.

All incubations were run in triplicates with 35 live worms added to each serum bottle, whereas control incubations contained 35 dead worms (see Supporting Information). 5 μl of wash water (see Supporting Information) or only ASW. To start the incubation, either 80 μl of CO (purity level 3.7; Air Liquide, Düsseldorf, Germany) or 80 μl of H$_2$ (purity level 5.0; Air Liquide) were injected into the headspace of the serum bottles; in the incubations without an external energy source nothing was added to the headspace. Serum bottles were stored at 22°C and gently tilted back and forth (18× per minute) to allow mixing and to reduce diffusion limitation. At given time points, subsamples from the headspace were taken with gas-tight syringes and CO and H$_2$ concentrations were measured with a Shimadzu GC-8A gas chromatograph equipped with a Molecular Sieve 5A column and an RGD2 Reduction Gas Detector (Trace Analytical, Menlo Park, CA, USA) as described previously (Pohorelic et al., 2002). To stay within the linear range of the detector samples were diluted with pure nitrogen gas if needed. H$_2$ and CO standards were produced from pure H$_2$ (purity level 5.0; Air Liquide) and CO gas (purity level 3.7; Air Liquide) in pure nitrogen gas (purity level 5.0; Air Liquide). To control for potential instrument drift, standards were measured in each measurement run and used to calculate sample concentrations within each run (Table S2). After H$_2$ was completely consumed, we added an additional 80 μl of H$_2$ to the serum bottles containing live worms.
worms for a better time resolution of H₂ consumption by the *O. algarvensis* symbiosis. At the end of the incubations, worms were processed for bulk tissue analyses and nanoSIMS (see below). CO and H₂ consumption rates were calculated based on the linear CO consumption between 65 and 87 h (Fig. 2) and the linear H₂ consumption during the last 17.5 h of the incubation using the average wet weight of one worm (0.5 mg) and the molar volume of an ideal gas at 22 °C (24.54 l mol⁻¹).

**Bulk analysis of ¹³C-incorporation in whole worms**

To determine incorporation of ¹³C-labelled bicarbonate in whole worms, eight worms from each replicate were killed in 3 ml of ASW and 100 µl of aqueous zinc chloride (ZnCl₂) solution (50% v/w). Worms were washed in ASW, rinsed briefly in 0.1% HCl to remove unixed labelled bicarbonate, washed again in ASW, placed in tin cups and their wet weight measured. Dead worms from control incubations were treated the same way. Tin cups with worms were dried over night at 70°C and stored at room temperature until further processing. Carbon isotope composition of the worms was analysed using an automated elemental analyzer (Thermo Flash EA 1112) coupled to an isotopic ratio mass spectrometer (Thermo Delta Plus XP, Thermo Fisher Scientific) ¹³C isotope content in the worms was calculated as atom percent (AT% = ¹³C/(¹³C + ¹²C) × 100). Caffeine (Sigma-Aldrich) was used as a standard for isotope calibration and quantification (Table S3).

**nanoSIMS analysis of ¹³C-incorporation into single symbiont cells**

To determine the amount of ¹³C assimilated by each symbiont we analysed the carbon isotope composition of single symbiont cells using CARD-FISH combined with nanoSIMS imaging (see Supporting Information for more details) (Musat et al., 2012; Polerecky et al., 2012).

**Incubation experiments with ¹³CO**

To investigate whether CO consumption was caused by oxidation to CO₂ or by CO assimilation, labelled ¹⁰⁰CO (99 AT% ¹³C, < 5 AT% ¹⁸O, Sigma-Aldrich Cat. No. 388505) was added to incubations that were prepared as described above but with unlabelled ¹²C-bicarbonate (Sigma-Aldrich) added to the ASW. Incubations were done in 12 ml glass vials (Exetainers, Labco, High Wycombe, UK) without headspace, with four parallel incubations for each time point. Four worms were placed in an incubation vial and the incubation started by adding CO to a final concentration of 7 µM. At given time points, samples were killed by addition of ZnCl₂ (50% w/v). Six milliliters of medium were transferred to 6 ml glass vials (Exetainers). Two milliliters of medium were taken with pure helium (He) gas (purity level 5.0; Air Liquide) and transferred to new He-flushed 6 ml glass vials (Exetainers). For outgasing of CO₂ into the headspace 0.2 ml of 85% phosphoric acid were injected. Two hundred fifty microlitres of the headspace were analysed with a gas chromatograph – isotope ratio mass spectrometer (VG Optima, Manchester, UK). Pure CO₂ (purity 4.5; Air Liquide) was used as a standard for isotope calibration and quantification.

CO end-point concentrations were measured after outgasing of CO into the 2 ml He headspace created in the 12 ml glass vials after the medium for the CO₂ analyses was removed. The same measurement setup as for the CO and H₂ worm incubations was used. The average CO concentration in the incubations with worms was blank corrected using the average CO concentration in control vessels without added CO to account for CO production by the rubber septa after killing of the worms.

**Measurement of CO and H₂ concentrations in the worms’ habitat**

Sediment pore water and seawater above the sediment were collected at the worm collection site by scuba diving and measured as previously described (Kleiner et al., 2012b). Nine profiles were sampled within an area of approx. one hundred square metres at sediment depths of 15 cm, 25 cm and in the dead seagrass rhizomes (Fig. 1). Seawater samples were collected –5 cm above the sediment surface. H₂ and CO concentrations were measured with an RGA3 reduction gas analyser (Trace Analytical) using ultra pure nitrogen (purity level 5.0; SOL s.p.a., Monza, Italy) as carrier gas. To control for potential instrument drift, standards were measured in each measurement run and used to calculate sample concentrations within each run (Table S2).

Eight blanks with ddH₂O were created, processed and measured in the same way as the pore water samples. Average CO concentrations (3.1 nM) and H₂ concentrations (21.5 nM) in blanks were used for blank correction of pore water and seawater concentrations. All concentrations in this study are given as blank-corrected values.

**CO and H₂ production of different components from the *O. algarvensis* habitat**

To identify the sources of CO and H₂ in the *O. algarvensis* environment, we incubated dead seagrass rhizome, sediment and seawater from six different locations at the worm collection site (Fig. 1). From each location, 70–100 g (wet weight) rhizome material (collected from the layer of dead rhizome mats at ≥ 25 cm sediment depth), 250 ml of sediment or unfiltered seawater were added to 0.5 l Schott bottles. We filled bottles completely with sterile filtered seawater, closed them with rubber septa and then withdrew 20 ml of seawater using a syringe to create a headspace. Bottles with ddH₂O were used as controls. Bottles were incubated in a water bath set at 23°C for ~ 3.5 h in the dark to avoid photochemical CO production (King and Weber, 2007). Ten minutes before measuring H₂ and CO concentrations, bottles were shaken thoroughly to allow produced H₂ and CO gas to equilibrate with the headspace. H₂ and CO were measured using the same setup as for the pore water samples (see above). Rhizome samples and sediment samples were rinsed in freshwater and dried after the incubation to determine their dry weight.

To distinguish biotic from abiotic CO and H₂ production, samples were either incubated in their native state or...
amended with ZnCl₂ (50% w/v) to a final concentration of 61 mM to stop biological activity. The pH was adjusted to the pH of the native seawater (pH 7.7–7.8) in ZnCl₂-amended samples. The minimum inhibitory concentration of ZnCl₂ for microbial activity is about 1 mM (Winslow and Haywood, 1931; He et al., 2002; Choi et al., 2010). We are therefore confident that the 61-fold higher concentration in our experiments efficiently stopped most biological activity.

Acknowledgements

We thank the team of the HYDRA Institute on Elba for their extensive support with sample collection and onsite experiments; Lubos Polerecky for help with the Look@NanoSIMS software; all members of the Symbiosis Group for helping sorting out worms of the sediment; Silke Wetzel, Agnes Zimmer and Nadine Lehnen for excellent technical assistance; Hannah Marchant and Tim Kalvelage for help with GC-measurements; Richard Hahnke for his help with everyday challenges; and Anne-Christine Kreutzmann for valuable comments on the experiments. Alex Copeland is acknowledged for assembling the CODH operon of the γ-symbiont as part of a CSP2012 project. We also thank the two anonymous reviewers for detailed and thoughtful feedback that helped improve this manuscript significantly. Sequencing was conducted by the U.S. Department of Energy Joint Genome Institute and is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. CW and MK were supported by scholarships of the Studienstiftung des deutschen Volkes. Funding for this study was provided by the Gordon and Betty Moore Foundation through Grant No. GBMF3811 to ND and the Max Planck Society.

References

Alcoverro, T., Manzanera, M., and Romero, J. (2001) Annual metabolic carbon balance of the seagrass Posidonia oceanica: the importance of carbohydrate reserves. Mar Ecol Prog Ser 211: 105–116.

Boudouresque, C.F., Bernard, G., Pergent, G., Shili, A., and Verlaque, M. (2009) Regression of Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: a critical review. Botanica Marina 52: 395–418.

Choi, E.K., Lee, H.H., Kang, M.S., Kim, B.G., Lim, H.S., Kim, S.M., and Kang, I.C. (2010) Potentiation of bacterial killing activity of zinc chloride by pyrrolidine dithiocarbamate. J Microbiol Biotechnol 48: 40–43.

Conrad, R., and Seiler, W. (1980) Role of microorganisms in the consumption and production of atmospheric carbon monoxide by soil. Appl Environ Microbiol 40: 437–445.

Conrad, R., and Seiler, W. (1982) Arid soils as a source of atmospheric carbon-monoxide. Geophys Res Lett 9: 1353–1356.

Conrad, R., and Seiler, W. (1985) Characteristics of abiological carbon monoxide formation from soil organic matter, humic acids, and phenolic compounds. Environ Sci Technol 19: 1165–1169.

Cypionka, H., Meyer, O., and Schlegel, H.G. (1980) Physiological characteristics of various species of strains of carboxydotrobacira. Arch Microbiol 127: 301–307.

Day, D.A., and Falloona, I. (2009) Carbon monoxide and chromophoric dissolved organic matter cycles in the shelf waters of the northern California upwelling system. J Geophys Res Oceans 114: doi: 10.1029/2007JC004590.

DeChaine, E.G., and Cavanaugh, C.M. (2006) Symbioses of methanotrophs and deep-sea mussels (Mytilidae: Bathymodiolinae). Prog Mol Subcell Biol 41: 227–249.

Diekert, G.B., and Thauer, R.K. (1978) Carbon monoxide oxidation by Clostridium thermoaceticum and Clostridium formicoaceticum. J Bacteriol 136: 597–606.

Duarte, C.M. (2002) The future of seagrass meadows. Environ Conserv 29: 192–206.

Dubilier, N., Mulders, C., Ferdelman, T., de Beer, D., Pernthaler, A., Klein, M., et al. (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature 411: 298–302.

Dubilier, N., Blazejak, A., and Rühland, C. (2006) Symbioses between bacteria and gutless marine oligochaetes. In Molecular Basis of Symbiosis. Overmann, J. (ed.). Berlin, Heidelberg: Springer-Verlag, pp. 251–275.

Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nat Rev Microbiol 6: 725–740.

Fraune, S., and Bosch, T.C.G. (2010) Why bacterium matter in animal development and evolution. Bioessays 32: 571–580.

Giere, O., and Erseus, C. (2002) Taxonomy and new bacterial symbioses of gutless marine Tubificidae (Annelida, Oligochaeta) from the Island of Elba (Italy). Org Divers Evol 2: 289–297.

Goodwin, S., Conrad, R., and Zeikus, J.G. (1988) Influence of pH on microbial hydrogen metabolism in diverse sedimentary ecosystems. Appl Environ Microbiol 54: 590–593.

Gutiérrez, J.L., Jones, C.G., Byers, J.E., Arkema, K.K., Berkenbusch, K., Commoto, J.A., et al. (2011) Functioning of estuaries and coastal ecosystems. In Treatise on Estuarine and Coastal Science. Heip, C.H.R., Philippart, C.J.M., and Middelburg, J.J. (eds). Elsevier, doi: 10.1016/B978-0-12-374711-2.00705-1.

Hardy, K.R., and King, G.M. (2001) Enrichment of high-affinity CO oxidizers in Maine forest soil. Appl Environ Microbiol 67: 3671–3676.

Häring, V., and Conrad, R. (1991) Kinetics of H₂-oxidation in respiring and denitrifying Paracoccus denitrificans. FEMS Microbiol Lett 78: 259–264.

He, G., Pearce, E.I.F., and Sissons, C.H. (2002) Inhibitory effect of ZnCl₂ on glycolysis in human oral microbes. Arch Oral Biol 47: 117–129.

Hendrickson, O.Q., and Kubisies, T. (1991) Soil microbial activity at high-levels of carbon-monoxide. J Environ Qual 20: 675–678.

Kerby, R.L., Ludden, P.W., and Roberts, G.P. (1995) Carbon monoxide-dependent growth of Rhodospirillum rubrum. J Bacteriol 177: 2241–2244.

King, G.M. (2007) Microbial carbon monoxide consumption in salt marsh sediments. FEMS Microbiol Ecol 59: 2–9.

King, G.M., and Weber, C.F. (2007) Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. Nat Rev Microbiol 5: 107–118.
Kleiner, M., Woyke, T., Ruehl, C., and Dubilier, N. (2011) The \textit{Olavius algarvensis} metagenome revisited: lessons learned from the analysis of the low-diversity microbial consortium of a gutless marine worm. In \textit{Handbook of Molecular Microbial Ecology II: Metagenomics in Different Habitats}. de Bruijn, F.J. (ed.). New York: Wiley-Blackwell, pp. 321–333.

Kleiner, M., Petersen, J.M., and Dubilier, N. (2012a) Convergent and divergent evolution of metabolism in sulfur-oxidizing symbionts and the role of horizontal gene transfer. \textit{Curr Opin Microbiol} 15: 621–631.

Kleiner, M., Wentrup, C., Lott, C., Teeling, H., Wetzel, S., Young, J., et al. (2012b) Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy use. \textit{Proc Natl Acad Sci USA} 109: E1173–E1182.

Klüber, H.D., and Conrad, R. (1993) Ferric iron-reducing \textit{Shewanella putrefaciens} and \textit{N}_{2}-fixing \textit{Bradyrhizobium japonicum} with uptake hydrogenase are unable to oxidize atmospheric \textit{H\textsubscript{2}}. \textit{FEMS Microbiol Lett} 111: 337–341.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Loso, T., Douglas, A.E., et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. \textit{Proc Natl Acad Sci USA} 110: 3229–3236.

McFall-Ngai, M.J. (2002) Unseen forces: the influence of bacteria on animal development. \textit{Dev Biol} 242: 1–14.

Maness, P.C., Huang, J., Smolinske, S., Tek, V., and Vanzin, G. (2005) Energy generation from the CO oxidation–hydrogen production pathway in \textit{Rubrivivax gelatinosus}. \textit{Appl Environ Microbiol} 71: 2870–2874.

Martin-Cuadrado, A.B., Ghai, R., Gonzaga, A., and Rodriguez-Valera, F. (2009) CO dehydrogenase genes found in metagenomic fosmid clones from the deep Mediterranean Sea. \textit{Appl Environ Microbiol} 75: 7436–7444.

Mateo, M.A., Romero, J., Perez, M., Littler, M.M., and Littler, D.S. (1997) Dynamics of millenary organic deposits resulting from the growth of the Mediterranean seagrass \textit{Posidonia oceanica}. \textit{Estuar Coast Shelf Sci} 44: 103–110.

Meyer, O., and Schlegel, H.G. (1983) Biology of aerobic carbon monoxide oxidizing bacteria. \textit{Annu Rev Microbiol} 37: 277–310.

Moersdorf, G., Frunzke, K., Gadkari, D., and Meyer, O. (1992) Microbial growth on carbon monoxide. In \textit{Prokaryotes}. In \textit{Annu Rev Microbiol} 36: 285–304.

Moran, A.N., and Miller, W.L. (2007) Resourceful heterotrophs make the most of light in the coastal ocean. \textit{Nat Rev Microbiol} 5: 792–800.

Moran, N.A. (2007) Symbiosis as an adaptive process and source of phenotypic complexity. \textit{Proc Natl Acad Sci USA} 104: 8627–8633. doi: 10.1073/pnas.0611659104

Moya, A., Pereto, J., Gil, R., and Latorre, A. (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. \textit{Nat Rev Genet} 9: 218–229.

Musat, N., Foster, R., Vagner, T., Adam, B., and Kuypers, M.M. (2012) Detecting metabolic activities in single cells, with emphasis on nanoSIMS. \textit{FEMS Microbiol Rev} 36: 486–511.

Musat, N., Stryhanyuk, H., Bombach, P., Lorenz, A., Audinot, J.N., and Richnow, H.H. (2014) The effect of FISH and CARD-FISH on the isotopic composition of \textit{^{13}C}- and \textit{^{15}N}-labeled \textit{Pseudomonas putida} cells measured by nanoSIMS. \textit{Syst Appl Microbiol} 37: 267–276.

Novelli, P.C., Michelson, A.R., Scranton, M.I., Banta, G.T., Hobbie, J.E., and Howarth, R.W. (1988) Hydrogen and acetate cycling in 2 sulfate-reducing sediments – Buzzards Bay and Town Cove, Mass. \textit{Geochim Cosmochim Acta} 52: 2477–2486.

Oelgeschlager, E., and Rother, M. (2008) Carbon monoxide-dependent energy metabolism in anaerobic bacteria and archaea. \textit{Arch Microbiol} 190: 257–269.

Perner, M., Petersen, J.M., Zielinski, F., Gennerich, H.H., and Seifert, R. (2010) Geochemical constraints on the diversity and activity of \textit{H\textsubscript{2}}-oxidizing microorganisms in diffuse hydrothermal fluids from a basalt- and an ultramafic-hosted vent. \textit{FEMS Microbiol Ecol} 74: 55–71.

Petersen, J.M., Zielinski, F.U., Pape, T., Seifert, R., Moraru, C., Amann, R., et al. (2011) Hydrogen is an energy source for hydrothermal vent symbioses. \textit{Nature} 476: 176–180.

Pohorelic, B.K.J., Voordouw, J.K., Lojou, E., Dolla, A., Harder, J., and Voordouw, G. (2002) Effects of deletion of genes encoding Fe-only hydrogenase of \textit{Desulfovibrio vulgaris} Hildenborough on hydrogen and lactate metabolism. \textit{J Bacteriol} 184: 679–686.

Polerecky, L., Adam, B., Milucka, J., Musat, N., Vagner, T., and Kuypers, M.M. (2012) Look@NanoSIMS – a tool for the analysis of nanoSIMS data in environmental microbiology. \textit{Environ Microbiol} 14: 1009–1023.

Quaiser, A., Zivanovic, Y., Moreira, D., and Lopez-Garcia, P. (2011) Comparative metagenomics of bathypelagic plankton and bottom sediment from the Sea of Marmara. \textit{ISME J} 5: 285–304.

Revsbech, N.P. (1989) An oxygen microsensor with a guard cathode. \textit{Limnol Oceanogr} 34: 474–478.

Ruehl, C., Blazejak, A., Lott, C., Loy, A., Erseus, C., and Dubilier, N. (2008) Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean seagrass sediments. \textit{Environ Microbiol} 10: 3404–3416.

Schwartz, E., and Friedrich, B. (2006) The \textit{H\textsubscript{2}}-metabolizing Prokaryotes. In \textit{The Prokaryotes}, Dworkin, M., Falkow, S.I., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds.). New York: Springer, pp. 496–563.

Smedile, F., Messina, E., La Cono, V., Tsoy, O., Monticelli, L.S., Borghini, M., et al. (2013) Metagenomic analysis of hadopelagic microbial assemblages thriving at the deepest part of Mediterranean Sea, Matapan–Vavilov Deep. \textit{Environ Microbiol} 15: 167–182.

Stewart, F.J., Newton, I.L., and Cavanaugh, C.M. (2005) Chemoaerobic endosymbioses: adaptations to oxic–anoxic interfaces. \textit{Trends Microbiol} 13: 439–448.

Tolli, J.D., Sievert, S.M., and Taylor, C.D. (2006) Unexpected diversity of bacteria capable of carbon monoxide oxidation in a coastal marine environment, and contribution of the \textit{Roseobacter}-associated clade to total CO oxidation. \textit{Appl Environ Microbiol} 72: 1966–1973.

Vignais, P.M., and Billoud, B. (2007) Occurrence, classification, and biological function of hydrogenases: an overview. \textit{Chem Rev} 107: 4206–4272.

Walker, A., and Crossman, L.C. (2007) This place is big enough for both of us. \textit{Nat Rev Microbiol} 5: 90–92.
Weber, C.F., and King, G.M. (2010) Distribution and diversity of carbon monoxide-oxidizing bacteria and bulk bacterial communities across a succession gradient on a Hawaiian volcanic deposit. *Environ Microbiol* **12**: 1855–1867.

Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The Prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer, pp. 3352–3378.

Winslow, C.-E.A., and Haywood, E.T. (1931) The specific potency of certain cations with reference to their effect on bacterial viability. *J Bacteriol* **22**: 49–69.

Woebken, D., Burow, L.C., Behnam, F., Mayali, X., Schintlmeister, A., Fleming, E.D., et al. (2015) Revisiting *N2* fixation in Guerrero Negro intertidal microbial mats with a functional single-cell approach. *ISME J* **9**: 485–496.

Woyke, T., Teeling, H., Ivanoa, N.N., Huntemann, M., Richter, M., Gloeckner, F.O., et al. (2006) Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature* **443**: 950–955.

Zhang, Y., Xie, H.X., Fichot, C.G., and Chen, G.H. (2008) Dark production of carbon monoxide (CO) from dissolved organic matter in the St. Lawrence estuarine system: implication for the global coastal and blue water CO budgets. *J Geophys Res Oceans* **113**: DOI: 10.1029/2008JC004811.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Epifluorescence images of *O. algarvensis* symbionts on a filter. Symbiont cells are the same as in Fig. 6. Images in the left and right columns show in the top row the composite CARD-FISH signals of all fluorescence channels, followed in the second row by the epifluorescence images of symbiont cells with the sulfur-oxidizing symbionts targeted by the gammaproteobacterial probe (Gam42a) in red. The third row shows epifluorescence images of all symbiont cells targeted by the general eubacterial probe (EUB338I-III) in green and the fourth row epifluorescence images of the general DNA stain DAPI in blue.

**Fig. S2.** Comparison of the aerobic form I CODH operons in known CO oxidizers and the γ3-symbiont. While the metagenomic sequences of the γ3-symbiont CODH genes were fragmented and distributed among two genome contigs (Woyke et al., 2006; Kleiner et al., 2012), we recovered the complete and uninterrupted CODH operon of the γ3-symbiont as part of our 2012 Community Sequencing Project (CSP) with the US Department of Energy Joint Genome Institute (see Acknowledgments). The bottom three microorganisms are known to oxidize CO at very low concentrations (<1000 ppm). Bold letters highlight the functional subunits of the CODH (coxMSL), while the other genes are accessory proteins (e.g. coxDEF). Genes shown in white do not belong to the CODH operon and genes labeled with # indicate a gene found in many CODH operons, but not in the *O. carboxidovorans* genome. This gene (#) is annotated as ‘CTP:molybdopterin cytidylytransferase’ according to RAST. Genes are not drawn to scale. The CSP 2012 contig is available upon request.

**Fig. S3.** Comparison of 13C isotope content in white and pale worms after incubation with 13C-labeled bicarbonate, nitrate and oxygen, but no additional external energy source for 36 h. The γ1-symbionts in white worms contain large amounts of stored elemental sulfur, which they use for CO2 fixation under oxic conditions (Giere, 2006). In pale worms, the sulfur stores of the γ1-symbionts are reduced or depleted leading to less CO2 fixation. Pale worms were obtained by oxic pre-incubations (see Experimental procedures). Mean values and standard deviations of five (for the white worms) and three (for the pale worms) independent incubations are shown. 13C isotope content values are given in atomic percentage (AT% = (13C/(12C + 13C))x100).

**Table S1.** 13C isotope fraction data for individual regions of interest (ROIs).

**Table S2.** Values for individual measurements of CO and H2 standards.

**Table S3.** Results from bulk analyses of whole worms.