

1 **Communal metabolism by *Methylococcaceae* and *Methylophilaceae***  
2 **is driving rapid aerobic methane oxidation in sediments of a**  
3 **shallow seep near Elba, Italy**

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## 40 **Originality-Significance Statement**

41 Methane is a potent greenhouse gas contributing substantially to global warming, and emissions  
42 from marine seeps contribute up to 10% of methane in the atmosphere. Methanotrophic  
43 microorganisms can use methane as carbon and energy source, and thus significantly mitigate global  
44 methane emissions from seep areas, acting as an important 'benthic filter'. This study reports on the  
45 efficiency and function of the 'benthic filter' at a shallow methane seep, by quantifying the rates of  
46 methane oxidation, identifying the microbial key players involved in this process and assessing their  
47 function. Compared to the well-studied deep-sea seeps, shallow seeps represent distinct  
48 hydrogeochemical settings, where the risk of emitted methane reaching the atmosphere is much  
49 higher. The findings we present are highly relevant to evaluate the impact of shallow seeps on global  
50 atmospheric methane budgets.

53 **Abstract**

54 Release of abiotic methane from marine seeps into the atmosphere is a major source of this potent  
55 greenhouse gas. Methanotrophic microorganisms in methane seeps use methane as carbon and  
56 energy source, thus significantly mitigating global methane emissions. Here we investigated  
57 microbial methane oxidation at the sediment-water interface of a shallow marine methane seep.  
58 Metagenomics and metaproteomics, combined with <sup>13</sup>C-methane stable isotope probing,  
59 demonstrated that various members of the gammaproteobacterial family *Methylococcaceae* were  
60 the key players for methane oxidation, catalyzing the first reaction step to methanol. We observed a  
61 transfer of carbon to methanol-oxidizing methylotrophs of the betaproteobacterial family  
62 *Methylophilaceae*, suggesting an interaction between methanotrophic and methylotrophic  
63 microorganisms that allowed for rapid methane oxidation. From our microcosms, we estimated  
64 methane oxidation rates of up to 871 nmol of methane per gram sediment and day. This implies that  
65 more than 50% of methane at the seep is removed by microbial oxidation at the sediment-water  
66 interface, based on previously reported *in situ* methane fluxes. The organic carbon produced was  
67 further assimilated by different heterotrophic microbes, demonstrating that the methane-oxidizing  
68 community supported a complex trophic network. Our results provide valuable eco-physiological  
69 insights into this specialized microbial community performing an ecosystem function of global  
70 relevance.

## 74 **Introduction**

75 Methane is the most abundant hydrocarbon in the atmosphere, and acts as a harmful greenhouse  
76 gas (Reeburgh, 2007). Approximately one third of the global methane flux to the atmosphere is  
77 derived from natural sources (Judd et al., 2002b). Reports on the contribution of oceanic methane  
78 emissions, primarily originating from natural cold seeps along continental margins (Etiope, 2012),  
79 vary from 1 to 10% of the total flux (Kvenvolden et al., 2001; Judd et al., 2002b). The methane flux  
80 from the subsurface sea bed, however, is even higher (Reeburgh, 2007). Biological activity of  
81 methane-oxidizing microorganisms in seafloor sediments and the water column considerably reduces  
82 the amount of methane that reaches the atmosphere. These microorganisms, termed  
83 methanotrophs, use methane as their sole carbon and energy source. The methanotrophs act as a  
84 'benthic filter' (Boetius and Wenzhöfer, 2013) modulating methane emission from the sea, and  
85 supply methane-derived carbon to a broad range of other organisms. Hence, in the seep  
86 environment, methanotrophs carry out a key role in the microbial community that is comparable to  
87 autotrophic primary producers, and their activity is affected by the microbial satellite community  
88 present (Yu and Chistoserdova, 2017). To understand the modulation of methane emission by the  
89 benthic filter, various studies have targeted microbial communities at methane seep areas, especially  
90 in the deep sea (see (Boetius and Wenzhöfer, 2013) for a review). Deep-sea sediments are typically  
91 characterized by fine-grain particles that limit the circulation of pore water. As the deep-sea seafloor  
92 is not influenced by hydrodynamic forces from waves or tidal movement, stable layers with steep  
93 hydrogeochemical gradients exist. Oxygen is consumed within the first few millimeters of the  
94 sediment through the degradation of organic matter deposited by sedimentation of particulate  
95 organic carbon (de Beer et al., 2006; Glud, 2008). Aerobic methane oxidation is hence restricted to a  
96 thin layer of sediment, or occurs in microbial mats covering the sediment (Boetius and Wenzhöfer,  
97 2013; Ruff et al., 2016; Paul et al., 2017). In subsurface layers, anaerobic oxidation of methane (AOM)  
98 by methanotrophic archaea in combination with sulfate-reducing bacteria takes place, typically

99 representing the predominant process for methane removal beneath the seafloor (Knittel and  
100 Boetius, 2009; Boetius and Wenzhöfer, 2013).

101 Shallow methane seeps, in contrast, can feature highly permeable sandy sediments, which allow  
102 advection-driven pore water circulation that introduces oxygen into deeper layers. The gas flow  
103 upwards additionally leads to a downstream of oxic sea water (O'Hara et al., 1995). Further,  
104 hydrodynamic forces result in mixing of the sediment and impede the formation of overlying  
105 microbial mats. Hence, in contrast to the stable conditions in deep-sea sediments, shallow sediments  
106 comprise a highly variable and heterogeneous environment with fluctuating oxygen concentrations.  
107 The frequent influx of oxygen restricts the highly oxygen-sensitive AOM consortia to deeper  
108 sediment layers (Knittel and Boetius, 2009). Thus, aerobic methane oxidation in the upper layers and  
109 at the sediment-water interface might be the predominant process for methane removal at shallow  
110 seeps.

111 Methane originating from depths below 100 m typically does not reach the sea surface due to  
112 dissolution processes of methane bubbles and oxidation of dissolved methane (Schmale et al., 2005;  
113 McGinnis et al., 2006). Hence, deep-sea seeps play little to no role in atmospheric methane emission.  
114 For shallow methane seeps, models suggest site specific parameters such as depth and initial bubble  
115 size along with aqueous methane concentration and upwelling flows to be major factors determining  
116 methane emission (Leifer and Patro, 2002; McGinnis et al., 2006). Emission from such shallow seeps  
117 has been estimated as  $310 \text{ g CH}_4 \text{ m}^{-2} \text{ year}^{-1}$  at the Kattegat coast, Denmark (Dando et al., 1994), up to  
118  $550 \text{ g CH}_4 \text{ m}^{-2} \text{ year}^{-1}$  at Torry Bay, UK (Judd et al., 2002a),  $260 \text{ g CH}_4 \text{ m}^{-2} \text{ year}^{-1}$  at Isla Mocha, Chile  
119 (Jessen et al., 2011), and  $400 \text{ g CH}_4 \text{ m}^{-2} \text{ year}^{-1}$  at the Santa Barbara Channel, CA, USA (Luyendyk et al.,  
120 2003). The total emissions of the small Kattegat and Torry Bay seeps, covering an area of only a few  
121 thousand square meters, are in the range of one metric ton per year, while the Isla Mocha and Santa  
122 Barbara Channel seep, covering several square kilometers, are estimated to release 800 to 7200  
123 metric tons of methane per year into the atmosphere.

124 Little is known about the identity and filter function of aerobic methanotrophic bacteria in such  
125 shallow seep areas. In this study, we investigated the diversity and function of aerobic  
126 methanotrophs at a shallow methane seep located off the coast of the Island of Elba, Italy, at only 12  
127 meters depth. Discovered in 1995, the Elba shallow methane seep is located in a tectonically-active  
128 site (Greve et al., 2014) and is characterized by a gentle, constant bubbling of gas, consisting of up to  
129 73% (Meister et al., 2018) to more than 85% abiotic methane (Ruff et al., 2016; Sciarra et al., 2019),  
130 leading to an efflux of  $145 \text{ g CH}_4 \text{ m}^{-2} \text{ year}^{-1}$  into the water column (Sciarra et al., 2019). A previous  
131 investigation of AOM at the seep site revealed predominantly sulfur-coupled methane oxidation by  
132 consortia resembling those found in deep-sea seeps, but restricted to sediment layers more than 20  
133 cm below the seafloor (Ruff et al., 2016). AOM exhibited only a low methane removal efficiency, and  
134 the authors concluded that aerobic methane oxidation is probably more important at this site (Ruff  
135 et al., 2016).

136 Here, we explored the microbial community in the top 2-3 centimeters of the sediment at the Elba  
137 methane seep, and its potential for methane oxidation. The aims of our study were (I) to determine  
138 the activity of aerobic methanotrophs and estimate their efficiency in methane removal, (II) to  
139 identify the key players of methane oxidation active in the oxic sediments, and (III) to follow the flux  
140 of methane-derived carbon through the microbial community, assessing the role of methanotrophs  
141 as key suppliers of organic carbon at the seep. We combined a  $^{13}\text{C}$ -methane stable isotope probing  
142 (SIP) approach with metagenomics, to obtain metagenome-assembled genomes (MAGs) of the  
143 microorganisms present, as well as metaproteomics, to verify their predicted metabolic functions  
144 and assess their activity. This allowed us to gain an understanding of structure and function of the  
145 specialized, methanotrophy-driven microbial community at the methane seep.

## 146 Results

### 147 Activity of methanotrophs in microcosms and estimation of the benthic 148 filter efficiency

149 A rapid consumption of methane was observed in microcosms containing sediment and water from  
150 the Elba shallow methane seep, when supplemented with 1% (v:v, headspace) of  $^{12}\text{C}$ - or  $^{13}\text{C}$ -  
151 methane. Methane consumption started immediately after setup of the microcosms. After 7 days of  
152 incubation, methane consumption rates of  $439 \pm 42 \text{ nmol d}^{-1} \text{ g sediment}^{-1}$  (average of microcosms  
153 with  $^{12}\text{C}$  and  $^{13}\text{C}$  methane,  $n = 12$ ,  $\pm \text{SD}$ ) were observed, with no difference between  $^{12}\text{C}$  and  $^{13}\text{C}$   
154 incubations (Figure 1). As the high consumption rates led to frequent depletion of methane, we  
155 increased the headspace concentration to 2% after 25 days of incubation. This resulted in a  
156 significant increase ( $p < 0.001$ , Student's  $t$ -test) of methane consumption to  $871 \pm 123 \text{ nmol d}^{-1} \text{ g}$   
157  $\text{sediment}^{-1}$  (average of microcosms with  $^{12}\text{C}$  and  $^{13}\text{C}$  methane,  $n = 8$ ,  $\pm \text{SD}$ ) (Figure 1). For individual  
158 microcosms, methane consumption up to  $2.26 \mu\text{mol d}^{-1} \text{ g sediment}^{-1}$  was observed (Dataset S1). In  
159 comparison, reported methane consumption rates for AOM at the same site were only up to  
160  $200 \text{ nmol d}^{-1} \text{ g sediment}^{-1}$  under 1.5 atmospheres of  $\text{CH}_4:\text{CO}_2$  (90:10) (Ruff et al., 2016).

161 Using the average rate of methane consumption for 2% headspace concentration, we estimated the  
162 annual methane consumption in the Elba methane seep. Based on the sediment porosity given in  
163 (Ruff et al., 2016), we calculated a methane consumption of approximately  $12 \text{ mol m}^{-2} \text{ year}^{-1}$   
164 (Supplementary Information). Previous studies have reported a gas flow of  $0.72 \text{ L m}^{-2} \text{ d}^{-1}$  from the  
165 sediment (Sciarra et al., 2019), containing approximately 85% (v:v) methane, resulting in a release of  
166  $9 \text{ mol m}^{-2} \text{ year}^{-1}$  methane into the water column. Hence, based on our estimated rates, more than  
167 50% of the methane flowing through the sediment is consumed at the sediment water interface.  
168 Indeed, this is likely a considerable underestimation of the *in situ* methane consumption. The  
169 methane concentration in the water phase of our microcosms was approximately  $22 \mu\text{M}$  (2%  
170 methane), according to calculations based on Henry's Law (Supplementary Information). *In situ*



171 concentrations at the Elba methane seep are up to one order of magnitude higher, with 50  $\mu\text{M}$  to  
172 550  $\mu\text{M}$  reported for pore water (Ruff et al., 2016). Considering the increase of methane  
173 consumption observed in our microcosms when increasing the headspace methane concentration  
174 from 1% to 2%, *in situ* consumption could be considerably higher than our estimates. Given that this  
175 aerobic removal of methane at the sediment-water-interface exceeds previously reported AOM rates  
176 (Ruff et al., 2016), we aimed to explore the function of the underlying microbial methane oxidizing  
177 processes.

## 178 **Identifying the key methane oxidizers**

179 We used an integrated approach combining different 'omics' techniques with SIP to elucidate the key  
180 players responsible for the methane consumption observed in our microcosms. Taxonomic profiles of  
181 the microbial communities in the microcosms sampled after 25, 45 and 65 days were investigated by  
182 metaproteomics to determine the dominant microbial taxa. The majority of peptides identified were  
183 consistently related to *Proteobacteria*, with *Alphaproteobacteria* and *Gammaproteobacteria*  
184 (including *Betaproteobacteriales*, based on the current Silva taxonomy release 132 (Quast et al.,  
185 2013)) being the dominant classes (Figure S1). At the family level, the presence of various taxa  
186 implicated in  $\text{C}_1$  metabolism was revealed, including *Methylococcaceae* (*Gammaproteobacteria*),  
187 *Methylophilaceae* (*Betaproteobacteriales*) and *Rhodobacteraceae* (*Alphaproteobacteria*)  
188 (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Ruff et al., 2015). To identify the active  
189 methanotrophs,  $^{13}\text{C}$  incorporation in peptides extracted from the microcosms amended with  $^{13}\text{C}$ -  
190 methane was investigated. Peptides related to *Methylococcaceae* as well as *Methylophilaceae*  
191 showed  $^{13}\text{C}$  relative isotope abundances (RIA) and incorporation patterns suggesting a direct uptake  
192 of  $^{13}\text{C}$  from methane (Figure 2, Figure S2). Peptides of *Rhodobacteraceae*, however, as well as those  
193 of several other taxa, showed incorporation patterns that suggested  $^{13}\text{C}$  uptake by cross-feeding  
194 rather than by direct uptake of a  $^{13}\text{C}$ -labelled substrate. The  $^{13}\text{C}$  isotopologue patterns acquired using  
195 SIP-metaproteomics allow a differentiation between such modes of carbon assimilation (Seifert et  
196 al., 2012; Taubert et al., 2012).

197 Furthermore, PCR analysis targeting key functional genes for C<sub>1</sub> metabolism was linked with DNA-SIP  
198 by investigating the heavy DNA fractions obtained from <sup>13</sup>C microcosms. The presence of *pmoA*,  
199 encoding the small subunit of the copper-dependent particulate methane monooxygenase (pMMO),  
200 as well as of *xoxF*, encoding a lanthanide-dependent methanol dehydrogenase (MDH) (Keltjens et al.,  
201 2014; Taubert et al., 2015; Howat et al., 2018) were observed. However, no *mmoX* encoding the  
202 alpha-subunit of soluble methane monooxygenase (sMMO), or *mxoF*, encoding a calcium-dependent  
203 MDH were found. Interestingly, *pmoA* sequences were exclusively affiliated with *Methylococcaceae*,  
204 while *xoxF* sequences were mainly affiliated with *Methylococcaceae*, *Betaproteobacteriales* and  
205 *Rhodobacteraceae* (Figure S3). Complementary functional analysis of the metaproteomes likewise  
206 revealed that peptides of the pMMO, covering all three subunits PmoCAB, were exclusively affiliated  
207 to *Methylococcaceae*. No peptides of other methane oxidizing enzymes, such as sMMO or methyl-  
208 coenzyme M reductase (Friedrich, 2005), were found. Peptides of methanol dehydrogenases were  
209 exclusively related to XoxF and not to MxoF, and were affiliated to multiple taxonomic groups,  
210 including *Methylococcaceae*, *Methylophilaceae* and different *Alphaproteobacteria* (Figure 3). Hence,  
211 while multiple taxa were potentially involved in downstream functions like the oxidation of methanol  
212 to formaldehyde, only *Methylococcaceae* were able to catalyze the first step in methane  
213 degradation, the oxidation of methane to methanol.

214 To explore the key players for methane oxidation more closely, we conducted SIP-metagenomics by  
215 Illumina MiSeq sequencing of the DNA obtained from heavy fractions of the <sup>13</sup>C microcosms. Ten  
216 million MiSeq reads were assembled and binned, resulting in 99 metagenome-assembled genomes  
217 (MAGs), with two MAGs considered complete genome drafts (> 90% completeness, < 5%  
218 contamination (Parks et al., 2015; Vollmers et al., 2017a)) and another eight intermediate quality  
219 genome drafts (> 70% completeness, < 10% contamination (Bishara et al., 2018) (Figure S4).  
220 Surprisingly, eighteen different MAGs affiliated with *Methylococcaceae* were found (Table 1),  
221 indicating multiple closely related methane oxidizers. To provide a more accurate taxonomic  
222 classification and to estimate relatedness between the different *Methylococcaceae* MAGs, we

223 performed phylogenetic analysis based on amino acid sequences of single copy marker genes (SCMG)  
224 (Wu et al., 2013). All *Methylococcaceae* MAGs contained marker genes that were most closely  
225 related to those of *Methylomonas* spp., creating a sister lineage of this genus (Figure 4A). The amino  
226 acid identity between the MAGs was typically less than 85%, indicating that indeed multiple closely  
227 related species were present.

228 Genes encoding subunits of pMMO, i.e., *pmoC*, *pmoA* and *pmoB*, were present exclusively in MAGs  
229 affiliated with *Methylococcaceae*. The same MAGs typically also contained genes of an ortholog to  
230 the *pmoCAB* operon, dubbed *pxmABC* (Figure S5). These orthologs also encode copper-dependent  
231 monooxygenases, which are potentially involved in methane oxidation under oxygen limited and  
232 nitrite rich conditions (Kits et al., 2015b; Kits et al., 2015a). Potentially linked to these putative  
233 alternative pMMOs, several MAGs contained genes involved in denitrification, such as *narG* and  
234 *napABC*, encoding nitrate reductases, and *nirS*, encoding nitrite reductase. The expression of the  
235 *pmoCAB* genes was confirmed for multiple MAGs (Table 2, Table S1), but no expression of *pxmABC*  
236 genes, as well as of the genes involved in denitrification, was observed. No other functional genes for  
237 methane-oxidizing enzymes were observed in the metagenomes. Based on both genomic and  
238 proteomic data, these bacteria utilized XoxF-type MDHs for oxidation of methanol to formaldehyde.  
239 The classification of the MDH genes was verified by phylogenetic analysis using a custom reference  
240 database of *xoxF* and *mxoF* genes, clearly placing the detected genes in the *xoxF5* clade (Figure S6).  
241 Furthermore, genes of the tetrahydromethanopterin (H<sub>4</sub>MPT) pathway for formaldehyde oxidation,  
242 as well as key genes of the ribulose monophosphate (RuMP) cycle for formaldehyde assimilation, 3-  
243 hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase, were expressed. The  
244 identified key players hence showed the typical metabolic traits of type I methanotrophs, in  
245 agreement with their taxonomic affiliation within the *Gammaproteobacteria* (Trotsenko and Murrell,  
246 2008).

247 The gene expression profiles of the different *Methylococcaceae*, as well as the enrichment of their  
248 DNA in the heavy fraction and the <sup>13</sup>C incorporation in their peptides, demonstrated that several of

249 these closely related bacteria were active and responsible for methane oxidation in the microcosms.  
250 Considering the heterogeneity of the sediment present at the methane seep, these bacteria can have  
251 differing environmental preferences, and so their distribution might be driven by hydrogeochemical  
252 factors beyond the availability of methane. Hence, despite their taxonomic similarity, these bacteria  
253 might inhabit different environmental niches.

## 254 **Role of non-methanotrophic methylotrophs**

255 In addition to the key methanotrophs, non-methanotrophic organisms affiliated with  
256 *Methylophilaceae* were also found to be highly active in the microcosms, as deduced from <sup>13</sup>C  
257 incorporation. Despite their lack of the ability to oxidize methane, evident from metaproteomic,  
258 metagenomic and functional gene data, the <sup>13</sup>C incorporation patterns in their peptides were  
259 indistinguishable from those of the methanotrophic *Methylococcaceae* (Figure 2, Figure S2),  
260 resembling a direct uptake of a <sup>13</sup>C labelled substrate (Seifert et al., 2012). Phylogenetic analysis of  
261 the six MAGs related to *Methylophilaceae* in our metagenomic dataset, based on amino acid  
262 sequences of SCMGs, demonstrated an affiliation with *Methylophilus* spp. and *Methylotenera* spp.  
263 (Figure 4B). Functional classification of peptides identified in the metaproteomics analysis showed  
264 the presence of XoxF-type methanol dehydrogenases affiliated with the *Methylophilaceae* (clades  
265 XoxF4 and XoxF1, Figure S6), as well as enzymes of the H<sub>4</sub>MPT pathway for formaldehyde oxidation  
266 and the RuMP cycle for formaldehyde assimilation, supporting a methylotrophic lifestyle. The  
267 identified peptides could be mapped to several of the six *Methylophilaceae* MAGs observed (Table 2,  
268 Table S1), indicating that also from this taxon, different methylotrophs were active in our  
269 microcosms.

270 As no genes or proteins involved in methane oxidation in the *Methylophilaceae* in our microcosms  
271 were present, we can exclude that these organisms used methane directly as a carbon source, and  
272 instead have more likely been labelled by cross-feeding. For cross-feeding organisms, a shift in the  
273 peptide RIA with incubation time can often be detected when newly synthesized, <sup>13</sup>C-labelled  
274 compounds from the primary consumers mix with pre-existing, unlabelled compounds (Seifert et al.,

275 2012; Taubert et al., 2012). In our study, we observed such shifts, for instance, in autotrophic  
276 *Nitrospirales* (Figure S2) that became labelled due to the enrichment of the carbonate pool in the  
277 incubations by  $^{13}\text{C}\text{O}_2$  released from  $^{13}\text{C}$ -methane oxidation. However, a low concentration of the  
278 respective pre-existing compound, e.g., caused by a starvation period or a rapid uptake by the cross-  
279 feeding organisms, will not result in sufficient amounts of intermediately labelled peptides to be  
280 detected by metaproteomics analysis. Given the presence of key methylotrophic functions in the  
281 *Methylophilaceae*, the most likely explanation for the  $^{13}\text{C}$  labelling of these organisms is the uptake of  
282  $^{13}\text{C}$  methanol released from the methanotrophic *Methylococcaceae*, implying a transfer of carbon  
283 from methanotrophs to methylotrophs.

284 Interestingly, further putative methylotrophs related to the alphaproteobacterial family  
285 *Rhodobacteraceae* were present and active in our microcosms, but showed only indirect  $^{13}\text{C}$   
286 incorporation patterns slowly increasing in RIA over time (Figure 2). The low  $^{13}\text{C}$ -labelling ratio  
287 observed indicated a much slower growth rate than for *Methylophilaceae*. Of 14 MAGs affiliated with  
288 *Alphaproteobacteria*, seven were related to the *Roseobacter* clade within the *Rhodobacteraceae*,  
289 while the remaining were related to *Hyphomonadaceae*, *Stappiaceae* and an unknown  
290 *Rhodobacterales* family (Table 1, Figure S7). Only for one of the MAGs affiliated with the *Roseobacter*  
291 clade was a gene encoding a *xoxF5*-type MDH found, as well as the corresponding gene product,  
292 indicating that the majority of these bacteria were not able to utilize methanol. Nevertheless, most  
293 of the 14 MAGs revealed a metabolic potential for  $\text{C}_1$  utilization, typically including glutathione- and  
294 tetrahydrofolate-(THF)-dependent pathways for  $\text{C}_1$  oxidation/reduction as well as key genes of the  
295 serine cycle for formaldehyde assimilation, including hydroxypyruvate reductase, glycerate 2-kinase,  
296 malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA reductase.  
297 Furthermore, in two of the MAGs affiliated with the *Roseobacter* clade, a gene encoding ribulose  
298 biphosphate carboxylase required for  $\text{CO}_2$  fixation was present. The coverage of our metaproteomic  
299 analysis was insufficient to verify the metabolism of these alphaproteobacterial organisms. The  
300 potential for  $\text{C}_1$  utilization suggested that they might assimilate other  $\text{C}_1$  compounds potentially

301 derived from methane oxidation, such as formaldehyde. However, the  $^{13}\text{C}$  RIA in the peptides  
302 affiliated with *Alphaproteobacteria* was significantly lower than that of *Methylophilaceae* ( $p < 0.001$   
303 for all time points, Student's *t*-test), while not significantly different to the autotrophic *Nitrospirales*.  
304 This suggested that some of these organisms could have assimilated carbon from  $\text{CO}_2$ , while using  $\text{C}_1$   
305 compounds as energy source (Figure 5). However, the lower RIA observed for *Alphaproteobacteria*  
306 might also result from recycling of unlabelled organic compounds in the microcosms. Hence, while  
307 our results strongly indicate that the different alphaproteobacterial taxa were continuously active  
308 and oxidized  $\text{C}_1$  compounds to gain energy in our microcosms, the nature of their carbon source  
309 remains uncertain.

## 310 **Discussion**

311 Previous studies indicated that the activity of methane oxidizing microorganisms leads to a massive  
312 reduction of methane emission from marine seeps. Boetius and Wenzhöfer summarized that  
313 between 20 and 80% of methane released from cold seeps of continental slopes is removed by this  
314 process, depending on the seep environment, with fluid flow rate and oxygen availability as  
315 influential parameters (Boetius and Wenzhöfer, 2013). Here we confirmed that this notion holds true  
316 for a shallow methane seep near Elba, characterized by highly permeable sandy sediment that allows  
317 an increased oxygen circulation into deeper layers. The methane oxidation potential estimated at 12  
318  $\text{mol m}^{-2} \text{ year}^{-1}$ , based on rate measurements in microcosms, was in the same range as the methane  
319 flux in the water column of 9  $\text{mol m}^{-2} \text{ year}^{-1}$ , measured *in situ* (Sciarra et al., 2019), indicating that a  
320 major portion of the methane is removed at the sediment-water interface before reaching the water  
321 column (Figure 5).

322 We identified members of the *Methylococcaceae* within the order *Methylococcales* as the key  
323 methane oxidizers. Previous studies indicated that *Methylococcales* are typically found at high  
324 relative abundance at methane seeps, independent of seep hydrogeochemistry and geographic  
325 location (Ruff et al., 2015). Here we showed that the key methane oxidizers present at the Elba seep

326 formed a sister lineage to *Methylomonas* sp. within the *Methylococcaceae*, potentially comprising a  
327 new genus, and that multiple closely related organisms of this taxon were present. This co-  
328 occurrence of bacteria from the same functional guild suggests the existence of different niches for  
329 methane oxidizers at the sediment-water interface. Parameters like the availability of oxygen and  
330 other electron acceptors, the methane concentration and the presence of alternative reduced  
331 molecules might drive the distribution of methane oxidizers with different metabolic capabilities. The  
332 presence of *pxmABC* genes hints to the potential for nitrite-dependent methanotrophy in the Elba  
333 sediments, given suitable conditions (Kits et al., 2015b; Kits et al., 2015a). Furthermore, the  
334 substrate-specificity of pMMO-like proteins is often not clear (Tavormina et al., 2013; Khadka et al.,  
335 2018), so some *Methylococcaceae* might additionally be capable of oxidizing alternative compounds  
336 like short chain alkanes. These divergent metabolic traits would allow the methanotrophs to occupy  
337 various niches and thrive under different biogeochemical conditions. Such a functional redundancy  
338 provides multiple advantages for ecosystem functions, such as enhanced stability against  
339 environmental disturbances (Griffiths and Philippot, 2013). In the shallow, sandy sediment,  
340 disturbances can easily occur, e.g., by hydrodynamic forces like waves and currents, or by seasonal  
341 changes (Ruff et al., 2016). Moreover, the adaptation of microorganisms to specific environmental  
342 niches optimizes their function and hence results in a fine-tuning of the methane oxidation  
343 machinery.

344 Furthermore, the association of methanotrophs with non-methanotrophic methylotrophs seems to  
345 be of major importance for the efficiency of methane oxidation. Our results suggested a transfer of  
346 methane-derived carbon from the *Methylococcaceae* to methylotrophs related to *Methylotenera*  
347 spp. and *Methylophilus* spp. of the *Methylophilaceae*. Interactions of *Methylococcaceae* with other  
348 bacteria, e.g., leading to aggregate formation, have been previously reported at deep-sea methane  
349 seeps (Ruff et al., 2013). Typically, *Methylophaga* spp. or other gamma- and alphaproteobacterial  
350 species are the most abundant methylotrophs associated with the methanotrophic  
351 *Methylococcaceae* (Lösekann et al., 2007; Ruff et al., 2013; Ruff et al., 2015; Paul et al., 2017).

352 *Methylophilaceae* related to *Methylothera*/*Methylophilus* spp., in contrast, are only rarely observed  
353 at marine methane seeps (Ruff et al., 2013; Paul et al., 2017). With the notable exception of the  
354 OM43 clade (Giovannoni et al., 2008), members of the *Methylophilaceae* family are typically not  
355 abundant in marine environments, and seem to prefer environments with lower salinity such as  
356 estuaries or freshwater (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Deng et al., 2018).  
357 Intriguingly, in sediments of Lake Washington (WA, USA), a well-studied freshwater lake featuring  
358 high methane fluxes, cooperations between *Methylococcaceae* and *Methylophilaceae* have been  
359 observed as well (Kalyuzhnaya et al., 2008; Beck et al., 2013). Incubation experiments revealed  
360 specific relationships between *Methylosarcina* spp. and *Methylophilus* spp. at high oxygen  
361 concentrations, as well as *Methylobacter* spp. and *Methylothera* spp. at lower oxygen  
362 concentrations (Hernandez et al., 2015). Synthetic culture experiments with methanotrophic and  
363 non-methanotrophic isolates from Lake Washington also revealed *Methylomonas* spp. to be included  
364 in such partnerships, and to be highly competitive (Yu et al., 2017). While the non-methanotrophic  
365 partners of such interactions obviously benefit from the release of methanol from the  
366 methanotrophs, the gain for the methanotrophs is still unclear. An exchange of public goods, such as  
367 vitamin B12, or interspecies electron transfer contributing to methane activation have been  
368 discussed (Yu and Chistoserdova, 2017). Regardless, the interaction of methanotrophs and  
369 methylotrophs is a common theme across various environments featuring high methane fluxes, and  
370 seems to be a major factor for efficient functioning of the benthic methane filter (Ho et al., 2014).

371 Methanol and other C<sub>1</sub> compounds are typically produced in marine environments as byproducts of  
372 algal growth or decomposition of organic compounds such as osmolytes, resulting in concentrations  
373 in the nM to μM range (Naqvi et al., 2005; Beale et al., 2015). Hence, methylotrophs that degrade  
374 these compounds are commonly found in marine habitats. These methylotrophs, however, are  
375 distinctly different from those present at methane seeps, and are typically dominated by members of  
376 the *Roseobacter* clade, the *Methylophilaceae* group OM43 or the SAR11 clade (Giovannoni et al.,  
377 2008; Sun et al., 2011; Zhuang et al., 2018). In our microcosms, we found members of the



378 *Roseobacter* clade and other *Alphaproteobacteria* with the genetic potential for C<sub>1</sub> utilization. These  
379 bacteria showed low, but consistent activity throughout 65 days of incubation. To succeed in the  
380 open sea water, these bacteria are optimized for the uptake of the low concentrations of organic  
381 compounds present, and usually utilize various C<sub>1</sub> compounds as well as multi-carbon substrates  
382 (Brinkhoff et al., 2008), and typically exhibit slow growth rates. In our microcosms, we observed an  
383 uptake of methane-derived carbon by these bacteria, but were unable to discern whether they  
384 assimilated methanol or other C<sub>1</sub> compounds as byproducts of methane oxidation, or multi-carbon  
385 compounds released by the primary C<sub>1</sub> utilizers, or if they fixed CO<sub>2</sub> and used organic carbon  
386 compounds solely as energy sources. Such a chemoorganoautotrophic lifestyle, often supported by  
387 anoxygenic photosynthesis, has been reported for various marine methylophils, termed  
388 “methylophils” (Sun et al., 2011; Pinhassi et al., 2016). Hence, although the methane seep recruits a  
389 distinct and specific community of C<sub>1</sub>-utilizing organisms, apparently the typical marine  
390 methylophils can also sustain their activity in this environment, and potentially benefit from the  
391 increased levels of organic compounds produced by the methanotrophs.

392 Interestingly, all methanotrophs and methylophils of the *Methylococcaceae*, *Methylophilaceae* and  
393 other *Alphaproteobacteria* detected in our incubations employed lanthanide-dependent, XoxF-type  
394 methanol dehydrogenases instead of the calcium-dependent methanol dehydrogenase MxaFI. The  
395 high diversity of *xoxF* gene sequences in marine habitats, especially *xoxF4* and *xoxF5*, as well as their  
396 prevalence over *mxoF* gene sequences, has previously been described (Ramachandran and Walsh,  
397 2015; Taubert et al., 2015). The lanthanides required for these enzymes, belonging to the rare earth  
398 elements, are typically present in sufficient concentrations in coastal environments from sediments  
399 or coastal run-off, despite their low solubility (Elderfield et al., 1990; Keltjens et al., 2014).

400 In summary, we showed that the microbial community present in the oxic sediments at the Elba  
401 methane seep is highly efficient in methane removal, exceeding the methane oxidation rates  
402 reported for AOM at this site (Ruff et al., 2016), likely due to the high oxygen levels in the sediment  
403 precluding AOM. We identified members of the *Methylococcaceae* as the key players of aerobic

404 methane oxidation, and obtained several genome drafts of different active, closely related members  
405 of this group. We observed a tight association of these methanotrophs with non-methanotrophic  
406 methylotrophs of the *Methylophilaceae*, likely through exchange of methanol, contributing to the  
407 efficiency of methane oxidation. Finally, methane-derived carbon was also transferred to other  
408 microorganisms not able to utilize methanol, supporting the hypothesis that methanotrophs fuel a  
409 complex trophic network and can be considered as primary producers in the methane seep  
410 environment. The gain of knowledge on methane removal by the 'benthic filter' at shallow seeps  
411 provided by our study will facilitate future estimations of the global methane budget, and highlights  
412 the relevance of methanotrophs as model systems to study principles of microbial interactions.

## 413 **Experimental Procedures**

### 414 **Sample collection and microcosm setup**

415 Samples of oxic sediment from the top 2-3 cm and water were collected in May 2014 by divers from  
416 a shallow methane seep located off the coast of Elba, Italy (42° 44.628' N, 10° 07.094' E), in 12 m  
417 water depth. Five 50 ml BD Falcon™ tubes were filled with ~100 g of sediment each, and two 1 L  
418 bottles were filled with seawater from a maximum of 50 cm above the sediment surface. Samples  
419 were transported and stored at 4°C until the start of the SIP experiments at the University of East  
420 Anglia, United Kingdom, four days after sampling. Microcosms were set up in 120 ml serum bottles  
421 with 20 g of sediment and 25 ml of seawater each, and marine ammonium mineral salts (MAMS)  
422 were added to a final concentration of 1% of full-strength medium. Microcosms were spiked with 1%  
423 (v:v, headspace) <sup>13</sup>C-labelled or unlabelled (<sup>12</sup>C) methane (six of each), and incubated at 25°C in a  
424 shaking incubator (50 rpm). Headspace methane concentrations were monitored using gas  
425 chromatography (Supplementary Information). When the headspace concentrations in all  
426 microcosms were below 0.1% (v:v), additional methane (1-2%, v:v) was added. Duplicate <sup>12</sup>C and <sup>13</sup>C  
427 microcosms were sacrificed for DNA and protein extraction after 25, 45 and 65 days of incubation.

## 428 **DNA and protein extraction and DNA-SIP**

429 Combined DNA and protein extractions were performed from microcosms as well as from untreated  
430 sediment (T0) according to a previously described protocol (Taubert et al., 2012) with minor  
431 modifications (Supplementary Information). Extracted DNA was subjected to fractionation using CsCl  
432 gradients, and fractions containing <sup>13</sup>C-labelled DNA were selected as previously described (Neufeld  
433 et al., 2007; Grob et al., 2015) with minor modifications (Supplementary Information).

## 434 **Amplicon and metagenomic sequencing**

435 PCR amplicons for 454 sequencing were obtained from selected fractions using the following primer  
436 sets and conditions: The *pmoA* gene encoding the β-subunit of particulate methane monooxygenase  
437 was amplified by nested PCR using primer pairs A189F/A682R (Holmes et al., 1995) and  
438 A189F/mb661R (Costello and Lidstrom, 1999) as previously described (Horz et al., 2005). The *mmoX*  
439 gene encoding soluble methane monooxygenase subunit A was amplified by nested PCR using primer  
440 pairs mmoX166f/mmoX1401r (Auman et al., 2000) and mmoX206f/mmoX886r (Hutchens et al.,  
441 2004) as described. The *xoxF4*, *xoxF5* and *mxoF* genes encoding different methanol dehydrogenases  
442 were amplified using primer pairs *xoxF4f/r*, *xoxF5f/r* (Taubert et al., 2015) and  
443 *mxoF1003f/mxoF1555r* (McDonald and Murrell, 1997) using PCR conditions as described by these  
444 authors. Combined and purified triplicate PCR products were subjected to 454 pyrosequencing (GS  
445 FLX Titanium system, MR DNA, Shallowater, TX, USA). Sequencing data were processed using mothur  
446 (v.1.35.1) (Schloss et al., 2009) for quality control, demultiplexing, and removal of barcodes and  
447 primers as previously described for other functional genes (Taubert et al., 2015). Sequences were  
448 binned to OTUs with a 97% identity threshold and chimeras were removed using USEARCH  
449 (v7.0.1090) (Edgar, 2013). Phylogeny was assigned using Megan (v.5.1.5) (Huson et al., 2011) and a  
450 previously described pipeline for functional genes (Dumont et al., 2014). Raw data are available at  
451 the National Center for Biotechnology Information (NCBI) database under bioproject PRJNA524087.

452 For metagenomic sequencing, separate libraries were prepared from total DNA from untreated  
453 sediment (T0) as well as from <sup>13</sup>C-labelled DNA obtained from the duplicate microcosms of each of  
454 the three time points. Metagenomic DNA was sheared using a Covaris S220 sonication device  
455 (Covaris Inc., MA, USA) with the following settings: 55 s 175 W, 5% Duty factor, 200 cycles of burst,  
456 55.5 µl. Library preparation was done using the NEBNext® DNA Library Prep kit for Illumina® (E6040,  
457 New England BioLabs® Inc., Ipswich, MA, USA). Sufficient material for sequencing (15 - 20 µg) was  
458 obtained from SIP fractions without further amplification. Metagenome sequencing was then  
459 performed on an Illumina MiSeq machine using v3 chemistry (600 cycles).

460 Metagenome reads were adapter clipped and quality trimmed using Trimmomatic v0.32 (Bolger et  
461 al., 2014). Low complexity reads were removed using the DUST approach of prinseq-lite v0.20.4  
462 (Schmieder and Edwards, 2011) with a cutoff of 15, and residual phiX-contaminants were filtered out  
463 using FastQ Screen (Wingett and Andrews, 2018). Overlapping read pairs were then merged using  
464 FLASH 1.2.11 (Magoč and Salzberg, 2011).

465 For each time point and for the untreated samples, an individual metagenome assembly was  
466 produced by coassembling the corresponding libraries from experimental replicates using megahit  
467 v1.0.5 (Li et al., 2015). Read coverage of assembled contigs was determined by mapping using  
468 Bowtie2 (Langmead and Salzberg, 2012). Each metagenome was then binned using Maxbin v.2.1.1  
469 (Wu et al., 2016). Bins were subsequently decontaminated using a z-score based differential  
470 coverage approach previously described (Vollmers et al., 2017b; Pratscher et al., 2018). Bins with a  
471 high likelihood of originating from the same species were identified based on similarity of coverage  
472 profiles across all time points and subsamples, as well as by the presence of nearly identical universal  
473 marker genes. Any such related bins were merged and coassembled by extracting the respective  
474 reads from all corresponding time points and reassembly using megahit. Completeness and potential  
475 contamination of the final binned MAGs was estimated using CheckM (Parks et al., 2015).

476 Phylogenetic trees to elucidate taxonomic relationships for metagenome-assembled genomes based  
477 on concatenated amino acid alignments of taxon-specific single copy marker genes were constructed

478 using the ezTree pipeline (Wu, 2018). The shotgun metagenome reads, corresponding assemblies, as  
479 well as binned MAGs with estimated completeness > 70% and contamination < 10% are available at  
480 the NCBI database under bioproject PRJNA522277.

## 481 **SIP-metaproteomics**

482 Sample preparation for metaproteomics analysis was done as previously described (Grob et al.,  
483 2015). Mass spectrometry was performed on an Orbitrap Fusion MS (Thermo Fisher Scientific,  
484 Waltham, MA, USA) (Supplementary Information).

485 Proteome Discoverer (v1.4.0288, Thermo Scientific) was used for protein identification and the  
486 acquired MS/MS spectra were searched against the NCBI nr database with taxonomy set to Archaea  
487 and Bacteria using the Mascot algorithm, and against protein sequences derived from all acquired  
488 MAGs using the SequestHT algorithm. Trypsin was chosen as cleavage enzyme, allowing a maximum  
489 of two missed cleavages. The precursor mass tolerance (MS) was set to 10 ppm, the fragment mass  
490 tolerance (MS/MS) was 0.05 Da. Carbamidomethylation of cysteine was considered as fixed and  
491 oxidation of methionine was set as dynamic modification. Peptide spectrum matches (PSMs) were  
492 validated using Percolator (v2.04) with a false discover rate (FDR) < 1% and quality filtered for XCorr  
493  $\geq 2.25$  (for charge state +2) and  $\geq 2.5$  (for charge state +3). Identified proteins were grouped by  
494 applying the strict parsimony principle (Nesvizhskii and Aebersold, 2005). The mass spectrometry  
495 proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-  
496 Riverol et al., 2019) partner repository with the dataset identifier PXD013378.

497 Taxonomic classification of peptides was done by the lowest common ancestor method using  
498 UniPept (Mesuere et al., 2018). Identification of  $^{13}\text{C}$ -labelled peptides and quantification of  $^{13}\text{C}$   
499 incorporation was done by comparing measured and expected isotopologue patterns,  
500 chromatographic retention times and fragmentation patterns as previously described (Seifert et al.,  
501 2012; Taubert et al., 2012). For each taxonomic group of interest,  $^{13}\text{C}$  incorporation was quantified in  
502 10 peptides per time point, 5 from each replicate microcosm.

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726 nitrogen cycling. *Environ Microbiol* **20**: 4543-4554.

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## 731 **Figure and Table Legends**

732 **Figure 1: Methane consumption in microcosms with sediment from the Elba methane seep.** Values  
733 given are the cumulative amount of methane consumed in the microcosms. Separate averaged  
734 values for microcosms with  $^{12}\text{C}$ -methane and microcosms with  $^{13}\text{C}$ -methane are depicted by cross  
735 and diamond symbols, respectively. Error bars indicate standard deviation. Arrows indicate time  
736 points of methane addition. Brackets display the amount of methane (% headspace, v:v) of each  
737 addition and number of replicate microcosms (n) each supplemented with  $^{12}\text{C}$ - or  $^{13}\text{C}$ - methane.

738 **Figure 2:  $^{13}\text{C}$  incorporation into peptides of different bacterial taxonomic groups.** Values depict (A)  
739 the  $^{13}\text{C}$  relative isotope abundance (RIA), i.e., the amount of carbon replaced by  $^{13}\text{C}$ , and (B) the  
740 labelling ratio, i.e., the abundance of  $^{13}\text{C}$ -labelled compared to unlabelled molecules, of peptides  
741 specific to the given taxonomic groups after incubation of sediment for 25, 45 and 65 days with  $^{13}\text{C}$ -  
742 methane. Values are based on n = 10 peptides per time point, error bars show standard deviation.

743 **Figure 3: Functional classification of identified peptides.** The numbers of peptides affiliated to  
744 different enzymes and pathways of different functional categories relevant for  $\text{C}_1$  metabolism are  
745 shown. Colors depict the taxonomic distribution of the peptides in each functional category based on  
746 the lowest common ancestor of each peptide. Peptide identification is based on metaproteomics  
747 analysis of samples from microcosms with  $^{12}\text{C}$ -methane of all three time points (n = 6). The peptides  
748 were identified using NCBI nr and the metagenome-assembled genomes obtained in this study as  
749 reference databases. MMO: methane monooxygenase, MDH: methanol dehydrogenase, FAE:  
750 formaldehyde-activating enzyme, H4MPT: tetrahydromethanopterin pathway for formaldehyde  
751 oxidation, THF: tetrahydrofolate pathway for formaldehyde oxidation, glutathione: glutathione  
752 pathway for formaldehyde oxidation, formate DH: formate dehydrogenase, RuMP: ribulose  
753 monophosphate pathway, based on the key enzymes 3-hexulose-6-phosphate synthase and 3-  
754 hexulose-6-phosphate isomerase. For the serine cycle, the key enzymes hydroxypyruvate reductase,

755 glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA  
756 reductase were taken into account. For the Calvin cycle, the key enzyme ribulose-1,5-bisphosphate  
757 carboxylase/oxygenase was taken into account.

758 **Figure 4: Phylogenetic affiliation of the key methanotrophs and methylotrophs identified at the**  
759 **Elba methane seep.** (A) Phylogenetic tree representing key methanotrophs, based on a concatenated  
760 amino acid alignment of 36 single copy marker genes with a total of 6 329 positions. Only  
761 metagenome-assembled genomes (MAGs) related to *Methylococcaceae* with at least 50%  
762 completeness are shown. *Pseudomonas oryzae* (*Pseudomonadales*) was included as an outgroup to  
763 root the tree. (B) Phylogenetic tree representing key methylotrophs, based on a concatenated amino  
764 acid alignment of 94 single copy marker genes with a total of 21 475 positions. Only MAGs related to  
765 *Methylophilaceae* with at least 35% completeness are shown. *Sulfuricella denitrificans*  
766 (*Gallionellaceae*) was included as an outgroup to root the tree. Both trees were inferred with the  
767 Approximately-Maximum-Likelihood approach of FastTree using the JTT-CAT model for amino acid  
768 evolution, local support values were calculated using the Shimodaira-Hasegawa test from 1 000  
769 resamples. The scale bars indicate the number of amino acid changes per site.

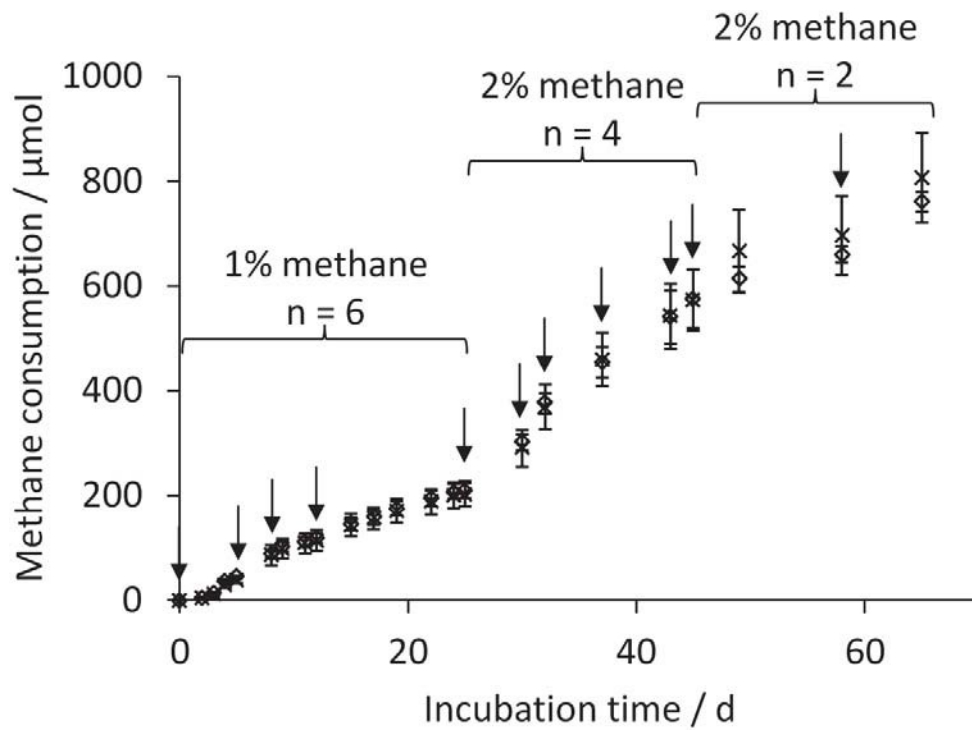
770 **Figure 5: Conceptual overview of communal methane metabolism at the Elba seep.** The character C  
771 in red indicates methane-derived carbon. OC: organic carbon compounds released from the primary  
772 methane utilizing community of *Methylococcaceae* and *Methylophilaceae*. \*Methane consumption  
773 of the microbial community estimated based on average consumption rates in microcosms from this  
774 study. †Methane flux from sediments to hydrosphere as reported in Ruff et al., 2015 (Ruff et al.,  
775 2015).

776 **Table 1: Statistics for metagenome-assembled genomes affiliated with *Methylococcaceae*,**  
777 ***Methylophilaceae* and other *Alphaproteobacteria*.** Taxonomic relationships were elucidated based  
778 on concatenated amino acid alignments of taxon-specific single copy marker genes using the ezTree

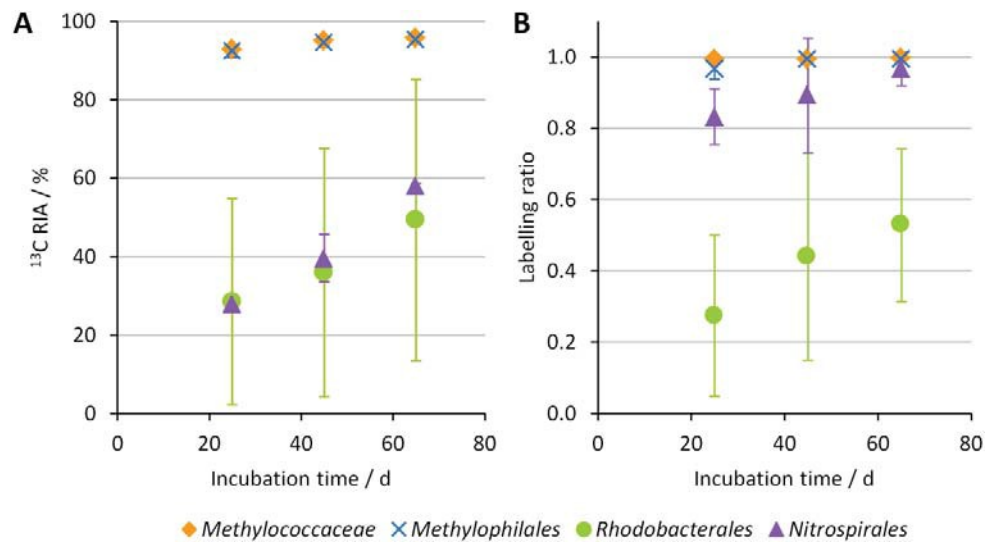


779 pipeline (Wu, 2018). <sup>1</sup>Based on CheckM analysis (Parks et al., 2015). N50: 50% of the genome  
780 assembly is contained in scaffolds equal to or larger than this value.

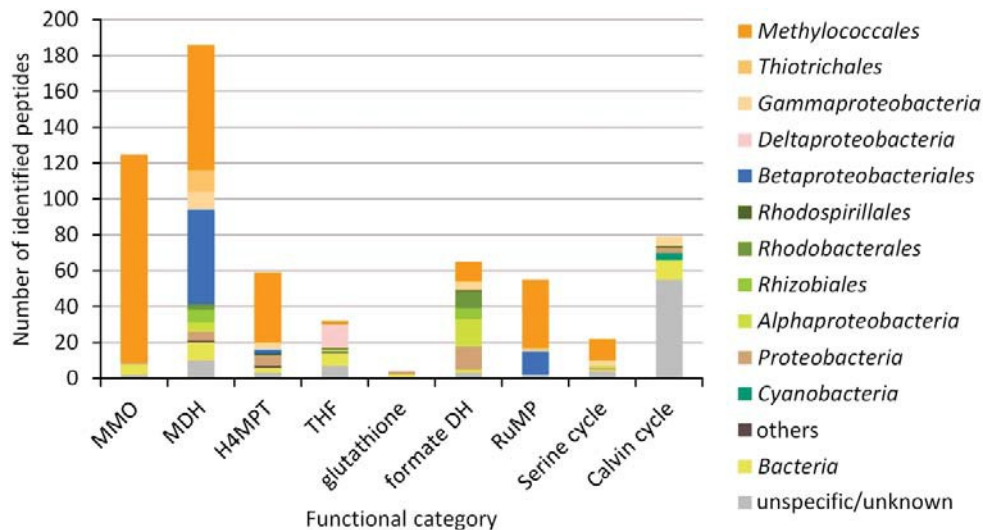
781 **Table 2: Presence and expression of functional genes for C<sub>1</sub> metabolism in metagenome-assembled**  
782 **genomes.** White fields indicate presence of functional genes for the respective function, red fields  
783 indicate expression of the encoded enzymes based on metaproteomics analysis. Numbers in the  
784 fields indicate number of genes expressed / number of genes present. <sup>1</sup>Based on key genes 3-  
785 hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase. <sup>2</sup>Based on key genes  
786 hydroxypyruvate reductase, glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate  
787 lyase and crotonyl-CoA reductase. <sup>3</sup>Based on key gene ribulose-1,5-bisphosphate  
788 carboxylase/oxygenase.



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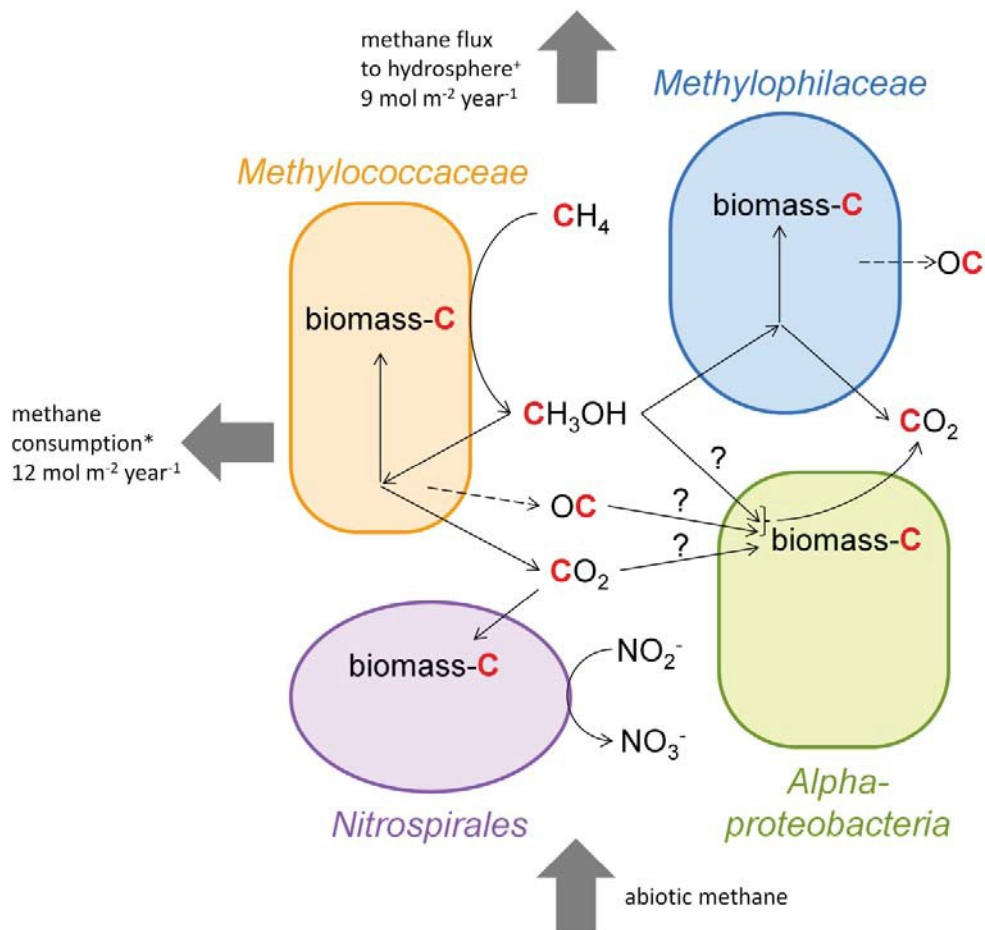


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**Figure 3: Functional classification of identified peptides.** The numbers of peptides affiliated to different enzymes and pathways of different functional categories relevant for C<sub>1</sub> metabolism are shown. Colors depict the taxonomic distribution of the peptides in each functional category based on the lowest common ancestor of each peptide. Peptide identification is based on metaproteomics analysis of samples from microcosms with <sup>12</sup>C-methane of all three time points (n = 6). The peptides were identified using NCBI nr and the metagenome-assembled genomes obtained in this study as reference databases. MMO: methane monooxygenase, MDH: methanol dehydrogenase, FAE: formaldehyde-activating enzyme, H4MPT: tetrahydromethanopterin pathway for formaldehyde oxidation, THF: tetrahydrofolate pathway for formaldehyde oxidation, glutathione: glutathione pathway for formaldehyde oxidation, formate DH: formate dehydrogenase, RuMP: ribulose monophosphate pathway, based on the key enzymes 3-hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase. For the serine cycle, the key enzymes hydroxypyruvate reductase, glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA reductase were taken into account. For the Calvin cycle, the key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase was taken into account.





**Figure 5: Conceptual overview of communal methane metabolism at the Elba seep.** The character C in red indicates methane-derived carbon. OC: organic carbon compounds released from the primary methane utilizing community of *Methylococcaceae* and *Methylophilaceae*. \*Methane consumption of the microbial community estimated based on average consumption rates in microcosms from this study.

+Methane flux from sediments to hydrosphere as reported in Ruff et al., 2015 (Ruff et al., 2015).

Table 1

MAG ID	Completeness <sup>1</sup> / %	Contamination <sup>1</sup> / %	Genome size / bp	# scaffolds	N50 / bp	Longest scaffold / bp	GC-content / %	Taxonomy	
T2.024_T3.008	100	1.7	3683201	376	34899	93907	44.2	<i>Methylococcaceae</i>	
T1.009_T2.004_T3.006	83	0.0	2300148	655	4880	22077	41		
T1.003_T2.003_T3.002	83	1.7	3077222	293	75471	205488	45.4		
T2.010	79	12.9	2913017	643	6449	30650	43.2		
T3.010	70	1.7	1589959	663	2776	11559	39.9		
T1.027_T2.045	54	0.0	2190210	498	6013	23572	50.7		
T1.007_T2.016_T3.009	48	21.9	2527797	1237	2234	22330	46.4		
T1.019_T2.038	44	10.0	890090	349	3371	21537	41		
T1.030	32	0.0	555941	75	11159	37635	43.5		
T1.008	28	0.0	1494445	617	3100	17312	40		
T2.043	22	0.0	362264	189	2000	6705	43.8		
T3.014	19	0.0	758301	465	1669	5485	42.2		
T2.026	18	0.0	717869	348	2219	9852	42.4		
T1.022	17	1.9	412297	304	1307	3810	40.5		
T2.011	17	3.5	444422	193	2739	15981	45		
T1.002	13	1.7	389904	210	1836	8026	45.4		
T2.056	12	0.0	433371	233	1946	5949	41.5		
T1.005	12	1.7	357089	217	1700	5296	42.9		
T2.027	90	7.6	1688246	192	19805	82017	43.8		<i>Methylophilaceae</i>
T2.008_T3.001	84	8.6	2242488	316	40778	107604	44.5		
T2.019	36	0.0	1151259	154	18334	44712	45.4		
T2.058	20	0.0	313978	123	2860	10416	44.3		
T2.018	16	0.0	672373	229	3746	22975	44.5		
T2.053	8	2.7	237367	104	2715	8566	44.5		
T2.009	91	0.0	2013842	46	107948	212351	54.8	<i>Rhodobacterales</i>	
T2.007	88	0.0	2945199	280	15984	62688	57.7	<i>Rhodobacterales</i>	
T2.006_T3.003	71	1.7	3450492	222	63712	159910	56.7	<i>Rhodobacterales</i>	
T2.015	57	0.3	2302051	202	19356	68546	59.1	<i>Hyphomonadaceae</i>	
T2.014	47	1.7	1805201	302	10348	38924	58.2	<i>Rhodobacterales</i>	
T2.023	45	7.5	1925340	1179	1653	8054	54.3	<i>Alphaproteobacteria</i>	
T2.054	42	0.0	1519802	590	3097	12667	50.4	<i>Alphaproteobacteria</i>	
T3.011	36	0.0	1629932	595	3299	11661	57.4	<i>Hyphomonadaceae</i>	
T1.014	35	0.0	826762	285	3403	12803	49.6	<i>Rhodobacterales</i>	
T2.029	25	1.0	1825719	1003	1936	7230	56.9	<i>Labrenzia</i>	
T1.018	18	3.8	803406	573	1353	4436	57.9	<i>Hyphomonadaceae</i>	
T1.016	17	0.0	1413249	817	1778	10583	57.9	<i>Rhodobacterales</i>	
T2.033	13	3.5	251432	198	1245	2899	58.2	<i>Alphaproteobacteria</i>	
T1.015_T2.022	4	0.0	593602	408	1414	5020	64.1	<i>Rhodobacterales</i>	

Table 2

	MAG ID	methane oxidation			methanol oxidation		C <sub>1</sub> oxidation				C <sub>1</sub> assimilation		
		methane monooxygenase/ammonia monooxygenase ( <i>pmo</i> )	soluble methane monooxygenase ( <i>mmo</i> )	methyl-coenzyme m reductase ( <i>mcr</i> )	methanol dehydrogenase ( <i>mxoA</i> F )	methanol dehydrogenase ( <i>xox</i> F )	tetrahydromethanopterin (H4MPT) pathway for formaldehyde oxidation	tetrahydrofolate (THF) pathway for formaldehyde oxidation	glutathione pathway for formaldehyde oxidation	formate dehydrogenase	ribulose monophosphate pathway <sup>1</sup>	serine pathway <sup>2</sup>	Calvin-Benson-Bassham cycle <sup>3</sup>
<i>Methylococcaceae</i>	T2.024_T3.008	3 / 3				1 / 1	3 / 14	0 / 3			3 / 3	0 / 4	
	T1.009_T2.004_T3.006	2 / 5				1 / 1	3 / 14	0 / 2		0 / 2	2 / 4	0 / 4	
	T1.003_T2.003_T3.002	1 / 8				1 / 1	1 / 9	0 / 3	0 / 1	1 / 1	1 / 2	0 / 4	
	T2.010	0 / 6					1 / 21	0 / 4	0 / 2	0 / 1	1 / 1	0 / 7	
	T3.010						2 / 9	0 / 1		0 / 1		0 / 3	
	T1.027_T2.045	0 / 2				1 / 1	1 / 8	0 / 3		0 / 1	2 / 2	0 / 1	
	T1.007_T2.016_T3.009	0 / 7					1 / 23	0 / 9	0 / 1	0 / 3		0 / 1	
	T1.019_T2.038					2 / 2	0 / 9			0 / 1		0 / 2	
	T1.030											0 / 3	
	T1.008	1 / 1				1 / 1	0 / 1	0 / 1				0 / 3	
	T2.043						0 / 2					0 / 3	
	T3.014	2 / 4					0 / 4	0 / 1				0 / 3	
	T2.026						1 / 5	0 / 2				0 / 2	
	T1.022	0 / 1					0 / 2	0 / 1		0 / 1		0 / 4	
	T2.011	0 / 9					0 / 5	0 / 1		0 / 1			
	T1.002	3 / 4					0 / 3						
	T2.056						0 / 2	0 / 1					
T1.005	1 / 3						0 / 1						
<i>Methylophilaceae</i>	T2.027					1 / 1	1 / 9	0 / 3		0 / 1	0 / 3		
	T2.008_T3.001					2 / 4	0 / 7	0 / 3		0 / 1	2 / 3		
	T2.019					0 / 1	0 / 7	0 / 2		0 / 2	0 / 1		
	T2.058							0 / 1					
	T2.018						0 / 3	0 / 3		0 / 1			
	T2.053					2 / 2	1 / 4			0 / 1	0 / 1		
<i>Alphaproteobacteria</i>	T2.009							0 / 2	0 / 1			0 / 3	
	T2.007							0 / 3	0 / 2	1 / 2		0 / 7	0 / 1
	T2.006_T3.003							0 / 3	1 / 3			0 / 12	0 / 1
	T2.015							0 / 2	0 / 2			1 / 5	
	T2.014							0 / 2	0 / 2	0 / 1		0 / 3	
	T2.023							0 / 2	0 / 2	1 / 3		0 / 3	
	T2.054							0 / 2	0 / 2	0 / 1		0 / 1	
	T3.011							0 / 1	0 / 3			0 / 4	
	T1.014							0 / 1				0 / 1	
	T2.029							0 / 2	0 / 2	2 / 2		0 / 2	
	T1.018							0 / 1	0 / 3			0 / 5	
	T1.016							0 / 2				0 / 3	
	T2.033											0 / 1	
	T1.015_T2.022					1 / 1	1 / 1	1 / 3				0 / 2	