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Published in:
Environmental Microbiology

DOI (link to publication from Publisher):
[10.1111/1462-2920.14728](https://doi.org/10.1111/1462-2920.14728)

Publication date:
2019

Document Version
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

Citation for published version (APA):
Taubert, M., Grob, C., Crombie, A., Howat, A. M., Burns, O. J., Weber, M., Lott, C., Kaster, A.-K., Vollmers, J., Jehmlich, N., von Bergen, M., Chen, Y., & Murrell, J. C. (2019). Communal metabolism by Methylococcaceae and Methylophilaceae is driving rapid aerobic methane oxidation in sediments of a shallow seep near Elba, Italy. *Environmental Microbiology*, 21(10), 3780-3795. <https://doi.org/10.1111/1462-2920.14728>

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

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36 Running title: (50 characters): Aerobic methane oxidation at a shallow seep

37 The authors declare no conflict of interest.

38

39 **Originality-Significance Statement**

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

50

51

52 **Abstract**

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

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73 Introduction

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

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

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

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

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

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

145 Results

146 Activity of methanotrophs in microcosms and estimation of the benthic filter efficiency

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

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

170 550 μM reported for pore water (Ruff et al., 2016). Considering the increase of methane
171 consumption observed in our microcosms when increasing the headspace methane concentration
172 from 1% to 2%, *in situ* consumption could be considerably higher than our estimates. Given that this
173 aerobic removal of methane at the sediment-water-interface exceeds previously reported AOM rates
174 (Ruff et al., 2016), we aimed to explore the function of the underlying microbial methane oxidizing
175 processes.

176 Identifying the key methane oxidizers

177 We used an integrated approach combining different 'omics' techniques with SIP to elucidate the key
178 players responsible for the methane consumption observed in our microcosms. Taxonomic profiles of
179 the microbial communities in the microcosms sampled after 25, 45 and 65 days were investigated by
180 metaproteomics to determine the dominant microbial taxa. The majority of peptides identified were
181 consistently related to *Proteobacteria*, with *Alphaproteobacteria* and *Gammaproteobacteria*
182 (including *Betaproteobacteriales*, based on the current Silva taxonomy release 132 (Quast et al.,
183 2013)) being the dominant classes (Figure S1). At the family level, the presence of various taxa
184 implicated in C_1 metabolism was revealed, including *Methylococcaceae* (*Gammaproteobacteria*),
185 *Methylophilaceae* (*Betaproteobacteriales*) and *Rhodobacteraceae* (*Alphaproteobacteria*)
186 (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Ruff et al., 2015). To identify the active
187 methanotrophs, ^{13}C incorporation in peptides extracted from the microcosms amended with ^{13}C -
188 methane was investigated. Peptides related to *Methylococcaceae* as well as *Methylophilaceae*
189 showed ^{13}C relative isotope abundances (RIA) and incorporation patterns suggesting a direct uptake
190 of ^{13}C from methane (Figure 2, Figure S2). Peptides of *Rhodobacteraceae*, however, as well as those
191 of several other taxa, showed incorporation patterns that suggested ^{13}C uptake by cross-feeding
192 rather than by direct uptake of a ^{13}C -labelled substrate. The ^{13}C isotopologue patterns acquired using
193 SIP-metaproteomics allow a differentiation between such modes of carbon assimilation (Seifert et
194 al., 2012; Taubert et al., 2012).

195 Furthermore, PCR analysis targeting key functional genes for C₁ metabolism was linked with DNA-SIP
196 by investigating the heavy DNA fractions obtained from ¹³C microcosms. The presence of *pmoA*,
197 encoding the small subunit of the copper-dependent particulate methane monooxygenase (pMMO),
198 as well as of *xoxF*, encoding a lanthanide-dependent methanol dehydrogenase (MDH) (Keltjens et al.,
199 2014; Taubert et al., 2015; Howat et al., 2018) were observed. However, no *mmoX* encoding the
200 alpha-subunit of soluble methane monooxygenase (sMMO), or *mxoF*, encoding a calcium-dependent
201 MDH were found. Interestingly, *pmoA* sequences were exclusively affiliated with *Methylococcaceae*,
202 while *xoxF* sequences were mainly affiliated with *Methylococcaceae*, *Betaproteobacteriales* and
203 *Rhodobacteraceae* (Figure S3). Complementary functional analysis of the metaproteomes likewise
204 revealed that peptides of the pMMO, covering all three subunits PmoCAB, were exclusively affiliated
205 to *Methylococcaceae*. No peptides of other methane oxidizing enzymes, such as sMMO or methyl-
206 coenzyme M reductase (Friedrich, 2005), were found. Peptides of methanol dehydrogenases were
207 exclusively related to XoxF and not to MxoF, and were affiliated to multiple taxonomic groups,
208 including *Methylococcaceae*, *Methylophilaceae* and different *Alphaproteobacteria* (Figure 3). Hence,
209 while multiple taxa were potentially involved in downstream functions like the oxidation of methanol
210 to formaldehyde, only *Methylococcaceae* were able to catalyze the first step in methane
211 degradation, the oxidation of methane to methanol.

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

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

226 Genes encoding subunits of pMMO, i.e., *pmoC*, *pmoA* and *pmoB*, were present exclusively in MAGs
227 affiliated with *Methylococcaceae*. The same MAGs typically also contained genes of an ortholog to
228 the *pmoCAB* operon, dubbed *pxmABC* (Figure S5). These orthologs also encode copper-dependent
229 monooxygenases, which are potentially involved in methane oxidation under oxygen limited and
230 nitrite rich conditions (Kits et al., 2015b; Kits et al., 2015a). Potentially linked to these putative
231 alternative pMMOs, several MAGs contained genes involved in denitrification, such as *narG* and
232 *napABC*, encoding nitrate reductases, and *nirS*, encoding nitrite reductase. The expression of the
233 *pmoCAB* genes was confirmed for multiple MAGs (Table 2, Table S1), but no expression of *pxmABC*
234 genes, as well as of the genes involved in denitrification, was observed. No other functional genes for
235 methane-oxidizing enzymes were observed in the metagenomes. Based on both genomic and
236 proteomic data, these bacteria utilized XoxF-type MDHs for oxidation of methanol to formaldehyde.
237 The classification of the MDH genes was verified by phylogenetic analysis using a custom reference
238 database of *xoxF* and *mxoF* genes, clearly placing the detected genes in the *xoxF5* clade (Figure S6).
239 Furthermore, genes of the tetrahydromethanopterin (H₄MPT) pathway for formaldehyde oxidation,
240 as well as key genes of the ribulose monophosphate (RuMP) cycle for formaldehyde assimilation, 3-
241 hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase, were expressed. The
242 identified key players hence showed the typical metabolic traits of type I methanotrophs, in
243 agreement with their taxonomic affiliation within the *Gamma*proteobacteria (Trotsenko and Murrell,
244 2008).

245 The gene expression profiles of the different *Methylococcaceae*, as well as the enrichment of their
246 DNA in the heavy fraction and the ¹³C incorporation in their peptides, demonstrated that several of

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

252 Role of non-methanotrophic methylotrophs

253 In addition to the key methanotrophs, non-methanotrophic organisms affiliated with
254 *Methylophilaceae* were also found to be highly active in the microcosms, as deduced from ^{13}C
255 incorporation. Despite their lack of the ability to oxidize methane, evident from metaproteomic,
256 metagenomic and functional gene data, the ^{13}C incorporation patterns in their peptides were
257 indistinguishable from those of the methanotrophic *Methylococcaceae* (Figure 2, Figure S2),
258 resembling a direct uptake of a ^{13}C labelled substrate (Seifert et al., 2012). Phylogenetic analysis of
259 the six MAGs related to *Methylophilaceae* in our metagenomic dataset, based on amino acid
260 sequences of SCMGs, demonstrated an affiliation with *Methylophilus* spp. and *Methylotenera* spp.
261 (Figure 4B). Functional classification of peptides identified in the metaproteomics analysis showed
262 the presence of XoxF-type methanol dehydrogenases affiliated with the *Methylophilaceae* (clades
263 XoxF4 and XoxF1, Figure S6), as well as enzymes of the H_4MPT pathway for formaldehyde oxidation
264 and the RuMP cycle for formaldehyde assimilation, supporting a methylotrophic lifestyle. The
265 identified peptides could be mapped to several of the six *Methylophilaceae* MAGs observed (Table 2,
266 Table S1), indicating that also from this taxon, different methylotrophs were active in our
267 microcosms.

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

273 2012; Taubert et al., 2012). In our study, we observed such shifts, for instance, in autotrophic
274 *Nitrospirales* (Figure S2) that became labelled due to the enrichment of the carbonate pool in the
275 incubations by $^{13}\text{C}\text{O}_2$ released from ^{13}C -methane oxidation. However, a low concentration of the
276 respective pre-existing compound, e.g., caused by a starvation period or a rapid uptake by the cross-
277 feeding organisms, will not result in sufficient amounts of intermediately labelled peptides to be
278 detected by metaproteomics analysis. Given the presence of key methylotrophic functions in the
279 *Methylophilaceae*, the most likely explanation for the ^{13}C labelling of these organisms is the uptake of
280 ^{13}C methanol released from the methanotrophic *Methylococcaceae*, implying a transfer of carbon
281 from methanotrophs to methylotrophs.

282 Interestingly, further putative methylotrophs related to the alphaproteobacterial family
283 *Rhodobacteraceae* were present and active in our microcosms, but showed only indirect ^{13}C
284 incorporation patterns slowly increasing in RIA over time (Figure 2). The low ^{13}C -labelling ratio
285 observed indicated a much slower growth rate than for *Methylophilaceae*. Of 14 MAGs affiliated with
286 *Alphaproteobacteria*, seven were related to the *Roseobacter* clade within the *Rhodobacteraceae*,
287 while the remaining were related to *Hyphomonadaceae*, *Stappiaceae* and an unknown
288 *Rhodobacterales* family (Table 1, Figure S7). Only for one of the MAGs affiliated with the *Roseobacter*
289 clade was a gene encoding a *xoxF5*-type MDH found, as well as the corresponding gene product,
290 indicating that the majority of these bacteria were not able to utilize methanol. Nevertheless, most
291 of the 14 MAGs revealed a metabolic potential for C_1 utilization, typically including glutathione- and
292 tetrahydrofolate-(THF)-dependent pathways for C_1 oxidation/reduction as well as key genes of the
293 serine cycle for formaldehyde assimilation, including hydroxypyruvate reductase, glycerate 2-kinase,
294 malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA reductase.
295 Furthermore, in two of the MAGs affiliated with the *Roseobacter* clade, a gene encoding ribulose
296 biphosphate carboxylase required for CO_2 fixation was present. The coverage of our metaproteomic
297 analysis was insufficient to verify the metabolism of these alphaproteobacterial organisms. The
298 potential for C_1 utilization suggested that they might assimilate other C_1 compounds potentially

299 derived from methane oxidation, such as formaldehyde. However, the ^{13}C RIA in the peptides
300 affiliated with *Alphaproteobacteria* was significantly lower than that of *Methylophilaceae* ($p < 0.001$
301 for all time points, Student's *t*-test), while not significantly different to the autotrophic *Nitrospirales*.
302 This suggested that some of these organisms could have assimilated carbon from CO_2 , while using C_1
303 compounds as energy source (Figure 5). However, the lower RIA observed for *Alphaproteobacteria*
304 might also result from recycling of unlabelled organic compounds in the microcosms. Hence, while
305 our results strongly indicate that the different alphaproteobacterial taxa were continuously active
306 and oxidized C_1 compounds to gain energy in our microcosms, the nature of their carbon source
307 remains uncertain.

308 Discussion

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

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

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

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

350 *Methylophilaceae* related to *Methylothera*/*Methylophilus* spp., in contrast, are only rarely observed
351 at marine methane seeps (Ruff et al., 2013; Paul et al., 2017). With the notable exception of the
352 OM43 clade (Giovannoni et al., 2008), members of the *Methylophilaceae* family are typically not
353 abundant in marine environments, and seem to prefer environments with lower salinity such as
354 estuaries or freshwater (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Deng et al., 2018).
355 Intriguingly, in sediments of Lake Washington (WA, USA), a well-studied freshwater lake featuring
356 high methane fluxes, cooperations between *Methylococcaceae* and *Methylophilaceae* have been
357 observed as well (Kalyuzhnaya et al., 2008; Beck et al., 2013). Incubation experiments revealed
358 specific relationships between *Methylosarcina* spp. and *Methylophilus* spp. at high oxygen
359 concentrations, as well as *Methylobacter* spp. and *Methylothera* spp. at lower oxygen
360 concentrations (Hernandez et al., 2015). Synthetic culture experiments with methanotrophic and
361 non-methanotrophic isolates from Lake Washington also revealed *Methylomonas* spp. to be included
362 in such partnerships, and to be highly competitive (Yu et al., 2017). While the non-methanotrophic
363 partners of such interactions obviously benefit from the release of methanol from the
364 methanotrophs, the gain for the methanotrophs is still unclear. An exchange of public goods, such as
365 vitamin B12, or interspecies electron transfer contributing to methane activation have been
366 discussed (Yu and Chistoserdova, 2017). Regardless, the interaction of methanotrophs and
367 methylotrophs is a common theme across various environments featuring high methane fluxes, and
368 seems to be a major factor for efficient functioning of the benthic methane filter (Ho et al., 2014).
369 Methanol and other C₁ compounds are typically produced in marine environments as byproducts of
370 algal growth or decomposition of organic compounds such as osmolytes, resulting in concentrations
371 in the nM to μM range (Naqvi et al., 2005; Beale et al., 2015). Hence, methylotrophs that degrade
372 these compounds are commonly found in marine habitats. These methylotrophs, however, are
373 distinctly different from those present at methane seeps, and are typically dominated by members of
374 the *Roseobacter* clade, the *Methylophilaceae* group OM43 or the SAR11 clade (Giovannoni et al.,
375 2008; Sun et al., 2011; Zhuang et al., 2018). In our microcosms, we found members of the

376 *Roseobacter* clade and other *Alphaproteobacteria* with the genetic potential for C₁ utilization. These
377 bacteria showed low, but consistent activity throughout 65 days of incubation. To succeed in the
378 open sea water, these bacteria are optimized for the uptake of the low concentrations of organic
379 compounds present, and usually utilize various C₁ compounds as well as multi-carbon substrates
380 (Brinkhoff et al., 2008), and typically exhibit slow growth rates. In our microcosms, we observed an
381 uptake of methane-derived carbon by these bacteria, but were unable to discern whether they
382 assimilated methanol or other C₁ compounds as byproducts of methane oxidation, or multi-carbon
383 compounds released by the primary C₁ utilizers, or if they fixed CO₂ and used organic carbon
384 compounds solely as energy sources. Such a chemoorganoautotrophic lifestyle, often supported by
385 anoxygenic photosynthesis, has been reported for various marine methylotrophs, termed
386 “methylovores” (Sun et al., 2011; Pinhassi et al., 2016). Hence, although the methane seep recruits a
387 distinct and specific community of C₁-utilizing organisms, apparently the typical marine
388 methylotrophs can also sustain their activity in this environment, and potentially benefit from the
389 increased levels of organic compounds produced by the methanotrophs.

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

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

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

411 [Experimental Procedures](#)

412 [Sample collection and microcosm setup](#)

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

426 DNA and protein extraction and DNA-SIP

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

432 Amplicon and metagenomic sequencing

433 PCR amplicons for 454 sequencing were obtained from selected fractions using the following primer
434 sets and conditions: The *pmoA* gene encoding the β-subunit of particulate methane monooxygenase
435 was amplified by nested PCR using primer pairs A189F/A682R (Holmes et al., 1995) and
436 A189F/mb661R (Costello and Lidstrom, 1999) as previously described (Horz et al., 2005). The *mmoX*
437 gene encoding soluble methane monooxygenase subunit A was amplified by nested PCR using primer
438 pairs *mmoX166f/mmoX1401r* (Auman et al., 2000) and *mmoX206f/mmoX886r* (Hutchens et al.,
439 2004) as described. The *xoxF4*, *xoxF5* and *mxαF* genes encoding different methanol dehydrogenases
440 were amplified using primer pairs *xoxF4f/r*, *xoxF5f/r* (Taubert et al., 2015) and
441 *mxαF1003f/mxαF1555r* (McDonald and Murrell, 1997) using PCR conditions as described by these
442 authors. Combined and purified triplicate PCR products were subjected to 454 pyrosequencing (GS
443 FLX Titanium system, MR DNA, Shallowater, TX, USA). Sequencing data were processed using *mothur*
444 (v.1.35.1) (Schloss et al., 2009) for quality control, demultiplexing, and removal of barcodes and
445 primers as previously described for other functional genes (Taubert et al., 2015). Sequences were
446 binned to OTUs with a 97% identity threshold and chimeras were removed using *USEARCH*
447 (v7.0.1090) (Edgar, 2013). Phylogeny was assigned using *Megan* (v.5.1.5) (Huson et al., 2011) and a
448 previously described pipeline for functional genes (Dumont et al., 2014). Raw data are available at
449 the National Center for Biotechnology Information (NCBI) database under bioproject PRJNA524087.

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

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

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

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

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

479 SIP-metaproteomics

480 Sample preparation for metaproteomics analysis was done as previously described (Grob et al.,
481 2015). Mass spectrometry was performed on an Orbitrap Fusion MS (Thermo Fisher Scientific,
482 Waltham, MA, USA) (Supplementary Information).

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

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

501 Acknowledgements

502 The authors are grateful for use of the analytical facilities of the Centre for Chemical Microscopy
503 (ProVIS) at the Helmholtz-Centre for Environmental Research, which is supported by European
504 Regional Development Funds (EFRE – Europe funds Saxony) and the Helmholtz-Association. We
505 thank the HYDRA team for supporting the field sampling campaign. This work was supported by the
506 Gordon and Betty Moore Foundation Marine Microbiology Initiative Grant GBMF3303 to J. Colin
507 Murrell and Yin Chen and through the Earth and Life Systems Alliance, Norwich Research Park,
508 Norwich, UK and by a Leverhulme Trust Early Career Fellowship to Andrew T. Crombie (ECF2016-
509 626).

510 The authors declare no conflict of interest.

511 Supplementary information is available at ISME Journal's website.

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729 Figure and Table Legends

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

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

741 **Figure 3: Functional classification of identified peptides.** The numbers of peptides affiliated to
742 different enzymes and pathways of different functional categories relevant for C_1 metabolism are
743 shown. Colors depict the taxonomic distribution of the peptides in each functional category based on
744 the lowest common ancestor of each peptide. Peptide identification is based on metaproteomics
745 analysis of samples from microcosms with ^{12}C -methane of all three time points (n = 6). The peptides
746 were identified using NCBI nr and the metagenome-assembled genomes obtained in this study as
747 reference databases. MMO: methane monooxygenase, MDH: methanol dehydrogenase, FAE:
748 formaldehyde-activating enzyme, H4MPT: tetrahydromethanopterin pathway for formaldehyde
749 oxidation, THF: tetrahydrofolate pathway for formaldehyde oxidation, glutathione: glutathione
750 pathway for formaldehyde oxidation, formate DH: formate dehydrogenase, RuMP: ribulose
751 monophosphate pathway, based on the key enzymes 3-hexulose-6-phosphate synthase and 3-
752 hexulose-6-phosphate isomerase. For the serine cycle, the key enzymes hydroxypyruvate reductase,

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

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

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

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

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

779 **Table 2: Presence and expression of functional genes for C₁ metabolism in metagenome-assembled**
780 **genomes.** White fields indicate presence of functional genes for the respective function, red fields
781 indicate expression of the encoded enzymes based on metaproteomics analysis. Numbers in the
782 fields indicate number of genes expressed / number of genes present. ¹Based on key genes 3-
783 hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase. ²Based on key genes
784 hydroxypyruvate reductase, glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate
785 lyase and crotonyl-CoA reductase. ³Based on key gene ribulose-1,5-bisphosphate
786 carboxylase/oxygenase.