Biogeochemical and omic evidence for multiple paradoxical methane production mechanisms in freshwater lakes

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Abstract

Aquatic ecosystems are globally significant sources of the greenhouse gas methane (CH_4) to the atmosphere. However, CH_4 is produced 'paradoxically' in oxygenated water via at least three mechanisms, fundamentally limiting our understanding of overall CH_4 production. Here we resolve these CH_4 production mechanisms through CH_4 measurements, $\delta^{13}CH_4$ analyses, 16S rRNA sequencing, and metagenomics/transcriptomics applied to freshwater incubation experiments with multiple time points and treatments (addition of a methanogenesis inhibitor, dark, high-light). We captured significant paradoxical CH_4 production, but show that methanogenesis was an unlikely CH_4 source. In contrast, abundant freshwater bacteria metabolized methylphosphonate—similar to observations in marine ecosystems. Metatranscriptomics and stable isotopic analyses applied to experimental treatments also identified a potential CH_4 production mechanism linked to (bacterio)chlorophyll metabolism by Cyanobacteria and especially Proteobacteria. Variability in these mechanisms across experiments indicates that multiple, widely-distributed bacterial groups and pathways can produce substantial quantities of CH_4 in aquatic ecosystems.

Introduction

Atmospheric concentrations of the potent greenhouse gas methane (CH_4) have increased significantly due to anthropogenic activity, representing an important component of climate change (IPCC 2014). However, these increases are superimposed on substantial natural variability. Of all natural CH_4 sources to the atmosphere, freshwater lakes are particularly important but poorly understood, with their estimated contribution ranging from 6 to 16% of all natural CH_4 emissions—despite accounting for only ~0.9% of the Earth's surface area (Bastviken et al. 2004). CH_4 emissions from lakes are conventionally viewed to be regulated by CH_4 production (occurring predominantly in anoxic sediments) and subsequent CH_4 oxidation in surface sediments and the water column (Bastviken 2009). However, oversaturation of CH_4 has been consistently observed in oxygenated waters of aquatic systems (Tang et al. 2016). This observation indicates that CH_4 is produced under oxic conditions, and that the rate of CH_4 production exceeds CH_4 oxidation. Since archaeal methanogenesis is an obligate anaerobic process (Hoehler et al. 2018), oxic CH_4 production is typically referred to as the "methane paradox," and has been observed in oceans (Karl et al. 2008, Damm et al. 2010), lakes (Grossart et al. 2011, Bogard et al. 2014, Tang et al. 2014), and aerobic wetland soils (Angle et al. 2017). Notably, paradoxical CH_4 production occurs near the surface, and so any produced CH_4 may readily flux to the atmosphere. Identifying which mechanisms produce CH_4 in oxygenated waters is therefore essential for our understanding of CH_4 fluxes and their contribution to climate change.

Although multiple mechanisms for paradoxical aerobic CH_4 production have been proposed, the degree to which these are active in freshwater lakes remains unknown. Initial studies suggested that CH_4 production under oxygenated conditions could be occurring in anoxic microsites in the water column—such as fecal pellets, detritus, and the gastrointestinal tracts of larger organisms such as fish or zooplankton (Oremland 1979, Angelis & Lee 1994, Karl & Tilbrook 1994). Several studies have also demonstrated a correlation between phytoplankton or primary production and CH_4 production (Grossart et al. 2011, Bogard et al. 2014, Tang et al. 2014). While phytoplankton metabolites can be the source for methanogenesis in oxygenated water, Grossart et al. (2011) showed that methanogens may reside on the surface of phytoplankton cells and produce CH_4 in presumably anoxic microsites. Bogard et al. (2014) and Donis et al. (2017) also hypothesized that several groups of methanogens have oxygen-tolerant

60 or detoxifying pathways that could aid in CH_4 production in the presence of oxygen. For
61 example, Angle et al. (2017) characterized a methanogen candidate that possesses the enzymes
62 to detoxify oxygen and produce CH_4 under aerobic conditions.

63 In contrast, the current prevailing view of marine ecosystems is that methylphosphonate
64 (MPn) is the main precursor of CH_4 production under oxic conditions, particularly in phosphorus
65 (P)-stressed ecosystems such as the open ocean (Karl et al. 2008, Repeta et al. 2016). MPn is the
66 simplest form of organic carbon (C)-P bonded compounds in aquatic ecosystems; microbial
67 utilization of MPn, and the consequent breakdown of the C-P bond, releases CH_4 as a by-product
68 (Kononova & Nesmeyanova 2002, Karl et al. 2008, Repeta et al. 2016). A broad range of marine
69 and freshwater bacteria have the genomic potential to metabolize MPn and produce CH_4, based
70 on the presence of the multi-gene C-P lyase pathway in their genomes. This includes multiple
71 groups of Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi, and Cyanobacteria (Kononova
72 & Nesmeyanova 2002, Huang et al. 2005, Sosa et al. 2019). While expression of this pathway is
73 thought to be regulated by phosphate availability (Kononova & Nesmeyanova 2002, Huang et al.
74 2005, Sosa et al. 2019), the degree to which this occurs in freshwater ecosystems is not well
75 known (Yao et al. 2016, Wang et al. 2017, Li et al. 2020). Finally, recent work indicates that
76 pure cultures of marine and freshwater Cyanobacteria can directly produce CH_4 (Bižić et al.
77 2020). However, the exact mechanism by which this occurs remains unknown, and this has not
78 been directly measured in aquatic ecosystems.

79 These proposed mechanisms for CH_4 production—(1) methanogenesis aided by
80 detoxifying genes or in anoxic microsites, (2) CH_4 production by breakdown of methylated
81 compounds, and (3) CH_4 production by Cyanobacteria—point to multiple mechanisms by which
82 CH_4 can be produced under oxygenated conditions. Many of these are recently discovered and
83 therefore poorly understood, and the degree to which they occur within different aquatic
84 ecosystems is largely unknown. We developed an experimental approach to disentangle these
85 mechanisms and determine which may produce CH_4 in freshwater lakes. We conducted
86 incubation experiments using surface waters from high-elevation lakes, in order to rule out
87 physical transport and focus on potential biological mechanisms of oxic CH_4 production. We
88 investigated specific mechanisms using a combination of CH_4 measurements over time,
89 experimental treatments and inhibitors, and 16S rRNA gene and transcript sequencing, while
90 also applying stable isotope analyses and metagenome and metatranscriptome sequencing to

selected experiments. Paradoxical CH_4 production was evident in multiple experiments and experimental treatments, and could be attributed to MPn breakdown via widely-distributed members of the *Comamonadaceae* family. However, experimental treatments, stable isotope $\delta^{13}C$ signatures of CH_4, and metatranscriptomic data also point to a new potential mechanism of aerobic CH_4 production carried out by Cyanobacteria and Proteobacteria.

Results and discussion

Our experiments provide evidence for widespread paradoxical CH_4 production in freshwater lakes. Out of 19 total experiments conducted in five lakes in Yosemite National Park, 26% of experiments showed unequivocal, monotonic CH_4 production in unamended controls; 16% showed net oxidation in controls; several exhibited significant nonlinear patterns; and multiple experimental treatments also showed significant production (see below; based on replicate bottles incubated for at least 24 hours; Fig. 1). We observed particularly high CH_4 production rates in Lukens Lake (e.g., L1 and L2 experiments), as well as consistent production in Lower Gaylor Lake (LG1, LG3, and LG4) and Upper Cathedral Lake (UC1 and UC3). Net CH_4 production rates ranged from 0.98 to 22.8 nM/h, with the majority of values <4 nM/h. These rates are consistent with the limited experiments previously conducted in other freshwater lakes—e.g., 0.1-2.5 nM/h and 3.7 in Lake Stechlin, Germany (Grossart et al. 2011 and Tang et al. 2014, respectively)—and, on the higher end, our values are similar to the range reported by Bogard et al. (2014) for experimental manipulations in Lac Cromwell (2.08-8.33 nM/h). CH_4 turnover rates also ranged from 5 to 146 days (with in situ concentrations ranging from 309-2839 nM), consistent with turnover rates of ~18 days in Lake Stechlin (CH_4 concentration ~430 nM, Grossart et al. 2011), ~2.2 days in Lac Cromwell (CH_4 concentration ~200 nM, Bogard et al. 2014), and 67 days in Yellowstone Lake (CH_4 concentration of 46.3 nM, Wang et al. 2017).

Our study is the first confirmation of the CH_4 paradox in high elevation lakes, demonstrating significant CH_4 production under oxygenated conditions. Incubations were monitored to confirm that they were under oxic conditions at all times, and observed CH_4 production is likely biotic, as sterile treatments (filtered lake water) did not show significant CH_4 production or oxidation over time. Methane accumulation was consistently observed in multiple lakes, occurring across different experiments conducted in different years (2016, 2017 and 2018). However, our experimental results are also clearly indicative of natural spatial and

temporal variation in CH_4 production and oxidation. For example, variations over time within incubation experiments, as well as variation between and within experimental treatments, sometimes led to a lack of statistical significance—e.g., LG2 showed notable accumulation of CH_4 which was not statistically significant due to variation across experimental replicates.

However, variations within and across experiments can provide insight into different CH_4 production mechanisms, and their relation to CH_4 consumption. Eleven experiments did not show monotonic increases or decreases, which could reflect either (1) a complete absence of CH_4 metabolism at the time of sampling, or, more likely, (2) variations in the balance between co-occurring CH_4 production and consumption over time. For example, initial CH_4 production could be followed by oxidation once CH_4 concentrations reach a certain threshold required for oxidation (Grossart et al. 2011). Conversely, initial decreases due to oxidation may be followed by eventual production—for example through the development of P limitation that triggers CH_4 production via MPn metabolism (Karl et al. 2008). It is also possible that production and oxidation proceed at similar rates, leading to no net change in concentrations, even though CH_4 is actively cycled. In this case, molecular data are useful for providing insight into the underlying dynamics.

We tested for nonlinear patterns using ANOVA, and found two cases of initial CH_4 production followed by oxidation (LG1 and UC3; SI text). We also examined CH_4 oxidation—manifested by the expression of the particulate methane monooxygenase gene *(pmoA)* in metatranscriptomes—and found that CH_4 oxidation occurred in the majority of the incubations surveyed (SI Text). These data, as well as observed variations in CH_4 concentrations, are indicative of active CH_4 oxidation that may obscure paradoxical production. Put another way, CH_4 is clearly produced in incubations showing initial increases (UC3, LG1) or significant net increases (L1, L2, LG3, LG4 and UC1); but it may also actively occur in experiments showing no significant change (or consumption) over time, if CH_4 oxidation rates are equal to or greater than production rates. We therefore evaluated potential paradoxical CH_4 production mechanisms across experiments using multiple experimental treatments (BES, dark, high-light), and applied 16S rRNA sequencing, metagenomics, metatranscriptomics, and $\delta^{13}C$ stable isotopic analyses to samples collected during these experiments.

153 *Methanogenesis*

154 We used these multiple approaches to test for phytoplankton- or particle-based methanogenesis,
155 as initial observations in freshwater lakes showed methanogens attached to phytoplankton
156 (Grossart et al. 2011), while other work has suggested CH_4 production can occur in anoxic
157 microsites on particles (Oremland 1979, Angelis & Lee 1994, Karl & Tilbrook 1994). In all
158 experiments from 2017 and 2018, we included a treatment that consisted of the addition of the
159 methanogenesis inhibitor BES (2-bromoethanesulphonate). While there are some caveats with its
160 use (Oremland & Capone 1988), BES is widely used, including earlier tests of the CH_4 paradox
161 (Grossart et al. 2011, Tang et al. 2014). In our experiments, BES had an inhibitory effect on CH_4
162 production in four experiments, but actually significantly increased CH_4 production rates in four
163 experiments, and had no significant effect in the five remaining experiments. In parallel, 16S
164 rRNA sequencing of DNA from experiments L1-6 and LG1-4 recovered no methanogen 16S
165 sequences (out of 5.4 million total sequences; Table 1). Sequencing of 16S rRNA transcripts in
166 RNA samples from experiments L5-6, LG5-6, and UC3-4 recovered methanogen sequences in
167 only two samples (dark treatments from L5 and LG5), but total numbers were only 0.014% and
168 0.008% of all 16S rRNA sequences (Table 1). Analysis of metatranscriptomes likewise showed
169 that methyl coenzyme A (*mcrA*; responsible for methanogenesis) transcripts were absent in LG6
170 and UC4(tf) incubations and minimal in LG5, L7 and UC4(t0) (Fig. 2). *mcrA* genes were absent
171 in all metagenomes, with the sole exception of the L6(tf) incubation (Fig. 2). Collectively these
172 data indicate that water column-based methanogenesis is not a substantial source of CH_4, as BES
173 effects were mixed (Fig. 3), methanogens were almost entirely absent and inactive based on 16S
174 data (Table 1), and *mcrA* transcripts and genes were scantly expressed in metatranscriptomes and
175 rarely detected in metagenomes (Fig. 2).

176

177 *Methylphosphonate breakdown as a source of methane*

178 Instead, analysis of metatranscriptomes and metagenomes showed potential for CH_4 production
179 mechanisms other than conventional methanogenesis. In particular, we found evidence for
180 microbial metabolism of MPn based on the universal expression of alpha-D-ribose 1-
181 methylphosphonate 5-phosphate C-P lyase (*phnJ*) transcripts in metatranscriptomes (Fig. 2).
182 *phnJ* is responsible for the cleavage of the C-P bond that results in CH_4 production from MPn;
183 this mechanism is thought to be the dominant paradoxical CH_4 production mechanism in the

184 ocean (Repeta et al. 2016), but has been documented in only a limited number of freshwater
185 lakes (Yao et al. 2016, Wang et al. 2017, Li et al. 2020). In addition to *phnJ*, phosphonate-
186 binding periplasmic protein (*phnD)* genes (involved in the binding component of phosphonate
187 uptake; Rizk et al. 2006) were also universally expressed (Fig. 2). *phnJ* and *phnD* genes were
188 also recovered in all metagenomes (Fig. 2), and the majority of the genes were present in
189 organisms of the same genera as the metatranscriptomes—indicating that P assimilation from
190 MPn is broadly distributed among surface water microbes in these lakes.
191 Related to this, we examined whether transcripts and genes involved in the production of
192 MPn were also present and active. Transcripts of phosphoenolpyruvate mutase (*pepM*) and
193 phosphonopyruvate decarboxylase (*ppd*)—involved in phosphonate biosynthesis (Yu et al.
194 2013)—were present in some of the metatranscriptomes (Fig. 2), which suggests that
195 phosphonate may be synthesized in situ. Moreover and unexpectedly, genes involved in the
196 DMSP demethylation pathway (*dmdB*: 3-methylmercaptopropionyl-CoA ligase, *dmdC*: 3-
197 methylmercaptopropionyl-CoA dehydrogenase and, *dmdD*: Methylthioacryloyl-CoA hydratase)
198 were expressed in some of the metatranscriptomes. The role of DMSP degradation pathways in
199 the CH_4 paradox in the ocean has already been hypothesized—for example, the *dmdD* gene
200 involved in the production of methanethiol (MeSH) has been proposed to be a precursor for CH_4
201 paradox in polar waters subject to nutrient availability (Kiene et al. 2000, Damm et al. 2010,
202 Moran et al. 2012). *dmdD* was expressed in one of our incubations (LG5) and not detected in any
203 metagenomes, whereas *dmdC* was more commonly expressed (exceptions LG5 and UC4-HL)
204 and present in all metagenomes (Fig 2). Dimethylsulfonioproprionate demethylase (*dmdA*;
205 involved in the first step in DMSP breakdown) and *dmdB* genes were also present in L6 and LG2
206 incubations (Fig 2). Few studies have examined DMSP degradation in lakes, although DMS
207 concentrations in freshwater can be similar to those found in oceans (Sela-Adler et al.
208 2015). Our findings suggest that members of the Actinobacteria phylum and the
209 betaproteobacterial *Comamonadaceae* family may be involved in DMSP transformations in lakes
210 based on metatranscriptomic data, and at least raise the possibility of DMS and CH_4 production
211 from DMSP.
212 The majority of the *phnJ* transcripts and genes were also found among organisms in the
213 *Comamonadaceae* family: while >40 different bacterial genera expressed or possessed *phnJ*
214 genes, multiple *Comamonadaceae* genera accounted for 20.9% of *phnJ* transcripts in

215 metatranscriptomes and 30.4% of *phnJ* genes in metagenomes. *phnJ* transcripts and genes from
216 *Sphingobacteriales* were also abundant in several samples (LG5 and LG6 metatranscriptomes,
217 L6 metagenomes). *Comamonadaceae* transcripts and genes showed notably high identity to
218 database sequences, with the majority of sequences showing >92% and up to 100% amino acid
219 identity to *Acidovorax, Hydrogenophaga*, *Limnohabitans, Polaromonas*, *Rhodoferax*, and
220 *Variovorax phnJ* sequences.

221 16S data are consistent with this and demonstrate that *Comamonadaceae* were
222 universally present and abundant across experiments. In 16S rRNA gene sequence libraries from
223 experiments L1-6, LG1-4, and UC1, *Comamonadaceae* ranged from 2% (L6) to 12% (UC1) of
224 all sequences (Table 1), and were abundant in several incubations with significant CH_4
225 production (L1, L2, UC1). Sequencing of 16S rRNA transcripts in RNA samples from
226 experiments L5-6, LG5-6, and UC3-4 showed similar values (Table 1), and *Comamonadaceae*
227 reached 15% of all 16S rRNA transcripts in the UC3 and UC4 experiments (where CH_4 was
228 significantly produced in some treatments). Similar to *phnJ* results, 16S sequences from the
229 dominant *Comamonadaceae* ASVs showed high (98.8-100%) nucleotide sequence identity to
230 16S rRNA genes in sequenced genomes.

231 Finally, we assembled and annotated metatranscriptomes and metagenomes to examine
232 whether particular groups possess and express multiple *phn* genes in parallel, as the genes
233 involved in phosphonate acquisition and utilization often occur in clusters (Huang et al. 2005).
234 We found that *phnJ* genes commonly clustered with other *phn* genes on the same contigs,
235 particularly those from the *Comamonadaceae*. For example, contigs >50 kb containing *phnC*,
236 *phnD*, *phnE*, *phnI*, *phnJ*, *phnK*, *phnL*, *phnM* were present in metagenomes from the LG2 and L6
237 incubations, and were affiliated with the abundant freshwater bacterial group *Limnohabitans*
238 (Newton et al. 2011, Kasalický et al. 2018, Imhoff et al. 2019), as well as other members of the
239 *Comamonadaceae,* such as *Hydrogenophaga* and *Acidovorax.* Several longer contigs also
240 contained *pst* or *pho* genes involved in P uptake and metabolism.

241 P limitation is common in freshwater lakes, as it is in the open ocean (Björkman & Karl
242 2003, Sickman et al. 2003, Elser et al. 2009). High-elevation lakes in the Sierra Nevada are
243 typically oligotrophic (Sickman et al. 2003, Moser et al. 2019), and inorganic phosphate
244 concentrations in the lakes sampled here are consistently near the limits of detection (100 nM) by
245 colorimetric techniques, with the vast majority of measurements <200 nM (Hayden & Beman

246 2016, Perez-Coronel et al. in review) Under these conditions, organic P compounds—including
247 phosphonates—could serve as important sources of P. Our findings are consistent with this idea,
248 and the presence and expression of *phnJ* genes indicates that several microbial groups are
249 capable of releasing CH_4 through the cleavage of the C-P bond in MPn (Fig. 2). Our data in
250 particular implicate multiple genera within the *Comamonadaceae* in MPn metabolism, given
251 high *phnJ* and 16S identities, recovery of contigs containing *phn* and other genes involved in P
252 acquisition and metabolism, and their widespread presence and abundance in all experiments
253 (Table 1).
254
255 *Evidence for methane production by Cyanobacteria and Proteobacteria*
256 In addition to microbial use of MPn, Cyanobacteria were recently identified as a possible source
257 of paradoxical CH_4 production—yet this has not been specifically investigated in the
258 environment, and the pathway by which Cyanobacteria ultimately produce CH_4 remains
259 unknown (Bižić et al. 2020). Based on 16S rRNA genes and transcripts (Table 1), and
260 metagenomes and metatranscriptomes, Cyanobacteria were common in our samples. However,
261 Cyanobacteria were also variable across lakes, over time in individual experiments, and
262 especially between experimental treatments. We evaluated whether these variations could be
263 used to determine whether Cyanobacteria are a significant source of paradoxical CH_4 production
264 in freshwater.
265 In particular, two additional treatments were used to partition the relative effects of CH_4
266 production vs. oxidation and evaluate Cyanobacteria as a potential source of CH_4. First, we
267 conducted light versus dark treatments in some experiments, as light has been shown to inhibit
268 CH_4 oxidation (Dumestre et al. 1999, Murase & Sugimoto 2005, Thottathil et al. 2018), and CH_4
269 production in cyanobacterial cultures was positively correlated with light (Bižić et al. 2020). As a
270 result, dark treatments would be expected to have higher CH_4 oxidation rates, lower
271 cyanobacterial CH_4 production, and therefore lower CH_4 concentrations compared with
272 controls. Additionally, we increased light intensity in four experiments, which may have a two-
273 fold effect: (1) greater inhibition of CH_4 oxidation, and (2) increased rates of CH_4 production
274 from Cyanobacteria or other phytoplankton (Grossart et al. 2011, Bogard et al. 2014, Bižić et al.
275 2020). For both of these reasons, we expected to observe higher CH_4 production rates under
276 higher light levels.

Dark bottles showed mixed results, with four cases of CH_4 consumption (L4, LC1, and LG6 at 24 hours and LG5 at 86 hours; all $p<0.05$), production in two cases (UC2 at 24 hours and L5 at 74 hours; both $p<0.05$), and no significant difference in four experiments (L3, L7, LG4 and UC3). In two of four experiments with high light treatments, we observed increased CH_4 production (L2 and UC4; both $p<0.05$). The UC4 experiment was particularly illuminating, as higher light intensity significantly boosted CH_4 production rates by 62-fold compared with the control (up to 3.2 nM/h; $p<0.0005$). This was among the strongest treatment effects observed across all experiments (Fig. 3). BES also increased CH_4 production rate by 49-fold compared with control in this experiment (up to 2.5 nM/h; $p<0.0005$, Fig 3).

To examine this in greater detail, we analyzed both the stable isotope composition of CH_4 produced during the UC4 experiment, as well as changes in gene expression in response to experimental treatments in metatranscriptomes. $\delta^{13}C$ of CH_4 from multiple sampling time points in the BES and HL treatments was compared with controls in the UC4 experiment. $\delta^{13}C$ values in the LG6 and L7 experiments—which did not show CH_4 production in treatments—were also analyzed for purposes of comparison. Consistent with a lack of CH_4 production in these experiments, $\delta^{13}C$ of CH_4 showed no significant variation between treatments in the LG6 and L7 experiments (Fig. 4).

UC4 data presented a clear contrast, as $\delta^{13}CH_4$ values were significantly lower in the BES and HL treatments compared with controls (Fig. 4). Along with increased CH_4 concentrations, these data are indicative of production of isotopically-depleted CH_4 in the BES and HL treatments, which we calculate to be -56.9‰ to -57.0‰ based on isotopic mass balance. Traditionally this could be interpreted as methanogenesis, as the $\delta^{13}C$ of biogenic CH_4 (-110 to -50‰) is lower than terrestrial organic material (-28 to -26‰ for C3 plants) or lake primary production (-35 to -25‰) (Grey 2016). While methanogenesis cannot be entirely ruled out, very low levels of *mcrA* expression (Fig. 2) in these treatments suggest another source. Strong isotopic fractionation from MPn also seems unlikely, as the mean isotopic fractionation for CH_4 derived from MPn by the cleavage of the C-P bond is only 1.3‰ (Taenzer et al. 2020). MPn could be allochthonous (terrestrial) or autochthonous (based on *pepM* results), but in either case, is unlikely to be so ^{13}C-depleted. For example, in marine systems, CH_4 production from MPn actually increases $\delta^{13}C$ of CH_4 (Repeta et al. 2016). *phnJ* was also downregulated in both the

307 BES and high-light treatments (Fig. 5), further suggesting that MPn breakdown may not be the
308 major source of CH_4 in these treatments.
309 Instead, our isotopic data and metatranscriptomic data are consistent with Cyanobacteria
310 as a source of CH_4 in the UC4 experiment. In cyanobacterial cultures, produced CH_4 was
311 isotopically-depleted, with values (-45 to -55‰; Bižić et al. 2020) similar to those we
312 observed. Only the addition of ^{13}C-labeled dissolved inorganic carbon (DIC) to cyanobacterial
313 cultures resulted in higher $\delta^{13}C$-CH_4 values, demonstrating that CH_4 was ultimately derived from
314 DIC. Although the pathway by which Cyanobacteria produce CH_4 remains unidentified, Bižić et
315 al. (2020) hypothesized that CH_4 is produced during the photosynthetic process owing to positive
316 correlation between light and CH_4. However, we found no significant differences in carbon
317 fixation ($p>0.05$) or photosynthesis ($p>0.05$) KEGG Pathway categories between treatments in
318 the UC4 experiment.
319 We consequently used metatranscriptomic data to identify specific functions that were
320 significantly different in the treatments vs. the control, and that may explain differences in CH_4
321 concentrations and isotopic composition. This is an advantage of 'omic data, as it is possible to
322 examine previously unidentified potential mechanisms for CH_4 production. Through comparison
323 of all functions across metatranscriptomes from the UC4 experiment, we identified two
324 functions, both involved in porphyrin and chlorophyll metabolism, that were upregulated:
325 ferredoxin:protochlorophyllide reductase (DPOR) and chlorophyllide a reductase (COR) were
326 significantly different between the control and the BES ($p<0.0005$) and HL ($p<0.005$) treatments
327 (Fig. 5). While not directly involved in the photosynthesis pathway, DPOR is found in
328 photosynthetic bacteria, Cyanobacteria, and green algae, and is involved in the light independent
329 reduction of protochlorophyllide (Fujita et al. 1993, Nomata et al. 2008); COR catalyzes the first
330 step in the conversion of chlorin to a bacteriochlorin ring during bacteriochlorophyll biosynthesis
331 (Nomata et al. 2006, Chew & Bryant 2007, Tsukatani et al. 2013). Notably, both DPOR and
332 COR are nitrogenase-like enzymes (Nomata et al. 2006, Chew & Bryant 2007, Tsukatani et al.
333 2013), and both were abundant in all metatranscriptomes and metagenomes. DPOR and COR
334 were particularly common within the *Limnohabitans* and *Polynucleobacter* genera, as some
335 strains of these abundant freshwater bacteria are capable of performing aerobic anoxygenic
336 photosynthesis (Kasalický et al. 2018, Imhoff et al. 2019, Hahn et al. 2017). DPOR exclusively

337 was also found in several cyanobacterial groups—such as *Pseudanabaena*, *Dolichospermum*,
338 *Gloeomargarita* and *Oscillatoria*—in the metatranscriptome data.
339 Based on these findings, we propose two potential mechanisms that could be involved in
340 paradoxical CH_4 production in oxic surface waters of freshwater lakes: (1) methane may be
341 produced from methoxyl groups present in (bacterio)chlorophyll precursors, or (2) methane
342 production may be catalyzed by DPOR and COR enzymes. In terrestrial plants, CH_4 is thought to
343 be produced aerobically from structural components—such as pectin, lignin and cellulose
344 (Keppler et al. 2006, Messenger et al. 2009, Vigano et al. 2008)—mainly in plants under stress
345 (such as increased temperature, UV radiation or physical damage; Bruhn et al. 2012).
346 Protochlorophyllide, chlorophyllide, and other bacteriochlorophyll precursors contain methoxyl
347 groups that have been shown to serve as precursors of CH_4 in plants (Keppler et al. 2008), and a
348 similar mechanism may be active in Cyanobacteria and/or Proteobacteria under stress in our
349 experimental treatments.
350 Alternatively, CH_4 production may be catalyzed by DPOR and COR enzymes.
351 Nitrogenases reduce a range of multibond compounds (Hu et al. 2011, Yang et al. 2011, Lee et
352 al. 2010, Zheng et al. 2018), and this quality is shared across different nitrogenases (Seefeldt et
353 al. 2020). Although DPOR and COR were expressed and present in all metatranscriptomes and
354 metagenomes, expression of both was an order of magnitude higher in the UC4 experiment
355 compared with the LG5, LG6, and L7 experiments (with the exception of COR in LG5). This
356 suggests that this mechanism can be variable, but is highly consistent with the lack of a $\delta^{13}CH_4$
357 isotopic signal in the LG6 and L7 experiments compared with the UC4 experiment (Fig. 4). We
358 propose that, similar to the findings of Zheng et al. (2018), the nitrogenase-like enzymes DPOR
359 and COR may be able to catalyze the reduction of CO_2 into CH_4. Importantly, these enzymes are
360 present in Cyanobacteria as well as the abundant and ubiquitous freshwater bacterial groups
361 *Limnohabitans* and *Polynucleobacter* (Kasalický et al. 2018, Imhoff et al. 2019, Hahn et al.
362 2017).
363
364 *Multiple mechanisms produce methane in freshwater lakes*
365 Our combined results indicate that multiple paradoxical CH_4 production mechanisms are active
366 in freshwater ecosystems, and have several implications for our understanding of aquatic CH_4
367 production. First, confirmation of the CH_4 paradox in freshwater is still limited in scope and

368 relatively recent (Grossart et al. 2011, Bogard et al. 2014, Bižić et al. 2020). We conducted
369 multiple experiments in multiple lakes, and measured significant CH_4 production in 37% of
370 experiments (Fig. 1). In many of the remaining experiments, variations over time, across
371 experimental treatments, and in *pmoA* gene expression patterns suggest that CH_4 production
372 occurs in parallel with coupled CH_4 oxidation (Fig. 2). Because CH_4 oxidation may obscure CH_4
373 production, use of different experimental treatments combined with 'omic data is required to
374 identify paradoxical CH_4 production. Our findings therefore expand on limited data and
375 demonstrate that paradoxical CH_4 production is common, but variable, in freshwater.
376 Given the ubiquity of *phnJ* genes in our data (Fig. 2), their presence on contigs from
377 abundant organisms, and the potential for P limitation in these lakes, microbial metabolism of
378 MPn may represent an important baseline CH_4 production mechanism in freshwater lakes. This
379 fits with current understanding of marine ecosystems (Karl et al. 2008, Repeta et al. 2016).
380 Metatranscriptomic and metagenomic data also indicate that, like the ocean, DMSP
381 transformations may be active in freshwater—with potential implications for both DMS and CH_4
382 production. Our data further demonstrate that MPn may itself be produced within lakes based on
383 *ppd* and *pepM* expression (Fig. 2). Quantifying the relative contributions of allochthonous vs.
384 autochthonous MPn production—as well as broader P acquisition dynamics—is therefore
385 essential to understanding CH_4 production in freshwater. Phosphonate compounds were once
386 thought to be resistant to breakdown and their formation remains poorly understood (Kafarski
387 2019). Under P limiting conditions, competition for these and other P compounds among
388 multiple groups of organisms will be intense and potentially variable in space and time. Along
389 with variations in CH_4 oxidation relative to production rates, this can drive variations in CH_4
390 production such as those evident in our data.
391 As a newly-identified CH_4 source that has not been examined in the environment and
392 occurs via an unknown pathway (Bižić et al. 2020), we used a combination of approaches to
393 identify whether cyanobacterial CH_4 production occurs in freshwater, and by what means.
394 Although independent data types (CH_4 concentrations, treatment effects, isotopic data, 'omic
395 data) may be interpreted in multiple ways, the combined data are consistent with cyanobacterial
396 production in the UC4 experiment. For example, the high light and BES treatments boosted CH_4
397 concentrations (Fig. 3), but this CH_4 is unlikely to have been the result of methanogenesis or
398 MPn breakdown given low and decreasing *mcrA* and *phnJ* expression (Fig. 5). If methanogenesis

399 is a source of CH_4 in these lakes, per transcript/gene rates would have to be remarkably high
400 given 16S rRNA and *mcrA* numbers. Instead, Cyanobacteria were present and responsive to
401 treatments as shown by metagenomic, metatranscriptomic, and 16S data. Our data are further
402 indicative of an isotopically light C source (Fig. 4)—consistent with $\delta^{13}C$ values from
403 cyanobacterial cultures reported by Bižić et al. 2020).
404 Notably, these patterns were most clearly evident in experimental treatments,
405 highlighting the efficacy of including different treatments to identify production
406 mechanisms. We leveraged 'omic data from these different treatments to identify potential
407 mechanisms of CH_4 production—providing an experimental approach, additional isotopic
408 signature data, and two potential gene targets to examine in other aquatic ecosystems. We found
409 that this mechanism was not limited to the Cyanobacteria, as the majority of DPOR and COR
410 transcripts and genes were derived from several members of the Proteobacteria. This has
411 multiple implications, as *Limnohabitans* and *Polynucleobacter* are among the most abundant and
412 ubiquitous bacteria found in freshwater ecosystems (Newton et al. 2011, Kasalický et al. 2018,
413 Imhoff et al. 2019, Hahn et al. 2017). Although there is significant genomic diversity within
414 these groups (Newton et al. 2011, Kasalický et al. 2013, Hahn et al. 2016), this suggests that the
415 potential for CH_4 production via this pathway may be widespread in freshwater. *Limnohabitans*
416 also expressed *phnJ* genes, indicating that they can play a dual role in paradoxical CH_4
417 production.
418 Ultimately, our combined dataset indicates that paradoxical CH_4 production is complex,
419 with multiple interacting groups and processes, each affected by multiple environmental
420 variables (e.g., P availability, light), and all producing CH_4 to differing degrees. Understanding
421 this complexity is essential, as multiple paradoxical CH_4 production mechanisms are widely-
422 distributed, and so may be important sources of CH_4 to the atmosphere.
423
424 **Materials and methods**
425 *Field site, sample collection, and experimental set up*
426 Water samples were collected in 2016-2018 in five high-elevation lakes in Yosemite National
427 Park, and used in incubation experiments. Samples were collected in Lukens (L), Lower
428 Cathedral (LC), Upper Cathedral (UC), Lower Gaylor (LG), and Upper Gaylor (UG) Lakes;
429 incubations are denoted by lake abbreviation and sequential numbering (Table 2). Water samples

430 were collected in the littoral and limnetic zones of the lakes at 0.1 m depth with previously acid-
431 washed plastic cubitainers, and then kept on ice or refrigeration to maintain in-lake temperature
432 until laboratory incubations were established within 24 hours of samples collection. Temperature
433 and dissolved oxygen were measured at the time of sample collection using a ProODO YSI
434 probe (YSI Inc., Yellow Springs, OH, USA).

435 Water collected in the lakes was transferred to 170 or 300 ml Wheaton bottles, capped
436 and crimped, and a known volume of air was introduced to generate a headspace for sampling.
437 Initial CH_4 samples were collected, and bottles were incubated in water baths at constant
438 temperature. All incubations were run in triplicate and set to the temperature at which water
439 samples were collected in the field. The different treatments used in the experiments were:

440 - Control: unamended lake water following a natural day-night set up (water bath lid was
441 opened at 7:00 hours and closed at 18:00 hours).
442 - Dark: unamended lake water; bottles were kept in the dark during the whole incubation
443 time.
444 - BES: lake water was amended with 2-bromoethanesulphonate (BES) to a final
445 concentration of $5x10^{-4}$ M. This concentration has been established to inhibit
446 methanogens (Oremland & Capone 1988) and has been used in methane paradox
447 experimentation before (Grossart et al. 2011). This treatment followed the same natural
448 daylight set up than the control.
449 - High light: unamended lake water was subjected to 500 mmol/m^2 inside a growth
450 chamber and followed the same natural daylight set up than the control (light would turn
451 on at 7:00 hours and off at 18:00 hours).

452 For all incubation types, gas samples were taken from the headspace every 6-24 hours for up to
453 96 hours with a syringe and immediately transferred to exetainers for later analyses in a gas
454 chromatograph. Not all treatments were tested in each incubation experiment. Temperature and
455 oxygen concentrations were monitored at each sampling point. Optical sensor spots (Fibox,
456 Loligo Systems, Viborg, Denmark) were used to measure oxygen concentrations during
457 incubations (detection limit of 100 nM) and make sure that the water did not go anoxic at any
458 time. Temperature was measured with a Fibox temperature sensor and kept constant during the
459 incubation time. Water samples were filtered at the beginning and end of the incubation for DNA
460 and RNA sampling.

461

462 *Methane measurements*

463 Methane concentrations were measured via headspace equilibration and gas chromatography.
464 Headspace gas samples from incubations were collected with a gas-tight syringe into 12-mL
465 Labco Exetainer vials (Labco Ltd., Lampeter, Ceredigion, UK) after incubations bottles were
466 shaken for 2 minutes to reach equilibration. Samples were subsequently analyzed using a
467 Shimadzu GC-2014 gas chromatograph with flame ionization detection (FID) for CH_4 (Weiss
468 1981). Headspace CH_4 concentration measurements were then used to calculate CH_4
469 concentration in lake water based on Henry's law of equilibrium (Yamamoto et al. 1976).

470

471 *DNA and RNA Extraction*

472 Water samples were filtered through 0.22 µm (Millipore, Darmstadt, Germany) then DNA filter
473 samples were preserved in Sucrose-Tris-EDTA (STE) buffer in pre-prepped Lysis Matrix E
474 tubes and frozen at -80°C until extraction. RNA samples were preserved in RNA*later*®
475 (Ambion™, AM7021) in pre-prepped Lysing Matrix E tubes (MP Bio, Eschwege, Germany),
476 and frozen at -80°C until extraction. DNA was extracted using the Qiagen DNeasy Blood &
477 Tissue Kit with a modified protocol from Beman et al. (2012). Briefly, samples were lysed with
478 100µL 10% sodium dodecyl sulfate (SDS), DNA gets separated from proteins and cellular debris
479 using proteinase K (20mg mL^{-1}; Qiagen, Inc., Valencia, CA, USA), precipitated with ethanol and
480 cleaned up. After extraction samples were preserved at -80°C until further analyses. RNA was
481 extracted using a *mir*Vana miRNA Isolation Kit (Ambion™, AM1560) with a modified protocol
482 from Huber & Fortunato (2017). Briefly, samples are lysed with the kit's lysing matrix, then
483 subjected to an organic extraction with phenol chloroform followed by a wash to obtain RNA.
484 Immediately after RNA extraction we used the SuperScript III First-Strand Synthesis System for
485 RT-PCR (Life Technologies Corporation, Carlsbad, CA, USA) to synthesize first-strand cDNA
486 and samples were preserved at -80°C until further analyses. DNA, RNA and cDNA purity was
487 measured using a Biospectrometer (Eppendorf AG, Hamburg, Germany) and the concentrations
488 were quantified using PicoGreen Quant-iT dsDNA quantitation assay (ThermoFisher Scientific,
489 USA) for DNA samples and the MaestroNano Pro (Maestrogen Inc., Taiwan) for RNA and
490 cDNA samples.

491

492 *16S sequencing*

493 DNA and RNA extracted from filtered water samples were diluted to a common concentration
494 (1ng/ul) and sent for 16S rRNA amplicon sequencing on an Illumina MiSeq (Illumina, San
495 Diego, CA, USA) according to Earth Microbiome protocols. We used the universal primers
496 515F-Y (5'- GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT).
497 DNA samples were sequenced at the Joint Genome Institute (Berkeley, CA, USA) and RNA
498 samples at the Argonne National Laboratory (Lemont, IL, USA).

499 ASVs were generated from 16S rDNA and rRNA sequence data using the Divisive
500 Amplicon Denoising Algorithm (DADA2; Callahan et al. 2016) as implemented in QIIME 2
501 (Bolyen et al. 2019), and then used for subsequent analyses. After import and demultiplexing,
502 read quality was visualized using the 'qiime tools view' command. Reads were then processed
503 using the 'qiime dada2 denoise-paired' command, with 13 bp trimmed from both the forward
504 and reverse reads, truncation of reverse to 169 bp (due to the well-known decline in sequence
505 quality observed for MiSeq reverse reads), and training of the denoising algorithm on 1 million
506 reads. Classification of ASVs was conducted in mothur (Schloss et al. 2009) using the SILVA
507 (version 128) database.

508

509 *Metatranscriptomes and metagenomes*

510 Metatranscriptomes and metagenomes were generated from later experiments in order to
511 examine potential production mechanisms and coupled methane oxidation. Following
512 extraction, DNA and RNA samples were sent for metagenome/metatranscriptome sequencing in
513 the Vincent J. Coates Genome Sequencing Laboratory (GSL) at the University of California,
514 Berkeley (https://genomics.qb3.berkeley.edu/), which is supported by NIH S10 OD018174
515 Instrumentation Grant. For each DNA sample, 250 ng of genomic DNA was sheared and
516 libraries were prepared using the KAPA HyperPrep Kit (Kapa Biosystems, Wilmington, MA,
517 USA). For each RNA sample, ~800 ng of total RNA was depleted of rRNA using the Ribo-Zero
518 rRNA Removal Kit (Illumina, Inc., San Diego, CA, USA), sheared, and libraries were prepared
519 using the KAPA RNA HyperPrep Kit (Kapa Biosystems, Wilmington, MA, USA). 12 samples
520 were pooled into a single lane and sequenced via 150-cycle paired-end sequencing on the
521 Illumina HiSeq 4000 platform (Illumina, Inc., San Diego, CA, USA).

522 Data were demultiplexed by the GSL and reads were filtered and trimmed using BBDuk
523 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) with the following
524 parameters: maq=8, maxns=1, minlen=40,minlenfraction=0.6, k=23, hdist=1, trimq=12,
525 qtrim=rl. Forward and reverse reads were then merged using PANDASeq
526 (https://github.com/neufeld/pandaseq; Masella et al. 2012) with default parameters. Merged
527 reads were subsequently annotated in DIAMOND (http://diamondsearch.org/; Buchfink et al.
528 2015) using the NCBI NR database (accessed February 11th, 2020) with the following search
529 criteria: maximum number of target sequences = 1, bit-score > 40. In order to quantify
530 functional gene abundances, we filtered the DIAMOND annotation data (Percent identity > 60%,
531 bit score > 100) to find the number of functional genes of interest present in each metagenome or
532 metatrascriptome. We calculated percent abundance based on the total number of reads and the
533 number of targeted genes in each metagenome or metatranscriptome.

534 UC4 metatranscriptomes were uploaded to the Joint Genome Institute (JGI;
535 https://img.jgi.doe.gov/) Integrated Microbial Genomes & Microbiomes platform for further
536 analyses. We used the function comparisons tool to identify functions that were significantly
537 different in the treatments vs. the control, and the function category tool identify differences in
538 KEGG pathway categories among the treatments. Moreover, we used the phylogenetic
539 distribution tool to quantify the abundance of different phylogenetic groups of potential
540 importance in the methane paradox.
541
542 *Stable isotopic measurements*
543 Headspace of incubation bottles was transferred to evacuated exetainers and sent to the UC
544 Davis Stable Isotope Facility (Davis, CA) for analysis. Measurements of stable isotope ratios of
545 carbon ($\delta^{13}C$) in CH_4 were conducted using a ThermoScientific Precon concentration unit
546 interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer
547 (ThermoScientific, Bremen, Germany). In brief, gas samples are passed through a H_2O/ CO_2
548 scrubber and a cold trap, and CH_4 is then separated from other gases and oxidized to CO_2. Pure
549 CO_2 reference gas is used to calculate provisional δ values, and final δ values are calculated after
550 correcting for changes in linearity and instrumental drift, and expressed relative to the Vienna
551 PeeDee Belemnite (V-PDB) standard.
552

Statistical analyses

Spearman's correlation test was used to assess the relationship between time (h) and CH_4 concentration in all incubations and across all treatments. A priori significance level was defined as $\alpha<0.05$. A significantly positive relationship between time and CH_4 was considered as net CH_4 production in the incubation, whereas a significantly negative relationship was considered as net CH_4 consumption; otherwise we defined the incubation as not having significant net production or consumption over time. We subsequently used ANOVA and Tukey honestly significant difference (HSD) post-hoc tests to test for cases where CH_4 production or oxidation varied significantly between sampling time points, resulting in nonlinear patterns in CH_4 concentrations over the course of the experiment. For example, initial CH_4 production could be followed by subsequent oxidation, resulting in significant CH_4 increases followed by significant decreases.

To express the different responses in terms of CH_4 concentration over time among the different experiments treatments we first calculated the response ratios (lnRR) by the following equation:

$$lnRR = ln\ \frac{mean\ CH_4\ in\ treatment}{mean\ CH_4\ in\ control}$$

Additionally, we calculated which treatments were significantly different from each other and the control using ANOVA and Tukey honestly significant difference (HSD) post-hoc tests. All statistical analyses and figures were done in the R statistical environment (RStudioVersion 1.2.5001).

574 **Tables**

575 **Table 1.** Relative abundance of methanogens, Cyanobacteria, and *Comamonadaceae* as a % of
576 16S rRNA genes and transcripts sequenced in different experiments and treatments.
577

Exp.	Treatment	Time (h)	Methanogens	Cyanobacteria	*Comamonadaceae*
			% of 16S rRNA genes		
L1	Control	0	0	0.902	9.17
L2	Control	0	0	1.10	6.03
	Control	24	0	1.65	5.20
L3	Control	0	0	0.061	4.56
L4	Control	0	0	2.38	7.97
L6	Control	0	0	5.55	2.03
	BES	45	0	12.6	0.338
LG1	Control	0	0	0.646	5.32
	Control	24	0	0.377	2.04
LG2	Control	0	0	0.795	11.1
LG3	Control	24	0	0.246	0.881
LG4	Control	0	0	5.79	4.35
UC1	Control	0	0	0.880	11.6
			% of 16S rRNA transcripts		
L5	Control	0	0	46.0	6.43
	Control	74	0	28.8	6.66
	Dark	74	0.014	58.5	4.70
	BES	74	0	24.9	8.65

L6	Control	0	0	8.34	6.27
	Control	55	0	0.800	5.42
	BES	55	0	0	5.20
L7	Dark	96	0	65.5	0.841
	BES	96	0	46.4	1.95
	High light	96	0	45.0	1.06
LG5	Control	0	0	17.3	12.8
	Control	86	0	2.00	12.0
	Dark	86	0.008	19.2	10.2
	BES	86	0	1.04	25.6
LG6	Control	0	0	3.05	6.14
	Control	96	0	2.45	8.69
	Dark	96	0	2.65	2.53
	BES	96	0	2.28	5.02
	High light	96	0	7.18	2.64
UC3	Control	0	0	0.227	7.98
	Control	24	0	0.275	8.72
	Control	48	0	0.221	15.2
	Control	57	0	0	12.3
	Dark	57	0	0	8.66
	BES	57	0	0	10.9
UC4	Control	96	0	3.148	14.0
	High light	96	0	0.474	7.25

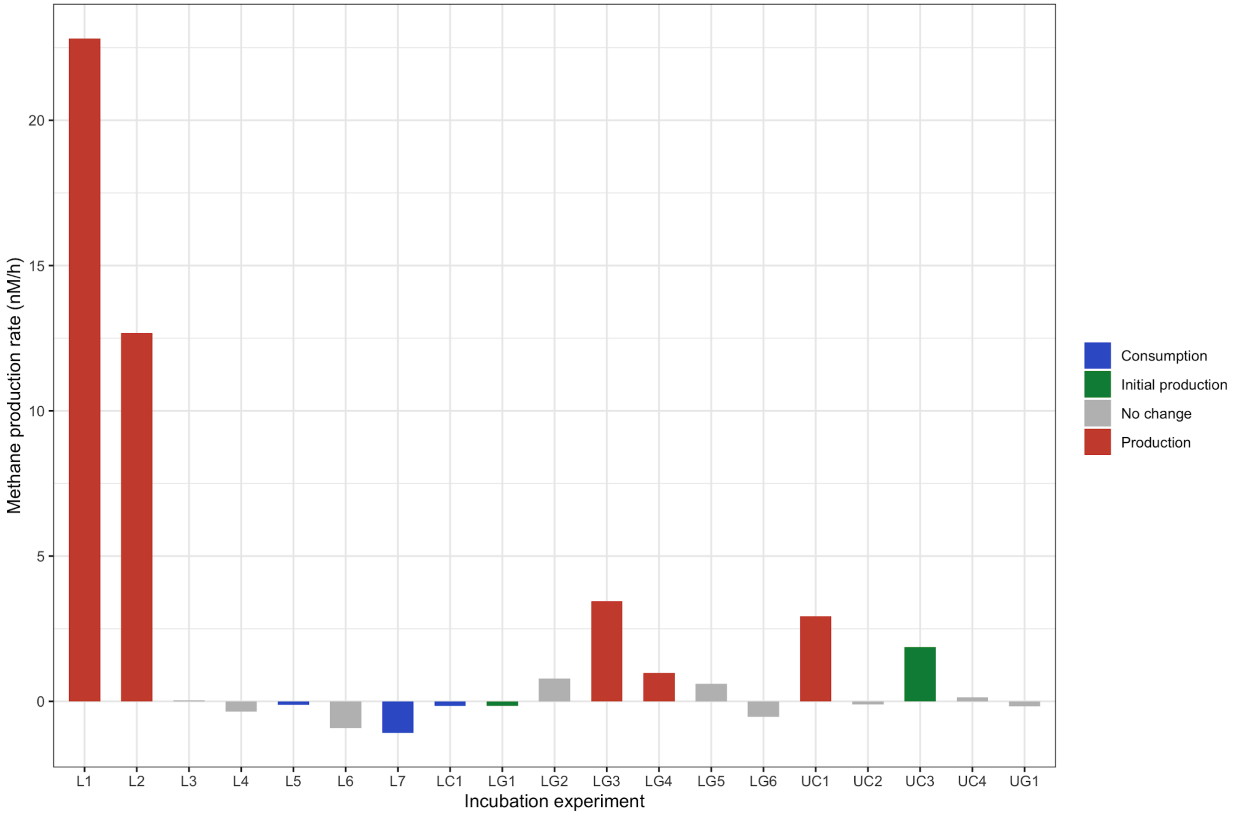
578 **Table 2.** Experimental incubations characteristics. BES corresponds to the addition of 2-
579 bromoethanesulphonate as a methanogenesis inhibitor, Dark represents experiments incubated in
580 total darkness and HL (high-light) are experiments subjected to high-light intensity during the
581 incubation.
582

Exp.	Y	M	D	CH_4	*P*	16S rDNA	16S rRNA	Metat	Metag	Length (h)	Treatments
L1	2016	8	12-13	+	0.02	X				24	
L2	2016	9	11-12	+	0.02	X				24	HL
L3	2017	7	28-30			X				48	BES, Dark
L4	2017	8	8-10			X				48	BES, Dark
L5	2017	9	26-29	-	0.02	X	X			74	BES, Dark
L6	2017	11	13-15			X	X	X	X	55	BES
L7	2018	9	23-28	-	0.02		X	X		96	BES, Dark, HL
LG1	2016	8	8-9	+/-	0.02	X				24	
LG2	2016	9	9-10			X			X	24	
LG3	2016	10	7-8	+	0.02	X				24	
LG4	2017	8	24-26	+	0.02	X				48	BES, Dark

LG5	2017	10	1-4				X	X		86	BES, Dark
LG6	2018	9	10-14				X	X		96	BES, Dark, HL
UC1	2016	8	10-11	+	0.02					24	
UC2	2017	8	10-12							48	BES, Dark
UC3	2017	10	8-11	+/-	0.006		X			57	BES, Dark
UC4	2018	10	8-12				X	X		96	BES, Dark, HL
LC1	2017	8	26-28	-	0.02					48	BES, Dark
UG1	2017	8	12-14							48	BES

583
584

585 **Figures**
586



587
588
589 **Figure 1.** CH_4 production or consumption rates across incubation experiments. Different colors
590 represent significant linear increases (production; red), linear decreases (consumption; blue),
591 nonlinear patterns (green), or no significant change in CH_4 concentrations (grey) over 24 hours in
592 unamended controls.
593
594
595
596
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599
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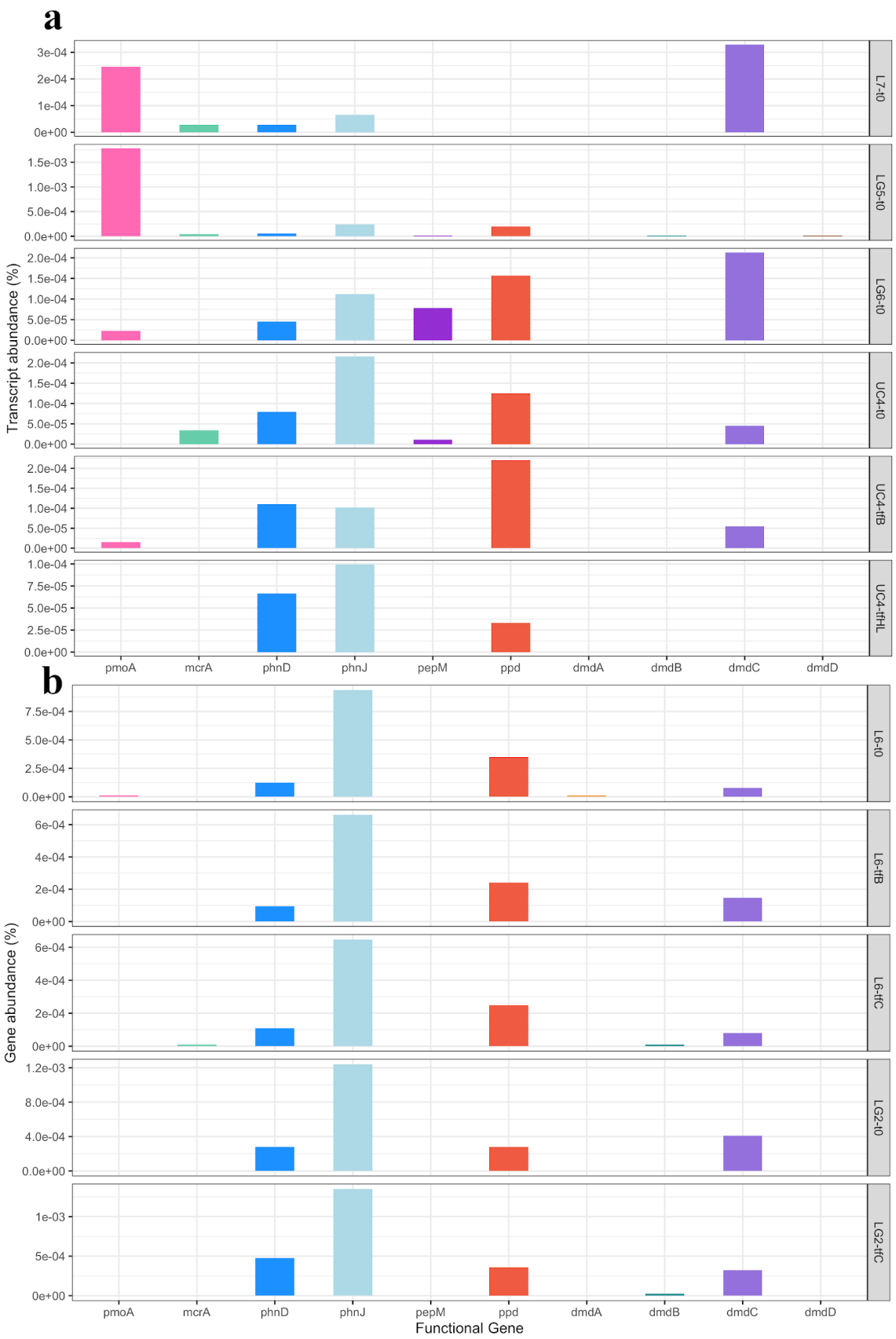
602
603
604
605 **Figure 2.** Variations across experiments and treatments in (a) transcript abundance (% of total
606 reads of metatranscriptomes) and (b) gene abundance (% of total reads of metagenomes) for
607 functional genes quantified involved in methane oxidation (*pmoA*), methanogenesis
608 (*mcrA*), phosphonate assimilation (*phnD*, *phnJ*), phosphonate synthesis (*ppd*, *pepM*), and DMSP
609 metabolism (*dmdA*, *dmdB*, *dmdC*, *dmdD*).


a
Transcript abundance (%)
L7-t0
LG5-t0
LG6-t0
UC4-t0
UC4-tfB
UC4-tfHL
pmoA
mcrA
phnD
phnJ
pepM
ppd
dmdA
dmdB
dmdC
dmdD
b
Gene abundance (%)
L6-t0
L6-tfB
L6-tfC
LG2-t0
LG2-tfC
Functional Gene

610

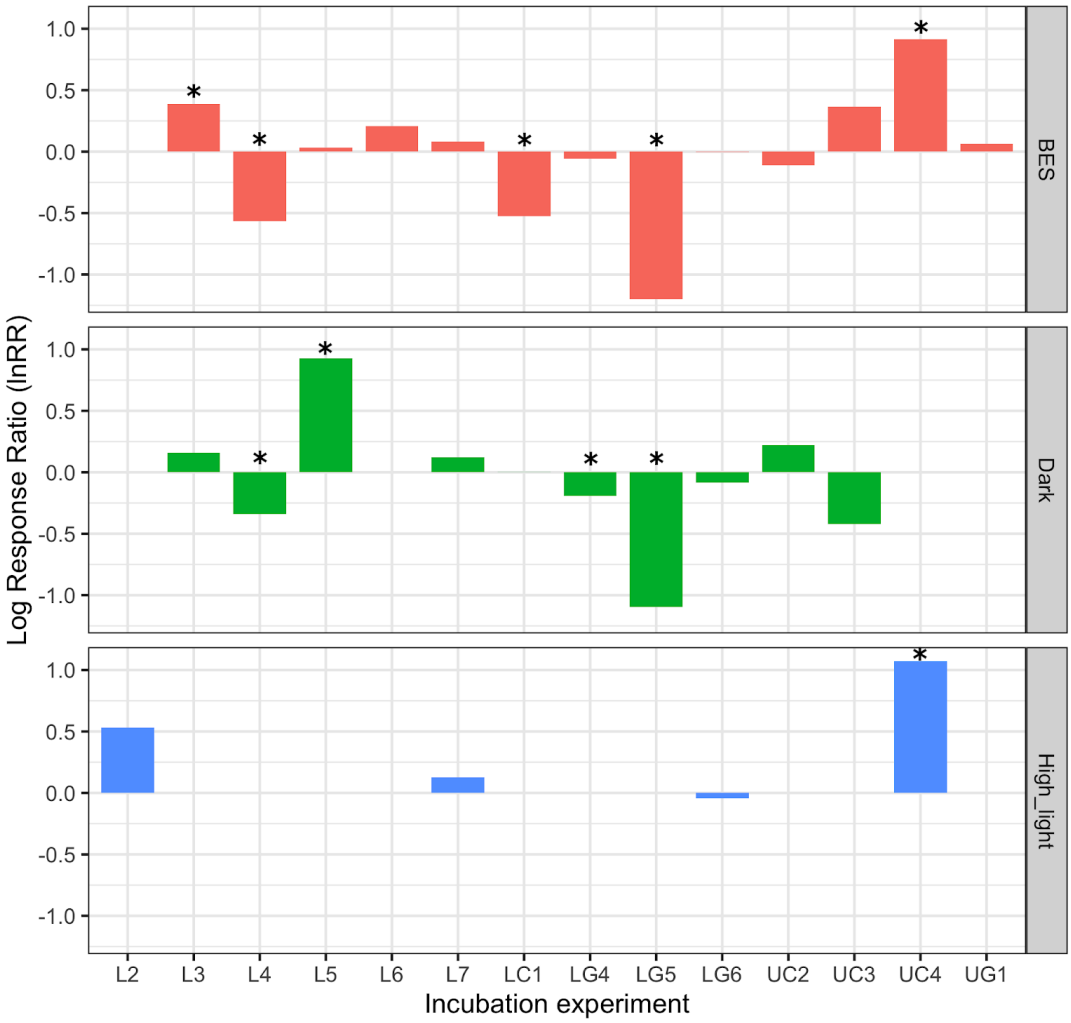


Figure 3. Log response ratios for experimental treatments at 24 hours. Asterisks denote experimental treatments that were significantly different from the control. Experiments L3, L5, LG5, and UC3 were longer experiments and treatment effects are shown for 36, 74, 86 and 57 hours respectively.

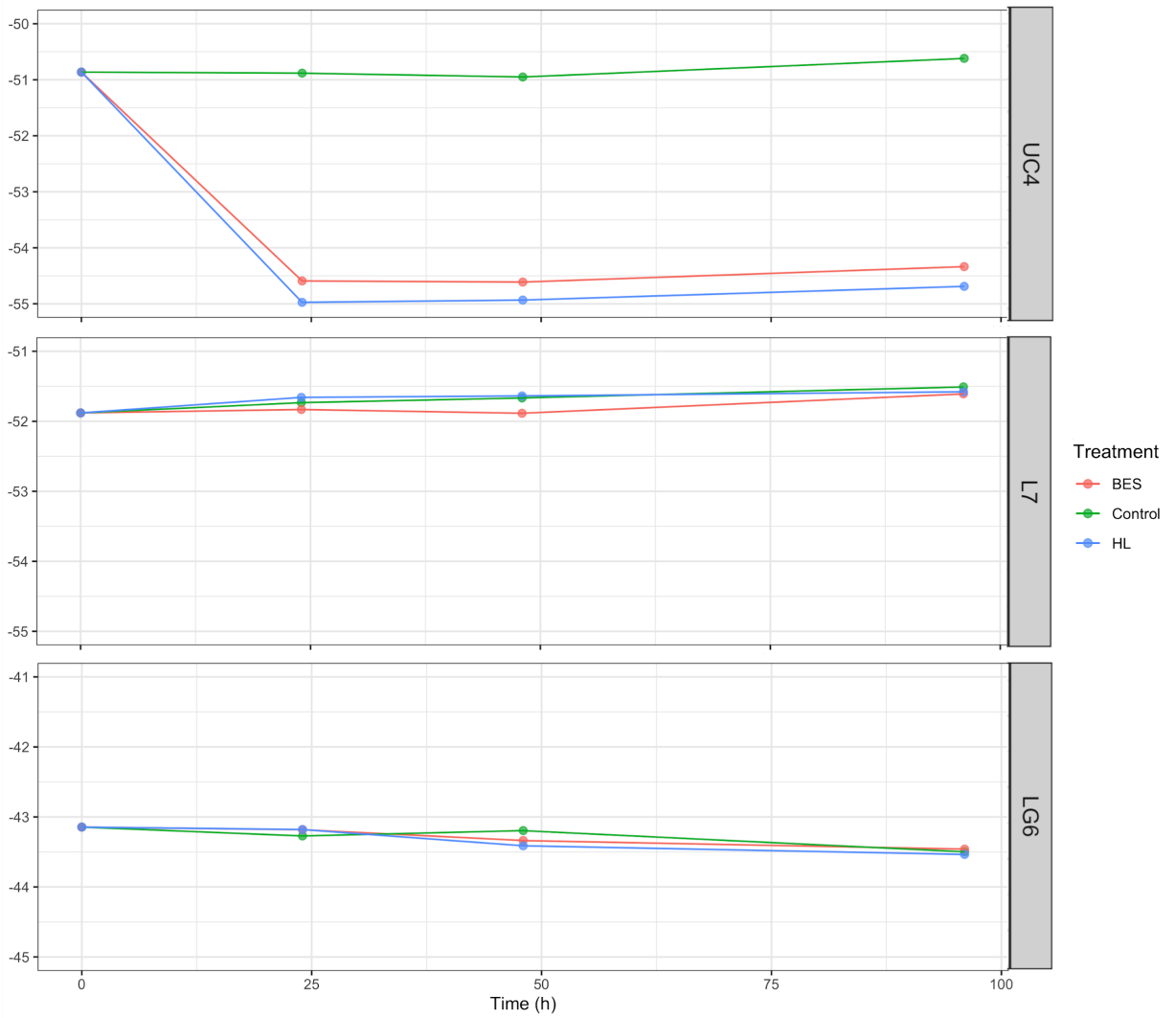


Figure 4. Contrasts in $\delta^{13}C$ values of CH_4 over time in experimental treatments in the UC4, L7, and LG6 experiments. Different colors denote different experimental treatments. Scales of the vertical axes differ between experiments due to different patterns in $\delta^{13}CH_4$.

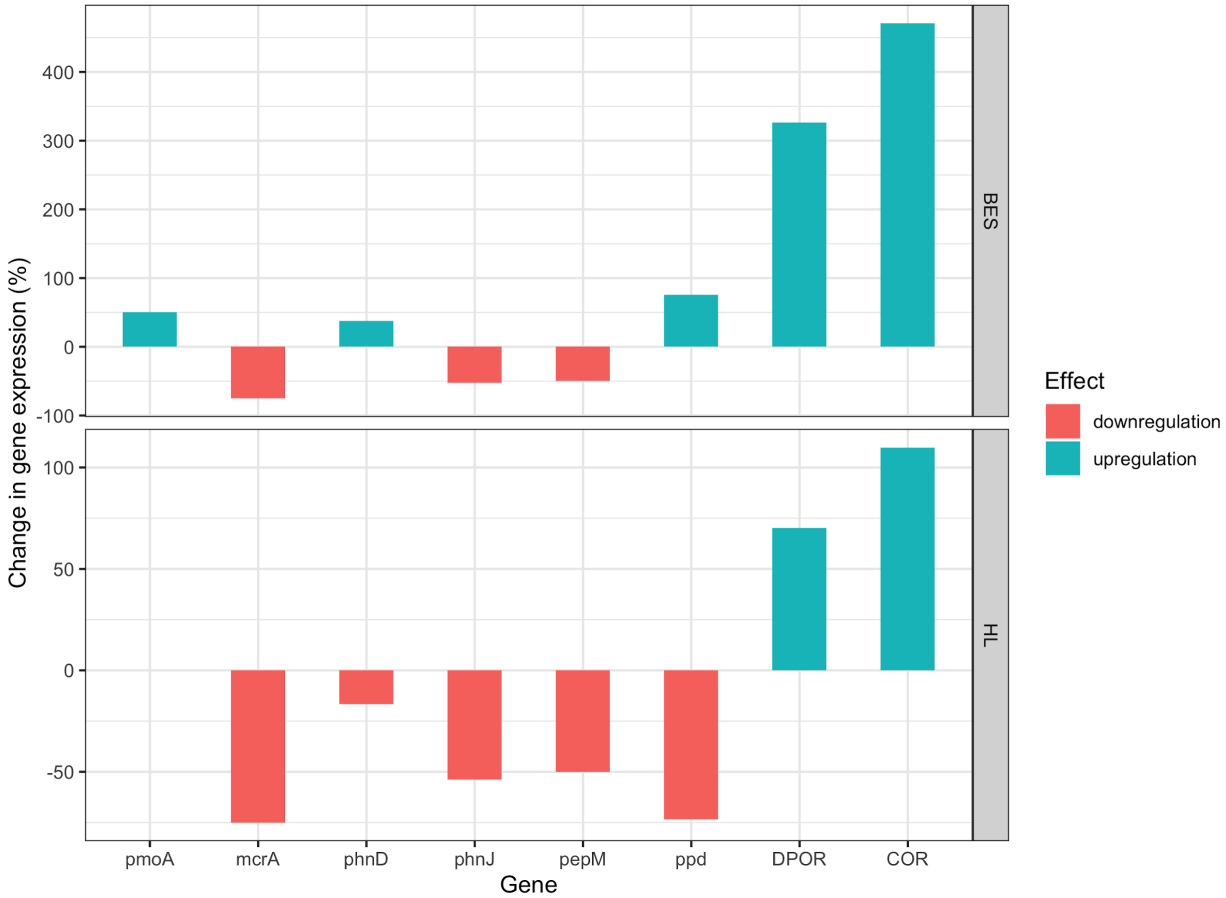


Figure 5. Upregulation and downregulation of genes in UC4 experimental treatments. Percentage change in expression is shown for genes involved in methane oxidation (*pmoA*), methanogenesis *(mcrA*), phosphonate assimilation (*phnD*, *phnJ*), phosphonate synthesis (*pepM*, *ppd*), and porphyrin and chlorophyll metabolism (DPOR, COR). BES treatment is shown in the top panel and the high-light treatment in the bottom panel. Colors denote the effect of the treatment on the different genes quantified.

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