Aquatic and terrestrial *Cyanobacteria* produce methane

Cyanobacteria, the most ancient and abundant photoautotrophs on Earth produce the greenhouse gas methane during photosynthesis.

Short title

- Methanogenesis by Cyanobacteria

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Abstract

Evidence is accumulating to challenge the paradigm that biogenic methanogenesis, considered a strictly anaerobic process, is exclusive to *Archaea*. We demonstrate that *Cyanobacteria* living in marine, freshwater and terrestrial environments produce methane at substantial rates under light, dark, oxic and anoxic conditions, linking methane production with light driven primary productivity in a globally relevant and ancient group of photoautotrophs. Methane production, attributed to cyanobacteria using stable isotope labelling techniques, was enhanced during oxygenic photosynthesis. We suggest that formation of methane by *Cyanobacteria* contributes to methane accumulation in oxygen-saturated marine and limnic surface waters. In these environments, frequent cyanobacterial blooms are predicted to further increase due to global warming potentially having a direct positive feedback on climate change. We conclude that this newly identified source contributes to the current natural methane budget and most likely has been producing methane since *Cyanobacteria* first evolved on Earth.

MAIN TEXT

Introduction

Methane (CH_4) is the second most important anthropogenic greenhouse gas after CO_2 and is estimated to have 28-34 times higher warming effect than the latter over a 100-year period (*1*). The mixing ratio of CH_4 in the troposphere has increased from 715 ppbv in the preindustrial era to currently 1,865 ppbv (Feb. 2019 NOAA). Estimated global CH_4 emissions to the atmosphere average at ca. 560 Tg per year ($1 Tg = 10^{12}$ g) exceeding the current estimated sinks by ca. 13 Tg per year (*2*). Thus, to mitigate the constant increase in atmospheric CH_4 a comprehensive understanding of global CH_4 sources and the environmental parameters that affect them is necessary.

Traditionally, biogenic methanogenesis is the formation of methane under strictly anoxic conditions by microbes from the domain *Archaea* (phylogenetically distinct from both eukaryotes and *Bacteria*). However, in the past decade there has been growing evidence that also eukaryotes such as algae (*3*), plants (*4*), animals (*5*), fungi (*6*) and probably humans (*7*) produce methane under oxic conditions albeit at considerably lower rates. These recent findings suggest that the phenomenon may not be solely limited to methanogenic *Archaea* and could include new metabolic pathways. For example, the conversion of methylated substrates such as methylphosphonates to CH_4 by *Bacteria* has been extensively addressed in recent years with regards to the "methane paradox" (*8, 9*). Recently, Zheng *et al.* (2018) have shown CH_4 formation by *Rhodopseudomonas palustris* during N_2 fixation. Methane emission was also detected from cryptogamic covers, i.e. phototrophic assemblages on plant, rock and soil surfaces (*11*).

Accumulation of CH_4 in oxygenated freshwater environments has been repeatedly associated with the presence of *Cyanobacteria* (see also Fig. S1). Methane production by *Cyanobacteria* has been attributed to either demethylation of methylphosphonates (*12–14*) or to the association of filamentous *Cyanobacteria* with methanogenic *Archaea*, providing the latter with the necessary hydrogen for CH_4 production (*15*). *Cyanobacteria* are ubiquitous, found literally in any illuminated environment as well as unexpectedly in some dark subsurface ones as well (*16, 17*). Furthermore, this phylum predominated Earth whilst the environment was still reductive and ca. 1.3 billion years prior to the great oxygenation event which occurred 2.4 billion years ago (*18*). Therefore, we tested whether this phylum contributes to the global CH_4 budget independent of naturally occurring, extra-cellular precursor substances or the presence of methanogenic *Archaea*. If so, their ubiquitous nature, their expected future increase in abundance (*19, 20*) and their proximity to the interface with the atmosphere makes them potential key players in the global CH_4 cycle.

101 Here we demonstrate that unicellular as well as filamentous, freshwater, marine and terrestrial
102 members of the prominent and ubiquitous phylum *Cyanobacteria*, a member of the domain
103 *Bacteria*, produce CH_4 at substantial rates under light and dark as well as oxic and anoxic
104 conditions.
105
Results and Discussion

107 To test the hypothesis that *Cyanobacteria* produce CH_4 independent of known methylated
108 precursors (e.g. methylphosphonates) in ambient water, thirteen different filamentous and
109 unicellular cyanobacterial cultures (for details of chosen cultures see Table 1) that are known to
110 grow in marine, freshwater and terrestrial environments were incubated under sterile conditions
111 with ^{13}C labelled sodium hydrogen carbonate ($NaH^{13}CO_3$) as carbon source. All investigated
112 cyanobacterial cultures showed CH_4 production with increasing stable isotope values ($\delta^{13}C$-CH_4
113 values) clearly indicating that ^{13}C carbon was incorporated into CH_4, whereas no ^{13}C enrichment
114 occurred in the control experiments (Fig. 1). These results unambiguously show that *Cyanobacteria*
115 produce CH_4 *per se* and that the process is most likely linked to general cell metabolism such as
116 photoautotrophic carbon fixation. The different enrichment of ^{13}C indicated by $\delta^{13}C$-CH_4 values
117 ranging from 1.71 to 1337 ‰ observed in the different cultures is a result of different production
118 rates as well as differences in biomass. The involvement of methanogenic *Archaea* in this process
119 can be ruled out. First, five of the cultures were axenic. Second, the oxygen concentrations during
120 CH_4 production were in most cases above saturation level (Fig. 2 and Fig. S2) and while
121 methanogenic *Archaea* were recently reported from oxic environments (*21*), their activity is
122 attributed to anoxic microniches. Peters and Conrad (*22*) have additionally shown that while
123 methanogens are abundant in oxic environments, methanogenesis remains inactive. Furthermore,
124 transcriptomic data from two experiments we conducted over the course of 24 h with non-axenic
125 cultures of *Anabaena* sp. PCC 7120 and *Microcystis* sp. PCC 7806 have revealed no expression of
126 genes known to be related to classical methanogenic activity. Third, sequencing analysis of non-
127 axenic cultures and quantitative real-time PCR of the methyl coenzyme M reductase gene (*mcrA*)
128 showed methanogenic *Archaea* are either absent or present in negligible numbers (Fig. S3).
129 Furthermore, for the following reasons demethylation of methylphosphonates from the spent
130 growth medium is unlikely to be the mechanism involved in this instance even though some
131 *Cyanobacteria* do possess the necessary enzymatic machinery (*12*, *13*). First, thus far,
132 demethylation of methylphosphonates has been shown to occur only under phosphorus starvation,
133 which was highly unlikely in this study since the culture medium contained ca. 200 µmol P L^{-1}.
134 Publicly available transcriptomic data for *Anabaena* sp. PCC 7120 (*23*, *24*) and *Trichodesmium*
135 *erythraeum* (*25*) show that the phosphonate C-P lyase genes are not expressed under standard (P-
136 rich) culture conditions. This was further corroborated by our own transcriptomes of cultures of
137 *Anabaena* sp. PCC 7120 and *Microcystis* sp. PCC 7806 where no expression of the C-P lyase genes
138 was detected by the *Cyanobacteria* or the accompanying microorganisms. Second, some of the
139 *Cyanobacteria* used in this study (i.e. *Microcystis aeruginosa* PCC 7806, *Synechococcus* WH7803
140 and WH8102, as well as all sequenced species of *Chroococcidiopsis* sp., *Leptolyngbya* sp.,
141 *Phormidium* sp. and *Prochlorococcus* sp.) do not possess the known C-P lyase genes necessary for
142 conversion of methylphosphonates to CH_4. The lack of the C-P lyase genes, responsible for
143 conversion of methylphosphonates to CH_4, was demonstrated to be a common feature of the genus
144 *Prochlorococcus* (*26*). A recent study looking at the processing of methylphosphonates by
145 *Prochlorococcus* (27) revealed an alternative pathway where methylphosphonates are oxidized to
146 formate. *T. erythraeum* was shown to internally produce phosphonates as P storage later to be freed
147 by demethylation (*28*), a process that is likely to release CH_4. Nevertheless, the same study shows,
148 though not focusing on cyanobacteria alone, that marine unicellular organisms such as
149 *Synechococcus* and *Crocosphaera*, do not contain any detectable phosphonate storage.

150 Despite the recent finding of CH_4 production during N_2 fixation by *Rhodopseudomonas palustris*
151 (*10*) we suggest that this is not the pathway leading to CH_4 production in our experiments. First,
152 most *Cyanobacteria* used in this study are unable (i.e marine *Synechococcus*, *Prochlorococcus*,
153 *Microcystis aeruginosa*) or unknown (*Leptolyngbya* sp., *Phormidium persicinum*) to fix N_2.
154 Second, all experiments were conducted in NO_3^- or NH_4^+ rich, fresh, media, and therefore N_2
155 fixation in capable cyanobacteria is likely to be inhibited to a certain degree (*29*). Thus, given the
156 rapid and tight response of CH_4 production with the onset of light, we consider that the mechanism
157 by which *Cyanobacteria* readily convert fixed CO_2 to CH_4 under light conditions must revolve
158 around the photosynthesis process. Inhibitors of photosynthesis such as Atrazine, DBMIB (2,5-
159 Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone) and HQNO (N-oxo-2-heptyl-4-
160 Hydroxyquinoline) inhibit the CH_4 production under light conditions (Fig. S4) and suggest a
161 connection to the photosynthetic electron transfer chain. However, the exact biochemical
162 pathway(s) involved in light-driven cyanobacteria-derived CH_4 formation remains so far unknown
163 and thus requires further investigation.
164 Patterns and rates of CH_4 production were investigated in seventeen cultures (the above mentioned
165 13 and additional 4) over several days of continuous measurement of CH_4 concentration using a
166 membrane inlet mass spectrometry system (MIMS). Our measurements, lasting 2-5 days, showed
167 that CH_4 production occurs both under light and dark conditions (Fig. 2 and Fig. S2). This is evident
168 by a positive production rate at almost all times in all experiments. Replicate experiments revealed
169 that, while *Cyanobacteria* repeatedly produced CH_4, rates and patterns were not consistent,
170 particularly so for production during the periods of darkness. Often, a period with lower rates of
171 CH_4 production was observed between light and dark phases (Fig. 2 and Fig. S2). The latter is
172 evidenced as a decrease in CH_4 concentration resulting from degassing of our incubation system.
173 This suggests that different mechanisms may be involved in CH_4 production under light and dark
174 conditions, presumably dependent on freshly generated photosynthetic products during light and
175 on storage compounds during dark periods. Fermentation of storage compounds by *Cyanobacteria*
176 has been previously described and known to produce among other compounds acetate and hydrogen
177 which are known precursors of acetoclastic CH_4 formation (*30*). Interestingly, most of the genes
178 required for methanogenesis are present in non-methanogenic organisms, including *Cyanobacteria*.
179 Nevertheless, in this instance since the methyl-coenzyme reductase (*mcr*) gene is absent this would
180 suggest that if *Cyanobacteria* produce CH_4 via conventional pathways, an ortholog of the *mcr* gene
181 exists, a rather unlikely option considering current knowledge from cyanobacterial genomes.
182
183 Methane production rates (Fig. 3) were calculated using the slope of CH_4 profiles and were
184 normalized on a cyanobacterial biomass dry weight basis for larger cyanobacteria or cell counts for
185 small-celled marine picophytoplankton. The latter was used to obtain high accuracy for the small-
186 cell-sized picophytoplankton, *Synechococcus* and *Prochlorococcus*. Hourly CH_4 production rates
187 across cultures of larger cyanobacteria were in the range of 0.1 to 3.4 µmol g^{-1} h^{-1} in individual
188 experiments and a mean of 0.51 ± 0.26 µmol g^{-1} h^{-1}. Among the marine picophytoplankton,
189 *Synechococcus* sp. exhibited low rates ranging between 0.01 and 0.6 pmol CH_4 per 10^6 cells h^{-1},
190 while *Prochlorococcus* cultures produced methane at rates ranging from 0.8 to 110 pmol CH_4 per
191 10^6 cells h^{-1}. When compared to production rates of typical methanogenic *Archaea*, CH_4 production
192 rates of freshwater, soil and large marine cyanobacteria are three to four orders of magnitude lower
193 than the CH_4 production rates noted for typical methanogenic *Archaea* in culture under optimal
194 conditions (oxygen free) but one to three orders of magnitude higher than rates observed in
195 eukaryotes (Fig. S5). In our experiments, *Prochlorococcus* and *Synechococcus* cultures produced
196 CH_4 only at light intensities above 20 µmol quanta m^{-2} s^{-1} and therefore, it is likely that only
197 *Procholorococcus* and *Synechococcus* communities in the upper water layers contribute to the
198 oceanic CH_4 flux to the atmosphere.
199

200 Methane production in oxic soils has been previously discussed and attributed mainly to abiotic
201 factors (*31*) or methanogenic *Archaea* (*32*), although the latter was thought unlikely (*31, 33*). Here
202 we show that a typical desert crust *cyanobacterium* (identified in this study as *Leptolyngbya* sp.),
203 as well as the most common endolithic cyanobacterium *Chroococcidiopsis* (*34*) produce CH_4 both
204 under light and dark conditions (Fig. 1, Fig. S2), thus inferring a new but as yet unknown and
205 unaccounted for source of CH_4 from oxic soils.

207 *Cyanobacteria* are ubiquitous in nature and their presence in aquatic systems is expected to increase
208 with eutrophication and rising global temperatures (*19*). The "methane paradox" describing the
209 production of CH_4 in oxic water layers has been known for four decades (*35*). Though values may
210 vary between water bodies, a recent study suggests that up to 90 % of CH_4 emitted from freshwater
211 lakes can be produced in the oxic layer (*36*) with *Cyanobacteria* often being associated with
212 elevated CH_4 concentration in oxygen supersaturated freshwater systems (*37*). In open oceanic
213 environments, distant from any coast, the contribution of lateral transport from anoxic environments
214 is expected to be absent. Nevertheless, based on the emission rates from our laboratory
215 investigations it is difficult to extrapolate the contribution of *Cyanobacteria* to marine, freshwater,
216 terrestrial, and finally to the global CH_4 budget. First, only one attempt has been performed to
217 estimate the global cyanobacterial biomass (*34*). That study does not account for the increase in
218 blooms of toxic and non-toxic *Cyanobacteria* in freshwater systems (*19, 20, 38–40*), nor for less
219 monitored cyanobacterial environments such as those under the ice-cover of frozen lakes (*41*).
220 Recent estimations of *Prochlorococcus* (*42*) suggest a global biomass of 3.4×10^{27} cells globally,
221 larger by 33 % than estimated in 2003 by Garcia-Pichel *et al* (*34*). Second, while our experiments
222 demonstrate unambiguously the ability of all investigated *Cyanobacteria* to produce CH_4 we cannot
223 account for the effect of nutrient concentrations and environmental factors such as light and
224 temperature to control emissions in the natural environment. Temperature alone was shown to have
225 a major effect on the *Prochlorococcus* growth rates (*43*). Nevertheless, considering the combined
226 day and night average CH_4 production rates of high and low light *Prochlorococcus* species (~9
227 pmol CH_4 h^{-1} 10^{-6} cells) which prevail in the upper 100 m of the oceans, and an average abundance
228 of 10^8 cells L^{-1}, the gross daily CH_4 production by *Prochlorococcus* is ~22 nmol L^{-1}.This
229 concentration is around one order of magnitude higher than concentrations of CH_4 found in
230 seawater that is at atmospheric equilibrium (~2 nmol L^{-1}). However, CH_4 in the mean mixed layer
231 of the Atlantic Ocean and surface waters of the Mediterranean Sea have been reported to be often
232 supersaturated reaching maximum CH_4 values up to 7 and 25 nmol L^{-1}, respectively (*44, 45*). This
233 simplified calculation demonstrates that *Prochlorococcus* alone (aside from other marine
234 *Cyanobacteria*) might contribute substantially to the observed oceanic CH_4 supersaturation.
235 In this study, we show that *Cyanobacteria* can readily convert fixed inorganic carbon directly to
236 CH_4 and emit the potent greenhouse gas under both light and dark conditions. This is in addition to
237 the already established ability of *Cyanobacteria* to produce CH_4 by the demethylation of
238 methylphosphonates (*12, 13*). *Cyanobacteria* as a phylum are the most ubiquitous group of
239 organisms on Earth, thriving in most, naturally and artificially, illuminated environments almost
240 regardless of temperatures, salinity and nutrient concentrations. Accordingly, their ability to
241 produce CH_4 via different pathways, likely related to their surroundings, makes them important to
242 the present and future global CH_4 cycle and budget. Even more so, as blooms of cyanobacteria are
243 increasing with eutrophication and rising global temperatures (*19, 20*). A recent study,
244 independently predicts that eutrophication resulting in a strong increase in photoautotrophs in the
245 water column (chlorophyll a), will lead to an increase of 30-90 % in freshwater CH_4 emissions (*46*).
246 According to Fletcher and Schafer (*47*) the drastic increase in atmospheric CH_4 concentrations since
247 2007 coupled to the decrease in $\delta^{13}C$-CH_4 values presumable caused by the increase in ^{12}C-enriched
248 biogenic CH_4 sources is difficult to explain with our current understanding of known CH_4 sources
249 and sinks of the global CH_4 budget. This further highlights the need to fully understand the

250 cyanobacterial contribution to the global methane budget and to identify the isotopic signatures of
251 the various CH_4 production pathways they might harbor. Additionally, as phototrophic prokaryotes
252 such as *Cyanobacteria* have been inhabiting Earth for more than 3.5 billion years (*48*, *49*) they may
253 have had a major contribution to Earth's CH_4 cycle such as during the great oxygenation event or
254 even earlier when the conditions on Earth were more reductive favoring CH_4 production.
255 Further research will elucidate the biochemical pathways of CH_4 formation in *Cyanobacteria* and
256 fully assess the environmental factors affecting it, clarifying its relevance for ecology and the global
257 CH_4 budget throughout Earth history, how it might change in the future, and how this CH_4 source
258 can be mitigated.
259
260
261 **Materials and Methods**
262
263 As part of the routine monitoring at Lake Stechlin a YSI probe Model 6600V2 was deployed to
264 record dissolved oxygen, and other physico-chemical parameters which are not displayed here.
265 Concentrations of taxon-specific phytoplankton pigments were measured by a BBE Moldaenke
266 Fluoroprobe.
267
268 Methane Concentrations in the Lake Stechlin were determined as follows. Water was transferred
269 from a Limnos Water Sampler to 60 ml serum bottles (clear borosilicate glass, $\geq$ 88 % transmission
270 of PAR spectrum), 3 times flushed and crimp-closed (PTFE-butyl septa, aluminum caps) without
271 gas bubbles. Dissolved CH_4 was extracted using head space (He) displacement method and
272 measured by a Shimadzu 14A GC/FID (35 °C Permabond FFAP column on N_2, split-less injection
273 and detection at 140 °C). Headspace CH_4 was converted to dissolved CH_4 concentrations based on
274 Henry's Law and standard conditions.
275
276 **Cyanobacterial cultures**
277 Seventeen different cyanobacterial cultures were obtained from various sources and grown using
278 the media described in Table 1. All the cultures were used for membrane inlet mass spectrometry
279 measurements while 13 cultures were used for stable isotope labelling experiments.
280
281 **Stable isotope labeling experiments**
282 **Culturing and treatments**
283 To investigate the production of *Cyanobacteria*-derived CH_4, 60 ml vials with 40 ml liquid and 20
284 ml head space volume (laboratory air) were used and sealed with septa suitable for gas sampling.
285 For the ^{13}C labelling experiments $NaH^{13}CO_3$ (99 % purity, Sigma-Aldrich, Germany) was added
286 amounting to 10 % of the initial dissolved inorganic carbon (DIC) in BG11 (*50*) (DIC = 0.4 mM,
287 enriched by added $NaHCO_3$; pH $\approx$ 7.0) and 4.5 % of the DIC in f/2 medium (*51*) (DIC = 2.01 mM;
288 pH $\approx$ 8.2) and 1 % of the DIC in the Pro99 (*52*) based medium used for axenic *Synechococcus* and
289 *Prochlorococcus* cultures. Four different examination groups were used: (1) Sterile medium; (2)
290 Sterile medium with $NaH^{13}CO_3$; (3) Sterile medium with culture; (4) Sterile medium with culture
291 and $NaH^{13}CO_3$; Four replicates of each cyanobacteria culture (n = 4).
292 The cultures were grown under a light–dark cycle of 16 and 8 hours at 22.5 °C at a light intensity
293 of $\approx$ 30 µmol quanta m^{-2} s^{-1} for a total period of 3 days.
294
295 **Continuous-flow isotope ratio mass spectrometry (CF-IRMS)**
296 CF-IRMS was employed for measurement of the $\delta^{13}C$-CH_4 values in the head space gas above the
297 cultures. Head space gas from exetainers was transferred to an evacuated sample loop (40 mL) and
298 interfering compounds were then separated by GC and CH_4 trapped on Hayesep D. The sample was
299 then transferred to the IRMS system (ThermoFinniganDeltaplus XL, Thermo Finnigan, Bremen,

300 Germany) via an open split. The working reference gas was carbon dioxide of high purity (carbon
301 dioxide 4.5, Messer Griesheim, Frankfurt, Germany) with a known $\delta^{13}C$ value of -23.64 ‰ relative
302 to Vienna Pee Dee Belemnite (V-PDB). All $\delta^{13}C\text{-}CH_4$ values were corrected using three CH_4
303 working standards (isometric instruments, Victoria, Canada) calibrated against IAEA and NIST
304 reference substances. The calibrated $\delta^{13}C\text{-}CH_4$ values of the three working standards were -23.9 $\pm$
305 0.2 ‰, -38.3 $\pm$ 0.2 ‰, and -54.5 $\pm$ 0.2 ‰. The average standard deviations (n = 3) of the CF-IRMS
306 measurements were in the range of 0.1 to 0.3 ‰. All ^{13}C / ^{12}C-isotope ratios are expressed in the
307 conventional δ notation in per mille (‰) vs. V-PDB, using the equation 1.

309 $$\delta = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} \right] - 1 \qquad\qquad\qquad (1)$$

Membrane inlet mass spectrometer experiments

312 Experiments were conducted using a Bay Instruments (MD, USA) Membrane Inlet Mass
313 Spectrometer (MIMS) consisting of a Pfeiffer Vacuum HiCube 80 Eco turbo pumping station
314 connected to a QMG 220 M1, PrismaPlus®, C-SEM, 1-100 amu, Crossbeam ion source mass
315 spectrometer (Pfeiffer Vacuum, Germany). Culture samples were pumped (Minipuls3, peristaltic
316 pump, Gilson) through a capillary stainless tubing connected to Viton® pump tubing as described
317 in Kana *et al.* (*53*). The coiled stainless-steel tubing was immersed in a water bath to stabilize the
318 sample temperature. Temperatures were set according to the growth conditions of the different
319 cultures. Inside the vacuum inlet the sample passed through an 8 mm long semipermeable
320 microbore silicone membrane (Silastic®, DuPont) before exiting the vacuum and returning to the
321 culture chamber forming a closed system with respect to liquids. This required a 3.5 ml
322 experimental chamber which consisted of an inner chamber where cultures were placed, and an
323 isolated outer chamber connected to a water bath to maintain the culture at a constant temperature.
324 The experimental chamber was placed on a magnetic stirrer and the cultures were continuously
325 stirred for the duration of the experiments to prevent the formation of concentration gradients.
326 Cultures were transferred to fresh medium before the onset of each experiment after which 3.5 ml
327 of the culture were transferred to the experimental chamber and an equal volume was used for
328 determination of cell counts or dry weight. The latter was determined by filtering the samples on
329 pre-weighed combusted GFF filters (Millipore) and drying at 105 °C for 48 h. In the case of non-
330 homogenous cultures, the biomass from the experimental chamber was used at the end of the
331 experiment for determination of dry weight. Marine picophytoplankton cultures were normalized
332 by cell counting using a FACSAria II flow cytometer (BD Biosciences, Heidelberg, Germany) at a
333 flow rate of 23.5 µl / min for 2.5 min. Autofluorescence was used to separate cells from salt
334 precipitates in the medium. Cells for counting were collected from the same batch used in the
335 experimental chamber.
336 The light regime for the experiments was as follows: dark from 19:30 to 09:00 then light intensity
337 was programmed to increase to 60, 120, 180, 400 µmol quanta m^{-2} s^{-1} with a hold time of 1.5 h at
338 each intensity. After maximum light the intensity was programmed to decrease in reverse order
339 with the same hold times until complete darkness again at 19:30. Experiments lasted a minimum of
340 48 h with at least one replicate longer than 72 h. A minimum of 3 replicate experiments were
341 conducted for each culture.
342 As negative controls ultrapure water as well as autoclaved cyanobacterial biomass were measured
343 to test for non-biogenic methane production by the experimental system (Fig. S2).

Experiments using photosynthesis inhibitors

346 Three photosynthesis inhibitors were used to observe their effect on methane production by
347 *Cyanobacteria*. All three were dissolved in water rather than ethanol as the latter results has an
348 ionization pattern in the masking that of CH4. Atrazine (Cat: 45330-250MG-R, Sigma-Aldrich) has

349 a solubility of 33 mg L^{-1} (153 µM) in water and was used at final concentrations of 5 and 10 µM.
350 DBMIB (2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone) (Cat: 271993-250MG, Sigma-
351 Aldrich) has a solubility 132 mg L^{-1} (410 µM) and was used at final concentrations of 5, 10 and 40
352 µM. HQNO (2-n-Heptyl-4-hydroxyquinoline N-oxide) (Cat: sc-202654A, Santa Cruz
353 Biotechnology) is described as only very slightly soluble in water without any numerical
354 information. We used 0.1 mg mL^{-1} culture yet have noticed that the powder grains remained nearly
355 intact. Nevertheless, given an observed effect on the culture, we conclude that part of the material
356 dissolved in the culture media. For the experiments, cultures of *Anabaena* sp. PCC 7120 were
357 concentrated and resuspended in fresh BG11 medium and incubated in a 10 mL analysis chamber
358 connected via circular flow to the MIMS. Inhibitors were introduced via injection of a concentrated
359 solution at 11:00 after 24 h from the experiment start to allow the culture to acclimate. The light
360 regime used was the same as described above.

361
362

363 **Methane calculations**
364 Methane (and oxygen) concentrations were calculated using the ratio relative to the inert gas Ar
365 (m/z 40). Methane concentration was deduced from mass 15 (see Fig. S6 for the ionization pattern
366 of CH_4) which does not overlap with other gases in the sample (*54*). The CH_4, O_2 and Ar
367 concentration in the different media were calculated based on known solubility constants (*55*) and
368 were calibrated to the measured signals in MQ water and growth media at different temperatures.
369 To further calibrate the $CH_{4(mz15)}$/Ar ratio, measurements were conducted on air saturated water at
370 different salinities (Fig. S7).
371 Methane production rates were calculated as the 1st derivative of the Savizky-Golay (*56*) smoothed
372 data using the sgolay function in the R package signal (http://r-forge.r-project.org/projects/signal/).
373 To account for the continuous degassing from the CH_4 supersaturation experimental chamber, the
374 degassing rate was determined experimentally using rapid heating of air-saturated water from 18 to
375 30 °C leading to an instant (super)saturation of 127 % and 130 % for CH_4 and Ar, respectively.
376 This procedure was repeated under two mixing conditions: I) mixing was applied via magnetic
377 stirring as conducted for most cultures; II) mixing occurred only via the cyclic pumping of sample
378 through the MIMS membrane as applied to *Synechococcus* and *Prochlorococcus* cultures. The
379 change in concentration of CH_4 was monitored and a linear ($R^2 = 0.95$) saturation degree dependent
380 rate was determined. The determined rate given in Equations 2 and 3 for type I and type II mixing,
381 respectively, was similar to that determined by comparing the most negative slopes of the culture
382 experiments, when cyanobacterial production rates are expected to be minimal or zero, and the
383 supersaturation state of the culture. Final rates were calculated by adding the absolute values of the
384 local CH_4 slope (1st derivative) and the local degassing rate (equations. 2,3).

385

386 $$R_{degassing} = -2.2365 \times 10^{-12} \times Sat_{(CH_4)} + 2.12656 \times 10^{-12} \qquad (2)$$
387 $$R_{degassing} = -8.8628 \times 10^{-14} \times Sat_{(CH_4)} + 3.5819 \times 10^{-14} \qquad (3)$$

388

389 Where: $R_{degassing}$ is the degassing rate in mol CH_4 sec^{-1} and $Sat_{(CH4)}$ is the fraction CH_4
390 saturation state >1 (and <1.3) determined by measured concentration vs. calculated
391 solubility.

392

393 **Transcriptome experiment**
394 Samples for transcriptome analyses were collected in triplicates at 5 time points during a 24 h
395 experiment with *Anabaena* sp. PCC 7120 and *Microcystis aeruginosa* PCC 7806. For this
396 experiment a non-axenic culture of *Anabaena* sp. PCC7120 was used. The cultures were divided
397 into 16, 60 mL bottles. One of the bottles was connected to the MIMS as described above while the
398 remaining fifteen were incubated under identical light and temperature conditions. The bottles were

399 incubated for 24-36 h of acclimation before T0 was collected on the day of the experiment. T1 was
400 sampled at 09:30, half an hour after the first light was turned on (60 µmol quanta m^{-2} s^{-1}). T2 was
401 sampled at 12:30, half an hour after the third light was turned on (180 µmol quanta m^{-2} s^{-1}). T3 was
402 sampled at 20:00, half an hour after all lights were turned off. T4 was collected at 00:00. Collected
403 samples were immediately filtered on polycarbonate filters (pore size 0.8 µm; Millipore) and frozen
404 at -80 °C till further processing.
405
406 **DNA and RNA extraction and sequencing**
407 To evaluate the presence of methanogenic *Archaea* in non-axenic cultures DNA was extracted as
408 described in Nercessian *et al.* (*57*). The resulting DNA was sent for Illumina sequencing at MrDNA
409 (Shallowater, TX, USA) on a Miseq platform (2x300 bp) using the Arch2A519F (CAG CMG CCG
410 CGG TAA) and Arch1041R (GGC CAT GCA CCW CCT CTC) primers (*58*). These primers with
411 a barcode on the forward primer were used in a PCR using the HotStarTaq Plus Master Mix Kit
412 (Qiagen, USA) under the following conditions: 94 °C for 3 minutes, followed by 30 cycles of 94
413 °C for 30 seconds, 56 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step
414 at 72 °C for 5 minutes was performed. After amplification, PCR products were checked in 2%
415 agarose gel to determine the success of amplification and the relative intensity of bands. The
416 samples were pooled together in equal proportions based on their molecular weight and DNA
417 concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled
418 and purified PCR product were used to prepare an Illumina DNA library. Sequencing was
419 performed on a MiSeq following the manufacturer's guidelines. Archaeal community composition
420 was analyzed using the SILVA-NGS pipeline (*59*) (Fig. S3). After a standard PCR for the *mcrA*
421 gene resulted in no visible products from any of the cultures, a qPCR assay was conducted as well.
422 For RNA extraction a similar protocol as above was used followed by DNA removal using the
423 Turbo DNA Free kit (Cat: AM1907, Thermo Fisher Scientific). Ribosomal RNA was removed from
424 the RNA samples using the Ribominus kit (Thermo Fisher Scientific) following the manufacturer's
425 instructions. The mRNA enriched samples were sequenced at MrDNA (Shallowater, TX, USA).
426 There the RNA samples were resuspended in 25 ul of nuclease free water and further cleaned using
427 RNeasy PowerClean Pro Cleanup Kit (Qiagen). The concentration of RNA was determined using
428 the Qubit® RNA Assay Kit (Life Technologies). 60-500 ng of RNA was used to remove the DNA
429 contamination using Baseline-ZERO™ DNase (Epicentre) following the manufacturer's
430 instructions followed by purification using the RNA Clean & Concentrator columns (Zymo
431 Research). DNA free RNA samples were used for library preparation using the KAPA mRNA
432 HyperPrep Kits (Roche) by following the manufacturer's instructions. The concentration of double
433 strand cDNA was evaluated using the Qubit dsDNA HS Assay Kit (Life Technologies). 25 ng DNA
434 was used to prepare the libraries. The protocol starts with enzymatic fragmentation to produce
435 dsDNA fragments followed by end repair and A-tailing to produce end-repaired, 5'-phosphorylated,
436 3'-dA-tailed dsDNA fragments. In the adapter ligation step, dsDNA adapters are ligated to 3'-dA-
437 tailed molecules. The final step is library amplification, which employs high fidelity, low-bias PCR
438 to amplify library fragments carrying appropriate adapter sequences on both ends. Following the
439 library preparation, the final concentration of all the libraries was measured using the Qubit®
440 dsDNA HS Assay Kit (Life Technologies), and the average library size was determined using the
441 Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries were then pooled in equimolar
442 ratios of 2nM, and 8pM of the library pool was clustered using the cBot (Illumina) and sequenced
443 paired end for 125 cycles using the HiSeq 2500 system (Illumina).
444 The resulting raw read data is deposited at the European Nucleotide Archive (ENA) under project
445 number PRJEB32889.
446
447 **Transcriptome analysis**

448 The fifteen paired end libraries generated from each experiment were trimmed for quality and
449 sequencing adapters using Trimommatic V 0.39 (*60*). For purposes of annotation two strategies
450 were used. For the first approach, all trimmed sequences from a single culture were co-assembled
451 using Trinity V2.8.4 (*61*). The assembled transcripts were then annotated following the Trinotate
452 pipeline (*62*) replacing BLAST with Diamond V0.9.22 (*63*). For the second approach, the trimmed
453 reads from each sample were mapped to the genes of either *Anabaena* sp. PCC 7120
454 (GCA_000009705.1) or *Microcystis aeruginosa* PCC 7806 (GCA_002095975.1).
455 The full results of these experiments including differential expression will be eventually published
456 separately.
457
458 **Quantitative Real Time PCR assay**
459
460 The abundance of methanogens was measured with quantitative PCR specific for the *mcrA* gene
461 encoding the alpha subunit of methyl coenzyme M reductase, the key enzyme of archaeal
462 methanogenesis. The primer pair mlas-mod for (GGY GGT GTM GGD TTC ACM CAR TA) /
463 mcrA- rev (CGT TCA TBG CGT AGT TVG GRT AGT) (*64*) was used. The assay was performed
464 on a qTOWER 2.2 instrument (Analytic Jena, Germany). Assays were performed in 15 µl volume
465 containing 7.5 µl of innuMIX qPCR MasterMix SyGreen (Analytic Jena), 0.75 µl of each primer
466 (10 pmol/µl), 5 µl of ddH2O and 1 µl of template DNA. Assay conditions were 95°C initial
467 denaturation 5 min, 35 cycles of denaturation (95 °C) 30 s, annealing (60 °C) 45 s and elongation
468 (72 °C) 45 s, followed by melting curve analysis. Triplicate assays were performed for each sample,
469 with template DNA concentrations of 10ng/µl. A parallel standard curve of *mcrA* genes was
470 recorded. The standard curve DNA fragment consisted of the *mcrA* gene of a
471 *Methanomassiliicoccus* sp. from cow rumen fluid cloned into the pGEM®-Teasy vector system
472 (Promega, Mannheim, Germany), amplified with vector-specific primers sp6 and T7. The qPCR
473 assay parameters were as follows: slope 3.55, efficiency 0.91, R2 > 0.99.
474
475
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Competing interests: The authors declare no conflict of interest.

Data and materials availability: The sequences were submitted to the European Nucleotide Archive under project numbers: PRJEB25851 and PRJEB32889.
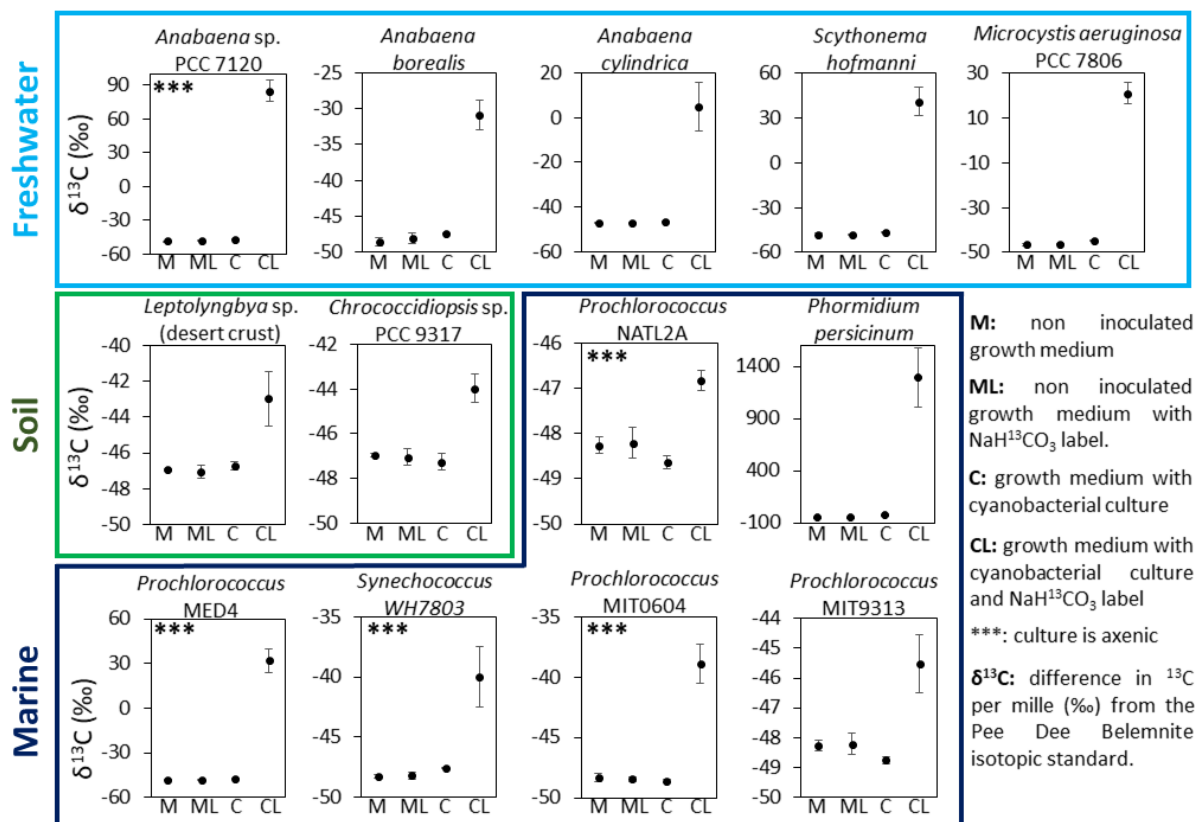
Figures and Tables

Figure 1. δ^{13}C-CH$_4$ values measured during incubation experiments of thirteen different filamentous and unicellular freshwater, soil and marine cyanobacterial cultures with and without NaH^{13}CO$_3$ supplementation. All cyanobacterial cultures produced CH$_4$. Using NaH^{13}CO$_3$ as carbon source (CL) resulted in increasing stable δ^{13}C-CH$_4$ values as compared to the starting condition. This establishes the direct link between carbon fixation and CH$_4$ production. The ^{13}C enrichment is not quantitative and thus not comparable between cultures. Error bars represent standard deviation (n=4).
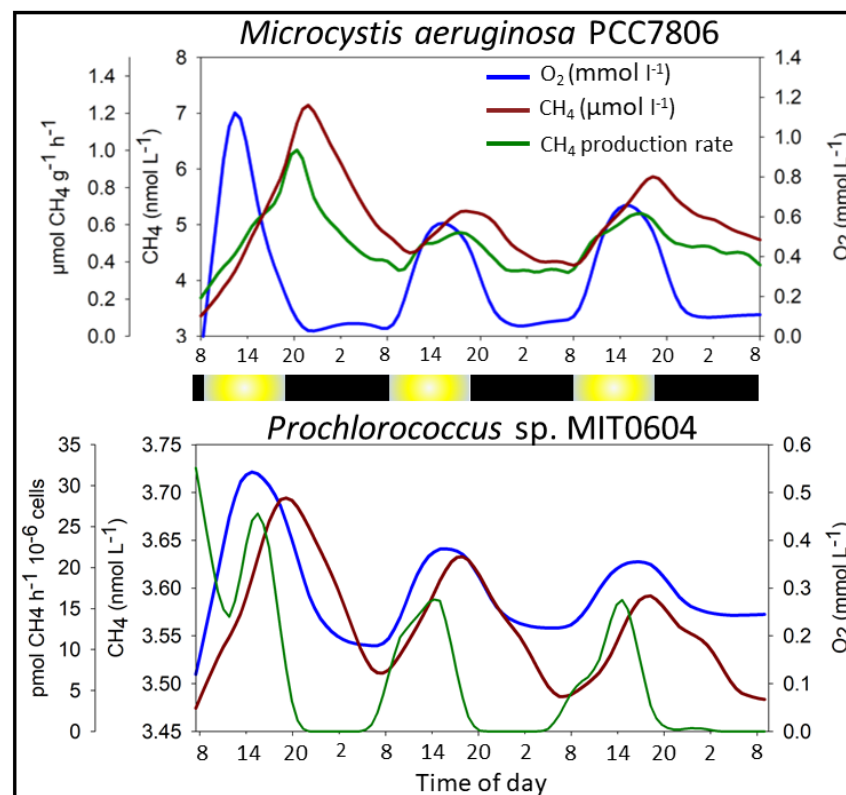
Figure 2. Continuous measurements of CH_4 and oxygen under light/dark periods using a membrane inlet mass spectrometer (MIMS). Examples are shown for two cultures. Data for other cultures can be found in Fig. S2. A decrease in CH_4 concentration is a result of either reduced, or no, production coupled with degassing from the supersaturated, continuously-mixing, semi-open incubation chamber towards equilibrium with atmospheric CH_4 (2.5 nM and 2.1 nM for freshwater and seawater, respectively). Calculated CH_4 production rates account for the continuous emission of CH_4 from the incubation chamber for as long as the CH_4 concentrations are supersaturated. The light regime for the experiments was as follows: dark (black bar) from 19:30 to 09:00 then light intensity (yellow bar) was programmed to increase to 60, 120, 180, 400 µmol quanta m^{-2} s^{-1} with a hold time of 1.5 h at each light intensity. After the maximum light period the light intensity was programmed to decrease in reversed order with the same hold times until complete darkness again at 19:30.
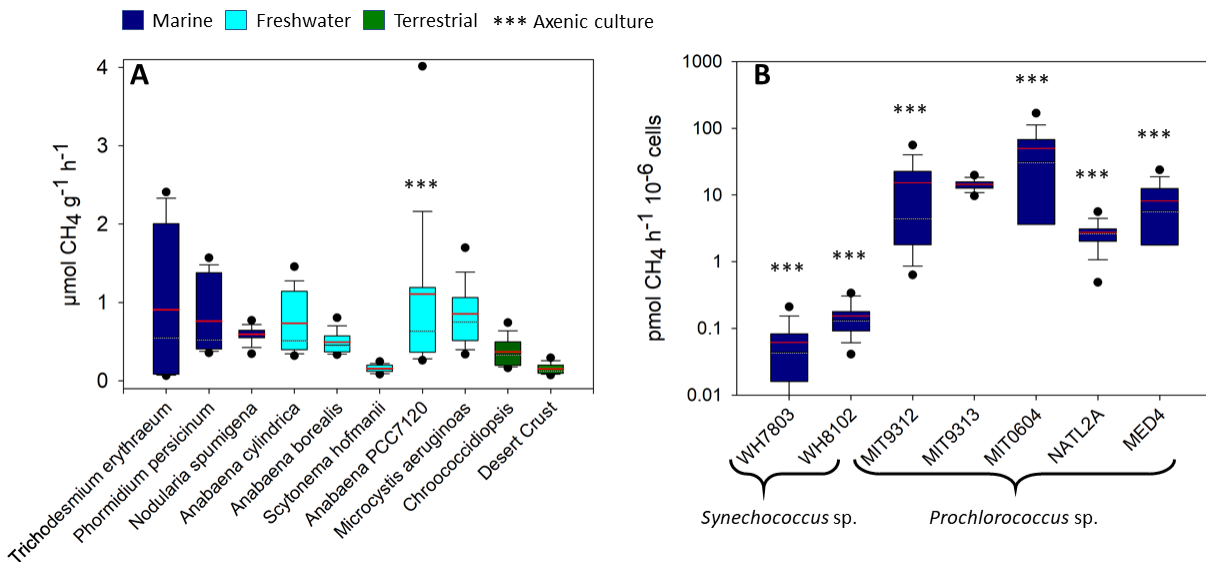
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Figure 3. Average CH_4 production rates obtained from multiple long-term measurements (2-5 days) using a membrane inlet mass spectrometer. The rates are designated by color according to the environment from which the *Cyanobacteria* were originally isolated; dark blue, light blue and green for marine, freshwater and soil environments, respectively. Gray and red lines represent median and mean values, respectively. Rates for the larger cyanobacteria in panel (A) are given in μmol g DW^{-1} h^{-1} and rates for the picocyanobacterial (B) are given in pmol h^{-1} 10^{-6} cells.

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687 **Table 1.** Cyanobacterial cultures used in this study and their growth conditions.

Strain name	Source	Morphology	Growth medium	Incubation Temp. °C
Anabaena sp. PCC7120	IBVF	Filamentous	BG11	30
Anabaena cylindrica ATCC29414	IBVF	Filamentous	BG11	30
Anabaena borealis	CCALA	Filamentous	BG11	30
Scytonema hofmanni PCC7110	IBVF	Filamentous	BG11	30
Leptolyngbya sp. (desert crust)	HUJI	Filamentous	BG11	30
Phormidium persicinum	IBVF	Filamentous	f/2	26
Trichodesmium erythraeum	MPI-MM	Filamentous	YBCII	26
Nodularia spumigena	IOW	Filamentous	f/2 (8 psu)	20
Chroococcidiopsis sp. PCC9317	IBVF	Unicellular	BG11	30
Microcystis aeruginosa PCC7806	IGB	Unicellular	BG11	30
Prochlorococcus sp. MIT9313	Uni Freiburg	Unicellular	AMP1	22
Prochlorococcus sp. MIT9312	Haifa Uni	Unicellular	ASW-Pro99	22
Prochlorococcus sp. MIT0604	Haifa Uni	Unicellular	FSW-Pro99	22
Prochlorococcus sp. NATL2A	Haifa Uni	Unicellular	ASW-Pro99	22
Prochlorococcus sp. MED4	Haifa Uni	Unicellular	ASW-Pro99	22
Synechococcus sp. WH7803	Haifa Uni	Unicellular	ASW-Pro99	22
Synechococcus sp. WH8102	Haifa Uni	Unicellular	ASW-Pro99	22

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689 Shaded cultures are fully axenic while others are mono-algal. Sources abbreviations: IBVF:
690 Culture collection of the Institute for Plant Biochemistry and Photosynthesis, Sevilla Spain;
691 CCALA: Culture collection of autotrophic organisms; HUJI: Laboratory of Aaron Kaplan,
692 Hebrew University of Jerusalem, Jerusalem Israel; IOW: Laboratory of Falk Pollehne,
693 Leibniz Institute for Baltic Sea research, Warnemünde, Germany; MPI-MM: Max Planck
694 Institute for Marine Microbiology, Bremen, Germany; IGB: Leibniz Institute of Freshwater
695 Ecology and Inland Fisheries, Neuglobsow, Germany; Uni. Freiburg, Laboratory of Claudia
696 Steglich, Freiburg University, Freiburg, Germany. Haifa University, Laboratory of Daniel
697 Sher. Media source: BG11 (*50*); f/2 (*51*); YBCII (*65*); AMP1 (*52*); Filtered sea water (FSW)
698 / Artificial sea water Pro99 (*52*).
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Supplementary Materials
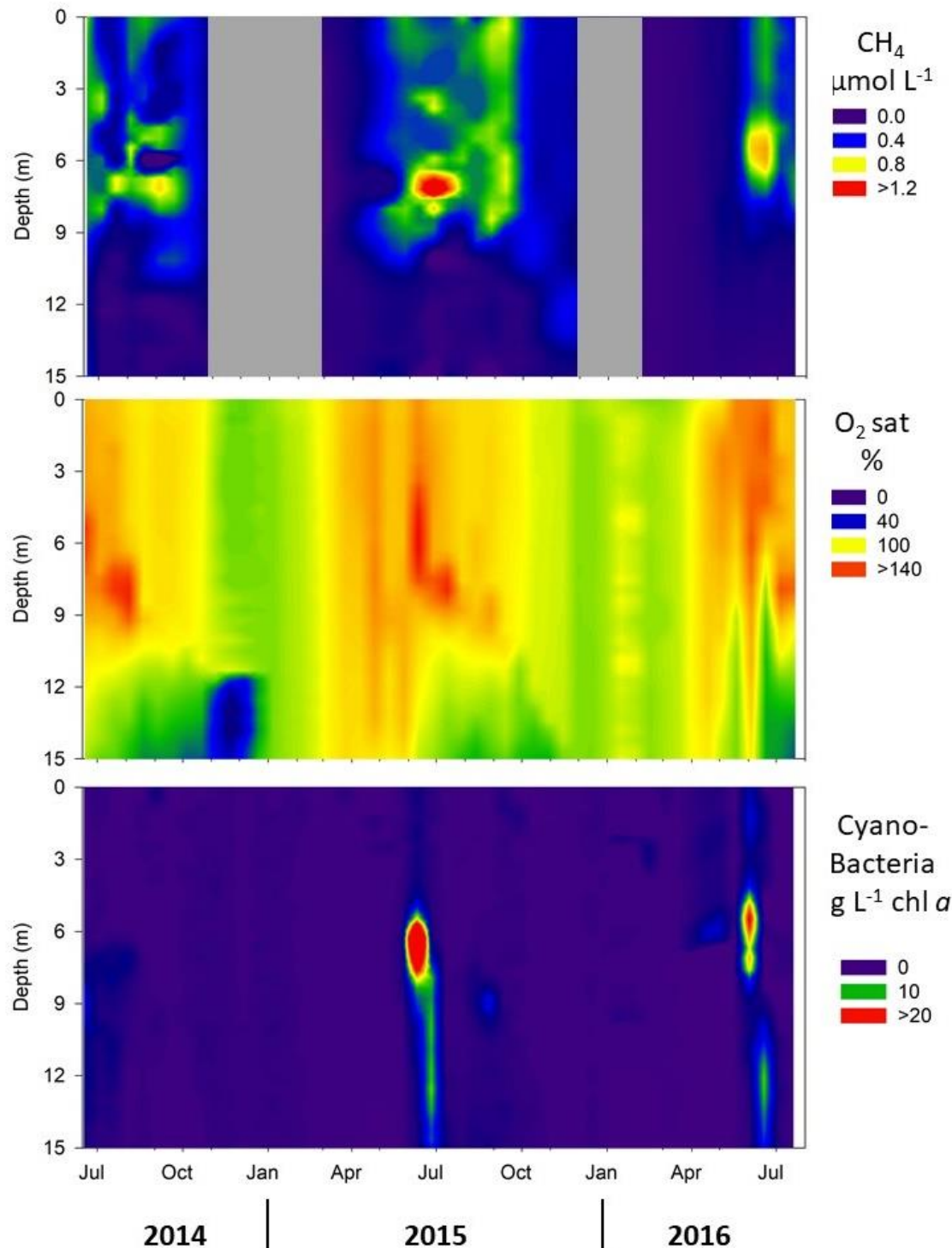
Fig. S1. Temporal profiles of CH4, O2 and cyanobacterial derived Chl a between July 2014 and July 2016. The CH4 data was measured every 1 - 4 weeks depending on season using a GC-FID as described in Grossart *et al*. (*37*). O2 and Chl a were measured hourly using a YSI and a BBE probe (see www.lake-lab.de), respectively.
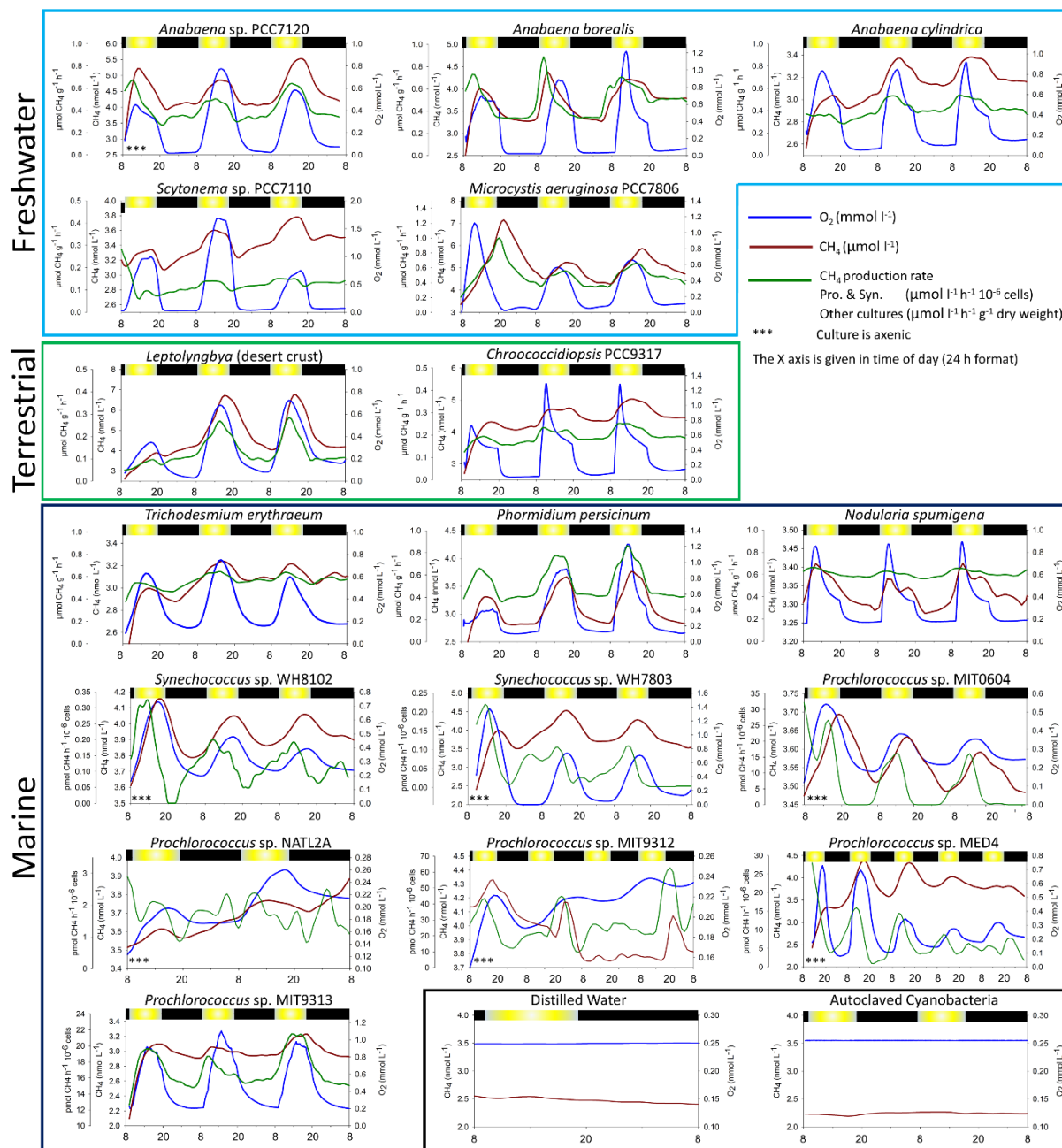
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Fig. S2. Continuous measurements of CH₄ and oxygen under light/dark cycles using a membrane inlet mass spectrometer (MIMS) in 17 different cyanobacterial cultures. A decrease in CH₄ concentration is a result of reduced (or no) production coupled with degassing from the supersaturated, continuously-mixing, semi-open incubation chamber towards equilibrium with atmospheric CH₄ (2.5 nM and 2.1 nM for freshwater and seawater, respectively). Calculated CH₄ production rates account for the continuous emission of CH₄ from the incubation chamber for as long as the CH₄ concentrations are supersaturated. The light regime for the experiments was as follows: dark (black bar) from 19:30 to 09:00 then light intensity (yellow bar) was programmed to increase to 60, 120, 180, 400 µmol quanta m⁻² s⁻¹ with a hold time of 1.5 h at each intensity. After maximum light period the intensity was programmed to decrease in reverse order with the same hold times until complete darkness again at 19:30.
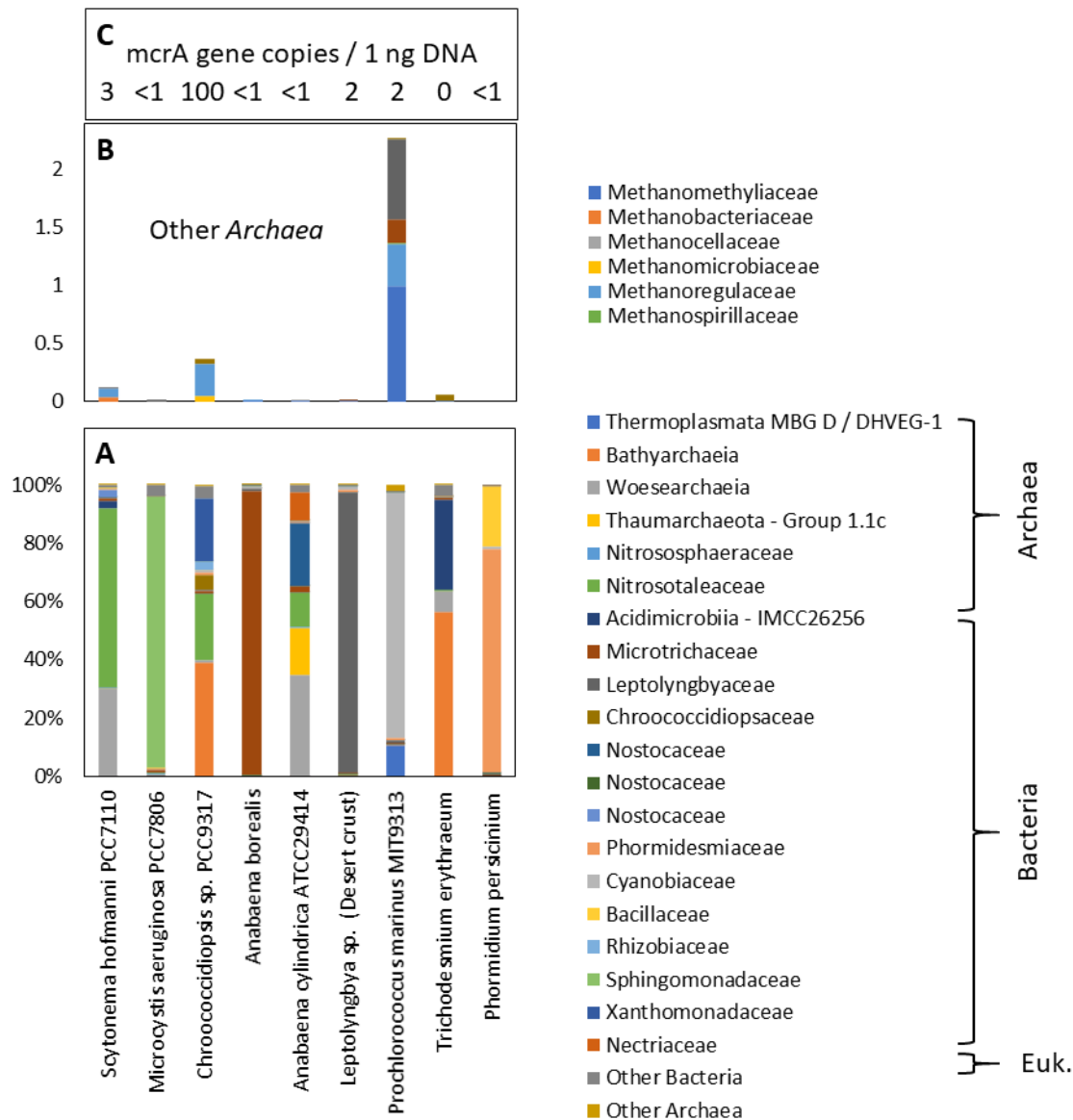
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Fig. S3. Community composition of the cyanobacterial cultures as obtained when sequenced using *Archaea* **specific** primers resulting in 71981 reads for PCC7110, 5442 reads for PCC 7806, 14,489 reads for PCC 9317, 91,105 reads for *A. borealis*, 13,173 reads for ATCC29414, 133,336 reads for *Leptolyngbia* sp., 172,112 reads for MIT9313, 35,257 for *T. erythraeum* and 82,190 reads for *P. persicinum* out of 200,000 commissioned reads. The variability in reads abundance alongside false positive results, suggest a low abundance of *Archaea*. Families of *Archaea* and false positive Bacteria making up more than 2% of the reads are shown in panel (A). Low abundance *Archaea*, comprising of methanogens are shown in panel (B). The background presence or complete absence of methanogens was confirmed by qPCR of the *mcrA* gene (C).
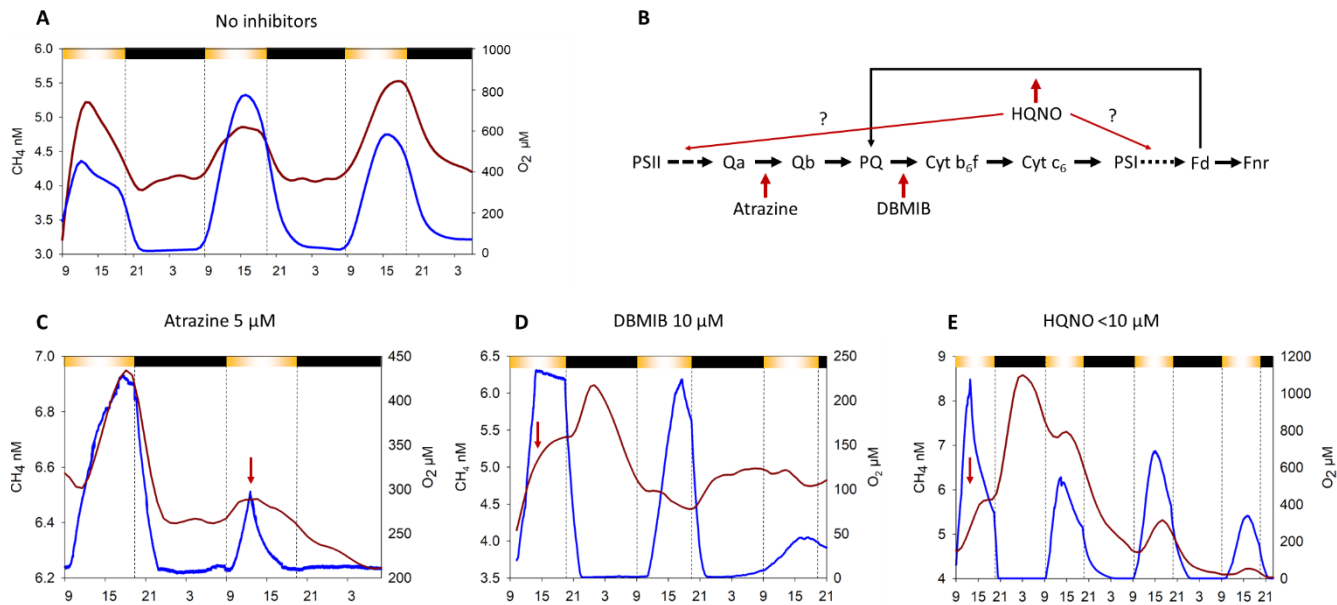
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Fig S4. Under standard experimental conditions the diurnal CH_4 (red lines) and O_2 (blue lines) profiles measured in a culture of *Anabaena* sp. PCC7120 look as in panel (A). To test the link between the photosynthetic system and CH_4 production, a series of known photosynthesis inhibitors (B) were used. The inhibitors were added at 11:00 am (red arrows) after the culture was exposed to 2 h of illumination and the expected increase in CH_4 concentration was observed. Atrazine (C) blocks immediately both O_2 and CH_4 production leading to the subsequent death of the culture. DBIMB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone) (D), results in an immediate decrease in CH_4 production rate under oxic conditions and a burst in CH_4 production when the culture turns anoxic. In subsequent days a decrease in methane concentration is observed immediately upon illumination. This is a result of cease of production coupled with degassing of the CH_4 from the semi-open experimental system. Since DBMIB can be reduced and it's blocking site bypassed, the culture resumes normal activity within few days. HQNO (2-Heptyl-4-hydroxyquinoline n-oxide) (E) has a similar effect as DBMIB in a dark anoxic culture. Upon re-illumination CH_4 production is delayed till stronger light intensities are applied.
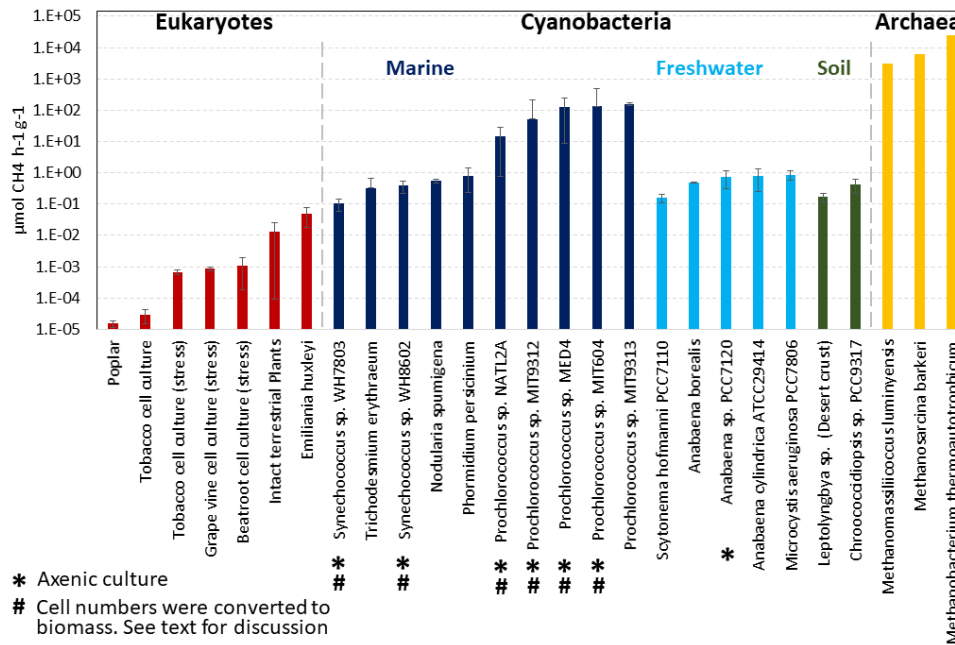
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750 **Fig. S5.** Average CH_4 production rates (μmol gDW^{-1} h^{-1}) obtained from multiple long-term
751 measurements (2-5 days) with a membrane inlet mass spectrometer. The rates are shown by color
752 according to the environment from which the *Cyanobacteria* were originally isolated; dark blue,
753 light blue and green for marine, freshwater and soil environments, respectively. The rates are
754 presented in comparison to three known methanogens. Rates for the methanogens were obtained
755 from references: Mountfort and Asher (*66*), Kröninger *et al.* (*67*) and Gerhard *et al.* (*68*). Rates for
756 eukaryotes including marine algae and terrestrial plants were taken from Lenhart *et al.* (*3*), Keppler
757 *et al.* (*4*), Brüggemann *et al.* (*69*), Wishkermann *et al.* (*70*) and Qaderi *et al.* (*71*) No emission rates
758 (on a dry weight basis) are available for fungi and animals.

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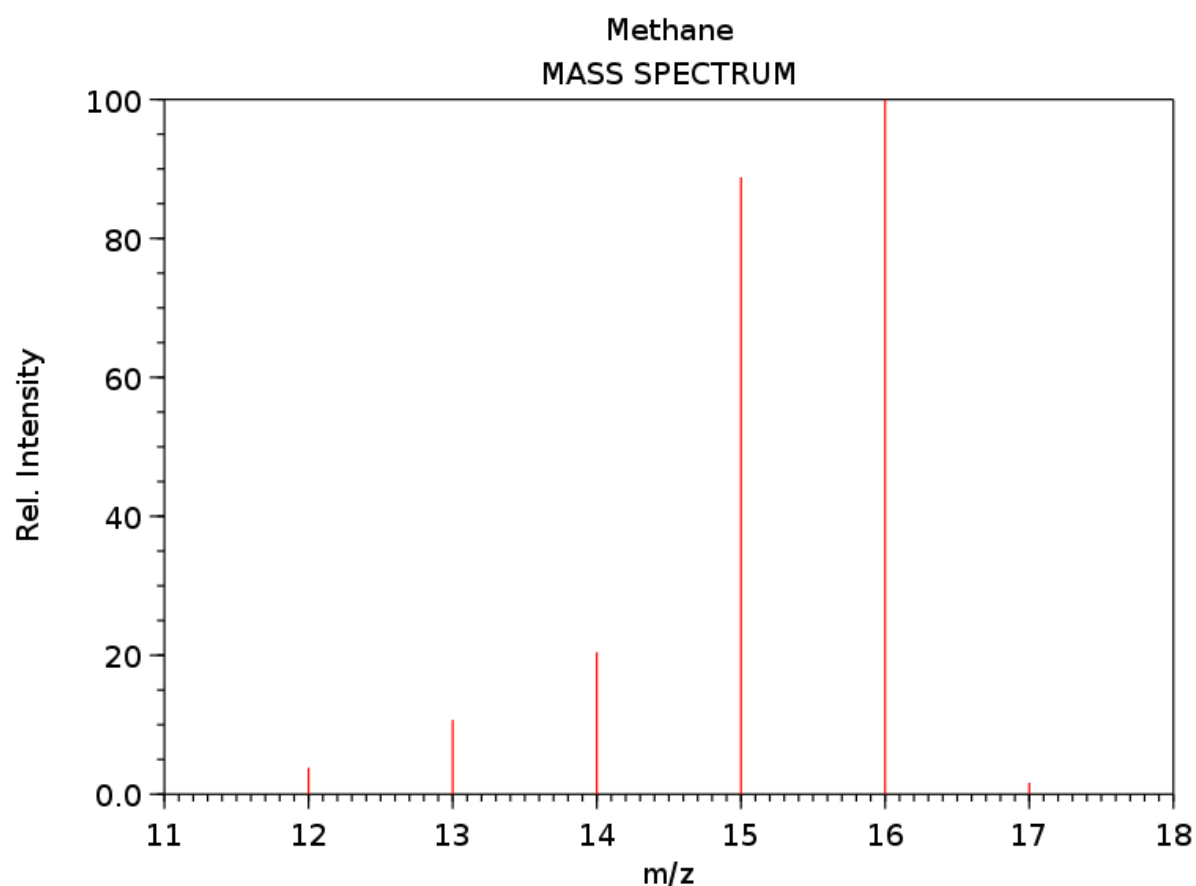
Fig S6. Ionization spectrum (mass spectrum) of methane. The peak at m/z 15 is used for analyses with the membrane inlet mass spectrometer and is ca. 90% of the main peak at m/z 16.
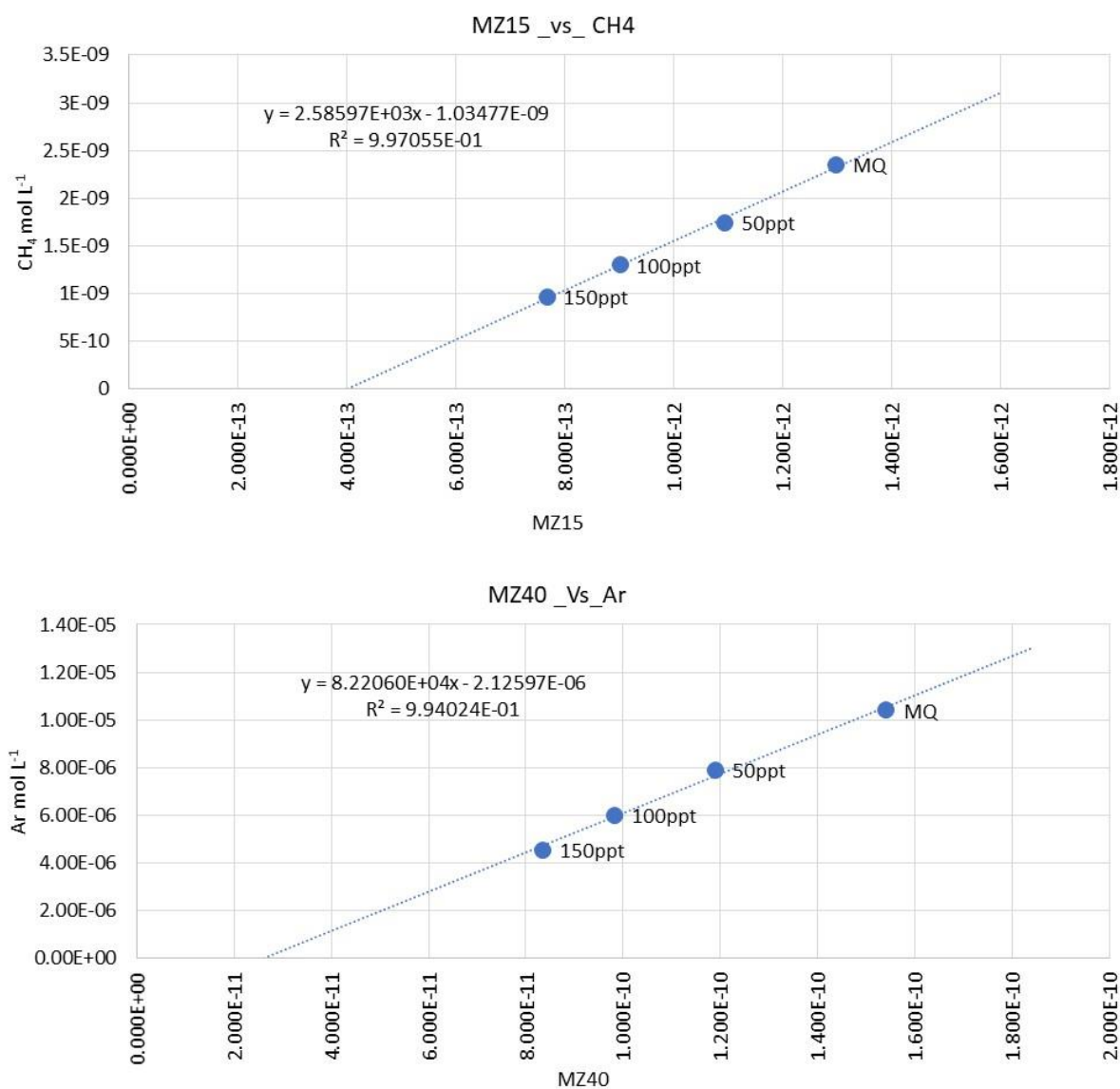
MZ15 _vs_ CH4

$y = 2.58597E+03x - 1.03477E-09$
$R^2 = 9.97055E-01$

MZ40 _Vs_Ar

$y = 8.22060E+04x - 2.12597E-06$
$R^2 = 9.94024E-01$

Fig. S7. Raw signal obtained from mass 15 (CH_4) and mass 40 (Argon) plotted against the calculated solubility at different salinities at 30 °C. The signal in both cases is linearly correlated to the concentration of the dissolved gas. The ratio between the two masses was extrapolated between 0 and 50 ppt and was used for calculating the CH_4 concentration.