Aquatic and terrestrial cyanobacteria produce methane

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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 labeling techniques, was enhanced duringoxic photosynthesis. We suggest that the 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 because of 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.

INTRODUCTION

Methane (CH₄) is the second most important anthropogenic greenhouse gas after CO₂ and is estimated to have 28 to 34 times higher warming effect than the latter over a 100-year period (1). The mixing ratio of CH₄ in the troposphere has increased from 715 parts per billion volume (ppbv) in the preindustrial era to currently 1865 parts ppbv (February 2019 National Oceanic and Atmospheric Administration). Estimated global CH₄ emissions to the atmosphere average at ca. 560 Tg year⁻¹ (1 Tg = 10¹² g), exceeding the current estimated sinks by ca. 13 Tg year⁻¹ (2). Thus, to mitigate the constant increase in atmospheric CH₄, a comprehensive understanding of global CH₄ sources and the environmental parameters that affect them is necessary.

Traditionally, biogenic methanogenesis is the formation of CH₄ under strictly anaerobic 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 CH₄ 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 alternative, yet to be identified metabolic pathways. For example, the conversion of methylated substrates, such as methylphosphonates to CH₄ by bacteria, has been extensively addressed in recent years with regard to the “methane paradox” (8, 9). Recently, Zheng et al. (10) have shown CH₄ formation by Rhodopseudomonas palustris during N₂ fixation. Methane emission was also detected from cryptogamic covers, i.e., phototrophic assemblages on plant, rock, and soil surfaces (11).

Accumulation of CH₄ 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 association of filamentous cyanobacteria with methanogenic archaea, providing the latter with the necessary hydrogen for the CH₄ production (15). Cyanobacteria are ubiquitous, found literally in any illuminated environment and, unexpectedly, in some dark subsurface ones (16, 17). Furthermore, this phylum predominated Earth while the environment was still reductive, ca. 1.3 billion years before the great oxygenation event, which occurred 2.4 billion years ago (18). Therefore, we tested whether this phylum contributes to the global CH₄ budget independent of naturally occurring, extracellular precursor substances or the presence of methanogenic archaea. If so, their ubiquitous nature, expected future increase in abundance (19, 20), and proximity to the interface with the atmosphere make them potential key players in the global CH₄ cycle.

Here, we demonstrate that unicellular and filamentous, freshwater, marine, and terrestrial members of the prominent and ubiquitous phylum Cyanobacteria, a member of the domain bacteria, produce CH₄ at substantial rates under light and dark and oxic and anoxic conditions.

RESULTS AND DISCUSSION

To test the hypothesis that cyanobacteria produce CH₄ independent of known methylated precursors (e.g., methylphosphonates) in ambient water, 13 different filamentous and unicellular cyanobacterial cultures (for details of chosen cultures, see Table 1) that are known to grow in marine, freshwater, and terrestrial environments were incubated under sterile conditions with 13C-labeled sodium hydrogen carbonate (NaH¹³CO₃) as carbon source. All investigated cyanobacterial cultures showed the CH₄ production with increasing stable isotope values (δ¹³C-CH₄ values), indicating that ¹³C carbon was incorporated into CH₄ whereas no ¹³C enrichment occurred in the control experiments (Fig. 1). These results unambiguously show that cyanobacteria produce CH₄ per se and that the process is most likely linked to general cell metabolism. The different enrichment
were axenic. Second, the oxygen concentrations during the CH$_4$ production were, in most cases, above saturation level (Fig. 2 and fig. S2); while methanogenic archaea were recently reported from methanogenic activity. Third, sequencing analysis of nonaxenic cultures and quantitative real-time polymerase chain reaction (PCR) analyses we conducted over the course of 24 hours with nonaxenic cultures of Microcystis sp. PCC 7120 and Microcystis sp. PCC 7806, where expression of the C-P lyase genes was not detected either in the cyanobacteria or in the accompanying microorganisms. Second, some of the cyanobacteria used in this study (i.e., Prochlorococcus sp. MIT9312*, Prochlorococcus sp. MIT0604*, Prochlorococcus sp. NATL2A*, and Synechococcus sp. MED4*) show that the phosphonate C-P lyase genes are not expressed under standard (P-rich) culture conditions. This was further corroborated by our own transcriptomes of cultures of Anabaena sp. PCC 7120 and Microcystis sp. PCC 7806, where expression of the C-P lyase genes was not detected either in the cyanobacteria or in the accompanying microorganisms. Second, some of the cyanobacteria used in this study (i.e., Microcystis aeruginosa PCC 7806, Synechococcus WH7803 and WH8102, and all sequenced species of Chroococcidiopsis sp., Leptolyngbya sp., Phormidium sp., and Prochlorococcus sp.) do not have the known C-P lyase genes necessary for the conversion of methylphosphonates to CH$_4$. The lack of C-P lyase genes was demonstrated to be a common feature of the genus Prochlorococcus (26). A recent study looking at the processing of methylphosphonates by Prochlorococcus (27) revealed an alternative pathway where methylphosphonates are oxidized to formate. T. erythraeum was shown to internally produce phosphonates as P storage later to be freed by demethylation (28), a process that is likely to release CH$_4$. Nevertheless, the same study shows, although not focusing on cyanobacteria alone, that marine unicellular organisms, such as Synechococcus and Crocospheira, do not contain any detectable phosphonate storage.

Despite the recent finding of CH$_4$ production during N$_2$ fixation by R. palustris (10), we suggest that N$_2$ fixation is not the source of CH$_4$ production detected in our experiments. First, most cyanobacteria used in this study are unable (i.e., marine Synechococcus, Prochlorococcus,

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

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Source</th>
<th>Morphology</th>
<th>Growth medium</th>
<th>Incubation temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabaena sp. PCC 7120*</td>
<td>IBVF</td>
<td>Filamentous</td>
<td>BG11</td>
<td>30</td>
</tr>
<tr>
<td>Anabaena cylindrica ATCC29414</td>
<td>IBVF</td>
<td>Filamentous</td>
<td>BG11</td>
<td>30</td>
</tr>
<tr>
<td>Anabaena borealis</td>
<td>CCALA</td>
<td>Filamentous</td>
<td>BG11</td>
<td>30</td>
</tr>
<tr>
<td>Syctonema hofmanni PCC 7110</td>
<td>IBVF</td>
<td>Filamentous</td>
<td>BG11</td>
<td>30</td>
</tr>
<tr>
<td>Leptolyngbya sp. (desert crust)</td>
<td>HUJI</td>
<td>Filamentous</td>
<td>BG11</td>
<td>30</td>
</tr>
<tr>
<td>Phormidium persicinum</td>
<td>IBVF</td>
<td>Filamentous</td>
<td>f/2</td>
<td>30</td>
</tr>
<tr>
<td>Trichodesmium erythraeum</td>
<td>MPI-MM</td>
<td>Filamentous</td>
<td>YBCII</td>
<td>26</td>
</tr>
<tr>
<td>Nodularia spumigena</td>
<td>IOW</td>
<td>Filamentous</td>
<td>f/2 [8 PSU (practical salinity unit)]</td>
<td>20</td>
</tr>
<tr>
<td>Chroococcidiopsis sp. PCC 9317</td>
<td>IBVF</td>
<td>Unicellular</td>
<td>BG11</td>
<td>30</td>
</tr>
<tr>
<td>Microcystis aeruginosa PCC 7806</td>
<td>IGB</td>
<td>Unicellular</td>
<td>AMP1</td>
<td>22</td>
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<tr>
<td>Prochlorococcus sp. MIT9313</td>
<td>University of Freiburg</td>
<td>Unicellular</td>
<td>ASW-Pro99</td>
<td>22</td>
</tr>
<tr>
<td>Prochlorococcus sp. MIT9312*</td>
<td>Haifa University</td>
<td>Unicellular</td>
<td>ASW-Pro99</td>
<td>22</td>
</tr>
<tr>
<td>Prochlorococcus sp. MIT0604*</td>
<td>Haifa University</td>
<td>Unicellular</td>
<td>FSW-Pro99</td>
<td>22</td>
</tr>
<tr>
<td>Prochlorococcus sp. NATL2A*</td>
<td>Haifa University</td>
<td>Unicellular</td>
<td>ASW-Pro99</td>
<td>22</td>
</tr>
<tr>
<td>Prochlorococcus sp. MED4*</td>
<td>Haifa University</td>
<td>Unicellular</td>
<td>ASW-Pro99</td>
<td>22</td>
</tr>
<tr>
<td>Synechococcus sp. WH7803*</td>
<td>Haifa University</td>
<td>Unicellular</td>
<td>ASW-Pro99</td>
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</tr>
<tr>
<td>Synechococcus sp. WH8102*</td>
<td>Haifa University</td>
<td>Unicellular</td>
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</table>

of $^{13}$C indicated by $\delta^{13}$C-CH$_4$ values ranging from 1.71 to 1337‰ observed in the different cultures is a result of different production rates and differences in biomass. The involvement of methanogenic archaea in this process can be ruled out. First, five of the cultures were axenic. Second, the oxygen concentrations during the CH$_4$ production were, in most cases, above saturation level (Fig. 2 and fig. S2); while methanogenic archaea were recently reported from aerobic environments (21), their activity is attributed to anoxic microchambers. Peters and Conrad (22) have additionally shown that while methanogens are abundant inoxic environments, methanogenesis remains inactive. Furthermore, transcriptomic data from two experiments we conducted over the course of 24 hours with nonaxenic cultures of Anabaena sp. PCC 7120 and Microcystis sp. PCC 7806 have revealed no expression of genes known to be related to classical methanogenic activity. Third, sequencing analysis of nonaxenic cultures and quantitative real-time polymerase chain reaction (PCR) of the methyl coenzyme M reductase gene (mcrA) showed that methanogenic archaea are either absent or present in negligible numbers (fig. S3).

Furthermore, for the following reasons, demethylation of methylphosphonates from the spent growth medium is unlikely to be the mechanism involved in this instance, although some cyanobacteria do have the necessary enzymatic machinery (12, 13). First, thus far, demethylation of methylphosphonates has been shown to occur only under phosphorus starvation, which was highly unlikely in this study since the culture medium contained P (ca. 200 µmol liter$^{-1}$). Publicly available transcriptomic data for Anabaena sp. PCC 7120 (23, 24) and Trichodesmium erythraeum (25) show that the phosphonate C-P lyase genes are not expressed under standard (P-rich) culture conditions. Despite the recent finding of CH$_4$ production during N$_2$ fixation by R. palustris (10), we suggest that N$_2$ fixation is not the source of CH$_4$ production detected in our experiments. First, most cyanobacteria used in this study are unable (i.e., marine Synechococcus, Prochlorococcus,

and M. aeruginosa) or unknown (Leptolyngbya sp., P. persicum) to fix N2. Second, all experiments were conducted in NO3−- or NH4+-rich, fresh media; therefore, N2 fixation in capable cyanobacteria is likely to be inhibited to a certain degree (29). Thus, given the rapid and tight response of the CH4 production with the onset of light, we consider that the mechanism by which cyanobacteria readily convert fixed CO2 to CH4 under light conditions might be closely linked to the photosynthesis process. Inhibitors of photosynthesis, such as atrazine, DBMIB (2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone), and HQNO (2-heptyl-4-quinolinol 1-oxide), inhibit the CH4 production under light conditions (fig. S4) and suggest a connection to the photosynthetic electron transfer chain, although related downstream processes might also be involved in the CH4 formation. However, the exact biochemical pathway(s) involved in light-driven cyanobacteria-derived CH4 formation remains, so far, unknown and thus requires further investigation.

Patterns and rates of the CH4 production were investigated in 17 cultures (the above-mentioned 13 and additional 4) over several days of continuous measurement of CH4 concentration using a membrane inlet mass spectrometry (MIMS) system. Our measurements, lasting 2 to 5 days, showed that the CH4 production occurs both under light and dark conditions (Fig. 2 and fig. S2). This is evident by a positive production rate at almost all times in all experiments. Replicate experiments revealed that, while cyanobacteria repeatedly produced CH4, rates and patterns were not consistent, particularly so for production during the periods of darkness. Often, a period with lower rates of the CH4 production was observed between light and dark phases (Fig. 2 and fig. S2). The latter is evidenced as a decrease in CH4 concentration resulting from degassing of our incubation system. This suggests that different mechanisms may be involved in the CH4 production under light and dark conditions, presumably dependent on freshly generated photosynthetic products during light and on storage compounds during dark periods. Fermentation of storage compounds by cyanobacteria has been previously described and is known to produce, among other compounds, acetate and hydrogen, which are known precursors of acetoclastic CH4 formation (30). Most of the genes required for methanogenesis are present in nonmethanogenic organisms, including cyanobacteria. Nevertheless, in this instance, since the methyl coenzyme reductase (mcr) gene is absent, this would suggest that if cyanobacteria produce CH4 via conventional pathways, then an ortholog of the mcr gene exists, a rather unlikely option considering current knowledge from cyanobacterial genomes.

Methane production rates (Fig. 3) were calculated using the slope of CH4 profiles and were normalized on a cyanobacterial biomass dry weight basis for larger cyanobacteria or cell counts for small-celled marine picophytoplankton. The latter was used to obtain high accuracy for the small cell–sized picophytoplankton, Synechococcus and Prochlorococcus. Hourly CH4 production rates across cultures of larger cyanobacteria were in the range of 0.1 to 3.4 μmol g−1 hour−1 in individual experiments and a mean of 0.51 ± 0.26 μmol g−1 hour−1. Among the marine picophytoplankton, Synechococcus sp. exhibited low rates ranging between 0.01 and 0.6 pmol CH4 per 10⁶ cells.
**Fig. 2.** Continuous measurements of CH$_4$ and oxygen under light/dark periods using a 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, semiopen incubation chamber toward equilibrium with atmospheric CH$_4$ (2.5 and 2.1 nM for fresh water 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 bars) from 19:30 to 09:00, and then light intensity (yellow bars) was programmed to increase to 60, 120, 180, 400 μmol quanta m$^{-2}$ s$^{-1}$ with a hold time of 1.5 hours 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.

The production of CH$_4$ in oxic water layers has been known for four decades (35). Although values may vary between water bodies, a recent study suggests that up to 90% of CH$_4$ emitted from freshwater lakes can be produced in the oxic layer (36), with cyanobacteria often being associated with elevated CH$_4$ concentration in oxygen supersaturated freshwater systems (37). In open oceanic environments, distant from any coast, the contribution of lateral transport from anoxic environments is expected to be absent. Nevertheless, on the basis of the emission rates from our laboratory investigations, it is difficult to extrapolate the contribution of cyanobacteria to marine, freshwater, terrestrial, and lastly to the global CH$_4$ budget. First, only one attempt has been performed to estimate the global cyanobacterial biomass (34). That study does not account for the increase in blooms of toxic and nontoxic cyanobacteria in freshwater systems (19, 20, 38–40) nor for less monitored cyanobacterial environments such as those under the ice cover of frozen lakes (41). Recent estimations of Prochlorococcus (42) suggest a global biomass of 3.4 × 10$^{27}$ cells globally, larger by 33% than estimated in 2003 by Garcia-Pichel et al. (34). Second, while our experiments unambiguously demonstrate the ability of all investigated cyanobacteria to produce CH$_4$, we cannot account for the effect of nutrient concentrations and environmental factors, such as light and temperature, to control emissions in the natural environment. Temperature alone was shown to have a major effect on the Prochlorococcus growth rates (43). Nevertheless, considering the combined day and night average CH$_4$ production rates of high and low light Prochlorococcus species (~9 pmol CH$_4$ per 10$^{-6}$ cells hour$^{-1}$), which prevail in the upper 100 m of the oceans, and an average abundance of 10$^6$ cells liter$^{-1}$, the gross daily CH$_4$ production by Prochlorococcus is ~22 nM. This concentration is around one order of magnitude higher than concentrations of CH$_4$ found in seawater that is at atmospheric equilibrium (~2 nM). However, CH$_4$ in the mean mixed layer of the Atlantic Ocean and surface waters of the Mediterranean Sea have been reported to be often supersaturated, reaching maximum CH$_4$ values up to 7 and 25 nM, respectively (44, 45). This simplified calculation demonstrates that Prochlorococcus alone (aside from other marine cyanobacteria) might contribute substantially to the observed oceanic CH$_4$ supersaturation. Moreover, table S1 provides, for estimation purposes, literature-derived estimates of global cyanobacteria biomass divided into several compartments alongside suggested minimum and maximum CH$_4$ production rates derived from the laboratory experiments obtained in our study. These global extrapolations span a wide range depending on the assumptions made, and we would like to note that this simple approach neglects the complexity of aquatic and terrestrial ecosystems; hence, these calculations should only be considered as first rough estimates.

In this study, we show that cyanobacteria can readily convert fixed inorganic carbon into CH$_4$ and emit this potent greenhouse gas under both light and dark conditions. This is in addition to the already established ability of cyanobacteria to produce CH$_4$ by the demethylation of methylphosphonates (12, 13). As a phylum, the Cyanobacteria are the most ubiquitous group of organisms on Earth, thriving in most naturally and artificially illuminated environments almost regardless of temperatures, salinity, and nutrient concentrations. Accordingly, their ability to produce CH$_4$ via different pathways, likely related to their surroundings, makes them important to the present and future global CH$_4$ cycle and budget, even more so as blooms of cyanobacteria are increasing with eutrophication and rising global temperatures (19, 20). A recent study independently predicts
that eutrophication resulting in a strong increase in photoautotrophs in the water column (chlorophyll a) will lead to an increase of 30 to 90% in freshwater CH₄ emissions (46). According to Fletcher and Schaefer (47), the marked increase in atmospheric CH₄ concentrations since 2007 coupled to the decrease in δ¹³C-CH₄ values presumably caused by the increase in δ¹³C-enriched biogenic CH₄ sources is difficult to explain with our current understanding of known CH₄ sources and sinks of the global CH₄ budget. This further highlights the need to fully understand the cyanobacterial contribution to the global CH₄ budget and to identify the isotopic signatures of the various CH₄ production pathways they might harbor. In addition, as phototrophic prokaryotes such as cyanobacteria have been inhabiting Earth for more than 3.5 billion years (48, 49), they may have had a major contribution to Earth’s CH₄ cycle such as during the great oxygenation event or even earlier when the conditions on Earth were more reductive favoring the CH₄ production.

Further research will elucidate the biochemical pathways of CH₄ formation in cyanobacteria and fully assess the environmental factors affecting it, clarifying its relevance for ecology and the global CH₄ budget throughout Earth history, how it might change in the future, and how this CH₄ source can be mitigated.

MATERIALS AND METHODS
As part of the routine monitoring at Lake Stechlin, a YSI probe model 6600V2 was deployed to record dissolved oxygen and other physicochemical parameters, which are not displayed here. Concentrations of taxon-specific phytoplankton pigments were measured by a BBE Moldaenke FluoroProbe.

Methane concentrations in Lake Stechlin were determined as follows. Water was transferred from a LIMNOS Water Sampler to 60-ml serum bottles (clear borosilicate glass, ≥88% transmission of photosynthetically active radiation spectrum), flushed three times, and crimp-closed (polytetrafluoroethylene-butyl septa and aluminum caps) without gas bubbles. Dissolved CH₄ was extracted using headspace (He) displacement method and measured by a Shimadzu 14A gas chromatography (GC)/flame ionization detector (35°C Permabond free fatty acid phase (FFAP) column on N₂, split-less injection, and detection at 140°C). Headspace CH₄ was converted to dissolved CH₄ concentrations based on Henry’s law and standard conditions.

Cyanobacterial cultures
Seventeen different cyanobacterial cultures were obtained from various sources and grown using the media described in Table 1. All the cultures were used for MIMS measurements, while 13 cultures were used for stable isotope labeling experiments.

Stable isotope labeling experiments
Culturing and treatments
To investigate the production of cyanobacteria-derived CH₄, 60-ml vials with 40 ml of liquid and 20 ml of headspace volume (laboratory air) were used and sealed with septa suitable for gas sampling. For the δ¹³C labeling experiments, NaH¹³CO₃ (99% purity; Sigma-Aldrich, Germany) was added, amounting to 10% of the initial dissolved inorganic carbon (DIC) in BG11 (DIC = 0.4 mM, enriched by added NaHCO₃; pH 7.0) (50), 4.5% of the DIC in f/2 medium (DIC = 2.01 mM; pH 8.2) (51), and 1% of the DIC in the Pro99-based medium (52) used for axenic Synechococcus and Prochlorococcus cultures. Four different examination groups were used: (i) sterile medium, (ii) sterile medium with NaH¹³CO₃, (iii) sterile medium with culture and NaH¹³CO₃; four replicates of each cyanobacterial culture (n = 4).

The cultures were grown under a light-dark cycle of 16 and 8 hours at 22.5°C at a light intensity of ~30 μmol quanta m⁻² s⁻¹ for a total period of 3 days.

Continuous-flow isotope ratio mass spectrometry
Continuous-flow isotope ratio mass spectrometry (CF-IRMS) was used for measurement of the δ¹³C-CH₄ values in the headspace gas above the cultures. Headspace gas from exetainers was transferred to an evacuated sample loop (40 ml), and interfering compounds were then separated by GC, and CH₄ was trapped on HayeSep D. The sample was then transferred to the IRMS system (Thermo Finnigan DELTAplus XL, Thermo Finnigan, Bremen, Germany) via an open split. The working reference gas was carbon dioxide of high purity (carbon dioxide 4.5, Messer Griesheim, Frankfurt, Germany) with a known δ¹³C value of ~23.64‰ relative to Vienna

Fig. 3. Average CH₄ production rates obtained from multiple long-term measurements (2 to 5 days) using a MIMS. The rates are designated by color according to the environment from which the cyanobacteria were originally isolated. Dark blue, marine environment; light blue, freshwater environment; green, soil environment. Gray and red lines represent median and mean values, respectively. Rates for the larger cyanobacteria in (A) are given in μmol g dry weight⁻¹ hour⁻¹ and rates for the picocyanobacteria (B) are given in pmol per 10⁶ cells per hour⁻¹.
Pee Dee belemnite (V-PDB). All \( \delta^{13} \text{C}-\text{CH}_4 \) values were corrected using three \( \text{CH}_4 \) working standards (Isometric Instruments, Victoria, Canada) calibrated against the International Atomic Energy Agency and the National Institute of Standards and Technology reference substances. The calibrated \( \delta^{13} \text{C}-\text{CH}_4 \) values of the three working standards were \(-23.9 \pm 0.2\%\), \(-38.3 \pm 0.2\%\), and \(-54.5 \pm 0.2\%\). The average SDs \((n = 3)\) of the CF-IRMS measurements were in the range of 0.1 to 0.3\%. All \( ^{13}/^{12} \)\text{C} isotope ratios were expressed in the conventional \( \delta \) notation in per mil (\( \% \)) versus V-PDB using Eq. 1

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

**MIMS experiments**

Experiments were conducted using a Bay Instruments (MD, USA) MIMS consisting of a Pfeiffer Vacuum HiCube 80 Eco Turbo pumping station connected to a QMG 220 M1, PrismaPlus, C-SEM, 1 to 100 atomic mass unit, crossbeam ion source mass spectrometer (Pfeiffer Vacuum, Germany). Culture samples were pumped (peristaltic pump, Minipuls 3, Gilson) through a capillary stainless tubing connected to Viton pump tubing, as described by Kana et al. (53). The coiled stainless steel tubing was immersed in a water bath to stabilize the sample temperature. Temperatures were set according to the growth conditions of the different cultures. Inside the vacuum inlet, the sample passed through an 8-mm-long semipermeable microbore silicone membrane (Silastic, DuPont) before exiting the vacuum and returning to the culture chamber, forming a closed system with respect to liquids. This required a 3.5-ml experimental chamber, which consisted of an inner chamber where cultures were placed, and an isolated outer chamber connected to a water bath to maintain the culture at a constant temperature. The experimental chamber was placed on a magnetic stirrer, and the cultures were continuously stirred for the duration of the experiments to prevent the formation of concentration gradients.

Cultures were transferred to a fresh medium before the onset of each experiment after which 3.5 ml of the culture was transferred to the experimental chamber, and an equal volume was used for determination of cell counts or dry weight. The latter was determined by filtering the samples on preweighed combusted GF/F filters (Millipore) and drying at 105°C for 48 hours. In the case of nonhomogeneous cultures, the biomass from the experimental chamber was used at the end of the experiment for determination of dry weight. Marine picophytoplankton cultures were normalized by cell counting using a FACSAria II flow cytometer (BD Biosciences, Heidelberg, Germany) at a flow rate of 23.5 \( \mu \text{l} \text{ min}^{-1} \) for 2.5 min. Autofluorescence was used to separate cells from salt precipitates in the medium. Cells for counting were collected from the same batch used in the experimental chamber.

The light regime for the experiments was as follows: Dark from 19:30 to 09:00 and then light intensity was programmed to increase to 60, 120, 180, 400 \( \mu \text{mol quanta m}^{-2} \text{ s}^{-1} \), with a hold time of 1.5 hours at each intensity. After maximum light, the intensity was programmed to decrease in reverse order with the same hold times until complete darkness again at 19:30. Experiments lasted a minimum of 48 hours with at least one replicate longer than 72 hours. A minimum of three replicate experiments were conducted for each culture. As negative controls, ultrapure water and autoclaved cyanobacterial biomass were measured to test for nonbiogenic \( \text{CH}_4 \) production by the experimental system (fig. S2).

**Experiments using photosynthesis inhibitors**

Three photosynthesis inhibitors were used to observe their effect on cyanobacterial \( \text{CH}_4 \) production. All three inhibitors were dissolved in water rather than ethanol as the latter results in an ionization pattern that masks that of \( \text{CH}_4 \). Attrazine (catalog number 45330-250MG-R, Sigma-Aldrich) has a solubility of 33 mg liter\(^{-1}\) (153 \( \mu \text{M} \)) in water and was used at final concentrations of 5 and 10 \( \mu \text{M} \). DBMIB (catalog number 271993-250MG, Sigma-Aldrich) has a solubility of 132 mg liter\(^{-1}\) (410 \( \mu \text{M} \)) and was used at final concentrations of 5, 10, and 40 \( \mu \text{M} \). HQNO (catalog number sc-202654A, Santa Cruz Biotechnology) was described as only very slightly soluble in water without any numerical information. We used culture (0.1 mg ml\(^{-1}\)) yet noticed that the powder grains remained nearly intact. Nevertheless, given an observed effect on the culture, we concluded that part of the material dissolved in the culture media. For the experiments, cultures of *Anabaena* sp. PCC 7120 were concentrated and resuspended in fresh BG11 medium and incubated in a 10-ml analysis chamber connected via circular flow to the MIMS. Inhibitors were introduced via injection of a concentrated solution at 11:00 after 24 hours from the experiment start to allow the culture to acclimate. The light regime used was the same as described above.

**Methane calculations**

Methane (and oxygen) concentrations were calculated using the ratio relative to the inert gas Ar [mass/charge ratio \((m/z)\), 40]. Methane concentration was deduced from mass 15 (see fig. S7 for the ionization pattern of \( \text{CH}_4 \)), which does not overlap with other gases in the sample (54). The \( \text{CH}_4 \), \( \text{O}_2 \), and Ar concentrations in the different media were calculated on the basis of known solubility constants (55) and were calibrated to the measured signals in Milli-Q water and growth media at different temperatures. To further calibrate the \( \text{CH}_4[/15]/\text{Ar} \) ratio, measurements were conducted on air-saturated water at different salinities (fig. S8).

Methane production rates were calculated as the first derivative of the Savitzky-Golay (56) smoothed data using the sgolay function in the R package signal (http://r-forge.r-project.org/projects/signal/). To account for the continuous degassing from the \( \text{CH}_4 \) supersaturation experimental chamber, the degassing rate was experimentally determined using rapid heating of air-saturated water from 18° to 30°C, leading to an instant (super)saturation of 127 and 130% for \( \text{CH}_4 \) and Ar, respectively. This procedure was repeated under two mixing conditions: (i) Mixing was applied via magnetic stirring as conducted for most cultures, and (ii) mixing occurred only via the cyclic pumping of sample through the MIMS membrane as applied to *Synechococcus* and *Prochlorococcus* cultures. The change in concentration of \( \text{CH}_4 \) was monitored, and a linear \((R^2 = 0.95)\) saturation degree–dependent rate was determined. The determined rates given in Eqs. 2 and 3 for type I and type II mixing, respectively, were similar to those determined by comparing the most negative slopes of the culture experiments, when cyanobacterial production rates were expected to be minimal or zero, and the supersaturation state of the culture. Final rates were calculated by adding the absolute values of the local \( \text{CH}_4 \) slope (first derivative) and the local degassing rate (Eqs. 2 and 3)

\[
R_{\text{degassing}} = -2.2365 \times 10^{-12} \times \text{Sat}_{(\text{CH}_4)} + 2.12656 \times 10^{-12} \tag{2}
\]

\[
R_{\text{degassing}} = -8.8628 \times 10^{-14} \times \text{Sat}_{(\text{CH}_4)} + 3.5819 \times 10^{-14} \tag{3}
\]

where \( R_{\text{degassing}} \) is the degassing rate in mol \( \text{CH}_4 \) s\(^{-1} \) and \( \text{Sat}_{(\text{CH}_4)} \) is the fraction \( \text{CH}_4 \) saturation state >1 (and <1.3) determined by measured concentration versus calculated solubility.
**DNA and RNA extraction and sequencing**

Further processing.

DNA was extracted as described by Nercessian et al. (57). The resulting DNA was sent for Illumina sequencing at MR DNA (Shallowater, TX) on a MiSeq platform (2 × 300 base pairs) using the Arch2AS19F (CAGCGMCGGCGGTAA) and ArchI041R (GGCCATGACCCWCCCTTCTC) primers (58). These primers with a barcode on the forward primer were used in a PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 40 s, and 72°C for 1 min, after which a final elongation step was performed at 72°C for 5 min. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. The samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated AMPure XP beads. Then, the pooled and purified PCR products were used to prepare an Illumina DNA library. Sequencing was performed on a MiSeq following the manufacturer’s guidelines. Archaeal community composition was analyzed using the SILVAngs pipeline (fig. S3) (59). After a standard PCR for the mcrA gene resulted in no visible products from any of the cultures, a quantitative PCR (qPCR) assay was conducted as well.

For RNA extraction, a similar protocol as above was used, followed by DNA removal using the TURBO DNA-free Kit (catalog number AM1907, Thermo Fisher Scientific). Ribosomal RNA was removed from the RNA samples using the RiboMinus Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. The mRNA-enriched samples were sequenced at MR DNA (Shallowater, TX). There, the RNA samples were resuspended in 25 μl of nuclease-free water and further cleaned using the RNeasy PowerClean Pro Cleanup Kit (Qiagen). The concentration of RNA was determined using the Qubit RNA Assay Kit (Life Technologies). Sixty to 500 ng of RNA were used to remove the DNA contamination using Baseline-Zero DNase (Epicycle) following the manufacturer’s instructions, followed by purification using the RNA Clean & Concentrator columns (Zymo Research). DNA-free RNA samples were used for library preparation using the KAPA mRNA HyperPrep Kits (Roche) following the manufacturer’s instructions. The concentration of double-stranded complementary DNA was evaluated using the Qubit dsDNA HS Assay Kit (Life Technologies). Twenty-five nanograms of DNA was used to prepare the libraries. The protocol starts with enzymatic fragmentation to produce double-stranded DNA (dsDNA) fragments followed by end repair and A-tailing to produce end-repaired, 5’-phosphorylated, 3’-tailed dsDNA fragments. In the adapter ligation step, dsDNA adapters were ligated to 3’-tailed molecules. The final step is library amplification, which uses high fidelity, low-bias PCR to amplify library fragments carrying appropriate adapter sequences on both ends. Following the library preparation, the final concentrations of all the libraries were measured using the Qubit dsDNA HS Assay Kit (Life Technologies), and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries were then pooled in equimolar ratios of 2 nM, and 8 pmol of the library pool was clustered using the cBot (Illumina) and sequenced paired end for 125 cycles using the HiSeq 2500 system (Illumina).

The resulting raw read data were deposited at the European Nucleotide Archive under project number PRJEB32889.

**Quantitative real-time PCR assay**

The abundance of methanogens was measured with qPCR specific for the mcrA gene encoding the alpha subunit of methyl coenzyme M reductase, the key enzyme of archaeal methanogenesis. The primer pair mlas-mod forward (GGYGGTGTMGGDTTCACMCARTA)/mcrA-reverse (CGTTCATBGCGTAGTTVGGRTAGT) (64) was used. The assay was performed on a qTOWER 2.2 instrument (Analytik Jena, Germany). Assays were performed in a 15-μl volume containing 7.5 μl of innuMIQ qPCR MasterMix SyGreen (Analytik Jena), 0.75 μl of each primer (10 pmol/μl), 5 μl of ddH2O, and 1 μl of template DNA. ASSAY conditions were 95°C initial denaturation for 5 min, 35 cycles of denaturation (95°C) for 30 s, annealing (60°C) for 45 s, and elongation (72°C) for 45 s, followed by melting curve analysis. Triplicate assays were performed for each sample, with template DNA concentrations of 10 ng/μl. A parallel standard curve of mcrA gene was recorded. The standard curve DNA fragment consisted of the mcrA gene of a Methanomasillilicoccus sp. from cow rumen fluid cloned into the pGEM-T Easy Vector System (Promega, Mannheim, Germany), amplified with vector-specific primers sp6 and T7. The qPCR assay parameters were as follows: slope, 3.55; efficiency, 0.91; R² > 0.99.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/3/eaax5343/DC1

Fig. S1. Temporal profiles of CH4, O2, and cyanobacterial derived chlorophyll a between July 2014 and 2016.

Fig. S2. Continuous measurements of CH4 and oxygen under light/dark cycles using a MIMS in July 2014 and 2016.

Fig. S3. Community composition of the cyanobacterial cultures as obtained when sequenced in triplicates at 09:30, half an hour after the first light was turned on (60 μmol quanta m⁻² s⁻¹).

Fig. S4. Temporal profiles of CH4, O2, and cyanobacterial derived chlorophyll a between July 2014 and 2016.

The 15 paired-end libraries generated from each experiment were trimmed for quality and sequencing adapters using Trimmomatic v0.39 (60). For purposes of annotation, two strategies were used. For the first approach, all trimmed sequences from a single culture were coassembled using Trinity v2.8.4 (61). The assembled transcripts were then annotated following the Trinotate pipeline (62), replacing BLAST1 with Diamond v0.9.22 (63). For the second approach, the trimmed reads from each sample were mapped to the genes of either Anabaena sp. PCC 7120 (GCA_000009705.1) or M. aeruginosa PCC 7806 (GCA_002095975.1). The full results of these experiments including differential expression will be eventually published separately.
showing the potential contribution of cyanobacteria in global CH$_4$ cycling.

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View/request a protocol for this paper from Bio-protocol.

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