

## MICROBIOLOGY

## 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 during oxygenic 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 (CH<sub>4</sub>) is the second most important anthropogenic greenhouse gas after CO<sub>2</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> emissions to the atmosphere average at ca. 560 Tg year<sup>-1</sup> (1 Tg = 10<sup>12</sup> g), exceeding the current estimated sinks by ca. 13 Tg year<sup>-1</sup> (2). Thus, to mitigate the constant increase in atmospheric CH<sub>4</sub>, a comprehensive understanding of global CH<sub>4</sub> sources and the environmental parameters that affect them is necessary.

Traditionally, biogenic methanogenesis is the formation of CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> by bacteria, has been extensively addressed in recent years with regard to the “methane paradox” (8, 9). Recently, Zheng *et al.* (10) have shown CH<sub>4</sub> formation by *Rhodospseudomonas palustris* during

N<sub>2</sub> fixation. Methane emission was also detected from cryptogamic covers, i.e., phototrophic assemblages on plant, rock, and soil surfaces (11).

Accumulation of CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> 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 CH<sub>4</sub> at substantial rates under light and dark and oxic and anoxic conditions.

## RESULTS AND DISCUSSION

To test the hypothesis that cyanobacteria produce CH<sub>4</sub> 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 <sup>13</sup>C-labeled sodium hydrogen carbonate (NaH<sup>13</sup>CO<sub>3</sub>) as carbon source. All investigated cyanobacterial cultures showed the CH<sub>4</sub> production with increasing stable isotope values (δ<sup>13</sup>C-CH<sub>4</sub> values), indicating that <sup>13</sup>C carbon was incorporated into CH<sub>4</sub>, whereas no <sup>13</sup>C enrichment occurred in the control experiments (Fig. 1). These results unambiguously show that cyanobacteria produce CH<sub>4</sub> per se and that the process is most likely linked to general cell metabolism. The different enrichment

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**Table 1. Cyanobacterial cultures used in this study and their growth conditions.** Cultures marked with an asterisk are fully axenic, while others are monoalgal. IBVF: Culture collection of the Institute for Plant Biochemistry and Photosynthesis, Sevilla Spain; CCALA: Culture collection of autotrophic organisms; HUJI: Laboratory of A. Kaplan, Hebrew University of Jerusalem, Jerusalem Israel; IOW: Laboratory of F. Pollehne, Leibniz Institute for Baltic Sea research, Warnemünde, Germany; MPI-MM: Max Planck Institute for Marine Microbiology, Bremen, Germany; IGB: Leibniz Institute of Freshwater Ecology and Inland Fisheries, Neuglobsow, Germany; University of Freiburg, Laboratory of Claudia Steglich, Freiburg University, Freiburg, Germany; Haifa University, Laboratory of D. Sher. Media source: BG11: (50); f/2: (51); YBCII: (65); AMP1: (52); filtered sea water (FSW)/artificial sea water Pro99: (52).

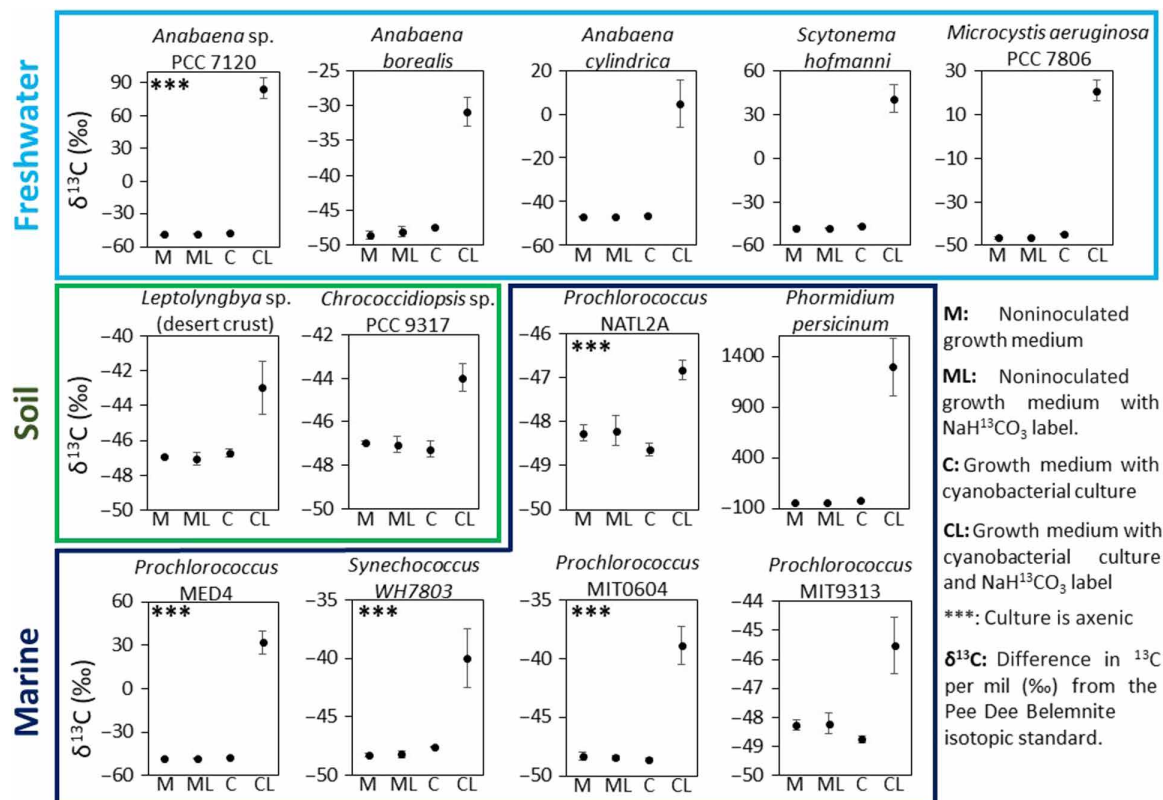
Strain name	Source	Morphology	Growth medium	Incubation temperature (°C)
<i>Anabaena</i> sp. PCC 7120*	IBVF	Filamentous	BG11	30
<i>Anabaena cylindrica</i> ATCC29414	IBVF	Filamentous	BG11	30
<i>Anabaena borealis</i>	CCALA	Filamentous	BG11	30
<i>Scytonema hofmanni</i> PCC 7110	IBVF	Filamentous	BG11	30
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

of <sup>13</sup>C indicated by δ<sup>13</sup>C-CH<sub>4</sub> 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<sub>4</sub> production were, in most cases, above saturation level (Fig. 2 and fig. S2); while methanogenic archaea were recently reported from oxic environments (21), their activity is attributed to anoxic microniches. Peters and Conrad (22) have additionally shown that while methanogens are abundant in oxic 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<sup>-1</sup>). 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. 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<sub>4</sub>. 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<sub>4</sub>. Nevertheless, the same study shows, although not focusing on cyanobacteria alone, that marine unicellular organisms, such as *Synechococcus* and *Crocospaera*, do not contain any detectable phosphonate storage.

Despite the recent finding of CH<sub>4</sub> production during N<sub>2</sub> fixation by *R. palustris* (10), we suggest that N<sub>2</sub> fixation is not the source of CH<sub>4</sub> production detected in our experiments. First, most cyanobacteria used in this study are unable (i.e., marine *Synechococcus*, *Prochlorococcus*,



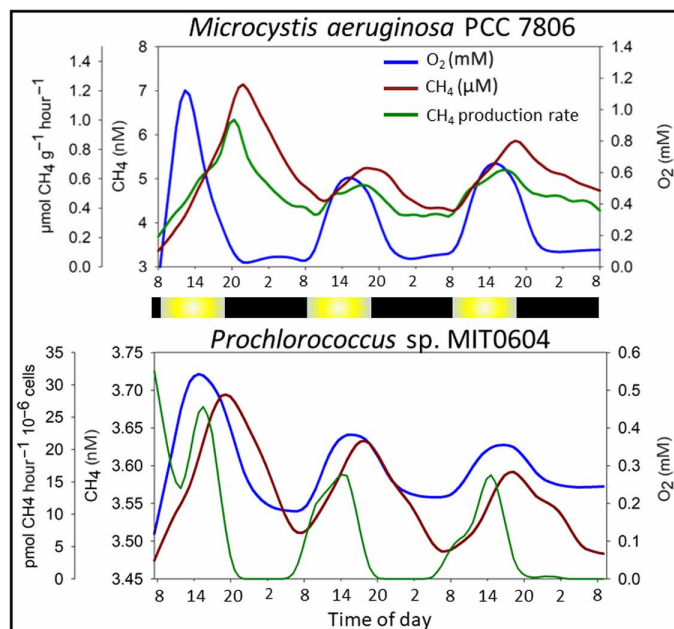
**Fig. 1.  $\delta^{13}\text{C}$ - $\text{CH}_4$  values measured during incubation experiments of 13 different filamentous and unicellular freshwater, soil, and marine cyanobacterial cultures with and without  $\text{NaH}^{13}\text{CO}_3$  supplementation.** All cyanobacterial cultures produced  $\text{CH}_4$ . Using  $\text{NaH}^{13}\text{CO}_3$  as carbon source (CL) resulted in increasing stable  $\delta^{13}\text{C}$ - $\text{CH}_4$  values as compared to the starting condition. This establishes the direct link between carbon fixation and  $\text{CH}_4$  production. The  $^{13}\text{C}$  enrichment is not quantitative and thus not comparable between cultures. Error bars represent SD ( $n = 4$ ).

and *M. aeruginosa*) or unknown (*Leptolyngbya* sp., *P. persicinum*) to fix  $\text{N}_2$ . Second, all experiments were conducted in  $\text{NO}_3^-$ - or  $\text{NH}_4^+$ -rich, fresh media; therefore,  $\text{N}_2$  fixation in capable cyanobacteria is likely to be inhibited to a certain degree (29). Thus, given the rapid and tight response of the  $\text{CH}_4$  production with the onset of light, we consider that the mechanism by which cyanobacteria readily convert fixed  $\text{CO}_2$  to  $\text{CH}_4$  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  $\text{CH}_4$  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  $\text{CH}_4$  formation. However, the exact biochemical pathway(s) involved in light-driven cyanobacteria-derived  $\text{CH}_4$  formation remains, so far, unknown and thus requires further investigation.

Patterns and rates of the  $\text{CH}_4$  production were investigated in 17 cultures (the above-mentioned 13 and additional 4) over several days of continuous measurement of  $\text{CH}_4$  concentration using a membrane inlet mass spectrometry (MIMS) system. Our measurements, lasting 2 to 5 days, showed that the  $\text{CH}_4$  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  $\text{CH}_4$ , rates and patterns were not consistent, particularly so for production during the periods of darkness. Often, a period with lower rates of the  $\text{CH}_4$  production was ob-

served between light and dark phases (Fig. 2 and fig. S2). The latter is evidenced as a decrease in  $\text{CH}_4$  concentration resulting from degassing of our incubation system. This suggests that different mechanisms may be involved in the  $\text{CH}_4$  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  $\text{CH}_4$  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  $\text{CH}_4$  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  $\text{CH}_4$  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  $\text{CH}_4$  production rates across cultures of larger cyanobacteria were in the range of  $0.1$  to  $3.4 \mu\text{mol g}^{-1} \text{hour}^{-1}$  in individual experiments and a mean of  $0.51 \pm 0.26 \mu\text{mol g}^{-1} \text{hour}^{-1}$ . Among the marine picophytoplankton, *Synechococcus* sp. exhibited low rates ranging between  $0.01$  and  $0.6 \text{ pmol CH}_4 \text{ per } 10^6 \text{ cells}$



**Fig. 2. Continuous measurements of CH<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> (2.5 and 2.1 nM for fresh water and seawater, respectively). Calculated CH<sub>4</sub> production rates account for the continuous emission of CH<sub>4</sub> from the incubation chamber for as long as the CH<sub>4</sub> 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  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  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.

hour<sup>-1</sup>, while *Prochlorococcus* cultures produced CH<sub>4</sub> at rates ranging from 0.8 to 110 pmol CH<sub>4</sub> per 10<sup>6</sup> cells hour<sup>-1</sup>. When compared to the production rates of typical methanogenic archaea, CH<sub>4</sub> production rates of freshwater, soil, and large marine cyanobacteria are three to four orders of magnitude lower than the CH<sub>4</sub> production rates noted for typical methanogenic archaea in culture under optimal conditions (oxygen free) but one to three orders of magnitude higher than the rates observed in eukaryotes (fig. S5). In our experiments, *Prochlorococcus* and *Synechococcus* cultures produced CH<sub>4</sub> only at light intensities above 20  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ; therefore, it is likely that only *Prochlorococcus* and *Synechococcus* communities in the upper water layers contribute to the oceanic CH<sub>4</sub> flux to the atmosphere.

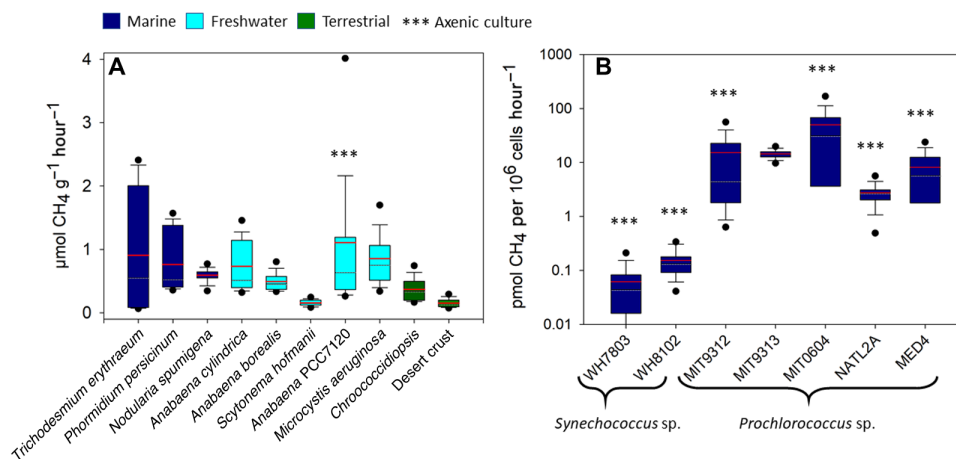
Methane production in oxic soils has been previously discussed and attributed mainly to abiotic factors (31) or methanogenic archaea (32), although the latter was thought unlikely (31, 33). Here, we show that a typical desert crust cyanobacterium (identified in this study as *Leptolyngbya* sp.), as well as the most common endolithic cyanobacterium *Chroococcidiopsis* (34), produces CH<sub>4</sub> both under light and dark conditions (Fig. 1 and fig. S2), thus inferring a new, but as yet, unknown and unaccounted for source of CH<sub>4</sub> from oxic soils.

Cyanobacteria are ubiquitous in nature, and their presence in aquatic systems is expected to increase with eutrophication and rising global temperatures (19). The “methane paradox” describing

the production of CH<sub>4</sub> 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<sub>4</sub> emitted from freshwater lakes can be produced in the oxic layer (36), with cyanobacteria often being associated with elevated CH<sub>4</sub> 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<sub>4</sub> 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  $3.4 \times 10^{27}$  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<sub>4</sub>, 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<sub>4</sub> production rates of high and low light *Prochlorococcus* species ( $\sim 9$  pmol CH<sub>4</sub> per 10<sup>6</sup> cells hour<sup>-1</sup>), which prevail in the upper 100 m of the oceans, and an average abundance of 10<sup>8</sup> cells liter<sup>-1</sup>, the gross daily CH<sub>4</sub> production by *Prochlorococcus* is  $\sim 22$  nM. This concentration is around one order of magnitude higher than concentrations of CH<sub>4</sub> found in seawater that is at atmospheric equilibrium ( $\sim 2$  nM). However, CH<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> via different pathways, likely related to their surroundings, makes them important to the present and future global CH<sub>4</sub> 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





**Fig. 3. Average CH<sub>4</sub> 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  $\mu\text{mol g dry weight}^{-1} \text{ hour}^{-1}$  and rates for the picocyanobacteria (B) are given in  $\text{pmol per } 10^6 \text{ cells per hour}^{-1}$ .

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 CH<sub>4</sub> emissions (46). According to Fletcher and Schaefer (47), the marked increase in atmospheric CH<sub>4</sub> concentrations since 2007 coupled to the decrease in  $\delta^{13}\text{C-CH}_4$  values presumably caused by the increase in <sup>12</sup>C-enriched biogenic CH<sub>4</sub> sources is difficult to explain with our current understanding of known CH<sub>4</sub> sources and sinks of the global CH<sub>4</sub> budget. This further highlights the need to fully understand the cyanobacterial contribution to the global CH<sub>4</sub> budget and to identify the isotopic signatures of the various CH<sub>4</sub> 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 CH<sub>4</sub> cycle such as during the great oxygenation event or even earlier when the conditions on Earth were more reductive favoring the CH<sub>4</sub> production.

Further research will elucidate the biochemical pathways of CH<sub>4</sub> formation in cyanobacteria and fully assess the environmental factors affecting it, clarifying its relevance for ecology and the global CH<sub>4</sub> budget throughout Earth history, how it might change in the future, and how this CH<sub>4</sub> 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,  $\geq 88\%$  transmission of photosynthetically active radiation spectrum), flushed three times, and crimp-closed (polytetrafluoroethylene-butyl septa and aluminum caps) without gas bubbles. Dissolved CH<sub>4</sub> 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 N<sub>2</sub>, split-less injection,

and detection at 140°C). Headspace CH<sub>4</sub> was converted to dissolved CH<sub>4</sub> 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 CH<sub>4</sub>, 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 <sup>13</sup>C labeling experiments, NaH<sup>13</sup>CO<sub>3</sub> (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 NaHCO<sub>3</sub>; 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 NaH<sup>13</sup>CO<sub>3</sub>, (iii) sterile medium with culture, and (iv) sterile medium with culture and NaH<sup>13</sup>CO<sub>3</sub>; 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  $\approx 30 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  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  $\delta^{13}\text{C-CH}_4$  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 CH<sub>4</sub> 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  $\delta^{13}\text{C}$  value of  $-23.64\%$  relative to Vienna

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  $-23.9 \pm 0.2\text{‰}$ ,  $-38.3 \pm 0.2\text{‰}$ , and  $-54.5 \pm 0.2\text{‰}$ . The average SDs ( $n = 3$ ) of the CF-IRMS measurements were in the range of 0.1 to 0.3‰. All  $^{13}\text{C}/^{12}\text{C}$  isotope ratios were expressed in the conventional  $\delta$  notation in per mil (‰) versus V-PDB using Eq. 1

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

### 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  $105^\circ\text{C}$  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 FACS Aria II flow cytometer (BD Biosciences, Heidelberg, Germany) at a flow rate of  $23.5 \mu\text{l min}^{-1}$  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  $\text{CH}_4$ . Atrazine (catalog number 45330-250MG-R, Sigma-Aldrich) has a solubility of  $33 \text{ mg liter}^{-1}$  ( $153 \mu\text{M}$ ) 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  $132 \text{ mg liter}^{-1}$  ( $410 \mu\text{M}$ ) 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 ( $0.1 \text{ mg ml}^{-1}$ ) 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  $\text{CH}_4(mz15)/\text{Ar}$  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  $18^\circ$  to  $30^\circ\text{C}$ , 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)

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

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

where  $R_{\text{degassing}}$  is the degassing rate in  $\text{mol CH}_4 \text{s}^{-1}$  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.

### Transcriptome experiment

Samples for transcriptome analyses were collected in triplicates at five time points during a 24-hour experiment with *Anabaena* sp. PCC 7120 and *M. aeruginosa* PCC 7806. For this experiment, a nonaxenic culture of *Anabaena* sp. PCC 7120 was used. The cultures were divided into 16 60-ml bottles. One of the bottles was connected to the MIMS as described above, while the remaining 15 were incubated under identical light and temperature conditions. The bottles were incubated for 24 to 36 hours of acclimation before T0 was collected on the day of the experiment. T1 was sampled at 09:30, half an hour after the first light was turned on ( $60 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). T2 was sampled at 12:30, half an hour after the third light was turned on ( $180 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). T3 was sampled at 20:00, half an hour after all lights were turned off. T4 was collected at 00:00. Collected samples were immediately filtered on polycarbonate filters (pore size,  $0.8 \mu\text{m}$ ; Millipore) and frozen at  $-80^\circ\text{C}$  until further processing.

### DNA and RNA extraction and sequencing

To evaluate the presence of methanogenic archaea in nonaxenic cultures, DNA was extracted as described by Nercissian *et al.* (57). The resulting DNA was sent for Illumina sequencing at MR DNA (Shallowater, TX) on a MiSeq platform ( $2 \times 300$  base pairs) using the Arch2A519F (CAGCMGCCGCGGTAA) and Arch1041R (GGCCATGCACCWCCTCTC) 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:  $94^\circ\text{C}$  for 3 min, followed by 30 cycles of  $94^\circ\text{C}$  for 30 s,  $56^\circ\text{C}$  for 40 s, and  $72^\circ\text{C}$  for 1 min, after which a final elongation step at  $72^\circ\text{C}$  for 5 min was performed. 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 SILVA 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  $25 \mu\text{l}$  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 (Epicentre) 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'-dA-tailed dsDNA fragments. In the adapter ligation step, dsDNA adapters were ligated to 3'-dA-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.

### Transcriptome analysis

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 BLAST 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.

### 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 (GGYGGTGTMGDDTTACMCARTA)/*mcrA*-reverse (CGTTCATBGCCTAGTTVGGRTAGT) (64) was used. The assay was performed on a qTOWER 2.2 instrument (Analytik Jena, Germany). Assays were performed in a  $15\text{-}\mu\text{l}$  volume containing  $7.5 \mu\text{l}$  of innuMIX qPCR MasterMix SyGreen (Analytik Jena),  $0.75 \mu\text{l}$  of each primer ( $10 \text{ pmol}/\mu\text{l}$ ),  $5 \mu\text{l}$  of ddH<sub>2</sub>O, and  $1 \mu\text{l}$  of template DNA. Assay conditions were  $95^\circ\text{C}$  initial denaturation for 5 min, 35 cycles of denaturation ( $95^\circ\text{C}$ ) for 30 s, annealing ( $60^\circ\text{C}$ ) for 45 s, and elongation ( $72^\circ\text{C}$ ) for 45 s, followed by melting curve analysis. Triplicate assays were performed for each sample, with template DNA concentrations of  $10 \text{ ng}/\mu\text{l}$ . A parallel standard curve of *mcrA* genes was recorded. The standard curve DNA fragment consisted of the *mcrA* gene of a *Methanomassiliicoccus* 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;  $R_2 > 0.99$ .

### 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 CH<sub>4</sub>, O<sub>2</sub>, and cyanobacterial derived chlorophyll a between July 2014 and 2016.

Fig. S2. Continuous measurements of CH<sub>4</sub> and oxygen under light/dark cycles using a MIMS in 17 different cyanobacterial cultures.

Fig. S3. Community composition of the cyanobacterial cultures as obtained when sequenced using archaea-specific primers resulting in 71,981 reads for PCC 7110, 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* of 200,000 commissioned reads.



Fig. S4. Effects of photosynthesis inhibitors on cyanobacterial CH<sub>4</sub> production.

Fig. S5. Average CH<sub>4</sub> production rates (μmol g dry weight<sup>-1</sup> hour<sup>-1</sup>) obtained from multiple long-term measurements (2 to 5 days) with a MIMS.

Fig. S6. Methane ionization pattern.

Fig. S7. Raw signal obtained from mass 15 (CH<sub>4</sub>) and mass 40 (Argon) plotted against the calculated solubility at different salinities at 30°C.

Table S1. Estimated global CH<sub>4</sub> production rates divided into various cyanobacterial habitats showing the potential contribution of cyanobacteria in global CH<sub>4</sub> cycling.

References (66–71)

[View/request a protocol for this paper from Bio-protocol.](#)

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