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Microbial methane production in oxygenated water column of an oligotrophic lake

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The prevailing paradigm in aquatic science is that microbial methanogenesis happens primarily in anoxic environments. Here, we used multiple complementary approaches to show that microbial methane production could and did occur in the well-oxygenated water column of an oligotrophic lake (Lake Stechlin, Germany). Oversaturation of methane was repeatedly recorded in the well-oxygenated upper 10 m of the water column, and the methane maxima coincided with oxygen oversaturation at 6 m. Laboratory incubations of unamended epilimnetic lake water and inoculations of photoautotrophs with a lake-enrichment culture both led to methane production even in the presence of oxygen, and the production was not affected by the addition of inorganic phosphate or methylated compounds. Methane production was also detected by in-lake incubations of lake water, and the highest production rate was 1.8–2.4 nM h−1 at 6 m, which could explain 33–44% of the observed ambient methane accumulation in the same month. Temporal and spatial uncoupling between methanogenesis and methanotrophy was supported by ﬁeld and laboratory measurements, which also helped explain the oversaturation of methane in the upper water column. Potentially methanogenic Archaea were detected in situ in the oxygenated, methane-rich epilimnion, and their attachment to photoautotrophs might allow for anaerobic growth and direct transfer of substrates for methane production. Speciﬁc PCR on mRNA of the methyl coenzyme M reductase A gene revealed active methanogenesis. Microbial methane production in oxygenated water represents a hitherto overlooked source of methane and can be important for carbon cycling in the aquatic environments and water to air methane ﬂux.

epilimnic methane peak | methanogens

Although methane makes up <2 parts per million by volume (ppmv) of the atmosphere, it accounts for 20% of the total radiative forcing among all long-lived greenhouse gases (1). In the aquatic environments, methane is also an important substrate for microbial production (2). The prevailing paradigm is that microbial methanogenesis occurs primarily in anoxic environments (3, 4). A commonly observed paradox is methane accumulation in well-oxygenated waters (2, 5), which is often assumed to be the result of physical transport from anoxic sediment and water (6–8) or in situ production within microanoxic zones (9–11). Two recent studies challenged this paradigm and suggested that microbes in oligotrophic ocean can metabolize methylated compounds and release methane even aerobically (12, 13). These claims are not without caveats, because the amounts of methylated compounds added [1–10 μM methylphosphonate (12) and 50 μM dimethyl sulfoxonipropionate (13)] were far higher than their environmental concentrations, and therefore, the ecological relevance remains obscure. Moreover, dissolved oxygen (DO) was not monitored during the long incubation (5–6 d), and the possibility that the experimental setups had become anoxic before methane production could not be dismissed. Despite the uncertainty, if microbial methane production can occur in oxygenated water, it will have profound implications for carbon cycling and climate.
bacteria (MOB) were not detectable in the epilimnion, whereas at 18 m, a single species of MOB type I (99% similarity to *Methyllobacter tundripaludum*) was detected. MOB type II were not detectable.

Repeated measurements in September of 2010 showed temporal regression of the methane peak at 6 m, decreasing to only 0.10 μM by September 12. The reverse temporal development was observed in 2011, when the methane peak at 6 m increased from 0.18 μM in May to 1.25 μM in June (Fig. S2). As in the previous year, the methane maxima in 2011 coincided with the thermocline and DO oversaturation. We detected the active methyl coenzyme M reductase A (*mcrA*) gene for methanogenesis based on mRNA at 6 m in May and June of 2011. The *mcrA* gene was also present at 18–20 m, but it was undetectable at 0 m in May (Table 1).

**Methane Production in Laboratory Incubation Experiments.** Laboratory incubation of unamended water samples from discrete depths in July showed positive methane production (Fig. 2C). Significantly higher production rates were observed when daytime water samples were incubated in the dark rather than when nighttime samples were incubated in the light. Water samples from the upper 8 m where cyanobacteria and green algae were abundant showed higher methane production rates (maximum = 3.40 nM h⁻¹), which corresponded with the high in situ methane concentrations (Fig. 2C). The water in the incubation bottles never became anoxic, and the DO was at 94–97% saturation at the end of the experiment. Despite the earlier reports (12, 13), addition of inorganic phosphate, methylphosphonate, or trimethylamine to lake samples was incubated in the light. Water samples from the upper 6 m in July showed positive methane production (Fig. 2C). Methane production was observed again in the second experiment (June 13) (Fig. 2B). The gross methane production rate varied significantly among water samples suspended at different depths but not between the light and dark treatments. The highest average production rates were at 1.2–1.8 nM h⁻¹ at 6 m. Methane production was observed again in the second experiment (June 15) at an even higher average rate of 2.4 nM h⁻¹ at 6 m (Fig. 2B) when in situ methane concentrations had further increased.

**Uncoupled Methane Oxidation in Laboratory Experiments.** When unamended Lake Stechlin surface water samples were incubated over a longer time, methane concentrations in all three replicates increased at an average rate of 52 nM h⁻¹ (Fig. S3). Two of the replicates were killed for DO measurement, which was at ≥80% saturation. Methane concentration in the remaining replicate subsequently decreased at a rate of 52 nM h⁻¹ (Fig. S3). Threshold-dependent methane oxidation was observed in another experiment. In unamended lake water and water with added methane and oxidation inhibitor, methane concentrations did not change significantly over time; in the sample augmented with >50 μM methane, methane oxidation proceeded at a rate of 0.19 μM h⁻¹ (Fig. S4).

**Enrichment Culture Experiments.** Microbial enrichment culture established from lake water was used to inoculate cultures of three photoautotrophs: (i) *A. flos-aquae* (SAG 31.87), (ii) axenic *Microcystis aeruginosa* (HUB W333), and (iii) a xenic Chlorella-like green alga from Lake Stechlin. In all cases, inoculation led to significantly higher methane production under well-oxygenated conditions compared with those conditions without the inoculum or the inoculum alone (Table S2). FISH revealed direct attachment of potentially methanogenic Archaea to the photoautotrophic cells (Fig. 3).

**Discussion**

Our study showed that epilimnetic methane oversaturation is a recurring phenomenon in Lake Stechlin during the stratified season. Similar temporal development of epilimnetic methane oversaturation has also been observed in Lake Constance, which was assumed to be caused by anoxic aggregates within the water column (11). Here, we showed positive methane production in both laboratory and in-lake incubation experiments with unamended lake water excluding any external methane sources. Although internal sources caused by microoxic zones could

**Table 1. Results of specific PCR on cDNA of the mcrA gene**

<table>
<thead>
<tr>
<th>Date</th>
<th>Sample (m)</th>
<th>PCR products</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 14, 2011</td>
<td>0</td>
<td>--/--</td>
</tr>
<tr>
<td>May 14, 2011</td>
<td>6</td>
<td>+/-</td>
</tr>
<tr>
<td>May 14, 2011</td>
<td>18</td>
<td>+/-</td>
</tr>
<tr>
<td>June 3, 2011</td>
<td>6</td>
<td>+/-</td>
</tr>
<tr>
<td>June 3, 2011</td>
<td>20</td>
<td>+/-</td>
</tr>
<tr>
<td>June 13, 2011</td>
<td>6</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Equal amounts (5 μL) of the respective PCR products were added to the 2% agarose gel together with negative and positive controls plus the DNA size standard. Samples were tested in parallels; presence (+) and absence (–) of PCR products are given for each parallel.
Gene transcription, and strong DO

was positively, not negatively, related to oxygen availability. Our results, therefore, provide direct empirical evidence of microbial methanogenesis. The methane production rate was highest at 6 m in both in-lake incubation experiments, consistent with the strong expression of the mcrA gene for methanogenesis (Table 1). Based on the ambient profiles, the methane concentration at 6 m increased from 0.59 to 1.25 mM between June 10 and 15, 2011 (Fig. S2), which is equivalent to an average accumulation rate of 5.5 nM h⁻¹. The estimated average production rates (1.8–2.4 nM h⁻¹ at 6 m) (Fig. 2B), therefore, suggest that in situ methane production could account for 33–44% of the observed methane accumulation at that depth in mid-June when assuming the total absence of methane oxidation.

In the presence of oxygen, methane production needs to be uncoupled from methane oxidation to produce the observed methane accumulation. Our longer-term incubation experiment with surface water showed that average methane increase rate during the first 51 h was the same as the average methane decrease rate in the subsequent 42 h. This finding suggests that, although methane oxidation could potentially balance methane production, there was a temporal uncoupling between the two processes, which could result from a time lag in growth of methanotrophs, inhibitory effects of initially high DO concentration (17), or a threshold methane concentration required by methanotrophs (18). Threshold-dependent methane oxidation was also supported by the additional experiment in which methane oxidation was detectable only in the water spiked with >50 μM methane, suggesting that methane oxidation only proceeds at an appreciable rate when the ambient methane concentration has reached a high level. In situ methane oxidation may also be inhibited by nitrate, nitrite, and ammonium released by grazing and microbial degradation of organic matter in water layers with high algal and cyanobacterial biomass (19).

Spatial uncoupling between methanogenesis and methanotrophy within the water column was supported by our molecular analysis. Methanogenic Archaea were present, but MOB were not detectable in the epilimnion in July of 2010 when methane oversaturation was observed. Active methanogens, especially at 6-m depth, were also confirmed by our mRNA data. MOB were detectable only below the thermocline (e.g., at 18- and 20-m depth), where ambient methane concentrations were low but mcrA gene expression was still detectable. This spatial separation between methanogenic Archaea and MOB could also contribute to the accumulation of methane in the epilimnion. Our field measurements showed that the methane peak at 6 m tended to develop in May to July and then decline in September (Fig. S2). This wax and wane pattern may be related to temporal changes in the abundance and activities of methanogens (production) and methanotrophs (consumption). Understanding what factors determine these temporal variations will be an important topic for future studies.

Several mechanisms have been proposed for methanogenesis in oxygenated waters (20): cleavage of methyl-esters by UV radiation, hypoxia-induced methane production in mitochondria,

The term aerobic methane production strictly means that the production process requires oxygen, which has not been shown in this study or earlier studies (12, 13); hence, we describe our results as methane production under oxygenated conditions.

Fig. 2. (A) Methane production by Lake Stechlin water collected from different depths in laboratory experiments in July of 2010. Excess methane was stripped by vigorous shaking before incubation. Daytime water samples were incubated in the dark, and nighttime water samples were incubated in the light (43 μmol photons m⁻² s⁻¹) for 8 h at room temperature. Final DO was 7.4–7.7 mg L⁻¹ (94–97% saturation). Methane production in dark incubation was significantly higher than production in light incubation (paired t test; n = 7, P = 0.022). Ambient methane concentration profiles are included for comparison. (B) Methane production by Lake Stechlin water during in-lake incubation experiments. Water was collected from different depths on June 13, 2011 and stripping of excess methane. Afterward, the water was used to fill up three sets of gas-tight bottles in triplicate (n = 3). One set was used as the light treatment, one set was wrapped in aluminum foil as the dark treatment, and one set was spiked with >10⁻⁶ M 2-bromoethanesulfonic acid (BES) to inhibit methanogenesis. The three sets of bottles were then suspended at their original depths for 8.5 h. At the end of the incubation, dissolved methane concentrations in the bottles were measured by headspace analysis. Gross methane production rates were calculated as the difference between the light or dark treatment and the BES treatment divided by the incubation time. Two-way ANOVA test indicated that methane production rate varied significantly with depth (n = 8, P = 0.0001) but not between the light and dark treatments (n = 8, P = 0.188). The in-lake incubation experiment was repeated on June 15 at the 6-m depth with light and BES treatments, and methane production was calculated in the same manner.

Fig. 3. Direct attachment of methanogenic Archaea (green, FITC-labeled oligonucleotide probe) to autotrophs (red, autofluorescence) observed by FISH. (Upper Left) A single Chlorella-like algal cell. (Right) A colony of Chlorella-like green alga. (Lower Left) A filament of the cyanobacterium A. flos-aquae.
methane release by organisms under oxidative stress, and microbial decomposition of methylated compounds. In oligotrophic lakes such as Lake Stechlin, UV penetration is limited to <2.0 m (21), much shallower than the observed methane peak. UV was also absent in our laboratory experiments where methane production was observed. The overlapping between the methane peak and the oxygen peak in situ suggests that hypoxia is not a factor. It is also unlikely that epilimnetic organisms would suffer from strong oxidative stress, because they frequently experience high oxygen concentration and have multiple mechanisms to cope with it (22). Although conversion of methylated compounds such as methanethiol to methane can be bioenergetically favorable for methanogenic Archaea (13), neither methanethiol nor other methylated compounds are commonly found in high concentrations in oxygenated epilimnion in lakes (23–25). In our experiments, the addition of neither inorganic phosphate nor methylated compounds affected methane production; hence, pelagic methanogenesis in Lake Stechlin did not seem to depend on phosphate or methylated substrates like other systems (12, 13).

The alternatives are hydrogenotrophic and aceticlastic methanogenesis, both of which are common among methanogenic Archaea (26). The Archaea in Lake Stechlin belong to groups that have the ability to perform hydrogenotrophic or aceticlastic methanogenesis (27–29). Many photoautotrophs can produce H2 through both direct and indirect photolysis (30). Cyanobacteria can also increase H2 yield through nitrogen-fixing activities at night (31). Indeed, the slightly higher methane production rates in our dark vs. light incubations (Fig. 2A) suggest that H2 production through intracellular fermentation using storage photosynthates (32) or nitrogen fixation in the dark may be particularly important for pelagic methanogenesis in Lake Stechlin. Contrary to common belief, many methanogens can tolerate and survive oxygen exposure (33), which helps explain their methanogenic activity within the water column. It has been shown that Methanosarcina and Methanococcales in desert soils can transcribe the merA gene even under oxic/oxygenic conditions and at the same time, actively transcribe the gene for oxygen-detoxifying catalase (34). It is also noteworthy that particle-associated Methanoseta were present in the epilimnion in Lake Stechlin (Fig. S1). This group of methanogens is known to tolerate oxygen exposure (35) and is particularly well-adapted to low acetate environments (28). The co-occurrence of high numbers of cyanobacteria, algae, and attached Archaea within the epilimnion in our study may enable a direct transfer of H2 or acetate from the autotrophs to the methanogenic Archaea to support methane production. Although our field observations suggest that, in Lake Stechlin, this methane production was closely associated with cyanobacteria, our laboratory experiments confirmed that methane production could also be supported by other photoautotrophs in the presence of methanogenic Archaea (Table S2). Given the ubiquitous distribution of Archaea and photoautotrophs in the aquatic environments, this methane production process may be more widespread than previously recognized.

Inverse modeling has been used to explain the observed atmospheric methane concentrations by constraining the uncertainties in known sources and sinks of methane, but this approach does not consider methane production in oxygenated water bodies (36). It is estimated that freshwater environments contribute >70% of the natural source of methane to the atmosphere (37). Because of the traditional emphasis on anaerobic methanogenesis, this methane is assumed to originate from anoxic sediments and bottom waters (38). Our study provides compelling evidence that microbial methane production can also take place within the oxygenated water and is high enough to contribute substantially to epilimnetic methane accumulation. Quantifying the fate of this methane source—whether through water to air flux or carbon cycling within the water column—may improve our understanding of the global methane budget and climate.

Materials and Methods

Study Site. Lake Stechlin is a dimictic oligotrophic lake in northeastern Germany (53° 10′ N, 13° 02′ E) with low anthropogenic impact (39). The lake has been continuously studied for almost 50 y, and it serves as a reference lake for the European Water Framework Directive. The lake has a maximum depth of 69.5 m and an area of 4.3 km², with a hypolimnetic oxygen saturation level of up to 60%.

In Situ Methane and Hydrographical Profiles. Water samples for dissolved methane were taken in 2-m depth intervals filled into 120-mL preweighed gas-tightrimp bottles. Within 1–2 h after sampling, dissolved methane was determined by headspace analysis (40) on a gas chromatograph with flame ionization detector (Shimadzu). Concurrent DO and water temperature were measured with an in situ probe (Wissenschaftlich Technische Werkstätten). CH4 profiles were measured by a fluorescence probe (Hardt). Attached and free-living microbes were operationally separated by a 5.0-μm polycarbonate membrane (Table S3) measured by DAPI direct count (41) at 1,000× magnification with an epifluorescence microscope (Zeiss).

Laboratory Incubation Experiments. Laboratory incubation experiments were conducted to test for methane production in July of 2010 (Fig. 2B). To test for the effects of inorganic phosphate and methylated compounds, surface water samples were incubated with or without the addition of 3 μM inorganic phosphate, 1 μM methylphosphonate, or 1 μM trimethylamine, and the methane concentrations were assessed over 48 h (Fig. 2C). The test for temporal decoupling between methanogenesis and methanotrophy, methane concentrations in triplicate bottles of unamended surface lake water samples were monitored over a longer time, and the average rate of methane increase in the first 51 h was compared with the average rate of methane decrease in the next 42 h (Fig. S3). To test for threshold-dependent methane oxidation, surface lake water samples were incubated for 58 h with added methane or methane plus the inhibitor difluoromethane to prevent methane oxidation (42). Unamended lake water sample was used as the control (Fig. S4).

In-Lake Incubation Experiments. The first in-lake incubation experiment was conducted on June 13, 2011 to test for methane production under near in situ condition. Water samples collected from different depths were divided into excess methane by shaking and incubated at the original depth with light, without light, or with added 2-bromoethane sulfonic acid (BES; final concentration >10−4 M) to inhibit methanogenesis (43). Gross methane production rates were calculated as the difference between the light or dark treatment and the BES treatment divided by the incubation time. The experiment was repeated on June 15 at 6-m depth with light and BES treatments (Fig. 2).

Enrichment Culture Experiments. A microbial enrichment culture was established by inoculating Lake Stechlin water with Z-Medium at 26 °C and 72 umol photon m−2 s−1 with a 16:8 light:dark cycle. The enrichment culture was monovoluminated in monochromatic light (chlorophyll-specific cultured under N-containing Z-Medium in a microorganism beads (44). The reaction mixtures for PCR amplification contained 2 μl template DNA, 200 nM each of the appropriate primers (Table S3), 250 μM each deoxyribonucleoside triphosphate, 3 mM MgCl2, 5 μl 10× PCR buffer, and 0.5 U BIOTAQ Red DNA polymerase (Bioline) in a total volume of 50 μl. Cloning of partial 16S rRNA genes was done using primers specific for Archaea including methanogenic Archaea (Table S3) and the pGEM-T–vector system II (Promega) according to the manufacturer’s protocol. Insert length was checked by primers T7 and SP6. Of each clone library, 20–30 clones were picked and sequenced. PCR products of clones were purified using an established protocol (45). Sequences are deposited in GenBank.
under the accession numbers JF510050–JF510160. To test for active methanogenesis as indicated by mRNA expression, samples for RNA extraction were taken in May and June of 2011; 1 L water from selected depths (0, 6, 18, 60 m) was filtered over a PES filter (Millipore). The samples were stored at −80°C. Extraction of DNA and RNA was used an established protocol (46). DNA was then removed by digestion with a Turbo DNA-Free Kit (Ambion) using the manufacturer’s instructions. The digestion was proofed by a PCR with universal primers. cDNA synthesis from extracted RNA was performed with random oligo(dT) primers and the Array Script (Ambion) using the manufacturer’s instructions. Specific primers (Table S3) were then used in a nested PCR approach to target cDNA from mRNA for mcrA. PCR conditions were the same as above. For FISH, photoautotrophic cells were embedded on slides (47) and fixed with 70% ethanol for 1.5 min. Hybridization was done for 2 h using the probe MG3 for predominantly methanogens (48) and used here to target potentially methanogenie Archaea. The probe was labeled with fluorescein-isothiocyanate (FITC). Afterward, hybridized cells were analyzed with an epifluorescence microscope using the FITC filter set (Leica).

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