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Effects of algal and terrestrial carbon on methane production rates and methanogen community structure in a temperate lake sediment

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SUMMARY

1. Sources of atmospheric CH_4 are both naturally occurring and anthropogenic. In fact, some anthropogenic activities may influence the production of CH_4 from natural sources, such as lakes.
2. Ongoing changes in the catchment of lakes, including eutrophication and increased terrestrial organic carbon export, may affect CH_4 production rates as well as shape methanogen abundance and community structure. Therefore, inputs from catchments to lakes should be examined for their effects on CH_4 production.
3. We added algal and terrestrial carbon separately to lake sediment cores and measured CH_4 production. We also used quantitative polymerase chain reaction and terminal restriction fragment length polymorphism to determine the effects of these carbon additions on methanogen abundance and community composition.
4. Our results indicate that CH_4 production rates were significantly elevated following the addition of algal biomass. Terrestrial carbon addition also appeared to increase methanogenesis rates; however, the observed increase was not statistically significant.
5. Interestingly, increased CH_4 production rates resulted from increases in per-cell activity rather than an increase in methanogen abundance or community compositional shifts, as indicated by our molecular analyses.
6. Overall, anthropogenic impacts on aquatic ecosystems can influence methanogenesis rates and should be considered in models of global methane cycling and climate.

Keywords: community, eutrophication, greenhouse gases, lakes, profundal

Introduction

Methane (CH_4), a potent greenhouse gas, continues to increase in concentration in our atmosphere. Current atmospheric CH_4 concentrations of 1774 ppbv are more than double pre-industrial levels of 350–800 ppbv (Loulergue *et al.*, 2008). Freshwater ecosystems may contribute up to 103 teragrams of CH_4 per year, 6–16% of global CH_4 emissions, and are not currently included in global greenhouse gas budgets (Bastviken *et al.*, 2004, 2011). To predict, and perhaps mitigate, future increases in atmospheric CH_4, we must understand factors that regulate the contribution of inland waters to the global CH_4 cycle and how these factors will change under future global change scenarios.

It is well accepted that lake-dwelling organisms and the processes they mediate are sensitive to anthropogenic activities in the surrounding catchment. Therefore, it is to be expected that aquatic methanogenic archaea, and methanogenesis rates, will be influenced by catchment change. Two pervasive and ongoing catchment-derived impacts on lakes are the transport of nutrients and organic matter (Schindler, 1974; Monteith *et al.*, 2007). Critically evaluating how these important drivers of global change in aquatic ecosystems, eutrophication and 'browning' (Roulet & Moore, 2006) may impact lake CH_4 dynamics is

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an important component of global climate change predictions.

Excess nutrient run-off from non-point sources into receiving aquatic systems has been an environmental concern for decades (Edmondson & Lehman, 1981; Smith, 2003; Bosch *et al.*, 2009). Lakes as well as other aquatic systems receiving subsurface and surface run-off of the limiting nutrient phosphorus are characterised by accelerated eutrophication (Sharpley *et al.*, 1994; Schindler *et al.*, 2008). Subsequent sinking and decay of algal blooms creates a nutrient-rich anoxic environment suitable for fermentation and the production of acetate and H_2/CO_2. These organic precursors, without biogeochemical competition, can be anaerobically reduced to CH_4 by methanogens (Conrad, 1999). As eutrophication, caused by anthropogenic sources, probably provides a greater supply of substrate for methanogenesis, we anticipate increased CH_4 production rates within lakes under eutrophic conditions.

Environmental change not only increases nutrient concentration in lakes, it may also result in an increase in terrestrial dissolved organic carbon (DOC) in many aquatic ecosystems. In Europe and North America, freshwater ecosystems have experienced increased DOC leaching from soil (Hejzlar *et al.*, 2003; Worrall *et al.*, 2004; Monteith *et al.*, 2007). Elevated export of DOC from upland soils over the past two decades is thought to have resulted from decreased soil acidity and ionic strength following policy changes for fossil fuel combustion and sulphur emissions in the 1990s (Monteith *et al.*, 2007). Increased soil pH and lower ionic strength enhance the solubility and mobility of organic acids in soil, which allows for greater DOC export to surface waters (Driscoll *et al.*, 2003; Evans *et al.*, 2006). However, other studies have hypothesised that climate change (Freeman *et al.*, 2001, 2004; Worrall, Burt & Shedden, 2003), nitrogen deposition (Findlay, 2005) and land use change (Garnett, Ineson & Stevenson, 2000) all have contributed to increased DOC export to lakes. Regardless of the reason, terrestrial organic carbon concentrations within lakes are increasing, and these terrestrial subsidies can be a source of energy for microbial populations (Bergstrom & Jansson, 2000; Kritzberg *et al.*, 2004), perhaps including methanogens.

Observed trends in lake trophic status around the globe suggest that many lakes are changing towards a more eutrophic or humic state (Vollenweider, 1989; Smith, 2003; Monteith *et al.*, 2007). Here, we ask whether we can expect these primarily human-driven changes to affect surface water contributions to the global CH_4 cycle. To evaluate whether algal or terrestrial carbon inputs could enhance CH_4 production rates, we conducted a laboratory-scale manipulation of carbon substrate supply to anoxic lake sediments. Any change in methanogenesis in response to substrate amendment would result from changes in the methanogenic archaeal community in the lake sediments. The enhanced methanogenic activity could be mediated by one or more scenarios of methanogen community change: (i) increased abundance of existing methanogens, (ii) increased per-cell activity of existing methanogens, but no change in abundance or (iii) shifts in methanogen community composition to a more efficient or active set of organisms. To assess what scenario might accompany any changes in methanogenesis activity in response to our carbon substrate supply manipulations, we used a combination of quantitative polymerase chain reaction (qPCR) and terminal restriction fragment length polymorphism (T-RFLP), targeting a gene integral to the methanogenesis biochemical pathway (*mcrA*).

Methods

Sampling site

Diamond Lake is an oligotrophic lake (DOC = 5.3 mg L^{-1}, TP = 23.7 μg L^{-1} and pH = 8.6) located in southwest Michigan, U.S.A. (41.9°N; 85.9°W). On 29 September 2010, we collected nine replicate intact sediment cores, including overlying water, with a Kajak–Brinkhurst corer (Wildlife Supply Co., Yulee, FL, U.S.A.) at the deepest point in the lake and returned them to the laboratory. Prior to collection of our cores, we measured profiles of water temperature, dissolved oxygen and CH_4 concentrations.

Core amendments, incubations and CH_4 measurements

All sediment cores contained approximately 200 mL of water, 600 mL of sediment and 100 mL of headspace, which was purged with N_2 gas. The nine cores were divided evenly into two treatments (algal and terrestrial) and a control. We added 7 g m^{-2} of dried *Scenedesmus obliquus* (Turpin) Kützing 1833 to the three algal cores. Each of the terrestrial cores was amended with 7 g m^{-2} of dried, ground maple leaves; for this purpose, freshly fallen maple leaves were collected and then dried at 60 °C. After drying, the leaves were ground to a particle size comparable to algal cells using a Thomas Wiley Mini-mill with size 60 mesh (Thomas Scientific, Swedesboro, NJ, U.S.A.) and a Wig-L-Bug grinding mill (Dentsply Rinn Corp., York, PA, U.S.A.). Although freshly fallen leaves are probably of higher quality than most terrestrial particulate carbon reaching lake sediments, we chose

fresh leaves because they are more comparable to the algal additions in terms of quality. The three control cores were treated in a similar manner to the algal and terrestrial cores, but no additional carbon substrate was supplied. The cores, sealed with a rubber stopper fit with valved sampling ports, were incubated at about 15 °C in the dark for 2.5 weeks. Approximately every 4 days, a 5-mL sample was taken from each core's headspace and replaced with 5 mL of N_2. The CH_4 concentration in each sample was measured with an Agilent 6890 Gas Chromatograph equipped with a flame ionising detector, using a GS carbon plot column with a length, diameter and filter size of 30 mm, 0.32 mm and 3.0 μm, respectively (Agilent Technologies, Santa Clara, CA, U.S.A.). CH_4 concentration increased linearly during the entire 2.5-week incubation in the nine sediment cores. Methanogenesis rates were inferred from the slope of linear regression fits to the time courses of CH_4 concentrations.

Molecular analyses

Following 2.5 weeks of incubation, overlying water and sediments were collected for DNA extractions. Filtered planktonic biomass and sediment DNA extractions were performed according to manufacturer's protocol with a Mo-Bio DNA power soil kit (Mo Bio, Carlsbad, CA, U.S.A.). Extracted DNA served as template for quantitative PCR (qPCR) targeting the alpha subunit of methyl coenzyme reductase (*mcrA*). Many previous studies have used *mcrA* as a genetic marker to determine methanogen abundance as well as community composition (Luton *et al*., 2002; Earl *et al*., 2003; Freitag *et al*., 2010; Milferstadt, Youngblut & Whitaker, 2010). This gene is an excellent marker as all known methanogens possess methyl coenzyme reductase, which is responsible for the conversion of a methyl group to CH_4 (Grabarse *et al*., 2001).

mcrA was amplified in a 20-μL qPCR in an ep gradient S realplex^2 master cycler (Eppendorf, Hauppauge, NY, U.S.A.), using SYBR Green as the reporter dye. Each reaction contained 1 μL of 1/100 diluted Diamond lake DNA template, 1× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.), 0.25 μM of each primer targeting *mcrA*: mcrAqF (5′-AYGGTATGGARCAGTACGA-3′) and mcrAqR (5′-TGVAGRTCGTABCCGWAGAA-3′) (W.E. West and S.E. Jones, in prep.). Thermocycling conditions for the *mcrA* qPCR were as follows: an initial denaturation at 94 °C for 1 min, followed by 40 cycles of 94 °C denaturation for 40 s, 54 °C annealing for 30 s, 72 °C elongation for 30 s and a fluorescent detection at 85 °C for 20 s. Melting curves were run to ensure absence of non-specific amplification. Amplification, fluorescence data collection and initial data analysis were all performed by the Eppendorf realplex^2 software (Eppendorf).

An environmental *mcrA* clone (TOPO TA cloning kit; Invitrogen, Grand Island, NY, U.S.A.) was used to construct a standard curve for our quantitative PCR. To obtain high concentrations of a portion of the *mcrA* gene containing our qPCR target, we amplified the *mcrA* gene insert and vector tag ends using *M13* vector primers. Each 25-μL PCR contained 1 × 1.5 mM $MgCl_2$ PCR buffer (Idaho Technology Inc., Salt Lake City, UT, U.S.A.), 0.5 mM dNTPS, 1 unit of Taq, 1 μM of M13F (5′-GTAAAACGACGGCCAG-3′) and 1 μM of M13R (5′-CAGGAAACAGCTATGAC-3′). Thermocycling conditions for the *M13* PCR were as follows: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C denaturation for 35 s, 55 °C annealing for 45 s, 72 °C elongation for 2 min, followed by a final elongation at 72 °C for 2 min. After amplification, the PCR product was cleaned using a Purelink PCR clean up kit (Invitrogen) as per manufacturer's instructions. After the clean-up, the PCR product was quantified using Invitrogen's Qubit technology (Invitrogen).

Terminal restriction fragment length polymorphism PCR was run in an ep gradient pro realplex^2 master cycler (Eppendorf) and contained 1 μL of 1/100 diluted DNA sample from an incubated core, 1 × 1.5 mM $MgCl_2$ PCR buffer (Idaho Technology Inc.), 0.4 mM dNTPS, 1 unit of Taq, 0.2 μM mcrA-FAM-labelled (5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′) and 0.2 μM mcrA-R (5′-TTCATTGCRTAGTTWGGRTAGTT-3′) (Luton *et al*., 2002). Thermocycling conditions for the *mcrA* PCR were as follows: an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C denaturation for 1 min, 53.5 °C annealing for 1.5 min, 72 °C elongation for 1.5 min, followed by a final elongation at 72 °C for 10 min. Each T-RFLP PCR was followed by a precipitation clean-up with ethanol. After a precipitation clean-up, 1× Buffer E (Promega, Madison, WI, U.S.A.), 5 units of TaqI restriction enzyme and 0.2 μL of 10 mg mL^{-1} BSA were added to each purified FAM-labelled PCR product for a 25-μL total digestion volume. Enzyme digestion was performed at 65 °C for 2 h. Each digestion sample was then cleaned up with an ethanol precipitation, and the University of Notre Dame Genomics Core conducted fragment analysis with an ABI 3730xl DNA analyser (Applied Biosystems, Carlsbad, CA, U.S.A.).

Statistical analysis

One-way ANOVA was used to test the significance of our carbon addition treatments on CH_4 production rates. A

post hoc test, Tukey's multiple comparisons of means with a 95% family-wise confidence level, was performed to compare mean CH_4 production rates from carbon-amended cores. A two-way ANOVA was used to investigate the influence of our treatments and water versus sediment effects on *mcrA* $\log_{10}$ gene abundance. Non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) were used for visualisation and to test for treatment effects on methanogen community composition. Significance of the ANOSIM statistic was evaluated using a 1000 permutations of the pairwise Bray–Curtis dissimilarity matrix. All statistical analyses were conducted in the R statistical environment using the base and vegan packages (R Development Core Team, 2008).

Results

Diamond Lake profile

Diamond Lake is a seasonally stratified lake. However, during our sampling, the lake had experienced autumn turnover. Thus, we observed a relatively consistent dissolved oxygen and temperature profile (Fig. 1). Methane concentrations ranged from 5 to 7.6 μM from the surface to 11 m. A sharp increase in CH_4 concentrations was observed below 11 m (Fig. 1).

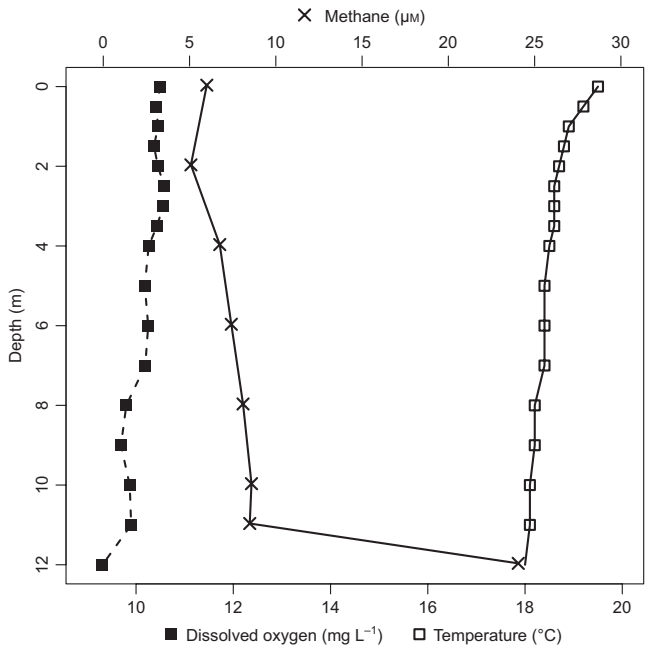

Fig. 1 Diamond Lake depth profile of dissolved oxygen, CH_4 concentrations and water temperature (°C). Methane concentrations (solid line and x points) are indicated on the upper horizontal axis. Temperature and dissolved oxygen are marked with open and closed squares and are associated with the lower horizontal axis.

CH_4 production rates

Overall, we observed significant effects of carbon addition on CH_4 production rates (one-way ANOVA, $F = 32.5$, $P = 0.007$, d.f. = 2, 6). Algal cores had the highest CH_4 production rate during the incubation period and were significantly higher than control cores and those receiving terrestrial carbon (Fig. 2). The average CH_4 production rate of the terrestrial cores was higher than the control, but not significantly different (Fig. 2).

Methanogen abundance and community composition

All sediments and overlying water contained *mcrA* at the end of our incubations. Per-gram sediment concentrations of *mcrA* were significantly higher than overlying water *mcrA* concentration (per millilitre) by about 100-fold (two-way ANOVA, $F = 17.7$, $P = 0.001$, d.f. = 1, 12; Fig. 3). However, there was not a significant difference in *mcrA* gene copies between algal, terrestrial and control sediment cores in either water or sediment samples (two-way ANOVA, $F = 0.22$, $P = 0.80$, d.f. = 2, 12).

Terminal restriction fragment length polymorphism revealed nine different operational taxonomic units (OTU) in the incubated cores. Analysis of similarity revealed no effect of treatment on *mcrA* composition within sediment samples (ANOSIM, $R = 0.05$; $P = 0.35$). A single *mcrA* OTU was observed in all overlying water samples.

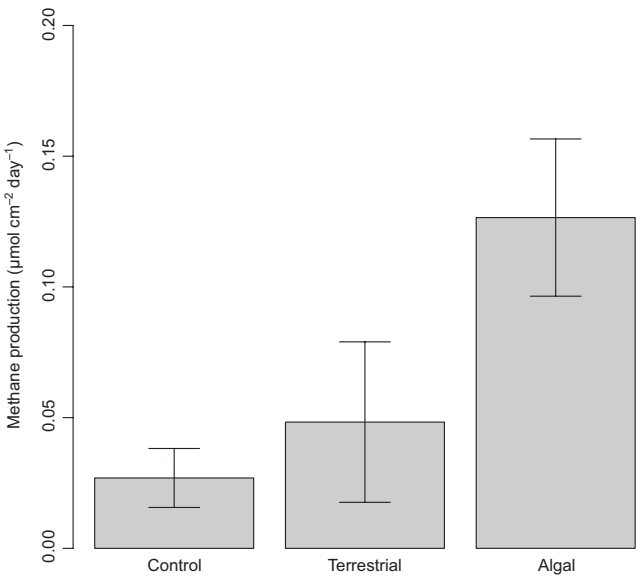

Fig. 2 Mean CH_4 production rates of Diamond Lake sediment cores with added algal biomass, added terrestrial carbon and control (one-way ANOVA, $F = 12.5$, $P = 0.007$, d.f. = 2, 6). Error bars indicate one standard deviation of the mean.

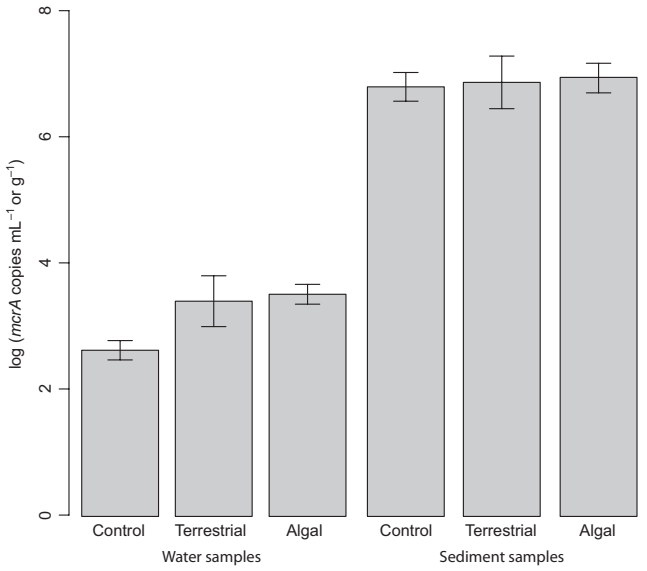

Fig. 3 *mcrA* copies of carbon treatments in sediment (per gram) and overlying water samples (per millilitre). Error bars represent 1 standard deviation. Methanogen abundance was not affected by algal or terrestrial carbon addition. (two-way ANOVA, $F = 0.22$, $P = 0.8$, d.f. = 2, 12). However, methanogen abundance in sediments was significantly higher than in the overlying water (two-way ANOVA, $F = 17.7$, $P = 0.001$, d.f. = 1, 12).

Discussion

Increased nutrient and terrestrial carbon loading have substantial impacts on aquatic ecosystems and drive lakes towards more eutrophic and humic states, respectively (Vollenweider, 1989; Sharpley *et al.*, 1994; Monteith *et al.*, 2007). Microbial populations and their processes may be sensitive to changes in lake trophic status (Lilkanen & Martikainen, 2003). Thus, increased concentrations of algal or terrestrial carbon may stimulate microbial growth and transcriptional activity. Our goal was to determine whether these carbon sources enhance CH_4 production rates and what sort of methanogen community change enables an increase in methanogenesis. We observed that algal biomass rapidly enhanced methanogenesis rates when compared to the control (Fig. 2). However, methanogen abundance based on *mcrA* remained similar amongst the incubations. This suggests per-cell transcription of cellular machinery required for the production of methane, and therefore, methanogenesis activity was probably higher in incubations with increased algal biomass.

It has been previously suggested that DOC sources (terrestrial versus algal) may impart selective forces on bacterial community composition (Newton *et al.*, 2006; Jones, Newton & McMahon, 2009). However, in our study, there was no shift in methanogen community composition in response to algal and terrestrial carbon amendments. The lack of change in methanogen community composition amongst algal and terrestrial cores may result from the limited diversity of metabolic substrates used by methanogens (primarily acetate and H_2/CO_2) (Deppenmeier, Müller & Gottschalk, 1996; Roy & Conrad, 1999). The fermentative processes that produce these precursors may negate the differences between algal and terrestrial carbon. Thus, it may be that changes in community composition occur at the level of the fermentative bacterial community and not within methanogen communities. Further experimentation is needed to investigate the role of fermenter community composition in controlling substrate availability for methanogens.

A similar increase in CH_4 production was observed in cores and slurries amended with algal biomass of *Peridinium gatunense* Nygaard 1925 from Lake Kinneret (Schwarz, Eckert & Conrad, 2008). However, their data suggest that 16s rRNA-based community composition changed significantly after 6 days of incubation with additional algal carbon. It is possible that differences in methodology can account for the apparent disagreement between our results. For example, we carried out qPCR and T-RFLP based on the *mcrA* gene, whereas their T-RFLP was carried out using 16s rRNA. These two loci may provide different taxonomic resolutions. However, previous studies have demonstrated that *mcrA* is a sensitive genetic marker for methanogen community composition and phylogenetic studies (Luton *et al.*, 2002; Earl *et al.*, 2003; Milferstadt *et al.*, 2010). In addition, the use of general bacterial and archaeal primers by Schwarz *et al.* (2008) seems to have detected changes in non-methanogenic components of the community, perhaps including fermenters. Nonetheless, both the Schwarz study (2008) and our findings confirm that CH_4 production increases with algal amendments, and both studies suggest that eutrophication of lakes on regional and global scales may contribute to the global CH_4 budget. However, we suggest that shifts in methanogen community composition and abundance (Fig. 2) do not account for increased methanogenesis rates.

Many bacterial communities preferentially utilise more labile algal carbon than recalcitrant terrestrial carbon because it promotes higher growth efficiencies (Kritzberg *et al.*, 2004, 2005). Additionally, other work has demonstrated the influence of resource quality on CH_4 production in sediments (Kankaala, Kaki & Ojala, 2003). A rapid response of CH_4 production to algal carbon addition probably reflects the high lability of algal biomass (Updegraff *et al.*,1995). The relatively recalcitrant nature of terrestrial carbon may lead to slower mineralisation by fermenters and a reduced, but perhaps extended, metha-

nogenesis response. Our data are in line with the possibility of this hypothetical dichotomy in lability and timing of enhanced methanogenesis whilst using algal and terrestrial carbon substrate. Labile algal carbon was readily fermented, resulting in significant increases in methanogenesis over the 2.5-week incubation. In comparison, terrestrial carbon sustained a lower increase in methanogenesis that may have persisted for a longer period of time. Alternatively, fermenters may utilise terrestrial carbon at a lower efficiency, and subsequently, a lower supply of substrate may be available for methanogens to use. In future, extended incubation periods would enable a test of this interesting hypothesis.

Overall, our study suggests that algal carbon sources are readily fermented into substrates that are reduced by methanogens, resulting in an increase in CH_4 production. Consequently, eutrophication may lead to higher CH_4 production rates on a whole-lake scale. Globally, more lakes are becoming vulnerable to the effects of anthropogenic nutrient loading (Smith, 2003), and eutrophication could play a major role in global CH_4 budgets. However, further investigation, at appropriate scales of inference, is needed to account for complex feedback that can be limited in microcosm studies. Future studies should focus on eutrophication and humic state dynamics within lakes on larger, and potentially longer, scales to examine the effects of nutrients and terrestrial DOC directly as well as indirectly on microbial biogeochemical interactions, CH_4 production and net CH_4 flux.

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