

**This is an electronic reprint of the original article.  
This reprint *may differ* from the original in pagination and typographic detail.**

**Author(s):** Rissanen, Antti; Karvinen, Anu; Nykänen, Hannu; Peura, Sari; Tirola, Marja; Mäki, Anita; Kankaala, Paula

**Title:** Effects of alternative electron acceptors on the activity and community structure of methane-producing and -consuming microbes in the sediments of two shallow boreal lakes

**Year:** 2017

**Version:**

**Please cite the original version:**

Rissanen, A., Karvinen, A., Nykänen, H., Peura, S., Tirola, M., Mäki, A., & Kankaala, P. (2017). Effects of alternative electron acceptors on the activity and community structure of methane-producing and -consuming microbes in the sediments of two shallow boreal lakes. *FEMS Microbiology Ecology*, 93(7), fix078.  
<https://doi.org/10.1093/femsec/fix078>

All material supplied via JYX is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.

**Effects of alternative electron acceptors on the activity and  
community structure of methane-producing and -consuming  
microbes in the sediments of two shallow boreal lakes**

Antti J. Rissanen<sup>1,3,\*</sup>, Anu Karvinen<sup>2</sup>, Hannu Nykänen<sup>3,4</sup>, Sari Peura<sup>3,5</sup>, Marja  
Tirola<sup>3</sup>, Anita Mäki<sup>3</sup>, Paula Kankaala<sup>2</sup>

1) Tampere University of Technology, Laboratory of Chemistry and Bioengineering, Tampere, Finland

2) University of Eastern Finland, Department of Environmental and Biological Sciences, Joensuu, Finland

3) University of Jyväskylä, Department of Biological and Environmental Science, Jyväskylä, Finland

4) University of Eastern Finland, Department of Environmental and Biological Sciences, Kuopio, Finland

5) Swedish University of Agricultural Sciences, Science for Life Laboratories, Department of Forest Mycology and Plant Pathology, Uppsala, Sweden

**\*Corresponding author:** Tampere University of Technology, Laboratory of Chemistry and Bioengineering, P.O. Box 541, FI-33101, Tampere, Finland

E-mail: antti.rissanen@tut.fi, Tel: +358 40 1981145, Fax: +358 3 3641392

**One sentence summary:** Anaerobic methane oxidation did not have any ecological relevance in the methane consumption in the sediments of two shallow boreal lakes.

## ABSTRACT

The role of anaerobic CH<sub>4</sub> oxidation in controlling lake sediment CH<sub>4</sub> emissions remains unclear. Therefore, we tested how relevant EAs (SO<sub>4</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup>, Fe<sup>3+</sup>, Mn<sup>4+</sup>, O<sub>2</sub>) affect CH<sub>4</sub> production and oxidation in the sediments of two shallow boreal lakes. The changes induced to microbial communities by the addition of Fe<sup>3+</sup> and Mn<sup>4+</sup> were studied using next-generation sequencing targeting the 16S rRNA and methyl-coenzyme M reductase (*mcrA*) genes and *mcrA* transcripts. Putative anaerobic CH<sub>4</sub> oxidizing archaea (ANME-2D) and bacteria (*NC 10*) were scarce (up to 3.4% and 0.5% of archaeal and bacterial 16S rRNA genes, respectively), likely due to the low environmental stability associated with shallow depths. Consequently, the potential anaerobic CH<sub>4</sub> oxidation (0–2.1 nmol g<sup>-1</sup><sub>dry weight (DW)</sub>d<sup>-1</sup>) was not enhanced by the addition of EAs, nor important in consuming the produced CH<sub>4</sub> (0.6–82.5 nmol g<sup>-1</sup><sub>DW</sub>d<sup>-1</sup>). Instead, the increased EA availability suppressed CH<sub>4</sub> production via the outcompetition of methanogens by anaerobically respiring bacteria and via the increased protection of organic matter from microbial degradation induced by Fe<sup>3+</sup> and Mn<sup>4+</sup>. Future studies could particularly assess whether anaerobic CH<sub>4</sub> oxidation has any ecological relevance in reducing CH<sub>4</sub> emissions from the numerous CH<sub>4</sub>-emitting shallow lakes in boreal and tundra landscapes.

Keywords: lake, sediment, methanogenesis, methane oxidation, 16S rRNA, *mcrA*

## INTRODUCTION

Lake sediments are globally important carbon stores (Molot and Dillon 1996; Kortelainen *et al.* 2004), but they are also important contributors of methane (CH<sub>4</sub>) to the atmosphere (Bastviken *et al.* 2004). CH<sub>4</sub> emissions from these environments are controlled by both methanogenesis and CH<sub>4</sub> oxidation. Methanogenesis is the final step in organic matter (OM) degradation, during which methanogenic archaea produce CH<sub>4</sub> from either acetate via the acetoclastic pathway or by oxidizing H<sub>2</sub> using CO<sub>2</sub> as an electron acceptor (EA) via the hydrogenotrophic pathway (Conrad 1999). Some archaea can also produce CH<sub>4</sub> from other methyl compounds (e.g. methanol) via the methylotrophic pathway (Conrad 1999; Borrel *et al.* 2013). The substrates necessary for methanogenesis are produced by fermentative and syntrophic bacteria (Conrad 1999). In oxic conditions, CH<sub>4</sub> is efficiently consumed through aerobic oxidation (MO) by methanotrophic bacteria (MOB) utilizing O<sub>2</sub> as an EA (e.g. Hanson and Hanson 1996). In anoxic conditions, CH<sub>4</sub> consumption can potentially proceed through anaerobic oxidation (AOM) by anaerobic methanotrophic archaea (ANME archaea) utilizing various inorganic (Beal, House and Orphan 2009; Knittel and Boetius 2009; Haroon *et al.* 2013; Ettwig *et al.* 2016) and organic (Scheller *et al.* 2016) compounds or bacteria of the *NC 10* phylum utilizing NO<sub>2</sub><sup>-</sup> (Ettwig *et al.* 2010) as EAs. While AOM coupled with SO<sub>4</sub><sup>2-</sup> - reduction dominates anaerobic CH<sub>4</sub> oxidation in marine sediments (Knittel and Boetius 2009), the limited existing evidence suggests that AOM, coupled with the reduction of various inorganic EAs, i.e. SO<sub>4</sub><sup>2-</sup> (Timmers *et al.* 2016), NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> (Deutzmann *et al.* 2014; á Norði and Thamdrup 2014), Fe<sup>3+</sup> (Sivan *et al.* 2011) and Mn<sup>4+</sup> (Segarra *et al.* 2013), could be important in freshwater systems. Furthermore, some methanogens oxidize small amounts of CH<sub>4</sub> without external EAs

during trace methane oxidation (TMO) due to enzymatic backflux (Moran *et al.* 2005; Timmers *et al.* 2017).

In temperate and boreal lakes, the sediment EA availability varies seasonally. During thermal stratification periods, the oxygenation of the sediment is inhibited, which leads to the exhaustion of sediment EAs (other than CO<sub>2</sub>) by microbial processes (Bedard and Knowles 1991; Mattson and Likens 1993). In contrast, sediment oxygenation, which is induced by water-column mixing during spring and autumn, as well as by biological and physical turbation, activates the biogeochemical processes that regenerate the sediment EA pool. The increasing availability of EAs (other than CO<sub>2</sub>) in sediments can mitigate CH<sub>4</sub> emissions in several ways. EAs can directly inhibit methanogenesis (Klüber and Conrad 1998) or decrease/suppress it by diverting the flow of electrons (from H<sub>2</sub>, volatile fatty acids, alcohols) generated by fermentative bacteria from methanogenic to other anaerobic respiration pathways (Klöpffel *et al.* 2014). EAs can also increase CH<sub>4</sub> consumption via CH<sub>4</sub> oxidation (á Norði and Thamdrup 2014). However, Fe<sup>3+</sup> and Mn<sup>4+</sup> (generated via Fe<sup>2+</sup> and Mn<sup>2+</sup> oxidation), by means of reacting with OM, can also increase OM recalcitrance and protect it from microbial degradation (Lalonde *et al.* 2012; Estes *et al.* 2017). This could also suppress methanogenesis via decreasing the availability of methanogenic substrates. The recently reported Fe<sup>3+</sup>-induced suppression of both CH<sub>4</sub> and CO<sub>2</sub> production in boreal lake sediments and peats (Karvinen, Lehtinen and Kankaala 2015) indeed implies that this process could play an important role in reducing CH<sub>4</sub> emissions from freshwater systems. It is well known that MO in oxic sediment layers represents an efficient CH<sub>4</sub> sink in lakes (Hanson and Hanson 1996) and that increased EA availability reduces methanogenesis in freshwater sediments (Segarra *et al.* 2013; Karvinen, Lehtinen and Kankaala 2015). However, the roles of the different EA-induced mechanisms that reduce methanogenesis (i.e. direct

inhibition, increased anaerobic respiration, or increased OM recalcitrance) and the effects of increased EA availability on AOM activity in freshwater sediments remain unclear.

In this study, we hypothesized that, in addition to aerobic MO, AOM, coupled with the reduction of  $\text{Fe}^{3+}$ ,  $\text{Mn}^{4+}$ ,  $\text{NO}_3^-$ , or  $\text{SO}_4^{2-}$ , takes place in boreal lake sediments. We further hypothesized that increased  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  decrease both  $\text{CH}_4$  and  $\text{CO}_2$  production in lake sediments via increasing OM recalcitrance and protection from microbial degradation. We conducted two sets of sediment slurry experiments with an increased concentration of either  $\text{Fe}^{3+}$ ,  $\text{Mn}^{4+}$ ,  $\text{SO}_4^{2-}$ ,  $\text{NO}_3^-$ , or  $\text{O}_2$  and analyzed the changes induced by the increase in each of the EAs in the potential rates of  $\text{CH}_4$  oxidation and production and total inorganic carbon (TIC) production. Furthermore, the effects of the increased  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  availability on the structure of the bacterial and archaeal communities were assessed using next-generation sequencing (NGS) of the ribosomal 16S rRNA genes. The  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$ -induced effects on the community structure and activity of methanogenic/methanotrophic archaea were also specifically studied by NGS of methyl-coenzyme M reductase (*mcrA*) genes and *mcrA* transcripts, respectively.

## **MATERIALS AND METHODS**

### **Sediment sampling**

The sediment samples for this study were collected from two sites in September 2012, namely a *Phragmites australis*-dominated littoral site (depth ca. 0.8 m) in a mesotrophic lake (L. Orivesi; 62°31'N, 29°22'E) and a profundal site (depth 7.5 m) in another mesotrophic lake (L. Ätäskö; 62°02'N, 29°59'E). Samples from the profundal site were also obtained in June 2014. These two sites were selected due to previously determined background information, that is, high  $\text{CH}_4$  emission rates were documented at the littoral site of L. Orivesi (Juutinen *et al.* 2003), while for L. Ätäskö, the postglacial sediment accumulation rate with  $^{14}\text{C}$ -based depth-zone dating was

available (Pajunen 2004; Table 1). The water columns in the study sites are not thermally stratified during the open water season. Based on measurements taken during sampling in 2012, the water above the sediment at both sites was oxic ( $O_2 > 265 \mu\text{mol L}^{-1}$ ).  $O_2$  was not measured in 2014. However, open data available from the Finnish Environment Institute show that the water column of the profundal site is oxic until the bottom during the open water season. The uppermost samples, i.e. 0–10 cm (sample code is P<sub>0-10</sub>) (six cores in 2012 and 10 cores in 2014) and 10–30 cm (P<sub>10-30</sub>) (six cores in 2012 and 10 cores in 2014) layers at the profundal site and a 0–25 cm (L<sub>0-25</sub>) (six cores in 2012) layer at the littoral site, were collected using a Kajak-type gravity corer ( $\varnothing = 54$  mm). The deeper samples, 90–130 cm (P<sub>90-130</sub>) (four cores in 2012) and 390–410 cm (P<sub>390-410</sub>) (three cores in 2012) layers at the profundal site, were collected using a Livingston-type piston corer ( $\varnothing = 54$  mm) (Table 1). Sediments were sectioned into depth layers and packed in gas-tight, sterile plastic bags already in the field, except for the sediment cores for P<sub>390-410</sub>, which were sealed and transported to the laboratory as a whole. Sediments from the different cores were pooled for P<sub>0-10</sub>, P<sub>10-30</sub>, and L<sub>0-25</sub>, whereas those for P<sub>90-130</sub> were not pooled but handled and packed individually. Contact with the air was minimized during the collection and handling procedures. Samples were transported to the laboratory on ice and covered from light.

### ***In vitro* incubations to determine the potential CH<sub>4</sub> oxidation and net production rates of CH<sub>4</sub> and total inorganic carbon (TIC)**

#### Slurry preparation and incubation

The experimental setup consisted of testing the effects of different EAs and CH<sub>2</sub>F<sub>2</sub> on CH<sub>4</sub> and TIC production and CH<sub>4</sub> consumption potentials (Table 2) as well as the determination of

potential  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  reduction (Table 3). Setting up the incubations took place in a glove bag under a  $\text{N}_2$  gas flow. The cores for  $\text{P}_{390-410}$  were first sectioned and put into sterile plastic bags, and similar to  $\text{P}_{90-130}$  samples, the sediments from the different cores were not pooled but handled individually. The middle parts of the sediments from the bags were collected for incubations, avoiding the outer sediment surfaces, which might have been exposed to air during sampling. The sediments were homogenized and weighted into  $\text{N}_2$ -flushed, pre-sterilized (by autoclaving) glass incubation bottles. In 2012, ~60 g of wet sediment from  $\text{L}_{0-25}$  and  $\text{P}_{10-30}$ , as well as ~50 g and ~40 g of sediment from  $\text{P}_{90-130}$  and  $\text{P}_{390-410}$ , respectively, were weighted into 150 ml bottles (12–16 bottles per sediment type, 59 bottles altogether). In 2014, wet sediment from the profundal site, ~50 g from  $\text{P}_{0-10}$ , and ~60 g from  $\text{P}_{10-30}$  was weighted into 300 ml bottles (38 and 40 bottles of  $\text{P}_{0-10}$  and  $\text{P}_{10-30}$ , respectively) (Table 2). Soon thereafter, the sediments obtained in 2014 were slurried by adding 50 ml  $\text{O}_2$ -free MQ- $\text{H}_2\text{O}$  to each bottle, followed by brief, vigorous shaking. All bottles were then re-flushed with  $\text{N}_2$  gas and closed with gas-tight caps and rubber septa. The sediments obtained in 2012 were pre-incubated to remove any traces of  $\text{O}_2$  by storing them in the dark at a temperature of  $+4^\circ\text{C}$  for seven days. Headspaces were then flushed with He (with three cycles of vacuum/He flushing). The sediments were slurried by the addition of  $\text{O}_2$ -free, sterilized artificial porewater (146 mM NaCl, 13.4 mM  $\text{MgCl}_2$ , 0.3 mM  $\text{CaCl}_2$ , 0.8 mM KCl, and 2.9 mM  $\text{NaHCO}_3$ ) at a 1:2 ratio of porewater (V/w) to each bottle followed by brief, vigorous shaking.

The bottles containing the 2012 sediments were then divided into four treatments: (1)  $^{13}\text{CH}_4$ , (2)  $^{13}\text{CH}_4 + 2 \text{ mM } \text{SO}_4^{2-}$  (as  $\text{Na}_2\text{SO}_4$ ), (3)  $^{13}\text{CH}_4 + 2 \text{ mM } \text{NO}_3^-$  (as  $\text{NaNO}_3$ ), and (4)  $^{13}\text{CH}_4 + \text{O}_2$ , with three to four replicate bottles included in each treatment for each of the four sediment types ( $\text{L}_{0-25}$ ,  $\text{P}_{10-30}$ ,  $\text{P}_{90-130}$ ,  $\text{P}_{390-410}$ ). Replicates for  $\text{P}_{90-130}$  and  $\text{P}_{390-410}$  represented the different

sampling cores. The bottles containing the 2014 sediments were divided into five treatments: (1)  $^{13}\text{CH}_4$ , (2)  $^{13}\text{CH}_4 + 10 \text{ mM Fe}^{3+}$  [amorphous  $\text{Fe}(\text{OH})_3$ ; Lovley and Phillips 1986], (3)  $^{13}\text{CH}_4 + 10 \text{ mM Mn}^{4+}$  (solid  $\text{MnO}_2$ ; Lovley and Phillips 1988), (4)  $^{13}\text{CH}_4 + \text{O}_2$ , and (5)  $^{13}\text{CH}_4 + \text{CH}_2\text{F}_2$ , as well as five corresponding treatments with non-labelled  $\text{CH}_4$ , with three to four replicate bottles included in each treatment for both sediment types ( $\text{P}_{0-10}$ ,  $\text{P}_{10-30}$ ) (Table 2).

The *in situ* porewater concentrations of the EAs were not measured, except for the Mn concentration in  $\text{P}_{0-10}$  ( $\sim 27 \mu\text{M}$ ), which is within the typical range ( $2\text{--}40 \mu\text{M}$ ) reported in surface sediments of arctic lakes (Bretz and Whalen 2014). However, in the 0–2 cm surface sediment layer of a boreal (Finnish) high- $\text{NO}_3^-$  lake (L. Pääjärvi,  $\text{NO}_3^- > 65 \mu\text{M}$  in the water above the sediment), the porewater  $\text{NO}_3^-$  concentration was previously found to only reach  $14 \mu\text{M}$  during the growing season (Rissanen *et al.* 2013). The porewater  $\text{SO}_4^{2-}$  concentration typically ranges from  $\sim 20$  to  $\sim 300 \mu\text{M}$  in the sediments of oligotrophic and mesotrophic lakes (Holmer and Storkholm 2001), while the porewater Fe concentration has been reported to only reach  $\sim 400 \mu\text{M}$  in previously studied northern lakes (Huerta-Diaz, Tessier and Carignan 1998; Bretz and Whalen 2014). This suggests that all EAs were added in significantly higher concentrations when compared to *in situ* conditions. Thus, this approach should show the effect of increased EA availability on the consumption and production of  $\text{CH}_4$ , as well as the TIC production processes, at the level of process potentials.

After preparing the EA amendments, all bottles in the anaerobic treatments (1 to 3 in 2012, 1 to 3 and 5 in 2014) were flushed with He (in 2012) or  $\text{N}_2$  (in 2014), followed by the addition of He or  $\text{N}_2$  overpressure, while the bottles in the aerobic treatments were treated similarly with air. The headspaces were then amended with 1 ml of non-labelled  $\text{CH}_4$  (in 2014), 0.6 ml (in 2012), or 1 ml (in 2014) of  $^{13}\text{C}$ -enriched  $\text{CH}_4$  ( $^{13}\text{C}$  percentage was 8.2% in 2012 and

10.9% in 2014), followed by the addition of 0.5 ml of CH<sub>2</sub>F<sub>2</sub> to treatment 5 in 2014 (headspace concentration ~0.25%). The <sup>13</sup>C-enriched CH<sub>4</sub> mixture was made by mixing CH<sub>4</sub> (99.95% purity, INTERGAS, UK) with <sup>13</sup>CH<sub>4</sub> (99.5% purity, 99.9% <sup>13</sup>C, Cambridge Isotope Laboratories, Inc., USA) in an He-flushed, O<sub>2</sub>-free glass bottle with NaOH powder to remove any contaminating CO<sub>2</sub>. The bottles were taken from the glove bag and incubated in the dark at +4°C for up to 14 months in 2012, and at +10°C for up to four months in 2014. During the incubation period, the headspace concentration of CH<sub>4</sub> was measured five times in 2012 and four to five times in 2014, while those of the CO<sub>2</sub> and the <sup>13</sup>C content of CO<sub>2</sub> were measured six times in 2012 and four times in 2014.

CH<sub>2</sub>F<sub>2</sub> inhibits CH<sub>4</sub> oxidation via its effect on methane mono-oxygenase (inhibited at a concentration > 0.03%). It also inhibits acetate-consuming methanogenesis (inhibited at a concentration > 0.1%; Miller, Sasson and Oremland 1998). Since ANME archaea use the H<sub>2</sub>-consuming methanogenesis pathway in reverse (Timmers *et al.* 2017), it can be speculated that CH<sub>2</sub>F<sub>2</sub> would inhibit CH<sub>4</sub> oxidation by MOBs and *NC 10* bacteria (since both use methane mono-oxygenase), but not AOM by ANME archaea. Furthermore, experiments conducted with *Methanosarcina acetivorans* suggest that TMO by acetate-consuming methanogens transfers methane-C to acetate, but not to CO<sub>2</sub> (Moran *et al.* 2007). As we did not measure acetate, TMO by acetate-consuming methanogens and its possible inhibition goes undetected in this study. Thus, the possible inhibition of CH<sub>4</sub> oxidation (to CO<sub>2</sub>) by CH<sub>2</sub>F<sub>2</sub> could specifically indicate MOB or *NC 10* bacteria activity during the incubations.

### **Determination of CH<sub>4</sub> and CO<sub>2</sub> concentrations and the isotopic composition of CO<sub>2</sub>**

The gas concentrations were measured in 2012 using an Agilent 6890N (Agilent Technologies, USA) gas chromatograph (GC) with an electron capture detector for CO<sub>2</sub> and a

flame ionization detector (FID) for CH<sub>4</sub> (first and second sampling), an Autosystem XL (Perkin Elmer, USA) GC with a thermal conductivity detector (TCD) for both gases (third sampling), an infrared Calanus gas analyzer (according to the method of Salonen 1981) for CO<sub>2</sub> (fourth to sixth sampling), an Agilent 6890N GC with a FID (fourth sampling), and a Clarus 500 (Perkin Elmer, USA) GC with a FID for CH<sub>4</sub> (sixth sampling). In 2014, the CH<sub>4</sub> and CO<sub>2</sub> concentrations were determined using a TurboMatrix and a Clarus 580 GC (Perkin Elmer, USA), a headspace sampler, and a GC equipped with a FID and a nickel catalyst for converting carbon dioxide to CH<sub>4</sub>. The same standards were used each time.

The fractional abundance of <sup>13</sup>C in the CO<sub>2</sub> gas, <sup>13</sup>F,

$$^{13}F = \frac{^{13}\text{C}}{(^{13}\text{C} + ^{12}\text{C})} \quad (1)$$

was analyzed with a Gasbench II (Thermo Fisher Scientific, Bremen, Germany), an online gas preparation and introduction system for isotope ratio mass spectrometry, coupled with a Delta Plus Advantage isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scientific).

### ***In vitro* incubations to determine the reduction of Fe<sup>3+</sup> and Mn<sup>4+</sup>**

The Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction incubations with samples from L<sub>0-25</sub> and P<sub>0-10</sub> in 2012 were set up similarly to the gas incubations described above. Approximately 9 g of wet sediment was slurried with 34 ml of N<sub>2</sub>-flushed MQ-H<sub>2</sub>O in 300 ml bottles (nine bottles from both L<sub>0-25</sub> and P<sub>0-10</sub>), which were then divided into three treatments: (1) no additions, (2) 10 mM Fe<sup>3+</sup>, and (3) 10 mM Mn<sup>4+</sup>, with three replicate bottles included in each treatment for both sediment types (Table 3). Following the N<sub>2</sub> flushing, the bottles were taken from the glove bag and incubated in darkness at +10°C for 4.5 months. Slurry samples for the determination of the Fe<sup>3+</sup> and Mn<sup>4+</sup>

reduction were taken from the bottles through a septum eight times during the incubation period. The bottles were briefly shaken before each sampling.

The  $\text{Fe}^{3+}$  reduction activity was measured as  $\text{Fe}^{2+}$  production using a ferrozine-based assay (Lovley and Phillips 1986), as previously described by Karvinen, Lehtinen, and Kankaala (2015). The  $\text{Mn}^{4+}$  reduction activity was measured by the accumulation of soluble  $\text{Mn}^{2+}$ . The slurry samples were acidified (pH 2) with HCl and then centrifuged (4500 RPM, 15 min). The Mn concentration of the supernatant was measured using an AAnalyst 300 atomic absorption spectrophotometer (Perkin Elmer, USA).

### **Other analyses**

The oxidation reduction potential (ORP) and pH of the sediment slurries collected in 2012 and 2014 were measured at the end of the incubations using a pH 3110 unit (WTW) equipped with a SenTix ORP electrode and a SenTix 41 pH electrode (WTW). In the 2012 experiment, pH was recorded at the start of the incubations, and a linear change in the  $[\text{H}^+]$  was assumed for the whole incubation period in order to enable the TIC calculations (Table S1, Supporting Information). In 2014, pH and ORP changes were monitored alongside the incubations (measurements at four to five time points) using an additional batch of slurry samples, with one replicate per each treatment: (1) no amendments, (2)  $\text{Fe}^{3+}$ , (3)  $\text{Mn}^{4+}$ , (4)  $\text{O}_2$ , and (5)  $\text{CH}_2\text{F}_2$  (Fig. S1, Supporting Information).

Dry weight (DW) of the sediments was analyzed by drying at 105 °C for 24 h. C and N contents of the sediment were measured from dry sediment using Thermo Finnigan Flash EA1112 elemental analyzer (Thermo Fisher Scientific). Total Fe content of the sediments was determined as in Karvinen, Lehtinen, and Kankaala (2015). Total Mn content of the sediment

supernatant (pH 2) was determined as explained above for the  $\text{Mn}^{4+}$  reduction analyses (Table 1).

## Calculations

The concentration of  $\text{CH}_4$  and  $\text{CO}_2$  dissolved in the slurry phase of the incubation bottles was calculated from their partial pressures in the gas phase (headspace) using Henry's law. This allowed for the calculation of the total amount of  $\text{CH}_4$  in the incubation bottles (gas phase + slurry phase). The amount of bicarbonate dissolved in the slurry phase was calculated from the amount of dissolved  $\text{CO}_2$  using the dissociation constant of bicarbonate (Stumm and Morgan 1981) and the pH of the sediment slurries (see above). The total inorganic carbon (TIC) was defined as the sum of bicarbonate, gaseous, and dissolved  $\text{CO}_2$ . The potential net production rates of  $\text{CH}_4$  and TIC, as well as the production rates of  $\text{Fe}^{2+}$  (potential  $\text{Fe}^{3+}$  reduction) and  $\text{Mn}^{2+}$  (potential  $\text{Mn}^{4+}$  reduction), were measured for each incubation bottle as a linear increase in the total amount of these substances with time.

Potential  $\text{CH}_4$  oxidation was measured for each incubation bottle as a linear increase in the amount of  $\text{CH}_4$  oxidized with time. This was calculated for each time point using a modification of the equation used by Blazewicz *et al.* (2012) and Moran *et al.* (2005):

$$N_{\text{oxCH}_4} = [(^{13}F_L - ^{13}F_n) \times N_{\text{LTIC}}] \times \frac{(N_{\text{iCH}_4} + N_{\text{bCH}_4})}{(F_{\text{iCH}_4}N_{\text{iCH}_4} + F_{\text{bCH}_4}N_{\text{bCH}_4})} \quad (2)$$

where  $N_{\text{oxCH}_4}$  is the amount of  $\text{CH}_4$  oxidized in the incubation bottle,  $N_{\text{LTIC}}$  is the amount of TIC in the incubation bottle with the added  $^{13}\text{CH}_4$ ,  $N_{\text{iCH}_4}$  is the amount of  $\text{CH}_4$  initially in the incubation bottle (day 1),  $N_{\text{bCH}_4}$  is the amount of biogenic  $\text{CH}_4$  produced during incubation,  $F_{\text{iCH}_4}$  is the initial fractional abundance of  $^{13}\text{C}$  in  $\text{CH}_4$  (same as the  $^{13}\text{C}$ -label percentage, see above),

$F_{bCH_4}$  is the fractional abundance of  $^{13}C$  in biogenically produced  $CH_4$ ,  $^{13}F_L$  is the fractional abundance of  $^{13}C$  in the  $CO_2$  in the incubation bottle with the added  $^{13}CH_4$ , while  $^{13}F_n$  is the fractional abundance of  $^{13}C$  in the  $CO_2$  in the incubation bottle of the respective treatment with the added non-labelled  $CH_4$  (for the 2014 incubations) or the fractional abundance of  $^{13}C$  in the  $CO_2$  initially in the incubation bottle with added  $^{13}CH_4$  (for the 2012 incubations). The left side of the equation (left from the central multiplication [x] sign) estimates the amount of  $^{13}CO_2$  produced from the added  $^{13}CH_4$  for each time point, which is multiplied by the right side of the equation estimating the ratio of total  $CH_4$  to  $^{13}C-CH_4$ . Both the  $^{13}F_L$  and  $^{13}F_n$  were determined using IRMS (from the gas phase  $CO_2$ ), while the  $N_{LTIC}$  and  $N_{iCH_4}$  were determined using GC (see above). The  $N_{bCH_4}$  was calculated as the difference between the total amount of  $CH_4$  at a given time point and the  $N_{iCH_4}$ . The effects of the isotopic fractionation associated with  $CO_2$  dissolution and bicarbonate formation (Stumm and Morgan 1981) were considered to be negligible in the calculations, since the incubations were labelled with  $^{13}C$ . The  $CH_4$  oxidation in this study was determined solely based on the transfer of  $^{13}C$  from  $CH_4$  to TIC, and hence, the proportion of  $CH_4-C$  bound to the biomass was not taken into account. As in the study by Blazewicz *et al.* (2012), it was assumed that the biogenic  $CH_4$  produced during the incubation had  $\delta^{13}C = -50\text{‰}$  ( $F_{bCH_4} = 0.01051$ ). The chosen value represents the typical values of boreal lakes, since it is in the middle of the total range,  $-20\text{‰}$  to  $-81\text{‰}$ , as measured previously from the water columns of two boreal lakes (Kankaala *et al.* 2007; Nykänen *et al.* 2014). All the process rates were expressed as per gram of dry weight and per  $cm^3$  of wet sediment.

### **Molecular analyses**

In 2012, 15 ml of wet sediment taken from L<sub>0-25</sub>, P<sub>0-10</sub> and P<sub>10-30</sub> prior to the incubations was stored at  $-20^\circ C$  and subsequently freeze-dried for molecular analyses. In 2014, ~500 mg

sediment aliquots were taken for molecular analyses from P<sub>0-10</sub> and P<sub>10-30</sub> prior to the incubations and from the sediment slurry of the treatments after the incubations and then stored at -80°C.

DNA was extracted from the freeze-dried sediment collected in 2012 using the PowerSoil DNA isolation kit (MOBIO). In 2014, DNA and RNA were simultaneously extracted from the frozen sediment samples using the method of Griffiths *et al.* (2000).

General microbial communities were studied by NGS of bacterial and archaeal 16S rRNA gene amplicons. Furthermore, potential and active methanogenic/methanotrophic archaea were specifically studied by NGS of *mcrA* from DNA and mRNA, respectively. Primer pairs utilized for PCR amplification of the 16S rRNA gene were Arch340F (5'-CCCTAYGGGGYGCASCAG-3')/Arch1000R (5'-GGCCATGCACYWCYTCTC-3') (Gantner *et al.* 2011) for archaea and 27F (5'-AGAGTTTGATCMTGGCTCAG)/338R (5'-TGCTGCCTCCCGTAGGAGT-3') for bacteria, while those utilized for PCR amplification of the *mcrA* gene were *mcrA* forward (5'-GGTGGTGMTGGATTCACACAR-3')/*mcrA* reverse (5'-TCATTGCRTAGTTWGGRTAGTT-3') (Beal, House and Orphan 2009). PCR of 16S rRNA and *mcrA* genes, reverse-transcriptase PCR (RT-PCR) of *mcrA*, preparation of sequence libraries as well as sequencing (Ion Torrent™ Personal Genome Machine) are described in detail in Supplemental Methods (Supplemental Methods 1, Supporting Information).

Mothur (Schloss *et al.* 2009) was used in all subsequent sequence analyses unless otherwise reported. The barcodes and primer sequences, as well as low-quality sequences (containing sequencing errors in the primer or barcode sequences, ambiguous nucleotides, and homopolymers longer than eight nucleotides), were removed. Framebot (FunGene website, <http://fungene.cme.msu.edu/FunGenePipeline>; Wang *et al.* 2013) was used to correct frameshift errors in the *mcrA* reads. The 16S rRNA gene sequences were assigned to taxonomies with a

naïve Bayesian classifier (bootstrap value cut off = 75%) (Wang *et al.* 2007) using the Greengenes database, which was amended by sequences of the recently described archaeal phyla *Verstraetearchaeota* presented in Vanwonterghem *et al.* (2016). Unclassified sequences as well as the sequences classified as chloroplasts, mitochondria, and eukaryota were removed. Furthermore, the bacterial sequences were removed from the archaeal libraries and *vice versa*. The taxonomic assignment for *mcrA* sequences was done similarly to the procedure for the 16S rRNA sequences, albeit with a custom-made database, whose preparation is described in Supplemental Methods (Supplemental Methods 2).

The 16S rRNA gene sequences were aligned using Silva reference alignment (Release 119), while the alignment of the *mcrA* sequences was conducted using a set of aligned *mcrA* sequences retrieved from the FunGene website ([http://fungene.cme.msu.edu/hmm\\_detail.spr?hmm\\_id=16](http://fungene.cme.msu.edu/hmm_detail.spr?hmm_id=16)). Chimeric sequences, denoted using Mothur's implementation of Uchime (Edgar *et al.* 2011), were removed from the data. In addition, Schloss, Gevers and Westcott's (2011) modification of the Huse *et al.* (2010) pre-clustering algorithm was used to reduce the effect of potential sequencing errors. After these steps, the final datasets from the years 2012/2014 contained 24013/212584 (length = ~307 bp), 23507/77038 (~223 bp), and 2167/82818 (~263 bp) bacterial 16S rRNA gene, archaeal 16S rRNA gene, and *mcrA* sequence reads, respectively.

The sequences were divided into operational taxonomic units (OTUs) at 97% and 96% similarity levels for the 16S rRNA and *mcrA* genes, respectively. For the beta-diversity and taxonomic analyses, rare OTUs, that is, OTUs with  $\leq 26$  and  $\leq 37$  of 16S rRNA and *mcrA* sequences, respectively, were removed (thus, those OTUs with less than one sequence per sample on average were removed). Each sample was subsampled to the size of the smallest

sample (=3580/4223 bacterial and 4541/1123 archaeal 16S rRNA gene reads and 479/1180 *mcrA* reads per sample in the 2012/2014 datasets, respectively). The OTUs were assigned consensus taxonomies. In addition, a phylogenetic tree analysis was performed to further validate and fine-tune the classification of the *mcrA*-OTUs (Supplemental Methods 3 and Fig. S2, Supporting Information).

The bacterial and archaeal OTUs were classified into functional groups. For the archaea, these were: (1) acetate, (2) H<sub>2</sub> + CO<sub>2</sub>, (3) methyl compound (Nazaries *et al.* 2013), (4) H<sub>2</sub> + methyl compound consuming methanogens (Borrel *et al.* 2013; Vanwonterghem *et al.* 2016), and (5) anaerobic methanotrophic archaea (Knittel and Boetius 2009). For the bacteria, the functional groups were: (1) fermentative (fermentative and syntrophic bacteria) (Herlemann *et al.* 2009; Yamada and Sekiguchi 2009; Kallistova, Goel and Nozhevnikova 2014; Wasmund *et al.* 2014; Wrighton *et al.* 2014; Zheng *et al.* 2016), (2) Fe<sup>3+</sup>/Mn<sup>4+</sup>-reducing (Finneran, Johnsen and Lovley 2003; Lovley 2006), (3) aerobic methanotrophic (Chowdhury and Dick 2013), and (4) anaerobic methanotrophic bacteria (Ettwig *et al.* 2010). Furthermore, representative 16S rRNA gene sequences of the *MCG* OTUs were searched against a *MCG* database consisting of the sequences of putative methanogens (Evans *et al.* 2015) and sequences classified into previously defined *MCG* sub-groups (Lazar *et al.* 2015) using BLASTN (Altschul *et al.* 1990).

The sequencing data were deposited into the NCBI's Sequence Read Archive (Study accession SRP091914).

## **Statistical analyses**

Differences in the process rate variables, the relative abundance of the functional groups of bacteria and archaea, and the relative activity (relative *mcrA* transcript abundance) of the functional groups of methanogenic/methanotrophic archaea among the treatments were tested for

each sediment layer using a t-test or one-way ANOVA. When necessary, the variables were log<sub>10</sub> transformed to fulfil the ANOVA assumptions. The one-way ANOVA was followed by pair-wise post-hoc tests using the least significant difference (LSD) technique with Hochberg-Bonferroni-corrected  $\alpha$ -values. The correlations among the relative activity of the functional groups of archaea and the process rates were tested using Spearman's rank correlation analysis. The ANOVA, t-tests, and correlation analysis were conducted using IBM SPSS Statistics version 23.

Variations in the beta diversity (Bray-Curtis distance metric) were visualized by non-metric multidimensional scaling (NMS) and tested among the treatments using a one-way permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001; McArdle and Anderson 2001). The NMS was conducted using PC-ORD version 6.0 (MjM Software, Gleneden Beach, Oregon, USA) (McCune and Mefford 2011). The PerMANOVA was conducted using PAST version 3.06 (Hammer, Harper and Ryan 2001). Furthermore, differences in the abundance of each OTU among the treatments were tested using Mothur's implementation of the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata *et al.* 2011).

## **RESULTS**

### **Potential net production rates of CH<sub>4</sub> and TIC and the potential CH<sub>4</sub> oxidation**

The potential net production rates of CH<sub>4</sub> and TIC in anaerobic conditions ranged from 0.6 to 82.5 nmol CH<sub>4</sub> g<sup>-1</sup><sub>DW</sub> d<sup>-1</sup> (0.1–11.7 nmol CH<sub>4</sub> cm<sup>-3</sup> d<sup>-1</sup>) and from 19.7 to 318.7 nmol TIC g<sup>-1</sup><sub>DW</sub> d<sup>-1</sup> (3.0–45.1 nmol TIC cm<sup>-3</sup> d<sup>-1</sup>), respectively (Table 2). They were one order higher at the littoral site than at the profundal site (when compared in 2012), and also differed among the depth layers at the profundal site (Table 2). There were differences between the 2012 and 2014 rates, which

were likely caused by the different incubation setups (see the Materials and Methods section), and hence, are not considered further. The major focus in this study was on the effects of increased EAs and CH<sub>2</sub>F<sub>2</sub>, that is, on the differences between the CH<sub>4</sub> treatment only and the other treatments (CH<sub>4</sub> + EAs and CH<sub>4</sub> + CH<sub>2</sub>F<sub>2</sub>) (Table 2).

The addition of Fe<sup>3+</sup> and Mn<sup>4+</sup> decreased the potential net CH<sub>4</sub> production by 30% and 77% in P<sub>0-10</sub>, respectively. In P<sub>10-30</sub>, the addition of Fe<sup>3+</sup> did not decrease it statistically significantly, although the addition of Mn<sup>4+</sup>, NO<sub>3</sub><sup>-</sup>, and SO<sub>4</sub><sup>2-</sup> decreased it by 83%, 60%, and 60%, respectively (Table 2). In contrast, NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> did not affect net CH<sub>4</sub> production in the deeper layers, P<sub>90-130</sub> and P<sub>390-410</sub>, at the profundal site (Table 2). The addition of SO<sub>4</sub><sup>2-</sup> and NO<sub>3</sub><sup>-</sup> also decreased the potential net CH<sub>4</sub> production by 66% and 40% in L<sub>0-25</sub> (Table 2), respectively. The addition of Fe<sup>3+</sup> and Mn<sup>4+</sup> also reduced the potential net TIC production by 60% and 42% in P<sub>0-10</sub>, respectively, although none of the added anaerobic EAs affected it in the other layers of the profundal site, nor at the littoral site (Table 2). The potential net CH<sub>4</sub> and TIC production rates were generally similar between the CH<sub>4</sub> and CH<sub>4</sub>+CH<sub>2</sub>F<sub>2</sub> treatments (Table 2). However, during the first week of incubation, CH<sub>2</sub>F<sub>2</sub> inhibited CH<sub>4</sub> production in two out of six and three out of eight incubation bottles containing sediment from P<sub>0-10</sub> and P<sub>10-30</sub>, respectively (Figs. S3A and C, Supporting Information). The relative expression of the *mcrA* (relative abundance of *mcrA* transcripts) of the acetate-consuming methanogens decreased with the increasing potential net CH<sub>4</sub> production ( $\rho = -0.709$ ,  $p < 0.05$ ), whereas no other correlations between the *mcrA* expression of the archaeal functional groups and the potential net production rates of CH<sub>4</sub> and TIC were detected.

The addition of O<sub>2</sub> generally resulted in the highest potential net TIC production. It also led to CH<sub>4</sub> consumption, except for the deepest layer, P<sub>390-410</sub>, of the profundal site, where no

CH<sub>4</sub> production or consumption occurred (Table 2). <sup>13</sup>C labelling confirmed that the CH<sub>4</sub> consumption was due to aerobic CH<sub>4</sub> oxidation ranging from 0 to 49.3 nmol g<sup>-1</sup><sub>DW</sub> d<sup>-1</sup> (0–7.0 nmol cm<sup>-3</sup> d<sup>-1</sup>) and also varying between sites (when compared in 2012) and depth layers (Table 2). The potential CH<sub>4</sub> oxidation in the anaerobic incubations ranged from 0 to 2.1 nmol g<sup>-1</sup><sub>DW</sub> d<sup>-1</sup> (0–0.3 nmol cm<sup>-3</sup> d<sup>-1</sup>). It was not affected by the addition of NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, Fe<sup>3+</sup>, or Mn<sup>4+</sup> at the profundal site, although NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> suppressed it at the littoral site (Table 2). The potential CH<sub>4</sub> oxidation did not generally differ between the CH<sub>4</sub> and CH<sub>4</sub>+CH<sub>2</sub>F<sub>2</sub> treatments (Table 2). However, during the first week of incubation, CH<sub>2</sub>F<sub>2</sub> inhibited it in two out of three and two out of four incubation bottles containing sediment from P<sub>0-10</sub> and P<sub>10-30</sub>, respectively (Figs. S3B and D). The potential CH<sub>4</sub> oxidation activity in the anaerobic treatments increased as the net TIC production increased ( $\rho = 0.524$ ,  $p < 0.05$ ), but it was not correlated with the net CH<sub>4</sub> production rate, nor with the relative expression of *mcrA* of the functional groups of archaea.

### **Potential rates of Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction**

The natural concentrations of Fe and Mn were higher in P<sub>0-10</sub> than in L<sub>0-25</sub> (Table 1). The average potential Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction rates ranged from 16.1 to 913.3 nmol g<sup>-1</sup><sub>DW</sub>d<sup>-1</sup> (3.3–129.1 nmol cm<sup>-3</sup> d<sup>-1</sup>) and 13.5 to 1066.4 nmol g<sup>-1</sup><sub>DW</sub>d<sup>-1</sup> (2.8–221 nmol cm<sup>-3</sup> d<sup>-1</sup>), respectively. The natural (non-amended) rate of anaerobic Fe<sup>3+</sup> reduction was considerably higher in P<sub>0-10</sub> than in L<sub>0-25</sub>, whereas the natural Mn<sup>4+</sup> reduction was only slightly higher in P<sub>0-10</sub> (Table 3). As expected, the addition of Mn<sup>4+</sup> increased the Mn<sup>4+</sup> reduction rate significantly at both sites (Table 3). Further, the addition of Fe<sup>3+</sup> increased the Fe<sup>3+</sup> reduction rate significantly in L<sub>0-25</sub>, although a much smaller increase was observed in P<sub>0-10</sub> (Table 3).

Since the Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction assays conducted in P<sub>0-10</sub> were performed in similar conditions to the gas production/consumption incubations conducted in 2014, the contribution of

Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction to C mineralization could be roughly estimated by assuming a 4:1 ratio for Fe<sup>3+</sup> reduction:TIC production and 2:1 for Mn<sup>4+</sup> reduction:TIC production (Hiscock and Bense 2014). The natural (non-amended) Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction contributed to 63.4% and 3.5% of TIC production, respectively. However, when Fe<sup>3+</sup> and Mn<sup>4+</sup> were added, OM oxidation coupled with dissimilatory Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction would have produced two to three times more TIC than was observed. This indicates that a large part of the Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction in the Fe<sup>3+</sup>- and Mn<sup>4+</sup>-amended treatments was not coupled with the processes that produce TIC.

### **Microbial community structure**

The sample storage and DNA extraction methods differed between the study years (see Materials and Methods), which precludes comparisons of microbial composition between 2012 and 2014. The structure of the microbial communities differed between the littoral and profundal sites and between P<sub>0-10</sub> and P<sub>10-30</sub> at the profundal site (Figs. 1 and 2, Figs. S4, S5, and S6, Supporting Information). Methanogens dominated the archaeal community at the littoral site, while their contribution was lower at the profundal site (Fig. 1A). *Methanobacteriaceae* that consume H<sub>2</sub>+CO<sub>2</sub> were more abundant at the littoral site than at the profundal site, whereas an opposite pattern was observed for acetate-consuming *Methanosaetaceae* and H<sub>2</sub>+CO<sub>2</sub>-consuming *Methanoregulaceae* (Fig. 1). The other detected but less abundant methanogenic groups were H<sub>2</sub>+CO<sub>2</sub>-consuming *Methanocellales* and H<sub>2</sub>+methyl-compound-consuming *Methanomassiliicoccales* and *Verstraetearchaeota* (Fig. 1). The anaerobic methane-oxidizing archaea all belonged to the ANME-2D archaea and were present in low abundance (0.02–3.4% of archaeal 16S rRNA and 0–3.9% of *mcrA* gene sequences) (Fig. 1). They had a higher relative abundance at the profundal site, especially in P<sub>10-30</sub> (Fig. 1). Of the methanogenic archaea actively expressing their *mcrA* gene in P<sub>0-10</sub>, *Methanosaetaceae* and *Methanoregulaceae* were

dominant, followed by *Methanobacteriaceae* (Fig. 1B), with much less of a contribution from *Methanomassiliicoccales* (2–4% of *mcrA* transcripts) and *Verstraetearchaeota* (0.1–0.4% of *mcrA* transcripts). Based on the much higher relative abundance of their *mcrA* at the mRNA level than at the DNA level, the ANME-2D archaea were especially active in the non-incubated sample (12% of *mcrA* transcripts), while their *mcrA* expression decreased considerably during the incubations (2.5–4.5% of *mcrA* transcripts after incubations) (Fig. 1B). Of the other archaea, the *MCG* archaea dominated at both sites, with less of a contribution from *Parvarchaea* and *DHVEG-1* (a family within *Thermoplasmata*) (Fig. S4). The *MCG* OTUs were not closely affiliated with the putative methanogenic/methanotrophic *MCG* (Evans *et al.* 2015) and mainly belonged to the *MCG* subgroups 6, 1, 15, 7/17, and 11 (based on Lazar *et al.* 2015).

*Bacteroidetes*, *Chloroflexi*, and *Deltaproteobacteria* (*Desulfobacteraceae*, *Syntrophaceae*, *Syntrophobacteraceae*, *Syntrophorhabdaceae*) dominated the putative fermentative bacterial community at both sites, while the other putative fermentative bacteria belonging to *Clostridia*, *Microgenomates*, *SR 1*, *TM 6*, *Saccharibacteria*, *Elusimicrobia*, and *Parcubacteria* constituted a much smaller segment of the bacterial community (Fig. 2A). Of the putative  $\text{Fe}^{3+}/\text{Mn}^{4+}$ -reducing bacteria, *Geobacter* dominated, with a lower contribution from *Thiobacillus* and *Geothrix*. The  $\text{Fe}^{3+}/\text{Mn}^{4+}$ -reducing bacteria were generally found with a very low abundance ( $\leq 1.1\%$ ), except in the  $\text{CH}_4+\text{Fe}^{3+}$  and  $\text{CH}_4+\text{Mn}^{4+}$  treatments in P<sub>10-30</sub>, in which the *Geobacter* were enriched (up to 5.9%) (Fig. 2B). The methanotrophic bacterial community consisted of *Methylococcales*, *Methylocystacea*, and *Methylacidiphilae* (Fig. 2C). As was the case with the ANME-2D archaea, the putative anaerobic methanotrophic bacteria within the *NC 10* phyla were present with a low relative abundance (0.1–0.5%) and were also more abundant at the profundal site (Fig. 2C).

Based on the analyses at the DNA level, the general and *mcrA*-carrying archaeal community did not differ among the CH<sub>4</sub>, CH<sub>4</sub>+Fe<sup>3+</sup>, and CH<sub>4</sub>+Mn<sup>4+</sup> treatments after the incubations (PerMANOVA,  $p > 0.05$ ). In addition, the relative abundance of the functional groups of archaea did not differ among the treatments (one-way ANOVA,  $p > 0.05$ ) (Fig. 1). Yet, according to the LEfSe analyses, some acetate-consuming *Methanosaetaceae* OTUs were more abundant in either the CH<sub>4</sub>+Fe<sup>3+</sup> or CH<sub>4</sub>+Mn<sup>4+</sup> treatments, while one H<sub>2</sub>+CO<sub>2</sub>-consuming *Methanoregulaceae* OTU, based on the 16S rRNA gene, exhibited its highest relative abundance in the CH<sub>4</sub> treatment in P<sub>0-10</sub> (Figs. S7 and S8, Supporting Information). One H<sub>2</sub>+methyl-compound-consuming *Verstraetearchaeota* OTU, based on the 16S rRNA gene, exhibited its highest relative abundance in the CH<sub>4</sub>+Fe<sup>3+</sup> treatment in P<sub>0-10</sub>, while two H<sub>2</sub>+CO<sub>2</sub>-consuming *Methanomicrobiales* OTUs, based on the *mcrA* gene, exhibited their highest relative abundance in the CH<sub>4</sub>+Mn<sup>4+</sup> treatment in P<sub>10-30</sub> (Figs. S7 and S8). Only one ANME-2D OTU, based on the *mcrA* gene, exhibited its highest relative abundance in the CH<sub>4</sub>+Mn<sup>4+</sup> treatment in P<sub>10-30</sub> (Fig. S8).

In contrast to the analyses at the DNA level, the relative *mcrA* expression of the different *mcrA*-carrying archaeal OTUs (mRNA level) differed between the CH<sub>4</sub> and CH<sub>4</sub>+Mn<sup>4+</sup> treatments (PerMANOVA,  $p < 0.05$ ) (Fig. S6E). In addition, the relative *mcrA* expression of the H<sub>2</sub>+methyl-compound-consuming methanogens was lower in the CH<sub>4</sub>+Fe<sup>3+</sup> treatment than in the other treatments (one-way ANOVA,  $p < 0.05$ ), while the *mcrA* expression of the other functional groups did not differ among the treatments (one-way ANOVA,  $p > 0.05$ ) (Fig. 1B). However, all the *mcrA* OTUs that exhibited their highest relative activity in the CH<sub>4</sub> treatment were H<sub>2</sub> consumers (Fig. 3A). In contrast, the relative expression of *mcrA* of one acetate-consuming *Methanosaetaceae* OTU was higher in the CH<sub>4</sub>+Fe<sup>3+</sup> and CH<sub>4</sub>+Mn<sup>4+</sup> treatments than in the CH<sub>4</sub>

treatment (Fig. 3B). Only one H<sub>2</sub>+CO<sub>2</sub> – consuming *Methanoregulaceae* OTU had its highest relative activity in CH<sub>4</sub>+Mn<sup>4+</sup> - treatment (Fig. 3C). The relative *mcrA* expression of the ANME-2D OTUs did not differ among the treatments.

In contrast to the archaea, the overall bacterial community differed between all three treatments in P<sub>0-10</sub> (PerMANOVA,  $p < 0.05$ ), while the CH<sub>4</sub>+Mn<sup>4+</sup> treatment differed from the other treatments in P<sub>10-30</sub> ( $p < 0.05$ ) (Figs. S6A and B). In addition, the relative abundance of the fermentative bacteria was higher in the CH<sub>4</sub>+Mn<sup>4+</sup> treatment than in the CH<sub>4</sub>+Fe<sup>3+</sup> treatment in P<sub>0-10</sub> (one-way ANOVA,  $p < 0.05$ ), while the metal-reducing bacteria differed in their relative abundance between treatments in P<sub>10-30</sub>, with the highest relative abundance being seen in the CH<sub>4</sub>+Mn<sup>4+</sup> treatment, the second highest in the CH<sub>4</sub>+Fe<sup>3+</sup> treatment, and the lowest in the CH<sub>4</sub> treatment (Figs. 2A and B). Neither the MOB<sub>s</sub>, nor the *NC 10* bacteria differed in their relative abundance between the treatments. There were, however, considerable differences in the responses of the OTUs to the treatments between the depth layers (Fig. 4). In P<sub>10-30</sub>, the OTUs that exhibited their highest abundances in the CH<sub>4</sub>+Fe<sup>3+</sup> and CH<sub>4</sub>+Mn<sup>4+</sup> treatments were mostly *Geobacter* and, for the CH<sub>4</sub>+Fe<sup>3+</sup> treatment, the *WS3* group (Figs. 4E and F). In contrast, in P<sub>0-10</sub>, the OTUs that had their highest abundance in the CH<sub>4</sub>+Fe<sup>3+</sup> and CH<sub>4</sub>+Mn<sup>4+</sup> treatments were mostly *Chloroflexi*, for the CH<sub>4</sub>+Fe<sup>3+</sup> treatment, *Betaproteobacteria*, and for the CH<sub>4</sub>+Mn<sup>4+</sup> treatment, *Elusimicrobia* (Figs. 4B and C). Of the known sulfur-cycling bacteria, one OTU within *Desulfobulbaceae* exhibited its highest abundance in the CH<sub>4</sub>+Mn<sup>4+</sup> treatment in P<sub>0-10</sub> (Fig. 4C).

## DISCUSSION

### CH<sub>4</sub> oxidation

The potential CH<sub>4</sub> oxidation in the anaerobic incubations was not enhanced by the increased availability of EAs, which contradicts our hypothesis that AOM coupled with the reduction of Fe<sup>3+</sup>, Mn<sup>4+</sup>, NO<sub>3</sub><sup>-</sup>, or SO<sub>4</sub><sup>2-</sup> would occur within the studied boreal lake sediments. The potential anaerobic CH<sub>4</sub> oxidation (0–0.3 nmol cm<sup>-3</sup> d<sup>-1</sup>) in the two investigated lakes was also very low when compared to those measured from other freshwater systems, including lakes (0–44 nmol cm<sup>-3</sup> d<sup>-1</sup>; Sivan *et al.* 2011; á Norði, Thamdrup and Schubert 2013; Deutzmann *et al.* 2014), nitrate-enriched pond microcosms (43–400 nmol cm<sup>-3</sup> d<sup>-1</sup>; á Norði and Thamdrup 2014), and wetland sediments (~0–30 nmol cm<sup>-3</sup> d<sup>-1</sup>; Segarra *et al.* 2013). It was only up to 2.5% of the maximum potential net CH<sub>4</sub> production (treatments amended with only CH<sub>4</sub>), whereas the aerobic MOBs could consume up to 60% of the maximum potential net CH<sub>4</sub> production.

CH<sub>4</sub> oxidation in anaerobic conditions can be mediated through AOM or TMO (Timmers *et al.* 2016). The independency of the potential CH<sub>4</sub> oxidation from the net potential CH<sub>4</sub> production in anaerobic conditions would suggest that CH<sub>4</sub> oxidation mainly took place through AOM at the profundal site. In contrast, the concurrent EA-induced decrease in the potentials of both CH<sub>4</sub> oxidation and net CH<sub>4</sub> production would suggest that CH<sub>4</sub> oxidation mainly took place through TMO at the littoral site (Table 2) (Moran *et al.* 2005; Meulepas *et al.* 2010; Timmers *et al.* 2016). However, the results of the CH<sub>2</sub>F<sub>2</sub> experiments necessitate a more in-depth discussion of the contribution of the different processes to CH<sub>4</sub> oxidation.

In the headspace concentrations used in this study (0.25%), CH<sub>2</sub>F<sub>2</sub> inhibits both methane mono-oxygenase and acetate-consuming methanogenesis (Miller, Sasson and Oremland 1998). Furthermore, CH<sub>2</sub>F<sub>2</sub> is rapidly depleted after only a few days of incubation, leading to the recovery of the inhibited processes (Miller, Sasson and Oremland 1998; Vicca *et al.* 2009), which agrees with our finding that CH<sub>2</sub>F<sub>2</sub>-induced inhibition of the CH<sub>4</sub> processes only took

place during the first week of incubation. Since ANME archaea use the  $H_2+CO_2$ -consuming methanogenesis pathway in reverse (Timmers *et al.* 2017), it can be speculated that their activity would not be inhibited by  $CH_2F_2$ . As  $NO_3^-$ , which is also rapidly reduced to  $NO_2^-$  in anoxic sediments, did not enhance  $CH_4$  oxidation, it can be suggested that the  $CH_2F_2$ -induced inhibition of the potential  $CH_4$  oxidation was not due to the inhibition of  $NO_2^-$  using *NC 10* bacteria, but rather due to the inhibition of aerobic MOB. Indeed, a risk of minor  $O_2$  contamination from the substrate/tracer injection or due to diffusion from the rubber septa or silicon sampling ports during incubations, which could induce microaerobic  $CH_4$  oxidation, has been acknowledged in previous incubation studies of anoxic freshwater samples (Blees *et al.* 2014; á Norði and Thamdrup 2014). It is, therefore, possible that the diffusion of trace amounts of  $O_2$  from the septa during the incubation period could not be fully prevented in this study, either. Although the observed net  $CH_4$  production and the measurements of ORP confirmed that anaerobic conditions, which are optimal for AOM, prevailed (Table S1, Fig. S1), it is possible that some active MOB that used the trace  $O_2$  were present on the surfaces of the sediment slurries. Thus, the inhibition of  $CH_4$  oxidation by  $CH_2F_2$  might indicate that besides AOM and TMO, MO could also have at least partially contributed to the  $CH_4$  oxidation. Therefore, our potential anaerobic  $CH_4$  oxidation rates could be better considered as overestimates, rather than underestimates, due to the possible contribution of MO. The possible MO activity also makes it impossible to separate TMO and AOM using the aforementioned criteria regarding the dependency between  $CH_4$  production and oxidation (Meulepas *et al.* 2010).

The relative abundance of anaerobic  $CH_4$  oxidizing bacteria (*NC 10*) and archaea (ANME-2D) was low, and neither their relative abundance, nor the relative abundance of the mRNA transcripts of ANME-2D was generally affected by increased  $Fe^{3+}$  or  $Mn^{4+}$ . Although

ANME-2D is generally found in various types of freshwater ecosystems (Welte *et al.* 2016), including lake sediments (L. Cadagno; Schubert *et al.* 2011), no comparable studies concerning its relative abundance in lake sediments exist. The relative abundance of *NC 10* bacteria (0.1–0.5% of bacteria) in the study lakes was within the same range (0.1–0.7 %) reported for aquaculture pond sediments, but it was lower than that measured from the sediments of a freshwater reservoir (1.0–1.5%) (Shen *et al.* 2016). In L. Constance, the relative abundance of *NC 10* bacteria was within the same range at a 12 m depth (~0–0.2%), but was substantially higher at ~80 m deep profundal depths (0.8–6.2%), where active  $\text{NO}_3^-/\text{NO}_2^-$ -driven AOM was detected as well (Deutzmann *et al.* 2014). The MOB also substantially outnumbered the *NC 10* in our study, whereas the opposite was true in the profundal sediments of L. Constance (Deutzmann *et al.* 2014). However, due to possible bias in reducing the *NC 10* sequences when using universal 16S rRNA gene primers (Ettwig *et al.* 2009), the relative abundance of the *NC 10* bacteria may be underestimated in our study. Our data do not allow for a direct comparison between the numbers of ANME-2D archaea and MOB. However, the total abundance of bacteria was more than ten times higher than that of archaea in previously studied lake sediments (Chan *et al.* 2005; Conrad *et al.* 2007; Conrad *et al.* 2010; Borrel *et al.* 2012), which strongly suggests that the ANME-2D archaea were substantially outnumbered by the MOB in our study.

Taken together, these results indicate that anaerobic  $\text{CH}_4$  oxidation is not important in mitigating  $\text{CH}_4$  emissions from the investigated lakes. This finding contrasts with previously studied freshwater sediments, where the volumetric AOM rates were >15% of the  $\text{CH}_4$  production rates or even larger than the  $\text{CH}_4$  production (Sivan *et al.* 2011; á Norði, Thamdrup and Schubert 2013; Segarra *et al.* 2013; á Norði and Thamdrup 2014). Thus, anaerobic  $\text{CH}_4$  oxidation can consume a substantial part of diffusive  $\text{CH}_4$  flux before it reaches the sediment-

water interface (á Norði, Thamdrup and Schubert 2013; Deutzmann *et al.* 2014; á Norði and Thamdrup 2014). However, apart from the 4.5 m depth in the iron-rich L. Ørn (á Norði, Thamdrup and Schubert 2013), lake sediment AOM activity has been detected from sites that are deeper and, therefore, probably more environmentally stable than our study sites, for instance, from a 37 m depth in L. Kinneret (Sivan *et al.* 2011) and a ~80 m depth in L. Constance (Deutzmann *et al.* 2014), as well as from a nitrate-enriched stable pond sediment microcosm (á Norði and Thamdrup 2014). Furthermore, AOM organisms (*NC 10* bacteria) were much more abundant in deep profundal sediments (at > 40 m and 80 m depths) than in shallower sites in L. Biwa and L. Constance, respectively (Kojima *et al.* 2012; Deutzmann *et al.* 2014), as well as in the sediments of a large reservoir than in small ponds (Shen *et al.* 2016). Accordingly, the higher abundance of AOM organisms at the profundal study site compared with the littoral study site is probably due to its greater depth. Thus, a plausible explanation for the discrepancy between our study and previous lake AOM studies could be variations in ecosystem stability, since slow-growing AOM microbes require stable environmental conditions for growth and to maintain their populations (Deutzmann *et al.* 2014).

Previous studies of ANME-2D archaea enrichment cultures have shown their potential to use  $\text{NO}_3^-$  (Haroon *et al.* 2013; Ettwig *et al.* 2016),  $\text{Fe}^{3+}$ , and  $\text{Mn}^{4+}$  (Ettwig *et al.* 2016) as EAs in anaerobic  $\text{CH}_4$  oxidation, while *NC 10* bacteria use  $\text{NO}_2^-$  (Ettwig *et al.* 2010). Environmental studies also suggest that ANME-2D are involved in  $\text{SO}_4^{2-}$ -dependent AOM (Timmers *et al.* 2016, 2017). However, our results showed that AOM organisms did not use inorganic EAs in the study sediments. We acknowledge that  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  (but not  $\text{SO}_4^{2-}$  and  $\text{NO}_3^-$ ) were the only EAs tested with the anaerobic samples from P<sub>0-10</sub> (Table 2). However, as the ANME-2D archaea had a higher relative abundance in P<sub>10-30</sub>, where the whole set of inorganic EAs were tested, the

possible effects of the EAs would have been even more likely to be detected. In addition, the *mcrA* expression of the ANME-2D was high *in situ* in P<sub>0-10</sub>, and it decreased during the incubations, irrespective of the treatment. This suggests that exhaustion of some important substrate other than CH<sub>4</sub> or inorganic EAs took place during the incubations. This could actually imply that ANME-2D archaea utilized organic EAs (e.g. humic compounds), which were not tested in this study. Despite organic EAs being very likely generated by the reduction of Mn<sup>4+</sup> or Fe<sup>3+</sup> (Lovley *et al.* 1996; Kappler *et al.* 2004) during the incubations, their quantity or quality (e.g. oxidation stage) was probably not sufficient to support AOM. Alternatively, it can be speculated that ANME-2D archaea did not drive AOM, but instead performed methanogenesis in the investigated sediments. Yet, previously studied ANME-2D archaea were not capable of methanogenesis (Ding *et al.* 2016; Timmers *et al.* 2017). More studies are definitely needed to reveal the metabolic capabilities of different ANME-2D species.

### **Production of CH<sub>4</sub> and TIC**

In contrast to the potential CH<sub>4</sub> oxidation, the potential net CH<sub>4</sub> production rates (0.6–82.5 nmol g<sup>-1</sup><sub>DW</sub>d<sup>-1</sup>; 0.1–11.7 nmol cm<sup>-3</sup> d<sup>-1</sup>) in the anaerobic incubations of the sediment slurries from the study lakes fell well within the range of reported CH<sub>4</sub> production rates in previous lake sediment studies: ~0–312 nmol g<sup>-1</sup><sub>DW</sub> d<sup>-1</sup> (Schulz, Matsuyama and Conrad 1997; Marotta *et al.* 2014; Karvinen, Lehtinen and Kankaala 2015) and ~1–23 nmol cm<sup>-3</sup> d<sup>-1</sup> (Sivan *et al.* 2011; á Norði, Thamdrup and Schubert 2013). The potential net CH<sub>4</sub> production rates also well represent the magnitude and variation of the estimated potential gross CH<sub>4</sub> production rates, since anaerobic CH<sub>4</sub> oxidation was always a minor fraction of the potential net CH<sub>4</sub> production (Table 2). As suggested by the lower C:N ratio (Table 1), the vegetated littoral site had a higher availability of labile OM than the profundal site. This explained the higher potential CH<sub>4</sub> and TIC production,

as well as the higher relative abundance of methanogens, at the littoral site. Furthermore, the higher and lower relative abundance of *Methanobacteriaceae* and *Methanoregulaceae*, respectively, at the littoral site was most likely due to differences in the adaptation of these two major H<sub>2</sub>+CO<sub>2</sub>-consuming freshwater methanogenic groups to variations in the substrate supply (Borrel *et al.* 2011). Together with the acetate-consuming *Methanosaetaceae*, these families were also the dominant methanogens in the investigated lakes, which agrees with the results obtained from many freshwater lakes (Borrel *et al.* 2011). The generally lower abundance and activity of the H<sub>2</sub>+methyl-compound-consuming methanogens also agrees with previous results obtained from lakes and ponds (Crevecoeur, Warwick and Lovejoy 2016; Fan and Xing 2016). Yet, to the best of our knowledge, this is the first report showing active *mcrA* expression (albeit low) by the very recently described phyla *Verstraetearchaeota* (Vanwonterghem *et al.* 2016) in any environment, as well as the second report after a study from Lake Pavin (Biderre-Petit *et al.* 2011), to show the active *mcrA* expression of *Methanomassiliicoccales* in lake sediments.

One possible mechanism behind the EA-induced decrease in methanogenesis is the direct inhibition of methanogens, as has been shown with NO<sub>3</sub><sup>-</sup> (Klüber and Conrad 1998) and Fe<sup>3+</sup> (van Bodegom, Scholten and Stams 2004), which could be expected to also take place with Mn<sup>4+</sup> (either directly or via the Mn<sup>4+</sup>-driven oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup>). However, the increased availability of NO<sub>3</sub><sup>-</sup> did not inhibit CH<sub>4</sub> production in the two deepest layers at the profundal site (P<sub>90-130</sub> and P<sub>490-310</sub>), as would have been expected, if it had directly inhibited the methanogens. In addition, neither the relative abundance, nor the *mcrA* expression of the H<sub>2</sub>+CO<sub>2</sub>-consuming methanogens were affected by Fe<sup>3+</sup> or Mn<sup>4+</sup>, as would have been expected, given that they are more sensitive than acetate-consuming methanogens to the inhibitory effects of Fe<sup>3+</sup> (van Bodegom, Scholten and Stams 2004). Yet, the Fe<sup>3+</sup>- and Mn<sup>4+</sup>-induced changes in the abundance

and activity of some individual methanogenic OTUs indicated that the slight inhibition of H<sub>2</sub>+CO<sub>2</sub>-consuming methanogens may have taken place, although it could not have been predominantly responsible for such large reductions in the potential net CH<sub>4</sub> production. The *mcrA* expression data also suggest that the H<sub>2</sub>+methyl-compound-consuming methanogens were slightly inhibited by Fe<sup>3+</sup>. However, due to their low abundance, the inhibition of their activity could not have resulted in the observed decreases in CH<sub>4</sub> production. Thus, the inhibition of methanogens was not an important factor underlying the decrease in the potential net CH<sub>4</sub> production.

The EA-induced decrease in CH<sub>4</sub> production was much more likely due to outcompetition of the methanogens for methanogenic substrates by anaerobically respiring bacteria (Klöpffel *et al.* 2014). The larger Fe<sup>3+</sup>- and Mn<sup>4+</sup>-induced changes seen in the bacterial communities rather than in the archaeal communities also support this view. The reduction of Mn<sup>4+</sup>, Fe<sup>3+</sup>, and NO<sub>3</sub><sup>-</sup> could also produce SO<sub>4</sub><sup>2-</sup>, S<sup>0</sup> (e.g. the cryptic sulfur cycle; Holmkvist, Ferdelman and Jørgensen 2011; Pester *et al.* 2012) and organic EAs (Lovley *et al.* 1996; Kappler *et al.* 2004), while that of Mn<sup>4+</sup> and NO<sub>3</sub><sup>-</sup> could produce Fe<sup>3+</sup> (Canfield, Kristensen and Thamdrup 2005). Therefore, at least part of the Fe<sup>3+</sup>-, Mn<sup>4+</sup>-, and NO<sub>3</sub><sup>-</sup>-induced decrease in CH<sub>4</sub> production could also be due to anaerobic respiration coupled with EAs with a lower reduction potential. The negligible effect of NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> on the potential net CH<sub>4</sub> production in the two deepest layers (P<sub>90-130</sub> and P<sub>390-410</sub>) of the profundal zone (Table 2) is probably due to the relatively low number of competing anaerobically respiring bacteria. Unfortunately, molecular data were not collected from these layers. It can, however, be speculated that the populations of anaerobically respiring bacteria in the deep layers is low due to the lack of EAs. As EAs are already very efficiently consumed in the surface sediments, the two deepest layers of the

profundal zone have not been exposed to oxidized forms of nitrogen and sulfur for at least 1000 years.

As hypothesized, both  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  decreased the potential net production rates of  $\text{CH}_4$  and TIC in  $\text{P}_{0-10}$ . This strongly suggests that besides increasing anaerobic respiration,  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  also induced decreases in methanogenesis via increasing OM recalcitrance and protecting it from microbial degradation (Lalonde *et al.* 2012; Karvinen, Lehtinen and Kankaala 2015; Estes *et al.* 2017). The effects of  $\text{Mn}^{4+}$  could have also been partially caused by  $\text{Fe}^{3+}$  produced via the  $\text{Mn}^{4+}$ -induced oxidation of the indigenous  $\text{Fe}^{2+}$  (Canfield, Kristensen and Thamdrup 2005). Furthermore, the decreased heterotrophic respiration processes might have led to an increase in the rate of the  $\text{CO}_2$ -fixing chemolithoautotrophic processes (via competitive release), which further contributed to the reduction in the potential net TIC production. Indeed, the uncoupling of a large part of the  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  reduction from the TIC production in the  $\text{Fe}^{3+}$ - and  $\text{Mn}^{4+}$ -amended treatments indicates that a significant chemolithoautotrophic and abiotic  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  reduction took place. The susceptibility of the sediment OM to the effects of  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  probably depends on the sediment type, as indicated by the lack of a  $\text{Fe}^{3+}$ - and  $\text{Mn}^{4+}$ -induced decrease in the potential net TIC production in  $\text{P}_{10-30}$  (Table 2). As highly labile OM fractions (proteins and carbohydrates) were suggested to preferentially bind to iron in the sediments (Lalonde *et al.* 2012), the difference between the study layers was probably due to the higher lability of OM (as indicated by the lower C:N ratio; Table 1) in  $\text{P}_{0-10}$ .

In accordance with the differences in the effects of increased  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  on  $\text{CH}_4$  and TIC production, the responses of the bacterial communities also differed between the study layers ( $\text{P}_{0-10}$  and  $\text{P}_{10-30}$ ) at the profundal site. There are several mechanisms which could explain this and thus are briefly discussed here. The lack of a  $\text{Fe}^{3+}$ - and  $\text{Mn}^{4+}$ -induced increase in the

relative abundance of the most typical metal-reducing sediment bacterial genus in P<sub>0-10</sub>, *Geobacter* (Coates *et al.* 1996; Lovley 2006), could be due to the decreased OM availability in that layer. OTUs belonging to *Chloroflexi*, *Betaproteobacteria*, and *Elusimicrobia* were probably more competitive than *Geobacter* in low OM conditions. Most known species of *Chloroflexi* (e.g. Yamada and Sekiguchi 2009) and *Elusimicrobia* (e.g. Herlemann *et al.* 2009) are fermentative. Therefore, besides being more competitive than *Geobacter* in low OM conditions, their increase implies that they were fermenting and hence gained an advantage over the other fermenters by using Fe<sup>3+</sup> and Mn<sup>4+</sup> as minor electron sinks (Lovley 2006). However, the metabolic capabilities of many phyla are not sufficiently understood. For example, *Chloroflexi* may also harbor species capable of Fe<sup>3+</sup> respiration (Kawaichi *et al.* 2013), while the Fe<sup>3+</sup> respiration capability is dispersed into at least three orders within *Betaproteobacteria* (Pronk *et al.* 1992; Cummings *et al.* 1999; Finneran, Johnsen and Lovley 2003). Thus, it is possible that OTUs showing a Fe<sup>3+</sup>- or Mn<sup>4+</sup>-induced increase in their relative abundance in P<sub>0-10</sub> were also using Fe<sup>3+</sup> or Mn<sup>4+</sup> as EAs in anaerobic respiration.

However, as discussed above, the suppression of heterotrophic processes could have led to an increase in Fe<sup>3+</sup>- and Mn<sup>4+</sup>-reducing chemolithoautotrophic processes in P<sub>0-10</sub>. Consequently, differences in the responses of the bacterial communities between the investigated layers may partially reflect the higher contribution of chemolithoautotrophic organisms in P<sub>0-10</sub>. Furthermore, both chemolithoautotrophic and abiotic Fe<sup>3+</sup> and Mn<sup>4+</sup> reduction can increase the availability of SO<sub>4</sub><sup>2-</sup>, S<sup>0</sup>, and organic EAs (Lovley *et al.* 1996; Kappler *et al.* 2004; Canfield, Kristensen and Thamdrup 2005; Pester *et al.* 2012). Therefore, the differences in the Fe<sup>3+</sup>- and Mn<sup>4+</sup>-induced responses of the bacterial communities between the study layers could also be partially due to the larger contribution of anaerobically respiring bacteria capable of reducing

$\text{SO}_4^{2-}$ ,  $\text{S}^0$ , and organic EA in  $\text{P}_{0-10}$ . Only one OTU related to a taxon that contains species capable of  $\text{SO}_4^{2-}$  reduction and  $\text{S}^0$  disproportionation (*Desulfobulbaceae*; Finster 2008) was slightly affected by  $\text{Mn}^{4+}$  in  $\text{P}_{0-10}$ . However, many freshwater  $\text{SO}_4^{2-}$  reducers belong to unknown phylogenetic lineages (Pester *et al.* 2012). Furthermore, besides the known  $\text{Fe}^{3+}$ -reducing bacteria (Lovley 2006), the bacteria that utilize organic EAs are mostly unknown. In fact, a large fraction of the bacteria that reduced organic EAs (e.g. humic acids) could not use  $\text{Fe}^{3+}$  in a previously studied lake sediment (Kappler *et al.* 2004). Further studies are therefore needed to assess the role of each discussed mechanism in explaining the bacterial community variations induced by the increased  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$ . These studies could use, for example,  $^{13}\text{C}$  labelling to reveal the role of chemolithoautotrophy ( $^{13}\text{C}$  transfer to bulk biomass) and the active chemolithoautotrophic organisms (stable isotope probing of DNA and RNA). The studies could also individually test the effects of the addition of each inorganic EA ( $\text{Fe}^{3+}$ ,  $\text{Mn}^{4+}$ ,  $\text{SO}_4^{2-}$ , and  $\text{S}^0$ ) and some organic EA compounds (e.g. humic acids; anthraquinone-2, -6, disulfonate) on the microbial community structure, as well as on the potential (metagenomics) and actively expressed (metatranscriptomics) metabolic pathways, for example, the pathways of fermentation and anaerobic respiration.

## CONCLUSION

This study tested the effects of increased  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  availability on the structure of microbial communities, as well as the effects of increased  $\text{Fe}^{3+}$ ,  $\text{Mn}^{4+}$ ,  $\text{NO}_3^-$ , and  $\text{SO}_4^{2-}$  on the potential  $\text{CH}_4$  oxidation and net production rates of  $\text{CH}_4$  and TIC in boreal lake sediments for the first time.

The results suggest that anaerobic  $\text{CH}_4$  oxidation (via AOM or TMO) was not an important factor in reducing  $\text{CH}_4$  emissions from the sediments of the two shallow study lakes. The results

further suggest that the regeneration of the sediment EA pool during oxidation events via water-column mixing, as well as via biological and physical turbation, may suppress CH<sub>4</sub> emissions from the lake sediments by decreasing methanogenesis and increasing MO, but not by increasing AOM. Comparing our results with those of previous lake sediment AOM studies also suggests that the abundance and activity of AOM microbes might decrease with the decreasing environmental stability associated with decreasing water column depth. In addition to the outcompetition of methanogens for methanogenic substrates by anaerobically respiring bacteria, this study suggests that increased protection of OM from microbial degradation (i.e. increased OM recalcitrance by Fe<sup>3+</sup> and Mn<sup>4+</sup>) is also a very important component of the Fe<sup>3+</sup>- and Mn<sup>4+</sup>-induced reduction in methanogenesis in lake sediments. Yet, the magnitude and controlling factors (e.g. sediment OM quality as well as Fe and Mn content) of these two mechanisms, which decrease methanogenesis, remain unclear. In order to better constrain both global and regional CH<sub>4</sub> budgets, it is especially important to determine whether AOM has any ecological relevance as well as to find out the importance and controlling factors of the different methanogenesis-decreasing mechanisms in the numerous small and shallow lakes and ponds in boreal and tundra landscapes, which represent globally significant sources of CH<sub>4</sub> to the atmosphere (Wik *et al.* 2016). Future studies should also specifically address the role of organic EAs in affecting methane production and consumption in lake sediments.

This study offers new insights into the mechanisms preserving OM in boreal lake sediments. They are effective C sinks due to the low temperature and recalcitrant nature of OM, which serve to constrain the microbial metabolism (Kortelainen *et al.* 2004; Gudasz *et al.* 2012). As noted here and in previous studies (Lalonde *et al.* 2012; Estes *et al.* 2017), the reactions of OM with Fe<sup>3+</sup> and Mn<sup>4+</sup> further increase OM preservation. However, the observed increase in C

degradation (TIC production), which was solely driven by the addition of O<sub>2</sub> (and not by the addition of any other EA) in the study sediments, also explains why anoxia plays an important role in retaining C in boreal lake sediments. This suggests that O<sub>2</sub>-consuming microbial processes are crucial in inducing sediment C storage. Besides generating anoxia, they increase OM recalcitrance via consuming labile OM and via oxidizing Fe<sup>2+</sup> and Mn<sup>2+</sup> to Fe<sup>3+</sup> and Mn<sup>4+</sup>, which react with OM (Lalonde *et al.* 2012; Estes *et al.* 2017). Whether or not the Fe<sup>3+</sup>- and Mn<sup>4+</sup>-induced inhibition of heterotrophic OM degradation processes could also benefit the chemolithoautotrophic processes that additionally increase sediment C storage via CO<sub>2</sub> fixation requires further study.

## **FUNDING**

This work was supported by The Academy of Finland [136455 and 140964 to HN, 286642 to AJR, and 260797 to MT] and Maj & Tor Nessling foundation [AK and PK].

## **ACKNOWLEDGEMENTS**

We thank S. Hietanen and B. Thamdrup for helpful comments on this manuscript and K. Ratilainen for his help with collecting sediment samples. We also thank the two anonymous reviewers and the editor for their constructive comments.

*Conflict of interest.* None declared.

## **REFERENCES**

- Altschul SF, Gish W, Miller W *et al.* Basic local alignment search tool. *J Mol Biol* 1990;**215**:403-410.
- Anderson MJ. A new method for non-parametric multi-variate analysis of variance. *Austral Ecol* 2001;**26**:32-46.
- Bastviken D, Cole J, Pace M *et al.* Methane emissions from lakes: dependence of lake characteristics, two regional assessments, and a global estimate. *Global Biogeochem Cy* 2004;**18**:GB4009.
- Beal EJ, House CH, Orphan VJ. Manganese- and iron-dependent marine methane oxidation. *Science* 2009;**325**:184-187.
- Bedard C, Knowles R. Hypolimnetic O<sub>2</sub> consumption, denitrification, and methanogenesis in a thermally stratified lake. *Can J Fish Aquat Sci* 1991;**48**:1048–1054.
- Biderre-Petit C, Jézéquel D, Dugat-Bony E. *et al.* Identification of microbial communities involved in the methane cycle of a freshwater meromictic lake. *FEMS Microbiol Ecol* 2011;**77**:533-545.
- Blazewicz SJ, Petersen DG, Waldrop MP *et al.* Anaerobic oxidation of methane in tropical and boreal soils: ecological significance in terrestrial methane cycling. *J Geophys Res* 2012;**117**:G02033.
- Blees J, Niemann H, Wenk CB *et al.* Micro-aerobic bacterial methane oxidation in the chemocline and anoxic water column of deep south-Alpine Lake Lugano (Switzerland). *Limnol Oceanogr* 2014;**59**:311-324

- Borrel G, Jézéquel D, Biderre-Petit C *et al.* Production and consumption of methane in freshwater lake ecosystems. *Res Microbiol* 2011;**162**:832-847.
- Borrel G, Lehours A-C, Crouzet O *et al.* Stratification of archaea in the deep sediments of a freshwater meromictic lake: vertical shift from methanogenic to uncultured archaeal lineages. *PLoS One* 2012;**7**:e43346.
- Borrel G, O'Toole PW, Harris HMB *et al.* Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. *Genom Biol Evol* 2013;**5**:1769-1780.
- Bretz KA, Whalen SC. Methane cycling dynamics in sediments of Alaskan Arctic Foothill lakes. *Inland Waters* 2014;**4**:65-78.
- Canfield DE, Kristensen E, Thamdrup B. *Aquatic Geomicrobiology*. San Diego: Elsevier Academic Press, 2005.
- Chan OC, Claus P, Casper P *et al.* Vertical distribution of structure and function of the methanogenic archaeal community in Lake Dagow sediment. *Environ Microbiol* 2005;**7**:1139-1149.
- Chowdhury TR, Dick RP. Ecology of aerobic methanotrophs in controlling methane fluxes from wetlands. *Appl Soil Ecol* 2013;**65**:8-22.
- Coates JD, Phillips EJP, Lonergan DJ *et al.* Isolation of *Geobacter* species from diverse sedimentary environments. *Appl Environ Microbiol* 1996;**62**:1531-1536.
- Conrad R. Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiol Ecol* 1999;**28**:193-202.

Conrad R, Chan OC, Claus P *et al.* Characterization of methanogenic Archaea and stable isotope fractionation during methane production in the profundal sediment of an oligotrophic lake (Lake Stechlin, Germany). *Limnol Oceanogr* 2007;**52**:1393-1406.

Conrad R, Klose M, Claus P *et al.* Methanogenic pathway, <sup>13</sup>C isotope fractionation, and archaeal community composition in the sediment of two clear-water lakes of Amazonia. *Limnol Oceanogr* 2010;**55**:689-702.

Crevecoeur S, Warwick VF, Lovejoy C. Environmental selection of planktonic methanogens in permafrost thaw ponds. *Sci Rep* 2016;**6**:31312.

Cummings DE, Caccavo Jr F, Spring S *et al.* *Ferribacterium limneticum*, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Arch Microbiol* 1999;**171**:183-188.

Deutzmann JS, Stief P, Brandes J *et al.* Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake. *PNAS* 2014;**111**:18273-18278.

Ding J, Fu L, Ding Z-W *et al.* Experimental evaluation of the metabolic reversibility of ANME-2d between anaerobic methane oxidation and methanogenesis. *Appl Microbiol Biotechnol* 2016;**100**:6481-6490.

Edgar RC, Haas BJ, Clemente JC *et al.* UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011;**27**:2194-2200.

Estes ER, Andeer PF, Nordlund D *et al.* Biogenic manganese oxides as reservoirs of organic carbon and proteins in terrestrial and marine environments. *Geobiology* 2017;**15**:158-172.

Ettwig KF, Butler MK, Le Paslier D *et al.* Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 2010;**464**:543-548.

Ettwig KF, van Alen T, van de Pas-Schoonen KT *et al.* Enrichment and molecular detection of denitrifying methanotrophic bacteria of the *NC10* phylum. *Appl Environ Microbiol* 2009;**75**:3656-3662.

Ettwig KF, Zhu B, Speth D *et al.* Archaea catalyze iron-dependent anaerobic oxidation of methane. *PNAS* 2016;**113**:12792-12796.

Evans PN, Parks DH, Chadwick GL *et al.* Methane metabolism in the archaeal phylum *Bathyarchaeota* revealed by genome-centric metagenomics. *Science* 2015;**350**:434-438.

Fan X, Xing P. Differences in the composition of archaeal communities in sediments from contrasting zones of Lake Taihu. *Front Microbiol* 2016;**7**:1510.

Finneran KT, Johnsen CV, Lovley DR. *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *Int J Syst Evol Microbiol* 2003;**53**:669-673.

Finster K. Microbiological disproportionation of inorganic sulfur compounds. *J Sulfur Chem* 2008;**29**:281-292.

Gantner S, Andersson AF, Alonso-Sáez L *et al.* Novel primers for 16S rRNA based archaeal community analyses in environmental samples. *J Microbiol Meth* 2011;**84**:12-18.

Griffiths RI, Whiteley AS, O'Donnell AG *et al.* Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* 2000;**66**:5488–5491.

Gudasz C, Bastviken D, Premke K *et al.* Constrained microbial processing of allochthonous organic carbon in boreal lake sediments. *Limnol Oceanogr* 2012;**57**:163-175.

Hammer Ø, Harper DAT, Ryan PD. PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 2001;**4**:9pp.

Hanson RS, Hanson TE. Methanotrophic bacteria. *Microbiol Rev* 1996;**60**:439-471.

Haroon MF, Hu S, Shi Y *et al.* Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 2013;**500**:567-570.

Herlemann DPR, Geissinger O, Ikeda-Ohtsubo W *et al.* Genomic analysis of “*Elusimicrobium minutum*,” the first cultivated representative of the phylum “*Elusimicrobia*” (Formerly Termite Group 1). *Appl Environ Microbiol* 2009;**75**:2841-2849.

Hiscock K, Bense V. *Hydrogeology: Principles and Practice*. John Wiley & Sons, 2014.

Holmer M, Storkholm P. Sulphate reduction and sulphur cycling in lake sediments: a review. *Freshwater Biology* 2001;**46**:431-451.

Holmkvist L, Ferdelman TG, Jørgensen BB. A cryptic sulfur cycle driven by iron in the methane zone of marine sediment (Aarhus Bay, Denmark). *Geochim Cosmochim Acta* 2011;**75**:3581-3599.

Huerta-Diaz MA, Tessier A, Carignan R. Geochemistry of trace metals associated with reduced sulfur in freshwater sediments. *Applied Geochemistry* 1998;**13**:213-233.

Huse SM, Welch DM, Morrison HG *et al.* Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ Microbiol* 2010;**12**:1889–1898.

Juutinen S, Alm J, Larmola T *et al.* Major implication of the littoral zone for methane release from boreal lakes. *Global Biogeochem Cy* 2003;**17**:1117.

Kallistova AY, Goel G, Nozhevnikova AN. Microbial diversity of methanogenic communities in the systems for anaerobic treatment of organic waste. *Microbiology* 2014;**83**:462-483.

Kankaala P, Taipale S, Nykänen H *et al.* Oxidation, efflux, and isotopic fractionation of methane during autumnal turnover in a polyhumic, boreal lake. *J Geophys Res* 2007;**112**:G02033.

Kappler A, Benz M, Schink B *et al.* Electron shuttling via humic acids in microbial iron(III) reduction in a freshwater sediment. *FEMS Microbiol Ecol* 2004;**47**:85-92.

Karvinen A, Lehtinen L, Kankaala P. Variable effects of iron (Fe (III)) additions on potential methane production in boreal lake littoral sediments. *Wetlands* 2015;**35**:137-146.

Kawaichi S, Ito N, Kamikawa R *et al.* *Ardenticatena maritima* gen. nov., sp. nov., a ferric iron- and nitrate-reducing bacterium of the phylum ‘*Chloroflexi*’ isolated from an iron-rich coastal hydrothermal field, and description of *Ardenticatena* classis nov. *Int J Syst Evol Microbiol* 2013;**63**:2992-3002.

Klüber HD, Conrad R. Effects of nitrate, nitrite, NO and N<sub>2</sub>O on methanogenesis and other redox processes in anoxic rice field soil. *FEMS Microbiol Ecol* 1998;**25**:301-318.

- Klüpfel L, Piepenbrock A, Kappler A *et al.* Humic substances as fully regenerable electron acceptors in recurrently anoxic environments. *Nature Geosci* 2014;**7**:195-200.
- Knittel K, Boetius A. Anaerobic oxidation of methane: progress with an unknown process. *Annu Rev Microbiol* 2009;**63**:311-334.
- Kojima H, Tsutsumi M, Ishikawa K *et al.* Distribution of putative denitrifying methane oxidizing bacteria in a sediment of a freshwater lake, Lake Biwa. *Syst Appl Microbiol* 2012;**35**:233-238.
- Kortelainen P, Pajunen H, Rantakari M *et al.* A large carbon pool and small sink in boreal Holocene lake sediments. *Glob Change Biol* 2004;**10**:1648-1653.
- Lalonde K, Mucci A, Ouellet A *et al.* Preservation of organic matter in sediments promoted by iron. *Nature* 2012;**483**:198–200.
- Lazar CS, Biddle JF, Meador TB *et al.* Environmental controls on intragroup diversity of the uncultured benthic *archaea* of the miscellaneous Crenarchaeotal group lineage naturally enriched in anoxic sediments of the White Oak River estuary (North Carolina, USA). *Environ Microbiol* 2015;**17**:2228-2238.
- Lovley D. Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. *Prokaryotes* 2006;**2**:635-658.
- Lovley DR, Coates JD, Blunt-Harris EL *et al.* Humic substances as electron acceptors for microbial respiration. *Nature* 1996;**382**:445-448.
- Lovley DR, Phillips EJP. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl Environ Microbiol* 1986;**51**:683-689.

Lovley DR, Phillips EJP. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* 1988;**54**:1472-1480.

Marotta H, Pinho L, Gudasz C *et al.* Greenhouse gas production in low-latitude lake sediments responds strongly to warming. *Nature Climate Change* 2014;**4**:467-470.

Mattson MD, Likens GE. Redox reactions of organic matter decomposition in a soft water lake. *Biogeochem* 1993;**19**:149–172.

McArdle BH, Anderson MJ. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* 2001;**82**:290–297.

McCune B, Mefford MJ. *PC-ORD. Multivariate analysis of ecological data.* Version 6. MjM Software, Gleneden Beach, Oregon, USA, 2011.

Meulepas RJW, Jagersma CG, Zhang Y *et al.* Trace methane oxidation and the methane dependency of sulfate reduction in anaerobic granular sludge. *FEMS Microbiol Ecol* 2010;**72**:261-271.

Miller LG, Sasson C, Oremland RS. Difluoromethane, a new and improved inhibitor of methanotrophy. *Appl Environ Microbiol* 1998;**64**:4357-4362.

Molot LA, Dillon PJ. Storage of terrestrial carbon in boreal lake sediments and evasion to the atmosphere. *Global Biogeochem Cy* 1996;**10**:483-492.

Moran JJ, House CH, Freeman KH *et al.* Trace methane oxidation studied in several Euryarchaeota under diverse conditions. *Archaea* 2005;**1**:303-309.

- Moran JJ, House CH, Thomas B *et al.* Products of trace methane oxidation during nonmethylophilic growth by *Methanosarcina*. *J Geophys Res* 2007;**112**:G02011.
- Nazaries L, Murrell JC, Millard P *et al.* Methane, microbes and models: fundamental understanding of the soil methane cycle for future predictions. *Environ Microbiol* 2013;**15**:2395-2417.
- á Norði K, Thamdrup B. Nitrate-dependent anaerobic methane oxidation in a freshwater sediment. *Geochim Cosmochim Acta* 2014;**132**:141-150.
- á Norði K, Thamdrup B, Schubert CJ. Anaerobic oxidation of methane in an iron-rich Danish freshwater lake sediment. *Limnol Oceanogr* 2013;**58**:546-554.
- Nykänen H, Peura S, Kankaala P *et al.* Recycling and fluxes of carbon gases in a stratified boreal lake following experimental carbon addition. *Biogeosci Discuss* 2014;**11**:16447–16495.
- Pajunen H. *Järvisedimentit kuiva-aineen ja hiilen varastona*. Geological Survey of Finland, Report of Investigation 160, 2004. (In Finnish with English summary: Lake sediments as a store of dry matter and carbon)
- Pester M, Knorr K-H, Friedrich MW *et al.* Sulfate-reducing microorganisms in wetlands – fameless actors in carbon cycling and climate change. *Front Microbiol* 2012;**3**:72.
- Pronk JT, de Bruyn JC, Bos P *et al.* Anaerobic growth of *Thiobacillus ferrooxidans*. *Appl Environ Microbiol* 1992;**58**:2227-2230.
- Rissanen AJ, Tirola M, Hietanen S *et al.* Interlake variation and environmental controls of denitrification across different geographical scales. *Aquat Microb Ecol* 2013;**69**:1-16.

Salonen, K. 1981. Rapid and precise determination of total inorganic carbon and some gases in aqueous solutions. *Water Res* 1981;**15**:403-406.

Scheller S, Yu H, Chadwick GL *et al.* Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. *Science* 2016;**351**:703-707.

Schloss PD, Gevers D, Westcott SL. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS One* 2011;**6**:e27310.

Schloss PD, Westcott SL, Ryabin T *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;**75**:7537-7541.

Schulz S, Matsuyama H, Conrad R. Temperature dependence of methane production from different precursors in a profundal sediment (Lake Constance). *FEMS Microbiol Ecol* 1997;**22**:207-213.

Schubert CJ, Vazquez F, Lösekann-Behrens T *et al.* Evidence for anaerobic oxidation of methane in sediments of a freshwater system (Lago di Cadagno). *FEMS Microbiol Ecol* 2011;**76**:26–38.

Segarra KEA, Comerford C, Slaughter J *et al.* Impact of electron acceptor availability on the anaerobic oxidation of methane in coastal freshwater and brackish wetland sediments. *Geochim Cosmochim Acta* 2013;**115**:15-30.

Segata N, Izard J, Waldron L *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;**12**:R60.

- Shen L, Wu H, Gao Z *et al.* Comparison of community structures of *Candidatus* Methyloirabilis oxyfera-like bacteria of NC10 phylum in different freshwater habitats. *Sci Rep* 2016;**6**:25647.
- Sivan O, Adler M, Pearson A *et al.* Geochemical evidence for iron-mediated anaerobic oxidation of methane. *Limnol Oceanogr* 2011;**56**:1536-1544.
- Stumm W, Morgan JJ. *Aquatic chemistry. An introduction emphasizing chemical equilibria in natural waters.* New York: John Wiley & Sons, 1981.
- Timmers PH, Suarez-Zuluaga DA, van Rossem M *et al.* Anaerobic oxidation of methane associated with sulfate reduction in a natural freshwater gas source. *ISME J* 2016;**10**:1400-1412.
- Timmers PHA, Welte CU, Koehorst JJ *et al.* Reverse methanogenesis and respiration in methanotrophic archaea. *Archaea* 2017; **2017**:article ID 1654237
- Van Bodegom PM, Scholten JCM, Stams AJM. Direct inhibition of methanogenesis by ferric iron. *FEMS Microbiol Ecol* 2004;**49**:261-268.
- Vanwonterghem I, Evans PN, Parks DH. *et al.* Methyloirabilis-like methanogenesis discovered in the archaeal phylum Verstraetearchaeota. *Nat Microbiol* 2016;**1**:16170; doi:10.1038/nmicrobiol.2016.170.
- Vicca S, Flessa H, Lofthfield N *et al.* The inhibitory effect of difluoromethane on CH<sub>4</sub> oxidation in reconstructed peat columns and side-effects on CO<sub>2</sub> and N<sub>2</sub>O emissions at two water levels. *Soil Biol Biochem* 2009;**41**:1117-1123.
- Wang Q, Garrity GM, Tiedje JM *et al.* Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;**73**:5261-5267.

Wang Q, Quensen III JF, Fish JA *et al.* Ecological patterns of *nifH* genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. *mBio* 2013;**4**:e00592-13.

Wasmund K, Schreiber L, Lloyd KG *et al.* Genome sequencing of a single cell of the widely distributed marine subsurface *Dehalococcoidia*, phylum *Chloroflexi*. *ISME J* 2014;**8**:383-397.

Welte CU, Rasigraf O, Vaksmaa A *et al.* Nitrate- and nitrite-dependent anaerobic oxidation of methane. *Environ Microbiol Rep* 2016;**8**:941-955.

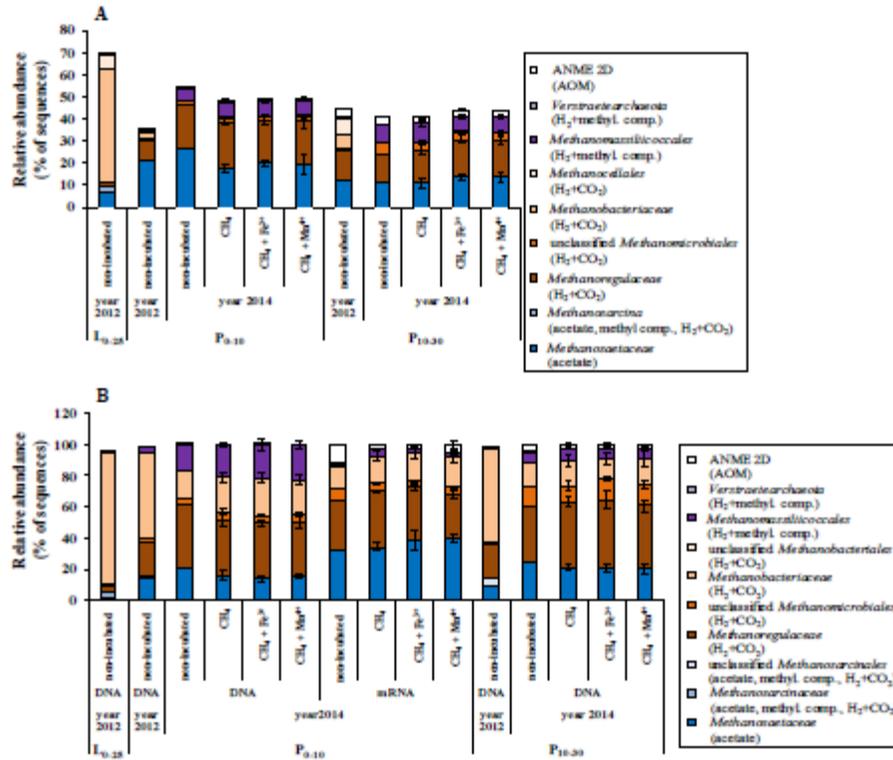
Wik M, Varner RK, Anthony KW *et al.* Climate sensitive northern lakes and ponds are critical components of methane release. *Nature Geosci* 2016;**9**:99-105.

Wrighton KC, Castelle CJ, Wilkins MJ *et al.* Metabolic interdependencies between phylogenetically novel fermenters and respiratory organisms in an unconfined aquifer. *ISME J* 2014;**8**:1452-1463.

Yamada T, Sekiguchi Y. Cultivation of uncultured *Chloroflexi* subphyla: Significance and ecophysiology of formerly uncultured *Chloroflexi* ‘Subphylum I’ with natural and biotechnological relevance. *Microbes Environ* 2009;**24**:205-216.

Zheng H, Dietrich C, Radek R *et al.* *Endomicrobium proavitum*, the first isolate of *Endomicrobia* class. nov. (phylum *Elusimicrobia*) – an ultramicrobacterium with an unusual cell cycle that fixes nitrogen with a Group IV nitrogenase. *Environ Microbiol* 2016;**18**:191-204.

Fig 1.



**Figure 1.** Relative abundances (average +/- SD) of the taxonomic groups of (A) methanogenic/methanotrophic archaea based on 16S rRNA sequencing and (B) methanogenic/methanotrophic archaea (DNA) and active methanogenic/methanotrophic archaea (mRNA) based on *mcrA* gene and *mcrA* mRNA transcript sequencing before (non-incubated) and after incubations with either CH<sub>4</sub>, CH<sub>4</sub>+Fe<sup>3+</sup> or CH<sub>4</sub>+Mn<sup>4+</sup> amendments. L<sub>0-25</sub>, P<sub>0-10</sub> and P<sub>10-30</sub> denote 0 – 25 cm layer of the littoral and 0 – 10 cm and 10 – 30 cm layers of the profundal study site, respectively. The substrates for methanogenesis as well as the potential AOM function are shown in brackets after the taxonomic information.

Fig. 2.

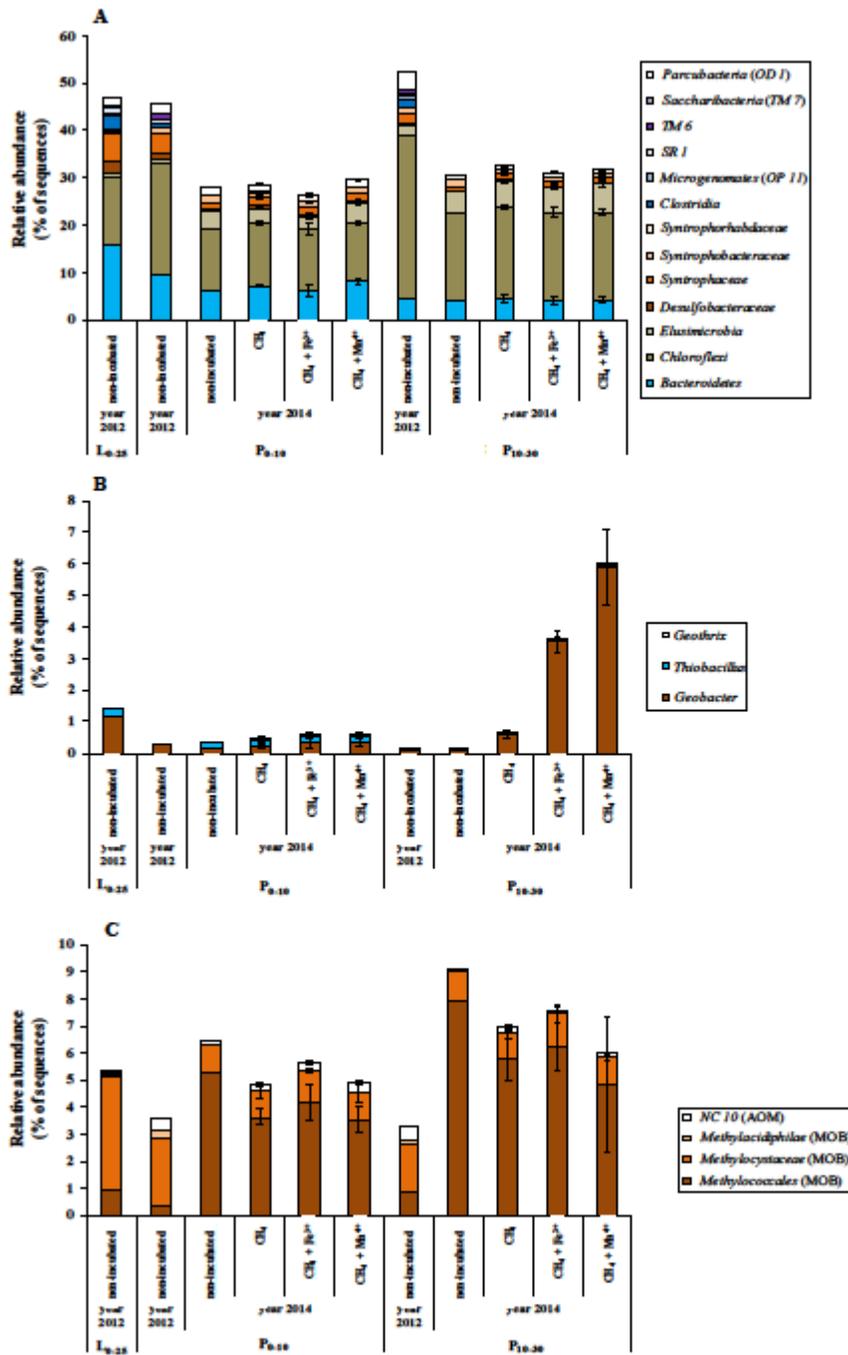
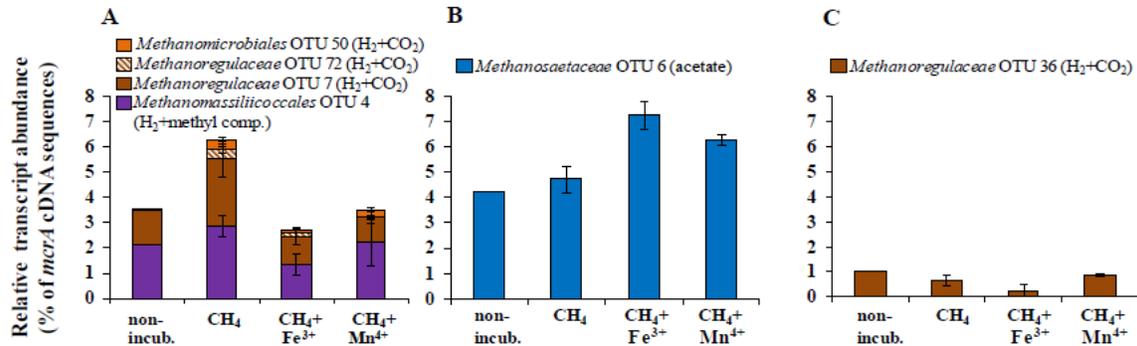


Figure 2. Relative abundances (average +/- SD) of the taxonomic groups of bacteria divided into (A)

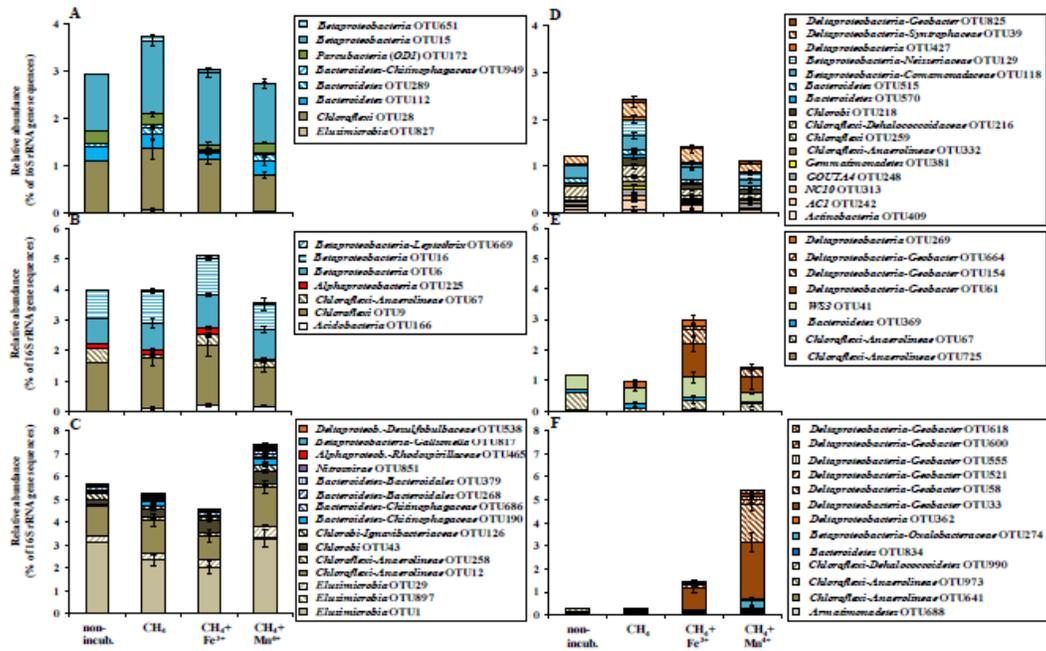
fermentative bacteria, (B)  $\text{Fe}^{3+}/\text{Mn}^{4+}$  (metal) reducing bacteria, and (C) aerobic (MOB) and anaerobic (AOM) methanotrophic bacteria before (non-incubated) and after incubations with either  $\text{CH}_4$ ,  $\text{CH}_4+\text{Fe}^{3+}$  or  $\text{CH}_4+\text{Mn}^{4+}$  amendments. L<sub>0-25</sub>, P<sub>0-10</sub> and P<sub>10-30</sub> denote 0 – 25 cm layer of the littoral and 0 – 10 cm and 10 – 30 cm layers of the profundal study site, respectively.

Fig. 3.



**Figure 3.** Relative activity (average  $\pm$  SD) and taxonomic affiliations (and methanogenic substrate) of the *mcrA* OTUs (based on the mRNA transcripts) that differed in their relative activity among the  $\text{CH}_4$ ,  $\text{CH}_4+\text{Fe}^{3+}$ , or  $\text{CH}_4+\text{Mn}^{4+}$  treatments after the incubation of sediment slurry samples from P<sub>0-10</sub> (linear discriminant analysis [LDA] effect size [LEfSe] method  $p < 0.05$ ). The OTUs are grouped into three subfigures according to the treatment in which they exhibited the highest relative abundance in comparison to the other treatments: (A)  $\text{CH}_4$ , (B)  $\text{CH}_4+\text{Fe}^{3+}$ , or (C)  $\text{CH}_4+\text{Mn}^{4+}$ .

Fig. 4.



**Figure 4.** Relative abundance (average  $\pm$  SD) and taxonomic affiliations of the bacterial 16S rRNA gene OTUs that differed in their relative abundance among the CH<sub>4</sub>, CH<sub>4</sub>+Fe<sup>3+</sup>, or CH<sub>4</sub>+Mn<sup>4+</sup> treatments after the incubation of sediment slurry samples from P<sub>0-10</sub> (A–C) and P<sub>10-30</sub> (D–F) (linear discriminant analysis [LDA] effect size [LEfSe] method  $p < 0.05$ ). The OTUs are grouped into three rows according to the treatment (CH<sub>4</sub>, CH<sub>4</sub>+Fe<sup>3+</sup>, or CH<sub>4</sub>+Mn<sup>4+</sup>) in which they exhibited the highest relative abundance in comparison to the other treatments: (A)/(D) CH<sub>4</sub>, (B)/(E) CH<sub>4</sub>+Fe<sup>3+</sup>, or (C)/(F) CH<sub>4</sub>+Mn<sup>4+</sup>.

**Table 1.** Sample codes and characteristics of the study sediments. Age is estimated according to Pajunen (2004). n.d. = not determined.

Sample code	Site	Layer (cm)	Age (y)	dry matter (%)	C (%)	N (%)	C:N	[Fe] ( $\mu\text{mol g}^{-1}_{\text{DW}}$ )	[Mn] ( $\text{nmol g}^{-1}_{\text{DW}}$ )
L <sub>0-25</sub>	Littoral	0-25	n.d.	19.4	4.80	0.48	10.0	130	100
P <sub>0-10</sub>	Profundal	0-10	0 – 50	13.4	7.97	0.67	11.9	270	340
P <sub>10-30</sub>		10-30	50 – 150	21.8	7.22	0.46	15.7	n.d.	n.d.
P <sub>90-130</sub>		90-130	1700 – 2500	14.2	11.63	0.71	16.4	n.d.	n.d.
P <sub>390-410</sub>		390-410	6800 – 7000	24.8	7.49	0.56	13.4	n.d.	n.d.

**Table 2.** Potential net production rates of CH<sub>4</sub> and TIC as well as the potential CH<sub>4</sub> oxidation rates in the incubations of the sediment slurry samples subjected to different treatments in 2012 and 2014. Significant differences among the treatments at each depth layer are shown on the right side of the values with a differing letter (one-way ANOVA,  $p < 0.05$ ). The values are shown as averages  $\pm$  SD,  $n = 3-4$ . See Table 1 for a definition of the sample codes.

Year	Sample code	Treatment	net CH <sub>4</sub> prod. ( $\text{nmol g}^{-1}_{\text{DWd}^{-1}}$ )		net TIC prod. ( $\text{nmol g}^{-1}_{\text{DWd}^{-1}}$ )		CH <sub>4</sub> ox. ( $\text{nmol g}^{-1}_{\text{DWd}^{-1}}$ )	
2012 <sup>1</sup>								
	L <sub>0-25</sub>	CH <sub>4</sub>	40.8 $\pm$ 6.8	a	132.4 $\pm$ 20.2	a	0.7 $\pm$ 0.2	a
		CH <sub>4</sub> +SO <sub>4</sub> <sup>2-</sup>	13.9 $\pm$ 6.1	b	133.5 $\pm$ 10.7	a	0	0
		CH <sub>4</sub> +NO <sub>3</sub> <sup>-</sup>	24.4 $\pm$ 2.9	c	124.7 $\pm$ 4.7	a	0	0
		CH <sub>4</sub> +O <sub>2</sub>	-0.8 $\pm$ 1.0	d	253.8 $\pm$ 65.5	b	4.1 $\pm$ 2.7	b
	P <sub>10-30</sub>	CH <sub>4</sub>	4.1 $\pm$ 1.5	a	38.2 $\pm$ 10.9	a	0	0
		CH <sub>4</sub> +SO <sub>4</sub> <sup>2-</sup>	1.6 $\pm$ 0.3	b	44.5 $\pm$ 4.4	a	0	0
		CH <sub>4</sub> +NO <sub>3</sub> <sup>-</sup>	1.8 $\pm$ 0.3	b	37.4 $\pm$ 4.2	a	0	0
		CH <sub>4</sub> +O <sub>2</sub>	-1.5 $\pm$ 0.2	c	44.6 $\pm$ 8.1	a	1.2 $\pm$ 1.2	a
	P <sub>90-130</sub>	CH <sub>4</sub>	5.5 $\pm$ 1.4	a	54.1 $\pm$ 3.8	a	0	0
		CH <sub>4</sub> +SO <sub>4</sub> <sup>2-</sup>	4.1 $\pm$ 1.1	a	67.4 $\pm$ 12.5	a	0	0
		CH <sub>4</sub> +NO <sub>3</sub> <sup>-</sup>	4.7 $\pm$ 1.1	a	51.4 $\pm$ 11.3	a	0	0
		CH <sub>4</sub> +O <sub>2</sub>	-5.0 $\pm$ 0.9	b	199.3 $\pm$ 39.3	b	1.7 $\pm$ 0.1	a
	P <sub>390-410</sub>	CH <sub>4</sub>	0.9 $\pm$ 0.04	a	27.7 $\pm$ 3.4	a	0	0
		CH <sub>4</sub> +SO <sub>4</sub> <sup>2-</sup>	0.9 $\pm$ 0.3	a	19.7 $\pm$ 7.2	a	0	0
		CH <sub>4</sub> +NO <sub>3</sub> <sup>-</sup>	0.6 $\pm$ 0.4	a	25.0 $\pm$ 8.9	a	0	0
		CH <sub>4</sub> +O <sub>2</sub>	0	0	98.2 $\pm$ 27.4	b	0	0
2014 <sup>1</sup>								
	P <sub>0-10</sub>	CH <sub>4</sub>	82.5 $\pm$ 15.4	a	294.9 $\pm$ 44.3	a	0.9 $\pm$ 0.2	a
		CH <sub>4</sub> +Fe <sup>3+</sup>	58.1 $\pm$ 6.6	b	118.7 $\pm$ 9.1	b	0.6 $\pm$ 0.2	a
		CH <sub>4</sub> +Mn <sup>4+</sup>	20.9 $\pm$ 8.4	c	172.5 $\pm$ 62.5	b	2.1 $\pm$ 1.9	a
		CH <sub>4</sub> +O <sub>2</sub>	-72.7 $\pm$ 3.7	d	782.4 $\pm$ 96.1	c	49.3 $\pm$ 16.9	b

		CH <sub>4</sub> +CH <sub>2</sub> F <sub>2</sub>	82.3±16.5	a	318.7±60.0	a	0.6±0.1	a
	P <sub>10-30</sub>	CH <sub>4</sub>	80.1±13.9	a	120.5±5.2	a	0.2±0.1	a
		CH <sub>4</sub> +Fe <sup>3+</sup>	67.2±24.5	a	125.2±9.1	a	0.1±0.05	a
		CH <sub>4</sub> +Mn <sup>4+</sup>	13.8±5.6	b	140.8±10.4	a	0.1±0.1	a
		CH <sub>4</sub> +O <sub>2</sub>	-18.3±3.5	c	274.6±47.1	b	18.0±14.5	b
		CH <sub>4</sub> +CH <sub>2</sub> F <sub>2</sub>	72.1±12.1	a	130.3±9.0	a	0.2±0.1	a

1) Incubations were done at +4°C for up to 14 months in 2012 and at +10°C for up to 4 months in 2014

**Table 3.** Potential Fe<sup>3+</sup> reduction rate (denoted as Fe<sup>2+</sup> production rate) in the incubations of the sediment slurry samples amended with or without Fe<sup>3+</sup>, as well as the potential Mn<sup>4+</sup> reduction rate (denoted as Mn<sup>2+</sup> production rate) in the incubations of the sediment slurry samples amended with or without Mn<sup>4+</sup> in 2012. Significant differences among the treatments at each depth layers are shown on the right side of the values with a differing letter (t-test, p < 0.05). The values are shown as averages ± SD, n = 3. n.d. = not determined. See Table 1 for a definition of the sample codes.

Sample code <sup>1</sup>		Fe <sup>2+</sup> production (nmol g <sup>-1</sup> <sub>DW</sub> d <sup>-1</sup> )		Mn <sup>2+</sup> production (nmol g <sup>-1</sup> <sub>DW</sub> d <sup>-1</sup> )	
L <sub>0-25</sub>	no addit.	16.1±8.7	a	13.5±0.2	a
	Fe <sup>3+</sup>	827.0±101.6	b	n.d	
	Mn <sup>4+</sup>	n.d		1066.4±231.3	b
P <sub>0-10</sub>	no addit.	748.4±66.8	a	20.8±3.8	a
	Fe <sup>3+</sup>	913.3±76.0	b	n.d	
	Mn <sup>4+</sup>	n.d		1009.7±113.1	b

1) Incubations were done at +10°C for up to 4.5 months