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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

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One sentence summary: Anaerobic methane oxidation did not have any ecological relevance in the methane consumption in the sediments of two shallow boreal lakes.
The role of anaerobic CH$_4$ oxidation in controlling lake sediment CH$_4$ emissions remains unclear. Therefore, we tested how relevant EAs (SO$_4^{2-}$, NO$_3^-$, Fe$^{3+}$, Mn$^{4+}$, O$_2$) affect CH$_4$ production and oxidation in the sediments of two shallow boreal lakes. The changes induced to microbial communities by the addition of Fe$^{3+}$ and Mn$^{4+}$ were studied using next-generation sequencing targeting the 16S rRNA and methyl-coenzyme M reductase (*mcrA*) genes and *mcrA* transcripts. Putative anaerobic CH$_4$ 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$_4$ oxidation (0–2.1 nmol g$^{-1}$ dry weight (DW)d$^{-1}$) was not enhanced by the addition of EAs, nor important in consuming the produced CH$_4$ (0.6–82.5 nmol g$^{-1}$ DWd$^{-1}$). Instead, the increased EA availability suppressed CH$_4$ production via the outcompetition of methanogens by anaerobically respiring bacteria and via the increased protection of organic matter from microbial degradation induced by Fe$^{3+}$ and Mn$^{4+}$. Future studies could particularly assess whether anaerobic CH$_4$ oxidation has any ecological relevance in reducing CH$_4$ emissions from the numerous CH$_4$-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$_4$) to the atmosphere (Bastviken et al. 2004). CH$_4$ emissions from these environments are controlled by both methanogenesis and CH$_4$ oxidation. Methanogenesis is the final step in organic matter (OM) degradation, during which methanogenic archaea produce CH$_4$ from either acetate via the acetoclastic pathway or by oxidizing H$_2$ using CO$_2$ as an electron acceptor (EA) via the hydrogenotrophic pathway (Conrad 1999). Some archaea can also produce CH$_4$ 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$_4$ is efficiently consumed through aerobic oxidation (MO) by methanotrophic bacteria (MOB) utilizing O$_2$ as an EA (e.g. Hanson and Hanson 1996). In anoxic conditions, CH$_4$ 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$_2^-$ (Ettwig et al. 2010) as EAs. While AOM coupled with SO$_4^{2-}$ - reduction dominates anaerobic CH$_4$ 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$_4^{2-}$ (Timmers et al. 2016), NO$_3^-/NO_2^-$ (Deutzmann et al. 2014; á Norði and Thamdrup 2014), Fe$^{3+}$ (Sivan et al. 2011) and Mn$^{4+}$ (Segarra et al. 2013), could be important in freshwater systems. Furthermore, some methanogens oxidize small amounts of CH$_4$ 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$_2$) 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$_2$) in sediments can mitigate CH$_4$ 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$_2$, volatile fatty acids, alcohols) generated by fermentative bacteria from methanogenic to other anaerobic respiration pathways (Klüpfel et al. 2014). EAs can also increase CH$_4$ consumption via CH$_4$ oxidation (á Norði and Thamdrup 2014). However, Fe$^{3+}$ and Mn$^{4+}$ (generated via Fe$^{2+}$ and Mn$^{2+}$ 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$^{3+}$-induced suppression of both CH$_4$ and CO$_2$ 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$_4$ emissions from freshwater systems. It is well known that MO in oxic sediment layers represents an efficient CH$_4$ 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 Fe$^{3+}$, Mn$^{4+}$, NO$_3^-$, or SO$_4^{2-}$, takes place in boreal lake sediments. We further hypothesized that increased Fe$^{3+}$ and Mn$^{4+}$ decrease both CH$_4$ and 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 Fe$^{3+}$, Mn$^{4+}$, SO$_4^{2-}$, NO$_3^-$, or O$_2$ and analyzed the changes induced by the increase in each of the EAs in the potential rates of CH$_4$ oxidation and production and total inorganic carbon (TIC) production. Furthermore, the effects of the increased Fe$^{3+}$ and 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 Fe$^{3+}$ and 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 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 $^{14}$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\textsubscript{2} > 265 \textmu mol L\textsuperscript{-1}). O\textsubscript{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\textsubscript{0-10}) (six cores in 2012 and 10 cores in 2014) and 10–30 cm (P\textsubscript{10-30}) (six cores in 2012 and 10 cores in 2014) layers at the profundal site and a 0–25 cm (L\textsubscript{0-25}) (six cores in 2012) layer at the littoral site, were collected using a Kajak-type gravity corer (Ø = 54 mm). The deeper samples, 90–130 cm (P\textsubscript{90-130}) (four cores in 2012) and 390–410 cm (P\textsubscript{390-410}) (three cores in 2012) layers at the profundal site, were collected using a Livingston-type piston corer (Ø = 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\textsubscript{390-410}, which were sealed and transported to the laboratory as a whole. Sediments from the different cores were pooled for P\textsubscript{0-10}, P\textsubscript{10-30}, and L\textsubscript{0-25}, whereas those for P\textsubscript{90-130} 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\textsubscript{4} oxidation and net production rates of CH\textsubscript{4} and total inorganic carbon (TIC)**

Slurry preparation and incubation

The experimental setup consisted of testing the effects of different EAs and CH\textsubscript{2}F\textsubscript{2} on CH\textsubscript{4} and TIC production and CH\textsubscript{4} consumption potentials (Table 2) as well as the determination of
potential Fe$^{3+}$ and Mn$^{4+}$ reduction (Table 3). Setting up the incubations took place in a glove bag under a N$_2$ gas flow. The cores for P$_{390-410}$ were first sectioned and put into sterile plastic bags, and similar to 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 N$_2$-flushed, pre-sterilized (by autoclaving) glass incubation bottles. In 2012, ~60 g of wet sediment from L$_{0-25}$ and P$_{10-30}$, as well as ~50 g and ~40 g of sediment from P$_{90-130}$ and 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 P$_{0-10}$, and ~60 g from P$_{10-30}$ was weighted into 300 ml bottles (38 and 40 bottles of P$_{0-10}$ and P$_{10-30}$, respectively) (Table 2). Soon thereafter, the sediments obtained in 2014 were slurried by adding 50 ml O$_2$-free MQ-H$_2$O to each bottle, followed by brief, vigorous shaking. All bottles were then re-flushed with 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 O$_2$ by storing them in the dark at a temperature of +4°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 O$_2$-free, sterilized artificial porewater (146 mM NaCl, 13.4 mM MgCl$_2$, 0.3 mM CaCl$_2$, 0.8 mM KCl, and 2.9 mM 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) $^{13}$CH$_4$, (2) $^{13}$CH$_4$ + 2 mM SO$_4^{2-}$ (as Na$_2$SO$_4$), (3) $^{13}$CH$_4$ + 2 mM NO$_3^-$ (as NaNO$_3$), and (4) $^{13}$CH$_4$ + O$_2$, with three to four replicate bottles included in each treatment for each of the four sediment types (L$_{0-25}$, P$_{10-30}$, P$_{90-130}$, P$_{390-410}$). Replicates for P$_{90-130}$ and P$_{390-410}$ represented the different
sampling cores. The bottles containing the 2014 sediments were divided into five treatments: (1) $^{13}$CH$_4$, (2) $^{13}$CH$_4$ + 10 mM Fe$^{3+}$ [amorphous Fe(OH)$_3$; Lovley and Phillips 1986], (3) $^{13}$CH$_4$ + 10 mM Mn$^{4+}$ (solid MnO$_2$; Lovley and Phillips 1988), (4) $^{13}$CH$_4$ + O$_2$, and (5) $^{13}$CH$_4$ + CH$_2$F$_2$, as well as five corresponding treatments with non-labelled CH$_4$, with three to four replicate bottles included in each treatment for both sediment types (P$_{0-10}$, P$_{10-30}$) (Table 2).

The *in situ* porewater concentrations of the EAs were not measured, except for the Mn concentration in P$_{0-10}$ (~27 µM), which is within the typical range (2–40 µM) 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-NO$_3^-$ lake (L. Pääjärvi, NO$_3^-$ > 65 µM in the water above the sediment), the porewater NO$_3^-$ concentration was previously found to only reach 14 µM during the growing season (Rissanen *et al*. 2013). The porewater SO$_4^{2-}$ concentration typically ranges from ~20 to ~300 µM in the sediments of oligotrophic and mesotrophic lakes (Holmer and Storkholm 2001), while the porewater Fe concentration has been reported to only reach ~400 µM 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 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 N$_2$ (in 2014), followed by the addition of He or 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 CH$_4$ (in 2014), 0.6 ml (in 2012), or 1 ml (in 2014) of $^{13}$C-enriched CH$_4$ ($^{13}$C percentage was 8.2% in 2012 and
10.9% in 2014), followed by the addition of 0.5 ml of CH$_2$F$_2$ to treatment 5 in 2014 (headspace concentration ~0.25%). The $^{13}$C-enriched CH$_4$ mixture was made by mixing CH$_4$ (99.95% purity, INTERGAS, UK) with $^{13}$CH$_4$ (99.5% purity, 99.9% $^{13}$C, Cambridge Isotope Laboratories, Inc., USA) in an He-flushed, O$_2$-free glass bottle with NaOH powder to remove any contaminating CO$_2$. 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$_4$ was measured five times in 2012 and four to five times in 2014, while those of the CO$_2$ and the $^{13}$C content of CO$_2$ were measured six times in 2012 and four times in 2014.

CH$_2$F$_2$ inhibits CH$_4$ 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$_2$-consuming methanogenesis pathway in reverse (Timmers et al. 2017), it can be speculated that CH$_2$F$_2$ would inhibit CH$_4$ 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$_2$ (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$_4$ oxidation (to CO$_2$) by CH$_2$F$_2$ could specifically indicate MOB or NC 10 bacteria activity during the incubations.

**Determination of CH$_4$ and CO$_2$ concentrations and the isotopic composition of CO$_2$**

The gas concentrations were measured in 2012 using an Agilent 6890N (Agilent Technologies, USA) gas chromatograph (GC) with an electron capture detector for CO$_2$ and a
flame ionization detector (FID) for CH\(_4\) (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\(_2\) (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\(_4\) (sixth sampling). In 2014, the CH\(_4\) and CO\(_2\) 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\(_4\). The same standards were used each time.

The fractional abundance of \(^{13}\text{C}\) in the CO\(_2\) gas, \(^{13}\text{F}\),

\[
^{13}\text{F} = \frac{^{13}\text{C}}{(^{13}\text{C} + ^{12}\text{C})}
\]  

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\(^{3+}\) and Mn\(^{4+}\)**

The Fe\(^{3+}\) and Mn\(^{4+}\) reduction incubations with samples from L\(_{0-25}\) and P\(_{0-10}\) 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\(_2\)-flushed MQ-H\(_2\)O in 300 ml bottles (nine bottles from both L\(_{0-25}\) and P\(_{0-10}\)), which were then divided into three treatments: (1) no additions, (2) 10 mM Fe\(^{3+}\), and (3) 10 mM Mn\(^{4+}\), with three replicate bottles included in each treatment for both sediment types (Table 3). Following the N\(_2\) 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\(^{3+}\) and Mn\(^{4+}\)
reduction were taken from the bottles through a septum eight times during the incubation period. The bottles were briefly shaken before each sampling.

The Fe\textsuperscript{3+} reduction activity was measured as Fe\textsuperscript{2+} production using a ferrozine-based assay (Lovley and Phillips 1986), as previously described by Karvinen, Lehtinen, and Kankaala (2015). The Mn\textsuperscript{4+} reduction activity was measured by the accumulation of soluble Mn\textsuperscript{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 AAAnalyst 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 [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) Fe\textsuperscript{3+}, (3) Mn\textsuperscript{4+}, (4) O\textsubscript{2}, and (5) CH\textsubscript{2}F\textsubscript{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 Mn$^{4+}$ reduction analyses (Table 1).

**Calculations**

The concentration of CH$_4$ and 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 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 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 CO$_2$. The potential net production rates of CH$_4$ and TIC, as well as the production rates of Fe$^{2+}$ (potential Fe$^{3+}$ reduction) and Mn$^{2+}$ (potential Mn$^{4+}$ reduction), were measured for each incubation bottle as a linear increase in the total amount of these substances with time.

Potential CH$_4$ oxidation was measured for each incubation bottle as a linear increase in the amount of 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):

$$N_{\text{oxCH}_4} = \left[\left(13F_L - 13F_n\right) \times N_{\text{TIC}}\right] \times \frac{\left(N_{\text{iCH}_4} + N_{b\text{CH}_4}\right)}{\left(F_{\text{iCH}_4}N_{\text{iCH}_4} + F_{b\text{CH}_4}N_{b\text{CH}_4}\right)}$$

where $N_{\text{oxCH}_4}$ is the amount of CH$_4$ oxidized in the incubation bottle, $N_{\text{TIC}}$ is the amount of TIC in the incubation bottle with the added $^{13}$CH$_4$, $N_{\text{iCH}_4}$ is the amount of CH$_4$ initially in the incubation bottle (day 1), $N_{b\text{CH}_4}$ is the amount of biogenic CH$_4$ produced during incubation, $F_{\text{iCH}_4}$ is the initial fractional abundance of $^{13}$C in CH$_4$ (same as the $^{13}$C-label percentage, see above).
$F_{bCH4}$ 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 $N_{iCH4}$ were determined using GC (see above). The $N_{bCH4}$ was calculated as the difference between the total amount of CH$_4$ at a given time point and the $N_{iCH4}$. 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‰ ($F_{bCH4}$ = 0.01051). The chosen value represents the typical values of boreal lakes, since it is in the middle of the total range, -20‰ to -81‰, 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$_{0.25}$, P$_{0.10}$ and P$_{10.30}$ prior to the incubations was stored at -20°C and subsequently freeze-dried for molecular analyses. In 2014, ~500 mg
sediment aliquots were taken for molecular analyses from $P_{0-10}$ and $P_{10-30}$ 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’-CCCTAYGGGGYGASCAG-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’-GTTGTTGTGTTGATCTACAC-3’)/mcrA reverse (5’-TCATTGCRAGTTGGGTAGTT-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*
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 ≤ 26 and ≤ 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$_2$ + CO$_2$, (3) methyl compound (Nazaries et al. 2013), (4) H$_2$ +
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 Nozhevnigova 2014; Wasmund et
al. 2014; Wrighton et al. 2014; Zheng et al. 2016), (2) Fe$^{3+}$/Mn$^{4+}$-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 log10 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 α-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 CH₄ and TIC and the potential CH₄ oxidation

The potential net production rates of CH₄ and TIC in anaerobic conditions ranged from 0.6 to 82.5 nmol CH₄ g⁻¹DW d⁻¹ (0.1–11.7 nmol CH₄ cm⁻³ d⁻¹) and from 19.7 to 318.7 nmol TIC g⁻¹DW d⁻¹ (3.0–45.1 nmol TIC cm⁻³ d⁻¹), 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$_2$F$_2$, that is, on the differences between the CH$_4$ treatment only and the other treatments (CH$_4$ + EAs and CH$_4$ + CH$_2$F$_2$) (Table 2).

The addition of Fe$^{3+}$ and Mn$^{4+}$ decreased the potential net CH$_4$ production by 30% and 77% in P$_{0-10}$, respectively. In P$_{10-30}$, the addition of Fe$^{3+}$ did not decrease it statistically significantly, although the addition of Mn$^{4+}$, NO$_3^-$, and SO$_4^{2-}$ decreased it by 83%, 60%, and 60%, respectively (Table 2). In contrast, NO$_3^-$ and SO$_4^{2-}$ did not affect net CH$_4$ production in the deeper layers, P$_{90-130}$ and P$_{390-410}$, at the profundal site (Table 2). The addition of SO$_4^{2-}$ and NO$_3^-$ also decreased the potential net CH$_4$ production by 66% and 40% in L$_{0-25}$ (Table 2), respectively. The addition of Fe$^{3+}$ and Mn$^{4+}$ also reduced the potential net TIC production by 60% and 42% in P$_{0-10}$, 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$_4$ and TIC production rates were generally similar between the CH$_4$ and CH$_4$+CH$_2$F$_2$ treatments (Table 2). However, during the first week of incubation, CH$_2$F$_2$ inhibited CH$_4$ production in two out of six and three out of eight incubation bottles containing sediment from P$_{0-10}$ and P$_{10-30}$, 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$_4$ 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$_4$ and TIC were detected.

The addition of O$_2$ generally resulted in the highest potential net TIC production. It also led to CH$_4$ consumption, except for the deepest layer, P$_{390-410}$, of the profundal site, where no
CH₄ production or consumption occurred (Table 2). ^13^C labelling confirmed that the CH₄ consumption was due to aerobic CH₄ oxidation ranging from 0 to 49.3 nmol g⁻¹DW d⁻¹ (0–7.0 nmol cm⁻³ d⁻¹) and also varying between sites (when compared in 2012) and depth layers (Table 2). The potential CH₄ oxidation in the anaerobic incubations ranged from 0 to 2.1 nmol g⁻¹DW d⁻¹ (0–0.3 nmol cm⁻³ d⁻¹). It was not affected by the addition of NO₃⁻, SO₄²⁻, Fe³⁺, or Mn⁴⁺ at the profundal site, although NO₃⁻ and SO₄²⁻ suppressed it at the littoral site (Table 2). The potential CH₄ oxidation did not generally differ between the CH₄ and CH₄+CH₂F₂ treatments (Table 2).

However, during the first week of incubation, CH₂F₂ inhibited it in two out of three and two out of four incubation bottles containing sediment from P₀–10 and P₁₀–30, respectively (Figs. S3B and D). The potential CH₄ oxidation activity in the anaerobic treatments increased as the net TIC production increased (ρ = 0.524, p < 0.05), but it was not correlated with the net CH₄ production rate, nor with the relative expression of mcrA of the functional groups of archaea.

**Potential rates of Fe³⁺ and Mn⁴⁺ reduction**

The natural concentrations of Fe and Mn were higher in P₀–10 than in L₀–25 (Table 1). The average potential Fe³⁺ and Mn⁴⁺ reduction rates ranged from 16.1 to 913.3 nmol g⁻¹DW d⁻¹ (3.3–129.1 nmol cm⁻³ d⁻¹) and 13.5 to 1066.4 nmol g⁻¹DW d⁻¹ (2.8–221 nmol cm⁻³ d⁻¹), respectively. The natural (non-amended) rate of anaerobic Fe³⁺ reduction was considerably higher in P₀–10 than in L₀–25, whereas the natural Mn⁴⁺ reduction was only slightly higher in P₀–10 (Table 3). As expected, the addition of Mn⁴⁺ increased the Mn⁴⁺ reduction rate significantly at both sites (Table 3). Further, the addition of Fe³⁺ increased the Fe³⁺ reduction rate significantly in L₀–25, although a much smaller increase was observed in P₀–10 (Table 3).

Since the Fe³⁺ and Mn⁴⁺ reduction assays conducted in P₀–10 were performed in similar conditions to the gas production/consumption incubations conducted in 2014, the contribution of
Fe\(^{3+}\) and Mn\(^{4+}\) reduction to C mineralization could be roughly estimated by assuming a 4:1 ratio for Fe\(^{3+}\) reduction:TIC production and 2:1 for Mn\(^{4+}\) reduction:TIC production (Hiscock and Bense 2014). The natural (non-amended) Fe\(^{3+}\) and Mn\(^{4+}\) reduction contributed to 63.4% and 3.5% of TIC production, respectively. However, when Fe\(^{3+}\) and Mn\(^{4+}\) were added, OM oxidation coupled with dissimilatory Fe\(^{3+}\) and Mn\(^{4+}\) reduction would have produced two to three times more TIC than was observed. This indicates that a large part of the Fe\(^{3+}\) and Mn\(^{4+}\) reduction in the Fe\(^{3+}\)- and Mn\(^{4+}\)-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\(_{0-10}\) and P\(_{10-30}\) 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\(_2\)+CO\(_2\) were more abundant at the littoral site than at the profundal site, whereas an opposite pattern was observed for acetate-consuming *Methanosetaeaceae* and H\(_2\)+CO\(_2\)-consuming *Methanoregulaceae* (Fig. 1). The other detected but less abundant methanogenic groups were H\(_2\)+CO\(_2\)-consuming *Methanocellales* and H\(_2\)+methyl-compound-consum ing *Methanomassiliicoccales* and Verstraetearchaeota (Fig. 1). The anaerobic methane-oxidizing archa eall belonged to the ANME-2D archa eae 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\(_{10-30}\) (Fig. 1). Of the methanogenic archa eae actively expressing their mcrA gene in P\(_{0-10}\), *Methanosetaeaceae* 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 Fe$^{3+}$/Mn$^{4+}$-reducing bacteria, *Geobacter* dominated, with a lower contribution from *Thiobacillus* and *Geothrix*. The Fe$^{3+}$/Mn$^{4+}$-reducing bacteria were generally found with a very low abundance (≤ 1.1%), except in the CH$_4$+Fe$^{3+}$ and CH$_4$+Mn$^{4+}$ treatments in P$_{10-30}$, 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$_4$, CH$_4$+Fe$^{3+}$, and CH$_4$+Mn$^{4+}$ 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 Methanosetaeaceae OTUs were more abundant in either the CH$_4$+Fe$^{3+}$ or CH$_4$+Mn$^{4+}$ treatments, while one H$_2$+CO$_2$-consuming Methanoregulaceae OTU, based on the 16S rRNA gene, exhibited its highest relative abundance in the CH$_4$ treatment in P$_{0-10}$ (Figs. S7 and S8, Supporting Information). One H$_2$+methyl-compound-consuming Verstraetearchaeota OTU, based on the 16S rRNA gene, exhibited its highest relative abundance in the CH$_4$+Fe$^{3+}$ treatment in P$_{0-10}$, while two H$_2$+CO$_2$-consuming Methanomicrobiales OTUs, based on the mcrA gene, exhibited their highest relative abundance in the CH$_4$+Mn$^{4+}$ treatment in P$_{10-30}$ (Figs. S7 and S8). Only one ANME-2D OTU, based on the mcrA gene, exhibited its highest relative abundance in the CH$_4$+Mn$^{4+}$ treatment in P$_{10-30}$ (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$_4$ and CH$_4$+Mn$^{4+}$ treatments (PerMANOVA, p < 0.05) (Fig. S6E). In addition, the relative mcrA expression of the H$_2$+methyl-compound-consuming methanogens was lower in the CH$_4$+Fe$^{3+}$ 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$_4$ treatment were H$_2$ consumers (Fig. 3A). In contrast, the relative expression of mcrA of one acetate-consuming Methanosetaeaceae OTU was higher in the CH$_4$+Fe$^{3+}$ and CH$_4$+Mn$^{4+}$ treatments than in the CH$_4$.
treatment (Fig. 3B). Only one H$_2$+CO$_2$– consuming Methanoregulaceae OTU had its highest relative activity in CH$_4$+Mn$^{4+}$ - 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$_{0-10}$ (PerMANOVA, p < 0.05), while the CH$_4$+Mn$^{4+}$ treatment differed from the other treatments in P$_{10-30}$ (p < 0.05) (Figs. S6A and B). In addition, the relative abundance of the fermentative bacteria was higher in the CH$_4$+Mn$^{4+}$ treatment than in the CH$_4$+Fe$^{3+}$ treatment in P$_{0-10}$ (one-way ANOVA, p < 0.05), while the metal-reducing bacteria differed in their relative abundance between treatments in P$_{10-30}$, with the highest relative abundance being seen in the CH$_4$+Mn$^{4+}$ treatment, the second highest in the CH$_4$+Fe$^{3+}$ treatment, and the lowest in the CH$_4$ treatment (Figs. 2A and B). Neither the MOBs, 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$_{10-30}$, the OTUs that exhibited their highest abundances in the CH$_4$+Fe$^{3+}$ and CH$_4$+Mn$^{4+}$ treatments were mostly Geobacter and, for the CH$_4$+Fe$^{3+}$ treatment, the WS3 group (Figs. 4E and F). In contrast, in P$_{0-10}$, the OTUs that had their highest abundance in the CH$_4$+Fe$^{3+}$ and CH$_4$+Mn$^{4+}$ treatments were mostly Chloroflexi, for the CH$_4$+Fe$^{3+}$ treatment, Betaproteobacteria, and for the CH$_4$+Mn$^{4+}$ treatment, Elusimicrobia (Figs. 4B and C). Of the known sulfur-cycling bacteria, one OTU within Desulfobulbaceae exhibited its highest abundance in the CH$_4$+Mn$^{4+}$ treatment in P$_{0-10}$ (Fig. 4C).

DISCUSSION

CH$_4$ oxidation
The potential CH₄ 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 Fe³⁺, Mn⁴⁺, NO₃⁻, or SO₄²⁻ would occur within the studied boreal lake sediments. The potential anaerobic CH₄ oxidation (0–0.3 nmol cm⁻³ d⁻¹) in the two investigated lakes was also very low when compared to those measured from other freshwater systems, including lakes (0–44 nmol cm⁻³ d⁻¹; Sivan et al. 2011; á Norði, Thamdrup and Schubert 2013; Deutzmann et al. 2014), nitrate-enriched pond microcosms (43–400 nmol cm⁻³ d⁻¹; á Norði and Thamdrup 2014), and wetland sediments (~0–30 nmol cm⁻³ d⁻¹; Segarra et al. 2013). It was only up to 2.5% of the maximum potential net CH₄ production (treatments amended with only CH₄), whereas the aerobic MOBs could consume up to 60% of the maximum potential net CH₄ production.

CH₄ oxidation in anaerobic conditions can be mediated through AOM or TMO (Timmers et al. 2016). The independency of the potential CH₄ oxidation from the net potential CH₄ production in anaerobic conditions would suggest that CH₄ oxidation mainly took place through AOM at the profundal site. In contrast, the concurrent EA-induced decrease in the potentials of both CH₄ oxidation and net CH₄ production would suggest that CH₄ 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 CH₂F₂ experiments necessitate a more in-depth discussion of the contribution of the different processes to CH₄ oxidation.

In the headspace concentrations used in this study (0.25%), CH₂F₂ inhibits both methane mono-oxygenase and acetate-consuming methanogenesis (Miller, Sasson and Oremland 1998). Furthermore, CH₂F₂ 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 CH₂F₂-induced inhibition of the CH₄ processes only took
place during the first week of incubation. Since ANME archaea use the \( \text{H}_2+\text{CO}_2 \)-consuming methanogenesis pathway in reverse (Timmers et al. 2017), it can be speculated that their activity would not be inhibited by \( \text{CH}_2\text{F}_2 \). As \( \text{NO}_3^- \), which is also rapidly reduced to \( \text{NO}_2^- \) in anoxic sediments, did not enhance \( \text{CH}_4 \) oxidation, it can be suggested that the \( \text{CH}_2\text{F}_2 \)-induced inhibition of the potential \( \text{CH}_4 \) oxidation was not due to the inhibition of \( \text{NO}_2^- \) using \( \text{NC} 10 \) bacteria, but rather due to the inhibition of aerobic MOBs. Indeed, a risk of minor \( \text{O}_2 \) contamination from the substrate/tracer injection or due to diffusion from the rubber septa or silicon sampling ports during incubations, which could induce microaerobic \( \text{CH}_4 \) 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 \( \text{O}_2 \) from the septa during the incubation period could not be fully prevented in this study, either. Although the observed net \( \text{CH}_4 \) 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 MOBs that used the trace \( \text{O}_2 \) were present on the surfaces of the sediment slurries. Thus, the inhibition of \( \text{CH}_4 \) oxidation by \( \text{CH}_2\text{F}_2 \) might indicate that besides AOM and TMO, MO could also have at least partially contributed to the \( \text{CH}_4 \) oxidation. Therefore, our potential anaerobic \( \text{CH}_4 \) 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 \( \text{CH}_4 \) production and oxidation (Meulepas et al. 2010).

The relative abundance of anaerobic \( \text{CH}_4 \) oxidizing bacteria (\( \text{NC} 10 \)) 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 \( \text{Fe}^{3+} \) or \( \text{Mn}^{4+} \). 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 NO$_3$/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 MOBs. 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 MOBs in our study.

Taken together, these results indicate that anaerobic CH$_4$ oxidation is not important in mitigating CH$_4$ emissions from the investigated lakes. This finding contrasts with previously studied freshwater sediments, where the volumetric AOM rates were >15% of the CH$_4$ production rates or even larger than the CH$_4$ production (Sivan et al. 2011; á Norði, Thamdrup and Schubert 2013; Segarra et al. 2013; á Norði and Thamdrup 2014). Thus, anaerobic CH$_4$ oxidation can consume a substantial part of diffusive 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. Órn (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 NO$_3^-$ (Haroon et al. 2013; Ettwig et al. 2016), Fe$^{3+}$, and Mn$^{4+}$ (Ettwig et al. 2016) as EAs in anaerobic CH$_4$ oxidation, while NC 10 bacteria use NO$_2^-$ (Ettwig et al. 2010). Environmental studies also suggest that ANME-2D are involved in 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 Fe$^{3+}$ and Mn$^{4+}$ (but not SO$_4^{2-}$ and NO$_3^-$) were the only EAs tested with the anaerobic samples from $P_{0-10}$ (Table 2). However, as the ANME-2D archaea had a higher relative abundance in $P_{10-30}$, 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 P0-10, and it decreased during the incubations, irrespective of the treatment. This suggests that exhaustion of some important substrate other than CH4 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 Mn4+ or Fe3+ (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 CH4 and TIC

In contrast to the potential CH4 oxidation, the potential net CH4 production rates (0.6–82.5 nmol g−1DW d−1; 0.1–11.7 nmol cm⁻³ d⁻¹) in the anaerobic incubations of the sediment slurries from the study lakes fell well within the range of reported CH4 production rates in previous lake sediment studies: ~0–312 nmol g⁻¹DW d⁻¹ (Schulz, Matsuyama and Conrad 1997; Marotta et al. 2014; Karvinen, Lehtinen and Kankaala 2015) and ~1–23 nmol cm⁻³ d⁻¹ (Sivan et al. 2011; á Norði, Thamdrup and Schubert 2013). The potential net CH4 production rates also well represent the magnitude and variation of the estimated potential gross CH4 production rates, since anaerobic CH4 oxidation was always a minor fraction of the potential net CH4 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 CH4 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$_2$+CO$_2$-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$_2$+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$_3^−$ (Klüber and Conrad 1998) and Fe$^{3+}$ (van Bodegom, Scholten and Stams 2004), which could be expected to also take place with Mn$^{4+}$ (either directly or via the Mn$^{4+}$-driven oxidation of Fe$^{2+}$ to Fe$^{3+}$). However, the increased availability of NO$_3^−$ did not inhibit CH$_4$ production in the two deepest layers at the profundal site ($P_{90−130}$ and $P_{490−310}$), 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$_2$+CO$_2$-consuming methanogens were affected by Fe$^{3+}$ or Mn$^{4+}$, as would have been expected, given that they are more sensitive than acetate-consuming methanogens to the inhibitory effects of Fe$^{2+}$ (van Bodegom, Scholten and Stams 2004). Yet, the Fe$^{3+}$- and Mn$^{4+}$-induced changes in the abundance
and activity of some individual methanogenic OTUs indicated that the slight inhibition of
H₂+CO₂-consuming methanogens may have taken place, although it could not have been
predominantly responsible for such large reductions in the potential net CH₄ production. The
mcrA expression data also suggest that the H₂+methyl-compound-consuming methanogens were
slightly inhibited by Fe³⁺. However, due to their low abundance, the inhibition of their activity
could not have resulted in the observed decreases in CH₄ production. Thus, the inhibition of
methanogens was not an important factor underlying the decrease in the potential net CH₄
production.

The EA-induced decrease in CH₄ production was much more likely due to
outcompetition of the methanogens for methanogenic substrates by anaerobically respiring
bacteria (Klüpfel et al. 2014). The larger Fe³⁺- and Mn⁴⁺-induced changes seen in the bacterial
communities rather than in the archaeal communities also support this view. The reduction of
Mn⁴⁺, Fe³⁺, and NO₃⁻ could also produce SO₄²⁻, S⁰ (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 Mn⁴⁺ and NO₃⁻ could produce Fe³⁺ (Canfield, Kristensen and
Thamdrup 2005). Therefore, at least part of the Fe³⁺-, Mn⁴⁺-, and NO₃⁻-induced decrease in CH₄
production could also be due to anaerobic respiration coupled with EAs with a lower reduction
potential. The negligible effect of NO₃⁻ and SO₄²⁻ on the potential net CH₄ production in the two
deepest layers (P₉₀–₁₃₀ and P₃₉₀–₄₁₀) 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 $P_{0-10}$. 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 $P_{10-30}$ (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 $P_{0-10}$.

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 ($P_{0-10}$ and $P_{10-30}$) 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$_{0-10}$, 

_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$^{3+}$ and Mn$^{4+}$ 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$^{3+}$ respiration (Kawaichi _et al_. 2013), while the Fe$^{3+}$ 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$^{3+}$- or Mn$^{4+}$-induced increase in their relative abundance in P$_{0-10}$ were also using Fe$^{3+}$ or Mn$^{4+}$ as EAs in anaerobic respiration.

However, as discussed above, the suppression of heterotrophic processes could have led to an increase in Fe$^{3+}$- and Mn$^{4+}$-reducing chemolithoautotrophic processes in P$_{0-10}$.

Consequently, differences in the responses of the bacterial communities between the investigated layers may partially reflect the higher contribution of chemolithoautotrophic organisms in P$_{0-10}$.

Furthermore, both chemolithoautotrophic and abiotic Fe$^{3+}$ and Mn$^{4+}$ reduction can increase the availability of SO$_4^{2-}$, S$^0$, 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$^{3+}$- and Mn$^{4+}$-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
SO\textsubscript{4}\textsuperscript{2-}, S\textsuperscript{0}, and organic EA in P\textsubscript{0–10}. Only one OTU related to a taxon that contains species capable of SO\textsubscript{4}\textsuperscript{2-} reduction and S\textsuperscript{0} disproportionation (Desulfobulbaceae; Finster 2008) was slightly affected by Mn\textsuperscript{4+} in P\textsubscript{0–10}. However, many freshwater SO\textsubscript{4}\textsuperscript{2-} reducers belong to unknown phylogenetic lineages (Pester \textit{et al.} 2012). Furthermore, besides the known Fe\textsuperscript{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 Fe\textsuperscript{3+} in a previously studied lake sediment (Kappler \textit{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 Fe\textsuperscript{3+} and Mn\textsuperscript{4+}. These studies could use, for example, \textsuperscript{13}CO\textsubscript{2} labelling to reveal the role of chemolithoautotrophy (\textsuperscript{13}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 (Fe\textsuperscript{3+}, Mn\textsuperscript{4+}, SO\textsubscript{4}\textsuperscript{2-}, and S\textsuperscript{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 Fe\textsuperscript{3+} and Mn\textsuperscript{4+} availability on the structure of microbial communities, as well as the effects of increased Fe\textsuperscript{3+}, Mn\textsuperscript{4+}, NO\textsubscript{3}-, and SO\textsubscript{4}\textsuperscript{2-} on the potential CH\textsubscript{4} oxidation and net production rates of CH\textsubscript{4} and TIC in boreal lake sediments for the first time. The results suggest that anaerobic CH\textsubscript{4} oxidation (via AOM or TMO) was not an important factor in reducing CH\textsubscript{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$_4$ 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$^{3+}$ and Mn$^{4+}$) is also a very important component of the Fe$^{3+}$- and Mn$^{4+}$-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$_4$ 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$_4$ 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$^{3+}$ and Mn$^{4+}$ further increase OM preservation. However, the observed increase in C
degradation (TIC production), which was solely driven by the addition of O₂ (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 O₂-consuming microbial processes are crucial in inducing sediment C storage. Besides generating anoxia, they increase OM recalcitrance via consuming labile OM and via oxidizing Fe²⁺ and Mn²⁺ to Fe³⁺ and Mn⁴⁺, which react with OM (Lalonde et al. 2012; Estes et al. 2017). Whether or not the Fe³⁺- and Mn⁴⁺-induced inhibition of heterotrophic OM degradation processes could also benefit the chemolithoautotrophic processes that additionally increase sediment C storage via CO₂ fixation requires further study.

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*Conflict of interest.* None declared.

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


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

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Site</th>
<th>Layer (cm)</th>
<th>Age (y)</th>
<th>dry matter (%)</th>
<th>C (%)</th>
<th>N (%)</th>
<th>C:N</th>
<th>[Fe] (µmol g\textsuperscript{-1}DW)</th>
<th>[Mn] (nmol g\textsuperscript{-1}DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L0-25</td>
<td>Littoral</td>
<td>0-25</td>
<td>n.d.</td>
<td>19.4</td>
<td>4.80</td>
<td>0.48</td>
<td>10.0</td>
<td>130</td>
<td>100</td>
</tr>
<tr>
<td>P0-10</td>
<td>Profundal</td>
<td>0-10</td>
<td>0 – 50</td>
<td>13.4</td>
<td>7.97</td>
<td>0.67</td>
<td>11.9</td>
<td>270</td>
<td>340</td>
</tr>
<tr>
<td>P10-30</td>
<td>Profundal</td>
<td>10-30</td>
<td>50 – 150</td>
<td>21.8</td>
<td>7.22</td>
<td>0.46</td>
<td>15.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>P90-130</td>
<td>Profundal</td>
<td>90-130</td>
<td>1700 – 2500</td>
<td>14.2</td>
<td>11.63</td>
<td>0.71</td>
<td>16.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>P390-410</td>
<td>Profundal</td>
<td>390-410</td>
<td>6800 - 7000</td>
<td>24.8</td>
<td>7.49</td>
<td>0.56</td>
<td>13.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Table 2. Potential net production rates of CH$_4$ and TIC as well as the potential CH$_4$ 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 ± SD, n = 3–4. See Table 1 for a definition of the sample codes.

<table>
<thead>
<tr>
<th>Year</th>
<th>Sample code</th>
<th>Treatment</th>
<th>net CH$_4$ prod. (nmol g$^{-1}$DW d$^{-1}$)</th>
<th>net TIC prod. (nmol g$^{-1}$DW d$^{-1}$)</th>
<th>CH$_4$ ox. (nmol g$^{-1}$DW d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L$_{0.25}$</td>
<td>CH$_4$</td>
<td>40.8±6.8 a</td>
<td>132.4±20.2 a</td>
<td>0.7±0.2 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_4$+SO$_4^{2-}$</td>
<td>13.9±6.1 b</td>
<td>133.5±10.7 a</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_4$+NO$_3^{-}$</td>
<td>24.4±2.9 c</td>
<td>124.7±4.7 a</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_4$+O$_2$</td>
<td>-0.8±1.0 d</td>
<td>253.8±65.5 b</td>
<td>4.1±2.7 b</td>
</tr>
<tr>
<td></td>
<td>P$_{10-30}$</td>
<td>CH$_4$</td>
<td>4.1±1.5 a</td>
<td>38.2±10.9 a</td>
<td>0 b</td>
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<td></td>
<td></td>
<td>CH$_4$+SO$_4^{2-}$</td>
<td>1.6±0.3 b</td>
<td>44.5±4.4 a</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_4$+NO$_3^{-}$</td>
<td>1.8±0.3 b</td>
<td>37.4±4.2 a</td>
<td>0 b</td>
</tr>
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<td>CH$_4$+O$_2$</td>
<td>-1.5±0.2 c</td>
<td>44.6±8.1 a</td>
<td>1.2±1.2 b</td>
</tr>
<tr>
<td></td>
<td>P$_{90-130}$</td>
<td>CH$_4$</td>
<td>5.5±1.4 a</td>
<td>54.1±3.8 a</td>
<td>0 b</td>
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<td></td>
<td></td>
<td>CH$_4$+SO$_4^{2-}$</td>
<td>4.1±1.1 a</td>
<td>67.4±12.5 a</td>
<td>0 b</td>
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<tr>
<td></td>
<td></td>
<td>CH$_4$+NO$_3^{-}$</td>
<td>4.7±1.1 a</td>
<td>51.4±11.3 a</td>
<td>0 b</td>
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<td></td>
<td></td>
<td>CH$_4$+O$_2$</td>
<td>-5.0±0.9 b</td>
<td>199.3±39.3 b</td>
<td>1.7±0.1 a</td>
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<tr>
<td></td>
<td>P$_{390-410}$</td>
<td>CH$_4$</td>
<td>0.9±0.04 a</td>
<td>27.7±3.4 a</td>
<td>0 b</td>
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<td></td>
<td></td>
<td>CH$_4$+SO$_4^{2-}$</td>
<td>0.9±0.3 a</td>
<td>19.7±7.2 a</td>
<td>0 b</td>
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<tr>
<td></td>
<td></td>
<td>CH$_4$+NO$_3^{-}$</td>
<td>0.6±0.4 a</td>
<td>25.0±8.9 a</td>
<td>0 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_4$+O$_2$</td>
<td>0 0</td>
<td>98.2±27.4 b</td>
<td>0 b</td>
</tr>
<tr>
<td>2014</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P$_{0-10}$</td>
<td>CH$_4$</td>
<td>82.5±15.4 a</td>
<td>294.9±44.3 a</td>
<td>0.9±0.2 a</td>
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<td>CH$_4$+Fe$^{3+}$</td>
<td>58.1±6.6 b</td>
<td>118.7±9.1 b</td>
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<td>CH$_4$+Mn$^{4+}$</td>
<td>20.9±8.4 c</td>
<td>172.5±62.5 b</td>
<td>2.1±1.9 a</td>
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<td>CH$_4$+O$_2$</td>
<td>-7.2±3.7 d</td>
<td>782.4±96.1 c</td>
<td>49.3±16.9 b</td>
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<td></td>
<td>P$_{10-30}$</td>
<td>CH$_4$</td>
<td>80.1±13.9 a</td>
<td>120.5±5.2 a</td>
<td>0.2±0.1 a</td>
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<td></td>
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<td>CH$_4$+Fe$^{3+}$</td>
<td>67.2±24.5 a</td>
<td>125.2±9.1 a</td>
<td>0.1±0.05 a</td>
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<td>CH$_4$+Mn$^{4+}$</td>
<td>13.8±5.6 b</td>
<td>140.8±10.4 a</td>
<td>0.1±0.1 a</td>
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<td>274.6±47.1 b</td>
<td>18.0±14.5 b</td>
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<td>CH$_4$+CH$_2$F$_2$</td>
<td>72.1±12.1 a</td>
<td>130.3±9.0 a</td>
<td>0.2±0.1 a</td>
</tr>
</tbody>
</table>

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\(^{3+}\) reduction rate (denoted as Fe\(^{2+}\) production rate) in the incubations of the sediment slurry samples amended with or without Fe\(^{3+}\), as well as the potential Mn\(^{4+}\) reduction rate (denoted as Mn\(^{2+}\) production rate) in the incubations of the sediment slurry samples amended with or without Mn\(^{4+}\) 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.

<table>
<thead>
<tr>
<th>Sample code(^1)</th>
<th>Fe(^{3+}) production (nmol g(^{-1})DW d(^{-1}))</th>
<th>Mn(^{4+}) production (nmol g(^{-1})DW d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(_{0.25})</td>
<td>no addit. 16.1±8.7</td>
<td>a 13.5±0.2 a</td>
</tr>
<tr>
<td></td>
<td>Fe(^{3+}) 827.0±101.6</td>
<td>b n.d b</td>
</tr>
<tr>
<td></td>
<td>Mn(^{4+}) n.d</td>
<td>1066.4±231.3 b</td>
</tr>
<tr>
<td>P(_{0.10})</td>
<td>no addit. 748.4±66.8</td>
<td>a 20.8±3.8 a</td>
</tr>
<tr>
<td></td>
<td>Fe(^{3+}) 913.3±76.0</td>
<td>b n.d n.d</td>
</tr>
<tr>
<td></td>
<td>Mn(^{4+}) n.d</td>
<td>1009.7±113.1 b</td>
</tr>
</tbody>
</table>

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

**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 CH₄, CH₄+Fe³⁺ or CH₄+Mn⁴⁺ amendments. L₀–25, P₀–10 and P₁₀–30 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.

**Figure 2.** Relative abundances (average +/- SD) of the taxonomic groups of bacteria divided into (A) fermentative bacteria, (B) Fe³⁺/Mn⁴⁺ (metal) reducing bacteria, and (C) aerobic (MOB) and anaerobic (AOM) methanotrophic bacteria before (non-incubated) and after incubations with either CH₄, CH₄+Fe³⁺ or CH₄+Mn⁴⁺ amendments. L₀–25, P₀–10 and P₁₀–30 denote 0–25 cm layer of the littoral and 0–10 cm and 10–30 cm layers of the profundal study site, respectively.

**Figure 3.** Relative activity (average +/- SD) and taxonomic affiliations (and methanogenic substrate) of the mcrA OTUs (based on the mRNA transcripts) that differed in their relative activity among the CH₄, CH₄+Fe³⁺, or CH₄+Mn⁴⁺ treatments after the incubation of sediment slurry samples from P₀–10 (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) CH₄, (B) CH₄+Fe³⁺, or (C) CH₄+Mn⁴⁺.

**Figure 4.** Relative abundance (average +/- SD) and taxonomic affiliations of the bacterial 16S rRNA gene OTUs that differed in their relative abundance among the CH₄, CH₄+Fe³⁺, or CH₄+Mn⁴⁺ treatments after the incubation of sediment slurry samples from P₀–10 (A–C) and P₁₀–30 (D–F) (linear discriminant analysis [LDA] effect size [LEfSe] method p < 0.05). The OTUs are grouped into three rows according to the treatment (CH₄, CH₄+Fe³⁺, or CH₄+Mn⁴⁺) in which they exhibited the highest relative abundance in comparison to the other treatments: (A)/(D) CH₄, (B)/(E) CH₄+Fe³⁺, or (C)/(F) CH₄+Mn⁴⁺.
Fig. 1.
Fig. 2.
Fig. 3.

A  Methanomicrobiales OTU 50 (H$_2$+CO$_2$)
    Methanoregulaceae OTU 72 (H$_2$+CO$_2$)
    Methanomassiliicoccales OTU 4
    (H$_2$+methyl comp.)

B  Methanaetaeae OTU 5 (acetate)
    Methanaetaeae OTU 36 (H$_2$+CO$_2$)

C  Methanaetaeae OTU 36 (H$_2$+CO$_2$)
Fig. 4.

Relative abundance (% of 16S rRNA gene sequences)

- Prosporomicrobacteria OTU15
- Prosporomicrobacteria OTU151
- Paracoccus OTU152
- Rickettsiales-Christinophagaceae OTU409
- Rickettsiales OTU302
- Rickettsiales OTU112
- Chloroflexi OTU28
- Elusimicrobia OTU417

- Prosporomicrobacteria OTU152
- Prosporomicrobacteria OTU151
- Paracoccus OTU152
- Rickettsiales-Christinophagaceae OTU409
- Rickettsiales OTU302
- Rickettsiales OTU112
- Chloroflexi OTU28
- Elusimicrobia OTU417

- Prosporomicrobacteria OTU152
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- Paracoccus OTU152
- Rickettsiales-Christinophagaceae OTU409
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Supplemental Methods 1:

PCR, RT-PCR, preparation of the sequence libraries and sequencing

For each PCR amplicon library, two PCR reactions were performed. In the first round, PCR of the 16S rRNA gene amplicons was performed using the domain-specific primer pairs Arch340F (5’-CCCTAYGGGYGASCAG-3’)/Arch1000R (5’-GGCCATGCACYWCYTCTC-3’) (Gantner et al. 2011) for archaea and 27F (5’-AGAGTTTGATCMTGGCTCAG)/338R (5’-TGCTGCTCCTCCGATTAGAGT-3’) for bacteria. PCR of the mcrA gene amplicons was performed using the primer pair mcrA forward (5’-GGTGGTGTMGGATTCACACAR-3’)/mcrA reverse (5’-TCATTGCRTCAGTGTGRC steady-3’) (Beal, House and Orphan 2009). The M13 sequence (5’-TGTAAAACGACGGCCAGT-3’) linker was attached to the 5’ end of both Arch340F and the mcrA forward primers (Mäki, Rissanen and Tiirila 2016). In the first reaction for each gene, ~6 ng (2012), ~12 ng [2014, 0–10 cm layer of the profundal site (P0-10)], or ~3 ng [2014, 10–30 cm layer of the profundal site (P10-30)] of DNA was used as a template in a 25µl mixture containing 0.25 mM of dNTPs, 0.25 µM of both primers, 1 x Phusion Buffer, and 0.03 U/µl Phusion High-Fidelity DNA Polymerase. PCR amplification was performed in a C1000™ Thermal Cycler (Bio-Rad), with an initial denaturation step at 98ºC for 30 sec and 30 cycles of amplification (98ºC for 10 sec, 53ºC for 30 sec, and 72ºC for 60 sec) for the bacterial 16S rRNA gene amplicons and 35 cycles of amplification (98ºC for 10 sec, 55ºC for 30 sec, and 72ºC for 60 sec) for the archaeal 16S rRNA gene amplicons. There was an initial denaturation step at 98ºC for 60 sec and 29 cycles of amplification (98ºC for 60 sec, 50ºC for 60 sec, and 72ºC for 60 sec) for the mcrA gene amplicons. In the second PCR, 1 µl of first
round PCR products was used as a template in a 20 µl PCR mixture. The PCR conditions were similar to those described above, except that only six amplification cycles were run, an M13 primer was used as a forward primer in the amplification of the archaeal 16S rRNA and mcrA gene amplicons (Mäki, Rissanen and Tiirola 2016), and all the primers except for the Arch1000R included sequencing adaptors at the 5’ end (A adaptor linked with forward primers 27F and M13; P1 adaptor linked with reverse primers 338R and mcrA reverse), as well as nucleotide barcodes incorporated between the A adapter and the forward primers to distinguish samples in the mixed reaction. PCR products were purified with Agencourt AMPure XP (Beckman Coulter), DNA concentration was measured using a Qubit 2.0 Fluorometer and a dsDNA HS Assay Kit (Thermo Fisher), and the samples were pooled in equal DNA amounts for each gene. The amplicon pool was further purified using AMPure XP.

The active methanogenic and methanotrophic archaeal population in P0-10 in 2014 was studied using the reverse transcriptase PCR (RT-PCR) of mcrA. DNAse treatment to remove the DNA, followed by an RT reaction with random hexamers to transcribe the RNA (10–14 ng per 10 µl reaction) into cDNA, was performed using a Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher Scientific) according to the manufacturer’s instructions. The PCR conditions for the cDNA were similar to those described above for the mcrA gene except that a lower concentration of polymerase was used (0.02 U/µl). In addition, 2 µl of RT product or RT controls (1. RTneg: DNAse + RT procedure without RT enzyme; 2. DNAse: DNAse treated sample without RT reaction) was utilized as a template in the first PCR reaction, from which 2 µl was utilized in the second PCR in a 21 µl total volume.

In order to adjust the length of the archaeal 16S rRNA gene amplicon (~660 bp) for the Ion Torrent™ sequencing (recommended ~400 bp), the fragments were cut shorter (Mäki,
Rissanen and Tiirola 2016). No fragmentation was performed for the bacterial 16S rRNA gene (< 400 bp) or mcrA gene amplicons (~500 bp). Although this might have slightly reduced the length and quality of the mcrA sequence reads (Mäki, Rissanen and Tiirola 2016), there were enough high-quality sequences left for the final analyses after post-sequencing quality control was performed (see below). Size selection, emulsion PCR, sequencing [Ion Torrent™ Personal Genome Machine (PGM)], and sequence filtering using PGM software were performed as described in the study by Mäki, Rissanen and Tiirola (2016).

Supplemental Methods 2:
Preparation of the mcrA gene database

The custom-made mcrA gene database was compiled of our initial in-house database and the database published by Yang et al. (2014). The sequences for our initial in-house database were retrieved from the EMBL ALL database (via the SRS server LION, http://www.dkfz.de/srs) using the search query “description = mcrA BUTNOT un,” which excluded hits from uncultured and unidentified microbes. The taxonomies of these sequences were then retrieved from the databases using an in-house script in EMBOSS (European Molecular Biology Open Software Suite). Furthermore, mcrA sequences of the ANME archaea, Methanomassiliicoccales, Verstraetearchaeota, and Miscellaneous Crenarchaeota group (MCG) (also referred to as Bathyarchaeota), as well as environmental sequences classified in those groups in the phylogenetic trees of previous papers (Ettwig et al. 2008; Lloyd, Alperin and Teske 2011; Yanagawa et al. 2011; Biddle et al. 2012; Borrel et al. 2013; Haroon et al. 2013; Evans et al. 2015; Vanwonterghem et al. 2016), were manually collected from the databases. This initial in-house database was then combined with that of Yang et al.
(2014), which included the mcrA sequences of only cultured methanogens and environmental sequences without taxonomies. Any duplicate sequences and sequences without taxonomies were removed from the combined database.

Supplemental Methods 3:
Validation and fine-tuning of the mcrA classification by phylogenetic tree analysis

A phylogenetic tree analysis (neighbor-joining method) of the amino acid sequences deduced from the mcrA gene using the representative sequences of the OTUs (in P0-10 and P10-30 in 2014) and a subset of the database sequences was performed with MEGA 6 (Tamura et al. 2013) to further validate and fine-tune the mcrA-classification (see Fig. S2, Supporting Information). This analysis assigned two OTUs and one OTU with only a kingdom-level classification (Archaea) into order Methanomassiliicoccales and phyla Verstraetearchaeota, respectively, two OTUs with only an order-level classification (Methanosarcinales) into ANME-2D, and four OTUs with only an order-level classification (Methanomicrobiales) into the Methanoregulaceae family.

References:


Supporting information. Table S1.

Table S1. Oxidation reduction potential (ORP) and pH at the end of the incubations of the sediment slurry samples subjected to different treatments in 2012 and 2014, as well as the pH at the start of the incubations in 2012. The values are shown as averages ± SD, n = 3–4. L₀₋₂₅, P₀₋₁₀, P₁₀₋₃₀, P₉₀₋₁₃₀, P₃₉₀₋₄₁₀ denote 0 – 25 cm layer of the littoral and 0 – 10 cm, 10 – 30 cm, 90 – 130 cm and 390 – 410 cm layers of the profundal study site, respectively.

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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.
Supporting information. Figures S1-S8

**Figure S1.** pH at four time points during the incubation of sediment slurries collected from the (A) 0–10 cm (P0-10) and (B) 10–30 cm (P10-30) layers of profundal sediments in 2014 and amended without anything (no addition) or with Fe$^{3+}$, Mn$^{4+}$, O$_2$, or CH$_2$F$_2$. (C) and (D) are the same as (A) and (B), but for the oxidation reduction potential (ORP).
Figure S2. Phylogenetic tree (neighbor-joining method) of the amino acid sequences deduced from the methyl-coenzyme M reductase (mcrA) gene, which includes database sequences and the representative sequences of the operational taxonomical units (OTUs) (at a 96% similarity level) detected in 0 – 10 cm (P₀₋₁₀) and 10 – 30 cm (P₁₀₋₃₀) layers of the profundal site in 2014. The names of the phylogenetic clusters harboring the OTU sequences are underlined.
Figure S3. Cumulative (A) CH$_4$ production and (B) CH$_4$ oxidation (based on $^{13}$CO$_2$ production) in the incubation bottles of sediment slurries collected from the 0–10 cm layer ($P_{0-10}$) of the profundal site and amended with CH$_4$+CH$_2$F$_2$ ($^{13}$C-labelled CH$_4$ and CH$_4$ with no label). (C) and (D) are the same as (A) and (B), but for the 10–30 cm layer ($P_{10-30}$) of the profundal site. Bottles where the CH$_4$ production or oxidation was inhibited by CH$_2$F$_2$, that is, where it did not increase between the first two time points, are indicated in the symbol description. The CH$_4$ concentration measurements for bottles $^{13}$C-CH$_4$ 2 and $^{13}$C-CH$_4$ 3 of $P_{0-10}$ on day 1 failed (in A). However, based on the relatively high CH$_4$ concentration during the second time point, it is very likely that CH$_4$ production was not inhibited between the first two time points in these bottles.
**Figure S4.** Relative abundances (average +/- SD) of the taxonomic groups of dominant non-methanogenic archaea in the study sediments based on 16S rRNA gene sequencing. L\(_{0-25}\), P\(_{0-10}\) and P\(_{10-30}\) denote 0 – 25 cm layer of the littoral and 0 – 10 cm and 10 – 30 cm layers of the profundal study site, respectively.
Figure S5. Relative abundances (average +/- SD) of the taxonomic groups of the other abundant bacteria. $L_{0-25}$, $P_{0-10}$ and $P_{10-30}$ denote 0 – 25 cm layer of the littoral and 0 – 10 cm and 10 – 30 cm layers of the profundal study site, respectively.
Figure S6. Non-metric multidimensional scaling analyses of the variation in the community structure of (A–B) bacteria (16S rRNA gene), (C–D) archaea (16S rRNA gene), and (E) methanogenic/methanotrophic archaea (*mcrA* gene), as well as the active methanogenic/methanotrophic archaea (mRNA transcripts of *mcrA*) before (non-incubated sample) and after the incubations of sediment slurries collected from the 0–10 cm (*P*<sub>0-10</sub>) (A, C, E) and 10–30 cm (*P*<sub>10-30</sub>) layers (B, D, E) of the profundal site in 2014 and amended with CH<sub>4</sub>, CH<sub>4</sub>+Fe<sup>3+</sup>, or CH<sub>4</sub>+Mn<sup>4+</sup>. Due to the substantial differences between *P*<sub>0-10</sub> and *P*<sub>10-30</sub> in the primary NMS ordinations of the archaeal and bacterial 16S rRNA gene sequence data (data not shown), the final NMS ordination was performed separately for the two depth layers (A–D).
Figure S7. Relative abundance (average +/- SD) and taxonomic affiliations (and methanogenic substrate) of the archaeal 16S rRNA gene OTUs that differed in relative abundance among the CH₄, CH₄+Fe³⁺, or CH₄+Mn⁴⁺ treatments after the incubation of sediment slurry samples from the 0–10 cm (P₀₋₁₀) (A–C) and 10–30 cm (P₁₀₋₃₀) (D–F) layers (linear discriminant analysis [LDA] effect size [LEfSe] method p < 0.05). The OTUs are grouped into three rows according to the treatment in which they exhibited the highest relative abundance in comparison to the other treatments: (A)/(D) CH₄, (B)/(E) CH₄+Fe³⁺, or (C)/(F) CH₄+Mn⁴⁺.
Figure S8. Relative abundance (average +/- SD) and taxonomic affiliations (and methanogenic substrate) of the mcrA gene OTUs that differed in their relative abundance among the CH₄, CH₄+Fe³⁺, or CH₄+Mn⁴⁺ treatments after the incubation of sediment slurry samples from the 0–10 cm (P₀₋₁₀) (B) and 10–30 cm (P₁₀₋₃₀) (A, C) layers (linear discriminant analysis [LDA] effect size [LEfSe] method p < 0.05). The OTUs are grouped into two rows according to the treatment in which they exhibited the highest relative abundance in comparison to the other treatments: A) CH₄+Fe³⁺ or (B)/(C) CH₄+Mn⁴⁺.