Multiple groups of methanotrophic bacteria mediate methane oxidation in anoxic lake sediments

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Abstract

Freshwater lakes represent an important source of the potent greenhouse gas methane (CH_4) to the atmosphere. Methane emissions are regulated to large parts by aerobic (MOx) and anaerobic (AOM) oxidation of methane that are important sinks in lakes. In contrast to marine benthic environments, our knowledge about the modes of AOM and the related methanotrophic microorganisms in anoxic lake sediments is still rudimentary. Here we demonstrate the occurrence of AOM in the anoxic sediments of Lake Sempach (Switzerland), with maximum in situ AOM rates observed within the surface sediment layers in presence of multiple groups of methanotrophic bacteria and various oxidants known to support AOM. However, substrate-amended incubations (with NO_2^-, NO_3^-, SO_4^{2-}, Fe^{3+} and Mn^{4+}) revealed that none of the electron acceptors previously reported to support AOM enhanced methane turnover in Lake Sempach sediments under anoxic conditions. In contrast, the addition of oxygen to the anoxic sediments resulted in an approximately tenfold increase in methane oxidation relative to the anoxic incubations. Phylogenetic and isotopic evidence indicate that both Type I and Type II aerobic methanotrophs were growing on methane under both oxic and anoxic conditions, although methane assimilation rates were an order of magnitude higher under oxic conditions. While the anaerobic electron acceptor responsible for AOM could not be identified, these findings expand our understanding of the metabolic versatility of canonically aerobic methanotrophs under anoxic conditions, with important implications for future investigations to identify methane oxidation processes. Bacterial AOM by facultative aerobic methane oxidizers might be of much larger environmental significance in reducing methane emissions than previously thought.

Introduction

Methane (CH_4) is a powerful greenhouse gas in the atmosphere and a major portion of this gas in aquatic and terrestrial ecosystems is produced biologically in anoxic environments (e.g., anoxic waters and sediments) by anaerobic methanogens. In contrast to oceans, freshwater habitats such as lakes cover only a small portion of the earth's surface (0.9%; Downing et al. 2006), yet, they contribute a significant part to the global emissions of methane to the atmosphere (6-16%; Bastviken et al. 2004). The comparatively low methane concentrations in both surface and bottom waters of lakes (Blees et al. 2015; Donis et al. 2017) relative to those (mM ranges) in anoxic sediments (Blees et al. 2014) may suggest that methane in lake sediments is mostly consumed before released to the water column.

In freshwater lakes, high activities of methane oxidation is often thought to occur at the oxic-anoxic transition zones (Lidstrom and Somers 1984; Kuivila et al. 1988; Frenzel et al. 1990; Bender and Conrad 1994; He et al. 2012). In this scenario, methane oxidation is usually carried out by the conventionally known aerobic methane-oxidizing bacteria (MOB), which is an important methane sink in lacustrine environments. In anoxic lake waters, this pathway can be fueled by oxygen diffusion or intrusion events from the oxic water column (Hanson and Hanson 1996; Bastviken et al. 2002; Pasche et al. 2011; He et al. 2012; Oswald et al. 2016), or through oxygenic photosynthesis in shallow lakes (Oswald et al. 2015; Milucka et al. 2015; Rissanen et al. 2018). On the other hand, true anaerobic oxidation of methane (AOM) in anoxic environments is usually performed by anaerobic methanotrophs (ANMEs) that are able to couple methane oxidation to the reduction of sulfate (Boetius et al. 2000; Orphan et al. 2002; Knittel et al. 2005; Milucka et al. 2012), nitrite (Raghoebarsing et al. 2006; Ettwig et al. 2008), nitrate (Haroon et al. 2013), metal oxides (Beal et al. 2009; Ettwig et al. 2016; Cai et al. 2018; Leu et al. 2020) or in a few cases, organic electron acceptors such as humic substances (Scheller et al. 2016). In anoxic lake sediments, pathways of AOM such as nitrate/nitrite-dependent methane oxidation may sometimes be masked by aerobic process due to the close proximity of nitrate/nitrite- and oxygen-consumption zones near the sediment-water interfaces, thus making it difficult to distinguish between true AOM and aerobic methane oxidation. Interestingly, nitrite-dependent AOM employs the pathway similar to canonical aerobic methane oxidation that involves soluble and/or particulate methane

monooxygenase enzymes and is catalyzed by *Candidatus* Methylomirabilis that are capable of producing oxygen intracellularly via the dismutation of nitric oxide (Ettwig et al. 2010). Such denitrifying methanotrophic bacteria have been recently reported for both lake sediments (Kojima et al. 2012; Deutzmann et al. 2014) and water column (Graf et al. 2018; Mayr et al. 2020). In contrast to marine ANMEs that use sulfate to oxidize methane (Boetius et al. 2000; Orphan et al. 2002; Knittel et al. 2005; Milucka et al. 2012), a recently identified anaerobic methanotrophic archaea in freshwater lake sediments that are phylogenetically closely related to *Candidatus* Methanoperedens nitroreducens (i.e., nitrate-dependent methanotrophs or ANME-2d, Haroon et al. 2013), was demonstrated to also perform methane oxidation coupled to sulfate reduction (Su et al. 2020).

Methane oxidation seems to be ubiquitous in strictly anoxic lake sediments (Martinez-Cruz et al. 2018), however, evidence of the occurrence of true AOM that involves anaerobic electron acceptors and related microorganisms, is still sparse (Schubert et al. 2011; Crowe et al. 2011; Sivan et al. 2011; Norði et al. 2013; Weber et al. 2017; Bar-Or et al. 2017; Su et al. 2020). Moreover, the role of aerobic methanotrophs in anoxic sediments and their contribution to the observed AOM is still not well understood. Indeed, under oxygen limitation, some gamma-proteobacterial MOB (i.e., *Methylomonas* and *Methylomicrobium*) have the capacity to oxidize methane with nitrate/nitrite as terminal electron acceptors (Kits et al. 2015a; b). In addition, *Methylobacter* was recently shown to utilize methane for lipid synthesis during long-term anoxic incubation although the responsible electron acceptors for AOM were not investigated (Martinez-cruz et al. 2017). This genus was also observed in deep anoxic hypolimnion of a permanently stratified lake with high methane oxidation rates (Blees et al. 2014), yet, the exact mechanism of this apparent AOM by aerobic methane oxidizers and the electron acceptor remained enigmatic. Another putative aerobic methanotroph lineage-*Crenothrix* (Stoecker et al. 2006), was also shown to have the potential to catalyze methane oxidation coupled to nitrate reduction under anoxic conditions (Oswald et al. 2017).

It appears that aerobic methanotrophs are versatile in electron acceptors use and able to conduct anaerobic respiration in oxygen-deficient or anoxic environments. In this study, we investigated methane oxidation in anoxic sediments of Lake Sempach in Switzerland, with the particular goal to elucidate the potential electron acceptors and microbial players involved and to verify whether the apparent anoxic methane

sink represents true AOM. Towards this goal, we combined sediment pore water hydrochemical data with in situ AOM rate measurements using radio-labeled methane, as well as slurry incubation experiments to study the impact of alternative oxidants (i.e., sulfate, iron, manganese, nitrite and nitrate) on AOM. In addition, we characterized the microorganisms that are involved in methane oxidation using approaches including lipid biomarker and 16S rRNA gene sequencing.

Materials and Methods

Study Site and sampling. Lake Sempach is a eutrophic lake and located in central Switzerland. Since decades ago, the hypolimnion of this lake has been artificially aerated, leading to complete mixing of water column (Stadelmann 1988). The oxygenation of the hypolimnion leads to oxic condition in the bottom waters and may potentially introduce alternative oxidants for methane oxidation in the sediment. In March 2015, sediment cores (inner diameter 62 mm) were recovered with a gravity corer from the deepest site of Lake Sempach. Samples for dissolved methane concentrations were collected onsite with cut-off syringes. 3 mL of sediment samples were fixed with 7.0 mL 10% NaOH in 20 mL glass vials, which were then immediately sealed with thick butyl rubber stoppers. Porewater was extracted by centrifuging the 1 or 2 cm segments of the sediment core under N_2 atmosphere and filtering the supernatant through 0.45 µm filters, and stored at 4 °C after fixation for respective analysis. Samples for DNA extraction and particulate iron/manganese were collected at 1 or 2 cm intervals of the sediment core, and stored frozen at -20 °C until further processing.

Chemical analyses. Methane concentrations in the headspace of NaOH-fixed samples were measured using a gas chromatography (GC, Agilent 6890N) with a flame ionization detector, and helium as a carrier gas. Nitrite was determined colorimetrically using Griess reagent (Hansen and Koroleff (1999). Concentrations of sulfate, nitrate and ammonium were analyzed by ion chromatography (881 IC compact plus, Metrohm, Switzerland). Samples for dissolved iron (Fe^{2+}) and manganese (Mn^{2+}) were fixed with HCl (6 M) after filtration through a 0.45 µm membrane filter and measured using inductively coupled plasma optical emission spectrometry (ICP-OES). Filtered samples for sulfide concentrations were stabilized

immediately after sampling with 200 µL zinc acetate (5% w/w) and analyzed in the laboratory photometrically (Cline 1969). Reactive iron oxide in the solid phase was extracted using 0.5 M HCl and then reduced to Fe(II) with 1.5 M hydroxylamine. Concentrations of Fe(II) were then determined photometrically using the ferrozine assay (Stookey 1970). Particulate reactive iron was calculated from the difference between the total Fe(II) concentrations after reduction, and the dissolved Fe(II) in the filtered sample. A total carbon analyzer (Shimadzu, Corp., Kyoto, Japan) was used to quantify dissolved inorganic carbon (DIC) concentrations. To determine the penetration depth, oxygen gradients in surface sediments were measured with a Clark-type microelectrode sensor (Ox 10; Unisense A/S, Denmark).

In situ AOM rate measurements. To determine depth-specific in situ AOM rates in Lake Sempach sediments, subcore incubation was applied (Su et al. 2019). Briefly, subcores were taken by inserting three small plexiglass tubes (inner diameter 16 mm) into the fresh sediment core and both ends of the core tubes were sealed securely with black rubber stoppers. After ~1 h preincubation, 20µL of $^{14}CH_4$ solution (~2.5kBq) was injected through the predrilled, silicone-sealed ports at 1.5 cm intervals. Subcores were incubated at in situ temperature (4 °C) in the dark for 2 days. At the end of the incubation, subcores were extruded and subsamples were collected at 1 or 2 cm segments, and directly transferred into 100 ml vials containing 20 ml of the aqueous NaOH (5% w:w) to terminate microbial activity. Radioactivities of different ^{14}C pools in the incubation samples and AOM rates were determined according to Su et al. (2019).

Substrate-amended slurry incubations. Experiment 1 (sulfate, iron and manganese-addition experiments with ^{14}C-labelled methane). Anoxic slurries of ~8 L were prepared by purging a mixture of sediments (surface 10 cm × 4 sediment cores, ~1500 cm^3) and autoclaved anoxic artificial sulfate-free medium (Su et al. 2020). Homogenized slurries of ~100 mL were subsequently dispensed into 120-mL serum bottles and sealed with butyl rubber stoppers. To the electron acceptor-supplemented bottles, stock solutions of sulfate, ferrihydrite and birnessite were added to final concentrations of 2 mM, 5 mM and 5 mM, respectively. For the inhibitor-addition experiment, molybdate was added to a final concentration of 4 mM. Base-killed controls (pH > 13) were also prepared and incubated in parallel. All bottles were

169 further purged with N_2 to remove any traces of oxygen. After 24-h preincubation, 1.5
170 ml pure CH_4 was injected into the headspace of each bottle, which was gently shaken
171 for 24 h, resulting in a final concentration of 100 μmol/L in the slurries. Finally, all
172 incubation bottles were transported to anoxic chamber and filled bubble-free with
173 anoxic medium (100 μmol/L CH_4) before injecting $^{14}CH_4$ gas tracer. At different time
174 points (30 and 60 days), incubation samples were stopped by adding 5 ml saturated
175 NaOH after creating 20 ml headspace. AOM rates were analyzed and calculated as
176 described previously (Su et al. 2019).

177 Experiment 2 (nitrite-, nitrate-addition and oxic experiments with ^{13}C-labelled
178 methane). To further test the role of nitrite/nitrate in AOM, we performed slurry
179 incubation experiments with a mixture of sediment and anoxic bottom lake water
180 taken in November 2020. Briefly, 100 ml mixed slurries (1.5 g dw sediment per 120-
181 mL bottle) were degassed with He to remove any traces of oxygen and background
182 methane before transporting to anoxic bag. 5 mL of pure ^{13}C-labelled methane (99.8
183 atom %, Campro Scientific) was injected into the headspace of bottles, which were
184 amended with anoxic stock solutions of nitrite and nitrate, with final concentrations of
185 0.48 mM and 2.4 mM, respectively. Nitrite and nitrate were replenished when
186 consumed or remained at low concentrations. Killed controls (autoclaved and $^{13}CH_4$),
187 live controls (only $^{13}CH_4$) and controls with only ^{13}C-labelled bicarbonate were also
188 incubated in parallel at room temperature. For oxic experiments, slurries were spiked
189 with pure oxygen and then injected with 5 mL of pure $^{13}CH_4$. Oxygen was replenished
190 regularly to maintain oxic condition in the incubation bottles. At different time points,
191 incubation slurries were homogenized and 5 mL of the supernatant was collected
192 under N_2 atmosphere and filtered with a 0.45 μm membrane filter for subsequent
193 sulfate, nitrate, nitrite, DIC concentration and stable carbon isotope ratio analyses. To
194 determine the carbon isotope composition of DIC, 0.2-1 mL of water sample was
195 transferred into a 12 mL He-purged exetainer (Labco Ltd) containing 200 μL zinc
196 chloride (50 % w/v), and then acidified with ~200 μL 80% H_3PO_4. The liberated CO_2
197 was analyzed in the headspace using a purge-and-trap purification system (Gas Bench)
198 coupled to a GC-IRMS (Thermo Scientific, Delta V Advantage). Absolute ^{13}C-DIC
199 abundances were determined from the DIC concentrations and the $^{13}C/^{12}C$ ratios
200 converted from $\delta^{13}C$-DIC values (Oswald et al. 2015). The temporal change in ^{13}C-
201 DIC with incubation time was then used to calculate slurry-incubation-based potential
202 methane oxidation rates.

Microbial lipid extraction and analysis. After 160 days of incubation with $^{13}CH_4$ (Experiment 2), slurries of triplicate bottles were combined and freeze-dried. Samples were then homogenized for lipid extraction using an Accelerated Solvent Extraction system (ASE 350, Dionex Corp., Sunnyvale, CA, USA). Samples were extracted with a solvent mixture of dichloromethane and methanol (9:1, vol/vol) over three extraction cycles at 100 °C and maximum pressure of 1500 psi. Total lipid extracts (TLEs) were collected in 60 mL glass vials and concentrated using a vacuum evaporator system (Rocket Synergy Evaporator, Genevac Ltd., Ipswich, UK). Prior to extraction, an internal standard mix (5α-Cholestane, Nonadecanoic acid and 1-Nonadecanol) was added to each sample for the quantification of individual biomarkers. The TLEs were further evaporated to dryness and then saponified with methanolic KOH-solution (12%) at 80 °C for 3 h. Neutral compounds were extracted by liquid-liquid extraction using hexane. After extracting the neutral fraction, fatty acids (FAs) were extracted from the remaining aqueous phase with hexane after acidification (pH ~ 1). The fatty acid fraction was methylated using BF_3 in methanol (14% v/v, Sigma Aldrich) at 80 °C for 2 h, and analyzed later as fatty acid methyl esters (FAMEs). Neutral compounds were further separated into hydrocarbon and alcohol fractions over silica glass gel columns, as described by Sicre et al. (1994) with following modifications. The neutral fraction was dissolved in heptane and transferred onto the wet column. Aliphatic, cyclic and aromatic hydrocarbons were eluted with 2 mL heptane and 2 mL heptane/toluene (3:1 v/v), ethers and ketones with 2 mL heptane/toluene (1:1 v/v) and 2 mL heptane/ethyl acetate (95:5 v/v), and finally alcohols with 2 mL heptane/ethyl acetate (85:15 v/v) and heptane/ethyl acetate (80:20 v/v). The alcohol fraction was methylated with 100 µL pyridine and 50 µl bis(trimethylsilyl)trifluoracetamide (BSTFA) at 70 °C for 1 h.

All fractions were quantified using a TRACE GC Ultra gas chromatograph equipped with a flame ionization detector (GC-FID) (Thermo Scientific, Waltham, MA, USA). Individual lipid compounds were quantified by normalization to the internal standard and identified by comparing their retention times to those of laboratory standard mixtures, or by a gas chromatograph mass spectrometry (GC-MS, Thermo Scientific DSQ II Dual Stage Quadrupole) and the acquired mass spectra were identified through comparison with published data. Compound-specific stable carbon isotope ratios were determined using a gas chromatograph with split/splitless

injector, connected on-line via a GC-Isolink combustion interface to a ConFlo IV and Delta V Advantage isotope ratio mass spectrometer (GC-IRMS, Thermo Scientific, Bremen, Germany). Both concentrations and $\delta^{13}C$ values of lipids were corrected for the introduction of carbon atoms during derivatization.

DNA extraction, PCR amplification, Illumina sequencing and data analysis. DNA was extracted from both samples of Lake Sempach core sediments as well as from the slurry sediments at the end of incubations (Experiment 2), using a FastDNA SPIN Kit (MP Biomedicals) following the manufacturer's instructions. A two-step PCR approach was applied in order to prepare the library for sequencing at the Genomics Facility Basel (https://www.bsse.ethz.ch/genomicsbasel). Briefly, a first PCR (25 cycles) was performed using primers 515F-Y and 926R targeting the V4 and V5 regions of the 16S rRNA gene (Parada et al. 2016). Sample indices and Illumina adaptors were added in a second PCR of eight cycles. Purified indexed amplicons were finally pooled at equimolar concentration into one library and sequenced on an Illumina MiSeq platform using the 2×300 bp paired-end protocol (V3 kit). After sequencing, quality of the raw reads was checked using FastQC (v 0.11.8) (Andrews 2010). Paired-end read merger (usearch v11.0.667_i86linux64) was used to merge forward and reverse reads into amplicons of about 419 bp length, allowing a minimum overlap of 40 nucleotides and a mismatch density of 0.25. Quality filtering (min Q20, no Ns allowed) was carried out using PRINSEQ (Schmieder and Edwards 2011). Amplicon sequence variants were determined by denoising using the UNOISE algorithm (UNOISE3, usearch v11.0.667_i86linux64) and are herein referred to as ZOTU (zero-radius OTU). Taxonomic assignment was done using SINTAX v11.0.240_i86linux64 (Edgar 2016) and the SILVA 16S rRNA reference database v128 (Quast et al. 2013). Subsequent data analyses were carried out with Phyloseq (McMurdie and Holmes 2013) in the R environment (R Core Team, 2014) (http://www.r-project.org/).

Data availability. Raw reads are deposited at the NCBI Sequence Read Archive (SRA) in the BioProject PRJNA759259, and can be accessed under the accession numbers from SRR15689022- SRR15689034.

Results

Rates of AOM and geochemistry of Lake Sempach sediments. Concentration of methane in the sediments increased with depth, reaching a maximum of ~1.75 mM at 10 cm depth. Methane oxidation were observed throughout the sediment core and the activity decreased with depth, with the maximum AOM rates (68.0 ± 19.0 nmol cm^{-3} d^{-1}) measured in the upper 0-2 cm (Fig. 1B). Nitrate was only detected above 3 cm (up to 5 μM) and the sulfate concentration decreased downward from 78 μM at the sediment-water interface to 7 μM at 10 cm. Both sulfide and nitrite were below detection at all depths. Reducible iron and manganese oxides were most abundant in surface sediments (24.3 and 30.8 μmol g^{-1} sediment respectively), and concentrations of both dissolved iron (Fe^{2+}) and manganese (Mn^{2+}) increased steadily with depth. Under the condition of fully oxygenated bottom waters, oxygen was rapidly consumed in the uppermost sediment layers and penetrated to a maximum depth of 3 mm (Fig. 1F).

Diversity and abundance of methanotrophs in lake sediments. Analysis of sediment microbial community, based on 16SrRNA gene sequencing, revealed the presence of multiple groups of aerobic methanotrophs, including Type I gamma- and Type II alpha-proteobacterial MOB (Fig. 1G-H). Type I gamma-MOB consist of the conventionally known *Methylococcaceae*, of which the genus *Methylobacter* dominated this cluster. The relative abundances of *Methylococcaceae* range from 0.07% to 0.23%, with peak abundance occurring at 3 cm depth. In comparison, Type II alpha-MOB *Methylocystaceae* was found at much lower relative abundances, with a maximum of 0.04% observed at 6 cm depth. A large number of 16S rRNA gene sequences (0.17–1.25%, Fig. 1I), belonged to *Crenotrichaceae*, of which *Crenothrix* were the predominant genus and the amplified sequence variants shared very high sequence similarities (96-100%) with what has been described recently as major methane oxidizers in stratified lakes (Oswald et al. 2017). In addition, a very small proportion of sequences (up to 0.03%) were affiliated with *Methylacidiphilaceae* within the *Verrucomicrobia* phylum and were reported to perform aerobic methane oxidation in acidic geothermal environments (Islam et al. 2008; Carere et al. 2017). A canonical correspondence analysis (CCA) was performed to show correlation between the concentrations of potential electron acceptors, methane oxidation rates and

303 specific methanotrophic species (Fig. 1K). The CCA triplot shows that members
304 belonging to *Crenothrix* or *Methylobacter* were positively correlated to the methane
305 oxidation rates and negatively correlated to methane concentrations. Interestingly, the
306 plot also shows that *Crenothrix* and *Methylobacter* were more likely found in surface
307 sediments with high concentrations of potential electron acceptors (i.e., oxygen,
308 nitrate, sulfate, iron and manganese).
309
310 **Effect of potential electron acceptor addition on AOM rates.** To investigate the
311 potential electron acceptors for AOM, sediments of top 10 cm where in situ rates were
312 measured (Fig. 1B), were incubated with different electron acceptors. In the first
313 attempted experiment with ^{14}C-labelled methane, no apparent increase in AOM rate
314 was observed in any amendment with potential electron acceptors compared with the
315 controls (no electron acceptors added, Fig. 2). Incubations of both 30 and 60 days
316 revealed very similar potential AOM rates between the untreated controls and
317 amendments with sulfate and molybdate. Interestingly, the addition of iron oxides to
318 the slurries did not stimulate methane oxidation but rather resulted in systematically
319 lower AOM rates relative to the control experiments. More surprisingly, incubations
320 with manganese oxides completely inhibited methane oxidation. In the second
321 experiment using ^{13}C-labelled methane, we further tested the roles of nitrate/nitrite
322 and compared with aerobic methane oxidation. Similar to ^{14}C-labelled methane
323 incubation experiments, AOM was occurring in the live controls (without electron
324 acceptors addition). Incubations with nitrate and nitrite additions showed a similar
325 trend, but did not stimulate methane oxidation when compared with the live controls
326 (Fig. 3). For both untreated controls and nitrate/nitrite amendments, AOM rates were
327 low over the first 4 days but then increased dramatically between 4 and 8 days and
328 afterwards remained low again. In contrast, methane oxidation was steady throughout
329 the incubation period under oxic condition and the rates were almost 10 time higher
330 than the controls. Strikingly, sulfate was depleted after 8 days in live controls and
331 there was extensive sulfate production in incubation bottles amended with both nitrate
332 or oxygen.
333
334 **Lipid biomarkers and microbial communities in incubations with $^{13}CH_4$.** At the
335 end of the incubation, abundance and carbon isotopic composition of fatty acids and
336 alcohols were examined to constrain methanotrophs and infer carbon flow through the

337 microbial population. The concentrations of lipid biomarkers in different treatments
338 were comparable, except for incubation with oxygen showing overall lower values
339 than the other treatments (Fig. 4). In the untreated control and nitrite amendment, the
340 most strongly ^{13}C-enriched fatty acids were monounsaturated hexadecanoic acids
341 ($C_{16:1\omega7}$ and $C_{16:1\omega5}$) and (ω-1)-OH-$C_{28:0}$, ranging from 531‰ to 1558‰. By
342 comparison, the highest ^{13}C incorporation was observed for (ω-1)-OH-$C_{28:0}$ in
343 incubation with nitrate (1524‰), followed by 10-Methyl-hexadecanoic acid (10-Me-
344 $C_{16:0}$, 367‰). Most strikingly, $\delta^{13}C$ measurements of fatty acids from the oxic
345 incubation revealed significantly stronger ^{13}C labeling of most fatty acids (values
346 between 6800 and 14500‰, Fig. 4) than both the control and incubations with
347 nitrite/nitrate. In order to reveal the effect of autotrophic carbon assimilation, we
348 incubated the slurries with ^{13}C-labelled bicarbonate only. The results showed that no
349 incorporation was observed for (ω-1)-OH-$C_{28:0}$ (-39‰) and $\delta^{13}C$ value of 10-Me-$C_{16:0}$
350 was 164‰. In addition, ^{13}C enrichments in slurry sample with ^{13}C-bicarbonate only
351 were insignificant for both $C_{16:1}$ and (ω-1)-OH-$C_{28:0}$ (ranging from 18 to 77‰)
352 relative to $^{13}CH_4$ incubations with nitrite/nitrate.
353 In the initial slurry (Slurry T0), *Euryarchaeota* and *Woesearchaeota* were the
354 most abundant phyla (Fig. 5A). Archaeal community structure in both the live control
355 and incubation with ^{13}C-bicarbonate was similar to that of Slurry T0. While nitrite
356 addition enhanced the percentage of *Bathyarchaeota* within archaea, nitrate seemed to
357 favor the growth of *Woesearchaeota*. A notable change in the archaeal community
358 was observed in oxic incubation, with *Thaumarchaeota* being predominant at the end
359 of incubation. As for bacterial composition at the class level (Fig. 5B), relative
360 abundances of both *Clostridia* and *Thermoleophilia* increased significantly in live
361 control and ^{13}C-bicarbonate relative to Slurry T0. *Gammaproteobacteria* comprised a
362 smaller proportion after nitrite/nitrate addition but members within *Anaerolineae* and
363 *Ignavibacteria* were boosted. In oxic slurries, *Alphaproteobacteria* accounted for a
364 larger proportion of the bacterial population. At family level, *Crenotrichaceae* and
365 *Methylococcaceae* were the most abundant groups among the known methanotrophic
366 communities in the initial slurry sample, representing 0.97% and 0.24% of total
367 sequences, respectively. It is surprising to observe that relative abundances of both
368 *Crenotrichaceae* and *Methylococcaceae* were higher in live control (3.67% and
369 1.14%) and ^{13}C-bicarbonate amendment (4.14% and 1.36%) when compared to Slurry
370 T0. In contrast, relative abundances of these two clusters were much lower in

371 nitrite/nitrate additions relative to Slurry T0, comprising 0.28% and 0.07% in
372 incubation with nitrite and 0.26% and 0.07% for nitrate amendment. Similar to lake
373 sediments, *Crenothrix* was the dominant genus within *Crenotrichaceae*, accounting
374 for 76-90% in different treatments. In addition, *Candidate* Methylomirabilis was
375 detected in incubation with nitrate only, but in very low abundance (0.005% of total
376 sequences).
377

378 **Discussion**

379 In Lake Sempach with a permanently oxic hypolimnion, oxygen was rapidly
380 depleted in the uppermost sediment layers and penetrated to a maximum depth of 3
381 mm. As revealed by in situ rate measurements, the maximum of methane oxidation
382 activity was located at depths near water-sediment interface and apparent AOM with
383 overall lower rates was also occurring in the deeper, anoxic sediment layers. This is
384 different from marine settings where highest AOM rates took place within sulfate
385 methane transition zones that were well below the sediment surface (Iversen and
386 Jørgensen 1985). In oxic-anoxic transition zones of lake sediments, oxygen may serve
387 as the most important electron acceptors and aerobic methanotrophs play a dominant
388 role in reducing the methane fluxes (Bender and Conrad 1994; He et al. 2012). In
389 anoxic sediments, however, the pathway of methane oxidation with regard to the
390 electron acceptors involved remains to be fully explored. Notably, the highest AOM
391 rates in surface sediment layers corresponded to the concentration maxima of nitrate,
392 sulfate, iron and manganese, suggesting their potential roles for AOM. Despite the
393 clear evidence of AOM in anoxic zones, no marine ANME-1 or 2 were detected
394 throughout the sediment core by 16S rRNA gene sequence with the primers that
395 match with a large fraction of the deposited sequences of anaerobic methanotrophic
396 archaeal clades (Su et al. 2020). No *Ca.* Methylomirabilis and *Ca.* Methanoperedens
397 were detected either (Ettwig et al. 2010; Haroon et al. 2013; Su et al. 2020). Instead,
398 the anoxic sediments contained abundant gamma-proteobacterial *Methylococcaceae*
399 and some alpha-proteobacterial *Methylocystacea* that are traditionally considered
400 aerobic methanotrophs (Hanson and Hanson 1996). In addition, the sediments also
401 contained an abundance of filamentous methanotroph *Crenothrix* found in drinking
402 water systems (Stoecker et al. 2006). The relative abundances of *Methylococcaceae*,
403 *Crenothrix* and *Methylacidiphilaceae* were higher in upper sediment layers, while

404 *Methylocystacea* were more abundant in lower parts of the sediments. The vertical
405 distribution of various methanotrophic groups may suggest their different affinities
406 for methane, as evidenced, at least in part, by some studies showing that Type I
407 methanotrophs were able to outcompete Type II MOB at low concentrations of
408 methane (Amaral and Knowles 1995; Henckel et al. 2000). However, the presence of
409 diverse aerobic methanotrophs in anoxic sediments led us to speculate that some of
410 them, if not all, might have an alternative anaerobic lifestyle.

411 Indeed, increasing evidence has demonstrated the occurrences of gamma-MOB
412 in anoxic lake waters and sediments (Blees et al. 2014; Milucka et al. 2015; Oswald et
413 al. 2016; Martinez-cruz et al. 2017). We could additionally show active growth of
414 gamma-MOB in the apparent absence of oxygen by incubating Lake Sempach
415 sediments with $^{13}CH_4$ and analyzing lipid biomarkers for methane assimilation. The
416 monounsaturated fatty acids $C_{16:1\omega7}$ and $C_{16:1\omega5}$, which were typically indicative of
417 Type I aerobic methanotrophs (Bowman et al. 1994; Hanson and Hanson 1996), were
418 most enriched in ^{13}C. Although the produced $^{13}CO_2$ from methane oxidation could be
419 assimilated into the biomass by autotrophic microorganism such as sulfate reducing
420 bacteria (SRB). It has been reported that lipids such as $C_{16:1\omega7}$ and $C_{16:1\omega5}$ might be
421 also associated with SRB at methane-rich seeps that were involved in sulfate-
422 dependent AOM (Elvert et al. 2003). However, no or very low incorporation of ^{13}C
423 into these lipid biomarkers in the incubation with ^{13}C-labelled bicarbonate only
424 indicates that they were not derived from SRBs and were thus signals of Type I
425 gamma-MOB. The results further confirmed the direct assimilation of methane rather
426 than DIC by these methanotrophic bacteria. The uptake of labeled methane by alpha-
427 MOB was also observed in the anoxic sediments as revealed by the strong ^{13}C-
428 enrichment of long-chain (ω-1)-hydroxy fatty acids, that is, (ω-1)-OH-$C_{28:0}$. This lipid
429 compound was mainly found in type II methane-utilizing bacteria including
430 *Methylosinus* and *Methylocystis* (Skerratt et al. 1992). These findings agreed with the
431 composition of methanotrophic community in the incubation slurry of live control that
432 contained both *Methylococcaceae* and *Methylocystaceae*. Similar to initial sediment
433 samples, *Methylococcaceae* were dominated by *Methylobacter* and *Methylocystis* was
434 the most abundant genus within *Methylocystaceae* (data not shown). With respect to
435 *Crenothrix*, we were not able to clearly verify the methane assimilation into biomass
436 since no diagnostic lipids were ever reported for them, but due to their capacity to
437 oxidize methane anaerobically and the high abundances in anoxic sediments, we

438 cannot rule out that this methanotrophic group was not one of the most important
439 methane consumers. Overall, our molecular data and lipid biomarker analysis provide
440 some of the first evidence that AOM in anoxic incubations as well as in Lake
441 Sempach sediments was most likely mediated by aerobic methanotrophs.

442 According to the CCA triplot, members of *Crenothrix* and *Methylobacter* were
443 more likely found at depths with higher methane oxidation rates and associated with
444 the detected electron acceptors in anoxic sediments. However, slurry incubations with
445 either $^{13}CH_4$ (with nitrite/nitrate additions) or $^{14}CH_4$ (with sulfate, iron or manganese
446 additions) demonstrate that common anaerobic electron acceptor amendments did not
447 stimulate AOM, providing further evidence that the AOM pathway in Lake Sempach
448 sediments was distinguished from those of previously investigated systems (Boetius
449 et al. 2000; Orphan et al. 2002; Raghoebarsing et al. 2006; Vaksmaa et al. 2017; Su et
450 al. 2020). On the contrary, the additions of metal oxides led to systematically lower
451 AOM rates (iron) or complete inhibition (manganese). These observations appear at
452 first sight at odds with recent studies showing the stimulation of both iron and
453 manganese additions for aerobic methanotrophs under anoxic conditions (Oswald et
454 al. 2016; Zheng et al. 2020). The inhibition of AOM as a result of adding external
455 anaerobic electron acceptors was also observed in previous studies (Segarra et al.
456 2013; Milucka et al. 2015; Rissanen et al. 2018). Extra addition of metal oxides in the
457 slurries may change the redox condition of the systems and likely favors the growth
458 of iron- or manganese-utilizing microorganisms that could outcompete methane
459 oxidizers for essential nutrients, but further investigation is needed to reveal their
460 effect on AOM. Notably, in $^{13}CH_4$ incubations without electron acceptors added, the
461 co-occurrence of increasing $^{13}CO_2$ and decreasing sulfate concentrations over the first
462 8 days of incubation suggests a possible link between methane oxidation and sulfate
463 reduction. However, the extensive amounts of sulfate produced in nitrate-amended
464 bottles did not continuously stimulate $^{13}CO_2$ production significantly when compared
465 with the live controls. This inconsistence confirmed that sulfate was not the electron
466 acceptor for AOM in Lake Sempach sediments, which was further supported by the
467 similar rates observed in amendments with molybdate, a competitive inhibitor for
468 microbial sulfate reduction (Wilson and Bandurski 1958). Despite that aerobic
469 methanotrophs are obligate aerobes, some of them were able to survive long periods
470 of oxygen starvation (Roslev and King 1995), and even switch from respiring oxygen
471 to nitrite or nitrate for methane oxidation (Kits et al. 2015a; b; Oswald et al. 2017).

472 Both Type I MOB and *Crenothrix* were detected in our incubations, however, the
473 AOM pathway involving nitrite or nitrate respiration seemed unlikely as neither
474 enhanced methane oxidation rates significantly, indicating an as-yet-unidentified
475 electron acceptor. Interestingly, nitrite-dependent *Ca.* Methylomirabilis was not
476 detected in nitrite bottles but present in incubation with nitrate addition, though in
477 very low abundance (1.36% of known methanotrophic community). This finding
478 agreed with the lipid data in nitrate amendments that displayed a stronger enrichment
479 of the fatty acid 10-methylhexadecanoic acid (10-Me-$C_{16:0}$), which was found in *Ca.*
480 Methylomirabilis oxyfera (Kool et al. 2012). It is likely that these denitrifying
481 methanatrophic bacteria contributed a very small proportion of the oxidized methane.
482 However, when considering their low abundance, the consumption of nitrite/nitrate
483 was mostly resulting from canonical denitrification (Zumft 1997).
484 At this point, we are not able to constrain the electron acceptors involved in the
485 anoxic lake sediments based on the performed slurry incubation experiments, as none
486 of the anaerobic electron acceptors used resulted in the stimulation of AOM.
487 Interestingly, a recent study suggests that AOM via "reverse methanogenesis" can
488 proceed in the absence of inorganic electron acceptors (Blazewicz et al. 2012). This
489 pathway of AOM may offer a possible explanation for the ineffectiveness of all tested
490 electron acceptors and we cannot conclude that AOM via reverse methanogenesis was
491 not important in Lake Sempach sediments. In addition, alternative electron acceptors
492 that were already present in the lake sediments (e.g., organic matter) could play a role.
493 Indeed, humic substances have been recognized as a viable electron acceptor through
494 the reduction of their quinone moieties (Lovley et al. 1996; Scott et al. 1999) and
495 organic electron acceptors in the wetlands such as humic acids were suggested to play
496 a role in AOM (Smemo and Yavitt 2011; Blodau and Deppe 2012). In addition,
497 anaerobic methanotrophic archaea from marine sediments were recently demonstrated
498 to use humic substance analogues (i.e., AQDS and humic acids) as electron acceptors
499 in the absence of sulfate (Scheller et al. 2016). Humic substances represent a large
500 proportion of lake sediments (Ishiwatari 1985), and have the potential to be the
501 electron acceptors for methane oxidation in anoxic sediments. If true, such mode of
502 AOM may play an underappreciated role in reducing methane emissions, given the
503 widespread distribution of humic substances in lacustrine environments. However,
504 further investigations are apparently required to explore the role of humic substances
505 and the biochemical mechanism apparently by aerobic methanotrophs in anoxic lake

sediments.

Most strikingly, additions of oxygen to our incubations with $^{13}CH_4$ resulted in an approximately tenfold increase in $^{13}CO_2$ production relative to the live controls or amendments with nitrite/nitrate. This clearly shows that aerobic methanotrophs were actively involved in methane oxidation when replenished with oxygen. Likewise, active growth of these methanotrophic bacteria was evidenced by the substantial ^{13}C-enrichment of the diagnostic fatty acids, with the methane assimilation rates at least ten times higher than those in anoxic incubations. This is expected since aerobic respiration generates much more energy than anaerobic metabolism. The result of oxic incubation further indicates that aerobic methane oxidation is a much more efficient methane filter than AOM pathways, consuming most of the methane flux in the oxic-anoxic transition zones and thus controlling the emission of this greenhouse gas from lake waters to the atmosphere. However, given that the vast majority of methane is produced and stored in anoxic lake sediments, the pathway of AOM is also an important process in reducing methane emissions and the widespread occurrence of AOM in anoxic sediments is of much larger geochemical and environmental significance than previously thought.

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786 **FIGURES LEGENDS**

787

788 **Figure 1.** (A-F) Depth profiles of methane, methane oxidation rates, porewater
789 nitrate/sulfate, particular iron/manganese oxides, dissolved iron/manganese and
790 oxygen in the sediment of Lake Sempach. Error bars represent standard deviations of
791 triplicate rate measurements. No nitrite was detected. (G-J) Relative abundances of
792 known metahnotrophic families and their subgroups at the genus level. (G)
793 Methylococcaceae or Type I gamma-proteobacterial MOB. (H) Methylocystaceae or
794 Type II alpha-proteobacterial MOB. (I) Crenotrichaceae. (J) Methylacidiphilaceae. (K)
795 Canonical correspondence analysis (CCA) showing the relationship between MOR
796 and methanotrophic species and the effect of nutrient concentrations on their
797 distribution. ZOTU abundances were normalized and Hellinger transformed prior to
798 CCA analysis. A total of 42 ZOTUs accounting for > 92% of methanotrophic
799 community were shown on the plot and bold text represents taxa belonging to the
800 genus *Methylobacter* or *Crenothrix*. Black triangles denote sediment samples at
801 different depths.

802

803 **Figure 2.** Methane oxidation in anoxic slurries with ^{14}C-labeled methane and
804 different electron acceptors (sulfate, amorphous iron and manganese oxides) over 30
805 and 60 days. Control incubations were conducted without electron acceptors. No
806 methane oxidation rates were detected in base-killed controls (pH>13).

807

808 **Figure 3.** Concentrations of produced $^{13}CO_2$ and sulfate in anoxic slurries amended
809 with ^{13}C-labeled methane and electron acceptors (nitrite, nitrate and oxygen). (A, B)
810 Controls including killed controls (autoclaved) and live controls (without electron
811 acceptors added). (C, D) Nitrite addition, nitrite was replenished after 17 days. (E, F)
812 Nitrate addition, nitrate was replenished after 17 days. (G, H) Oxic incubations. No
813 nitrite and nitrate was detected in both controls and slurries with oxygen.

814

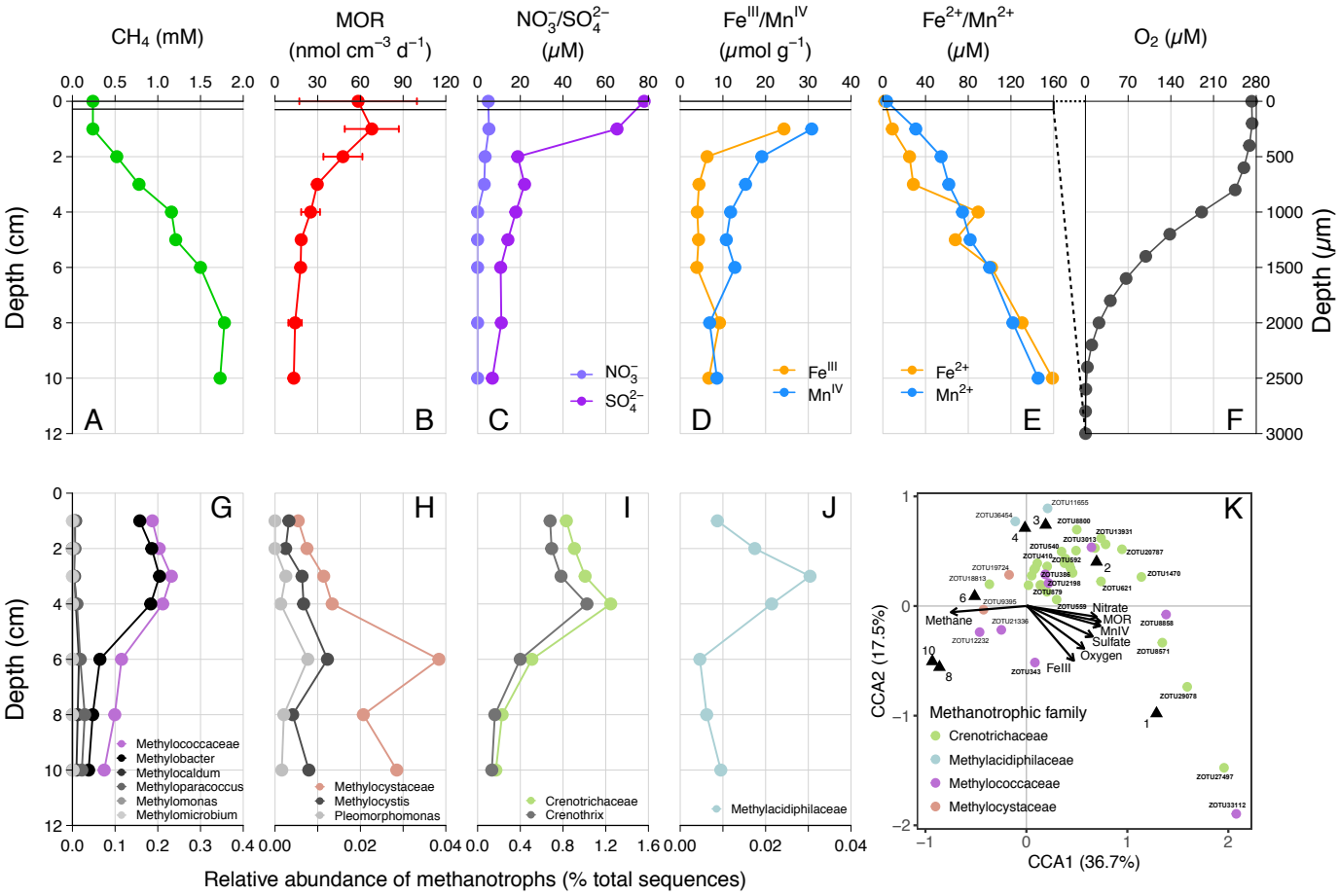
815 **Figure 4.** Concentrations and compound-specific $\delta^{13}C$ values of fatty acids diagnostic
816 for putative methanotrophs in initial slurry sample (Slurry T0) and $^{13}CH_4$ incubations
817 with different treatments after 160 days.

818

Figure 5. Microbial communities in slurry samples before (Slurry T0) and after incubation with different treatments. (A) Relative abundances of the most abundant phyla within archaea. (B) Relative abundances of top bacterial classes. (C) Fractional abundances of known methanotrophic families. Data are based on read abundances of 16S rRNA gene sequences.

FIGURES

Figure 1



FIGURES

Figure 2

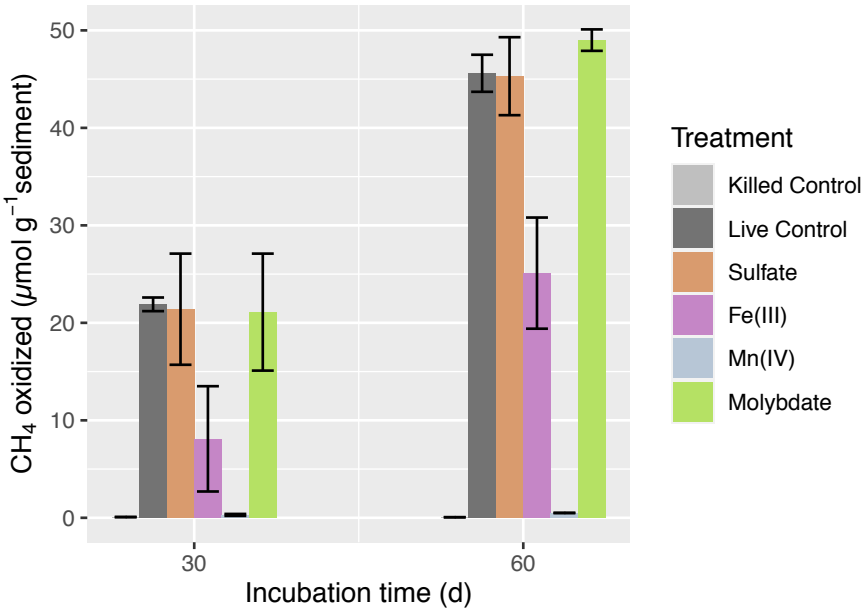
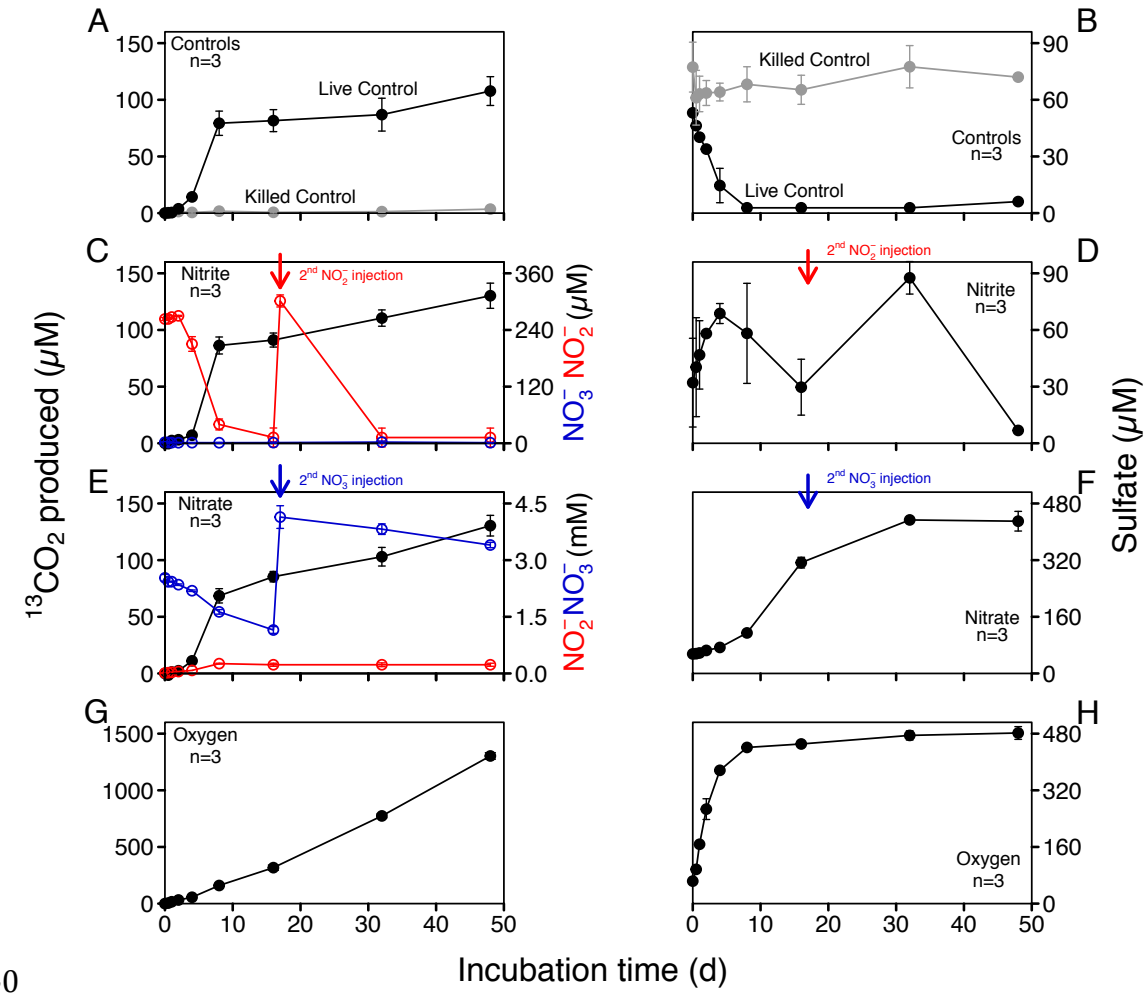


Figure 3



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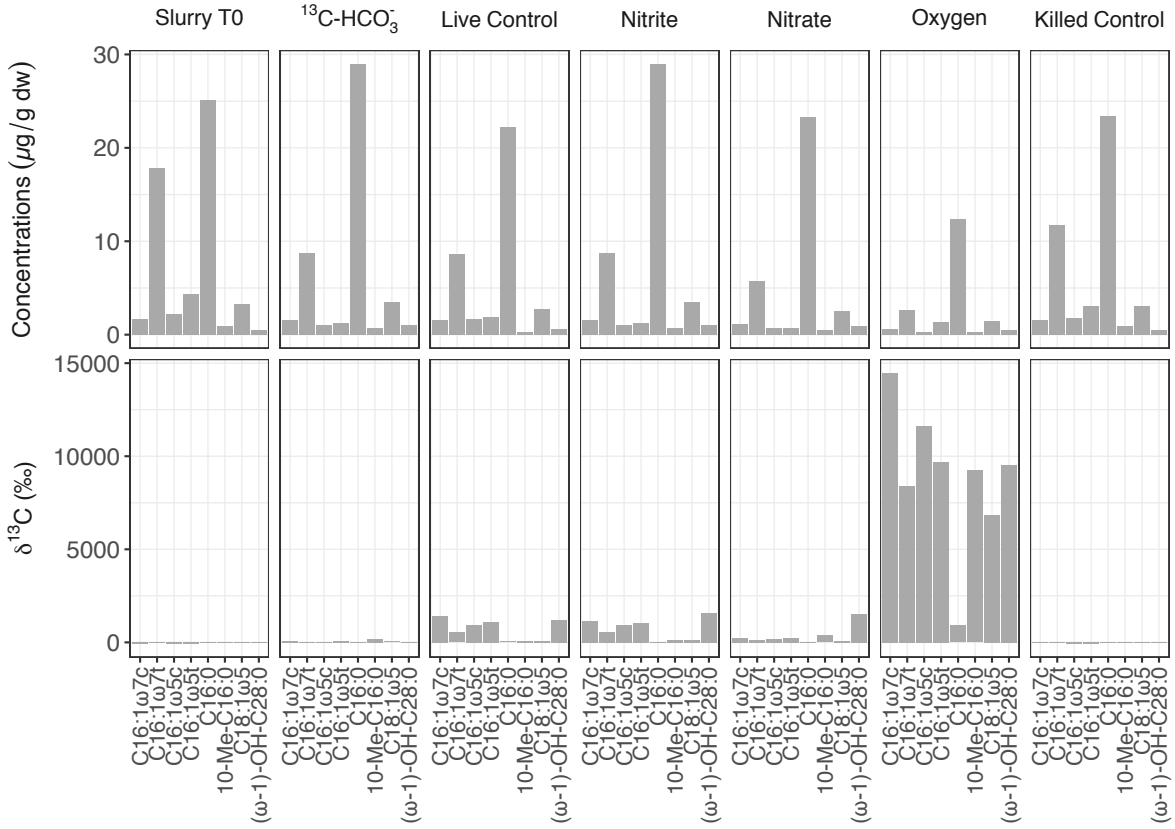

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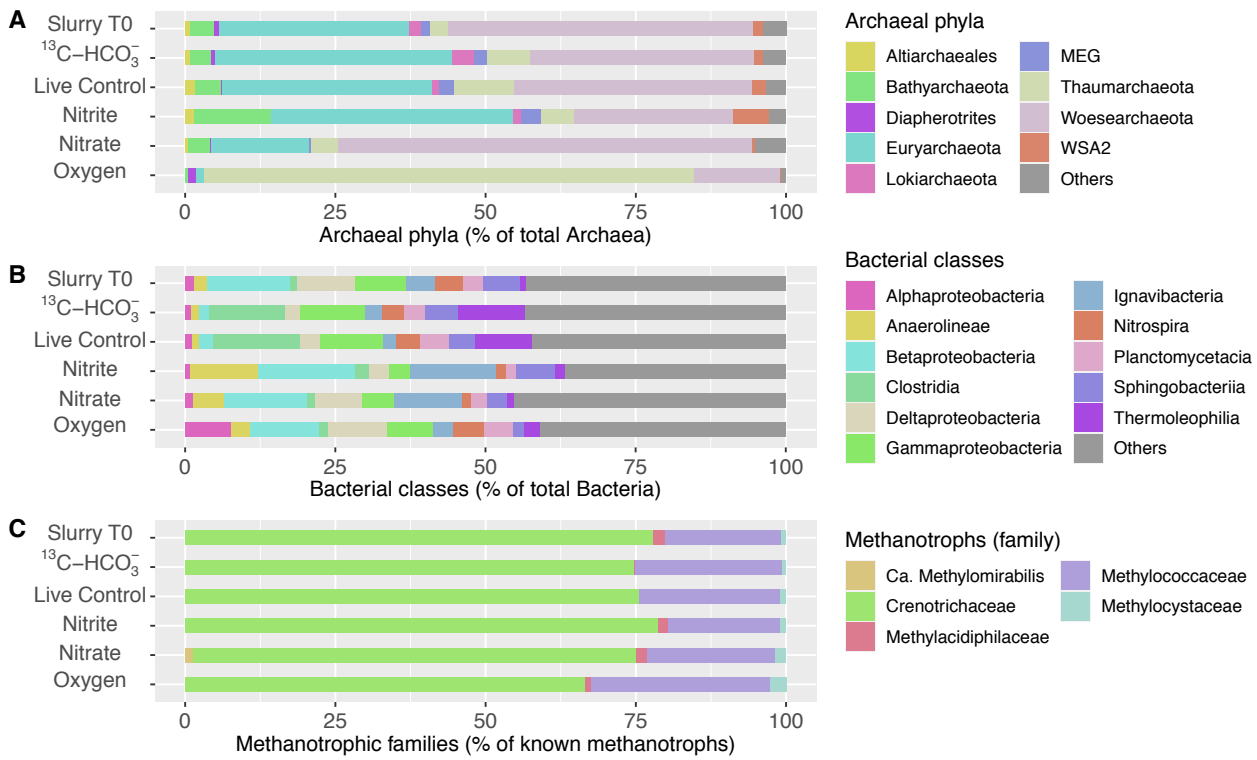
844 **Figure 5**