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 in anoxic sediments (mM range, e.g. Blees et al. 2014) suggest that methane in lake sediments is largely consumed before released to the water column. However, the primary sink for biogenic methane in anoxic lake sediments is still not well understood, and the physiological mechanisms and modes of benthic methane oxidation by microbes have not yet been unraveled.

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). Here, methane oxidation is usually carried out by aerobic methane-oxidizing bacteria (MOB), and the methane oxidation with oxygen as electron acceptor (MOx) is considered the prime methane sink in lacustrine environments, and thus mitigator of lacustrine methane emissions. 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). True anaerobic oxidation of methane (AOM) in anoxic environments is usually performed by anaerobic methanotrophic archaea (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), 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). Methane oxidizing microorganisms may be metabolically quite

adaptable. For example, recently identified anaerobic methanotrophic archaea in lake sediments, which are phylogenetically closely related to *Candidatus* Methanoperedens nitroreducens (i.e., nitrate-dependent methanotrophs or ANME-2d, Haroon et al. 2013), were reported to also perform methane oxidation coupled to sulfate reduction (Su et al. 2020). In addition to archaeal AOM, AOM can be performed by bacteria. Bacteria of the NC10 phylum (i.e., *Candidatus* Methylomirabilis), for example, can couple aerobic methane oxidation to nitrite reduction (Raghoebarsing et al. 2006; Ettwig et al. 2008), whereby the bacteria produce oxygen for methane oxidation intracellularly via the dismutation of nitric oxide (Ettwig et al. 2010). Thus, nitrite-dependent AOM can employ a pathway similar to that of canonical aerobic methane oxidation, which involves soluble and/or particulate methane monooxygenase enzymes. 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). Irrespective of the microbial players involved, in anoxic lake sediments, pathways of AOM like nitrate/nitrite-dependent methane oxidation may be masked by aerobic process, due to the close proximity of the nitrate/nitrite- and oxygen-consumption zones near sediment-water interfaces, thus making it difficult to distinguish between true AOM and aerobic methane oxidation.

Methane oxidation may be ubiquitous in strictly anoxic lake sediments (Martinez-Cruz et al. 2018), yet, evidence for the occurrence of true AOM (i.e., by the above-mention AOM-performing microbes, involving electron acceptors other than oxygen) 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 traditionally aerobic methanotrophs in anoxic sediments and their contribution to AOM is still not well understood. Under oxygen limitation, some gamma-proteobacterial MOB (i.e., *Methylomonas* and *Methylomicrobium*) seem capable of oxidizing methane with nitrate/nitrite as terminal electron acceptors (Kits et al. 2015a; b), however, the initial attack of methane by methane monooxygenase still seems to be dependent on oxygen. Similarly, *Methylobacter* was recently shown to utilize methane for lipid synthesis during long-term anoxic incubations although the responsible electron acceptors for AOM were not investigated (Martinez-Cruz et al. 2017). High abundances of *Methylobacter* were also observed in the deep anoxic hypolimnion of a permanently stratified lake, in association with high methane

oxidation rates (Blees et al. 2014), yet, the exact mechanism or mode of this apparent AOM by aerobic methane oxidizers, and the electron acceptor involved remained enigmatic. Finally, another putatively gammaproteobacterial lineage, *Crenothrix* was shown to have the potential to catalyze methane oxidation coupled the reduction of nitrate to N_2O under oxygen-deficient conditions (Oswald et al. 2017). It appears that aerobic methanotrophs are versatile in their electron acceptor requirements, capable 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 benthic 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 located in central Switzerland. For more than three decades, the hypolimnion of this lake has been artificially aerated to maintain bottom water conditions oxic during summer thermal stratification, and to support mixing throughout the water column in winter (Stadelmann 1988). Alternative oxidants for methane oxidation (e.g. nitrate, nitrite, Fe^{3+}, Mn^{4+}) are found in the shallow sediments and porewaters. 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 through pre-drilled, tape-covered holes in the sediment core tube. 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 (Niemann et al. 2015). Porewater was extracted by centrifuging the 1 or 2 cm segments of a second sediment core under N_2 atmosphere, and filtering the supernatant through 0.45 μm filters. Sample aliquots for sulfide concentration measurements were fixed with Zn acetate (5%) immediately

after filtration. Sample aliquots for dissolved iron (Fe^{2+}) and manganese (Mn^{2+}) were fixed with HCl (6 M). The fixed or untreated filtered sample aliquots were then stored at 4 °C. Sediment samples for DNA extraction and particulate iron/manganese analyses were collected from a third sediment core, also at 1- or 2-cm intervals, 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 the Griess reaction (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 analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES). Sulfide concentrations were 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. The penetration depth of oxygen into the surface sediments was determined using a Clark-type microelectrode sensor (Unisense A/S, Denmark).

AOM rate measurements

To determine depth-specific AOM rates in Lake Sempach sediments, the subcore incubation approach was applied (Su et al. 2019). Briefly, subcores were collected 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, 20 μL of $^{14}CH_4$ solution (~2.5kBq) was injected through each of 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

170 segments, and directly transferred into 100 ml vials containing 20 ml of the aqueous
171 NaOH (5% w:w) to terminate microbial activity. Radioactivities of different ^{14}C pools
172 in the incubation samples and AOM rates were determined according to Su et al.
173 (2019).
174
175 **Substrate-amended slurry incubations**
176 *Experiment 1 (sulfate, iron and manganese-addition experiments with ^{14}C-labelled*
177 *methane)*. Anoxic slurries of ~8 L were prepared by purging a mixture of sediments
178 (surface 10 cm × 4 sediment cores, ~1500 cm^3) and autoclaved anoxic artificial
179 sulfate-free medium (Su et al. 2020). Homogenized slurries of ~100 mL were
180 subsequently dispensed into 120-mL serum bottles and sealed with butyl rubber
181 stoppers. For the electron acceptor amended incubations, stock solutions of sulfate,
182 ferrihydrite and birnessite were added to final concentrations of 2 mM, 5 mM and 5
183 mM, respectively. For the inhibitor-addition experiment, molybdate was added to a
184 final concentration of 4 mM. Base-killed controls (pH > 13) were also prepared and
185 incubated in parallel. All bottles were further purged with N_2 to remove any traces of
186 oxygen. After 24-h preincubation, 1.5 ml pure CH_4 was injected into the headspace of
187 each bottle, which was gently shaken for 24 h, resulting in a final concentration of
188 100 μmol/L in the slurries. Finally, all incubation bottles were transported to an
189 anoxic chamber and filled bubble-free with anoxic medium (100 μmol/L CH_4) before
190 injecting $^{14}CH_4$ gas tracer. At different time points (30 and 60 days), incubation
191 samples were stopped by adding 5 ml saturated NaOH after creating 20 ml headspace
192 of N_2. AOM rates were analyzed and calculated as described previously (Su et al.
193 2019).
194 *Experiment 2 (nitrite-, nitrate-addition and oxic experiments with ^{13}C-labelled*
195 *methane).* To further test specifically the role of nitrite/nitrate in AOM, we performed
196 slurry incubation experiments with a mixture of sediment and anoxic bottom lake
197 water taken in November 2020. Briefly, 100 ml mixed slurries (1.5 g dw sediment per
198 120-mL bottle) were degassed with He to remove any traces of oxygen and
199 background methane before transfer to an anoxic bag with continuous N_2 flow. 5 mL
200 of pure ^{13}C-labelled methane (99.8 atom %, Campro Scientific) was injected into the
201 headspace of the bottles, which were amended with anoxic stock solutions of nitrite
202 and nitrate, with final concentrations of 0.48 mM and 2.4 mM, respectively.
203 Incubations took place at room temperature under N_2 atmosphere. Nitrite and nitrate

204 were replenished, when consumed or when they remained at low concentrations.
205 Killed controls (autoclaved and $^{13}CH_4$), live controls (only $^{13}CH_4$) and controls with
206 only ^{13}C-labelled bicarbonate were also incubated in parallel under the same
207 conditions. For oxic experiments, slurries were spiked with pure oxygen and then
208 injected with 5 mL of pure $^{13}CH_4$. Oxygen was replenished regularly to maintain oxic
209 condition in the incubation bottles. At different time points, incubation slurries were
210 homogenized and 5 mL of the supernatant was collected under N_2 atmosphere, and
211 filtered with a 0.45 µm membrane filter for subsequent sulfate, nitrate, nitrite, DIC
212 concentration and stable carbon isotope ratio analyses to determine the transfer of ^{13}C
213 into the product DIC pool. For the latter, 0.2-1 mL of water sample was transferred
214 into a 12 mL He-purged exetainer (Labco Ltd) containing 200 µL zinc chloride (50 %
215 w/v), and then acidified with ~200 µL 80% H_3PO_4. The liberated CO_2 was then
216 analyzed in the headspace using a purge-and-trap purification system (Gas Bench)
217 coupled to a GC-IRMS (Thermo Scientific, Delta V Advantage). Absolute ^{13}C-DIC
218 abundances were determined from the DIC concentrations and the $^{13}C/^{12}C$ ratios
219 converted from $\delta^{13}C$-DIC values (Oswald et al. 2015). The temporal change in ^{13}C-
220 DIC with incubation time was then used to calculate slurry-incubation-based potential
221 methane oxidation rates. Temporal solute-concentration and 13C-DIC changes were
222 monitored over a total experimental period of 48 days. Thereafter, incubations were
223 continued without further subsampling. After a total incubation period of
224 approximately 5 months, slurry incubations were sacrificed for lipid analysis and
225 DNA extraction (see below).
226
227 **Microbial lipid extraction and analysis**
228 At the end of the incubation with $^{13}CH_4$ (Experiment 2), after 160 days, triplicate
229 slurries were combined and freeze-dried. Samples were then homogenized for lipid
230 extraction using an Accelerated Solvent Extraction system (ASE 350, Dionex Corp.,
231 Sunnyvale, CA, USA). Samples were extracted with a solvent mixture of
232 dichloromethane and methanol (9:1, vol/vol) over three extraction cycles at 100 °C
233 and maximum pressure of 1500 psi. Total lipid extracts (TLEs) were collected in 60
234 mL glass vials, and concentrated using a vacuum evaporator system (Rocket Synergy
235 Evaporator, Genevac Ltd., Ipswich, UK). Prior to extraction, an internal standard mix
236 (5α-Cholestane, Nonadecanoic acid and 1-Nonadecanol) was added to each sample
237 for the quantification of individual biomarkers. The TLEs were further evaporated to

238 dryness, and then saponified with methanolic KOH-solution (12%) at 80 °C for 3 h.
239 Neutral compounds were extracted by liquid-liquid extraction using hexane. After
240 extracting the neutral fraction, fatty acids (FAs) were extracted from the remaining
241 aqueous phase with hexane after acidification (pH ~ 1). The fatty acid fraction was
242 methylated using BF_3 in methanol (14% v/v, Sigma Aldrich) at 80 °C for 2 h, and
243 analyzed later as fatty acid methyl esters (FAMEs). Neutral compounds were further
244 separated into hydrocarbon and alcohol fractions over silica glass gel columns, as
245 described by Sicre et al. (1994) with the following modifications. The neutral fraction
246 was dissolved in heptane and transferred onto the wet column. Aliphatic, cyclic and
247 aromatic hydrocarbons were eluted with 2 mL heptane and 2 mL heptane/toluene (3:1
248 v/v), ethers and ketones with 2 mL heptane/toluene (1:1 v/v) and 2 mL heptane/ethyl
249 acetate (95:5 v/v), and finally alcohols with 2 mL heptane/ethyl acetate (85:15 v/v)
250 and heptane/ethyl acetate (80:20 v/v). The alcohol fraction was methylated with 100
251 µL pyridine and 50 µL bis(trimethylsilyl)trifluoracetamide (BSTFA) at 70 °C for 1 h.
252 All fractions were quantified using a TRACE GC Ultra gas chromatograph
253 equipped with a flame ionization detector (GC-FID) (Thermo Scientific, Waltham,
254 MA, USA). Individual lipid compounds were quantified by normalization to the
255 internal standard and identified by comparing their retention times to those of
256 laboratory standard mixtures, or by a gas chromatograph mass spectrometry (GC-MS,
257 Thermo Scientific DSQ II Dual Stage Quadrupole) and the acquired mass spectra
258 were identified through comparison with published data. Compound-specific stable
259 carbon isotope ratios of individual compounds were determined using a gas
260 chromatograph with split/splitless injector, connected on-line via a GC-Isolink
261 combustion interface to a ConFlo IV and Delta V Advantage isotope ratio mass
262 spectrometer (GC-IRMS, Thermo Scientific, Bremen, Germany). Both concentrations
263 and $\delta^{13}C$ values of lipids were corrected for the introduction of carbon atoms during
264 derivatization.
265
266 **DNA extraction, PCR amplification, Illumina sequencing and data analysis**
267 DNA was extracted from both samples of Lake Sempach core sediments, as well as
268 from the slurry sediments at the end of incubations (Experiment 2), using a FastDNA
269 SPIN Kit (MP Biomedicals) following the manufacturer's instructions. A two-step
270 PCR approach was applied in order to prepare the library for sequencing at the
271 Genomics Facility Basel. Briefly, a first PCR (25 cycles) was performed using

272 primers 515F-Y and 926R targeting the V4 and V5 regions of the 16S rRNA gene
273 (Parada et al. 2016). Sample indices and Illumina adaptors were added in a second
274 PCR of eight cycles. Purified indexed amplicons were finally pooled at equimolar
275 concentration into one library, and sequenced on an Illumina MiSeq platform using
276 the 2×300 bp paired-end protocol (V3 kit). After sequencing, quality of the raw reads
277 was checked using FastQC (v 0.11.8) (Andrews 2010). Paired-end read merger
278 (usearch v11.0.667_i86linux64) was used to merge forward and reverse reads into
279 amplicons of about 419 bp length, allowing a minimum overlap of 40 nucleotides and
280 a mismatch density of 0.25. Quality filtering (min Q20, no Ns allowed) was carried
281 out using PRINSEQ (Schmieder and Edwards 2011). Amplicon sequence variants
282 were determined by denoising using the UNOISE algorithm (UNOISE3, usearch
283 v11.0.667_i86linux64) and are herein referred to as ZOTU (zero-radius OTU).
284 Taxonomic assignment was done using SINTAX v11.0.240_i86linux64 (Edgar 2016)
285 and the SILVA 16S rRNA reference database v128 (Quast et al. 2013). Subsequent
286 data analyses were carried out with Phyloseq (McMurdie and Holmes 2013) in the R
287 environment (R Core Team, 2014) (http://www.r-project.org/).
288
289 **Data availability.** Raw reads are deposited at the NCBI Sequence Read Archive
290 (SRA) in the BioProject PRJNA759259, and can be accessed under the accession
291 numbers SRR15689022- SRR15689034.
292

293 ## Results

294 **Rates of AOM and geochemistry of Lake Sempach sediments**

295 Concentration of methane in the sediments increased with depth, reaching a
296 maximum of ~1.75 mM at 10 cm depth. Methane oxidation was observed throughout
297 the sediment core. Activity decreased with depth, with the maximum AOM rates
298 (68.0 ± 19.0 nmol cm^{-3} d^{-1}) measured in the upper 0-2 cm (Fig. 1B). Nitrate was only
299 detected above 3 cm (up to 5 μM) and the sulfate concentration decreased downward
300 from 78 μM at the sediment-water interface to 7 μM at 10 cm. Both sulfide and nitrite
301 were below the detection limit at all depths. Reducible iron and manganese oxides
302 were most abundant in surface sediments (24.3 and 30.8 μmol g^{-1} sediment
303 respectively), and concentrations of both dissolved iron (Fe^{2+}) and manganese (Mn^{2+})
304 increased steadily with depth. Even under the condition of fully oxygenated bottom

305 waters, oxygen was rapidly consumed in the uppermost sediment layers and
306 penetrated to a maximum depth of 3 mm (Fig. 1F).
307
308 **Diversity and abundance of methanotrophs in lake sediments**
309 Analysis of sediment microbial community, based on 16SrRNA gene sequencing,
310 revealed the presence of multiple groups of aerobic methanotrophs, including Type I
311 gamma- and Type II alpha-proteobacterial MOB (Fig. 1G-H). Type I gamma-MOB
312 consist of *Methylococcaceae*, of which the genus *Methylobacter* was the dominating
313 cluster. The relative abundances of *Methylococcaceae* range from 0.07% to 0.23%,
314 with peak abundance occurring at 3 cm depth. In comparison, Type II alpha-MOB
315 *Methylocystaceae* was found at much lower relative abundances, with a maximum of
316 0.04% observed at 6 cm depth. A large number of 16S rRNA gene sequences (0.17–
317 1.25%, Fig. 1I), belonged to *Crenotrichaceae*, of which *Crenothrix* represented the
318 predominant genus and the amplified sequence variants shared very high sequence
319 similarities (96-100%) with what has been described recently as major methane
320 oxidizers in stratified lakes (Oswald et al. 2017). In addition, a very small proportion
321 of sequences (up to 0.03%) were affiliated with *Methylacidiphilaceae* within the
322 *Verrucomicrobia* phylum. *Methylacidiphilaceae* were reported to perform aerobic
323 methane oxidation in acidic geothermal environments (Islam et al. 2008; Carere et al.
324 2017). A canonical correspondence analysis (CCA) was performed to show any given
325 correlation between the concentrations of potential electron acceptors, methane
326 oxidation rates and specific methanotrophic species (Fig. 1K). The CCA triplot shows
327 that members belonging to *Crenothrix* or *Methylobacter* were positively correlated to
328 the methane oxidation rates, and negatively correlated to methane concentrations.
329 Interestingly, the plot also demonstrates that *Crenothrix* and *Methylobacter* were
330 more likely to be found in surface sediments with higher concentrations of potential
331 electron acceptors (i.e., oxygen, nitrate, sulfate, iron and manganese).
332
333 **Effect of potential electron acceptor addition on AOM rates**
334 To investigate the potential electron acceptors for AOM, sediments of the top 10 cm
335 (i.e., where in situ rates were measured; Fig. 1B) were incubated with different
336 electron acceptors. In the first experiment with ^{14}C-labelled methane, no apparent
337 increase in AOM rate was observed with any potential electron acceptor amendment,
338 compared with the live controls (no electron acceptors added, Fig. 2A). In fact,

339 incubations of both 30 and 60 days revealed very similar potential AOM rates
340 between the untreated controls and amendments with sulfate and molybdate.
341 Interestingly, the addition of iron oxides to the slurries did not stimulate methane
342 oxidation but rather resulted in systematically lower AOM rates relative to the control
343 experiments. Even more surprisingly, in incubations with added manganese oxide,
344 methane oxidation was completely inhibited and substantial amounts of sulfate was
345 produced. In the second experiment using ^{13}C-labelled methane, we further tested the
346 role of nitrate/nitrite in AOM, in direct comparison with aerobic methane oxidation
347 after O_2 injection. Similar to ^{14}C-labelled methane incubation experiments, AOM
348 occurred in the anoxic live controls (without any electron acceptors added).
349 Incubations amended with nitrate and nitrite showed similar trends with respect to the
350 control, hence, there was no obvious stimulation of methane oxidation by either of
351 the NOx compounds (Fig. 3). For both untreated controls and nitrate/nitrite
352 amendments, AOM rates were low during the first four days of the incubation period,
353 but then increased dramatically between 4 and 8 days. Thereafter, they remained
354 relatively low again (i.e., only subtle ^{13}DIC accumulation between Day 8 and the end
355 of the incubation). In contrast, methane oxidation was linear throughout the
356 incubation period under oxic conditions, and the rates were almost an order of
357 magnitude higher than the maximum rate in the control and NOx-amended
358 incubations. Strikingly, sulfate was fully depleted after 8 days in the live controls (but
359 not in the killed control), whereas there was extensive sulfate production in incubation
360 bottles amended with both nitrate or oxygen. When nitrite was added, net sulfate
361 production was also observed, at least during phases (e.g., initially and between Day
362 17 and 40; Fig. 3D), and at lower rates.
363
364 **Lipid biomarkers and microbial communities in incubations with $^{13}CH_4$**
365 At the end of the $^{13}CH_4$ incubations (after160 days), the abundance and carbon
366 isotopic composition of MOx/AOM diagnostic biomarkers (specific fatty acids and
367 alcohols) were examined in the slurry sediments to constrain the methanotrophs
368 involved and to infer carbon flow through the microbial community. The overall
369 concentrations of lipid biomarkers in the different treatments were quite similar,
370 except for incubations with oxygen, where concentrations were somewhat lower than
371 in the other treatments (Fig. 4). In the untreated control and the nitrite-amended
372 experiment, the most strongly ^{13}C-enriched fatty acids were monounsaturated

373 hexadecanoic acids ($C_{16:1\omega7}$ and $C_{16:1\omega5}$) and (ω-1)-OH-$C_{28:0}$, with $\delta^{13}C$ values ranging
374 from 531‰ to 1558‰. By comparison, the highest ^{13}C incorporation in incubations
375 with nitrate was observed for (ω-1)-OH-$C_{28:0}$ (1524‰), followed by 10-Methyl-
376 hexadecanoic acid (10-Me-$C_{16:0}$, 367‰). Most strikingly, $\delta^{13}C$ analyses of biomarker
377 extracts from the oxic incubations revealed significantly stronger ^{13}C labeling of most
378 fatty acids (compound-specific $\delta^{13}C$ values between 6800 and 14500‰; Fig. 4)
379 compared to the controls and to incubations with nitrite/nitrate. In order to assess
380 concurrent autotrophic carbon assimilation, we performed additional slurry
381 incubations with ^{13}C-labelled bicarbonate only. No ^{13}C incorporation at all was
382 observed for (ω-1)-OH-$C_{28:0}$ (-39‰), and the $\delta^{13}C$ value of 10-Me-$C_{16:0}$ (164‰)
383 suggests only minor ^{13}C-label incorporation. Generally, $\delta^{13}C$ values of compounds
384 diagnostic for methanotrophic microorganisms in incubations with ^{13}C-bicarbonate
385 were significantly lower than those in $^{13}CH_4$ incubations with nitrite/nitrate (Fig. 4).
386 Initially (Slurry T0), *Euryarchaeota* was the most abundant archaeal phyla (Fig.
387 5A). The archaeal community structure in both the live control and incubation with
388 ^{13}C-bicarbonate, after 160 days of incubation, was similar to that of Slurry T0.
389 However, No sequences of typical anaerobic archaeal methanotrophs such as ANME-
390 1, -2, -3 and *Candidatus* Methanoperedens have been detected. As for bacterial
391 composition at the class level (Fig. 5B), g*ammaproteobacteria* comprised a smaller
392 proportion after nitrite/nitrate and oxygen additions. In oxic slurries,
393 *Alphaproteobacteria* accounted for a larger proportion of the bacterial population
394 compared to the pre-incubation microbial abundances. At family level,
395 *Crenotrichaceae* and *Methylococcaceae* were the dominant groups among the known
396 methanotrophs in the initial slurry sample (Fig. 5C), consistent with the diversity and
397 abundance of methanotrophs in the sediment profiles (see Fig. 1G-J). After 160 days,
398 *Crenotrichaceae* and *Methylococcaceae* remained dominant within the known
399 methanotrophic families in all the treatments. Although representing a minor
400 proportion, the relative abundances of *Methylocystaceae* slightly increased in
401 incubations with nitrate/nitrite and oxygen when compared to Slurry T0. In oxic
402 slurries, fractional abundance of *Methylococcaceae* was higher relative to Slurry T0
403 and the other treatments and the dominance of *Crenotrichaceae* seemed to be reduced
404 (Fig. 5C). In addition, the anaerobic nitrite-dependent methanotroph *Candidatus*
405 Methylomirabilis sp. was detected in incubation with nitrate and at a low abundance
406 (1.36% of known methanotrophs; Fig. 5C).

Discussion

In Lake Sempach, despite the aeration of the deep hypolimnion, oxygen is rapidly depleted in the uppermost sediment layers (maximum O2 penetration depth of 3 mm). Based on the measured methane turnover rates as a function of depth (Fig. 1b), maximum methane oxidation activity was located at depths near the water-sediment interface, but apparent AOM (though at lower rates) was also indicated in the deeper, anoxic sediment layers. This is different from marine settings and some exceptional freshwater settings (e.g., Su et al. 2020) where highest AOM rates take place within the sulfate methane transition zones, well below the sediment surface (Iversen and Jørgensen 1985). Within oxic-anoxic transition zones of lake sediments, oxygen likely serves as the most important electron acceptors and aerobic methanotrophs play a dominant role in reducing the overall methane fluxes (Bender and Conrad 1994; He et al. 2012). In anoxic sediments, however, the pathway of methane oxidation with regard to the electron acceptors involved remains to be fully explored. Notably, the highest AOM rates in surface sediment layers corresponded to the concentration maxima of nitrate, sulfate, iron(III)- and manganese(IV) oxides, suggesting their potential roles for AOM. Despite the clear evidence of AOM in anoxic zones, no ANME-1 or 2, key microbial drivers of AOM in marine settings, were detected throughout the sediment core by 16S rRNA gene amplicon sequencing with the primers that match with a large fraction of the deposited sequences of anaerobic methanotrophic archaeal clades (Su et al. 2020). Neither could we detect molecular evidence for the presence of *Ca.* Methylomirabilis and *Ca.* Methanoperedens were detected either (Ettwig et al. 2010; Haroon et al. 2013; Su et al. 2020). Instead, the anoxic sediments contained high abundances of gamma-MOB and some alpha-MOB, which both are traditionally considered aerobic methanotrophs (Hanson and Hanson 1996). Most importantly, the sediments contained high relative abundances of *Crenothrix*, also gamma-MOB thought to be aerobes (i.e., requiring oxygen for methane activation), and regularly found as contaminant in drinking water systems (Stoecker et al. 2006). The relative abundances of *Methylococcaceae*, *Crenothrix* and *Methylacidiphilaceae*, respectively, were higher in upper sediment layers, while *Methylocystacea* were more abundant in lower portions of the sediments. The vertical distribution of various methanotrophic groups may indicate

440 their different affinities towards methane, as evidenced, at least in part, by some
441 studies showing that Type I methanotrophs were able to outcompete Type II MOB at
442 low concentrations of methane (Amaral and Knowles 1995; Henckel et al. 2000).
443 However, the presence of diverse aerobic methanotrophs in anoxic sediments led us to
444 speculate that some of them, if not all, might have an alternative anaerobic lifestyle.
445 Indeed, increasing evidence has demonstrated the occurrences of gamma-MOB
446 in anoxic lake waters and sediments (Blees et al. 2014; Milucka et al. 2015; Oswald et
447 al. 2016; Martinez-Cruz et al. 2017). Also for Lake Sempach sediments, we were able
448 to demonstrate active growth of gamma-MOB in absence of oxygen. That is, in O2-
449 free incubations with Lake Sempach sediments and $^{13}CH_4$,, the incorporation of the
450 $^{13}CH_4$-carbon into specific lipid biomarkers provides clear evidence for microbial
451 methane assimilation. The monounsaturated fatty acids $C_{16:1\omega7}$ and $C_{16:1\omega5}$, which are
452 typically indicative of Type I aerobic methanotrophs (Bowman et al. 1994; Hanson
453 and Hanson 1996), were most enriched in ^{13}C. In theory, the ^{13}C enrichment could
454 also be attributed to the assimilation of $^{13}CO_2$ from methane oxidation into the
455 biomass by autotrophic microorganism, as for example found for certain sulfate
456 reducing bacteria (SRB) (Kellermann et al. 2012). Indeed, it has been reported that
457 specific lipids such as $C_{16:1\omega7}$ and $C_{16:1\omega5}$ are associated with SRB that were involved
458 in sulfate-dependent AOM at methane-rich seeps (Elvert et al. 2003). However, no or
459 very low incorporation of ^{13}C into these lipid biomarkers was observed in the
460 incubation with ^{13}C-labelled bicarbonate only. This indicates that the ^{13}C-enriched
461 lipids in the $^{13}CH_4$ incubations were not derived from bicarbonate-assimilating SRB,
462 but can rather be attributed to the direct assimilation of methane-derived carbon by
463 Type I gamma-MOB. Direct uptake of ^{13}C-labeled methane by alpha-MOB in the
464 anoxic sediments was also observed, evidenced by the strong ^{13}C-enrichment of long-
465 chain (ω-1)-hydroxy fatty acids, such as (ω-1)-OH-$C_{28:0}$. This lipid compound is
466 mainly found in type II methane-utilizing bacteria including *Methylosinus* and
467 *Methylocystis* (Skerratt et al. 1992). These are consistent with our findings regarding
468 the composition of the methanotrophic community in the live control incubation
469 slurry, which comprised relatively high abundances of both *Methylococcaceae* and
470 *Methylocystaceae*. *Methylococcaceae* were dominated by *Methylobacter* and
471 *Methylocystis* was the most abundant genus within *Methylocystaceae* (data not
472 shown). However, we were not able to verify methane assimilation into the biomass
473 of *Crenothrix*, because no diagnostic lipids have been reported for them to date.

474 Moreover, *Crenotrichaceae* likely also include bacteria that are not strict
475 methanotrophs. Yet, due to the apparent capacity of at least some of the
476 *Crenotrichaceae* genera to also oxidize methane anaerobically (Oswald et al. 2017),
477 and given their high relative abundances in the anoxic sediments, however, we
478 speculate that this methanotrophic group may comprise the most important methane
479 consumers in Lake Sempach sediments. Overall, our molecular data and lipid
480 biomarker analysis provide multiple lines of evidence that AOM in Lake Sempach
481 sediments is most likely mediated by methanotrophs canonically considered aerobes.
482 According to the CCA triplot (Fig. 1K), members of *Crenothrix* and
483 *Methylobacter* were more abundantly found at sediment with higher methane
484 oxidation rates, and were associated with elevated concentrations of potential
485 alternative electron acceptors in the anoxic sediments. However, in none of the
486 electron acceptor-amended slurry incubations, i.e. neither with $^{13}CH_4$ (nitrite/nitrate
487 added) nor with $^{14}CH_4$ (sulfate, iron or manganese added), stimulation of anaerobic
488 methane turnover by any of the common anaerobic electron acceptors was indicated.
489 This suggests an AOM pathway in Lake Sempach sediments that may be different
490 from AOM reported for other investigated systems (Boetius et al. 2000; Orphan et al.
491 2002; Raghoebarsing et al. 2006; Vaksmaa et al. 2017; Su et al. 2020). In fact, the
492 addition of metal oxides led to systematically lower AOM rates (iron) or complete
493 inhibition (manganese). These observations appear, at first sight, at odds with recent
494 studies showing the stimulation of both iron and manganese additions for aerobic
495 methanotrophs under anoxic conditions (Oswald et al. 2016; Zheng et al. 2020). Yet,
496 the inhibition of AOM as a result of excess "anaerobic electron acceptors" was also
497 observed in previous studies (Segarra et al. 2013; Milucka et al. 2015; Rissanen et al.
498 2018). The addition of metal oxides to the slurries may change the redox condition of
499 the systems, and may favor the growth of iron- or manganese-utilizing
500 microorganisms that could outcompete methane oxidizers for essential nutrients, but
501 further investigation is needed to reveal their effect on AOM. Notably, in $^{13}CH_4$
502 incubations without electron acceptors added, the co-occurrence of increasing $^{13}CO_2$
503 and decreasing sulfate concentrations over the first 8 days of incubation suggests a
504 possible link between methane oxidation and sulfate reduction. On the other hand, the
505 relatively large amounts of sulfate produced in nitrate-amended bottles, likely by the
506 oxidation of sulfide with nitrate (Su et al. 2020), did not stimulate $^{13}CO_2$ production
507 significantly, when compared to the live controls, suggesting that sulfate was at least

508 not a limiting electron acceptor for AOM in Lake Sempach sediments. The fact that
509 sulfate reduction is not directly involved in driving AOM in Lake Sempach sediments
510 is further supported by the observation that similar methane turnover rates were
511 measured for incubations with and without molybdate, a competitive inhibitor for
512 microbial sulfate reduction (Wilson and Bandurski 1958).

513 Despite the fact that aerobic methanotrophs are obligate aerobes, some of them
514 seem to be able to survive long periods of oxygen starvation (Roslev and King 1995;
515 Blees et al. 2014), and may even switch from respiring oxygen to nitrite or nitrate for
516 methane oxidation (Kits et al. 2015a; b; Oswald et al. 2017). Both Type I MOB and
517 *Crenothrix* were detected in our incubations, and their preference for O_2 as oxidant of
518 methane was clearly demonstrated for the Lake Sempach Sediments (Fig. 3G).
519 However, how they (and/or other methane oxidizing microorganisms in the Lake
520 Sempach sediments) drive methane oxidation in the absence of O_2 remains uncertain.
521 As outlined before, the AOM pathway involving nitrite or nitrate respiration seemed
522 unlikely as neither enhanced methane oxidation rates significantly, indicating an as-
523 yet-unidentified electron acceptor. While nitrite-dependent *Ca.* Methylomirabilis was
524 not detected at all in any of the nitrite additions, it was present after incubation with
525 nitrate addition, though at very low abundance. This finding is also consistent with the
526 lipid data for the nitrate-amended anoxic incubations , which revealed a stronger 13C
527 enrichment in the fatty acid 10-methylhexadecanoic acid (10-Me-$C_{16:0}$), putatively
528 diagnostic for *Ca.* Methylomirabilis oxyfera (Kool et al. 2012). It is plausible that
529 these denitrifying methanatrophic bacteria contributed a small portion to the overall
530 methane oxidation.

531 At this point, based on our slurry incubation results, we are not able to better
532 constrain the electron acceptors involved in AOM in Lake Sempach sediments, and
533 we can only speculate about what sustains the observed methane turnover in anoxic
534 incubations. A recent study suggests that AOM can proceed in the absence of
535 inorganic electron acceptors, via "reverse methanogenesis" (Blazewicz et al. 2012).
536 This AOM pathway may offer a possible explanation for the observed
537 "ineffectiveness" of all tested electron acceptors to stimulate AOM. Alternatively,
538 other electron acceptors that were already present in the lake sediments (e.g., organic
539 matter) could be involved. Indeed, humic substances have been recognized as a viable
540 electron acceptor through the reduction of their quinone moieties (Lovley et al. 1996;
541 Scott et al. 1999), and organic electron acceptors such as humic acids were suggested

542 to play a role in AOM in wetlands (Smemo and Yavitt 2011; Blodau and Deppe
543 2012). In addition, anaerobic methanotrophic archaea in marine sediments were
544 recently demonstrated to use humic-substance analogues (i.e., AQDS and humic
545 acids) as electron acceptors in the absence of sulfate (Scheller et al. 2016). If humic
546 (or similar organic) substances can indeed serve as methane oxidizing agent, and
547 given the widespread distribution of these compounds in freshwater environments as
548 important component of the sedimentary organic matter pools (Ishiwatari 1985), such
549 mode of AOM may play an underappreciated role in reducing methane emissions
550 anoxic lake sediments. Clearly, further investigations are required to explore their role
551 in lacustrine AOM.

552 Most strikingly, additions of oxygen to our incubations with $^{13}CH_4$ resulted in an
553 approximately tenfold increase in $^{13}CO_2$ production relative to the live controls or
554 amendments with nitrite/nitrate. This clearly shows that aerobic methanotrophs were
555 actively involved in methane oxidation when replenished with oxygen, and growth of
556 these methanotrophic bacteria was evidenced by the substantial ^{13}C-enrichment of
557 diagnostic fatty acids. Our results thus highlight that there can be a strong potential
558 for MOx in anoxic sediments, which, at least in Lake Sempach, seems to be mostly
559 sustained by aerobic gamma proteobacteria (Type I MOB and *Crenothrix*). The fact
560 that these microbes are found in sediments where O_2 is fully depleted, and that they
561 can resume full aerobic metabolic activity upon the addition of O_2, attests to their
562 resilience against O_2 limitation. We propose that a potential capacity to switch to
563 anaerobic modes of methane oxidation may be key to this resilience, allowing them to
564 metabolize, at least at low level, also in the absence of O_2.

565 Methane carbon assimilation rates in the oxic incubations were an order of
566 magnitude higher than those in anoxic incubations. This is expected, since aerobic
567 respiration provides much more energy for growth than respiration with alternative
568 electron acceptors. While the result of the oxic incubation experiment confirm that
569 aerobic methane oxidation is much more efficient than AOM in consuming methane,
570 it needs to be noted that the vast majority of methane is produced, and accumulates, in
571 the anoxic part of lake sediments. Here, AOM, just as in the marine environment, may
572 serve as the important first step in a two-component benthic methane filter system. In
573 this context of this ecosystem function, the metabolic versatility and apparent capacity
574 of canonically aerobic methanotrophs to perform AOM has important implications for
575 the natural mitigation of CH_4 emission from lake waters to the atmosphere.

Acknowledgements

We thank Thomas Kuhn and Judith Kobler Waldis for technical support in the lab. Thanks also go to Robert Lovas, Jana Tischer, Adeline Cojean and Yuki Weber for their great help during the campaigns on Lake Sempach. This research was supported by the China Scholarship Council (CSC) and additional funds from the Department of Environmental Sciences, University of Basel.

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853 **FIGURES LEGENDS**

854

855 **Figure 1.** (A-F) Depth profiles of methane, methane oxidation rates, porewater
856 nitrate/sulfate, particular iron(III)/manganese(IV) oxides, dissolved
857 iron(II)/manganese(II) and oxygen in the sediment of Lake Sempach. Error bars
858 represent standard deviations of triplicate rate measurements. No nitrite was detected.
859 (G-J) Relative abundances of known families OF metahnotrophes and their subgroups
860 at the genus level: (G) Methylococcaceae or Type I gamma-proteobacterial MOB, (H)
861 Methylocystaceae or Type II alpha-proteobacterial MOB, (I) Crenotrichaceae, (J)
862 Methylacidiphilaceae, (K) Canonical correspondence analysis (CCA), showing the
863 relationship between MOR and methanotrophic species and the effect of nutrient
864 concentrations on their distribution. ZOTU abundances were normalized and
865 Hellinger-transformed prior to CCA analysis. A total of 42 ZOTUs accounting for >
866 92% of the methanotrophic community were shown on the plot and bold text
867 represents taxa belonging to the genus *Methylobacter* or *Crenothrix*. Black triangles
868 denote sediment samples at different depths.

869

870 **Figure 2.** (A) Methane oxidation in anoxic slurries with ^{14}C-labeled methane and
871 different electron acceptors: Sulfate (2 mM), amorphous iron(III) oxides (5 mM) and
872 manganese(IV) oxides (5 mM) after 30 and 60 days, respectively. (B) Sulfate
873 concentrations in the incubation supernatants of different treatments after 30 and 60
874 days. Control incubations were conducted without electron acceptors added. No
875 methane oxidation rates were detected in base-killed controls (pH>13). Error bars
876 represent standard error of mean (n=3). Sulfate was below the detection limit in the
877 live controls and in incubations with Fe(III) (points are overlapping).

878

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

886

887 **Figure 4.** Concentrations and compound-specific $\delta^{13}C$ values of fatty acids diagnostic
888 for putative methanotrophs in the initial slurry sample (Slurry T0), and in $^{13}CH_4$
889 incubations with different treatments after 160 days.
890
891 **Figure 5.** Microbial communities in slurry samples before (Slurry T0) and after
892 incubation (160 days) with different treatments. (A) Relative abundances of the most
893 abundant phyla within archaea. (B) Relative abundances of top bacterial classes. (C)
894 Fractional abundances of known methanotrophic families. Data are based on read
895 abundances of 16S rRNA gene sequences.
896
897
898
899 **FIGURES**
900
901 **Figure 1**

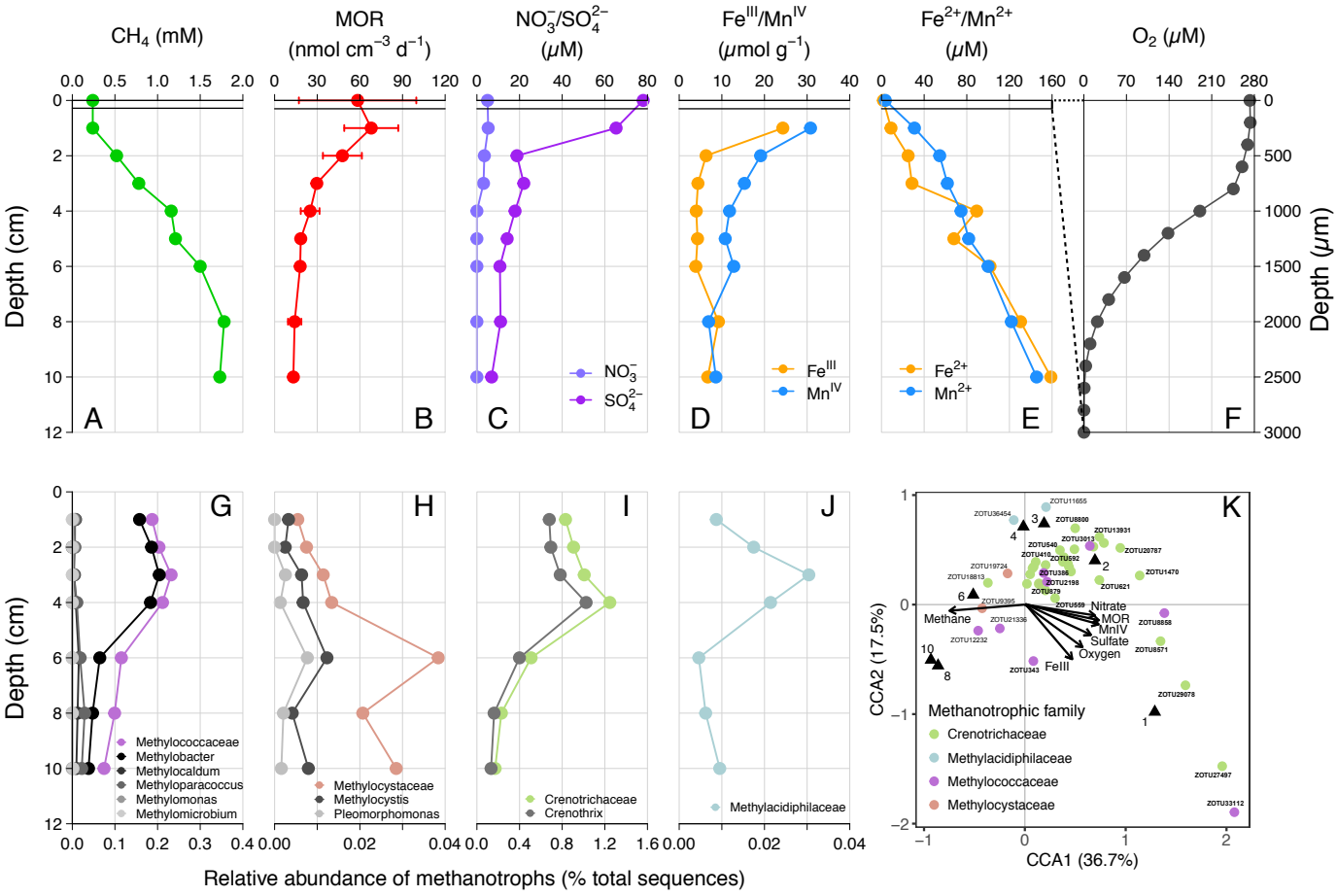

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Figure 2

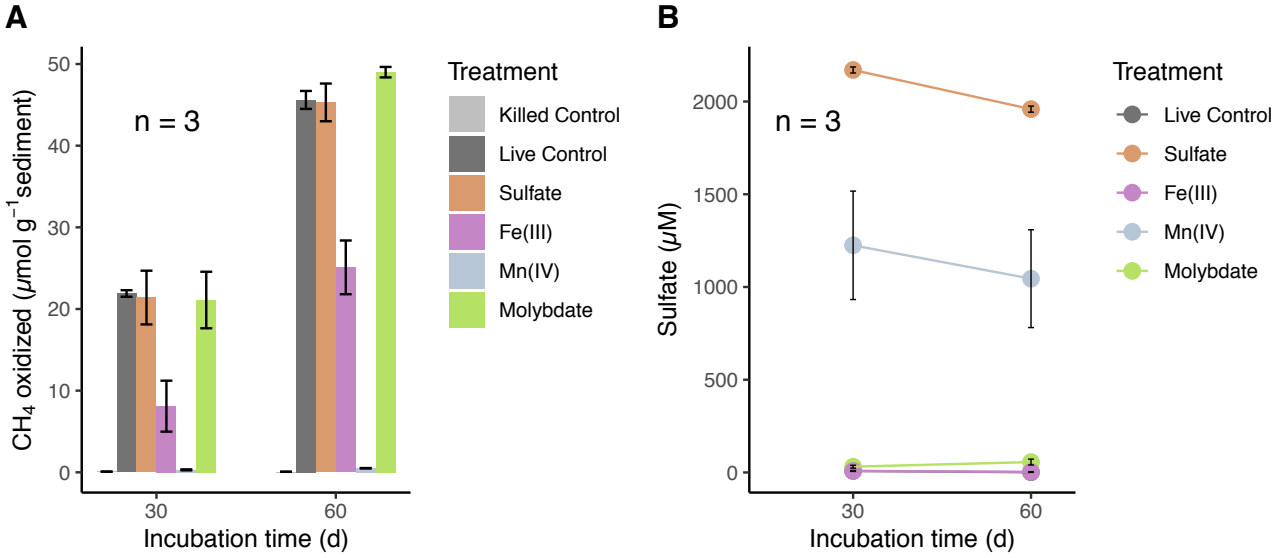

Figure 3

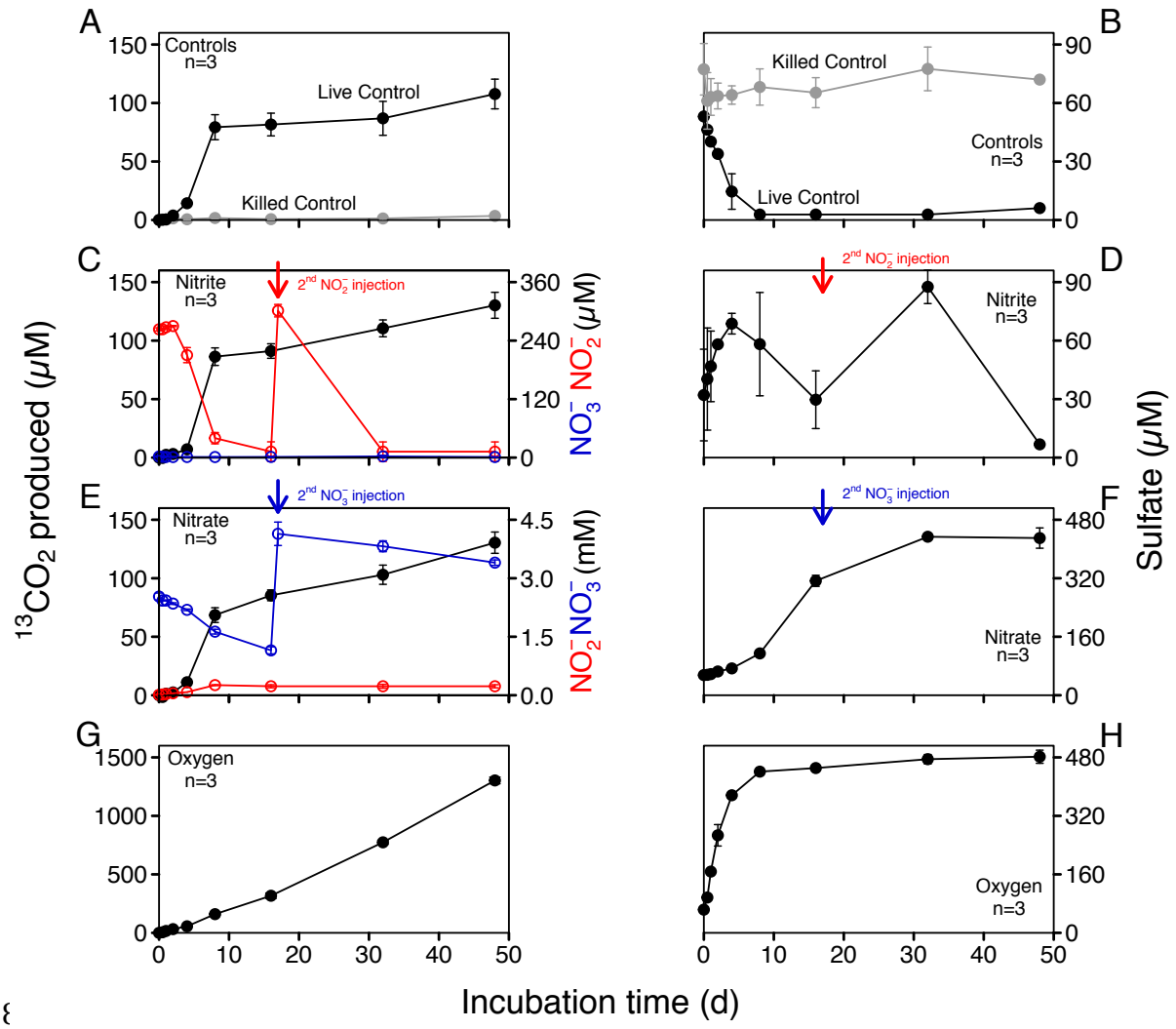

Figure 4

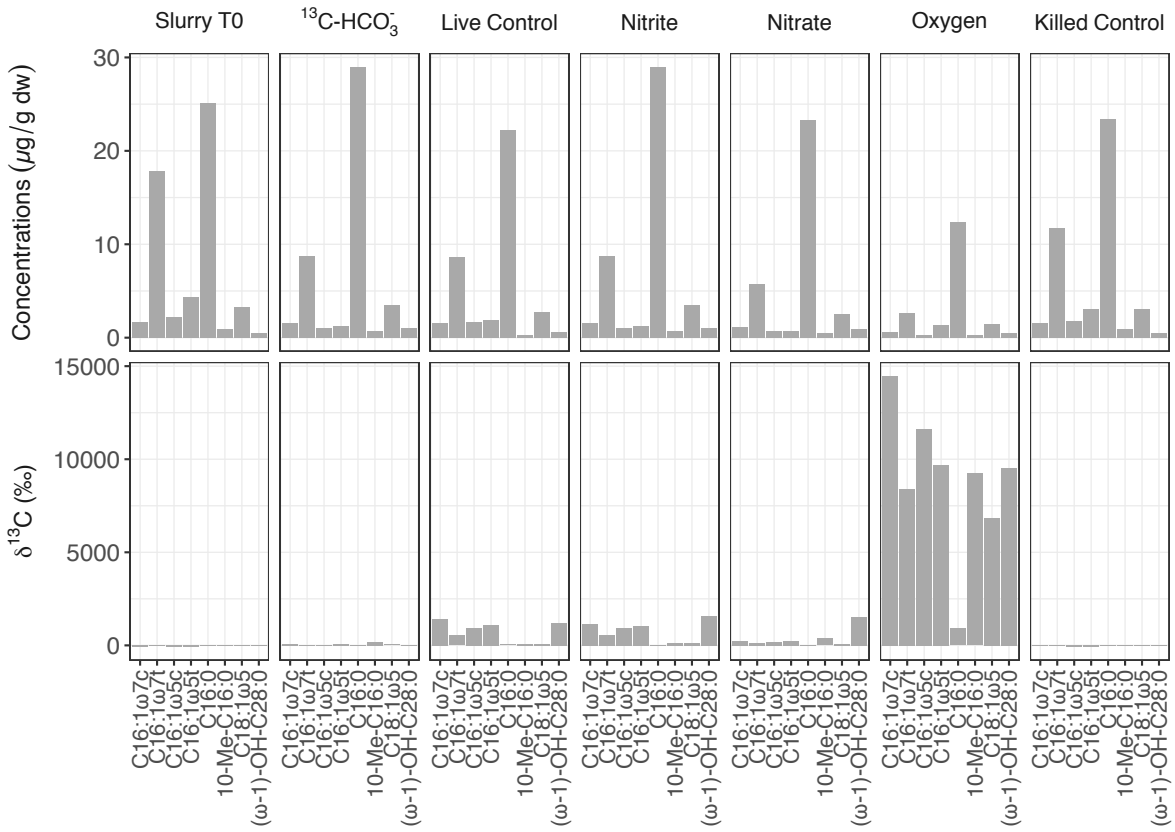
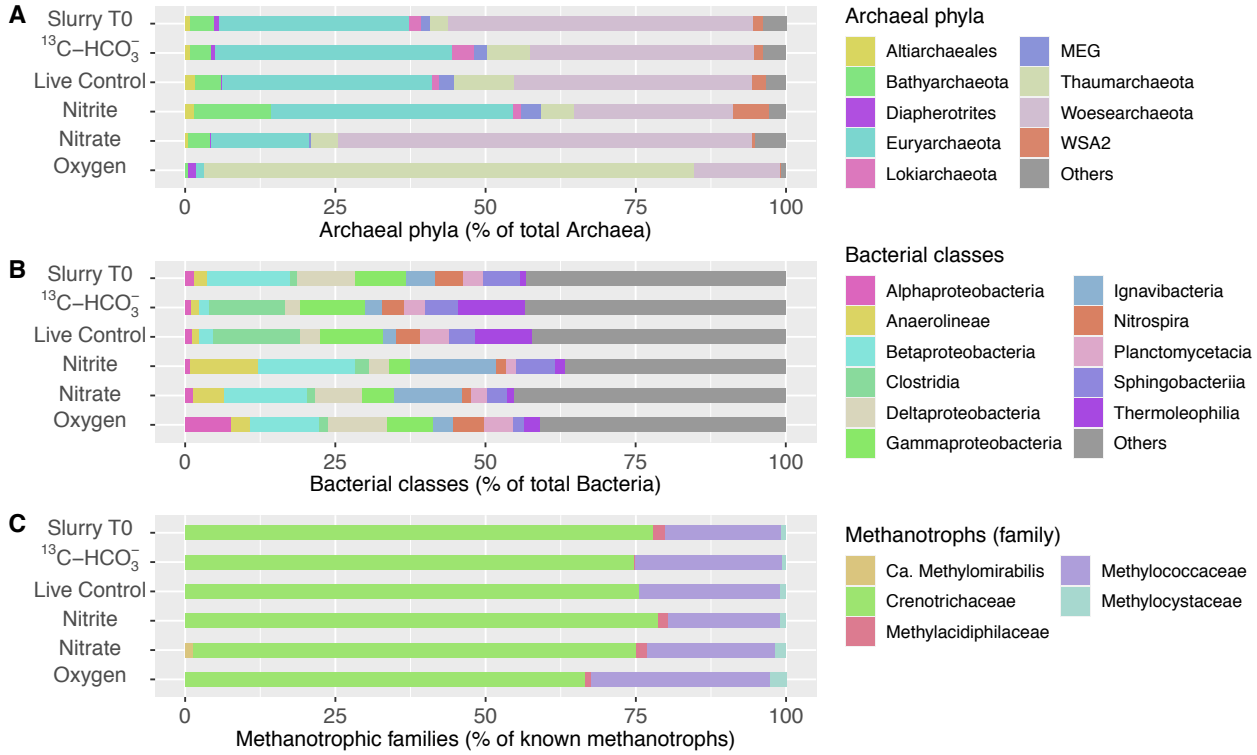
Figure 5