1 **Biogenic methane cycling is controlled by microbial cohorts**

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21 **Abstract**

22 The generation and consumption of methane by aquatic microbial communities is an
 23 important contribution to the global carbon budget. We sought to broaden understanding of
 24 consortia members and interactions by combining multiple methods including analysis of
 25 natural and cultivated microbial communities. By analysing the microbial community
 26 composition and co-occurrence patterns of a lake time-series we were able to identify
 27 potential consortia involved in methane cycling. In combination with methane flux, we also
 28 analysed the community composition and co-occurrence patterns of reduced microbial model
 29 communities with inoculum from the same lake. While the network analyses confirmed many
 30 known associations, when combined with results from mixed cultures, we noted new players
 31 in methane cycling. Cultivated model communities were shown to be an effective method to
 32 explore the rarer but still important players in methane cycling and for identifying new
 33 putative members. Here we show that using multiple methods to approach the complex
 34 problem of methane cycling consortia yields not just insights into the known taxa but
 35 highlights potential new members creating new hypotheses to be tested.

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38 Introduction

39 Methane emissions from biogenic sources are determined by the ratio and activity of
 40 methanogenic microbes producing and methanotrophic microbes consuming this powerful
 41 greenhouse gas. Considering that methane is responsible for 17% to 20% of the radiative
 42 forcing component of global warming (1, 2), elucidating all players in the methane cycle
 43 involved in the net methane emissions from various biogenic sources is of utmost importance.
 44 It is known that methanotrophs can oxidize between 30 and 99% of CH₄ produced in the
 45 sediments and the water column before it reaches the atmosphere (3–6). However, the
 46 detection of methanotrophs in an environment does not necessarily correlate with
 47 methanotrophic activity, and other factors may determine whether methane is oxidized (7).
 48 One factor that may affect the rate or existence of methanotrophy is the composition of the
 49 microbial community. For example, it has been shown that aerobic methanotrophs can be
 50 active in anoxic waters when co-occurring with photosynthetic organisms (8), suggesting that
 51 the methanotrophs utilize oxygen liberated by these phototrophs for methane oxidation.
 52 However, discovering and pinpointing more of these symbiotic interactions is difficult in
 53 natural environments as the number of possible interacting organisms is very high. Methods
 54 to cultivate communities with reduced diversity have been developed (9) to overcome this
 55 limitation and study organisms in a model communities in conditions mimicking their natural
 56 habitat. In this method, environmental samples are diluted in filtered water from their own
 57 environment and microorganisms subsampled for cultivation. These so-called model
 58 communities are a powerful way to study the microbial interaction in a semi-natural
 59 environment (10). Model communities also provide tools for building hypotheses that could
 60 then be further tested by looking into interaction networks in time series of natural
 61 environments (11).
 62 For this study, we established 177 dilution-cultures model communities using samples from

63 Lake Lomtjärnan, a lake in Sweden with high methane concentrations in the water column to
64 examine the methane cycling capacity of microbial assemblages. We also utilized a 2-week
65 depth discrete timeseries taken from March to April 2016 to examine the network around the
66 methane oxidizing communities in the actual lake. We hypothesize that the capacity to
67 produce or oxidize methane is not only related to the presence of organisms with the known
68 ability to produce or use methane, but that their cohorts might be key controllers in methane
69 cycling.

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71 **Materials and methods**

72 *Lake water collection and media preparation*

73 Environmental samples for the time-depth-series were collected and published previously
74 (12). In brief, a sampling campaign including six time points was done in winter the last week
75 of March and the first week of April 2016 on Lake Lomtjärnan. This small forest lake had an
76 ice cover at the time of sampling and is located in central west region (Jämtland) of Sweden
77 (Fig 1A). The surface area of the lake is about 1 ha, and the maximum depth is 3.5 m. The
78 lake is located on a mire surrounded by a coniferous forest. At each sampling occasion,
79 samples at six different depths (0.65 m, 1.0 m, 1.35 m, 1.85 m, 2.35 m, 2.75 m) were taken to
80 create a time-depth-series of the lake totalling 35 samples. The deepest depth was not taken
81 on the first sampling because samples were taken in a location where the max depth was
82 around 2.35 m.

83 Water for the time-depth-series was collected using a depth-discrete Limnos tube-sampler
84 (Limnos, Poland) and the water was subsequently filtered through a Sterivex filter (0.22 µm)
85 and the filters were stored immediately in liquid nitrogen for later DNA extraction. Water to
86 be used for both growth media and inoculum was collected from the anoxic layer on the sixth
87 sampling occasion of the lake in April 2016 and again in September 2017.

88 The water for media was collected using the same depth-discrete Limnos tube-sampler
 89 (Limnos, Poland) and was quickly poured into 1 litre Schott bottles which were filled and let
 90 to overflow to remove all the oxygenic water prior to closing the bottles. These bottles were
 91 then kept in the dark at 4°C for 2 days to ensure anoxia. The water for media was filtered
 92 twice through 0.2 µm Sterivex filters (Millipore) inside the anaerobic glove box, and the
 93 filtered-sterilized media was collected in a sterile Schott bottle and closed. These bottles were
 94 further exposed to UV light for 10 min.

95 The inoculum for the cultures was collected using the Limnos tube-sampler in a falcon tube
 96 and was then flash frozen with liquid nitrogen and kept in -80 °C until the cultures were
 97 established. The following steps of the preparation were performed under anoxic atmosphere
 98 using an anaerobic glove box.

99

100 *Preparation of serum bottles, cell inoculant and cultures*

101 20 ml serum bottles, crimps, and stoppers were autoclaved at 120°C for 20 min. To limit the
 102 risk of potential bactericides such as benzyltoluenes and phenylalkanes, the stoppers were
 103 autoclaved submerged in deionized water, and subsequently rinsed and boiled in sterile
 104 deionized water and finally cooled in sterile deionized water. After autoclaving, the serum
 105 bottles were sealed with these sterilised and detoxified butyl rubber stoppers and aluminium
 106 crimps. The incubation vessels were assembled under a laminar flow, using autoclaved tools,
 107 before being flushed at least 3 times with nitrogen to remove O₂. To maintain the sterility of
 108 the bottles during the flushing operation, new sterile needles were used for each bottle and the
 109 stoppers were carefully disinfected with 90% ethanol. Furthermore a 0.2 µm filter was used
 110 to prevent contamination from the N₂ gas flow.

111 The sealed sterile and anaerobic serum bottles were supplemented with 10 ml of media,
 112 a.k.a. filtered lake water. Subsamples of the unfiltered lake water from Lomtjärnan April

2016 (average 5×10^6 cells/ml) and September 2017 were run through a flow cytometer to estimate cell concentration. Based on those values, another subsample, not exposed to the atmosphere was diluted with lake-water-media to approximately 50 cells/ml. To inoculate the bottles, 1 ml of this 50 cells/ml solution was then injected using sterile needles and syringes into the 20 ml serum bottles. The number of cultures prepared were: 98 from 2016 and 79 from 2017. A few bottles were incubated without inoculum as media control cultures. To test for the potential for anoxic methane oxidation 4 ml of a mix of CH₄ and CO₂ with a ratio of 80/20 % were injected into each bottle (Figure 1B).

Bottles were then incubated at 11°C for two months in the dark, to mimic lake conditions, for the samples taken in the autumn and under dim light for the samples collected in the spring. After the two-month incubation, 1 ml of the headspaces was sampled for gas analysis. Methane content was measured using the gas analyser Biogas 5000 (Geotechnical Instruments, UK) and gas chromatography (Clarus 500, Perkin Elmer, USA, Polyimide Uncoated capillary column 5m x 0.32mm, FID detector). At the same time as gas sampling 200 µl of the cultures were collected and preserved at -80°C for DNA analysis.

16S rRNA gene amplicon preparation and sequencing

DNA was amplified directly from 1 µl of culture. Library preparation for 16S rRNA gene analysis was done following a two-steps Polymerase Chain Reaction (PCR) protocol, as described in Mondav et al 2020 (11). All PCRs were conducted in 20 µl of volume using 0.02 U/µl Phusion high fidelity DNA polymerase, 1X Q5 reaction buffer (NEB, UK), 0.25 µM primers and 200 µM dNTP mix and 1 µl mixed culture template. The first step was performed in triplicate with primers 341F (3'-CCTACGGGNGGCWGCAG-5') and 805NR (3'-GACTACNVGGGTATCTAA-5') (13). The thermal program consisted of 20 cycles with an initial 98°C denaturation step for 10 min, a cycling program of 98°C for 10 seconds, 48°C

for 30 seconds, and 72°C for 30 seconds and a final elongation step at 72°C for 2 minutes. Triplicate PCR reactions were then pooled and purified with magnetic beads (Sera-Mag™ Select, GE Healthcare, Chicago, United States of America), and 2 µl of the purified products were used at a template for a second stage PCR, where indexed primers were added. The second thermal program consisted of 15 cycles with an initial 98°C denaturation step for 30 seconds, a cycling program of 98°C for 10 seconds, 66°C for 30 seconds, and 72°C for 30 seconds and a final elongation step at 72°C for 2 minutes. Following amplification, PCR products were again purified with magnetic beads and quantified with Qubit™ using the Qubit™ dsDNA HS Assay Kit (Invitrogen™). Finally, 15.6 µg of each indexed and purified PCR product were pooled before submission of the sample to the Science for Life Laboratory SNP/SEQ sequencing facility hosted by Uppsala University (Uppsala, Sweden). Sequencing was done using Illumina Miseq in paired-end mode with 300bp and v3 chemistry.

Amplicon bioinformatics

Sequence processing was performed with Mothur 1.41.0 following the MiSeq SOP (14), with the exception that clustering to operational taxonomic units (OTUs) was done using VSEARCH (15) as implemented in Mothur (16). Taxonomy was assigned against the Silva 132 database (17). Amplicon sets were separated into groups of sample or inoculant origin: time-depth-series lake communities, anaerobic lake cultures 2016, anaerobic lake cultures 2017, and 2 un-inoculated media controls. Nine lake-2017 cultures produced only a few sequences (<20) and were removed from the dataset. Remaining sequences were then subsampled once using Qiimes (18) single rarefaction to an even depth of 600 reads per sample for the un-inoculated media, and 2000 reads per sample for all others. This resulted in final sample counts: 35 lake time-depth-series, 93 lake-2016 cultures and 70 lake-2017 cultures. These normalized OTU tables were used for compositional and comparative

analyses. Column graphs of the ten most abundant phylotypes from each dataset were made for compositional analyses. OTU tables were processed and visualised in R 4.0.3 (19) using phyloseq 1.34.0 (20) and ggplot2 (21).

Prior to co-occurrence analysis for network visualisation, OTUs present in less than 10% of each culture group, 50% of the lake water samples, or with a relative abundance always below 1 % were removed. This was done to reduce sparsity to below 50%. A network ensemble approach was used where any correlation (edge) between OTUs (node) that was found in at least two of the three following methods was included in the final visualisation: SPIEC-EASI (22), SparcCC (23), and Pearsons correlation. All co-occurrence methods were implemented in R 4.0.3 using spiec-easi 1.1.1 for SPIEC-EASI, SparcCC and the psych 2.1.3 package for Pearsons correlation. Networks were visualised in cytoscape.

To identify communities that had unbalanced methane cycling (flux), all cultures that had a recorded change in methane concentration and all cultures that had at least one phylotype associated with methane generation or consumption were retained and analysed for OTU co-occurrence.

Data availability and source code

All scripts used to calculate network correlations are available at: https://github.com/rmondav/methane_cycling_networks, v1.0.0 recorded at <https://doi.org/10.5281/zenodo.5531947>. Time-depth-series amplicons are available under BioProject PRJEB27633 (12). Mixed culture sequence data has been deposited at the European Nucleotide Archive (ENA) at EMBL-EBI under accession number BioProject PRJEB48661 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB48661>).

Results

Change in dominant lake community with depth

The most abundant OTUs in the lake time-depth-series belonged to just three phyla: Bacteroidetes, Chloroflexi, and Proteobacteria. The surface lake microbial community was dominated by the α -proteobacterial Rhodospirillum OTU25 and Polaromonas OTU11 and Methylobacteriaceae OTU24 while the deeper lake community was increasingly dominated by a Chlorobium OTU9 and Oscillochloris OTU21 (Fig. 1C). Polynucleobacter OTU2 was highly abundant throughout the lake water (Fig 1C) showing no preference for depth. It was also abundant in most of the mixed cultures (Fig 1D) and was present at low abundance in media controls from Sept 2017 (0.2 μ m filterable microbes).

Communities in lakes associated with methane cycling

Known methanotrophs (obligate methane consumers) in the lake time-depth-series network were associated with either a phototrophic cluster of bacteria (green circle top left) or clustered around a methanogen (gold circle top centre, Figure 2). Carbon fixing phototrophs as detected in this cluster, methanotrophs, and the methanogen are all obligate C1-carbon molecule cyclers. Sulphate-reducing bacteria were also associated with these two clusters. The Oscillochloris OTU21 and Chlorobium OTU9 were dominant phylotypes of deeper lake (green circle Figure 2). The methanotroph Methylobacteriaceae OTU24 and Polaromonas OTU11 (grey circle bottom Figure 2) were dominant in lake Lomtjärnan surface waters (Fig 1B). This cluster (grey circle) was negatively correlated to the other methanotrophs, the phototrophs and the methanogen. Methylobacteriaceae (facultative methane consumers) had no clear association with any of these metabolic clusters.

Percentage of mixed cultures with growth varied with inoculum source

All inoculated cultures from the 2016 inoculum grew and produced sequences, while 71% of the cultures from 2017 sampling produced sequences. Despite similar culture conditions, there were distinct differences in the dominant genera in the cultures inoculated with water samples from early spring 2016 compared to autumn 2017 (Fig. 1D).

Abundant lineages can both pass through and be retained by 0.22 µm filters

On average half of the top ten most abundant phylotypes in the time-depth-series and the 2017 culture-series were also detected in the media control sequenced from 2017. Specifically, five of the most prevalent phylotypes in the 2017 cultures (Pelomonas, Betaproteobacteriales - T34, Polynucleobacter, Sphingomonas, and Staphylococcus) were also detected in the 2017 media control. Seven of the top 25 most abundant OTUs in the time-depth series (Polaromonas, Polynucleobacter, Rhodoferax, Betaproteobacteriales - T34, an unclassified genus of the Sporichthyaceae family, Flavobacterium, and an unclassified genus of Burkholderiaceae) were abundant in media controls.

Detection of methane cycling phylotypes does not always correlate to methane flux measurements

The majority (91%) of cultures had no detectable change in methane concentration or (60%) had no known methane cycling phylotypes (Figure 3A). Of note in our search for new organisms with a putative role in methane cycling were the 5% of cultures that had methane flux but no methane cycling phylotypes detected. Also of note were the 36 % of mixed cultures where obligate methane cycling phylotypes were detected but no methane flux was recorded. These two culture groups were noted and selected for further investigation.

Putative cohorts and potential newcomers to methane cycling

Due to the poor correlation between methane flux and methane cycling phylotypes, the co-occurrence patterns of the cultures of note were examined. Organisms detected uniquely in cultures that had unexplained methane concentration were recorded and a putative role in methane cycling proposed (Table 1). Eight microbes associated with the production of methane without detection of archaeal methanogens were noted by “CH₄ production without methanogen”. One of these candidates, *Rhodopseudomonas*, is already annotated as a putative methane producer via the recently documented Fe-only nitrogenase methane release pathway (24, 25). Another cohort candidate, *Desulfobulbaceae*, is a putative sulphate reducer. Many cultures had abundant obligate methane consumers but no change in methane concentration, twenty-five microbes were uniquely associated with these cultures and were noted with “CH₄ balance in the presence of methanotroph”. There were many *Patescibacteria* and one putative ammonia oxidizer, *Nitrosomonadaceae*, in this group. Six bacteria associated with consumption of methane without detection of methane consumers were noted by “CH₄ consumption without CH₄ consumer”. In total, 39 lineages were identified as potential contributors to methane cycling assemblages.

Discussion

Assembly of microbial communities is cell density and diversity dependent

The 71 % to 100 % success rate of culturing in this experiment is in stark contrast to the 10% to 30% success rate of dilution to extinction where only a few to maybe 20 cells are used in inoculant mixed cultures (11, 26). It therefore seems that around 50 cells of a natural community per inoculum (for anaerobic cultures from Lake Lomtjärnan) capture sufficient metabolic breadth to form a functional self-sustaining non-photoc community, or cohorts. This for the first time places an upper limit on the minimum metabolic diversity required for functional (anaerobic) aquatic cohorts (27, 28).

262

263 *Filterable microbes play a significant role in natural and laboratory systems*

264 A growing literature has discussed and evaluated the aquatic microbial filterable microbes in
 265 relation to the concept and efficacy of filter sterilisation of media used for laboratory
 266 culturing and environmental role (29–32). Here we also found that some phylotypes e.g.,
 267 Polynucleobacter, can both pass through filters to be detected in filter ‘sterilised’ media, and
 268 be captured on filters as seen here in the time-depth-series. While the debate over whether
 269 filter sterilisation works is mostly complete, a discussion on the effect on abundance
 270 estimates of the loss of the filterable microbes might be worth re-opening.

271

272 *Associations between methanotrophs and other lifestyles in the lake*

273 The lake time-series network shows an association between photoautotrophic Chlorobium, a
 274 methanotrophs, and sulphate reducing bacteria. This connection could be, for example, via
 275 carbon fixation by the autotrophic bacteria which also oxidise sulphur species to sulphate
 276 which is then consumed by the sulphate reducers. The Methylobacter phylotype, based on
 277 associations visualised in the network, is not reliant on proximity to a methanogen to thrive
 278 but rather is integral to the phototrophic cohort. The remainder of the methanotrophs in the
 279 cluster are associated with a methanogen and sulphate reducers. One of these sulphate
 280 reducers, Desulfobulbaceae is a putative methane consumer cohort member, while the other,
 281 Geobacter, is a known consort of Methanotrix (synonym Methanosaeta (40)) whereby
 282 direct electron transfer allows the methanogen to switch to CO₂ utilisation for methane
 283 generation (44, 45). Methane is then released into the water and consumed by the cohort of
 284 methanotrophs.

285 Except for two cyanobacteria OTUs (Planktothrix and Pseudoanabaena) most of the taxa in
 286 the green and gold circles are associated with oxic-anoxic interfaces. Besides the phototrophs

all of the taxa present in the upper cohort and correlated to methanotrophs are potential microaerophiles e.g. Polyangjaceae or Gallionalaceae (33–35). Also included in the circles are potential anaerobe with known ability to live in microaerophile condition like Geobacter, or Rhodocyclaceae a taxa known to use a wide range of electron acceptor, including but not limited to O₂ (36). These correlations between methanotrophs and taxa associated with the oxic anoxic interface is in line with the fact that this interface is a hotspot for methanotrophy (37–39). Based only on correlation it is impossible to tell if the cooccurrence of different OTU is due to shared environmental preference of by necessary interactions. The fact that all the organisms represented in the gold and green circles appear to favorize low oxygen environment suggests that the cooccurrence might be driven by environmental preferences. It is nevertheless interesting to observe that three different OTUs attributed to methanotrophic taxa are directly correlated to a methanogen, Methanothrix (synonym Methanosaeta (40)). The presence of an archaeal methanogen in a potentially oxic environment might seem surprising, but again there is also mounting evidence that methanogenesis isn't limited to anoxic environments (41, 42). Furthermore Methanothrix is among the methanogen with a potential to thrive in oxic conditions (42, 43). Interestingly though the Methanothrix OTU correlates with two OTUs that lean more on the anaerobic side of the interface (Geobacter and Crenothrix).

Others interesting correlation are the ones with iron cycling phylotypes (i.e Geobacter and Gallioneallaceae). Indeed both methanogen and methanotrophs have high iron demand for cofactor production (46). Furthermore, it has been suggested that interactions can be favorable to methanotrophs (47, 48). But if notable it is, again, impossible to clearly state the nature of the relation between those methane cycling taxa with the iron cycling ones.

All in all, the clusters in the top of the figure suggest that most of the methanotrophs detected are attached to an environment with little oxygen favorizing taxa with the ability to deal with change in O₂ concentration through mobility (like Gallionella or Chlorflexi) or metabolic plasticity (e.g Chloroflexi or Rhodocycladeae). Interestingly, the Methylococcales OTU abundances correlates with both anoxic leaning OTU (Chrenothrix, Methanothrix, Geobacter) and an oxygenic phototroph (Planktothrix and Pseudanabaena, both cyanobacteria). This again suggest microoxic condition, and is in line with work suggesting that aerobic methanotrophy can be enhanced by phototroph in low oxygen environment (49).

The most probable explanation for the negative correlation between the upper circle clusters (Figure 2) and the grey circle cluster in the bottom of the figure are due to spatial separation likely controlled by temperature. Several OTU present in the grey cluster belongs to taxa including both aerobic and anaerobic species like Polaromonas, Flavobacterium or Burkholderiaceae. Both Polaromonas and Flavobacterium are generally considered psychrophilic and psychrotolerant taxa respectively (50, 51) (52). Furthermore Both Pedobacter and Rhodoferax also include psychrophilic taxa (53, 54). The association of these psychrophilic and tolerant taxa with the surface water, might be explained considering that sampling was performed during the ice-covered season it make sense as during winter, stratification is inverted, with the coldest water found at the surface directly beneath ice. It seems therefor very likely that the grey cluster is associated with cold temperature compared to rather than by oxygen concentration.

Associations between methanotrophs and other lifestyles in the cohorts in model communities

Over the last two decades there has been a large increase in the catalogued number of microbes and metabolic pathways capable of generating, releasing, or consuming methane

(55, 56). Both increases are likely to continue as we explore deeper genomic and metabolic space. For these reasons it is not a surprise that methane consumption and production did not correlate clearly with detection of known methane cycling phylotypes. It was surprising however, the degree to which these were decoupled. It therefore not clear if this uncoupling is due to poor flux detection, or low abundance of methane cycling organisms. Previously, it has been demonstrated that methanotroph abundance can be uncoupled from methane oxidation rates (58–60).

The generation here of a list of 39 novel phylotypes that may be directly or indirectly involved in methane cycling is a mere indicator of how much more work can be done to identify key players effecting cycling of methane and other C1 molecules.

We hypothesize that the participate in methane cycle is not only related to the presence of organisms with the known ability to produce or use methane, but that their cohorts as found in the model communities might be key controllers in methane cycling.

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SP, GM, SLG conceived of and implemented field and laboratory research. SP and SLG obtained financial and material resources while SP and RM obtained computational resources. RM and SLG wrote and implemented scripts for analysis and visualization of results. RM and SP drafted the manuscript and all authors contributed to discussions, editing, and approval of final manuscript. RM curated data and scripts. SP and SLG supervised and SLG coordinated the project.

References

1. Forster P, Ramaswamy V, Artaxo P, Bernsten T, Betts R, Fahey DW, Haywood J, Lean J, Lowe DC, Myhre G, Nganga J, Prinn R, Raga G, Schulz M, Dorland R Van. 2007. Changes in Atmospheric Constituents and in Radiative ForcingClimate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, AR4 ed. Cambridge University Press, Cambridge.
2. Fernández-Amador O, Francois JF, Oberdabernig DA, Tomberger P. 2020. The methane footprint of nations: Stylized facts from a global panel dataset. Ecol Econ 170:106528.
3. Frenzel P, Thebrath B, Conrad R. 1990. Oxidation of methane in the oxic surface layer of a deep lake sediment (Lake Constance). FEMS Microbiol Ecol 73:149–158.
4. Kankaala P, Huotari J, Peltomaa E, Saloranta T, Ojala A. 2006. Methanotrophic activity in relation to methane efflux and total heterotrophic bacterial production in a stratified, humic, boreal lakeLimnol. Oceanogr.
5. Bastviken D, Tranvik LJ, Downing JA, Crill PM, Enrich-prast A. 2011. the Continental Carbon Sink. Science (80-) 331:6.

- 384 6. Mayr M, Zimmermann M, Dey J, Wehrli B, Bürgmann H. 2020. Lake mixing regime
385 selects methane-oxidation kinetics of the methanotroph assemblage. *Biogeosciences Discuss*
386 1–15.
- 387 7. Sawakuchi HO, Martin G, Peura S, Bertilsson S, Karlsson J, Bastviken D. 2021.
388 Phosphorus Regulation of Methane Oxidation in Water From Ice-Covered Lakes. *J Geophys*
389 *Res Biogeosciences* 126:e2020JG006190.
- 390 8. Oswald K, Milucka J, Brand A, Littmann S, Wehrli B, Kuypers MMM, Schubert CJ.
391 2015. Light-dependent aerobic methane oxidation reduces methane emissions from
392 seasonally stratified lakes. *PLoS One* 10:e0132574.
- 393 9. Garcia S. 2016. Mixed cultures as model communities: hunting for ubiquitous
394 microorganisms, their partners, and interactions. *Aquat Microb Ecol* 77:79–85.
- 395 10. Garcia SL, Buck M, McMahon KD, Grossart H-P, Eiler A, Warnecke F. 2015.
396 Auxotrophy and intrapopulation complementary in the ‘interactome’ of a cultivated
397 freshwater model community. *Mol Ecol* 24:4449–4459.
- 398 11. Mondav R, Bertilsson S, Buck M, Langenheder S, Lindström ES, Garcia SL. 2020.
399 Streamlined and abundant bacterioplankton thrive in functional cohorts. *mSystems* 5:e00316-
400 20.
- 401 12. Garcia SL, Szekely AJ, Bergvall C, Schattenhofer M, Peura S. 2019. Decreased Snow
402 Cover Stimulates Under-Ice Primary Producers but Impairs Methanotrophic Capacity.
403 *mSphere* 4.
- 404 13. Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF. 2011.
405 Transitions in bacterial communities along the 2000-km salinity gradient of the Baltic Sea.
406 *ISME J* 5:1571–9.
- 407 14. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development
408 of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon

409 Sequence Data on the MiSeq Illumina Sequencing Platform. *Appl Environ Microbiol*
410 79:5112–5120.

411 15. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. 2016. VSEARCH: a versatile open
412 source tool for metagenomics. *PeerJ* 4:e2584.

413 16. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski
414 RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ,
415 Weber CF. 2009. Introducing mothur: Open-source, platform-independent, community-
416 supported software for describing and comparing microbial communities. *Appl Environ*
417 *Microbiol* 75:7537–7541.

418 17. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.
419 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-
420 based tools. *Nucleic Acids Res* 41:D590-6.

421 18. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK,
422 Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE,
423 Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR,
424 Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME
425 allows analysis of high- throughput community sequencing data. *Nat Methods* 7:335–336.

426 19. R Core Development Team. 2015. R: a language and environment for statistical
427 computing, 3.2.1. Doc Free available internet <http://www.r-project.org>.

428 20. McMurdie PJ, Holmes S. 2013. phyloseq: An R Package for Reproducible Interactive
429 Analysis and Graphics of Microbiome Census Data. *PLoS One* 8:e61217.

430 21. Ginestet C. 2011. ggplot2: Elegant Graphics for Data Analysis. *J R Stat Soc Ser A*
431 (Statistics Soc 174:245–246.

- 432 22. Kurtz ZD, Müller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA. 2015.
433 Sparse and Compositionally Robust Inference of Microbial Ecological Networks. PLOS
434 Comput Biol 11:e1004226.
- 435 23. Friedman J, Alm EJ. 2012. Inferring Correlation Networks from Genomic Survey
436 Data. PLoS Comput Biol 8:e1002687.
- 437 24. Fixen KR, Zheng Y, Harris DF, Shaw S, Yang Z-Y, Dean DR, Seefeldt LC, Harwood
438 CS. 2016. Light-driven carbon dioxide reduction to methane by nitrogenase in a
439 photosynthetic bacterium. Proc Natl Acad Sci 113:10163–10167.
- 440 25. McRose DL, Zhang X, Kraepiel AML, Morel FMM. 2017. Diversity and activity of
441 alternative nitrogenases in sequenced genomes and coastal environments. Front Microbiol.
- 442 26. Henson MW, Lanclos VC, Pitre DM, Weckhorst JL, Lucchesi AM, Cheng C,
443 Temperton B, Thrash JC. 2020. Expanding the diversity of bacterioplankton isolates and
444 modeling isolation efficacy with large-scale dilution-to-extinction cultivation. Appl Environ
445 Microbiol 86.
- 446 27. Yu X, Polz MF, Alm EJ. 2019. Interactions in self-assembled microbial communities
447 saturate with diversity. ISME J 13:1602–1617.
- 448 28. Pascual-García A, Bonhoeffer S, Bell T. 2020. Metabolically cohesive microbial
449 consortia and ecosystem functioning. Philos Trans R Soc Lond B Biol Sci 375:20190245.
- 450 29. Hahn MW. 2004. Broad diversity of viable bacteria in “sterile” (0.2 µm) filtered
451 water. Res Microbiol 155:688–691.
- 452 30. Nakai R. 2020. Size matters: Ultra-small and filterable microorganisms in the
453 environment. Microbes Environ.
- 454 31. Ghuneim LAJ, Distaso MA, Chernikova TN, Bargiela R, Lunev EA, Korzhenkov
455 AA, Toshchakov S V., Rojo D, Barbas C, Ferrer M, Golyshina O V., Golyshin PN, Jones

DL. 2021. Utilization of low-molecular-weight organic compounds by the filterable fraction of a lotic microbiome. *FEMS Microbiol Ecol* 97:1–16.

32. Maejima Y, Kushimoto K, Muraguchi Y, Fukuda K, Miura T, Yamazoe A, Kimbara K, Shintani M. 2018. Proteobacteria and Bacteroidetes are major phyla of filterable bacteria passing through 0.22 µm pore size membrane filter, in Lake Sanaru, Hamamatsu, Japan. *Biosci Biotechnol Biochem* 82:1260–1263.

33. Pedersen K. 2011. Gallionella, p. 411–412. In Reitner, J, Thiel, V (eds.), *Encyclopedia of Geobiology*. Springer Netherlands, Dordrecht.

34. Hanert HH. 2006. The Genus Gallionella, p. 990–995. In Dworkin, M, Falkow, S, Rosenberg, E, Schleifer, K-H, Stackebrandt, E (eds.), *The Prokaryotes: Volume 7: Proteobacteria: Delta, Epsilon Subclass*. Springer, New York, NY.

35. Garcia R, Müller R. 2014. The Family Polyangiaceae, p. 247–279. In Rosenberg, E, DeLong, EF, Lory, S, Stackebrandt, E, Thompson, F (eds.), *The Prokaryotes: Deltaproteobacteria and Epsilonproteobacteria*. Springer, Berlin, Heidelberg.

36. Oren A. 2014. The Family Methanoregulaceae, p. 253–258. In Rosenberg, E, DeLong, EF, Lory, S, Stackebrandt, E, Thompson, F (eds.), *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*. Springer, Berlin, Heidelberg.

37. Rissanen AJ, Saarenheimo J, Tirola M, Peura S, Aalto SL, Karvinen A, Nykanen H. 2018. Gammaproteobacterial methanotrophs dominate methanotrophy in aerobic and anaerobic layers of boreal lake waters. *Aquat Microb Ecol* 81:257–276.

38. Reis PCJ, Thottathil SD, Ruiz-González C, Prairie YT. 2020. Niche separation within aerobic methanotrophic bacteria across lakes and its link to methane oxidation rates. *Environ Microbiol* 22:738–751.

39. Samad MS, Bertilsson S. 2017. Seasonal Variation in Abundance and Diversity of Bacterial Methanotrophs in Five Temperate Lakes. *Front Microbiol* 8.

- 481 40. Boone DR. 1991. Strain GP6 is proposed as the neotype strain of *Methanothrix*
482 *soehngeni*(VP) pro synon. *Methanothrix concilii*(VP) and *Methanosaeta concilii*(VP):
483 Request for an opinion. *Int J Syst Bacteriol* 41:588–589.
- 484 41. Bogard MJ, del Giorgio PA, Boutet L, Chaves MCG, Prairie YT, Merante A, Derry
485 AM. 2014. Oxic water column methanogenesis as a major component of aquatic CH₄ fluxes.
486 *Nat Commun* 5:5350.
- 487 42. Angle JC, Morin TH, Solden LM, Narrowe AB, Smith GJ, Borton MA, Rey-Sanchez
488 C, Daly RA, Mirfenderesgi G, Hoyt DW, Riley WJ, Miller CS, Bohrer G, Wrighton KC.
489 2017. Methanogenesis in oxygenated soils is a substantial fraction of wetland methane
490 emissions. *Nat Commun* 8:1567.
- 491 43. Jetten MS, Stams AJ, Zehnder AJ. 1989. Purification and characterization of an
492 oxygen-stable carbon monoxide dehydrogenase of *Methanothrix soehngeni*. *Eur J Biochem*
493 181:437–441.
- 494 44. Holmes DE, Shrestha PM, Walker DJF, Dang Y, Nevin KP, Woodard TL, Lovley
495 DR. 2017. Metatranscriptomic evidence for direct interspecies electron transfer between
496 *Geobacter* and *Methanothrix* species in methanogenic rice paddy soils. *Appl Environ*
497 *Microbiol* 83.
- 498 45. Liu C, Sun D, Zhao Z, Dang Y, Holmes DE. 2019. *Methanothrix* enhances biogas
499 upgrading in microbial electrolysis cell via direct electron transfer. *Bioresour Technol*
500 291:121877.
- 501 46. Johnson C, England A, Munro-Ehrlich M, Colman DR, DuBois JL, Boyd ES. 2021.
502 Pathways of Iron and Sulfur Acquisition, Cofactor Assembly, Destination, and Storage in
503 Diverse Archaeal Methanogens and Alkanotrophs. *J Bacteriol* 203:e0011721.

- 504 47. Ho A, de Roy K, Thas O, De Neve J, Hoefman S, Vandamme P, Heylen K, Boon N.
505 2014. The more, the merrier: heterotroph richness stimulates methanotrophic activity. ISME J
506 8:1945–1948.
- 507 48. Iguchi H, Yurimoto H, Sakai Y. 2011. Stimulation of Methanotrophic Growth in
508 Cocultures by Cobalamin Excreted by Rhizobia. Appl Environ Microbiol 77:8509–8515.
- 509 49. van Grinsven S, Oswald K, Wehrli B, Jegge C, Zopfi J, Lehmann MF, Schubert CJ.
510 2021. Methane oxidation in the waters of a humic-rich boreal lake stimulated by
511 photosynthesis, nitrite, Fe(III) and humics. Biogeosciences 18:3087–3101.
- 512 50. Darcy JL, Lynch RC, King AJ, Robeson MS, Schmidt SK. 2011. Global Distribution
513 of Polaromonas Phylotypes - Evidence for a Highly Successful Dispersal Capacity. PLoS
514 One 6:e23742.
- 515 51. Michaud L, Caruso C, Mangano S, Interdonato F, Bruni V, Lo Giudice A. 2012.
516 Predominance of Flavobacterium, Pseudomonas, and Polaromonas within the prokaryotic
517 community of freshwater shallow lakes in the northern Victoria Land, East Antarctica. FEMS
518 Microbiol Ecol 82:391–404.
- 519 52. Bernardet J-F, Bowman JP. 2006. The Genus Flavobacterium, p. 481–531. In
520 Dworkin, M, Falkow, S, Rosenberg, E, Schleifer, K-H, Stackebrandt, E (eds.), The
521 Prokaryotes: Volume 7: Proteobacteria: Delta, Epsilon Subclass. Springer, New York, NY.
- 522 53. Viana AT, Caetano T, Covas C, Santos T, Mendo S. 2018. Environmental superbugs:
523 The case study of Pedobacter spp. Environ Pollut 241:1048–1055.
- 524 54. Finneran KT, Johnsen C V., Lovley DRY 2003. Rhodoferax ferrireducens sp. nov., a
525 psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of
526 Fe(III). Int J Syst Evol Microbiol 53:669–673.
- 527 55. Kurth JM, Op den Camp HJM, Welte CU. 2020. Several ways one goal—
528 methanogenesis from unconventional substrates. Appl Microbiol Biotechnol 104:6839–6854.

56. Berghuis BA, Yu FB, Schulz F, Blainey PC, Woyke T, Quake SR. 2019. Hydrogenotrophic methanogenesis in archaeal phylum Verstraetearchaeota reveals the shared ancestry of all methanogens. *Proc Natl Acad Sci U S A* 116:5037–5044.
58. DeBruyn JM, Leigh-Bell JA, McKay RML, Bourbonniere RA, Wilhelm SW. 2004. Microbial Distributions and the Impact of Phosphorus on Bacterial Activity in Lake Erie. *J Great Lakes Res* 30:166–183.
59. Vrede K, Vrede T, Isaksson A, Karlsson A. 1999. Effects of nutrients (phosphorous, nitrogen, and carbon) and zooplankton on bacterioplankton and phytoplankton—a seasonal study. *Limnol Oceanogr* 44:1616–1624.
60. Zheng Y, Zhang L, He J-Z. 2013. Immediate effects of nitrogen, phosphorus, and potassium amendments on the methanotrophic activity and abundance in a Chinese paddy soil under short-term incubation experiment. *J Soils Sediments* 13:189–196.

Figures and Table

Figure 1. Sample location [A], schematic of dilution and set up of model communities [B], most abundant OTUs detected in lake timeseries [C], and most abundant OTUs from model communities [D]. The legend in C is also for D.

Figure 2. Lake time-depth-series network. Filled node-circles are OTUs with the following color codes: methanogens-red, methanotrophs-orange, methylotrophs-golden, C-fixing phototrophs-bright green, other phototrophs-pale green, sulphate reducers-yellow. Grey lines show positive correlations and pink-dotted lines show negative correlations. Green, golden, and grey bigger circles designate cohort clusters. We designated putative roles in methane consumption from observations in the dilution model communities and they are shown in not-filled node-circles in peach and green.

Figure 3. Venn diagram of all cultures in this study [A], cultures from April 2016 [B] and cultures from September 2017 [C]. In orange the cultures with measured methane flux and in grey the cultures with detection of (known) methane cycling phylotypes.

567 **Table 1.** Phylotypes in this study with a potential role in methane cycling.

phylum	family	genus	metabolism	culture note	putative role in CH ₄ cycling
Patescibacteria	Ca.Vogelbacteria_fa	Ca.Vogelbacteria_ge	unknown	CH ₄ production without methanogen	CH ₄ producer or their cohort
Nanoarchaea	Woesearchaeia_fa	Woesearchaeia_ge	unknown	CH ₄ production without methanogen	CH ₄ producer or their cohort
Patescibacteria	Ca.Adlerbacteria_fa	Ca.Adlerbacteria_ge	unknown	CH ₄ production without methanogen	CH ₄ producer or their cohort
Patescibacteria	Ca.Staskawiczbacteria_fa	Ca.Staskawiczbacteria_ge	unknown	CH ₄ production without methanogen	CH ₄ producer or their cohort
Proteobacteria	Burkholderiaceae	Burkholderiaceae_ge	unknown	CH ₄ production without methanogen	CH ₄ producer or their cohort
Patescibacteria	Parcubacteria_fa	Parcubacteria_ge	unknown	CH ₄ production without methanogen	CH ₄ producer or their cohort
Proteobacteria	Desulfobulbaceae	Desulfobulbaceae_ge	SO ₄ _redctn	CH ₄ production without methanogen	methanogen cohort
Proteobacteria	Xanthobacteraceae	Rhodopseudomonas	put N ₂ , CH ₄	CH ₄ production without methanogen	CH ₄ producer
Actinobacteria	Corynebacteriaceae	Corynebacterium_1	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Actinobacteria	Kineosporiaceae	Kineosporiaceae_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Actinobacteria	Micrococcaceae	Rothia	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Actinobacteria	Streptomycetaceae	Streptomyces	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Bacteroidetes	SB-5	SB-5_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Firmicutes	Erysipelotrichaceae	Solobacterium	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Nanoarchaea	Woesearchaeia_fa	Woesearchaeia_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Berkelbacteria_fa	Berkelbacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Adlerbacteria_fa	Ca.Adlerbacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Azambacteria_fa	Ca.Azambacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Kaiserbacteria_fa	Ca.Kaiserbacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Moranbacteria_fa	Ca.Moranbacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Nomurabacteria_fa	Ca.Nomurabacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Staskawiczbacteria_fa	Ca.Staskawiczbacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Woesebacteria_fa	Ca.Woesebacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Ca.Yanofskybacteria_fa	Ca.Yanofskybacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	CPR2_fa	CPR2_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	Parcubacteria_fa	Parcubacteria_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Patescibacteria	WWE3_fa	WWE3_ge	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Planctomycetes	Pirellulaceae	uncultured	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Proteobacteria	Burkholderiaceae	Acidovorax	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Proteobacteria	Burkholderiaceae	Ralstonia	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Proteobacteria	Coxiellaceae	Coxiella	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Proteobacteria	Hyphomonadaceae	Hirschia	unknown	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Proteobacteria	Nitrosomonadaceae	uncultured	NH ₃ oxidizer	CH ₄ balance in the presence of methanotroph	CH ₄ producing cohort or methanotroph inhibitor
Bacteroidetes	Weeksellaceae	Cloacibacterium	unknown	CH ₄ consumption without CH ₄ consumer	CH ₄ consumer or their cohort
Proteobacteria	Burkholderiaceae	Tepidimonas	unknown	CH ₄ consumption without CH ₄ consumer	CH ₄ consumer or their cohort
Firmicutes	Clostridiaceae_1	Fonticella	unknown	CH ₄ consumption without CH ₄ consumer	CH ₄ consumer or their cohort
Proteobacteria	Xanthomonadaceae	Pseudoxanthomonas	unknown	CH ₄ consumption without CH ₄ consumer	CH ₄ consumer or their cohort
Actinobacteria	Corynebacteriaceae	Lawsonella	unknown	CH ₄ consumption without CH ₄ consumer	CH ₄ consumer or their cohort
Proteobacteria	Beijerinckiaceae	Beijerinckiaceae_ge	unknown	CH ₄ consumption without CH ₄ consumer	CH ₄ consumer or their cohort