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2 Title: Niche partitioning of methane-oxidizing bacteria along the
3 oxygen-methane counter gradient of stratified lakes

4 Running title: Methanotrophs in the oxygen-methane counter gradient

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13 Supplementary Methods

14 *Analysis of methane by gas chromatography*

15 For methane measurements 20 ml lake water was filled into sealed 40 ml serum bottles purged with
16 N₂ gas, containing 4 g of NaOH pellets (≥98%, Sigma-Aldrich, Darmstadt, Germany) to stop biological
17 activity. A gas chromatograph (Agilent 6890N, Agilent Technologies, Santa Clara, CA, USA) with
18 Carboxen 1010 column (30 m x 0.53 mm x 30 µm, Supelco, Bellefonte, PA, USA) equipped with a flame
19 ionization detector and 1 ml injection volume was used to quantify headspace methane
20 concentrations. Column temperature was ramped from 50°C (4 min) to 140°C within 4 min. Calibration
21 standards were prepared by diluting pure methane (99.5%) with nitrogen gas. Triplicate dilutions were
22 measured to obtain a calibration curve, which was checked daily prior to measurements with a
23 commercial standard (100 ppm). The relative method standard deviation for calibration with our set-
24 up is 6%. Samples above 10 000 ppm (outside linear range) were diluted prior to measurement. In
25 control experiments, we found that 4 g of NaOH pellets release 32.6 nmol of methane on average,
26 which we subtracted from the total methane in the bottle. Methane concentrations in water were
27 calculated according to Wiesenburg and Guinasso (1979) [1].

28 *Potential methane oxidation rates*

29 We used a modified protocol [2, 3] to measure potential methane oxidation rates. Autoclaved 60 ml
30 serum vials were filled with lake water, closed with butyl stoppers and stored cool and dark. On the
31 same day, the water was purged with N₂ gas to remove the methane. To supply non-limiting O₂
32 concentrations (~50 µmol L⁻¹) a 10 ml subsample from each vial was equilibrated with air by gentle
33 shaking in a syringe half-filled with air and then added back to the incubation. A non-limiting ¹³C-CH₄
34 concentration (final concentration of ~80 µmol L⁻¹) was supplied by adding about 1 ml sterile anoxic
35 Nanopure-purified water (Nanopure, Thermo Fischer Scientific) saturated with ¹³C-CH₄ (99 at%,
36 Campro Scientific, Berlin, Germany). Nanopure with ¹³C-CH₄ was prepared in a serum vial (120 ml) with
37 100 ml boiled, N₂-purged and autoclaved Nanopure by adding 60 ml of labelled gas into the headspace.
38 Five subsamples of each water sample were transferred into 6 ml Exetainers (Labco, Lampeter, UK)

without headspace and incubated close to *in situ* temperature of the respective lake (Rotsee 11°C, Greifensee 13°C, Lake Zug 5°C, Lake Lugano 11°C) in the dark on a shaker. The subsamples were killed sequentially with 100 µl ZnCl₂ 50% w/v after 0, 3, 9, 21 and 44 hours to measure ¹³C-CO₂ production over time. Isotopic ratios of CO₂ were measured with GC-IRMS (IsoPrime, Micromass, Wilmslow, UK), equipped with a column (2.5 m x 1/8 inch x 2 mm, Restek, Bellefonte, PA, USA) packed with HayeSepp Q and a 60 - 80 mesh. The oven temperature was set to 100°C and the valve temperature to 80°C. Rates were calculated with linear regression from ¹³C-CO₂ production. 100 µl of H₃PO₄ were added to a 3.7 ml Exetainer and the headspace was exchanged with He with a final pressure of about 700 mbar. 1.5 ml of sample were transferred into the 3.7 ml Exetainer and incubated for at least one hour to allow the CO₂ to move into the headspace. The amount of produced ¹³C-CO₂ was calculated from δ¹³C-CO₂ (‰) and the concentration of DIC in the sample. ETH Zurich LSI/SIL Carrara marmor with a δ¹³C of 2.1‰ was used as a standard.

Library preparation and sequencing

To prepare the 16S rRNA gene/rRNA and *pmoA* DNA/mRNA libraries for Illumina MiSeq sequencing a two-step PCR using NEBNext Q5® Hot Start HiFi PCR Master Mix (New England BioLabs, Hitchin, UK) was performed. For the first step tailed forward and reverse primers (0.3µM; 16S rRNA gene/rRNA [4]: S-D-Bact-0341-b-S-17, D-Bact-0785-a-A-21, *pmoA* DNA/mRNA [5]: 189f, mb661) were used. The first PCR was performed in triplicate: initial denaturation 30 s, 98 °C, denaturation 10 s, 98 °C, annealing 35 s, 54 °C, extension 35 s, 65 °C and final extension 5 min, 65 °C; 17 cycles for 16S rRNA gene/rRNA and 25 cycles for *pmoA* DNA/mRNA. Products of replicates were pooled and cleaned with Agencourt AMPure XP kit (Beckman coulter, Indianapolis, IN, USA), in a second PCR (8 cycles, annealing at 55°C) Illumina barcodes and adapters were attached with Nextera XT Index Kit set A and D (Illumina Inc., San Diego, CA, USA) and cleaned again. Libraries were quantified with Qubit DNA BR reagents (Thermo Fischer Scientific) on a microplate reader (Spark M10, Tecan, Männedorf, Switzerland). Pooled libraries were inspected with Tape Station 2200 (Agilent Genomics, USA). We sequenced twice on an Illumina

MiSeq platform (Illumina Inc.) with 600-cycle MiSeq reagent kit v3 (Illumina Inc.) and 10% PhiX at the Genetic Diversity Centre (GDC) of ETH Zurich to obtain at least 10000 reads per sample.

Sequence analyses

For analysis of 16S rRNA and 16S rRNA gene sequences primers and adapters were removed. To infer amplicon sequence variants (ASVs) we used DADA2 [6], which was recommended to replace OTU (operational taxonomic unit) based approaches [7, 8]. DADA2 resolves exact sequence variants without grouping sequences into OTUs based on a similarity cut-off and strongly reduces spurious sequences based on error rates. With DADA2 (version 1.6.0) [6] in R (3.4.2) [9] forward and reverse reads were trimmed to 270 nucleotides (nt) and 210 nt, respectively. Reads were truncated when reaching a quality score of two and removed if reads contained ambiguous bases or an expected error rate above three. Then error rates were calculated, filtered reads were dereplicated and the dada denoising algorithm was applied to infer exact sequence variants. Forward and reverse reads were merged and chimera removed. 16S rRNA ASVs were classified based on the SILVA database (v132) [10]. With phyloseq package (1.24.2) [11] in R (3.5.0), sequences affiliated to mitochondria and chloroplasts were removed and read counts transformed to relative abundance. Further analyses included ASVs affiliated with known methanotrophic groups (Order *Methylococcales*, Genus *Methylocystis*, Genus *Candidatus Methyloirabilis*) based on the SILVA SSU reference database [10] that reached >0.2% relative abundance in at least two samples from the respective lake. Within the order *Methyloacidiphilales* (*Verrucomicrobia*) no closely related sequences to MOB were detected.

For *pmoA* gene and transcript analysis primers were trimmed prior to the DADA2 workflow [6]. Using DADA2 (version 1.6.0) in R (3.4.2) [9] *pmoA* DNA/mRNA reverse reads were trimmed to 235 nt. Read quality control and inferring amplicon sequence variants was done using the same configurations as for 16S rRNA analysis (see above). Forward and reverse reads were merged, sequences with desired length (471 nt) kept and chimera removed. Sequence variants were transformed to amino acid sequences (aaASV) in MEGA7 [12]. Sample reads were transformed to relative abundance and only aaASVs reaching >2% at least once in the respective lake were retained.

Canonical correspondence analysis (CCA) with available environmental variables

An additional CCA (Supplementary Figure S3) was performed based on the available environmental variables (temperature, pH, salinity, oxygen, methane, sulfate, phosphor, nitrate and ammonium concentration). To find a parsimonious set of variables forward selection based on the MOB 16S rRNA gene data (ordiR2step, vegan 2.5.2, R) and removal of highly correlated variables (variance inflation factor >10) was conducted. This resulted in a subset of environmental variables (temperature, oxygen, methane, nitrate and sulfate concentration). Nitrite was not included in the analysis, because it was not measured in Lake Lugano. First pH was removed as it was not significant ($p > 0.05$). Ammonium had the highest variance inflation factor (vif) and was therefore removed from the data set (collinear with methane). The results of ordiR2step were used to select nitrate and to remove salinity due to its collinearity with nitrate, and to select temperature and sulfate, but to remove phosphor because of its high vif (collinear with temperature and sulfate). The reduced set of environmental variables was also applied to the *pmoA* mRNA data. In both CCAs (16S rRNA gene and *pmoA* mRNA) all five axes were significant, and the first three axes are shown in the Supplementary Figure S3. The environmental variables were centered and scaled (values <LOQ set to zero) prior to analysis.

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