The ISME Journal - Supplementary Methods

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

Running title: Methanotrophs in the oxygen-methane counter gradient

Magdalena J. Mayr[1,2], Matthias Zimmermann[1,2], Carole Guggenheim[2], Andreas Brand[1,2], Helmut Bürgmann[1]

[1]Department of Surface Waters—Research and Management, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland

[2]Institute of Biogeochemistry and Pollutant Dynamics, Department of Environmental Systems Science, ETH Zurich, Swiss Federal Institute of Technology, Zurich, Switzerland

*Corresponding author: Magdalena J. Mayr, Seestrasse 79, 6047 Kastanienbaum, +41 58 765 2142, magdalena.mayr@eawag.ch

Supplementary Methods

Analysis of methane by gas chromatography

For methane measurements 20 ml lake water was filled into sealed 40 ml serum bottles purged with N_2 gas, containing 4 g of NaOH pellets (≥98%, Sigma-Aldrich, Darmstadt, Germany) to stop biological activity. A gas chromatograph (Agilent 6890N, Agilent Technologies, Santa Clara, CA, USA) with Carboxen 1010 column (30 m x 0.53 mm x 30 µm, Supelco, Bellefonte, PA, USA) equipped with a flame ionization detector and 1 ml injection volume was used to quantify headspace methane concentrations. Column temperature was ramped from 50°C (4 min) to 140°C within 4 min. Calibration standards were prepared by diluting pure methane (99.5%) with nitrogen gas. Triplicate dilutions were measured to obtain a calibration curve, which was checked daily prior to measurements with a commercial standard (100 ppm). The relative method standard deviation for calibration with our set-up is 6%. Samples above 10 000 ppm (outside linear range) were diluted prior to measurement. In control experiments, we found that 4 g of NaOH pellets release 32.6 nmol of methane on average, which we subtracted from the total methane in the bottle. Methane concentrations in water were calculated according to Wiesenburg and Guinasso (1979) [1].

Potential methane oxidation rates

We used a modified protocol [2, 3] to measure potential methane oxidation rates. Autoclaved 60 ml serum vials were filled with lake water, closed with butyl stoppers and stored cool and dark. On the same day, the water was purged with N_2 gas to remove the methane. To supply non-limiting O_2 concentrations (~50 µmol L^{-1}) a 10 ml subsample from each vial was equilibrated with air by gentle shaking in a syringe half-filled with air and then added back to the incubation. A non-limiting ^{13}C-CH_4 concentration (final concentration of ~80 µmol L^{-1}) was supplied by adding about 1 ml sterile anoxic Nanopure-purified water (Nanopure, Thermo Fischer Scientific) saturated with ^{13}C-CH_4 (99 at%, Campro Scientific, Berlin, Germany). Nanopure with ^{13}C-CH_4 was prepared in a serum vial (120 ml) with 100 ml boiled, N_2-purged and autoclaved Nanopure by adding 60 ml of labelled gas into the headspace. 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 $ZnCl_2$ 50% w/v after 0, 3, 9, 21 and 44 hours to measure ^{13}C-CO_2 production over time. Isotopic ratios of CO_2 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 ^{13}C-CO_2 production. 100 µl of H_3PO_4 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 CO_2 to move into the headspace. The amount of produced ^{13}C-CO_2 was calculated from $\delta^{13}C$-CO_2 (‰) and the concentration of DIC in the sample. ETH Zurich LSI/SIL Carrara marmor with a $\delta^{13}C$ 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* Methylomirabilis) 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 *Methylacidiphilales* (*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 (aaOTU) in MEGA7 [12]. Sample reads were transformed to relative abundance and only aaOTUs 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 *pmo*A 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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The ISME Journal – Supplementary Figures

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

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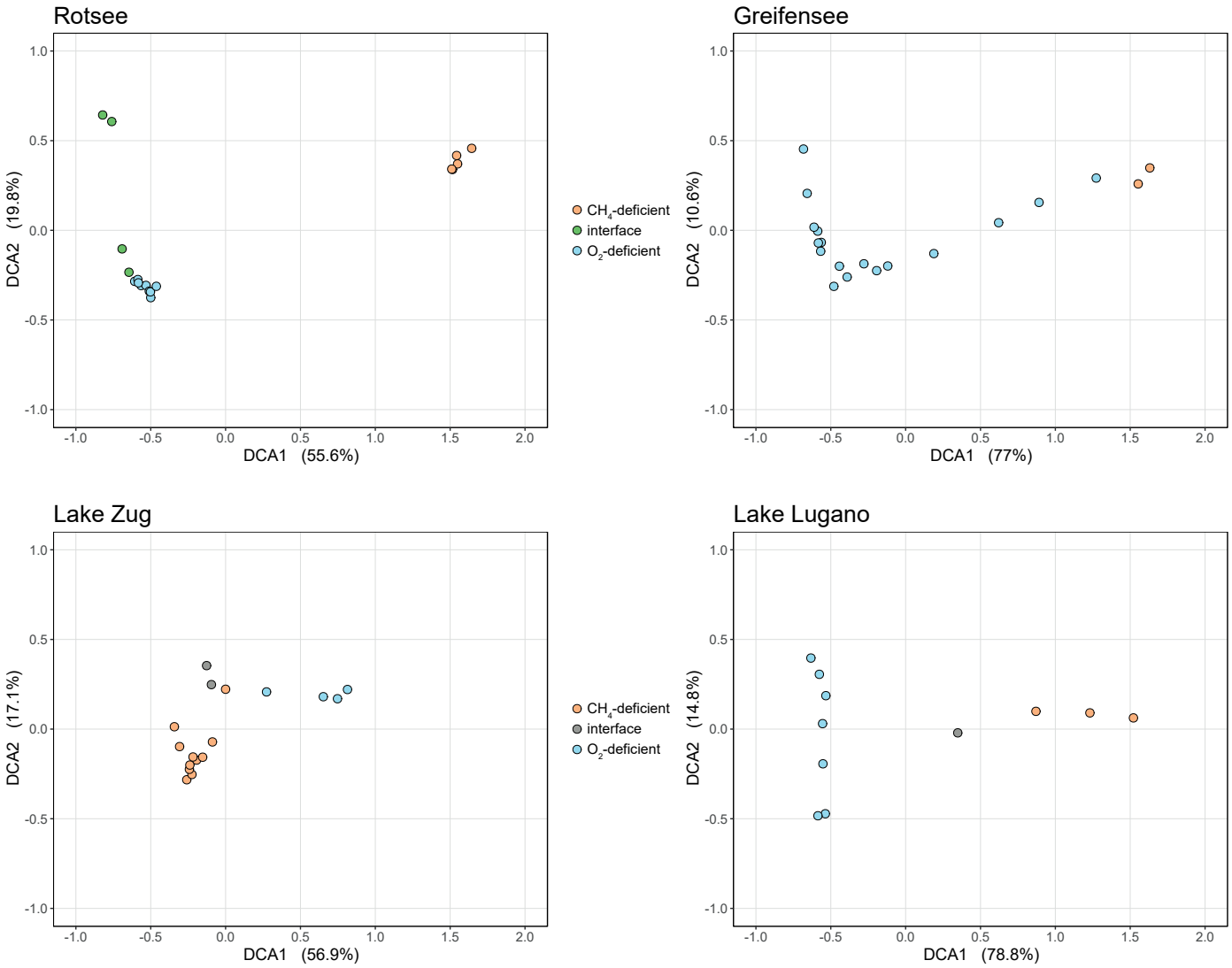
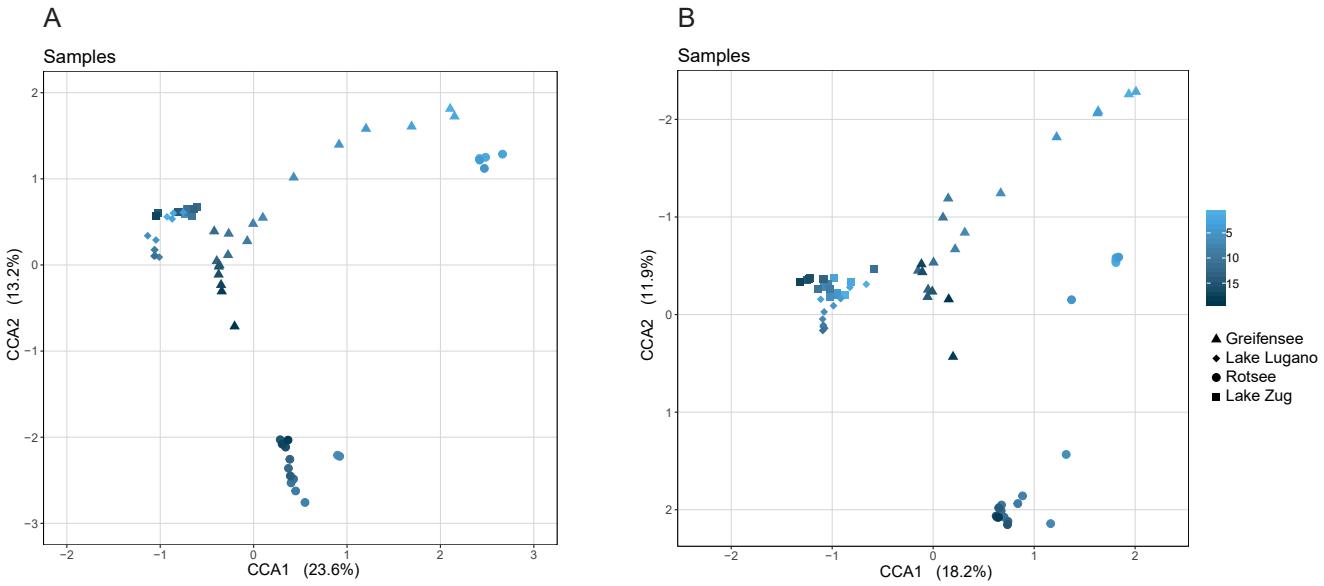
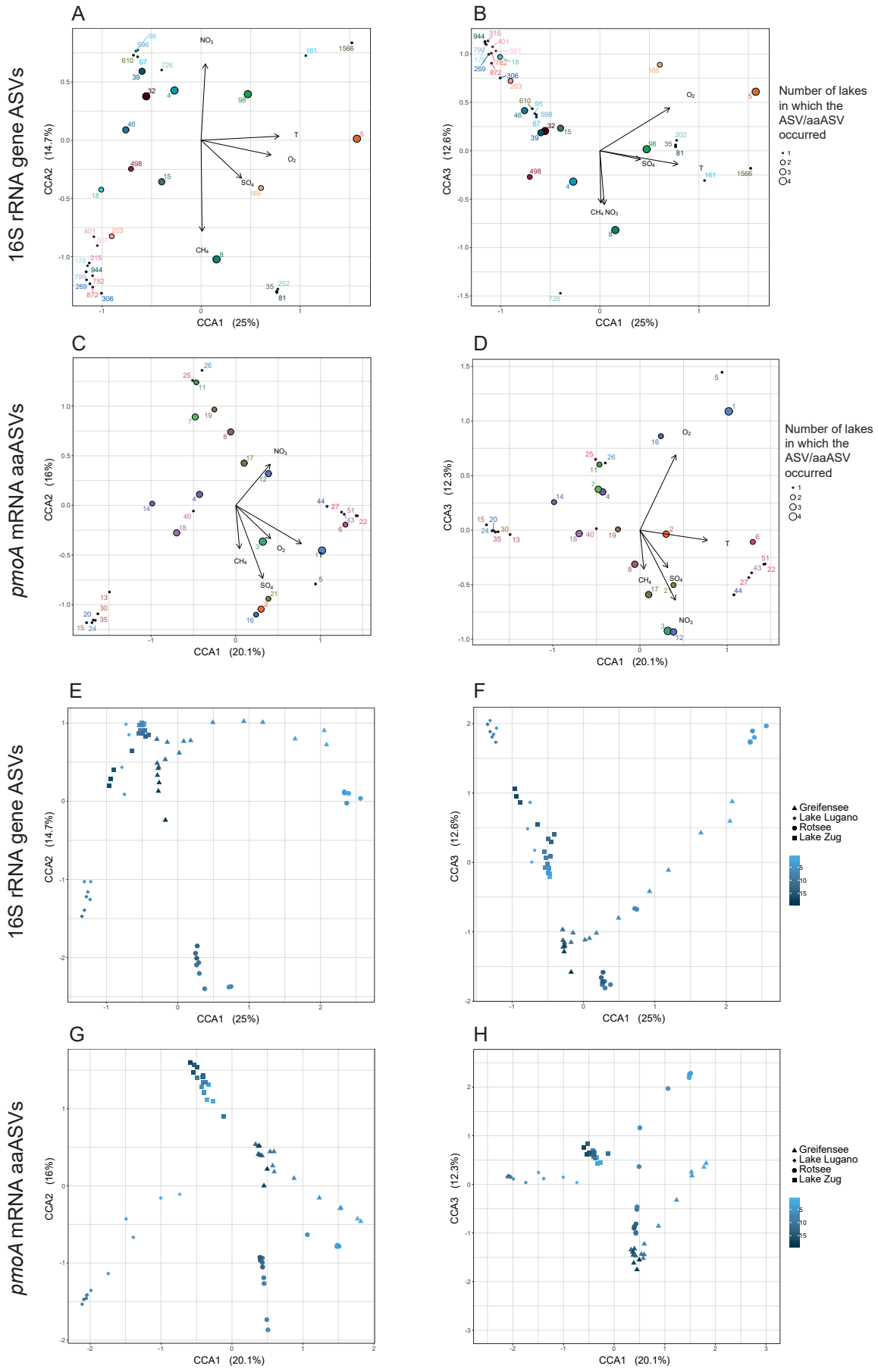


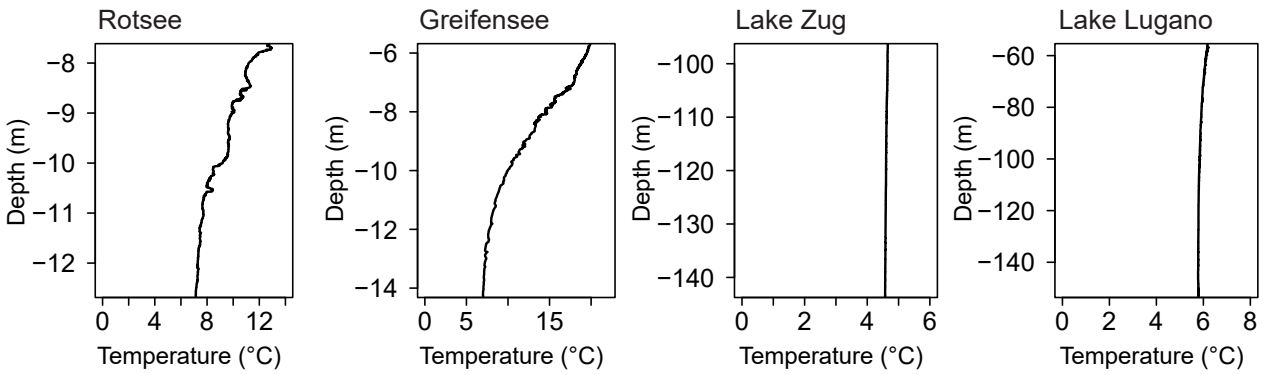
Supplementary Figure S1 Detrended correspondence analysis (DCA) calculated based on a Bray-Curtis dissimilarity matrix using relative abundances of MOB 16S rRNA gene ASVs compared to all MOB ASVs (sample sum=1). Samples are shown separately for each lake and were colored according to their corresponding niche (see Figure 1 and 2), which are based on oxygen and methane availability. Please note that in Greifensee only methane-deficient and oxygen-deficient were captured.



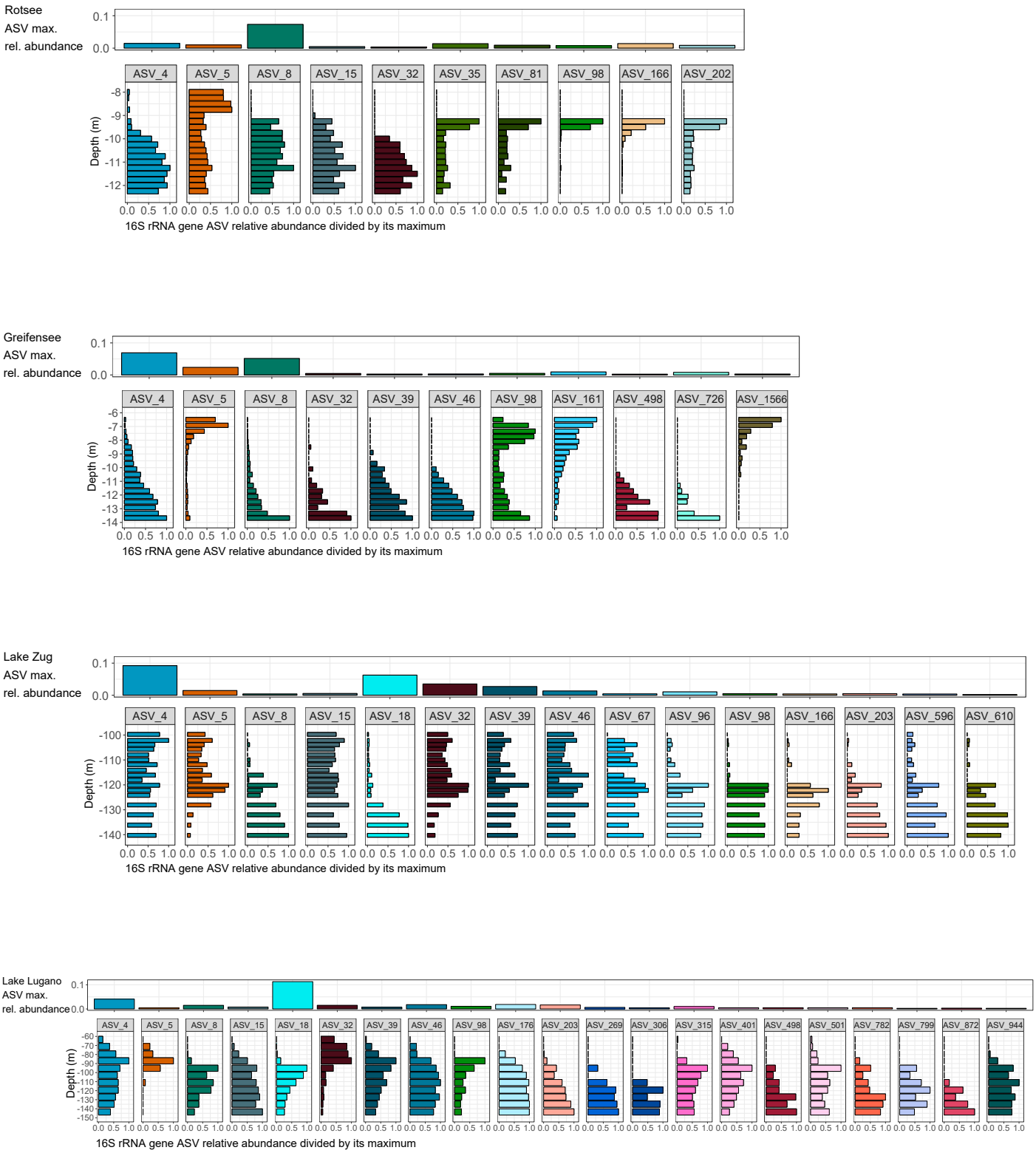
Supplementary Figure S2 Canonical correspondence analysis (CCA) calculated based on a Chi-square dissimilarity matrix using relative abundances compared to all MOB sequences in a sample (sample sum=1) and standardized physicochemical parameters (T=Temperature). **A**) samples scores of 16S rRNA gene ASVs **B**) samples scores of *pmoA* mRNA aaASVs. The color gradient corresponds to ranked depths of each lake, from the shallowest (light blue) to the deepest (dark blue) depth.



Supplementary Figure S3 Canonical correspondence analysis (CCA) calculated based on a Chi-square dissimilarity matrix using relative abundances compared to all MOB sequences in a sample (sample sum=1) and selected physicochemical variables (T=temperature). The first three axes are shown. Taxa scores of 16S rRNA gene ASVs are shown in A and B, corresponding sample scores in E and F. Taxa scores of *pmoA* mRNA aaASVs are shown in C and D and corresponding sample scores in G and H. A-D colors are taxon specific and the dot size shows the number lakes in which the the ASV/aaASV occurred. E-H the color gradient corresponds to ranked depths of each lake form the shallowest (light blue) to the deepest (dark blue) depth.

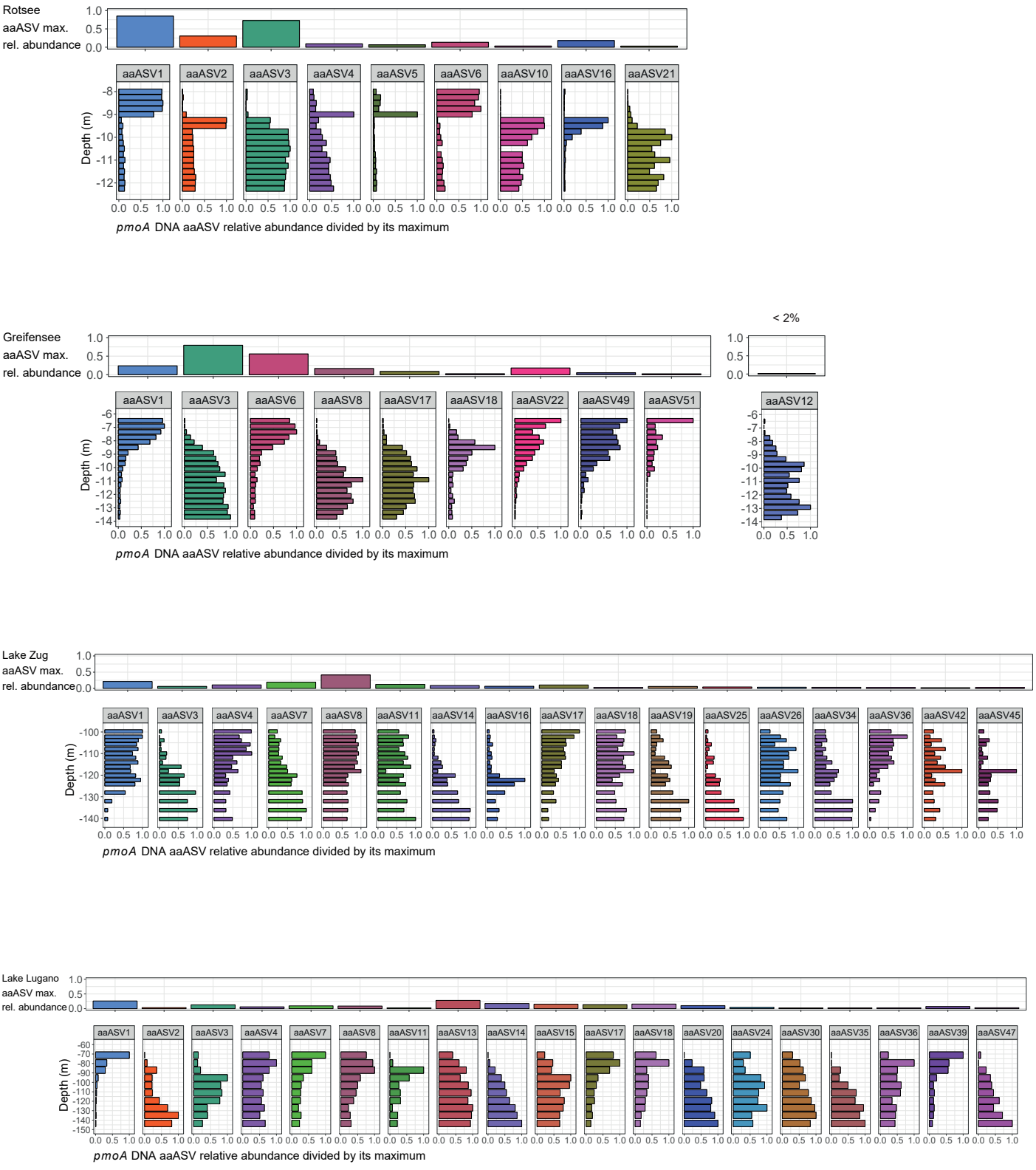


Supplementary Figure S4 Vertical profiles of temperature in Rotsee, Greifensee, Lake Zug and Lake Lugano across the oxygen-methane counter gradient.

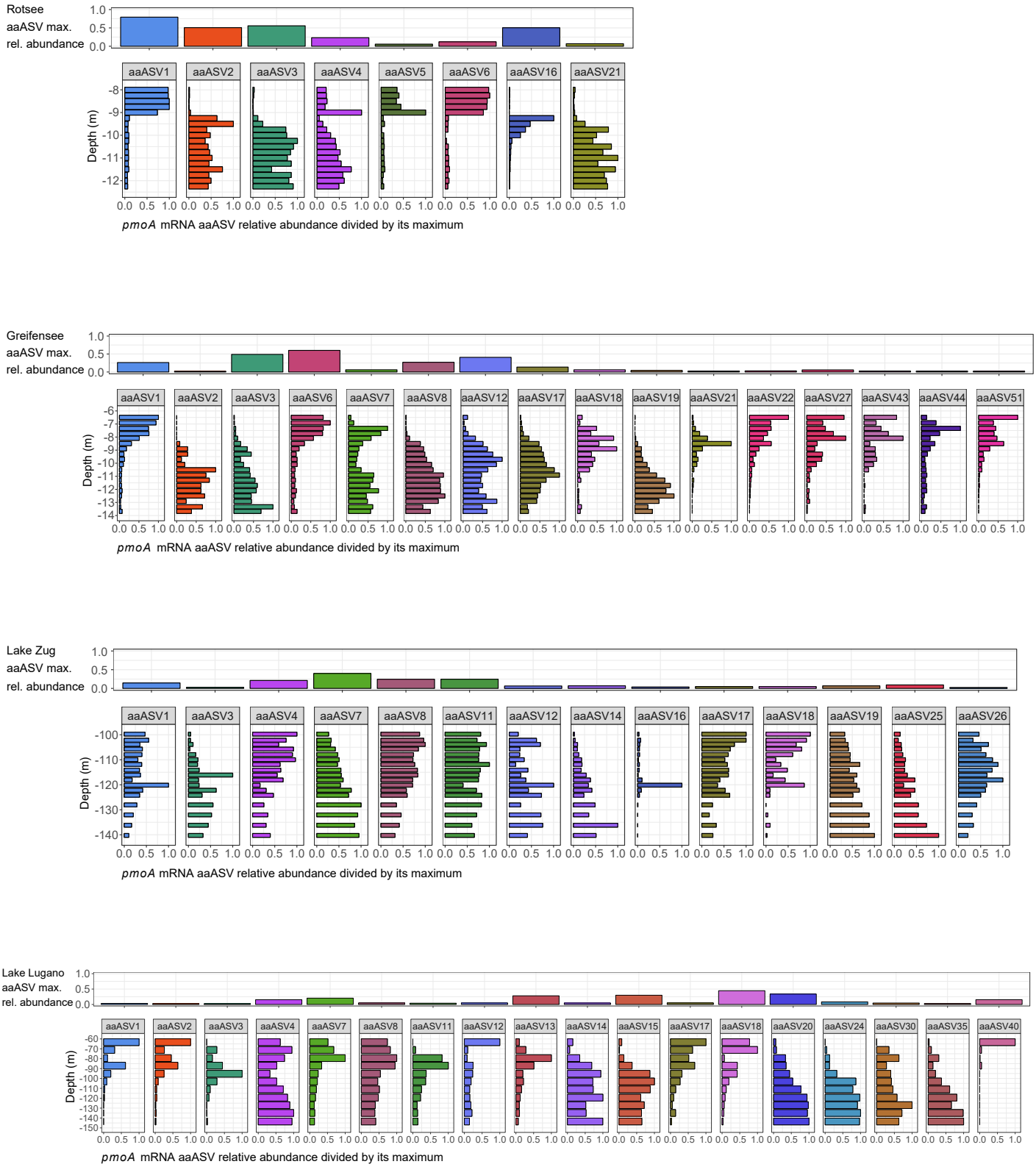


Supplementary Figure S5 Vertical distribution patterns of MOB 16S rRNA gene ASVs in Rotsee, Greifensee, Lake Zug and Lake Lugano along the oxygen-methane counter gradient. On top the maximum relative abundance compared to all bacterial 16S rRNA gene sequences for each ASV is shown. Colors are ASV specific. ASVs are sorted according to their numbering.

Supplementary Figure S6 Vertical distribution patterns of MOB 16S rRNA ASVs in Rotsee, Greifensee, Lake Zug and Lake Lugano along the oxygen-methane counter gradient. On top the maximum relative abundance compared to all bacterial 16S rRNA sequences for each ASV is shown. Colors are ASV specific. ASVs in grey do not have a corresponding 16S rRNA gene ASV with applied filtering thresholds. ASVs are sorted according to their numbering.



Supplementary Figure S7 Vertical distribution patterns of *pmoA* DNA aaASVs in Rotsee, Greifensee, Lake Zug and Lake Lugano along the oxygen-methane counter gradient. On top the maximum relative abundance compared to all *pmoA* DNA sequences for each aaASV is shown. Colors are aaASV specific. Note that *pmoA* DNA from the shallowest depth (63 m) of Lake Lugano could not be amplified. aaASVs are sorted according to their numbering.



Supplementary Figure S8 Vertical distribution patterns of *pmoA* mRNA aaASVs in Rotsee, Greifensee, Lake Zug and Lake Lugano along the oxygen-methane counter gradient. On top the maximum relative abundance compared to all *pmoA* mRNA sequences for each aaASV is shown. Colors are aaASV specific. aaASVs are sorted according to their numbering.

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[2]Institute of Biogeochemistry and Pollutant Dynamics, Department of Environmental Systems Science, ETH Zurich, Swiss Federal Institute of Technology, Zurich, Switzerland

*Corresponding author: Magdalena J. Mayr, Seestrasse 79, 6047 Kastanienbaum, +41 58 765 2142, magdalena.mayr@eawag.ch

Supplementary Table 1 Summary of environmental parameters measured in Rotsee, Greifensee, Lake Zug and Lake Lugano in 2015 for each depth. Measurements below the limit of quantification (LOQ) were indicated by "<" and the value of the LOQ. LOQ of methane is the equivalent dissolved concentration of the lowest calibration concentration; LOQ of ammonium is equivalent to the lowest calibration concentration; LOQ of nitrite and nitrate was derived from the FIA instrument baseline. In case of oxygen the limit of detection is given (<20 nmol L^{-1}). NA are missing values. T = Temperature, NPOC = non-purgeable organic carbon.

Rotsee depth (m)	T (°C)	Methane (µmol L^{-1})	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Sulfate (µmol L^{-1})	NPOC (mg L^{-1})	Oxygen (µmol L^{-1})
8.00	10.93	1.0	2	<0.4	<4	120	3	390.43
8.25	10.90	1.1	<0.5	<0.4	<4	121	NA	390.09
8.50	10.70	<0.05	<0.5	<0.4	<4	121	NA	343.87
8.75	10.08	0.1	<0.5	<0.4	<4	122	NA	306.52
9.00	9.87	0.6	3	<0.4	<4	121	2	282.80
9.25	9.61	2.1	18	<0.4	<4	124	3	261.89
9.50	9.76	7.2	20	<0.4	<4	125	3	241.02
9.75	9.62	41.7	63	<0.4	<4	120	3	39.85
10.00	9.21	113.1	77	<0.4	<4	105	3	4.88
10.25	8.40	122.9	84	<0.4	<4	100	2	0.13
10.50	8.44	129.4	88	<0.4	<4	100	2	0.41
10.75	7.65	154.4	93	<0.4	<4	91	3	0.38
11.00	7.71	141.7	84	<0.4	<4	94	3	0.03
11.25	7.42	160.2	85	<0.4	<4	97	3	0.03
11.50	7.47	182.4	NA	NA	NA	NA	3	0.03
11.75	7.35	199.2	NA	NA	NA	NA	2	0.03
12.00	7.30	207.1	NA	NA	NA	NA	2	0.03
12.25	7.27	227.7	NA	NA	NA	NA	2	0.06

Greifensee depth (m)	T (°C)	Methane (µmol L^{-1})	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Sulfate (µmol L^{-1})	NPOC (mg L^{-1})	Oxygen (µmol L^{-1})
6.5	18.48	<0.05	12	4	55	125	3	47.32
6.9	18.00	<0.05	12	6	51	124	3	2.00
7.3	16.61	0.7	10	7	50	123	3	<0.02
7.7	15.29	1.0	8	6	49	121	3	<0.02
8.1	14.28	3.7	7	5	49	122	3	<0.02
8.5	13.32	3.5	5	4	53	121	3	<0.02
8.9	12.55	7.8	5	3	51	121	3	<0.02
9.3	11.37	9.4	6	3	51	121	3	<0.02
9.7	10.39	8.3	3	3	54	121	3	<0.02
10.1	9.80	8.4	4	3	57	121	3	<0.02
10.5	9.12	5.5	3	3	61	122	3	<0.02
10.9	8.61	6.3	4	2	60	122	3	<0.02
11.3	8.32	7.2	2	3	62	122	3	<0.02
11.7	7.97	12.5	4	3	60	123	3	<0.02
12.1	7.65	11.5	2	3	61	121	NA	<0.02
12.5	7.37	11.1	2	3	62	122	3	<0.02
12.9	7.26	14.3	4	3	62	122	3	<0.02
13.3	7.15	13.3	6	3	66	122	3	<0.02
13.7	7.12	14.2	<0.5	3	67	122	3	<0.02

Lake Zug depth (m)	T (°C)	Methane (µmol L^{-1})	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Sulfate (µmol L^{-1})	NPOC (mg L^{-1})	Oxygen (µmol L^{-1})
100	4.65	<0.05	1	<0.4	22	55	2	37.03
102	4.64	<0.05	1	<0.4	21	56	2	32.41
104	4.64	<0.05	1	<0.4	21	55	2	23.85
106	4.63	<0.05	1	<0.4	21	55	2	24.78
108	4.62	<0.05	1	<0.4	21	54	2	21.06
110	4.62	<0.05	1	<0.4	21	54	2	15.59
112	4.61	<0.05	1	<0.4	20	54	2	15.03
114	4.61	<0.05	1	<0.4	20	54	2	12.41
116	4.60	<0.05	1	<0.4	20	55	3	8.78
118	4.60	<0.05	1	<0.4	21	54	3	3.66
120	4.60	<0.05	1	<0.4	19	54	2	2.38
122	4.60	<0.05	1	0.6	19	54	2	0.06
124	4.59	<0.05	1	0.5	18	55	2	<0.02
128	4.59	0.1	<0.5	0.4	16	54	2	0.03
132	4.58	0.8	1	<0.4	14	54	2	0.03
136	4.58	3.2	2	<0.4	14	54	2	<0.02
140	4.58	4.2	3	<0.4	11	54	3	<0.02

Lake Lugano depth (m)	T (°C)	Methane (µmol L^{-1})	Ammonium (µmol L^{-1})	Nitrite (µmol L^{-1})	Nitrate (µmol L^{-1})	Sulfate (µmol L^{-1})	NPOC (mg L^{-1})	Oxygen (µmol L^{-1})
63	6.08	0.3	<0.5	NA	26	117	2	102.04
71	5.99	0.4	<0.5	NA	22	113	2	55.88
79	5.93	0.3	<0.5	NA	17	116	2	23.72
87	5.89	0.2	1	NA	12	116	2	0.03
95	5.86	4.0	3	NA	<4	116	2	0.03
103	5.83	9.5	5	NA	<4	115	1	0.03
111	5.81	14.4	7	NA	<4	113	2	<0.02
119	5.79	23.5	8	NA	<4	116	2	<0.02
127	5.79	31.3	10	NA	<4	112	2	<0.02
135	5.78	36.2	12	NA	<4	110	2	<0.02
143	5.78	39.2	13	NA	<4	109	2	0.03