The following supplements accompany the article

Gammaproteobacterial methanotrophs dominate methanotrophy in aerobic and anaerobic layers of boreal lake waters

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SUPPLEMENT 1

Supplemental Methods

Sampling

The vertical O_2 and temperature profiles were measured at 20 and 50 cm intervals in Mekkojärvi and Alinen-Mustajärvi, respectively, using a YSI model 55 (Yellow Springs Instruments). Subsequently, three specific sampling depths were determined for Mekkojärvi: 1.2 m, 1.6 m, and 2.8 m, representing the epi-, meta-, and hypolimnion, respectively.

The water for analysis of other background variables was collected using a Limnos water sampler (Limnos.pl, Poland). The vertical profiles of the oxidation-reduction potential (ORP) and pH were measured in the field from inside the sampler at 50-cm depth intervals in Alinen-Mustajärvi, while ORP was measured at 20-80 cm intervals and pH from the epi-, meta-, and hypolimnion in Mekkojärvi. The ORP and pH measurements were performed using a pH 3110 (WTW) unit and the SenTix ORP and SenTix 41 pH electrodes (WTW). Furthermore, 30 mL water samples for the measurement of CH_4 and CO_2 concentrations as well as $^{13}C/^{12}C$ of CH_4 (only in Alinen-Mustajärvi) from the epi-, meta-, and hypolimnion in Mekkojärvi (n = 2 per depth) and at 50 cm intervals in Alinen-Mustajärvi (n = 1 per depth) were taken into gas-tight 60 mL polypropylene syringes that were kept on ice prior to headspace equilibration and injection of syringe headspace into pre-evacuated gas-tight glass vials (Labco, 12 mL) (Ojala et al. 2011). Similarly, samples for $^{13}C/^{12}C$ of dissolved inorganic C (DIC) were taken from the same depths by injecting 5 mL of water into glass vials (Labco, 12 mL) with a helium atmosphere and 0.3 mL (Mekkojärvi) or 0.15 mL (Alinen-Mustajärvi) of H_3PO_4 and stored upside down at RT before analyses. The samples for dissolved sulfide and DNA and RNA-based amplicon sequencing analyses were collected from epi-, meta and hypolimnion in Mekkojärvi and at 50 – 150 cm depth intervals in Alinen-Mustajärvi, whereas samples for shotgun metagenomic analyses were collected at 20-100 cm depth intervals in Alinen-Mustajärvi. Sulfide samples were collected by overflowing water (2-3 times the vial volume) into empty gas-tight glass vials (Labco, 12 mL), which were stored on ice before analyses (within 1 h from collection). For DNA and RNA-based amplicon sequencing, pre-filtered (in the field, through 50 µm plankton net) water was stored in 100 mL vials on ice before the subsequent steps (within 1 - 3 h), that were filtering of the biomass on 0.22 µm filters (Disposable filter, MO BIO Laboratories) and freezing of the filters at -80°C for Mekkojärvi samples and freezing of the water samples at -20°C for Alinen-Mustajärvi samples. Water samples for shotgun metagenomic analyses in Alinen-Mustajärvi were filtered on 0.2 µm polycarbonate filters that were stored at -80°C. In addition, pre-filtered (50 µm) samples were collected from epi-, meta- and hypolimnion in

Mekkojärvi for determination of total dissolved Fe and Mn, while in Alinen-Mustajärvi, samples for analyses of inorganic nutrients (NO_3^-+NO_2^-, NH_4^+, PO_4^{2-}), SO_4^{2-}, total N, total P, dissolved organic carbon (DOC) and particulate organic carbon (POC) were collected at 50-150 cm depth intervals. The samples were stored on ice before filtering through 0.7 μm filter (GF-F, Whatman) for Fe, Mn, inorganic nutrients, SO_4^{2-}, and DOC analyses. The biomass retained on the filter was used in POC analyses.

Incubation samples in Mekkojärvi were collected in 600 mL hard plastic vials with gas-tight caps from the epi-, meta-, and hypolimnion (epi-, n = 3; meta-, n = 9, and hypolimnion, n = 9) using a sampler, which was a combination of a rubber hose connected via tri-tap to a 60 mL syringe, thoroughly checked for any leakage of air (Basnet 2014). Before sampling, the bottles were made anoxic, evacuated below atmospheric pressure by He-flushing and vacuuming, and amended with different EAs as stock solutions (NO_3^- and SO_4^{2-}) or as colloidal [Fe^{3+} as $Fe(OH)_3$ colloid (Leibl et al. 1999)] and solid forms [Mn^{4+} as MnO_2 (Lovley & Phillips 1988), organic EA: disodium anthraquinone-2,6-disulfonate]. To consume possible traces of O_2, the bottles were stored in the dark at 4°C for two days prior to the onset of the experiments.

Incubation samples in Alinen-Mustajärvi were collected by overflowing prefiltered (50 μm) water from the hypolimnion (5.5 m depth) into plastic cans (V = 8 L, n = 12). Each sample was concentrated in ~400 mL using tangential flow filtration (Durapore cassette, pore size 0.22 μm; Millipore, USA) and transferred into glass vials (V = 715 mL), which were then closed with caps and rubber septa. The samples were degassed (made anoxic) by vacuuming the headspace for 5 min followed by flushing and the addition of overpressure with N_2 gas. The overpressure was released by avoiding direct contact with the atmosphere by using a needle connected to a tube whose other end was submerged in water.

In vitro determination of potential CH_4 oxidation

The EA experiments of Mekkojärvi were initiated under an N_2-atmosphere in a glove bag. The bottles were injected with 5 mL of either $^{13}CH_4$ (99.9% ^{13}C, see above) or $^{14}CH_4$ (ARC1297 Methane [^{14}C] 50 mCi/mmol, American Radiolabeled Chemicals, Inc.). There were three treatments: 1. CH_4, 2. CH_4 + mixture of inorganic EAs (5 mM NO_3^-, 1 mM SO_4^{2-}, 10 mM Mn^{4+}, and 0.5 mM Fe^{3+}) and 3. CH_4 + organic EA [4 mM (= 1.6 g L^{-1}) disodium anthraquinone-2,6-disulfonate], which were tested at the three depths (i.e. epi-, meta-, and hypolimnion), except for the epilimnion, where only treatment 1 was tested. There were two and one replicates in treatments with $^{13}CH_4$ and $^{14}CH_4$, respectively. Incubations took place in the dark at 10 °C for 21 days. The bottles were positioned upside down, partially submerged in water to prevent air exposure of the caps, and gently shaken once a week during the incubation. The sampling for $^{13}C/^{12}C$ of DIC (4 mL as explained above), concentrations of CH_4 and CO_2 (30 mL as explained above), as well as microbial nucleic acids (100 mL filtered on 0.2 μm filters, as explained above), was done once, on the last day of incubations.

The headspaces of the pre-incubation bottles of the Alinen-Mustajärvi experiment were amended either with nothing (three bottles), with 10 mL of isotopically natural CH_4 (99.95 % purity, INTERGAS, UK) (three bottles), or $^{13}CH_4$ (99.5% purity, 99.9% ^{13}C, Cambridge Isotope Laboratories, Inc., USA) (six bottles). The incubation was carried out in the dark at 7 °C for a total of ~9 months, but the EA and light experiments were initiated already after 6.5 months. Three bottles (one with isotopically natural CH_4 and two with $^{13}CH_4$) were sacrificed already after six days of incubation. The samples for temporal monitoring of CH_4-concentration (0.1 mL) were taken 15 times from the headspace, while those for ^{13}C-DIC (1 mL to 12 mL glass vials containing H_3PO_4, as explained above) and

sulfide (4 mL) were taken six and two times, respectively, from the water phase of the bottles amended with $^{13}CH_4$ or isotopically natural CH_4, during the whole 9-month incubation period. The first measurement of ^{13}C-DIC and sulfide included only the sacrificed samples.

For EA and light experiments in Alinen-Mustajärvi, 4 mL subsamples of concentrated water from one of the pre-incubation bottles amended with isotopically natural CH_4 was injected into gas-tight glass vials (Labco 12 mL) (altogether 63 vials). The vials had been degassed (made anoxic) by repeated cycles of vacuuming/He-flushing, left with a He-overpressure and amended with different EAs as anoxic stock solutions (NO_3^- as $NaNO_3$, SO_4^{2-} as Na_2SO_4) or as amorphous [Fe^{3+} as $Fe(OH)_3$ (Lovley & Phillips 1986)] and solid forms (humic acid sodium salt, Sigma Aldrich H1675-2) (see further). To consume possible traces of O_2, the samples were further incubated underwater in the dark at 4 °C for 4 days. Thereafter, 0.2 mL of either ^{13}C-labeled CH_4 (33 % ^{13}C-label, made by mixing the abovementioned $^{13}CH_4$ and isotopically natural CH_4) or isotopically natural CH_4 was injected into each vial followed by an injection of 1 mL of air into aerobic treatment (treatment 7, next sentence). This initiated the experiments with the following treatments: 1) CH_4, 2) CH_4 + 3 mM Fe^{3+}, 3) CH_4 + 1 g L^{-1} humic acid salt, 4) CH_4 + 1 g L^{-1} humic acid salt + 3 mM Fe^{3+}, 5) CH_4 + 1 mM NO_3^-, 6) CH_4 + 1 mM SO_4^{2-}, 7) CH_4 + O_2, which were incubated underwater in the dark at 6.1 °C, as well as 8) CH_4, which was incubated underwater under white light at 6.1 °C, or 9) under red light at 6.5 °C. There were five and two replicates in treatments with $^{13}CH_4$ and isotopically natural CH_4, respectively. The incubations lasted for 134 days, except for O_2 treatment that lasted for 27 days. Photosynthetically active radiation (PAR), measured using LI-COR model LI-185B and LI-COR quantum sensor, was adjusted to ~0.3 µmol photons m^{-2} s^{-1}. PAR was adjusted by placing the samples in dark blue buckets and shielding them from the light source (MEGAMAN ESL 9W in both treatments) with perforated tinfoil. To produce the red light in treatment 9, the samples in the bucket were further covered with a red plastic membrane. Furthermore, the zone between the tinfoil and the light source in treatment 9 was protected from scattered light using non-perforated tinfoil, which very likely led to the slight increase in T (~0.4 °C) compared to other treatments. Sampling for the ^{13}C-content of CO_2 was done four times during the incubation period by taking 1 mL of headspace gas into an evacuated and He-flushed glass vial (Labco, 12 mL). To avoid O_2 contamination during the EA-experiment, injections were always done submerged in water using He-flushed syringes and needles.

Concentration analyses

Dissolved sulfide was measured with the LCK 653 kit (Hach) using Dr. Lange Lasa 100 – spectrophotometer (Hach Lange). Nutrients, DOC, POC, Fe, Mn, and SO_4^{2-} were measured using standard methods (Finnish Standards Association, www.sfs.fi). Briefly, POC was measured from water samples filtered through a pre-ignited glass fiber filter (GF-F, Whatman) using a high-temperature combustion method (Salonen 1979). The DOC concentration was analyzed from the filtrates (<0.7 µm) with a SHIMADZU TOC-5000A Total Organic Carbon Analyzer. A flow injection analyzer (QuikChem®8000, Lachat Instruments) was used to measure PO_4^{2-} (Murphy & Riley 1962), NH_4^+ (Solorzano 1969), and NO_3^-+NO_2^- (Wood et al. 1967) from the filtrates, whereas the total P and N were determined from the un-filtered samples. Furthermore, SO_4^{2-}, as well as the total dissolved Fe and Mn, were determined from the filtrates using the DIONEX 2000i ion chromatography system, a Perkin Elmer Lambda 12 UV/VIS Spectrometer, and a Perkin Elmer AAnalyst 300 atomic absorption spectrophotometer, respectively.

Isotopic calculations

Water column isotope results are expressed as

$$\delta^{13}C = \left(\frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{standard}} - 1 \right) \times 1000 ,$$

where the standard material is Vienna Pee Dee Belemnite (VPDB). Furthermore, the isotopic analysis results were also converted into the fractional abundance of ^{13}C in CO_2 or DIC,

$$^{13}F = \frac{^{13}C}{(^{13}C + ^{12}C)} ,$$

which was used in the calculations of excess concentrations of ^{13}C-CO_2 or ^{13}C-DIC for each time point during the incubations:

$$\text{Excess } ^{13}C\text{-}CO_2 \text{ or } ^{13}C\text{-DIC} = (^{13}F_L - ^{13}F_n) \times C_{conc} ,$$

where C_{conc} is the concentration of CO_2 or DIC, and $^{13}F_L$ is the fractional abundance of ^{13}C in ^{13}C-labeled samples, while $^{13}F_n$ denotes that in non-labeled (Alinen-Mustajärvi) or ^{14}C-labeled (thus, not labeled with ^{13}C) (Mekkojärvi) samples. The excess ^{13}C-CO_2 or ^{13}C-DIC is produced solely from the added ^{13}C-CH_4. Thus, this approach accounts for the natural background variation in $^{13}C/^{12}C$ of CO_2 and DIC. It gives conservative estimates of CH_4 oxidation potential, since the incorporation of CH_4-C into the biomass is not accounted for.

PCR, RT-PCR, preparation of the amplicon sequence libraries, and sequencing

The primer pair utilized for PCR amplification of the bacterial 16S rRNA gene was 27F (5’ – AGAGTTTGATCMTGGCTCAG) / 338R (5’ – TGCTGCCTCCCGTAGGAGT – 3’), whereas the PCR of archaeal *mcrA* gene was done using the primer pair *mcrA* forward (5’-GGTGGTGTMGGATTCACACAR-3’)/ *mcrA* reverse (5’-TCATTGCRTAGTTWGGRTAGTT-3’) (Beal et al. 2009), and that of bacterial *pmoA* was done using A189F (5’ – GGNGACTGGGACTTCTGG) / Mb601-r (5’ – ACRTAGTGGTAACCTTGYAA – 3’) (Kolb et al. 2003). For each PCR amplicon library, two PCR reactions were done. M13 linker (5’-TGTAAAACGACGGCCAGT-3’) was attached to 5’ – the end of both *mcrA* forward and A189F primers (Mäki et al. 2016). In the first reaction for each gene, ~1 ng (Alinen-Mustajärvi) or ~5 ng (Mekkojärvi) of DNA were used as a template in a 25 µl mixture containing Maxima SYBR Green qPCR master mix (2X) (ThermoFisher Scientific), H_2O, and 0.5 µM of both primers. PCR amplification was performed in a C1000™ Thermal Cycler (Bio-Rad) with an initial denaturation step at 98ºC for 30 sec, and 30 cycles of amplification (98ºC for 10 sec, 53ºC for 30 sec, 72ºC for 60 sec) for 16S rRNA, 29 cycles of amplification (98ºC for 60 sec, 50ºC for 60 sec, 72ºC for 60 sec) for *mcrA,* and 35 cycles of amplification (95ºC for 30 sec, 54ºC for 35 sec, 72ºC for 45 sec) for *pmoA* genes. In the second PCR, 1 µl of the first round of PCR products was used as a template in a 20 µl PCR mixture. PCR conditions were similar as above, except that only six amplification cycles were run for 16S rRNA, but eight cycles for *mcrA* and *pmoA* products. In addition, the M13-primer was used as a forward primer in the amplification of *mcrA* and *pmoA* gene amplicons, and all primers included sequencing adaptors at the 5’ end as well as nucleotide barcodes incorporated between the adapter and forward primers to distinguish samples in the mixed reaction (Mäki et al. 2016). The PCR products were checked for correct size using agarose gel electrophoresis, purified with Agencourt AMPure XP (Beckman Coulter), measured for their DNA concentration using a Qubit 2.0 Fluorometer and a dsDNA HS Assay Kit (Thermo Fisher), and pooled in equal DNA amounts for each gene. The amplicon pool was further purified using AMPure XP. Emulsion PCR, sequencing [Ion

Torrent Personal Genome Machine (PGM)], and sequence filtering by the PGM software were done as described in Mäki et al. (2016).

Reverse-transcriptase PCR (RT-PCR) was utilized with Mekkojärvi samples to study the bacterial 16S rRNA, as well as the active methanogenic/methanotrophic archaea and the active methanotrophic bacteria, by targeting mRNA transcripts of *mcrA* and *pmoA*, respectively. The DNAse I treatment to remove DNA was done already during the RNA isolation step. The RT reaction with random hexamers to transcribe RNA (8-12 ng per 9 µl reaction) into cDNA was done using the RevertAid Kit (Thermo Fisher Scientific). The PCR of cDNA products and sequencing proceeded similarly as above. The effectiveness of the DNAse I treatment to remove all the DNA was confirmed by negligible PCR amplification of the DNAse I-treated nucleic acid extracts that had not been subjected to RT reaction.

Shotgun metagenomic sequencing

Shotgun metagenomic libraries were prepared from 10 ng of genomic DNA. First, the genomic DNA was sheared using a focused-ultrasonicator (Covaris E220), and subsequently, sequencing libraries were prepared with the Thruplex FD Prep kit (Rubicon Genomics), according to the manufactures protocol (R40048-08, QAM-094-002). The library size selection was made with AMPure XP beads (Beckman Coulter) in 1:1 ratio. The prepared sample libraries were quantified using next-generation sequencing library qPCR kit (KAPA Biosystems) and run on a StepOnePlus (Life Technologies) real-time PCR instrument. The quantified libraries were then prepared for sequencing on the Illumina HiSeq sequencing platform with a TruSeq paired-end cluster kit, v3, and Illumina's cBot instrument to generate a clustered flowcell for sequencing. The sequencing of the flow cell was performed on the Illumina HiSeq2500 sequencer using Illumina TruSeq SBS sequencing kits, v3, following a 2x100 indexed high-output run protocol.

LITERATURE CITED

Basnet S (2014) Measuring anaerobic primary production by green sulfur bacteria in humic lakes. MSc thesis. University of Jyväskylä, Jyväskylä, Finland

Beal EJ, House CH, Orphan VJ (2009) Manganese- and iron-dependent marine methane oxidation. Science 325:184–187 doi:10.1126/science.1169984

Kolb S, Knief C, Stubner S, Conrad R (2003) Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl Environ Microbiol 69:2423–2429 doi:10.1128/AEM.69.5.2423-2429.2003

Leibl H, Tomasits R, Brühl P, Kerschbaum A, Eibl MM, Mannhalter JW (1999) Humoral and cellular immunity induced by antigens adjuvanted with colloidal iron hydroxide. Vaccine 17:1017–1023 doi:10.1016/S0264-410X(98)00201-1

Lovley DR, Phillips EJP (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol 51:683–689 PubMed

Lovley DR, Phillips EJP (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54:1472–1480 PubMed

Mäki A, Rissanen AJ, Tiirola MA (2016) Practical method for barcoding and size-trimming PCR templates for amplicon sequencing. Biotechniques 60:88–90 doi:10.2144/000114380

Murphy J, Riley JP (1962) A modified single solution method for determination of phosphate in natural waters. Anal Chim Acta 27:31–36 doi:10.1016/S0003-2670(00)88444-5

Ojala A, Bellido JL, Tulonen T, Kankaala P, Huotari J (2011) Carbon gas fluxes from a brown-water and a clear-water lake in the boreal zone during a summer with extreme rain events. Limnol Oceanogr 56:61–76 doi:10.4319/lo.2011.56.1.0061

Salonen K (1979) A versatile method for the rapid and accurate determination of carbon by high temperature combustion. Limnol Oceanogr 24:177–183 doi:10.4319/lo.1979.24.1.0177

Solorzano L (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol Oceanogr 14:799–801 doi:10.4319/lo.1969.14.5.0799

Wood ED, Armstrong FA, Richards FA (1967) Determination of nitrate in sea water by cadmium-copper reduction to nitrite. J Mar Biol Assoc UK 47:23–31 doi:10.1017/S002531540003352X

SUPPLEMENT 2

Supplementary figures S1-S10:

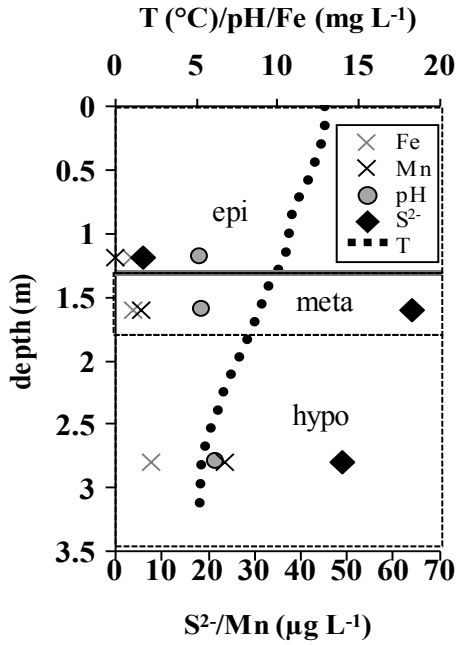

Fig. S1. Vertical depth profiles of temperature (T), pH, Fe, Mn, and S^{2-} in Lake Mekkojärvi. Oxycline depth is denoted with a grey line. The epi- (above the oxycline) as well as meta- and hypolimnion (below the oxycline) zones are indicated with dashed line boxes

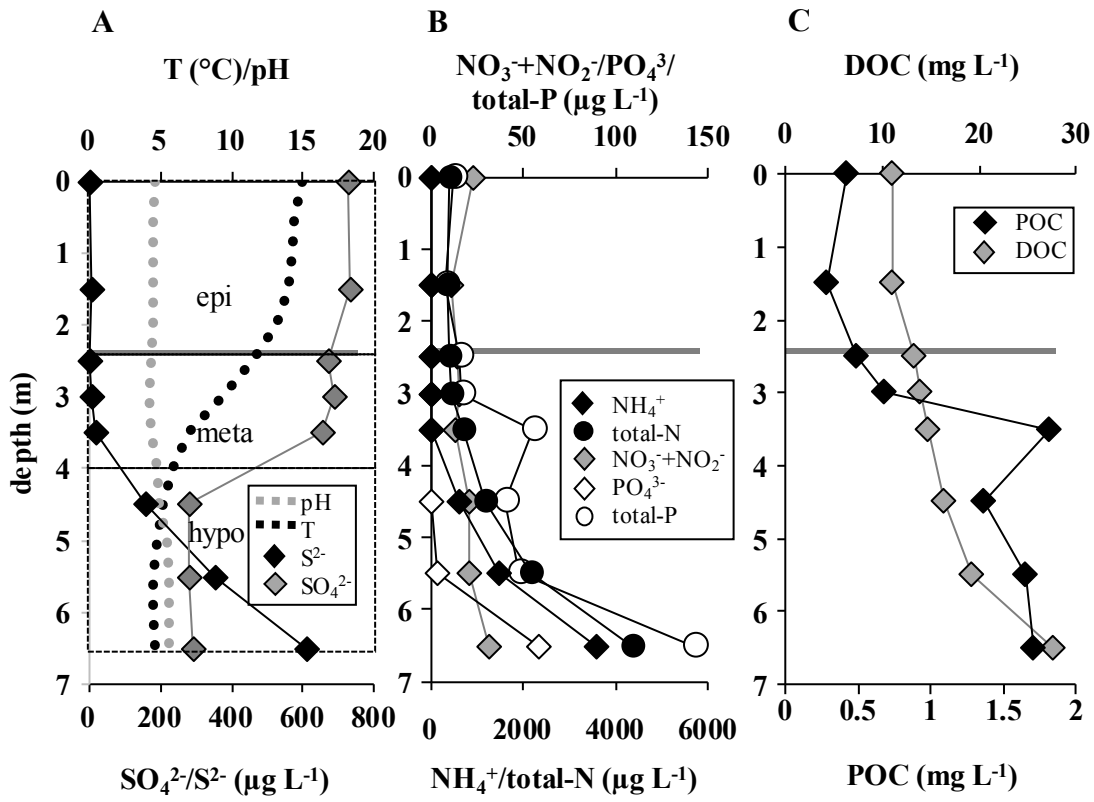

Fig. S2. Vertical depth profiles of A) temperature (T), pH, S^{2-}, and SO_4^{2-}, B) NH_4^+, total-N, $NO_3^-+NO_2^-$, PO_4^{3-}, total-P, and C) particulate organic carbon (POC) and dissolved organic carbon (DOC) in Lake Alinen-Mustajärvi. Oxycline depth is denoted with a grey line. The epi- (above the oxycline) as well as meta- and hypolimnion (below the oxycline) zones are indicated with dashed line boxes

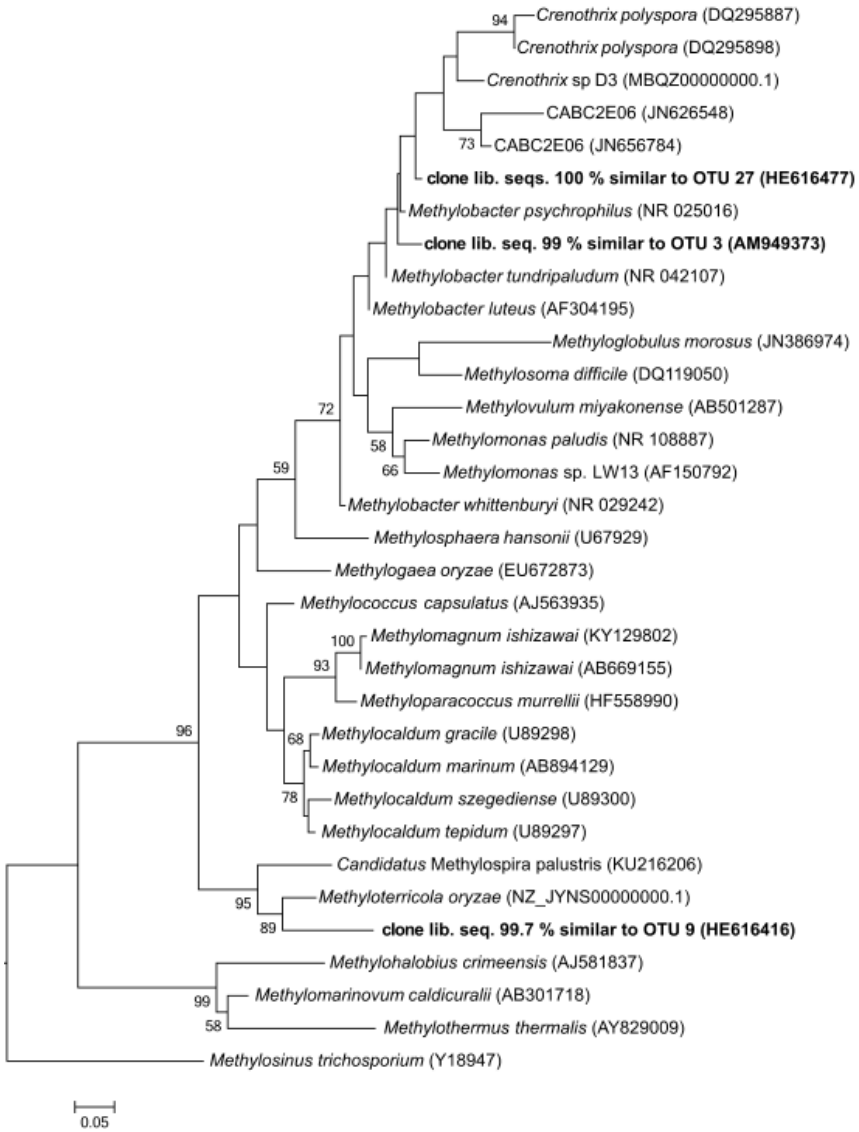

Fig. S3. Phylogenetic tree of 16S rRNA gene sequences of *Methylococcales* (i.e. type I MOB) showing the phylogenetic positions of clone library sequences (from Alinen-Mustajärvi and Mekkojärvi) collected previously, which are highly similar to representative sequences of OTUs 3, 9, and 27. The tree was constructed using the maximum-likelihood algorithm with the GTR model. The length of nucleotide sequences varies from 390 to 444 bp. The sequence from alphaproteobacterial methanotrophic bacteria (i.e. type II MOB) was used to root the tree. The scale bar indicates the number of substitutions per site. The numbers at the nodes indicate the percentage of occurrence in 100 bootstrapped trees (bootstrap values >50 % are shown)

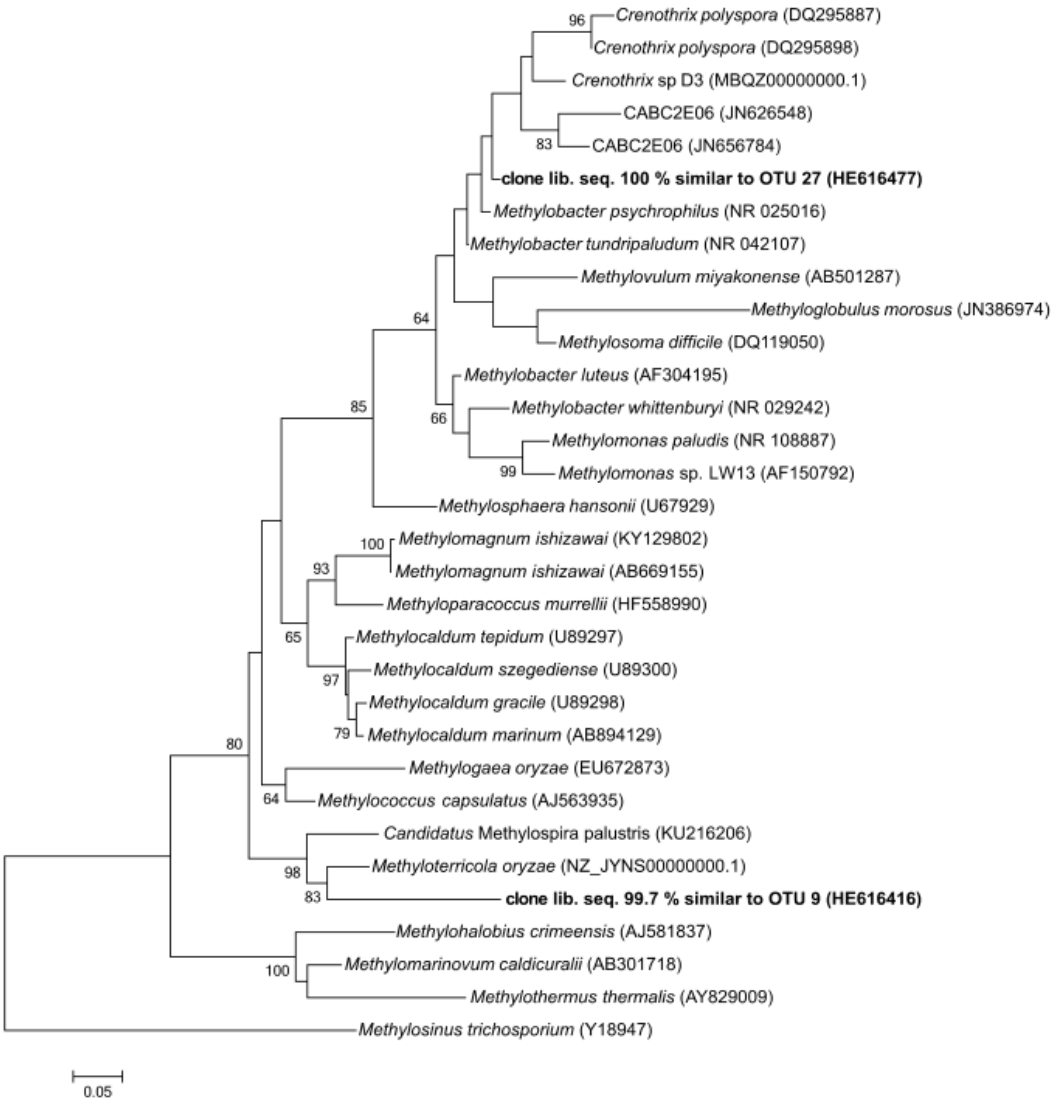

Fig. S4. Phylogenetic tree of 16S rRNA gene sequences of *Methylococcales* (i.e. type I MOB) showing the phylogenetic positions of clone library sequences (from Alinen-Mustajärvi) collected previously, which are highly similar to representative sequences of OTUs 9 and 27. The tree was constructed using the maximum-likelihood algorithm with the GTR model. The length of nucleotide sequences varies from 747 to 803 bp. The sequence from alphaproteobacterial methanotrophic bacteria (i.e. type II MOB) was used to root the tree. The scale bar indicates number of substitutions per site. The numbers at the nodes indicate the percentage of occurrence in 100 bootstrapped trees (bootstrap values >50 % are shown)

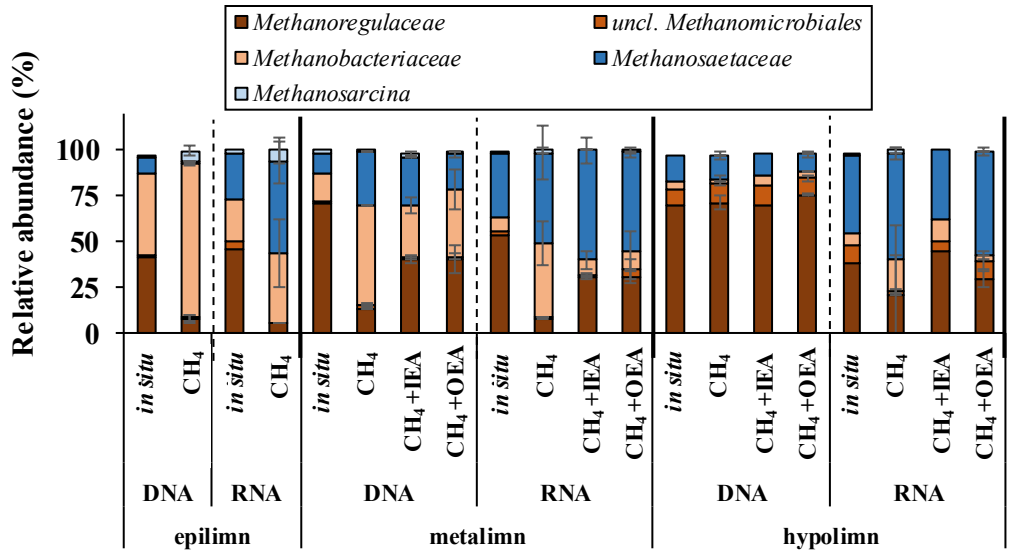

Fig. S5. The relative abundance of methanogenic archaea based on the *mcrA* gene and mRNA transcripts, respectively, *in situ* and after experimental incubation (21 days), of water samples collected from the epilimnion, metalimnion, and hypolimnion of Lake Mekkojärvi and amended with $^{13}CH_4$, $^{13}CH_4$+mixture of inorganic EAs (IEA: NO_3^-, SO_4^{2-}, Fe^{3+} and Mn^{4+}), and $^{13}CH_4$+organic EA (OEA: di-sodium anthraquinone-2,6-disulfonate). Data is presented as average ± SD when n = 2, otherwise n = 1

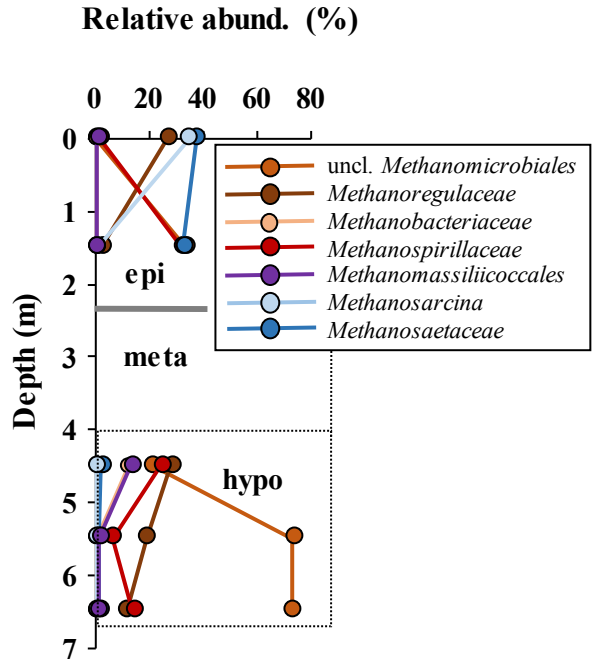

Fig. S6. Vertical depth profiles of methanogenic archaea (% of *mcrA* gene amplicons) in Lake Alinen-Mustajärvi. Oxycline depth is denoted with a grey line. The epi- (above the oxycline) as well as meta- and hypolimnion (below the oxycline) zones are indicated with dashed line boxes. The PCR for the *mcrA* gene was not successful in metalimnion samples

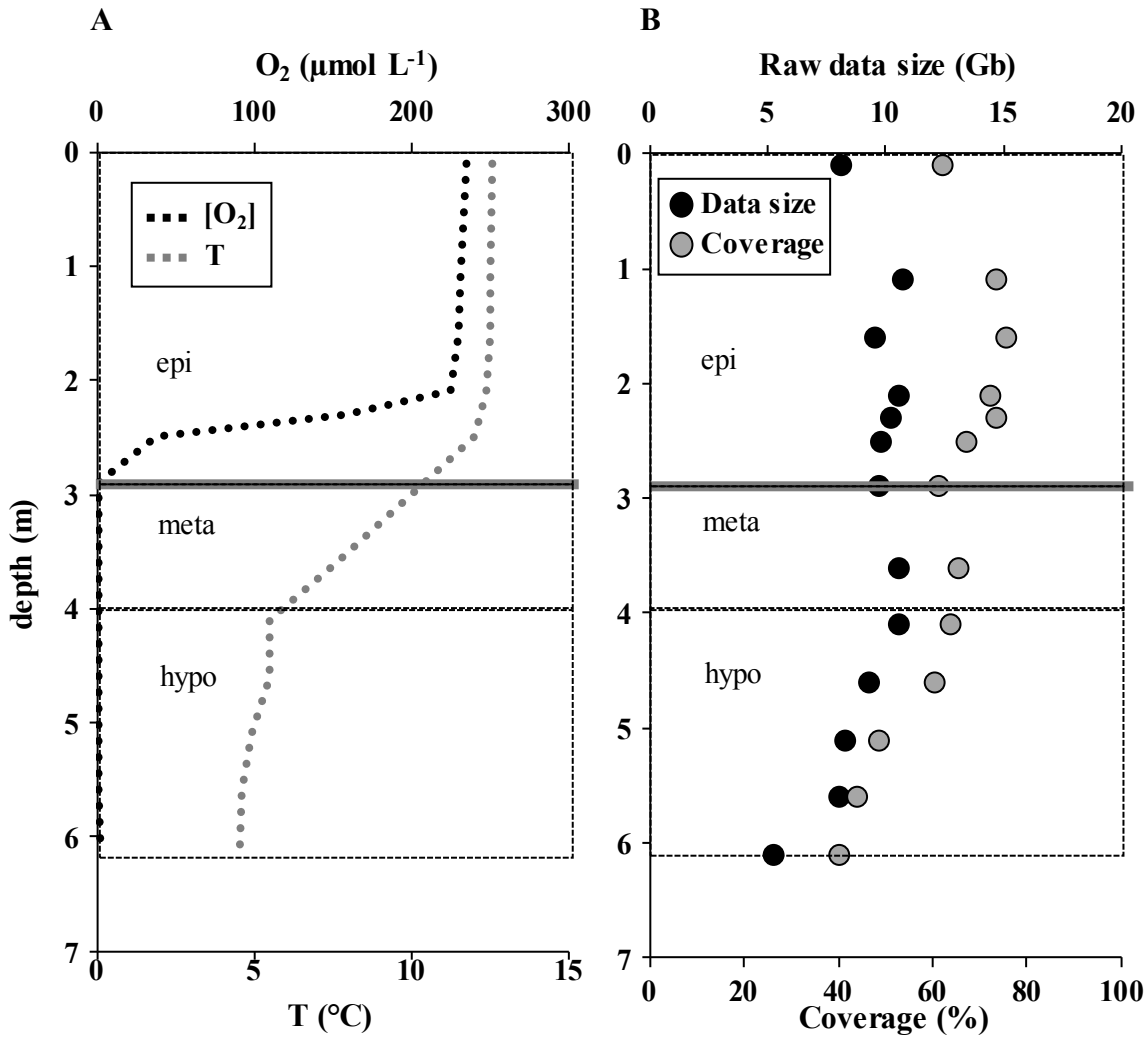

Fig. S7. Vertical depth profiles of A) O_2 and T, as well as B) size and coverage of shotgun metagenomic libraries for Alinen-Mustajärvi on 23rd September 2013, thus, two weeks after sampling for biogeochemical profiles, incubations, and amplicon sequencing

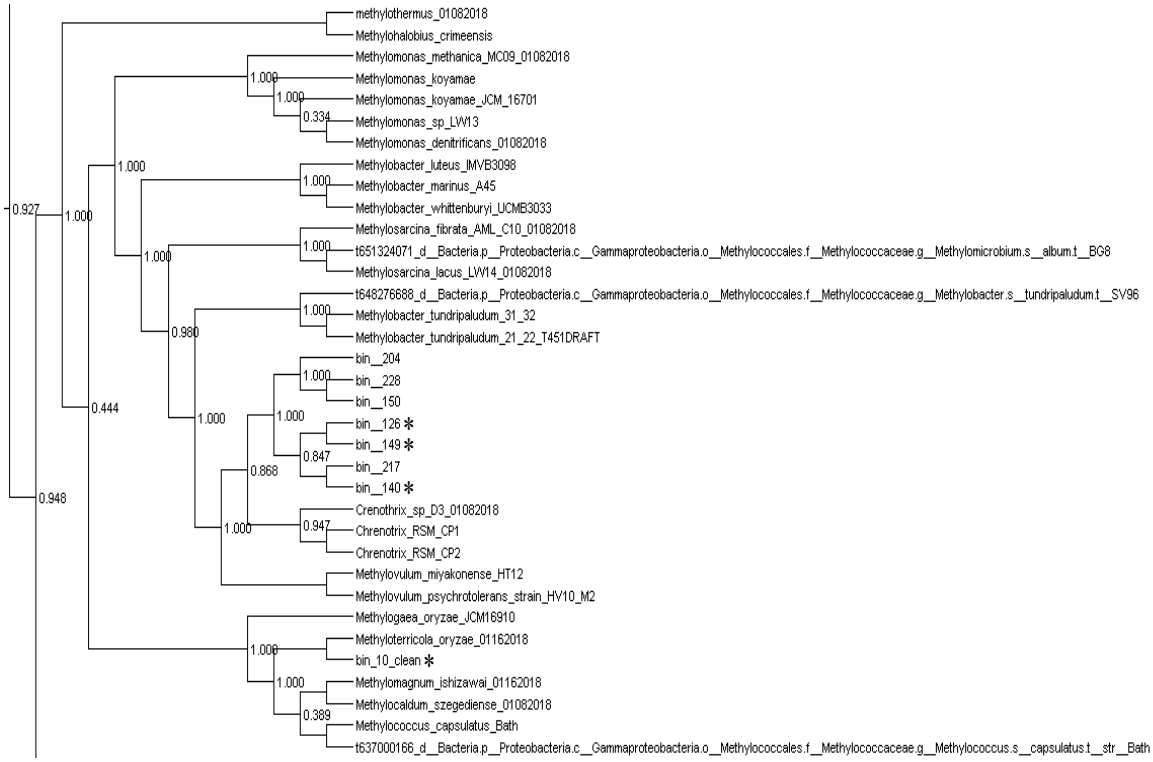

Fig. S8. Excerpt from Phylophlan tree showing the metagenome assembled genomes (MAGs, i.e. metagenomics bins) of *Methylococcales* in Alinen-Mustajärvi based on shotgun metagenomic analysis. High-quality metagenomic bins considered in this study are marked with the * sign

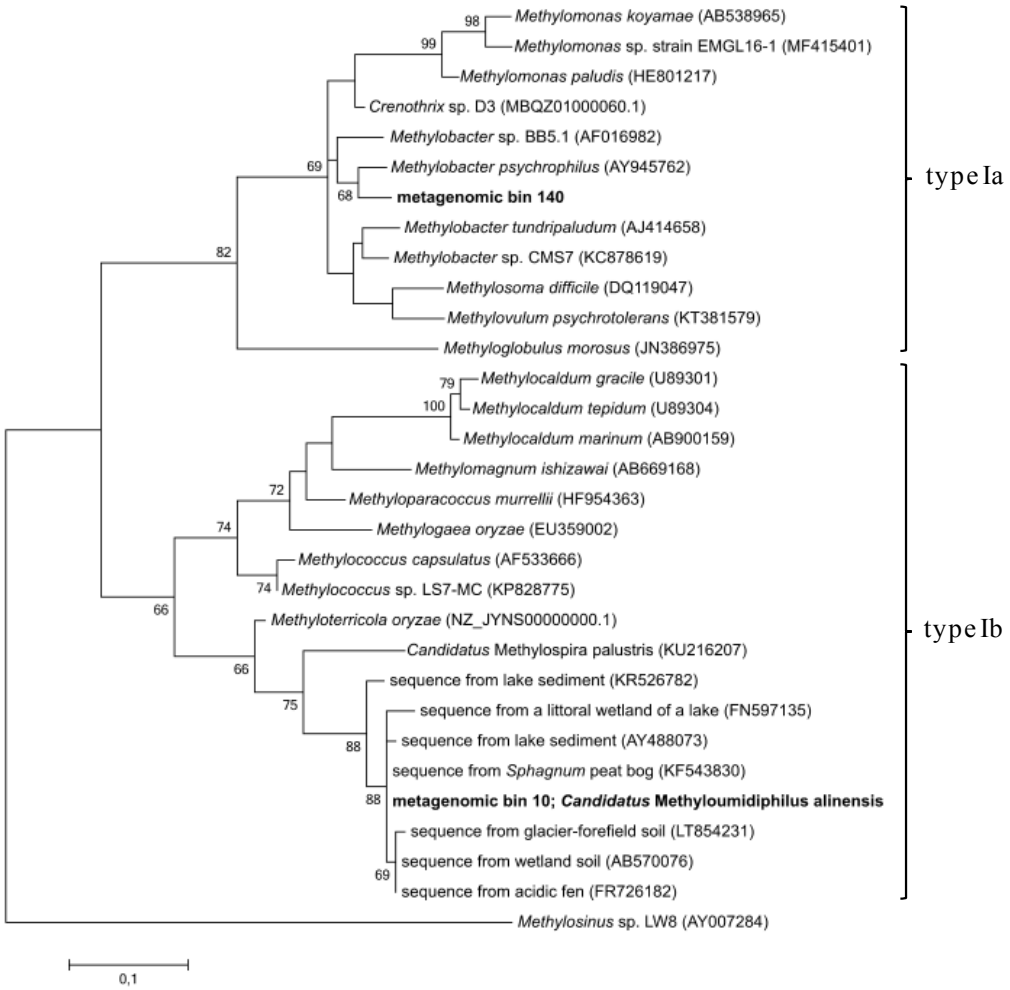

Fig. S9. Phylogenetic tree of deduced amino acid sequences of the *pmoA* gene of *Methylococcales* (i.e. type I MOB), showing the phylogenetic positions of the metagenomic bins from Alinen-Mustajärvi. The tree was constructed using the maximum-likelihood algorithm with the JTT substitution model. The length of amino acid sequences is 141. The sequence from the alphaproteobacterial methanotrophic bacteria (i.e. type II MOB) was used to root the tree. The scale bar indicates the number of substitutions per site. The numbers at the nodes indicate the percentage of occurrence in 100 bootstrapped trees (bootstrap values >50 % are shown)

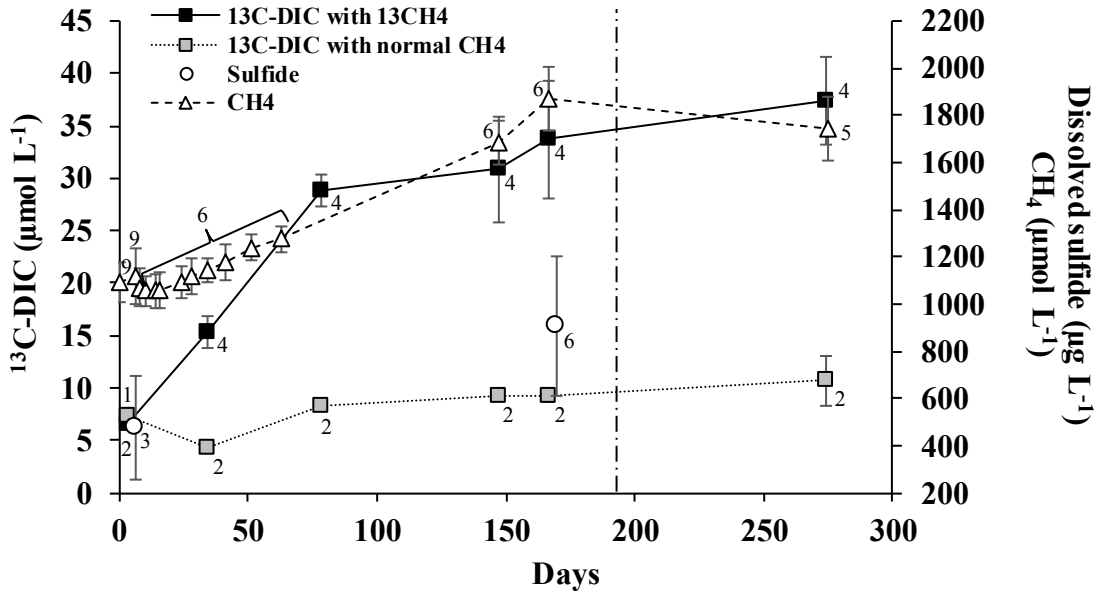

Fig. S10. Accumulation of ^{13}C-DIC, total CH_4, and dissolved sulfide [average ± SD (SD is shown when n ≥ 2), n = 1 – 9, shown in the figure] during anaerobic pre-incubation (see Materials and methods and Supplement 1) of concentrated water samples collected from the hypolimnion (5.5 m) of Lake Alinen-Mustajärvi and amended with ^{13}C-labeled CH_4 or isotopically natural CH_4. ^{13}C-DIC is shown separately for $^{13}CH_4$ and normal CH_4-treatments. The dashed vertical line denotes the time point (after 6.5 months of pre-incubation), when the actual light and EA-addition experiments were started with concentrated water from one of the pre-incubated samples that had received isotopically natural CH_4. Three incubation bottles (two with $^{13}CH_4$ and one with normal CH_4) were sacrificed during day 6. The first measurement of ^{13}C-DIC and sulfide included only the sacrificed samples