Effects of nitrogen load on the function and diversity of methanotrophs in the littoral wetland of a boreal lake

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Methane (CH4) is the second most abundant greenhouse gas in the atmosphere. A major part of the total methane emissions from lake ecosystems is emitted from littoral wetlands. Methane emissions are significantly reduced by methanotrophs, as they use methane as their sole energy and carbon source. Methanotrophic activity can be either activated or inhibited by nitrogen. However, the effects of nitrogen on methanotrophs in littoral wetlands are unknown. Here we report how nitrogen loading in situ affected the function and diversity of methanotrophs in a boreal littoral wetland. Methanotrophic community composition and functional diversity were analyzed with a particulate methane monooxygenase (pmoA) gene targeted microarray. Nitrogen load had no effects on methane oxidation potential and methane fluxes. Nitrogen load activated pmoA gene transcription of type I (Methyllobacter, Methylomonas, and LW21-freshwater phylotypes) methanotrophs, but decreased the relative abundance of type II (Methylcystis, Methylosinus trichosporium, and Methylosinus phylotypes) methanotrophs. Hence, the overall activity of a methanotroph community in littoral wetlands is not affected by nitrogen leached from the catchment area.

Keywords: methane, littoral wetland, methanotrophs, nitrogen, pmoA gene, pmoA transcript, pmoA microarray

INTRODUCTION

Methane (CH4) is the second most abundant greenhouse gas in the atmosphere after carbon dioxide. It is 25 times more efficient than carbon dioxide (Denman et al., 2007). Methane is mostly emitted from lakes (Juutinen et al., 2003). Nitrogen (N) can either inhibit (Steudler et al., 1989) or stimulate (Bodelier et al., 2000) CH4 oxidation and subsequently cause higher or lower CH4 emissions. Both responses are possible in upland and wetland soils but the mechanisms behind the different effects are not fully understood (Bodelier and Laanbroek, 2004). Littoral wetlands, which are under the influence of the fluctuating water levels of lakes, are the target of N leached from the catchment. If N inhibits CH4 oxidation, the CH4 emissions from littoral wetlands can increase. However, the effects of nitrogen on the function and diversity of methanotrophs at the species level in littoral wetlands are unknown.

We studied the effects of experimental nitrogen loading in situ on the function and diversity of methanotrophs and fluxes of CH4 in a boreal littoral wetland during a growing season. The relative abundance of pmoA genes and gene transcripts was examined with a pmoA targeting diagnostic microarray (Bodrossy et al., 2003).

MATERIALS AND METHODS

STUDY SITE

The studied littoral wetland of the hypereutrophic Lake Kevätön is located in Eastern Finland (63°6′N, 27°37′E). Since...
spatial variation contributes to the function and diversity of methanotrophs, six (three for N fertilization and three for control) study plots of 1.44 m² were randomly established in the “intermediate” area of the wetland, i.e., in the area 7–10 m from the shoreline (Siljanen et al., 2011). This area has only minor spatial variation in hydrology and distribution of vegetation. The vegetation consists mainly of sedges and it did not vary among the study plots (variances were tested by the Kruskal Wallis rank sum test, P > 0.35). The water level variation did not differ statistically significantly between the control and manipulated plots (Mixed model, P > 0.124...0.421) although the N-treated plots had a slightly lower water table than the control plots (Figure A1 in Appendix).

SOIL SAMPLING, NITROGEN LOAD, BIOGEOCHEMICAL ANALYSES, AND NUCLEIC ACID EXTRACTIONS FROM THE SOILS

Soil samples were taken on June 7, July 7, and August 16, 2007 from triplicate nitrogen and control plots. Nitrogen treatment (NH₄NO₃ dissolved in distilled H₂O, total dose 10 g N m⁻², corresponds with 100 kg N ha⁻¹) was done four times during the 2007 growing season with 1 week intervals (2.5 g N m⁻² each dose). Control plots received similar amounts of water (distilled H₂O) as the nitrogen treated plots. The first soil samples were taken 14 days after the first nitrogen dose. The second soil samples were taken 14 days after the start of the nitrogen loading, when 50% (5.0g N m⁻²) of the total nitrogen dose was applied (Figure A1 in Appendix). The last soil samples were collected 6 days after the nitrogen loading. Soil profiles were taken with a box corer (diameter 8 cm × 8 cm) from the plots and divided into 0–2, 2–10, and 10–20 cm layers. Methane fluxes, CH₄ oxidation potential, and soil chemical characteristics (nitrate and ammonium concentrations) were determined as described previously (Siljanen et al., 2011). From each soil layer, 15 ml sub-samples were collected for molecular analyses of the methanotrophic community and were frozen immediately with dry ice at the study site. Soil was freeze-dried (−50°C, 48 h). DNA extractions were performed as described previously (Siljanen et al., 2011). RNA extractions and clean-up were done according to a protocol described by Steenbergh et al. (2010) with minor modifications: contaminating DNA was removed according to the manufacturer’s instructions with DNase I and cDNA synthesis with RevertAid MuLV–H reverse transcriptase, both provided by Fermentas.

MOLECULAR ANALYSES OF METHANOTROPHS

PCR products of pmoA genes for microarray analysis were amplified with a semi-nested approach with reaction mixtures and cycling conditions, as described earlier (Siljanen et al., 2011). The primers used for the semi-nested approach were A189 (5′-GGNGACTGGGACTTGTGG-3′), A682-T7 (5′-TTATAGCCTACTAAGGAAGCGCAGGAGG-3′), and mb661-T7 (5′-TTATAGCCTACTAGAAGCGCGGMGCAA GCATTCATAC-3′). The relative abundance of pmoA genes and gene transcripts was examined with a pmoA microarray as described previously (Bodrossy et al., 2003). The presence of Methylocella and Methylocella-like methanotrophs was determined with a PCR method (Rahman et al., 2011). For amplification, 2× Premix F (Epicentre), 1 unit of Taq polymerase (Invitrogen), and 50 ng of template DNA or cDNA were used. For amplification of pmoA genes 25 pmol of each primer was used, and 40 pmol of each primer was used for mmoX genes. Reactions were carried out in 50 μl volume. The PCR cycling conditions for Methylocella primers were the following: denaturation 95°C, 15 s, annealing 68°C, 1 min, elongation 72°C, 1 min for 45 cycles. The primers used for Methylocella PCR were mmoXLF (5′-GAAGATTGGGGCGCAGCTCTG-3′) and mmoXLR (5′-CACAATCACTGCCTAGAAAGGAT-3′; Rahman et al., 2011). Cloned fragments of mmoX genes of Methylocella palustris were used as a positive control for the assay. For analysis of the diversity of Methylocella methanotrophs, PCR products were ligated to a pDRIVE vector and cloned, as described previously (Siljanen et al., 2011). Clones were subjected to restriction fragment length polymorphism (RFLP). In RFLP analysis, DNA of clones was digested with SalI and BamHI restriction enzymes and restriction patterns were visualized with electrophoresis in a 2.5% (w/v) agarose gel. Clones displaying identical restriction patterns were grouped into operational taxonomical units (OTUs). One to two clones per OTUs were sequenced. DNA sequencing was performed at the University of Eastern Finland Sequencing Laboratory with the MegaBACE 750 analysis system with a DYEnamic™ET Terminator Cycle Sequencing Kit. The identity of clones was examined by BLASTn searches of the GenBank database (Altschul et al., 1990).

STATISTICAL ANALYSIS

The effects of the manipulation on CH₄ fluxes, CH₄ oxidation, and on the responses of community composition and functional diversity of methanotrophs at the species level were analyzed with a mixed-effect model (proportional to a repeated measure ANOVA; Laird and Ware, 1982). Amplification of pmoA genes for four cDNA replicates (10–20 cm layer, mainly clay with negligible CH₄ oxidation potential) did not succeed even though re-extraction was performed. In these cases, duplicates instead of triplicates were used for the analysis. For evaluating the effect of nitrogen at the species level of methanotrophs, 1020 mixed-effect models were calculated, one model for both manipulated and control plots and for each of 85 microarray probes showing positive signals. Prior to the analysis, the microarray data were square-root transformed. The effects of nitrogen loading were evaluated with the difference between the models of the manipulated and control plots (see example of model results in Figure A3 in Appendix). The normality of residuals was tested for each variable group to fulfill the requirements of the analysis set-up. Mixed-effect model tests were done with the statistical program SPSS 17.0 (SPSS Inc., USA). The relation between the change in the methanotroph community (both community composition and functional diversity), CH₄ oxidation and nitrogen load was studied with constrained correspondence analysis (CCA). CCA analyses were performed for those microarray probes showing change. The analysis included the probes Mb271, Mb C11-403, Mm531, MmESS46, Ia 193, Ia 193, Ia 575, LW21-374, LW21-391, Ib453, Mcy233, Mcy413, Mcy264, Mcy459, Mcy255, McyM309, MsT214, MsS314, MsI423, MsI294, and NMsiT-271. The probes targeting the RA14 group (probe RA14-591) and Methycapsa (probes B2all343, B2all341) were omitted from the analysis because of lack of hybridization to species-specific probes (RA14-594, B2-400). Constrained
correspondence analyses were conducted with the VEGAN (Oksanen et al., 2010) add-on package in the R 2.12.0 statistical program (R Development Core Team, 2010). The Pearson correlation coefficients between nitrate and ammonium concentrations, CH$_4$ oxidation potential, and microarray data were also calculated with the R program.

**RESULTS**

**EFFECTS OF NITROGEN ON CH$_4$ OXIDATION AND CH$_4$ FLUXES**

Nitrogen loading increased nitrate ($F_{\text{nitrate}} = 12.792$, $P < 0.005$, maximum in loaded plots was 6 $\mu$g NO$_3^-$ N cm$^{-3}$) concentration in the 0- to 2-cm soil layer (Figure A2 in Appendix). Ammonium concentration increased also slightly in this layer ($F_{\text{ammonium}} = 4.366$, $P = 0.059$, the maximum in the loaded plots was 25 $\mu$g NH$_4^+$ N cm$^{-3}$) as well as in the deeper layers (2–10 cm layer $F_{\text{ammonium}} = 3.409$, $P = 0.090$; 10–20 cm layer $F_{\text{ammonium}} = 3.825$, $P = 0.076$; Figure A2 in Appendix). Nitrogen load did not affect CH$_4$ oxidation potential statistically significantly but CH$_4$ oxidation increased during the experimental season in the 0- to 2-cm layer in both control and manipulated plots (Figure 1) as a result of changing environmental conditions (natural lowering in water table, Figure A4 in Appendix). Nitrogen loading had no significant effect also on the CH$_4$ fluxes which decreased in both control and manipulated plots toward autumn as a result of the decrease in water level (Figure A4 in Appendix). The relative decrease in methane fluxes was higher in the N-treated plots (Figure 2) also indicating that nitrogen load did not inhibit methane oxidation.

**EFFECTS OF NITROGEN ON THE METHANOTROPHIC COMMUNITY**

The methanotrophic community structure was close the same in the manipulated and control plots before nitrogen loading, only a few phylotypes showed some variation (14 days before fertilization started; Figure 3A). Nitrogen loading changed the community structure and functional diversity of methanotrophs as revealed by mixed-effect models (Figure 3A) and CCA analysis (Figures 3B–D). When the site had received 50% of the total nitrogen load there was a decrease in the relative abundance of pmoA genes of type II (Methylocystis, Methylosinus trichosporium, and Methylosinus phylotypes, $P < 0.05$) methanotrophs in the 0- to 10-cm soil layers (Figure 3A; Figure A3A in Appendix). There was also an increase in the relative abundance of pmoA transcripts of type I (Methylobacter, Methylomonas, and LW21-freshwater phylotypes, $P < 0.05$) methanotrophs in the 2- to 10-cm soil layer (Figure 3A; Figure A3B in Appendix). CCA multivariate ordination analysis revealed a correlation between the concentrations of ammonium and nitrate and microarray data measured during nitrogen loading in affected soil layers and gene pools, as samples during the experiment are clustered together with the ordinated arrows for ammonium and nitrate (Figures 3B–D). Manipulation had the strongest effect on the community in the 2- to 10-cm soil layer (Figures 3C,D), and nitrate also had an effect on functional diversity after the experiment (Figure 3D).
However, methanotrophic community also shows seasonal variation in the littoral wetland (Siljanen et al., 2012), and this variation was taken into account by comparing the nitrogen loading plots with non-treated control plots.

The effects of nitrogen loading on the methanotrophic community were also examined more deeply with correlation analysis for the relative abundance of pmoA genes and gene transcripts (Table A1 in Appendix). The relative abundance of the pmoA genes and gene transcripts of type I methanotrophs correlated positively with the content of ammonium and nitrate, while pmoA transcripts of type II methanotrophs correlated negatively with ammonium (Table A1 in Appendix). Across the experiment, type I and type II methanotrophs had opposite correlations with CH4 oxidation, suggesting differences in the reactions of the taxonomical groups to nitrogen (Table A1 in Appendix).

**DISCUSSION**

Methane fluxes declined similarly in the control and manipulated plots over the study period because of the decrease in water level toward autumn. Nitrogen load had no statistically significant effects on either CH4 oxidation potential or CH4 fluxes in the littoral wetland, suggesting that the overall activity of methanotrophic communities there was not disturbed by nitrogen, although the methanotrophic community structure was affected. Similar observations on the tolerance of methane oxidation against nitrogen have been made previously in other soil types (Dunfield et al., 1995; Delgado and Mosier, 1996; Cai and Yan, 1999; Bykova et al., 2007).

As in our littoral wetland, nitrogen has been reported to inhibit type II methanotrophs in forest (Mohanty et al., 2006) and field soils (Cébron et al., 2007). The activity of type I methanotrophs was stimulated by nitrogen load in the present study as has been found in rice field soil (Bodelier et al., 2000; Mohanty et al., 2006; Noll et al., 2008; Shrestha et al., 2010) and in forest soil at high methane concentrations (Mohanty et al., 2006). Nitrogen leached from agricultural soils (Riley et al., 2001; Pare et al., 2006) to littoral wetlands evidently does not inhibit CH4 oxidation because methanotrophic communities there are dominated by type I methanotrophs (Siljanen et al., 2011).

In the littoral wetland, pmoA gene transcription of type I methanotrophs was stimulated but not the relative abundance of these methanotrophs, indicating the existence of factors limiting the increase in their number. It can be that type I
methanotrophs are nitrogen limited in the littoral wetland and subsequently stimulated by nitrogen load (see later) similarly to the rhizosphere of rice (Bodelier et al., 2000). Selective grazing by protists on type I methanotrophs (Murase and Frenzel, 2008) may be another reason for the lack of increase in their relative abundance.

It has been suggested that the inhibition of type II methanotrophs by nitrogen is due to competition between different types of methanotrophs (Cébron et al., 2007). In nitrogen-rich conditions, type I methanotrophs could outcompete type II methanotrophs. This can be associated to the better ability of type II methanotrophs to fix molecular nitrogen, which lowers their need for ammonium and nitrate (Murrell and Dalton, 1983).

Thus, type I methanotrophs can increase their CH₄ oxidation activity by nitrogen addition in nitrogen-limited environments. Biomass production of wetland plants in the littoral wetland studied is high (Larmola et al., 2003) causing high demand for nitrogen, and nitrogen can also be efficiently removed by denitrification in wetland. Competition for nitrogen there is thus high.

A similar inhibitory effect of nitrogen on type II methanotrophs, as in the littoral wetland here, has been detected among Methylocystis methanotrophs (Mohanty et al., 2006; Cébron et al., 2007). In the littoral wetland, Methylosinus and M. trichosporium methanotrophs were also inhibited (Figure 3A). However, nitrogen loading increased the relative abundance of pmoA transcripts of one Methylosinus phylotype (Msi294). The results of the present study support the findings that nitrogen can reduce CH₄ oxidation if type II methanotrophs dominate the methanotrophic community (Mohanty et al., 2006).

Since the microarray method depicts the relative abundance in methanotrophic communities, a change in the relative abundance of type II methanotrophs could be a result either of an increase in the relative abundance of type I methanotrophs over type II methanotrophs, or a decrease in the relative abundance of type II methanotrophs. However, microarray data indicated no distinctive co-increase of type I methanotrophs during the experiment when inhibition of type II methanotrophs took place (Figure A3A in Appendix), suggesting that type II methanotrophs have been inhibited by nitrogen load as such, not through competition between type I and type II methanotrophs. However, it is important to note that the methanotrophic community of the littoral wetland reacted rapidly to nitrogen load and acclimated to the prevailing conditions. The shift in the methanotrophic community took place within 14 days after the start of the nitrogen loading, and the community recovered soon after the loading ended (Figure 3A).

This reveals the ability of methanotrophic community in the littoral wetland to withstand environmental changes and perturbations.

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Figure 3A

The semi-nested PCR approach and microarray probe set-up targeted type I and type II methanotrophs as well as RA14 members of upland soil cluster α (USCa) methanotrophs and Methylocapsa methanotrophs but excluded Crenothrix, Methylocella, and Verrucomicrobia methanotrophs. However, it was proven by analysis of A682 PCR products with the pmoA microarray (detects Crenothrix, Siljanen et al., 2011) that Crenothrix methanotrophs were not present in that part of the wetland studied here (data not shown). Thus, Crenothrix may play a role in littoral wetlands but only in the areas with a higher water table than that in the area used in this study (Siljanen et al., 2011). Methylocella specific primers mmoXLF/R (Rahman et al., 2011) showed only a few negligible and very faint products from DNA samples and none from RNA samples. Therefore, although Methylocella methanotrophs are found in the littoral wetland, they play only a limited role in the CH₄ oxidation.

The studied littoral wetland has a moderately high diversity of methanotrophs: 47 OTUs with 93% similarity (Siljanen et al., 2011), compared with other environments: 26 OTUs in temperate forest soils, 93% similarity (Degelmann et al., 2010), and about 35 OTUs, 90% similarity, in rice field soils (Lühr et al., 2010). Since the sub-communities of this diverse community in the littoral wetland react differently to nitrogen load, the overall effect of nitrogen loading was neutral, causing no change in CH₄ oxidation potential or CH₄ fluxes.

There are only a few studies where the effects of nitrogen on CH₄ fluxes and the methanotrophic community composition in situ have been studied simultaneously. Previous studies have investigated the effects of nitrogen load on the functioning and diversity of methanotrophs using microcosms and incubation experiments (Bodelier et al., 2000; Mohanty et al., 2006; Cébron et al., 2007; Noll et al., 2008; Shrestha et al., 2010). Here we provide new insights into how the nitrogen load affects the methanotrophic community and its functioning in situ.

In conclusion, methane oxidation in boreal littoral wetland tolerates nitrogen load as a result of diverse methanotrophic community. Although some methanotrophs are suffered by nitrogen, there are methanotrophs responding positively to extra nitrogen.

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## APPENDIX

Table A1 | Pearson correlation co-efficients across the experiment between methanotroph relative abundance of *pmoA* genes/*pmoA* transcripts, CH₄ oxidation potential, and concentration of nitrate/ammonium (*n* = 18).

| CH₄ oxidation | [NO₃⁻] | [NH₄⁺] |
|---------------|--------|--------|
| DNA: 0–2 cm layer | Type I probes: Mb_C11-403, *r* = 0.70, *P* < 0.01 | Type I probes: BB51-299, Mb_SL#3-300, DS3-446, *r* = 0.77...0.89, *P* < 0.001 |
| DNA: 2–10 cm layer | Type I probes: fw1-641, P_LVW21-391, LK580, Ib453, *r* = −0.54...−0.63, *P* < 0.05 | Type I probe: Mb_SL#3-300, *r* = 0.71, *P* < 0.01 |
| RNA: 0–2 cm layer | Type I probes: Mm451, 501-375, fw1-641, *r* = 0.63...0.89, *P* < 0.05 | Type II probes: Mcy413, Mcy622, Mcy459, Ms232, *r* = −0.49...−0.53, *P* < 0.05 |
| RNA: 2–10 cm layer | Type I probes: Mb282, Mb_C11-403, Mm275, *r* = 0.50...0.52, *P* < 0.05 | Type I probe: Mmb303, *r* = 0.57, *P* < 0.05 |
| Only significant correlations are shown. | | |

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| Name               | Intended specificity                          | Name               | Intended specificity                          |
|--------------------|----------------------------------------------|--------------------|----------------------------------------------|
| BB51-302           | Methylobacter                                | fw1-286            | fw1 group: M. coccus–M. caldum related marine |
| Mb292              | Methylobacter                                | fw1-286            | and freshwater sediment clones                |
| Mb282              | Methylobacter                                | LVW21-374          | LVW21 group                                   |
| Mb_URC278          | Methylobacter                                | LVW21-391          | LVW21 group                                   |
| Mb267              | Methylobacter                                | OSC220             | Finnish organic soil clones and related     |
| 511-436            | Methylobacter                                | OSC300             | Finnish organic soil clones and related     |
| MbA486             | Methylobacter                                | JRC3-535           | Japanese Rice Cluster #3                     |
| MbA557             | Methylobacter                                | LK580              | fw1 group + Lake Konstanz sediment cluster    |
| Mb_SL#3-300        | Methylobacter                                | JRC2-447           | Japanese Rice Cluster #2                     |
| Mb460              | Methylobacter                                | M90-574            | M. coccus–M. caldum related marine and freshwa-|
| Mb_LW12-211        | Methylobacter                                | M90-253            | ter sediment clones                          |
| Mb_C11-403         | Methylobacter                                | M90-253            | M. coccus–M. caldum related marine and freshwa-|
| Mb271              | Methylobacter                                | M90-253            | ter sediment clones                          |
| PS80-291           | Clone PS80                                   | Mth413             | Methylotrophus                                |
| ESt514             | Methylomicrobium-related clones              | lb453              | Type I b (M. thermus–M. coccus–M. caldum and  |
| Mm_pe467           | Methylomicrobium pelagicum                   | lb559              | related)                                      |
| Mb_SL#299          | Soda lake Methylobacter isolates and clones  |                    |                                              |
| Mb_SL#1-418        | Soda lake Methylobacter isolates and clones  |                    |                                              |
| DS1_401            | Deep sea cluster #1                          |                    |                                              |
| Mm531              | Methylomonas                                 |                    |                                              |
| Mm_ES294           | Methylomonas                                 |                    |                                              |
| Mm_ES543           | Methylomonas                                 |                    |                                              |
| Mm_ES546           | Methylomonas                                 |                    |                                              |
| Mm_M430            | Methylomonas                                 |                    |                                              |
| Mm_MV421           | Methylomonas                                 |                    |                                              |
| Mm275              | Methylomonas                                 |                    |                                              |
| Mm451              | Methylomonas                                 |                    |                                              |
| peat_1-3-287       | Methylomonas-related peat clones             |                    |                                              |
| Jpn284             | Clone Jpn 07061                              |                    |                                              |
| Mmb303             | Methylomicrobium album                       |                    |                                              |
| Mmb259             | Methylomicrobium album + Landfill M. microbia|                    |                                              |
| Mmb_M266           | Mmb. album and Methylosarcina                |                    |                                              |
| LP20-444           | Methylomicrobium-related clones              |                    |                                              |
| la183              | Type I a (M. bacter–M. monas–M. microbium)  |                    |                                              |
| la575              | Type I a (M. bacter–M. monas–M. microbium–M. |                    |                                              |
| sarcinal           |                                              |                    |                                              |
| JRC4-432           | Japanese rice cluster #4                     |                    |                                              |
| MciT272            | Methylcaldium tepidum                       |                    |                                              |
| McIG281            | Methylcaldium gracile                       |                    |                                              |
| McIE302            | Methylcaldium E10                           |                    |                                              |
| McIS402            | Methylcaldium szegediense                    |                    |                                              |
| McI048             | Methylcaldium                                |                    |                                              |
| S01-376            | Methylcoccus-related marine and freshwater sediment clones |
| S01-286            | Methylcoccus-related marine and freshwater sediment clones |
| USC3-305           | Upland soil cluster #3                       |                    |                                              |
| Mc398              | Methylcoccus                                 |                    |                                              |
| fw1-639            | fw1 group: M. coccus–M. caldum related marine and freshwater sediment clones |
| fw1-641            | fw1 group: M. coccus–M. caldum related marine and freshwater sediment clones |

(Continued)
| Name | Intended specificity |
|------|---------------------|
| LP21-190 | Novel pmoA copy of type II and related environmental clones |
| LP21-260 | Novel pmoA copy of type II and related environmental clones |
| NMCy1-247 | Novel pmoA copy of *M. cystis* #1 (*) |
| NMCy2-262 | Novel pmoA copy of *M. cystis* #2 (*) |
| NMSi-271 | Novel pmoA copy of *M. sinus trichosphonium* (*) |
| LP21-232 | Novel pmoA copy of type II and related environmental clones |
| RA14-594 | RA14 related clones |
| RA14-591 | RA14 related clones |
| Wsh1-566 | Watershed + flooded upland cluster 1 |
| Wsh2-491 | Watershed + flooded upland cluster 2 |
| Wsh2-450 | Watershed + flooded upland cluster 2 |
| B2rel251 | Methylocapsa-related clones |
| B2-400 | Methylocapsa |
| B2all343 | Methylocapsa and related clones |
| pmoAMO3-400 | Clone pmoA-MO3 |
| ESR-579 | ESR (Eastern Snake River) cluster |
| TUSC409 | Tropical upland soil cluster #2 |
| TUSC502 | Tropical upland soil cluster #2 |
| mtrof173 | Methanotrophs |
| mtrof362-I | Methanotrophs |
| mtrof681 | Methanotrophs |
| mtrof682-I | Methanotrophs |
| mtrof656 | Methanotrophs |
| NmNc53 | Methanotrophs |
| Nsm_eut | Nitrosomonas–Nitrosococcus |
| PB5-226 | Nitrosomonas–Nitrosococcus related clones |
| P6-306 | Nitrosomonas–Nitrosococcus related clones |
| NSv207 | Nitrosospira–Nitrosovibrio |
| NSv363 | Nitrosospira–Nitrosovibrio |
| Nitr_rel47 1 | AOB related clones/probably methanotrophs |
| Nitr_rel22 3 | AOB related clones/probably methanotrophs |
| ARC529 | AOB related clones/probably methanotrophs |
| Nitr_rel47 0 | AOB related clones/probably methanotrophs |
| Nitr_rel35 1 | AOB related clones/probably methanotrophs |
| Nit_rel30 4 | Crenothrix and related |
| M84P105-451 | Environmental clones of uncertain identity |
| WC306_54-385 | Environmental clones of uncertain identity |
| M84P22-514 | Environmental clones of uncertain identity |
| gp23-454 | Environmental clones of uncertain identity |
| MR1-348 | Environmental clones of uncertain identity |
| gp619 | Environmental clones of uncertain identity |
| gp391 | Environmental clones of uncertain identity |
| gp2-581 | Environmental clones of uncertain identity |
| RA21-466 | Clone RA21 – environmental clone of uncertain identity |

*Assignment based on limited information from cultivated methanotrophs.*
Nitrogen loading:
- 2.5 g N m$^{-2}$ (NH$_4$NO$_3$) dose loaded four times in growing season.
- Equal volume of H$_2$O loaded at the same time to control plots.

Soil sampling before manipulation: Jun 7th
Soil sampling during manipulation: Jul 17th
Soil sampling after manipulation: Aug 16th

FIGURE A1 | (A) The littoral wetland of Lake Kevätönn in July 2007. For experiment, three control and three manipulated plots of 1.44 m$^2$ were established to an area having equal water level and vegetation. The chambers for measurements of in situ CH$_4$ fluxes were inserted into study plots 2 weeks before the experiment. Soil sampling and in situ CH$_4$ flux measurements were taken from boardwalks to omit disturbance of the soil. (B) Soil sampling and nitrogen loading scheme. Time points of soil samplings and nitrogen/water loading are colored with brown and blue respectively. Methane fluxes were measured three times before, during and after the nitrogen loading (with 1–2 week intervals). During the N loading period, fluxes were measured before addition of NH$_4$NO$_3$ solution or distilled water.
FIGURE A2 | Nitrogen content of soil. Means and SDs of triplicates are shown. The asterisk indicates the difference between control and manipulation ($P < 0.01$).
FIGURE A3 | Microarray results of community composition [(A), DNA] and functional diversity [(B), RNA] of methanotrophs before, during, and after nitrogen loading. Averages of triplicate plots are shown. A value of 100 (purple) indicates the maximum and a value of 0 (yellow) indicates the minimum signal intensity of a probe against reference hybridizations determined for each probe individually (Bodrossy et al., 2003). Only probes having positive hybridization are shown. N denotes nitrogen loading plots, NC control plots, L1 0–2 cm layer, L2 2–10 cm layer, and L3 10–20 cm layer.
FIGURE A4 | The water table in the study plots. The water table was measured from perforated plastic tubes inserted in soil inside the study plots. The ratio of mean water level of nitrogen loaded and control plots is marked on bottom of the figure.

FIGURE A5 | Example of a mixed-effect model result for the microarray probe Mb271 (RNA samples, 2–10 cm). Mixed-effect model comparison evaluated the difference between two fitted models, the control and the manipulation model. In Figure 3A, the result of each comparison is shown in color, thus, no effect on studied microarray probe by nitrogen = yellow, stimulated effect = green, inhibited effect = red, and for negative probes = blue.