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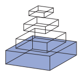

Effects of nitrogen load on the function and diversity of methanotrophs in the littoral wetland of a boreal lake

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Methane 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 (*Methylobacter*, *Methylomonas*, and LW21-freshwater phylotypes) methanotrophs, but decreased the relative abundance of type II (*Methylocystis*, *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 (CH_4) is the second most abundant greenhouse gas in the atmosphere after carbon dioxide. It is 25 times more efficient (with a time horizon of 100 years) as a greenhouse gas than carbon dioxide (Denman et al., 2007). It accounts for about 20% of the radiative forcing (warming effect) of the atmosphere (Denman et al., 2007). In oxic surface layers of wetlands, methanotrophs, which are aerobic methane oxidizing bacteria, can consume more than 90% of the CH_4 produced in deeper anoxic layers (Oremland and Culbertson, 1992), implementing an important ecosystem service.

Taxonomically, methanotrophs belong to two phyla, Verrucomicrobia and Proteobacteria (Semrau et al., 2010). Verrucomicrobial methanotrophs have been shown to live only in extreme conditions in geothermal environments (Op den Camp et al., 2009), so they are not of particular interest to this study. Proteobacterial methanotrophs are divided into two classes, Gammaproteobacteria and Alphaproteobacteria (often referred to as type I and type II methanotrophs, respectively) on the basis of phylogeny, physiology, morphology, and biochemistry (Semrau et al., 2010). Type I methanotrophs are further divided into type Ia and type Ib subgroups based on their phylogeny (Bodrossy et al., 2003).

Methane mono-oxygenases (MMO) of methanotrophs are the key enzymes in the CH_4 oxidation process, and the phylogeny of MMO genes corresponds well with 16S rRNA gene-based phylogeny. The gene fragments of the particulate form of MMO, *pmoA*, and soluble form of MMO, *mmoX*, can be used for the detection of a diversity of methanotrophs and their CH_4 oxidation activity. Almost all methanotrophs possess the *pmoA* gene, and strains lacking it can be detected by *mmoX*-targeted approaches (Dedysh et al., 2005; Rahman et al., 2011; Vorobev et al., 2011). Most methanotrophs use only methane as their carbon and energy source, but some strains of Alphaproteobacteria methanotrophs have been shown to grow also with C_2 substrates (Dedysh et al., 2005; Dunfield et al., 2010; Belova et al., 2011).

A littoral zone can contribute as much as 70% of the total CH_4 emissions of lakes (Juutinen et al., 2003). Nitrogen (N) can either inhibit (Steudler et al., 1989) or stimulate (Bodelier et al., 2000) CH_4 oxidation and subsequently cause higher or lower CH_4 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 CH_4 oxidation, the CH_4 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 CH_4 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 m^2 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, $P > 0.124\ldots\ 0.421$) although the N-treated plots had a slightly lower water table than the control plots (**Figure A4** 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 (NH_4NO_3 dissolved in distilled H_2O, 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 H_2O) as the nitrogen treated plots. The first soil samples were taken 14 days before the first nitrogen dose. The second soil samples were taken 14 days after the start of the nitrogen loading, when 50% (5.0 g 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, CH_4 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′-GGNGACTGGGACTTCTGG-3′), A682-T7 (5′-TAATACGACTCACTATAGGAASGCNGAGAAGAASGC-3′), and mb661-T7 (5′-TAATACGACTCACTATAGCCGGMGCAA CGTCYTTACC-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′-GAAGATTGGGGCGGCATCTG-3′) and mmoXLR (5′-CCCAATCATCGCTGAAGGAGT-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 *Sal*I and *Bam*HI 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 Dye 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 CH_4 fluxes, CH_4 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 CH_4 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), CH_4 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, MmES546, Ia 193, Ia 575, LW21-374, LW21-391, Ib453, Mcy233, Mcy413, Mcy264, Mcy459, Mcy255, McyM309, MsT214, Msi269, 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

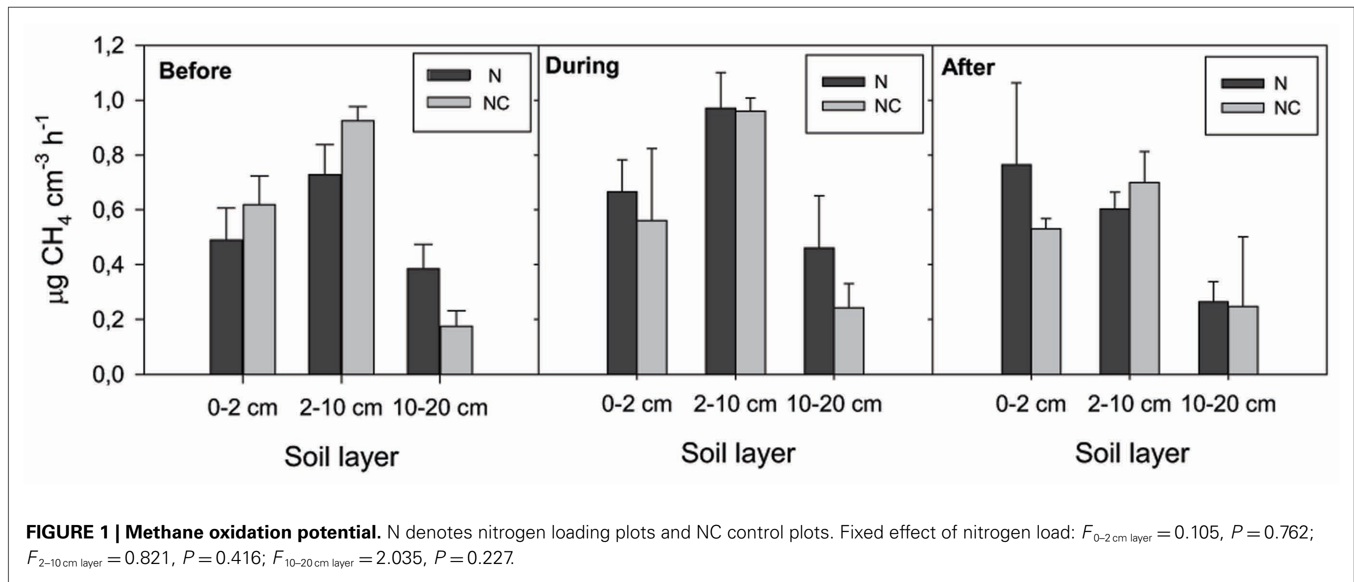

FIGURE 1 | Methane oxidation potential. N denotes nitrogen loading plots and NC control plots. Fixed effect of nitrogen load: $F_{0\text{–}2\,\text{cm layer}} = 0.105$, $P = 0.762$; $F_{2\text{–}10\,\text{cm layer}} = 0.821$, $P = 0.416$; $F_{10\text{–}20\,\text{cm layer}} = 2.035$, $P = 0.227$.

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.

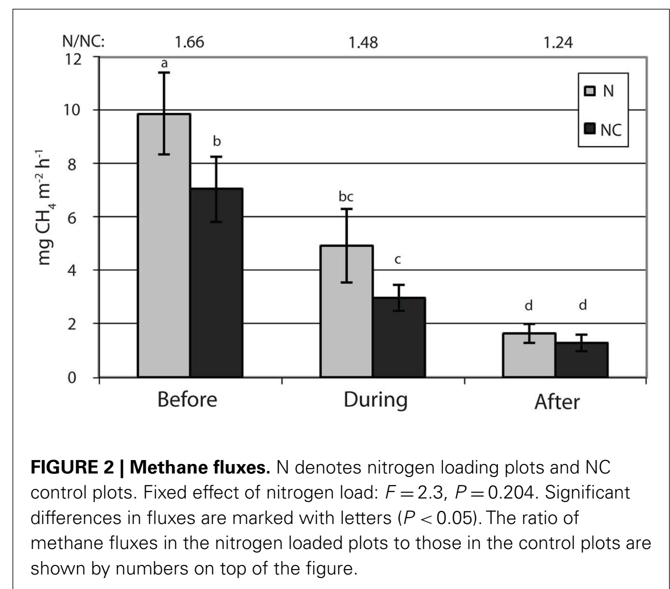

FIGURE 2 | Methane fluxes. N denotes nitrogen loading plots and NC control plots. Fixed effect of nitrogen load: $F = 2.3$, $P = 0.204$. Significant differences in fluxes are marked with letters ($P < 0.05$). The ratio of methane fluxes in the nitrogen loaded plots to those in the control plots are shown by numbers on top of the figure.

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**).

FIGURE 3 | The effect of nitrogen loading on methanotrophs. The species level effects of nitrogen loading on relative abundance of *pmoA* genes (DNA) and relative abundance of *pmoA* transcripts (RNA) of methanotrophs. **(A)** The table summarizes the mixed-effect model tests between manipulated and control plots. Soil samples were taken from control and manipulated plots before, during and after the nitrogen loading for methanotroph community analysis, and the effects of manipulation were evaluated with mixed-effect models. Only probes with signals are shown. **Table A2** in Appendix shows complete list of microarray probes used and specificity of them. Effects of nitrogen load on microarray probes in manipulated plots compared with control plots ($df_1 = 1$, $df_2 = 12$, $P < 0.05$) are marked at the each time point. Probe intensity inhibitions compared to control plots, are marked with red and stimulations with green. Yellow color means that probe is positive (but not affected by nitrogen) and blue that probe is negative. **(B)** Constrained correspondence analysis of nitrogen affected community, nitrogen content and CH_4 oxidation in the 0- to 2-cm layer, and **(C)** in the 2- to 10-cm layer of DNA and **(D)** in the 2- to 10-cm layer of RNA samples. The percentages at the axes shows the proportion of constrained inertia explained by the constrained axis and inertia explained by unconstrained axis, respectively.

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 CH_4 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 CH_4 oxidation potential or CH_4 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 CH_4 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 CH_4 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 CH_4 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 methanotroph 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.

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 (USCα) 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 CH_4 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 (Lüke 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 CH_4 oxidation potential or CH_4 fluxes.

There are only a few studies where the effects of nitrogen on CH_4 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, CH_4 oxidation potential, and concentration of nitrate/ammonium ($n = 18$).

	CH_4 oxidation	$[NO_3^-]$	$[NH_4^+]$
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, $r = 0.77 \ldots 0.89$, $P < 0.001$	
DNA: 2–10 cm layer	Type I probes: fw1-641, P_LW21-391, LK580, Ib453. $r = -0.54 \ldots -0.63$, $P < 0.05$ Type II probes: Mcy_264, Msi_294, II509, $r = -0.51 \ldots -0.57$, $P < 0.05$	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, $r = 0.63 \ldots 0.89$, $P < 0.05$	Type II probes: Mcy413, Mcy522, Mcy459, Msi232, Peat264, $r = -0.49 \ldots -0.53$, $P < 0.05$
RNA: 2–10 cm layer	Type I probes: Mb282, Mb_C11-403, Mm275, $r = 0.50 \ldots 0.52$, $P < 0.05$	Type I probe: Mmb303, $r = 0.57$, $P < 0.05$	Type I probes: Mb282, b_C11-403, Mm275, $r = 0.70 \ldots$ 0.78, $P < 0.01$

Only significant correlations are shown.

Table A2 | The microarray probe set used in the study and probe specificity.

Name	Intended specificity
BB51-302	*Methylobacter*
Mb292	*Methylobacter*
Mb282	*Methylobacter*
Mb_URC278	*Methylobacter*
Mb267	*Methylobacter*
511-436	*Methylobacter*
MbA486	*Methylobacter*
MbA557	*Methylobacter*
Mb_SL#3-300	*Methylobacter*
Mb460	*Methylobacter*
Mb_LW12-211	*Methylobacter*
Mb_C11-403	*Methylobacter*
Mb271	*Methylobacter*
PS80-291	Clone PS80
Est514	*Methylomicrobium*-related clones
Mm_pel467	*Methylomicrobium pelagicum*
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*
Mmb562	*Mmb. album* and *Methylosarcina*
LP20-644	*Methylomicrobium*-related clones
Ia193	Type I a (*M. bacter–M. monas–M. microbium)*
Ia575	Type I a (*M. bacter–M. monas–M. microbium–M. sarcina)*
JRC4-432	Japanese rice cluster #4
MclT272	*Methylocaldum tepidum*
MclG281	*Methylocaldum gracile*
MclE302	*Methylocaldum* E10
MclS402	*Methylocaldum szegediense*
Mcl408	*Methylocaldum*
501-375	*Methylococcus*-related marine and freshwater sediment clones
501-286	*Methylococcus*-related marine and freshwater sediment clones
USC3-305	Upland soil cluster #3
Mc396	*Methylococcus*
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
fw1-286	fw1 group: *M. coccus–M. caldum* related marine and freshwater sediment clones
LW21-374	LW21 group
LW21-391	LW21 group
OSC220	Finnish organic soil clones and related
OSC300	Finnish organic soil clones and related
JRC3-535	Japanese Rice Cluster #3
LK580	fw1 group + Lake Konstanz sediment cluster
JRC2-447	Japanese Rice Cluster #2
M90-574	*M. coccus–M. caldum* related marine and freshwater sediment clones
M90-253	*M. coccus–M. caldum* related marine and freshwater sediment clones
Mth413	*Methylothermus*
Ib453	Type I b (*M. thermus–M. coccus–M. caldum* and related)
Ib559	Type I b (*M. thermus–M. coccus–M. caldum* and related)
DS3-446	Deep sea cluster #3
JR2-409	JR cluster #2 (California upland grassland soil)
JR2-468	JR cluster #2 (California upland grassland soil)
JR3-505	JR cluster #3 (California upland grassland soil)
JR3-593	JR cluster #3 (California upland grassland soil)
Nc_oce4 26	*Nitrosococcus oceani*
USCG-225	Upland soil cluster Gamma
USCG-225b	Upland soil cluster Gamma
Mcy233	*Methylocystis*
Mcy413	*Methylocystis*
Mcy522	*Methylocystis* A + peat clones
Mcy264	*Methylocystis*
Mcy270	*Methylocystis*
Mcy459	*Methylocystis*
Mcy255	*M. cystis B (parvus/echinoides/* strain M)
McyM309	*M. cystis strain M and related*
McyB304	*M. cystis B (parvus/echinoides/* strain M)
MsT214	*Methylosinus trichosporium* OB3b and rel.
Msi520	*M. trichosporium*
Msi269	*M. trichosporium*
MsS314	*Methylosinus sporium*
MsS475	*Methylosinus sporium*
Msi263	*Methylosinus sporium* + 1 *Msi. trichosporium subclaster*
Msi423	*Methylosinus*
Msi294	*Methylosinus*
Msi232	*M. sinus* + most *M. cystis*-considered as additional type II probe
Peat264	Peat clones
II509	Type II
II630	Type II
xb6-539	Novel *pmoA* copy of type II and related environmental clones

(Continued)

Table A2 | 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* (*)
NMsiT-271	Novel *pmoA* copy of *M. sinus trichpsporium* (*)
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
B2all341	*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	Universal
mtrof362-I	Methanotrophs
mtrof661	Methanotrophs
mtrof662-I	Methanotrophs
mtrof656	Methanotrophs
NmNc53 3	*Nitrosomonas–Nitrosococcus*
Nsm_eut 381	*Nitrosomonas eutropha*
PS5-226	*Nitrosomonas–Nitrosococcus* related clones
Pl6-306	*Nitrosomonas–Nitrosococcus* related clones
NsNv207	*Nitrosospira–Nitrosovibrio*
NsNv363	*Nitrosospira–Nitrosovibrio*
Nit_rel47 1	AOB related clones/probably methanotrophs
Nit_rel22 3	AOB related clones/probably methanotrophs
ARC529	AOB related clones/probably methanotrophs
Nit_rel47 0	AOB related clones/probably methanotrophs
Nit_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.*

B

Nitrogen loading:

- 2.5 g N m ² (NH_4NO_3) dose
loaded four times in growing season.
- Equal volume of H_2O 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

Soil sampling from control and manipulated plots:

FIGURE A1 | (A) The littoral wetland of Lake Kevätön in July 2007. For experiment, three control and three manipulated plots of 1.44 m^2 were established to 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_4NO_3 solution or distilled water.

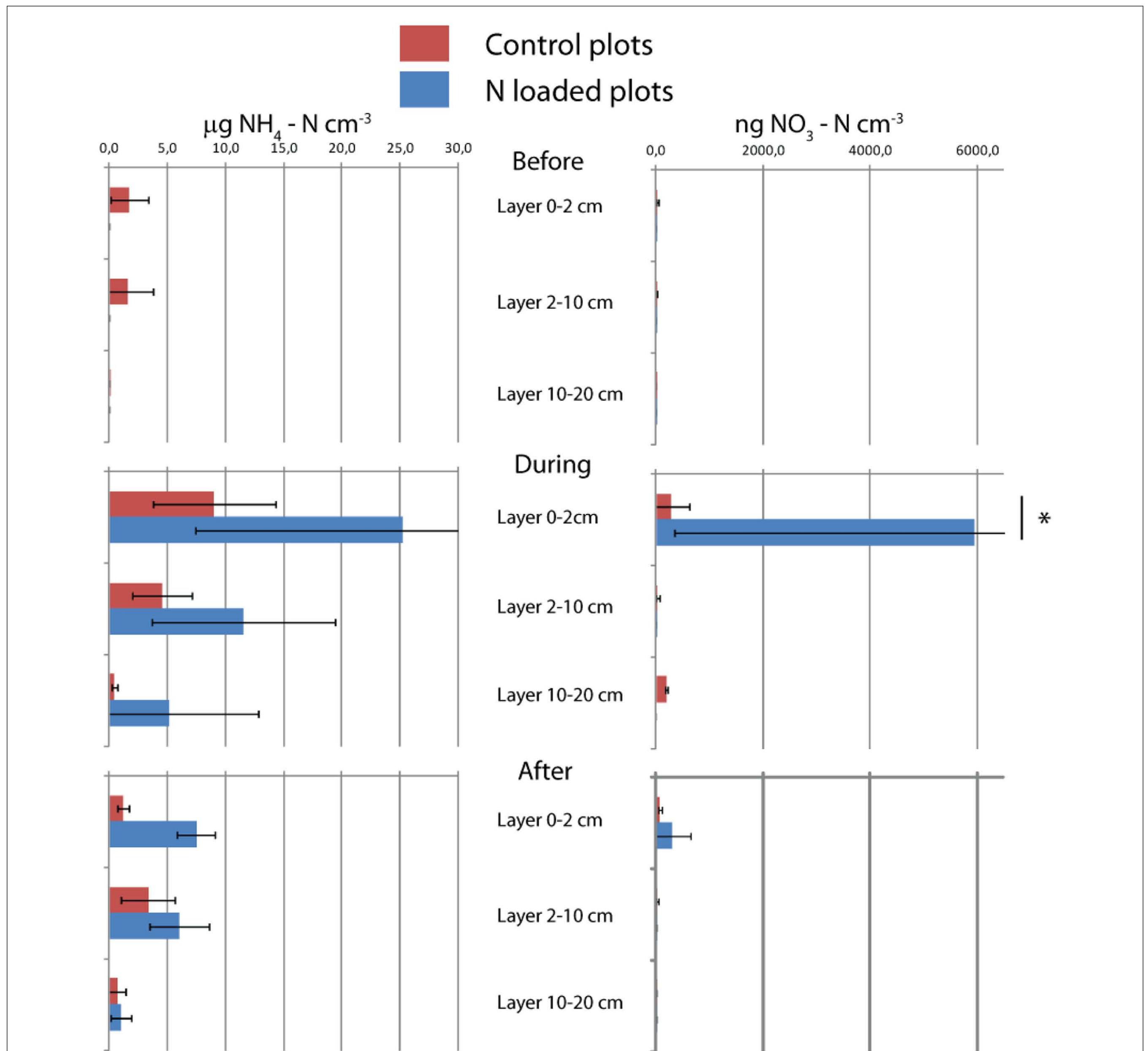

FIGURE A2 | Nitrogen content of soil. Means and SDs of triplicates are shown. The asterisk indicates the difference between control and manipulation ($P < 0.01$).

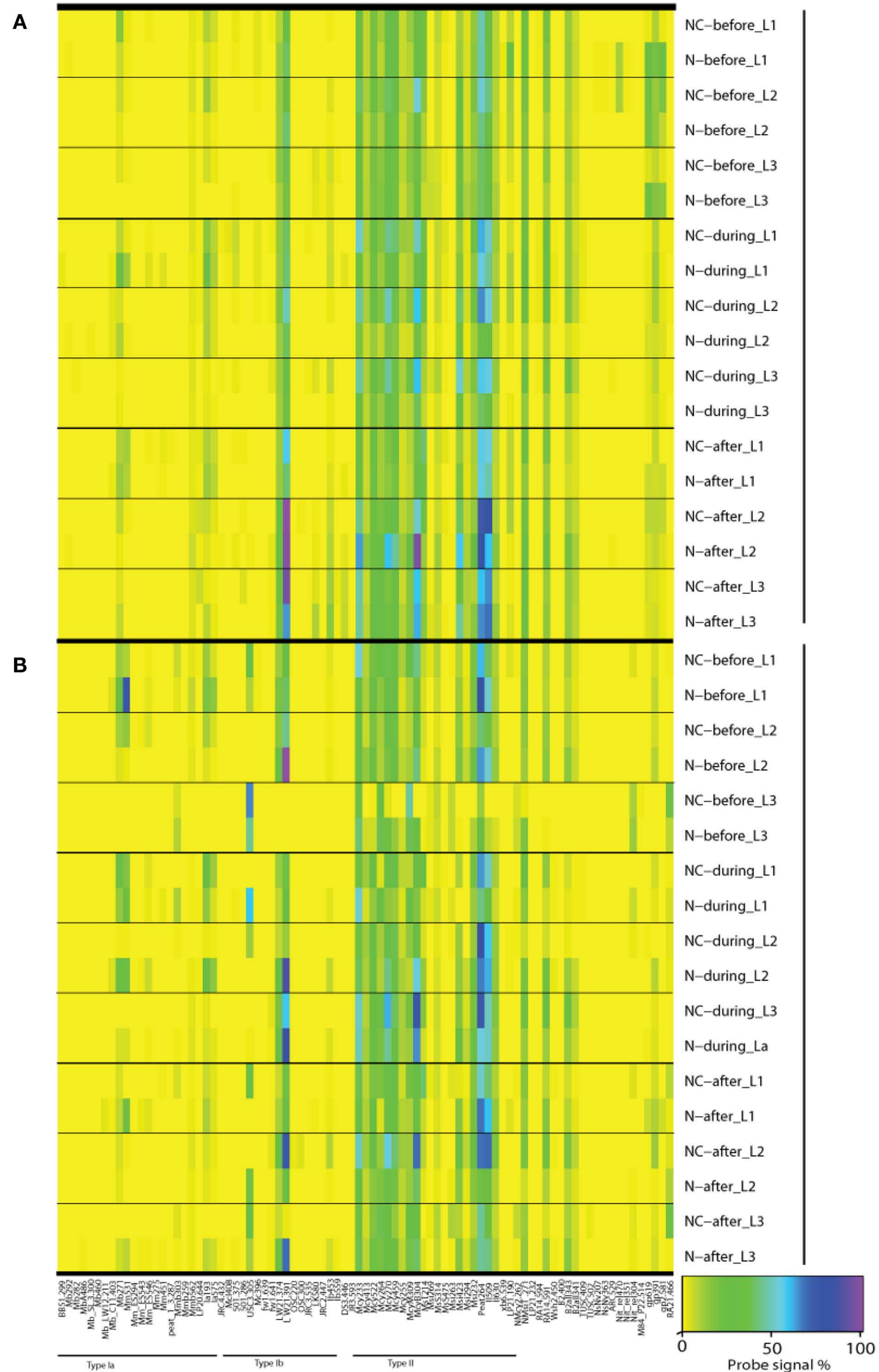

FIGURE A3 | Microarray results of community composition [(A), DNA] and functional diversity [(B), RNA] of methanotrophs before, during, and after nitrogen loading. Averages of triplicate plots are shown. A value of 100 (purple) indicates the maximum and a value of 0 (yellow) indicates the minimum signal 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.

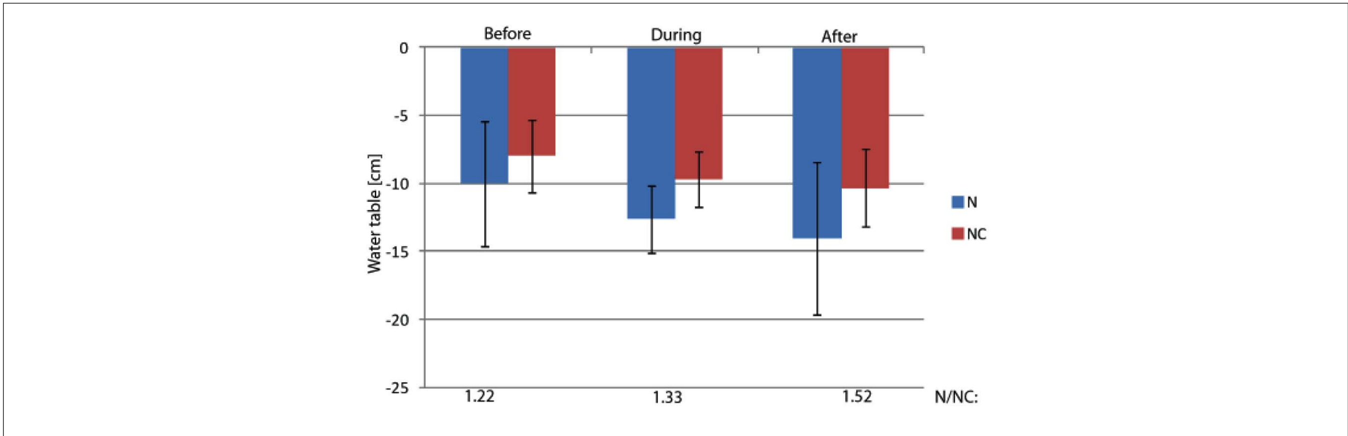

FIGURE A4 | The water table in the study plots. The water table was measured from perforated plastic tubes inserted in soil inside the study plots. The ratio of mean water level of nitrogen loaded and control plots is marked on bottom of the figure.

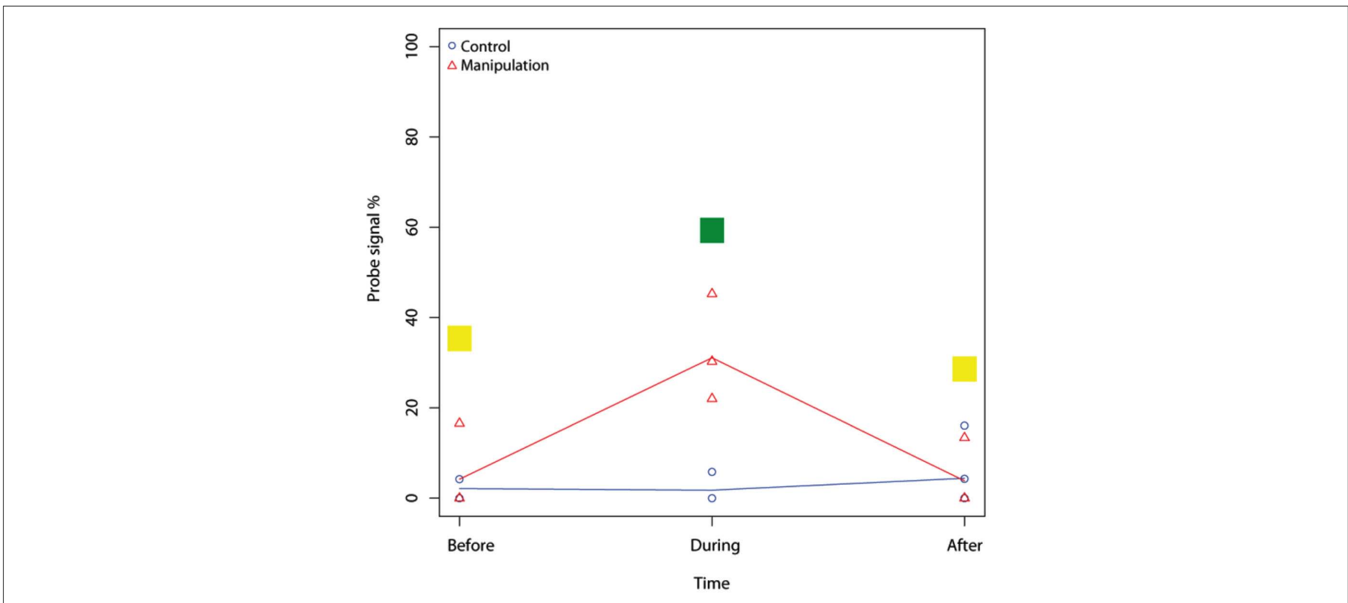

FIGURE A5 | Example of a mixed-effect model result for the microarray probe Mb271 (RNA samples, 2–10 cm). Mixed-effect model comparison evaluated the difference between two fitted models, the control and the manipulation model. In Figure 3A, the result of each comparison is shown in color, thus, no effect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