Attenuation of Methane Oxidation by Nitrogen Availability in Arctic Tundra Soils

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Jaehyun Lee[1], Jeongeun Yun[1], Yerang Yang[1], Ji Young Jung[2], Yoo Kyung Lee [2], Junji Yuan[3], Weixin Ding[3], Chris Freeman[4], Hojeong Kang[1]*

[1]School of Civil and Environmental Engineering, Yonsei University, Seoul 03722, South Korea.
[2]Korea Polar Research Institute, Incheon 21990, South Korea.
[3]State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China.
[4]School of Natural Sciences, Bangor University, Bangor LL57 2UW, UK.

*** Corresponding author**

Professor Hojeong Kang

School of Civil and Environmental Engineering

Yonsei University

Seoul, 03722

South Korea

E-mail: hj_kang@yonsei.ac.kr

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Abstract

CH_4 emission in the Arctic has large uncertainty due to the lack of mechanistic understanding of the processes. CH_4 oxidation in Arctic soil plays a critical role in the process, whereby removal of up to 90% of CH_4 produced in soils by methanotrophs can occur before it reaches the atmosphere. Previous studies have reported on the importance of rising temperatures in CH_4 oxidation, but because the Arctic is typically an N-limited system, fewer studies on the effects of inorganic nitrogen (N) have been reported. However, climate change and an increase of available N caused by anthropogenic activities has recently been reported, which may cause drastic change of CH_4 oxidation in Arctic soils. In this study, we demonstrate that excessive levels of available N in soil cause an increase of net CH_4 emissions via reduction of CH_4 oxidation in surface soil in Arctic tundra. *In-vitro* experiments suggested that N in the form of NO_3^- is responsible for the decrease in CH_4 oxidation via influencing soil bacterial and methanotrophic communities. Findings of our meta-analysis suggest that CH_4 oxidation in the boreal biome is more susceptible to addition of N than other biomes. We provide evidence that CH_4 emissions in Arctic tundra can be enhanced by an increase of available N, with profound implications for modeling of CH_4 dynamics in Arctic regions.

Synopsis

In this study, in-situ, in-vitro manipulation experiments, and global meta-analysis were used to examine the response of CH_4 oxidation in soil to an increase of available nitrogen in Arctic tundra.

1. Introduction

In the northern permafrost region, approximately 1330–1580 Pg of carbon (C) is stored in soil, 1/3 of global storage of soil organic carbon (SOC).[1,2] However, due to the permafrost thaw induced by warming, SOC stored in this region can be released to the atmosphere in the form of CO_2 and CH_4.[3–7] Our current knowledge of this issue and our ability to make predictions are limited, and there is great uncertainty due to the lack of understanding of the response of northern permafrost zone ecosystems, particularly microbial processes, to future climate change.[8–10]

The permafrost region is classified as an N-limited environment due to its low N mineralization rate[11], low atmospheric N deposition (~ 10 kg N ha^{-1} yr^{-1}; Geels et al.[12]), and the rapid uptake of the small amount of available N by plants and microbes.[13] However, findings from recent studies have suggested that an increase of soil available N may occur through several potential mechanisms: (1) Increased soil temperature can stimulate decomposition of soil organic matter (SOM)[14,15]; (2) Liberation of inorganic and organic N may occur through permafrost thaw[16,17]; (3) Expansion of the extent of shrub vegetation harboring N-fixing symbionts could occur through an increase of soil temperature [18–20]; (4) Extreme N deposition events with occurrence of over 40% of regional annual N deposition within a few days could become more frequent.[21] Choudhary et al.[22] suggested that N resulting from extreme deposition is largely retained within the tundra regardless of the N forms and remains there over two growing seasons. Permafrost regions are defined as N-limited ecosystems, thus substantial changes in various ecosystem processes can be induced by increases in available N.[23–25]

CH_4 is oxidized in soil by methane-oxidizing bacteria using oxygen as a terminal electron

acceptor. Aerobic methanotrophs belong to the Gammaproteobacteria (Type I, Methylococcaceae and Methylothermaceae families), Alphaproteobacteria (Type II, Methylocystaceae and Beijerinckiaceae families), and the Verrucomicrobia phyla (Methylacidiphilaceae family)[26,27]. Verrucomicrobial methanotrophs are known to play an important role in methane oxidation in extreme environments [28,29] whereas proteobacterial methanotrophs are found in moderate and extreme environments.[26,30,31] Methanotrophs that are adapted to oxidize low concentrations of CH_4 are called high-affinity methanotrophs and those that are adapted to the high concentration of CH_4 are called low-affinity methanotrophs.[32–34] Most soils exhibit both affinities of oxidation with a transition from high-affinity to low-affinity CH_4 oxidation observed between 100 and 1000 ppm CH_4.[35]

CH_4 oxidation in surface soil plays an important role in determining CH_4 fluxes in the ecosystem, as illustrated by the fact that oxidation of up to 90% of CH_4 produced by methanogenesis in deep soils by methanotrophs can occur before it reaches the atmosphere.[36,37] Moist acidic tundra provides a favorable condition for methanogenesis, and large amounts of CH_4 are released to the atmosphere.[38–41] Thus, a decrease in the CH_4 oxidation process in soil in the tundra can cause a substantial increase in CH_4 emission. Inorganic N such as NH_4^+ and NO_3^- is an important environmental variable controlling CH_4 oxidation in soil.[42] The effects of these forms of N are dependent on the types of ecosystems and management schemes.[42] For example, in forest soil, CH_4 oxidation is usually inhibited by NH_4^+ through competition with CH_4 for the active site of methane monooxygenase (MMO) enzyme.[43–48] Inhibition of CH_4 oxidation by NO_3^- occurs through production of NO_2^-, which is toxic to methanotrophs.[49–51] NO_3^- can also stimulate CH_4 oxidation by releasing N limitation in forest soil.[44,52,53] In wetlands, NH_4^+ and NO_3^- have shown inhibitory effects [54–56], stimulatory effects[35,57], or no effect.[58] In addition to the form of inorganic N, van Dijk et al.[59] suggested that the effect of

ammonium is different between soluble ammonium and exchangeable ammonium. Although several studies have examined the effect of inorganic N on CH_4 oxidation in soil in diverse ecosystems, studies on the response of Arctic tundra have rarely been reported, limiting our capability for estimation of CH_4 dynamics in this ecosystem.

The different responses of soil CH_4 oxidation to inorganic N may be explained in part by differences in methanotrophic community composition. For example, findings from pure-culture studies have demonstrated that the effects of inorganic N enrichment on the growth rate and activity of methanotrophs are species-specific.[60,61] The different responses of methanotrophic activity between type I and type II methanotrophs to N addition were reported.[62–64] For example, Mohanty et al.[63] suggested that type II methanotrophs can be more vulnerable to N addition than type I methanotrophs. In addition to the methanotrophic community, recent studies have shown that the activity and growth rate of methanotrophs are significantly promoted by the diversity of heterotrophic bacteria through generation of products that are beneficial for methanotrophs or by removal of inhibitory products.[65–70] In other words, the response of CH_4 oxidation to addition of inorganic N can also be affected by different heterotrophic communities and the diversity of each ecosystem. However, the majority of previous studies focused on physico-chemical characteristics of soil, and did not include a detailed analysis of the microbial community, thus these conclusions may underestimate the significance of microbial biodiversity.

Recent studies have proposed that CH_4 uptake by high-affinity methanotrophs will increase as temperatures rise in Arctic mineral cryosols.[71,72] However, this hypothesis only considers the effect of NH_4^+ provided by deposition whereby the effect of NO_3^- and other inorganic N sources (e.g. N mineralization, permafrost thaw) were not considered. This study

aimed to examine the effect of an increase of inorganic N (NH_4^+ and NO_3^-) on CH_4 oxidation in soil in Arctic tundra. *In-situ* and *in-vitro* manipulation experiments were performed to determine the effect of inorganic N on (1) CH_4 fluxes in the field, (2) soil CH_4 oxidation in different vegetation types, and (3) the composition and diversity of the methanotrophic and bacterial community. In the *in-vitro* experiment, the response of high-affinity and low-affinity CH_4 oxidation was examined separately because their response to N can differ.[32] We hypothesized that an increase in available N would result in substantial attenuation of CH_4 oxidation in soil in Arctic tundra by (1) enzymatic competition and/or production of NO_2^-, (2) causing changes to the composition of the methanotrophic community, and (3) causing a decrease of non-methanotrophic bacterial diversity[73,74]. In addition, a meta-analysis was performed in order to examine the response of CH_4 oxidation in soil to inorganic N in different ecosystems and biomes, to elucidate general patterns with regard to the effects of N on CH_4 oxidation.

2. Methods

2.1 Site description

Samples were collected from an Arctic region of moist acidic tundra located in Council, Alaska (64°51′N, 163°39′W). The dominant vegetation within the site was either tussock (*Eriophorum vaginatum*), dwarf shrub (*Vaccinium uliginosum*), lichen (*Cladonia spp.*), or moss (*Sphagnum spp*.).[75] The mean annual temperature and precipitation at the site were −2.6°C and 428 mm, respectively, and the depth of the soil active layer was 45-70 cm during the sampling period (September 2017). The soil was classified as Histic-turbic Cryosols using a WRB system (Typic Histoturbels with a US soil Taxonomy).

2.2 Field manipulation experiment

In September 2016, a field manipulation experiment was conducted in order to examine the effect of N addition on CH_4 flux. We established four plots (10m×10m) with relatively uniform vegetation types (tussock and moss dominant) and white-colored opaque PVC chambers (30 cm diameter×30 cm height, cylindrical shape) were installed. Installing a chamber on the soil surface causes pressure downward, which can pull out CH_4 captured in the subsoil layer. Additionally, physical disturbance to the plant can affect CH_4 flux immediately after installation. To minimize the disturbance effect on soil CH_4 flux, chambers were stabilized for 10 days. After stabilization, N was added to each plot as follows: control (distilled water), low N (0.02g N m^{-2}), and high N (0.2g N m^{-2}). N fertilizer was applied as a form of NH_4NO_3. Low N was applied to simulate extreme N deposition events where up to 40% of the annual atmospheric N deposition occurs in less than a week (Hodson et al., 2005). High N was applied

149 to simulate the combined effect of extreme N deposition and other inorganic N sources (e.g.
150 permafrost thaw, warming-induced mineralization, shrub expansion). The distance between
151 each treatment was at least 5 m and the fertilizer was added into the flux chamber to prevent
152 cross-contamination. Three treatments (control, low N, high N) were added to each plot, thus
153 the number of replicates for each treatment was 4. Measurements of CH_4 fluxes were performed
154 immediately following additions (D0), and seven days after (D7). A closed static chamber
155 method was used, and the headspace gas samples were transferred to a pre-evacuated glass vial
156 every 15 minutes for 60 minutes. The air temperature inside the chamber was measured before
157 and after gas measurement and considered in gas flux calculation. A GC-FID (CP-3800 Varian,
158 CA, USA; PORAPAK Q 80/100 column, with the detector operated at 150°C, carrier gas: N_2)
159 apparatus was then used for measurement of CH_4 concentration and calculation of CH_4 fluxes
160 using the following equation:

161 $$F_{CH_4} = \frac{dC_{CH_4}}{dt} \times \frac{V \times M_{CH_4} \times T}{A \times V_{mol} \times T_0} \quad (1)$$

162 where F_{CH_4} is the flux of CH_4 gas (μg CH_4 m^{-2} min^{-1}); dC_{CH_4}/dt is the rate of change in CH_4
163 concentration (10^{-6} min^{-1}) over time; V is the headspace volume of the chamber (m^3); M_{CH_4} is
164 the molecular weight of CH_4 (16 g mol^{-1}); A is the surface area of the chamber (m^2); V_{mol} is
165 the volume of a mole at a certain temperature (22.4L mol^{-1}).
166

167 ## *2.3 Incubation experiment setup*

168 Soils for incubation experiment were collected in September 2017 in areas consisting of three
169 different vegetation types (shrub, tussock, and moss) according to the soil moisture gradient
170 (Table S1). Prior to sampling, the aboveground vegetation and Oi layer were removed, and

samples were collected from a depth of 0-20 cm in the organic layer (three samples per vegetation type). Most mineral A horizons were found below a depth of 50 cm. Collected samples were placed in plastic zipper bags and transported to the laboratory on ice. Samples were stored at −20°C until soil analysis and incubation experiments.

Frozen samples of each vegetation type (shrub, tussock, and moss) were placed in a 4°C refrigerator for 14 days to prevent the effect of rapid thaw. Following gentle homogenization, the samples were placed in 120 ml vials (5 g fresh soil) and 946 ml Mason jars (30 g fresh soil). Although the use of homogenized soil samples may have a limitation in that it does not represent actual field conditions, it is still a widely used method for measuring specific microbial activity (e.g. CH_4 oxidation) and gives us a clear result. Vials were capped with butyl rubber stoppers and Mason jars were closed tightly and sealed with Teflon tape to ensure gas tightness. In the first phase (induction), CH_4 was injected at an initial headspace concentration of 50 ppm into the Mason jars (high-affinity CH_4 oxidation) and at 1000 ppm into the vials (low-affinity CH_4 oxidation). Measurement of the headspace CH_4 concentrations was performed every 72 h until the concentration had decreased to 2 ppm for high-affinity oxidation, and 100 ppm for low-affinity oxidation, which took 15-21 days. After induction, containers were flushed with room air. Nitrogen was then added to the jars as $(NH_4)_2SO_4$ (AS), KNO_3 (PN), and NH_4NO_3 (AN) at 10, 50, or 100 μg N g dry soil^{-1}. The maximum level of N (100 μg N gdw^{-1}) was approximately four times the peak extractable N concentration observed in moist acidic tundra soil.[76] Treatment with distilled water only was used as a control. Triplicate samples were used in performance of each treatment. CH_4 was injected and measurement of the headspace concentrations was performed every 48 hours until the CH_4 concentration had decreased to certain levels, as described above. Headspace gas was collected

from Maison jar (8mL) and vial (2mL) and transferred to pre-evacuated 5mL exetainer. Gas sample from vial was diluted with 6mL N_2 to meet the volume requirement for GC measurement (5mL). The same volume of collected gas was compensated by synthetic air (20% O_2 & 80% N_2) and accounted for in calculations. All incubations were performed at 15°C in the dark. Incubation of additional jars was performed without CH_4 enrichment in order to verify the influence of methanogenesis; changes in headspace CH_4 concentration were minor, suggesting that the effect of methanogenesis was negligible. In addition, three empty Maison jars and vials were incubated without soil to assess the occurrence of leakage during the incubation.

Headspace CH_4 concentration was analyzed using GC-FID (CP-3800 Varian, CA, USA; PORAPAK Q 80/100 column, with the detector operated at 150°C, carrier gas: N_2). Calculation of the potential CH_4 oxidation rate was based on the following equation:

$$\text{Potential } CH_4 \text{ oxidation rate } (\mu g\ CH_4\ gdw^{-1}\ d^{-1}) = \frac{dCH_4}{dt} \times \frac{V \times M_{CH_4}}{gdw \times V_{mol}} \quad (2)$$

where dCH_4/dt is changes in methane concentration over time (10^{-6} d^{-1}); V is headspace volume of vial or jar (L); M_{CH_4} is the molecular weight of CH_4 (16 g mol^{-1}); gdw is dry weight of soil (g); and V_{mol} is the volume of a mole in a certain temperature (22.4L mol^{-1}). In addition, calculation of the inhibition rate was based on the following equation:

$$\text{Inhibition rate } (\%) = \frac{P_c - P_t}{P_c} \times 100 \quad (3)$$

where P_c is the potential CH_4 oxidation rate of control; P_t is the potential CH_4 oxidation rate for each treatment.

In addition to the effect of inorganic N, we further performed supplementary experiments to investigate the effect of salt (K_2SO_4) and glucose, which can be indirect

controlling factors induced by inorganic N. Detailed methods are described in Supporting Information (Text S1, S2, S3).

Although the microbial community can be changed during the sample storage at -20 °C since microbes in Arctic regions are cold-tolerant, soil samples were frozen for 8 months (September 2017 – May 2018), which matches the frozen season of the study site. Thus, we assume that our sample storage cycle follows the actual cycle of *in-situ* conditions. In addition, we performed 3 steps of preincubation to let the microbes adapt to the new temperatures.

2.4 Chemical analysis

Analysis of chemical characteristics of both fresh and post-incubation soil was performed. Oven-drying was performed at 105°C for 24 h for measurement of soil moisture content, and the loss-on-ignition method was used for determination of soil organic matter content. Measurement of soil pH was performed using a pH meter after mixing soil:distilled water in a ratio of 1:5 and centrifugation was performed for precipitation of soil particles. Extraction of soil NO_2^- and NO_3^- was performed by mixing soil with distilled water as described above and the extract was subsequently filtered through a 0.2-µm filter, followed by analysis using ion chromatography (ICS-1100; Dionex, Sunnyvale, CA, USA). Extraction of soil NH_4^+ was performed using 2 M KCl and measurements were performed using the indophenol blue method.[77]

2.5 DNA extraction and T-RFLP analysis of methanotrophs

Subsampling of soil samples was performed after incubation, followed by storage at

238 −80°C until DNA extraction. Subsamples of 0.4 g were placed in the bead tubes supplied with
239 the PowerSoil DNA Extraction Kit (Qiagen, Chatsworth, CA, USA) and extraction of DNA
240 was performed according to the manufacturer's instructions. Terminal restriction fragment
241 length polymorphism (T-RFLP) analysis was performed in order to examine the composition
242 and diversity of the methanotrophic community. In brief, amplification of the *pmoA* gene was
243 performed using the fluorescence labeled forward primer A189f (5′-[6FAM]-
244 GGNGACTGGGACTTCTGG-3′) and reverse primer A682r (5′-GAASGCNGAG
245 AAGAASGC-3′).[78] A detailed description of the T-RFLP method is provided in Supporting
246 Information (Text S4).

247

248 *2.6 16S rRNA sequencing*

249 16S rRNA sequencing was performed in order to examine the soil bacterial community.
250 In brief, the forward primer 337F (5′-CCTACGGGNGGCWGCAG-3′) and reverse primer
251 805R (5′-GACTACHVGGGTATCTAAT CC-3′) were used to amplify the V3–V4 region of the
252 bacterial 16S rRNA gene.[79] Preparation of libraries was based on the Illumina 16S
253 Metagenomic Sequencing Library protocol. Sequencing of the libraries was performed by
254 Macrogen Inc. (Seoul, Republic of Korea) using an Illumina (San Diego, CA, USA) MiSeq
255 platform v3 technology (2 × 300 bp, paired-end). A detailed description of the 16S rRNA
256 sequencing method is provided in Supporting Information (Text S5).

257

258

2.7 Meta-analysis

A meta-analysis was conducted by extracting potential CH_4 oxidation data from published journal articles that reported data on both control and added N treatments in order to examine the effect of N addition on CH_4 oxidation in soil. In brief, a search of the literature was conducted in an online database (ISI Web of Science, Science Direct, Google and Google Scholar) for collection of studies; the following search terms were used in any combination: nitrogen, N, addition, fertilization, CH_4, CH_4 oxidation, methanotroph. Data were extracted from tables and figures. In total, 192 paired measurements were collected from 22 publications (Supporting data); each pair contained potential CH_4 oxidation rate values for single N addition and control. A detailed description of the meta-analysis is provided in Supporting Information (Text S6).

For each paired measurement, the response ratio was used as the effect size measure as follows:

$$\text{Response ratio, } \ln RR = \ln(X_t/X_c) \quad (4)$$

where X_c and X_t are paired average potential CH_4 oxidation rate values for the control (X_c) and N addition (X_t). The percentage change in CH_4 oxidation in response to addition of N was calculated as follows:

$$\% \text{ change} = (\exp(\ln RR) - 1) \times 100\% \quad (5)$$

2.8 Statistical analysis

SPSS 23 (SPSS Inc., Chicago, IL, USA) was used in performance of all statistical analyses.

Analysis of the differences in potential CH_4 oxidation rate, inhibition rate, field CH_4 flux, and Shannon diversity index of each treatment was performed using the one-way ANOVA test. Two-way ANOVA was used to examine the difference in inorganic N content with treatment and vegetation type after incubation. Polynomial regression analysis was performed in order to determine the relationship between potential CH_4 oxidation rate and bacterial Shannon diversity index. Linear regression analysis was performed in order to determine the relationship between CH_4 oxidation inhibition rate and increase of NO_3^- content in soil. Pearson correlation analysis was performed in order to determine the correlation between NO_3^- content and bacterial Shannon diversity index.

3. Results

3.1 Effects of N fertilization on soil CH_4 flux in the field

Overall, the study site was a net CH_4 source, where positive CH_4 fluxes were measured across the time and treatment (Figure 1). On day 0, low N (1.38 μg CH_4 m^{-2} min^{-1}) and high N treatment (1.78 μg CH_4 m^{-2} min^{-1}) showed similar CH_4 fluxes compared with control (0.54 μg CH_4 m^{-2} min^{-1}). On day 7, similar CH_4 fluxes were observed in control (0.71 μg CH_4 m^{-2} min^{-1}) and low N treatment (0.41 μg CH_4 m^{-2} min^{-1}) while high N treatment showed a substantially higher CH_4 flux (5.71 μg CH_4 m^{-2} min^{-1}).

3.2 Effect of N on the behaviors of CH_4 and microbial community

3.2.1 Soil inorganic N contents

Soil inorganic N contents (NH_4^+ and NO_3^-) after incubation are shown in Figure S1. During the incubation experiment, an increase in the soil NH_4^+ content of control was observed in comparison with fresh soil ($P < 0.01$) indicating that there was net N mineralization. The highest NH_4^+ content was observed in AS100, followed by AS50 and AN100, while no significant increase of the content was observed in AN10 and AS10 compared with the control.

No significant change of the soil NO_3^- content of the control was observed in comparison with fresh soil. At 50 ppm CH_4, the highest NO_3^- content was generally observed in shrub soil and the lowest in moss soil. The highest NO_3^- content was observed in PN100, followed by AN100 and PN50 in all vegetation types at both 50 and 1000 ppm CH_4. In PN50 and PN100, only a slight decrease of added NO_3^- was observed in shrub and tussock soils while

311 ~50% added NO_3^- reduced in moss.

312

313 *3.2.2 Effect of inorganic N on CH_4 oxidation*

314 Effect of inorganic N on CH_4 oxidation is shown in Table S2. Potential high-affinity CH_4
315 oxidation rates of control were 0.64, 0.59, and 0.50 μg CH_4 gdw^{-1} d^{-1} in shrub, tussock, and
316 moss soil, respectively, showing significant differences ($P < 0.001$). Inhibitory effects of
317 inorganic N were only observed in PN100 in shrub and tussock soils, with inhibition of 25.9%
318 and 25.0%, respectively (Figure 2).

319 Potential low-affinity CH_4 oxidation rates of control were 15.3, 11.91, and 9.16 μg
320 CH_4 gdw^{-1} d^{-1} in shrub, tussock, and moss soil, respectively, showing significant differences
321 ($P < 0.001$). CH_4 oxidation was significantly inhibited by PN50 and PN100 in all vegetation
322 types at 1000 ppm CH_4 (Figure 2). The strongest inhibitory effect was observed in shrub soil
323 (73% inhibition in PN100) and the weakest in moss soil (36% inhibition in PN100). NH_4^+ had
324 no significant effects on low-affinity CH_4 oxidation even in AS100. The combined effect of
325 NH_4^+ and NO_3^- was only significant in AN100; this inhibitory effect was similar to that
326 observed in PN50.

327

328 *3.2.3 Methanotrophic community composition*

329 Relative abundances of 14 terminal restriction fragments (TRFs) are shown in Figure 3. Among
330 14 different TRFs, a 245-bp fragment associated with *Methylocystis* and *Methylosinus* (Table
331 S3) was the most abundant (38%–82%) in all vegetation types. According to the 16S rRNA

sequence results, *Methylocystis rosea* was generally the most predominant species (16.72 to 88.85%) followed by *Methylosinus trichosporium* (5.57 to 63.51%.), which is in accordance with T-RFLP results that showed predominance of *methylocystis* and *methylosinus* (Figure 3). Under 50ppm CH_4, the relative abundance of *Methylocystis rosea* was highest in tussock (56.8%) followed by moss (42.4%) and lowest in shrub (21.2%). The relative abundance of *Methylosinus trichosporium* was not significantly different among species. *Methylocystis hirsute* was only detected in shrub (27.8%). Under 1000ppm CH_4, the relative abundance of *Methylocystis rosea* was highest in tussock (70.3%) while statistically similar in moss (54.5%) and shrub (42.6%). The relative abundance of *Methylosinus trichosporium* was not significantly different among species. *Methylocystis hirsute* was only detected in shrub (8.3%). Overall, predominance of type II methanotrophs was observed at our study site while dominance of type I was minor. Methanotrophic community structure was separated by vegetation types whereas fertilizer's types and concentrations did not affect methanotrophic community (Figure S2).

3.2.4 Correlation between CH_4 oxidation activity and bacterial diversity

The results of regression analysis showed overall positive correlations between soil CH_4 oxidation rate and bacterial diversity (Shannon index value) at 50 ppm CH_4 (Figure 4a; R^2 = 0.46, $P < 0.001$) and 1000 ppm CH_4 (Figure 4b; $R^2 = 0.39$, $P < 0.001$). We also found that CH_4 oxidation rate and bacterial diversity showed significant positive correlations in tussock soil at 50 ppm CH_4, shrub soil at 1000 ppm CH_4, and moss soil at 1000 ppm CH_4 within each vegetation and affinity type (Figure S3). No significant correlation was observed in moss soil at 50 ppm, shrub soil at 50 ppm, and tussock soil at 1000 ppm CH_4. In addition, significant

355 negative correlations were found between NO_3^- content in soil and bacterial diversity with the
356 exception of moss soil at 50 ppm CH_4 and tussock soil at 1000 ppm CH_4 (Table S4).
357

358 ### *3.3 Response of CH_4 oxidation to N fertilizer in diverse ecosystems: Meta-*
359 ### *analysis*

360 The results of a meta-analysis of changes in the CH_4 oxidation rate in response to addition of
361 N are shown in Figure 5. Overall, the effect of N addition on the potential CH_4 oxidation rate
362 was negligible, however, CH_4 oxidation was significantly affected by the type of fertilizer (P
363 = 0.011). Unlike previous studies that reported competitive inhibition by NH_4^+ as a major
364 inhibitory mechanism, we found that CH_4 oxidation was significantly inhibited by NO_3^-
365 (−17.3%), while it was stimulated by addition of NH_4^+ (4.9%) and urea (37.6%). Strong
366 inhibitory effects of N addition were observed in peatland (−71.6%), forest (−39.7%), tundra
367 (−34.7%), and farm (−22.3%) while a weak inhibitory effect was observed in grassland soils
368 (−12.0%). By contrast, CH_4 oxidation was significantly stimulated by addition of N in landfill
369 (61.9%) and paddy (96.6%). Biome type was also a significant factor (P = 0.006), where N
370 addition significantly inhibited soil CH_4 oxidation in boreal regions (−39.7%) but stimulated it
371 in tropical regions (52.1%) whereas the minor effect was observed in temperate regions (7.4%).
372

4. Discussion

4.1 Enhanced CH_4 emission by N fertilizer in the field

In the field, the effect of both low N and high N treatments on CH_4 emissions was negligible in comparison with control on day 0 (Figure 1). On day 7, high N treatment resulted in 704% higher CH_4 emission compared to control, while no significant difference was observed between CH_4 emission with low N treatment and control. It appeared that the majority of N added in the low N treatment was immobilized by plants and microbes, or absorbed into the soil[59] while surface layer CH_4 oxidation was suppressed by residual N in the high N treatment, increasing net emission of CH_4. N addition may also stimulate methanogenesis in deeper soil. However, inhibition of methanogenesis by N in various wet ecosystems has been demonstrated[80,81] and development of the nutritional effects of added N on slow-growing methanogens would take longer than the direct inhibitory effects on methanotrophs[82], suggesting that reduced CH_4 oxidation in surface soil is primarily responsible for the enhanced emission of CH_4 in fertilized treatment.

4.2 Effect of inorganic N on CH_4 oxidation

In our study, CH_4 oxidation was substantially attenuated by the high concentration of NO_3^- whereas NH_4^+ did not show a significant effect. Previous studies have reported on several inhibitory mechanisms of NO_3^- on CH_4 oxidation. The majority of studies suggested that the denitrification-induced production of NO_2^- inhibits CH_4 oxidation in soil.[50,51,56,83,84] In this study, NO_2^- contents in soil after incubation were below the detection limit (data not shown), suggesting that production of NO_2^- from NO_3^- is unlikely to be the inhibitory mechanism.

NO_3^- can increase the C mineralization rate and stimulates the heterotrophic microbial process, consequently suppressing methanotrophic activity. For example, the addition of glucose resulted in a reduction of CH_4 oxidation in forest soil by 83% compared with control, and by 99.4% when combined with NO_3^- addition.[43] The authors suggested that the glucose addition stimulates the heterotrophic microbial processes and suppresses methanotrophic activity. We hypothesized that NO_3^- indirectly inhibits CH_4 oxidation activities by stimulating C mineralization and heterotrophic microbial processes. Potential CH_4 oxidation was not inhibited by the addition of glucose in comparison with the control (Figure S4). The addition of NO_3^- also had no effect on dissolved organic carbon content (Figure S5), suggesting that an increase of labile C compounds also did not have an underlying effect on the inhibition of CH_4 oxidation in our study.

Osmotic stress is induced by the addition of high concentrations of salts, which can thus inhibit methanotrophic activity.[46,50,54,56,85] King et al.[86] examined the effect of ammonium and non-ammonium salt addition on soil CH_4 oxidation activity and found that non-ammonium salt significantly inhibited CH_4 oxidation activity by inducing osmotic stress to methanotrophs. The addition of salt resulted in slight inhibition of high-affinity CH_4 oxidation, however, inhibition of low-affinity CH_4 oxidation was observed in shrub soil only (Table S5). CH_4 oxidation activity could be inhibited by the addition of KNO_3 through a decrease in pH.[56] However, no changes in soil pH were observed in N treatments compared with control (data not shown). Production of NH_4^+ from NO_3^- by dissimilatory nitrate reduction to ammonium[87] could also inhibit CH_4 oxidation. NH_4^+ inhibits CH_4 oxidation by inducing competition between methanotroph and ammonium oxidizer, and Ho et al.[88] suggested that this competition is significantly affected by the concentration of NH_4^+. However, we found a negligible effect of

418 NH_4^+ on CH_4 oxidation, suggesting that NH_4^+ produced from NO_3^- was not the inhibitory
419 mechanism.

420

421 ### *4.3 Methanotrophic composition determines the responses to NO_3^- addition*

422 While the mechanisms previously established by other research (e.g. NO_2^- production,
423 enzymatic competition, osmotic stress) were not observed in our study, we propose that,
424 nevertheless, microbial changes have significant influence on the inhibitory effect of NO_3^- on
425 CH_4 oxidation. Mediation of CH_4 oxidation occurs through the activity of microbes and tundra
426 is generally an N-limited environment; significant change in microbial community structure as
427 well as a decrease in microbial diversity and activity including methanotrophs can be induced
428 by an increase in available N.[89,90]

429 *Methylocystis* and *Methylosinus* were predominant methanotrophs in our study site
430 (Figure 3). According to previous studies of Arctic peatland and wetland, which have
431 ecosystems similar to that of moist acidic tundra, *Methylocystis* is the most active and
432 predominant methanotroph species.[91,92] Several characteristics enable *Methylocystis* to adapt
433 and thrive in such an environment. First, *Methylocystis* species, which are facultative
434 methanotrophs, are able to utilize other C compounds such as methanol, ethanol, and acetate
435 as their C sources, rather than CH_4.[93–96] This ability may enable survival of *Methylocystis* under
436 CH_4-limited conditions. Second, the genome sequence of *Methylocystis* suggests that it has the
437 ability to fix atmospheric N.[97,98] N fixation can enable better survival of *Methylocystis*
438 compared with other methanotrophs in N-limited Arctic ecosystems. *Methylosinus* also has the
439 ability to fix atmospheric N[99], implying that *Methylocystis* and *Methylosinus* are adopted to an

N-limited condition and their growth rate and activity can be significantly reduced by excessive N. In addition, some studies have reported that type II methanotrophs, including *Methylocystis* and *Methylosinus* are inhibited by excessive N while type I methanotrophs are stimulated.[100–102] Our results showing the predominance of type II methanotrophs (Figure 3) suggest that addition of NO_3^- resulted in significant inhibition of CH_4 oxidation due to the vulnerability of type II methanotrophs to excessive N.

According to findings from pure culture studies of methanotrophs, *Methylocystis* is more vulnerable to NO_3^- than to NH_4^+, while other methanotrophs bind preferentially to NO_3^-.[60,61] Therefore, we can assume that significant inhibition of CH_4 oxidation by addition of NO_3^- can be explained by the dominance of *Methylocystis* at our study site. In addition, another pure culture study reported a lack of inhibition of the growth rate and activity of *Methylocystis* under a high concentration of NH_4^+ [(103)], supporting our finding showing the effects of the absence of NH_4^+ on CH_4 oxidation. Previous studies reported that *Methylocystis* is the most active and predominant methanotrophic species in Arctic wetlands[91,92], therefore, it is plausible that an increase in NO_3^- content in soil could result in significantly reduced CH_4 oxidation in surface soil in such an environment.

This study used 16S amplicon sequencing and T-RFLP to investigate the methanotrophic community. These methods may provide relatively lower-resolution data compared to the amplicon sequencing of the specific functional genes. However, Cai et al.[104] examined the methanotrophic community of 20 forests in China and suggested that high-throughput sequencing of the 16S rRNA gene is a good alternative to *pmoA* sequencing to characterize soil methanotrophic community. In addition, Lindström et al.[105] suggested that T-RFLP can be trusted to show similar general community patterns as Illumina MiSeq. Our

results also show that the general pattern of the methanotrophic community (*Methylocystis* and *Methylosinus* dominant, community structure) is similar between T-RFLP and 16S rRNA sequencing results, suggesting that T-RFLP is still a feasible method to investigate the pattern of the methanotrophic community in the soil environment.

4.4 Positive correlation between CH_4 oxidation rate and bacterial diversity

Significant positive correlations were observed between soil CH_4 oxidation rate and bacterial diversity (Shannon index value) across all vegetation types ($P < 0.0001$; Figure 4). These data indicate the strong influence of bacterial diversity on CH_4 oxidation in soil. In addition, a significant negative correlation was found between bacterial diversity and NO_3^- content in soil with the exception of moss soil under 50 ppm CH_4 and tussock soil under 1000 ppm CH_4 (Table S4). These results suggest that the inhibitory effect of NO_3^- may depend on a decrease in bacterial diversity. In moss soil under 50 ppm CH_4, the inhibitory effect of NO_3^- addition on the oxidation rate of CH_4 may have been negated by the fact that there were no changes in bacterial diversity. CH_4 oxidation has previously been associated exclusively with methanotrophs, because they are the only functional group responsible for the process. However, findings from recent studies have suggested that methanotrophs and heterotrophs are mutually co-dependent, and that CH_4 oxidation may also be affected by general microbial diversity.[65–69] For example, a previous study reported a higher CH_4 oxidation activity for cultured methanotrophs with diverse heterotrophs compared to samples incubated with methanotrophs only.[65] It was suggested that this mutual relationship was based on the fact that certain heterotrophs produce essential metabolites for methanotrophs, thus stimulating CH_4 oxidation activity. In order to provide evidence for the symbiotic relationship between

methanotroph and non-methanotrophic bacteria, we further conducted the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis based on 16S rRNA amplicon sequencing data. The functional annotation of PICRUSt predictions was obtained based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Text S7). MMO showed overall positive correlations with methanol dehydrogenase and formate dehydrogenase (Figure S6). Krause et al.[68] suggested that the symbiotic relationship between methanotrophs and non-methanotrophs occurs through cross-feeding of methanol. Positive correlations observed between MMO and methanol dehydrogenase indicate a symbiotic relationship between these two microbial processes. Growth of methanotrophs may also be enhanced by formaldehyde produced by methanol dehydrogenase, a form of carbon assimilated by methanotrophs. A positive correlation between MMO and formate dehydrogenase may be related to the removal of alternative carbon compounds. Fender et al.[49] suggested that CH_4 oxidation activity was significantly inhibited by alternative carbon compounds including formate because methanotrophs utilize alternative carbon rather than CH_4. CH_4 is a more efficient carbon source for methanotrophic growth, thus the population of methanotrophs can be decreased by utilization of alternative carbon compounds.[106,107]

Reduction of soil bacterial diversity due to N fertilization has been reported, and, based on the findings of a meta-analysis, the inhibitory effect of NO_3^- is stronger than that of NH_4^+.[108,109] For example, Berthrong et al.[74] suggested that due to high energy cost, N_2 fixing microbes may decline with increasing N availability and microbes less tolerant to high osmotic stress may be killed, both resulting in the decline of microbial diversity. We found an overall negative relationship between soil NO_3^- content and bacterial diversity (Table S4). Our data indicate that the potential CH_4 oxidation rate is inhibited by addition of NO_3^- via a reduction

in bacterial diversity of soil, although the precise mechanism of the mutual relation between methanotrophic and non-methanotrophic bacteria has yet to be established.

4.5 Differences in responses according to vegetation type

We observed that the responses to addition of NO_3^- varied with different vegetation types (Figure 2). Strong inhibition by NO_3^- in shrub soil can be explained by osmotic stress. Findings from our supplementary study showed that addition of K_2SO_4 resulted in significant inhibition of CH_4 oxidation in shrub soil at 1000 ppm CH_4 (Table S5), indicating that CH_4 oxidation in shrub soil is severely inhibited by a combined effect of NO_3^- and osmotic stress. The weakest inhibitory effect was observed in moss soil, where no effect was observed at 50 ppm CH_4. This finding could be explained by the significant loss of NO_3^- within moss soil, compared to minor losses in shrub and tussock soils (Figure S1). The highest soil moisture content was observed in moss soil (Table S1), which is a favorable condition for denitrification or nitrate reduction, resulting in a substantial decrease in NO_3^-.[110–112]

4.6 Implications of the study: Comparison with meta-analysis result

While details regarding the inhibitory mechanism of NH_4^+ have been described in previous research, the precise mechanism of inhibition of CH_4 oxidation by NO_3^- has remained unclear. However, our incubation study and meta-analysis suggest that NO_3^- is strong inhibitor of soil CH_4 oxidation (Figure 2 and 5). A significantly higher proportion of NO_3^- compared with NH_4^+ in atmospheric N deposition has been reported in the Arctic[21], suggesting that considerable

reduction of CH_4 oxidation in soil can occur by way of extreme N deposition events in Arctic regions.

The current CH_4 emission estimates for Arctic regions are regarded as controversial.[113,114] Findings from recent studies have suggested that warmer temperatures in the high Arctic would cause an increase of atmospheric uptake of CH_4, thus offsetting the increased emission of CH_4 as a result of permafrost thaw.[71,72] However, this hypothesis was proposed without considering the inhibitory effect of NO_3^-, which is regarded as a strong inhibitor of CH_4 oxidation in Arctic tundra. Thus, a part of increased CH_4 oxidation caused by warming can be offset by considering the inhibitory effect of NO_3^- on CH_4 oxidation in Arctic tundra. Our experimental data and results of meta-analysis suggest that CH_4 oxidation in surface soil could be significantly reduced by an increase in available N in tundra ecosystems (Figure 6).

Soil microbial diversity is associated with ecosystem processes such as C decomposition and nitrification.[115–117]. Due to climate change occurring across the globe, significant changes in soil microbial diversity may occur, ultimately altering the function of diverse ecosystems.[118,119] Our study found a significant positive correlation between bacterial diversity of soil and CH_4 oxidation rate, indicating that alteration of soil microbial diversity induced by climate change can also affect the CH_4 cycle of the ecosystem. This finding is in accordance with findings from previous culture studies that showed a mutual relationship between methanotrophic and non-methanotrophic bacteria, indicating that our study is the first soil-based experimental study to demonstrate this relationship. However, understanding of the underlying mechanisms of this symbiotic relationship remains limited. Conduct of studies for further identification of the specific bacteria and metabolites that are beneficial to methanotrophs will be required in order to obtain more information about the relationship

554 between methanotrophs and non-methanotrophs. Overall, the results of our study provide
555 evidence that CH_4 oxidation in surface soil can be significantly attenuated by an increase of
556 available N in soil via climate change, ultimately resulting in enhanced net emission of CH_4 in
557 Arctic tundra.
558

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Author Contributions

J.L. conceived and co-designed the study, conducted field and incubation experiments, and led the writing of the paper. H.K co-conceived and co-designed the study and contributed to writing. J.Y. contributed to formal analysis and visualization. Y.Y. contributed to microbial analysis. J.Y.J. contributed to field survey and writing. Y.K.L contributed to field survey and writing. J.J., W.D., and C.F. contributed to reviewing and writing the manuscript.

Data Accessibility Statement

Supporting Information is available online. The dataset used for meta-analysis can be found in Data file S1. Sequencing data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the BioSample numbers of SAMN22374711 (shrub), SAMN22374589 (tussock), and SAMN22374646 (moss).

Supporting Information

T-RFLP of methanotrophs; 16s rRNA sequencing; Meta-analysis; Salt addition experiment; Glucose addition experiment; KEGG analysis; Fresh soil chemical characteristics; Correlation between CH_4 oxidation and bacterial diversity; Correlation between bacterial diversity and NO_3^- content.

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Figure captions

Figure 1. The effects of N addition on *in-situ* CH_4 flux. Fluxes were measured immediately after (day 0) and seven days after (day 7) N application. N was applied as a form of NH_4NO_3 (control = distilled water added; Low N = 10kg N ha^{-1} yr^{-1} added; High N = 100kg N ha^{-1} yr^{-1} added). Fluxes are presented as log (CH_4 flux + 10). Small letter and capital letter denotes statistically significant differences between the treatment on day 0 and day 7, respectively, and error bars indicate standard error of mean (N = 4, P < 0.05).

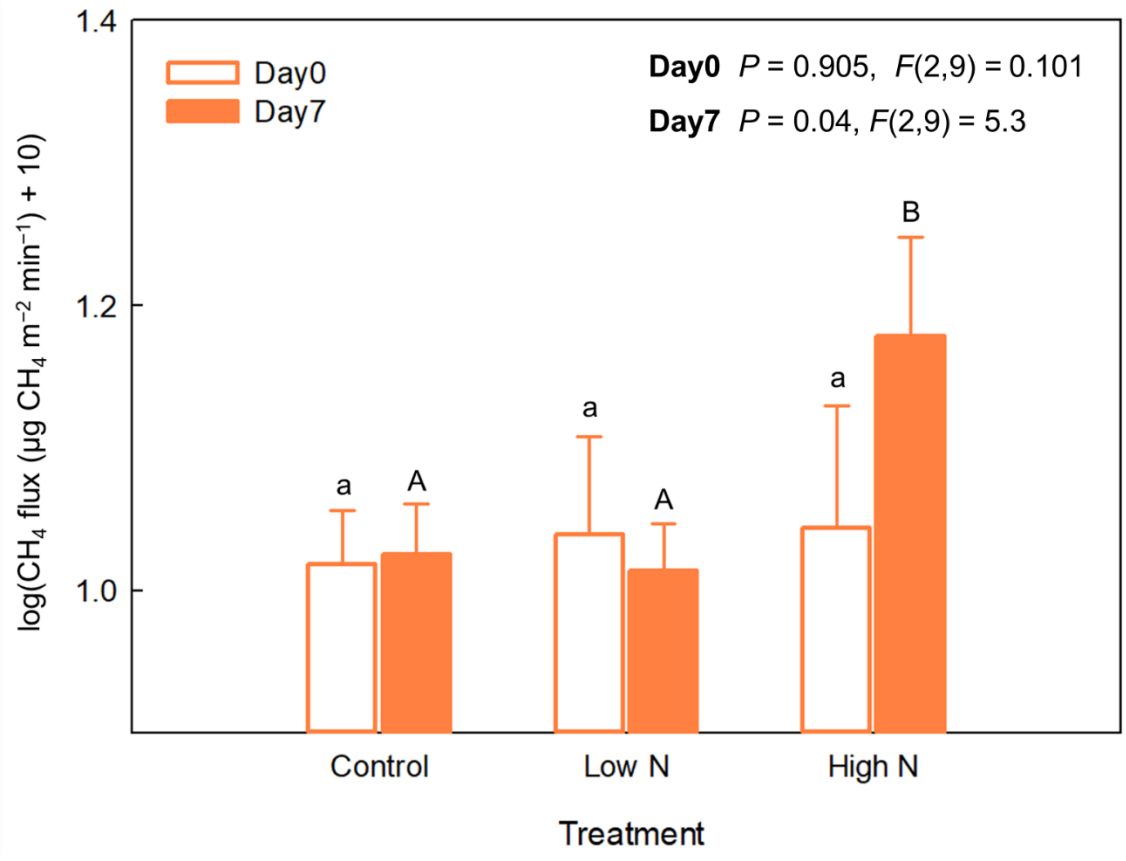
Figure 2. Inhibition of CH_4 oxidation rate at 50 ppm CH_4 (a to c) and 1000 ppm CH_4 (d to f) with different N form (AN = NH_4NO_3; AS = $(NH_4)_2SO_4$; PN = KNO_3). Differing letters denote statistically significant differences and error bars indicate standard error of mean (N = 3, P < 0.05).

Figure 3. Relative abundance of methanotrophs result from *pmoA* T-RFLP and 16S rRNA sequence. Phylogenetic affiliation of T-RFs is shown in Table S3.

Figure 4. Relationship between potential CH_4 oxidation rate and bacterial diversity at (a) 50 ppm CH_4 (N = 90, P < 0.0001) and (b) 1000 ppm CH_4 (N = 90, P < 0.0001). The shaded area shows the 95% confidence interval of the correlation.

Figure 5. Changes in soil CH_4 oxidation rate by addition of inorganic N (NH_4^+, NO_3^-, urea) with different types of fertilizer, ecosystem, and biome. Positive values correspond to stimulation by addition of inorganic N and negative values correspond to inhibition by inorganic N. Differing letters denote significant differences among fertilizer, ecosystem, and biome type (P < 0.05).

Figure 6. Schematic diagram of the study.



1075

1076 Figure 1

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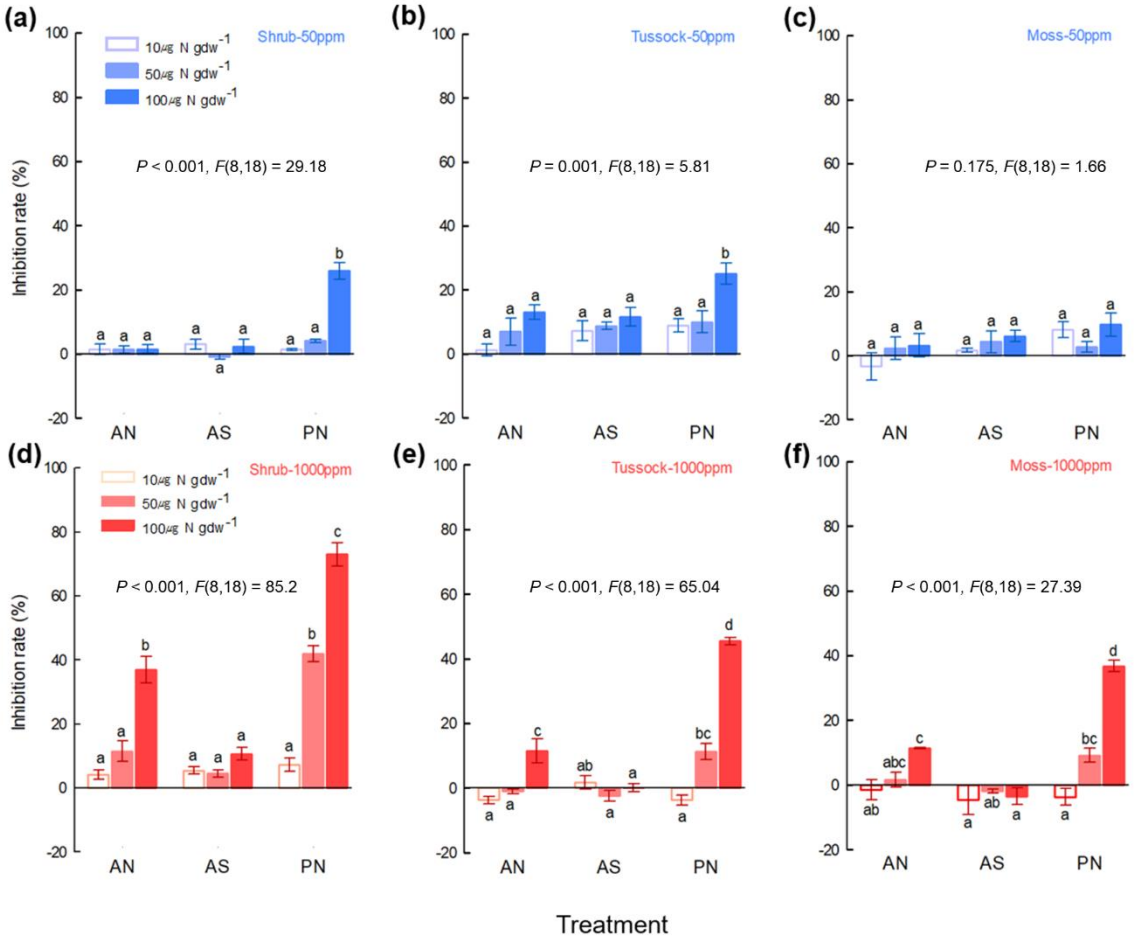

1078

1079 Figure 2

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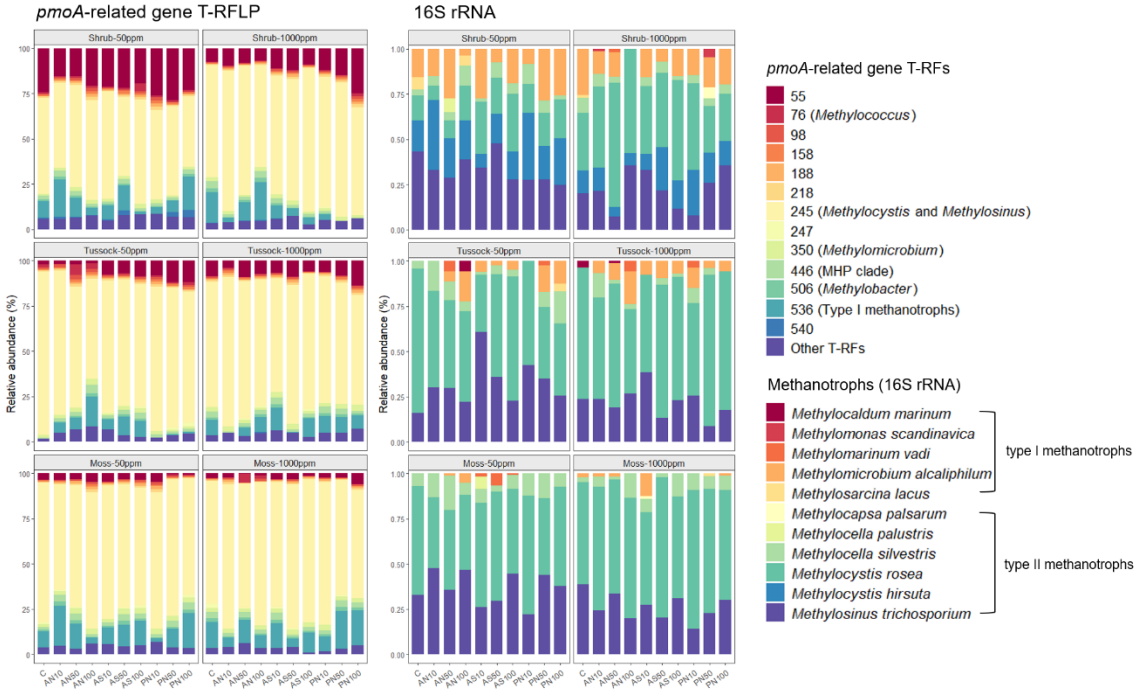
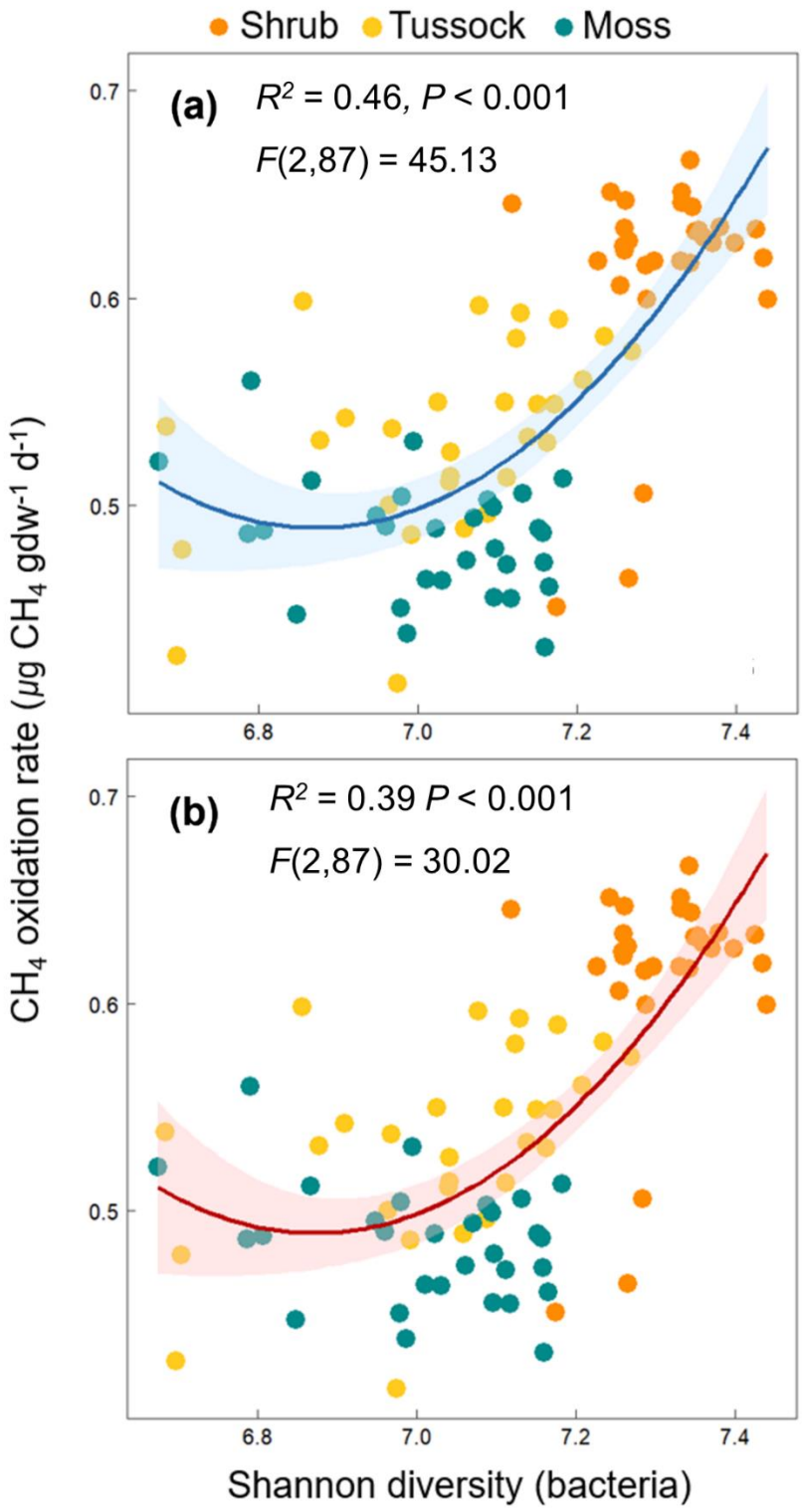

pmoA-related gene T-RFLP
16S rRNA
Shrub-50ppm
Shrub-1000ppm
Tussock-50ppm
Tussock-1000ppm
Moss-50ppm
Moss-1000ppm
Relative abundance (%)
C
AN10
AN60
AN100
AS10
AS60
AS100
PN10
PN60
PN100
pmoA-related gene T-RFs
55
76 (Methylococcus)
98
158
188
218
245 (Methylocystis and Methylosinus)
247
350 (Methylomicrobium)
446 (MHP clade)
506 (Methylobacter)
536 (Type I methanotrophs)
540
Other T-RFs
Methanotrophs (16S rRNA)
Methylocaldum marinum
Methylomonas scandinavica
Methylomarinum vadi
Methylomicrobium alcaliphilum
Methylosarcina lacus
type I methanotrophs
Methylocapsa palsarum
Methylocella palustris
Methylocella silvestris
Methylocystis rosea
Methylocystis hirsuta
Methylosinus trichosporium
type II methanotrophs

Figure 3



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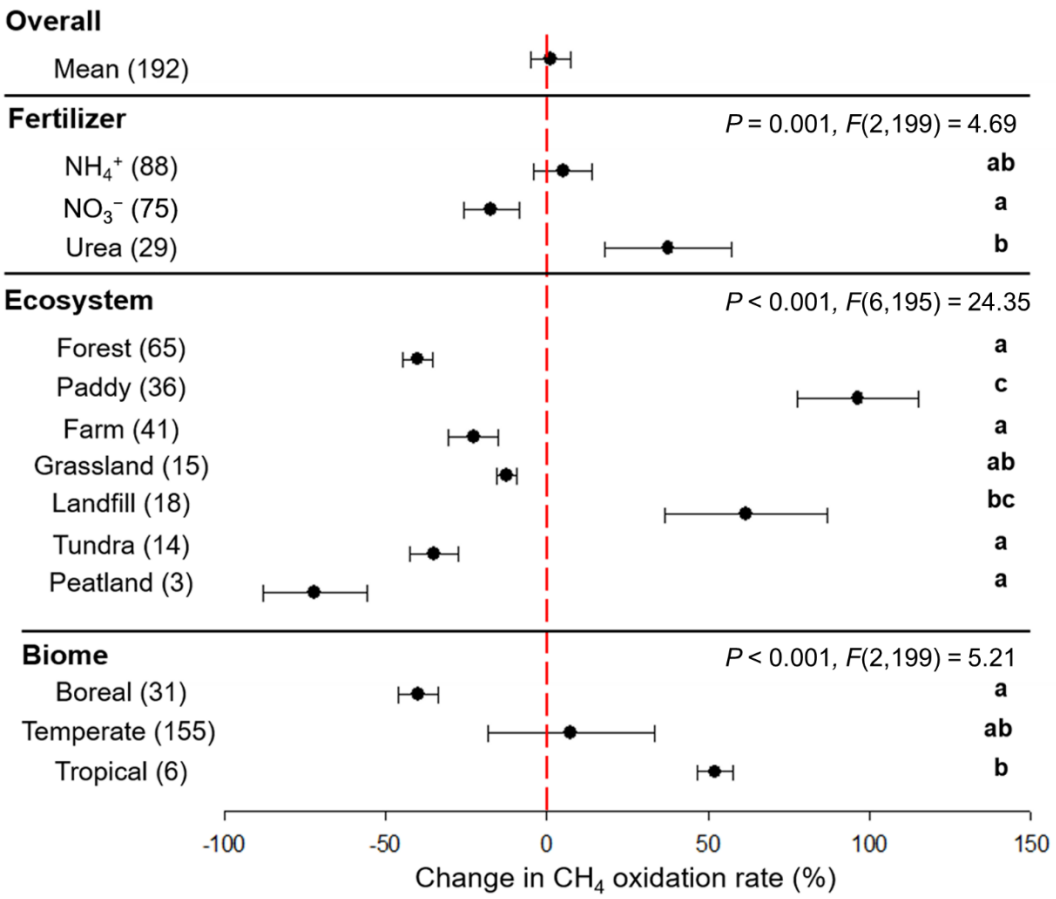
1085 Figure 4



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1087 Figure 5

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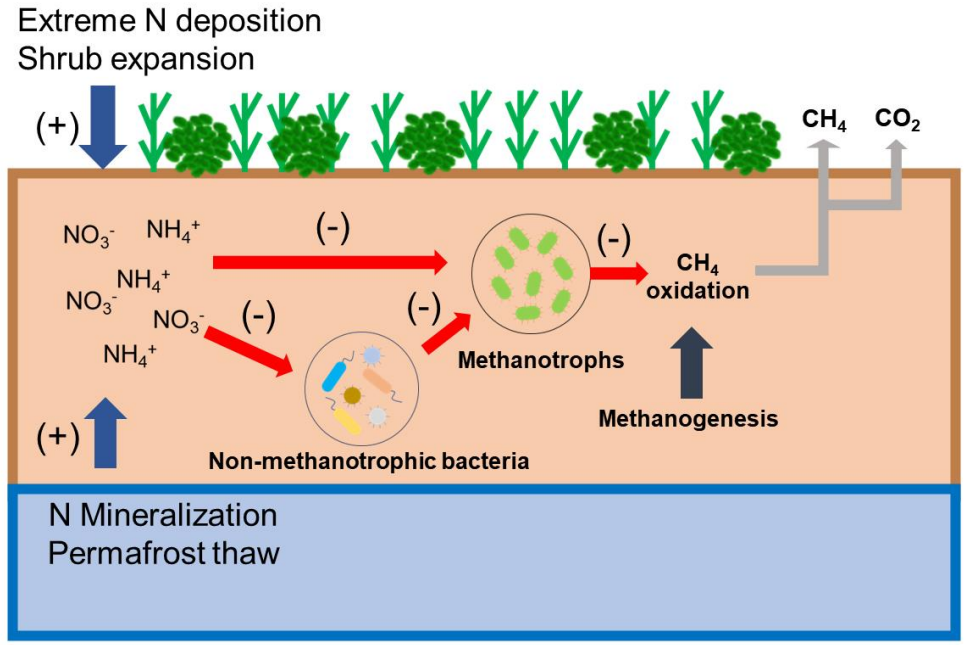

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1090 Figure 6

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