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Environmental Science and Technology

DOI: 10.1021/acs.est.2c05228

Published: 14/02/2023

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA): Lee, J., Yun, J., Yang, Y., Jung, J. Y., Lee, Y. K., Yuan, J., Ding, W., Freeman, C., & Kang, H. (2023). Attenuation of Methane Oxidation by Nitrogen Availability in Arctic Tundra Soils. Environmental Science and Technology, 57(6), 2647-2659. https://doi.org/10.1021/acs.est.2c05228

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Title: Attenuation of methane oxidation by nitrogen availability in Arctic tundra soils

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21	Key words: methane oxidation, available N, methanotrophs, bacterial diversity, moist acidic
22	tundra, Arctic region

23 Abstract

CH₄ emission in the Arctic has large uncertainty due to the lack of mechanistic understanding 24 of the processes. CH₄ oxidation in Arctic soil plays a critical role in the process, whereby 25 removal of up to 90% of CH₄ produced in soils by methanotrophs can occur before it reaches 26 the atmosphere. Previous studies have reported on the importance of rising temperatures in 27 CH₄ oxidation, but because the Arctic is typically an N-limited system, fewer studies on the 28 effects of inorganic nitrogen (N) have been reported. However, climate change and an increase 29 of available N caused by anthropogenic activities has recently been reported, which may cause 30 31 drastic change of CH₄ oxidation in Arctic soils. In this study, we demonstrate that excessive 32 levels of available N in soil cause an increase of net CH₄ emissions via reduction of CH₄ 33 oxidation in surface soil in Arctic tundra. In-vitro experiments suggested that N in the form of NO₃⁻ is responsible for the decrease in CH₄ oxidation via influencing soil bacterial and 34 35 methanotrophic communities. Findings of our meta-analysis suggest that CH₄ oxidation in the boreal biome is more susceptible to addition of N than other biomes. We provide evidence that 36 CH₄ emissions in Arctic tundra can be enhanced by an increase of available N, with profound 37 38 implications for modeling of CH₄ dynamics in Arctic regions.

39

40 Synopsis

In this study, in-situ, in-vitro manipulation experiments, and global meta-analysis were used to
examine the response of CH₄ oxidation in soil to an increase of available nitrogen in Arctic
tundra.

45 **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}

53 The permafrost region is classified as an N-limited environment due to its low N mineralization rate¹¹, low atmospheric N deposition (~ $10 \text{ kg N ha}^{-1} \text{ yr}^{-1}$; Geels et al.¹²), and the 54 rapid uptake of the small amount of available N by plants and microbes.¹³ However, findings 55 56 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 57 soil organic matter (SOM)^{14,15}; (2) Liberation of inorganic and organic N may occur through 58 permafrost thaw^{16,17}; (3) Expansion of the extent of shrub vegetation harboring N-fixing 59 symbionts could occur through an increase of soil temperature ^{18–20}; (4) Extreme N deposition 60 events with occurrence of over 40% of regional annual N deposition within a few days could 61 become more frequent.²¹ Choudhary et al.²² suggested that N resulting from extreme deposition 62 is largely retained within the tundra regardless of the N forms and remains there over two 63 growing seasons. Permafrost regions are defined as N-limited ecosystems, thus substantial 64 changes in various ecosystem processes can be induced by increases in available N.²³⁻²⁵ 65

66

CH4 is oxidized in soil by methane-oxidizing bacteria using oxygen as a terminal electron

67 acceptor. Aerobic methanotrophs belong to the Gammaproteobacteria (Type I, Methylococcaceae and Methylothermaceae families), Alphaproteobacteria (Type II, Methylocystaceae and Beijerinckiaceae 68 families), and the Verrucomicrobia phyla (Methylacidiphilaceae family)^{26,27}. Verrucomicrobial 69 methanotrophs are known to play an important role in methane oxidation in extreme environments ^{28,29} 70 whereas proteobacterial methanotrophs are found in moderate and extreme environments.^{26,30,31} 71 Methanotrophs that are adapted to oxidize low concentrations of CH₄ are called high-affinity 72 methanotrophs and those that are adapted to the high concentration of CH₄ are called low-73 affinity methanotrophs.^{32–34} Most soils exhibit both affinities of oxidation with a transition from 74 high-affinity to low-affinity CH₄ oxidation observed between 100 and 1000 ppm CH₄.³⁵ 75

CH₄ oxidation in surface soil plays an important role in determining CH₄ fluxes in the 76 ecosystem, as illustrated by the fact that oxidation of up to 90% of CH₄ produced by 77 methanogenesis in deep soils by methanotrophs can occur before it reaches the atmosphere.^{36,37} 78 79 Moist acidic tundra provides a favorable condition for methanogenesis, and large amounts of CH₄ are released to the atmosphere.^{38–41} Thus, a decrease in the CH₄ oxidation process in soil 80 in the tundra can cause a substantial increase in CH₄ emission. Inorganic N such as NH₄⁺ and 81 NO₃⁻ is an important environmental variable controlling CH₄ oxidation in soil.⁴² The effects of 82 these forms of N are dependent on the types of ecosystems and management schemes.⁴² For 83 example, in forest soil, CH₄ oxidation is usually inhibited by NH₄⁺ through competition with 84 CH₄ for the active site of methane monooxygenase (MMO) enzyme.^{43–48} Inhibition of CH₄ 85 oxidation by NO₃⁻ occurs through production of NO₂⁻, which is toxic to methanotrophs.^{49–51} 86 NO₃⁻ can also stimulate CH₄ oxidation by releasing N limitation in forest soil.^{44,52,53} In 87 wetlands, NH4⁺ and NO₃⁻ have shown inhibitory effects ^{54–56}, stimulatory effects^{35,57}, or no 88 effect.⁵⁸ In addition to the form of inorganic N, van Dijk et al.⁵⁹ suggested that the effect of 89

ammonium is different between soluble ammonium and exchangeable ammonium. Although
several studies have examined the effect of inorganic N on CH₄ oxidation in soil in diverse
ecosystems, studies on the response of Arctic tundra have rarely been reported, limiting our
capability for estimation of CH₄ dynamics in this ecosystem.

The different responses of soil CH₄ oxidation to inorganic N may be explained in part 94 by differences in methanotrophic community composition. For example, findings from pure-95 culture studies have demonstrated that the effects of inorganic N enrichment on the growth rate 96 and activity of methanotrophs are species-specific.^{60,61} The different responses of 97 methanotrophic activity between type I and type II methanotrophs to N addition were 98 reported.^{62–64} For example, Mohanty et al.⁶³ suggested that type II methanotrophs can be more 99 vulnerable to N addition than type I methanotrophs. In addition to the methanotrophic 100 community, recent studies have shown that the activity and growth rate of methanotrophs are 101 102 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, 103 the response of CH₄ oxidation to addition of inorganic N can also be affected by different 104 105 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 106 detailed analysis of the microbial community, thus these conclusions may underestimate the 107 significance of microbial biodiversity. 108

109 Recent studies have proposed that CH_4 uptake by high-affinity methanotrophs will 110 increase as temperatures rise in Arctic mineral cryosols.^{71,72} However, this hypothesis only 111 considers the effect of NH_4^+ provided by deposition whereby the effect of NO_3^- and other 112 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 (NH₄⁺ and NO₃⁻) on CH₄ oxidation 113 in soil in Arctic tundra. In-situ and in-vitro manipulation experiments were performed to 114 115 determine the effect of inorganic N on (1) CH₄ fluxes in the field, (2) soil CH₄ oxidation in different vegetation types, and (3) the composition and diversity of the methanotrophic and 116 bacterial community. In the *in-vitro* experiment, the response of high-affinity and low-affinity 117 CH₄ oxidation was examined separately because their response to N can differ.³² We 118 hypothesized that an increase in available N would result in substantial attenuation of CH4 119 120 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 121 decrease of non-methanotrophic bacterial diversity^{73,74}. In addition, a meta-analysis was 122 performed in order to examine the response of CH₄ oxidation in soil to inorganic N in different 123 ecosystems and biomes, to elucidate general patterns with regard to the effects of N on CH₄ 124 oxidation. 125

127 **2. Methods**

128 2.1 Site description

Samples were collected from an Arctic region of moist acidic tundra located in Council, Alaska ($64^{\circ}51'N$, $163^{\circ}39'W$). 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 $-2.6^{\circ}C$ 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).

136

137 2.2 Field manipulation experiment

138 In September 2016, a field manipulation experiment was conducted in order to examine the effect of N addition on CH₄ flux. We established four plots (10m×10m) with relatively uniform 139 vegetation types (tussock and moss dominant) and white-colored opaque PVC chambers (30 140 cm diameter×30 cm height, cylindrical shape) were installed. Installing a chamber on the soil 141 surface causes pressure downward, which can pull out CH₄ captured in the subsoil layer. 142 Additionally, physical disturbance to the plant can affect CH₄ flux immediately after 143 installation. To minimize the disturbance effect on soil CH₄ flux, chambers were stabilized for 144 10 days. After stabilization, N was added to each plot as follows: control (distilled water), low 145 N (0.02g N m⁻²), and high N (0.2g N m⁻²). N fertilizer was applied as a form of NH₄NO₃. Low 146 N was applied to simulate extreme N deposition events where up to 40% of the annual 147 atmospheric N deposition occurs in less than a week (Hodson et al., 2005). High N was applied 148

149 to simulate the combined effect of extreme N deposition and other inorganic N sources (e.g. permafrost thaw, warming-induced mineralization, shrub expansion). The distance between 150 each treatment was at least 5 m and the fertilizer was added into the flux chamber to prevent 151 cross-contamination. Three treatments (control, low N, high N) were added to each plot, thus 152 153 the number of replicates for each treatment was 4. Measurements of CH4 fluxes were performed immediately following additions (D0), and seven days after (D7). A closed static chamber 154 method was used, and the headspace gas samples were transferred to a pre-evacuated glass vial 155 every 15 minutes for 60 minutes. The air temperature inside the chamber was measured before 156 and after gas measurement and considered in gas flux calculation. A GC-FID (CP-3800 Varian, 157 CA, USA; PORAPAK Q 80/100 column, with the detector operated at 150°C, carrier gas: N₂) 158 apparatus was then used for measurement of CH₄ concentration and calculation of CH₄ fluxes 159 using the following equation: 160

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

where F_{CH_4} is the flux of CH₄ gas (µg CH₄ m⁻² min⁻¹); dC_{CH₄}/dt is the rate of change in CH₄ concentration (10⁻⁶ min⁻¹) over time; V is the headspace volume of the chamber (m³); M_{CH₄} is the molecular weight of CH₄ (16 g mol⁻¹); A is the surface area of the chamber (m²); V_{mol} is the volume of a mole at a certain temperature (22.4L mol⁻¹).

166

167 2.3 Incubation experiment setup

Soils for incubation experiment were collected in September 2017 in areas consisting of three different vegetation types (shrub, tussock, and moss) according to the soil moisture gradient (Table S1). Prior to sampling, the aboveground vegetation and Oi layer were removed, and 171 samples were collected from a depth of 0-20 cm in the organic layer (three samples per 172 vegetation type). Most mineral A horizons were found below a depth of 50 cm. Collected 173 samples were placed in plastic zipper bags and transported to the laboratory on ice. Samples 174 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 175 4°C refrigerator for 14 days to prevent the effect of rapid thaw. Following gentle 176 homogenization, the samples were placed in 120 ml vials (5 g fresh soil) and 946 ml Mason 177 jars (30 g fresh soil). Although the use of homogenized soil samples may have a limitation in 178 that it does not represent actual field conditions, it is still a widely used method for measuring 179 specific microbial activity (e.g. CH₄ oxidation) and gives us a clear result. Vials were capped 180 181 with butyl rubber stoppers and Mason jars were closed tightly and sealed with Teflon tape to ensure gas tightness. In the first phase (induction), CH₄ was injected at an initial headspace 182 183 concentration of 50 ppm into the Mason jars (high-affinity CH₄ oxidation) and at 1000 ppm into the vials (low-affinity CH₄ oxidation). Measurement of the headspace CH₄ concentrations 184 was performed every 72 h until the concentration had decreased to 2 ppm for high-affinity 185 oxidation, and 100 ppm for low-affinity oxidation, which took 15-21 days. After induction, 186 containers were flushed with room air. Nitrogen was then added to the jars as (NH₄)₂SO₄ (AS), 187 KNO₃ (PN), and NH₄NO₃ (AN) at 10, 50, or 100 µg N g dry soil⁻¹. The maximum level of N 188 (100 µg N gdw⁻¹) was approximately four times the peak extractable N concentration observed 189 in moist acidic tundra soil.⁷⁶ Treatment with distilled water only was used as a control. 190 Triplicate samples were used in performance of each treatment. CH₄ was injected and 191 192 measurement of the headspace concentrations was performed every 48 hours until the CH4 concentration had decreased to certain levels, as described above. Headspace gas was collected 193

194 from Maison jar (8mL) and vial (2mL) and transferred to pre-evacuated 5mL exetainer. Gas sample from vial was diluted with 6mL N₂ to meet the volume requirement for GC 195 measurement (5mL). The same volume of collected gas was compensated by synthetic air (20% 196 O₂ & 80% N₂) and accounted for in calculations. All incubations were performed at 15°C in 197 the dark. Incubation of additional jars was performed without CH₄ enrichment in order to verify 198 the influence of methanogenesis; changes in headspace CH₄ concentration were minor, 199 suggesting that the effect of methanogenesis was negligible. In addition, three empty Maison 200 jars and vials were incubated without soil to assess the occurrence of leakage during the 201 incubation. 202

Headspace CH₄ 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: N₂). Calculation of the potential CH₄ oxidation rate was based on the following equation:

206 Potential CH₄ oxidation rate (
$$\mu g CH_4 g dw^{-1} d^{-1}$$
) = $\frac{dCH_4}{dt} \times \frac{V \times M_{CH_4}}{g dw \times V_{mol}}$ (2)

where dCH₄/dt is changes in methane concentration over time ($10^{-6} d^{-1}$); V is headspace volume of vial or jar (L); M_{CH₄} is the molecular weight of CH₄ (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:

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

where P_c is the potential CH₄ oxidation rate of control; P_t is the potential CH₄ 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 $-20 \,^{\circ}C$ 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.

223

224 2.4 Chemical analysis

Analysis of chemical characteristics of both fresh and post-incubation soil was performed. 225 Oven-drying was performed at 105°C for 24 h for measurement of soil moisture content, and 226 227 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 228 229 ratio of 1:5 and centrifugation was performed for precipitation of soil particles. Extraction of soil NO₂⁻ and NO₃⁻ was performed by mixing soil with distilled water as described above and 230 the extract was subsequently filtered through a 0.2-µm filter, followed by analysis using ion 231 chromatography (ICS-1100; Dionex, Sunnyvale, CA, USA). Extraction of soil NH4⁺ was 232 performed using 2 M KCl and measurements were performed using the indophenol blue 233 method.⁷⁷ 234

235

236 2.5 DNA extraction and T-RFLP analysis of methanotrophs

237

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

247

248 2.6 16S rRNA sequencing

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

257

A meta-analysis was conducted by extracting potential CH₄ oxidation data from published 260 journal articles that reported data on both control and added N treatments in order to examine 261 the effect of N addition on CH₄ oxidation in soil. In brief, a search of the literature was 262 conducted in an online database (ISI Web of Science, Science Direct, Google and Google 263 Scholar) for collection of studies; the following search terms were used in any combination: 264 265 nitrogen, N, addition, fertilization, CH₄, CH₄ oxidation, methanotroph. Data were extracted from tables and figures. In total, 192 paired measurements were collected from 22 publications 266 (Supporting data); each pair contained potential CH₄ oxidation rate values for single N addition 267 and control. A detailed description of the meta-analysis is provided in Supporting Information 268 (Text S6). 269

270 For each paired measurement, the response ratio was used as the effect size measure271 as follows:

272 Response ratio,
$$\ln RR = \ln(X_t/X_c)$$
 (4)

where X_c and X_t are paired average potential CH₄ oxidation rate values for the control (X_c) and N addition (X_t). The percentage change in CH₄ oxidation in response to addition of N was calculated as follows:

276 % change =
$$(\exp(\ln RR) - 1) \times 100\%$$
 (5)

277

278 *2.8 Statistical analysis*

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

Analysis of the differences in potential CH₄ oxidation rate, inhibition rate, field CH₄ flux, and 280 Shannon diversity index of each treatment was performed using the one-way ANOVA test. 281 282 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 283 determine the relationship between potential CH₄ oxidation rate and bacterial Shannon 284 diversity index. Linear regression analysis was performed in order to determine the relationship 285 between CH₄ oxidation inhibition rate and increase of NO₃⁻ content in soil. Pearson correlation 286 analysis was performed in order to determine the correlation between NO3⁻ content and 287 bacterial Shannon diversity index. 288

290 **3. Results**

291 3.1 Effects of N fertilization on soil CH_4 flux in the field

Overall, the study site was a net CH₄ source, where positive CH₄ fluxes were measured across the time and treatment (Figure 1). On day 0, low N (1.38 μ g CH₄ m⁻² min⁻¹) and high N treatment (1.78 μ g CH₄ m⁻² min⁻¹) showed similar CH₄ fluxes compared with control (0.54 μ g CH₄ m⁻² min⁻¹). On day 7, similar CH₄ fluxes were observed in control (0.71 μ g CH₄ m⁻² min⁻¹) and low N treatment (0.41 μ g CH₄ m⁻² min⁻¹) while high N treatment showed a substantially higher CH₄ flux (5.71 μ g CH₄ m⁻² min⁻¹).

298

299 3.2 Effect of N on the behaviors of CH_4 and microbial community

300 *3.2.1 Soil inorganic N contents*

Soil inorganic N contents (NH₄⁺ and NO₃⁻) after incubation are shown in Figure S1. During the incubation experiment, an increase in the soil NH₄⁺ content of control was observed in comparison with fresh soil (P < 0.01) indicating that there was net N mineralization. The highest NH₄⁺ 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 CH₄, 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 CH₄. In PN50 and PN100, only a slight decrease of added NO_3^- was observed in shrub and tussock soils while

313 *3.2.2 Effect of inorganic N on CH*⁴ oxidation

Effect of inorganic N on CH₄ oxidation is shown in Table S2. Potential high-affinity CH₄ oxidation rates of control were 0.64, 0.59, and 0.50 μ g CH₄ gdw⁻¹ d⁻¹ in shrub, tussock, and moss soil, respectively, showing significant differences (*P* < 0.001). Inhibitory effects of inorganic N were only observed in PN100 in shrub and tussock soils, with inhibition of 25.9% and 25.0%, respectively (Figure 2).

Potential low-affinity CH₄ oxidation rates of control were 15.3, 11.91, and 9.16 µg 319 CH₄ gdw⁻¹ d⁻¹ in shrub, tussock, and moss soil, respectively, showing significant differences 320 (P < 0.001). CH₄ oxidation was significantly inhibited by PN50 and PN100 in all vegetation 321 types at 1000 ppm CH₄ (Figure 2). The strongest inhibitory effect was observed in shrub soil 322 (73% inhibition in PN100) and the weakest in moss soil (36% inhibition in PN100). NH_4^+ had 323 no significant effects on low-affinity CH₄ oxidation even in AS100. The combined effect of 324 NH4⁺ and NO3⁻ was only significant in AN100; this inhibitory effect was similar to that 325 326 observed in PN50.

327

328 *3.2.3 Methanotrophic community composition*

Relative abundances of 14 terminal restriction fragments (TRFs) are shown in Figure 3. Among 14 different TRFs, a 245-bp fragment associated with *Methylocystis* and *Methylosinus* (Table S3) was the most abundant (38%–82%) in all vegetation types. According to the 16S rRNA 332 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 333 with T-RFLP results that showed predominance of *methylocystis* and *methylosinus* (Figure 3). 334 Under 50ppm CH₄, the relative abundance of *Methylocystis rosea* was highest in tussock 335 (56.8%) followed by moss (42.4%) and lowest in shrub (21.2%). The relative abundance of 336 Methylosinus trichosporium was not significantly different among species. Methylocystis 337 hirsute was only detected in shrub (27.8%). Under 1000ppm CH₄, the relative abundance of 338 *Methylocystis rosea* was highest in tussock (70.3%) while statistically similar in moss (54.5%) 339 and shrub (42.6%). The relative abundance of Methylosinus trichosporium was not 340 significantly different among species. *Methylocystis hirsute* was only detected in shrub (8.3%). 341 Overall, predominance of type II methanotrophs was observed at our study site while 342 dominance of type I was minor. Methanotrophic community structure was separated by 343 vegetation types whereas fertilizer's types and concentrations did not affect methanotrophic 344 community (Figure S2). 345

346

347 *3.2.4 Correlation between CH*⁴ *oxidation activity and bacterial diversity*

The results of regression analysis showed overall positive correlations between soil CH₄ oxidation rate and bacterial diversity (Shannon index value) at 50 ppm CH₄ (Figure 4a; $R^2 =$ 0.46, P < 0.001) and 1000 ppm CH₄ (Figure 4b; $R^2 = 0.39$, P < 0.001). We also found that CH₄ oxidation rate and bacterial diversity showed significant positive correlations in tussock soil at 50 ppm CH₄, shrub soil at 1000 ppm CH₄, and moss soil at 1000 ppm CH₄ 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 CH₄. In addition, significant negative correlations were found between NO_3^- content in soil and bacterial diversity with the exception of moss soil at 50 ppm CH₄ and tussock soil at 1000 ppm CH₄ (Table S4).

357

358 3.3 Response of CH₄ oxidation to N fertilizer in diverse ecosystems: Meta-359 analysis

The results of a meta-analysis of changes in the CH₄ oxidation rate in response to addition of 360 N are shown in Figure 5. Overall, the effect of N addition on the potential CH₄ oxidation rate 361 was negligible, however, CH₄ oxidation was significantly affected by the type of fertilizer (P 362 363 = 0.011). Unlike previous studies that reported competitive inhibition by NH_4^+ as a major inhibitory mechanism, we found that CH₄ oxidation was significantly inhibited by NO₃⁻ 364 (-17.3%), while it was stimulated by addition of NH₄⁺ (4.9%) and urea (37.6%). Strong 365 inhibitory effects of N addition were observed in peatland (-71.6%), forest (-39.7%), tundra 366 (-34.7%), and farm (-22.3%) while a weak inhibitory effect was observed in grassland soils 367 (-12.0%). By contrast, CH₄ oxidation was significantly stimulated by addition of N in landfill 368 (61.9%) and paddy (96.6%). Biome type was also a significant factor (P = 0.006), where N 369 addition significantly inhibited soil CH₄ oxidation in boreal regions (-39.7%) but stimulated it 370 371 in tropical regions (52.1%) whereas the minor effect was observed in temperate regions (7.4%).

373 **4. Discussion**

4.1 Enhanced CH₄ emission by N fertilizer in the field

In the field, the effect of both low N and high N treatments on CH₄ emissions was negligible 375 in comparison with control on day 0 (Figure 1). On day 7, high N treatment resulted in 704% 376 higher CH₄ emission compared to control, while no significant difference was observed 377 between CH₄ emission with low N treatment and control. It appeared that the majority of N 378 added in the low N treatment was immobilized by plants and microbes, or absorbed into the 379 soil⁵⁹ while surface layer CH₄ oxidation was suppressed by residual N in the high N treatment, 380 increasing net emission of CH₄. N addition may also stimulate methanogenesis in deeper soil. 381 However, inhibition of methanogenesis by N in various wet ecosystems has been 382 demonstrated^{80,81} and development of the nutritional effects of added N on slow-growing 383 methanogens would take longer than the direct inhibitory effects on methanotrophs⁸², 384 suggesting that reduced CH₄ oxidation in surface soil is primarily responsible for the enhanced 385 emission of CH₄ in fertilized treatment. 386

387

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. 395 NO₃⁻ can increase the C mineralization rate and stimulates the heterotrophic microbial process, consequently suppressing methanotrophic activity. For example, the addition of 396 397 glucose resulted in a reduction of CH₄ oxidation in forest soil by 83% compared with control, and by 99.4% when combined with NO₃⁻ addition.⁴³ The authors suggested that the glucose 398 addition stimulates the heterotrophic microbial processes and suppresses methanotrophic 399 activity. We hypothesized that NO₃⁻ indirectly inhibits CH₄ oxidation activities by stimulating 400 C mineralization and heterotrophic microbial processes. Potential CH₄ oxidation was not 401 402 inhibited by the addition of glucose in comparison with the control (Figure S4). The addition of NO₃⁻ also had no effect on dissolved organic carbon content (Figure S5), suggesting that an 403 increase of labile C compounds also did not have an underlying effect on the inhibition of CH4 404 oxidation in our study. 405

Osmotic stress is induced by the addition of high concentrations of salts, which can 406 thus inhibit methanotrophic activity.^{46,50,54,56,85} King et al.⁸⁶ examined the effect of ammonium 407 and non-ammonium salt addition on soil CH₄ oxidation activity and found that non-ammonium 408 salt significantly inhibited CH₄ oxidation activity by inducing osmotic stress to methanotrophs. 409 410 The addition of salt resulted in slight inhibition of high-affinity CH₄ oxidation, however, inhibition of low-affinity CH₄ oxidation was observed in shrub soil only (Table S5). CH₄ 411 oxidation activity could be inhibited by the addition of KNO₃ through a decrease in pH.⁵⁶ 412 However, no changes in soil pH were observed in N treatments compared with control (data 413 not shown). Production of NH4⁺ from NO3⁻ by dissimilatory nitrate reduction to ammonium⁸⁷ could 414 also inhibit CH₄ oxidation. NH₄⁺ inhibits CH₄ oxidation by inducing competition between 415 methanotroph and ammonium oxidizer, and Ho et al.⁸⁸ suggested that this competition is 416 significantly affected by the concentration of NH₄⁺. However, we found a negligible effect of 417

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

While the mechanisms previously established by other research (e.g. NO_2^- production, enzymatic competition, osmotic stress) were not observed in our study, we propose that, nevertheless, microbial changes have significant influence on the inhibitory effect of NO_3^- on CH₄ oxidation. Mediation of CH₄ oxidation occurs through the activity of microbes and tundra is generally an N-limited environment; significant change in microbial community structure as well as a decrease in microbial diversity and activity including methanotrophs can be induced by an increase in available N.^{89,90}

Methylocystis and Methylosinus were predominant methanotrophs in our study site 429 (Figure 3). According to previous studies of Arctic peatland and wetland, which have 430 ecosystems similar to that of moist acidic tundra, Methylocystis is the most active and 431 predominant methanotroph species.^{91,92} Several characteristics enable *Methylocystis* to adapt 432 and thrive in such an environment. First, Methylocystis species, which are facultative 433 methanotrophs, are able to utilize other C compounds such as methanol, ethanol, and acetate 434 as their C sources, rather than CH4.93-96 This ability may enable survival of *Methylocystis* under 435 CH₄-limited conditions. Second, the genome sequence of *Methylocystis* suggests that it has the 436 ability to fix atmospheric N.^{97,98} N fixation can enable better survival of *Methylocystis* 437 compared with other methanotrophs in N-limited Arctic ecosystems. Methylosinus also has the 438 ability to fix atmospheric N⁹⁹, implying that *Methylocystis* and *Methylosinus* are adopted to an 439

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 CH₄ oxidation due to the vulnerability of type II methanotrophs to excessive N.

According to findings from pure culture studies of methanotrophs, Methylocystis is 446 more vulnerable to NO_3^- than to NH_4^+ , while other methanotrophs bind preferentially to 447 NO₃^{-.60,61} Therefore, we can assume that significant inhibition of CH₄ oxidation by addition of 448 NO₃⁻ can be explained by the dominance of *Methylocystis* at our study site. In addition, another 449 pure culture study reported a lack of inhibition of the growth rate and activity of Methylocystis 450 under a high concentration of NH4^{+ (103)}, supporting our finding showing the effects of the 451 absence of NH4⁺ on CH4 oxidation. Previous studies reported that *Methylocystis* is the most 452 active and predominant methanotrophic species in Arctic wetlands^{91,92}, therefore, it is plausible 453 that an increase in NO₃⁻ content in soil could result in significantly reduced CH₄ oxidation in 454 455 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 highthroughput 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.

467

468 4.4 Positive correlation between CH₄ oxidation rate and bacterial diversity

Significant positive correlations were observed between soil CH₄ oxidation rate and bacterial 469 diversity (Shannon index value) across all vegetation types (P < 0.0001; Figure 4). These data 470 471 indicate the strong influence of bacterial diversity on CH₄ oxidation in soil. In addition, a significant negative correlation was found between bacterial diversity and NO₃⁻ content in soil 472 with the exception of moss soil under 50 ppm CH₄ and tussock soil under 1000 ppm CH₄ (Table 473 474 S4). These results suggest that the inhibitory effect of NO₃⁻ may depend on a decrease in bacterial diversity. In moss soil under 50 ppm CH₄, the inhibitory effect of NO₃⁻ addition on 475 the oxidation rate of CH₄ may have been negated by the fact that there were no changes in 476 bacterial diversity. CH₄ oxidation has previously been associated exclusively with 477 methanotrophs, because they are the only functional group responsible for the process. 478 479 However, findings from recent studies have suggested that methanotrophs and heterotrophs are 480 mutually co-dependent, and that CH₄ oxidation may also be affected by general microbial diversity.^{65–69} For example, a previous study reported a higher CH₄ oxidation activity for 481 cultured methanotrophs with diverse heterotrophs compared to samples incubated with 482 methanotrophs only.⁶⁵ It was suggested that this mutual relationship was based on the fact that 483 certain heterotrophs produce essential metabolites for methanotrophs, thus stimulating CH₄ 484 485 oxidation activity. In order to provide evidence for the symbiotic relationship between

486 methanotroph and non-methanotrophic bacteria, we further conducted the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis 487 based on 16S rRNA amplicon sequencing data. The functional annotation of PICRUSt 488 predictions was obtained based on Kyoto Encyclopedia of Genes and Genomes (KEGG) 489 database (Text S7). MMO showed overall positive correlations with methanol dehydrogenase 490 and formate dehydrogenase (Figure S6). Krause et al.⁶⁸ suggested that the symbiotic 491 relationship between methanotrophs and non-methanotrophs occurs through cross-feeding of 492 493 methanol. Positive correlations observed between MMO and methanol dehydrogenase indicate 494 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 495 496 assimilated by methanotrophs. A positive correlation between MMO and formate dehydrogenase may be related to the removal of alternative carbon compounds. Fender et al.⁴⁹ 497 suggested that CH₄ oxidation activity was significantly inhibited by alternative carbon 498 compounds including formate because methanotrophs utilize alternative carbon rather than 499 500 CH₄. CH₄ 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} 501

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, N₂ 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 CH₄ 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 betweenmethanotrophic and non-methanotrophic bacteria has yet to be established.

511

512 *4.5 Differences in responses according to vegetation type*

We observed that the responses to addition of NO_3^- varied with different vegetation types 513 (Figure 2). Strong inhibition by NO₃⁻ in shrub soil can be explained by osmotic stress. Findings 514 515 from our supplementary study showed that addition of K₂SO₄ resulted in significant inhibition of CH₄ oxidation in shrub soil at 1000 ppm CH₄ (Table S5), indicating that CH₄ oxidation in 516 shrub soil is severely inhibited by a combined effect of NO₃⁻ and osmotic stress. The weakest 517 inhibitory effect was observed in moss soil, where no effect was observed at 50 ppm CH₄. This 518 finding could be explained by the significant loss of NO₃⁻ within moss soil, compared to minor 519 losses in shrub and tussock soils (Figure S1). The highest soil moisture content was observed 520 in moss soil (Table S1), which is a favorable condition for denitrification or nitrate reduction, 521 resulting in a substantial decrease in NO₃^{-.110-112} 522

523

524 4.6 Implications of the study: Comparison with meta-analysis result

525 While details regarding the inhibitory mechanism of NH_4^+ have been described in previous 526 research, the precise mechanism of inhibition of CH_4 oxidation by NO_3^- has remained unclear. 527 However, our incubation study and meta-analysis suggest that NO_3^- is strong inhibitor of soil 528 CH_4 oxidation (Figure 2 and 5). A significantly higher proportion of NO_3^- compared with NH_4^+ 529 in atmospheric N deposition has been reported in the Arctic²¹, suggesting that considerable reduction of CH₄ oxidation in soil can occur by way of extreme N deposition events in Arctic
regions.

The current CH₄ emission estimates for Arctic regions are regarded as 532 controversial.^{113,114} Findings from recent studies have suggested that warmer temperatures in 533 the high Arctic would cause an increase of atmospheric uptake of CH₄, thus offsetting the 534 increased emission of CH₄ as a result of permafrost thaw.^{71,72} However, this hypothesis was 535 proposed without considering the inhibitory effect of NO_3^- , which is regarded as a strong 536 inhibitor of CH₄ oxidation in Arctic tundra. Thus, a part of increased CH₄ oxidation caused by 537 warming can be offset by considering the inhibitory effect of NO₃⁻ on CH₄ oxidation in Arctic 538 tundra. Our experimental data and results of meta-analysis suggest that CH₄ oxidation in 539 surface soil could be significantly reduced by an increase in available N in tundra ecosystems 540 (Figure 6). 541

Soil microbial diversity is associated with ecosystem processes such as C 542 decomposition and nitrification.^{115–117}. Due to climate change occurring across the globe, 543 significant changes in soil microbial diversity may occur, ultimately altering the function of 544 diverse ecosystems.^{118,119} Our study found a significant positive correlation between bacterial 545 diversity of soil and CH₄ oxidation rate, indicating that alteration of soil microbial diversity 546 induced by climate change can also affect the CH₄ cycle of the ecosystem. This finding is in 547 548 accordance with findings from previous culture studies that showed a mutual relationship 549 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 550 551 underlying mechanisms of this symbiotic relationship remains limited. Conduct of studies for further identification of the specific bacteria and metabolites that are beneficial to 552 methanotrophs will be required in order to obtain more information about the relationship 553

between methanotrophs and non-methanotrophs. Overall, the results of our study provide evidence that CH₄ oxidation in surface soil can be significantly attenuated by an increase of available N in soil via climate change, ultimately resulting in enhanced net emission of CH₄ in Arctic tundra.

559 Funding

This study was supported by the Ministry of Science and ICT of Korea (2020R1I1A2072824,
2020M1A5A1110494, PN20081, 2021M1A5A1075508, PN22012, 2022R1I1A1A01071925),
Ministry of Environment of Korea (2022003640002), and the Ministry of Education of Korea
(2019H1A2A1076239, 2021R1A6A3A03039376).

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565 Author Contributions

566 J.L. conceived and co-designed the study, conducted field and incubation experiments, and led 567 the writing of the paper. H.K co-conceived and co-designed the study and contributed to 568 writing. J.Y. contributed to formal analysis and visualization. Y.Y. contributed to microbial 569 analysis. J.Y.J. contributed to field survey and writing. Y.K.L contributed to field survey and 570 writing. J.J., W.D., and C.F. contributed to reviewing and writing the manuscript.

571

572 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).

578 Supporting Information

- 579 T-RFLP of methanotrophs; 16s rRNA sequencing; Meta-analysis; Salt addition experiment;
- 580 Glucose addition experiment; KEGG analysis; Fresh soil chemical characteristics; Correlation
- 581 between CH₄ oxidation and bacterial diversity; Correlation between bacterial diversity and
- 582 NO_3^- content.

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1051 Figure captions

- 1052 Figure 1. The effects of N addition on *in-situ* CH₄ flux. Fluxes were measured immediately
- 1053 after (day 0) and seven days after (day 7) N application. N was applied as a form of NH₄NO₃
- 1054 (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 (CH₄ flux + 10). Small letter and capital letter denotes
- statistically significant differences between the treatment on day 0 and day 7, respectively, and
- 1057 error bars indicate standard error of mean (N = 4, P < 0.05).
- 1058 Figure 2. Inhibition of CH₄ oxidation rate at 50 ppm CH₄ (a to c) and 1000 ppm CH₄ (d to f)
- 1059 with different N form (AN = NH_4NO_3 ; AS = (NH_4)₂SO₄; PN = KNO₃). Differing letters denote
- 1060 statistically significant differences and error bars indicate standard error of mean (N = 3, P <
- 1061 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.
- 1064 Figure 4. Relationship between potential CH₄ oxidation rate and bacterial diversity at (a) 50
- 1065 ppm CH₄ (N = 90, P < 0.0001) and (b) 1000 ppm CH₄ (N = 90, P < 0.0001). The shaded area
- shows the 95% confidence interval of the correlation.
- Figure 5. Changes in soil CH₄ oxidation rate by addition of inorganic N (NH₄⁺, NO₃⁻, 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).
- 1072 Figure 6. Schematic diagram of the study.
- 1073







1079 Figure 2



1082 Figure 3







1087 Figure 5



1090 Figure 6