Effects of Nitrogen Deposition on Soil Methanotrophs in Temperate Forests: A Postprint

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Abstract

Numerous studies have demonstrated that nitrogen deposition affects forest methane uptake, yet the underlying microbial driving mechanisms remain poorly understood. Using quantitative PCR and clone sequencing techniques, we investigated the seasonal variation characteristics of the abundance and community composition of forest soil methanotrophs under long-term application of different nitrogen forms ((NH4)2SO4, NH4Cl, and KNO3) in a typical temperate forest of Changbai Mountain based on a long-term nitrogen deposition simulation experimental platform. The results showed that in summer, the pmoA gene abundance of forest soil methanotrophs exhibited no significant differences among different nitrogen addition treatments ($1.54\$\times10^6 - 3.20\times10^6 copies per gram dry soil$); $in autumn, the pmoA gene abundance in plots treated with NH4Cl and (NH4)2S$ $7.6\times10^5 copies per gram dry soil) decreased compared with the control ((4.03\times10^6 \pm 1.2\times10^6) copies per gram dry soil$ while the relative abundance of Methylococcus-group (Type I) showed an increasing trend in soils treated with (NH4)2SO4 and NH4Cl. These results indicate that ammonium nitrogen fertilizer addition exerts an inhibitory effect on the growth of methanotrophs in temperate forest soils and causes alterations in their community structure; influenced by summer temperature and moisture, this inhibitory effect was more pronounced in autumn, whereas NO3–N addition had no significant effect on the community composition and abundance of soil methanotrophs. These results explain previous observations that ammonium nitrogen fertilizer addition significantly reduced net methane uptake by temperate forest soils in autumn but had no significant effect in summer, thereby elucidating the microbial mechanisms by which long-term nitrogen deposition affects forest soil methane uptake.

Full Text

Preamble

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Effects of Nitrogen Addition on Methanotrophs in Temperate Forest Soil

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Abstract

Numerous studies have shown that increasing nitrogen deposition affects methane uptake in forest ecosystems; however, the underlying microbial mechanisms remain unclear. Using a long-term nitrogen deposition simulation experimental platform in a typical temperate forest of Changbai Mountain and employing real-time PCR, cloning, and sequencing techniques, this study investigated the seasonal variation in methanotroph abundance and community composition under long-term application of different nitrogenous compounds ($(NH_4)_2SO_4$, NH_4Cl, and KNO_3 at 45 kg N ha^{-1} yr^{-1}). In summer, methanotroph *pmoA* gene abundances were similar across all fertilized and control plots, ranging from 1.54$×10^{6}$ to 3.20$×10^{6}$ copies g^{-1} dry soil. In fall, however, abundance in the $(NH_4)_2SO_4$ treatment (4.61$×10^{6}$ ± 2.61$×10^{6}$ copies g^{-1} dry soil) was significantly lower than in control plots (4.03$×10^{6}$ ± 1.2$×10^{6}$ copies g^{-1} dry soil). In both seasons, methanotroph communities were dominated by Type I methanotrophs, particularly the *Methylobacter*-group (Type I), which accounted for 70.6%-85.4% of the community. The relative abundance of this dominant clade varied from 55.1% to 91.7%. While no significant differences were observed among treatments in summer, fall samples showed significantly lower proportions in $(NH_4)_2SO_4$ (52.7% ± 6.5%) and NH_4Cl (56.1% ± 8.9%) treatments compared to control. Conversely, the *Methylococcus*-group (Type I) exhibited an increasing trend in $(NH_4)_2SO_4$ and NH_4Cl treatments in fall. These results demonstrate that ammonium-based nitrogen addition inhibits methanotroph growth and alters

community composition in temperate forest soils, with the inhibitory effect being more pronounced in fall than summer. Nitrate addition showed no significant impact on methanotroph abundance or community composition. These findings align with previous observations that ammonium-based fertilizers significantly reduce net methane uptake from temperate forest soils in fall but not summer, providing insights into the microbial mechanisms driving methane uptake under long-term nitrogen deposition.

Keywords: methanotrophs; *pmoA* gene; forest soil; nitrogen deposition

Introduction

Methane (CH_4) is the third most important greenhouse gas after water vapor and carbon dioxide, contributing approximately 20% to global warming (IPCC, 2007). The rise in atmospheric CH_4 concentration is attributable to both increased sources and reduced sinks. Unsaturated soils represent a crucial atmospheric methane sink, absorbing an estimated 20–60 Tg of methane annually. Forest soils are considered the most effective methane sink, yet their methane oxidation capacity exhibits high variability due to environmental factors including precipitation, nitrogen deposition, and forest management practices. Elucidating the microbial mechanisms underlying these environmental effects is essential for comprehensive assessment and management of forest soil methane oxidation capacity.

Over the past 150 years, emissions of nitrogen compounds from fossil fuel combustion and agricultural fertilizer use have surged, substantially increasing atmospheric nitrogen deposition. Forest ecosystems are major recipients of this deposition, with temperate forests receiving 5–15 kg N ha^{-1} yr^{-1}, and some regions exceeding 50 kg N ha^{-1} yr^{-1}. Consequently, nitrogen deposition has become a critical environmental concern in forest ecosystems, prompting numerous studies on its effects on soil methane oxidation. However, most research has focused on flux dynamics and absorption kinetics, while investigations of the key microbial mechanisms remain limited, particularly in China.

The primary microorganisms responsible for forest soil methane oxidation are methanotrophs—bacteria that utilize methane as their sole carbon and energy source. Through methane monooxygenase (MMO), they oxidize methane to methanol, which is subsequently metabolized to CO_2 and H_2O. Based on physiological, biochemical, and metabolic differences, methanotrophs are classified as Type I (γ-Proteobacteria) or Type II (α-Proteobacteria). While previous studies have documented methanotroph abundance and community composition in various soils and their responses to temperature, moisture, vegetation type, and fertilization, research on responses to increased nitrogen deposition remains scarce, especially long-term field studies.

The broadleaf-Korean pine mixed forest in Changbai Mountain represents an important temperate forest ecosystem in northeastern China. This study utilized a long-term nitrogen deposition simulation platform to investigate seasonal vari-

ations in methanotroph abundance and community composition under different nitrogen forms, and their relationships with soil properties, to reveal the microbial mechanisms through which long-term nitrogen deposition affects temperate forest soil methane oxidation.

1. Materials and Methods

1.1 Soil Collection and Physicochemical Analysis

Soil samples were collected from the long-term nitrogen deposition simulation experimental platform in the broadleaf-Korean pine mixed forest of Changbai Mountain (42°24 N, 127°06 E), established in 2005. The region exhibits a typical temperate continental mountain climate influenced by monsoons, with cold, prolonged winters and a mean annual temperature of 2.5–5.5°C. Annual precipitation ranges from 600–900 mm. The soil is mountain dark brown soil developed from volcanic ash parent material through organic matter accumulation and weak leaching. Dominant vegetation includes *Pinus koraiensis*, *Quercus mongolica*, and *Fraxinus mandshurica*.

Samples were collected during two distinct periods: summer (coinciding with the rainy season) and fall (dry period). High-dose nitrogen treatments (45 kg N ha^{-1} yr^{-1}) of three forms—$(NH_4)_2SO_4$, NH_4Cl, and KNO_3—and a non-fertilized control were selected, each with replicate plots. In each plot, stainless steel soil corers were used to randomly collect 0–10 cm depth samples, which were thoroughly mixed and transported on ice.

One subsample was immediately flash-frozen in liquid nitrogen and stored at -80°C for molecular analysis. Another was stored at 4°C for physicochemical analysis. Soil ammonium and nitrate were extracted with 1 mol/L KCl (5:1 water:soil ratio) and measured using a continuous flow analyzer (SKALAR, Netherlands). Soil pH was measured in deionized water (2.5:1 water:soil ratio). Dissolved organic carbon (DOC) was extracted with 0.5 mol/L K_2SO_4 and measured using a TOC analyzer (Shimadzu TOC-Vcsh/TN, Japan).

1.2 DNA Extraction and Real-time Quantitative PCR

DNA was extracted from 0.5 g soil using the MoBio UltraClean Soil DNA Kit (San Diego, CA) following manufacturer protocols, with cell disruption at 5.0 m/s for 30 s. DNA was diluted 10-fold before analysis.

The methanotroph *pmoA* gene was targeted using primers A189 (5 -GGNGACTGGGACTTCTGG-3) and mb661 (5 -CCGGMGCAACGTCTTACC-3). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) in 25 L reactions containing 12.5 L 2×SYBR Premix Ex Taq, 0.5 L each of 10 mol/L primers, 2 L DNA template, and 9.5 L ddH_2O. Cycling conditions on an iCycler IQ thermocycler (Bio-Rad) were: 94°C for 5 min; followed by 5 cycles of 94°C for 45 s, 63°C for 45 s (decreasing 1°C per cycle), and 72°C for 1

min; then 35 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min. Data were analyzed using iCycler software.

1.3 Methanotroph Community Composition Analysis

Community composition was analyzed via *pmoA* gene cloning and sequencing. PCR products were purified using the Wizard SV Gel & PCR Clean-Up System (Promega, USA), ligated into pGEM-T Easy Vector (Promega, Madison, WI), and transformed into *E. coli* JM109 competent cells. Transformants were selected on LB agar with ampicillin/IPTG/X-Gal and incubated for 16–18 h.

White colonies were randomly selected and screened by colony PCR with T7/SP6 primers. Approximately 50 positive clones per library were sequenced. Sequences were aligned using ClustalX2, clustered into OTUs at 97% similarity using mothur, and used to construct a neighbor-joining phylogenetic tree in MEGA 5.0 with bootstrap analysis (1000 replicates). Reference sequences were obtained from GenBank via BLAST.

1.4 Statistical Analysis

Statistical analyses were performed in SPSS 19.0. Inter-group differences were assessed using one-way ANOVA with Duncan' s test ($P < 0.05$). Relationships between community composition and soil properties were analyzed by redundancy analysis (RDA) in CANOCO 4.5. Spearman correlation analysis was applied to clone library data.

2. Results

2.1 Soil Physicochemical Properties

Basic soil properties are presented in Table 1. Soil pH in NH_4Cl treatment (4.54–5.84) was significantly lower than in other treatments (5.08–6.51) in both seasons. However, different nitrogen forms had no significant effects on soil moisture, DOC content, or the DOC:mineral N ratio.

Soil basic physicochemical properties (mean±SE)

2.2 Methanotroph Abundance and Relationships with Soil Properties

Real-time PCR analysis revealed no significant differences in *pmoA* gene abundance among nitrogen treatments in summer ($1.54\$\times10^{\{6\}-3.20\times10}\{6\}\$$ copies g^{-1} dry soil). In fall, however, abundance differed significantly, with the $(NH_4)_2SO_4$ treatment ($4.61\$\times10\hat{}\{6\}\$ \pm 2.61\$\times10\hat{}\{6\}\$$ copies g^{-1} dry soil) being significantly lower than the control ($4.03\$\times10\hat{}\{6\}\$ \pm 1.2\$\times10\hat{}\{6\}\$$ copies g^{-1} dry soil).

Correlation analysis indicated that *pmoA* gene abundance was significantly positively correlated with soil pH ($r = 0.74$, $P < 0.05$), but showed no significant

correlation with NH_4^+-N, NO_3^--N, DOC content, DOC:mineral N ratio, or soil moisture across all treatments.

2.3 Methanotroph Community Composition

Clone sequencing analysis showed that methanotroph communities were dominated by Type I methanotrophs in both seasons, comprising 70.6%–85.4% of the community. The dominant Type I clade was *Methylobacter*-group, with relative abundance ranging from 55.1% to 91.7%.

In summer, *Methylobacter*-group relative abundance showed no significant differences among treatments. In fall, however, it was significantly lower in $(NH_4)_2SO_4$ (52.7% ± 6.5%) and NH_4Cl (56.1% ± 8.9%) treatments compared to the control (77.0% ± 2.9%). Conversely, *Methylococcus*-group (Type I) abundance increased in $(NH_4)_2SO_4$ and NH_4Cl treatments in fall.

RDA analysis revealed that methanotroph community composition was significantly positively correlated with DOC:mineral N ratio ($r = 0.49$, $P < 0.05$), but not with soil moisture or pH. PerMANOVA indicated that sampling time (season) significantly affected community composition ($P < 0.1$).

[Figure 1: see original paper] The abundance of soil methanotrophic *pmoA* gene under different nitrogenous compound amendments in Summer and Fall

[Figure 2: see original paper] The community composition of methanotroph under different nitrogenous compound amendments in Summer and Fall

[Figure 3: see original paper] Phylogenetic tree of methanotroph *pmoA* gene sequences

[Figure 4: see original paper] Redundancy analysis (RDA) between methanotroph community and environmental variables

3. Discussion

3.1 Methanotroph Abundance Response to Nitrogen Addition

Methanotrophs oxidize methane via methane monooxygenase (MMO), with the *pmoA* gene serving as a key molecular marker. Our summer results showed no treatment differences in *pmoA* abundance, coinciding with high precipitation (soil moisture 68.3%–81.2%) that can inhibit aerobic methane oxidation. This aligns with field observations of non-significant methane flux differences among treatments during summer.

In fall, significantly lower *pmoA* abundance in the $(NH_4)_2SO_4$ treatment corresponded with reduced methane uptake, indicating that ammonium-based fertilizers inhibit methanotroph populations. Ammonium competes with methane for the MMO active site due to structural similarity, while nitrite and hydroxylamine produced during nitrification can be toxic. This inhibition is typically

more pronounced in acidic soils, though our results demonstrate effects across the pH range studied.

3.2 Community Composition Response to Nitrogen Addition

Type I methanotrophs, which dominate in high-oxygen environments using the ribulose monophosphate pathway, were overwhelmingly dominant (70.6%–85.4%) in both seasons. The *Methylobacter*-group, as the primary Type I clade, showed seasonal differences in response to nitrogen: no summer treatment effects but significant reductions in fall under ammonium-based fertilizers, accompanied by increased *Methylococcus*-group abundance.

This shift suggests that ammonium addition selectively inhibits certain Type I methanotrophs while potentially stimulating others, altering community structure without completely eliminating the Type I dominance.

3.3 Seasonal Modulation of Nitrogen Effects

The pronounced seasonal difference in nitrogen response—significant effects in fall but not summer—can be attributed to interacting environmental factors. Summer temperatures (20–30°C) in Changbai Mountain are optimal for most methanotrophs, potentially enhancing activity enough to mask nitrogen inhibition. Additionally, vigorous summer plant growth increases root exudates, providing supplementary carbon that may alleviate nitrogen stress on methanotrophs.

In fall, cooler temperatures (5–15°C) and reduced plant activity likely diminish methanotroph metabolic rates and carbon availability, making the community more susceptible to nitrogen inhibition. This seasonal interaction explains why long-term nitrogen deposition effects on methane uptake are more detectable in fall.

4. Conclusion

This study demonstrates that long-term nitrogen deposition significantly affects methanotroph abundance and community composition in temperate forest soils, with effects modulated by season. Key conclusions include:

1. **Seasonal differences in abundance**: Summer *pmoA* gene abundances were unaffected by nitrogen treatments, while fall application of $(NH_4)_2SO_4$ and NH_4Cl significantly reduced methanotroph numbers.
2. **Community structure shifts**: Type I methanotrophs dominated year-round (70.6%–85.4%), with *Methylobacter*-group as the primary clade (55.1%–91.7%). Ammonium-based fertilizers significantly reduced this group' s abundance in fall while increasing *Methylococcus*-group representation.

3. **Mechanistic drivers**: The stronger nitrogen effects in fall reflect: (a) optimal summer temperatures promoting methanotroph activity that overrides inhibition, and (b) enhanced summer root exudates providing additional carbon resources.

These findings elucidate the microbial mechanisms underlying observed seasonal patterns in forest soil methane uptake responses to nitrogen deposition, highlighting the importance of considering seasonal dynamics when assessing nitrogen deposition impacts on soil methane cycling.

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Note: Figure translations are in progress. See original paper for figures.