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Long-term nitrogen and phosphorus fertilization modulates methane uptake and microbial community dynamics in subtropical forest soils

Wanying DONG[1], Wenyan LI[1], Suwen FENG[1], Yongcui DENG[2], Zemei ZHENG[1,3] and Sascha M. B. KRAUSE[1,3,*]

[1]*School of Ecological and Environmental Sciences, East China Normal University, Shanghai 200241 (China)*
[2]*School of Geography, Nanjing Normal University, Nanjing 210097 (China)*
[3]*Tiantong National Station of Forest Ecosystem, Chinese National Ecosystem Observation and Research Network, Ningbo 315132 (China)*

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ABSTRACT

Subtropical forests play a crucial role in reducing atmospheric methane (CH_4) levels; however, how long-term nitrogen and phosphorus enrichment regulates CH_4 uptake at low CH_4 levels remains poorly understood. This study combined laboratory microcosms, CH_4 uptake potential measurements, DNA-stable isotope probing, quantitative polymerase chain reaction, and Illumina sequencing of 16S rRNA gene to assess atmospheric CH_4 uptake at low levels of CH_4 and the dynamics of CH_4-consuming communities in subtropical forest soils under long-term nitrogen and phosphorus fertilization. While three-month microcosm incubation did not reveal consistent patterns in CH_4 uptake with nitrogen fertilization, the increase in phosphate (PO_4^{3-}) availability stimulated CH_4 uptake, suggesting alleviation of phosphorus limitation. The abundance of upland soil cluster α methanotrophs, crucial for atmospheric CH_4 uptake, exhibited sensitivity to high nitrogen levels, potentially influencing CH_4 dynamics in the forest ecosystems. Furthermore, the genus *Methylocystis* was identified as the predominant active methanotroph in the plots with high nitrogen and phosphorus levels, suggesting its adaptation to metabolize atmospheric CH_4 under nutrient-rich conditions. These findings underscore the complex interplay between nutrient availability, microbial communities, and atmospheric CH_4 dynamics in subtropical forest soils under ongoing nutrient deposition. Further studies are needed to investigate the underlying mechanisms and their implications for ecosystem functions.

Key Words: climate change, greenhouse gas, methanotrophs, nutrient deposition, stable isotope probing

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INTRODUCTION

Methane (CH_4) is a potent greenhouse gas with a significantly higher global warming potential than carbon dioxide (CO_2), contributing substantially to anthropogenic climate change (Saunois *et al*., 2020; IPCC, 2021). Despite its short atmospheric lifetime, the impact of CH_4 on radiative forcing highlights the urgency of developing effective mitigation strategies (Saunois *et al*., 2020).

Forests play a crucial role in reducing atmospheric CH_4 concentrations by accounting for up to 62% of the global soil microbial CH_4 sink (Dutaur and Verchot, 2007; Feng *et al*., 2023). However, there is significant uncertainty in microbial CH_4 uptake due to the influence of environmental factors such as soil pH, moisture, temperature, and nutrient availability (Kolb, 2009; Chen *et al*., 2010; Gao *et al*., 2014; Ni and Groffman, 2018; Deng *et al*., 2019). Thus, it is crucial to gain a better understanding of the mechanisms driving the environmental impacts on forest microbial atmospheric CH_4 uptake.

Aerobic CH_4-oxidizing bacteria (methanotrophs) convert CH_4 into CO_2 and represent the only known biological pathway for atmospheric CH_4 removal (Murguia-Flores *et al*., 2021). The first step in this pathway is catalyzed by methane monooxygenase (MMO). The alpha subunit of particulate MMO (pMMO), encoded by *pmoA* gene, is present in nearly all methanotrophs and serves as a functional marker gene to quantify methanotroph populations in various environments (Kolb *et al*., 2003). Methanotrophs are found in the phyla Verrucomicrobiota, Proteobacteria, and Actinobacteria and in the candidate division NC10 (Guerrero-Cruz *et al*., 2021; van Spanning *et al*., 2022).

Within proteobacterial aerobic methanotrophs, Gammaproteobacteria (type I methanotrophs) primarily use the ribulose monophosphate cycle for assimilation, while Alphaproteobacteria (type II methanotrophs) use the serine cycle (Trotsenko and Murrell, 2008). While CH_4 oxidation by methanotrophs in high CH_4 soil ecosystems, known as low-affinity methanotrophy, has been well studied over the years (Guerrero-Cruz *et al*., 2021), high-affinity methanotrophs, active in different soils and capable of oxidizing atmospheric

*Corresponding author. E-mail: ssa@des.ecnu.edu.cn.

CH_4, were not identified until 2003. These methanotrophs were classified as upland soil cluster α (USCα) and upland soil cluster γ (USCγ) (Knief *et al*., 2003). Recent studies have demonstrated that conventional type II methanotrophs, such as *Methylocystis*, *Methylosinus*, and *Methylocapsa*, are also responsible for atmospheric CH_4 uptake (Kravchenko *et al*., 2010; Cai *et al*., 2016; Tveit *et al*., 2019).

Nitrogen (N) is an essential nutrient, and its deposition has substantially increased in many ecosystems including forests, due to global population growth, industrialization, and agricultural intensification (Bodelier and Laanbroek, 2004; Larssen *et al*., 2011; Fowler *et al*., 2013; Xu *et al*., 2019; Chen *et al*., 2020). High concentrations of inorganic N, such as ammonium (NH_4^+) or nitrate (NO_3^-), have been shown to inhibit methanotrophy and methanogenesis, respectively (Suwanwaree and Robertson, 2005; Zhang *et al*., 2011; Fender *et al*., 2012). Additionally, N addition has been reported to stimulate CH_4 uptake (Maljanen *et al*., 2006), which may be related to the reduction of CH_4 production by NO_3^- inhibiting the activity of methanogens (Klüber and Conrad, 1998).

In addition to N availability, phosphorus (P) is another key nutrient for methanotrophy. However, information regarding the impact of P availability on forest soil CH_4 fluxes is limited (Veraart *et al*., 2015; Martinson *et al*., 2021). Previous research indicates that the addition of P to forest ecosystems can be both stimulatory and inhibitory for CH_4 uptake, but this effect is ecosystem-specific (Zhang *et al*., 2011; Zheng *et al*., 2016; Yu *et al*., 2017). For example, the observed stimulating effect of P addition on CH_4 uptake has been attributed to an increased CH_4 sink capacity, because P addition relieves N inhibition of microbial CH_4 oxidation (Yu *et al*., 2017). Therefore, further research is needed to understand the complex interactions between N and P availability and their combined effects on CH_4 fluxes in forest ecosystems.

Subtropical forests are important ecosystems that contribute approximately 19% to the global forest soil CH_4 uptake and are characterized by rich biodiversity and high productivity (Feng *et al*., 2023). In this study, we combined microcosm incubations and CH_4 uptake measurements in the laboratory with DNA-stable isotope probing (SIP), quantitative polymerase chain reaction (qPCR), and 16S rRNA gene Illumina sequencing to assess CH_4 uptake and CH_4-consuming community dynamics in subtropical forest soils under long-term N and P fertilization. We selected 12 parts per million by volume (ppmv) as a concentration of CH_4 that is metabolically unfavorable, similar to 1.92 ppmv, in that it limits methanotrophic activity but still permits some labeling in SIP. We hypothesized that i) N addition inhibits and P addition promotes soil CH_4 uptake and ii) these nutrient enrichments modulate the diversity, abundance, and community structure of methanotrophs.

MATERIALS AND METHODS

Soil sampling

In December 2021, soil samples were collected from the plots with long-term N and P fertilization at the Zhejiang Tiantong Forest Ecosystem National Observation Research Station, East China (29°52′ N, 121°39′ E), which were established in December 2010. This evergreen broadleaved forest region is characterized by a typical subtropical monsoon climate with a mean annual temperature of 16.2 °C and annual precipitation of 1 374 mm (Bu *et al*., 2018). The dominant tree species include *Schima superba*, *Castanopsis carlesii*, *Lithocarpus glaber*, and *Castanopsis fargesii* (Wang *et al*., 2007). The soil is characterized as an Acrisol with medium-heavy loam texture and a 5 cm top organic layer (Gao *et al*., 2014).

In this study, four fertilization treatments were designed, including no addition of nutrients (control, CK) and additions of 100 kg N ha^{-1} $year^{-1}$ (high N, HN), 50 kg N ha^{-1} $year^{-1}$ (low N, LN), and 15 kg P ha^{-1} $year^{-1}$ from 2010 to 2014 and 50 kg P ha^{-1} $year^{-1}$ since 2014 (PP). The experiment was set up in plots with a size of 20 m × 20 m, with each treatment replicated three times in a completely randomized design. The polyvinyl chloride boards were inserted 60 cm into the soil to enclose each plot, which were spaced at least 10 m apart. Fertilizers (NH_4NO_3 for N and NaH_2PO_4 for P) were applied monthly, starting in January 2011, by distributing the solutions over the litter layer, with the control plots receiving equivalent amounts of water to account for throughfall differences (Gao *et al*., 2014; Zheng *et al*., 2017).

Three replicates were sampled from the HN, LN, PP, and CK treatments, referred to as HN1–HN3, LN1–LN3, PP1–PP3, and CK1–CK3, respectively. Each soil sample consisted of three randomly collected subsamples from each plot using a standard soil auger (10 cm in depth and 2.5 cm in diameter). Afterward, the soil samples were homogenized and sieved through a 2-mm screen to remove debris. A portion of the soil samples was stored in a 4 °C freezer for subsequent soil physicochemical analyses and microcosm experiments, and the remaining part was frozen at −20 °C for subsequent molecular biology analysis.

Microcosm setup and DNA-SIP experiment

Approximately 10 g (dry weight equivalent) of fresh wet soil was weighed and placed into 1-L corrective media bottles sealed with a 5-mm rubber septum for gas sampling (24 bottles in total). Deionized water was added to each soil sample until reaching 60% of the maximum water-holding capacity. Given that previous research has confirmed the importance of lanthanum in CH_4 oxidation (Skovran *et al*., 2019; Liu *et al*., 2024), all microcosms were supplemented with lanthanum in the form of lanthanum chloride ($LaCl_3$,

14.4 μmol). All microcosms were pre-incubated for a week to minimize the effect of soil disturbance on the measurement of CH_4 uptake rate.

After one week, the microcosms were divided into two groups. In one group, $^{13}CH_4$ was added to the microcosms with a mixture of 6 ppmv $^{12}CH_4$ (99.999%, Shanghai Jiaya Chemical Co., Ltd., China) and 6 ppmv $^{13}CH_4$ (99%, Shanghai Reer Technology Co., Ltd., China), while the other group served as the control group ($^{12}CH_4$ incubation), receiving only 12 ppmv $^{12}CH_4$. A previous study suggested that methanotrophs have a slight preference for the lighter isotope (^{12}C), so we first tried a mixture of $^{12}CH_4$ and $^{13}CH_4$ to boost atmospheric CH_4 consumption in the labeled group during the first 2 months instead of using only $^{13}CH_4$ (Kuloyo *et al*., 2020).

The headspace concentration of CH_4 was regularly monitored by gas chromatography (Nexis GC-2030, Shimadzu, Japan), equipped with a flame ionization detector (FID) to ensure that CH_4 is utilized as a substrate by methanotrophs. The CH_4 uptake rate (MUR, μg kg^{-1} h^{-1}) was calculated using the formula:

$$\mathrm{MUR} = \frac{\Delta m}{M\Delta t} = \frac{\rho v \Delta C}{M\Delta t} \tag{1}$$

where $\Delta m/\Delta t$ is the change in CH_4 mass within the incubation time, $\Delta C/\Delta t$ is the change in CH_4 concentration within the incubation time, and M, ρ, and v refer to soil sample mass, gas density, and bottle volume, respectively.

The headspace CH_4 concentration was regulated to 12 ppmv by replenishing the headspace with either $^{12}CH_4$ or $^{13}CH_4$ after the first and second months of incubation as described above. In the third month, the control group of soil samples was exposed to 12 ppmv $^{12}CH_4$, while the labeled group received 12 ppmv $^{13}CH_4$ for complete labeling, with this procedure repeated weekly. After three months, soil samples were collected, and DNA was extracted and then stored in a −80 °C freezer.

Determination of soil physicochemical properties

A range of soil physicochemical parameters were measured, including soil moisture, pH, total carbon (TC), dissolved organic carbon (DOC), soil organic carbon (SOC), total N (TN), total P (TP), NH_4^+-N, NO_3^--N, potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), and copper (Cu). Soil moisture was determined by the difference between the wet and dry weights of 6 g of soil. Soil pH was measured in a mixture with distilled water using a pH meter (S220-K, Mettler-Toledo, Switzerland). All other properties were measured by Sangon Biotech (Shanghai) Co., Ltd., China, according to the following standards: HJ 695-2014 for TC, HJ 501-2009 for DOC, NY/T 1121.6-2006 for SOC, LY/T 1228-2015 for TN, HJ-632-2011 for TP, HJ 974-2018 for micronutrients, and HJ 634-2012 for NH_4^+-N and NO_3^--N (Ministry of Ecology and Environment of the People's Republic of China, 2024).

DNA extraction and SIP fractionation

Soil genomic DNA was extracted from the samples previously stored at −20 °C and from the soils after three months of incubation using the DNeasy PowerSoil® Pro kit (Qiagen, Germany). The DNA concentrations were measured with a NanoDrop™ One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The DNA samples after incubation were subjected to SIP fractionation using the procedures described previously (Lu and Jia, 2013) with minor modifications.

For each sample, 2.0 μg of purified DNA was mixed with cesium chloride (CsCl) to an average density of 1.725 g mL^{-1}. Ultra-centrifugation was performed using 8-mL polyallomer UltraCrimp tubes (Sorvall, USA) in a VTi 65.2 vertical rotor (Beckman Coulter, USA) at 190 000 × *g* for 44 h at 20 °C. A total of 15 fractions from each sample were obtained from the buoyant density gradient, and the refractive index of each fraction was determined using an AR200 refractometer (Reichert, USA). The fractionated DNA was recovered by polyethylene glycol 6000 precipitation, dissolved in 20 μL of elution buffer (Qiagen, Germany), and then used for qPCR and 16S rRNA gene sequencing.

qPCR of pmoA gene and 16S rRNA gene

Microbial abundances were measured in the DNA-SIP fractions, starting samples (before incubation), and microcosm soil samples. All analyses were conducted by Sangon Biotech (Shanghai) Co., Ltd., China. In brief, the abundance of the *pmoA* gene in methanotrophs was quantified using the primer pair A189F/A650R (Bourne *et al*., 2001). The polymerase chain reaction (PCR) was performed in a 10 μL mixture containing 5 μL of 2 × SybrGreen qPCR Master Mix, 0.2 μL of each primer, 1 μL of DNA template, and 3.6 μL of double distilled H_2O. The reaction conditions were set as follows: pre-denaturation at 95 °C for 3 min; denaturation at 95 °C for 15 s; and annealing and extension at 60 °C for 30 s, repeated for 45 cycles. The qPCR run was performed on a LightCycler480 II fluorescence qPCR instrument (Roche, Switzerland). Serially diluted PCR plasmids containing respective *pmoA* gene fragments were used as standards. The average amplification efficiency was 94.2%, with an R^2 value of 0.994 6.

The abundance of the 16S rRNA gene in bacteria was quantified using the primer pair 357F/518R (Turner *et al*., 1999; Lee *et al*., 2010). The PCR reaction setup, conditions, and instrument used were the same as described above. Serially diluted PCR plasmids containing respective 16S rRNA gene fragments were used as standards. The average amplification efficiency was 103.4%, with an R^2 value of 0.992 8.

16S rRNA gene Illumina high-throughput sequencing analysis

The microbial community structure in the DNA-SIP fractions, starting samples, and microcosm soil samples was assessed by the 16S rRNA gene sequencing. The primers 341F/805R were selected for amplification of V3–V4 regions of the 16S rRNA gene (Herlemann *et al*., 2011). The PCR amplification of 16S rRNA gene, library preparation, quantification, and sequencing were performed using MiSeq Illumina technology and 2 × 300 nucleotide paired-end sequencing at Sangon Biotech (Shanghai) Co., Ltd., China (Liu *et al*., 2024). All sequences are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive under BioProject ID PRJNA1124617.

The DADA2 pipeline version 1.26.0 in R 4.2.2 (R Core Team, 2021) was used to process raw sequences and has been described in detail elsewhere (Callahan *et al*., 2016). In brief, 16S rRNA gene forward and reverse reads were trimmed at positions 270 and 240 from the end, or any position with a trunc(quality-score) of 2, for the 36 soil samples from the starting samples and microcosm incubations. For the 12 selected fractions from the DNA-SIP, reads were trimmed at positions 250 and 220 from the end or any position with a trunc(quality-score) of 2. Forward reads over two expected errors and reverse reads over five expected errors, or any ambiguous bases, were removed. Error models were constructed with random sample picking. The SILVA ribosomal RNA database SSU version 138 (Pruesse *et al*., 2012) was used to classify the resulting amplicon sequence variances (ASVs) taxonomically. The ASVs that were identified as mitochondrial or chloroplast sequences or not classified into prokaryotic phyla were removed from the dataset. The ASVs occurring at less than 0.001% relative abundance were removed, resulting in 5 136 ASVs and retention of 86.87% of total reads.

Statistical analysis

Differences between the treatments in CH_4 uptake rate, qPCR gene copy numbers, and Shannon diversity index were tested using one-way analysis of variance in IBM SPSS Statistics 23.0 (IBM Corp., USA). Shannon diversity index was calculated to assess alpha diversity using the vegan package (version 2.6.4) (Oksanen, 2022) in R 4.2.2 (R Core Team, 2021). Relative abundance plots were produced using the R packages reshape2 version 1.4.4 (Wickham, 2007), dplyr version 1.1.4 (Wickham *et al*., 2023), and tidyverse version 2.0.0 (Wickham *et al*., 2019).

RESULTS

N and P additions change soil physicochemical properties

As shown in Table I, soil pH and TC content in the PP treatment were higher than those in the LN treatment. Compared to CK, the DOC content was lower in the HN treatment, whereas the SOC content was lower in the LN treatment. As expected, the HN treatment led to numerical increases in soil TN, which differed significantly from those in the LN and PP treatments. Similarly, the NH_4^+-N content was the highest in the HN treatment, while the NO_3^--N content was the highest in the LN treatment. However, the highest TP content was observed in the PP treatment. Measured micronutrient levels varied significantly between the treatments and were higher in CK, except for K.

TABLE I

Soil physicochemical properties in the long-term (2010–2021) fertilization treatments with no addition of nutrients (control, CK) and additions of 100 kg N ha^{-1} $year^{-1}$ (high N, HN), 50 kg N ha^{-1} $year^{-1}$ (low N, LN), and 15 kg P ha^{-1} $year^{-1}$ from 2010 to 2014 and 50 kg P ha^{-1} $year^{-1}$ since 2014 (PP) at the Zhejiang Tiantong Forest Ecosystem National Observation Research Station, China

Property[a)]	CK	HN	LN	PP
Moisture (%)	34.2 ± 5.6[b)] a[c)]	34.7 ± 2.5a	36.7 ± 4.8a	42.9 ± 4.5a
pH	4.4 ± 0.10ab	4.4 ± 0.10ab	4.3 ± 0.12b	4.5 ± 0.08a
TC (g kg^{-1})	32.2 ± 1.2ab	31.1 ± 2.2ab	27.8 ± 4.0b	36.0 ± 2.4a
DOC (mg kg^{-1})	539.6 ± 68.4a	418.9 ± 37.8b	488.6 ± 19.0ab	471.7 ± 77.3ab
SOC (g kg^{-1})	31.0 ± 1.0a	29.5 ± 1.5ab	26.0 ± 1.5b	34.0 ± 2.0a
TN (g kg^{-1})	2.4 ± 0.08ab	2.5 ± 0.09a	2.02 ± 0.29c	2.1 ± 0.06bc
TP (mg kg^{-1})	666.0 ± 84.7ab	609.3 ± 59.5b	594.7 ± 56.0b	801.7± 51.8a
N:P ratio	3.7 ± 0.56a	4.15 ± 0.39a	3.38 ± 0.24ab	2.63 ± 0.13b
NH_4^+ (mg kg^{-1})	11.3 ± 1.2b	17.0 ± 0.0a	12.2 ± 2.2b	11.8 ± 1.9b
NO_3^- (mg kg^{-1})	15.7 ± 0.8c	18.7 ± 0.9b	21.9 ± 1.3a	15.9 ± 0.8c
K (g kg^{-1})	7.9 ± 0.25b	8.2 ± 0.21ab	8.4 ± 0.11a	8.3 ± 0.31ab
Ca (mg kg^{-1})	296.7 ± 17.0a	284 ± 20.8a	216 ± 26.5b	180 ± 20.1b
Mg (mg kg^{-1})	779.7 ± 21.8a	774.7 ± 120.4ab	681.3 ± 168.3ab	587.7 ± 80.5b
Fe (g kg^{-1})	28.3 ± 0.7a	27.2 ± 1.9ab	27.5 ± 0.3a	25.5 ± 0.2b
Cu (mg kg^{-1})	15.4 ± 0.8a	12.5 ± 1.9ab	14.9 ± 1.9ab	12.2 ± 1.9b
Zn (mg kg^{-1})	48.2 ± 1.6a	41.3 ± 2.9ab	42.1 ± 5.9ab	34.3 ± 4.9b

[a)] TC = total C; DOC = dissolved organic C; SOC = soil organic C; TN = total N; TP = total P.
[b)] Means ± standard deviations ($n = 3$).
[c)] Means followed by different letters within each row are significantly different at $P < 0.05$.

P addition increases CH_4 uptake rate

Overall, the CH_4 uptake rate showed similar patterns between the microcosms containing $^{12}CH_4$ and $^{13}CH_4$ (Fig. 1). On average, the samples in the PP treatment depicted the highest CH_4 uptake rate (0.62 μg kg^{-1} h^{-1}), followed by CK, HN, and LN treatments. In addition, the CH_4 uptake rate in the third month depicted the highest value in this experiment (Fig. 1). Interestingly, the LN treatment exhibited the lowest CH_4 uptake rate among all treatments, especially in the third month with the $^{13}CH_4$-only incubation (Fig. 1b).

N and P additions change total abundances of soil bacteria and methanotrophs

Overall, the total 16S rRNA gene abundance did not change significantly over time (Fig. 2, Table SI, see Supplementary Material for Table SI). However, a significant decline in 16S rRNA gene abundance was observed over time in the soils from the LN treatment (Fig. 2, Table SI).

The *pmoA* gene abundance showed distinct results before and after three-month incubation in different treatments (Fig. 3, Table SI). In the soil from CK, the *pmoA* gene abundance decreased significantly after three months of incubation with either $^{12}CH_4$ or $^{13}CH_4$ at low level. The *pmoA* gene abundance was generally lower in the N and P fertilization treatments compared to CK in the starting samples (before incubation), indicating negative effects of N and P additions on the abundance of methanotrophs. In the soil from the HN treatment, the *pmoA* gene abundance was up to 100 times lower than that in CK and did not increase with incubation time. In the soil from the LN treatment, the *pmoA* gene abundance significantly decreased (by 20 times) after three months of incubation with either $^{12}CH_4$ or $^{13}CH_4$ at low level.

N and P additions change soil microbial community composition, but not alpha diversity measure

Illumina sequencing of 16S rRNA gene in soil samples, both before and after incubation, identified a total of 34

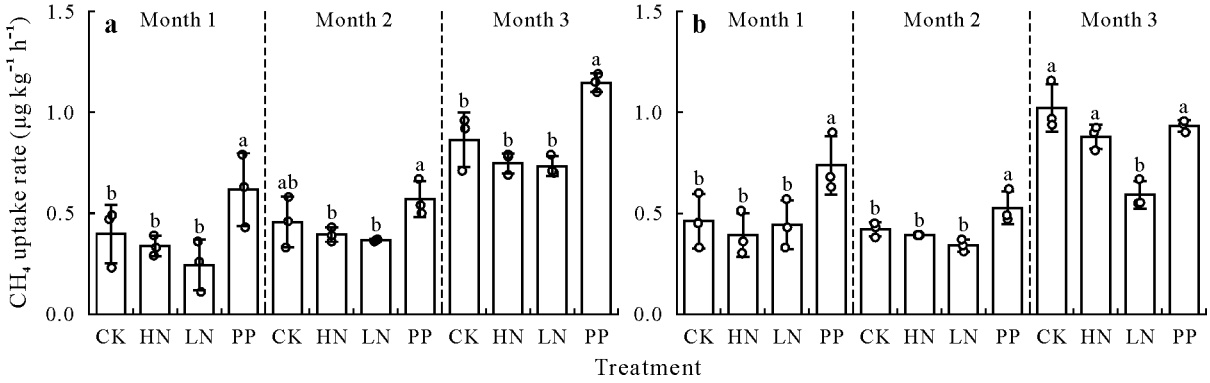

Fig. 1 CH_4 uptake rates in the microcosms containing $^{12}CH_4$ (a) and $^{13}CH_4$ (b) during the three-month incubation period using soil of the long-term (2010–2021) fertilization treatments with no addition of nutrients (control, CK) and additions of 100 kg N ha^{-1} $year^{-1}$ (high N, HN), 50 kg N ha^{-1} $year^{-1}$ (low N, LN), and 15 kg P ha^{-1} $year^{-1}$ from 2010 to 2014 and 50 kg P ha^{-1} $year^{-1}$ since 2014 (PP) at the Zhejiang Tiantong Forest Ecosystem National Observation Research Station, China. Vertical bars indicate standard deviations of the means ($n = 3$). Bars with different letters for a given month are significantly different at $P < 0.05$.

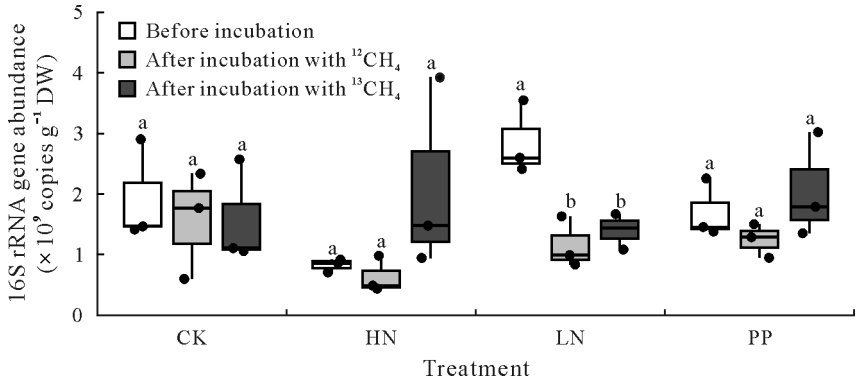

Fig. 2 Box plots of abundances of 16S rRNA gene in bacteria in the microcosms containing $^{12}CH_4$ or $^{13}CH_4$ before and after three-month incubation using soil of the long-term (2010–2021) fertilization treatments with no addition of nutrients (control, CK) and additions of 100 kg N ha^{-1} $year^{-1}$ (high N, HN), 50 kg N ha^{-1} $year^{-1}$ (low N, LN), and 15 kg P ha^{-1} $year^{-1}$ from 2010 to 2014 and 50 kg P ha^{-1} $year^{-1}$ since 2014 (PP) at the Zhejiang Tiantong Forest Ecosystem National Observation Research Station, China. Boxes with different letters for a given treatment are significantly different at $P < 0.01$. DW = dry weight.

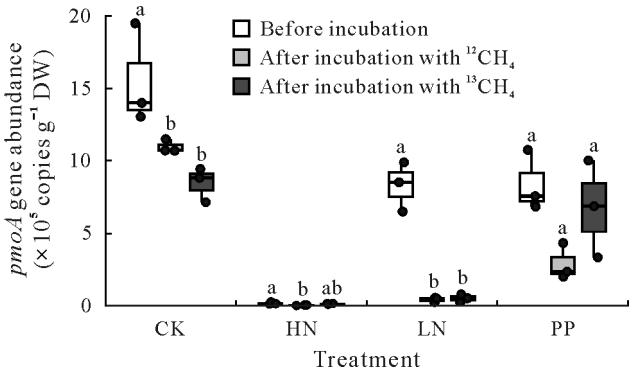

Fig. 3 Box plots of abundances of *pmoA* gene in methanotrophs in the microcosms containing $^{12}CH_4$ or $^{13}CH_4$ before and after three-month incubation using soil of the long-term (2010–2021) fertilization treatments with no addition of nutrients (control, CK) and additions of 100 kg N ha^{-1} year^{-1} (high N, HN), 50 kg N ha^{-1} year^{-1} (low N, LN), and 15 kg P ha^{-1} year^{-1} from 2010 to 2014 and 50 kg P ha^{-1} year^{-1} since 2014 (PP) at the Zhejiang Tiantong Forest Ecosystem National Observation Research Station, China. Boxes with different letters for a given treatment are significantly different at $P < 0.05$ for CK and HN and $P < 0.001$ for LN. DW = dry weight.

phyla-level and 434 genera-level ASVs. The genera *Candidatus*_Xiphinematobacter and *Burkholderia-Caballeronia-Paraburkholderia* were the most abundant bacterial groups, comprising 5.79% and 5.30% of the total bacterial community, respectively (Fig. 4). Methanotrophic populations, as expected, constituted a small fraction of the total bacterial community. Among the 60 most dominant genera, two methanotrophic genera, *Methylocapsa* (0.09%) and *Methylovirgula* (0.11%), were detected. Additionally, *Methylocella*, *Methyloferula*, and *Methylocystis* were also found in our samples, albeit in low relative abundance (data not shown). We observed the presence of *Mycobacterium* (phylum Actinobacteria), a genus recently confirmed to live on CH_4 as the sole energy and carbon source but not belonging to any of the known methanotrophic clades (van Spanning *et al*., 2022). It ranked surprisingly high, representing 2.51% of the total genera (Fig. 4).

Microbial Shannon diversity index, ranging from 5.68 ± 0.51 to 5.95 ± 0.04, did not differ between N and P fertilization treatments and CK before and after three-month incubation with $^{12}CH_4$. However, in the $^{13}CH_4$ incubation microcosms, the Shannon diversity index was significantly higher than that in the $^{12}CH_4$ incubation microcosms for CK and the LN treatment (Fig. S1, see Supplementary Material for Fig. S1).

During the experiment, approximately 0.77 μmol of CH_4 was consumed in each microcosm. We performed qPCR analysis targeting the *pmoA* gene on SIP fractions 2–14 (Fig. S2, see Supplementary Material for Fig. S2). The highest *pmoA* gene abundance was found in the heavy fraction with a buoyant density of 1.714 g mL^{-1} on average. Specifically, this was observed in replicates CK1 and CK3 in CK, HN2 and HN3 in the HN treatment, and PP2 and PP3 in the PP treatment. In contrast, the *pmoA* gene abundance from the $^{12}CH_4$ microcosms was predominantly enriched in the light fraction with a buoyant density of 1.699 g mL^{-1} on average (Fig. S2). However, the LN treatment, where ^{12}C and ^{13}C were not separated, indicated a lower CH_4 uptake rate and a low abundance of methanotrophs (Figs. 1 and 3).

Consequently, we selected the peak fractions from the $^{12}CH_4$ and $^{13}CH_4$ incubation microcosms in the 6 replicates

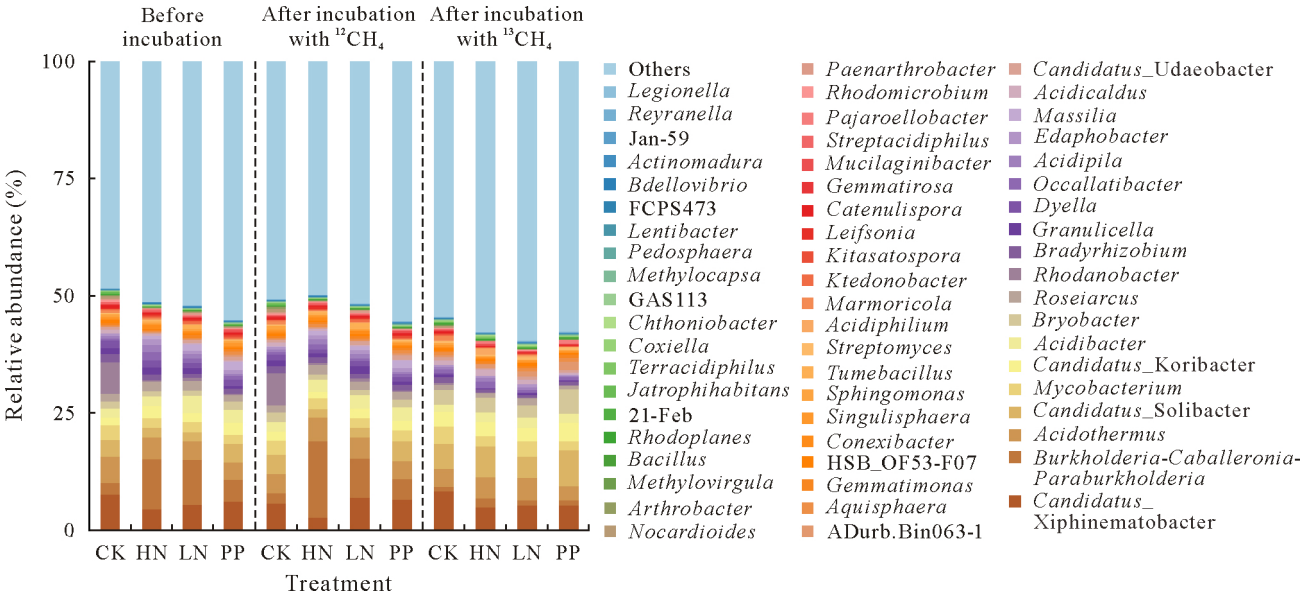

Fig. 4 Top 60 bacterial genera based on amplicon sequence variants from 16S rRNA gene sequencing in the microcosms containing $^{12}CH_4$ or $^{13}CH_4$ before and after three-month incubation using soil of the long-term (2010–2021) fertilization treatments with no addition of nutrients (control, CK) and additions of 100 kg N ha^{-1} year^{-1} (high N, HN), 50 kg N ha^{-1} year^{-1} (low N, LN), and 15 kg P ha^{-1} year^{-1} from 2010 to 2014 and 50 kg P ha^{-1} year^{-1} since 2014 (PP) at the Zhejiang Tiantong Forest Ecosystem National Observation Research Station, China.

CK1, CK3, HN2, HN3, PP2, and PP3. These fractions were then subjected to high-throughput sequencing of the 16S rRNA gene.

Among the most dominant genera, we identified four types of methanotrophic bacteria: *Methylocystis*, *Methylovirgula*, *Methylocapsa*, and the members of family Methyloligellaceae, although they could not be further resolved taxonomically (Fig. S3, see Supplementary Material for Fig. S3). Notably, *Methylocystis* accounted for 10.3% in the heavy fraction compared to 0.06% in the light fraction in replicate PP2, and 2% in the heavy fraction compared to 0.01% in the light fraction in replicate HN2 (Fig. S3). This indicates significantly active assimilation of ^{13}C by the methanotrophic microbial community, despite the challenge of incorporating $^{13}CH_4$ into the microbial community at low levels of CH_4.

DISCUSSION

Despite amending the forest soils with high (100 kg N ha^{-1} $year^{-1}$) and low (50 kg N ha^{-1} $year^{-1}$) doses of N, we did not find consistent trends in atmospheric CH_4 uptake over three months of incubation (Fig. 1). These results were surprising, because an earlier meta-analysis demonstrated that N addition in temperate, tropical, and subtropical forest soils significantly decreased CH_4 uptake (Xia *et al*., 2020). In line with another meta-analysis (Aronson and Helliker, 2010), Chang *et al*. (2021) further showed that N addition at 40 kg N ha^{-1} $year^{-1}$ decreased CH_4 uptake, whereas N addition at 8.0 kg N ha^{-1} $year^{-1}$ stimulated CH_4 uptake in montane forest soil.

These findings suggest that the effect of N addition on CH_4 uptake is dose-dependent and requires further research, perhaps exploring additional factors or longer incubation periods, to elucidate the underlying mechanisms driving CH_4 uptake dynamics in response to N amendments in these forest soils. Another possible explanation is that the studied microorganisms were limited by P deficiency rather than N deficiency. In this context, an increase in N concentration could exacerbate P limitation by raising the N:P ratio (Table SI). This is supported by previous work in lake sediments, where alleviating P limitation stimulated CH_4 oxidation (Nijman *et al*., 2022).

In the three-month microcosm incubations, an increased PO_4^{3-} content led to an increase in potential CH_4 uptake rate at low levels of CH_4 (Fig. 1). The observed decrease in $^{13}CH_4$ uptake rate during the third month can be attributed to fluctuating microbial activities and a slight preference for the lighter ^{12}C isotope (Kuloyo *et al*., 2020). This preference affected the overall performance in the ^{13}C-only setups (Fig. 1b). The stimulation of CH_4 uptake is in line with previous studies in different types of sediments and tropical forest soils, showing a higher rate of CH_4 oxidation with higher PO_4^{3-} availability (Zhang *et al*., 2011; Veraart *et al*., 2015; Nijman *et al*., 2022). Zhang *et al*. (2011) suggested that the addition of P in soil can enhance plant water uptake, resulting in reduced soil moisture and higher levels of CH_4 diffusing from the atmosphere into the soil. However, there were no plants in our model system, and soil moisture was kept equal between treatments, which makes it unlikely that the increased soil CH_4 uptake could be attributed to increased pore space in the soil. A study by Nijman *et al*. (2022) suggested that higher availability of PO_4^{3-} can promote CH_4 oxidation directly by stimulating the growth of methanotrophs, since they could use more P to invest in rRNA to increase their growth rate. We did not find any support for this mechanism, because the qPCR of both 16S rRNA and *pmoA* genes did not show any significant increase in their abundances as a proxy for microbial biomass in the PP treatment compared to CK (Figs. 2 and 3). However, a significant increase in microbial biomass might simply take more than 3 months, as shown for a recently identified atmospheric CH_4 oxidizer called *Methylocapsa gorgona*, which required more than 120 d to form colonies on floating filters under atmospheric air (Tveit *et al*., 2019). In addition, methanotrophs might not have shown any growth, because they utilized common carbon storage compounds like poly-β-hydroxybutyrate built up during periodic exposures to higher concentrations of CH_4 prior to the sampling for our incubation (Dunfield, 2007; Degelmann *et al*., 2010; Cai *et al*., 2016). Finally, increased P concentration in the soil may shift the methanotrophic community structure, favoring species or strains with higher CH_4 oxidation efficiency, even without a corresponding increase in *pmoA* gene abundance.

The abundance of USCα, a potential atmospheric CH_4 oxidizer, was up to 100 times lower in the HN and LN treatments than in CK (Table SI). It has been previously shown that atmospheric CH_4 uptake can already be affected by relatively small amounts of N addition (Adamsen and King, 1993), indicating that USCα-like populations are sensitive to both high and low N levels. In our case, the LN treatment resulted in a measurable reduction in USCα abundance, though less dramatic than that in the HN treatment. This suggests that, while low N inputs might not push USCα methanotrophs to low population levels, they still lead to a decline in population size and activity. In addition, CH_4 and NH_4^+ have a similar molecular structure, so that MMO can oxidize both CH_4 (to CH_3OH) and NH_4^+ (to NO_2^-). Therefore, CH_4 and NH_4^+ may compete for the same MMO and exacerbate substrate competition for the MMO (Gulledge *et al*., 2004). It is important to note that the NH_4^+ content was significantly high in the HN treatment, which was not the case for the LN treatment, where the NH_4^+ content did not differ significantly from CK (Table I). This suggests that substrate competition between CH_4 and NH_4^+ for the MMO in the LN treatment may not be the primary driver behind

the reduction in *pmoA* gene abundance. Instead, other factors could be at play, such as indirect effects of N addition or shifts in soil microbial community structure. These changes might still suppress methanotroph populations in the LN treatment despite the lack of a significant NH_4^+ increase.

Several different primers have been used for the amplification of *pmoA* gene fragments, with the first oligonucleotide primer pair A189F/A682R widely adopted in environmental studies (McDonald *et al*., 2008). However, they also amplify the *amoA* gene of ammonia-oxidizing bacteria due to the high similarity between pMMO and ammonia monooxygenase (AMO) enzymes (Bourne *et al*., 2001). To avoid the co-amplification, the primer pair A189F/mb661R was designed, but it discriminates against or poorly amplifies certain atmospheric methanotrophs such as USCα and type IIb methanotrophs (Bourne *et al*., 2001; Deng *et al*., 2013). In this experiment, we exclusively utilized the primer set A189F/A650R, which is not perfect but has shown a preference for USCα, a group of atmospheric CH_4 oxidizers dominant in forest soils, while discriminating against other conventional methanotrophs like *Methylocystis* (Cai *et al*., 2020). Hence, the observed discrepancy between *pmoA* gene abundance and CH_4 activity at high P levels may be due to primer selection. In addition, three other methanotrophs, *Methyloferula*, *Methylovirgula*, and *Methylocella*, identified in our study, do not contain the classic pMMO enzyme and thus could not be detected using *pmoA*-based primer system.

Major differences were not observed in the overall community composition and diversity in different treatments (Fig. 4). Two main methanotrophic populations were detected, namely *Methylovirgula* (0.11%) and *Methylocapsa* (0.09%), both of which belong to the family Beijerinckiaceae (type II). The *Methylovirgula* genus is typically found in acidic soils and decaying wood (Vorob'ev *et al*., 2009). However, there is only one known methanotrophic species in this genus, named *Methylovirgula thiovorans* HY1, which can aerobically oxidize CH_4 and reduce sulfur compounds for growth (Gwak *et al*., 2022). The *Methylocapsa* genus was confirmed to have the ability to live on trace atmospheric CH_4 as the sole carbon source. Tveit *et al*. (2019) isolated a strain of *Methylocapsa gorgona* MG08 that oxidizes atmospheric CH_4, has been detected globally in different soils, and is closely related to the uncultured members of the USCα clade.

It has long been believed that only the members of Proteobacteria and Verrucomicrobia can perform methanotrophic growth under aerobic conditions. However, a recent study by van Spanning *et al*. (2022) demonstrated that a representative of the genus *Mycobacterium*, referred to as *Candidatus* Mycobacterium methanotrophicum, can utilize CH_4 as the sole carbon source and is globally distributed. We found ASVs belonging to *Mycobacterium* (2%–5% relative abundance) in the microbial communities in our forest soils. These sequences were compared with the known *Candidatus* M. methanotrophicum sequences using the BLAST search at the NCBI. It showed 98.1%–99.52% identity over a 420 bp coverage, indicating *Mycobacterium* detected in our study is closely related to *Candidatus* M. methanotrophicum at the 16S rRNA gene level. Due to the differences in sequences, we cannot conclude that they were the same species, but there was a possibility that the *Mycobacterium* found in the present study was a potential methanotroph and deserved further investigation.

Stable isotope probing using low CH_4 levels successfully distinguished between heavy and light fractions (Fig. S3). The DNA-SIP results showed that the genus *Methylocystis* (type II methanotroph) was the main active methanotroph at low CH_4 levels under long-term N and P additions (Fig. S3). This group has long been hypothesized to be atmospheric CH_4 oxidizers, because they contain a second copy of pMMO, which is expressed at trace level of atmospheric CH_4 (Baani and Liesack, 2008; Kravchenko *et al*., 2010). It has been shown that the representatives of *Methylocystis* genus can be responsible for atmospheric CH_4 uptake in periodically drained ecosystems by flush-feeding the soil with elevated CH_4 (Cai *et al*., 2016). In addition, Krause *et al*. (2015) demonstrated that *Methylocystis*-dominated microbial communities in restored peatlands oxidized CH_4 at a wide range of concentrations due to water-table fluctuations and suggested a very pronounced flexibility and persistence of this group under land use change. Another study demonstrated that the *Methylocystis* genus was also the dominant CH_4 oxidizer in nutrient-rich grasslands (Wang *et al*., 2023). Together with our findings, it indicates that the *Methylocystis* genus has a wide adaptability in nutrient-rich environments as well.

CONCLUSIONS

This study demonstrated complex dynamics of atmospheric CH_4 uptake in subtropical forest soils under long-term N and P fertilization. While we did not observe consistent trends in atmospheric CH_4 uptake with varying N levels, we found that increased P availability stimulated CH_4 oxidation rate, suggesting a potential relief of P limitation in these soils, and that USCα methanotrophs were highly sensitive to N addition. The DNA-SIP results indicated the *Methylocystis* genus as an important key player at low levels of CH_4 and supported the idea that the metabolic flexibility of *Methylocystis* (Krause *et al*., 2015; Cai *et al*., 2016) is also involved in ensuring ecosystem functionality in subtropical forest soils under long-term N and P fertilization. While our study employed three replicates per treatment, a standard approach in many ecological field studies, we acknowledge that larger sample sizes could enhance the robustness of the statistical analyses. Further research, along with increased replication, is needed to investigate the crucial role of CH_4 uptake in forest and agricultural soils.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article can be found in the online version.

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