Abstract

Increased global nitrogen (N) and phosphorus (P) inputs caused by human activities can significantly impact methane (CH_4) uptake in terrestrial ecosystems. Forest soils, as the largest CH_4 sink among terrestrial ecosystems, play a crucial role in mitigating global warming. However, the effects of long-term N and P additions on CH_4 sink and the associated microbial mechanisms in subtropical forest soils remain unclear. To address this knowledge gap, we conducted a one-year *in-situ* field observations of soil CH_4 fluxes in a long-term N and P addition experimental platform in subtropical forest, focusing community structure and abundance of methanotrophs. Our findings revealed that long-term N addition significantly reduced the forest CH_4 sink, which was attributed mostly to a decrease in the CH_4 oxidation potential and the abundance of methanotrophs. Conversely, long-term P addition significantly enhanced the forest CH_4 sink due to an increase in the CH_4 oxidation potential and abundance of methanotrophs. Furthermore, we found a significant interactive effect of long-term N and P additions on forest CH_4 sink, with P mitigating the inhibitory effects of N addition on soil CH_4 sink. Overall, our results underscore the importance of understanding the interactive effects of long-term N and P additions on CH_4 sink in forests. This knowledge will enhance the accuracy of model predictions regarding atmospheric CH_4 dynamics amidst future global changes in N and P inputs.

Keywords

N addition; P addition; Methane process model; Methane sink; Methanotrophic activity; Methanotrophic abundance

1 Introduction

Methane (CH_4) is recognized as a significant greenhouse gas, contributing approximately 25-30% to global warming, with a global warming potential (GWP) 28 times greater than that of carbon dioxide (CO_2) (Saunois et al., 2020). Global atmospheric CH_4 concentrations represent a dynamic balance between CH_4 sources and sinks, which have continued to rise since the industrial revolution (Kim and Yi, 2009). Terrestrial ecosystems, particularly forest ecosystems, serve as the primary biotic CH_4 sink, with forest ecosystems accounting for around 52% of the global terrestrial CH_4 sink, estimated at 14.2 ± 15.5 Tg annually (Malyan et al., 2016). A decline in the CH_4 sink capacity of forest ecosystems, due to increasing temperatures, has the potential to influence atmospheric CH_4 concentrations by creating a feedback loop that would exacerbate global warming (Ni and Groffman, 2018).

In forest ecosystems, both soil and tree woody surfaces effectively absorb CH_4 (Gauci et al., 2024). The CH_4 efflux in forest soils results from the combined processes of soil CH_4 oxidation and CH_4 production (Zhou et al., 2021). Generally, the former significantly outweighs the latter, leading to a net phenomenon known as CH_4 uptake (Tate, 2015, Zhou et al., 2021). Numerous studies have demonstrated that CH_4 uptake

45 is notably affected by soil nutrient content (Song et al., 2024). Nitrogen (N) and
46 phosphorus (P) are essential limiting nutrients for plant growth and play fundamental
47 roles in biogeochemical cycles. These nutrients interact with one another, jointly
48 influencing CH_4 uptake in forest soils (Du et al., 2020). The inputs of N and P into
49 terrestrial ecosystems has accelerated due to increased global deposition caused by
50 human activities (Galloway et al., 2004, Elser et al., 2007). Thus, quantifying the
51 impacts of N and P inputs on forest soil CH_4 uptake is crucial for informing the global
52 CH_4 budget, advancing developments of CH_4 process models, and providing valuable
53 data for intergovernmental climate negotiations.

54 In recent decades, an increasing number of experiments simulating atmospheric N
55 and P inputs have been conducted to investigate their effects on forest soil CH_4 uptake.
56 Most studies indicate that N addition inhibits CH_4 uptake in forest soils (Zhang et al.,
57 2008, Yang et al., 2017, Chen et al., 2019a). However, this inhibitory effect appears to
58 be influenced by the duration of the fertilization. A meta-analysis by Chen et al. (2019)
59 quantified the effects of N addition on global upland soil CH_4 uptake and found that the
60 inhibitory effects of N initially increase before declining after 25 years of application.
61 Nonetheless, this relationship remains considerably uncertain due to a lack of long-term
62 N addition studies in forests where CH_4 uptake has been assessed. In contrast to N
63 addition, the effects of P addition on forest soil CH_4 uptake exhibit considerable
64 variability across studies, and the long-term effects remain unclear (Veraart et al., 2015).
65 Additionally, Kim et al. (2015) emphasized the interactive effects of combined N and
66 P additions on soil CH_4 uptake compared to the effects of N or P addition alone. These

two nutrients interact to affect the substrate availability and, consequently, the uptake of CH_4. Zhang et al. (2011) reported that combined N and P additions enhanced forest soil CH_4 uptake in comparison to N addition alone in a native forest, suggesting that P may mitigate the inhibitory effects of N addition. Conversely, Zheng et al. (2016) found that P did not mitigate the inhibitory effects in a human-disturbed forest. These contradictory findings may stem from different responses of forest soil CH_4 uptake processes to N and P addition. Therefore, it is essential to further investigate the underlying mechanisms involved in CH_4 uptake responses to long-term N and P additions.

Uptake of CH_4 is primarily mediated by a specialized group of microorganisms known as methanotrophs, which convert CH_4 into CO_2 using methane monooxygenase (MMO) (Tate, 2015). Generally, short-term high N addition can lower soil pH, thereby reducing CH_4 oxidation potentials (Tian et al., 2018). Additionally, elevated N levels can indirectly inhibit CH_4 uptake by increasing soil ammonium (NH_4^+) concentrations. Given that NH_4^+ has a chemical structure similar to that of CH_4, NH_4^+ can competes for the MMO reaction sites with CH_4. Additionally, NO_2^-, NO_3^-, and cations bound to NO_3^- produced during this process can also be toxic to methanotrophs (Nyerges and Stein, 2009). However, this inhibitory effect may diminish and become negligible over time as the soil methanotrophic community adapts to prolonged N addition (Chen et al., 2021). Zhang et al. (2011) investigated the mechanisms by which P addition promotes CH_4 uptake in N-saturated tropical forests in southern China, suggesting that P addition may enhance methanotrophic potential. Nevertheless, previous studies have not

thoroughly examined the microbial processes involved in CH_4 uptake in forest soil subjected to long-term N and P additions.

To fill this knowledge gap, we conducted an experiment to investigate *in-situ* soil CH_4 fluxes in a long-term (13-year) N and P addition platform in a subtropical forest using the static chamber approach. On this basis, we utilized *in-situ* CH_4 observations to simulate daily soil CH_4 flux in the study plots for a year using the methanotrophic process model (Memo) (Murguia-Flores et al., 2018). Additionally, we analyzed the effects of long-term N and P additions on methanotrophic abundance, methanotrophic community structure, and CH_4 oxidation potential through incubation experiments and metagenomic analysis. Our study aims to achieve two primary objectives: (1) to clarify the effects of long-term N and P additions on CH_4 sink in subtropical forest soils, and (2) to explore the microbial mechanisms influencing how long-term N and P additions impact CH_4 sink in subtropical forest soils. Our hypotheses are: (1) long-term N addition may reduce forest soil CH_4 sink to some extent, while P addition could mitigate the inhibitory effects of N addition; and (2) N and P addition could modify forest soil CH_4 sink by altering soil CH_4 oxidation potential.

2 Materials and methods

2.1 *Site description*

The experimental platform was established in December 2010 at the Tiantong Forest Ecosystem National Observation and Research Station (29°40′N, 121°47′E) in

111 Zhejiang Province, China. This area has a subtropical monsoon climate, characterized
112 by an average annual temperature of 16.2°C and annual precipitation of 1374.7 mm,
113 with the majority of rainfall occurring in the summer months (June to August),
114 accounting for 35% to 40% of total yearly precipitation. The annual N and P inputs
115 rates in this region ranges from 16.87 – 26.64 kg N ha^{-1} and 0.28 – 0.68 kg P ha^{-1},
116 respectively (Zhu et al., 2016, Chen et al., 2019b). The dominant soil type in this region
117 is Acrisol, featuring a medium-heavy loam texture. The dominant tree species include
118 *Schima superba*, *Lithocarpus glaber*, and *Camellia fraterna*.
119
120 2.2 *Experimental design*
121 Twelve experimental plots, each measuring 20 m × 20 m, were established in a
122 randomized block design, spaced 10 m apart (see Fig. 1). Those plots were enclosed
123 with 3 mm thick PVC boards, which were inserted into the soil to a depth of 60 cm to
124 minimize the mutual interference between plots resulting from fertilizer application.
125 Based on the current atmospheric N and P inputs rates in the region, along with the soil
126 N and P ratios in the sample plots, and following the international practice of doubling
127 the N application rate while maintaining a N and P fertilization ratio of 2:1, we
128 implemented four treatments with three replicates : Ambient control (no N or P
129 addition); N addition (100 kg N ha^{-1} yr^{-1}); P addition (50 kg P ha^{-1} yr^{-1}); and N and P
130 additions (100 kg N ha^{-1} yr^{-1} + 50 kg P ha^{-1} yr^{-1}) (Zheng et al., 2017). Considering the
131 background nutrient inputs rates in this region, the annual N and P inputs to the four
132 plots were approximately as follows: 20 kg N ha^{-1} yr^{-1} + 0.5 kg P ha^{-1} yr^{-1} for ambient

133 control, 120 kg N ha^{-1} yr^{-1} + 0.5 kg P ha^{-1} yr^{-1} for the N addition, 20 kg N ha^{-1} yr^{-1} +
134 50.5 kg P ha^{-1} yr^{-1} for the P addition, 120 kg N ha^{-1} yr^{-1} + 50.5 kg P ha^{-1} yr^{-1} for the N
135 and P additions, respectively. The experiment commenced in January 2011 and is still
136 ongoing, with a cumulative fertilizer application period of 13 years to date. Every
137 month, NH_4NO_3 or NaH_2PO_4 was dissolved in 20 L of water and evenly sprayed under
138 the canopy in the plots using a backpack sprayer. Simultaneously, the ambient plots
139 received an equal amount of water to ensure consistency in rainfall and biogeochemical
140 cycling across different treatments.
141
142 2.3 *Measurement of in-situ soil CH_4 fluxes and soil sampling*
143
144 *In-situ* soil CH_4 fluxes were measured five times from May 2023 to March 2024
145 using a static chamber approach (Zheng et al., 2008, Stiles et al., 2018). The sampling
146 dates were mid-May 2023, mid-July 2023, mid-October 2023, late December 2023, and
147 early March 2024, respectively. The sampling chamber, constructed from PVC,
148 comprised a bottomless box (30 cm in diameter and 40 cm in height) and a base fixed
149 into the soil at a depth of 8 cm. The top of the chamber featured a three-way valve for
150 gas sampling. All gas samples were collected between 9:00 AM and 12:00 PM local
151 time. For gas flux measurement from each sample plot, 30 ml gas samples were
152 extracted from the inner chamber using a syringe and injected into a vacuum tube at
153 10-minute intervals over a 40 minute period following deployment. Concurrently, soil
154 moisture at a depth of 10 cm was manually measured using a handheld Time-Domain

Reflectometry (TDR) sensor (FieldScout TDR 100, Spectrum Technologies, USA), while the soil temperature at a depth of approximately 5 cm, ambient air temperature, and intra-chamber air temperature were recorded with a handheld digital thermometer. Gas samples were transported to the laboratory and analyzed using gas chromatography (Agilent 7890B GC, USA) within 48 hours. The gas chromatograph was equipped with a flame ionization detector to measure CH_4 concentration, with N_2 utilized as the carrier gas. The *in-situ* soil CH_4 flux was calculated using the following equation:

$$f_{field} = \frac{\Delta m}{A\Delta t} = \frac{\rho v \Delta C}{A\Delta t} = \rho h \frac{\Delta C}{\Delta t}$$

where (f_{field}) represents the *in-situ* soil CH_4 flux, ($\Delta m/\Delta t$) denotes the change in CH_4 mass over time within the static chamber, ($\Delta C/\Delta t$) signifies the change in CH_4 concentrations within the static chamber, ρ represents the density of CH_4 within the static chamber, and (v), (A), and (h) represent the volume, bottom area, and height of the static chamber, respectively (Zhou et al., 2021).

Soil samples were collected twice: once in July 2023 and again in March 2024. Following a diagonal sampling pattern (i.e., one point at each corner and one point in the center of each plot), five sampling points were established within each sample plot prior to soil core collection. Using a soil auger with a diameter of 2.5 cm, approximately 500 grams of soil samples were extracted from a depth of 0 - 10 cm per plot and stored in a freezer at 4°C until they could be thoroughly mixed for subsequent analysis. The samples were cleaned of any plant residues and stones using a 2 mm sieve. Portions of the samples were stored at 4°C, while another portion was stored at -20°C for DNA extraction.

Soil pH was measured using the potentiometric method with a water-dry soil ratio of 2.5:1. Total P content in the soil was determined colorimetrically after acidified ammonium persulfate digestion. Total carbon (C) and N content were analyzed using a Vario Micro Cube elemental analyzer (Elementar, Germany). Extractable organic carbon (EOC), extractable organic nitrogen (EON) content, and NH_4^+-N content were measured using the hot water extraction method (Zhou et al., 2013, Wang et al., 2020).

2.4 *Measurement of oxidation potential, abundance, community structure of methanotrophs*

Soil samples were incubated in the laboratory to assess CH_4 oxidation potential. Approximately 10 g of soil samples were weighed into a 25 ml plastic cup and then placed in a 1 L glass bottle. To prevent water loss during incubation, a wet tissue was positioned at the bottom of the bottle. The bottles containing the soil samples were kept in a dark environment at 22°C for one week to ensure consistent methanotrophic activity. After this initial period, the bottles were flushed with fresh air, and the incubation continued for an additional week under the same conditions. At the beginning and end of the incubation period, we collected 30 ml of gas from the bottles using a syringe and injected it into a vacuum tube. The CH_4 concentration was analyzed using a gas chromatograph (GC9890B, Agilent). The soil CH_4 oxidation potential was determined based on the difference in CH_4 concentrations at the top of the bottles during the incubation process (Zhou et al., 2018).

199 The abundance of the *pmoA* gene and the methanotrophic community structure
200 were measured through metagenomic analysis. The abundance of *pmoA* is indicative of
201 methanotrophic abundance, as it is a key gene associated with these organisms. Soil
202 total genomic DNA was extracted from soil samples stored in a -20°C freezer according
203 to established procedures and subsequently sequenced using an Illumina HiSeq 4000
204 sequencer (Zhou et al., 2021). To obtain high-quality filtered data, the sequenced data
205 underwent quality control using fastp. The high-quality reads were then reassembled
206 into contigs using Megahit version 1.0.6 with default parameters (sequence identity
207 cutoff of 0.95 and minimum coverage cutoff of 0.9) (Li et al., 2015). Contigs longer
208 than 500 bp were utilized in Metagenemark, and the clustered gene sequences predicted
209 from all samples were compiled into a nonredundant gene catalog using CD-HIT (Li
210 and Godzik, 2006). Gene taxonomy classification in our catalog was established by
211 searching the NCBI-NR database (June 2017 version) (Xue et al., 2020). To determine
212 the abundance of species in each sample at the taxonomic level, the nonredundant gene
213 catalog was compared with the Kyoto Encyclopedia of Genes and Genomes (KEGG)
214 database using DIAMOND, with the Blast comparison parameter set at an e-value
215 threshold of 10^{-5} (Buchfink et al., 2015).
216
217 2.5 *Statistical analysis*
218
219 Daily soil CH_4 fluxes in the Tiantong subtropical evergreen forest during the
220 experimental period from March 2023 to March 2024 were simulated using the Memo

model (Zhou et al., 2024). Memo is a process-based model for estimating the CH_4 sink in soils, and soil temperature and moisture content are the main factors affecting the soil CH_4 sink in this model. Based on the *in-situ* soil CH_4 fluxes data, as well as daily soil temperature and moisture content data measured in these plots during experimental period, we simulated daily CH_4 flux in the Tiantong subtropical evergreen forest during the experimental period from March 2023 to March 2024. Subsequently, we calculated the annual CH_4 uptake by summing the daily soil CH_4 fluxes.

All statistical analyses were performed in R (Grunsky, 2002). In the process of data analyses, we utilize blocks as random factors to enhance the accuracy and effectiveness of our analysis. One-way analysis of variance (ANOVA) was performed to assess differences in average soil characteristics (pH, TC, TN, TP, EOC, EON, NH_4^+-N) among different treatments. Two-way ANOVA was employed to investigate both the individual and interactive effects of N and P additions on annual CH_4 uptake and methanotrophic abundance. Repeated-measures analysis of variance was used to analyze the effects of N and P additions on *in-situ* soil CH_4 fluxes, CH_4 oxidation potential, and the abundance of *pmoA*. Differences among treatments were compared using Tukey's honestly significant difference (HSD) test, with a significance threshold set at $P < 0.05$. The P-value for the main effect of each factor (e.g., N or P) indicates whether the independent effect of that factor on the dependent variable is significant, while accounting for the interaction effect. The P-value of the interaction effect is used to determine whether there is a significant interactive effect between the two treatment factors. If $P < 0.05$, it suggests that a significant interactive effect exists between the

two factors.

Additionally, structural equation modeling using maximum likelihood estimation was performed using R software to explore the causal relationships among N and P additions, soil physical properties (including soil moisture, soil temperature, pH), and soil chemical properties (including soil total C, N and P contents, EOC and EON content), soil NH_4^+ content, soil CH_4 oxidation potential, methanotrophic abundance and soil CH_4 uptake (Zhou et al., 2017, Gu et al., 2022). The model utilized data from four sample plots, each with three replicates. CH_4 fluxes were independently observed for five times and soil samples were collected independently twice in each plot, resulting in a total of 120 data points. Considering the random sampling and repeated measurements, we estimated variability among sample plots using a mixed-effects model before constructed the structural equation modeling. We also assessed the fitness of the model by testing the model fit parameters (χ^2/ df and Normed Fit Index). In this model, N addition and P addition are considered endogenous variables, while all other variables are exogenous variables.

3 Results

3.1 *Effects of N and P additions on soil CH_4 fluxes in subtropical forest*

The soil CH_4 fluxes in this subtropical forest soil exhibited considerable variability during the sampling period, ranging from -48.06 ± 6.31 μg m^{-2} h^{-1} to -4.37 ± 1.56 μg m^{-2} h^{-1}. This indicates that the forest soil predominantly acts as CH_4 sinks (see Fig. 2a).

Model simulations indicated that plots with N addition exhibited lower CH_4 uptake over the entire year, whereas plots with P addition showed higher CH_4 uptake compared to control plots. Notably, there was considerable variation in simulated daily CH_4 flux within the P addition plots. During the growing season (April 2023 - October 2023), soil CH_4 uptake was higher in the N and P additions plots relative to the N addition plots (see Fig. 2b).

Annual calculations of CH_4 uptake indicated that N addition significantly reduced the forest soil's CH_4 sink to 0.13 ± 0.03 g CH_4 m^{-2} $year^{-1}$. In contrast, P addition significantly increased the forest soil's CH_4 sink to 0.54 ± 0.14 g CH_4 m^{-2} $year^{-1}$ compared to control plots, which had a sink of 0.45 ± 0.04 g CH_4 m^{-2} $year^{-1}$. The N and P additions also exhibited a significant interactive effect on the forest soil's CH_4 sink, suggesting that P alleviated the inhibitory effect of N on forest soil CH_4 uptake (see Fig. 2c).

3.2 *Effects of N and P additions on the CH_4 oxidation potential, methanotrophic abundance and community in subtropical forest*

Compared to control plots, N addition significantly decreased soil CH_4 oxidizing potential, while P addition significantly enhanced it (Fig. 3a). Additionally, there was a notable interactive effect between N and P addition on soil CH_4 oxidizing potential (see Fig. 3a). Metagenomic analysis revealed that N addition significantly reduced the abundance of methanotrophs compared to the control treatment, whereas P addition did

not have a significant impact on methanotrophic abundance. However, N and P additions exhibited a significant increased interactive effect on the abundance of these microorganisms ($P < 0.01$) (refer to Fig. 3b).

We explored the relative changes in other functional genes involved in soil CH_4 oxidation. The results indicated that N addition, P addition, and N and P additions all resulted in a decrease in the abundance of the *pmoA*, *maxF*, and *fdhA* genes compared to the control treatment, with the reduction in *maxF* abundance being more pronounced than that of *fdhA* (see Fig. 3b and Table S1).

The metagenomic analysis showed that the methanotrophic community structure was relatively similar across different treatments (see Table S2), with type II methanotrophs dominating this plot (see Fig. 4a). A total of 13 species of methanotrophs were identified in the soil, with *Methylocapsa* and *Methylocystis* being the most abundant type II methanotrophs, while *Methylobacter* was the most abundant type I methanotroph (see Fig. 4a). Variance analysis further indicated that N and P additions had a significant interactive effect on the abundance of *Methylocapsa*. Specifically, N addition significantly reduced the abundance of *Methylocystis* ($P < 0.001$), whereas P addition increased the abundance of both methanotrophs compared to control conditions (see Fig. 4b).

3.3 *The key factor driving subtropical forest CH_4 sink*

Structural equation modeling results showed that N addition significantly altered

soil physical properties and increased soil NH_4^+ concentrations, subsequently modifying soil chemical properties as well (notable increases in nutrient content). P addition, on the other hand, significantly decreased both soil NH_4^+ concentrations and nutrient content, without significantly affecting soil pH. Furthermore, the changes in soil physical properties and NH_4^+ concentrations significantly enhanced CH_4 oxidation potential and methanotrophic abundance, leading to increased soil CH_4 uptake. However, the decrease in soil pH and the rise in NH_4^+ concentrations due to N addition directly inhibited soil CH_4 uptake. Importantly, P addition mitigated the inhibitory effects of N on soil CH_4 uptake by reducing NH_4^+ content and enhancing CH_4 oxidation potential and methanotrophic abundance (see Fig. 5).

4 Discussion

4.1 *Effects of long-term N and P additions on subtropical forest soil CH_4 sink*

Field observations of *in-situ* CH_4 fluxes, alongside model simulations, demonstrated that N addition significantly inhibited CH_4 uptake in subtropical forest soils. This finding aligns with previous studies (Zhang et al., 2017, Stiles et al., 2018, Chen et al., 2019a). Chen et al. (2021) illustrated that N application notably increased inorganic nitrogen levels in the soil in a short time, which in turn inhibited soil CH_4 uptake due to the accumulation of N. The inhibitory effect of N application on CH_4 uptake became less significant over time, attributed to the adaptation of methanotrophs to prolonged N addition (Gulledge et al., 2004).

However, our findings reveal that long-term N addition continues to significantly inhibit soil CH_4 uptake *via* two mechanisms. First, long-term N addition decreases soil pH and increases NH_4^+ content, thereby inhibiting CH_4 oxidation potential (refer to Table 1 and Fig. 3a). Second, it alters the methanotrophic community, resulting in a lower abundance of type II methanotrophs in the soil (refer to Fig. S1).

P addition was found to enhance forest soil CH_4 uptake, consistent with previous findings (Hütsch, 1996, Mori et al., 2003, Zhang et al., 2011). The underlying mechanism involves long-term P addition increasing CH_4 oxidation potential (refer to Fig. 3). As an essential element for organism growth and energy synthesis, P plays a crucial role in sustaining microbial metabolic activities (Holford, 1997). Subtropical forests have historically experienced N saturation and P limitation (Zhang et al., 2011, Du et al., 2020), meaning P addition alleviates this limitation. This increased availability of P enhances CH_4 oxidation potential and, consequently, promotes soil CH_4 uptake.

Moreover, N and P additions exhibited an interactive effect on CH_4 uptake (see Fig. 2), with P addition significantly alleviating the inhibitory effects of N addition, thus providing robust support for our first hypothesis. Our results align with previous N and P additions experiments (Zhang et al., 2011, Zhang et al., 2020). We also observed that CH_4 uptake was higher in N and P addition plots during the growing season (April 2023 - October 2023) compared to N addition plots. However, soil CH_4 uptake levels were similar in both sample plots during the non-growing season, indicating that the alleviating effects of P addition is closely correlated with plant aboveground biomass.

353 Soil fertility is essential for sustaining aboveground plant biomass, as the total
354 contents of carbon, nitrogen, and phosphorus in the soil underpins nutrient availability
355 (Ågren, 2008). In the context of P addition alleviating P limitation in subtropical forests,
356 the increase in soil carbon, nitrogen, and phosphorus contents significantly enhances
357 aboveground biomass. This boost in aboveground biomass results in greater resources
358 allocation to belowground substrates, thereby facilitating soil CH_4 uptake (refer to Table
359 1; Table S3).
360
361 4.2 *The microbial processes of long-term N and P additions affects subtropical forest*
362 *soil CH_4 sink*
363
364 Long-term N and P additions have the potential to alter soil physicochemical
365 properties, which may subsequently affect soil CH_4 uptake. These alterations can also
366 directly affect CH_4 oxidation potential, methanotrophic abundance and community
367 structure, further influencing soil CH_4 uptake (see Fig. 5). While numerous studies have
368 examined the effects of N addition on CH_4 oxidation potential (He et al., 2019, Zeng et
369 al., 2019), no research has concurrently investigated the effects of both N and P
370 additions on CH_4 uptake in forest soils, along with the associated changes in CH_4
371 oxidation potential and methanotrophic abundance. Our study is the first to explore the
372 microbial mechanisms by which long-term N and P additions influence CH_4 uptake in
373 subtropical forest soils.
374 Soil CH_4 uptake is a microbial process mediated by specific functional

375 microorganisms known as methanotrophs. The structure and abundance of the
376 methanotrophic community are critical biological factors affecting this process (Tate,
377 2015, Gu et al., 2019). Our findings indicate that the effects of long-term N and P
378 additions on CH_4 oxidation potential and methanotrophic abundance were consistent
379 with their impacts on soil CH_4 uptake, thereby supporting our second hypothesis.

380 On one hand, long-term N addition can alter soil physicochemical properties such
381 as pH and nutrient availability, which in turn affects the physiological activity of
382 methanotrophs (Ågren, 2008, Tian et al., 2018). On the other hand, ammonium nitrate
383 (NH_4NO_3) is typically converted to nitrite (NO_2^-) or hydroxylamine (NH_2OH) in soil
384 (Nyerges and Stein, 2009), both of which are toxic to methanotrophs. We observed a
385 significant decrease in soil CH_4 oxidation potential and methanotrophic abundance due
386 primarily to effective reductions in soil pH in N-saturated subtropical forests (Table 1).
387 Soil organic extractable carbon and nitrogen, as active substrates utilized by
388 microorganisms (Zhou et al., 2012), can enhance CH_4 oxidation potential to some extent.
389 However, this promoting effect may be overshadowed by the detrimental impacts of
390 decreased pH and NO_2^- toxicity. In contrast, long-term P addition increased CH_4
391 oxidation potential, likely by alleviating P limitation for methanotrophs and enhancing
392 their utilization efficiency of soluble soil organic carbon (refer to Table 1). Furthermore,
393 long-term P addition may improve the stability of aluminum ion (Al^{3+}) in the soil
394 through adsorption, thereby mitigating its toxic effects on methanotrophs (Nanba and
395 King Gary, 2000, Zhang et al., 2011).

396 Differences in methanotrophic community structure are a critical factor

influencing CH_4 oxidizing capability (Singh and Tate, 2007). Thus, understanding the response of the methanotrophic community structure to long-term N and P additions is essential for estimating the global CH_4 sink in forests. Our study revealed that the methanotrophic community was predominantly composed of type II methanotrophs, consistent with previous research on methanotrophic communities in global forest soils (Kolb, 2009). Soil pH emerged as the primary factor influencing methanotrophic community structure in this experiment (Nguyen et al., 2018). The abundance of *Methylocapsa* within methanotrophic communities was linked soil pH (which ranged from 3.88 to 4.37) (Kolb et al., 2005, Täumer et al., 2021). Generally, the abundance of type II methanotrophs is influenced by their tolerance to inhibition by NH_4^+ and nitrate (NO_3^-) to some extent (Nyerges and Stein, 2009). While the abundance of *Methylocapsa* was not significantly altered by long-term N addition or P addition, the abundance of *Methylocystis*, *Methyloferula*, and *Methylosinus* was decreased as a result of long-term N addition. This effect may be attributed to the adaptation of *Methylocapsa* to low pH environments, as this acidophilic bacterium might mitigate the inhibitory effects of NH_4^+ and NO_3^- (Nyerges and Stein, 2009).

5 Conclusions

Our results indicate that long-term N addition significantly reduces forest soil CH_4 sink by inhibiting CH_4 oxidation potential and decreasing methanotrophic abundance. In contrast, long-term P addition significantly enhances forest soil CH_4 sink by

increasing CH_4 oxidation potential and the abundance of methanotrophs. Notably, long-term P addition also decreased soil NH_4^+ content, alleviating the inhibitory effects of N addition on CH_4 oxidation potential and, consequently, on the forest soil CH_4 sink. Therefore, to improve the accuracy of terrestrial ecosystem models in estimating global atmospheric CH_4 concentrations, it is recommended that these models account for how P mitigates the inhibitory effects of N addition on forest soil CH_4 sink. In conclusion, our findings advance our understanding of soil CH_4 uptake in forests and provide theoretical support for managing forest soil carbon sink.

CRediT author contribution statement

Xiaoqi Zhou: Conceptualization, Funding acquisition, Methodology, Validation, Writing – review and editing. **Yanjun Liu**: Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review and editing. **Wensheng Xiao**: Formal analysis. **Jing Feng**: Formal analysis. **Marcos Fernández-Martínez**: Validation, Writing – review and editing. **Carly Stevens**: Validation, Writing – review and editing. **Zemei Zheng**: Resources, Project administration.

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Data availability statement

All the data necessary to evaluate the conclusions are included in the paper and/or the Supplementary Materials.

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692 Table 1 Variations in soil characteristics of Tiantong evergreen forests (0 - 10 cm)
693 under long-term N and P additions (mean ± standard errors, n = 3).

694

Soil properties	Control	N	P	NP
pH	4.18±0.03b	3.88±0.05c	4.37±0.03a	3.94±0.03c
TC (g kg^{-1})	24.8±1.43c	30.1±0.38b	32.4±0.31b	37.9±0.87a
TN (g kg^{-1})	1.80±0.12c	2.10±0.06b	2.29±0.01ab	2.39±0.01a
TP (g kg^{-1})	0.28±0.01b	0.27±0.01b	0.49±0.04a	0.46±0.07a
EOC (mg kg^{-1})	763.24±15.56b	957.74±21.24a	897.49±22.86a	925.06±26.27a
EON (mg kg^{-1})	65.50±2.94a	72.47±2.66a	67.53±1.54a	71.41±1.65a
NH_4^+-N (mg kg^{-1})	24.23± 2.24a	19.58 ±1.77a	12.23 ±0.16b	11.57 ±0.17b

695
696 TC, total carbon; TN, total nitrogen; TP, total phosphorus; extractable organic carbon
697 (EOC), extractable organic nitrogen (EON), NH_4^+.
698 Different lowercase letters represent significant differences ($P < 0.005$) between the
699 treatments. N indicates N addition; P indicates P addition; NP indicates combined
700 addition of N and P.
701

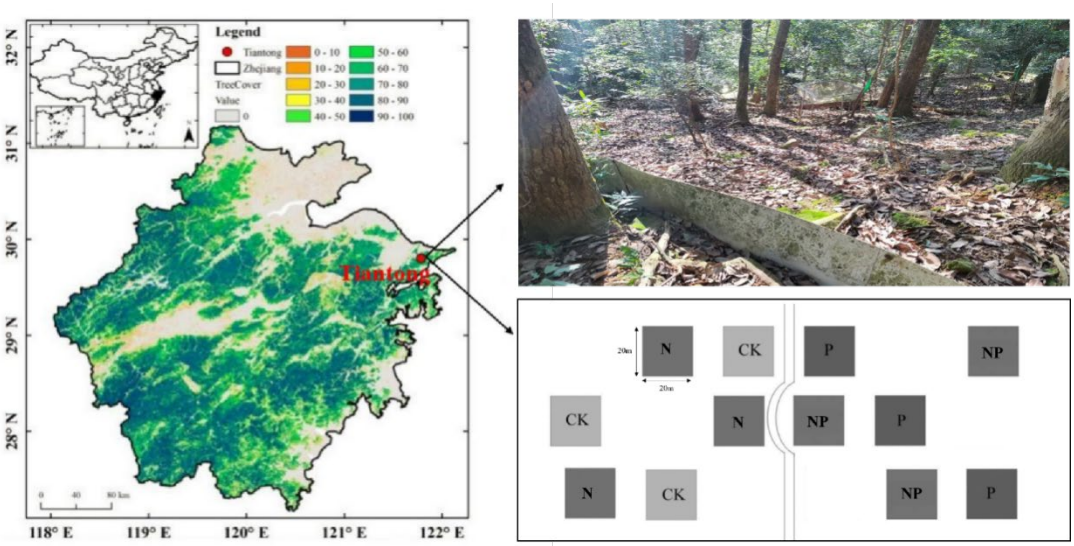

Fig.1 Location and summary of the long-term nitrogen and phosphorus addition experimental platform in Tiantong Forest Ecosystem National and Research Station in Zhejiang province, China. The diagram on the left shows the location of the experimental plots. The top right image is a field picture of the sample plots, and the bottom right shows the distribution of the sample plots in this region. N indicates N addition; P indicates P addition; NP indicates combined addition of N and P.

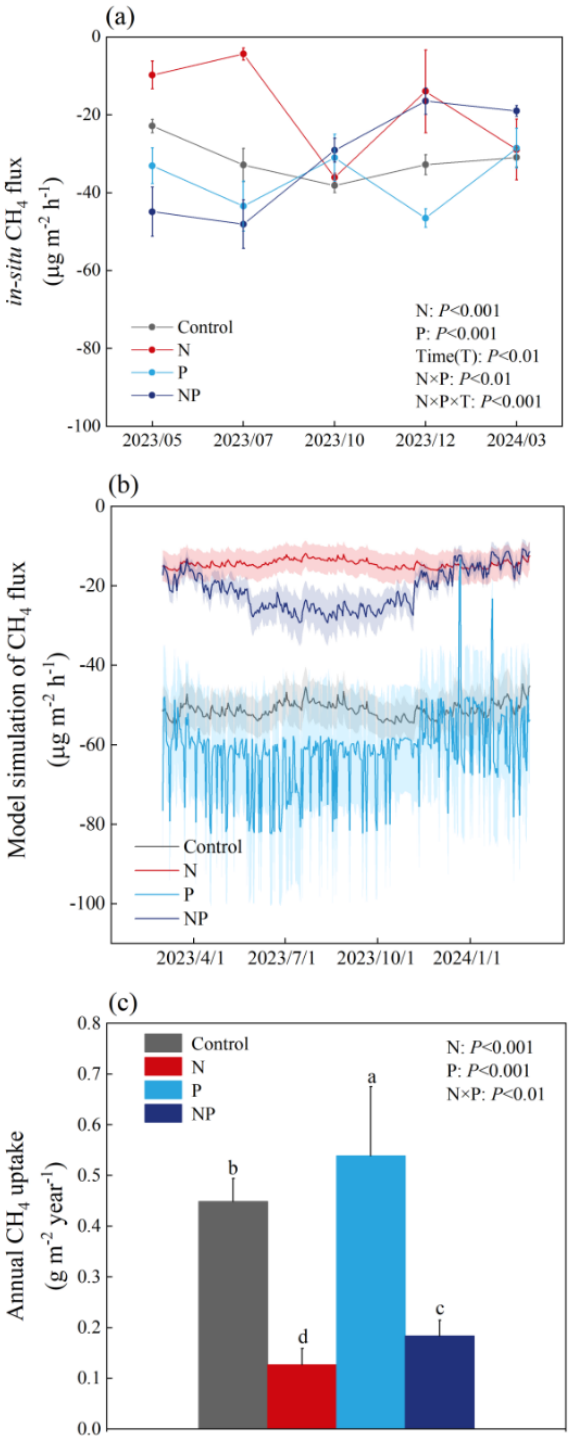

Fig.2 Variations in (a) soil *in-situ* CH_4 flux between March 2023 and March 2024, (b) daily CH_4 flux simulated by the Memo model, and (c) annual CH_4 uptake in Tiantong evergreen forests. The shaded portion of Fig. b indicates the standard deviation in the model estimates. The values in the figure are presented as mean ± standard error, with n = 3. Different lowercase letters represent significant differences ($P < 0.005$) between the treatments. N indicates N addition; P indicates P addition; NP indicates combined addition of N and P.

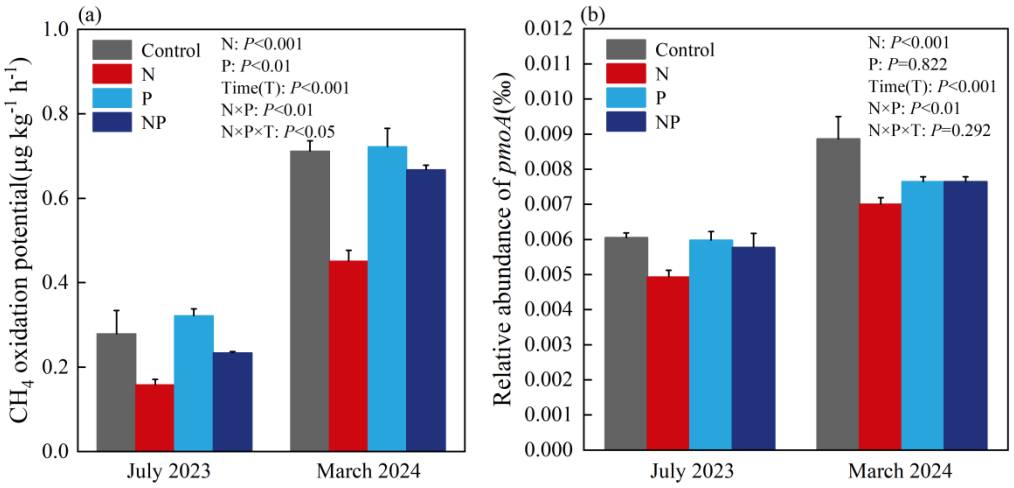

Fig.3 Changes in CH_4 oxidation potential (a) and the abundance of *pmoA* (b) in Tiantong evergreen forests under long-term N and P additions (mean ± standard errors, n=3). N indicates N addition; P indicates P addition; NP indicates combined addition of N and P.

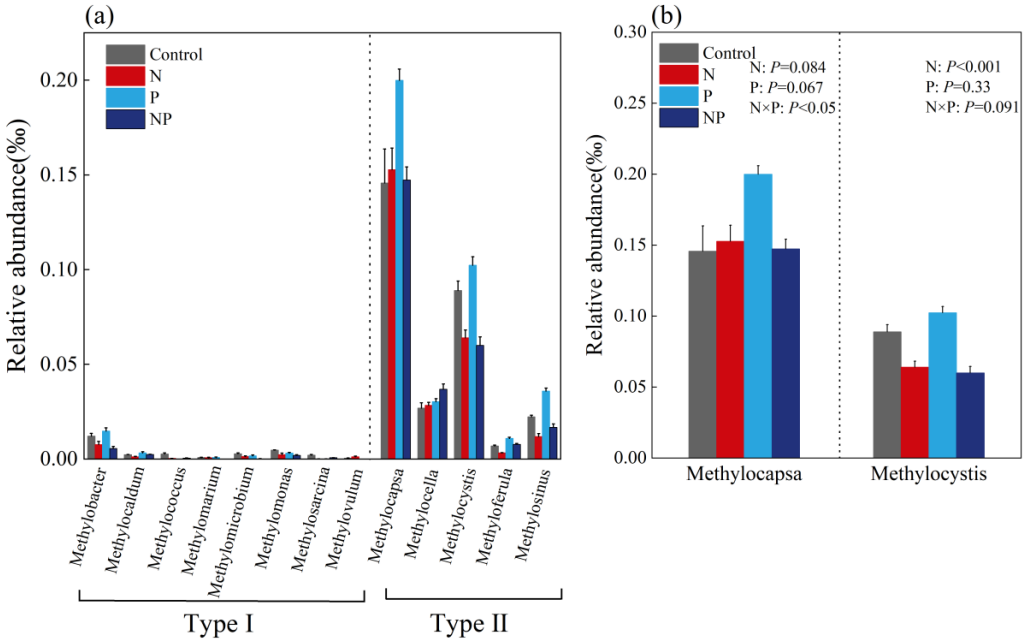

Fig.4 Relative abundance of different genus of methanotrophs (a) and changes in the relative abundance of main methanotrophic genus of *Methylocapsa* and *Methylocystis* (b) in Tiantong evergreen forests under long-term N and P additions (mean ± standard errors, n=3). N indicates N addition; P indicates P addition; NP indicates combined addition of N and P.

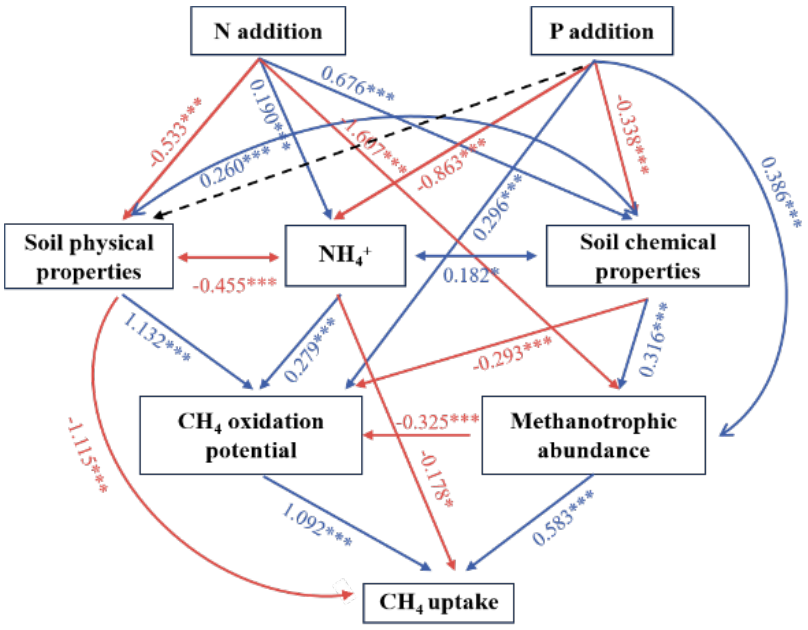

Fig.5 Structural equation modeling was used quantify the effects of N and P additions on soil CH_4 uptake. The model fitting results indicated a good model fit to the data, with a χ^2/df ratio of 0.997 and a Normed Fit Index (NFI) of 0.976. In the model, the blue arrow represents a significant positive effect, while the red arrow indicates a significant negative effect. The gray dotted arrow denotes a non-significant effect. *, **, and *** represent significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.