Soil Biology and Biochemistry 184 (2023) 109098

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

N-induced soil acidification triggers metal stimulation of soil methane oxidation in a temperate steppe ecosystem

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ARTICLE INFO

Keywords:
Nitrogen addition
pH
CH_4 oxidation
Metal element

ABSTRACT

Methane (CH_4) is a potent greenhouse gas, and it is well established that low nitrogen (N) stimulates- and high N suppresses CH_4 oxidation in grassland ecosystems. In this study, we examined the response of CH_4 uptake to long-term (>10 years) multi-level N additions in a temperate steppe of northern China. The N impacts on CH_4 uptake transitioned from positive to negative at the N addition rate of 4 g N m^{-2} y^{-1}. However, the high-N suppression on CH_4 uptake was partially relieved when the continuous (>10 years) high N inputs (16 g N m^{-2} y^{-1} to 64 g N m^{-2} y^{-1}) caused soil pH drop to < 5.7. Further experiments revealed that continuous high-N inputs acidified soil to pH < 5.7 that released metal ions to stimulate monooxygenase enzyme activity; therefore, the CH_4 oxidation suppression was partially relieved under continuous N loading. Structural equation model results confirmed that metal ions, such as Iron (Fe^{3+}), Manganese (Mn^{4+}), and Copper (Cu) desorbed by soil particles, were negatively correlated with soil pH while positively correlated with CH_4 oxidation under high N inputs. These analyses suggested that continuous high N inputs-induced soil acidification can release previously bounded metal elements that partially alleviated the N suppression on CH_4 uptake. The N-induced soil acidification impacts on CH_4 uptake might play a critical role in global CH_4 cycling and deserve further investigation as external N inputs continue climbing.

1. Introduction

Semiarid grasslands account for ~6% of the global sink of atmospheric methane (CH_4), the second most potent greenhouse gas (Dalal et al., 2008; Dijkstra et al., 2013). External nitrogen (N) inputs have been reported to stimulate (Bodelier et al., 2000) or suppress (Tlustos et al., 1998) CH_4 uptake, depending on the rate and forms of the N added. Low N inputs typically stimulate the growth of methanotrophs that are always N-limited, thus enhancing the CH_4 oxidation in grasslands (King, 1992; Peng et al., 2019). On the contrary, high N inputs typically suppress CH_4 oxidation via the competition between CH_4 and NH_4^+ molecules for CH_4 monooxygenases (Zhang et al., 2020), as confirmed by a continuous decline in CH_4 uptake under high N inputs in grasslands (Peng et al., 2019). Meanwhile, high N inputs acidify soils, thereby altering soil physical and chemical properties (Breemen et al., 1982; Horswill and Nauseef, 2008). Soil pH drop induced by continuous N might contribute to the suppression of CH_4 oxidation (Saarnio et al., 2004). However, the mechanisms of CH_4 oxidation suppression caused by N addition-induced soil pH drop remain elusive (Ambus and Robertson, 2006; Zhang et al., 2014).

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https://doi.org/10.1016/j.soilbio.2023.109098
Received 18 February 2023; Received in revised form 7 June 2023; Accepted 11 June 2023
Available online 17 June 2023

One possible mechanism is acidification caused-metal elements desorption from soil particles. For example, metal elements have been proven to affect CH_4 oxidation due to their impacts on methanotrophs and methanogens, thus mediating CH_4 flux (Stein, 2020). Iron (Fe), Manganese (Mn), and Copper (Cu) have direct and indirect impacts on microbial methanotrophy (Semrau et al., 2010; Semrau et al., 2013; Oni and Friedrich, 2019), and therefore CH_4 uptake at the ecosystem level. The immediate impact is through the positive impacts of Fe and Mn on CH_4 oxidation (Hakemian and Rosenzweig, 2007; Oni and Friedrich, 2019), likely due to their roles as alternative electron acceptors (Stein, 2020). The indirect effects occur by promoting enzyme activity (Krause et al., 2017). Through these direct and indirect impacts, soil microbes affect CH_4 flux as regulated by metal elements (e.g., Cu) (Semrau et al., 2010, 2013). For example, the activity of Clade II nitrous oxide reductase (*NosZ*) is directly related to the CH_4 cycling because some methanotrophs produce a copper chelator, named methanobactin, that has been shown to compete with NH_4^+, thereby increasing CH_4 oxidation effectively (DiSpirito et al., 2016).

The soil consumption of CH_4 is functionally intertwined with a range of other biogeochemically-active molecules, resulting in CH_4 flux variations. Previous studies have suggested various mechanisms via which N affects soil CH_4 uptake (Gulledge et al., 2004; Zhang et al., 2020), with an overall unimodal response to N additions (Bodelier and Laanbroek, 2004; Peng et al., 2019; Zhang et al., 2020). Although high-N inhibition on CH_4 uptake over a short term has been widely accepted (Peng et al., 2019; Zhang et al., 2020), whether the suppression persists or there was a new transition along the N cumulative over time remains unknown. Integrating existing information to develop a holistic understanding of CH_4 oxidation under N inputs is urgent to enhance our predictability of CH_4 flux in terrestrial ecosystems under changing environments.

To address those gaps, we made use of a long-term field experiment (initialized in 2003) in which a semiarid grassland was exposed to a gradient of N applications (0, 1, 2, 4, 8, 16, 32, 64 g N m^{-2} y^{-1}) to investigate the mechanisms of CH_4 uptake under a gradient of N inputs over time. The primary objective was to explore the mechanisms via which N addition affects CH_4 uptake along an N gradient. We hypothesized that (a) soil acidification caused by long-term N application (Tian et al., 2016; Malik et al., 2018; Xiao et al., 2020) enhanced the availability of metal elements such as Fe, Mn, Cu, and other soil ions (Ernani et al., 2002); and (b) higher metal availability stimulates methane monooxygenase (MMO) enzyme activity, thus partially relieving the N suppression on CH_4 oxidation; this is built on the reported correlations between methane monooxygenase (MMO) enzyme activity and Cu (Hakemian and Rosenzweig, 2007), Fe, and Mn (Oni and Friedrich, 2019) concentration. The major implication is to clarify the role of soil acidification-related microbial methanotrophs acted in mitigating CH_4 emissions to the atmosphere.

2. Materials and methods

2.1. Study site

This study was conducted in Duolun County (42°02′N, 116°70′E, 1324 m a.s.l.), a semiarid temperate steppe in Inner Mongolia, China. The mean annual precipitation and mean annual temperature are approximately 385 mm and 2.1 °C, respectively. Low foothills characterize the topography at elevations of 1150–1800 m. The soil type is *Calcis-orthic Aridisol* according to the US Soil Taxonomy classification, with 62.75 ± 0.04% sand, 20.30 ± 0.01% silt, and 16.95 ± 0.01% clay. The dominant plant species are *Stipa Krylov, Artimesia frigida, Potentilla acaulis, Cleistogenes squarrosa, Allium bidentate,* and *Agropyron cristattum*.

2.2. Experimental design

The N fertilization experiment was established in 2003. Seven N treatments (1, 2, 4, 8, 16, 32, and 64 g N m^{-2} y^{-1}) and control (CK) with eight replicates were randomly arranged in the areas of homogenous block to avoid heterogeneity within the plots, with 10 × 15 m plots for each treatment. A 4 m wide buffer zone was set up to separate the adjacent blocks. The fields were annually fertilized by the urea, and the levels of N addition amounted to 0, 1, 2, 4, 8, 16, 32, and 64 g N m^{-2} y^{-1}, respectively, in the middle of July. Because the local fertilization time was in the growing season (May–September) and this study aimed to develop a mechanistic understanding rather than quantifying the annual budget, we did the measurements and observations in the growing season only.

2.3. Measurement of ecosystem CH_4 flux

We used a static chamber technique to measure the CH_4 flux (Livesley et al., 2009) from 2013 to 2016. Briefly, three stainless steel permanent bases (50 × 50 × 12 cm) with a 3-cm-deep groove for sealing water were inserted into soils down to 12 cm in the plots approximately a month before the measurement each year (Zhang et al., 2012). A stainless-steel top chamber (50 cm height) with heat-isolating and light-impenetrable material was installed outside the base, with the downside into the groove. The grooves were filled with water to seal the chamber. Two electric fans were installed on the inside top of the chamber to mix the air in the headspace continuously and thoroughly during the measurements (Zhang et al., 2014, 2020). Sixty-ml gas samples were collected at 10-min intervals for 30 min using sixty-ml syringes with airtight stopcocks. Simultaneously, the air temperature and pressure in the chamber were measured, and the soil temperature and moisture were measured at 5- to 10-cm depths using a thermometer (Spectrum Technologies, Inc. East - Plainfield, Illinois) and portable soil moisture measuring kit ML2x (ThetaKit, Delta-T Devices, Cambridge, UK), respectively. The CH_4 concentrations of the gas samples were analyzed using a gas chromatograph (Agilent 7860, Agilent Technologies) equipped with a flame ionization detector (FID) using 60–80 mesh 13 XMS column (2 mm inner diameter and 2 m long), with an oven temperature of 55 °C. Nitrogen with a flow speed of 30 mL min^{-1} was used as a carrier gas, and the CH_4 flux was determined from the linear slope ($r^2 \geq 0.95$) of the mixing ratio changes in four samples taken at 0, 10, 20, and 30 min after chamber closure. The CH_4 fluxes were measured weekly from May to September in 2013, 2014, 2015, and 2016, and the positive fluxes indicated uptake.

2.4. Measurements of soil properties

Soil samples were taken to measure the soil total carbon (STC), total soil nitrogen (TN), total soil phosphorus (P), microbial biomass carbon (MBC), nitrogen (MBN), trace elements, and other physical properties (soil pH, texture, etc.). We took three soil cores to a depth of 10 cm from each plot once every month from May to September 2013–2016. The samples were mixed uniformly for each plot and separated into two sets. One set was stored at 4 °C in the laboratory to determine MBC and MBN using the chloroform fumigation extraction method (Liu et al., 2014). The other was air-dried, sieved (2 mm), ground, and assessed for the determination of total C, N, P on a CNS elemental analyzer (Variomax CNS Analyser, Elementar GmbH, Hanau, Germany) and metal element (Fe, Mn, Cu, see the next section). Soil pH, NH_4^+, and NO_3^- were also measured in the laboratory. The soil samples stored at −20 °C were used for metagenomic analysis. The abundance of the *pmo*A gene was quantified by qPCR using primer pairs A189F/Mb601R (Cai et al., 2016). The PCR was performed in a 20-μl mixture containing 10.0 μl SYBR Premix Ex Taq (Takara), 0.5 μM each primer, 2 μl of DNA template, and ddH_2O 7 μl. All qPCR assays were performed on an ABI7500 Real-Time Detection System (Applied Biosystems, Inc., CA, USA). The relative abundance of methanotrophs was measured by Illumina Miseq, using primers A189F (5′-GGNGACTGGGACTTCTGG-3′) and A601R (5′-CCGGMGCAACGTCYTTACC-3′). 644 genus microbial groups were detected and including three that participated in the CH_4 oxidation

process (i.e., Methylosarcina, Methylocaldum, and Methylocystis).

2.5. Incubation experiments

To validate the metal impacts on CH_4 oxidation, we designed an incubation experiment to quantify the impacts of Fe, Mn, and Cu on the CH_4 oxidation rate with and without N addition. Meanwhile, we designed another incubation experiment to quantify the multi-N level impacts on CH_4 oxidation rate, in which the Fe^{2+} and Mn^{2+} were measured, while Fe^{3+} was calculated as total Fe – Fe^{2+}, and Mn^{4+} was calculated as total Mn – Mn^{2+} (Lindsay and Norvell, 1978; Ferri et al., 2012; Wang and Hu, 2016) (the details are described in the section of 2.6). The soils for those incubation experiments were sampled from the adjacent sites that are 10 m away from the field experiment site. Four replicates of fresh soil, approximately 100 g each (moisture:10.1%), were sealed in 450 mL bottles. A total of 64 soils were filled with a plastic lid fitted with a rubber stopper and were incubated at 30 °C (the optimum temperature for CH_4 oxidation) (Visvanathan et al., 1999). For the trace elements, 0.01 g N (applied as NH_4NO_3), 0.286 g Fe_2O_3 (0.2g Fe^{3+}, 2 g/kg), 0.331g MnO_2 (0.2g Mn^{4+}, 2 g/kg) and 0.2g Cu (2 g/kg) (applied as copper powder), were added to each treatment, the amounts of added Fe, Mn, and Cu are based on the elemental variation concentration at the field site.

To ensure a sufficient mixture of the soil samples and metal, we put these reagents into 20 mL (water content kept at ~30% field capacity) deionized water (0.01 mol L^{-1} NH_4NO_3). All samples were incubated airtightly at 30 °C incubators. During incubation, we collected the gases in a wild-mouth bottle and measured CH_4 concentration by meteorological chromatography (Ambient CH_4 was used in these CH_4 oxidation experiments) within 48 h.

The other 18 soil samples were incubated with 0, 0.01, 0.02, 0.05, 0.1, and 0.2 g N (0, 0.00179, 0.00357, 0.00893, 0.0179 and 0.0357 mol L^{-1} NH_4NO_3 solution with 20 mL, equals 0, 4.5, 9, 22.5, 45, 90 g N m^{-2} in field experiment state) with three replicates. Gas was extracted, and the CH_4 concentration was measured by gas chromatography at the incubation time of 0, 3, 6, 12, 24, and 48 h. Then the CH_4 oxidation rate was calculated with the fitted equation of $y = ax + b$, y is the CH_4 concentration, and x is the incubation time, the parameter a represents the CH_4 oxidation rate (Fig. S8).

2.6. Metal measurements

The total Fe, Mn, and Cu were measured with the ICPS-500 (Shimadzu, Japan). Specifically, 0.5g air-dried soil samples were digested by HNO_3–HF–H_2O_2 (10:8:5). After digestion and complete deacidification, 20 mL HNO_3 (1:4) and 10 mL HCl (1:1) were added to the solution and heated for 2 h. After filtering the sediments, the solution was diluted with deionized water to 50 mL. Finally, the ICP-500 was used to measure the metallic concentrations of the solution.

The Fe^{2+} and Mn^{2+} were measured by spectrophotometer (UV2550) (Beal et., 2009; Amos et al., 2012). Specifically, 10.0000g fresh soil samples were put into a 250 mL conical flask, and 200 mL 0.1 mol L^{-1} Al_2SO_4 was added. After stirring thoroughly, the solution was diluted to 50 mL and added with 1 mL hydroxylamine hydrochloride. After being kept for a few minutes, 5 mL phenanthroline solution was drawn and used to measure the concentration of Fe^{2+} by using the spectrophotometer at the wavelength of 520 nm. Similarly, Mn^{2+} in soil was extracted by 0.1 mol L^{-1} Al_2SO_4, Mn^{2+} was oxidized to MnO^{4-} by KIO_4 in a neutral solution (pH = 7.0) and was measured by using the spectrophotometer at the wavelength of 525 nm.

The NH_4^+, NO_3^-, TN, and TP were measured by a Flow analyzer (Seal AA3, Germany) (Yang et., 2020). Specifically, 10 g sieved fresh soil samples were put into the centrifuge tubes, and 40 mL 2.0 mol L^{-1} KCl solution was added. After oscillation and centrifugation, the supernatant was extracted to measure the NH_4^+-nitrogen concentration. TN and TP were determined colorimetrically using a heating digestion method with $HClO_4$–H_2SO_4. 0.5 g air-dried soil samples and 1g catalyzer mixed with K_2SO_4 and $CuSO_4$ (10:1) were put into digestion tubes and mixed with 5 mL H_2SO_4 (Yang et., 2020). After digestion, the solution was extracted to measure the concentration of N and P. The TC was measured using a Multi N/C 2100 TOC analyzer (Analytikjena, Germany) with a high temperature (1100 °C) combustion method. 0.1 g air-dried soil was placed into the combustion chamber and was kept for 1 h. We calculated soil TC as the weight loss as CO_2. After incubation, all the experimental and undisturbed soils were used for qPCR and sequencing analysis, following the same method described above.

2.7. Data analysis

Structural Equation Modelling (SEM) was performed to understand the N impacts on the CH_4 oxidation and soil-microbe-metal interactions. Based on our knowledge of N impacts on CH_4 oxidation, we developed a priori model, allowing a hypothesized causal interpretation of the linkages between soil acidification, microbe, metal elements, and CH_4 oxidation. To test the hypothesis, we constructed conceptual models and determined the best fit with the SEM analysis. The hypothetical pathways of N impacts on CH_4 oxidation were based on previous studies: (a) CH_4 oxidation is primarily controlled by soil microbial and biochemical conditions (Hütsch, 2001), which is affected by N addition (Reischer et al., 2013); (b) CH_4 oxidation is a biological process adjusted by N input-induced soil acidification (Tian et al., 2016). (c) Soil acidification induced by N addition triggers metal element desorption from soil particles, leading to the enrichment of metal elements that can alter CH_4 oxidation (Hakemian and Rosenzweig, 2007; Oni and Friedrich, 2019). We used the abundances of the *pmo*A gene and CH_4-oxidizing bacteria as indicators for soil microbial activity (PC). The functional gene abundance represents the CH_4 oxidation potential and its association with the CH_4 oxidation rate at the ecosystem level in this study. Before running SEM, the N addition levels, pH, and CH_4 oxidation were log-transformed to ensure normality. Analysis of variance (ANOVA) followed by Duncan's posthoc test was performed to test the N impacts on soil acidification, microbial activities, and metal elements. Differences were deemed to be significant if $p < 0.05$. Results from the best-fitting regression models were presented. SEM analyses were performed using the "lavaan" package in the R program (3.2 version).

3. Results

3.1. Nonlinear response of CH_4 uptake to long-term N addition

The field measurements showed that the semi-arid grassland functioned as a CH_4 sink during the growing seasons (from May to September) of 2013 (10th year) - 2016 (13th year) (Fig. S1 a-d). The annual CH_4 uptake ranged 1.40–4.47 kg ha^{-1} y^{-1}, with a strong interannual variability across the N gradient (Fig. S2). A humpback model that best characterized the relationship between CH_4 response ratio (defined as log (CH_4_treatment/CH_4_control * 100%)) and N addition rate in each of four measured years (Fig. 1a; Fig. S1 a-d), but the response ratio started to climb at the end of the treatment under the treatments of 16 g m^{-2} y^{-1} to 64 g m^{-2} y^{-1} (Fig. 1a), showing the alleviated-suppression from 10th to 13th year (Fig. 1b). The inhibition impact on CH_4 uptake (expressed as log(CH_4)) decreased from 0.37 in the 10th year to 0.22 in the 11th year, 0.03 in the 12th year, and 0.025 in 13th year of N addition, suggesting the N-suppression on CH_4 uptake was relieved from the 10th year to 13th year of N addition (Fig. 1b). At the level of 16 g N m^{-2} y^{-1}, CH_4 uptake rates were 11.9% (2013), 18.2% (2014), 20.0% (2015) and 21.2% (2016) lower than those in control plots (Fig. S2 a-d). At the level of 32 g N m^{-2} y^{-1}, CH_4 uptake rates were 37.3% (2013), 33.4% (2014), 20.8% (2015), and 21.7% (2016) lower than those in the control treatment (Fig. S2 a-d). Under the treatment of 64 g N m^{-2} y^{-1}, CH_4 uptake rates were 47.0% (2013), 39.4% (2014), 23.2% (2015), and 23.9% (2016) lower than the control treatment

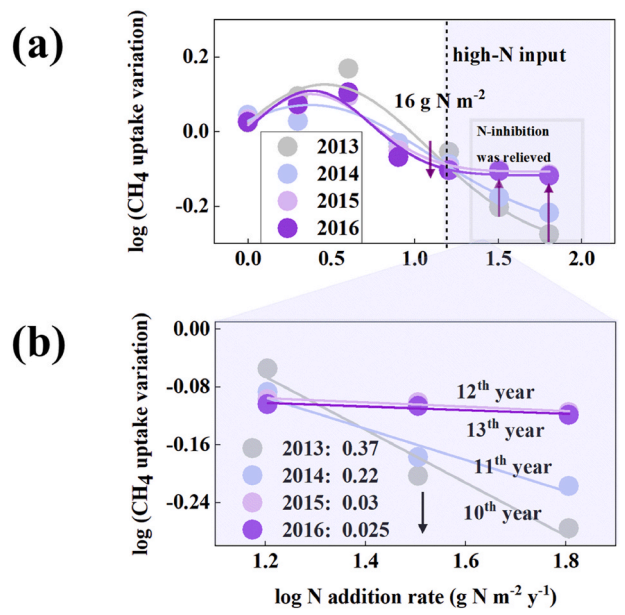

Fig. 1. N induced variation of CH_4 oxidation in the grassland sites. Relationships between the N addition rate and the CH_4 oxidation response ratio (the natural logarithm of the ratio between the treatment and control groups) over the four experimental years (2013–2016). The vertical line and light pink area indicate the second N threshold (from 16 g N m^{-2} y^{-1}) that alleviates the high N-suppression over time (**a**), and that was zoomed as (**b**); The Gaussian equation estimated the relationship: $y = y0+a*\exp(-0.5*((x-x0)/b)^2)$, where y is CH_4 oxidation response ratio, x is the natural logarithm of N application rate, y0, a, b, and x0 are the fitted coefficients (**a**). The alleviation rate was shown as (**b**).

(Fig. S2 a-d).

We further explored the indirect impacts of N addition on CH_4 uptake through its effects on soil properties and conditions. Across all N addition treatments, increases in soil NH_4^+ (Fig. 2a) and NO_3^- (Fig. 2b) and decreases in soil microbial biomass carbon (MBC, Fig. 2c) and soil pH (Fig. 2e) were observed. In contrast, soil microbial nitrogen (MBN) exhibited a humpback-shaped response curve with a maximum at 8 g N m^{-2} y^{-1} N-addition (Fig. 2d). It was noted that soil pH decreased exponentially from 6.9 to 5.3, following: $y = 4.95e^{5/(14.8+x)}$, where x is the N addition rate, y is the pH value (Fig. S3 a). A significant difference was found between soil iron (Fe) content (Fig. S4 a) and manganese ion (Mn) (Fig. S4 b) in the field experiment, while small differences were found among the N treatments in soil copper (Cu) (Fig. S4 c). Strong stimulating impacts were found for Fe, Mn, Cu on CH_4 uptake in the field experiment (Fig. 3a–c).

3.2. Impacts of elements (Fe, Mn, and Cu) on CH_4 oxidation

Results from the incubation experiment were consistent with those from the field experiments regarding metal impacts on CH_4 oxidation. For instance, we found that Fe, Mn, and Cu stimulated the CH_4 oxidation (Fig. 4a), promoted the abundances of *pmo*A (a genetic indicator for microbial oxidation strength (Boiesen et al., 1993; Ettwig et al., 2010)) (Fig. 4b) and CH_4-oxidizing bacteria (Fig. 4c). Specifically, increasing Fe, Mn, and Cu individually enhanced the CH_4 oxidation rate by 85.2%, 85.6%, and 57.5%, respectively. At the same time, N addition (0.00893 mol L^{-1} with 20 mL solution, equivalent to 22.5 g N m^{-2} in the field experiment) suppressed the CH_4 oxidation rate by 79.3%. Concurrent applications of Fe and N, Mn, and N, or Cu and N enhanced the CH_4 oxidation rate by 131.2%, 131.3%, and 142.2%, respectively (Fig. 4a). Adding Fe, Mn, and Cu alone increased the *pmo*A abundance by 44%, 43.4%, and 33.1%, respectively (Fig. 4b). In comparison, N-only addition decreased the CH_4 oxidation rate by 2.5% (Fig. 4a). In agreement with the field observations of CH_4 uptake, concurrent additions of Fe and N, Mn and N, Cu and N enhanced the CH_4 oxidation rate by 106.7%, 117.4%, and 136.5%, respectively (Fig. 4a). Adding Fe, Mn, and Cu alone increased the CH_4-oxidizing bacteria abundance by 19.9%, 32.2%, and 54.2%, respectively, while adding N decreased the CH_4-oxidizing bacteria abundance by 6.7% (Fig. 4c). At the same time, concurrent additions of Fe and N, Mn and N, or Cu and N enhanced the CH_4-oxidizing bacteria abundance by 153.7%, 165.7%, and 212.9% (Fig. 4c),

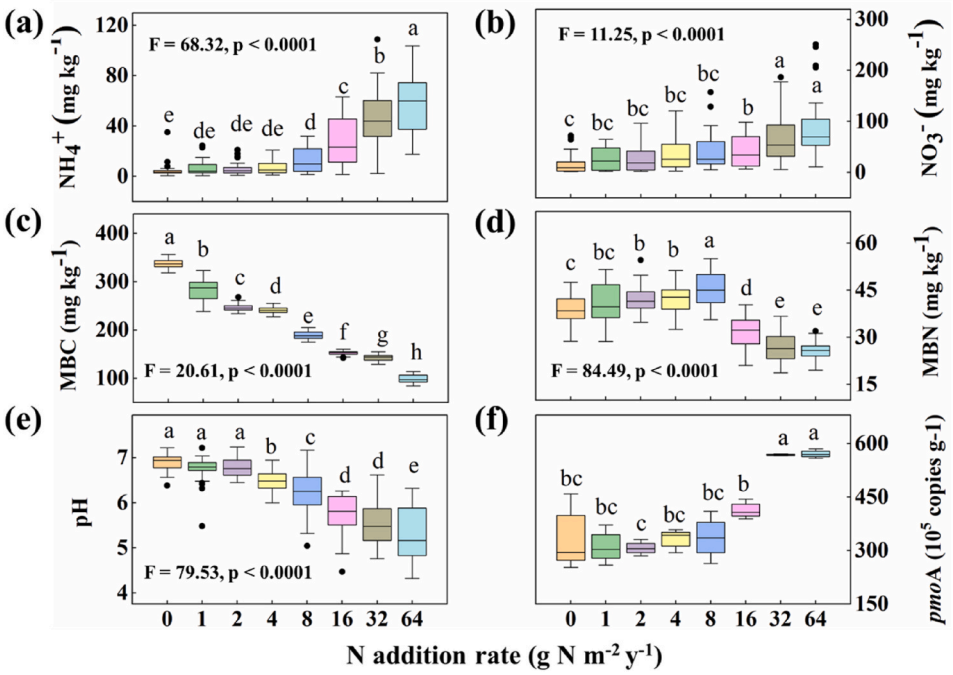

Fig. 2. N induced changes in other parameters in the grassland sites. N addition impacts on soil NH_4^+-N (**a**), NO_3^--N (**b**), MBC (**c**), MBN (**d**), pH (**e**), *pmo*A copies (**f**). All data are presented as the mean ± SE (n = 4, 1 measured for every season). Box plots show the median, first quartile, and third quartile; Different letters indicate the significance between N addition treatments at P = 0.05 level.

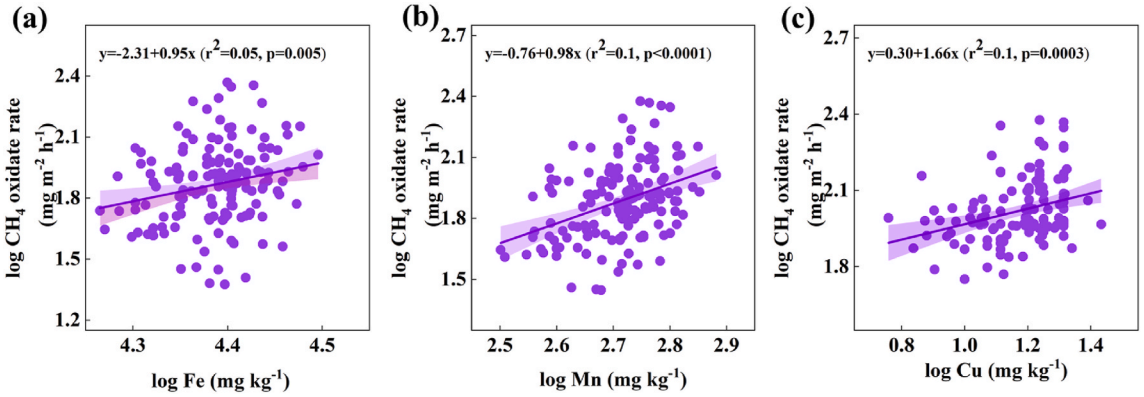

Fig. 3. The relationship between Fe (**a**), Mn (**b**), Cu (**c**), and CH_4 uptake rate in the field experiment.

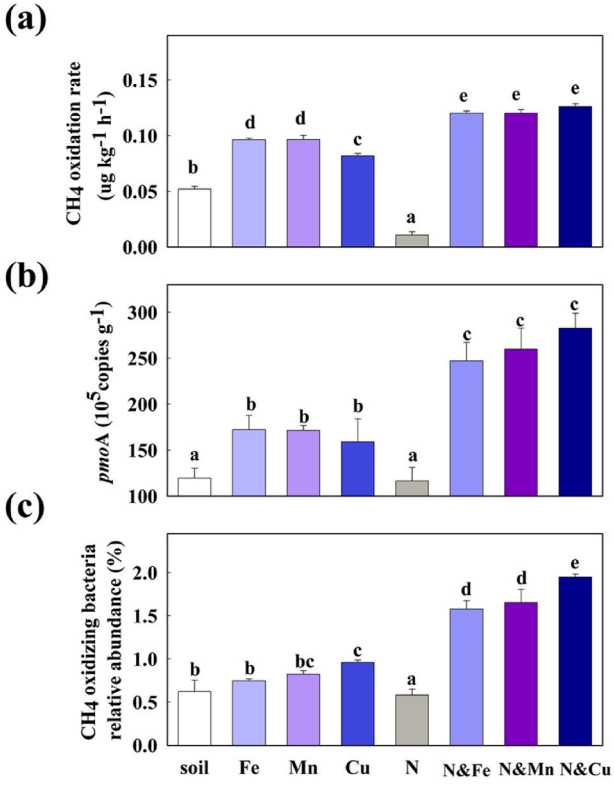

Fig. 4. The variation of CH_4 oxidation rate (**a**), *pmo*A copies (**b**) and CH_4 oxidizing bacteria relative abundance (**c**) under Fe, Mn, Cu, and N additions and their interactions in incubation. All data are presented as the mean ± SE (n = 4).

respectively.

3.3. A mechanistic understanding of CH_4 oxidation

A structural equation modeling (SEM) approach was applied to investigate the roles of Fe, Mn, and Cu in the microbial mechanisms of N impacts CH_4 oxidation. The abundances of *pmo*A gene (Fig. 4b) and CH_4-oxidizer (Fig. 4c) were included in our models, given their established effects on CH_4 oxidation and their association with N addition (Fig. 4b and c). Consistent with the observational results (Fig. 2e; Fig. S3 a), the SEMs confirmed the N-induced soil acidification. However, there was a slight difference in how soil acidification (pH decrease) affected CH_4 oxidation among metal ions (Fe, Mn, and Cu) (Fig. 5a–c).

For Fe, a direct negative effect of N (β = −0.61) and a positive effect of microbe (β = 0.49) on CH_4 oxidation were observed (Fig. 5a). Soil acidification (pH drop) indirectly affected the CH_4 oxidation by strongly suppressing soil microbial activity (β = −0.42) and increasing Fe^{3+} (β = 0.1). Microorganisms promoted CH_4 oxidation by taking Fe^{3+} and converting them into Fe^{2+}, inferred from the negative correlation between Fe^{3+} and microorganisms. The positive correlation between microorganisms and Fe^{2+} (Fig. 5a) can be inferred from the negative correlation between Fe^{3+} and CH_4 oxidation rate (Fig. S5c) and the positive correlation between Fe^{2+} and CH_4 oxidation rate (Fig. S5a).

For Mn, a direct positive effect of soil microbial activity, i.e., *pmo*A abundance and CH_4-oxidizer abundance (β = 0.42), and a direct negative impact of N (β = −0.61) on CH_4 oxidation were observed (Fig. 5b). Meanwhile, N also indirectly suppressed CH_4 oxidation by strongly suppressing soil microbial activity (β = −0.84) and indirectly affected soil Mn^{4+} by acidifying soil (β = −0.95, but the path is not significant), which positively affected Mn^{4+} (β = 0.55) (Fig. 5b). Soil acidification (pH drop) indirectly affected CH_4 oxidation by strongly enhancing soil microbial activity (β = 0.83). Microorganisms promoted CH_4 oxidation by converting Mn^{4+} to Mn^{2+}, which can be inferred from the negative correlation between Mn^{4+} and microorganisms (β = −0.86), and the positive correlation between microorganisms and Mn^{2+} (β = 0.12) (Fig. 5b), expressed as the negative correlation between Mn^{4+} and CH_4 oxidation rate (Fig. S5d), and the positive correlation between microorganisms, Mn^{2+} and CH_4 oxidation rate (Fig. S5b).

For Cu, a direct negative effect of N (β = −0.6) and a positive effect of microbe (β = 0.42) on CH_4 oxidation were observed (Fig. 5c). Soil acidification (pH drop) indirectly affected the CH_4 oxidation by increasing soil Cu content (β = 0.56), which further increased CH_4 oxidizers (β = 0.25) and thus CH_4 oxidation (β = 0.42) (Fig. 5c). Taken all together, metal elements mediated the N impact on CH_4 oxidation through their effects on soil microbes, but their mechanisms are different (Fig. 5a–c).

4. Discussion

4.1. Impacts of continuous high-N inputs on CH_4 uptake

This study quantitatively characterized the relationship between CH_4 uptake and N levels (Fig. 1a; Fig. S1 a-d; Fig. S2 a-d) and found a stimulating impact on CH_4 uptake under low N input while suppression impacts on CH_4 uptake under high N inputs (Fig. 1b). The finding is consistent with previous studies, and it might be associated with N limitation on CH_4 oxidation and competition theory (Zhang et al., 2020). A similar response of N impacts on CH_4 uptake was recently reported for an alpine steppe on the Tibetan Plateau (Fig. S6) (Peng et al., 2019). These data prove that N availability strongly affects CH_4 uptake, with the response magnitude depending on N addition rates. A meta-analysis reported that low N addition (<10 g N m^{-2} y^{-1}) stimulated CH_4 uptake

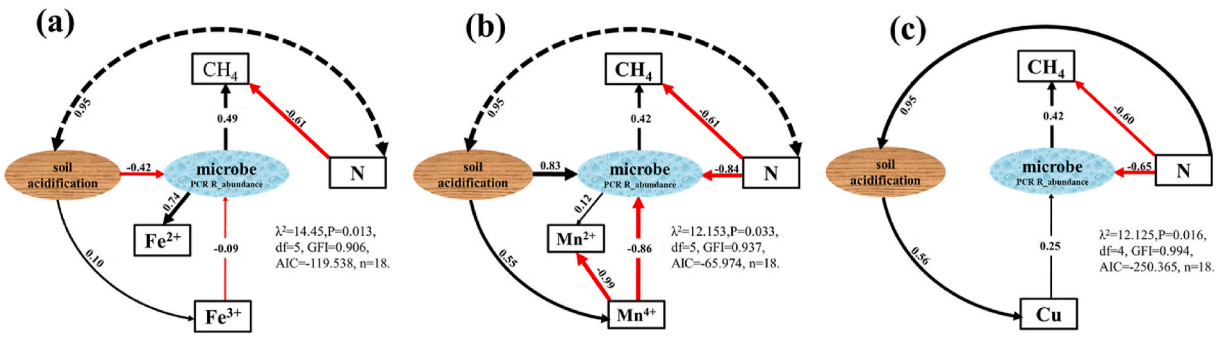

Fig. 5. Structural equation modeling **diagrams illustrating the mechanism of soil acidification triggers trace element stimulation on CH_4 oxidation**. SEM of the N addition impacts on CH_4 oxidation via the pathways of N addition, soil microbial activity (microbe), soil acidification (pH), and trace elements (Fe(**a**), Mn(**b**), Cu(**c**)), Microbe is the first principal component by soil microbial CH_4-oxidizing activity (*pmo*A abundance and CH_4-oxidizing bacteria abundance); **a**, Fe: $\chi^2 = 14.45$, $p = 0.013$, $df = 5$, Goodness of Fit Index (GFI) = 0.91, Akaike Information Criterion (AIC) = −119.54, and n = 18; **b**, Mn: $\chi^2 = 12.15$, $p = 0.033$, $df = 5$, Goodness of Fit Index (GFI) = 0.94, Akaike Information Criterion (AIC) = −65.97, and n = 18; **e**, Cu: $\chi^2 = 12.13$, $p = 0.016$, $df = 5$, Goodness of Fit Index (GFI) = 0.99, Akaike Information Criterion (AIC) = −250.37, and n = 18; Red and black solid arrows represent significant negative and positive effects ($p < 0.05$), respectively, dotted lines represent the insignificant paths. Values associated with the arrows represent standardized path coefficients).

(Aronson and Helliker, 2010), while high N addition (>10 g N m^{-2} y^{-1}) inhibited CH_4 uptake. In another study (Geng et al., 2017), CH_4 uptake peaked at a lower N addition rate (1 g N m^{-2} $year^{-1}$), which was explained by N limitation. Under N-limited conditions, N application was found to either relieve the N limitation of methanotrophic community or stimulate the biosynthesis of the enzymes involved in CH_4 oxidation (King, 1992). The reported declining trend in CH_4 uptake may have occurred due to the competition between NH_4^+ and CH_4 (Dunfield and Knowles, 1995; Schimel and Gulledge, 1998) or N's toxic impact on methanotrophs (Dunfield and Knowles, 1995). Soil NH_4^+ concentration is directly associated with soil CH_4 oxidation rate. The NH_4^+ molecule inhibits CH_4 oxidation by competing for the binding site of the CH_4 mono-oxygenase enzyme (Bodelier and Laanbroek, 2004). In the present study, a negative relationship between inorganic N and CH_4 uptake (Fig. 6f) was found. The suppressed CH_4 uptake may be attributed to the inhibitory effects of hydroxylamine and nitrite inhibitory, two intermediate products of nitrification (Bodelier and Laanbroek, 2004). The nonlinear response pattern of soil bacterial diversity (including Chao1, observed_species, pD_whole_tree, and Shannon diversity index) might be relevant to the reported unimodal response to N addition by methanotrophic bacteria (Fig. S7). Further investigations to examine the mechanisms of microbial regulation of CH_4 oxidation are deemed critical for

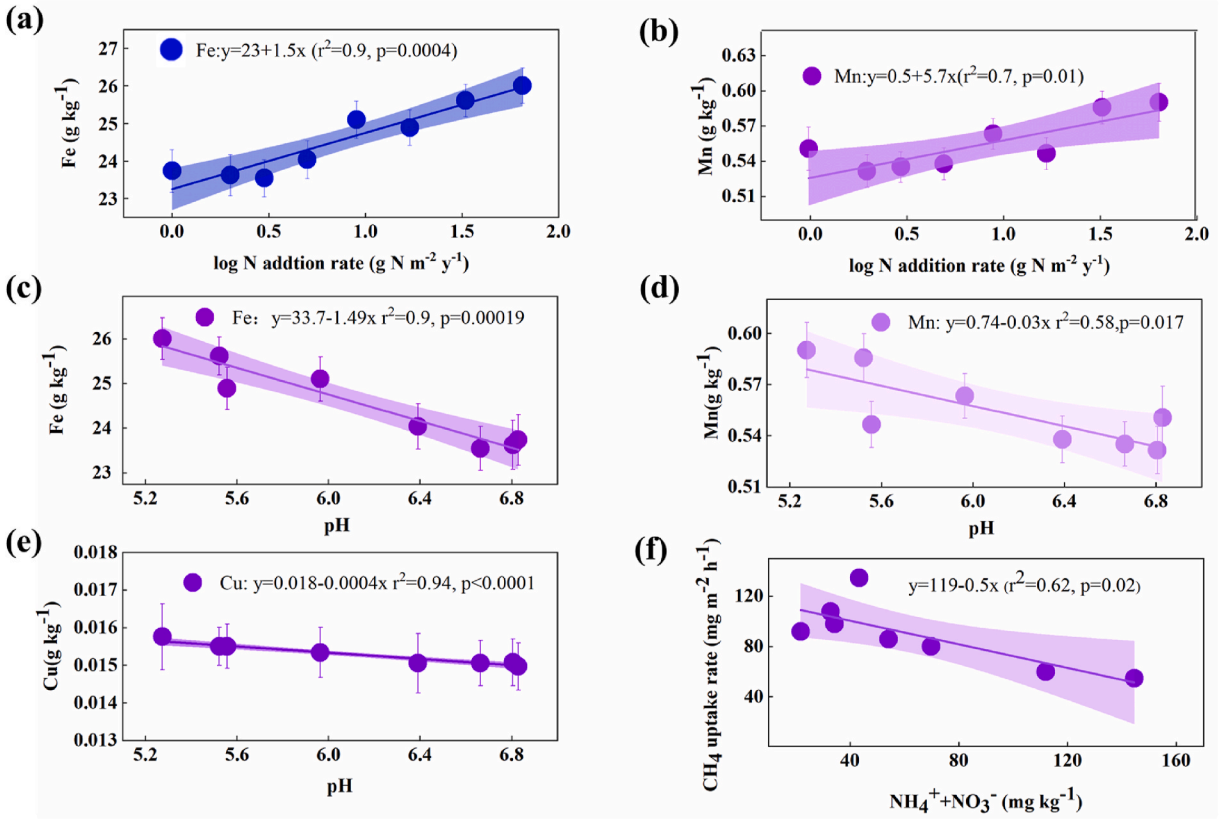

Fig. 6. The increase in soil Fe (**a**) and Mn(**b**) content with nitrogen application rate in a field experiment. The relationship between Fe (**c**), Mn (**d**), Cu (**e**), and pH across control and all nitrogen addition treatments. The relationship between NH_4^++NO_3^- and CH_4 uptake rate to N addition across control and all nitrogen addition treatments (**f**).

understanding the N impacts on CH_4 cycling.

4.2. Soil acidification triggered the trace element's stimulation on CH_4 oxidation

Based on our experimental results and theoretical framework, we speculated three mechanisms to explain the N-induced acidification triggered the stimulation of CH_4 oxidation. First, long-term N addition acidified soils, leading to soil pH < 5.7 which corresponded to the threshold of N impacts on CH_4 (Fig. 2e; Fig. S3 a). Under pH < 5.7, soil metal ions were desorbed from soils particles, leading to an accumulation of soil Fe, Mn (Fig. 6a and b; Fig. S5), which was in agreement with studies in the same site (Tian et al., 2016), and other regions (Ernani et al., 2002; Raza et al., 2020). Soil pH is a critical factor controlling CH_4 oxidation (Saarnio et al., 2004) and was positively correlated with the CH_4 uptake along N application (Fig. S3 b). In our study, the positive relationship between Fe^{2+} and Mn^{2+} with CH_4 oxidation rate in the incubation experiment (Figs. S5a and b), the negative relationship between Fe and Mn element with pH (soil acidification stimulated elemental release from soil minerals) in the field experiment were reported (Fig. 6c and d).

Second, increased N addition promoted the abundance of CH_4 oxidation gene *pmo*A (Fig. 2f). N also shifted bacterial communities (Fig. S7) toward more putatively copiotrophic bacterial taxa (Liu et al., 2020). This community shift may have contributed to the increased enzymes that can oxidize CH_4 with Fe (III) and Mn (IV) minerals as electron acceptors (Oni and Friedrich, 2019). Therefore, we conjectured that an unknown methanotrophic bacteria might have participated in the CH_4 oxidization with Fe (III) and Mn (IV) minerals as electron acceptors (Oni and Friedrich, 2019). The inconsistent effects of N application on CH_4 uptake and *pmo*A in the experiment (Fig. 1a; Fig. 2f) are noteworthy and can be explained by the competition between NH_4^+ and CH_4 for *pmo*A (Schimel and Gulledge, 1998). Due to the microbial N transformations with N additions, both NH_4^+ and NO_3^- contents increased (Fig. 2a and b). As external NH_4^+ entered the soils (Fig. 2a), methanotrophic bacteria converted ammonia to nitrite, consuming oxygen (O_2) and leading to an anaerobic microenvironment (Fig. 7), thus leading to CH_4 oxidization with Fe (III) and Mn (IV) minerals as electron acceptors (Fig. 7). And this can be confirmed by our incubation experiments, in which the positive relationship between Fe^{2+}, Mn^{2+}, and CH_4 oxidation rate, and the negative relationship between Fe^{3+}, Mn^{4+}, and CH_4 oxidation rate were observed (Fig. S5).

A third potential mechanism is the positive impact of Cu on CH_4 monooxygenase (MMO) (Hakemian and Rosenzweig, 2007; Stein, 2020). This is built upon the positive correlation between concentrations of the *pmo*A transcript and the Cu levels (Choi et al., 2003), as confirmed in this study by the positive correlation between Cu content and CH_4 uptake rate (Fig. 3c) and negative correlation between pH and Cu content (Fig. 6e). Soil acidification enhanced the activity of copper-containing enzymes or compounds (such as methanobactin) produced by methanotrophs (Chang et al., 2018). The production and release of reactive intermediates (related enzymes or compounds) by microorganisms can be strongly enhanced by copper availability, further altering the rate of CH_4 oxidation by activating copper-containing enzymes (Beal et al., 2009; Ettwig et al., 2016).

4.3. A conceptual model for trace-elements mediation of CH_4 oxidation

We proposed a conceptual framework to summarize the previous mechanisms to explain the relieved suppression of CH_4 oxidation under cumulative-high N addition (Fig. 7). After long-term N addition, H^+ accumulated and pH declined (Schroder et al., 2011; Tian et al., 2016; Xiao et al., 2020) (Fig. 2e). With declining soil pH, exchangeable soil cations such as Fe, Mn, and Cu desorbed from the cation exchange sites (Schroder et al., 2011; Rezapour, 2014; Zhang et al., 2017; Yang et al., 2018) (Fig. 7; Fig. 5a–c; Fig. S4). Soil acidification has been found to enhance the availability of metal elements ($\beta = 0.1$ for Fe^{3+}, $\beta = 0.55$ for Mn^{4+}, $\beta = 0.56$ for Cu) (Fig. 5a–c; Fig. 6 c-e). Moreover, the *pmo*A gene (Fig. 2f; Fig. S3 c), the concentrations of soil NH_4^+ (Fig. 2a) and NO_3^- (Fig. 2b) were observed significantly increase with the N application rate while CH_4 uptake (Fig. 1a) first increased then decreased with the N application rate. And Fe, Mn, and Cu have stimulated impaction on the CH_4 oxidation (Fig. 4a), the abundances of *pmo*A gene (Fig. 4b) and CH_4-oxidizers (Fig. 4c). Based on the above results and previous studies (Ettwig et al., 2010; Xiao et al., 2020), the hypothesis that higher available metal elements stimulate CH_4 oxidation was supported as indicated by the *pmo*A expression (Fig. 7). The rapid O_2 consumption by other microbes may have created anoxic microenvironments and provided abundant substrates for acetoclastic methanogenesis (Angle et al., 2017). Therefore, metal elements, such as Fe, Mn are electron acceptors substituting oxygen to complete the CH_4 oxidation process (Stein, 2020).

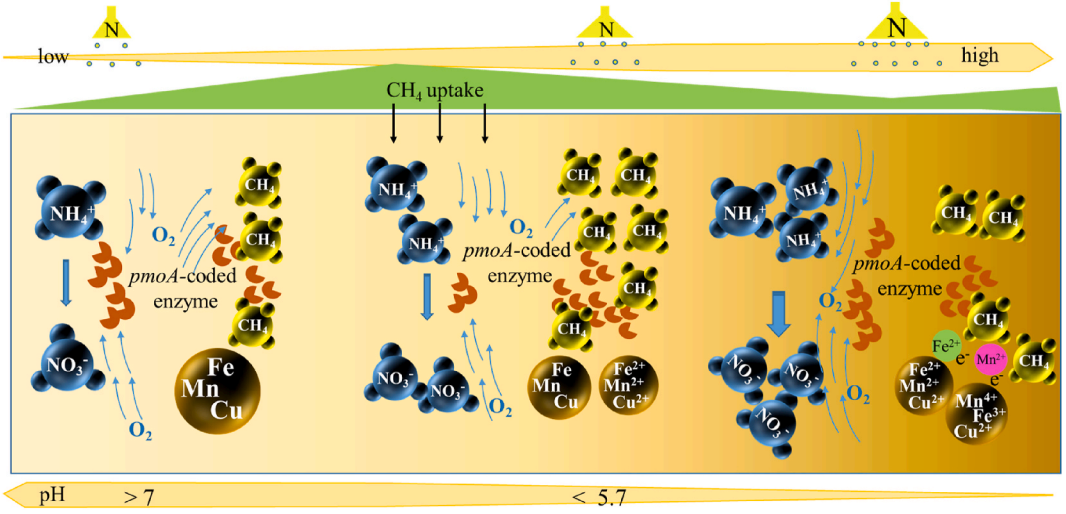

Fig. 7. Conceptual diagram illustrating the mechanisms of soil acidification triggered trace element mediating on CH_4 oxidation along high N accumulation. Long-term N addition induced soil acidification, stimulating the release of soil ions from soil particles. The oxidation of elevated NH_4^+ to NO_3^- consumed much oxygen. Then, activated trace elements due to soil acidification could be used as an alternative electron acceptor for the CH_4 oxidation during long-term N enrichment.

4.4. Limitations and future work

Although the substantial impact of N addition on CH_4 oxidation has been well established (Stein, 2020), this study reported a novel finding on the N impact on CH_4 oxidation through changing metal element availabilities. Two major limitations have been identified and must be addressed in future work. First, no experimental studies have been conducted to characterize soil metal elements along an N addition gradient over the long term and focused on how eventual concentration changes affect CH_4 oxidation across N limitation to saturation scenarios (Peng et al., 2019). The question remains whether there is a general cognition on the relationship between CH_4 oxidation and soil metal element along the N addition gradient (Hakemian and Rosenzweig, 2007) in semiarid grassland ecosystems. The significant impacts of Fe, Mn, and Cu on CH_4 oxidation, *pmo*A, and CH_4-oxidizing bacteria abundance (Fig. 4a–c) and the correlation between Fe, Mn, and CH_4 oxidation rate (Fig. S5) in our incubation experiment confirmed our conceptual framework and offering reliable foundation.

Second, we used the functional gene abundance to represent the CH_4 oxidation potential and its association with the CH_4 oxidation rate at the ecosystem level. We focused on the total CH_4-oxidizer community and used the abundances of *pmo*A and CH_4-oxidizer as indicators for soil microbial activity (PC); the specific genes related to the enzymes for Fe, Mn, and Cu association with CH_4 oxidation were not measured in the present study and will be investigated in the future.

5. Conclusion

This study established an ecosystem-scale mechanistic link between N-led soil acidification and CH_4 uptake transition under long-term N addition, implying the vital role of metal elements on CH_4 oxidation during soil acidification. The pH value of 5.7 during soil acidification represents a critical threshold of metal elements affecting CH_4 cycling in soils. We thus highlighted the importance of metal elements in mediating CH_4 oxidation under future elevated N deposition, which is critical for understanding the CH_4 cycle under future N deposition.

To the best of our knowledge, this study represented the first attempt to report the metal element-mediated CH_4 oxidation in the aerobic environment of a semi-arid grassland. Consequently, the novel findings of continuous high N-input in alleviating N suppression of CH_4 oxidation contribute to better predictability of the CH_4 cycle under changing environments, particularly considering the projected climbing atmospheric N depositions. The metal impact on CH_4 oxidation has yet to be incorporated into process-based CH_4 models (Xu et al., 2016); this study provides mechanistic knowledge to fill this gap.

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.

Data availability

Data will be made available on request.

Acknowledgments

This study was partially supported by the National Natural Science Foundation of China (32271681, 41125001, 4137111, 32171873) and the Joint Funds of China's National Natural Science Foundation (U2006215), and by Key Laboratory of Ecology and Environment in Minority Areas (Minzu University of China), National Ethnic Affairs Commission (KLEEMA202206). X.X. acknowledged the financial assistance provided by the National Science Foundation (2145130) and SPRUCE and NGEE Arctic projects, supported by the Office of Biological and Environmental Research in the Department of Energy Office of Science. I.A.J. acknowledges support from the European Research Council Synergy Grant IMBALANCE-P (ERC-2013-SyG-610028).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109098.

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