

Effects of electron acceptors on CH₄ emission in alpine wetlands

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SUMMARY

Alpine wetlands are an important source of methane (CH₄) and play a key role in the global carbon cycle. Their CH₄ emissions largely depend on microbial CH₄ production and oxidation processes that involve external electron acceptors. Seasonal precipitation drives redox cycles of humic acids (HAs), iron oxide and sulfur species, which will in turn affect CH₄ production and oxidation. To investigate the effects of electron acceptors on CH₄ emissions, soil samples from a typical alpine wetland on the Tibetan Plateau were incubated with the addition of ferrihydrite (HFO), HAs, sodium sulfate (SO₄²⁻) or combinations (HAs-HFO, HAs-SO₄²⁻ and HAs-HFO-SO₄²⁻). During long-term anaerobic incubation, CH₄ concentrations showed similar trends, increasing rapidly from 0 to 60 days, decreasing from 60 to 240 days, and finally slowly increasing again after 240 days, in all treatments except the sterilised control. Thus, the incubation period was divided into the production, consumption and reproduction phases. The addition of HFO, HAs or HAs-containing electron acceptors promoted the rates of both production and consumption of CH₄, increasing the production potential of CH₄ by 65–100 % and the oxidation potential of CH₄ by 58–115 %. On the other hand, SO₄²⁻ inhibited the production and consumption of CH₄, reducing the production potential by 35 % and the oxidation potential by 50 %. Electron acceptors such as HFO, HAs and SO₄²⁻ play important roles in CH₄ emissions. HAs are the dominant factor affecting CH₄ emissions in alpine wetlands. From a broader ecological perspective, organic and inorganic electron acceptors play a key role in CH₄ production and oxidation under anaerobic conditions, influencing CH₄ emissions from alpine wetlands.

KEY WORDS: CH₄ oxidation, CH₄ production, methane, peat soil, swamp meadow, Tibetan Plateau

INTRODUCTION

Methane (CH₄) is the second most important greenhouse gas after carbon dioxide (CO₂) (Weller *et al.* 2015, Shakoor *et al.* 2020). CH₄ currently contributes approximately 22 % of global warming, and the atmospheric CH₄ concentration continues to increase at a rate of 1.0–1.2 % per year (Shakoor *et al.* 2020). Wetlands account for only 5–8 % of the earth's land area but contain more than 30 % of the world's soil carbon (Malone *et al.* 2013). Correspondingly, CH₄ emissions from wetlands account for 70 % of all CH₄ emissions from natural sources and 24.8 % of global CH₄ emissions (Zhang *et al.* 2002).

CH₄ production is driven by the anaerobic degradation of organic matter in environments that are depleted in oxygen (O₂) and the other electron acceptors, e.g., nitrite/nitrate (NO₂⁻/NO₃⁻), tetravalent manganese and ferric ions (Mn⁴⁺/Fe³⁺) and sulfate (SO₄²⁻) (Fan *et al.* 2020). On the other hand, CH₄ can

be oxidised, aerobically or anaerobically, by bacteria utilising electron acceptors such as O₂, NO₂⁻, NO₃⁻ (Ettwig *et al.* 2010, Haroon *et al.* 2013), Mn⁴⁺, Fe³⁺ (Valenzuela *et al.* 2019), SO₄²⁻ and HAs (humic acids; Keller *et al.* 2009). When CH₄ production exceeds oxidation, CH₄ is emitted into the atmosphere. Thus, the levels of electron acceptors are a key factor in determining the emission of CH₄ (Niu *et al.* 2022). Previous studies have shown that the addition of electron acceptors such as hydrous ferric oxide (HFO) and SO₄²⁻ inhibits CH₄ production (Peters & Conrad 1996, Chen *et al.* 2022) and promotes the anaerobic oxidation of CH₄ (Chen *et al.* 2018). HFO or HAs may also serve as electron shuttles in microbially mediated reactions that accelerate CH₄ production or oxidation (W.Q. Wang *et al.* 2018).

Naturally, microbial processes drive the consumption and formation of these electron acceptors. Thermodynamically, microbes prefer to use electron acceptors with high redox potential in

microbial respiration to harvest more energy. In terms of redox potential, O₂ is the most powerful electron acceptor in wetland soils, followed by NO₃⁻, Mn⁴⁺, Fe³⁺, SO₄²⁻ and CO₂. Kinetically, however, microbial respiration is also strongly regulated by the kinetics (rates) of electron transfer between electron donors and acceptors. Electron acceptors such as Mn⁴⁺ and Fe³⁺ are in the solid phase, which limits their microbial accessibility (Dong *et al.* 2022). Electron transfer through direct contact commonly occurs via three pathways, through the microbial membrane or via nanowires and electron shuttles. Reduction rate and extent of solid electron acceptors such as Mn⁴⁺ and Fe³⁺ are mainly constrained by electron shuttles, and even by the crystal structure of minerals bearing Mn⁴⁺ and Fe³⁺ such as nontronite, illite, chlorite and magnetite (Dong *et al.* 2003, Zhang *et al.* 2006, 2007, 2009, 2011). Interactions amongst microbial functional groups also affect the consumption of electron acceptors. NO₃⁻-reducing bacteria are capable of reducing NO₃⁻ and oxidising Fe²⁺ to harvest energy (Straub *et al.* 1996). Several studies have found that NO₃⁻-reducing bacteria can also reduce NO₂⁻ and oxidise Fe²⁺ (Violaine & Holmes 2012), and have also reported bacteria oxidising NH₄⁺ and reducing Fe³⁺ (Oshiki *et al.* 2016). The addition of Fe³⁺ inhibits SO₄²⁻ reduction, and the electron flow of SO₄²⁻ reduction decreases while the electron flow of Fe³⁺ reduction increases correspondingly (Lovley & Phillips 1987). Thus, the elemental cycling of N, C, S and iron in their different valency states is tightly networked together via different microbial functional groups (Karimian *et al.* 2018). Many species of bacteria can harvest energy via two or more metabolic pathways, which complicates the interactions among bacteria (Hemme *et al.* 2010).

Soil from the alpine wetlands on the Tibetan plateau contains 4.7–40 % organic matter and 3.5–9.5 % iron by dry weight (Sheng *et al.* 2012, Chen *et al.* 2013), and these are the most abundant potential electron acceptors apart from O₂ (Zhou *et al.* 2014, Valenzuela *et al.* 2019). HAs are irregular complex macromolecules containing multiple functional groups including hydroxybenzene, quinone and aromatic amino carboxylic acid, with a broad range of redox potential (-0.48–0.70 V). Thus, HAs can be utilised as electron donors by metal, SO₄²⁻ or NO₃⁻-reducing bacteria (Roden *et al.* 2010) and can also accept electrons from metals via the same types of bacteria (Bradley *et al.* 1998, Coates *et al.* 2002). HAs that can be reduced and re-oxidised in redox zonation and electron-accepting capacities make up 27–63 % of the total (Klüpfel *et al.* 2014). Fan *et al.* (2020) found that HAs were one of the most effective

electron donors in the CH₄ production phase, increasing CH₄ production by 5–6 times during 84 days of anaerobic incubation. Abiotic O₂, UV light and aerobic respiration by bacteria oxidise humic acids (Page *et al.* 2012, Sharpless *et al.* 2014), while anaerobic microbial respiration can reduce humic acids in anoxic environments. Redox oscillation of the environment leads to redox cycling of humic acids (Ratasuk & Nanny 2007, Michael *et al.* 2010) and thus plays a complex role in CH₄ emission (Van der Zee & Cervantes 2009). Therefore, it is worthwhile to evaluate the roles of humic acids, Fe³⁺ and SO₄²⁻ in CH₄ generation.

Wetland soils are enriched with organic matter and a variety of electron receptors (Valenzuela *et al.* 2017). Subtle changes in soil electron acceptors due to intermittent rainfall have been mostly neglected as drivers of the carbon cycle which affect microbe-mediated CH₄ emissions (Deppe *et al.* 2010, Yasin *et al.* 2022). Previous studies have reported that CH₄ emission is controlled by soil aeration, i.e., the aerobic:anaerobic ratio in wetland soil (Voigt *et al.* 2017). However, studies on the interaction of electron acceptors with CH₄ emissions from alpine wetlands are still scarce and they need to be investigated further. In this study, we investigated the roles of the major electron acceptors (HAs, HFO and SO₄²⁻) in CH₄ production and consumption in alpine wetlands.

METHODS

Study area and sampling

An alpine wetland was selected, located at 4500 m a.s.l. in the Nagqu region of the Tibetan Plateau (31° 17' 59.76" N, 91° 54' 1.87" E) (Figure 1). The study area belongs to a seasonally frozen soil area, with a soil depth of 1–2 m. The wetland is an alpine swamp meadow with a peat soil. At this location, the mean annual temperature is -2.9–3.4 °C and the mean annual precipitation is 406 mm (Luosang *et al.* 2014). The majority of precipitation on the wetland occurs during the summer monsoon season from June to September. The vegetation of this wetland is mainly alpine meadow with the dominant species *Kobresia pygmaea*, *Kobresia schoenoides*, *Koeleria litwinowii* and *Stipa purpurea*. In the study area, wetland hollows are submerged all year round while the hummocks are semi-submerged under the influence of seasonal precipitation. The average water-table depth in the sampling site from July to October 2020 was about 12 cm. After persistent heavy rain, the site becomes completely submerged. Three topsoil samples (0–8 cm) were collected at the end of

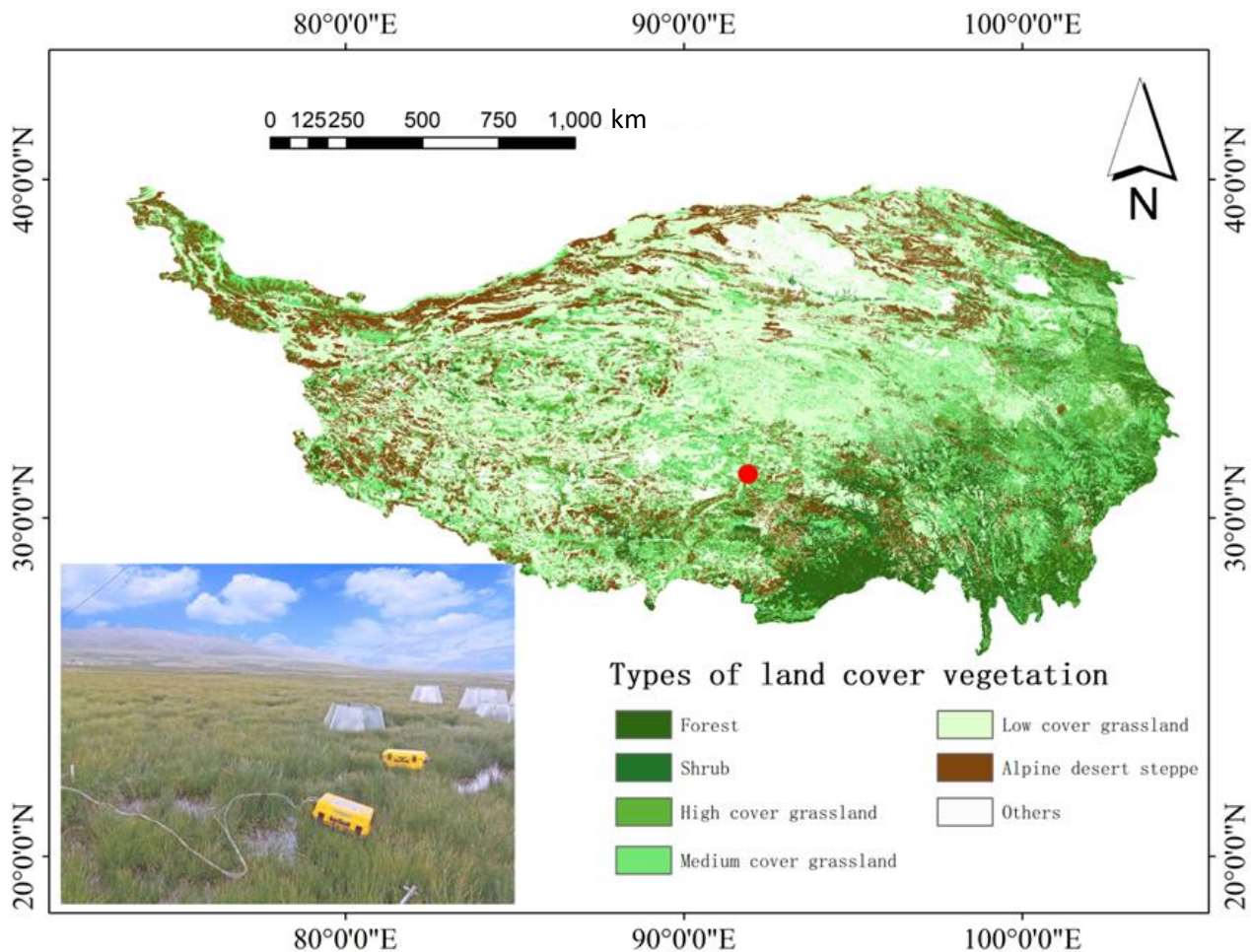


Figure 1. Geographical location map of the study area and general view across the wetland on the Qinghai-Tibet Plateau.

September 2020 from a hummock with a water-table depth of around 17 cm, using a corer fitted with a PVC pipe of internal diameter 5 cm. All samples were enclosed in PVC pipes to protect them from light and oxidation. The samples were packed in ice for transport to the laboratory, where they were homogenised and subsampled for subsequent incubation experiments in an anaerobic glove box.

Anaerobic incubations

The anaerobic incubation experiment was designed to test the effects of different electron acceptors (HAs, HFO, SO₄²⁻ or combinations) on CH₄ production in an anaerobic environment. To simulate alpine wetland conditions, the artificial pore water used in the incubations contained 57 μM potassium chloride, 100 μM magnesium chloride, 323 μM calcium chloride, 2 mM sodium bicarbonate, 500 μM ammonium chloride and 30 μM potassium phosphate (Cervantes *et al.* 2000, Segarra *et al.* 2013). HAs extracted from the soil in the study area (Keller *et al.* 2009) were added to achieve a concentration of

25 g L⁻¹. HFO was synthesised by neutralising a solution of 0.05 M FeCl₃·6H₂O with 1M NaOH (Blazewicz *et al.* 2012), and SO₄²⁻ was added as Na₂SO₄ solution (Sahrawat 2004).

Sub-samples of soil (about 1.6 g dry weight) were placed in 50 mL sterile serum bottles with 7 mL of artificial pore water and sealed with thick butyl rubber stoppers (Lovley & Phillips 1987), after which the headspace gas was replaced with N₂. The additions of electron acceptors in the main experimental treatments were as follows: blank control (CK; no additions), HFO (10 mM), HAs (5 g L⁻¹), SO₄²⁻ (1 mM), SO₄²⁻ (10 mM), HAs-HFO (2.5 g L⁻¹ HAs and 5 mM HFO), HAs-SO₄²⁻ (2.5 g L⁻¹ HAs and 0.5 mM SO₄²⁻), HAs-HFO-SO₄²⁻ (2.5 g L⁻¹ HAs, 5 mM HFO and 0.5 mM SO₄²⁻). A sterilised-soil-sample treatment was also applied. This was prepared in the same way as the blank control, except that the soil was first autoclaved at 121 °C for 20 min. The amounts of electron acceptors selected were based on field measurements in alpine wetlands. There are various

types of wetlands on the Qinghai-Tibet Plateau, including many lakeside wetlands. Most of the lakes on the Tibetan Plateau are saline-alkali lakes, which are high in SO₄²⁻ (ranging from < 1 mM to > 100 mM) (Liu *et al.* 2016). Tibetan lakes have been growing in area over the last 30 years, and this has affected the lakeside wetlands. Given the scenario of persistent global warming, it is necessary to evaluate the effect of high concentrations of SO₄²⁻. Accordingly, two concentrations of SO₄²⁻ (1 mM and 10 mM) were applied in this study. The average monthly temperature in Nagqu region in summer is 12–16 °C, and the highest temperature reached is 23.3 °C (Lin *et al.* 2016). Therefore, the *in situ* conditions were simulated at 15 °C. The serum bottles, including four replicates for each treatment, were incubated at 15 °C for 540 days. Methane concentrations were measured on the 30th, 38th, 60th, 140th, 240th and 540th days of the incubation.

Analytical methods

Total organic carbon (TOC) was determined by an elemental analyser (Vario Macro Cube, Elementar, Germany) after removing inorganic carbon with HCl according to previous literature (Hou *et al.* 2014). The average total organic carbon (TOC) content of the soil was 255.8 g kg⁻¹ and the soil pH was about 6.80. Fe²⁺ and Fe³⁺ ions in fresh soil samples were determined by UV-visible Spectrophotometer (UV-2550) (Hu *et al.* 2021). Pore water was obtained by centrifugation. SO₄²⁻, NO₃⁻ and NO₂⁻ in pore water were determined by ion chromatography (Dionex ICS-600) (Hou *et al.* 2013). Dissolved organic carbon (DOC) and dissolved nitrogen (DN) were measured with a C/N analyser (Multi N/C 2100, Jena, Germany). CH₄ concentrations were measured with a gas chromatograph (Agilent 7890A) by flame ionisation detector (Andersen *et al.* 1998, Zhu *et al.* 2021). During the measurement, 100 µL of gas from the bottle headspace was tested twice, and the difference between the two results was less than 5 %. The change rate of CH₄ concentration was expressed as the change in concentration between time points (30, 38, 60, 140, 240 and 540 days) per mole of soil organic carbon (in mmol mol⁻¹C_{org} d⁻¹) using the following equation:

$$v = \frac{dc}{dt} \cdot \frac{V_H}{TOC} \cdot \frac{M_M}{M_V} \quad [1]$$

where v (mmol mol⁻¹C_{org} d⁻¹) is the daily change rate of CH₄, dc/dt is the change of CH₄ concentration per unit time (mmol mol⁻¹C_{org} d⁻¹), V_H is the headspace volume of the incubation bottle (L), TOC represents the soil organic carbon content per unit mass

(mol mol⁻¹C_{org}), M_M is the molecular weight of CH₄ (g mol⁻¹); and M_V is the volume of 1 mol of gas in the standard state (L).

Calculations for meta-analysis

To further evaluate the influence of the addition of electron acceptors on CH₄ production and consumption, we calculated the weighted mean effect sizes of different treatments on CH₄ production or consumption. We used an effective model to estimate the size of the overall weighted average effect of electron acceptors on CH₄ production or oxidation (Hedges *et al.* 1999). The rates of CH₄ production and consumption over 540 days of incubation in the six treatments HFO, HAs, SO₄²⁻, HAs-HFO, HAs-SO₄²⁻ and HAs-HFO-SO₄²⁻ were compared with the blank control (CK). The effect size of the CH₄ response ratio (RR) metric was calculated as the logarithmic change of the means between treatment and CK.

$$RR = \ln \frac{\bar{X}_t}{\bar{X}_c} \quad [2]$$

where \bar{X}_t and \bar{X}_c are the average CH₄ production or oxidation rates of the experimental treatment and CK. To correct for potential bias introduced by small sample sizes, we used the bias correction metric RR^{Δ} (Lajeunesse 2015). For each pair of treatment-control comparisons, the effect size RR^{Δ} was calculated as:

$$RR^{\Delta} = RR + \frac{1}{2} \left(\frac{SD_t^2}{N_t \cdot \bar{X}_t^2} - \frac{SD_c^2}{N_c \cdot \bar{X}_c^2} \right) \quad [3]$$

where SD_t and SD_c are the standard deviations of the experimental treatment and CK, and N_t and N_c are the repetition times of the experimental treatment and CK. Sample size and standard deviation were used to quantify the sampling variation in RR in the effect size in each comparison. For each comparison, the variance (va) of RR^{Δ} was calculated as:

$$va = \left(\frac{SD_t^2}{N_t \cdot \bar{X}_t^2} + \frac{SD_c^2}{N_c \cdot \bar{X}_c^2} \right) + \frac{1}{2} \left(\frac{SD_t^4}{N_t^2 \cdot \bar{X}_t^4} + \frac{SD_c^4}{N_c^2 \cdot \bar{X}_c^4} \right) [4]$$

The CH₄ production or oxidation potential per mass of soil organic carbon (P ; mmol mol⁻¹C_{org}) during a specified phase of the incubation (Wassmann *et al.* 1998) was calculated as:

$$P = \Delta c \cdot \frac{V_H}{W_S} \cdot \frac{M_M}{M_V} \quad [5]$$

where Δc is the overall change in CH₄ concentration during the phase (per unit mass of soil organic carbon; mol mol⁻¹C_{org}); and W_S is the mass of soil organic carbon (mol) (= $m \cdot TOC$; see Equation 1).

Statistical analysis

A one-way ANOVA (analysis of variance) was applied to compare the rates of change of CH₄ concentrations amongst different sampling times and treatments with CK. Linear regression analysis was used to assess the correlations between electron acceptors and CH₄ production and oxidation. Both ANOVA and linear regression were conducted with SPSS Statistics 19 (IBM Inc., Chicago IL, USA), and the results were considered significant at $P < 0.05$. The graphical analysis was performed in R Studio software (version 3.6.3), and the R package “ggplot2” was used for visualisation.

RESULTS

Geochemical conditions of the samples and variations in electron acceptors during incubation

Before incubation, the organic carbon content in the soil samples was relatively high, accounting for more than 25 % of dry weight. In pore water, the

concentration of DOC was 33.9 mg L⁻¹ and DN was 3.35 mg L⁻¹. The measurable electron acceptors in pore water included 0.01 mM Fe³⁺, 0.05 mM SO₄²⁻, 0.03 mM NO₃⁻ and 0.002 mM NO₂⁻. In solid soil, the concentration of Fe³⁺ was 94.9 μmol g⁻¹ in dry weight. For the purpose of this study, the concentration of SO₄²⁻ was amended to 0.5 mM, 1 mM or 10 mM in different treatments. On the 240th day, almost all SO₄²⁻ in the microcosms had been reduced, but some Fe³⁺ remained in the solid phase (Figure 2).

Effects of electron acceptors on the concentrations of CH₄ production and consumption

Overall, during the long-term anaerobic incubation, CH₄ concentrations increased rapidly during the first 60 days then gradually decreased at 60–240 days and finally slowly increased again at 240–540 days, showing a similar pattern in the seven treatments and CK although not in the sterilised control (Figure 3). CH₄ concentration did not change in the sterilised incubation, which confirmed that the variations of CH₄ were related to microbial activities (Figure 3).

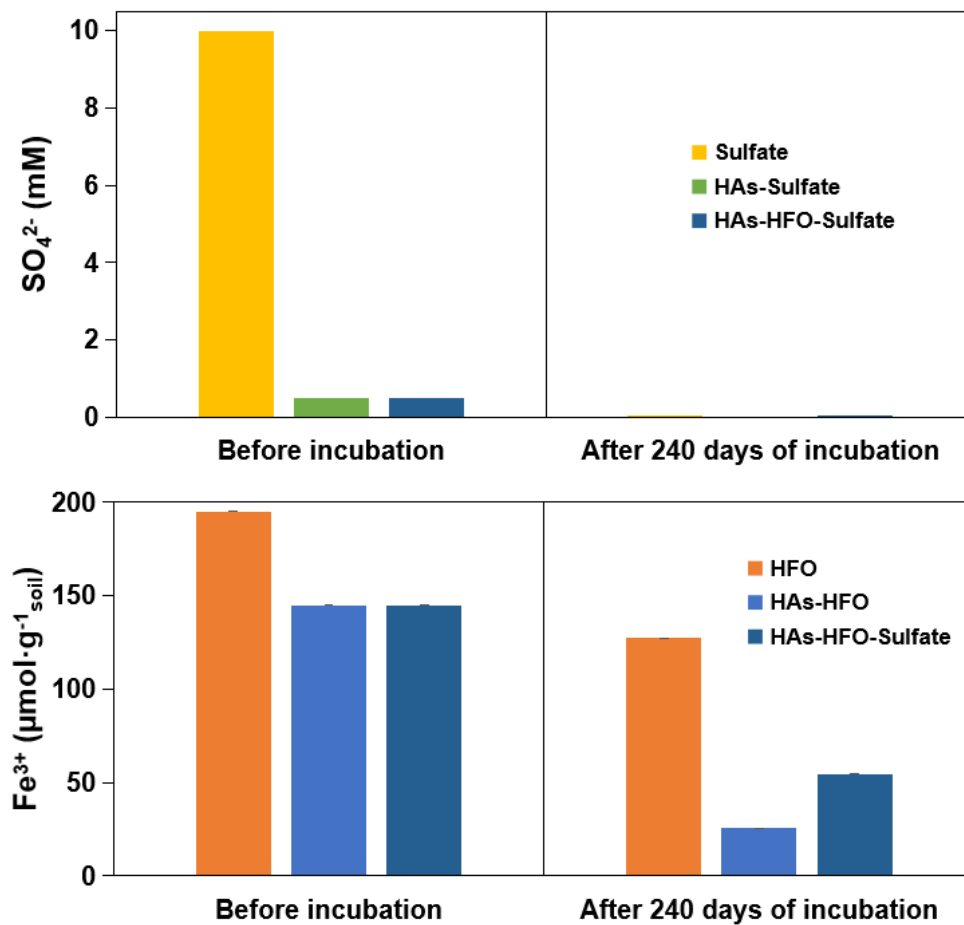


Figure 2. Concentrations in the microcosms of sulfate in the pore water (above) and Fe³⁺ in the solid phase (below), before incubation (left) and after 240 days of incubation (right).

Figure 4 shows that the variations of CH₄ were categorised into three phases, namely the production phase, the consumption phase and the reproduction phase. During the CH₄ production phase, SO₄²⁻ addition significantly reduced the average CH₄ production rate, and the addition of HFO, HAs and HAs-containing combinations significantly increased it. The average CH₄ production rates of the HFO, HAs, HAs-HFO, HAs-SO₄²⁻ and HAs-HFO-SO₄²⁻ treatments were 0.22, 0.26, 0.22, 0.23 and 0.26 mmol mol⁻¹C_{org} d⁻¹, respectively (Figure 4). The average CH₄ production rate in CK (3.3 mmol mol⁻¹C_{org} d⁻¹) was lower than in the five treatments with HAs and/or HFO, indicating that these electron acceptors promoted the production of CH₄ (Figure 4). However, the average rate of CH₄ produced by 1 mM SO₄²⁻ treatment was slightly higher than in CK, and the average rate of CH₄ production in the treatment with 10 mM SO₄²⁻ (0.09 mmol mol⁻¹C_{org} d⁻¹) was significantly lower than in CK, indicating an inhibitory influence of SO₄²⁻ on CH₄ production. In the CH₄ consumption phase the lowest rates of CH₄ production, reached at 60–240 days, were -0.06, -0.07, -0.06, -0.06 and -0.07 mmol mol⁻¹C_{org} d⁻¹ in the HFO, HAs, HAs-HFO, HAs-SO₄²⁻ and HAs-HFO-SO₄²⁻ treatments, respectively. During this phase (240–540 in Figure 4), CH₄ production in the treatments containing only HAs and/or HFO was significantly lower than that in CK (-0.05 mmol mol⁻¹C_{org} d⁻¹), indicating that the consumption of CH₄ was promoted by the HAs and HFO treatments; whereas in the two treatments containing SO₄²⁻, the

rates were not significantly different from CK, perhaps indicating that SO₄²⁻ was depleted. In the CH₄ reproduction phase, the average rates of CH₄ production were low compared to the production phase and were not significantly different amongst all treatments and CK at 240–540 days.

The weighted mean effect sizes, calculated by the method of meta-analysis of response ratio, are shown in Figure 5. During the CH₄ production phase, the effects of adding electron acceptors on CH₄ production were generally significant, consistent and synchronous. In the 0–38 day part of the incubation the effects of HFO, HAs and HAs-containing treatments increased significantly, with average effect sizes approximately three times higher than in CK (Figure 5a). The SO₄²⁻ treatment also promoted CH₄ production at 0–38 days, but significantly reduced CH₄ production at 38–60 days. The effect sizes for other treatments were not significantly different from CK at 38–60 days (Figure 5a). In the CH₄ consumption phase, effect sizes decreased and became asynchronous but remained significant. During this phase, the average effect sizes of the HFO, HAs, HAs-HFO, HAs-SO₄²⁻ and HAs-HFO-SO₄²⁻ treatments were 58–115 % higher than in CK, demonstrating promoted CH₄ consumption (Figure 5b). In the CH₄ reproduction phase, the effect sizes of HFO, HAs-HFO and SO₄²⁻ addition all decreased. With the exhaustion of the electron acceptors, the effect sizes of HFO, HAs-HFO and SO₄²⁻ additions were 10–50 % and significantly lower than in CK (Figure 5c).

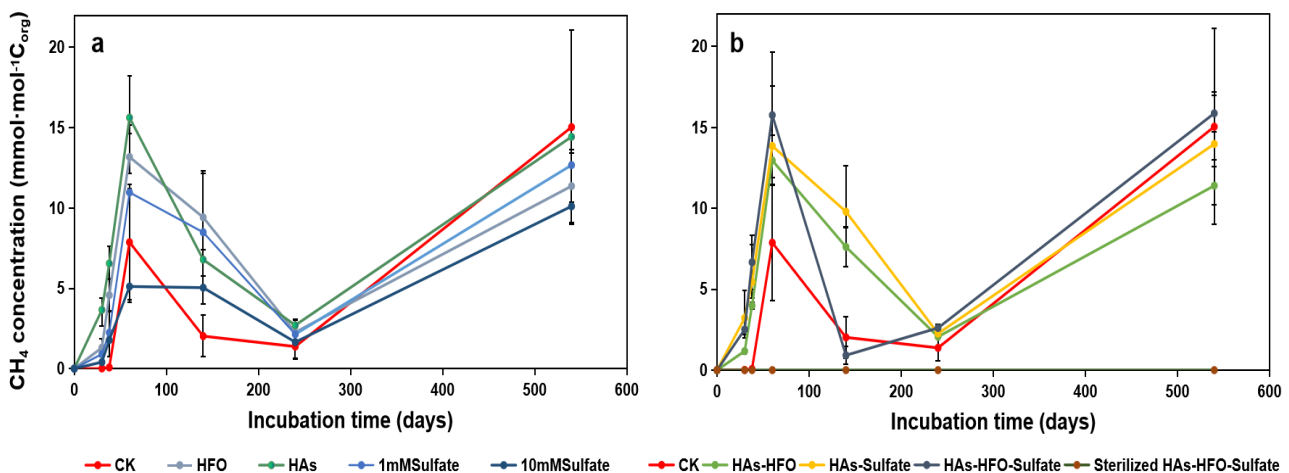


Figure 3. Concentrations of CH₄ over 540 days of incubation in the headspace of microcosms with different electron acceptor treatments: (a) blank control (CK), HFO, HAs, 1 mM Sulfate and 10 mM Sulfate; (b) the combinations HAs-HFO, HAs-Sulfate, HAs-HFO-Sulfate and Sterilised HAs-HFO-Sulfate.

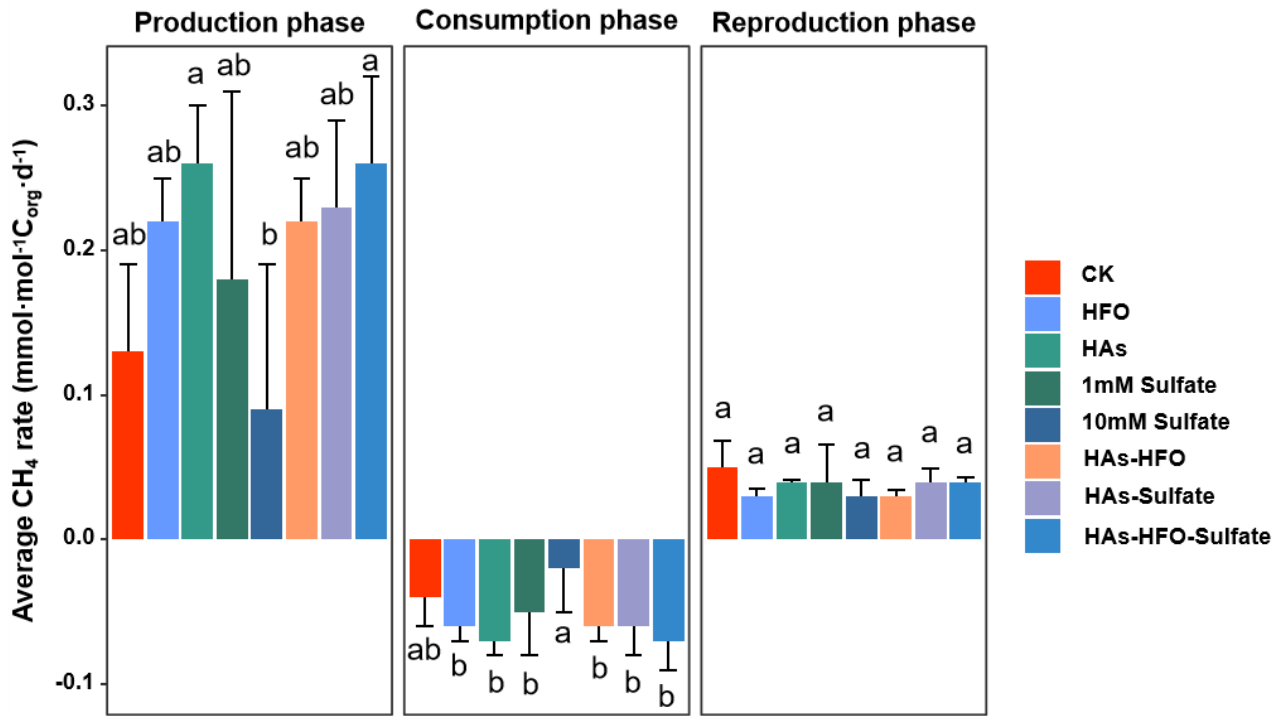


Figure 4. Average rates of CH₄ production or oxidation over 540 days of incubation in the blank control (CK) and seven different electron acceptor treatments (HFO, HAs, 1mM Sulfate, 10mM Sulfate, HAs-HFO, HAs-Sulfate and HAs-HFO-Sulfate). The three panels show different phases of the incubation, namely: production phase (left; 0–60 days), consumption phase (middle; 60–240 days) and reproduction phase (right; 240–540 days). Different letters (a, b) indicate significant differences between treatments ($P < 0.05$).

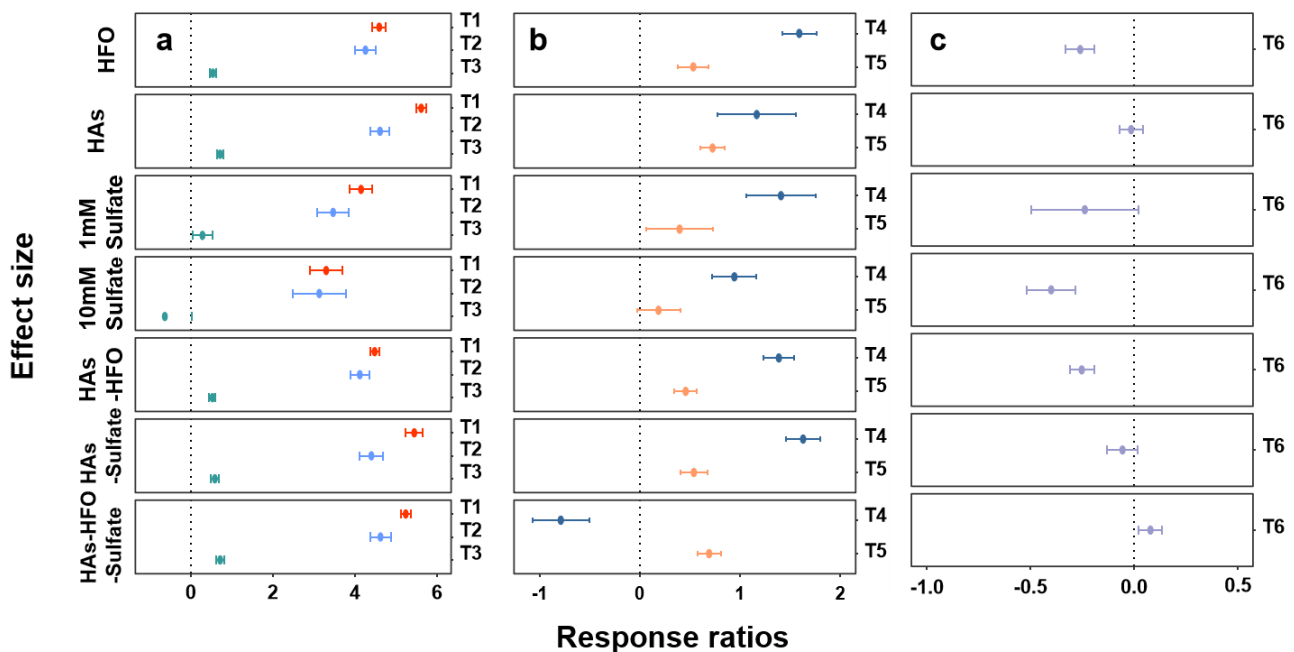


Figure 5. Effect sizes of the seven electron acceptor treatments on CH₄ production and consumption during the three phases: (a) CH₄ production phase (T1: 0~30 days, T2: 30~38 days, T3: 38~60 days); (b) CH₄ consumption phase (T4: 60~140 days, T5: 140~240 days); (c) CH₄ reproduction phase (T6: 240~540 days).

Effects of electron acceptors on CH₄ production and oxidation potential

With the addition of electron acceptors, the CH₄ production potential during the 60-day CH₄ production phase ranged from 0.09 to 0.26 mmol mol⁻¹C_{org} d⁻¹ (Figure 6). The addition of HFO and HAs significantly increased CH₄ production to 0.22 and 0.26 mmol mol⁻¹C_{org} d⁻¹, respectively ($P < 0.05$). CH₄ production with the addition of 10mM SO₄²⁻ was lower than in CK (0.13 mmol mol⁻¹C_{org} d⁻¹), and the CH₄ production potential decreased significantly to 0.09 mmol mol⁻¹C_{org} d⁻¹. The HAs-containing combinations (HAs-HFO-SO₄²⁻, HAs-SO₄²⁻, and HAs-HFO) also increased the CH₄ production potential, to 0.26, 0.23 and 0.22 mmol mol⁻¹C_{org} d⁻¹, respectively ($P < 0.05$).

During the CH₄ consumption phase, the CH₄ oxidation potential ranged from -0.19 to -0.02 mmol mol⁻¹C_{org} (Figure 6). The addition of HFO and HAs reduced the CH₄ oxidation potential to -0.06 mmol mol⁻¹C_{org} and -0.12 mmol mol⁻¹C_{org} d⁻¹, respectively, and the HAs-HFO-SO₄²⁻, HAs-SO₄²⁻ and HAs-HFO treatments had similar effects, whereas the addition of 10mM SO₄²⁻ raised the CH₄ oxidation potential to -0.02 mmol mol⁻¹C_{org} d⁻¹. During the CH₄ reproduction phase, the CH₄ production potentials were significantly lower than those during the first stage, and no significant differences were observed amongst all treatments (Figure 6).

DISCUSSION

Effects of electron acceptors on CH₄ production and oxidation

In this study we investigated whether electron acceptors (e.g., HAs, HFO, SO₄²⁻) have a positive effect on CH₄ production and consumption in alpine wetlands. Previous studies have shown that electron acceptors promote CH₄ production (Wang *et al.* 2009, Li *et al.* 2016). Our study showed that the addition of HAs and HA-containing combinations promoted CH₄ production (Figure 3) and increased the rate of CH₄ production by 3.7–5.6 times compared to CK (Figure 4). In contrast, Ye *et al.* (2016) observed inhibitory effects of HAs on CH₄ production that depended on the characteristics of the HAs. These conflicting observations suggest that HAs play a complex role, serving either as an electron donor or as an electron acceptor (Keller *et al.* 2009). The addition of HAs increased CH₄ production, indicating that HAs could stimulate the decomposition of organic matter to produce CH₄. Electron acceptors also play an important role in CH₄ oxidation (Liu *et al.* 2020), and peat-related CH₄ oxidation occurs ubiquitously across the globe (Kip *et al.* 2010). Our study observed that the rate of CH₄ oxidation during the stage of CH₄ consumption with the addition of HAs and HAs-containing electron acceptors was significantly higher than CK,

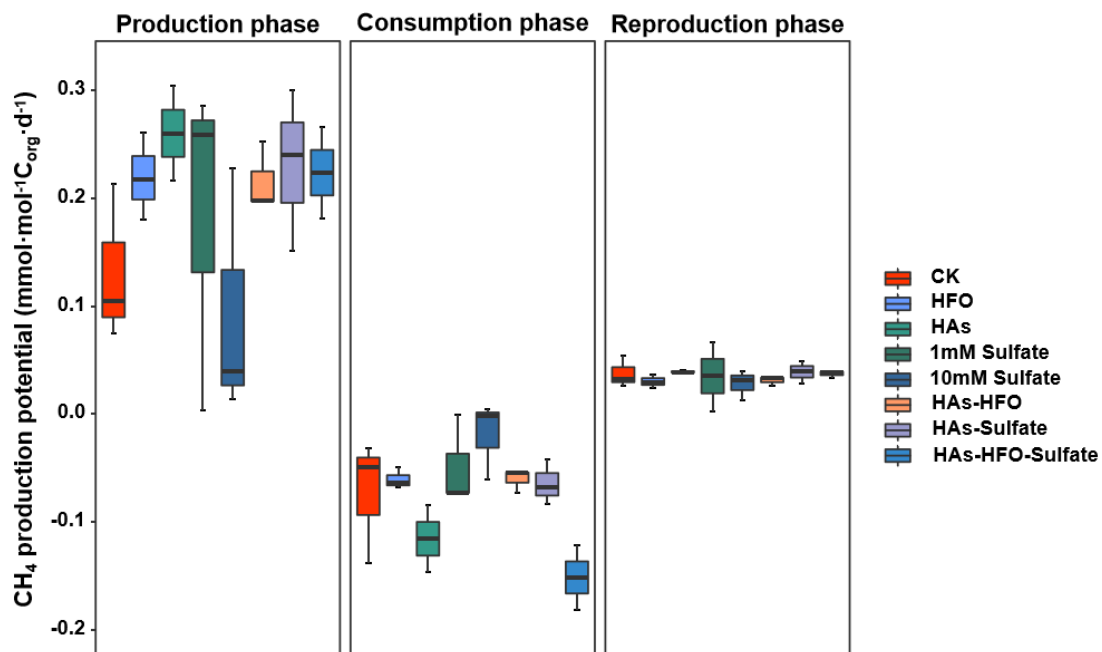


Figure 6. Box plots of CH₄ production and oxidation potential during 540 days of microcosm incubation for the seven different treatments plus sterilised control (CK) during the CH₄ production phase (0–60 days), the CH₄ consumption phase (60–240 days) and the CH₄ reproduction phase (240–540 days).

indicating that HAs promoted the oxidation of CH₄ in this stage (Figure 3). HAs can also be used as a potential electron acceptor and is one of the most effective electron acceptors for CH₄ oxidation (Cervantes *et al.* 2000). When HAs was added to organic carbon-enriched sediments, microorganisms anaerobically oxidised CH₄ to CO₂ (Bradley *et al.* 1998). HAs can also theoretically promote CH₄ oxidation by serving as the terminal electron acceptor for microbial respiration (Lovley *et al.* 1996, Scott *et al.* 1998). The dual roles of HAs in CH₄ production and oxidation are a particularly important aspect that might be applied in environmental management to reduce CH₄ emissions from wetlands.

The addition of Fe³⁺ can promote the CH₄ production process (Siegert *et al.* 2011), and the addition of a small amount of Fe³⁺ can stimulate the production of CH₄ (Dettling *et al.* 2006, Karvinen *et al.* 2014). Other studies suggest that the addition of Fe³⁺ inhibits CH₄ production (Lovley & Phillips 1987, Peters & Conrad 1996); but as they studied low-altitude sites with subtropical or temperate climates, it is most likely that the differences are due to different soil types and environments. The study area of Siegert *et al.* (2011) was similar to ours in that both belonged to alpine regions with long-term frozen soil. Aqueous soluble Fe²⁺ can facilitate direct electron transfer between iron-reducing microorganisms and methanogenic bacteria to promote methanogenesis (Liu *et al.* 2013) and promote direct electron transfer for CH₄ production in anaerobic environments (Kato *et al.* 2012, Zhang & Lu 2016). Anaerobic oxidation of CH₄ coupled with heavy metal reduction has also been found in wetlands (Segarra *et al.* 2013, Dang *et al.* 2021). In the consumption phase, the rate of CH₄ oxidation was significantly higher with HFO than in CK (Figure 4), indicating that HFO promoted the oxidation of CH₄ (Figure 3). This result is in line with previous findings that iron-mediated oxidation of CH₄ effectively facilitated the occurrence of oxidation (Egger *et al.* 2015, Oni & Friedrich 2017). It also proved that Fe³⁺ promoted CH₄ oxidation under anaerobic conditions (Kumaraswamy *et al.* 2001).

Our research showed that the addition of SO₄²⁻ inhibited CH₄ production, in contrast to the promoting effects of HAs and HFO (Figure 5), which is consistent with previous studies (Achnich *et al.* 1995, Zeng *et al.* 2008). The reduction process of SO₄²⁻ inhibited the activity of methanogens as the competitive ability of methanogens in obtaining electrons is weaker than that of SO₄²⁻-reducing bacteria. The activity of heterotrophic microorganisms limited the production of CH₄. The reduction of SO₄²⁻ reduced both CH₄ production (Holmer & Kristensen 1994) and the potential for

CH₄ production. Some studies showed that SO₄²⁻ inhibited CH₄ oxidation (Blazewicz *et al.* 2012), even up to 3.4–5.5 times for anaerobic oxidation of CH₄ (Fan *et al.* 2020). In our study, the oxidation of CH₄ in the SO₄²⁻ treatment was not significantly different from the control without added electron acceptors (Figure 4). This was potentially caused by the depletion of SO₄²⁻ after long-term cultivation.

The addition of HAs-HFO-SO₄²⁻, HAs-SO₄²⁻ and HAs-HFO promoted CH₄ production and oxidation (Figure 4) which involved complex interactions between different electron acceptors. HAs have high reactivity with metal oxides (Cervantes *et al.* 2002), and HFO with high surface area strongly absorbs HAs, leading to coprecipitation of HAs and HFO (Liu *et al.* 2017). Thus, Fe³⁺ inhibits the effectiveness of HAs under anaerobic conditions (Azman *et al.* 2015). A high concentration of dissolved HAs is toxic to both methanogenic and SO₄²⁻-reducing bacteria (Minderlein & Blodau 2010, Zuo *et al.* 2020). On the other side, Fe²⁺ promoted the activity of SO₄²⁻-reducing bacteria and methanogens by scavenging toxic H₂S (Xia *et al.* 2021). HAs could serve as an electron shuttle to accelerate the bio-reduction rate of Fe³⁺ and reduce the effect of Fe³⁺ on the anaerobic oxidation of CH₄ (M.W. Wang *et al.* 2018). HFO decreased CH₄ production but enhanced SO₄²⁻ reduction and increased the secretion of HAs to enhance electron transfer capacity (Jin *et al.* 2020). These complex interactions amongst electron acceptors contributed to their effects on the rates of methane production and oxidation (Figure 5).

Interestingly, our long-term experiments showed that CH₄ concentration increased initially, then decreased, and finally increased again, indicating CH₄ redox oscillations in an enclosed environment except in sterilised controls (Figure 3). Redox oscillations occur widely with natural environmental perturbation. Similar redox cycling of Fe²⁺/Fe³⁺ was also observed in the batch experiment (Zhang *et al.* 2007). Unfortunately, we did not determine the redox state of HAs in this study, so it is difficult to speculate about CH₄ redox oscillations, which need further study.

Effects of electron acceptors on CH₄ production and oxidation potential

HFO, HAs and HAs-containing combinations increased the production potential of CH₄ by 65–100 % ($P < 0.05$, Figure 6) and the oxidation potential of CH₄ by 58–115 % ($P < 0.05$, Figure 6). The addition of SO₄²⁻ reduced the production potential of CH₄ by 35 % ($P < 0.05$, Figure 6) and the oxidation potential of CH₄ by 50 % ($P < 0.05$; Figure 6). The production or oxidation potential of

CH₄ was consistent with the rate of CH₄ production or oxidation. The production and oxidation potentials of CH₄ are influenced by soil organic matter content and electron acceptor types and concentrations in different environments (Segers 1998). We found that the addition of electron acceptor as 10 mM Fe³⁺ promoted CH₄ production (Fig 3). These results are consistent with the findings of Luo *et al.* (2021), who found that adding Fe³⁺ increased CH₄ production (Luo *et al.* 2021). Studies have shown that HAs have a significant inhibitory effect on methanogenesis, through competition with substrates or by direct toxic effects (Keller *et al.* 2009, Minderlein & Blodau 2010) as reduction of HAs is thermodynamically more favourable than methanogenesis (Cervantes *et al.* 2000, Ye *et al.* 2012). The addition of 30 mM SO₄²⁻ has an inhibitory effect on CH₄ oxidation, as in our results (Mostovaya *et al.* 2021). Our results also showed the addition of electron acceptors greatly decreased the potential for CH₄ production in wetlands, which provide a reference for the management of alpine wetland subject to methane emission (Yang *et al.* 2022, Zhang *et al.* 2022).

Concluding remarks

CH₄ production and anaerobic oxidation strongly depend on the type of electron acceptors in Nagqu alpine wetlands. Regardless of the addition of electron acceptors, the wetland soil demonstrated CH₄ production, consumption and reproduction phases in long-term anaerobic incubation in the laboratory. Addition of the electron acceptors HFO and HAs promoted the production and oxidation potentials of CH₄, while high-concentration SO₄²⁻ (10mM) inhibited the two potentials. HAs tended to be the dominant factor affecting CH₄ emissions as electron acceptors in alpine wetlands because of their high concentration. Our research results are of reference value in further revealing the role of electron receptors in CH₄ emission processes and provide important practical guidance towards reducing global CH₄ emissions.

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AUTHOR CONTRIBUTIONS

GZ conceived the study, which was designed by GZ, WH, BN and YY. HJ and YL conducted soil column sampling in the field. YY, BN, GH and AY conducted the laboratory experiments. YY and WH analysed the data and wrote the manuscript. All authors developed and approved the manuscript.

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