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ORIGINAL PAPER

Sources and intensity of CH_4 production in paddy soils depend on iron oxides and microbial biomass

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Abstract

A paddy soil, with microbial biomass considerably reduced by chloroform fumigation, was treated with low-crystalline ferrihydrite and high-crystalline goethite and with ^{13}C-labeled acetate. In the first 10 days of the incubation, CH_4 was produced mainly from the added acetate (56−91%). After day 30, however, 3−11% of the total CH_4 emissions originated from the added acetate. Chloroform fumigation reduced the microbial biomass by 43−87%, leading to the decrease in the CH_4 emission from the fumigated soil for 352−1127 times compared to that from the unfumigated soil. Acetate only contributed to 0−6% of the total CH_4 emission from the fumigated soil during the entire incubation period. Thus, chloroform fumigation largely reduced the abundance of methanogens, and the reduction in the abundance of acetotrophic methanogens was high. Iron oxide additions reduced CH_4 emissions from the added acetate and from other sources. The reduction was stronger in the fumigated soil compared to that in the unfumigated soil because the lower abundance of methanogens in the fumigated soil decreased the competition for substrates with iron reducers. The effect of ferrihydrite on CH_4 emission from non-acetate sources was stronger than that of goethite before day 6; however, this effect became weaker thereafter, because of the reduced number of reactive sites after acetate sorption by ferrihydrite. We conclude that the marked reduction in the microbial biomass, and especially methanogens, decreased the methane production, changed the CH_4 sources, and increased the relative effects of iron oxides on CH_4 production.

Keywords Iron oxidation–reduction · Methanogenesis · Chloroform fumigation · Microbial biomass · Acetoclastic pathway · ^{13}C isotope labeling

Introduction

Methane (CH_4) contributes to 16% of total anthropogenic greenhouse gas emissions (IPCC 2014). Rice fields, accounting for 9% of the global cropland area, contribute to 11% of global anthropogenic CH_4 emissions (IPCC 2013; Ge et al. 2017; Liu et al. 2021; Wei et al. 2021). In soils under anoxic conditions, organic matter is first converted by hydrolysis and fermentation into acetate and H_2, which serve as substrates for methanogens (Malyan et al. 2016; Li et al. 2020a,

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c; Xu et al. 2020). Acetotrophic and hydrogenotrophic pathways using acetate and H_2 are major pathways for CH_4 production. Theoretically, the contribution of acetate to methanogenesis during anaerobic degradation of carbohydrates can minimally be 67% of the total CH_4 formed (Conrad 1999), as proved by studies of methanogenic environments (Palmer and Reeve 1993; Chin and Conrad 1995). Therefore, studies on regulation of methanogenesis, especially acetotrophic methanogenesis, are of great importance for the mitigation of CH_4 emissions in paddy soils.

Iron, as a redox-sensitive and abundant element in paddy soils, participates in redox processes and influences CH_4 emissions (Achtnich et al. 1995; Chidthaisong and Conrad 2000; Hori et al. 2010; Han et al. 2018). CH_4 emission from soil with a high iron content after straw addition was lower than that from soil with a low iron content (Hu et al. 2020). Application of iron, such as (oxyhydr)oxides, ferrous iron, ferrihydrite, and even iron-rich wastes, reduces CH_4 emissions in paddy soils (Furukawa and Inubushi 2004; Jäckel et al. 2005; Hu et al. 2020). The reduced iron content closely correlates with CH_4 emissions from paddy soils (Peng et al. 2015; Sun et al. 2019). Iron reduction occurs when Eh < 100 mV (Thamdrup 2000), whereas methanogenesis occurs when Eh < − 100 mV, and the reduction of CO_2 to CH_4 requires an Eh < − 200 mV (Tyagi et al. 2010; Ali et al. 2019). Iron reducers can compete with methanogens for acetate and H_2 (Achtnich et al. 1995; Chidthaisong and Conrad 2000; Roden and Wetzel 2003). Some mesophilic and thermophilic methanogens are capable of reducing iron by using H_2 as the reducing equivalent, and the diversion of electrons from the CO_2 reduction to iron reduction suppresses methanogenesis (Zhang et al. 2012; Yamada et al. 2014). Furthermore, iron oxides can affect the C cycle in soil by sorption/desorption with organic C (Dippold et al. 2014; Ye and Horwath 2017; Li et al. 2021; Wei et al. 2022).

The role of iron in SOC decomposition and methanogenesis has been intensively studied. However, when new C sources such as straw, litter, dead roots, and rhizodeposits supplement the C pool of paddy soils, they cause pulses of CH_4 emissions and influence CH_4 production from soil organic C (SOC), i.e., priming effect (PE) (Zhu et al. 2016; Ye and Horwath 2017; Zhou et al. 2020). However, it is unclear how iron oxides influence CH_4 source partitioning and PE. Further, paddy soils show taxonomic redundancy of microbial groups involved in methanogenesis (Liu et al. 2019). However, it is unknown if the role of iron oxides on CH_4 emission remains the same when the methanogen abundance is reduced. Iron oxides in paddy soils are present in a variety of forms, ranging from amorphous minerals (such as ferrihydrite) to crystals (such as goethite) (Cornell and Schwertmann 2003). Ferrihydrite has higher specific surface area and more rapid reduction rate under anoxic condition than goethite (Hansel et al. 2004; Kaiser et al. 2007; Hanke et al. 2014). Studies using various iron oxides have mainly compared organic matter adsorption capacity, reducing rate, and crystallization processes (Hansel et al. 2004; Hori et al. 2010; Vogelsang et al. 2016). However, the influence of these processes on methanogenesis remains undetermined.

Therefore, we used ^{13}C-labeled substrate to study the effects of iron oxide addition on methanogenesis and PE in anoxic paddy soils, with both original and greatly reduced microbial biomass. Ferrihydrite and goethite were used because of their differences in crystallinity. We chose acetate as the substrate to eliminate the effects of iron oxides on the fermentation of substrates when interpreting the data and study methanogenesis (mainly acetoclastic) exclusively. We reduced the soil microbial biomass by chloroform fumigation (Wu et al. 1990) because chloroform can inhibit acetoclastic and hydrogenotrophic methanogenesis and its effect on iron reducers is weak (Chidthaisong and Conrad 2000). We tested the following hypotheses: (1) the addition of iron oxides reduces CH_4 emission and weakens the PE because iron reducers compete for substrates with methanogens; (2) the reduction effect of ferrihydrite on CH_4 emission and PE is stronger than that of goethite because the reduction of ferrihydrite is more rapid and complete than that of goethite; and (3) a large decrease in the microbial biomass (e.g., after soil fumigation with chloroform) changes the portion of total CH_4 emission derived from acetate and increases the relative effects of iron oxides on methanogenesis and PE because the marked decrease in methanogenesis by chloroform fumigation makes iron reduction more competitive for substrates.

Materials and methods

Soil description

Soil was sampled from the 20-cm-deep plow layer of a rice paddy field located in Hunan, China (113° 18′ 53″ E, 27° 15′ 21″ N; 118 m.a.s.l.). The climate in the study site is subtropical, with a mean annual temperature of 17.5 °C, annual precipitation of 1300 mm, a total of 1663 annual sunlight hours, and a frost-free period of up to 274 days. The soil was carbonate-free silt-loam (sand 28.1%, silt 65.7%, and clay 6.2%) with a mean organic C content of 32.8 g kg^{-1}, total N content of 2.4 g kg^{-1}, total P content of 0.6 g kg^{-1}, total iron content of 25.8 g Fe_2O_3 kg^{-1}, and pH of 4.7 (soil:H_2O, 1:2.5).

Iron oxide preparation

Ferrihydrite was synthesized by titrating a solution of 0.4 M $FeCl_3 \cdot 6H_2O$ using 1 M NaOH until a pH of 7.0 was attained (Schwertmann and Cornell 2000). After aging for 6 h,

the suspension was washed with double deionized water. Goethite was synthesized by titrating a solution of 1 M $FeCl_3 \cdot 6H_2O$ using 1 M NaOH until a pH of 12.0 was attained (Atkinson et al. 1967). After aging at 70 °C for 60 h, the suspension was washed with double deionized water. The specific surface areas of the prepared ferrihydrite and goethite were measured by Brunauer–Emmett–Teller gas adsorption analysis and were 268 and 39 $m^2\ g^{-1}$, respectively.

Soil fumigation

Prior to the actual incubation, sieved soils (< 2 mm) were preincubated under flooded conditions (i.e., water 3 cm above the surface) at 25 °C for 14 days in darkness. Next, the soils were treated with chloroform ($CHCl_3$) fumigation or were untreated. The water above the flooded soil was discarded before fumigation. For the fumigation, soil (approximately 1.5 kg fresh weight) was spread in a thin layer across four shallow plates, which were then placed in a desiccator with 100 mL of ethanol-free $CHCl_3$. The desiccator was evacuated until the $CHCl_3$ had boiled for 10 min, after which the plates were incubated in darkness at 25 °C for 24 h (Wu et al. 1990). After fumigation, the residual $CHCl_3$ in the soil was removed through repeated evacuations. Two desiccators were used to fumigate approximately 3 kg of fresh soil. After fumigation, the soil from the two desiccators was thoroughly mixed.

Soil incubation

Three factors were designed in this experiment, i.e., iron oxide addition (ferrihydrite, goethite, none), acetate addition (acetate, none), and chloroform fumigation (fumigated, unfumigated). Thus, there were 12 treatments in total, each with three replicates. The treatments are herein referred to as ferrihydrite, goethite, acetate, ferrihydrite + acetate, goethite + acetate, and control, for both unfumigated and fumigated soils. The treated soils were incubated anaerobically for 100 days in darkness at 25 °C.

Briefly, the soils with ferrihydrite and ferrihydrite + acetate (fumigated or unfumigated) treatments were thoroughly mixed with ferrihydrite (0.9 g Fe kg^{-1} soil), corresponding to 5% of the total soil iron content prior to flooding and incubation. Goethite was added at the same rate as ferrihydrite. Soil (equivalent to 20 g dry soil) was weighed into 600-mL stopped glass bottles and then flooded with sterilized distilled water (water 3 cm above the surface). The ^{13}C-acetate (uniformly labeled, > 99 atom% 1, 2-^{13}C2, Cambridge Isotope Laboratories, Germany) and ^{12}C-acetate (> 99.5%, Sinopharm Chemical Reagent Co., Ltd., China) were mixed to reach 4 atom% ^{13}C. In the acetate, ferrihydrite + acetate, and goethite + acetate soils, ^{13}C-labeled acetate (4 atom% ^{13}C) was added to each bottle at a rate of 2% of the soil organic C (SOC) content (656 mg C kg^{-1} soil). The molar ratio of the added Fe to acetate was 1:3.4. Soil with unlabeled acetate was also prepared to measure naturally occurring ^{13}C. Soil without iron oxides or acetate was incubated; this served as the control soil. The bottles were repeatedly evacuated and filled with N_2 gas to remove O_2.

Gas and soil sampling and analyses

Approximately 30 mL of headspace gas was sampled using a gas-tight syringe every 1−15 days. This gas was then stored in pre-evacuated glass bottles (LabCo, High Wycombe, UK). CH_4 fluxes were measured using a gas chromatograph (Agilent 7890A; Agilent Technologies, Palo Alto, USA) equipped with a thermal conductivity detector. CH_4 concentrations (mol L^{-1}) were quantified by comparing the peak areas with those of the calibration gasses. The emission rates were calculated as mg C kg^{-1} day^{-1} using Eq. (1):

$$R_{CH4} = M \times C \times V \times (273/(273 + T)) \times \left(P/P_0\right)/22.4/W/t \quad (1)$$

where R_{CH4} is the CH_4 emission rate (mg C kg^{-1} day^{-1}), M is the molecular weight, C is the CH_4 concentration (mol L^{-1}), V is the volume of the incubation bottle, T is the incubation temperature (25 °C), P_0 and P are the standard and real atmospheric pressure values, respectively, W is the weight (g) of soil in the bottle, and t is the time (day) for gas cumulation, i.e., the period between sampling dates. The ^{13}C abundance of CH_4 in each headspace sample was analyzed using a MAT253 isotope mass spectrometer coupled with a GasBench (Thermo Fisher Scientific, Waltham, USA) and expressed as δ (‰), relative to the Pee Dee Belemnite standard and then converted to atom%.

Soils were sampled on day 100. Dissolved organic C (DOC) was extracted using 0.5 M K_2SO_4 and measured using a total organic C (TOC) analyzer (TOC-VWP; Shimadzu, Kyoto, Japan). Soil microbial biomass C (MBC) was determined using the chloroform-fumigation-extraction method (Wu et al. 1990). Briefly, a soil sample (15 g) was extracted with 60 mL of 0.5 M K_2SO_4 solution, and another subsample (15 g) was fumigated with ethanol-free $CHCl_3$ under darkness for 24 h. It was then extracted with 60 mL of 0.5 M K_2SO_4 solution. The TOC concentration for each extract was analyzed using a TOC analyzer (TOC-VWP; Shimadzu). Soil MBC was calculated as the difference in TOC contents between the fumigated and unfumigated sample extracts, which was adjusted by a 0.45 extraction efficiency coefficient (Wu et al. 1990).

Calculations

The ^{13}C content (mg C kg^{-1}) in the evolved CH_4 was calculated using Eq. (2):

$$^{13}CH_{4(sample)} = (\text{atom\% } CH_{4(L)} - \text{atom\% } CH_{4(UL)}) \times CH_{4(sample)}/100 \quad (2)$$

where atom% $CH_{4\ (L)}$ and atom% $CH_{4\ (UL)}$ are the $^{13}CH_4$ atom% in the samples from the labeled and unlabeled acetate-added soils, respectively, and $CH_{4\ (sample)}$ is the total CH_4 content (mg C kg^{-1}) of the sample.

Acetate-derived CH_4 emissions (mg C kg^{-1}) were calculated using Eq. (3), as reported by Rochette et al. (1999):

$$\begin{aligned}\text{Acetate-}CH_{4\ (+Acetate)} = {} & CH_{4\ (+Acetate)} \\ & \times (\text{atom\%}CH_{4\ (+Acetate)} - \text{atom\%}CH_{4\ (-Acetate)}) \\ & /(\text{atom\%}C_{(acetate)} - \text{atom\%}C_{(soil)})\end{aligned} \quad (3)$$

where Acetate-$CH_{4\ (+Acetate)}$ and $CH_{4\ (+Acetate)}$ are the CH_4 emissions derived from acetate (mg C kg^{-1}) and the total CH_4 emissions derived from both acetate and SOC (mg C kg^{-1}) in the soil with acetate (i.e., acetate, ferrihydrite + acetate, and goethite + acetate), respectively. Atom% $CH_{4\ (+Acetate)}$ and atom% $CH_{4\ (-Acetate)}$ are the ^{13}C atom% of the CH_4 emissions in soil with labeled acetate and acetate-free soil (i.e., control, ferrihydrite, and goethite), respectively. Atom% $C_{\ (acetate)}$ and atom% $C_{\ (soil)}$ are the ^{13}C atom% of the added labeled acetate and soil, respectively.

The CH_4 emissions from non-acetate C sources (mg C kg^{-1}) were calculated using Eq. (4):

$$\text{Non-acetate-}CH_{4\ (+Acetate)} = CH_{4\ (+Acetate)} - \text{Acetate-}CH_{4\ (+Acetate)} \quad (4)$$

where non-acetate-$CH_{4\ (+Acetate)}$ is the CH_4 emissions (mg C kg^{-1}) from C sources other than the added acetate. In the unfumigated soil, the non-acetate C source comprised SOC and microbial metabolic products. In the fumigated soil, because labile C was released from the killed microorganisms, these non-acetate C sources included not only SOC and microbial metabolic products but also C from dead cells.

The cumulative PE (mg kg^{-1}) of CH_4 emission from non-acetate C sources was calculated using Eqs. (5) and (6):

$$PE_{(Iron\ oxides)} = CH_{4(Iron\ oxides)} - CH_{4(Control)} \quad (5)$$

$$PE_{(+Acetate)} = Non - acetate - CH_{4(+Acetate)} - CH_{4(Control)} \quad (6)$$

where $PE_{\ (Iron\ oxides)}$ was used for the soil with ferrihydrite and goethite, whereas $PE_{\ (+Acetate)}$ was used for the soil with acetate. $CH_{4\ (Iron\ oxides)}$ denotes the cumulative CH_4 emissions (mg C kg^{-1}) from the ferrihydrite and goethite soils. $CH_{4\ (Control)}$ is the cumulative CH_4 emissions of the control. Two separate controls were used for the unfumigated and fumigated soils.

The effect of the iron oxides on CH_4 emissions was calculated using Eq. (7):

$$\text{Iron oxide effect} = (CH_{4(+Iron\ oxides)} - CH_{4(-Iron\ oxides)}) / CH_{4(-Iron\ oxides)} \times 100\% \quad (7)$$

where $CH_{4\ (+Iron\ oxides)}$ is the cumulative CH_4 emissions (mg C kg^{-1}) in the soils with iron oxide addition (i.e., ferrihydrite, goethite, ferrihydrite + acetate, and goethite + acetate) and $CH_{4\ (-Iron\ oxides)}$ is the cumulative CH_4 emissions (mg C kg^{-1}) from the soil without iron oxide addition (i.e., control and acetate).

Statistical analysis

Data were tested for normality (Shapiro–Wilk) and homogeneity of variance (Levene's test) and transformed when necessary. One-way analysis of variance (ANOVA) was performed to analyze the effects of the iron oxide addition on CH_4 emissions from added acetate and non-acetate C sources. Multiple comparisons between the ferrihydrite, goethite, and control soils were performed using ANOVA with Tukey pairwise post-hoc testing. Effects were considered significant at $p < 0.05$. Statistical analyses were conducted using SPSS v. 22 (IBM Inc., Armonk, USA).

Results

CH_4 emissions from added acetate and non-acetate C sources in the unfumigated soil

Cumulative CH_4 emission from the unfumigated soil without acetate addition was 137 mg kg^{-1} after 100 days of incubation (Fig. 1). Following acetate addition (656 mg C kg^{-1}), the cumulative CH_4 emission increased by 318 mg C kg^{-1}. Based on ^{13}C labeling, the cumulative CH_4 emission derived from acetate (acetate-CH_4) was 221 mg kg^{-1} after 100 days of incubation (Fig. 1). Cumulative non-acetate-CH_4 emission was 235 mg kg^{-1} (Fig. 1) and originated from SOC and microbial metabolic products. In the fumigated soil, the non-acetate C also included organics released from dead cells killed by chloroform fumigation. Thus, we used the term "non-acetate C sources" instead of SOC to represent C sources other than the added acetate in both unfumigated and fumigated soils. The non-acetate-CH_4 emission in the unfumigated soil with acetate was 98 mg kg^{-1}, which was greater than the CH_4 emission from soil without acetate. Therefore,

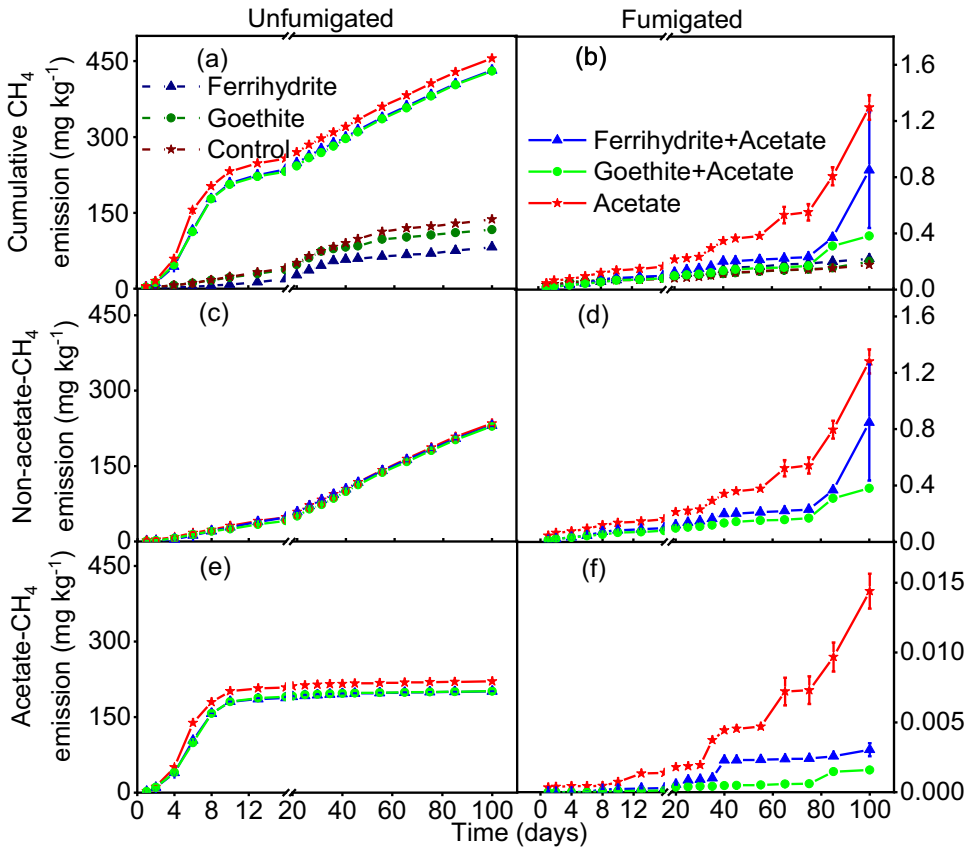

Fig. 1 Cumulative CH_4 emissions in the **a** unfumigated and **b** fumigated soils, without (control) and with additions of ferrihydrite, goethite, and acetate (ferrihydrite, goethite, acetate, ferrihydrite + acetate, and goethite + acetate, respectively). Cumulative CH_4 emissions derived from added acetate and non-acetate sources (acetate-CH_4, non-acetate-CH_4, respectively) (left, mg kg^{-1} soil; right, % of initial acetate/SOC) in the **c**, **e** unfumigated and **d**, **f** fumigated soils with acetate (acetate, ferrihydrite + acetate, and goethite + acetate). Values represent the means ± standard errors ($n = 3$). Note the different y-axis scales for the unfumigated (left) and fumigated (right) soils. For better illustration of the early stage when acetate was rapidly consumed, a break was added between days 16–20, and the scale before day 16 was enlarged

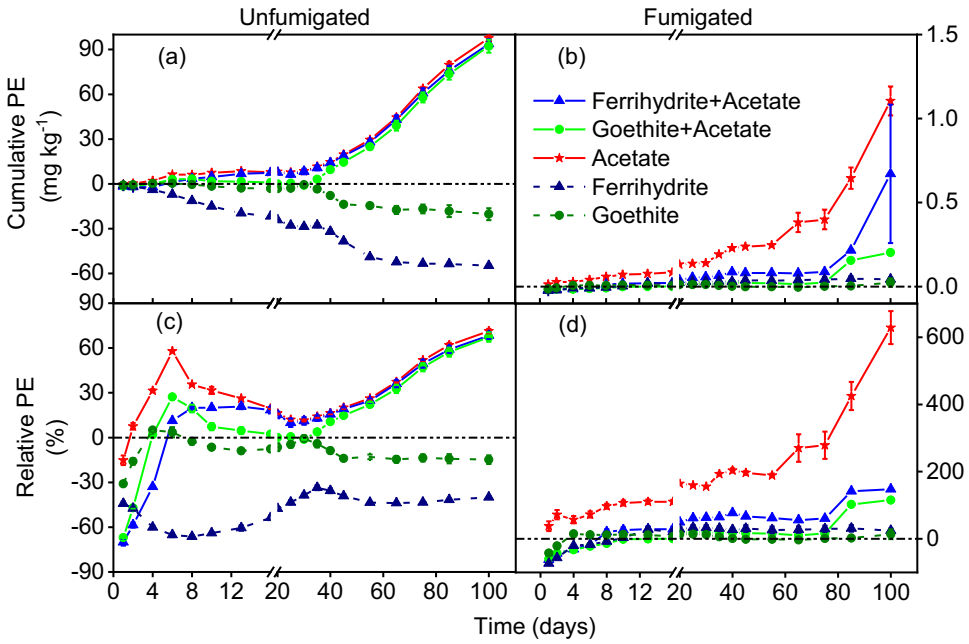

Fig. 2 Cumulative priming effect (PE, mg kg^{-1}) and relative PE (% of control) values caused by acetate, ferrihydrite, and goethite in the **a**, **c** unfumigated and **b**, **d** fumigated soils of ferrihydrite, goethite, acetate, ferrihydrite + acetate, and goethite + acetate treatments. Values represent the means ± standard errors ($n = 3$). Note that a break was added between days 16–20 and the scale before day 16 was enlarged

the relative PE of acetate on CH_4 emissions (measured as a percentage relative to the control) was 71% (Fig. 2).

In the soil with acetate, the acetate-CH_4 emission rate was relatively rapid in the first 10 days and peaked at approximately 44 mg kg^{-1} day^{-1} (6.7% of acetate day^{-1}) on day 6. After day 10, the acetate-CH_4 emission rate suddenly slowed down and fell below 0.6 mg kg^{-1} d^{-1} (0.009% of acetate day^{-1}) on day 100. In the first 10 days, 30.6% of added acetate was converted to CH_4, and only 3.0% was converted to CH_4 in

the last 90 days. In contrast, the non-acetate-CH_4 emission rate from soil with acetate was relatively stable (1.7–4.2 mg kg^{-1} d^{-1}) and higher than the acetate-CH_4 emission rate after day 10. Acetate caused positive PE, which constantly increased after day 30 (Fig. 2). Acetate-CH_4 emissions accounted for the major proportion (56−91%) of total daily CH_4 emissions in the first 10 days and peaked on day 6 (91%). This percentage decreased sharply from day 10 (74%) to day 30 (11%) and gradually after day 30 until it was only 3% on day 100 (Fig. 3).

Effects of iron oxides on CH_4 emissions

Iron oxide addition alone to the unfumigated soil reduced CH_4 emissions compared with the control (Fig. 1). Ferrihydrite caused stronger negative PE (− 55 mg kg^{-1}) than goethite (− 20 mg kg^{-1}) (Fig. 2). With the addition of acetate, iron oxides reduced CH_4 emissions from acetate and non-acetate sources by 9–29% and 0.3–65% during incubation, respectively (Fig. 4). The effect of iron oxides was stronger on acetate-CH_4 emissions than on non-acetate-CH_4 emissions (Fig. 4e and g). Ferrihydrite and goethite showed no significant differences in their effects on acetate-CH_4 emissions during incubation (Fig. 4c and g). Ferrihydrite reduced more CH_4 emissions from non-acetate sources than goethite in the first 6 days but less after day 10 (Fig. 4a and e). Iron oxides decreased the PE induced by acetate. Acetate addition with ferrihydrite caused a lower PE than acetate addition with goethite in the first 6 days and a higher PE after day 10. In the last 60 days, acetate with ferrihydrite and goethite caused equally positive PE in the unfumigated soil (Fig. 2).

CH_4 emissions from the fumigated soil

The cumulative CH_4 emissions from the fumigated soil at the end of the 100-day incubation were 352−1127 times lower than those from the unfumigated soil, i.e., only 0.18–0.22 mg kg^{-1} (Fig. 1). Acetate addition only increased cumulative CH_4 emissions from the fumigated soil by 0.18–1.12 mg kg^{-1} on day 100 (Fig. 1). Compared to those from acetate and non-acetate C sources from the unfumigated soil, CH_4 emissions from acetate and non-acetate C sources from the fumigated soil were extremely low. Approximately 29–34% of acetate was consumed for CH_4 production in the unfumigated soil, whereas only 0.02–0.04% of acetate contributed to CH_4 emissions from the fumigated soil (Fig. 1). In the unfumigated soil, 0.70–0.72% of the non-acetate C sources was consumed for CH_4 production; in the fumigated soil, 0.0012–0.0039% of the non-acetate C sources was transformed to CH_4. In addition, cumulative acetate-CH_4 emissions from the fumigated soil were much lower than non-acetate-CH_4 emissions. This was in contrast to the results of the unfumigated soil (Fig. 1). Of the total daily CH_4 emissions, 0–6% was from acetate during the entire incubation period (Fig. 3). In contrast to those in the unfumigated soil, the acetate-CH_4 and non-acetate-CH_4 emission rates in the fumigated soil increased over time.

The relatively low CH_4 emissions in the fumigated soil meant that the reduction effects of iron oxides on acetate-CH_4 and non-acetate-CH_4 emissions were much smaller than those in the unfumigated soil (Fig. 4). However, in terms of their relative reduction ratios, the fumigated soil (48–100% of acetate-CH_4; 33–73% of non-acetate-CH_4) was more affected than the unfumigated soil (9–29% of acetate-CH_4; 0.3–65% of non-acetate-CH_4) for most of the incubation period (Fig. 4). Similarly, the PE in the fumigated soil,

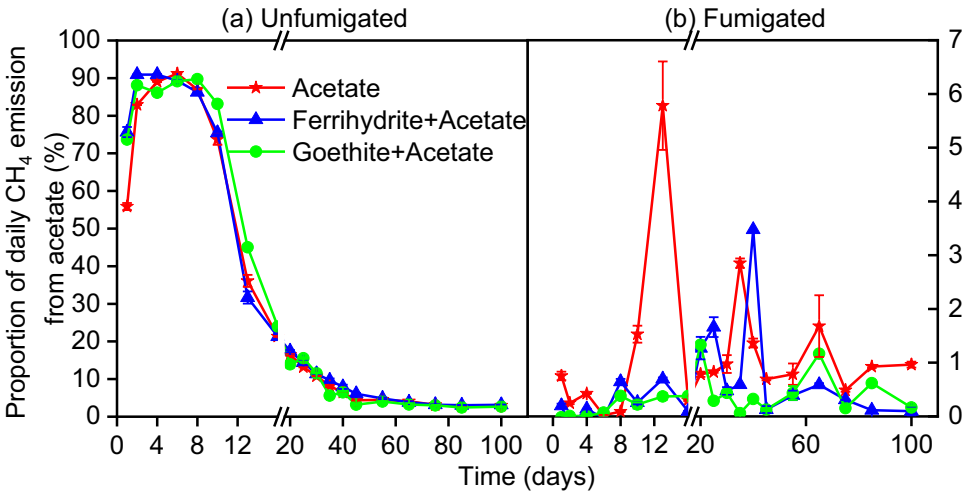

Fig. 3 Proportion of daily CH_4 emission derived from acetate in the **a** unfumigated and **b** fumigated soils for the acetate, ferrihydrite + acetate, and goethite + acetate treatments. Values represent the means ± standard errors ($n = 3$). Note the different y-axis scales for the unfumigated (left) and fumigated (right) soils. Note that a break was added between days 16−20 and the scale before day 16 was enlarged

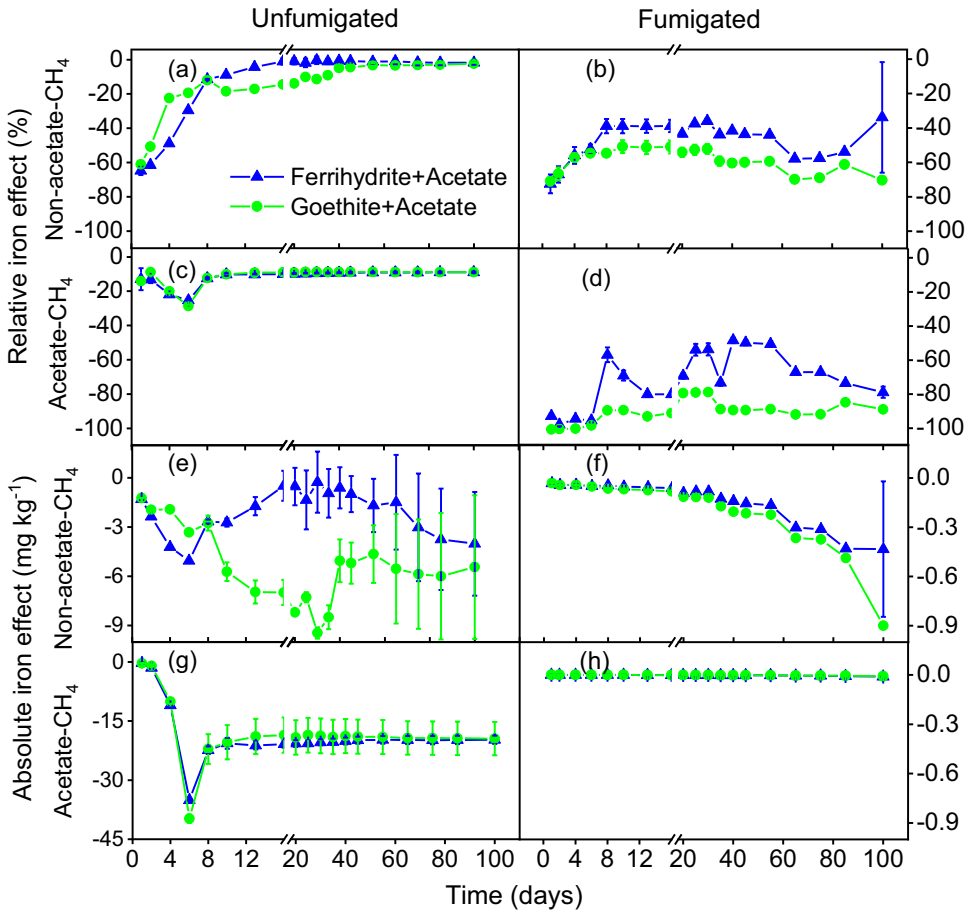

Fig. 4 Iron oxide effects (mg kg^{-1}) and relative effects (% control or % acetate) on CH_4 emissions derived from acetate and non-acetate C sources in the **a**, **c** unfumigated and **b**, **d** fumigated soils for the acetate, ferrihydrite + acetate, and goethite + acetate treatments. The iron oxide effect was calculated as the difference in cumulative CH_4 emissions (from acetate or non-acetate sources) between treatments with and without added iron oxides, viz., $CH_{4\ (Iron\ oxide\ +\ Acetate)} - CH_{4\ (Acetate)}$ and $(CH_{4\ (Iron\ oxide\ +\ Acetate)} - CH_{4\ (Acetate)})/\ CH_{4\ (Acetate)}$. Values represent the means ± standard errors, $n = 3$. Note that a break was added between days 16–20 and the scale before day 16 was enlarged

both with and without iron oxides, was much lower than that in the unfumigated soil. The relative PE (as a percentage relative to the control) was much higher in the fumigated soil than in the unfumigated soil (Fig. 2). Similar to the unfumigated soil with acetate, ferrihydrite reduced less CH_4 emissions from non-acetate sources than goethite after day 8. Ferrihydrite and goethite showed no differences in their effects on acetate-CH_4 emissions in the fumigated soil (Fig. 4).

Discussion

Effects of iron oxides on methanogenesis

The addition of iron oxides reduced the acetate-CH_4 emissions in the unfumigated soil ($p < 0.05$) (Fig. 4); this result is consistent with our first hypothesis and similar to the findings of other studies (Chidthaisong and Conrad 2000; Hori et al. 2010; Kato et al. 2012). Iron reduction and methanogenesis competed for acetate as electron donors (Achtnich et al. 1995; Chidthaisong and Conrad 2000). Dissimilatory iron-reducing bacteria can prevail over methanogens in the presence of iron oxides and become the predominant acetate-consuming microorganisms (Hori et al. 2010). Acetate addition without iron oxides resulted in positive PE regarding CH_4 production because the acetate activated the methanogens to use SOC and other microorganisms to produce compounds for methanogenesis. The addition of iron oxides decreased this positive PE, as suggested in the first hypothesis, because of the competition between iron reduction and methanogenesis.

The methanogenesis originated from SOC and microbial metabolic products in the unfumigated soil without acetate addition. It included three distinct pathways: acetoclastic, methylotrophic, and hydrogenotrophic pathways using

acetate, methanol/methylamines, and $H_2/CO_2/CO$/formate as substrates, respectively (Fenchel et al. 2012). Acetate can contribute to approximately two-thirds of the CH_4 production in soil (Chin and Conrad 1995), whereas other substrates such as formate and H_2/CO_2 contribute 10–30% (Palmer and Reeve 1993; Conrad 1999). Thus, the preceding discussion regarding acetate-sourced methanogenesis can also largely explain the observed reductions in CH_4 emissions from soil without acetate following the addition of iron oxides (Fig. 1). Besides acetate, competition between iron reduction and methanogenesis for other substrates and electron donors also contributed to this reduction (Chidthaisong and Conrad 2000; Zhang et al. 2012; Yamada et al. 2014).

Ferrihydrite addition reduced more CH_4 emissions from the unfumigated soil without acetate addition than goethite, i.e., it caused a lower PE (Fig. 2) because ferrihydrite can be reduced more rapidly and completely than the more crystalline goethite (Bose et al. 2009; Shimizu et al. 2013; Adhikari et al. 2017). However, ferrihydrite and goethite showed no difference in reducing CH_4 emissions from acetate in soil, and this is unexpected according to the second hypothesis. Ferrihydrite had a stronger reduction effect on non-acetate-CH_4 emissions than goethite when acetate-CH_4 emission was rapid and a weaker effect when acetate-CH_4 emission became slow (Fig. 4). The molar ratio of the added Fe/acetate was 1:3.4, which was much lower than the ratio required for iron reduction (1:0.125, $8FeOOH + CH_3COOH + 16H^+ \rightarrow 8Fe^{2+} + 2CO_2 + 14H_2O$). Abundant acetate supported iron reduction. Ferrihydrite reduction was more rapid than goethite reduction, resulting in stronger negative effect on non-acetate-CH_4 emissions in the early stage. The excessive acetate, however, was adsorbed onto the surface of the poorly crystalline ferrihydrite, and this reduced its available reactive sites (Kalbitz et al. 2005; Dippold et al. 2014). This resulted in the lower-than-expected effects of ferrihydrite on acetate-CH_4 and non-acetate-CH_4 emissions (Fig. 5). This interpretation was supported by the higher incorporation of acetate into SOC in soil with ferrihydrite than in soil with goethite (Table S1).

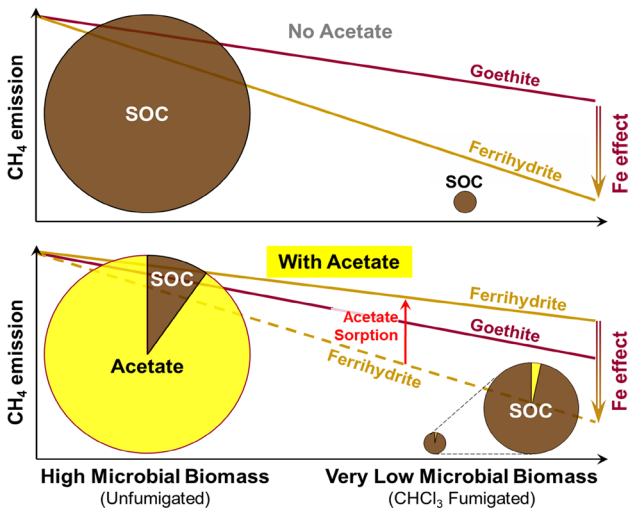

Fig. 5 Concept of C sources of CH_4 emissions in an anaerobic paddy soil depending on microbial biomass and the addition of iron oxides. The pie chart of CH_4 emission from soil with low microbial biomass was enlarged to show the source partition. Note that the arrow of iron effect represents reduction and when the arrow is pointing downward, it represents stronger reduction. The dash ginger line represents the initial effect of ferrihydrite. After acetate sorption, ferrihydrite effect is represented as the solid ginger line. SOC represents non-acetate C sources including soil organic C and organics released from dead cells killed by chloroform fumigation here

Effect of microbial biomass and iron oxides on methanogenesis

Fumigation reduced MBC by 43–87% (Table S2) and reduced cumulative CH_4 emissions by 352−1127 times (Fig. 1). This indicated that methanogens were mostly killed by fumigation, and, thus, methanogenesis likely ceased. We conducted the same incubation, but without any added substances, for 78 days in a previous study (unpublished). Soils were preincubated under flooding conditions for 10 days and then treated with or without chloroform fumigation, similar to this study. The Eh of the unfumigated soil was lower than that of the fumigated soil (Fig. S1). The Eh was − 32.5 mV on day 4 of the incubation of the unfumigated soil and then decreased to − 201.5 mV on day 16 and was even lower thereafter (Fig. S1). However, the Eh in the fumigated soil was almost 300 mV at the beginning of the incubation and then decreased to − 113 mV at the end of the incubation (Fig. S1). Generally, CH_4 production occurs at Eh values < − 100 mV or − 150 mV in soil (Masscheleyn et al. 1993; Wang et al. 1993; Tyagi et al. 2010). Thus, the redox potential mainly explained the minor CH_4 production in the fumigated soil. The Eh represents the ratio of the activities of oxidants and reductants and can reflect the reduction intensity (DeLaune and Reddy 2004; Reddy and DeLaune 2008). Biologically, the reduction intensity is the intensity of electron dispersion by microorganisms when they oxidize an energy source (Reddy and DeLaune 2008). In the fumigated soil, compared to the unfumigated soil, functional microbes capable of reducing oxidants such as nitrate, ferric, and sulfate had very low microbial biomass and activity. This resulted in a low intensity of electron dispersion, a high ratio of oxidants to reductants, and, consequently, a higher Eh in the fumigated soil than in the unfumigated soil. The CH_4 emission rate decreased over time in the unfumigated soil, whereas it increased in the fumigated soil. This increase in the emission in the fumigated soil was likely associated with the slow and delayed decrease in Eh. Notably, $CHCl_3$ inhibits all bacteria using the acetyl-CoA pathway for acetate

consumption, including methanogens (Scholten et al. 2000). $CHCl_3$ also reduces the activity of methyl-coenzyme M reductase, which is an essential enzyme for methanogenesis (Rospert et al. 1991). Although we removed $CHCl_3$ after 24 h of fumigation by repeated evacuation, such damage may not have been reversible.

CH_4 produced from acetate in the unfumigated soil accounted for the major proportion of total daily CH_4 emissions (56−91%) in the first 10 days when acetate was rapidly consumed. As fumigation reduced more CH_4 produced from acetate than from non-acetate sources, acetate contributed to only a minor proportion of CH_4 emissions from the fumigated soil during the incubation period (Fig. 3). In fact, the non-acetate C sources in the unfumigated soil were SOC and microbial metabolic products, whereas non-acetate C also included C released from dead cells killed by chloroform fumigation in the fumigated soil. SOC and new C from dead cells can produce CH_4 through several pathways (including acetoclastic, methylotrophic, and hydrogenotrophic pathways), whereas acetate can only be used by the acetoclastic methanogenesis. Therefore, the results indicated that acetoclastic methanogenesis, which dominated CH_4 production in the unfumigated soil, was more damaged by chloroform fumigation than the other pathways. Alternatively, it was more impacted by the increased Eh caused by chloroform fumigation. In addition, when microbial activity was low, adsorption between acetate and iron oxides may have prevailed in soil, thus limiting acetate accessibility to methanogens. Furthermore, $-CH_3$ and -COOH groups in acetate are the sources of CH_4 and CO_2 production, respectively. The ratio of CH_4 to CO_2 produced through acetoclastic methanogenesis is 1:1. Approximately 29–34% of acetate was consumed for CH_4 production (Fig. 1), and 36–39% was for CO_2 production in the unfumigated soil (Li et al. 2020b). These values were 0.02–0.04% of CH_4 production and 18–26% of CO_2 production in the fumigated soil (Fig. 1; Li et al. 2020b). Most of acetate in the unfumigated soil was utilized by methanogens, whereas in the fumigated soil, other microorganisms were more competitive for acetate than methanogens. Chloroform fumigation increased the DOC content by 114%, mainly due to organic C from dead cells, which accounted for 53% of the DOC content of the fumigated soil. As the organic C released from dead cells was more labile than SOC, it was the major source for non-acetate-CH_4 emissions in the fumigated soil. Thus, we speculate that dead cells contributed to 53–100% of the non-acetate-CH_4 emissions from the fumigated soil. In summary, fumigation altered C source consumption and pathways for methanogenesis (Fig. 5).

As the absolute amounts of CH_4 emissions were much lower in the fumigated soil than in the unfumigated soil, the reductions in the CH_4 emissions resulting from the addition of iron oxides were low. However, the relative effects of iron oxides were higher in the fumigated soil than in the unfumigated soil, as proposed by the third hypothesis (Fig. 5). This was because of the uneven impact of $CHCl_3$ fumigation on iron reduction and methanogenesis. $CHCl_3$ inhibits acetate consumption by methanogenesis but has little effect on acetate consumption by iron reduction (Chidthaisong and Conrad 2000). Firmicutes are one of the dominant groups of iron reducers in paddy soils (Li et al. 2011; Zhuang et al. 2015a,b) and are dominant in the bacterial community after $CHCl_3$ fumigation (Dominguez-Mendoza et al 2014; Chen et al. 2016). In addition, iron reduction occurs when $Eh < 100$ mV (Thamdrup 2000), whereas methanogenesis occurs when $Eh < -100$ mV, and the reduction of CO_2 to CH_4 requires an $Eh < -200$ mV (Tyagi et al. 2010; Ali et al. 2019). Thus, under the high Eh of the fumigated soil, iron reduction could occur more successfully than methanogenesis (Fig. S1). Therefore, the addition of iron oxides had a stronger effect in the fumigated soil than in the unfumigated soil. The presence of iron oxides increases the soil redox potential (van Bodegom et al. 2004). Unfortunately, in this study, we did not determine whether the addition of iron oxides affected the Eh, which thereafter could have affected CH_4 production. The relative iron effect was stronger on acetate-CH_4 than on non-acetate-CH_4 emissions. As acetate is a preferable C source for iron reducers and has a high affinity to iron oxides, acetate-derived methanogenesis was more reduced by iron oxides than other C substrates, such as SOC and dead cells.

Conclusions

To the best of our knowledge, this is the first study to use $CHCl_3$ fumigation to analyze the effects of microbial biomass reduction and iron oxides on the intensity and sources of CH_4 production from paddy soil. The reduction of CH_4 production after $CHCl_3$ fumigation compared to that in the unfumigated soil (> 370 times) was much higher than the reduction of the microbial biomass (< 8 times). Acetate was the main source of CH_4 production from the unfumigated soil in the first 10 days. Its contribution, however, became lower in the fumigated soil because (i) large amounts of other available C was released from dead microbial cells during $CHCl_3$ fumigation and (ii) acetoclastic methanogenesis was damaged by $CHCl_3$ fumigation, likely stronger than other pathways. Ferrihydrite and goethite both reduced CH_4 production and priming effect, mainly because of the competition for substrates between iron reduction and methanogenesis. The larger surface area of ferrihydrite than that of goethite markedly decreased CH_4 production. However, the effects of ferrihydrite and goethite on CH_4 production from ^{13}C-labeled acetate were similar. Ferrihydrite reduced the non-acetate-CH_4 emissions more than goethite in the first 6 days; however, this effect was weaker thereafter. The change in the effect of ferrihydrite over

time was mainly due to the decrease in the number of reactive sites of ferrihydrite by acetate sorption, and therefore, this favored methanogenesis. The relative effects of iron oxides were stronger in the soil with reduced microbial biomass than in the unfumigated soil, indicating that the lower abundance of methanogens in the fumigated soil decreased the competition of methanogens for substrates with iron reducers. Summarizing, the abundance of methanogens was reduced more than the total microbial biomass by $CHCl_3$ fumigation, leading to the marked decrease in methanogenesis, changes in its sources, and an increase in the effects of iron oxide on CH_4 production.

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Declarations

Conflict of interest The authors declare no competing interests.

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