

Iron oxidation affects nitrous oxide emissions via donating electrons to denitrification in paddy soils



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ABSTRACT

Paddy soils are important source of nitrous oxide (N₂O), which production is mainly regulated through redox processes and electron transfer. Ferrous iron [Fe(II)] oxidation coupled to denitrification is ubiquitous in paddy soils, which could affect N₂O production via donating electrons to denitrification. To clarify the effects of Fe(II) oxidation on denitrification and N₂O emissions, a flooding experiment was conducted in two paddy soils with contrasting Fe(II) levels. The soil with high Fe(II) concentration emitted less N₂O than did the other soil with low Fe(II) concentration. Nitrate addition decreased Fe(II) concentration and stimulated N₂O production in both soils, suggesting that Fe(II) oxidation is coupled to denitrification. The stoichiometry of electron transfer between nitrate reduction and Fe(II) oxidation demonstrated that the percentage of electrons contributed by Fe(II) to denitrification accounted for 16.2% and 32.9%, and the ratios of the electrons donated by Fe(II) to the electrons accepted by nitrate for N₂O production were 43.7% and 130.7% in the two soils with low and high Fe(II) concentration, respectively. The ratio beyond 100% implies that the electrons donated by high Fe(II) concentration exceeded the electron demand for N₂O production, which lead to the further reduction of N₂O to N₂. In conclusion, Fe(II) oxidation coupled to denitrification affects N₂O emissions via electron donation, and Fe(II) in a high concentration bears great potential for efficient denitrification and low N₂O emissions from paddy soils.

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1. Introduction

Atmospheric nitrous oxide (N₂O) contributes to global warming and stratospheric ozone depletion (Wuebbles, 2009). As a long-lived trace gas in the atmosphere (Butterbach-Bahl et al., 2013), N₂O is the third most important greenhouse gas next to carbon dioxide (CO₂) and methane (CH₄), and it is also recognized as the dominant ozone-depleting substance emitted in the 21st century (Ravishankara et al., 2009). The threat of N₂O emission to the environment motivates the managements of N₂O production and emission (Kanter et al., 2013).

Agricultural soils are major N₂O sources because of intensive nitrogen (N) fertilization (Hoben et al., 2011; Shcherbak et al., 2014). Irrigation management is also an important factor in N₂O emissions from agricultural soils (Liu et al., 2011), especially the flooding and drainage regime in paddy soils with specific redoximorphic features (Kögel-Knabner et al., 2010). The total annual N₂O emissions from paddy soils in China were determined to be about 29.0 Gg N₂O-N (Zou et al.,

2007). Hence, the studies of N₂O emissions from paddy soils are particularly important.

N₂O is a byproduct of nitrification and an obligate intermediate of denitrification processes (Canfield et al., 2010), but N₂O emissions from paddy soils mainly derive from denitrification (Liu et al., 2012). In flooded paddy soils, redox processes strongly affect denitrification and electron transfer. Organic carbon frequently serves as the dominant electron donor to denitrification under anoxic conditions (Burford and Bremner, 1975; Cayuela et al., 2014; Melton et al., 2014). In addition, ferrous iron [Fe(II)] also donates electrons to denitrification through oxidation reaction. Previous research demonstrated that Fe(II) oxidation coupled to denitrification was energetically viable at neutral pH (Klueglein and Kappler, 2013; Melton et al., 2014; Weber et al., 2006b), which suggested that Fe(II) could affect N₂O emissions.

Iron (Fe) is the fourth most abundant element in the Earth's crust, and the redox transition between ferrous and ferric valences plays a fundamental role in modern environmental biogeochemistry (Melton et al., 2014; Weber et al., 2006a). With the periodic drying–wetting alternation in paddy soils, Fe is subjected to various oxidation state changes and interacts with the dynamics of N through redox reactions (Kögel-Knabner et al., 2010), such as nitrate-dependent Fe(II) oxidation (NDFO) (Ratering and Schnell, 2001; Weber et al., 2001). Mixotrophic

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nitrate-dependent Fe(II) oxidation is restricted to anoxic conditions and regulates the oxidation of Fe(II) to Fe(III) in the upper few millimeters of sediments (Melton et al., 2012). Most nitrate-reducing Fe(II)-oxidizing bacteria can reduce NO_3^- to NO_2^- and further denitrification intermediates and products, including the gaseous species NO, N_2O and N_2 (Chakraborty and Picardal, 2013; Melton et al., 2014; Muehe et al., 2009). As NO_2^- is an intermediate of denitrification and also a strong chemical oxidant for Fe(II), Fe(II) oxidation by the reactive NO_2^- intermediate can be a significant indirect abiotic process which is involved in NDFO (Melton et al., 2014). Thus, both biotic and abiotic NDFO processes play an important role in global Fe cycles, and also regulate denitrification in soils (Canfield et al., 2006; Melton et al., 2014; Weber et al., 2006a). As an intermediate of denitrification and a potential chemical oxidant of Fe(II), N_2O can be further reduced to N_2 by Fe(II) in the NDFO processes. This implies that, in paddy soils, N_2O emissions from denitrification are related to Fe(II). However, the effect of Fe(II) on N_2O emissions from soils has not been completely uncovered. The elucidation of the relevance of Fe(II) with denitrification and N_2O emissions is beneficial to take measures to mitigate the threat of N_2O emission to global climate change.

In this study, we hypothesized that Fe(II) regulated N_2O emissions by donating electrons to denitrification in paddy soils and a high Fe(II) concentration could decrease N_2O emissions through the further reduction of N_2O to N_2 . In order to assess the effects of Fe(II) on denitrification and N_2O emissions, we focused on the interactions between nitrate and Fe(II) in two paddy soils with contrasting Fe(II) concentrations under flooding conditions by a nitrate addition experiment.

2. Materials and methods

2.1. Soil

Two paddy soils were taken from the top layer (0–20 cm) at Xianning, Hubei Province, China (30°1.1' N, 114°22.1' E, called S1) and Yueyang, Hunan Province, China (28°42.9' N, 112°55.6' E, called S2). Both soils develop from the quaternary red earth. Soil samples were air-dried and then ground to pass through a 20-mesh sieve for the incubation experiment. The geochemical properties of the soils are presented in Table 1.

2.2. Experimental design and treatments

In this study, the variations of Fe(II) concentration, nitrate content, and N_2O emission were detected after nitrate addition in the flooded soils. Fe(II) oxidation and nitrate reduction were described by the decrease of Fe(II) concentration and nitrate content, respectively. The electron transfer between Fe(II) oxidation and nitrate reduction was calculated to clarify the contribution of electrons donated by Fe(II) to denitrification and N_2O production. The experiment was conducted as follows.

Air-dried soil samples (S1 and S2) were activated at 20% soil moisture content at 25 °C for 3 days. Subsequently, aliquots (18.75 g) of the activated soils corresponding to 15 g dry soil were respectively loaded into 132 bottles with the capacity of 145 mL each. All soil samples in

the bottles were preincubated under submerged conditions (dry soil: water = 1:1.25 (w/v)) at 25 °C for 7 days to create a stable soil environment.

Two treatments were established: (i) nitrate treatment performed by adding KNO_3 solution to the preincubated soils with the N fertilizer rate of 100 mg N kg^{-1} dry soil (corresponding to 225 kg N ha^{-1} year $^{-1}$); (ii) control treatment performed by adding deionized water to the soils. The final ratio of soil to water was 1:1.5 (w/v). Each treatment was conducted with three replications. The soils then were incubated in the dark at 25 °C for 40 days. Soil and water were stratified without disturbance during the incubation, and so O_2 diffusion was limited. The soil layer was about 8 mm and the water layer was 10 mm.

The headspace gases in the bottles were sampled every day for the analysis of N_2O concentration. N_2O fluxes were determined as the changes of N_2O concentration in the headspace gases within 1 h. Soil pH and redox potential (Eh) were detected in situ without stirring the soil layer and water layer. After the gas sampling and soil pH and Eh measurements, the flooded soils were mixed homogeneously to obtain slurries for the other property analyses. The destructive sampling was carried out to determine dissolved organic carbon (DOC), ammonium, nitrate, nitrite, Fe(II), and Fe(III) on day 0, 1, 3, 6, 10, 14, 18, 22, 27, 32, and 40, respectively. Each time the destructive sampling consumed 12 bottles (3 for the nitrate treatments and 3 for the controls in the two soils).

2.3. Analytical methods

N_2O concentrations of the gas samples were analyzed by GC (Agilent 7890A, Agilent Technologies Inc., Santa Clara, CA, USA) with an electron capture detector (ECD). Soil Eh was measured with an oxidation-reduction potentiometer (Nanjing Jaoyuan Analytical Instrument Company Limited, China) by using a platinum composite electrode. The pH was determined with a pH meter (Sartorius, Basic pH Meter PB-10, Germany). DOC was extracted with water from soil slurry samples and analyzed by a TOC analyzer (vario TOC cube, Elementar, Germany). Soil total N was determined by an elemental analyzer (vario MAX CN, Elementar, Germany), and soil organic N was calculated as the difference between total N and inorganic N. Inorganic N was extracted with 1 M KCl from soil slurry, ammonium was analyzed by the phenol hypochlorite method (Scheiner, 1976), and nitrite was analyzed by the modified Griess-Ilosvay method (Moorcroft et al., 2001).

Nitrate was analyzed by the ultraviolet spectrophotometry method (Cawse, 1967). To remove the interference of dissolved organic matter (DOM) and Fe(III) on soil nitrate analysis, 5 ml aliquots of soil extracts were mixed with 20 ml of alumina suspension and spinned in a centrifuge. Alumina suspension was prepared as the following procedure: (i) 30 g of potassium aluminum sulfate was dissolved in 1 L of deionized water, and then the suspension was filtered; (ii) the filtrate was added to a mixture of 225 ml of deionized water and 25 ml of ammonia solution to promote aluminum hydroxide free precipitating from sulfate; and (iii) finally the suspension of the precipitate was diluted to 1 L for nitrate analysis (Cawse, 1967). The final pH of alumina suspension was about 6.8, and alumina suspension was well agitated during use. In addition, 100 μL of 1 M Na_2HPO_4 solution was added to 5 mL of soil

Table 1
The geochemical properties of the two paddy soils.

| Sample sites | pH (H_2O) | Organic C (g C kg^{-1}) | Total N (g N kg^{-1}) | NH_4^+ (mg N kg^{-1}) | NO_3^- (mg N kg^{-1}) | Total Fe (g kg^{-1}) | 0.5 M HCl-extracted Fe (g kg^{-1}) | Texture | | |
|------------------|--------------------------------|--------------------------------------|------------------------------------|---|---|-----------------------------------|---|----------------|----------------|---------------|
| | | | | | | | | Slit (%) | Clay (%) | Sand (%) |
| Xianning (S1) | 5.3 (0.00) | 10.5 (0.05) | 1.3 (0.02) | 46.5 (0.76) | 15.9 (0.83) | 22.5 (0.25) | 1.8 (0.07) | 69.6 (0.74) | 28.1 (1.08) | 2.3 (0.42) |
| Yueyang (S2) | 5.2 (0.00) | 19.2 (0.09) | 2.3 (0.11) | 82.0 (0.63) | 8.7 (0.55) | 24.6 (0.81) | 3.1 (0.03) | 60.1 (1.92) | 34.5 (1.28) | 5.4 (0.82) |

Standard errors (n = 3 replicate samples) are shown in parentheses.

extract and the resulting Fe precipitate is removed by membrane filtration to tackle the interference of Fe(II) on nitrate analysis (Yang et al., 2012).

Fe(II) and Fe(III) were extracted with 0.5 M HCl from soil slurry, and both the sampling and extraction were conducted in the anaerobic glovebox. The extracted Fe(II) was analyzed by the ferrozine method (Viollier et al., 2000). The total extracted Fe was determined by the same procedure with the exception that hydroxylamine hydrochloride was added to the soil extracts to transform Fe(III) to Fe(II). The amount of Fe(III) was calculated as the difference between total extracted Fe and Fe(II).

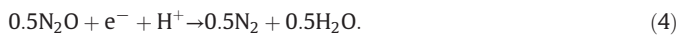
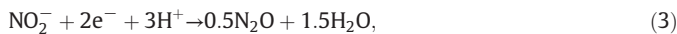
2.4. Calculations and statistical analyses

The soil N₂O fluxes were calculated as follows:

$$F = \rho \times V/m \times \Delta c/\Delta t \times 273/T \times 28/44.$$

Where F ($\mu\text{g N kg}^{-1} \text{ h}^{-1}$) is the soil N₂O flux, ρ is the density of N₂O (1.98 kg m^{-3}) under standard conditions (273 K, $1.013 \times 10^6 \text{ Pa}$), V (m^3) is the headspace volume of the bottle, m (kg) is the mass of dry soil in the bottle, Δc ($\text{m}^3 \text{ m}^{-3}$) is the change of N₂O concentration in the bottle during the period Δt (h), T (K) is the absolute temperature, and 28/44 is the conversion factor from N₂O to N. The cumulative N₂O emission is equal to the sum of all the products of the flux in each sampling multiplied by the time interval between when each sample was taken.

Many soil processes (such as mineralization, immobilization, nitrification, and denitrification) are involved in the dynamics of N and Fe. However, in the flooded soils with nitrate addition, denitrification is the dominant N process outcompeting the other soil processes. Therefore, we focused on the effect of denitrification on the concentration of nitrate and Fe(II) to explore the NDFO process, and assumed that the influence of other soil processes could be neglected here. In light of the instability and negligible amount of nitric oxide (NO) from denitrification, we merely considered N₂O and N₂ as the N losses caused by denitrification. Based on the stoichiometry of electron transfer between nitrate reduction and Fe(II) oxidation, the following equations were derived:



On the basis of the reaction equations above and the analytical method of Pyzola (2013), the electron contribution of Fe(II) oxidation to denitrification with N₂O and N₂ production (P_1), and the ratio of the electrons donated by Fe(II) to the electrons accepted by nitrate for N₂O production (P_2) were calculated as follows:

$$P_1 = \frac{\Delta\text{Fe}^{2+} \times 1}{\Delta\text{NO}_3^- \times [4 \times (\text{N}_2\text{O})\% + 5 \times (\text{N}_2)\%]}$$

$$P_2 = \frac{\Delta\text{Fe}^{2+} \times 1}{\Delta\text{NO}_3^- \times 4 \times (\text{N}_2\text{O})\%}$$

Where P_1 is the proportion of the electrons donated by Fe(II) to denitrification and the total accepted electrons in the course of denitrification, P_2 is the proportion of the electrons donated by Fe(II) and the electrons accepted by nitrate for N₂O production, ΔFe^{2+} is the rate of Fe(II) oxidation determined by the slope of the linear least squares fit of the decrease of Fe(II) concentration (Pyzola, 2013), 1 represents the number of electrons transferred in the process of oxidation from

1 mol Fe(II) to 1 mol Fe(III), ΔNO_3^- is the rate of nitrate reduction described by the decrease rate of nitrate content (Achnich et al., 1995) and is determined as the slope of the linear least squares fit of nitrate consumption (Pyzola, 2013), 4 represents the number of electrons transferred in the process of reduction from 1 mol NO₃⁻ to 0.5 mol N₂O, (N₂O)% is the ratio of N₂O/(N₂ + N₂O) in denitrification, 5 represents the number of electrons transferred in the process of the reduction from 1 mol NO₃⁻ to 0.5 mol N₂, and (N₂)% is the ratio of N₂/(N₂ + N₂O) in denitrification.

Statistical analyses were conducted by using SAS 8.1 (SAS Institute, Cary, NC, USA). Flooded soils subjected to the control and nitrate treatments were used as experimental units to test treatment effects. These effects were tested by using the mixed model at $\alpha = 0.05$. Pearson correlation analysis was conducted to evaluate the dependence of N₂O emission on soil properties such as ammonium, nitrate, nitrite, and Fe(II) concentrations.

3. Results

3.1. Soil N₂O emissions

N₂O fluxes from the two paddy soils without nitrate addition were almost zero, but nitrate addition boosted N₂O emissions from both soils (Fig. 1). N₂O fluxes rapidly peaked on the first two days after nitrate addition, and fluctuated at a high level in S1 until day 22, while sharply decreased to zero in S2 on day 6 (Fig. 1a). Maximal N₂O flux (Fig. 1a) in S1 with nitrate addition treatment was smaller than that in S2, but the cumulative N₂O emissions (Fig. 1b) in S1 were higher than that in S2. The total N₂O emissions of the 40 days' incubation in S1 and S2 were 42.7 ± 2.8 and $28.3 \pm 1.2 \text{ mg N kg}^{-1}$ dry soil, respectively. N₂O fluxes were positively correlated with nitrate and nitrite content in all soil samples (Table 2), which demonstrates that denitrification is the dominant process responsible for N₂O production.

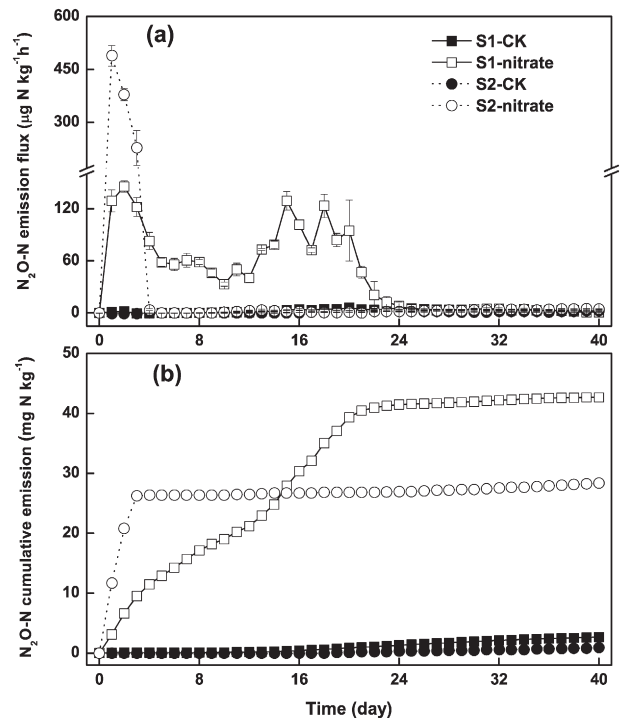


Fig. 1. N₂O emission rate (a) and cumulative emission (b) from two soils with and without nitrate addition [the controls: S1-CK and S2-CK; nitrate amendments: S1-nitrate and S2-nitrate]. The values present the mean of three replicates, and the error bars represent standard errors.

Table 2
Correlation matrix of the chemical properties in all soil samples.

| | N ₂ O | NO ₃ ⁻ N | NO ₂ ⁻ N | NH ₄ ⁺ -N | Fe(II) | Fe(III) | pH | Eh | DOC |
|---------------------------------|------------------|--------------------------------|--------------------------------|---------------------------------|----------|----------|----------|----------|-------|
| N ₂ O | 1.000 | | | | | | | | |
| NO ₃ ⁻ N | 0.701** | 1.000 | | | | | | | |
| NO ₂ ⁻ N | 0.884** | 0.407** | 1.000 | | | | | | |
| NH ₄ ⁺ -N | 0.093 | -0.105 | 0.291 | 1.000 | | | | | |
| Fe(II) | -0.116 | -0.286 | 0.075 | 0.842** | 1.000 | | | | |
| Fe(III) | 0.236 | 0.357* | 0.026 | -0.741** | -0.924** | 1.000 | | | |
| pH | 0.034 | -0.203 | 0.245 | 0.959** | 0.860** | -0.791** | 1.000 | | |
| Eh | 0.022 | 0.151 | -0.165 | -0.941** | -0.911** | 0.828** | -0.920** | 1.000 | |
| DOC | -0.280 | -0.331* | -0.127 | 0.546** | 0.763** | -0.701** | 0.634** | -0.641** | 1.000 |

Sample size: n = 160.

* p ≤ 0.05.

** p ≤ 0.01.

3.2. Soil pH, Eh and DOC

Nitrate addition had no significant effect on soil pH. The pH value of S1 was lower than that of S2 (Fig. 2a). Meanwhile, N₂O emissions from S1 exceeded those from S2, which implies that low pH contributes to N₂O emission from soils. The pH of S1 decreased to a minimum value of 4.5 from day 0 to day 22. However, there was little change in S2 during the first 4 weeks, and then the pH of S2 decreased to 5.5 from day 27 to day 40. The pH was positively correlated with Fe(II) and ammonium content, while it negatively correlated with Fe(III) concentration (Table 2).

Nitrate addition slightly increased Eh (Fig. 2b) in both soils in the first 18 days, and then the effect of nitrate addition on Eh gradually weakened. No significant difference in Eh was found between the control and nitrate treatment in the whole period of incubation. Eh was positive in S1, but negative in S2 (Fig. 2b). During the incubation, average Eh values of S1 were respectively 147 ± 24 mV and 180 ± 22 mV in the control and nitrate treatments, but these were much lower in S2 (-133 ± 10 mV in the control, -111 ± 10 mV in the nitrate treatment). Eh was positively correlated with Fe(III) concentration, while negatively correlated with Fe(II) concentration, ammonium content, and pH (Table 2).

There was no significant difference in DOC content between the control and nitrate treatments in both soils. However, DOC content in S1 was lower than that in S2 during the incubation (Fig. 3c). In addition, DOC content was positively correlated with pH and Fe(II) concentration, but negatively correlated with Eh and Fe(III) concentration (Table 2).

3.3. Soil inorganic nitrogen

Nitrate and nitrite contents in both soils with nitrate addition were higher than those without nitrate addition (Fig. 3a,b). After nitrate addition, nitrate content in S1 slowly decreased to the same nitrate level in the control treatment from day 0 to day 26, while in S2 it rapidly decreased from day 0 to day 6. The periods of nitrate decrease in both soils were almost consistent with the periods of soil N₂O emissions in the nitrate treatment. This indicates that nitrate decrease is accompanied by N₂O emissions from soils. Nitrite content (Fig. 3b) in both soils was negligible as compared with nitrate and ammonium content. Ammonium content in S2 was more than 2 folds higher than that in S1 (Table 1, Fig. 3c). After a slight increase in the first 6 days, ammonium content in both soils decreased (Fig. 3c).

Considering the simultaneity of N₂O emissions and nitrate decrease in the nitrate treatment, the specific analysis phases of nitrate reduction along with N₂O production in S1 and S2 were designated as from day 0 to day 22 and from day 0 to day 6, respectively. Linear fitting functions for nitrate decrease of the specific phases in S1 and S2 were

$y = -0.35x + 7.6$ ($R^2 = 0.907$) and $y = -1.17x + 6.6$ ($R^2 = 0.729$) (Fig. 3a), respectively. The slopes of the two lines representing the rates of nitrate consumption demonstrated that nitrate reduction in S1 was slower than that in S2.

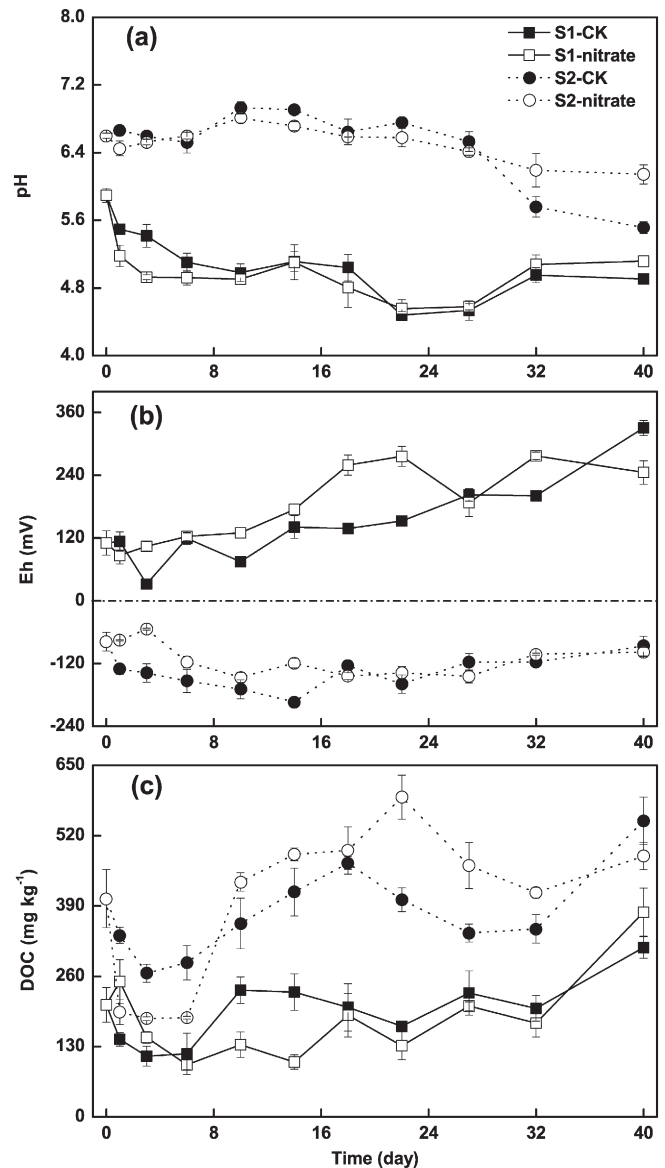


Fig. 2. Soil pH (a), Eh (b) and DOC (c) in two soils with and without nitrate addition [the controls: S1-CK and S2-CK; nitrate amendments: S1-nitrate and S2-nitrate]. The values present the mean of three replicates, and the error bars represent standard errors.

3.4. Dynamics of Fe redox speciation

Although the total Fe content in the two soils had no significant difference, HCl-extracted Fe in S2 was higher than that in S1 (Table 1). Moreover, Fe(II) concentration (Fig. 4a) in S2 was approximately 2 folds higher than that in S1 during the incubation, while Fe(III) concentration (Fig. 4b) in S2 was about half of that in S1. In addition, nitrate addition to the flooded soils led to the decrease of Fe(II) concentration (Fig. 4a) and the increase of Fe(III) concentration (Fig. 4b), which implies that nitrate reduction is coupled with Fe(II) oxidation. Nevertheless, the increasing amount of Fe(III) was not equal to the decreasing amount of Fe(II), which is possibly attributed to the formation of poorly soluble Fe(III) products.

Fe(II) concentration in S1 with nitrate addition decreased from day 0 to day 22, and reached the minimum value on day 22, but in S2 it decreased from day 0 to day 6 and reached the minimum on day 6. The periods of Fe(II) concentration decrease in both soils were in agreement with the periods of nitrate decrease and N₂O emissions, which reveals that Fe(II) oxidation is simultaneous with nitrate reduction accompanied by N₂O production.

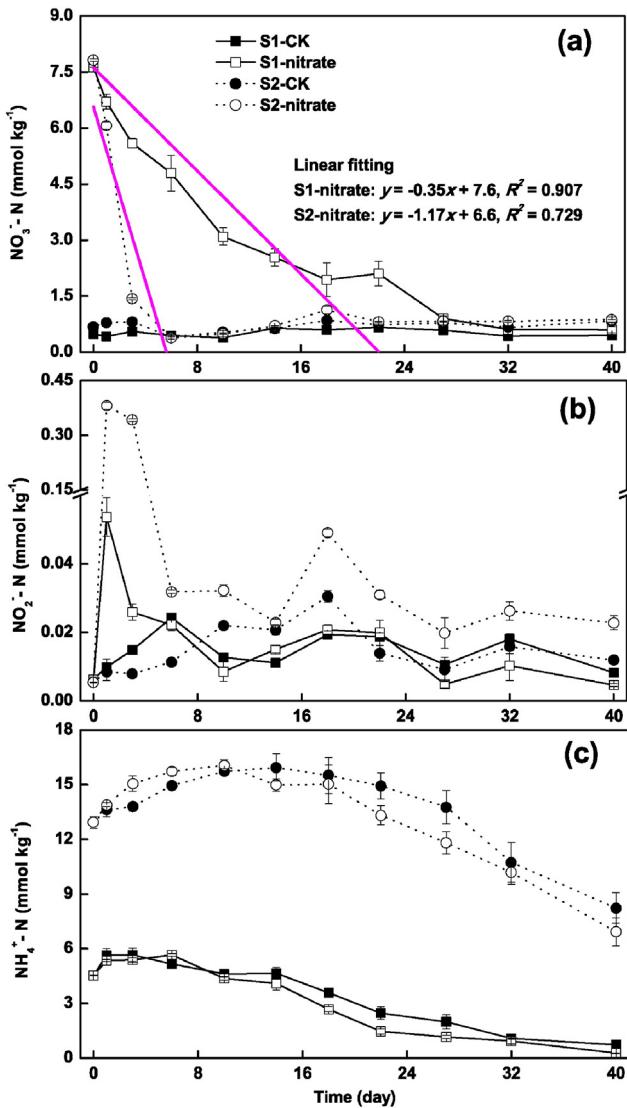


Fig. 3. Nitrate (a), nitrite (b) and ammonium (c) contents in two soils with and without nitrate addition [the controls: S1-CK and S2-CK; nitrate amendments: S1-nitrate and S2-nitrate]. Nitrate linear fittings (a) of the specific phases (from day 0 to day 22 in S1 and from day 0 to day 6 in S2) in the nitrate treatment are displayed in the figure. The values present the mean of three replicates, and the error bars represent standard errors.

Considering the simultaneity of nitrate reduction and Fe(II) decrease after nitrate addition in the soils, we also chose the specific phases of Fe(II) oxidation with nitrate consumption accompanied by N₂O production in S1 and S2 from day 0 to day 22 and from day 0 to day 6, respectively. Fe(II) linear fitting functions of the specific phases in S1 and S2 were $y = -0.26x + 11.2$ ($R^2 = 0.858$) and $y = -1.81x + 35.4$ ($R^2 = 0.940$), respectively (Fig. 4a). The slopes of the two lines representing Fe(II) decrease rates indicated that Fe(II) oxidation in S1 was slower than that in S2.

3.5. Nitrate and Fe(II) reaction stoichiometry

In view of the simultaneity of nitrate reduction, Fe(II) oxidation and N₂O emissions in the specific phases, we calculated the N budgets in S1 from day 0 to day 22 and in S2 from day 0 to day 6, respectively. The changes of N content from the beginning to the end of the specific phases are displayed in Fig. 5. Compared with other forms of N, nitrite content was negligible. N losses in S1 and S2 with nitrate addition were respectively 93.8 mg N kg⁻¹ and 89.2 mg N kg⁻¹ higher than that in the control treatments. Because denitrification is the main process in flooding conditions, we assumed that soil N was lost through N₂O and N₂ emissions. N₂O emissions in S1 and S2 with nitrate addition were respectively 39.9 mg N kg⁻¹ and 26.4 mg N kg⁻¹ higher than that in the control treatments. Therefore, the ratios of N₂O emissions to soil N loss in S1 and S2 were respectively 42.5% and 29.6% (calculated by 39.9/93.8 and 26.4/89.2).

According to the slopes of the linear fitting of nitrate and Fe(II) decrease in Figs. 3 and 4, the rates of nitrate and Fe(II) consumption were as follows:

$$\Delta\text{NO}_3^-_{\text{S1}} = 0.35 \text{ mmol kg}^{-1} \text{ day}^{-1}, \Delta\text{NO}_3^-_{\text{S2}} = 1.17 \text{ mmol kg}^{-1} \text{ day}^{-1},$$

$$\Delta\text{Fe}^{2+}_{\text{S1}} = 0.26 \text{ mmol kg}^{-1} \text{ day}^{-1}, \Delta\text{Fe}^{2+}_{\text{S2}} = 1.81 \text{ mmol kg}^{-1} \text{ day}^{-1}.$$

The rate of nitrate reduction in S1 was lower than those in the research of Achtnich et al. (1995) and Pyzola (2013), but in S2 it was higher than the results of these two studies. Their results were 0.84 mmol kg⁻¹ day⁻¹ and 0.027 μmol g⁻¹ h⁻¹ (equal to 0.648 mmol kg⁻¹ day⁻¹).

In light of the minor concentration and instability of nitrite in the acid soils, the reaction stoichiometry of nitrite with Fe(II) was negligible in the calculation of the contribution of Fe(II) oxidation to denitrification. The results of the calculation were as follows:

S1-nitrate treatment:

$$P_{1-S1} = \frac{\Delta\text{Fe}^{2+} \times 1}{\Delta\text{NO}_3^- \times [4 \times (\text{N}_2\text{O})\% + 5 \times (\text{N}_2)\%]}$$

$$= \frac{0.26 \times 1}{0.35 \times [4 \times 42.5\% + 5 \times (1-42.5\%)]} = 16.2\%$$

S2-nitrate treatment:

$$P_{1-S2} = \frac{\Delta\text{Fe}^{2+} \times 1}{\Delta\text{NO}_3^- \times [4 \times (\text{N}_2\text{O})\% + 5 \times (\text{N}_2)\%]}$$

$$= \frac{1.81 \times 1}{1.17 \times [4 \times 29.6\% + 5 \times (1-29.6\%)]} = 32.9\%.$$

The proportion of the electrons donated by Fe(II) and the electrons accepted by nitrate for N₂O production was as follows:

S1-nitrate treatment:

$$P_{2-S1} = \frac{\Delta\text{Fe}^{2+} \times 1}{\Delta\text{NO}_3^- \times 4 \times (\text{N}_2\text{O})\%} = \frac{0.26 \times 1}{0.35 \times 4 \times 42.5\%} = 43.7\%$$

S2-nitrate treatment:

$$P_{2-S2} = \frac{\Delta Fe^{2+} \times 1}{\Delta NO_3^- \times 4 \times (N_2O)\%} = \frac{1.81 \times 1}{1.17 \times 4 \times 29.6\%} = 130.7\%.$$

According to these calculations, the contributions of Fe(II) oxidation to the whole denitrification processes in S1 and S2 were 16.2% and 32.9%, respectively. The proportions of the electrons donated by Fe(II) and the electrons used for N₂O production in denitrification in S1 and S2 were 43.7% and 130.7%, respectively. The ratio of the electrons donated by Fe(II) oxidation and the electrons used for N₂O production in S2 was above 100%, which implies that more electrons are donated by Fe(II) than are used for N₂O production.

Because the direct measurement of N₂ produced from denitrification was not possible in this study, N₂ production was calculated as the difference between N loss and N₂O emission from the soils. This estimation may cause some uncertainties in the calculation of the contribution of electrons donated by Fe(II) to N₂O production and whole denitrification. The N losses from soils include N₂O and N₂ but also many other gaseous species NO, NO₂, N₂O₃, N₂O₄, etc. This leads to the overestimation of N₂ production from denitrification and thus the underestimation of the contribution of electrons donated by Fe(II) to the whole denitrification (P1). In addition, N₂O emission is the result of N₂O production and consumption. In these calculations, the description of N₂O production as N₂O emission results in the overestimation of the contribution of electrons donated by Fe(II) to N₂O production (P2).

4. Discussion

4.1. Soil mineral N and Eh

The decrease of nitrate content (Fig. 3a) is attributed to denitrification during the incubation under the submerged condition. In addition, the consumption of ammonium (Fig. 3c) implies that nitrification also occurred. This is consistent with previous research concluding that nitrification and denitrification are ongoing simultaneously in flooded paddy soils (Reddy and Patrick, 1986). Although both nitrification and denitrification produce N₂O, the positive correlation between nitrate and N₂O flux and the non-significant correlation between ammonium and N₂O flux (Table 2) reveals that denitrification is the dominant process for N₂O production in the studied soils. After day 6 of the incubation, the sharp decrease of ammonium and little change of nitrate content (Fig. 3) illustrate that nitrate production from nitrification is nearly equal to the nitrate consumption from denitrification. In addition, the negligible nitrite content (Fig. 3b) is due to the instability of nitrite in acid soils as an intermediate product of N turnover (Shen et al., 2003).

The increase of the soil Eh (Fig. 2b) was caused by nitrate addition, which is ascribed to the high standard potential of NO₃⁻ to change to its reduced forms (Wang et al., 1992). However, the effect of nitrate addition on Eh gradually weakened (Fig. 2b) because of the nitrate consumption in denitrification. Because microbial reduction processes sequentially use NO₃⁻, Mn⁴⁺, Fe³⁺, and SO₄²⁻ as electron acceptors in flooded paddy soils (Kögel-Knabner et al., 2010), nitrate prevents Fe(III) reduction and increases Eh value. In addition, a previous study demonstrated that soil Eh was regulated by the exchange of electrons in redox couples such as Fe(II)/Fe(III) during the reduction and oxidation process (Fiedler et al., 2007). Based on the positive correlation between Eh and Fe(II) and the negative correlation between Eh and Fe(II)/Fe(III), further analysis of the relationship between Eh and Fe(II)/Fe(III) indicated that $Eh = -116 \ln[Fe(II)/Fe(III)] + 102$ ($R^2 = 0.80$, $p < 0.01$). This signifies the effect of Fe(II)/Fe(III) ratio on the Eh value and also explains the reason for the positive Eh value in S1 (ratio [Fe(II)/Fe(III)] < 1). The denitrification activity highly depends on Eh, and low Eh promotes the fast denitrification rates (Seo and DeLaune, 2010). The lower Eh with the high rate of nitrate reduction in S2 than that in S1 (Fig. 3) reveals the indirect effect of Fe on denitrification through the regulation of Fe(II)/Fe(III) ratio on Eh.

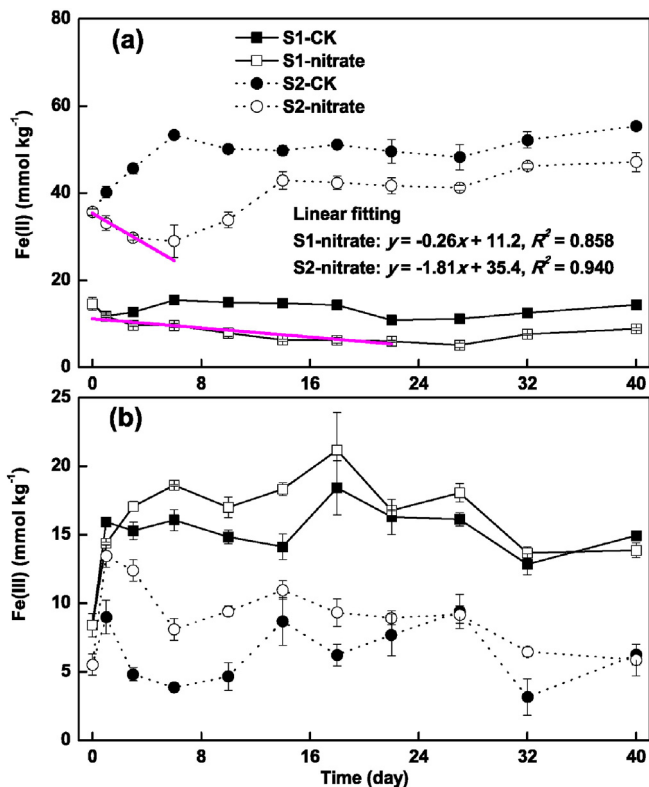


Fig. 4. The concentration of Fe(II) (a) and Fe(III) (b) during the incubation in two soils with and without nitrate addition [the controls: S1-CK and S2-CK; nitrate amendments: S1-nitrate and S2-nitrate]. Fe(II) linear fittings (a) of the specific phases (from day 0 to day 22 in S1 and from day 0 to day 6 in S2) in nitrate treatment are displayed in the figure. The values present the mean of three replicates, and the error bars represent standard errors.

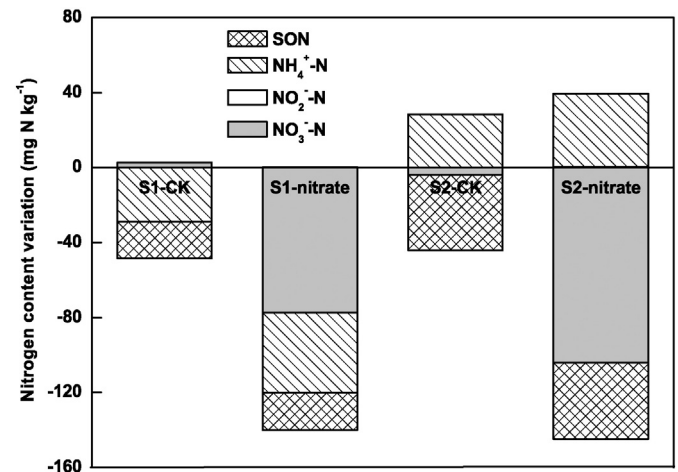


Fig. 5. The changes of N species in the specific phases (from day 0 to day 22 in S1 and from day 0 to day 6 in S2) in two soils with and without nitrate addition [the controls: S1-CK and S2-CK; nitrate amendments: S1-nitrate and S2-nitrate]. Nitrite values are negligible and invisible in the graph. N content variations were calculated as the end N contents minus the beginning values of the specific phases. The columns below the horizontal axis indicate N decrease, and those above the horizontal axis indicate N increase.

4.2. Soil N₂O emissions

The fact that N₂O emissions in the nitrate treatment were higher than that in the control treatment (Fig. 1) results from denitrification with increased nitrate loading under flooding conditions (Beaulieu et al., 2011). The sharp increase of N₂O emissions (Fig. 1) indicates that denitrification occurs immediately after nitrate is added (Hibiya et al., 2003). Positive correlations between N₂O flux and nitrate as well as nitrite (Table 2) demonstrate that denitrification is the main source of N₂O. With the same amount of nitrate addition to both soils, N₂O emissions from S2 were lower than those from S1. This implies that denitrification performs more efficiently in S2 than in S1, as N₂O and N₂ are the main N losses from denitrification in flooded soils. In addition, the shorter N₂O emission process (Fig. 1) in S2 also indicates the more efficient denitrification in S2 than in S1.

The efficiency of denitrification is related to soil reducing strength and capacity, which is contributed by electron donors such as organic carbon and Fe(II) (Burford and Bremner, 1975; Cayuela et al., 2014). Higher DOC and Fe(II) concentration (Fig. 2) in S2 relative to those in S1 promote the further reduction of N₂O to N₂ and the acceleration of denitrification. That is why denitrification proceeded more completely in S2 than in S1. In addition to organic carbon as the dominant electron donor in denitrification (Burford and Bremner, 1975; Cayuela et al., 2014), Fe(II) is also used as an electron donor in denitrification (Melton et al., 2014). The contributions of Fe(II) in donating electrons to denitrification in S1 and S2 were respectively 16.2% and 32.9%, which reveals that the important role of Fe(II) in denitrification cannot be neglected.

4.3. Nitrate-dependent Fe(II) oxidation

Lower Fe(II) concentration in the nitrate treatment than that in the control treatment (Fig. 4) indicates that nitrate acting as an oxidant drives the oxidation of Fe(II) and decreases Fe(II) concentration. This finding is consistent with previous research that demonstrated nitrate-driven iron-redox cycling was used to address the important biochemical processes in subsurface environments (Roden, 2012). All these findings point to the conclusion that NDFO [Fe(II) oxidation coupled to denitrification] exists in paddy soils (Ratering and Schnell, 2001; Weber et al., 2006a). However, the increase of Fe(III) concentration with nitrate addition was not equivalent to the decrease of Fe(II) concentration (Fig. 4). The unequal change amount of Fe(II) and Fe(III) is attributed to the fact that NDFO microbes oxidized both soluble and insoluble Fe(II) to produce a variety of poorly soluble iron mineral products in flooded soils (Chaudhuri et al., 2001; Weber et al., 2006a; Weber et al., 2001). In general, iron in both aqueous and solid phases is likely to be involved in iron redox cycling (Roden, 2012).

The electron contributions of Fe(II) to denitrification in the two soils (16.2% and 32.7%) indicate that Fe(II) oxidation affect denitrification. Fe(II) can be oxidized by a variety of denitrifying bacteria with the production of N₂O and N₂ (Roden, 2012). In addition, most denitrifying bacteria require an organic co-substrate, such as acetate, to oxidize Fe(II) to Fe(III), which is termed mixotrophic NDFO (Muehe et al., 2009). This reveals that the higher DOC (Fig. 2) promotes the larger electron contribution of Fe(II) to denitrification in S2 than in S1.

In addition, the chemical reaction between nitrite and Fe(II) generates nitric oxide (NO) and N₂O in a process called chemodenitrification (Brons et al., 1991; Kampschreur et al., 2011). The rate of reaction between Fe(II) and nitrite is fast at acidic pH and increases in more acidic conditions (Picardal, 2012). This suggests that the lower pH facilitates the faster rate of reaction between Fe(II) and nitrite and higher N₂O production in S1 than in S2. Nevertheless, the minor concentration of nitrite (Fig. 3b) implies that nitrate plays a dominant role in the production of N₂O and N₂ accompanied by Fe(II) oxidation (Weber et al., 2001). As both abiotic and biotic reactions between nitrate and Fe(II) are involved in the NDFO processes (Carlson et al., 2013; Klueglein et al., 2014;

Picardal, 2012; Weber et al., 2006a), both chemical and biological NDFO processes contribute to the production of N₂O. The linear regressions over the specific periods (22 and 6 days) in the two soils illustrated the faster rate and the shorter time of the reaction between nitrate and Fe(II) in S2 than in S1. This suggests that the dominant process in S2 is abiotic NDFO while in S1 is biotic NDFO. However, the proportion of the contribution of abiotic and biotic NDFO processes to N₂O production in each soil is beyond the results of this research. This topic should be explored in future studies.

In the NDFO processes, direct electron donation from Fe(II) to nitrate respiration is a possible mechanism, if the appropriate electron acceptors are available to nitrate-reducing bacteria (Carlson et al., 2013). N₂O as an available electron acceptor can react with Fe(II) in the NDFO processes. The ratio of the electrons donated by Fe(II) and the electrons accepted by nitrate for N₂O production in S2 was 130.7%, which indicates that electrons donated by Fe(II) exceed the demand of the electrons used for N₂O production. N₂O could accept the superfluous electrons donated by Fe(II) and was further reduced to N₂ (Eq. (4) in the section of Materials and methods). Therefore, the comparison of the electrons donated by Fe(II) and the electrons accepted by nitrate for N₂O production implies that a high concentration of Fe(II) in flooded soils facilitates the thorough performance of denitrification to produce N₂ and decrease N₂O emission. As a forgotten driver of N₂O production (Zhu et al., 2013), Fe plays an important role in the regulation of N₂O emission in denitrification of paddy soils.

5. Conclusions

N₂O emission from flooded paddy soils mainly derives from denitrification. Fe(II) oxidation coupled to denitrification (the NDFO process) exists in paddy soils. A high Fe(II) concentration in soil promotes more efficient denitrification and contributes larger percentage of electrons to denitrification than does a low Fe(II) concentration in soil. The percentage of electrons contributed by Fe(II) to denitrification can reach up to as high as 30% in the studied soils. Fe(II) acting as an electron donor plays an important role in denitrification, and Fe(II) in a high level can decrease N₂O emissions from flooded soils. These conclusions imply that NDFO is a significant contributor to N₂O emissions from paddy soils, and Fe(II) could be a potential regulator of N₂O emissions from denitrification, especially in Fe(II)-rich systems.

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