Nitrogen Loss through Anaerobic Ammonium Oxidation Coupled to Iron Reduction from Paddy Soils in a Chronosequence

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Supporting Information

ABSTRACT: Anaerobic ammonium oxidation coupled to iron(III) reduction (termed Feammox) with dinitrogen, nitrite, or nitrate as the end-product is a recently discovered process of nitrogen cycling. However, Feammox has not been described in paddy soils, which are rich in iron(III) oxides and subjected to intensive nitrogen fertilization. Here, evidence for Feammox in a paddy soil chronosequence with a gradient of microbially reducible iron(III) levels was obtained in Southern China using ¹⁵N-labeled ammonium-based isotopic tracing and acetylene inhibition techniques. Our study demonstrated the occurrence of Feammox in the chronosequence, and direct dinitrogen production was shown to be the dominant Feammox pathway. Within the chronosequence, three paddy soils with higher microbially reducible iron(III) levels had higher Feammox rates (ranged from 0.17 to 0.59 mg N kg⁻¹ d⁻¹) compared to an uncultivated soil (0.04 mg N kg⁻¹ d⁻¹). It is estimated that a loss of 7.8–61 kg N ha⁻¹ year⁻¹ is associated with Feammox in the examined paddy soils. Overall, we discover that rice cultivation could enrich microbially reducible iron(III), accelerate Feammox reaction and thus fuel nitrogen loss from soils, and suggest that Feammox could be a potentially important pathway for nitrogen loss in paddy soils.

INTRODUCTION

Rice is a global staple food which is grown on approximately 155 million hectare (ha) of the Earth's surface and feeds more than half of the world's population.¹ Nitrogen (N) fertilizers (usually as urea and ammonia) have been applied intensively in paddy soils to obtain high yields over the last decades, especially in China, which is one of the major rice producers in the world and has become the largest producer and consumer of N fertilizers so far, accounting for one-third of world fertilizer production in 2010.² The amounts of N fertilizers applied to paddy soils are likely to further increase to feed growing populations. However, the excessive use of N fertilizers has not only decreased N-utilization efficiency by crops, but also increased N loss to the environment, leading to pollution in atmosphere and water systems.^{3,4} Therefore, concerns regarding N pollution of the environment have been raised for decades, and numerous studies have been focused on pathways of N cycling in paddy soils.^{1,4–7}

Traditionally, three distinct microbial processes, denitrification, codenitrification, and anaerobic ammonium (NH₄⁺) oxidation (anammox), are usually involved in the N loss from terrestrial ecosystems via the generation of nitrous oxide (N₂O)

or dinitrogen gas (N₂).^{8–10} In addition to these processes, it has recently been discovered that anaerobic NH₄⁺ oxidation could be coupled to ferric iron (Fe(III)) reduction (termed Feammox) with either N₂ (eq 1),¹¹ nitrite (NO₂⁻) (eq 2),^{11–14} or nitrate (NO₃⁻) (eq 3)¹¹ as the end-product in several environments. For example, anaerobic oxidation of NH₄⁺ to NO₂⁻ has been detected under iron-reducing conditions in wetland soils.^{12,14} Moreover, Yang et al.¹¹ found the evidence of N₂, NO₂⁻, or NO₃⁻ production via Feammox in tropical forest soils. These new findings implicate alternative pathways of N loss from soils. However, few studies have focused on soil indigenous microbially reducible iron(III) utilization as the sole electron acceptor during the Feammox process, and to date there has been no report on the occurrence of Feammox in paddy soils. Whether Feammox could occur and make a contribution to total N loss in paddy soils is unknown at the moment. Considering that paddy soils are one of the most

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important N sinks in terrestrial ecosystems,¹⁵ Fe(III) is by far the most abundant oxidant in paddy soils, and the periodic alternation between oxic and anoxic conditions in paddy soils can promote Fe redox reactions, we hypothesize that paddy soils have the potential to support Feammox.

The primary objective of this study was thus to quantitatively determine if Feammox occurs in paddy soils using a chronosequence in Southern China. The relative contribution of the Feammox pathways was also identified by combining ¹⁵N-labeled NH₄⁺ (¹⁵NH₄⁺)-based isotopic tracing and acetylene (C₂H₂) inhibition techniques.

MATERIALS AND METHODS

Site Description and Soil Sampling. Paddy terraces in the hilly regions of Southern China, with increasing age of cultivation from the top to the bottom of the slopes, provide soil chronosequences. A paddy soil chronosequence developed from Quaternary red clays (RC) was selected for our study. The chronosequence is located in Jinxian, central Jiangxi Province of China (between 28°10′–28°45′ N, 116°1′–116°34′ E), which represents a typical hilly region for rice cultivation in subtropical Southern China. Within the chronosequence, four representative soils were selected for sampling. Three cultivated soils at the top, middle, and bottom of the slope with increasing age of rice cultivation were named as RC11 (100 years), RC12 (100–300 years), and RC13 (300 years) soils, respectively. An uncultivated soil (time zero) at the highest slope position were named as RC10 soil. The sampling positions and image of the chronosequence are shown in Supporting Information (SI) Figure S1. Details of the sampling site and chronosequence have been described in a previous study.¹⁶ Surface soil samples (0–10 cm depth) were collected after rice harvest when fields had been drained for about 7 up to 10 days. Each soil sample was partitioned into two subsamples: one was air-dried, passed through a 2.0 mm sieve for soil chemical properties analyses, and the second was used for isotope tracer incubations. The analytical methods of soil chemical properties are given in the SI.

Isotope Tracer Incubations. The entire incubation experiment was performed in an ultrahigh purity helium (He)-filled anaerobic glovebox (Shel Lab Bactron IV, U.S.A.) equipped with a solution of resazurin as the redox indicator. In our study, the resazurin solution remained colorless throughout the experiment indicating the anoxic conditions in the glovebox. Prior to incubation, soil slurries were prepared by adding sterile anoxic deionized water to the soils (oven-dry equivalent) at a ratio of 3:1 (v/w), and preincubated anoxically in the dark at 25 °C for 2 days to remove indigenous oxygen (O₂) and NO_x[−] (i.e., NO₂[−] and NO₃[−]) (see SI). After the preincubation, indigenous microbially reducible Fe(III) in all soil slurries remained almost unreduced (data not shown). Aliquots (6 g) of the homogenized slurries were transferred into 60 mL serum vials, which were then sealed with butyl rubber septa and crimped with aluminum caps. The headspace

of soil slurries was flushed with ultrahigh purity He. Three treatments (*n* = 6 per treatment) were established: (i) control treatment with sterile anoxic deionized water instead of ¹⁵NH₄Cl; (ii) ¹⁵NH₄Cl addition (¹⁵N at 99%, Cambridge Isotope Laboratories, Andover, U.S.A.; ¹⁵NH₄⁺); and (iii) ¹⁵NH₄Cl and C₂H₂ addition (¹⁵NH₄⁺ + C₂H₂). The final concentration of ¹⁵NH₄Cl was 90 mg N kg^{−1} soil dry weight, by injecting 0.5 mL of ultrahigh purity He-purged stock solution. The amount of ¹⁵NH₄⁺ amended was chosen based on the actual amount of chemical N fertilizer usually applied to paddy soils in China (about 200 kg N ha^{−1} year^{−1}).^{4,17} For the C₂H₂ treatment, 23 mL headspace gas in each vial was removed and replaced with 23 mL C₂H₂ to reach 30% (v/v) C₂H₂ in the headspace. All vials were shaken vigorously to homogenize the treatment solutions and dissolve the C₂H₂.

The destructive sampling was carried out at 1 day intervals up to 6 days from each vial. Before sampling, each vial was shaken vigorously to equilibrate the gas between dissolved and gaseous phases. For analysis of ¹⁵N-N₂ and ¹⁵N-N₂O, 12 mL gas samples were immediately collected by gastight syringes and then injected into 12 mL pre-evacuated glass vials (Exetainer, Labco, U.K.). To prevent atmospheric contamination, these sampling processes were conducted in the He-filled anaerobic glovebox. The ¹⁵N enrichment in N₂ was determined by isotope ratio mass spectrometry (IRMS, Thermo Finnigan Delta V Advantage, Bremen, Germany) coupled with GasBench II, and the ¹⁵N enrichment in N₂O was determined by IRMS coupled with a preconcentration unit.¹⁸ Headspace N₂ and N₂O concentrations were directly measured using a robotized sampling and analyzing system,¹⁹ which was composed of an autosampler with a peristaltic pump, a thermostatic water bath and an Agilent 7890 gas chromatography (Santa Clara, CA, U.S.A.). ¹⁵N₂ concentration was calculated as the product of N₂ concentration and ¹⁵N-N₂ atom % excess above its natural abundance.²⁰ ¹⁵N₂O concentration was also calculated as the product of N₂O concentration and ¹⁵N-N₂O atom% excess above its natural abundance.²¹ ¹⁵N₂ and ¹⁵N₂O production rates were calculated from the linear change in ¹⁵N₂ and ¹⁵N₂O concentrations in the vial headspace between two given time points. Potential Feammox rates were conservatively estimated from the difference in vial headspace ³⁰N₂ production alone with and without ¹⁵NH₄⁺ addition.

After the gas sampling, the slurries were subsampled for analysis of HCl-extractable Fe(II) and total extractable Fe using the procedure slightly modified from Lovley et al.²² Briefly, 1.0 g of soil slurry sample was extracted with 5 mL 0.5 M HCl for 2 h at room temperature, and extracted Fe(II) was determined using the ferrozine method. Total extractable Fe in the slurry sample was analyzed by the same procedure with the exception that the extractant was 5 mL 0.25 M hydroxylamine hydrochloride in 0.25 M HCl. The amount of microbially reducible Fe(III) (considered as hydroxylamine-reducible Fe(III)) was calculated as the difference between total extractable Fe and Fe(II).²² All extractions were conducted in the anaerobic glovebox. Fe(III) reduction rates were calculated from the linear change in Fe(II) concentrations between two given time points.

Statistical Analyses. SPSS (version 16.0; SPSS Inc., Chicago, IL, U.S.A.) software was used to perform standard statistical tests, including analysis of variance (ANOVA) and Pearson correlation analysis. We performed two-tailed student's *t* tests to compare ¹⁵N₂ or ¹⁵N₂O production rates, and Fe(II)

Table 1. Chemical Characteristics of the Paddy Soil Chronosequence^a

soil	pH	microbially reducible Fe(III) (g kg ⁻¹)	total Fe (g kg ⁻¹)	TOC ^b (g kg ⁻¹)	TN ^b (g kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO _x ⁻ -N ^b (mg kg ⁻¹)	DOC ^b (mg kg ⁻¹)
RC10	4.7 ± 0.01 D ^c	0.54 ± 0.03 D	65 ± 2.2 A	8.7 ± 0.38 D	0.80 ± 0.03 D	7.0 ± 0.21 D	1.2 ± 0.06 D	72 ± 3.3 D
RC11	4.9 ± 0.01 C	1.6 ± 0.06 C	26 ± 1.2 D	19 ± 0.41 B	1.9 ± 0.06 B	15 ± 0.40 B	2.1 ± 0.07 C	165 ± 6.9 B
RC12	5.3 ± 0.01 B	4.5 ± 0.16 A	47 ± 1.5 B	16 ± 0.33 C	1.4 ± 0.04 C	10 ± 0.31 C	2.9 ± 0.09 A	126 ± 5.7 C
RC13	5.7 ± 0.02 A	3.2 ± 0.11 B	35 ± 1.3 C	24 ± 0.73 A	2.2 ± 0.08 A	21 ± 0.65 A	2.4 ± 0.09 B	218 ± 9.4 A

^aThe paddy soil chronosequence contains an uncultivated soil (RC10, time zero) and three cultivated paddy soils at the top (RC11, 100 years), middle (RC12, 100–300 years), and bottom (RC13, 300 years) of the slope with increasing cultivation age. ^bTOC: Total organic carbon; TN: Total nitrogen; NO_x⁻: NO₂⁻ and NO₃⁻; DOC: Dissolved organic carbon. ^cMean ± standard error (*n* = 6). The different capital letters within the same column indicate statistically significant (*P* < 0.05) differences among different soils.

Figure 1. Mean headspace ¹⁵N₂ and ¹⁵N₂O production rates for the examined paddy soil chronosequence. (a–d), mean headspace ³⁰N₂ (a), ²⁹N₂ (b), ⁴⁶N₂O (c), and ⁴⁵N₂O (d) production rates in the control, ¹⁵NH₄⁺ and ¹⁵NH₄⁺ + C₂H₂ treatments. The different capital letters above the horizontal line denote statistically significant (*P* < 0.05) differences among different soils regardless of the treatments, using two-way analysis of variance. Error bars represent standard errors (*n* = 6). nd = not detectable within a detection limit of 0.008 mg ¹⁵N kg⁻¹ d⁻¹.

production between treatments with ¹⁵NH₄⁺ addition (¹⁵NH₄⁺ and ¹⁵NH₄⁺ + C₂H₂) and control to determine whether significant NH₄⁺ oxidation and promotion of Fe(II) production occurred in the presence of ¹⁵NH₄⁺. We also compared ¹⁵N₂ or ¹⁵N₂O production rates between ¹⁵NH₄⁺ and ¹⁵NH₄⁺ + C₂H₂ treatments in each soil using two-tailed student's *t* tests. Moreover, we compared ¹⁵N₂ or ¹⁵N₂O production rates among different soils regardless of the treatments, using two-way analysis of variance. Mean values are shown in the text followed by standard errors (±SE). Statistical significance was denoted at *P* < 0.05 unless otherwise noted.

RESULTS

Chemical Characteristics of the Paddy Soil Chronosequence. For the examined chronosequence, which was composed of an uncultivated soil (RC10 soil) and three cultivated paddy soils at the top (RC11 soil), middle (RC12 soil), and bottom (RC13 soil) of a slope with increasing cultivation age, the measurements of microbially reducible Fe(III), total Fe, and other chemical properties including pH,

total organic carbon (TOC), total nitrogen (TN), NH₄⁺, NO_x⁻ (i.e., NO₂⁻ and NO₃⁻), and dissolved organic carbon (DOC) are shown in Table 1. A gradient of microbially reducible Fe(III) levels was an important characteristic of the chronosequence, varying considerably from the lowest (0.54 ± 0.03 g kg⁻¹) in the RC10 soil to the highest (4.5 ± 0.16 g kg⁻¹) in the RC12 soil. In contrast, the concentration of total Fe in the uncultivated soil (i.e., RC10 soil) was much higher than in all paddy soils (i.e., RC11, RC12, and RC13 soils). These indicated that long-term rice cultivation could enrich amorphous Fe(III) oxides while decreasing total iron. Furthermore, the pH value and concentrations of TOC, TN, NH₄⁺, NO_x⁻, and DOC in all paddy soils were significantly (*P* < 0.05) higher than that in the uncultivated soil, indicating that long-term rice cultivation could modify the soil pH to neutral values and elevate soil nutrient levels.

¹⁵N₂ and ¹⁵N₂O Production from Anoxic Incubations. For all soils, significant amount of ³⁰N₂ (¹⁵N¹⁵N) was detected in treatments with ¹⁵NH₄⁺, and ¹⁵NH₄⁺ and C₂H₂ (¹⁵NH₄⁺ + C₂H₂), but was not detectable in the control (without ¹⁵NH₄⁺)

after 6-day anoxic incubation (Figure 1a). This demonstrated the occurrence of Feammox in both uncultivated and paddy soils, as N_2 directly from Feammox or Feammox-produced NO_2^- or NO_3^- followed by anammox or denitrification are the sole potential sources of $^{30}N_2$ under anoxic conditions (Table 2). In the $^{15}NH_4^+$ treatment, the mean $^{30}N_2$ production rates in

Table 2. Possible Processes for $^{30}N_2$ and $^{29}N_2$ Generation from $^{15}NH_4^+$ under Anaerobic Conditions (after Yang et al.,¹¹ Modified)^a

product	nitrogen atom source 1	nitrogen atom source 2	process
$^{30}N_2$	amended $^{15}NH_4^+$	amended $^{15}NH_4^+$	Feammox to N_2
	amended $^{15}NH_4^+$	Feammox-produced $^{15}NO_2^-$	Anammox
	Feammox-produced $^{15}NO_2^-/^{15}NO_3^-$	Feammox-produced $^{15}NO_2^-/^{15}NO_3^-$	denitrification
$^{29}N_2$	amended $^{15}NH_4^+$	indigenous $^{14}NH_4^+$	Feammox to N_2
	amended $^{15}NH_4^+$	indigenous $^{14}NO_2^-$	Anammox
	Feammox-produced $^{15}NO_2^-$	indigenous $^{14}NH_4^+$	Anammox
	Feammox-produced $^{15}NO_2^-$	indigenous amino compounds	codenitrification
	Feammox-produced $^{15}NO_2^-/^{15}NO_3^-$	indigenous $^{14}NO_2^-/^{14}NO_3^-$	denitrification

^aIndigenous $^{14}NO_2^-/^{14}NO_3^-$ or Feammox-produced $^{15}NO_2^-/^{15}NO_3^-$ could be converted into $^{14}NH_4^+$ or $^{15}NH_4^+$ through dissimilatory NO_3^- reduction to NH_4^+ , respectively. Therefore, no additional processes for $^{30}N_2$ and $^{29}N_2$ generation from $^{15}NH_4^+$ were established.

^bFeammox can produce $^{15}NO_2^-$ or $^{15}NO_3^-$ from amended $^{15}NH_4^+$.

all paddy soils (i.e., RC11, RC12 and RC13 soils) varied from 0.17 ± 0.01 to 0.59 ± 0.03 mg N kg⁻¹ d⁻¹, which were significantly ($P < 0.05$) higher than that in the uncultivated soil (0.04 ± 0.01 mg N kg⁻¹ d⁻¹ for the RC10 soil) (Figure 1a). Furthermore, the presence of C_2H_2 caused a decrease (by 0.01 – 0.14 mg N kg⁻¹ d⁻¹, about 22–33%; $P < 0.05$ for all soils except RC10 soil) in the $^{30}N_2$ production rates in all soils. Compared to the $^{30}N_2$ formation, greater $^{29}N_2$ ($^{14}N^{15}N$) formation was observed in all soils following the addition of $^{15}NH_4^+$ and $^{15}NH_4^+ + C_2H_2$ (Figure 1b). Within the chronosequence, the $^{29}N_2$ production rates showed similar trends to those observed in the $^{30}N_2$ production rates in the $^{15}NH_4^+$ or $^{15}NH_4^+ + C_2H_2$ treatments.

In addition to $^{15}N_2$ (i.e., $^{30}N_2$ and $^{29}N_2$), headspace $^{15}N_2O$ (i.e., $^{46}N_2O$ and $^{45}N_2O$) significantly ($P < 0.05$) accumulated only in the presence of C_2H_2 in all soils, and $^{46}N_2O$ accumulated to a lesser extent compared to the $^{45}N_2O$ (Figure 1c,d). Moreover, for all soils, the extent of headspace $^{46}N_2O$ accumulation (by 0.01 – 0.10 mg N kg⁻¹ d⁻¹) was slightly lower than the extent of decrease in $^{30}N_2$ production (by 0.01 – 0.14 mg N kg⁻¹ d⁻¹) after C_2H_2 addition, probably resulting from the partial dissolution of $^{46}N_2O$ produced in the liquid phase. Within the chronosequence, the lowest headspace $^{46}N_2O$ and $^{45}N_2O$ production rates were observed in the uncultivated RC10 soil, while the highest were in the RC12 soil.

Iron Reduction during Anoxic Incubations. For all soils, the amount of ferrous iron (Fe(II)) was significantly increased in all treatments after the 6-day incubation, and the extent of increase in the $^{15}NH_4^+$ and $^{15}NH_4^+ + C_2H_2$ treatments was significantly ($P < 0.05$) greater than in the control (Figure 2). Total extractable Fe in all treatments was fairly constant throughout the incubation (Figure 2). These results indicated

Figure 2. Kinetics of ferrous iron and total extractable iron during the anoxic incubation of slurries within the chronosequence. Error bars represent standard errors ($n = 6$).

that part of the soil indigenous microbially reducible Fe(III) was reduced to Fe(II), and $^{15}NH_4^+$ addition promoted Fe(II) production. Moreover, in the $^{15}NH_4^+$ and $^{15}NH_4^+ + C_2H_2$ treatments, the extent of increase in Fe(II) concentration in the RC10 soil was 130 ± 4.1 – 139 ± 5.7 mg kg⁻¹ (increased by approximately 44%) after the incubations, which were considerably ($P < 0.05$) lower than in the other soils (1130 ± 22 – 3615 ± 54 mg kg⁻¹, by about 151–215%).

Correlation Analysis of Iron Reduction and $^{15}N_2$ Production Rates. Linear regression analysis was used to study the relationship between Fe(III) reduction and $^{15}N_2$ (i.e., $^{30}N_2$ and $^{29}N_2$) production rates. For the chronosequence, Fe(III) reduction rate showed a significant and positive correlation with the $^{30}N_2$ ($R^2 = 0.878$) and $^{29}N_2$ ($R^2 = 0.933$) production rate, respectively, further indicating the occurrence of Feammox in both uncultivated and paddy soils (Figure 3).

Correlation Analysis of Rate Measurements and Soil Chemical Characteristics. Of all the soil chemical properties examined, the $^{30}N_2$ production rates exhibited a strong correlation only with soil microbially reducible Fe(III) levels (Pearson correlation coefficient 0.967, $P < 0.01$; SI Table S1). Likewise, the $^{29}N_2$ production rates also showed a significant and positive correlation (Pearson correlation coefficient 0.987, $P < 0.01$) with the levels of microbially reducible Fe(III). Both $^{46}N_2O$ and $^{45}N_2O$ production rates were positively correlated with NO_x^- ($P < 0.05$) and microbially reducible Fe(III) ($P < 0.01$) concentrations.

Figure 3. Relationships between iron reduction rate and $^{30}\text{N}_2$ (a), $^{29}\text{N}_2$ (b) production rate. Linear regression was used to test the correlation between the iron reduction and $^{15}\text{N}_2$ production rates. Adjusted R^2 values with the associated P -values are shown.

DISCUSSION

Evidence for the Occurrence of Feammox in the Paddy Soil Chronosequence. Feammox is likely to occur in acidic paddy soils derived from quaternary red clay, which are rich in Fe(III) oxides and experience periodic anoxia. We thus estimated the potential for Feammox in such a paddy soil chronosequence with a gradient of microbially reducible Fe(III) levels through anoxic slurry incubations with $^{15}\text{NH}_4^+$ tracer. Significant $^{30}\text{N}_2$ production in the presence of $^{15}\text{NH}_4^+$ in all soils provides direct evidence for the occurrence of Feammox in the examined chronosequence (Figure 1a and Table 2). All soil slurries were preincubated anoxically to remove indigenous O_2 and NO_x^- . The residual NO_x^- concentrations in all soil slurries after the preincubation were 0.72–1.6 mg N kg^{-1} . This presence of NO_x^- may be resulted from indigenous NO_3^- and/or NO_2^- generation through Feammox. Strict anaerobic procedures were used throughout the experiment, and we believe that aerobic nitrification was negligible in this study (see SI). Under these conditions, N_2 directly from Feammox or Feammox-generated NO_2^- or NO_3^- followed by anammox or denitrification are the only potential pathways for $^{30}\text{N}_2$ production (Table 2). All of these pathways, however, require the occurrence of Feammox at first. Codenitrification, which often co-occurs with denitrification and produces N_2 ,²³ can be ruled out as a potential source of $^{30}\text{N}_2$ in this study, given that other ^{15}N -labeled nitrogen compounds including hydrazine and amino compounds, which could reduce Feammox-produced $^{15}\text{NO}_2^-$ to $^{30}\text{N}_2$ were unavailable.⁹ Furthermore, the promotion of Fe(III) reduction after $^{15}\text{NH}_4^+$ addition, together with the positive ($P < 0.0001$) correlation between Fe(III) reduction and $^{30}\text{N}_2$ production rates, further provides strong evidence for the occurrence of Feammox in the chronosequence (Figures 2 and 3a).

Potential Feammox rates are conservative estimates calculated from $^{30}\text{N}_2$ production alone.¹¹ The potential Feammox rates (i.e., $^{30}\text{N}_2$ production rates) in all paddy soils ranged from 0.17 to 0.59 mg N $\text{kg}^{-1} \text{d}^{-1}$ (Figure 1a), which were comparable to that reported in tropical forest soils (about 0.32 mg N $\text{kg}^{-1} \text{d}^{-1}$) by Yang et al.¹¹ Also, this order of magnitude was comparable to that of anammox¹ and denitrification rates¹⁷ reported in paddy soils of China. Furthermore, the potential Feammox rates were found to be positively correlated with microbially reducible Fe(III) levels ($P < 0.01$; SI Table S1), indicating that soil microbially reducible Fe(III) level was a key factor controlling the Feammox activity, though microbially reducible Fe(III) is insoluble in soils with

pH > 4.²⁴ Various strategies, such as direct contact, have been proposed to be potentially used by microorganisms to access insoluble Fe(III) oxides and then facilitate Fe(III) reduction.²⁵ Furthermore, it has been suggested that Feammox could be mediated by microorganisms,¹¹ and different microbial populations might be involved in different Feammox pathways (i.e., produce N_2 , NO_2^- or NO_3^- via Feammox). We thus hypothesize that a higher microbially reducible Fe(III) level might provide more surface area for microbial contact, and thus potentially accelerate Feammox reaction, since the added $^{15}\text{NH}_4^+$ was sufficient enough to drive this reaction in all soils. Within the examined chronosequence, rice cultivation markedly enriches amorphous Fe(III) oxides. This presumably results from the enhancement of soil DOC after rice cultivation (Table 1), since that high concentrations of dissolved organic matter during flooding seasons could promote the release of structural Fe in clay minerals and support the formation of amorphous Fe(III) oxides.¹⁵ High amorphous Fe(III) oxide concentrations and water-logged oxygen-limiting conditions under rice cultivation could promote Feammox reaction and thus potentially accelerate gaseous N loss.

It is noteworthy that, $^{29}\text{N}_2$ also significantly accumulated in all soils amended with $^{15}\text{NH}_4^+$ (Figure 1b), though the added $^{15}\text{NH}_4^+$ pool (90 mg N kg^{-1}) was much larger than the indigenous $^{14}\text{NH}_4^+$ pool in all soils (7.0–21 mg N kg^{-1} after the preincubation), indicating that the indigenous $^{14}\text{NH}_4^+$ and added $^{15}\text{NH}_4^+$ pools were completely mixed. Several possible pathways exist for $^{29}\text{N}_2$ production: the utilization of indigenous $^{14}\text{NH}_4^+$ in combination with added $^{15}\text{NH}_4^+$ in Feammox to N_2 , Feammox to NO_2^- followed by anammox or codenitrification to N_2 , or Feammox to NO_2^- or NO_3^- followed by denitrification to N_2 (Table 2). Nevertheless, all of these pathways are based on anaerobic NH_4^+ oxidation. As with the potential Feammox rates (i.e., $^{30}\text{N}_2$ production rates), the $^{29}\text{N}_2$ production rates were also positively correlated with Fe(III) reduction rates ($P < 0.0001$) and microbially reducible Fe(III) ($P < 0.01$) levels (Figure 3b and SI Table S1), this further indicates that Feammox played an important part in anaerobic NH_4^+ oxidation, irrespective of the ultimate pathway for $^{29}\text{N}_2$ production. Considering the generation of both $^{30}\text{N}_2$ and $^{29}\text{N}_2$, anaerobic NH_4^+ oxidation consumed 0.40–0.60% and 2.1–9.1% of the added $^{15}\text{NH}_4^+$ label in the uncultivated and paddy soils after the incubations, respectively.

Relative Contribution of Feammox Pathways. N_2 directly from Feammox accounted for 67 (± 2.4) to 78 (± 4.5) % of $^{30}\text{N}_2$ loss in all soils treated with $^{15}\text{NH}_4^+$ alone, and there was no significant difference in this proportion among different soils. We used C_2H_2 to distinguish direct N_2 generation via Feammox from gaseous N loss via anammox and/or denitrification of Feammox-produced NO_2^- and NO_3^- . C_2H_2 has been reported to not only block the reduction of N_2O to N_2 ,²⁶ resulting in the accumulation of N_2O produced from denitrification, but also could inhibit anammox²⁷ and the first step of aerobic nitrification.²⁸ In our study, aerobic nitrification was negligible (see SI), and the final concentration of C_2H_2 in the headspace was 30% (v/v; about 11.5 mM), and therefore, well above the reported threshold for nearly complete inhibition of N_2O reduction ($\geq 290 \mu\text{M}$)^{26,27,29} and anammox ($\geq 30 \mu\text{M}$).²⁷ Therefore, N_2 directly from Feammox was thought to be the sole pathway of $^{30}\text{N}_2$ production in the $^{15}\text{NH}_4^+ + \text{C}_2\text{H}_2$ treatment (Table 2). A decrease in $^{30}\text{N}_2$ production caused by C_2H_2 addition in all soils indicating

that about 22–33% of $^{30}\text{N}_2$ loss in both uncultivated and paddy soils was ascribed to anammox and/or denitrification of Feammox-produced $^{15}\text{NO}_2^-$ and $^{15}\text{NO}_3^-$, the remainder being due to N_2 directly from Feammox. This suggests that direct N_2 production is dominantly via Feammox in the chronosequence. Theoretically, N_2 directly from Feammox is also energetically more favorable than Feammox to NO_2^- or NO_3^- and occurs under a wider range of conditions (e.g., a wider pH range).¹¹

We also detected significant headspace $^{46}\text{N}_2\text{O}$ accumulation in all soils only in the presence of C_2H_2 (Figure 1c). N_2O is produced in soils during various microbial processes, including aerobic nitrification, codenitrification, and denitrification.^{9,30} In our study, aerobic nitrification was negligible, and codenitrification is also excluded as a potential source of $^{46}\text{N}_2\text{O}$ since other ^{15}N -labeled nitrogen compounds which could reduce Feammox-produced $^{15}\text{NO}_2^-$ to $^{46}\text{N}_2\text{O}$, such as hydrazine and azide, were unavailable.⁹ Thus, the $^{46}\text{N}_2\text{O}$ accumulation in the headspace with C_2H_2 only came from the denitrification of Feammox-produced $^{15}\text{NO}_2^-$ and/or $^{15}\text{NO}_3^-$. On the basis of the total production rates of $^{46}\text{N}_2\text{O}$ in the gas and liquid phases, approximately $0.01\text{--}0.14\text{ mg N kg}^{-1}\text{ d}^{-1}$ in all soils was oxidized to $^{15}\text{NO}_2^-$ or $^{15}\text{NO}_3^-$ via Feammox at first and subsequently reduced to $^{46}\text{N}_2\text{O}$ via denitrification (see SI), occupying 19–28% of $^{30}\text{N}_2$ loss in all soils. This proportion supported the result calculated from the difference in $^{30}\text{N}_2$ production with and without C_2H_2 .

Proportion of Iron Reduction Associated with Feammox. Interestingly, the molar ratio of measured $^{30}\text{N}_2$ to Fe(II) production following the addition of $^{15}\text{NH}_4^+$ alone does not match the stoichiometry shown in eq 1 in any of the soils, probably because only a minor proportion of the Fe(III) reduced was linked to the NH_4^+ oxidation in the examined chronosequence.¹⁴ Therefore, we determined the proportion of Fe(III) reduction attributed to Feammox in the $^{15}\text{NH}_4^+$ treatment in all soils using the theoretical ratio of 3–6 mol of Fe(III) reduced per mole of NH_4^+ oxidized according to the thermodynamic calculations (see SI). On the basis of this approach, only 2.0–4.0% and 0.81–2.2% of Fe(III) reduction is associated with Feammox in the uncultivated and paddy soils, respectively. It is assumed that the majority of Fe(III) reduced was potentially involved in the oxidation of soil organic matter, given that organic compounds are served as common electron donors for Fe(III) reduction under anaerobic conditions in addition to NH_4^+ .³¹ This assumption might explain why the highest proportion of Fe(III) reduction associated with Feammox was in the uncultivated soil (2.0–4.0%), which has the lowest TOC content, while the lowest proportion was in the paddy soil RC13 (0.81–1.6%) with the highest TOC content (Table 1). Furthermore, the proportions of Fe(III) reduction associated with Feammox in the examined chronosequence were higher than previously reported (0.4–0.8%) by Yang et al.¹¹ This may also be related to the difference in soil TOC content, as the upland soils used in that study contained about 12% of organic carbon,³² whereas the chronosequence used in this study contained only 0.87–2.4% (Table 1). Therefore, it is suggestive to hypothesize that soils deficient in organic carbon might utilize NH_4^+ as an electron donor more readily than those rich in organic carbon when Fe(III) oxide is served as sole electron acceptor. This is particularly important in the subtropical region of China, where soil fertility (such as organic carbon levels) is often very low while amorphous Fe(III) oxides are abundant, due to heavy weathering and nutrient leaching.³³

Estimation of Ecosystem Ammonia Loss through Feammox

On the basis of rates obtained from slurry incubations and soil bulk density, it is estimated that the potential ammonia loss via Feammox (0–10 cm depth) in all paddy soils was $7.8\text{--}61\text{ kg N ha}^{-1}\text{ year}^{-1}$, accounting for approximately 3.9–31% of the N fertilizer usually applied to paddy soils in China (about $200\text{ kg N ha}^{-1}\text{ year}^{-1}$),^{4,17} which was much greater than in the uncultivated soil ($2.1\text{--}8.6\text{ kg N ha}^{-1}\text{ year}^{-1}$; 1.0–4.3%) (see SI), indicating that rice cultivation could facilitate more N loss via Feammox. The potential N loss via Feammox in the chronosequence is comparable to that reported for NH_3 volatilization (9–40% of the applied N fertilizer), leaching (3–9%), runoff (5–7%), denitrification (up to 40%) and anammox (about 23%) from paddy soils in China.^{1,3–5,17} This suggests that Feammox could potentially be an important pathway for N loss in paddy soils, and change our current estimates of total N losses from terrestrial ecosystems. However, the estimate of the environmental importance of Feammox process as an important pathway for N loss in paddy soils still appears to be linked with a uncertainty because slurry incubations in this study might overestimate the actual in situ activity due to the anoxic conditions and release of soil labile organic C via slurrying that stimulate Fe(III) reduction. Therefore, more studies that simulate the in situ conditions are needed to quantitatively determine the importance of Feammox as an alternative N loss pathway in paddy soils.

In summary, for the first time, we provide direct evidence for the occurrence of Feammox in paddy soils, and found that direct N_2 production via Feammox is the dominant pathway of gaseous N loss. Furthermore, rice cultivation could progressively enrich indigenous microbially reducible Fe(III), therefore accelerating the Feammox reaction, resulting in more gaseous N loss. It is estimated that a total loss of $7.8\text{--}61\text{ kg N ha}^{-1}\text{ year}^{-1}$ is linked to Feammox in paddy soils, accounting for about 3.9–31% of the N fertilizer usually applied to paddy soils in China. This study reveals that rice cultivation, in which a large portion of global synthesized N is used, could fuel N loss from soils via Feammox, and provides insights into alternative N loss from terrestrial ecosystems. This study demonstrates a poorly understood yet important process of N cycling, and warrants extensive investigation at different scales.

■ ASSOCIATED CONTENT

Supporting Information

Details on supportive methods and discussion; the sampling design and image of the examined paddy soil chronosequence; and the Pearson correlations between soil chemical characteristics and rate measurements within the chronosequence. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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