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RNA Stable Isotope Probing of Potential Feammox Population in Paddy Soil

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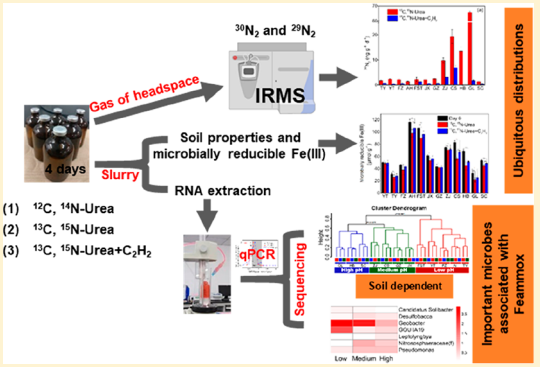
Supporting Information

ABSTRACT: Anaerobic ammonium oxidation coupled to iron reduction (Feammox) is a recently discovered pathway contributing to nitrogen loss in various ecosystems such as paddy soils and sediments. However, little is known about the microbes driving Feammox in an agricultural ecosystem. Here, we demonstrated the occurrence of Feammox in paddy soils of Southern China using a ^{15}N isotopic tracing technique, and examined the microbial communities associated with Feammox using RNA based stable isotope probing (RNA-SIP) combined with Illumina sequencing. Feammox was detected in all collected soils with direct N_2 production as the dominant Feammox pathway. It was estimated that approximately 6.91% of the applied nitrogen fertilizers were lost through Feammox in the paddy soils. RNA-SIP results showed that the composition of enriched active microbial communities were dependent on soil properties, especially the soil pH and grain size. *Geobacter* were enriched in most soils across various properties. The abundance of enriched GOUTA19 were significantly higher in soils with low pH than those in soils with medium pH and high pH, and the relative abundance of active Nitrososphaeraceae and *Pseudomonas* only increased in soils with medium and high pH during 4-day of incubation. These results suggested Feammox is a ubiquitous and important process for N loss. Geobacter, GOUTA19, Nitrososphaeraceae and *Pseudomonas* were active during the incubation that favored Feammox and the growth of Feammox microbes, suggesting these microbes were potentially associated with Feammox in natural agricultural soils.



INTRODUCTION

Nitrogen (N) fertilizers are intensively applied in paddy soils to increase rice production, however only around 35% of the applied N fertilizers are actually utilized by the crops.[1] The excessive use of N fertilizers increases nitrogen (N) emission to the environments, leading to N pollution in various ecosystems.[2] Investigators have focused on N cycling pathways in paddy soils for decades[3−7] in order to increase N utilization and decrease N pollution of the environments.

Anaerobic ammonium oxidation coupled to Fe(III) reduction (Feammox) has been proposed as a pathway for N loss in terrestrial ecosystems in addition to denitrification, codenitrification, nitrification and normal anaerobic ammonium oxidation (anammox).[8−10] Feammox has been detected in various environments including forest soils, wetland and enrichment cultures, with either dinitrogen gas (N_2), nitrite (NO_2^-) or nitrate (NO_3^-) as the end-products.[8,11−13] Periodic flooding of paddy fields leads to repeated shifts between oxic and anoxic conditions in paddy soils, which promotes Fe transformation between Fe(II) and Fe(III). Paddy soils are characterized by an abundance of Fe(III) and high concentrations of NH_4^+, and thus may be important habitats for microorganisms associated with the Feammox process. However, only limited studies have explored the Feammox process in paddy soils,[9] and the microbial communities associated with Feammox in various paddy soils are still unclear.

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A major challenge of studying Feammox lies in the identification and characterization of microorganisms associated with Feammox. Li et al.[11] have suggested that *Geobacter* and *Shewanella* might drive the Feammox process due to the significantly positive correlation between the abundance of these taxa, Fe(III) reduction rates and $^{30}N_2$ production rates in periodic dry and wet sediments solely amended with $^{15}NH_4^+$. Other studies have in addition speculated that *Geobacter*[12] and *Acidimicrobiaceae*[14] might play an important role in driving the Feammox process in paddy soils and soils from a forested riparian wetland based on increasing relative abundances in microcosm experiments, while so far only one isolate, namely *Acidimicrobiaceae* sp. A6, has been identified to perform the Feammox process[15]Furthermore, Dissimilatory iron reducing bacteria such as *Geothrix*,[16] *Geobacter*,[17] and *Shewanella*[18,19] are suggested to be potential microorganisms driving the Feammox process because of their capabilities in Fe(III) reduction. However, recent investigations have employed either incubation experiment with limited soil samples, or DNA derived 16S rRNA gene- and functional gene-based sequencing and quantitative PCR technologies, which determine the total microbes including dead microbes[20] and therefore would include redundant and imprecise information for characterizing the active microbes in various ecosystems. Active microbes are the drivers of microbial processes such as Feammox, anammox, nitrification, and denitrification. RNA-based techniques are more sensitive to investigate active microbial communities compared with DNA-based analysis.[21] Thus, in order to better understand the function of the active microbial community, RNA-based SIP and 16S rRNA gene sequencing have been employed to identify iron-reducing microorganisms in previous study.[22] Until now, it has not been possible to clearly determine the functional microorganisms involved in the Feammox process due to the absence of findings concerning the Feammox functional genes, which are key elements for investigating the diversity and abundance of Feammox-associated microorganisms in different ecosystems. RNA-SIP combined with high-throughput sequencing are an optional tool for linking microbial community functions to the phylogeny of the organisms accountable for it.[17,22−25] Hence, the objectives of this study were (i) to characterize Feammox in paddy soils of Southern China with different properties; (ii) to identify the key microorganisms involved in Feammox in paddy soils using RNA-SIP combined with Illumina sequencing techniques.

MATERIALS AND METHODS

Soil Sampling and Isotope Tracer Incubations. Samples were collected in July 2013 from the surface (0−20 cm) of 12 paddy soils from Southern China (Supporting Information, SI, Figure S1) with broad-scale variability in soil properties (e.g., grain size, pH, and organic matters, and so forth). Four subsamples of soil were collected at each site and were subsequently mixed to obtain one representative soil sample per site. The fresh soil samples were placed in sterile plastic bags, sealed without air, and transported to the laboratory on ice. Each composite soil sample was partitioned, with one subsample being air-dried and passed through a 2 mm sieve for soil property analysis, and the remaining fresh soil was stored at 4 °C and subsequently used for isotope tracer incubation. The chemical properties of the paddy soils used in this study have been described in our previous study.[26] Briefly, the soils examined in this study were grouped into three clusters, namely low pH (YT, TY, FZ, AH, and FST, pH < 6), medium pH (CS, ZJ, JX, and GZ, pH 6−7), and high pH (SC, GL, and HB, pH > 7) samples.

Stable Isotope Tracer Incubation and ^{15}N-Labeled Gas Analysis. Approximately 10 g of fresh soil was well mixed with three volumes of sterile MiliQ water and transferred into 120 mL serum vials, which were then sealed with butyl rubber septa and crimped with aluminum caps. The headspace air in the serum vials was replaced with 99.999% helium (He), and the soil slurries were preincubated in the dark at 25 °C for 5 to 7 days to deplete indigenous electron acceptors.[26] After the preincubation, the headspace gas in the serum vials was replaced with 99.999% He, and 1 mL of soil slurry from each serum vial was sampled for microbially reducible Fe(III) analysis by using a sterile syringe. Three treatments were set up in quadruplicates: (1) unlabeled urea (^{12}C, ^{14}N-urea) addition; (2) ^{13}C, ^{15}N-urea addition (^{13}C at 99%, ^{15}N > 98%, Cambridge Isotope Laboratories, Andover, U.S.A, $^{13}CH_4{}^{15}N^{15}NO$); (3) ^{13}C, ^{15}N-urea and C_2H_2 addition, mainly for determining the contributions of N_2 production directly via Feammox to total N_2 production. In the treatment with C_2H_2 addition, the N_2 production pathway of N_2O to N_2 (important step of denitrification)[27] and anammox are blocked.[9] Thus, the contribution of each pathway of Feammox to N_2 production could be calculated by comparing the ^{15}N-labeled N_2 gas between the two labeled treatments. We should note that there is a possibility that some of Feammox microbes are also inhibited by C_2H_2, however, due to the limited knowledge on Feammox microbes, the potential effect of C_2H_2 on Feammox was not included in this study. The final concentration of urea-N was 70 mg kg^{-1} (wet weight), which represents the typical amount of chemical N fertilizer applied to rice fields in China. For the C_2H_2 treatment, C_2H_2 was injected into the serum vials to reach 30% (v/v) C_2H_2 in the headspace. All vials were shaken thoroughly to homogenize the mixtures and to dissolve the C_2H_2, which were incubated at 25 °C in the dark for 4 days.

After incubation, 1 mL of gas from the headspace of the vials was transferred into a 12 mL glass vial (Exetainer, Labco, High Wycombe, Buckinghamshire, U.K.) filled with gaseous helium by using a gastight syringe, and was subsequently used for $^{15}N-N_2$ analysis. The concentrations of N_2 and $^{15}N-N_2$ atom % were determined by using a GasBench II/isotope ratio mass spectrometry (IRMS, Thermo Finnigan Delta V Advantage, Bremen, Germany). The $^{15}N-N_2$ concentration and Feammox rates were calculated according to Ding et al.[9] After gas sampling, the soil slurries were subsampled for microbially reducible Fe(III) analysis according to a modified protocol of Lovely et al.[28] Briefly, 1.0 g of soil slurry was incubated with 5 mL of 0.25 M HCl with 0.25 M hydroxylamine hydrochloride in an anaerobic glovebox for 2 h at 25 °C, and the total Fe was subsequently determined using the ferrozine method. HCl-Fe(II) was analyzed using the same procedure except that the extractant was 5 mL 0.5 M HCl. The microbially reducible Fe(III) was calculated as the difference between total Fe and HCl-Fe(II). The pH values of the soil slurries were determined by using a pH analyzer, and the remaining slurries were stored at −80 °C for RNA extraction.

RNA Extraction and Stable Isotope Probe Gradient Fractionation. Total RNA was extracted according to the protocol described by Ding et al.[17] In brief, after extraction using phenol-chloroform-isoamyl alcohol [25:24:1 (v/v/v)] (pH 8.0) and chloroform-isoamyl alcohol [24:1 (v/v)], the

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total nucleic acid pellets were washed with precooled 70% ethanol, air-dried, and finally resuspended in 50 μL of DEPC water. Co-extracted DNA was removed from the extracted RNA by using DNase I and the RNA was subsequently purified using the RNeasy mini Kit (Qiagen, Hilden, Germany). The concentration and quality of the purified RNA were measured using a NanoDrop-1000 (Wilmington, DE, U.S.A.). Approximately 500 ng of purified RNA was mixed with cesium trifluoroacetate gradients to achieve an initial buoyant density of 1.790 g mL^{-1}. The mixture was centrifuged at 130 000*g*, 20 °C for 65 h. The centrifuged RNA gradients (about 400 μL) were fractionated using an automatic sampling instrument (model BSZ-100), and the buoyant density of each fraction was determined as previously described.[29] The complementary DNA (cDNA) for each fraction was synthesized using the total RNA as a template according to the protocol provided by PrimeScript RT-PCR Kit (TaKaRa Bio, Japan). The copy numbers of the 16S rRNA gene in each fraction was quantified by real-time PCR, employing the primer set of 515*f*/907*r*[30] with synthesized cDNA as template in a Roche 480II Thermocycler. A standard curve was constructed using a serial of 10-fold diluted plasmids harboring the 16S rRNA gene. Real-time PCR was performed in triplicates for all samples including the plasmids.

16S rRNA Gene Illumina Sequencing. Selected fractions of RNA were reversely transcribed into cDNA by utilizing the random primers of PrimeScript first strand cDNA synthesis kit (TaKaRa Bio, Japan) following the manufacturer's instructions. A primer set of 515*f*/907*r* was used to amplify the V4−V5 hypervariable regions of the 16S rRNA genes. To identify sequences from different samples, 6-bp barcodes were tagged at the 5′ end of each reserve primer. Amplifications were conducted in 50 μL reaction mixtures containing 25 μL of Premix Taq (Ex Taq Version 2.0, TaKaRa Bio, Japan), 1.0 μM of each primer, 1.0 μL of bovine serum albumin (BSA, 20 mg/mL) and 2 μL of cDNA as template. The nontemplate control reactions were performed using 2 μL sterile PCR-grade water as template. Amplifications were performed using a Thermal cycler (LifePro Thermal Cycler, Bioer Serves Life) following the thermal condition described previously.[31] Target amplicons were gel purified and quantified using the NanoDrop-1000 (Wilmington, DE, U.S.A.). Subsequently, equal amounts of the purified amplicons with different barcodes were mixed and sent to Novogene Beijing (Beijing, China) for Illumina sequencing on a Hiseq 2500 platform.

Illumina Data Processing and Statistical Analysis. Raw sequences were processed using the Quantitative Insights Into Microbial Ecology toolkit-version 1.9.0[32] following a procedure reported previously.[31] Briefly, after removal of ambiguous and low-quality reads (length <150bp, Q30 < 70%), the sequences with 97% nucleic acid similarity were clustered into an operational taxonomic unit (OTU). The representative sequence with the most abundance for each OTU was assigned to a taxonomy using an RDP classifier (Version 11, http://rdp.cme.msu.edu) with a minimum confidence threshold of 80%.[33] Sequences identified as chimeras, singletons, mitochondria, and chloroplast were removed from the final data. Enriched OTUs of each detected fraction in labeled treatments were identified by comparing the relative abundance of OTUs in "heavy" RNA fractions with the same density from labeled and unlabeled treatments using fold change (FC) = 2 and $P < 0.05$, and gathered as the enriched OTUs in labeled treatments. After detection of enriched OTUs, the representative sequence for each enriched OTU was assigned to a taxonomy using an RDP classifier (Version 11, http://rdp.cme.msu.edu).

The α (Shannon and Phylogenetic Diversity_Whole tree) and β diversity were calculated by a random and equal sequencing depth of 21 998 sequences across different samples. SPSS software (SPSS Inc., Chicago, III, U.S.A.) was used to perform two-way analysis of variance (ANOVA) and Pearson correlation analysis on the soil biogeochemical data. For the 16S rRNA gene sequencing data, adonis test, and constrained canonical analysis of principal coordinates (CAP) were performed using R (version 3.4.0) software with the vegan package.[34] The plots in this work were generated using Origin Lab version 2018.

Accession Number of 16S rRNA-Based Sequences. The nucleic acid reads based on 16S rRNA genes have been deposited at NCBI GenBank database with the accession number of SRP116171.

RESULTS

Iron Reduction and ^{15}N-Labeled Gas Production after Incubation. Addition of urea significantly decreased the concentration of microbially reducible Fe(III) in the soil slurries after incubation. However, the decrease of microbially reducible Fe(III) in the urea+C_2H_2 amended soils (0.91−17.38 μmol g^{-1}) were generally lower than in soils amended with urea only (1.83−27.00 μmol g^{-1}) (Figure 1). Following

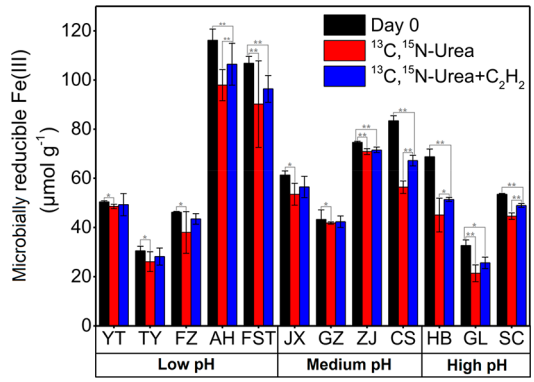

Figure 1. Concentration of microbially reducible iron in paddy soils during incubation experiment. Error bar was calculated based on 4 replicates for one treatment. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

anoxic incubation, production of ^{15}N-labeled N_2 ($^{29}N_2$ and $^{30}N_2$) was detected in soils amended with ^{13}C, ^{15}N-urea and ^{13}C, ^{15}N-urea+C_2H_2 (Figure 2a) but not in soils amended with $^{12}C,^{14}N$-urea. The production rate of $^{30}N_2$ in soils amended with ^{15}N-labeled urea ($^{13}C,^{15}N$-urea treatment and $^{13}C,^{15}N$-urea+C_2H_2 treatment) ranged from 1.40 to 65.68 ng N g^{-1} d^{-1}, which was significantly ($P < 0.005$) lower than $^{29}N_2$ production rates (23.85−284.58 ng N g^{-1} d^{-1}) (Figure 2b). The ^{15}N-labeled N_2 ($^{29}N_2$ and $^{30}N_2$) production rates were significantly ($P < 0.05$) higher in $^{13}C,^{15}N$-urea amended soils as compared to $^{13}C,^{15}N$-urea+C_2H_2 amended soils. Microbially reducible Fe(III) reduction rates showed a significant and positive correlation with $^{30}N_2$ production rates in soils amended with ^{13}C, ^{15}N-urea ($r = 0.65$, $P < 0.05$, Figure 3a) and ^{13}C, ^{15}N-urea +C_2H_2 ($r = 0.78$, $P < 0.005$, Figure 3b). However, no

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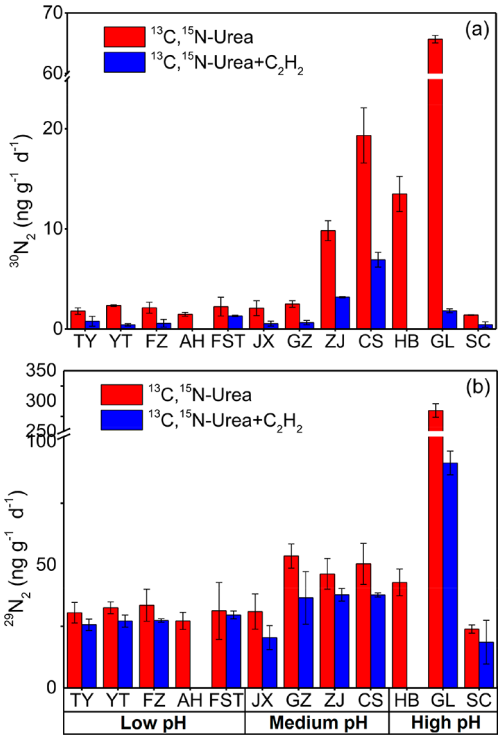

Figure 2. Production rates of $^{30}N_2$ (a) and $^{29}N_2$ (b) in paddy soils amended with ^{13}C,^{15}N-urea only and ^{13}C,^{15}N-urea+C_2H_2. Error bar was calculated based on 4 replicates for one treatment. The labeled N_2 ($^{29}N_2$ and $^{30}N_2$) in soils amended with ^{13}C, ^{15}N-urea+C_2H_2 were missed due to the fault during detection in this study.

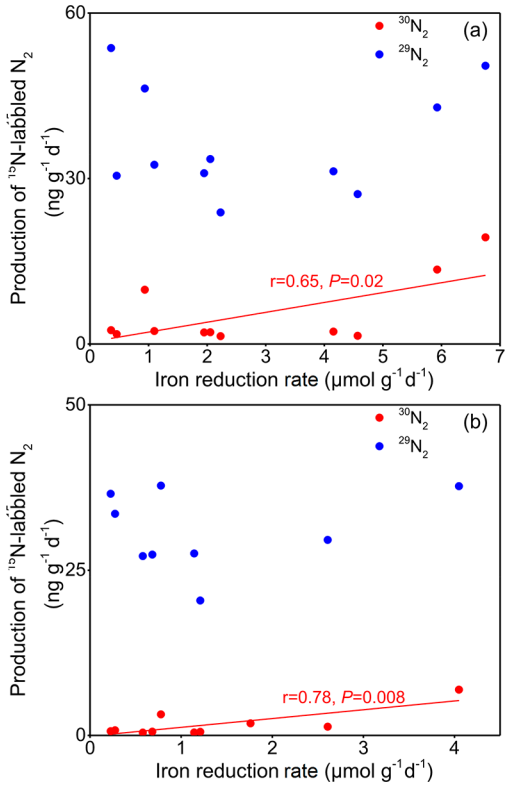

Figure 3. Relationships between iron reduction rate and $^{30}N_2$, $^{29}N_2$ production rate in ^{13}C,^{15}N-urea (a) and ^{13}C,^{15}N-urea+C_2H_2 (b) treatments. Linear regression was used to test the correlation between ^{15}N–N_2 production rates and iron reduction rate. Adjusted R^2 values with the associated *P*-values are shown.

significant correlation was observed between the $^{29}N_2$ production rate and Fe(III) reduction rate in this study.

Distributions of Microorganisms Associated with Feammox. Extracted RNA was subjected to ultracentrifugation to identify ^{13}C labeled active microorganisms. Quantification of 16S rRNA genes showed a clear shift in distribution along the density gradient between labeled treatments and corresponding unlabeled controls, indicating that active microorganisms were labeled during incubation (SI Figure S2).

The 16S rRNA gene amplicons in "heavy" RNA fractions of the labeled treatment and corresponding fraction of unlabeled controls (shaded area, SI Figure S2) were sequenced to identify the community compositions of active microorganisms. A total of 7.1 million high-quality sequences were obtained and were clustered into 126 700 OTUs. Of the dominant 30 genera, *Geobacter* was the most abundant genus accounting for 2.67% of the relative abundance, followed by *Thiobacillus* (1.74%), *Desulfobacca* (1.73%) and *Anaeromyxobacter* (1.68%) (Figure 4). Cluster analysis showed that community compositions of active microorganisms in "heavy" RNA fractions were separated distinctly by soil pH (adonis analysis, $P < 0.001$, 999 permutations, Figure 5). Constrained canonical analysis of principal coordinates (CAP) indicated that the compositions of active microorganisms were dependent on soil properties (Figure 6), especially on pH and grain size, which explained 9.95% and 9.48% of observed microbial community variation, respectively, followed by NH_4^+ (5.19%) and C/N ratio (3.70%). The determined soil properties explained 62.83% (including 22.27% interactional explanation) of the observed variations in active microbial communities between soils (Figure 6).

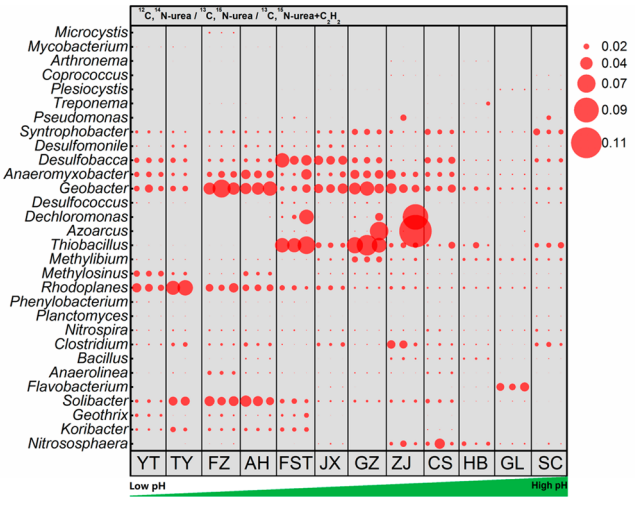

Figure 4. Top 30 genus detected in soils with different treatments. The bubble indicts the relative abundance (average of 4 replicates for one treatment).

Potential Microorganisms Associated with Feammox in Paddy Soils. The addition of C_2H_2 would change the communities of bacteria in soils, and the detected functional microbes in ^{13}C,^{15}N-urea + C_2H_2 treatments may not reflect the function microbes associated with Feammox in natural environment, thus we only determined the enriched genera in ^{13}C,^{15}N-urea treatment compared with ^{12}C,^{14}-urea treatment. Since pH played a key role in shaping the microbial

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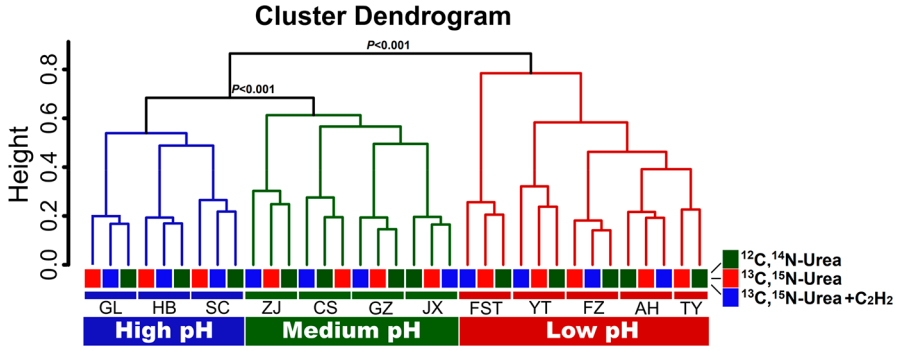

Figure 5. Cluster analysis based on Bray−Curtis distances of microbial communities in RNA fractions of different samples. The branches with different colors indicate the soils with different pH (red: low pH; green: medium pH; and blue: high pH). The rectangle with different colors indicate different treatments (green: ^{12}C, ^{14}N-urea; red: ^{13}C, ^{15}N-urea; and blue: ^{13}C, ^{15}N-urea+C_2H_2).

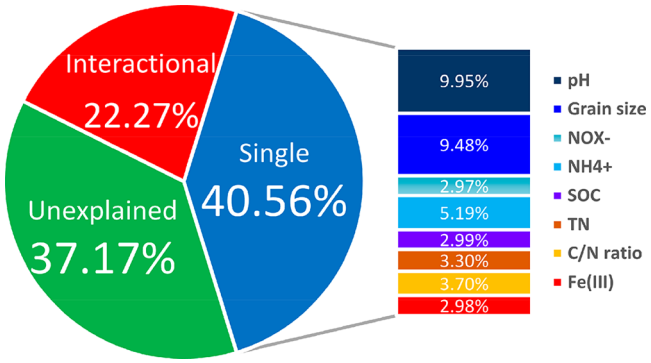

Figure 6. Contributions of soil properties to bacterial community variations calculated by constrained canonical analysis of principal coordinates (CAP). Interactional explanation indicate the cocontribution of detected soil properties to bacterial community variations.

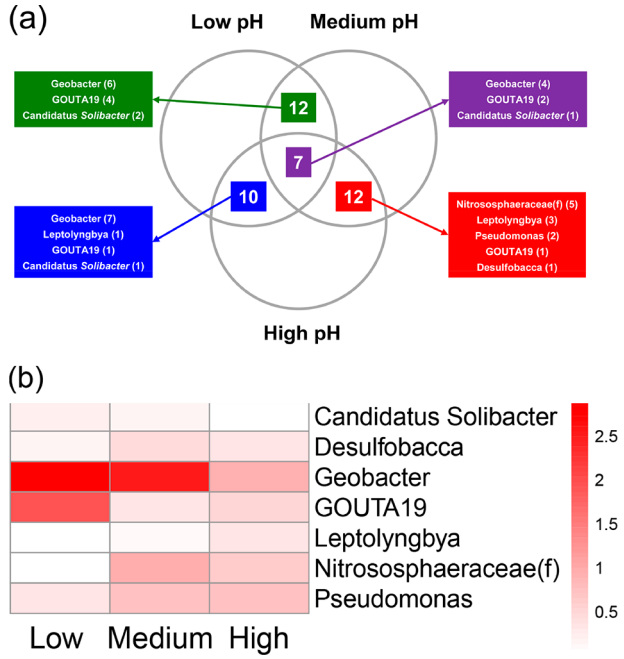

Figure 7. Significant enriched microbes in soils amended with ^{13}C,^{15}N-urea compared to ^{12}C,^{14}N-urea control. A Venn plot (a) showing the numbers of shared OTUs significantly enriched in soils with varying pH (low, medium and high pH). The classification of enriched OTUs are showed in block diagram, and the number in bracket after each genus indicates the number of enriched OTUs within this genus; A heatmap (b) showing the relative abundance of significant enriched genus ($P < 0.05$, FC ≥ 2), where the relative abundance of each genera is the sum of OTUs enriched in at least two groups soil samples with different pH level.

communities associated with Feammox, the collected soils were separated into three groups with different levels of pH (low pH, medium pH, and high pH) to excavate potential functional microorganisms driving Feammox. Several OTUs were significantly enriched (FC = 2, $P < 0.05$) in the labeled soils amended solely with urea under the different pH conditions, as compared to unlabeled treatments. In low pH soil, 69 OTUs mainly affiliated to *Geobacter* spp. (40.58%), Candidatus Solibacter spp. (28.99%) and GOUTA19 (17.39%) were significantly enriched (SI Table S1). In medium pH soil, 118 OTUs mainly affiliated to Nitrososphaeraceae(f) (30.51%) and Geobacter spp. (12.71%) were enriched (SI Table S1), whereas 74 OTUs majorly affiliated to Geobacter spp. (20.27%) and Leptolyngbya spp. (16.22%) were significantly enriched in the high pH soil (SI Table S1). Seven OTUs (Figure 7a) affiliated within *Geobacter*, GOUTA19 group or Candidatus Solibacter were enriched in all soils samples across various pH, and 12 OTUs (Figure 7a) affiliated within Nitrososphaeraceae(f), *Desulfobacca*, GOUTA19, *Leptolyngbya*, and *Pseudomonas* were enriched in soils with medium pH and high pH (Figure 7a). Most of enriched OTUs affiliated within Geobacter and GOUTA19 were enriched at least in two group of soil samples with different pH level (SI Table S1). The relative abundance of enriched *Geobacter* was the highest among the enriched genera, ranging from 2.89% in low pH soils to 0.90% in high pH soils, and was negatively correlated with soil pH (Figure 7b). The enriched GOUTA19 group was more abundant ($P < 0.01$) in soils with low pH (1.93%) comparted with those in soils with medium (0.33%) or high pH (0.53%) (Figure 7b). The average relative abundance of Nitrososphaeraceae was only 0.02% in soils with low pH, while it increased to 0.94% and 0.65% in soils with medium pH and high pH, respectively. Of the enriched genera in soils with medium pH and high pH, Nitrososphaeraceae was the most abundant, followed by *Pseudomonas* and *Desulfobacca* (Figure 7b).

DISCUSSION

Evidence for the Occurrence of Feammox in Paddy Soils from Southern China. Following anaerobic preincubation, the residual NO_x^- concentration in all soil slurries of this study was below the detection limits of an ion

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chromatograph (ICS-3000, Dionex). Hence, the production of $^{30}N_2$ in the soils amended with $^{13}C^{15}N$-urea only and $^{13}C,^{15}N$-urea+C_2H_2 could only be derived from Feammox-produced NO_x^- followed by denitrification or anammox under strict anaerobic condition. Furthermore, a positive correlation between $^{30}N_2$ production and Fe(III) reduction was detected during the incubation, providing an evidence for the occurrence of Feammox in all examined paddy soils. Feammox is likely to occur in soils rich in microbially reducible Fe(III) and NH_4^+ under strict anaerobic condition.[9,11] Paddy soils generally receive large amounts of chemical N fertilizer and are subjected to long-term flooding during rice cultivation, which provide conditions that highly favor Feammox.

In this study, the Feammox rate, calculated based on the $^{30}N_2$ production rate, ranged from 0.42 to 65.68 ng N g^{-1} d^{-1}. This is lower than previously reported in tropical forest soils (~0.32 ng N g^{-1} d^{-1}),[8] paddy soils (0.17−0.59 ng N g^{-1} d^{-1})[9] and sediments (0.24−0.36 ng N g^{-1} d^{-1}),[11] but are comparable to rates reported in tropical forest soils at an initial pH of 6.12.[8] Meanwhile, the Feammox rates were significantly ($P < 0.01$) lower than anammox-and denitrification rates detected in these paddy soils.[26] The Fe(III) reduction rates (averaging 123 mg kg^{-1} d^{-1}) in this study were furthermore significantly lower than in previous studies,[9,11] which might explain the observed relatively lower Feammox rates. The highest $^{30}N_2$ production rate was detected in GL soil, which might be attributed to the higher TOC.[26] In fact, organic matters have been reported to increase the formation of Fe(III) oxides by releasing structural Fe from clay minerals, and can subsequently promote the Feammox process.[35]

Estimation of Nitrogen Loss through Feammox in Paddy Soils. The Feammox process contains three pathways: (1) $3Fe(OH)_3 + 5H^+ + NH_4^+ = 3Fe^{2+} + 9H_2O + 0.5N_2$ $\Delta G = -245$ kJ mol^{-1}; (2) $6Fe(OH)_3 + 10H^+ + NH_4^+ = 6Fe^{2+} + 16H_2O + NO_2^-$ $\Delta G = -164$ kJ mol^{-1}; (3) $8Fe(OH)_3 + 14H^+ + NH_4^+ = 8Fe^{2+} + 21H_2O + NO_3^-$ $\Delta G = -207$ kJ mol^{-1} [8]. In this study, the contribution of each pathway to the Feammox process was evaluated by comparing ^{15}N-labeled N_2 production in $^{13}C,^{15}N$-urea amended- and $^{13}C,^{15}N$-urea +C_2H_2 amended soils. In the soils amended with C_2H_2(30%, v/v), the reduction of N_2O to N_2(one important pathway of denitrification producing N_2)[36] and the anammox process[37] would be blocked by the C_2H_2. Furthermore, codenitrification could also be ruled out since the amino compounds were much lower than NH_4^+ in the examined paddy soils. Thus, in this study, Feammox could be considered the only source of $^{30}N_2$ and $^{29}N_2$ production in the $^{13}C^{15}N$-urea+C_2H_2 treatment. The contribution of each Feammox pathway to the N_2 production was calculated based on the production rate of $^{15}N-N_2$ ($^{29}N_2$ and $^{30}N_2$) in the $^{13}C,^{15}N$-urea amended (25.25−350.26 ng N g^{-1} d^{-1}) -and $^{13}C,^{15}N$-urea+C_2H_2 amended soils (18.96−93.17 ng N g^{-1} d^{-1}). Results revealed that direct production of N_2 by Feammox was accountable for 26.60−92.12% (average of 69.94%) of the total N_2 production, whereas the remaining could be attributed to anammox or/and denitrification using Feammox-produced NO_x^-. The high percentage of directly produced N_2 suggests that direct N_2 production is the dominant pathway of Feammox in paddy soils from Southern China. Ding et al. [9] have also demonstrated that most $^{30}N_2$ production (67−78%) in paddy soils can be attributed to direct N_2 production by Feammox. Theoretically, direct N_2 production in Feammox is more energetically favorable under a wider range of conditions than NO_x^- production, which yields less energy but consumes more Fe(III)-oxides.[8]

On the basis of the $^{15}N-N_2$ production rates measured during the anoxic incubation experiments, Feammox associated N loss from paddy soils (0−20 cm) was estimated at 1.63 Tg N per year, which accounts for ~6.91% of the applied N fertilizer in rice fields (23.62 Tg N per year in 2015) (China Statistical Yearbook, Agriculture section, http://www.stats.gov.cn/tjsj/ndsj/2016/indexeh.htm). Although the potential Feammox associated N loss in the paddy soils was lower compared to anammox (~12%),[26] Feammox is still an important pathway for N loss in paddy soils, and can improve our understanding of the N cycle in terrestrial ecosystems. However, the environmental importance of Feammox in paddy soils remains uncertain since the occurrence of anaerobic methane oxidation and anaerobic organic matter degradation, can affect the Feammox process in paddy soils by contributing to Fe(III) reduction.[38−41] Meanwhile, incubation of soil slurries with urea could provide excessive NH_4^+, which might overestimate the actual Feammox activity, and future studies are therefore needed to accurately estimate the in situ contributions of Feammox to N loss in paddy soils.

Identification of Functional Microorganisms Related to Feammox. RNA-SIP combined with 16S rRNA gene amplicon sequencing was adopted to identify functional microorganisms related to Feammox. *Geobacter* spp. were found to be abundant in the expressed community and were significantly enriched in the 16S rRNA gene pool from the $^{13}C^{15}N$-enriched RNA than in the $^{12}C^{14}N$ RNA during the anaerobic incubation across all examined paddy soils. Although *Geobacter* spp. were enriched in all soil, their phylotypes were different in response to various soil properties (i.e., pH). *Geobacter* spp. have been detected in various ecosystems including paddy soils,[22,42] sediments,[43] groundwater,[44] and biofilms.[45] *Geobacter* spp. are considered the most abundant putative iron-reducers in paddy soils[17,22] and also dominate the enrichments favoring Feammox process in paddy soil[12] due to its strong capacity of electron transfer in Fe(III) reduction.[46] *Geobacter* spp. can use various substrates (e.g., acetate, toluene, NH_4^+, and H_2, and so forth)[12,47,48] as electron donors and their pathways to obtain energy are highly dependent on different environmental settings.[49]

In addition to *Geobacter*, some genotypes affiliated within GOUTA19 group and Candidatus *Solibacter* were also enriched in all soils with different pH during 4-day incubation. The relative abundance of GOUTA19 was abundant with 1.93% in soils with low pH, but decreased to about 0.33% and 0.53% in soils with medium pH and high pH, indicating that GOUTA19 group were active during incubation, especially in the soils with low pH. A large number of GOUTA19 could be classified into Fe- and/or S-related bacteria.[50,51] GOUTA19 species were seldom detected in rice paddy soils, but dominated in paddy soils irrigated by acid mine drainage contaminated water,[50] which was consistent with this study. Interestingly, Nitrososphaeraceae, a family of archaea, was enriched during the incubation, especially in the soils with medium and high pH. Nitrososphaeraceae is an abundant ammonia-oxidizing archaea (AOA), which plays a key role in ammonia oxidation.[5,52,53] From this result, we suggested that archaea such as AOA may be associated with Feammox. However, the enrichment of Nitrososphaeraceae in heavy fractions from $^{13}C,^{15}N$-urea treatments could also attributed to carbon fixation of urea derived CO_2 during incubation.

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Environ. Sci. Technol. 2019, 53, 4841−4849

Pseudomonas have been reported as iron-reducing bacteria in many studies[54] and in this study *Pseudomonas* were active in soils with medium pH and high pH because of their abundance increase in ^{13}C,^{15}N-urea treatments compared with ^{12}C,^{14}N-urea treatments. Previous studies[15,55,56] indicated that members of *Acidimicrobiaceae* were active Feammox microbes in soil, however, *Acidimicrobiaceae* were not detected in this study. These results suggested that enriched active microbes were diverse and pH dependent during incubation, members from *Geobacter*, Nitrososphaeraceae, GOUTA19, and *Pseudomonas* may be potential drivers of Feammox in paddy soils.

Cluster analysis showed that these enriched active microbes that were potentially associated with Feammox were clearly separated into three groups according to soil pH. CAP analysis showed that pH and grain size were the most important factors influencing the microbial compositions associated with Feammox, followed by NH_4^+, C/N ratio, and total nitrogen (TN). Soil pH has been demonstrated to be a key factor controlling Feammox[55] by affecting the bioavailability of Fe(III) in soils.[57] Low soil pH would accelerate the release of Fe(III) and NH_4^+ ion, which is the substrates for Feammox. Yang et al.[8] have suggested that NO_2^- and NO_3^- production by Feammox might not occur in soils with pH > 6.5. However, in this study NO_x^- produced by Feammox was observed in all examined soils. Grain size would affect soil aeration, carbon availability and the transformation of nitrogen and iron, which could affect the community structure and activity of bacteria.[56,58] Higher soil organic matter ($P < 0.05$)) and C/N ratio had been detected in soils with high pH in our previous study,[26] which can increase the formation of Fe(III) oxides from clay minerals and can subsequently promote Fe(III) reduction[35] and would further promote the Feammox process and shape the microbial communities associated with Feammox in anoxic environments. In addition to the release of iron, high labile organic C and C/N ratios would also increase the activity of microbes such as anammox bacteria[26,59,60] and iron-reducing bacteria.[61]

Although RNA-SIP combined with 16S rRNA gene high-through sequencing is promising to identify active microbes in soil, there were limitations when this technique was adopted to identify Feammox microbes. The incorporated ^{13}C in active microbes should derived from $^{13}CO_2$ produced during hydrolysis of urea. Feammox microbes, anammox bacteria, and other carbon fixing bacteria such as Nitrososphaeraceae could incorporate $^{13}CO_2$ into cells. Nevertheless, the growth of anammox bacteria need the production of NO_2^- by Feammox microbes, and enrichment of anammox bacteria were not detected in this study. Carbon fixing bacteria could be another group of labeled microbes and would be identified as active microbes but might not participate in Feammox. Although previous studies[17,22] have identified some potential functional microbes associated with iron-reduction in paddy soils and even rare functional microbes associated with pollutant degradation[62] using this technique, RNA-SIP results of this study can only identify active microbes under the conditions that favored Feammox and the growth of Feammox microbes. These active microbes may contribute to Feammox in paddy soil, however, isolation of pure strain or metagenomic analysis of enrichment cultures are needed to identify active microbes responsible for Feammox and the mechanisms underlying Feammox.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b05016.

Figure S1. Sampling sites of paddy soil collected in this study; and Figure S2. Quantitative distribution of 16S rRNA genes across the entire bouyant density gradient of the RNA fractions from soil microcosms (PDF)

Table S1. Classification of each enriched OTU in low pH, medium pH, and high pH amended with ^{13}C,^{15}N-urea (XLSX)

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