

Article

Effects of Soil pH on Gaseous Nitrogen Loss Pathway via Feammox Process

Ding Ma ¹, Jin Wang ², Jun Xue ³, Zhengbo Yue ^{2,*}, Shaofeng Xia ³, Lei Song ³ and Hongwen Gao ^{1,*}

¹ College of Environmental Science and Engineering, Tongji University, Shanghai 200092, China; 1610377@tongji.edu.cn

² School of Resources and Environmental Engineering, Hefei University of Technology, Hefei 230009, China; sophiawj@hfut.edu.cn

³ Hefei Municipal Design & Research Institute Co., Ltd., Hefei 230009, China; xueyeah2020@163.com (J.X.); xshf1225@163.com (S.X.); songlei3424@163.com (L.S.)

* Correspondence: zbyue@hfut.edu.cn (Z.Y.); hwgao@tongji.edu.cn (H.G.)

Abstract: The application of N fertilizer is one of the most critical soil acidification factors in China, and soil acidification significantly alters biogeochemical processes such as N loss. Anaerobic ammonium oxidation coupled with iron reduction (Feammox) is an important biological process for N loss in natural environments, with the end-products of N₂, NO₂⁻ and NO₃⁻. However, the response of Feammox pathways to soil pH fluctuation has not been thoroughly studied. In the current study, Feammox pathways and microbial communities were explored through a slurry culture experiment with an artificially adjusted pH combined with a ¹⁵N isotope tracing technique and molecular biotechnology. Results showed significant differences in the gaseous N loss through Feammox (0.42–0.97 mg N kg⁻¹ d⁻¹) under different pH conditions. The gaseous N loss pathways were significantly affected by the pH, and Feammox to N₂ was the predominant pathway in low-pH incubations. The proportion of N loss caused by Feammox coupled with denitrification increased as the soil pH increased. The gaseous N loss through Feammox increased by 43.9% when the soil pH decreased from 6.5 to 5.0. Fe-reducing bacteria, such as *Ochrobactrum*, *Sphingomonas*, and *Clostridium* increased significantly in lower pH incubations. Overall, this study demonstrated the effects of soil pH on Feammox pathways and extended the understanding of the N biogeochemical cycle in acidic soil.

Keywords: Feammox; soil pH; acidification; N loss



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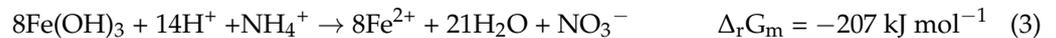
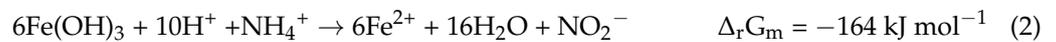
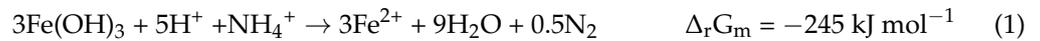
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1. Introduction

With the decrease of arable land area and the decrease of soil fertility, the dependence of agriculture on chemical fertilizers is increasingly serious [1]. The overuse of Nitrogen (N) fertilizer has caused critical environmental problems, such as soil acidification [2]. It was found that the soil pH in the major grain-producing regions in China has decreased significantly since 1980, and the contribution of fertilization to H⁺ is much higher than that of acid deposition [3]. Soil acidification has adverse effects on terrestrial ecosystems and causes a series of secondary problems, such as the deterioration of soil properties and the activation of heavy metals [4].

A large amount of N fertilizer is applied to cultivated land in the agricultural production process. However, only about 35% of the N can be used by crops, and the remaining N enters the water and atmosphere through leaching and microbial metabolism [5]. Recently, anaerobic ammonium oxidation coupled with iron reduction (Feammox) has been reported as a novel microbial process involved in the soil N cycle [6–9]. NH₄⁺ is directly oxidized to N₂ (Equation (1)), NO₂⁻ (Equation (2)), and NO₃⁻ (Equation (3)), by microorganisms using Fe(III) as an electron acceptor under anaerobic conditions in the Feammox process [10,11]. Compared to the nitrification-denitrification process, the Feammox process shortens the N

cycle time, and makes a great contribution to N loss in ecosystems. It was estimated that a loss of 7.8–61.0 kg N ha⁻¹ y⁻¹ was associated with Feammox, representing 3.9–31.0% of the N fertilizer applied to paddy soils in southern China [7].



As an important environmental factor, the soil pH significantly influences the microbial N cycle, including the processes of aerobic ammonia oxidation, denitrification, nitrification, and anammox. Denitrification and anammox processes are generally inhibited under acidic conditions, whereas Feammox might favor lower-pH environments as the process needs a ready supply of H⁺ ions [12,13]. A canonical correlation analysis showed that pH is one of the key factors affecting the rate of Feammox according to the analysis of 66 soil samples collected from different soil/sediments [14]. Moreover, *Acidimicrobiaceae* A6 has been identified as the only Feammox functional microorganism which can oxidize NH₄⁺ to NO₂⁻, was also isolated from acidic wetland sediment and showed the highest NH₄⁺ oxidation rates at pH 4 [11]. Nevertheless, there are three end-products (N₂, NO₂⁻ and NO₃⁻) in Feammox process (Equations (1)–(3)), and the effect of soil pH changes on Feammox pathways has not been thoroughly studied. According to thermodynamic calculations, Feammox to N₂ is more likely to occur than the other two pathways (Equation (1)). Although the above conclusion is supported by recent studies, two other Feammox pathways with the end-products of NO₂⁻ and NO₃⁻ are noteworthy [6,7]. Especially in acidic soils/sediments, the activities of microorganisms such as denitrification and anammox are inhibited, and NO₂⁻ produced through Feammox is prone to accumulation, which can further lead to water pollution or greenhouse gas (e.g., N₂O) emission [12]. With the burning of fossil fuels and the extensive use of chemical fertilizers, the situation of soil acidification of cultivated land is gradually increasing [3]. Therefore, it is of great significance to explore the influence of soil pH changes on the rates and pathways of Feammox for controlling N loss in agricultural fertilization and reducing the risk of environmental pollution.

Red soil is one of the most important soil types in South China, and its high content of iron oxides lays a foundation for the occurrence of Feammox. Meanwhile, soil acidification is common in southern China, and it restricts crop growth and poses a severe threat to food security [3]. The main objectives of this study were to explore the effects of soil pH on the pathways of Feammox and the subsequent N transformation. The microbial communities of Feammox and their responses to soil pH also have been investigated.

2. Materials and Methods

2.1. Isotopic Tracer Incubation

Soil samples were collected from Yiliang County, Kunming, China (24°47' N, 103°1' E) in November 2017. The average annual temperature of the site is 16.3 °C, and the annual precipitation is 1000 mm. The region has a subtropical monsoon and the soil type is red soil, with long-term crop cultivation (mainly dryland crops). Five samples were collected within a range of 10 × 10 m in the area (0–10 cm depth) and then mixed together. The samples were transported to the laboratory in an icebox within 24 h and divided into 3 subsamples. One subsample was air-dried for analyzing the soil properties, the second subsample was used for isotope incubation, and the remaining subsample was stored in the freezer at −80 °C. The soil pH was 6.5 ± 0.3, and the water content was 48.8 ± 0.2%. The total Fe, TOC, NH₄⁺-N, and NO₃⁻-N contents were 87.7 ± 3.5 g·kg⁻¹, 70.0 ± 0.8 g·kg⁻¹, 40.9 ± 0.3 mg·kg⁻¹, and 1.9 ± 0.2 mg·kg⁻¹, respectively. NO₂⁻-N was not detectable in the soil.

Isotope tracer incubation was conducted in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI, USA) filled with ultra-pure He using the procedure slightly modified from Ding et al. [7]. Prior to incubation, sterile anoxic deionized water was added to the

soils at a ratio of 3:1 (*v/w*) and pre-incubated in an anaerobic glove box in the dark at 20 °C for 1 week to remove background O₂ and NO_x⁻. After pre-incubation, the O₂ and NO_x⁻ concentration was maintained at a low level (<0.1 mg·L⁻¹ and <1 mg·kg⁻¹, respectively).

After pre-incubation, 21.5 g of homogenized slurries were transferred into 50 mL serum vials. The soil acidification process was simulated by manually adjusting the slurry pH. Three pH groups (5.0 ± 0.1, 6.5 ± 0.1, and 8.0 ± 0.1) were set in the incubation, representing acidic, weakly acidic, and alkaline soil pH. 1 mM NaOH and HCl solutions were used to adjust the pH of the slurries to the preset values. The pH of the slurries was adjusted again to the set values after 12 h to reduce the influence of the soil acid-base buffer on the experiment. In each pH group, 3 treatments (*n* = 5 per treatment) were established to detect Feammox, namely (1) the control with only sterile anoxic deionized water; (2) ¹⁵NH₄Cl addition (¹⁵NH₄⁺, 99 atom %; Sigma-Aldrich, St. Louis, MO, USA); and (3) ¹⁵NH₄Cl and C₂H₂ addition (¹⁵NH₄Cl + C₂H₂). It has been reported that C₂H₂ (acetylene) can inhibit the reduction of N₂O to N₂ in denitrification and the activity of anammox bacteria [15,16]. The serum vials were then sealed with butyl rubber stoppers, and the headspace was exchanged with ultra-pure Ar for 5 min. The ¹⁵NH₄Cl solution was prepared using sterile anoxic deionized water, and 0.5 mL of ¹⁵NH₄Cl solution was injected into the serum vials with sterile syringes so that the final concentration of ¹⁵NH₄Cl was 5 mM. For the C₂H₂ treatment, 7.5 mL of headspace gas in each vial was removed and replaced with 7.5 mL of C₂H₂ purified by H₂SO₄ to reach 30% (*v/v*) C₂H₂ in the headspace [7]. Isotope tracer incubations were operated at 20 °C in a constant temperature incubator, and all the vials were shaken vigorously to homogenize the treatment solutions daily. A set of vials was destructively sampled in an anaerobic glove box at 0, 10, 20, and 30 d. All the vials were shaken vigorously before sampling to equilibrate the gas between the dissolved and gaseous phases. Five milliliters of the headspace gas were extracted with sterile syringes and injected into a 12 mL pre-vacuumed glass tube (Labco Co., Ltd., High Wycombe, UK). The ¹⁵N enrichment in N₂ was determined by isotope ratio mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations of ¹⁵N₂ were calculated according to the production of N₂ and its concentrations above the natural abundance [7].

The potential Feammox rate was conservatively estimated based on the difference in the ³⁰N₂ production between the treatment with ¹⁵NH₄Cl and the control. The N₂ production rates were calculated from the changes in the N₂ concentrations between two given times. The Feammox pathways were distinguished by adding C₂H₂, and the concentration of C₂H₂ used in this study was able to completely inhibit the denitrification and anammox processes [7]. The Feammox-N₂ rate (Equation (1)) was estimated by the ³⁰N₂ production rate in the ¹⁵NH₄⁺ + C₂H₂ treatment [6].

2.2. Anaerobic Chemodenitrification Experiment

In order to explore the effects of pH on the chemodenitrification reaction under anaerobic conditions, a series of experiments was conducted. The Fe²⁺ and NO₂⁻ solutions were prepared with anoxic deionized water. A 10 mL 5 mM Fe²⁺ solution was injected into 50 mL serum vials, and then the initial pH was adjusted to 4.0–7.0 using NaOH and HCl solutions. After exchanging the headspace with ultra-pure Ar, the serum vials were sealed with butyl rubber stoppers. An NO₂⁻ solution was added into the serum vials with sterile syringes to make the concentration of NO₂⁻ in the reaction system reach 2 mM. The serum vials with only NO₂⁻ or Fe²⁺ were set as controls at pH 5.0. The experiments were repeated in triplicate. The serum vials were incubated at 30 °C in a constant temperature incubator in the dark for 100 h. The supernatant was extracted at setting time, and the concentration of the NO₂⁻ was determined by ion chromatography (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Physicochemical Analysis

The soil pH was determined using a pH meter (Mettler-Toledo, Zurich, Switzerland), and the ratio of deionized water to soil was 2.5:1 (*v/w*). The total organic C (TOC) content was determined by the potassium dichromate oxidation method [17]. The NH_4^+ and NO_x^- were extracted with a 2 M KCl solution for 1 h and determined by a continuous-flow nutrient auto-analyzer (Skalar Analytic, Breda, The Netherlands). The total extractable Fe(III) and Fe(II) in the soil were determined according to the slightly modified method of Ding et al. [7]. Briefly, 1.0 g of soil was extracted with 5 mL of 0.50 M HCl and 5 mL of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl for 2 h at 30 °C in an anaerobic glove box. The ferrozine method was used to measure the total Fe and Fe(II) contents [18]. The ^{15}N enrichment in the N_2 was determined by isotope ratio mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Microbial Community Analysis

DNA samples were extracted from 1.0 g of soil slurry collected after 30 d of incubation and amplified using the primer pair 515F and 806R for the V4 region of the 16S rRNA gene as described by the manufacturer's protocol. Sequencing was conducted at a commercial company (Majorbio, Shanghai, China). The microbial community functional profiles were predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). DNA samples were extracted from 1.0 g of soil slurry collected after 30 days' incubation using a Fast DNA SPIN Kit for soil (MP Biomedical, Solon, OH, USA), according to the manufacturer's protocol. The quality and purity of the extracted DNA were determined using the NanoDrop-2000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA) and then stored in the refrigerator at -20 °C. The extracted DNA was amplified using the primer pair 515F and 806R for V4 region of the 16S rRNA gene. PCRs were performed in a total volume of 30 μL containing 15 μL . 2 \times Taq master Mix, 1 μL Bar-PCR primer F (10 μM), 1 μL Primer R (10 μM), 10–20 ng Genomic DNA, dd H_2O added to 30 μL . The PCR program was: 94 °C for 3 min (Initial steps), 28 cycles of 94 °C for 30 s (Melt), 53 °C for 40 s (Anneal), 72 °C for 5 min (Extend). A mixture of the 16S rRNA PCR products was used for sequencing on the Illumina Miseq platform. Sequencing was conducted at a commercial company (Majorbio, Shanghai, China).

The microbial community functional profiles were predicted by using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). The 16S rRNA gene sequences were compared in the KEGG database (www.genome.jp/kegg/pathway.html, (accessed on 7 June 2018)) to analyze the relative abundance of the KEGG orthologous groups associated with the N cycle in metabolic pathways. The predicted functional data were clustered according to the abundance distribution of functional groups or the similarity between samples. The functional groups and samples were sorted according to the clustering results and then presented by the heat map. R-project was used for cluster analysis and heat map drawing (www.R-project.org, (accessed on 20 June 2018)).

2.5. Statistical Analyses

One-way analysis of variance and Pearson's correlation analysis were conducted using SPSS 22.0 (SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was set to denote the significance level. One-way ANOVA analyses were used to calculate the variance between obtained data under different pH values. Pearson's correlation analysis was performed to evaluate the relationships among the nitrite concentrations, Feammox rates, and Fe(III) reduction rates. The figures in this study were drawn using Origin 9.0 (Origin Lab, Northampton, MA, USA).

3. Results and Discussion

3.1. pH-Dependent Feammox Rates and Pathways

For all the pH groups, a significant amount of $^{30}\text{N}_2$ was detected in the $^{15}\text{NH}_4^+$ and $^{15}\text{NH}_4^+ + \text{C}_2\text{H}_2$ treatments, whereas no $^{30}\text{N}_2$ accumulated in the control (Figure 1a). The

$^{30}\text{N}_2$ production rates were significantly ($p < 0.05$) higher in the 5.0 pH group than those in the 6.5 and 8.0 pH groups, varying from $0.94 \pm 0.08 \text{ mg N kg}^{-1} \text{ d}^{-1}$ in the 5.0 pH group to $0.42 \pm 0.03 \text{ mg N kg}^{-1} \text{ d}^{-1}$ in the $^{15}\text{NH}_4^+$ addition treatments in the 8.0 pH group. The variations in the $^{29}\text{N}_2$ production rate with pH were identical to those of the $^{30}\text{N}_2$. However, the $^{29}\text{N}_2$ production rates were higher than those of the $^{30}\text{N}_2$ in all the groups.

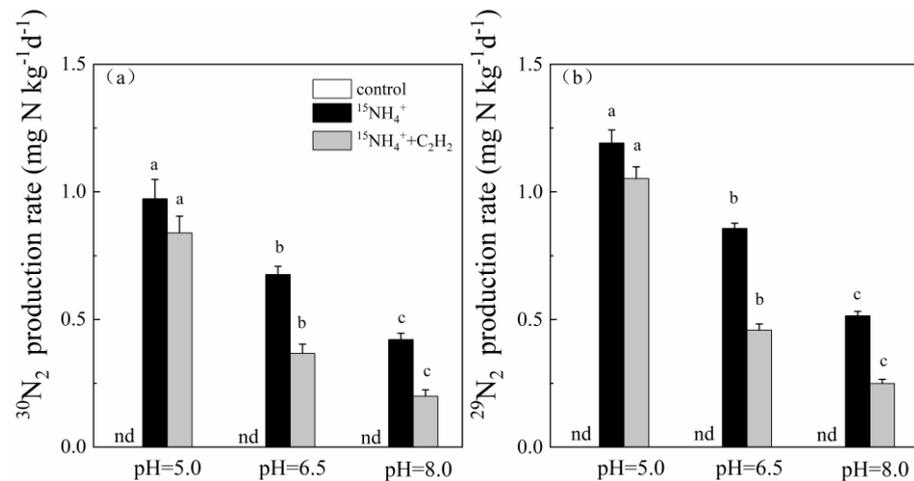


Figure 1. Mean headspace $^{30}\text{N}_2$ (a) and $^{29}\text{N}_2$ (b) production rates in the control, $^{15}\text{NH}_4^+$ and $^{15}\text{NH}_4^+ + \text{C}_2\text{H}_2$ treatments under three different pH. The different small letters above the column denote statistically significant ($p < 0.05$) differences in different pH groups.

In this study, the soil slurries were strictly pre-incubated in an anoxic environment to remove indigenous molecular O_2 , NO_3^- , and NO_2^- . Strict anaerobic conditions were maintained during the ^{15}N isotope incubation. Meanwhile, the 16S rRNA sequencing results showed that no typical anammox bacteria were found in any of the experimental treatments. Therefore, the aerobic nitrification and anammox processes can be ruled out. Under these conditions, the Feammox to N_2 (Equation (1)) and Feammox-generated $^{15}\text{NO}_x^-$ (Equations (2) and (3)) reduction by denitrification are the only potential biological pathways for $^{30}\text{N}_2$ production. Moreover, $^{15}\text{NH}_4^+$ promoted the Fe(III) reduction rates in the incubations, thereby further providing robust evidence for the occurrence of the Feammox process (Supplementary Figure S1). The Feammox rates in our incubation ($0.42\text{--}0.94 \text{ mg N kg}^{-1} \text{ d}^{-1}$) were of the same order of magnitude as those reported in natural environments (Figure 1 and Supplementary Table S1). The production rates of $^{30}\text{N}_2$ and $^{29}\text{N}_2$ in the 5.0 pH groups (with and without C_2H_2) were significantly ($p < 0.05$) higher than those of the other pH groups, and there were higher initial pH values with lower Feammox rates (Figure 1). The significantly higher Feammox rates in the acid red soil than those in natural environments indicated that acid soil is more beneficial to the occurrence of Feammox (Supplementary, Table S1). However, it has been reported that a higher pH and lower Eh are beneficial to Feammox in mangrove wetlands [19], and the Feammox process also occurs in alkaline sediments (e.g., the intertidal wetlands of the Yangtze Estuary) [8]. The responses of the Feammox to soil pH fluctuations are not thoroughly understood. Fe oxides in the soil will release more soluble Fe(III) when the pH decreases [20], and there might be higher microbially reducible Fe(III) contents in acidic soil, which can be beneficial for providing more electron acceptors for the Feammox.

The thermodynamic calculations showed that the Feammox- N_2 pathway could be conducted under a wide range of pH conditions, but the Feammox- NO_2^- pathway occurs only when the pH is less than 6.5 [6]. Previous studies have shown that Feammox- N_2 is the main pathway of gaseous N loss in the Feammox process (>50%), but the effect of soil pH on the Feammox pathways has been rarely reported. The Feammox- N_2 pathway accounted for 47.4–86.2% of the total $^{30}\text{N}_2$ loss in all the pH groups, and the proportion was significantly ($p < 0.05$) higher in the low-pH group (Supplementary Figure S2). The gaseous N loss ratio

contributed by the Feammox coupled with denitrification (Feammox-denitrification) was 52.6% at a pH of 8.0. Although the PICRUST results showed a significant increase in the denitrifying microbial abundance at a low pH, the gaseous N loss rate from the Feammox-denitrification pathway remained low in the pH 5.0 group (Supplementary Figure S2). This may be due to the large amount of NO_2^- produced by Feammox under low pH conditions, which promoted the growth of denitrifying bacteria. However, the optimal pH value of the denitrifying bacteria was 7.0–9.0, and the activity of the denitrifying bacteria decreased under low pH conditions [12].

3.2. Potential Transformation Pathways of NO_2^-

The concentrations of NO_3^- -N and NO_2^- -N in the serum samples were measured at the end of the incubation (Figure 2). The concentrations of NO_2^- -N were significantly ($p < 0.05$) higher than that of the NO_3^- -N in each pH group. For the three different pH groups, the concentrations of NO_2^- -N in the $^{15}\text{NH}_4^+$ and $^{15}\text{NH}_4^+ + \text{C}_2\text{H}_2$ treatments at a pH of 5.0 were $4.84 \pm 0.46 \text{ mg}\cdot\text{kg}^{-1}$ and $6.13 \pm 0.47 \text{ mg}\cdot\text{kg}^{-1}$, respectively, which were significantly higher than those in the other pH conditions ($p < 0.05$). The linear regression analysis showed a positive correlation between the NO_2^- -N contents and the Feammox rate of $^{30}\text{N}_2$ in all the treatments (Supplementary Figure S3), which further indicated that NO_2^- is an important intermediate product of the Feammox- N_2 pathway (Equation (2)). NO_2^- is one of the primary end-products of Feammox in natural and wastewater treatment systems and plays a vital role in the transformation of the Fe cycle [10,21,22]. The Feammox- NO_2^- pathway and the further transformation process of NO_2^- are worthy of attention.

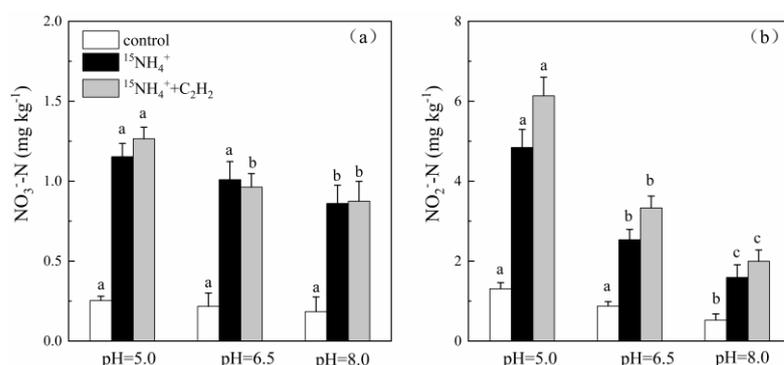


Figure 2. Concentration of NO_3^- -N (a) and NO_2^- -N (b) in anaerobic incubations on day 30. The different small letters above the column denote statistically significant ($p < 0.05$) differences in different pH groups.

Previous studies have shown that the Fe(III) reduction rates are significantly positively correlated with the N_2 production rates [6,7,9]. The fluctuation in the Fe(III) reduction rate was not clear among the three pH groups in our study (Supplementary Figure S1). However, the $^{30}\text{N}_2$ production rate was significantly increased in the 5.0 pH groups. The gaseous N production rate in the $^{15}\text{NH}_4^+ + \text{C}_2\text{H}_2$ treatment was also significantly increased at a pH of 5.0. This meant that the chemodenitrification reaction occurred and played an important role in the 5.0 pH groups because the biological reduction of NO_x^- was completely inhibited. It has been reported that NO_2^- can be reduced to N_2 with Fe(II) through chemodenitrification under anaerobic conditions (Equation (4)), which may be one of the potential pathways of N_2 loss in the Feammox process [21,23]. The kinetics of the chemodenitrification reaction under different pH values indicated that the NO_2^- reduction rates increased with the decrease in the pH (Figure 3). It has been demonstrated that chemodenitrification makes a greater contribution to NO_2^- decomposition in sterilized acid soil [24]. The increase in the NO_2^- reduction rate at a low pH may be due to the higher proportion of HNO_2 in the liquid phase. The protonation of NO_2^- promotes the fracture of N-O, thereby resulting in a more robust oxidation of HNO_2 than NO_2^- [25].

This confirmed that Feammox-generated Fe(II) was more likely to react with NO_2^- in the low-pH groups and further promote the N and Fe biogeochemical cycles.

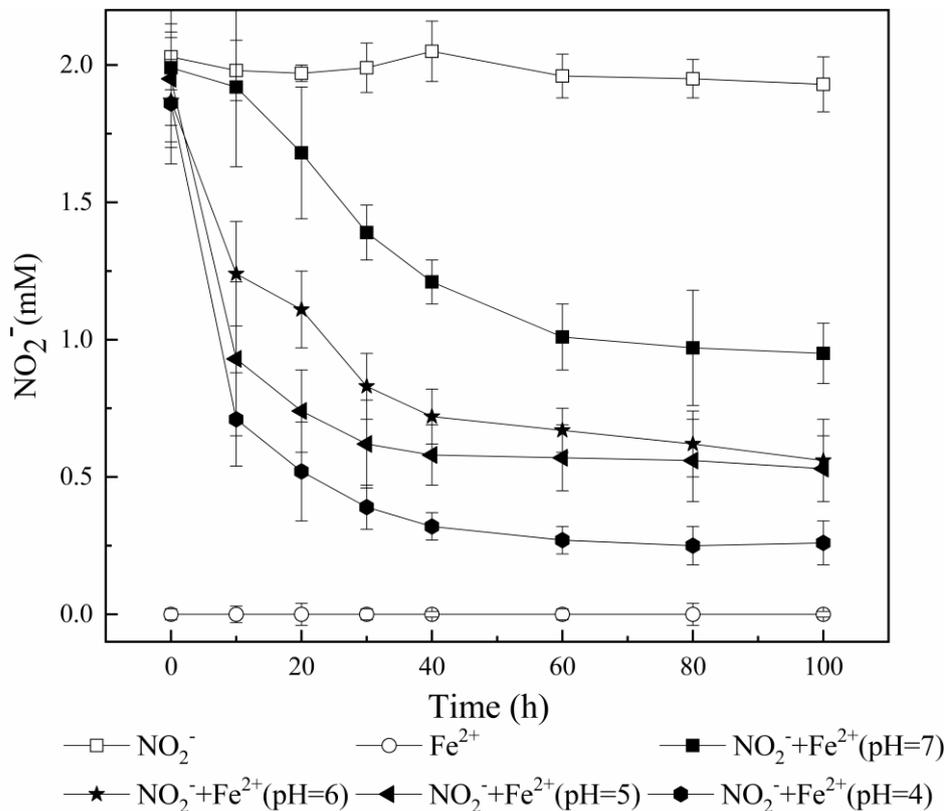
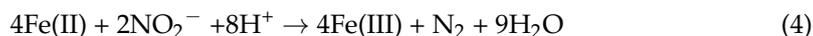


Figure 3. Anaerobic chemodenitrification at different pH values.

Amorphous Fe oxides in the soil will release more soluble Fe(III) when the pH decreases [20], which would promote the Feammox process and provide more Fe(II). Studies have shown that the surface of soil minerals can reaggregate the Fe(II) produced by microorganisms and accelerate the transfer of electrons to NO_2^- . Meanwhile, the Fe(II) adsorbed on the soil minerals has a higher oxidizability in the reaction between Fe(II) and NO_2^- compared with that of dissolved Fe^{2+} [26]. Therefore, soil acidification will facilitate Fe(II) generation in the Feammox process and the chemical reduction of NO_2^- induced by Fe(II). The Fe(III) reduction was initiated by the Feammox process, and the generated Fe(II) was oxidized to Fe(III) by the NO_2^- -N reduction. Owing to the lack of continuous data on the NO_2^- -N concentration, the extent of Feammox- NO_2^- could not be accurately calculated in our study.

3.3. Evolution of the Microbial Community in Feammox

Proteobacteria was the dominant phylum in the 5.0 pH group in this study, with a relative abundance of 43.3–44.9% (Figure 4). It has been reported that *Ochrobactrum* and *Sphingomonas* from Proteobacteria might contribute to a metals and NO_x^- reduction, and are more abundant in acidic incubations [27–30]. The relative abundance of Firmicutes significantly increased ($p < 0.05$) at a pH of 5.0. *Clostridium* accounted for 6.68–15.59% of all the microorganisms, which was possibly because the genus is more suitable to more acidic environments. *Clostridium* species have been reported to reduce Fe(III) and NO_3^- using acetate as an electron donor [31,32]. Our results also demonstrated that the Acidimicrobiaceae family had a higher abundance in the low-pH group. Although the genus information could not be obtained at the 97% OTU similarity level for Acidimicrobiaceae in

this study, Acidimicrobiaceae has been classified as Fe-reducing and Feammox bacteria [22]. *Acidimicrobiaceae* bacterium A6 has been widely reported and identified as the functional microorganism responsible for the Feammox process [14,22].

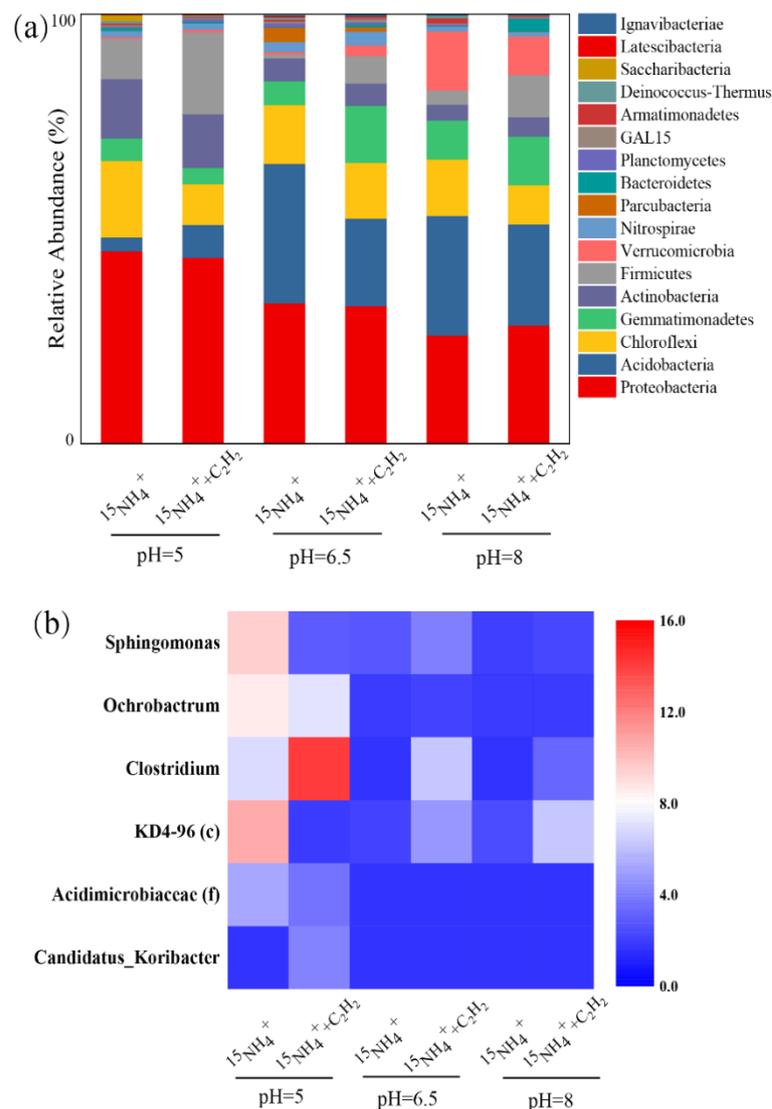


Figure 4. Relative abundance of main phyla (a) and genera (b) in the incubations.

Owing to the positive relationship between Fe-reducing bacteria and Feammox rates, researchers believe that Fe-reducing bacteria play a vital role in Feammox. The reported Fe-reducing bacteria have shown diverse communities in ecosystems, including *Geobacter*, *Shewanella*, *Anaeromyxobacter*, and *Pseudomonas* [9,33,34]. However, the Feammox-N₂ functional microorganisms have not yet been identified. *Geobacter* and *Shewanella* were not detected in any of the treatments in this study. *Geobacter* would disappear during long-term Feammox culture [22]. In this study, the diversity of Fe-reducing bacteria changed significantly with the soil pH. *Ochrobactrum*, *Sphingomonas*, and *Clostridium* may play significant roles in Fe reduction in the Feammox culture system at a pH of 5.0.

The effects of pH on the microbial community functional profiles were investigated using PICRUSt (Figure 5). The results showed that the relative abundances of the clusters of orthologous groups in the 5.0 pH groups, which were associated with NO₃⁻ and NO₂⁻ reduction or transformation (K00370–K00374, K00376, and K02305), were significantly higher than those at other pH values ($p < 0.05$). In this study, the detected NO_x⁻ in the incubation was generated from the Feammox process. The low environmental pH promoted

the Feammox- NO_2^- pathway (Equation (2)) and stimulated denitrifying microbial growth, which was consistent with the PICRUSt results. Although most denitrifiers have been reported in neutral and weakly alkaline environments, acidic environments can stimulate the growth of specific denitrifiers, such as *Ochrobactrum* and *Clostridium* [28,32]. In addition, the long-term culture of the Feammox system also showed that the abundance of the *nirS* and *nirK* genes increased, thereby demonstrating that denitrification was also active during the Feammox process [14,22].

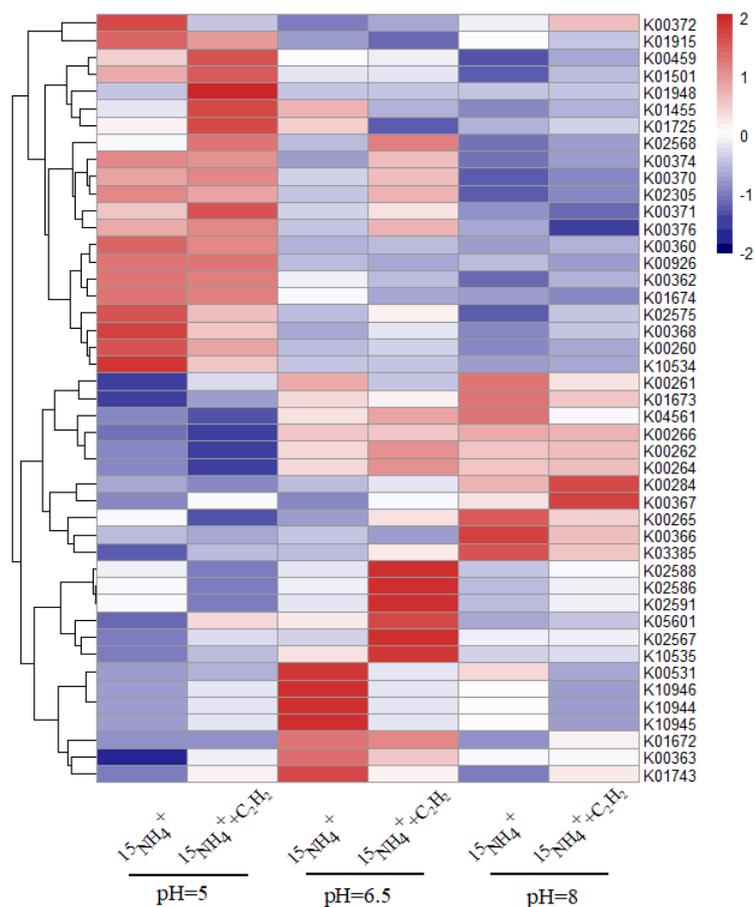


Figure 5. Heat map of KO (KEGG orthology) abundance associated with the nitrogen cycle based on PICRUSt.

3.4. Environmental Implications

Compared with that of previous studies, the high Feammox rate could be attributed to the low pH and high Fe content in the soil explored in this study. The soil was collected from the subtropical region of Southern China, which has a severe soil acidification problem. Based on the rates obtained from isotope incubations, the gaseous N loss via the Feammox process increased by 43.9% when the pH decreased from 6.5 to 5.0 in red soils. This result indicated that Feammox could be a vital pathway for N loss in acidic soils. However, the strict anaerobic conditions and high NH_4^+ concentrations in the incubations possibly led to the overestimation of the Feammox rates, and precise evaluation is needed to perform in situ studies.

Theoretically, the oxidation of 1 mol of NH_4^+ will consume 5–14 protons in the Feammox process, and the efficiency of neutralizing H^+ is much higher than that of lime, which was generally used to mitigate soil acidification (Equation (1)). However, the excessive lime application reduces the availability of nutrients in the soil, and additional agricultural measures are required to thoroughly mix the lime with the soil [35,36]. It has been reported that the pH value increases with the incubation and NH_4^+ dosage in the Feammox process [10]. Similarly, the pH increased significantly at the end of our

incubation in the 5.0 pH group (data not shown). Therefore, the Feammox process may play an essential role in the acid-base equilibrium in soils. Feammox is theoretically feasible for remediating acidified soil, and its effects still need to be investigated through further experiments.

4. Conclusions

This study demonstrated that Feammox is a crucial gaseous N loss pathway in acidic upland soil ($0.42\text{--}0.97\text{ mg N kg}^{-1}\text{ d}^{-1}$), and the Feammox pathways were also affected by the soil pH. Specifically, N_2 was mainly directly generated by the Feammox process in acidic soils, while most of the N_2 loss was attributed to Feammox-coupled denitrification in high-pH soils. The gaseous N loss through Feammox increased by 43.9% when the soil pH decreased from 6.5 to 5.0. In addition, NO_2^- is an important end-product of Feammox in acidic soil and is possibly reduced by Fe(II) through the abiotic/biotic process. Moreover, the relative abundances of Acidimicrobiaceae and Fe-reducing bacteria increased significantly under acidic conditions. This study provides experimental evidence of the influence of soil acidification on the Feammox pathways and new insights into the interaction between N application and soil acidification.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/su131810393/s1>, Figure S1: Fe(III) reduction rates measured in the isotope tracer incubations, Figure S2: The contribution of Feammox to N_2 pathway (Feammox- N_2) to gaseous N loss, Figure S3: Pearson's correlations of nitrite concentrations with both Feammox rates (a) and Fe(III) reduction rates (b), Table S1: Research on Feammox in natural environments.

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