Transformation of Nitrogen and Iron Species during Nitrogen Removal from Wastewater via Feammox by Adding Ferrihydrite

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ABSTRACT: Fe(III) reduction coupled to anaerobic ammonium oxidation, namely, Feammox, plays an important role in the Fe/N cycle in natural environments. However, it has been rarely studied in wastewater treatment systems. To date, transformation of nitrogen and iron species of Feammox during anaerobic digestion remain unknown. In this study, ferrihydrite was supplemented (50 mM) in an anaerobic digester to remove ammonium through Feammox. Results showed that the ammonium removal efficiency after 63 days reached 69.49%, significantly higher than that of the control (35.63%). X-ray diffraction analysis showed that ferrihydrite was transformed into magnetite and akaganeite after the experiment. Further study found that Fe(II) could be oxidized by NO$_3^-$ in the inoculants taken from the ferrihydrite-supplemented group, i.e., possible NO$_3^-$-dependent Fe(II) oxidation could provide the possibility for further Feammox. Microbial analysis showed that iron-reducing bacteria and iron-oxidizing bacteria were both detected in two groups.

KEYWORDS: Feammox, NO$_3^-$-dependent Fe(II) oxidation, Anaerobic digestion, Nitrogen loss

INTRODUCTION

In general, the nitrification–denitrification and anaerobic ammonium oxidation (anammox) processes have been considered as the two main approaches to biological nitrogen removal. The nitrification–denitrification process operated in separate aerobic and anaerobic tanks is energy intensive and requires amendment with extra carbon sources in many low C/N wastewaters. The anammox process, which removes ammonium with nitrite as the electron acceptor under anoxic conditions (NH$_4^+ + NO_2^- \rightarrow N_2 + 2\text{H}_2\text{O}$), is sensitive to the environmental conditions, and the proliferation of anammox bacteria is slow.

Recently, Feammox, i.e., ferric iron [Fe(III)] reduction coupled to anaerobic ammonium oxidation, has been reported to play an important role in the nitrogen cycles of natural environments. In Feammox, Fe(III) is reduced to Fe(II), accompanied by oxidation of NH$_4^+$ to N$_2$, NO$_3^-$, and NO$_2^-$ (eqs 1–3). Feammox has been estimated to metabolize 7.8–61 (3.9%–31% of nitrogen fertilizer loss) kg of NH$_4^+$/ha/year in paddy soil. In our previous study, it was found that 20.1% total nitrogen removal was achieved when supplementing Fe(OH)$_3$ in a high-ammonium anaerobic sludge digester. Although the efficiency was not high, Feammox had showed its potential to run as an in-site anaerobic ammonium treatment process. However, little is known about the transformation and possible interactions of nitrogen and iron species during Feammox as well as the potential effects on the nitrogen removal in anaerobic wastewater treatment. For example, NO$_3^-$ as a product of Feammox was reported to be capable of oxidizing Fe(II) to Fe(III), termed as NO$_3^-$-dependent Fe(II) oxidation (NDFO). In other words, Fe(III) is likely regenerated by NDFO.

Therefore, the aim of this study was to investigate the effects of Feammox on nitrogen removal, including the following contents: (1) changes in the nitrogen and iron species during Feammox, (2) possible interaction during ferrihydrite related Feammox process and their potential effects on the nitrogen removal, and (3) bacterial communities analysis.
was synthesized by adding Fe(III) chloride to water that was maintained at a pH of 6.8–7.2.\textsuperscript{12} Morphology of the synthesized ferrihydrite characterized by a scanning electron microscope is shown in Figure S1. Prior to incubation, the sterile anoxic denitized water (autoclaved at 120 °C for 20 min) was added into the inoculant sludge at a ratio of 5:1 (v/v) and preincubated anoxically in the dark at 25 °C for 1 day statically; then, the supernatant after centrifugation was poured out to remove indigenous NH₄⁺ and NO₃⁻. The above procedure was repeated three times. Then, the prepared sludge was used as the inoculant sludge.

Two batch experiments were conducted in 200 mL serum vials: a control group and a ferrihydrite group. Here, 10 mL of prepared inoculant sludge was added into each vial as the seed sludge. Ferrihydrite was supplemented in the ferrihydrite group with a dose of 50 mM, while Fe(III) was not supplemented in the control. Then, NH₄⁺-containing medium was added to ensure that the initial volume of each serum vial was 150 mL. The medium (pH 5.0) contains MgCl₂·6H₂O (0.4 g/L), CaCl₂·2H₂O (0.1 g/L), NH₄Cl (0.027 g/L), KH₂PO₄ (0.6 g/L), 1 mL/L of a vitamin solution, 1 mL/L of a trace element solution, and 30 mM bicarbonate buffer.\textsuperscript{17} Afterward, the serum vials were sealed with butyl rubber septa and crimped with aluminum caps. The headspace of the vial was flushed with N₂ and CO₂ (80%/20%) for 30 min. Then, the vials were incubated statically at 25 °C in the dark for 87 days. The experiments were repeated in triplicate. The initial parameters of the mixture in the control and ferrihydrite groups (before adding ferrihydrite) are shown in Table 1.

### Table 1. Main Initial Characteristics before Incubation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Initial value</th>
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<tr>
<td>TCOD\textsuperscript{a}</td>
<td>4515 ± 1.62 mg/L</td>
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<tr>
<td>Total polysaccharide</td>
<td>306.56 ± 11.69 mg/L</td>
</tr>
<tr>
<td>Total protein</td>
<td>191.87 ± 14.20 mg/L</td>
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<tr>
<td>NH₄⁺-N</td>
<td>10.14 ± 0.51 mg/L</td>
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<tr>
<td>NO₃⁻-N</td>
<td>Undetected</td>
</tr>
<tr>
<td>NO₂⁻-N</td>
<td>Undetected</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>106.48 ± 12.65 mg/L</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>72.87 ± 13.35 mg/L</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>49.85 ± 7.17 mg/L</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Average data and standard deviation obtained from triplicate tests. \textsuperscript{b}TCOD: total chemical oxygen demand.

An anaerobic NDFO experiment was conducted using a homogenized slurry collected from the ferrihydrite group after the above-mentioned experiment. This experiment was operated under the following three culture conditions: (i) 1 mL of slurry + 1.2 mM NO₃⁻, (ii) 1 mL of slurry + 1.2 mM NO₂⁻, and (iii) 1 mL of slurry + sterilized denitized water. These three cultures were added in three 100 mL serum vials containing 50 mL of sterilized medium. After flushing the headspace of the vials with N₂ and CO₂ (80%/20%), the vials were incubated statically at 25 °C in the dark for 30 days. Prior to sampling, the vials were thoroughly mixed to ensure sampling equally. The experiments were repeated in triplicate.

### Analysis Methods

The pH was determined with a dual-channel pH-ion-concentration-dissolved oxygen meter (X60, Fisher Scientific). The total chemical oxygen demand (TCOD) was analyzed according to a reference.\textsuperscript{17} The contents of protein, polysaccharide, and total nitrogen were determined based on the method described in our previous report.\textsuperscript{13} The well-mixed slurry was sampled inside the anaerobic chamber and centrifuging (10,000 rpm) for 5 min. The supernatant was filtered through a 0.22 μm PTFE filter. The filtrate was then applied for measuring NH₄⁺, NO₃⁻, and NO₂⁻ using a Dionex DX100 ion chromatograph (IC) system. The well-mixed slurry was dried in a desiccator located inside the anoxic chamber to avoid oxidation. The dried solids were analyzed by X-ray diffraction (XRD)\textsuperscript{18,19} and X-ray photoelectron spectroscopy (XPS)\textsuperscript{20–22} to identify the crystalline iron phases present in the sample. An XRD measurement was carried out via an XRD diffractometer (Empyrean, Panalytical, Netherlands) with Cu Kα radiation at 45 kV and 200 mA, and the XRD pattern was analyzed by the software Jade. An XPS measurement was performed using a 250Xi system (Escalab 250Xi, Thermo, America), and XPSPEAK4.1 software was used for pattern analysis.

Changes in the Nitrogen Species during the Experiment. Figure 1a demonstrates the changes in the NH₄⁺-N content during the experiment. In the initial 20 days, the NH₄⁺-N content increased drastically from 10.14 to 66.15 mg/L in the ferrihydrite group, while that in the control group increased slightly to 21.82 mg/L, indicating that ferrihydrite accelerated the release of ammonium from the decomposition of proteins that was a major organic component of sludge.\textsuperscript{23} As shown in Figure 1b, the protein of the ferrihydrite group on day 37 was undetectable, while 21.1 mg/L protein was still observed in the control, meaning that protein removal efficiency was higher with amending ferrihydride. These results agreed well with previous reports that Fe(III) enhanced the anaerobic decomposition of protein.\textsuperscript{24} After the first 20 days, the NH₄⁺-N content of the ferrihydrite group exhibited an obvious downward trend to 20.05 mg/L on day 87. In contrast, the NH₄⁺-N content of the control increased to 35.62 mg/L on day 87; this content was higher than that of the ferrihydrite group. Ammonium is an intermediate product of protein degradation that might be consumed by the potential Feammox process, which could explain the fluctuation in the ammonium content observed in the two groups. In addition to ammonium, organic matters could also provide electrons for the microbial dissimilatory Fe(III) reduction. From the thermodynamic point of view, organic substrates are more suitable to use as electron donors in the Fe(III) reduction than ammonium.\textsuperscript{25,26} Therefore, as the organic matters were consumed over time, the dissimilatory Fe(III) reduction was more inclined to use ammonium as the electron donor, which could be a main reason for the lag in ammonium removal (Figure 1a). The control group contained 49.85 mg/L of endogenous Fe(III) (Table 1), which also resulted in nitrogen loss through Feammox, although the nitrogen loss was obviously lower than that in the ferrihydrite group.

Notably, the ammonium content of the ferrihydrite group showed less changes during the period from day 36–47, while the content decreased again after sodium bicarbonate (NaHCO₃) was added into each serum vial to a final content of 1.5 mM on day 49. This change was consistent with Feammox being inorganically autotrophic\textsuperscript{27,28} and requiring a sufficient amount of inorganic carbon as the carbon source. To further verify that ammonium was removed in the presence of ferrihydrite, on the 87th day, an extra 73.2 mg/L of NH₄⁺-N was added to the ferrihydrite group. Meanwhile, an additional...
bicarbonate was also added into the system as the carbon source (final content was 1.5 mM). At this time, the content of Fe(III) in the ferrihydrite group was 1294.5 mg/L. As shown in Figure 1a, the NH$_4^+$-N content dropped again to 54.5 mg/L after the following 30 days. The pH of the two systems ranged from 5.0 to 6.2 during the experiment, and these weakly acidic conditions prevented volatilization of the ammonium. An abiotic experiment with no inoculant sludge was conducted in parallel and showed that the ammonium content almost remained unchanged, i.e., 7.43 ± 0.08 mg/L, when adding ferrihydrite alone, further indicating that ammonium removal here was a biotic process (Figure 1c).

In Feammox, NH$_4^+$ was directly converted to N$_2$ or oxidized to NO$_x^-$, which might be further denitrified into N$_2$. Considering that NH$_4^+$ is an intermediate in the anaerobic decomposition of protein, the total nitrogen, including the organic and inorganic nitrogen of a well-mixed slurry, was determined to further clarify the Feammox process. On day 37, the total nitrogen in the control decreased from the initial level of 106.48 to 96.54 mg/L, while the total nitrogen in the ferrihydrite group decreased from 106.48 to 68.01 mg/L (Figure 1d). Namely, on day 37, the total nitrogen removal efficiency in the two systems was 9.33% and 36.13%, respectively. On day 63, the removal efficiency of total nitrogen was 35.63% in the control, while that in the...
ferricyanide group reached 69.49%. This difference indicated that ferricyanide effectively promoted nitrogen removal. Remarkably, the nitrogen removal efficiency in the control group was 9.33% on day 37 and rose to 35.63% on day 63. This was related to the presence of endogenous Fe(III) (49.85 mg/L) in the sludge, which was mainly originated from the sludge dewatering process that used iron salts as coagulants. NO$_3^-$ and NO$_2^-$ are also potential products of Feammox (eqs 2 and 3); however, these species were nearly undetected in this study (Figures S2 and S3). One reason for this result was that N$_2$ was the dominant product of Feammox thermodynamically (eqs 1–3), which lowered the production of NO$_3^-$. Zhou et al. also found that NO$_3^-$ was nearly undetected during the Feammox process in paddy soil. The N$_2$ directly produced from Feammox was reported to account from 67% to 78% of the total N$_2$ emission. The NO$_3^-$ generated during Feammox was also observed in sediment.16 The other hand, the NO$_3^-$ generated was easily reduced by organic matters (namely, denitrification) and Fe(II) (E(NO$_3^-$/N$_2$O) = 1.31 V vs E(Fe$^{III}$/Fe$^{II}$) = 0.2 V, pH 7).

Changes in Iron. As a product of Feammox driven by dissimilatory Fe(III) reduction, the Fe$^{II}$ content could be used to quantify the extent of Fe(III) reduction. As shown in Figure 2a, on day 6, the Fe$^{II}$ content in the ferricyanide group was 1026.78 mg/L, approximately 10-fold higher than that in the control group (106.98 mg/L). Besides more nitrogen removal in the ferricyanide group (Figure 1d), TCOD removal efficiency reached 82.22%, compared with 48.52% in the control (Figure 3). Therefore, the generation of more Fe(II) was consistent with the more nitrogen and TCOD removal in the ferricyanide group compared with the control.

**Figure 3.** TCOD contents in the different groups on day 37. Error bars represent standard deviations.

Figure 2b illustrates the changes in the Fe(III) content of the control. As shown in Figure 2a and b, the contents of Fe(II) and Fe(III) fluctuated. In this anoxic system, the dissimilatory Fe(III) reduction by organic matters and NH$_4^+$ (i.e., Feammox) as electron donors was responsible for the generation of Fe(II). The absence of detectable NO$_3^-$ in this study did not necessarily mean that no NO$_3^-$ was produced. Instead, anaerobic NDFO (eqs 4 and 5) might explain the increase in Fe(III) and decrease in Fe(II) (Figure 2a and b). However, 8 or 6 mol Fe(II) could be produced when producing 1 mol NO$_3^-$ or NO$_2^-$ according to eqs 2 and 3, while only 5 mol (eq 4) and 2 mol (eq 5) Fe(III) could be produced when even all NO$_3^-$ and NO$_2^-$ were reduced to N$_2$ via NDFO. The imbalance of electron transfer between Fe(III) reduction and Fe(II) oxidation might lead to less and less NO$_3^-$ production in each circle and then gradually weaken NDFO and Fe(III) regeneration. This meant that the produced Fe(II) cannot be completely transformed back to Fe(III) when only considering Feammox and subsequent NDFO. Regarding the fluctuant changes in the Fe(II) and Fe(III) contents (Figure 2), it was assumed that other original metal oxides were involved in the N/Fe cycle. From EDX results (Figure S4), besides iron, heavy metals Cr, Pd, etc. were detected in the sludge. These polyvalent metal oxides were reported capable of inducing dissimilatory metal reduction, thereby likely participating in ammonium oxidation like Feammox$^{31–33}$ which is required to be further studied. If this assumption was true, the extra NO$_3^-$ might be produced from ammonium oxidation by these polyvalent metal oxides, further oxidizing Fe(II). Similar results of Fe(III) and Fe(II) during Feammox were also observed in sediment.16

$$10\text{Fe}^{II} + 2\text{NO}_3^- + 12\text{H}^+ \rightarrow 10\text{Fe}^{III} + 3\text{N}_2 + 6\text{H}_2\text{O}$$ (4)

$$4\text{Fe}^{II} + 2\text{NO}_2^- + 8\text{H}^+ \rightarrow 4\text{Fe}^{III} + 3\text{N}_2 + 4\text{H}_2\text{O}$$ (5)

**Potential NDFO Process.** NDFO was possible in this study when considering that NO$_3^-$ was the product of Feammox (eqs 2 and 3). Particularly with the consumption of organics, denitrification gradually decreased, and NDFO likely gradually increased. It means that Fe(III) is likely regenerated by NDFO in this study. To further identify the iron transformation during NDFO, a NDFO experiment in the inoculants taken from the ferricyanide-supplemented group was conducted. As shown in Figure 4a, the NO$_3^-$ content decreased rapidly from the initial level of 17.1 to 3.09 mg/L on day 2, resulting in a decrease in the Fe(II) content from the initial level of 51.32 to 10.98 mg/L on day 5, while the Fe(II) content of the NO$_3^-$-free group remained nearly unchanged (Figure 4b). Similar results also occurred in the reactor amended with NO$_3^-$. E.g., the Fe(II) content also decreased sharply when supplementing NO$_3^-$. This result indicated that NDFO occurred in the presence of NO$_3^-$ and Fe(II) in this system, which was consistent with the results of Bao and Li,34 who found that Fe(II) was oxidized to Fe(III) when NO$_3^-$ was added to an Fe(II)-containing anoxic system. Fe(II) cannot be oxidized to Fe(III) under anoxic conditions unless stronger oxidizing agents, e.g., NO$_3^-$, are present (eqs 4 and 5). The NO$_3^-$ species produced from Feammox likely served as an electron acceptor in the oxidation of Fe(II) through NDFO. In turn, the possible regenerated Fe(III) could likely participate in the Feammox process again, resulting in further nitrogen removal. The further solid evidence supporting NDFO occurrence in this system will be explored in future studies.

**Iron Speciation during Incubation.** The physiochemical nature of the Fe (hydr)oxide, including Fe(III) reduction and possible NDFO, could influence Fe (hydr)oxide transformation.35 As demonstrated in Figure 5a, the XRD pattern of the original sludge showed characteristic peaks at 21.2°, 26.3°, 36.4°, 46.8°, and 60.19° ascribed to the (1052), (2874), (772), (706), and (662) planes, respectively, corresponding to the standard card of iron hydroxide (Fe(OH)$_3$) (JCPDS Card No. 38-0032). It indicated that the endogenous Fe(III) in the original sludge from the dewatering process was in the form of iron hydroxide. The peaks located at 709.9 and 724.0 eV contributed to the formation of Fe(III) hydroxide. Reduction of Fe(III) cannot possibly occur in the Fe(III)-containing anoxic system, as shown in Figure 5b (Figure S5). This result also indicated that NDFO occurred in the presence of NO$_3^-$ and Fe(II) in this system, which was consistent with the results of Bao and Li,34 who found that Fe(II) was oxidized to Fe(III) when NO$_3^-$ was added to an Fe(II)-containing anoxic system. Fe(II) cannot be oxidized to Fe(III) under anoxic conditions unless stronger oxidizing agents, e.g., NO$_3^-$, are present (eqs 4 and 5). The NO$_3^-$ species produced from Feammox likely served as an electron acceptor in the oxidation of Fe(II) through NDFO. In turn, the possible regenerated Fe(III) could likely participate in the Feammox process again, resulting in further nitrogen removal. The further solid evidence supporting NDFO occurrence in this system will be explored in future studies.

**Figure 4.** Fe(III) content in the different groups on day 5. Error bars represent standard deviations.
indicated the presence of Fe(II) and Fe(III), consistent with that Fe(II) and Fe(III) both existed in original sludge (Table 1). The Fe(III) added as ferrihydrite in the ferrihydrite group was often inadequately described as Fe(OH)₃ because these two iron compounds have similar physicochemical properties. A slight change in the XRD spectrum of the control group was observed after the experiment; i.e., besides iron hydroxide (Fe(OH)₃) (Figure 6a), a new iron species, iron tartrate hydrate (C₄H₄FeO₆·H₂O) (Figure 6b), was formed. Specifically, the characteristic peaks at 21.2°, 26.3°, 36.4°, 60.19°, and 67.92° ascribed to the (1002), (3728), (1057), (618), and (599) planes, respectively, of iron hydroxide (JCPDS Card No. 38-0032), and the characteristic peaks at 26.2°, 32.4°, 50.07° were ascribed to the (3728), (1006), and (889) planes, respectively, corresponding to the standard card of iron tartrate hydrate (JCPDS Card No. 23-0299). While the XRD pattern of the iron minerals in the slurry of the ferrihydrite group after the experiment showed that the characteristic peaks at 30.09°, 35.42°, and 37.05° were ascribed to the (1504), (1602), and (1602) planes, respectively, corresponding to the standard card of magnetite (JCPDS Card NO. 19-0629) (Figure 7a), and the characteristic peaks at 26.72°, 35.16°, and 39.22° were ascribed to the (1438), (1326), and (1282) planes, respectively, of akaganeite (β-FeOOH) (Figure 7b) (JCPDS Card No. 34-1266); i.e., magnetite and akaganeite were formed after experiment.

Figure 4. Changes in the (a) NO₃⁻ and (b) Fe(II) contents with and without NO₃⁻ under anaerobic conditions.

Figure 5. XRD (a) and Fe 2p XPS (b) spectrum patterns of the original sludge. The XRD pattern for standard rutile is shown as straight lines (a).

Figure 6. XRD pattern of the iron minerals in the sludge of the control group after the experiment. The XRD pattern for standard rutile is shown as straight lines [iron hydroxide (a) and iron tartrate hydrate (b)].
meant that the original ferrihydrite transformed into magnetite and akaganeite. This result was consistent with those of previous studies, showing that ferrihydrite could be converted to magnetite and other Fe(III) oxides. Besides possible NDFO, Boland et al. indicated that with the presence of aqueous Fe(II) ferrihydrite could be chemically transformed to more crystalline minerals. The observed changes in the crystalline iron, especially in the ferrihydrite group, indicated Fe(III) reduction and regeneration.

**Proportion of Iron Reduction Associated with Feammox.** Under anoxic conditions, the potential of assimilation by microbes, anammox, denitrification, and possible NDFO could contribute to nitrogen loss (Table 2). However, for the assimilation, NH$_4^+$ could be converted by microbes to nitrogenous organic matters such as proteins, which might be mostly decomposed into NH$_4^+$ again. The reminder still stayed in the sludge slurry with no way to become N$_2$ to generate nitrogen loss during assimilation. For denitrification, anammox, and NDFO, these processes relied on Feammox to generate NO$_3^-$.

Table 2. Potential Pathway of Nitrogen Loss under Anoxic Conditions

<table>
<thead>
<tr>
<th>N loss Mechanism</th>
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<tr>
<td>Feammox</td>
<td>eqs 1–3</td>
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<tr>
<td>assimilation by microbes</td>
<td>NH$_4^+$ + NO$_3^-$ → N$_2$ + 2H$_2$O</td>
</tr>
<tr>
<td>denitrification</td>
<td>Organic act as electron donors</td>
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<tr>
<td>NDFO</td>
<td>eqs 4 and 5</td>
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  - assimilation by microbes: NH$_4^+$ + NO$_3^-$ → N$_2$ + 2H$_2$O
  - denitrification: Organic act as electron donors
  - NDFO: eqs 4 and 5

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Therefore, the nitrogen loss could result from the following two pathways:

1. N$_2$ directly produced from Feammox.
2. Feammox-generated NO$_3^-$ followed by denitrification, possible NDFO, or anammox (anammox was ignored based on microbiological analysis below).

In other words, the nitrogen loss in this study was caused by Feammox, regardless of directly producing N$_2$ or first producing NO$_3^-$ then being reduced to N$_2$ (Figure 8).

Organic materials and ammonium can both serve as electron donors in the dissimilatory Fe(III) reduction. On the basis of thermodynamics, the majority of Fe(III) reduction was involved preferentially with the oxidation of organics rather than with ammonium. Therefore, the proportion of Fe(III) generated from Feammox was not high. Ding et al. found that only 0.81%–2.2% of the Fe(III) reduction in paddy soils was associated with Feammox and increasing the organic content led to a decrease in the amount of Fe(III) generated from Feammox.

The organic content in the wastewater treatment system in this study was much higher than that in the natural environment; thus, the proportion of Fe(III) generated from Feammox should be lower in this study than in that by Ding et al. In contrast, based on the nitrogen loss and the stoichiometric Fe/N ratio of 3 (eq 1), Fe(II) generated from Feammox in the ferrihydrite group accounted for 30.3% and 65.0% of the total Fe(II) on days 37 and 63, respectively, which were much higher than the proportions reported in previous studies (0.81%–2.2% and 0.4%–6.1%).

It was likely because that NDFO was indispensable to reproduce Fe(III) (Figure 8), consistent with an NO$_3^-$-reducing Fe(II) oxidizer (see Microbial Community below) detected in systems, leading to the more nitrogen removal and higher proportion of Fe(III) reduction through Feammox. Notably, the endogenous Fe(III) content of 49.85 mg/L in the control was not sufficient for removing such a large amount of nitrogen by day 63. Specifically, only 4.15 mg/L of nitrogen could theoretically be removed via Feammox based on the 49.85 mg/L Fe(III) content in the control based on the stoichiometric

![Figure 8. Diagram of nitrogen removal in this study.](image-url)
Fe/N ratio of 3 (eq 1). Nevertheless, 37.94 mg of nitrogen was removed on day 63 in the control, which was more than the theoretical amount, implying that polyvalent metal oxides existing in the sludge (Figure S4) was likely involved in the nitrogen removal. Importantly, the proportion of Fe(III) reduction associated with Feammox in the ferrihydrite group increased over time, e.g., from 30.3% on day 37 to 65.0% on day 63. As the organic matters were depleted during digestion, ammonium gradually became the main electron donor for Fe(III) reduction.

Microbial Community. To date, the underlying microbiological process of Feammox has not been clarified. Most researchers have suggested that iron-reducing bacteria participate in Feammox (Figure S6). For example, Zhou et al. ascribed the Feammox process in paddy soil to the function of Geobacter, an iron-reducing bacterium.16 Huang and Jaffe stated that an uncultured Acidimicrobiaceae bacterium capable of reducing Fe(III) played a key role in Feammox in a forested riparian wetland.27 In this study, the iron-reducing bacteria were also enriched. As shown in Figure 9a, Deltaproteobacteria, which contains iron-reducing bacteria, e.g., Geobacter, accounted for 6.02% of the bacteria 16S rRNA gene sequences at the class level in the control, while this class accounted for 12.8% of the bacteria in the ferrihydrite-supplemented group. Further identification at the genus level showed that Geobacter made up 0.04% and 0.48% of the total sequences in the control and ferrihydrite groups, respectively (Figure 9b). The low abundance of Geobacter in the sludge agreed with the results of previous reports,38 indicating that the inoculant sludge was not an ideal system for enriching Geobacter compared with the natural habitat. This low abundance of Geobacter was probably related to the low efficiency of nitrogen removal in this study (69.49% on day 63 in the ferrihydrite group, Figure 1d).

The NO3−-reducing Fe(II) oxidizer Acidovorax (Figure 9b) and the family Comamonadaceae (Figure 9c), belonging to iron-oxidizing bacteria, were also detected in both groups. Particularly, the abundance of Acidovorax was 1.18% and 0.81% in the control and ferrihydrite groups, respectively, and the abundance of Comamonadaceae was 1.28% and 0.44%. Putative denitrifiers, including Acinetobacter, Rhodooplanes, Bacillus, and Pseudomonas,39 were detected in the two groups, indicating that denitrification of NO3− from Feammox was an alternative to the consumption of NO3− (Figures S2 and S3).

Notably, one microbe at the genus level, Aminicenantes_genera_incertae_sedis, was abundantly enriched in the control (19.88%) and the ferrihydrite group (40.34%) (Figure 9b). Aminicenantes has been found to adapt and utilize a variety of complex sugar polymers and amino acids, such as glycine, glutamate, and aspartate,40 which was consistent with the observed polysaccharide and protein removal (Figure 1b and Figure S7). The genes for the assimilatory acquisition of nitrogen were also identified in Aminicenantes. However, the ability of Aminicenantes_genera_incertae_sedis to reduce Fe(III), even when mediated by Feammox, is unknown.

IMPLICATION

Ammonium is one of mainly concerned pollutants due to the risk to cause eutrophication. Feammox has increasingly been reported in natural systems. However, this nitrogen removal model has rarely been investigated in wastewater treatment systems. In this study, the nitrogen removal efficiency in the ferrihydrite group was significantly higher than that of the control (Figure 1d), implying that ferrihydrite induced anaerobic NH4+ oxidation and further nitrogen removal. Although the nitrogen removal efficiency was not high, e.g., 69.49% of nitrogen was removed during 63 days when supplementing ferrihydrite, the study still demonstrated the possibility of application of Feammox in anaerobic nitrogen removal from wastewater. Although the results suggested that Feammox was related to nitrogen removal, the direct evidence still lacked. 15N-labeled isotopic is an ideal tool to characterize nitrogen loss in Feammox, which will be investigated in our next study.
CONCLUSIONS
In this study, ferricydrite was supplemented in an anaerobic wastewater treatment reactor to investigate its effects on nitrogen removal. The results showed that nitrogen removal efficiency increased by 33.86% after 63 days compared to the control. As part products of Feammonox, Fe(II) and NO$_3^-$ likely reacted together in this study, resulting in NDFO to generate microbial community analysis compounds, magnetite and akaganeite, were detected during Fe(III). XRD results showed that new species of Fe(III) compounds, magnetite and akaganeite, were detected during Fe (Hydr)oxide transformation. Microbial community analysis demonstrated that iron-reducing bacteria and iron-oxidizing bacteria were enriched in the two groups, indicating that the cooperation of two bacteria was essential for the ammonium removal in Fe(III)-containing wastewater treatment systems.

ASSOCIATED CONTENT
1. Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b03083.

2. Notes
The authors declare no competing financial interest.

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