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Oxidation of ammonium in aerobic wastewater by anoxic ferric iron-dependent ammonium oxidation (Feammox) in a biofilm reactor

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ABSTRACT

Anoxic ferric iron-dependent ammonium oxidation (Feammox) is a novel biological nitrogen removal process that oxidizes ammonium to NO_2^-, NO_3^- or N_2 using ferric iron as the electron acceptor. In this study, the Feammox process was successfully developed in a biofilm reactor using sponge iron as a Fe source feeding ammonium-containing aerobic water. The results showed that NH_4^+ was oxidized to NO_3^- by Feammox. During 300 d of operation, the bioreactor demonstrated an average NH_4^+ oxidation efficiency and rate of 42.3% and 32.2 mg N L^{-1} d^{-1}, respectively. Sponge iron was oxidized to ferric by dissolved oxygen (DO) in the influent and provided ferric and anaerobic conditions for Feammox. Only a small proportion of the generated NO_3^- was reduced to N_2 by nitrate-dependent Fe(II) oxidation causing nitrogen removal in the Feammox biofilm reactor. A distinct inhibition of Feammox was observed when DO increase to 1.0 mg L^{-1}. Microbiological composition analysis of the Feammox biomass showed that the dominant iron-reducing bacteria were *Geobacter* spp., *Fervidicella*, and *Geothrix*, which may contribute to Feammox. This study provides information on the process development of Feammox.

Keywords: Feammox; Biofilm reactor; NFO; Nitrogen removal; Sponge iron

1. Introduction

The anoxic ferric iron-dependent ammonium oxidation (Feammox) process is the anoxic oxidation of ammonium with ferric iron as an electron acceptor [1,2]. The oxidation of ammonium by Feammox is a microbially mediated reaction under anaerobic conditions [1–3]. The possible product of ammonium oxidation by Feammox could be N_2 [4,5], nitrite (NO_2^-) [2,3,6,7] or nitrate (NO_3^-) [4], and Fe(III) is reduced to Fe(II). Feammox has been detected in several systems, such as forest soils [4,6], riparian sediment [2,8,9], paddy soils [5], river sediment [10], and marine sediment [11,12]. Feammox has also been found in activated sludge systems [3,8,13]. Researchers have started to develop Feammox technology for potential applications in wastewater treatment. NH_4^+ was oxidized to NO_2^- in a fixed-bed reactor supplying ammonium and Fe(III) Fe(III) ethylenediaminetetraacetic acid Na (Fe(III) EDTA Na) Na after 42 d of anoxic operation [3]. NH_4^+ was oxidized to NO_3^- by Feammox coupled with anaerobic ammonium oxidation (Anammox) in anaerobic sludge from a piggery wastewater treatment plant, and ferric citrate was used as an electron acceptor [8]. Simultaneous NH_4^+ and NO_3^- removal was obtained by Feammox coupled with nitrate-dependent Fe(II) oxidizing (NFO) and Anammox using ferric chloride as a catalyst in an anaerobic microbial culture

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tank [14,15]. NO_2^- and NO_3^- were generated from the oxidation of NH_4^+ by Feammox in anaerobic digestion of a sludge system supplemented with magnetite, Fe_2O_3, and $Fe(OH)_3$ [13]. Accordingly, Feammox and the Feammox–Anammox, Feammox-denitrification, and Feammox–NFO coupling processes can be used as the effective new ammonium removal technologies in wastewater treatment.

$$3Fe(OH)_3 + 5H^+ + NH_4^+ \rightarrow 3Fe^{2+} + 9H_2O + 0.5N_2 \quad (1)$$

$$6Fe(OH)_3 + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 16H_2O + NO_2^- \quad (2)$$

$$8Fe(OH)_3 + 14H^+ + NH_4^+ \rightarrow 8Fe^{2+} + 21H_2O + NO_3^- \quad (3)$$

Although Feammox is an anaerobic microbial nitrogen conversion process, environments with fluctuating redox conditions, such as intertidal wetland, are favorable for the occurrence of the microbial Feammox process [16]. The generated Fe(II) from the Feammox process can be oxidized to Fe(III) by dissolved oxygen (DO) in a water column or sediment. Therefore, DO may contribute to the occurrence of Feammox in natural ecosystems. As an anaerobic microbial process, the Feammox process could be inhibited by DO. N_2 stripping was used to remove DO in the influent to weaken the inhibition of DO on the Feammox during the operation of the Feammox-activated sludge system [3,8,14]. However, N_2 stripping increased the energy consumption of the system. It seems difficult to cultivate Feammox feeding with aerobic influent. Also, few studies have focused on the effect of DO on Feammox biomass.

Fe(III) EDTA Na, ferric citrate, $FeCl_3$, and ferrihydrite were used as electron acceptors for Feammox [3,13,14]. High concentrations of Fe(II) or Fe(III) in the effluent may negatively affect the receiving water. It is important to choose the appropriate iron as the electron acceptor for this anaerobic biological process. Comparing to chelate iron and other dissolved iron, solid iron can be used as a carrier for microorganisms. Also, the relatively low iron release rate from solid iron would result in a low negative effect on the receiving water. Solid iron has been used for the remediation of nitrate contaminated groundwater [17], phosphorus removal [18,19], the deoxygenation of supply water of industrial boilers [20,21], hexavalent chromium removal [22] and dissolved chlorinated solvents removal [23]. Therefore, we expect that solid iron might be used as a source of Fe to strengthen the microbially mediated Feammox process and restore ammonium-contaminated wastewater, shallow groundwater, and agricultural subsurface drainage.

The two primary objectives of this study were to (i) develop a Feammox biofilm reactor (FeBR) for the oxidation of ammonium with sponge iron as the Fe source and (ii) investigate the effect of DO on the performance of the Feammox bioreactor and the resistance of Feammox biomass to DO. To reveal the characteristics of biological nitrogen transformation processes, several groups of batch tests were designed. Also, the microbial community structure of the Feammox biomass was investigated. We hope that the results presented in this study will provide information to further develop Feammox technology.

2. Materials and methods

2.1. Feammox biofilm reactor

A schematic representation of the Feammox methyl methacrylate bioreactor (8 cm i.d. × 120 cm) is shown in Fig. 1. The (FeBR1 and FeBR2) were covered with black cloth to prevent the production of oxygen by phototrophic organisms and the inhibition by light. The anaerobic digestion granular sludge used in this study was collected from a municipal wastewater treatment plant (Nanjing, China). Sponge iron (Φ5–8 mm) was purchased from Gongyi City Mingyue Corundum Co. Ltd., (Gongyi City, Henan Province, China). The bottom part of FeBR1, from port A to the bottom, was filled with sponge iron. The upper part of the column (from port A to the top) was filled with biofilm carriers, anaerobic digestion granular sludge, and sponge iron, and the volume ratio was 8:1:2. These carriers were made of polypropylene with a diameter of only 10 mm and

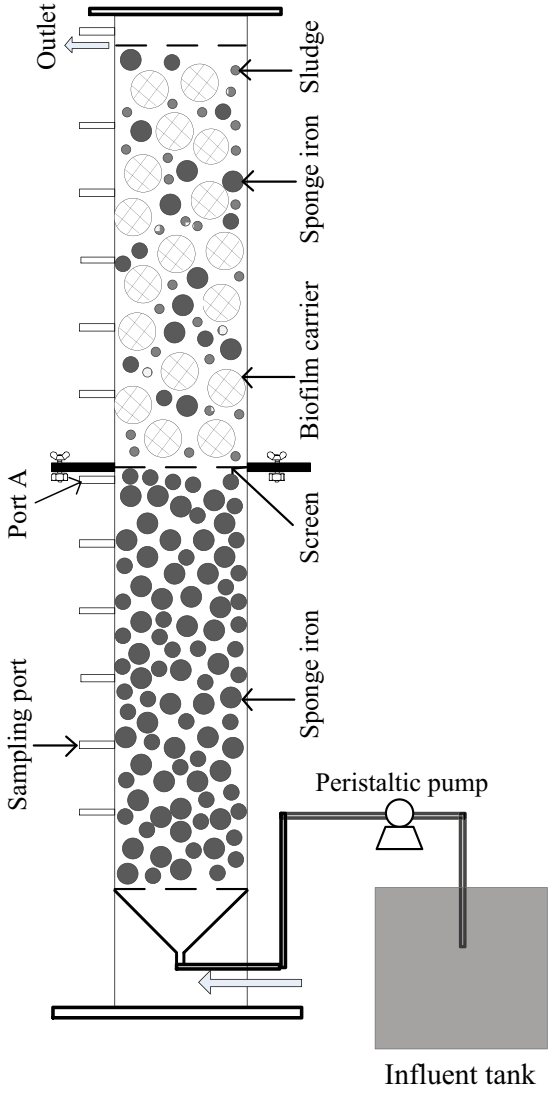

Fig. 1. Schematic diagram of the Feammox biofilm bioreactor in this study.

could be recycled and reused. No sponge iron was filled in the control reactor (FeBR2), and DO in the influent was removed by N_2 stripping. The upflow configurations were fed synthetic wastewater using a peristaltic pump.

The influent of FeBR1 and FeBR2 was prepared with NH_4Cl; $NaHCO_3$, 1.5 g L^{-1}; $CaCl_2{\cdot}2H_2O$, 0.18 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 0.12 g L^{-1}; KH_2PO_4, 0.027 g L^{-1}; and 1.0 ml L^{-1} of trace elements solution I and II. Trace elements solution I consisted of EDTA, 5.0 g L^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.43 g L^{-1}; $CoCl_2{\cdot}6H_2O$, 0.24 g L^{-1}; $MnCl_2{\cdot}4H_2O$, 0.99 g L^{-1}; $CuSO_4{\cdot}5H_2O$, 0.25 g L^{-1}; $NaMoO_4{\cdot}2H_2O$, 0.22 g L^{-1}; $NiCl_2{\cdot}6H_2O$, 0.19 g L^{-1}; $Na_2SeO_4{\cdot}10H_2O$, 0.21 g L^{-1}; and H_3BO_4, 0.014 g L^{-1}, and trace elements solution II consisted of $FeSO_4$, 5 g L^{-1}; and EDTA, 5.0 g L^{-1}. The temperature of the two reactors was maintained at 28°C ± 1°C using a water bath. The hydraulic retention time of the two bioreactors was maintained at 6 h.

2.2. Batch tests

The batch tests were conducted to investigate the characteristics of the Feammox process and the interplay of Fe and N cycling in the bioreactor. The experimental conditions are shown in Table 1. Batch tests A1 (Fe^{3+} + NH_4^+ + activated sludge under anaerobic conditions), A2 (Fe^{3+} + NH_4^+ + inactivated sludge under anaerobic conditions), A3 (NH_4^+ + activated sludge under anaerobic conditions), A4 (Fe^{2+} + NH_4^+ + activated sludge under anaerobic conditions), A5 (sponge iron + NH_4^+ + activated sludge under anaerobic conditions), A6 (sponge iron + NH_4^+ + activated sludge under aerobic conditions), A7 (Fe^{3+} + $^{15}NH_4^+$ + activated sludge under anaerobic conditions) and A8 (Fe^{3+} + $^{15}NH_4^+$ + C_2H_2 + activated sludge under anaerobic conditions) were conducted to investigate the characteristics of the Feammox process in FeBR1. Batch tests B1 (NH_4^+ + NO_2^- + activated sludge under anaerobic conditions), B2 (NO_3^- + CH_3COONa + activated sludge under anaerobic conditions) and B3 (NO_3^- + Fe(II) + activated sludge under anaerobic conditions) focused on the transformations of Fe and N in FeBR1. The effect of DO on the Feammox process was investigated in a batch test. For the $^{15}NH_4Cl$ and C_2H_2 treatment (A7), sterile anoxic deionized water with dissolved C_2H_2 was prepared using 100 mL serum vials by adding 70 mL of sterile anoxic deionized water; 30 mL of headspace gas in each serum vial was replaced with C_2H_2 for 24 h [9]. The addition of acetylene can completely inhibit the production of N_2 by denitrification [24] and Anammox [25]. Gas samples were collected for analyzing $^{30}N_2$ by an isotope ratio mass spectrometer (IRMS, Finnigan-MAT 253, Thermo Fisher Scientific, Bremen, Germany).

The biomass sample (biofilm and granule sludge) used in the batch tests was taken from FeBR1 at day 180 and washed five times with a nutrient solution before the batch tests. The component of the nutrient solution was the same as the influent of the FeBR1 elements except for ammonium. The washed biomass was transferred into a 150 mL serum vial under helium in an anaerobic glove box. The serum vial was immediately sealed with a butyl rubber cap and an aluminum cap. For batch-test A2, the biomass was inactivated by autoclaving (121°C, 1.5 bar) for 1 h, followed by a second autoclaving (15 min) on the next day [26]. $^{15}NH_4Cl$ was added to the serum vial in batch tests A7 and A8 (^{15}N at 99.0% was purchased from Shanghai Aladdin Bio-Chem Technology Co. Ltd., (Shanghai, China). After 6 h of incubation, gas samples were collected for analyzing $^{30}N_2$ by an isotope ratio mass spectrometer (IRMS, Finnigan-MAT 253, Thermo Fisher Scientific, Bremen, Germany). The pH was measured using a pH electrode (S220-K, Mettler, Switzerland) and was adjusted to 6.5 by adding 1 mol L^{-1} HCl and 1 mol L^{-1} NaOH. The temperature of the batch test was maintained at 28°C ± 1°C using a water bath.

2.3. Sampling and analysis

Batch test samples were taken from the serum vial every hour using syringes. According to the APHA [27] protocols, ammonium, nitrate, and nitrite were determined by flow injection analysis (Smartchem 200, AMS Alliance, Italy), and the chemical oxygen demand was analyzed using colorimetric methods. After extraction by 0.5 M HCl, the total extractable Fe and Fe(II) in the sediment was measured using the ferrozine method described in a previous study [28].

Table 1
Experimental conditions for each batch test

Batch tests	Sponge iron g	$FeCl_3$ mM	NH_4^+ mg N L^{-1}	NO_2^- mg N L^{-1}	NO_3^- mg N L^{-1}	CH_3COONa mg L^{-1}	$FeCl_2$ mmol L^{-1}	C_2H_2 mM	Remarks
A1	/	2.0	20	/	/	/	/	/	Activated sludge, anaerobic
A2	/	2.0	20	/	/	/	/	/	Inactivated sludge, anaerobic
A3	/	/	20	/	/	/	/	/	Activated sludge, anaerobic
A4	/	/	20	/	/	/	1.0	/	Activated sludge, anaerobic
A5	10	/	20	/	/	/	/	/	Activated sludge, anaerobic
A6	10	/	20	/	/	/	/	/	Activated sludge, aerobic
A7		2.0	$^{15}NH_4^+$, 20	/	/	/	/	/	Activated sludge, anaerobic
A8		2.0	$^{15}NH_4^+$, 20	/	/	/	/	180	Activated sludge, anaerobic
B1		/	10	10	/	/	/	/	Activated sludge, anaerobic
B2		/	/	/	20	70	/	/	Activated sludge, anaerobic
B3		/	/	/	20	/	2.0	/	Activated sludge, anaerobic

The amount of microbially reducible Fe(III) was calculated from the difference between the content of the total extractable Fe and Fe(II).

2.4. Deoxyribonucleic acid extraction and quantitative real-time polymerase chain reaction

Microbial communities of biomass sampled on day 180 and seed sludge were determined, which represent different stages of the FeBR operation. Microorganisms attached to the biofilm carriers were detached and concentrated after sonication and filtration. Deoxyribonucleic acid (DNA) was extracted from the biomass samples using a Power Soil DNA Isolation Kit (MoBio, USA) according to the manufacturer's instructions. The DNA concentration was determined on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, DE). 16S ribosomal ribonucleic acid (rRNA) was partially amplified from the DNA extracts using a nearly universal bacterial primer set and processed by the Magigene Biotechnology Company (Guangzhou, China) for high-throughput DNA sequencing with the Illumina MiSeq System (Illumina, San Diego, U.S.A.). To quantify the relative abundance of denitrifying microorganisms, polymerase chain reaction (qPCR) for the nitrate-reductase-encoding genes (*napA* and *narG*) as well as for the nitrite-reductase-encoding genes (*nirK* and *nirS*) were performed on the DNA samples. qPCR was performed on an ABI StepOne Plus detection system (Applied Biosystems, USA) using SYBR green-based detection. The *napA* primers were *V17* m (TGGACVATGGGYTTYAAYC) and *napA*4r (ACYTCRCGHGCVGTRCCRCA). The *narG* primers were *narG*-f (TCGCCSATYCCGGCSATGTC) and *narG*-r (GAGTTGTACCAGTCRGCSGAYTCSG). The protocols used for the quantification of *napA* and *narG* were described in a previous study [29]. The protocols used for the quantification of *nirK* (*nirK*876c ATYGGCGGVCAYGGCGA and *nirK*1040 GCCTCGATCAGRTTRTGGTT) and *nirS* (*nirS*Cd3aF AACGYSAAGGARACSGG and *nirS*R3cd GAST TCGGRTGSGTCTTSAYGAA) were described in previous studies [30,31].

3. Results and Discussion

3.1. Performance of the FeBR

NH_4^+ in the effluent gradually decreased to 6.4 mg N L^{-1} from day 1 to day 120 in FeBR1 (Fig. 2). In contrast, NO_3^- in the effluent gradually increased to 15.2 mg N L^{-1}. After 120 d, the FeBR1 ran stably for approximately 70 d. The average NH_4^+ removal rate and NO_3^- accumulation rate were 53.1 and 45.5 mg N L^{-1} d^{-1} during 70 d, respectively (Fig. 2). These results showed that NH_4^+ was oxidized to NO_3^- in the FeBR1 reactor. After 190 d the concentration of NH_4^+ in the effluent increased gradually to 19.5 mg N L^{-1}, and the concentration of NO_3^- in the effluent progressively decreased to 5.5 mg N L^{-1}. There was no obvious NO_2^- in the effluent during the operation of FeBR1. The total nitrogen (TN) removal rate and efficiency profiles of the bioreactor followed a similar pattern to the trend of NH_4^+ in the effluent. The TN removal rate and efficiency were 6.0–9.6 mg N L^{-1} d^{-1} and 8.1%–12.3% from day 120 to 190, respectively (Fig. 2). The pH of the effluent increased with the NH_4^+ removal rate and ranged from 8.39 to 9.67. According to the Feammox reaction (Eqs. (1)–(3)), protons are consumed, increasing pH.

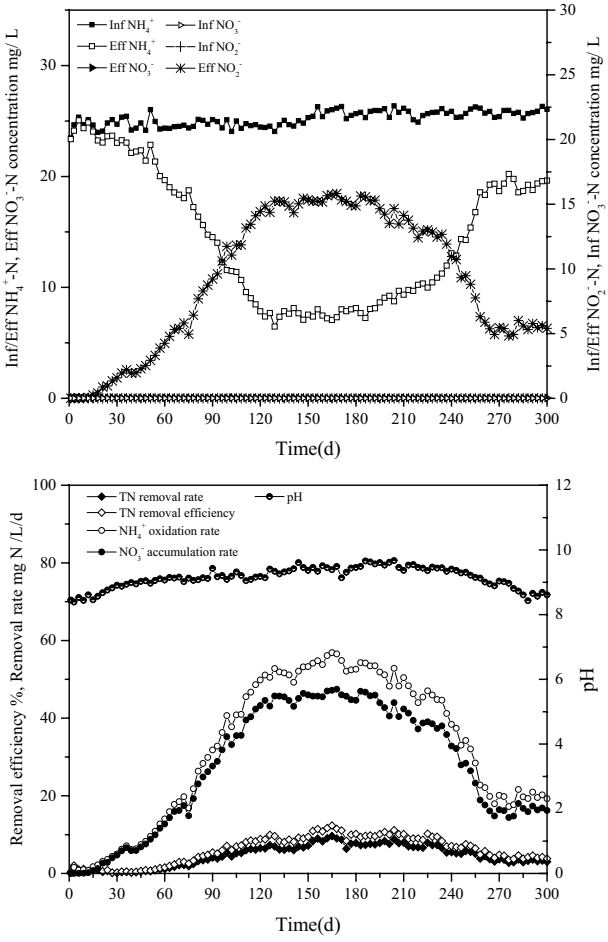

Fig. 2. Performance of FeBR1.

As shown in Fig. 3, obvious Fe(II) and Fe(III) in the effluent were measured during the operation of FeBR1. The Fe(II) in the effluent gradually increased to 0.52 mM with experiments and then decreased to 0.07 mM after 210 d. The Fe(III) in the effluent ranged from 0.07–0.43 mM during the initial periods and then steadily decreased to 0.05 mM in the last 114 d. These results indicated that Fe was released from the sponge iron to the liquid phase in the bioreactor. The influent of the bioreactor was aerobic synthetic wastewater. Fe(II) was released from the chemical reaction between the sponge iron and DO. The produced Fe(II) was further oxidized to Fe(III). NO_3^- in the effluent of the bioreactor was therefore produced from the oxidation of NH_4^+ through Feammox.

The concentration of NH_4^+ in the effluent of FeBR2 without sponge iron filled in the bioreactor was kept constant (Fig. 4). No obvious NO_3^- or NO_2^- was detected in the effluent. The volatile suspended solids of FeBR2 decreased considerably during the initial 30 d. These results indicated that NH_4^+ could not be oxidized in FeBR2, and the bioreactor could not run stably. The operation results of FeBR2, together with the NH_4^+ oxidation in FeBR1, revealed that NH_4^+ was oxidized to NO_3^- by sponge iron in FeBR1. There are three

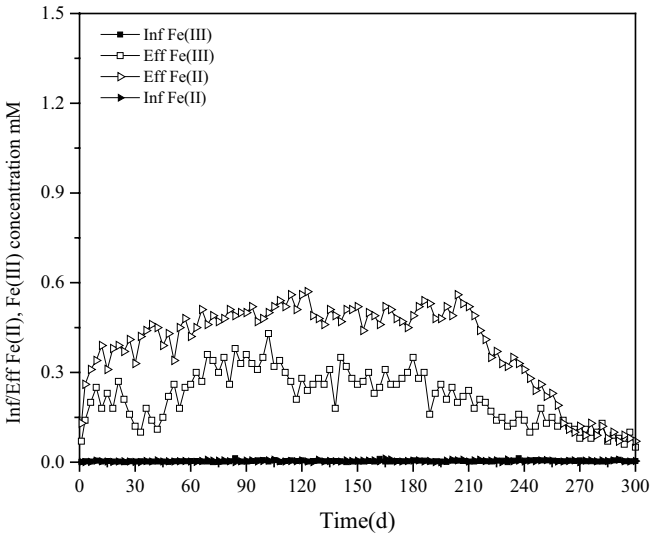

Fig. 3. Fe(III) and Fe(II) concentration profile during the operation of FeBR1.

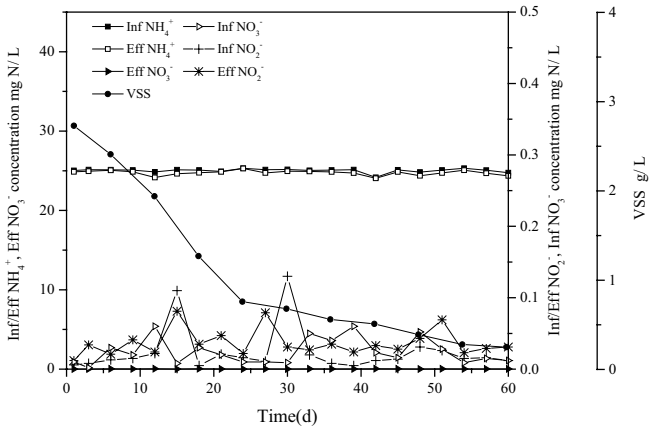

Fig. 4. Performance of FeBR2.

possible reasons for the removal of TN in FeBR1. First, N_2 is directly produced from the oxidation of NH_4^+ by Feammox [4]. Second, the oxidation of NH_4^+ to NO_3^- by Feammox is followed by denitrification [16] or nitrate-dependent Fe(II) oxidation [32]. Third, Feammox to NO_2^- followed by denitrification or Anammox [16].

3.2. Characteristics of nitrogen and Fe transformation

To investigate the characteristics of nitrogen and Fe transformation inFeBR1, two groups of batch tests were devised (Table 1). There was no change in the concentration of NH_4^+ in the $FeCl_3$ and NH_4^+ addition with inactivated biomass under anaerobic conditions (test A2, Fig. 5b), NH_4^+ addition with activated biomass under anaerobic conditions (test A3, Fig. 5c), and $FeCl_2$ and NH_4^+ addition with activated biomass treatment under anaerobic conditions (test A4, Fig. 5d). Distinct NH_4^+ decreases and NO_3^- production in the $FeCl_3$ and NH_4^+ addition with activated biomass under anaerobic conditions were observed (test A1, Fig. 5a), demonstrating that the oxidation of NH_4^+ to NO_3^- was $FeCl_3$-dependent and mediated by microorganisms.

There was also no change in the concentration of NH_4^+ in the sponge iron and NH_4^+ addition with activated biomass under anaerobic conditions (test A5, Fig. 5e) or under aerobic conditions (test A6, Fig. 5f). However, the concentration of Fe(III) increased gradually in test A6 with sponge iron addition under aerobic conditions. Combined with the results from tests A1-A5, these results illustrated that the Fe(III) released from the oxidation of sponge iron in aerobic conditions oxidized NH_4^+ to NO_3^- in the Feammox bioreactor.

Except for the Feammox process, nitrate can also be produced and is associated with the growth of anaerobic ammonium oxidizing bacteria (AnAOB) [33]. However, the concentration of NH_4^+ did not change with NO_2^- addition under anaerobic conditions (test B1, Fig. 6a). The results indicated that the activity of AnAOB was extremely low in FeBR1 and made little contribution to the production of NO_3^- and TN removal. Feammox could thus be considered the only pathway of NO_3^- generation in FeBR1. Little NO_3^- removal was observed in the test with CH_3COONa addition under anaerobic conditions (test B2, Fig. 6b), revealing that the activity of heterotrophic denitrifying bacteria was particularly low in FeBR1 and could not result in significant NO_3^- removal.

Two possible processes could be the reasons for TN removal: N_2 is directly generated from the oxidation of NH_4^+ by Feammox or the reduction of NO_3^- by autotrophic denitrification. Acetylene is known as an inhibitor of N_2O reductase [34] and has no obvious inhibitory effect on Fe(III) reduction [5,16]. Therefore, $^{15}NH_4^+$ isotope tracer incubation coupled with C_2H_2 inhibition was conducted to investigate the possible TN removal process (batch test A7 and A8, Figs. 5g and h). Although NH_4^+ was oxidized to NO_3^- by Feammox in tests A7 with C_2H_2 addition, little $^{30}N_2$ was measured. On the contrary, the concentration of $^{30}N_2$ increased gradually after 6 h of incubation in test A8 without C_2H_2 addition. These results indicated that TN removal in FeBR1 was the result of autotrophic denitrification.

NFO is hypothesized to reduce NO_3^- to N_2 in FeBR1. To investigate the contribution of NFO to nitrogen transformation, NO_3^- and Fe(II) were added to the Feammox sludge system without Fe(III) and NH_4^+ (batch test B3, Fig. 6c). The NO_3^- concentration decreased to 13.2 mg N L^{-1} during the 6 h operation. Similar to the trend of NO_3^-, the Fe(II) concentration progressively declined from 2.7 to 1.8 mM. Concurrently, the Fe(III) concentration gradually increased in the anaerobic system. The results showed that nitrate reduction was concomitant with Fe(II) oxidation throughout the incubation. NO_3^- can be reduced to N_2 by NFO, resulting in nitrogen removal [35]. The production of NO_3^- and Fe(II) in FeBR1 was favorable for the growth of microorganisms catalyzing NFO.

3.3. Effect of DO on the Feammox process

As an anaerobic biological reaction, the Feammox process might be inhibited by high concentrations of DO. The batch tests were designed to investigate the effect of DO on Feammox biomass. The NH_4^+ oxidation rate and TN removal rate decreased rapidly when the concentration of DO increased from 0 to 1.0 mg L^{-1} in the batch test (Fig. 7).

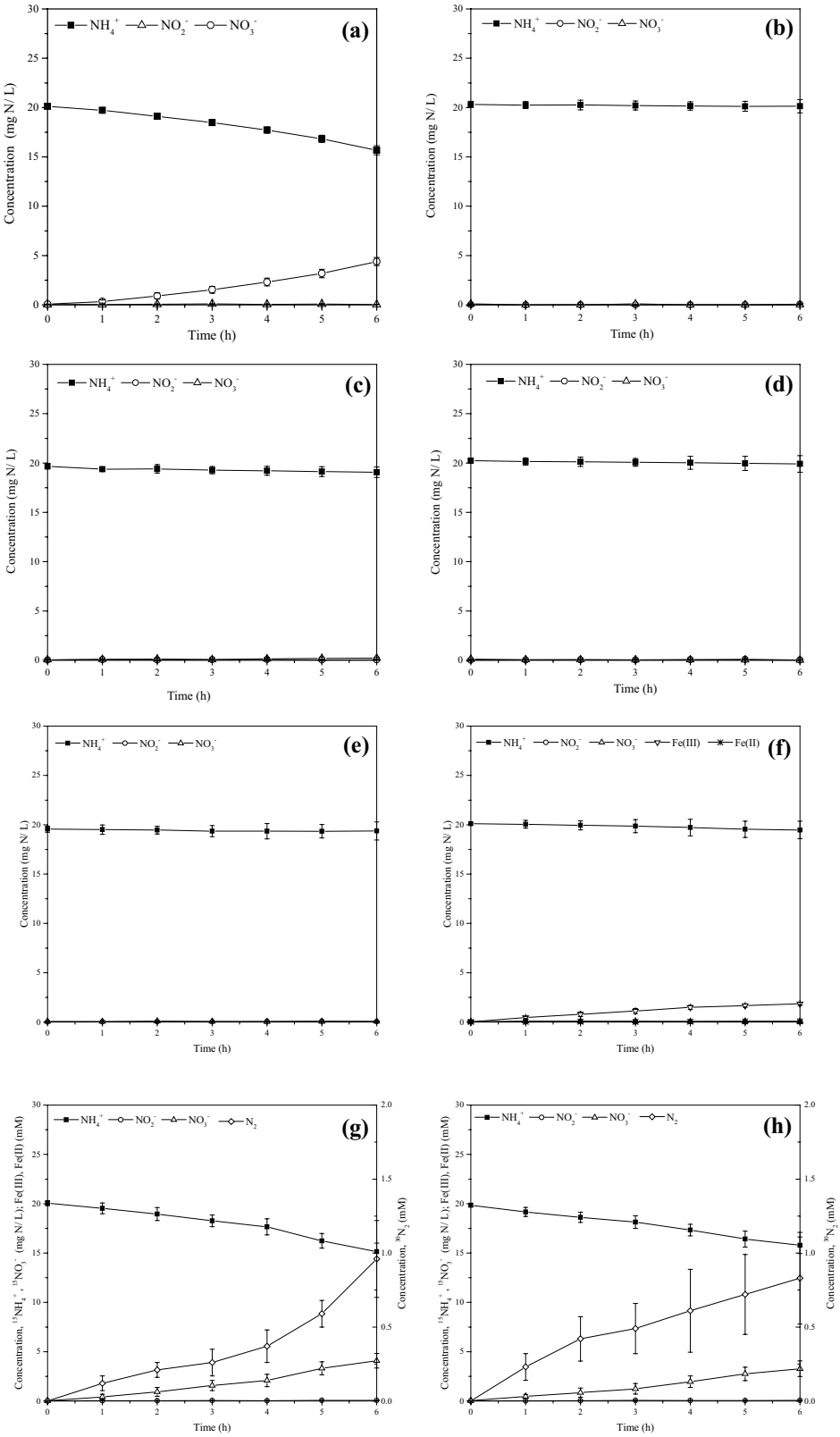

Fig. 5. Nitrogen conversion properties of Feammox biomass in batch tests. (a) Batch test A1, $FeCl_3$ + NH_4^+ + activated sludge, anaerobic, (b) batch test A2, $FeCl_3$ + NH_4^+ + inactivated sludge, anaerobic, (c) batch test A3, NH_4^+ + activated sludge, anaerobic, (d) batch test A4, $FeCl_2$ + NH_4^+ + activated sludge, anaerobic, (e) batch test A5, sponge iron + NH_4^+ + activated sludge, anaerobic, (f) batch test A6, sponge iron + NH_4^+ + activated sludge, aerobic, (g) batch test A7, $FeCl_3$ + $^{15}NH_4^+$ + activated sludge, anaerobic, and (h) batch test A8, $FeCl_3$ + $^{15}NH_4^+$ + C_2H_2 + activated sludge, anaerobic.

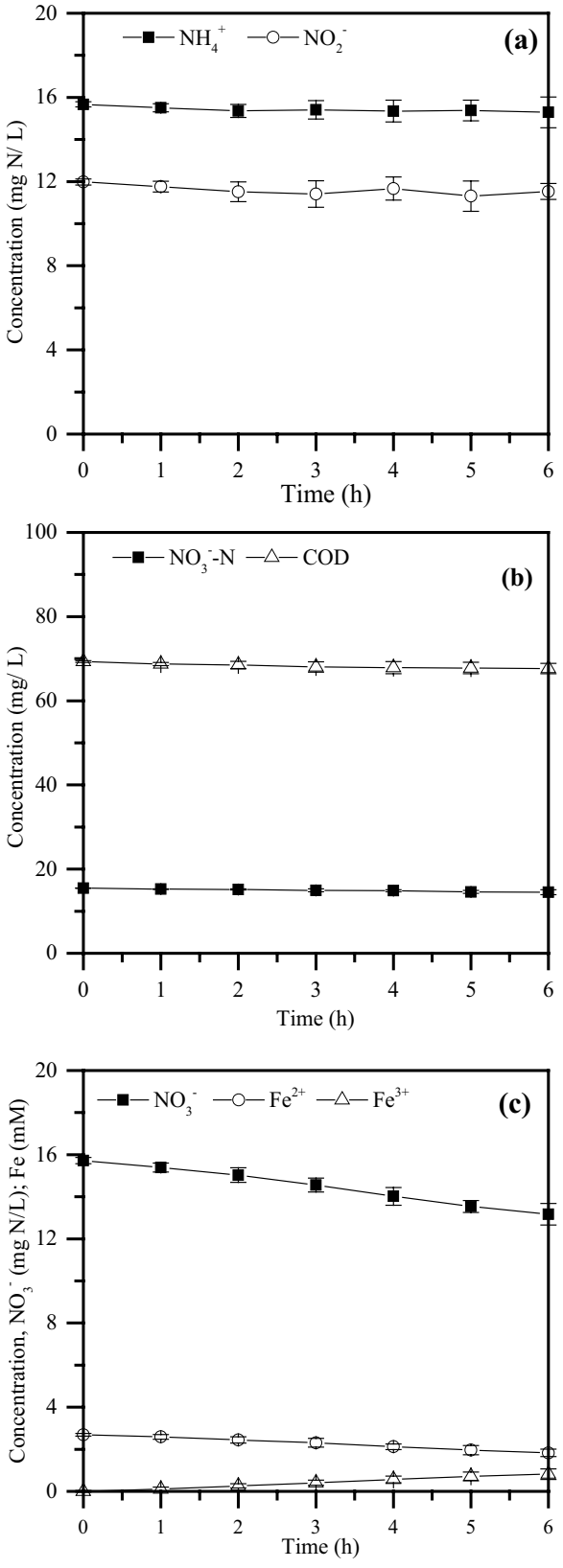

Fig. 6. Nitrogen and Fe conversion properties of different biological processes in batch tests. (a) Batch test B1, NH_4^+ + NO_2^- + activated sludge, (b) batch test B2, NO_3^- + CH_3COONa + activated sludge, and (c) batch test B3, NO_3^- + Fe(II) + activated sludge.

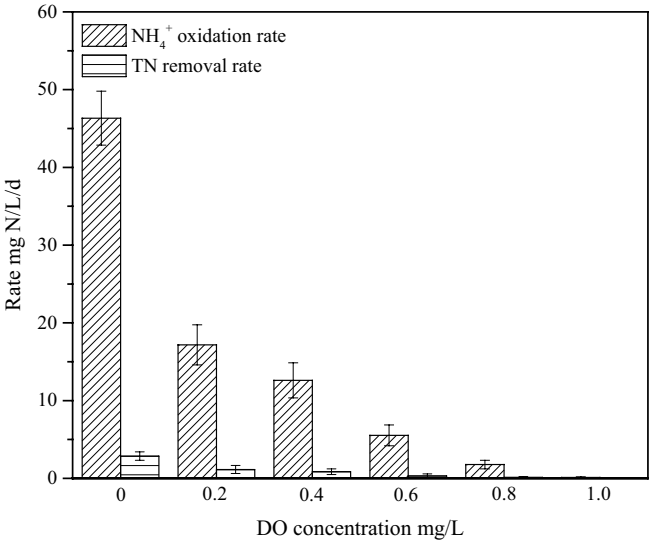

Fig. 7. NH_4^+ oxidation rate and TN removal rate at different DO concentrations. TN consisted of NH_4^+–N, NO_2^-–N and NO_3^-–N; the NH_4^+ oxidation rate, NO_3^- accumulation rate and TN removal rate were obtained by dividing the slopes of the linear regressions with the experimental results (measured concentrations) from the volume of biofilm carrier, and the linear correlation coefficients were all greater than 90%.

After 200 d of experiments, the NH_4^+ oxidation rate and TN removal rate decreased with an increase in DO in the effluent. The most possible reason for the decrease in the NH_4^+ oxidation rate and TN removal rate was that the Feammox process was inhibited by DO in the influent. The formation of surface films may cause long-term problems by reducing the activity of the metal surface [23,36]. In this study, the sponge iron in the lower part of the reactor kept the top portion of the reactor containing the biofilm carriers and sponge iron at an anaerobic condition for approximately 200 d total. Therefore, the sponge iron should be replaced according to the distribution of DO in the reactor. In addition, more studies that remove the surface film on the granular iron are needed to prolong the successive operating time of the FeBR.

3.4. Microbial community analysis

The microbial communities in the biomass collected from different stages (seed sludge and biomass on day 180) of FeBR1 are depicted at the phylum level in Fig. 8a. *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Spirochaetes*, *Proteobacteria*, and *Actinobacteria* were the dominant phyla of the seed sludge, and the abundances were 45.37%, 17.55%, 14.87%, 9.26%, 4.83%, and 2.46%, respectively. The relative abundance of *Bacteroidetes*, *Chloroflexi*, *Spirochaetes*, and *Actinobacteria* decreased to 20.53%, 15.39%, 2.16% and 0.47% with the time, respectively. Meanwhile, the relative abundances of *Proteobacteria*, *Chlorobi*, *Elusimicrobia*, *Gemmatimonadetes*, and *Verrucomicrobia* increased to 29.31%, 0.17%, 0.41%, 7.96% and 0.89%, respectively. The dominant phyla on day 180 were *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Firmicutes*, and *Gemmatimonadetes*. *Bacteroidetes* were also found in the system of the Sulfammox [12]. Also, *Bacteroidetes* has been

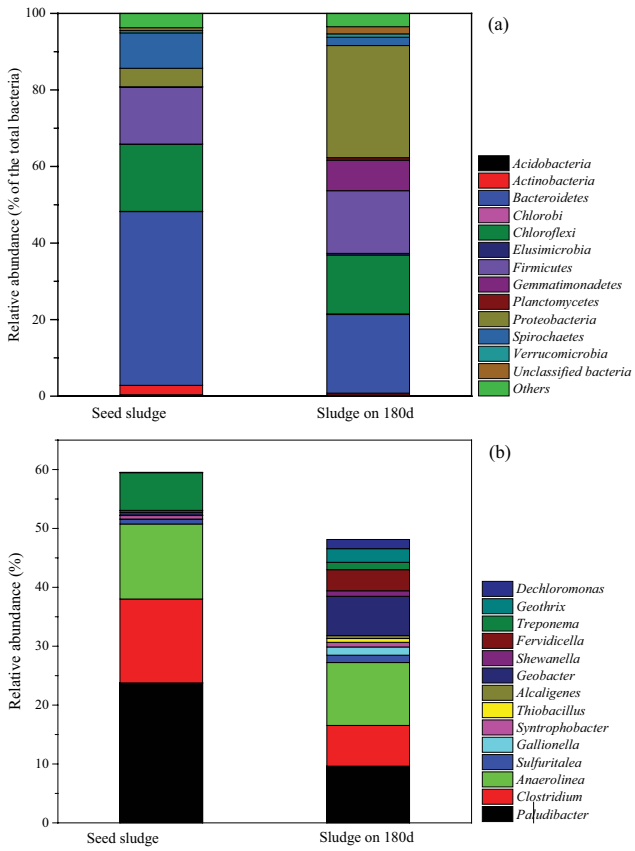

Fig. 8. Relative abundances (percent) of microbial groups at the phylum level (a) and the dominant genera (b) in biomass collected from FeBR1 at day 180 and in the seed sludge.

documented in many sulfate reduction environments and may be involved in the sulfur reduction process [37,38]. It was also demonstrated that the sulfur cycle could drive the Feammox process [39]. Sulfate in the influent may propel the nitrogen cycle in the reactor due to the presence of *Bacteroidetes*. The role of *Bacteroidetes* in the reactor requires further study.

Fig. 8b summarizes the relative abundances of the dominant identified bacteria at the genus level in the seed sludge and biomass on day 180. The bacteria community of the putative Anammox coupled to Fe(III) was composed of five genera, including *Geobacter* spp., *Geothrix*, *Shewanella* spp., *Fervidicella*, and *Clostridium*. These five genera can reduce Fe(III) to Fe(II) [40–43]. Among these genera, the relative abundances of *Geobacter* spp., *Fervidicella* and *Geothrix* in biomass on day 180 were significantly higher than those in the seed sludge. It is noteworthy that *Fervidicella*, which is capable of Fe(III) reduction [41], was detected as one of the most abundant populations (3.59%) in biomass on day 180. Also, *Geobacter* spp. and *Shewanella* spp. have been reported to be related to Feammox in intertidal wetland soil [16] and riparian sediment [9]. *Geothrix* was reported in paddy soil amended with electron shuttles to enhance Feammox [44]. Although the functional Feammox bacteria could not be identified directly, the characterization of bacterial communities in the FeBR1 might provide microbial evidence for the importance of iron-reducing bacteria in the Feammox process.

The relative abundance of *Dechloromonas*, which are associated with nitrate-dependent Fe(II) oxidation [45], was higher in biomass on day 180 than in seed sludge. To quantify the relative abundances of denitrifying microorganisms, qPCR for the nitrate-reductase-encoding genes *napA* and *narG* as well as for the nitrite-reductase-encoding genes *nirK* and *nirS* were performed on DNA samples. As shown in Fig. 9, although the abundances of *napA*, *narG*, *nirK*, and *nirS* were higher in seed sludge than in the biomass collected on day 180, obvious abundances of nitrate-reductase-encoding genes and nitrite-reductase-encoding genes were observed in the biomass collected on day 180. Also, NO_3^- could be reduced to N_2, causing TN removal, and extremely low activity of heterotrophic denitrification was observed. The observation of the abundances of nitrate-reductase-encoding genes *napA* and *narG* and the nitrite-reductase-encoding genes *nirK* and *nirS* in the biomass collected on day 180 may be due to autotrophic nitrate-reducing Fe(II) oxidation bacteria. Previous studies have reported that the presence of Fe(II) can enhance the nitrate reduction rate [45,46] and increase the expression of nitrate- and nitrite-reductase-encoding genes [47]. Anaerobic nitrate-dependent Fe(II) oxidizing microorganisms accept electrons from Fe(II), gaining a growth advantage and reduce nitrate and nitrite [48,49]. Therefore, the cooperative behavior of microorganisms catalyzing Feammox and NFO converts NH_4^+ to N_2 in anaerobic conditions with Fe as an electron shuttle.

3.5. Application of the Feammox bioreactor

The results of this study show that the Feammox process was successfully developed in a biofilm reactor using sponge iron as a Fe source. The oxidation of NH_4^+ to NO_3^- by Feammox under anaerobic conditions was evident in

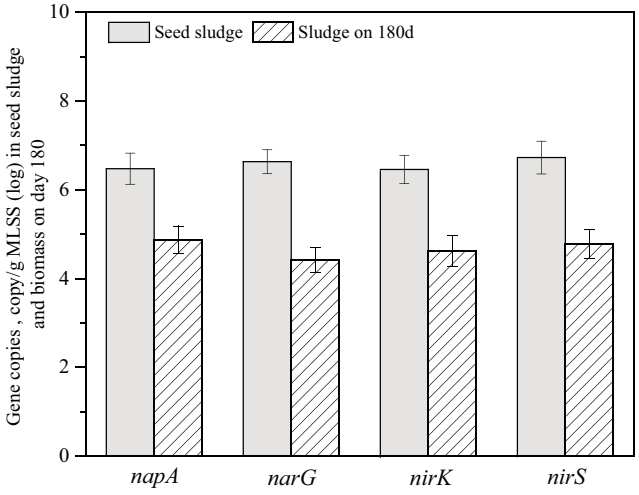

Fig. 9. Gene copy numbers of nitrate-reductase-encoding genes (*napA* and *narG*) and nitrite-reductase-encoding genes (*nirK* and *nirS*) in biomass collected from FeBR1 at day 180 and in the seed sludge. Values are represented by average values from three replications, and bars indicate standard errors.

our experiments. Although poor TN removal efficiency was obtained in the system, the experimental results also confirmed the possibility of coupling Feammox with NFO for nitrogen removal. The DO in the wastewater of this study was consumed by sponge iron, making the system suitable for anaerobic biological processes or coupling with anaerobic biological processes, such as denitrification and NFO. Sulfur-based and pyrite-based autotrophic denitrification can reduce nitrate to achieve nitrogen removal. It has been reported that pyrite can be used to achieve nitrate removal [50]. Therefore, the addition of pyrite may increase the nitrogen removal capacity of the reactor. Although the nitrogen removal rate caused by NFO in the Feammox system of this study was very low, enhancing the NFO process seems to be a direct and effective way to improve the nitrogen removal capacity. Researchers have tried to achieve nitrogen removal by coupling Feammox with NFO [14]. Extensive studies are still required to increase the ammonia oxidation rate and nitrogen removal rate of the reactor. We hope that the discussion presented in this paper provides a possible approach for developing this Feammox system further.

4. Conclusion

In conclusion, this study successfully developed a FeBR using sponge iron as the Fe source feeding of aerobic influent water. Sponge iron reacted with DO in the influent, which caused anaerobic conditions and provided Fe(III) for Feammox. NH_4^+ was oxidized to NO_3^- by Feammox in the FeBR. The average NH_4^+ oxidation rate and TN removal rate reached 32.2 mg N L^{-1} d^{-1} and 4.6 mg N L^{-1} d^{-1} during the 300 d operation, respectively. The Feammox process could be inhibited significantly even when the concentration of DO increased to 1.0 mg L^{-1}. Only a small fraction of the generated NO_3^- was reduced to N_2 by NFO causing nitrogen removal. The FeBR could be an effective technology for NH_4^+ oxidation. The coupled microbially driven Fe and N redox transformation may provide new insights into the development of ammonium removal technology. Extensive studies are still required to increase the ammonia oxidation rate and nitrogen removal rate of the reactor.

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