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Effect of Fe^{2+} on ANAMMOX Granular Sludge Cultured in a Biased Acidic Influent and Dynamic Environment

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Abstract: A continuous stirred tank reactor (CSTR) was utilized in this study to enrich and cultivate anaerobic ammonia oxidation process (ANAMMOX) granular sludge by gradually decreasing its pH, and to investigate the effects of different concentrations of ferrous ions (Fe^{2+}) on the activity of ANAMMOX granular sludge cultivated under biased acidic conditions. The final nitrogen removal of ANAMMOX deteriorated at pH 6.30–6.50 after 220 days of continuous operation, but the nitrogen removal of ANAMMOX was favorable at pH 6.50–7.00. This indicates that a slightly acidic environment (pH = 6.50–7.00) promotes the activity of ANAMMOX, but the pH should not be too low (pH = 6.30–6.50). In the reactor, *Candidatus Kuenenia* was consistently the dominant ANAMMOX genus and its abundance declined from 11.70% on day 1 to 10.44% on day 220. As Fe^{2+} concentrations were increased (10, 20, 30 mg/L) in ANAMMOX granular sludge cultured in an acidic environment, the nitrogen removal effects gradually increased. In addition, with the increase in Fe^{2+} concentrations, the total nitrogen removal load (NRL) in the reactor was increased from 1.16 kg/(m^3/d) to 1.42 kg/(m^3/d). Increases in Fe^{2+} concentration did not result in inhibition of ANAMMOX, which may be attributed to the morphology of sludge and the shape of the reactor. As a result of the present study, new insights were gained into the physiological characteristics of ANAMMOX in an acidic environment over the long term, and how Fe^{2+} affects its ability to remove nitrogen from the environment.

Keywords: ferrous ion; ANAMMOX; acidic environment; pH; nitrite; CSTR

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1. Introduction

Water eutrophication is caused by the excessive concentration of nitrogen and phosphorus in water [1], and has gradually become a global environmental issue [2]. Water ecosystems have been attracting widespread attention in terms of nitrogen removal pathways [3]. The emerging ANAMMOX (Equation (1)) can significantly reduce energy consumption and organic carbon demand compared to traditional nitrification–denitrification technology [4–7]. The stability of partial nitrification (PN) is crucial to maintaining a continuous supply of nitrite (NO_2^--N). However, in lower ammonia (NH_4^+-N) concentrations water, the provision of stable NO_2^--N through PN and partial denitrification (PD) is difficult to achieve [8,9]. In addition, the instability of PN and PD will lead to nitrification/denitrification in the whole process, and also consume a part of NO_2^--N which will lead to the lack of reaction substrates in ANAMMOX [10].

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

$$2Fe^{2+} + NO_3^- + 3H_2O \rightarrow 2FeOOH + NO_2^- + 4H^+ \quad (2)$$

Fe^{2+} could stimulate the enzyme activity and accelerate the specific growth rate of ANAMMOX, which is favorable for the rapid enrichment of ANAMMOX [11]. In the ANAMMOX system, Fe^{2+} can trigger other pathways for nitrogen removal, thereby enhancing nitrogen removal [12]. Furthermore, Fe^{2+} [13] in an acidic environment has a strong reducing ability for nitrate (NO_3^--N) (Equation (2)), and NO_2^--N is produced during this reaction. According to Zhang et al. [13], NO_2^--N concentrations were detected when NO_3^--N was reduced by Fe^{2+}. Therefore, the reduction of NO_3^--N to NO_2^--N using Fe^{2+} will provide a reaction substrate for the ANAMMOX reaction, which not only saves carbon source and reduces the residual sludge discharge, but also allows the NO_3^--N produced by ANAMMOX to then continue to participate in the nitrogen removal process. Reduction of NO_3^--N with Fe^{2+} produces a number of gaseous intermediates, such as NO [12], N_2O [14], and N_2 [15]. In addition, iron is involved in almost all nitrogen transformations in the processes in which ANAMMOX bacteria are involved. For example, iron is an important component of heme. Each hydroxylamine oxidase contains twenty-six heme [16]; each hydrazine oxidase contains eight heme [17]; each hydroxylamine dehydrogenase contains seven heme [18]; and each cytochrome contains one heme [19]. It can be seen that Fe^{2+} is not only able to reduce NO_3^--N, but also is able to promote the metabolism and activity of the ANAMMOX, which improves the performance of the reactor in removing nitrogen.

When cultured ANAMMOX, it was almost in a static state in the reactor, and most of the studies indicated that ANAMMOX bacteria are suitable for survival in alkaline biased environments. One such study is Anja et al. [20], which indicated that the appropriate pH range of ANAMMOX is 7.00–7.50 and Li et al. [21] indicated that the appropriate pH range of ANAMMOX is 7.50–8.00. In addition, Fe^{2+} concentrations have been investigated as a potential promoter of ANAMMOX nitrogen removal [22,23]. But most of the ANAMMOX they used was cultured in a biased alkaline environment for enrichment. However, it has not been studied how ANAMMOX initiates in a dynamic and under long-term acidic conditions, or how Fe^{2+} affects the activity of the strain cultivated under such conditions.

Therefore, this study was conducted in order to investigate the nitrogen removal performance of ANAMMOX under acidic conditions and to determine the effect of Fe^{2+} on the performance of this ANAMMOX in nitrogen removal. Specifically, it was divided into (1) exploring the long-term operational effects of ANAMMOX by gradually lowering the pH in the system in the dynamic environment of the CSTR; (2) changes in biodiversity and dominant genera within the reactor were explored by 16sRNA high-throughput sequencing; (3) ANAMMOX granular sludge with successfully initiated nitrogen removal was investigated at different Fe^{2+} concentrations.

2. Materials and Methods

2.1. Wastewater Feed and Sludge Source

Synthetic wastewater was prepared with tap water. The system used NH_4Cl and $NaNO_2$ as a nitrogen source, and NaH_2PO_4 as a phosphorus source. Suitable amounts of trace element solution were added to the reactor [24]. Table 1 presents the parameters that influence reactor operation. A rising filter column cultured by the group was used to inoculate sludge taken from ANAMMOX granular sludge. Total nitrogen removal rate (NRR) in the rising filter column averaged 71.15%. The sludge was brownish red in color due to the multiple dead zones in the reactor. In order to investigate the nitrogen removal performance of ANAMMOX under acidic environment, organic carbon source was not added in this study.

Table 1. A list of the reactor's influential parameters.

Time/ (d)	HRT/ (h)	pH	rpm/ (r/min)	NH_4^+-N/ (mg/L)	NO_2^--N/ (mg/L)	NO_3^--N/ (mg/L)	PO_4^{3-}-P/ (mg/L)	Trace Element/ (ml/L)	DO/ (mg/L)
1–31	4.0	7.50–7.70	20	180–250	200–250	5–12	10	1	0.30
32–73	4.0	7.10–7.50	30	165–240	210–280	5–13	10	1	0.16
74–108	4.0	7.10–7.50	40	180–205	155–235	10–18	10	1	0.15
109–152	5.7	6.50–7.50	40	150–220	175–255	8–16	10	1	0.11
153–223	7.0	6.30–6.50	40	170–200	205–270	8–12	10	1	0.09

2.2. Reactor Configuration and Operation

In this study, ANAMMOX was continuously stirred in a continuous stirred tank reactor (CSTR) in order to enrich it and observe the effect of Fe^{2+} on the performance of nitrogen removal (Figure 1). The reactor had an effective volume of 7 L and the temperature inside the reactor was maintained at 30 ± 1 °C by heating it in a water bath. The CSTR was operated continuously for 223 days, and the speed was gradually increased from 20 to 40 rpm using the motor (Table 1). The water feed to the reactor was continuous. The CSTR was activated by adjusting the influent water quality and hydraulic residence time (HRT) and mixing speed to make the granular sludge mix well with the sewage.

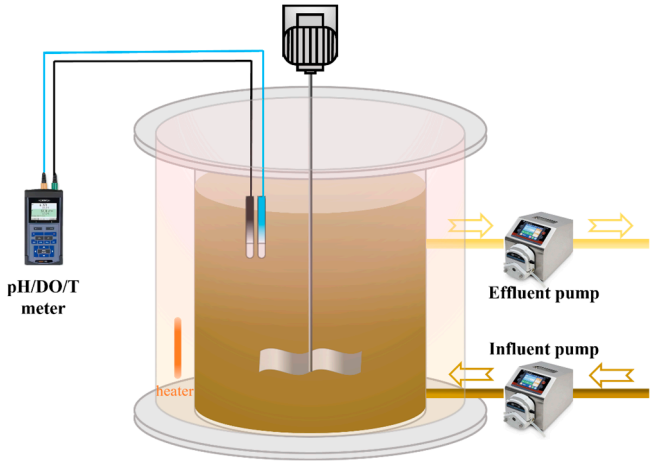

Figure 1. Reaction device diagram of CSTR.

2.3. Microbial Diversity

We used the Alpha diversity index to assess the diversity of the sludge, and high-throughput sequencing was used to identify the microbial populations in the sludge. On day 1 and day 220, samples of sludge were taken from the reactor. DNA was extracted from the CSTR soil samples using an E.Z.N.A Soil DNA Kit (OMEGA, New York, NY, USA) in accordance with the manufacturer's instructions. Using the primer sets 341F (5′-ATGCGTAGCCGACCTGAGA-3′) and 805R (5′-CGTCAGACTTTCGTCCATTGC-3′), the V3-V4 region of the universal 16S rDNA gene was amplified. A two-step multiplex PCR protocol was used to prepare the 16S rDNA amplicon library. The first round of PCR reactions was conducted with a mixture of 15 L (2.00 L) of Hieff® PCR Master Mix (Yousen Biotech Co., Ltd., Shanghai, China), 1 L Bar-PCR primer F and 1 L Index-PCR primer R, combined with 10–20 ng of template DNA and PCR-grade water, reaching a total volume of 30 µL. For the second round of PCR, 15 mL of two-factor Hieff® PCR Master Mix (Yousen Biotech Co., Ltd., Shanghai, China), one mL of primer Bar-PCR F, one mL of Index-PCR-Primer R, 20–30 ng of PCR products, and PCR-grade water were used, resulting in a final volume of 30 µL. There were five cycles of PCR amplification: an initial step of denaturing at 94 °C for 3 min, an annealing stage at 55 °C for 20 s, an extension stage at 72 °C for 5 min, and a final extension stage at 72 °C for 10 min. A 1% (*w*/*v*) agarose gel in

TBE buffer (Tris, boric acid, EDTA) was used to electrophorese the PCR products, which were stained with ethidium bromide and examined under ultraviolet illumination.

2.4. Influence of Different Fe^{2+} Concentrations on the Nitrogen Removal Effect

The experiment was conducted using ANAMMOX granular sludge successfully initiated in CSTR. This section examined the effects of different Fe^{2+} concentrations on the activity of ANAMMOX as well as the effectiveness of nitrogen removal. The temperature was controlled at 30 ± 1 °C and pH 7.50 ± 0.10 during the experiments. Table 2 provides a detailed description of the experimental conditions.

Table 2. Experimental operation conditions.

Time/(d)	Fe^{2+}/(mg/L)	HRT/(h)	rpm/(r/min)
224–243	10	7.00	40
244–263	20	7.00	40
264–283	30	7.00	40

2.5. Analytical Methods

A sample of water was collected from influent and effluent water of the CSTR. Standard methods have been used to monitor the concentrations of NH_4^+-N, NO_2^--N, and NO_3^-N [25]. An HI 931700 pH meter and an HI 2400 DO meter were used to measure pH and dissolved oxygen (DO), respectively. On day 220, a fluorescence microscope (BX 41, OLYMPUS) was used to observe the sludge morphology in reactors.

2.6. Calculation

The total nitrogen (TN), total nitrogen influent loading (NIL), ammonia removal efficiency (ARE), NRL, and NRR were calculated by Equations (3)–(8) [8,26–28] as follows:

$$TN = C_{NH_4^+-N} + C_{NO_2^--N} + C_{NO_3^--N} \quad (3)$$

$$NIL = \frac{TN_{in}}{HRT} \quad (4)$$

$$NRL = \frac{TN_{in} - TN_{eff}}{HRT} \quad (5)$$

$$ARE = \frac{NH_4^+{}_{Inf} - NH_4^+{}_{Eff}}{NH_4^+{}_{Inf}} \quad (6)$$

$$NRR = \frac{TN_{in} - TN_{eff}}{HRT} \quad (7)$$

where $C_{NH_4^+-N}$, $C_{NO_2^--N}$, and $C_{NO_3^--N}$ are the concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N, respectively; NIL and NRL are total nitrogen influent loading and total nitrogen removal loading in kg/(m^3/d), respectively; ARE and NRR are the ammonia removal efficiency and TN removal rate in %; TN_{in} and TN_{eff} represent total nitrogen concentrations in influent and effluent, respectively; and HRT represents hydraulic retention time in hours. $NH_4^+{}_{Inf}$, $NH_4^+{}_{Eff}$, $NO_2^-{}_{Inf}$, $NO_2^-{}_{Eff}$ are the concentration of NH_4^+-N and NO_2^--N in the influent and effluent in mg/L, respectively.

3. Results and Discussion

3.1. The Enrichment of ANAMMOX in CSTR by Gradual Reduction of pH

During the initial period of enrichment (1–31 d), the stirring speed was controlled to 20 r/min and pH was controlled at 7.50–7.70 in the influent water (Figure 2c). The ARE and NRR in the reactor on day 1 were only 46.5% and 45.75% (Figures 2a and 3a), respectively,

which was not good compared to the inoculated sludge. NH_4^+-N concentration was 27 mg/L in the effluent from the inoculated sludge reactor, and ARE and NRR were 85% and 79%, respectively. Since much of the granular sludge was stirred up and turned into flocculent sludge in the CSTR, the efficiency of nitrogen removal was reduced. A similar phenomenon occurred in Tang et al. [29] at the beginning of the ANAMMOX startup, where the ANAMMOX granular sludge changed to flocs after agitation. Due to the flocculent nature of the ANAMMOX sludge at this time, and with the process of reforming granular sludge in progress, artificial sludge refluxing was started on day 4 to avoid significant sludge loss.

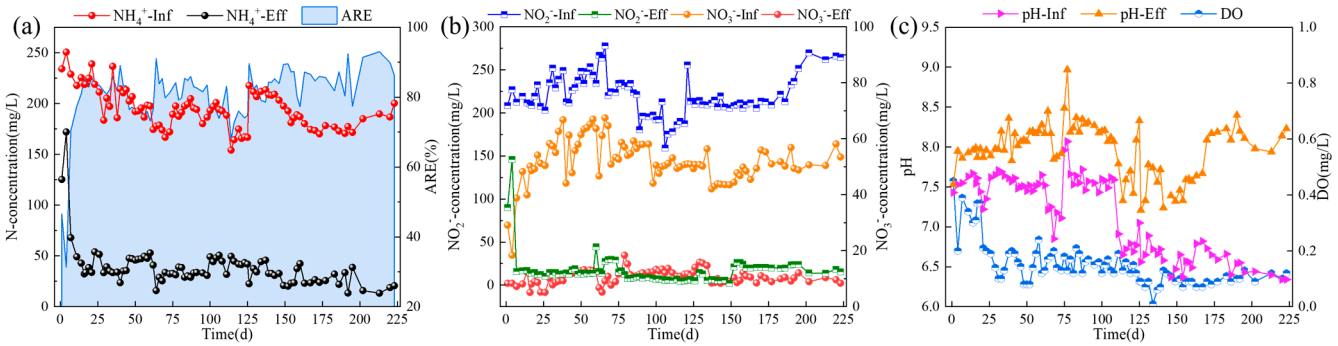

Figure 2. (**a**) Changes in NH_4^+-N concentrations in influent and effluent; (**b**) changes in NO_2^--N and NO_3^--N concentrations in influent and effluent; (**c**) changes of pH and DO in influent and effluent in the reactor.

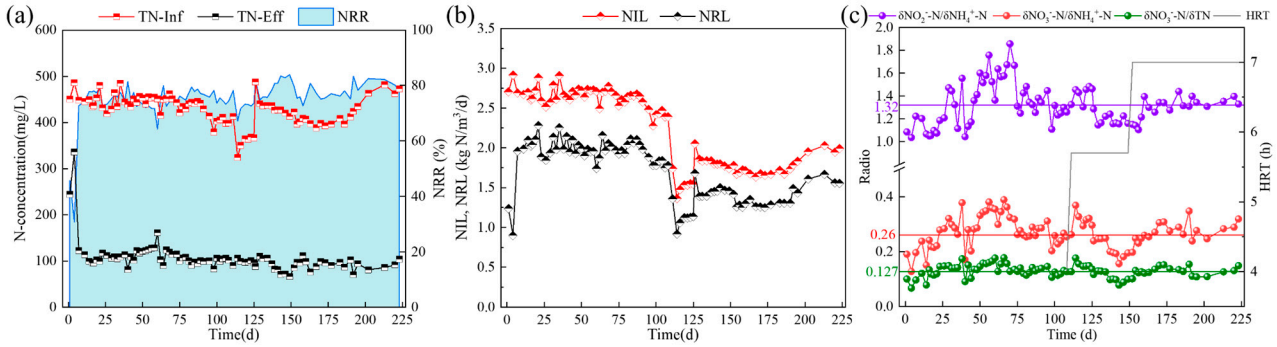

Figure 3. (**a**) Changes of TN concentrations in influent and effluent; (**b**) changes of NIL and NRL; (**c**) changes of the ratio of the difference between the influent and effluent of each nitrogen.

In addition to the negative effects of agitation, higher DO in the reactor may also affect ANAMMOX activity, resulting in poor nitrogen removal performance. There are three main reasons for the elevated DO concentration. On the one hand, the tap water distribution made the influent have close to saturated DO concentration and no measures were taken to remove DO from the influent at the beginning of the startup period; on the other hand, poor reactor sealing with agitation caused atmospheric oxygen to enter the system. Last but not least, the artificial sludge refluxing was also a significant reason for DO concentration in the reactor. By reducing the concentration of DO in the reactor, it may be possible to reduce the abundance of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). To minimize the DO content of the feed water, nitrogen blowdown was used in the formulation of the feed water in order to provide ANAMMOX with a suitable growth environment. DO had decreased to 0.21 mg/L in the reactor on day 21, and then the value of DO in the system was still decreasing.

At the beginning of the reaction (1–4 d), the average NRR in the reactor was only 38.23%, and the average NRR increased rapidly to 75.92% after increasing reflux (4–31 d) (Figure 3a). The NRL in the reactor also increased from 0.90 kg/(m^3/d) to 1.24 kg/(m^3/d) at this time (Figure 3b). In addition, the ratios of the difference in influent and effluent for

each nitrogen, δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN were 1.138, 0.195, and 0.101 (Figure 3c), respectively, which deviated from their theoretical ratios of 1.32, 0.26, and 0.127 (Equation (1)). It can be seen that, at the beginning of the reaction, the ANAMMOX sludge in the reactor was unable to adjust to the CSTR's operating mode, and ANAMMOX activity was low due to the effects of agitation and DO.

On days 32 to 73, the pH of the influent water was reduced to 7.10–7.50 and the stirring speed was increased to 30 r/min. The concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N in effluent gradually stabilized at 38.31 mg/L, 17.91 mg/L, and 59.20 mg/L, respectively. The concentration of DO stabilized at about 0.16 mg/L. The NRL showed a gradual increasing trend (Figure 3b), with a maximum of 2.26 kg/(m^3/d) and an average NRR of 74.83% in the reactor. At this time, δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN were 1.460, 0.310, and 0.146, respectively, and the ratios were all higher than the theoretical values (Figure 3c). It is speculated that AOB and NOB can grow in the reactor because there was always a low DO concentration in the reactor. The results were obtained by oxidizing NH_4^+-N, NO_2^--N to NO_3^--N, which resulted in values that were higher than predicted. Despite this, the NRR and NRL continued to increase in the reactor. When starting the ANAMMOX process, Nutchanat and Suwanchai [30] also found that the system enters a stagnation phase after going through the cell lysis phase. At this time, the ANAMMOX activity was slowly increasing, and then it stepped into a period of increased activity. Thus, the ANAMMOX activity in this study was gradually stabilizing and increasing slowly at this time.

On days 74 to 108, the pH was controlled at 7.10–7.50 and the stirring rate was increased to 40 r/min. There was a reduction in effluent concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N in the reactor, with average values of 36.88 mg/L, 11.03 mg/L, and 53.60 mg/L (Figure 2c), respectively, and the DO concentration was stabilized at about 0.15 mg/L. The NRR in the reactor continued to increase up to 79.41% and the NRL stabilized with an average value of 1.78 kg/(m^3/d) (Figure 3a). At this time, δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN were 1.353, 0.273, and 0.128, respectively. In this stage, the concentration of the reactor effluent was gradually stabilized (Figure 3c), the effect of nitrogen removal gradually increased, and all the ratios were close to the theoretical ratios. It shows that the operation of the reactor tends to stabilize, and the ANAMMOX had good activity and played a dominant role.

On days 109 to 152, the stirring rate was kept constant at 40 r/min, and in order to continue reducing pH, the HRT in the reactor was increased to 5.7 h. This resulted in a pH reduction of 6.50–7.00 in the influential. The effluent concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were reduced to 34.63 mg/L, 7.13 mg/L, and 48.56 mg/L, and the DO concentration was stabilized at about 0.11 mg/L. The NRR in the reactor continued to increase during this phase, with a maximum of 83.90%, and the NRL stabilized at about 1.42 kg/(m^3/d) (Figure 3b). At this time, δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN were 1.283, 0.253, and 0.124 (Figure 3c), respectively, and all ratios were close to the theoretical ones. The results indicate that the ANAMMOX nitrogen removal process is performing well at this stage. Blum et al. [31] found that the ANAMMOX activity remained at a high level and a gradual emergence of denitrifying bacteria in the system with decreasing pH when culturing ANAMMOX. From the influent and effluent about the pH of the reactor, the influent pH was slightly lower, but the effluent pH was relatively higher (Figure 2c). This may be related to the occurrence of denitrification reactions and thus alkali production within the system (Equation (8)), which provides suitable space for ANAMMOX to survive. It can be seen that the ANAMMOX activity in the reactor was favorable and still dominant at the influent pH of 6.50–7.00.

$$NO_3^- + 5/6CH_3OH \rightarrow 1/2N_2 + 5/6CO_2 + 7/6H_2O + OH^- \quad (8)$$

On days 153 to 223, in order to further reduce the pH to 6.30–6.50, the HRT in the reactor was increased to 7.0 h. Figure 2a shows that the effluent NH_4^+-N concentration gradually decreased to 25.53 mg/L on average, whereas NO_2^--N and NO_3^--N concentra-

tions in the system effluent increased to 15.08 mg/L and 51.79 mg/L on average. The NRR fluctuated at this stage, with an average NRR of around 77.85% (Figure 3a). And the NRL gradually increased up to 1.67 kg/(m^3/d). The δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN were 1.375, 0.271, and 0.129 (Figure 3c), respectively, and the ratios are all higher than the theoretical ratio. According to the experimental results, ANAMMOX activity in the system decreased with further reduction in pH. A possible explanation for this may be the stirring action of the system after influent caused rapid contact between the ANAMMOX and the lower pH influent water in the system, which had a strong effect on the ANAMMOX. And other strains may have emerged as well, but the ANAMMOX system has not completely collapsed. Therefore, further lowering the pH of the influent water may have caused changes in colony composition within the reactor, though the nitrogen removal capacity was still high.

ANAMMOX granular sludge morphology was observed at day 220, as shown in Figure 4. ANAMMOX granular sludge has a large particle size and a bright red color. Due to the favorable gas production of the sludge, there was sludge uplift after a short resting period. This suggests that ANAMMOX was not immediately inactivated despite experiencing an acidic environment for a certain period of time, and that ANAMMOX may still have some dominance at this time.



Figure 4. The morphology of ANAMMOX granular sludge outside the reactor (**a**) and in a measuring cylinder (**b**) on day 220.

3.2. Microbial Diversity Analysis

In order to further clarify the relationship between the microbial community and the nitrogen removal performance within the CSTR, high-throughput sequencing was used to study the structural changes of the microbial population within the reactor. Inoculated sludge from the first day and ANAMMOX granular sludge from the CSTR on day 220 were selected for comparative analysis. As shown in Table 3, Alpha diversity indices were calculated. According to this sequencing result, the Coverage value indicates whether each sample is covered, and the Coverage values of 1.00 and 0.97 indicate that the sample is representative of the microbial diversity in the reactor. Shannon index and Simpson index indicate a high degree of biodiversity in the microbial community, as the Shannon index is higher and Simpson index is lower. With the gradual decrease in pH, the Shannon index decreased from 0.11 to 0.03, and the Simpson index increased from 3.58 to 4.26. This suggests that the decrease in pH led to the creation of new communities of organisms in the system. Moreover, the Simpson index provides information regarding the proportion of dominant genera as well. The Simpson index on day 220 was smaller than that on the first day, which shows that although the study obtained a favorable nitrogen removal effect by adopting the initiation strategy of gradually lowering pH, it made the proportion occupied by the dominant strain decrease.

Table 3. Statistical results for the Alpha diversity index.

Sample	OTU	Simpson	Shannon	ACE	Chao1	Coverage
day1	962	0.11	3.58	1322	1238.07	1.00
day220	1729	0.03	4.26	24967.40	12446.44	0.97

3.3. Microbial Community Evolution at Phylum and Genus Level

As shown in Figure 5a, there were 15 strains detected at the common phylum level for both samples, which indicates that although the two samples were in different states within the reactor, the dominant strains remained essentially the same. *Proteobacteria*, *Planctomycetes* and *Chloroflexi* were the dominant species at the phylum level in two samples. *Proteobacteria* has been considered to the common functional phyla in WWTPs [32]. The *Proteobacteria* contains AOB and NOB [33]; some NOB are present in the *Nitrospirae* [34], and ANAMMOX bacteria are mainly found in the *Planctomycetes* [35,36]. *Planctomycetes* dominated on day 220 with a percentage of 34.08%, which was 18.25% higher than inoculated sludge. This indicates that changing the sludge from the stationary to fluidized state led to the successful enrichment of *Planctomycetes*. It is hypothesized that the reason for this may be due to the fact that mixing allows the particulate sludge to come into fuller contact with the substrate in the water, thus increasing the sludge activity and metabolism and allowing for the enrichment of the *Planctomycetes*. The Proteobacteria where AOB and NOB were located decreased by 24.72% in the reactor after successful startup.

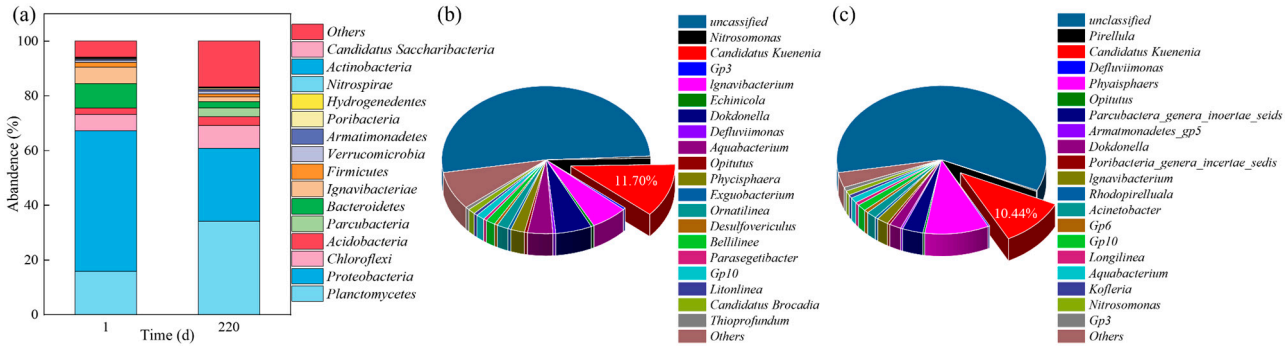

Figure 5. (**a**) A comparison of the relative abundances of microbial communities at the phylum level on day 1 and day 220. (**b**) The relative abundance of microbial communities at the genus level on day 1. (**c**) The relative abundance of microbial communities at the genus level on day 220.

In order to further understand the microbial composition within the reactor before and after startup, the genera associated with nitrogen removal at the genus level in the reactor were analyzed. As shown in Figure 5b, the dominant genus of granular sludge in the CSTR was *Candidatus Kuenenia*. The largest percentage of ANAMMOX on both Day 1 and Day 220 was *Candidatus Kuenenia* with 11.70% and 10.44%, respectively (Figure 5b,c). On day 220 *Phycisphaera* (9.88%) emerged in the reactor, and this genus was a major component of nitrogen removal bioreactor [37]. This is an important reason why the pH of the effluent was able to rise. However, no study has been carried out to show the relationship between *Phycisphaera* and influent pH. In this study, it seems that a decrease in the pH of the influent water may stimulate the reproduction of *Phycisphaera*. Comparison of the percentage of *Nitrosomonas* (a typical AOB genus) in the two samples shows that the abundance of *Nitrosomonas* increased from 0.41% on day 1 to 0.99% on day 220. The NOB genera, such as *Nitrospira*, were not detected on day 220. Nitrogen blowdown failed to remove all of the DO, and low DO was still present in the system. This is an important reason for the deviation of δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN from the theoretical values in the final system.

In general, the nitrogen removal effect was still well, although the proportion of ANAMMOX decreased slightly in the reactor. It is hypothesized that the two reasons are (1)

the fluidized ANAMMOX granular sludge in the CSTR had full contact with the effluent throughout the reactor and thus promoted nitrogen removal; (2) the reduction of pH in the influent allowed *Phycisphaera* to be enriched and could reduce NO_3^--N into a reaction substrate for ANAMMOX bacteria.

3.4. Influence of Different Fe^{2+} Concentrations on the Nitrogen Removal Effect

The changes in the indicators in the reactor during operation are shown in Figures 6 and 7. A concentration of Fe^{2+} of 10 mg/L was used on days 224 to 243. The concentration of NH_4^+-N and NO_2^--N effluents were 3.18 mg/L and 5.79 mg/L, respectively (Figure 6b). ARE increased from 94.69% to 98.15% and NRR increased from 84.28% to 97.30%. At this time, the NRR was 83.84% and the aver NRL was 1.16 kg/(m^3/d) (Figure 7b), and all the ratios were close to the theoretical ratios (Figure 7c). It can be seen that the addition of Fe^{2+} led to an increase in the activity of ANAMMOX bacteria in the reactor and a decrease in the concentrations of NH_4^+-N and NO_2^--N in the effluent, while keeping the influent load basically unchanged. This can be explained in two ways. On the one hand, the increase in Fe^{2+} concentration in the system will stimulate ANAMMOX bacteria to take up iron ions in the environment, which is used to synthesize hydroxylamine oxidase [16], hydrazine oxidase [17], hydroxylamine dehydrogenase [18] and heme [19] in ANAMMOX bacteria. On the other hand, increased Fe^{2+} concentrations can not only form ferritin in ANAMMOX bacteria [19], but also thicken the surface and increase the density of ANAMMOX bacteria to make the bacteria more dense [38], thus enhancing the efficiency of nitrogen removal.

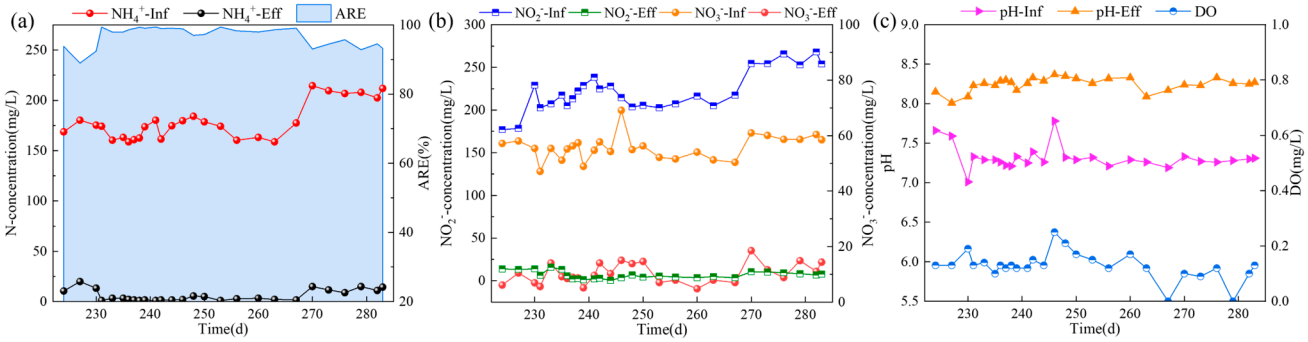

Figure 6. (**a**) Changes in NH_4^+-N concentrations in influent and effluent. (**b**) Changes in NO_2^--N and NO_3^--N concentrations in influent and effluent. (**c**) Changes of pH and DO in influent and effluent in the reactor.

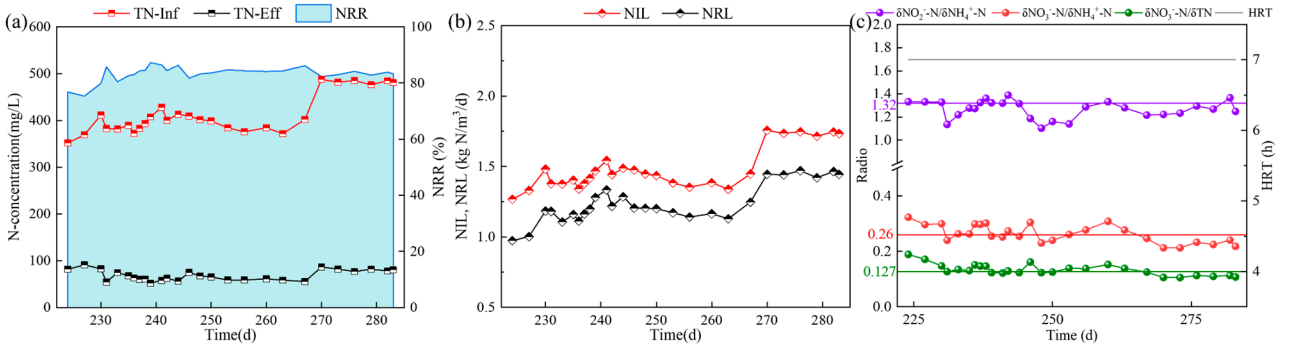

Figure 7. (**a**) Changes of TN concentrations in influent and effluent. (**b**) Changes of NIL and NRL. (**c**) Changes of the ratio of the difference between the influent and effluent of each nitrogen.

On days 244 to 263, the influent Fe^{2+} concentration was increased to 20 mg/L, and the influent NH_4^+-N and NO_2^--N concentrations remained the same as in the previous stage (Figure 6a,b). The treatment effect was good in this stage, with the concentrations in effluent of NH_4^+-N and NO_2^--N as low as 2.67 mg/L and 4.32 mg/L. At this time, the NRR was 84.55% (Figure 7a), the aver NRL increased up to 1.19 kg/(m^3/d), and the ratios were close

to the theoretical ratios during the reaction (Figure 7b). Zhang et al. [38] concluded when the concentrations of Fe^{2+} reached 1–5 mg/L, it will favor the promotion of biological activity and nitrogen removal efficiency, while an increase to 10–30 mg/L produced an inhibitory effect during the experiment using an ANAMMOX biofilm reactor. While further increasing the influent Fe^{2+} concentration in this experiment did not inhibit ANAMMOX bacteria, but rather led to a continuous enhancement of the activity of ANAMMOX bacteria in the reactor. There are two possible reasons for this difference: (1) Increased concentrations of Fe^{2+} can cause the system to generate many iron oxides wrapped around the biofilm affecting the contact of the ANAMMOX sludge with the NH_4^+-N and NO_2^--N [39], thus reducing the performance of nitrogen removal [40]. In this experiment, granular sludge with small relative specific surface area was used. It was therefore minorly affected by the increase in Fe^{2+} concentration. (2) A CSTR reactor was used in this experiment, and the stirring action made it difficult for iron oxides to adhere to the sludge surface [41]. Thus, the increased Fe^{2+} concentration did not have an adverse effect.

On days 264 to 283, the influent concentration of Fe^{2+} increased to 30 mg/L, and to provide adequate substrate, the influent NH_4^+-N and NO_2^--N concentrations increased to 208.88 mg/L and 258.32 mg/L. The effluent concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were higher than before (Figure 6a,b). The average NRR in the reactor during this phase was 83.43% and the average NRL increased to 1.42 kg/(m^3/d) (Figure 7b). It is important to note that, although the influence load was increased, the increase in the concentration of Fe^{2+} in the influential allowed for continued enhancement of ANAMMOX bacterial activity in the reactor. This improved the reactor's ability to remove nitrogen. A further increase in Fe^{2+} concentration did not inhibit the ANAMMOX granular sludge. At this time, the δNO_2^--N/δNH_4^+-N and δNO_3^--N/δNH_4^+-N ratios were low (Figure 7c). Li et al. [21] proved that the joint reduction of NH_4^+-N and NO_3^--N during their experiments may be due to the Fe^{3+} reduction coupled with anaerobic ammonia oxidation (Feammox) of the production. This may occur in the reduction of NO_3^--N to NO_2^--N by Fe^{2+} and thus further participate in the ANAMMOX reaction, and there may the oxidization of NH_4^+-N to NO_3^--N or N_2 by Fe^{3+} [42]. A low ratio of each nitrogen in the reactor at this time may be due to the possibility that a portion of the NH_4^+-N in the system was oxidized by Fe^{3+} and did not participate in the ANAMMOX process resulting in a low δNO_2^--N/δNH_4^+-N ratio. Remaining NH_4^+-N reacted with NO_2^--N in the influencer and NO_2^--N produced by reduction of NO_3^--N by Fe^{2+} in the ANAMMOX reaction, leading to low ratios of both NO_2^--N/NH_4^+-N and NO_3^--N/NH_4^+-N. Therefore, although excessive Fe^{2+} concentration will increase the NRR, it will lead to a deviation in the nitrogen consumption ratio of the ANAMMOX reaction.

4. Conclusions

(1) The temperature was controlled at 30 ± 1 °C and pH was gradually decreased during the incubation of ANAMMOX. The average NRR in the CSTR was 82.39% and the average NRL was 1.78 kg/(m^3/d) after stable operation. When the pH was adjusted to the range of 6.50–7.00, the nitrogen removal effect gradually increased, and the δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN ratios were close to the theoretical ratios. When further lowering the influent pH to the range of 6.30–6.50, the ratios were all higher than the theoretical ratio, which caused the composition of the community in the reactor to change, but ANAMMOX bacteria were always dominant.

(2) After the reactor reached stabilization, *Candidatus Kuenenia* occupied the dominant genus in ANAMMOX, while *Phycisphaera*, a genus under the phylum *Planctomycetes*, appeared due to the decrease in pH in the system.

(3) Increasing the influent Fe^{2+} concentration to 10 mg/L and 20 mg/L led to favorable removal rates of NH_4^+-N, NO_2^--N and TN, as well as closer to theoretical rates of δNO_2^--N/δNH_4^+-N, δNO_3^--N/δNH_4^+-N, and δNO_3^--N/δTN. As Fe^{2+} concentrations increased in the influential water to 30 mg/L, the δNO_2^--N/δNH_4^+-N and δNO_3^--N/δNH_4^+-N

decreased to 1.105 and 0.197, respectively, due to the reaction of Fe^{2+} with NO_3^--N and Fe^{3+} with NH_4^+-N.

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