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Effect of Sulfur Autotrophic Denitrification Sludge on the Start-Up Characteristics of Anaerobic Ammonia Oxidation

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Abstract: In this study, we used a two-stage experiment in order to investigate the effect of the inoculation with elemental sulfur-based autotrophic denitrification (S^0-SADN) sludge on the start-up characteristics of Anaerobic ammonia oxidation (ANAMMOX). In the first stage, we attempted direct enrichment with an elemental sulfur and pyrite filler in a S^0-SADN reactor, which retained stable operation, and adjusted the nitrogen source components of the influent at different times. In the second stage, we replaced the original filler with Kaldnes filler, and set the influent component to be divided into NH_4^+-N and NO_2^--N. The ANAMMOX process could not be started in the 80-day S^0-SADN stage despite the 0.8% abundance of *Candidatus Kuenenia*; however, after changing the original filler, the reactor showed obvious ANAMMOX reaction characteristics after day 44, and under the condition of an influent TIN load of 0.36 $kg(m^3 \cdot d)^{-1}$, the reactor TIN removal rate was stable at more than 80% after day 55. The main ANAMMOX bacteria in the reactor were *Candidatus Brocadia* (1.08%) and *Candidatus Kuenenia* (0.96%). The results show that it is feasible to initiate the ANAMMOX process by inoculating the S^0-SADN sludge; however, it is not suitable to start the ANAMMOX and the stable operation of the S^0-SADN simultaneously. The ANAMMOX process can be started first under the condition of no sulfur source, which takes little time. After initiating the ANAMMOX process, the coupling S^0-SADN process can be re-considered given an excessive accumulation of S^0-SADN bacteria in the system.

Keywords: ANAMMOX; start up; inoculated sludge; S^0-SADN; microbial communities

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1. Introduction

The conventional heterotrophic denitrification process typically involves the oxidation of NH_4^+-N to NO_3^--N by autotrophic nitrifying bacteria in the aeration tank [1,2], followed by the entry of NO_3^--N into the anoxic tank as an electron acceptor for the heterotrophic denitrification process. In this process, a carbon source is used as an electron donor, and NO_3^--N is reduced to N_2. The reaction equation for the traditional nitrogen removal process are as Equations (1) and (2):

$$NH_4^+\text{-N} + 2O_2 + 2HCO_3^- = NO_3^- + 2CO_2 + 3H_2O \quad (1)$$

$$4NO_3^- + 5C + 2H_2O^- = 2N_2 + 4HCO_3^- + CO_2 \quad (2)$$

Compared with the traditional denitrification process, anaerobic ammonia oxidation (ANAMMOX) has the advantages of less aeration, high nitrogen removal efficiency and low sludge yield [3–5], which reduces energy consumption and is in line with the concept of sustainable development [6]. However, while the ANAMMOX process consumes NH_4^+-N and NO_2^--N, it also produces NO_3^--N as a byproduct, with a theoretical yield of about 11% relative to the total nitrogen influent [7]. The reaction equation of the ANAMMOX

process is shown in Equation (3). In practice, due to the presence of nitrite oxidizing bacteria, the effluent NO_3^--N concentration of the actual process will be higher than the theoretical value [8], which directly affects the discharged effluent quality.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (3)$$

To solve this problem, elemental sulfur-based autotrophic denitrification (S^0-SADN) is often coupled with the ANAMMOX process as it provides similar advantages, including no requirement for the addition of organic matter and high nitrogen removal efficiency [9,10]. The reaction equation of S^0-SADN is shown in Equations (4) and (5). Most importantly, a number of studies have established that S^0-SADN preferentially utilizes NO_3^--N, then utilizes NO_2^--N [11]. Therefore, since the S^0-SADN process treats the NO_3^--N produced by the ANAMMOX, it can avoid competition for NO_2^--N [12], and the combination of these two reactions can achieve an increase in the total nitrogen removal rate (NRE). At the same time, this is also beneficial for maintaining the pH balance during the reactor's operation, as the S^0-SADN process is an acid-producing reaction and the ANAMMOX is an alkali-producing reaction [13].

$$S^0 + 3NO_3^- + HCO_3 \rightarrow SO_4^{2-} + 3NO_2^- + CO_2 + H^+ \quad (4)$$

$$S^0 + 2NO_2^- \rightarrow SO_4^{2-} + N_2 \quad (5)$$

To start the coupling process of ANAMMOX and S^0-SADN, most scientists choose to inoculate with ANAMMOX sludge. Although the time required for the introduction of the S^0-SADN process by inoculation with ANAMMOX sludge is short, the coupling process still remains challenging due to the relatively slow growth rate of ANAMMOX bacteria [14].

Previous studies have identified two methods for initiating the coupling process of ANAMMOX and S^0-SADN. The first method involves adding a sulfur source to the stable reactor of the ANAMMOX to cultivate the S^0-SADN bacteria [15,16]. The second method involves inoculating the ANAMMOX and S^0-SADN simultaneously to directly couple the two processes [17]. However, these two different start-up methods all require a large number of ANAMMOX bacteria, which is challenging given their slow growth and long generation time. Obtaining a sufficient number of ANAMMOX bacteria is difficult and can adversely affect the start of the coupling process.

Therefore, we considered starting the coupling process of ANAMMOX and S^0-SADN without inoculating with ANAMMOX bacteria. The decision to start the ANAMMOX process by inoculating the S^0-SADN sludge was based on the following considerations: (1) Although many studies have investigated the impact of sludge on the starting process of ANAMMOX [18–24], using S^0-SADN bacteria to initiate the process is a novel approach. If successful, it could provide a new option to inoculate sludge during the ANAMMOX. (2) Previous researchers have shown that coupling the ANAMMOX and S^0-SADN processes without inoculation of the S^0-SADN sludge typically takes 30 days to domesticate and cultivate the S^0-SADN bacteria in the ANAMMOX system [15,16]. However, when S^0-SADN sludge is added, the system may maintain a certain level of S^0-SADN abundance without the need for additional inoculation or cultivation of S^0-SADN bacteria.

To sum up, to solve the difficultly of obtaining ANAMMOX sludge, we planned to only inoculate the S^0-SADN bacteria once, and to then try to start the ANAMOX process. At the same time, the most ideal condition is that there is also a portion of S^0-SADN sludge in the system, so as to quickly start the S^0-SADN and ANAMMOX coupling process. Although the optimal growth temperature range for ANAMMOX bacteria is 30–38 °C [25], in order to simulate a more realistic situation, the reactor operated at ambient temperature (19.8–26.2 °C) throughout the whole process. During reactor operation, the microbial

community structure in the sludge were analyzed by high throughput at different stages, and then the characteristics of inoculating the S^0-SADN sludge to start-up the ANAMMOX process were explored, in order to promote the application of the coupling of S^0-SADN and ANAMMOX in the field of sewage treatment.

2. Materials and Methods

2.1. Start-Up ANAMMOX Using a Two-Stage Experiment

2.1.1. The S^0-SADN Operation Stage

First, we attempted to enrich the ANAMMOX bacteria directly in the reactor during the S^0-SADN operation stage by coupling the ANAMMOX and S^0-SADN. To achieve this, NH_4^+-N was added, which is necessary for ANAMMOX but not required for the S^0-SADN process; NO_2^--N is required for both the ANAMMOX process and the S^0-SADN process. Once the ANAMMOX process has been successfully introduced at this stage, it is possible to initiate the coupling of S^0-SADN and ANAMMOX.

2.1.2. The ANAMMOX Start-Up Stage

Because the initial attempt to introduce the ANAMMOX process failed. In the second stage, the original packing was replaced to remove the sulfur source in the reactor. This can achieve an inhibition of the S^0-SADN process and avoid competition between the S^0-SADN bacteria and the ANAMMOX bacteria for NO_2^--N; the environment will be conducive to the growth of ANAMMOX bacteria. However, compared to the first method and due to the lack of a sulfur source after the ANAMMOX start-up, the absence of S^0-SADN bacteria in the system cannot be ensured. Therefore, the focus of the second stage was on quickly starting the ANAMMOX process.

2.2. Test Apparatus

The experiment was divided into two stages—the S^0-SADN operation stage and the ANAMMOX start-up stage (Figure 1). The reactor was an up-flow biological filter made of acrylic sheets, with an inner diameter of 9.0 cm, effective height of 58.5 cm, and an effective volume of 3.7 L. The reaction system was composed of an inlet bucket, peristaltic pump and the ascending biological filter column reactor, and the specific processes of the different stages were as follows.

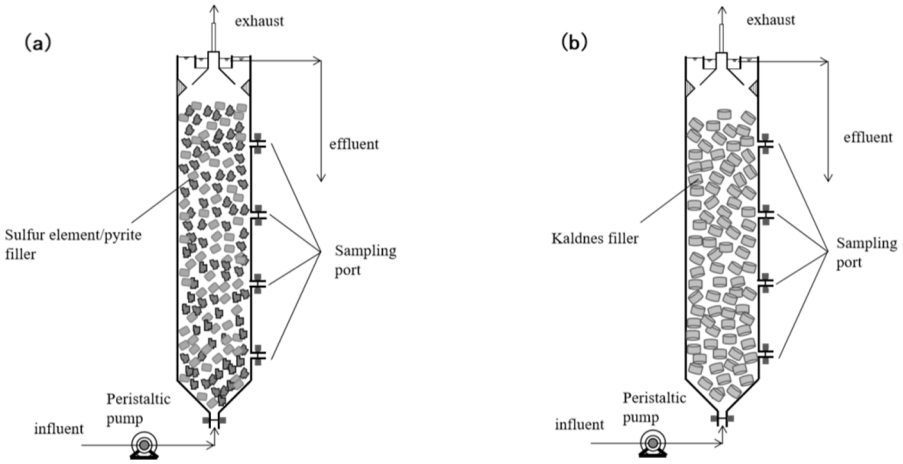


Figure 1. Schematic diagram of the test apparatus: (**a**) The S^0-SADN operation stage; (**b**) The ANAMMOX start-up stage.

2.3. Filler

2.3.1. The S^0-SADN Operation Stage

This stage was carried out in the existing stable operation of the S^0-SADN reactor, filled with elemental sulfur particles with a diameter of 6–8 mm (elemental sulfur con-

tent > 99.00%) and pyrite particles with a diameter of 5–8 mm. The reason for being filled with elemental sulfur and pyrite was to provide a S source for the S^0-SADN process and for the growth of microorganisms. The ratio of the different fillers in the reactor was 1:1 and the filler morphology is shown in Figure 2a,b. Prior to this stage, the S^0-SADN reactor had been successfully started, the NO_3^--N removal rate had reached 80%, and the removal load was 0.08 $kg \cdot (m^3 \cdot d)^{-1}$.

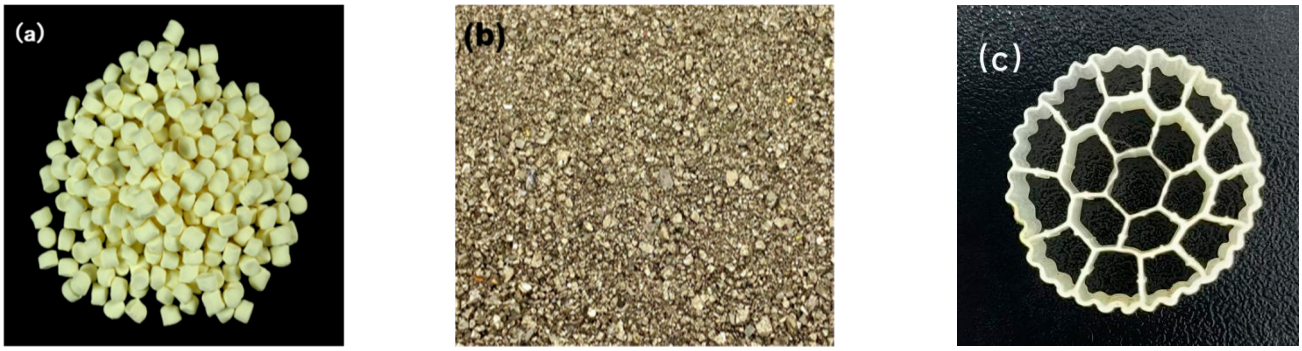


Figure 2. Filler morphological diagram: (**a**) Elemental sulfur particles; (**b**) Pyrite particles; (**c**) Kaldnes filler.

2.3.2. The ANAMMOX Start-Up Stage

At this stage, the S^0-SADN sludge was washed out of the elemental sulfur and the pyrite filler, and the filler was replaced with a Kaldnes filler with a diameter of about 20 mm. This filler was cylindrical, with an inside support structure, and the tail fin was extended radially on the side to increase the specific surface area (Figure 2c), so microorganisms could attach and growth on the surface.

2.4. Synthetic Wastewater Composition

The synthetic wastewater contained NO_3^--N, NO_2^--N, NH_4^+-N in the form of $NaNO_3$, $NaNO_2$, NH_4Cl, $NaHCO_3$, which was used as an inorganic carbon source. Na_2HPO_4 was used as a phosphorus source.

The S^0-SADN operation stage changed the water inlet conditions of the original S/pyrite reactor with only NO_3^--N as the nitrogen source, and the culture of ANAMOX bacteria in the ANAMMOX start-up stage adjusted the filler while changing the water inlet conditions. The components of the influent nitrogen source, set at different times, are shown in Table 1, and the specific formulas of the trace elements are referred to in the literature [26].

Table 1. Experimental operation conditions.

Operation Stage	Time (d)	NH_4^+-N ($mg \cdot L^{-1}$)	NO_2^--N ($mg \cdot L^{-1}$)	NO_3^--N ($mg \cdot L^{-1}$)
S^0-SADN operation stage	1–66	22 ± 5	-	12–35
	67–79	18 ± 2	25 ± 5	-
ANAMMOX start-up stage	1–10	27 ± 3	33 ± 2	-
	11–30	50 ± 5	75 ± 10	-
	31–75	65 ± 5	75 ± 5	-

2.5. Water Quality Measurement

The determination of the conventional pollution indicators in water samples followed the national standard method [27]. NO_3^--N, NH_4^+-N, NO_2^--N, SO_4^{2-} concentrations were measured using ion chromatography (HACH DR6000, Loveland, CO, USA). The NO_3^--N

determination used ultraviolet spectrophotometry; NH_4^+-N determination was based on the Nahrenherin reagent method; NO_2^--N determination was achieved by 1-Amino-2-(α-naphthylamino)ethane dihydrochloride spectrophotometry; SO_4^{2-} determination followed the ISO/DIS15923-1 standard method; both the pH and DO (Dissolved Oxygen) were determined using the electrode method (WTW FDO925, Xylem, Germany).

2.6. High-Throughput Sequencing

Polymerase chain reaction amplification of the V3-V4 region of the 16S rRNA gene was conducted using bacterial primer pairs 338F(5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R(5′GGACTACCAGGGTATCTAAT-3′). Then, three independent PCR products were pooled in equal amounts and purified with an Axy-Prep DNA Gel Extraction Kit (Axgen, Palo Alto, CA, USA), and then quantified with QuantiFluor™-ST (Promega, Madison, WI, USA) according to the manufacturer's instructions. Finally, amplicon libraries (300 bp paired end reads) were constructed and sequenced on a MiSeq Illumina platform by the Majorbio Company (Shanghai, China).

2.7. Batch Beaker Test

This involved taking a certain amount of sludge from the reactor and washing the sludge at the beginning of the reaction from the reactor with deionized water to wash away the remaining substrate. Subsequently, a specific volume of pharmaceutical wastewater was injected into the bottle to acquire the initial NO_2^--N concentrations and NH_4^+-N concentrations. Then, we blew off the mixture with nitrogen for 20 min, put the serum bottle into a thermostatic incubator for the reaction, measured the changes of NO_2^--N, NH_4^+-N and NO_3^--N during the test, and performed a linear fitting of its change trend.

2.8. Statistical Treated of Data

Samples were collected and analyzed regularly to evaluate the treatment performance. In the experiment, Origin software was selected for the data analysis and plot processing. During the batch experiment, the mean and standard deviation of various indicators in the batch experiment were calculated, and the least square method was used to perform the curve fitting on the batch experiment results.

2.9. TIN Measurement Method

The TIN (total inorganic nitrogen) concentration was calculated according to Equation (6):

In the equation: C_{TIN} is the total nitrogen concentration, $mg \cdot L^{-1}$; $C_{NH_4^+\text{-}N}$ is the NH_4^+-N concentration, $mg \cdot L^{-1}$; $C_{NO_2^-\text{-}N}$ is the NO_2^--N concentration, $mg \cdot L^{-1}$; $C_{NO_3^-\text{-}N}$ is the NO_3^--N concentration, $mg \cdot L^{-1}$.

$$C_{TIN} = C_{NH_4^+\text{-}N} + C_{NO_2^-\text{-}N} + C_{NO_3^-\text{-}N} \quad (6)$$

3. Results

3.1. S^0-SADN Operation Stage

In this stage, the reactor tries to combine the ANAMMOX process with the S^0-SADN process. Theoretically, the NH_4^+-N required for the ANAMMOX process is provided by the influent and NO_2^--N can be provided by the sulfur autotrophic partial denitrification process. For days 1–66, the nitrogen sources in the influent included both NH_4^+-N and NO_3^--N, and for days 1–53, their concentrations in the influent were approximately 22.0 $mg \cdot L^{-1}$ and 30.0 $mg \cdot L^{-1}$, respectively. The NO_3^--N removal rate of the system was rapidly reduced to 79.3% on day 1, was only 22.4% on day 45, and on day 49, the NO_3^--N of the reactor effluent exceeded 20 $mg \cdot L^{-1}$, while simultaneously, no NO_2^--N was detected. On days 55–65, the concentration of NO_3^--N in the influent was reduced to about 15.0 $mg \cdot L^{-1}$. On day 55, the NO_3^--N removal rate momentarily increased but soon decreased again to below 50%.

To utilize the ANAMMOX process, on days 67–79, the nitrogen source components of the influent were adjusted, with NH_4^+-N and NO_2^--N concentrations about 22.0 mg·L^{-1} and 30.0 mg·L^{-1}, respectively. The NH_4^+-N consumption in the system was about 7.3 mg·L^{-1}, with no significant fluctuations (Figure 3). On days 67–77, there was a certain amount of NO_2^--N in the effluent of the system but its concentration decreased with time until by day 75 it was not clearly detectable.

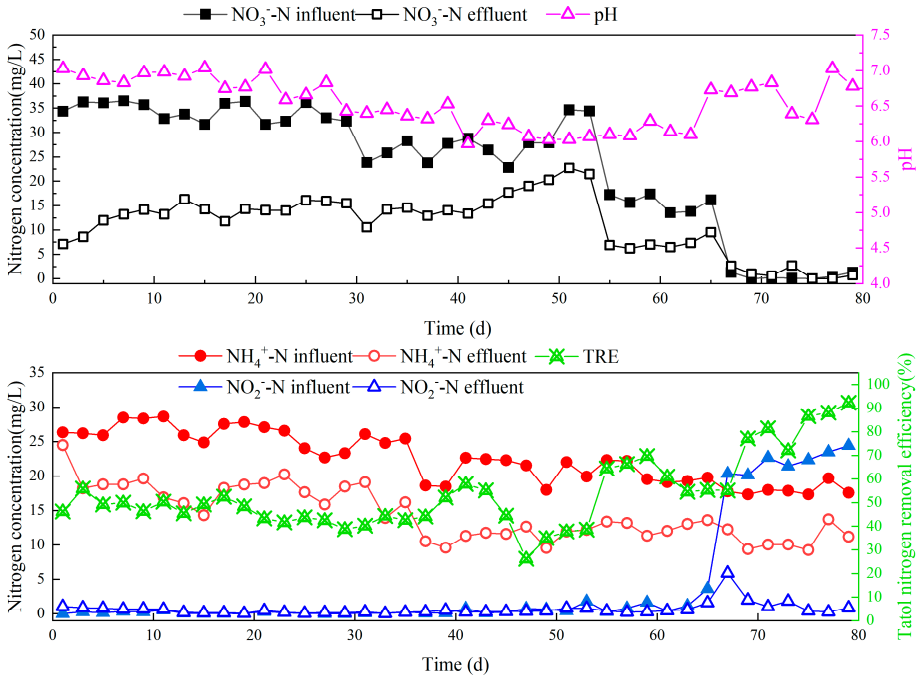


Figure 3. Variations of water quality and nitrogen removal efficiency during the S^0-SADN phase.

3.2. ANAMMOX Start-Up Stage

Attempts were made to enrich the ANAMMOX bacteria directly in the reactor during the S^0-SADN operation stage by the coupling of ANAMMOX and S^0-SADN; however, these were unsuccessful. In the second stage, the filler was replaced in order to remove the sulfur source of the reactor.

For days 1–10, the NH_4^+-N and NO_2^--N concentrations in the effluent were significantly reduced compared to the influent, standing at approximately 20.8/26.0 mg·L^{-1} and 12.5/30.0 mg·L^{-1}, respectively (Figure 4), while the TIN removal rate started at 47.5% and increased over time. For this period, the concentration of NO_3^--N in the reactor did not change significantly (Figure 4).

For days 11–30, the NH_4^+-N and NO_2^--N concentrations in the influent increased, while the TIN removal rate fluctuated over time (Figure 4). On day 14, the TIN removal rate reached a maximum of 77.6% and then started to decrease, reaching its minimum at only 13.2% on day 21. For days 21–30, it gradually increased again, while simultaneously, the effluent NH_4^+-N and NO_2^--N concentrations decreased. Starting from day 28, the concentration of NO_3^--N in the effluent was significantly higher than that in the influent.

For days 31–75, the NH_4^+-N influent concentration increased to an average of 65.0 mg·L^{-1}, while the NO_2^--N influent concentration was approximately 72.0 mg·L^{-1} (Figure 4). The TIN removal rate decreased slightly with the increase in the influent load, and then started to rise, reaching 87.7% on day 44, suggesting the successful start-up of the ANAMMOX. For days 45–50, both the TIN removal rate and the NO_3^--N concentration in the reactor effluent decreased. For days 55–75, the removal rates of NH_4^+-N and NO_2^--N in the reactor reached (99.1 ± 1.5) and (99.0 ± 0.9), respectively, and the TIN removal rate was (83.0 ± 2.6%). The average total nitrogen load rate (TINLR) was 0.36 kg(m^3·d)$^{-1}$, while the average total nitrogen removal rate (TINRR)

reached 0.30 kg(m^3·d)$^{-1}$, and the activity of the ANAMMOX bacteria in the reactor was restored.

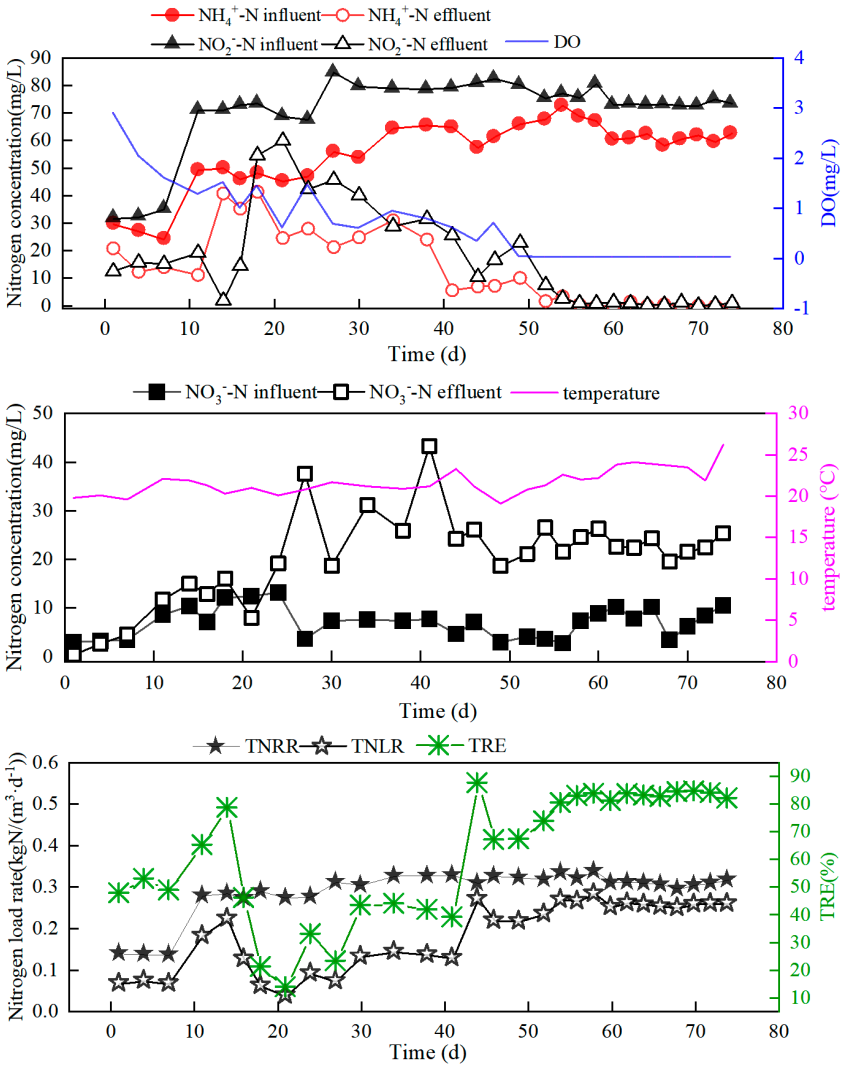


Figure 4. Variations of water quality and nitrogen removal efficiency during the ANAMMOX start-up stage.

3.3. Microbial Communities

The start-up of the ANAMMOX reactor signified the beginning of the process of ANAMMOX bacteria enrichment. To further analyze the effects of the two different initiation methods on the microbial communities within the system, the high-throughput sequencing technology was performed at the beginning (i.e., at the end of the S^0-SADN operation stage) and on day 60 of the ANAMMOX start-up stage (i.e., when the process within the reactor was stable).

The abundance of microbial communities was characterized using Shannon, operational taxonomic units (OTUs), Chao, ACE and Simpson indices. A comparison between the end of the S^0-SADN operation stage and the ANAMMOX process start-up indicated significantly higher values for microbial abundance and the diversity of the bacterial communities after the ANAMMOX start-up (Table 2).

Table 2. Statistical results of the alpha diversity index in different sludge samples.

Sludge Sample	Sequences	OTUs	Shannon	Chao	ACE	Simpson
0 d	25.115	318	3.05	367	359	0.019
60 d	23.159	467	4.58	467	467	0.029

Authors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

After the end of the S^0-SADN operation stage, the genus with the highest proportion detected in the reactor was *Thiobacillus*, with a relative abundance of 45.06% (Figure 5). Members of *Thiobacillus* are obligate autotrophic bacteria with denitrification functions, widely distributed in soil and water, that can assimilate CO_2 and oxidize the reduced sulfide to SO_4^{2-} [28]. *Ignavibacterium* (11.11%) comprises green sulfur bacteria, most of which can oxidize sulfur or thiosulfate under anaerobic conditions, and so the genus has a role in S^0-SADN. *Rhodanobacter* (6.99%) is a genus of both nitrification and denitrification bacteria, some of which can use sulfides as electron donors for autotrophic denitrification reactions [29,30]; it is suitable for a low pH, high nitrate concentration growth environment [31]. *Thermomonas* (2.43%) is a genus of heterotrophic aerobic denitrifying bacteria [32], which are common in water treatment systems [33]. *Dokdonella* (1.84%) is also a genus of heterotrophic denitrifying bacteria [34], which can use organic matter as a carbon source for denitrification. The reactor was stocked with a 0.80% abundance of the ANAMMOX functional bacteria *Candidatus Kuenenia*.

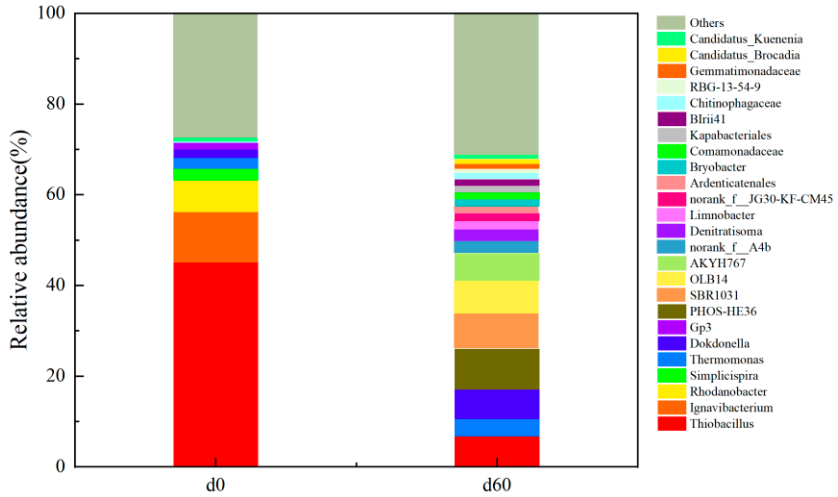


Figure 5. Comparison of the composition of the microbial communities.

After 60 days of incubation in the ANAMMOX start-up stage, the abundance of S^0-SADN-related genera decreased significantly. The abundance of *Thiobacillus* in the system decreased from 45.09% to 7.22%, and *Ignavibacterium* and *Rhodanobacter* were not detected at all (Figure 5). At the same time, *PHOS-HE36* (9.59%), *SBR1031* (8.23%), *OLB14* (7.90%), and *AKYH767* (6.48%), which were not detected at the S^0-SADN stage, became the dominant bacteria in the system. *PHOS-HE36* was the dominant bacterium in this system, with the highest abundance in the reactor; however, its function is yet unclear, and some studies speculate that it has some denitrification functions [35,36]. *SBR1031* belongs to anaerobic microorganisms with the ability to ferment carbohydrates, and its metabolic function is related to ammonia nitrogen [37,38]. *OLB14* belongs to the *Chloroflexi* phylum, and it is the core genus for anaerobic digestion [39]. At the same time, the abundance of *Thermomonas*

in the system increased from 2.43% to 4.13%, and the abundance of *Dokdonella* from 1.84% to 6.94%.

The abundance of ANAMMOX functional bacteria was significantly higher in the sample from day 60. Among them, the abundance of *Candidatus Kuenenia* had increased from 0.80% to 0.96%, and the abundance of *Candidatus Brocadia* went from undetected to 1.08%. This, combined with the TIN removal rate (see Figure 4), indicate the successful start-up of the ANAMMOX process by day 60.

3.4. Batch Beaker Test Results

At the end of the S^0-SADN stage, the fitting results of the batch beaker test were as follows: for the first six hours, the NH_4^+-N change trend fitted the one-time function and the slope was −0.033, R^2 = 0.027; the NO_2^--N change trend fitted the primary function and the slope was 0.11, R^2 = 0.297; the NO_3^--N change trend fitted the one-time function and the slope was 0.008, R^2 = 0.008 (Figure 6a). The concentrations of NO_3^--N, NO_2^--N and NH_4^+-N in the system did not change significantly and followed no obvious trend. Although a small amount of ANAMMOX bacteria (*Candidatus Kuenenia*) was found by high-throughput sequencing, any obvious characteristics of the ANAMMOX were not found at the macroscopic level.

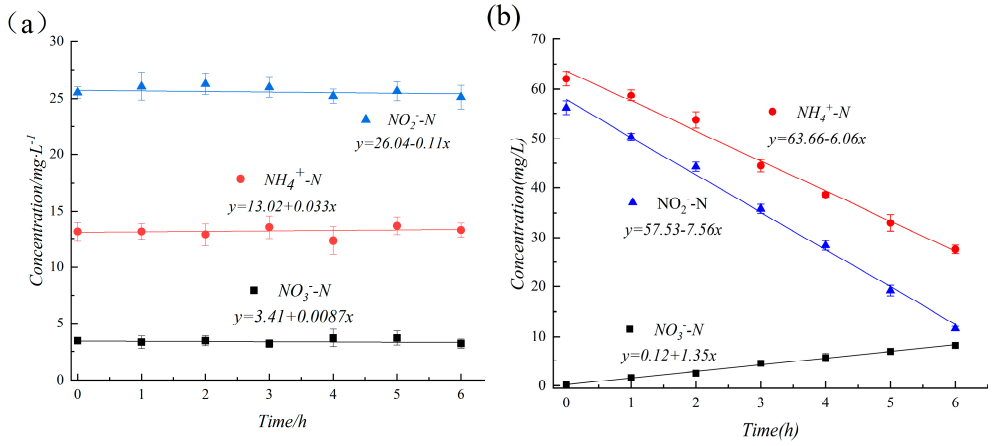


Figure 6. Variations of NO_3^--N, NO_2^--N and NH_4^+-N in the system under no DO conditions: (**a**) The S^0-SADN operation stage; (**b**) The ANAMMOX start-up stage.

At the end of the ANAMMOX start-up stage, the fitting results of the batch beaker test were as follows: for the first six hours, the change trend of NH_4^+-N fitted a linear function with a slope of −6.06 and R^2 = 0.990; the change trend of NO_2^--N fitted a linear function with a slope of −7.56 and R^2 = 0.994; the change trend of NO_3^--N fitted a linear function with a slope of 1.35 and R^2 = 0.993 (Figure 6b). The concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were linear for time, which conforms to the zero order reaction trend. At the same time, the ratio for the absolute values of slope fitting for changes in NH_4^+-N, NO_2^--N and NO_3^--N was 1:1.25:0.22, close to the theoretical ratio of 1:1.32:0.26. The results demonstrate that NH_4^+-N, NO_2^--N and NO_3^--N react in proportion and present the characteristics of an ANAMMOX process.

3.5. Morphology Change of Sludge

At the end of the S^0-SADN operation stage, the sludge was grayish black with more biomass and a loose structure (Figure 7a). After long-term S^0-SADN stage culture, the reactor enriched a large number of relevant functional bacteria. With the successful start-up of the ANAMMOX, the biomass in the reactor decreased and a large number of S^0-SADN bacteria with denitrification-related functions had died and autolyzed. At the same time, although the color change of the biofilm was not obvious (Figure 7b), the thallus structure

was more compact (Figure 8b). The sludge did not display the characteristic red color of ANAMMOX bacteria.

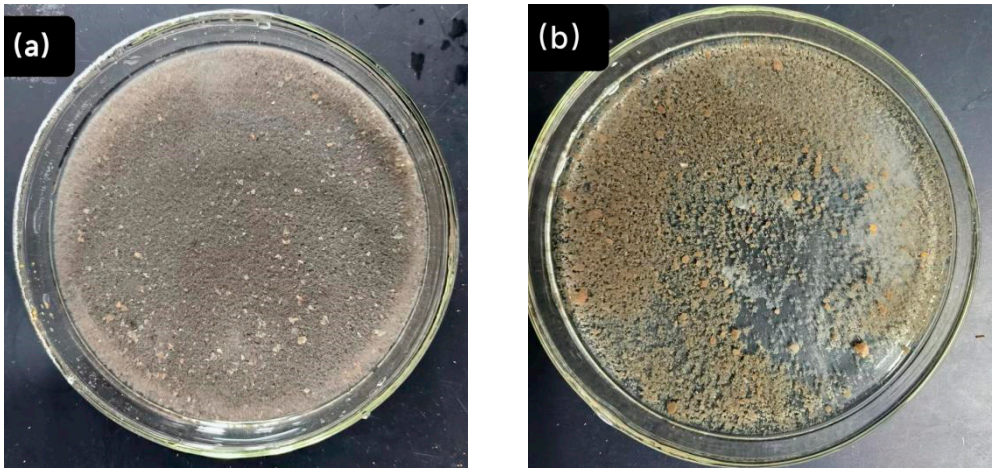
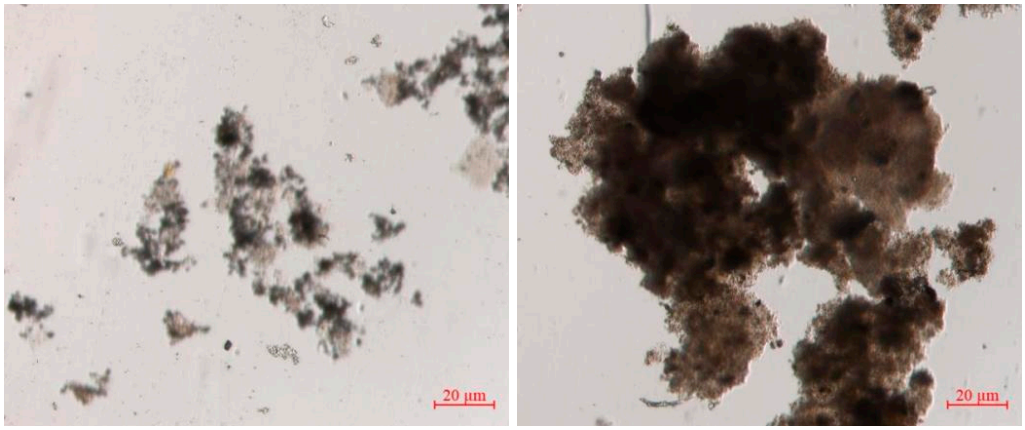


Figure 7. Variations of sludge morphology: (**a**) The S^0-SADN operation stage; (**b**) The ANAMMOX start-up stage.



(**a**) (**b**)

Figure 8. Microscopic observation of the thallus: (**a**) The S^0-SADN operation stage; (**b**) The ANAMMOX start-up stage.

4. Discussion

4.1. S^0-SADN Operation Stage

During days 1–66, the NH_4^+-N removal amount of the system was much higher than expected, which might have been due to the presence of dissolved oxygen in the influent. The reactor always operated under the condition of low dissolved oxygen, with AOB (ammonia oxidizing bacteria) in the system, so the NH_4^+-N was oxidized to NO_2^--N and then removed by the S^0-SADN. For days 67–79, the removal amount of NH_4^+-N did not change significantly, as the influent nitrogen source components were adjusted to NH_4^+-N and NO_2^--N. If the ANAMMOX process existed in the system and the ANAMMOX had an electron receptor that could be directly used after changing the nitrogen source, it would not have relied on the S^0-SADN and the consumption of NH_4^+-N would have increased. Combined with the batch test results, this indicates that the system had not yet developed the ANAMMOX process at the macroscopic level.

At this stage, pyrite and sulfur simple fillers were used in the reactor. Previous studies have demonstrated that pyrite is not easily consumed by sewage microorganisms and is added mainly for the purpose of facilitating their attachment [40]. Although sulfur was the main element that served as an energy substrate for the S^0-SADN, there is evidence that

the key to a successful coupling of S^0-SADN and ANAMMOX is to effectively solve the competition between the ANAMMOX bacteria and the S^0-SADN bacteria for NO_2^--N. The sulfur source in the system was sufficient at this stage, regardless of whether the influent nitrogen source components were NH_4^+-N and NO_3^--N or NH_4^+-N and NO_2^--N, and it was difficult to observe significant NO_2^--N accumulation in the system. According to our analyses, the sulfur autotrophic denitrifying bacteria can easily further reduce NO_2^--N to N_2, provided that the elemental sulfur in the reactor is sufficient. Consequently, it is difficult for ANAMMOX bacteria to obtain the NO_2^--N they need through substrate competition, which is the reason for the absence of the ANAMMOX process in the S^0-SADN stage of the reactor.

4.2. ANAMMOX Start-Up Stage

The TIN removal rate of the reactor changed three times during this stage, initially increasing (days 1–14), then decreasing for a short period (days 15–21), and finally increasing again until it reached stable levels (days 21–44).

For days 1–14, the denitrification effect was mainly due to the removal of NO_2^--N and NH_4^+-N from the system, while there were no significant changes in the concentration of the NO_3^--N inlet and outlet water. The process is predominantly driven by the combined effect of AOB, S^0-SADN bacteria and heterotrophic denitrification bacteria.

The removal of NO_2^--N was mainly due to the combined effect of sulfur autotrophic denitrifying bacteria, which use residual sulfur for autotrophic denitrification, and heterotrophic bacteria, which use carbon generated by sludge digestion for heterotrophic denitrification.

Because the reactor was not sealed, and there was a certain amount of dissolved oxygen in the water inlet, the system was in an environment with low dissolved oxygen. Our analysis revealed that the main driver of NH_4^+-N removal was the ammonia oxidation reaction carried out by AOB in the system.

The denitrification effect decreased on days 15–21. It was hypothesized that the decrease in the NO_2^--N removal rate may be due to the gradual reduction of sources of residual sulfur and carbon caused by microbial digestion and autolysis with the progress of the reaction. At 24 days, the DO concentration in the system increased (0.59 to 1.45), which may have been due to the low amount of organic matter produced by microbial autolysis in the reactor, the slowing down of the oxygen consumption rate by heterotrophic aerobic bacteria, or the decrease in temperature (21 to 20.1), resulting in a decrease in the activity of nitrifying bacteria and heterotrophic aerobic bacteria; thus, a slowing down of the oxygen consumption rate.

Higher NO_2^--N concentration in the reactor inhibits the activity of AOB and causes a reduction in the NH_4^+-N removal rate. At the same time, the original sulfur autotrophic denitrifying bacteria in the reactor gradually died off and dissolved, producing NH_4^+-N and organic matter. The production of NH_4^+-N directly leads to an increase in NH_4^+-N in the effluent, and the production of organic matter promotes the growth of heterotrophic bacteria, which compete with AOB for dissolved oxygen in the system; thus, inhibiting the nitrification process. For days 1–21, the ANAMMOX bacteria were in the adaptation period, the denitrification effect of the reactor fluctuated greatly, and the ANAMMOX characteristics were not obvious. For days 21–44, the TIN removal rate increased continuously, while both NH_4^+-N and NO_2^--N were removed synchronously, indicating the gradual start of the ANAMMOX reaction in the reactor. Temperature was the main cause for the decline in the TIN removal rate for days 45–50, as the temperature in the reactor decreased from 23.3 °C on day 44 to 18.8 °C on day 49. This had a pronounced inhibitory effect on the ANAMMOX bacteria.

A number of laboratory studies have used different inoculation sludge to start-up an ANAMMOX reactor: Ni et al. [18] inoculated anaerobic granular sludge in the Up-flow Anaerobic Sludge Bed (UASB) reactor, and successfully started and maintained the process for 140 days; Xiong et al. [19] achieved similar results with leachate sludge, successfully

running the process for 100 days. A large number of studies have shown that the realization of a rapid start-up of the ANAMMOX process is closely related to the type of inoculated sludge, although the rate of initiation of ANAMMOX by different types of inoculated sludge is different: anaerobic sludge [20], nitrified sludge [21], aerobic pool biofilm [22], and a small amount of mixed sludge [23] are among the types of sludge that have been successfully used as inoculation sludge. Compared with other types of inoculated sludge, the time required to start the ANAMMOX process by inoculating the S^0-SADN sludge is short. The start-up of the ANAMMOX process was carried out under ambient temperature and in the formal cultivation stage, it started successfully in only 44 days. Although after the process start-up, the ANAMMOX bacteria abundance in the system was only 2.04%, these bacteria had high activity. It has been established that during the process of starting the ANAMMOX reaction using inoculation with S^0-SADN sludge, a large number of associated bacteria readily grow in the reactor. These include anaerobic bacteria with functions related to digestion and denitrification, which create a suitable environment for ANAMMOX bacteria to grow and reproduce, improving the nitrogen removal effect of the ANAMMOX process [40]. The test also revealed that the ANAMMOX process can be started with S^0-SADN sludge as the inoculation sludge, and it is possible to carry this out without a sulfur source, effectively avoiding the inhibition of the ANAMMOX process due to competition between the ANAMMOX bacteria and the S^0-SADN bacteria.

4.3. The Mass Balance of Nitrogen Species in Both Systems

The Variations of TIN and TRE in the influent and effluent during the S^0-SADN operation stage and the ANAMMOX start-up stage are shown in Figure 9.

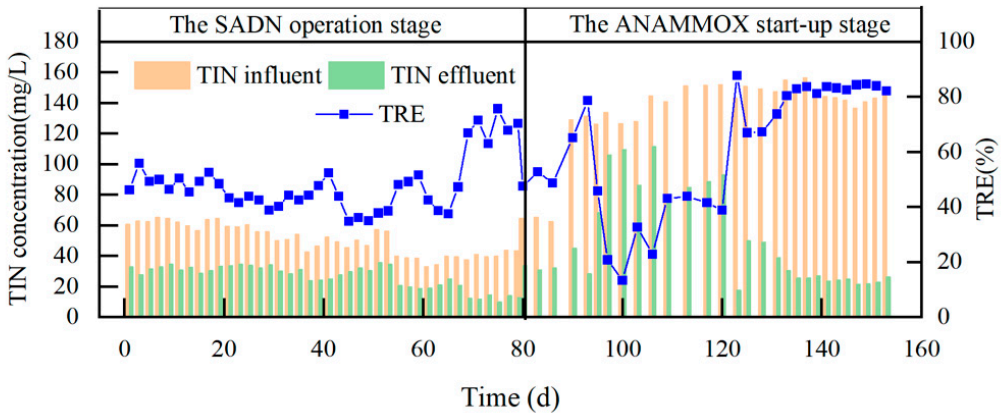


Figure 9. Variations of TIN and TRE during the S^0-SADN operation stage and the ANAMMOX start-up stage.

In the reactor, the reaction processes related to nitrogen conversion include the microbial autolysis hydrolysis process, autotrophic nitrification process, heterotrophic denitrification process, S^0-SADN process, and ANAMMOX process. Among them, only the heterotrophic denitrification process (Equation (2)), S^0-SADN process (Equations (4) and (5)), and ANAMMOX process (Equation (3)) can convert nitrogen containing compounds into N_2, achieve the removal of TIN, and affect the nitrogen balance of the system.

Due to the simultaneous existence of multiple denitrification paths in the reactor, it is difficult to quantitatively analyze the contribution of each denitrification path to TIN removal. However, qualitative analysis of the nitrogen balance can be attempted. During the operation of these two stages, the system always removed TIN. According to the results of the batch experiments in Section 3.4 and the discussions in Sections 4.1 and 4.2, during the S^0-SADN phase, there was no ANAMMOX process in the system, and the reduction in TIN mainly comes from the S^0-SADN process. Among them, for days 1–66, the S^0-SADN bacteria utilized nitrate nitrogen for the reaction, and for days 67–79, utilized nitrite nitrogen for the reaction. During the initial ANAMMOX start-up stage (days 80–100), the decrease in

TIN mainly comes from the heterotrophic denitrification process, and the decrease in TIN mainly comes from the ANAMMOX process in the middle and late ANAMMOX start-up stages (days 101–154).

4.4. Matrix and Product Metering Ratio

At the beginning of the ANAMMOX start-up phase, the ratio of the NO_2^--N removal amount (ΔNO_2^--N) to the NH_4^+-N removal amount (ΔNH_4^+-N) fluctuated greatly and gradually stabilized at 1.15 during days 54–75. According to the ANAMMOX reaction equation (Equation (3)), the theoretical ratio of ΔNO_2^--N to ΔNH_4^+-N is 1.32, and the ratio in this study is lower than the theoretical value.

Other studies have produced similar results [41], and our analysis suggests the following possible reasons: first, because of the dissolved oxygen in the influent, AOB could grow in the system, causing part of the NH_4^+-N to be converted into NO_2^--N; second, the bacteria may release oxidants during enrichment. Some studies have demonstrated that when an ANAMMOX bacteria culture has a low concentration, the system will produce oxidants (superoxide or hydroxyl radical) due to the reduction in the concentration of bacteria, resulting in an increase in the consumption of NH_4^+-N [42,43].

At the same time, there was no obvious change in the concentration of NO_3^--N in the inlet and outlet water on days 1–24. The concentration of NO_3^--N in the outlet water increased significantly during days 25–41, even surpassing the value reached during the stable ANAMMOX reaction (days 54–75). The analysis revealed that as the activity of NOB in the system gradually increased, oxidation of NO_2^--N to NO_3^--N during the ANAMMOX reaction released new NO_3^--N. The ANAMMOX process competes with NOB for NO_2^--N, leading to the inhibition of NOB activity and the ratio of NO_3^--N removal amount (ΔNO_3^--N) to ΔNH_4^+-N is about 0.25, close to the theoretical value of 0.26.

4.5. High-Throughput Result Analysis

(1) ANAMMOX bacteria: *Candidatus Brocadia* and *Candidatus Kuenenia* are considered to be the main ANAMMOX bacteria in laboratory reactors [30]. At the end of the S^0-SADN stage, the ANAMMOX bacteria in the system were mainly *Candidatus Kuenenia*. After the ANAMMOX process was successfully started and stabilized, the abundance of *Candidatus Kuenenia* did not change significantly, while that of *Candidatus Brocadia* increased from 0% to 1.08%. This different growth rate at different stages can be explained by the fact that *Candidatus Kuenenia* can utilize a low concentration of nitrite nitrogen and is more tolerant to inhibition [44], while *Candidatus Brocadia* has a weak substrate affinity but a strong reproductive capacity [43]. At the S^0-SADN stage, the addition of ammonia nitrogen in the influent water stimulated the growth of the ANAMMOX to some extent but the growth process was slow and the activity of the ANAMMOX bacteria was inhibited as the system was dominated by the autotrophic denitrification culture. At the end of this stage, despite the 0.80% abundance of *Candidatus Kuenenia* in the system, there was no ANAMMOX process at the macroscopic level.

(2) S^0-SADN bacteria: this group dominated the S^0-SADN operation stage. After 60 days of the ANAMMOX start-up stage, there were still some functional bacteria of this group in the system, proving that it is possible to initiate the ANAMMOX process using inoculation with sulfur autotrophic denitrifying bacteria. First, the ANAMMOX can be started using inoculating sulfur autotrophic denitrifying bacteria, and then the sulfur autotrophic denitrifying process can be coupled.

(3) Heterotrophic denitrifying bacteria: although in the S^0-SADN operation stage there was a certain amount of heterotrophic denitrification process in the system, as no organic matter was added to the influent water, endogenous denitrification mainly occurred after the ANAMMOX process was successfully started and stabilized. The number of heterotrophic denitrification bacteria increased after the death of the original S^0-SADN bacteria in the reactor, as this produced a large amount of organic matter.

Concerning the comprehensive high-throughput inspection results and the reactor operation, the reasons for the enrichment of a small amount of ANAMMOX bacteria in the first stage and the fast start-up of ANAMMOX in the second stage are analyzed as follows:

(1) A small amount of ANAMMOX bacteria were enriched in the first stage of the S^0-SADN operation. The ANAMMOX process takes NO_2^--N as the electron acceptor, while the S^0-SADN takes NO_3^--N or NO_2^--N as the electron acceptor. During the S^0-SADN process, NO_3^--N will be first reduced to NO_2^--N; on the other hand, ANAMMOX needs to use NH_4^+-N as the electron donor, while S^0-SADN uses a sulfur simple substance or a sulfur ion as the electron donor, and the two are different. However, some ANAMMOX bacteria can conduct a DNRA reaction [45], directly reducing NO_2^--N or NO_3^--N to NH_4^+-N, and the microorganisms will also generate part of the NH_4^+-N after autolysis of the bacteria. Therefore, sulfur autotrophic denitrifying bacteria and ANAMMOX bacteria have certain similarities in growth conditions, which make the sulfur autotrophic denitrifying sludge form a micro-concentration of ANAMMOX bacteria during the operation process.

(2) The autolysis process of the bacteria in the reactor is slow. In other research reports [21,46–48], at the initial stage of the start-up of the anaerobic ammonia oxidation process, the accumulation of NH_4^+-N and the removal of a large amount of NO_2^--N occurred in the reactor due to the influence of the bacterial autolysis and endogenous denitrification and denitrification processes. In the studies by Chen et al. [46] and Wang et al. [47], this process lasted about 20 days. In the second stage, during the start-up of the ANAMMOX using inoculating sulfur autotrophic denitrifying sludge in this experiment, there was no large accumulation of NH_4^+-N at the initial stage, and NH_4^+-N consumption began to occur at the initial stage of the reactor start-up. It was analyzed that the sulfur autotrophic denitrifying bacteria had a strong ability to adapt to the new environment without sulfur sources, the amount of NH_4^+-N produced by the autolysis of the bacteria was less, and the oxidation consumption of NH_4^+-N by AOB was presented. At the same time, the slowing down of the autolysis process also inhibits the endogenous denitrification process to a certain extent and reduces the competition between the endogenous denitrifying bacteria and ANAMMOX bacteria. Moreover, the slow autolysis of sulfur autotrophic denitrifying bacteria is conducive to the retention of some sulfur autotrophic denitrifying bacteria in the system after the start of the ANAMMOX, creating the possibility of reintroducing the sulfur autotrophic denitrifying process without the re-culture or inoculation of the sulfur autotrophic denitrifying bacteria.

5. Conclusions

(1) When pyrite was used as a filler and elemental sulfur was used for autotrophic denitrification, a certain amount of NH_4^+-N was added to the influent water. Through high-throughput sequencing, it was established that although there were a certain amount of ANAMMOX bacteria, they did not have the characteristics of an ANAMMOX process at the macroscopic level.

(2) Under the condition that there is no sulfur source in the system, the start-up time required to initiate the ANAMMOX process by inoculating the S^0-SADN sludge was short, so this type of sludge can reduce the cultivation time of the ANAMMOX bacteria. After 60 days without a sulfur source in the system, there were also sulfur autotrophic denitrifying functional bacteria with an abundance of 7.22% in the system, belonging to Thiobacillus.

(3) When the influent nitrogen source components were NH_4^+-N and NO_2^--N, with no external sulfur source, at day 44 of the ANAMMOX start-up stage, the total nitrogen removal rate reached 87.66%, and the TIN removal load reached 0.30 $kg(m^3 \cdot d)^{-1}$. After 55 days, the removal rates of NH_4^+-N and NO_2^--N in the reactor were 99.1% and 99.0%, respectively, and the removal rates of TIN were 83.0%. *Candidatus Brocadia* and

Candidatus Kuenenia were the main genera of ANAMMOX bacteria, with abundances of 1.08% and 0.96%, respectively.

(4) The experimental results indicate that it is feasible to initiate the ANAMMOX process by inoculating the S^0-SADN sludge. However, simultaneous initiation of the ANAMMOX and the stable operation of the S^0-SADN is not feasible. It was recommended to start-up the ANAMMOX under the condition of no sulfur source, which can be accomplished in a short period. Despite the successful start-up of the ANAMMOX, there remained an excessive abundance of S^0-SADN bacteria within the system, which suggest that is worth considering reinitiating the coupled S^0-SADN process at a later time; this is greatly beneficial for further removing TIN effluent.

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