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Start-up Strategies for Anaerobic Ammonia Oxidation (Anammox) in In-Situ Nitrogen Removal from Polluted Groundwater in Rare Earth Mining Areas

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Abstract: The tremendous input of ammonium and rare earth element (REE) ions released by the enormous consumption of $(NH_4)_2SO_4$ in in situ leaching for ion-adsorption RE mining caused serious ground and surface water contamination. Anaerobic ammonium oxidation (anammox) was a sustainable in situ technology that can reduce this nitrogen pollution. In this research, in situ, semi in situ, and ex situ method of inoculation that included low-concentration (0.02 mg·L^{-1}) and high-concentration (0.10 mg·L^{-1}) lanthanum (La)(III) were adopted to explore effective start-up strategies for starting up anammox reactors seeded with activated sludge and anammox sludge. The reactors were refrigerated for 30 days at 4 °C to investigate the effects of La(III) during a period of low-temperature. The results showed that the in situ and semi in situ enrichment strategies with the addition of La(III) at a low-concentration La(III) addition (0.02 mg·L^{-1}) reduced the length of time required to reactivate the sludge until it reached a state of stable anammox activity and high nitrogen removal efficiency by 60–71 days. The addition of La(III) promoted the formation of sludge floc with a compact structure that enabled it to resist the adverse effects of low temperature and so to maintain a high abundance of AnAOB and microbacterial community diversity of sludge during refrigeration period. The addition of La(III) at a high concentration caused the cellular percentage of AnAOB to decrease from 54.60 ± 6.19% to 17.35 ± 6.69% during the enrichment and reduced nitrogen removal efficiency to an unrecoverable level to post-refrigeration.

Keywords: anammox; start-up strategy; rare earth elements; La

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1. Introduction

Rare earth elements (REEs) are used extensively in high-technology industries, by military and in sustainable clean energy applications. There has been a worldwide surge in demand for heavy REEs [1,2], and this demand is expected to increase rapidly within the next two decades [3–5]. China has been the dominant force in the global REE market as both a consumer and a supplier since 2000 [6,7]. China produced over 70.59% of the global supply of REEs, of which 14.51% originated from ion-adsorption deposits in South China in 2019 [8,9]. The REE-enriched ion-adsorption deposits were in situ mined by ammonium salt leaching agents. A large amount of ammonium residues were produced that were subsequently converted into nitrite and nitrate, which was transported into soil, rivers, and groundwater [4,10,11]. As documented in report, aqueous ammonium and nitrate levels exceeded by tens or hundreds of times the allowable limits set out in the Chinese National Standard for Groundwater Quality(grade III) [12] and Surface Water [13] in areas adjacent to REE extraction operations using in situ leaching [14]. The recovery technology is inefficient [15], and thus REEs have often become the principal metallic pollutant in waters close to REE mining areas [16–18]. There is an urgent need to remediate the ammonium, and REEs ions that have seriously contaminated the surface and underground water [15].

The use of microbial denitrification technology is at present the preferred method of reducing pollution and protecting water quality, such as endogenous denitrification [19], autotrophic nitrogen removal [20], and conventional nitrification-denitrification process [21]. Anaerobic ammonium oxidation (anammox), in which ammonium reacts with nitrite or nitrate to form nitrogen gas under anaerobic conditions, is widely considered to be the basis for the most sustainable in situ technology to alleviate the ammonium pollution resulting from rare earth extraction. It outcompeted traditional wastewater treatments for being economical, energy-saving, and yields less sludge than other processes [22–25]. Over 100 projects have been instigated to construct wastewater treatment plants that use the anammox technology [26]. However, this promising way has difficulty in enriching anammox bacteria (AnAOB) during the start-up phase since AnAOB have a long doubling time [27]. Besides, it is well documented that REE ions also present problems for anammox processes as the ions affect AnAOB [28–31].

In our previous, we found that AnAOB showed resistance to lanthanum (La)(III)—a major REE found in stream and groundwater nearby REE mining areas, which indicated that anammox may make difference in the in situ bioremediation [22]. However, anammox wastewater treatment reactors are usually started up by inoculating with enough anammox sludge, partial nitrification sludge, ordinary nitrification sludge or their mixture, which were harvested from some other stably operated reactor or plants [26,32–36]. Thus, low-temperature preservation during long-distance is inevitable. Together, difficulties in enrichment and transportation limit the application of anammox in the in situ bioremediation of ammonia nitrogen pollution in underground water nearby rare earth mining areas. It is therefore a priority to devise a strategy for enriching sludge with a sufficient quantity of AnAOB that are adapted to wastewater that contains REEs and ammonium in order to promote the application of anammox in in situ bioremediation of REE-contaminated groundwater rich in ammonium. However, there has been little research into such AnAOB enrichment.

The goal of this study is to investigate viable inoculation techniques for the in situ use of anammox to reduce the ammonium content of wastewater produced by REE mining. Considering the growth prospect of market demand for REEs, La was identified as a typical REEs found in the groundwater and was selected to explore the effects of REEs on AnAOB enrichment process containing a low-temperature preservation. Start-up strategies included in situ enrichment (enrich AnAOB with water containing La(III), ammonium and nitrite), semi in situ enrichment (enrich AnAOB with water containing ammonium and nitrite, and then add La(III) when the system shows anammox activity), and ex-situ enrichment (start up the reactor with mature anammox flocs and media containing La(III), ammonium and nitrite). The experiments were conducted using sequencing batch reactors under dark and anaerobic conditions. Nitrogen removal performance and sludge characteristics were measured and observed. The evolution of microbial community was determined by 16S rRNA high-throughput sequencing. Furthermore, the recovery performance of the reactors was investigated after refrigeration for one-month at 4 °C to determine the effect of a low-temperature environment on anammox activity.

2. Materials and Methods

2.1. The Start-Up Strategies

Five sequencing batch reactors (SBRs) were used in the experiments. Different start-up strategies were used for each SBR as shown in Table 1. In-situ, semi-in-situ, and ex-situ start-up strategy were designed to simulate the use of activated sludge, activated sludge with anammox activity, and mature anammox flocs for in- situ bioremediation of ammonia nitrogen pollution in groundwater found near rare earth mining areas. The control reactor was inoculated with synthetic media (presented in Table S1 in the Supplementary Materials). Concentrations of ammonium and nitrite aried depending on the nitrogen removal performance in the reactor.

Table 1. Start-up features of in-situ, semi-in-situ, and ex-situ enrichment strategies.

Strategy	Reactor	Seed Sludge	Operating Period	La(III) Addition Period	La(III) Concentration in Reactor	Ammonium Consumption Appearance	Anammox Activity Inflexion
Control	R0	Activated sludge	Days 1–108	-	-	Day 26	Day 86
In-situ	R1	Activated sludge	Days 1–108	Days 1–108	0.02 mg L^{-1}	Day 25	Day 60
Semi in-situ	R2	Activated sludge	Days 1–108	Days 61–108	0.02 mg L^{-1}	Day 28	Day 71
Semi in-situ	R3	Activated sludge	Days 1–108	Days 61–108	0.10 mg L^{-1}	Day 30	Day 70
Ex-situ	R4	Anammox flocs	Days 30–108	Days 30–108	0.10 mg L^{-1}	Day 1	Day 1

We maintained the temperature of the reactors bleow 4 °C for 30 days without medium replacement immediately after the 108-day enrichment period to simulate the low-temperature preservation during the long-distance transportation. At the end of the refrigeration period, we reactivated the reactors to investigate the effects of La(III) on the fermatation. Reactor R3 experienced mechanical failure at after 15days post-refrigeration.

2.2. Reactor Operation

The activated sludge (Seed 1) was harvested from the secondary sedimentation tank of an anaerobic-anoxic(A2/O) process in a wastewater treatment plant in Nanchang, China. The anammox flocs (Seed 2) were from a lab scale SBR, which is fed with synthetic wastewater and operated stably for half a year. The inoculants were washed three times with 0.8% saline and then mixed with 1000 mL media. The original MLVSS content within the bottles was set as 4800 mg L^{-1}. Reactor operation is demonstrated in Text S1 in the Supplementary Materials.

La content in stream water close to mineral areas was >0.02 mg L^{-1} [37]. Considering that environmental release of REEs is likely to increase due to the potential centraliation of production, we also investigated the potential effects of a higher concentrations of La(III) (0.10 mg L^{-1}). We used La(III) concentrations of 0, 0.02, and 0.10 mg L^{-1} La(III) to investigate the effects of La(III) on start-up. Lanthanum chloride ($LaCl_3 \cdot 6H_2O$, 99.99% metals basis, Macklin, China) stock solution (10,000 mg L^{-1} La(III)) was adopted to supply La ions in the media.

2.3. Morphological Observation

The morphologies of microorganisms in the media pre- andpost-preservation, and in the seed sludge were observed using a scanning electron microscopy (SEM). Inoculants Seed 1 and Seed 2 were observed. Samples R0A, R1A, R2A, R3A, and R4A were collected from relevant reactors pre-refrigeration, and samples R0B, R1B, R2B, and R4B were collected post-preservation. Sample R3B was not used.

In each case thesludge was rinsed with distilled water three times, fixed with 2.5% (w/v) glutaraldehyde at 4 °C for 2h, then dehydrated in a graded ethanol series (15 min in each of 20%, 40%, 50%, 60%, 70%, 80%, 90% (w/v) ethanol, then triple rinsed in anhydrous ethanol to remove the final traces of water). The dehydrated sludge was thenplaced into mixture of anhydrous ethanol and isoamyl acetate (1:1 v/v) and then isoamyl acetate each for 15 min. After drying for 8 h, the sample was coated with gold by vacuum coater (Cressington108 SPUTTER COATER, UK). The surface and cross-sectional morphology of samples were examined by scanning electron microscopy (FEI Quanta200F, The Netherlands).

2.4. FISH Analysis

Fluorescence in situ hybridization (FISH) was used to characterize microbe samples. FISH protocol followed was adapted from Suárez-Ojeda et al. [38]. The Seed 1 and Seed 2 were sampled for FISH analysis. Samples R0A, R1A, R2A, and R4A were collected from reactors pre-refrigeration and samples R0B, R1B, R2B, and R4B were sampled post-preservation. Samples R3A and R3B werenot used.

Group-specific probes for AnAOB (AMX820) [39,40] were carried out simultaneously with probes for nitrite oxidizing bacteria (NOB) (NIT 3) [41] and ammonia oxidizing bacteria (AOB) (NSO190) [42,43]. DAPI staining (blue in images) was used to quantify the total number of cells. All probes were purchased from Invitrogen (USA), and synthesized with 5′-Cy5 (purple), 5′-FITC (green), and 5′-Cy3 (red) labels, respectively. Details of probes and base sequences are given in Table 2. FISH images were captured by a laser scanning confocal microscopy (Zeiss LSM 710, Germany) coupled with an image acquisition system (Zeiss ZEN 2.3, Germany). Software Image J (version 1.51, National Institutes of Health, USA) was used for FISH quantification of hybridized cells. AnAOB, AOB, and NOB relative cell percentage were expressed as the percentage of AMX820-positive, NIT3-positive, and NSO190-positive cells out of DAPI-positive cells, respectively, meanwhile each result was given as mean ± standard deviation of data from twenty fields of vision. Then, the relative enrichment or diminution of the relative percentage of cells were ranked using odds ratio (OR). Formula (1) shows the OR is calculation, where φ represents the relative percentage of cells and S, A, B refer to the sample collection time points (S for seed, A and B for pre-and post-refrigeration, respectively). The differences in cell percentage were defined using logarithm (Ln) of the OR of one given functional bacterial, where a positive Ln means an enrichment, whereas a negative Ln is indicative of a diminution [44].

$$OR^{P_A/P_B} = \frac{\varphi_A/(1-\varphi_A)}{\varphi_{B/}(1-\varphi_B)}$$

Table 2. Information of probe used for fluorescence in situ hybridization (FISH) detection.

Probe	Specificity	Label	Color in Images	Sequence(5′-3′)
Amx820	AnAOB	Cy5	purple	AAAACCCCTCTACTTAGTGCCC
NIT3	NOB	FITC	green	CCTGTGCTCCATGCTCCG
NSO190	AOB	Cy3	red	CGATCCCCTGCTTTTCTCC

2.5. DNA Extraction, Amplification, and High-Throughput Sequencing

To study the variations of microbial community in each reactor, the sludge samples were extracted from five reactors and then detected on an Illumina Mi Seq platform at Shanghai OE BioTech. Co., Ltd. in Shanghai, China. Inoculum Seed 1 and Seed 2 were involved. Samples R0A, R1A, R2A, and R4A were collected from the corresponding reactor before the refrigeration while R0B, R1B, R2B, and R4B were sampled after the low-temperature preservation. Unfortunately, sample R3A and R3B were lost by mistake. For bacterial diversity analysis, V3-V4 variable regions of 16S rRNA genes was amplified with universal primers 343F (5′- TACGGRAGGCAGCAG -3′) and 798R (5′-AGGGTATCTAATCCT-3′). More details about bioinformatic analysis are presented in Text S2 in the Supplementary Materials.

2.6. Other Analytical Procedures

Based on the standard methods [45], the concentrations of TN, NO2-, NO3-, NH4+ in inlet and outlet, volatile suspended solids (VSS), suspended solids (SS) and pH were determined.

3. Results and Discussion

3.1. Nitrogen Removal Performance

3.1.1. Nitrogen Removal Performance in Enrichment Period

As can be seen from Figure 1, all activated sludge samples showed positive anammox activity throughout the 108-day enrichment period irrespective of La(III) content. This is evidence to support the efficacy of the ammonium and nitrite feeding methods used for AnAOB enrichment. The biomass maintained an overall appearance of activated sludge, with a brownish color at the end of enrichment, and there was little difference between

samples from different reactors. There were differences in activity levels corresponding to the different start up strategies. Most notable was that there were four phases of enrichment: autolysis (P1, days 1–19), lag (P2, days 20–60), increased activity (P3, days 61–98) and stationary (P4, days 99–108).

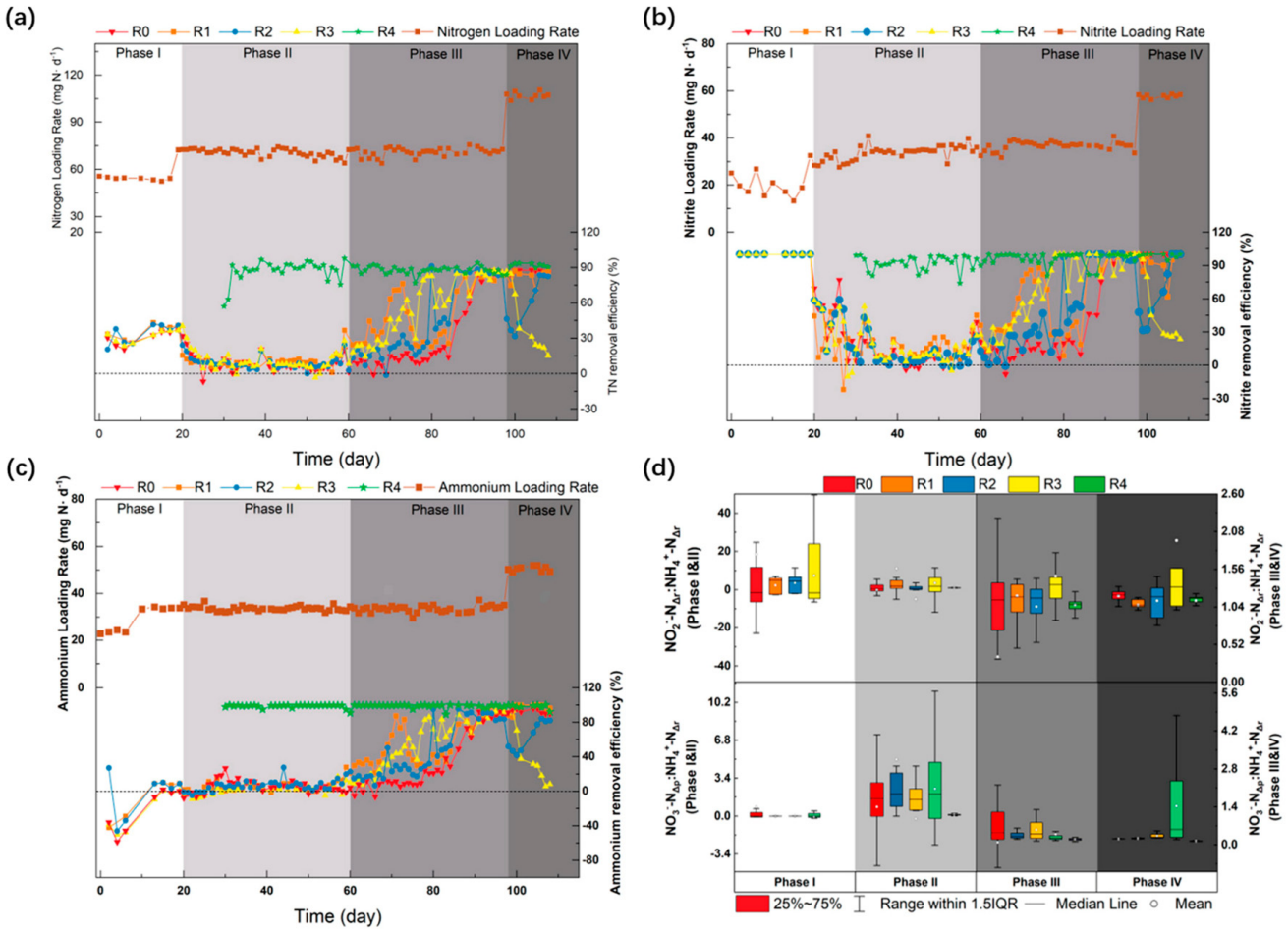

Figure 1. Nitrogen removal performance and evolution of stoichiometric ratio during enrichment period. (**a**) The nitrogen loading rate and TN removal efficency pre-preservation; (**b**) The nitrite loading rate and nitrite removal efficency pre-preservation; (**c**) The ammonium loading rate and ammonium removal efficency pre-preservation; (**d**) Stoichiometric ratio for ΔNO_2^-–N_r:ΔNH_4^+–N_r and ΔNO_3^-–N_p:ΔNH_4^+–N_r pre-preservation.

In phase P1 there was negative ammonium removal and almost complete nitrite removal. There was little nitrite remaining at the end of P1, and NH_4^+–N concentration in the effluent increased slightly during incubation due to hydrolysis of the remaining organic matter in Seed 1. This indicated that endogenous heterotrophic denitrification was the dominant process. Cell decomposition caused by changes in the inoculant sludge also increased the accumulation of NH_4^+–N. The stoichiometric ratios ΔNO_2^-–N_r:ΔNH_4^+–N_r and ΔNO_3^-–N_p:ΔNH_4^+–N_r both deviated from the stoichiometric values during P1.

In P2, in contrast, the NO_2^-–N concentration in the effluent increased and there was a slight decrease in NH_4^+–N concentration. This was because there was less residual organic matter, which reduced denitrification. The additional ammonia nitrogen and nitrite loading were beneficial to AnAOB proliferation.

In P3, there was evidence of simultaneous NH_4^+–N and NO_2^-–N consumption with noticeable anammox activity in the later stage of the phase. The sudden onset of greater anammox activity was consistent with the results of previous studies in which anammox reactors were variously inoculated with anaerobic granular sludge [46], denitrification sludge [47], activated sludge [48], and their mixture [33,34]. We infer that this increased

activity was due to quorum sensing by AnAOB, in which anammox activity occurs only when the cell density is higher than a threshold value (10^{10}–10^{11}/mL) [49]. The ranges of the ratios ΔNO_2^-–N_r:ΔNH_4^+–N_r and ΔNO_3^-–N_p:ΔNH_4^+–N_r were narrower and closer to stoichiometric values than in other phases.

In P4, nitrogen loading was increased to 107.12 ± 2.16 g/m^3/d N. This loading increased anammox activity in reactors R0 and R1, as evidenced by unchanged TN and ammonium and nitrite removal. Samples from reactors R2 and R3 showed differences due to loading shock. In the initial stage of P4, ammonium and nitrite concentrations increased in reactors R2 and R3, which were started using the semi-in situ method, and subsequent nitrogen removal during the later stage of P4 was observed in the R2 sample, but anammox performance did not recover in R3.

3.1.2. Nitrogen Removal in the Recovery Period

We investigated changes in N-form concentrations in the reactors to identify the effects of different start up methods on nitrogen removal post-refrigeration. Figure 2 shows that there were three phases of the post-refrigeration period: activity resumption (P1, days 1–9), increased activity (P2, days 10–34) and load reduction (P3, days 35–52). Recovery showed different characteristics across reactors.

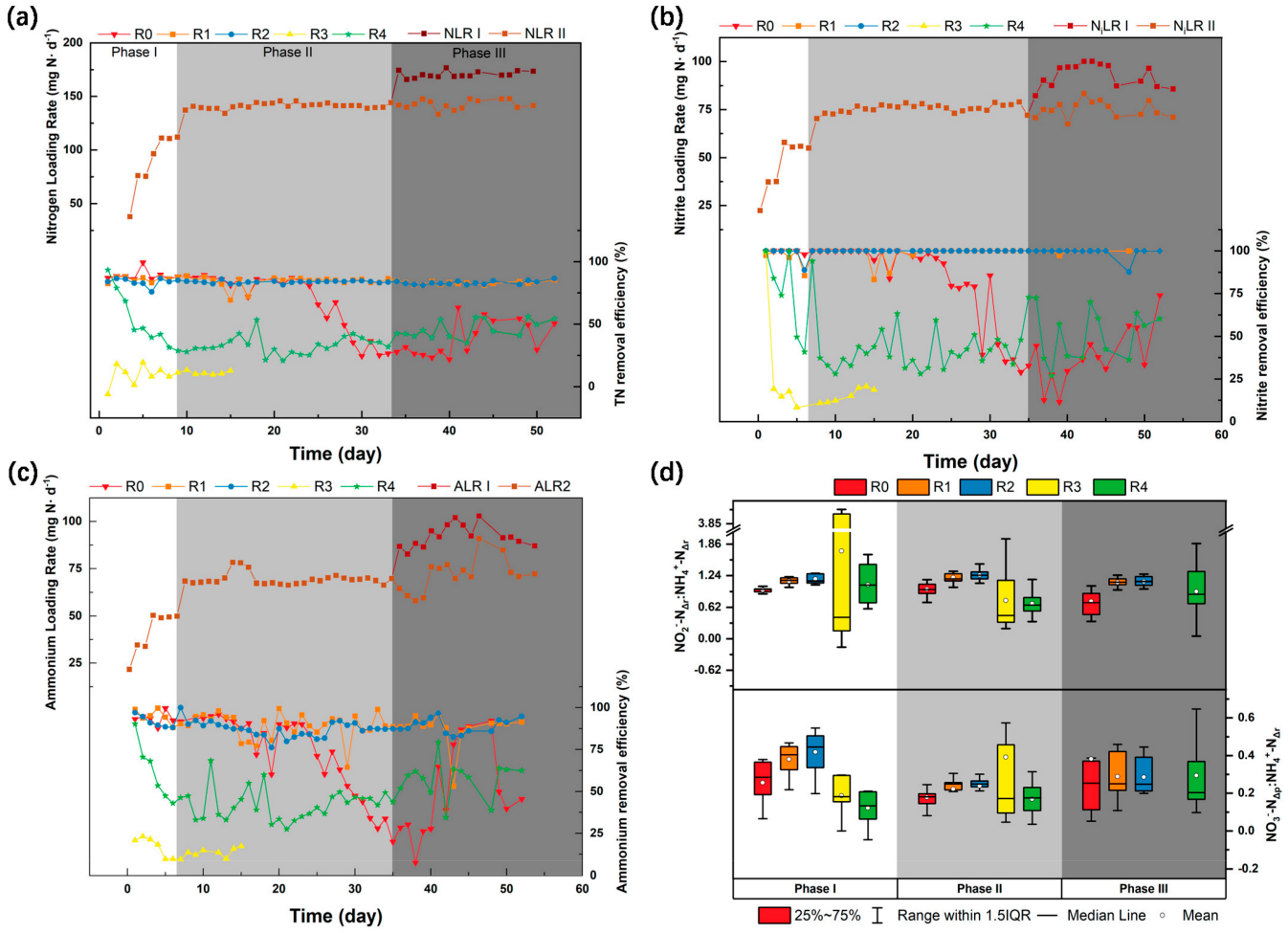

Figure 2. The nitrogen removal performance of R0, R1, R2, R3, and R4 during recovery period. (**a**) The nitrogen loading rate and TN removal efficency post-preservation; (**b**) The nitrite loading rate and nitrite removal efficency post-preservation; (**c**) The ammonium loading rate and ammonium removal efficency post-preservation; (**d**) Stoichiometric ratio for ΔNO_2^-–N_r:ΔNH_4^+–N_r and ΔNO_3^-–N_p:ΔNH_4^+–N_r post-preservation.

In P1, microorganism activity was adjusted briefly. The control group R0, R1 started up with in situ strategy and R2 started-up with semi in situ strategy containing low-concentration La(III) all demonstrated swift rejuvenated performance whose TN removal efficiency were 88.74 ± 4.12%, 86.04 ± 2.14%, and 83.68 ± 3.32%, respectively. However, R3 started-up with semi in situ strategy by an increase in ammonium and nitrite in the effluent and R4 started-up with ex situ strategy and suffered the downward fluctuation in nitrogen removal efficiency; what both have in common is that being affected by the high-concentration La(III) (0.010 mg L^{-1}). In this stage, the ΔNO_2^--N_r:ΔNH_4^+-N_r of R0, R1, and R2 reactors were slightly lower than the theoretical value but the ΔNO_3^--N_p:ΔNH_4^+-N_r was the other way around. Both stoichiometric ratios of R3 and R4 deviated from the theoretical value, indicating their system instability. When the nitrogen loading was increased in P2, the nitrogen removal performance in R4 was still in low-level fluctuation. On the contrary, R1 and R2 still maintain a high TN removal efficiency as 84.08 ± 4.45% and 83.73 ± 0.96% respectively under the shock of nitrogen loading. Notably, the 25th day was a watershed point for nitrogen removal performance in R0 where R0 had a precipitous drop in nitrogen removal efficiency from then on. During P2 the ΔNO_2^--N_r:ΔNH_4^+-N_r of R1 and R2 got stabler and moved closer to the theoretical value. In P3, with the further increasing in nitrogen loading in R1 and R2, the TN removal efficiency maintained as 82.79 ± 1.29% and 82.97 ± 1.50% while in R0 and R4 the nitrogen removal efficiency kept oscillating in low range.

In enrichment period, the startup characteristics of R4 were similar with those of reactors started-up with anammox sludge despite of the La(III) addition. This indicated that 0.010 mg L^{-1} La(III) hardly affected the anammox flocs nitrogen removal efficiency. Start-up performance of reactor R0, R1, R2, and R3 which all were inoculated with activated sludge Seed 1 showed a similar trend despite of the in situ and semi in situ enrichment strategies adopted. Nevertheless, time for detecting stable NH_4^+-N consumption was variable, ranging from 25 days in R1 to 30 days in R3. Although there was little difference in time needed to develop NH_4^+-N consumption among reactors, R1 executing in situ enrichment strategy had the shortest one. In the terms of time-consuming of anammox activity inflexion, R1 with in situ strategy took the shortest one, followed by R2 and R3 with semi in situ enrichment strategy, and the control group took the longest one. Compared with the control group R0, the addition of La(III) helped shorten the time needed for occurring anammox activity striking in the reactors thus speeding up the startup process. In addition, R2 and R3 started the La(III) addition at the 61st day where R3 exposed to high-concentration La(III) (0.10 mg L^{-1}) and R2 was fed with low-concentration La(III) (0.02 mg L^{-1}), while both reactors appeared anammox activity boom synchronously. Besides, low-concentration La(III) addition in R2 was as the same as R1 which was started up with in situ strategy, but R1 with a longer-time La(III) addition showed the surge in anammox activity on the 60th day which is earlier than the 71st day in R2. Hence, it can be inferred that the duration of La(III) addition had a greater impact on shortening the time-consumption of anammox activity inflexion rather than the concentration.

During the recovery period in this article, R1 performed in situ strategy with a low concentration of La (0.02 mgL^{-1}) and R2 adopted semi in situ strategy achieved high and stale anammox activity after preservation. However, the nitrogen removal performance of R4 with high-concentration La(III) addition was much lower and did not recover to the level before the preservation. In 30-days low temperature preservation, AnAOB in R1, R2, R3 and R4 was under the triple effects of low temperature, starvation and La(III). Related studies on AnAOB cryopreservation [50] showed that the anammox activity retention rate after storage at 4 °C for 1 month was only 89.7% of that before refrigeration, and the special particle structure of AnAOB as well as the large amount of EPS covered on its surface enhanced its ability to resist external environmental disturbances despite of 15 mmol·L^{-1} nitrite in media which can inhibit AnAOB activity. Research on reactor started up by anammox flocs and showed that the anammox flocs still can start the reactor even after starvation, but 50-day continuous cultivation was not enough for the anammox activity

inflexion to occur [51], which was in response to the R4 in this article. As previously reported, it is believed that REEs could replace Ca^{2+} and Mg^{2+} in various cell functions since their radius is like that of Ca^{2+} and Mg^{2+} [52,53]. Under low temperature stress, the addition of low-concentration Ca^{2+} promotes the anammox activity while nitrogen removal performance showed a downward trend after adding more than 0.04 mmol·L^{-1} Ca^{2+} [54]. Another study demonstrated that high concentrations of Mg^{2+} inhibited the activity of anammox bacteria, resulting in reduced nitrogen removal efficiency and growth rate [55].

3.2. La(III) Effects on Sludge's Morphology

The effect of La(III) on microstructures of sludge in five reactors were examined through SEM before and after refrigeration, and seed sludge Seed 1 and Seed 2 were also involved. As shown in Figure 3a, Seed 1, the activated sludge which was inoculated in R0, R1, R2, and R3, was coated with massive number of EPS and with varied shaped. While Seed 2 (Figure 3b), the inoculum of R4, had abundant crevices and pores on the surface, which are spherical bacteria, embedded in EPS, dominated, and scattered the clustered "cauliflower" structure which were regarded as the typical AnAOB morphology. Before the refrigeration, R0A, R1A, R2A, R3A, and R4A (Figure 3c–g) were dominated by loosely distributed spheroidal bacteria of which some showed crater-like depressions on both sides. This indicated that all the four start-up strategies applied in this research were feasible to help sludge achieve typical AnAOB structures after 108-day enrichment culture, which were also supported by the nitrogen removal performance of five reactors. Meanwhile, obvious rod-like species and filamentous bacteria extruding out from the surface were observed in the images Figure 3c–g, which showed multiple morphological and functional bacteria coexist. It is interesting to note that, as shown in Figure 3h–k, the sludge in each reactor demonstrated more obvious conglomeration aggregated by massive subunits after 30 days of low-temperature refrigeration. The aggregation was similar to the results of previous study of Wang et al., in which the aggregation of bacteria inside the anammox particles visualized after 5 months preservation at 4 °C and the degree of agglomeration is inversely proportional to the anammox activity [56]. This suggested that bacterial aggregation might be one of the responses of AnAOB facing with the disturbance of the low temperature environment to minimize the cryo-injury [57]. However, compared with R0B (Figure 3h), the sample of control group after refrigeration, the number of spherical bacterial in R1B and R2B was less but more obviously aggregated into clusters (Figure 3i,j). The difference between R0B, R1B, and R2B implied the positive effects of La(III) exposure on aggregation formation, which might be related to the EPS embedded on the surface. Generally, EPS and cell surfaces are negatively charged, while La^{3+} is positive. Adding low-concentration La(III) neutralized part of the negative charge on the surface of the colony reduced the repulsion of the same charge and made the sludge easier to aggregate into agglomerates. In addition, studies showed that a variety of low-concentration metal ions, including rare earth ions, can increase the number of EPS skeletons on the surface of the bacterial cluster and promote the secretion of polysaccharides, an important component of EPS [22,55,58,59], which can indirectly promote the aggregation of microorganisms through the bridging effect [60]. The EPS shell on the aggregated anammox granular surface was more conducive to alleviating the adverse effects of low temperature on AnAOB bacteria. Hence, together with the results of nitrogen removal performance, low-concentration La(III) exposure was beneficial to the low-temperature preservation of sludge with anammox activity.

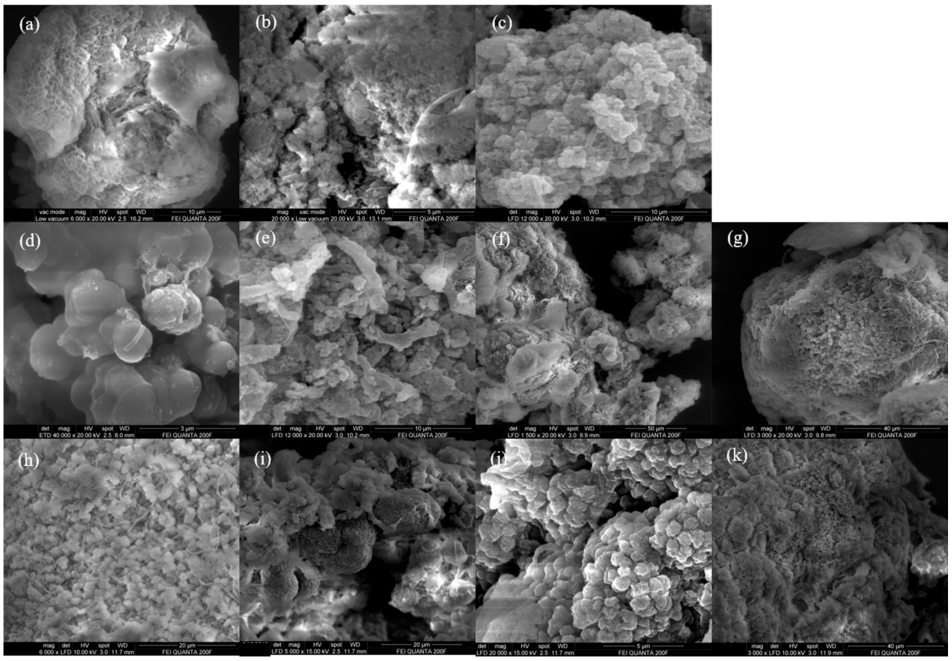

Figure 3. Morphology images of seed 1 (**a**), seed 2 (**b**), sludges sampled before 4 °C preservation in R3 (**c**), R0 (**d**), R1 (**e**), R2 (**f**), and R4 (**g**), as well as sludges sampled after 4 °C preservation in R0 (**h**), R1 (**i**), R2 (**j**), and R4 (**k**).

3.3. FISH Analysis

The evolution of sludge was assessed by FISH technique at the end of the experiment to investigate the relative abundance of functional bacteria in total bacteria. AMX820 probe was used to target AnAOB, meanwhile NIT3 and NSO190 probes were employed to target NOB and AOB, respectively. In addition, DAPI was applied to label total bacteria.

A positive test for aboriginal AnAOB bacteria (colored in purple) was identified (Figures S1 and S2 in the Supplementary Materials), which was regarded as the basis for the success of subsequent AnAOB enrichment in R0, R1, R2, and R3 [61]. After 108-day enrichment, the reactors adopting different enrichment strategies have formed a community structure with AnAOB as the dominant strain when they were started up with activated sludge. The cell relative percentage of AnAOB in sample R0A, R1A, and R2A was larger than Seed 1, and R1A adopting in situ enrichment strategy had the maximum followed by R2A, as a result R0A had the least AnAOB among samples started-up with activated sludge before the refrigeration. It was indicated that the addition of 0.02 mgL^{-1} La(III) could help AnAOB gain an advantage in the enrichment period when started up with activated sludge, which was supported by the nitrogen removal performance. Besides, the relative abundance of NOB has increased significantly due to anaerobic environment, from 2.51 ± 1.55% at the time of inoculation to 12.27 ± 5.66% (R0A), 20.97 ± 8.85% (R1A) and 15.09 ± 3.41% (R3A) (Table S2 in the Supplementary Materials), respectively. It was worth noting that under the stress of high concentration of La(III) (0.010 $mg{\cdot}L^{-1}$), the abundance of AnAOB bacteria in ANAMMOX sludge dropped sharply from 54.60 ± 6.19% (Seed 2) to 17.35 ± 6.69% (R4A) (Table S2 in the Supplementary Materials), however, the nitrogen removal performance of the R4 reactor did not show a corresponding decrease. The asynchronous changes may be related to the high ANAMMOX activity of Seed 2 sludge that allows R4 show resistance to La(III) exposure. The flocculent seed sludge was taken from a lab-scale parent SBR reactor, whose history has exceeded half a year at a steady nitrogen removal rate of about 5.04 $kgN{\cdot}m^3{\cdot}d^{-1}$ with the specific anammox activity (SAA) of 649.0 ± 56.65 mg total nitrogen (TN) g^{-1} VSS d^{-1}, which was much higher than the feed water load of the R4 reactor and the concentration of ammonia and nitrite has not reached the limit of its treatment capacity.

Then, the relative enrichment or diminution of functional bacterial was ranked using odd ratio (Figure 4). During the enrichment period, the changes of AOB and NOB in R1, R2 and R4 with La(III) addition show a consistent trend where the relative proportions of AOB and NOB were increased, while that of AnAOB, the main functional bacteria in R4, decreased. However, without La(III), the change trend of functional bacteria AnAOB, AOB and NOB in the control group R0 was opposite to that of R1, R2, and R4 with La(III). After 30-day low-temperature preservation, without La(III) addition, AnAOB and AOB in R0 were at a lower abundance, but those of AnAOB in the R1, R2, and R4 reactors using the La(III) addition strategy were relatively enhanced. This result is consistent with the SEM image analysis. The protection of AnAOB by La(III) addition may benefit from the combination of the promotion of EPS secretion induced by La(III) and the enhanced cell aggregation. Promoted EPS secretion allowed a better wrap around AnAOB to resist the adverse effects of low temperature, which is similar to the effect of Ca^{2+} on anammox [54].

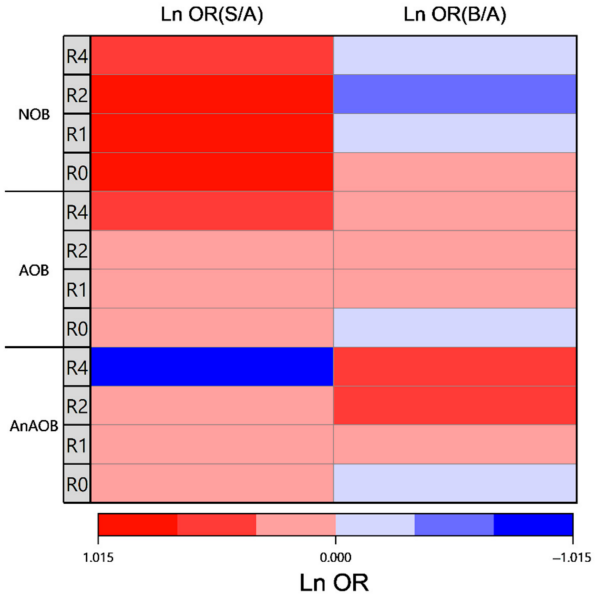

Figure 4. The relative abundance evolution of functional bacterial based on FISH analysis.

3.4. Microbial Community Analysis

3.4.1. Diversity Changes in the Microbial Community

As summarized in Table 3, a total of 2.8 × 105 sequencing reads were obtained and clustered into 915 OTUs. The coverage indices, representing sequencing depth, of all 10 samples were >0.99. The OTUs and the indices acquired in samples from R1 were consistent with those collected from R2. Nevertheless, for R0A, although its Chao1 index was higher than that in Seed 1, its Simpson and Shannon indices were lower than those for Seed 1. At the end of enrichment period, community richness and diversity showed decrease when reactors started up with activated sludge, and the inhibited aerobic bacteria and the toxic effect of nitrite in the medium on microorganisms were responsible for the elimination of some bacteria, which was consistent with the studies of Ding et al. and *Kocamemi* et al. [32,33]. While increase in community richness and diversity was demonstrated when anammox sludge was adopted to start-up R4. When R0 persevered in 4 °C without La(III), marked decreases in the Chao1, Simpson and Shannon indices, as well as in the OTU numbers, were observed after refrigeration while the addition of La(III) increased community richness but decreased species diversity in R1, R2, and R4. Under cold storage and starvation stress, microorganisms might endogenously consume and degrade intracellular macromolecules, thereby changing the biodiversity and community structure in the sludge [62,63].

Table 3. Bacterial diversity indices of sludge samples.

Sample ID	Reads	OUTs	Chao1	Shannon	Simpson	Coverge
Seed1	25,513	554	593.91	6.903113	0.969402	0.9972
R0A	31,415	550	616.23	5.744148	0.950788	0.9958
R0B	30,986	452	512.73	5.42796	0.950321	0.9959
R1A	31,214	519	597.47	5.725502	0.955701	0.9956
R1B	32,783	520	559.44	5.252368	0.904126	0.9962
R2A	32,278	528	574.37	5.431604	0.935977	0.9958
R2B	32,850	544	591.52	5.372883	0.922735	0.9960
Seed2	31,559	309	347.61	4.615046	0.905782	0.9972
R4A	32,186	333	353.62	4.945635	0.930457	0.9979
R4B	31,487	369	407.44	4.62402	0.886857	0.9971

3.4.2. Changes in Microbial Composition at Phylum Level

The bacterial community structure of sludge at the end of enrichment and refrigeration period was unraveled by high-throughput sequencing. Seed sludges were also involved in the analysis. As shown in Figure 5, top fifteen primary phyla were identified in samples, including *Proteobacteria*, *Chlorobi*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Gemmatimonadetes*, *Armatimonadetes*, *Acidobacteria*, *Planctomycetes*, *Nitrospirae*, *Chloroflexi*, *Spirochaetae*, *TA06*, *Verrucomicrobiacea*, and *TM6*. The phylum *Proteobacteria* are the dominant in the seed sludge (Seed 1 and 2) and sludge before refrigeration (R0A, R1A, R2A, and R4A), which is consistent with the bacterial community distribution in most anammox reactors [34,48]. Among the top fifteen primary phyla in the samples, the *Proteobacteria*, *Nitrospirae*, and *Planctomycetes* were involved in nitrogen removal progress, which have antagonistic demand for oxygen.

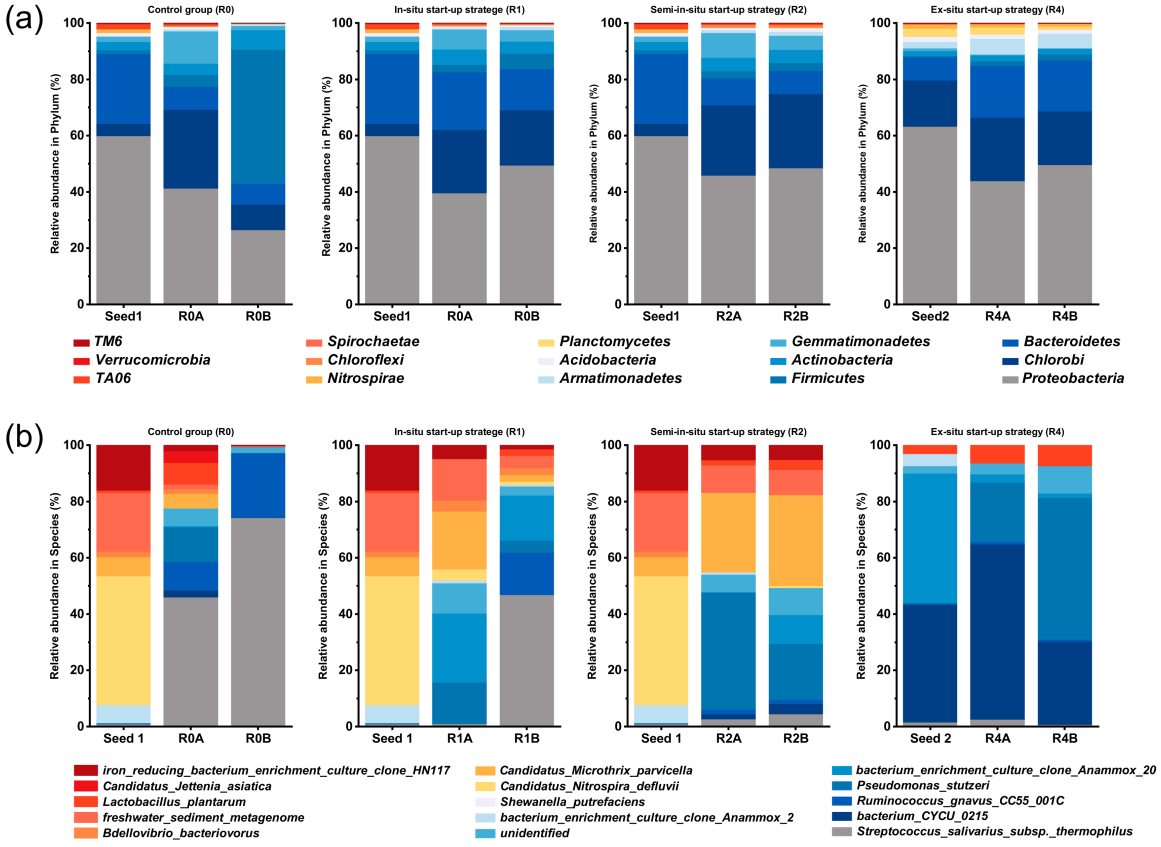

Figure 5. Microbial community structure of the sludge in reactors started up with different strategies. The relative abundance at the phylum (**a**) or class (**b**) level was defined as the number of sequences assigned to a given taxon divided by the total number of sequences per sample.

Notably, after 108-day enrichment, the relative abundance of *TM6*, *TA06*, *Spirochaetae*, *Nitrospirae*, *Acidobacteria*, *Bacteroidetes*, and *Proteobacteria* in R0, R1, and R2 decreased, while the abundance of *Planctomycetes, Chlorobi*, *Firmicutes, Actinobacteria, Gemmatimonadetes, Armatimonadetes, Chloroflexi* and *Verrucomicrobiacea* increased instead, which showed a pattern of "waxing and waning." It has been found that the population of *Proteobacteria, Nitrospirae, Bacteroidetes,* and *Planctomycetes* correlated significantly with the nitrogen removal performance of biological reactors [64]. The phylum *Proteobacteria*, which most of AOB belongs to, were common and the predominate phylum of the sludge community in anaerobic condition, due to its tight link to the metabolism of C, N, and S [65–67]. The phylum *Proteobacteria* was repressed but still dominant during enrichment. The phylum *Nitrospirae* containing massive NOB, which inhabited in anaerobic reactors relying on trace oxygen carried in by the influent, performed important functions in the nitrogen cycle. The presence of NOB consumed the entire oxygen carried in during culture medium replacement thus maintaining the anaerobic environment, which was important to the AnAOB enrichment. The phylum *Nitrospirae* gradually eliminated within enrichment period might be due to the inhibition caused by decreasing dissolved oxygen. The repressed *Nitrospirae* benefited the AnAOB enrichment. *Bacteroidetes* is consisted of some microorganisms that are able to digest high molecular weight compounds [68] and can perform denitrification of nitrite under anoxic conditions [69], in which way they may also be potential competitors for nitrite with AnAOB in the nitrogen cycle [70]. To the end of enrichment period, the organic matter in reactor was degraded until exhausted, thus the abundance of *Bacteroidetes* decreased due to the shortage of carbon source. AnAOB, the major nitrogen removal contributor, belongs to the phylum *Planctomycetes*, whose increase in the relative abundance was the main reason for the enhancement of the nitrogen removal performance of the R0, R1, and R2 reactors. The decrease in the *Proteobacteria, Nitrospirae,* and *Bacteroidetes* indicated that the operation condition kept anaerobic without organic input during the enrichment period suppressed nitrification, and denitrification processes, leading to the transformation of nitrogen removal process from the coexistence of multiple processes to predominantly anammox process. This indication was consistent with the increment in the phylum *Planctomycetes*. Notably, *Chloroflexi* is another phylum that is frequently detected in the anammox reactors. Members of the *Chloroflexi* phylum have been reported to have the ability to degrade a wide range of complex organic matters [71], indicating their promoting effect on the anammox enrichment, but the exact path is still unclear. Besides, with the addition of La(III), the relative abundance of *Nitrospirae* and *Bacteroides* in R1A and R2A were lower than the control group R0B. Nevertheless, the community composition of R0A, R1A, and R2A was similar with each other and the differences in abundance of *Nitrospirae* and *Bacteroides* were indistinctive. The community evolution trend of R0, R1, and R2 started-up with activated sludge kept synchronous and the phylum *Planctomycetes* in R0, R1 and R2 had similar relative abundances, which is consistent with the similar nitrogen removal performance in the later stage of the enrichment period. These results inferred that La(III) exposure hardly affected the formation of the predominant phylum in the AnAOB enrichment processing when the reactors started-up with activated sludge. When it comes to the addition of high concentration of La(III), the relative abundance of *Nitrospirae*, *Proteobacteria,* and *Planctomycetes* in R4A significantly decreased, indicating 0.10 $mg{\cdot}L^{-1}$La(III) had an effect on nitrogen removal bacteria, such as AOB, NOB, and AnAOB, and had an inhibitory effect on denitrification, nitrification, anammox, and other nitrogen removal processes, which are supported by the FISH image results. Macroscopically, the nitrogen removal performance of the R4 at the later enrichment period did not significantly reduce due to the low nitrogen loading rate and its unsaturated nitrogen removal capacity. The study revealed hormesis condition upon La(III) dosage, thus, dose-response phenomenon exhibited sludge stimulation at low La(III) dosage (0.02 $mg{\cdot}L^{-1}$) and inhibition at high-dose (0.10 $mg{\cdot}L^{-1}$), similar with the study of Hao et [72].

At the end of refrigeration period, *Firmicutes* (47.55%), instead of *Proteobacteria* (41.19%), had been observed as the dominant phylum in the sludge sample collected from the control

group (R0B) and *Planctomycetes*, the second predominated phyla, was downregulated to 26.51%, which is consistent with the trend of the relative proportion of AnAOB in FISH analysis. The transformation of dominant phylum might be responsible for the loss of process stability of R0 in the phase II of recovery period. Besides, the relative abundance of *Planctomycetes* in R0 reactor decreased from 0.06% to 0.05%. It was reported that inhibited activity, lower growth rate, and downregulated metabolic rate of AnAOB at the individual level appeared when the anammox sludge cultivated at low temperature [73,74]. Refrigeration also brought microbial community shifts on anammox sludge, which will further induce the alteration of sludge structure macroscopically and cause a decrease in the nitrogen removal efficiency of the reactor as a result [73,74]. After refrigeration, the abundances of *Proteobacteria, Nitrospirae,* and *Planctomycetes* in R1 and R2 were greater, which was verified from the nitrogen removal performance, sludge morphology, and FISH analysis results of R1 and R2 during the recovery period. While the growing trend of *Proteobacteria, Nitrospirae,* and *Planctomycetes* in reactors with La(III) addition was contrast with the control group R0. The contradictory evolution of the three core functional bacterial phyla among R0, R1, and R2 implied that 0.02 mg·L^{-1} La(III) had a certain protective effect on bacteria associated with nitrogen removal under low temperature stress. Meanwhile, the abundance of *Nitrospirae* and *Planctomycetes*, which are involved in the nitrogen removal process in R4, decreased after low-temperature refrigeration, indicating that 0.10 mg·L^{-1} La(III) might inhibit anammox activity.

3.4.3. Evolution of Microbial Composition at Class Level

Figure 5b showed the top 15 groups at the class level of sludge samples. It demonstrated that *Candidatus Nitrospira defluvii* which is related to nitrite metabolism, *bacterium enrichment culture clone Anammox 20*, *bacterium enrichment culture clone Anammox 2,* and *Candidatus Jettenia asiatica* who are responsible for anammox process, as well as *iron reducing bacterium enrichment culture clone HN117*, *Streptococcus salivarius subsp. Thermophilus, bacterium CYCU 0215, Ruminococcus gnavus CC55 001C, Pseudomonas stutzeri*, *Shewanella putrefaciens*, *Candidatus Microthrix parvicella*, *Bdellovibrio bacteriovorus*, *freshwater sediment metagenome, Lactobacillus plantarum* were observed in samples. The class *Candidatus Nitrospira defluvii* dominated in activated sludge Seed 1, while anammox flocculent sludge Seed 2 contained predominantly bacterium enrichment culture clone Anammox 20. The relative abundances of the AnAOB in both seed sludge were consistent with the FISH results.

After enrichment period, Streptococcus salivarius subsp. thermophilus in the R0 replaced Candidatus Nitrospira defluvii as the dominant class. In the term of AnAOB, Candidatus Jettenia asiatica surpassed bacterium enrichment culture clone Anammox 2 and predominated in R0, while in R1 and R2 with La(III) exposure Candidatus Jettenia asiatica advantaged. Under ex situ start-up strategy, the abundance of bacterium enrichment culture clone Anammox 20 in R4 was kept as the dominate AnAOB even though it decreased. Notably, bacterium enrichment culture clone Anammox 2 shared a downtrend among R0, R1, R2, and R4, but the addition of La(III) offered a smaller decline.

At the end of refrigeration period, *Streptococcus salivarius subsp. Thermophilus* and *Ruminococcus gnavus CC55 001C* in R0 were enriched while others weaken rapidly. The dominant class in R1 was transformed from *bacterium enrichment culture clone Anammox 20* to *Streptococcus salivarius subsp. Thermophilus,* even though the abundance of *bacterium enrichment culture clone Anammox 20* increased. The dominant bacteria transformation caused by low-temperature refrigeration was similar to the results of previous studies [62,63]. The abundance upsurge of *bacterium enrichment culture clone Anammox 20* also occurred in R2 and R4, which were supported by the increase in AnAOB proportion in FISH results and more compact coccus aggregation in SEM images. It is speculated that the addition of La(III) promoted the aggregation of AnAOB and then enhanced its resistance to low-temperature environments, which increased the abundance of AnAOB wrapped inside the granule as a result.

4. Conclusions

The in situ, semi in situ, and ex situ start-up strategies adopted in enrichment period started up the anammox reactors smoothly, but the in situ and semi in situ enrichment strategies with low-concentration La(III) addition (0.02 $mg \cdot L^{-1}$) shortened the time-consumption to form stable anammox activity and high nitrogen removal efficiency to 60–71 days. In addition, the effect of low-temperature preservation on the anammox activity was investigated, and the recovery performance of the reactors was tested after one-month cold storage at 4 °C. The results showed that R1 and R2, the reactors with low concentration of La(III), restored the anammox activity and obtained stable and high nitrogen removal performance within 10 days.

Morphological observation found that the low concentration La(III) addition promoted the formation of a compact structure of sludge to resist the adverse effects of low temperature. Meanwhile, with the help of FISH images and 16S rRNA sequencing, it showed that 0.02 $mg \cdot L^{-1}$ La(III) was beneficial to maintain AnAOB high abundance and microbacterial community diversity in refrigeration period.

On summary, the in situ and semi in situ enrichment start-up strategy using low-concentration La(III) addition had the potential to start the anammox reactor and reduce the adverse effects of low-temperature during long-distance transportation of anammox sludge, which was beneficial to promote the application of the anammox process in the in situ remediation of ammonia pollution in rare earth mines areas regarded as a feasible strategy for starting the anammox in situ remediation process. Further, studies focused on the fast start-up of anammox reactor are priority in in situ bioremediation of REE-contaminated nitrogen pollution in groundwater, and continuous efforts should be made on understanding the effects of other REEs on anammox.

Supplementary Materials: The following are available online at https://www.mdpi.com/2071-1050/13/8/4591/s1. Text S1: B Reactor operation. Text S2: Bioinformatic analysis. Table S1: Concentration and composition of the synthetic wastewater. Table S2: The relative fluorescence area ratio of AnAOB, AOB, and NOB. Figure S1: The FISH images of seed 1(a), seed 2(b), R0A(c), R0B(d), and R1A(e). Figure S2: The FISH images of R1B(f), R2A(g), R2B(h), R4A(i), and R4B(j).

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