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The Recovery of a Sequencing Biofilm Batch Reactor—Anammox System: Performance, Metabolic Characteristics, and Microbial Community Analysis

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Abstract: The mainstream application of the anammox process is often hindered by its long recovery phase and instability under disturbance. In this study, a lab-scale anammox sequencing biofilm batch reactor (SBBR) was rapidly recovered within 85 days by gradually increasing the influent nitrogen concentration, and the total inorganic nitrogen (TIN) removal efficiency achieved 83.44 ± 0.03%. During the recovery process, the nitrogen removal rate (NRR) increased from 0.05 to 0.34 kg/(m^3·d), with the nitrogen loading rate (NLR) changing from 0.08 to 0.40 kg/(m^3·d) in the anammox system. The activities of hydrazine oxidase (HZO) in the biofilm also increased from 0.17 to 10.80 μmol Cyt-c/(g VSS·s). Also, the dominant anammox genera in the biofilm were *Candidatus Kuenenia* and *Candidatus Brocadia*. The results of this study suggested that an SBBR–anammox system filled with a microbial carrier could facilitate the enrichment of anammox bacteria and contribute to performance recovery.

Keywords: anammox; metabolic enzyme activities; microbial community; rapid recovery; SBBR system

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1. Introduction

Excessive nitrogen emissions pose a significant challenge to the global environment, leading to severe water pollution issues [1]. Traditional nitrogen removal processes suffer from various drawbacks, including high energy consumption for aeration and high sludge yield. As a result, these processes fail to achieve energy-neutral wastewater treatment [2]. An earlier study revealed that carbon emissions from the sewage treatment industry account for 1–2% of total societal emissions, placing it among the top ten carbon-emitting sectors [3]. Traditional wastewater treatment processes might not be conducive to the implementation of sustainable development strategies. Anaerobic ammonium oxidation (anammox) technology is an emerging biological nitrogen removal technology with great promise, with the simultaneous removal of ammonia (NH_4^+-N) and nitrite nitrogen (NO_2^--N) without an external carbon source, which has been regarded as a way to remove nitrogen from wastewater sustainably [4,5].

Numerous studies have demonstrated that the practical application of the anammox process has improved energy conservation and treatment efficiency, fostering an ecological environment conducive to sustainable development [6]. Liang et al. [7] confirmed that the anammox process could be widely applied to the nitrogen removal of mature landfill leachate. Deng et al. [8] also found that anammox bacteria could adapt to a certain range of salinity and heavy metals after a period of accumulation. Regardless of the type of

anammox process, the results showed that the required nitrogen removal rate could be achieved under the premise of normal operation. The study of Tsai and Chen [9] showed that the efficiency of anammox sludge to remove nitrogen wastewater was above 90%. In conclusion, anammox bacteria would use ions in industrial wastewater as electron acceptors to remove nitrogen, including mature landfill leachate, sludge digestion wastewater, and semiconductor wastewater, to achieve environmental sustainability [10].

However, anammox bacteria are slow-growing autotrophic bacteria with specific requirements for environmental conditions such as temperature, pH, and nutrient availability [11]. Normally, the doubling period of anammox bacteria is approximately 11 days [12]. Due to seasonal maintenance or long periods of storage, anammox sludge would face starvation periods. Extended periods of storage could result in unfavorable conditions or nutrient depletion, which could negatively impact the viability and activity of anammox bacteria [13]. Thus, a deep understanding of the recovery process of the anammox-based process after a long period of starvation is essential for its practical application. A previous study reported that the effective interception and growth of anammox bacteria were key issues to realizing the successful recovery of anammox systems as well as maintaining high efficiency and stability [14–16]. Restoring the biomass of anammox bacteria to its maximum extent is crucial for effectively recovering the biological activity and nitrogen removal performance of anammox sludge following long-term starvation [17]. The sequencing biofilm batch reactor (SBBR)–anammox system improved the biomass and species of attached biofilms by conducting microbial culture on the suspended vector added [18,19], which is an ideal choice for mainstream anammox [20,21]. Compared to flocculent sludge, biofilm provides a relatively dense form of anammox bacteria, which is considered an essential advantage for reactivating anammox systems during chronic hunger conditions [22–26]. Nevertheless, relevant research about the recovery performance and mechanism of anammox bacteria after long-term starvation is still lacking, especially for the SBBR–anammox system, which might not be conducive to the promotion and application of SBBR–anammox technology.

Moreover, diverse microbial communities are abundant in the SBBR–anammox system [27,28]. Cai et al. studied the mixed bacterial community diversity structure in the SBBR–anammox system. The study found that *Candidatus Brocadia* was the dominant anammox genus, with a relative abundance of 1.60% [29]. In addition, Miao et al. found that the dominant anammox taxon was *Candidatus Kuenenia* in an SBBR–anammox system treating landfill leachate, with a relative abundance of 20.40% [30]. The study of Liu et al. also reported that the relative abundance of *Candidatus Kuenenia* increased to 20.1% under low COD/N ratio conditions in the simultaneous nitrification, anammox, and denitrification (SNAD) system during the operation of the SBBR reactor [31]. The study of Chen et al. confirmed that the SBBR system has great potential to enrich anammox functional bacteria, in which the relative abundance of *Candidatus Brocadia* was 0.1%, representing successful enrichment [32]. In conclusion, the variation in anammox abundance in the SBBR–anammox system is widely known, but the variations in microbial community structure during the recovery process in the SBBR–anammox system have not been investigated.

Therefore, it would be necessary to explore how to quickly and efficiently restore the performance of the SBBR–anammox system, so as to provide information about the microbial structure and mechanisms during anammox performance recovery for related research, further promoting the practical application of the anammox process. The objectives of this paper were to (1) summarize the reactor's performance under a rapid recovery process in the SBBR–anammox system, (2) analyze the specific physicochemical properties of biofilm, including EPS contents and key enzyme activities, and (3) investigate concrete changes in microbial community structure during operation of the SBBR–anammox system.

2. Materials and Methods

2.1. Experimental Set-Up

A 14.50 L lab-scale SBBR–anammox reactor was used and the stirring device adopted an electric mixer and stirring paddle with a rotation speed of 65 rpm (Figure 1). In the SBBR–anammox system, K1 porous plastic carriers, made of HDPE, were utilized as the type of carriers. The specific surface area of the carriers used in this experiment was 800 m^2/m^3, with a specific density of 0.95 g/cm^3. With the addition of trace elements such as hydrophilic groups, it took only 3–15 days to hang the film. The temperature in the SBBR–anammox system was controlled by heating rods. The dissolved oxygen (DO) concentration and temperature were controlled below 0.05 mg/L and 31.2 ± 0.6 °C, respectively.

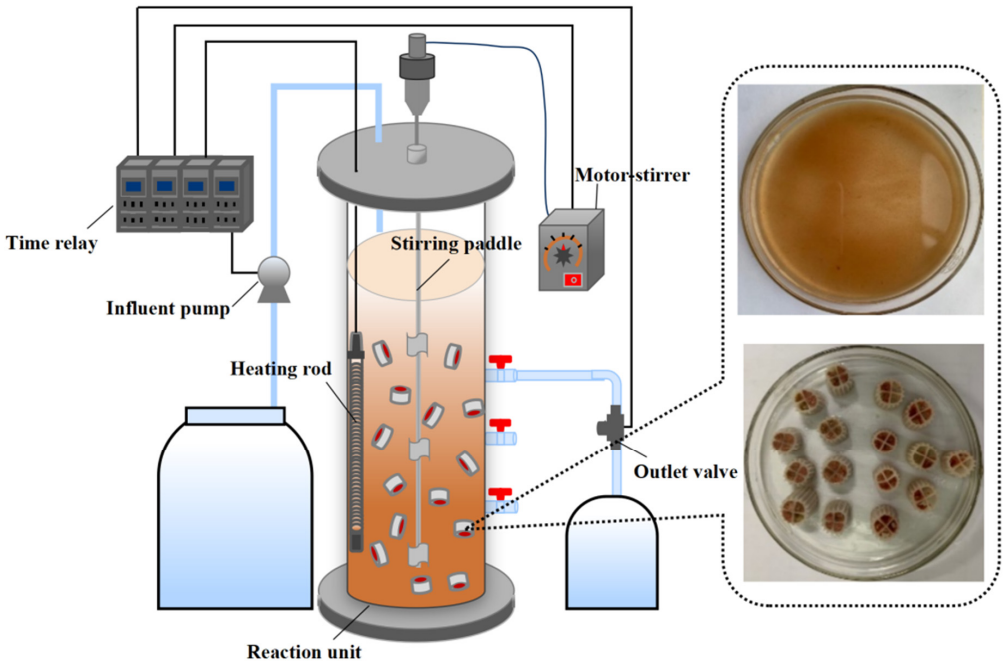

Figure 1. Schematic representation of the SBBR–anammox system.

The running cycle during the recovery of SBBR–anammox is shown in Table 1. The whole restoration process is divided into four stages, including the adaptation stage (1~7 d), the load lifting stage (8~29 d), the rapid recovery stage (30~59 d), and the stable stage (60~85 d).

Table 1. Cycle parameters during operation of the SBBR–anammox system.

Period	Water Inlet	Anaerobic Reaction	Sedimentation	Water Outlet	Idle
Time	15 min	360 min	80 min	15 min	10 min

2.2. Substrate Composition and System Properties Analysis

NH_4^+-N and NO_2^--N were provided by NH_4HCO_3 and $NaNO_2$, respectively, in synthesis wastewater. Generally, the influent volume was controlled at 30 L. The compositions of the mineral medium of synthetic influent were 50 mg/L $CaCl_2{\cdot}2H_2O$, 25 mg/L KH_2PO_4, 200 mg/L $MgSO_4{\cdot}7H_2O$, and 1 mL/L trace element. The specific recipe elements were as follows: trace elements I and II (I: 5.0 g/L $Na_2{\cdot}EDTA{\cdot}2H_2O$, and 5.0 g/L $FeSO_4{\cdot}7H_2O$, and II: 15.0 g/L $Na_2{\cdot}EDTA{\cdot}2H_2O$, 0.43 g/L $ZnSO_4{\cdot}7H_2O$, 0.24 g/L $CoCl_2{\cdot}6H_2O$, 0.99 g/L $MnCl_2{\cdot}4H_2O$, 0.25 g/L$CuSO_4{\cdot}5H_2O$, 0.22 g/L $NaMO_4{\cdot}2H_2O$, 0.19 g/L $NiCl_2{\cdot}6H_2O$, 0.21 g/L $NaSeO_4{\cdot}10H_2O$, and 0.014 g/L H_3BO_3).

Furthermore, the collection of effluent samples was filtered with a membrane filter (0.45 μm). Total inorganic nitrogen (TIN) was calculated with the sum of NH_4^+-N, NO_2^-N, and NO_3^--N [33]. Based on the stoichiometric formulas of Vázquez-Padín et al. [34], the TIN removal rate, free ammonia (FA), free nitrite (FNA), nitrogen removal rate (NRR), and nitrogen load rate (NLR) were calculated by relevant methods, shown as follows:

$$TIN_{in}\ (mg\ N/L) = [NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in} + [NO_3^-\text{-}N]_{in} \quad (1)$$

$$TIN_{out}\ (mg\ N/L) = [NH_4^+\text{-}N]_{out} + [NO_2^-\text{-}N]_{out} + [NO_3^-\text{-}N]_{out} \quad (2)$$

$$\Delta N\ (mg\ N/L) = [TIN]_{in} - [TIN]_{out} \quad (3)$$

$$TIN\ removal\ rate\ (\%) = \frac{\Delta N}{[TN]_{in}} \times 100\% \quad (4)$$

$$NLR\ (kg\ N/(m^3 \cdot d)) = \frac{[TN]_{in}}{HRT} \quad (5)$$

$$NRR\ (kg\ N/(m^3 \cdot d)) = \frac{\Delta N}{HRT}$$
$$FA(mg\ N/L) = \frac{17}{14} \frac{[NH_4^+\text{-}N]_{out} \times 10^{pH}}{exp(\frac{6334}{273+T}) + 10^{pH}} \quad (6)$$

$$FNA\ (mg\ N/L) = \frac{47}{14} \frac{[NO_2^-\text{-}N]_{out}}{exp(\frac{-2300}{273+T}) \times 10^{pH}} \quad (7)$$

where $[NH_4^+\text{-}N]_{in}$, $[NO_2^-\text{-}N]_{in}$, and $[NO_3^-\text{-}N]_{in}$ represent the influent NH_4^+-N, NO_2^--N, and NO_3^--N concentration (mg N/L), respectively; $[NH_4^+\text{-}N]_{out}$, $[NO_2^-\text{-}N]_{out}$, and $[NO_3^-\text{-}N]_{out}$ represent the effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentration (mg N/L), respectively; and pH and T are the value of pH and temperature (°C) in the SBBR–anammox system, respectively.

2.3. Extracellular Polymeric Substances (EPS) Content

The method of extracting EPS is ultrasonic crushing combined with cation exchange resin (CER). Concisely, the first step was centrifugation for the biofilm at 4000 rpm for 20 min and then, we obtained a loose extracellular polymer (LB-EPS) after filtration with filter membranes of 0.45 μm. The biofilm was resuspended to the original volume (25 mL) with PBS solution and then crushed by ultrasonic treatment (interval time of 3 s, working time of 3 s, and total time of 6 min), and then CER was added to stir for 4 h. After stirring, the solution was centrifuged for 20 min at 12,500× *g* rpm and 4 °C in 1.5 mL centrifuge tubes to obtain the supernatant. Finally, a tight extracellular polymer (TB-EPS) was obtained after filtration with filter membranes of 0.45 μm. The phenol–sulfuric acid method and Lowry method could be used to test the contents of polysaccharides (PS) and protein (PN), respectively.

2.4. Metabolic Enzyme and Electron Transport System Activities

The extraction method of crude hydrazine oxidase (HZO) was as follows [35]. At the beginning, the sample of 25 mL was centrifuged for 10 min at 4000 rpm. The procedure was repeated three times. Then, the sediment was resuspended with a PBS buffer solution to 25 mL after pouring the supernatant. Finally, the sample was centrifuged for 20 min at 15,000× *g* after stirring for 1.5 h at 500× *g* rpm. The liquid supernatant obtained after centrifugation was the crude enzyme extract. The method of detecting HZO activity was referenced by the study of Zhang et al. [36].

According to the method described in the study of Maurines-Carboneill et al. [37], the activity of the electron transport system (ETSA) was tested in this study, and the detailed

steps are illustrated as follows. Firstly, the sample of 10 mL was centrifuged for 10 min at 4000× *g* rpm, and the supernatant was discarded. Then, 2 mL Tris-HCl buffer solution and 0.5 mL 0.2% INT solution were added successively, and the prepared sample was wrapped with black cloth and placed in an air bath shaker at (35 ± 1) °C for oscillating culture for 30 min. Next, the sample with the addition of 1 mL 37% pure formaldehyde was centrifuged for 15 min at 4000× *g* rpm. The sediment was added into 5 mL methanol and pumped for 10 min in an air bath shaking bed at (35 ± 1) °C. Finally, the liquid supernatant centrifuged for 8 min at 3000× *g* rpm was detected with an ultraviolet spectrophotometer at 485 nm. The specific computational formula of ETSA is as follows:

$$\text{ETSA } (\mu\text{g/min·g MLSS}) = \frac{\text{D} \times \text{V}}{\text{K} \times \text{W} \times \text{t}} \quad (8)$$

where D, V, K, W, and t represent the absorbance of the extract at wavelength 485 nm, the extractant volume (mL), the slope of the standard curve, the experimental sludge dry weight (g MLSS), and the culture time (min), respectively, in the SBBR–anammox system.

2.5. 16S rRNA Amplicon Sequencing

To analyze the changes in microbial community diversity at different recovery stages (stage I, II, III, and IV) in the SBBR–anammox system, biofilm samples were extracted and tested on the Illumina MiSeq platform at Sangon Biotech Co., Ltd. in Shanghai. The PCR amplifiers (primers 341F: CCTACGGGNGGCWGCAG and 805R: GACTACHVGGGTATC-TAATCC) belonged to the V3–V4 hypervariable region for high-throughput sequencing. All sequences were divided by operational taxonomic units (OTUs) at a 97% similarity level, and statistical analysis was carried out.

3. Results and Discussion

3.1. SBBR–Anammox System Performance

The anammox system performance of the gradual recovery stages of the SBBR system is shown in Figure 2a. In stage I, the recovery experiment was conducted with a low NLR of 0.08 kg/(m^3·d), and the TIN removal rate was recovered to around 56.67%. In this stage, the normal physiological activities of anammox bacteria were inhibited due to poor adaptability to the changing environment [38]. At the beginning of stage II, the NLR had increased to 0.10 kg/(m^3·d) and the TIN removal rate increased to 76.62% (day 29), showing that the removal performance of ammonia nitrogen and nitrous in the SBBR–anammox system was gradually restored [11].

Then, the NLR continued to increase from 0.12 to 0.33 kg/(m^3·d) in stage III; also, the TIN removal efficiency gradually increased from 76.62% (day 29) to 82.94% (day 59). It was noted that the pH value was 8.60 on average (day 54), which exceeded the optimal value of 7.80–8.50 for anammox bacteria [39]. Meanwhile, FA attained the highest value of 8.94 mg/L (day 54), leading to fluctuations in anammox system performance, and the TIN removal rate dropped to 74.74%. The deterioration of the nitrogen removal effect was attributed to the high concentration of FA, which would cause certain negative effects on recovery performance in the SBBR–anammox system, which is consistent with a previous study by Chang et al. [40] (Figure 2c). The result demonstrated that a high concentration of FA would not be conducive to stability in the recovery stages of an anammox reaction [41]. Therefore, it is important to constantly adjust the anammox reactor operating environment during the recovery process, considering the high sensitivity of anammox bacteria to changing environments. This might require us to duly choose and optimize effective strategies ahead of schedule for anammox bacteria preservation, such as liquid paraffin, liquid nitrogen, refrigeration, freezing, lyophilization, gel encasement, and so on. It is worth noting that relatively proper storage methods could greatly accelerate the start-up and recovery stage of the anammox system. In stage IV, the NLR increased from 0.32 to 0.41 kg/(m^3·d), and the TIN removal rate was maintained at 83.44% on average. This result

illustrated that the SBBR–anammox system would have a great impact on resistance load with nitrogen load shock.

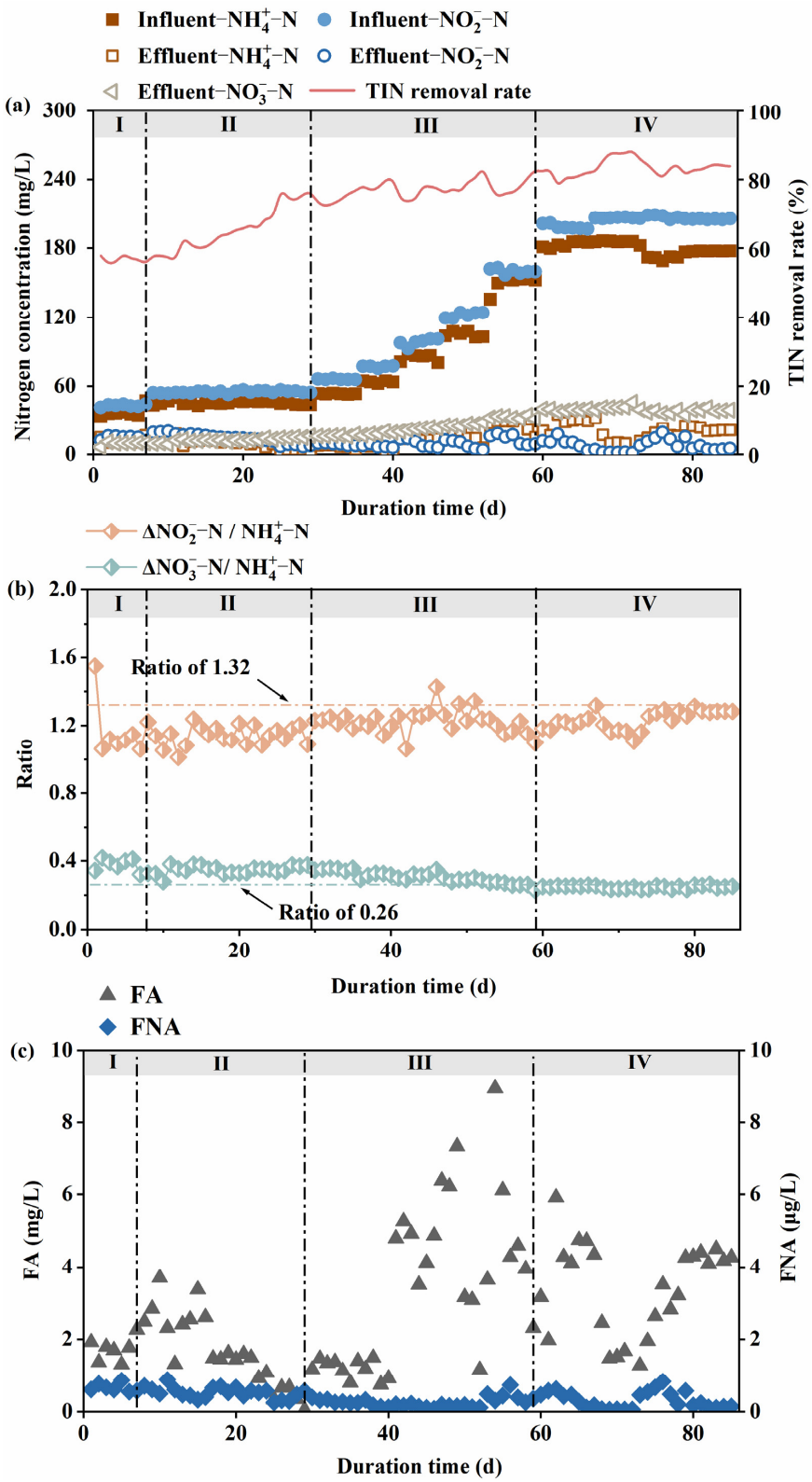

Figure 2. Recovery performance of the SBBR–anammox system at different operation stages. (**a**) Changes in the nitrogen concentration and TIN removal rate. (**b**) Ratio of ΔNO_2^--N/ΔNH_4^+-N, ΔNO_3^--N/Δ NH_4^+-N. (**c**) Concentration of FA and FNA.

A previous study suggested that the stoichiometry ratios of ΔNO_3^--N/ΔNH_4^+-N and ΔNO_2^--N/ΔNH_4^+-N are the key indicators for evaluating the nitrogen removal contribution rate of anammox bacteria during the operation of mixed anammox systems [23]. As shown in Figure 2b, the ratios of ΔNO_3^--N/ΔNH_4^+-N and ΔNO_2^--N/ΔNH_4^+-N were 0.38 and 1.16 during stage I on average, respectively, which deviated from the expected values of 0.26 and 1.32, respectively. The results suggested that the activity of anammox bacteria would decrease after prolonged storage, while AOB and NOB exhibited stronger activity and competed with anammox bacteria for substrates. Figure 2b shows that the ratio of ΔNO_3^--N/ΔNH_4^+-N fluctuated and gradually approached the theoretical value of 0.26 during stage III, which indicated that reactor performance in the SBBR–anammox system would be gradually recovered. In stage IV, the ratio of ΔNO_2^--N/ΔNH_4^+-N was 1.28 from day 74 to day 85 on average, which is close to the theoretical value of 1.32. The study of Du and Peng [3] showed that the ratio of ΔNO_2^--N/ΔNH_4^+-N was close to 1.32 only when the influent NH_4^+-N and NO_2^--N were bio-converted by the anammox process, indicating that anammox bacteria occupied a dominant position in the SBBR–anammox system. The stoichiometric ratio of ΔNO_3^--N/ΔNH_4^+-N was 0.25 on average during stage IV, less than 0.26, indicating the presence of denitrification during the anammox recovery process.

Overall, the SBBR system had carried out a TIN removal rate of 83.84% on day 85 with the NLR increasing to 0.41 kg/(m^3·d), indicating successful recovery in the SBBR–anammox system. This indicated that the substrate supply would have a significant effect on the activity of anammox bacteria. A sufficient substrate concentration could affect the metabolic process of anammox bacteria, improve the activity of bacteria, and thus increase the biomass of anammox biofilms.

3.2. Variation in Metabolic Characteristics

3.2.1. Changes of EPS Contents

A previous study reported that EPS would affect the physicochemical properties of anammox sludge, such as microbial structure, sedimentation performance, adsorption capacity, etc. [42]. The secretion of endogenous EPS could help anammox bacteria to resist adverse external environments and maintain cell activity [43] and promote cell aggregation to form granular sludge [44,45]. In this study, the concentration of TB-EPS increased from 181.16 during stage I to 404.91 mg/g VSS during stage IV (Figure 3a), with an increase in the NLR. The previous study demonstrated that a high proportion of TB-EPS would improve the structural stability of sludge, which was further conducive to the improvement of nitrogen removal performance [46]. Moreover, EPS has a tendency to mediate the dense formation of anammox biofilms, further promoting the enrichment of anammox bacteria and providing corresponding substances and required enzymes [47,48]. A previous study found that the performance recovery of the anammox system would be further enhanced, which was conducive to the improvement of EPS content with the increase in the NLR [49].

As shown in Figure 3a, the values of PN/PS increased from 3.86 during stage I to 5.38 during stage IV in the TB-EPS, which could assess the stability of biofilm in the anammox system. Additionally, the PN/PS value during stage III was 5.04, which was close to 5.38 during stage IV in the TB-EPS. This illustrated that the PN/PS value would tend to be at a stable level with the improvement in recovery ability, which was beneficial to the resistance of adverse external environments for anammox bacteria. This result demonstrated that the higher PN/PS value was beneficial to improve the hydrophobicity of the anammox biofilms, thus enhancing stability in the anammox system [50]. The study of Zhang et al. evaluated the feasibility of exogenous EPS as a protective agent for the preservation of anammox sludge. The results concluded that the PN content of EPS was a key factor in optimizing the preservation, which led to the 90.6% NRE of the anammox system after 90 days of preservation [51].

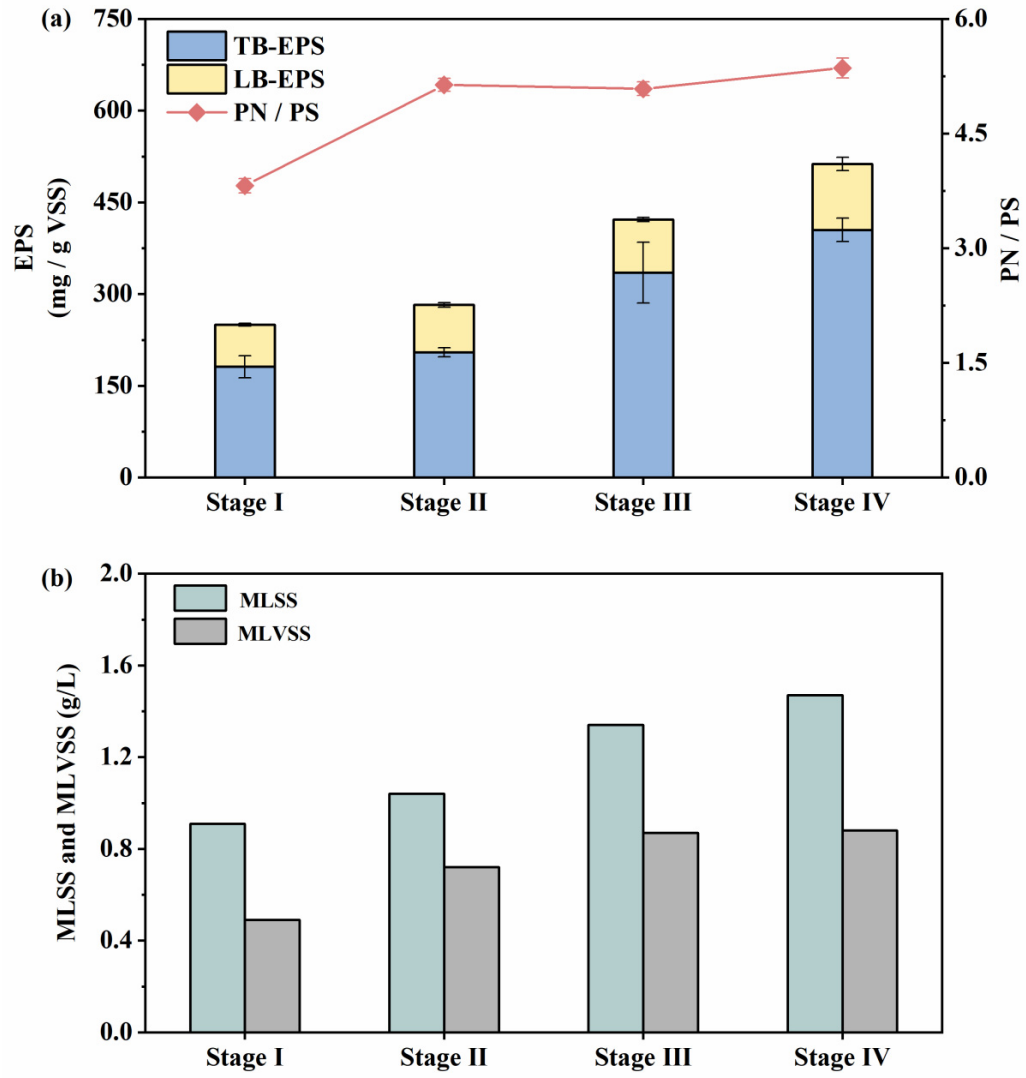

Figure 3. Variations in biofilm characteristics. (**a**) Variations in EPS production. (**b**) MLSS and MLVSS.

The MLSS and MLVSS concentrations in different recovery stages are shown in Figure 3b.

3.2.2. Metabolic Enzyme Activity

The enzyme activity indicated the working state of the anammox reaction system to some extent. Figure 4 shows the activity of the HZO enzyme and the ETS of the anammox bacteria. Actually, HZO is a crucial enzyme in the conversion of hydrazine to nitrogen during the anammox reaction, which is only found in anammoxosome inside anammox bacteria [52]. The HZO activity of the biofilm increased from 7.11 during stage I of the experiment to 26.78 μmol Cyt-c/(g VSS·s) during stage IV. It could be seen that loading a certain number of microbial carriers in an anammox system could improve the enrichment of microorganisms and also the expression level of nitrogen metabolism, which played a crucial role in the rapid recovery and maintenance of good stability for the entire sludge system.

The activity of electron transport systems (ETSAs) is considered to be a concrete indicator of microbial respiration [37] and could also be used as one of the indexes to evaluate nitrogen removal performance in the anammox system. Previous studies showed that the anammox process had a complete electron transport chain [53]. In anammox bacteria, NO_2^- absorbed $4e^-$ and was reduced to hydroxylamine (NH_2OH). NH_2OH reacted with NH_4^+ to form hydrazine (N_2H_4), which was oxidized to N_2 by HZO. At the

same time, $4H^+$ and $4e^-$ were released. In the final step, the $4e^-$ released by the reaction passed down the electron transport chain, and a new round of anammox began. The ETSA of the biofilm increased from 52.94 μg/(min·g MLSS) during stage I of the experiment to 155.97 μg/(min·g MLSS) during stage IV, increasing by 103.02 μg/(min·g MLSS), indicating that the activity of the anammox bacteria was gradually recovered. Overall, the key enzyme activities increased gradually with the recovery process of the SBBR–anammox system in this study.

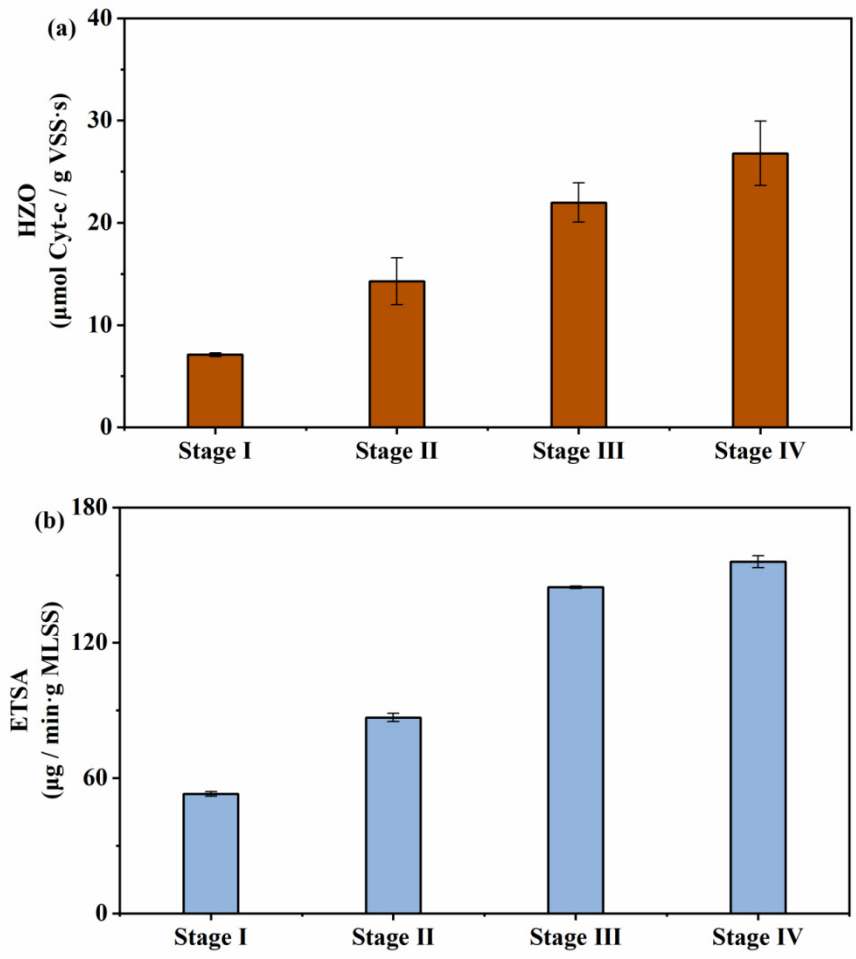

Figure 4. Variation in metabolic enzyme activities: (**a**) HZO and (**b**) ETSA.

In fact, the connection between the nitrogen removal performance, EPS contents, key enzyme activities, and microbial structure is still not sufficiently understood. Further understanding of these interactions would promote the practical application of the SBBR–anammox process. In addition, the expression level of genes related to nitrogen metabolism should be studied in-depth.

3.3. Microbial Community Analysis

3.3.1. Microbial Community Dynamics

In this study, 16S rRNA gene-targeted amplicon sequencing was used to analyze the succession dynamics of microorganisms at different recovery stages to determine the relationships among key bacterial communities. The main bacteria at the phylum level are Proteobacteria, Planctomycetes, and Chloroflexi (Figure 5a). Anammox bacteria belonged to Planctomycetes and metabolized very slowly, and their relative abundance in the biofilm increased from 4.36% during stage I to 22.25% during stage IV. Planctomycetes is a typical phylum representing anammox microorganisms [54]. It has demonstrated that Planctomycetes could be used for the initial breakdown of various highly complex

polysaccharides, thus making them available to other microbiomes and then playing a key role in the biogeochemical transformation of the carbon and nitrogen cycles [55]. The increasing abundance was associated with the improvement performance of nitrogen removal during the recovery stage. A previous study also reported that Planctomycetes possess special characteristics, such as a dynamic intimal system, a peptidoglycan cell wall (Gram-negative), a separator structure, the presence of C1 transferase, ladane lipids, and the ability to oxidize ammonia anaerobically [56]. This indicated that anammox bacteria have diverse and complex sludge characteristics. Moreover, Chen et al. [57] found that Planctomycetes-affiliated differentially expressed proteins showed a wide range of functional potential and activities by using proteomic techniques, especially the presence of a large number of metal ion binding proteins in the Planctomycetes. The existence of metal ion binding proteins might trap metal ions in the anammox system, which could trigger biofilm formation or cell aggregation. This indicated that anammox bacteria harbored strong adhesion and could easily aggregate on the carrier to form biofilm. This laterally confirmed that the increase in the relative abundance of Planctomycetes would enhance the density of anammox biofilm, thus improving the mechanical strength of anammox sludge, which was consistent with the results of this study. In summary, it was the main feature of performance recovery of the anammox system that Planctomycetes at the phylum level gradually became the dominant flora during the recovery stages [58] (Figure 5a). Additionally, Chloroflexi is a generally used metabolite among microorganisms to grow [59,60]. The relative abundance of Chloroflexi in biofilm increased from 6.91% during stage II to 7.04% during stage IV. The results suggested that the metabolic flexibility and adaptability of microorganisms were enhanced, and the substrates provided for Chloroflexi had increased with gradual recovery of the anammox system.

As shown in Figure 5b, *Cadidatus Brocadia* was the dominant anammox bacteria, especially during stages II–IV. The result was consistent with the study of Zheng et al. which demonstrated that *Cadidatus Brocadia* preferred to live in biofilms [61]. The relative abundance of *Cadidatus Brocadia* in biofilms was 19.47% during stage IV. Previous studies have reported that the half-saturation constant (Ks) for NH_4^+-N of *Cadidatus Brocadia* was more than that of *Cadidatus Kuenenia* [62], suggesting that *Cadidatus Kuenenia* could not adapt to the environment as well, with high NH_4^+-N load. The relative abundance of AOB decreased from 0.35% during stage I to 0.07% during stage IV. This suggested that the competitiveness of AOB decreased and the contribution rate of anammox bacteria to nitrogen removal had improved with a gradual recovery in the SBBR–anammox system. Moreover, the recovery stages of the anammox system have other complex competition mechanisms among microbial communities, which was also one of the challenges for the stability of the anammox system, including the competition of nitrites between NOB and anammox bacteria [63]. The relative abundance of NOB dropped from 0.18% during stage I to 0.03% during stage IV. To sum up, the SBBR–anammox system provided conditions for the effective enrichment of anammox bacteria and facilitated rapid recovery during the anammox process. However, the synergistic communication and cross-feeding between anammox bacteria and symbiotic bacteria still need to be further discussed. Relatively speaking, the existence of biofilms is helpful to enhance the recovery ability of an anammox system, but the systematic explanation of metabolic response is still lacking. Further investigation is needed to understand how these exogenous agents interact with the microbial community and contribute to the recovery process. Additionally, the addition of exogenous agents might also accelerate the recovery of the anammox system, such as hydrazine, acetate, folate, signaling molecules, etc., and the related metabolic details contained in them still need to be further clarified.

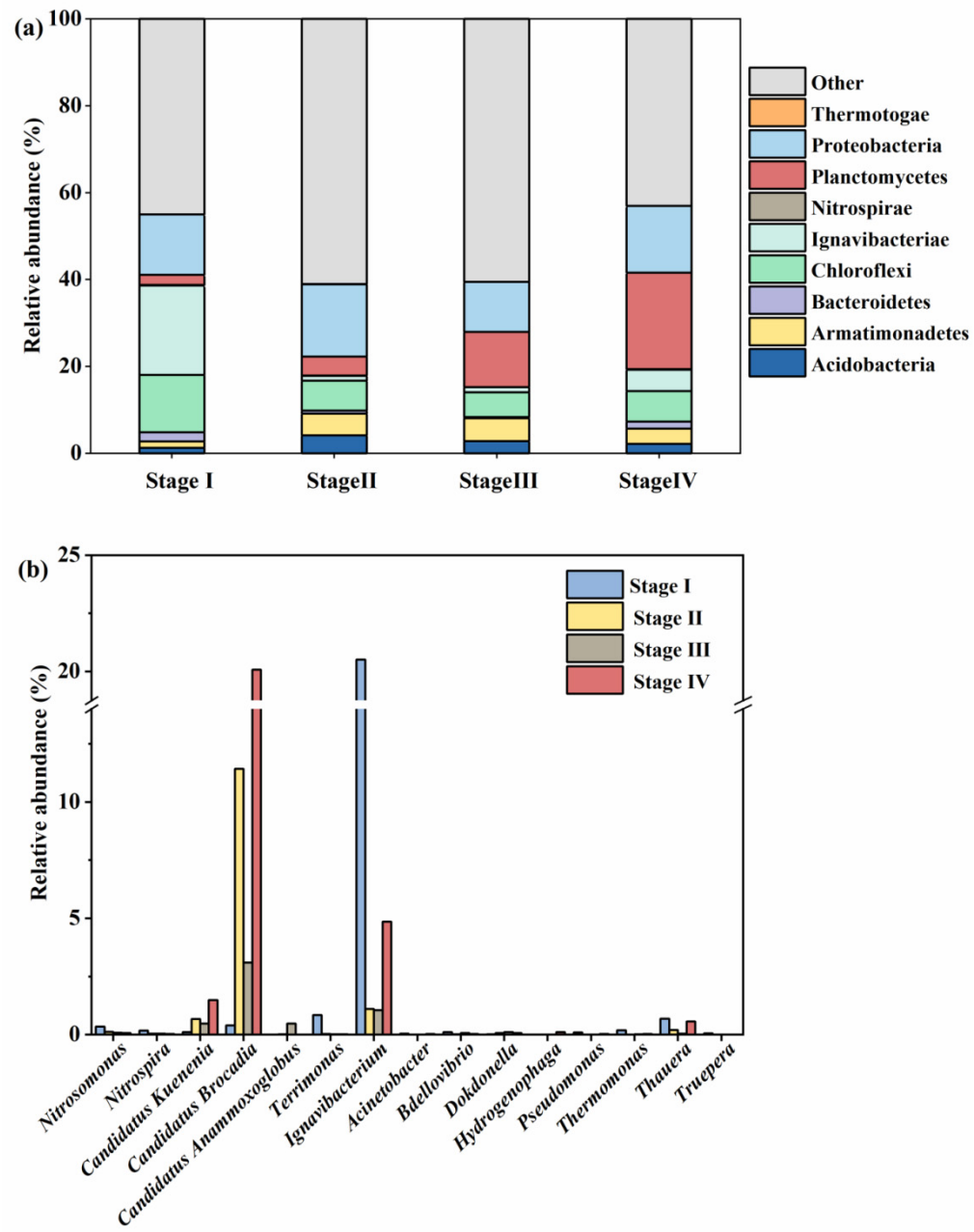

Figure 5. Microbial community composition. (**a**) The relative abundance of different recovery stages at the phylum level. (**b**) The relative abundance of different recovery stages at the genus level.

3.3.2. Microbial Community Analysis

A total of 173,907 valid sequences were acquired from four biofilms samples at different recovery stages in the SBBR–anammox system (Table 2). It is well known that the Shannon and Simpson indices can express microbial community diversity in the anammox system [64]. The low Simpson index and high Shannon index indicate low microbial diversity and high microbial richness, respectively. The Simpson index decreased from 0.17 during stage I to 0.08 during stage IV. Conversely, the Shannon index increased from 3.09 during stage I to 3.60 during stage IV. This result implied that the microbial community diversity gradually dropped with the gradual recovery of the SBBR–anammox system, i.e., the anammox bacteria gradually took the dominant position, and other bacteria had been eliminated.

Table 2. Diversity index and OTU richness of the biofilm at different recovery stages.

Samples	Reads	OTU (97%)	Shannon Index	Simpson Index
Stage I	40,582.0	425.0	3.09	0.17
Stage II	48,778.0	487.0	3.45	0.11
Stage III	63,248.0	508.0	3.44	0.12
Stage IV	21,299.0	414.0	3.60	0.08

Principal component analysis (PCA) was used for analyzing the similarities in microbial community diversity in four biofilm samples at different recovery stages. Figure 6 shows that the closer the distance between the different stages, the smaller the differences in community composition between the different stages. The results of the PCA showed that the dataset variance was explained by PC1 and PC2. In addition, the contribution rates of PC1 and PC2 were 43.8% and 33.6%, respectively (Figure 6), which showed that the SBBR–anammox system at stage I and stage IV had a similar community composition. In addition, data analysis also played an important role during the recovery stage of the anammox process, and the corresponding mathematical model could be established to continuously optimize the operation parameters of the anammox system. It was found that the corresponding rules among microbial communities could be further explored, which contributed to achieving cost-saving and energy-saving treatment technology in the anammox process.

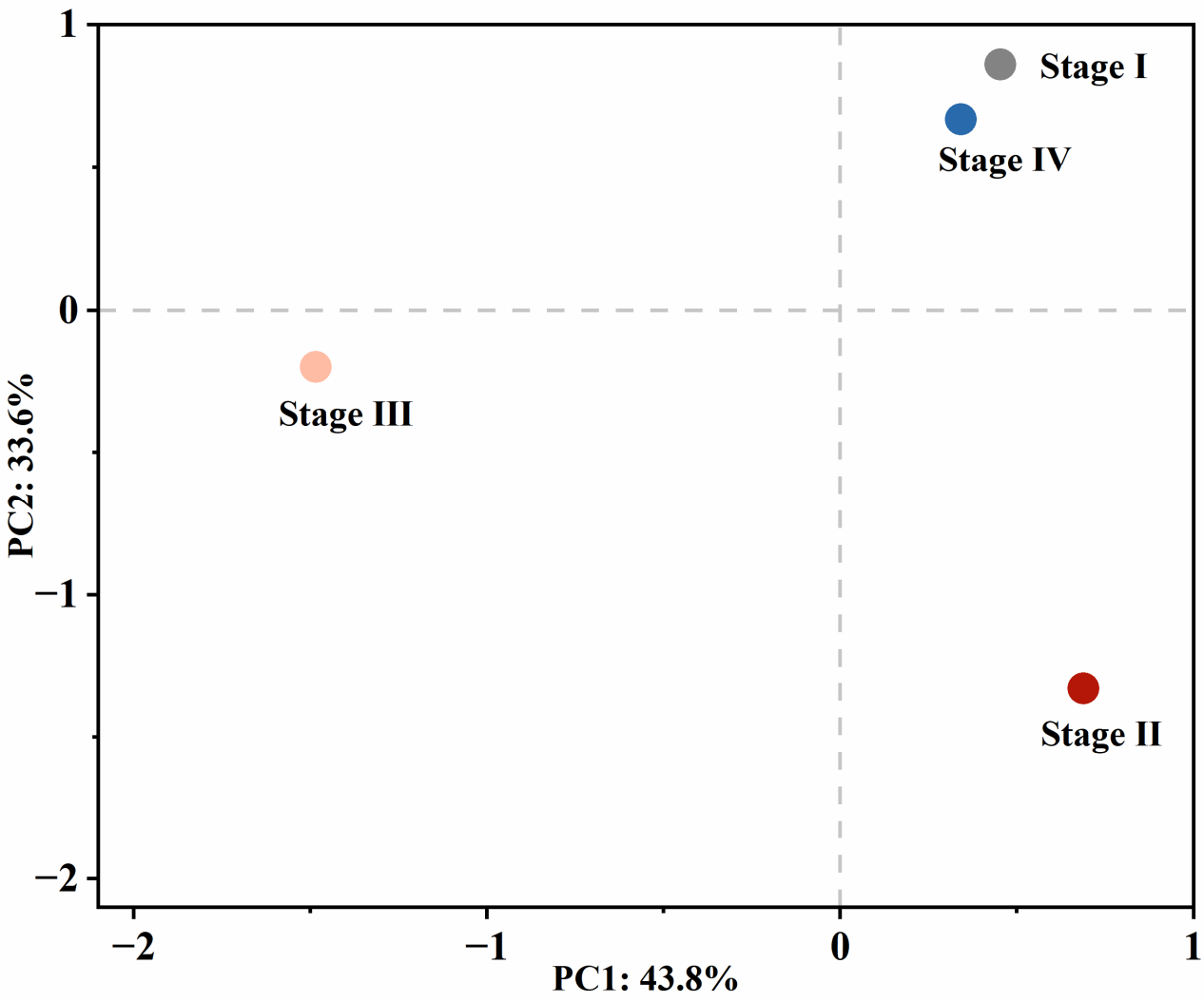

Figure 6. PCA of the SBBR–anammox system at different recovery stages.

4. Conclusions

In this study, an SBBR–anammox system was recovered within 85 days after long-term storage with an NRR of 0.34 kg/(m^3·d). The concentration of TB-EPS increased from 181.16 to 404.91 mg/g VSS, and the HZO activity of the biofilm increased from 7.11 to 26.78 μmol Cyt-c/(g VSS·s) with the increase in the NLR. The results showed that the EPS contents and key enzyme activities increased gradually with the recovery process of the SBBR–anammox system. The anammox bacteria became dominant during the recovery process, indicating the successful recovery of the system. This study has provided a strategy for the rapid recovery of anammox system performance after long-term storage and insights into the variation of sludge characteristics and microbial structure and mechanisms during anammox performance recovery. Nevertheless, it is necessary to further achieve the large-scale application of the anammox process in sewage treatment plants; the detailed restoration mechanism of the anammox system still needs to be carefully studied.

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