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Effects of Ca^{2+} Concentration on Anaerobic Ammonium Oxidation Reactor Microbial Community Structure

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Abstract: The anaerobic ammonium oxidation (anammox) reaction removes nitrogen from wastewater, the performance of which is influenced by Ca^{2+} ; however, the effect of Ca^{2+} on microbial community structure is unclear. Therefore, the effects of Ca^{2+} concentration on the treatment performance of an anammox reactor and microbial community structure of anammox sludge were investigated. Ca^{2+} concentration minimally influenced the removal efficiency of NO_2^- -N and NH_4^+ -N, but substantially influenced total N removal. Changing the Ca^{2+} concentration (between 25 and 125 mg/L) caused the average removal rate of total nitrogen to fluctuate by 3.3 percentage points. There were five major bacterial phyla in the anammox sludge: Proteobacteria, Chloroflexi, Acidobacteria, Planctomycete, and Chlorobi. Microbiological analysis revealed that the genera *Acidobacterium*, *Anaerolinea*, and *Denitratisoma* were positively correlated with Ca^{2+} concentration, and improved treatment performance of the anammox reactor. Moreover, uncultured *Chlorobi* bacterium clone RUGL1-218 (GQ421108.1) and uncultured *sludge bacterium* A21b (KT182572.1) may be key microorganisms for the immobilization of anammox bacteria. These findings offer a theoretical basis for improved wastewater treatment using the anammox process.

Keywords: anammox; calcium concentration; microbial community structure

1. Introduction

Anaerobic ammonium oxidation (anammox) bacteria can convert NO_2^- -N and NH_4^+ -N into N_2 using NO_2^- -N as the electron acceptor and NH_4^+ -N as the electron donor under anaerobic conditions [1]. Traditionally, biological nitrogen removal was performed mainly using the nitrification–denitrification process, where nitrification involves the conversion of NH_4^+ -N into NO_2^- -N or NO_3^- -N by nitrifying bacteria under aerobic conditions [2], and denitrification is the process by which denitrifying bacteria convert NO_2^- -N or NO_3^- -N into N_2 gas [3]. In comparison, the anammox process realizes the efficient use of the internal carbon source of wastewater with high NH_4^+ -N concentrations, and effectively solves some of the shortcomings of the traditional biological nitrogen removal technology [4]. Overall, the anammox process has the advantages of low residual sludge production, low energy consumption, and no need for additional carbon sources [5]. The anammox *Planctomycetes* has a unique metabolism and generates energy via oxidation of ammonia without oxygen (i.e., the “anammox” process, that is, anaerobic ammonia oxidation). This process is important for global nitrogen cycling, is used for environmental cleanup of nitrogen in wastewater [6,7], and may in future considerably reduce the energy costs of sewage cleanup [8].

Calcium and other polyvalent cations are important for bioflocculation and granulation, because they can form bridges between bacteria and bioflocs that are negatively charged due to the formation of extracellular polymeric substances [9]. Calcium indirectly maintains the biomass of the bacteria by immobilizing the cells. Cell immobilization technologies are also the basis of process engineering applications [10,11]. For instance, de Graaff et al. [12] studied the autotrophic removal of N from black water, and showed that the addition of extra calcium was necessary to sustain the anammox biomass in the reactor. In addition, Jiang et al. [13] investigated specific cultured microbial particles in sludge bed reactors, and noted that divalent metal cations such as Ca^{2+} had an important role in microbial biomass immobilization. In particular, an increase in Ca^{2+} concentration from 80 to 150 mg/L promoted anammox granule formation during start-up. Liu et al. [14] studied the effect of Ca^{2+} on the recovery of damaged anammox bacteria, and showed that a certain concentration of Ca^{2+} could increase the strength and density of anammox granules, ensuring good sedimentation and high biomass. Moreover, Ca^{2+} precipitated on the particles reduced the effective mass transfer of the sludge [14]. These and other studies underline the importance of understanding cell immobilization in anammox processes, including granulation and biofilm, to improve the treatment performance of the reactor without changing the original microbial community structure [15–21].

To this end, a relatively large body of research has investigated the promotion of the anammox process and nitrogen removal by Ca^{2+} . However, Hyun et al. [22] studied the effects of Ca^{2+} on biological nitrogen removal in a reverse osmosis concentrate and adsorption treatment, according to the specific denitrification rate, and observed an interesting trend. Below 500 mg/L, Ca^{2+} had a beneficial effect on denitrification, and the transport of anion NO_3^- -N and a carbon source by denitrifying microorganisms was facilitated by injection of Ca^{2+} . In contrast, at Ca^{2+} concentrations above 500 mg/L, denitrification was suppressed, and the efficiency was lowered; this was caused by a large amount of precipitate formed by Ca^{2+} , which adsorbed onto the surface of microorganisms, reducing microbial activity. Although the application of immobilized cells has been studied for 20 years, its application to the anammox process has focused on the removal performance of the reactor. By contrast, no studies have investigated the changes in microbial community structure involved in the anammox reaction during the immobilization process.

Therefore, this study investigated the effect of Ca^{2+} concentration on the nitrogen removal performance of an anammox reactor to determine the optimal concentration of Ca^{2+} , specifically in the removal of TN, NH_4^+ -N, and NO_2^- -N. In addition, combined with molecular biology methods, the microbial community structure changes and dominant species of anammox sludge under different Ca^{2+} concentrations were analyzed. These findings provide a theoretical basis for engineering applications of the anammox process.

2. Materials and Methods

2.1. Experimental Setup

A schematic diagram of the experimental reactor used in this study is shown in Figure 1. Sequencing batch reactors made of plexiglass were used in this study. The whole device was wrapped with black cloth to occlude light, which can have a negative impact on bacteria [23,24]. The reactor was filled with the seed anammox sludge with the 1–3 mm (in length) granular activated carbon carriers, which were cultured in a 50 L reactor for 6 years at Guilin University of Technology, China, and showed high biological activity [25–29]. The working volume was 300 mL, and the experimental wastewater enters the reactor through a peristaltic pump. The effluent was drained from the upper area after passing through the sludge layer. The experiment was conducted at 32 ± 1 °C, controlled using a water bath. The influent pH was adjusted at 7.4–7.6 by adding 0.5 mol/L H_2SO_4 [30]. The dissolved oxygen (DO) concentrations of the feeding synthetic wastewater were kept below 0.5 mg/L using N_2 gas purging for approximately 1 h [31].

Zhang et al. [25] showed that 35 days was sufficient to successfully start-up and restore the activity of stored anaerobic ammonium oxide sludge. Therefore, six reactors were operated simultaneously, with a running time of 35 days; and analyzed the denitrification performance. Different Ca^{2+} concentrations have an effect on anammox bacteria, and the effect of Ca^{2+} concentration on anammox process is manifested by the denitrification during the reaction.

Figure 1. Schematic diagram showing the experimental anaerobic ammonium oxidation (anammox) reactor.

2.2. Artificial Wastewater

Artificial wastewater was used as the experimental influent. The contents of the wastewater are listed in Tables 1 and 2. Of note, $\text{NH}_4^+\text{-N}$ was supplied as NH_4HCO_3 at a concentration of 20 mg/L, and $\text{NO}_2^-\text{-N}$ was supplied as NaNO_2 at a concentration of 30 mg/L, which were provided at a molar ratio of $\text{NH}_4^+\text{-N}$ to $\text{NO}_2^-\text{-N}$ of 1:1.5. KH_2PO_4 was added as the phosphorus source, and the concentration of phosphorus is about 7 mg/L. CaCl_2 was added to final influent Ca^{2+} concentrations of 25, 50, 75, 100, and 125 mg/L. The synthetic wastewater was purged with N_2 and a small amount of anhydrous sodium sulfite was added to control the dissolved oxygen (DO) content below 0.5 mg/L.

Table 1. Components of the influent.

Component	Concentration
NH_4HCO_3	0.11 g/L
NaNO_2	0.12 g/L
KH_2PO_4	0.03 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g/L
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.09–0.46 * g/L
$\text{Na}_2\text{S}_2\text{O}_3$	0.04 * g/L
EDTA	0.25 * mL/L
$\text{Na}_2\text{S}_2\text{O}_3$	0.01 mL/L
Trace elements	0.5 mL/L

* $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added at prescribed experimental concentrations to yield final Ca^{2+} concentrations of 0.025–0.125 g/L; EDTA, 5 mg/L; $\text{Na}_2\text{S}_2\text{O}_3$, 24.81 mg/L.

Table 2. Trace elements in the reactor influent.

Component	Concentration (mg/L)
FeSO ₄ ·7H ₂ O	10,000
C ₁₀ H ₁₄ N ₂ Na ₂ O ₃	5600
MnCl ₂ ·4H ₂ O	352
CoCl ₂ ·6H ₂ O	96
NiCl ₂ ·6H ₂ O	80
CuSO ₄ ·5H ₂ O	100
ZnSO ₄ ·7H ₂ O	172
NaMoO ₄ ·2H ₂ O	110

2.3. Analytical Methods

2.3.1. Wastewater Samples

After the reactor was started, wastewater samples were collected daily. NH₄⁺-N and NO₂⁻-N were measured according to the Standard Methods protocol (APHA, 1995) [32]. Total nitrogen (TN) was determined using the persulfate method (APHA, 1995) [32]. NO₃⁻-N was calculated as the difference between TN and the sum of NH₄⁺-N and NO₂⁻-N. The Ca²⁺ concentration was determined using EDTA titration (ISO 6058-1984). pH was measured using a pH meter (9010; Jenco Instruments, San Diego, CA, USA), and DO was measured using a DO meter (6010; Jenco Instruments, San Diego, CA, USA).

2.3.2. Microbial Analysis

Sludge samples were collected from the reactors containing different Ca²⁺ concentrations. After dewatering, the sludge samples were sealed in a centrifuge tube and stored in a refrigerator at −20 °C. The PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) was used to extract DNA from the sludge samples. After verifying the purity (OD₂₆₀/OD₂₈₀: 1.6–1.8), the DNA was amplified by polymerase chain reaction (PCR). Using PCR, amplification of the target fragment was performed simultaneously with cleavage of the target DNA fragment. The primers used in this experiment were 968F and 1401R, and the amplified DNA fragment was the V6 region of 16S rDNA of bacteria. The sequence length was about 430 bp.

The target DNA fragment was isolated and purified via denaturing gradient gel electrophoresis (DGGE), where the DNA fragments of different microorganisms were fixed at different positions of the gel. After each step of gelation, individual bands were recovered and re-dissolved for purification of the target DNA fragment. The DGGE results were cloned and using high-throughput molecular approaches sequenced by Bioengineering Biotechnology (Shanghai) Co., Ltd. The sequences were identified with DNASTAR® software and the vector sequences at both ends were removed. The sequenced genes were compared with the National Center for Biotechnology Information (NCBI) GenBank database using BLAST analysis, and DGGE images were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.3.3. Data Analysis

Data analysis was carried out using Origin 2017 software (Origin Lab, Northampton, MA, USA). To explore the correlation between microbial diversity and environmental factors, detrended correlation analysis was performed on the species, after which redundancy analysis (RDA) was performed.

3. Results

3.1. Effect of Ca^{2+} Concentration on Nitrogen Removal

The results of nitrogen removal after 35 days of operation at different Ca^{2+} concentrations are shown in Figure 2. Wastewater distribution and wastewater exchange were carried out in a cycle of 3 days. For each wastewater exchange, wastewater samples were collected. The indicators NH_4^+-N , NO_2^--N , and TN were measured, and their removal rates were analyzed.

Figure 2. Removal of (a) NH_4^+-N , (b) NO_2^--N , and (c) TN from the anammox reactor after 35 days of operation at different Ca^{2+} concentrations.

The Ca^{2+} concentration had little effect on NH_4^+-N and NO_2^--N removal (Figure 2). Nevertheless, Ca^{2+} concentration had a substantial influence on TN removal. For both NH_4^+-N and NO_2^--N , the removal rates were maintained around 95% with increasing Ca^{2+} concentration. In the reactor containing a Ca^{2+} concentration of 100 mg/L, the NH_4^+-N removal rate fluctuated greatly (as shown by the length of the error bars in Figure 2), indicating that the presence of Ca^{2+} altered the community structure of microbes involved in its reaction. However, once the microorganisms adapted to the environment, the removal rate was basically maintained at 95%. According to the length of the error bars in Figure 2, the removal rate fluctuated little in the reactor containing a Ca^{2+} concentration of 125 mg/L; however, the microbial activity decreased when Ca^{2+} concentration changed, indicating that high concentrations of Ca^{2+} affected the environment required to maintain sludge particle structure and microbial activity.

The overall nitrogen removal rate fluctuated continuously (Figure 2), indicating that the microbial community structure associated with nitrogen removal changed constantly over time, adapting to the concentrations of Ca^{2+} in the reactor. Optimal Ca^{2+} concentrations can increase the strength and density of anammox sludge granules, ensuring good sedimentation and high biomass retention [14]. However, excessive Ca^{2+} will form precipitates and reduce the effective mass transfer of sludge. This could explain the different degrees of fluctuation in nitrogen removal at different Ca^{2+} concentrations. For instance, at a Ca^{2+} concentration of 100 mg/L, the removal rate fluctuated minimally, ultimately

reaching 95%, indicating that the microorganisms at this concentration were better adapted to the environment, resulting in minimal changes to the community structure.

The removal of TN was affected by $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$, and overall first declined and then increased. Changing the Ca^{2+} concentration (between 25 and 125 mg/L) caused the average removal rate of TN to fluctuate by 3.3 percentage points. The main inhibiting factor was precipitate formed by Ca^{2+} , which could adhere to the surface of the microorganisms involved in the reaction [33], hindering the availability of matrix nutrients to anammox bacteria, resulting in their death and thereby reducing the removal rate. Subsequently, microbial activity was restored, and the removal rate increased after the anammox bacteria lost their activity; therefore, $\text{NO}_3^-\text{-N}$ accumulated as a product of the anammox reaction around the bacteria. In areas where $\text{NO}_3^-\text{-N}$ concentrated, some denitrifying bacteria could grow, consuming $\text{NO}_3^-\text{-N}$ as a substrate, whereas the inactivated anammox bacteria provided nutrients; therefore, denitrification could occur. Therefore, the TN removal rate showed an increasing trend in the later stage of the reaction.

3.2. DGGE

Figure 3 shows the DGGE electropherogram, which is capable of separating organisms at the genus level, where the bright and dark bands represent the dominant and inferior microbial taxa, respectively. From the changes in bright and dark bands among the reaction conditions, dominant and inferior taxa varied by Ca^{2+} concentration. Moreover, some bright bands were only observed at a single Ca^{2+} concentration. For example, band 1 was bright at all Ca^{2+} concentrations, indicating that it was a dominant species. Band 3 was also present at all Ca^{2+} concentrations, but exhibited a lower brightness, indicating that it was an inferior taxon. Band 9 was only bright (i.e., dominant) in the initial sludge, and showed low brightness at all Ca^{2+} concentrations. Compared to the microbial population of the initial sludge, addition of, and changes in, Ca^{2+} concentration resulted in substantial changes in microbial population structure.

Figure 3. Denaturing gradient gel electrophoresis (DGGE) gel imaging. From right to left, sample 1, initial sludge; Sample 2, (Ca^{2+}) = 25 mg/L; Sample 3, (Ca^{2+}) = 50 mg/L; Sample 4, (Ca^{2+}) = 75 mg/L; Sample 5, (Ca^{2+}) = 80 mg/L; Sample 6, (Ca^{2+}) = 100 mg/L; and Sample 7, (Ca^{2+}) = 125 mg/L.

3.3. Microbial Community Structure Analysis

Microbial diversity was analyzed using the Shannon–Weaver index and the results of the seven sludge samples are presented in Table 3. Although there were slight differences in the microbial

diversity index values among the seven samples, all were greater than 2, indicative of relatively high microbial population richness, and relatively uniformity of microorganisms among the seven samples.

Table 3. Microbial diversity index of sludge samples from the seven reactors.

Calcium Ion Concentration (mg/L)	Diversity Index
Initial sludge	2.327
25	2.443
50	2.231
75	2.345
80	2.131
100	2.350
125	2.367

The relative proportions of the dominant taxa in the seven samples are shown in Figure 4. The microbial community structures differed substantially between the initial sludge and sludge containing added Ca^{2+} , as well as among the samples containing different Ca^{2+} concentrations.

Figure 4. Relative proportions of the dominant microbial populations from the denaturing gradient gel electrophoresis bands in sludge samples from anammox reactors containing different Ca^{2+} concentrations. The numbers in the legends refer to the band numbers in Figure 3. Sample 1, initial sludge; Sample 2, (Ca^{2+}) = 25 mg/L; Sample 3, (Ca^{2+}) = 50 mg/L; Sample 4, (Ca^{2+}) = 75 mg/L; Sample 5, (Ca^{2+}) = 80 mg/L; Sample 6, (Ca^{2+}) = 100 mg/L; Sample 7, (Ca^{2+}) = 125 mg/L.

The relative concentrations of the different taxa (based on the DGGE separation) in each sample and the relative concentrations of the same taxa in different samples can be divided into several cases, as follows. First, bands 2, 6, 7, 8 and 10 showed similar degrees of dominance in all seven samples. Second, bands 1, 11 and 13 were dominant taxa in all seven samples, albeit present in varying proportions, with the maximum proportions of bands 1, 11, and 13 observed in samples 3, 5, and 5, respectively. Third, bands 5 and 9 were present in all seven samples, but were dominant taxa in samples 1 and 2; the band 5 taxon was dominant in samples 2 and 6, whereas the band 9 taxon was dominant in sample 1. Fourth, the band 3, 4 and 12 taxa were dominant in samples 1, 2, 4, 5, 6 and 7, but were not observed in sample 3. Overall, these results indicated that Ca^{2+} concentration influenced the microbial community structures of the anammox reactors.

The 13 bands in the DGGE results were cloned and the BLAST (Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov>) results with a similarity of 92%–100% are shown in Table 4. The microbial composition and dominant species in the activated sludge were analyzed by DGGE. The number and brightness of the bands were statistically different during the periods with high and low Ca^{2+} concentrations, indicating that the addition of Ca^{2+} had a significant effect on the microbial community structure. In total, 13 main bands were collected and sequenced. The resulting sequences in the anaerobic anammox sludge mainly comprised five bacterial phyla: Proteobacteria (46%), Chloroflexi, Acidobacteria, Planctomycete, Chlorobi, as well as some uninformable untrained microorganisms (UUMs). UUMs are complex and may play a variety of roles in the anammox system. No Firmicutes or Bacteroides were detected in any samples.

Table 4. Homology search results for 16S rRNA gene sequences of the main bacterial members in the anammox sludge community.

Band No.	Strains	Gene Band No.	Similarity	Phylum
1	<i>Ignavibacterium</i> sp.	JQ724348.1	98%	Chlorobi
2	Uncultured <i>Acidobacteria</i> bacterium clone 3F2	KC442541.1	97%	Acidobacteria
3	Uncultured <i>Chlorobi</i> bacterium clone RUGL1-218	GQ421108.1	99%	Chlorobi
4	<i>Denitratisoma oestradiolicum</i> clone 20b_15	KF810114.1	99%	Proteobacteria
5	Uncultured <i>Chloroflexi</i> bacterium clone MA-R101	JN038662.1	98%	Chloroflexi
6	Uncultured <i>Chlorobi</i> bacterium	CU918838.1	92%	Chlorobi
7	Uncultured <i>planctomycete</i> clone 5GA_Pla_HKP_08	GQ356155.1	100%	Planctomycete
8	Uncultured <i>Anaerolinea</i> sp.	EF636836.1	94%	Chloroflexi
9	Uncultured <i>Denitratisoma</i> sp. clone as185	KF287743.1	98%	Proteobacteria
10	<i>Denitratisoma oestradiolicum</i> clone 20b_2	KF810120.1	99%	Proteobacteria
11	Uncultured <i>beta</i> proteobacterium clone B-AB39	AY622250.1	99%	Proteobacteria
12	Uncultured sludge bacterium A21b clone MBR Ca 300ppm 19	KT182572.1	99%	Proteobacteria
13	Uncultured <i>gamma</i> proteobacterium clone 428	AB252885.1	98%	Proteobacteria

The Proteobacteria in the reactor could be divided into two classes: β -Proteobacteria and γ -Proteobacteria. In some DGGE bands, the bacteria could be identified to the genus and species levels; however, some of these could not be matched with known bacteria, indicating that there were undescribed strains present in the anaerobic anammox reactors. It is generally considered that a sequence homology of less than 97% is representative of different populations [34]. Analysis of the five major bacterial phyla were identified in the anaerobic anammox sludge. The microbes in band 1 belonged to the genus *Ignavibacterium* (Chlorobi). The microbes in band 2 belonged to *Acidobacterium* (Acidobacteria). The microbes in bands 4, 9, and 10 belonged to *Denitratisoma* (β -Proteobacteria); those in band 7 belonged to *Planctomycete* (Planctomycetes). Microbiologists have found Planctomycetes in the environment via culturing using media containing antibiotics, since Planctomycetes are inherently resistant to penicillin, or via clone libraries generated from DNA followed by PCR and sequencing of 16S rRNA genes. The microbes in band 8 belonged to *Anaerolinea* (Chloroflexi). Finally, the microbes in band 13 belonged to *Steroidobacter* (γ -Proteobacteria). The activities of these bacteria in sludge reactors are described in the Discussion below (Section 4).

4. Discussion

Redundancy analysis (RDA) is a sorting method developed based on correspondence analysis (CA). Correspondence analysis and multivariate regression analysis are combined, and regression is carried out with environmental factors in each step, also known as multivariate direct gradient analysis. Detrended correspondence analysis of the species data showed that the maximum gradient length in the four axes was 0.610; because this value was less than 3, linear RDA was performed. According to the experimental conditions, we selected Ca^{2+} concentration (E1), TN (E2), NH_4^+ -N (E3), and NO_2^- -N (E4) as the environmental factors. In addition, we used TN removal rate (X1), NH_4^+ -N removal rate

(X2) and NO_2^- -N removal rate (X3) to analyze the denitrification efficiency. The RDA results are shown in Figure 5.

Figure 5. Correlations between microbial community structure of the anammox sludge with (A) environmental factors and (B) nitrogen removal rate and between microbial community structure based on the denaturing gradient gel electrophoresis bands with (C) environmental factors and (D) nitrogen removal rate. The black numbers in (A, B) refer to the experimental reactors: 1, (Ca^{2+}) = 25 mg/L; 2, (Ca^{2+}) = 50 mg/L; 3, (Ca^{2+}) = 75 mg/L; 4, (Ca^{2+}) = 80 mg/L; 5, (Ca^{2+}) = 100 mg/L; 6, (Ca^{2+}) = 125 mg/L; 7, initial sludge. The black numbers in (C, D) refer to the band numbers in Figure 3. E1, Ca^{2+} concentration; E2, total nitrogen content; E3, NH_4^+ -N content; E4, NO_2^- -N content; X1, NO_2^- -N removal rate; X2, NH_4^+ -N removal rate; X3, TN rate.

Figure 5 show the correlations of microbial community structure with the environmental factors and nitrogen removal rates, respectively. Based on the distribution of the samples, the samples containing added Ca^{2+} were relatively far from the initial sludge (sample 7) (Figure 5A). In addition, the samples containing Ca^{2+} showed some degree of separation, indicating that changes in environmental factors resulted in substantial differences in microbial community structure. For instance, a change in Ca^{2+} concentration had a degree of influence on microbial community structure. The Ca^{2+} concentration was related to the total TN content, but showed little correlation with NH_4^+ -N and NO_2^- -N contents; therefore, the Ca^{2+} concentration likely influenced the microbial community structure through interactions with TN (Figure 5A).

In terms of nitrogen removal, the Ca^{2+} concentration was correlated the TN removal rate (Figure 5B), confirming the results described above. The Ca^{2+} concentration influenced the anammox

treatment performance, but had little correlation with the average $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal rates. This indicates that Ca^{2+} concentration may be related to $\text{NO}_3^-\text{-N}$ removal, subsequently affecting TN removal. The average TN removal rates from the reactors containing different Ca^{2+} concentrations followed the order $125 \text{ mg/L Ca}^{2+} > 75 \text{ mg/L Ca}^{2+} > 80 \text{ mg/L Ca}^{2+} > 100 \text{ mg/L Ca}^{2+} > 25 \text{ mg/L Ca}^{2+} > 50 \text{ mg/L Ca}^{2+}$. Although the average TN removal rate fluctuated with Ca^{2+} concentration, overall, the TN removal rate increased with increasing Ca^{2+} concentration.

Figure 5C,D show the correlation of microbial community structure with environmental factors and nitrogen removal rate by DGGE band. Bands 3 and 8 were positively correlated with all environmental factors, indicating that the taxa in bands 3 and 8 were positively correlated with Ca^{2+} concentration (Figure 5C). The microorganisms in band 3 belonged to the phylum Chlorobi but could not be identified to the genus or species levels; because only a part of the RUGL1-218 gene was cloned, it could not be further studied. However, it has been shown that Ca^{2+} has an important role in the immobilization of anammox sludge [13], and that bacteria of the phylum Chloroflexi have a certain effect on the granulation of anaerobic anammox sludge. Therefore, it can be speculated that the microorganisms in band 3 may be key microorganisms for the immobilization of anammox sludge. In addition, the microorganisms in band 8 belonged to the genus *Anaerolinea*, which has been shown not to have nitrogen-removal functions [35]. However, the RDA results suggested that *Anaerolinea* had an important role in the environment in which Ca^{2+} interacted with other environmental factors, such as TN, $\text{NH}_4^+\text{-N}$, and $\text{NO}_2^-\text{-N}$. Therefore, these microbes may also be related to bacteria with denitrification functions, and may be related to immobilization of anammox bacteria.

Bands 2, 3, 9 and 12 were positively correlated with all nitrogen removal rates (Figure 5D). As the Ca^{2+} concentration changed, the microorganisms in band 2, 3, 9 and 12 promoted denitrification and were all related to the immobilization of anammox bacteria. The microorganisms in band 12 belonged to β -Proteobacteria, but could not be identified to the genus and species level; however, a previous study showed such microbes to be related to Ca^{2+} [36]. The microorganism in band 2 belonged to *Acidobacterium*, and was not a main denitrifier based on the phylogenetic tree. However, from the RDA, it was speculated that increases in Ca^{2+} concentration would affect *Acidobacterium* levels, which would subsequently influence the removal rates of TN, $\text{NH}_4^+\text{-N}$, and $\text{NO}_2^-\text{-N}$. Therefore, *Acidobacterium* may comprise important symbiotic bacterial species. The microorganisms in band 9 belonged to *Denitratisoma*, within the family Rhodocyclaceae. Fahrback et al. [36] isolated microbes from activated sludge from municipal wastewater treatment plants, using 17- β -estradiol as a carbon source for the reduction of $\text{NO}_2^-\text{-N}$ into N_2O . With reference to their findings, under conditions of different Ca^{2+} concentrations, *Denitratisoma* species are key microorganisms affecting anammox denitrification performance.

In this study, an improved TN removal rate was observed with increasing Ca^{2+} concentration. Therefore, the removal of residual TN is a research topic for future anammox applications, and the present findings are expected provide direction for enhancing TN removal using the anammox process.

5. Conclusions

In the experimental anammox reactors, the removal of TN was affected by $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$, and overall first declined and then increased. And when the calcium ion concentration was 100 mg/L, the removal rate of TN was the highest and the denitrification effect was the best. Ca^{2+} concentration was positively related to TN removal rate, but showed no obvious effects on $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal. The microbial analysis indicated that the microorganisms in the sludge samples could be divided into five phyla (Proteobacteria, Chloroflexi, Acidobacteria, Planctomycete, and Chlorobi), as well as unknown bacteria. From the RDA, *Acidobacterium* and *Denitratisoma* were positively correlated with Ca^{2+} concentration, and were confirmed to be important for promoting the immobilization of anammox bacteria; *Acidobacterium* are important symbiotic bacteria and *Denitratisoma* are critical to anammox denitrification. These findings offer a theoretical basis to improve wastewater treatment using the anammox process.

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