Treatment of Low C/N Ratio Wastewater by a Carbon Cloth Bipolar Plate Multicompartment Electroenhanced Bioreactor (CBM-EEB)

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ABSTRACT: The traditional biological denitrification process has the problems of low removal rates and lack of a carbon source when treating wastewater with high ammonia nitrogen concentration and a low carbon–nitrogen ratio. Based on a bio-electrochemical system (BES), a novel carbon cloth bipolar plate multicompartment electroenhanced bioreactor (CBM-EEB) system was constructed. In this study, nitrogen removal efficiency and enrichment of functional bacteria using CBM-EEB under different voltage conditions were investigated. The results from next-generation sequencing indicated that the CBM-EEB included heterotrophic nitrification and aerobic denitrification (HNAD) and was dominated by heterotrophic nitrification aerobic denitrifying bacteria (HNADB). The applied voltage was confirmed as having the ability to regulate the microbial community structure and abundance of functional genes, thereby further enhancing the nitrogen removal efficiency of the system. The total nitrogen removal efficiency was 77.70 ± 1.14, 87.10 ± 0.56, 86.40 ± 0.59, and 89.30 ± 0.53% under applied voltages of 0.4, 0.7, 1.0, and 1.3 V, respectively. All values were significantly higher than the control group (62.86 ± 2.06%). HNADB had the highest abundance among the 17 detected genera related to nitrogen metabolism. Facultative denitrifying bacteria, Pseudoxanthomonas, along with key bacteria of HNADB, such as Flavobacterium, constructed a shortcut simultaneous nitrification–denitrification (SND) process. Poisson analysis and redundancy analysis (RDA) showed that the applied voltage improved the denitrification efficiency by changing the microbial community structure, reducing the abundance of heterotrophic bacteria, and increasing the unit abundance of key functional genes so that less organics were required for the denitrification process. The increased nitrogen removal efficiency in the experimental group was mainly related to simultaneous nitrification–denitrification process and cooperation of microbial communities in the anode and the cathode. This study highlighted the feasibility of CBM-EEB to enhance the HNAD reaction and the response of wastewater with a low C/N ratio to enhance the abundance of microbial bacteria and their functional gene abundance.

1. INTRODUCTION

Pig breeding is one of the main sources of global meat consumption. China has the largest scale of pig breeding in the world, accounting for 56.6% of the global total production. Total meat consumption in China is expected to reach 57.6 million tons in 2020, accounting for 52% of the total amount raised in large-scale farming. However, a large amount of wastewater is produced by large-scale farming, which poses a serious threat to the environment and human beings. Biogas production is generally considered an effective way to treat wastewater from large-scale farms. However, the effluent biogas slurry (BS) produced after anaerobic fermentation still contains a high concentration of inorganic nitrogen, which needs further treatment before returning to the field or being discharged into natural water. Nitrification and denitrification...
treatments are commonly applied to remove nitrogen from the BS, which contains high concentrations of ammonia nitrogen, organic carbon, and complex organic matter including recalcitrant cellulose. A low C/N ratio of wastewater (representing an insufficient carbon source) and a low concentration of organics (representing a deficiency in nutrients and electron requirements) results in an incomplete denitrification reaction. To solve the problem of nitrification and denitrification of wastewater with a low concentration of carbon and nitrogen, organic matter (e.g., methanol and acetic acid) has been incorporated by previous studies to supplement the carbon source and electron donors. However, this would increase the treatment cost and organic loading of the wastewater.

In recent decades, a large number of new processes derived from traditional nitrification and denitrification methods have been proposed to improve the treatment efficiency of wastewater with a low C/N ratio and reduce treatment costs. These processes include: (1) novel biological denitrification processes based on dominant bacteria, such as anammox and heterotrophic nitrification and aerobic denitrification (HNAD);5,6 and (2) biological systems stimulated with electrochemical methods, such as microbial fuel cells (MFC) and microbial electrolysis cells. Despite extensive studies of anammox, there are still limitations of the activities of anaerobic ammonia oxidation microorganisms due to the reproduction of heterotrophic microorganisms in wastewater with high concentrations of organic carbon (e.g., glucose, organic acids, and other organic matter). For traditional MFC, the direct transfer of electrons to electrodes requires electricitgens. This would compete with contaminant removal bacteria when the concentrations of nitrate and ammonia nitrogen are relatively low, which leads to a slow growth rate of bacteria and an unstable efficiency of electricity generation. However, it is difficult to realize effective treatment of wastewater when the concentration of organic matter is high.7,8 Therefore, it seems that both anammox and MFCs are not the best choices for treating wastewater containing high concentrations of ammonia nitrogen and organic carbon.

As a new green denitrification reactor based on a bioelectrochemical system (BES), microbial electrolysis cells can use electrical energy input to achieve multifunctional output, such as resource recovery and pollutant removal from various types of wastewater.9,10 It has been proven that an applied electric field can enhance the pollutant removal efficiency of wastewater with a low C/N ratio in the microbial electrolysis cells.11,12 However, a microbial electrolysis cell system still has a series of problems, e.g., high operating cost, poor microbial adhesion performance, and poor design of electrode plates.13,14 Compared with traditional noble metal electrodes, carbon-based materials have low cost and rough surfaces that are easy for microorganisms to attach. Therefore, the microbial electrolysis cells that use carbon as the electrode material have become the research hot spots. Slembo et al. improved the microbial adhesion to the electrode by modifying the surface of the carbon electrode.15 The application of carbon-based materials such as carbon fiber cloth could effectively solve the problems of high operating cost of the microbial electrolysis cell system and poor electrode performance of microbial adhesion. Fan et al. used J-cloth as a separator to make the anode and the cathode into an integral electrode plate.16 The J-cloth separated the cathode and the anode, which prevented direct contact between the cathode and the anode, thus avoiding a short circuit in the electrochemical system. It also minimized the distance between the two electrodes and greatly reduced the diffusion of oxygen between the two electrodes.

In this study, a new reactor model based on BES was established: carbon cloth bipolar plate multicompartment electroenhanced bioreactor (CBM-EEB). The study aimed to: (1) investigate the effects of different conditions on the removal of pollutants via HNAD; (2) explore the effects of applied voltage on the structure and diversity of the microbial community in the reactor; (3) determine functional bacteria,

Figure 1. Removal efficiency of the CBM-EEB system. (a) Concentrations of COD in the influent and effluent under different direct current (DC) power supplies (0.4, 0.7, 1.0, and 1.3 V). (b) Concentrations of NH4+-N in the influent and effluent under different DC power supplies (0.4, 0.7, 1.0, and 1.3 V). (c) Concentrations of NO3− in the influent and effluent under different DC power supplies (0.4, 0.7, 1.0, and 1.3 V). The result is presented as the mean value ± standard deviation.
Table 1. ANOVA of COD and TN Values of Four Groups of Voltages

| Voltage | COD (±) | TN (±) | F (3,56) | P     |
|---------|---------|--------|----------|-------|
| 0.4 V   | 97.23 ± 47.15  | 12.02 ± 3.80 | 1.661    | 0.186 |
| 0.7 V   | 74.41 ± 50.08  | 8.91 ± 4.88  | 3.286    | 0.027 |
| 1.0 V   | 102.92 ± 43.61 | 7.40 ± 4.25  |          |       |
| 1.3 V   | 1.1284 ± 90.01 | 0.34 ± 0.50  |          |       |

biological enzymes, and nitrogen metabolic pathways associated with denitrification; and (4) clarify the relationship between functional bacteria and nitrogen removal mechanisms based on the removal of nitrogen metabolic enzymes and pollutants.

2. RESULTS

2.1. Effect of Voltage on Wastewater Treatment under Aerobic Conditions. Figure 1 shows the removal efficiencies of chemical oxygen demand (COD), ammonia, nitrate, and nitrite nitrogen. The COD removal ranged from 80.44 ± 1.28 to 92.42 ± 0.34% under different conditions. The highest COD removal (80.44 ± 1.28%) in the control group was achieved at day 15, corresponding to the lowest COD concentration of 101 ± 6.4 mg/L. The COD removal of groups with applied voltages of 0.4 and 1.3 V was lower than 90%, and their values were significantly higher than the control group by 6.97 and 5.61%, respectively (p < 0.01). In comparison, the COD removal of groups with applied voltages of 0.7 and 1.0 V reached up to 92.42 ± 0.34 and 90.01 ± 0.50% at day 15, respectively. In general, the applied voltage enhanced the COD removal, and the optimal voltage for COD removal was 0.7 V. At the same time, we analyzed the COD and TN of four groups of voltage by analysis of variance (ANOVA) analysis, and the results are shown in Table 1. There was a significant difference in TN among the four groups, but no significant difference in COD.

The applied voltage significantly enhanced the ammonia removal (Figure 1b), i.e., ammonia removal significantly increased as the voltage increased (p < 0.05), and the highest value of 93.82 ± 0.48% was achieved at a voltage of 1.3 V. However, the rate of increase slowed at the end of the test. In particular, the ammonia removal increased by 7% when the voltage increased from 0.4 to 0.7 V, but increases of only 1.0 and 1.2%, respectively, were observed when the voltage increased from 0.7 to 1.0 and 1.3 V. This indicated that the ammonia removal rate of increase was slowed down when the applied voltage was higher than 0.7 V, and the marginal returns decreased as the voltage decreased. Hence, a voltage of 0.7 V was recommended since it was more cost effective than others.

This study focused on aerobic denitrifying microorganisms. In comparison to the anaerobic denitrification process, nitrite nitrogen did not accumulate during the aerobic denitrification process, and it only existed at a low concentration. Hence, the denitrification efficiency under aerobic conditions was analyzed using the sum of the nitrate concentration and the nitrite concentration (NO3−). The initial nitrate concentration was 20 mg/L and no nitrite was present in the influent. As shown in Figure 1c, there was no significant difference between the concentrations of nitrate and nitrite in the applied voltage group or the control group in the first 72 h. However, the concentrations of nitrate and nitrite in the effluent of the applied voltage group were significantly lower than that of the control group after the enrichment of the nitrification and denitrification microbial communities. In particular, the sum of the concentrations of nitrate and nitrite in the effluent of the groups with applied voltages of 0.7, 1.0, and 1.3 V was lower than 5 mg/L on day 15, and the values in the control group were higher than 9 mg/L. This indicated that denitrification of the CBM-EEB system was strengthened by the applied voltage. Future research should focus on the optimization of the treatment conditions to promote the removal efficiency and further decrease the operation cost.

2.2. Sequencing Results of Microbial Communities. The microbial community distribution at the phylum level is shown in Figure 2a. There were four dominant microbes (abundances higher than 0.007) in all samples, including Proteobacteria, Bacteroidetes, Verrucomicrobia, and Firmicutes. Proteobacteria, which belongs to the category of facultative heterotrophic bacteria, has respiratory metabolism and fermentation metabolism capabilities, and it is responsible for the degradation of organics and the removal of pollutants. The detected Proteobacteria included 4 classes, 18 orders, 34 families, and 95 genera. The relative abundance of bacteria in Proteobacteria is shown in Figure 2a. Group 1A had the highest abundance of Proteobacteria (0.611) in the highest abundance of Proteobacteria (0.894), which was 58.51% higher than that of the control group. In comparison, the abundance of Proteobacteria in other groups with an applied voltage was lower than that of the control group. As for samples in the cathode nonaeration area, group 1B had the highest abundance of bacteria in Proteobacteria (0.894), which was 8.51% higher than that of the control group. The distributions of Proteobacteria in groups 2B and 3B were also slightly higher than that of the control group. The result indicated that the microorganisms in Proteobacteria had a stronger competitive advantage.
advantage in the cathode area, which may be due to the weakened aerobic oxidation ability of organic matter and the higher concentration of organic matter compared with that of the anode area. However, the abundance of Proteobacteria in group 4B decreased by 28.01% as the applied voltage increased, suggesting that the high applied voltage was unfavorable to the enrichment of Proteobacteria. The distribution of Bacteroides was related both to environmental conditions of the electrode plate and the competition with Proteobacteria. In particular, the abundance of Bacteroides remained at a low level when Proteobacteria was the dominant group, and Bacteroides increased as the presence of the Proteobacteria group decreased.

At the genus level, 17 genera of microorganisms related to denitrification were detected. As shown in Figure 2b, 10 genera with heterotrophic nitrification aerobic denitrification ability were detected (relative abundance > 0.7%). All of them were dominant species, and the abundances of autotrophic nitrifying bacteria Nitrosira, Nitrosossira, and Nitrosomonas were lower than 0.2%, indicating that domestication had been achieved. Flavobacterium and Cloacibacterium, which belong to Bacteroidetes, were found in heterotrophic nitrification aerobic denitrifying bacteria (HNADB), and their abundance reached up to 47.48 and 51.44%, respectively, under an applied voltage of 1.3 V. 18, 19 The total abundances of samples 1A, 2A, and 3A obtained in the anode aeration area reached up to 22.16, 22.22, and 30.31%, respectively, and all of them were higher than the value (15.72%) of the control group DAZ. As for the cathode non-aeration area, all samples except for group 4B under an applied voltage of 1.3 V showed a relatively lower abundance than that of the control group. Although both Pseudomonas and Enterobacter belong to HNADB, they exhibited different distributions in all samples. 20, 21 In particular, the abundance of Pseudomonas in the control group was 13.78% (DZA) and 14.57% (DZB), respectively. In comparison, the abundances in the other eight samples were all lower than 9%, and the lowest distribution was 1.49% in group 4A. The abundance of Enterobacter in the control group was lower than 1.5%, and its abundance in the groups with applied voltages of 0.4, 0.7, and 1.0 V ranged from 4 to 6%. Pseudoxanthomonas and Thermomonas were the main facultative anaerobic denitrifying bacteria (FADB) in this study. 22 The abundance of Pseudoxanthomonas in the control group reached up to 9.23%, but it remained less than 1% in the experimental groups. Pedobacter and Azospirillum, which belong to the class of heterotrophic nitrogen-fixing bacteria (HNFB), were found in the bioreactor. 23 This indicated that ammonia nitrogen removal in this study depended not only on nitrification and denitrification but also the assimilation of microorganisms.

2.3. Distribution of Gene and Functional Proteins Related to Nitrogen Metabolism. Based on the sequencing

Figure 3. Relative abundance of genes related to nitrogen metabolism. (a) Genes related to the ammonia function. (b) Genes related to the assimilation function. (c) Genes related to the denitrification function. (d) Genes related to the ammoxidation function. (e) Genes related to the azotiﬁcation function. (f) Genes related to the respiration function.
of 10 samples, a total of 15,039 functional protein-coding genes and gene abundances were predicted by PICRUSt based on the KEGG database. Further screening of enzymes and functional proteins related to nitrogen metabolism in 15,039 functional protein-coding genes is shown in Figure 3.

Hydroxylamine reductase catalyzed the conversion of hydroxylamine to ammonia nitrogen, which was involved in ammoniation (Figure 3a). The gene abundance of hydroxylamine reductase in group 1B was the highest, reaching up to 3884, while that in group 1A was the lowest (344). The average abundance of the hydroxylamine reductase gene in the other samples with an applied voltage was 65.3%, which was lower than that of the control group.

The abundances of genes of nitrate reductase (NAS), nitrite reductase (Nir/ccNir), and glutamine synthetase (GS) are shown in Figure 3b. The shift in the abundance of NAS and Nir/ccNir exhibited a similar trend, in which a high voltage reduced their abundance and a low voltage increased their abundance. In the total 10 samples, there were more than 25,000 copies of the GS gene, and the copies of GS reached up to 40,137 (group 4A), which indicated robust activities of microbes in the bioreactors.

The detected enzymes involved in denitrification mainly included periplasmic nitrate reductase (NAP), allosteric nitrous-oxide reductase (Nir-NO-forming), nitric-oxide reductase (NorBC-1 and NorBC-2) and nitrous-oxide reductase (NosZ). NorBC-1, NorBC-2, and NosZ shared a similar trend with the change of the applied voltage. In particular, there was a positive relationship between the three enzymes and the applied voltages in the anode aeration area. However, the NosZ group with an applied voltage of 0.7 V demonstrated a lower abundance than the control group. As for the cathode nonaeration area, the group with an applied voltage of 0.4 V exhibited the highest enzyme abundance, and the applied voltage promoted the presence of three enzymes. Nir-NO-forming was responsible for transforming nitrite into nitric oxide and continuing denitrification. The highest numbers of copies of the coding genes were achieved in groups 4A and 4B with an applied voltage of 1.3 V, and the abundance was 6522 and 2943, respectively, which were 155.5 and 41.7% higher than those in groups DZA and DZB, respectively.

The copy number coding gene peaks in the anode and the cathode. However, the applied voltage of 1.0 V were higher than those of the control group. Only the copies of the glucokinase gene with an applied voltage of 1.0 V were higher than those of the control group. Pyruvate kinase was the key enzyme of glycolysis, and pyruvate was the final product of glucose respiration. The abundance of pyruvate kinase in the groups with applied voltages of 0.4 and 0.7 V was 42.1% higher than that of the groups with applied voltages of 1.0 and 1.3 V. In addition, the copies of the NADH dehydrogenase gene in the anode area were 27.1% higher than that of the cathode area, and the abundance in the experimental group with an applied voltage was 20.2% higher than that of the control group. This suggested that the applied voltage could effectively increase the copy number of the key biological enzyme gene in the electronic donor, and the CBM-EEB system could effectively handle the low C/N ratio wastewater with a shortage of denitrification electron donors.

2.4. Statistical Analysis. Figure 4 shows the correlation analysis results of 17 enzymes involved in nitrogen metabolism and total nitrogen removal. The color intensity of the scale indicates the intensity of the correlation.
On the other hand, the Pearson correlation index of the NAP gene abundance and the total nitrogen removal rate was $-0.996$, which was significantly negative at the level of 0.01. For the whole CBM-EEB system, the Pearson correlation index of the NAP gene abundance and total nitrogen removal rate was $-0.758$, which was significantly negative at the level of 0.05.

The relationship between the denitrification bacteria, the applied voltage, dissolved oxygen (DO) concentration, and pH were determined based on redundancy analysis (RDA) results. As shown in Figure 5a, the angle between the arrow and genus indicated that most of the enriched bacteria were negatively correlated with the applied voltage, DO concentration, and pH, including Pseudomonas and Zoogloea, which functioned as HNAD organisms. In comparison, dominant aerobic denitrification bacteria Flavobacterium and Cloacibacterium were positively correlated with the applied voltage, DO concentration, and pH. In addition, the abundance of Nitrosospira and Nitrosomonas was positively correlated with the applied voltage.

RDA results showed that the genes amoA, pMMO, NIR, Nor, and Nosz, which were related to the HNAD process, were positively correlated with the applied voltage (Figure 5b). In comparison, the genes glucokinase, pyruvate kinase, and glutamic acid synthetase, which were closely related to respiration, were positively correlated with the DO concentration and pH. On the other hand, the transformation from inorganic nitrogen to organic nitrogen does not involve a redox reaction, and there was no significant correlation with the applied electric field. However, copies of the GS gene in the aeration area were found to be significantly higher than that of the nonaeration area, which indicated that the microbes at a higher dissolved oxygen content were more active.

3. DISCUSSION

3.1. Treatment of Wastewater with a Low C/N Ratio by HNADB in a CBM-EEB System. According to the result of next-generation sequencing and the 16s + PICRUSt prediction, HNADB were the key functional microbial community in this system and exhibited excellent performance at removing nitrogen in wastewater with a low ratio of C/N, as observed before.25 The HNAD is a nitrogen removal process dominated by HNADB, which simultaneously complete HNAD reactions. Simultaneous nitrification–denitrification (SND) exhibits a high nitrogen removal ability and stable environmental adaptability with an appropriate dissolved oxygen concentration.26 During the heterotrophic nitrification process, ammonia nitrogen is converted to hydroxylamine under the action of heterotrophic bacteria, which is catalyzed by AMO or pMMO, followed by the conversion of hydroxylamine into NO$_2^-$ and NO catalyzed by HAO. Aerobic denitrification can be catalyzed by NAP, NIR, nitric-oxide oxidase (NOD), and nitric-oxide reductase (NOS) under microaerobic conditions.27–31 In addition, inorganic nitrogen in wastewater can be removed by assimilation of HNADB, which was different in comparison to traditional autotrophic bacteria.32

From day 4, the removal efficiencies of ammonia nitrogen, COD, and other pollutants significantly improved. Heterotrophic denitrification bacteria had a shorter growth period than autotrophic denitrification bacteria, which indicated that HNADB were more suitable for treating wastewater with a high organic concentration and a low C/N ratio. The microbial structure was obviously affected by the applied voltage. According to the microbial distribution results, the 10 samples shared similar species of denitrification bacteria. The dominant strains in the control group, Pseudomonas and Zoogloea, which were responsible for denitrification, were inhibited by the applied voltage based on RDA results. In comparison, the abundance of Flavobacterium and Cloacibacterium, which were the dominant bacteria in the experimental groups, was positively correlated with the applied voltage. However, these two strains only had the capability of undergoing aerobic denitrification but needed to cooperate with other microorganisms to complete the whole denitrification process. Moreover, the abundance of these microorganisms decreased with the increase of the applied voltage, indicating a low voltage was more conducive to the enhancement of the Enterobacter abundance. Enterobacter, a species capable of HNAD, can secrete extracellular polymeric substances (EPS) to condense dispersed particles, microorganisms, and salt particles into activated sludge.33 This indicated that the activated sludge had a more stable morphology at a low applied voltage (0.4–0.7 V).

3.2. Nitrogen Metabolic Pathways in a CBM-EEB System. The functional protein gene in the system can be obtained by comparing nitrogen metabolism reactions and
related biological enzyme species. The nitrogen metabolism pathway in this system is shown in Figure 6. In this system, synthetic wastewater was used and ammonia nitrogen and nitrate served as the nitrogen sources. Generally, two denitrification pathways were speculated (Figure 6). Pathway 1 was typical complete denitrification, i.e., $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$, where nitrate was the starting compound and nitrite was the key intermediate. Pathway 2 was a process of denitrification: $\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2$, where ammonia nitrogen was the starting compound. Meanwhile, nitrite was difficult to be converted into nitrate due to the absence of the nitrite oxidoreductase (NXR) enzyme. Thus, this reaction can be concluded to be a short-range SND reaction. The assimilation of ammonia nitrogen by microorganisms was also critical to the fate of nitrogen. Specifically, ammonium can be assimilated or directly acquired by microorganisms such as heterotrophic nitrogen-fixing bacteria and then the needed organic matter for the growth of the microorganisms can be synthesized.

**3.3. Mechanisms of Nitrogen Metabolism in a CBM-EEB System.** The same categories but different amounts of denitrogenase existed in the 10 samples from different groups and two sampling areas. This result demonstrated the existence of the complete denitrification pathway in every region of the system. The mechanisms can be divided into three denitrification processes and can be sorted (from a high to low level) accordingly: the SND process between two-compartment communities at the anode and the cathode, the SND process between single-compartment communities, and the HNAD process of single strains.

Based on the results of the Poisson analysis, in the anode of the CBM-EEB system, HAO and pMMO genes that were positively correlated with denitrification efficiency belonged to the class of compounds known as ammonia oxidase, and the functional genes were critical to catalyzing the nitrification reaction. Meanwhile, in the cathode, the NIR (NO) gene was positively correlated with denitrification efficiency and played a critical role in denitrification, where nitrite was converted into nitric oxide. The correlation analysis results indicated that the SND reaction in the anode and the cathode was beneficial to the improvement of the denitrification efficiency. The oxidizability was enhanced in the anode aeration area and the concentration of dissolved oxygen was elevated, which was conducive to the occurrence of the denitrification reaction. The promoted reduction and low dissolved oxygen concentration in the cathode nonaeration area were conducive to the occurrence of the denitrification reaction. The major reason for the higher nitrogen removal efficiency of the experimental group than the control group was the existence of the SND denitrification mechanism in the anode and cathode compartments. NAP was the starting enzyme of the nitrogen metabolism pathway 1, and it demonstrated a drastic negative correlation with the nitrogen removal efficiency, indicating that this path will reduce the denitrification efficiency of the whole system, thereby emphasizing the importance of establishing the shortcut pathway for nitrification and denitrification efficiencies.

According to the RDA analysis results, HNAD is a mechanism of denitrification dominated by functional bacteria. In the system, the HNADB such as *Pseudomonas* and *Zoogloea* were inhibited by the applied voltage, which can be concluded from the lower abundance in the experimental groups compared with the control groups. In the experimental groups, the dominant strains, *Flavobacterium* and *Cloacibacterium*, have the capability of undergoing aerobic denitrification but cannot complete the nitrification process. However, the RDA results showed that the abundances of the denitrification genes were positively correlated with the applied voltage, which implied that the abundance of both the aerobic denitrification functional gene and the heterotrophic nitrification functional gene were improved. In summary, the structure of the microbial community can be regulated, and the activities of the denitrifying microorganisms can be improved with the applied voltage.

It is not appropriate to compare the total abundance of functional genes under different experimental conditions because the nitrogen metabolisms were significantly different for different functional bacteria. A lower abundance of heterotrophic functional bacteria augmented the abundance of functional genes per microbe, and wastewater with a lower C/N ratio presented a higher denitrification efficiency. In the control groups, the nitrogen removal efficiency based on the denitrification mechanism of *Pseudomonas* and *Zoogloea* was low. In comparison, in the experimental group, heterotrophic bacteria such as *Pseudomonas* and *Zoogloea* were used to complete nitrification at the anode, and then the products were driven by aeration and an applied electric field to enter the cathode and complete denitrification by aerobic denitrifying bacteria. Therefore, a higher denitrification efficiency can be obtained through the SND mechanism.

**4. CONCLUSIONS**

The CBM-EEB system established in this study achieved a stable operation of short-range SND dominated by HNAD. The abundance of the microbial community and the functional genes as well as the efficiency of nitrogen removal were significantly influenced by the applied voltage. *Pseudomonas* and *Zoogloea* were the dominant bacteria and readily cooperated with other microbes to complete nitrogen removal, so the highest nitrogen removal efficiency was obtained during the SND process, which was higher than that of the single denitrification process of *Pseudomonas* and *Zoogloea* in the control group. The applied voltage had a significant effect on the structure of HNAD, and the most economical and efficient condition for running the CBM-EEB system was 0.7 V based on the results of denitrification efficiency and functional gene abundance. Our design of the CBM-SEC system will bring scientific perspectives and inspirations to future research.
on wastewater treatment, especially the mechanism associated with bacteria and gene analysis.

5. EXPERIMENTAL SECTION

5.1. Construction Settings and Operation of a CBM-EEB System. The CBM-EEB system as the bio-electrochemical denitrification reactor was constructed in a rectangular poly(methyl methacrylate) (PMMA) container (L × W × H = 100 mm × 150 mm × 200 mm). In the reactor, several insulating porous plates (n > 1, Shengcai Textile Company, Hebei, China) were planted. Two equivalent pieces of carbon cloths (CFS-I-300, Kaben Technology Company, Tianjin, China) as electrodes (L × W × H = 10 mm × 100 mm × 150 mm) were sewn on an insulating porous plate using nylon rope. The insulating porous plates were made of polypropylene (PP) material to prevent the direct contact of two carbon cloths, which could result in a short circuit. The insulating porous plates were arranged in parallel, dividing the water tank into n + 1 separate multicompartments. In each compartment, two electrodes were set opposite to each other. As a result, the cathode and anode chambers were alternately arranged in the reactor, and thus aerobic aeration zones in anode potential and anoxic zones in cathode potential were constructed (Figure 7). An aeration pipe was set in each anode chamber, while no aeration device was provided in the cathode chamber, making two adjacent compartments continuously circulating. The aeration in the anode chamber generates an upflow thrust, which will drive the solution from the anode chamber to the cathode chamber, forming a circulation outlet.

The wastewater of a low C/N ratio used in this study was simulated wastewater, which was prepared using deionized (DI) water, glucose, ammonium chloride, and nitrate. The quality of the prepared wastewater was detailed as follow: C/N = 3:1, [w/w]; TOC: 187.5; COD: 500 mg/L; NH₄⁺: 50 mg/L; and NO₃⁻: 20 mg/L. Trace elements (Zn²⁺, Mn²⁺, Cu²⁺, Ni²⁺, Co²⁺, and Mg²⁺) were also supplemented as described in a previous study to ensure the growth of microorganisms. In our previous studies, if the initial C/N ratio was set too low to support the growth of the microorganisms, the dead bacteria culture would decompose and influence the normal function of the system. Therefore, we chose a rational and representative low C/N ratio to carry out our experiments. All chemicals used in this study were purchased from Aladdin Biochemical (Shanghai, China). The sludge used in the experiment was taken from the sewage treatment reactor of Tianjin University (Shanghai, China). The aerobic activated sludge in the reactor was obtained from Gandu Environmental Engineering Co., Ltd. (Shanghai, China). After inoculating the sludge, the reactor was kept continuously aerated for 3 days with an aeration rate of 100 mL/min. During the aeration, the feed water (C/N = 5:1) was supplemented. Finally, the simulated wastewater was added into the reactor after discharging the incubation solution. The reactor was connected to 0.4, 0.7, 1.0, and 1.3 V voltage by four DC power supplies. The reactors were connected to different DC power supplies (0.4, 0.7, 1.0, and 1.3 V), respectively.

The reactors adopted the pattern of a sequential batch feed with a hydraulic retention time of 12 h. The experiment was performed at room temperature (20 ± 2 °C). The reactor without the extra DC power supply was set as the control. The experimental settings and the control were conducted in triplicate.

5.2. Sample Collection and Analytical Methods. The water samples were collected from the CBM-EEB reactors every 24 h. Before sampling, the aeration device was stopped and kept at rest for 2 h. Then, a quantitative filter paper was used to remove the impurities from the samples. The standard test protocols of ammonia nitrogen, nitrate, COD, pH, dissolved oxygen, and other experimental index are described in previous studies. The results of concentrations of COD, ammonia nitrogen, and NO₃⁻ of the influent and effluent water were used to represent the pollution level and treatment efficiency of the CBM-EEB system. The measurement of the water index of the experimental groups was conducted in duplicate, while the measurement of that of the control group was conducted every 7 days. Calculations related to removal efficiencies (Rᵢᵣ, %), which reflected the performance of pollution removal by the CBM-EEB system, were performed according to the following formula

\[ Rᵢᵣ = \frac{C_{\text{influent}} - C_{\text{effluent}}}{C_{\text{influent}}} \times 100\% \]

5.3. Microbial Analysis and Prediction of a Functional Protein. A total of 10 sludge samples were collected from the aerated anode area and the nonaerated cathode area in the reactors for further microbiological analysis. Among these samples, four samples were collected from the aerated anode area (1A, 2A, 3A, and 4A) of experimental groups; four samples were collected from the nonaerated cathode area (1B, 2B, 3B, and 4B) of experimental groups; while the DZA and DZB were derived from the aerated anode and nonaerated cathode areas of the control group, respectively. Labeled numbers 1, 2, 3, and 4 represented the operating voltages of 0.4, 0.7, 1.0, and 1.3 V, respectively.

Microbial DNA from the sludge samples was extracted using the E.Z.N.A. soil DNA Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer’s protocols. The final DNA concentration and purification were determined by a Nano-
Chimeric sequences were identified and removed using the UPARSE method applying Human Microbial Community and Soil Composition of the functional genes could be well detected. The sequence of the functional gene was predicted on the basis of the microbial composition in the sample. Then, the functional differences between different samples was determined via the abundance of the functional genes. In previous studies, the accuracy of this method analyzing human microbial communities and soil microbial communities was close to 95%, indicating that the composition of the functional genes could be well detected.

5.4. Statistical Analysis. Statistical significance was analyzed using IBM SPSS Statistics 21 software package (IBM, New York) and Python (Python 3.6.2, Python Software Foundation, Available at http://www.python.org). One-way ANOVA was performed, followed by pairwise comparisons using Student’s t-test to assess differences in the removal efficiency between multiple conditions. The results were shown in the mean value ± standard deviation. The relationship between the removal rate of TN, the relative abundance of functional bacteria, and functional genes was tested by Poisson analysis. Redundancy analysis (RDA) was also performed to determine the relationship between the bacterial community, functional genes, and environmental factors to detect the influence of reaction conditions on the abundance of functional bacteria and functional genes.

Notes
The authors declare no competing financial interest.

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