Intensified nitrogen removal in the tidal flow constructed wetland-microbial fuel cell: Insight into evaluation of denitrifying genes

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A novel tidal flow constructed wetland coupled with a microbial fuel cell system (TFCW-MFC), using the influent chemical oxygen demand (COD)/total nitrogen (TN) ratio of 10:1 (Device A) and 5:1 (Device B), systematically assessed nitrogen attenuation and power production performance; the spatiotemporal distribution characteristics of denitrifying functional genes and their relationship with nitrogen removal were also determined. The results showed that the TFCW-MFC achieved high removal efficiencies for COD and TN, with both devices above 95% and 83%, respectively. The maximum power density showed a notable increase from 16.97 in Device B to 25.78 mW/m³ in Device A. The distribution of the Shannon index indicated that the diversity of napA, nirK, and nirS were higher at the cathode layers in two devices. The high COD/TN ratio obviously increased the nirK diversity in anode on the 30th day, while a low COD/TN ratio apparently promoted the diversities increase of narG, nirK, and nirS in upper or bottom layers. Proteobacteria was the dominant phylum in both devices, and the composition differentiation of the dominant denitrifying genera was mainly affected by the space variation, specifically the nitrogen concentration, pH, dissolved oxygen, and their collaborative roles, rather than the COD/TN ratio. Furthermore, TN removal was very significantly positively correlated with voltage and the relative abundance of Rhodanobacter. In summary, this study provided an insight for the key functional genes shaping the enhanced nitrogen removal by the newly designed TFCW-MFC system.

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1. Introduction

Anthropogenic activities, such as agriculture, have considerably increased the amount of nitrogen entering our soils, rivers, lakes, seas, and oceans. The accumulation of these compounds, including ammonia (NH₄⁺-N), nitrite (NO₂⁻-N), and nitrate (NO₃⁻-N), can lead to serious environmental concerns such as eutrophication and declining drinking water quality (Aljerf, 2018). Studies identifying methods to enhance nitrogen removal from watercourses have intensified in recent years. Novel technologies employing a diverse array of techniques, including physical, chemical, biological method, or a combination method, have been explored to treat nitrogenous pollutants in wastewater (Vymazal and Kroepfelova, 2011a; Wu et al., 2017). However, considering the high cost and necessary expertise to operate most of these technologies, identifying more economical systems to treat nitrogenous wastewater is crucial.

Constructed wetlands (CWs), engineered treatment systems that use processes akin to those occurring in natural wetlands, have become a widespread, easily maintained, cheap means to reduce nitrogen from wastewater (Vymazal, 2013). However, the total nitrogen (TN) removal efficiency in CW is usually in the range of 40%–70%, which is mainly due to the lack of carbon source in the denitrification process. Moreover, oxygen transport to the media bed is often limited in CWs and can lead to an insufficient biological nitrification-denitrification in nitrogen removal (Lu et al., 2016).

Tidal flow constructed wetlands (TFCWs) that generate sequential, rhythmic “feed/flood,” and “drain/rest” cycles have been
considered as an efficient modification to improve nitrogen removal in CWs (Zhi and Ji, 2014). Although many studies have shown that TN and NH₄⁻N can be removed effectively by TFCWs (Zhao et al., 2004), little has been done to investigate the removal mechanisms of nitrogen in TFCW at the molecular level.

Aside from TFCWs, the simultaneous removal of organic and nitrogen through microbial fuel cells (MFCs) has also been investigated. Organic degradation occurs in the anodic compartment (Gude, 2016), with nitrification occurring through the higher oxygen supply and denitrification by the lower oxygen supply in the cathodic compartment (Virdis et al., 2010), whereby completes the redox systems through the external load to generate energy at the same time (Walter et al., 2018). Recently, the coupling of a CW with an MFC (CW-MFC) into one expanded system, allowing for wastewater treatment and simultaneous electricity generation, has been explored (Peter et al., 2007). Sharing the naturally existing redox gradient, an aerobic cathode and anaerobic anode among CWs and MFC, the produced electricity via MFC can directly enhance the power generation performance of MFC in the TFCW system, however, are still uncertain.

This study explores the operational adaptability and feasibility of treating wastewater samples with two different C/N ratios in the same operator mode by the TFCW-MFC, and to quantitatively analyze the underlying molecular mechanisms for the first time. Three specific objectives were pursued: (1) to evaluate the treatment performance of COD, TN, NO₃⁻N, NO₂⁻N, and NH₄⁺-N removal, and bioelectricity generation; (2) to discern the temporal and spatial distribution characteristics of the relative abundance and community structure for five denitrifying functional genes (napA, narG, nirK, nirS, and qnrB) at different C/N ratios; and (3) to identify the key functional genes that affect the nitrogen removal rates as well as the relationships between environmental factors and structure of five denitrifying genes.

### 2. Material and methods

#### 2.1. TFCW-MFC configuration

A TFCW-MFC coupling system, with a diameter of 50 cm and a height of 80 cm, was made from plexiglass; it was principally composed of an inlet pool, a TFCW configuration (Fig. 1); the support layer was filled with fine gravels (0.5–1.0 cm in diameter) and a height of 10 cm of cobbles (1.5–3.0 cm in diameter) to prevent clogging at the bottom of the system. For the MFC graphite electrodes (0.3–0.5 cm in diameter), a stainless steel mesh was interwoven through the felt to serve as a current collector. The cathode was located at the air-water interface, and the anode was buried under the cathode. The distance between the anode and cathode was approximately 25 cm, with the two connected by insulated titanium wire (1 mm in diameter) through an external
circuit with a load of 1000 Ω. From the top of the device, six evenly-spaced water sampling points (S1, S2, S3, S4, S5, S6) were marked and designed, and a substrate sampling port was respectively arranged at the cathode layer (c), upper filter layer (u), anode layer (a), and bottom filter layer (b). The TFCW-MFC system was surrounded by black shading membranes to prevent formation of microalgae.

2.2. Operation of the system

The TFCW-MFC system worked under the “tidal flow” principle (feed-flood-drain-rest) (Fig. 1). Wastewater was constantly pumped into the TFCW-MFC from the holding tank with a hydraulic load of 0.66 m³/(m² d) until the water reached the effective volume (40 l). The bed was kept saturated for 10 h after the construction was filled. After that, all of the wastewater was rapidly drained (0.2 h) into the recycling tank and the bed maintained 5.5 h in an empty state. The treated water in the circulating pool was then discharged into the coupling device again through the peristaltic pump (Longer Pump BT100-2J). After circulating two times in sequence, all the water was discharged. The inlet was positioned at the bottom of the TFCW-MFC which allowed the system to run in a continuous rise mode. The operation was controlled by peristaltic pumps as well as a programmable timer and ran continuously for 90 d.

One month was used for the inoculation of the cathodic compartment with activated sludge and of the anodic compartment with anaerobic digestion sludge sourced from Nanjing Sewage Treatment Plant before the system start-up. Next, ten strains of cattails (Typha latifolia L.) were planted in the cathode layer of the TFCW-MFC after they had been cultivated for more than 1 month at room temperature. Following inoculation, simulated wastewater was pumped into each device. The synthetic domestic wastewater composition included glucose, NH4Cl, NaNO3, NaNO2, KH2PO4, and some trace elements. The pH of wastewater was maintained at approximately 7.5 using HCl or NaOH. Two types of wastewater were investigated to determine if nitrate removal and bioelectricity generation were affected by C/N in the TFCW-MFC (Table 1). Wastewater under a C/N ratio of nearly 10:1 (Device A) or 5:1 (Device B) was provided to analyze the effect of carbon resource in the denitrification process.

Fig. 1. Schematic of the TFCW-MFC during a typical cycle, including the feed, flood, drain, and rest phases.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C/N = 10:1</th>
<th>C/N = 5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
<td>mg/L</td>
<td>513.95 ± 26.90a</td>
</tr>
<tr>
<td>TP</td>
<td>mg/L</td>
<td>6.32 ± 3.09</td>
</tr>
<tr>
<td>NH4-N</td>
<td>mg/L</td>
<td>55.35 ± 7.63</td>
</tr>
<tr>
<td>NO3-N</td>
<td>mg/L</td>
<td>14.28 ± 1.78</td>
</tr>
<tr>
<td>NO2-N</td>
<td>mg/L</td>
<td>6.09 ± 1.42</td>
</tr>
<tr>
<td>TN</td>
<td>mg/L</td>
<td>59.94 ± 8.87</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.65 ± 0.52</td>
</tr>
<tr>
<td>DO</td>
<td>mg/L</td>
<td>4.12 ± 1.31</td>
</tr>
<tr>
<td>T</td>
<td>ºC</td>
<td>16.15 ± 4.27</td>
</tr>
</tbody>
</table>

Note: * values are presented as the mean standard ± deviation.
2.3. Physical and chemical analyses

The influent water was changed after every two cycles; then, water samples were collected synchronously. Effluent samples were also collected from both devices after every two cycles, and each sample was taken in triplicate. The concentrations of COD, TN, NH₄⁻N, NO₃⁻N, NO₂⁻N, dissolved oxygen (DO), and pH were determined for all samples. The DO and pH were measured using a DO meter (HI 9146, Hanna Instruments, USA) and an S–3C model pH meter, respectively. The soluble COD was determined with a HACH DR2800 (HACH, Loveland, Colorado, USA); TN, NH₄⁺-N, NO₃⁻-N, and NO₂⁻-N were measured by a UV–1800 spectrophotometer (Shimadzu, Kyoto, Japan). All the above variables were analyzed according to standard analytical procedures (APHA, 1995).

2.4. Bioelectricity generation monitoring and measurement

The voltage of the MFC between the external resistors was continuously measured by a multimeter with a data acquisition system (2700 Keithley Instruments, USA). The volumetric current density \( I_a \) and the volumetric power density \( P \) were calculated using the basic electrical formulas (Eqs. (1) and (2)) (Logan et al., 2006). The polarization curves were obtained by changing the resistance from 1000 \( \Omega \)–50 \( \Omega \) at the end of each cycle. The polarization curve slope method was used to obtain the internal resistance \( R_{\text{int}} \) of the MFC, and the absolute value of fitting curve slope is regarded as the \( R_{\text{int}} \) of the MFC (Aelterman et al., 2006).

\[
I_a = \frac{U_{\text{cell}}}{V_{\text{ca}}} = \frac{1}{R_{\text{ex}}V_{\text{ca}}}
\]

(1)

\[
P = \frac{IU_{\text{cell}}}{V_{\text{ca}}} = \frac{U_{\text{cell}}^2}{R_{\text{ex}}V_{\text{ca}}}
\]

(2)

where \( I_a \) corresponds to the volumetric current density (mA/m³), \( I \) is the current (A), \( V_{\text{ca}} \) is the anode effective volume (m³), \( U_{\text{cell}} \) corresponds to the cell voltage (mV), \( R_{\text{ex}} \) is the external resistance (Ω), \( P \) is the volumetric power density (mW/m³).

2.5. Microbial community analysis

The microbial community samples were collected from the electrode and filter layers after 30 d and 90 d in both C/N conditions. A total of 16 samples were collected, and Device A (10:1 C/N ratio) and B (5:1 C/N ratio) filter media were gathered on the 30th day from the cathode layer (Ac1 & Bc1), upper layer (Au1 & Bu1), anode layer (Aa1 & Ba1), and bottom layer (Ab1 & Bb1) sampling holes; the samples collected in the different layers on the 90th day were labeled as sample 2 (e.g., Ac2 & Bc2). Five denitrifying reductases, \( \text{naph} \), \( \text{narg} \), \( \text{nirK} \), \( \text{nirS} \), and \( \text{gqorB} \), were amplified by PCR using the bacterial primers (Table 2); the nosZ gene was not detected, perhaps owing to its sensitivity to the environment or because the content was too low. The sequences generated in this study have been stored in the National Center for Biotechnology Information under accession number SRP148957 for \( \text{naph} \), SRP148946 for \( \text{narg} \), SRP150133 for \( \text{nirK} \), SRP147964 for \( \text{nirS} \), and SRP150135 for \( \text{gqorB} \). High-throughput data were used to analyze the basic biological characteristics of the five denitrifying genes and were expressed via the relative abundance, alpha diversity, redundancy analysis (RDA), and variance partitioning analyses (VPA). Related PCR reaction condition and microbiological analysis methods were shown in Text S1.

2.6. Statistical analyses

The difference of water parameters in effluents and denitrify microorganisms in different experimental conditions were evaluated by the analysis of variance (ANOVA) with the LSD test (SPSS 19.0) with \( P < 0.05 \) as the significance level. Pearson correlation analyses were performed among dominant denitrifying genera, voltage, and removal rate of nitrogen in the system.

3. Results and discussion

3.1. Treatment performance of TFCW-MFC

Dissolved oxygen (DO) and pH are two important factors that affect the effectiveness of NO₃⁻-N removal in CW. The pH value in Device A had a certain decrease with time, which was relatively stable in Device B. Moreover, the difference of DO was small in both devices, while the DO in Device A was generally lower than that of Device B (Fig. 1Sa, b). The lower amount of effluent DO in Device A is due to a large amount of DO consumed by oxidation of substantial organic matter; whereas the decrease of pH in Device A may be caused by more organic acids generated by microbial degradation and physiological activities of plants (Chen et al., 2014). Therefore, the pH value gradually decreases as the depth of the device increases (Fig. S1c). Additionally, DO also showed a downward trend as the depth of the device increased. The higher DO at the top of the device may be caused by the release of oxygen from the root system, but the contribution of the roots to the oxygen in the wetland system is somewhat controversial (Vymazal, 2011).

The total COD removal rate (97.96%) in Device A was consistent with that in Device B (97.54%) (Fig. 2), which implied that the available carbon to support microbial activities could be fully oxidized in the environment with sufficient oxygen in both systems. It was considered that the C/N ratio in the influent (with glucose as carbon source) was 4.2 as the cut-off point, beyond which the COD removal rate did not obviously fluctuate in the CW-MFC (Tao et al., 2020). Moreover, the removal rates of COD in the upper part of the two devices were higher than those in the lower part, which was similar to the effluent concentration of NH₄⁻-N. This is predominantly because of the role that DO plays in removing pollutants, such as COD and NH₄⁻-N in the CW. The surface removal load of COD in Device A and Device B reached 41.85 and 14.45 g/(m² d), respectively. Previous studies have applied intermittent aerated CWs for treating various wastewaters to obtain a high surface loading removal rate (SLRR) of COD (28.17 g/(m² d) (Avsar et al., 2007), 29.20 g/(m² d) (Komerup et al., 2009)). Obviously, compared with previous studies, the present TFCW with an MFC provided a higher SLRR of COD.

The removal rates of TN and NH₄⁻-N in Device A (TN: 83.98%; NH₄⁻-N: 97.84%) and Device B (TN: 84.26%; NH₄⁻-N: 98.85%) at the total water outlet were similar (Fig. 2). It is generally believed that the lower removal efficiency of TN in the CW is mainly due to the lack of carbon source in the denitrification process, therefore, the

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Table 2

<table>
<thead>
<tr>
<th>Specific primer</th>
<th>Specific primer sequence</th>
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<tbody>
<tr>
<td>( \text{naph} )</td>
<td>5'-TCGGGACCATGGGGGTTTACCAAC-3'</td>
</tr>
<tr>
<td>( \text{narg} )</td>
<td>5'-ACGACACCCGGCGACGCCCAACG-3'</td>
</tr>
<tr>
<td>( \text{nirK} )</td>
<td>5'-TAWGTCGGCCAGGARAAAACCTG-3'</td>
</tr>
<tr>
<td>( \text{nirS} )</td>
<td>5'-GGTAAAGCTTGAAGCAAGG-3'</td>
</tr>
<tr>
<td>( \text{gqorB} )</td>
<td>5'-CCAGAGTCATTACGATGCATTCCGGGCAATTGCT-3'</td>
</tr>
</tbody>
</table>

Spreadsheet data for all figures and tables are available in the SI.
higher C/N ratio promotes the TN removal. Nevertheless, the TN removal rate was not reduced in a lower C/N ratio in this study, which revealed that the MFC reduced the dependence of the denitrification process on organic carbon sources in TFCW systems. Therefore, the tidal flow operation mode coupled with an MFC is beneficial to the TN removal. Wang et al. (2019a) also confirmed that the average TN removal rates were barely increased in the closed-circuit CW-MFC when the influent C/N ratio exceeded 3.

Compared with other research, the TFCW-MFC had a higher TN removal rate than that of the CW-MFC (74% (Wang et al., 2017b); 69% (Wu et al., 2017)), which further showed that the innovative bioreactor had a unique effect on nitrogen removal in aerobic conditions. Besides, the TN effluent concentration also increased with the depth of Device A, which was similar to the removal trend of NH4+-N. However, the removal rate of NO3-/NO2-N was mostly opposite to that of TN and NH4+-N (Fig. 2), which was gradually reduced from 99.74%, 95.95% at the bottom to 47.54%, 48.04% at the top of Device A and B, suggesting that the lower part of the device was more suitable for the survival of denitrifying bacteria. NO3-/NO2-N can be utilized as an electron acceptor in the MFC cathode region; nevertheless, O2 can compete with NO3-N as a terminal electron acceptor in the oxidation of the organic substrate (Zhi et al., 2015). Obviously, it was confirmed that O2 had a greater effect on the removal of NO3-N compared with the MFC cathode region, therefore, the NO3-N removal rates were reduced in the upper part of both devices. The average concentration of effluent NO3-N was composed of influent NO3-N, nitrification of NH4-N, and denitrification of NO3-N. Under two C/N ratios, NO3-N effluent concentration in the upper part was higher in two devices (Fig. S2), indicating that nitrification of NH4-N had a greater influence on the NO3-N effluent concentration. Previous studies had reported that the accumulation of NO2-N was controlled by the C/N ratio (Zhi and Ji, 2014). For example, denitrification at a C/N ratio of 6:1 was sufficient to completely remove nitrogen from the nitrification in wastewater (Zhi et al., 2015); yet, other studies showed that the complete removal of NO3-N and negligible accumulation of NO2-N were only achieved at C/N ratios between 8 and 13.33 (Münch et al., 1996; Chen et al., 2017). Although the removal rates of NO3-N were not much different in this study, there was still a slight accumulation in the upper part of the two devices, indicating that the optimal C/N ratio should be above 10:1; Chen and Ni (2011) similarly suggested that C/N should be above 10:1.

3.2. Effect of C/N ratio on bioelectricity generation

The maximum power density of the Device A and B was respectively 25.78 and 16.97 mW/m³ (Fig. 3). The corresponding current densities were 0.11 and 0.18 A/m³, and the corresponding internal resistance of the system with Device A was 167 Ω and with Device B was 157 Ω. The influent C/N ratio affected the power density and internal resistance of the devices to some extent, and high C/N was beneficial for the system power generation. Organic matter can be used by microorganisms in the anode for respiratory metabolism and electricity generation (Corbella et al., 2014), therefore, it is an indispensable part of the electricity production process; however, it is not the higher the concentration, the more favorable for electricity production. The optimal C/N ratio for higher electrical performance still needs further studies. The power generation performance in this study was lower than that (31.04 mW/m³) of Srivastava et al. (2017), who employed a CW–MFC device, with Canna indica plants and intermittent aeration. This may be due to air diffusion to the anode zone in the process of instantaneous
empting wastewater, which destroys the required redox conditions. Here, the high removal of available organic might reduce the food substrate for electricity generating bacteria, resulting in poor voltage output. Besides, the decreased power production in this study may be also related to the farther electrode distance (25 cm), which was confirmed that the external resistance had an extremely significant and decisive influence on the power density (Wang et al., 2019b).

3.3. Alpha diversity of denitrifying functional bacteria in TFCW-MFC

The alpha diversity indexes of the five denitrifying bacteria were shown in Tables S1–S5. To obtain a more vivid picture of the temporal and spatial trends of the species diversity of the five denitrifying bacteria, the relative values of the Shannon indexes were compared and analyzed (Fig. 4). It is worth noting that, the Shannon values of the different sampling points in two devices did not significantly change with time but were closely related to the depth gradient. It indicates that the different filter layer environments of the two devices, such as DO, pH, nitrogen remaining concentration, etc., had a greater influence on denitrifying bacteria, which will be further discussed later. The diversity of napA, nirK, and nirS were higher at the cathode layers in two devices, which indicates that the rapid transfer of electrons in the cathode region promoted the growth of these denitrifying bacteria, and most of the denitrifying bacteria were more abundant in a relatively oxygen-rich environment. Besides, compared with napA and qnorB, the C/N ratio had a greater influence on napG, nirK, and nirS. Specifically, the high C/N ratio obviously increased the nirK diversity in anode on the 30th day, which may be due to the fact that the dominant bacteria of nirK are more likely to survive in electrode region and anaerobic environments; while the low C/N ratio apparently promoted the increase in diversities of napG, nirK, and nirS in the upper or bottom layer. This may be due to the higher abundance of the three types of bacteria in the electrode layer at high C/N ratio, thereby reducing the relative abundance of the upper and bottom layers. Previous studies also showed that the C/N ratio had a certain influence on the diversity of denitrifying bacteria (Luo et al., 2017). COD was the major driver shaping rare sub-community, hence, most of the genera were significantly related to the C/N ratio in the transient sub-community (Shu et al., 2018). Moreover, the C/N ratio not only affected directly the distribution of dominant genera, but also influenced their distribution by changing the surrounding environment indirectly. Furthermore, the quantity of nirK was lower than that of nirS (Tables S3 and S4), indicating that nirS had played an essential role in the denitrification process where NO2 was converted to NO (Chen et al., 2016). This may also because nirK is more sensitive to the C/N ratio than nirS (Chen et al., 2010), thus reducing the diversity of nirK.

3.4. Community structure of denitrifying bacteria at different C/N ratios

The relative abundance of the five kinds of denitrifying genera in different samples of the two devices was analyzed and expressed by a histogram of community structure (Fig. 5). The dominant denitrifying bacteria in Device A and B belonged within Proteobacteria, which is a phylogram of gram negative bacteria that play important roles in removing COD, NO2-N, and NO3-N from wastewater treatment systems (Chen et al., 2017). Besides, the spatial and temporal distribution of the main denitrifying genera was mainly affected by the depth gradient rather than the C/N ratio. This indicates that the distribution of different denitrifying genera was affected by different factors such as DO, pH and so forth. In terms of the distribution difference of the dominant denitrifying genera, the change in the different samples for napA was mainly reflected in the abundance and the dominant genera were Magnetospirillum, Escherichia, Sinorhizobium, and Azospira (Fig. 5a). It is worth noting that the abundance of Magnetospirillum was higher at the anode and bottom layer, indicating that Magnetospirillum was more likely to get electrons at the anode to reduce NO3-N. Few genera with napG were not detected, and the only genera with a comparatively high abundance were Methyllobacterium, Acidovorax, Aquimonas, and Oligotropha (Fig. 5b). Compared with Device B, the samples of Device A had higher abundance; in particular, the distribution difference of Methyllobacterium in each layer of two devices was small, and the relative abundance on the 90th day was lower than on the 30th day. Methyllobacterium, a proteotherian bacterium can grow using one-carbon compounds and reduce NO3-N to NO2-N, has been identified in various denitrification systems (Wang et al., 2018). The temporal and spatial distribution characteristics of Magnetospirillum and Methyllobacterium in the two devices indicate that they played a key role in the reduction of NO3-N.

The nirK gene was abundant in the microbial community, and the dominant genera were Rhodopseudomonas, Bradyrhizobium, Pseudomonas, and Mesorhizobium (Fig. 5c). It was reported that Rhodopseudomonas could readily take advantage of organic carbon for efficient denitrification (Ginge et al., 2005). Therefore, the relative abundance of Rhodopseudomonas in the same period and the filter of Device A is higher than that of Device B. Furthermore, the relative abundance of Rhodopseudomonas in the bottom layer was significantly higher, indicating that the bacteria were more suitable for growth under anaerobic conditions. The relative abundance of Bradyrhizobium at the cathode and anode layer was relatively high, owing to the presence of sufficient electron donors.

![Image](image-url)
The two principal genera possessing nirS were *Pseudomonas* and *Rhodanobacter* (Fig. 5d). *Pseudomonas* is a heterotrophic nitrifying and aerobic denitrifying bacterium that usually found in simultaneous nitrification and denitrification (SND) systems (Su et al., 2006). The abundance of *Pseudomonas* within nirS and nirK was high at the anode layer and upper layer, respectively, which indicated that this genus could utilize a variety of electron donors to perform denitrification. Furthermore, a large number of *Rhodanobacter* indicated that NO2/C0-N was used as the preferred electron acceptor (Kostka et al., 2012). The abundance of qnorB gene was generally low, and most of the genera were not detected; its main microorganisms included *Stenotrophomonas* and *Chitinophaga* (Fig. 5e). The abundance of *Stenotrophomonas* in the cathode samples was relatively high, which indicated that the cathode used NO3-N as an electron acceptor and effectively enhanced denitrification. The abundance of *Chitinophaga* in the anode and bottom filter was high, which may be related to the anaerobic environment.

### 3.5. Influence of environmental variables on the distribution of denitrifying functional gene community

It has been reported that C/N ratio, pH, DO, temperature, nitrite/nitrate concentrations are potentially important environmental controls on the denitrification (Kraft et al., 2014); however, it still remains unknown which of the above factors are most important...
Fig. 5. (continued).
due to the highly variable structure of the microbial community. The correlation between the five denitrifying bacteria and environmental factors was analyzed in terms of the general chemical indexes (pH, DO), temperature (T), nitrogen concentration and voltage (Fig. 6). Our variation partitioning analysis (VPA) showed that nitrogen concentration (13%–14%), general chemical indexes (7%–10%), and their combined effects (11%–16%) on the five denitrifying bacteria were significant; whereas, voltage and T had little effect on these parameters. The combination of T and nitrogen concentration had a greater impact on the five denitrifying bacteria than the effect of individual T. This may be due to the inconsistent effects of T on denitrifying bacteria, and the indirect effects of nitrogen removal by denitrifying bacteria are still significant. The roles of NO3⁻-N, DO and pH in controlling denitrification rates have been studied for many years, and they were considered to be the key regulators of denitrification rates at any denitrification process (Wallensten et al., 2006). To further analyze the correlation between the main environmental factors and relative abundance of the five bacteria under different C/N ratios, RAD analysis was conducted. The five denitrifying bacteria at the cathode and the upper layer of the device were positively correlated to NO3⁻-N, DO, pH, and negatively correlated with TN, NO2⁻-N, and NH4⁺-N; this trend was reversed at the anode and bottom layer. DO provided by the plant during photosynthesis and the tidal flow operation was used by biodegradation of organic matter, nitrifiers for nitrification process, and as a terminal electron acceptor for electricity production (Oon et al., 2014). As a result, the increased DO content in the upper part of TFCW-MFC systems was beneficial to the oxidation of NH4⁺-N, while the decreased DO content in the lower part of the device promoted the denitrification of NO3⁻-N, and this was also verified by Fig. 2. It is generally known that pH value affects the denitrifier community and the denitrification rate. Moreover, there was a positive correlation between alkaline environment and denitrifier community activity (Chen et al., 2014), which was consistent with the results presented in Fig. S1c and Fig. 5. The influence of nitrogen form on the distribution of denitrifying bacteria is complicated, closely related to the dominant species of denitrifying bacteria, and needs to be further discussed.

3.6. Relationship between the main denitrifying bacteria and nitrogen removal

The bacterial genera that accounted for more than 5% of the total number of individuals were identified, and Pearson correlation analyses were performed with the removal rate of nitrogen and voltage in the system. There was a significant positive correlation between removal rate of TN and Rhodanobacter and voltage (R = 0.548 and 0.513, P < 0.01), and a significant negative correlation with Methylobacterium (R = −0.440, P < 0.05) (Table 3). NH4⁺-N was significantly negatively correlated with Methylobacterium, Rhodopseudomonas, Bradyrhizobium, and NO3⁻-N (R = −0.631, −0.668, −0.577, and −0.814; P < 0.01), and significantly positively correlated with Rhodanobacter, and TN (R = 0.677 and 0.657; P < 0.01), as well as voltage (R = 0.249; P < 0.05). NO3⁻-N was significantly positively correlated with Bradyrhizobium and voltage (R = 0.684 and 0.477; P < 0.01), and significantly positively correlated with Methylobacterium, Rhodo pseudomonas, and Rhodanobacter (R = 0.515, 0.486, and 0.415; P < 0.05). NO2⁻-N was significantly negatively correlated with Rhodanobacter (R = −0.423; P < 0.05). Besides, the Magnetospirillum genus of napA and Sternotrophomonas genus of qnorB bacteria are not significantly related to nitrogen removal.

Oxidized nitrogen (NO3⁻-N and NO2⁻-N) is converted to NO, N2O, and N2 by dissimilatory reduction of selected bacteria and archaea (Spain and Krumholz, 2011). NO3⁻-N can be reduced to NO2⁻-N by Methylobacterium, whose high relative abundance in Ab1 could result in the low NO3⁻-N concentration of bottom effluents in Device A. Its positive correlation with NO3⁻-N removal rate revealed that Methylobacterium served as the critical index of NO3⁻-N reduction. Although there was no apparent difference of Methylobacterium in Device B as the spatial variation, it also was used to predict the TN and NH4⁺-N in effluents, corresponding with the significant relationship between them presented in Table 3. It is worth noting that Rhodanobacter, possessing NH4⁺-N degradation and converting NO3⁻-N to N2 via heterotrophic denitrification in aerobic conditions (Prakash et al., 2012; Hu et al., 2016), had a significant correlation with concentrations of all forms of nitrogen in effluents. The occurrence might be caused by the complex ecological niche in this
Fig. 6. VPA and RDA analysis curves of napA (a, b), narG (c, d), nirK (e, f), nirS (g, h), qnorB (i, j). Symbols show the samples, arrows represent environmental variables; General chemical indexes: pH and DO; Nitrogen concentrations: TN, NH4\textsuperscript{+}-N, NO3\textsuperscript{-}-N, NO2\textsuperscript{-}-N. Group A represents Device A (C/N ratio = 10:1), and Group B represents Device B (C/N ratio = 5:1). Environmental variables were selected based on significance calculated from the individual RDA results.
Fig. 6. (continued).
Fig. 6. (continued)
Fig. 6. (continued).
system, i.e. interaction among nitrification, denitrification, SND and bioelectricity generation, making the prominent role of *Rhodanobacter* in the nitrogen removal. It was also reported that a large number of *Rhodanobacter* demonstrated that NO$_2$-N was used as the preferred electron acceptor (Kostka et al., 2012). Thus, there was a negative correlation between *Rhodanobacter* and NO$_2$-N removal. With regard to *Rhodopseudomonas* and *Bradyrhizobium*, they could simultaneously reduce NO$_3$-N to NH$_4$-N and N$_2$ when Fig. 6. (continued).
Table 3

Pearson correlation analysis between dominant denitrifying genera, voltage, and removal rate of nitrogen.

<table>
<thead>
<tr>
<th></th>
<th>Methylobacterium</th>
<th>Rhodopseudomonas</th>
<th>Bradyrhizobium</th>
<th>Pseudomonas</th>
<th>Rhodanobacter</th>
<th>Stenotrophomonas</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO3 Removal rate</td>
<td>0.232</td>
<td>0.340</td>
<td>0.271</td>
<td>0.029</td>
<td>0.469</td>
<td>0.329</td>
</tr>
<tr>
<td>Voltage</td>
<td>0.321</td>
<td>0.396</td>
<td>0.111</td>
<td>0.345</td>
<td>0.384</td>
<td>0.548</td>
</tr>
<tr>
<td>TN</td>
<td>0.405</td>
<td>0.469</td>
<td>0.405</td>
<td>0.469</td>
<td>0.562</td>
<td>0.164</td>
</tr>
<tr>
<td>NO3</td>
<td>0.218</td>
<td>0.266</td>
<td>0.218</td>
<td>0.266</td>
<td>0.384</td>
<td>-0.022</td>
</tr>
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<td></td>
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</tbody>
</table>

- The coefficient of association (r) is significant at P < 0.05, and **significant at P < 0.01.
- Coefficient of association (r) in a single sample; "significant at P < 0.05; **significant at P < 0.01.
- The selected genera are more than 5% of the total number of individuals in a single sample.

Nitrate was used as terminal electron acceptor (Vairinhos et al., 1989; Xing et al., 2008), which explained why the high relative abundance of these two genera significantly decreased NO3-N but increased NH4-N concentration. Meanwhile, their absolute dominance at the anode and bottom-layer filter also justified the high removal rate of NO3-N but not NH4-N in here. Generally, Pseudomonas species are widely present in the environment, especially with the denitrification potential, as identified in previous studies (Hosono et al., 2015). Some bacteria belonging to the genus Pseudomonas are capable of autotrophic and heterotrophic denitrification. Based on the changeable and flexible characteristic of this genus, it is only negatively correlated with the NH4-N removal rate, while the correlation with other forms of N is insignificant.

Voltage was significantly positively correlated with NO3-N and TN (P < 0.01). This is mainly because organic matter can be oxidized by electrons on the anode of the MFC, and that the generated electrons can flow to the cathode through the external circuit; then, the denitrifying bacteria can reduce NO3-N and NO2-N to N2 using the electrons on the cathode (Puig et al., 2012), which was verified here by the higher alpha diversity of denitrifying bacteria on the cathode (Fig. 4). Therefore, the MFC increased the removal rate of TN and NO3-N in TFCW by enhancing electric transmission capability.

4. Conclusions

Considerable efficient removal of COD and TN in the TFCW-MFC system for wastewater treatment was obtained regardless of high or low C/N ratio. The peak voltage of Device A was 25.78 mW/m², and cathode region was conducive to the diversity increase of denitrifying functional genes (napA, nirK, and nirS). Compared with diversities, C/N ratio had a weaker influence on denitrifying community structures, while the depth change resulted in their composition differences, namely pH, DO, nitrogen concentration and their combined function in affecting denitrifying community distribution. Briefly, adding the relative abundance of Rhodanobacter, reducing that of Methyllobacterium, or increasing power generation, might be effective methods to boost the performance of TN removal using the TFCW-MFC. The results of this study provide a valuable reference for understanding the bioelectrochemical denitrification process, as well as the responsible bacterial communities in the removal of different nitrogen ions. Due to the complicated operation and poor power output in this system, its practical application is hindered. Moreover, the TWCF-MFC should be further investigated to combat the two major bottlenecks: simplifying the running mode for reducing the operational cost, and improving bioenergy recovery on the premise of ensuring effective pollutant removal.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Longmian Wang: Formal analysis, Data curation, Writing - original draft. Ying Zhou: Funding acquisition, Formal analysis, Data curation, Writing - original draft. Fuquan Peng: Data curation, Writing - original draft. Aiguo Zhang: Conceptualization, Writing - original draft. Qingqing Pang: Data curation, Writing - original draft. Jianjun Lian: Supervision, Writing - original draft, Project administration. Yimin Zhang: Conceptualization, Writing - original draft.
Fei Yang: Data curation, Writing - original draft. Yueming Zhu: Writing - original draft. Chengcheng Ding: Data curation, Writing - original draft. Lixiao Ni: Writing - original draft. Yibin Cui: Supervision, Writing - original draft, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2020.121580.

References