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Bacterial community and nitrate removal by simultaneous heterotrophic and autotrophic denitrification in a bioelectrochemically-assisted constructed wetland

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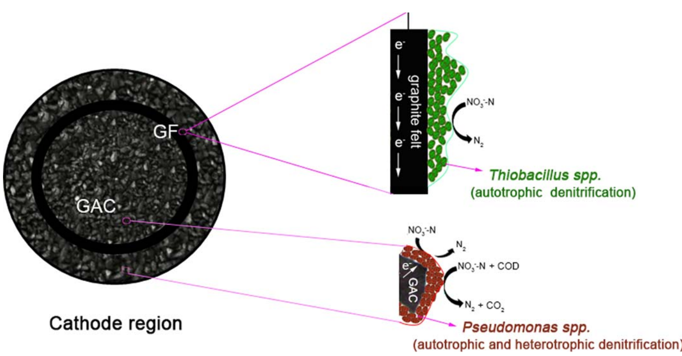
GRAPHICAL ABSTRACT



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ABSTRACT

To enhance nitrate removal in constructed wetlands (CWs), a bioelectrochemically-assisted CW (BECW) integrating a three-dimensional biofilm-electrode reactor (3D-BER) into the CW was evaluated for the effectiveness of combined autotrophic and heterotrophic denitrification in the presence of organic matter and applied current. The effects of COD/N ratios on nitrate removal were investigated, and the bacterial communities in the granular active carbon (GAC) and graphite felt (GF) in the reactor's cathode region were compared. The highest NO_3^--N and TN removal efficiencies of 91.3 ± 7.2% and 68.8 ± 7.9% were obtained at the COD/N ratio of 5. According to the results of high-throughput sequencing analysis, sample GAC was enriched with a high abundance of *Pseudomonas* (17.29%) capable of autotrophic and heterotrophic denitrification, whereas autotrophic bacteria *Thiobacillus* (43.94%) was predominant in sample GF. The synergy between heterotrophic and autotrophic denitrification bacteria is believed to cause the high and stable nitrogen removal performance.

1. Introduction

Owing to uncontrolled discharge of wastewater and intensive use of fertilizers in agriculture, nitrate pollution in water resource has become a serious threat to human health and water ecosystem. Intake of high nitrate concentration in drinking water may be linked to infant methemoglobinemia and bladder cancer (Weyer et al., 2001). The discharge of wastewater containing excessive nitrate levels into aquatic

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systems (e.g., lakes, rivers or seas) can lead to adverse effects associated with eutrophication, including algal blooms, changes in biodiversity and bottom anoxia (Maier et al., 2009). Extensive research has been done on the development of technologies for eliminating nitrate from wastewater and water. Physicochemical methods are not economically feasible for large applications because of high cost and the need for waste brine disposal (Park & Yoo, 2009). As an alternative, biological denitrification is considered the most promising approach for nitrate removal because of its effective performance and low cost.

Constructed wetlands (CWs) have become increasingly and extensively used as an alternative treatment method for nitrate-rich water and wastewater (Almeida et al., 2017), owing to their favorable purification efficiency, low cost, simple operation and maintenance, and environmental friendliness. Nitrate nitrogen (NO_3^--N) in CWs is removed mainly by conventional heterotrophic denitrification (Zhi & Ji, 2014), in which organic carbon compounds are combined with electron acceptors (NO_3^-) to yield oxidized carbon (CO_2), a reduced product (N_2), and energy. Thus, it is often difficult to maintain high nitrate removal efficiency when organic carbon in wastewater is insufficient. To date, numerous studies have been undertaken to enhance denitrification in CWs when treating low chemical organic demand to nitrogen (COD/N) ratio wastewaters, which mainly include looking for cheap and abundantly available alternative carbon sources (e.g., various plant materials) (Hang et al., 2016) and incorporating autotrophic denitrification into CWs (Park et al., 2015; Song et al., 2016a). However, directly adding plant carbon source into CWs is increasingly unfavorable because of its low effective utilization compared to soluble substrates (e.g., methanol), need for optimization of plant biomass dosage and dosing position, as well as potential risk of secondary pollution resulting from excessive organic carbon (Hang et al., 2016). Autotrophic denitrification can be attained by using a variety of inorganic reduced compounds (e.g., sulfur-reduced compounds, ferrous iron, and hydrogen gas) as electron donors to reduce nitrate (Park & Yoo, 2009; Song et al., 2016a; Xu et al., 2016). Contrary to heterotrophic denitrification, autotrophic denitrification has the unique advantages of lower cost and risk of adding organic carbon compounds, as well as effectively mitigating the clogging problem of CWs (Xu et al., 2016). Thus, some newly intensified CWs associated with various autotrophic denitrification processes (e.g., sulfur-based and hydrogenotrophic denitrification), have been receiving more attention in recent years (Song et al., 2016a; Xu et al., 2016).

Biofilm-electrode reactors (BERs) are one of the most extensively studied autotrophic denitrification technologies owing to their advantages of harmless products, lack of need to add reduced inorganic compounds, and precise control of the electron donor (Park & Yoo, 2009). In a BER, hydrogen gas is produced by water electrolysis, which is used as an electron donor for autotrophic denitrification by denitrifying bacteria immobilized on the cathode surface. In particular, the novel three-dimensional BER (3D-BER) was developed with the objective to improve denitrification performance and reduce electrical energy consumption. In a 3D-BER, granular activated carbon (GAC) or GAC mixed with other particulate matters is introduced and packed in a common two-dimensional BER (2D-BER) to act simultaneously as a third bipolar electrode and a biocarrier, which provides a large surface area for biomass growth and attachment as well as makes high H_2 production possible (Hao et al., 2016). Previous studies of 2D-BER or 3D-BER technologies have mainly focused on the optimization of reactor design and operating factors (Capua et al., 2015; Mousavi et al., 2012); a few studies had revealed the information of associated bacterial community. Moreover, the studies of bacterial community in 3D-BERs only focused on the 3D electrode (Hao et al., 2016), the comparative analysis of the bacterial community enriched on the 3D electrode (e.g., GAC or AC) and the conventional 2D electrode (e.g., graphite felt (GF), carbon rod, or graphite plate) is lacking. Recently, a preliminary study of a bioelectrochemically-assisted constructed wetland (BECW) integrating a 3D-BER into a CW, constructed by using GAC as the 3D electrode and GF as the 2D electrode both in the anode and cathode regions, was presented (Xu et al., 2017). Completely autotrophic denitrification (78.92 ± 3.12% of nitrate removal) was obtained in the BECW system with an applied current of 15 mA. However, in fact, most of the low COD/N ratio wastewaters still contain some organic carbon, such as some domestic sewage and secondary domestic/municipal wastewater. The presence of organic matters in wastewaters would reduce the consumption of electrical energy and enhance the denitrification behavior, as well as influence the distributions of denitrifying bacteria in the 2D and 3D electrodes.

Thus, the objective of this study was to investigate the effect of COD/N ratios on BECW nitrate removal performance under the mixotrophic condition of organic matter and applied current. Moreover, to elucidate the denitrification mechanism and optimize reactor performance further, the bacterial community attached to GAC and GF biofilms in the cathode region were compared using high-throughput 16S rDNA pyrosequencing.

2. Materials and methods

2.1. BECW reactors set-up and operation

As illustrated in Fig. 1, the three BECW reactors consisted of polyvinyl chloride (PVC) columns (700 mm height, 160 mm in diameter), a 25 L holding bucket, and a peristaltic pump connected to inlet pipes at the bottom of the reactors. The constructional details and start-up procedures of the reactors have been described in a previous study (Xu et al., 2017). Briefly, the reactors were alternately filled with gravel media (particle size of 8–16 mm) and GAC (diameter 3–5 mm) from the bottom upward: gravel (100 mm in depth), GAC (100 mm in depth), gravel (100 mm in depth), GAC (100 mm in depth), followed by the gravel planted with *Canna indica* var. *flava* (200 mm in depth). The upper layer filled with GAC acted as the cathode region for auto-hydrogenotrophic denitrification, whereas the lower GAC layer acted as the anode region. In addition, GFs (300 mm length × 100 mm width × 6 mm thickness) were inserted into the randomly packed GAC to collect or release electrons. To induce cathode reaction, a constant current of 15 mA was applied to the circuit by connecting the positive pole of a DC power supply (LongWei PS-305DM, Shenzhen, China) to the anode, and the negative pole to the cathode.

At the beginning of autotrophic denitrifier enrichment, each reactor

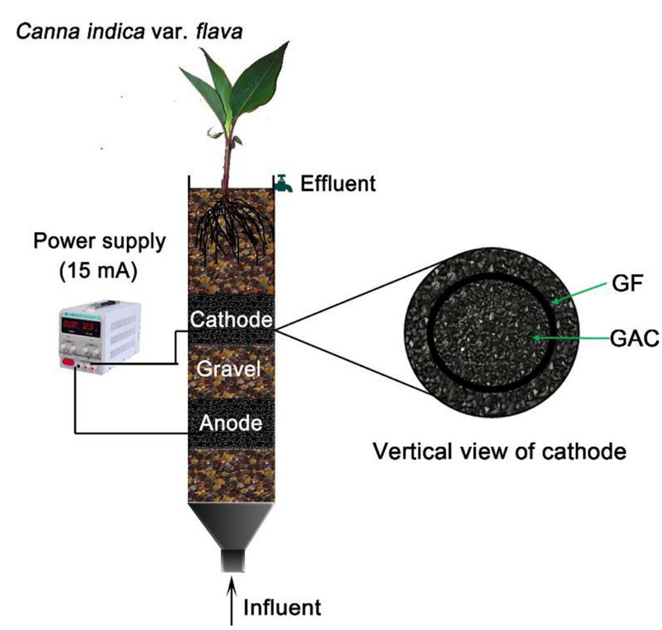

Fig. 1. Schematic representation of BECWs.

was inoculated with anaerobic activated sludge (30% v/v) taken from a local domestic wastewater treatment plant (Wuchang Zone, Wuhan, China) and filled with synthetic wastewater augmented with sodium nitrate to a target concentration of 30 mg L^{-1} without supplemental organic carbon. The ingredients of the synthetic wastewater were as follows (L^{-1}): 0.182 g $NaNO_3$, 0.045 Na_2HPO_4, 0.5 g NaCl, 0.0068 g $CaCl_2 \cdot 6H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.45 g $NaHCO_3$ and 0.1 mL trace nutrient solution (Sayess et al., 2013). The pH of the synthetic wastewater was adjusted to 7.30–7.40 using 1 N HCl. Four COD/N ratios of synthetic wastewater (approximately 1, 2, 3, and 5) were tested in this study by adjusting the sodium acetate dosage. The reactors were operated stably for 5 days at each COD/N ratio. All reactors were operated indoors in continuous up-flow mode with a hydraulic retention time (HRT) of 24 h. The air temperature was in the range of 15–20 °C.

2.2. Sampling and chemical analysis

Water samples were collected from the BECW effluents to determine the concentrations of chemical oxygen demand (COD), total-N (TN), ammonium-N (NH_4^+-N), nitrite-N (NO_2^--N) and nitrate-N (NO_3^--N). The inorganic nitrogen concentration was measured using a spectrophotometer (UV-1800, Shimadzu, Japan) according to the standard methods as follows: TN (alkaline potassium persulfate digestion), NH_4^+-N (Nessler's reagent), NO_2^--N (N-(1- naphthyl)-ethylenediamine dihydrochloride), and NO_3^--N (ultraviolet colorimetric methods). The COD concentration was measured with a Hach DR2800 colorimeter according to the instrument's standard calibration and operation.

2.3. Biomass sampling, DNA extraction, PCR amplification and pyrosequencing

At the end of the experiment, both the GAC and GFs were collected from the cathode region to compare bacterial community differences, namely GACc and GFc, respectively. All the samples were stored in sealed bags at −80 °C until analysis. Before DNA extraction, the GFs were cut into small pieces with sterilized scissors so that the bacteria attached to the sample surface could contact the lysate better and release more DNA. The genomic DNA was extracted using an extraction kit (E.Z.N.ATM Mag-Bind Soil DNA Kit, Omega, USA) according to the manufacturer's instructions. The V3-V4 region of the bacterial 16S rDNA gene was PCR-amplified using the universal primers 341F (CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGC AG) and 805R (GACTGGAGTTCCTTGGCACCCGAG AATTCCAGACTA CHVGGGTATCTAATCC). Then, the pyrosequencing proceeded according to the approach described by Xu et al. (2017).

2.4. Analyses of sequence data

Operational taxonomic units (OTUs), rarefaction curves, and diversity indices were determined based on the OTUs by Mothur ver. 1.30.1. The network was analyzed via QIIME and drawn using R code based on the obtained OTUs. The sequences obtained were allocated phylogenetically down to the phylum, class, and genus levels at 97% similarity for community composition analysis. For taxonomic analysis, the representative sequences from each OTU were subjected to the RDP-II Classifier of the Ribosomal Database Project (RDP) and National Center for Biotechnology Information (NCBI) BLAST. After clustering, a heat map was drawn using R code to reflect similarities and differences in community composition among bacterial samples at the genus level.

3. Results and discussion

3.1. Effect of COD/N ratios on the denitrification performance of BECW

To examine simultaneous heterotrophic and autotrophic denitrification performance under mixotrophic conditions, synthetic wastewater at four

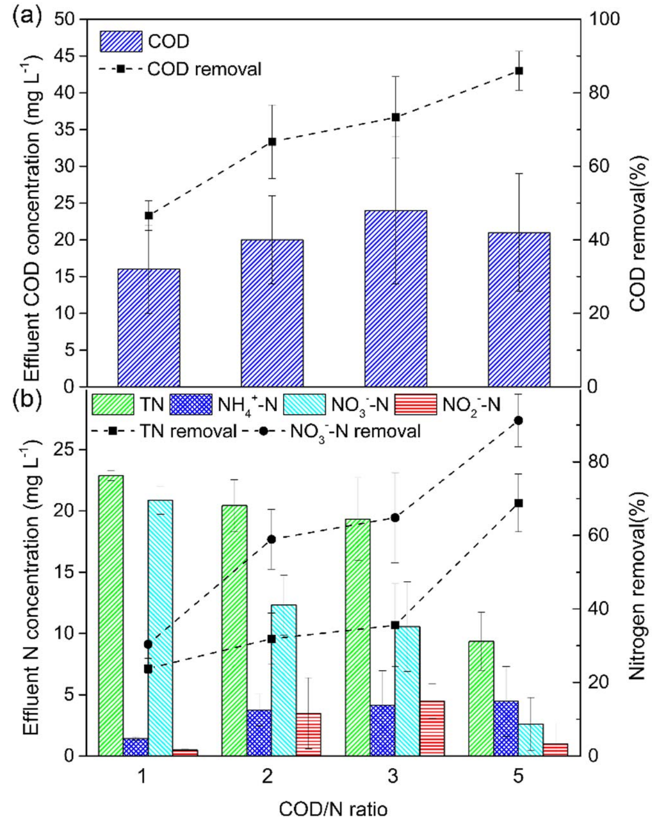

Fig. 2. COD removal (a) and nitrogen removal performance (b) of BECWs with different influent COD/N ratios.

different initial COD/N ratios (approximately 1, 2, 3, and 5) were studied in BECWs, which were constructed by combining CWs with 3D-BERs. The experiment was operated under the following conditions: a constant current of 15 mA, an HRT of 24 h, and an average air temperature of 15–20 °C. In autotrophic denitrification processes, the hydrogen produced from water electrolysis acts as an electron donor, and nitrate acts as an electron acceptor. In heterotrophic denitrification, the organic matter serves as an electron donor and then reduces nitrate into nitrogen gas. The COD removal efficiencies were positively correlated with the COD/N ratios (shown in Fig. 2a). When the COD/N ratio increased from 1 to 5, the COD removal efficiency increased from 46.6 ± 4.0% to 86.0 ± 5.3%. The anode facilitated the oxidation of organic matters, and then the produced electrons were delivered to the cathode acting as an electron donor for autotrophic denitrification (Gregory et al., 2004). In addition, an increase in influent C/N ratio can accelerate the growth of heterotrophic denitrification bacteria, but its effect on autotrophic denitrifiers remains unknown and needs to be considered based on the results from the bacterial community structure analysis.

The effluent characteristics of NH_4^+-N, NO_3^--N, NO_2^-N, and TN during the experiment are shown in Fig. 2b. The removal efficiencies of NO_3^--N and TN were significantly increased with increasing COD/N ratio. As shown in Fig. 2b, the removal of NO_3^--N and TN were not significant under an initial COD/N ratio of 1; only 23.8 ± 1.3% of the initial TN and 30.4 ± 3.9% of the initial NO_3^--N were removed after an HRT of 24 h. According to the results of a previous study (Xu et al., 2017), the highest denitrification efficiency (78.92 ± 3.12%) was obtained in the BECW with organic-free influent under the following conditions: 15 mA, HRT of 48 h, and close to 30 °C. Except for the shorter HRT and lower operating temperature, the inhibition of autotrophic denitrification by organic matters could also be one of the reasons leading to low denitrification efficiency in this experiment. This also indicated that the COD/N ratio of 1 was inadequate for establishing an efficient denitrification process. By increasing the COD/N ratio, clear and consistent trends of increasing NO_3^--N and TN removal efficiencies were

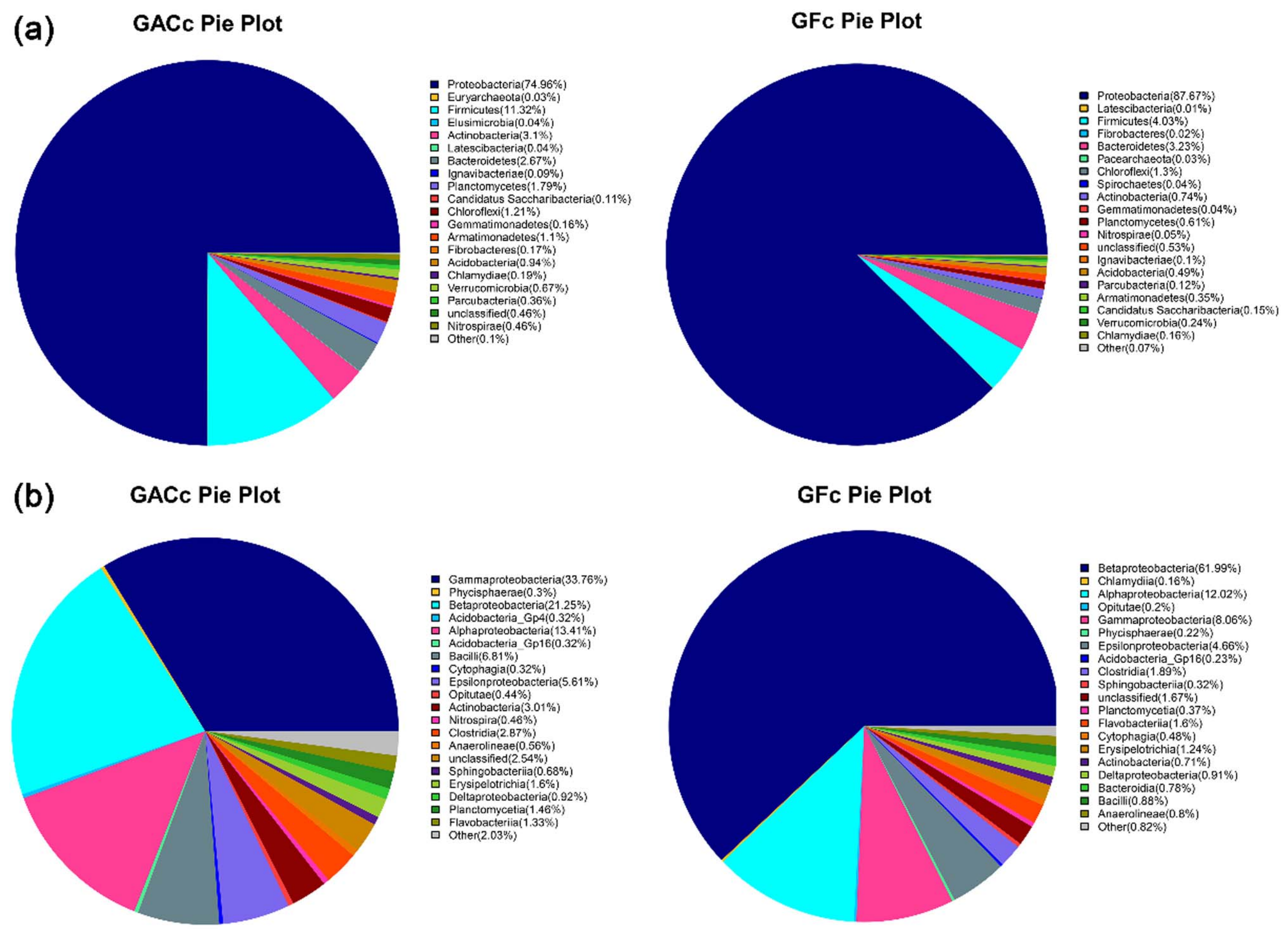

Fig. 3. Taxonomic classification of bacterial 16S rRNA gene reads at phylum level (a) and class level (b) retrieved from the cathode electrodes of BECWs.

presented, which is consistent with previous studies (He et al., 2016; Wang et al., 2017; Zhi & Ji, 2014). Heterotrophic denitrification is highly related to the oxidation of organic matter, which usually requires above 3.0 g of COD to remove 1.0 g of NO_3^--N owing to the presence of oxygen and the cell synthesis of microorganisms (Lee et al., 2001). Moreover, microbial heterotrophic denitrification is considered the dominant N sink in many conventional CWs (Zhi & Ji, 2014), and an optimal COD/N ratio ranging from 5 to 12 was proposed to achieve good nitrogen removal performance (Pous et al., 2014; Song et al., 2016b; Zhao et al., 2011). Thus, the reason for the increased NO_3^--N and TN removal with the rise of COD/N ratios could be ascribed to sufficient carbon source supply, which can accelerate the growth of heterotrophic denitrifying bacteria, thus promoting the total denitrification rate. As the COD/N ratio changed from 2 to 5, the effluent TN concentration decreased from 20.43 ± 2.10 to 9.35 ± 2.36 mg L^{-1} and the corresponding removal gradually increased from 31.9 ± 7.0% to 68.8 ± 7.9%, whereas the effluent NO_3^--N concentration decreased from 12.32 ± 2.45 to 2.62 ± 2.15 mg L^{-1} and the corresponding removal gradually increased from 58.9 ± 8.2% to 91.3 ± 7.2%. It is obvious that the removal of NO_3^--N was higher than that of TN permanently removed from BECWs, especially when the COD/N ratio was relatively high. As the COD/N ratio increased, the NO_3^--N removed was gradually converted to NH_4^+-N and NO_2^--N, and thus the TN removal efficiency decreased. The effluent NH_4^+-N concentration gradually increased from 1.42 ± 0.14 to 4.45 ± 2.86 mg L^{-1} as the COD/N ratio increased from 1 to 5. The accumulation of NH_4^+-N in BECWs may result from two factors: (1) the nitrate electrochemical reduction in the cathode region and (2) the occurrence of dissimilatory nitrate reduction to ammonium (DNRA). As the effluent concentration of NH_4^+-N increased with the rise of COD/N ratio, the occurrence of DNRA might be a possible electron sink during heterotrophic and autohydrogenotrophic denitrification under a mixotrophic condition in BECWs. The occurrence of DNRA has also been observed in the cathodic chamber of bio-electrochemical systems performing denitrification (Huang et al., 2013; Sander et al., 2015), suggesting that microorganisms could use electrodes as a sole electron donor for DNRA as well as denitrification. Regarding nitrite accumulation, its trend was rather different from that of NH_4^+-N (Fig. 2b). As the COD/N ratio increased from 1 to 4, the NO_2^--N effluent concentration increased from 0.48 ± 0.08 to 4.48 ± 1.40 mg L^{-1} but then decreased to 1.01 ± 1.70 mg L^{-1} at the COD/N ratio of 5. This COD/N ratio and its resulting pH change could be associated with nitrite buildup (Karanasios et al., 2016; Lee & Rittmann, 2003).

3.2. Richness and diversity of the bacterial community

In total, 67,056 raw sequences and 59,739 high-quality reads were obtained for the identification of microbial communities from samples GACc and GFc, with an average length of ~423 bp. Then these gene sequences were assigned to conduct downstream analyses and 1275 and 897 OTUs were obtained at a 97% sequence identity threshold for GACc and GFc, respectively. This indicated that the microbial community in sample GACc was more complex than that in sample GFc. Good's coverage estimator ≥0.99 suggested that bacterial OTUs in the two samples were well represented by the collected gene sequences. It is obvious that the Chao 1 and Shannon indices of sample GACc were higher than those of sample GFc, indicating higher richness and diversity of the bacterial community in GACc. Moreover, the rarefaction curves for samples GACc and GFc showed a decreasing rate of accumulation of OTUs but did not reach saturation, suggesting that the sequencing obtained a large proportion of the diversity of the electrode communities.

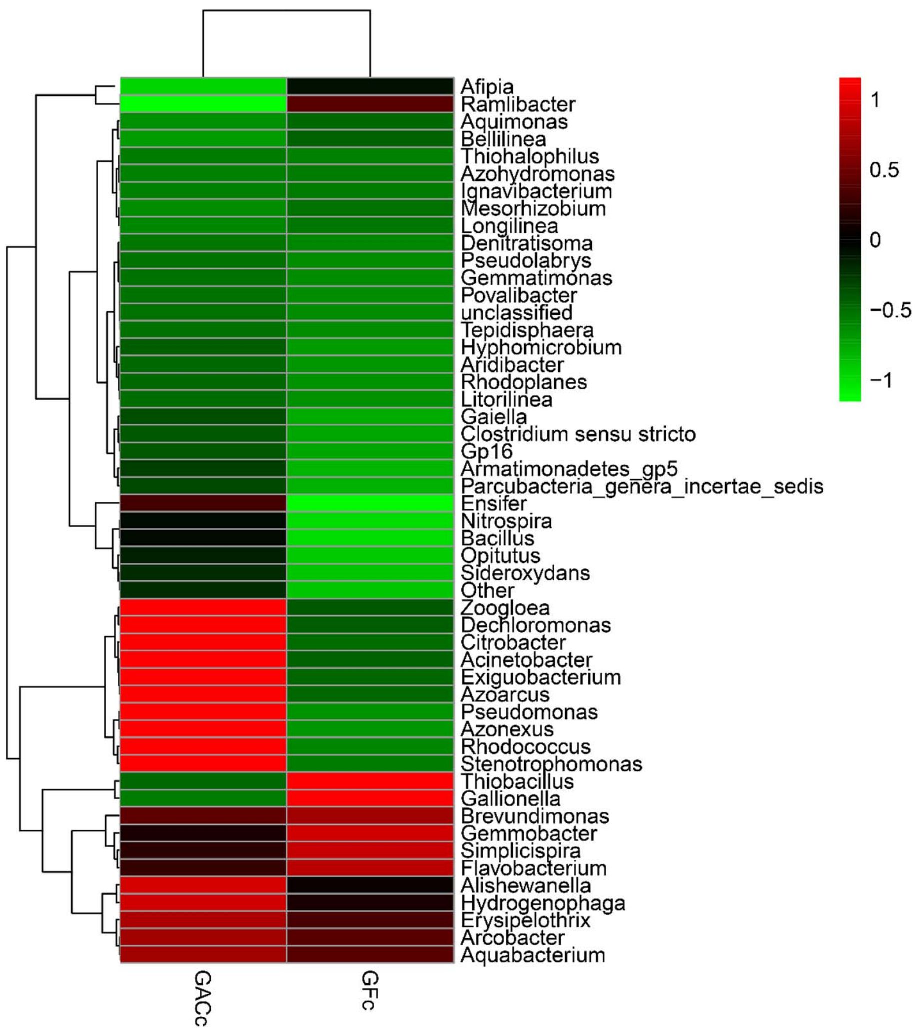

Fig. 4. Heat map of hierarchy cluster for the top fifty genera. The color intensity in each panel respects the similarity characteristic between the two samples. Red and green color respectively mean the high or poor enrichment of a genus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

According to the Venn diagram, the numbers of shared OTUs between samples GACc and GFc was 652, accounting for 51.1% in GACc and 72.7%, in GFc. These results indicated that the microbial compositions of samples GACc and GFc were highly similar, but some unique microorganisms still existed in each sample. The network analysis of the bacterial communities showed that the majority of shared OTUs had higher abundances than those of unshared ones. In addition, many shared OTUs showed quite different distributions for these two samples, such as Otu0, Otu5, Otu11, and Otu12.

3.3. Bacterial community structure associated with nitrogen removal

The 30,174 classifiable sequences from sample GACc were affiliated with 27 phyla, 48 classes, and 318 genera, whereas the 29,565 classifiable sequences from sample GFc were affiliated with 27 phyla, 42 classes, and 220 genera. The phylogenetic classification of sequences at the phylum and class levels from the two samples is shown in Fig. 3. Proteobacteria, Firmicutes, and Bacteroidetes were detected as the dominant phyla in both GACc and GFc, but their relative abundances in each sample were different (Fig. 3a). For example, Proteobacteria was the most dominant phylum both in sample GACc and GFc, but Proteobacteria was more abundant in sample GFc (87.67%) than in sample GACc (74.96%). In contrast, Firmicutes was less abundant in sample GFc (2.67%) than in sample GACc (11.32%). Based on the relative abundance at the class level of taxonomic classifications from Fig. 3b, the classes with an average abundance > 2% in sample GACc were γ-Proteobacteria (33.76%), β-Proteobacteria (21.25%), α-Proteobacteria (13.41%), Bacilli (6.81%), δ-Proteobacteria (5.61%), Actinobacteria (3.01%) and Clostridia (2.87%), whereas those in sample GFc were β-Proteobacteria (61.99%), α-Proteobacteria (12.02%) and δ- Proteobacteria (4.66%). It is obvious that the distributions of the major classes were significantly different for these two samples. In particular, the abundance of β-Proteobacteria in sample GFc was much higher than that in sample GACc, whereas the distributions of γ-Proteobacteria, Bacilli, and Actinobacteria in sample GFc were relatively low compared to those of sample GACc.

To gain deeper insight into the similarities and differences between samples GACc and GFc, a heat map of hierarchical clustering for the 50 abundant genera is shown in Fig. 4. Among the abundant genera with relative abundance > 2%, a significantly higher abundance of *Pseudomonas* (17.29%) was observed in sample GACc, whereas its relative abundance in sample GFc was only 2.12%. Generally, *Pseudomonas* species are widely found in the environment, particularly with regard to their denitrification potential, as identified in previous studies, e.g., *Pseudomonas aureofaciens* (Hosono et al., 2015). Moreover, some bacteria belonging to the genus *Pseudomonas* are capable of both autotrophic and heterotrophic denitrification, such as *Pseudomonas stutzeri* (Szekeres et al., 2002), and *Pseudomonas* sp. C27 (Chen et al., 2013), which can utilize a variety of electron donors (e.g., H_2, reduced sulfur compounds or organic carbon) to perform denitrification. The increasing COD/N ratio could affect the performance of microorganism involved in autohydrogenotrophic denitrification. As is known, the growth yield of autotrophic denitrifiers is lower than that of heterotrophic denitrifiers. When the COD/N ratio was gradually increasing, the autohydrogenotrophic denitrifier may have been gradually domesticated into heterotrophic denitrifier for nitrate removal. Thus, the high abundance of *Pseudomonas* in sample GACc suggested that high similarity with organisms capable of autotrophic and heterotrophic denitrification could be affiliated with the genus *Pseudomonas* in the presence of organic matter and applied current. In addition, a significantly higher abundance of *Exiguobacterium* was also found in sample GACc (6.26%) compared to that in sample GFc (0.73%). *Exiguobacterium* is a potential genus for facilitating sulfur-based denitrifying system (Sahinkaya et al., 2013) and bioelectrochemical denitrification reactors (Chen et al., 2016), which could be involved in the denitrification process of BECWs. Contrary to sample GACc, *Thiobacillus* (43.94%) and *Gallionella* (8.59%) were the two most dominant genera in sample GFc, whereas their abundances in sample GACc were only 5.24% and 0.1%, respectively. *Thiobacillus* has been considered a typical autotrophic bacterium over the last decades, which is closely related to oxidizing the reducing inorganic sulfur (Capua et al., 2016; Liu et al., 2015). In recent years, it has been suggested that some species belonging to the genus *Thiobacillus* play a key role in the cathode-driven bioelectrochemical reduction of nitrate (Chen et al., 2016; Nguyen et al., 2016). More specifically, *Thiobacillus denitrificans* has the ability to assimilate electrons directly from the electrode to facilitate nitrate reduction (Yu et al., 2015). Recently, the high abundance of *Thiobacillus* (59.81%) was enriched in the cathode electrode (carbon fiber felt) collected from a 2DBER coupled with a CW reactor, which is in accordance with the results for sample GFc obtained in this study. In addition, *Gallionella* could also be responsible for autotrophic denitrification process because bacteria affiliated with the genus *Gallionella* have been reported as ferrous-iron-oxidizing nitrate-reducing bacteria (Nordhoff et al., 2017).

It can be concluded that different denitrifying bacteria were enriched in the packed particle electrode (3D electrode) and the plate electrode (2D electrode). The excellent nitrate removal ability of the BECWs was ascribed to the synergy between the predominant *Pseudomonas* in sample GACc and *Thiobacillus* in sample GFc, which facilitated the combined autotrophic and heterotrophic denitrification process. More specifically, the bacteria enriched in the GAC could perform both autotrophic and heterotrophic denitrification, whereas the bacteria enriched in the GFs were mainly involved in autotrophic denitrification. However, to elucidate the synergy mechanism between the 3D electrode and the 2D electrode further, more studies should be conducted to reveal the characteristics of denitrifiers in 3D-BERs and combined systems of such.

4. Conclusion

The denitrification performance of BECW significantly increased with the increasing COD/N ratio, and the highest NO_3^--N and TN removal efficiencies were 91.3 ± 7.2% and 68.8 ± 7.9%, respectively, at the COD/N ratio of 5. High-throughput sequencing technologies were used to characterize the cathodic microbial communities of the GAC and GF electrodes under the mixotrophic condition and revealed different denitrifiers were enriched in the 3D electrode (GAC) and the 2D electrode (GF). Sample GACc was enriched with a high abundance of *Pseudomonas* (17.29%) capable of autotrophic and heterotrophic denitrification, whereas autotrophic bacteria *Thiobacillus* (43.94%) was predominant in sample GFc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.09.045.

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