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Bacterial communities in a novel three-dimensional bioelectrochemical denitrification system: the effects of pH

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Abstract Influence of pH on the dominant microbial community structure in bioelectrochemical system (BES) for nitrate removal is poorly understood. Here, the dynamics and variations of microbial communities were investigated with pH varied from 6.0 to 9.0 in a novel three-dimensional BES (3D-BES). The maximum nitrate removal efficiencies 97.58 and 96.36 % were obtained at pH 7.0 and 8.0, due to the main contributions of bacterial phylum *Firmicutes* and class *Clostridia*. The abundances of dominant phyla and classes tended to decrease under pH 6.0 and 9.0 conditions. Additionally, phylum *Proteobacteria* and class *Gammaproteobacteria* preferred acid environment in the BES, while phylum *Chloroflexi* and class *Bacilli* and *Betaproteobacteria* preferred alkaline environment. Furthermore, the excellent nitrate removal ability of the 3D-BES was ascribed to the presences of genera *Exiguobacterium*, *Proteiniclasticum*, *Pseudomonas*, *Planococcus*, *Thauera*, *Azoarcus*, *Thiobacillus*, etc. These genera facilitated the combined autotrophic denitrification process so that this system achieved excellent nitrate degradation efficiency.

Keywords Microbial communities · 3D-BES · Nitrate · *Firmicutes* · *Clostridia*

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Introduction

Because excessive nitrate in waters always leads to human diseases such as gastrointestinal cancer, blue baby syndrome, and methemoglobinemia (Zhang et al. 2014a), besides high amounts of nitrate in natural waters often cause eutrophication (Wang et al. 2014), numerous kinds of technologies such as reverse osmosis; ion exchange; electrodialysis; biological denitrification; and physicochemical, chemical, and electrochemical processes (Epsztein et al. 2015; Kondaveeti et al. 2014) are conducted to eliminate nitrate in waters. However, considering the operating cost and removal efficiency, bioelectrochemical process is deemed as an effective and economical method for nitrate remediation (Kondaveeti et al. 2014).

Nowadays, bioelectrochemical systems have been widely used for contaminant degradation. Lim and Kim (2015) developed an integrated ion exchange and bioelectrochemical system for organic matter and total nitrogen removal and obtained excellent removal efficiencies. Guo and coworkers (Guo et al. 2015) established a bioelectrochemical reactor to degrade 4-chloronitrobenzene and achieved 93.7 % removal efficiency at initial concentration 20 mg/L with 0.5 V voltage. Bonmati et al. (2013) prepared a continuous bioelectrochmical reactor to removal oxalate and demonstrated that anode microbial community showed a shift during the start-up phase. In the research of Zhang and coworkers (Zhang et al. 2014b), a new bioelectrochmical reactor was prepared for nutrient removal, which showed that phosphate was removed by ion exchange and nitrogen was removed by current generation. Li et al. (2014) developed a fluidized bed membrane bioelectrochemical reactor for energy-efficient wastewater treatment process and obtained satisfied effects. Huang et al. (2013) discussed the effect of C/N ration on nitrogen removal using a bioelectrochemical reactor and evaluated the removal rates and anode transformation

efficiency. Wang and coworkers (Wang et al. 2015a) summarized the developments and advantages of bioelectrochemical systems for environmental remediation. In the study of Ghafari and coworkers (Ghafari et al. 2008), numerous bioelectrochemical systems for nitrate removal were discussed and analyzed. Kong et al. (2014) used bioelectrochemical system for 4-chlorophenol dechlorination process and obtained excellent removal efficiency. Although numerous researches had investigated the pollutant removal efficiencies, fewer studies had explored the bacterial communities and structures to deeply discuss the relationship between pollutant removal and dominant bacterial community in bioelectrochemical system (BES).

In the present study, a novel three-dimensional bioelectrochemical denitrification system was developed to achieve high concentration of nitrate removal and evaluate the microbial phylogenetic and dominant communities under different pH condition through Illumina Miseq pyrosequencing. The novel BES system possessed not only the advantage of balanced pH environment but also the excellent nitrate removal efficiency due to the reason that H^+ produced from sulfur-based autotrophic denitrification process would be utilized by hydrogen-based autotrophic denitrification process, and the concurrence of two autotrophic denitrification processes significantly improved nitrate removal ability. The exploration of this work would profoundly reveal the removal mechanisms of BES.

Materials and methods

Experimental setup

The continuous three-dimensional BES (3D-BES) (working volume 3.0 L) was developed using carbon fiber felt as cathode, graphite rod (diameter 8 mm) as anode, and sulfur granule (diameter 5–8 mm) as particle carrier (Fig. 1). The cathode and anode were connected by a direct current power.

At the initial phase, the continuous-flow reactor was started for 60 days at current 10 mA, hydraulic retention time (HRT) 16 h, pH 7.0, and temperature 25 °C condition with anaerobic sludge (MLSS 3500 mg/L) collected from Erlangmiao Municipal Wastewater Treatment Plant, Wuhan, China. The medium was NO_3^--N 50 mg/L, HCO_3^- 70 mg/L, $MgCl_2$ 10 mg/L, $ZnCl_2$ 0.50 mg/L, $CoCl_2$ 2.00 mg/L, $MnSO_4$ 1.00 mg/L, $NiCl_2$ 0.30 mg/L, $CuCl_2$ 0.30 mg/L, $FeSO_4$ 0.20 mg/L, $CaCl_2$ 0.50 mg/L, and Na_2MoO_4 0.30 mg/L.

Reactor operation

The BES was operated for 240 days by adjusting pH from 6.0 to 9.0 to evaluate the effect of pH on the BES. The initial nitrate concentration was maintained at high value 200 mg/L
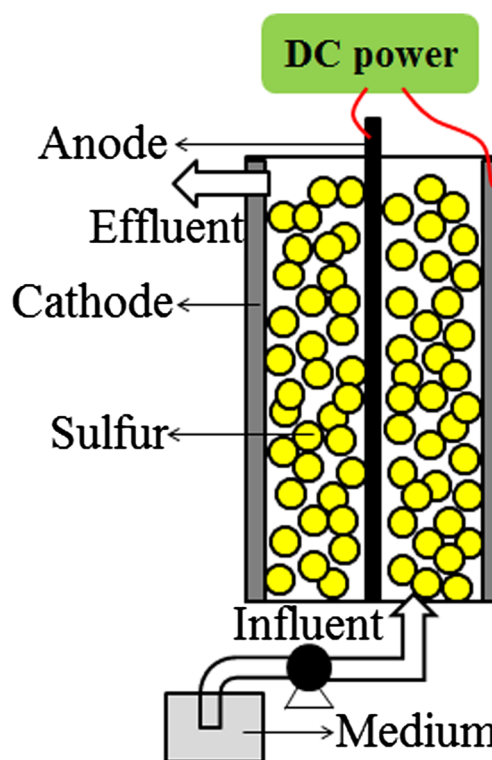

Fig. 1 Schematic diagram of the BES

with current 100 mA, HRT 16 h, and temperature 25 °C during the experiment. The operational procedure of the reactor was summarized in Table 1. The concentrations of NO_3^-, NO_2^-, N_2, and SO_4^{2-} were measured to assess nitrate removal efficiency of this system under different pH environment.

Analytical methods

The pH value in the reactor was adjusted using NaOH and HCl. The samples during the experiments were filtered by 0.30-μm membrane by a suction filter machine. NO_3^--N was measured by ultraviolet spectrophotometric method using spectrophotometer at 220–275 × 2 nm. NO_2^--N was measured by N-(1-naphthyl) ethylenediamine dihydrochloride spectrophotometric method using spectrophotometer at 540 nm. Nitrogen gas (N_2) was measured by an Agilent HP4890D gas chromatography. NH_4^+-N was measured by Nessler's reagent spectrophotometer method at 420 nm. SO_4^{2-}-S

Table 1 Operational procedure of the reactor

Operation	pH	Nitrate concentration (mg/L)	Current (mA)	HRT (h)	Temperature (°C)
Day 0–60	6.0	200	100	16	25
Day 61–120	7.0	200	100	16	25
Day 121–180	8.0	200	100	16	25
Day 181–240	9.0	200	100	16	25

concentration was measured by ion chromatograph (881 Compact IC Pro, Metrohm, Switzerland). The pH was measured using a pH meter (PHS-3C, Kexiao Instrument, China). The water temperature was measured by a thermometer (TM827, Zhugongda Instrument, China).

DNA extraction and Miseq pyrosequencing

Four biofilm samples, D1, D2, D3, and D4 at pH 6.0, 7.0, 8.0, and 9.0 conditions, were collected, and the bacterial genomic DNA was extracted by PowerSoil DNA Extraction Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The PCR amplification, pyrosequencing procedure, and sequence analyses followed the method described in our previous study (Chen et al. 2015). Especially, the primer sequences were 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 16S rRNA gene sequences of the four samples are available at NCBI Sequence Read Archive (accession number SRX1611191).

Results

BES performances with different pH environment

The concentrations of NO_3^--N, NO_2^--N, N_2, and SO_4^{2-}-S in the effluent were given in Fig. 2. The average nitrate removal efficiencies were 77.12, 97.58, 96.36, and 88.48 % at pH 6.0, 7.0, 8.0, and 9.0, respectively, which indicated that 200 mg/L nitrate was almost completely degraded under pH 7.0–8.0 condition in the BES. Besides, the contents of intermediate product nitrite were near zero at pH 7.0–8.0 condition, while the accumulated nitrite (maximum value 8.51 mg/L) was observed at pH 6.0 and 9.0. In addition, when pH varied from
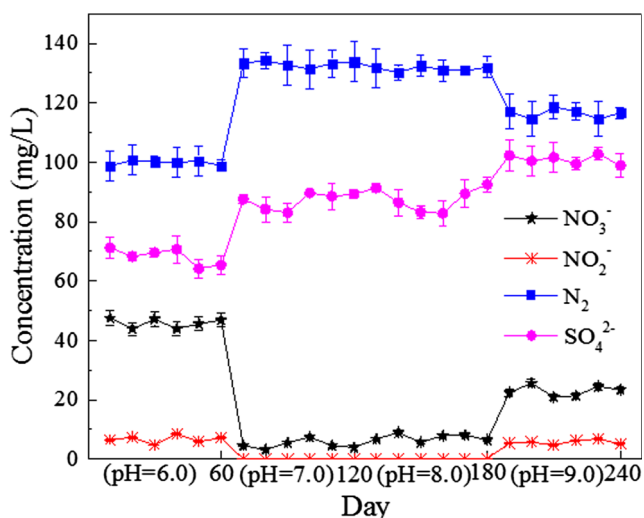

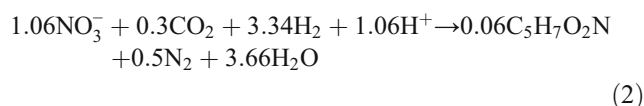**Fig. 2** Concentrations of main components in the effluent under different pH conditions

6.0 to 9.0, the produced N_2 concentrations reached 99.79, 133.08, 131.33, and 116.42 mg/L, which confirmed that the main ultimate product was nitrogen gas in this novel 3D-BES. Moreover, the average SO_4^{2-}-S concentrations were 68.23, 87.02, 87.62, and 100.91 mg/L with pH increase from 6.0 to 9.0, which was lower than the limited value 250 mg/L (China EPA). The concentrations of NH_4^+-N were varied from 0.02 to 0.64 mg/L.

In this 3D-BES, the main nitrate degradation process was displayed in Eqs. (1) and (2).

Sulfur-based autotrophic denitrification process (S-process)

$$1.06NO_3^- + 1.11S + 0.3CO_2 + 0.785H_2O \rightarrow 0.06C_5H_7O_2N \\ + 0.5N_2 + 1.11SO_4^{2-} + 1.16H^+$$

$$(1)$$
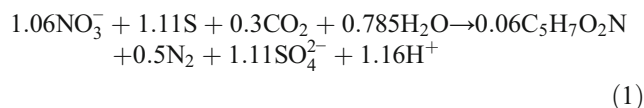
Hydrogen-based autotrophic denitrification process (H-process)

$$1.06NO_3^- + 0.3CO_2 + 3.34H_2 + 1.06H^+ \rightarrow 0.06C_5H_7O_2N \\ + 0.5N_2 + 3.66H_2O$$

$$(2)$$

On the one hand, a portion of nitrate was converted to nitrogen gas by consuming S and then generated SO_4^{2-} and H^+ ions. On the other hand, the produced H_2 on the cathode was used as electron donor to reduce the other part of nitrate to nitrogen gas, and H^+ ion produced by S-process was consumed during the H-process. And because of this, the novel 3D-BES possessed promising nitrate removal efficiency and sustainable operation.

Equations (1) and (2) also show that pH played a vital role in the 3D-BES because the nitrate removal process would generate H^+ ions and consume H^+ ions. Furthermore, pH always affected the BES process through influencing the electric conductivity by decomposing the carbonate ions (Mook et al. 2013). Under alkaline environment, OH^- could react with carbonate ions and then generated carbonate ion, while carbon dioxide was produced under acid environment so that the inorganic carbon for combined autotrophic denitrification process was decreased and thus decreasing the nitrate removal efficiency. Additionally, the unsuitable pH would inhibit the activity of autotrophic microorganism, thus affecting the denitrification efficiency (Mousavi et al. 2012). However, in this novel BES, when initial pH was 7.0–8.0, the effluent pH was maintained at 6.8–7.8 (Fig. 3), suggesting that this BES could control the system pH at neutral condition and thus facilitating the denitrification efficiency. Besides, the average effluent pH were 6.6 and 8.3 at initial pH 6.0 and 9.0.

Moreover, the variation of effluent SO_4^{2-} concentrations showed that pH value would influence the proportions of S-process and H-process in the BES. At acid environment, the S-
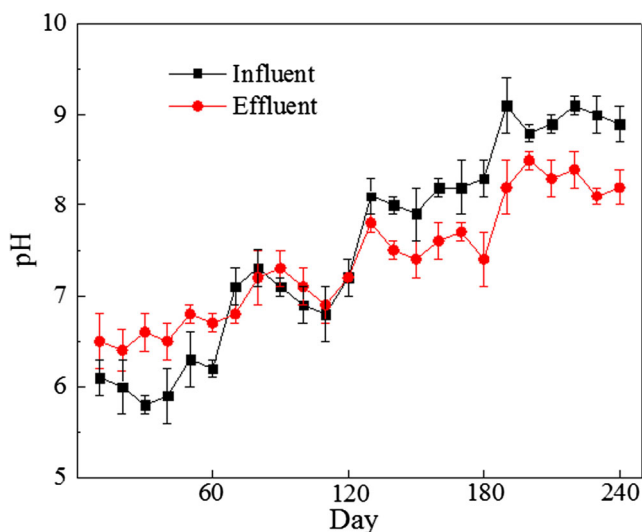
Fig. 3 pH values in the influent and effluent

process was weakened because excessive H^+ ions would reduce the S-process (Eq. (1)) whereas enhance the H-process (Eq. (2)) so that SO_4^{2-} concentrations were relatively lower. However, the S-process efficiency was improved and the SO_4^{2-} concentrations were higher at pH 9.0 due to the increasing OH^- ions.

Bacterial diversity and community composition

In order to further investigate the microbial community diversity, phylogenetic analyses of the gene sequences were performed in phylum, class, genus, and species levels. The identified phyla of sample D1–D4 were shown in Fig. 5a. Overall, most of the identified phyla of the four samples were *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. For D1 at pH 6.0 condition, the abundances of the three major phyla were *Proteobacteria* (40.05 %), *Firmicutes* (39.64 %), and *Actinobacteria* (12.93 %). At pH 7.0, the main phyla were *Firmicutes* (68.60 %), *Proteobacteria* (23.71 %), and *Actinobacteria* (3.48 %), while the proportions of the prominent phyla were *Firmicutes* (72.39 %), *Proteobacteria* (19.54 %), and *Actinobacteria* (3.87 %) under pH 8.0 condition. Besides, the phyla distribution turned to *Firmicutes* (48.09 %), *Proteobacteria* (35.80 %), and *Actinobacteria* (8.20 %) when pH was 9.0. Because the best nitrate removal

efficiencies were achieved at pH 7.0–8.0 environment in the BES, phylum *Firmicutes* was considered to be the most dominant community in this novel BES.

Using the Miseq high-throughput pyrosequencing 30,244, 35,572, 40,329, and 42,483 high-quality reads for samples D1, D2, D3, and D4 were obtained. Besides, the corresponding number of operational taxonomic units (OTUs), Ace, Chao, Shannon, Simpson, and Coverage were calculated and given in Table 2. The Coverage values of four samples were greater than 99 %, suggesting that the diversities of microbial communities in the BES were covered by the sequences (Shu et al. 2015). The OTU numbers of D1 and D4 were 646 and 603, which were greater than that of D2 429 and D3 358, which not only demonstrated that the BES possessed abundant bacterial communities but also indicated that the community abundances under pH 6.0 and 9.0 were higher than that of pH 7.0–8.0. In addition, the values of Ace, Chao, Shannon, and Simpson of D1–D4 showed the similar trends as OTU, which also proved that there were more communities in the BES at pH 6.0 and 9.0 environments. The reason for this phenomenon might be that more communities would appear under extreme pH environment in this bioelectrochemical system, which also indicated that this system possessed abundant communities under extreme pH environment. Figure 4 displayed the Venn diagram of the four samples, which showed that the four samples shared only 248 OTUs (12.18 % of the total OTUs) in the total of classified 2036 OTUs, indicating that the microbes in the four samples were rather different from each other in the BES at different pH condition. Based on the richness and abundance analyses, it could be concluded that the novel BES contained abundant bacteria and pH condition was important for the bacteria to degrade nitrate.

On class level, the major classes of the four samples were *Clostridia*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, and *Bacilli* (Fig. 5b). For D1, the abundances of the major classes were *Clostridia* (32.32 %), *Alphaproteobacteria* (19.51 %), *Gammaproteobacteria* (11.93 %), *Actinobacteria* (7.52 %), *Betaproteobacteria* (7.08 %), *Bacilli* (5.45 %), and *Erysipelotrichia* (1.81 %). For D2, the abundances were *Clostridia* (56.54 %), *Gammaproteobacteria* (10.51 %), *Bacilli* (7.93 %), *Alphaproteobacteria* (7.04 %), *Betaproteobacteria* (6.00 %), and *Actinobacteria* (2.30 %). The proportions of the main classes were *Clostridia*

Table 2 Similarity-based OTUs, species richness, and diversity estimation of the determined bacteria on sample D1–D4

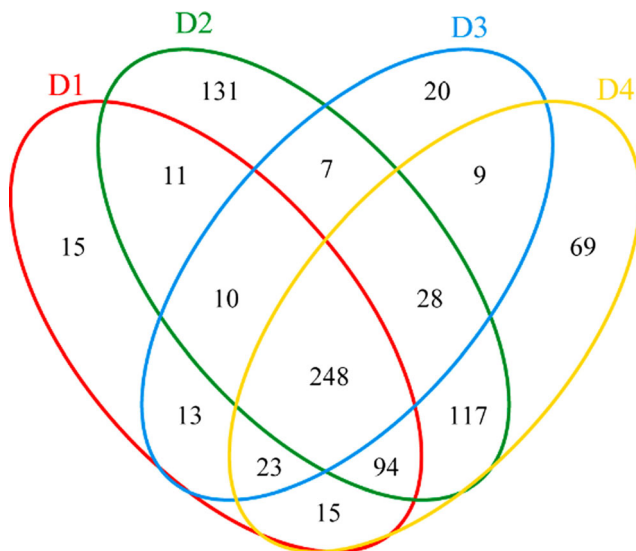| Sample	Reads	OTU	Ace	Chao	Shannon	Simpson	Coverage
D1	30,244	646	740.4480	752.4189	4.446025	0.033110	0.995834
D2	35,572	429	554.0507	585.0222	3.304665	0.095149	0.996655
D3	40,329	358	427.2634	441.0000	2.787227	0.173897	0.997942
D4	42,483	603	704.0160	724.2273	4.020901	0.071139	0.997011

Fig. 4 Venn diagram of shared OTUs between D1, D2, D3, and D4

(51.47 %), *Bacilli* (20.75 %), *Gammaproteobacteria* (8.91 %), *Alphaproteobacteria* (5.75 %), *Betaproteobacteria* (4.67 %), and *Actinobacteria* (2.86 %) for D3 under pH 8.0 condition. While, under pH 9.0, the main components were *Bacilli* (27.51 %), *Betaproteobacteria* (21.10 %), *Clostridia* (19.25 %), *Alphaproteobacteria* (8.79 %), *Gammaproteobacteria* (4.75 %), and *Actinobacteria* (4.25 %). Based on the class abundance results, *Clostridia* and *Bacilli* were the dominant classes due to the reason that the maximum nitrate removal efficiency was obtained at pH 7.0–8.0, and the most abundant classes were *Clostridia* and *Bacilli* at pH 7.0–8.0 as well.

Discussion

As reported by some literatures, *Firmicutes* and *Proteobacteria* often participated in the denitrification process. In the research of Wang et al. (2015b), the dominant phyla of the denitrification system contained *Proteobacteria* and *Firmicutes*, which confirmed that *Proteobacteria* and *Firmicutes* exhibited potential nitrate removal abilities. Liu and coworkers (Liu et al. 2014) discovered that *Proteobacteria*, *Firmicutes*, and *Actinobacteria* in a bioelectrochemical system harbored nitrate reductive genes and thus possessed denitrification abilities. In the study of Liu et al. (2015), *Proteobacteria* phylum was proved to be the most abundant denitrification bacteria in the nitrogen removal system. Zhang and coworkers (Zhang et al. 2015) demonstrated that *Proteobacteria* was the dominant phylum in a sulfur-based autotrophic system. In the present study, the most important contributor for nitrate removal through combined sulfur-based and hydrogen-based autotrophic denitrification process was phylum *Firmicutes* in this novel BES due to the

fact that *Firmicutes* was the most abundant phylum when highest nitrate removal efficiency was achieved at pH 7.0–8.0 in this system. In addition, *Proteobacteria* and *Actinobacteria* also showed good nitrate removal abilities because 77.12 and 88.48 % nitrate removal efficiencies were obtained under pH 6.0 and 9.0 condition. The above analyses also indicated that *Firmicutes* was the dominant community for denitrification process in the BES and worked best under pH 7.0–8.0 environment. Otherwise, *Proteobacteria* and *Actinobacteria* preferred acid or alkaline environment in the BES.

Clostridia and *Alphaproteobacteria* might have significant contributions to nitrate removal. Lee et al. (2013) demonstrated that class *Clostridia* was the main community in autotrophic denitrification process and proved that *Clostridia* exhibited denitrifying activity in the bioelectrochemical denitrification system. Mao et al. (2013) indicated that most reported hydrogenotrophic denitrification bacteria belonged to α-, β-, and γ-*proteobacteria* classes, and α-*proteobacteria* was the dominant class in the hydrogen-oxidizing autotrophic denitrifying system. In the study of Kondaveeti et al. (2014), class *Alphaproteobacteria* played a vital role in the nitrate reduction process in a bioelectrochemical denitrification system. In this BES, *Clostridia* were the most important contributor for highest nitrate removal under pH 7.0–8.0 condition, followed by the class *Bacilli*, *Gammaproteobacteria*, *Alphaproteobacteria*, etc. Additionally, class *Bacilli* tended to prefer alkaline environment so that the most abundant class for pH 9.0 condition was *Bacilli*, while *Gammaproteobacteria* was the most abundant class under pH 6.0 environment, meaning that acid environment was more suitable for class *Gammaproteobacteria* in the BES. The above analyses suggested that pH 7.0–8.0 was most suitable for effective class *Clostridia*, and thus facilitating autotrophic denitrification efficiency, which also demonstrated that class *Clostridia* was the most important and dominant communities for achieving highly effective nitrate degradation in this novel BES.

The similarity and differences of the four community structures were investigated using the microbial community heat map analysis (Fig. 6). Results showed that the most abundant genera of the four samples were different from each other, suggesting that pH had obvious effect on bacterial community in this BES. The major genus of D1–D3 was *Peptostreptococcaceae_incertae_sedis*, while *Anaerobacillus* was the most abundant genus in D4. In addition, *Exiguobacterium*, *Proteiniclasticum*, *Pseudomonas*, *Planococcus*, *Thauera*, *Azoarcus*, *Thiobacillus*, etc. also played vital roles during the combined autotrophic denitrification process in the BES. As reported, *Peptostreptococcaceae* was always excited in anaerobic environment (Lin et al. 2015), which might be essential for nitrate removal in the BES. *Exiguobacterium* was proven to be a potential and promising genus for
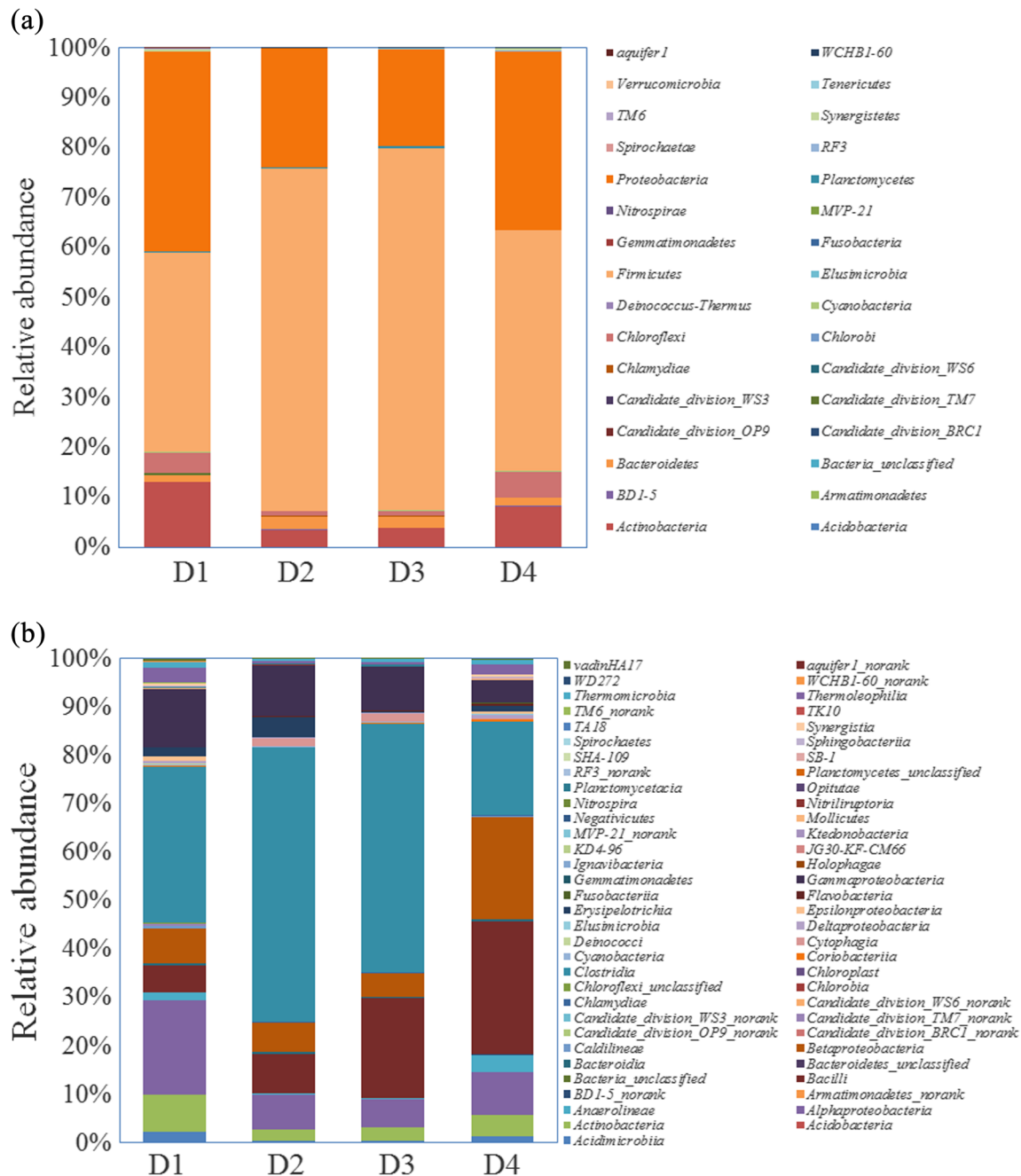
(a)

(b)

Fig. 5 Taxonomic classification of bacterial 16S rRNA gene reads at **a** phylum level and **b** class level

denitrification process in the denitrifying sulfide system by the study of Huang et al. (2015). Similarly, Sahinkaya and coworkers (Sahinkaya et al. 2013) indicated that genus *Exiguobacterium* played a key role in a sulfur-based denitrification process. Thus, the genus *Exiguobacterium* in this BES could degrade nitrate and performed good sulfur-based autotrophic denitrification. *Thauera* and *Thiobacillus* were both considered as the main contributors for nitrate removal in the research of Huang and colleague (Huang et al. 2015). Yu et al. (2015) also suggested that *Thiobacillus* was an effective autotrophic

denitrification bacterium in the bioelectrochemical system. Christianson and coworkers (Christianson et al. 2015) demonstrated that *Azoarcus* sp. (KH32C and BH72) and *Thauera* sp. MZ1T appeared more frequently in the sulfur-based autotrophic denitrification biofilters. In the research of Hosono and colleagues (Hosono et al. 2015), *Pseudomonas* and *Thiobacillus* contributed essential roles for denitrification process. In this novel BES, the observed excellent nitrate removal ability was ascribed to the presences of these reported genera *Exiguobacterium*, *Proteiniclasticum*, *Pseudomonas*, *Planococcus*, *Thauera*, *Azoarcus*, *Thiobacillus*, etc. These
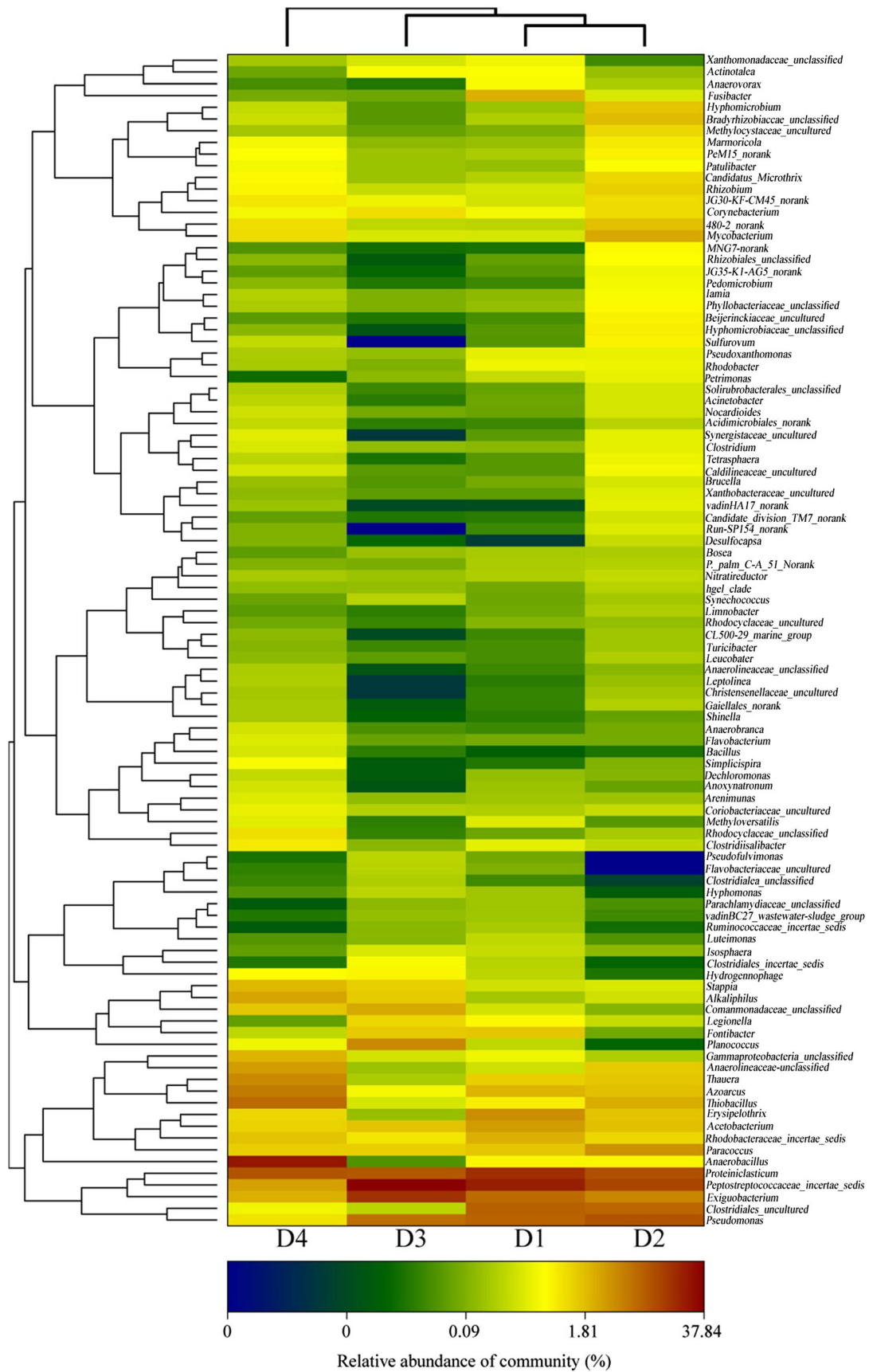
Fig. 6 Richness heat map of microbial communities at genus level

genera coexisted in the BES, facilitating the combined autotrophic denitrification process so that the BES achieved excellent nitrate degradation efficiency. Furthermore, *Proteiniclasticum*, *Planococcus*, and *Anaerobacillus* genera might be valuable for the BES and needed to conduct much deep research.

Based on the evaluation of nitrate removal, pH played a vital role in the 3D-BES. At acid environment, the S-process was weaker than H-process, while H-process was enhanced under alkaline environment. The dominant bacterial phyla and classes showed obvious migrations with pH varied from 6.0 to 9.0. The highest nitrate removal efficiency was achieved at pH 7.0–8.0, which was ascribed to the most abundant phylum *Firmicutes* and class *Clostridia*. Thus, phylum *Firmicutes* and class *Clostridia* were the most important contributors for nitrate degradation in this novel 3D-BES.

Compliance with ethical standards This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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