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Simultaneous degradation of anodic nitrate and cathodic sulphate in a bioelectrochemical reactor: evaluation of degradation efficiency and characterization of microbial communities

Jing Chen[a,b], Hao Xu[c,d,*], Na Tong[c,d], Shu He[e], Shaobin Huang[c,d,*], Yongqing Zhang[c,d]

[a]*School of Hydraulic Engineering, Changsha University of Science & Technology, Changsha 410114, China*
[b]*Key Laboratory of Water-Sediment Sciences and Water Disaster Prevention of Hunan Province, Changsha 410114, China, email: 1016620087@qq.com*
[c]*School of Environment and Energy, South China University of Technology, Higher Education Mega Center, Guangzhou 510006, China, emails: Eden_Hao_Xu@163.com (H. Xu), 1224013722@qq.com (N. Tong), chshuang@scut.edu.cn (S. Huang), zhangyq@scut.edu.cn (Y. Zhang)*
[d]*Guangdong Provincial Key Laboratory of Atmospheric Environment and Pollution Control, Guangzhou 510006, China*
[e]*School of Environment, Harbin Institute of Technology, Harbin 150000, China, email: sharon_he0@163.com*

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ABSTRACT

Sulphate and nitrate as respiratory electron acceptors widely exist in various water bodies and are potentially harmful contaminants. In this study, we hypothesized that simultaneous removal of anodic nitrate and cathodic sulphate in a bioelectrochemical reactor (BER) can be accomplished. Results indicated that the average nitrate removal efficiency, sulphate removal efficiency, nitrite production efficiency, and ammonia production efficiency were 38.84, 22.10, 4.56 and 0.38 mg/L, respectively. Simultaneous degradation of anodic nitrate and cathodic sulphate in BER was accomplished, although the obtained results suggested that the removal efficiencies of nitrate and sulphate were not as good as those of conventional biological treatments. The existence of dominant species *Pseudomonas* and *Azoarcus* in the anode proved that nitrate was reduced by nitrate-reducing bacteria with acetate as the electron donor. In the cathode, *Desulfomicrobium* and *Thauera* were the main functional bacteria for sulphate reduction.

Keywords: Bioelectrochemical reactor (BER); Anodic nitrate removal; Cathodic sulphate removal; Bacterial community structure

1. Introduction

Sulphate and nitrate as respiratory electron acceptors widely exist in industrial and municipal wastewater and natural water bodies and are potentially harmful contaminants [1]. Nitrate is responsible for the eutrophication of aquatic systems and can be converted into nitrites, which can cause methemoglobinemia and gastric cancer [2]. Sulphate is not considered a threat to animal or human health, but its main reduction products (including SO_2 and H_2S) can pose problems to the ecological environment and human health [3,4]. Therefore, given the increase in nitrate and sulphate concentrations in various water bodies, treatment of these contaminants has become an urgent problem. Biological treatments for nitrate- and sulphate-containing wastewater have been extensively investigated. For example, the expanded granular sludge blanket reactor was developed to accomplish simultaneous removal of sulphate and nitrate under different dissolved oxygen (DO) concentrations [5–7]; the results indicated that an equilibrium relationship exists between optimal DO concentration and nitrate and sulphate reduction. In addition, a novel biological nitrogen removal process called nitrate reduction, autotrophic denitrification, and nitrification integrated process was proposed and scaled up to a

* Corresponding authors.

800–1,000 m^3/d full-scale demonstration plant [8,9]. However, in several cases, nitrate and sulphate are not contained in one water body and separated instead; in such cases, two types of wastewater (containing sulphate or nitrate) exist. In this study, we hypothesized that sulphate- and nitrate-containing wastewater can be simultaneously treated in one bioreactor and need not be premixed. The bioelectrochemical reactor (BER) may be a good approach for this hypothesis because it was developed for different operating modes required in different applications [10]. BER consists of an anode chamber and a cathode chamber; two types of wastewater can be added to the two separate chambers to accomplish the removal of target pollutants.

Previous reports have indicated that anode-respiring bacteria (ARB) possess nitrate reduction capabilities because several of them, including *Geobacter* spp. and *Shewanella* spp., have an alternative dissimilatory metabolism for electrode reduction [11,12]. This finding proves the feasibility of anodic nitrate degradation. In addition, Kashima and Regan [12] indicated that anode-reducing biofilms facultatively reduce nitrate at all anode potentials (−150 to +900 mV vs. standard hydrogen electrode) with a rapid metabolic shift. Sulphate can also be reduced by sulphate-reducing bacteria (SRB) in BER biocathode [13]. Thus, this study attempted to add nitrate-containing wastewater into the anode and sulphate-containing wastewater to the cathode for further degradation by various functional microorganisms. With regard to anodic nitrate reduction by ARB, acetate as an organic carbon resource can make the nitrate reduction process relatively stable (stable current and biomass production) [12]. Thus, in this study, acetate as an organic carbon resource was used for nitrate and sulphate reduction. In addition, BER can be applied at a potential of 0.2 V vs. Ag/AgCl to the anode, and this value is optimal for the growth of electrochemical active bacteria [14,15].

Simultaneous removal of various pollutants in BER has been widely investigated. For example, simultaneous nitrification, denitrification (at the cathode), and carbon removal (at the anode) in BER [16]; simultaneous phenol removal, nitrification, and denitrification in biocathode [17]; and complete nitrogen removal and electricity production in *Thauera*-dominated air-cathode single-chambered BER [18] have been accomplished. However, to the best of the authors' knowledge, only a few studies have focused on the simultaneous removal of anodic nitrate and cathodic sulphate. Thus, in this study, indexes, such nitrate removal efficiency (NRE), sulphate removal efficiency (SRE), nitrite production efficiency (NPE), and ammonia production efficiency (APE), were estimated to evaluate treatment performance. Illumina high-throughput sequencing technology was used to characterize the bacterial community quantitatively.

2. Materials and methods

2.1. Setup of reactors

The BER, as described in detail in an earlier study [19], is H-shaped with two chambers separated by a cation-exchange membrane (Nafion®N-117 membrane, 0.180 mm thick, ≥0.90 meq/g exchange capacity, CAS: 31175-20-9·d. 1.98). The working volume of each chamber is 100 mL. A CHI1000C potentiostat (Shanghai CH Instrument Company, China) connected to a three-electrode system was used to control the different external voltages. The cathode was used as the working electrode, and the anode served as the counter electrode. The Ag/AgCl electrode (CHI111, Shanghai CH Instrument Company, China) was placed in the cathode chamber as the reference electrode. Carbon felt was chemically treated as described in a previous report [20] and used as the cathode material. A graphite plate was utilized as the anode material. Unless otherwise stated, all potentials reported throughout this paper are relative to that of the Ag/AgCl electrode.

Each of the cathode chambers was inoculated with 30 mL of activated sludge obtained from a sludge-thickening tank at Guangzhou Lijiao Sewage Treatment Plant in Guangdong, China. Numerous microorganisms, which exhibited high activity in the inoculated sludge, were observed by microscopic examination. The microorganisms showed a relatively clear (transparent) appearance and structure. The value of the mixed liquor volatile suspended solids (MLVSS)/mixed liquor suspended solids (MLSS) was 0.605. The components of the basal medium in the cathode were 100 mM phosphate buffer solution (PBS), 50 mg L^{-1} of SO_4^{2-}, 10 mL/L of vitamin solution, and 20 mL/L of mineral solution. The vitamin and mineral solutions were formulated as instructed in a previous report [17]. The components of the basal medium in the anode were as follows: 100 mM PBS, 50 mg L^{-1} of NO_3^-, 10 mL/L of vitamin solution and 20 mL/L of mineral solution. Before placing the synthetic wastewater in the reactor, N_2 was added continuously for more than 30 min to reduce the DO concentration. Anaerobic conditions were maintained. All experiments were performed at a laboratory temperature (26°C ± 3°C), and pH was maintained within the range of 6.5–7 for all experiments. The operation time of all reactors was 140 d, and each operation cycle lasted for 7 d. After each operation cycle, the water exchange rate of the anode and cathode was 80% and 80%, respectively.

2.2. Measurements and analysis

All samples were filtered through a 0.22 μm filter before analysis. The concentrations of NH_4^+, NO_3^-, and NO_2^- in the influent and effluent were measured periodically through a previously established method [21–23]. The sulphate concentration in the aqueous phase was measured using an ICS-1000 ion chromatography system (Dionex, USA) with an IonPac AS14 anion column.

2.3. Bacterial analysis using high-throughput sequencing

The sludge samples from BER were labelled as R-S and R-N, and the inoculated sludge was labelled as seed. The genomic DNA of each sample was extracted using an E.Z.N.ATM Mag-Bind Soil DNA Kit (Omega Bio-tek, USA). The integrity of the extracted DNA was checked via agarose gel electrophoresis. A Qubit2.0 DNA kit (Life Technologies, China) was used for precise quantification of the genomic DNA and to control the amount of DNA added to the mixture for the polymerase chain reaction (PCR). A set of primers was utilized to amplify the hypervariable V3–V4

region of the bacterial 16S rRNA gene. After amplification, the PCR products were purified with Agencourt AMPure XP Beads (Beckman Coulter Inc., Brea, California, USA), and the DNA concentrations in the purified products were measured using a Qubit2.0 DNA kit (Life Technologies, China). High-throughput gene sequencing was performed with the Illumina MiSeq platform manufactured by Sangon Biotechnology, Co., Ltd. (Shanghai, China). Raw sequence data were quality filtered and analyzed using QIIME v1.8.0. High-quality representative sequences for each operational taxonomic units (OTUs) were assigned using Usearch v5.2.236 with 97% sequence identity. A Venn diagram was created using VennDiagram v1.6.16. Principal component analysis was performed to evaluate the differences in microbial community of the samples using R v3.2.

3. Results and discussion

3.1. Performance

As shown in Fig. 1a, the average NRE, SRE, NPE and APE were 38.84, 22.10, 4.56 and 0.38 mg/L, respectively. Although the removal efficiencies of nitrate and sulphate were not as excellent as those of conventional biological treatments [24], simultaneous degradation of anodic nitrate and cathodic sulphate in BER was accomplished. In the cathodic sulphate degradation process, the removal efficiency of sulphate was lower than that of nitrate because compared with SRB, nitrate-reducing bacteria (NRB) can better obtain electrons. SRB grows proportionally slower than NRB, and NRB can potentially obtain more energy than SRB [23,25].

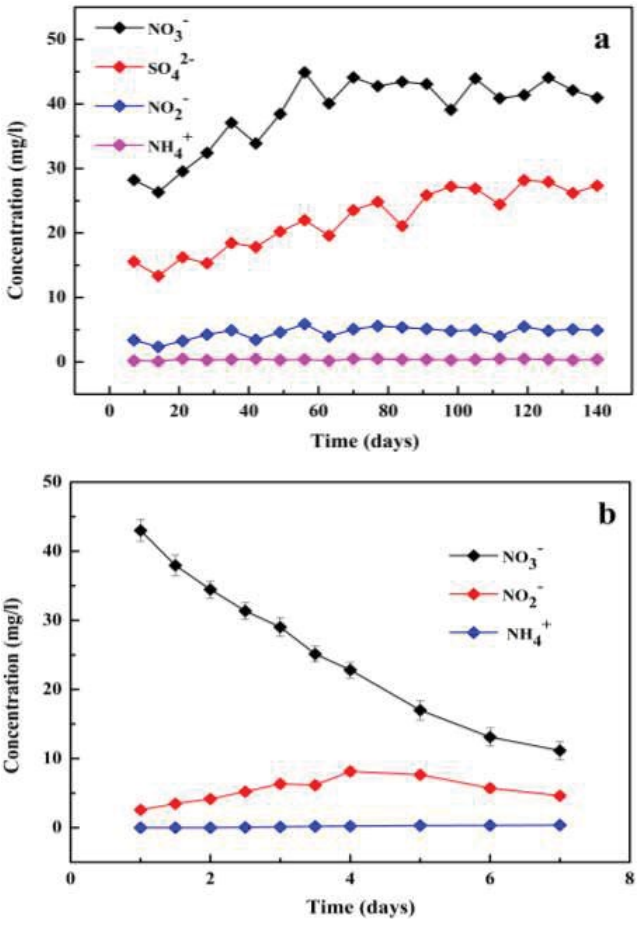

Fig. 1. (a) Degraded concentrations of nitrate and sulphate and the produced concentrations of nitrite and ammonia and (b) removal profile of nitrate and producing profiles of nitrite and ammonia in a running cycle.

In the anodic nitrate degradation process, the produced results are in agreement with previous studies' findings that the production of nitrite and ammonia exists [11,12]. Nitrite is an important intermediate in nitrate degradation regardless of denitrification. Fig. 1 shows a significant accumulation of nitrite concentration in running cycles; it increased in the beginning then decreased. Meanwhile, the existence of ammonia suggests that nitrate was reduced to ammonia via dissimilatory nitrate reduction to ammonia (DNRA). DNRA is a sequential two-step reduction of nitrate to ammonia with nitrite as the intermediate, and reports have suggested that energy generation is mostly from the first step, and the second step is used to dump excess electrons and degrade toxic nitrite [12,26]. Thus, the increased nitrite concentration in the beginning was due to denitrification and DNRA. Subsequently, the concentration decreased because it was further reduced to N_2 or ammonia. Kashima and Regan [12] indicated that the nitrite formed via nitrate reduction is preferentially reduced over the anode and/or nitrite and inhibits the anode reduction reaction. In this study, the production of nitrite was more than 10 times that of ammonium; these results suggest that although denitrification and DNRA are potentially degrading to the introduction of nitrate into the anode, the primary degradation of nitrate was undoubtedly mainly due to the heterotrophic denitrification in this mixed-culture system [11]. In this system, although the concentration of ammonium was low, it increased continuously in each cycle.

3.2. Bacterial community diversity

Three anaerobic activated sludge samples, namely, Seed, R–S, and R–N, were collected at the end to identify the dominant strains and changes in the microbial community structure by performing Illumina high-throughput sequencing on the V3–V4 region of 16S rRNA. Table 1 lists the effective read, OTU, Shannon diversity, ACE, Simpson index, Chao1 richness, and Good's coverage values of all

Table 1
Diversity indices of bacterial communities in three sludge samples

Sample ID	Seed	R–S	R–N
No. of reads	48,124	45,517	51,562
No. of OTUs	2,358	1,457	925
Shannon	5.73	4.75	3.70
ACE	2,985.09	2,063.16	1,183.24
Simpson	0.01	0.03	0.07
Chao1	2,858.88	2,006.19	1,128.62
Coverage	0.99	0.99	0.99

samples. The Shannon indexes in Seed, R–S and R–N were 5.73, 4.75 and 3.70, respectively. The Simpson indexes in Seed, R–S and R–N were 0.01, 0.03 and 0.07, respectively. Generally, a high Shannon index suggests high community diversity, and a high Simpson index suggests low community diversity. Thus, the microbial community biodiversity in R–S was higher than that in R–N. The Good's coverage estimator (≥0.99) suggests that the bacterial OTUs in the seven samples were well represented by the collected gene sequences. Compared with Seed, R–S and R–N showed decreased microbial community biodiversity possibly due to the selective responses to different growth conditions. In Fig. 2, the rarefaction curves for three sludge samples 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 sludge communities [27]. The similarity of the OTUs of the three sludge samples at the community level was illustrated with Venn diagrams (Fig. 3) that describe shared and unique OTUs. The three samples shared 363 OTUs or <50% of the total number of OTUs.

3.3. Bacterial community structure

As shown in Fig. 4a, the bacterial community structures of the three sludge samples were dominated by the following phyla: Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Planctomycetes, Synergistetes, Actinobacteria and Acidobacteria. Compared with Seed, R-N and R-S showed that the relative abundances of Proteobacteria, Firmicutes and Synergistetes increased significantly, and the relative abundances of Chloroflexi, Planctomycetes, Actinobacteria and Acidobacteria decreased significantly. Proteobacteria, Actinobacteria, Euryarchaeota and Firmicutes are widely distributed in acetate-fed BER [28,29]. Proteobacteria and Bacteroidetes could play key roles in electricity generation [30], especially the beta-subclass of Proteobacteria, which was the most abundant division in the present sample. This genus has been found in iron-oxidizing cultures [31] and can deliver electrons from Fe(II) to other electron acceptors, such as nitrate [32,33]. In addition, Proteobacteria is the dominant phylum in petroleum refineries, acrylic polymers, pharmaceutical industry, whey processing, steel and pet food industrial wastewater treatment plants, and sewage [34,35]. Bacteria belonging to Betaproteobacteria could use electrodes as their electron donor to reduce other substances [33]. Firmicutes (Sedimentibacter) is known for its ability to degrade a wide range of hydrocarbons under anaerobic conditions [36]. The electrode biofilm community might provide Firmicutes with anaerobic conditions, which are favourable for its growth [34].

The class-level identification of bacterial communities in the three activated sludge samples is illustrated in Fig. 4b. The dominant classes included Clostridia, Betaproteobacteria, Gammaproteobacteria, Bacteroidia, Deltaproteobacteria, Anaerolineae, Planctomycetia and Alphaproteobacteria. Compared with Seed, R-S and R-N showed that the relative abundances of Clostridia, Bacteroidia and Betaproteobacteria increased significantly, whereas the relative abundances of Planctomycetia and Alphaproteobacteria decreased significantly. In addition, the relative abundances of Deltaproteobacteria and Anaerolineae increased in R-S but decreased in R-N. A previous study has shown that Alphaproteobacteria and Betaproteobacteria are predominant in acetate-fed BER [28]. Betaproteobacteria is a versatile organic degrader existing in various microbial communities from phenol-, PAHs- and PCP-degrading bacteria [37]. Alphaproteobacteria has been reported as the major class for degrading PCP using acetate as the co-substrate [38]. In addition, Firmicutes, Chloroflexi and Alphaproteobacteria contain heterotrophic bacteria, while Betaproteobacteria includes several groups of nitrifiers, denitrifiers and other N-cycle-related microorganisms [22]. Anaerolineae is a member of the phylum Chloroflexi, a core microbial population in anaerobic digesters [39]. Although members of Anaerolineae are obligate anaerobes, genes for aerobic respiration have been recovered from the genomes of several Anaerolineae members [23,40]. Deltaproteobacteria can degrade other microbes by secreting hydrolytic exoenzymes [41]; in addition, it is

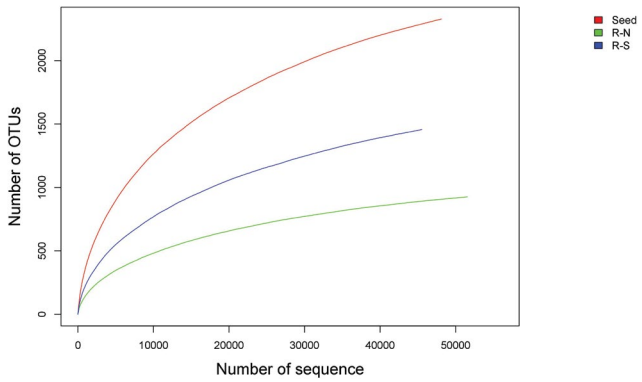

Fig. 2. Rarefaction curves based on the OTUs in three anaerobic sludge samples.

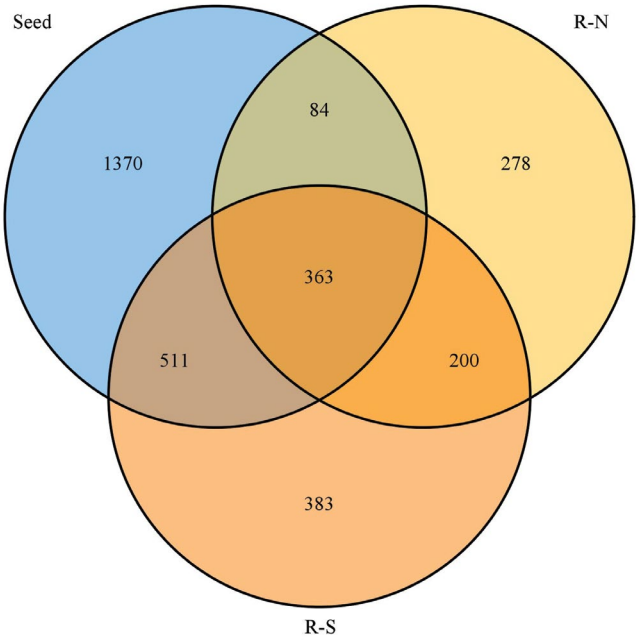

Fig. 3. Venn diagram based on the OTUs in three anaerobic sludge samples.

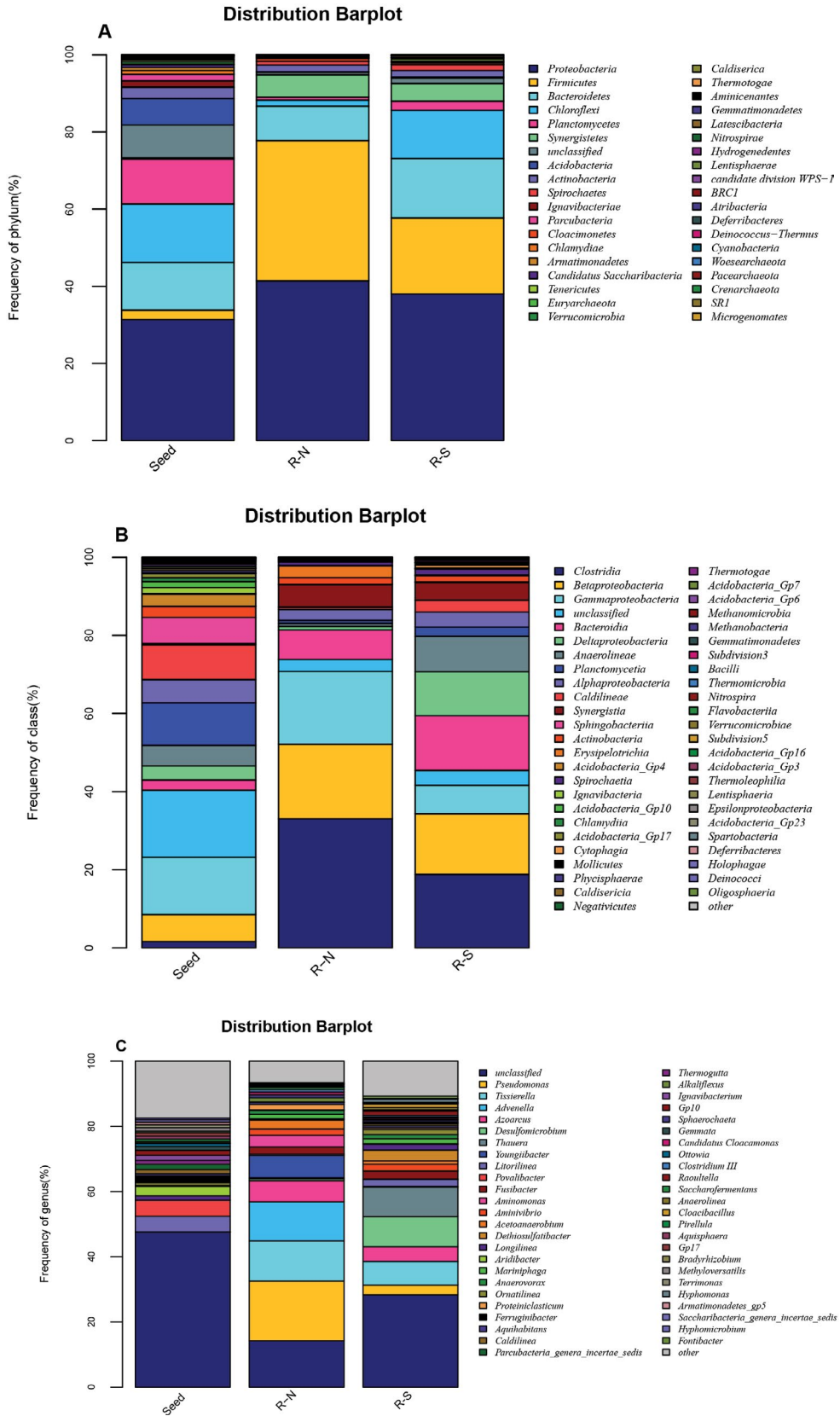

Fig. 4. Relative abundance of the dominant bacterial at (a) phylum level, (b) class and (c) genus level in three activated sludge samples (Seed, R–N and R–S).

believed to be responsible for direct electron transfer to the electrode. Enrichment of Deltaproteobacteria could enhance the direct electron transfer efficiency between bacteria and electrode [42].

Sequences from dominant bacterial communities were analyzed at the genus level to obtain insights into the microbial community structure of the three activated sludge samples. Only taxa that accounted for >1% of the relative abundance of the total bacterial communities were considered. Fig. 4c presents the differences in community structures at the genus level in the three sludge samples.

In R-N, the dominant genera included *Pseudomonas*, *Tissierella*, *Advenella*, *Youngiibacter*, *Azoarcus*, *Aminomonas* and *Acetoanaerobium*. *Pseudomonas* is abundant in the environment, particularly with regard to their denitrification potential [43]. Some bacteria belonging to the genus *Pseudomonas* are capable of autotrophic and heterotrophic denitrification and can utilize various electron donors (e.g., H_2, reduced sulphur compounds or organic carbon) to perform denitrification [27,44]. The existence of *Pseudomonas* suggests that nitrate is reduced by NRB using acetate as the electron donor in the anode. *Pseudomonas* is a gram-negative rod bacterial genus that can achieve extracellular electron transfer via electron shuttles from cells to electrodes, thereby showing high bioelectrochemical activity in BER [45]. *Azoarcus* can degrade lactate to CO_2 and consume N-compound [6]. In addition, the relative abundance of several genera was lower than that of others, and their existence is also essential. For example, *Ignavibacterium*, which possesses capabilities of electron transfer and aromatic hydrocarbon/azo dye transformation, was also enriched at the anode in previous studies [45].

In R-S, the dominant genera included *Desulfomicrobium*, *Thauera*, *Tissierella*, *Azoarcus*, *Dethiosulfatibacter*, *Pseudomonas* and *Fusibacter*. The genus *Desulfomicrobium* belongs to the class *Deltaproteobacteria*, which has been reported to be commonly found in sulphate-reducing processes [42]. *Thauera* transforms SO_4^{2-} to S^{2-} with organic matter as electron donors and carbon sources [6]. *Desulfomicrobium* and *Thauera* are probably the functional genus in charge of sulphate reduction in the cathode. In addition, the relative abundance of several genera was lower than that of others, and their existence is also essential. For example, *Clostridium* is an anaerobic gram-negative strain that can use organic carbon sources, such as glucose, maltose, starch, or lactic acid, to produce volatile fatty acids, and several strains can reduce sulphate to sulphide [46]. *Longilinea* is a filamentous strict anaerobe that can ferment various carbohydrates [47]. *Tissierella* exists in R-N and R-S, and it can grow on urine by metabolizing creatinine as the sole carbon source producing acetate, methylamine, ammonia and carbon dioxide [48]. The functional genus for the simultaneous degradation of anodic nitrate and cathodic sulphate has been indicated in the description above, and its existence suggests the feasibility of the original idea of this study. After applying 0.2 V of external voltage in the anode, nitrate degraded in the anode and sulphate degraded in the cathode.

4. Conclusion

The average NRE, SRE, NPE and APE were 38.84, 22.10, 4.56 and 0.38 mg/L, respectively. Although denitrification and DNRA are potential degrading processes to the introduction of nitrate into the anode, the primary degradation of nitrate was undoubtedly mainly due to the heterotrophic denitrification in this mixed-culture system. The existence of dominant species *Pseudomonas* and *Azoarcus* in the anode proved that nitrate was reduced by NRB using acetate as the electron donor. In the cathode, *Desulfomicrobium* and *Thauera* were the main functional bacteria for sulphate reduction. The existence of major functional bacteria confirms the feasibility of this study.

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