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Effects of different external voltages on simultaneous heterotrophic removal of sulphate and nitrate in a bioelectrochemical reactor: degradation efficiency, formation of elemental sulphur and bacterial community structure

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ABSTRACT

A bioelectrochemical reactor (BER) with an anaerobic biocathode was used for simultaneous heterotrophic removal of nitrate and sulphate. Different external voltages in range from 0.1 to 0.5 V were applied to investigate its effects on the degradation efficiencies, the formation of elemental sulphur and bacterial community structure. The results indicated that the effects of external voltage on the removal efficiencies of targeted pollutants were unsimilar. Chemical oxygen demand (COD) was highly affected and nitrate was the lowest affected by the variation in the external voltage. Furthermore, the removal rate of sulphate was attributed to the fact that sulphate was not affected entirely by voltage. Notably, the removal efficiency did not continuously increase with increasing voltage. The dominant genera in the reactors were *Pseudomonas*, *Comamonas*, *Chryseobacterium*, *Stenotrophomonas*, *Desulfococcus* and *Sphingobacterium*, respectively. Despite the similarities between the dominant genera, the application of external voltages resulted in variation of abundance of each microorganism in each BER. The results indicated the possible formation of elemental S during the reduction of sulphate.

Keywords: Bioelectrochemical reactor; External voltage; Nitrate; Sulphate; Elemental S; Bacterial community

1. Introduction

Sulphate-rich wastewater, a common pollutant produced by industrial processes, such as pulp and paper production and food processing [1]; is loaded with complex and potentially harmful ingredients. It also contains high concentrations of organic matter [2]. Large quantity of untreated sulphate-rich wastewater is discharged into environment; which not only increases the sulphate in water bodies but also causes several environmental issues resulting ecological instability and corrosion [3]. Sulphate concentrations in water body greater than 250 mg/L can cause health impacts, such as diarrhoea in humans [4]. Thus, various technologies for removing sulphate, including chemical sedimentation, ion exchange, adsorption and biological methods, have been developed and utilized. In particular, biological

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treatments have been widely utilized in recent years. With the application of sulphate-reducing bacteria (SRB) or through the oxidation of intermediate products using sulphide-oxidizing bacteria (SOB) under oxygen-limited conditions [1], biological treatment reduces sulphate to other sulphur compounds. However, these reactions only change the form of the existing sulphur compounds. Moreover, majority of the products obtained from this process, such as SO_2 and H_2S, may be detrimental to the environment. Hence, an effective method for removing sulphate should result in elemental S (S^0) formation, which is less harmful to environment than the other intermediate products, and this method can be performed under micro-oxygen conditions [5]. S^0 can further be recovered as a renewable resource for use in the fertilizer industry, in sulphuric acid production and as a substrate in bioleaching processes [6]. The optimum condition for the oxidation of sulphide (S^{2-}) to S^0 in a fed-batch reactor has been reported to be $O_2/S^{2-} = 0.7$ at a dissolved oxygen (DO) concentration of less than 0.1 mg/L [7]. The higher efficiency of sulphide oxidation (>98%) followed by conversion to S^0 (>75%) with a sulphide loading of less than 1.7 mol/m^2·d; and with an O_2 pressure sufficient enough to provide an O_2 flux of at least 1.5 mol/m^2·d in the membrane biofilm reactor was achieved simultaneously (Erkan). The results of this study suggest that the sulphide oxidation depends on the oxygen supply [8]. Sulphate-removal efficiency was attained up to 81.5% along with the recovery of S^0 at a peak of >70% with a DO concentration of 0.10–0.12 mg/L in an expanded granular-sludge-bed reactor [9]. Hence, control of the DO concentration is crucial for the reduction of sulphate to S^0.

Meanwhile, nitrate is a typical co-contaminant that appears with sulphate in various waste streams. Excess nitrate can cause gastric cancer, methemoglobinemia in infants and Hodgkin's lymphoma; thus, the World Health Organization has stipulated that the concentration of nitrate in drinking water should be less than 10 mg/L [10,11]. Denitrification involves biological degradation, and it is commonly applied in various wastewater treatment fields. This process involves the use of four enzymes and has been shown to result in the complete reduction of nitrate (NO_3^-) to nitrogen gas (N_2) [12–15].

So, complex integrated processes are required for the biological removal of high concentrations of sulphate and nitrate that are simultaneously present in the waste stream. In recent years, bioelectrochemical reactor (BER) is garnering increasing attention as a novel and promising green approach because of the use of microbial catalysis with electrochemically active microorganisms adhering to the surface of working electrode. The increasing attention on BERs is also mainly due to its environmental friendly operation [3,15]. The basic principle of operation of BER involves redox reaction and electron transport between two electrodes. The principle has been applied for electricity generation, energy recovery, degradation and desalination [16]. Moreover, it is also applied in the simultaneous removal of chlorinated phenol, production of hydrogen peroxide [16], simultaneous denitrification and biohydrogen production [17] and simultaneous removal of sulphate and zinc [18]. For treating complex wastewater, the BER is an excellent choice to accomplish high-efficiency simultaneous removal of two targeted pollutants in one reactor.

It is inevitably crucial for a BER because the external voltage could promote or inhibit the bacterial activity to further affect the removal of targeted pollutants. Hence, this study aimed to determine the effects of different external voltages on the removal efficiency of the target pollutants in a BER using anaerobic biocathode. The results obtained from these experiments will provide a theoretical basis for future research on the simultaneous heterotrophic removal of nitrate and sulphate from a single chamber in the BER. All BERs were operated under a batch mode which involved a 24-h cycle. The input time was 20 min, followed by operation for 23 h, again setting for 20 min and effluent application for 20 min. The hydraulic retention time was 23 h. After each operation cycle, the water exchange rate of anode and cathode was 100% and 80%, respectively. Indices such as the COD removal rate (CRR), sulphate-reducing rate (SRR) and nitrate-reducing rate (NRR) were estimated to evaluate the effects of external voltage on treatment efficiency. In addition, Illumina high-throughput sequencing technology was utilized to characterize the bacterial community quantitatively.

2. Materials and methods

2.1. Reactors setup

The BER, as described in an earlier study [1], 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 was 100 mL. A CHI1000C potentiostat (Shanghai CH Instrument Company, Shanghai, China) connected to a three-electrode system was used to control the different external voltage. The cathode was used as the working electrode; the anode as the counter electrode, and the Ag/AgCl electrode (CHI111, Shanghai CH Instrument Company, China) was placed into cathode chamber as the reference electrode. Carbon felt was chemically treated as per the study by Zhu et al. [19] and was used as the cathode material. A graphite plate was used as the anode material. Unless otherwise stated, all the potentials reported throughout this study were relative to that of the Ag/AgCl electrode.

The cathode chambers were inoculated with 30 mL of activated sludge which was extracted from the sludge-thickening tank of Guangzhou Lijiao sewage treatment plant in Guangdong, China. The components of the basal medium in cathode were as follows: 100 mM phosphate buffer solution (PBS), 300 mg·L^{-1}·d^{-1} SO_4^{2-}; 100 mg·L^{-1}·d^{-1} NO_3^-; 1,000 mg·L^{-1}·d^{-1} COD; 10 mL/L of vitamin solution and 20 mL/L of mineral solution (the vitamin and mineral solutions were formulated as instructed by Feng et al. [20] in a study), and CH_3COONa as organic carbon source. Before the synthetic wastewater was put into the reactor, N_2 was added continuously for more than 30 min to reduce the DO concentration while maintaining anaerobic conditions. The anode chamber was fed with only a 100 mM PBS solution (pH 7.0). The operating temperature was room temperature (24°C ± 3°C), with pH in range of 6.5–7 for all experiments. Five BERs were run in parallel under various different external voltages: 0.1 (R1), 0.2 (R2), 0.3 (R3), 0.4 (R4) and 0.5 (R5) V under anaerobic conditions.

2.2. Measurements and analysis

All samples were filtered through a 0.22 μm filter before analysis. The COD and the NO_3^- concentration of influent and effluent both were measured periodically using a previously established method [21,22]. The sulphate concentration in the aqueous phase was measured using an ICS-1000 ion chromatography system (Dionex, USA) with an IonPac AS14 anion column. The S^{2-} concentration was measured with a portable S^{2-} rapid-checking instrument (321-S, Shanghai Bante Instrument Limited, Shanghai, China). An assorted micro-sensor multimeter (Unisense Co., Denmark) was used to detect the concentration of total dissolved H_2S concentration. A confocal laser-scanning microscope (CLSM) (Leica TCS SP8, Leica Microsystems, Germany) was used to observe the distribution of microorganisms on the surface of carbon felt. Prior to that, samples were stained using a LIVE/DEAD® BacLight™ bacterial viability kit [23].

2.3. Statistical analyses

The SPSS for Windows v23.0.0.0 (SPSS, Inc.) was used for statistical analysis. The differences between five BERs in CRR, NRR and SRR were estimated via Duncan's multiple range test and least significant difference test (LSD) at a significance level of 0.05.

2.4. Bacterial analysis using high-throughput sequencing

The sludge samples taken from the five reactors were named R1-0.1, R2-0.2, R3-0.3, R4-0.4 and R5-0.5. The genomic DNA of each sample was extracted using an E.Z.N.A™ Mag-Bind Soil DNA Kit (Omega Bio-tek, USA) according to the manufacturer's guidance. 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 genomic DNA and to control the amount of DNA added to the mixture for polymerase chain reaction (PCR). A set of primers was used to amplify the hypervariable V3–V4 region of the bacterial 16S rRNA gene. The primers were designed using a blend of universal primers used in MiSeq sequencing platforms: primer 341F (CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG) and primer 805R (GACTGGAGTTCCTTGGCACCCGAGAATTCCAGACTACHVGGGTATCTAATCC).

Two PCR reactions were performed. In first reaction, the mixture contained 15 μL of 2 × Taq master mix, 1 μL of 10 μM bar-PCR primer F, 1 μL of 10 μM primer R, 10–20 ng genomic DNA and H_2O to complete the volume to 30 μL. As described in the manufacturer's guidelines, the amplifications were run under the following cycling conditions: 1 × 94°C for 3 min, 5 × 94°C for 30 s, 5 × 45°C for 20 s, 5 × 65°C for 30 s, 20 × 94°C for 20 s, 20 × 55°C for 20 s, 20 × 72°C for 30 s and 1 × 72°C for 5 min followed by a final soak at 10°C. In the second reaction, the mixture contained 15 μL of the 2 × Taq master mix, 1 μL of 10 μM primer F, 1 μL 10 μM primer R, 20 ng of genomic DNA and H_2O to complete the volume to 30 μL. The amplifications were run under the following cycling conditions: 1 × 94°C for 3 min, 5 × 94°C for 30 s, 5 × 55°C for 20 s, 5 × 72°C for 30 s and 1 × 72°C for 5 min with a final soak at 10°C. After amplification, the PCR products were purified and DNA concentrations in the purified products were measured using a Qubit2.0 DNA kit (Life Technologies, China). An Illumina MiSeq (Illumina, USA) instrument was used for pooling and sequencing of PCR products with equal DNA content for each sample. The 16S rRNA gene sequences were classified into operational taxonomic units (OTUs) with 97% similarity. Sequencing data were deposited in the NCBI Sequence Read Archive under the accession number SRP130367 (PRJNA430952).

3. Results and discussion

Because there existed two targeted pollutants as electron acceptors, nitrate and sulphate, can be reduced by the electrons provided by an external electric field or not. Thus, it is important to investigate the effects of different external voltages on the removal efficiency of these two targeted pollutants via external electric field without the activated sludge. Thus, the prepared basal medium was added to the reactors at a time, and various different external voltages were applied: 0.1, 0.2, 0.3, 0.4 and 0.5 V. The results indicate that the application of an external voltage has not promoted the reduction of targeted pollutants; which is not the main reason for removal of targeted pollutants. The removal mainly depended on the biodegradation in this system. In addition, the removal efficiencies of targeted pollutants in BER were more effective than control reactor without current flow when the carbon source was CH_3COONa [24]; so, in current study, no control reactor without current flow was operated parallel.

3.1. Performance under different external voltages

The CRR is commonly used as an indicator for the capacity of a bioreactor to treat wastewater. Five reactors were operated in parallel under anaerobic conditions, with the application of different voltage to each reactor. The average CRR in R1, R2, R3, R4 and R5 were 541.75, 571.60, 599.66, 630.32 and 599.33 $mg \cdot L^{-1} \cdot d^{-1}$, respectively (Figs. 1(a) and (d)). On increasing voltage from 0.1 V in R1 to 0.4 V in R4, the CRR increased by 13%–14%. The highest CRR was obtained in reactor R4 with 0.4 V. However, the CRR decreased by approximately 7% on further increasing the voltage from 0.4 (R4) to 0.5 V (R5). Significant differences existed between each two BERs by increasing external voltage: R1 vs. R2 ($p < 0.05$); R2 vs. R3 ($p < 0.05$); R3 vs. R4 ($p < 0.05$); R4 vs. R5 ($p < 0.05$) (Tables 1 and S1). This indicated that external voltage made a bigger influence in CRR. Figs. 1(a) and (d) indicate that external voltage made a bigger influence in CRR. The increased voltage could lead a rapid CRR because of improved high oxidation–reduction potential values for promoting microorganisms to be utilized in metabolic processes, which was directly proportional to the potential energy difference between electron donor and electron acceptor; simply, the redox reactions were boosted [25–27]. However, a high voltage may inhibit bacterial activity, so the CRR has not further increased with an additional increase in voltage. Hence, it is crucial to tune the external voltage in order to attain the optimal adaptive growth of microorganisms in BERs.

The average NRR values in R1, R2, R3, R4 and R5 were 95.80, 96.62, 97.07, 95.78 and 95.35 $mg \cdot L^{-1} \cdot d^{-1}$, respectively

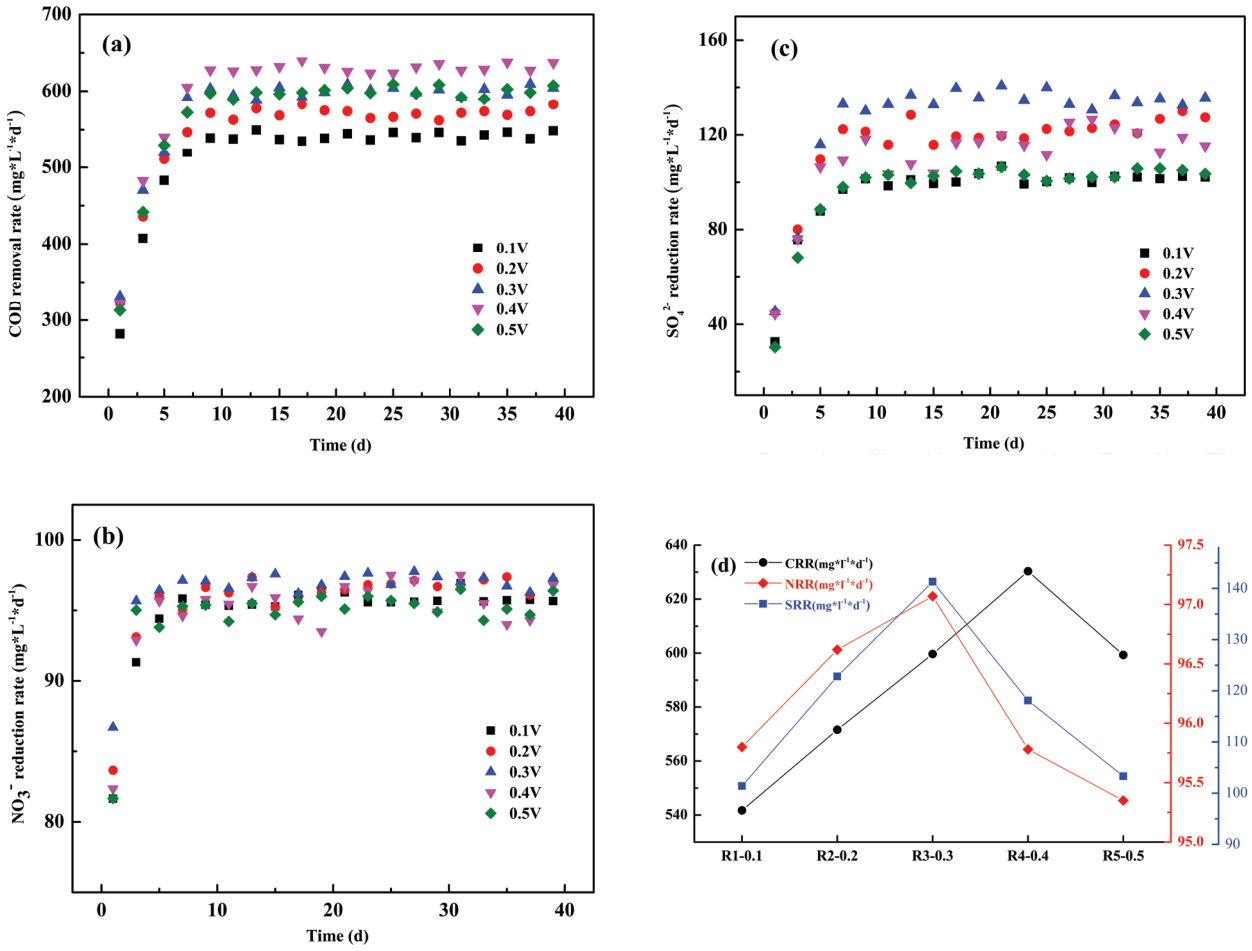

Fig. 1. (a), (b) and (c) the removal ability of three target pollutants (organic matter, nitrate and sulphate) in each BER; (d) the average value of the removal ability of three target pollutants in each BER.

Table 1
Least significant difference test (LSD) for significant differences between five BERs in CRR, NRR and SRR

Samples in different BES reactor	CRR	NRR	SRR
R1 vs. R2	*p*=0.000	*p*=0.006	*p*=0.000
R1 vs. R3	*p*=0.000	*p*=0.000	*p*=0.000
R1 vs. R4	*p*=0.000	*p*=0.930	*p*=0.000
R1 vs. R5	*p*=0.000	*p*=0.117	*p*=0.252
R2 vs. R3	*p*=0.000	*p*=0.118	*P*=0.000
R2 vs. R4	*p*=0.000	*p*=0.004	*p*=0.006
R2 vs. R5	*p*=0.000	*p*=0.000	*p*=0.000
R3 vs. R4	*p*=0.000	*p*=0.000	*p*=0.000
R3 vs. R5	*p*=0.876	*p*=0.000	*p*=0.000
R4 vs. R5	*p*=0.000	*p*=0.139	*p*=0.000

Note: The mean difference is significant at the 0.05 level.

(Figs. 1(b) and (d)). On increasing voltage from 0.1 (R1) to 0.3 V (R3), the NRR increased; however, it decreased as the voltage further increased from 0.3 (R3) to 0.5 V (R5). For five BERs, the NRRs were more than 95%. However, for nitrate reduction, no obvious differences were caused by external voltage, which is elaborated in Tables 1 and S2, R1 vs. R2 ($p > 0.05$); R2 vs. R3 ($p > 0.05$); R3 vs. R4 ($p < 0.05$); R4 vs. R5 ($p > 0.05$). For nitrate reduction process, no obvious differences were caused by external voltage (Figs. 1(b) and (d)). It involves an electron transfer process, in the presence of an external electric field, H^+ is produced due to the electrolysis of water at the anode, and it subsequently diffuses from the anode to cathode. The free electrons provided by the external electric field can be utilized by microorganisms to reduce nitrate into nitrite, and nitrite is further reduced to form nitrogen gas (N_2) through a series of reactions at the cathode. However, because organic compounds can also serve as electron donors in the denitrification process, the electron donors do not serve as a unique alternative.

The SRR varied with the external voltage in a trend similar to that of the NRR. The average SRR values in R1, R2, R3, R4 and R5 were 101.40, 122.81, 141.32, 118.08 and 103.32 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, respectively (Figs. 1(c) and (d)); while R3 (0.3 V) showed the highest SRR. The sulphate reducing process was similar to COD removal process and it was affected by external voltage (Tables 1 and S3). As discussed above, two other electron donors were present in the system which contributed to SRR. However, it is important to note that the

nitrate and sulphate were in competition for electron donors in this complex reaction system. It was observed that even with excess presence of electron donors, sulphate reduction begins only after almost complete denitrification; in other words, sulphate reduction is strictly inhibited when the concentration of nitrate in the effluent is ≥0.1 mg N/l Aura [28]. However, the rate of sulphate reduction is relatively moderate and that the strict sequential reduction of nitrate followed by sulphate reduction does not occur Xu [4]. Whether these processes occur sequentially or simultaneously, the critical factor is the promotion of nitrate reduction to improve the SRR. Thus, an external voltage of 0.3 V (R3) is optimal for the reduction of both nitrate and sulphate as observed through highest SRR as well as highest NRR. In addition, the corresponding generated current is shown in Fig. S1. It can be seen that different current was produced due to different external electric field.

3.2. Possibility of elemental S formation

The majority of the SRR in each reactor was greater than 100 mg·L^{-1}·d^{-1}. Hence, several compounds of S may have been produced by the redox reaction, including sulphide (S^{2-}), thiosulphate ($S_2O_3^{2-}$), dissolved hydrogen sulphide (H_2S), H_2S gas and sulphite. However, the concentrations of some of these compounds, such as thiosulphate, sulphite and H_2S gas, were relatively low or were undetectable; hence, they can be considered negligible [1,9]. Meanwhile, the metabolism by microorganisms is inevitable and may partially explain the consumption of sulphate. In addition, there was no diffusion of sulphate from the cathode to anode; and the sulphate reducing process solely occurred in cathode. However, these processes were not the main reason for the imbalance between average SRR and the amount of dissolved H_2S produced in each reactor, which was nearly 20 times greater (Fig. 2). Thus, the possible formation of elemental S cannot be disregarded. Meanwhile, elemental S may be less severe than the other S compounds in terms of environmental pollution. Some crystals were deposited on the granular electrodes but only 2% sulphur precipitate was detected by Coma et al. [29]. This result did not prove the elemental S formation, but it provided motivation for further investigation. Sulphur

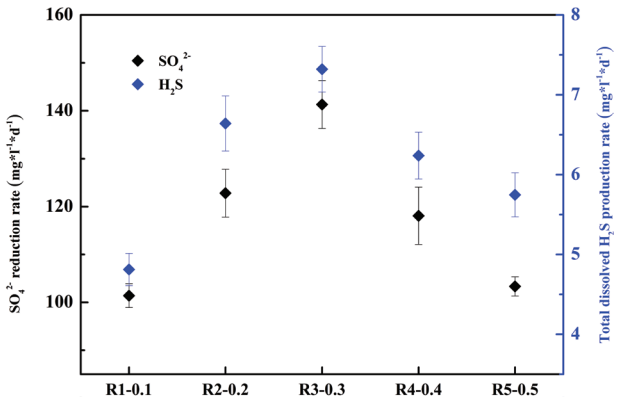

Fig. 2. Average value of sulfate-reduction ability and the average value of production rate of dissolved hydrogen sulfide when the operation reached a steady condition in each BER.

imbalance may occur if the elemental S formation is ignored based on the analysis of all products which may be produced during reduction Enric [1]. In addition, energy-dispersive X-ray spectroscopy revealed that S^0 was the main component of the solids deposited on the cathode surface. Hence, the elemental S formation must be taken into account while examining the complete circulation of sulphur in the system.

For elemental S formation, controlling the concentration of added oxygen is an effective method for the recovery of elemental S via a sulphate-reducing process in conventional reactors [30]. However, for BERs, this approach would add complexity and increase the cost of the process. Thus, the external voltage increase was leveraged to promote the production of oxygen in the anode via water hydrolysis, and the oxygen partially diffused through the membrane to the cathode [1]. However, in this case, the external voltages were far from those needed for hydrolysis and oxygen production; so, the experiments were conducted under anaerobic conditions with oxygen-free environment in reactors. Thus, it can be assumed that oxygen was not the electron donor involved in the recovery of elemental S in present study. However, when both S^{2-} and NO_3^- are present, SOB can convert S^{2-} to S^0 using NO_3^- as an electron acceptor (Eq. (1)):

$$S^{2-} + 1.25CH_3COOH + 2.4NO_3^- + 2.4H^+ \rightarrow S^0 + 2.5CO_2 + 1.2N_2 + 2.7H_2O + 2OH^- \quad (1)$$

This reaction may be the main pathway for the formation of S^0 in the present study. In addition, at the end of each operation cycle, sulphide (S^{2-}) was not detected; thus, it is likely that S^{2-} was used for the elemental S formation. However, the yield of S^0 did not appear to be correlated with the external voltage in this complicated system; thus, further investigations are required.

3.3. Analysis of microbial community

3.3.1. CLSM images

After the application of different external voltages, a CLSM was used to observe the growth of microorganisms adhered to the carbon-felt surface of cathode. Distinct differences in the abundance of metabolically active (green) cells to inactive (red) cells at different voltages were observed (Fig. 3). The carbon felt can be regarded as a substrate for bacterial growth. Significant bacterial activity was observed in large number of microorganisms growing on the carbon-felt surface. The CLSM images revealed different trends in the relative distribution of living and dead cells in different reactors, which may have relation to the different external voltages. In addition, the attachment morphology in biofilm was observed. The microorganisms adhering on the carbon-felt surfaces in R2, R3 and R4 were more than those in R1 and R5. Hence, to a certain extent, the microbial growth is attributed to external voltage.

3.3.2. Analysis of bacterial communities in activated sludge

Illumina high-throughput sequencing was used to assess microbial diversity and community structure in five BERs.

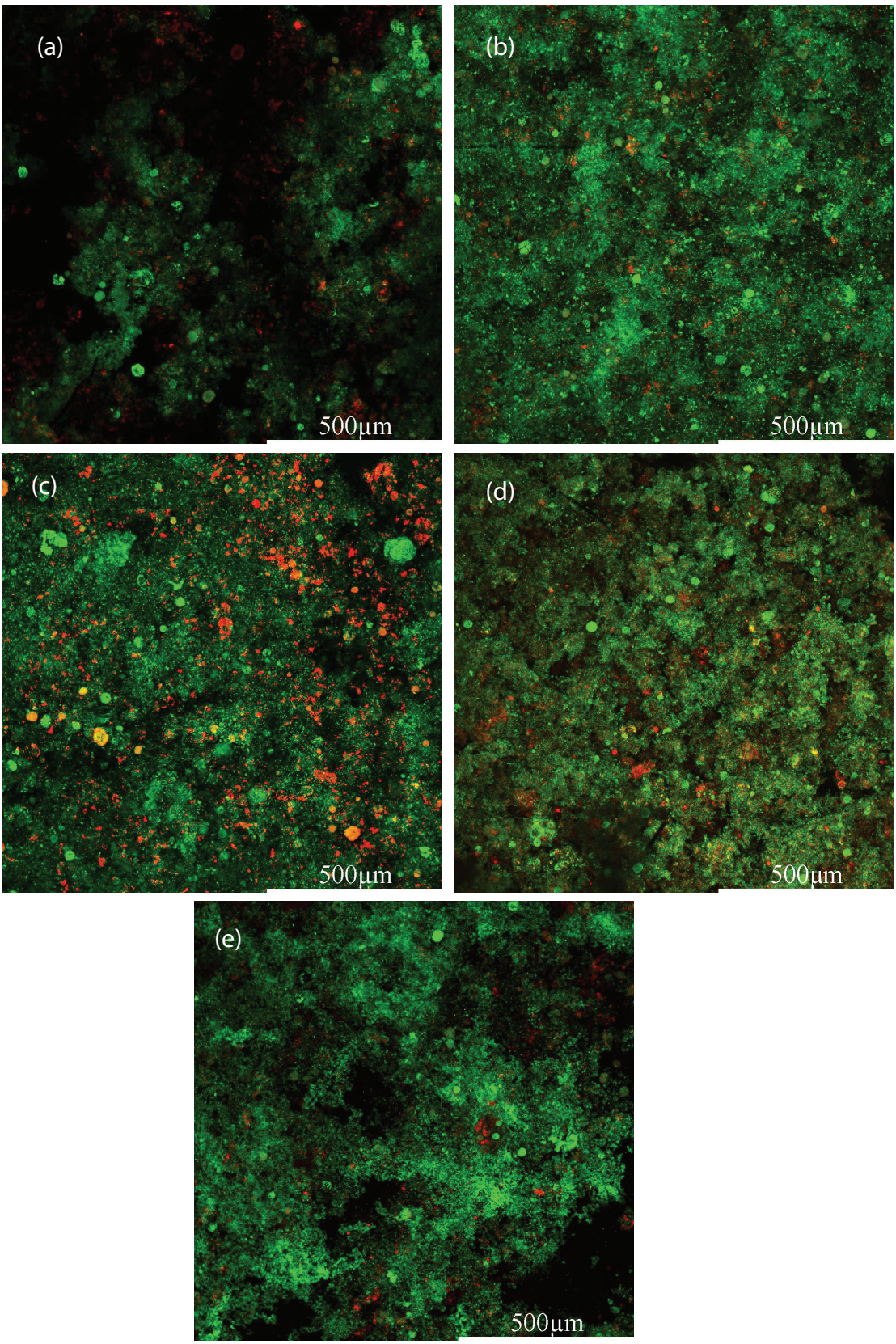

Fig. 3. Confocal laser scanning microscopy images of cathodic biofilms adhering to the surface of carbon felt under different external voltage: (a) 0.1 V, (b) 0.2 V, (c) 0.3 V, (d) 0.4 V, (e) 0.5 V. The images are to help to distinguish bacteria with metabolically active (green) and inactive cells (red). The cells were scrutinized in all three-dimensional views of different dimensions.

Five sludge samples were collected from the five BERs after the experiments had stopped. Diversity indices of bacterial communities are shown in Table 2. The total genomic DNA was extracted and the V3–V4 region of the 16S rRNA gene was amplified, leading to a high-quality pyrosequencing reads in range of 24,177–21,008 with an average of 22,812. The Shannon provides information of species diversity in a sample, and its value for sludge samples in all reactors was not same. As shown in Table 2, the value for R5 was highest and the value for R2 was lowest, which indicated a higher microbial diversity in R5 and a lowest microbial diversity in R2. The result was also confirmed by Simpson index. The coverage for each sample showed high values indicating the number of sequences to be sufficiently high to obtain an accurate and complete understanding of bacterial diversity. Fig. 4 presents the exclusive or shared OTU number between samples, and there were 40 OTUs shared by all five samples.

The results of non-metric multi-dimensional scaling (NMDS) analysis suggest variations in bacterial communities of different samples. As shown in Fig. 5, the comparison of unweighted UniFrac distance between samples in different categories revealed that sludge samples R1-0.1, R2-0.2 and R3-0.3 had a similar bacterial community in comparison of other two reactors. However, for R4-0.4 and R5-0.5, a significant difference in community composition was observed. The results indicated that external voltage could make an influence on bacterial community composition when it increased further from 0.4 to 0.5 V.

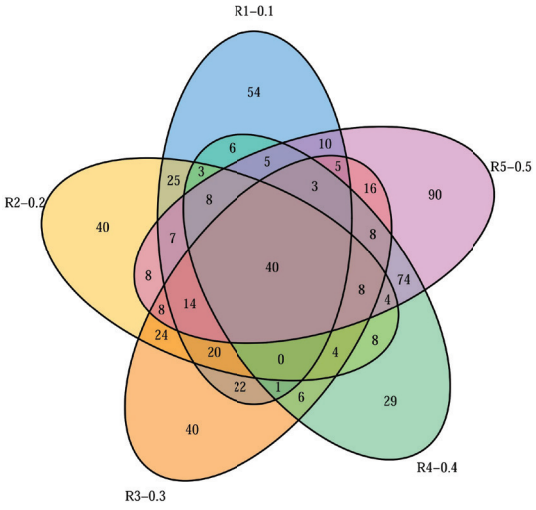

Fig. 4. Exclusive and shared OTU in sludge samples of five BERs.

To further compare the differences in microbial communities, as shown in Fig. 6, the taxonomic classifications of the dominant phylogenetic groups were characterized at genus level. The total number of genera detected in each sample was greater than 30. The dominant genera in the reactors were *Pseudomonas*, *Comamonas*, *Chryseobacterium*, *Stenotrophomonas*, *Desulfococcus* and *Sphingobacterium*,

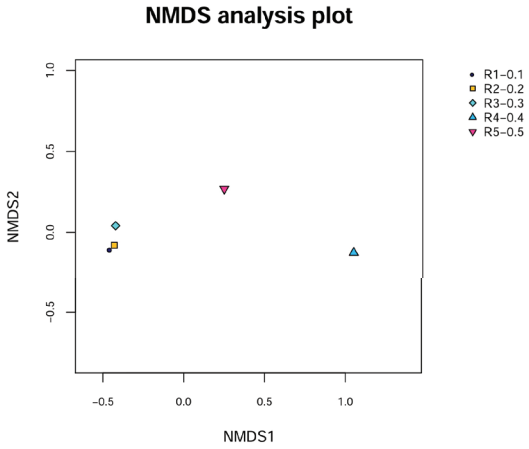

Fig. 5. Non-metric distance scaling (NMDS) analysis of the weighted Unifrac distance indices of the bacterial community at OTU level. Different symbols and colours are used for different sludge samples of five BERs.

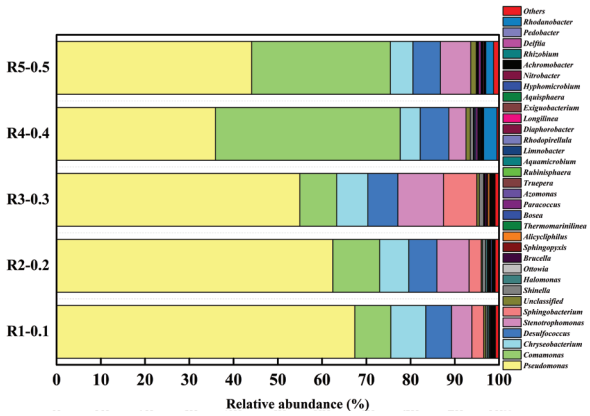

Fig. 6. Taxonomic classification of 16S rRNA gene from bacterial communities of sediment samples at genus level: relative abundance defined as the percentage of the same taxon to the corresponding total sequences for each sample.

Table 2
Diversity indices of bacterial communities in sludge samples of five BERs with different external voltage (i.e., 0.1, 0.2, 0.3, 0.4 and 0.5) after 40 d of operation

Sample ID	No. of reads	No. of OTUs	Shannon	ACE	Simpson	Chao1	Coverage
R1-0.1	21,008	223	1.37	334.52	0.53	304.45	1.00
R2-0.2	23,183	221	1.34	292.63	0.55	282.39	1.00
R3-0.3	24,177	219	1.64	298.28	0.40	280.67	1.00
R4-0.4	21,857	207	1.55	358.72	0.48	288.16	1.00
R5-0.5	23,836	308	2.00	384.64	0.29	361.64	1.00

respectively. It is worth mentioning that different population distributions were caused by different external voltages. In the five BERs, *Pseudomonas*, *Comamonas*, *Chryseobacterium*, *Stenotrophomonas*, *Sphingobacterium* and *Desulfococcus* bacteria were found at highest abundance at voltages of 0.1, 0.4, 0.1, 0.3, 0.3 and 0.3 V. It was found that on increasing voltage from 0.1 to 0.3 V, the abundance of *Pseudomonas* decreased and the abundance of *Sphingobacterium* increased; however, on further increasing voltage from 0.4 to 0.5 V, the abundance of *Pseudomonas* decreased rapidly and *Sphingobacterium* disappeared. For *Comamonas*, it gained higher abundance under higher external voltage (0.4 and 0.5 V). The SRB, *Desulfococcus*, was present at its highest ratio in R3, resulting in the highest SRR of five reactors. This higher SRR was likely due to the higher NRR. In terms of removal efficiencies of pollutants and abundance of functional bacteria, the optimal external voltage was 0.3 V in present study.

For presented functional bacteria in this investigation (Fig. 6), *Pseudomonas* and *Comamonas* have been reported as the most common denitrifiers. *Pseudomonas* are gram-negative, rod-shaped and polar-flagellated with some sporulating species, which are able to use the aromatic compounds as organic carbon source; in addition, it also could be regarded as a sulfide-oxidizing denitrifier, which can oxidize sulfide with the denitrification of nitrate to NO_2 gas [31–33]. *Chryseobacterium* can respire with nitrate as the terminal electron acceptor. And *Sphingobacterium* has the ability to reduce nitrogen to nitrogen gas [17]. *Stenotrophomonas* as a heterotrophic denitrifier can convert nitrate to nitrite [33]. *Desulfococcus* bacteria have crucial roles in the reduction of sulphate [34]. In addition, other bacteria also played an essential role in this investigation. *Halomonas* was alkaliphilic and halotolerant denitrifying bacteria capable of expressing all enzymes necessary for the complete reduction of nitrate to N_2 [35]. *Ottowia* can grow well through nitrate reduction [17]. *Longilinea* have been shown to be anaerobic filamentous bacteria capable of utilizing both acetate and propionate [36]. *Paracoccus* could utilize inorganic sulphur compounds such as sulfide, sulphur and thiosulfate as electron donors and nitrate as an electron acceptor to drive denitrification [33]. This indicated that it is a cooperative process for targeted pollutants removal.

4. Conclusion

In summary, external voltage had the most significant influence on organic matter, and it had the least effect on nitrate. Despite the similarities between these dominant strains, their relative percentages varied when different external voltages were applied. In addition, the elemental S formation during the reduction of sulphate in the BERs should be further investigated as a beneficial alternative to other intermediates.

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Supplementary material

Table S1
Duncan's multiple range test for significant differences between five BERs in CRR

R		N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	R1	20	541.75			
	R2	20		571.60		
	R5	20			599.33	
	R3	20			599.66	
	R4	20				630.32
	Sig.		1.000	1.000	0.876	1.000

Means for groups in homogeneous subsets are displayed.
[a]Uses harmonic mean sample size = 20.000.

Table S2
Duncan's multiple range test for significant differences between five BERs in NRR

R		N	0.05	
			1	2
Duncan[a]	R5	20	95.53	
	R4	20	95.78	
	R1	20	95.80	
	R2	20		96.62
	R3	20		97.07
	Sig.		0.139	0.118

Means for groups in homogeneous subsets are displayed.
[a]Uses harmonic mean sample size = 20.000.

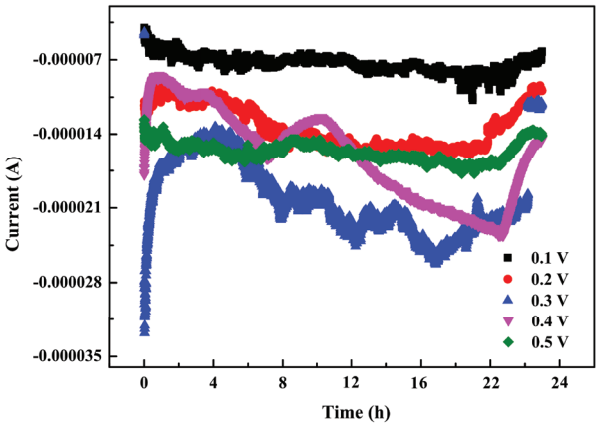

Fig. S1. Representative cathode current generation curves recorded in five BERs in the end of experiment.

Table S3
Duncan's multiple range test for significant differences between five BERs in SRR

R		N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	R1	20	101.40			
	R5	20	103.32			
	R4	20		118.08		
	R2	20			122.81	
	R3	20				141.32
	Significance		0.252	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
[a]Uses harmonic mean sample size = 20.000.