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The potential of electrotrophic denitrification coupled with sulfur recycle in MFC and its responses to COD/SO_4^{2-} ratios

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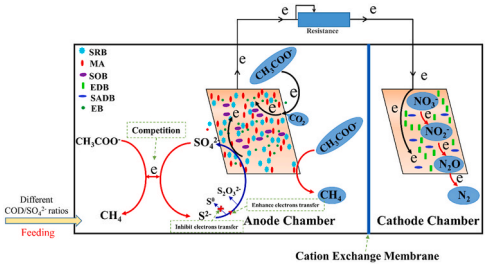
HIGHLIGHTS

- The electrons generated from sulfate-sulfide cycle could contribute to the denitrification.
- MFC could enhance the electrons transfer for electrotrophic denitrification.
- Anodic influent COD/SO_4^{2-} ratios impacted the sulfate reduction and bio-cathode denitrification performance.
- The *Thiobacillus* in cathodic biofilm strengthened by the electrons from anode could promote the denitrification process.

GRAPHICAL ABSTRACT



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ABSTRACT

Electrotrophic denitrification is a promising novel nitrogen removal technique. In this study, the performance and the mechanism of electrotrophic denitrification coupled with sulfate-sulfide cycle were investigated under different anodic influent COD/SO_4^{2-} ratios. The results showed that electrotrophic denitrification contributed to more than 22% total nitrogen removal in cathode chamber. Higher COD/SO_4^{2-} ratios would deteriorate the sulfate reduction but enhance methane production. Further mass balance indicated that the electron flow utilized by methanogenic archaea (MA) increased while that utilized by sulfate-reducing bacteria (SRB) decreased as the COD/SO_4^{2-} ratio increased from 0.44 to 1.11. However, higher COD/SO_4^{2-} ratios would produce more electrons to strengthen electrotrophic denitrification. Microbial community analysis showed that the biocathode was predominantly covered by *Thiobacillus* that encoded with *narG* gene. These findings collectively suggest that electrotrophic denitrification could be a sustainable approach to simultaneously remove COD and nitrogen under suitable COD/SO_4^{2-} ratio based on sulfur cycle in wastewater.

1. Introduction

Conventional heterotrophic denitrification (HD) is widely applied for mainstream nitrogen removal in domestic sewage treatment (Xu et al., 2015). However, it would be limited by the low chemical oxygen demand (COD) concentration in wastewater. To achieve highly efficient

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nitrogen contaminants removal, exogenous carbon source (e.g., glucose) needs to be dosed into wastewater to meet the requirement of HD process (Cui et al., 2019b). Dosing organic carbons would cause an increase of the treatment cost. Moreover, large amounts of excessive activated sludge waste would lead to the subsequent sludge disposal problem, which would in turn increase the overall operational cost again (Cui et al., 2019a). Therefore, it is deemed necessary to find a more cost-effective and sustainable approach to mainstream nitrogen removal.

Electrotrophic denitrification, a cutting-edge technology for nitrogen removal from wastewater, is promising to become an alternative way for the traditional denitrification (Clauwaert et al., 2007). Electrotrophic denitrification, coupling with the bio-cathode which was attached the functional microorganisms on the surface, can utilize electricity from anode as electron donor to reduce the nitrate to nitrogen gas in the cathode chamber (Ghazouani et al., 2014; Nguyen et al., 2016; Al-Mamun et al., 2020). Compared to traditional HD removal techniques, electrotrophic denitrification could achieve high nitrogen removal efficiency even under a low C/N ratio due to electrotrophic denitrification can obtain and utilize electrons from diverse donors. For instance, it was reported that a novel denitrifying sulfide removal microbial fuel cell (MFC) could use sulfide as the anodic electron donor to achieve to a biocathodic nitrate removal rate of 12.1–17.4 g NO_3^--N/m^3 NC/d with more than 71.9% of the anodic sulfide oxidized (Zhong et al., 2017). Virdis et al. used acetate as the electron donor in the anode chamber to study the electron loss in the biological cathode denitrification process, and found that although 40.1% of the acetate in the anode chamber was consumed by methanogens to produce methane, the nitrate removal rate was 0.327–0.484 mM NO_3^--N/h (Virdis et al., 2009). These studies together demonstrated that both organics and sulfide can be further utilized to enhance denitrification by transferring electrons to the electrode. However, the sulfur in domestic sewage is commonly exists as sulfate, and usually in a concentration range of 20–60 mg/L (Van Doan et al., 2013; Lv et al., 2017). Most of these studies used substrates such as acetate and sulfide as anode electron donors to investigate the biocathode denitrification performance, which leads to low practical applications for engineering.

It is commonly accepted that sulfate could act as the electron acceptor of SRB (Fang et al., 2020). The end-product, sulfide, can be further re-oxidized by sulfur-oxidizing bacteria coupled with electrons generation (Huang et al., 2015). If these electrons could be directly transferred and utilized by nitrate, this would vastly improve the denitrification efficiency and electrons utilization efficiency (Tesoriero et al., 2000). MFC, is a new technology that can generate electricity from oxidation of various organic and inorganic while accomplishing pollutants removal (Logan, 2006), may provide a more suitable approach to investigate the electron flow and electron loss during electrotrophic denitrification process. Furthermore, in anaerobic system, many microorganisms (e.g., MA and SRB) has a similar ecological niche and co-exist with each other. Although they co-exist, they also internally compete. For example, MA would digest organic matter to produce methane and SRB would consume organic matter to generate sulfide. Although SRB can use a wide range of carbon sources as electron donors and produce hydrogen sulfide (H_2S) with a high growth rate and good substrate affinity, enabling rapid organic removal (Colleran, 1995). The other studies reported that COD/SO_4^{2-} ratio could disturb the microbial community structure through altering the relative abundance of MA and SRB, which would further change the electrons flow and reactor performance (Ren et al., 2007; Lu et al., 2018). However, the effects of COD/SO_4^{2-} ratio on electrotrophic denitrification have not been systematically studied.

Therefore, it was hypothesized that SRB in anodic biofilm can effectively reduce sulfate to sulfide, and the produced sulfide can then be used as an electron donor for nitrogen removal in electrotrophic denitrification system. In addition, given the anodic influent COD/SO_4^{2-} ratio plays an important role in the competition of those functional microorganisms for common electron donors, further affecting the denitrification performance in cathode chamber. It is further hypothesized that higher COD/SO_4^{2-} ratio in turn deteriorate overall nitrate removal. To verify these hypotheses, a bio-cathode denitrification MFC controlled by SRB-mediated anodic MFC was constructed and operated under different anodic influent COD/SO_4^{2-} ratios. The main aims of the work were to evaluate the possibility of electrotrophic denitrification coupled with sulfate reduction under different anodic influent COD/SO_4^{2-} ratios and to elucidate underlying mechanisms of the biocathode denitrification. The knowledge of this work could provide a comprehensive environmental evaluation concerning the electrode denitrification performance in the biocathode MFC and shed new light on further exploring the denitrification performance based on sulfur recycle in the wastewater treatment plants (WWTPs).

2. Materials and methods

2.1. Bio-cathode microbial fuel cell construction and operation

Two-chamber MFCs comprising anode and cathode cuboid chambers were separated by a cation exchange membrane cation exchange membrane (CEM) (CMI-7000). Both the anode and cathode electrodes were made of carbon felt. The configurations, sizes and other specifications, were shown in our previous study (Ai et al., 2020).

The four identical MFCs (i.e., MFC1, MFC2, MFC3 and MFC4) started operating in batch mode. During the startup stage, the anode chamber was inoculated with aerobic active sludge, while the cathode chamber was seed with a microbial consortium previously operating in an anodic mixotrophic denitrification MFC (Ai et al., 2020). The influent anolyte was modified based on the Postgate C culture medium but compromising with the characteristics of this experiment (Postgate, 1963). Specifically, the synthetic anodic feed contained NH_4Cl (1 g/L), KH_2PO_4 (0.5 g/L), $CaCl_2{\cdot}6H_2O$ (60 mg/L), $MgCl_2{\cdot}6H_2O$ (60 mg/L), $FeSO_4{\cdot}7H_2O$ (4 mg/L) and 1 mL/L trace element solution (Ai et al., 2020). The cathodic feed consisted of the same medium in the anodic influent (no sulfide) with the previous study (Ai et al., 2020). The nitrate concentration in the cathodic influent was fixed at 20 mg/L. To investigate the effect of anodic COD/SO_4^{2-} ratios on the biocathode denitrification performance, CH_3COONa and Na_2SO_4 with different concentrations were added into the anode chamber. The sulfate concentration was fixed at 288 mg/L, while the acetate concentration was respectively 164 mg/L, 246 mg/L, 328 mg/L and 410 mg/L to make the corresponding COD/SO_4^{2-} ratio of 0.44, 0.67, 0.88 and 1.11 in the four MFCs, respectively (i.e., MFC1, MFC2, MFC2, MFC4). During the whole operation process, pH of the synthetic wastewater was kept at 8.00 ± 0.21. After successfully start-up, the biocathode MFCs were operated in fed-batch mode under ambient condition for 10 batches. In the 5th batch, the MFC was operated with open circuit, and the anode and cathode were disconnected, so no electron donor flowed from anode to the bio-cathode. Unless otherwise specifically indicated, the MFCs were operated with closed circuit.

2.2. Analytical measurements and calculation

The influent and effluent acetate, nitrate, nitrite, ammonia, sulfide, sulfate, thiosulfate and sulfite concentration were analyzed according to our previous study (Ai et al., 2020). Surface elements of the used and clean anodes were analyzed employing XRF (X-ray Fluorescence Spectrometry, XRF-1800). Assuming that COD was mainly used for sulfate reduction, methane production and electricity generation, and the rest COD that consumed by bacterial growth was grouped into “others”. Those fractions of COD consumed by SRB and MA were stoichiometric calculated according a previous study (Wang et al., 2019). In this system, electrogenesis contributions to COD removal that is equal to the columbic efficiency (CE) of the system (Li et al., 2012).

Voltage and current output were recorded and calculated as

previously described (Ai et al., 2020). The CE was calculated by dividing the amount of electric charge (integrating current and time) by total electric charge input based on acetate (Lee et al., 2012). The electron utilization efficiency of denitrification was defined as the ratio of the total coulombs of the electron donors required to reduce nitrate to the total coulombs of the electron donors generated by the external circuit in the MFC (Wu et al., 2017).

2.3. Genomic DNA extraction and 16S rRNA gene-based amplicon sequencing

Genomic DNA of anodic and cathodic biofilm was extracted by the PowerSoil ®DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA). The extracted genomic DNA was further applied to microbial community analysis and Real-time polymerase chain reaction (qPCR) analysis. For microbial community analysis, PCR amplification of the 16S rRNA genes was applied with 515F (5′- Illumina overhang- GTGY-CAGCMGCCGCGGTAA- 3′) and 907R (5′- Illumina overhang-CCCCGYCAATTCMTTTRAGT- 3′). All amplicons were of the anticipated size of approximately 550 bp and the negative control had no amplification. PCR amplicons were cleaned up by AMPure XP beads (Beckman Coulter, CA, USA). After that, index PCR was conducted to attach dual indices provided by the Nextera XT Index Kit (Illumina Inc, San Diego, CA, USA) based on the manufacturer's protocol. Indexed PCR amplicons were cleaned up by AMPure XP beads (Beckman Coulter, CA, USA). Equimolar concentrations of the samples were mixed and submitted to Sangon Biotech Co. (Shanghai) for Illumina MiSeq sequencing. The analytical procedures and detailed microbial community analysis were according to a previous study (Ai et al., 2019).

2.4. Real-time polymerase chain reaction analysis

A real-time PCR system, StepOne Plus™ (Applied Biosystems, USA) was used to quantitatively determine the abundance of denitrification associated genes. Those primes for those functional genes were shown in Table S1. Each 20 μL reaction volume consist of 16.5 μL ChamQ SYBR Color qPCR Master Mix, 1 μL of each primer (10 μM), 0.8 μL respective probe and 2 μL of DNA template. The thermal cycling profile was as follows: hold for 5 min 95 °C, and then 40 cycles of melt for 20s at 95 °C, anneal for 30s at 58 °C and extend for 40s at 72 °C. After that, the reactions was conducted on 96-well thermal cycler block in fluorescence ration PCR instrument (ABI 7500). Each sample was performed in triplicate.

2.5. Statistical analysis

All experimental parameters were tested in triplicate to the result accuracy. All significance testing was analyzed by one-way analysis of variance (ANOVA). Data analyses were conducted by using SPSS 22.0. Results were regarded as statistically significant when $p < 0.05$ (at confidence level of 95%).

3. Results and discussion

3.1. Denitrifying performance of biocathodes

All MFCs were operated for 20 days with 10 batches. As it was shown in Fig. 1, during the whole operation except the 5th open circuit batch, the performance of all MFCs kept stable and the average nitrate concentrations in effluents of the four MFC were all less than 5.50 mg/L (i.e., 5.23 mg/L, 4.98 mg/L, 4.38 mg/L and 4.08 mg/L from MFC1, MFC2, MFC3 and MFC4, respectively). Further statistics analysis indicates that

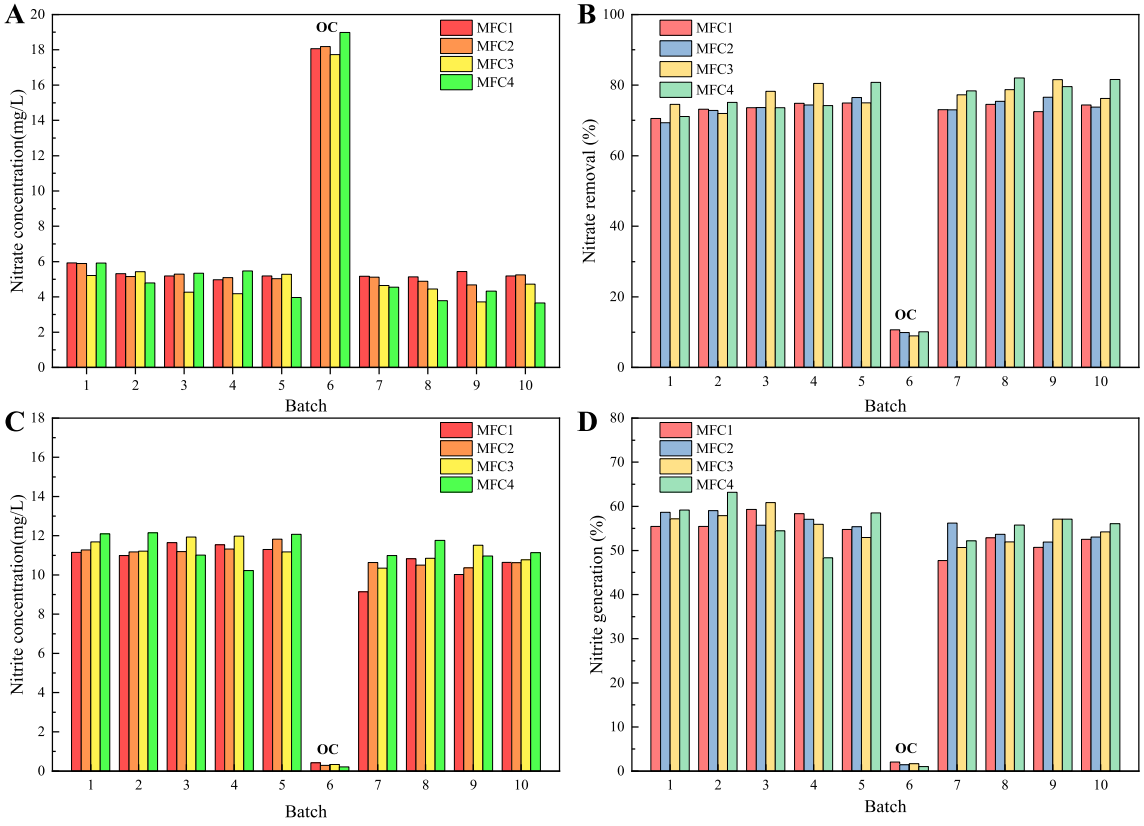

Fig. 1. The nitrate and nitrite concentration in effluent during the 10 batches. (A) Nitrate concentration; (B) Nitrate removal efficiency; (C) Nitrite concentration; (D) Nitrite generation rate. OC (open circuit) indicates that anode and cathode were disconnected, so there was no electron flow from anode to the bio-cathode.

the nitrate concentration significantly decreased from MFC1 to MFC4 (ANOVA, P = 0.03) (Fig. 1A). Given the original feeding nitrate concentration was approximately 20 mg/L, the average nitrate removal rates were up to 73.50%, 73.93%, 77.11% and 77.40%, respectively (Fig. 1B), showing a good nitrate removal efficiency. Concurrently with nitrate reduction, nitrite accumulated in the effluents ranged from 10.53 mg/L to 11.22 mg/L (Fig. 1C), resulting in a nitrite accumulation percent as high as 52.89%, 53.46%, 53.49% and 55.28% in the four MFCs (Fig. 1D). Ammonia in effluent was under detectable limitation during the whole operational process. In contrast, another common denitrification intermediate (N_2O), was present in the headspace of anode chamber with the accumulation concentration of 0.29 mg/L, 0.34 mg/L, 0.38 mg/L and 0.48 mg/L in the four MFCs, respectively (Fig. S1). Previous studies have demonstrated that nitrite and N_2O in the cathodic chamber can be further reduced to nitrogen gas by using current as the sole electron donor (Liang et al., 2019; Prokhorova et al., 2020). However, those accumulated denitrification intermediates in the biocathode chamber would significantly reduce the electrons utilization efficiency (Virdis et al., 2009). After calculating the nitrogen element in chamber, it was found that all MFCs could achieve to more than 22.46%–26.47% of total nitrogen removal.

To further evaluate the contribution of electrons from anode to denitrification in bio-cathode, the wire line was disconnected between anode and cathode at the 5th batch, forming an open circuit. It was found that the nitrate removal efficiency were deteriorated to 10.68%, 9.91%, 8.98% and 10.1%, which is much lower than those in closed circuit batches. However, the nitrate removal efficiency immediately recovered to more than 72.9% after connecting anode and cathode at the 6th batch, suggesting that electrotrophic denitrification contributed more than 62% nitrate removal. Similarly, the nitrite accumulation efficiencies of four MFCs were all less than 2.1% and much lower than that in closed circuit batches (Fig. 1). These various nitrate removal efficiency and nitrite accumulation between open and closed circuit implied that electrons from anode contributing a lot to denitrification process in cathodic chamber. Therefore, it could be concluded that those denitrifying bacteria attached on cathodic biofilms utilized the electrons from the electrode to successfully accomplish the electrotrophic denitrification.

3.2. *Effects of COD/SO_4^{2-} on sulfate reduction and methane production in anodic chamber*

Fig. S2 and Fig. 2 depicted the impact of COD/SO_4^{2-} ratio on COD and sulfate removal performance, respectively. As can be seen, the COD removal efficiency decreased from 100% to 71.14% when the COD/SO_4^{2-} ratio increased from 0.44 to 1.11 (Fig. S2). Similarly, the sulfate removal efficiency significantly decreased from 45.78% in MFC1 to 31.14% in MFC4 (ANOVA, P < 0.01), resulting in an average sulfide concentration decreased from 9.19 mg/L to 1.28 mg/L (Fig. 2). Meanwhile, the thiosulfate concentration was also descended from 10.60 mg/L to 2.67 mg/L, demonstrating that the sulfide produced by sulfate reduction can be re-oxidized to thiosulfate (Lee et al., 2012). Moreover, a portion of sulfide would be re-oxidized to sulfate again, and thus only an average low sulfate removal efficiency (from 31.14% to 45.78%) was achieved (Sangcharoen et al., 2015).

The methane production was monitored and to determine the methanogenesis capacity. As it was illustrated in Fig. S3, the higher COD/SO_4^{2-} ratios would significantly strengthen the methane production (ANOVA, P < 0.01). The average methane production increased from 0.86 mg/L to 5.05 mg/L when the COD/SO_4^{2-} ratio increased from 0.44 in MFC1 to 1.11 in MFC4, indicating that high COD/SO_4^{2-} ratios was more beneficial for MA to compete for electron donors to produce methane (Silva et al., 2020). Methane is greenhouse gas and could emit from the system. Additionally, methane production would inevitably resulted in electrons loss by directly competing with electricigens for electron donors (Virdis et al., 2009). Therefore, it can be concluded that high COD/SO_4^{2-} ratios would go against to the electron utilization in the biocathode denitrification MFC.

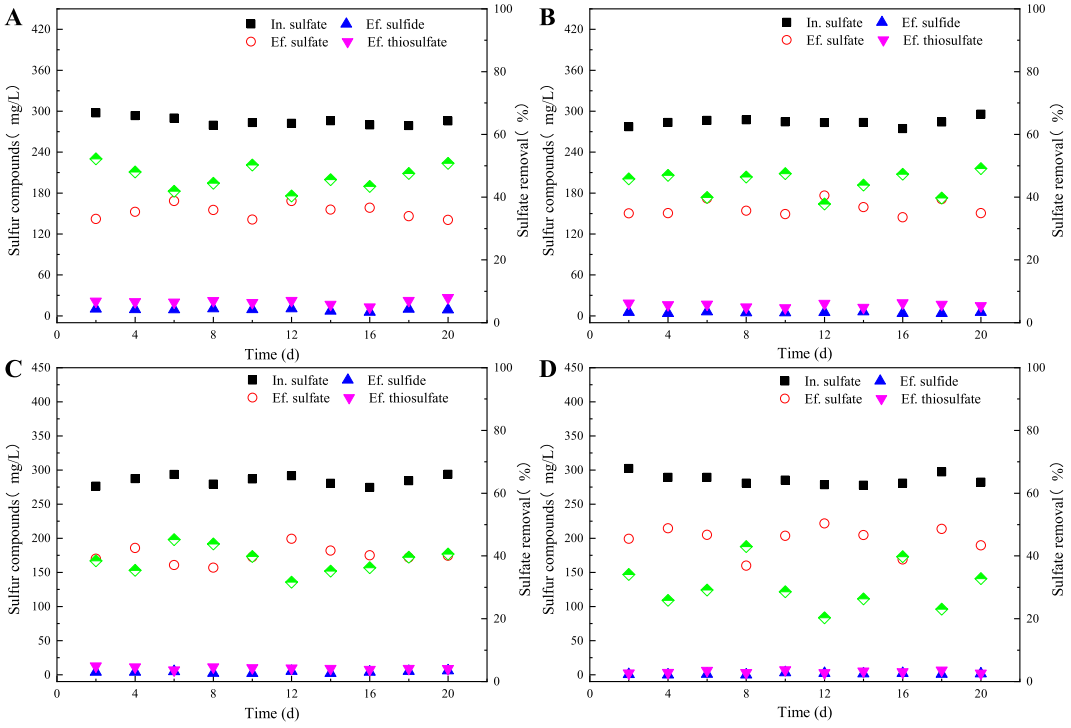

Fig. 2. The monitoring of sulfur concentration during the 10 batches operation under different COD/SO_4^{2-} ratios. (A) MFC1 with COD/SO_4^{2-} ratio of 0.44; (B) MFC2 with COD/SO_4^{2-} ratio of 0.67; (C) MFC3 with COD/SO_4^{2-} ratio of 0.88; (D) MFC3 with COD/SO_4^{2-} ratio of 1.11.

3.3. Mass balance and electron flow analysis of the bio-cathode MFC

3.3.1. Mass balance calculation of COD and sulfur

The mass balance of COD and sulfur in the anodic chamber were calculated at different COD/SO_4^{2-} ratios. The distribution of electrons generated by consuming COD at different anodic influent COD/SO_4^{2-} ratios is depicted in Table 1. When a COD/SO_4^{2-} ratio of 0.44 was applied in MFC1, up to 70.67% of the total COD was consumed by sulfate reduction, whereas only 2.51% was consumed by MA to produce methane and 12.42% of COD was utilized by electroactive bacteria for electricity generation. When COD/SO_4^{2-} ratio increased to 1.11 in MFC4, only 26.53% COD was estimated to consume by sulfate reduction, a slightly increased COD (9.40%) was released as methane, while 9.86% of COD was converted into current. Higher methane production (Fig. S3) along with lower sulfate reduction (Fig. 2) were observed at higher COD/SO_4^{2-} ratios, indicating that the increased influent COD concentration strengthen the activity of MA but deteriorate the sulfate reduction. It is interestingly to note that sulfate reduction is always dominant in the process of competing with methanogenesis for electron donors (Table 1). Similarly, the presence of a substantial SRB population in the inoculum was a main cause for SRB to outcompete MA (Omil F. et al., 1998).

The sulfur balance at different COD/SO_4^{2-} ratios is shown in Fig. 3. The sulfur concentration in the effluents consists of four parts: (1) reduced sulfide in the liquid phase; (2) residual sulfate in the effluent; (3) thiosulfate re-oxidized by sulfide in the effluent; (4) element sulfur re-oxidized by sulfide in the effluent and on the anode. Assuming that those thiosulfate and element sulfur was re-oxidized by sulfide instead of sulfate reduction. During the experiments at fixed COD/SO_4^{2-} ratio, higher sulfate removal efficiency were observed at lower COD/SO_4^{2-} ratios. As depicted in Fig. 3, a maximum sulfate removal of 47.62% was achieved at COD/SO_4^{2-} ratio of 0.44, which was higher than that in COD/SO_4^{2-} ratio of 1.11 (31.49%). The reduction products of sulfate would be sulfide, thiosulfate, and sulfur. Because pH in the influent was controlled at about 8.0, thus the sulfide produced by sulfate reduction was in the form of HS^- (Lu et al., 2016). The proportion of sulfide that reduced from sulfate in the effluent accounted for 8.83%–1.30%, whereas thiosulfate fluctuated between 2.80% and 11.11%. Note that the element sulfur would precipitate on the surface of electrodes and its concentration would change over time, the content of elemental sulfur was not quantitatively monitored (Tang et al., 2010). However, the element compositions analysis conducted by XRF qualitatively demonstrated the presence of element sulfur (Fig. S4). The proportion of element sulfur (ranging from 27.39% to 30.57% in Fig. 3) was calculated by subtracting the content of the total soluble sulfur compounds from the initial sulfate content (Klok et al., 2012).

3.3.2. Electron flow analysis of the bio-cathode MFC

The current generated in the MFC was supplied as the sole electron donor for electrotrophic denitrification, and thus the electricity generation performance would inevitably affect the denitrification performance. The variation of output voltage under different COD/SO_4^{2-} ratios are detailed in Fig. 4. Up to 564 mV of average peak voltage was obtained at COD/SO_4^{2-} ratio of 1.11 in MFC4, which corresponded to a COD removal of 71.14%. A lower average peak voltage of 465 mV was obtained at COD/SO_4^{2-} ratio of 0.44 in MFC1, which resulted in a COD removal rate of 88.47%. To consolidate the understanding of the bio-cathode denitrification performance, the electron transfer and balance under different COD/SO_4^{2-} ratios are calculated. The electric charge generated by the current of the four MFCs were 76.18C, 79.89C, 90.56C and 98.43C, respectively (Table S2). Higher nitrate removal efficiency as well as higher electric charge were observed at higher influent COD/SO_4^{2-} ratios (Fig. 1), indicating that the increase of the external electric charge provides more electron donors for the cathode, and thus promoting the electrotrophic denitrification performance. Similarly, better nitrate removal performance was also obtained in a MFC for biocathode denitrification at higher applied current (Virdis et al., 2008; Zhang et al., 2013). As current was the sole electron donor for biological cathode denitrification, so the electrons consumed by electrotrophic denitrification could be calculated by the denitrified nitrate (Virdis et al., 2011). The electron donor required for nitrate reduction was theoretically

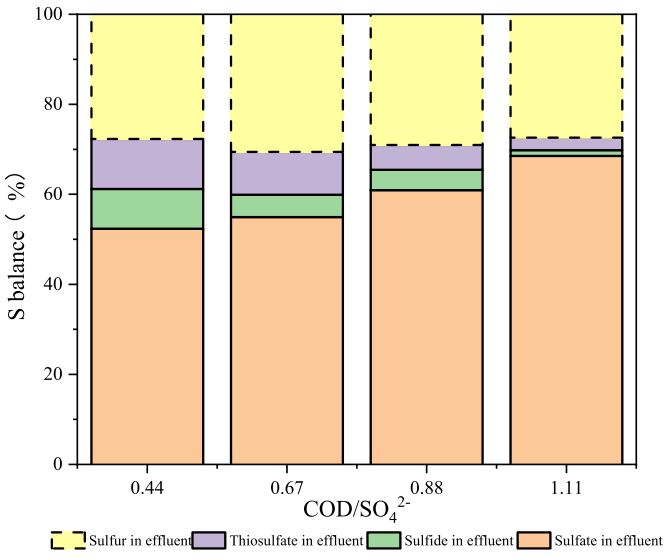

Fig. 3. The sulfur mass balance under different COD/SO_4^{2-} ratios.

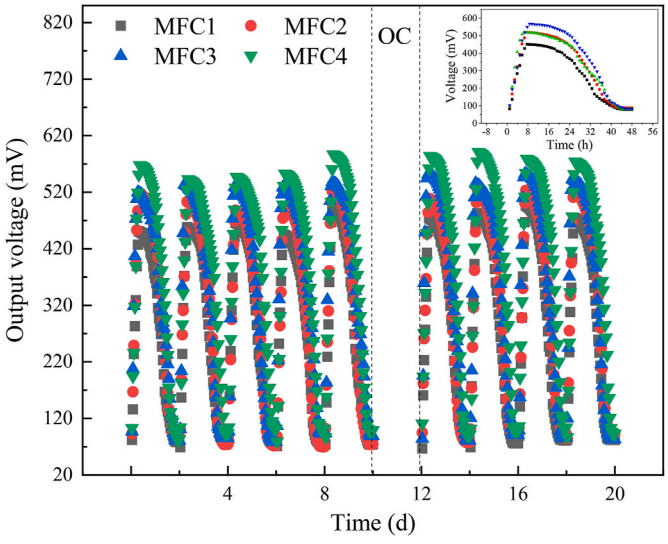

Fig. 4. Variation of output voltage during the 10 batches under different COD/SO_4^{2-} ratios. OC (open circuit) indicates that anode and cathode were disconnected, so there was no electron flow from anode to the bio-cathode.

Table 1
COD mass balance at different COD/SO_4^{2-} ratios.

MFCs	Acetate removal contribution in the anode chamber (%)			
	Methanogenesis	Sulfate reduction	Electricity generation	Others[a]
MFC1	2.51%	70.67%	12.43%	<14.40%
MFC2	4.47%	52.58%	11.17%	<31.78%
MFC3	7.22%	40.71%	10.75%	<41.31%
MFC4	9.40%	26.53%	9.86%	<54.22%

[a] The attribution of others (bacterial growth) for COD removal = 100%-contribution of electricity generation (%)-contribution of sulfate reduction (%)-contribution of methanogenesis (%).

71.58C, 72.35C, 81.26C and 86.1C, which corresponded to an average of 93.97%, 90.56%, 89.73% and 87.5% electron utilization efficiency under different COD/SO_4^{2-} ratios in the four MFCs. As expected, the electric charge generated by the current was all higher than the electrons consumed to reduce nitrate. Apart from those electrons used for denitrification, the electron utilization efficiency in MFCs was decreased by processes like the accumulation of intermediates (e.g. nitrite and N_2O) and bacterial growth (Virdis et al., 2009; Cui et al., 2019b).

3.4. Microbial community in cathodic and anodic biofilms

To investigate the microbial mechanism of electrotrophic denitrification, both cathodic and anodic biofilm microbial community were explored. The detailed microbial community of cathodic biofilm at phylum and genus levels are shown in Fig. S5A and Fig. 5A. The phylum *Proteobacteria* was exclusively enriched as consistent with many other cathodic denitrifying biofilms (Xu et al., 2019). As shown in Fig. 5A, further classified at genus level, the dominant genus in cathodic biofilms is *Thiobacillus*, with relative abundances of 67.30%, 74.35%, 75.83% and 79.70% in the four MFCs, respectively. *Thiobacillus* was multi-functional microbes which could not only use sulfide as the electron donors for denitrification when sulfide present in the influent, but also could make use of electrode as the sole electron donors for denitrification when sulfide absent in the influent (Yu et al., 2015; Zhang et al., 2018). It was reported that the relative abundance of *Thiobacillus* was up to 68% in a strict autotrophic denitrification system with sulfide as the sole electron donor (Huang et al., 2017). Moreover, it has been demonstrated that *Thiobacillus* was able to accomplish denitrification by directly using the electrons from an electrode as the sole electron donors (Van Doan et al., 2013; Yu et al., 2015; Liang et al., 2019). Similarly, current generated by the MFC was the sole electron donor for biocathode denitrification, and *Thiobacillus* was the dominated microbes that characterized with the potential of denitrification on the cathodic biofilms. Thus, it can be concluded that *Thiobacillus* played a vital role in electrotrophic denitrification in the biocathode MFC. It is important to note that the relative abundance of *Thiobacillus* increased as the COD/SO_4^{2-} ratios increased. This could be explained by the fact that higher COD/SO_4^{2-} ratios would provide more electrons (Fig. 4) to promote propagation of those denitrifying bacteria (Fig. 5A), which would finally lead to a high nitrate removal efficiency (Fig. 1). However, limited by the electron transfer ability of *Thiobacillus* (Liang et al., 2019), the accumulation of nitrite in the effluent was observed (Fig. 1).

For anodic biofilm microbial community, the microbial community was much more diversity. The most predominant phylum was also *Proteobacteria* and occupied approximately 52.27%, followed by *Chloroflexi* (22.12%), *Firmicutes* (7.65%), *Bacteroidetes* (4.67%) (Fig. S5B). Similarly, *Proteobacteria*, *Chloroflexi*, and *Firmicutes* were also observed as the dominant bacterial phyla in the MFC system (Deng et al., 2020). COD/SO_4^{2-} ratio played a key role in the competition between MA and SRB on microbial community structure. The total abundance of the five MAs of *Methanosarcina*, *Methanosaeta*, *Methanobacterium*, *Methanobrevibacter*, and *Methanolinea* in the four MFCs slightly increased from 4.47% to 5.63% (Table S3). Among them, *Methanosarcina* and *Methanosaeta* were the only two known MA that can utilize acetate for methane generation (Liu and Whitman, 2008). The total relative abundance of those two acetotrophic MA with COD/SO_4^{2-} ratio of 1.11 was found to be higher than that in other COD/SO_4^{2-} ratios (Table S3), indicates that the growth of MA was promoted at higher COD/SO_4^{2-} ratio, which was consistent with the performance of methane production in the reactor (Fig. S3). The dynamics of seven SRBs with relative abundance ≥0.1% were analyzed and presented in Table S4. By contrast, decreasing the COD/SO_4^{2-} ratio from 0.44 to 1.11 abate the total relative abundance of these SRB decreased from 8.07% to 4.71%. This decrease was caused by higher COD/SO_4^{2-} ratio would make SRB in the competition for electron donors more disadvantaged than MA (Chou et al., 2008). The complete oxidizing SRB of *Desulfobacter* and *Desulfococcus*, which can utilize acetate directly as electron donors for sulfate reduction (Tang et al., 2009), were the most dominant, and its abundance also decreased from 3.31% to 1.92%–0.53% and 0.48%, respectively. This result is in agreement with the performance of sulfate reduction (Fig. 2), suggesting that those two complete oxidizing SRB play a vital role in sulfate reduction in this system.

3.5. Nitrogen associated genes evaluations

In order to explore more on nitrogen metabolism function, the

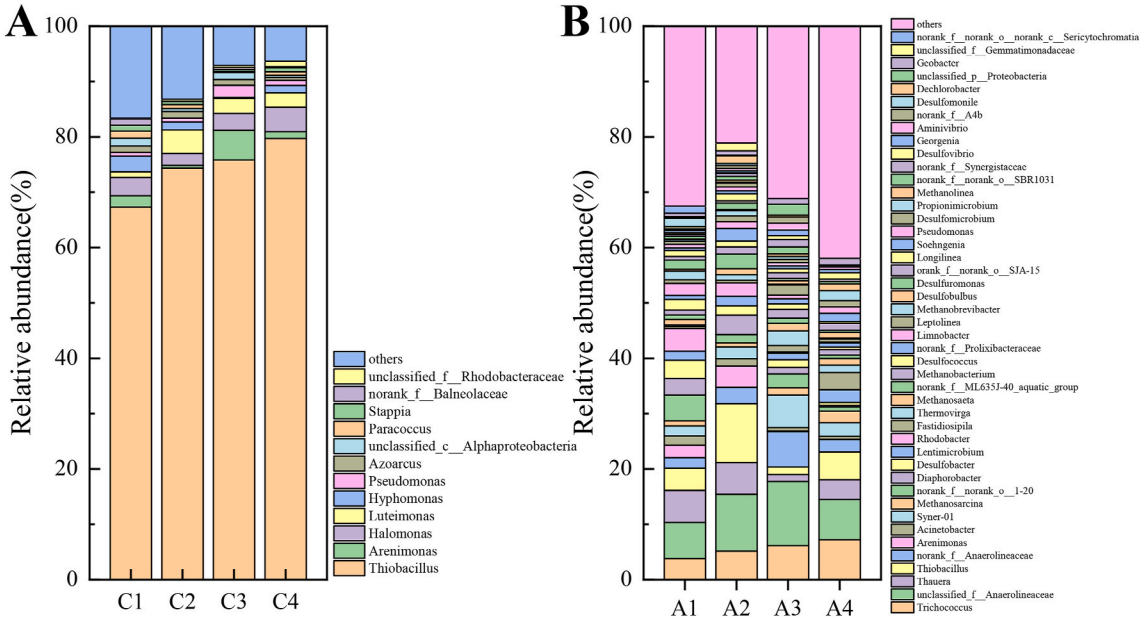

Fig. 5. The relative abundance of microbial community at genus level in cathode and anode biofilms with different COD/SO_4^{2-} ratios. (A) Cathode biofilms; (B) Anode biofilms. Genera with relative abundance lower than 1% were classified into group "others". C1, C2, C3 and C4 correspond to the cathode biofilms collected from the MFCs at COD/SO_4^{2-} ratios of 0.44, 0.67, 0.88 and 1.11, respectively. A1, A2, A3 and A4 correspond to the anode biofilms collected from the MFCs at COD/SO_4^{2-} ratios of 0.44, 0.67, 0.88 and 1.11, respectively.

related denitrification functional genes such as *narG*, *nirK*, *nirS* and *nosZ*, were analyzed by q-PCR. As it was shown in Fig. 6, the abundance of nitrate reductase gene (i.e., *narG*) increased from 3.9145×10^9 copies/g packing to 7.5220×10^9 copies/g packing when the feeding COD/SO_4^{2-} ratio increased (Fig. 6A). It was found that the dominated *Thiobacillus* which encoded with *narG* gene contribute a lot to biocathode denitrification (Pous et al., 2014). Similarly, the abundance of *nirS* gene also increased from 5.7926×10^8 copies/g packing to 2.7703×10^9 copies/g packing when increasing the feeding COD/SO_4^{2-}ratio (Fig. 6C). However, *nirK* gene showed decreasing tendency when applied a higher COD/SO_4^{2-}ratio (Fig. 6B). In the cases of *nirK* and *nirS*, their gene expressions were weaker than the *narG*. This indicates that the rate of nitrate reduction was faster than nitrite reduction, and thus leading to the accumulation of nitrite in the effluent (Fig. 1C). For nitrous oxide reductase gene (i.e., *nosZ*), the abundance fluctuated between 1.3401×10^7 copies/g packing and 6.0055×10^7 copies/g packing in the four MFCs (Fig. 6D). N_2O accumulated has been confirmed with positively correlation with *nosZ* gene in denitrification reactors (Liu et al., 2018).

3.6. Environmental implication

Sulfur is always present in sewage, but few studies have paid attention to it. It has been reported that electron transfer from the sulfur cycle could, in theory, be exploited by denitrification. Although sulfate reduction also needs some carbon sources as electron donors, the oxidation of sulfide can provide electrons to nitrate, coupling with sulfur element recycle in the system. However, the key to such recycle is to prevent the formation of solid sulfur. Therefore, through the design of the reactor, the electrode is applied to enhance the electron transfer and utilization efficiency, which can not only utilize the sulfur circulation, but also promote the denitrification process in the absence of carbon sources. This idea can open up a new way for nitrogen removal when low C/N ratio becomes a common problem, and can also provide technical support for reducing CO_2 emissions and achieving carbon neutral.

4. Conclusion

In this study, the effect of anodic influent COD/SO_4^{2-} ratios on electrotrophic denitrification was explored. Results showed that electrotrophic denitrification contributed to more than 22% total nitrogen removal in cathode chamber, and electrons supplied by re-oxidizing sulfide reduced from sulfate reduction certainly contributed to electrotrophic denitrification. The nitrate removal could be attributed to the predominant *Thiobacillus* grew on cathodic biofilms, and the expression of nitrate associated genes. Higher COD/SO_4^{2-} ratios would make MA more competitive in competing with SRB for the common electron donors, demonstrated by lower relative abundance of SRB at higher COD/SO_4^{2-} ratios, and thus leading to higher methane production but with lower sulfate reduction. Overall, the findings suggested that the use of two-chamber bio-cathode MFCs can act as a potentially sustainable approach to accomplish simultaneous COD and nitrogen removal through sulfur recycle.

Credit author statement

Tao Ai: Conceptualization, Methodology, Validation, Investigation,

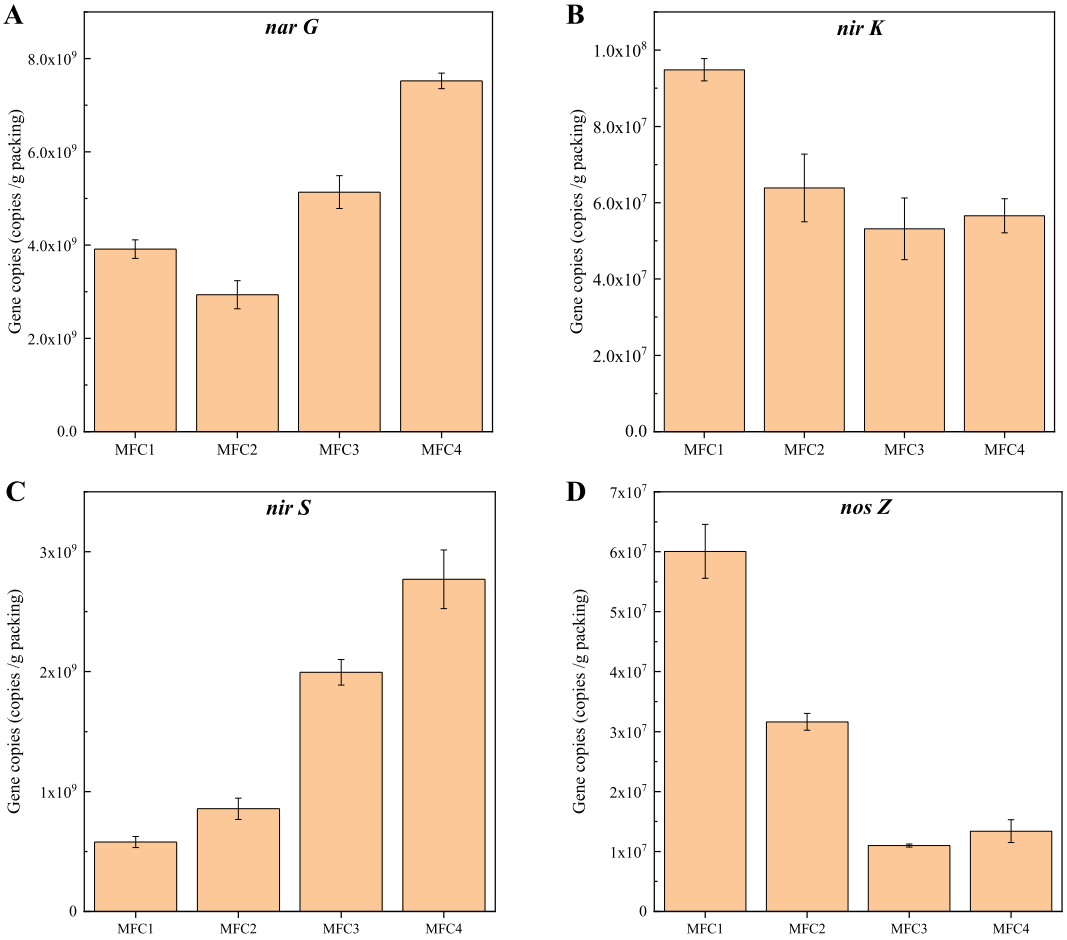

Fig. 6. The expression of denitrification associated genes in the cathodic biofilms. (A) *nar G,* (B)*nir K,* (C)*nir S,* (D)*nos Z.* The initial COD/SO_4^{2-} ratio were 0.44, 0.67, 0.88 and 1.11 in the batch tests of MFC1, MFC2, MFC3 and MFC4, respectively.

Writing – original draft, Visualization. Linzhi Zou: Software, Formal analysis, Writing – review & editing. Hong Cheng: Writing – review & editing, Visualization. Zhongwu Luo: Investigation, Writing – review & editing. Wisam S. Al-Rekabi: Writing – review & editing. Hua Li: Writing – review & editing, Software, Formal analysis. Qibin Fu: Writing – review & editing. Qiang He: Writing – review & editing, Formal analysis. Hainan Ai: Conceptualization, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.132149.

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