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RESEARCH ARTICLE

Impacts of electron donor and acceptor on the performance of electrotrophic denitrification

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Abstract Electrotrophic denitrification is a novel nitrogen removal technique. In this study, the performance and the mechanism of electrotrophic denitrification were investigated at different nitrate concentrations and current intensities. The results showed that the performance of electrotrophic denitrification was good with a sludge loading of 0.39 kg N/kg VSS day. The half-saturation constant for nitrate-N was 1894.03 mg/L. The optimal nitrate-N concentration and current intensity were 1500 mg/L and 20 μA, respectively. Electrotrophic denitrification was defined as the process of direct use of electron for nitrate reduction, and electrotrophic denitrifier was proposed to be the microbe of using electricity as energy source directly. The present work will benefit the development and application of electrotrophic denitrification.

Keywords Bio-cathode · Electrotrophic denitrification · Denitrification performance · Electron donor · Electron acceptor · Microbial mechanism

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Introduction

Nitrogen pollution from various sources has become a serious issue worldwide (Wang and Chu 2016), causing eutrophication and toxic algal blooms in receiving waters (Ghafari et al. 2008). According to the government annual report in 2015, a total of 2.299 million tons of ammonia-N was discharged in China (Ministry of Environmental Protection 2015). The nitrogen discharge has resulted in the deterioration of water quality and in potential hazard to human and animal health (Sumino et al. 2006). Nitrate is the product of N ammonia oxidation. It is often removed by heterotrophic denitrification (Cherchi et al. 2009). However, the application of heterotrophic denitrification is limited by the increasing cost and the risk of secondary pollution when treating wastewaters with low COD/N ratio (Lin et al. 2002; Li et al. 2016).

Electrotrophic denitrification is a new technology for nitrogen removal from wastewaters, which is promising to become an alternative way for the traditional denitrification (Clauwaert et al. 2007). In electrotrophic denitrification, functional microorganisms grow on the bio-cathode, and use electricity from anode as electron donor (Vijay et al. 2016; Oon et al. 2016). Nitrate is reduced to nitrogen gas in the cathode chamber, and accepts the electrons from the cathode (Ghazouani et al. 2014; Prosnansky et al. 2002; Nguyen et al. 2016). The current electron could be generated from biological reaction, chemical reaction, or direct electricity. For heterotrophic denitrification, however, organic matter is necessary.

Electron plays a key role in denitrification. In the electrotrophic denitrification, electron is transported from electricity (through direct contact, nanowire, or electron mediator) into the denitrifying cell (Logan 2009). With the help of the electron transport chain inside the denitrifying cell, the electron is accepted by nitrate, nitrite, nitric oxide, or nitrous

oxide (Chen and Strous 2013). During the electron transport, adenosine triphosphate (ATP) is supposed to synthesize via oxidative phosphorylation, which supports the growth of denitrifying cell.

It is well known that both ends of the electron transfer chain (from electricity to nitrate) were crucial for the electrotrophic denitrification. So far, however, the main attention was focused on the reactor construction and the electrode modification. Rare information is available about the effect of electricity and nitrate on the performance of electrotrophic denitrification.

The objective of this study is to investigate the performance of electrotrophic denitrification and to reveal the effects of electron donor (electricity) and electron acceptor (nitrate) on the performance of electrotrophic denitrification.

Materials and methods

Bio-cathode preparation

Two-chamber microbial fuel cell (MFC) was applied in this study. The configuration of the MFC was similar to that in previous study (Xie et al. 2013). The two identical cylindrical chambers (anode chamber and cathode chamber, with effective volume of 400 mL each) were made of plexiglass, and they were separated by cation exchange membrane (CEM, 28.3 cm^2, Ultrax CMI-7000, Membranes International, USA). The MFC was placed on the platform of magnetic stirrers (HJ-2, Wenhua Instruments Ltd., China). Anode and cathode were connected through titanium wire along with a resistance of 500 Ω. The bio-cathode was prepared in an enrichment reactor established in a previous study. The cathode was made of carbon felt (7 cm × 5 cm × 2 mm) with the effective area of 70 cm^2. The denitrifying aggregates on the bio-cathode were red in color and dense in structure. The red color was considered to be the indication of cytochrome *c* which served as the electron mediator (unpublished). The dominant genera on the bio-cathode were *Acholeplasma* and *Azoarcus*, which were suggested to be the functional microorganisms.

Experiment procedure

The experiment was divided into three sections. In the first section, the MFC was operated in batch mode. Thirteen batches were carried out. The culture medium in the cathode chamber contained 2.0 g/L $NaHCO_3$, 6.0 g/L $NaNO_3$, and 0.18 g/L KH_2PO_4, while the culture medium in the anode chamber was 3.0 g/L CH_3COONa, 0.4 g/L NH_4Cl, and 0.18 g/L KH_2PO_4. The hydraulic retention time (HRT) was set at 8 h in all 13 batches. The temperature was maintained at 25 ± 1 °C, and the pH was controlled at 7.5 ± 0.1. In the 1st, 2nd, and 12th batch, the MFC was operated with open circuit, during which the anode and cathode were disconnected so no electron donor flowed to the bio-cathode microorganism. In the other batches, the MFC was operated with closed circuit, during which the anode and cathode were connected by wire, and the bio-cathode microorganism could gain electron from the anode.

In the second section, HRT was prolonged to 24 h. The component of the culture medium in both catholyte and anolyte was kept the same as those in the first section, except for the nitrate concentration. The nitrate-N concentration was set about 500, 1000, 1500, 2000, and 2500 mg/L in the cathode chamber, respectively. The experiment was conducted in one reactor for three batches at each nitrate concentration when the performance was stable.

In the third section, nitrate concentration was set at 1500 mg/L. The culture medium and the operation mode were the same as those in the second section. An electrochemical workstation was used to adjust the electricity to 5, 10, 20, 50, and 100 μA. The experiment was also conducted in three batches under each electricity condition.

Analytical methods

Chemical and electrochemical analysis

In the first section, water sample was collected every hour, and in the second and third sections, it was collected before and after each batch. All the water samples were filtered through 0.22-μm filters (Labmax, China) before detection. Nitrate concentration was determined by ion chromatography (DIONEX ICS-1000, USA) (Zhang et al. 2016). The ion chromatography was equipped with an AS16 column and AG16 precolumn. The KOH eluent concentration was 7.5 mM in the first 10 min and increased to 30 mM within 1 min. The eluent concentration returned to 7.5 mM after running at 30 mM for 5 min. The total running time was 23 min, and the flow rate was 1.0 mL/min. The real-time output voltage (V) of MFC was monitored by a data acquisition system (Agilent 34970A, Agilent Technologies, USA). The bio-cathode potential, open circuit voltage, and cyclic voltammetry curve were measured using an electrochemical workstation (CHI660d, China).

Specific activity analysis

The nitrate removal in the first hour was supposed to reach the maximum reaction rate (mg/L h). The biomass in the bio-cathode and cathode was tested at the same time, and the difference value was calculated. The specific activity of bio-

cathode microorganisms was obtained by the ratio of maximum reaction rate to biomass weight.

Extracellular polymeric substance determination

Extracellular polymeric substance (EPS) in filtrated water samples was determined using the method described by Huang et al. (2011). Protein and carbohydrate concentration of EPS were determined with bovine serum albumin (BSA) and glucose as the standard references, respectively. The absorbance was measured by a spectrophotometer (UV2450, China).

Functional gene test

The biofilm was scraped with a sterile scalpel from the bio-cathodes at the end of each batch. Genomic DNA was extracted from the biofilm samples by the 3S DNA Isolation Kit (Bocai, China) according to the manufacturers' instructions and stored at −20 °C. One percent agarose gel electrophoresis was used to test the integrity of genomic DNA (average length ~23 kb). The DNA was then used to make quantitative polymerase chain reaction analyses (Wcgene Biotechnology Co., Ltd., Shanghai, China), and the kit was SYBR Premix Ex Taq II (Takara Bio Inc., Japan). The typical functional genes of denitrification, including nitrate reductase gene (*napA* and *narG*), nitrite reductase gene (*nirS* and *nirK*), and nitrous oxide reductase gene (*nosZ*), were determined. The function gene primers and programs are shown in Table 1. Relative abundance of functional gene was calculated by the ratio of functional gene abundance to 16*S* rRNA gene abundance.

Data analysis

All the data was analyzed by Origin 8.0.

Results

Nitrogen removal performance of electrotrophic denitrification

The MFC was operated for a long term (13 batches) to investigate the efficiency and the stability of electrotrophic denitrification. In the 1st, 2nd, and 12th batch, the MFC was in open circuit (no transfer of electron to cathode); while in the other batches, the MFC was under closed circuit condition (with transfer of electron to cathode). In the state of open circuit, no nitrate was reduced. On the contrary, in the state of closed circuit, nitrate was reduced. As shown in Fig. 1, the bio-cathode displayed stable denitrification performances in all the ten batches with closed circuit. Nitrate was converted by 80 to 100 mg/L in 8 h. Interestingly, no reduction of nitrate was observed in the 12th batch with open circuit, but the denitrification performance resumed immediately in the next batch with closed circuit. In other words, the performance of bio-cathode denitrification was electricity-dependent.

Kinetic property of electrotrophic denitrification

Different nitrate (electron acceptor) concentrations and electricity (electron donor) intensity were applied to study the kinetics of electrotrophic denitrification. As Fig. 2a shows, the nitrate removal efficiency rose from 0 to 16.78% with the nitrate-N concentration increasing from 500 to 1500 mg/L. However, the nitrate removal efficiency declined to around 13.87% when the nitrate-N concentration further increased to 2500 mg/L. As Fig. 2c displays, the reaction rate was increased from 0 to 42.21 mg/L h with the increase of nitrate concentration. The fitted curve of the experimental data was in accordance with the Michaelis-Menten equation, whose correlation coefficient R^2 was 0.944 (Table 2). Based on the fitted equation, the half-saturation constant K_m was determined as

Table 1 Primes and programs of denitrification functional genes

Target	Sequence (5′-3′)	Thermal profile for qPCR (40 cycles)	Reference
nirS	F: GTSAACGTSAAGGARACSGG R: GASTTCGGRTGSGTCTTGA	95 °C/5 min; 95 °C/60 s, 59 °C/60 s, 72°C/60 s	Michotey et al. (2000)
nirK	F: ATCATGGTSCTGCCGCG R: GCCTCGATCAGRTTGTGGTT	95 °C/3 min; 95 °C/40 s, 62.5 °C/40 s, 72 °C/60 s	Hallin and Lindgren (1999)
nosZ	F: CGCRACGGCAASAAGGTSMSSGT R: CAKRTGCAKSGCRTGGCAGAA	95 °C/5 min; 95 °C/60 s, 60 °C/60 s, 72 °C/60 s	Henry et al. (2006)
napA	F: AAYATGGCVGARATGCACCC R: GRTTRAARCCCATSGTCCA	95 °C/5 min; 95 °C/30 s, 57 °C/45 s, 72 °C/60 s	Henry et al., (2008)
narG	F: TAYGTSGGGCAGGARAAACTG R: CGTAGAAGAAGCTGGTGCTGTT	95 °C/5 min; 95 °C/20 s, 59 °C/30 s, 72 °C/40 s	Gregory et al. (2000)

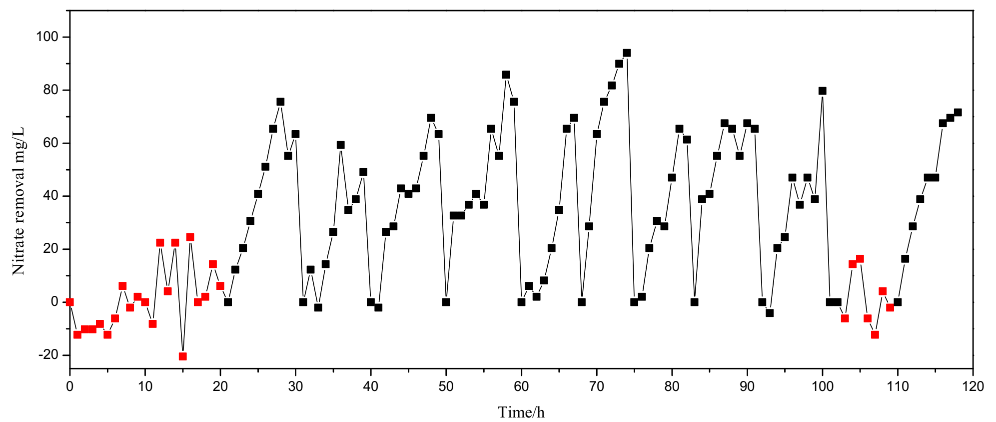

Fig. 1 The performance of electrotrophic denitrification during a long-term operation. *Black dots* indicate that anode and cathode were connected by wire, and the bio-cathode microorganism could gain electron donor from anode. *Red dots* indicate that anode and cathode were disconnected, so there was no electron donor flow to bio-cathode microorganism

1894.03 mg/L (by nitrate-N), and the maximum reaction rate r_{max} was determined as 74.17 mg/L h (by nitrate-N). Compared with the parameters in literatures, the affinity of electrotrophic denitrification for nitrate was relatively weak, but the tolerance to high nitrate concentration was considerably strong with the optimal nitrate-N concentration of 1500 mg/L.

Figure 2b illustrates the effect of electricity intensity on nitrate removal efficiency. During the increase of electricity intensity from 5 to 100 μA, the nitrate removal efficiency first went up from 14.28 to 17.76% and then went down to 13.47%. The optimal electricity intensity was about 20 μA with the highest nitrate removal efficiency. The fitted curve of the experimental data was in accordance with the Haldane model, whose correlation coefficient R^2 was 0.970 (Fig. 2d). According to the fitted equation, the half-saturation constant K_m achieved 1.99 μA, the half inhibition constant was 158.71 μA, and the maximum reaction rate r_{max} was

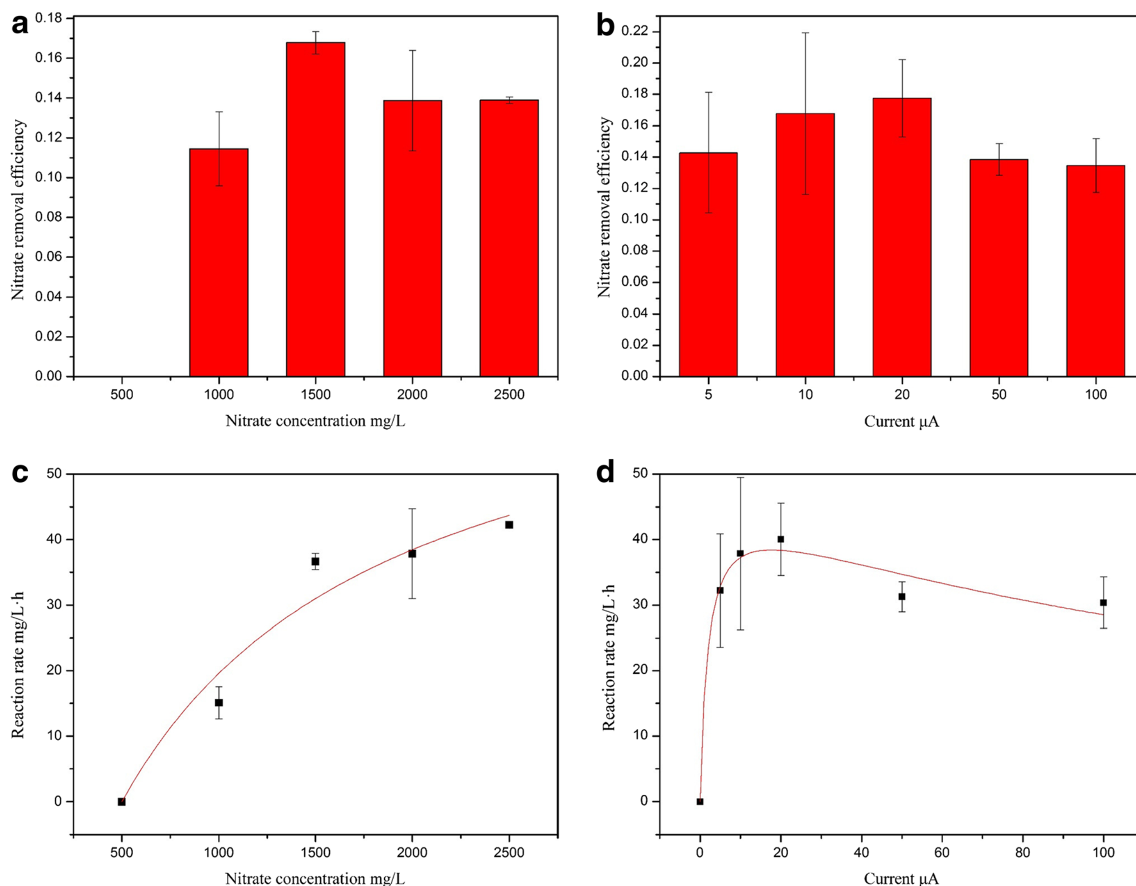

Fig. 2 **a** Nitrate removal efficiency at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **b** Nitrate removal efficiency at 5, 10, 20, 50, and 100 μA current. **c** Nitrate removal rate at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **d** Nitrate removal rate at 5, 10, 20, 50, and 100 μA current

Table 2 Kinetic parameter at different nitrate concentrations

Kinetic parameter	r_{max}	K_m
Value	74.17	1894.03
Fitted equation	$r = 74.17 \times ([S] - 500) / (1894.03 + [S])$	
R^2	0.944	

47.02 mg/L h (by nitrate-N) with the optimal electricity intensity of 20 μA (Table 3).

Electrochemical performance of electrotrophic denitrification

The electrochemical performance was investigated at different nitrate concentrations and current intensity. As Fig. 3a shows, the bio-cathode potential showed a similar trend of firstly going up and then going down during any batch with one of the five nitrate concentrations. Although the peak potential was nearly the same, the terminal potential was different. The open circuit voltage (OCV) at different nitrate concentrations is shown in Fig. 3b, and it went up from 73.27 to 224.35 mV with the increase of nitrate concentration.

The electrochemical performance at different electricity intensity showed a good consistency with its kinetic property. The cathode potentials at the electricity intensity of 50 and 100 μA were far lower than those at the current intensity of 5, 10, and 20 μA (Fig. 3c). The OCV rose from 15.28 to 18.49 mV at the low electricity intensity and declined immediately to 14.25 and 12.69 mV at the high electricity intensity of 50 and 100 μA (Fig. 3d).

Microbial mechanism of electrotrophic denitrification

Specific activity of bio-cathode microorganisms

Specific activity is the key characteristic of bio-cathode microorganisms, which plays a decisive role in the performances of electrotrophic denitrification. The specific activity of microbial biofilm under different conditions is shown in Fig. 4. With the increase of nitrate-N concentration from 500 to 1500 mg/L, the specific activity of bio-cathode biofilm jumped from 0 to 0.74 mg N/g biofilm h and then obtained a steady state with value of 0.75 mg N/g biofilm h when the nitrate concentrations were between 2000 and 2500 mg/L.

Table 3 Kinetic parameter at different electricity intensity

Kinetic parameter	r_{max}	K_m	K_i
Value	47.02	1.99	158.71
Fitted equation	$r = 47.02/(1 + 1.99 / [S] + [S] / 158.71)$		
R^2	0.970		

The denitrification activity of bio-cathode microorganism reached the top level when the nitrate concentration was above 1500 mg/L. The specific activity first went up and then down with the increase of electricity intensity. The maximal specific activity was 0.80 mg N/g biofilm h at 20 μA.

EPS of bio-cathode microorganisms

EPS plays an important role for the formation and function of bio-cathode. Polysaccharide and protein concentrations at different nitrate concentrations and electricity intensity are shown in Fig. 5. As illustrated in Fig. 5a, nitrate concentration had a great impact on polysaccharide rather than the protein. Polysaccharide concentration grew from 4.35 to 23.39 mg/L with the increase of nitrate concentration from 500 to 2500 mg/L. Similarly, electricity intensity had no influence on protein, but had a large influence on polysaccharide. Protein concentration kept the same under all the five electricity intensities, while polysaccharide concentration went up from 3.65 to 7.05 mg/L at 5 and 10 μA. With the further increase of electricity intensity, polysaccharide concentration slightly went down to 6.05 mg/L at 20 μA but remarkably rose to 30.83 mg/L at 100 μA.

Functional genes of bio-cathode microorganisms

Functional genes are the basis of microbial denitrification, and their relative abundance is affected by environmental conditions. Five functional genes of denitrifying bacteria were determined at different nitrate-N concentrations and electricity intensity, and the results are shown in Fig. 6. *napA* and *narG* were typical genes for nitrate reductase. With the increase of nitrate-N concentration, *napA* rose from 23.42 to 30.09% at 2000 mg/L and slightly decreased to 29.8% at 2500 mg/L. However, no *narG* could be detected. With the increase of nitrate-N concentration, the gene for nitrite reductase *nirS* first increased then decreased. The maximum fraction achieved 26.22% at the nitrate-N concentration of 1500 mg/L. *nirK* was another gene for nitrite reductase, and its maximum fraction was 34.55% at the nitrate-N concentration of 1000 mg/L. *nosZ* was the gene for nitrous oxide reductase, and its fraction reached up to 18.88% at the nitrate-N concentration of 1000 mg/L.

With the increase of electricity intensity, the fraction of different functional genes varied obviously. The relative abundance of functional genes rose when the electricity intensity was increased from 5 to 20 μA. However, it declined when the electricity intensity was increased to 50 and 100 μA. The relative abundance of *napA*, *nirS*, and *nirK* at 20 μA were 1.17, 1.11, and 1.37 times, respectively, higher than those at 50 μA and 1.21, 5.80, 1.63, and 1.82 times higher than those at 100 μA.

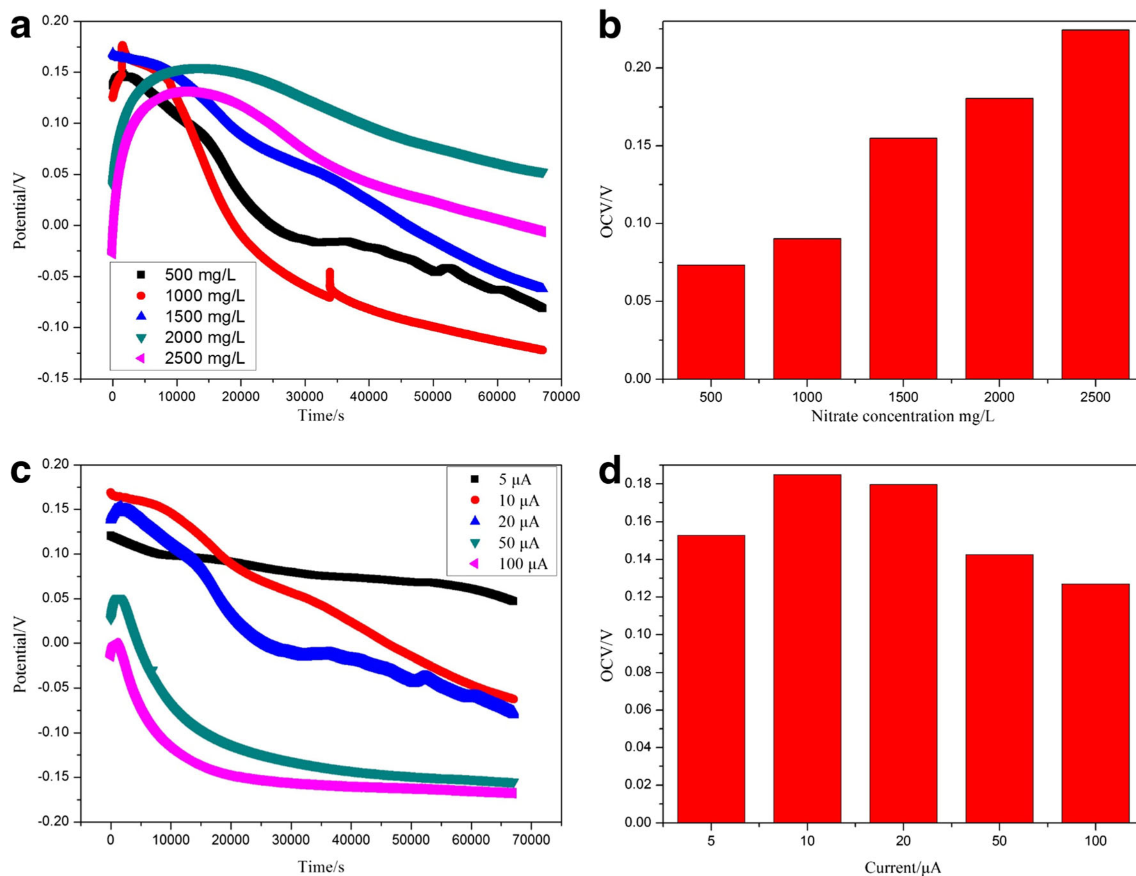

Fig. 3 **a** The change of cathode potential at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **b** The change of OCV at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **c** The change of cathode potential at 5, 10, 20, 50, and 100 μA current. **d** The change of OCV at 5, 10, 20, 50, and 100 μA current

Discussion

The typical characteristics of electrotrophic denitrification

The electrotrophic denitrification had two typical characteristics, i.e., strong stability and high nitrate removal capacity. As shown in Fig. 1, no electron was supplied for denitrification, and so, no significant nitrate reduction was observed in the state of open circuit. On the contrary, electricity was supplied in the state of closed circuit; the electron from the electricity was directly used in denitrification, and so, a large amount of nitrate was converted. The electrotrophic denitrification stopped when the MFC was changed from closed circuit to open circuit, but it resumed immediately after the MFC was returned from open circuit to closed circuit. The long-term operation of MFC demonstrated the performance stability of electrotrophic denitrification.

It has been reported that the sludge loading of heterotrophic denitrification was 0.58 kg N/kg VSS day (Li et al. 2014) and that of autotrophic denitrification was 0.003 kg N/kg VSS day (Zhang et al. 2015). In our work, the sludge loading of electrotrophic denitrification was determined as 0.39 kg N/kg VSS day, which was very competent. In a previous study on bio-cathode denitrification, the nitrate removal was about 0.1–0.4 kg N/(m^3 day), while it was about 0.25 kg N/(m^3 day) in this work (Clauwaert et al. 2007; Zhang and Angelidaki 2013). For heterotrophic denitrification, the electron could only be obtained from organic matters, and the oxidation of organic matters often occurred with the reduction of nitrate in the same chamber. For electrotrophic denitrification, however, the electron could be supplied by various sources such as acetate oxidation, iron oxidation, or electricity. The oxidation of fuels and reduction of nitrate could be separated in different chambers, which could extend the application field of electrotrophic denitrification. Since electricity is a clean electron donor without secondary pollution, the electrotrophic denitrification is promising to be an alternative to traditional denitrification.

The activity of bio-cathode microorganisms

Nitrate concentration and current intensity represented the availability of electron acceptor and electron donor in the denitrification. The performance of electrotrophic denitrification was improved with the increase of nitrate concentration. In our

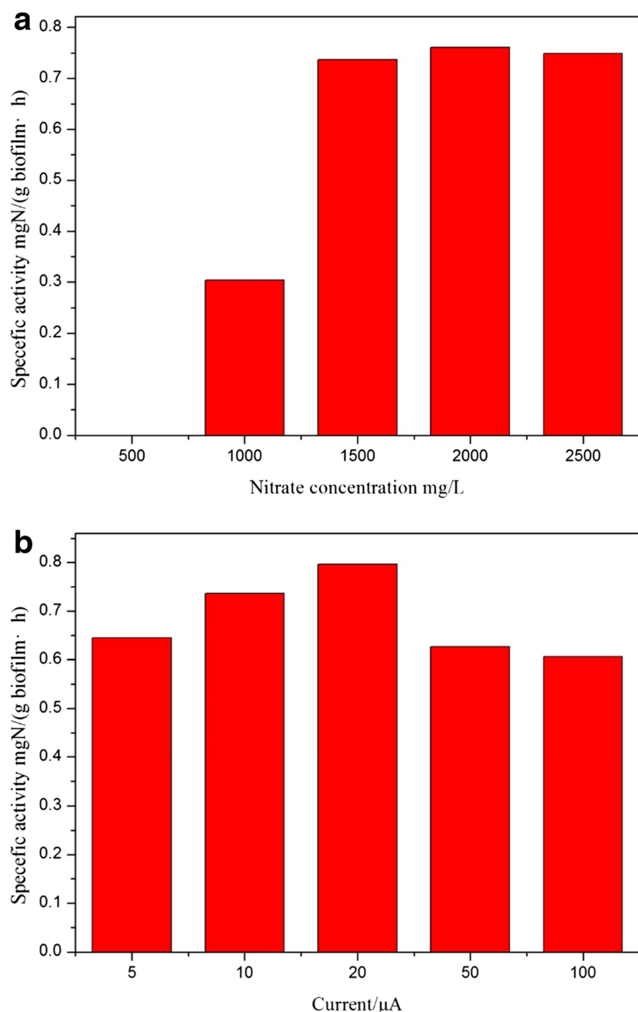

Fig. 4 **a** The specific activity of bio-cathode microorganisms at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **b** The specific activity of bio-cathode microorganisms at 5, 10, 20, 50, and 100 μA current

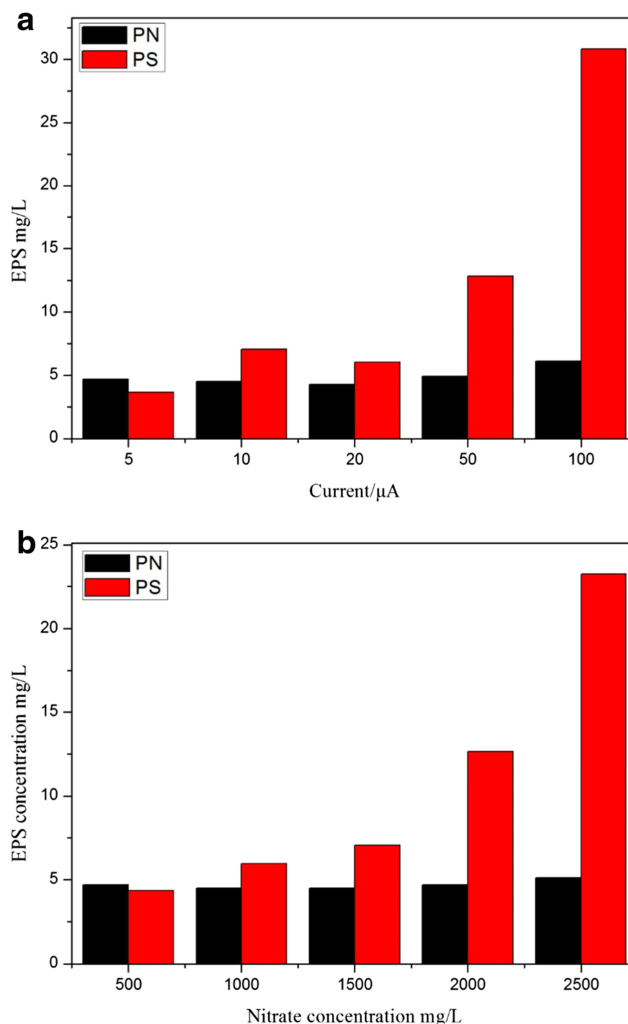

Fig. 5 **a** The change of protein and polysaccharide at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **b** The change of protein and polysaccharide at 5, 10, 20, 50, and 100 μA current

work, the floor level of nitrate-N concentration was determined at 500 mg/L, which can get an explanation from the high half-saturation constant K_m of 1894.03 mg/L. It has been reported that in the heterotrophic denitrification, the K_m for nitrate-N was about 63.00 mg/L, while in the autotrophic denitrification, it was about 3.66 mg/L (Ivanov et al. 2015; Kim et al. 2005). The affinity for nitrate in the electrotrophic denitrification was far weaker than those in the traditional denitrification. Therefore, the electrotrophic denitrification might be more suitable for wastewaters with high nitrate concentration. Because the electrotrophic denitrification occurred on the cathode, electrochemical performance could be used to indicate denitrification performance. OCV reflected the potential difference between anode and cathode, which could be an indicator for the reaction difficulty in the thermodynamics. A larger OCV means an easier reaction.

Microorganism was the performer of electrotrophic denitrification, and its activity decided the performance of electrotrophic denitrification. In autotrophic conditions, bio-cathode microorganism synthesizes organic matter from CO_2 with electricity as sole energy source, so organic matter like EPS can reflect the general activity of bio-cathode microorganism. In this work, the concentration of polysaccharide, the main component of EPS, rose obviously with the increase of nitrate concentration and electricity intensity, indicating that electricity can indeed serve as energy source.

The response of functional genes to environmental conditions

napA, *narG*, *nirS*, *nirK*, and *nosZ* were typical functional genes for denitrification. The relative abundance of these denitrifying genes can indicate the potential activity of bio-cathode microorganisms. *narG* was below detection limit. Compared with the counterparts at 500 mg/L, the relative abundance of four denitrifying genes generally gave a positive

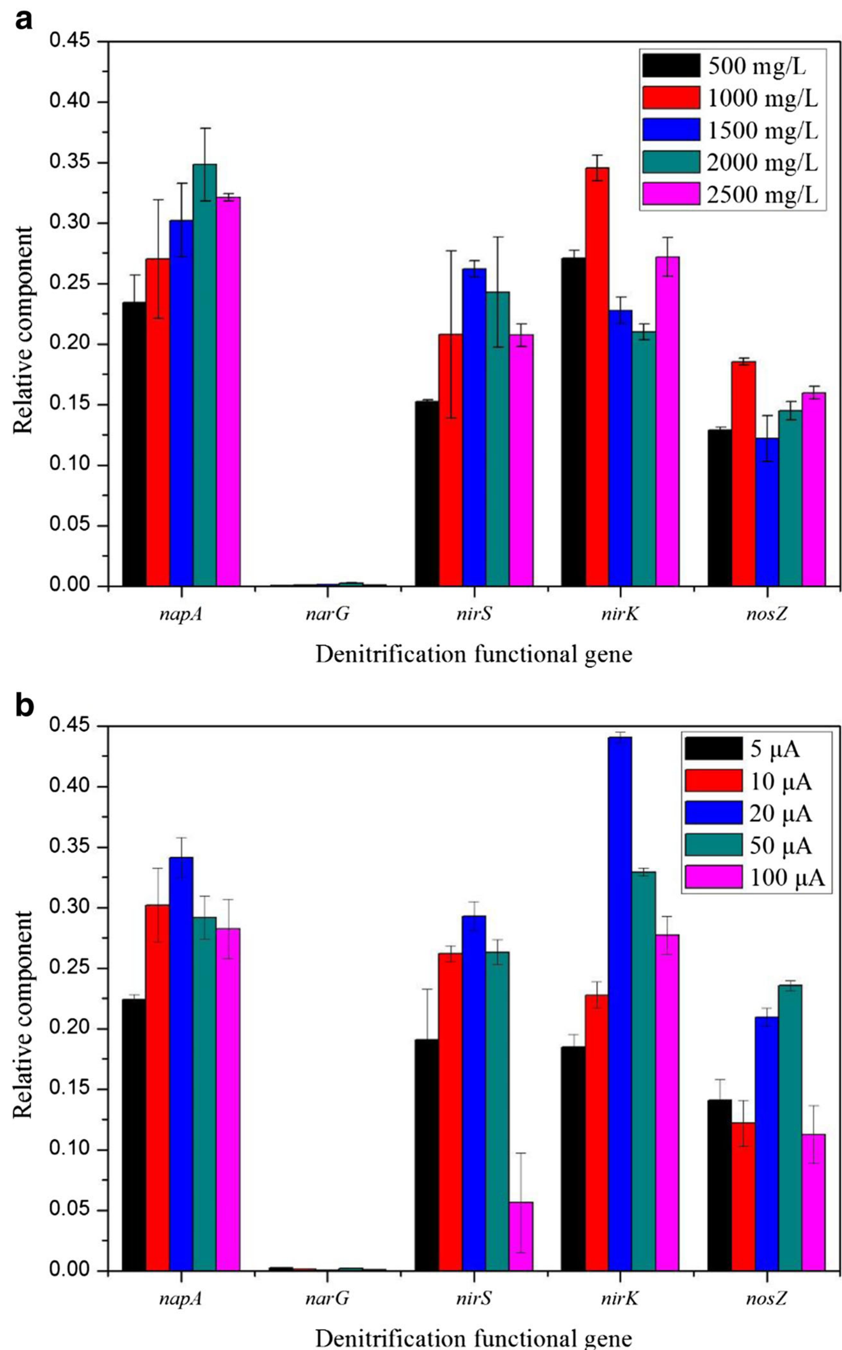

Fig. 6 **a** The functional genes of bio-cathode microorganisms at 500, 1000, 1500, 2000, and 2500 mg/L nitrate concentration. **b** The functional genes of bio-cathode microorganisms at 5, 10, 20, 50, and 100 μA current

response to the increase of nitrate (electron acceptor) concentration, and the optimal nitrate concentration for *napA* (2000 mg/L), *nirS* (1500 mg/L), *nirK* (1000 mg/L), and *nosZ* (1000 mg/L) gradually decreased. Compared with the counterparts at 5 μA, the relative abundance of *napA* and *nirK* gave a positive response to the increase of electricity (electron donor) intensity, but the level of *nirS* and *nosZ* gave a changeable response. The relative abundance of *napA*, *nirS*, and *nosZ* decreased in order at the optimal electricity intensity as well as *nirK*. In a previous study, it was found that the relative abundance of *nirK* in bio-cathode denitrifier was reported much higher than that in the control reactor (no electricity electron was supplied to bio-cathode microorganism), and the relative abundance could reach 40%, which was in agreement with our results (Li et al. 2015).

In this work, the experimental data showed that the electrotrophic denitrification is electricity-dependent, indicating that the electron could be directly used to reduce nitrate. The experimental data displayed that EPS content is proportional with electricity intensity, indicating that the electricity could serve as energy source for cell anabolism. The experimental data also demonstrated that the relative abundance of

four denitrifying genes (*napA*, *nirS*, *nirK*, *nosZ*) were positive responses to the increase of nitrate concentration and electricity intensity, which implied that the electricity could serve as energy source to support the microbial growth. In addition, the electrotrophic denitrification was stable for the long-term operation, which confirmed that the electricity can serve as electron donor to drive denitrification and serve as energy source to support the microbial growth.

It is well known that organic and inorganic matters in their reduced state can serve as electron donor to provide electron for denitrification or nitrate respiration, generating ATP to support the growth of denitrifying cells. In this work, we proved that the electron from cathode could be directly used for denitrification, possibly synthesizing ATP to support the growth of bio-cathode microorganisms. Since the bio-cathode microorganisms carry out denitrification, they are called as denitrifiers; since they directly use electron to reduce nitrate, they are called electrotrophic denitrifiers. The electrotrophic denitrification and the electrotrophic denitrifier are two great discoveries in the field of environmental microbiology.

Conclusions

1. Electrotrophic denitrification is a novel process which is effective and stable. The sludge loading was 0.39 kg N/kg VSS day. The performance could stay at a high level for 13 successive batches.
2. The fitted equation between nitrate reduction rate and nitrate concentration is $r = 74.17 \times ([S] - 500) / (1894.03 + [S])$; the fitted equation between nitrate reduction rate and current intensity is $r = 47.02 / (1 + 1.99 / [S] + [S] / 158.71)$. The optimal nitrate-N concentration and electricity intensity for electrotrophic denitrification are 1500 mg/L and 20 μA, respectively.
3. In electrotrophic denitrification process, electron could be directly used to reduce nitrate from cathode, and bio-cathode microorganism (electrotrophic denitrifier) could be directly used as current electron for microbial catabolism and anabolism.

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