Effect of electro-stimulation on activity of heterotrophic denitrifying bacteria and denitrification performance

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HIGHLIGHTS
- Nitrate could be effectively removed in developed bio-electrochemical reactor.
- Heterotrophic denitrifying bacterial activity could be enhanced by electro-stimulation.
- High ATP aggregate level was obtained at the current density of 200 mA/m².

ABSTRACT
The effects of electro-stimulation on heterotrophic denitrifying bacterial activity and nitrate removal were investigated using a bench-scale bio-electrochemical reactor in this study. Results showed that the maximum nitrate removal efficiency was 100% at the optimum current density of 200 mA/m², at which low nitrite production and high ATP aggregate level were obtained. The activity of denitrifying bacteria was highest at the range densities of 200–250 mA/m², although the terminative pH increased to 8.62 at 200 mA/m² and 9.63 at 250 mA/m². This demonstrates that suitable current densities could improve the activity of denitrifying bacteria. Therefore, this study provides a number of useful information to improve the bio-electrochemical reactor designs and promote the removal efficiency of pollutants.

1. Introduction
Nitrate contamination in groundwater, a main source of drinking water, has recently become an increasingly serious problem, resulting from inputs of over fertilization in farms, industrial effluents, animal and human wastes (Ghafari et al., 2008). High concentration of nitrate in drinking water not only causes serious illnesses such as methemoglobinemia or blue baby syndrome (Mousavi et al., 2012), but could also lead to gastric cancer (Ghafari et al., 2009). Therefore, the maximum contaminant level (MCL) for nitrate stipulated by the World Health Organization (WHO, 2008) is at 11.29 mg-N/L in drinking water, and the value proposed by China is 10 mg-N/L (Standards for Drinking Water Quality, China: GB5749-2006).

Biofilm-electrode reactor (BER) has attracted considerable interests in treating polluted water by nitrate removal. Islam and Suidan (1998) used a continuous flow BER to evaluate biological denitrification at different currents (0–100 mA), and obtained 98% nitrate removal efficiency at 20 mA current. Ghafari et al. (2009) also treated contaminated water containing 20 mg/L NO3–N in an upflow bioelectrochemical reactor (UBER) at currents 0–20 mA, and reported that the nitrate removal efficiency was 100% at optimum electric currents 10–16 mA. Zhou et al. (2009) found that concentrations of nitrate and nitrite decreased greatly to around 2 mg/L at 15 mA in a three-dimensional bio-electrochemical reactor (3D-BER). However, traditional BERs consume a lot of electrical energy to produce CO2 and H2 (Mousavi et al., 2012). In order to reduce the electric energy consumption and addition of organic carbon source, and to improve denitrification efficiency, Zhao et al. (2011) developed a HAD system using fiber threads as independent carrier with carbon rods as the anode and stainless steel wire as the cathode to produce H2 for autotrophic bacteria, and they reported that the nitrate...
removal efficiency was over 97% at current of 40 mA and C/N of 0.75. Tong et al. (2013) used a similar HAD system but with a stainless steel rod as anode, which could avoid the generation of carbon dioxide during electrolysis process, and further observed 99.9% nitrate removal efficiency at current density of 200 mA/m². Hao et al. (2013) developed a three-dimensional biofilm-electrode reactor (3D-BER) with cooperative heterotrophic and autotrophic denitrification of wastewater treatment plant (WWTP) effluent, and they investigated the effects of C/N ratio and HRT on the denitrification performance and bacterial community under constant 40 mA supply. However, in previous study, the authors only reported the optimum current or set a constant amount of current as experimental conditions, and showed that the high current could inhibit microbial activity, or even kill microorganisms.

The potential effects of low current and suitable current to improve microbial activity has been rarely reported or tested. Also, knowledge on the mechanisms of how current affects activity of the denitrifying bacteria remains to be understood. In fact, the effect of electric current on bacterial activity and viability is a major concern in applying bio-electrochemical method. Some studies pointed out that the electro-stimulation could affect microbial growth and activity, and proper electro-stimulation could promote microbial metabolism (Thrash and Coates, 2008). Kojima et al. (1992) used 0.2–0.6 V potential to stimulate MKN45 cells (Carcinoma cells) in a culture dish with Pt as the anode and cathode, and showed that current stimulation could change the DNA, protein synthesis, membrane permeability and cell growth. Furthermore, heterotrophic bacterial viability was also observed to be not significantly affected when the applied electric current density was less than 6.2 A/m², but was partly inactivated at current densities more than 12.3 A/m² in a membrane reactor (Wei et al., 2011). The same observation was also true for the autotrophic species where the denitrification and viability drastically decreased at electrical stimulation with densities more than 16 mA/cm² but remained active and viable at 2 and 4 mA/cm² (Safari et al., 2013).

In this study, to further explore the effects of electro-stimulation on the activity of heterotrophic denitrifying bacteria and denitrification efficiency, a small bench-scale bio-electrochemical reactor was developed. Specifically, this study aims to: (1) determine the optimum current density by investigating nitrate removal efficiency under different current densities, (2) investigate the activity of heterotrophic denitrifying bacteria under different current densities, and (3) elucidate the mechanisms of electro-stimulation on the activity of denitrifying bacteria in a bio-electrochemical nitrate treatment.

2. Methods

2.1. Synthetic groundwater preparation

Synthetic groundwater was prepared by dissolving 0.304 g NaNO₃, 0.044 g KH₂PO₄, and addition of 0.21 mL methanol (CH₃OH) in a liter of tap water. The concentration of NO₂⁻–N was prepared as 50.00 mg/L. The initial pH was around the range of 7.25 ± 0.02 and needed no further adjustment.

All chemical reagents used in the experiments were analytical grade.

2.2. Experimental apparatus

The experimental apparatus consisted of a bench-scale bio-electrochemical reactor, a DC regulated power supply and a constant temperature magnetic stirrer. The bio-electrochemical reactor composed of a conical flask (1 L), an anode and a cathode. The anode was a stainless steel rod (diameter 3 mm, length 250 mm), while the cathode was made of a spiral iron wire (iron wire diameter 1 mm, spiral diameter 35 mm and spiral height 30 mm). The cathode was concentrically installed in the conical flask, and the anode was fixed in the center of the cathode. The conical flask was then sealed with a rubber plug through which a sampling pipe was inserted. A constant temperature magnetic stirrer (Hijiu, H01-1C, China) was used to keep the synthetic groundwater at constant temperature of 30 ± 2 °C and ensure homogeneous distribution of the bacterial suspension in the reactor during the experiments. A DC regulated power supply (PS-302D, Shenzhen, China) was used to provide a constant DC for the different runs.

2.3. Acclimation of sludge

Activated sludge for inoculation of denitrifying bacteria was collected from the Qinghe wastewater treatment plant (Beijing, China), and was washed three times with tap water prior to cultivation in 1 L liquid nutrient medium and incubation at 30 °C. The media was also supplemented with nutrients such as NaNO₃ (7 mM), KH₂PO₄ (0.3 mM) and glucose (10 mM), and was replaced every 4 days. Acclimation was considered successful when the nitrate removal efficiency became stable (maintained at >95%) and also when the sludge turned dark grey.

2.4. Experimental start-up

The acclimated sludge (4 mL) and synthetic groundwater (1 L) were added to the bio-electrochemical reactors at the beginning of each experiment. Each group of experiment was independent and the acclimated sludge in each experiment was re-added to inhibit growth of autotrophic denitrifying bacteria.

To investigate the effects of different current densities and initial pH on nitrate removal efficiency and bacterial activity, the applied current densities were set as 0, 50, 100, 150, 200, 250, 300, 350 and 400 mA/m² at initial pH 7.25 ± 0.02, and initial pH was adjusted to 7.25, 8.25, 9.25, 10.25 and 11.25 by 1 mol/L NaOH solution at the observed optimum current density. This pH set-point was based on the terminative pH (i.e. pH at the end of the experiment) which was observed under the different current densities. The reactor was operated for 14 days for each experimental condition, and 8 mL of sample was taken from the sampling port every 24 h.

To ascertain the potential electrochemical nitrate reduction, electrochemical experiments were carried out without bacteria inoculation. The applied current densities were also gradually increased from 50 to 400 mA/m² at an interval of 50 mA/m².

2.5. Analytical methods

NO₃⁻–N, NO₂⁻–N and NH₄⁺–N were determined by ultraviolet spectrophotometer (DR6000, HACH, US) according to the Water and Wastewater Monitoring Analysis Method (SEPA, 2002). Adenosine triphosphate (ATP) content was measured by ATP meter (AF-100, TOA-DKK, Japan). The standard deviations were analyzed at a confidence level of 90%, and all analyses were carried out in Origin 8.0 (OriginLab, trial version). The pH was determined by pH meter (Seven Multi S40, Mettler Toledo, Switzerland).

Scanning electron microscope (SEM) was performed to observe the induced changes in cell shape and extracellular polymers under 0, 50, 200 and 400 mA/m². At the end of each experiment, bacterial samples were washed gently with a saline (0.9% NaCl, v/v) and fixed for 2 h with 2.5% glutaraldehyde. The samples were dehydrated by using sequential ethanol concentrations from 30%, 50%, 75%, 90% to 100%, and exposure for 15–20 min per concentration.
Then the acetate isoamyl ester was added to samples. The samples were dried with CO₂ critical point dryer (HCP-2, Hitachi, Japan). Finally, the samples were treated by sputter coating with gold in an ion coater for 5 min (E-1010, Hitachi, Japan), and then examined with a scanning electron microscope (S-3000N, Hitachi, Japan).

3. Results and discussion

3.1. Effect of electrochemical reduction on nitrate removal

As shown in Fig. 1, almost no nitrate was reduced at current densities of 50–200 mA/m², and a small amount of nitrate was removed at the current density of 250–400 mA/m², the maximum nitrate removal efficiency was 10.32% at 400 mA/m². The increase in nitrate removal efficiency at high current density was probably due to the electrochemical reduction by the cathode (Li et al., 2009a). In this study, relatively small amount of nitrate was removed by electrolysis and thus, electrochemical effect could be ignored, especially at 50–200 mA/m². Zhao et al. (2012) also showed that the maximum nitrate removal efficiency was only 5.7% by electrolysis and electrochemical reductions in an intensified biofilm-electrode reactor (IBER) when applied currents from 50 to 500 mA.

3.2. Biological denitrification

As shown in Fig. 1, NO₃⁻–N naturally decreased slowly to 16.55 ± 1.34 mg/L during the 14 days with no electrical stimulation. NO₃⁻–N decreased to 13.43 ± 0.37, 7.34 ± 0.29, 5.04 ± 0.03, 0.00 ± 0.01, 0.00 ± 0.03, 1.25 ± 0.13, 3.01 ± 0.03 and 5.05 ± 0.05 mg/L, then kept stable at current densities of 50, 100, 150, 200, 250, 300, 350 and 400 mA/m², respectively. These results demonstrate that the maximum NO₃⁻–N reduction efficiency (~100%) was achieved at current densities of 200 and 250 mA/m², no matter higher or lower than this value, removal efficiency would decline. On the other hand, the NO₃⁻–N removal rates at different current densities were also computed (determination coefficients $R^2 > 0.9$) following the first-order kinetics model. Results revealed that when no current is applied, nitrate removal rate constant was 0.10 d⁻¹. When the current density was 200 mA/m², the nitrate removal rate constant was 0.31 d⁻¹, higher than 0.15, 0.17, 0.18, 0.22, 0.26, 0.22 and 0.20 d⁻¹ at current densities of 50, 100, 150, 200, 250, 300, 350 and 400 mA/m², respectively.
similarly, Park et al. (2005) reported that the maximum nitrate removal efficiency reached 98% at current density of 200 mA/m². Tong et al. (2013) also observed 99.9% nitrate removal efficiency at the current density of 200 mA/m² after testing different current densities from 0 to 320 mA/m² in a heterotrophic/biofilm-electrode autotrophic denitrification reactor (HAD-BER). Furthermore, they pointed out the optimum current density was 239.6 mA/m² using central composite design (CCD) and response surface methodology (RSM) in a HAD-BER (Tong et al., 2014).

Variation of NO₂⁻–N concentrations in different applied current densities was also tested and showed in Fig. 1. The initial NO₂⁻–N concentration was less than 0.02 mg/L in all set-ups. When no current applied, the NO₂⁻–N concentration first increased then decreased, with the maximum accumulations of 4.22 ± 0.25 mg/L. When the applied current densities were 50, 100, 150, 200, 250, 300, 350 and 400 mA/m², NO₂⁻–N concentration increased to 4.59 ± 0.51, 13.22 ± 0.19, 12.86 ± 0.15, 2.40 ± 0.05, 5.17 ± 0.29, 2.24 ± 0.02, 4.08 ± 0.09 and 2.62 ± 0.53 mg/L, respectively, then decreased and kept below 0.20 mg/L during 14 days. Stimulation at different current densities seemed to have various results, such as, the maximum NO₂⁻–N accumulations were higher at 100 and 150 mA/m² than other current densities, it might be explained by the sensitivity of the enzyme nitrate reductase to such current levels, affecting their activity leading to the large amount of NO₂⁻–N to be reduced to NO₃⁻–N. Nitrite accumulated on the first days of the experiment largely due to the activity of nitrate reductase and lack of further reduction at all current densities (Komer and Zumft, 1989).

Then, the nitrite later decrease might be due to the onset of nitrite reduction later than the onset of nitrate reduction (Rivett et al., 2008). Results also showed that without electrical stimulation, both rates of increase or decrease of nitrite were generally lower than other conditions that were applied with current. This suggests that the current had a stimulating effect on both nitrate reductase and nitrite reductase. In addition, no nitrite was detected in the electro-chemical reduction without bacteria, further implying that the microorganisms were the main reason for NO₂⁻–N accumulation.

Furthermore, it was also showed in Fig. 1, that NH₄⁺–N concentration increased rapidly under different current densities during 14 days. The maximum NH₄⁺–N concentration in the reactor was 16.59 ± 0.07 mg/L without applied current. When current densities were 50, 100, 150, 200, 250, 300, 350 and 400 mA/m², the NH₄⁺–N concentration rapidly increased to 15.80 ± 0.26, 14.73 ± 0.20, 16.04 ± 0.83, 25.57 ± 0.20, 29.08 ± 0.11, 28.92 ± 0.24, 16.62 ± 0.02 and 18.59 ± 0.29 mg/L, respectively, and kept stable. The NH₄⁺–N concentration was highest at 200, 250 and 300 mA/m². The accumulation of NH₄⁺–N in the reactor might be due to the following reasons: (1) Fe cathode had the high selectivity of nitrate reduction to ammonia (Li et al., 2009b), especially the current density was further raised to higher than 250 mA/m². (2) dissipatory nitrate reduction to ammonium (DNRA) reaction occurred. Nitrate reduction processes include denitrification and DNRA, which are both microbial process, and DNRA occurs under high available C/N ratio conditions, while denitrification occurs at lower carbon concentrations (Nogaro and Burgin, 2014). In addition, Cheng and Lin, 1993 reported that the complete denitrification occurred when C/N was 0.71 where heterotrophic denitrification used methanol as the organic carbon source. In this study, the C/N was 1.25 which was higher than the theoretical value of 0.71 which could have resulted to NH₄⁺–N accumulation. Moreover, almost no NH₄⁺–N was detected in electrochemical reduction at 50 to 200 mA/m², and only a small amount of NH₄⁺–N accumulated and changed between 1.41 ± 0.01 and 3.63 ± 0.12 mg/L when the current density increased from 250 to 400 mA/m². This suggested that the NH₄⁺–N accumulation was mainly caused by the effect of microorganism.

3.3. Variation of ATP content under different applied current densities

ATP is one of the most important smaller molecules in living organisms. ATP generated through respiration by metabolically active cells and is necessary for organisms’ survival, growth and replication (Mempin et al., 2013), i.e., ATP reflects the activity of microbial growth. As shown in Fig. 2, ATP content increased at first and then decreased under different current densities. When no current applied, ATP content only reached as high as 14.87 ± 0.12 nM on the seventh day. When the applied current density was increased incrementally from 50 to 400 mA/m², the maximum values of ATP content were 21.53 ± 0.40, 21.74 ± 0.17, 23.40 ± 0.52, 26.40 ± 0.52, 28.27 ± 0.64, 28.92 ± 0.02, 30.34 ± 0.04 and 29.51 ± 0.12 nM, respectively. Hence, this suggests that increase in the amount of current density applied to the bioreactor (i.e. 50 to 400 mA/m²) increases ATP production.

However, when no current applied, ATP content decreased slowly to 4.47 ± 0.46 nM during the last 7 days, it might be due to lack of nutrition and inhibited microbial activities. At the end of experiments, ATP content changed between 4.33 ± 0.60 and 5.33 ± 0.58 nM at 0–250 mA/m², higher than between 2.51 ± 0.44 and 0.57 ± 0.19 nM at 300–400 mA/m². This difference suggests that higher than the suitable current density range could inhibit microbial activity. Compared with Fig. 1, at the beginning of each run, ATP inversely increases with the concentration of NO₃⁻–N, and decreased when the NO₃⁻–N concentration of the latter remained unchanged towards the end of the experiment. These results further suggested that NO₃⁻–N removal efficiency is closely related to the activity of denitrifying bacteria (Safari et al., 2013).

Integral theorem states that the absolute area under the ATP curve could express the ATP aggregate level. ATP aggregate level was computed in this study using Origin 8.0. The ATP aggregate levels reached the highest 195.41 ± 3.04 and 175.97 ± 5.03 nM d at 200 and 250 mA/m², higher than 128.01 ± 1.73, 163.41 ± 4.26, 159.33 ± 0.58, 168.63 ± 4.04, 142.49 ± 2.65, 136.27 ± 3.77 and 129.48 ± 3.74 nM d at 0, 50, 100, 150, 300, 350 and 400 mA/m², respectively. These results indicate that decline in ATP production was observed in both levels higher or lower than optimum current density range.

These results demonstrate that suitable electro-stimulation has a positive effect on bacteria by promoting their metabolism and activity. Matsumoto et al. (1999) reported that the growth period of Thiobacillus ferrooxidans could be extended three times as well as significantly increase its count with continuous electro-stimulation. Chen et al. (2006) demonstrated that electro-stimulation at a certain voltage (normally 0.2–1 V) could lead to increased pore formation in the cell membrane allowing more permeability to ions, molecules and even macromolecules. Similarly, it was suggested that an appropriate amount of current could enhance transport of nutrients into the cells in this study.

On the other hand, electro-stimulation could also have negative effects on bacteria. Electric fields could directly oxidize intracellular constituents without destroying their membranes, leading to cell death (Matsunaga et al., 1992). High electric current could also cause irreversible permeabilization of the cell membrane and subsequent leakage of essential cytoplasmic constituents (Dreesa et al., 2003). Luo et al. (2005) reported that when 20 mA current was applied to phenol-degrading bacteria, it could increase the surface hydrophobicity and flatten the cell shape, and that 40 mA current could change the bacterial cell’s surface properties, physiology, shape and movement. Furthermore, electrolysis generates by-products like oxygen radicals (i.e. OH⁻, H₂O₂ and O₂), which are also potentially harmful to bacterial cells (She et al., 2006).

Studies also showed that many products of abiotic reactions at the electrode surface could influence microbial metabolism, such as hydrogen peroxide, which could inhibit some of the microbial
metabolic activities (Thrash and Coates, 2008). Electrolytic reactions could also vary the pH and thereby affect microbial activity. For example, Wei et al. (2011) observed that when the applied current density was 12.3 A/m², the pH of liquid medium shifted to around 10, which might be partially responsible for the bacterial inactivation. However, electric current impact and antimicrobial oxidants could act synergistically to inactivate microbes (Costerton et al., 1994).

3.4. Change in cell shape and extracellular substances under different applied current densities

The SEM images showed that the changes in bacterial cell shape and extracellular substances under 0, 50, 200 and 400 mA/m² (images not shown). The denitrifying bacteria were mainly short rods, a small amount of cells were rods and cocci when no current applied, and most bacteria looked like wrapped in the sludge. When current density was 50 mA/m², the majority of the bacteria were the same shape as the bacteria without current applied. When 200 mA/m² of current density was applied, most of the denitrifying bacteria were short rods, in particular, exhibited the bacteria uniform distribution and growing well. However, when 400 mA/m² current density was applied, the majority of the bacteria were short rods and roughened due to the presence of exudates on the cell surface. These results were consistent with Section 3.3, indicated that the microbial inactivation may be resulted from irreversible permeabilization of the cell membrane with a high electric current, then induced change in the cell shape (Luo et al., 2005). In addition, the bacteria clustered together under 200 and 400 mA/m² suggest that the current could promote the bacteria growing in clusters.

3.5. pH variation under different applied current densities

The initial pH of synthetic groundwater was stabilized between 7.23 and 7.27 under different current densities. The terminative pH gradually increased from 7.93 to 8.62 with increasing amount of applied stimulation from 0 to 200 mA/m². However, when the applied current density was further raised from 250 to 400 mA/m², the pH increased significantly and changed between 9.23 to 11.14. Although the pH in the reactor has actually gradually increased with the time of experiments to the terminative value during the tests, pH was only measured at the end of the experiments because the experiment apparatus was sealed and the volume of synthetic groundwater was also limited in this study. The increase of pH in the reactor might be due to the following reasons: (1) part of the OH⁻ was produced due to electrolysis of water, (2) OH⁻ was produced in the denitrification process, which increased the alkalinity of the water (Zhao et al., 2011). (3) by experimental observation, the anode corroded when applied current density increased from 250 to 400 mA/m², which could be partially responsible for the mixed liquid medium being shifted to alkaline in the reactor. To study the effect of pH on nitrate removal and bacterial activity, set up a series of pH test as follows.

3.6. Effect of initial pH on nitrate removal and ATP content

This study was designed with an initial pH based on the terminative pH observed under different current densities. And the current density was set as 200 mA/m² according to the optimum current density in above experiments. Fig. 3 shows the nitrate removal and ATP content in the reactor under different initial pH. The nitrate was completely removed at pH 7.25 and 8.25 with the maximum ATP contents generally higher than those at pH 9.25, 10.25 and 11.25. This result was similar to the previous study which showed that pH between 7 and 8 are an optimal pH range for denitrification systems (Qambrani et al., 2013). However, compared to the traditional biological denitrification (without electro-simulation), the nitrate removal efficiency reached 80%, microorganism still had good activity, and almost no NO₂⁻–N accumulation occurred (data not shown) at the pH of 9.25 in this study.
This result may suggest that electro-stimulation could enhance alkali-resistance of the nitrate reducing bacteria and the nitrate reducing bacteria under optimal current density. However, the nitrate removal efficiency at pH 10.25 and 11.25 were only 30.25% and 26.37%, respectively. This might be associated with increase in pH which makes the medium strongly alkaline that inhibits nitrate reduction (Ghafari et al., 2009). Also, as discussed in the previous sections, the pH in the reactor reached 10.04 and 11.14 at 300 and 400 mA/m², which possibly contributed to bacterial inactivation in the latter part of the experiment. The ATP content decreased at the beginning of the experiments under pH of 10.25 and 11.25, and then increased towards the end of the study. This result was postulated that continuous current stimulation enhances the activities of some bacteria with strong alkali-resistance.

4. Conclusions

In this study, it was demonstrated that nitrate could be effectively removed with a maximum nitrate reduction efficiency of 100% using electro-stimulation at 200 and 250 mA/m². And at the optimum current density range, the denitrification exhibited low nitrite production, high ATP aggregate level, and suitable pH. However, when the current density was 300–400 mA/m², the ATP content sharply decreased in the latter part of the experiments due to current impact and obviously higher pH. The results obtained in this study have far-reaching significance to improve the efficiency of applying bio-electrochemical technologies into the wastewater treatment processes.

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