Characteristics of heterotrophic/biofilm-electrode autotrophic denitrification for nitrate removal from groundwater

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HIGHLIGHTS

- Nitrate is effectively removed in developed HAD–BER.
- The optimum current density of HAD–BER is 200 mA/m².
- Heterotrophic and autotrophic bacteria are quantitatively analyzed.
- CO₂ generated by heterotrophic process is used by autotrophic bacteria.
- There is synergistic interaction between heterotrophic and autotrophic bacteria.

ABSTRACT

A heterotrophic/biofilm-electrode autotrophic denitrification reactor (HAD-BER) was developed to improve denitrification efficiency and reduce the consumption of organic carbon source. Maximum nitrate removal efficiency of 99.9% was gained under the optimum current density of 200 mA/m². The number of heterotrophic denitrification bacteria (HDB) 2.0×10⁵ and hydrogen autotrophic denitrification bacteria (ADB) 2.0×10³ in per milliliter biofilm solution were observed by the most probable number (MPN) culture, demonstrating that HDB and ADB coexist in the HAD-BER. The inorganic carbon source for autotrophic denitrification was supplied by the dissolved carbon dioxide (CO₂) evolved from the heterotrophic denitrification process, indicating that there was synergistic interaction between the HDB and ADB, i.e., the organic carbon source used for denitrification could be decreased in the HAD-BER. Therefore, the developed HAD-BER would be a promising approach for nitrate removal from groundwater.

1. Introduction

Groundwater is one of the main water resources of human. It is widely used as drinking water in the most countries of the world (Wang et al., 2009). However, in recent decades, nitrate concentration in groundwater has become increasingly serious in most parts of the world as a result of discharge of domestic and industrial wastewater and increased usage of nitrogenous fertilizers (Park et al., 2005). Nitrate is a substance that threatens humans and animals (Kross et al., 1993). Nitrate is reverted to toxic nitrite by microorganisms in human body, which reacts with the hemoglobin in blood, and then it converts the hemoglobin into methemoglobin, which cannot carry oxygen to cell tissues and results in methemoglobinemia (Mousavi et al., 2012). Nitrate is also known to cause gastric cancer (Mirvish, 1977). Therefore, the maximum contaminant levels (MCL) of nitrate are stipulated to be 10 mg NO₃⁻-N/L by USEPA and WHO, the same values are also adopted by China (Standards for Drinking Water Quality, GB5749-2006). Nitrite is closely related to nitrate, which is an even more serious cause of methemoglobinemia; the MCL of nitrite by WHO is 0.03 mg NO₂⁻-N/L, whereas MCL in the USEPA and China (China Standard examination methods for drinking water, GB5750-2006) are both 1 mg/L.

Various physical–chemical methods have been developed for nitrate removal, such as ion exchange (Bae et al., 2002), reverse osmosis (Schoeman and Steyn, 2003), electro-dialysis (Peel et al., 2003) and catalysis (Pintar et al., 2004). Among them, the physical–chemical methods are only able to separate nitrate from...
polluted groundwater, and a cumbersome treatment is inevitable (Ghafari et al., 2008). While biological processes have high potential for the elimination of nitrate with high stability and reliability (Foglär et al., 2005), exhibiting a promising process for controlling nitrate pollutions.

Biological denitrification can be involved in heterotrophic and autotrophic modes (Soares, 2000). Nitrate heterotrophic denitrification is based on organic carbon source as the electron donor, and has drawn lots of attentions (Karanasios et al., 2010), while autotrophic denitrification is based on inorganic carbon source, including hydrogen gas and sulfur as the electron donor. Recently, biofilm-electrode reactor (BER) combined biological and electrochemical methods is one of effective nitrate removal devices based on hydrogen autotrophic denitrification, which involves microorganisms on the cathode, where hydrogen produced from electrolysis of water is used as an electron donor for autotrophic denitrification (Sakakibara and Kuroda, 1993; Zhang et al., 2011). However, traditional BERs consume a lot of electric energy to produce CO2 and H2 (Mousavi et al., 2012), and have relatively lower efficiency compared with heterotrophic denitrification (Tang et al., 2012), while heterotrophic denitrification also has the problem of secondary pollution from added organic carbon source due to the lack of carbon source in the groundwater (Zhang et al., 2012).

Hence, in order to improve denitrification efficiency and reduce the consumption of electric energy and addition of organic carbon source, an intensified biofilm-electrode reactor (IBER) has been developed to treat nitrate polluted groundwater in our previous works (Zhao et al., 2011, 2012). However, carbon rods are employed as anode in the IBER, carbon dioxide (CO2) may be produced by both heterotrophic denitrification and anodization of carbon rods (Mousavi et al., 2012). CO2 utilized by autotrophic denitrification has not been clarified in the previous work (Zhao et al., 2012), while heterotrophic denitrification bacteria (HDB) and hydrogen autotrophic denitrification bacteria (ADB) have not been quantitatively analyzed in the IBER.

In this study, in order to elucidate symbiotic environment of HDB and ADB, namely whether the carbon dioxide produced by heterotrophic denitrification could be used by ADB, a heterotrophic/biofilm-electrode autotrophic denitrification reactor (HAD-BER) was developed. Firstly, the synthetic groundwater containing NO3-N was used to evaluate the performance of HAD-BER at different current densities to determine the optimum current density. Then, in order to confirm the symbiosis between HDB and ADB in HAD-BER, the number of HDB and ADB was analyzed by using the method of most probable number (MPN), and inorganic carbon in effluent was also investigated.

2. Methods

2.1. Experimental set-up

The configuration of the HAD-BER was shown in Fig. 1. The HAD-BER consisted of the cylinder, anode and cathode module. The cylinder was a plexiglass (180 mm in diameter, 250 mm in height), avoiding the generation of carbon dioxide during the electrolysis process, which was different from the anode composed of carbon rod that reported in the previous work (Zhao et al., 2011). The cathode module was made up of perforated plexiglass column, stainless steel mesh cathode and biological carriers. The perforated plexiglass column was used as bracket for the cathode and carriers. The bracket was drilled with 10 mm diameter holes and 20 mm distance between the hole centers. The cathode was a column stainless steel mesh (grids, 10 mm × 10 mm) with 110 mm in diameter and 220 mm in height, which was wrapped around the bracket. The total effective area of the cathode was 0.25 m². Similar to the previous work (Zhao et al., 2011), fiber threads containing about 80% cotton and 20% terylene were used as carriers for nitrate-reducing micro-organisms growing. The carriers were tightly surrounded by the outer of the cathode and the bracket.

Anode was fixed in the center of the reactor, and cathode module concentrically installed in the cylinder. The total volume of plexiglass cylinder was 7.5 L and the working volume was 5 L. The solution flowed from the bottom and overflowed from the upper. Influent, which was pumped into the reactor by a peristaltic pump (Longer Pump, LEAD-2, China), was stored in an influent tank with a volume of 25 L, and replaced every 2 days.

2.2. Experimental procedure

2.2.1. Experimental start-up

Synthetic groundwater (per liter of tap water) contained 0.304 g NaNO3, 0.044 g KH2PO4, and 0.21 ml CH3OH. The concentration of NO3-N was prepared as 50 mg/L. All chemical reagents used in the experiment were analytical grade.

The inoculums were collected from anaerobic sludge taken from the Qinghe Sewage Treatment Plant (Beijing, China). Seed sludge was acclimated in HAD-BER for 7 days in the batch mode, with the addition of 2.0 g glucose every day. Afterwards, microbes in the reactor were further acclimated for 30 days in the continuous mode. The hydraulic retention time (HRT) was set at 8 h and the temperature of HAD-BER was maintained at 30 °C similar to our previous work (Zhao et al., 2011).

In the cathode module, biofilm was observed at the fiber threads covered with a dark grey color, and it was considered as growing well when the nitrate in the effluent was kept at less than 2 mg/L.

2.2.2. Experimental run

To determine the optimum current density, the HAD-BER was run over a period of 80 days. Current densities gradually increased from 0 to 320 mA/m² at an interval of 40 mA/m². The reactor ran 10 days at every current density. After determined the optimum current density, HAD-BER was run at 0 mA/m² and the optimum current density to determine the relationship between the production of inorganic carbon source and consumption of organic carbon source in HAD-BER. The temperature and HRT were the same with the above description (Section 2.2.1).
2.3. Culture for HDB and ADB

The population densities of HDB and ADB were determined by MPN technique. In general this approach was applied to quantify nitrate reducers, such as denitrifiers in soil and in solution after incubation at specific temperature and enriched with nitrate (McCarty et al., 2007). This approach was used instead of the commonly quantitative PCR method because it additionally allowed isolating denitrifiers that can subsequently be genetically and functionally characterized. (Braker et al., 2010).

The biofilm solution for MPN count was extracted from the HAD-BER operated at optimum condition. The biofilm solution was taken from the outer surface of the cathode module and the sampling point was located at 150 mm from the bottom. Then sludge samples were divided into two aliquots used for MPN test of HDB and ADB, respectively, with control of growth medium. A 10-fold dilution series of each aliquot was prepared using deionized water, and 10 ml of each dilution was added into 100 ml of the sterilized growth medium in 250 ml conical flasks (four replicates per dilution stage). The flasks of HDB and ADB were aerated by nitrogen and hydrogen, and then incubated in a constant temperature Horizontal Shaking Bath container (30 °C, 160 rpm; Tai-chang, DDHZ-300, China) for 14 days. NO$_3^-$-N concentration in the culture media was measured after incubation. The number of positive wells for each dilution had been used to estimate population size using MPN value table, which was based on MPN statistics and the cut-off probability theory calculated as described by Briones and Reichardt (1999) and Kohno and Fukumaga (1998).

The growth medium (per liter of deionized water) for HDB contained 2.0 g KNO$_3$, 5.0 g C$_6$H$_{12}$Na$_2$O$_7$, 0.2 g MgSO$_4$·7H$_2$O, 1.0 g K$_2$HPO$_4$, 1.0 g KH$_2$PO$_4$ and the pH was adjusted to 7.2 by 1 mol/L HCl or NaOH solution, while that for ADB contained 2.0 g KNO$_3$, 9.8 g NaHCO$_3$ (the same C/N with HDB medium), 0.2 g MgSO$_4$·7H$_2$O, 1.0 g K$_2$HPO$_4$, 1.0 g KH$_2$PO$_4$ and the pH was also adjusted to 7.2. Both of them were sterilized at 121 °C for 30 min in autoclave.

As the comparison, 10 ml deionized water was added into 100 ml of the sterilized growth medium in 250 ml conical flasks (4 replicates). The concentration of NO$_3^-$-N in the blank test was 300 ± 3 mg/L.

2.4. Analytical methods

NO$_3^-$-N, NO$_2^-$-N and NO$_2^-$-N both in influent and effluent were determined by ultraviolet spectrophotometer (HACH, DR 5000, USA) according to standard methods, with two duplications for influent and three dilutions for effluent every 24 h. NO$_3^-$-N in MPN culture media were also measured by taking three parallel samples.

Chemical oxygen demand (COD) was measured by the standard method of potassium dichromate. Dissolved CO$_2$ was monitored by the carbon dioxide detector (TDG, FC-100, China). COD and dissolved CO$_2$ in effluent were also measured every 24 h, and three parallel samples were tested every time.

3. Results and discussion

3.1. Biological nitrate reduction

Fig. 2 showed that nitrate could be reduced under different current densities. When the applied current density was increased incrementally up to 200 mA/m$^2$, NO$_3^-$-N concentration in effluent was decreased accordingly. The maximum nitrate reduction efficiency was 99.9%. When the applied current density was further raised to 320 mA/m$^2$, the average NO$_3^-$-N concentration in effluent increased, especially when the current density increased to 320 mA/m$^2$, the concentration of NO$_3^-$-N was raised rapidly. Consequently, nitrate reduction efficiency was decreased. Little nitrate was removed by electrolysis and electrochemical effect could be ignored according to our previous study (Zhao et al., 2012). Therefore, the variation of NO$_3^-$-N concentration was caused by the effect of current density on microbial denitrification. This variation trend was consistent to the results reported by Park et al. (2005) who used a BER with a 1 m$^2$ cathode as direct electron donor and employed the current density from 0 to 600 mA/m$^2$. In their study, the maximum nitrate removal efficiency of 98% was emerged when the current density was 200 mA/m$^2$. Rodziewicz et al. (2011) obtained the same optimized current density in a rotating biological reactor. These results indicated that the current density on the impact of microorganisms was basically similar in different BERs, optimal current density was almost 200 mA/m$^2$. It seemed that microorganisms were impacted via the high current density, independent of voltage. No matter higher or lower than this value, removal efficiency would decline. This might be due to that the low current could not produce the required sufficient amount of hydrogen, while the high current might inhibited microbial activities, even killed microorganisms in the reactor (Flora et al., 1994).

Some BERs with different designs had been reported within these decades. Feleke and Sakakibara (2002) discussed a BER coupled with absorber (granular activated carbon and silicone resin) for the nitrate removal. Approximately 10.2% of NO$_3^-$-N was removed through the BER process. Prosnansky et al. (2002) developed a multi-cathode BER combined with microfiltration to treat nitrate-contaminated water. When the initial NO$_3^-$-N concentration was 25 mg/L, the maximum nitrate removal efficiency of 80% was obtained. The maximum nitrate removal efficiency reported by Park et al. (2005) was 98%, as previously described. Compared to traditional BERs, the nitrate removal efficiency in developed HAD-BER was substantially improved to almost 100%.

When the current density was lower than 200 mA/m$^2$, NO$_3^-$-N concentration in effluent was kept below 0.03 mg/L, indicating few NO$_3^-$-N accumulated in the reactor. However, when the current density was larger than 200 mA/m$^2$, NO$_3^-$-N began to accumulate. Particularly when the current density was 320 mA/m$^2$, NO$_3^-$-N concentration ranged from 0.07 to 0.28 mg/L. Except for the direct influence of current, nitrite reductase might be inhibited by higher concentration of hydrogen gas. Ghafari et al. (2009) also concluded that the current, as the hydrogen generator, must be controlled at an appropriate value. Adequate amount of hydrogen must be supplied to cope with the susceptibility of denitrification process. In other words, electric current, as the in situ hydrogen generator, must be controlled at an appropriate magnitude.

Moreover, Fig. 2 showed ammonia increased firstly and then decreased. When current densities varied at the range from 120 to 200 mA/m$^2$, NO$_3^-$-N concentration in effluent was the highest and changed between 3.54 and 6.23 mg/L. The accumulation of NO$_3^-$-N in HAD-BER might be due to the following reasons: (1) the nitrate electrochemical reduction in cathode (Li et al., 2009); (2) the occurrence of dissimilatory nitrate reduction to ammonium (DNRA) (Zhang et al., 2012). Firstly, Li et al. (2009) discussed electrochemical reduction of nitrate using Fe as cathode, which was similar to the cathode in HAD-BER. When the NO$_3^-$-N concentration was 100 mg/L and current density was 20 mA/cm$^2$, the accumulation of NO$_3^-$-N within 180 min was 51.1 mg/L. So the cathodic reduction might lead to the accumulation of NO$_3^-$-N in our system. Secondly, Zhang et al. (2012) investigated the behaviour of solid carbon sources for biological denitrification in groundwater remediation, they found that DNRA was seen as a counterproductive process in denitrification studies and the balance between denitrification and DNRA depended on conditions of the reactor, in which, C/N was an important factor. Zhao et al. (2012) also discussed the
concentration of NO$_3^-$-N in the effluent, and concluded that NO$_3^-$-N concentration decreased with the reduce of C/N. Hence, the occurrence of DNRA might be a key reason for NO$_3^-$-N accumulation in this study. Furthermore, it is necessary to detect DNRA in the HAD-BER in the future. Although ammonia in drinking water standards was a sensory traits, excessive ammonia would not only bring discomfort to people on the senses (Standards for Drinking Water Quality, GB5749-2006), but also nitrogen deposition might affect the acidity of sensitive habitats and harm human health (Webb et al., 2005). Therefore, optimizing conditions in HAD-BER should be applied to avoid this problem in the future studies.

3.2. Enumeration of denitrifying Microorganisms

The positive/negative identification of MPN flasks for HDB was shown in Fig. 3(a), which showed that all four from $10^{-1}$ to $10^{-4}$ diluted MPN flasks were positive; three of the four $10^{-5}$ diluted samples were positive; only two of the four $10^{-6}$ diluted samples were positive; and all four $10^{-7}$ diluted samples were negative. Besides, the positive/negative identification of MPN flasks for ADB was shown in Fig. 3(b). It could be seen that the positive/negative identification of MPN flasks for ADB was shown in Fig. 3(b). It could be seen that all the $10^{-1}$ and $10^{-2}$ diluted MPN samples were positive; only two of the four $10^{-3}$ diluted samples were positive; and all four $10^{-4}$ diluted samples were positive; three of the four $10^{-5}$ diluted samples were positive; only one of the four $10^{-6}$ diluted samples were positive; and all four $10^{-7}$ diluted samples were negative. These findings were summarized in Table 1.

Table 1 illustrated that the quantitative indicator of HDB was ‘432’. It was about 20.0 according to the MPN value table, and then it was multiplied by the dilution multiple of the first digit (the dilution multiple of $10^{-4}$ is 10,000). Therefore, the MPN value of HDB was calculated as $2.0 \times 10^5$. Additionally, nitrate concentration varied widely even in samples at the same dilution stage. The similar result was also observed by Hirooka et al. (2009). This might be due to sample probability after diluted. Although there were differences in the number of viable cells contained in the four replicates of the same dilution, it had no effect on the result of MPN counts because microbial growth was judged only via the nitrate degradation.

According to the MPN count rule, no matter how many repetitions, the quantitative indicator must be a three-digit number, and the first number must be all positive (4 in our study). If the following dilution was still growing, the number could be added to the third adjacent digit. Hence, Table 1 showed that the quantitative indicator of ADB was ‘424’, which was corresponded to 20.0 according to the MPN value table. It was multiplied by the dilution multiple of the first digit (the dilution multiple of $10^{-2}$ is 100), $2.0 \times 10^3$ of the MPN value of ADB was obtained.

Unlike HDB media, NO$_3^-$-N concentration in ADB media varied narrowly (from 10 to 25 mg/L) even among all the dilution stage. It might be due to the lower denitrification rates of ADB. Tang et al. (2012) also found the efficiency of HDB was much higher than ADB. Because of the slower chemosynthetic and metabolic processes, ADB were much less inefficient. Therefore, only part of the nitrate in culture medium was metabolized by living ADB in 14 days.

Based on the results mentioned above, it could be found that HAD-BER was a symbiotic system for HDB and ADB. The culture method for ADB as mentioned in Section 2.3, pure culture of the bacteria for heterotrophic denitrification was tested before energized, as a result, no autotrophic denitrification bacteria was ob-

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**Fig. 2.** Effect of current densities on nitrate removal, nitrite and ammonia accumulation in HAD-BER (NO$_3^-$-N = 50 mg/L, C/N = 1.25, HRT = 8 h).
served. Therefore, it was proved that the symbiotic system for HDB and ADB was formed after long-term domestication in HAD-BER. However, the number of HDB was significantly higher than the number of ADB due to that the activity of HDB was much higher at C/N of 1.25. C/N of 1.25 was considered as an optimum value for the HDB growth by Wang et al. (2009). At the same time, Lee et al. (2013) detected the coexist of autotrophic and heterotrophic denitrification bacteria under the C/N of 0.8. Hao et al. (2013) also found both heterotrophic and autotrophic bacteria responsible for nitrate removal through the microbial community analysis under the C/N of 1.5. Therefore, the optimum C/N in the symbiotic system would be studied in the future research.

### 3.3. Production and consumption of inorganic carbon source

In order to further analyze the relationship between the production of inorganic carbon source and consumption of organic carbon source in HAD-BER, COD and inorganic carbon in the effluent were monitored. Inorganic carbon (dissolved CO$_2$) consisted of CO$_3^{2-}$, HCO$_3^{-}$ and CO$_2$ molecules in the effluent. Fig. 4 showed the change in COD and dissolved CO$_2$ in effluent when the current densities increased from 0 to 200 mA/m$^2$.

When the current density was 0 mA/m$^2$, the average concentration of dissolved CO$_2$ was 130.0 ± 11.0 mg/L, and COD was 8.0 ± 1.0 mg/L. When the current density increased to 200 mA/m$^2$, dissolved CO$_2$ decreased to 73.4 mg/L and COD rapidly increased to 20.3 mg/L. In general, the bacteria requires an adjustment when the condition changes. The concentration of NO$_3^-$-N was detected a temporary increase in the effluent, suggesting the biofilm in the reactor might also need a short adjustment period when the current was just switched on. As time going on, the average of dissolved CO$_2$ concentration in the HAD-BER was kept at 104.3 ± 11.0 mg/L, reduced by 25.7 mg/L compared to the 0 mA/m$^2$. However, COD was 9.0 ± 0.5 mg/L, increased by about 1.0 mg/L. Equivalent carbon dioxide produced by heterotrophic denitrification was decreased by about 1.4 mg/L. So about 24.3 mg/L dissolved CO$_2$ was used. The rise in COD might be due to that a part of nitrate was consumed by ADB when the current density existed, consequently the consumption of organic carbon was reduced. Besides, the nitrate removal efficiency remained basically stable throughout the experiment.

On basis of the above analysis, it could be concluded that the part of inorganic carbon evolved from the heterotrophic denitrification, was used as inorganic carbon source by ADB for autotrophic denitrification. In addition, Ghafari et al. (2010) found sodium bicarbonate could be used as inorganic carbon source for ADB when its concentration ranged from 20 to 2000 mg/L, which implied that dissolved CO$_2$ (about 130 mg/L) produced by heterotrophic denitrification in HAD-BER reached the level that can be

### Table 1

| Species | Dilution | Replicates | Positive | MPN count
|---------|----------|------------|----------|-------------
|         | 10$^{-1}$ | 10$^{-2}$  | 10$^{-3}$ | 10$^{-4}$  | 10$^{-5}$  | 10$^{-6}$  | 10$^{-7}$ |
| HDB     |          | 4          | 4         | 4         | 4         | 4         | 4         |
| ADB     |          | 4          | 4         | 2         | 3         | 1         | 0         | 0         |

![Fig. 3. NO$_3^-$-N concentration of the media for denitrification bacteria. (a) HDB and (b) ADB.](image-url)
exploited. The two denitrification processes were described as follows (Wang et al., 2009; Xia et al., 2010):

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\begin{align*}
6\text{NO}_3^- + 5\text{CH}_3\text{OH} & \rightarrow 3\text{N}_2 + 7\text{H}_2\text{O} + 5\text{CO}_2 + 6\text{OH}^- \\
2.16\text{NO}_3^- + 7.24\text{H}_2 + 0.8\text{CO}_2 & \rightarrow 0.16\text{CH}_3\text{H}_2\text{O}_2\text{N} + \text{N}_2 + 5.6\text{H}_2\text{O} + 2.16\text{OH}^- 
\end{align*}
\]

According to Eqs. (1) and (2), there are two ways to increase the utilization of inorganic carbon in HAD-BER: increase the amount of hydrogen, namely increased the current density of HAD-BER; and reduce the dosage of organic carbon. However, the high current might inhibited microbial activities as mentioned above. So reducing the dosage of organic carbon, scilicet decreasing C/N, was a more favorable choice to improve the carbon source utilization in the HAD-BER, which was consistent with Section 3.2. Assuming nitrate was denitrified completely at the current density of 0 mA/m², 130.9 mg/L CO₂ was calculated according to Eq. (1), and the detected dissolved CO₂ was 119.0–141.0 mg/L, which indicated the carbon balance was constructed.

Therefore, heterotrophic and hydrogen autotrophic denitrification bacteria coexisted in the developed HAD-BER, synergistic interaction between heterotrophic and autotrophic microorganisms was confirmed in the HAD-BER.

4. Conclusions

Nitrate could be effectively reduced in the proposed HAD-BER. The minimum NO₃⁻-N concentration in effluent was 0.05 mg/L by the maximum nitrate removal efficiency of 99.9% at an applied current density of 200 mA/m². Through the MPN culture, per milliliter bacteria liquid contained the number of HDB 2.0 × 10^9 and ADB 2.0 × 10^7, respectively, demonstrating that HAD-BER was a symbiotic system for HDB and ADB. Dissolved CO₂, generated by heterotrophic denitrification was used as inorganic carbon source by ADB. There was synergistic interaction between HDB and ADB in the developed HAD-BER.

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