Effect of nitrate concentration, pH, and hydraulic retention time on autotrophic denitrification efficiency with Fe(II) and Mn(II) as electron donors
Jun-feng Su, Jing-xin Shi, Ting-lin Huang, Fang Ma, Jin-suo Lu and Shao-fei Yang

ABSTRACT
The role of electron donors (Fe\(^{2+}\) and Mn\(^{2+}\)) in the autotrophic denitrification of contaminated groundwater by bacterial strain SY6 was characterized based on empirical laboratory-scale analysis. Strain SY6 can utilize Fe\(^{2+}\) more efficiently than Mn\(^{2+}\) as an electron donor. This study has shown that the highest nitrate removal ratio, observed with Fe\(^{2+}\) as the electron donor, was approximately 88.89%. An immobilized biological filter reactor was tested by using three levels of influent nitrate (10, 30, and 50 mg/L), three pH levels (6, 7, and 8), and three levels of hydraulic retention time (HRT; 6, 8, and 12 h), respectively. An optimal nitrate removal ratio of about 95% was achieved at pH 6.0 using a nitrate concentration of 50 mg/L and HRT of 12 h with Fe\(^{2+}\) as an electron donor. The study showed that 90% of Fe\(^{2+}\) and 75.52% removal of Mn\(^{2+}\) were achieved at pH 8.0 with a nitrate concentration of 50 mg/L and a HRT of 12 h. Removal ratio of Fe\(^{2+}\) and Mn\(^{2+}\) is higher with higher influent nitrate and HRT. A weakly alkaline environment assisted the removal of Fe\(^{2+}\) and Mn\(^{2+}\).

Key words | autotrophic denitrification, ferrous-dependent denitrification, immobilized biological filter reactor, manganese-dependent denitrification, nitrate

INTRODUCTION
Nitrate contamination groundwater is becoming more and more serious in many countries (Sahinkaya & Kilic 2014). Groundwater contamination by nitrate usually originates from anthropogenic sources, mainly as a result of wastewater discharges and intensive application of fertilizers and animal manure to agricultural land (Qambrani et al. 2013).

In China, the nitrate contamination of groundwater has increased rapidly, and nitrate concentration in some groundwater source is higher than 30 mg/L (Fan & Qu 2001). It is recommended by the World Health Organization (WHO) that the nitrate concentration in drinking water be lower than 10 mg/L (Xing et al. 2016).

Biological denitrification is the most significant removal process in natural environments (Canziani & Bonomo 1998). A biological denitrification process, which comprises autotrophic denitrification and heterotrophic denitrification, is considered an efficient technology to treat nitrate contaminated water (Su et al. 2015b). Heterotrophic denitrification is very efficient in nitrate removal if adequate amounts of organic carbon are available. However, in groundwater treatment, insufficient organic carbon may limit the application of heterotrophic denitrification unless external organic carbon sources are supplied (Straub et al. 1996). Autotrophic denitrification has increased recently due to two major advantages compared with heterotrophic denitrification: (1) no external carbon is needed, which reduces the cost and risks related to the operation; and (2) less sludge is produced, which minimize the bacteria in the effluent (Kappler & Straub 2005).

It has been reported that most of the bacterial in activated sludge systems are capable of denitrification (Nielsen & Nielsen 2002). Furthermore, with the development of molecular techniques, more and more studies have been carried out to identify the microbial community structure under nitrate reducing conditions (Fernández et al. 2008). Autotrophic denitrification is an alternative to heterotrophic denitrification of wastewater that has a low
carbon content. The energy required by these microorganisms is derived from the oxidation of certain reduced inorganic compounds (Fe$^{2+}$, Mn$^{2+}$, S$^{2-}$, H$_2$) coupled with the reduction of nitrate or nitrite (Hashimoto et al. 1987; Zhang et al. 2014).

With the objective to evaluate the effectiveness of autotrophic denitrification using Fe$^{2+}$ and Mn$^{2+}$ as electron donors, respectively, an immobilized biological filter reactor was used herein to assess the effect of the nitrate concentration, pH, and hydraulic retention time (HRT) on the ratio of nitrate removal from contaminated groundwater using bacterial strain SY6. In addition, the study aimed to find out the most suitable environment for the removal of nitrate, ferrous ion and manganese.

**MATERIALS AND METHODS**

**Culture medium preparation**

An autotrophic denitrifying bacterium SY6 was isolated from a sediment (Su et al. 2015a). Bacteria were grown in 1,000 mL bottles containing 900 mL medium. The following microbial culture media were used:

The basal medium (BM) used in this study was comprised of the following reagents per L: 0.5 g of NaHCO$_3$; 0.1 g of NaNO$_3$; 0.1 g of KH$_2$PO$_4$; 0.05 g of MgSO$_4$·7H$_2$O; 0.05 g of CaCl$_2$ and 2 mL of trace elements solution. The components of trace elements in the solution were as follows: 0.5 g L$^{-1}$ MgSO$_4$·7H$_2$O, 1.0 g L$^{-1}$ EDTA, 0.2 g L$^{-1}$ ZnSO$_4$, 0.1 g L$^{-1}$ MnCl$_2$·4H$_2$O, 0.5 g L$^{-1}$ FeSO$_4$·7H$_2$O, 0.5 g L$^{-1}$ CuSO$_4$·5H$_2$O and 0.2 g L$^{-1}$ CoCl$_2$·6H$_2$O.

The final pH of BM was adjusted to 6.5 and then put into a portable pressure steam sterilization at 121°C for 30 min. Finally, the BM was removed from the pressure cooker, autoclaved Fe$^{2+}$ or Mn$^{2+}$ sources were added from a concentrated anoxic sterile stock solution to achieve a final concentration and incubated at 30°C.

**Influence of different electron donors on nitrate removal by the bacterium SY6**

The experiment was performed with contaminated groundwater after sterilization by adding extra Fe$^{2+}$, Mn$^{2+}$. The 250 mL bottles were incubated at 30°C with 10% of inoculum (v/v). The electron donor additive quantity was two times the initial concentration of the nitrate nitrogen. The bottles were placed in an incubator, for a period of 168 h, at a constant temperature of 30°C. Samples were taken from the bottles periodically to determine the nitrate, nitrite, Fe$^{2+}$, Mn$^{2+}$, pH and alkalinity.

**Immobilized biological filter reactor set up**

The biological filter reactor was operated with total and working volumes of 5 L and 3.5 L. The diameter and the total height of the reactor were 0.08 m and 1.0 m. The reactor was filled with silica sand filtering medium to 0.7 m of the column height. A lower support layer of 6 cm of gravel was packed at the bottom of the column. The reactor was fed in a ‘flow down’ mode. Three sampling ports allowed sampling at the bottom of the columns (Figure 1). The porosity of the column bed was around 40%, and then recycled for 10 d for the microorganisms to attach onto the surface of the silica sand filtering medium.

The medium used in this biological filter reactor was comprised of the following reagents per L: 0.5 g of NaHCO$_3$; 0.1 g of KH$_2$PO$_4$; 0.05 g of MgSO$_4$·7H$_2$O and 0.05 g of CaCl$_2$. The medium needed to be added with different concentrations of Fe$^{2+}$, Mn$^{2+}$ and nitrate. The medium was pumped from the influent tank into the reactor through the top inlet. The liquid flowed past the biofilm and then the nitrate was denitrified by autotrophic bacteria. Effluent was drawn from the water collector. The effluent of the column were sampled once a day to measure nitrate, nitrite, Fe$^{2+}$, Mn$^{2+}$, and pH. Different HRTs, pH and influent nitrate concentrations needed to be adjusted.

**Effect of nitrate concentration, pH value and HRT on the biofilm reactor nitrate removal ratio**

The experiment was performed in an immobilized biological filter reactor. In order to investigate the influence of nitrate concentration, pH value and HRT on the biofilm reactor nitrate removal ratio, the experiments were carried out with different nitrate concentrations, pH values and HRT. The influence of nitrate concentration was examined over a range of 10–50 mg/L. Su et al. (2015b) observed that the optimal range of pH for autotrophic denitrifier was 6.0–8.0 in the biological filter reactor. When the pH exceeded 8.0 and was below 6.0, the autotrophic denitrification bacterium cannot grow much and autotrophic denitrification efficiency cannot achieve the ideal effect. Therefore, the influence of the initial pH was examined over a range of 6.0–8.0. The influence of HRT was examined over a range of 6–12 h (Table 1). The effluents of the reactor were sampled once
a day to measure nitrate, nitrite, Fe$^{2+}$, Fe$^{3+}$, Mn$^{2+}$ and pH value.

**Analytical measurements**

The nitrate concentration was determined using a UV spectrophotometric screening method and calculating the difference between OD$_{220}$ and $2 \times$ OD$_{275}$. The nitrite concentration was determined by colorimetry at a wavelength of 540 nm according to the State Environmental Protection Administration of China (State Environmental Protection Administration of China 2002). The Fe$^{2+}$ concentration was measured spectrophotometrically with phenanthroline at 510 nm (Gendel & Lahav 2008). The presence of dissolved Fe$^{3+}$ on the biofilm reactor was measured as the difference between dissolved Fe$^{2+}$ concentrations

![Diagram of the experimental system](image)

**Table 1** | Overview of the operation mode in different periods

<table>
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<tr>
<th>Phase</th>
<th>HRT during the test [N]</th>
<th>pH during the test</th>
<th>Initial NO$_x$-N [mg/L]</th>
<th>Initial Fe$^{2+}$ or Mn$^{2+}$ [mg/L]</th>
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*Reactor preformed with Fe$^{2+}$ and Mn$^{2+}$ as electron donor in the 75 days.
after and before reduction with excess hydroxylamine hydrochloride. Mn\textsuperscript{2+} was determined according to standard method-potassium periodate spectrophotometric method at 530 nm (APHA-AWWA-WPCF 1989).

**Statistical analysis**

The denitrification, Fe\textsuperscript{2+} and Mn\textsuperscript{2+} removal rate formula is (C\textsubscript{0} - C\textsubscript{n})/h. C\textsubscript{0} is the initial concentration and C\textsubscript{n} is the final concentration of NO\textsubscript{3}-N, Fe\textsuperscript{2+} or Mn\textsuperscript{2+}. h is the time of reactor treatment. The nitrate, Fe\textsuperscript{2+} and Mn\textsuperscript{2+} removal ratio formula is (C\textsubscript{0} – C\textsubscript{n})/C\textsubscript{0} × 100%. C\textsubscript{0} is the initial concentration and C\textsubscript{n} is the final concentration of NO\textsubscript{3}-N, Fe\textsuperscript{2+} or Mn\textsuperscript{2+}. Data in this experiment were analyzed by Microsoft Excel and Origin 8.5 software.

**RESULTS AND DISCUSSION**

**Effect of different electron donors on autotrophic denitrification of contaminated groundwater by bacterial strain SY6**

The effect of different electron donors (Fe\textsuperscript{2+}, Mn\textsuperscript{2+}, and a mixture of electron donors) on autotrophic denitrification of contaminated groundwater by bacterial strain SY6 was empirically assessed after sterilization of the water sample.

**Single electron donor**

The suitability of different electron donors for supporting nitrate reduction was evaluated as shown in Figure 2(a).

![Figure 2](https://iwaponline.com/wst/article-pdf/74/5/1185/459217/wst074051185.pdf)

Figure 2 | (a) The effects of different electron donors on the denitrification efficiency; (b) the characteristics of denitrification using Fe\textsuperscript{2+}, Mn\textsuperscript{2+} as electron donor simultaneously.
During the initial period, denitrification occurred slowly as the strain SY6 had to adapt to the new water environment. After 48 h, the concentration of nitrate, Fe$^{2+}$, and Mn$^{2+}$ declined substantially. The nitrate removal ratios were noticeably different depending on the supplied electron donors, and the highest nitrate removal ratio of approximately 88.89% was achieved with the use of Fe$^{2+}$ as the electron donor. A nitrate removal ratio of 73.69% was achieved when Mn$^{2+}$ was used as the electron donor. The oxidation ratio changed significantly with the use of different electron donors. The concentration of ferrous ions decreased rapidly from 5.79 mg/L to 0.62 mg/L due to many reasons, including oxidation, with a removal ratio of 89.29%. The concentration of Mn$^{2+}$ declined from 5.78 mg/L to 3.28 mg/L, with a removal ratio of 43.25%. The aforementioned results indicated that the denitrification efficiency and the removal ratio of the electron donor were higher when Fe$^{2+}$ was used as the electron donor than when Mn$^{2+}$ was used as the electron donor.

In accordance with the study presented by Su et al. (2015b), strain SZF15 reduced 80.86% of the nitrate and oxidized 75.53% of the Fe$^{2+}$ over 72 h in anaerobic conditions. Herein, the experiment employing Fe$^{2+}$ as the electron donor led to a higher removal of nitrate under anaerobic conditions, indicating the potential applicability of Fe$^{2+}$ to groundwater treatment.

**Combined Fe$^{2+}$ and Mn$^{2+}$ electron donor**

Fe$^{2+}$ and Mn$^{2+}$ acted in combination as electron donors in the autotrophic denitrification. As shown in Figure 2(b), the nitrate concentration decreased rapidly in the presence of Fe$^{2+}$ and Mn$^{2+}$, with a removal ratio of 87.5%. Concomitantly, the concentration of Fe$^{2+}$ also declined rapidly, from 5.79 mg/L to 0.83 mg/L. However, compared with nitrate and Fe$^{2+}$, the concentration of Mn$^{2+}$ changed relatively slowly, moving only from 5.96 mg/L to 3.80 mg/L. This suggests that the SY6 strain can make full use of Fe$^{2+}$; however, in the presence of a large number of Fe$^{2+}$ ions, the strain SY6 will not completely utilize Mn$^{2+}$ as an electron donor. In other words, the strain SY6 may use Fe$^{2+}$ as an electron donor more easily than Mn$^{2+}$.

**Effect of the influent nitrate concentration on nitrate removal in the reactor**

The effect of Fe$^{2+}$ as a donor on nitrate removal in the reactor was evaluated, as shown in Figure 5(a) and 3(c). The concentration of nitrate was varied to evaluate the suitability of Fe$^{2+}$ as a donor in supporting nitrate reduction. The nitrate removal ratio was noticeably dependent on the initial nitrate concentration, and the highest nitrate removal ratio of approximately 95% was achieved with an initial nitrate concentration of 50 mg/L in the biofilm reactor with the removal ratio of Fe$^{2+}$ being 87%, simultaneously. The removal ratios of nitrate and Fe$^{2+}$ were respectively about 90% and 82% for the initial nitrate concentration of 30 mg/L. When the influent concentration was reduced to...
10 mg/L, the removal ratios of nitrate and Fe$^{2+}$ were only about 70% and 55%.

The influence of Mn$^{2+}$ as an electron donor is summarized in Figure 3(a) and 3(c). The highest nitrate removal ratio of approximately 80.25% was obtained with an initial nitrate concentration of 50 mg/L in the biofilm reactor, accompanied by a Mn$^{2+}$ removal ratio of 70.78%. No nitrite accumulation was observed for the duration of the experiment (Figure 3(b)). The removal ratios of nitrate and Mn$^{2+}$ were respectively about 72.26% and 63.53% for the initial nitrate concentration of 30 mg/L. When the influent concentration was reduced to 10 mg/L, the removal ratios of nitrate and Fe$^{2+}$ were only about 66.45% and 60.55%, respectively.

The experimental data showed that the nitrate removal ratio was higher when the nitrate concentration was higher (up to 50 mg/L), regardless of the type of electron donor (Fe$^{2+}$ or Mn$^{2+}$). The highest nitrate, Fe$^{2+}$, and Mn$^{2+}$ removal ratios were achieved when the influent nitrate concentration was 50 mg/L; almost complete denitrification efficiency was achieved (Sahinkaya et al. 2015). However, the rate of nitrate removal was higher when Fe$^{2+}$ was used as an electron donor than with Mn$^{2+}$. Moreover, the bacteria in the reactor utilized Fe$^{2+}$ more efficiently than Mn$^{2+}$ as an electron donor at a nitrate concentration of 50 mg/L.

A nitrate removal rate of 1.112 mg NO$_3^-$-N L$^{-1}$·h$^{-1}$ was obtained with an influent nitrate concentration of 50 mg/L. A nitrate removal rate of 0.186 mg NO$_3^-$-N L$^{-1}$·h$^{-1}$ was observed when the influent nitrate concentration was 10 mg/L. Overall, the data indicated that the speed of nitrate reduction was slightly faster at high nitrate concentrations than at low concentrations. Zhou et al. (2011) also reached the same conclusion. Simultaneously, the Fe$^{2+}$ removal ratio increased when the influent nitrate concentration increased. However, similar results were not obtained for the removal ratio of Mn$^{2+}$ with increasing nitrate concentration. The results indicated that a higher concentration of nitrate was beneficial for Fe$^{2+}$ removal, whereas for Mn$^{2+}$ removal, the impact was not as pronounced.

**Effect of HRT on nitrate removal in the reactor**

Fe$^{2+}$ as an electron donor: The HRT is a very important consideration in laboratory-scale tests or in real applications. Based on the current experimental data, the maximum denitrification activity was observed at an HRT of 12 h. A nitrate removal ratio of 95% was obtained at an HRT of 12 h. A nitrite accumulation was observed over the course of the experiment (Figure 3(b)). The removal rate of nitrate decreased when the HRT was changed from 12 h to 6 h. A nitrate removal ratio of 69.54% was obtained at an HRT of 6 h. Increasing the HRT from 6 h to 12 h caused the removal ratio of Fe$^{2+}$ to increase from 60% to 90%.

When Mn$^{2+}$ was used as the electron donor, based on the data from this experiment (Figure 3(a) and 3(c)), the maximum denitrification activity was observed at an HRT of 12 h. A nitrate removal ratio of 80.25% was obtained at an HRT of 12 h. The removal rate of nitrate decreased when the HRT was changed from 12 h to 6 h. A nitrate removal ratio of 60.54% was obtained at an HRT of 6 h. When the HRT increased from 6 h to 12 h, the removal ratio of Mn$^{2+}$ increased from 59.10% to 70.78%.

Autotrophic denitrification using sulfur limestone as the electron donor was demonstrated to be feasible for removing the nitrate and nitrite from aqueous systems. More time was required to achieve good removal efficiency in the treatment of water containing much more nitrate or nitrite (Zhou et al. 2011). Further, a nitrate removal ratio of 90% was obtained by using sulfide sulfur as an electron donor (Wang et al. 2009), and a nitrate removal ratio of 95% was achieved with the use of hydrogen as an electron donor (Sunger & Bose 2009). Herein, a similar trend was observed with longer HRTs in the reactor, where the removal ratio of Fe$^{2+}$ and Mn$^{2+}$ increased with higher HRT. Moreover, the bacteria employed in this reactor utilized Fe$^{2+}$ more efficiently than Mn$^{2+}$ as an electron donor at an HRT of 12 h. This conclusion is similar to that drawn in the section ‘Immobilized biological filter reactor set up’.

**Effect of pH on nitrate removal in the reactor**

Different species of bacteria function optimally at different pH values, and there is no single effective pH range for all strains. Growth of microorganisms is favored at pH levels ranging from 6.0 to 9.0 (Liu et al. 2009). The activities and functions of most microorganisms are impaired in overly basic or acidic conditions (Yu et al. 2014).

The effect of the initial pH on nitrate removal in the reactor using Fe$^{2+}$ as an electron donor is summarized in Figure 3(a) and 3(c). The initial pH of the medium was set to 6.0, 7.0, and 8.0. After a process of time 10 d, the nitrate removal ratio at pH 6.0 was approximately the same as the ratio at pH 7.0. A nitrate removal ratio of 95% was obtained at pH 6.0–7.0, and improved reduction of Fe$^{2+}$ was observed in the pH range of 7.0–8.0. The maximum Fe$^{2+}$ removal ratio of 90% was obtained at pH 8.0, which may be ascribed to the fact that Fe$^{2+}$ is more likely to be oxidized into Fe$^{3+}$ in a weak alkaline environment.
Mn$^{2+}$ as electron donor: As shown in Figure 3(a) and 3(c), a nitrate removal ratio of 72.77% was obtained at pH 6.0. The corresponding removal ratio of Mn$^{2+}$ was 59.92%. The removal ratios of nitrate and Mn$^{2+}$ were about 80.25% and 70.78%, respectively, at pH 7.0 and about 70.45% and 75.52%, correspondingly, at pH 8.0.

In a weakly acidic environment, the influent nitrate was reduced more quickly than in weakly alkaline environment (Zhou et al. 2011). Furthermore, the optimal denitrification performance was obtained under conditions of pH 6.8–7.0 (Wang et al. 2014). In another study, autotrophic denitrifying bacteria showed good denitrification capability under neutral or weakly acidic conditions (Su et al. 2015c). The results also showed that a weakly alkaline environment was beneficial for the removal of Fe$^{2+}$ and Mn$^{2+}$, which may be the case since chemical oxidation is more probable in a weakly alkaline environment.

The effluent nitrate concentrations obtained after most of the present experiments were below the drinking water standard of 10 mg/L NO$_3$-N. No nitrite effluent accumulation was observed because a low nitrate concentration was obtained regardless of the influent nitrate concentration, HRT, or pH. These are advantages of the autotrophic denitrification processes. However, the quantity of electron donor (Fe$^{2+}$ or Mn$^{2+}$) used in the biofilm reactor was 100 mg/L; this value is somewhat high, which is the major limitation of the study.

CONCLUSIONS

Laboratory experiments were performed to characterize the roles of electron donors (Fe$^{2+}$ and Mn$^{2+}$) in autotrophic denitrification. Fe$^{2+}$ was compared with Mn$^{2+}$ as an electron donor. The highest nitrate removal ratio was observed with Fe$^{2+}$ as the electron donor at approximately 88.89%. Strain SY6 can utilize Fe$^{2+}$ as an electron donor more efficiently than Mn$^{2+}$ as an electron donor. The experiments were carried out in an immobilized biological filter reactor and showed that at a pH6.0, a nitrate concentration of 50 mg/L and HRT of 12 h, the reactor had the highest removal ratio of nitrate with Fe$^{2+}$ as an electron donor at about 95%. For the higher concentration of nitrate removal, longer HRT was necessary. With a higher influent nitrate and HRT, the removal ratio of Fe$^{2+}$ and Mn$^{2+}$ was higher. A weakly alkaline environment was helpful for the removal of Fe$^{2+}$ and Mn$^{2+}$.

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