Performance of an autotrophic denitrification process with mixed electron donors and a functional microbial community

Xianxin Luo, Junfeng Su, Han Liu, Tinglin Huang, Li Wei, Jiawei Nie, Hanyu Gao and Dongpeng Li

ABSTRACT

A moving bed biofilm reactor (MBBR) using Mn(II) and Fe(II) as mixed electron donors was designed for nitrate removal. The optimal state, as determined by response surface methodology, was an Fe(II):Mn(II) molar ratio of 0.62, electron donor:electron acceptor molar ratio of 2.62 and hydraulic retention time of 10.88 h. Subsequently, the MBBR was applied to groundwater treatment and demonstrated a final nitrate-N removal efficiency of 99.5% with a nitrite-N accumulation rate of 0.0706 mg-N·L⁻¹·h⁻¹. Furthermore, high-throughput sequencing was employed to characterize bacterial communities in the MBBR. Results showed that the genera of Pseudomonas and Acinetobacter may make a contribution to the nitrate removal.

Key words | autotrophic denitrification, microbial community, mix electron donors, nitrate

INTRODUCTION

Nitrate-nitrogen (NO₃⁻-N) contamination of groundwater has become an environmental and public health issue in both developing and developed countries. High nitrate concentrations in drinking water sources are generally anthropogenic, and are chiefly caused by fertilizers, explosives, metal finishings, and nuclear industries. Elevated nitrate levels could threaten human health and other life-forms, such as with methemoglobinemia (Mousavi et al. 2012); thus, the removal of nitrate from drinking water has become a necessity. Accordingly, permissible nitrate levels have been set at 10 mg/L NO₃⁻-N in the USA and 12 mg/L in Europe (USEPA 1987; Karanasios et al. 2010).

As an alternative selection to remove the nitrogen source, autotrophic denitrification is a promising technology, which leads to low biomass production, decreased risk of bacterial contamination and reduced operational cost (Sahinkaya et al. 2016). Nevertheless, because of the low solubility (1.6 mg/L at 20 °C) and high flammability in hydrogenotrophic denitrification application (Mansell & Schroeder 2002), and sulfate and acid generation in the sulfur-based autotrophic denitrification process (Sahinkaya et al. 2016), a new type of denitrification process involved in other electron donors is urgently to be proposed. In the natural groundwater body, high concentrations of Fe(II)
and Mn(II) often occur simultaneously (Bai et al. 2016), thus, Fe(II) and Mn(II) need to be further removed from the groundwater body to maintain the safety of drinking water. In 1996, Straub et al. (1996) reported a new bioprocess for removal of nitrate using ferrous iron as electron donor. Thereafter, Fe(II)-iron-mediated autotrophic denitriﬁcation has been studied intensively and has drawn wide attention due to its novelty and ecological effect during the past decades (Kiskira et al. 2017). Ferrous iron-based chemautotrophic denitriﬁcation (Fe-CAD) is more promising than heterotrophic denitriﬁcation due to the lower C/N ratio that is required and ferrous salt which is much cheaper than methanol or acetate (Li et al. 2014). Wang et al. (2017) operated a Fe-CAD reactor to treat the nitrate-contaminated wastewater, and the results showed that the reactor was operated successfully with the volumetric loading rate and volumetric removal rate of NO3 being 0.26 ± 0.01 kgN/(m3/d) and 0.09 ± 0.03 kgN/(m3/d). Su et al. (2017) found that nitrate could be depleted completely in the immobilized biological ﬁlter using Fe(II) and Mn(II) as mixed electron donors. In addition, Su et al. (2016) also established a moving bed bioﬁlm reactor (MBBR) to remove nitrate with high nitrogen removal performance, and results indicated that it is feasible to carry out an autotrophic denitriﬁcation process using Mn(II) as electron donor.

However, there have been few reports on the study of the removal of nitrate, Fe(II) and Mn(II) simultaneously. For this purpose, we proposed a new type of autotrophic denitriﬁcation by using Fe(II) and Mn(II) as electron donors simultaneously in this study. Meanwhile, a denitriﬁcation reactor was reported in 1975 for the ﬁrst time (Miyaji & Kato 1975). It has become a promising technology for denitriﬁcation with high treatment performance and avoiding microbial contamination (Sahinkaya et al. 2016). Thus, the bio-reactor performance for nitrogen removal by mixed electron donors was employed by immobilizing two strains (strain Pseudomonas sp. SZF15 and Acinetobacter sp. SZ28) in this study.

To evaluate the effects of the mixed electron donors, Fe(II) and Mn(II), on autotrophic denitriﬁcation, a MBBR employing simultaneous nitrate removal, was proposed. Thus, the overall objectives of this study were to: (i) test the performance of Fe(II) and Mn(II) based on autotrophic denitriﬁcation and by analyzing the effects of various hydraulic retention times (HRTs), electron donor:electron acceptor molar ratios, and Fe(II):Mn(II) molar ratios on nitrate removal; and (ii) investigate the microbial communities in the MBBR.

MATERIALS AND METHODS

Microbial culture

Pseudomonas sp. SZF15 (Su et al. 2015a) and Acinetobacter sp. SZ28 (Su et al. 2015b) were isolated from the sediment of Tang Yu reservoir and Shi Bian Yu oligotrophic reservoir located in Xi’an City, Shaanxi Province, China, respectively. Bacteria were grown in inorganic basal medium (IM) at pH 6.5, 30 °C and dose of 2 ml trace element solution, where Vbacteria/Vmedium was chosen to be 10%. Bacteria could be harvested after 7 days, corresponding to the OD600 of 0.280. The components of IM and trace element solution were followed by Su et al. (2016). Freshly prepared cell suspensions were used for inoculation through a sterile individual ﬂow cell channel by a sterile syringe. The ﬂow cells were maintained in a horizontal position for 8 h, with no ﬂow after inoculation to allow initial bacterial adhesion. The inoculated ﬂow cell system was then placed at 30.0 °C.

Reactor set-up

Two bio-reactors were operated simultaneously. One reactor acted as the control with only carrier media; the other acted as the experimental reactor with immobilized bacteria. Both MBBRs were ﬁlled with 40% bulk volume (Vsupport/Vreactor) Yu Long plastic carrier media (Yixing City Yulong F.P. Co., Ltd, China). Detailed information on Yu Long plastic carrier media, as well as the detailed composition and effective volumes of the bio-reactor system are as previously described by Su et al. (2016). Firstly, 10 L of sterile IM were prepared in the feed tank to simulate the polluted groundwater. Inﬂuent was pumped into the MBBR at the desired ﬂow rate and Fe(II) and Mn(II) were added as inorganic electron donors.

To determine suitable operating conditions, effects of Fe(II):Mn(II) molar ratios (0.11, 0.43 and 1.0), electron donor:electron acceptor molar ratios (0.5, 1.75 and 3.0)
and HRTs (8 h, 10 h and 12 h) were studied. According to Subha & Muthukumar (2012), the response surface methodology (RSM) is a statistical approach incorporating the analysis of both experiential and modeling data. Each operating condition was identified according to the RSM to operate over a 7-day period. The MBBRs operated continuously for 194 days, including 119 days of RSM experimentation, 63 days under optimal conditions and 12 days of groundwater treatment testing. Groundwater was collected from Pucheng County, Weinan City, Shaanxi Province, China.

## Analytical methods

The liquid samples collected during the experiments were filtered through a 0.45 μm membrane filter in order to remove the suspended solids. Then, 1 mL samples were used to analyse the concentrations of nitrate-N, nitrite-N, Mn(II) and Fe(II) by the methods of ultraviolet spectrophotometry, N-(1-naphthalene)-diaminoethane spectrophotometry, potassium periodate spectrophotometry and phenanthroline, respectively (DR5000, HACH, USA) (APHA 2005).

## DNA extraction, PCR amplification and high-throughput sequencing

The total microbial DNA of the samples was extracted using a DNA extraction kit protocol (MO BIO Laboratories, Inc., Carlsbad, CA). The biofilm sample of YL1 was collected from the control reactor. Samples of YL2, YL3 and YL4 were collected from the experimental reactor under the stable periods when the Fe(II):Mn(II) were 0.11, 0.43 and 1.0, respectively. V1 and V3 hypervariable regions of the bacterial 16S rRNA gene were targeted by polymerase chain reaction (PCR) amplification, with the primers of 338F (5’-ACTCCTACGGGAGGCAGCA-3’) and 806R (5’-GG ACTACHVGGGTWT-CTA-AT-3’), respectively. The pyrosequencing procedure, the program of PCR amplification and the analysis of the sequence were followed by Chen et al. (2015). Then, RDP-II Classifier of the Ribosomal Database Project (RDP) and the National Center for Biotechnology Information (NCBI) BLAST were used to analysis the taxonomy data base. Finally, a new generation of high throughput sequencing machine (Illumina MiSeq PE300) was used to contrast the denitrifier bacteria microbial community.

## RESULTS AND DISCUSSION

### Model fitting by RSM

#### Determination of optimum operating conditions

To determine suitable reactor operating conditions, the effects of Fe(II):Mn(II) molar ratios, electron donor:electron acceptor molar ratios and HRTs on nitrate-N concentrations were investigated; the initial nitrate-N concentration was 16.5 mg-N/L and the level of Fe(II) and Mn(II) in the manuscript were varied and could be calculated according to the values of electron donor:electron acceptor molar ratio. Analysis of variance (ANOVA) was applied to the results (Table 1), where 'Prob > F' values less than 0.05 were considered significant (Khuri & Mukhopadhyay 2010). The ANOVA revealed a significant RSM with a high $R^2$ (0.9885), along with $P < 0.0001$ and lack-of-fit of 0.0077, indicating a highly significant model. Similarly, the p-values were less than 0.05 for the Fe:Mn ratio, electron donor:electron acceptor ratio, and HRT. Furthermore, they were also less than 0.05 for both the mutual interaction between the Fe:Mn ratio and electron donor:electron acceptor ratio, and between the electron donor:electron acceptor ratio and the HRT, indicating that they exhibited significant

<table>
<thead>
<tr>
<th>Source</th>
<th>Regression coefficient</th>
<th>F value</th>
<th>p-value</th>
<th>prob &gt; F</th>
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<tbody>
<tr>
<td>Model</td>
<td>86.99</td>
<td>66.73</td>
<td>&lt;0.0001***</td>
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</tr>
<tr>
<td>A – Fe:Mn ratio</td>
<td>12.16</td>
<td>82.40</td>
<td>&lt;0.0001***</td>
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<tr>
<td>B – Electron donor:</td>
<td>24.41</td>
<td>332.37</td>
<td>&lt;0.0001***</td>
<td></td>
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<tr>
<td>electron acceptor ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-HRT</td>
<td>7.33</td>
<td>29.96</td>
<td>0.0009***</td>
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<tr>
<td>AB</td>
<td>4.59</td>
<td>5.88</td>
<td>0.0457*</td>
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</tr>
<tr>
<td>AC</td>
<td>–1.11</td>
<td>0.35</td>
<td>0.5755</td>
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</tr>
<tr>
<td>BC</td>
<td>5.61</td>
<td>8.76</td>
<td>0.0211*</td>
<td></td>
</tr>
<tr>
<td>A²</td>
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<td>11.51</td>
<td>0.0116*</td>
<td></td>
</tr>
<tr>
<td>B²</td>
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<td>116.75</td>
<td>&lt;0.0001***</td>
<td></td>
</tr>
<tr>
<td>C²</td>
<td>–3.86</td>
<td>4.38</td>
<td>0.0746</td>
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</tbody>
</table>

*Significant (0.01 < p value < 0.05).
**Very significant (0.001 < p value < 0.01).
***Vitally significant (p value < 0.001).
nitrate-N removal. The optimum level for each variable with respect to denitrification efficiency (98.82%) occurs at an Fe(II):Mn(II) molar ratio of 0.62, electron donor:electron acceptor molar ratio of 2.62 and HRT of 10.88 h.

**Effect of process variables on the denitrification process**

Response contour plots were used to visualize the relationships between the operating factor levels and the denitrification process. Significant interactive effects on nitrate-N removal efficiency were observed between Fe(II):Mn(II) molar ratios and electron donor:electron acceptor molar ratios (Figure 1(a)). Nitrate-N reduction first increased and then stabilized as the initial Fe(II):Mn(II) molar ratio increased from 0.11 to 1.0 and the electron donor:electron acceptor molar ratio increased from 0.50 to 3.00. A high Fe(II):Mn(II) molar ratio is maybe more conducive to the autotrophic denitrification process because bacteria will preferentially use Fe(II) over Mn(II) as an electron donor since it more readily loses an electron, allowing for easier oxidation and, thus, a greater energy to be gained by the bacteria during denitrification. Furthermore, the effect of electron donor:electron acceptor molar ratios on nitrate removal was greater than that of the Fe(II):Mn(II) molar ratios, indicating that the denitrification process is maybe limited when the system lacks an electron donor. This is supported by the observation of Chung et al. (2014), whose denitrification stopped after 120 h when the S:N ratio was depleted beyond 1.5, which is below the stoichiometric requirements of the electron donor.

The mutual interaction between HRTs and Fe(II):Mn(II) molar ratios on nitrate-N removal were investigated

![Figure 1](https://iwaponline.com/ws/article-pdf/19/2/434/592007/ws019020434.pdf)

*Figure 1* | Contour plots of nitrate-N removal efficiency as a function of: (a) electron donor:electron acceptor ratio and Fe(II):Mn(II) ratio; (b) HRT and electron donor:electron acceptor ratio; (c) HRT and Fe(II):Mn(II) ratio.
(Figure 1(b)). Results indicate that the nitrate-N removal ratio was enhanced from 80% to 99.50% by increasing the HRT from 8 h to 12 h. Increasing residence times in the reactor allows for greater microbial nitrate consumption. Based on previous research (Su et al. 2016), the appropriate HRT of Mn(II)-based autotrophic denitrification is 10.96 h. Zhao et al. (2011) reported that a 100% nitrate removal efficiency was achieved in a biofilm–electrode reactor when the HRT was longer than 8 h. Similarly, Wu et al. (2005) reported that the same HRT was appropriate for being used in their biofilm–electrode reactor.

Optimization of reactor conditions

The optimal combination of variables was determined in terms of maximizing the desired objective response yield. These conditions were then employed to test the performance of the reactor as a function of HRT. The HRT was gradually increased from 4 h to 11 h over a period of 56 days (Figure 2). During the first 4 days, slight fluctuations of nitrate in the effluent were observed at the beginning of each phase because the variable flow rate, caused by the change in HRT, impacted the biofilm reactor. At an HRT of 11 h, the nitrate-N concentration in the effluent was measured as 0.23 mg/L, indicating a good rate of autotrophic denitrification in the system under the optimal conditions ((Fe(II):Mn(II) molar ratio of 0.62, electron donor:electron acceptor molar ratio of 2.62) (Figure 2(a)). Increasing the HRT from 4 to 11 h resulted in a gradual decrease of nitrate-N in the effluent and a corresponding increase in the nitrate removal ratio from 46.26% to 98.9%. Since the HRT determines the treatment capacity of the reactor at a given influent velocity, the increased residence time in the reactor allows the bacteria to remove nitrate to a greater extent. For comparison, Wang et al. (2017) operated a Fe-CAD reactor to treat nitrate-contaminated wastewater with an HRT of 16 h, and the results showed that when the influent nitrate-N concentration was above 50 mg/L, the nitrogen removal efficiency would be decreased, indicating that a higher nitrate content is unfavorable for the denitrification process.

Conversely, nitrite-N in the effluent showed an upward trend, with slight fluctuations (Figure 2(b)). The highest nitrite-N concentration in the effluent was observed at approximately 0.42 mg/L when the HRT was set at 11 h, which remains below the permissible nitrite-N level of 1 mg-N/L. In all cases, as the HRT gradually reduced, the nitrite-N in the effluent gradually increased. This is due to a two-step mechanism in which nitrate-N reduction simultaneously occurs with nitrite-N accumulation (Xia et al. 2010). Sun & Nemati (2012) reported that nitrate reduction results in formation of nitrite, and that high nitrate reduction occurs as soon as nitrate is completely utilized; thus, a short HRT might lead to nitrate transformation into nitrite. However, an appropriate nitrate concentration range is a reliable indication that the microorganisms have adapted to their surroundings and that the biofilm has grown to maturity over the course of gradual acclimation (Zhao et al. 2011). Meanwhile, oxidation of 61.87% of the Mn(II) and 100% of Fe(II) was observed in the MBBR (Figure 2(c) and 2(d)). The reactor exhibited simultaneous removal efficiencies of nitrate, Fe(II) and Mn(II) in nitrate-contaminated water.

Performance of groundwater treatment

The denitrification performances were monitored in stable MBBRs that were adapted to treat groundwater (Figure 3). The quality of raw water was: pH of 7.05, temperature of 24.30 °C, nitrate-N of 12.05 mg/L, nitrite-N of 0.012 mg/L, ammonium of 0.06 mg/L, total organic carbon of 0.86 mg/L. The operating conditions used for studying the performance of groundwater treatment were: temperature of 25 °C, pH of 7.00, Fe(II):Mn(II) molar ratio of 0.62, electron donor:electron acceptor molar ratio of 2.62, HRT of 11 h. An initial adjustment period was observed in the MBBRs as the microorganisms adjusted to the new environment. During this phase, the microorganisms need some time to adjust to the new environment, and the nitrate-N concentrations showed a gradually decreasing trend from 12.01 mg-N/L to 0.13 mg-N/L, with a corresponding increase in the removal efficiency from 45.9% to 98.91%. Under optimal conditions, the nitrate-N removal efficiency from groundwater increased to 99.5%, which is similar to the value in the synthetic groundwater treatment experiment, thereby supporting the feasibility of using this system for treating nitrate-contaminated groundwater. Meanwhile, the highest nitrite-N accumulation rate of
Figure 2 | Effect of HRTs on the denitrification process. Concentration profiles of (a) nitrate-N, (b) nitrite-N, (c) Mn(II) and (d) Fe(II) in the influent and effluent.
0.0706 mg-N·L⁻¹·h⁻¹ was obtained in the groundwater treatment.

**Microbial community analysis: phylum- and genus-level identification**

Since the behaviors and functions of bacteria influenced the activity of MBBR, further investigation by MiSeq high-throughput sequencing was conducted to analyze the community structures of the denitrifying bacteria. For the purpose, DNA for the amplification and subsequent sequencing of the 16S rRNA gene was extracted from the reactor under the operating conditions in terms of Fe(II):Mn(II) molar ratio (YL1, YL2, YL3 and YL4, respectively).

In total, 14 phyla, excluding unclassified taxa, were identified in the four samples (Figure 4(a)). The results show that the initial Fe(II):Mn(II) molar ratio had a significant affect on the abundance of the dominant phyla within the bio-reactor. In the control sample (YL1), the relative abundance of Proteobacteria was 21.13%. The percentages of Proteobacteria in samples YL2, YL3 and YL4 were 30.87%, 39.38% and 49.23%, respectively, thus indicating that this phylum was enriched throughout the experiment, suggesting a positive relationship between Proteobacteria and the Fe(II):Mn(II) molar ratio. Actinobacteria, the second most dominant overall phylum, was detected at 43.67% in YL1, 11.25% in YL2, 20.22% in YL3, and 3.31% in YL4. The Firmicutes phylum was also detected at 10.15% in YL1, 6.73% in YL2, 16.22% in YL3, and 17.31% in YL4. In addition, the Fusobacteria and Bacteroidetes phyla were detected in YL1, YL2, YL3 and YL4. It is well known that Proteobacteria is the prominent phylum in the denitrification community, and according to the previous study (Su et al. 2016), Pseudomonas sp. SZF15 and Acinetobacter sp. SZ28 belong to phylum Proteobacteria, suggesting that the environment is conducive to the growth of the phylum Proteobacteria. Among the many studies reported, most of the denitrifiers belong to phylum Proteobacteria (Karanasios et al. 2010).

Further analysis was done to distinguish the relative microbial variation associated with the increasing Fe(II):Mn(II) molar ratio at the genus level. In total, 50 genera...
were identified (Figure 4(b)). *Pseudomonas* and *Acinetobacter* were detected in all three experimental samples, but not the control (YL1). The proportion of *Pseudomonas* and *Acinetobacter*, which have denitrifying capabilities (Su et al. 2016), in samples YL2, YL3, YL4 were 5.22% and 2.13%, 9.21% and 4.67%, and 14.69% and 11.37%, respectively. These results indicate that endogenous denitrifying bacteria were enriched as the Fe(II):Mn(II) molar ratio condition increased, subsequently improving the denitrification efficiency. This also demonstrates that the genera of *Pseudomonas* and *Acinetobacter* can grow in this environment, and that they may play a role in the denitrification process. Additionally, the relative abundance of *Pseudoxanthomonas* showed a similar trend as the molar ratio of Fe(II):Mn (II) increased. This genus has been reported in several denitrification processes (Sahinkaya et al. 2013) and contributes to nitrate removal in the reactor. Furthermore, the denitrification-related genera *Sphingomonas*, *Bacteroides*, *Bacillus*, *Rhizobium* and *Lactobacillus* were also detected in the experimental samples YL2, YL3, YL4. Specifically, *Lactobacillus* contributes to the conversion of NO3 to N2O (Dodds & Collins-Thompson 1985). Therefore, there were several denitrifying bacteria with multiple species of Fe(II) and Mn(II) serving as electron donors that contributed to nitrate removal and played an important role in the reactor. Conversely, the relative abundance of *Azospirillum* and *Acidovorax* decreased as the Fe(II):Mn(II) molar ratio increased. This observation may be attributed to competitive interactions between microbes in the reactor.

**CONCLUSIONS**

Analysis using RSM identified the optimum level of each variable in MBBR with the Fe(II):Mn(II) molar ratio of 0.62, electron donor:electron acceptor molar ratio of 2.62 and HRT of 10.88 h. Subsequently, the optimum condition was employed to test the performance of the reactor, and
results suggested that the reactor could remove nitrate completely. Furthermore, the MBBR was applied to groundwater treatment, and results demonstrated a final nitrate-N removal efficiency of 99.5% with the nitrite-N accumulation rate of 0.0706 mg-N·L\(^{-1}\)·h\(^{-1}\). High-throughput sequencing was employed to profile the microbial communities of denitrifying bacteria in the reactor, and results suggested that a higher Fe(II):Mn(II) molar ratio environment is conducive to the growth of the genera of *Pseudomonas* and *Acinetobacter*.

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