Multifunctional sugar-cube-like Fe$_3$O$_4$@Cu/PVA biomaterials for enhanced removal of nitrate and Mn(II) from moving bed biofilm reactor (MBBR)

Jun Feng Su, Yi chou Gao, Dong hui Liang, Li Wei, Xue chen Bai and Hai rong Zhu

ABSTRACT

A novel Fe$_3$O$_4$@Cu/PVA biomaterial as a new adsorbent and bacterial cell immobilized carrier was synthesized in this work. The structure and morphology were characterized by scanning electron microscopy (SEM). Effects of factors on Mn(II)-based autotrophic denitrification were investigated in a moving bed biofilm reactor (MBBR). The results indicate that the highest nitrate removal and Mn(II) oxidation efficiency occurred under the conditions of initial Mn(II) concentration of 80 mg·L$^{-1}$, hydraulic retention time (HRT) of 10 h and pH 7. Meteorological chromatography analysis showed that N$_2$ was produced as an end-product, and that gas compositions were different depending on the concentration of Mn(II) in the MBBR. The community diversity in the MBBR was markedly influenced by the concentration of Mn(II) and Pseudomonas sp. H-117 played a primary role in the process of nitrate removal and Mn(II) oxidation.

INTRODUCTION

Nitrate-nitrogen contamination of groundwater has become an environmental and public health issue in developed and developing countries (Su et al. 2018). Several abiotic and biotic techniques for the removal of nitrates from water have been proposed in recent years (Wang et al. 2018). Among them the reduction of nitrate into harmless nitrogen gas (i.e., denitrification) by biological processes has become the most popular technology (Ghafari et al. 2008). However, low carbon-to-nitrogen ratios in many wastewaters require a supply of external carbon to meet complete heterotrophic denitrification (Wei et al. 2017). Autotrophic denitrification has received significant attention lately due to several inherent merits compared with conventional heterotrophic denitrification (Yang et al. 2016). Compared with heterotrophic denitrification, autotrophic denitrification has associated low operational costs and reduced sludge production autotrophic denitrifying (Yang et al. 2017). However, bacteria have a relatively low growth rate (Montalvo et al. 2016). To address these issues, immobilized cell technology can be applied in autotrophic denitrification.

Polymer brush-grafted magnetic nanoparticles for highly effective adsorption of heavy metal ions (Bae et al. 2016), and Fe$_3$O$_4$ nanoparticles also have potential for re-collection of metal ions. The objectives of this work were to: (1) determine nitrate removal and Mn(II) oxidation capability of the Fe$_3$O$_4$@Cu/PVA immobilized cells and removal mechanism; (2) evaluate the nitrate removal and Mn(II) oxidation capability under conditions of different pH, hydraulic retention time (HRT) and initial Mn(II) concentrations in the moving bed biofilm reactor (MBBR); (3) investigate the impacts of Mn(II) concentration on the microbial community and metabolic activity in the MBBR.
MATERIALS AND METHODS

Media and cultivation

Strain H-117 (Su et al. 2017) was isolated from the Shi Bian Yu reservoir, and grown in an anaerobic autotrophic medium (AM). The ingredients of the AM and trace element solution (TE) are presented in Table 1.

Reactor operation and evaluation of the optimum conditions

The polyvinyl alcohol (PVA) sponge was purchased from Xiya Chemical Industry Co., Ltd (Shandong, China). The preparation of the Fe₃O₄@Cu/PVA functionalized biomaterials was according to Nodeh et al. (2016) and Azizi et al. (2014). The bioreactor system consisted of three modules: feed tank, pump, and MBBR. The MBBR was a 0.10 m organic glass column of 0.25 m inner diameter. The total volume was 4.5 L and the working volume was 4.0 L. The working volume was filled to a depth of 0.06 m with Fe₃O₄@Cu/PVA biomaterials (1 x 1 x 1 cm), and void ratio of 95%. The reactor was sealed by a gasket. The experiment involved running two parallel reactors, one reactor with no immobilization as a control, the other as the immobilized reactor. The MBBR was continuously and steadily operated for 115 days. As shown in Table 1, the operation of the MBBR was divided into 15 stages. Firstly, AM were prepared in the feed tank and the AM was pumped into the MBBR at the desired flow rate. The inorganic electron donor (Mn(II)) was prepared by diluting MnSO₄·H₂O in distilled water to the desired concentration. The reactor was operated at 30 ± 2°C. The effluent port was set in the bottom of the MBBR and measured the nitrate-N, Mn(II), and nitrite-N concentrations each day.

Determination of nitrate removal and Mn(II) oxidation ability was performed by variation of HRT, pH and initial Mn(II) concentration, and the MBBR was employed to optimize the operating variables. The optimum conditions could be confirmed by the above 15 stages. After the preliminary study, the MBBR was operated under the optimum conditions.

Analytical methods

In all experiments, the effluent samples were filtered by a membrane (0.45 um pore-size) through a suction filter machine. The concentrations of nitrate, nitrite and Mn(II) were measured according to the standard methods. Water pH was measured by a bench-top pH meter (MM110, HACH, USA). The morphological properties of Fe₃O₄@Cu/PVA biomaterials and the bacteria were characterized by a scanning electron microscopy (SEM, JSM-5800, Japan JEOL) system, and the crystallographic structure of the Fe₃O₄@Cu/PVA biomaterials was determined by X-ray diffraction (XRD, Rigaku Ultima IV). The gases were analyzed immediately after collection using gas chromatography (Agilent6890, Japan; and PerkinElmer clarus 600, USA). Bacterial genomic DNA in the MBBR was extracted from the biofilm samples for bacterial community analysis.

Table 1 | The components of the media used in the autotrophic denitrification and operational conditions of the MBBR

<table>
<thead>
<tr>
<th>TE</th>
<th>AM</th>
<th>TE g·L⁻¹</th>
<th>AM g·L⁻¹</th>
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<td>FeCl₂·4H₂O</td>
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<tr>
<td>MnSO₄·7H₂O</td>
<td>KH₂PO₄</td>
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<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>ZnCl₂</td>
<td>CaCl₂</td>
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<td>0.05</td>
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<tr>
<td>H₃BO₃</td>
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<table>
<thead>
<tr>
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<th>Initial pH</th>
<th>HRT (h)</th>
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<tr>
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<tr>
<td>Period 3</td>
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RESULTS AND DISCUSSION

SEM photographs and XRD spectra of Fe₃O₄@Cu/PVA

The SEM images of Fe₃O₄@Cu/PVA and bacteria are shown in Figure 1. The Fe₃O₄@Cu/PVA exhibited a 3D interconnected network skeleton and a bumpy surface morphology (Figure 1(a)). After 10 days of incubation, strain H-117 was wrapped on the bumpy surface of the Fe₃O₄@Cu/PVA (Figure 1(c)). Comparing Figure 1(a) and 1(c), it is observed that strain H-117 was immobilized on the surface of the Fe₃O₄@Cu/PVA. The sediments of the experimental groups were also observed by SEM (Figure 1(b)).

In order to determine the crystal structure and the composition of the as-prepared samples, the PVA before and after the adsorption of Fe₃O₄@Cu was subjected to XRD analysis under a wide range of scans. Figure 1(d) shows the XRD patterns of PVA, Fe₃O₄@Cu, and Fe₃O₄@Cu/PVA, and these data indicate the presence of Fe₃O₄@Cu on the surface of the PVA. The Fe₃O₄@Cu/PVA shows four strong peaks at 30.19 (2.96 Å), 35.62 (2.52 Å), 56.98 (1.61 Å), and 62.78 (1.48 Å), which correspond to peaks [220], [311], [511], and [440] of Fe₃O₄@Cu.

Denitrification and Mn(II) oxidation in the MBBR

The experimental and control groups were operated in parallel under period 1–15 conditions for 90 days (Table 1). In the first three periods, the effect of decreasing HRT from 10 h (period 1) to 6 h (period 3) was tested. The nitrate removal ratio decreased from 100% (1.61 mg·L⁻¹·h⁻¹) in period 1 to 91% (2.31 mg·L⁻¹·h⁻¹) in period 3, and the nitrate removal ratio reached a maximum value of 100% when the HRT was 10 h (period 1). The data demonstrate that the denitrification efficiency was improved when the HRT increased. For Mn(II)-based autotrophic
denitrification, Mn(II) was oxidized during nitrate removal. The Mn(II) oxidation ratio decreased from 74.83% (period 1) to 64.90% (period 2) and 60.32% (period 3) with the decrease in the HRT from 10 to 8 and 6 h, respectively (Figure 2(a)), which is in good agreement with the theoretical predictions. The Mn(II) could not be completely oxidized by microorganisms when the HRT was 6 h, resulting in a high concentration of Mn(II) in the effluent. The results suggest that good Mn(II) oxidation efficiency was obtained with long HRT in the MBBR. This may be because a long HRT provides bacteria with enough time to adapt to the new environment and Mn(II) had enough time to degrade. Bai et al. (2016) reported that a longer HRT may increase the reaction time, and thus improve the treatment effect. Compared with the removal of nitrate and Mn(II) oxidation, the accumulation of nitrite followed a different rule, and the concentration of nitrite remained below 2.5 mg L\(^{-1}\) during the experiment.

In periods 4–5, pH in the feed was increased from 6 (period 4) to 8 (period 5). In order to recover the decrease in the HRT from 10 to 8 and 6 h, respectively, the Mn(II) oxidation ratio increased when the Mn(II) concentration was kept at 60 mg L\(^{-1}\) during period 5, pH in the feed was increased from 6.0, 7.0, and 8.0, respectively. These data indicate that the Mn(II)-based autotrophic denitrification process was easily influenced by pH and that 16 mg L\(^{-1}\) nitrate was almost completely degraded at pH 7.0 in the MBBR. Ghafari et al. (2010) also showed that the optimal pH value for the denitrification process was 7.5–8.0. The Mn(II) concentration is also shown in Figure 2(a). The Mn(II) oxidation ratios were 74.83% (pH 7.0), 64.90% (pH 6.0), and 60.32% (pH 8.0). The data can be explained by the fact that Mn(II) oxidation bacteria are more efficient in neutral pH conditions, which might be attributed to low or high pH values inhibiting Mn(II) oxidation microbial communities in the MBBR. Chen et al. (2015) also reported that low pH values (pH 6.0) inhibited the denitrification process due to the reactivity of hydrogenotrophic denitrifying bacteria being affected by the decomposition of carbonate ions in this environment. Nitrite concentration remained at very low levels (<2.5 mg L\(^{-1}\)).

From period 6 to period 10, the Mn(II) concentration was kept at 60 mg L\(^{-1}\). As shown in Figure 2(b), the highest nitrate removal (1.44 mg L\(^{-1}\)h\(^{-1}\)) and Mn(II) oxidation ratio (4.20 mg L\(^{-1}\)h\(^{-1}\)) occurred during period 6 (HRT 10.0 h and pH 7.0). From period 11 to period 15, the Mn(II) concentration was kept at 40 mg L\(^{-1}\). As shown in Figure 2(c), the maximum Mn(II) oxidation (1.26 mg L\(^{-1}\)h\(^{-1}\)) and denitrification efficiency (2.30 mg L\(^{-1}\)h\(^{-1}\)) were observed during period 11 (HRT 10 h and pH 7.0). Comparing period 1 (80 mg L\(^{-1}\) Mn(II)), period 6 (60 mg L\(^{-1}\) Mn(II)) and period 11 (40 mg L\(^{-1}\) Mn(II)), the nitrate removal ratio increased when the Mn(II) concentration increased, and the maximum nitrate removal ratio of 100% (1.61 mg L\(^{-1}\)h\(^{-1}\)) was obtained at 80 mg L\(^{-1}\) Mn(II). The results show that autotrophic denitrification ability is affected by Mn(II) concentration (Mn(II) as electron donor) and strain H-117 could tolerate and oxidize Mn(II) at concentrations as high as 80 mg L\(^{-1}\).

Based on data in Figure 2, the strain H-117 may conduct the following sequential Mn(II)-based autotrophic denitrification reactions (Swathi et al. 2016):

\[
\begin{align*}
\text{NO}_3^- + \text{Mn}^{2+} + \text{H}_2\text{O} &= \text{NO}_2^- + \text{Mn}^{4+} + 2\text{OH}^- \quad (1) \\
2\text{NO}_2^- + \text{Mn}^{2+} + 2\text{H}_2\text{O} &= 2\text{NO}^- + \text{Mn}^{4+} + 4\text{OH}^- \quad (2) \\
2\text{NO}^- + \text{Mn}^{2+} + \text{H}_2\text{O} &= \text{N}_2\text{O} + \text{Mn}^{4+} + 2\text{OH}^- \quad (3) \\
\text{N}_2\text{O} + \text{Mn}^{2+} + \text{H}_2\text{O} &= \text{N}_2 + \text{Mn}^{4+} + 2\text{OH}^- \quad (4) \\
5\text{Mn}^{2+} + 2\text{NO}_3^- + 4\text{H}_2\text{O} &= 5\text{MnO}_2 + \text{N}_2 + 8\text{H}^+ \quad (5)
\end{align*}
\]

\[\Delta G = -14.35 \text{kJ mol}^{-1}\]

Detection of gaseous nitrogen compounds under different conditions

To confirm the nitrogen removal mechanism of the MBBR, gas chromatography (GC) was used to monitor the gas composition produced by the MBBR. Figure 3 shows that there was 99.86% nitrogen in the MBBR when the Mn(II) concentration was 80 mg L\(^{-1}\) (period 1), 96.53% nitrogen when 60 mg L\(^{-1}\) Mn(II) was used (period 6), and 88.76% nitrogen with 40 mg L\(^{-1}\) Mn(II) (period 11). Meanwhile, 79% nitrogen was detected in the atmospheric background. It could be concluded that the gas compositions were
Figure 2 | Nitrate, nitrite, Mn(II) variations in the MBBR and control group: (a) Mn(II) = 80 mg·L\(^{-1}\), (b) Mn(II) = 60 mg·L\(^{-1}\), (c) Mn(II) = 40 mg·L\(^{-1}\).
different in the various Mn(II) concentrations. The maximum N₂ emission (99.86%) was obtained when 80 mg·L⁻¹ Mn (II) was used in the MBBR as the only electron donor. The result is in accordance with the nitrate removal ratio.
in the MBBR because N₂ emission increased with the increased nitrate removal ratio. Zhou et al. (2017) also reported that a higher excess N₂ concentration indicated a higher denitrification rate during NPKM treatment.

At the end of the experiment, N₂O was not detected under anaerobic conditions when the concentration of the electron donor was 80 mg·L⁻¹, which is in agreement with the results found by Ribera-Guardia et al. (2014). However, a low concentration of N₂O was detected by the PE600 at 60 mg·L⁻¹ Mn(II) (1.68 mg·L⁻¹ N₂O) and 40 mg·L⁻¹ Mn(II) (3.09 mg·L⁻¹ N₂O). These results suggest that NO₂ levels in the MBBR were slightly different at different Mn(II) concentrations, and the highest concentration of NO₂ was found when the Mn(II) concentration was 60 mg·L⁻¹. Itokawa et al. (2001) reported that limited availability of the electron donor could cause N₂O accumulation. In this sense, there may be more chance of N₂O accumulation in the MBBR with a low concentration of Mn(II). The results indicated that the MBBR shows good superiority in practical applications due to its negligible N₂O emissions.

The MBBR performance under optimum conditions

As shown above, the optimum conditions for nitrate removal were initial Mn(II) concentration of 80 mg·L⁻¹, HRT of 10 h, and pH of 7, and these conditions were used to carry out the following experiment. As shown in Figure 3(d), the average nitrate removal and Mn(II) oxidation efficiency of the MBBR were not stable during the first 4 days of the experiment due to the MBBR being in the start-up stage. After the fourth day, the nitrate removal and Mn(II) oxidation efficiency were 100.00% (1.59 mg·L⁻¹·h⁻¹) and 78.50% (6.30 mg·L⁻¹·h⁻¹), which are higher than in the other periods.

Pyrosequencing analysis of bacterial community structures

The bacterial community structure varied when different Mn(II) concentrations were applied to the MBBR. In this study, the samples were collected at initial Mn(II) concentrations of 80 mg·L⁻¹ (FM1), 60 mg·L⁻¹ (FM2), 40 mg·L⁻¹ (FM3) and 80 mg·L⁻¹ (control group FM4).

Altogether 19 bacterial phyla were detected in FM1, FM2 and FM3, and only 14 bacterial phyla were detected in FM4. These results indicate that bacteria could tolerate and grow at concentrations as high as 80 mg·L⁻¹. The results in Figure 4(a) show that Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Chloroflexi played a major role in the MBBR, and phylum Proteobacteria was the most dominant community in the MBBR. Comparing the relative abundance of Proteobacteria in FM1, FM2, and FM3 indicates that the Proteobacteria were accustomed to the high Mn(II) concentration (80 mg·L⁻¹) and contributed to nitrate removal. Sun et al. (2017) also indicated that Proteobacteria were the main denitrification bacteria.

At the molecular level, 16S rRNA sequences belonging to 43 classes were identified in the biofilm of the MBBR. As shown in Figure 4(b), the major classes of the four samples were Gammaproteobacteria, Actinobacteria, Clostridia and Bacilli. According to our previous study, H-117 belongs to Gammaproteobacteria class, and the sum abundances of Gammaproteobacteria in samples FM1, FM2, FM3 and FM4 were 38.54%, 29.66%, 27.11%, and 18.46%, respectively. These results indicate that the relative abundance of Gammaproteobacteria increased as the Mn(II) concentration increased. It was shown above that the nitrate removal and Mn(II) oxidation ratio increased when the Mn(II) concentration increased, which suggests that class Gammaproteobacteria was important and the dominant community for nitrate removal and Mn(II) oxidation in the MBBR. Other studies have also indicated that Gammaproteobacteria play a vital role in the denitrification process and perform with nitrate removal abilities in a CBSAD reactor (Chen et al. 2018). Meanwhile, Pseudomonas was present in the Gammaproteobacteria, which is known to be an Mn(II) oxidizer and detected in oligotrophic environments (Cao et al. 2015).

The microbial community heatmap (Figure 4(c)) shows the similarity and abundances of the four samples; Pseudomonas (6.40%) was the most abundant genus in FM1, and FM2, FM3, FM4 showed different relative abundances of Pseudomonas (4.96% in FM2, 4.25% in FM3 and 1.57% in FM4). It could be concluded that Pseudomonas showed an increasing trend when the Mn(II) concentration was increased. Meanwhile, as the most dominant genus in the MBBR, Pseudomonas may make a significant contribution.
Figure 4  | Taxonomic classification of bacterial 16S rRNA gene reads at (a) phylum level and (b) class level of samples; (c) genus levels FM1–FM4.
to nitrate removal and Mn(II) oxidation in the water treatment process. Villalobos et al. (2005) reported that Pseudomonas has the ability to oxidize Mn(II). Diep et al. (2009) reported that Pseudomonas can be used to remove nitrate from wastewater. Most of the iron-oxidizing bacteria detected in the biofilter, such as Pseudomonas, Crenothrix, Gallionella, and Bacillus, had the ability to oxidize manganese (Cheng et al. 2017). Acinetobacter, Chryseobacterium and Rhodococcus were also detected in the four samples and these bacteria also play important roles in the process of nitrate removal and Mn(II) oxidation.

CONCLUSIONS

The Fe₃O₄@Cu/PVA biomaterials inoculated with the nitrate-removing and Mn(II)-oxidizing bacteria Pseudomonas sp. H-117 effectively removed nitrate and oxidized Mn(II) under anaerobic conditions. Fe₃O₄@Cu/PVA-immobilized cells were used for nitrate removal in the MBBR, and the highest nitrate removal efficiency conditions were an initial Mn(II) concentration of 80 mg·L⁻¹, HRT of 10 h, and pH 7. Meteorological chromatography analysis suggested that N₂ and NO₃ levels varied depending on the concentration of Mn(II). High-throughput sequencing suggested that Pseudomonas sp. H-117 was the dominant contributor for effective removal of nitrate and oxidation of Mn(II) in the MBBR.

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