

## Nitrate removal during Fe(III) bio-reduction in microbial-mediated iron redox cycling systems

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### ABSTRACT

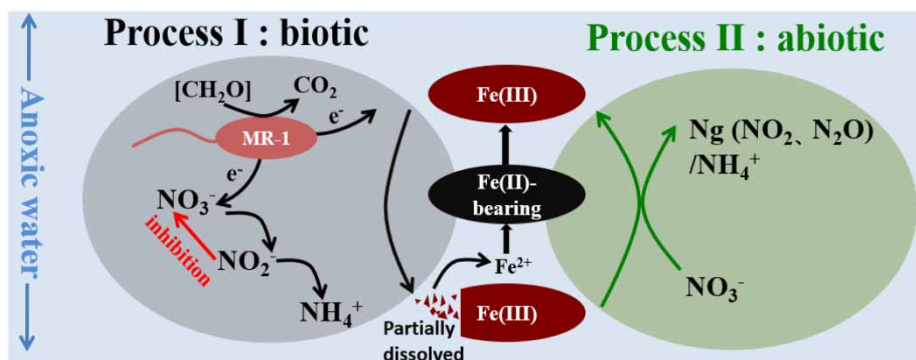
Fe(III) bio-reduction provides a prospect of applying the iron redox cycle to nitrate remediation in the aquatic environment. The objective of this study was to realize multiple nitrate removals in the system containing *Shewanella oneidensis* MR-1 (*S. oneidensis* MR-1) and ferrihydrite or magnetite. The results showed that with three periods of 30 mg·L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N addition, all nitrate reduction was completed within 170 h. In the first period (0–30 h) of nitrate addition, the main contribution of nitrate removal was due to the biological reduction process by *S. oneidensis* MR-1, accompanied by the reduction of Fe(III). During the second (45–90 h) and third periods (100–170 h) of nitrate addition, oxidation of biogenic Fe(II) coupled with the reduction of nitrate took place. This redox reaction resulted in the production of gaseous nitrogen of 47.33% and 16.8% for ferrihydrite/*S. oneidensis* MR-1 and magnetite/*S. oneidensis* MR-1 systems, respectively. In addition, nitrite, as an intermediate product, accumulated and negatively affected nitrate removal after the third addition of nitrate. By comparing the patterns of X-ray diffraction of the iron minerals before and after the bio-reduction, it was found that ferrihydrite was transformed into magnetite, while magnetite kept its original crystal form.

**Key words:** biogenic Fe(II), denitrification, gaseous nitrogen, iron minerals, iron-reducing bacteria

### HIGHLIGHTS

- Low-concentration nitrate did not inhibit the bio-reduction of Fe(III).
- Nitrate was removed continuously in a microbial Fe redox cycle.
- Abiotic reduction with biogenic Fe(II) was the sole process to remove TN.
- Nitrite accumulated might have a negative effect on nitrate removal.

### GRAPHICAL ABSTRACT



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## 1. INTRODUCTION

Nitrogen is an indispensable nutrient in the process of human survival. However, extensive application of nitrogen fertilizers, leakage of landfill leachate and septic tanks can lead to the accumulation of nitrate in surface water and groundwater, causing a series of environmental and health issues (Howarth 2004). Remediation of nitrate contaminated water becomes a significant environmental challenge worldwide (Wu 2020). Among the numerous remediation techniques, bioremediation is a process of removing nitrate through microbial metabolisms, commonly known as denitrification. However, the lack of available organic carbon is recognized as the largest hindrance to denitrification (Cao & Zhou 2019). By comparison, chemical reductions such as those facilitated with zero-valent iron ( $\text{Fe}^0$ ) have been widely applied due to the strong reduction ability (Park *et al.* 2009). However, a previous study found that ammonium ( $\text{NH}_4^+$ ) was the end-product of the nitrate reduction by  $\text{Fe}^0$  (Suzuki *et al.* 2012), making it an incomplete nitrogen removal process. It there is an urgent need to develop an environment friendly, sustainable and effective method for removing nitrate.

Fe(II), which is both reductive and oxidative, plays an important role in the removal of pollutants, and is easily oxidized (Melton *et al.* 2014). Moreover, in nature, iron mainly exists in the form of different iron minerals, among which ferrihydrite and magnetite are the typical representatives of weakly crystalline and strongly crystalline iron minerals, respectively. The application of dissimilatory iron reduction bacteria (DIRB) can transform structured Fe(III) into Fe(II) and improve the utilization rate of iron minerals (Kappler *et al.* 2021). Knowledge of microbial-mediated Fe(III) reduction process has grown over the past few decades, and is mostly a process of reducing Fe(III) to Fe(II) coupled with organic compound oxidation under anoxic conditions, driven by DIRB (Pan *et al.* 2019). During dissimilatory iron reduction, DIRB solubilise Fe(III) oxides as Fe(II), known as biogenic Fe(II) (Roden & Urrutia 2002). With the reduction of Fe(II), sulfide, heavy metals and organic pollutants can be reduced to restore water (Melton *et al.* 2014). They can also be used to form vivianite combined with phosphate and to generate high economic profits (Wang *et al.* 2018). Therefore, the iron bio-reduction process has promising potential for nitrate contaminated water remediation.

Among these iron-reducing bacteria, *Geobacter* sp. and *Shewanella* sp. have been widely used (Lovley *et al.* 2004). The iron bio-reduction process is relatively slow, so it is necessary to add electron shuttles to accelerate the occurrence of the iron bio-reduction process (Zhou *et al.* 2016; Yang *et al.* 2019). It was also found that *S. oneidensis* MR-1 bacteria themselves could secrete electron shuttles (Marsili *et al.* 2008; Peretyazhko *et al.* 2010). *S. oneidensis* MR-1 could use Fe(III) and nitrate as electron acceptors, while high-concentration nitrate inhibited the growth of *S. oneidensis* MR-1 (Myers & Nealson 1990). Li and his colleagues also found that high concentrations of nitrate as electron acceptors could inhibit the bio-reduction of iron minerals (Li *et al.* 2015). The DIRB/nitrate-dependent Fe(II)-oxidizing coculture system could undergo three redox cycles during a period of 220 days with nontronite NAu-2 (total Fe content of NAu-2 is 24%, of which 99.4% is Fe(III)) and biogenic Fe(II) serving as the electron acceptor and donor, respectively (Zhao *et al.* 2015). This result indicated that the biotic process achieved multiple removal cycles over an extended period of time. Our previous work showed that microbial-mediated multiple iron redox cycles played an important role in nitrite removal with a low nitrite concentration of  $30 \text{ mg}\cdot\text{L}^{-1}$  (Lu *et al.* 2017). However, the above-mentioned studies mainly focused on nitrite through denitrification by microbial-mediated iron reduction (Lu *et al.* 2020), while few studies have been carried out on nitrate. Additionally, in nitrate-rich systems, the intermediate product nitrite would be produced, which inhibits microbial activity and negatively affects the removal of nitrate (Cooper *et al.* 2003). The removal of nitrite by *S. oneidensis* MR-1-mediated iron mineral reduction is in the early stages of being studied. Within 180 h, both the systems of *S. oneidensis* MR-1 with ferrihydrite and *S. oneidensis* MR-1 with magnetite could complete at least four and six cycles, respectively (Lu *et al.* 2017). Therefore, it would be expected to remove nitrate in an iron minerals system containing iron-reducing bacteria based on the iron recycling process. However, nitrite can react with both biogenic Fe(II) and non-biological  $\text{Fe}^{2+}$ , but nitrate can react with biogenic Fe(II) (Melton *et al.* 2014; Kappler *et al.* 2021). The questions of nitrate removal by iron reduction processes, then, are: (1) whether the mechanism of nitrate removal through iron minerals reduction mediated by *S. oneidensis* MR-1 is the same as that of nitrite removal, and (2) how many iron cycles can be achieved within 180 h. Understanding the mechanisms of nitrate removal would also be helpful in improving the application prospect of denitrification by iron minerals. Therefore, it is important to investigate the role of biogenic Fe(II) in nitrate removal. The objectives of this study, were to investigate: (1) the biological and abiotic processes of nitrate removal, (2) the nitrogen transformation pathway and the iron minerals transformation, and (3) the mechanisms underlying nitrate and nitrite removal in the iron mineral reduction process mediated by *S. oneidensis* MR-1.

## 2. MATERIALS AND METHODS

### 2.1. Minerals and cultures preparation

In this study, iron oxide ferrihydrite or magnetite was used for nitrate removal. Briefly, ferrihydrite was synthesized by neutralization of the  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  ( $n_{\text{Fe}^{2+}} = 0.4 \text{ M}$ ) solution with  $\text{NaOH}$  ( $n_{\text{OH}^-} = 1 \text{ M}$ ), for which the final pH was 7.0, and the above solution was centrifuged (4,000 rpm, 10 min), then the supernatant was removed and washing with deionized water was repeated two or three times (Lovley & Phillips 1986). Magnetite was prepared by the co-precipitation method of  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  ( $n_{\text{Fe}^{2+}} = 0.05 \text{ M}$ ) and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  ( $n_{\text{Fe}^{2+}} = 0.1 \text{ M}$ ), and dissolved under vigorous stirring at  $80^\circ\text{C}$  under a nitrogen atmosphere. After that,  $\text{NH}_4\text{OH}$  (25 wt.%) was added and continuously stirred for 30 min, then centrifuged (10,000 rpm, 10 min) and washed three or four times with deionized water (Hu *et al.* 2014).

*S. oneidensis* MR-1, as the model bacteria for DIRB, was provided by the Marine Culture Collection of China (MCCC). The cells were incubated in Luria-Bertani medium ( $5 \text{ g} \cdot \text{L}^{-1}$  NaCl,  $10 \text{ g} \cdot \text{L}^{-1}$  tryptone,  $5 \text{ g} \cdot \text{L}^{-1}$  yeast extract) at  $30 \pm 0.5^\circ\text{C}$  in a dark, constant temperature, incubator shaker. Bacteria were collected by centrifugation at 10,000 rpm for 10 min when the cell growth reached the mid-log phase (around 18 h). After washing with 0.9% NaCl three times, and diluted with 0.9% NaCl, a cell suspension was obtained (Xu *et al.* 2014). The final concentration of *S. oneidensis* MR-1 bacteria was about  $5 \times 10^7$  cells  $\cdot \text{mL}^{-1}$  in all experimental systems in this study. The procedure for the experimental process is shown in Figure 1. A series of batch experiments was set up as follows with iron minerals and *S. oneidensis* MR-1 bacteria.

### 2.2. Batch experiments

All the experimental systems were established in 100 mL anaerobic serum bottles, and the volume of the final culture system was 80 mL. Lactate ( $\text{C}_3\text{H}_5\text{O}_3\text{Na}$ ) 50 mM was added as the electron donor, ferrihydrite or magnetite ( $2,500 \text{ mg} \cdot \text{L}^{-1}$  total Fe) was added as the electron acceptor. In addition, sodium anthraquinone-2,6-disulfonate (AQDS,  $100 \mu\text{M}$ ) was added as the electron shuttle to enhance the effects on biotic reduction. For studying the removal of nitrate of different concentrations, the concentration of  $\text{NO}_3^- \text{-N}$  was controlled at 30, 70, 140, and  $280 \text{ mg} \cdot \text{L}^{-1}$ . In the iron minerals-only system, the volume of the system was adjusted to 80 mL with a mineral medium ( $5.85 \text{ g} \cdot \text{L}^{-1}$  NaCl,  $0.67 \text{ g} \cdot \text{L}^{-1}$   $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ,  $0.3 \text{ g} \cdot \text{L}^{-1}$  NaOH,  $0.097 \text{ g} \cdot \text{L}^{-1}$  KCl and  $1 \text{ mL} \cdot \text{L}^{-1}$  of trace elements solution (Lu *et al.* 2017)). In the culture system for iron minerals and microorganisms, the culture system was adjusted to 40 mL with the same mineral medium, before 40 mL of cell suspension was added. All systems were then capped with a rubber stopper after a 5-min anaerobic treatment (99.99% He gas), then incubated in a thermostatic shaker (100 rpm) and maintained at  $30 \pm 0.5^\circ\text{C}$  in the dark. The final pH was 6.8. In the iron cycle experiment, the method of system establishment was the same as above. The timings of the  $30 \text{ mg} \cdot \text{L}^{-1}$  nitrate addition were 0, 45 and 100 h. The first and second additions of nitrate were termed the first period and the operational period, respectively. All materials except iron minerals were autoclaved at  $121^\circ\text{C}$  for 30 min before use. Each treatment in this study had three replicates.

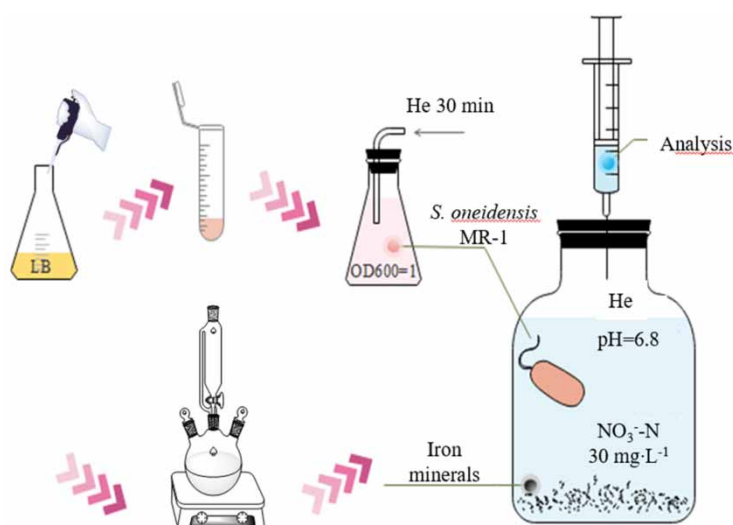


Figure 1 | Flow diagram of experiment.

### 2.3. Analytical methods

The reaction mixtures were sampled by syringes and filtered through 0.22  $\mu\text{m}$  PTFE (polytetrafluoroethylene) filters to remove iron oxides and organic components. Standard methods were used to measure  $\text{NO}_2^-$ -N,  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N (Peretyazhko *et al.* 2010; Lu *et al.* 2017). The biogenic Fe(II) produced by the iron reduction process contained various forms, not only dissolved, but also colloidal, particle, adsorption, etc. Different types of Fe(II) particle size were present. In the measurement for Fe(II), the filter head was used to filter the organic matter to not affect the measurement of Fe(II). The extraction process could decompose Fe(II) with large particle size into small Fe(II), reducing the measurement error of Fe(II) concentration (Peretyazhko *et al.* 2010), as determined by the ferrozine method. Here, a 1 mL aliquot was extracted for 1.0 h in 6 M HCl to dissolve the insoluble iron oxide complete.

For gaseous nitrogen measurements, the air from the headspace of the serum bottle was detected by gas chromatography (GC 2030 plus, Shimadzu, Japan) with an SH-Rtx-5 column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ) and an FID detector. The operation conditions included an oven temperature of 60  $^\circ\text{C}$  and an FID temperature of 330  $^\circ\text{C}$ . The external standard method was used to quantify gas concentrations. The peak was  $\text{CO}_2$  in 2.40 min,  $\text{N}_2\text{O}$  in 3 min and  $\text{NO}_2$  in 1.40 min. The change of ferrihydrite or magnetite during redox cycling of nitrate was analyzed from their X-ray diffraction (XRD) patterns, which were recorded on a Rigaku D/max RBX X-ray diffractometer with Cu-K $\alpha$  radiation ( $\lambda = 0.154$  nm) and a scanning rate of 8 $^\circ\cdot\text{min}^{-1}$  in the  $2\theta$  range of 5–80 $^\circ$ . After each experimental run, the serum bottles were allowed to settle for 10 min. The supernatant was then gently poured into a 50 mL sterile centrifuge tube for high-speed centrifugation (10,000 rpm, 10 min). The supernatant was discarded and the bacterial precipitate was collected. The activity of the cells was then observed through a laser confocal microscope (Olympus FV1200).

The data were statistically analyzed using Origin (version 9.0, Massachusetts, OriginLab, USA) and results were expressed as the mean  $\pm$  standard deviation (SD). Analysis of variance (ANOVA) with Duncan test was performed at the significance level of  $p < 0.05$  using SPSS (version 18.0, Chicago, Illinois, USA). The removal rate of nitrate and the proportion of different kinds of nitrogen compounds in the transformation products of nitrate are shown in Equations (1) and (2), respectively.

$$\text{Nitrate removal rate (\%)} = \frac{(C_0 - C) \times 100}{C_0} \quad (1)$$

$$\text{Content proportion (\%)} = \frac{C_t \times 100}{(C_0 - C)} \quad (2)$$

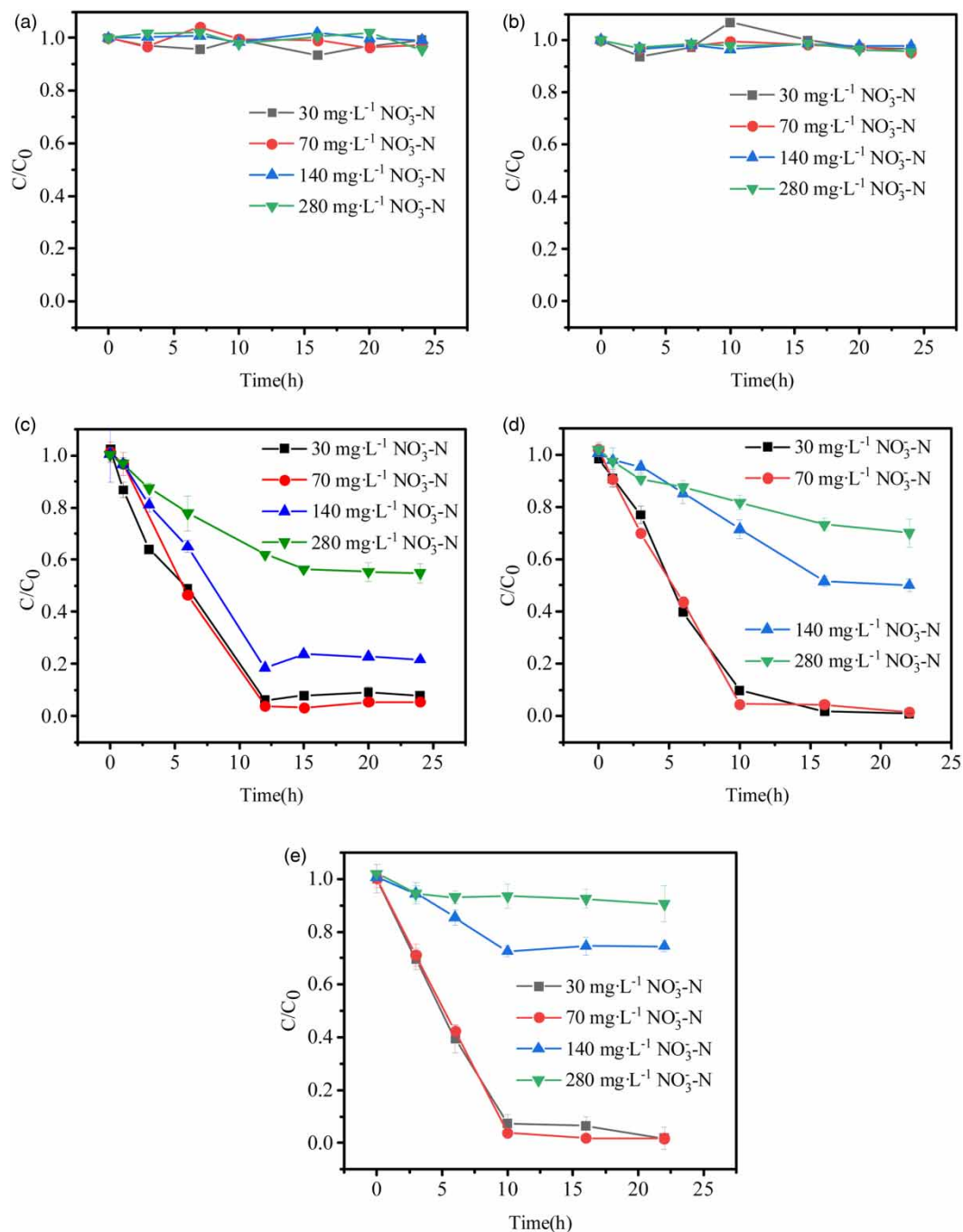
in which,  $C_0$  is the initial concentration of nitrate ( $\text{mg}\cdot\text{L}^{-1}$ );  $C$  is the concentration of nitrate in the reaction process ( $\text{mg}\cdot\text{L}^{-1}$ ); and  $C_t$  is the concentration of different nitrogen compounds ( $\text{mg}\cdot\text{L}^{-1}$ ).

## 3. RESULTS AND DISCUSSION

### 3.1. Comparison of nitrate removal with or without iron oxides

No significant nitrate reduction was observed in the control systems only with ferrihydrite or magnetite (Figure 2(a) and 2(b)), illustrating that iron minerals had no reductive activity for nitrate (Zhu *et al.* 2014). When *S. oneidensis* MR-1 bacteria incubated with nitrate and lactate, 90% of  $\text{NO}_3^-$ -N was reduced in 12 h with initial concentrations of 30  $\text{mg}\cdot\text{L}^{-1}$  and 70  $\text{mg}\cdot\text{L}^{-1}$  (Figure 2(c)), indicating that nitrate can be used as electron acceptor for the growth of *S. oneidensis* MR-1 bacteria (Lovley *et al.* 2004). Inhibition of microbial activities could be detected with the increase in  $\text{NO}_3^-$ -N concentration (Figure 2(c)). This was likely to be because nitrate could enter the periplasmic nitrate reductase system (NAP) located in *S. oneidensis* MR-1 cells and reduced nitrate to nitrite, which was then reduced to ammonium by the nitrite reductase system (NRF) (Cruz-Garcia *et al.* 2007; Simpson *et al.* 2010). During this process, nitrite accumulated, and the toxicity of nitrite inhibited the biological activity of the cells (Li *et al.* 2015).

In the presence of both iron oxide (either ferrihydrite or magnetite) and *S. oneidensis* MR-1 bacteria, reduction of nitrate with different dosages was detected (Figure 2(d) and 2(e)). In both systems, the removal efficiency of  $\text{NO}_3^-$ -N was increased to more than 95% within 10 h at initial concentrations of 30  $\text{mg}\cdot\text{L}^{-1}$  and 70  $\text{mg}\cdot\text{L}^{-1}$ . When  $\text{NO}_3^-$ -N concentration was 30  $\text{mg}\cdot\text{L}^{-1}$ , the final removal efficiencies of  $\text{NO}_3^-$ -N were 99% and 98.20% in the system for *S. oneidensis* MR-1 bacteria with ferrihydrite and magnetite, respectively. Compared to the removal efficiency of nitrate (92.25%) in a single microbial



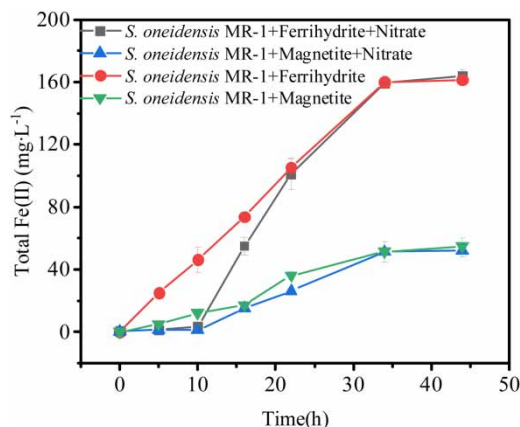
**Figure 2** | Reduction of 30, 70, 140, and 280  $\text{mg}\cdot\text{L}^{-1}$  of  $\text{NO}_3^-$ -N in setups containing ferrihydrite (a), magnetite (b), *S. oneidensis* MR-1 (c), *S. oneidensis* MR-1 incubated with magnetite (d), and *S. oneidensis* MR-1 incubated with ferrihydrite (e).  $C/C_0$  represents the ratio of the reduced  $\text{NO}_3^-$ -N to the initial concentration of  $\text{NO}_3^-$ -N added.

system (Figure 2(c)), the removal efficiency was indeed improved, suggesting that the addition of iron minerals greatly participated in the nitrate removal process (Zhu *et al.* 2013).

### 3.2. Formation of biogenic Fe(II) during the Fe(III) bio-reduction process

Biogenic Fe(II) accumulation synchronized with Fe(III) reduction. To evaluate the influence of nitrate on *S. oneidensis* MR-1 reduced iron minerals, a set of experiments of Fe(III) bio-reduction in the presence or absence of nitrate was performed (Figure 3). Here, 30  $\text{mg}\cdot\text{L}^{-1}$   $\text{NO}_3^-$ -N was added in the experiments for the purpose of achieving high nitrate removal efficiency



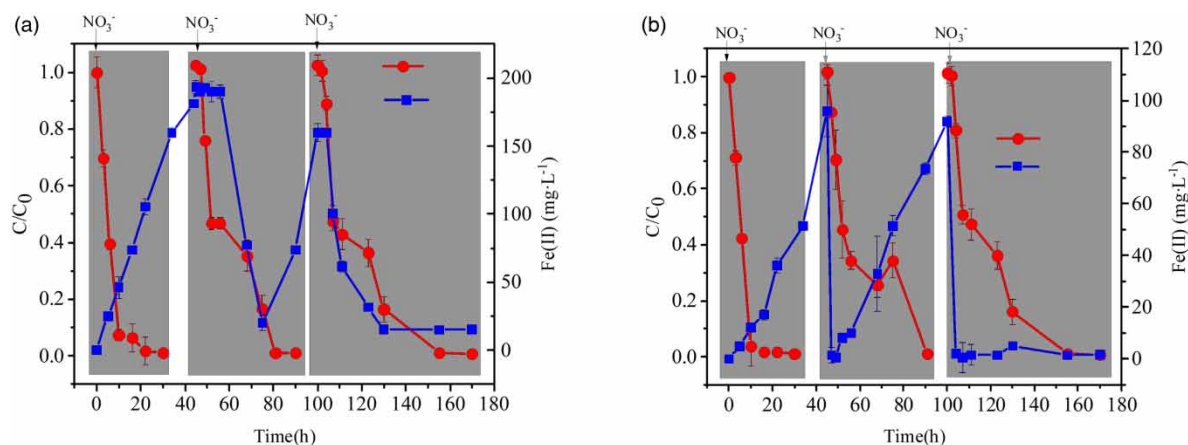


**Figure 3** | The accumulation of total Fe(II) in setups containing *S. oneidensis* MR-1 incubated with iron minerals (with or without adding nitrate) with incubation time.

with a low initial concentration and being consistent with the previous study for nitrite (Lu *et al.* 2017). The average rates of biogenic Fe(II) formation were  $4.70 \pm 0.12$  ( $\text{mg}\cdot\text{L}^{-1}$ )  $\text{h}^{-1}$  for ferrihydrite and  $1.51 \pm 0.01$  ( $\text{mg}\cdot\text{L}^{-1}$ )  $\text{h}^{-1}$  for magnetite within 34 h. In the system containing ferrihydrite and *S. oneidensis* MR-1 bacteria, biogenic Fe(II) concentration was about three times higher than that from magnetite bio-reduction, indicating that the difference in particle size and crystallinity can influence the extent of Fe(III) bio-reduction (Xie *et al.* 2017). It was noted that, in the presence of  $\text{NO}_3^-$ -N, the reduction of both ferrihydrite and magnetite, as mediated by *S. oneidensis* MR-1 bacteria, hardly occurred in the first 10 h. This was attributable to the preferred reduction of  $\text{NO}_3^-$ -N by *S. oneidensis* MR-1 bacteria over Fe(III) (Lovley *et al.* 2004). However, in both cases the concentration of biogenic Fe(II) gradually increased after 10 h and reached the same maximum and at about the same time as before the addition of nitrate. These results showed that the addition of  $\text{NO}_3^-$ -N was able to delay the onset of biogenic Fe(II) formation, but did not prevent the system from reaching the same final Fe(II) concentration or in a same length of time. In other words, there was no inhibition effect observed for  $\text{NO}_3^-$ -N on Fe(III) bio-reduction in this work.

### 3.3. Nitrate removal in multiple redox cycles of Fe(III) bio-reduction

In the system for iron oxide (either ferrihydrite or magnetite) associated with *S. oneidensis* MR-1 bacteria, three periods of nitrate reduction were completed within 170 h (Figure 4). During the first period in the first 30 h, biogenic Fe(II) was generated gradually in both systems accompanied by the reduction of nitrate. The Fe(II) concentration reached its maximum at 45 h at  $193.47 \pm 4.73$   $\text{mg}\cdot\text{L}^{-1}$  and  $95.93 \pm 10.09$   $\text{mg}\cdot\text{L}^{-1}$  for the ferrihydrite and magnetite systems, respectively. In the second period, the oxidation of Fe(II) to Fe(III) coupled with the reduction of  $\text{NO}_3^-$  commenced, followed by a gradual reduction in Fe(III) by *S. oneidensis* MR-1 bacteria that resulted in a relatively high concentration of Fe(II) before the

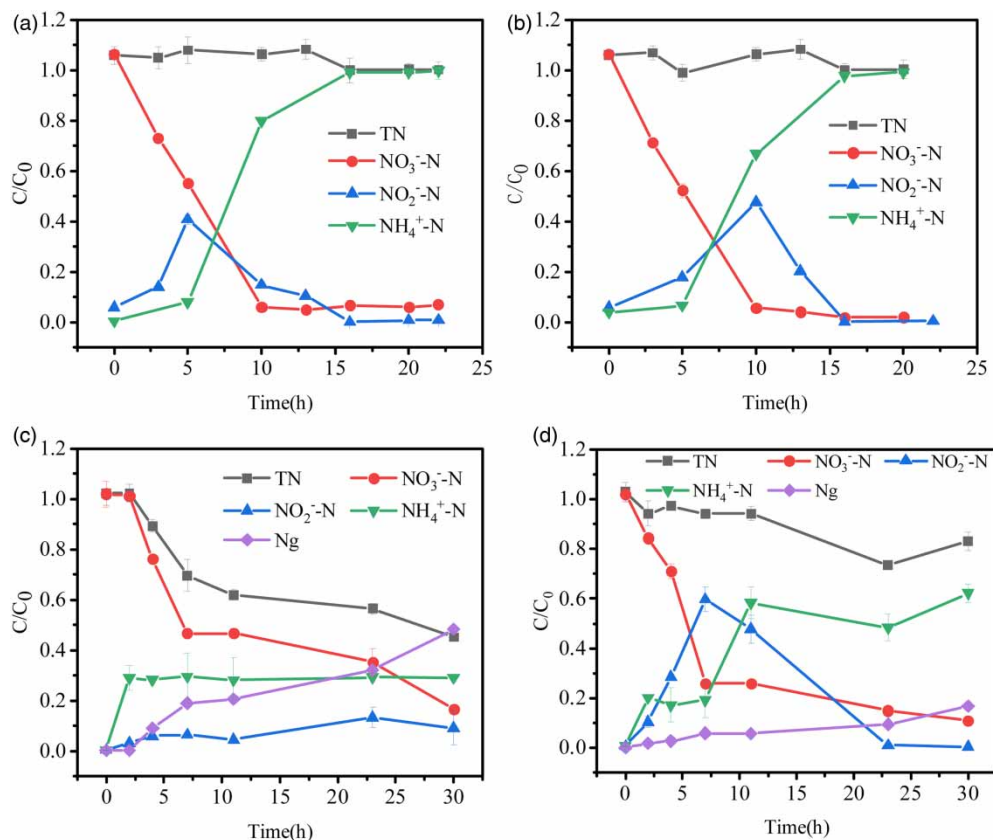


**Figure 4** | Time-course changes for  $\text{NO}_3^-$ -N and Fe(II) through multiple redox cycles associated with ferrihydrite (a) and magnetite (b) reduction by *S. oneidensis* MR-1. The arrows indicate the addition of nitrate ( $30 \text{ mg}\cdot\text{L}^{-1}$ ).

third period started. By comparison, in the control experiments, the reduction of  $\text{NO}_3^-$  by *S. oneidensis* MR-1 bacteria took more than 110 h during the second period (Fig. S1). In contrast, nitrate was removed three times during the biotic process in 220 days in the coculture system of DIRB/nitrate-dependent Fe(II)-oxidization, which had a long removal cycle (Zhao *et al.* 2015). However, after the last nitrate addition at the beginning (4 h) of the third period, the rate of nitrate removal was significantly decreased. The removal rates of nitrate in the three stages were  $3.61 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ,  $1.98 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ , and  $1.01 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$  in the ferrihydrite system, respectively, and  $4.13 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ,  $2.36 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ , and  $1.53 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$  in the magnetite system. Furthermore, it was noted that dissimilatory iron reduction by *S. oneidensis* MR-1 bacteria was not observed after three periods of  $\text{NO}_3^-$  removal, similarly shown by previous studies (Zhao *et al.* 2015). In comparison with the previous work on continuous nitrite removal (Lu *et al.* 2017), the number of periods for nitrate removal under the same conditions decreased. A possible reason was the accumulation of nitrite as an intermediate product of nitrate reduction. The bio-toxicity of nitrite, combined with cell encrustation caused by the abiotic reaction of Fe(II) and nitrite, may have resulted in cell death or non-growth of the *S. oneidensis* MR-1 bacteria population (Zhao *et al.* 2013).

### 3.4. Contribution of biotic and abiotic reductions for nitrate removal

To investigate the role of biotic and abiotic processes in nitrogen transformation, reductive transformation of  $\text{NO}_3^-$ -N in both iron oxide/*S. oneidensis* MR-1 bacteria systems during the first and second nitrate reduction periods was analyzed (Figure 5). In the first period, the  $\text{NO}_3^-$ -N removal efficiencies were 94.4% for ferrihydrite and 98.1% for magnetite, while 99% of the final product almost was  $\text{NH}_4^+$ -N, similar to the results in the control experiment with only the microbe present (Table 1). Notably,  $\text{NO}_2^-$ -N accumulated as an intermediate at the beginning of the reaction (Figure 5(a) and 5(b)), indicating that the reduction pathway from  $\text{NO}_3^-$ -N to  $\text{NH}_4^+$ -N by *S. oneidensis* MR-1 bacteria was a stepwise process with  $\text{NO}_2^-$ -N as an intermediate (Yoon *et al.* 2015). Upon the second addition of nitrate, biogenic Fe(II) produced from the first addition reacted with both of the newly added  $\text{NO}_3^-$ -N and  $\text{NO}_2^-$ -N as an intermediate (Figure 5(c) and 5(d)).



**Figure 5** | Reductive transformation of  $\text{NO}_3^-$ -N in '*S. oneidensis* MR-1/ferrihydrite' (a) and '*S. oneidensis* MR-1/magnetite' (b) systems during the first cycle and both of them underwent the second cycle for (c) and (d), respectively. Ng is the sum of  $\text{N}_2\text{O}$  and  $\text{NO}_2$ , TN is the sum of  $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N,  $\text{NH}_4^+$ -N and Ng.

**Table 1** | Content of various types of nitrogen (% of total N) after nitrate reduction by *S. oneidensis* MR-1 only and in the ferrihydrite- and magnetite-associated *S. oneidensis* MR-1 systems at the end of the first (1st) and second (2nd) nitrate addition periods

System	$C_t/C_0$ (%)				
	$\text{NO}_3^- \text{-N}$	$\text{NH}_4^+ \text{-N}$	$\text{NO}_2^- \text{-N}$	$\text{N}_2\text{O}$	$\text{NO}_2$
<i>S. oneidensis</i> MR – 1	3.11	96.12	0.77	ND <sup>a</sup>	ND
Ferrihydrite/ <i>S. oneidensis</i> MR – 1 (1st)	5.64	93.56	0.80	ND	ND
Magnetite/ <i>S. oneidensis</i> MR – 1 (1st)	1.93	97.57	0.50	ND	ND
Ferrihydrite/ <i>S. oneidensis</i> MR – 1 (2nd)	14.44	29.15	9.08	25.14	22.19
Magnetite/ <i>S. oneidensis</i> MR – 1 (2nd)	10.94	62.15	10.11	10.13	6.67

<sup>a</sup>ND means not detected.

As shown in Table 1, in the ferrihydrite/*S. oneidensis* MR-1 bacteria system, 85.6%  $\text{NO}_3^- \text{-N}$  was removed and gaseous nitrogen ( $\text{N}_g$ ) emission was 47.3% of the initial total N in the second period. The  $\text{N}_g$  emission contained 25.1%  $\text{N}_2\text{O}$  and 22.2%  $\text{NO}_2$  according to GC analysis (Fig. S2). In comparison,  $\text{NO}_3^- \text{-N}$  removal efficiency was 89.1% and yield of  $\text{N}_g$  was 16.8% (contained 10.1%  $\text{N}_2\text{O}$  and 6.67%  $\text{NO}_2$ ) in the magnetite/*S. oneidensis* MR-1 bacteria system. Moreover, both systems contained around 10% of  $\text{NO}_2^- \text{-N}$ , which might have brought negative effects on  $\text{NO}_3^- \text{-N}$  removal. Given that total nitrogen in solution in the first period remained the same, indicating no gaseous nitrogen production, it was reasonable to believe abiotic nitrate/nitrite reduction by biogenic Fe(II) contributed to all the gaseous nitrogen production in the second nitrate addition period.

In the first period, both nitrate and Fe(III) can be used as electron acceptor for the growth of DIRB.  $\text{NH}_4^+ \text{-N}$  and biogenic Fe(II) gradually formed as the product of microbial nitrate and Fe(III) reduction, respectively. These two reactions were biotic processes. Microbial-mediated Fe(III) reduction is the key for the start-up of redox cycle in the first period. It can be seen from Fig. S3 that  $\text{Fe}^{2+}$  prepared by dissolution of  $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ , did not react abiotically with nitrate. Therefore, biogenic Fe(II) generated from the biotic process played a dominant role in nitrogen removal from the system.

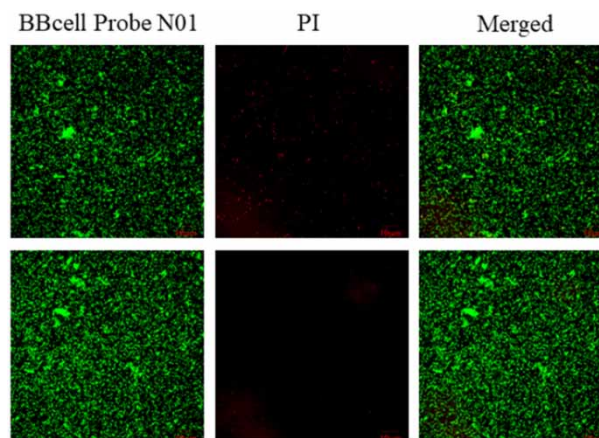
### 3.5. Mineral conversion

Change of ferrihydrite and magnetite during redox cycling of nitrate was measured by XRD (Fig. S4). The XRD pattern after 170 h reaction of ferrihydrite shows the characteristic peak of magnetite. The results demonstrated that ferrihydrite is gradually converted into magnetite during the process of microbial-mediated Fe(III) reduction. Magnetite, on the other hand, remained structurally consistent throughout. Fe(II) obtained by the reduction of structural state Fe(III) by iron-reducing bacteria would undergo a series of complex reactions with iron (hydrogen) oxides, including adsorption, electron transfer, reduction dissolution, atomic exchange and crystal phase recombination, thus transformed to secondary minerals (Zachara *et al.* 2002). The biogenic Fe(II) was oxidized with the added  $\text{NO}_3^- \text{-N}$ , thus completing one iron redox cycle. Therefore, it was the biotic reduction of Fe(III) that started the iron redox cycle. Reaction equations of these redox cycles were consistent with the previously published research (Lu *et al.* 2017). As a whole, both iron minerals showed excellent cycling.

### 3.6. Relationship and difference between nitrate and nitrite

*S. oneidensis* MR-1 can use nitrate and nitrite as its electron acceptor, and the product is  $\text{NH}_4^+ \text{-N}$  (Cruz-Garcia *et al.* 2007; Simpson *et al.* 2010). In nitrate removal by biotic Fe(II), however, it is necessary to first convert nitrate into nitrite (Cooper *et al.* 2003). That will lead to the accumulation of part of the nitrite produced, and a longer removal time for nitrate than nitrite. Using the same experimental system, we previously demonstrated that a reduction in iron could still be observed when the reaction time was 180 h, which indicated that biological reduction was still in progress (Lu *et al.* 2017). In the current experiment, staining of living and dead cells showed a large number of active microorganisms within 72 h after the end of the experiment (Figure 6). After the third addition of nitrate, no reduction of iron minerals was observed, indicating that the accumulation of nitrite inhibited the activity of *S. oneidensis* MR-1. A high  $\text{NH}_4^+ \text{-N}$  concentration will also inhibit the activity of microorganisms (Zhang *et al.* 2020). Therefore, the effective removal of nitrate, like nitrite, depends on the presence of biogenic Fe(II) but, in the cycling system, the accumulation of nitrite and  $\text{NH}_4^+ \text{-N}$  inhibits the activity of *S. oneidensis* MR-1, thus hindering iron reduction. Although the removal of nitrate was different from that of nitrite, after the iron reduction process, ferrihydrite was transformed into magnetite, and magnetite still maintained its own crystal structure.





**Figure 6** | Staining images of *S. oneidensis* MR-1 after the end of the experimental cycle system. The upper part is the ferrihydrite system and the lower part is the magnetite system.

#### 4. CONCLUSION

In this study, continuous nitrate removal at a low concentration ( $30 \text{ mg}\cdot\text{L}^{-1}$ ) using the iron redox cycle mediated by *S. oneidensis* MR-1 bacteria was investigated. It was found that nitrate was removed continuously. Microbial-mediated Fe(III) reduction was the key for the start-up of the redox cycle. The Fe(II) concentration reached its maxima at 45 h at  $193.47 \pm 4.73 \text{ mg}\cdot\text{L}^{-1}$  and  $95.93 \pm 10.09 \text{ mg}\cdot\text{L}^{-1}$  for the ferrihydrite and magnetite systems, respectively. The removal mechanism of nitrate was the same as that of nitrite, i.e. abiotic oxidization of biogenic Fe(II) coupled with the reduction in  $\text{NO}_3^-/\text{NO}_2^-$  was the sole process responsible for the removal of TN. The difference was that, in the nitrate system, especially after the third addition of nitrate, reduction of iron minerals can no longer be observed due to the accumulation of the intermediate product nitrite from nitrate reduction. However, biogenic Fe(II) produced from the initial microbial-mediated Fe(III) reduction reacted with nitrite to remove it from the system. Time allowed for this reaction may be increased to complete the removal of nitrite before the addition of more nitrate. Overall, the excellent  $\text{Fe}^{3+}/\text{Fe}^{2+}$  cycling of the two iron oxides tested in this study provide a potential application for continuous nitrate removal in the aquatic environment.

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#### DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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