Nitrate reduction in the interactive reaction system of L17 and soil minerals

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Abstract
In this study, the potential for microbially catalyzed NO_3^- reduction with iron oxide was examined using *Klebsiella pneumoniae* strain L17 and four types of iron oxides under anaerobic conditions. The results showed that L17 had the capacity of nitrate reduction, and iron oxides can accelerate the reduction rate significantly. The biogenic Fe(II) contributed to the acceleration slightly, which was not enough to reduce so much nitrogen. To investigate the role of iron oxide for the nitrate reduction, a series of minerals but iron oxides with L17 were combined for nitrate reduction, and the results showed all the oxides can accelerate the reduction rate, indicating that the electron might transfer to nitrate through the oxides but not experiencing iron oxide's reduction. Hence, besides the well-known mechanism: direct microbial reduction and reduction by the biogenic Fe(II), a new mechanism was proposed whereby soil minerals can mediate electron transfer to accelerate the microbial nitrate reduction. This study could be helpful in understanding the relationship between the redox cycles of Fe and N in subsurface sedimentary environments.

Key Words
Nitrogen cycle, iron cycle, electron transfer, denitrification, dissimilatory iron reduction.

Introduction

Nitrogen is an essential element for living organisms, and the availability of a suitable nitrogen source often limits primary productivity in both natural environments and agriculture (Cabello *et al.* 2004). It is well known that the natural nitrate reduction is mainly attributed to the biotic process of direct enzyme catalysis by nitrate reduction bacteria (Gonzalez *et al.* 2006), while various abiotic and biotic-abiotic combined processes have also been reported to be responsible for the natural reduction of nitrate in anoxic environment (Jørgensen *et al.* 2009). Firstly, the biotic processes involve (i) the anaerobic reduction of NO_3^- to N_2O and N_2 (denitrification), (ii) the conversion of NO_3^- into ammonia (dissimilatory ammonification), and (iii) the conversion of nitrate to ammonia, which is used by the cell to incorporate nitrogen into biomolecules (assimilation) (Gonzalez *et al.* 2006). Secondly, it is suggested that ferrous iron as electron donors is capable of reducing nitrate in anaerobic, sedimentary environments (Jørgensen *et al.* 2009). Reduction of nitrate to ammonia can proceed at appreciable rates in abiotic systems in the presence of green rust compounds at circumstance pH (Ottley *et al.* 1997). The presence of crystalline iron oxide (lepidocrocite and goethite) surfaces accelerates low-temperature reduction of NO_3^- coupled to Fe(II) oxidation at pH values greater than 8.0 (Hansen *et al.* 2009). Thirdly, microbially catalyzed nitrate reduction coupled to Fe(II) oxidation under anaerobic environment has also been reported (Straub *et al.* 1996; Weber *et al.* 2001), and the role of biogenic Fe(II) was taken into consideration for nitrogen cycling.

The occurrence of biological Fe(II)-dependent nitrate reduction in a variety of natural systems suggests that this reaction may play a significant role in coupling the redox cycles of Fe and N in sedimentary environments. However, it is also observed that the molar ratios of NO_3^- consumed to Fe(II) oxidized exceeded the theoretical stoichiometry (Straub *et al.* 1996; Weber *et al.* 2001; Nielsen *et al.* 1998). The reason for this disagreement is unclear in these previous studies. Hence, the study was aim at explaining the reason of above disagreement in the simulated system of bacteria/soil mineral/nitrate, and the mechanism will be further discussed.

Methods

Materials

$NaNO_3$ (≥99.0%) were purchased from Sigma-Aldrich without further purification. Other chemicals being of analytical grade were purchased from Guangzhou Chemical Reagent Factory, China. *Klebsiella pneumoniae* strain L17 was a dissimilatory iron reducing bacterium (DIRB), isolated subterranean forest sediment in Zhaoqing, China (Li *et al.* 2009). Goethite (α-FeOOH), Lepidocrocite (γ-FeOOH), hematite (α-Fe_2O_3), and Maghemite (γ-Fe_2O_3) were synthesized according to procedures as previously described (Li *et al.* 2009),

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while Bismuth oxide (Bi_2O_3), Aluminium oxide (Al_2O_3), Neodymium oxide (Nd_2O_3), Zirconium dioxide (ZrO_2), and Titanium dioxide (TiO_2) were purchased from Sigma-Aldrich without further purification.

Experimental procedure

To avoid the interference of other inorganic anions in detection, the anaerobic $NaHCO_3$-buffered (30 mM, pH 6.8, N_2:CO_2 (80:20) atmosphere) medium only contained 7.5 mM $NaNO_3$ or $NaNO_2$ as electron accepter and 5 mM glucose as electron donor, and harvested cells of *K. pneumoniae* L17 were added with final concentration of ca. 10^7 cells/ml. Cells were grown in nutrient broth under aerobic conditions on a rotary shaker at 180 rev/min at 30°C, and harvested by centrifugation at 6,900 × *g* for 10 min at 4 °C when it approached the exponential phase. The pellets were washed three times and resuspended in sterile fresh basal medium to an optical density of 0.7 to 1.1 (λ = 600 nm). The density of 1.1 corresponded to approximately 1.4×10^8 cells/ml, based on preliminary experiments that correlated culture optical density with viable cell counts determined by serial dilution and plating. Several batch experiments for NO_3^-/NO_2^- reduction including controls were conducted in this study: (1) Fe^{2+} (0.3 mM); (2) α-FeOOH (25 mM); (3) α-FeOOH (25 mM) + Fe^{2+} (0.3 mM); (4) L17; (5) L17 + α-FeOOH, γ-FeOOH, α-Fe_2O_3, γ-Fe_2O_3, Bi_2O_3, Al_2O_3, Nd_2O_3, ZrO_2, or TiO_2 (4.5 g/L). Standard anaerobic techniques were used throughout all experiments as previously described (Li *et al.* 2009). Inoculation and sampling were conducted by using sterile syringes and needles. All vials were conducted in duplicate and incubated in a BACTRON Anaerobic/Environmental Chamber II (SHELLAB, Sheldon Manufacturing Inc.) at 30°C in dark.

Analytical methods.

To remove the cells and oxide, samples for determination of NO_3^-/NO_2^- must be filtrated using a 0.22-μm syringe filter after centrifugation at 8,500 × *g* for 20 min. The concentration of NO_3^-/NO_2^- was determined by ion chromatography (Dionex ICS-90) with an ion column (IonPac AS14A 4 × 250 mm). A mobile phase consisting of Na_2CO_3 (8.0 mM) and $NaHCO_3$ (1.0 mM) solutions was operated at a flow rate of 1.0 mL/min. The total concentration of Fe(II), including dissolved and sorbed Fe(II), was determined by extracting Fe(II) from the samples using 0.5 mol/L HCl for 1.5 h and assaying the extract using 1,10-phenanthroline colorimetric assay. Dissolved Fe(II) was determined by removing the mineral and adsorbed Fe(II) from the aqueous phase using a 0.22-μm syringe filter and then assaying the filtrate by 1,10-phenanthroline. Adsorbed Fe(II) was calculated as the difference between the total and dissolved Fe(II).

Results

NO_3- reduction

Figure 1a showed that L17 can reduce nitrate efficiently from 7.5 mM to 0 mM in 4 days, while the NO_3^- reduction rate was obviously accelerated by the addition of iron oxides, and the total NO_3^- (7.5 mM) disappeared completely just in 2 days. The first-order-rate constants (k) in Figure 1c suggested that the k values of L17/α-FeOOH, L17/γ-FeOOH, L17/α-Fe_2O_3, and L17/γ-Fe_2O_3 were 2.1732/d, 2.3132/d, 2.6806/d, and 2.8828/d, much higher than that of L17 alone (0.6503/d). To illustrate the role of biogenic Fe(II) from dissmilitaroy iron reduction, the controls of Fe^{2+}, α-FeOOH, and Fe^{2+}/α-FeOOH were simultaneously conducted, and the results showed that no significant reduction of NO_3^- was observed with Fe^{2+} or α-FeOOH alone, while 10% of NO_3^- was reduced in the Fe^{2+}/α-FeOOH system after 4 days reaction, indicating that the biogenic Fe(II) just had minor contribution to the nitrate reduction in the L17/IOs system. Hence, the acceleration of nitrate reduction might be due to not only the biogenic Fe(II) but also some other unexpected factors. Herein we proposed a hypothesis that the IOs, as an semiconductor oxides, can transfer electron from L17, to confirm this hypothesis, a series of minerals but not iron oxides (non-IOs) were used in this reaction system, such as Bi_2O_3, Al_2O_3, Nd_2O_3, ZrO_2, and TiO_2. As shown in Figure 1b, in comparison with L17 alone, the NO_3^- reduction rate was accelerated by the addition of non-IOs, and The first-order-rate constants (k) in Figure 1c suggested that the k values of L17/Bi_2O_3, L17/Al_2O_3, L17/Nd_2O_3, L17/ZrO_2, and L17/TiO_2 were 0.766/d, 1.1243/d, 1.2695/d, 1.4583/d and 1.6038/d, higher than that of L17 alone (0.6503/d). The above important finding suggested that the acceleration of nitrate reduction might be attributed to the possible mechanism that the electron from L17 can be transferred through the semiconductor, which can lead an increase of electron transfer.

Proposed mechanisms and reduction pathways

Based on the above discussion, the electron transfer from cell to nitrate/nitrite may have three ways (Figure 2), (i) the direct reduction via the metabolism of the bacterium, (ii) the reduction by biogenic adsorbed Fe(II) of dissimilatory iron reduction, besides these two ways, a new way was proposed as (iii) the semiconductor-

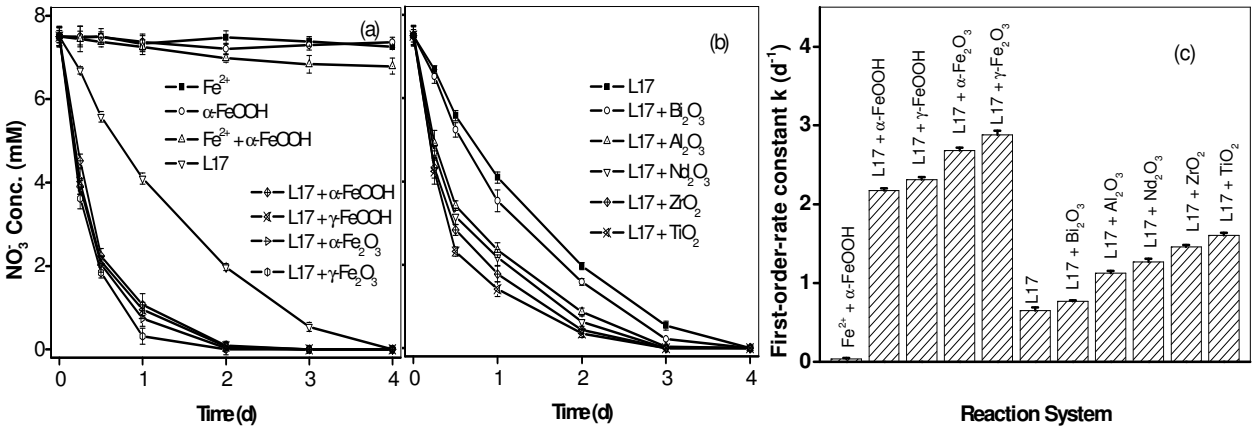

Figure 1. NO_3^- reduction in the reaction system of (a) L17 + iron oxides (L17/IOs), (b) L17 + minerals but not iron oxides (L17/non-IOs), and (c) the first-order-rate constants of various reaction systems (k,/d).

mediated electron transfer process. It must be clear that the electron transfer for the reduction of nitrate and Fe(III) oxide is originally driven by the microbe L17. And the biogenic adsorbed Fe(II) can also contribute to the denitrification slightly, while a large fraction of electron from L17 was directly transferred to nitrogen through the iron oxide, which lead a significant enhancement of nitrate/nitrite reduction. Regarding to semiconductor-mediated electron transfer process, an important question was raised how the electron from the L17 transfer through the semiconductor. Herein we proposed another hypothesis, which was described as: the electron from cells can be injected to the conduction band of semiconductors, and then transferred to the surface, finally, it can be accepted by the surface reducible species, including nitrate, Fe(III) and so on. Next work will be focused on proving this hypothesis.

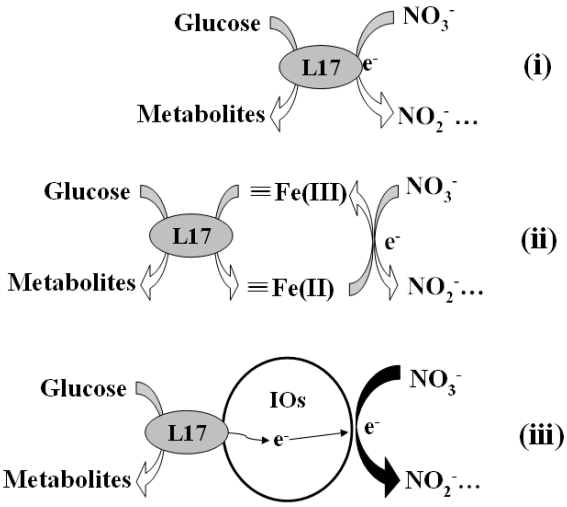

Figure 2. The proposed mechanism of NO_3^- reduction in the system of L17 and soil minerals: (i) direct microbial reduction, (ii) reduction by the biogenic Fe(II), (iii) reduction by mineral-mediated electron transfer.

Conclusion

The study showed that L17 had the capacity of nitrate reduction, and iron oxides can accelerate the reduction rate significantly. But the biogenic Fe(II) contributed to the acceleration slightly, which was not enough to reduce so much nitrogen. A series of non iron oxides with L17 were combined for nitrate reduction, and the results showed all the oxides can accelerate the reduction rate, indicating that the electron might transfer to nitrate through the oxides but not experiencing iron oxide's reduction. Hence, besides the well-known mechanism: direct microbial reduction and reduction by the biogenic Fe(II), a new mechanism was confirmed as soil mineral can mediate electron transfer to accelerate the microbial nitrate reduction.

References

Cabello P, Roldan MD, Moreno-Vivian C (2004) Nitrate reduction and the nitrogen cycle in archaea. *Microbiology* **150**, 3527–3546.

Gonzalez PJ, Correia C, Moura I, Brondino CD, Moura JJG (2006) Bacterial nitrate reductases: Molecular

and biological aspects of nitrate reduction. *Journal of Inorganic Biochemistry* **100**, 1015–1023.

Hansen HCB, Koch CB, Nancke-Krogh H, Borggaard OK, Sørensen J (1996) Abiotic nitrate reduction to ammonium: Key role of green rust. *Environmental Science and Technology 30*, 2053–2056.

Jørgensen CJ, Jacobsen OS, Elberling B, Aamand J (2009) Microbial oxidation of pyrite coupled to nitrate reduction in anoxic groundwater sediment. *Environmental Science & Technology* **43**, 4851–4857.

Li XM, Zhou SG, Li FB, Wu CY, Zhuang L, Xu W, Liu L (2009) Fe(III) oxide reduction and carbon tetrachloride dechlorination by a newly isolated *Klebsiella pneumoniae* strain L17. *Journal of Applied Microbiology* **106**, 130–139.

Liu L, Li FB, Feng CH, Li XZ (2009) Microbial fuel cell with an azo-dye-feeding cathode. *Applied Microbiology and Biotechnology* **85**, 175–183.

Nielsen JL, Nielsen PH (1998) Microbial nitrate-dependent oxidation of ferrous iron in activated sludge. *Environmental Science and Technology* **32**, 3556–3561.

Ottley CJ, Davison W, Edmunds WM (1997) Chemical catalysis of nitrate reduction by iron(II). *Geochim. Cosmochim. Acta* **61**, 1819–1828.

Straub KL, Benz M, Schink B, Widdel F (1996) Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl. Environ. Microbiol.* **62**, 1458–1460.

Weber KA, Picardal FW, Roden EE (2001) Microbially catalyzed nitrate-dependent oxidation of biogenic solid-phase Fe(II) compounds. *Environmental Science and Technology* **35**, 1644–1650.