SOILS, SEC 3 • REMEDIATION AND MANAGEMENT OF CONTAMINATED OR DEGRADED LANDS • RESEARCH ARTICLE

Electron shuttle-mediated microbial Fe(III) reduction under alkaline conditions

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Received: 5 April 2017 / Accepted: 14 May 2017

Responsible editor: Kitae Baek

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Abstract

Purpose Extracellular Fe(III) reduction plays an important role in a variety of biogeochemical processes. Several mechanisms for microbial Fe(III) reduction in pH-neutral environments have been proposed, but pathways of microbial Fe(III) reduction within alkaline conditions have not been clearly identified. Alkaline soils are vastly distributed thus a better understanding of microbial Fe(III) reduction under alkaline conditions is of significance. The purpose of this study is to explore the dominant mechanism of bacterial iron reduction in alkaline environments.

Materials and methods We used antraquinone-2,6-disulfonate (AQDS) as a representative of quinone moities of humic substances and elemental sulfur and sulfate as sulfur species to investigate the potential role of humic substances and sulfur species in mediating microbial Fe(III) reduction in alkaline environments. We carried out thermodynamic calculations to predict the ability of bacteria to reduce Fe(III) (oxyhydr)oxides under alkaline conditions and the ability of AQDS and sulfur species to serve as electron acceptors for microbial anaerobic respiration in an assumed alkaline soil environments. A series of incubation experiments with two model dissimilatory metal reducing bacteria, *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA as well as mixed bacteria enriched from a soil were performed to confirm the contribution of AQDS and sulfur species to Fe(III) reduction under alkaline conditions.

Results and discussion Based on thermodynamic calculations, we predicted that, under alkaline conditions the enzymatic reduction of Fe(III) (oxyhydr)oxides would be thermodynamically feasible but very weak. In our incubation experiments, the reduction of ferrihydrite by anaerobic cultures of *Shewanella oneidensis* MR-1, *Geobacter sulfurreducens* PCA or microbes enriched from a soil was significantly increased in the presence of S^0 or AQDS. Notably, AQDS contributed more to promoting Fe(III) reduction as a soluble electron shuttle than S^0 did under the alkaline conditions probably because of different mechanisms of microbial utilization of AQDS and S^0.

Conclusions These results suggest that microbial reduction of Fe(III) (oxyhydr)oxides under alkaline conditions may proceed via a pathway mediated by electron shuttles such as AQDS and S^0. Considering the high ability of electron shuttling and vast distribution of humic substances, we suggest that humic substances-mediated Fe(III) reduction may potentially be the dominant mechanism for Fe(III) reduction in alkaline environments.

Keywords Alkaline conditions • AQDS and sulfur • Electron shuttles • Humic substances • Microbial Fe(III) reduction • Usable thermodynamic energy

1 Introduction

Dissimilatory reduction of Fe(III) (oxyhydr)oxides, one of the most predominant terminal electron acceptors of extracellular respiration, is of significance in various biogeochemical processes, including cycling of elements like carbon and nitrogen (Bickle 2009; Ding et al. 2014), and biomineralization and decomposition of minerals (Köhler et al. 2013). Dissimilatory iron-reducing bacteria can reduce various substrates to support their growth, but their ability to metabolize a certain substrate is controlled by the thermodynamic energy available from the metabolic reaction (Nealson et al. 2002). The thermodynamic energy for microorganisms depends on the standard Gibbs formation energy and activities of the substrates and products (Bethke et al. 2011). Microbial reduction of Fe(III) (oxyhydr)oxides such as ferrihydrite and goethite in acid-neutral (pH = 4-7) environments is usually feasible because of enough usable energy, but it is less thermodynamically favorable under alkaline conditions because proton activity at alkaline pH is very weak to drive forward the reactions (Flynn et al. 2014). The distribution of alkaline (pH > 7.0) soils (38.23 million hm^2) is vast, accounting for 25.7 % of the total terrestrial area in the world (Malcolm and Sumne 1998). Alkaline soils are widely distributed in China (4.49 million hm^2) as well, accounting for 46.8 % of the total terrestrial area of China (IGBP-DIS 1998). The investigation of the mechanism for Fe(III) reduction under alkaline conditions is therefore important., Microbial Fe(III) (oxyhydr)oxide reduction has been observed in some alkaline environments such as soils, sediments, groundwater and lakes (Zavarzina et al. 2006; Burke et al. 2012; Thorpe et al. 2012; Chao et al. 2014), but the mechanism has not been clearly identified. Elemental sulfur (S^0) was reported to be an electron shuttle to expedite goethite reduction by *Shewanella oneidensis* MR-1 at pH 9.0 and this S^0-mediated electron transfer pathway was postulated to be an important mechanism for iron reduction under alkaline conditions with a relatively high S^0 level (Flynn et al. 2014). However, the concentration and activity of S^0 are very low (usually below detection limit) in some natural environments, especially in soils. The function of S^0 to mediate Fe(III)

reduction is likely to be limited in alkaline soils (Nealson 1997). It is likely that there are other compounds that promote iron reduction under alkaline conditions.

Humic substances are ubiquitous and rich in terrestrial environments, with concentrations ranging from 0.1 mg C/kg to several hundreds of mg C/kg (Aiken et al. 1985). It has been widely accepted that humic substances could act as electron shuttles to facilitate microbial Fe(III) oxide reduction at near-neutral environments (Klüpfel et al. 2014). Quinone moieties, represented by anthraquinone-2,6-disulfonate (AQDS), are recognized in humic substances as redox-active units that transfer electrons between microorganisms and Fe(III) (oxyhydr)oxides (Scott et al. 1998; Straub and Schink 2003). A vast number of dissimilatory iron reducing bacteria have been identified in soil environments (Wang et al. 2009; Yuan et al. 2016), and almost all microorganisms capable of reducing Fe(III) can also reduce humic substances (including AQDS) (Lovley et al. 1999). It was found that humic substances including AQDS promoted the reduction of Fe(III) (oxyhydr)oxides driven by alkaliphilic bacteria isolated from extreme environments (pH 10.0 microbial fuel cells) and in uranium-contaminated sediments of pH 10.0-10.5 (Ma et al. 2012; Wu et al. 2013; Williamson et al. 2014). The alkaliphilic bacteria could not represent microorganisms in natural soils and it is still ambiguous whether humic substance-mediated Fe(III) reduction is pervasive by common microorganisms in alkaline soils. It seemed that both humic substances and S^0 could mediate iron reduction in alkaline soil environments. However which one is the predominant mediator is unknown.

This study aimed to investigate the main mechanism of Fe(III) (oxyhydr)oxide reduction under alkaline conditions. We used AQDS as a representative of quinone moieties and elemental sulfur and sulfate as sulfur species. We first predicted the potential ability of AQDS and sulfur species to serve as electron acceptors for microbial anaerobic respiration in an assumed alkaline soil environment through thermodynamic calculation. To confirm the contribution of AQDS and sulfur species to Fe(III) reduction in alkaline soil environments, we

performed a series of incubation experiments with two model dissimilatory metal reducing bacteria, *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA as well as mixed bacteria enriched from soil.

2 Materials and methods

2.1 Thermodynamic calculation

We calculated the energy liberated from a series of redox reactions (Table 1) occurring in an anoxic electron-donor-limited environment ΔG_r using Eq. (1):

$$\Delta G_r = \Delta G_r^0 + RT\ln Q \qquad (1)$$

Where ΔG_r^0 is the standard Gibbs free energy change of the reaction and R stands for the gas constant which equals to 8.314 J/mol and T denotes absolute temperature (298.15 Kelvin), respectively. Q represents the ion activity product computed from Eq. (2):

$$Q = \frac{\Pi {a_p}^{vp}}{\Pi {a_s}^{vs}} \qquad (2)$$

Where a_p and a_s are the activities of the products and substrates; v_p and v_s denote their stoichiometric coefficients. We assumed the anoxic environment at 298.15 K in the pH range of 4-10 consisting of ferrihydrite ($Fe(OH)_3$), goethite (α-FeOOH), elemental sulfur (S^0) in solid state, and constant concentrations of 1 mM SO_4^{2-}, HCO_3^-, 1 μM Fe^{2+}, HS^-, AQDS and AH_2QDS (reduced AQDS); and 1 μM either acetate (CH_3COO^-) or formate ($CHOO^-$) as an electron donor.

The activity values of the aqueous species involving in the reaction we used in the calculations were set to concentrations of ions. We defined the values of activities of solids and water as 1 (Donald 1997). We considered only the activities of the dominant species in the calculations, e.g., HCO_3^- and HS^- dominate over CO_2 (aq) and H_2S (aq) respectively in the pH range of 4-10. Although the values of activities would be lower than values of molar concentrations, the difference of using values of activities or concentrations would not influence the trend

of the data distribution significantly.

When a microbe respires through a certain metabolic pathway, it captures a portion of energy liberated from the reaction by synthesis of adenosine triphosphate (ATP) to maintain its own growth (including cellular matter synthesis, motility, ion transport and other metabolic processes) which is named "maintenance energy" (Thauer et al. 1977). The remaining part of energy liberated, called usable energy ΔG_U, is utilized by microorganisms to drive forward the catalytic reaction which can be computed according to Eq. (3) (Jin and Bethke 2009):

$$\Delta G_U = - \Delta G_r - m\Delta G_P \qquad (3)$$

Where ΔG_P represents the free energy change of ATP synthesis, which is equal to 45 kJ per mole of ATP under environmental conditions (Thauer et al. 1977). The value of *m* is the number of ATP created per turnover of the redox reaction. In this case, we used 1.25 for iron reducers, 1 for sulfate and 2.67 for sulfur reducers for 8 electron transfer reaction in accordance with previous reports (Schauder and Kröger 1993; Bethke et al. 2011; Rabus et al. 2013). As for AQDS reducers, no reported value of *m* is available. Since the value of *m* is associated with the respiration chain and the extracellular respiration of Fe(III) and AQDS applies similar electron transfer pathways, we assign to *m* for AQDS reducers with 1.25, the value of *m* of Fe(III) reducers (Jin and Bethke 2005; Lies et al. 2005; Voordeckers et al. 2010).

The redox reactions will run in order of the thermodynamic ladder controlled by the values of ΔG_U. That is, the higher ΔG_U of the reaction, the more likely the reaction is to happen. A ΔG_U *value* below zero means there is not enough energy released from the reaction to support the growth of microorganisms, thus this reaction would not be catalyzed by microorganisms.

2.2 Preculture of bacteria and soil

Shewanella oneidensis MR-1 (ATCC 70050) was from our laboratory stock; *Geobacter sulfurreducens* PCA

(ATCC 51573) was obtained from the Institute of Urban Environment, Chinese Academy of Sciences. *S. oneidensis* was grown anaerobically in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) at 30 °C in the dark; *G. sulfurreducens* was grown anaerobically in NBAF medium (Coppi et al. 2001) (pH = 7.0) at 30 °C in the dark for preculture of bacteria. *S. oneidensis* MR-1 and *G. sulfurreducens* PCA were chosen because these two strains are among the best-characterized iron-reducing bacteria, extracellular respiration of which have been investigated enzymatically and genetically. *S. oneidensis* MR-1 was originally isolated from an alkaline lake (pH = 9.1) and showed similar growth under neutral and alkaline pH ranges (6.5-6.9 and 8.5-9.0) (Tang et al. 2006). The optimum pH for growth of *G. sulfurreducens* PCA is not known and it is usually cultured at neutral pH. Here we used these two strains to investigate and compare the differed strategies they take when reducing Fe(III) at differed pHs.

Samples of paddy soil (0-20 cm depth) were collected from Enshi, Hubei province, China (30°16' N, 109°46' E). Soil samples were air-dried (plant litter including leaves, stems and roots were picked out), passed through a 2.0 mm sieve and stored in the dark before use. Soil (10 g) was inoculated into a sterilized 125 mL serum vial containing 100 mL LB medium (pH = 7.0), flushed with oxygen-free N_2 for 45 min and sealed with butyl rubber stoppers. The soil-medium mixture was cultured in an incubator at 30 °C in the dark for 24 h for preculture of bacteria.

2.3 Ferrihydrite synthesis

Ferrihydrite was synthesized through the addition of NaOH to $FeCl_3$ solution according to the method published by Schwertmann and Cornell (2000). The synthesised ferrihydrite was rinsed 5 times with deionized water, freeze-dried, ground using a mortar and pestle and sieved to < 75 μm. Ferrihydrite was kept at 4 °C to reduce the rate of spontaneous transformation to goethite. Ferrihydrite was used in this experiment within one month after

synthesis. We chose ferrihydrite as the representative of Fe(III) minerals because of its predominance in the anaerobic soil environment, especially in paddy fields (Wang et al. 1993).

2.4 Batch Incubation Experiment

Each incubation bioreactor was set up in a 125 mL serum bottle with 100 mL medium containing 1 mM formate for *S. oneidensis* or 1 mM acetate for *G. sulfurreducens* and mixed microbes from a soil as electron donors respectively. Six treatments were established for mixed microbes from a soil: (1) ferrihydrite only (Fe);

(2) ferrihydrite and S^0 (Fe+S^0);

(3) ferrihydrite and AQDS (Fe+AQDS);

(4) ferrihydrite and S^0 and AQDS (Fe+S^0+AQDS);

(5) ferrihydrite and Na_2SO_4 (Fe+SO_4^{2-});

(6) ferrihydrite and Na_2SO_4 and AQDS (Fe+SO_4^{2-}+AQDS).

Given that *S. oneidensis* and *G. sulfurreducens* cannot metabolize sulfate, only treatments (1-4) were set up for *S. oneidensis* and *G. sulfurreducens*. The final concentration of ferrihydrite (solid), S^0 (solid) and Na_2SO_4 (aqueous) was 10 mM and AQDS (aqueous) was 0.1 mM as high concentration (10 mM) of AQDS can potentially harm microorganisms (Nevin and Lovley 2000).

The pH of the medium was adjusted to 6.8 or 9.0 with 30 mM bis-tris propane (BTP, with pK_a values of 6.8 and 9.0 (Lide 2012)) for near neutral and alkaline conditions respectively according to previous researches (Flynn et al. 2014). The serum bottles capped with butyl rubber stoppers were flushed with oxygen-free nitrogen for 45 min and then autoclaved at 114 °C (S^0 has a melting point at 117 °C) for 30 min before bacteria inoculation.

Late-log-phase cells of *S. oneidensis* MR-1, *G. sulfurreducens* PCA and mixed microbes enriched from a soil were separately collected from preculture through centrifugation at 8000 rpm for 10 min. Cells were

anaerobically washed using sterilized anaerobic buffer solution of BTP (30 mM, pH 6.8 or 9.0) for three times and concentrated to a volume of ~20 mL. One mL cell suspension was anaerobically added to a bioreactor to give a final OD_{600} of ~0.5 ($5\times10^{\wedge8}$ cells/mL) separately. The bioreactors were placed in an incubator at 30 °C in the dark and agitated at 130 rpm. 121 °C heat-killed and uninoculated controls were set up and all the batch incubations were performed in triplicate. The pH of the medium was measured in the bioreactors at day 1 and day 9 in the pre-experiment. The actual pH of all near neutral/alkaline treatments in the bioreactors at day 9 was maintained in the range of 6.8-7.0/8.5-8.8.

2.5 Measurement of total Fe(II)

Total Fe(II) in the bioreactors was measured through ferrozine assay at day 0, 1, 2, 3, 6 and 9 during the incubation (Stookey 1970). Each bioreactor was shaken by hand to homogenize the media in the nitrogen-purged anoxic glove box. A sample (0.3 mL) of solid-liquid mixture was taken out from the bioreactor with a syringe, and 0.2 mL of the subsample was immediately transferred to an anoxic tube containing 6 M HCl with pipette. After dissolution of the iron minerals for 24 h in the anoxic capped tubes, samples were centrifuged at 8000 rpm for 3 minute and the supernatants were passed through a 0.22 μm filter each. A 0.2 mL filtered supernatant was then added to 3.8 mL 0.5 g/L ferrozine reagent (buffered to pH 7.0 with 50 mM HEPES). The absorbance at 562 nm was determined on a spectrophotometer.

3 Results and discussion

3.1 Thermodynamic calculation

We introduced an index of ΔG_U, the potential usable energy liberated from redox reactions, to estimate the feasibility of corresponding microbial reduction of Fe(III) (oxyhydr)oxides, sulfur and AQDS with either acetate

or formate as an electron donor across a range of pH in a hypothesized anoxic soil environment. The enzymatic reduction of ferrihydrite and goethite using either electron donor would generate less energy as the pH increases (Fig. 1). Iron reduction with formate or acetate as an electron donor is strongly favored by acidic conditions because 16 or 15 H^+ are consumed during reduction of 8 Fe(III) to 8 Fe(II) (formula 1, 2, 6, 7 in Table 1). These models predict that there is little energy to drive forward the microbial reduction of goethite above pH 7.0, or ferrihydrite above pH 9.0 (Fig. 1). Of the common Fe(III) containing minerals in the natural environment, ferrihydrite reduction yields the highest energy (Majzlan et al. 2004). Even so, very little energy can be captured from its reduction to support growth of microorganisms at highly alkaline conditions. In contrast to iron reduction, AQDS (formula 5, 10 in Table 1) and sulfate reducers (formula 4, 9 in Table 1) acquire similar, although relatively low, usable energy across the almost entire pH range of 4-10 tested in the hypothesized anoxic soil environment (Fig. 1), which means enzymatic reduction of AQDS and sulfate would not be influenced much by pH. More usable energy (ΔG_U) can be obtained by sulfur reducers at high pH through the proton-producing reactions (formula 3, 8 in Table 1).

3.2 Ferrihydrite reduction by model dissimilatory metal reducing bacteria

The amount of Fe^{2+} produced by *S. oneidensis* and *G. sulfurreducens* under alkaline conditions (pH = 8.5-8.8 during 9 day incubation) was about 2 and 4.5 folds higher in bioreactors containing ferrihydrite and AQDS compared with ferrihydrite-only bioreactors at day 9 (Fig. 2A and Fig. 2C), indicating that AQDS improved iron reduction for both microorganisms. The rate of Fe^{2+} production in the presence of AQDS at pH = 9.0 was dramatically promoted, most likely because of the electron shuttling via cycling between AQDS and its reducing product AH_2QDS. Fe^{2+} was produced from the chemical reduction of ferrihydrite by AH_2QDS (Fig. 3). Though enzymatic reduction of ferrihydrite and AQDS yields similar thermodynamic energy at pH 9.0 (Fig. 1),

AQDS-mediated ferrihydrite reduction might proceed much faster than direct microbial reduction of ferrihydrite. It was reported that microbial reduction of humic substances and chemical reduction of ferrihydrite by microbially reduced humic substances were at least 27 and 7 times faster than direct microbial reduction of ferrihydrite at neutral pH (Jiang and Kappler 2008).

Addition of S^0 to cultures of *S. oneidensis* and *G. sulfurreducens* under alkaline conditions produced 1.7 and 1.8 folds higher amount of Fe^{2+} than ferrihydrite-only treatments, suggesting that the presence of S^0 also enhanced ferrihydrite reduction (Fig. 2A and Fig. 2C), in agreement with the report of Flynn et al. (2014). The iron reduction with S^0 addition was likely the result of abiotic reduction of ferrihydrite by sulfide produced from the enzymatic reduction of S^0 as a black suspension was observed in the medium which was likely to be FeS according to early reports (Fig. 3) (Lohmayer et al. 2014). The formation of FeS may decrease free Fe^{2+} level in the solution, hence driving forward Fe(III) reduction (Flynn et al. 2014).

The final Fe^{2+} concentration in Fe+AQDS bioreactors was 0.1 mM higher than that in the Fe+S^0 bioreactors in *S. oneidensis* incubation and approximately three times higher than that in *G. sulfurreducens* incubation at pH 9.0 (Fig. 2A and Fig. 2C). These results suggest that AQDS plays a more important role in mediating iron reduction than sulfur in alkaline environments. The difference of Fe^{2+} production in Fe+S^0 bioreactors between the two bacteria species was likely because of their efficiency at metabolizing S^0 and utilization of different electron donors (Jr et al. 1994; Moser and Nealson 1996). The different contribution between AQDS and S^0 to the improvement of Fe(III) reduction may be because of different mechanisms of microbial utilization of AQDS and S^0. The reduction of AQDS seems to occur on the outer membrane of cells according to most previous studies though there are still controversies on the mechanism of microbial AQDS reduction (Piepenbrock and Kappler 2013). AQDS is highly soluble and has a fast diffusion rate and cycling speed (Arnold et al. 1986). Microbial sulfur respiration occurs on the cytoplasmatic membrane predominately via the soluble intermediate of

polysulfide (S_n^{2-}), which is chemically formed by the reaction of insoluble sulfur (S^0) and sulfide (Schauder and Kröger 1993). Due to the poor solubility of elemental sulfur (5 μg/L at 25 °C) (Boulegue 1973) and evidence that *S. oneidensis* S^0 reduction does not occur through direct contact with the insoluble form (Moser and Nealson 1996), direct microbial S^0 reduction is limited. In alkaline cultures supplemented with sulfur as an electron acceptor some of the available soluble sulfur must first be reduced to sulfide and then combine with more sulfur to form polysulfide (Ma and Adams 1993). The gradually elevating enzymatic reduction of S^0, or more properly, S_n^{2-}, therefore may be relatively slower than the reduction of AQDS. In addition, Fe^{2+} would combine with HS^- to form FeS, competing available HS^- for polysulfide formation (Saalfield and Bostick 2009). All in all, AQDS reduction has kinetic advantages compared to S^0 reduction under environmental alkaline conditions and is therefore more likely to participate in the microbial reduction of Fe(III) (oxyhydr)oxides.

The amount of Fe^{2+} produced in Fe+AQDS bioreactors was comparable to that in Fe+AQDS+S^0 bioreactors inoculated with *S. oneidensis* or *G. sulfurreducens* (Fig. 2A and Fig. 2C), indicating that the AQDS present in the bioreactors was sufficient to support microbial Fe(III) respiration, and that the supplemental S^0 did not significantly affect the rate of respiration, making AQDS the primary electron carrier between microbes and ferrihydrite under alkaline conditions.

The concentration of Fe^{2+} in Fe+AQDS bioreactors at pH 9.0 (Fig. 2A and Fig. 2C) was approximately half of that at pH 6.8 for both *S. oneidensis* and *G. sulfurreducens* (Fig. 2B and Fig. 2D). It might be explained by the lower reaction rate between ferrihydrite and AH_2QDS under alkaline conditions since this chemical step is the rate-limiting step in the whole process of AQDS-mediated bacterial iron reduction (Jiang and Kappler 2008). Fe(III) reduction by AH_2QDS at alkaline pH can be constrained thermodynamically since the energy yield of this abiotic reaction is getting smaller with increasing pH. Increased adsorption of Fe^{2+} on the surface of iron minerals at high pH might also block Fe(III) reduction (Orsetti et al. 2013). The different pH values (pH =6.8

and 9.0) were likely to affect related enzyme activities of the two strains and further their ability to reduce Fe(III) as well although *S. oneidensis* MR-1 grew similarly under neutral and alkaline pH ranges (6.5-6.9 and 8.5-9.0) according to Tang et al. (2006).

In ferrihydrite-only bioreactors, much less Fe^{2+} was produced at pH 9.0 (0.71 and 0.38 mM for *S. oneidensis* and *G. sulfurreducens*) (Fig. 2A and Fig. 2C) than at pH 6.8 (2.18 and 2.90 mM for *S. oneidensis* and *G. sulfurreducens*) (Fig. 2B and Fig. 2D), which is in correspondence with our theoretical calculation. Higher Fe^{2+} was observed in the incubation of *S. oneidensis* than *G. sulfurreducens* was likely because of different efficiencies of electron shuttles such as flavins secreted by the bacteria. Although both the bacteria were reported to be able to release flavins, flavins might not be utilized as equivalently efficient electron shuttles in both cases (Marsili et al. 2008; Okamoto et al. 2014). The amount of flavins released from *S. oneidensis* MR-1 and *G. sulfurreducens* PCA reached 0.1 ~ 0.6 μM and 0.1 μM respectively after 24-hour and 87-hour culture at neutral pH (Marsili et al. 2008; Okamoto et al. 2014). It was also reported that alkaliphilic bacteria could release riboflavins at pH = ~ 9 and the addition of 100 μM riboflavins raised the rate of ferrihydrite reduction by bacteria community in alkaline sediments (pH = ~ 11.8) (Fuller et al. 2014; Williamson et al. 2013). But the thermodynamic feasibility of the reduction of flavins ($E^{0'}$ = -0.21 V) was higher with formate than acetate as an electron donor at pH 9.0 (Marsili et al. 2008). Nevertheless, even if electron shuttles like flavins secreted by microorganisms played a role in facilitating Fe(III) reduction at pH 9.0, Fe(II) production was much slower in the absence than presence of exogenous electron shuttles such as AQDS and S^0. The 3.03 mM (and 3.12 mM) of Fe^{2+} produced in Fe+AQDS (and Fe+S^0+AQDS) amended treatments at pH 6.8 exceeded the maximum theoretical value (2 mM) that can be reduced from the oxidation of 1 mM formate. The extra ca. 1 mM Fe^{2+} production is likely to be associated with the reducing power stored in the harvested cells or scavenging of organic redox-active compounds released from cell lysis (Schwarzenbach et al. 2005; Jiang and Kappler 2008).

It was reported that, 0.05-0.1 mM Fe^{2+} was produced in the absence of electron donors at pH 9.0 by the residual reducing power of *S. oneidensis* MR-1 ($1\times10^{\wedge8}$ cells/mL), which is comparable with that of the cells ($5\times10^{\wedge8}$ cells/mL) in our experiments (Flynn et al. 2014).

At neutral pH, enzymatic reduction of poorly crystalline Fe(III) (oxyhydr)oxides is thermodynamically favorable, and the presence of AQDS can further improve the rate and extent of iron reduction which is accepted widely and shown in this study (Fig. 2B and Fig. 2D). Poorly crystalline Fe(III) (oxyhydr)oxides such as ferrihydrire are likely to transform into more crystalline minerals and become more difficult to use for microorganisms over time. These crystalline Fe(III) containing minerals might be more available for microbial reduction when soluble electron shuttles such as quinones are present (Lovley et al. 2000). However, the production of Fe^{2+} in bioreactors containing ferrifydrite and S^0 at pH 6.8 was not improved compared to that of ferrihydrite only bioreactors (Fig. 2B and Fig. 2D) which is consist with Flynn et al. (2014), indicating that S^0 made no difference to Fe(III) reduction at neutral pH.

3.3 Ferrihydrite reduction within enriched communities from a soil

Incubation of enriched bacteria from a soil showed similar results to that of model bacteria. 1.5 folds higher Fe(II) was produced in the presence of AQDS than that of S^0, which further suggests that AQDS plays a more important role in mediating Fe(III) reduction than S^0 under alkaline conditions (Fig. 4A). Given that the source of S^0 is usually the oxidation of sulfide produced through sulfate respiration of sulfate reducing-bacteria (SRB), sulfate was amended in some of our bioreactors. We calculated the usable energy for microorganisms released from sulfate reduction and the result suggests that microbial sulfate reduction is thermodynamically feasible under alkaline conditions (Fig. 1). Fe(III) reduction was accelerated in $Fe+SO_4^{2-}$ bioreactors compared with ferrihydrite-only treatments, consistent with previous findings that sulfate could be reduced to sulfide by sulfate

reducing- but not iron reducing- microbes and reoxidized abiotically to elemental sulfur and even sulfate by Fe(III) (Saalfield and Bostick 2009). But the extent of Fe^{2+} in Fe+SO_4^{2-} bioreactors was similar to that in Fe+S^0 bioreactors at pH 9.0 and near half of that in AQDS-containing bioreactors. These results further confirm that AQDS contributes more than sulfur species on mediating Fe(III) (oxyhydr)oxide reduction under alkaline conditions.

3.4 Potential role of humic substances in mediating Fe(III) reduction in alkaline environments

In this study, AQDS was used as a representative for humic substances in terms of redox activity according to previous reports (Aulenta et al. 2010). Actually the reduction potential ($E^{0'}$ = -0.184 V) of AQDS cannot represent the wide range of reduction potential of humic substances. It was reported that humic acid has a standard reduction potential $E^{0'}$ in the range of -0.3 to +0.15 V (Aeschbacher et al. 2011). Humic acid quinones are the primary electron-accepting units for microbial humic respiration (Scott et al. 1998) and formatotrophic and acetotrophic reduction of humic acid quinones to hydroquinones can proceed when $E^{0'}$ is above ca. -0.25 V and -0.21 V under alkaline conditions according to our thermodynamic calculation. These results imply that a vast variety of humic substances can be microbially reduced under alkaline conditions like AQDS.

Although most humic substances components are not as highly soluble or diffusible as AQDS and humic substances of low concentration would inhibit iron reduction by absorption on surface of minerals while AQDS would not (Wolf et al. 2009), there is still evidence that humic substances can accelerate Fe(III) reduction when the dissolved humic substance concentration reaches a minimum of 5-10 mg C/kg, which is not rare in soil environments (Aiken et al. 1985; Jiang and Kappler 2008). Despite the fact that AQDS of high concentration is toxic for some microorganisms, humic substances have not been reported to have such toxic effect for microorganisms (Nevin and Lovley 2000). The strong reversibility and sustainability reflected by numerous

cycles between reduced and oxidized state of humic substances in temporarily anoxic systems render humic substances efficient electron shuttles mediating microbial iron reduction (Klüpfel et al. 2014). Humic acids, one of the main components of humic substance, showed an increased solubility at alkaline pH (Wu et al. 2013). As a consequence, alkaline conditions may be conducive to the microbial reduction of humic acids and further humic acids-mediated iron reduction. It was reported that the reduction rate and extent of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) were increased at high pH increase by AH_2QDS (Kwon and Finneran 2008). Additionally, it was shown that solid-phase humic substances in wetland sediments can serve as electron shuttles to accelerate Fe(III) (oxyhydr)oxide reduction attributed at least partly to quinone moieties. Solid-phase electron shuttles must be physically associated with Fe(III) (oxyhydr)oxides, but the solid-phase electron-accepting capacity was much higher than the dissolved-phase (Roden et al. 2010). We can speculate that the shuttling of humic substances may be a potential driver of extracellular iron reduction under alkaline conditions. Some units within dissolved humic substances can chelate Fe(III), raising Fe(III)/Fe(II) reduction potential and promoting microbial Fe(III) reduction thermodynamically (Ho et al. 2003), which may potentially play a part in particular at alkaline pH since humic material is better soluble at alkaline pH. The distribution of alkaline soils is very vast, therefore humic substances-mediated Fe(III) reduction may occupy a considerable portion among the different pathways of Fe(III) reduction. But the actual contribution of humic substances to mediate Fe(III) reduction in alkaline environments is still required to be explored.

4 Conclusions

In summary, through the thermodynamic calculation and incubation with both pure and mixed bacteria, we demonstrate that microbial iron reduction utilizes a mixed biotic-abiotic pathway mediated by electron shuttles such as AQDS and S^0 in alkaline environments (Fig. 3). Humic substances analogue AQDS has a higher ability

to promote Fe(III) reduction as an electron shuttle than S^0 does under alkaline condition. While S^0 may be a strong contributor for Fe(III) reduction in alkaline environments rich in S^0 such as marine sediments, humic substances can still potentially be a dominant mediator of Fe(III) reduction in most alkaline environments considering the high ability of electron shuttling and vast distribution of humic substances. Microorganisms may take different strategies to adapt to shifting environments and the co-occurrence of the ability to reduce Fe(III) (oxyhydr)oxides and humic substances allows these microorganisms a competitive advantage in various environments across a wide range of pH. This study gives insight in understanding extracellular Fe(III) reduction, especially in alkaline environment.

Notes The authors declare no competing financial interest.

Acknowledgements This work was financially supported by the State Key Program of Natural Science Foundation of China (No. 41430858) and the National Natural Science Foundation of China (No. 41371459).

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Figure captions

Fig. 1 The change of usable energy ΔG_U for microorganisms liberated from redox reactions with Fe(III) (oxyhydr)oxides (ferrihydrite and goethite), sulfur species (S^0 and SO_4^{2-}) or AQDS as an electron acceptor, and formate (**A**) or acetate (**B**) as an electron donor with pH in a hypothesized anoxic environment. A reaction catalyzed by microorganisms can proceed only when its ΔG_U is higher than 0 kJ/mol denoted by the dashed line

Fig. 2 Total Fe^{2+} production in bioreactors incubated of *S. oneidensis* MR-1 with 1 mM formate (**A** and **B**), *G. sulfurreducens* PCA with 1 mM acetate (**C** and **D**) at pH 9.0 or 6.8 amended with (1) ferrihydrite only (Fe); (2) ferrihydrite and S^0 (Fe+S^0); (3) ferrihydrite and AQDS (Fe+AQDS); (4) ferrihydrite and S^0 and AQDS (Fe+S^0+AQDS) during 9 days. Error bars represent standard deviations of triplicate bioreactors

Fig. 3 Proposed model for AQDS and S^0 mediated Fe(III) reduction under alkaline conditions. The arrows in royal and magenta represent the processes of Fe(III) reduction mediated by AQDS and S^0 respectively. The red arrows represent the process of direct enzymatic reduction of Fe(III) which is difficult to happen under alkaline conditions. Thickness of the arrow denotes the contribution of each pathway

Fig. 4 Total Fe^{2+} production in bioreactors incubated of soil inoculum with 1 mM acetate at pH 9.0 or 6.8 (**A** and **B**) amended with (1) ferrihydrite only (Fe); (2) ferrihydrite and S^0 (Fe+S^0); (3) ferrihydrite and AQDS (Fe+AQDS); (4) ferrihydrite and S^0 and AQDS (Fe+S^0+AQDS); (5) ferrihydrite and Na_2SO_4 (Fe+SO_4^{2-}); (6) ferrihydrite and Na_2SO_4 and AQDS (Fe+SO_4^{2-}+AQDS) during 9 days. Error bars represent standard deviations of triplicate bioreactors.