Environmental Science
Processes & Impacts

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Environmental impact statement

Chromium contamination is a significant problem worldwide, Many countries have already put chromium included in the list of priority pollutants. Chromium generally exists in two stable oxidation states, trivalent chromium Cr(III) and hexavalent chromium Cr(VI). Cr(VI) compounds are highly soluble in water and toxic due to their strong oxidizing nature. The most common approach to remediate Cr(VI) is through its reduction into chemically stable and relatively nontoxic Cr(III), followed by precipitation or adsorption of the cationic species. Compare to chemical method, the microbial reduction method has the advantages of good water quality, low operating cost and no secondary pollution, etc, making it become the research hotspot of chromium-containing wastewater treatment.

Enhanced biotic and abiotic transformation of Cr(VI) by quinone-reducing bacteria/dissolved organic matters/Fe(III) in anaerobic environment

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Abstract: This study investigated the simultaneous transformation of Cr(VI) via a closely coupled abiotic pathway in an anaerobic system of quinone-reducing bacteria/dissolved organic matters (DOM)/Fe(III). Batch studies were conducted with quinone-reducing bacteria to assess the influences of sodium formate (NaFc), electron shuttling compounds (DOM) and Fe(III) on Cr(VI) reduction rates as these chemical species are likely to be present in the environment during in situ bioremediation. Results indicated that the concentration of sodium formate and anthraquinone-2-sodium sulfonate (AQS) had apparently effect on Cr(VI) reduction. The fastest decrease in rate for incubation supplemented with 5 mM sodium formate and 0.8 mM AQS showed that Fe(III)/DOM significantly promoted the reduction of Cr(VI). Presumably due to the presence of more easily utilizable sodium formate, DOM and Fe(III) have indirect Cr(VI) reduction capability. The coexist cycles of Fe(II)/Fe(III) and DOM(ox)/DOM(red) exhibited higher redox function than the individual cycle, and their abiotic coupling action can significantly enhance Cr(VI) reduction by quinone-reducing bacteria.

Keywords: Cr(VI); Bioremediation; Quinone-reducing bacteria; Electron shuttle; Dissolved organic matter

1 Introduction

Chromium contamination is a significant problem worldwide, China and many other countries have already put chromium included in the list of priority pollutants[1]. Chromium generally exists in two stable oxidation states, trivalent chromium Cr(III)

and hexavalent chromium Cr(VI)[2]. Cr(VI) compounds are highly soluble in water and toxic due to their strong oxidizing nature[3]. The most common approach to remediate Cr(VI) is through its reduction into chemically stable and relatively nontoxic Cr(III), followed by precipitation or adsorption of the cationic species[4]. Compare to chemical method, the microbial reduction method has the advantages of good water quality, low operating cost and no secondary pollution, etc., making it become the research hotspot of chromium-containing wastewater remediation[5].

Biological Cr(VI) reduction is mediated principally by direct enzymatic reduction and indirect chemical reduction[6]. Direct microbial Cr(VI) reduction through enzymatic mechanisms is a slow process when compared to chemical reduction[4] and therefore, the presence of additional electron donors and shuttles may play an important role in Cr(VI) reduction in situ.

Essentially, the transformation of Cr(VI) in anaerobic environments is a microbial quinone respiration induced electron transfer metabolic process, during which the Cr(VI) served as electron acceptors[7]. This process is largely regulated by the coordination and competition of the available electron donors and acceptors[8]. Some organic acids with low molecular weights, such as sodium formate and sodium acetate, usually act as electron donors in the form of readily metabolizable carbon sources[9], and may play a key role in Cr(VI) reduction rates in situ. More over, various ionic species and dissolved organic matter (DOM) usually act as competitive electron acceptors by microorganisms during quinone respiration[10].

Ionic species, such as Fe(III), have been shown to influence the ability of microorganisms to reduce them[11]. Microbial reduction of Fe(III) is considered especially important in Cr(VI) contaminated aquifers as it has been shown that Fe(II) has the ability to directly reduce Cr(VI), often at rates much higher than enzymatic processes[12]. The mechanism of indirect biological Cr(VI) reduction involves the reaction of Cr(VI) with the produced metabolite or Fe(II) generated by iron-reducing bacteria[13]. The produced Fe(II) can be recycled back to Fe(III), there by acting as an electron shuttle between the bacteria and Cr(VI)[14].

Besides, DOM, such as humic acid (HA) and fulvic acid (FA)[15], has been shown to influence the reduction of oxidized environmental contaminants including reducible heavy metals, for example Cr(VI)[12]. DOM acts as electron shuttle during this process, which can abiotically reduce organic pollutants as well as Fe(III) oxides[16]. DOM and ionic species coexist in the anaerobic water environment, and can mediate microbial reduction of Cr(VI). However, the abiotic coupling mechanism is not very clear. Therefore, we need to build up a system to investigate the simultaneous transformation of Cr(VI) via a closely coupled, abiotic pathway in an anaerobic system of quinone-reducing bacteria/DOM/Fe(III).

The aims at this study were to isolate quinone-reducing bacteria from sediment, and a series of batch experiments were performed to investigate the capacity of sediment enriched quinone-reducing bacteria to remediate Cr(VI) contamination. The influences of sodium formate and electron shuttling compounds (DOM models, HA and FA) on Cr(VI) reduction were tested. The redox functions of Fe(III)/Fe(II) cycles and DOM(ox)/DOM(red) cycles, as well as their abiotic coupling action to Cr(VI) reduction by quinone-reducing bacteria were also investigated.

2 Materials and methods

2.1 Chemicals and DOM preparation

DOM models anthraquinone-2-sodium sulfonate (AQS) and humic acid (HA) were purchased from Sigma Aldrich (USA), and the other reagents were purchased in analytical grade from Sinopharm Chemical Reagent Co., Ltd., China. Sediment was obtained from ErHai Lake in Dali, Yunnan, China, the physical and chemical characteristics were shown in Table 1. DOM was extracted from the collected sediment. 20 kg of sediments were gathered and kept it cold back to the laboratory, and then freeze-dried. It was griddled by a 4.0 mm sieve, then manually removed impurities such as gravel and plants extracted lake humic acid (LHA) and lake fulvic acid (LFA) in strict accordance with the method recommended by International Humic Substance Society (IHSS) (Fig. 1). Finally, the prepared LHA and LFA stock solutions were

stored in the polyethylene containers and kept at 4 °C in dark for using within 3 weeks.

<Table 1>

<Fig. 1>

2.2 Enrichment and isolation of quinone-reducing bacteria

A method of sediment slurry incubation was used to estimate the AQS reduction efficiency of sediment bacterial communities supplied with sodium formate as carbon substrate. The bacterias were isolated from the sediment at the end of the sodium formate incubation. Standard anaerobic culturing techniques were used throughout the study, and the basal medium was modified from Lovley and Phillips[17], which contained (mg/L): NaCl, 1000; NH_4Cl, 800; KH_2PO_4, 500; K_2HPO_4, 600; $MgCl_2$, 200; $CaCl_2 \cdot 2H_2O$, 50, and then 5 mL each of a vitamin solution and a trace mineral solution were added[18]. In addition, AQS (1 mM) was used as electron acceptor and sodium formate (5 mM) as carbon substrate. pH values for the beginning of enrichment medium and subsequent reaction medium were adjusted to 7.0. The media were sterilized by autoclaving for 20 min and cooled to room temperature under a constant stream of 80% N_2 and 20% CO_2. Sediment (1 mL), as mentioned above, was transferred into sterilized serum bottles, capped with butyl rubber stoppers, and then incubated at 30 ^oC in the dark. During incubation, the percentage reduction of AQS reached 80%, the mixture was transferred into new media at a volume of 10% as inoculum (*v*/*v*). After continuous inoculation three to five times, a stable microbial culture with the potential to reduce AQS was obtained. The absorbance of AQS and reduced AQS (AH_2QS) were measured at 336 and 398 nm by UV-Vis spectrophotometer, respectively.

To isolate the quinone reducing bacteria, the incubation solution was diluted serially and plated on agar plates containing sodium formate and AQS. Selected well developed colonies were streaked three times onto new agar before further study. For identification of the quinone reducing bacteria, genomic DNA was extracted from the microbial cells grown on agar plates with standard extraction procedures. The amplification of 16S-rRNA genes was performed in a total volume of 25 μL, containing the universal primers 11F and 1387R. The amplification product (PCR) was purified

with the GENECLEAN Kit (Sango); the recovered fragments were cloned using a pEASYTM-T1 Cloning Kit. The 16S-rRNA gene sequences were compared to known sequences available in the Gen Bank using the BLAST program. The best matching sequences obtained from the Gen Bank were analyzed with CLUSTAL 2.0 and then a phylogenetic tree was constructed using the MEGA version 4. The resultant tree was evaluated using the bootstrap values based on 1000 replicates.

2.3 Hexavalent chromium reduction experiments

All the experiments were conducted statically in anaerobic condition and at constant temperature (30 ^{o}C). Sterile controls were prepared under the same conditions and autoclaved at 121 ^{o}C for 25 min. Anaerobic batch experiments were set up in 250 mL serum bottle N_2/CO_2 (80: 20) mixed gas headspace and capped with butyl rubber stoppers. Treatments were inoculated with the quinone-reducing bacteria, and at the start of each experiment, the initial Cr(VI) concentration was about 0.2 mM. In order to explore the effects of sodium formate, AQS, and Fe(III) on the reduction of Cr(VI), batch experiments were performed as follows: (1) Effect of sodium formate concentration on enhance Cr(VI) biotic transformation was tested by quinone-reducing bacteria in a sodium formate concentration range of 0-10 mM. (2) Effect of AQS concentration on enhance Cr(VI) abiotic transformation was tested by quinone-reducing bacteria in a AQS concentration range of 0-1.0 mM. (3) Abiotic coupling of Fe(III)/AQS on Cr(VI) reduction was tested by quinone-reducing bacteria in a Fe(III) concentration of 0.5 mM. (4) Abiotic coupling of Fe(III)/DOM (DOM included: AHA, LHA and LFA) on Cr(VI) reduction was tested by quinone-reducing bacteria in a Fe(III) concentration of 0.5 mM. All the Cr(VI) reduction experiments were conducted in triplicate.

2.4 Analytical methods

Samples were withdrawn from pressure tubes flushed with N_2 using a needle and a syringe through 0.45 μm glass fiber filters (GF/F, Millipore Corp., USA) which were prebaked at 450 ℃ for 4 h.The absorbance of AQS and reduced AQS (AH_2QS) were measured at 336 and 398 nm by UV-Vis spectrophotometer, respectively. Besides, the

concentration of AH_2QS can also be calculated by the change of AQS[7].

$$AH_2QS = C_{[AQS]0} - C_{[AQS]t} \quad (1)$$

$C_{[AQS]0}$: the initial concentration of AQS; $C_{[AQS]t}$: the concentration of AQS for a period of time.

Cr(VI) concentration was determined colorimetrically by using diphenylcarbazide reagent in the supernatant[19]. The concentrations of Fe(II) was determined based on the 1,10-phenanthroline colorimetric method after extracting Fe(II) from the samples using 0.5 mM HCl for 24 h at room temperature. Every sample was triplicate, and statistical analysis was performed using SPSS 20 for windows, all data were expressed as means ± standard error of mean.

3 Results and discussion

3.1 Isolation of quinone-reducing bacteria

The purpose was to isolate anaerobic bacterium and assess its ability for AQS reduction and microbial reduction of Cr(VI) in anaerobic water environment. In support of this, quinone-reducing bacteria was isolated and incubated with AQS and carbon substrate. Phylogenetic analysis indicated that the closest relative of strain was *Shewanella. strain*. with 99% 16r RNA gene sequence similarity (Fig. 2). The strain Y2 represents the genus *Shewanella* within the family *Shewanella* in the phylum firmicutes. It was reported that the representatives of genus *Shewanella* were able to degrade xenobiotics[20]. Some other species known as anaerobic reduction of Cr(VI) are *Shewanella. putrefaciens. CN-32*[21], *Clostridium beijerinckii Z*[22]. Besides, *Shewanella* can reduce many heavy metal pollutants[23]. For instance, *Shewanella oneidensis MR-1* mutants selected for their inability to produce soluble organic-Fe(III) complexes are unable to respire Fe(III) as anaerobic electron acceptor[24]. Burnes et al. isolated *Shewanella putrefaciens* to reduce Mn(IV) by two rapid screening techniques[25]. It indicated that the quinone-reducing bacteria could be applied to the reduction of Cr (VI) experiments, which was mediated principally through direct enzymatic reduction

mechanisms [17]. However, the direct microbial Cr(VI) reduction without external active substance was a slow process. Therefore, the presence of additional carbon source as electron donor and DOM (included DOM models) as electron shuttle may enhance the ability of biotic and abiotic transformation of Cr(VI).

<Fig. 2>

3.2 Effect of sodium formate on enhance Cr(VI) biotic transformation

Carbon sources play an extremely important role in microbial growth to promote the process of restore heavy metal pollutants[26]. Whether sodium formate can improve biological transformation of chromium by quinone-reducing bacteria is not clear. The effect of sodium formate concentrations on Cr(VI) reduction by the isolated bacteria with 0.8 mM AQS was shown in Fig. 3A. Results indicated that the reduction rates of Cr(VI) with 2, 5, 8, and 10 mM sodium formate as carbon source were much faster than the control without sodium formate. In the absence of sodium formate, Cr(VI) was also reduced by quinone-reducing bacteria, because AH_2QS was produced by endogenous respiration (Fig. 3B). Sodium formate at 2 mM, the reduction rate of Cr(VI) was slower than other levels. The results might have been caused by insufficient sodium formate for simultaneous AH_2QS generation (Fig. 3B). Besides, the effect of sodium formate concentrations (5, 8, and 10 mM) on Cr(VI) reduction rate were almost the same, but sodium formate at 5 mM on Cr(VI) reduction rate was the fastest. Compared with the 5 mM sodium formate, the concentration of sodium formate at 8 or 10 mM will inhibit the reduction of chromium.

Researches show that microbes make use of carbon source for quinone breathing process coupling the reduction of quinone material, the greater reduction rate of quinone material, the faster reduction rate of heavy metal pollutants[27], but excessive concentration of carbon source would inhibit quinone respiration[28]. The results indicated that exogenous carbon was one of the crucial factors in the process of Cr(VI) reduction, and 5 mM sodium formate was the optimal concentration for AQS reduction in this study (Fig. 3B). An appropriate external carbon source was also found to be important for AQS and Cr(VI) reduction by quinone-reducing bacteria.

<Fig. 3(A, B)>

3.3 Effect of AQS on enhancement of Cr(VI) abiotic transformation

Apart from carbon source, DOM and its models could be served as electron shuttle substances to improve abiotic transformation of heavy metals[26], which has very important significance for accelerating chromium reduction. Cr(VI) reduction in the absence and presence of AQS by quinone-reducing bacteria was shown in Fig. 4A. It indicated that the reduction rates of Cr(VI) with different concentration of AQS as electron shuttling compound were much faster than the control without AQS. So the adding external AQS played an important role in Cr(VI) abiotic transformation, which was in accordance with other studies[22,26,29]. Field et al. found that anthraquinone-2-6-sulfonate (AQDS) promoted Cr(VI) reduction by *Cellulomonassp. strain ES6*[26]. Brose and James reported that the addition of AQDS enhanced Cr(VI) reduction by *Shewanella oneidensis* in soil[29]. Xu et al. also showed that the additional AQDS could accelerate the reduction of Fe(III) by *Clostridium beijerinckii Z* in sediment[22].

In addition, no AH_2QS was detected in the incubated sample without AQS in the reaction medium (Fig. 4B). After 60 h, the transformation of Cr(VI) effected by different concentration of AQS was ranked in the following order: AQS at 0.8 mM > AQS at 0.5 mM > AQS at 1.0 mM, with Cr(VI) reduction rate of 97.32%, 91.52%, 86.34%, respectively (Fig. 4A). The fastest decrease in rate for incubations supplemented with 0.8 mM AQS showed that AQS significantly promoted the reduction of Cr(VI). Low concentration of AQS (0.5 mM) might lead to insufficient AH_2QS by microbial quinone respiration, but when the AQS concentration reached to 1.0 mM, inhibiting effects on Cr(VI) reduction were observed (Fig. 4B). Wolf et al. found that both AQDS and 2-hydroxy-1,4-naphthoquinone (lawsone, LQ) showed strong accelerating effects on ferrihydrite reduction by *Geobacter metallireducens* at concentrations ranging from 0.1 to 100 μM. On the other hand, inhibitory effects were found with the addition of higher concentration of quinones, the inhibition effects of quinones were generally attributed to its toxicity to bacterial cells[30].

<Fig. 4 (A, B)>

3.4 Abiotic coupling mechanism for Cr(VI) reduction by quinone-reducing bacteria/DOM/Fe(III)

Essentially, the transformation of Cr(VI) in anaerobic environments is a microbial quinone respiration induced electron transfer metabolic process[7]. The process is largely regulated by the coordination and competition of the available acceptors. Fe(III) usually act as active substance competitive electron acceptors by microbial metabolic process[8]. However, the abiotic coupling mechanism is not very clear. Therefore, in order to improve the microbial reduction of Cr(VI), we need further to study abiotic coupling mechanism for Cr(VI) reduction by quinone-reducing bacteria/DOM/Fe(III). Cr(VI) reduction in the co-exist Fe(III)/AQS by quinone-reducing bacteria was shown in Fig. 5.

<Fig. 5>

As shown in Fig. 5, it demonstrated that AQS and Fe(III) have no apparently effect on Cr(VI) reduction under the condition of without microorganisms. The addition of quinone-reducing bacteria could increase Cr(VI) reduction rates with the value ranged from 42.6% to 45.2% after 60 h even in the absence of AQS and Fe(III). However, Fe(III) has a great influence on microbial reduction of Cr(VI), which can enhance the reduction rate in the range of 58.3%-59.5% after 60 h. In the process, Fe(III) obtained electrons from bacteria prior to Cr(VI) and can be reduced to Fe(II) (Fig. 6). The cycles of Fe(II)/Fe(III) has a promoting effect on Cr(VI) compared to direct microbial chromium reduction. Similar to Fe(III), the cycles of AQS(ox)/AQS(red) can also facilitate Cr(VI) reduction with the rate ranges of 93.3%-95.8% after 60 h. In this cycle, the quinone in DOM was reduced to hydroquinone by microbial metabolism (Fig. 6), which has a stronger reduction ability to Cr(VI). At the same time of reducing Cr(VI), hydroquinone was oxidized into quinone.

<Fig. 6>

However, Cr(VI) reduction by quinone-reducing bacteria was a complex process along with Fe(III) and DOM in anaerobic environment. When Fe(III) and DOM were simultaneously exposed to microbial Cr(VI) reduction, the coexist cycles of

Fe(II)/Fe(III) and AQS(ox)/AQS(red) exhibited higher reduction efficiency on Cr(VI) than the individual cycle, with the value ranged from 96.3% to 98.8% after 40 h. Because the occurrence of AQS(ox)/AQS(red) cycles can promote Fe(II)/Fe(III) cycles by electron transfer (Fig. 6). It demonstrated that AQS will priority acquire electron to accelerate the cycles of Fe(II)/Fe(III), which expedite the reduction of chromium. Besides, it was found that the redox function of AQS(ox)/AQS(red) cycle was more effective than Fe(II)/Fe(III) cycle, which is most likely due to the electron gain ability of AQS superior to Fe(III). The microbial reduction mechanism of chromium impacted by Fe(III) and DOM was summarized in Fig. 7, their abiotic coupling action can remarkably improve Cr(VI) reduction by quinone-reducing bacteria.

<Fig. 7>

AQS is a model of DOM, however, whether natural DOM (such as LHA and LFA) extracted from the sediment have the same effect to enhance the reduction of Cr(VI) need to be further discussed. Cr(VI) reduction in the co-exist Fe(III)/DOM by quinone-reducing bacteria was shown in Fig. 8. It indicated that DOM and Fe(III) have no effect on Cr(VI) reduction without quinone-reducing bacteria. LFA demonstrated the best stimulating effects among the DOMs tested. Compared to that obtained in the absence of DOM (k=4.21E-11), the k value was increased almost 6.14-fold (k= 2.58E-10) by the addition of 5 mgC·L^{-1} LFA, and the addition of 5 mgC·L^{-1} LHA and 5 mgC·L^{-1} HA could also result in 3.76-fold and 2.86-fold increase of k value, respectively. The results showed that DOM extracted from the sediment played a significant promoting role to the reduction of Cr(VI). Besides, the addition of 0.5 mM Fe(III) could increase 1.55-fold of k value (Fig. 8). The coexist cycles of Fe(II)/Fe(III) and DOM(ox)/DOM(red) manifested higher reduction efficiency on Cr(VI) than the individual cycle, with the k value of Fe/LHA, Fe/LFA and Fe/HA was 2.21E-10, 3.63E-10 and 1.56E-10, respectively. We gained the conclusion that the abiotic coupling action of Fe(II)/Fe(III) and DOM(ox)/DOM(red) cycles can significantly enhance Cr(VI) reduction by quinone-reducing bacteria.

<Fig. 8>

5 Conclusions

The results of this study demonstrated that Cr(VI) remediation was achieved through quinone-reducing bacteria enriched and isolated from sediment. The addition of electron donor could increase Cr(VI) biotic transformation process, and AQS enhance abiotic reduction of Cr(VI) by quinone-reducing bacteria. The fastest decrease in rate for incubations supplemented with 5 mM sodium formate and 0.8 mM AQS showed that Fe(III)/AQS significantly promoted the reduction of Cr(VI). DOM extracted from actual aquatic environment and served as electron shuttles can significantly enhance the ability of quinone-reducing bacteria to reduce Cr(VI). The influence of DOM was more remarkable than the other factors investigated here. The coexist cycles of Fe(II)/Fe(III) and DOM(ox)/DOM(red) exhibited higher redox function than the individual cycle, and their abiotic coupling action can significantly enhance Cr(VI) reduction by quinone-reducing bacteria. This study has provided comprehensive information on possible microbial and geochemical interactions of chromium pollutant in anaerobic environments. It has therefore furthered the understanding of the multiple environmental functions of DOM and reducing species.

Acknowledgments

This project was sponsored by the National Natural Science Foundation of China (Grant no. 41401558) and China Postdoctoral Science Foundation (Grant no. 2014T70887).

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Table 1 The physical and chemical characteristics of the sediment sample

Name	Sediment
Sampling site	99°32'46.11' E, 25°25'32.47'N
Organic matter (g kg^{-1})	23.64
Cation exchange capacity (mmol kg^{-1})	312
Sand: silt: clay ratio(%)	16: 32: 21
pH	8.37
Total nitrogen (g kg^{-1})	0.84
Total phosphorus (g kg^{-1})	0.72
Depth of water (m)	11.26

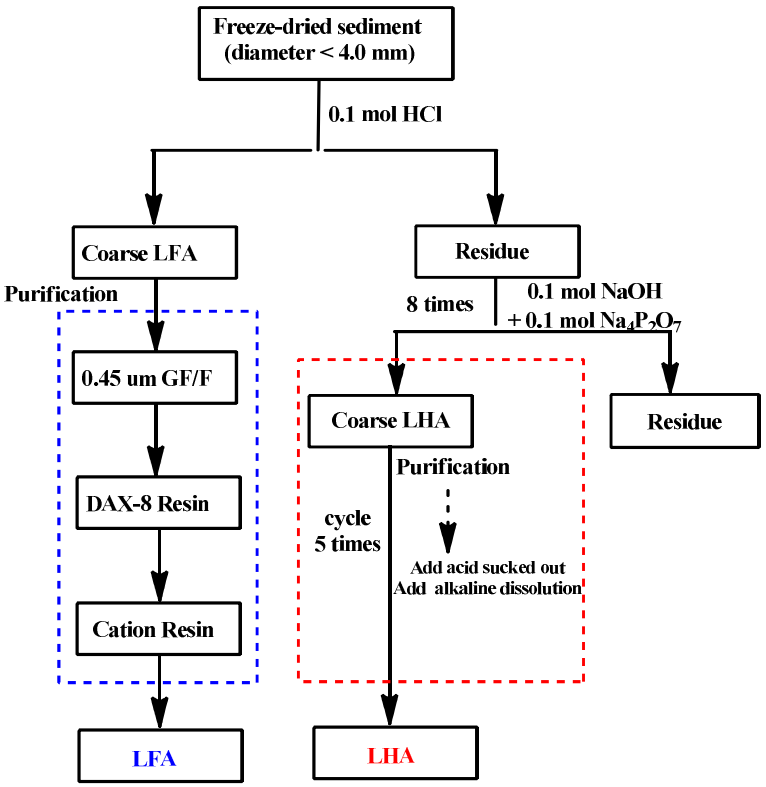


Fig. 1 Procedures of lake humic acid (LHA) and lake fulvic acid (LFA) extraction from the collected sediments.

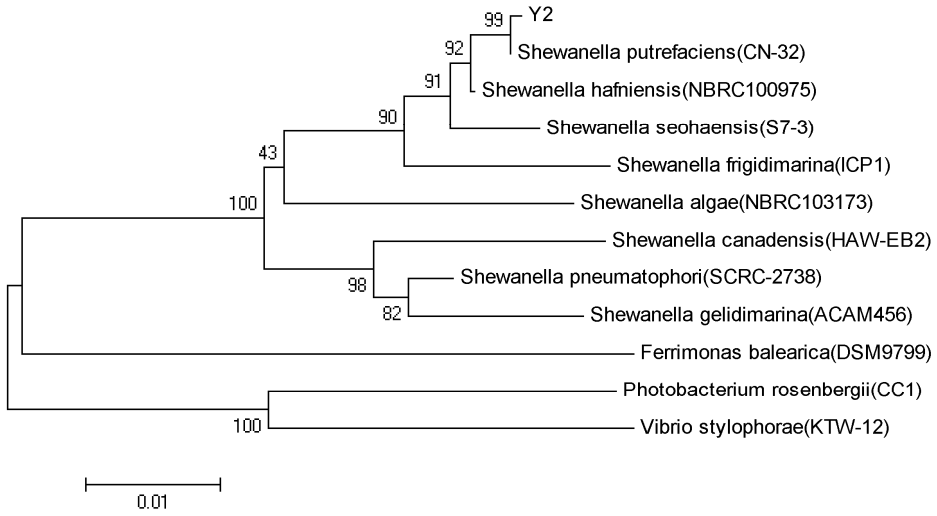
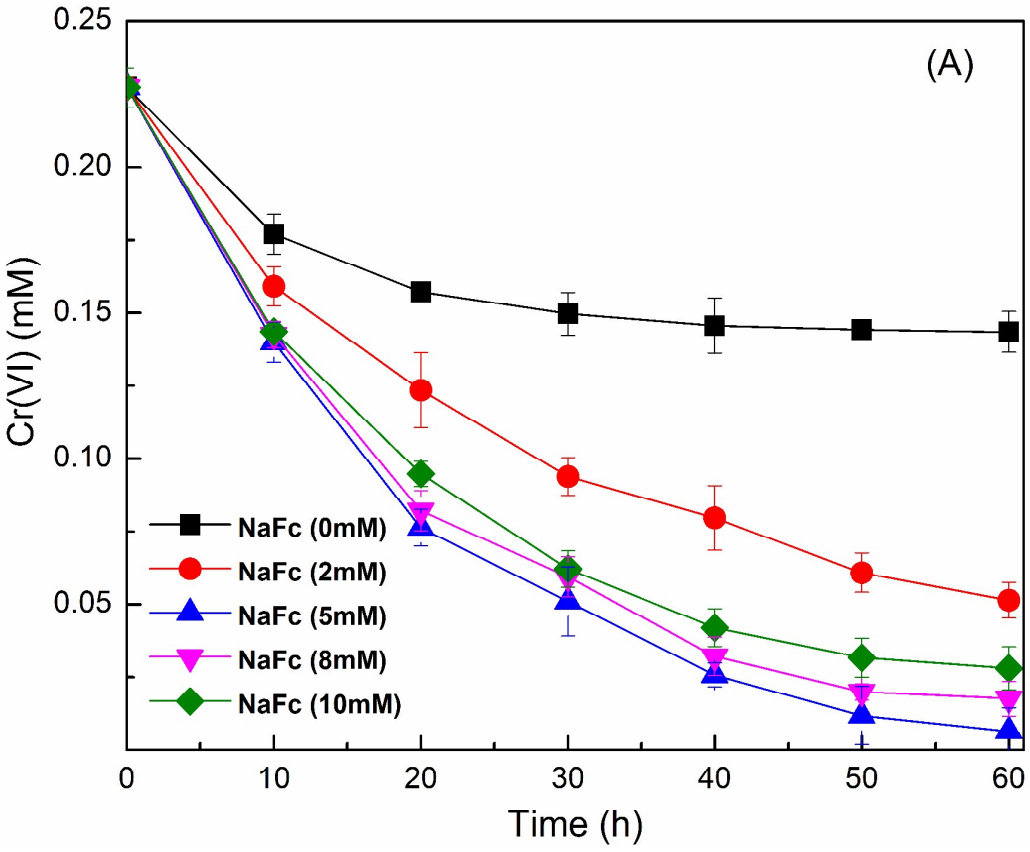


Fig. 2 Phylogenetic tree constructed by neighbor-joining algorithm based the partial 16S rRNA gene sequences and 1000 bootstrap replicates, showing the position of Y2 in relation to members of the genus *Shewanella*.



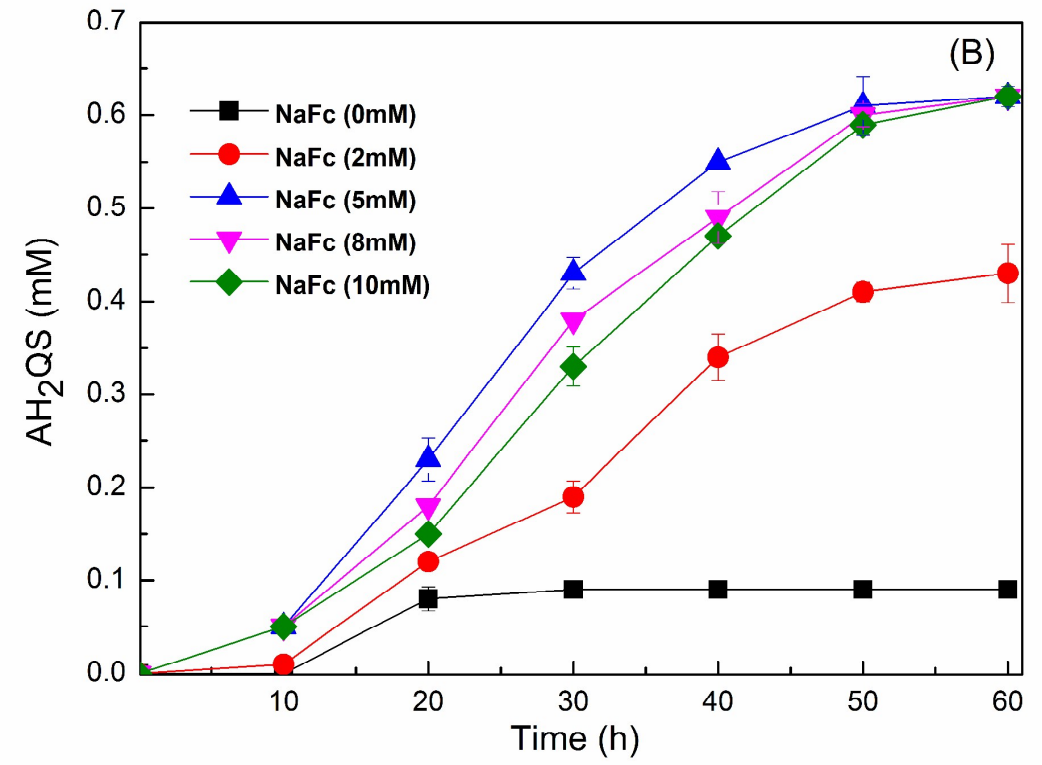


Fig. 3 The effects of sodium formate (NaFc) (as electron donor) concentration on Cr(VI) reduction and AH_2QS production by quinone-reducing bacteria (0.8 mM AQS). (A): the reduction of Cr(VI), (B): the production of AH_2QS.

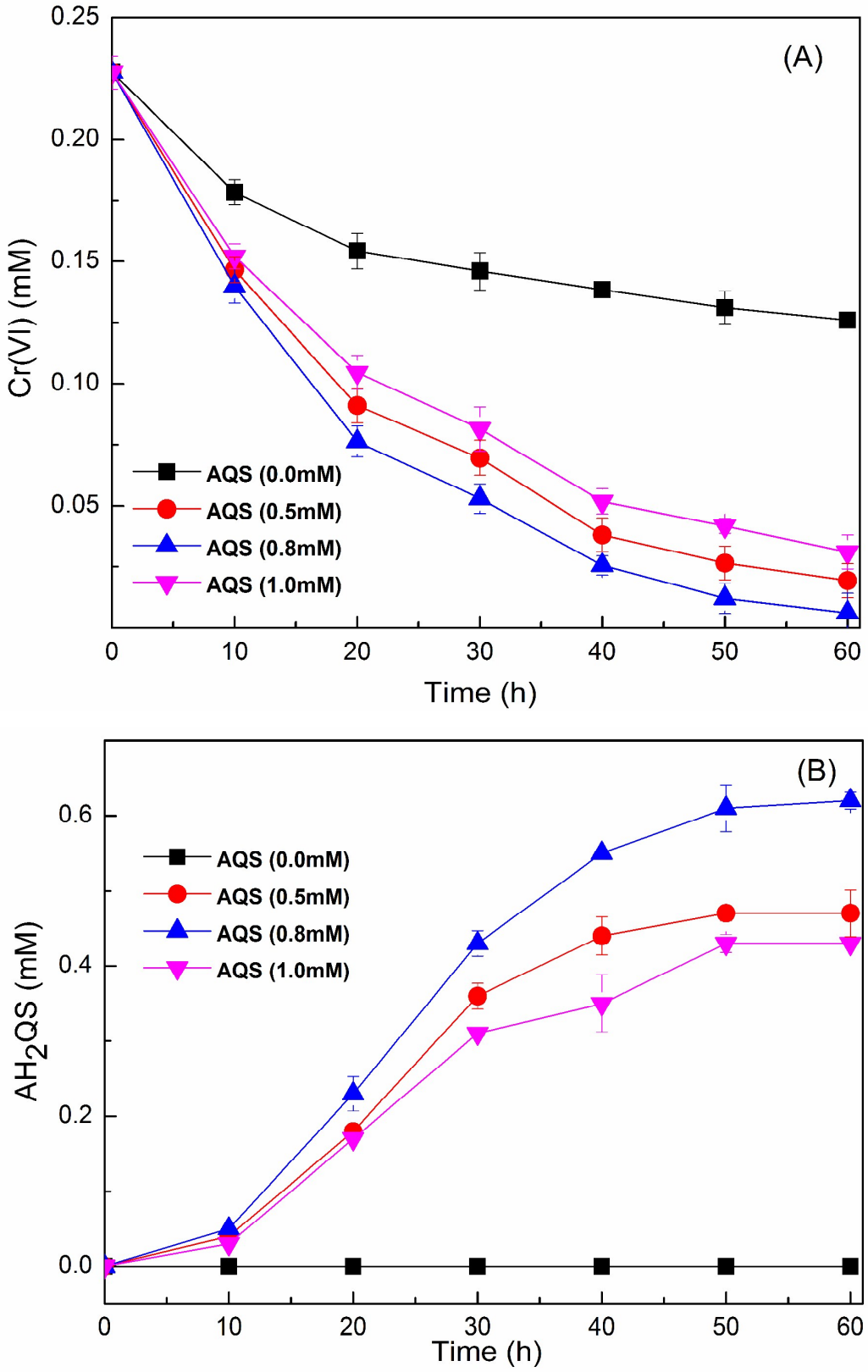


Fig. 4 The effects of AQS (as electron shuttle) concentration on Cr(VI) reduction and AH_2QS production by quinone-reducing bacteria; (A): the reduction of Cr(VI), (B): the production of

AH_2QS.

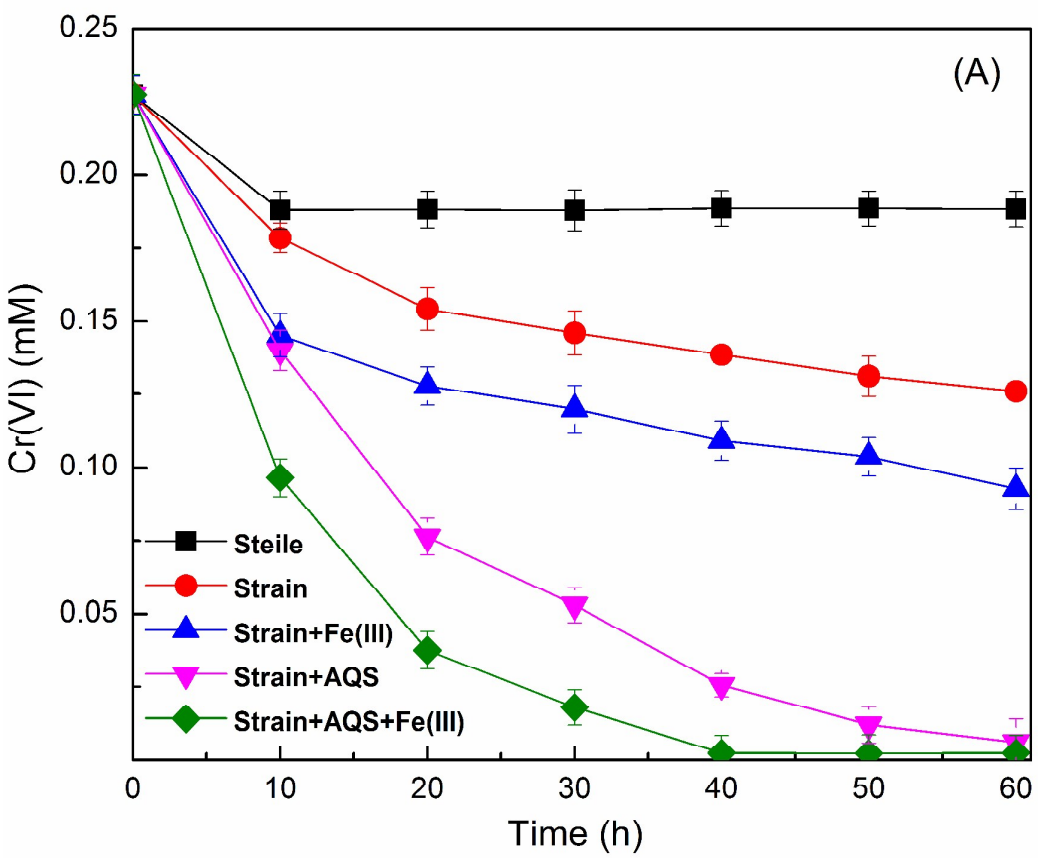


Fig. 5 The coupling contribution for Cr(VI) reduction by quinone-reducing bacteria/AQS (0.8 mM)/Fe(III) (0.5 mM).

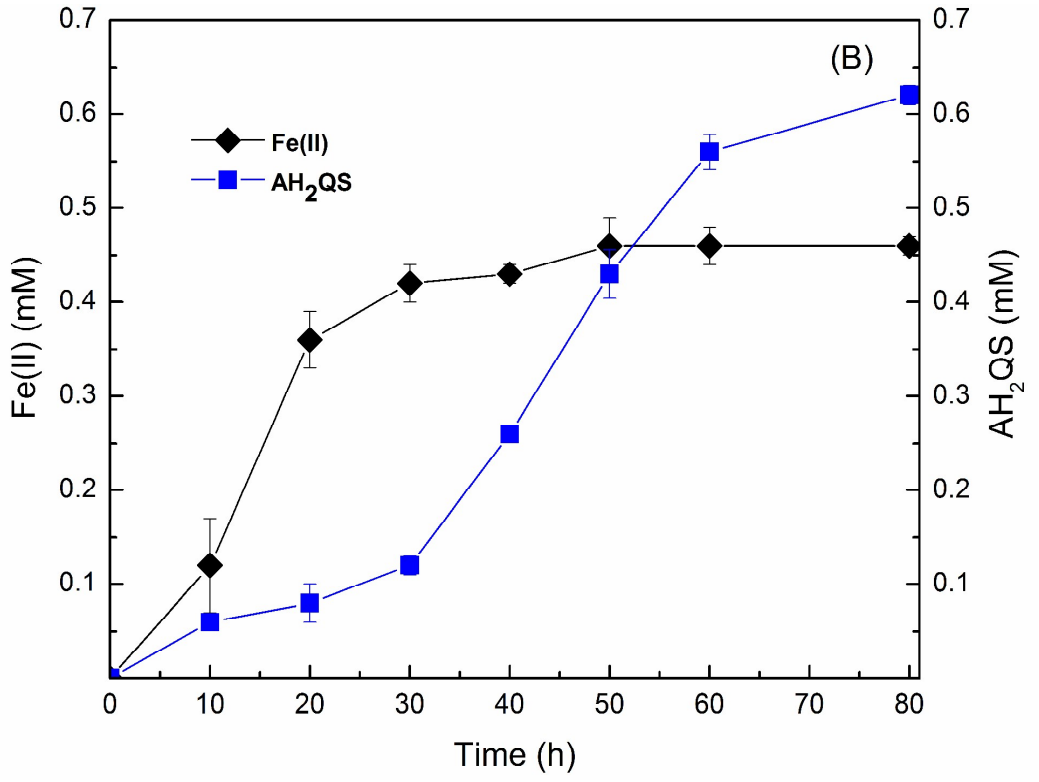


Fig. 6 The competitive ability of electron acceptor identified by quinone-reducing bacteria/AQS (0.8 mM)/Fe(III) (0.5 mM).

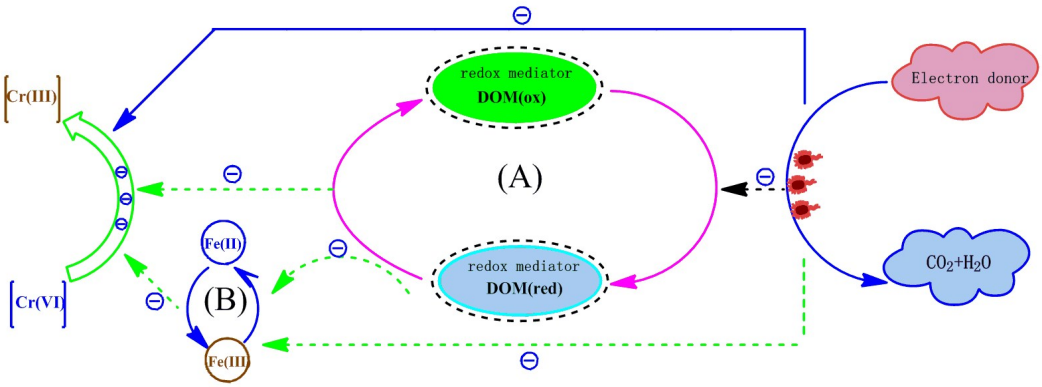


Fig. 7 Abiotic coupling mechanism for Cr(VI) reduction by quinone-reducing bacteria /DOM/Fe(III). (A): the cycles of DOM(ox)/DOM(red) ; (B): the cycles of Fe(II)/Fe(III).

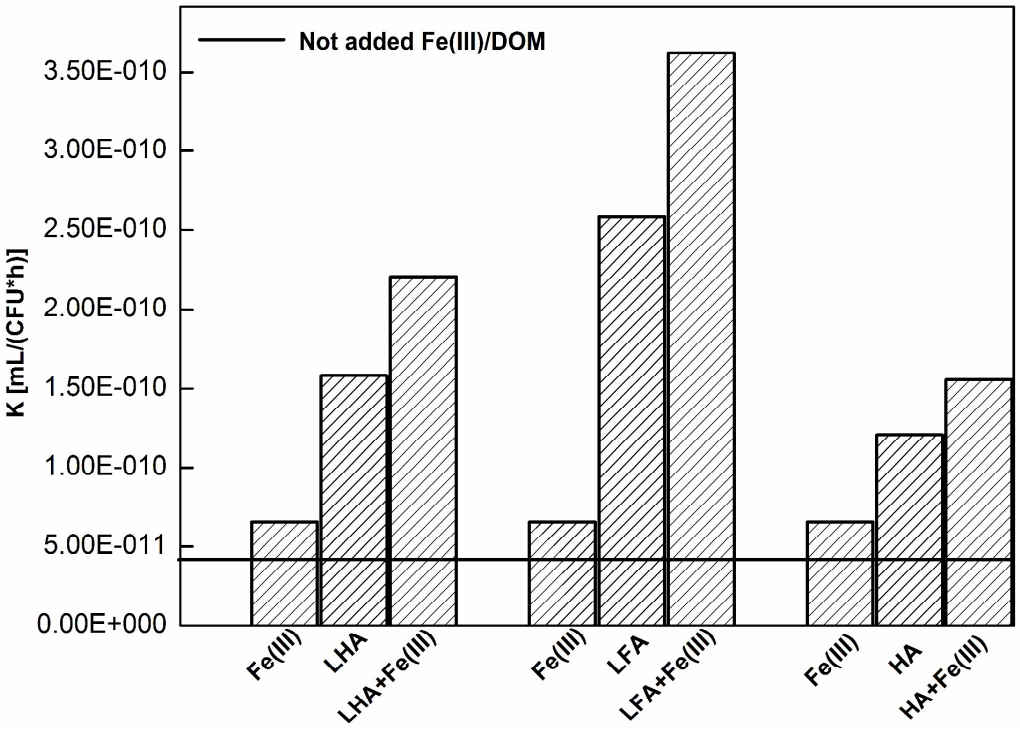


Fig. 8 The coupling contribution for Cr(VI) reduction by quinone-reducing bacteria/DOM (5 mgC/L)/Fe(III) (0.5 mM).