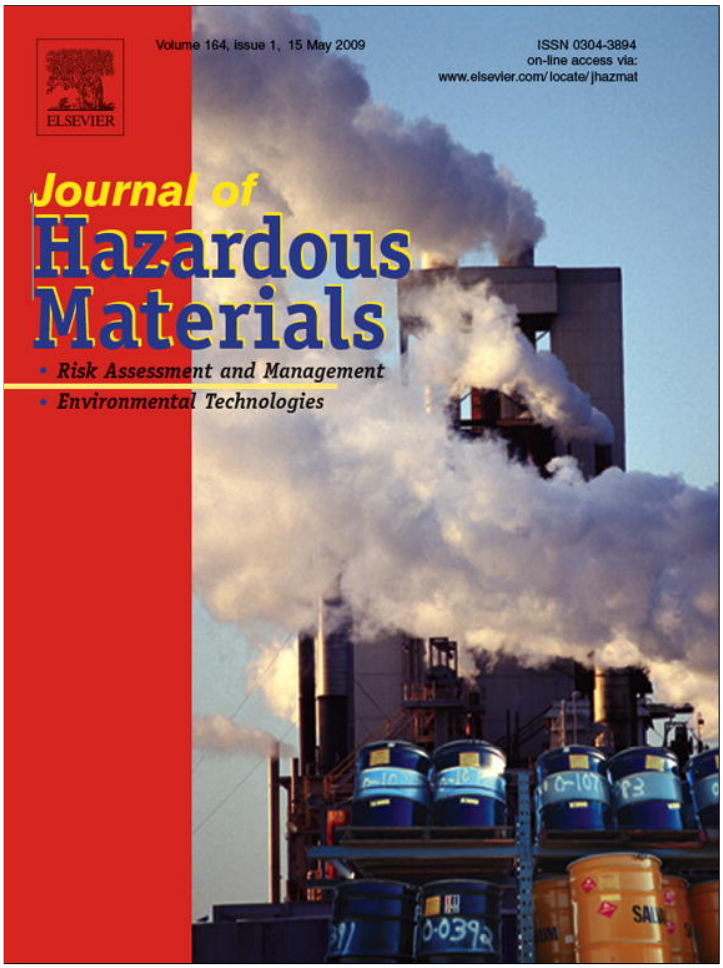

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# Transformation of carbon tetrachloride by biogenic iron species in the presence of *Geobacter sulfurreducens* and electron shuttles

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ABSTRACT

The transformation of carbon tetrachloride (CT) by biogenic iron species produced from the bioreduction of various Fe(III) oxides in the presence of *Geobacter sulfurreducens* and electron shuttles were investigated. Cysteine and anthraquinone-2,6-disulfonate (AQDS) at concentrations of 0.5 mM and 10 μM, respectively, were added as the electron shuttles. Addition of electron shuttles enhanced the extent of reduction and rate of ferric oxide reduction. The bioreduction extents of ferric oxides by *G. sulfurreducens* in the presence of electron shuttles were 22.8–48.3% for ferrihydrite, 6.5–17.2% for hematite, and 3.0–11.3% for goethite. After normalization to the surface areas, a higher rate of CT reduction was observed per unit of adsorbed Fe(II) on crystalline oxides. The produced biogenic Fe(II) from crystalline iron oxides was 2.8–7.6 times lower than that obtained from ferrihydrite, while the surface area-normalized rate constant for iron-mediated CT transformation in the presence of goethite and hematite were, by factors of 2–21, higher than that obtained using ferrihydrite. These results clearly depict that *G. sulfurreducens* drove the reduction of CT primarily through the formation of biogenic iron species in the presence of electron shuttle under iron-reducing conditions and that it is a surface area dependent process.



## 1. Introduction

Chlorinated hydrocarbons such as carbon tetrachloride (CT) and chloroform (CF) have been widely used in large quantities for different industrial and domestic purposes. Due to the frequent release to the environment by accidental leakage from tanks and waste disposal sites, these compounds are becoming the most often found recalcitrant compounds in contaminated environments [1–3]. In addition, the microbial reduction of ferric oxides by dissimilatory iron-reducing bacteria (DIRB) has recently been recognized as an important process for the generation of Fe(II) as well as the degradation of organic contaminants in anaerobic environments [4,5]. It is believed that the high reactivity of the heterogeneous Fe(II)–Fe(III) systems in the contaminated environments can be maintained over a long period of time because such Fe(II) species may be regenerated constantly through the sorption of Fe(II) from aqueous solution generated through microbial Fe(III) oxide reduction.

Laboratory and field studies have demonstrated the importance of surface-bound iron species in the reductive transformation of organic and inorganic compounds [6–8]. Iron minerals such as green rust, mackinawite, and goethite have been found to effectively reduce priority pollutants such as halogenated hydrocarbons [3,9–12] and nitroaromatic compounds [13,14]. Several factors including the pH value, surface density of Fe(II), available surface area of iron minerals, and contact time of Fe(II) with minerals have been shown to influence the reactivity of Fe(II) species bound with iron oxides [7,15,16]. Amonette et al. [16] dechlorinated CT in a Fe(II)-amended goethite system and found that the density of sorbed Fe(II) over goethite surface was the major factor on which the rate of dechlorination was dependent. Iron oxides are ubiquitous in the environment. This gives great impetus to employ the surface-bound iron species for removal of chlorinated hydrocarbons by adding Fe(II) to the contaminated sites or stimulating the generation of biogenic Fe(II) by DIRB in the presence of electron shuttling compounds.

It is generally accepted that the use of electron transfer mediators such as humic acid and quinone moieties can significantly enhance the transformation efficiency and rate of organic contaminants [17–19] as well as stimulate the reductive dissolution of Fe(III) oxides [20–22]. Current evidence suggests that anthraquinone-2,6-disulfonate (AQDS) is an effective electron shuttle for enhancing the reductive dissolution of Fe(III) oxides by DIRB [20,23,24]. More recently, addition of AQDS was found to enhance the degradation efficiency of some priority pollutants including phenol and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in the presence of DIRB under anaerobic conditions [4,25].

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In addition to AQDS, cysteine has been used as a reductant to decrease the redox potentials of solutions. Straub et al. [26] reported that cysteine could be used to lower the redox potential of solution for growth of anaerobic microorganisms. Our previous study showed that cysteine in the concentration range 0.5–2 mM is an effective mediator to transfer electrons between *Geobacter sulfurreducens* and ferrihydrite [27]. High concentrations of cysteine and AQDS have also been used as reductants for the abiotic dechlorination of CT under anoxic conditions [1,17,18]. These results clearly show that both AQDS and cysteine can be used in chemical and biological reactions for degradation of priority pollutants under anaerobic conditions. Tuntoolavest and Burgos [25] depicted that the oxidation of phenol by *Geobacter metallireducens* can be slightly enhanced when 25 μM AQDS were added to the hematite suspensions. The dechlorination of CT by *Shewanella putrefaciens* or *G. metallireducens* was enhanced in the presence of ferrihydrite or magnetite, and that the increased transformation rates were suggested to be due to the production of biogenic surface-bound or structural Fe(II) [28–30]. However, the biotic and abiotic reactions of chlorinated hydrocarbons mediated by various biogenic iron species in the presence of electron shuttles and *G. sulfurreducens* under iron-reducing conditions have received less attention.

The objective of this study was to investigate the bioreduction extent of different iron oxides by *G. sulfurreducens* in the presence of electron mediators, and the potential of utilizing the biogenic iron species for the removal of CT under iron-reducing conditions. Three ferric oxides, ferrihydrite, goethite and hematite, were used to examine the influence of types of ferric oxides on the biotransformation of CT. Cysteine and AQDS were selected as electron shuttling compounds to enhance the electron transfer rate to the solid Fe(III) minerals and CT. In addition, the kinetics for biogenic Fe(II) formation and CT transformation in the presence of different iron oxides and electron shuttles were examined and compared.

## 2. Materials and methods

### 2.1. Chemicals

Carbon tetrachloride ($CCl_4$, >99.8%, GC grade) and chloroform ($CHCl_3$, >99.8%, GC grade) were purchased from Merck Co. (Darmstadt, Germany). Fumarate disodium salt ($C_4H_2O_4Na_2$, 99%), *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanosulfonic acid) (HEPES, 99.5%), L-cysteine hydrochloride ($C_3H_7NO_2SCl$) (>98%), ferrozine monosodium salt ($C_{20}H_{13}N_4O_6S_2Na$), and anthraquinone-2,6-disulfonic acid disodium salt (AQDS, $C_{14}H_6O_8S_2Na_2$, >98%) were purchased form Sigma–Aldrich Co. (Milwaukee, WI). All other chemicals were of analytical grade and were used as received without further purification. Solutions were prepared with deoxygenated deionized water using a vacuum and high-purity $N_2$ purging system [31].

Ferric oxides were synthesized according to the methods of Schwertman and Cornell [32], and were characterized using X-ray powder diffractometry (XRPD) and surface area analyzer. The precipitates were washed four times with bidistilled water to remove dissolved ions. The resulting ferric oxide suspensions were degassed and gassed with $N_2$ gas several times and maintained under $N_2$ atmosphere in tightly sealed serum bottles. The ferric oxide suspensions were autoclaved afterwards and stored at room temperature under anoxic conditions. After the microwave digestion, the total Fe concentrations of ferric oxides were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). The surface areas of ferric oxides, determined by a BET $N_2$ adsorption surface area analyzer (Micromeritics, ASAP 2020), were 325 $m^2\,g^{-1}$ for ferrihydrite, 39.4 $m^2\,g^{-1}$ for hematite, and 28.8 $m^2\,g^{-1}$ for goethite.

### 2.2. Microorganism and cultivation

*G. sulfurreducens*, a courtesy from Dr. B. Schink, University of Konstanz, Germany, were cultivated in bicarbonate-buffered mineral medium (pH 7.2 ± 0.1) as previously reported [27]. The compositions of mineral media used for growth contained the following mineral salts ($g\,L^{-1}$): $NH_4Cl$, 0.25; NaCl, 1.0; $MgCl_2{\bullet}6H_2O$, 0.4; KCl, 0.5; $CaCl_2{\bullet}2H_2O$, 0.15; $KH_2PO_4$, 0.2. After autoclaving and cooling under an atmosphere of $N_2/CO_2$ (80/20, v/v), 30 mM sodium bicarbonate buffer solution, 1 mL of trace mineral, vitamin, and selenite–tungstate solutions were added per liter. 20 mM acetate and 40 mM fumarate solutions were added as electron donor and acceptor, respectively. All cultures were incubated at 25 ± 1 °C in the dark, and the purity was checked by optical microscopy at regular intervals. In addition, the optical density at 660 nm ($OD_{660}$) was used to monitor the growth of *G. sulfurreducens* during the incubation period. The $OD_{660}$ could reach 0.5–0.6 after incubation of 3 d, and suspensions were then used for further experiments.

### 2.3. Reduction of Fe(III) oxides and carbon tetrachloride

Batch experiments for microbial reduction of Fe(III) oxides and CT dechlorination were conducted under anoxic conditions using 70-mL serum bottles. High-purity $N_2$ was introduced into the bottles to maintain the anoxic conditions during the experimental periods. Anoxic 50 mL of HEPES buffer solutions (10 mM) were used to maintain the solution pH at 7.0 ± 0.1 and to prevent the precipitation of siderite ($FeCO_3$). The 10 mM Fe(III) oxides and 20 mM acetate were introduced into serum bottles using $N_2$-purged sterilized syringes. The chemical structures of $Fe(OH)_3$, $Fe_2O_3$, and α-FeOOH were used to calculate the molarities of ferrihydrite, hematite, and goethite, respectively. In addition, 10 μM AQDS or 0.5 mM cysteine were added as electron shuttle. One milliliter of *G. sulfurreducens* without the washing procedure was then injected into the serum bottles, which means that small amounts of fumarate would be introduced into the serum bottles. For CT dechlorination experiments, an appropriate amount of the CT stock solution dissolved in deoxygenated methanol was delivered into the serum bottle through a sterilized gas-tight glass syringe to obtain a final concentration of 3.5 μM, which corresponds to the actual aqueous concentration of 2.2 μM when considering the partitioning of CT between gas and aqueous phases at 25 °C [33]. Both *G. sulfurreducens* and CT were added at the beginning of the experiment. The total volume of the liquid phase in the serum bottle was maintained at 50 mL, resulting in a 20-mL volume available for headspace analysis. In sterilized experiments, media were introduced into the serum bottles through 0.2-μm sterilized PTFE membrane filters to understand the effect of solution ingredients on abiotic reduction of iron oxides. In addition, parallel control experiments in the absence of Fe(III) oxides, electron shuttle and/or bacteria (sterilized control) were also performed to evaluate the reactivity of *G. sulfurreducens* and electron shuttles toward CT dechlorination. All serum bottles were incubated in the dark on an orbital shaker at 150 rpm and 25 ± 1 °C.

### 2.4. Analytical methods

The headspace analytical technique was used for the determination of chlorinated hydrocarbons [2,3,17]. The concentrations of CT and byproducts in the headspace of the test bottles were monitored by withdrawing 50 μL of gas from the headspace and then injecting into a gas chromatograph (GC, Perkin-Elmer, Autosystem) equipped with an electron capture detector (ECD). A 60-m VOCOL megabore capillary column (0.545 mm × 3.0 μm, Supelco Co.) was used to separate the chlorinated compounds. The column temper-

ature was isothermally maintained at 90 °C using ultra-high purity $N_2$ (>99.9995%) as the carrier gas. Concentrations of chlorinated hydrocarbons in aqueous solutions were then calculated using the external standard method by preparing the known concentrations of chlorinated hydrocarbons in aqueous solutions. The relative standard deviation for GC analysis was controlled within 10%. The limits of detection for CT and CF were 0.04 and 0.1 μM, respectively.

Concentrations of total HCl-extractable Fe(II) in the serum bottles were monitored by withdrawing 0.5 mL of well-mixed suspension using $N_2$-purged syringes, and were immediately acidified with 1 mL of 1 M HCl [3,27]. After mixing vigorously for 5 min, the acidified samples were centrifuged at 14,000 × *g* for 10 min to remove particles, and Fe(II) contents in the supernatants were determined colorimetrically with ferrozine at 562 nm. To determine the dissolved fraction of Fe(II) in serum bottles, aliquots were withdrawn with a 1 mL $N_2$-purged plastic syringe, and then immediately filtered into a 0.5-mL acidic solution using a 0.2 μm acidified filter cartridge. The filter cartridge was acidify with 0.5 mL of 1 N HCl prior to the filtration to avoid the oxidation of Fe(II) in solution. The concentration of the dissolved Fe(II) in the filtrate was determined by the ferrozine method, and the sorbed Fe(II) concentration was calculated from the difference between the total and dissolved concentrations.

## 3. Results and discussion

### 3.1. Reduction of various Fe(III) oxides

Fig. 1 shows the bioreduction of 10 mM (0.56 g-Fe/L) various Fe(III) oxides by *G. sulfurreducens* in the presence of electron shuttles at neutral pH. In the absence of an electron shuttle (cysteine or AQDS), the bioreduction extent of ferrihydrite by *G. sulfurreducens* alone was only 0.63 ± 0.09 mM (6.3 ± 0.9%) after incubation of 20 d. Addition of 0.5 mM cysteine enhanced the bioreduction extent of ferrihydrite, and the maximum concentration of Fe(II) in the suspension was 2.28 ± 0.11 mM (22.8 ± 1.1%) after incubation of 20 d. A previous study [27] investigated the reductive dissolution of ferrihydrite by *G. sulfurreducens* in 30 mM bicarbonate buffer with 0.5 mM cysteine as an electron shuttling compound, and found that the Fe(II) concentrations was 2.69 mM after incubation of 22 d. In addition, the reduction of ferrihydrite by 0.5 mM cysteine in the absence of *G. sulfurreducens* (sterilized control) was only 0.52 ± 0.05 mM, which is consistent with the stoichiometric relationship of one-electron transfer between cysteine and ferrihydrite [34]. These results clearly indicate that the generation of Fe(II) is mainly due to the microbial Fe(III) reduction in the presence of cysteine. Different from the reduction of poorly crystalline ferric oxide, however, only slight bioreduction of goethite and hematite was observed. The detected Fe(II) concentrations were 0.30 ± 0.03 and 0.65 ± 0.09 mM for goethite and hematite, respectively, which are also higher than those in sterilized controls (0.027 ± 0.003 and 0.17 ± 0.01 mM).

Similar to the results obtained in the presence of cysteine, ferrihydrite was the most reactive Fe(III) oxide towards bioreduction in the presence of 10 μM AQDS (Fig. 1b). The Fe(II) concentration increased rapidly within the first 2 d and then reached a maximum concentration of 4.83 ± 0.12 mM after incubation of 9 d. Fredrickson et al. [35] compared the effect of AQDS on dissolution efficiency of ferrihydrite by *S. putrefaciens* in PIPES and bicarbonate buffer solutions and found that the reduction of ferrihydrite was greater in the bicarbonate buffer than in the PIPES-buffered medium. In this study, the produced Fe(II) concentration from ferrihydrite was 5.45 ± 0.29 mM when changing buffer solution to 10 μM AQDS (data not shown), suggesting that HEPES would slightly decrease

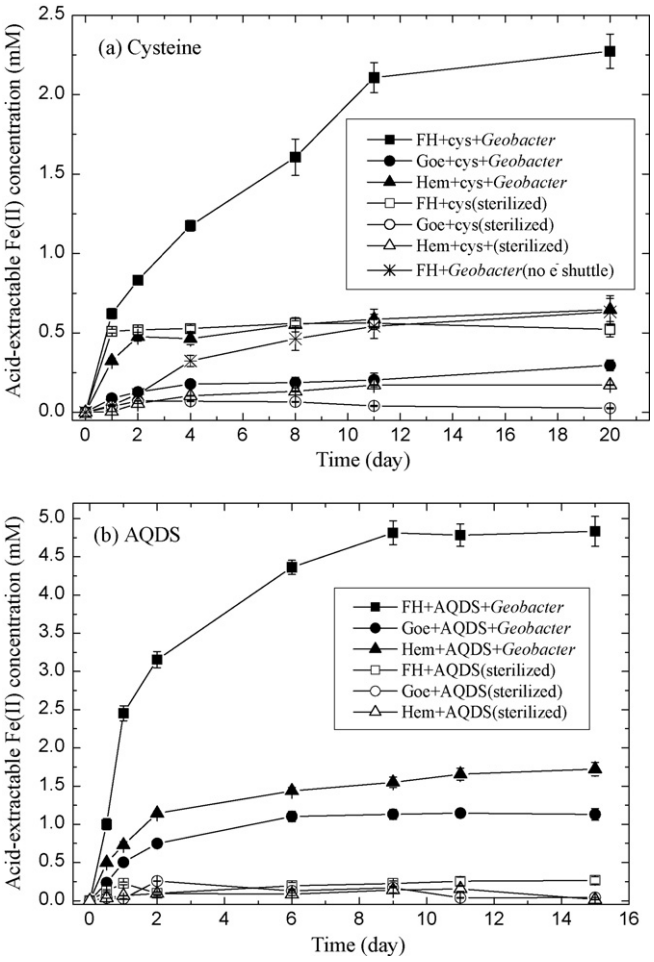


**Fig. 1.** Reduction of 10 mM ferric oxides by *Geobacter sulfurreducens* in the presence of (a) cysteine and (b) AQDS at neutral pH. The ferric oxides used in this study included ferrihydrite (FH), goethite (Goe) and hematite (Hem). Concentrations of cysteine and AQDS were 0.5 mM and 10 μM, respectively.

the reduction efficiency of Fe(III) oxides under anaerobic conditions.

The reduction of the crystalline Fe(III) oxides by *G. sulfurreducens* was slow. After incubation of 15 d, the Fe(II) concentrations produced from goethite and hematite were 1.13 ± 0.08 and 1.72 ± 0.09 mM, respectively. In sterilized controls, the produced Fe(II) concentrations were all lower than 0.25 mM, indicating that the increased Fe(II) concentration in the presence of *G. sulfurreducens* and AQDS was due to the microbial Fe(III) reduction.

To confirm the mineral phase and the morphology of the products, XRPD and SEM were used to characterize the biologically produced solids. Fig. 2 shows the SEM images and XRPD patterns of the ferric oxide minerals before and after bioreduction of goethite, hematite and ferrihydrite by *G. sulfurreducens*. The XRPD patterns of ferric oxide minerals after bioreduction of ferrihydrite showed peaks at 35.46°, 43.31°, 56.96°, and 62.79° (2$\theta$), which are consistent with both magnetite ($Fe_3O_4$) and maghemite ($\gamma$-$Fe_2O_3$). After bioreduction of ferrihydrite, the original dark-brown color of ferrihydrite gradually transformed to a black-colored product, and could be collected magnetically, suggesting that the produced solids could be magnetite. SEM images showed that the particle sizes of biogenic iron oxides ranged between 50 and 120 nm. In addition, XRD patterns of goethite and hematite before and after bioreduction were similar. This means that no secondary mineral

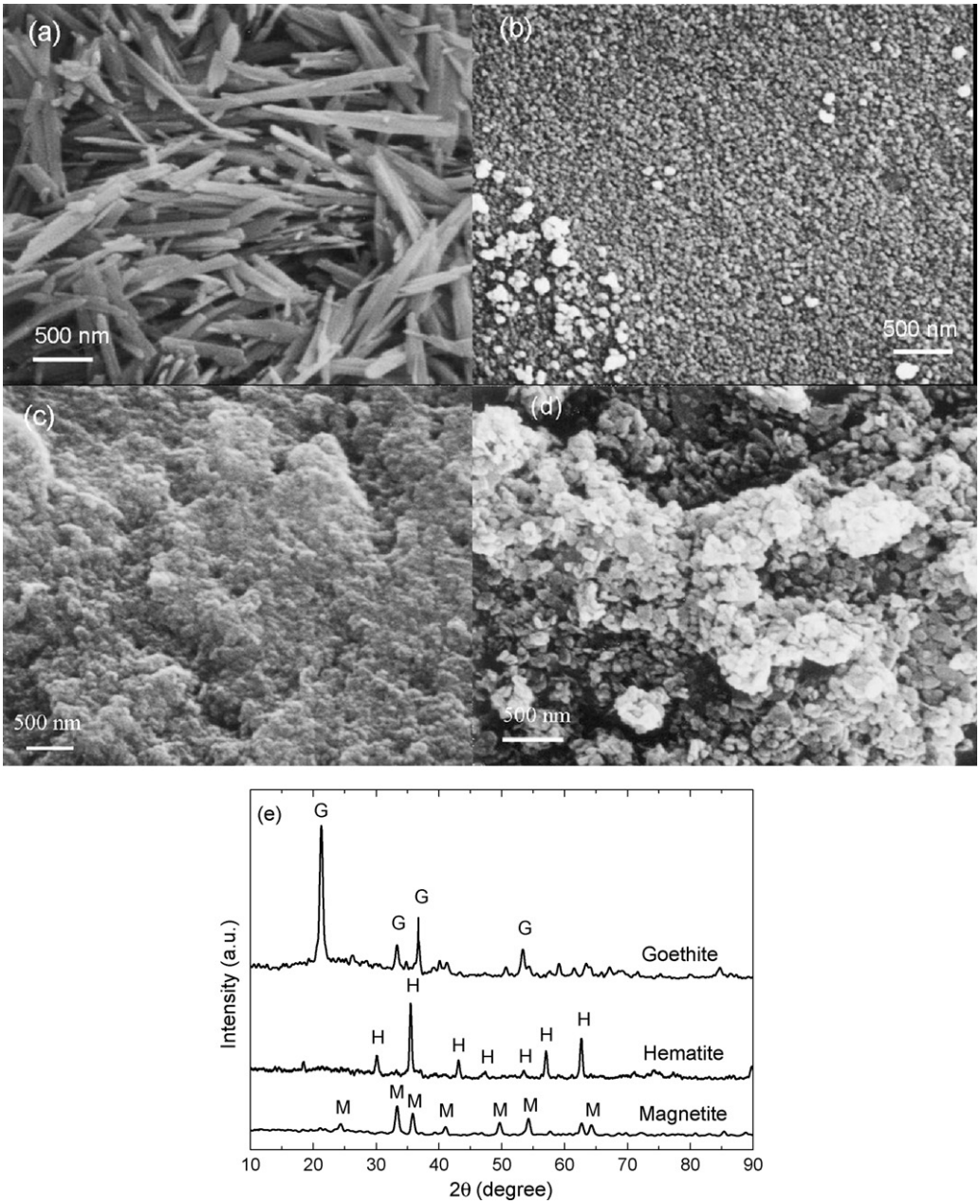


**Fig. 2.** SEM images and XRD patterns of ferric oxide minerals before and after bioreduction by *G. sulfurreducens*. Figures (a), (b) and (c) are SEM images of goethite, hematite and ferrihydrite, respectively, figure (d) is magnetite after bioreduction of ferrihydrite, while figure (e) is the XRD patterns of the biogenic ferric oxides. The unit of intensity (a.u.) means arbitrary unit.

phase of goethite and hematite is formed after bioreduction of 20 d, which is consistent with previous findings [36].

### 3.2. Kinetics of ferric oxide reduction

The production of total acid-extractable Fe(II) can be described by a pseudo-first-order rate equation in which Fe(III) reduction depends on the free reduction sites on the Fe(III) oxide surfaces [27,34]:

$$\frac{\mathrm{dFe(II)}}{\mathrm{d}t} = k_{\mathrm{obsFe}}\mathrm{Fe(III)} \quad (1)$$

$$\mathrm{Fe(II)}_t = \mathrm{Fe(III)}_0[1 - \exp(-k_{\mathrm{obsFe}}\, t)] \quad (2)$$

where $\mathrm{Fe(II)}_t$ is the concentration of acid-extractable Fe(II) at time $t$, $\mathrm{Fe(III)}_0$ is the initial concentration of ferric oxides, and $k_{\mathrm{obsFe}}$ is the pseudo-first-order rate constant for total acid-extractable Fe(II) formation. Table 1 shows the pseudo-first-order rate constants ($k_{\mathrm{obsFe}}$) for acid-extractable Fe(II) formation in the presence of *G. sulfurreducens* and electron shuttles. In the absence of electron shuttles, the $k_{\mathrm{obsFe}}$ values for total acid-extractable Fe(II) formation were $0.006 \pm 0.001$ $\mathrm{d}^{-1}$ for ferrihydrite. Addition of cysteine or AQDS accelerated the bioreduction rate of ferric oxides, and the $k_{\mathrm{obsFe}}$ values for ferrihydrite, goethite, and hematite reduction in the presence of 0.5 mM cysteine were $0.023 \pm 0.001$ ($r^2 = 0.863$), $0.005 \pm 0.001$ ($r^2 = 0.843$), and $0.006 \pm 0.001$ ($r^2 = 0.953$) $\mathrm{d}^{-1}$, respectively. Higher bioreduction rates of ferric oxides were observed when AQDS was used as the electron shuttle. The $k_{\mathrm{obsFe}}$ values for total acid-extractable

**Table 1**
The pseudo-first-order ($k_{obsFe}$) and surface area-normalized rate constants for ferric oxide reduction by *G. sulfurreducens* in the presence of electron shuttles at neutral pH

| Ferric oxide | Rate constants for ferric oxide reduction ($d^{-1}$) | | | | |
|---|---|---|---|---|---|
| | In the absence of electron shuttle | Pseudo-first-order rate constant ($k_{obsFe}$) | | Surface area-normalized rate constants ($d^{-1}\ m^{-2}$) | |
| | | Cysteine | AQDS | Cysteine | AQDS |
| Ferrihydrite | 0.006 ± 0.001 | 0.023 ± 0.001 | 0.208 ± 0.012 | 0.001 ± 0.0003 | 0.012 ± 0.001 |
| Goethite | N/A | 0.005 ± 0.001 | 0.042 ± 0.004 | 0.004 ± 0.0007 | 0.033 ± 0.003 |
| Hematite | N/A | 0.006 ± 0.001 | 0.066 ± 0.006 | 0.002 ± 0.0008 | 0.021 ± 0.001 |

Concentrations of cysteine and AQDS were 0.5 mM and 10 μM, respectively. N/A: The reduction extent was lower than 5%.

Fe(II) formation in the presence of AQDS were 0.208 ± 0.012 $d^{-1}$ ($r^2$ = 0.923) for ferrihydrite, 0.042 ± 0.004 $d^{-1}$ ($r^2$ = 0.959) for goethite, and 0.066 ± 0.006 $d^{-1}$ ($r^2$ = 0.939) for hematite.

The bioreduction extent of crystalline Fe(III) was found to be lower than that of poorly crystalline Fe(III) oxide. Roden [37] reported that both crystalline and amorphous iron oxides could be reductively dissolved to Fe(II) by *S. putrefaciens* under anaerobic conditions. The reported reduction efficiencies of hematite, goethite, and ferrihydrite were 1.5–12.6%, 0.64%, and 19.8–44.1%, respectively, which are similar to the results obtained in this study. In addition, the long-term extent of Fe(III) reduction of various ferric oxides by *Shewanella* species was found to be correlated to their surface areas. In the present study, the reduction efficiency of ferric oxides mediated by *G. sulfurreducens* in the presence of electron shuttles also correlates positively with the surface areas of the Fe(III) oxides (Fig. 3), showing that the surface area is an important factor controlling the reductive dissolution of ferric oxides by *G. sulfurreducens*. After normalization to the surface areas of ferric oxides, the normalized pseudo-first-order rate constants for Fe(II) formation in the presence of electron shuttles were 0.001–0.012 $d^{-1}m^{-2}$ for ferrihydrite, 0.004–0.033 $d^{-1}\ m^{-2}$ for goethite, and 0.002–0.021 $d^{-1}\ m^{-2}$ for hematite (Table 1). These results indicate that electron mediators may shuttle electrons to crystalline ferric oxides more efficiently than ferrihydrite.

Different electron shuttling compounds have different enhancement effects on the bioreduction of ferric oxides by *G. sulfurreducens*. Cysteine has been used to lower the redox potential both in biotic and abiotic solutions [26]. Interactions between cysteine and iron species have been addressed in several studies [34,38]. The overall reaction between cysteine and Fe(III) is a one-electron transfer process, and results in the reduction of Fe(III) to total acid-extractable Fe(II) and the oxidation of cysteine to cystine [34]. However, the role of cysteine in the electron transport system of *G. sulfurreducens* is not yet clear. On the contrary, AQDS is a more efficient electron shuttle than cysteine to enhance the bioreduction extent and rate of ferric oxides. AQDS has been proven to be an effective electron shuttling compound to increase the electron transfer rate from DIRB to iron minerals [20,21,39]. Several studies have depicted that the electron transfer of *G. sulfurreducens* to ferric oxides is through direct mineral surface–protein interaction using cytochrome-dependent and NAD-dependent Fe(III) reductase emplaced on the outer membrane [40–42]. Fig. 4 shows the concentration effect of AQDS on the bioreduction of ferrihydrite by *G. sulfurreducens* at neutral pH. The bioreduction extent of ferrihydrite increases rapidly from 20.1 ± 1.3% to 51.2 ± 2.4% upon increasing AQDS concentration from 0.05 to 10 μM, and then leveled off to a plateau (52.4 ± 3.1%) when further increased the concentration to 50 μM, clearly showing that small amounts of AQDS are sufficient to enhance the microbial reduction efficiency of ferric oxides.

### 3.3. Dechlorination of CT by biogenic iron

The production of biogenic iron species by *G. sulfurreducens* in the presence of CT and AQDS was investigated to understand the possible inhibition effect of CT on the bioreduction activity of *G. sulfurreducens*. As depicted in Fig. 5, the maximum concentrations of Fe(II) produced from ferrihydrite, goethite and hematite in the presence of CT and AQDS were 4.89 ± 0.16, 1.47 ± 0.05, and 2.04 ± 0.05 mM, respectively. These Fe(II) concentrations are comparable with those obtained in the absence of CT, which shows that the existence of CT has little effect on the bioreduction activity of *G. sulfurreducens* at the tested aqueous concentrations. It is noted that the Fe(II) concentration produced from goethite decreased slightly from 1.47 ± 0.05 mM at 6 d to 1.06 ± 0.09 mM at 15 d, presumably

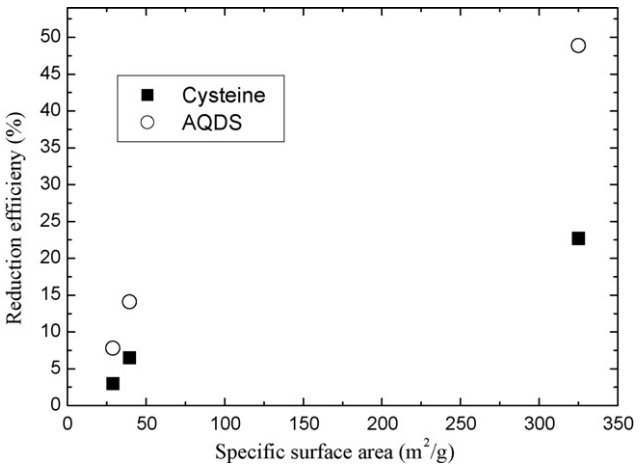


**Fig. 3.** The bioreduction extent of ferric oxides as a function of specific surface area in the presence of electron shuttles.

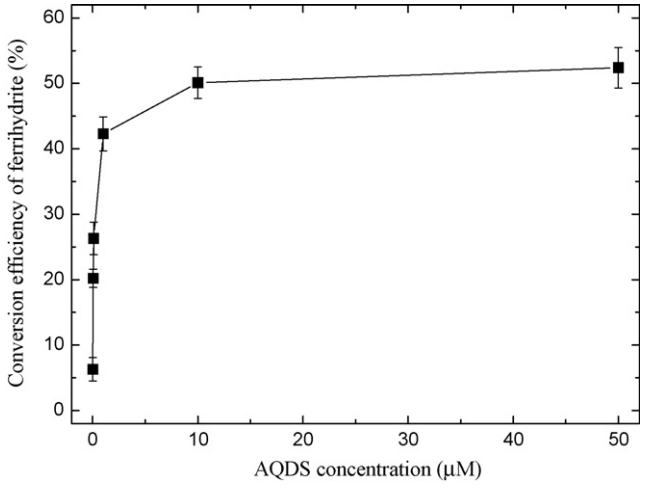


**Fig. 4.** Fraction of ferrihydrite reduced after 9 d by *G. sulfurreducens* in the presence of various concentrations of AQDS.

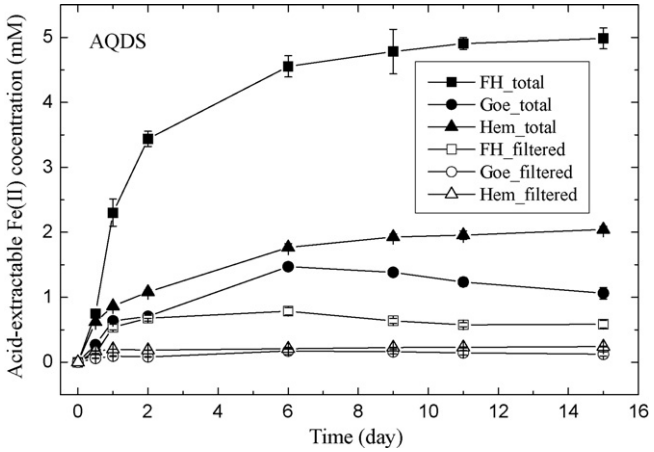


**Fig. 5.** Concentrations of Fe(II) produced from the bioreduction of 10 mM iron minerals by *G. sulfurreducens* in the presence of 2.2 μM CT (aqueous concentration) and 10 μM AQDS at neutral pH. The iron minerals used were ferrihydrite (FH, $Fe(OH)_3$), goethite (Goe, FeOOH), and hematite (Hem, $Fe_2O_3$).

due to the re-adsorption of solubilized Fe(II) onto the surface of goethite. In addition, the dissolved Fe(II) concentrations were $0.59 \pm 0.06$ mM for ferrihydrite, $0.13 \pm 0.03$ mM for goethite, and $0.24 \pm 0.02$ mM for hematite, clearly depicting that the biogenic Fe(II) is mainly adsorbed onto the surface of secondary iron oxide mineral, resulting in the formation of reactive mineral-bound Fe(II) species.

Fig. 6 shows the transformation of 3.5 μM CT in the presence of *G. sulfurreducens* and electron shuttle at neutral pH. The residual concentration of CT in ferrihydrite-amended suspensions in the presence of *G. sulfurreducens* and cysteine were 0.25 μM after incubation of 20 d, which corresponds to 92% of CT reduction. A 59% decrease in CT concentration resulting from the abiotic reduction by cysteine alone was also observed in the absence of *G. sulfurreducens*. In addition, the change in CT concentrations in the presence and absence of *G. sulfurreducens* were similar when crystalline ferric oxides including goethite and hematite were added to the suspensions, depicting that microbial activity has little effect on the transformation of CT in the presence of crystalline iron oxide when cysteine was added as the electron shuttle.

The ability of *G. sulfurreducens* to reduce CT in the presence of Fe(III) oxides and 10 μM AQDS was examined. No significant decrease in CT concentration was observed when 10 μM AQDS was added to the solutions in the absence of *G. sulfurreducens* and ferric oxides (Fig. 6b), depicting that AQDS alone cannot facilitate the transformation of CT under anaerobic conditions. However, 31% of CT was transformed by *G. sulfurreducens* in the absence of ferric oxide but contained AQDS. In addition, less than 5% of CT was reduced by *G. sulfurreducens* after incubation of 20 d in the absence of AQDS (data not shown), which means that AQDS can be reduced by *G. sulfurreducens* to form AHQDS, and then the reduced AQDS serves as an effective reductant to directly transfer electrons to CT for transformation. Moreover, addition of ferric oxides accelerated the extent and rate of CT dechlorination mediated by *G. sulfurreducens* and AQDS. The transformation efficiencies of CT in the presence of ferrihydrite, goethite and hematite were 59, 85, and 93%, respectively, after incubation of 16 d, which reflects the fact that the transformation of CT in the presence of ferric oxides is primarily attributed to the formation of mineral-bound Fe(II) species. It is noted that the added concentrations of electron shuttles (10 μM and 0.5 mM) were higher than that of CT (3.5 μM). The AQDS and cysteine could be reduced by *G. sulfurreducens* at a single time and can reduce CT without diminishing their concentration too much,

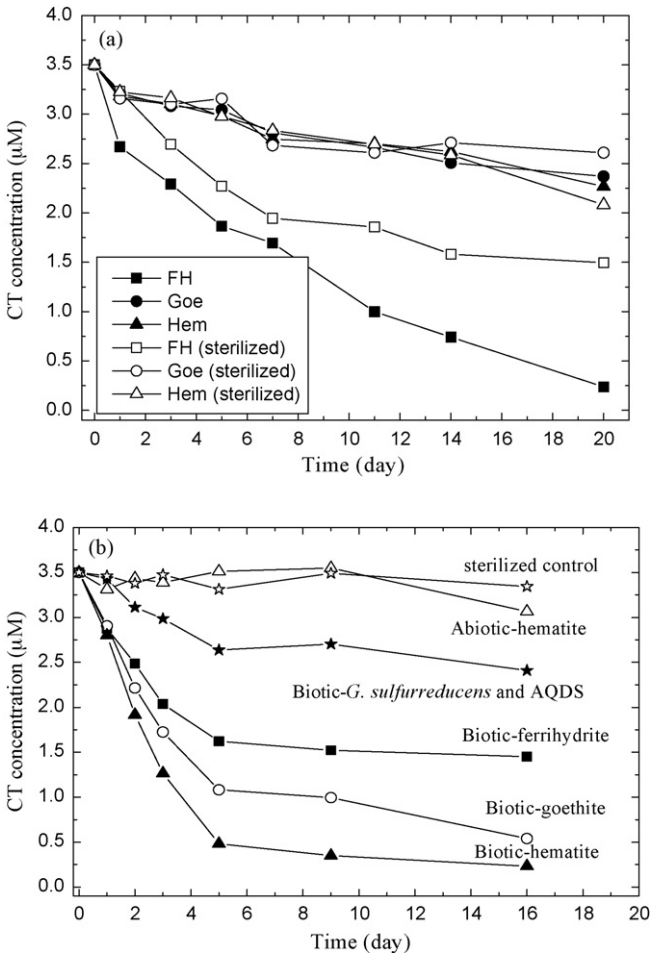


**Fig. 6.** Transformation of 3.5 μM CT mediated by *G. sulfurreducens* in the presence of (a) cysteine and (b) AQDS at neutral pH. The microbial system contained 20 mM acetate and 10 mM iron minerals (ferrihydrite, goethite, and hematite). Concentrations of cysteine and AQDS were 0.5 mM and 10 μM, respectively.

which means that the shuttling effect of AQDS and cysteine on CT reduction in this study was not obvious.

Chloroform was found to be the only identified chlorinated product in the *G. sulfurreducens* suspensions. Formation of chloroform accounted for 16–31% of the consumed CT (Fig. 7). The

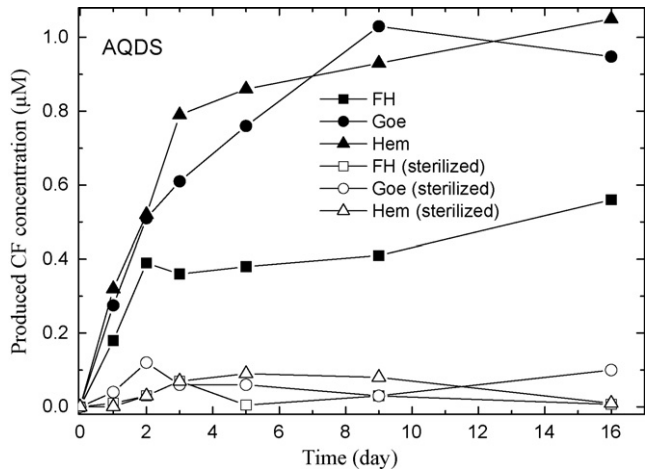


**Fig. 7.** Concentration profiles of CF ($CHCl_3$) produced from the reductive dechlorination of CT in the ferric oxide suspensions containing *G. sulfurreducens* and AQDS.

**Table 2**
The pseudo-first-order ($k_{obsCT}$) and surface area-normalized rate constants for CT reduction in the presence of *G. sulfurreducens* and electron shuttles at neutral pH

| Ferric oxide | The rate constants for CT reduction ($d^{-1}$)[a] | | | | | |
|---|---|---|---|---|---|---|
| | | | Biotic reaction | | | |
| | Abiotic reaction | | Pseudo-first-order rate constant ($k_{obsCT}$) | | Surface area-normalized rate constants ($d^{-1}\,m^{-2}$) | |
| | Cysteine | AQDS | Cysteine | AQDS | Cysteine | AQDS |
| Ferrihydrite | 0.057(0.085)[b] | N/A[c] | 0.119 (0.177) | 0.163 (0.243) | 0.007 (0.011) | 0.009 (0.014) |
| Goethite | 0.013(0.019) | N/A | 0.018 (0.027) | 0.241 (0.359) | 0.014 (0.021) | 0.188 (0.280) |
| Hematite | 0.018(0.027) | N/A | 0.019 (0.028) | 0.354 (0.527) | 0.006 (0.009) | 0.112 (0.167) |

Concentrations of cysteine and AQDS were 0.5 mM and 10 μM, respectively.
[a] The $k_{obsCT}$ for CT reduction in the presence of *G. sulfurreducens* and AQDS but contained no ferric oxide was 0.055 $d^{-1}$.
[b] The value in parentheses is the headspace partitioning corrected pseudo-first-order rate constants for CT reduction ($k'_{obsCT}$).
[c] N/A: The reduction extent was lower than 5%.

low carbon mass balance (40–56%) through biotic pathways in the *G. sulfurreducens* suspensions suggests the formation of non-chlorinated compounds that cannot be detected by GC–ECD in headspace. When GC–MS and GC–FID were used to identify the formation products during the CT transformation experiment, however, no product was found in headspace samples, presumably due to the formation of non-volatile products. Several studies have reported the formation of low concentrations of CF, dichloromethane ($CH_2Cl_2$), methane ($CH_4$), cell-bound products, and other unidentified products from the studies of CT dechlorination by the cultures of *G. metallireducens* and *S. putrefaciens* [28,29]. McCormick and Adriaens [29] investigated the dechlorination of 13 μM CT by nanoscale biogenic magnetite/maghemite particles produced by the *G. metallireducens* and found that CF, CO, $CH_4$, and a trace amount of tetrachloroethylene were identified as the products. This results implies that processes other than reductive dechlorination are also involved in CT transformation mediated by *G. sulfurreducens* and AQDS under iron-reducing conditions.

The kinetics of biological degradation of organic compounds generally followed the Monod kinetics. Fredrickson et al. [35] investigated the reduction of ferrihydrite by *S. putrefaciens* in the presence of phosphate, and found that the enhanced reduction of Fe(II) was not due to changes in cells population density. In addition, previous studies suggested that the increased transformation of CT by *S. putrefaciens* or *G. metallireducens* was due to the production of biogenic surface-bound or structural Fe(II). Therefore, the pseudo-first-order reaction kinetics was used to describe the transformation of CT by *G. sulfurreducens*:

$$\ln\left(\frac{C_{CTt}}{C_{CT0}}\right) = -k_{obsCT}\,t \quad (3)$$

where $C_{CT0}$ is the initial concentration of CT, $C_{CTt}$ is the concentration of CT at time $t$, and $k_{obsCT}$ is the pseudo-first-order rate constants for CT transformation. Several studies [43,44] have corrected values of $k_{obsCT}$ to account for the effects of partitioning of chlorinated hydrocarbons between the aqueous and gas phases. The corrected $k_{obsCT}$ values ($k'_{obsCT}$)can be given by:

$$k'_{obsC} = k_{obsCT}\left(1 + H_c\frac{V_g}{V_w}\right) \quad (4)$$

where $H_c$ is the dimensionless Henry's law constant for CT (1.244 at 25 °C) [33], $V_g$ and $V_W$ are the volumes of the gas and aqueous phases. Table 2 shows the $k_{obsCT}$ ($k'_{obsCT}$) values for CT transformation in the presence of *G. sulfurreducens* and electron shuttles. In the presence of 0.5 mM cysteine, the $k_{obsCT}$($k'_{obsCT}$) for CT transformation in goethite- and hematite-amended solution in the presence and absence of *G. sulfurreducens* were similar (0.018–0.019 $d^{-1}$) (0.027–0.028 $d^{-1}$). This may be due to the low bioreduction extent of crystalline ferric oxides by *G. sulfurreducens*, and results in the production of small amount of biogenic Fe(II)–Fe(III) species for CT transformation. On the contrary, the $k_{obsCT}$($k'_{obsCT}$) for CT transformation in the presence of ferrihydrite and *G. sulfurreducens* was 0.119(0.177) $d^{-1}$ ($r^2$ = 0.973), which is 2 times higher than that in the absence of *G. sulfurreducens* (0.057(0.085) $d^{-1}$, $r^2$ = 0.931), proving again that biogenic iron species play a crucial role in facilitating the transformation reaction of CT under iron-reducing conditions.

Addition of AQDS produced higher Fe(II) concentrations than cysteine to form the reactive surface-bound iron species for CT transformation. In the absence of ferric oxide, the $k_{obsCT}$($k'_{obsCT}$) for CT biotransformation mediated by *G. sulfurreducens* in the presence of AQDS was 0.055 $d^{-1}$ ($r^2$ = 0.974), while the $k_{obsCT}$($k'_{obsCT}$) values in the ferrihydrite-, goethite-, and hematite-containing solutions were 0.163(0.243) ($r^2$ = 0.982), 0.241(0.359) ($r^2$ = 0.986), and 0.354(0.527) $d^{-1}$ ($r^2$ = 0.935), respectively, when 10 μM AQDS was added as the electron shuttle.

Although the total acid-extractable Fe(II) produced from ferrihydrite was higher than those from goethite and hematite, its capability in the reduction of CT is low. The produced concentration of biogenic Fe(II) by crystalline iron oxides was found to be 2.8–7.6 times lower than that obtained in the ferrihydrite suspensions. However, the surface area-normalized rate constants for CT transformation were 0.007–0.009 (0.011–0.014), 0.014–0.188 (0.021–0.280), and 0.006–0.112 (0.009–0.167) $d^{-1}m^{-2}$ when solutions contained ferrihydrite, goethite and hematite, respectively (Table 2). These results were, by factors of 2.0–21, higher than that obtained using ferrihydrite. Maithreepala and Doong [45] reported that addition of 3 mM Fe(II) to different iron oxide suspensions significantly enhanced the degradation rate of CT, and the rate constant for CT dechlorination followed the order hematite, goethite, magnetite, and then ferrihydrite. In this study, the XRPD patterns and SEM images showed that ferrihydrite was biotransformed to magnetite, while goethite and hematite remained their original mineral phases after bioreduction by *G. sulfurreducens*. This depicts that goethite and hematite have high reactivity towards the transformation of CT in the presence of *G. sulfurreducens* under iron-reducing conditions. It is noted that the transformation efficiencies of CT in the presence of Fe(III) oxides were 3.0–6.4 times higher than that mediated by *G. sulfurreducens* and AQDS in the absence of ferric oxide. This finding indicates that the transformation of CT in the presence of *G. sulfurreducens* and iron minerals may be attributed to the transformation by biogenic iron species.

## 4. Conclusions

In this study, we demonstrated the contribution of abiotic and biotic reactions of various ferric oxide minerals to the transformation of CT. Both cysteine and AQDS were found to enhance the reduction efficiency of ferric oxides by *G. sulfurreducens*. The bioreduction extent of ferric oxides by *G. sulfurreducens* followed the order ferrihydrite > hematite > goethite. After normalization to the

surface area, goethite was found to be the most effective ferric oxide to reduce CT via biogenically produced surface-bound iron species. Although the total amounts of the produced biogenic Fe(II) by crystalline iron oxides (0.30–1.72 mM) were 2.8–7.6 times lower than that obtained in the ferrihydrite suspensions (2.27–4.83 mM), the surface area-normalized rate constants for CT transformation in solutions containing goethite and hematite were 2–21 times higher than those obtained for ferrihydrite. In the natural environment, bicarbonate ion is the naturally occurring buffer system, and the HEPES buffered solution used in this study are not directly applicable to natural systems in which carbonate is likely to be present. However, results obtained in this study are still helpful in facilitating the development of processes that could be useful for the enhanced transformation of chlorinated hydrocarbons in the contaminated sites containing ferric oxides and DIRB.

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