The ISME Journal (2009) 3, 466–476

www.nature.com/ismej

ORIGINAL ARTICLE

Thermodynamic targeting of microbial perchlorate reduction by selective electron donors

James Ian Van Trump and John D Coates
Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA

Here we describe 2,6-anthrahydroquinone disulfonate (AH_2DS) as a model thermodynamically 'targeting' electron donor capable of selectively stimulating respiratory processes relevant to the bioremediation of perchlorate. Pure cultures of *Dechloromonas aromatica*, *Dechloromonas agitata* and *Azospira suillum*, as well as uncharacterized microbial consortia, were capable of stoichiometrically reducing perchlorate to chloride upon oxidation of AH_2DS to the corresponding quinone 2,6-anthraquinone disulfonate (AQDS). No degradation of the anthraquinone structure was observed, and no organism tested grew by this metabolism. Thermodynamic calculations suggest that AH_2DS oxidation should support nitrate and perchlorate reduction, whereas sulfate reduction and methanogenesis are predicted to be unfavorable. Mixed community microcosms oxidizing AH_2DS reduced nitrate and perchlorate, whereas sulfate reduction never occurred. In contrast, microcosms amended with acetate respired nitrate, perchlorate and sulfate, as would be predicted by thermodynamic calculation. Our results suggest that the thermodynamic properties of hydroquinones allow for targeted stimulation of only a subset of potential respiratory processes. This observation could help improve enhanced *in situ* bioremediation of perchlorate by negating many of the detrimental aspects of biofouling.
The ISME Journal (2009) **3,** 466–476; doi:10.1038/ismej.2008.119; published online 18 December 2008
Subject Category: microbial engineering
Keywords: perchlorate; bioremediation; hydroquinone; humic; nitrate; respiration

Introduction

Perchlorate (ClO_4^-) is a common environmental contaminant that adversely affects mammalian thyroid gland function (Stanbury and Wyngaarden, 1952). Although chemically stable, a wide variety of microorganisms can reduce ClO_4^- to chloride by respiration, supporting energy generation and growth (Coates and Achenbach, 2004, 2006). Such dissimilatory ClO_4^--reducing bacteria (DPRB) are considered ubiquitous (Coates *et al.*, 1999; Coates and Achenbach, 2004). This observation, combined with the efficacy of ClO_4^- respiration to produce innocuous end products, is the basis of bioremediative strategies to effectively ameliorate ClO_4^- contamination in the environment.

DPRB metabolism *in situ* can be stimulated through addition of electron donors such as acetate, citrate or ethanol to the contaminated area. ClO_4^- reduction is inhibited at the genetic level by the presence of oxygen and, to some extent, nitrate in many bacteria (Bender *et al.*, 2002; Chaudhuri *et al.*, 2002; O'Connor and Coates, 2002; Coates and Achenbach, 2006). Therefore, initial electron donor additions are generally devoted to biologically removing these competing electron acceptors. Once this is accomplished, optimal ClO_4^- reduction can commence. Unfortunately, repeated addition of excessive amounts of electron donors to the environment can complicate the remediation process (Coates and Jackson, 2008). Continuous amendments with labile organic substrates can create conditions within injection wells and surrounding filter packs conducive to the outgrowth of non-ClO_4^--reducing microorganisms, resulting in the establishment of iron-reducing, sulfate-reducing or methanogenic populations within the treatment area (Coates and Achenbach, 2006). This results in an ineffective treatment of the target contaminant, inefficient use of electron donor, plugging of the near-well aquifer matrix, changes in mineral content, hydraulic conductivity, and pH, and a reduction in water quality through direct or indirect release of undesirable end products (Fe(II), HS^-, CH_4 sorbed heavy metals, and so on.) (Ponnamperuma, 1972, 1984; Taylor and Jaffe, 1990; Cunningham *et al.*, 1991; Vandevivere

Correspondence: J Coates, Department of Plant and Microbial Biology, University of California, Berkeley, 271 Koshland Hall, Berkeley, CA 94720, USA.
E-mail: jcoates@nature.berkeley.edu
Received 16 May 2008; revised 2 November 2008; accepted 4 November 2008; published online 18 December 2008

and Baveye, 1992; Coates and Achenbach, 2001, 2006; Khan and Spalding, 2003).

Therefore, control of the respiratory activity in the course of electron donor addition is of paramount importance. An ideal donor would stimulate reduction of electron acceptors relevant to the remediation of ClO_4^- (oxygen, nitrate and ClO_4^-), while precluding undesirable respiratory activities (iron, sulfate and CO_2 reduction) and preventing gross overgrowth of microbial biomass (Coates and Jackson, 2008). Efforts, including pulsed injection of electron donor (Khan and Spalding, 2003, 2004), use of slowly hydrolyzing organic polymers (Wu *et al.*, 2001) or utilization of inorganic electron donors such as Fe(II) (Bruce *et al.*, 1999; Coates *et al.*, 1999; Chaudhuri *et al.*, 2001; Lack *et al.*, 2002), reduced or elemental sulfur compounds (Bruce *et al.*, 1999; Coates *et al.*, 1999; Ju *et al.*, 2007) or H_2 gas (Miller and Logan, 2000; Nerenberg and Rittmann, 2004; Yu *et al.*, 2006; Thrash *et al.*, 2007), have been utilized in attempts to resolve some of these concerns. In a similar vein, this study illustrates that microbial respiratory activity can be simultaneously stimulated and controlled based on the reduction potential of a thermodynamically poised electron donor. In this method, the thermodynamics of the respiratory process are harnessed to restrict microbial respiration to the desired electron-accepting processes.

To target stimulation of desired respiratory activities only, the electron donor should have an intermediate reduction potential sufficiently electronegative to stimulate reduction of oxygen, nitrate and ClO_4^-, but sufficiently electropositive to bar reduction of compounds such as sulfate and carbon dioxide. Such a donor would support biological removal of ClO_4^- from the area of contamination, and then diffuse to other areas containing oxygen, nitrate or ClO_4^- without contributing to an alternative respiratory activity. Reduced quinones (hydroquinones) represent thermodynamically selective electron donors of this type. Many bacteria are capable of oxidizing hydroquinones to quinones in a non-degradative electron transfer, supporting processes such as nitrate reduction (Lovley *et al.*, 1999; Lack *et al.*, 2002). The redox potential of the quinone/hydroquinone couple varies with the overall structure of the parent molecule. Therefore, a quinoid molecule containing a reduction potential ideal for ClO_4^- reduction could potentially be selected.

In this study, the model hydroquinone, 2,6-anthrahydroquinone disulfonate (AH_2DS), is shown to function as an electron donor supporting ClO_4^- reduction by both pure DPRB cultures and uncharacterized microbial consortia. This donor stimulates microbial activity while limiting microbial growth, and furthermore, exhibits redox characteristics allowing the compound to selectively stimulate only a portion of potential respiratory processes. The midpoint reduction potential of 2,6-anthraquinone disulfonate (AQDS)/AH_2DS ($E_o = -0.184$ V) is capable of stimulating nitrate and ClO_4^- reduction, but is too electropositive to support sulfate reduction or methanogenesis (< -0.217 V). Our results suggest that the reduction potential of electron donors influence the types of microbial respiration supported, and imply a possible mechanism for controlling respiratory behavior.

Materials and methods

Media techniques

All experiments were carried out in sealed N_2–CO_2 (80:20) sparged vessels containing anoxic basal bicarbonate-buffered freshwater media (pH = 6.8–7.0) (Bruce *et al.*, 1999). Media containing AH_2DS was prepared by adding AQDS (1, 5 or 10 mM) to basal media, then reducing the anthraquinone with H_2 in the presence of palladium as previously described (Coates *et al.*, 2001a, b). Subsequent sparging of the media with N_2–CO_2 for 20 min served to remove dissolved H_2. Additions of electron donors or acceptors were from anoxic sterile stock solutions of the respective sodium salts.

Analytical techniques

Anions were quantified by ion chromatography on a Dionex DX500 system using a CD20 conductivity detector suppressed by an ASRS–ULTRA II 4-mm suppressor system. ClO_4^- samples were resolved on an IonPac AS16 anion exchange column as previously described (Thrash *et al.*, 2007). Nitrate and sulfate analyses were performed using an IonPac AS9-HC anion exchange column, with a 9 mM Na_2CO_3 mobile phase at a flow rate of 1 ml min^{-1}. Acetate was quantified by high-performance liquid chromatography analysis using an Aminex Fast Acid column with a 0.02 N sulfuric acid mobile phase coupled to ultraviolet irradiation detection at 210 nm by a Shimadzu SPD-10A UV-VIS detector.

Reducing equivalents of AH_2DS were quantified by ferric citrate back titration or spectrophotometric determination at 450 nm as previously described (Lovley *et al.*, 1996, 1998; Coates *et al.*, 1998). Total and ferrous soluble iron concentrations were determined as described earlier (Coates *et al.*, 1998). Total sulfide analysis was accomplished by acidification of a sample with 3 N HCl in a sealed anaerobic bottle of known volume. Gas samples were subjected to total sulfide determination (Cline, 1969).

Pure culture incubations

In the case of *Dechloromonas aromatica* and *Dechloromonas agitata*, 45 ml of basal media containing AH_2DS, AQDS (10 mM total anthraquinone) or without quinone were prepared and anaerobically dispensed into 120 ml serum bottles. Incubations were amended with ClO_4^- where noted, as well as 0.1 mM acetate as a potential carbon source. Similar experiments were performed with *Azospira suillum*

in pressure tubes filled with 10 ml of media (5 mM total anthraquinone). Incubations were maintained in the dark at 30 °C, and concentrations of AH_2DS and ClO_4^- were monitored over time. Cells were counted by phase-contrast microscopy with a Hausser Scientific counting chamber. Experiments examining the dual oxidation of AH_2DS and acetate by pure cultures of *D. aromatica* were performed as described above, except that additional acetate was added to incubations as indicated. To investigate degradation of AQDS by pure cultures of DPRB, total anthraquinone concentrations were resolved by absorbance at 320 nm of aerated samples shaken for 30 s as described earlier (Coates *et al.*, 2002).

Oxidation of AH_2DS and ClO_4^- reduction by sediment microorganisms

Sediments were collected from the bank of Strawberry Creek on the UC Berkeley Campus, within 25 cm from the edge of the creek. These sediments were mixed with those collected from the bed of the creek itself, about 25 cm within the water's edge. Sediments were diluted 1:10 by mixing with sterilized low-Fe Abbot Pit sand to lower the labile organic content of the sediments. Each of the series of serum bottles received approximately 20 g of the sand/sediment mixture. These were then degassed with N_2–CO_2 (80:20) for 20 min and sealed. Basal medium (20 ml) with or without AH_2DS (10 mM total quinone) and amended with ClO_4^- was added. Bottles were incubated in the dark at 30 °C, and tested for ClO_4^- reduction and hydroquinone oxidation.

Iron-oxidizing cell suspensions

Anoxic serum bottles filled with 45 ml of basal medium containing AH_2DS (1 mM total quinone) or lacking anthraquinone were amended with ClO_4^-. Fe(III) as synthetic HFO (hydrous ferric oxide) (Lovley and Phillips, 1986) was aliquoted from 50 mM stock solutions to the bottles (final concentration, approximately 0.5 mM Fe(III)). Fe species were allowed to react chemically with the AH_2DS and/or media components overnight in the dark at 30 °C before addition of cells. Subsequently, 1 ml of a washed cell suspension of *A. suillum* strain PS, generated as described earlier (Thrash *et al.*, 2007), was used to inoculate experimental bottles. Heat-killed controls were boiled for 10 min before inoculation. AH_2DS and ClO_4^- concentrations were determined in subsamples collected by N_2-flushed syringes. Similarly, Fe(II) concentrations were determined by ferrozine assay (Stookey, 1970) in 1 ml subsamples after 1 h extraction with 0.1 ml of anoxic 6 N HCl in nitrogen-sparged serum bottles.

Targeting microcosms

Prepared microcosms were inoculated with cells washed from Strawberry Creek sediment. Approximately 3 g of fresh Strawberry Creek sediments were transferred to a sterile 120 ml serum bottle, and aseptically purged with N_2–CO_2 (80:20). Aliquots (20 ml) of basal medium containing 0.1% (v/v) sodium pyrophosphate were added to sediments, which were shaken in the dark at room temperature for 1 h. The liquid phase was then transferred to a sterile, N_2–CO_2-sparged bottle. 5 ml of this suspension was autoclaved for 30 min to serve as a sterilized control. Microcosms consisted of serum bottles containing 45 ml of anaerobic basal medium with an N_2–CO_2 (80:20) headspace. Medium was amended with AH_2DS, AQDS (5 mM total anthraquinone) or lacked anthraquinone entirely. Microcosms were amended with nitrate, ClO_4^-, sulfate and 250 μM acetate before any analysis. An acetate-based microcosm was amended with additional acetate to a total 1.25 mM to match the reducing equivalents present in AH_2DS-containing microcosms. Before adding additional Fe(III), AH_2DS and 0.5 N HCl-extractable Fe(II) were quantified through the ferrozine assay and back titrations. After quantification, Fe(III) chelated with nitrilotriacetic acid (Fe(III)-NTA) was added to each microcosm, which were then incubated in the dark at 30 °C overnight. Subsequently, AH_2DS and 0.5 N HCl-extractable Fe(II) were again quantified immediately after inoculation with either viable or autoclave-killed cells. Concentrations of AH_2DS, total anthraquinone, nitrate, nitrite, ClO_4^-, sulfate, acetate, 0.5 N HCl-extractable Fe(II) and acid volatile (3 N HCl) sulfide were monitored over time.

Results

Oxidation of AH_2DS coupled to ClO_4^- reduction

Active cultures of *D. aromatica*, *D. agitata* and *A. suillum*, three representatives of environmentally dominant DPRB, readily reduced ClO_4^- to chloride in the presence of AH_2DS, as shown in Figure 1 for *A. suillum* strain PS. Heat-killed controls neither oxidized AH_2DS nor reduced ClO_4^-, indicating that this reaction was mediated enzymatically (Figure 1). Under ClO_4^--limiting conditions, oxidation of AH_2DS by strain PS ceased on depletion of ClO_4^- from the medium (Figure 1 Supplementary information); conversely, ClO_4^- reduction halted on complete oxidation of available acetate and AH_2DS (Figure 2). For all tested DPRB, total anthraquinone concentrations remained constant throughout the experiment, suggesting that neither AH_2DS nor AQDS were biodegraded (data not shown). The oxidation of 766 μM AH_2DS resulted in the reduction of 211 μM ClO_4^- beyond AH_2DS-free controls (data not shown), giving a stoichiometry of 3.6 ± 0.6 (mean ± s.d., $n = 3$), which is 92.5% of the theoretical molar ratio of 4 according to

$$4C_{14}H_8O_8S_2^{2-}(AH_2DS) + ClO_4^- \rightarrow 4C_{14}H_6O_8S_2^{2-}(AQDS) + Cl^- + 4H_2O \quad (1)$$

Interestingly, none of the pure cultures tested were observed to grow by AH_2DS-dependent ClO_4^-

reduction, even when 0.1 mM acetate was provided as a suitable carbon source (Figure 2 Supplementary information). Cell numbers observed between AH_2DS-oxidizing cultures and cultures containing 0.1 mM acetate alone were not reliably statistically different for all DPRB tested. This result was unexpected, as earlier studies have shown that several organisms, including DPRB, rapidly grow with AH_2DS as electron donor and nitrate as the electron acceptor (Coates *et al.*, 2001a, b, 2002), (JI Van Trump, unpublished data).

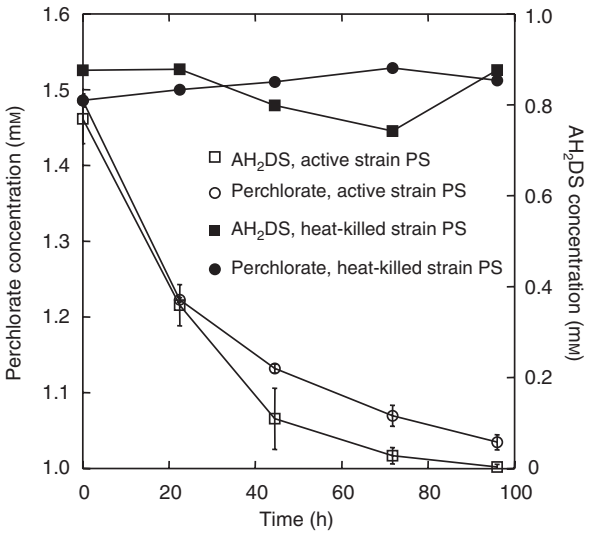

Figure 1 Microbial AH_2DS oxidation coupled to perchlorate reduction by *Azospira suillum* strain PS. Live cultures of PS oxidized AH_2DS (open squares) concomitant with perchlorate reduction (open circles). Heat-killed cultures of PS neither oxidized AH_2DS (closed squares) nor reduced perchlorate (closed circles).

Simultaneous oxidation of acetate and AH_2DS by D. aromatica

Whereas acetate can be utilized as both a carbon source and an electron donor for microbial respiration, AH_2DS is utilized solely as an electron donor. To determine whether or not DPRB would preferentially utilize acetate over AH_2DS, an active anaerobic culture of *D. aromatica* was used to inoculate anaerobic medium supplemented with a mixture of acetate and AH_2DS. A rapid simultaneous oxidation of both electron donors was observed (Figures 2a and b), and approximately 1.4 mM acetate (Figure 2a) and 3.3 mM AH_2DS (Figure 2b) were oxidized within 22 h with 3.5 mM ClO_4^- (Figure 2c). The presence of the AH_2DS showed no significant effect on the rate of oxidation of the acetate (Figure 2a), whereas the extra electron-donating capacity of the mixed acetate/AH_2DS medium significantly enhanced the

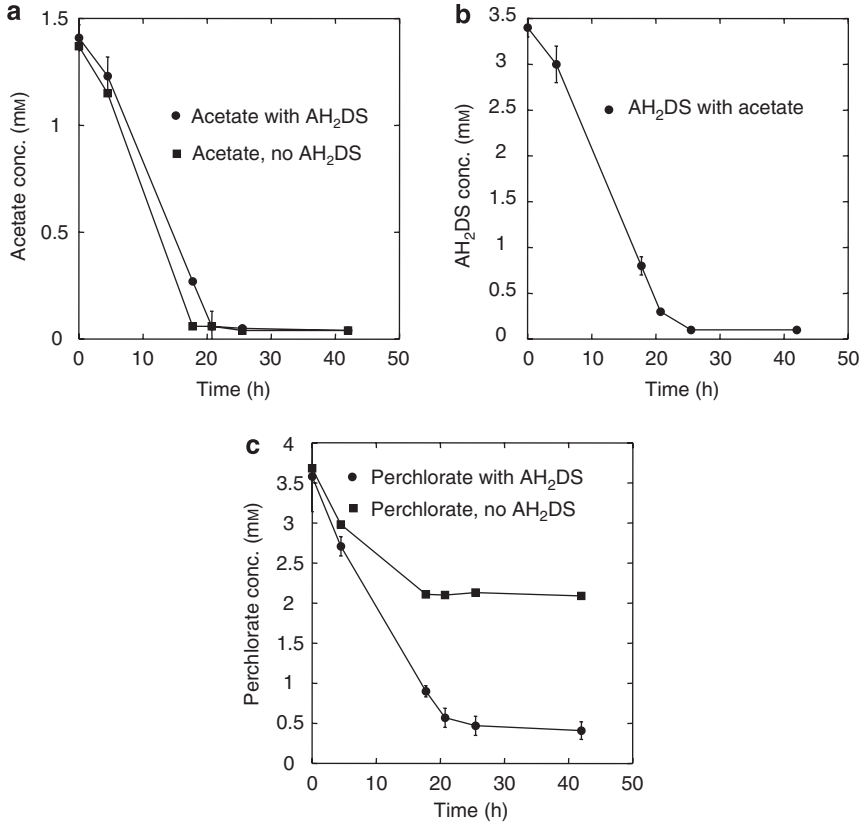

Figure 2 Relative rates of acetate and AH_2DS oxidation by *Dechloromonas aromatica* RCB in the presence of perchlorate. (**a**) Oxidation of acetate by strain RCB in the presence of AH_2DS (closed circles) or in absence of the hydroquinone (closed squares). (**b**) Oxidation of AH_2DS by RCB over the same time period. (**c**) Total perchlorate reduction in the presence of AH_2DS (closed circles) and with acetate only (closed squares).

extent of ClO_4^- reduction (Figure 2c). Specifically, the presence of 3.5 mM AH_2DS resulted in the reduction of an additional 1.5 mM ClO_4^- over acetate-only treatments (Figure 2c), which is more than that would be predicted on the basis of equation (1).

Oxidation of AH_2DS by an uncharacterized microbial community

Microcosm studies were used to show that AH_2DS could stimulate ClO_4^- reduction with an undefined microbial community present in sediments. Rapid and sustained oxidation of 3.8 mM AH_2DS was observed (Figure 3a), with concomitant reduction of almost 1.15 mM ClO_4^- (Figure 3b). Some ClO_4^- reduction (0.26 mM) was observed in control microcosms not amended with AH_2DS, indicating the presence of an electron donor intrinsic to the sediment (Figure 3b). When the indigenous electron-donating capacity of the sediment is taken into account, the ratio of AH_2DS oxidized to ClO_4^- reduced was 4.4 ± 0.8 ($n = 3$), in close agreement with the theoretical molar ratio of 4 shown in equation (1) above.

The role of Fe(III)

AH_2DS is sufficiently electronegative to abiotically reduce several Fe(III) mineral forms (Weber *et al.*, 2006). This reaction could potentially prevent the reducing equivalents of AH_2DS from acting as electron donors for microorganisms *in situ*. However, reaction of AH_2DS with Fe(III) generates Fe(II), and all DPRB screened to date, including members of both the *Dechloromonas* and *Azospira* genera, can oxidize Fe(II) (Lack *et al.*, 2002). As expected, the addition of Fe(III) to sterile media containing AH_2DS resulted in the oxidation of AH_2DS to AQDS and concomitant reduction of Fe(III) to Fe(II) (Figure 4a). Interestingly, oxidation of the initial 165 μM AH_2DS was incomplete with the addition of approximately 500 μM Fe(III)-HFO, indicating that some of the iron species were not readily reduced by AH_2DS. After 27.5 h incubation, the sterile microcosms were inoculated with an active washed cell suspension of *A. suillum* strain PS. Addition of strain PS resulted in rapid and complete oxidation of the remaining AH_2DS (Figure 4b). Consistent with earlier findings for DPRB (Chaudhuri *et al.*, 2001; Lack *et al.*, 2002), Fe(II) generated by the AH_2DS/Fe(III)-HFO reaction was simultaneously oxidized along with the residual AH_2DS by strain PS (Figure 4a). In contrast, no Fe(II) or AH_2DS oxidation was observed in heat-killed controls. Oxidation of Fe(II) and AH_2DS resulted in complete reduction of ClO_4^- to chloride (Figure 4c), and Fe(II) oxidation halted on depletion of ClO_4^- from the medium (Figures 4a and c). Total oxidation of AH_2DS and Fe(II) accounted for 83% of the additional ClO_4^- reduction observed in AH_2DS-containing bottles relative to bottles lacking hydroquinone, indicating that the reducing equivalents abiotically transferred to Fe(III) were still bioavailable for the microbial reduction of ClO_4^-.

Electron donor 'targeting'

To show that AH_2DS could selectively stimulate microbial ClO_4^- reduction, sediment microcosms were amended with a mixture of nitrate, ClO_4^-, Fe(III)–NTA, and sulfate as potential electron acceptors. AH_2DS-based and acetate-based microcosms contained equivalent electron-donating capacity based on a theoretical two-electron oxidation of AH_2DS to AQDS and an eight-electron oxidation of acetate to CO_2. As such, the type of electron donor, rather than the extent of electron-donating capacity, was compared. Importantly, AH_2DS microcosms contained a small amount (250 μM) of acetate to aid

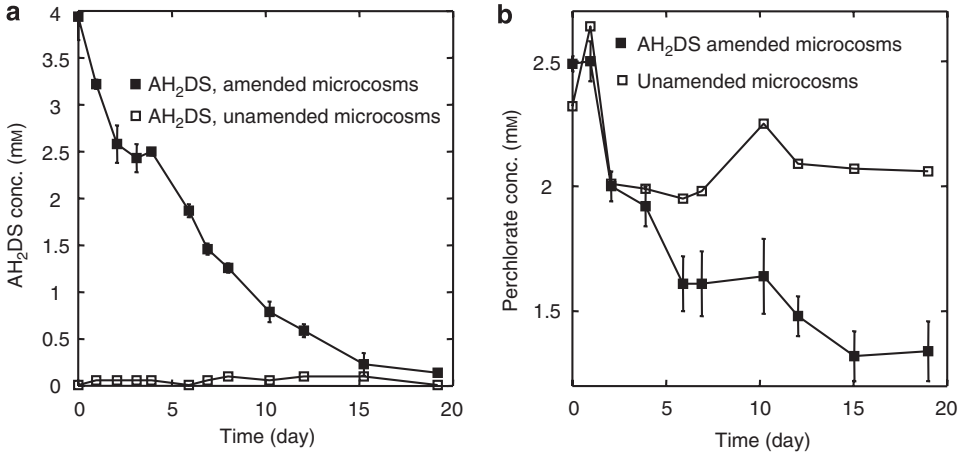

Figure 3 Oxidation of AH_2DS coupled to perchlorate reduction in uncharacterized microbial community microcosms. (**a**) AH_2DS concentrations in microcosms amended with AH_2DS (closed squares) and those without externally added electron donor (open squares). (**b**) Perchlorate reduction in sediment microcosms amended with AH_2DS (closed squares) and without donor amendment (open squares).

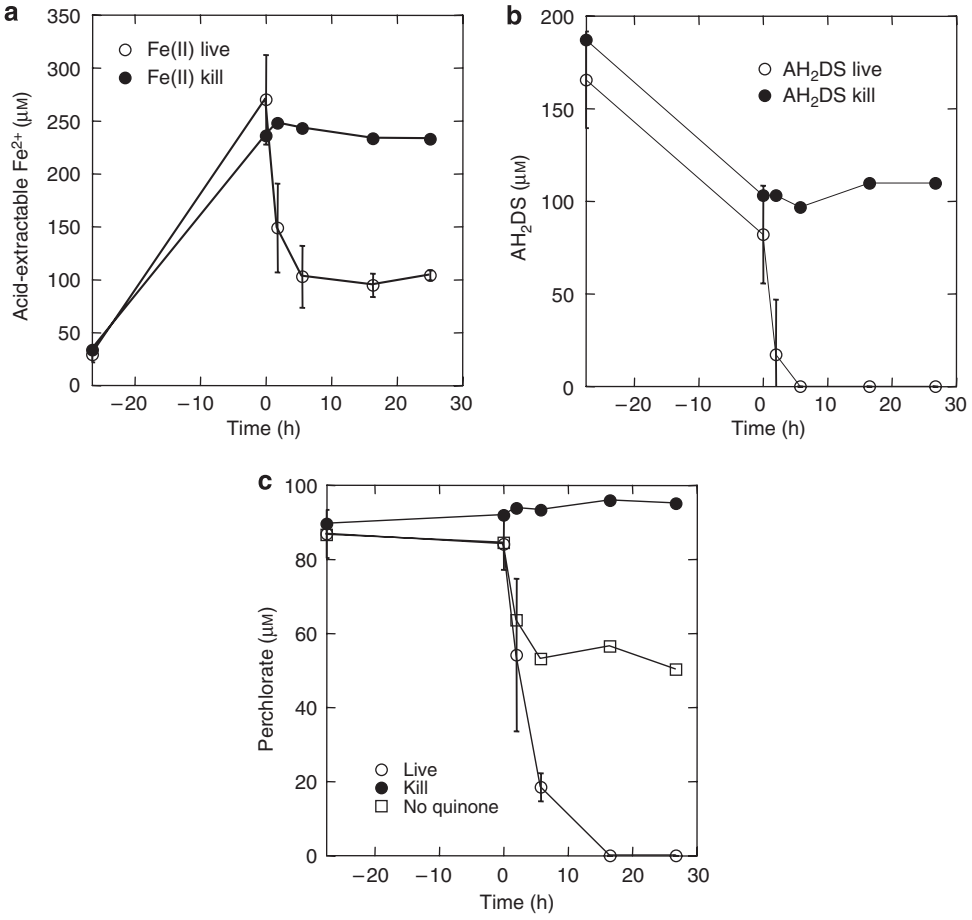

Figure 4 Oxidation of AH_2DS and Fe(II) coupled to perchlorate reduction by strain PS. (**a**) Fe(II) generated by Fe(III) reduction by AH_2DS, and oxidation of that iron by live (open circle) and heat-killed (closed circle) strain PS. (**b**) AH_2DS oxidation by Fe(III) and live (open circle) or heat-killed (closed circle) strain PS. (**c**) Perchlorate reduction by live (open circle) or heat-killed (closed circle) strain PS, and by strain PS in the absence of hydroquinone (open squares).

in the establishment of suitable microbial communities. This acetate was accounted for in electron balance calculations.

A. AH_2DS-containing microcosms

Before inoculation, Fe(III)–NTA (E_0 of Fe(III)/Fe(II) = +0.2 V) was added to the sterile AH_2DS microcosms, resulting in the immediate oxidation of approximately 0.7 mM AH_2DS (Figure 5a). This abiotic redox reaction accounted for an immediate reduction of more than 82% of the total 0.5 N HCl-extractable Fe in the killed microcosm (1156 μM) and 96% of the total 0.5 N HCl-extractable Fe in the viable microcosms (1077 ± 165 μM) (Figure 5b). Little or no Fe(II) production was observed in samples amended with AQDS or those lacking anthraquinone entirely, indicating the dependence of this reaction on AH_2DS (Figure 5b, and data not shown). Little further oxidation of AH_2DS was observed in the heat-killed control after this initial reaction with Fe(III)–NTA (Figure 5a). Furthermore, the total quinone concentration remained constant over the experimental time period in all treatments, indicating no observable biotic or abiotic degradation of the anthraquinone (Figure 5a).

After inoculation, the viable microcosms continued to oxidize AH_2DS, although at a slower visual rate, over the next 5 days of incubation (Figure 5a). During this phase, concomitant nitrate reduction was observed with transient production of 50 μM nitrite (Figure 5d). No nitrate reduction, nitrite production or AH_2DS oxidation was observed in heat-killed controls (Figure 5c). As expected in viable microcosms, ClO_4^- reduction was initiated after depletion of nitrate (Figure 5d), with concomitant oxidation of the AH_2DS at a slower visual rate than that observed under nitrate reduction (Figure 5a). This phase of AH_2DS oxidation coupled to ClO_4^- reduction continued for the total 94 days of the incubation. Over 40 days after ClO_4^- depletion, microcosms exhibited no further AH_2DS oxidation, indicating that excess AH_2DS was not a suitable electron donor for alternative respiratory processes (data not shown). Limited ClO_4^- reduction (114 μM) occurred in the killed microcosm within the first 20

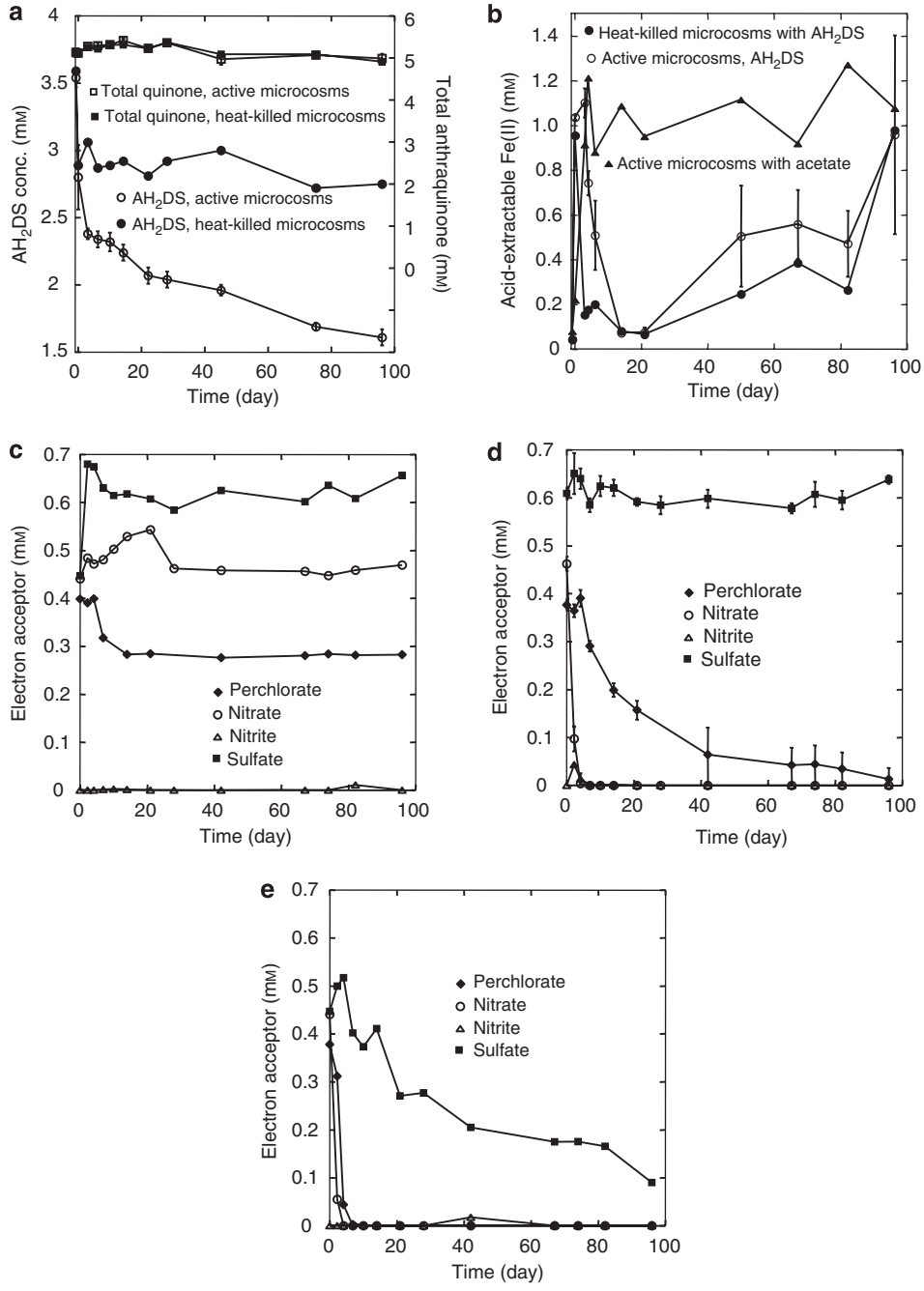

Figure 5 AH_2DS oxidation coupled to alternative electron accepting processes by natural communities showing thermodynamic targeting. (**a**) AH_2DS and total quinone concentrations in viable AH_2DS-containing microcosms (open circles and squares, respectively) and heat-killed AH_2DS-containing microcosms (closed circles and squares, respectively); (**b**) 0.5 N HCl-extractable Fe(II) in AH_2DS-containing viable (open circles) and heat-killed (closed circles) microcosmsand acetate-containing microcosms (closed triangles); (**c**) AH_2DS-containing heat-killed microcosm, showing reduction of nitrate (open circles), nitrate (open triangles), perchlorate (closed diamonds) and sulfate (closed squares); (**d**) AH_2DS as the main electron donor in viable microcosms; (**e**) acetate as the soil-amended electron donor in viable microcosms.

days of incubation (Figure 5c). This period of abiotic ClO_4^- reduction corresponded to the period of Fe(II) oxidation (approximately 889 μM) in the killed microcosm (Figure 5b). Interestingly, the amount of ClO_4^- reduced stoichiometrically balanced with the Fe(II) oxidized over this time period (887 μequiv. Fe(II) oxidized/908.8 μequiv. ClO_4^- reduced = 0.98). Although abiotic reduction of ClO_4^- by Fe(II) in electrolyte solutions containing 1 M ClO_4^- has been observed earlier (Prinz and Strehblow, 1998) such a mechanism is unlikely to be significant at the much lower ClO_4^- concentrations used in the current studies.

Neither sulfate reduction nor significant sulfide production was observed with AH_2DS (Figure 5d, Supplementary Figure 3). Even after more than 130

days of incubation, no sulfate reduction, further AH_2DS oxidation or anthraquinone degradation had occurred (data not shown), further illustrating the stability of AH_2DS as an electron donor in systems depleted of more electropositive electron acceptors. In contrast, in the presence of excess acetate (5 mM), active microcosms containing AH_2DS readily supported reduction of sulfate to sulfide, indicating that presence of the hydroquinone did not inhibit sulfate reduction (data not shown). Similarly, when AH_2DS was replaced with AQDS, microcosms first reduced AQDS to AH_2DS and then used the residual acetate to reduce sulfate (data not shown).

AH_2DS-based microcosms identical to those described earlier but lacking Fe(III) amendment also sequentially reduced nitrate and ClO_4^- reduction, but never respired sulfate (data not shown). Although some Fe was transferred to microcosms via the inoculum, these data suggest that the thermodynamic targeting capabilities of AH_2DS are not dependent on high local Fe concentrations.

B. Acetate-containing microcosms

In contrast to the results observed with AH_2DS, the microcosm amended with acetate as the sole electron donor showed the expected sequential utilization of all of the added electron acceptors. Nitrate was quickly depleted with no observable production of nitrite (Figure 5e), and ClO_4^- and Fe(III) reductions were initiated subsequently (Figures 5b and e). The observed rate of ClO_4^- reduction was significantly higher than that observed in microcosms with AH_2DS, and the ClO_4^- was removed to below detection (4 $\mu g\,l^{-1}$) within 7 days of incubation. Once ClO_4^- and Fe(III) reduction was complete, sulfate reduction was initiated and continued until depletion of the acetate (Figure 5e). At the end of the experimental time period, 220 μm total sulfide was detectable in acetate-containing microcosms (Supplementary Figure 3).

Electron balance calculation

Calculation of an electron balance in the AH_2DS-amended microcosms accounted for 96% of the expected theoretical value (Table 1) based on the balanced equations for oxidation of AH_2DS and acetate coupled to each of the respective electron acceptors. The viable AH_2DS microcosms reduced a total 6132 μequiv. of the suite of electron acceptors, and oxidized a total 3860 μequiv. of AH_2DS. Similarly, 2000 μequiv. (250.0 μM) acetate was detectably oxidized in the viable AH_2DS microcosm based on high-performance liquid chromatography analysis (data not shown). When summed, the oxidation of acetate and total AH_2DS together account for 5860 μequiv. of the total 6132 μequiv. measured by the depletion of electron acceptors (Table 1).

Discussion

These results show that both DPRB cultures and uncharacterized microbial communities can oxidize the model hydroquinone AH_2DS to its quinone analog concomitant with ClO_4^- reduction. These results also illustrate that AH_2DS stimulates some respiratory processes such as nitrate and ClO_4^- reduction, while barring reduction of more electronegative electron acceptors such as sulfate. To our knowledge, this is the first demonstration of the utilization of thermodynamic properties of the electron donor to selectively stimulate specific respiratory processes. Although earlier studies have

Table 1 Total reducing equivalents transferred to electron acceptors in microcosm treatments

Electron donor treatment	*Total μequiv. consumed by nitrate reduction*	*Total μequiv. consumed by perchlorate reduction*	*Total μequiv. consumed by Fe(III) reduction*	*Total μequiv. transferred to electron acceptors*
AH_2DS (viable)	2312	2905	915	6133
AH_2DS (non-viable)	−62	930	934	1802
AQDS (viable)	2093	1377	654	4124
No donor added (viable)	2261	1068	564	3898

Abbreviations: AH_2DS, 2,6-anthrahydroquinone disulfonate; AQDS, 2,6-anthraquinone disulfonate.
All values are expressed as microequivalents transferred to the relevant electron acceptor (μequiv.). An electron balance is carried out to correlate biotic AH_2DS oxidation to total electron acceptor reduction.

Calculation of electron balance:
Total electron donation in viable AH_2DS microcosms:

$$3860\,\mu\text{equiv.}\,(AH_2DS) + 2000\,\mu\text{equiv.}\,(\text{acetate}) = 5860\,\mu\text{equiv.}$$

Total electron acceptor reduction in viable AH_2DS microcosms:

$$6133\,\mu\text{equiv.}$$

Balance between expected electron donor oxidation and electron acceptor reduction:

$$(5860\,\mu\text{equiv.}/6133\,\mu\text{equiv.})\times 100 = 95.6\%\,\text{electron balance}$$

used AH_2DS as a reductant for transformation of contaminants, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (Kwon and Finneran, 2006) and carbon tetrachloride (Doong and Chiang, 2005), data presented here indicate that microbial AH_2DS oxidation can support bioremediative processes as well.

Pure culture studies

The ability of bacteria, including DPRB, to oxidize AH_2DS coupled to the reduction of nitrate has been illustrated earlier (Coates *et al.*, 2002). However, this is the first in-depth study on AH_2DS oxidation under ClO_4^--reducing conditions. Our results highlight notable differences between organisms coupling AH_2DS oxidation to nitrate reduction and ClO_4^- reduction. The first obvious difference between these two respiratory schemes is the rate at which they proceed. Although these experiments were not normalized for cell number with earlier studies, qualitative observations indicate that AH_2DS oxidation proceeds more rapidly under nitrate-reducing conditions (observed in earlier studies (Coates *et al.*, 2002) than during ClO_4^- reduction. This qualitative rate difference was observed repeatedly. In addition, although many organisms, including *D. aromatica* and closely related DPRB (Coates *et al.*, 2002), (JI Van Trump, unpublished data), have been shown to grow by AH_2DS oxidation with nitrate and 100 μM acetate, growth was not observed in any ClO_4^--respiring DPRB tested. This lack of growth may, at least in part, help to explain the rate differences of AH_2DS oxidation under nitrate and ClO_4^--reducing conditions. Similar electron acceptor-specific effects were observed earlier with DPRB using Fe(II) as an electron donor (Lack *et al.*, 2002).

The inability of AH_2DS-oxidizing and ClO_4^--respiring bacteria to grow by this metabolism suggests that insufficient energy for cellular replication is produced, even though these organisms readily grow by AH_2DS oxidation in the presence of nitrate. The rapid abiotic reduction of molecular oxygen by AH_2DS may explain these contrasting observations. Molecular oxygen is produced as a transient intermediate during microbial ClO_4^- reduction, and is subsequently reduced to H_2O (Coates and Achenbach, 2004, 2006). If O_2 reduction is an important energy-generating step for DPRB during ClO_4^- respiration, it is likely that abiotic reactions between AH_2DS and O_2 would compete with the enzymatic reduction of O_2, resulting in short circuiting or impairment of the electron transport chain. Such impairment would not occur under nitrate-reducing conditions, as molecular O_2 is not produced as an intermediate of this metabolism.

Community studies

Our results suggest that microbial consortia readily utilize AH_2DS as an electron donor for ClO_4^- respiration. This observation is perhaps unsurprising, given that ClO_4^--reducing microorganisms, as well as organisms capable of oxidizing AH_2DS and reduced humic materials, seem to be ubiquitous (Coates *et al.*, 1999, 2002). Our data confirm this prevalence, and indicate that cell populations capable of AH_2DS oxidation would likely be present within a contaminated area, precluding the need for bioaugmentation procedures. If augmentation is required, a contaminated area could be pulsed with an electron donor such as acetate, which could increase the microbial populations to the desired level. As shown for *D. aromatica*, DPRB simultaneously oxidize acetate and AH_2DS without significant rate impacts on acetate oxidation, suggesting that AH_2DS-oxidizing cells could compete for simple carbon substrates in the presence of AH_2DS. As AH_2DS oxidation does not seem to support growth of the model DPRB under ClO_4^--reducing conditions, low-level acetate spikes could therefore also aid in maintaining AH_2DS-oxidizing DPRB populations during a treatment process.

Thermodynamically targeted ClO_4^- reduction

It is well established that thermodynamic considerations impact microbial respiratory behaviors. For example, if electron donors are amended to an environment with multiple potential terminal electron acceptors, the microbial community respires the more electropositive compounds first, before proceeding to less thermodynamically favorable respiratory processes (at least in the absence of overriding genetic regulation) (Ponnamperuma, 1972; Yoshida, 1975; Champ *et al.*, 1979; Lovley and Goodwin, 1988; Chapelle *et al.*, 1997; Coates and Achenbach, 2001). Similarly, data shown here indicate that the thermodynamic characteristics of electron-donating compounds also influences the types of respiratory processes supported.

Acetate-containing microcosms in this study exhibited a sequential, structured respiratory pattern (nitrate, then ClO_4^- and iron, and then sulfate) that would be predicted based on the thermodynamics of the terminal electron acceptors (Coates and Jackson, 2008), with the caveat that nitrate is known to inhibit ClO_4^- reduction (Coates and Achenbach, 2004, 2006). In contrast, AH_2DS-oxidizing microcosms showed a very different pattern of electron acceptor use. AH_2DS supported the immediate abiotic reduction of Fe(III) to Fe(II). Once cells were introduced, nitrate reduction, with a transient spike of nitrite, occurred alongside oxidation of AH_2DS and the small amount of acetate present. ClO_4^- reduction then began and proceeded slowly over the experimental time period. AH_2DS oxidation halted upon depletion of ClO_4^- from the system, and degradation of the parent anthraquinone was never observed under anaerobic conditions, in agreement with similar studies (Seignez *et al.*,

1996). Sulfate reduction was not observed in this microcosm, even several months after ClO_4^- depletion. In the presence of excess acetate, neither AQDS nor AH_2DS precluded sulfate reduction, suggesting that these compounds exhibited neither toxicity nor redox-buffering events preventing the respiration from occurring.

AH_2DS and amended acetate were found to be the ultimate electron donors for the respiratory and abiotic reductive reactions observed in the AH_2DS-containing microcosms, as indicated by the calculated electron balance. Interestingly, Fe(II) generated from Fe(III) reduction by AH_2DS is also an electron donor known to support reduction of nitrate and ClO_4^- (Bruce *et al.*, 1999; Coates *et al.*, 1999; Chaudhuri *et al.*, 2001; Weber *et al.*, 2001; Lack *et al.*, 2002). Therefore, it is possible that Fe(II) acted as an electron shuttle between AH_2DS and microbial cells, with a given Fe atom being potentially subjected to multiple redox cycles. Results shown here confirm that Fe(II) generated from reduction of Fe(III) by AH_2DS is indeed bioavailable for ClO_4^- reduction. The experimental setup of targeting microcosms was unable to distinguish this possibility in microcosms systems, although the calculated electron balance again suggests that AH_2DS functioned as the primary electron donor in either case. A hydroquinone with a reduction potential more electropositive than the Fe(III)/Fe(II) couple may represent a more ideal electron donor than AH_2DS for the remediation of ClO_4^- to avoid Fe(III) reduction entirely. Therefore, AH_2DS can be seen as a model compound showing the concept of thermodynamically selective electron donors supporting ClO_4^- bioremediation, rather than the ideal compound to achieve this remediation strategy.

Taken together, results from the microcosm studies show that oxidation of hydroquinones allows for the thermodynamic targeting of respiratory processes useful to the bioremediation of ClO_4^-, while more electronegative electron donors support the respiration of more electronegative terminal electron-accepting processes.

Significance

These results suggest that the thermodynamic properties of hydroquinone electron donors can be used to stimulate and control specific microbial metabolisms. This observation may be of use in designing *in situ* bioremediative treatment strategies that necessitate electron donor addition. This observation may also speak to the electron-donating behavior of other common environmental compounds that admit of intermediate reduction potentials, such as reduced humic substances and Fe(II).

Acknowledgements

We thank Dr David Watson for the collection and shipping of sediment samples. Research on the microbial interactions with humic substances in the laboratory of JDC is supported by grant funding from the Natural Research Initiative of the USDA through Grant no. 2005-35107-16237.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)