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Successful microcosm demonstration of a strategy for biodegradation of a mixture of carbon tetrachloride and perchloroethene harnessing sulfate reducing and dehalorespiring bacteria

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ABSTRACT

Carbon tetrachloride (CT) is known to inhibit the transformation of perchloroethene (PCE) to ethene by dehalorespiring bacteria, creating a challenge for the bioremediation of environments contaminated with both compounds. We report on the sequential use of sulfate reduction and dehalorespiration as a microbial strategy for the transformation of a mixture of CT (10 μM) and PCE (14 μM). Sulfide production in *Desulfovibrio vulgaris* cultures led to complete CT disappearance in as little as 12 days. The addition of amorphous ferric oxide decreased the proportion of chloroform (CF) produced from 65% to 30%. CT conversion rates were enhanced more than 13-fold where vitamin B_{12} (5 μM) was added. In vitamin B_{12}-containing *D. vulgaris* cultures, no chlorinated products were detected and carbon disulfide was the major product of CT transformation. PCE concentrations were not impacted upon by *D. vulgaris* activity. The subsequent inoculation of a PCE-respiring enrichment culture resulted in microbial PCE dechlorination to ethene.

1. Introduction

Chlorinated aliphatic hydrocarbons (CAHs) are highly toxic, recalcitrant substances which occur frequently in contaminated soil and groundwater sites worldwide. Due to the significant threat they pose to human and environmental health, CAHs rank highly on the US EPA's Priority List of Hazardous Substances [1]. In 2007, the CAHs perchloroethene (PCE) and carbon tetrachloride (or tetrachloromethane, CT) were ranked 33rd and 47th respectively out of 275 compounds [1], a reflection of pressing needs for their remediation.

In subsurface anaerobic environments, PCE and CT are susceptible to several microbially driven reactions, rendering their destruction amenable to bioremediation technologies. PCE is a metabolic electron acceptor for energy generation in dehalorespiring bacteria (DRB) in genera such as *Dehalococcoides*, *Desulfitobacterium* and *Dehalobacter* (reviewed in [2–4]). During growth, these bacteria reductively dechlorinate PCE in a stepwise process to yield the innocuous product ethene. They are however severely inhibited by low levels (1–10 μM) of CT and its partly dechlorinated intermediate chloroform (CF) [5,6], representing a significant challenge to the application of DRB at sites co-contaminated with PCE and CT or CF.

While CT cannot serve as a metabolic electron acceptor for any microbial species, several studies have reported its transformation in a range of anaerobic microbial cultures. Dechlorination was observed in methanogenic [7,8], denitrifying [9], sulfate-reducing [8] and fermentative conditions [10,11]. Studies with bacterial metallo-coenzymes elucidated the mechanism underlying this process, demonstrating that binding of CT to metal centers of these molecules followed by electron transfer from bulk reductants led to CT dechlorination [12–16]. A number of these redox-active molecules, termed electron shuttles (ES), were also shown to enhance CT transformation when added to live anaerobic bacterial cultures [17–19]. Vitamin B_{12} (B_{12}) amendments in particular cause dramatic CT conversion rate increases and divert products away from CF [19,20].

CT also reacts directly with naturally occurring reductants present in low-redox environments [21–23]. Minerals such as metal sulfides, green rust and other iron-bearing minerals transform CT to products including CF, dichloromethane, carbon disulfide, carbon monoxide, formate and CO_2 [21,24–26]. The proportion of ferrous iron in soil minerals in particular is correlated with their reactivity towards CT [23,27].

The CT transformation mechanisms described above point to the use of micro-organisms as remediation agents, as not only their redox-active cell components but also in some cases their reduced metabolic end-products can react with CT. This latter property is characteristic of iron- and sulfate-reducing bacteria (IRB and SRB respectively), ubiquitous in anaerobic subsurface

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environments [28,29]. The ferrous iron and sulfide they produce can serve to generate reactive mineral surfaces resulting in CT reduction. The ability of IRB and SRB to drive CT transformation through this process has been demonstrated [30–33], however data concerning the feasibility of this approach in terms of dovetailing the process with subsequent dehalorespiration remain scarce.

In this paper, we tested the hypothesis that sulfide production by the sulfate-reducing bacterium *Desulfovibrio vulgaris* could result in CT transformation and relieve inhibition of PCE respiration by DRB. The impact of adding iron oxide to precipitate sulfide and provide a reducing mineral surface was investigated. Vitamin B_{12} was chosen as a model compound for bacterial corrinoids, consistent with previous studies [14,16,34]. Results indicate that combining the two processes of sulfate reduction and dehalorespiration can result in the successful bioremediation of CT–PCE mixtures. This research extends our understanding of biogeochemical conditions which support the biological treatment of contaminant mixtures.

2. Materials and Methods

2.1. Micro-organisms and culture conditions

D. vulgaris was kindly provided by Dr Priyangshu Manab Sarma of The Energy and Research Institute (TERI), New Delhi, India. This organism was grown under a 4:1 mixture of N_2:CO_2 in a bicarbonate-buffered (pH 7) medium containing, per L: KH_2PO_4 0.3 g; NaCl 0.6 g; $MgCl_2{\cdot}6H_2O$ 0.1 g; $CaCl_2{\cdot}2H_2O$ 0.08 g; NH_4Cl 1.0 g; $KHCO_3$ 4.0 g; resazurin 1.0 mg; trace elements solution 10 mL; vitamin solution 10 mL; tungstate/selenium solution 1 mL. The trace elements solution contained, per L: nitrilotriacetic acid 1.5 g; $MgSO_4{\cdot}7H_2O$ 3.0 g; $MnSO_4{\cdot}H_2O$ 0.5 g; NaCl 1.0 g; $FeSO_4{\cdot}7H_2O$ 0.1 g; $CoSO_4{\cdot}7H_2O$ 0.18 g; $CaCl_2{\cdot}2H_2O$ 0.1 g; $ZnSO_4{\cdot}7H_2O$ 0.18 g; $CuSO_4{\cdot}5H_2O$ 0.01 g; $KAl(SO_4)_2{\cdot}12H_2O$ 0.02 g; H_3BO_3 0.01 g; $Na_2MoO_4{\cdot}2H_2O$ 0.01 g; $NiCl_2{\cdot}6H_2O$ 0.025 g. The vitamin solution contained, per L: biotin 2.0 mg; folic acid 2.0 mg; pyridoxine-HCl 10.0 mg; thiamine-HCl·$2H_2O$ 5.0 mg; riboflavin 5.0 mg; nicotinic acid 5.0 mg; D-Ca-pantothenate 5.0 mg; vitamin B_{12} 0.1 mg; *p*-aminobenzoic acid 5.0 mg; lipoic acid 5.0 mg. The tungstate/selenite solution contained, per L: NaOH 0.5 g; $Na_2SeO_3{\cdot}5H_2O$ 3 mg; $Na_2WO_4{\cdot}2H_2O$ 4 mg. Substrates added were 10 mM sodium lactate (1.12 g/L) and 5 mM sodium sulfate (0.71 g/L).

Cultures were grown in 120 mL serum bottles containing 80 mL of medium and sealed with Teflon-lined butyl rubber septa and aluminum crimp caps. A late-exponential phase *D. vulgaris* culture grown on lactate and sulfate was harvested by centrifugation and washed with sterile anoxic medium in order to eliminate any sulfide prior to inoculation (3.75% [v/v]). Unfed control cultures, inoculated with washed cells but containing no lactate and no sulfate, were also established. All treatments were performed in triplicate.

Amorphous ferric oxide (referred to hereafter as Fe(III)) was prepared as described by Lovley and Phillips [35]. Where applicable, 100 mg was added per culture, representing an excess of ferric iron (12 mM) over the potential 5 mM sulfide produced. Vitamin B_{12} was added at 5 μM where applicable.

A mixture of CT and PCE was added to each bottle from a concentrated methanolic stock solution immediately prior to inoculation with *D. vulgaris*, resulting in nominal concentrations of 10 μM CT and 14 μM PCE. Bottles were incubated quiescently in the dark at 30 °C, inverted with the liquid in contact with the septum in order to minimize losses through gas leakage at the vessel/septum interface.

A PCE-respiring culture was enriched in our laboratory from soil contaminated with a mixture of CAHs at the Botany Industrial Park in Sydney, Australia. Briefly, soil (~2 g) was added to sterile anoxic basal medium supplemented with acetate (5 mM) as a carbon source and 50 μM PCE (from a methanolic stock solution) as electron acceptor. Methanogenesis was inhibited by 1 mM bromoethanesulfonic acid (BES). This culture was subcultured twice in these conditions each time after complete conversion of PCE to stoichiometric amounts of ethene was observed. The resulting sediment-free active PCE-dechlorinating enrichment was inoculated in all test bottles 36 days after inoculation with *D. vulgaris*.

2.2. Analytical methods

Acetate levels were monitored with a Shimadzu gas chromatograph (GC) fitted with a ZB-FFAP column (30 m, 0.32 mm i.d., 0.25 μm film thickness) and a flame-ionization detector (FID). Liquid samples (0.2–0.8 mL) were centrifuged, diluted and acidified with formic acid (1%). The injection volume was 1 μL. The oven temperature program was as follows: 60 °C (1 min), increase to 250 °C at 10 °C/min, hold at 250 °C (1 min). Inlet and detector temperatures were 250 °C. The carrier gas was Helium (2 mL/min). The inlet was operated with a split ratio of 30:1. Acetate concentrations were determined by interpolation of a 5-point standard curve (0–4 mM).

PCE, trichloroethene (TCE), CT and CF were monitored using an Agilent 7890A GC equipped with a J&W DB-5 column (30 m, 0.32 mm i.d., 0.25 μm film thickness) and an electron capture detector. Liquid culture samples (100 μL) were withdrawn and added to 400 μL ethanol prior to injection (1 μL) with a split ratio of 20:1. The oven temperature program was as follows: 35 °C (1 min), increase to 130 °C at 10 °C/min, hold at 130 °C (1 min). The carrier gas was Helium (0.57 mL/min). Dichloroethene (DCE) isomers, vinyl chloride (VC), dichloromethane (DCM), chloromethane and ethene were monitored by GC-FID using a J&W GS-GASPRO column (60 m, 0.32 mm i.d., 0 μm film thickness). Headspace samples (100 μL) were removed by lockable syringe and injected into a split inlet (split ratio 10:1). Compounds were separated using the following oven temperature program: 50 °C (1 min), increase to 250 °C at 20 °C/min, hold at 250 °C (1 min). The inlet and detector were maintained at 250 °C. The carrier gas was helium (2.85 mL/min). Concentrations were determined using standards set up in identical bottles with the same volumes of medium and headspace (80 mL and 40 mL) as in test bottles. These standards were analyzed in the same way as test bottles, whether by headspace or liquid injections. As a result, all concentrations reported herein are nominal, consisting in the sum of moles of a compound present both in the gaseous and aqueous phases divided by liquid volume. This method takes into account gas–liquid partitioning.

Carbon disulfide was quantified with a Thermo Focus GC fitted with a GS-GASPRO column and a Thermo DSQII mass spectrometer. Liquid samples (0.5 mL) were withdrawn and placed in 12 mL headspace vials with 0.2 g Na_2SO_4. Vials were heated and agitated at 80 °C for 4 min prior to injection (0.4 mL). The inlet was maintained at 100 °C and operated with a split ratio of 10:1. The oven temperature program was as follows: 50 °C (1 min), increase to 140 °C at 10 °C/min, hold at 140 °C (1 min). The carrier gas was helium (2 mL/min). Both the MS transfer line and ion source were kept at 260 °C. The mass spectrometer was run in full scan mode in the mass range 46–100. CS_2 levels were also determined using standards made up in the same vessels with the same medium to headspace ratio as test bottles in order to account for partitioning.

Sulfide was determined using the method of Cline [36]. Briefly, 0.1 mL culture samples were diluted in 5 mL deoxygenated water to which 0.4 mL of a solution containing 50% HCl [v/v], 16 g/L *N*,*N*-diethyl-*p*-phenylenediamine sulfate and 24 g/L $FeCl_3{\cdot}6H_2O$ was added. Following color development, absorbance readings were

Table 1
Desulfovibrio vulgaris cultures established to minimize the production of chloroform and free sulfide from CT and sulfate reduction respectively.

Treatment	Lactate	Sulfate	Fe(III)	Vitamin B_{12}
Unfed controls	–	–	–	–
L-S	10 mM	5 mM	–	–
L-S-Fe(III)	10 mM	5 mM	12 mM	–
L-S-B12	10 mM	5 mM	–	5 μM
L-S-Fe(III)-B12	10 mM	5 mM	12 mM	5 μM

taken at 670 nm. Ferrous iron was measured using the ferrozine method of Stookey [37]. Culture samples (0.1 mL) were extracted in 0.5 N HCl (5 mL) and diluted 1:4 in a ferrozine solution (1 g/L 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine disodium salt in 50 mM HEPES at pH 7) before absorbance was measured at 562 nm.

3. Results

Carbon tetrachloride (CT) is recognized as a strong inhibitor of DRB. Whilst it is understood that CT can be dechlorinated by metal sulfides, offering a potential solution to the degradation of CAH mixtures containing CT, the resulting daughter product chloroform and free sulfide are also recognized as potent inhibitors of dehalorespiration. A set of *D. vulgaris* cultures containing CT and PCE were established to define environmental conditions (ferric iron and vitamin B_{12} combinations) under which this sulfate reducing bacterium can be used to remove CT without imposing subsequent inhibition of DRB by free sulfide or chloroform production. For clarity, the experimental layout is presented in Table 1.

Following inoculation, the turbidity of all cultures increased (except in unfed controls) indicative of growth of *D. vulgaris* on lactate as electron donor and sulfate as electron acceptor. Fig. 1 illustrates the production of sulfide from sulfate reduction, acetate from lactate oxidation and ferrous iron from ferric iron reduction in the cultures. The production of approximately 10 mM acetate in all fed cultures and of 5 mM free sulfide in cultures lacking Fe(III) indicated that all lactate and sulfate present in the culture medium had been consumed after 2 days incubation. In Fe(III)-amended cultures, a black precipitate characteristic of iron sulfides formed and no sulfide could be detected in solution (data not shown). This suggested that the Fe(III) present in the media had effectively precipitated the dissolved sulfide formed from sulfate reduction by *D. vulgaris*. In support of this Fe(II) was detected in Fe(III)-amended cultures, indicative of Fe(III) reduction by sulfide.

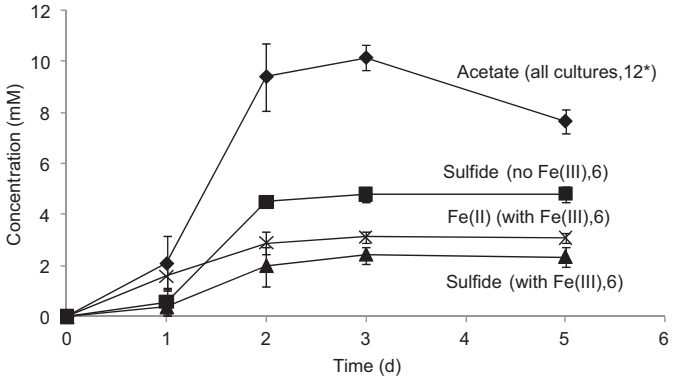

Fig. 1. Formation of acetate, sulfide and ferrous iron in *D. vulgaris* cultures. *"All cultures" refers to all cultures except unfed controls. "No Fe(III)" refers to cultures lacking Fe(III) addition. "With Fe(III)" refers to cultures amended with Fe(III). Sulfide and Fe(II) in Fe(III)-amended cultures correspond to precipitated forms. Data presented are averages of the number of cultures indicated in brackets. Error bars represent one standard deviation. No ferrous iron was detected in cultures without Fe(III) addition.

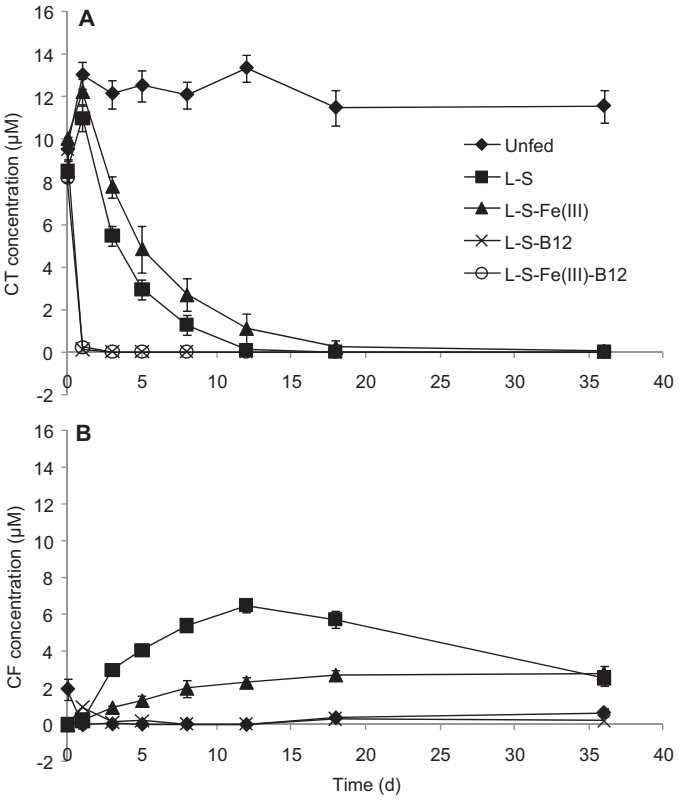

Fig. 2. Disappearance of carbon tetrachloride (A) and formation of chloroform (B) in *D. vulgaris* cultures (nominal concentrations). Data presented are average of triplicate cultures. Error bars represent one standard deviation.

In all cultures, excluding unfed controls in which no growth of *D. vulgaris* occurred, CT transformation was observed after day 1 (Fig. 2). The observed increase in CT during day 1 results from time zero samples being taken before full equilibrium between the liquid and gas phases was reached, compared with standard bottles. In cultures without B_{12}, rates of CT depletion were similar in the presence and absence of Fe(III) (Fig. 2A). CT was completely transformed within 12–18 days and followed first-order kinetics with rate constants of 0.201 (±0.05) d^{-1} and 0.266 (±0.034) d^{-1} in treatments with and without Fe(III) respectively (calculated using intervals d1–d8 and d1–d12 respectively). In cultures lacking Fe(III), CF was the major product, accounting for 65% of initial CT at day 12 and decreasing to 26% over 36 days (Fig. 2B). In Fe(III)-amended cultures, CF accounted for only 28% of CT products at day 12 though this was stable thereafter.

The presence of 5 μM B_{12} led to the extremely rapid disappearance of CT (Fig. 2A). All CT was transformed within 24 h, corresponding to at least a 13-fold increase in rate compared with *D. vulgaris* cultures not amended with B_{12}. Encouragingly, only trace quantities of CF were formed in B_{12}-amended cultures (Fig. 2B). The presence of Fe(III) had no impact on CT conversion rates or CF levels in the presence of B_{12}. The concentration of PCE in all cultures remained stable throughout the incubation period (data not shown).

A mass balance for CT transformation products was established after 36 days of incubation (Fig. 3). In B_{12}-free cultures, CF accounted for 26–28% of CT carbon independent of Fe(III) addition. Levels of CS_2 were also similar regardless of Fe(III) addition (20–25% of initial CT). The presence of Fe(III) did however decrease DCM production with 38% of CT carbon being recovered as DCM in cultures

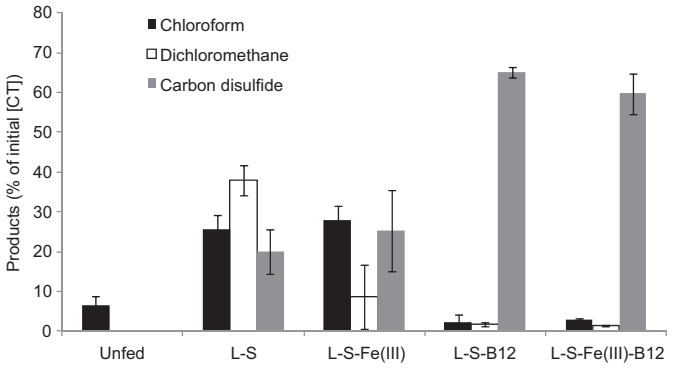

Fig. 3. Mass balance of CT products (chloroform, dichloromethane and carbon disulfide) in *D. vulgaris* cultures at day 36. No chloromethane was detected. CT mass recovery was as follows: unfed 100%; L-S 84%; L-S-Fe(III) 62%; L-S-B12 67%; L-S-Fe(III)-B12 61%. Data presented are average of triplicate cultures. Error bars represent one standard deviation.

lacking Fe(III) and only 9% in the presence of Fe(III). A higher proportion of unidentified products, considered as the remaining fraction of CT carbon not recovered as CF, CS_2 or DCM, occurred in Fe(III)-amended cultures (38%) compared with those lacking Fe(III) (16%). Chloromethane was not observed in any of the cultures. In B_{12}-amended treatments, the major product of CT conversion was CS_2, accounting for 60–65% of CT in the presence or absence of Fe(III) (Fig. 3). No chloromethane and only traces levels of CF and DCM were detected in B_{12}-amended treatments.

After 36 days incubation all cultures were inoculated with a mixed inoculum capable of PCE dechlorination. Unfed controls were supplemented with acetate (2.5 mM) in order to provide the same substrate as in spent *D. vulgaris* cultures where lactate oxidation had resulted in acetate accumulation. PCE dechlorination was observed only in treatments supplemented with Fe(III) (Fig. 4A), likely due to the precipitation and removal of dissolved sulfide by Fe(III).

TCE appeared as a transient product in cultures amended with Fe(III) alone or with Fe(III) and B_{12} (Fig. 4B). Dichloroethene was not observed in any of the cultures. Ten days after inoculation with the dehalorespiring community (46 days after the experiment commenced) VC was observed in Fe(III) amended cultures at a concentration of 9.2 ± 0.53 μM. Both VC (9.4 ± 0.37 μM) and ethene (3.7 ± 0.55 μM) were observed in cultures amended with both Fe(III) and B_{12} indicating that conditions enabling DRB reduction of PCE to ethene had been successfully achieved. No PCE dechlorination occurred in unfed control cultures in which CT concentrations remained high or in cultures lacking Fe(III) where dissolved sulfide concentrations remained high.

4. Discussion

Results of this investigation support the feasibility of combining sulfate reduction with dehalorespiration as a bioremediation strategy for the treatment of CT–PCE mixtures. Active cultures of an SRB completely transformed CT, with amendments of ferric iron (Fe(III)) and vitamin B_{12} impacting on soluble sulfide concentrations and CT products, thereby creating favorable conditions for the subsequent conversion of PCE to ethene by DRB.

Sulfide production by *D. vulgaris* co-occurred with CT transformation. The presence of Fe(III) had no effect on CT reaction rate. It is well-documented that CT reacts very slowly with dissolved sulfide in homogenous systems, with first-order rate constants in the order of 0.02 d^{-1} [26,38]. CT conversion rates in the order of 0.2 d^{-1} as observed in this study are thought to occur as surface-mediated reactions, often dependent on the presence of metal precipitates

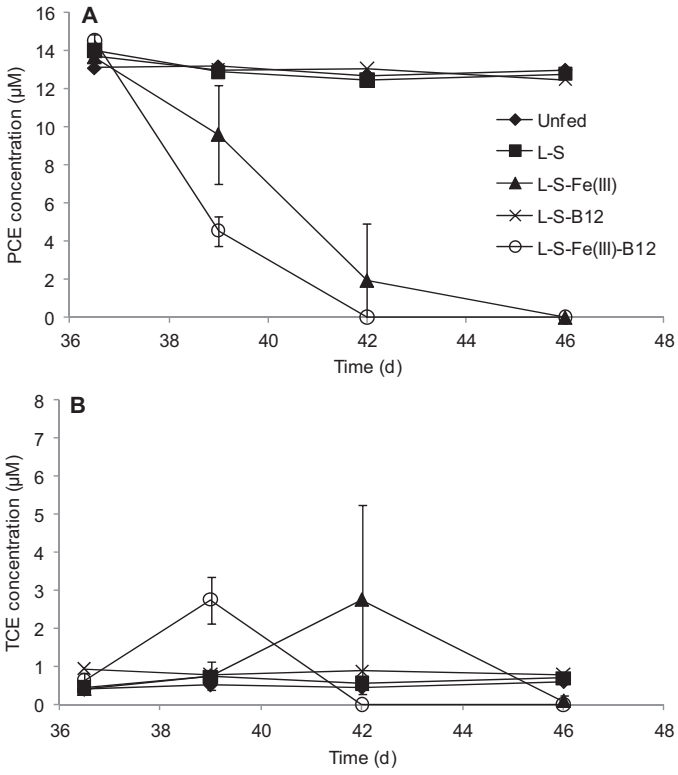

Fig. 4. Perchloroethene (A) and Trichloroethene (B) (nominal concentrations) in spent *D. vulgaris* cultures inoculated with a PCE-respiring enrichment culture on day 36. The legend applies to A and B. Data presented are average of triplicate cultures. Error bars represent one standard deviation.

[23,26,27,39]. It was thus expected that CT transformation would proceed faster in cultures with Fe(III) than in those without, given the relatively larger mineral surface area Fe(III) amendments provided. This was not observed, suggesting CT conversion was not limited by surface area in any of these cultures. Metal sulfides generally have very low aqueous solubility values, *e.g.* 2.51×10^{-9} M for FeS and 6.3×10^{-11} M for CoS [40]. As metal ions in the basal medium used here were far in excess of these concentrations (3.6×10^{-6} M for Fe(II) and 6.4×10^{-6} M for Co(II)), the mineral surface provided by these precipitates likely mediated CT removal.

Whilst not impacting on the reaction rate, the presence of Fe(III) led to a lower proportion of CF. It has been postulated that fully dechlorinated products of CT reduction are favored where reactive intermediates can be stabilized at a surface [26,41]. High concentrations of hydrogen-donating compounds, such as organic matter, are also known to lead to higher CF concentrations [41,42]. These two factors potentially explain the smaller proportion of CF formed in Fe(III)-amended cultures, where a higher mineral surface area to biomass ratio prevailed compared with cultures without Fe(III). Furthermore, certain oxidized sulfur-containing species such as pyrite (FeS_2) can lead to an increase in the ratio of CS_2 to CF formed from CT in FeS-containing systems [43]. In the current experiment, the reduction of Fe(III) by sulfide would have yielded oxidized sulfur species such as S^0, which can react with FeS to form pyrite, as given by $FeS + S^0 \rightarrow FeS_2$ [44]. Results presented here lend support to this hypothesis, as Fe(III)-containing treatments yielded a higher ratio of CS_2 to CF.

The decrease in CF observed in Fe(III)-free *D. vulgaris* cultures over 36 days can be attributed to reduction to DCM. Although the nature of the reductant(s) is unknown, this reduction was observed after growth had stopped and was possibly enhanced by porphyrin-type cell components released from inactive or decaying cells of *D. vulgaris*. Egli et al. [10] observed a similar phenomenon with

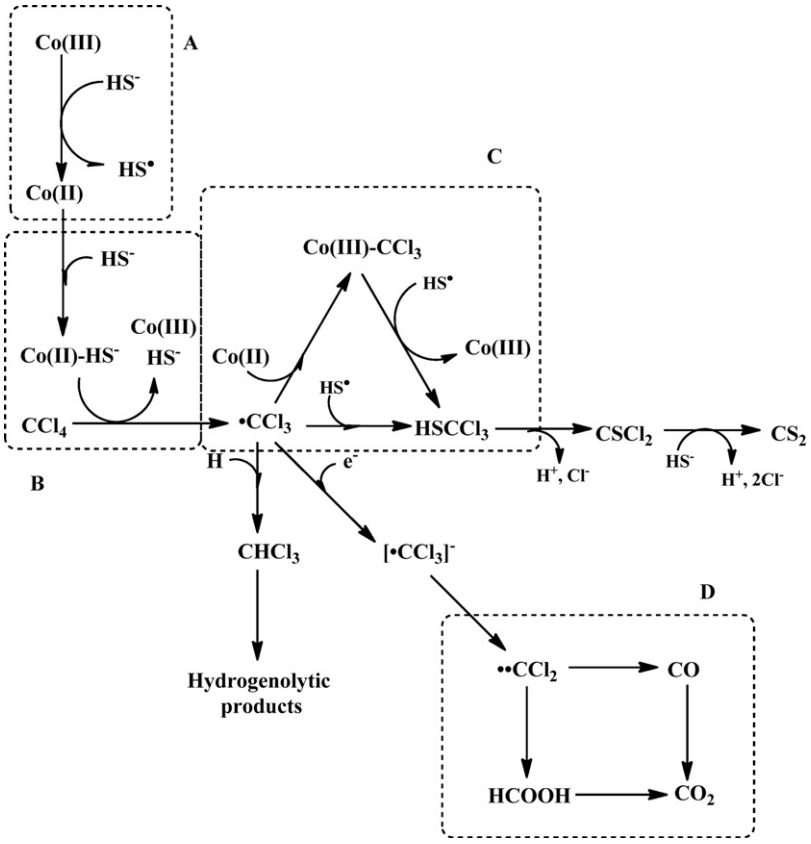

Fig. 5. Hypothetical pathways of CT transformation in reduced environments and role of B_{12}. Oxidized B_{12} is denoted by "Co(III)" and reduced B_{12} (to its Cobalt(II) form) by "Co(II)".
Adapted from references [26,48,49,62].

the sulfate reducer *Desulfobacterium autotrophicum*, where a small proportion of CF resulting from CT dechlorination was converted to DCM after prolonged incubation.

The presence of B_{12} accelerated CT degradation and altered products dramatically. CS_2 was the only detected product, and no chlorinated species were found. Similar rate increases were noted by Hashsham and Freedman following the addition of cobalamins to anaerobic fermenting cultures [19,45]. Products of CT dechlorination also shifted, with cobalamins leading to less CF and more CO_2 [19]. Cultures of the iron reducing bacterium *Shewanella alga* growing with lactate and 45 μM B_{12} converted CT to 1.4% CF and 92% CO [46]. In the current study, B_{12} increased the amount of CS_2 generated from CT. This could be due to a higher sulfide concentration (5 mM) than in studies cited. Importantly, CS_2 does not inhibit ORB activity and is known to hydrolyze to CO_2 at appreciable rates in groundwater [26].

A compilation of proposed CT transformation pathways in low-redox conditions is given in Fig. 5, which suggests three possible mechanisms by which B_{12} can lead to more CS_2 in high-sulfide systems. Firstly, owing to its high redox potential of +200 mV [47], B_{12} can be reduced by sulfide ($E^{0\prime}$ −270 mV) from its Co (III) form to its Co (II) form. This can occur as given by $2\ Co(III) + HS^- \rightarrow 2\ Co(II) + S^0 + H^+$ or through thiyl radical formation (Fig. 5, Process A). Secondly, as B_{12} in its Co(II) form itself is thought to be unreactive towards CT [48], we propose that a sulfide–B_{12} complex transferred electrons to CT (Process B), similarly to the cysteinate–B_{12} complex put forward by Chiu and Reinhard [48]. Thirdly, B_{12} is known to covalently bind to the trichloromethyl radical $^{\bullet}CCl_3$, yielding a trichloro-cobalamin intermediate (Process C). The cobalt–carbon bond breakage can be catalyzed by thiyl radicals existing in sulfidic systems [48], resulting in $HSCCl_3$ and forming CS_2. Given the high molar ratio of B_{12} to CT employed here (1:2), we suggest that B_{12} potentially prevented the formation of CF by establishing a Co–C complex with $^{\bullet}CCl_3$ and favoring the formation of $HSCCl_3$. Additionally, B_{12} possibly encouraged the formation of reactive thiyl radicals (Process A), causing a shift in the pathways of CT conversion towards CS_2. The ratio of B_{12} to CT is presumably important in the processes described above, as an excess of $^{\bullet}CCl_3$ radical is likely to lead to more chloroform, as less cobalamin is available to form a Co–C complex and prevent hydrogen or proton abstraction by radicals.

In all cultures, various proportions of CT dechlorination products remained unidentified. Carbon monoxide and formate may account for the missing carbon (Process D), as shown by Workman et al. [46]. Some authors also observed the formation of soluble and cell-bound material, possibly resulting from the coupling of radicals with biomass or reductants [32,49,50].

Upon inoculation of PCE-respiring bacteria on day 36, PCE could be dechlorinated only where Fe(III) was present. Several lines of evidence disprove the possibility of this reaction being caused by iron sulfide or disulfide (FeS and/or FeS_2). Firstly, PCE concentrations did not change during the first 36 days of experimentation while being in contact with iron sulfide. Secondly, PCE transformation rates measured in studies with FeS are much lower than noted here, with half-lives in the order of months [51,52]. Thirdly, products of iron sulfide-associated PCE conversions include a large proportion of acetylene and DCE isomers while no VC generally occurs [51,53]. In this study, PCE was completely transformed to daughter products within days, in accordance with microbial PCE reductive dechlorination pathways [54].

In *D. vulgaris* cultures that did not contain B_{12}, CT and/or its chlorinated products remained when PCE-respiring bacteria were

introduced. In unfed controls where no *D. vulgaris* growth had occurred, PCE dechlorination could not proceed despite the presence of acetate. The presence of 6.7 μM CT and 3.3 μM CF in these treatments likely accounted for the inhibition of DRB, concurring with previous findings concerning the deleterious effects of CT and/or CF in this concentration range on DRB activity [5,55–57]. In treatments with and without Fe(III), similar CF and CS_2 levels existed (2.7 and 2.3 μM respectively) and hence could not have been inhibitory to PCE respiration. The high concentration of DCM found only in Fe(III)-free cultures on the other hand could have contributed to the inhibition of DRB observed in these treatments. In Fe(III)-supplemented cultures, the accumulation of VC could be due to CF inhibiting its conversion to ethene, as observed by Duhamel et al. [55].

The addition of B_{12} to *D. vulgaris* cultures was highly beneficial, given no chlorinated products were formed from CT. As both sets of B_{12}-amended treatments contained the same level of CS_2, it is improbable that CS_2 prevented PCE respiration in Fe(III)-free cultures. As the only difference between the two treatments was Fe(III) addition, dissolved sulfide was most likely responsible for DRB inhibition, as noted by other authors [6,58,59]. This factor also most likely played a major role in inhibiting PCE dechlorination in cultures without Fe(III) and B_{12}, regardless of the presence of DCM.

Results presented here outline the importance of employing dechlorination strategies which do not rely on or generate conditions toxic to DRB in situations where their activity is required as a part of a process. In using a sulfate reducing bacterium to effect CT reduction, the presence of Fe(III) and B_{12} were necessary to generate conditions ultimately conducive to DRB activity. While several anaerobic microbial processes can result in CT dechlorination, sulfate reduction in particular presents several advantages. Sulfate-reducing bacteria are ubiquitous, diverse and fast-growing and utilize a wide range of substrates [60], making sulfate reduction a rapid and accessible means to produce reducing power. This metabolic pathway also consumes protons and hence counteracts acidification caused by reductive dechlorination. Sulfate reduction further leads to very low-redox conditions that lead to the reduction of CF to DCM. Dichloromethane is a fermentable substrate for *Dehalobacterium formicoaceticum* [61] and hence can be metabolized *in situ*.

5. Conclusions

This study provides evidence to support a two-stage microbially driven biogeochemical process to remediate mixtures of CT and PCE, previously perceived as recalcitrant to bioremediation. Sulfate reduction by *D. vulgaris* could remove CT, with amendments of ferric oxide and B_{12} improving rates and lowering or eliminating chloroform as a product. Subsequent dechlorination of PCE to ethene could proceed only where PCE-respiring DRB were shielded from the toxic effects of both CT dechlorination products and free sulfide.

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