Low and high acetate amendments are equally as effective at promoting complete dechlorination of trichloroethylene (TCE)

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Abstract Experiments with trichloroethylene-contaminated aquifer material demonstrated that TCE, cis-DCE, and VC were completely degraded with concurrent Fe(III) or Fe(III) and sulfate reduction when acetate was amended at stoichiometric concentration; competing TEAPs did not inhibit ethene production. Adding 10× more acetate did not increase the rate or extent of TCE reduction, but only increased methane production. Enrichment cultures demonstrated that ~90 μM TCE or ~22 μM VC was degraded primarily to ethene within 20 days with concurrent Fe(III) or Fe(III) + sulfate reduction. The dechlorination rates were comparable between the low and high acetate concentrations (0.36 vs 0.34 day^{-1}, respectively),

with a slightly slower rate in the 10× acetate amended incubations. Methane accumulated to 13.5 (±0.5) μmol/tube in the TCE-degrading incubations with 10× acetate, and only 1.4 (±0.1) μmol/tube with low acetate concentration. Methane accumulated to 16 (±1.5) μmol/tube in VC-degrading enrichment with 10× acetate and 2 (±0.1) μmol/tube with stoichiometric acetate. The estimated fraction of electrons distributed to methanogenesis increased substantially when excessive acetate was added. Quantitative PCR analysis indicated that 10× acetate did not enhance *Dehalococcoides* biomass but rather increased the methanogen abundance by nearly one order of magnitude compared to that with stoichiometric acetate. The data suggest that adding low levels of substrate may be equally if not more effective as high concentrations, without producing excessive methane. This has implications for field remediation efforts, in that adding excess electron donor may not benefit the reactions of interest, which in turn will increase treatment costs without direct benefit to the stakeholders.

Keywords Reductive dechlorination ·
Dehalococcoides · Trichloroethylene ·
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Introduction

Incomplete reduction of trichloroethylene (TCE) is often attributed to the availability of electron donors,

and adding substrates that ferment to hydrogen to stimulate reductive dechlorination in situ is an effective bioremediation strategy (Lee et al. 1998; Rodriguez et al. 2004; Scheutz et al. 2010). While the concentration of electron equivalents required should be based on a stoichiometry between the electrons liberated by oxidation and electrons that can be accepted by all available electron acceptors, the remediation industry has been presented numerous times with a "more time and more electrons" mantra to overcome the so-called competition from other electron acceptors, as reviewed by Lee et al. 1998. Excessive electron donor concentration is usually applied to compensate for this competition, in an effort to avoid incomplete dechlorination or "cis-dichloro-ethylene (DCE) stall" (Gibson et al. 1994; Heimann et al. 2007; Leahy and Shreve 2000). The debate is often framed by stating that the excess electron donor is added to compensate for unknowns related to solid electron acceptors such as ferric iron (Evans and Koenigsberg 2001; Kouznetsova et al. 1998; Zaa et al. 2010), or for concentration changes in aqueous acceptors like sulfate (Bennett et al. 2007; Lee et al. 1998). Alternatively, excess electron donor may be added to deliver a specific concentration along the entire plume, factoring in dilution as it moves with groundwater. While no single donor strategy is appropriate for every environment, it is reasonable to suggest that on a site-specific basis it is preferable to start at low concentration, and add more if necessary.

It is uncertain whether adding excess substrate (i.e. building in an amendment "factor of safety") actually stimulates complete dechlorination relative to low electron donor approaches because previous results have been inconsistent (additional discussion in the Supporting Information). In addition, there are undesirable effects due to excessive organic substrates, with high methane production (Bennett et al. 2007; Lee et al. 2004; Yang and McCarty 2002, 2003) being most prevalent. Methane is a potent greenhouse gas. Whether subsurface generation of methane is a critical issue is still debated, and does not currently attract regulatory attention. However, remediation efforts can generate significant methane, and stakeholders are obligated to decontaminate their sites without generating new problems for human health or the environment. Even if greenhouse gas generation is not a concern—it is pure waste. Electron donors that end up as methane have done nothing but consume the natural resources required to produce and deliver the amendment, without contributing electrons to the critical reactions. Cost is an additional factor for in situ remediation; the operation and maintenance cost over the lifetime of the remedial action is higher when excessive substrate is amended.

Our previous research demonstrated that acetate can serve as the sole electron donor for complete dechlorination, with H_2 as an intermediate formed by Fe(III)-reducing biomass (Wei and Finneran 2011). Fe(III) reducers and dechlorinators developed simultaneously, and VC was reduced to ethene concurrently with Fe(III) reduction. The specific influence of acetate concentration was not reported (Wei and Finneran 2011). Acetate will promote TCE → cis-DCE reduction by organisms such as *Desulfuromonas chloroethenica*, *Desulfuromonas michiganensis*, and *Geobacter lovleyi* (Krumholz 1997; Krumholz et al. 1996; Sung et al. 2006). However, H_2 is specifically required for *Dehalococcoides* species (DHC) to reduce cis-DCE and/or VC to ethene. Acetate concentration, therefore, should have a large impact on the rate and extent of complete dechlorination as well as DHC growth.

Acetate has previously been cited as a poor electron donor for complete dechlorination (Lee et al. 2007; He et al. 2002). cis-DCE was the primary daughter product that accumulated in acetate-only treatments, and the authors concluded acetate alone would not likely serve as reasonable electron donor for complete dechlorination. However, Fe(III) reduction was not reported for those experimental conditions, and it is possible that the material lacked the requisite Fe(III) reducers to promote H_2 generation from acetate, which we and others have reported (Cord-Ruwisch et al. 1998; Wei and Finneran 2011). It was suggested that remediation strategies be developed to better utilize acetate at the PCE/TCE → cis-DCE step, with H_2 being supplied to reduce cis-DCE → ethene (Lee et al. 2007). This is also a good approach, although data presented here and elsewhere (Wei and Finneran 2011; Sung et al. 2006) indicate that acetate is a good electron donor under conditions where H_2 generated from acetate oxidation is favorable.

The objective of this study was to determine the influence of electron donor concentration ($10\times$ necessary acetate versus stoichiometric acetate) on TCE and vinyl chloride (VC) reduction, and to further characterize how acetate can promote dechlorination

simultaneously with both Fe(III) and sulfate reduction. Both contaminated aquifer material incubations and enrichment cultures were used to compare the dechlorination rates and extents under conditions with or without Fe(III)/sulfate reduction, and to evaluate whether excessive electron donor addition increased either the rate or extent of complete dechlorination, and whether increasing electron donor concentration actually increases DHC biomass.

Materials and methods

Chemicals

TCE, *cis*-DCE, and VC (>99.5 % pure gas or 1,000 ppmv in nitrogen) were purchased from Sigma-Aldrich (Milwaukee, WI). Poorly crystalline Fe(III) (hydr)oxide was synthesized from ferric chloride as previously described (Lovley and Phillips 1986). [U-^{14}C]-VC (5 mCi/mmol, in hexadecane) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals used were of reagent grade quality or higher.

Sediment batch experiments

Sediment incubations were constructed using TCE-contaminated aquifer material provided by Camp, Dresser, and McKee (confidential site). The samples were processed immediately after they were shipped to our lab in anoxic, sealed containers. Total ferric iron was 2.4 μmol/g (wet mass), and sulfate concentration was <0.1 mM (nominal slurry concentration). The concentration of dechlorination daughter products was negligible.

Three aquifer-material experimental series were constructed using previously described anoxic techniques (Wei and Finneran 2009). Briefly, 30 g of saturated aquifer material and 10 ml of groundwater were mixed in a 125 ml (nominal volume) glass serum bottle and sealed under an anoxic nitrogen headspace. All amendments were added from anoxic, sterile stock solutions using a syringe and needle. The first batch had only TCE added as the electron acceptor; the second batch had TCE and poorly crystalline Fe(III) (hydr)oxide (ferrihydrite) ($\sim$20 mmol/l), and the third batch had TCE, Fe(III), and 10 mM sulfate. Acetate was the sole electron donor. Bottles were incubated at 18 °C, in the dark, without agitation. All experiments were conducted in triplicate.

There were two *electron donor strategies* for each series. The first donor strategy was operationally defined as "stoichiometric", in which the acetate concentration was balanced "electron to electron" with all electron acceptors present (as closely as possible). The final acetate concentrations were 0.5, 3, and 13 mM in the three batch experiments described above, respectively. The second approach was an "excessive" electron donor approach in which 10× the necessary stoichiometric amount of acetate was added. This batch was meant to mimic the "ten times factor of safety" amendment strategy, which has been employed at many TCE-contaminated sites where soluble electron donors are amended (Lee et al. 1998; Scheutz et al. 2010). Sterile controls were set up in the same way as experimental bottles and were autoclaved for 1 h each day for three consecutive days. To evaluate dechlorination of VC as parent compound, identical sediment batch experiments were conducted in which $\sim$50 μM VC was added in lieu of TCE.

Enrichment culture experiment

Previously reported liquid enrichment cultures (Wei and Finneran 2011) were used to further quantify the influence of electron donor concentration on chlorinated solvent transformation, and by-product (methane) formation. The medium used was a modified defined freshwater medium (Lovley et al. 1993) with 25 mM HEPES buffer and resazurin to indicate trace oxygen; the medium did not contain yeast extract or any alternative H_2-generating carbon sources (to prevent H_2 from yeast extract fermentation (Fennell et al. 1997). Each anoxic pressure tube (nominal volume 26 ml) contained 13.5 ml medium with a N_2 headspace (Wei and Finneran 2009). Two batches were set up, with either TCE or VC as the parent compound, respectively. Three treatments were applied for each batch: TCE or VC + Fe(III) + acetate at stoichiometric concentration, TCE or VC + Fe(III) + 10× acetate, and TCE-only or VC-only as controls (with acetate at stoichiometric level). 0.15 μl TCE (1:20 v/v in hexadecane) was added to deliver $\sim$1.5 μmol total mass per tube ($\sim$90 μM aqueous concentration); VC standard gas was added to deliver $\sim$0.7 μmol total mass per tube ($\sim$22 μM aqueous concentration). Fe(III) was in the form of poorly crystalline Fe(III) (hydr)oxide (ferrihydrite). Experiments began with a 10 % (v/v) inoculum and

tubes were incubated upside down, in the dark, at 18 °C without shaking. The parameters quantified are described below.

Electron equivalents calculations

The electron equivalents consumed in each terminal electron accepting processes (i.e. complete dechlorination (TCE → ethene), Fe(III) reduction, and methanogenesis) were calculated based on molar mass changes of each electron acceptor and production of the corresponding reduction products. The distribution of electron equivalents was estimated as the percentage of electron equivalents consumed in each TEAP divided by the total electron equivalents consumed. The ratio of electron equivalents consumed for each TEAP was calculated by dividing the equivalents consumed by each acceptor by the theoretical electron equivalents available in the added acetate. Acetate analysis was not performed, so this value is based on theoretical total acetate consumed rather than actual acetate consumed. Specific values used in the electron equivalents calculation are presented in the supporting information in Table S2.

^{14}C-VC anaerobic oxidation study

Anoxic aquifer material incubations were constructed to assess carbon mass balances using U-[^{14}C]-VC. ^{14}C-VC was added in the presence and absence of Fe(III) (20 mmol/l) or Fe(III) + sulfate; controls were ^{14}C-VC alone. Limited acetate was added (0.5 mM) as supplemental carbon. 14C-VC was added from an anoxic stock to deliver 1.8 μCi. Headspace samples were collected using a strict anoxic technique and ^{14}C-labeled degradation products including ^{14}C-ethene, $^{14}CO_2$, and $^{14}CH_4$ were quantified. Liquid phase ^{14}C compounds were measured with scintillation counter. Gas phase ^{14}C compounds were measured with a Hewlett Packard Series 6890A gas chromatograph equipped with gas proportional radiochromatography detector (IN/US Systems, Inc., GC-Ram) as previously described (Finneran and Lovley 2001).

Analytical methods

TCE, DCE isomers, VC, ethene, ethane, and methane were quantified with a gas chromatograph equipped with a flame ionization detector (Hewlett Packard Series 6890A); the column used was a 30 m by 0.53 mm GS-Q column (J

& W Scientific, Waldbronn, Germany). Headspace samples were taken using standard anoxic techniques (Finneran and Lovley 2001). Aqueous Fe(II) and total bioavail-able iron were quantified by the ferrozine assay as previously described (Lovley and Phillips 1987).

DNA extraction and quantitative PCR analysis of DHC

Samples from enrichment culture experiments were collected at days 1, 11, and 20. Genomic DNA was extracted using FastDNA SPIN Kits (MP Biomedicals, Solon, OH, USA).

Quantitative polymerase chain reaction (qPCR) analysis was conducted with a MiniOpticon qPCR detection system and IQ SYBR Green Supermix (BioRad, Hercules, CA). Specific primers were used to target and quantify copies of DHC 16S rRNA genes. The DHC 16S rRNA gene primers have been described previously (Ritalahti et al. 2006). Each 25 μl reaction contained 12.5 μl 2× IQ SYBR Green Supermix (Bio-Rad), 0.3 mM of forward and reverse primers each, 2 μl template DNA and sterile, nuclease-free water (Invitrogen). The PCR program for DHC 16S rRNA gene was as follows: 50 °C (2 min), 95 °C (10 min), 45 cycles of 95 °C (15 s) and 58 °C (1 min). Finally, a melting curve ranging from 55 to 99 °C was performed with steps of 1 °C and a hold of 5 s. Calibration curves were made by using serial dilutions of PCR amplified DNA samples from DHC sp. FL2 pure cultures. The amplification efficiencies (E) ranged between 1.87 and 1.92. Calibration curves had linear range over about seven orders of magnitude, from 10^9 to 10^2 gene copies per μl DNA template, and R^2 values >0.98. The gene copy numbers of the standard DNA template were calculated based on the equations and parameters described previously (Ritalahti et al. 2006). Each qPCR analysis was conducted in triplicate, and calibration curves and no-template controls were performed together with samples each time. Statistical t-tests were performed to quantify the significance of differences amongst the incubation conditions for each time point, as described previously (Wei and Finneran 2011).

Archaeal clone library and qPCR analysis

Partial Archaeal 16S rRNA genes in the extracted DNA samples were amplified by PCR with primer pair Arc25F (5′-CYG GTT GAT CCT GCC RG-3′)

(Elshahed et al. 2004) and Arc958R (5′-YCC GGC GTT GAM TCC AAT T-3′) (Delong 1992). PCR product purity and size were checked by electrophoresis on 2 % agarose gel.

Clone libraries were constructed using PCR products from the 10× acetate amended cultures following the previously described method (Wei and Finneran 2009) with a TOPO TA Cloning Kit for Sequencing and One Shot Mach1TM-T1 competent *E. coli* cells (Invitrogen Corporation, Carlsbad, CA, USA). Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed by digesting the M13-primer PCR products with two FastDigest® restriction enzymes, *Hha*I and *Msp*I (Fermentas). The digested products were separated on a 3 % Metaphor agarose gel (Cambrex Bio Science). The DNA band patterns were visualized and recorded using Molecular Imager Gel Doc XR System (Bio-RAD). Representative clones were sequenced at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois. Chimeric sequence check was performed with Chimera Check software on the RDP website (http://rdp.cme.msu.edu/). Sequences were compared with the GenBank database using nucleotide National Center for Biotechnology Information (BLAST).

The sequence reported in this study has been deposited in GenBank under accession number JQ345702.

Archaeal 16S rRNA gene primers were used in Sybr Green-I qPCR analysis to quantify methanogens in all samples (10× acetate versus stoichiometric acetate). The primer pairs used were Arc806F (5′-AT TAG ATA CCC SBG TAG TCC-3′) (Takai and Horikoshi 2000) and Arc958R. The PCR program was as follows: 50 °C for 2 min, 95 °C for 10 min, the 45 total cycles at 95 °C (15 s) and 54 °C (1 min). The amplification cycle was followed by a melting curve which ranged from 55 to 99 °C and was performed at 1 °C steps with a hold of 5 s at each step. Calibration curves were made with purified archaeal 16S rRNA gene PCR product. The amplification E value was 1.97. Calibration curves had a linear range from 10^9 to 10^2 gene copies per µl DNA template, and a R^2 value >0.99. Each qPCR analysis was conducted in triplicate, and calibration curves and no-template controls were performed together with samples. Statistical *t*-tests were performed to quantify the significance (or lack thereof) of differences amongst the incubation conditions for each time point as described previously (Wei and Finneran 2011).

Results and discussion

High versus low acetate as electron donor for dechlorination in aquifer material

TCE was reduced to ethene in sediment incubations with stoichiometric acetate concentration plus ferrihydrite (Fig. 1a). TCE was completely reduced in 10 days, and reduction continued if TCE, acetate, and Fe(III) were re-amended (data not shown). Ethene was the primary daughter product; *cis*-DCE and VC accumulation was minimal. Fe(II) concentration increased concurrently with ethene production (Fig. 1a). Methane concentration was near the detection limit (1.0 ppmv) in all incubations (Fig. 1b). The rates and extents of dechlorination were more sporadic in the TCE-alone incubations, and no Fe(III) was reduced (SI, Figure S1 and Table S1).

Excess (10×) acetate did not increase the rate or extent of TCE degradation or ethene production; in fact, the rate and extent of both was lower with more acetate (Fig. 2a, Table S1). Methane production, conversely, was significantly greater in the 10× acetate-amended incubations; CH_4 accumulated to nearly 30 µmol/bottle in one of the replicates while the other two produced 10 µmol/bottle (Fig. 2b).

Sediment incubations amended with *ferrihydrite + sulfate* reduced TCE to ethene with concurrent Fe(III) reduction and sulfate reduction (SI, Figure S2A); methane production was 1 µmol/bottle at most (SI, Figure S2B). In comparison, adding 10× acetate slowed dechlorination rates and did not increase Fe(III) reduction or sulfate reduction (SI, Figure S3A and Table S1). However, methane generation increased significantly (SI, Figure S3B).

We attributed the slower dechlorination rates (with excess donor) to increased competition for available substrates from methanogens (both acetate and hydrogen). As methanogens increased they directly competed for electrons with the DHC-like organisms, which was less prevalent in the low electron donor amended systems. As is presented below, it was not a case of fewer DHC-like cells, but more likely efficiency of the reactions. Methanogens consume H_2 at approximately 13 times the rate that dechlorinators do; 3 versus 40 µmol mg VSS^{-1} h^{-1} (Fennell and Gossett 1998).

Sediment incubations with VC as the starting chlorinated electron acceptor behaved in a very similar manner to the TCE-amended incubations (SI, Figures S4A and S4B) with or without Fe(III) amendment.
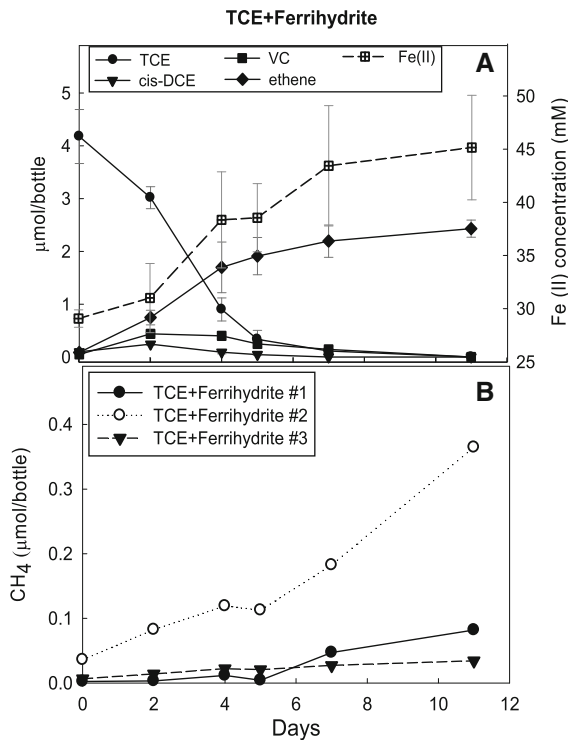
TCE+Ferrihydrite

Fig. 1 **a** TCE degradation, and Fe(II) accumulation in the aquifer sediment incubations amended withTCE + ferrihydrite + acetate (stoichiometric level). Results are the means of triplicate analyses. *Bars* indicate standard deviations. **b** Methane production in the sediment incubations amended with TCE + Fe(III) + stoichiometric level acetate. Results from each triplicate are shown individually

TCE+Ferrihydrite+10X acetate

Fig. 2 **a** TCE degradation, and Fe(II) accumulation in the sediment incubations amended with TCE + Fe(III) + 10× acetate. Results are the means of triplicate analyses. *Bars* indicate standard deviations. **b** Methane production in the sediment incubations amended with TCE + Fe(III) + 10× acetate. Results from each triplicate are shown individually

Excess electron donor is typically added as a "factor of safety" to account for electron accepting processes that may not have been identified in the original site characterization. The processes that are typically unaccounted are Fe(III) and/or Mn(IV) reduction (Lovley 1991; McLean et al. 2006). These processes are overlooked because aquifer solids are required, which increases the expense of acquiring the data. However, excessive electron donor did not stimulate the critical process—reductive dechlorination. The excess electron donor generated methane, which was not directly linked to TCE reduction.

Complete dechlorination has most often been linked to "highly reduction conditions" such as sulfate reduction and methanogenesis; though there are specific reports of complete dechlorination without concomitant methane production (Distefano et al. 1991; Duhamel et al. 2004). We suggest the alternative that methane
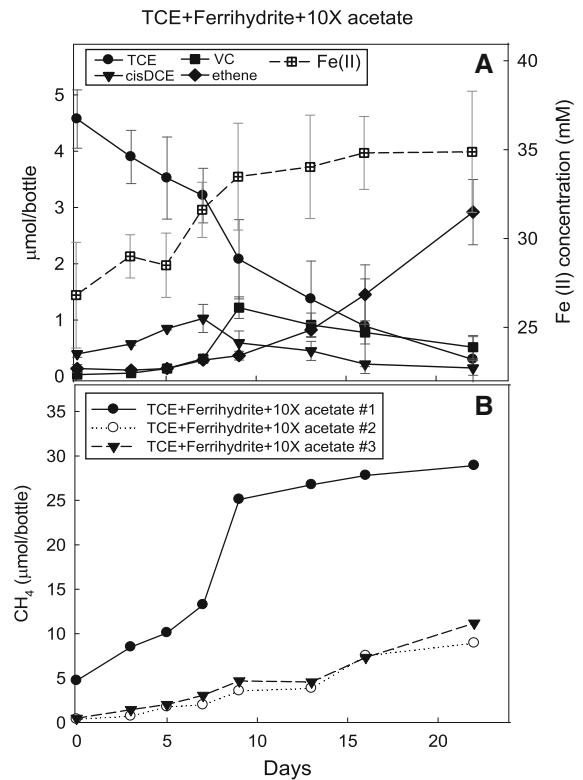
production is often the result of over-adding electron donor, and dechlorination is stimulated concurrently, though the processes are not necessarily linked and the "low environmental redox potential" is not required for complete dechlorination (Bennett et al. 2007; Cabirol et al. 1998a, b; Chapelle and Bradley 2000; Doong et al. 1996; Kennes et al. 1998). Previous reports do indicate that there is a dynamic amongst microbial populations present and electron donor concentration, and that methane production is not always related to excessive substrate (Fennell and Gossett 1998; Fennell et al. 1997; Fennell et al. 2001). However, in the vast majority of remediation cases, the result of excess substrate is methanogenesis (Illman and Alvarez 2009; Mulligan and Yong 2004; Perelo 2010; Rivett and Thornton 2008; Roling and van Verseveld 2002; Skubal et al. 2001). In addition, these previous reports focused on H_2-production kinetics rather than the relative quantity of DHC versus alternate microbial populations.

Our recent data with overlapping Fe(III) reduction demonstrate that there are at least cases where low redox potential is not necessary for complete dechlorination, despite past results suggesting that they are (Cabirol et al. 1996; Cabirol et al. 1998a). We capitulate to the fact that there will be cases where more, rather than less, electron donor will be required, but it should not be an a priori assumption for the site. But, as our knowledge about DHC-like organisms increases, we understand that they are more metabolically flexible than previously reported and that previous assumptions regarding pH, electron donor concentration, redox condition boundaries, etc., may need to be refined (Bratt et al. 2004; Freedman et al. 2011; Morris et al. 2007).

Field remediation realities will preclude use of this specific strategy in certain situations. Stakeholders may be reticent to use multiple, low concentration injections because it will cost more. This is especially true in environments with significant adsorbed mass, where multiple injections will be required as mass-transfer limited TCE dissolution limits the effectiveness of a soluble electron donor in a specific location for a defined time period before it flows out of the system. Finally, direct comparison of soluble acetate and lipid-based substrates is not reasonable, as their behavior in aquifer material is quite different. These studies will be repeated with several commercial oils to ascertain whether low lipid-based electron donor mass (i.e. 1:1 molar stoichiometry) still promotes equivalent dechlorination relative to higher lipid-based electron donor mass, taking into account the insoluble nature of the oils, and the initial degradation reactions that generate H_2 and acetate.

Irrespective of concentration acetate promoted complete dechlorination, and proliferation of DHC-like microorganisms as cis-DCE and VC were reduced to ethene. Previous data suggested that this is via Fe(III)-reduction mediated H_2 generation during acetate oxidation (Wei and Finneran 2011), which was likely the mechanism here. Regardless of the mechanism this suggests that acetate is a suitable electron donor for complete dechlorination in some conditions, which has practical implications for environmental remediation. The first is that acetate may directly be used as an electron donor in environments where alternative, soluble substrates are not appropriate. The second is that acetate may be one of several products generated during lipid decomposition, and it will be critical to understand what role acetate plays during

vegetable oil based remediation as the insoluble substrates decompose.

High versus low acetate as electron donor in enrichment cultures

Liquid enrichments were used to further investigate the effects of electron donor concentration on dechlorination and alternative TEAPs of interest. Similar to results with aquifer material, TCE was reduced to ethene in both stoichiometric and 10× acetate amended incubations, with concurrent Fe(III) reduction when Fe(III) was added (Fig. 3a–c). One notable difference was VC accumulation. All of the VC was depleted in the stoichiometric acetate amended incubations while the incubations with 10× acetate still had VC on day 20 (Fig. 3a vs b). Control incubations with TCE-alone (no acetate amendment) did not significantly reduce TCE (SI, Figure S6), confirming that acetate was used as the primary source of electrons in these cultures. Past reports (Fennell et al. 1997) suggested that yeast extract or other unaccounted carbon substrates in media recipes led to H_2 production, which we were careful to prevent here.

VC degradation rates, and ethene accumulation rate and extent, were similar amongst all treatments when VC was added as the primary chlorinated electron acceptor (Fig. 4). These data support the aquifer material experiments in that stoichiometric electron donor concentration was sufficient to promote complete dechlorination (starting with either TCE or VC) even when alternate electron acceptors were present, and that excess electron donor concentration did not actually increase the rate or extent of complete dechlorination. TCE reduction was slightly inhibited with excess acetate while VC reduction was not; this is possibly due to the fact that the VC-degrading enrichment cultures were more highly enriched in the two primary microbial populations, while the TCE-degrading enrichments were initially more diverse. Hence, there was more competition for acetate, or the H_2 generated from acetate. While not the primary focus of this report—sulfate reduction did not inhibit complete dechlorination (starting at 10 mM) when acetate was added at the lower, stoichiometric concentration. TCE was reduced to ethene as sulfate was simultaneously reduced (SI, Figure S2). This suggests that electron donor amendment strategies can be altered to accommodate multiple
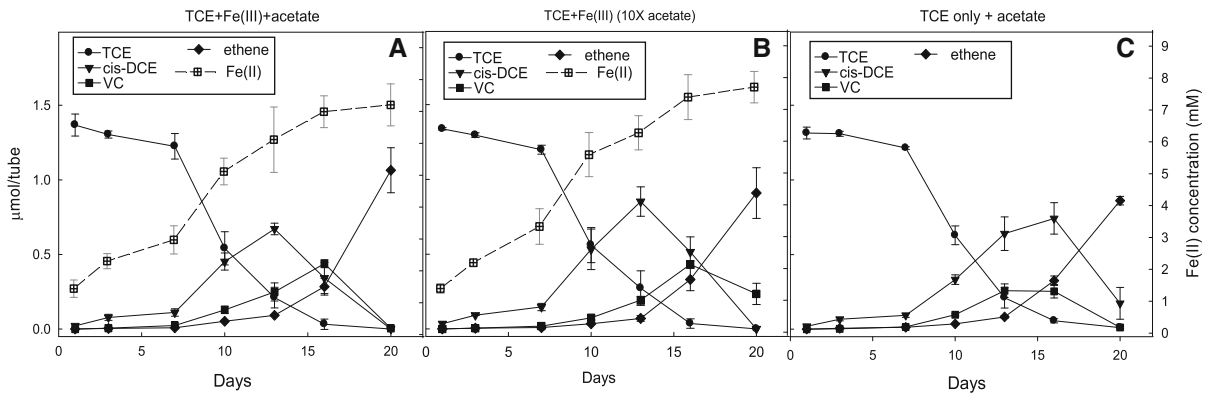
Fig. 3 TCE reductive dechlorination and Fe(II) accumulation in enrichment culture incubations with **a** TCE + ferrihyrite + stoichiometric acetate, **b** TCE + ferrihyrite + 10× acetate, **c** TCE only + acetate. Results are the means of triplicate analyses. *Bars* indicate standard deviations
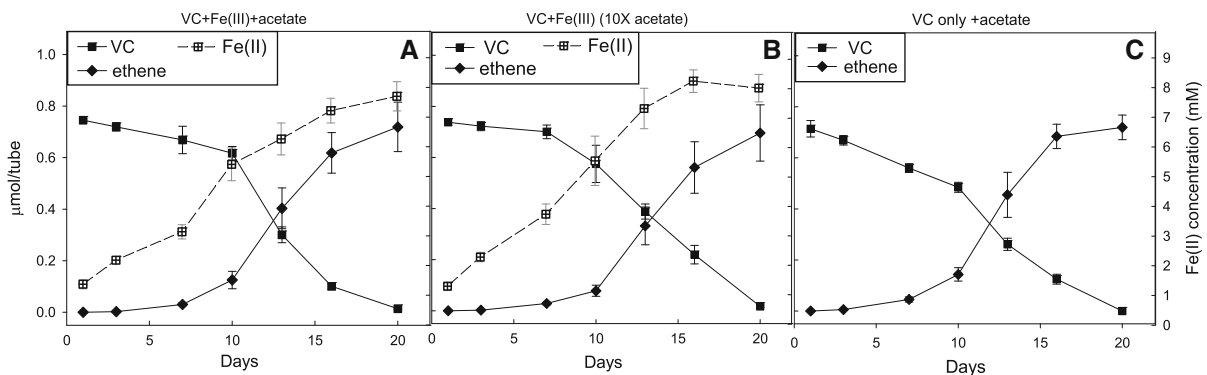

Fig. 4 VC dechlorination and Fe(II) accumulation in enrichment culture incubations with **a** VC + ferrihyrite + stoichiometric acetate, **b** VC + ferrihyrite + 10× acetate, C. VC only + acetate. Results are the means of triplicate analyses. *Bars* indicate standard deviations

electron acceptors, which will be useful to site remediation.

Electron donor concentrations and methanogenesis

As expected methane production increased significantly as acetate concentration increased (Fig. 5). Methane accumulated to 13.5 μmol/tube on average in the TCE-degrading incubations with 10× acetate addition, and to only 1.4 μmol/tube with low acetate concentration. (Fig. 5a). The VC degrading incubations had similar profile as to methane production under high versus low acetate conditions (16 μmol/tube and 2 μmol/tube on average in the incubations with 10× and stoichiometric acetate, respectively) (Fig. 5b). Fe(III) transiently inhib

-ited methanogenesis, but ultimately methane production was very high in all incubations that were amended with 10× the necessary acetate concentration.

These results suggest that methanogenesis and complete dechlorination were not linked. As indicated above complete dechlorination has often been directly linked to methanogenic conditions (Cabirol et al. 1998a, b; Doong et al. 1996; Kennes et al. 1998; Lee et al. 1998); although a specific mechanism for methanogens has not been identified. Previous studies (Distefano et al. 1991; Duhamel et al. 2004; Ballapragada et al. 1997; Kromann et al. 1998; Leahy and Shreve 2000) have suggested that dechlorination and methanogenesis may not be linked, and this report further demonstrates that increasing methane production (via excess electron donor amendment) did not
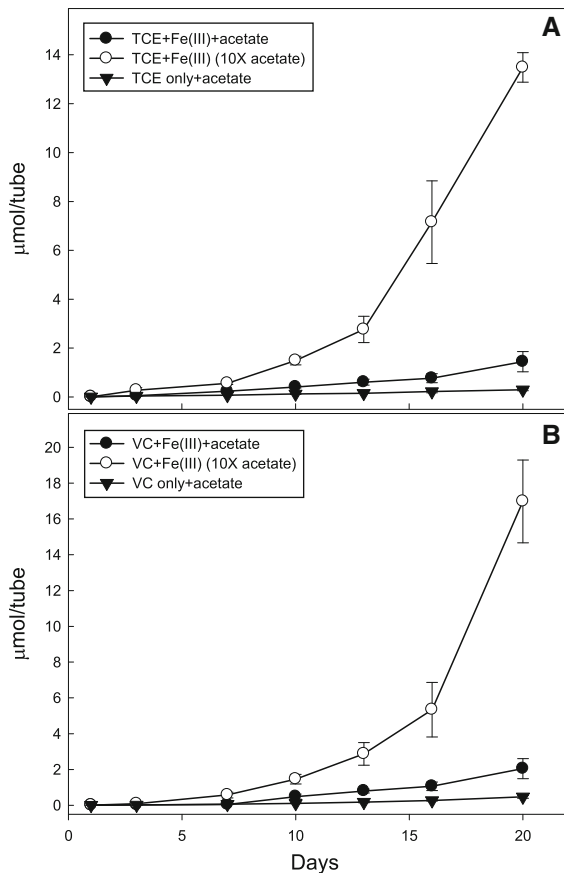
Fig. 5 Methane production in the TCE-degrading enrichment cultures (**a**) and VC-degrading enrichment cultures (**b**) under different incubations conditions. Results are the means of triplicate analyses. *Bars* indicate standard deviations

increase either the rate or extent of TCE, *cis*-DCE, or VC reduction.

Distribution of electron consumption in different TEAPs

The proportion of the electrons consumed by each of the three TEAPs was calculated based on theoretical complete consumption of acetate and compared amongst the conditions with different electron donor concentrations (Table 1). When acetate concentration was low, Fe(III) reduction consumed most of the electrons but enough were available for complete dechlorination, and only a small portion of electrons distributed to methanogenesis (Table 1, column A and C). When the high concentration of acetate was added, electron equivalents distributed to dechlorination did

not increase (i.e. total electron equivalents consumed by dechlorination), which is in agreement with the results of dechlorination rates and extents as discussed earlier. However, the fraction of electrons going to methanogenesis increased substantially. It increased to $\sim 36\%$ in TCE + Fe(III) + 10× acetate versus $\sim 6\%$ in stoichiometric acetate, and $\sim 42\%$ in VC + Fe(III) + 10× acetate versus $\sim 9\%$ in stoichiometric acetate.

It is also noteworthy that only a small fraction of total available electrons were used in the 10× acetate amended incubations (Table 1, column B and D). Admittedly, the calculation here may underestimate all electrons consumed as electron equivalents because processes such as biosynthesis were not taken into account. For example, only 51–53 % of the total electrons were "consumed" in the operationally defined stoichiometric batch. The general trend still holds, however, and increasing electron donor concentration did not increase electron flow to complete dechlorination, but rather methanogenesis.

DHC versus methanogens based on electron donor concentration

DHC spp. were previously identified as one of the dominant populations in the enrichment cultures used in these experiments (Wei and Finneran 2011). Changes in DHC and methanogen abundance were quantified using qPCR.

DHC abundance was similar irrespective of high versus low electron donor concentration (Fig. 6). In fact, the gene copy number of DHC at day 20 in the low acetate concentration incubations was statistically higher than those with the high acetate concentration ($P < 0.05$) (Fig. 6a). The presence of Fe(III) had little impact on the number of DHC gene copies. In the VC amended cultures, there was no significant difference among the different incubation conditions on day 20 ($P > 0.05$) (Fig. 6a). The microbial population data confirmed the rate/extent of dechlorination data, in that excessive acetate did not increase the total number of DHC, or activity amongst those cells present.

DHC gene copies (10^7–10^8 ml^{-1} slurry) in these laboratory incubations are comparable to previously reported data (Ritalahti et al. 2006). These data, as in that study, are significantly higher (4–6 orders of magnitude) than the number of DHC gene copies recovered in TCE-contaminated aquifer material, even

Table 1 Distribution of electron consumption in reductive dechlorination, Fe(III) reduction and methanogenesis in enrichment culture incubations with different starting acetate concentrations

TEAPs	A. TCE + Fe(III) + stoichiometric acetate		B. TCE + Fe(III) + 10× Acetate		C. VC + Fe(III) + stoichiometric acetate		D. VC + Fe(III) + 10× acetate	
	Distribution of e⁻ (%)	e⁻ use/total (%)	Distribution of e⁻ (%)	e⁻ use/total (%)	Distribution of e⁻ (%)	e⁻ use/total (%)	Distribution of e⁻ (%)	e⁻ use/total (%)
Dechlorination	7.01 (0.68)	3.53 (0.18)	4.71 (1.06)	0.38 (0.07)	1.64 (0.16)	0.88 (0.11)	0.86 (0.15)	0.08 (0.01)
Fe(III) reduction	86.77 (3.9)	44.04 (4.96)	58.84 (3.28)	4.85 (0.46)	89.79 (2.52)	48.04 (3.76)	57.42 (3.49)	5.17 (0.24)
Methanogenesis	6.22 (1.07)	3.20 (0.91)	36.45 (2.25)	2.99 (0.13)	8.57 (2.62)	4.54 (1.23)	41.72 (3.42)	3.77 (0.51)
e⁻ used/total e⁻ available		50.77		8.22		53.46		9.02

Results are presented as percent (%) of electron equivalents theoretically available from the complete oxidation of acetate. The values are the estimated fraction of electrons recovered in each terminal electron accepting process, with the remainder theoretically remaining as acetate

under engineered conditions (Major et al. 2002; Ritalahti et al. 2006). While these data can be extrapolated to field sites, the absolute biomass increases in the field will likely be much smaller.

BLAST results of the clone library generated indicated that the dominant Archaeal sequence was most closely related to uncultured clone ATB-KM-3231-A16 with 97 % similarity. The clone ATB-KM-3231-A16 was reported in a study that characterized methanogenic Archaea in biogas reactor (Klocke et al. 2008). This phylotype clusters within the currently uncharacterized CA-11 methanogen group (Klocke et al. 2008); it is uncertain whether they are hydrogenotrophic or aceticlastic methanogens. While the specific physiology of the dominant methanogen in our samples is uncertain, initial sediment characterization with 2-[^{14}C]-acetate demonstrated that methane was produced directly from the acetate carbon, which indicates that aceticlastic methanogenesis cannot be ruled out in these cultures. This Archaeal primer pair was then used to target and quantify methanogens using qPCR analysis. Until this methanogen group is further characterized we cannot know if it directly competed for acetate or hydrogen (with the DHC-like organisms). However, the end result is virtually the same because our previous data indicated that acetate metabolism was linked to hydrogen production (Wei and Finneran 2011)—competition at either point compromises electrons available for dechlorination.

The number of methanogen gene copies in the 10× acetate amended incubations was approximately one order of magnitude higher than the stoichiometric acetate incubations for the duration of the experiment, for both the TCE-degrading and VC-degrading cultures ($P > 0.05$) (Fig. 6b). The number of methanogens recovered in the Fe(III)-amended stoichiometric batch was statistically greater than the stoichiometric batch that had only TCE or VC as the primary electron acceptor ($P > 0.05$) (Fig. 6b). Since no direct mechanisms for Fe(III) reduction enhancing methanogenesis has been reported (iron is a trace nutrient for methanogens, but at the concentrations recovered in these cultures it is well above the stimulatory level and no data support excess iron increasing methanogenesis), we conclude that the increase is due to acetate added in excess of what was required to compensate for the Fe(III). As presented above (Table 1), approximately 51–53 % of the electrons were consumed in the stoichiometric batch, indicating that acetate was
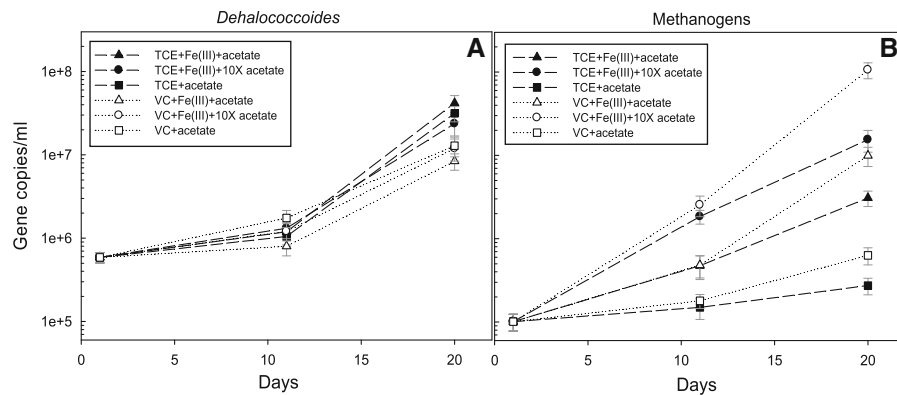
Fig. 6 a 16S rRNA gene copy numbers of *Dehalococcoides* organisms in the TCE-degrading or VC-degrading enrichment cultures under different incubation conditions. **b** Archaeal 16S rRNA gene copy numbers (representing methanogens) in the TCE-degrading or VC-degrading enrichment cultures under different incubation conditions. *Each point* represents the average of DNA samples from triplicate bottles under each incubation condition, each quantified in triplicate ($n = 9$). *Error bars* represent standard deviations. The gene copy numbers are given on a log scale

actually present in excess (despite the stoichiometric target). Any excess electron donor increased methane produced and the number of methanogens.

VC oxidation and carbon mass balance

Carbon mass could not be balanced strictly using ethene as an end product. ^{14}C-VC was used to determine whether some fraction of the carbon was being oxidized to $^{14}CO_2$, given that Fe(III) and sulfate reduction were simultaneously active during dechlorination. Twenty to forty percent of ^{14}C-VC was recovered as $^{14}CO_2$ when Fe(III) and/or sulfate were available as electron acceptors (SI, Figure S5), which effectively accounted for carbon transformation in these experimental systems. We understand recent data suggest *cis*-DCE and VC oxidation may be due to trace oxygen present in operationally defined anoxic systems. Cultures were incubated using strictly anoxic conditions, inside of a glove bag to further reduce the potential for oxygen contamination. However, in light of recent data (Gossett 2010) it is impossible to prove that oxygen was absent given the levels of oxygen required (by calculation) are below realistic thresholds for quantitative analyses. These data are further discussed in the supporting information.

In summary, these data demonstrate that targeting a stoichiometric concentration of electron donor will be adequate for efficient and complete TCE reduction during bioremediation, without producing excessive methane. The amount of organic substrate amended during chlorinated ethene bioremediation must be determined carefully by evaluating the actual impact with respect to stimulating dechlorination, as well as the potential undesirable side effects. We understand that these data may not apply to each and every site contaminated with TCE, and there will be cases where dechlorination rate is directly proportional to the concentration of electron donor added. But, this will be accompanied by increased methane, which conflicts with the remediation goals on the whole. At the very least electron donor amendment can start low and be increased, whereas the opposite is not true. We anticipate this may reduce the total mass of substrate required at many sites, which in turn increases the sustainability of chlorinated solvent bioremediation.

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