

Reductive dehalogenase gene biomarkers differentiate 1,1,1-trichloroethane from 1,1-dichloroethane dechlorination in microcosms and at a field site treated with granular ZVI and guar gum

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Running Title

Gene-based biomarkers to differentiate 1,1,1-TCA from 1,1-DCA dechlorination

Abstract

Chlorinated ethanes, such as 1,1,1-trichloroethane (1,1,1-TCA), are environmental pollutants frequently found in soil, groundwater and the atmosphere, and are commonly identified in many industrial contaminated sites. Removal of 1,1,1-TCA can be achieved via abiotic transformation or biodegradation by dechlorinating organisms such as *Dehalobacter* (*Dhb*). In this study, we analyzed samples from groundwater microcosms and an industrial site where a mixture of granular zero valent iron (ZVI) and guar gum was injected for 1,1,1-TCA remediation. Abiotic and biotic transformation products were monitored and microbial community changes were tracked using quantitative PCR (qPCR), with primers targeting the *Dehalobacter* 16S rRNA gene and two functional genes *cfrA* and *dcrA* encoding the reductive dehalogenases capable of converting 1,1,1-TCA to 1,1-dichloroethane (1,1-DCA) and 1,1-DCA to chloroethane (CA), respectively. The abundance of genes targeted by the *cfrA* and *dcrA* qPCR primers tracked dechlorination activity in a highly consistent and specific manner. The two reductive dechlorination steps were catalyzed by two distinct *Dehalobacter* populations harboring two closely related but distinct reductive dehalogenase genes. Using cell yields established in microcosms along with *Dhb* 16S rRNA, *Dhb cfrA*- and *dcrA*-like gene copies in site samples, the biotic component of 1,1,1-TCA transformation at a complex field site was estimated.

1. Introduction

Chlorinated aliphatic hydrocarbons, such as chlorinated ethanes and chlorinated ethenes, have been widely used as metal degreasers and cleaning agents, industrial solvents and adhesives, and reagents in various industries (Tobiszewski and Namieśnik, 2012). Among these compounds, 1,1,1-trichloroethane (1,1,1-TCA) was identified as an ozone-depleting compound under the Montreal Protocol of 1987, and subsequently its use was phased out starting in 1989 (Gu et al., 2011). Nevertheless, 1,1,1-TCA is still one of the most commonly identified organic pollutants in contaminated soil and groundwater at hazardous waste sites, e.g., 1,1,1-TCA was found at more than 393 of the 1315 active sites on the National Priorities List (US EPA database as of 2012). Exposure to 1,1,1-TCA might lead to damage to the central nervous system, heart and liver (ATSDR, 2006). The EPA sets the maximum contaminant level of 1,1,1-TCA in drinking water at 200 µg/L (EPA, 1995). Furthermore, 1,1,1-TCA is very volatile (124 mm Hg at 20°C) (Boublik et al., 1984), poorly soluble in water (1500 mg/L at 25°C) (Horvath, 1982) and sorbs to organic matter (log K_{oc}=2.02) (Friesel et al., 1984).

In groundwater, 1,1,1-TCA is prone to abiotic transformation to 1,1-dichloroethene (1,1-DCE) via elimination, or to 1,1-dichloroethane (1,1-DCA) via reduction catalyzed either by naturally occurring metals or added metals in engineered systems (Scheutz et al., 2011; Palau et al., 2016). In particular, the addition of zero valent iron (ZVI) has been tested at lab and pilot scales and implemented at various sites to reduce chlorinated compounds including 1,1,1-TCA (Fennell and Roberts, 1998; Agrawal et al., 2002; Lookman et al., 2004; Song and Carraway, 2005; Velimirovic et al., 2014; Wu et al., 2014), where 1,1-DCA was often found as the major daughter product and ethane as a minor by-product.

Biotransformation of 1,1,1-TCA occurs in anaerobic environments via reductive dechlorination (Fam et al., 2012). *Dehalobacter* sp. TCA1, isolated by Sun et al. (2002), was the first pure culture of an organohalide respiring bacterium that coupled growth to the sequential dechlorination of 1,1,1-TCA to 1,1-DCA and ultimately to chloroethane (CA). A few other strains, namely *Dehalobacter* sp. strain CF and strain DCA (Tang and Edwards, 2013B), *Dehalobacter* sp. strain UNSWDHB (Wong et al., 2016),

Dehalobacter sp. strain THM1 (Zhao et al., 2017), and *Desulfitobacterium* strain PR (Ding et al., 2014), have since been described or isolated with the ability to partially or completely dechlorinate 1,1,1-TCA and/or 1,1-DCA. All of these strains use either hydrogen or formate as electron donor. Six reductive dehalogenases (RDases) that catalyze these specific dechlorination reactions in these bacteria have also been identified, and are referred to as CtrA, TmrA, DcrA, CfrA and ThmA. These proteins and their corresponding genes form a clade of highly similar sequences belonging to Ortholog Group 46 (Hug et al., 2013). Interestingly, while the proteins are very similar (>95% amino acid identity), they do have distinct substrate preferences. For example, DcrA from *Dhb* strain DCA catalyzes dechlorination of 1,1-DCA to CA, but is not active on 1,1,1-TCA or chloroform (CF), while CfrA from *Dhb* strain CF and ThmA from *Dhb* strain THM1 are active on CF and 1,1,1-TCA but not on 1,1-DCA (Tang and Edwards, 2013; Tang and Edwards, 2013B; Zhao et al., 2017). In contrast, CtrA from *Desulfitobacterium* strain PR and TmrA from *Dhb* UNSWDHB are reported to be active on all three substrates to varying degrees (Ding et al., 2014; Wong et al., 2016). Primers to amplify the genes encoding these RDases have been incorporated into quantitative polymerase chain reaction (qPCR) approaches to measure the abundance of these organisms or RDase genes in the studies mentioned above.

Chlorinated ethane concentrations are frequently monitored to evaluate 1,1,1-TCA treatment efficiency at remediation sites, but concentration data are easily affected by site operations, making interpretation of data difficult, particularly in trying to distinguish between biotransformation and abiotic removal. Gene-based biomarkers (i.e., qPCR-based approaches) applied to field samples have the potential to monitor biological 1,1,1-TCA removal (Scheutz et al., 2011). To date, functional gene-based primers developed for organisms dechlorinating 1,1,1-TCA have been applied to microcosm and culture samples only, and have not been evaluated with actual field site samples in published work. Moreover, only a limited number of field studies reported using qPCR with primers to measure the 16S rRNA gene of *Dehalobacter* dechlorinating 1,1,1-TCA at remediation sites (Postiglione et al., 2006; Duchesneau et al., 2007; Damgaard et al., 2013).

In this study, we worked in collaboration with consultants from Pinchin Ltd. on a site contaminated with 1,1,1-TCA. The remedy selected for the site was injection of granular ZVI that was mixed with guar gum to stabilize the iron. Site samples were analyzed for chlorinated ethane concentrations and gene abundances using qPCR during the on-going remediation. In parallel, we also prepared microcosms with site groundwater to more definitively track both abiotic and biotic processes under defined conditions. The objectives of this research were to evaluate the use of the *Dhb* 16S rRNA gene and the *cfrA*- and *dcrA*-like dehalogenase genes as biomarkers for 1,1,1-TCA dechlorination and to investigate the impact of guar gum stabilized ZVI addition on the biodegradation of 1,1,1-TCA.

2. Materials and Methods

2.1 Site Description

The Site was a former adhesive manufacturing plant located in Southern Ontario and operated between 1950 and 1991. A spill of approximately five hundred litres of 1,1,1-TCA in the late 1980s was the only recorded release at the site. According to previous investigations, 1,1-DCA, 1,1-DCE, 1,1,1-TCA and dichloromethane (DCM) were the major contaminants detected between 1998 and 2014, with 1,1,1-TCA being at highest concentration (Table S1). A pump and treat system was operated between 1995 and 2015, and 4800 tonnes of impacted soil were excavated around year 2000. The stratigraphy information of the site is explained in Supporting Text S1 in the Supporting Information.

2.2 Granular ZVI Particles, Guar Gum, and On-Site ZVI Injection

Coarse ZVI filings (Cast Iron Aggregate Size 8/50, 0.37-2.36mm) were provided by Peerless Metal Powders & Abrasive. A suspension matrix (slurry) of water, guar gum gel (Biofrac G), cross-linker (Biofrac X) and a caustic pH buffer was provided by Frac Rite Environmental Ltd. Guar gum is a polysaccharide mixture extracted from guar beans and is predominantly composed of galactose and mannose. In late September 2015, the mixture of guar gum and ZVI was injected mainly along the

property boundary and in the plume area (Figure S1). One week after ZVI-guar gum injection, portions of the site were excavated and back-filled with ZVI alone (Figure S1). The details of the fracturing pressure, distance and intervals of injections, and the amounts of guar gum and ZVI injected and soil excavation are explained in Table S2 in the Supporting Information.

2.3 Groundwater Monitoring and Sample Collection

Between September 2015 and June 2016, groundwater samples were collected from three wells in the plume area, SW5, SW6 and MW202 (shown in Figure S1). All wells were developed and purged prior to sampling. Upon groundwater recovery, samples were collected using dedicated inertial pumps. Groundwater samples for chlorinated organic compound analysis were stored in 44 mL glass vials with screw caps with Teflon liners and zero headspace, and preserved with 0.2 g sodium bisulphate tablets. Samples for microbial analysis were collected in 250 mL sterilized plastic bottles containing sodium thiosulphate with zero headspace. All samples were placed on ice immediately after sampling and were stored at 4°C upon arrival at the lab at the University of Toronto.

2.4 Groundwater Microcosm Setup and Sampling

To evaluate *in situ* microbial activity with and without added ZVI, groundwater collected on day 253 from SW6 was used to set up microcosms. The groundwater (2 × 2 L in medium bottles) was purged with 80%/20% N₂-CO₂ gas mix for 1 hour to remove oxygen and residual volatile compounds. In a disposable glovebag (Atmosbag, Aldrich) filled with 80%/20% N₂-CO₂ gas mix, 150 mL of purged groundwater was dispensed into each sterile 250 mL glass bottle, and ZVI (8/50 Grade from Peerless Metal Powders & Abrasive) was added to a subset of the bottles (1 g per bottle). All bottles were then capped with Mininert septa screw caps (sterilized by wiping with ethanol) before the bottles were taken out of the glovebag. As detailed in Table S3, six treatments were established that included: Sterile (i.e., negative control by autoclaving); Sterile + ZVI; Active without ZVI; Active + ZVI; Active without TCA and ZVI; and bioaugmented with ACT3 + ZVI where ACT3 refers to a previously described enrichment

culture that dechlorinates 1,1,1-TCA via 1,1-DCA to CA (Tang and Edwards, 2013B). Neat 1,1,1-TCA (~4.25 μ L per bottle) was added for an initial liquid concentration of approximately 26 mg/L. ACT3 culture (0.1 mL) was anaerobically added to the ACT3 + ZVI set on Day 2 inside a lab glovebox. In most of the bottles, we did not add any additional electron donor, as the purged groundwater contained approximately 800 mg COD/L from residual guar gum and its degradation products. The only exceptions were bottle #8 in the Active set without ZVI and bottle #21 in the Active + ZVI set to which ethanol and lactate were added on day 149 at five times electron equivalent excess to the remaining 1,1-DCA (see Table S3). An extra serum bottle (capped with rubber stopper and crimped) containing the same amount of groundwater without any 1,1,1-TCA or ZVI was also included in the assay to evaluate methane production from the groundwater COD. All bottles were incubated statically, upside-down, in an anaerobic glovebox (Coy) filled with 5%-95% H_2 - N_2 gas mix at room temperature. Liquid samples were withdrawn from bottles (after shaking to mix) using gas-tight syringes and sterile needles for chemical and microbial analysis.

2.5 DNA Extraction and Microbial Analysis

Groundwater samples collected from the site for microbial analysis were transported, packed with ice, to our lab at the University of Toronto and filtered within 24 hours after sampling through sterile 0.22 μ m Sterivex filters using a vacuum pump (Ritalahti et al., 2009). The filters were frozen at -80°C immediately after filtration. Liquid samples from lab microcosms (1.6 mL) were transferred to 2 mL anaerobic screw-cap vials inside the glovebox. Samples were centrifuged at 13,000 \times g at 4°C. The supernatant was removed and the tubes with pellets were stored at -80°C until DNA extraction.

DNA extraction was conducted using the Mobio® PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA). For groundwater samples, filter membranes were removed from the plastic casings, and added to the bead beating tubes. For each microcosm sample, the beads and the solution in one bead beating tube were carefully transferred to the screw-cap vial containing the thawed cell pellet, and

vortexed until the cell pellet was completely re-suspended. Subsequent DNA extraction steps were performed following manufacturer instructions. A NanoDrop ND-1000 spectrophotometer was used to measure DNA concentration and quality.

Quantitative PCR (qPCR) was used to enumerate specific phylogenetic and functional genes in the DNA samples. Primers *Dhb477f/Dhb647r* were used to quantify the abundance of *Dehalobacter* 16S rRNA gene copies (Grostern and Edwards, 2006). Primers *cfrA413f/cfrA531r* and *dcrA424f/dcrA533r* were adapted to track two reductive dehalogenase genes *cfrA* and *dcrA* encoding the proteins catalyzing dechlorination of 1,1,1-TCA and 1,1-DCA respectively (Tang and Edwards, 2013B). Detailed recipe of the qPCR reactions and programs are explained in Text S2 in the Supporting Information.

The DNA extracted from site samples and groundwater microcosm samples was also submitted to McGill University and Genome Quebec Innovation Center for 16S rRNA gene amplicon sequencing. The primary goal of the amplicon sequencing was to determine the percentages of dechlorinating organisms in the microbial communities. Detailed information on the primers used to construct the library and the analysis of the sequencing data are presented in Text S3 in the Supporting Information.

To obtain the complete sequences of reductive dehalogenase genes from our samples, the DNA extracted from the bottle with the fastest degradation of 1,1,1-TCA in the Active set without ZVI (i.e., bottle #9 on day 49) was amplified with primers *rdhA23f/rdhA1383r* targeting the reductive dehalogenase. The PCR program, the primer sequences, the cloning procedures, the culturing of the *E. coli* cells and the procedures to extract plasmid DNA are described in the work conducted by Tang and Edwards (2013B). Five clone samples were submitted to the Centre for Applied Genomics (Toronto), and each was sequenced four times in both the forward and the reverse directions with the M13f and M13r primers. Using the Geneious program, the forward and reverse reads were merged to recreate the amplicons, and aligned with five gene sequences encoding known 1,1,1-TCA/1,1-DCA dehalogenases:

dcrA(JX282330.1), *cfrA*(JX282329.1), *ctrA*(KF155162.1), *tmrA* (NZ_AUUR01000010 REGION: 19936..21303) and *thmA* (KX344907.1).

2.6 Chemical Analysis of Groundwater and Microcosm Samples

Chlorinated ethane concentrations in field samples were measured within 48 hours after the samples arrived at the University. Groundwater microcosm samples were tested immediately after sample collection. Aliquots of 0.5-1 mL of groundwater or microcosm samples were added to acidified MilliQ water to a total liquid volume of 6 mL in 11 mL crimped-cap headspace autosampler vials and placed in the Agilent G1888 Headspace Sampler. Headspace in the crimped vials was analyzed using the Agilent 7890 gas chromatograph (GC) equipped with the GS-Q column and detected using a flame ionization detector (FID). External standards, purchased from Sigma, were first diluted with methanol, and then in acidified MilliQ water for calibration. A modified EPA 8021 method was used as per Kocur et al. (2015). Several groundwater samples were also analyzed for total COD according to the EPA Method 410.4 potassium dichromate/mercuric sulfate colorimetric method (600 nm) (USEPA, 1993).

3. Results and Discussion

The role of abiotic and biotic transformation in 1,1,1-TCA removal at the site are assessed from microcosms and field chemical and microbial analyses, and implications for field monitoring of abiotic and biotic transformation are discussed.

3.1 Dechlorination of 1,1,1-TCA in Groundwater Microcosms

Mole balances in groundwater microcosm bottles were calculated comparing consumption of electron donors and electron acceptors to evaluate the accuracy of our measured analytical data. A mole balance for all two carbon compounds (C2-compounds) was calculated, comparing data from time zero to what was measured subsequently considering all measured degradation products of 1,1,1-TCA including 1,1-

DCA, 1,1-DCE, cis-DCE, trans-DCE, 1,2-DCA, ethane and ethene. This mole balance revealed that total moles of C₂-compounds were conserved in the Sterile Control microcosms without ZVI, in the Active set without ZVI, and in the set with ACT3 culture and ZVI (ACT3 + ZVI) (Figure S2), implying that major transformation products were accounted for. In the Active + ZVI and Sterile +ZVI sets, measured degradation products did not completely account for substrate loss, likely due to products that were not measured (see below). Methane production was estimated and compared to the original COD measured in the groundwater. In the Active set without 1,1,1-TCA and ZVI, the methane produced accounted for approximately 90% of the original COD present in the groundwater (Table S4), demonstrating that although no extra electron donor was added to the microcosms, the residual guar-gum and its products were sufficient to drive methanogenesis and dechlorination. The lower methane production in bottles fed with 1,1,1-TCA (Table S4) reflects the well-documented inhibition of methanogenesis by compounds like 1,1,1-TCA (Grostern and Edwards, 2006).

Overall, 1,1,1-TCA was dechlorinated in all groundwater microcosm bottles except for the Sterile Controls without ZVI (Figure S3), but the rates and the extent of reductive dechlorination of 1,1,1-TCA varied considerably. The least activity was seen in the Sterile + ZVI bottles where dechlorination stopped at 1,1-DCA (Figure S3). In contrast, dechlorination of 1,1,1-TCA past 1,1-DCA to CA was observed in both ACT3 + ZVI bottles (Figure S4), in more than half of the bottles in the Active set without ZVI (Figure 1A) and the Active + ZVI set (Figure 5SA). Thus, dechlorination of 1,1-DCA to CA only occurred in bottles containing active organisms. In bottles containing ZVI, whether active or not, 1,1,1-TCA was quickly converted to 1,1-DCA, while in Active bottles without ZVI, a seven to twenty three day lag time was noted before dechlorination started (Figure S6). The rapid dechlorination to 1,1-DCA and lack of further dechlorination in microcosms with ZVI without active organisms were consistent with previous abiotic studies (Cwiertny et al., 2006; Wu et al., 2014). Among all active bottles, the ACT3 + ZVI bottles and bottle #9 in the Active set without ZVI exhibited the shortest lag times, the highest rates of dechlorination of 1,1,1-TCA, and the earliest onset of conversion of 1,1-DCA to CA. The

dechlorination rates of 1,1,1-TCA in other bottles were similar, with lag times being the only difference (Figure S6). Moreover, in the bottles with ZVI added, 20-30% mole loss was observed during the first two weeks, presumably due to unidentified products (Figure S2) such as 2-butyne or cis-2-butene as previously reported (Fennelly and Roberts, 1998).

3.2 Evaluation of the Specificity of the Primer Sets *cfrA* and *dcrA* Targeting RDase Functional Genes

16S rRNA gene Amplicon sequencing was conducted to identify the principal dechlorinating organisms in our samples. *Dehalobacter* were most abundant (up to 64.7% of the community in groundwater microcosm samples). *Sulfurospirillum*, which might carry the gene encoding dehalogenase to degrade tetrachloroethene (Hug et al., 2013), were also present at relative high percentages in microcosms (up to 40%, Table S7), but showed no correlation to our 1,1,1-TCA or 1,1-DCA concentration data. The percentages of other known dechlorinators (Hug et al., 2013) were very low. *Dehalococcoides* was not detected, and *Desulfitobacterium* and *Dehalogenimonas* each comprised less than 0.3% (Table S7). Therefore, we focused on quantifying *Dehalobacter* and examining the dynamics of the *Dehalobacter* population by conducting qPCR with primers targeting two functional genes encoding two distinct RDases, i.e., the *cfrA* and *dcrA* genes (Tang and Edwards, 2013B).

In order to evaluate how well the chosen *cfrA* and *dcrA* primers (designed based on known sequences) matched to the unknown *Dhb* at the site, we amplified DNA extracted from microcosm bottle #9 on day 49 to obtain nearly complete (1231bp) RDase genes. Three clones were successfully sequenced and were aligned and compared to five previously described genes coding RDases that are involved in the dechlorination of 1,1,1-TCA or 1,1-DCA: CtrA, TmrA, ThmA, CfrA and DcrA (Tang and Edwards, 2013B; Ding et al., 2014; Wong et al., 2016; Zhao et al., 2017). As detailed in the Supporting Information (Figure S7 and Table S5), Clone 3 was most similar (>99.5%) to the *dcrA* gene, and Clones 1 and 2 were most similar to the *thmA* gene which coded an RDase with substrate specificity highly similar

to CfrA (i.e., able to target 1,1,1-TCA but not 1,1-DCA) (Tang and Edwards, 2013B; Zhao et al., 2017). The alignments of the two tested primer sets to these RDase genes and our clones are demonstrated in Figure S7 and summarized in Table 1. Based on the alignment to the primer sets, the reference genes and our clones fall into two clays. Clones 1 and 2 are more similar to *crtA*, *tmrA*, *cfrA* and *thmA* genes with fewer mismatches to the *cfrA* primer set. In contrast, Clone 3 is more alike to the *dcrA* gene and shows complete alignment to the *dcrA* primer set. For convenience, in this study we call the genes amplified with the *cfrA* primer the *cfrA*-like genes. Similarly, the ones amplified with the *dcrA* primer were referred to as the *dcrA*-like genes.

3.3 Gene Abundances during Dechlorination in Groundwater Microcosms

The abundance of *Dehalobacter* (*Dhb*) 16S rRNA genes, as well as the abundance of the *cfrA*- and *dcrA*-like genes, was tracked over time in microcosms (Figures 1B, 1C, S5B and S5C). The efficiency, detection limits and melt curves are presented in Supporting Information Table S6 and Figure S8. When the signal was close to or less than the detection limit (particularly true for the *dcrA*-like gene), the data are flagged with ‘*’. In these flagged samples, melt curves and agarose gel images confirmed that products were different than those from standards. When gene copy numbers were well above the detection limit, melt curves of samples and standards were the same (Figure S8).

The abundance of *Dhb* 16S rRNA genes, in particular the *cfrA*- and *dcrA*-like genes, tracked dechlorination activity in a highly consistent and specific manner. As shown in the qPCR results (Figures 1B, 1C, S5B and S5C), in all active bottles when 1,1,1-TCA was dechlorinated to 1,1-DCA, the copies of the *Dhb* 16S rRNA gene and the *cfrA*-like gene increased significantly. In bottles #7, #8 and #9 in the Active set without ZVI and in bottles #18 and #21 in the Active + ZVI set, as 1,1-DCA was dechlorinated to CA, the copies of the *dcrA*-like gene increased significantly. The increases in the abundance of the genes amplified with the *cfrA* and *dcrA* primers coincided with 1,1,1-TCA dechlorination and 1,1-DCA dechlorination, respectively, indicating that these genes belonged to different sub-populations of

Dehalobacter. In other words, the data suggest that two distinct populations were involved, one in each of the two dechlorination steps, rather than one population carrying out both steps.

For further examination, the proportion of *Dehalobacter* carrying the *cfrA*-like genes and the proportion of *Dehalobacter* carrying the *dcrA*-like genes ($\%_{cfrA-Dhb}$ and $\%_{dcrA-Dhb}$) were estimated. According to the closed genomes of *Dehalobacter* sp. Strain DCA (NC_018866.1) and *Dehalobacter* sp. Strain CF (NC_018867.1), *Dehalobacter* usually contain three copies of the 16S rRNA genes in their genome, and only one copy of the *cfrA* or *dcrA* gene in each strain (Tang and Edwards, 2013B). Therefore, we included a factor of three when we calculated $\%_{cfrA-Dhb}$ and $\%_{dcrA-Dhb}$. As shown in Figure 2, the sum of these two percentages in the samples mostly fell between 75% and 150%, which is reasonable considering the errors of qPCR and that not all *Dehalobacter* contain three copies of the 16S rRNA gene (e.g., five copies in *Dehalobacter restrictus* strain PER-K23, NZ-CP007033 (Kruse et al., 2013)). When comparing these percentages to the degradation profiles, $\%_{cfrA-Dhb}$ increased when 1,1,1-TCA was dechlorinated to 1,1-DCA, and then declined while the $\%_{dcrA-Dhb}$ went up during the reduction of 1,1-DCA to CA. Given the observed trends of $\%_{cfrA-Dhb}$ and $\%_{dcrA-Dhb}$, we propose that there are two different groups of *Dehalobacter* originating from the site groundwater with one responsible for converting 1,1,1-TCA to 1,1-DCA and the other for converting 1,1-DCA to CA. The coexistence of two highly similar but distinct *Dehalobacter* strains in the dechlorination of 1,1,1-TCA has only been reported in the study conducted by Tang *et al.* (Tang et al., 2012; Tang and Edwards, 2013B; Tang et al., 2016), where the authors speculated that the switch might be an artifact of evolution during long term laboratory enrichment in the presence of ample electron acceptor and donor. This study has demonstrated that this kind of selection can also take place *in situ* at a contaminated field site, and also explains why the observed dechlorination proceeds in a highly sequential manner where the 1,1,1-TCA is first converted to 1,1-DCA before further dechlorination is observed. The second step requires growth of the second strain which lags behind and presumably competes with the first strain for available donor.

3.4 Biomass Yields Inferred from Microcosm Data

We estimated the cell (biomass) yield as the ratio between the change in the copies of the *Dhb* 16S rRNA gene and the moles of 1,1-DCA or CA produced, assuming that most of the bioreduction of 1,1,1-TCA and 1,1-DCA after the lag time was carried out by *Dehalobacter*. Since one chloride (Cl) is released from each reductive dechlorination step, the data are presented in terms of change in *Dhb* 16S rRNA gene copy number / μmol of Cl evolved (Supporting Information Table S8). Between days 0 and 49 when cells mainly grew on reduction of 1,1,1-TCA to 1,1-DCA, the cell yields ranged between 5×10^6 and 1×10^8 copies of *Dhb* 16S rRNA gene / μmol Cl evolved. Beyond day 49 when the conversion from 1,1-DCA to CA was the dominant reaction, the cell yields were between 2×10^7 and 9×10^7 copies of *Dhb* 16S rRNA gene / μmol Cl evolved. For the pure culture of *Dehalobacter* sp. UNSWDHB which was unable to effectively use 1,1,1-TCA and 1,1-DCA in respiration, the cell yields were estimated to be 1.1×10^7 and 2.1×10^7 copies of *Dhb* 16S rRNA gene / μmol Cl evolved respectively (Wong et al., 2016). When using acetate as substrates to convert 1,1,1-TCA to CA via 1,1-DCA, the overall cell yield of *Dehalobacter* sp TCA1 was estimated to be 2.4×10^8 copies of *Dhb* 16S rRNA gene / μmol Cl evolved (Sun et al., 2002). The enrichment culture ACT3 derived from a 1,1,1-TCA contaminated site and fed with methanol, ethanol, acetate and lactate had cell yields of 9.4×10^8 and 2.4×10^9 copies of *Dhb* 16S rRNA gene / μmol Cl evolved in 1,1,1-TCA and 1,1-DCA reduction correspondingly (Grostern and Edwards, 2006B). The yields in our microcosm study were at the low end as compared to the literature values, which might be explained by a few factors in the experimental setup. The studies with high cell yields used pure *Dehalobacter* or enrichment cultures in medium supplemented with vitamins or other nutrients, while we used groundwater as the inoculum in our microcosms. The electron donors (i.e., the leftover guar gum and degradation products in the groundwater) could result in lower yield, and no additional nutrients (e.g., vitamins) were added to the microcosms. Considering the impact of ZVI, when 1,1,1-TCA was being dechlorinated to 1,1-DCA in the Active + ZVI set (i.e., by day 49), the cell yields seemed to be slightly lower than those in the Active set without ZVI. Considering that some 1,1-DCA accounted for in the

calculation was produced from abiotic dechlorination of 1,1,1-TCA in the Active + ZVI set, we concluded that the actual cell yields in the active bottles with and without ZVI addition were not substantially different, suggesting that ZVI did not affect the growth of *Dehalobacter*.

3.5 Analysis of Dechlorination Time Profiles and Genes in Field Samples

After evaluating the *dcrA*- and *cfrA*-like genes in groundwater microcosm samples, we examined their presence in field samples to confirm their utility in monitoring remediation on site. Three wells were sampled extensively during this study, SW6, SW5 and MW202, all located in the plume area with high concentrations of 1,1,1-TCA (i.e., 60-160 mg 1,1,1-TCA/L groundwater) prior to guar gum-ZVI injection. Because of the extent and variety of treatments applied to the site, which included soil excavation, iron emplacement in trenches, injection and groundwater pumping, it was generally difficult to interpret the site concentration data. SW6 was the only well with a clear sign of transformation with a reasonable mass balance, so we decided to first focus on the results from this well. Data from other monitoring wells (i.e., SW5 and MW202) will also be presented to illustrate the importance of biomarkers in site assessment.

Regarding monitoring well SW6, the decline of 1,1,1-TCA coincided with the generation of 1,1-DCA after the injection of the guar gum-ZVI mixture (Figure 3A). The concentration of *Dhb* 16S rRNA and *cfrA*-like genes increased when 1,1,1-TCA was reduced to 1,1-DCA (Figure 3B), while the concentration of *dcrA*-like genes increased with a slight decline in the *cfrA*-like genes as 1,1-DCA was gradually converted to CA after day 202. These degradation profiles and correlations between the biomarker genes and the chlorinated ethane concentrations paralleled the observations in microcosm bottles in the Active + ZVI set (i.e., #18 and 21) where 1,1,1-TCA was transformed to 1,1-DCA and further to CA by active organisms at the presence of additional ZVI. Assuming a similar cell yield in SW6 to that in microcosms (i.e., bottles #7, 8 and 12 between days 0 and 49), approximately 30% and 100% of the 1,1,1-TCA removed between days 69 and 97 and days 97 and 133 was attributed to biodegradation by *Dehalobacter*

respectively (Table S9.1). This indicates that biotic dechlorination was playing an important role in removing 1,1,1-TCA in SW6.

In SW5 (Figures 3C and 3D) and MW202 (Figure S9), concentration data were difficult to interpret because the disappearance of 1,1,1-TCA did not coincide with the appearance of 1,1-DCA. However, as demonstrated for SW5 as an example (in Figure 3D), the concentration of the *Dhb* 16S rRNA genes and the *cfrA-like* genes increased by more than two orders of magnitude between days 40 and 97, indicating that biological dechlorination was occurring. Similar to the estimation done for SW6 above, based on the average cell yield in microcosms and the qPCR results of SW5, biological reductive dechlorination accounted for roughly 60% of 1,1,1-TCA removal between days 40 and 97 in SW5 (Table S9.2). Similarly in MW202, dechlorination by *Dehalobacter* was estimated to contribute to about 10% of the 1,1,1-TCA removed between days 40 and 97 (Table S9.3). Therefore, despite of the noisy concentration data, the qPCR results provided evidence that a significant amount of biotransformation was in fact occurring in SW5 and MW202 during the periods monitored.

3.6 Impact of Guar Gum and ZVI on Dechlorination of 1,1,1-TCA

ZVI was injected as a guar gum-based blend, creating a mobile mixture of coarse ZVI and guar gum that could also serve as carbon source. The addition of ZVI has been shown to enhance the biodegradation of various chlorinated compounds such as carbon tetrachloride, chloroform, 1,1,1-TCA and 1,2-dichloroethane (Zemb et al., 2010; Zhou et al., 2014; Lee et al., 2015). Three major mechanisms were proposed to explain the enhancement: ZVI provided an environment with lower redox potential and extra surface area for microbial colonization; the cathodic hydrogen released from the reaction of iron and water supported the growth of organisms involved in dechlorination; and ZVI served as a direct electron donor in the transformation of chlorinated compounds. In contrast to the studies just mentioned, Xiu et al. (2010) found that the addition of nano-scale ZVI (nZVI) to a *Dehalococcoides*-containing methanogenic consortium initially inhibited the reduction of trichloroethene, likely because the

attachment of nZVI to the cell surface led to disruption of bacterial membrane function. It is likely that the influence of ZVI on microbial activity and biodegradation of chlorinated compounds depends on the microbial community, the specific chlorinated compounds and the type and amount of injected ZVI.

Our microcosms included two active sets, one with ZVI addition and the other without, along with the qPCR data of site samples, allowing us to investigate the impact of the addition of ZVI on biotic 1,1,1-TCA dechlorination. The degradation profiles of the Sterile + ZVI set and the Active + ZVI set indicated that the impact of ZVI was noticeable only in the first few days after addition (Figure S6). The addition of ZVI decreased the lag time before onset of 1,1,1-TCA biodegradation. Ethene, ethane and unidentified compounds were produced within the first two weeks after ZVI addition, which ultimately resulted in lower concentrations of 1,1-DCA or CA. The addition of ZVI did not seem to positively or negatively affect *Dehalobacter* biomass growth or yield in our study. Based on the findings from our groundwater microcosms, as well as the degradation time profile and qPCR results from site samples, we proposed that the addition of ZVI mainly facilitated abiotic dechlorination at the site within the first few weeks of ZVI injection before significant growth of *Dehalobacter* occurred. This was also clear from amplicon sequencing data, where *Dehalobacter* made up a relative large fraction of the population at the site (i.e., up to ~8%). Since the 1,1,1-TCA concentration in SW5 and SW6 increased above 1.2 mM after ZVI-injection (possibly due to preferential flow caused by hydraulic fracturing), abiotic transformation of 1,1,1-TCA to 1,1-DCA might help reduce the possible negative impact of high concentrations of contaminants on microbial communities. However, the organisms might benefit more from the addition of guar gum than from the injected ZVI. Dechlorination of 1,1,1-TCA without addition of extra electron donors occurred in the groundwater microcosms. MW202 samples had both the lowest population of *Dehalobacter* and the lowest COD concentration (Figure S10). These two observations suggest that guar gum was the food for fermenting bacteria that produced the hydrogen that served as electron donor for reductive dechlorination of 1,1,1-TCA by *Dehalobacter*. Guar gum could also serve as the carbon source for these organisms.

3.7 Implications for Bioremediation

We propose that DNA-based biomarkers analyzed from groundwater samples are more stable than contaminant concentrations because microbes grow and repopulate as resident biofilm that is not easily washed out with groundwater. Yields determined from groundwater microcosm data (i.e., *in situ* conditions) were very useful for estimating the minimum amount of contaminant transformation that could be attributed to biodegradation.

Regarding the effectiveness of ZVI in remediation in this project, ZVI showed some activity at early time (initial abiotic reduction), but the greater and more persistent impact was through donor addition. While 1,1-DCA was the major product from abiotic degradation of 1,1,1-TCA, bioremediation further transformed 1,1-DCA to the less toxic CA. Based on the qPCR results, we can also propose a threshold concentration for activity. Our data suggest that active dechlorination might not proceed until each litre of groundwater contains at least 1×10^7 copy of functional genes such as *cfrA* or $\sim 3 \times 10^6$ copies of *Dehalobacter* 16S rRNA genes. In order to achieve such thresholds, electron donors, and in some cases bioaugmented culture (e.g., ACT3), should be supplied to sites for more complete and sustained bioremediation of 1,1,1-TCA.

Associated Content

Supporting Information

Figures S1-S10, Tables S1-S9 and Supporting Texts S1-S3 include site information (historical data, map, stratigraphy conditions, and injection information), qPCR-related information (recipe, running program, melt curves and efficiency), details in Illumina sequencing (primers, analysis method and OTU percentages), microcosm setup, chlorinated ethane concentrations and qPCR data for MW202 and microcosms samples that were not presented in the main text figures (Sterile, Sterile + ZVI, Active + ZVI and ACT3 + ZVI), methane production and mole balance of C2 compound in microcosms, initial 1,1,1-

420 TCA reduction to 1,1-DCA in microcosms, alignment of clone sequences to reference genes, COD
421 concentrations in field samples, yield calculation, and biotic dechlorination estimation for site samples.

422 **Notes**

423 The authors declare no competing financial interest.

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433

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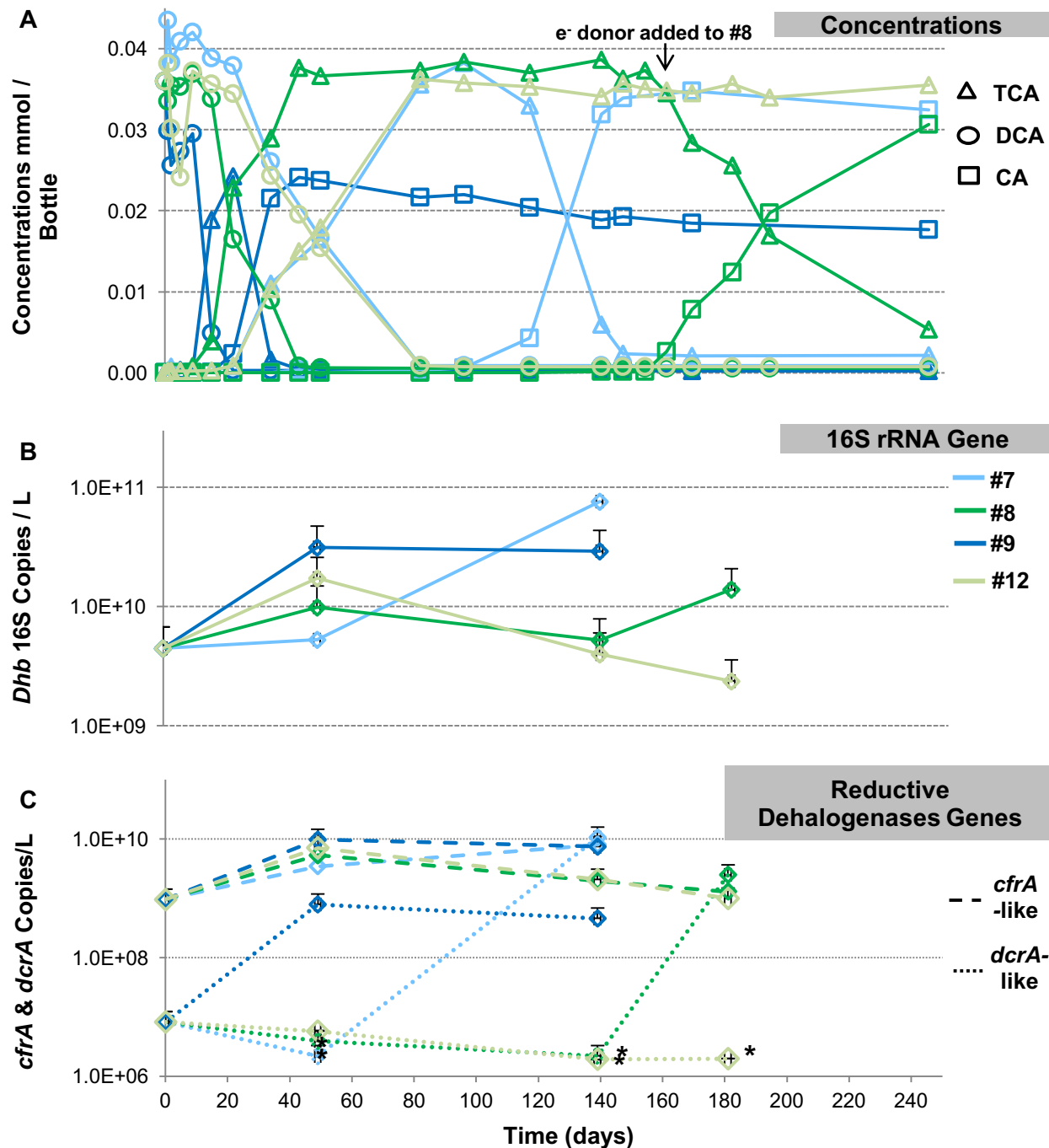


Figure 1. Trends in Chlorinated ethane concentrations and gene copies in groundwater microcosms without ZVI over 250 days. Although replicate microcosms were prepared, each bottle behaved a little differently and thus they are plotted separately. Panel A: Complete reduction of 1,1,1-TCA (circles) to 1,1-DCA (triangles) to CA (squares) was observed in bottle #8 (dark green) with electron donors added on day 140, and in bottles #7 (light blue) and #9 (dark green), while degradation stopped at 1,1-DCA in

bottle #12 (light green). Panel B: *Dhb* 16S gene copies first increased in bottle #9, then in #7, and later in #8 after adding electron donors. Panel C: early increases in the copy number of the *cfrA*-like genes in all bottles and increases in the copy number of the *dcrA*-like genes in bottles #9, #7 and #8 at various time points were observed. Data points marked with ‘*’ were below the lowest detection limit or showed nonspecific amplification. Error bars on qPCR data reflect typical error of about a half an order of magnitude from DNA extraction and qPCR.

Bottle	Process		
#7	TCA→DCA		DCA→CA
#8	TCA→DCA		* DCA→CA
#12	TCA→DCA		
#18	TCA→DCA	DCA→CA	
#21	TCA→DCA		* DCA→CA
#19	TCA→DCA		

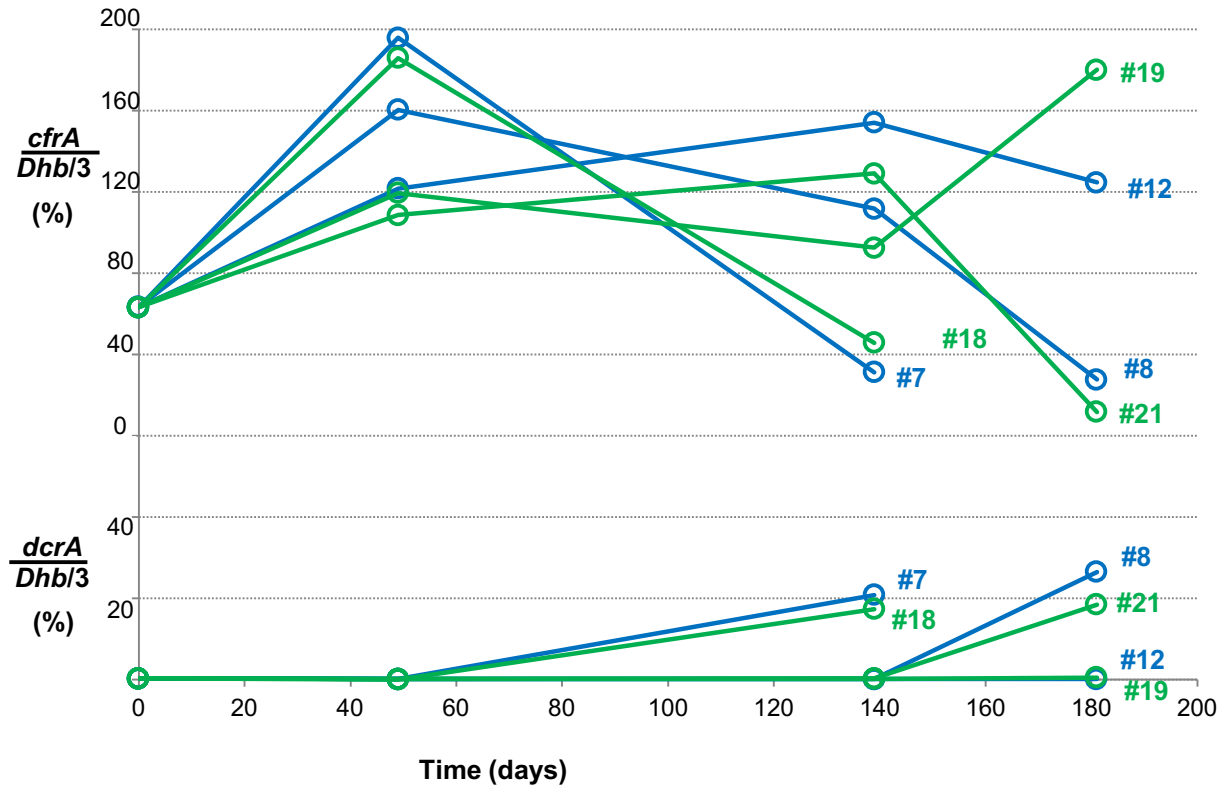


Figure 2. Proportions of *Dehalobacter* carrying the *cfrA*-like gene (top) and the *dcrA*-like gene (bottom) at different stages of 1,1,1-TCA dechlorination in microcosms without ZVI (blue) and in microcosms with ZVI (green). As 1,1,1-TCA was dechlorinated to 1,1-DCA, the percentage of *Dehalobacter* targeted by the *cfrA* primer pair increased. As 1,1-DCA was dechlorinated to CA, the percentage of *Dehalobacter* targeted by the *dcrA* primer pair increased while the percentage of

560 *Dehalobacter* targeted by the *cfrA* primer decreased. Electron donors were added to bottles #8 and #21 at
561 the time indicated by *.

562

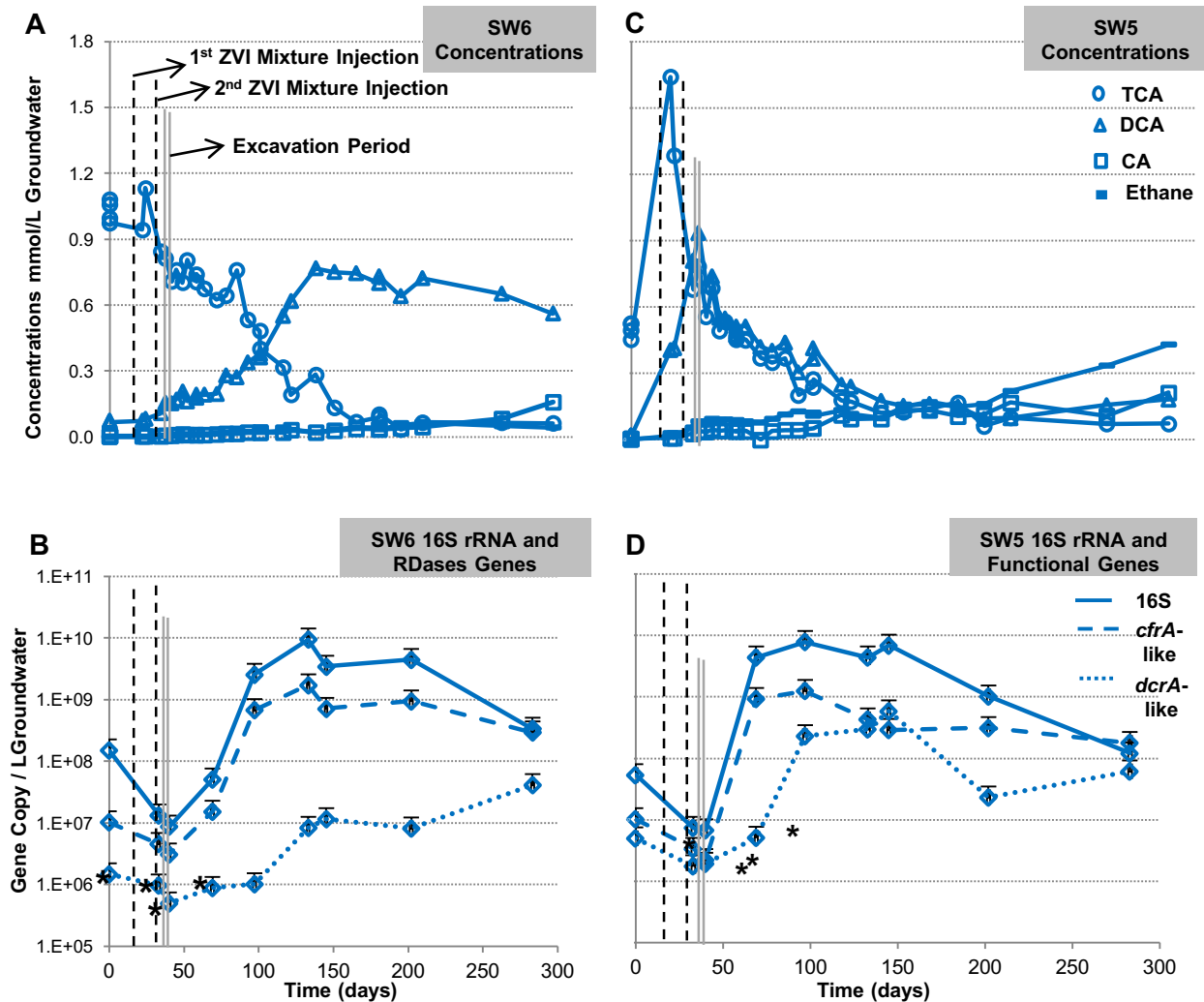


Figure 3. Chlorinated ethane concentrations and qPCR results in samples from wells SW6 and SW5. Panel A: 1,1,1-TCA concentration (circles) in well SW6 decreased while 1,1-DCA concentration (triangles) increased after excavation; a slight increase in CA (squares) appeared along with a minor decrease in 1,1-DCA beyond day 202. Panel B: qPCR results from well SW6 showed increases in the copy numbers of the *Dehalobacter* 16S rRNA gene (solid line) and the *crfA*-like (dashed line) and *dcrA*-like genes (dotted line). Panel C: in well SW5, 1,1,1-TCA (circles) and 1,1-DCA (triangles) spiked after ZVI injection, then both decreased. Panel D: qPCR result from well SW5 showed that *Dehalobacter* 16S rRNA gene copies (solid line) increased initially, along with *crfA*-like gene copies (dashed line), and *dcrA*-like gene copies also increased (dotted line) between days 40 and 97. Data points marked with ‘*’

574 were below the lowest detection limit or showed nonspecific amplification. Error bars on qPCR data
575 reflect typical error of about a half an order of magnitude from DNA extraction and qPCR.
576

577

Table 1. Summary of Number of Mismatches between Genes and Primers

Target	<i>cfrA</i> Forward	<i>cfrA</i> Reverse	<i>dcrA</i> Forward	<i>dcrA</i> Reverse
<i>Dhb</i> sp CF <i>cfrA</i> Gene (JX282329.1)	0	0	4	3
<i>Dsf</i> sp. PR <i>ctrA</i> Gene (KF155162.1)	2	0	4	3
<i>Dhb</i> sp UNSWDHB <i>tmrA</i> Gene (NZ_AUUR01000010 REGION: 19936..21303)	0	0	4	3
<i>Dhb</i> sp. SN <i>thmA</i> Gene (KX344907.1)	1	0	4	3
Microcosm Clone 1	1	0	4	4
Microcosm Clone 2	2	0	5	3
<i>Dhb</i> sp DCA <i>dcrA</i> Gene (JX282330.1)	3	3	0	0
Microcosm Clone 3	3	3	0	0

578

Note: all gene and clone sequences were trimmed down to 1236 nucleotides after sequence alignment for

579

comparison and tree construction. Detailed alignment and tree can be found in Figure 7S. The numbers of

580

mismatches among the reference genes and clone sequences are summarized in Table 6S.