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) (Boublík et al., 1984), poorly soluble in water (1500 mg/L at 25°C) (Horvath, 1982) and sorbs to organic matter (log Koc=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),

61 *Dehalobacter* sp. strain THM1 (Zhao et al., 2017), and *Desulfitobacterium* strain PR (Ding et al., 2014),
62 have since been described or isolated with the ability to partially or completely dechlorinate 1,1,1-TCA
63 and/or 1,1-DCA. All of these strains use either hydrogen or formate as electron donor. Six reductive
64 dehalogenases (RDases) that catalyze these specific dechlorination reactions in these bacteria have also
65 been identified, and are referred to as CtrA, TmrA, DcrA, CfrA and ThmA. These proteins and their
66 corresponding genes form a clade of highly similar sequences belonging to Ortholog Group 46 (Hug et
67 al., 2013). Interestingly, while the proteins are very similar (>95% amino acid identity), they do have
68 distinct substrate preferences. For example, DcrA from *Dhb* strain DCA catalyzes dechlorination of 1,1-
69 DCA to CA, but is not active on 1,1,1-TCA or chloroform (CF), while CfrA from *Dhb* strain CF and
70 ThmA from *Dhb* strain THM1 are active on CF and 1,1,1-TCA but not on 1,1-DCA (Tang and Edwards,
71 2013; Tang and Edwards, 2013B; Zhao et al., 2017). In contrast, CtrA from *Desulfitobacterium* strain PR
72 and TmrA from *Dhb* UNSWDHB are reported to be active on all three substrates to varying degrees
73 (Ding et al., 2014; Wong et al., 2016). Primers to amplify the genes encoding these RDases have been
74 incorporated into quantitative polymerase chain reaction (qPCR) approaches to measure the abundance of
75 these organisms or RDase genes in the studies mentioned above.

76 Chlorinated ethane concentrations are frequently monitored to evaluate 1,1,1-TCA treatment efficiency at
77 remediation sites, but concentration data are easily affected by site operations, making interpretation of
78 data difficult, particularly in trying to distinguish between biotransformation and abiotic removal. Gene-
79 based biomarkers (i.e., qPCR-based approaches) applied to field samples have the potential to monitor
80 biological 1,1,1-TCA removal (Scheutz et al., 2011). To date, functional gene-based primers developed
81 for organisms dechlorinating 1,1,1-TCA have been applied to microcosm and culture samples only, and
82 have not been evaluated with actual field site samples in published work. Moreover, only a limited
83 number of field studies reported using qPCR with primers to measure the 16S rRNA gene of
84 *Dehalobacter* dechlorinating 1,1,1-TCA at remediation sites (Postiglione et al., 2006; Duchesneau et al,
85 2007; Damgaard et al., 2013).

86 In this study, we worked in collaboration with consultants from Pinchin Ltd. on a site contaminated with
87 1,1,1-TCA. The remedy selected for the site was injection of granular ZVI that was mixed with guar gum
88 to stabilize the iron. Site samples were analyzed for chlorinated ethane concentrations and gene
89 abundances using qPCR during the on-going remediation. In parallel, we also prepared microcosms with
90 site groundwater to more definitively track both abiotic and biotic processes under defined conditions.
91 The objectives of this research were to evaluate the use of the *Dhb* 16S rRNA gene and the *cfrA*- and
92 *dcrA*-like dehalogenase genes as biomarkers for 1,1,1-TCA dechlorination and to investigate the impact
93 of guar gum stabilized ZVI addition on the biodegradation of 1,1,1-TCA.

94 ## 2. Materials and Methods

95 ### 2.1 Site Description

96 The Site was a former adhesive manufacturing plant located in Southern Ontario and operated between
97 1950 and 1991. A spill of approximately five hundred litres of 1,1,1-TCA in the late 1980s was the only
98 recorded release at the site. According to previous investigations, 1,1-DCA, 1,1-DCE, 1,1,1-TCA and
99 dichloromethane (DCM) were the major contaminants detected between 1998 and 2014, with 1,1,1-TCA
100 being at highest concentration (Table S1). A pump and treat system was operated between 1995 and
101 2015, and 4800 tonnes of impacted soil were excavated around year 2000. The statigraphy information of
102 the site is explained in Supporting Text S1 in the Supporting Information.

103 ### 2.2 Granular ZVI Particles, Guar Gum, and On-Site ZVI Injection

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

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

113 ## 2.3 Groundwater Monitoring and Sample Collection

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

122 ## 2.4 Groundwater Microcosm Setup and Sampling

123 To evaluate *in situ* microbial activity with and without added ZVI, groundwater collected on day 253
124 from SW6 was used to set up microcosms. The groundwater (2 × 2 L in medium bottles) was purged
125 with 80%/20% N_2-CO_2 gas mix for 1 hour to remove oxygen and residual volatile compounds. In a
126 disposable glovebag (Atmosbag, Aldrich) filled with 80%/20% N_2-CO_2 gas mix, 150 mL of purged
127 groundwater was dispensed into each sterile 250 mL glass bottle, and ZVI (8/50 Grade from Peerless
128 Metal Powders & Abrasive) was added to a subset of the bottles (1 g per bottle). All bottles were then
129 capped with Mininert septa screw caps (sterilized by wiping with ethanol) before the bottles were taken
130 out of the glovebag. As detailed in Table S3, six treatments were established that included: Sterile (i.e.,
131 negative control by autoclaving); Sterile + ZVI; Active without ZVI; Active + ZVI; Active without TCA
132 and ZVI; and bioaugmented with ACT3 + ZVI where ACT3 refers to a previously described enrichment

133 culture that dechlorinates 1,1,1-TCA via 1,1-DCA to CA (Tang and Edwards, 2013B). Neat 1,1,1-TCA
134 (~4.25 µL per bottle) was added for an initial liquid concentration of approximately 26 mg/L. ACT3
135 culture (0.1 mL) was anaerobically added to the ACT3 + ZVI set on Day 2 inside a lab glovebox. In most
136 of the bottles, we did not add any additional electron donor, as the purged groundwater contained
137 approximately 800 mg COD/L from residual guar gum and its degradation products. The only exceptions
138 were bottle #8 in the Active set without ZVI and bottle #21 in the Active + ZVI set to which ethanol and
139 lactate were added on day 149 at five times electron equivalent excess to the remaining 1,1-DCA (see
140 Table S3). An extra serum bottle (capped with rubber stopper and crimped) containing the same amount
141 of groundwater without any 1,1,1-TCA or ZVI was also included in the assay to evaluate methane
142 production from the groundwater COD. All bottles were incubated statically, upside-down, in an
143 anaerobic glovebox (Coy) filled with 5%-95% H_2-N_2 gas mix at room temperature. Liquid samples were
144 withdrawn from bottles (after shaking to mix) using gas-tight syringes and sterile needles for chemical
145 and microbial analysis.

146 ## 2.5 DNA Extraction and Microbial Analysis

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

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

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

160 Quantitative PCR (qPCR) was used to enumerate specific phylogenetic and functional genes in the DNA
161 samples. Primers *Dhb*477f/*Dhb*647r were used to quantify the abundance of *Dehalobacter* 16S rRNA
162 gene copies (Grostern and Edwards, 2006). Primers *cfrA*413f/*cfrA*531r and *dcrA*424f /*dcrA*533r were
163 adapted to track two reductive dehalogenase genes *cfrA* and *dcrA* encoding the proteins catalyzing
164 dechlorination of 1,1,1-TCA and 1,1-DCA respectively (Tang and Edwards, 2013B). Detailed recipe of
165 the qPCR reactions and programs are explained in Text S2 in the Supporting Information.

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

171 To obtain the complete sequences of reductive dehalogenase genes from our samples, the DNA extracted
172 from the bottle with the fastest degradation of 1,1,1-TCA in the Active set without ZVI (i.e., bottle #9 on
173 day 49) was amplified with primers *rdhA*23f/*rdhA*1383r targeting the reductive dehalogenase. The PCR
174 program, the primer sequences, the cloning procedures, the culturing of the *E. coli* cells and the
175 procedures to extract plasmid DNA are described in the work conducted by Tang and Edwards (2013B).
176 Five clone samples were submitted to the Centre for Applied Genomics (Toronto), and each was
177 sequenced four times in both the forward and the reverse directions with the M13f and M13r primers.
178 Using the Geneious program, the forward and reverse reads were merged to recreate the amplicons, and
179 aligned with five gene sequences encoding known 1,1,1-TCA/1,1-DCA dehalogenases:

180 *dcrA*(JX282330.1), *cfrA*(JX282329.1), *ctrA*(KF155162.1), *tmr*A (NZ_AUUR01000010 REGION:
181 19936..21303) and *thmA* (KX344907.1).

182 2.6 Chemical Analysis of Groundwater and Microcosm Samples

183 Chlorinated ethane concentrations in field samples were measured within 48 hours after the samples
184 arrived at the University. Groundwater microcosm samples were tested immediately after sample
185 collection. Aliquots of 0.5-1 mL of groundwater or microcosm samples were added to acidified MilliQ
186 water to a total liquid volume of 6 mL in 11 mL crimped-cap headspace autosampler vials and placed in
187 the Agilent G1888 Headspace Sampler. Headspace in the crimped vials was analyzed using the Agilent
188 7890 gas chromatograph (GC) equipped with the GS-Q column and detected using a flame ionization
189 detector (FID). External standards, purchased from Sigma, were first diluted with methanol, and then in
190 acidified MilliQ water for calibration. A modified EPA 8021 method was used as per Kocur et al. (2015).
191 Several groundwater samples were also analyzed for total COD according to the EPA Method 410.4
192 potassium dichromate/mercuric sulfate colorimetric method (600 nm) (USEPA, 1993).

193 3. Results and Discussion

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

197 3.1 Dechlorination of 1,1,1-TCA in Groundwater Microcosms

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

202 DCA, 1,1-DCE, cis-DCE, trans-DCE, 1,2-DCA, ethane and ethene. This mole balance revealed that total
203 moles of C2-compounds were conserved in the Sterile Control microcosms without ZVI, in the Active set
204 without ZVI, and in the set with ACT3 culture and ZVI (ACT3 + ZVI) (Figure S2), implying that major
205 transformation products were accounted for. In the Active + ZVI and Sterile +ZVI sets, measured
206 degradation products did not completely account for substrate loss, likely due to products that were not
207 measured (see below). Methane production was estimated and compared to the original COD measured
208 in the groundwater. In the Active set without 1,1,1-TCA and ZVI, the methane produced accounted for
209 approximately 90% of the original COD present in the groundwater (Table S4), demonstrating that
210 although no extra electron donor was added to the microcosms, the residual guar-gum and its products
211 were sufficient to drive methanogenesis and dechlorination. The lower methane production in bottles fed
212 with 1,1,1-TCA (Table S4) reflects the well-documented inhibition of methanogenesis by compounds like
213 1,1,1-TCA (Grostern and Edwards, 2006).

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

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

231 ### 3.2 Evaluation of the Specificity of the Primer Sets *cfrA* and *dcrA* Targeting RDase Functional
232 Genes

233 16S rRNA gene Amplicon sequencing was conducted to identify the principal dechlorinating organisms
234 in our samples. *Dehalobacter* were most abundant (up to 64.7% of the community in groundwater
235 microcosm samples). *Sulfurospirillum*, which might carry the gene encoding dehalogenase to degrade
236 tetrachloroethene (Hug et al., 2013), were also present at relative high percentages in microcosms (up to
237 40%, Table S7), but showed no correlation to our 1,1,1-TCA or 1,1-DCA concentration data. The
238 percentages of other known dechlorinators (Hug et al., 2013) were very low. *Dehalococcoides* was not
239 detected, and *Desulfitobacterium* and *Dehalogenimonas* each comprised less than 0.3% (Table S7).
240 Therefore, we focused on quantifying *Dehalobacter* and examining the dynamics of the *Dehalobacter*
241 population by conducting qPCR with primers targeting two functional genes encoding two distinct
242 RDases, i.e., the *cfrA* and *dcrA* genes (Tang and Edwards, 2013B).

243 In order to evaluate how well the chosen *cfrA* and *dcrA* primers (designed based on known sequences)
244 matched to the unknown *Dhb* at the site, we amplified DNA extracted from microcosm bottle #9 on day
245 49 to obtain nearly complete (1231bp) RDase genes. Three clones were successfully sequenced and were
246 aligned and compared to five previously described genes coding RDases that are involved in the
247 dechlorination of 1,1,1-TCA or 1,1-DCA: CtrA, TmrA, ThmA, CfrA and DcrA (Tang and Edwards,
248 2013B; Ding et al., 2014; Wong et al., 2016; Zhao et al., 2017). As detailed in the Supporting
249 Information (Figure S7 and Table S5), Clone 3 was most similar (>99.5%) to the *dcrA* gene, and Clones 1
250 and 2 were most similar to the *thmA* gene which coded an RDase with substrate specificity highly similar

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

259 **3.3 Gene Abundances during Dechlorination in Groundwater Microcosms**

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

267 The abundance of *Dhb* 16S rRNA genes, in particular the *cfrA*- and *dcrA-like* genes, tracked
268 dechlorination activity in a highly consistent and specific manner. As shown in the qPCR results (Figures
269 1B, 1C, S5B and S5C), in all active bottles when 1,1,1-TCA was dechlorinated to 1,1-DCA, the copies of
270 the *Dhb* 16S rRNA gene and the *cfrA*-like gene increased significantly. In bottles #7, #8 and #9 in the
271 Active set without ZVI and in bottles #18 and #21 in the Active + ZVI set, as 1,1-DCA was dechlorinated
272 to CA, the copies of the *dcrA*-like gene increased significantly. The increases in the abundance of the
273 genes amplified with the *cfrA* and *dcrA* primers coincided with 1,1,1-TCA dechlorination and 1,1-DCA
274 dechlorination, respectively, indicating that these genes belonged to different sub-populations of

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

277 For further examination, the proportion of *Dehalobacter* carrying the *cfrA*-like genes and the proportion
278 of *Dehalobacter* carrying the *dcrA*-like genes ($\%_{cfrA\text{-}Dhb}$ and $\%_{dcrA\text{-}Dhb}$) were estimated. According to the
279 closed genomes of *Dehalobacter* sp. Strain DCA (NC_018866.1) and *Dehalobacter* sp. Strain CF
280 (NC_018867.1), *Dehalobacter* usually contain three copies of the 16S rRNA genes in their genome, and
281 only one copy of the *cfrA* or *dcrA* gene in each strain (Tang and Edwards, 2013B). Therefore, we
282 included a factor of three when we calculated $\%_{cfrA\text{-}Dhb}$ and $\%_{dcrA\text{-}Dhb}$. As shown in Figure 2, the sum of
283 these two percentages in the samples mostly fell between 75% and 150%, which is reasonable considering
284 the errors of qPCR and that not all *Dehalobacter* contain three copies of the 16S rRNA gene (e.g., five
285 copies in *Dehalobacter restrictus* strain PER-K23, NZ-CP007033 (Kruse et al., 2013)). When comparing
286 these percentages to the degradation profiles, $\%_{cfrA\text{-}Dhb}$ increased when 1,1,1-TCA was dechlorinated to
287 1,1-DCA, and then declined while the $\%_{dcrA\text{-}Dhb}$ went up during the reduction of 1,1-DCA to CA. Given
288 the observed trends of $\%_{cfrA\text{-}Dhb}$ and $\%_{dcrA\text{-}Dhb}$, we propose that there are two different groups of
289 *Dehalobacter* originating from the site groundwater with one responsible for converting 1,1,1-TCA to
290 1,1-DCA and the other for converting 1,1-DCA to CA. The coexistence of two highly similar but distinct
291 *Dehalobacter* strains in the dechlorination of 1,1,1-TCA has only been reported in the study conducted by
292 Tang *et al*. (Tang et al., 2012; Tang and Edwards, 2013B; Tang et al., 2016), where the authors speculated
293 that the switch might be an artifact of evolution during long term laboratory enrichment in the presence of
294 ample electron acceptor and donor. This study has demonstrated that this kind of selection can also take
295 place *in situ* at a contaminated field site, and also explains why the observed dechlorination proceeds in a
296 highly sequential manner where the 1,1,1-TCA is first converted to 1,1-DCA before further
297 dechlorination is observed. The second step requires growth of the second strain which lags behind and
298 presumably competes with the first strain for available donor.

299 ### 3.4 Biomass Yields Inferred from Microcosm Data

300 We estimated the cell (biomass) yield as the ratio between the change in the copies of the *Dhb* 16S rRNA
301 gene and the moles of 1,1-DCA or CA produced, assuming that most of the bioreduction of 1,1,1-TCA
302 and 1,1-DCA after the lag time was carried out by *Dehalobacter*. Since one chloride (Cl) is released from
303 each reductive dechlorination step, the data are presented in terms of change in *Dhb* 16S rRNA gene copy
304 number / µmol of Cl evolved (Supporting Information Table S8). Between days 0 and 49 when cells
305 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
306 copies of *Dhb* 16S rRNA gene / µmol Cl evolved. Beyond day 49 when the conversion from 1,1-DCA to
307 CA was the dominant reaction, the cell yields were between 2×10^7 and 9×10^7 copies of *Dhb* 16S rRNA
308 gene / µmol Cl evolved. For the pure culture of *Dehalobacter* sp. UNSW*DHB* which was unable to
309 effectively use 1,1,1-TCA and 1,1-DCA in respiration, the cell yields were estimated to be 1.1×10^7 and
310 2.1×10^7 copies of *Dhb* 16S rRNA gene / µmol Cl evolved respectively (Wong et al., 2016). When using
311 acetate as substrates to convert 1,1,1-TCA to CA via 1,1-DCA, the overall cell yield of *Dehalobacter* sp
312 TCA1 was estimated to be 2.4×10^8 copies of *Dhb* 16S rRNA gene / µmol Cl evolved (Sun et al., 2002).
313 The enrichment culture ACT3 derived from a 1,1,1-TCA contaminated site and fed with methanol,
314 ethanol, acetate and lactate had cell yields of 9.4×10^8 and 2.4×10^9 copies of *Dhb* 16S rRNA gene / µmol
315 Cl evolved in 1,1,1-TCA and 1,1-DCA reduction correspondingly (Grostern and Edwards, 2006B). The
316 yields in our microcosm study were at the low end as compared to the literature values, which might be
317 explained by a few factors in the experimental setup. The studies with high cell yields used pure
318 *Dehalobacter* or enrichment cultures in medium supplemented with vitamins or other nutrients, while we
319 used groundwater as the inoculum in our microcosms. The electron donors (i.e., the leftover guar gum
320 and degradation products in the groundwater) could result in lower yield, and no additional nutrients (e.g.,
321 vitamins) were added to the microcosms. Considering the impact of ZVI, when 1,1,1-TCA was being
322 dechlorinated to 1,1-DCA in the Active + ZVI set (i.e., by day 49), the cell yields seemed to be slightly
323 lower than those in the Active set without ZVI. Considering that some 1,1-DCA accounted for in the

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

327 ### 3.5 Analysis of Dechlorination Time Profiles and Genes in Field Samples

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

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

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

350 In SW5 (Figures 3C and 3D) and MW202 (Figure S9), concentration data were difficult to interpret
351 because the disappearance of 1,1,1-TCA did not coincide with the appearance of 1,1-DCA. However, as
352 demonstrated for SW5 as an example (in Figure 3D), the concentration of the *Dhb* 16S rRNA genes and
353 the *cfrA-like* genes increased by more than two orders of magnitude between days 40 and 97, indicating
354 that biological dechlorination was occurring. Similar to the estimation done for SW6 above, based on the
355 average cell yield in microcosms and the qPCR results of SW5, biological reductive dechlorination
356 accounted for roughly 60% of 1,1,1-TCA removal between days 40 and 97 in SW5 (Table S9.2).
357 Similarly in MW202, dechlorination by *Dehalobacter* was estimated to contribute to about 10% of the
358 1,1,1-TCA removed between days 40 and 97 (Table S9.3). Therefore, despite of the noisy concentration
359 data, the qPCR results provided evidence that a significant amount of biotransformation was in fact
360 occurring in SW5 and MW202 during the periods monitored.

361 **3.6 Impact of Guar Gum and ZVI on Dechlorination of 1,1,1-TCA**

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

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

375 Our microcosms included two active sets, one with ZVI addition and the other without, along with the
376 qPCR data of site samples, allowing us to investigate the impact of the addition of ZVI on biotic 1,1,1-
377 TCA dechlorination. The degradation profiles of the Sterile + ZVI set and the Active + ZVI set indicated
378 that the impact of ZVI was noticeable only in the first few days after addition (Figure S6). The addition
379 of ZVI decreased the lag time before onset of 1,1,1-TCA biodegradation. Ethene, ethane and unidentified
380 compounds were produced within the first two weeks after ZVI addition, which ultimately resulted in
381 lower concentrations of 1,1-DCA or CA. The addition of ZVI did not seem to positively or negatively
382 affect *Dehalobacter* biomass growth or yield in our study. Based on the findings from our groundwater
383 microcosms, as well as the degradation time profile and qPCR results from site samples, we proposed that
384 the addition of ZVI mainly facilitated abiotic dechlorination at the site within the first few weeks of ZVI
385 injection before significant growth of *Dehalobacter* occurred. This was also clear from amplicon
386 sequencing data, where *Dehalobacter* made up a relative large fraction of the population at the site (i.e.,
387 up to ~8%). Since the 1,1,1-TCA concentration in SW5 and SW6 increased above 1.2 mM after ZVI-
388 injection (possibly due to preferential flow caused by hydraulic fracturing), abiotic transformation of
389 1,1,1-TCA to 1,1-DCA might help reduce the possible negative impact of high concentrations of
390 contaminants on microbial communities. However, the organisms might benefit more from the addition
391 of guar gum than from the injected ZVI. Dechlorination of 1,1,1-TCA without addition of extra electron
392 donors occurred in the groundwater microcosms. MW202 samples had both the lowest population of
393 *Dehalobacter* and the lowest COD concentration (Figure S10). These two observations suggest that guar
394 gum was the food for fermenting bacteria that produced the hydrogen that served as electron donor for
395 reductive dechlorination of 1,1,1-TCA by *Dehalobacter*. Guar gum could also serve as the carbon source
396 for these organisms.

397 ### 3.7 Implications for Bioremediation

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

403 Regarding the effectiveness of ZVI in remediation in this project, ZVI showed some activity at early time
404 (initial abiotic reduction), but the greater and more persistent impact was through donor addition. While
405 1,1-DCA was the major product from abiotic degradation of 1,1,1-TCA, bioremediation further
406 transformed 1,1-DCA to the less toxic CA. Based on the qPCR results, we can also propose a threshold
407 concentration for activity. Our data suggest that active dechlorination might not proceed until each litre
408 of groundwater contains at least 1×10^7 copy of functional genes such as *cfrA* or $\sim3\times10^6$ copies of
409 *Dehalobacter* 16S rRNA genes. In order to achieve such thresholds, electron donors, and in some cases
410 bioaugmented culture (e.g., ACT3), should be supplied to sites for more complete and sustained
411 bioremediation of 1,1,1-TCA.

412 ## Associated Content

413 ### Supporting Information

414 Figures S1-S10, Tables S1-S9 and Supporting Texts S1-S3 include site information (historical data, map,
415 stratigraphy conditions, and injection information), qPCR-related information (recipe, running program,
416 melt curves and efficiency), details in Illumina sequencing (primers, analysis method and OTU
417 percentages), microcosm setup, chlorinated ethane concentrations and qPCR data for MW202 and
418 microcosms samples that were not presented in the main text figures (Sterile, Sterile + ZVI, Active + ZVI
419 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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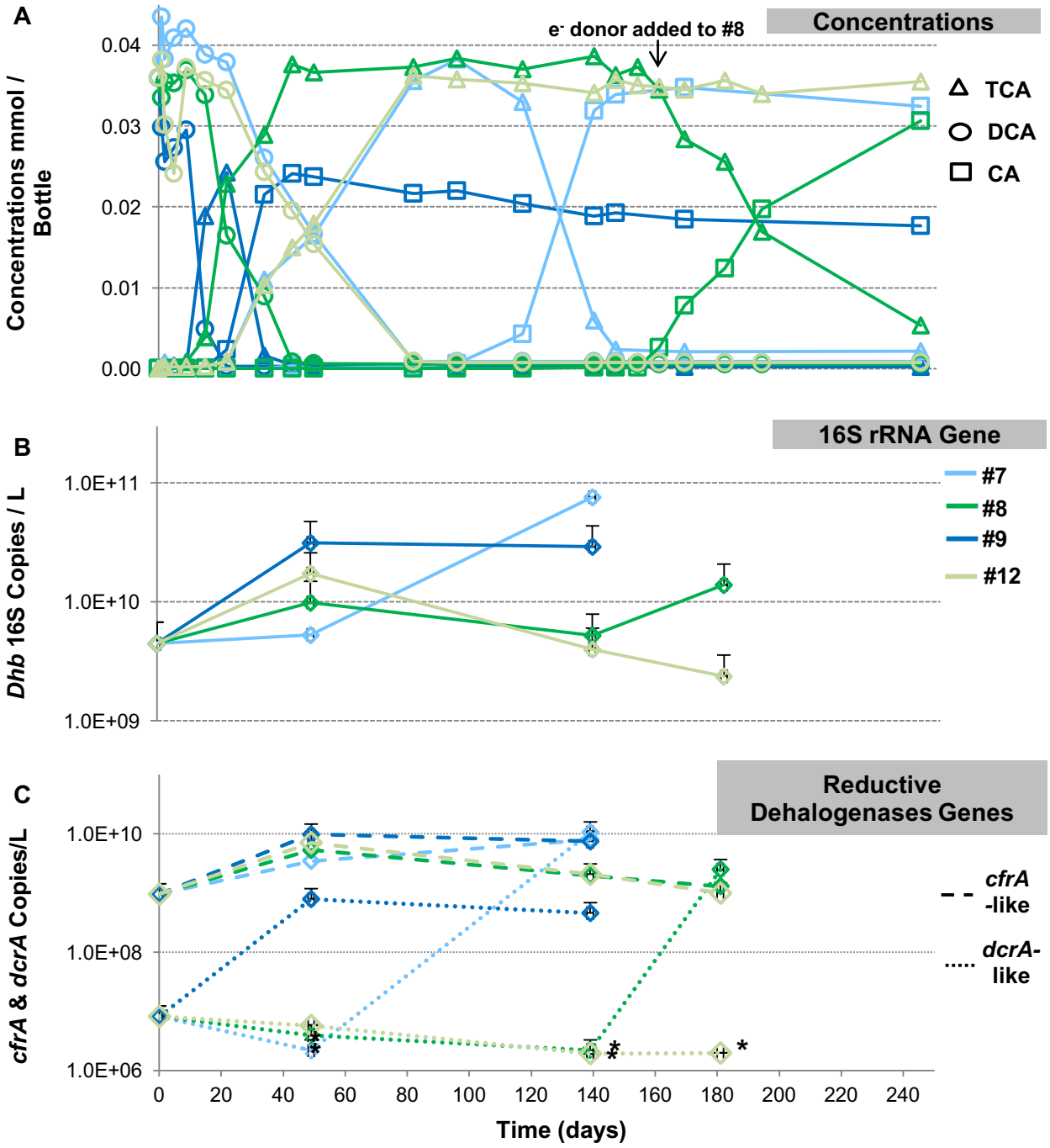
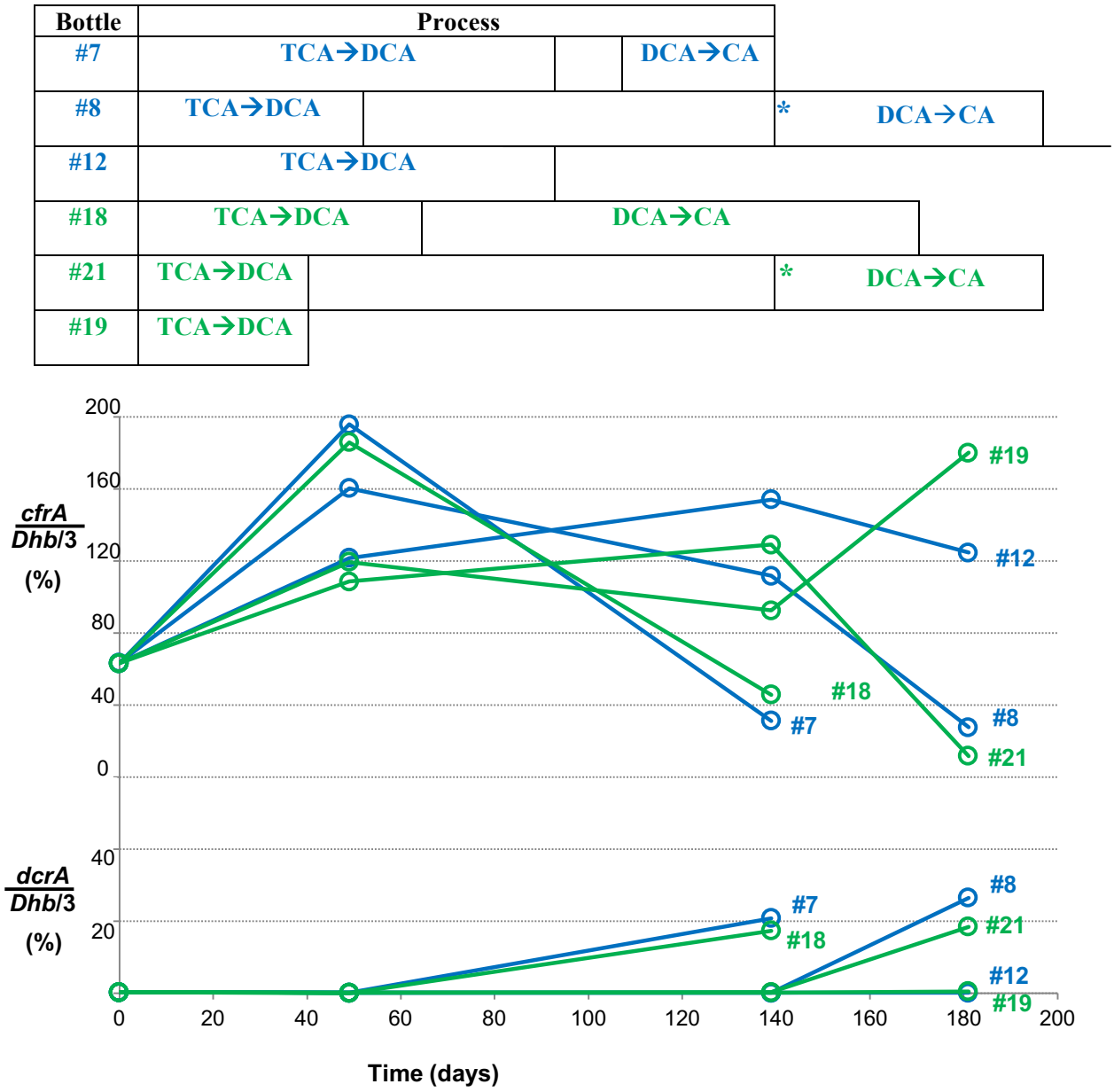


Figure 1. Trends in Chlorinated ethane concentrations and gene copies in groundwater microcosms without ZVI over 250 days. Although replicate microcosms were prepared, each bottle behaved a little differently and thus they are plotted separately. Panel A: Complete reduction of 1,1,1-TCA (circles) to 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

547 bottle #12 (light green). Panel B: *Dhb* 16S gene copies first increased in bottle #9, then in #7, and later in
548 #8 after adding electron donors. Panel C: early increases in the copy number of the *cfrA*–like genes in all
549 bottles and increases in the copy number of the *dcrA*-like genes in bottles #9, #7 and #8 at various time
550 points were observed. Data points marked with '*' were below the lowest detection limit or showed
551 nonspecific amplification. Error bars on qPCR data reflect typical error of about a half an order of
552 magnitude from DNA extraction and qPCR.
553



554

555 **Figure 2. Proportions of *Dehalobacter* carrying the *cfrA*-like gene (top) and the *dcrA*-like gene**

556 **(bottom) at different stages of 1,1,1-TCA dechlorination in microcosms without ZVI (blue) and in**

557 **microcosms with ZVI (green).** As 1,1,1-TCA was dechlorinated to 1,1-DCA, the percentage of

558 *Dehalobacter* targeted by the *cfrA* primer pair increased. As 1,1-DCA was dechlorinated to CA, the

559 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

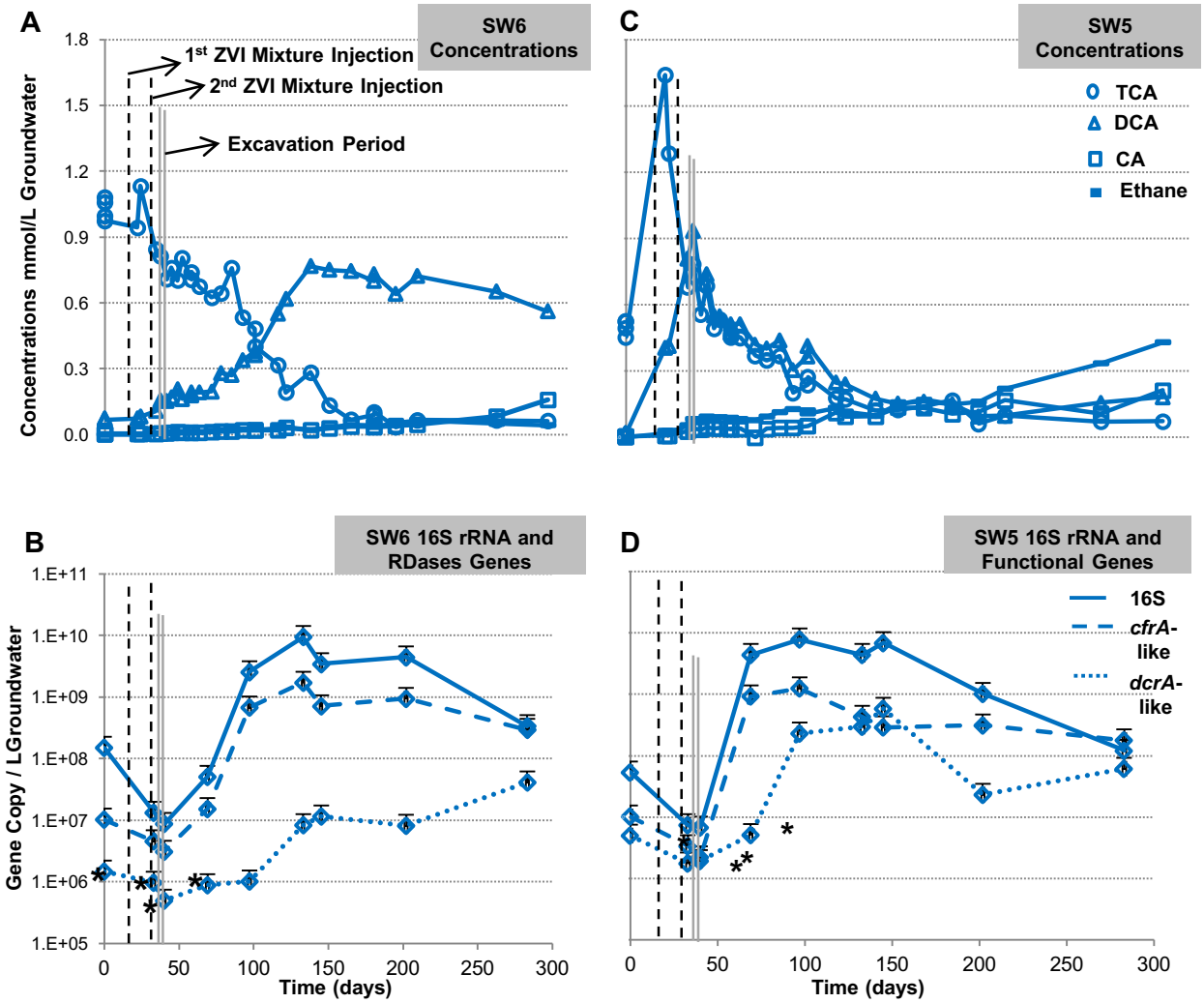


Figure 3. Chlorinated ethane concentrations and qPCR results in samples from wells SW6 and SW5. Panel A: 1,1,1-TCA concentration (circles) in well SW6 decreased while 1,1-DCA concentration (triangles) increased after excavation; a slight increase in CA (squares) appeared along with a minor decrease in 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	*cfrA* Forward	*cfrA* Reverse	*dcrA* Forward	*dcrA* Reverse
***Dhb* sp CF *cfrA* Gene (JX282329.1)**	0	0	4	3
***Dsf* sp. PR ctrA Gene (KF155162.1)**	2	0	4	3
***Dhb* sp UNSWDHB *tmrA* Gene (NZ_AUUR01000010 REGION: 19936..21303)**	0	0	4	3
***Dhb* sp. SN *thmA* Gene (KX344907.1)**	1	0	4	3
Microcosm Clone 1	1	0	4	4
Microcosm Clone 2	2	0	5	3
***Dhb* sp DCA *dcrA* 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.