Using cell yields and qPCR to estimate biotic contribution to 1,1,1-trichloroethane dechlorination at a field site treated with granular zero valent iron and guar gum

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Abstract

Chlorinated ethanes are environmental pollutants found frequently at many contaminated industrial sites. 1,1,1-Trichloroethane (1,1,1-TCA) can be dechlorinated and detoxified via abiotic transformation or biologically by the action of dechlorinating microorganisms such as *Dehalobacter* (*Dhb*). At a field site, it is challenging to distinguish abiotic vs biotic mechanisms as both processes share common transformation products. In this study, we evaluated using the *Dhb* 16S rRNA gene and specific reductive dehalogenase genes as biomarkers for 1,1,1-TCA and 1,1-dichloroethane (1,1-DCA) dechlorination. We analyzed samples from laboratory groundwater microcosms and from 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 the changes in dechlorinating organisms were tracked using quantitative PCR (qPCR) with primers targeting the *Dhb* 16S rRNA gene and two functional genes *cfrA* and *dcrA* encoding enzymes that dechlorinate 1,1,1-TCA to 1,1-DCA and 1,1-DCA to chloroethane (CA), respectively. The abundance of the *cfrA*- and *dcrA*-like genes confirmed that the two dechlorination steps were carried out by two distinct *Dhb* populations at the site. Using cell yields established in laboratory microcosms along with measured abundances of the *Dhb* 16S rRNA gene in site samples, biotic and abiotic transformation of 1,1,1-TCA at the site was estimated. The biomarkers used in this study proved useful for tracking biodechlorination of 1,1,1-TCA and 1,1-DCA where both abiotic (e.g. with ZVI) and biotic processes co-occur.

Keywords

Reductive Dehalogenases, *Dehalobacter*, Groundwater Remediation, Chlorinated Ethanes, Zero Valent Iron, 16S rRNA, Quantitative PCR

40 **Abbreviations**

41 1,1-DCA: 1,1-dichloroethane

42 1,1,1-TCA: 1,1,1-trichloroethane

43 CA: chloroethane

44 COD: chemical oxygen demand

45 *Dhb*: *Dehalobacter*

46 GC: gas chromatography

47 PCR: polymerase chain reaction

48 qPCR: quantitative polymerase chain reaction

49 RDases: reductive dehalogenases

50 ZVI: zero valent iron

51

52 ## 1. Introduction

53 Chlorinated aliphatic hydrocarbons, such as chlorinated ethanes and ethenes, have been widely used as
54 metal degreasers and cleaning agents, solvents and adhesives, and reagents in various industries
55 (Tobiszewski and Namieśnik, 2012). Of these compounds, 1,1,1-trichloroethane (1,1,1-TCA) was
56 identified as an ozone-depleting compound under the Montreal Protocol of 1987, and its use was phased
57 out starting in 1989 (Gu et al., 2011). Nevertheless, 1,1,1-TCA is still a common organic pollutant in
58 contaminated soil and groundwater at hazardous waste sites, with 1,1,1-TCA found at more than 393 of
59 the 1315 active sites on the National Priorities List (US EPA database as of 2012). Exposure to 1,1,1-
60 TCA may lead to damage to the central nervous system, heart and liver (ATSDR, 2006). The EPA has set
61 the maximum contaminant level of 1,1,1-TCA in drinking water at 200 μg/L (EPA, 1995).

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

69 Biotransformation of 1,1,1-TCA occurs in anaerobic environments via reductive dechlorination (Fam et
70 al., 2012). *Dehalobacter* (*Dhb*) sp. TCA1 was the first pure culture of an organohalide respiring
71 bacterium that coupled growth to the sequential dechlorination of 1,1,1-TCA to 1,1-DCA and ultimately
72 to chloroethane (CA) (Sun et al., 2002). A few other strains, namely *Dhb* sp. strain CF and strain DCA
73 (Tang and Edwards, 2013B), *Dhb* sp. strain UNSWDHB (Wong et al., 2016), *Dhb* sp. strain THM1 (Zhao
74 et al., 2017), and *Desulfitobacterium* strain PR (Ding et al., 2014), have since been described or isolated
75 with the ability to dechlorinate 1,1,1-TCA and/or 1,1-DCA. All of these strains use either hydrogen or
76 formate as electron donor. Five reductive dehalogenases (RDases) catalyzing these specific

77 dechlorination reactions in these bacteria have also been identified (i.e., CtrA, TmrA, DcrA, CfrA and
78 ThmA). These proteins form a clade of highly similar sequences belonging to Ortholog Group 46 as
79 defined by Hug et al.(2013) and updated by Molenda et al. (2020). Interestingly, while the proteins share
80 greater than 95% amino acid identity, they do have distinct substrate preferences. For example, DcrA
81 catalyzes dechlorination of 1,1-DCA to CA, but is not active on 1,1,1-TCA or chloroform, while CfrA
82 and ThmA are active on chloroform and 1,1,1-TCA but not on 1,1-DCA (Tang and Edwards, 2013; Tang
83 and Edwards, 2013B; Zhao et al., 2017). CtrA from *Desulfitobacterium* strain PR and TmrA from *Dhb*
84 UNSWDHB are reported to be active on all three substrates to varying degrees (Ding et al., 2014; Wong
85 et al., 2016).

86 Chlorinated ethane concentrations are frequently monitored to evaluate 1,1,1-TCA treatment efficiency at
87 remediation sites, but concentration data are easily affected by site operations, making interpretation of
88 data difficult, particularly in trying to distinguish between biotransformation and abiotic removal. Gene-
89 based biomarkers applied to field samples have the potential for monitoring biological 1,1,1-TCA
90 removal (Scheutz et al., 2011). However, only a limited number of field studies reported using
91 quantitative PCR (qPCR) with primers to measure the 16S rRNA gene of *Dehalobacter* dechlorinating
92 1,1,1-TCA at remediation sites (Postiglione et al., 2006; Duchesneau et al, 2007; Damgaard et al., 2013).
93 Moreover, to date, primers targeting functional genes from organisms dechlorinating 1,1,1-TCA have
94 only been applied to microcosm and culture samples, but not to field samples in published work. Without
95 specific biomarkers, estimating the fraction of 1,1,1-TCA removed by biodegradation remains
96 challenging.

97 In this study, we hypothesized that the change in chlorinated ethane concentration caused by
98 biodegradation would be closely reflected in the abundance of substrate-specific dehalogenase genes, and
99 that the fraction of 1,1,1-TCA removed by reductive dehalogenation at a contaminated site could be
100 estimated based on cells yields and the abundance of these biomarker genes. To test this hypothesis, we
101 worked in collaboration with consultants from Pinchin Ltd. on a site contaminated with 1,1,1-TCA. The

102 *in situ* remedy selected for the site was injection of granular ZVI mixed with guar gum. The objectives of
103 this research were to evaluate the use of the *Dhb* 16S rRNA gene and the *cfrA*- and *dcrA*-like
104 dehalogenase genes as biomarkers for 1,1,1-TCA and 1,1-DCA biodechlorination, and ultimately to
105 estimate the fraction of 1,1,1-TCA biotransformed at the site. Additionally, the impact of guar gum and
106 ZVI addition on the biodegradation of 1,1,1-TCA was assessed. This project consisted of the analysis of
107 site samples as well as a laboratory groundwater microcosm study. Site samples were collected to
108 measure chlorinated ethane concentrations and gene abundances using qPCR during the on-going
109 remediation. Microcosms prepared with site groundwater were used track both abiotic and biotic
110 processes under more controlled conditions, and to evaluate the specificity of the substrate-specific primer
111 sets, and to estimate approximate cell yields applicable to the corresponding site samples.

112 2. Materials and Methods

113 2.1 Site Description

114 The Site (Supplementary Fig. S1) was a former adhesive manufacturing plant located in Southern Ontario
115 and operated between 1950 and 1991. A spill of approximately five hundred litres of 1,1,1-TCA in the
116 late 1980s was the only recorded release at the site. Historical contaminant concentrations are presented
117 in Supplementary Table S1. Prior to the guar gum-ZVI injection, 1,1,1-TCA was the dominant
118 contaminant at the site, with concentrations approximately ten times greater than other compounds. The
119 stratigraphy of the site is provided in Supplementary Text S1.

120 2.2 Granular ZVI Particles, Guar Gum, and On-Site Guar Gum-ZVI Injection

121 In late September 2015, the mixture of guar gum and ZVI was injected mainly along the property
122 boundary and in the plume area (Supplementary Fig. S1). Guar gum is a polysaccharide mixture
123 extracted from guar beans and is predominantly composed of galactose and mannose. One week after
124 guar gum-ZVI injection, portions of the site were excavated and back-filled with ZVI alone. The details

125 of ZVI, guar gum matrix, fracturing pressure, distance and intervals of injections, and the amounts of guar
126 gum and ZVI injected and soil excavation are explained in Supplementary Table S2.

127 ## 2.3 Groundwater Monitoring and Sample Collection

128 Between September 2015 and June 2016, groundwater samples were collected from three wells in the
129 plume area, SW5, SW6 and MW202 (Supplementary Fig. S1). In the field study presented in this paper,
130 we defined Day 0 as the time when the first groundwater sample was collected prior to ZVI injection. All
131 wells were developed and purged prior to sampling. Upon groundwater recovery, samples were collected
132 using dedicated inertial pumps. Groundwater samples for chlorinated organic compound analysis were
133 stored in 44 mL glass vials with screw caps with Teflon liners and zero headspace, and preserved with 0.2
134 g sodium bisulphate tablets. Samples for microbial analysis were collected in 250 mL sterilized plastic
135 bottles containing sodium thiosulphate with zero headspace. All samples were placed on ice immediately
136 after sampling and arrived at our lab at the University of Toronto on the same day.

137 ## 2.4 Groundwater Microcosm Setup and Sampling

138 To evaluate *in situ* microbial activity with and without ZVI added, groundwater collected on Day 202
139 from SW6 was used to set up microcosms. The groundwater (2 × 2 L in medium bottles) was purged
140 with 80%/20% N_2-CO_2 gas mix for 1 hour to completely remove oxygen and residual volatile compounds.
141 In a disposable glovebag (Atmosbag, Sigma-Aldrich, Missouri, US) filled with 80%/20% N_2-CO_2 gas mix,
142 150 mL of purged groundwater was dispensed into each sterile 250 mL glass bottle, and one gram ZVI
143 (same type used at the site) was added to the bottles designated with ZVI addition. Mininert septa screw
144 caps were sterilized by wiping with ethanol. All bottles were then capped with the sterile Mininert caps
145 before leaving the glovebag. No other inoculum, electron donor or carbon source was added, unless
146 specified.

147 As detailed in Supplementary Table S3, six treatments were established, including: Sterile (i.e., negative
148 control by autoclaving); Sterile + ZVI; Active without ZVI; Active + ZVI; Active without TCA and ZVI;
149 and bioaugmented with ACT3 (ACT3 + ZVI) where ACT3 refers to a previously described enrichment
150 culture that dechlorinated 1,1,1-TCA via 1,1-DCA to CA (Tang and Edwards, 2013B). Neat 1,1,1-TCA
151 (~4.25 μL per bottle) was added for an initial liquid concentration of approximately ~0.2 mmol/L (0.036
152 mmol per bottle). In the groundwater microcosm experiment, Day 0 refers to the day when the batch
153 assays were set up and 1,1,1-TCA was added to the bottles. ACT3 culture (0.1 mL) was anaerobically
154 added to the ACT3 + ZVI set on Day 2 inside a lab glovebox, yielding an additional ~1×10^9 copies of the
155 *Dhb* 16S rRNA gene /L in these ACT3 + ZVI bottles. In most of the microcosm bottles, we did not add
156 any additional electron donor, as the purged groundwater contained approximately 800 mg/L COD from
157 residual guar gum and its degradation products. The only exceptions were bottle #8 in the Active set
158 without ZVI and bottle #21 in the Active + ZVI set. Ethanol and lactate were added to these two bottles
159 on Day 149 at five times electron equivalent excess to the remaining 1,1-DCA (Supplementary Table S3).
160 An extra bottle containing the same amount of groundwater without any 1,1,1-TCA or ZVI was also
161 included in the assay to evaluate methane production from the groundwater COD. All bottles were
162 incubated statically, upside-down and covered with black cloth in an anaerobic glovebox (Coy Laboratory
163 Products Inc, Michigan, US) filled with 5%-95% H_2-N_2 gas mix at room temperature.

164 **2.5 DNA Extraction and Microbial Analysis**

165 Groundwater samples collected from the site for microbial analysis were packed with ice and shipped to
166 our lab at the University of Toronto, and filtered within 24 hours after sampling through sterile 0.22 μm
167 Sterivex filters using a vacuum pump (Ritalahti et al., 2009). The filters were frozen at -80 °C
168 immediately after filtration. Liquid samples from lab microcosms (1.6 mL) were collected using sterile
169 plastic syringes and needles, and transferred to 2 mL anaerobic screw-cap vials inside the glovebox.
170 Samples were centrifuged at 13,000×g at 4 °C for 15 minutes. The supernatant was removed and the
171 vials with pellets were stored at -80 °C until DNA extraction.

172 DNA extraction was conducted using the Mobio® PowerSoil DNA isolation kit (MoBio Laboratories Inc.,
173 Carlsbad, CA). For groundwater samples, filter membranes were removed from the plastic casings, and
174 added to the bead beating tubes. For each microcosm sample, the beads and the solution in one bead
175 beating tube were carefully transferred to the screw-cap vial containing the thawed cell pellet, and
176 vortexed until the cell pellet was completely re-suspended. Subsequent DNA extraction steps were
177 performed following manufacturer instruction. A NanoDrop ND-1000 spectrophotometer was used to
178 measure DNA concentration.

179 Quantitative PCR (qPCR) was used to enumerate specific phylogenetic and functional genes in the DNA
180 samples. Primers *Dhb*477f ((5'-GATTGACGGTACCTAACGAGG-3') /*Dhb*647r (5'-
181 TACAGTTTCCAATGCTTTACGG-3') were used to quantify the abundance of the *Dhb* 16S rRNA gene
182 copies (Grostern and Edwards, 2006). Primers *cfrA*413f (5'- CCCGAACCTCTAGCACTTGTAG -3')
183 /*cfrA*531r (5'- ACGGCAAAGCTTGCACGA -3') and *dcrA*424f (5'- AGCACTCAGAGAGCGTTTTGC
184 -3') /*dcrA*533r (5'- CAACGGCCCAGCTTGCAT -3') were adapted to track two reductive dehalogenase
185 genes *cfrA* and *dcrA* encoding the proteins catalyzing dechlorination of 1,1,1-TCA and 1,1-DCA
186 respectively (Tang and Edwards, 2013B). The annealing temperature was set at 62.5 °C for the *Dhb* 16S
187 primer or at 60 °C for the *cfrA* and *dcrA* primer sets. Detailed recipe and information of the qPCR
188 reactions and detection are presented in Supplementary Text S2 and Fig. S2.

189 The DNA extracted from site and microcosm samples was also submitted to McGill University and
190 Genome Quebec Innovation Center for amplicon sequencing with the universal 16S rRNA gene primers
191 926f (5'-AAACTYAAAKGAATWGRCGG-3') and 1392r (5'- ACGGGCGGTGWGTRC-3') (Qiao et al.,
192 2020). The amplicon sequencing data were analyzed using the QIIME2 pipeline with the Dada2 package
193 (Bolyen et al., 2019, details in Supplementary Text S3).

194 To obtain nearly complete sequences of the reductive dehalogenase genes from our samples, the DNA
195 extracted from the bottle with the fastest degradation of 1,1,1-TCA (bottle #9 on Day 49) was amplified

196 with primers designed by Tang and Edwards (2013B) termed *rdhA*23f (5'-
197 AAGAGATTGTAhGAAGCAGCGG-3') and *rdhA*1383r (5'-CTTAGTAAATGGGCAAGCAGC-3')
198 targeting the RDase genes (the original numbering is a little mis-leading as the forward primer is actually
199 a little outside the coding region, as shown in Supplementary Text S4). The PCR program, the primer
200 sequences, the cloning procedures in *E. coli* and the procedures to extract plasmid DNA are described in
201 Tang and Edwards (2013B). Clones were submitted to The Centre for Applied Genomics (Toronto), and
202 each was sequenced four times in both the forward and reverse directions with the M13f and M13r
203 primers. The forward and reverse reads were assembled using Geneious and resulting contigs aligned
204 with the five known gene sequences encoding 1,1,1-TCA/1,1-DCA dehalogenases: *dcrA* (JX282330.1),
205 *cfrA* (JX282329.1), *ctrA* (KF155162.1), *tmr*A (NZ_AUUR01000010 REGION: 19936..21303) and *thmA*
206 (KX344907.1).

207 **2.6 Chemical Analysis of Groundwater and Microcosm Samples**

208 Chlorinated ethane concentrations in field samples were stored at 4 °C and were measured within 48
209 hours after the samples arrived at the University. Microcosm were first shaken and then sampled using
210 gas-tight glass syringes and sterile needles inside the glovebox, and analyzed immediately. Aliquots (0.5
211 - 1 mL) from groundwater or microcosm samples were added to acidified MilliQ water to a total liquid
212 volume of 6 mL in 11 mL crimped-cap headspace autosampler vials and placed in the Agilent G1888
213 Headspace Sampler. Headspace was analyzed using the Agilent 7890 gas chromatograph (GC) equipped
214 with the GS-Q column and detected using a flame ionization detector (FID). External standards,
215 purchased from Sigma, were first diluted with methanol, and then in acidified MilliQ water for calibration.
216 A modified EPA 8021 method was used as per Kocur et al. (2015). Several groundwater samples were
217 also analyzed for total COD according to the EPA Method 410.4 (USEPA, 1993).

218 ### 2.7 Accession Numbers

219 Raw amplicon sequences were submitted to the NCBI Sequence Read Archive (SRA, ID PRJNA634323),
220 with sample accession numbers from SAMN14988330 to SAMN14988369 (details in Supplementary
221 Text S3). The cloned sequences of the nearly complete RDase genes were deposited to GenBank with
222 accession numbers MT536772 for Clone 1, MT536773 for Clone 2 and MT536774 for Clone 3
223 (sequences also provided in Supplementary Text S4).

224 ## 3. Results and Discussion

225 We analyzed chlorinated solvent concentrations and gene abundances in both microcosm and
226 groundwater samples to investigate transformation of 1,1,1-TCA at a field site undergoing treatment with
227 guar gum stabilized ZVI. The microcosms provided a controlled experiment to distinguish
228 biodegradation from abiotic transformation, to obtain a correlation between depletion of chlorinated
229 ethanes and changes in biomarker abundance, and to aid in interpreting biodegradation at the site.
230 Microcosm results are presented first, followed by field data, and ending with an analysis of microbial
231 communities in both lab and field samples. The contributions of abiotic and biotic processes are also
232 discussed.

233 ### 3.1 Dechlorination of 1,1,1-TCA in Groundwater Microcosms

234 Six treatments were evaluated in microcosms prepared with site groundwater only (Supplementary Table
235 S3). To evaluate the accuracy of our measured concentration data, the sum of the moles of all two-carbon
236 compounds (C2-compounds) was tracked over time considering all possible products of 1,1,1-TCA
237 dechlorination. These included 1,1-DCA, 1,1-DCE, cis-DCE, trans-DCE, 1,2-DCA, CA, ethane and
238 ethene. The sum of moles of all C2-compounds was conserved in the Sterile Control microcosms without
239 ZVI, in the Active set without ZVI, and in the set with ACT3 culture plus ZVI (ACT3 + ZVI), confirming
240 that all major transformation products were being monitored (Supplementary Fig. S3) in this cases. In the

241 Active + ZVI and Sterile + ZVI sets, measured degradation products accounted for only 70-80% of
242 substrate (1,1,1-TCA) loss, indicating that some products were not being measured in these bottes
243 (discussed further below). Methane production was also monitored and compared to the original COD
244 concentration measured at Day 0 in microcosms (Supplementary Table S4A). In the absence of 1,1,1-
245 TCA and ZVI, methane production accounted for approximately 90% of the original COD present in the
246 bottle, indicating that although no extra electron donor was added to the microcosm bottles, residual guar-
247 gum and transformation products in the groundwater were sufficient to drive methanogenesis (and
248 dechlorination in the bottles fed with 1,1,1-TCA). Less methane was produced in bottles fed with 1,1,1-
249 TCA reflecting the well-documented inhibition of methanogenesis (Grostern and Edwards, 2006B). Mole
250 balances were closed in most cases establishing the accuracy of our analytical data.

251 Dechlorination of 1,1,1-TCA to 1,1-DCA occurred in all cases except for the Sterile Control bottles
252 without ZVI (Supplementary Fig. S4A). However, the extent of dechlorination, the lag time, and the end-
253 products varied considerably, even within replicates for a given treatment (Fig. 1 & Fig. S6). For
254 example, dechlorination to CA was observed in bottles #7 and #9 from the Active without ZVI treatment,
255 but not in bottle #8 from the same set until it was amended with electron donor after Day 150 (Fig. 1).
256 Dechlorination of 1,1-DCA to CA was also seen in bottle #18 of the Active + ZVI treatment, but not in
257 bottle #21 until it was amended with electron donor after Day 150 (Fig. S6). Dechlorination to CA was
258 also observed in the ACT3 + ZVI treatment (Fig. S5). In contrast, in the Sterile + ZVI bottles
259 (Supplementary Fig. S4B), dechlorination of 1,1,1-TCA stopped at 1,1-DCA, consistent with previous
260 studies reporting that 1,1-DCA is not reduced abiotically by ZVI (Cwiertny et al., 2006; Wu et al., 2014).
261 In all bottles containing ZVI, whether (biologically) active or not, dechlorination of 1,1,1-TCA to 1,1-
262 DCA was relatively rapid (i.e., completed in less than 50 days). In bottles without ZVI, lag times ranged
263 from 7 to 21 days before initiation of dechlorination, although once initiated, dechlorination rates were
264 similar in all these bottles. Ethane and ethene were only detected in the ZVI-containing bottles (Table
265 S4B), and these minor products appeared as early as Day 1, possibly via another abiotic dechlorination

266 pathway bypassing CA, as no CA was detected in the Sterile + ZVI set. Moreover, in the bottles
267 containing ZVI (sterile or not), we observed a 20-30% mole loss over the first two weeks (Supplementary
268 Fig. S3), presumably due to the formation of unidentified products such as 2-butyne or cis-2-butene, as
269 previously reported (Fennelly and Roberts, 1998). In summary, 1,1-DCA was the major final
270 dechlorination product from 1,1,1-TCA with ZVI alone. In the presence of active microorganisms,
271 dechlorination proceeded further to CA. The addition of ZVI reduced the lag time for 1,1,1-TCA
272 dechlorination to 1,1-DCA and generated other by-products that were not identified.

273 3.2 Evaluation of the Specificity of the Primer Sets Targeting the *cfrA*-like and *dcrA*-like RDase
274 Functional Genes

275 Certain *Dehalobacter restrictus* (*Dhb*) populations are well-known for catalyzing 1,1,1-TCA and 1,1-
276 DCA dechlorination, therefore we first established that this genus was present in the groundwater, and
277 then focussed on enumerating *Dhb* in the microcosms over time. We also tracked the abundance of the
278 corresponding functional genes *cfrA* and *dcrA* (Tang and Edwards, 2013B). We amplified and cloned the
279 DNA extracted from microcosm bottle #9 on Day 49 to retrieve nearly complete (1231bp) RDase genes.
280 Three clones were successfully sequenced, and their sequences were compared to the five genes encoding
281 RDases known to dechlorinate 1,1,1-TCA and/or 1,1-DCA, namely CtrA, TmrA, ThmA, CfrA and DcrA
282 (Tang and Edwards, 2013B; Ding et al., 2014; Wong et al., 2016; Zhao et al., 2017). Based on sequence
283 alignment and clustering (Fig. S7A and Table S5), Clone 3 was found to be most similar (>99.5%) to the
284 *dcrA* gene (encoding a 1,1-DCA dehalogenase), and Clones 1 and 2 were more similar to the *thmA* gene
285 which encodes an RDase that is active on 1,1,1-TCA but not 1,1-DCA, similar to CfrA (Tang and
286 Edwards, 2013B; Zhao et al., 2017). Next we aligned the cloned sequences to the *cfrA* and *dcrA* primer
287 sequences. As shown in Table 1 (detailed alignment in Fig. S7B). the alignment revealed two clades.
288 The first clade (1,1,1-TCA group) comprises Clones 1 and 2 and *ctrA*, *tmrA*, *cfrA* and *thmA* that have 2 or
289 fewer total mismatches to the *cfrA* primer pair. The second clade (1,1-DCA group) comprises Clone 3

290 and *dcrA* and both are a perfect match to the *dcrA* primer pair. We thus refer to the genes amplified with
291 the *cfrA* primers as "*cfrA*-like" genes, and those amplified with the *dcrA* primers as "*dcrA*-like" genes.

292 ### 3.3 Gene Abundances during Dechlorination in Groundwater Microcosms

293 We tracked the abundance of *Dehalobacter* (*Dhb*) 16S rRNA genes as well of the *cfrA*- and *dcrA*-like
294 genes using qPCR in microcosm samples over time. Melt curves were examined, particularly for data
295 close to or lower than the detection limit to confirm specific detection or not. When gene copy numbers
296 were well above the detection limit, melt curves of samples and standards were identical (Supplementary
297 Fig. S2).

298 The abundance the *cfrA*- and *dcrA-like* genes tracked dechlorination activity in a consistent and specific
299 manner. As 1,1,1-TCA was dechlorinated to 1,1-DCA, the copy numbers of the *Dhb* 16S rRNA gene and
300 the *cfrA*-like gene increased as well (Figs. 1 and S6). As 1,1-DCA was dechlorinated to CA in bottles #7,
301 #9 and #18, the copy number of the *dcrA*-like gene increased. In bottles #8 (Fig. 1B) and #21 (Fig. S6C),
302 when dechlorination was stuck at 1,1-DCA for more than 100 days, the copy number of the *dcrA*-like
303 gene remained low ($<10^7$ copies/ L). As soon as electron donor was added to these two bottles, 1,1-DCA
304 was dechlorinated to CA and at the same time, the *dcrA*-like gene abundance increased by two orders of
305 magnitude. Clearly, 1,1-DCA dechlorination was stalled due to lack of electron donor. We estimated the
306 relative proportions of *Dehalobacter* carrying the *cfrA*-like and *dcrA*-like genes from the ratio of each
307 functional gene to the 16S rRNA gene ($\%_{cfrA\text{-}Dhb}$ and $\%_{dcrA\text{-}Dhb}$). The $\%_{cfrA\text{-}Dhb}$ increased while 1,1,1-TCA
308 was being converted to 1,1-DCA, and then declined while the $\%_{dcrA\text{-}Dhb}$ went up during the reduction of
309 1,1-DCA to CA (Fig. S8), confirming that these two genes clearly belonged to two different *Dhb*
310 populations.

311 The coexistence of two highly similar but distinct *Dehalobacter* populations in the dechlorination of
312 1,1,1-TCA has only been reported in the study conducted by Tang *et al*. (Tang et al., 2012; Tang and
313 Edwards, 2013B; Tang et al., 2016), where the authors speculated that the speciation into two highly

314 specific strains in the ACT3 culture might be an artifact of laboratory cultivation over more than ten years
315 in the presence of ample electron acceptor and donor. The current study suggests that this kind of
316 speciation and selection can also take place *in situ* at a contaminated site. This realization also explains
317 why dechlorination proceeded in a highly sequential manner where 1,1,1-TCA was first converted to 1,1-
318 DCA before further dechlorination occurred. The second step required the growth of a distinct *Dhb*
319 population which lagged behind and presumably competed with the first population for available donor.
320 Consistently, the *dcrA*-containing population was at much lower abundance initially in all microcosms.

321 **3.4 Biomass Yields Estimated from Microcosm Data**

322 *Dehalobacter* cell yields, expressed as 16S rRNA copies per µmole chloride released for each
323 dechlorination step, were estimated from microcosm data and compared to previously published values
324 (Tables 2 and S6). Considering the many sources of possible errors in these measurements (including not
325 accounting for cell decay, DNA extraction efficiency and inherent qPCR error), the results are reasonably
326 consistent. Yields from microcosms with ZVI were slightly lower than those without ZVI, consistent
327 with some abiotic transformation. Calculated cell yields from this study were similar to the yield reported
328 for a pure culture of *Dehalobacter* sp. UNSWDHB (Wong et al., 2016), but are an order of magnitude
329 lower than the yields reported for *Dehalobacter* sp TCA1 (Sun et al., 2002) and of *Dehalobacter* in the
330 enrichment culture ACT3 (Grostern and Edwards, 2006B). The lower observed cell yields in this study
331 are perhaps the result of incubating in groundwater only with no added vitamins.

332 **3.5 Analysis of Dechlorination Time Profiles and Gene Abundances in Field Samples**

333 The abundances of *Dhb* 16S rRNA, *cfrA*-like and *dcrA*-like genes were determined in field samples to
334 compare changes in gene abundances over time to changes in contaminant concentrations. Three wells
335 were sampled extensively during this study, SW6, SW5 and MW202, all located in the plume area with
336 high concentrations of 1,1,1-TCA (i.e., 0.45 - 1.2 mmol 1,1,1-TCA/L) prior to guar gum-ZVI injection.
337 Because of the extent and variety of treatments applied to the site, including soil excavation, iron

338 emplacement in trenches, injection and groundwater pumping, it was generally difficult to interpret the
339 site concentration data.

340 SW6 was the only well with a clear pattern of transformation and a reasonable mole balance. After the
341 guar gum-ZVI was injected to SW6, the decline of 1,1,1-TCA coincided with the generation of 1,1-DCA
342 as well as increasing abundances of the *Dhb* 16S rRNA and *cfrA*-like genes (Fig. 2A). The abundance of
343 the *dcrA* -like genes increased with a slight decline in the *cfrA*-like genes when 1,1-DCA was gradually
344 converted to CA (after Day 250). These degradation profiles and correlations between the biomarker
345 genes and the chlorinated ethane concentrations paralleled the observations from microcosm bottles.
346 Assuming that the cell yield estimated from microcosms is applicable to the conditions in SW6,
347 approximately 30% of the 1,1,1-TCA removed between Days 69 and 97 and 100% of that removed
348 between Days 97 and 133 were attributed to dechlorination by *Dehalobacter* (Supplementary Table S7A).
349 Towards the end of the field study, roughly 0.15 mmol/L CA was measured in groundwater from SW6,
350 representing approximately 15% of the initial 1,1,1-TCA concentration. Since CA is strictly a biotic
351 product, this 15% CA production provides a minimum estimate of the fraction dechlorinated by
352 *Dehalobacter* carrying the *dcrA*-like gene. Therefore, biological dechlorination was responsible for a
353 significant fraction of the observed dechlorination, from a minimum of 15% overall to a maximum of 100%
354 at certain time intervals in SW6.

355 In monitoring wells SW5 (Fig. 2B) and MW202 (Supplementary Fig. S9), concentration data were
356 difficult to interpret because the disappearance of 1,1,1-TCA did not coincide with stoichiometric
357 production of 1,1-DCA. However, in SW5, the concentration of the *Dhb* 16S rRNA and *cfrA-like* genes
358 increased by more than two orders of magnitude between Days 40 and 97 (Fig. 2B), revealing substantial
359 biological dechlorination. Similar to the estimation done for SW6, based on the average cell yield in the
360 microcosms and the qPCR results for SW5, biodechlorination accounted for roughly 60% of 1,1,1-TCA
361 removed between days 40 and 97 in SW5 (Supplementary Table S7B). In MW202, biodechlorination
362 was estimated to be about 10% of the 1,1,1-TCA removed between days 40 and 97 (Supplementary Table

363 S7C). CA concentrations on Day 283 corresponded to 15% and 7% of the peak 1,1,1-TCA concentration
364 in SW5 and MW202, respectively. Therefore, despite the noisy concentration data, the increase in the
365 abundances of biomarker genes provided clear evidence that significant biological dechlorination was
366 occurring in SW5 and MW202 during the periods monitored.

367 Our site data suggest that there needs to be at least ~3×10^6 copies of *Dehalobacter* 16S rRNA genes / L
368 groundwater for measurable dechlorination of 1,1,1-TCA at the site. In addition, qPCR data from SW6
369 field samples (Fig. 2A) confirmed that indeed two distinct populations of *Dehalobacter* carrying two
370 different RDase genes were present at the site, and each was responsible for one of the two dechlorinating
371 steps. This observation is consistent with the observations in the groundwater microcosm studies
372 presented earlier, and in the study of enrichment culture ACT-3 (Tang and Edwards, 2013B).

373 ### 3.6 Microbial Communities in Microcosm and Site Samples

374 Amplicon sequencing based on the 16S rRNA gene was conducted to identify other organisms in the
375 microcosm and field samples. These results were filtered to reveal organisms with max abundance > 5%
376 in at least one sample (Table S8). *Dehalobacter* was certainly one of the dominant organisms in the
377 microcosms (up to 34%). Other possible dechlorinating organisms include *Sulfurospirillum* (max 29%),
378 *Dechloromonas* (max 7.6%) and *Geobacter* (max 3.6%, not shown in Table S8). Certain *Sulfurospirillum*
379 and *Geobacter* species have been shown to dechlorinate chlorinated ethenes (Buttet et al., 2013; Sung et
380 al., 2006), but not chlorinated ethanes. *Dechloromonas* was reported to dechlorinate chloroform (Lai et
381 al., 2019). We examined the relationship between *Sulfurospirillum* abundance and 1,1,1-TCA and 1,1-
382 DCA biodegradation. In microcosms (Figs S10A to S10C), *Sulfurosprillum* abundance did not increase
383 as 1,1-DCA was dechlorinated to CA. In MW202 (Fig S10D), *Sulfurosprillum* abundance increased
384 beyond Day 97 while no 1,1-DCA or CA was produced. Therefore, *Sulfurospirillum* was likely not
385 responsible for the reduction of 1,1,1-TCA or 1,1-DCA in this study. Similarly, the trends in *Geobacter*
386 and *Dechloromonas* did not correspond to the changes in the chlorinated ethane concentrations (Table S8).

387 Thus, we can confidently state that *Dehalobacter* was responsible for biological 1,1,1-TCA and 1,1-DCA
388 dechlorination in this study.

389 The amplicon sequencing data also provide some clues as to the likely organisms fermenting the guar
390 gum polysaccharide, as well as an indication of the different selection pressures in the groundwater
391 microcosm bottles versus the field samples. In the site samples, the relative abundance of several
392 organisms increased substantially after guar gum injection (Tables S8). These included *Paludibacter*,
393 *Trichococcus*, *Clostridium sensu stricto 1*, *Hypnocyclicus* and *Macellibacteroides* , all organisms known
394 to metabolize carbohydrates to produce volatile fatty acids and/or simple alcohols (Murali et al., 2017;
395 Wiegel et al., 2006; Roalkvam et al., 2015; Jabari et al., 2012; Liu et al., 2002). Interestingly, none of
396 these organisms, except *Paludibacter*, became abundant in microcosm bottles. Rather, *Sulfurospirillum*
397 increased substantially by day 49 in all batch bottles (Figs S10A to S10C) becoming one of the major
398 organisms in the communities, yet as mentioned above, their abundance was not correlated to 1,1,1-TCA
399 or 1,1-DCA dechlorination. Some *Sulfurospirillum* have been reported to ferment simple carbohydrates
400 (Kruse et al., 2018; Kodama et al., 2007). Therefore, in our microcosms, *Sulfurospirillum* was the most
401 likely fermenter to generate the H_2 required by *Dehalobacter*. Moreover, it is clear that the lab
402 microcosms were more reduced than the field samples, as shown by greater abundance of methanogens
403 and other strict anaerobes.

404 ### 3.7 Impact of Guar Gum and ZVI on Dechlorination of 1,1,1-TCA

405 Previous studies reported enhanced biodegradation of carbon tetrachloride, chloroform, 1,1,1-TCA and
406 1,2-dichloroethane after ZVI addition (Zemb et al., 2010; Zhou et al., 2014; Lee et al., 2015), while Xiu et
407 al. (2010) found that the addition of nano-scale ZVI to a *Dehalococcoides*-containing methanogenic
408 consortium initially inhibited trichloroethene dechlorination. In the current study, we did not observe
409 adverse impacts of ZVI on *Dehalobacter* growth. Based on our parallel microcosm and site studies, the
410 impact was most noticeable immediately after ZVI addition, with very rapid dechlorination of 1,1,1-TCA

411 to 1,1-DCA. We also found that ZVI addition led to formation of some ethane and ethene from 1,1,1-
412 TCA. While ethane concentration in MW202 and SW5 gradually increased to 0.2 - 0.4 mmol /L in over
413 280 days, ethane production in active bottles with ZVI addition mainly occurred on Day 1 and contributed
414 to less than ~10% of 1,1,1-TCA removal (Table S4B). Therefore, the reasons for the relatively high
415 concentration of ethane observed in SW5 and MW202 are not well understood at this time. Overall, the
416 main ZVI-related transformation production of 1,1,1-TCA was 1,1-DCA at the site, and further
417 transformation of 1,1-DCA to CA was entirely biological.

418 Guar gum and its degradation products were a critical source of electron donor for the dechlorinating
419 microbial communities in the field and in the microcosms. The monitoring well with lower COD
420 concentration also contained lower abundance of *Dehalobacter* (e.g., MW202 between Day 50 and 100,
421 Supplementary Fig. S11). In microcosms, the residual guar gum and fermentation products contained in
422 the groundwater were sufficient to support biological dechlorination of 1,1,1-TCA and 1,1-DCA.
423 Dechlorination stalled in only a few bottles, and in those cases, it was easily re-initiated by addition of
424 electron donor.

425 4. Conclusions

426 This study established that injection of granular ZVI with guar gum supported a very active and sustained
427 dechlorinating population that contributed to a large fraction of the observed dechlorination at the field
428 site. Using an estimate of cell yield (copies *Dehalobacter* 16S rRNA genes per mol chloride released) in
429 conjunction with qPCR abundance data we estimated the fraction of 1,1,1-TCA that was microbially
430 dechlorinated versus that which was abiotically reduced by ZVI in actual field samples. By tracking the
431 abundances of two specific functional genes (*cfrA*- and *dcrA*-like genes), we showed that two different
432 *Dehalobacter* populations participated in the successive dechlorination reactions at the field site. One
433 population was associated with dechlorination of 1,1,1-TCA to 1,1-DCA while the second was associated
434 with dechlorination of 1,1-DCA to CA. Temporal and spatial trends in DNA-based biomarkers from

435 groundwater samples provided an orthologous measure of biologically-mediated dechlorination reactions.
436 Cell yields for obligate dechlorinating organisms such as *Dehalobacter* were used together with measured
437 cell abundances to estimate contaminant transformation attributable to biodegradation. This is
438 particularly valuable to deconvolute simultaneous abiotic and biotic transformation processes.

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443 offering the ACT3 culture and sharing their knowledge of the functional genes and primers.

444 Declarations of Competing Interest

445 There is no conflict of interest.

446 Supporting Information

447 Figures and tables in the Supplementary Information include detailed site information, microcosm setup,
448 reaction recipe, and results of concentration data and sequence alignment that were not presented in the
449 main text figures and tables.

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584

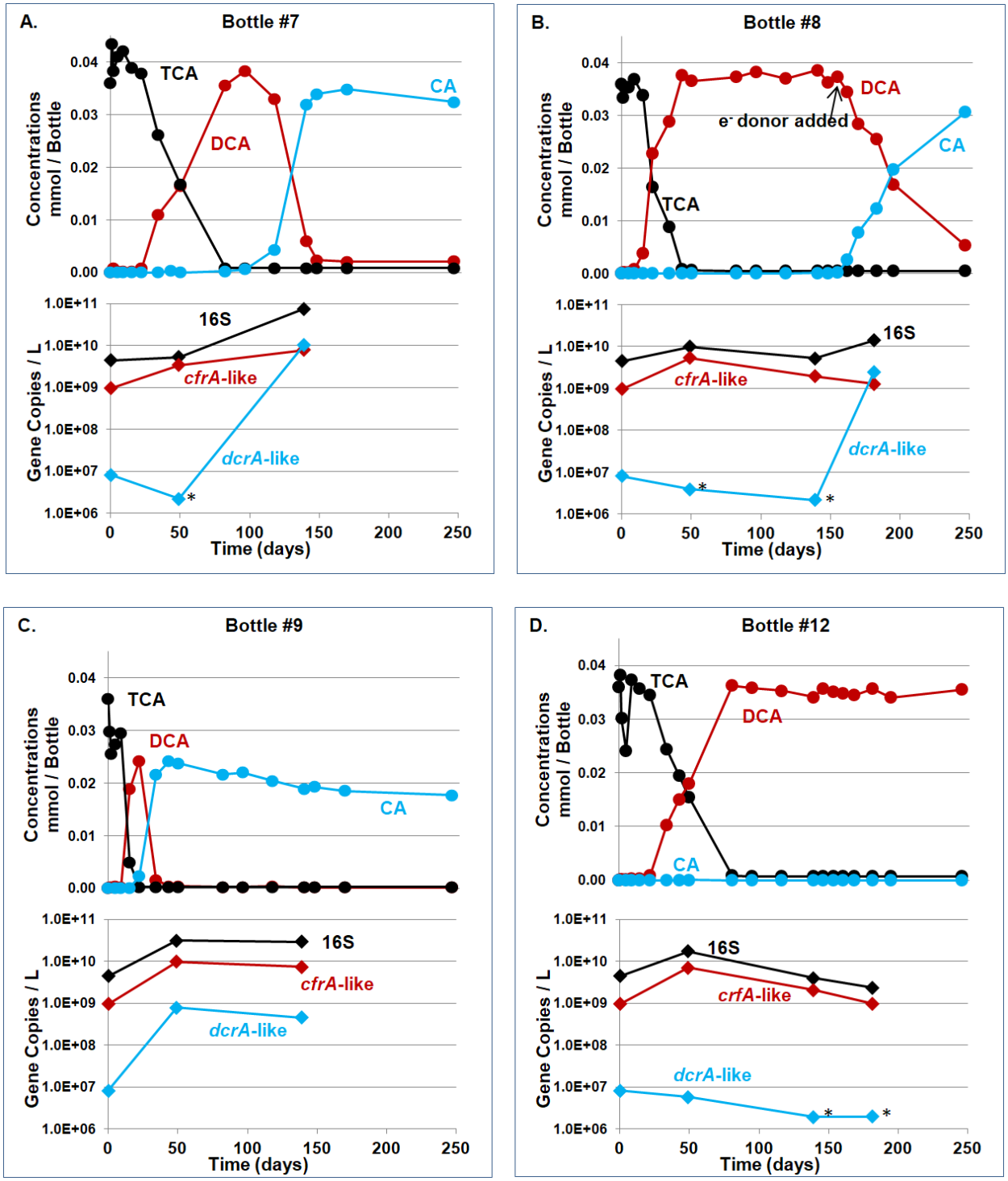

Fig. 1. Chlorinated ethane concentrations and gene copy numbers in groundwater microcosms without ZVI. Data from bottles #7, #8, #9 and #12 are presented in Panels A, B, C and D, respectively.

590 In each panel, the top figure shows chlorinated ethane concentrations (in circles, black for 1,1,1-TCA, red
591 for 1,1-DCA, and blue for CA) and the bottom figure displays the qPCR results in copies per L (in
592 diamonds, black for the *Dhb* 16S rRNA gene, red for the *cfrA*-like gene, and blue for the *dcrA*-like gene).
593 Gene copies are the average of three technical replicates, and qPCR data points marked with * are below
594 the lowest detection limit or showed nonspecific amplification.

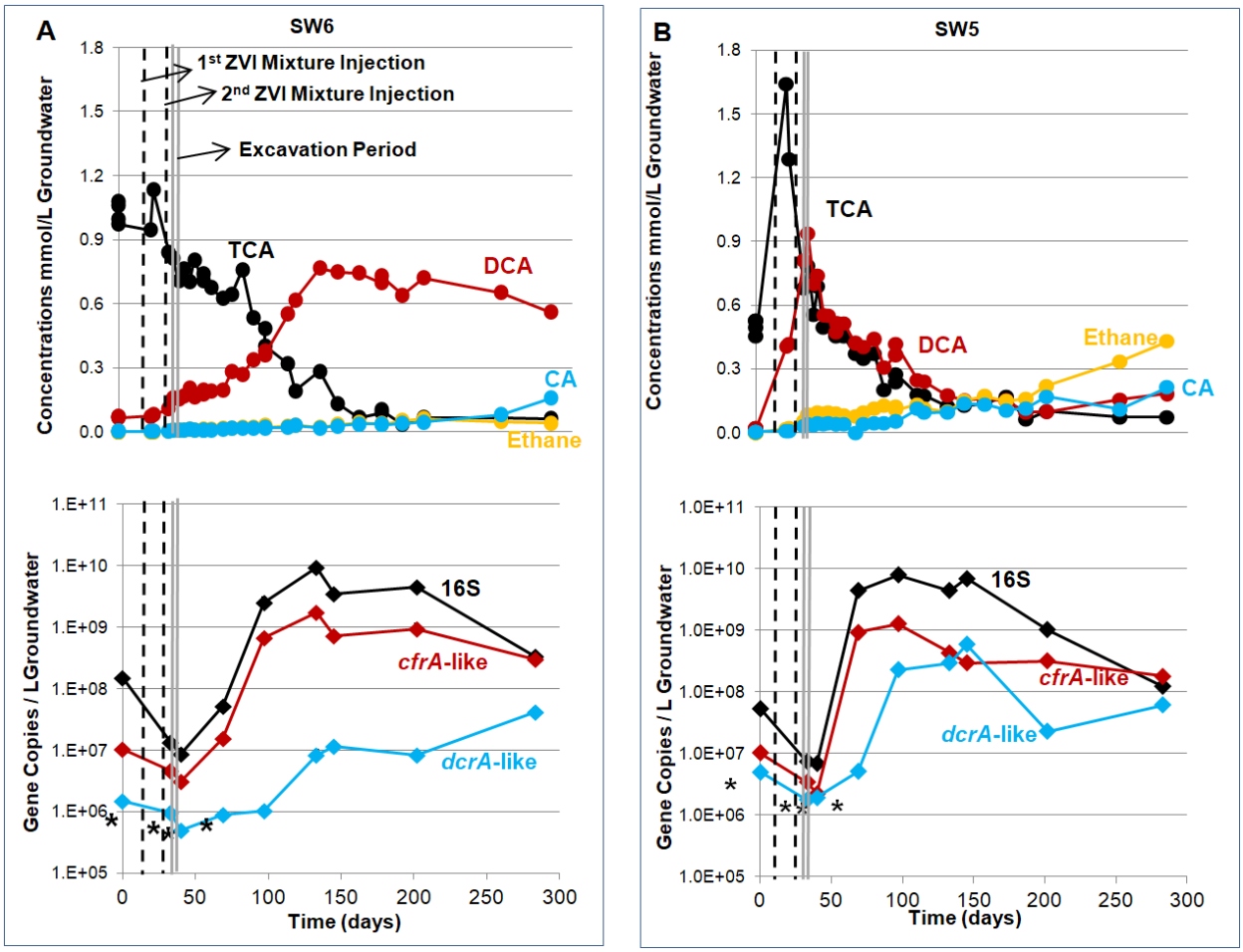

Fig. 2. Chlorinated ethane concentrations and qPCR results in samples from wells SW6 (Panel A) and SW5 (Panel B). In each panel, the top figure shows chlorinated ethane concentrations (in circles, black for 1,1,1-TCA, red for 1,1-DCA, blue for CA, and yellow for ethane) and the bottom figure displays the qPCR results (in diamonds, black for the *Dhb* 16S rRNA gene, red for the *cfrA*-like gene, and blue for the *dcrA*-like gene). The values in the gene copies are the average of three technical replicates, and qPCR data points marked with '*' are below the lowest detection limit or showed nonspecific amplification.

603 **Table 1. Number of mismatches between dehalogenase gene sequences and primers**

Target	*cfrA* forward	*cfrA* reverse	*dcrA* forward	*dcrA* reverse
***Dhb* sp CF *cfrA* gene (JX282329.1)**	0	0	4	3
***Desulfitobacterium* 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 Clone1	1	0	4	4
Microcosm Clone2	2	0	5	3
***Dhb* sp DCA *dcrA* gene (JX282330.1)**	3	3	0	0
Microcosm Clone3	3	3	0	0

604 Note: detailed alignments are shown in Fig. S7B.

605

606 **Table 2. Summary of cell yields (gene copies / µmol Cl^- evolved) for organisms growing on 1,1,1-**

607 **TCA and/or 1,1-DCA**

Organism/culture name	Pure culture	Yield on 1,1,1-TCA	Yield on 1,1-DCA	Reference
***Dhb*-UNSWDHB**	Yes	1.1×10^7	2.1×10^7	Wong et al., 2016
***Dhb*-MS (ACT3)**	No	9.4×10^8	2.4×10^9	Grostern and Edwards, 2006
***Dsb*-PR**	Yes	2.1×10^8	Data not available	Ding et al., 2014
***Desulfitobacterium* -TCA1**	Yes	2.4×10^8		Sun et al., 2002
Active without ZVI	No	Average = 4.2×10^7 [Bottles #7, 8 and 12, 5×10^6 to 1×10^8]	8.7×10^7 [Bottle #8]	This study
Active + ZVI	No	Average = 2.0×10^7 [Bottles #18 and 19, 3.5×10^7 to 5.6×10^6]	1.6×10^7 [Bottle #21]	This study

608

609 Note: details of calculations presented in Table S6.