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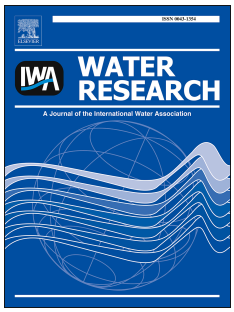

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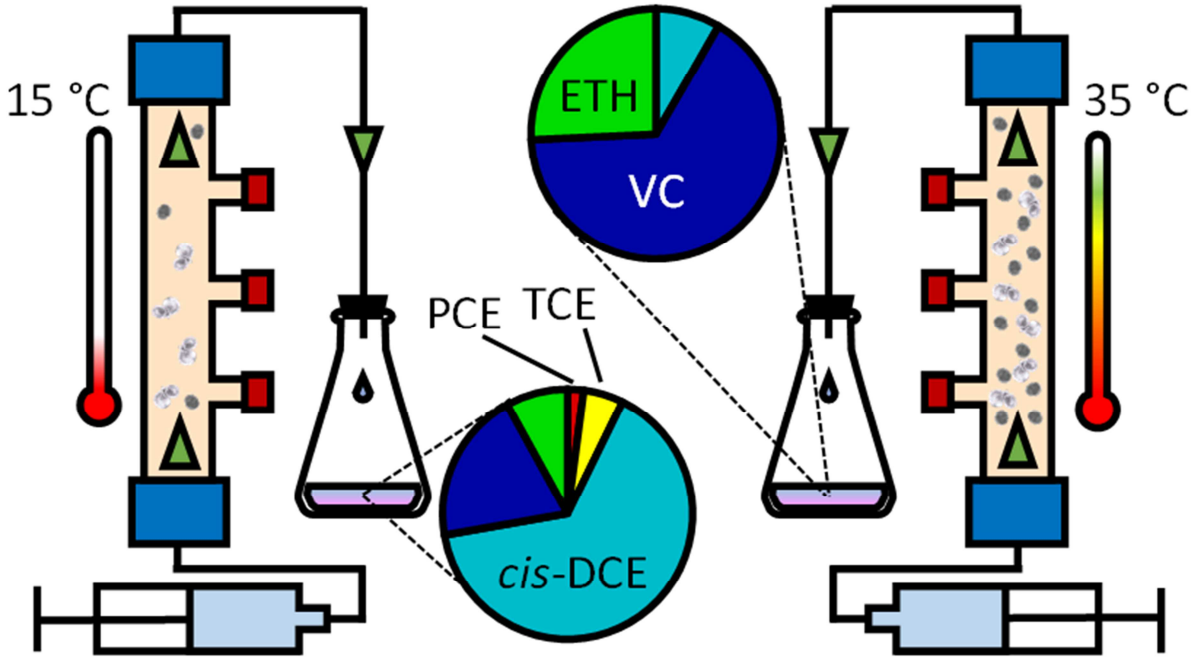

Impacts of Low-Temperature Thermal Treatment on Microbial Detoxification of Tetrachloroethene under Continuous Flow Conditions

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Abstract

Coupling in situ thermal treatment (ISTT) with microbial reductive dechlorination (MRD) has the potential to enhance contaminant degradation and reduce cleanup costs compared to conventional standalone remediation technologies. Impacts of low-temperature ISTT on *Dehalococcoides mccartyi* (*Dhc*), a relevant species in the anaerobic degradation of *cis*-1,2-dichloroethene (*cis*-DCE) and vinyl chloride (VC) to nontoxic ethene, were assessed in sand-packed columns under dynamic flow conditions. Dissolved tetrachloroethene (PCE; 258±46 μM) was introduced to identical columns bioaugmented with the PCE-to-ethene dechlorinating consortium KB-1®. Initial column temperatures represented a typical aquifer (15 °C) or a site undergoing low-temperature ISTT (35 °C), and were subsequently increased to 35 and 74 °C, respectively, to assess temperature impacts on reductive dechlorination activity. In the 15 °C column, PCE was transformed primarily to *cis*-DCE (159±2 μM), which was further degraded to VC (164±3 μM) and ethene (30±0 μM) within 17 pore volumes (PVs) after the temperature was increased to 35 °C. Regardless of the initial column temperature, ethene constituted >50 mol% of effluent degradation products in both columns after 73 – 74 PVs at 35 °C, indicating that MRD performance was greatly improved under low-temperature ISTT conditions. Increasing the temperature of the column initially at 35 °C resulted in continued VC and ethene production until a temperature of approximately 43 °C was reached, at which point *Dhc* activity substantially decreased. The abundance of the *vcrA* reductive dehalogenase gene exceeded that of the *bvcA* gene by 1 – 2.5 orders of magnitude at 15 °C, but this relationship was reversed at temperatures >35 °C, suggesting *Dhc* strain-specific responses to temperature. These findings demonstrate improved MRD performance with low-temperature thermal treatment and emphasize potential synergistic effects at sites undergoing ISTT.

Key words: chlorinated solvents, combined remedies, thermal treatment, bioremediation, microbial reductive dechlorination, reductive dehalogenase genes, *Dehalococcoides mccartyi*

1. Introduction

The coupling of in situ thermal treatment (ISTT) and microbial reductive dechlorination (MRD) has been proposed to improve remediation performance at sites contaminated with chlorinated solvents (Beyke and Fleming 2005, Christ et al. 2005, Stroo et al. 2012). The average groundwater temperature in the United States ranges from 3 °C in parts of Maine and Minnesota to 25 °C in southern Florida and Texas (USEPA 2016), but the key dechlorinating bacteria involved in reductive dechlorination of chlorinated solvents are mesophilic, with optimal growth temperatures ranging from 22 to 38 °C (Fletcher et al. 2011, Friis et al. 2007, Gerritse et al. 1996, Holliger et al. 1993, Löffler et al. 2013). A narrower optimal range of 25 to 30 °C has been identified for neutrophilic, strictly hydrogenotrophic *Dehalococcoides mccartyi* (*Dhc*) strains, which are essential bacteria for the reduction of *cis*-1,2-dichloroethene (*cis*-DCE) and vinyl chloride (VC) to nontoxic ethene (Löffler et al. 2013). Use of ISTT to achieve the optimal temperature for MRD could substantially impact in situ dechlorination rates. A batch culture study using the tetrachloroethene (PCE)-to-ethene dechlorinating consortium Bio-Dechlor INOCULUM (BDI) demonstrated *Dhc* activity at 40 °C, but sustained biotransformation of VC to ethene required temperatures not exceeding 35 °C (Fletcher et al. 2011), emphasizing the potential negative impacts of heating on the MRD process.

MRD has been studied extensively in static batch incubations, but system scale and conditions (i.e., presence of porous medium and dynamic flow conditions) have been shown to strongly

influence microbial activity, in some cases impacting microbial tolerance to environmental stressors. In a study designed to compare MRD in continuous flow versus batch reactor systems, *Dhc* dechlorination rates in columns exceeded those in batch reactors by more than 200 fold (Schaefer et al. 2009), highlighting the potential for system conditions (e.g., hydraulic parameters) to substantially affect microbial activity. Similarly, the PCE- and TCE-dechlorinating bacterium *Dehalobacter restrictus* strain PER-K23 did not grow in batch reactors maintained at 10 °C (Holliger et al. 1993), despite transforming PCE at a high rate of 3.7 µM/h in a continuous flow column operated at the same temperature (De Bruin et al. 1992). Contaminant toxicity has also been shown to vary with system scale and microbial diversity. For example, despite batch study results demonstrating that four pure cultures (e.g., *Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* strain BB1, *Geobacter lovleyi* strain SZ, and *Desulfitobacterium* sp. strain Viet1) were unable to dechlorinate PCE when concentrations exceeded 540 µM (Amos et al. 2007, Amos et al. 2008), microbial reduction dechlorination of PCE near the aqueous solubility ($C_{sat,PCE}$ ≈ 1,200 µM) has been reported in column (Isalou et al. 1998), aquifer cell (Cápiro et al. 2015), and pilot (Adamson et al. 2003) studies. The underlying reasons for these discrepancies are unclear, but geochemical conditions such as dissolved oxygen (Amos et al. 2008, Heavner et al. 2018), temperature (Bradley et al. 2005, Fletcher et al. 2011, Macbeth et al. 2012, Truex et al. 2007), pH (Yang et al. 2017a, Yang et al. 2017b), contaminant concentration gradients (Amos et al. 2008, Behrens et al. 2008, Cope and Hughes 2001, Sleep et al. 2006), or biofilms/microenvironments (Costerton et al. 1995, Lens et al. 1993) can have strain-specific impacts, leading to variability in microbial growth and dechlorination activity.

Several well-studied *Dhc* strains involved in the reductive dechlorination of chlorinated ethenes each possess a single copy of the *Dhc* 16S rRNA gene and a single copy of one of the reductive dehalogenase (RDase) genes implicated in *cis*-DCE or VC reduction: strains 195 and FL2 possess the *tceA* gene (TCE → DCEs → VC), strains VS and GT possess the *vcrA* gene (*cis*-DCE → VC → ethene), and strain BAV1 possesses the *bvcA* gene (DCEs → VC → ethene) (Löffler et al. 2013). The *vcrA* and *bvcA* genes are often monitored in groundwater via quantitative real-time polymerase chain reaction (qPCR) analysis because of their association with ethene formation (Holmes et al. 2006, Lee et al. 2008, Müller et al. 2004, Scheutz et al. 2008). Several studies have demonstrated that *Dhc* strains harboring the *vcrA* and *bvcA* genes appear to have different responses to geochemical conditions (Behrens et al. 2008, Heavner et al. 2018, van der Zaan et al. 2010, Yan et al. 2015, Yang et al. 2017a, Yang et al. 2017b). For example, shifts in the relative abundance of *Dhc* strains occurred during ISTT at a site in Fort Lewis, WA, wherein heating from ambient temperature (~12 °C) to 33 °C caused a relative increase of the *bvcA* gene versus the *vcrA* gene (Macbeth et al. 2012).

During field application of electrical resistance heating (ERH), a widely used ISTT technology, treatment zone temperatures of 80 – 110 °C are commonly targeted to achieve desorption and volatilization of organic contaminants, allowing for gas-phase recovery and subsequent above-ground treatment (Triplett Kingston et al. 2010). Laboratory-scale microcosm studies showed that these conditions lead to viability loss of bacteria capable of MRD (Fletcher et al. 2011, Friis et al. 2007); however, MRD activity could be retained or even increased in peripheral and down-gradient zones where temperatures were lower, but still elevated relative to ambient groundwater. Alternatively, ISTT could be specifically designed to moderately increase

groundwater temperatures with the goal of stimulating MRD. This strategy has substantially lower energy requirements than conventional ISTT and eliminates the need for costly gas-phase extraction and above-ground treatment systems (Beyke and Fleming 2005, Macbeth et al. 2012). Despite being a promising remedial strategy, the potential benefits of coupling low-temperature ISTT (e.g., up to 50 °C) with MRD have received little quantitative attention, particularly with respect to the simultaneous effects of variable temperature and flow through porous media (Macbeth et al. 2012, Truex et al. 2007). Given the potential impacts of system scale and flow conditions on microbial activity, the results of batch studies are not immediately transferrable to in situ conditions. Thus, the objectives of this study were to: a) assess the impacts of simultaneous low-temperature thermal treatment and 1-D flow through a porous medium on the extent of MRD of PCE and its chlorinated daughter products, and b) determine the impacts of increasing temperature on strain-specific growth and activity of *Dhc* in continuous flow, 1-D column systems. Dissolved PCE was continuously introduced to columns bioaugmented with a PCE-to-ethene dechlorinating consortium, and the columns were cooled or heated to represent groundwater temperatures at an unheated site or a site undergoing low-temperature ISTT, respectively. Chlorinated ethenes and ethene concentrations, as well as the abundance and distribution of *Dhc* 16S rRNA and RDase genes, were monitored to evaluate the potential benefits and impacts of low-temperature ISTT on MRD.

2. Materials and Methods

2.1. Materials

PCE (≥99%) and sodium lactate (60% w/w syrup) used in the column experiments were obtained from Sigma-Aldrich (St. Louis, MO). Gas chromatography (GC) standards were prepared with

PCE, TCE (≥99.5%; Sigma-Aldrich), *cis*-DCE (97%; Sigma-Aldrich), VC gas (VC; 99%; SynQuest Laboratories, Alachua, FL), and ethene gas (≥99.5%; Sigma-Aldrich). Ion chromatography (IC) standards were prepared with the sodium lactate syrup and the sodium salts of acetate (≥99%; EMD Millipore, Burlington, MA); butyrate (≥98%; Alfa Aesar, Haverhill, MA); formate (≥99%; Sigma-Aldrich); and propionate (≥99%; Sigma-Aldrich). All other chemicals satisfied the reagent grade purity requirements defined by the American Chemical Society (ACS). Unless specified otherwise, gases used for medium preparation, column operation, and analyses were of ultra-high purity and obtained from Airgas (Radnor, PA). Aqueous solutions were prepared using 18.2 MΩ deionized (DI) water (EMD Millipore).

2.2. Column design and preparation

Low-temperature thermal treatment experiments were performed using borosilicate glass chromatography columns (15 cm *l* × 2.5 cm *ID*) equipped with Teflon endplates (Kimble-Chase, Vineland, NJ). Each column was customized with three glass sampling ports sealed with rubber septa (Restek, Bellefonte, PA). Ports 1 and 3 were located 3.5 cm from the column influent and effluent endplates, respectively, and port 2 was located at the column midpoint (Figure S1). All components of the experimental setup were steam-sterilized for 30 min at 121 °C prior to use. The columns were packed in 1 cm increments with dry Federal Fine Ottawa sand (30 – 140 mesh; U.S. Silica, Frederick, MD), a quartz sand with mean grain size of 0.32 mm, permeability of 4.2×10^{-11} m^2, and total organic carbon content of <0.01 wt% (Suchomel et al. 2007). The packed columns were purged with filter-sterilized carbon dioxide (CO_2) gas and saturated with a sterile synthetic groundwater solution (see below). Non-reactive bromide tracer tests ($[Br^-]$ = 10 mM, flow rate (Q) = 0.02 mL/min) were completed prior to and following each column

experiment; breakthrough curve data were analyzed using the Code for Estimating Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITM) (Van Genuchten 1980) to determine porosity (n) and aqueous pore volume (PV).

2.3. Synthetic groundwater preparation and delivery

Reduced, synthetic groundwater was prepared by making the following modifications to the synthetic basal salt medium used to grow *Dhc* (Löffler et al. 2005): mineral salt and trace element concentrations were reduced by 90%; sodium sulfide nonahydrate concentration was increased from 0.2 to 1 mM; vitamin solution was eliminated; and the pH was adjusted to 7.1 – 7.2. Lactate (5 mM) served as both the electron donor and carbon sources. Synthetic groundwater was prepared in 1,600 mL batches, evenly dispensed into each of three 1,000 mL glass bottles (Chemglass, Vineland, NJ) under an 80 vol% nitrogen/20 vol% CO_2 headspace. Neat PCE (300 µL) was added to one of the three bottles to serve as a PCE-saturated stock solution, then each bottle was sealed with a rubber stopper and sterilized in an autoclave at 121 °C for 30 min. The concentration of the PCE-saturated stock was then measured by GC (see below), and the column influent solution was prepared by drawing appropriate volumes of the PCE-saturated stock and PCE-free synthetic groundwater into a sterile, 100 mL gas-tight syringe (SGE Analytical Science, Victoria, AU). The final PCE concentration of the influent solution was 258±46 µM. Column influent was delivered via a PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA) and syringes were refilled approximately every 3 PVs.

2.4. Column bioaugmentation, operation, and sampling

Two identical columns, hereinafter referred to as Column A_{i15} ($n_{A,initial}$ = 0.40 cm^3/cm^3, $PV_{A,initial}$ = 30.3 mL) and Column B_{i35} ($n_{B,initial}$ = 0.40 cm^3/cm^3, $PV_{B,initial}$ = 30.3 mL), were operated

similarly and in parallel, except with respect to temperature. A third column experiment, Column $C_{control}$ ($n_{C,initial}$ = 0.41 cm^3/cm^3, $PV_{C,initial}$ = 31.4 mL), was performed to verify that no abiotic contaminant degradation occurred in the absence of the KB-1® consortium (SiREM, Ontario, CA). Column $C_{control}$ methods and results are described in the Supplementary Materials (Appendix A). A recirculating water bath (Thermo Fisher, Waltham, MA) was used to cool Column A_{i15} and associated influent tubing (1.6 mm *OD*; Kimble-Chase) to an initial temperature of 15 °C, while a second recirculating water bath (VWR, Radnor, PA) was used to heat Column B_{i35} and associated influent tubing to an initial temperature of 35 °C. Columns were thoroughly wrapped with polyethylene pipe insulation (2.5 cm wall thickness; Grainger, Lake Forest, IL) to ensure that the target temperatures were not impacted by ambient laboratory conditions. A Type K thermocouple (VWR) was used approximately every 3 PVs to independently verify column temperatures and accuracy of the water bath digital temperature controllers. Synthetic groundwater was introduced to columns A_{i15} and B_{i35} at an initial pore water velocity of 15 cm/d (Q = 0.020 mL/min or 1 PV/d) for 17 PVs to establish reduced conditions and stable concentrations of dissolved PCE and lactate. Flow was then paused and 6 mL of the KB-1® consortium was injected via each column influent, sampling ports 1 – 3, and effluent, for a total of 30 mL per column. KB-1® is a widely used, methanogenic, PCE-to-ethene dechlorinating consortium predominantly comprised of *Acetobacterium* sp., *Geobacter* sp., and multiple *Dhc* strains harboring the *vcrA*, *bvcA*, and *tceA* RDase genes (Duhamel and Edwards 2006, Liang et al. 2015, Roberts 2017). Column flow was resumed after a 48-hour flow interruption that was designed to facilitate cellular attachment. For simplicity in all subsequent text and figures, this resumption of flow was considered PV = 0.

The experiment was completed in four phases, each involving one or both columns inoculated with the KB-1® consortium: in Phase I, columns A_{i15} and B_{i35} were held at their initial temperatures (15 and 35 °C, respectively) to allow for direct comparison of dechlorination activity at a typical ambient groundwater temperature versus an elevated temperature that might be targeted during low-temperature ISTT; in Phase II, Column A_{i15} was heated from 15 to 35 °C (+2 °C/PV) to assess dechlorination activity in a system initially at ambient groundwater temperature that later underwent low-temperature ISTT; in Phase III, Column B_{i35} was heated from 35 °C (+0.5 °C/PV) to determine the maximum temperature permissive of *Dhc* activity (i.e., *cis*-DCE and VC reduction) in a continuous flow system; in Phase IV, heating of Column B_{i35} was continued (+0.5 – 1 °C/PV) to determine the maximum temperature permissive of overall MRD activity (i.e., PCE and TCE reduction by bacteria other than *Dhc*). Aqueous effluent samples (5 mL) were collected from a stainless-steel sampling reservoir every 2 – 3 PVs using a 5 mL gas-tight syringe (Agilent, Santa Clara, CA). Triplicate 1 mL aqueous samples were immediately added to 20 mL headspace vials (Agilent) containing ambient air, thus preventing further microbial reductive dechlorination prior to the analysis of chlorinated ethenes and ethene. Effluent pH and oxidation-reduction potential (ORP) were measured in the remaining 2 mL sample volume, which was then transferred to a polypropylene microcentrifuge tube (VWR) and stored in a -20 °C freezer pending organic acid analysis. Influent and effluent system components (e.g., syringes, tubing, valves, sampling reservoirs) were periodically flushed with isopropanol (70 vol%) to prevent microbial growth outside of the temperature-controlled, sand-packed columns. Port samples (1 mL) were collected using a sterile syringe (BD, Franklin Lakes, NJ) at least once during each phase to determine aqueous abundances of the *Dhc* 16S rRNA, *tceA*, *bvcA*, and *vcrA* genes. Additional port samples were collected periodically to

231 determine the spatial distribution of PCE and its daughter products, and to confirm that reductive
232 dechlorination occurred within the columns rather than in the effluent sampling reservoirs.
233 Columns were destructively sampled (Figure S1) after the experiment to determine solid-phase
234 abundances of the *Dhc* 16S rRNA and RDase genes using qPCR (see Appendix A.
235 Supplementary Material).
236

237 **2.5. Analytical methods**

238 Concentrations of chlorinated ethenes and ethene in aqueous samples were measured using an
239 Agilent 7890B GC connected to a Teledyne Tekmar HT3 Headspace Analyzer (Teledyne
240 Technologies, Thousand Oaks, CA). The GC was equipped with an Agilent DB-624 capillary
241 column (60 m *length* × 320 µm *outside diameter*) and a flame ionization detector (FID). PCE,
242 TCE, and *cis*-DCE calibration standards were prepared by diluting a concentrated stock solution
243 (5,000 – 10,000 µg/mL) of each compound dissolved in methanol. VC and ethene calibration
244 standards were prepared by injecting a known volume of each gas into a sealed 160 mL culture
245 bottle containing 100 mL of 18.2 MΩ water and allowing them to equilibrate overnight. Details
246 of the GC-FID analytical method are provided in the Supplementary Material. The method
247 detection limits for each chlorinated ethene and ethene were 1 µM or lower and were determined
248 following the method described by Hubaux and Vos (1970).
249

250 Lactate, formate, acetate, propionate, and butyrate were measured using a Thermo Fisher ICS-
251 2100 ion chromatograph equipped with an IonPac AS11-HC analytical column (250 mm *l* × 4
252 mm *ID*). Analyte separation was achieved using a variable concentration potassium hydroxide
253 eluent generator and constant flow rate of 1.5 mL/min. The method detection limit for each

organic acid was determined according to the method described by Hubaux and Vos (1970), and ranged from 0.8 µM for propionate to 4.2 µM for formate.

Dhc 16S rRNA gene and RDase gene abundances were determined by qPCR analysis using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Analyses targeting the *Dhc* 16S rRNA, *tceA*, *bvcA*, and *vcrA* genes were completed in triplicate according to an established TaqMan-based qPCR protocol (Ritalahti et al. 2006). Primers and probes used in qPCR analyses were obtained from IDT Technologies (Coralville, IA) or Thermo Fisher. TaqMan Universal PCR Master Mix was obtained from Applied Biosystems. Standards were prepared by using a Qubit 2.0 Fluorometer (Thermo Fisher) to determine the concentration of plasmid DNA containing a single copy of each target gene, then diluting the plasmid DNA to 1 ng/µL and performing 10:1 serial dilutions in molecular biology-grade water (Thermo Fisher).

3. Results and Discussion

3.1. Inoculum characterization and column bioaugmentation

The KB-1® consortium used to bioaugment columns A_{i15} and B_{i35} contained $1.4\pm0.2 \times 10^7$ *Dhc* 16S rRNA gene copies/mL, $1.4\pm0.0 \times 10^7$ *vcrA* gene copies/mL, and $4.5\pm1.1 \times 10^4$ *bvcA* gene copies/mL. The culture also contained 77±1 µM ethene, but no chlorinated ethenes. Accounting for cells immediately washed out during bioaugmentation, the initial aqueous *Dhc* 16S rRNA gene abundance was $9.7\pm2.2 \times 10^6$ gene copies/mL in Column A_{i15} and $1.2\pm0.2 \times 10^7$ gene copies/mL in Column B_{i35}. More than 99% of *Dhc* cells retained in each column immediately following bioaugmentation possessed the *vcrA* gene, while only 0.3% possessed the *bvcA* gene. The *tceA* gene was not detected in the inoculum nor in any of the samples collected throughout

277 the experiment, consistent with previous studies demonstrating low relative abundance of *tceA*
278 compared to the *vcrA* and *bvcA* genes in the KB-1® consortium (Liang et al. 2015).
279
280 Influent and effluent pH values fell within the range of 7.0 – 7.2 for the experiment duration, and
281 measured ORP values were consistently less than -100 mV, regardless of changes in *Dhc*
282 abundance or system temperature. Within 17 PVs post-bioaugmentation, effluent lactate
283 concentrations decreased from 5.2 to 1.8 mM and 5.3 to 0.2 mM in columns A_{i15} and B_{i35},
284 respectively. However, lactate fermentation products, acetate (0.2 – 3.8 mM) and propionate (2.7
285 – 4.9 mM), were consistently detected, indicating that availability of carbon and fermentable
286 organic substrates were not limiting MRD during the experiment (Table S1).
287
288 **3.2. Effects of heating on dechlorination**
289 Phase I of the experiment assessed dechlorination activity (PCE_{IN} = 258±46 µM) at a typical
290 ambient groundwater temperature (15 °C, Column A_{i15}) versus a moderately elevated
291 temperature (35 °C, Column B_{i35}). Within the first 20 PVs following bioaugmentation, PCE and
292 TCE in the effluent of Column A_{i15} was transformed primarily to *cis*-DCE (127±0 µM), with
293 lower levels of VC (41±1 µM) and ethene (14±0 µM) (Figure 1a, 0 – 20 PVs). In contrast, PCE
294 and TCE were not detected in the effluent of Column B_{i35} post-bioaugmentation, and *cis*-DCE
295 (132±13 µM) initially detected in the Column B_{i35} effluent was rapidly degraded to VC (163±3
296 µM) and ethene (63±0 µM) within the same period (Figure 2a, 0 – 20 PVs). These results
297 indicate that a 20 °C temperature difference substantially impacts the extent of dechlorination
298 between otherwise equivalent continuous flow systems with comparable initial abundances of
299 *Dhc* cells.

300

301 During Phase II, the *cis*-DCE concentration in the Column A_{i15} effluent decreased from 142±2 to
302 86±1 µM as the system was heated from 15 to 35 °C (Figure 1a, 52 – 61 PVs), then fell below
303 the 1 µM detection limit over the next 17 PVs at 35 °C (Figure 1a, 61 – 78 PVs). Effluent VC
304 concentration rose from 31±0 to 74±1 µM over the duration of the temperature increase (Figure
305 1a, 52 – 61 PVs). The observed shift in dechlorination products was likely due to the increased
306 activity of existing *Dhc* in response to the temperature increase, as evidenced by the rapid
307 decline in *cis*-DCE despite consistent aqueous *Dhc* 16S rRNA gene abundances prior to (2.4±0.4
308 $\times 10^5$ – 1.0±0.1 $\times 10^6$ gene copies/mL; Figure 1c) and immediately following (1.3±0.0 $\times 10^5$ –
309 9.6±0.4 $\times 10^5$ gene copies/mL; Figure 1d) the temperature increase. However, the elevated
310 temperature also prompted the growth of new *Dhc* cells (Figures 1b-e), contributing to the
311 continued decrease in *cis*-DCE concentrations after the 35 °C target was reached. The same 15-
312 to-35 °C temperature increase that prompted rapid *cis*-DCE degradation had no immediately
313 apparent impact on VC-to-ethene dechlorination, potentially due to inhibition of *Dhc* by the *cis*-
314 DCE itself. Despite being reported in the literature (Amos et al. 2009, Azizian et al. 2008,
315 Sabalowsky and Semprini 2010, Yu et al. 2005, Yu and Semprini 2004), the mechanisms
316 governing the inhibitory effects of *cis*-DCE on *Dhc* dechlorination activity are poorly
317 understood; however, laboratory- and modeling-based kinetic studies indicate that major factors
318 may include acute toxic effects (i.e., increased cellular decay, decreased growth rates) and
319 preferential reduction of more highly chlorinated compounds (i.e., competitive inhibition)
320 (Adamson et al. 2004, Chu 2004, Cupples et al. 2004, Lee et al. 2004, Sabalowsky and Semprini
321 2010, Yu et al. 2005, Yu and Semprini 2004). After Column A_{i15} had been maintained at 35 °C
322 for approximately 60 PVs, the effluent ethene concentration began to rise (Figure 1a, 121 PVs)

and surpassed VC as the primary (>50 mol%) dechlorination product after 74 PVs at 35 °C (Figure 1a, 135 PVs). This phenomenon of rapid *cis*-DCE degradation with delayed ethene formation also occurred in Column B_{i35}, with effluent ethene concentration surpassing VC concentration only after Column B_{i35} had been at 35 °C for 73 PVs (Figure 2a, 73 PVs). The consistent duration of the lag period between complete *cis*-DCE reduction and rapid ethene formation suggests that high *cis*-DCE concentrations limited *Dhc* activity as observed previously, or that the abundance of the *Dhc* strain(s) primarily responsible for VC reduction at 35 °C was initially low in the KB-1® consortium used for bioaugmentation; this latter point is discussed in more detail in section 3.4. Furthermore, the similar duration of the lag period (74 and 73 PVs in Column A_{i15} and Column B_{i35}, respectively) demonstrates that the order of remedy application (i.e., heating following bioaugmentation versus bioaugmentation following heating) did not substantially impact the ultimate extent of dechlorination.

Concentrations of VC in both columns continued to decline during the periods of rapid ethene formation, but these reductions in VC mass accounted for only 18.2 and 18.4% of the increased ethene mass in Column A_{i15} (Figure 1a, 121 – 165 PVs) and Column B_{i35} (Figure 2a, 50 – 94 PVs), respectively. These mass balance discrepancies were attributed to equilibration between the aqueous phase and a visible gas phase that slowly developed in each column during their respective 35 °C phases. The discontinuity of each gas phase precluded direct sampling, but analyses of aqueous port samples collected from Column A_{i15} revealed that ethene accumulated in the column at 35 °C (Figure 3b), temporarily delaying ethene elution and inflating subsequent effluent concentrations. This hypothesis was supported by results of post-experiment tracer tests, which revealed markedly lower mobile porosities ($n_{A,final}$ = 0.33 cm^3/cm^3, $n_{B,final}$ = 0.30 cm^3/cm^3)

346 compared to initial column conditions ($n_{A,initial}$ = 0.40 cm^3/cm^3, $n_{B,initial}$ = 0.40 cm^3/cm^3),
347 consistent with formation of the visible gas phases within each column during prolonged heating
348 at 35 °C.
349
350 **3.3. Maximum temperatures permissive of dechlorination activity**
351 In order to assess whether the optimal (25 – 30 °C) (Löffler et al. 2013) and maximum
352 temperature (35 – 40 °C) (Fletcher et al. 2011) ranges determined for *Dhc* in batch reactors are
353 applicable to a continuous flow system, Column B_{i35} was gradually heated (Phase III). During
354 this period of gradual heating, starting at 35 °C, the effluent concentration of VC continued to
355 decrease from 115±3 to 61±1 µM (Figure 2a, 95 – 114 PVs); ethene concentration remained high
356 (>250 µM) and *cis*-DCE remained below detection, indicating continued *Dhc* activity. Heating
357 was paused at 40 °C when low concentrations (2 – 3 µM) of *cis*-DCE were again detected in
358 effluent samples (Figure 2a, 103 – 108 PVs), but resumed upon confirmation that VC (68±2 µM)
359 and ethene (285±5 µM) concentrations remained steady. However, once the temperature of
360 Column B_{i35} reached 43 °C, effluent VC and ethene concentrations declined sharply and *cis*-
361 DCE concentrations rebounded to 100±1 µM within 14 PVs (Figure 2a, 113 – 127 PVs),
362 suggesting that a maximum temperature permissive of *Dhc* activity had been reached.
363
364 Phase IV, like Phase III, was intended to determine a maximum temperature permissive of
365 dechlorination activity in a continuous flow system, but with focus on PCE and TCE
366 dechlorination (i.e., dechlorination by non-*Dhc* bacteria in KB-1®). Heating of Column B_{i35} was
367 continued from 43 °C until reaching a final temperature of 74 °C (Figure 2a, 134 – 169 PVs).
368 These high temperatures led to increased gas formation and loss of contaminant mass that did not

occur at lower temperatures (15 – 43 °C), limiting further quantitative assessment of PCE and its dechlorination daughter products. Losses were confirmed by results of aqueous port sample analyses of Column B_{i35} (Figure S2b) and Column $C_{control}$ (Figure S3), which demonstrated a decline in total concentrations of chlorinated ethenes and ethene in the direction of flow, consistent with volatile losses through the pierced septa. The result was an overall mass balance of 81% for Column B_{i35}, compared to 95% for Column A_{i15}. Despite these losses due to volatilization, low concentrations of *cis*-DCE persisted in the Column B_{i35} effluent, indicating that PCE-to-*cis*-DCE dechlorinating microbes remained active at temperatures exceeding 43 °C; however, lack of VC in effluent samples suggested that continued low concentrations of ethene were likely not indicative of continued *Dhc* activity, but instead resulted from the aqueous-gas equilibration discussed in section 3.2. These observations are consistent with the results of qPCR analysis of aqueous port samples, which demonstrated a steep decline of *Dhc* 16S rRNA gene and RDase gene abundances in Column B_{i35} following heating from 43 °C (Figure 2f) to 74 °C (Figure 2g). This decline is illustrated in port 3 samples, wherein the *Dhc* 16S rRNA gene abundance decreased from $5.5\pm2.1 \times 10^7$ to $3.7\pm0.3 \times 10^5$ gene copies/mL between 126 and 176 PVs (Figures 2f, g). These results demonstrate that *Dhc* retains metabolic activity at higher temperatures (35 – 43 °C) in continuous flow systems than previously reported for batch systems (Fletcher et al. 2011, Löffler et al. 2013). Caution is warranted, however, as even slight (i.e., 1 – 3 °C) exceedance of the operational temperature range may cause rapid cessation of *Dhc* activity.

3.4. Impacts of temperature on *Dehalococcoides* strains

Results of the qPCR analyses performed on aqueous port samples (Figures 1b-g, 2b-g) were consistent with dechlorination activity observed in columns A_{i15} and B_{i35}. For example, aqueous samples collected from port 3 (nearest to effluent) of the cooled Column A_{i15} at 18 PVs post-

bioaugmentation contained 2.5±0.3 × 10^5 *Dhc* 16S rRNA gene copies/mL (Figure 1b), compared to 7.1±0.9 × 10^6 gene copies/mL (Figure 2b) in corresponding samples collected from the heated Column B_{i35}. Furthermore, although *Dhc* 16S rRNA gene abundance continued to increase in both columns from 18 – 47 PVs, the average abundance over ports 1 – 3 increased only 3-fold in Column A_{i15} (Figures 1b, c) compared to a 12-fold increase in Column B_{i35} (Figure 2b, c). Following heating of Column A_{i15} from 15 to 35 °C (Figure 1a, 52 – 61 PVs), *Dhc* 16S rRNA gene abundance increased further, reaching a maximum of 5.0±0.2 × 10^7 gene copies/mL by the end of the experiment. This *Dhc* growth represents a 230-fold overall increase (Figures 1b, g) and demonstrates the relationship between *Dhc* 16S rRNA gene abundance and system temperature.

The column data also indicate that individual *Dhc* strains within the same culture (i.e., consortium KB-1®) respond differently to temperature changes. As noted previously, *Dhc* strains possessing the *vcrA* gene accounted for more than 99% of *Dhc* in the inoculum. This predominance of *vcrA* over *bvcA* was maintained throughout the entire 15 °C phase of Column A_{i15} and the beginning of the 35 °C phase (Figures 1b-d). However, *vcrA* gene abundance remained static or declined during heating from 15 °C (9.2±3.1 × 10^4 – 1.1±0.5 × 10^6 copies/mL) to 35 °C (1.1±0.0 × 10^5 – 4.2±0.2 × 10^5 copies/mL), despite increases in *Dhc* 16S rRNA gene abundance at every sampling port between 47 and 92 PVs (Figures 1c, e). Abundance of the *bvcA* gene increased from 2.8±0.9 × 10^3 – 1.3±0.2 × 10^4 copies/mL to 2.6±0.4 × 10^5 – 4.2±0.9 × 10^5 copies/mL during this period, indicating that growth of a *Dhc* strain carrying *bvcA* was positively impacted by the same temperature increase that limited growth of *Dhc* strains harboring the *vcrA* gene. Interestingly, effluent ethene concentration in Column A_{i15} exceeded

VC concentration (Figure 1a, 121 PVs) only after *bvcA* gene abundance surpassed that of the *vcrA* gene (Figure 1f). Over the remainder of the experiment, *vcrA* gene abundance in Column A_{i15} slowly began to increase, but still lagged behind *bvcA* gene abundance by an order of magnitude or greater (Figures 1f, g). These strain-specific responses were also observed in Column B_{i35}; ethene became the primary (>50 mol%) degradation product (Figure 2b, 73 PVs) after *bvcA* gene abundance exceeded *vcrA* gene abundance (Figures 2c, d), and *bvcA* gene abundance (averaged over ports 1 – 3) increased 3-fold during the 35-to-43 °C temperature increase (Figure 2a, 95 – 113 PVs) while average *vcrA* abundance remained unchanged (Figures 2e, f). This apparent correlation between increased *bvcA* gene abundance and ethene production at elevated temperatures is consistent with data obtained at the field scale (Macbeth et al. 2012). These findings support the mounting volume of evidence demonstrating that, despite coding for enzymes with seemingly identical function (i.e., *cis*-DCE and VC reduction), *Dhc* strains carrying *vcrA* versus *bvcA* genes are not equivalent in terms of in situ dechlorination performance under different environmental conditions (Behrens et al. 2008, Heavner et al. 2018, van der Zaan et al. 2010, Yan et al. 2015, Yang et al. 2017a, Yang et al. 2017b). The recognition that functionally equivalent (i.e., *cis*-DCE-to-ethene-dechlorinating) *Dhc* strains respond differently to environmental parameters is relevant, and suggests that further optimization of bioaugmentation consortia for site-specific treatment is possible.

Destructive sampling and subsequent qPCR analysis revealed that 95 – 98% of *Dhc* 16S rRNA gene copies in Column A_{i15} (35 °C at experiment conclusion) were associated with the solid phase (Figure 4a). This finding is generally consistent with results of a previous study quantifying solid-phase attachment rates in a room temperature (~21 °C) Federal Fine Ottawa

sand column, wherein 73 – 89% of *Dhc* cells were attached to the solid phase at the conclusion of the experiment (Cápiro et al. 2014). In the same study, a positive relationship was identified between extent of cellular attachment and dechlorination activity. Greater extents of cellular attachment (95 – 98%) and dechlorination in Column A_{i15} compared to the room temperature study support this relationship, and allude to a potentially complex link between system temperature, cellular attachment, and dechlorination activity. Furthermore, results of the Column A_{i15} qPCR analysis suggest that extent of *Dhc* attachment at 35 °C may be strain-specific. *Dhc* strains possessing the *bvcA* gene (averaged over ports 1 – 3) were less likely to attach to the solid phase (87±18% attachment) than strains possessing the *vcrA* gene (94±9% attachment). Given the importance of *Dhc* 16S rRNA and RDase genes in assessing likely indicators of ethene formation (e.g., aqueous *Dhc* 16S rRNA abundance >0.05% of total bacteria, $>10^3$ gene copies/mL, ≈ *vcrA* + *bvcA* abundance), further study is warranted to ensure the continued, accurate interpretation of these biomarkers. If elevated temperatures result in greater *Dhc* attachment and activity, for instance, then ethene formation may remain likely despite a lower abundance of *Dhc* cells in the aqueous phase.

Dhc 16S rRNA and RDase gene abundances were low in solid-phase samples collected 3 – 15 cm from the influent of Column B_{i35} (Figure 4b), which was held at 74 °C during the final stage of the experiment. The maximum total *Dhc* abundance was only $1.1±0.2 \times 10^6$ gene copies/g and RDase gene abundances were near or below the 1.0×10^4 gene copies/g detection limit for solid-phase samples. Solid-phase abundances of *Dhc* 16S rRNA genes ($1.2±0.3 \times 10^7$ gene copies/g), *vcrA* ($2.9±0.9 \times 10^5$ gene copies/g), and *bvcA* ($2.7±0.6 \times 10^5$ gene copies/g) at the Column B_{i35} inlet remained elevated. However, the lack of VC and ethene in port samples collected at the end

462 of the experiment (Figure S2b) indicated that these remaining cells were inactive and likely
463 trapped within immobile pore spaces that developed during formation of the gas phase.
464
465 **4. Conclusions**
466 For more than a decade, the coupling of ISTT with MRD has been proposed as an attractive
467 remedial alternative that retains beneficial aspects of each standalone technology, while
468 minimizing disadvantageous aspects such as high energy demand and prolonged cleanup time.
469 However, progression to rigorous field-scale application has been hindered by scarce and
470 sometimes conflicting reports, particularly with respect to the optimal and maximum
471 temperatures at which *Dhc* transforms *cis*-DCE to VC and ethene. Native microbes will respond
472 to physical-chemical in situ remediation approaches, and the responses of resident microbiology
473 must be understood to take full advantage of potential synergistic effects. The findings presented
474 here provide valuable insight to assist researchers and practitioners in the successful integration
475 of in situ thermal and microbial treatments under continuous flow conditions; most notably:
476 • The demonstration of sustained *Dhc* dechlorination activity at temperatures previously
477 considered to be inhibitory (>35 °C) emphasizes the critical importance of physical scale
478 and complexity (resembling that of the field with groundwater flow and porous medium)
479 of experimental systems (i.e., batch reactors versus continuous flow columns) when
480 assessing microbial tolerance thresholds and potential.
481 • The demonstration of rapid and substantial impact of increasing system temperature on
482 the extent of *cis*-DCE-to-VC dechlorination will provide remediation professionals an
483 additional tool to combat the *cis*-DCE stall that frequently occurs at contaminated sites
484 undergoing MRD.

- The preliminary results demonstrating that dechlorination extent was independent of the order of remedial application may provide practitioners flexibility to modify existing or underperforming standalone thermal or biological treatment systems.
- The evidence that *bvcA*-harboring *Dhc* strains can supersede *vcrA*-harboring strains as the key contributors to ethene production at elevated temperatures will allow for more effective monitoring of MRD performance in systems operating at elevated temperatures.
- The identification of system temperature control as a means to induce *Dhc* strain-specific responses provides opportunities to improve bioaugmentation cultures for site-specific applications.

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Appendix A. Supplementary materials

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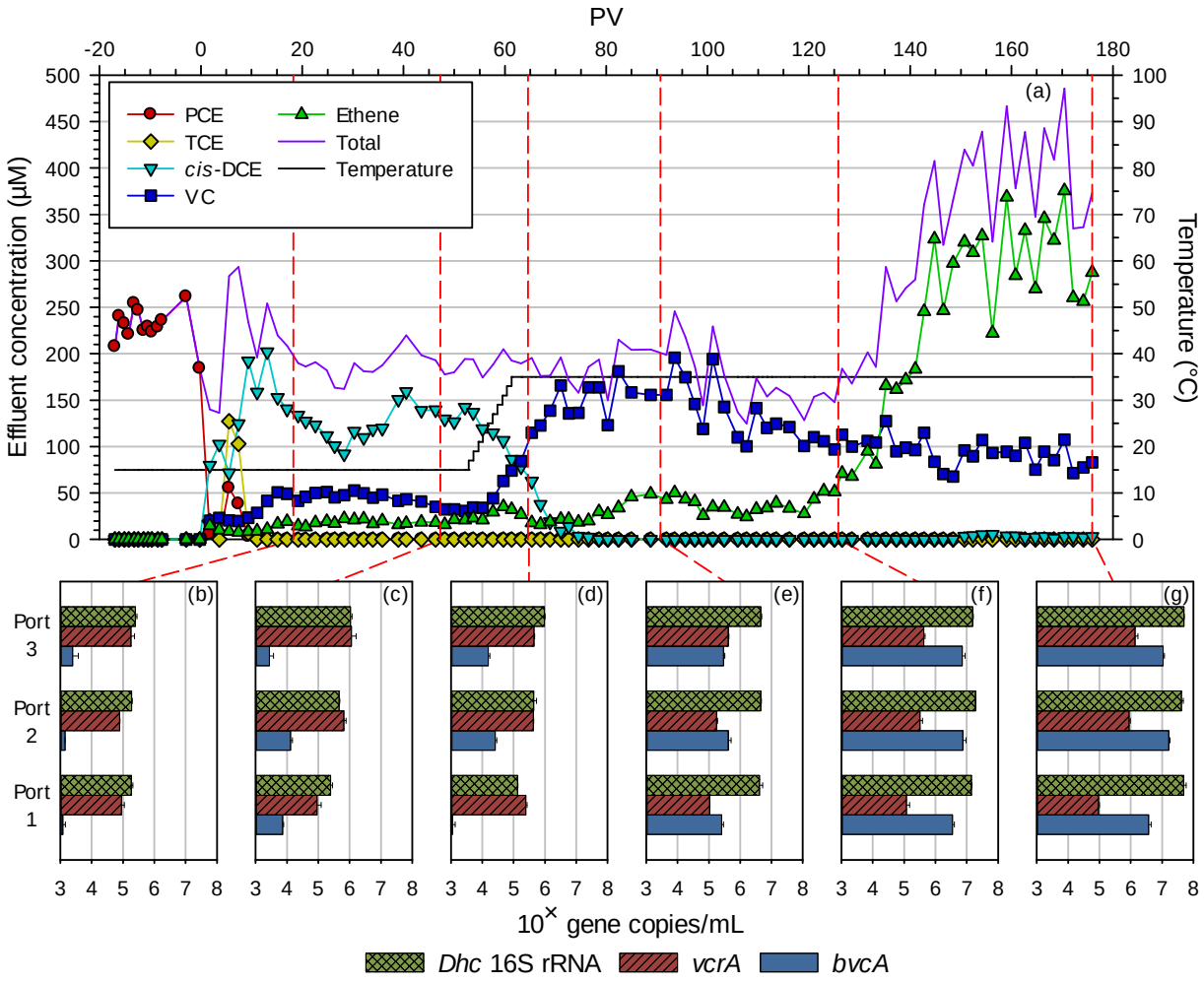

Figure 1. Column A_{i15} effluent concentrations of PCE and daughter products (a). "Total" line represents sum of PCE and daughter product concentrations. Bioaugmentation occurred at PV = 0. Dashed vertical lines link *Dhc* 16S rRNA gene and RDase gene abundances (b – g) in aqueous port samples to corresponding column PV. Error bars represent one standard deviation.

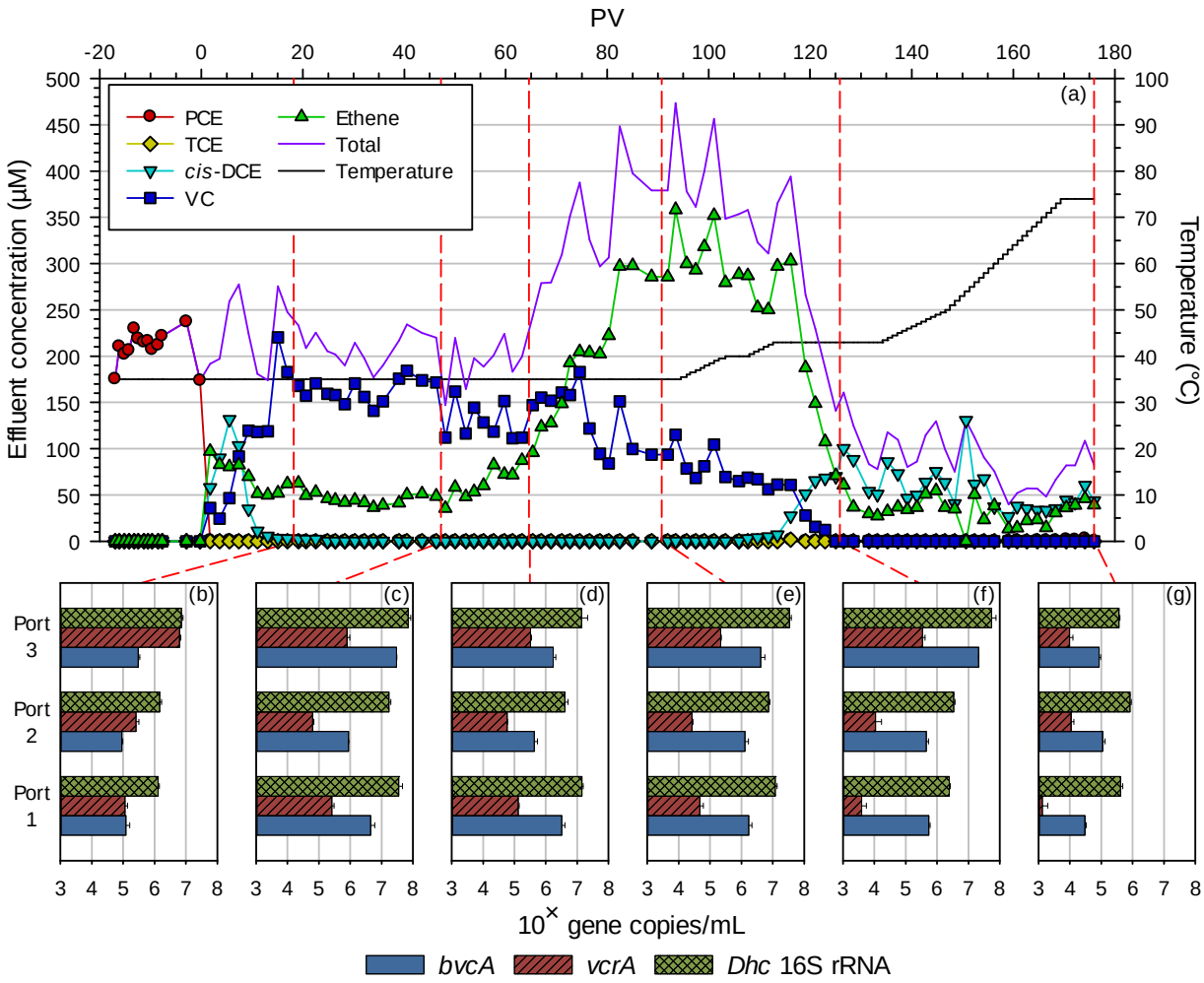

Figure 2. Column B_{i35} effluent concentrations of PCE and daughter products (a). "Total" line represents sum of PCE and daughter product concentrations. Bioaugmentation occurred at PV = 0. Dashed vertical lines link *Dhc* 16S rRNA gene and RDase gene abundances (b – g) in aqueous port samples to corresponding column PV. Error bars represent one standard deviation.

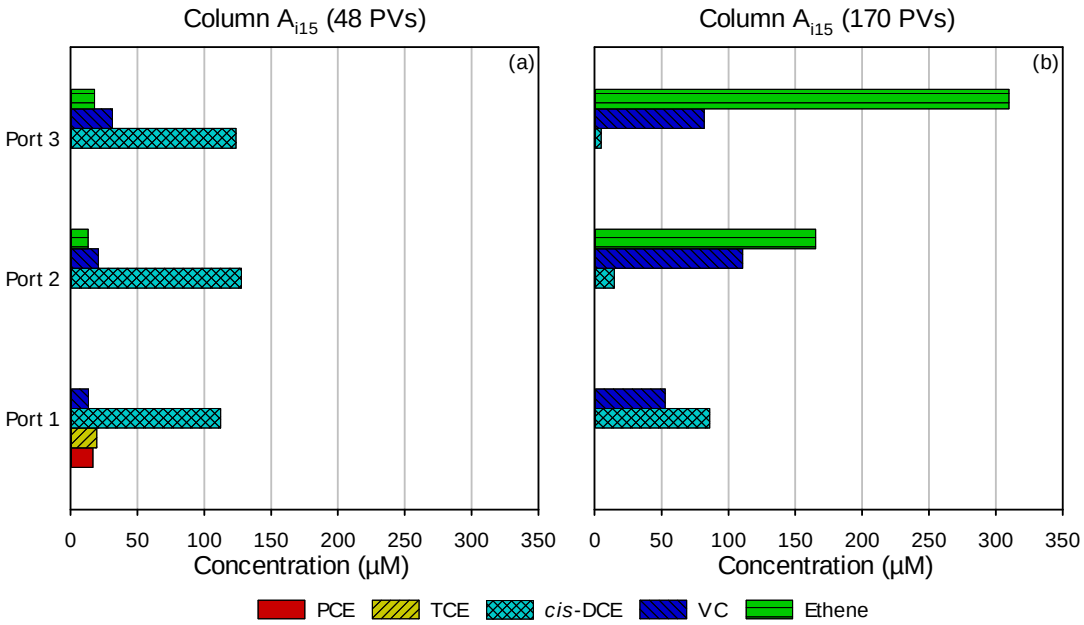

Figure 3. Concentrations of PCE and its daughter products in Column A_{i15} aqueous port samples collected at 15 °C prior to temperature increase (a) and following temperature increase to 35 °C, during rapid ethene formation (b).

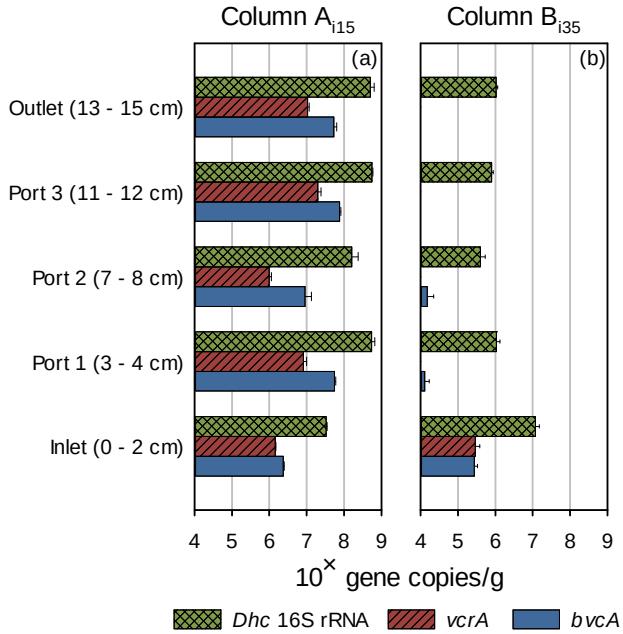

Figure 4. Solid-phase *Dhc* 16S rRNA gene and RDase gene abundances following destructive sampling of Column A_{i15} (a) and Column B_{i35} (b). Error bars represent one standard deviation.

Impacts of Low-Temperature Thermal Treatment on Microbial Detoxification of Tetrachloroethene under Continuous Flow Conditions

Highlights:

- Microbial reductive dechlorination of PCE was enhanced at temperatures up to 43°C.
- Impacts of heating on *Dhc* dechlorination activity were strain-specific.
- *Dhc* tolerates higher temperatures in flowing systems compared to batch reactors.
- Ethene formation was not sensitive to the order of thermal-bio remedy application.