



Chlorinated Electron Acceptor Abundance Drives Selection of *Dehalococcoides mccartyi* (*D. mccartyi*) Strains in Dechlorinating Enrichment Cultures and Groundwater Environments

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Dehalococcoides mccartyi (*D. mccartyi*) strains differ primarily from one another by the number and identity of the reductive dehalogenase homologous catalytic subunit A (*rdhA*) genes within their respective genomes. While multiple *rdhA* genes have been sequenced, the activity of the corresponding proteins has been identified in only a few cases. Examples include the enzymes whose substrates are groundwater contaminants such as trichloroethene (TCE), *cis*-dichloroethene (cDCE) and vinyl chloride (VC). The associated *rdhA* genes, namely *tceA*, *bvcA*, and *vcrA*, along with the *D. mccartyi* 16S rRNA gene are often used as biomarkers of growth in field samples. In this study, we monitored an additional 12 uncharacterized *rdhA* sequences identified in the metagenome in the mixed *D. mccartyi*-containing culture KB-1 to monitor population shifts in more detail. Quantitative PCR (qPCR) assays were developed for 15 *D. mccartyi* *rdhA* genes and used to measure population diversity in 11 different sub-cultures of KB-1, each enriched on different chlorinated ethenes and ethanes. The proportion of *rdhA* gene copies relative to *D. mccartyi* 16S rRNA gene copies revealed the presence of multiple distinct *D. mccartyi* strains in each culture, many more than the two strains inferred from 16S rRNA analysis. The specific electron acceptor amended to each culture had a major influence on the distribution of *D. mccartyi* strains and their associated *rdhA* genes. We also surveyed the abundance of *rdhA* genes in samples from two bioaugmented field sites (Canada and United Kingdom). Growth of the dominant *D. mccartyi* strain in KB-1 was detected at the United Kingdom site. At both field sites, the measurement of relative *rdhA* abundances revealed *D. mccartyi* population shifts over time as dechlorination progressed from TCE through cDCE to VC and ethene. These shifts indicate a selective pressure of the most abundant chlorinated electron acceptor, as was also observed in lab cultures. These results also suggest that reductive

dechlorination at contaminated sites is brought about by multiple strains of *D. mccartyi* whether or not the site is bioaugmented. Understanding the driving forces behind *D. mccartyi* population selection and activity is improving predictability of remediation performance at chlorinated solvent contaminated sites.

Keywords: bioaugmentation, *rdhA* gene, *Dehalococcoides*, real-time PCR, chlorinated ethenes (CE), groundwater bioremediation, bioestimulation, reductive dechlorination

INTRODUCTION

There are thousands of public and private sites with chlorinated solvent groundwater contamination problems (McCarty, 2010). Chlorinated volatile organic compounds (cVOCs) such as tetrachloroethene (PCE) and trichloroethene (TCE) as well as their daughter products including the isomers of dichloroethene (DCE) and vinyl chloride (VC) are highly toxic compounds and TCE and VC are recognized human carcinogens by the National Toxicology Program. Clean-up of groundwater contaminated with these compounds takes time and is costly. Biostimulation (electron donor addition) and bioaugmentation (addition of inoculum) have gained significant acceptance as viable approaches for treatment of chlorinated ethenes in the subsurface (Stroo et al., 2010). The primary biotransformation mechanism for chlorinated ethenes in groundwater is reductive dechlorination under anaerobic conditions, which involves a stepwise replacement of Cl atoms with H atoms following the sequence: PCE, TCE, DCE (mainly cDCE), VC and finally non-toxic ethene (Maymó-Gatell et al., 2001; Duhamel et al., 2002, 2004; Cupples et al., 2003).

Diverse anaerobic microorganisms (e.g., *Dehalococcoides*, *Desulfotobacterium*, *Dehalobacter*, *Sulfurospirillum*, *Desulfuromonas*, *Geobacter*, *Dehalogenimonas*) can couple reductive dechlorination of chlorinated ethenes with growth in a process called organohalide respiration (Gerritse et al., 1996; Krumholz, 1997; Holliger et al., 1998; Luijten et al., 2003; Sung et al., 2006a; Moe et al., 2009). Nonetheless, dechlorination beyond DCE and VC has only been shown so far for members of the *Dehalococcoidales*, namely *Dehalococcoides* and *Dehalogenimonas* (Maymó-Gatell et al., 1997; Cupples et al., 2003; He et al., 2003; Duhamel et al., 2004; Sung et al., 2006b; Manchester et al., 2012; Yang et al., 2017). In practice, owing to subsurface heterogeneity, natural reductive dechlorination is incomplete in some locations, resulting in the accumulation of the daughter products cDCE and the carcinogen VC (Henry, 2010). This is generally attributed to poor mixing, lack of appropriate *Dehalococcoides mccartyi* organisms or electron donor, or inhibition of terminal dechlorination steps (Stroo et al., 2010).

Biostimulation and bioaugmentation with mixed cultures containing *D. mccartyi* can overcome stalling at cDCE or VC and reduce the time to clean up (Ellis et al., 2000; Major et al., 2002; Lendvay et al., 2003; Hood et al., 2008; Stroo et al., 2010; Dugat-Bony et al., 2012; Pérez-de-Mora et al., 2014; Kocur et al., 2016). The abundance of *D. mccartyi* in groundwater is most often assessed via quantitative PCR (qPCR) of the 16S rRNA gene (Rahm et al., 2006a; Lee et al., 2008; Hatt and Löffler,

2012; Hatt et al., 2013). While the abundance of *D. mccartyi* is general strongly correlated with dechlorination, sometimes dechlorination is still incomplete even at high abundance. The dechlorinating abilities of *D. mccartyi* strains is determined by the its complement of reductive dehalogenase genes and their activity. Thus, *D. mccartyi* strains with identical 16S rRNA may differ in the chlorinated compounds that they can respire and dehalogenate. Reductive dehalogenase enzymes (RDases) catalyze the cleavage of the carbon-halogen bond, and thus are an additional biomarker for tracking *D. mccartyi* strains. RDases are heterodimeric, membrane-bound enzymes, consisting of a catalytic active A unit of about 500 amino acids (aa) anchored outside of the cytoplasmic membrane by a small (100 aa) predicted integral membrane B subunit. These subunits are encoded by the so-called *rdhA* and *rdhB* genes, respectively (Smidt and de Vos, 2004). Due to their hydrophobic nature, oxygen sensitivity and complex association, only a few RDases have been biochemically characterized to date. Among these are the enzymes catalyzing the conversion of PCE to cDCE (coded by the *pceA* gene) and TCE to VC (coded by the *tceA* gene), as well as the RDases catalyzing the conversion of cDCE to ethene (coded by the *bvcA* and *vcrA* genes) (Magnuson et al., 1998, 2000; Krajmalnik-Brown et al., 2004; Müller et al., 2004; Fung et al., 2007; Tang et al., 2016). Quantitative PCR methods that target these specific genes have been developed and are being increasingly used as prognostic and diagnostic tools in the field to overcome the limitations of the 16S rRNA gene (Rahm et al., 2006b; Ritalahti et al., 2006, 2010; Lee et al., 2012; Lu et al., 2015).

The genomes of more than 10 *D. mccartyi* isolates have now been sequenced. These genomes are highly streamlined (~1.4 Mb) and striking in their similarity, differing primarily in two regions termed High Plasticity Regions (HPR) on either side of the origin of replication (ORI). Each genome harbors many distinct full-length *rdhAB* homologous genes per genome (e.g., 17 in strain 195, 32 in strain CBDB1 and 36 in strain VS) (Kube et al., 2005; McMurdie et al., 2009; Seshadri et al., 2005). Hundreds if not thousands more putative *rdhAB* genes have been identified from metagenome sequencing efforts. Owing to the lack of functional characterization for most of this protein family, a sequence identity-based classification of orthologs into groups based on >90% aa identity was developed (Hug et al., 2013). This sequence-based classification was adopted prior to having a crystal structure to identify active site and other key residues. Fortunately, the two crystal structures recently solved (Bommer et al., 2014; Payne et al., 2015) support the original classification. The database of sequences and new ortholog groups continues to expand (Hug et al., 2013; Hug, 2016).

In this study, we aimed to distinguish different *D. mccartyi* strains from each other in mixed cultures and groundwater, where multiple *D. mccartyi* strains coexist. We define strains as genetic variants of *D. mccartyi* (e.g., differing in their *rdhA* complement) that have not necessarily been isolated as pure cultures. Our final aim was to better understand the contribution of native vs. introduced *D. mccartyi* to overall remediation at two contaminated sites where mixed dechlorinating culture KB-1 was used for bioaugmentation.

As it is very difficult to find unique regions within the genomes to distinguish strains, we decided to focus on quantifying a broader suite of *rdhA* genes, including both characterized and uncharacterized *rdhA* genes. We first compared the *rdhA* complement in the metagenome of the mixed culture KB-1 with those of eleven isolated *D. mccartyi* strains to identify *rdhA* sequences (and their corresponding Ortholog Groups) that are less commonly shared between strains. Methods for qPCR were developed for the selected *rdhA* genes and these assays were first tested in subcultures of KB-1 enriched on different chlorinated ethenes. Here, we hypothesized that a larger number of *D. mccartyi* strains should be present in the TCE-amended subcultures compared to those amended with cDCE, VC, or 1,2-DCA, because the latter have fewer dechlorination steps. Finally, we used the selected suite of *rdhA* genes to explore strain dynamics in groundwater samples from two sites that had been bioaugmented with KB-1.

MATERIALS AND METHODS

Cultures and Growth Conditions

The KB-1 consortium is a functionally-stable enrichment culture that originated from microcosms consisting of aquifer material from a TCE-contaminated site in southern Ontario (Duhamel et al., 2002). KB-1 is routinely maintained in batch mode with TCE as electron acceptor, and dechlorinates PCE through TCE, cDCE, and VC to ethene. A transfer from the original KB-1 culture has been grown and used commercially for more than a decade for bioaugmentation at cVOC-contaminated sites¹. The main organisms in the KB-1 culture have been investigated over the years via clone libraries, qPCR and metagenome sequencing (Duhamel and Edwards, 2006, 2007; Chan et al., 2011; Hug, 2012; Hug et al., 2012; Waller et al., 2012). Two dechlorinating genera have been identified in the culture, namely *Dehalococcoides* and *Geobacter*, that are supported by many other organisms such as fermenters, acetogens, and methanogens (Hug et al., 2012; Waller, 2009). Many years ago, the original TCE-fed KB-1 enrichment culture was used to inoculate various subcultures maintained on different terminal electron acceptors (TCE, cDCE, VC and 1,2-dichloroethane [1,2-DCA]). These subcultures have been maintained with methanol (M), hydrogen (H₂), or a mixture of methanol and ethanol (ME) as electron donor. Supplementary Table S1 summarizes the main features and growth conditions of all the different cultures studied with the name format indicating electron acceptor amended/donor

used and year created. The commercial KB-1 culture (KB-1[®]) is referred to as TCE/ME_2001_SiREM in Supplementary Table S1. All cultures were grown anaerobically in a defined minimum mineral medium (Duhamel et al., 2002). Cultures maintained at the University of Toronto are grown either in 0.25 L bottles sealed with screw caps with mininert valves or in 1 or 2 L glass bottles sealed with black butyl stoppers. Typically, bottles contained 10% by volume of headspace flushed with a N₂/CO₂ 80%/20% as needed. These cultures are kept in the dark in an anaerobic glovebox at room temperature (22–25°C). At SiREM, KB-1 is grown in 100 L stainless steel vessels at 22–25°C. At the University of Toronto, dechlorinating cultures were typically re-amended every 2–3 weeks. Cultures maintained at SiREM are re-amended more frequently, typically every 3 to 4 days, as substrate is depleted.

KB-1 Metagenome Data and *rdh* Sequences

The parent KB-1 culture has been maintained at the University of Toronto since 1998 with TCE as the electron acceptor and methanol as the electron donor. This culture is referred to as “TCE/M_1998_Parent” in Supplementary Table S1 and all other KB-1 enrichments originated from this parent culture. The partially assembled metagenome sequence of TCE/M_1998_Parent is publicly available at the Joint Genome Institute (JGI)². Details on the extraction of genomic DNA for sequencing and the assembly of the KB-1 metagenome and of a draft chimeric genome of *D. mccartyi* strains are provided elsewhere (Hug et al., 2012; Islam et al., 2014). As the core genome of all *D. mccartyi* strains is almost identical, the search for unique non-coding regions in the metagenome yielded no positive results. The only differences found during comparative metagenomics were associated with the coding regions of the *rdhA* genes of the HPR regions. A total of 31 distinct *rdhA* gene sequences have been identified in the KB-1 cultures from multiple investigations (Hug, 2012; Hug and Edwards, 2013). Thirty sequences are associated with *D. mccartyi*, whereas one sequence is from the *Geobacter* population present in KB-1. Supplementary Table S2 compiles all previously identified KB-1 *rdhAB* sequences, which include the original 14 sequences found by Waller et al. (2005) as well as those identified more recently from metagenome sequencing (Hug et al., 2012) where an additional 13 *rdhAB* full sequences and two *rdhA* partial sequences were annotated (Hug and Edwards, 2013; Hug et al., 2013). For ease of reference and consistency, these additional *rdhAB* sequences have been renamed and are now deposited to Genbank under the accession numbers KP085015–KP085029 to replace the previous JGI gene locus tags (Supplementary Table S2).

Phylogenetic Analysis of *rdhA* Sequences

A total of 249 *D. mccartyi* *rdhA* sequences from sequenced and characterized isolated strains were retrieved from NCBI

¹ www.siremlab.com

² https://genome.jgi.doe.gov/portal/BioGr_2013843002/BioGr_2013843002.download.html

for phylogenetic comparisons. These sequences include available sequences from eleven isolated and sequenced *D. mccartyi* strains, an incomplete set of 3 *rdhA* sequences from strain MB (available at the time of retrieval), and the *rdhA* sequences found in KB-1. The number of *rdhA* sequences contributed by each strain was as follows: 195 (17), Bav1 (10), BTF08 (20), CBDB1 (32), DCMB5 (23), FL2 (14), GT (20), GY50 (26), JNA (19), MB (3), VS (37), and KB-1 (28 [*Dhc*]+1 [*Geobacter*])). Two of the KB-1 *D. mccartyi* *rdhA* sequences and sequence VS_1308 are only partial sequences (less than 850 nucleotides) and were therefore not included in phylogenetic comparisons. Files containing all amino acid and nucleotide sequences as well as matching of tree nomenclature with protein names can be found in a folder labeled *rdhA_Dhc_seq150420* at the following address: <https://docs.google.com/folder/d/0BwCzK8wzlz8ON1o2Z3FTbHFPYXc/edit>.

Phylogenetic analysis of *rdhA* nucleotide sequences, including bootstrapping, was performed with the ClustalX free software (version 2.0.12; University College Dublin³). All sequences were compared to each other by separate pairwise comparisons using the dynamic programming method with a gap opening of 10 (range of 0–100) and a gap extension of 0.1 (range 0–100). Results were then computed into a DNA weight matrix that determines the similarity of non-identical bases. Based on the DNA weight matrix, a dendrogram was constructed which in turn serves as guide for the final multiple alignment. Settings for the final multiple alignment were as follows: a gap opening penalty of 10 (range 0–100), a gap extension penalty of 0.2 (range 0–100), a delay divergent sequences switch set at 30% divergence and a DNA transition weight of 0.5 (range 0–1). The IUB weight matrix was used for computation. A phylogenetic tree was built using the Neighbor Joining (NJ) Algorithm with the same program. The NJ-tree was visualized and further edited with Figtree (version 1.4.0.1; Institute of Evolutionary Biology, University of Edinburgh⁴). The midpoint criterion was used for rooting.

A phylogenetic analysis based on *rdhA* amino acid sequences was also computed using the ClustalX v2.0.12. For computing the alignment, the following protein gap parameter options were considered: (i) the residue specific and hydrophilic residue penalties options were enabled, (ii) a gap separation distance of 4 (range 0–100) and (iii) no end gap separation, which treats end gaps the same way as internal gaps, was allowed. The phylogenetic tree was built using the Neighbor Joining (NJ) Algorithm and further visualized and edited with Figtree v1.4.0. As for the nucleotide-based phylogenetic tree the midpoint criterion was used for rooting. Ortholog groups were highlighted on the trees with Adobe Illustrator.

Groundwater Sampling and Site Description

Groundwater was collected from two TCE-contaminated sites prior to and after bioaugmentation with KB-1. The first site was located in Southern Ontario (ISSO site) and was characterized

by contaminants in fractured bedrock. Supplementary Figure S1A shows details of relevant sampling dates and events related to this site. Samples corresponding to three different phases of the remediation were investigated: (i) predonor or pretreatment phase, (ii) biostimulation phase, consisting of the daily addition of ethanol as an electron donor and (iii) bioaugmentation phase, following the inoculation of KB-1 (Supplementary Figure S1A). At the ISSO site, a groundwater recirculation system consisting of two injection and three extraction wells was installed to improve the distribution of electron donor (ethanol) and microorganisms (Supplementary Figure S1B). During both the biostimulation and bioaugmentation phases, ethanol was added on a daily basis. Bioaugmentation with KB-1 consisted of one single addition of approximately 100 L of culture. Groundwater samples for molecular analysis were obtained from the composite pipeline, where groundwater from the three extraction wells was combined (Supplementary Figure S1B). Additional information on this site can be found in an earlier published study reporting on a microbial community survey at the site (Pérez-de-Mora et al., 2014).

The second site is located in the United Kingdom and consisted of a pilot test cell (30 m × 7 m × 4 m) for treatment of a DNAPL source area (~1000 kg of DNAPL within the cell). The cell was conceived as an “in-Situ Laboratory” for investigating source area bioremediation (SaBRE project⁵). The relevant sampling dates and events related to this site and a sketch with sampling locations are shown in Supplementary Figures S2A,B. Samples for investigation were collected: (i) prior to any remediation action; and (ii) after donor addition of one single dose of donor SRSTM, a commercially available emulsified vegetable oil (Terra Systems, Inc.) and bioaugmentation with KB-1 (Supplementary Figure S2A). Groundwater was collected from fully screened sampling wells (SW) at four locations within the test cell: (i) at the influent (INF); (ii) within the source zone (SW70); (iii) within the plume (SW75); and (iv) at the effluent (EFF), also within the plume. The test cell was operated initially for a 90-day baseline period to establish steady-state pre-treatment conditions. Groundwater was extracted at an average of 1.4 l per minute, corresponding to an average residence time within the cell of 45 days. A total of 2,400 kg of SRSTM at a 5% concentration was used as the electron donor and injected along the test cell. Two weeks after donor injection approximately 65 L of KB-1 was added using the same injection ports that were used to add electron donor. Within the test cell, both TCE and cDCE were the main cVOCs. A dissolved phase plume emanating from the source and extending more than 400 m away was further characterized by the presence of VC and ethene.

The starting point (Day 0) was defined as the day following the end of the emulsified oil injection, which took approximately 1 week to complete. Further details on the SaBRE site can be found in technical bulletins freely available on the SaBRE-CL:Aire website (see above).

³<http://www.clustal.org/clustal2/>

⁴<http://tree.bio.ed.ac.uk/software/figtree/>

⁵http://www.claire.co.uk/index.php?option=com_content&view=article&id=53&Itemid=47

Nucleic Acid Extraction

Samples were collected from eleven different enrichment cultures at one or two times each during 2011 (Supplementary Table S1). Frozen DNA samples (-80°C) from the TCE/ME_2001_SiREM culture collected over 5 years were obtained from SiREM. DNA was also extracted from groundwater samples from the two field sites described above. For extraction and isolation of genomic DNA (gDNA), samples from cultures (10–50 mL) or groundwater (200–1000 mL) were filtered through SterivexTM (Millipore, MA, United States) (0.22 μm pore size) filters using a centrifugal pump and dual-trap (two liquid traps in series) system. Filters were subsequently stored at -80°C until further processing. For gDNA extraction, the casing of the filter was opened and the filter cut in about 30 pieces of similar size. The latter were introduced into a 2 mL nucleic acid extraction tube containing buffers and beads (Mo Bio Laboratories UltraClean[®] Soil DNA Isolation Kit, CA, United States) and DNA extraction was completed following the manufacturer's instructions. Elution of nucleic acids from the silica membrane was performed using 50 μL of UltraPureTM DNase/RNase-Free distilled water (Invitrogen, CA) for samples from cultures as well as from the ISSO site in Canada, whereas 920 μL were used for elution of samples from the SaBRE site in the United Kingdom.

Quantitative PCR (qPCR) Amplification of Extracted DNA

Quantification of the 16S rRNA gene of *Dehalococcoides* and *Geobacter*-KB1 as well as a total of 15 *D. mccartyi* *rdhA* genes plus one *Geobacter* *rdhA* gene from the KB-1 culture was achieved via qPCR on an Opticon 2 thermocycler (MJ Research). Supplementary Table S3 provides details on the primer pairs used, their annealing temperatures and the length of the amplicon generated. With the exception of five genes (KB1-6/*bvcA*, KB1-14/*vcrA*, KB1-27/*tceA*, KB1-1 and KB1-5), new primers were designed for all other *rdhA* genes in this study. The specificity of the primers was tested *in silico* against the public NCBI NR nucleotide database and tested *in vitro* against DNA extracted from mixed dechlorinating and non-dechlorinating cultures. Supplementary Table S4 shows the number of mismatches for each primer pair with sequences belonging to the ortholog group of the targeted *rdhA* gene. The PCR reaction (20 μL) consisted of: 10 μL of SsoFastTM EvaGreen[®] Supermix (Bio-Rad, CA), 0.5 μL of each primer (10 mM), 7 μL of UltraPureTM DNase/RNase-Free distilled water (Invitrogen, CA) and 2 μL of template. The amplification program included an initial denaturation step at 98°C for 2 min followed by 40 cycles of 5 s at 98°C and 10 s at the corresponding annealing temperature. A final melting curve from 70 to 95°C degrees at increments of 0.5°C per second was performed. Since two concentrations were tested per template (undiluted and 10-fold diluted) for assessment of potential matrix-associated inhibitory effects, reactions for each dilution were performed in duplicate. The undiluted sample generally contained between 10–20 ng of DNA per μL as measured using a Nanodrop spectrophotometer (Thermo Scientific, DE). Generally, there was good agreement between the two measurements. Ten-fold serial dilutions of

plasmid DNA containing one copy of the 16S rRNA gene or the *rdhA* gene (~ 1000 –1500 bases) were used as calibrators. Plasmids for calibration were prepared by PCR amplifying the desired *rdhA* from KB-1, inserting the gene into the pCR 2.1 PCR vector and subsequently transforming the construct into TOP10 *Escherichia coli* competent cells (Invitrogen, CA) as per the TOPO TA cloning[®] kit (Invitrogen, CA). Plasmid DNA was purified using the GenEluteTM Plasmid Miniprep Kit following the manufacturer's instructions (Sigma-Aldrich, MO). DNA was eluted using 50 μL of Ultra Pure DNase/RNase-free distilled water (Invitrogen, CA). Standard curves exhibited linear behavior ($R^2 > 0.990$) when plotted on a logarithmic scale over seven orders of magnitude. Supplementary Table S5 provides the details on the standard curves for each primer pair, including slopes (efficiencies), *Y*-intercepts and calibration model fit. The specificity of the amplicons was checked by melt curve analysis as well as by agarose gel electrophoresis for selected samples. Non-template controls were included in each run. Absolute gene copy concentrations for all cultures and field samples are provided as Supplementary Tables S6–S8. The method detection limits (MDLs) were expressed in terms of gene copies per L of groundwater or per mL of culture and varied depending on the volume of sample filtered and the volume employed for elution of DNA from the purification column. The MDLs for each set of samples are provided in Supplementary Table S9.

Cluster Analysis of *rdhA* Sequences and Calculation of *rdhA*/16S rDNA *Dhc* Ratios

Phylogenetic analysis of *rdhA* nucleotide and amino acid sequences, including bootstrapping, was performed with the ClustalX free software (version 2.0.12; University College Dublin⁶). Protein sequences were clustered into ortholog groups as previously defined [90% pairwise identity in amino acid alignments as per (Hug et al., 2013)]. The similarity of the *Dehalococcoides* populations in the various KB-1 subcultures was investigated on the basis of their *rdhA* fingerprints (*rdhA*/16S rRNA gene *Dhc* ratios) by means of hierarchical cluster ordination analysis. The latter was performed with the free software Hierarchical Clustering Explorer (HCE) (version 3.5; Human-Computer Interaction Lab, University of Maryland⁷). The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used as linkage clustering method, whilst Pearson's correlation coefficients were used to construct the similarity distance matrix. Heatmaps generated with the HCE v3 software were further edited manually for scale optimization in Excel 2010.

RESULTS AND DISCUSSION

Reductive Dehalogenase (*rdhA*) Genes in the KB-1 Consortium

As the core genome of all *D. mccartyi* strains is almost identical, the metagenome search for unique non-coding regions that could

⁶<http://www.clustal.org/clustal2/>

⁷www.cs.umd.edu/hcil/hce/

differentiate different strains yielded no positive results. As a consequence, efforts were turned to the complement of *rdhA* genes, which does vary significantly between strains. To visualize sequence similarities, we constructed phylogenetic trees using 249 amino acid (Figure 1) and nucleotide (Supplementary Figure S3) sequences that included all full *RdhA* sequences found in the KB-1 metagenome as well as those identified in 11 isolated and sequenced *D. mccartyi* strains. Amino acid sequences belonging to the same ortholog group (OG), defined as having >90% PID (Hug et al., 2013), are highlighted in different colors on the

phylogenetic trees. These highlighted branches clearly support the classification and reveal how some *RdhA* sequences are common to all strains, while others only in a few (Figure 1). When the same sequences are compared at the nucleotide level (Supplementary Figure S3) identity within an ortholog group can be substantially lower than 90%. The terminology reductive dehalogenase *homologous* genes (*rdhA*) was adopted years ago, and perhaps implies more knowledge of common ancestry than is in fact known; still, it is not unreasonable to suspect that these genes arose from either speciation (orthologous) or duplication

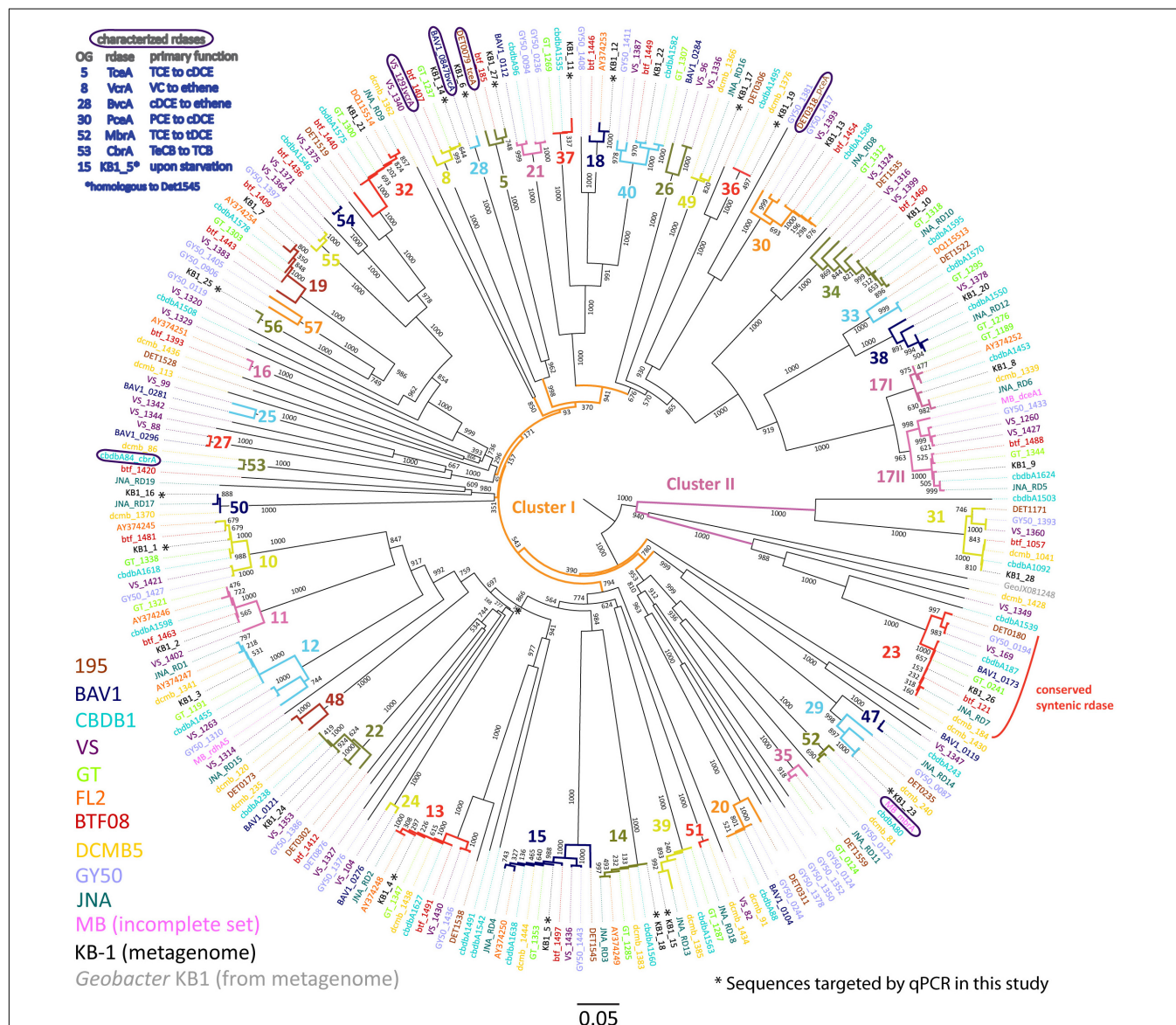


FIGURE 1 | Phylogenetic tree of *Dehalococcoides mccartyi* reductive dehalogenases based on amino acid sequences. Tree includes sequences predicted from 248 *rdhA* genes found in eleven isolated *D. mccartyi* strains as well as those found in the mixed dechlorinating culture KB-1, including the *rdhA* gene sequence found in *Geobacter* KB-1 as an outgroup. The scale is the number of substitutions per amino acid. Bootstraps are shown at tree nodes. Sequences names are colored corresponding to their source as shown in the legend at bottom left. The numbers and different branch colors highlight ortholog groups (OGs) where sequences have >90% PID. Sequences corresponding to characterized RDases are circled and their substrates shown in the top right legend (chlorinated substrate abbreviations as in text, plus TeCB, tetrachlorobenzene; TCB, trichlorobenzene).

(paralogous) events. Herein we use the term “ortholog” to refer to *rdhA* genes that group together according to the classification system cited above. The term “homologous” is used to refer to all *rdhA* genes regardless of which ortholog group they belong to.

The phylogenetic analysis of 249 *D. mccartyi* *RdhA* sequences generated a total of 43 ortholog groups (OG) including 6 previously un-described groups (OG 52 to 57). Of the 249 sequences analyzed in 2016, 37 sequences remained ungrouped, meaning without a single ortholog (Figure 1). At the amino acid level, none of the KB-1 *D. mccartyi* *RdhA* sequences is unique because each is more than 90% similar to a sequence in at least one other *D. mccartyi* strain. The existence of numerous shared *rdhA* genes among *D. mccartyi* is consistent with their co-localization with insertion sequences and other signatures for horizontal gene transfer, and within genomic islands in high plasticity regions (McMurdie et al., 2009).

Selection of Suite of Distinguishing KB-1 *rdhA* Genes

We selected a total of 15 characterized and uncharacterized *rdhA* sequences to track using qPCR. Ten uncharacterized *rdhA* sequences, KB1-25 (OG56), KB1-11 (OG37), KB1-12 (OG18), KB1-16 (OG50), KB1-17 (OG 49), KB1-19 (OG36), and KB1-23 (OG29), were selected as those with the fewest orthologs in other strains based on our trees (Figure 1 and Supplementary Figure S3). KB1-15 (OG39) and KB1-18 (OG14) were selected because they were found on the same contig in the KB-1 metagenome and both share homology to genes in strains GT, CBDB1, DCMB5, and JNA, therefore they may be mobilized together (Figure 1). We included KB1-4 (OG13) because it appears to have orthologs in all other strains and could be useful for normalization, similar to the 16S rRNA gene. To this set of 10 uncharacterized genes, we added five more genes of interest: KB1-1 (OG10), KB1-5 (OG15), KB1_14/*vcrA* (OG8), KB1_6/*bvcA* (OG28) and KB1_27/*tceA* (OG5). KB1-1 and KB1-5 were selected because their corresponding proteins have previously been detected in KB-1 (Tang et al., 2013; Liang et al., 2015). Furthermore, KB1-5 (OG 15) which is expressed upon starvation, is orthologous to DET1545 from Strain 195 (Rahm and Richardson, 2008; Tang et al., 2013). KB1_14, KB1_6 and KB_27 correspond to the functionally-characterized vinyl chloride and trichloroethene reductases *VcrA*, *BvcA*, and *TceA*, respectively. We designed qPCR primers to these 10 uncharacterized genes, and used previously designed primers for the remaining 5 genes (Supplementary Table S4). Next we monitored the abundance of this suite of *rdhA* genes in DNA samples from 11 different KB-1 enrichment sub-cultures maintained over years on different chlorinated electron acceptors (Supplementary Table S1).

Quantification of *rdhA* Genes in KB-1 Enrichments With Different Chlorinated Electron Acceptors

To reflect shifts in *D. mccartyi* populations, qPCR results for each sample are presented as *rdhA* gene copies divided by 16S rRNA gene copies to provide an approximation of the

relative proportion of each *rdhA* gene per *D. mccartyi* genome. *D. mccartyi* are known to harbor only one copy of the 16S rRNA gene per genome. *rdhA*/16S rRNA ratios were visualized using heatmaps with values ranging from above 0.6 (where more than 60% of all *D. mccartyi* genomes in the culture contains that gene) to less than 0.001 (where the gene is present in fewer than 0.1% of the *D. mccartyi* populations) (Figure 2). Although the primers were designed to target specific KB-1 *rdhA* sequences, most sequences within the same ortholog group would likely also be amplified. Supplementary Table S4 compiles all mismatches found between primers used and other sequences in the corresponding OG.

Dehalococcoides mccartyi *rdhA*/16S rDNA gene copy ratios measured in KB-1 enrichment cultures (Figure 2) revealed more variability of *D. mccartyi* strains than anticipated, considering the number of years of enrichment and the fact that they all originated from a common parent culture. Clustering analysis revealed *rdhA* genes common to all cultures and *rdhA* gene specific to a particular chlorinated electron acceptor. For instance, all subcultures were found to contain KB1-1 (OG10) (ratios all greater than 0.6), and to have consistently abundant *rdhA* sequences of KB1-4 (OG13) and KB1-5 (OG15), and KB1-14 (*vcrA*; OG8). The most striking finding (Figure 2A) is the clustering of specific patterns with terminal chlorinated electron acceptor. The VC enrichments show the lowest diversity of *rdhA* sequences and the most concordance between enrichments. These enrichments contain high abundances of 6 genes (KB1-1, KB1-14/*vcrA*, KB1-11, KB1-4, KB1-15, and KB1-18) with more variable but still abundant presence of KB1-5, KB1-23 and KB1-12. Other *rdhA* sequences are near or below detection. The cDCE enrichments form their own cluster, distinct from the VC enrichments, because they contain significant abundance of the KB1-6/*bvcA* gene, and much lower proportions of KB1-23 and KB1-12. These cDCE enrichments were the only ones consistently enriched in *bvcA*. The 1,2-DCA enrichments also formed a distinct cluster characterized by high ratios for KB1-16, KB1-17, KB1-19, KB1-25 as well as KB1-27/*tceA*. Finally, the TCE cultures reflect a blend of all the other enrichments, showing more variability, although favoring an *rdhA* ratio pattern most like the VC enrichments.

The clustering analysis also revealed co-variation among some of the *rdhA* genes regardless of the enrichment, suggesting co-location of these genes on the same genome as is the case of KB1-15 and -18, and possibly also KB1-16, -17, -19, and -25 (Figure 2A). The *Geobacter* *rdhA* gene was detected at similar abundance to the *Geobacter* 16S rRNA gene only in the TCE-amended cultures (at approximately 15–25% of *Dhc* abundance); in all other enrichments *Geobacter* 16S rDNA and *rdhA* genes were below the detection limits (Supplementary Tables S6, S7), as expected since *Geobacter* only dechlorinates PCE or TCE as far as cDCE. In control DNA samples from cultures without *D. mccartyi*, copies of *rdhA* genes were all below the MDL (data not shown).

The stability of the *rdhA* fingerprints over time was also assessed. For the VC, 1,2-DCA and cDCE enrichments, two samples from the same enrichment culture bottle were analyzed at 4 or 9 months intervals (January and October or June and

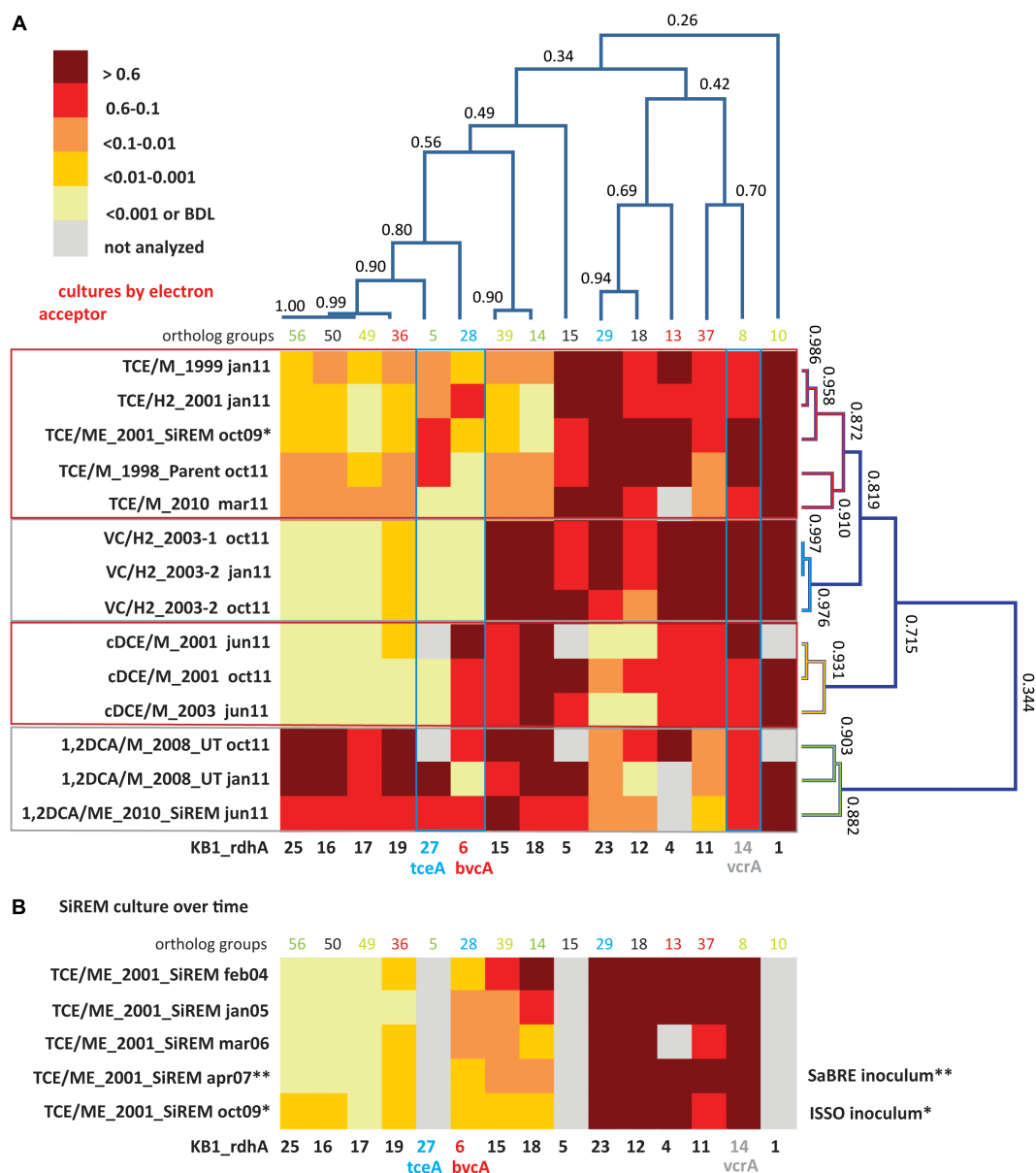


FIGURE 2 | Cluster analysis and heatmaps showing *D. mccartyi* *rdhA*/16S rRNA gene copy ratios measured in KB-1 enrichment cultures. **(A)** Shows how data clustered by the chlorinated electron acceptor amended to individual enrichment cultures as well as by *rdhA* sequence. The name format indicates electron acceptor amended/donor used_year created, followed by date sampled (e.g., TCE/M_1999 jan11 is a TCE and methanol enrichment culture first established in 1999 and sampled in January of 2011). The numbers on the cluster branches of both axes indicate the percentage similarity between samples based on the Pearson's correlation coefficient. **(B)** It is a heatmap of *rdhA*/16S rRNA gene copy ratios in the SIREM KB-1 culture over 5 years. Cultures indicated with * or ** are those used for bioaugmenting field sites in 2007 (SaBRE) and 2009 (ISSO).

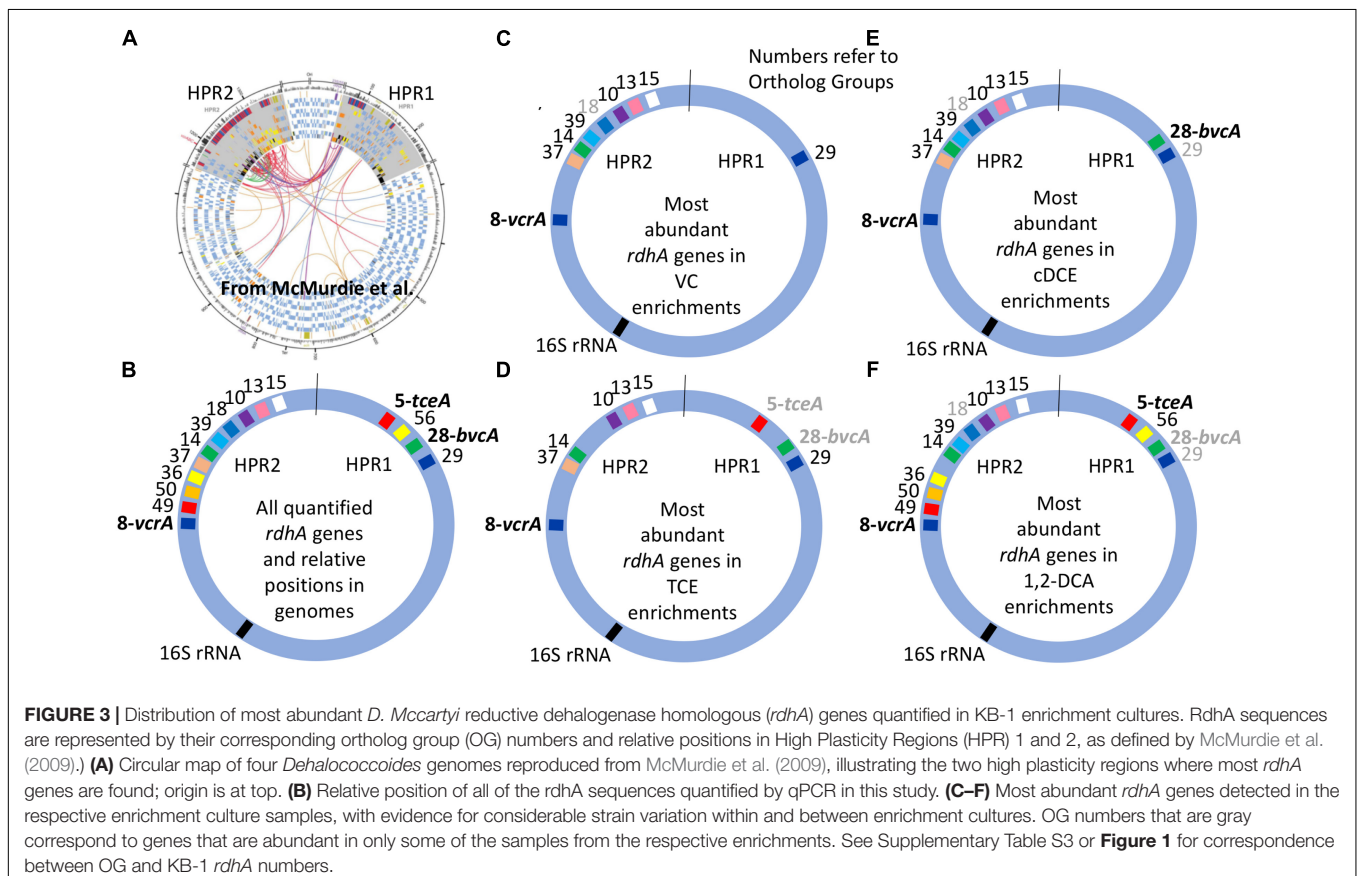
October, 2011; **Figure 2A**). There was good agreement between the two time points, although small differences likely reflect changes in relative abundance of *D. mccartyi* strains in batch incubation conditions. For the TCE/ME_2001_SiREM culture, five DNA samples over a time span of 5 years (2004, 2005, 2006, 2007, and 2009) were available, and were investigated using a subset of *rdhA* genes (**Figure 2B**). The *rdhA* fingerprint was relatively stable over time except for three *rdhA* genes, namely KB1-18, KB1-15, and KB1-6/*bvcA*. Ratios for KB1-18

and KB1-15 gradually shifted from greater than 0.6 in 2004 to less than 0.01 and even less than 0.001 by 2009. KB1-6/*bvcA* ratios fluctuated over this time period between 0.01 and 0.1. As noted already, KB1-18 and KB1-15 appear to co-vary, supporting their co-localization in the same genome. These two genes are abundant in VC, cDCE, and 1,2-DCA enrichment cultures but not in the TCE enrichments and diminished over time in the TCE/ME_2001_SiREM. These data reveal that the dominant VC-dechlorinating, *vcrA*-containing *D. mccartyi* strains are not

the same in the TCE and VC enrichment cultures. It appears that the dominant *D. mccartyi* strain more recently present in the VC/H₂ sub-cultures is more similar to the one that was originally present in the parent culture, and that this population has been gradually supplanted in TCE/ME₂₀₀₁_SiREM culture. This data indicates that a major shift can also happen in the *rdhA* fingerprint of a mixed culture even when maintained consistently on the same electron acceptor. This is different to the wholesale changes observed when the electron acceptor is changed, where a shift in the dominant *D. mccartyi* population may happen much faster, as demonstrated in studies by Mayer-Blackwell et al. (2014, 2016) where the dominant *D. mccartyi* population in a bioreactor shifted over a period of 50–100 days when the electron acceptor was switched for 1,2-DCA from TCE. In the field, significant changes in *Dhc* populations may also be expected within a few months due to changing conditions: for instance, in electron acceptor availability.

The clustered data for 15 *rdhA* genes across 14 DNA samples from 11 different cultures yielded four major groups corresponding to each of the four chlorinated electron acceptors (Figure 2A). To create a more visual representation of the distribution of these *rdhA* genes in the two High Plasticity Regions (HPRs) of *D. mccartyi* genomes, we mapped corresponding OG groups in the order and HPR they typically appear in published genomes (Figure 3). This map provides evidence not just of multiple strains but also of likely gene

insertions and/or deletions (e.g., *bvcA* in the HPR1). As indicated previously, the considerable variability in the *rdhA* to 16S ratios within each culture and electron acceptor group was at first very surprising, because the cultures all derive from the same parent and have been enriched for so long on the same substrate. We expected to see only one clearly dominant population, especially in the VC to ethene and 1,2-DCA to ethene cultures that involve only a single dechlorination step. What the data show instead is a more complex pattern in each group, suggesting the presence of more than one *D. mccartyi* population even in the single-dechlorination step, highly enriched VC/H₂ cultures. We repeated many of the DNA extractions and qPCR reactions to verify results, with no appreciable changes. Recent analysis of these enrichments yielded similar results (data not shown). Early experiments by Duhamel et al. (2002, 2004) and Waller et al. (2005) had identified at least two distinct *D. mccartyi* populations (KB1-PCE and KB1-VC), the former containing KB1-6 (*bvcA*) and the latter not; these two strains could actually also be distinguished by a single nucleotide difference in their 16S rRNA sequences (Duhamel et al., 2004). Subsequently, Hug (Hug, 2012) was unable to close the assembly of a *D. mccartyi* genome from the KB-1 TCE/M₁₉₉₈ parent culture metagenome, particularly in the high plasticity regions rich in *rdhA* genes, because of the presence of multiple, highly similar *D. mccartyi* populations. Considering the cluster diagram by enrichment culture (Figure 2A; right side), we can perhaps



infer at least four distinct populations based on *rdhA* distribution in the TCE enrichments, at least two in the VC enrichments (one with and one without *rdhA* KB1-12 (OG18), at least two in the cDCE enrichments and three in the 1,2-DCA enrichments (Figure 3). In an attempt to verify these results, we sequenced metagenomes from representative VC, cDCE, and 1,2-DCA enrichments. Sequencing has confirmed the presence of multiple distinct *D. mccartyi* genomes in all enrichment cultures, even in the VC enrichments, upholding these results (data not shown; manuscript in preparation). The co-existence of multiple *D. mccartyi* strains at different cell densities within these enrichments likely arises from subtle substrate preferences of the expressed reductive dehalogenases and competition for available nutrients and vitamins. Functional characterization of some reductive dehalogenases reveals substrate overlap yet specific substrate preferences as observed for *BvcA* and *VcrA* (Yan et al., 2012). Moreover, availability and type of corrinoid can alter rates of dechlorination for certain enzymes (Yan et al., 2016). Low abundance populations may persist in these cultures because they are maintained in batch mode, with infrequent medium changes and thus long residence times from 30 to 100 days.

Quantification of *rdhA* Genes in Site Groundwater

A suite of 12 *rdhA* genes representative of both dominant and minor *D. mccartyi* strains was used to monitor population shifts before and after bioaugmentation with KB-1 (TCE/ME_2001_SiREM) at two TCE-contaminated sites: one in Canada (ISSO) and another site in the United Kingdom (SaBRE). At the United Kingdom site, the abundance of *Geobacter* 16S rRNA and *rdh* genes were also monitored.

The results from the Canadian ISSO site revealed that the two most abundant *rdhA* genes in native populations (monitored prior to any treatment) were orthologs to KB1-4 and KB1-6/*bvcA*, but that their titers were barely above detection limits, at approximately 3×10^5 copies per L (Supplementary Table S8). Although the *bvcA*-encoded dehalogenase is known to catalyze the dechlorination of cDCE all the way to ethene (Holmes et al., 2006), there was little dechlorination beyond cDCE before electron donor amendment (Pérez-de-Mora et al., 2014). During biostimulation with electron donor prior to bioaugmentation, *D. mccartyi* and *vcrA* copy numbers increased significantly, reaching a *vcrA*/16S rRNA gene ratio near one (Figure 4A), indicating growth of *vcrA*-bearing native populations. The abundance of *rdhA* genes orthologous to KB1-11, KB1-15, KB1-18 also increased during this period (Figure 4A). After bioaugmentation with KB-1, the *rdhA* profile did not change substantially, even though higher ethene concentrations and faster dechlorination were observed (Figure 4A). A reduction of the *bvcA*/16S rRNA gene ratio from ~ 0.5 to 0.1 was the most notable change during this period (Figure 4A). The native populations that grew upon electron donor addition (prior to inoculation) harbored orthologs of 6 out of the 12 KB-1 *rdhA* genes monitored. From the data after bioaugmentation, it is clear that the dominant *D. mccartyi* populations from the KB-1 inoculum were not the ones responsible for the

enhanced ethene production observed because KB1-12 and KB1-23 sequences that were dominant in the inoculum were not enriched in samples from the site. Instead a low-abundance *D. mccartyi* population in the inoculum grew at the site or, alternatively, bioaugmentation may have provided supporting organisms that enhance dechlorination of VC by the native *D. mccartyi*. This result is in agreement with previous analyses of data from this site (Pérez-de-Mora et al., 2014) that suggested that growth of a *Bacteroidetes* present in KB-1 capable of ethanol fermentation enhanced ethenogenesis after bioaugmentation. Other fermenters or acetogens capable of producing the lower base of vitamin B12, namely dimethylbenzimidazole (DMB), which is required by *VcrA*, may have grown at the site, although this could not be shown for the known strains in KB-1.

The *rdhA* survey also revealed *D. mccartyi* population shifts as dechlorination progressed in the field (Figure 4A). Specifically, prior to any active treatment and when cDCE was the dominant cVOC, *bvcA* and KB1-4 were the most abundant *rdhA* genes found, a feature only seen with the cDCE enrichments in the KB-1 subculture survey. During biostimulation with electron donor, there was a gradual decrease in cDCE concentration and an increase in the concentration of VC and ethene. After 15 months of biostimulation (Month 18), the relative abundance of several other *rdhA* genes increased to above 10%, including genes similar to KB1-11, KB1-15, KB1-18, and especially *vcrA* (Figure 4A). This pattern of *rdhA* genes was also seen in the KB-1 VC enrichments. Three months after bioaugmentation (Month 22), the proportion of ethene relative to VC and cDCE further increased. Here, the *bvcA* ratio decreased to less than 0.1 while the *vcrA* ratio remained at approximately 1. At Month 30, when there was almost no VC or cDCE left, the *vcrA* ratio returned to below 0.6. At this time, relative abundance of KB1-12 decreased substantially; these *rdhA* was also found to vary between enrichment cultures.

Ratios describe shifts in the underlying *D. mccartyi* strains, but the absolute abundance of *D. mccartyi* is also relevant to observed dechlorinating activity. Major changes in absolute abundance of *rdhA* genes KB1-11, KB1-15, KB1-18, and KB1-14/*vcrA* were observed as well, starting from below detection limit ($<10^5$ copies L^{-1}) up to $0.9\text{--}3 \times 10^6$ copies L^{-1} (Supplementary Table S8). The abundances of *vcrA* and *D. mccartyi* 16S rRNA genes increased up to the end of the monitoring period (Month 30) reaching concentrations of 1.6×10^6 and 6×10^6 copies L^{-1} , respectively. For the *rdhA* genes KB1-12 and KB1-23, characteristic of the dominant *D. mccartyi* populations in the inoculum (Figure 4A), there was no such significant change, further indicating that this population did not contribute to the enhanced dechlorination observed after bioaugmentation.

The *rdhA* gene suite also was used to investigate groundwater from the SaBRE bioaugmentation trial within a test cell at a TCE-contaminated site in the United Kingdom. Prior to any treatment, *D. mccartyi* gene copy numbers were below the detection limit ($<6 \times 10^4$ gene copies L^{-1}) in all wells studied except the effluent (EFF) location where *D. mccartyi* titers of about 10^5 gene copies L^{-1} were detected (data not shown).

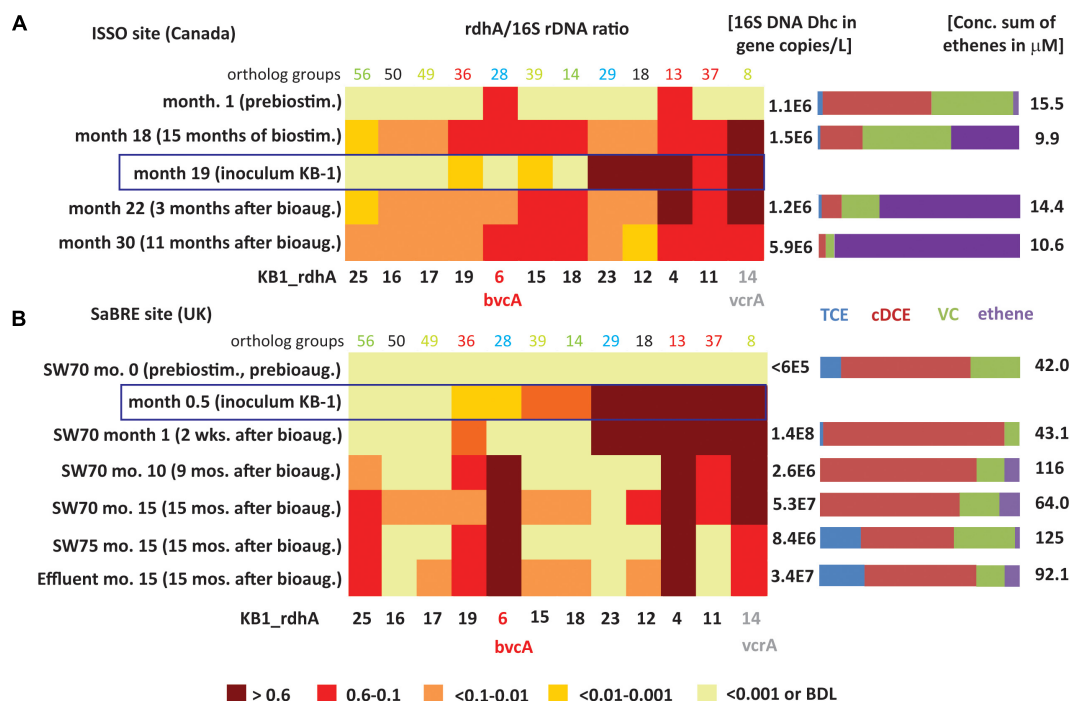


FIGURE 4 | Heatmaps showing *D. mccartyi* *rdhA*/16S rRNA gene copy ratios in field samples. **(A)** Canadian ISSO site. **(B)** United Kingdom SaBRE site.

Corresponding absolute copies of *D. mccartyi* per L and concentrations of chlorinated ethenes are represented on the right side of each panel. Data from the ISSO site are from the composite pipeline (see Supplementary Figure S1). Data from the SaBRE site are from the influent (INF), sampling wells (SW70 and SW75) and effluent (EFF) of the test plot (see Supplementary Figure S2).

No significant VC and ethene concentrations were measured in these wells prior to treatment. Unfortunately, no samples were collected during biostimulation prior to inoculation of KB-1 (all data provided in Supplementary Table S8). Two weeks after bioaugmentation with KB-1 and about a month after a single donor addition event, *D. mccartyi* titers increased by three orders of magnitude in SW70, reaching 10^8 gene copies L^{-1} (Supplementary Table S8). In a sample from this well, all KB-1 biomarkers characteristic of the original inoculum, that is KB1-4, KB1-11, KB1-12, KB1-14/*vcrA*, and KB-23 were found to have *rdhA* ratios greater than 0.6 (Figure 4B). The KB-1 *Geobacter* 16S rRNA gene and the *Geobacter rdhA* gene were also detected at significant titers in the range $0.8\text{--}4.6 \times 10^7$ copies L^{-1} (Supplementary Table S8). These data indicate that the dominant KB-1 *D. mccartyi* strain in the inoculum grew within the test cell, at least in the vicinity of this particular well (SW70).

In samples from SW-70 taken 10 and 15 months after donor addition, the ratios of KB1-11, KB1-12, and KB1-23, abundant in the inoculum and at 1 month, gradually decreased from values greater than 0.6 down to 0.1 (Figure 4B). The decrease of the ratio for KB1-23 was even more pronounced with a conservative estimate of 0.001 at month 15. Over the same time, the *vcrA* ratio remained greater than 0.6, while the *bvcA* ratio increased from less than 0.01 to about one (Figure 4B), indicating a dramatic shift in the dominant *D. mccartyi* strains with time in this well. A feature of this site was the consistently high

concentrations of cDCE that seems to have led to the growth of *bvcA*-containing strains. At month 15, downgradient sampling locations SW75 and EFF were dominated by KB1-6/*bvcA*, KB1-4 and KB1-14/*vcrA*, and were similar to well SW70. At the influent (INF) sample location, upgradient of where the inoculum was added, gene copy numbers of *D. mccartyi* and *rdhA* genes were all below their detection limits. Curiously, the *rdhA* and 16S rRNA genes from *Geobacter* were detected at levels just above the detection.

The Canadian ISSO and United Kingdom SaBRE sites differed in many ways. The former Ontario site had one order of magnitude lower cVOC concentrations, more complete dechlorination to ethene, a more-readily fermentable substrate (ethanol vs. emulsified oil) and a recirculation system that provided better mixing of substrates and microorganisms. The SaBRE site had higher concentrations of VOCs, giving rise to higher microbial and ethene concentrations than at the Canadian site, although the dominant compound at the site was cDCE. Particularly interesting at the SaBRE site was the co-presence of both *vcrA* (KB1-14) and *bvcA* (KB1-6) after month 10 at SW70 and after month 15 at both SW75 and Effluent. Co-presence of *bvcA* and *vcrA* at field sites has been reported in various studies, with greater abundance of *bvcA* over *vcrA* at some sites and vice versa at others (Lee et al., 2008; Carreon-Diazconti et al., 2009; van der Zaan et al., 2010; Atashgahi et al., 2017). Such differences have been attributed to redox potential, with *bvcA* perhaps more abundant under less reducing conditions

(van der Zaan et al., 2010). The redox potential at SaBRE was relatively low (−200 to −400 mV) and consistent over the length of the cell and the experimental time. Based on our laboratory cultures enriched with cDCE, it seems more likely that the high concentrations of cDCE and VC at a site exerted significant selective pressure on *D. mccartyi* strains harboring different *rdhA* genes, mimicking what we also observed in enrichment cultures.

Our results show that tracking specific *rdhA* fingerprints is challenging due to growth of minor populations of *D. mccartyi* either from native or inoculum-derived strains. Still, the approach employed in this study did reveal significant differences regarding the growth of the bioaugmented inoculum at the two sites. In future studies, it will be necessary to first identify site-specific *rdhA* genes that are not present at all in bioaugmentation cultures like KB-1, to track growth of indigenous populations. Perhaps a screening approach such as outlined in Hug and Edwards (2013) based on a set of 46 primer pairs to all known *rdhA* sequences, or the approach by Mayer-Blackwell et al. (2014) should be used to identify *rdh* genes in native strains prior to bioaugmentation. Then subsequent screens may be more discriminating.

More significantly, this study found clear selective pressure on *D. mccartyi* populations from the most abundant terminal chlorinated electron acceptor present. In both field samples and in well-established enrichment cultures, we found that *D. mccartyi* are not a homogeneous population but rather a complex and diverse mixture of strains harboring different complements of *rdhA* genes that respond and perhaps adapt selectively to external conditions – in particular electron acceptor – as revealed herein by monitoring *rdhA*/16S rRNA gene of *Dhc* ratios. The success of biostimulation and bioaugmentation approaches relies on the growth of populations with enzymes that actively convert chlorinated ethenes past VC to ethene. It seems that specific *rdhA* sequences confer populations and, by extent, mixed dechlorinating cultures, with fitness advantages depending on their local environment, and suggests that bioaugmenting with more refined cultures acclimated to intermediates like cDCE or VC that tend to accumulate at some sites may help to overcome stall and achieve complete dechlorination at some contaminated sites.

This study also underpins the need to further understand the role of horizontal gene transfer in these dechlorinating communities as suggested by the insertion/deletion of *rdhA* genes observed in the metagenomes of the KB-1 subcultures (Figure 3). These *rdhA* genes are often situated in regions of the genome that suggest potential for mobilization; yet we have no idea how

quickly and under what conditions *rdhA* gene transfer occurs in organohalide-respiring bacteria such as *Dehalococcoides*. This may be particularly relevant for bioaugmented sites where native and non-native populations coexist.

AUTHOR CONTRIBUTIONS

AP-d-M conceived of the experiments, carried out the molecular experiments, performed the comparative analyses, and co-drafted the manuscript. AL and XL assisted with the molecular experiments, culture maintenance, and helped to draft the manuscript. SD and MM conceived and led both field bioaugmentation trials, coordinated field monitoring, and helped to draft the manuscript. EE participated in the design of the study and co-drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.00812/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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