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Sequential anaerobic and aerobic bioaugmentation for commingled groundwater contamination of trichloroethene and 1,4-dioxane

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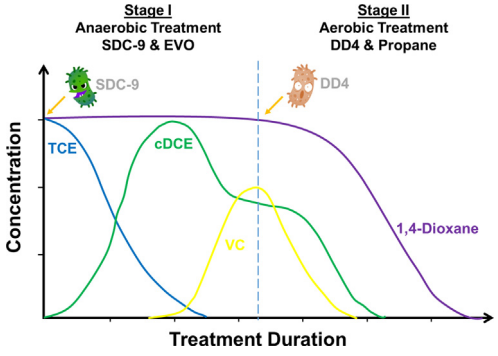
HIGHLIGHTS

- cDCE and/or VC may linger during slow or incomplete anaerobic treatment of TCE via reductive dehalogenation.
- *Azoarcus* sp. DD4 aerobically cometabolizes VC, cDCE, and 1,4-dioxane in tandem.
- Toluene monooxygenase and propane monooxygenase are responsible for cDCE and VC co-oxidation in DD4, respectively.
- Sequential anaerobic/aerobic bioremediation can effectively mitigate the co-contamination of cVOCs and 1,4-dioxane.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorinated solvents, notably trichloroethene (TCE), and the cyclic ether stabilizer, 1,4-dioxane (dioxane), have been frequently detected commingling in contaminated aquifers. Here we developed a sequential anaerobic and aerobic treatment strategy effective to mitigate the co-contamination of TCE and dioxane, particularly when dioxane is present at ppb levels relevant to many impacted sites. After the primary anaerobic treatment by a halorespiring consortium SDC-9, TCE was effectively removed, though lingering less-chlorinated metabolites, vinyl chloride (VC) and *cis*-dichloroethene (cDCE). Subsequent aerobic bioaugmentation with *Azoarcus* sp. DD4, a cometabolic dioxane degrader, demonstrated the ability of DD4 to degrade dioxane at an initial concentration of 20 μg/L to below 0.4 μg/L and its dominance (~7%) in microcosms fed with propane. Even better, DD4 can also transform VC and cDCE in tandem, though cDCE and VC at relatively high concentrations (e.g., 1 mg/L) posed inhibition to propane assimilation and cell growth of DD4. Mutagenesis of DD4 revealed group-2 toluene monooxygenase and group-5 propane monooxygenase are responsible for cDCE and VC co-oxidation, respectively. Overall, we demonstrated the feasibility of a treatment train combining reductive dehalogenation and aerobic co-oxidation processes in tandem to not only effectively clean up prevalent co-contamination of TCE and dioxane at trace levels but also mitigate persistent products (e.g., cDCE and VC) when complete reductive dehalogenation of less-chlorinated ethenes occurs slowly in the field.

1. Introduction

Chlorinated volatile organic compounds (cVOCs) are the most prevalently detected organic contaminants in aquifers, overburdens, and soils. Once released to the subsurface, cVOCs interact with aquifer materials through dynamic adsorption and desorption processes

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governed by their relatively low solubility and high hydrophobicity (USEPA, 1996). Trichloroethene (TCE) in particular is of great concern because it is a potent mutagen and can generate toxic metabolites, such as *cis*-dichloroethene (cDCE), *trans*-dichloroethene (tDCE), and the carcinogenic vinyl chloride (VC), via biotic and abiotic degradation (McCarty, 1993). Thus, United States Environmental Protection Agency (USEPA) has enforced a stringent regulation of TCE at a maximum contaminant level (MCL) of 5 μg/L (USEPA, 2004), stimulating extensive research and engineering efforts in TCE remediation.

With the discovery of reductive dehalogenation (Maymó-Gatell et al., 1997; Vogel and McCarty, 1985), anaerobic bioremediation has emerged as a feasible and economical alternative for in situ treatment of chlorinated solvents, particularly TCE. For instance, SDC-9 (Aptim, Inc., Lawrenceville, NJ) is a commercialized consortium consisting of halorespiring bacteria, which belong to genera *Dehalococcoides* and *Desulfitobacterium*, and many other associated microorganisms, enabling the sequential and complete reductive dehalogenation of TCE to cDCE, VC, and eventually the non-toxic ethene (Dang et al., 2018). SDC-9 thus has been widely used as the bioaugmentation inoculum for in situ bioremediation of TCE and other highly chlorinated cVOCs at hundreds of impacted sites with varying geochemical conditions (Vainberg et al., 2009; Schaefer et al., 2010).

However, two issues have been frequently reported at sites where anaerobic bioremediation is implemented, underscoring the need for effective solutions. First, once TCE is reduced, dehalogenation of cDCE and VC occurs at a slower pace in the field, conducive to the prolonged occurrence of these toxic degradation byproducts (Dang et al., 2018; Griffin et al., 2004; MacFarlane et al., 2011; Abe et al., 2009). Though the use of bioaugmentation with halorespiring cultures is quite effective in mitigating daughter product generation at many sites, lingering daughter products for months or years remains a concern at certain sites (Dang et al., 2018). This possibly pertains to the insufficiency of bacteria that are efficient in reducing cDCE or VC to ethene (Ellis et al., 2000), deficiency of electron donors (e.g., hydrogen) (Conrad et al., 2010; Panagiotakis et al., 2007), slow kinetics restricted by low concentrations of these intermediate compounds (Cupples et al., 2004), and/or competition with indigenous microorganisms for electron acceptors (e.g., sulfate and iron (III)) (Lovley et al., 1995; Pfennig et al., 1981). The other issue is the concurrence of trace levels of 1,4-dioxane (dioxane), an anthropogenic cyclic ether used for stabilizing chlorinated solvents (Mohr, 2004). Co-contamination of TCE and dioxane has been reported across the US and globally. Anderson reported that 93.5% (730 out of 781) of TCE-impacted sites were co-contaminated with dioxane based on the monitoring data from 4196 United States Air Force (USAF) sites (Anderson et al., 2012). Similarly, Adamson investigated over 2000 sites in California. Among the 605 sites with positive detection of dioxane, 94% were co-contaminated by TCE and/or 1,1,1-trichloroethane (TCA) (Adamson et al., 2014). Though many Actinomycetes, such as *Pseudonocardia dioxanivorans* CB1190 (Parales et al., 1994) and *Mycobacterium dioxanotrophicus* PH-06 (Kim et al., 2009; He et al., 2017), have been identified for their capability of metabolizing dioxane as the sole carbon and energy source, their viability and activity are much restricted by the low concentrations of dioxane prevailing in the field (Barajas-Rodriguez and Freedman, 2018; Barajas-Rodriguez et al., 2019). Further, the presence of cVOCs at high concentrations (e.g., >1 mg/L) can negatively affect the performance of aerobic dioxane degradation given their potency to inhibit key catalytic enzymes (e.g., soluble di-iron monooxygenases [SDIMOs]) (Mahendra et al., 2013; Li et al., 2019) and trigger universal cellular stress (Zhang et al., 2016). Therefore, elimination of co-occurring cVOCs, especially TCE, can be a prerequisite to achieving an efficient biotreatment of dioxane.

In this present study, we designed and demonstrated a sequential treatment strategy (Fig. 1) that can effectively reduce TCE first by SDC-9 under anaerobic conditions and then oxidize dioxane and other persisting cVOCs by *Azoarcus* sp. DD4 (Deng et al., 2018) under aerobic conditions. Polasko et al. reported a consortium mixed with KB-1 and CB1190 can degrade TCE and dioxane (at ~3.5 mg/L) in tandem with no accumulation of cDCE (Polasko et al., 2019). In the interim of this treatment, pure oxygen was injected as a secondary step for a quick transition from anaerobic to aerobic conditions so that the degradation of dioxane and cDCE can be initiated (Polasko et al., 2019). Unlike this previous work, our treatment train is technologically distinctive, because (1) DD4 is employed as a cometabolic dioxane degrader that is efficient to remove dioxane at low concentrations (e.g., 20 μg/L) relevant for many contaminated sites (Adamson et al., 2014; Stroo and Ward, 2010), (2) DD4 is inoculated after the completion of the initial anaerobic treatment, in which microcosms are air sparged without exposing DD4 to undesirable anaerobic conditions, and (3) DD4 exhibits several superior physiological properties suited for in situ applications (e.g., fast planktonic growth and compatibility with aquifer environments)

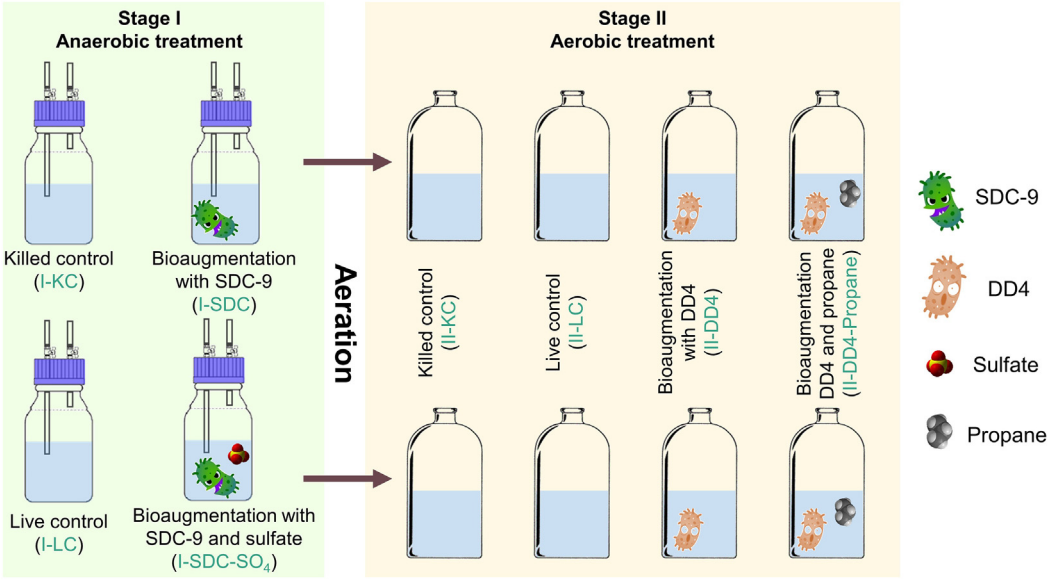

Fig. 1. Experimental scheme of the sequential anaerobic-aerobic treatment in this microcosm study. Killed control (KC) and live control (LC) were designed for both anaerobic (stage I) and aerobic (stage II) treatments. After the anaerobic treatment, samples from I-SDC or I-SDC-SO_4 were aerated, pooled, and split to prepare the stage II aerobic treatments.

(Deng et al., 2018) and expresses a diversity of SDIMOs that can degrade cVOCs (e.g., 1,1-DCE) and other co-existing contaminants (e.g., toluene) (Deng et al., 2019; Deng et al., 2020). In our microcosms mimicking the anaerobic/aerobic sequential treatment, abundances of key degraders are monitored using 16S rRNA gene amplicon sequencing and quantitative polymerase chain reaction (qPCR). Environmental implications and molecular foundations are further characterized for DD4 cometabolism using wild-type and mutant strains in which respective SDIMO genes were deleted using homologous recombination assisted by triparental mating. Collectively, our bioremediation treatment train combining reductive dehalogenation and cometabolic oxidation has broad application potentials for the cleanup of many sites where high concentrations of TCE and trace concentrations of dioxane co-occur with accelerated removal of undesirable biotransformation byproducts (e.g., cDCE and VC) when they persist in the field.

2. Materials and methods

2.1. Chemicals and cultures

Neat TCE (>99.5%), cDCE (>99.5%), and dioxane (>99.8%), as well as VC (2000 μg/mL in methanol), were purchased from Sigma-Aldrich (St. Louis, MO). Propane (>99.5%) was provided by Airgas (Radnor, PA). Slow-release substrate (SRS) emulsified vegetable oil (EVO) and SDC-9 bioaugmentation culture commercially marketed as TSI DC™ were supplied from Terra Systems Inc. (TSI, Claymont, DE). The SDC-9 culture was maintained on sodium lactate and PCE in the reduced anaerobic mineral medium (Schaefer et al., 2010; Schaefer et al., 2009; Kucharzyk et al., 2020) and shipped in sealed containers to our lab for immediate use. *Azoarcus* sp. DD4 was isolated by our lab from an activated sludge sample collected at a local wastewater treatment plant in Northern New Jersey (Deng et al., 2018). DD4 was grown in the nitrate mineral salts (NMS) medium supplemented with propane as the sole carbon and energy source (Deng et al., 2018). Chemicals (>99.0%) for preparing the culture media were all purchased from Sigma-Aldrich (St. Louis, MO) without further purification.

2.2. Anaerobic microcosm assays

Groundwater and bedrock core samples were collected from a site located in central New Jersey in April 2017. This site was operated by a gas company and has been historically impacted by TCE contamination in a deep bedrock aquifer up to 61 m below the ground surface (BGS). Approximately 20 L of groundwater was collected in compliance with the NJDEP Low Flow Purging and Sampling Guidance from a monitoring well with the highest TCE concentration at the site according to the monitoring data archived in December 2016. Field recording data revealed pH and specific conductivity stabilized around 6.5 and 475 μS/cm during the sampling. The aquifer was slightly aerobic with dissolved oxygen (OD) of 2.8–4.8 mg/L and redox potential of 4.9–14.6 mV. The baseline concentrations (Table S1) of TCE, cDCE, and dioxane were 296 μg/L, 96 μg/L, and 6.45 μg/L, respectively, as analyzed by a commercial analytical lab. TOC, dissolved iron, and dissolved manganese in the groundwater sample were 1.9 mg/L, 0.7 mg/L, and 12.5 μg/L, respectively. The bedrock cores between 11.0 and 14.6 m BGS were collected during the drilling of the injection well. In order to minimize exposure to oxygen and volatilization of cVOCs, cores were kept with dry ice and crushed under the nitrogen blanket into about 2.5-cm pieces. The groundwater and bedrock samples were separately stored in bottles with 10 min of filtered nitrogen purging. Bottles were sealed with PTFE caps on site, preserved at 4 °C on ice, and transported to New Jersey Institute of Technology (Newark, NJ).

Four anaerobic treatments were prepared as Fig. 1 and Table S2, including (1) killed control (I-KC), (2) live control (I-LC), (3) bioaugmentation with SDC-9 (I-SDC), and (4) bioaugmentation with SDC-9 and sulfate amended (I-SDC-SO_4). Considering aquifers in the northeast US are rich in iron minerals, especially in New Jersey (Tedrow and Krieger, 1986), we initially postulate the addition of sulfate may enhance or accelerate the total TCE removal since sulfate can be reduced by sulfate-reducing bacteria (SRB) in SDC-9 to sulfide, which couples with ferrous ions leached from the iron-rich bedrock to form FeS particulates that abiotically react with TCE (He et al., 2010). Each treatment was prepared in triplicate with 410 mL of groundwater and 275 g of bedrock sample, leaving approximately 50 mL of headspace. TCE was spiked to 8.5 mg/L (equivalent to 64.7 μM, if all dissolved in the groundwater), resulting in around 25 μM as measured as the initial TCE concentration in the aqueous phase in all treatments. This indicated that the majority of TCE was adsorbed to the bedrock and evaporated into the headspace when partitioning equilibrium was initially established in microcosms. EVO (1000 mg/L) and magnesium hydroxide (60 mg/L) were added as the exogenous carbon source and the alkaline reagent to maintain the neutral or slightly basic pH (7.3–7.6), respectively. SDC-9 was inoculated to a final cell density of 2×10^8 CFUs/mL. A high concentration (3000 mg/L) of sodium azide was added as a biocide in the killed control. Magnesium sulfate heptahydrate was selected as the source of dissolved sulfate (sulfate concentration equivalent to 584 mg/L). Microcosms were set quiescently at room temperature (i.e., 24 ± 3 °C). Concentrations of TCE, cDCE, and VC in microcosm bottles were analyzed at a commercial lab using the EPA Method 8260C. This is a standard method for quantifying a wide span of cVOCs and other VOCs in aqueous samples using purge-and-trap gas chromatography/mass spectrometry (GC/MS). cDCE concentrations in one of the triplicate microcosms were not in good consensus with the others since Week 11 in Treatments I-SDC and I-SDC-SO_4, probably due to the variance in bedrock samples. These data were excluded from the analysis. When the anaerobic treatments were terminated, genomic DNA from treatments I-SDC and I-SDC-SO_4 were extracted for 16S rRNA gene amplicon sequencing and taxonomic analysis (see supplementary data).

2.3. Aerobic microcosm assays

After the removal of TCE, two sets of anaerobic treatments, bioaugmentation of SDC-9 without amendment of sulfate (I-SDC) and bioaugmentation of SDC-9 with the amendment of sulfate (I-SDC-SO_4), were selected for sequential treatment of dioxane via aerobic cometabolism by DD4 (Fig. 1). Anaerobic bottles were uncapped and exposed to air for 30 min until the liquids became aerobic with positive oxidation-reduction potential (ORP) (>50 mV) and high dissolved oxygen (DO) (>8.0 mg/L) as measured by Xplorer GLX datalogger (PASCO scientific, Roseville, CA). For either anaerobic treatment (I-SDC or I-SDC-SO_4), groundwater and bedrock samples were removed from these triplicated anaerobic microcosms and pooled to result in approximately 700 mL of anaerobically treated groundwater and 750 g of bedrock. During aeration and sample assortment, loss of cVOCs and other compounds might occur due to volatilization.

Each aerobic microcosm was prepared in the 160-mL serum bottle containing 50 mL of water sample and 25 g of bedrock that have been previously treated under the anaerobic condition. The aqueous samples were spiked with dioxane to achieve an initial concentration of 20 μg/L. As shown in Fig. 1 and Table S3, four treatments were prepared, including killed control (II-KC), live control (II-LC), and DD4 bioaugmentation with (II-DD4-Propane) or without (II-DD4) propane amendment. All treatments were conducted in triplicate. DD4 was harvested at the exponential phase after being cultured in NMS media with propane as the sole carbon source. Cells were washed with the phosphate-buffered saline (PBS) buffer twice and resuspended to an OD_{600nm} of 2.0. For the two sets of DD4 bioaugmentation microcosms (II-DD4-Propane and II-DD4), 0.5 mL of the harvested cell were inoculated, resulting in an initial protein concentration of 0.17 mg per vial (equivalent to 1.5×10^6 CFU/mL). Propane (0.10% *v*/v equivalent to 2.0 mg/L in

headspace) was amended to one set of DD4 bioaugmentation microcosms (II-DD4-Propane) as the carbon supplement, while no additional substrates were added to the other bioaugmentation set (II-DD4). Live control (II-LC) was prepared without the addition of DD4 or propane (Table S3). Microcosms were incubated at room temperature (i.e., 24 ± 3 °C) while being shaken at 150 rpm. At selected intervals, liquid and headspace samples were collected for the analysis of dioxane and propane by GC/MS and GC (see supplementary data), respectively. The relative abundance of DD4 was enumerated by quantitative PCR (qPCR) analysis as detailed in the supplementary data.

2.4. Biotransformation of VC and cDCE by DD4 and their inhibitory effects to dioxane degradation in DD4

Given the observation of cDCE and VC being generated from the anaerobic treatment of TCE, growing and resting cells of DD4 were used to (1) investigate the degradation capability of DD4 on VC and cDCE and (2) assess their impacts on dioxane degradation. DD4 cells were prepared in 20 mL NMS medium in a 160-mL serum bottle with 4 mL propane (2.86% v/v equivalent to 57.1 mg/L in headspace) amended. Cells were harvested and resuspended with the fresh NMS medium to an OD_{600nm} of ~2.0. Growing cell assays were prepared with 0.1 mL of resuspended DD4, inoculated to 10 mL groundwater sample spiked with 10 mg/L of dioxane, 1 mg/L of cDCE, and 1 mg/L of VC. As an auxiliary substrate, 150 μL of propane was amended at the beginning and when propane concentration was lower than 0.2 mg/L in headspace. For resting cell assays, resuspended DD4 with an OD_{600nm} of 2.0 was exposed to dioxane of an initial concentration of 10 mg/L in 5 mL of the NMS medium in 30-mL serum bottles. cDCE or VC was spiked to reach an aqueous phase concentration of 0.35 mg/L. Control treatments for the growing and resting cell assays were prepared in identical setups, but with no inoculation of DD4. Concentrations of dioxane, cDCE, and VC were monitored by the analytical methods described in the supplementary data. Method detection limits for the analysis of dioxane, cDCE, and VC were estimated as 0.4, 50, and 50 μg/L, respectively.

2.5. cDCE and VC transformation assays in DD4 mutants

To investigate the genes responsible for cDCE and VC degradation in DD4, *prm*-, *bmo*-, and *tmo*-deleted mutants were constructed using in-frame deletion via homologous recombination. Experimental details were described in Deng et al. (Deng et al., 2020). Briefly, the recombinant insert carrying the upstream and downstream regions of the target gene was cloned into the suicide plasmid pK18mobsacB with kanamycin resistance and *sacB* counterselection. This plasmid with the recombinant insert was transformed into wild-type DD4 by triparental conjugation with the assistance of the helper strain *Escherichia coli* HB101 carrying the mobile plasmid pRK2013. Successful exconjugants were obtained through two rounds of screening: first on VM-ethanol agar plates containing 25 μg/mL kanamycin and then on VM-ethanol agar plates with 10% sucrose. Knock-out of the target gene in DD4 mutants was examined using colony PCR and gel electrophoresis.

After pre-growth with ethanol (200 mg/L) in NMS media, wild-type or mutant DD4 clones were cultivated and washed three times with 1 × PBS. Transformation assays were performed in 35-mL serum vials containing 4.5 mL of 1 × PBS and 0.5 mL of cell suspensions. The initial biomass was estimated as 1.5 mg total protein per vial. In the beginning, cDCE and VC were dosed at 1.0 and 0.3 mg/L as the equilibrium aqueous phase concentrations, respectively. Abiotic controls were prepared without cell suspensions. All treatments were conducted in triplicate. At selected incubation time, 100 μL headspace samples were removed and analyzed for the disappearance of cDCE and VC by GC/MS.

3. Results and discussions

3.1. TCE was transformed to cDCE and VC by SDC-9 in anaerobic microcosms

After amendment with SDC-9, TCE was completely reduced to cDCE and VC within the first 4 weeks of incubation (Fig. 2). No significant decrease of TCE was observed in killed or live controls, suggesting the lack of intrinsic abiotic reactions or biodegradation by indigenous halorespirers. In Week 4, TCE concentration in the SDC-9 augmented

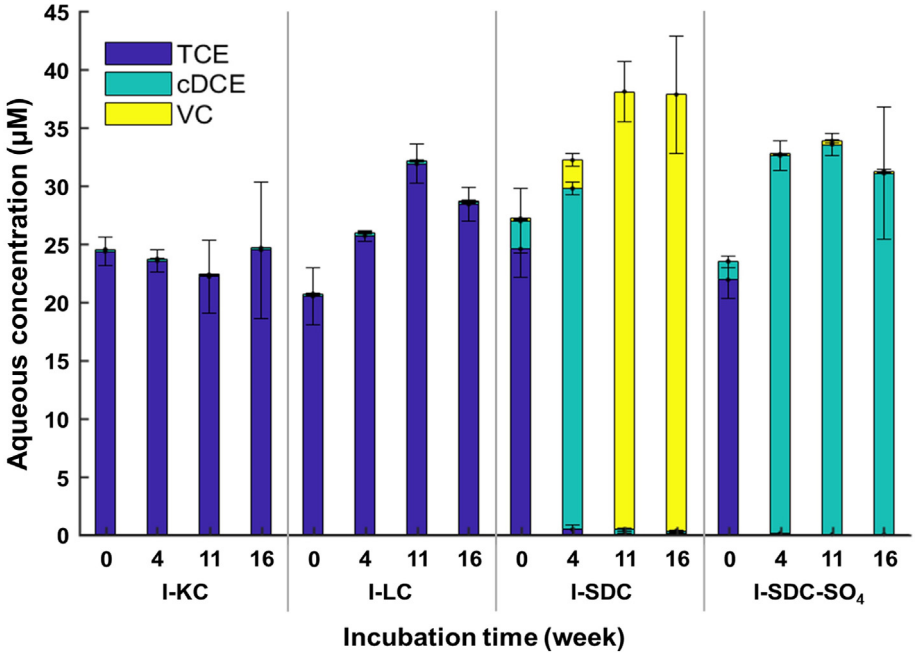

Fig. 2. cVOCs monitoring during the anaerobic treatments in killed control (I-KC), live control (I-LC), and bioaugmentation microcosms amended with SDC-9 (I-SDC), and with both SDC-9 and sulfate (I-SDC-SO_4), respectively. Blue, green, and yellow bars represent the concentration of TCE, cDCE, and VC in μM, respectively.

treatment (I-SDC) decreased from 24.56 ± 2.46 to 0.47 ± 0.38 μM with the formation of an equivalent molar amount of cDCE (~26.9 μM). SDC-9 was able to continue the reductive dehalogenation and transform cDCE mostly to VC. From Week 4 to Week 11, cDCE concentration decreased from 29.30 ± 0.58 μM to 0.39 ± 0.14 μM, while VC concentration increased from 2.43 ± 0.54 μM to the highest 37.61 ± 2.57 μM. Higher VC and cDCE observed in the aqueous phase than the initial TCE were probably due to their lower partitioning coefficiencies to the bedrock (NJDEP, 2010) and biotransformation of the back-diffused TCE when the aqueous TCE was being depleted by SDC-9. From Week 11 to Week 16, VC persisted in the SDC-9-bioagumented microcosms with no further significant concentration decrease. We thus decided to terminate the microcosms to assess the microbial communities and proceed with the secondary aerobic treatment (Fig. 1) as described in the following sections. The unconsumed VC over the last 4 weeks of the anaerobic incubation may reflect its slow transformation in line with the observations of the prolonged existence of VC for months to years at some sites (e.g., Indian Head, Md and Quantico, VA) where SDC-9 was employed for bioaugmentation (Dang et al., 2018). It is likely, based on the many successful experiences demonstrating fast kinetics and complete dechlorination by SDC-9 or associated consortia (Schaefer et al., 2010; Schaefer et al., 2009; Kucharzyk et al., 2020), that VC would be further reduced to ethene. Unfortunately, ethene was not monitored given the unavailability of appropriate analytical instrumentation during the period of this experiment. Three possible reasons may partially explain the slow or incomplete dehalogenation observed in our microcosms: (1) reduction of VC to ethene is thermodynamically less favorable and thus much slower compared to prior reduction steps (i.e., from TCE to cDCE and from cDCE to VC) (Smidt and de Vos, 2004; Futagami et al., 2008; Marcus and Bonds, 1999); (2) halorespirers are sensitive to certain geochemistry factors (e.g., pH and DO) and co-contaminants (e.g., heavy metals) in the environment (Vainberg et al., 2009; Townsend and Suflita, 1997; Liu et al., 2017; Dries et al., 2005); and (3) fastidious growth of VC degrading microorganisms can be restricted by the competition of indigenous strains (Maymó-Gatell et al., 2001). 16S rRNA gene amplicon sequencing analysis (Fig. S1) revealed the existence of two well-known halorespiring bacteria, *Dehalococcoides* and *Desulfuromonas*, reflecting their essential roles in reductive dechlorination of TCE to cDCE or VC (Dang et al., 2018). Some other bacteria (e.g., *Geobacter* and *Anaeromyxobacter*) that may assist dehalogenation in the I-SDC microcosms were further discussed in the supplementary data.

In the anaerobic treatment I-SDC-SO_4 that received both SDC-9 and sulfate (584 mg/L), TCE was rapidly transformed from 21.92 ± 1.61 to 0.14 ± 0.03 μM within the first four weeks. Concurrently, cDCE increased from the initial of 1.57 ± 0.50 μM to 32.46 ± 1.26 μM. However, neither reduction of cDCE nor generation of VC was noticed after Week 4. Therefore, the addition of sulfate may interfere with the sequential reduction of cDCE to VC, probably due to the outcompetition of halorespiring bacteria by sulfate reducing bacteria (SRB). After the addition of sulfate, the total SRB increased to nearly 7% of the total bacteria (Fig. S1), including *Desulfoprunum* (6.38%), *Desulfovirga* (0.33%), *Desulfovibrio* (0.16%), *Desulfbulbus* (0.03%), *Desulfatiferula* (0.03%). In contrast, in the I-SDC microcosms where sulfate was not amended, the relative abundance of total SRB was as low as 0.05%. Furthermore, the amendment of sulfate also greatly reduced the abundance of *Dehalococcoides* from 0.020% (in I-SDC) to 0.007% (in I-SDC-SO_4) (Fig. S1). As *Dehalococcoides* are key contributors to the reduction of cDCE to VC (Becker, 2006), the decrease of their abundance could be conducive to the absence of cDCE reduction or VC formation as observed in the I-SDC-SO_4 treatment. In these microcosms, though formation of FeS was observed as dark precipitates (Fig. S3), contribution of FeS-derived abiotic transformation of TCE was minor, contrary to our initial hypothesis (see detailed discussion in the supplementary data). Even worse, sulfate served as an alternative electron acceptor and exerted a significant selection on SRB, prohibiting reductive dechlorination due to their rapid and competitive utilization of electron donors (Aulenta et al., 2008; Drzyzga et al., 2001).

Dioxane was persistent over the course of anaerobic treatments as no significant dioxane concentration change was observed in all anaerobic microcosms (data not shown). To date, anaerobic treatment of dioxane remains elusive. Thus, a subsequent aerobic treatment by DD4 was conducted to mitigate dioxane residual after the anaerobic treatment of TCE.

3.2. DD4 effectively eliminated dioxane and sustained its abundance in aerobic microcosms

Dioxane in the field groundwater previously treated anaerobically with SDC-9 was efficiently removed by DD4 when propane was initially supplemented. Within 32 days of incubation, dioxane was degraded from 20.9 ± 0.1 μg/L to below our MDL (i.e., 0.4 μg/L), meeting stringent groundwater cleanup guidance in NJ (i.e., 0.4 μg/L) and the screening level suggested by USEPA (i.e., 0.67 μg/L). Propane (300 μL) was supplemented twice to achieve a complete dioxane removal (Figs. 3A and S2).

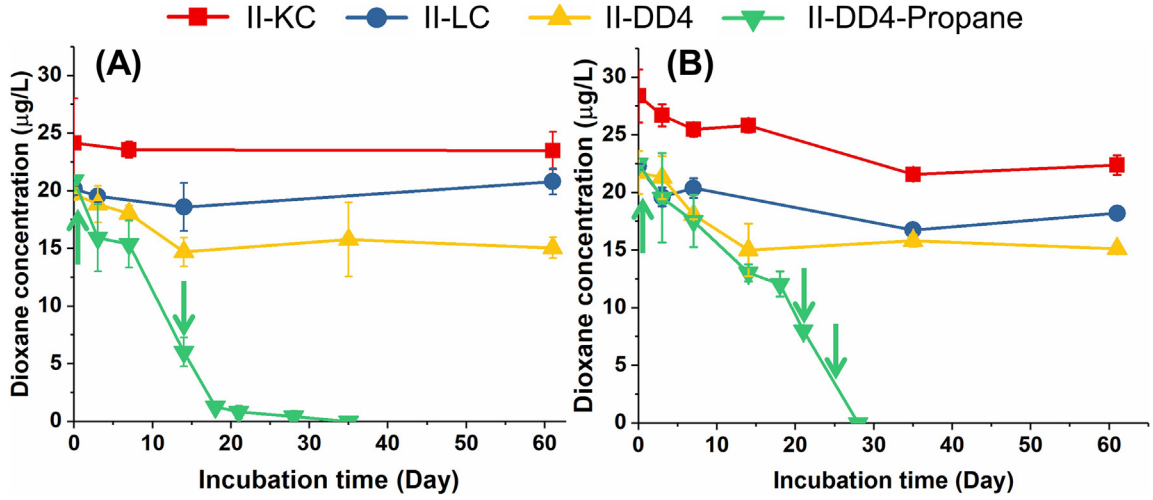

Fig. 3. Dioxane depletion in the aerobic treatments, including killed control (II-KC), live control (II-LC), bioaugmentation with DD4 (II-DD4), and bioaugmentation with DD4 and propane (II-DD4-Propane). The aerobic microcosms were prepared with samples from the previous anaerobic treatment of (A) SDC-9 without sulfate (I-SDC) and (B) SDC-9 with sulfate amended (I-SDC-SO_4). Green arrows indicate the addition of propane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Absolute qPCR analysis (Fig. 4) revealed a high abundance (6.7%) of DD4 over the course of bioaugmentation treatment with the propane supplement.

Though at a slowed degradation rate, complete dioxane was also achieved by propane-fed DD4 in the microcosms prepared with aquifer samples previously treated with both SDC-9 and sulfate (I-SDC-SO_4) (Fig. 3B). Interestingly, the initial propane consumption was unexpectedly fast. Around 77% of the initially dosed propane was quickly removed in the first 2 days of incubation (Fig. S2). Later, propane consumption was markedly slowed down, taking 21 days for DD4 to completely degrade the rest of propane that was amended at the beginning (Fig. S2). Concurrently, dioxane was degraded from 22.5 ± 0.4 μg/L to 8.1 ± 0.1 μg/L (Fig. 3B). In comparison, in aerobic microcosms prepared with the samples treated with SDC-9 but no sulfate (I-SDC), only 12 days were spent to fully deplete the initial propane with a similar dioxane removal to 8.4 ± 1.0 μg/L (Fig. 3A). The difference in the initial propane consumption in microcosms prepared with I-SDC and I-SDC-SO_4 samples cannot be explicitly explained, though may be related to certain abiotic or biotic factors derived from previous anaerobic treatment with the excessive sulfate supplement. Within 28 days of active treatment in the aerobic microcosms prepared with the I-SDC-SO_4 samples, propane was added three times resulting in a total amendment of 450 μL (Fig. S2). This third amendment of propane also greatly accelerated dioxane degradation from Day 25 (Fig. 3B) and enriched a higher abundance of DD4 which is 30.4% in I-SDC-SO_4 in comparison with 6.7% in I-SDC (Fig. 4). Though native propane-degrading aerobes might be present in DD4-bioaugmented microcosms, their contribution to propane consumption or dioxane removal was presumably minor considering the fact that groundwater and bedrock samples were previously anaerobically treated.

Interestingly, even without the amendment of propane as the exogenous carbon source, there was over 25% disappearance of dioxane in DD4 bioaugmented treatments (II-DD4) within the first 2 weeks of incubation (Fig. 3). However, dioxane degradation ceased after 14 days. This suggests DD4 may be able to exploit carbon residuals (e.g., EVO and its fermentation metabolites) and/or carry out endogenous growth (i.e., utilization of cell debris of SDC-9) (Adamson et al., 2011) from the previous anaerobic treatments to empower the co-oxidation of dioxane. qPCR analysis (Fig. 4) revealed a relatively low but stable abundance of DD4, which were 2.6% and 2.7% in microcosms that received I-SDC and I-SDC-SO_4 samples, respectively. No significant dioxane degradation was observed in either killed or live controls (Fig. 3).

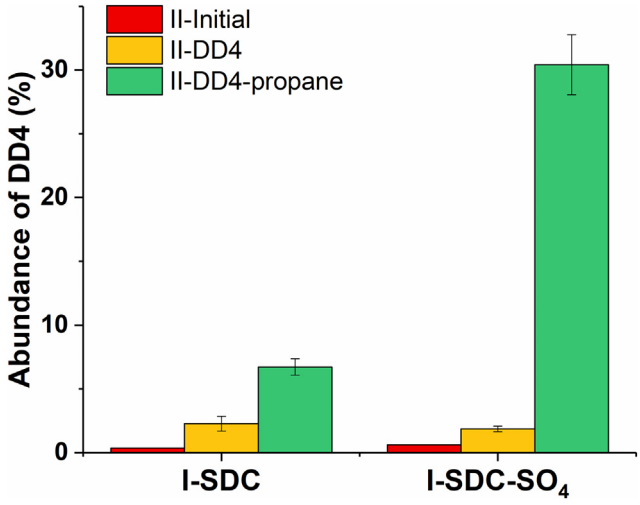

Fig. 4. Relative abundance of DD4 estimated by qPCR in microcosms at the beginning (II-Initial) and end of the aerobic treatments by DD4 (II-DD4) and DD4 with propane (II-DD4-propane). The x-axis indicates the samples were previously anaerobically treated by SDC-9 without sulfate (I-SDC) or with sulfate (I-SDC-SO_4).

Bioaugmentation with gaseous alkane degraders has been examined and employed at a few sites as a cost-efficient approach for in situ treatment of dioxane (Rolston et al., 2019; Hatzinger et al., 2017; Lippincott et al., 2015). However, it should be noted that gaseous cometabolism for dioxane removal remained at the research level and has been applied much less frequently than reductive dehalogenation for groundwater bioremediation (Zhang et al., 2017). In this study, we verified propane as an auxiliary substrate can provide sufficient energy for DD4 enabling it becomes the dominant bacteria among the indigenous community. Effectiveness of in situ bioaugmentation is affected by (1) the types of substrate delivery methods (solubilization in recirculated groundwater or sparging), (2) different propane concentrations, and especially (3) microbial inocula (Chu et al., 2018). Unlike the gram-positive propanotrophs, DD4 is a planktonic gram-negative microorganism that may exhibit better distribution and thus greater remediation radius once injected at contaminated sites (Deng et al., 2018). Further assessments engaging column studies and pilot- and field-scale tests are in need to warrant and optimize the in situ treatment performance of DD4 bioaugmentation.

3.3. DD4 entailed cometabolic degradation of cDCE and VC, two main accumulating products from TCE dehalogenation

During the aerobic treatments by DD4, it is interesting that residual cDCE and VC were also removed along with dioxane degradation. After the primary anaerobic treatments by SDC-9 and aeration, we detected 1.4 μg/L of VC and 6.7 μg/L of cDCE remained in the aqueous phase of the aerobic microcosms prepared with treated samples from I-SDC and I-SDC-SO_4, respectively. Notably, neither VC nor cDCE was detectable after the II-DD4-Propane treatment. To further verify the ability of DD4 to cometabolize cDCE or VC, biotransformation assays were employed using DD4 resting cells. After incubation, cDCE and VC were both fully degraded by DD4 within 20 h and 5 h, respectively, when their initial concentrations were dosed at around 0.35 mg/L (Fig. 5A). No significant loss of cDCE or VC was observed in the abiotic control treatments, therefore confirming that direct oxidization of cDCE and VC did not occur. Additional biotransformation assays revealed propane-fed DD4 was not able to cometabolize TCE (data not shown).

To further mimic the commingled contamination observed in the field, growing cell assays were further conducted with SDC-9-treated groundwater that was dosed with all three co-contaminants, VC (1 mg/L), cDCE (1 mg/L), and dioxane (10 mg/L). DD4 was inoculated at a relatively low concentration (0.0034 mg protein/mL) of DD4 and fed with 2 mg/L propane. Within the first 9 days of incubation, VC was primarily degraded from 1.11 ± 0.02 mg/L to 0.13 ± 0.08 mg/L, achieving 88.3% removal (Fig. 6A). Complete depletion of VC was observed later on Day 15. Concurrently, cDCE and dioxane were degraded much slower than VC. cDCE was degraded from 1.06 ± 0.02 mg/L to 0.80 ± 0.02 mg/L on Day 9, and 0.39 ± 0.11 mg/L on Day 15. Only 18.1% of dioxane was removed in the first 15 days. After cDCE was degraded to as low as 0.03 ± 0.01 mg/L on Day 18, dioxane degradation greatly accelerated. Dioxane was then degraded to below 0.1 mg/L on Day 30. This is the first report of a gram-negative propanotroph that can synchronize the removal of dioxane, cDCE, and VC.

A parallel treatment was dosed with dioxane only. Without the presence of cDCE or VC, complete dioxane removal was achieved within 5 days (Fig. 6B). The consumption of propane was also much faster, suggesting a greater growth of DD4 and steady enzyme expression. The initial dose of propane was fully depleted in 2 days when DD4 was not exposed to cDCE or VC. However, when both cDCE and VC were present, it took 15 days for the complete consumption of the same amount of propane. The prolonged propane consumption and dioxane degradation reflected the potential inhibitory efforts of cDCE and VC to DD4, even though both cVOCs can be fortuitously degraded by this propanotrophic bacterium via cometabolic pathways.

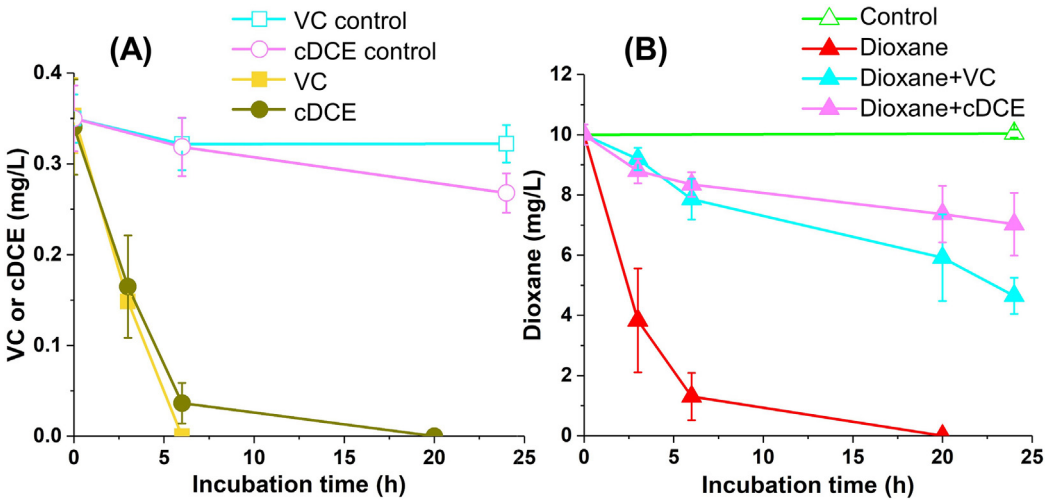

Fig. 5. Degradation of dioxane by DD4 resting cells with or without the presence of VC and cDCE. The concentrations of cDCE and VC were shown in (A) and dioxane concentration was shown in (B).

3.4. cDCE was more potent in inhibiting dioxane degradation by DD4 than VC

To assess the inhibitory effects of cDCE and VC, either compound was exposed to DD4 resting cells at an initial dosage of 0.35 mg/L. Significant inhibition to dioxane degradation was observed for both compounds in comparison with the control that received no cVOCs (Fig. 5B). Without the presence of VC or cDCE, the resting cells of DD4 completely degraded 10.0 ± 0.3 mg/L of dioxane in 20 h. However, in 24 h of incubation, dioxane concentration remained as high as 4.7 ± 0.6 mg/L and 7.0 ± 1.0 mg/L for VC-exposed and cDCE-exposed DD4 cells, respectively. Thus, cDCE posed a more potent inhibition to DD4 since the dioxane degradation rate was significantly slower ($p < 0.05$) than that observed in VC-exposed cells.

A number of previous investigations have reported the inhibitory effects of cDCE and VC on aerobic biodegradation of dioxane or other groundwater contaminants. It was reported that 5 mg/L of cDCE showed significant inhibition to dioxane degradation by CB1190, and dioxane degradation completely halted at 50 mg/L of cDCE. Inhibitory effects of cDCE may be attributed to universal stress triggered by this compound (Zhang et al., 2016). To date, no previous studies have reported the inhibitory effects of VC on dioxane biodegradation. As a mutagen, VC can disrupt bacterial metabolism (Chiang et al., 1997) and their abilities to degrade cVOCs (Gaza et al., 2019). A prior microcosm study observed reversible and irreversible inhibitions by VC (~5.0 mg/L) to aerobic cometabolism of TCE and cDCE, respectively (Schmidt et al., 2014). Similarly, Zhao et al. reported that the rate of cDCE (60 μM) degradation decreased with the increase of VC concentrations (from 10 to 110 μM) (Zhao et al., 2010). Collectively, degradation of VC that occurred prior to cDCE and dioxane as observed in our microcosms may result from the combination of high affinity to its degrading enzyme in DD4 and less potent inhibitory effects so cells can overcome the inhibition of VC first via cometabolic decomposition.

3.5. Tmo and prm are responsible for the co-oxidation of cDCE and VC

According to previous studies, the observed VC and cDCE cometabolism in DD4 may be attributed to the catalysis of SDIMOs (Fathepure et al., 2005). There exist five putative SDIMO-encoding genes in DD4, including *prmABCD* encoding a group-5 propane monooxygenase (MO), *bmoXYBZDC* encoding a group-3 butane MO, *tmoABCDEF* encoding a group-2 toluene MO, and *dmp1KLMNOP* and

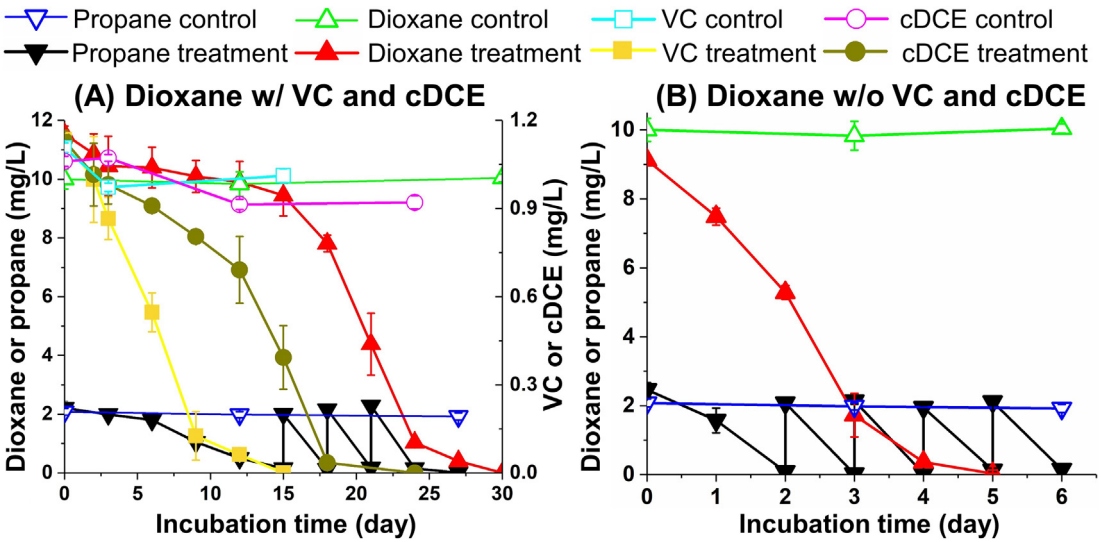

Fig. 6. Cometabolic degradation of dioxane (10 mg/L) by DD4 in anaerobically pretreated groundwater with (A) or without (B) the presence of VC (1 mg/L) and cDCE (1 mg/L).

dmp2KLMNOP encoding two group-1 phenol hydroxylases (Deng et al., 2019; Deng et al., 2020). In our recent work, three DD4 mutants were successfully created with the deletion of *prm*, *bmo*, and *tmo* gene clusters, respectively (Deng et al., 2020). To investigate the roles of these three SDIMOs in cDCE and VC degradation, these three knockout mutants were cultivated and exposed to cDCE or VC in comparison with wild-type DD4 as the positive control.

As shown in Fig. 7A, deletion of the *tmo* gene cluster was conducive to the complete loss of the ability to degrade cDCE. In contrast, no significant impact was observed in the other two knockout mutants compared to the wild-type DD4 within the first 6 h of incubation. These results revealed that *tmo*-encoding group-2 toluene MO is solely responsible for the co-oxidation of cDCE. This toluene MO also takes part in the initial catabolism of toluene and propane, as well as cometabolism of dioxane and 1,1-DCE (Deng et al., 2020). As this single toluene MO is in charge of co-oxidation of both cDCE and dioxane, they may compete for the available enzyme active sites when both contaminants co-exist in the environment, explaining the tandem degradation order for cDCE and dioxane as observed in wild-type DD4 (Fig. 6A). Furthermore, expression of this toluene MO can be upregulated by propane, toluene, and their primary hydroxylation products (Deng et al., 2020). Thus, propane and other inducing compounds can be effective auxiliary substrates that sustain the growth of DD4 and its degradation activity toward dioxane and other co-occurring chloroethenes (e.g., cDCE and 1,1-DCE) through cometabolic pathways.

Further assays with these mutant clones indicated VC degradation in DD4 is associated with the *prm* gene cluster, encoding the group-5 propane MO (Fig. 7B). The *prm*-deleted mutant exhibited no significant removal of VC compared to the abiotic control. However, wild-type DD4 can quickly degrade approximately 0.3 mg/L VC within 6 h. VC biotransformation in mutants detected with *tmo* or *bmo* was slightly slowed down with the complete removal prolonged to 20 h. This is probably because deletion of either gene may negatively affect the overall degradation performance of resting cells by diminishing cellular energy efficiency or sacrificing their ability to cope with VC as a potent mutagen. SDIMO-harboring bacteria are known for their capability of degrading VC. *Mycobacterium vaccae* JOB5 (Wackett et al., 1989) and *Burkholderia cepacia* G4 (Iwashita et al., 2002) are the two SDIMO-expressing bacteria that can transform VC to VC epoxide. *Rhodococcus rhodochrous* ATCC21197 and *Mycobacterium aurum* L1 were also reported to degrade VC aerobically (Malachowsky et al., 1994; Hartmans and De Bont, 1992). To our knowledge, this is the first study to prove a group-5 propane MO is responsible for VC co-oxidation in a gram-negative bacterium.

4. Conclusions

Co-contamination of TCE (~ ppm level) and dioxane (~ ppb level) in groundwater is prevailing at sites in the US and globally. This bench-scale microcosm study demonstrated an anaerobic and aerobic treatment train as a potential solution to mitigate this challenging co-contamination issue in an accelerated timeframe. In situ bioaugmentation with halorespiring cultures, such as SDC-9 and KB-1, have been widely used at thousands of TCE-impacted sites. After the primary anaerobic bioaugmentation treatment, aerobic bioaugmentation with DD4 can not only degrade the lingering dioxane but also expedite the removal of undesirable metabolites (e.g., cDCE and VC) commonly generated from reductive dehalogenation. As indicated in our previous work, DD4 has also demonstrated a spectrum of properties compatible with in situ remediation technologies (e.g., biosparging), spanning fast planktonic growth, and the ability to exploit trace nutrients and adapt to diverse aquifer environments. The endured viability and activity of this strain in environmental samples pretreated with anaerobic procedures were also validated in this study. Though high concentrations of cDCE and VC may negatively affect dioxane cometabolism in DD4, DD4's versatile catalytic capability allowed it to decompose these inhibiting compounds. Through engineering approaches (e.g., recirculation and air injection), the ability of DD4 to conquer field inhibitory factors can be reinforced to accelerate the site remediation and meet stringent cleanup goals for both cVOCs and dioxane. Further column and pilot studies are underscored for scaling up the application of DD4 bioaugmentation and optimizing its treatment effectiveness in the field.

Note that a few Actinomycetes (e.g., *Mycobacterium vaccae* JOB5) can concurrently degrade TCE and dioxane via cometabolism (Hand et al., 2015). However, limitations exist for the field application of Actinomycetes since they like to form clumps, hindering the subsurface distribution (Deng et al., 2018; Lippincott et al., 2015). Coexistence of TCE at ppm levels can also inhibit enzyme activities and their expression (Mahendra et al., 2013; Li et al., 2019; Zhang et al., 2016). Cometabolic degradation of TCE may also generate epoxide products that pose potent toxicity to the degraders (Hand et al., 2015). Thus, primary anaerobic treatment to eliminate TCE is likely to be beneficial to enhance the subsequent removal of dioxane when considering that reductive dechlorination has been and is being predominantly employed at numerous TCE-impacted sites worldwide. Further, some recent field studies demonstrated the effectiveness of biostimulation with propane and other alkane gases (e.g., isobutane) in removing dioxane, TCE, and some other co-existing contaminants (Chu et al., 2018; Hatzinger et al.,

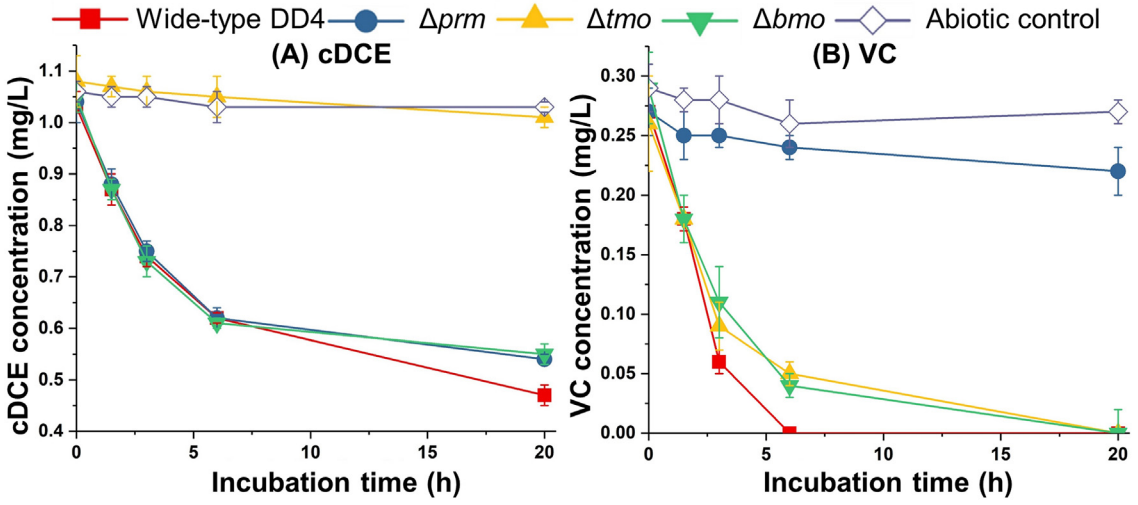

Fig. 7. cDCE and VC degradation by resting cells of wild-type and SDIMO gene-deleted DD4 that were precultured with ethanol. Abiotic controls were prepared without cells. Data are the average of triplicates and error bars indicate their standard deviation.

2018). It should be noted that such cometabolizers are not ubiquitous in aquifers and it often involves a long span of acclimation (typically a couple of months). Nonetheless, our treatment train provides an effective alternative to speed up the field remediation with minimal increment in engineering efforts.

At many bedrock formations like the one tested in this study, iron can be leached to the aqueous phase in the aquifer to supplement biotic and abiotic processes. Previous studies have demonstrated FeS can mediate abiotic transformation of TCE, contributing to the removal of this resistant compound. Through the addition of excessive sulfate, we intend to integrate this abiotic TCE removal into the anaerobic biotreatment. Unfortunately, the sulfate amendment in our bench-scale microcosm assays didn't appear sufficiently benefitial for cVOC or dioxane removal in the iron-rich bedrock samples collected from the site of interest. First, sulfate stimulated the growth of SRB in SDC-9. The dominated SRB outcompeted the halorespirers, especially those in charge of reducing cDCE to VC. Second, the production of reducing minerals may also hinder the growth and activity of DD4 in the subsequent aerobic treatment. Combining these lines, SDC-9 without sulfate is suggested in the sequential treatment for TCE and dioxane bioremediation.

CRediT authorship contribution statement

Fei Li: Conceptualization, Methodology, Investigation, Visualization, Validation, Data curation, Writing – original draft. **Daiyong Deng:** Conceptualization, Methodology, Investigation. **Lingke Zeng:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Stewart Abrams:** Conceptualization, Methodology, Supervision. **Mengyan Li:** Supervision, Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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We declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145118.

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