Successful operation of continuous reactors at short retention times results in high-density, fast-rate Dehalococcoides dechlorinating cultures

Anca G. Delgado, Devyn Fajardo-Williams, Sudeep C. Popat, César I. Torres & Rosa Krajmalnik-Brown

Applied Microbiology and Biotechnology

ISSN 0175-7598

Appl Microbiol Biotechnol DOI 10.1007/s00253-013-5263-5



Volume 97 Number 20 October 2013

Mini-Reviews

The DprE1 enzyme, one of the most vulnerable targets of Mycobacterium tuberculosis G. Riccardi - M.R. Pasca - L.R. Chiarelli - G. Manina - A. Mantevi - C. Binda - 8841

TRAIL and microRNAs in the treatment of prostate cancer therapeutic potential and role of nanotechnology

Challenges of formulation and quality of biofertilizers fo successful inoculation L. Herrmann · D. Lesueur 8859

Biotechnological products and process engineering

Synthesis of the building block 2-hydroxyisobutyrate from fructose and butyrate by Cupriaridus necator H16
D. Przybylski · T. Rohwerder · H. Harms · N. Yaneva · R.H. Müller 8875

Microbial monomers custom-synthesized to build true bio-derived aromatic polymers T. Fujita · H.D. Nguyen · T. Ito · S. Zhou · L. Osada · S. Tateyama · T. Kancko · N. Takaya 8887

Fermentation and quality of yellow pigments from golden brown ric solid culture by a selected Monascus mutant B. Yongsmith · P. Thongpradis · W. Klinsupa · W. Chantrapornchai · V. Haruthaithanasan 8895

Metabolic engineering of Aspergillus oryzae NRRL 3488 for increased production of L-malia caid S.H. Brown · L. Bashkirova · R. Berka · T. Chandler · T. Doty · K. McCall M. McCulloch · S. McFarland · S. Thompson · D. Yaver · A. Berry 8903

Effects of Lys and His supplementations on the regulation of nitrogen metabolism in lager year.

H. Lei: H. Li: F. Mov. L. Zhao; H. Zhao: M. Zhao 8913

Application of micro-segmented flow for two-dimensional characterization

Streptomyces strains
J. Cao · D. Kürsten · K. Krause · E. Kothe · K. Martin · M. Roth - J.M. Köhler 8923

Overproduction of poly(β-malic acid) (PMA) from glucose by a nove Autrobasidium sp. P6 strain isolated from mangrove system Y. Ma·G.-Y. Wang·G.-L. Liu·Z.-P. Wang·Z.-M. Chi 8931

Biotechnologically relevant enzymes and proteins

Comparative metagenomic analysis of microcosm structures and lignocellulolytic enzyme systems of symbiotic biomass-degrading consortia S. Wongwidawidni or L. Lochamacharcon - W. Mhuantong - S. Tangphatsornruang L. Eurwilaichitr · Y. Igarashi · V. Champreda 8941

Correlation of cell growth and heterologous protein production by Saccharomyces cerevisiae

Z. Liu, J. Hou, J. H. Murtinez, D. Petranovic, J. Nielsen, 8955

Structural and biochemical characterisation of a NAD*-dependent alcohol dehydrogenase from *Oenococcus oeni* as a new model molecule or industrial biotechnology applications S. Elleuche K. Fodor - B. Kilppel - A. von der Heyde - M. Wilmanns -

A 24.7-kDa copper-containing oxidase, secreted by *Thermobifida fusca* significantly increasing the xylanase/cellulase-catalyzed hydrolysis of sugarcane bagase.

C-Y, Chen - Z-S, Hsich - J, Cheepudom - C.-H, Yang - M, Meng 8977

ricinoleic acid by an oleate hydratase from Lysinibacillus fusiformis M.-H. Sco · K.-R. Kim · D.-K. Oh 8987

Expression of dehydrin gene from Arctic Cerastium arcticum increase shorts to the control of the control of

genetically engineered Saccharomyces cerevisiae laboratory strain L-S. Kim·H.-Y. Kim·Y.-S. Kim·H.-G. Choi·S.-H. Kang·H.-S. Yoon 89

Applied genetics and molecular biotechnology

Increase in rRNA content in a Saccharomyces cerevisiae suppressor strain from rrn10 disruptant by rDNA cluster duplication F. Khatun - Y. Sasano - M. Sugiyama - Y. Kaneko - S. Harashima 9011 Expression and assembly of Norwalk virus-like particles in plants using a virul RNA silencing suppressor area.

E.F. Noronha · T. Nagata 9021

Characterization of stable, constitutively expressed, chromosomal green and red fluorescent transcriptional fusions in the select agent bacterium, Francisella tularensis Schu S4 and the surrogate type B live vaccine

and red fluorescent transcriptional fusions in the select agent bacterium, Francisclia Luderasis Schu S4 and the surrogate type B live vaccine strain (LVS)

Su - R. Saldamha - A. Pemberton - H. Bangar - S.A. Kawamoto - B. Aronow D.J. Hassett - T.J. Lamkin 9029

(Continued on inside front cover)





Your article is protected by copyright and all rights are held exclusively by Springer-Verlag Berlin Heidelberg. This e-offprint is for personal use only and shall not be selfarchived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".



ENVIRONMENTAL BIOTECHNOLOGY

Successful operation of continuous reactors at short retention times results in high-density, fast-rate *Dehalococcoides* dechlorinating cultures

Anca G. Delgado • Devyn Fajardo-Williams • Sudeep C. Popat • César I. Torres • Rosa Krajmalnik-Brown

Received: 16 July 2013 / Revised: 11 September 2013 / Accepted: 12 September 2013 © Springer-Verlag Berlin Heidelberg 2013

Abstract The discovery of *Dehalococcoides mccartyi* reducing perchloroethene and trichloroethene (TCE) to ethene was a key landmark for bioremediation applications at contaminated sites. *D. mccartyi*-containing cultures are typically grown in batch-fed reactors. On the other hand, continuous cultivation of these microorganisms has been described only at long hydraulic retention times (HRTs). We report the cultivation of a representative *D. mccartyi*-containing culture in continuous stirred-tank reactors (CSTRs) at a short, 3-d HRT, using TCE as the electron acceptor. We successfully operated 3-d HRT CSTRs for up to 120 days and observed sustained dechlorination of TCE at influent concentrations of 1 and 2 mM TCE to ≥97 % ethene, coupled to the production of 10^{12} *D. mccartyi* cells L_{culture}⁻¹. These outcomes were possible in part by using a medium with low bicarbonate concentrations

 $(5 \, \mathrm{mM})$ to minimize the excessive proliferation of microorganisms that use bicarbonate as an electron acceptor and compete with D. mccartyi for H_2 . The maximum conversion rates for the CSTR-produced culture were $0.13\pm0.016,~0.06\pm0.018,$ and 0.02 ± 0.007 mmol Cl $^ L_{\mathrm{culture}}^{-1}$ h^{-1} , respectively, for TCE, cis-dichloroethene, and vinyl chloride. The CSTR operation described here provides the fastest laboratory cultivation rate of high-cell density Dehalococcoides cultures reported in the literature to date. This cultivation method provides a fundamental scientific platform for potential future operations of such a system at larger scales.

Keywords Chemostat \cdot *Dehalococcoides* \cdot *Geobacter* \cdot Organohalide respiration \cdot Bioremediation \cdot Microbial community management

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5263-5) contains supplementary material, which is available to authorized users.

A. G. Delgado (⋈) · D. Fajardo-Williams · S. C. Popat ·

C. I. Torres · R. Krajmalnik-Brown (⊠)

Swette Center for Environmental Biotechnology, Biodesign Institute, Arizona State University, P.O. Box 875701, Tempe,

AZ 85287-5701, USA e-mail: anca.delgado@asu.edu e-mail: dr.rosy@asu.edu

Published online: 02 October 2013

A. G. Delgado

School of Life Sciences, Arizona State University, Tempe, AZ, USA

D. Fajardo-Williams · R. Krajmalnik-Brown School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, AZ, USA

C. I. Torres

School for Engineering of Matter, Transport and Energy, Arizona State University, Tempe, AZ, USA

Introduction

In the US, at least 60 % of the National Priorities List Superfund sites and at least 17 % of groundwater sources have detectable levels of chlorinated solvents, including trichloroethene (TCE) and perchloroethene (PCE) (ATDSR 2011; Moran et al. 2007). The presence and persistence of these compounds in the environment is a major threat to public health. Biological reduction by members of the bacterial genus *Dehalococcoides* is a common and cost-effective avenue for in situ bioremediation of sites contaminated with chlorinated solvents. *Dehalococcoides mecartyi* strains provide a unique solution to remediating chlorinated ethenes as they can reductively dechlorinate PCE and TCE to the nontoxic end-product, ethene, with transient production of *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) (Ellis et al. 2000; Löffler et al. 2013; Maymó-Gatell et al. 1997).



The common laboratory cultivation method for TCE- and PCE-dechlorinating cultures containing *D. mccartyi* is in batch reactors under batch-fed conditions. To achieve high concentrations of *Dehalococcoides* (e.g., 10^{11} – 10^{12} cells L⁻¹), these cultures must be fed with high concentrations (millimolar range) of chlorinated ethenes. Batch systems can be cumbersome, as self or competitive inhibition of dechlorination, and toxicity of *Dehalococcoides* and other community members prevents feeding TCE or PCE in high concentrations (Chambon et al. 2013). Therefore, batch cultivation of *Dehalococcoides* entails receiving and reducing (mostly to ethene) several non-inhibitory, successive feeds of electron acceptors.

Often times, laboratory studies and especially bioaugmentation applications at contaminated sites require large volumes of culture containing high-density Dehalococcoides cells (Ellis et al. 2000; Steffan and Vainberg 2012; Tang et al. 2013). Continuous stirred-tank reactors (CSTRs) are well established sources for yielding large volumes of steady-state cells or proteins (Hoskisson and Hobbs 2005). Moreover, a shorthydraulic retention time (HRT) CSTR is an ideal tool for community-based transcriptomics or proteomics studies, which require cells constantly growing and at high densities. Unlike in a batch reactor, theoretically, inhibition or toxicity to microbial community members can be minimized in a CSTR by continuously maintaining low concentrations of TCE or PCE. These low concentrations should enable feeding higher concentrations of chlorinated solvents in the same time interval than in a batch reactor, thus achieving higher Dehalococcoides concentrations.

Kinetic parameters suggest the potential for culturing Dehalococcoides at a short HRT in a CSTR. Specifically, doubling times of ≤ 1 day have been reported for some D. mccartyi pure cultures (Cheng and He 2009; Maymó-Gatell et al. 1997) and D. mccartvi enrichment cultures (Vainberg et al. 2009). Furthermore, the low Monod half-maximum rate concentrations (K_s) for TCE, cis-DCE, and VC of D. mccartyi (<5 μM) (Popat and Deshusses 2011) indicate that these microbes should perform well in a continuous-flow reactor where the aqueous concentrations of electron acceptors are low. Despite these potential advantages, dechlorination studies using CSTRs are limited (Berggren et al. 2013; Carr et al. 2000; Drzyzga et al. 2001; Sabalowsky and Semprini 2010; Yang and McCarty 1998; Zheng et al. 2001). In the past two decades since the discovery of D. mccartyi, there has been little success in achieving sustainable growth of dechlorinating cultures that are able to reduce chloroethenes to mostly ethene. In fact, Yang and McCarty (1998) and Berggren et al. (2013) are the only two cases reported for the complete conversion of PCE and TCE to ethene in CSTRs (Dehalococcoides concentrations were not reported) operated at 36- and 50-d HRTs, respectively. These HRTs are even longer than those of methanogenic anaerobic digesters (Tchobanoglous et al. 2003),

even though the growth rates of *D. mccartyi* are faster than those of acetoclastic methanogens (Tchobanoglous et al. 2003).

A good understanding of the growth requirements and microbial interactions in dechlorinating cultures containing *Dehalococcoides* should allow for cultivation of *Dehalococcoides* in a high-growth rate system, such as a short-HRT CSTR. We hypothesized that culturing *Dehalococcoides* communities in a short-HRT CSTR has, thus far, been impeded for two major reasons. First is inhibition due to toxicity of the chlorinated electron acceptors. For growth of dechlorinating cultures to occur, a high enough concentration of chlorinated solvents must be fed to attain high concentrations of *Dehalococcoides*. Yet, very high removal of TCE or PCE to ethene must occur to avoid inhibition (the effluent concentrations of chlorinated ethenes must be low). Second is the stringent competition between *Dehalococcoides* and other community members for the obligate electron donor, H₂.

We report here the successful cultivation and performance of a D. mccartyi-containing culture in a CSTR operated at a 3-d HRT and fed with 1 and 2 mM TCE. To achieve this successful, proof-of-concept CSTR operation, we built upon data from prior CSTR runs in our laboratory and a systematic study evaluating HCO₃⁻ as a competing electron acceptor in microbial dechlorination of TCE (Delgado et al. 2012). In the previous CSTR runs, summarized in the Supplementary Material, we tested different operating conditions (TCE concentration, electron donor concentration, and HRT) in 30 mM bicarbonate (HCO₃⁻)-buffered medium. In the HCO₃⁻ study (Delgado et al. 2012), we saw that high HCO₃⁻ levels (i.e., 30 mM) increase the H₂ demand by stimulating homoacetogenesis and methanogenesis, two processes competing for H₂ and, therefore, potentially limiting reductive dechlorination of chloroethenes. Thus, the successful CSTR runs presented here were achieved with an optimized medium composition with a low bicarbonate concentration, thereby managing the microbial communities and achieving low effluent concentrations of chlorinated ethenes.

Materials and methods

Bioreactor design and operation

A schematic and a photograph of the reactor setup used (bioreactors 1 and 2) are shown in Fig. 1. Each reactor consisted of a 0.65-L glass bottle sealed with a butyl rubber stopper and a screw cap. The stopper was perforated to fit the influent and effluent lines, and a gas sampling port containing a removable septum (IceBlue® Septa, Restek, USA). The septum was changed several times throughout the runs. As a result of this, some losses of compounds in the headspace occurred due to brief flushing with ultra-high purity (UHP) N_2 , which resulted in a drop in ethene concentration and mol balances. The actual



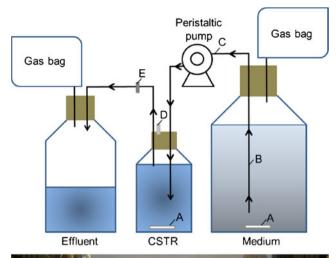




Fig. 1 Schematic (top panel) and photograph (bottom panel) of the experimental apparatus employed in this study. a magnetic stir bar, b stainless steel line, c Viton pump tubing, d gas sampling port, e liquid sampling port. The arrows denote the direction of the liquid flow

liquid and headspace operating volumes were 0.5 and 0.1 L, respectively. Each reactor was magnetically stirred at 200 rpm and submerged in a water bath set at 30 °C. Influent medium was pumped from 5-L glass bottles containing 4 L of medium with a Minipuls 3 peristaltic pump (Gilson, Inc., USA) to achieve a 3-d HRT. All lines and tubing used were 1/8" diameter stainless steel or Viton material. The liquid sampling port was located before the effluent collection bottle. The effluent culture was collected into 1-L glass bottles equipped with 1-L gas Tedlar bags (SKC Inc., USA) for gas collection.

Bioreactor 1 (presented in the main text) and bioreactor 2 (presented in the Supplementary Material) were operated under identical conditions for a total of 120 and 100 days, respectively. During this time, the bioreactors were fed TCE-containing medium continuously at all times, except for the initial four days after inoculation and for seven days in between switching the concentrations of TCE from 1 to 2 mM. Before increasing the TCE concentration in the influent medium, the bioreactors were also flushed with UHP N_2 to remove headspace gases.

Inoculum culture and medium composition

The culture employed for the studies was DehaloR^2 (Ziv-El et al. 2011), a TCE-to-ethene dechlorinating consortium containing *Dehalococcoides* and *Geobacter*. DehaloR^2 was initially grown in a CSTR fed with 3 mM TCE at a 4-d HRT (Table S1, run 1). The culture from this run was collected and stored at 4 °C for 15 months prior to inoculating the bioreactors presented herein. Here, 0.5 L DehaloR^2 culture (100 % vol/vol) per reactor was inoculated on day 0. Trace concentrations of *cis*-DCE and VC were present in this culture during storage; therefore, we added 2 mM lactate and kept the reactors in batch mode for ~4 days to reduce the chlorinated ethenes to ethene before proceeding to continuous operation.

We prepared reduced anaerobic mineral medium containing 1 mM TCE (aqueous concentration), 7.5 mM sodium DLlactate, 15 mM methanol, 15 mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), 5 mM NaHCO₃, 5 mL L⁻¹ ATCC® MD-VSTM vitamin supplement (ATCC, USA), 500 μ g L⁻¹ vitamin B₁₂, 0.25 μ g L⁻¹ resazurin, 0.2 mM L-cysteine, and 0.2 mM Na₂S × 9 H₂O. The salts and trace nutrients added per liter medium were those described in Delgado et al. (2012). In the medium with 2 mM TCE, lactate and HEPES were increased to 10 and 20 mM, respectively, NaCl was decreased to 0.1 g L⁻¹, and methanol was kept at 15 mM. The influent medium pH was adjusted to 7.5–7.8 with 10 N NaOH. The same base medium composition was used for previous CSTR runs presented in Table S1, except the noted differences summarized in the table. We first autoclaved the medium, boiled it under a stream of UHP N₂, and then added the reducing agents. To avoid fluctuations in TCE concentrations in the media bottles from changes in the liquid-headspace ratios during continuous operation, the bottles were fitted with collapsible 3-L gas Tedlar bags filled with UHP N₂. We added per bottle 515 or 1,030 µL neat TCE to achieve a concentration of 1 and 2 mM TCE in the liquid, respectively. Abiotic transformation of TCE or significant TCE losses did not occur in the influent medium.

Chemical analyses

We sampled gas from the reactors to quantify the concentrations in the headspace of TCE, cis-DCE, VC, ethene, methane, and H_2 . The methods for the Shimadzu gas chromatography instruments were previously described (Delgado et al. 2012). The concentrations of chlorinated ethenes and ethene in the liquid were calculated using Henry's constants (K_H) for each compound:

$$[Compound]_{liq} = [Compound]_{gas}/K_{H}$$
 (1)

We obtained dimensionless Henry's constants (mM $_{\rm gas}/$ mM $_{\rm liq}$, T=30 °C) experimentally for the mineral medium used



in this study for TCE (0.49), cis-DCE (0.17), VC (1.32), and ethene (9.00). Gas concentrations were used to estimate liquid concentrations based on the above Henry's constant. The flow of chlorinated ethenes and ethene out of the reactors was through both liquid and gas phases. When methane production was small, the flow of ethenes was mainly through the liquid. When methane production increased (especially in the 2-mM TCE run), the flow of these compounds through the gas phase also increased due to the higher gas volumes produced. The increase in methane production also led to dilution of ethene concentration in the headspace, thus also decreasing the liquid ethene concentrations (as shown in Figs. 2 and S1). Because of the small gas flow rates and the difficulty in separating liquid and gas effluents, we did not measure the total gas production, but estimated it according to the mol balance equation below:

$$[\text{TCE}]_{\text{in}} \times Q_{\text{liq}} = [\text{Ethenes}]_{\text{out gas}} \times Q_{\text{gas}} + [\text{Ethenes}]_{\text{out liq}} \times Q_{\text{liq}}$$
 (2)

in which [TCE] = TCE aqueous concentration (millimolar), [Ethenes] = cumulative concentration of chlorinated ethenes (TCE, cis-DCE, VC) and ethene in the reactor and effluent (millimolar), and Q = flow rate (milliliters per day).

We removed liquid samples to measure lactate, methanol, acetate, and propionate using high-performance liquid chromatography (Delgado et al. 2012). We used an Orion pH meter (Thermo Scientific, USA) to monitor the pH, which ranged from 6.3 to 7.5. We performed pH adjustments to \sim 7 with 10 N NaOH only when the pH inside the reactors dropped to 6.3.

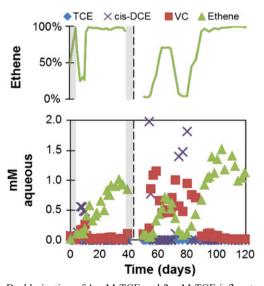


Fig. 2 Dechlorination of 1 mM TCE and 2 mM TCE influent and the corresponding percent ethene conversion (*top line graphs*) in a CSTR operated at a 3-d HRT. The *light gray shaded areas* are periods of batch operation, and the *dashed line* represents the start of the 2 mM TCE continuous feed

Microbial ecology

We extracted total genomic DNA from pellets made with 1.5 mL liquid samples according to the protocol previously published (Ziv-El et al. 2011). Quantitative real-time PCR (qPCR) assays were performed targeting the 16S rRNA genes of D. mccartvi, Geobacteraceae, and Archaea, and formyltetrahydrofolate synthase (FTHFS) (gene involved in the pathway for acetate production by homoacetogens) as described by Ziv-El et al. (2012b). We also performed qPCR tracking the reductive dehalogenase genes of D. mccartyi, tceA, vcrA, and bvcA, using the qPCR protocol, primers, probes, reagent concentrations, and PCR conditions detailed previously (Ziv-El et al. 2012b), except that each reductive dehalogenase gene was assayed separately. Enumeration of D. mccartyi was performed using the assumption that one Dehalococcoides cell has one copy of the 16S rRNA gene (Ritalahti et al. 2006).

Conversion rates and long-term viability of CSTR-grown culture

Once pseudo-steady state (defined as stable conversion of TCE to mostly ethene) was achieved for the 1- and 2-mM TCE continuous runs, we determined the maximum rates of conversion, R_{max} , for TCE, cis-DCE, and VC. We transferred 100 mL effluent culture to 160-mL serum glass bottles and flushed for 20 min with UHP N₂ gas to remove any carry-over ethenes. Then, we provided a chlorinated electron acceptor (0.5 mmol L_{culture}⁻¹ of either TCE, cis-DCE, or VC), 5 mM lactate, 12 mM methanol, and 10 mL H_2 (4.1 mmol $L_{culture}^{-1}$). The bottles were incubated at 30 °C on an orbital shaker set at 200 rpm. We measured the concentration of dechlorination products formed over short time intervals (5 h or less) in order to minimize increases in dechlorinating populations. qPCR tracking the D. mccartyi 16S rRNA gene confirmed that these bacteria had not grown significantly throughout the course of these short tests (data not shown). All R_{max} values were determined from at least triplicate experiments. The overall rate of dechlorination (in mmol Cl⁻ L_{culture} ⁻¹ h⁻¹) was calculated from TCE experiments as described above, by multiplying the concentrations of each dechlorination daughter product formed by their respective number of Cl released: cis-DCE, 1; VC, 2; and ethene, 3.

The culture produced in the CSTR from runs 1–2 and 7–8 in Table S1 was stored in a 4 °C refrigerator and periodically monitored for activity. Viability experiments consisted of transferring 10 mL stored culture to 160-mL serum bottles containing 90 mL anaerobic medium (10 % inoculum vol/vol), adding 0.5–1 mmol $L_{\rm culture}^{-1}$ TCE, 5 mM lactate, and 12 mM methanol, and monitoring TCE dechlorination to ethene in time course experiments.



Results

Dechlorination performance in a 3-d HRT CSTR fed with 1 or 2 mM TCE

We initially assessed the dechlorination activity (performance of the culture) in the CSTRs by measuring TCE and its dechlorination products using a GC. Figure 2 shows the performance of one replicate CSTR fed 1 mM TCE at a 3-d HRT (see Fig. S1 for data on the other replicate). *cis*-DCE and VC initially accumulated in the bioreactors within the first two HRTs; however, by day 11, ethene became the prevalent dechlorination end-product, and >90 % conversion of TCE to ethene was observed thereafter. Both bioreactors reached dechlorination pseudo-steady state after ~5.5 HRTs, which was maintained until the end of this continuous run.

When the influent was 2 mM TCE, the bioreactors exhibited the same conversion trends as when initially fed with 1 mM TCE (Figs. 2 and S1). For the first several HRTs, *cis*-DCE and VC were the main dechlorination products. Conversion to mostly ethene was achieved in ~14 days (day 65), but performance declined shortly after (Fig. 2). We believe this decline was due to an oxygen leak into the reactor from a damaged influent pump tubing. Once the tubing was replaced, the reactor recovered, and a pseudo-steady state with greater than 93 % conversion to ethene was achieved by day 94 with 2 mM TCE influent concentration and sustained for 9 subsequent HRTs (Fig. 2). The duplicate bioreactor presented in Fig. S1 also reached conversion to mostly ethene, with a pseudo-steady state of ~80 % reduction to ethene of 2 mM-fed TCE.

Growth of *Dehalococcoides* and enrichment of efficient dechlorinating microbial communities

The high conversion to ethene was coupled to increases in Dehalococcoides densities. We monitored the growth of D. mccartyi every HRT until pseudo-steady state was achieved. Figure 3a shows the initial concentration of D. mccartyi and the average pseudo-steady-state abundances of 1.3×10^{12} and 1.6×10^{12} cells $L_{culture}^{-1}$ when continuously feeding 1 or 2 mM TCE, respectively, at a rate of biomass production of 3.3×10^{11} D. mccartyi cells L_{culture}⁻¹ d⁻¹. In terms of D. mccartyi diversity/composition, the CSTR-grown culture contained the three previously identified reductive dehalogenase genes, tceA (Magnuson et al. 1998), vcrA (Muller et al. 2004), and bvcA (Krajmalnik-Brown et al. 2004). Figure 3b highlights that concentrations of the three reductive dehalogenase genes increased during operation, reaching their highest levels during the 2 mM TCE pseudo-steady state, with abundances of 10^{11} copies L⁻¹ for tceA and vcrA and 10^8 copies L⁻¹ for bvcA. Besides Dehalococcoides, DehaloR^2 culture contains only one other identified dechlorinating bacterial group most closely related to *Geobacter lovleyi* (Ziv-El et al. 2011; Ziv-El et al. 2012c), which only partially reduces TCE to *cis*-DCE (Sung et al. 2006). *Geobacter* was assayed using the family specific 16S rRNA gene of *Geobacteraceae*, as it is the only bacterial genus belonging to this family in DehaloR^2 inoculum (Ziv-El et al. 2011). As depicted in Fig. 3a, *Geobacter* also increased throughout the two operating conditions. The densities obtained for *Geobacteraceae* in our CSTRs were 6.4×10^{10} gene copies L_{culture}^{-1} . Data on growth of TCE/PCE-reducing *Geobacter* in CSTRs for pure culture or for mixed communities are absent from the literature; however, the abundances obtained for these microbes in our CSTRs are also on the high end compared to those in batch-fed mixed dechlorinating cultures (Duhamel and Edwards 2007; Ziv-El et al. 2011).

Fate of electron donors and H₂-induced microbial interactions

Consumption of the provided fermentable substrates is presented in Fig. 4a. Lactate was not detectable in all measurements at both influent TCE concentrations. Approximately half of the 15 mM methanol was consumed for the phase with 1 mM TCE influent, while close to complete methanol consumption was recorded at 2 mM TCE pseudo-steady state (Fig. 4a). The duplicate bioreactors behaved similarly in terms of lactate and methanol consumption in the 1-mM TCE and 2-mM TCE runs, although the bioreactor converting TCE to ~80 % ethene (Fig. S2A) did not completely consume the influent methanol until the last HRT.

As a result of lactate and methanol fermentation, H₂ concentrations in the headspace of the bioreactors were 0.3-0.5 mM for the 1-mM TCE run. However, with 2 mM TCE, H₂ was no longer detected in the headspace (the detection limit for our H₂ measurements was 0.018 mmol L⁻¹ gas concentration) despite a higher lactate concentration feed. One of the driving hypotheses of this study was that growth of Dehalococcoides coupling high cell densities to dechlorination of TCE to ethene can occur if competition for H₂ by non-dechlorinating populations is minimized. H₂-oxidizing methanogens, the only type of methanogens in the inoculum culture, were initially present at concentrations of 109 gene copies L_{culture}^{-1} and decreased by two orders of magnitude to 10^7 gene copies L_{culture}^{-1} during the 1-mM TCE run (Fig. 3a). The decrease in gene copies of methanogens was also corroborated by the gradual decrease in methane in the bioreactors, as seen in Figs. 4b and S2B. During the 2-mM TCE continuous run, the gene copies of methanogens per liter culture increased to 10⁸ (Fig. 3a), and hence, methane concentrations in the liquid were up to 1.3 mM (Figs. 4b and S2B). This was likely a consequence of the increase in lactate influent concentration from 7.5 to 10 mM, which subsequently yielded additional H₂ and HCO₃⁻/CO₂ as growth substrates for hydrogenotrophic methanogens. The FTHFS gene copies of



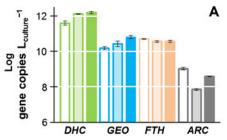
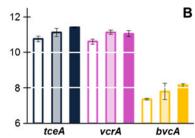


Fig. 3 Microbial population abundance in a 3-d HRT CSTR determined by qPCR at time 0 (*no-fill bars*), 1 mM TCE pseudo-steady state (*light-filled bars*), and 2 mM TCE pseudo-steady state (*dark-filled bars*). **a** Log concentrations of *Dehalococcoides mccartyi* (DHC), *Geobacteraceae* (GEO), FTHFS (FTH), and *Archaea* (ARC). **b** Log concentrations of *Dehalococcoides mccartyi* functionally defined reductive dehalogenase



genes, tceA, vcrA, and bvcA. All $error\ bars$ show standard deviations of replicate samples: $time\ \theta$, n=2 (bioreactors 1 and 2); $l\ mM\ TCE$ on day 24 and 36, n=4 (bioreactors 1 and 2); $l\ mM\ TCE$ on day 90, 94, and 118, $l\ m=3$ (bioreactor 1) and analytical qPCR reactions: $time\ \theta$, $l\ m=6$; $l\ mM\ TCE$, $l\ m=12$; $l\ mM\ TCE$, $l\ m$

hydrogenotrophic homoacetogens, another competing sink coupling H_2 oxidation to the reduction of HCO_3^- , were 5×10^{10} initially, decreased during the 1-mM run and remained fairly constant at the 2-mM pseudo-steady state (Fig. 3a).

Dechlorination kinetics of the CSTR-grown culture

Table 1 summarizes the maximum conversion rates, $R_{\rm max}$, at pseudo-steady state obtained from the culture produced in the CSTR grown with the two concentrations of TCE. Experimental data for the values in Table 1 were obtained from

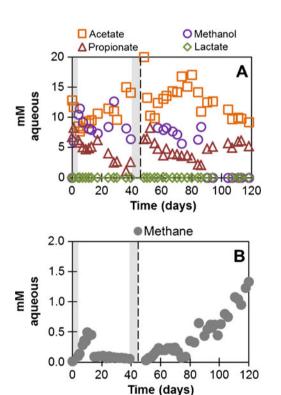


Fig. 4 Consumption of influent **a** lactate and methanol and production of acetate, propionate, and **b** methane during continuous feed of medium containing 1 mM TCE and 2 mM TCE in a 3-d HRT CSTR. The *light gray shaded areas* are periods of batch operation, and the *dashed line* represents the start of the 2 mM TCE continuous feed

separate short-term batch experiments for each individual chlorinated ethene (examples shown in Fig. S3). These experiments were performed to ensure that the rates of dechlorination were within the same order of magnitude for all chlorinated ethenes. With the culture produced when continuously feeding 2 mM TCE, we obtained an overall rate of dechlorination of 0.16 (±0.010) mmol Cl $^-$ Lculture $^{-1}$ h $^{-1}$. This rate surpasses the previously reported batch-grown DehaloR^2 maximum rate of 0.04 mmol Cl $^-$ released Lculture $^{-1}$ h $^{-1}$ (or 0.92 mmol Cl $^-$ Lculture $^{-1}$ d $^{-1}$) (Ziv-El et al. 2012a), which was obtained by feeding a total of 3 mmol Lculture $^{-1}$ TCE in three consecutive additions of 1 mmol L $^{-1}$.

The methodologies to determine culture rates vary between research groups, which makes comparisons challenging. Schaefer et al. (2009) employed a similar experimental approach as described in our study to determine maximum rates of conversion. As seen in Table 1 at 2 mM TCE influent, $R_{\rm max}$ values for the culture produced in this study are four times greater for TCE to cis-DCE and cis-DCE to VC than those reported by SDC-9 culture (in Schaefer et al. (2009), 0.04 and 0.02 Cl $^-$ mmol L_{culture} $^{-1}$ h $^{-1}$, respectively), while VC to ethene rates of DehaloR 2 measured here are lower than those of SDC-9 by a factor of two (in Schaefer et al. (2009), 0.04 mmol Cl $^-$ L_{culture} $^{-1}$ h $^{-1}$).

We predicted that $R_{\rm max}$ and D. mccartyi cell density would roughly double when the influent concentration of TCE was increased from 1 to 2 mM. Table 1 and Fig. S3 reveal that the rates of TCE and cis-DCE dechlorination were three times

Table 1 Maximum conversion rate $(R_{\rm max})$ of chloroethenes by DehaloR^2 culture produced in a CSTR fed with 1 mM TCE and 2 mM TCE influent concentrations. The $R_{\rm max}$ values are averages with standard deviations of at least triplicate experiments as those shown in Fig. S3

$[TCE]_{in} \\$	$R_{\rm max}$ (mmol Cl ⁻ L _{culture} ⁻¹ h ⁻¹)				
	TCE	cis-DCE	VC		
1 mM 2 mM	0.04 (±0.004) 0.13 (±0.016)	0.02 (±0.002) 0.06 (±0.018)	0.01 (±0.001) 0.02 (±0.007)		



greater, while VC dechlorination rates were two times greater at 2 mM TCE influent. Yet, *D. mccartyi* concentrations increased only from 1.3×10^{12} (1-mM TCE run) to 1.6×10^{12} cells L_{culture}^{-1} (2-mM TCE run). A plausible explanation for the apparent discordance between the higher rates of dechlorination and those of *D. mccartyi* cell copies is related to the growth of *Geobacter* dechlorinators in the culture. *Geobacter* increased ~2.5 fold when the CSTR medium contained 2 mM TCE (Fig. 3a). This suggests that the contribution of *Geobacter* in the reduction of TCE to *cis*-DCE increased significantly at 2 mM TCE feed, when compared to the contribution of *D. mccartyi* for this dechlorination step.

Culture viability after prolonged storage

An advantage in producing dense microbial cultures containing *Dehalococcoides* is that they can be cultured in the laboratory and stored for prolonged periods for later usage. Culture SDC-9 showed no significant loss in activity after ~40 days at 4 °C (Vainberg et al. 2009) and sustained ~60–70 % of its original PCE and *cis*-DCE dechlorinating activity even after seven months at this temperature (Steffan and Vainberg 2012). The culture initially produced in our CSTR (run 1, Table S1) was stored for extended periods at 4 °C. In support of previous findings on robust dechlorinating activity even after extended refrigeration, Fig. S4A shows that complete dechlorination of ~0.7 mmol L_{culture} ⁻¹ TCE occurred in 6 days after the CSTR-grown culture had been stored for seven months. After 15 months of storage, the same concentration of TCE was

reduced to 80 % ethene in 15 days (Fig. S4B), implying that, while some loss of activity will occur (due to cell decay), these cultures maintain good dechlorinating activity profiles when the appropriate conditions are provided for revival and growth.

Discussion

In this study, we show that using carefully selected conditions in a CSTR, cultivation of Dehalococcoides at short HRTs is feasible, resulting in robust communities capable of fast dechlorination. A compilation of previous CSTR studies on dechlorination of chlorinated ethenes is shown in Table 2. As revealed in Table 2, in most previous CSTR studies, the main reduced end-product of dechlorination of TCE and PCE was cis-DCE. This suggested that if present in the inocula used for these studies, D. mccartyi species respiring cis-DCE or VC to ethene were inhibited by high concentrations of chlorinated solvents, washed out, or outcompeted by other microbes. Our study documents conversion to mostly ethene in a CSTR at a 3-d HRT (Table 2). We also report for the first time pseudosteady-state densities and production rates for Dehalococcoides cultivated in a CSTR. Moreover, the values for D. mccartyi presented in Fig. 3a are close to the maximum ever reported for these microbes; the only past study to obtain growth to 10^{12} Dehalococcoides cells L_{culture}⁻¹ was Vainberg et al. (2009).

The community data regarding methanogens and homoacetogens abundances, in conjunction with the CSTR

Table 2 Summary of key parameters and microbial inocula employed in chlorinated ethene CSTR studies

Chlorinated ethene	e donor and C source	Buffer	HRT (d)	Major reduced product	Inoculum culture
1 mM TCE	7.5 mM lactate and 15 mM methanol	15 mM HEPES and 5 mM HCO ₃	3	Ethene	DehaloR^2 dechlorination culture converting TCE to ethene (this study)
2 mM TCE	10 mM lactate and 15 mM methanol	20 mM HEPES and 5 mM HCO ₃	3	Ethene	•
1.12 mM PCE	4.3 mM lactate	35 mM Na ₂ CO ₃ and 6 mM K ₂ HPO ₄	50–55	Ethene	Point Mugu (PM) dechlorinating culture converting PCE to ethene (Berggren et al. 2013)
7.4 mM TCE	25.6 mM lactate	CO ₃ ²⁻	5.9–25.3	cis-DCE	Evanite (EV) subculture converting PCE to <i>cis</i> -DCE (Sabalowsky and Semprini 2010)
0.52 mM PCE	52 mM methanol, 20 mM pyruvate or 80 % H ₂ / 20 % CO ₂ , and 2 mM acetate	90 mM HCO ₃ ⁻	11 5.8 2.9	VC cis-DCE cis-DCE	Methanol/PCE enrichment culture converting PCE to VC and ethene (Zheng et al. 2001)
≤ 50 mM PCE (nominal)	45 mM lactate	10 mM (NH ₄)H ₂ PO ₄ and 20 % CO ₂	~2	cis-DCE	Co-culture of <i>Desulfitobacterium frappieri</i> TCE1 and <i>Desulfovibrio</i> sp. strain SULF1 (Drzyzga et al. 2001)
0.2 g PCE in hexadecane NAPL	10 mM formate	10 mM HCO ₃	3	cis-DCE	Methanol/PCE enrichment culture converting PCE to VC and ethene (Carr et al. 2000)
0.98 mM PCE	1.7 mM benzoate	14 mM Na ₂ CO ₃ and 3 mM K ₂ HPO ₄	36	Ethene	Dechlorinating source culture converting PCE to ethene (Yang and McCarty 1998)



dechlorination performance and the concentrations of methane and acetate, support the fact that competing sinks for H_2 were minimized using our medium composition, thus allowing H_2 to be used optimally for dechlorination. Indeed, Dehalococcoides and Geobacter growth correlated with good TCE dechlorination performance in these continuous reactors. Moreover, recently, Geobacter was documented to provide D. mccartyi with required corrinoids for dechlorinating activity and cellular growth (Yan et al. 2012) and, therefore, may be a desired partner in Dehalococcoides-containing cultures.

The CSTR-produced culture exhibited very rapid rates of dechlorination, as shown in Table 1. The lower R_{max} for VC compared to TCE and cis-DCE (Table 1) implies that the limiting step in the CSTRs was dechlorination of VC. VC to ethene is commonly the slowest dechlorination step (Yu et al. 2005), which might explain some of the rate differences between VC and TCE and cis-DCE dechlorination. Another factor we identified that could have led to lower apparent rates for VC dechlorination is the poorer gas-liquid transfer properties of VC, given its higher Henry's constant. In abiotic batch experiments using our medium composition (data not shown), we determined that $0.5 \text{ mmol L}^{-1} \text{ VC}$ added as gas did not equilibrate between the liquid and gas within the time of the R_{max} experiments (5 h or less). Therefore, the slower dissolution of VC into the medium might have limited its bioavailability. Hence, the reported values for VC in Table 1 are the minimum R_{max} for this electron acceptor, with the possibility that the rates were higher as we did not observe significant VC accumulation during reactor operation (Fig. 2).

The high abundances of *D. mccartyi* obtained in our CSTRs (10¹² *Dehalococcoides* cells L⁻¹) clearly support the opportunity for their efficient cultivation in continuous reactors at short HRTs, which brings about numerous advantages when working with dechlorinating cultures. In the laboratory, such a system is ideal to provide a continuous supply of uniform culture for downstream applications requiring large volumes of cultures. These could include studies on microbial interactions, inhibition, transcriptomics and proteomics, experiments testing a large matrix of environmental conditions, or pilot-scale bioaugmentation applications. Moreover, a CSTR can minimize reactor size requirements and/or time of operation to achieve high-density *Dehalococcoides* cultures.

For field applications, a short-HRT CSTR would be ideal for production of robust cultures capable of fast rate of dechlorination containing high-cell density *Dehalococcoides*. Depending on the site to be remediated, bioaugmentation can require hundreds to thousands of liters of bioaugmenting culture (Aziz et al. 2012). Fast dechlorination rates linked to high concentrations of *D. mccartyi* have been demonstrated in few well-characterized, batch grown bioaugmentation cultures, including the commercially produced culture, SDC-9, where PCE is constantly supplied to batch fermenters and 10^{11} – 10^{12} *Dehalococcoides* cells are produced (Vainberg

et al. 2009). This study demonstrates that a similar outcome in terms of *Dehalococcoides* densities and rates of dechlorination can also be achieved using a continuous-flow bioreactor, and provides the first scientific platform for a potential future implementation of such system at a larger scale.

Acknowledgments The authors acknowledge Prathap Parameswaran and Jonathan P. Badalamenti for their expertise and help with quantitative PCR. This project was supported by National Science Foundation CAREER Award Number 1053939 to RKB and startup funds from the School for Engineering of Matter, Transport and Energy at Arizona State University to CIT.

References

- ATDSR (2011) Priority list of hazardous substances. Agency for Toxic Substances and Disease Registry
- Aziz CE, Wymore RA, Steffan RJ (2012) Bioaugmentation considerations. In: Stroo HF, Leeson A, Ward HC (eds) Bioaugmentation for groundwater remediation. Springer, New York, pp 141–169
- Berggren DRV, Marshall IPG, Azizian MF, Spormann AM, Semprini L (2013) Effects of sulfate reduction on the bacterial community and kinetic parameters of a dechlorinating culture under chemostat growth conditions. Environ Sci Technol 47(4):1879–1886
- Carr CS, Garg S, Hughes JB (2000) Effect of dechlorinating bacteria on the longevity and composition of PCE-containing nonaqueous phase liquids under equilibrium dissolution conditions. Environ Sci Technol 34(6):1088–1094
- Chambon JC, Bjerg PL, Scheutz C, Bælum J, Jakobsen R, Binning PJ (2013) Review of reactive kinetic models describing reductive dechlorination of chlorinated ethenes in soil and groundwater. Biotechnol Bioeng 110(1):1–23
- Cheng D, He JZ (2009) Isolation and characterization of "Dehalococcoides" sp strain MB, which dechlorinates tetrachloroethene to *trans*-1,2-dichloroethene. Appl Environ Microbiol 75(18):5910–5918
- Delgado AG, Parameswaran P, Fajardo-Williams D, Halden RU, Krajmalnik-Brown R (2012) Role of bicarbonate as a pH buffer and electron sink in microbial dechlorination of chloroethenes. Microb Cell Factories 11(128)
- Drzyzga O, Gerritse J, Dijk JA, Elissen H, Gottschal JC (2001) Coexistence of a sulphate-reducing *Desulfovibrio* species and the dehalorespiring *Desulfitobacterium frappieri* TCE1 in defined chemostat cultures grown with various combinations of sulphate and tetrachloroethene. Environ Microbiol 3(2):92–99
- Duhamel M, Edwards EA (2007) Growth and yields of dechlorinators, acetogens, and methanogens during reductive dechlorination of chlorinated ethenes and dihaloelimination of 1,2-dichloroethane. Environ Sci Technol 41(7):2303–2310
- Ellis DE, Lutz EJ, Odom JM, Buchanan RJ, Bartlett CL, Lee MD, Harkness MR, Deweerd KA (2000) Bioaugmentation for accelerated in situ anaerobic bioremediation. Environ Sci Technol 34(11): 2254–2260
- Hoskisson PA, Hobbs G (2005) Continuous culture—making a comeback? Microbiology 151:3153–3159
- Krajmalnik-Brown R, Holscher T, Thomson IN, Saunders FM, Ritalahti KM, Löffler FE (2004) Genetic identification of a putative vinyl chloride reductase in *Dehalococcoides* sp. strain BAV1. Appl Environ Microbiol 70(10):6347–6351
- Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Muller JA, Fullerton H, Zinder SH, Spormann AM (2013) Dehalococcoides mccartyi gen. nov., sp nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen



- cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov and family *Dehalococcoidaceae* fam. nov., within the phylum *Chloroflexi*. Int J Syst Evol Microbiol 63:625–635
- Magnuson JK, Stern RV, Gossett JM, Zinder SH, Burris DR (1998) Reductive dechlorination of tetrachloroethene to ethene by two-component enzyme pathway. Appl Environ Microbiol 64(4):1270–1275
- Maymó-Gatell X, Chien YT, Gossett JM, Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276(5318):1568–1571
- Moran MJ, Zogorski JS, Squillace PJ (2007) Chlorinated solvents in groundwater of the United States. Environ Sci Technol 41(1):74–81
- Muller JA, Rosner BM, von Abendroth G, Meshulam-Simon G, McCarty PL, Spormann AM (2004) Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp strain VS and its environmental distribution. Appl Environ Microbiol 70(8): 4880–4888
- Popat SC, Deshusses MA (2011) Kinetics and inhibition of reductive dechlorination of trichloroethene, cis-1,2-dichloroethene and vinyl chloride in a continuously fed anaerobic biofilm reactor. Environ Sci Technol 45(4):1569–1578
- Ritalahti KM, Amos BK, Sung Y, Wu QZ, Koenigsberg SS, Löffler FE (2006) Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. Appl Environ Microbiol 72(4):2765–2774
- Sabalowsky AR, Semprini L (2010) Trichloroethene and cis-1,2dichloroethene concentration-dependent toxicity model simulates anaerobic dechlorination at high concentrations. II: continuous flow and attached growth reactors. Biotechnol Bioeng 107(3):540–549
- Schaefer CE, Condee CW, Vainberg S, Steffan RJ (2009) Bioaugmentation for chlorinated ethenes using *Dehalococcoides* sp.: comparison between batch and column experiments. Chemosphere 75(2):141–148
- Steffan RJ, Vainberg S (2012) Production and handling of Dehalococcoides bioaugmentation cultures. In: Stroo HF, Leeson A, Ward CH (eds) Bioaugmentation for groundwater remediation. Springer, New York, pp 89–115
- Sung Y, Fletcher KF, Ritalaliti KM, Apkarian RP, Ramos-Hernandez N, Sanford RA, Mesbah NM, Löffler FE (2006) Geobacter lovleyi sp nov strain SZ, a novel metal-reducing and tetrachloroethenedechlorinating bacterium. Appl Environ Microbiol 72(4):2775–2782
- Tang SQ, Chan WWM, Fletcher KE, Seifert J, Liang XM, Löffler FE, Edwards EA, Adrian L (2013) Functional characterization of

- reductive dehalogenases by using blue native polyacrylamide gel electrophoresis. Appl Environ Microbiol 79(3):974–981
- Tchobanoglous G, Burton FL, Stensel HD (2003) Wasterwater engineering: treatment and reuse. The McGraw-Hill Companies, Inc., New York
- Vainberg S, Condee CW, Steffan RJ (2009) Large-scale production of bacterial consortia for remediation of chlorinated solvent-contaminated groundwater. J Ind Microbiol Biotechnol 36(9): 1180–1197
- Yan J, Ritalahti KM, Wagner DD, Löffler FE (2012) Unexpected specificity of interspecies cobamide transfer from Geobacter spp. to organohalide-respiring Dehalococcoides mccartyi strains. Appl Environ Microbiol 78(18):6630–6636
- Yang YR, McCarty PL (1998) Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. Environ Sci Technol 32(22):3591–3597
- Yu SH, Dolan ME, Semprini L (2005) Kinetics and inhibition of reductive dechlorination of chlorinated ethylenes by two different mixed cultures. Environ Sci Technol 39(1):195–205
- Zheng D, Carr CS, Hughes JB (2001) Influence of hydraulic retention time on extent of PCE dechlorination and preliminary characterization of the enrichment culture. Bioremediat J 5(2):159–168
- Ziv-El M, Delgado AG, Yao Y, Kang DW, Nelson KG, Halden RU, Krajmalnik-Brown R (2011) Development and characterization of DehaloR², a novel anaerobic microbial consortium performing rapid dechlorination of TCE to ethene. Appl Microbiol Biotechnol 92(5):1063–1071
- Ziv-El M, Delgado AG, Yao Y, Kang DW, Nelson KG, Halden RU, Krajmalnik-Brown R (2012a) Development and characterization of DehaloR², a novel anaerobic microbial consortium performing rapid dechlorination of TCE to ethene (vol 92, pg 1063, 2011). Appl Microbiol Biotechnol 95(1):273–274
- Ziv-El M, Popat SC, Cai K, Halden RU, Krajmalnik-Brown R, Rittmann BE (2012b) Managing methanogens and homoacetogens to promote reductive dechlorination of trichloroethene with direct delivery of H₂ in a membrane biofilm reactor. Biotechnol Bioeng 109(9): 2200–2210
- Ziv-El M, Popat SC, Parameswaran P, Kang DW, Polasko A, Halden RU, Rittmann BE, Krajmalnik-Brown R (2012c) Using electron balances and molecular techniques to assess trichoroethene-induced shifts to a dechlorinating microbial community. Biotechnol Bioeng 109(9):2230–2239

