BIOINDICATORS OF REDUCTIVE DECHLORINATION IN A *DEHALOCOCCOIDES ETHENOGENES*-CONTAINING MIXED CULTURE: TRANSCRIPTIONAL TRENDS WITH RESPECT TO SUBSTRATE TYPE, SUBSTRATE CONCENTRATION, AND CULTURE OPERATION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2008

BIOINDICATORS OF REDUCTIVE DECHLORINATION IN A *DEHALOCOCCOIDES ETHENOGENES*-CONTAINING MIXED CULTURE: TRANSCRIPTIONAL TRENDS WITH RESPECT TO SUBSTRATE TYPE, SUBSTRATE CONCENTRATION, AND CULTURE OPERATION

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Cornell University 2008

Expression levels of potentially important gene transcripts were examined in a *Dehalococcoides ethenogenes* strain 195 (DET)-containing mixed culture (D2) capable of reductively dechlorinating tetrachloroethene (PCE) successively to trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), vinyl chloride (VC), and non-toxic ethene using H_2 as electron donor. Gene transcripts associated with general cell activity, including those corresponding to 16S rRNA and subunits of RNA polymerase (RpoB) and an ATP synthase, and also multiple reductive dehalogenase (RDase), hydrogenase (H_2ase) and other oxidoreductase enzymes were selected for study as potential bioindicators of reductive dechlorination in DET. Quantitative reverse-transcriptase PCR (qRT-PCR) data on selected gene transcripts in batch PCE-fed microcosms indicated that a subset of targets, including RDases TceA, PceA, DET1559 and DET1545, the H_2ase Hup, and a gene annotated as formate dehydrogenase (Fdh), were highly up-regulated within 1 to 12 h after PCE feeding. Transcription profiles over time suggested that genes belonging to similar functional groups were regulated in similar ways. Expression studies in cultures fed either electron donor or acceptor showed that neither PCE nor H_2 alone was sufficient to signal up-regulation of chosen bioindicators.

Experiments in microcosms continuously fed medium containing PCE showed that pseudo-steady-state mRNA levels were achieved and that increases in PCE loading rate led to corresponding increases in chloroethene respiration rate. Regulation of steady-state transcript levels of most bioindicators was sensitive to chloroethene respiration rate and/or concentration. Within a limited range of respiration rates (1.5 – 4.8 µmol/L/hr), Fdh, Hup, TceA, PceA and DET1559 transcript levels displayed positive correlation with respiration rate, and could be well-fit with linear regression models (R^2 between 0.95 - 0.97). At high PCE respiration rates, however, most bioindicator levels reached a plateau or decreased, the reasons for which remain unknown. At very low PCE respiration rates, RDases DET1559 and DET1545 were the only potential bioindicators up-regulated above time-0 levels, suggesting they play key roles in reductive dechlorination when substrate concentrations are low. Fdh, Hup and TceA transcript abundances were also high in microcosms continuously fed medium containing TCE or cDCE. Experiments using these alternate electron acceptors indicated that while some RDase bioindicators could reflect substrate utilization, the H_2ase Hup was a more accurate and sensitive indicator of cellular respiration rate.

BIOGRAPHICAL SKETCH

Brian grew up in Irondequoit, New York and nearly missed graduating from West Irondequoit High School because he refused to wear a tie. He attended Cornell University, where he graduated in 1999 with a B.S. in biology. Unable to spark a music career in the crashing dot-com depression that was San Francisco in 2001, Brian decided to go back to school. In 2003, he completed an M.S. in civil and environmental engineering at the University of California at Berkeley, a valuable degree and super-cool autograph of then-governor Arnold Schwarzenegger.

A time and a place: this is for my grandparents

ACKNOWLEDGMENTS

I would like to thank all my friends who have made this time what it was. I would also like to thank Ruth Richardson for putting up with me, for working with me, and for driving me to Wegmans at 1 am to walk life off. To James Gossett and Stephen Zinder, who are pretty much the coolest advisors around. And to Summer for dealing with me while I write this.

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CHAPTER 1

Background and Objectives

This dissertation describes molecular biological analyses carried out on a *Dehalococcoides ethenogenes*-containing, reductively dechlorinating mixed culture under a variety of conditions, including different substrate types, substrate concentrations, and operational feeding regimens. As a papers-based thesis, each main chapter can be read as a discreet story that contains all the information necessary for understanding the observed results and conclusions, and which contextualizes these results within the literature. This background section provides a more complete historical context for the PCE-dechlorinating mixed culture examined here, and also summarizes work with the pure culture of *Dehalococcoides ethenogenes* strain 195 derived from it. Besides providing a reference of past work, this section presents a basic rationale for the hypotheses discussed throughout this text. Chapter 5 synthesizes the results and discussions of each chapter, highlighting the most important observations made during this research. Additionally, it contains a discussion of the many unanswered questions that have arisen, and tries to frame them in a way that facilitates meaningful future research.

1.A. A Brief History of the Mixed Culture

The anaerobic mixed culture upon which this dissertation is based has been operated and studied for about twenty years. It was initially derived from the anaerobic digester of the Ithaca wastewater treatment plant and displayed the ability to biologically dechlorinate tetrachloroethene (PCE), a pervasive organic groundwater contaminant commonly used as a solvent, to the non-toxic end product ethene under methanogenic conditions (*1*). Radiotracer experiments proved that conversion of PCE

to ethene occurred without significant transformation to carbon dioxide (CO_2) or methane (CH_4). It was also observed that the rate-limiting step was the conversion of vinyl chloride (VC), a known human carcinogen, to ethene. Methanol was initially provided as an electron donor, although hydrogen (H_2), formate, acetate and glucose were also found to be effective electron donor substrates. Later, it was shown that PCE dechlorination could be maintained even when solvent concentrations were increased to 550 μM, a concentration found to be inhibitory to methanogenesis (*2*). Under these conditions, electrons derived from methanol were shown to go to dechlorination (31%) and acetate production (69%), suggesting a prominent role for acetogens rather than methanogens. Hydrogen, most likely produced via acetogenic fermentation of provided methanol, was found to be the direct electron donor for PCE dechlorinators (*3*) and remains the only compound to serve this function. Initial attempts to describe the kinetics of this mixed culture effectively ruled out the possibility that reductive dechlorination was being mediated by transition metal cofactors, and instead found that the disappearance of PCE and several of its daughter products, trichloroethene (TCE) and 1,2-*cis*-dichloroethene (cDCE), occurred in near zero-order fashion (*4*). The conversion of VC to ethene, a critical step in the final detoxification of chloroethene contaminants, was observed to obey first-order kinetics, and was subject to what most likely appeared to be competitive inhibition by higher chlorinated ethenes.

Microbiological characterization of the methanol-PCE culture confirmed the presence of high levels of acetogens and decreased numbers of methanogens, in addition to sulfidogens and fermentative heterotrophs (*5*). Competition for H_2 electron donor between dechlorinators and methanogens was examined, with results indicating that dechlorination activity could be maximized by maintaining low H_2 partial pressures (*6*). The secondary electron donor substrate was subsequently switched

from methanol to butyrate, whose fermentation to H_2 is thermodynamically limited to relatively low H_2 levels. While several secondary electron donors were capable of supporting dechlorination on a long-term basis, further studies confirmed that slowly fermented donors that released low concentrations of H_2, such as butyrate and propionate, were optimally suited for this role (*7*). A Michaelis-Menten-type kinetic model was developed for the mixed culture that described electron donor fermentation, acetoclastic methane production, and also the competition for H_2 between dechlorinators and hydrogenotrophic methanogens (*8*). Active dechlorinating biomass parameters were estimated from measurements of volatile suspended solids, assumed yield coefficients, and properties of the reactor system such as mean cell residence time and substrate fluxes. Predicted dechlorination results of the proposed model were in good agreement with experimental data.

1.B. A Brief History of the Pure Culture

Initial attempts at isolation of the dechlorinating organism indicated that it required a complex nutritional supplement and had established a synergistic relationship with one or more organisms in the mixed culture (*3*). Experiments with vancomycin suggested that methanol-fermenting acetogens were producing H_2, which in turn was acting as the direct electron donor for dechlorinators (*5*). The mixed culture was enriched for the dechlorinating organism as knowledge of its nutritional requirements grew. H_2, rather than methanol, was used as a direct electron donor. Yeast extract was replaced with acetate – most likely a carbon source – while B_{12} was found to be the only vitamin supplement required for dechlorination. Isolation of the dechlorinating organism *Dehalococcoides ethenogenes* strain 195 (DET), an irregular coccoid microbe with a doubling time of approximately 19 h, was reported in 1997 (*9*).

This newly purified microbe was at first shown to grow on several chlorinated ethenes, including PCE, TCE, 1,1-dichloroethene (1,1-DCE), cDCE, and 1,2-dichloroethane (DCA) (*10,11*). Although DET demonstrated the ability to dechlorinate other compounds such as 1,2-*trans*-dichloroethene (tDCE) and VC, these were shown to be cometabolic substrates that did not support growth. It was later shown that DET could partially dechlorinate a diverse array of aromatic compounds, including chlorodibenzo-*p*-dioxins, chlorodibenzofurans, polychlorinated biphenyls, and chloronapthalenes, although it was not determined whether these processes were cometabolic (*12*). Growth appeared to be supported on some, but not all, chlorobenzene congeners.

Its unique and relevant metabolism made DET an attractive target for more detailed biochemical and molecular studies. This work began most notably in 1998 when Magnuson et al. found that dehalogenase and hydrogenase activity could be associated with the membrane fraction of cells taken from the methanol-PCE mixed culture (*13*). They found evidence for at least two enzymes catalyzing reductive dechlorination reactions, and added them to a newly described family of proteins called reductive dehalogenases (RDases). One enzyme (PCE-RDase) catalyzed the conversion of PCE to TCE, while another (TCE-RDase) was found to dechlorinate TCE, cDCE, tDCE, 1,1-DCE and VC, although its activity on tDCE and VC was significantly lower than for the other compounds. The TCE-RDase encoding gene (*tceA*) was subsequently cloned and characterized as producing a peripheral membrane bound protein (TceA) that shared limited homology to enzymes exhibiting similar function in phylogenetically unrelated PCE-to-cDCE dechlorinators (*14*). Observations in pure culture supported the new biochemical findings. cDCE accumulation was greater in cultures fed TCE than in PCE-fed cultures, an observation that could be explained by competition between cDCE and TCE for the same

enzymatic binding site (TceA) (*10*). More directly, experiments with methyl viologen, which cannot cross lipid bilayers, also supported the idea that the site of electron transfer for both reductase and hydrogenase reactions was outside the cytoplasmic membrane, and that this membrane contained all the needed components for effective electron transport between PCE and H_2 (*15*).

Completion of the DET genome revealed up to 17 RDase enzymes (plus two homologs predicted to be nonfunctional due to truncation or point mutations causing a premature stop codon) and a variety of other targets potentially involved in respiration and electron transport, including hydrogenases (H_2ase) and other oxidoreductases (*16*). With the exception of DET1545 and the previously characterized *tceA*, all potentially functional RDase genes were found to be in close proximity to potential transcription regulators. Among the five H_2ase genes detected, only the predicted NiFe-*hup* possessed a twin-arginine transport signal, suggesting it plays an important role in processing H_2 in the periplasm. Also, a gene encoding a putative formate dehydrogenase was detected, although DET has not been shown capable of using formate as an electron donor.

1.C. Rationale of Current Approach

Laboratory enrichment microcosm studies, together with a limited number of field-based analyses, have provided a relatively clear picture of the abilities of DET and related *Dehalococcoides* strains and the conditions under which they tend to flourish. Promoting these conditions and the biotransformation processes catalyzed by these organisms is desirable at field sites contaminated with certain halogenated organic compounds. A better understanding of the biochemistry and molecular biology underlying DET metabolism is envisioned to lead to more effective remediation strategies. Many recent studies on a variety of both pure and mixed

Dehalococcoides-containing cultures have been published that serve to provide a broader context for the work described here. Relevant research will be presented in the introductory and discussion sections of each main chapter.

This dissertation describes the detection and quantification of DET DNA and RNA macromolecules, and relates these measurements to chloroethene concentrations and respiration rates. Assays of nucleic acids, especially RNA, were chosen as the main tools of research based on the following rationale. We start with the assumption that all macromolecules examined here originate from DET cells, and that it is these, and only these, cells that are responsible for chloroethene reduction. Within DET cells, the central dogma of molecular biology provides a framework for understanding the flow of information that eventually leads to dechlorination activities and phenotypes. DNA, as a repository for information, acts as a blueprint for all cellular processes and is limited to describing what is possible. Assays of DNA, therefore, can confirm the presence and potential of DET, but cannot indicate activity. RNA is transcribed from DNA, usually in response to environmental and/or intracellular conditions, and acts as an intermediate between DNA and protein. Although it is not directly responsible for activity, RNA does reflect an initiation of protein synthesis. Generally, the translation of mRNA into protein results in the creation of functional enzyme, and protein assays provide direct evidence of specific DET activities.

In accordance with this biological dogma, therefore, DET dechlorination activity is a direct result of cellular protein composition, which is in turn related to the transcription of RNA from the DNA template. Protein assays, however, suffer from methodological limitations associated with extraction and quantification of specific proteins, particularly in complex populations found in most mixed cultures and field environments. RNA and DNA assays, on the other hand, utilize highly specific oligonucleotide sequences, and are capable of reliably detecting and quantifying

discreet targets even when culture complexity is high. As described above, cellular RNA content is regulated based on immediate environmental conditions and metabolic needs, while DNA content remains static even during periods of intense activity. It is for these reasons that the majority of data presented here will focus on DET nucleic acid characterization, particularly of RNA. It should be stressed, however, that making inferences about protein abundance and activity based on measurements of RNA has its limitations, a point that is discussed in more detail in Chapter 5.

1.D. Research Objectives

In this thesis research, bioindicators of reductive dechlorination are molecules (in this case RNA) whose presence and abundance reflect the substrate range and activity level of DET populations. Good RNA bioindicators have three characteristics: they should be specific, accurate and quantifiable. In other words, they should be specific to the activity of interest (reductive dechlorination), can be accurately correlated to that activity, and can be detected and measured with relative ease. Overall, the goal of this work is to enhance the understanding of DET-mediated reductive dechlorination in mixed culture through examination of potential bioindicator gene transcripts of known and putative respiratory chain components.

Main objectives include:

1. Identify specific DET gene transcripts that might be used as bioindicators of reductive dechlorination
2. Determine how chosen bioindicators are expressed under typical mixed culture conditions
3. Determine how changes in substrate concentration, substrate type and operational reactor conditions affect bioindicator expression

4. Relate bioindicator expression under various conditions to protein abundance and cellular respiration rates

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CHAPTER 2

Temporal Expression of Respiratory Genes in an Enrichment Culture Containing *Dehalococcoides ethenogenes* *

2.A. Abstract

Multiple reductive dehalogenase (RDase), hydrogenase (H_2ase), and other respiration-associated (RA) oxidoreductase genes have been identified in cultured representatives of *Dehalococcoides*. Although their products are likely to play key roles in the environmentally important process of reductive dechlorination, very little information is available about their regulation and specific functions. Here we show increased expression and temporal variability in the expression of five RDase genes and in the expression of genes for a putative formate dehydrogenase (Fdh) and two H_2ases, including a periplasmic [Ni/Fe]-H_2ase (Hup) and a cytoplasmic [Fe]-H_2ase (Vhu). mRNA transcripts extracted from PCE-dechlorinating mixed cultures corresponding to Fdh, the H_2ase Hup and the RDase targets TceA and DET0162 were expressed most highly, with average levels 34 (± 7.5), 23 (± 6.7), 16 (± 3.3) and 13 (± 3.3)-fold higher, respectively, than that for RNA polymerase (RpoB). H_2ase and RA transcripts reached their respective expression maxima within the first 2 hours after feeding. RDase transcripts, however, were most highly expressed after 3 hours, and exhibited greater temporal variability than other targets. Comparison with *D. ethenogenes* strain 195 pure culture expression levels indicated that RDase DET1545 was more highly expressed in mixed cultures where, on average, its transcript level was sixfold higher than that of RpoB. While the specific function of several of these

* Rahm, B. G.; Morris, R. M.; Richardson, R. E. Temporal expression of respiratory genes in an enrichment culture containing *Dehalococcoides ethenogenes*. *Appl. Environ. Microbiol.* **2006,** *72*, 5486-5491.

gene products remains elusive, the high expression levels and temporal variability reported here suggest that these groups of enzymes are metabolically important for the respiration of chlorinated ethenes in mixed cultures containing *Dehalococcoides*.

2.B. Introduction

Chlorinated ethenes are common groundwater contaminants that are found at more than half of the sites on the EPA's National Priorities List (*1*). A mixed enrichment culture capable of reductively dechlorinating tetrachloroethene (PCE) successively to trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), vinyl chloride (VC), and the harmless compound ethene was developed to study this microbially mediated process (*2*). The enrichment culture (D2) has been maintained for more than a decade and is currently the source of an extract required to grow pure cultures of *Dehalococcoides ethenogenes* strain 195, a member of the *Chloroflexi* phylum capable of complete anaerobic reductive dechlorination of PCE. *D. ethenogenes* uses H_2 as its sole electron donor; in the D2 enrichment culture, H_2 is formed from the anaerobic fermentation of butyric acid to acetate and H_2 (*3*,*4*). This organism, which was isolated from an earlier generation of the D2 enrichment culture, has been well characterized and its genome has been sequenced (*5*-*8*). Characterization of additional *Dehalococcoides* indicates that although all strains have multiple reductive dehalogenase (RDase) genes, both the number of RDase genes and the corresponding substrate ranges can vary by strain (*9*-*11*).

Of the 19 putative RDases identified in the genome of *D. ethenogenes* strain 195, only those corresponding to PceA and TceA, which are believed to catalyze the reductions of PCE to TCE (*12*) and of TCE to ethene (*13*), respectively, have been characterized. Very little is known about the specific functions of additional RDases and other putative respiratory enzymes in the D2 enrichment culture containing strain

195. Recent studies in both pure (J. Fung, personal communication) (*14*) and mixed (*14*) PCE-fed cultures containing strain 195 indicated that genes predicted to encode four RDases (TceA, DET0162, DET0318, and DET1559), a periplasmic [Ni/Fe]-H_2ase (Hup), and a putative formate dehydrogenase (Fdh) exhibited the highest overall expression levels. In another study, expression of multiple RDases in a chloroethene reducing *Dehalococcoides*-containing mixed culture (KB1) was induced by a single chlorinated substrate, suggesting that several RDase enzymes might contribute to chloroethene dechlorination (*11*). Although RDase DET1545 did not show increased expression levels in PCE-grown strain 195 pure cultures, Waller and colleagues reported expression of a closely related homolog (*11*), and peptides matching DET1545 were detected during proteomic analyses of strain 195 (R. Morris, unpublished data).

It has recently been shown for *Geobacter sulfurreducens* that molecular parameters can serve as bioindicators of interesting metabolic processes and that levels of mRNA transcripts can be correlated with rates of substrate reduction (*15*). In the present study, we targeted several genes from *D. ethenogenes* strain 195 that may serve as potential bioindicators of reductive dechlorination and describe their expression profiles over the course of a PCE feeding cycle. Targets included eight RDases that showed increased expression during growth on PCE in pure culture, a gene whose RDase was identified by proteomic analyses (DET1545), five H_2ase genes, and four additional respiration-associated (RA) transcripts (Table 2.1). These data provide novel insights into the relative expression levels and temporal expression variability of key *D. ethenogenes* respiratory oxidoreductase genes in mixed cultures and suggest that they may serve as good bioindicators of PCE reductive dechlorination.

2.C. Materials and Methods

2.C.1. Chemicals and stock solutions

Butyric acid (99%; Acros Organics) and PCE (99%; Alfa Aesar) were used as culture substrates and, in the case of PCE, as an analytical standard. TCE (99.5%; Fisher Scientific), *cis*-1,2-DCE (97%; Aldrich Chemical Co.), VC (99.5%; Matheson Gas Products) and ethene (Matheson Gas Products) were used for preparation of analytical standards. Yeast extract (Difco Laboratories) was used as a culture amendment.

2.C.2. Culture procedure

A PCE-butyrate enrichment culture containing *D. ethenogenes* strain 195, designated D2, was maintained as described previously (*16*,*17*). Ten percent of the culture was periodically wasted and replaced with fresh basal medium (*3*) to obtain an average hydraulic residence time of approximately 100 days. Expression studies were performed in triplicate 160 mL subculture serum bottles with a headspace-to-liquid ratio similar to the D2 enrichment culture. The D2 enrichment culture and each subculture were fed PCE (110 µM), butyric acid (440 µM) at a 2:1 ratio to PCE on an electron equivalents basis (with butyric acid defined as having 4 equiv/mol based on its fermentation to 2 moles acetate and 2 moles H_2 rather than its oxidation to CO_2), a vitamin solution (*18*), and yeast extract to obtain a concentration of 20 mg yeast extract/liter of culture. A subculture lacking PCE was set up as a control to determine whether the activity of other organisms in the enrichment culture might contribute to expression levels. Strain 195 pure culture was grown on PCE and H_2 as previously described (*5*,*6*). In short, culture inoculum sizes were 2% (vol/vol) in 27 mL culture tubes containing 10 mL of growth medium. Basal salts medium was amended with 2 mM acetate, a vitamin solution containing 0.05 mg of vitamin B_{12} per liter, 10%

(vol/vol) filter-sterilized anaerobic digestor sludge supernatant, and 1% (vol/vol) D2 enrichment culture extract. Culture tubes were sealed with Teflon-coated butyl rubber stoppers and incubated at 35 °C.

2.C.3. Gas chromatographic methods

Ethene and chlorinated ethenes were measured by taking 100-µL headspace samples via a gas-tight locking syringe, analyzed with a Perkin Elmer Autosystem Gas Chromatograph utilizing a 1/8-inch by 8-ft stainless-steel column packed with 1% SP-1000 on 60/80 Carbopak B (Supelco, Inc.) and routed to a flame-ionization detector as described previously (*2,17*). Column temperature was held at 90 °C for 2.5 min, subsequently ramped at 30 degrees per minute to 195 °C, and then held isothermally for 8 min. The flame ionization detector was isothermally held at 90 °C over the 14-minute run time. Standard curves for PCE, TCE, cDCE, VC and ethene were created by adding known amounts of each pure compound to 160-mL serum bottles containing 100 mL of distilled H_2O.

2.C.4. Sampling procedure and nucleic acid extraction

Liquid culture samples were taken from the D2 enrichment culture and from each subculture prior to feeding (time zero), and at selected times following feeding. A sterile syringe was purged three times with a 70% N_2-30% CO_2 gas mixture and used to withdraw either 1 or 2 mL of liquid culture for DNA or RNA analyses, respectively. The samples were placed in centrifuge tubes and immediately pelleted at 21,000 × *g* for 2 min at 4 °C. Supernatants were discarded and cell pellets were stored at –20 °C or –80 °C prior to DNA and RNA extraction, respectively. Pure culture cell pellets (3 mL) for RNA extractions were prepared by centrifugation at 4 °C for 10 min at 21,000 × *g*. The supernatant was discarded and cell pellets were stored at -20 °C.

DNA and RNA extractions were performed within 24 h using the UltraClean Microbial DNA Isolation (Mo Bio Laboratories) and RNeasy Mini (Qiagen) kits. To control for mRNA losses during sample preparation and inefficiencies in reverse transcription, a normalization protocol modified from that described in the work of Johnson et al. (*19*), in which 6 x 10^9 copies of luciferase control RNA (Promega) were added during the lysis step of each RNA extraction, was employed. DNA contamination was removed from RNA samples according to the optional on column RNase-free DNase I (Qiagen) digestion protocol. RNA was quantified using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). A second DNase treatment step lasting 30 min was performed using RQ1 RNase-free DNase (Fisher Scientific).

2.C.5. Quantitative reverse transcriptase PCR (qRT-PCR)

On average, about 1 µg of RNA was obtained per mL of culture collected. cDNA was synthesized from 0.2 µg of RNA using an iScript cDNA Synthesis kit (BioRad) according to the manufacturer's instructions. Gene transcripts were quantified by amplification of cDNA with iQ SYBR Green Supermix (BioRad) and primers specific for *D. ethenogenes* strain 195 gene targets and for the luciferase control. H_2ase primers were designed using the software package Beacon Designer 4 (Biosoft International) (*14*). Other *D. ethenogenes*-specific oligonucleotides were designed using PrimerQuest (*20*) and mFold software available at the IDT website (http://scitools.idtdna.com/Primerquest/) (J. Fung and S.H. Zinder, personal communication). Primer specificity was checked by BLAST analysis (*21*). Standard curves for *D. ethenogenes* targets and the luciferase control target (log DNA concentration versus cycle number at which fluorescence reaches an arbitrarily set cycle threshold value [C_t]) were generated using serial dilutions of DNA of known

concentration extracted from pure and mixed enrichment cultures (for *D. ethenogenes* targets), and luciferase control DNA (for the luciferase control target). Triplicate amplifications of all standards, unknowns, and controls were performed using an iCycler iQ Multicolor Real-Time PCR Detection System (BioRad); 25-µL reaction volumes contained 1x iQ SYBR Green Supermix, forward and reverse primer at a concentration of 700 nM, and approximately 3 ng of cDNA template. PCR conditions used for H_2ase primer sets were as follows: 2 min at 50 °C and 3 min at 95 °C, followed by 40 cycles of 1 min at 55 °C and 1 min at 95 °C. PCR conditions for all other primer sets were: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 1 min at 60 °C and 1 min at 95 °C. Melt curve analyses were performed after all runs to check for purity of amplicons. A pure culture DNA sample of known quantity was analyzed with each primer set and yielded the same abundance value regardless of the primer used, supporting the suitability of the mixed culture standard curves.

2.D. Results

2.D.1. Preliminary analysis of gene expression and target selection

qRT-PCR data were taken from the D2 enrichment culture over the course of a PCE-butyrate feed to identify temporal trends in gene regulation and to select highly expressed targets for further analyses (Figure 2.1; Table 2.1). Genes encoding four RDases (TceA, DET0162, DET0318, and DET1559), two H_2ases (Hup and Vhu), and two RA genes (Fdh and AtpA) exhibited the highest overall expression levels and were greater than that of the gene encoding RpoB, which was chosen as a "housekeeper." The RDase DET1545 was not targeted in the initial D2 expression study. Its product, however, was subsequently identified by liquid chromatography-tandem mass spectrometry proteomic approaches, and it was added to the list of potential bioindicators. A comparison of pure and mixed culture expression levels

Table 2.1. Genomic identification and description of qRT-PCR targets.

Locus tag [a]	Description	Abbreviation [b]	Class
0603	DNA-dependent RNA polymerase, beta subunit	RpoB	Housekeeper
0187	formate dehydrogenase, alpha subunit, putative	Fdh	Other respiration-associated
0926	proton-translocating NADH-quinone oxidoreductase, D subunit, putative	Nuo	
0562	ATP synthase, F1 alpha subunit	AtpA	
0103	molybdopterin oxidoreductase, iron-sulfur binding subunit, putative	Mod	
0110	[Ni/Fe] hydrogenase, group 1, large subunit, putative	Hup	Hydrogenase
0615	hydrogenase, group 3, VhuA subunit, putative	Vhu	
0867	hydrogenase, group 4, EchE subunit, putative	Ech	
1571	hydrogenase, group 4, HycE subunit, putative	Hyc	
0147	[Fe] hydrogenase, large subunit HymC, putative	Hym	
0079	trichloroethene reductive dehalogenase (*tceA*) gene	TceA	Reductive Dehalogenase
0318	reductive dehalogenase, putative	PceA [c]	
1545	reductive dehalogenase, putative		
0162	reductive dehalogenase, putative, auth pt mutation		
1559	reductive dehalogenase, putative		
0173	reductive dehalogenase, putative		
0876	reductive dehalogenase, putative		
0306	reductive dehalogenase, putative		
1519	reductive dehalogenase, putative		

[a] orf identification number from the *D. ethenogenes* strain 195 genome

[b] abbreviation of known or putative protein name

[c] Stephen H. Zinder, personal communication

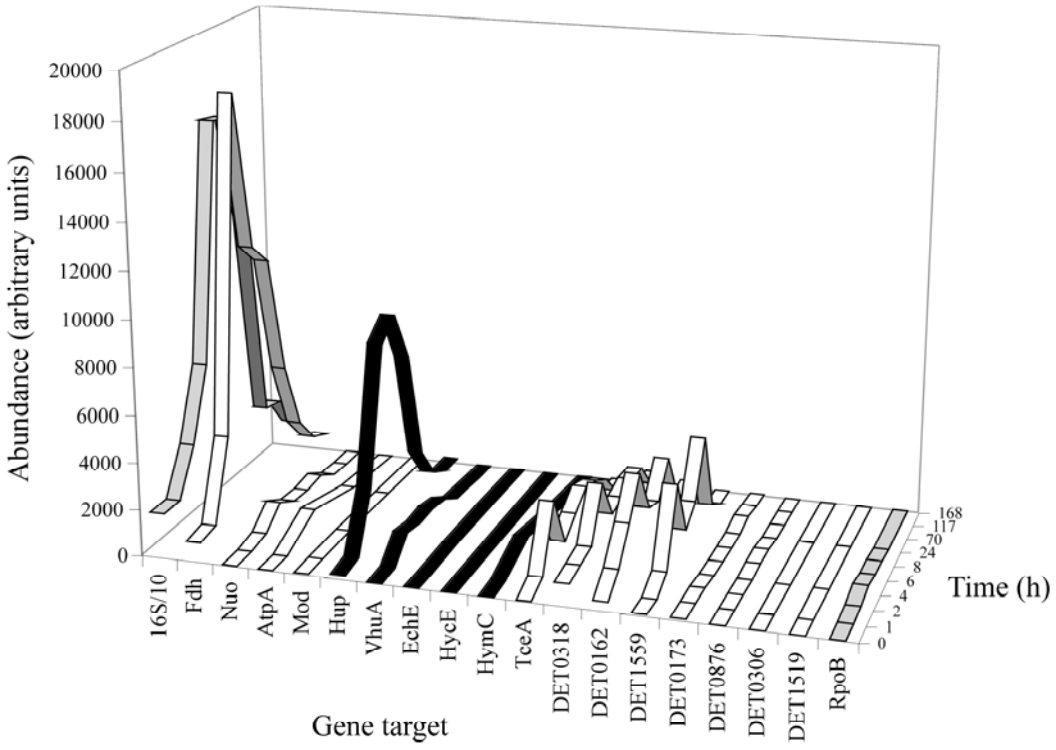


Figure 2.1. Expression profiles of potential bioindicator targets in the D2 enrichment culture. 16S rRNA and transcripts corresponding to Fdh, the H_2ase Hup, and the RDases TceA, DET0162, DET0318 and DET1559 are preferentially expressed.

indicated that DET1545 expression was lower than RpoB expression in pure cultures but increased by nearly 5.6 (± 3.4) fold relative to RpoB expression in mixed cultures. Genes encoding the H_2ase Hym and the RA target NADH-ubiquinone oxidoreductase (Nuo) had expression levels similar to that for the RpoB gene, and the expression levels of additional RDase, H_2ase, and RA targets, including the molybdopterin oxidoreductase (Mod) gene, were lower than that for the RpoB gene and were not included in additional expression studies reported here.

2.D.2. Expression and temporal variability

Preliminary qRT-PCR results (Figure 2.1) suggested that expression of some targets increased within 1 h of feeding and reached maximum observed levels within 12 h, after which point expression tended to slowly decline until returning to the hour 0 state. As a result, high-resolution qRT-PCR measurements of a subset of highly expressed targets were taken from triplicate subcultures inoculated with D2 and incubated for 12 h. With the exception of DET1545 and DET0318, all targets studied showed at least an order of magnitude increase in expression between 0 and 1 h. The Fdh target had average expression levels 34 (± 7.5) fold higher than that of the housekeeper RpoB, and was followed by the H_2ase Hup and the RDase targets TceA and DET0162, which had average expression levels 23 (± 6.7), 16 (± 3.3) and 13 (± 3.3) fold higher, respectively, than that for RpoB (Figure 2.2). Similarly high expression levels were observed for the Fdh target in a previous study, which indicated that the Fdh target was up-regulated in both batch-pure and mixed cultures (*14*); this result was surprising, given the evidence that *D. ethenogenes* strain 195 is not capable of using formate as an electron donor (X. Maymó-Gatell, Y. Chien, T. Anguish, J. M. Gossett, and S. H. Zinder, unpublished results). For all targets studied,

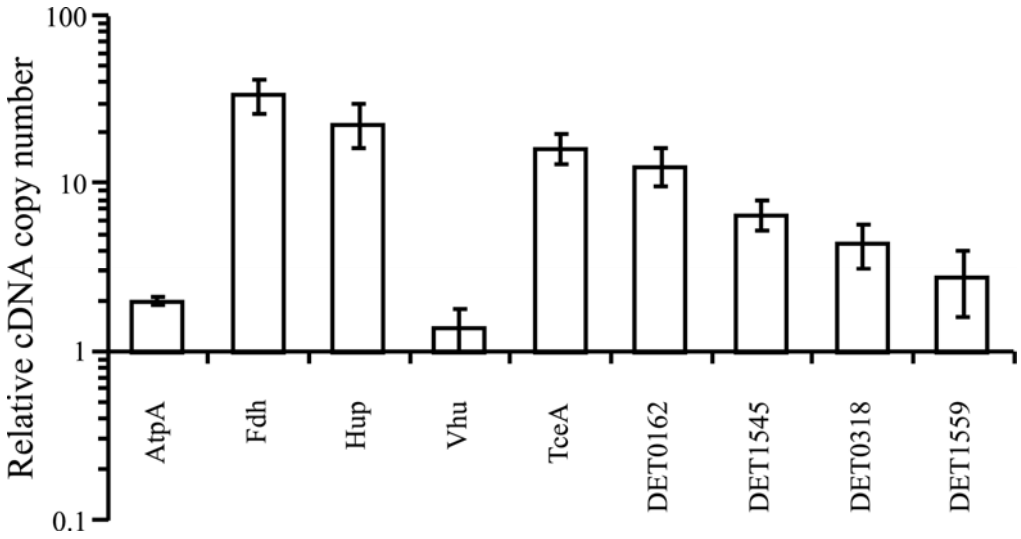


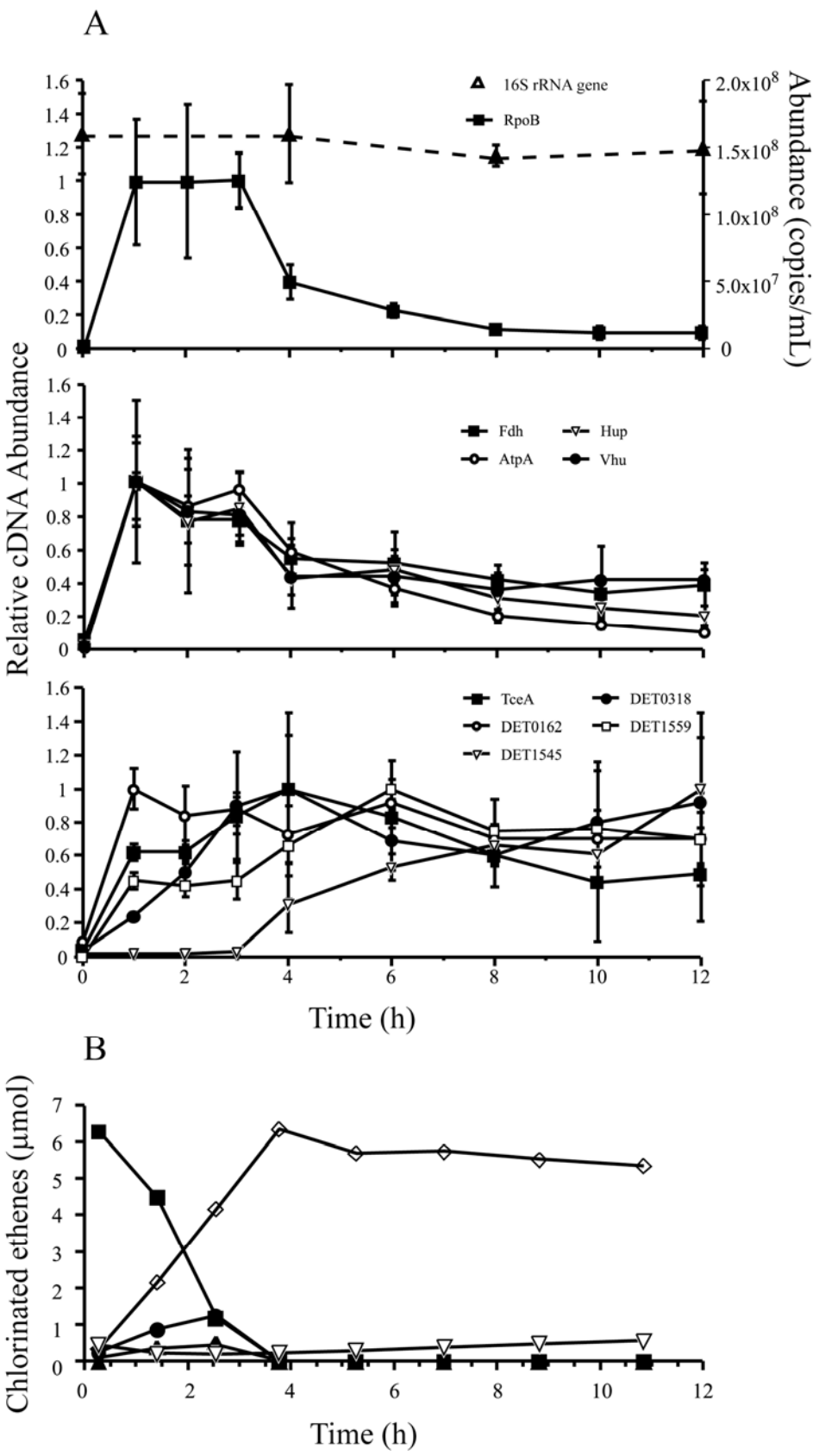
Figure 2.2. Expression level of each target in triplicate subcultures relative to that for the RpoB gene (data presented are averages of values from 1 to 12 h after PCE feeding). Targets are represented by their corresponding products. Error bars represent standard deviations.

average expression levels in the control subculture lacking PCE were approximately 2 orders of magnitude lower (2 % on average) than in PCE-fed subcultures.

No significant increases were observed in *D. ethenogenes* 16S rRNA gene abundance during the first 12 h after PCE feeding (Figure 2.3A). These data suggest that the population of *D. ethenogenes* stayed relatively constant throughout the experiment and that qRT-PCR trends reported here reflected differences in expression rather than cell numbers. Temporal variability in the expression profile of the RpoB gene, when plotted relative to its own expression maximum, indicates that the period of highest cellular activity is between 0 and 3 h (Figure 2.3A). Statistical comparison of three gene groups - RpoB, H2ase/RAs (Hup, Vhu, Fdh, and AtpA), and RDases - indicate that expression profiles differ across the three groups (One-way ANOVA showed significant interaction of time by gene group $F(8,2) = 4.612$; $P < 0.0001$). General trends in gene categories suggest that H_2ase and RA transcript levels were highest early during dechlorination (0 to 3 h), while RDase transcript levels increased more slowly, reached their maxima after 3 h, and persisted for up to 12 h (Figure 2.3A). RDase transcript levels were typically highest after TCE, cDCE, and VC daughter products had reached their maximum values (Figure 2.3A and B). Reductive dechlorination profiles resembled those previously reported for the D2 enrichment culture (*4*). Daughter products were observed within 0.3 h, and added PCE was dechlorinated by near-zero-order kinetics to VC and ethene within 4 to 5 h. Between 2 and 3 h, TCE and cDCE accumulated to their respective maxima of approximately 10 and 5 µmol/L. VC concentration increased for 3 to 4 h, at which point it was slowly converted to ethene for the duration of the experiment (Figure 2.3B).

Previous studies have suggested that expression levels of *Dehalococcoides* targets may vary over time and that conclusions based on any single time point may be confounding (*14*). Figure 2.4 indicates that, between hours 1 and 12, some targets

Figure 2.3. **(A)** 16S rRNA gene abundance (dashed line) and mRNA expression profiles (solid lines) of individual targets over time broken up into functional categories. Error bars represent standard deviations. Each mRNA target is plotted as a fraction of its maximum expression and is represented by its corresponding product. Expression of the RpoB gene, a central metabolic housekeeper, declines after 3 to 4 h, when a majority of respirable substrates are dechlorinated. The Fdh, AtpA, Hup and Vhu genes are grouped as targets associated with hydrogen metabolism and experience maximum expression within the first two hours. RDase genes are grouped together and tend to experience maximum expression after 3 h. **(B)** Dechlorination profiles in triplicate subculture bottles. PCE (■), TCE (●) and cDCE (▲) are fully dechlorinated within 4 to 5 h, followed by the slow conversion of VC (◇) to ethene (▽).


A
16S rRNA gene
RpoB
Abundance (copies/mL)
Relative cDNA Abundance
Fdh
Hup
AtpA
Vhu
TceA
DET0318
DET0162
DET1559
DET1545
Time (h)
B
Chlorinated ethenes (µmol)
Time (h)

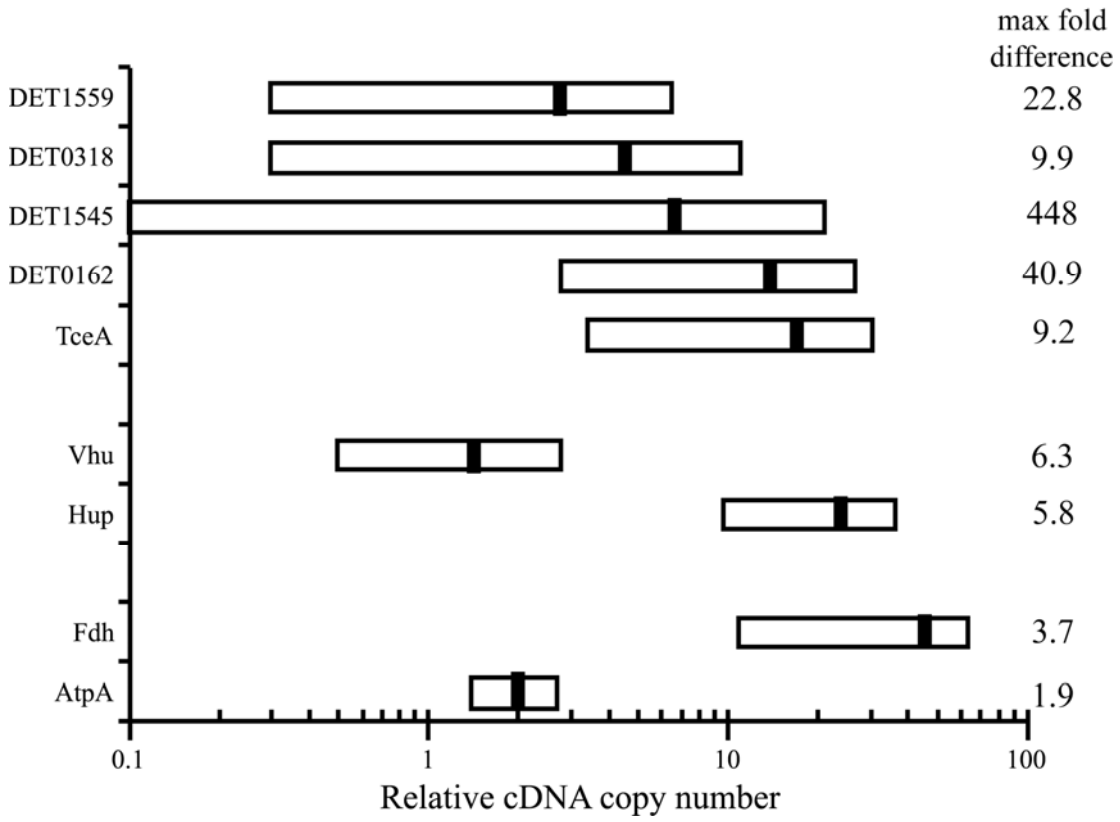


Figure 4.4. Temporal ranges of expression levels relative to that for the RpoB gene. Left and right ends of horizontal bars mark the levels of lowest and highest expression, respectively, for hours 1 through 12. Black hash marks within each bar indicate the average levels of relative expression during this time. The data column shows the difference (n-fold) between the highest and lowest expression level for each target; targets are represented by their corresponding products.

have a larger range of expression relative to that of the RpoB gene than others. The relative abundance of the TceA gene target at its maximum is 9.2 times higher than its lowest observed relative abundance during this time period. In contrast, the Hup gene target shows only a 5.8 fold difference in relative abundance between its highest and lowest expression levels. In general, group comparison indicates that RDase targets are expressed with greater variability than are H2ase or RA targets.

2.E. Discussion

Genes encoding Fdh, the H_2ase target Hup, and RDase targets TceA, DET0162, DET1545, DET0318 (tentatively identified as PceA - S.H. Zinder, personal communication), and DET1559 were transcribed at levels above that for the housekeeping gene encoding RpoB, and their corresponding proteins, with the exception of DET0162, were identified in a previous study using proteomic approaches (*14*). The DET0162 target, although highly expressed, contains a point mutation, has a partially truncated anchor protein, and is unlikely to code for a functional RDase (*8*). RDase and H_2ase targets tended to be expressed as groups of respiratory oxidoreductases that exhibited different patterns of temporal variability. Additionally, expression by gene category, particularly in the case of the RDase genes, varied significantly over time. It is likely that the products of these genes play important roles in reductive dechlorination. Further research into the relationship between expression levels and the catalytic functions of these enzymes is likely to provide novel insights into the signal(s) that controls their expression at field sites contaminated with chlorinated ethenes.

While increases in expression were observed within the first hour after PCE feeding, expression in no-PCE controls did not significantly increase, supporting the conclusion that expression levels are attributable to *D. ethenogenes* rather than to other

members of the community, such as fermenters or methanogens. Furthermore, measurements of relative cell numbers did not indicate a significant change in the population size during the time frame of this study (Figure 2.3A). Although some growth certainly occurred in these cultures, increases in cell numbers alone could not account for the order of magnitude increases in expression that were observed.

Two recent studies of *Dehalococcoides* have shown that expression levels of TceA and H_2ase targets can vary with time (*14*,*22*). Johnson et al. examined the RDase target TceA under a variety of conditions and found that its expression was independent of hydrogen concentration and chlorinated ethene concentration down to about 2 µM but that it varied according to the electron acceptor used (*22*). In general, they found that expression of the TceA target increased over time in response to growth-supporting substrates (TCE, cDCE, 1,1-DCE) as well as to *trans*-DCE, which does not support growth, but that PCE or VC did not lead to increased expression. Our results indicate that expression levels in batch cultures of many key respiratory targets, particularly RDases, depend on time of sampling and that individual target expression does not appear to correlate with the instantaneous dechlorination of specific chlorinated ethenes. Furthermore, results show distinct temporal patterns in the ways that RDases and H_2ases are expressed: H_2ases and RA targets (including Fdh and AtpA) tend to reach their maximum expression earlier in the feeding cycle, as does the housekeeper RpoB, while RDases tend to reach their maxima later. *D. ethenogenes* may maintain a relatively large "standing crop" of RDase enzymes which, though adequate for initial dechlorination, require augmenting as high concentrations of substrate persist. It is also possible that *D. ethenogenes* devotes its initial energy to the gathering of electrons, up-regulating RDase expression only after creating a sufficient pool of reducing energy (a high concentration of charged energy

carriers). Finally, the expression of RDase and H_2ase targets may be up-regulated as daughter products (such as TCE, cDCE, or VC) reach critical concentrations.

The variability in expression trends across functionally distinct gene categories (housekeeper, H_2ase, RA and RDase) suggest that transcriptional regulation is occurring at the group level. Housekeeper, H_2ase and RA target expression profiles share similar patterns of temporal variation that are different from those of RDase profiles (Figure 2.3A). While RDases as a group are up-regulated later than H_2ase and RA targets, the timing and extent of up-regulation varies for each RDase target. This suggests that unique regulatory pathways exist for each RDase gene and agrees with the prediction of two component regulatory elements (histidine kinases and response regulators) flanking most of the RDase genes in the available *Dehalococcoides* genomes (*8,10*). These regulatory elements may work in both *cis* and *trans* fashion to coordinate expression of multiple RDase genes.

Our goal was to identify possible bioindicators of reductive dechlorination and understand their expression over time in response to addition of growth-limiting substrates. A good bioindicator for ultimate field use should be specific (unique to the genes imparting the desired activity), accurate (correlated to the desired activity) and quantifiable (detectable and measurable). The housekeeper RpoB is an attractive option, since it is highly conserved among *Dehalococcoides* groups compared to more mobile and divergent targets such as the RDases. On the other hand, H_2ases and RDases, once a better understanding of their expression under various conditions is obtained, may have the ability to yield more information about dechlorination potential and rate. Also, the higher expression levels of some H_2ase and RDase targets may make them more attractive options at field sites where cell densities are low and detection limits are a major concern. Accurate and comprehensive documentation of in situ bioremediation at a field site will probably require a suite of bioindicators that

includes highly conserved, more metabolically central targets, such as RpoB, and targets more specific to reductive dechlorination, such as Hup and TceA. Here we report novel respiratory oxidoreductase expression data from a mixed dechlorinating community containing *D. ethenogenes*, identify temporal patterns in gene regulation, and suggest potential bioindicators.

2.F. Acknowledgments

This work was supported by the U.S. Department of Energy (DOE) Office of Cleanup Technologies and administered by the U.S. Department of Energy Savannah River (SR) Operations Office (Contract No. DE-AC09-96SR18500), and is a product of the Monitored Natural Attenuation/Enhanced Attenuation for Chlorinated Solvents Technology Alternative Project.

We thank Jennifer Fung and Stephen Zinder for RDase primer sequences and valuable advice, Jennifer Zabinsky for assistance with the D2 enrichment culture, and Dr. Merri Rosen for help with statistical analyses. Professor James Gossett also provided valuable advice on the manuscript.

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CHAPTER 3

Correlation of Respiratory Gene Expression Levels and Pseudo-Steady-State PCE Respiration Rates in *Dehalococcoides ethenogenes* *

3.A. Abstract

DNA and RNA transcripts, particularly of genes of functional importance in the reductively dechlorinating microbe *Dehalococcoides*, are increasingly being studied as potential molecular bioindicators of reductive dechlorination. Ideally, mRNA bioindicators would be informative both qualitatively (with respect to dechlorination endpoint and substrate range) and quantitatively (with respect to activity rates). Here, we examined pseudo-steady-state mRNA levels in *Dehalococcoides*-containing microcosms continuously fed PCE at various loading rates. We characterized gene transcript abundance of potential *Dehalococcoides* bioindicators of reductive dechlorination, including 16S rRNA, and genes encoding an annotated formate dehydrogenase (Fdh), the hydrogenase (H_2ase) Hup, and the reductive dehalogenases (RDases) TceA, DET1559, PceA and DET1545. Increases in steady PCE loading rate led to corresponding increases in PCE respiration rate (1.5 ± 0.1, 2.5 ± 0.3, 4.8 ± 0.1, and 9.2 ± 0.5 µmol/L/hr). We also observed that pseudo-steady-state expression levels of most functional targets increase linearly over PCE respiration rates of 1.5 to 4.8 µmol/L/hr, with Fdh, Hup and TceA transcripts increasing by approximately 2 x 10^{10} copies per mL of culture for every µmol/L/hr increase in chloroethene respiration rate, and DET1559 and PceA transcripts increasing by approximately 9 x 10^{9} copies per mL of culture, whereas increased

* Rahm, B. G.; Richardson, R. E. Correlation of respiratory gene expression levels and pseudo-steady-state PCE respiration rates in *Dehalococcoides ethenogenes*. *Environ. Sci. Technol.* **2007,** in press.

respiration rates of 9.2 µmol/L/hr did not necessarily lead to corresponding increases in transcript levels.

3.B. Introduction

Characterizing the microbially mediated reductive dechlorination of chlorinated ethenes at contaminated field sites is often limited to gas chromatographic assays of the chlorinated compounds themselves, and to measurements of geochemical parameters that may signal adequate reducing conditions (*1-6*). However, there is increasing evidence that molecular biological characterization of microbial populations may also be useful for characterization of contaminated sites and for making accurate predictions about microbial potentials and activities (*7-14*). Assays of specific microbial DNA and RNA sequences can be attractive to scientists and engineers who seek to enhance inferences based on geochemical data alone. To date, use of microbial bioindicators at chloroethene-contaminated field sites has been limited to assays for DNA signatures of *Dehalococcoides* (DHC) populations known to carry out reductive dechlorination. This is accomplished by targeting, and in some cases quantifying, DHC 16S rRNA genes, although there have been some efforts to target other gene sequences as well (*12,13,15-21*).

Expression studies in batch-fed, reductively dechlorinating cultures containing DHC strains have shown that certain "functional" targets – gene transcripts whose corresponding enzymatic proteins are potentially related to this group's unique respiration capabilities – are abundant and potentially valuable as bioindicators (*22-28*). These include reductive dehalogenases (RDases), which are responsible for the reductive dehalogenation of chlorinated organic compounds; hydrogenases (H_2ases), which oxidize hydrogen, the only known electron donor for DHC strains; and a putative formate dehydrogenase (Fdh). Up-regulation of bioindicator expression in

batch-fed enrichment and pure cultures can depend on substrate type and substrate concentration (*24-27*). Previous work in batch culture has suggested that RDase genes are transcriptionally regulated (*22,24-27*), a feature which is predicted from their proximity to regulatory genes in DHC genomes (*29,30*), and that multiple RDases are often co-expressed upon growth on a single chlorinated substrate (*26-28,31*). However, it is unclear whether results obtained under batch-fed conditions, in which cells experience periods of high chloroethene concentration followed by starvation, can be extrapolated to field environments. No studies have yet tested the relationship between steady-state respiration rates by DHC and functional gene expression levels.

Here we explore the behavior of a reductively dechlorinating mixed culture (D2) containing *Dehalococcoides ethenogenes* strain 195 (DET) as its sole dechlorinating constituent (*32,33*) by utilizing a pseudo-steady-state (PSS) reactor system in which PCE is continuously loaded at different rates for up to 24 hours. This system more closely reflects the continuous and relatively steady recharge of chlorinated ethenes seen by microbial populations at field sites and allows investigation into the transcription of bioindicator gene targets under conditions of low substrate concentration. In this study, data on DET genes previously found to be significantly up-regulated during the reductive dechlorination of PCE, including those corresponding to RDases TceA, DET1559, PceA (corresponding to DET0318 [*22*]) and DET1545, the H_2ase Hup, and Fdh (*26*), suggest that expression occurs at steady levels which correlate with chloroethene respiration rates within a limited range. Additionally, these findings support the idea that RDases are individually regulated and provide evidence that induction of expression occurs at lower chloroethene levels than previously tested.

3.C. Materials and Methods

3.C.1. Culture procedure

A DET-containing enrichment culture (D2) capable of reductively dechlorinating PCE was maintained as previously described (*26*,*34*). Briefly, the D2 enrichment culture was pulse-fed PCE (110 µM), butyric acid (440 µM) at a 2:1 ratio to PCE on an electron equivalent basis (with PCE defined as having 8 equivalents/mol based on its reductive dechlorination to ethene, and butyric acid defined as having 4 equivalents/mol based on its fermentation to 2 mol acetate and 2 mol H_2 rather than its oxidation to CO_2), a vitamin solution (*35*), and 20 mg yeast extract/liter of culture. Expression and chloroethene concentration data were obtained from triplicate 100 mL PSS subcultures established in 160 mL serum bottles. PSS microcosms were seeded with D2 culture that had been starved for 72 hours. They were then given an initial pulse-feeding of vitamin solution, yeast extract, and 44 µmoles butyric acid, the fermentation of which has been shown to facilitate the slow release of electron donor concentrations adequate for dechlorination for at least 20 hours (*36*). Culture bottles were then held upside-down at a 45º angle over a magnetic stir plate and stirred vigorously. Filtered basal culture medium (*37*) containing approximately 414 µM PCE was delivered at rates of 5, 10, 20 and 40 µL per min through the submerged top stopper via 10 mL gas-tight locking syringe dispensed by syringe pump. To allow for headspace sampling without disturbing the bottle, a second port was drilled into the side of each PSS serum bottle and fitted with a Teflon coated butyl stopper held in place with a screw tight ring clamp. Gas and liquid samples were extracted by syringe from the side port periodically, as was waste liquid as needed to maintain steady culture volume (100 mL).

3.C.2. Chemicals and analyses of chloroethenes

Butyric acid, chloroethenes and yeast extract were purchased and used as previously described (*26*). Gas chromatograph measurements were obtained by taking 100 µL headspace samples via gas-tight locking syringe, and were analyzed using a Perkin Elmer Autosystem gas chromatograph with a 1/8 inch by 8 ft stainless steel column packed with 1% SP-1000 on 60/80 Carbopak B (Supelco, Inc.), and routed to a flame ionization detector as previously described (*36*).

3.C.3. Sampling procedure and nucleic acid extraction

Prior to nutrient addition and initiation of PCE feeding (time 0) and at selected times thereafter, 1 and 1.5 mL liquid culture samples for DNA and RNA analyses, respectively, were taken from PSS microcosms and processed as previously described (*26*). Cell pellets for DNA and RNA analyses were stored at –20°C or –80°C, respectively. DNA and RNA extractions were performed within 24 hours. For RNA samples, reverse transcription inefficiencies and mRNA losses incurred during sample preparation were estimated using a modified normalization protocol (*38*) in which 6 x 10^9 copies of luciferase control RNA (Promega) were added to cell pellets prior to lysis. Sample processing then proceeded as described previously (*26*). RNA was quantified using the RNA 6000 Nano assay on an Agilent 2100 bioanalyzer (Agilent Technologies).

3.C.4. Quantitative reverse transcriptase PCR (qRT-PCR)

cDNA was synthesized from 0.1 µg of RNA with the iScript cDNA select synthesis kit using the provided random hexamer primers (Bio-Rad). Gene transcripts and added luciferase RNA were measured by amplification of corresponding cDNA using iQ SYBR Green Supermix (Bio-Rad) and DET-specific primers for genes

encoding subunits of RNA polymerase (RpoB), TCE-RDase (TceA), PCE-RDase (PceA), putative RDase DET1559 (*22*), an annotated formate dehydrogenase (Fdh), a nickel-iron H_2ase (Hup) (*28*), 16S rRNA (*31*), putative RDase DET1545 (this study), and the luciferase control sequence (*38*). Primers targeting DET1545 were designed by Robert Morris using Beacon Designer 4 (Biosoft International) (CCTCCACCTACAACTTCC and AAGAGGCAGGTCTGTTAAG). Amplifications were performed in triplicate using an iCycler iQ multicolor real-time detection system (Bio-Rad) under conditions previously described (*26*). Melt curve analysis confirmed the absence of primer-dimerization.

3.C.5. Expression data analysis

Expression levels were calculated using Data Analysis for Real-Time PCR (DART-PCR), a freely available Excel (Microsoft) based macro which determines threshold cycles, reaction efficiencies and relative cDNA starting quantities from raw fluorescence data (*39*) (http://www.gene-quantification.de/download.html#dart). Differences in amplification efficiency within and between tested groups of gene targets were assessed using one-way analysis of variance (ANOVA). Outlier samples were excluded from further analyses. Standard curves covering four orders of magnitude (R^2 values between 0.95 – 0.97) were constructed with serial dilutions of DNA of known concentration extracted from DET pure culture (for DET targets) and of luciferase control DNA (for the luciferase control target) (Promega), and were used to estimate transcript concentration (copies/mL culture) in PSS microcosms. cDNA concentrations in all samples were above the limit of detection, and average RNA recovery for all samples after RNA extraction and cDNA synthesis steps was determined to be 14 ± 4%.

3.D. Results

3.D.1. Chloroethene dechlorination in PSS microcosms

Reductive dechlorination of added PCE in PSS reactors is shown in Figure 3.1. Dechlorination occurred steadily throughout the experiment with increasing PCE feeding rates leading to corresponding increases in VC + ethene accumulation of 34 ± 1, 62 ± 5, 111 ± 1, and 207 ± 8 µmol/L, respectively, within 22 to 24 hours. Concentrations of PCE, TCE and cDCE reached steady levels within 2 to 3 hours that were either undetectable (<30 nM) or very low (Table 3.1). PCE respiration rates were calculated from the production of VC + ethene, rather than either compound alone, based on the following line of reasoning. The slow, cometabolic conversion of VC to ethene by DET does not yield energy (*40-42*), and therefore cannot be considered respiration. However, with respect to a chloroethene mole balance, both VC and ethene represent moles of fully respired PCE, and each compound must be accounted for in the calculation of molar respiration rates. Therefore, since production of both VC and ethene was observed to occur at nearly constant rates, a linear regression of VC + ethene concentrations over time was used to determine PCE respiration rate for each reactor. Microcosms operated at increasing PCE feeding rates had PCE respiration rates of 1.5 ± 0.1, 2.5 ± 0.3, 4.8 ± 0.1, and 9.2 ± 0.5 µmol/L/hr, respectively (Table 3.1).

3.D.2. Gene expression in PSS microcosms

For all targets studied, transcript levels increased over an order of magnitude above time 0 levels (time 0 is indicative of conditions after 72 hours starvation) within 2 hours of supplying PCE. Expression of transcripts corresponding to 16S rRNA, RpoB, RDases TceA, DET1559, PceA and DET1545, the H_2ase Hup and a putative Fdh, was observed to remain relatively steady after hour 6 regardless of PCE

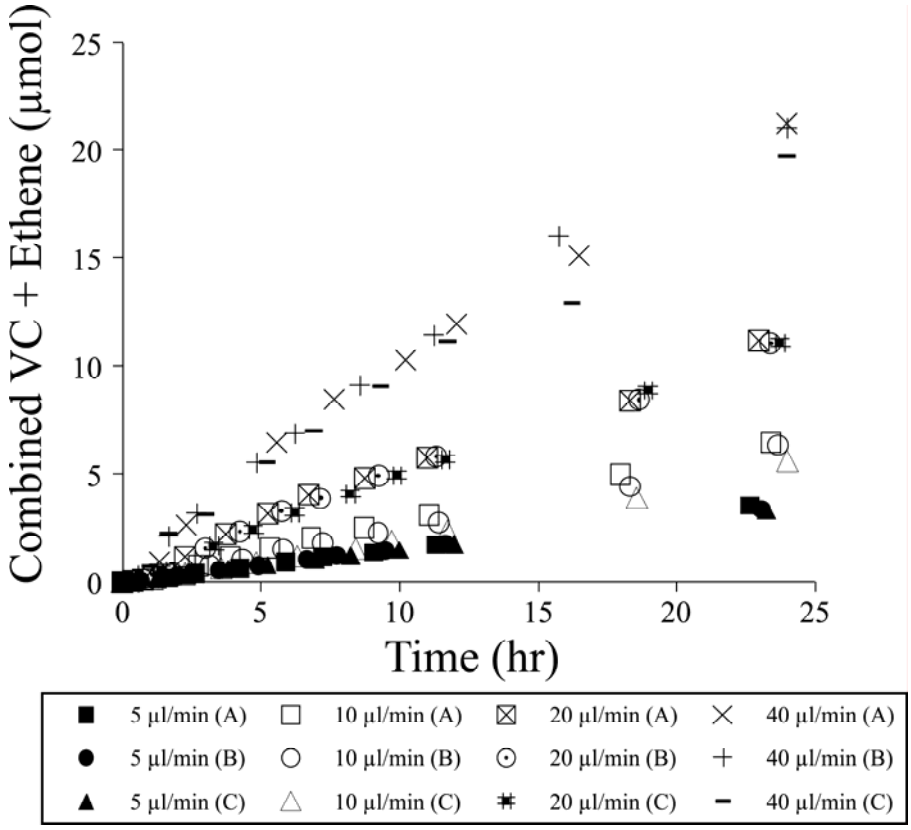

Figure 3.1. VC + ethene production in triplicate PSS microcosms continuously fed medium containing approximately 414 µM PCE at feeding rates of 5, 10, 20 and 40 µL/min. Portions of this data have been presented previously (*48*).

Table 3.1. Average PSS chloroethene concentrations and calculated respiration rates in triplicate microcosms.

PCE Feeding Rate (µL/min)	Average PSS Concentration (nM)			Average Respiration Rate
	PCE	TCE	cDCE	(µmol/L/hr)
5	BD[a]	20[b] ± 12	370 ± 30	1.5 ± 0.1
10	BD[a]	40 ± 1	550 ± 80	2.5 ± 0.3
20	140 ± 20	130 ± 4	780 ± 100	4.8 ± 0.1
40	20[b] ± 6	190 ± 50	1600 ± 90	9.2 ± 0.5

[a] BD: below limit of detection (<30 nM)

[b] Average contains data points which were below the detection limit and assigned a value of zero

± Standard deviation of triplicate means

feeding rates (Figure 3.2; Figure A4.1). Transcript levels of RDases DET1559 and DET1545 showed some variability (Figure A4.1). Expression of the gene for RpoB, chosen here as a general indicator of cellular activity, appeared to peak around hour 2, particularly at the higher PCE feeding rates (Figure 3.2), before settling down to steady levels.

For comparative analyses, we identified the time period between 6 and 18 hours as "pseudo-steady-state" (except in microcosms respiring PCE at 1.5 μmol/L/hr, for which data were only obtained through hour 12), since targets had relatively steady expression profiles during this time. Pseudo-steady-state expression was calculated by averaging observed transcript levels from hours 6, 12 and 18, and is shown in Figure 3.3. Data indicate that PCE feeding rate, and thus chloroethene respiration rate, can significantly affect expression levels. 16S rRNA was the most abundant RNA at each respiration rate and, like many of the enzyme-encoding gene transcripts, was significantly more abundant at rates of 4.8 and 9.2 μmol/L/hr. Genes for Fdh and the H_2ase Hup showed relatively high expression overall, particularly at higher PCE respiration rates. Among RDase targets, TceA was the most highly expressed in 3 of the 4 experiments, followed in each case by DET1559, and then either PceA or DET1545 (Figure 3.3). At the lowest PCE respiration rate, however, DET1559 was observed to have the highest expression, suggesting that RDases may be differentially transcribed depending on substrate concentration or per-cell respiration rate. Interestingly, DET1545 transcript levels were highest at 2.5 μmol/L/hr, but were comparatively low at 9.2 μmol/L/hr (Figure 3.3).

Measurements of 16S rRNA gene copy abundance in each microcosm at hours 0, 6, 8, 12 and 24 remained statistically unchanged and indicated a steady DET population size of approximately $5.2 \pm 1.3 \times 10^8$ cells/mL regardless of PCE respiration rate. Using published biomass yield values for related DHC strains BAV1

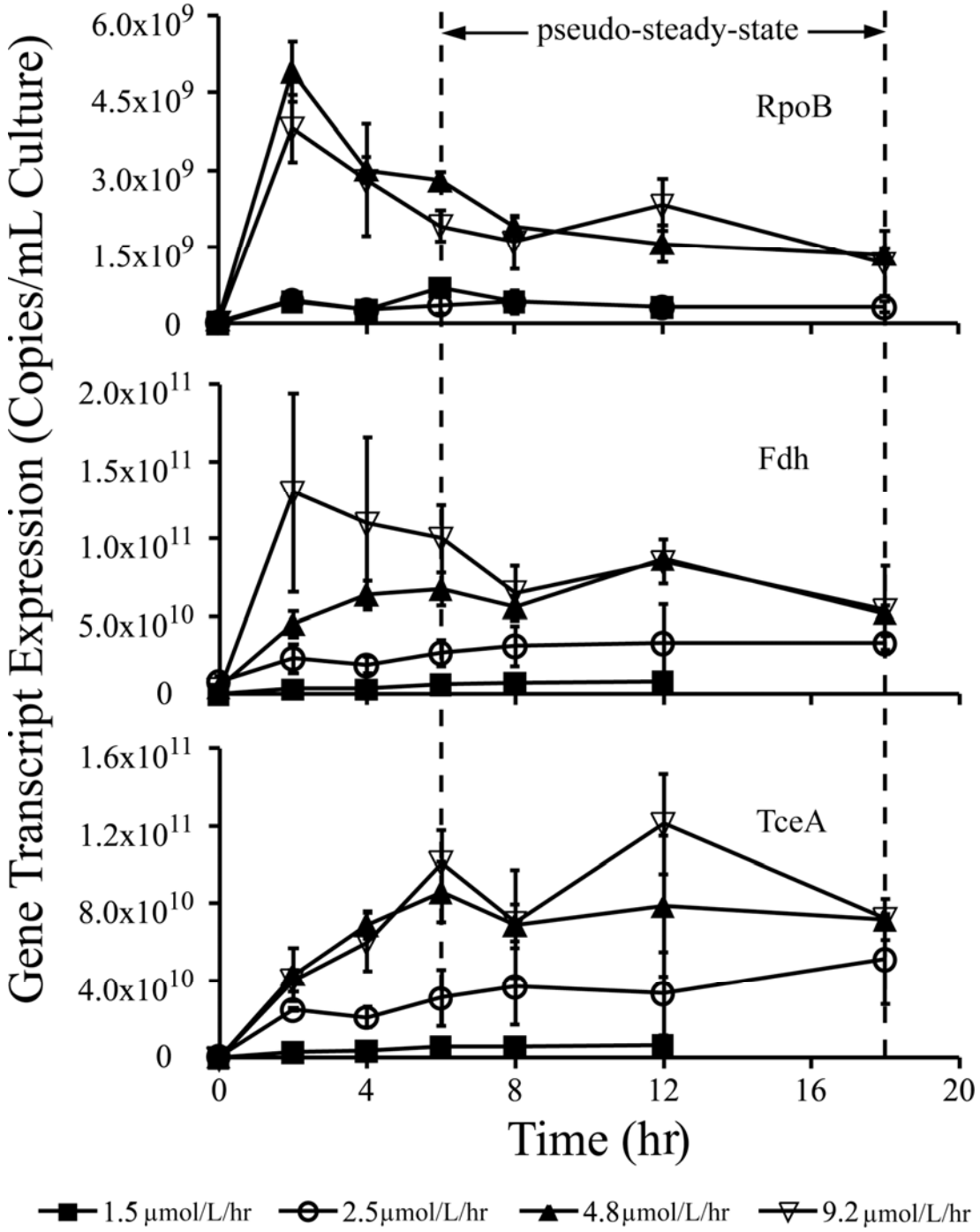

Figure 3.2. Expression profiles of selected gene transcripts over time in PSS microcosms. Hours 6 – 18 are identified as pseudo-steady-state. Error bars represent standard deviation of triplicate microcosms. Profiles of additional targets are provided in Figure A4.1.

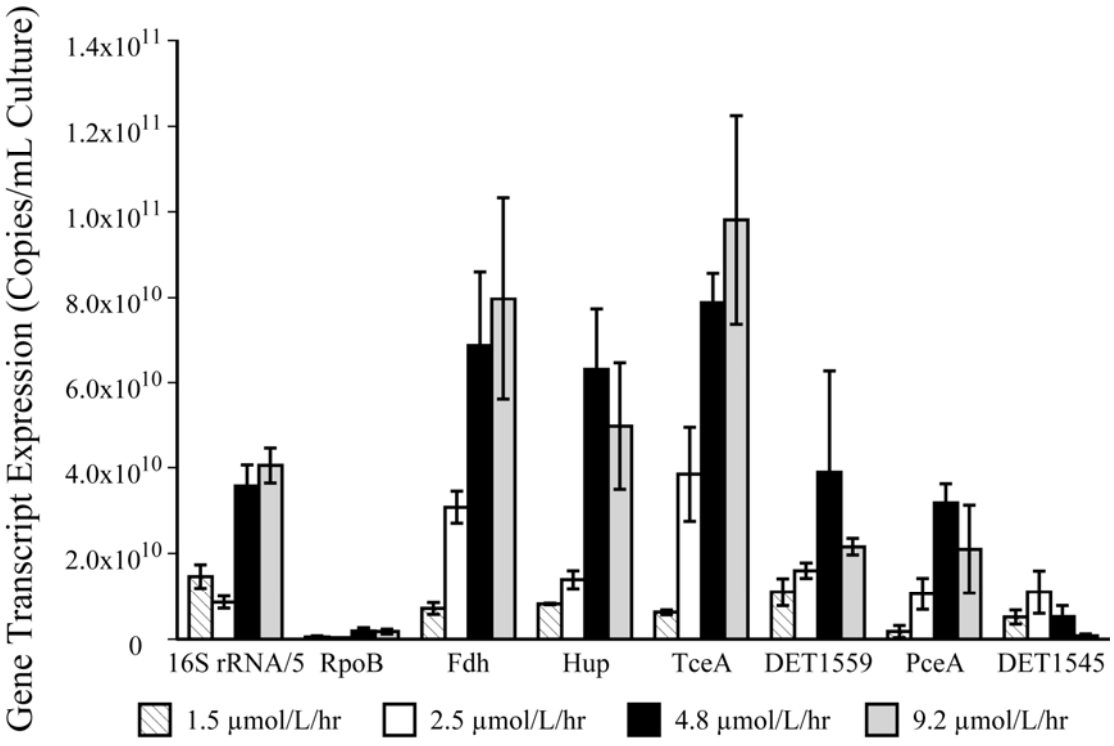

Figure 3.3. Average bioindicator gene expression levels during the period of pseudo-steady-state (hours 6-18) for all targets at all PCE respiration rates. Error bars represent standard deviation of the means of triplicate microcosms.

(*43*) and bacterium VS (*44*) of 6.0 x 10^7 and 5.2 x 10^8 16S rRNA gene copies per µmol Cl^- respired, respectively, we calculated potential increases in DET population size of between 7 and 62% in microcosms respiring PCE at 9.2 µmol/L/hr, the highest rate tested. However, the standard deviation of 16S rRNA gene copy values, as measured via qPCR, indicated fluctuations of about 25%. Therefore, although it is likely that cell growth in these microcosms occurred, it is possible that DET biomass yield values are more similar to those for BAV1 than for bacterium VS, and that the sensitivity of our quantitative tools was not sufficiently high to quantify growth.

Pseudo-steady-state expression levels of enzyme-encoding targets, with the exception of DET1545, increase steadily over PCE respiration rates of 1.5 to 4.8 µmol/L/hr (Figure 3.3). Correlation between PCE respiration rates within this range and expression level for these functional transcripts can be illustrated using a linear model (Figure 3.4), with the abundance of Fdh, Hup and TceA transcripts increasing by approximately 2 x 10^{10} copies per mL of culture for every µmol/L/hr increase in PCE respiration rate, and with DET1559 and PceA transcripts increasing by approximately 9 x 10^9 copies per mL of culture for every µmol/L/hr increase. However, expression of most of these targets shows no significant increase between PCE respiration rates of 4.8 and 9.2 µmol/L/hr. Only Fdh and the RDase TceA show additional increases in pseudo-steady-state expression with increasing rates of PCE respiration above 4.8 µmol/L/hr.

3.E. Discussion

The major goal of this study was to determine the relationship between pseudo-steady-state PCE respiration rates and potential DET bioindicator expression levels. Regulation of gene transcription is presumably based on the cell's ability to sense concentrations of available substrates and/or intracellular energy stores. Gene

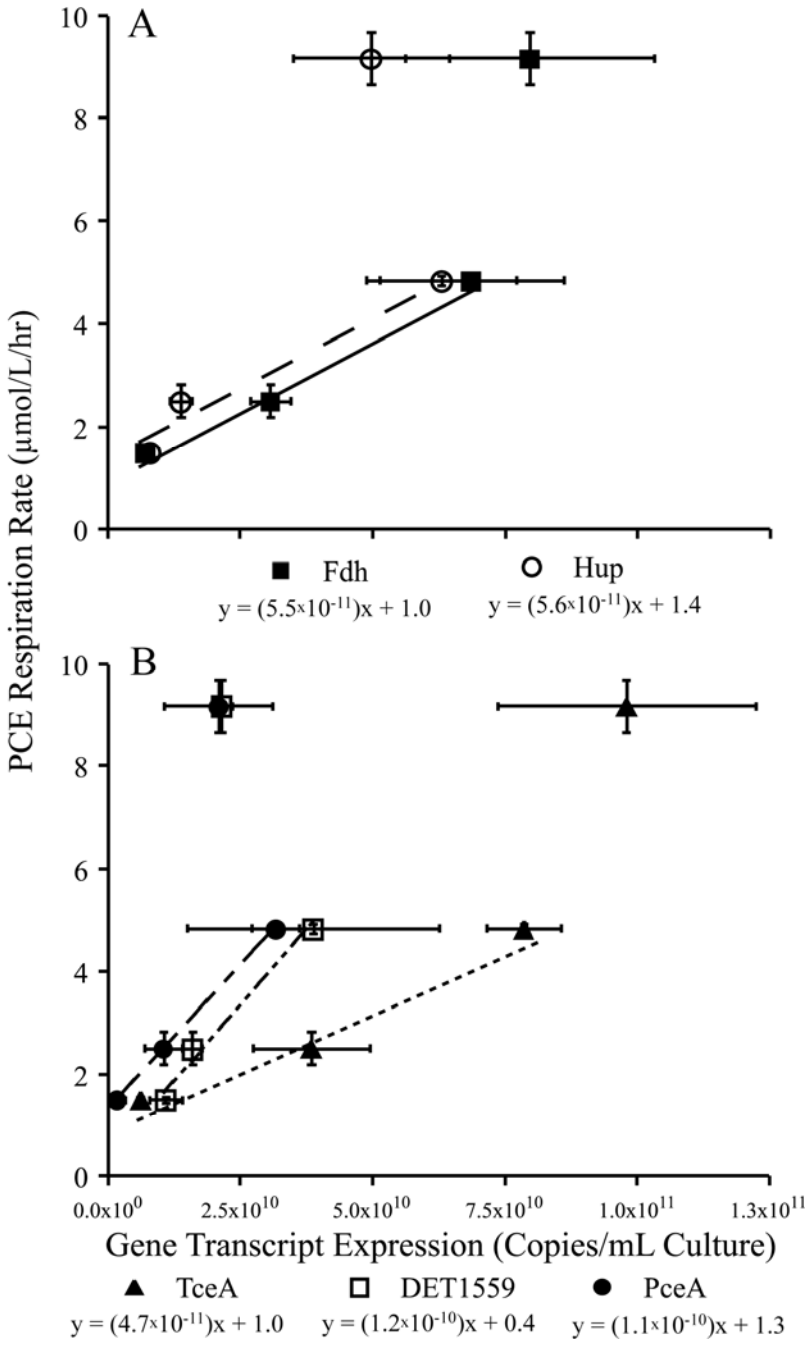

Figure 3.4. Correlation between PCE respiration rate and pseudo-steady-state gene transcript expression level for targets (A) Fdh and the H_2ase Hup and for (B) RDases TceA, DET1559 and PceA. Data for the three lowest PCE respiration rates are fit with a linear regression model. Error bars represent standard deviation of the mean of triplicate microcosms.

transcript levels, in turn, influence cellular concentrations of functional enzymes whose activities are directly responsible for observed respiration of PCE. As Figure 3.1 and Table 3.1 show, steady rates of VC + ethene production were achieved in all reactors, with higher PCE feeding rates leading to correspondingly higher rates of respiration without PCE buildup, indicating that PCE dechlorination kinetics were non-limiting. qRT-PCR results indicated that the abundance of functional transcripts corresponding to Fdh, Hup, TceA, DET1559 and PceA could be linearly correlated to PCE respiration rate within a limited range (Figure 3.4). If this linear model were extended to the y-axis, it could be postulated that a basal rate of respiration (approximately 1 µmol/L/hr) is required for cells at this DET population density (5 x 10^8 per mL) to actively up-regulate these transcripts. It is possible that PCE feeding rates corresponding to respiration rates below this threshold would not induce transcription of new functional RNA.

Functional targets Fdh and the RDase TceA have shown high relative expression in previous studies (*26*,*28*) and, unlike other targets studied here, are transcribed at levels that correlate with increasing PCE respiration rates over the entire range examined (1.5 to 9.2 µmol/L/hr). The lack of correlation between transcript levels of other targets and chloroethene respiration at the highest PCE feeding rate was unexpected. A possible explanation for this observation is that maximum per-cell transcription rates have been reached. Alternatively, transcription of biosynthesis related genes might be prioritized at the expense of respiratory gene expression as cells sense an opportunity for increased rates of growth. These hypotheses are hard to test given the relatively low predicted yield of DET, the short time scale of this study, and the previously discussed limitations in 16S rRNA gene quantitation. Lastly, there may be an unknown toxicity effect related to high PCE respiration rates that represses the expression of these functional targets. This seems unlikely as respiration of PCE

occurred steadily at 9.2 µmol/L/hr, and since pseudo-steady-state PCE concentrations remained low (20 nM) and steady. Moreover, previous studies have shown that DET activity is not inhibited by VC or ethene concentrations observed here (*35*,*42*).

RDase genes presumably reflect the substrate ranges of DHC organisms. However, for most putative RDases, little is known about their substrate specificity and transcriptional regulation. Results here suggest that expression of individual RDase genes is tightly regulated and highly sensitive to cellular respiration activity and/or substrate concentration. Previous studies have shown that transcription of the gene corresponding to TceA is induced by TCE + cDCE levels as low as 2 µM (*24*). Considering that PSS levels of TCE and cDCE in the 1.5 µmol/L/hr experiment were 0.02 and 0.4 µM, respectively, this induction range can be extended downward. Overall, TceA transcripts were most abundant among RDases at respiration rates of 2.5 to 9.2 µmol/L/hr. However, at the lowest rate of 1.5 µmol/L/hr, where PSS chloroethene concentrations were also lowest (Table 3.1), DET1559 was the most abundant RDase transcript (Figure 3.3), suggesting that relative quantities of RDase transcripts can change even when substrate type remains the same. Putative RDase DET1545, which has homologs in at least three other DHC strains (*27*,*29*,*45*), showed decreasing transcript levels as respiration rates increased from 2.5 to 9.2 µmol/L/hr (Figure 3.3; Figure A4.1), which is curious given the fact that it has no recognizable adjacent regulator (*30*). Interestingly, in previous batch-fed studies (*26*), DET1545 expression only increased above background after all measurable PCE had been consumed. These data suggest that DET1545 expression is either repressed by one or more of the respirable chloroethenes or up-regulated during substrate-limiting conditions by some as-yet unknown cellular signaling mechanism.

Challenges remain before these techniques can be confidently incorporated into remediation and modeling efforts at chloroethene-contaminated sites. While PSS

concentrations of respirable chloroethenes studied here (~0.4 – 2.1 μM) may reasonably reflect values observed in contaminated groundwater (*12,13,15*), the rates of respiration in this highly enriched culture are much faster than what can be expected at field sites. Studies with more dilute cultures and lower PCE loading rates are an important step in further development of these and other bioindicators. As for the feasibility of mRNA detection in the field, specific *Geobacteraceae* mRNAs were detected in groundwater at levels between 7 and 200 transcripts per ng of total extracted RNA during biostimulation at a uranium-contaminated site (*10*). Although detection of DHC transcripts in groundwater has not been reported, all mRNAs targeted in this study were found at concentrations greater than 500 transcripts per ng of total extracted RNA, supporting the possibility that methods used here could be adapted to field use.

Continued improvements in our understanding of the cellular regulation of dehalorespiring DHC strains are needed, as are improved methodologies for the extraction and quantification of RNA and other microbial macromolecules from field environments. Nevertheless, this study suggests that a variety of gene transcripts can serve as potential bioindicators of reductive dechlorination. Because of the high abundance of 16S rRNA transcripts, even after 72 hours of starvation, and because rRNA is generally more stable than mRNA, it remains a good option for initial detection of potentially active dechlorinating populations. Data presented here suggest that enzyme-encoding gene transcripts for Fdh, the H_2ase Hup, and the RDases TceA, DET1559, PceA and DET1545 could potentially be used to assay more specific signs of activity. Although the function of Fdh is not known, its high level of expression and conservation across observed DHC strains (*29,46*) could make it a good general indicator of dehalorespiration while RDases, particularly TceA, could serve as bioindicators of specific chloroethene conversion processes. DET1559 and DET1545

may be reasonable choices as bioindicators when chloroethene concentrations and/or respiration rates are low. However, until their substrate ranges are characterized, it is not possible to associate them with specific dehalorespiration processes. Other gene targets such as those for VC-RDases VcrA (*19,47*) and BvcA (*16,21*), which are important functional gene transcripts in VC-respiring cultures, must also be studied. Assays capable of targeting multiple DHC strains and that incorporate many of these potential bioindicators are needed before an overall picture of the relationships between gene transcript levels and dehalorespiration can be elucidated.

3.F. Acknowledgments

We thank Jennifer Fung and Robert Morris for assistance with primer design, and Professors James Gossett and Stephen Zinder for helpful advice regarding experimental design. We also thank Annette Rowe for help reviewing the manuscript, and three anonymous reviewers for their insightful comments. This project was supported through a fellowship to BG Rahm from the Initiative for Future Agriculture and Food Systems Grant no. 2001-52104-11484 from the USDA Cooperative State Research, Education, and Extension Service, and also by the U.S. Department of Energy (DOE) Office of Cleanup Technologies and administered by the U.S. Department of Energy Savannah River (SR) Operations Office (Contract No. DE-AC09-96SR18500), and is a product of the Monitored Natural Attenuation/Enhanced Attenuation for Chlorinated Solvents Technology Alternative Project.

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CHAPTER 4

Dehalococcoides' Gene Transcripts as Quantitative Bioindicators of PCE, TCE and cDCE Dehalorespiration Rates: Trends and Limitations. *

4.A. Abstract

Gene transcripts corresponding to 16S rRNA, a putative formate dehydrogenase (Fdh), the hydrogenase (H_2ase) Hup and reductive dehalogenases (RDases) TceA, PceA, DET1559 and DET1545 in *Dehalococcoides ethenogenes* strain 195 (DET) hold promise as potential bioindicators of the dehalorespiration of chlorinated ethenes. Here, we present quantitative reverse-transcriptase PCR (qRT-PCR) data taken from DET-containing mixed culture microcosms (4.4×10^8 DET 16S rRNA gene copies/mL) operated under continuous-feed conditions, with the aim of clarifying the relationship between potential bioindicator transcript abundance and respiration rate of various substrates, including tetrachloroethene (PCE), trichloroethene (TCE), and *cis*-1,2-dichloroethene (cDCE). Results from PCE-fed microcosms respiring at very low rates showed that an induction threshold for transcription of some bioindicator genes exists between chloroethene respiration rates of 2.1 and 9.5 μeeq/L/hr. Putative RDase genes DET1559 and DET1545, however, were up-regulated at low PCE respiration rates, suggesting that these enzymes may be functionally significant when substrate levels are low. Data from microcosms in which the DET population was respiring PCE at its maximum kinetic rate indicated that high respiration rate was not necessarily associated with correspondingly high bioindicator transcript abundance. From these microcosms operated at 30 °C we

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calculated an approximate yield value of 1.6 x 10^8 16S rRNA gene copies (cells) per µmol Cl^- released, and estimated a kmax of PCE respiration of 3 x 10^{-9} µmol Cl^- per 16S rRNA gene copy per day. TCE- and cDCE-fed microcosm studies indicated that Fdh, Hup and TceA transcripts would all make suitable choices as bioindicators of activity for these substrates. Hup transcripts could be positively correlated to respiration rate (between approximately 8 and 45 µeeq/L/hr), regardless of chloroethene substrate, with transcript levels predicted to increase by 1.8 x 10^9 copies/mL culture for every µeeq/L/hr increase in respiration rate (R^2 = 0.90). While RDase transcripts may provide information on substrate range, H_2ase transcripts may be better indicators of per cell respiration rates.

4.B. Introduction

Pure and mixed cultures containing *Dehalococcoides ethenogenes* strain 195 (DET) have the ability to reductively dechlorinate halogenated organic compounds including chlorinated ethenes (*1,2*) and various chloroaromatics (*3,4*). The dechlorinating capabilities of DET and other closely related *Dehalococcoides* strains have led to their usage in *in situ* bioremediation of chloroethene contaminants in groundwater (*5-7*). Reduction of chloroethenes by *Dehalococcoides* has been shown to be mediated by a group of membrane-bound reductive dehalogenase enzymes (RDases) (*8-10*), while hydrogenase enzymes (H_2ases) are likely involved in the stripping of electrons from H_2, their only known electron donor (*11,12*).

While many RDases have been identified in *Dehalococcoides* strains (*10,13-17*), very little is known about their specific functions, their substrate ranges, or the regulatory networks that govern their expression. In DET, RDases PceA and TceA have been shown to catalyze the reductions of tetrachloroethene (PCE) to trichloroethene (TCE) and TCE to ethene, respectively (*8*). However, recent studies

have shown that transcripts of several other RDases, including DET1559 and DET1545, can be detected at relatively high levels in actively respiring cultures fed PCE (*18,19*). Proteome characterization studies have also detected each of these proteins, PceA, TceA, DET1559 and DET1545 in mixed DET-containing cultures (*20,* J.J. Werner – unpublished results). In general, data indicate that transcript abundance of individual RDases depends in part on substrate type and concentration, and that co-transcription of multiple RDases is possible, even under single-substrate conditions (*3,14,17,19,21-23*).

Despite the apparent complexity of RDase regulation and function, their specificity to phenotypes of interest – the degradation of chloroethenes – and their potentially high expression make them good candidates as bioindicators of dehalorespiration. Here, a "bioindicator" is a biological molecule – in this case RNA – whose detection and quantity may reflect the substrate range and activity level of a DET population. Several other DET RNA targets corresponding to 16S rRNA, a putative formate dehydrogenase (Fdh), and the membrane bound H_2ase Hup also share these characteristics (*18,19,24*). Assays of mRNA are being increasingly utilized to evaluate and predict microbial potentials and activities at contaminated field sites (*25-28*). Determining how measurements of these molecular bioindicators are related to phenotypic states remains a major challenge.

In a previous study, we utilized a continuously fed mixed culture (D2) containing DET as its sole dechlorinating constituent (*29,30*) to explore the relationship between steady-state transcript levels of potential bioindicators and PCE respiration rates (*19*). Results suggested that a predictive relationship exists between transcript levels of certain targets and PCE respiration rate, but that this relationship is only valid within a limited range of respiration rates. The objectives of the present study are to further explore the relationship between transcription of bioindicator

targets and PCE respiration, both at very low rates that might describe conditions in groundwater containing dilute PCE-contaminants, and at very high rates that maximize the culture's kinetic potential. Additionally, we evaluate the transcription of bioindicators in D2 cultures continuously fed TCE or 1,2-*cis*-dichloroethene (cDCE). Data suggest that transcript abundance of each bioindicator gene target varies with respiration rate and that some bioindicators have a varied transcriptional response pattern when respiring different substrates.

4.C. Materials and Methods

4.C.1. Chemicals and analyses of chloroethenes

Chloroethenes and solutions used during culturing procedures were purchased and used as previously described (*18*). Gas chromatograph measurements were obtained by taking 100 µL headspace samples via gas-tight locking syringe, and were analyzed using a Perkin Elmer Autosystem gas chromatograph with a 1/8-inch by 8-ft stainless-steel column packed with 1% SP-1000 on 60/80 Carbopak B (Supelco, Inc.), and routed to a flame-ionization detector (*31*).

4.C.2. Calculation of dehalorespiration rates

In this study PCE was defined as having 8 electron equivalents per mole based on its respiration to VC and cometabolic conversion to ethene, while TCE, cDCE and VC were likewise defined as having 6, 4 and 2 electron equivalents per mole, respectively. Therefore, the following equations were used where r_{PCE}, r_{TCE} and r_{cDCE} represent respiration rates (µeeq/L/hr) of PCE, TCE and cDCE, respectively.

$$r_{PCE} = 8\frac{d(ethene)}{dt} + 6\frac{d(VC)}{dt} + 4\frac{d(cDCE)}{dt} + 2\frac{d(TCE)}{dt}$$

$$r_{TCE} = 6\frac{d(ethene)}{dt} + 4\frac{d(VC)}{dt} + 2\frac{d(cDCE)}{dt}$$

$$r_{cDCE} = 4\frac{d(ethene)}{dt} + 2\frac{d(VC)}{dt}$$

4.C.3. Culture procedure

The dehalorespiring enrichment culture D2, containing DET as its sole dechlorinator, was maintained as previously described (*18*), and was used as a source culture for all experiments. The D2 culture was consistently starved for approximately 72 hr prior to establishment of microcosms studied here. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and gas chromatograph measurements were taken from triplicate 100 mL pseudo-steady-state (PSS) microcosms operated at 30ºC as previously described (*19*) with the following exceptions. For low-rate PCE-fed microcosms (PCElow), filtered basal culture medium (*32*) containing approximately 410 µM dissolved PCE was delivered by syringe pump at a constant volumetric loading rate of 1.5 µL/min. TCE- and cDCE-fed microcosms were similarly given medium containing approximately 810 µM dissolved TCE and 1400 µM dissolved cDCE, respectively, by syringe pump at volumetric loading rates of 5, 10 and 20 µL/min. In this way, similar volumetric loading rates of each substrate – including PCE experiments reported previously (*19*) – delivered comparable amounts of respirable electron equivalents regardless of substrate type. For high-rate PCE-fed microcosms (PCEhigh), initial feeding rates (hours 1-5) failed to push the culture to its maximum kinetic potential as evidenced by a lack of detectable PCE build-up. Therefore, starting at hour 5, PCEhigh microcosms were amended with 2 µL pulses of neat PCE every 3 hours, thereby keeping the aqueous PCE concentration above 13 µM at all times. All microcosms were

administered initial pulse-feedings of vitamin solution (*33*), 20 mg yeast extract/liter of culture, and 44 µmoles butyric acid (with butyric acid defined as having 4 electron equivalents per mole based on its fermentation to 2 moles acetate and 2 moles H_2 rather than its oxidation to CO_2). Bottles were then held upside-down at a 45º angle over a magnetic stir plate and stirred vigorously. Additional pulses of nutrients and butyric acid were amended as dictated by established feeding procedures (*18*). Gas and liquid samples were extracted by syringe from a side port, as was waste liquid as needed to maintain steady culture volume (100 mL).

4.C.4. Nucleic acid extraction and qRT-PCR analysis

Liquid culture samples of 1 and 1.5 mL for DNA and RNA analyses, respectively, were taken from all microcosms prior to substrate addition and initiation of chloroethene feeding (time 0) and at selected times thereafter, and processed as previously described (*18*). Procedures for treatment of RNA samples and cDNA synthesis have been reported previously (*19*). Gene transcripts corresponding to 16S rRNA, the B subunit of RNA polymerase (RpoB), an annotated formate dehydrogenase (Fdh), a nickel-iron H_2ase (Hup), TCE-RDase (TceA), PCE-RDase (PceA), and putative RDases DET1559 and DET1545 were measured using iQ SYBR Green Supermix (Bio-Rad) and an iCycler iQ multicolor real-time detection system (Bio-Rad) as previously described (*18,19*). As before (*19*), transcript levels were determined with the help of Data Analysis for Real-time PCR (DART-PCR), available at http://www.gene-quantification.de/download.html#dart (*34*).

4.D. Results

4.D.1. Transcriptional regulation at low PCE feeding rates

Dehalorespiration in PCElow PSS reactors is illustrated in Figure 4.1A. Added PCE was respired at a steady rate of 2.1 ± 0.1 μeeq/L/hr (0.3 ± 0.0 μmol/L/hr) throughout the experiment (Table 4.1), which was well below our previously estimated threshold of 1 μmol/L/hr for the up-regulation of potential bioindicator transcripts in PSS cultures (*19*). Figure 4.2 compares the average 6-18 hour transcript abundance of bioindicator targets to their average time-0 levels and indicates that transcript levels corresponding to 16S rRNA, RpoB, Fdh, Hup, TceA and PceA remained statistically unchanged, a result which was verified using two-tailed Student's *t*-tests. Transcript levels of RDases DET1559 and DET1545, however, increased by approximately 25 ± 4.8 (*p*-value = 0.00006) and 2.8 ± 0.4 (*p*-value = 0.004) fold, respectively (Figure 4.2).

4.D.2. Relationship between transcript abundance and respiration at high PCE loading rates

Dehalorespiration in PCEhigh reactors is illustrated in Figure 4.1B. The purpose of PCEhigh microcosms was to evaluate transcript levels under conditions of maximum PCE respiration rate. Unlike other PSS trials, PCE concentrations were not allowed to equilibrate, but rather were maintained above 13 μM, forcing maximum kinetic rates on the DET population. As a result, TCE and cDCE concentrations reached steady levels that were significantly higher in these microcosms than in others (Table 4.1). After hour 5, GC data indicates that added PCE was steadily respired at a rate of 370 ± 5.9 μeeq/L/hr (64 ± 1.2 μmol/L/hr) (Table 4.1).

The relationship between potential bioindicator transcript abundance and respiration rate in PCEhigh microcosms is summarized in Figure 4.3, and is shown in

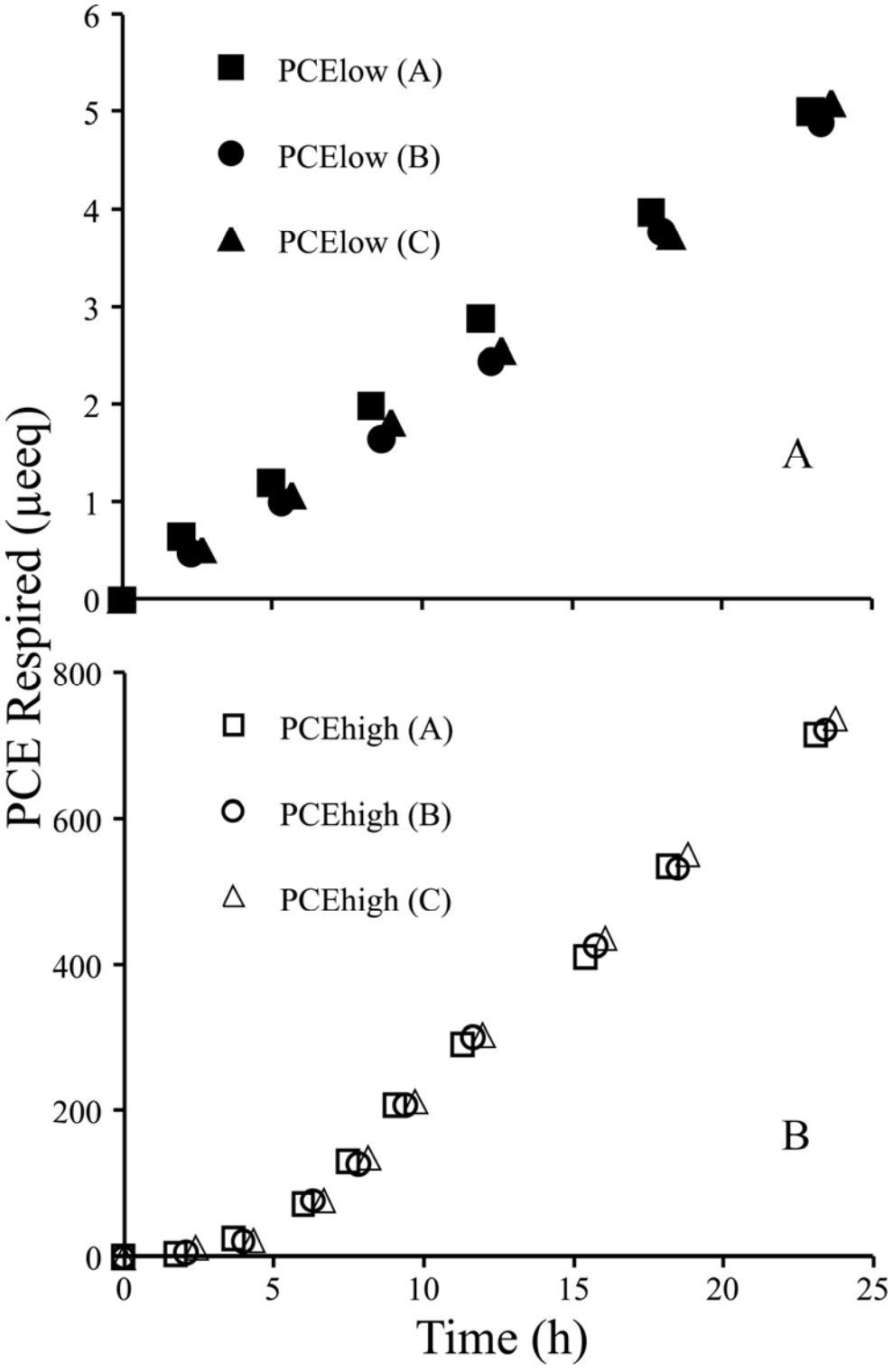

Figure 4.1. Respiration in triplicate (A) PCElow and (B) PCEhigh microcosms.

Table 4.1. Average PSS chloroethene concentrations and calculated respiration rates at 30 °C.

Substrate	Feeding Rate (µL/min)	Average PSS Concentration (nM)			Average Respiration Rate of Indicated Substrate		Respiration Rate Per Cell[a]	
		PCE	TCE	cDCE	(µmol/L/hr)	(µeeq/L/hr)	(amol/cell/hr)[b]	(aeeq/cell/hr)[b]
PCE	1.5	BD[c]	BD[c]	BD[c]	0.3 ± 0.0	2.1 ± 0.1	6.8 ± 0.2	4.8 ± 0.2
	high[d]	NA[e]	19000 ± 5600	15000 ± 3900	64 ± 1.2	370 ± 5.9	1500 ± 28[f]	840 ± 13[f]
TCE	5		BD[c]	300 ± 60	1.9 ± 0.1	8.3 ± 0.5	43 ± 2.3	19 ± 1.1
	10		140 ± 50	830 ± 40	4.9 ± 0.4	21 ± 2.1	110 ± 9.1	48 ± 4.8
	20		300 ± 40	3800 ± 20	10 ± 0.4	45 ± 2.0	230 ± 9.1	100 ± 4.6
cDCE	5			50 ± 20	3.7 ± 0.1	8.9 ± 0.1	84 ± 2.3	20 ± 0.2
	10			200 ± 10	8.5 ± 0.3	20 ± 0.9	190 ± 6.8	45 ± 2.1
	20			470 ± 20	19 ± 0.3	43 ± 0.9	430 ± 6.8	98 ± 2.1

[a] Based on average DHC population density of about 4.4×10^8 cells/mL for all microcosms

[b] Lowercase unit "a" refers to the prefix "atto" (10^{-18})

[c] Below limit of detection (30 nM)

[d] Cultures were fed with frequent, high doses of neat PCE so as to force maximum kinetic respiration rates and to maintain PCE concentrations above 13 µM at all times

[e] Not applicable. PCE concentrations were kept arbitrarily high and not allowed to equilibrate

[f] These values are probably overestimates, since population density was measurably increasing in PCEhigh microcosms

± Standard deviation of triplicate means

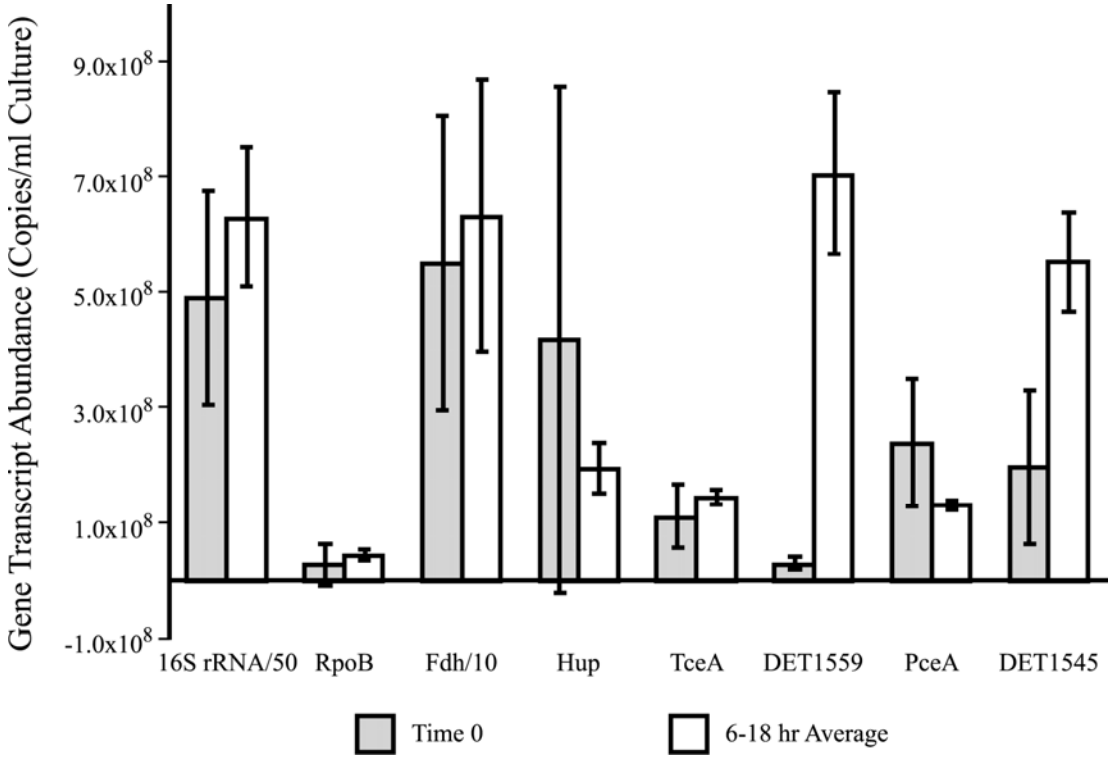

Figure 4.2. Transcript abundance in PCElow microcosms respiring PCE at the rate of 2.1 ± 0.1 µeeq/L/hr (0.3 ± 0.0 µmol/L/hr). Time-0 levels (after 72 h starvation), observed prior to addition of substrates, are compared to 6-18 hour average levels. To maintain scale, 16S rRNA and Fdh transcript levels are divided by 50 and 10, respectively. Error bars represent standard deviation of the mean of triplicate microcosms.

the context of other experiments performed here (PCElow) and in previous work (*19*). For each of the four RDases studied, 6-18 hour average transcript levels in PCEhigh cultures were either similar to (TceA & PceA) or lower than (DET1559 & DET1545) average transcript levels in previously studied cultures operated at the lower PCE respiration rate of 57 ± 3.2 μeeq/L/hr (9.2 ± 0.5 μmol/L//hr), suggesting RDase transcript abundance may plateau, or in some cases decrease, as respiration approaches maximum kinetic rates (Figure 4.3A). Transcripts corresponding to Fdh were more abundant in PCEhigh cultures than in any previously studied PSS culture, although the relationship between transcript abundance and respiration rate does not appear to be linear over the whole range (Figure 4.3B). 16S rRNA was also more abundant in PCEhigh cultures than in previous trials, and the full time-course shows that 16S rRNA levels continue to increase throughout the experiment (Figure 4.4). 16S rRNA gene-copy measurements indicated an approximate 79% increase in DET population size in PCEhigh microcosms (data not shown). However, given that many of the trends observed here occur over several orders of magnitude, this growth was determined to minimally affect measurements of transcript abundance.

4.D.3. Bioindicator performance in TCE-fed PSS cultures

Dehalorespiration in PSS reactors fed TCE at three different loading rates is illustrated in Figure 4.5A, with increasing loading rates leading to increasing dehalorespiration rates of 8.3 ± 0.5, 21 ± 2.1 and 45 ± 2.0 μeeq/L/hr (1.9 ± 0.1, 4.9 ± 0.4 and 10 ± 0.4 μmol/L/hr) (Table 4.1). In all TCE-fed microcosms, concentrations of TCE and cDCE reached steady levels that were either below detection (<30 nM) or very low (Table 4.1), indicating that TCE dechlorination kinetics were non-limiting. Average 6-18 hour transcript abundance for each potential bioindicator was significantly higher than time-0 levels at all three feeding rates (Figure 4.6A). In

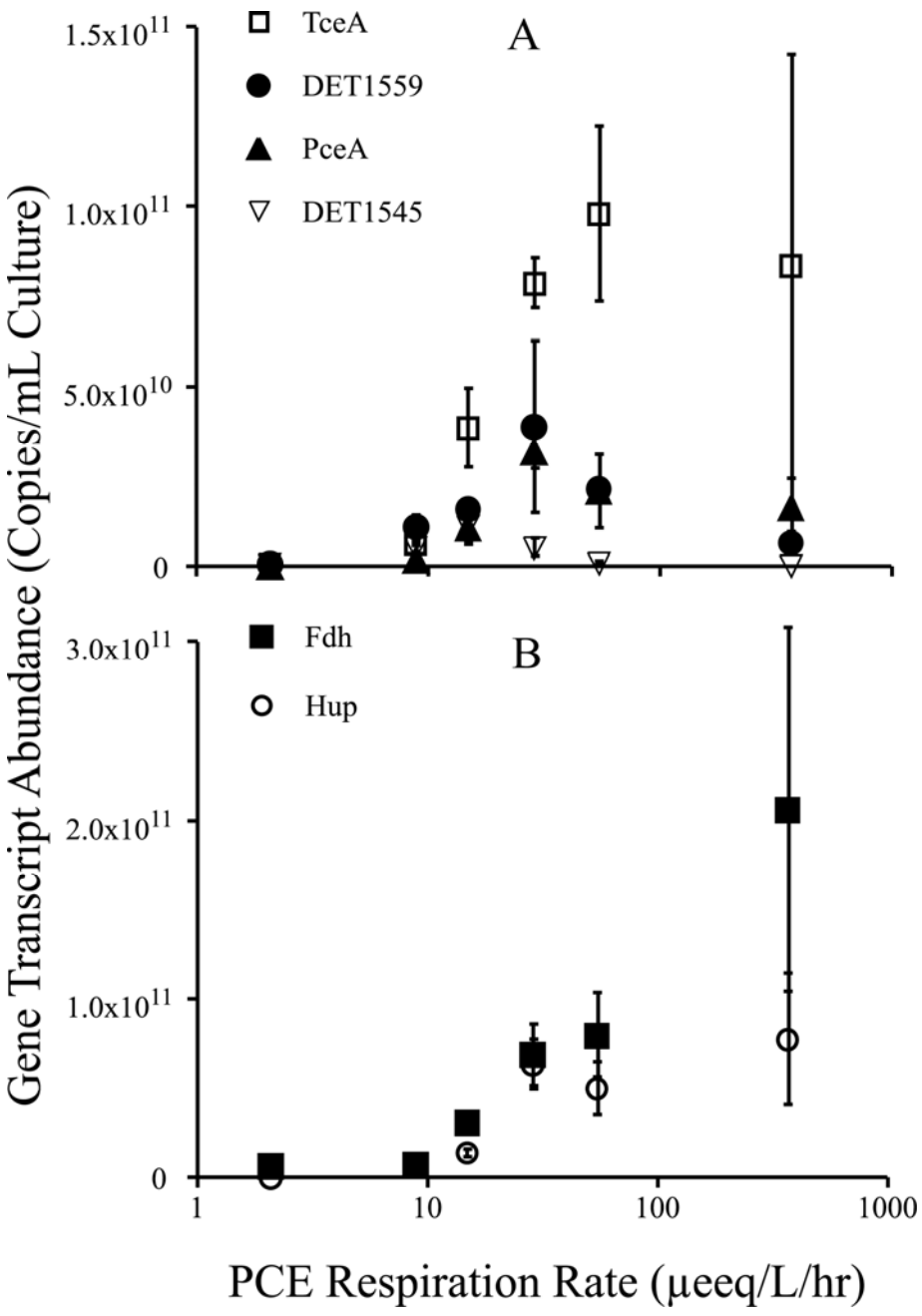

Figure 4.3. Relationship between PCE respiration rates and gene transcript levels of (A) RDases and (B) the H_2ase Hup and Fdh in PCE-fed PSS microcosms. Data are presented both from this and a previous study (*19*). Error bars represent standard deviations of the mean of triplicate microcosms. X-axis error bars are smaller than the symbols.

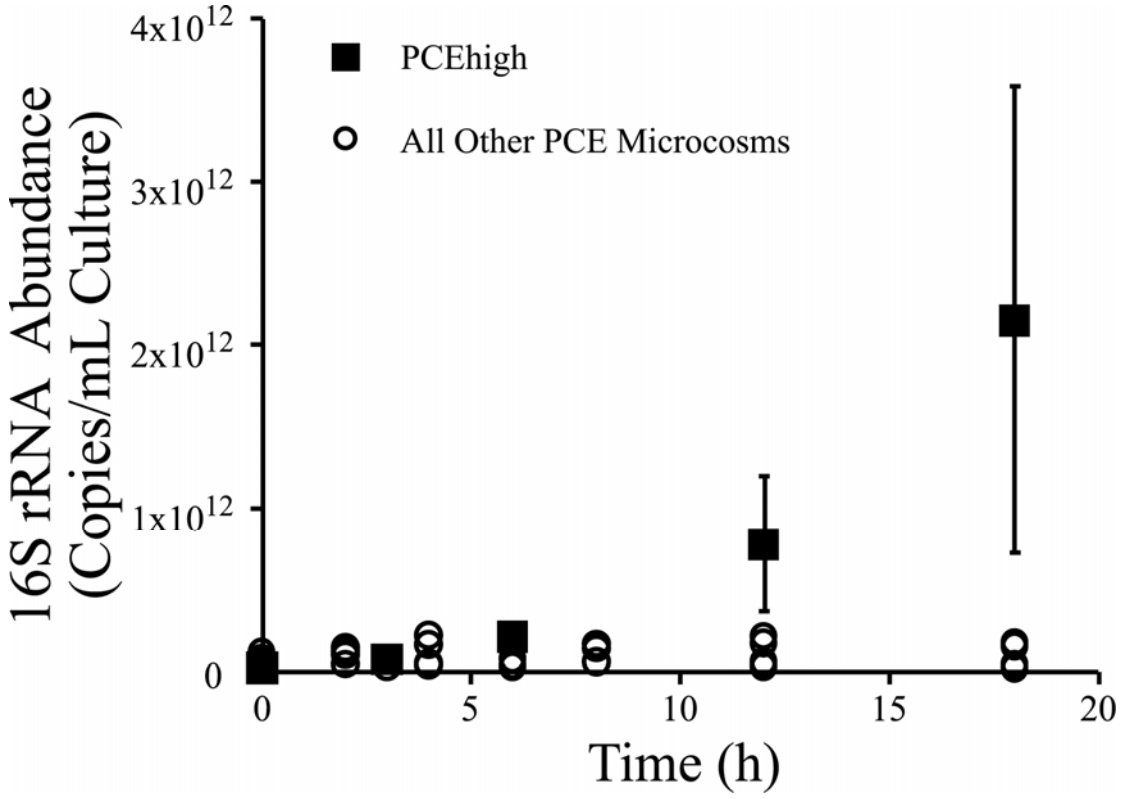

Figure 4.4. Abundance of 16S rRNA transcripts in PCE-fed microcosms over time. 16S rRNA transcript levels increase over two orders of magnitude during the time-course of PCEhigh experiments, while transcript levels in all other PCE-fed PSS microcosms remain relatively low and steady. Some data are reprinted from a previous report (*19*).

general, regardless of respiration rate, Fdh was observed to be the most abundant transcript overall, while TceA was consistently the most abundant RDase transcript. TceA transcript levels increased with increasing TCE respiration rate, and could be well-fit (R^2: 1.00) with a linear model predicting an additional 1.0 x 10^9 copies/mL culture for each µeeq/L/hr increase in TCE respiration, compared to increases of 3.3 x 10^9 copies/mL culture for each µeeq/L/hr increase in PCE respiration (within a similar respiration range [*19*]) (Figure 4.7A), while Fdh (R^2: 0.95) and Hup (R^2: 0.97) both increased by about 1.4 x 10^9 copies/mL culture for each µeeq/L/hr increase in TCE respiration rate (compared to 3.3 x 10^9 copies/mL culture during PCE respiration [*19*]) (Figure 4.7B). The RDase PceA showed the opposite relationship, with transcript abundance decreasing linearly (R^2: 0.99) with each successive increase in TCE respiration rate (Figure 4.7A). 16S rRNA was always relatively abundant, but did not correspond either positively or negatively to TCE respiration rates. DET population size, determined by 16S rRNA gene quantification, remained statistically unchanged during the course of the experiments.

4.D.4. Bioindicator performance in cDCE-fed PSS cultures

Increasing loading rates of cDCE-containing media led to continuous dehalorespiration rates of 8.9 ± 0.1, 20 ± 0.9 and 43 ± 0.9 µeeq/L/hr (3.7 ± 0.1, 8.5 ± 0.3 and 19 ± 0.3 µmol/L/hr) (Figure 4.5B; Table 4.1). Concentrations of cDCE were observed to reach steady levels (Table 4.1), indicating that cDCE kinetics were non-limiting. Average 6-18 hour transcript abundance for each potential bioindicator isshown in Figure 4.6B. Genes corresponding to Fdh, the H_2ase Hup and the RDase TceA all show relatively high transcript levels; however, only Hup transcript abundance increased successively with increasing cDCE respiration rate and could be well-fit (R^2: 0.97) with a linear model predicting an additional 1.6 x 10^9 copies/mL for

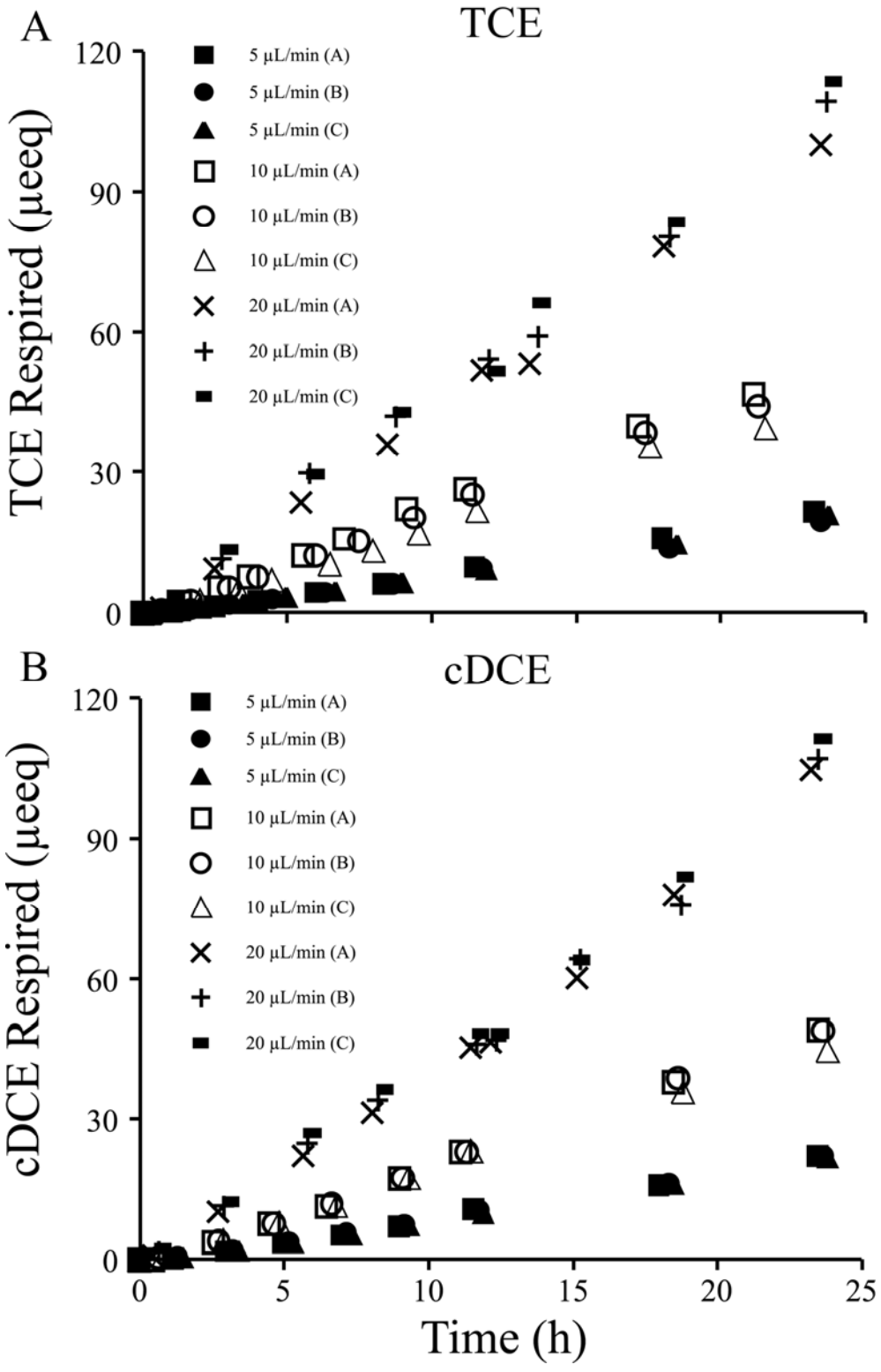

Figure 4.5. Respiration in triplicate (A) TCE-fed and (B) cDCE-fed microcosms.

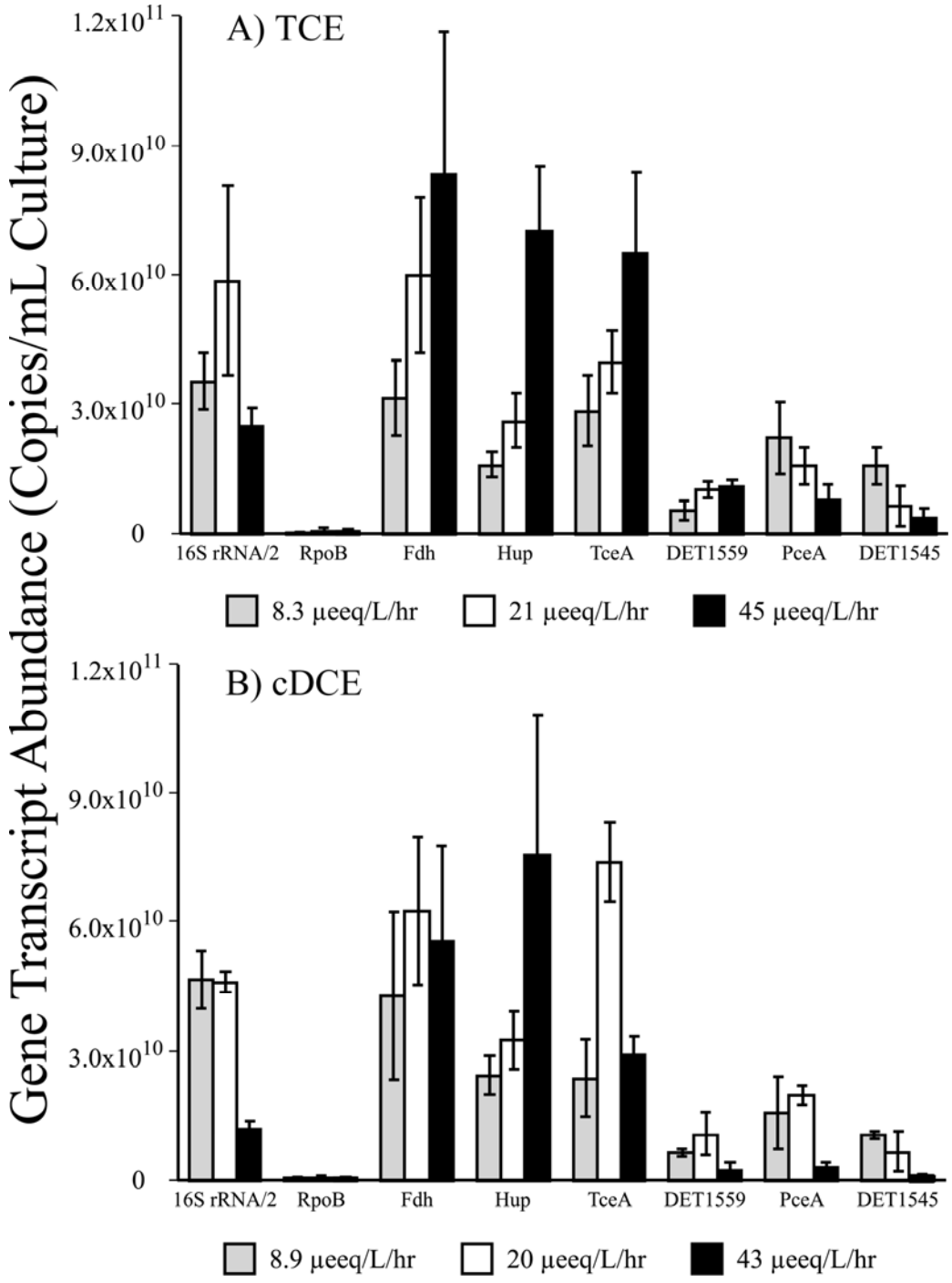

Figure 4.6. Average bioindicator transcript levels during the period of pseudo-steady-state (hours 6-18) in (A) TCE-fed and (B) cDCE-fed microcosms. To maintain scale, 16S rRNA level is divided by 2. Error bars represent standard deviations of the mean of triplicate microcosms.

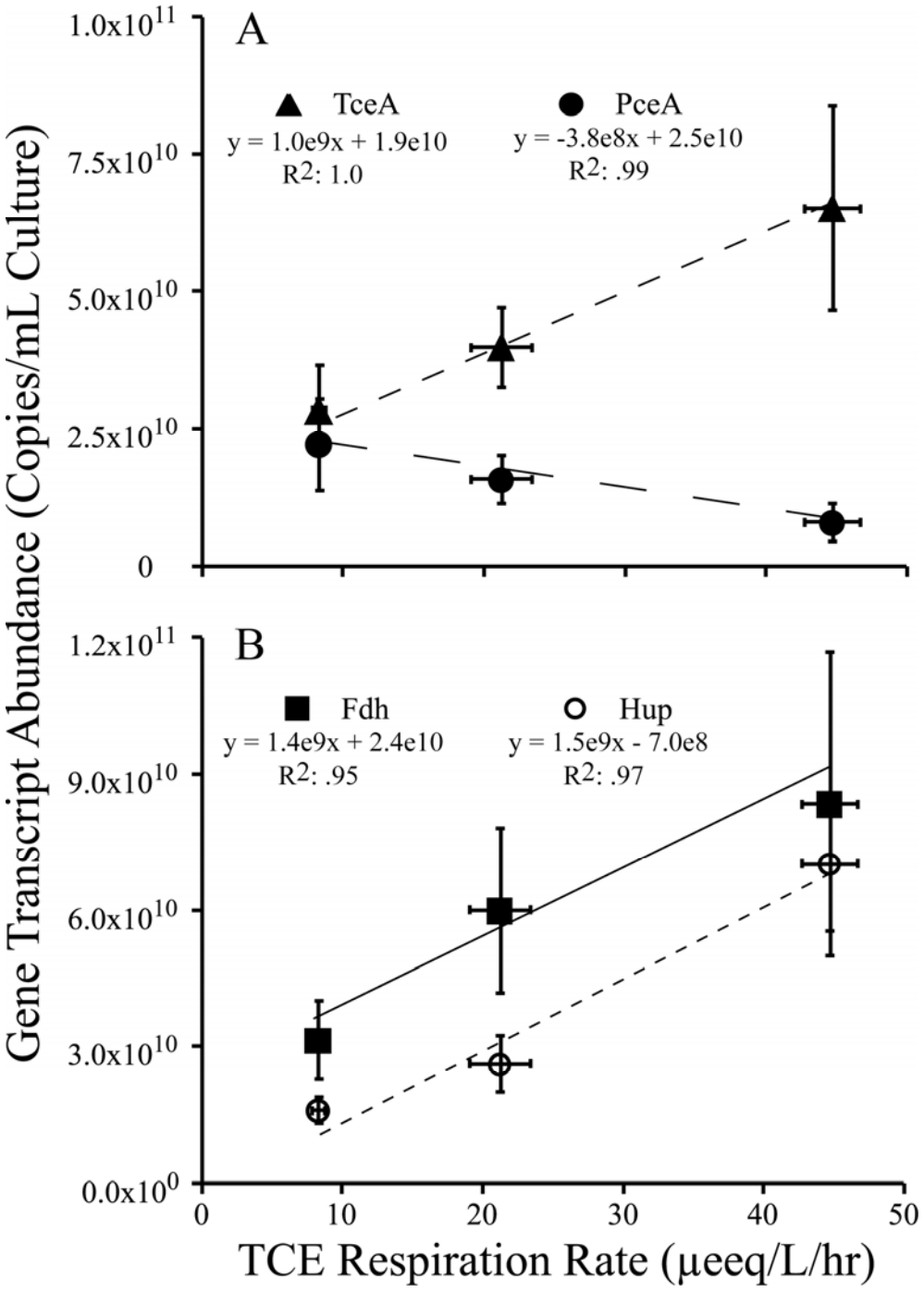

Figure 4.7. Correlation between TCE respiration rate and average pseudo-steady-state transcript abundance for (A) RDases TceA and PceA and (B) Fdh and the H_2ase Hup in TCE-fed microcosms. Error bars represent standard deviations of the mean of triplicate microcosms.

each μeeq/L/hr increase in cDCE respiration (data not shown). TceA was consistently the most abundant RDase, followed each time by PceA. For each RDase, abundance decreased significantly as cDCE respiration rate increased from 20 ± 0.9 to 43 ± 0.9 μeeq/L/hr. As with TCE-fed cultures, 16S rRNA was relatively abundant in cDCE-fed microcosms, but did not correspond to cDCE respiration rates, and growth measured via 16S rRNA gene quantification was negligible.

4.E. Discussion

Given that DET relies on halogenated organic compounds and H_2 for energy conservation, it is not surprising that RDase and H_2ase enzymes are important components of this organism's respiratory machinery and are transcriptionally regulated according to changes in substrate type and concentration. Indeed, the genome sequence reveals transcriptional regulator genes in close proximity to 15 of the 19 annotated RDase genes (*16*), and other possible regulatory candidates have been suggested (*21*). Nonetheless, how regulation of key respiratory genes is linked to aqueous substrate conditions and internal cell redox signals remains unknown. Previous work in PSS cultures respiring PCE at a rate of 9.5 μeeq/L/hr (1.5 μmol/L/hr) demonstrated significant increases in chosen RDase transcript levels at TCE + cDCE concentrations of 390 nM (*19*), almost an order of magnitude lower than previously reported inducing chloroethene concentrations (*22*), and also suggested that a threshold for up-regulation may exist at approximately 1 μmol PCE respired per liter per hour (*19*). GC data from PCElow microcosms studied here (Figure 4.1A) indicated that added PCE was steadily respired at a rate of 2.1 μeeq/L/hr (0.3 μmol/L/hr), well below the predicted threshold for transcriptional up-regulation, while TCE + cDCE concentrations were below the limit of detection (<30 nM). qRT-PCR data taken from these cultures indicate that only RDases DET1559 and DET1545 were

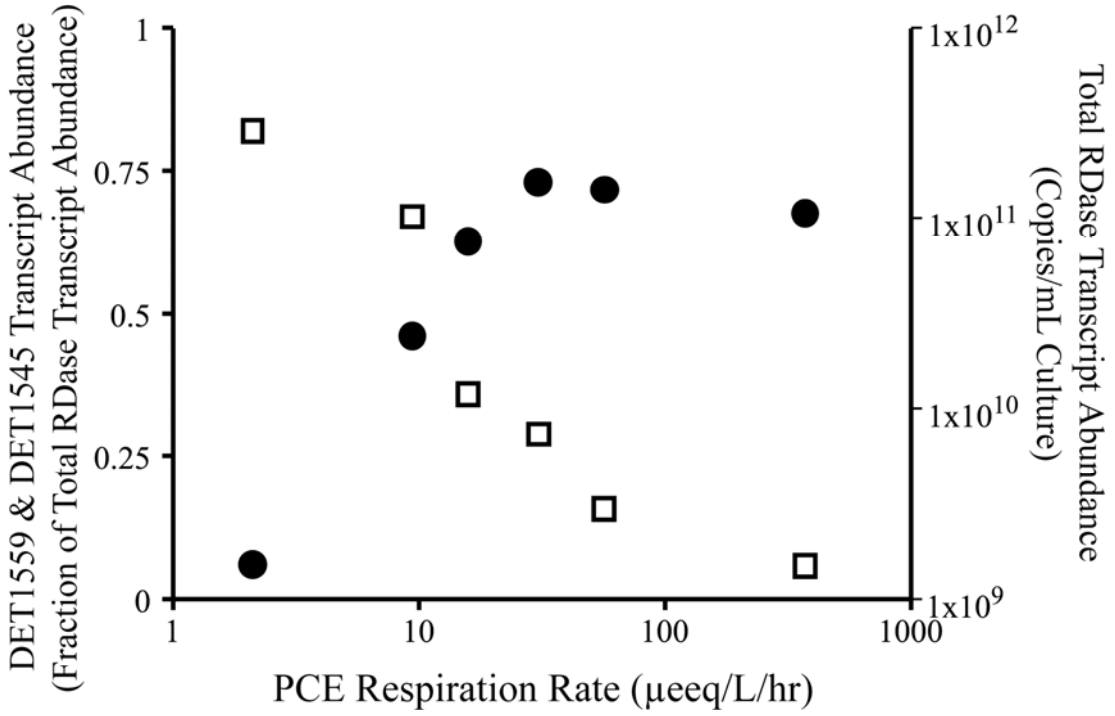

Figure 4.8. RDase DET1559 and DET1545 transcript levels in PCE-fed microcosms at 30 °C. Filled circles indicate total measured RDase transcript abundance (made up of TceA, PceA, DET1559 and DET1545 transcripts). Open squares represent the fraction of that total that is comprised of DET1559 and DET1545 transcripts at various PCE-respiration rates.

significantly up-regulated, while transcript levels of other targets remained at time-0 levels (indicative of conditions after 72 hr starvation), suggesting that different genes have different regulatory thresholds, and supporting previous evidence that these RDases may play important roles when chloroethene concentrations and/or respiration rates are low (*19*). To illustrate this point further, Figure 4.8 shows how RDases DET1559 and DET1545 make up a larger fraction of observed total RDase transcripts as PCE respiration rate decreases. In microcosms respiring PCE at 2.1 μeeq/L/hr, DET1559 + DET1545 account for 82% of measured RDase transcripts, while at 370 μeeq/L/hr, they drop to only 6%, the majority accounted for as TceA transcripts (78%). What functional roles DET1559 and DET1545 play in chloroethene conversion remain unknown.

The PCEhigh experiment pushed the DET population to respire PCE at maximum kinetic rates. Previous results showed lack of correlation between transcript levels of most potential bioindicators and PCE respiration as rates rose to 57 μeeq/L/hr (9.2 μmol/L/hr), leading to various hypotheses: a maximum per-cell transcription rate had been reached, transcription of biosynthesis related genes are prioritized at the expense of respiratory genes as cells sense an opportunity for increased rates of growth, or some unknown toxicity effect related to high PCE respiration rate was repressing transcription (*19*). PCE respiration rate in PCEhigh cultures reached 370 μeeq/L/hr (64 μmol/L/hr), roughly 6.5 times higher than in previously studied PSS cultures (*19*), effectively ruling out the toxicity hypothesis. During PCEhigh trials, however, 6-18 hour average transcript abundance of all four RDases was similar to, or lower than, abundance observed in microcosms respiring PCE at 57 μeeq/L/hr (9.2 μmol/L/hr) (Figure 4.3). While both Fdh and Hup transcripts were more abundant in PCEhigh cultures by factors of roughly 2.5 and 1.5, respectively, only the increase in Fdh transcript level was statistically significant. Furthermore, the higher Fdh and Hup

transcript levels were modest relative to the increase in respiration rate, suggesting that the slope and/or form of the correlation curves are not constant over the whole range of respiration rates studied. Interestingly, data from PCEhigh cultures indicated a significant increase in 16S rRNA level as the experiment proceeded (Figure 4.4). Although some increase in 16S rRNA abundance was expected due to the 79% increase in DET population, the modest growth observed cannot by itself account for the two orders of magnitude change in ribosome content. This suggests that the observed transcriptional "plateau" of functional RDase targets did not represent a limitation in per-cell transcription rates, but rather may have been due to a shift in transcription prioritization. It is also possible that 16S rRNA abundance became the controlling factor in translation of new RDase proteins at these higher respiration rates. Alternatively, or perhaps concomitant with this possibility, the controlling mechanism may be related to the regulation of protein stability.

A *Dehalococcoides* population's ability to respire chloroethenes at a given rate is dependent on its cell density. Microcosms studied here contain a relatively dense DET population ($4.4 \pm 1.2 \times 10^8$ cells/mL on average). While such high cell densities allowed us to operate these microcosms over a wide range of respiration rates, they also meant that accurate measures of growth would require very high respiration rates or experiment times. Growth observed in PCEhigh cultures at 30˚C, amounting to a 79% increase in DET abundance, allowed us to calculate an approximate yield of 1.6×10^8 16S rRNA gene copies (cells) per µmol Cl^- released (between PCE & VC), a value in between those found for *Dehalococcoides* strain CBDB1 grown at 29-32˚C on 2,3-dichlorophenol and other substrates (7.6×10^7 cells per µmol Cl^- released) (*3*), and strain KB1 grown at room temperature on cDCE and TCE (about 3.6×10^8 16S rRNA gene copies per µmol Cl^- released) (*35,36*), and about one-third the value found for bacterium VS grown at 20˚C on VC (5.2×10^8 16S rRNA gene copies per µmol Cl^-

released) (*37*). Also, given the evidence that PCEhigh cultures were operating at their maximum kinetic potential, we estimated a kmax for PCE respiration of about 3 x 10^{-9} µmol Cl^- per 16S rRNA gene copy per day. This value is about 4-fold greater than that reported for bacterium VS grown on VC (8 x 10^{-10} µmol Cl^- per 16S rRNA gene copy per day) (*37*).

Due to their high overall abundance and linear responses to changes in TCE respiration rate (within the range studied), Fdh, Hup and TceA targets, all of which made reasonable choices as bioindicators in PCE-fed cultures (*19*), are also candidates for bioindicators in TCE-fed cultures. Data for the RDase PceA suggested that cultures fed increasing amounts of TCE maintained lower steady-state levels of this transcript, thus making it unsuitable for use as a bioindicator when TCE is the most highly chlorinated substrate. Unlike in PCE-fed cultures, DET1559 was never the most abundant RDase transcript in any TCE-fed cultures, and its usefulness as a bioindicator under these conditions is questionable. DET1545 transcripts were found in relatively high numbers at the lowest TCE feeding rate, three-fold higher than in PCE-fed cultures respiring at comparable rates on an eeq basis (at the lowest feeding rate only). Thus, DET1545, which has homologs in most other studied *Dehalococcoides* strains, may be a viable bioindicator in TCE-fed cultures when the respiration rate is low.

With the exception of PceA, trends in the transcript abundance of many bioindicators were similar in both PCE- and TCE-fed cultures: Fdh, Hup, TceA and DET1559 transcript abundance tended to increase with increasing respiration rate (within a limited range) while DET1545 decreased. Transcript levels in cDCE-fed cultures, however, did not follow these trends so clearly. No RDase transcript, for example, increased monotonically with cDCE respiration rate. Only the H_2ase Hup showed increased transcript abundance with successive increases in cDCE respiration.

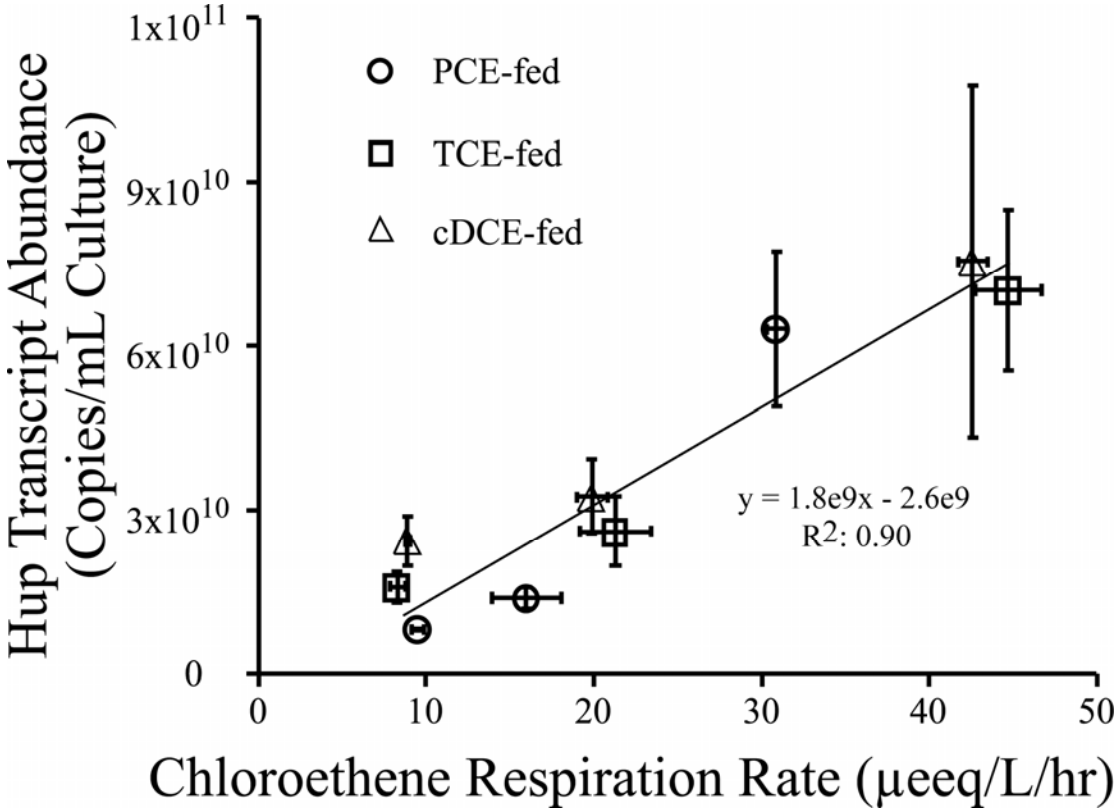

Figure 4.9. Dehalorespiration rates on all substrates (within the range of approximately 8 and 45 µeeq/L/hr), and average pseudo-steady-state transcript abundance of the H_2ase Hup in PCE-, TCE- and cDCE-fed cultures. Error bars represent standard deviations of the mean of triplicate microcosms. Portions of this data have been presented previously (*19*).

Interestingly, Hup seemed to respond similarly regardless of substrate, and a general relationship between chloroethene respiration and Hup transcript level could be described in which an additional 1.8×10^9 copies/mL culture are transcribed per μeeq/L/hr increase in respiration rate (between approximately 8 and 45 μeeq/L/hr) (Figure 4.9). This is an interesting result since provision of electron donor in all PSS cultures was presumed to supply a steady, non-limiting source of H_2 ($P_{H2} \geq 1 \times 10^{-5}$ atm) (*31*). Experiments in microcosms under conditions of higher H_2 partial pressures and also in H_2 limited cultures will be useful for determining the signals controlling H_2ase regulation.

High overall expression of the H_2ase Hup and its linear response to increases in cDCE respiration rate made it the only bioindicator candidate with diagnostic and predictive potential in cDCE-fed cultures. Despite their high levels, the lack of a good linear correlation between Fdh and TceA transcripts and cDCE respiration rate over the observed range prevents their use as rate-predictive bioindicators when cDCE is the key substrate. RDases PceA, DET1559, and DET1545, while detectable, were never the most abundant RDases found nor were their transcript levels proportional to cDCE respiration rates. Thus, these RDases do not seem ideal as bioindicators of cDCE respiration.

Interpretation of results presented here, and their application to phenotypes desired in field environments, is complicated by several factors. For example, there is not necessarily a direct relationship between transcript abundance and *in situ* concentration of functional enzyme. RDase and H_2ase enzyme concentration within active DET cells depends on protein translation rates, the cell's ability to properly fold and transport these enzymes to their functional destination in the periplasmic membrane space, and individual protein decay rates, factors which were beyond the scope of the current study. Moreover, the introduction of different substrates such as

TCE and cDCE to a culture accustomed to growth on PCE may create different patterns of transcriptional induction, the exact causes of which are currently poorly understood. More work is needed to define the causal relationships underpinning respiratory function and transcriptional regulation in DET and other *Dehalococcoides* strains. Despite these limitations, the current study provided new insight into how transcription of potential bioindicators is influenced by substrate type and respiration rate. Furthermore, data suggest that while RDase bioindicators may supply more specific information about what type of substrate is being dechlorinated, it may be H_2ase levels that more accurately reflect the instantaneous respiration activity of DET cells.

4.F. Acknowledgments

This project was supported by the U.S. Department of Energy (DOE) Office of Cleanup Technologies and administered by the U.S. Department of Energy Savannah River (SR) Operations Office (Contract No. DE-AC09-96SR18500), and is a product of the Monitored Natural Attenuation/Enhanced Attenuation for Chlorinated Solvents Technology Alternative Project. We thank Stephen Zinder and James Gossett for valuable conversations and for comments on this manuscript.

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CHAPTER 5

A Discussion of Results and Remaining Questions

5.A. Reiteration of Research Goals

The aim of this research was to gain insight into the transcription of genes employed during the process of anaerobic dehalorespiration in *Dehalococcoides ethenogenes* strain 195 by combining analytical observation of the reductively dechlorinating mixed culture D2 with DET molecular biological data. Specific research goals, as given in Chapter 1, are reiterated below.

1. Identify specific DET gene transcripts that might be used as bioindicators of reductive dechlorination
2. Determine how chosen bioindicators are expressed under typical mixed culture conditions
3. Determine how changes in substrate concentration, substrate type and operational reactor conditions affect bioindicator expression
4. Relate bioindicator expression under various conditions to protein abundance and cellular respiration rates

5.B. Summary of Findings

An initial list of DET genes potentially important in reductive dechlorination was assembled based on the annotated DET genome (*1*) and on qRT-PCR (*2*) and proteomic (*3*) analyses in both pure and mixed cultures. Preliminary screening of batch, PCE-fed D2 microcosms revealed that only a subset of these potentially important genes showed significant up-regulation relative to time-zero expression levels. Among these, the most highly expressed bioindicators were gene transcripts reflecting general levels of cellular activity, including 16S rRNA, RpoB and ATPase,

as well as transcripts corresponding to genes of known or putative respiratory function, including a putative Fdh, H_2ases Hup and Vhu, and RDases TceA, PceA, DET1559, DET1545 and DET0162. Other putative respiratory genes, including the H_2ase Hym as well as oxidoreductases Nuo and Mod, were up-regulated, but to a lesser extent.

In PCE-fed microcosms operated in typical batch fashion, temporal variability of bioindicator gene transcript levels was observed, particularly for RDases. Most targets were significantly up-regulated within 2 h of batch addition of substrates, and profiles of transcript levels over time indicated group-specific regulation. H_2ase (Hup and Vhu) and Fdh transcripts were observed to reach their maximum levels between 4 and 13 h after substrate addition and remained at relatively constant levels throughout 24 h. RDase transcripts (TceA, PceA, DET1559, DET1545 and DET0162), on the other hand, reached maximum levels between 11 and 24 h, and displayed an increased variability in individual RDase expression. The cause of this temporal discrepancy between maximal expression of H_2ases and RDases, which presumably work in concert to gain energy from the respiration of H_2 and chloroethenes, remains unclear.

Substrate variations were also observed to affect transcription of bioindicator genes. Data from batch-fed microcosms in which either PCE or butyrate was omitted from the medium indicated that neither substrate alone was able to induce significant up-regulation of gene expression (Appendix II). Rather, transcriptional regulation appeared to depend on a more complex signal indicative of growth-supporting conditions. Although some up-regulation of bioindicator transcription was observed in microcosms lacking butyrate, the transcriptional response was relatively weak and temporally late, and was likely due to H_2 released via endogenous decay of mixed culture biomass.

Results from PCE-fed PSS microcosms indicated that steady-state transcript levels had been achieved in individual reactors and that transcriptional regulation of bioindicator genes was sensitive to chloroethene respiration rates and/or concentrations. After a brief response period (<6 hours) transcript abundance in PSS microcosms generally did not change over time for any given experiment, regardless of the gene target. This result was not surprising since the PSS reactor system was setup to stabilize transcript levels at steady-state concentrations. Comparison across experiments showed that abundance of gene transcripts corresponding to Fdh, Hup, TceA, PceA, and DET1559 increased with successive increases in PCE respiration rate within a certain range (1.5 – 4.8 µmol/L/hr), and could be well-fit with a linear regression. However, at relatively high PCE respiration rates, including rates in kinetically limited cultures, transcript levels were observed to plateau, and in some cases decline, relative to levels observed in microcosms respiring at lower rates. These results indicate that some factor other than transcript level controls high rates of respiration. High levels of 16S rRNA in these microcosms suggest that activity may be regulated by ribosome content and/or translational kinetics. The abundance of DET1545 gene transcripts tended to decrease with each successive increase in PCE respiration rate. At very low respiration rates, the DET population did not significantly up-regulate the transcription of many bioindicator genes relative to time-0 levels (indicative of 72 h starvation), suggesting that some threshold may exist below which a DET population of this size does not actively respond. Interestingly, transcription of DET1559 and DET1545 genes were actively up-regulated even at these low levels of respiration, implying that enzymes corresponding to these genes may play important roles in reductive dechlorination when substrate concentrations are low.

PSS microcosms fed alternate substrates TCE and cDCE provided additional evidence that substrate type and concentration can affect bioindicator gene transcript levels. As in PCE-fed microcosms (within a range of respiration rates), abundance of gene transcripts corresponding to Fdh, Hup, and TceA increased with successive increases in TCE respiration rate (between 1.9 and 10 µmol/L/hr), and could be well-fit with a linear regression. In cDCE-fed microcosms, however, trends were not so clear. Although the abundance of Fdh and TceA gene transcripts was always relatively high, they could not be well correlated to cDCE respiration rates (between 3.7 and 19 µmol/L/hr). Only Hup gene transcript abundance could be positively correlated with cDCE respiration rate within this range.

Results from PSS microcosm studies generally supported previous substrate range characterizations of RDases PceA and TceA. The RDase PceA had been shown to catalyze the reduction of PCE to TCE (*2,4*). This was supported by data from PCE-fed PSS microcosms in which transcript levels of the gene corresponding to PceA were relatively high and showed positive correlation with increasing PCE respiration rate within a certain range. Conversely, PceA gene transcript abundance in TCE-fed microcosms was relatively low and showed a negative correlation to increasing TCE respiration. Gene transcripts for TceA, however, were relatively abundant in all PSS microcosms, and showed a positive correlation with increasing PCE and TCE respiration, supporting the claim that TceA catalyzes the reduction of both TCE and cDCE (*4,5*). RDase gene DET1559 showed similar transcript levels regardless of substrate respired, while transcription of DET1545 seemed to decrease with increasing respiration rate regardless of substrate. While both DET1559 and DET1545 tended to be more highly transcribed under conditions of low respiration, neither could be associated with the respiration of any specific chloroethenes.

5.C. Unanswered and Newly Discovered Questions

Initial studies in batch PCE-fed microcosms suggested that transcription of functionally related groups of bioindicator genes, such as H_2ases and RDases, were regulated differently from each other. It was later determined that neither electron donor nor acceptor alone could cause up-regulation of transcription. Work both here and elsewhere (*2,6*) has shown that some RDase genes are regulated in response to substrate type, but the presence of substrate alone does not seem to be enough. More research is needed in order to understand the environmental signals triggering these variable transcriptional responses, and the basis for group-specific regulation.

Later studies performed in PSS PCE-fed reactors demonstrated that the abundance of several bioindicator gene transcripts increased linearly with increasing PCE respiration rate within a certain range, but that higher rates of PCE respiration did not lead to correspondingly higher transcript levels. One possible hypothesis for the lack of apparent up-regulation at high respiration rates was that transcription of biosynthesis related genes was prioritized at the expense of respiratory gene transcription. Indeed, endpoint PCR followed by gel electrophoresis of cDNA corresponding to the *ftsA* gene, which has been shown to be involved in cell division in other organisms (*7,8*), qualitatively suggested that transcription increases successively with increasing PCE respiration rate, even at high rates (H. Fullerton, unpublished results). Additional studies on the transcription of a broader range of gene targets, including genes known or thought to participate in growth-related pathways, would help to evaluate this hypothesis. Microarray and proteomic techniques would help to identify the physiological differences between DET populations respiring PCE at rates above the linear range identified here. An evaluation of transcript and protein decay rates is also essential for accurately relating information from diverse macromolecule pools.

PSS PCE-fed microcosms respiring PCE at very low levels indicated that a respiration threshold exists for up-regulation of many chosen bioindicators. Interestingly, RDase genes DET1559 and DET1545 were up-regulated at these low respiration rates, even while other RDases were not, suggesting that their corresponding proteins may play important roles in reductive dechlorination when substrate concentrations are low. However, more research is needed to confirm this transcriptional threshold, and to clarify the roles of DET1559 and DET1545 in chloroethene respiration. Since inferring the substrate ranges of these RDases via data on transcript abundance has proven difficult, it may be necessary to purify these proteins and characterize them separately, as Magnuson et al. did for TceA and PceA (*4*,*5*). How the DET population was able to reductively dechlorinate PCE at low rates, despite the lack of up-regulation of many RDase genes, was not specifically determined. It is possible that the "standing crop" of RDase enzymes – carried over from the previous period of activity - was sufficiently large to reduce the substrate available. Better characterization of this standing crop, including its relationship to previous activity levels, is needed in order to clarify these results.

Studies in TCE- and cDCE-fed PSS microcosms yielded results that were in agreement with previous research on the characteristics of TceA and PceA mediated reductive dechlorination, but did not give any clear evidence about the substrate ranges of DET1559 or DET1545. Fung et al (2007) examined the transcription of all putative RDase genes in DET pure culture grown on TCE (*2*). However, nothing is known about the transcription of DET's full complement of RDase genes in cultures grown on cDCE. Expression studies in microcosms given cDCE and other halogenated organic compounds, including VC, chlorobenzenes and others, might enhance our ability to define specific RDase substrate ranges.

Much additional research is needed before the goals of this study are realized in a practical and meaningful way. Proteomics experiments are needed in order to address the relationship between bioindicator transcript levels and cellular enzyme concentrations, as well as to understand the effects of protein decay and translation kinetics on the regulation of enzyme pools. While initial proteomic studies suggest that relative protein levels change in agreement with bioindicator transcript abundance (J. Werner, unpublished results), these data need to be replicated and expanded to include additional experimental conditions. Also, data similar to that reported here for the D2 mixed culture should be collected from other culture environments, including the DET pure culture and other pure and mixed cultures with similar dechlorinating phenotypes. Useful translation of these results to complex field environments will require a robust understanding of various *Dehalococcoides* organisms. Lastly, interpretation of data presented here requires the elucidation of the specific functions of Fdh, DET1559 and DET1545.

5.D. A Brief Discussion of Methods: Advantages and Limitations

In these studies, conclusions rely heavily on RNA based assays, and it is worthwhile to recognize the advantages and limitations of such an approach. As discussed throughout this work, RNA was chosen as a bioindicator molecule for several reasons: specificity, accuracy, and ease of quantification. Here, chosen RNA bioindicators are specific to reductive dehchlorination activity and are not abundant unless conditions are favorable for growth on chloroethenes. Many of these bioindicators are also accurate assays of activity under some conditions, which is to say their abundance can be correlated to the respiration rate of one or more chloroethene substrates. Lastly, the relatively high transcript abundance of bioindicators under conditions examined here makes them easy to acquire using

current RNA isolation methods, while the wide range of transcript abundance of bioindicators under various conditions is readily differentiated using qRT-PCR techniques.

DNA assays, too, have their advantages. Relative to RNA, DNA is more resistant to degradation. Also, DNA extraction and quantification protocols are relatively simple and avoid reverse-transcription steps that can be variably inefficient. However, while detection of bioindicator genes via DNA confirms presence of DET and indicates the potential for reductive dechlorination, it does not reflect activity. Furthermore, since gene copy numbers do not change except in the case of DET population growth – a relatively slow process – there is little opportunity to observe the potentially dynamic relationship between chloroethene degradation and cellular metabolic regulation. DNA, therefore, was not a primary candidate for use as a bioindicator.

Theoretically, protein assays are the most direct way of measuring activity. DNA encodes the information needed to produce a functional enzyme, and RNA converts this information into an accessible blueprint, but neither molecule – in most cases – is endowed with a function that leads to a recognizable phenotype. Enzymatic proteins are the functional molecules of the cell and are specific for the reactions they catalyze. Their concentration, depending on the kinetics of a given reaction, may be directly proportional to activity rate. Despite these attractive characteristics, the extraction, detection and quantification of proteins remains difficult. It was for this reason that protein was not chosen as the primary bioindicator molecule in these studies.

Having established RNA as the bioindicator molecule of choice for these studies, it still must be stressed that making inferences about cellular activity based on RNA data alone is problematic. To begin with, gene expression profiles may be

altered by procedures such as cell pelleting and storage (*9*). Given that the half-life of TceA transcripts has been determined to be around 5 h (*10*), it is unlikely that these relatively quick procedures make a significant impact on bioindicator expression levels. Still, the recovery of an accurate and representative RNA sample remains a concern. Assuming that RNA has been extracted faithfully, making conclusions about specific enzymatic functions and activities remains tenuous. Transcripts corresponding to the DET0162 RDase gene were found in high abundance in batch PCE-fed microcosms; however, due to a point mutation, predicted DET0162 peptides were never detected in shotgun proteomic assays (*3,11*). Transcript abundance is not necessarily related to transcription rate or translation rate. Several unrelated species have all been shown to exhibit widely variable mRNA decay rates that are generally inversely proportional to mRNA abundance (*9,12,13*). This implies that identification of "important" gene transcripts on the basis of high abundance is misleading, and may not accurately reflect the translation process. Bioindicator transcripts found at relatively low levels, therefore, such as DET1559 and DET1545, may be among the most stable mRNAs, possibly supporting their role under basal metabolic and/or starvation conditions. Highly stable transcripts may also require longer time periods to reach steady-state concentrations, a possible explanation for the similar transcript abundance of DET1559 during PSS experiments under conditions of various PCE loading rates.

Despite the difficulties associated with protein extraction and detection mentioned above, a number of studies have recently stressed the need to combine RNA assays with proteomic techniques in order to gain an accurate understanding of cellular gene networks (*14*). In part, this is because the quantitative link between levels of mRNA and protein is not straightforward due to varying protein degradation rates and post-translational regulation strategies (*15*). In fact, there is now evidence

that mRNA and protein levels do not necessarily correspond to each other (*16,17*). Additional reasons for this include limitations in ribosome concentration, competition for tRNA, and constraints on the kinetics of translation (*17*).

5.E. Future Work: Context and Suggestions

Future experiments utilizing PSS microcosms can help resolve many of the questions posed above. In particular, microcosms amended with various substrates, including chloroethenes/ethanes, bromoethenes/ethanes, DCP and PCB electron acceptors, and H_2, butyrate, methanol and lactate electron donors, should help to define the substrate ranges and behaviors of various bioindicators. Experiments with even lower PCE loading rates, lower temperatures, and more dilute starting cell densities may more accurately reflect field environments in which DET populations and chloroethenes are less concentrated than in the enrichment D2 culture used for these studies. Also, as mentioned above, repeating these tests using different *Dehalococcoides* strains should show whether bioindicators studied here have homologs with similar transcriptional profiles, and will also help to identify and characterize other gene targets that may be important in other organisms. Ultimately, it will be important to apply these RNA assays to field samples and to combine them with estimates of field-observed chloroethene degradation rates.

Bringing together analytical data describing culture conditions, available substrates, intermediates, and products, along with molecular biological data describing DNA, RNA and protein concentrations, transcription thresholds and possible patterns of regulation, necessitates a systems biology framework. Work described here would most accurately fall into what Bernhard Palsson termed “step 1” of the systems biology paradigm (*18*): an enumeration of observed macromolecules under a variety of conditions that will hopefully lead to a reconstruction of the DET

biochemical reaction network. Additional experiments outlined above will help to continue this process.

High-throughput methodologies are critically important in the continued development of this systems biology foundation. qRT-PCR techniques, which have been used for the work presented here, are valuable for the study of relatively small groups of RNA targets under a limited range of conditions. Results are relatively quick and easy to obtain, and generally do not require elaborate validation or explanation. However, techniques utilizing microarrays and proteomic approaches have the ability to yield far more data, and are also recommended tools for incorporation into future research. Preliminary results using a *Dehalococcoides*-specific microarray, for example, were able to delineate and partially characterize a cobalamin (vitamin B_{12}) regulon in DET (*19*), and also described the up-regulation of many genes, including RDases, H_2ases, and ABC transporters in cultures reductively dechlorinating TCE (*20*). Proteomic techniques, as discussed before, have been able to confirm the presence of many bioindicator peptides in D2 cultures respiring PCE, and have also been used to identify additional peptides of interest, such as one tentatively thought to represent the S-layer protein (*3*). Current studies already underway should provide insight into protein decay half-lives and the correlation between bioindicator transcript abundance and protein abundance in PCE-, TCE- and cDCE-fed PSS microcosms. As information via high-throughput technologies such as microarrays and proteomics becomes easier, more reliable and cheaper, results obtained through these methodologies will continue to support and drive PSS microcosm and other studies.

It is hoped that a more detailed understanding of the expression of bioindicator targets in *Dehalococcoides* organisms will lead to accurate diagnostic assays of microbial activities at field sites contaminated with halogenated organic compounds.

Experiments described here have furthered this aim by identifying and partially characterizing the expression of several potential bioindicators in DET under a variety of conditions. Future work utilizing similar methods in combination with high-throughput techniques such as microarrays and proteomic assays will expand this research. All together, a more robust knowledge of bioindicator gene networks will allow engineers to more skillfully manipulate the microbial populations that are responsible for desirable remediation outcomes.

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APPENDIX I

Comparison of Bioindicator Transcript Levels in D1 and D2 Cultures

Transcript abundance of potential bioindicator genes in both of the mixed communities currently being maintained (D1 and D2) was evaluated in order to assess possible similarities and differences between the two dechlorinating populations. Transcript levels presented here for the D2 culture represent independent measurements from those reported in Chapter 2 under similar conditions. The D1 and D2 cultures were operated in parallel, and were treated identically as outlined in Chapter 2. Sampling procedures and processing, gas chromatograph analyses, and qRT-PCR were also performed as described in Chapter 2.

Dechlorination profiles of the D1 and D2 cultures are provided in Figure A1.1, and illustrate typical behavior. Added PCE was degraded sequentially to TCE, cDCE, VC and finally ethene. In both cultures, PCE persisted until about hour 10; TCE and cDCE were transiently observed at low concentrations until hour 4; VC production occurred rapidly until about hour 8, at which point it began to slow; ethene formation continued more or less steadily throughout the experiment.

Results of qRT-PCR analyses are summarized in Figure A1.2. Detailed timecourses of each target studied, which include genes for RpoB, ATPase, Fdh, the H_2ases Hup and Vhu, and the RDases TceA, DET0162, DET1559, and DET0318, are provided as well (Figure A1.3). Relative to that of the RpoB gene, which was used as an indicator of general DET activty, transcription levels of the Fdh, Hup, TceA and DET0162 genes were high in both cultures, while ATPase, which was also considered a general activity indicator, was transcribed just above levels of the RpoB gene. The Vhu gene transcript was found at comparable levels to the RpoB transcript in each

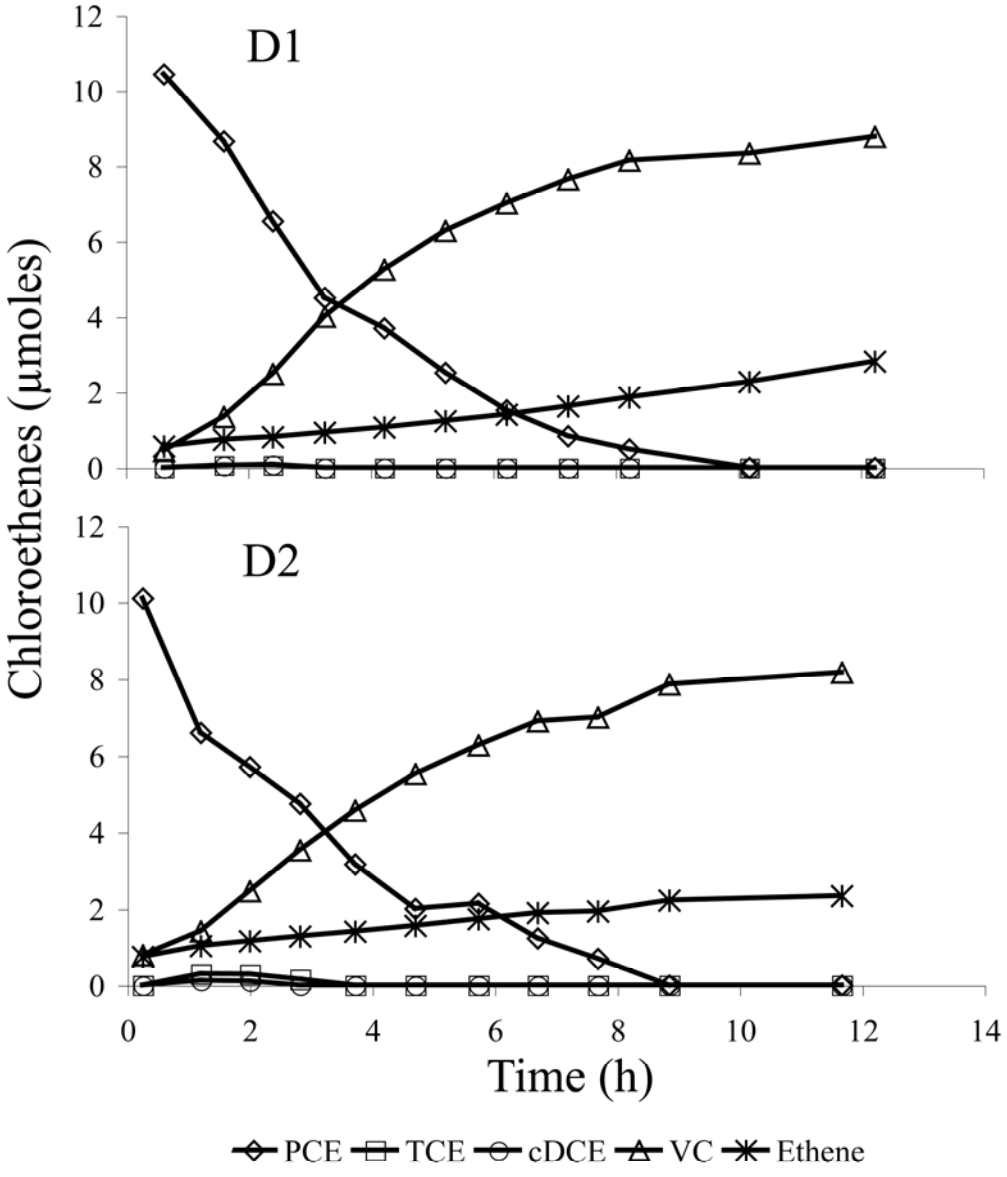


Figure A1.1. Dechlorination profiles in D1 and D2 enrichment cultures amended with approximately 11 µmoles PCE.

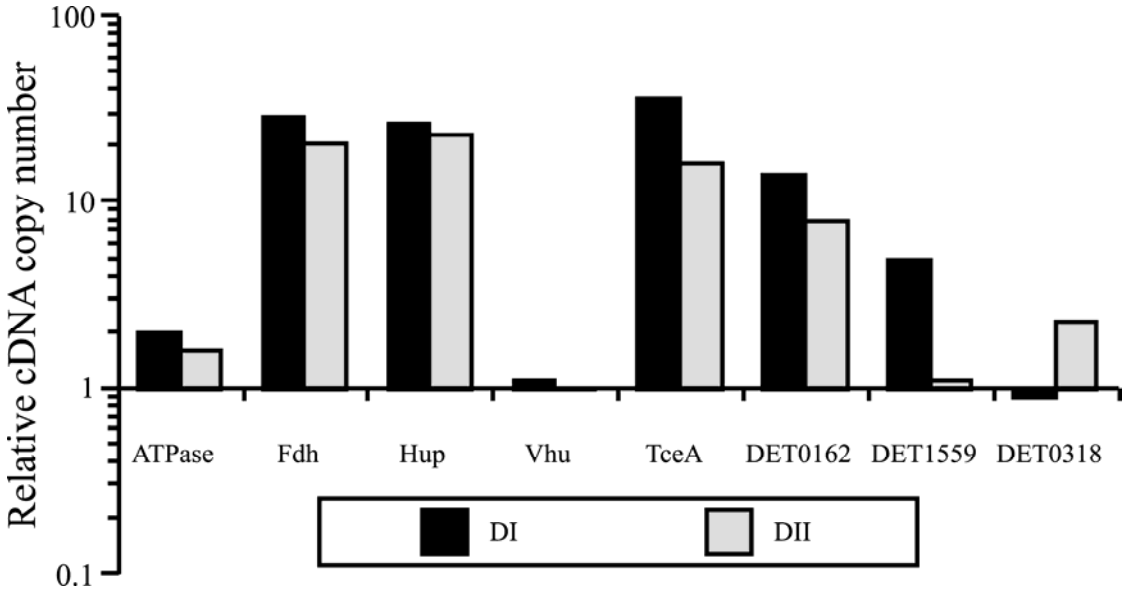


Figure A1.2. Transcript level of each target in D1 and D2 cultures relative to that for the RpoB gene (data presented are averages of values from 1 to 12 h after PCE feeding).

culture. Interestingly, D1 was found to contain relatively high levels of the RDase DET1559 compared to D2, while the opposite was true for the RDase DET0318.

Since the D1 and D2 cultures behave phenotypically similar with respect to the reductive dechlorination of PCE, it is not surprising that a comparison of their transcriptional profiles would yield many similarities. Indeed, transcript levels of genes for RpoB, ATPase, Fdh, Hup, Vhu, TceA and DET0162 are similar in both cultures and agree with other independent observations of the D2 culture (Chapter 2). Curiously, this data set indicates that the RDases DET1559 and DET0318 can be differentially expressed, raising the possibility that these two cultures contain very similar dechlorinating populations that utilize different combinations of RDase enzymes to achieve the same goal. However, data taken from D2 subcultures shown in Chapter 2 indicate a higher level of transcription for the DET1559 gene, which agrees more closely with the level observed in the D1 culture here. As a result, the idea that the D1 and D2 cultures express their RDases differently should be treated with some skepticism. More work, particularly with the D1 culture, is needed to verify such claims.

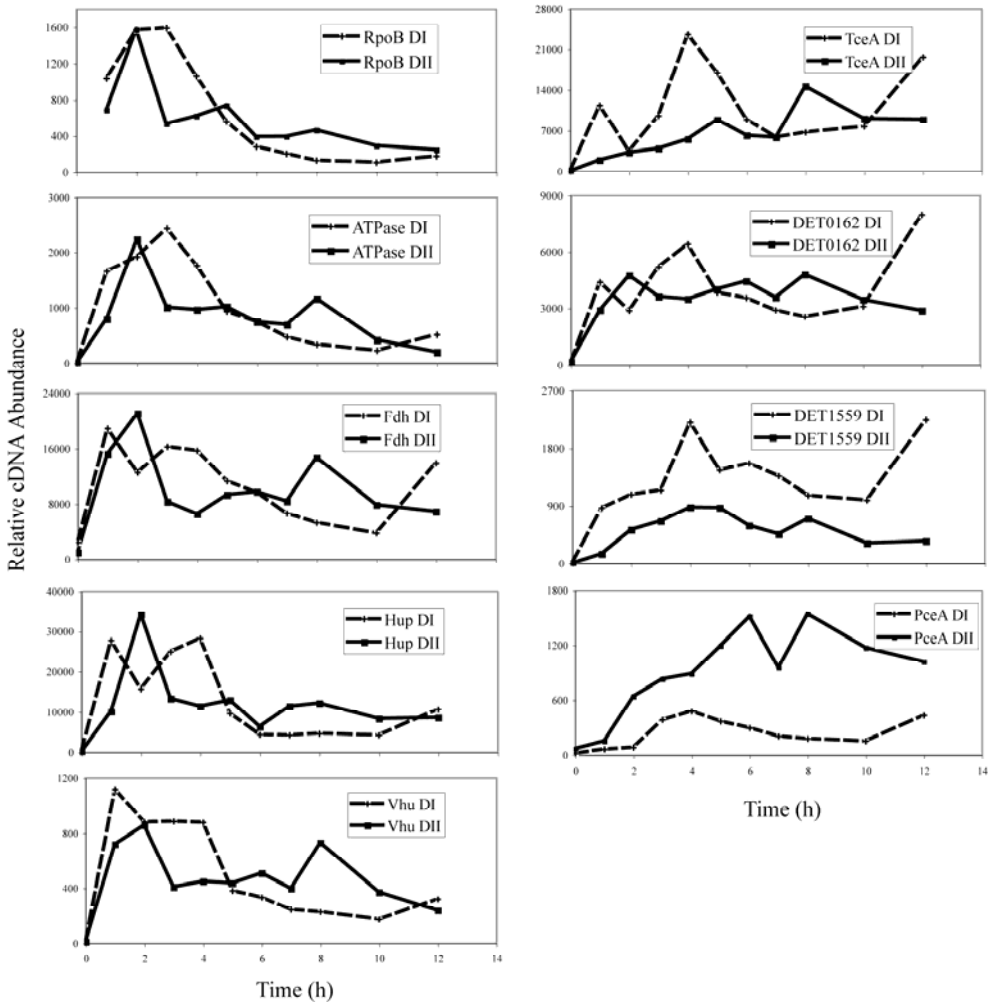


Figure A1.3. Transcript abundance timecourses for each target in D1 and D2 cultures. Data presented has been normalized according to luciferase reference target recovery, a procedure that helps to control for mRNA losses during sample preparation and inefficiencies in reverse transcription.

APPENDIX II

Bioindicator Expression in Amendment Microcosms

Amendment subcultures were established to determine what contribution each substrate, PCE and butyric acid, may have on expression of bioindicator targets. Additionally, a subculture amended with a half-dose of PCE was established to determine the effect of substrate concentration on expression levels. 100 mL amendment subcultures were established in 160 mL serum bottles and were fed either PCE (3 µmoles for the "PCE only" bottle), 44 µmoles butyric acid ("butyrate only"), or both (3 and 1.5 µmoles PCE for the "PCE+butyrate" and "PCE/2+butyrate" bottles, respectively). Additionally, all bottles were fed a vitamin mixture and 20 mg yeast extract/liter of culture. Sampling procedures and gas chromatograph measurements were performed as outlined in Chapter 2. qRT-PCR was also carried out as described in Chapter 2, except that luciferase reference RNA was not included in the analyses. As a result, transcript levels were not corrected for mRNA losses incurred during sample processing and cDNA synthesis, and are reported in arbitrary abundance units.

Reductive dechlorination of added PCE in amendment subcultures is shown in Figure A2.1. In the "PCE+butyrate" bottle, TCE was detected within 2 h, and added PCE was dechlorinated to VC and ethene within 10 h. VC concentration reached a maximum of 36 µmol/L around hour 7, at which point it was slowly converted to ethene. Similar trends were observed in the "PCE/2+butyrate" bottle where added PCE was dechlorinated to VC and ethene within 9 h. In the "PCE only" bottle, added PCE (30 µmol/L) was dechlorinated slowly to a final concentration of 19 µmol/L, while TCE concentration steadily increased to 4 µmol/L. cDCE and VC concentrations never rose above 1 µmol/L, and no ethene was detected. Dechlorination was still occurring when the experiment ended. In the absence of

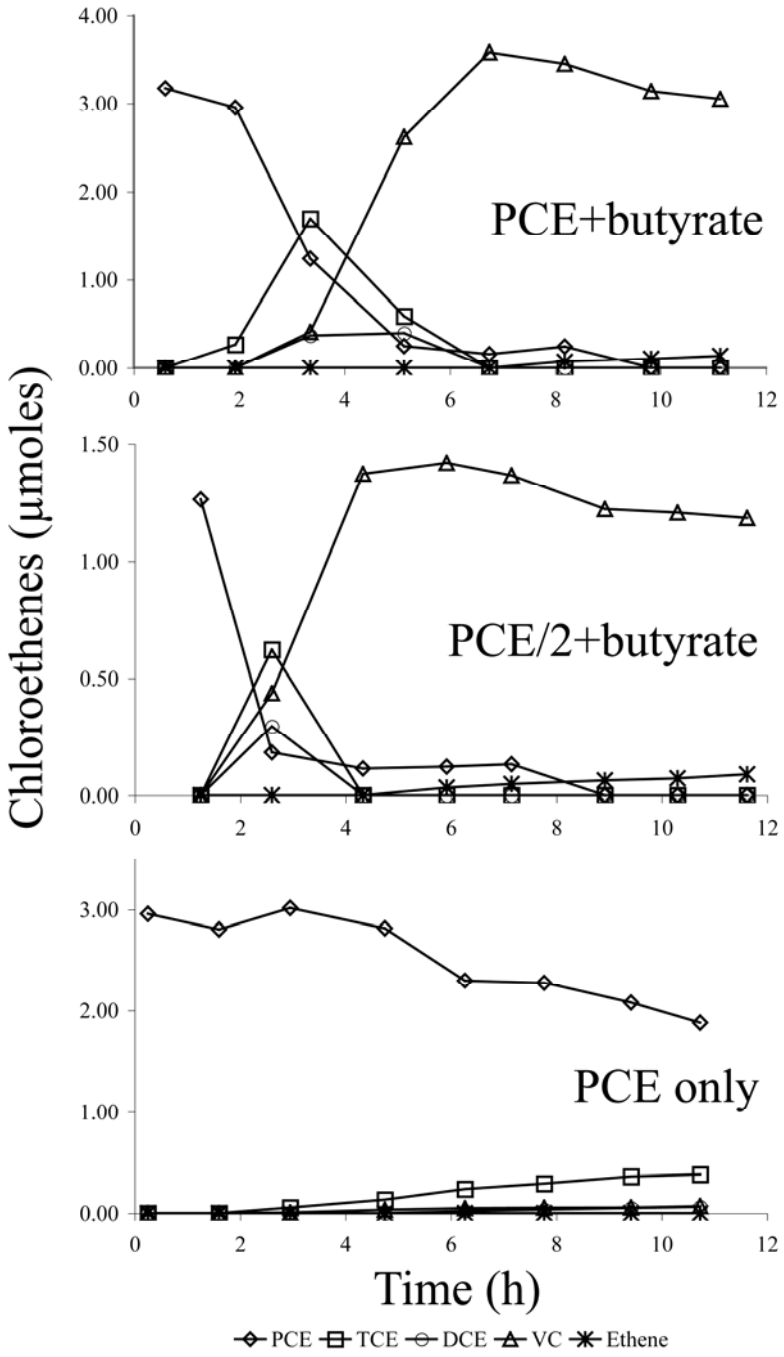


Figure A2.1. Dechlorination profiles of amendment subcultures. The "PCE+butyrate" and "PCE/2+butyrate" bottles shows degradation of all PCE, TCE and cDCE within 10 h, followed by the slow conversion of VC to ethene. The "PCE only" bottle shows modest PCE degradation with corresponding TCE accumulation, with negligible production of VC and ethene.

butyrate, dechlorination observed in the “PCE only” bottle was probably due to the generation of hydrogen via endogenous decay of biomass. No chloroethenes were detected in the “butyrate only” bottle.

To determine whether both electron acceptor and donor were required for expression of bioindicators, qRT-PCR data were taken from amendment subcultures over the course of 12 h. Figure A2.2 summarizes this data, and detailed timecourses of expression are provided in Figure A2.3 and A2.4. Generally, transcript levels were highest in cultures amended with both PCE and butyrate. Furthermore, while respirable chloroethenes (PCE, TCE, cDCE) were largely depleted by hour 6.5 and 4 in the “PCE+butyrate” and “PCE/2+butyrate” bottle, respectively, elevated levels of many transcripts continued until at least hour 12 when the experiment was terminated. Transcript levels in the “PCE only” bottle were generally low. However, levels of some transcripts, particularly for certain RDase genes, rose an order of magnitude or more above time 0 levels, although this increase was not observed until hour 6.5, indicating relatively late up-regulation. The late and reduced onset of RDase transcription increases in the “PCE only” bottle compared to bottles receiving PCE and butyrate, together with the lack of observable up-regulation of H_2ase targets (with the exception of Vhu) supports the suggestion that PCE degradation in this bottle was due to hydrogen produced by endogenous decay. In the “butyrate only” bottle, expression of transcripts never increased above time 0 levels.

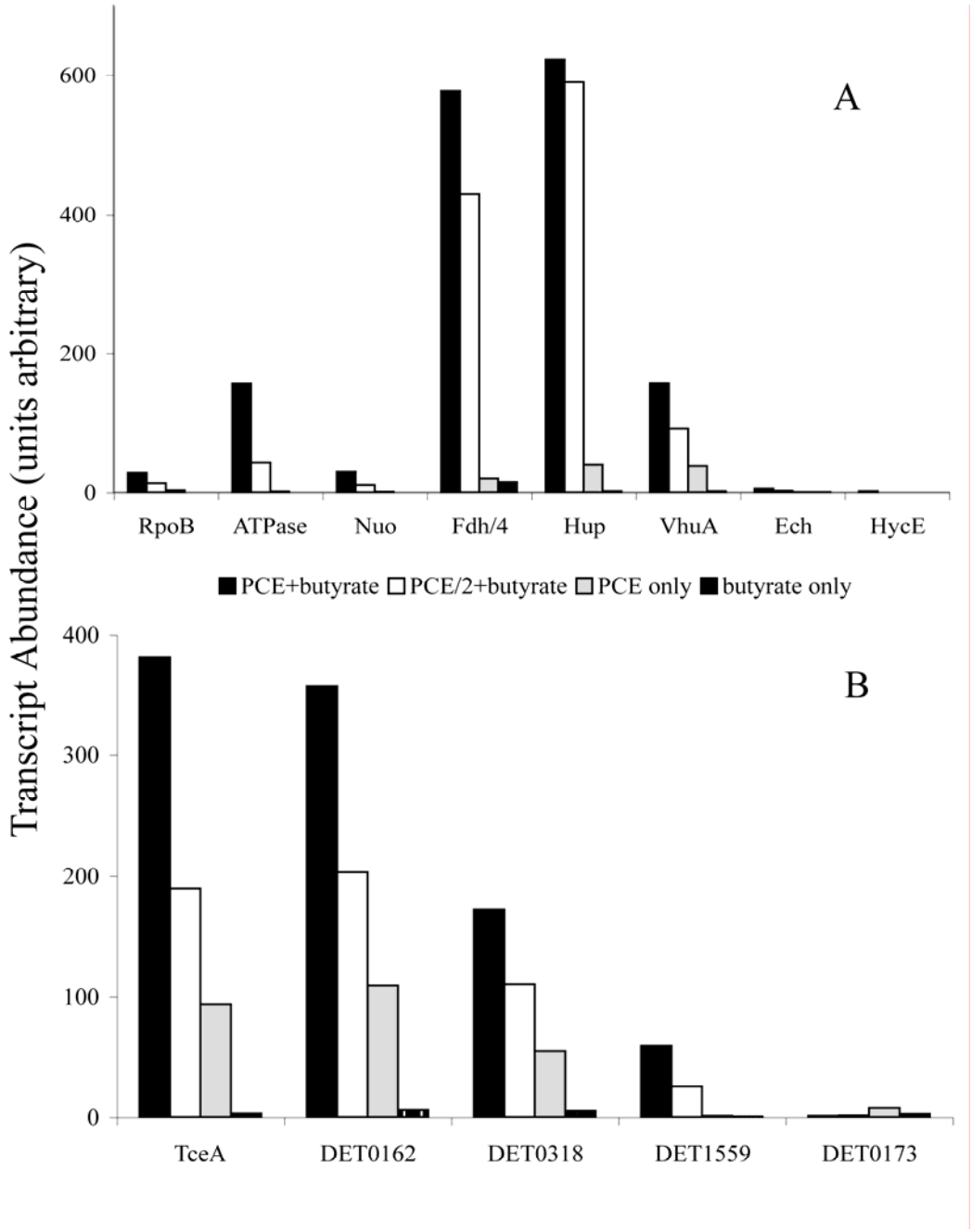


Figure A2.2. Average transcript abundance in amendment subcultures from 4 to 12 h (data from 0 to 4 h was excluded since transcription in all subcultures appeared to lag at time zero levels until 4 hours after amendment addition). Expression data is shown for (A) the housekeeper gene RpoB, ATPase, the oxidoreductase Nuo, the gene annotated as Fdh, and the H_2ases Hup, Vhu, Ech and HycE, as well as for (B) the RDase genes TceA, DET0162, DET0318, DET1559 and DET0173.

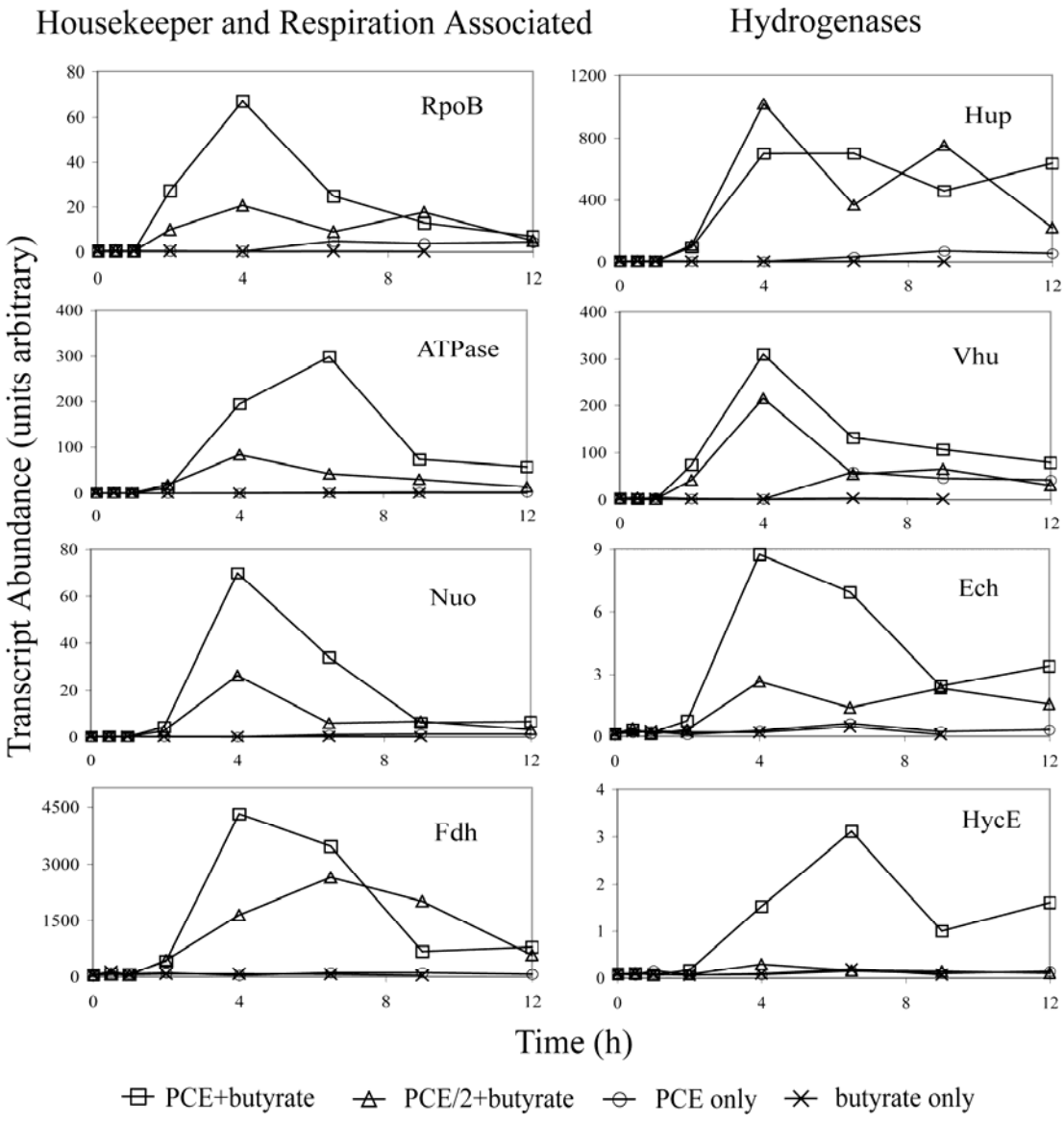


Figure A2.3. Timecourses of housekeeper, respiration associated and hydrogenase gene transcript abundance in amendment cultures.

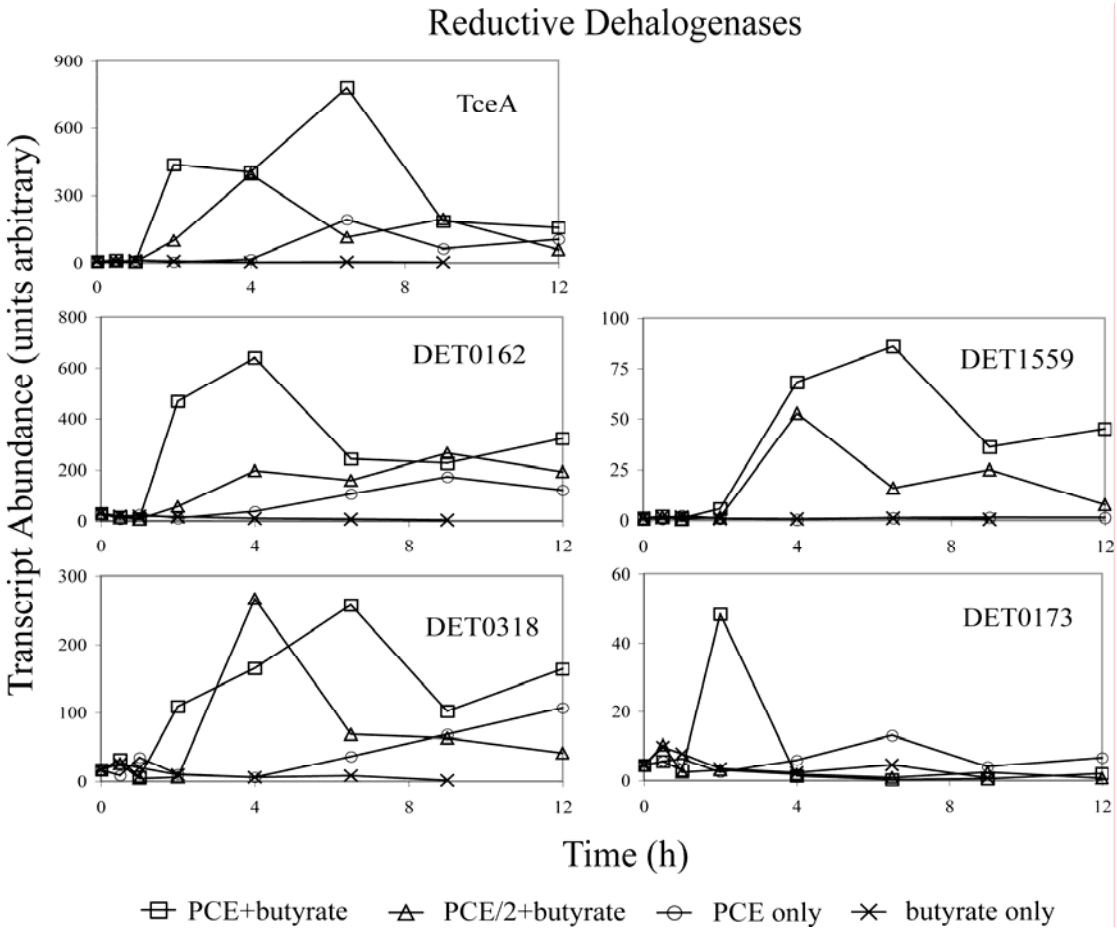


Figure A2.4. Timecourses of reductive dehalogenase gene transcript abundance in amendment cultures.

APPENDIX III

Supporting Timecourses for Chapter 2

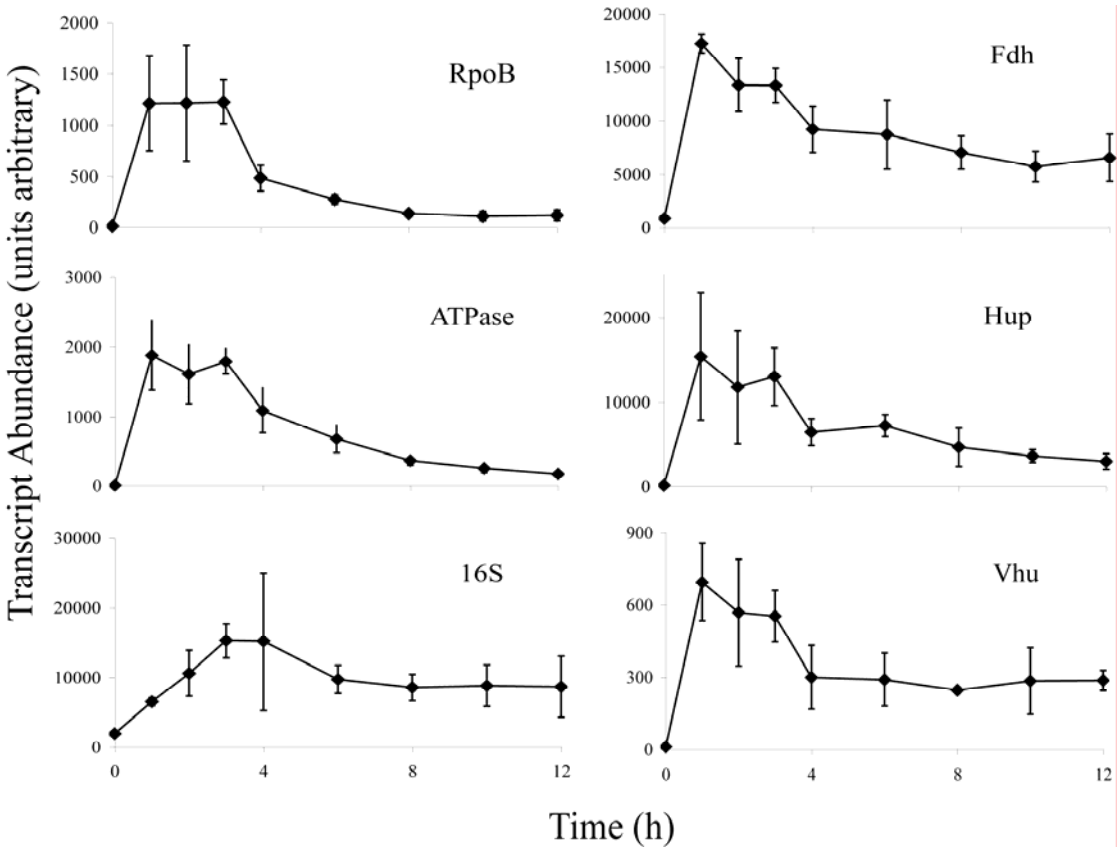


Figure A3.1. Timecourses of transcript abundance in triplicate D2 subcultures batch-fed PCE as described and summarized in Chapter 2. Data for gene transcripts, including RpoB, ATPase, 16S rRNA, Fdh and the H_2ases Hup and Vhu are presented normalized to luciferase reference RNA recovery in order to correct for mRNA losses incurred during sample processing and cDNA synthesis. Error bars represent standard deviations of triplicate cultures.

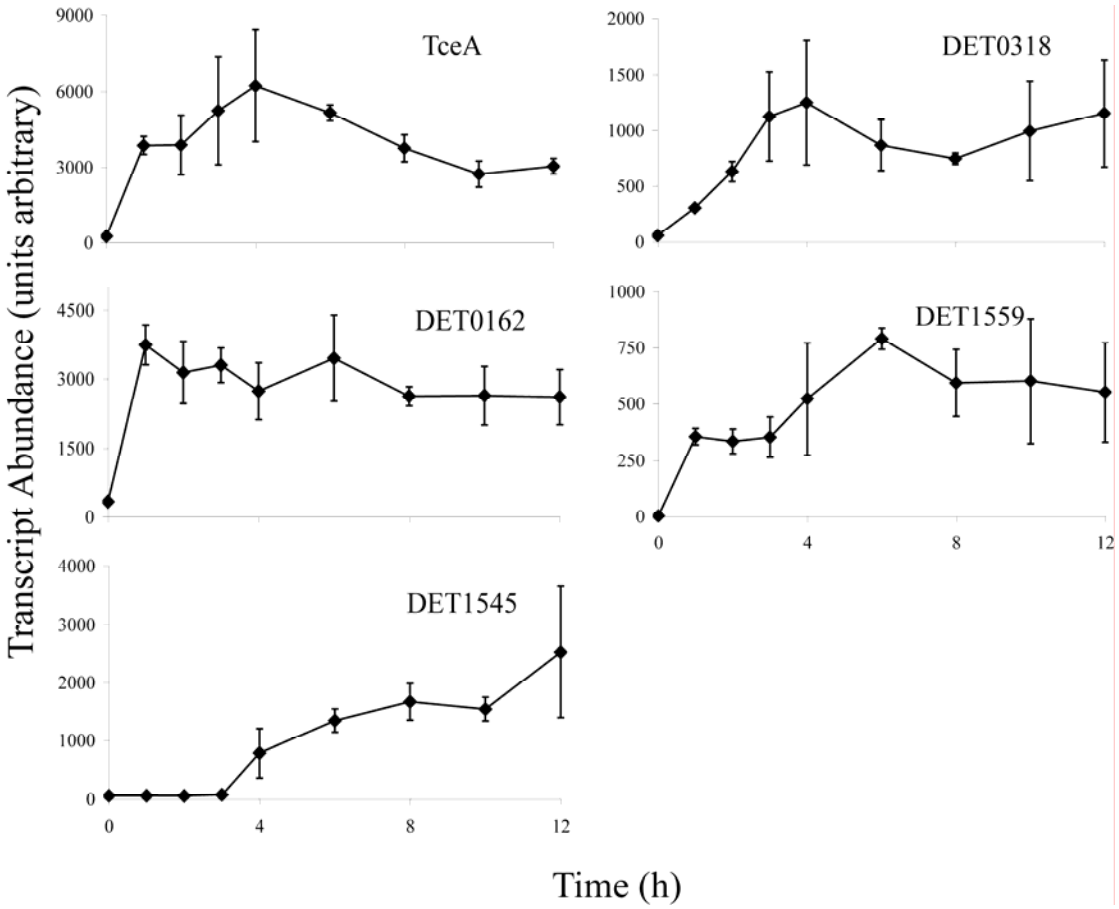
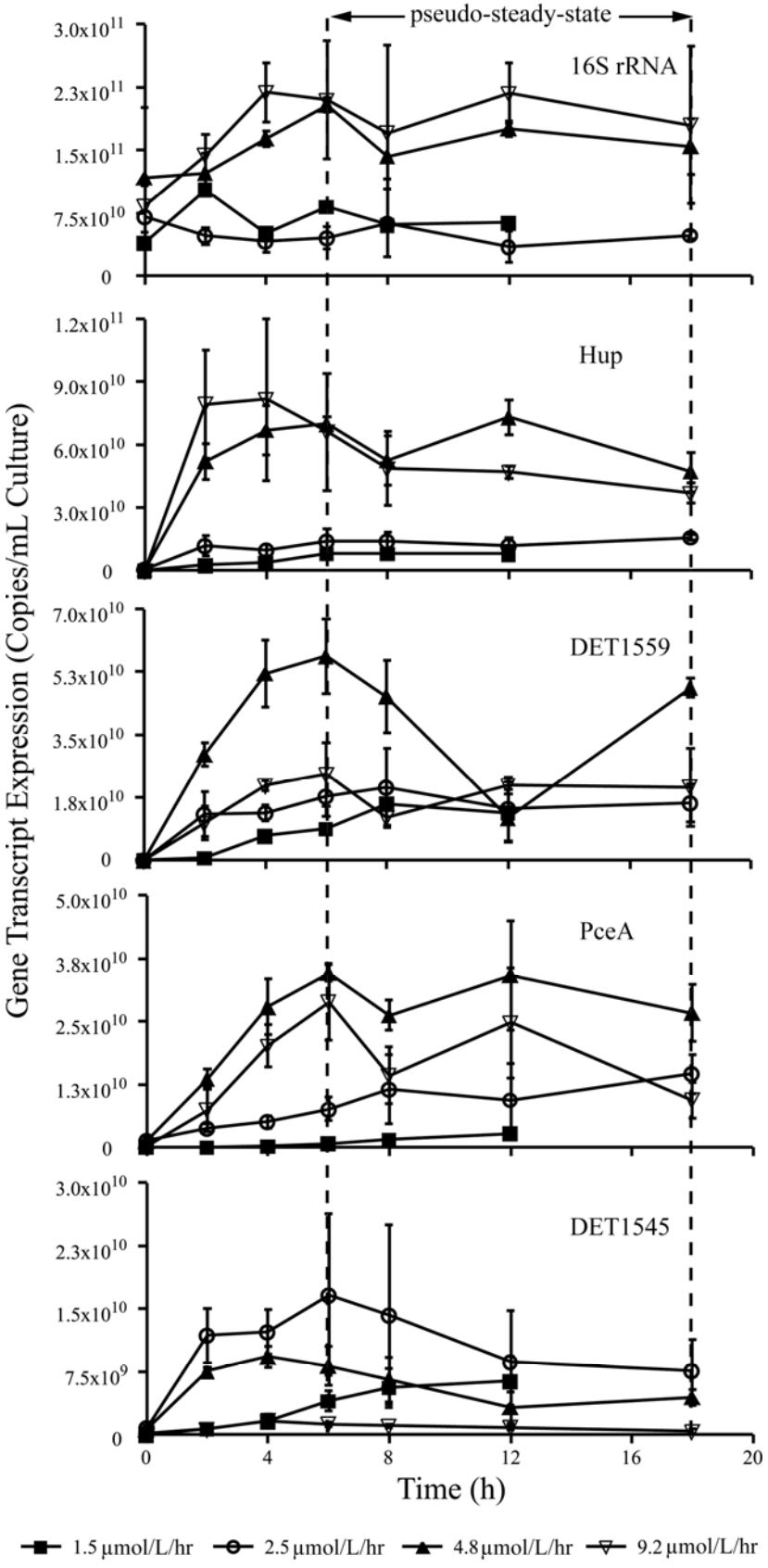


Figure A3.2. Timecourses of transcript abundance in triplicate D2 subcultures batch-fed PCE as described and summarized in Chapter 2. Data for gene transcripts, including RDases TceA, DET0162, DET1545, DET0318 and DET1559 are presented normalized to luciferase reference RNA recovery in order to correct for mRNA losses incurred during sample processing and cDNA synthesis. Error bars represent standard deviations of triplicate cultures.

APPENDIX IV

Supporting Timecourses for Chapter 3

Figure A4.1. Expression profiles of 16S rRNA and remaining functional transcripts over time. Hours 6 – 18 are identified as pseudo-steady-state. Error bars represent standard deviation of the mean of triplicate microcosms.


pseudo-steady-state
16S rRNA
Hup
DET1559
PceA
DET1545
Gene Transcript Expression (Copies/mL Culture)
Time (h)
1.5 µmol/L/hr
2.5 µmol/L/hr
4.8 µmol/L/hr
9.2 µmol/L/hr

APPENDIX V

Supporting Timecourses for Chapter 4

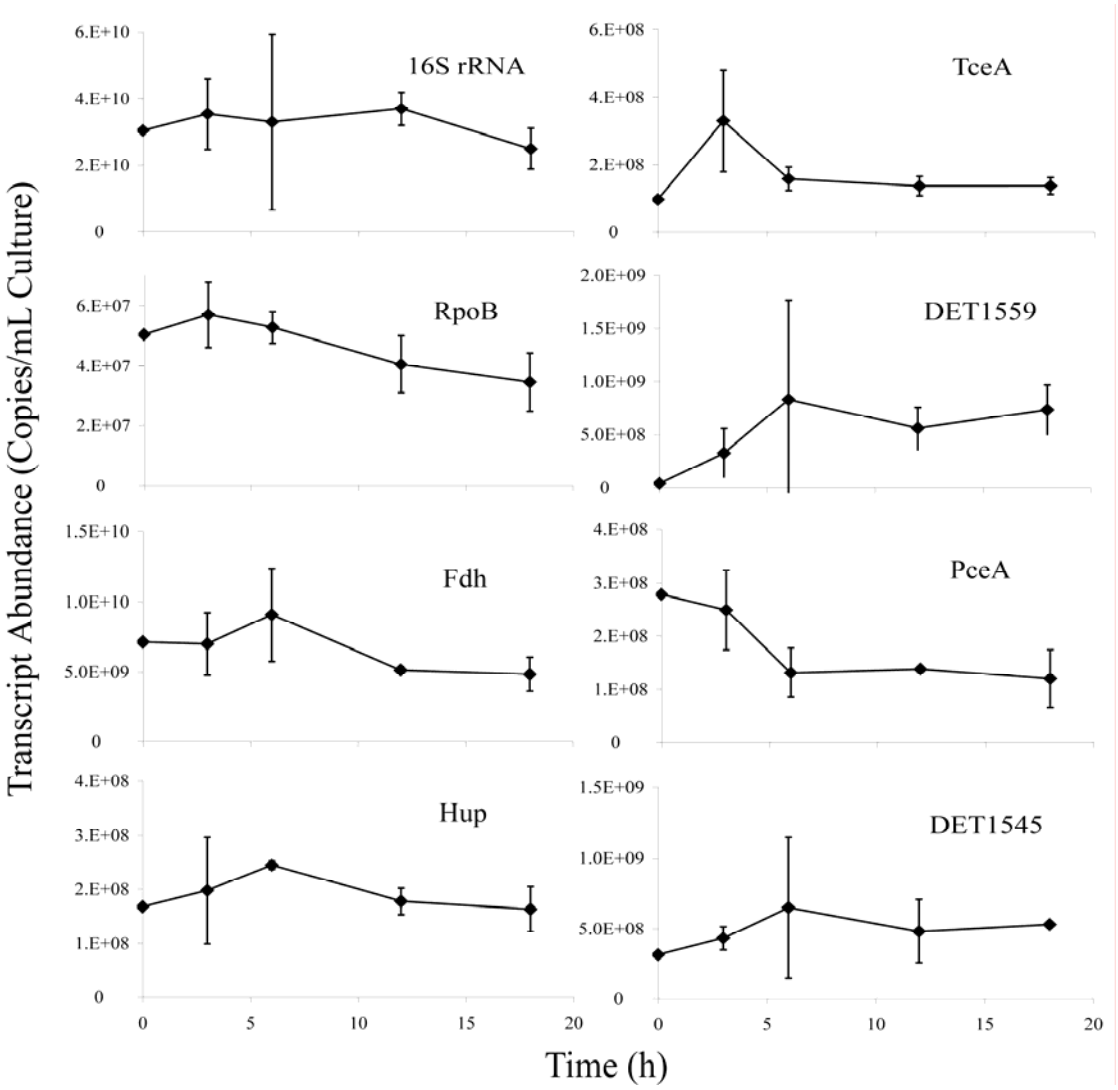


Figure A5.1. Expression profiles of bioindicator transcripts in PCElow microcosms respiring PCE at the rate of 2.1 ± 0.1 µeeq/L/hr (0.3 µmol/L/hr). Time-0 samples reflect culture conditions after 72 hours of starvation. Hours 6 – 18 are identified as pseudo-steady-state. Error bars represent standard deviation of the mean of triplicate microcosms.

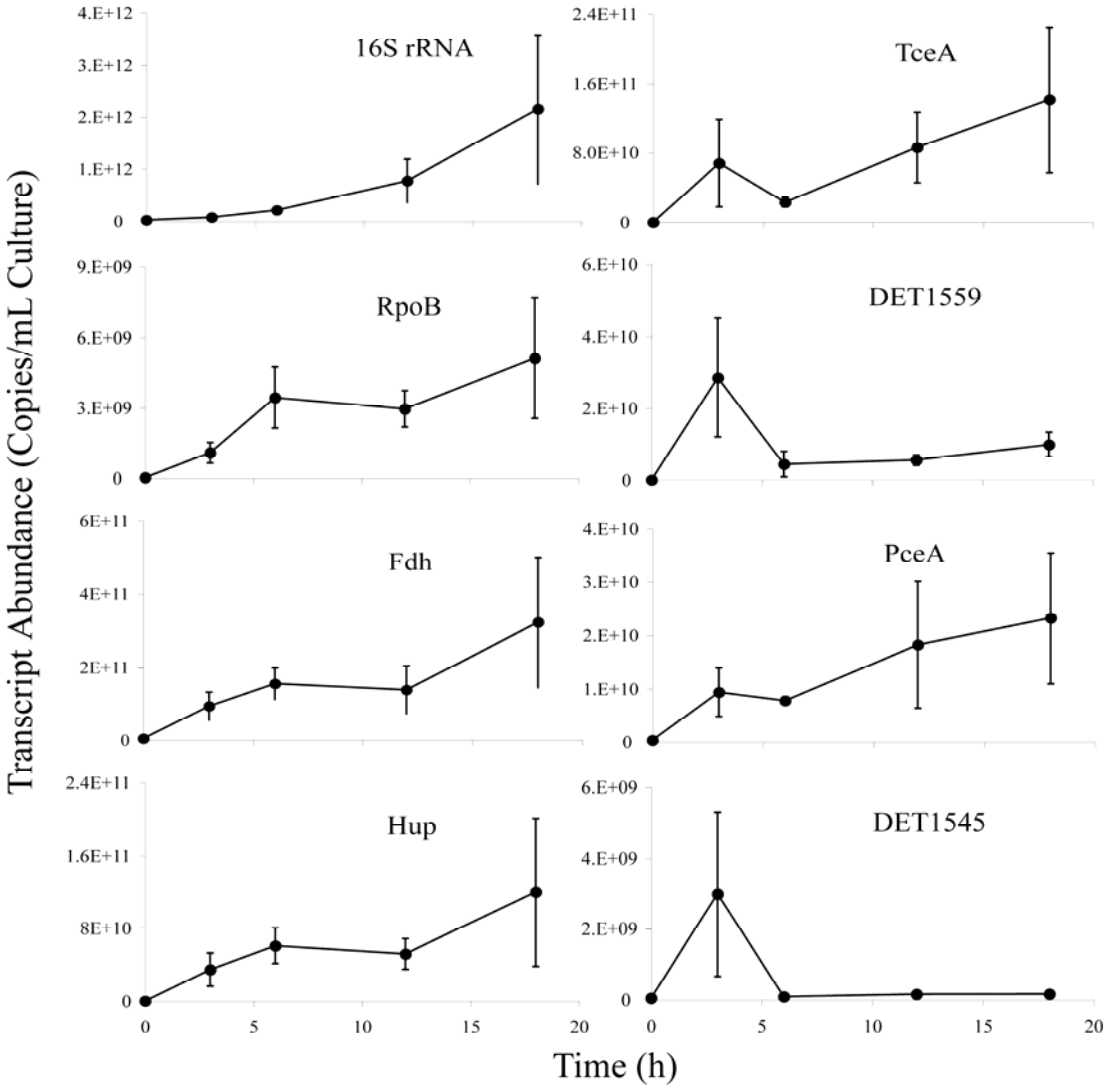
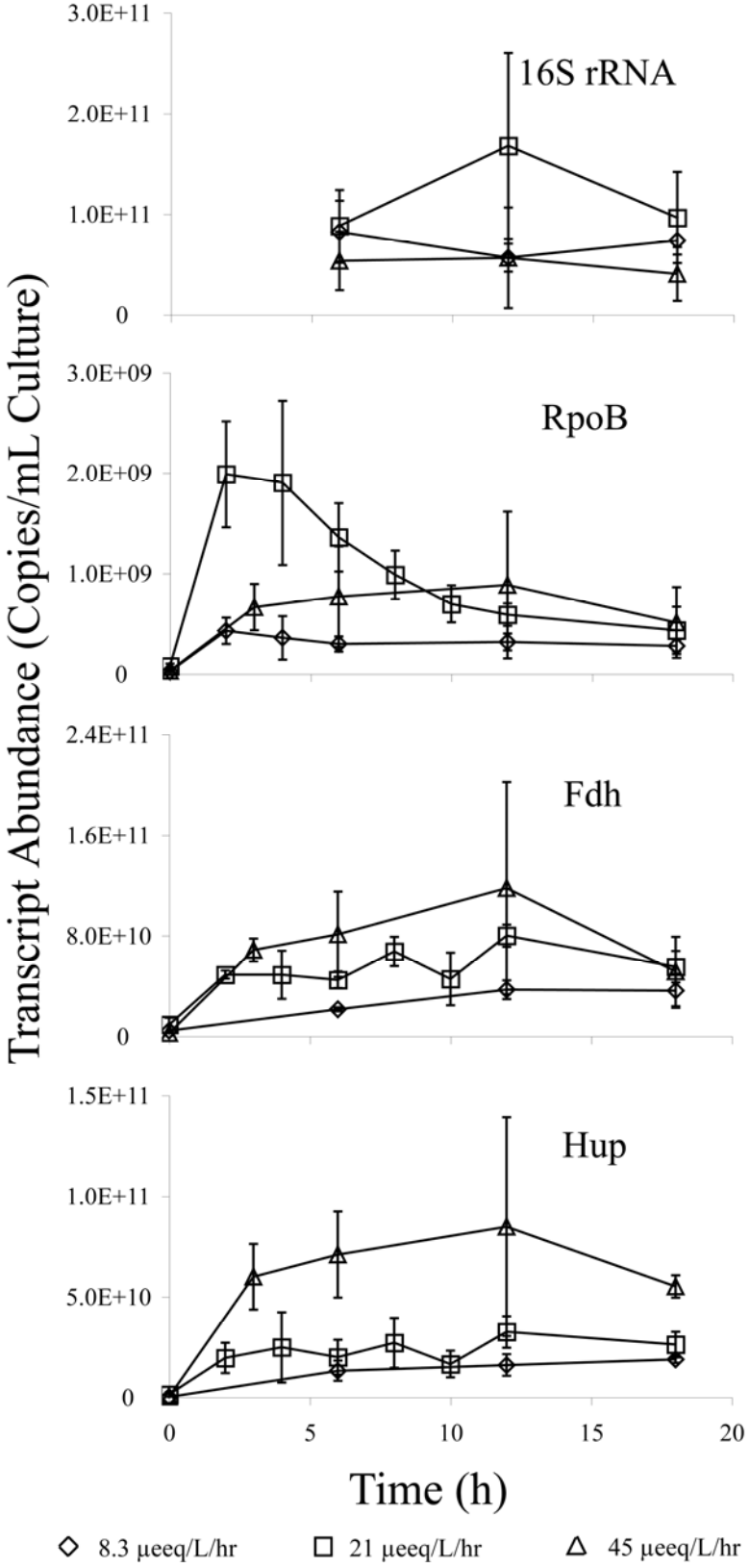


Figure A5.2. Expression profiles of bioindicator transcripts in PCEhigh microcosms respiring PCE at the rate of 370 ± 5.9 μeeq/L/hr (64 μmol/L/hr). Hours 6 – 18 are identified as pseudo-steady-state. Error bars represent standard deviation of the mean of triplicate microcosms.

Figure A5.3. Expression profiles of genes corresponding to (A) 16S rRNA, RpoB, Fdh and Hup and (B) RDases TceA, DET1559, PceA and DET1545 in TCE-fed microcosms respiring TCE at the rates of 8.3 ± 0.5, 21 ± 2.1 and 45 ± 2 μeeq/L/hr (1.9, 4.9 and 10 μmol/L/hr). Hours 6 – 18 are identified as pseudo-steady-state. Error bars represent standard deviation of the mean of triplicate microcosms.

A)


16S rRNA
RpoB
Fdh
Hup
Transcript Abundance (Copies/mL Culture)
Time (h)
3.0E+11
2.0E+11
1.0E+11
0
3.0E+09
2.0E+09
1.0E+09
2.4E+11
1.6E+11
8.0E+10
1.5E+11
5.0E+10
0
5
10
15
20
8.3 µeeq/L/hr
21 µeeq/L/hr
45 µeeq/L/hr

B)

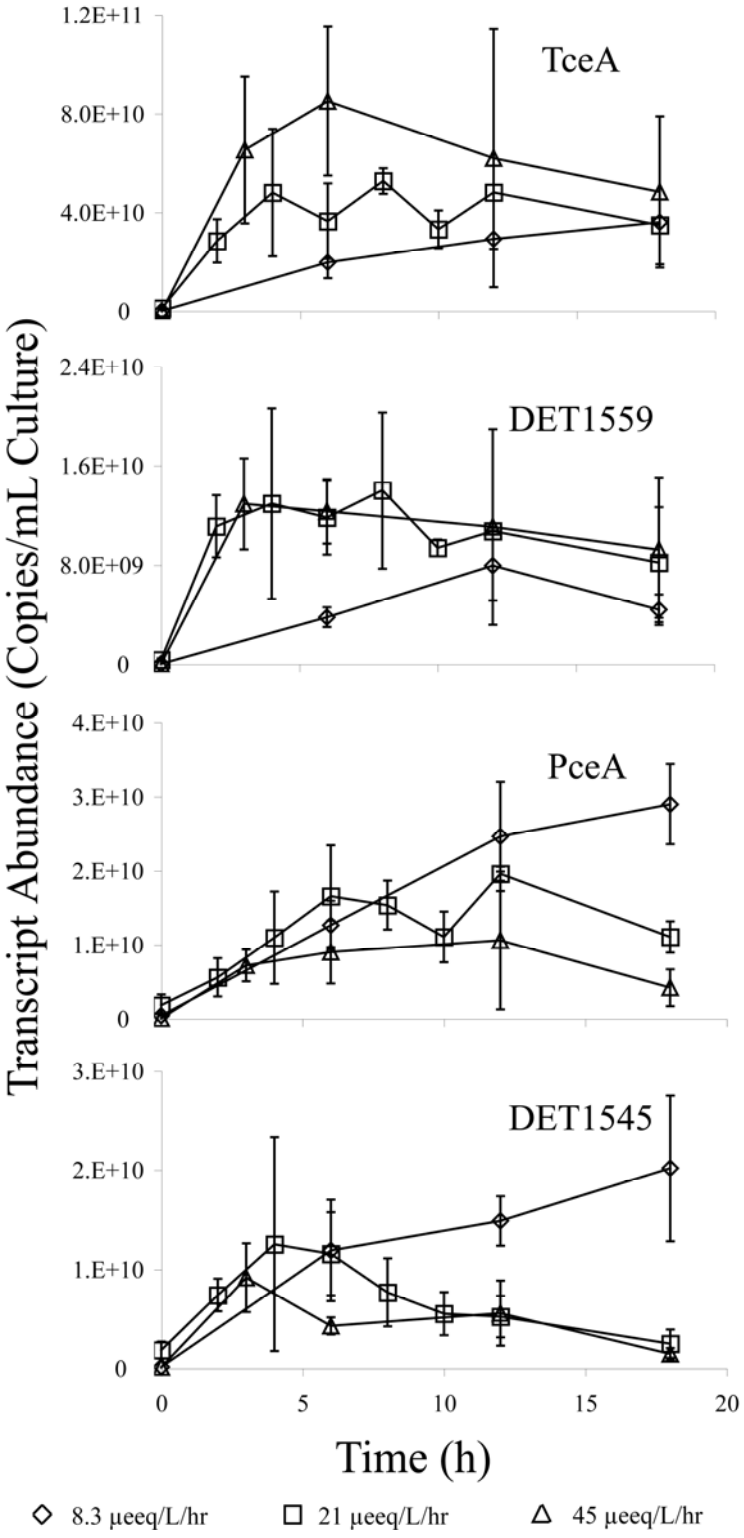
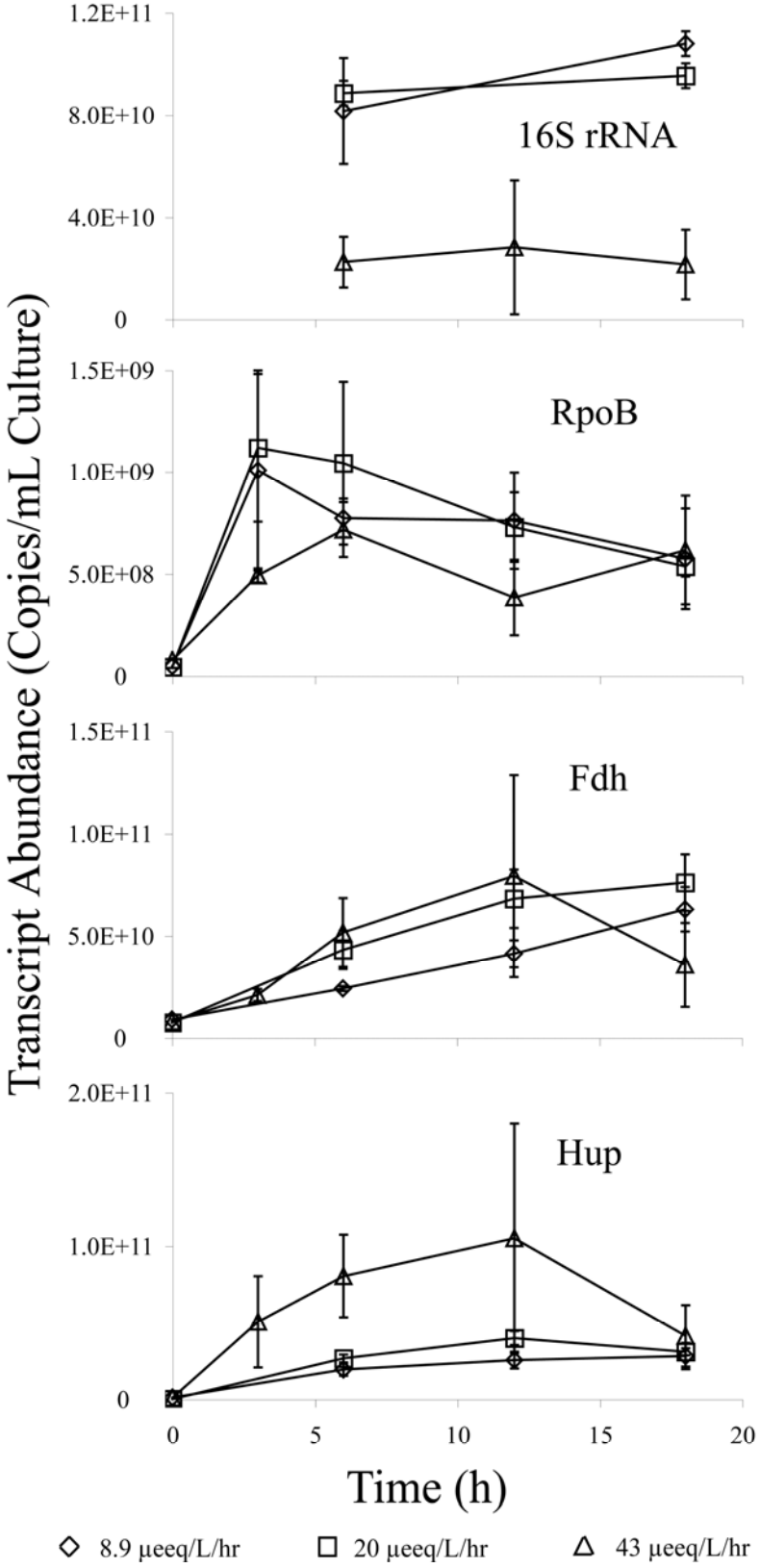
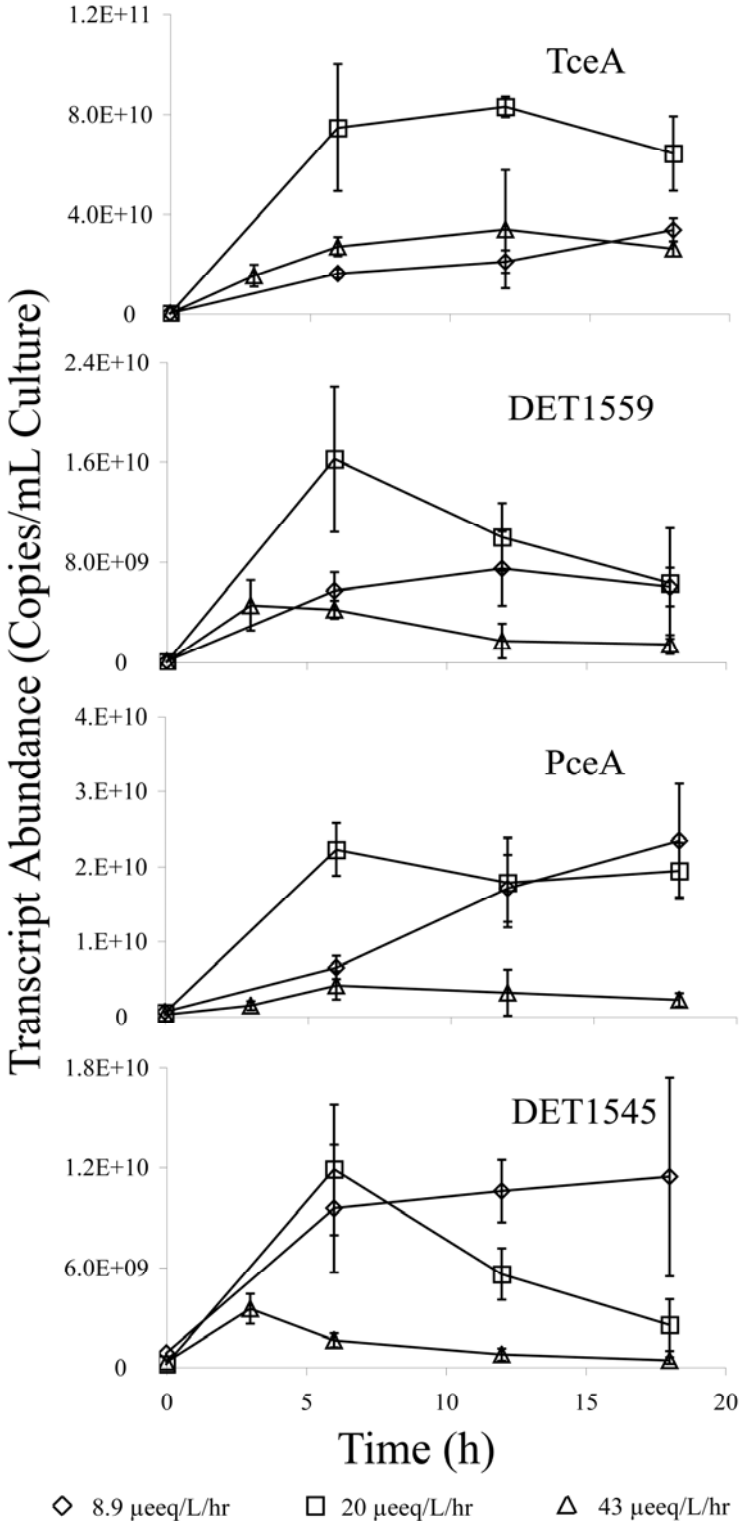

TceA
DET1559
PceA
DET1545
Transcript Abundance (Copies/mL Culture)
Time (h)
8.3 µeeq/L/hr
21 µeeq/L/hr
45 µeeq/L/hr

Figure A5.4. Expression profiles of genes corresponding to (A) 16S rRNA, RpoB, Fdh and Hup and (B) RDases TceA, DET1559, PceA and DET1545 in cDCE-fed microcosms respiring cDCE at the rates of 8.9 ± 0.1, 20 ± 0.9 and 43 ± 0.9 μeeq/L/hr (3.7, 8.5 and 19 μmol/L/hr). Hours 6 – 18 are identified as pseudo-steady-state. Error bars represent standard deviation of the mean of triplicate microcosms.

A)


1.2E+11
8.0E+10
4.0E+10
0
16S rRNA
1.5E+09
1.0E+09
5.0E+08
0
RpoB
1.5E+11
1.0E+11
5.0E+10
0
Fdh
2.0E+11
1.0E+11
0
Hup
Transcript Abundance (Copies/mL Culture)
0
5
10
15
20
Time (h)
8.9 µeeq/L/hr
20 µeeq/L/hr
43 µeeq/L/hr

B)


TceA
DET1559
PceA
DET1545
Transcript Abundance (Copies/mL Culture)
Time (h)
1.2E+11
8.0E+10
4.0E+10
0
2.4E+10
1.6E+10
8.0E+09
4.E+10
3.E+10
2.E+10
1.E+10
1.8E+10
1.2E+10
6.0E+09
0
5
10
15
20
8.9 µeeq/L/hr
20 µeeq/L/hr
43 µeeq/L/hr