Coupling Bioflocculation of *Dehalococcoides* to High-Dechlorination Rates for *Ex situ* and *In situ* Bioremediation

by

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ABSTRACT

Bioremediation of Trichloroethene (TCE) using *Dehalococcoides mccartyi*-containing microbial cultures is a recognized and successful remediation technology. Our work with an upflow anaerobic sludge blanket (UASB) reactor has shown that high-performance, fast-rate dechlorination of TCE can be achieved by promoting bioflocculation of *Dehalococcoides mccartyi*-containing cultures. The bioreactor achieved high maximum conversion rates of 1.63 ± 0.012 mmol Cl^- $L_{culture}^{-1}$ h^{-1} at an HRT of 3.6 hours and >98% dechlorination of TCE to ethene while fed 2 mM TCE. The UASB generated bioflocs from a microbially heterogeneous dechlorinating culture and produced *Dehalococcoides mccartyi* densities of $1.73x10^{-13}$ cells $L_{culture}^{-1}$ indicating that bioflocculation of *Dehalococcoides mccartyi*-containing cultures can lead to high density inocula and high-performance, fast-rate bioaugmentation culture for in situ treatment. The successful operation of our pilot scale bioreactor led to the assessment of the technology as an onsite ex-situ treatment system. The bioreactor was then fed TCE-contaminated groundwater from the Motorola Inc. 52nd Street Plant Superfund site in Phoenix, AZ augmented with the lactate and methanol. The bioreactor maintained >99% dechlorination of TCE to ethene during continuous operation and maximum conversion rates of 0.47 ± 0.01 mmol Cl^- $L_{culture}^{-1}$ h^{-1} at an HRT of 3.2 hours. These rates exceed those documented for commercially available dechlorinating cultures. Microbial community analysis under both experimental conditions reveal shifts in the community structure although maintaining high rate dechlorination. High density dechlorinating cultures containing bioflocs can provide new ways to 1) produce dense bioaugmentation cultures, 2) perform ex-situ bioremediation of TCE, and 3) increase our understanding of

Dehalococcoides mccartyi critical microbial interactions that can be exploited at contaminated sites in order to improve long-term bioremediation schemes.

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CHAPTER 1

INTRODUCTION

Chlorinated solvents are prevalent groundwater contaminants across the US with trichloroethene (TCE) being the most widely detected organic chemical at National Priority List (NPL) sites. According to the US EPA total on- and off-site release of PCE and TCE was 4 million and 11 million pounds, respectively from 1998 to 2001 (U.S. Environmental Protection Agency, 2003). Trichloroethene (TCE), perchloroethene (PCE), and the lower chlorinated ethene vinyl chloride (VC) rank 16th, 33rd and 4th, respectively on the EPA's National Priority List of Hazardous Substances. These chemicals are known or potential carcinogens, and pose multiple other health hazards (ATSDR 2013).

In situ remediation has been increasing in favor over pump and treat systems which have higher energy input demands. Within this category, bioremediation is one of the most frequently selected remediation technologies and has been increasing in favor (Table 1). Organohalide respiring bacteria that can reductively dechlorinate PCE and TCE to ethene have been effectively explored for bioremediation of chlorinated solvent contaminated sites. In situ bioremediation through bioaugmentation has proven to be a successful and environment-friendly remediation technique (US EPA, 2004; Interstate Technology & Regulatory Council, 2007; Ellis et al. 2000; Lendvay et al. 2003; Major et al. 2002).

Table 1 Groundwater remedy technologies selected in decision documents. Adapted from the Superfund Remedy Report, 14th Edition.

Technology	Percentage of Groundwater Decision Documents (FY 2005-2008)	Percentage of Groundwater Decision Documents (FY 2009-2011)
Pump and Treat	26%	22%
Groundwater Pump and Treat	25%	21%
Surface Water Collect and Treat	2%	<1%
In Situ Treatment	30%	38%
Bioremediation	19%	24%
Chemical Treatment	12%	14%
Air Sparging	3%	6%
Permeable Reactive Barrier	2%	4%
In-Well Air Stripping	0%	1%
Multi-Phase Extraction	<1%	1%
Phytoremediation	1%	0%
Fracturing	<1%	0%
MNA of Groundwater	36%	27%
Groundwater Containment (VEB)	5%	3%

Bioaugmentation involves the delivery of microbial consortia, as well as electron donor, pH buffering agents and other nutrients. Of these bacteria, the genus Dehalococcoides has been identified as crucial for bioremediation of chlorinated ethenes for their capability of complete dechlorination to ethene (He et al. 2003; He et al. 2005; Maymo-Gatell et al. 1997; Sung et al 2006). However, microbial consortia are required to create optimal growth conditions for the propagation Dehalococcoides and, therefore, to achieve complete dechlorination. Dehalococcoides are corrinoid auxotrophs and rely on corrinoid producing methanogenic Archaea and acetogenic Bacteria for growth (He et al. 2007; Yan et al. 2013; Yan et al. 2012). Fermenters provide acetate and H2 from

fermentable substrates; H2 is the only energy source utilized by Dehalococcoides whereas acetate is their carbon source. Acetogens can also provide acetate. As such, bioaugmentation with these mixed cultures provides more favorable conditions for effective in situ dechlorination where the indigenous microbial community may not favor complete dechlorination.

When performing bioaugmentation, inoculum persistence at the contaminated site directly affects the effective dechlorination achieved. Cell immobilization at the soil or sediment close to the contaminant source may increase effectiveness and reduce long lag periods preceding biodegradation (El Fantroussi & Agathos, 2005). Few publications report that Dehalococcoides can grow immobilized on a surface in multispecies biofilms (Popat & Deshusses, 2011; Ziv-El et al, 2012). Furthermore, little information is available on their capacity to aggregate and form bioflocs and how multispecies bioflocs can help promote high-performance dechlorination. Rowe et al. (2008) studied the distribution of Dehalococcoides and methanogens in bioflocs and postulated that the spatial distribution of the microorganisms likely proved beneficial for both because there is greater accessibility to key metabolites. Aggregation of these syntrophic communities can also provide robust cell-dense inocula for bioremediation, both in the laboratory, as well as in contaminated soils and sediments, resulting in faster higher rates of dechlorination. Our study focused on understanding biofloc formation by Dehalococcoides dechlorinating communities in a bioreactor and implications for bioremediation.

Delgado et al (2014a) showed that high density, high performance dechlorinating cultures can be produced in continuous reactors at HRTs as low as 3 days. Continuous

reactors can provide optimal growth conditions if high concentrations of chlorinated electron acceptors are coupled with high removal rates in order to stimulate growth but prevent inhibition due to toxicity. Upflow anaerobic sludge blanket (UASB) reactors are widely used to treat municipal and industrial wastewaters due to their great efficiency and short retention time. This is attributed to their high biomass retention due to the upflow design which also facilitates the aggregation of key microorganisms. Therefore, we hypothesized that a continuous upflow reactor could not only support high-performance dechlorination but also promote the aggregation of dechlorinating community members to form bioflocs.

In this study, Dehalococcoides mccartyi-containing bioflocs were generated in an upflow bioreactor. The reactor was modeled after a typical UASB but included a recycle stream to reintroduce washed-out biomass, which allowed for high-cell retention, leading to increased cell density aiding bioflocculation and high dechlorination rates. Past studies have explored the use of UASB reactors for bioremediation of PCE and TCE in a synthetic wastewater feed. However, these studies have shown incomplete dechlorination, often with residual PCE/TCE in the effluent and cis-DCE and vinyl chloride (VC) being the dominant products of reduction (Zhang, Wang, et al. 2015; Prakash and Gupta 2000; D. T. Sponza 2003; Hwu and Lu 2008; Ozdemir, et al. 2007; Tresse, et al. 2005). To our knowledge, UASB systems have not been employed to remediate TCE- or PCE-contaminated groundwater. Furthermore, no studies have been published which show >99% dechlorination of TCE to ethene in a UASB. In Phase I we focused on producing high-density culture capable of high conversions (i.e., >95%) of TCE to ethene by providing high concentrations of TCE (2 mM) and synthetic

groundwater. In Phase II, we explored the ability of the system to maintain high conversions of TCE when fed a continuous stream of contaminated groundwater (0.5 mM TCE).

CHAPTER 2

MATERIALS AND METHODS

2.1 Upflow Bioreactor Design

A schematic and photograph of the bioreactor is presented in Figure 1. The bioreactor was constructed using a 100-mL glass tube of 27 cm height. Butyl rubber stoppers were used to seal the bioreactor. These were drilled and fitted with a stainless steel for an effluent line at the top, and an influent line and sludge dispense line at the base of the bioreactor. Viton tubing and stainless steel connectors were used. The effluent line at the top of the bioreactor was split with a T-connector dividing the effluent between a 1-L collection bottle fitted with a 1-L Tedlar bag, and the recycle stream which was fed back into the bioreactor as seen in Figure 1. A Masterflex Easy-Load II (Cole-Parmer, Vernon Hills, IL) peristaltic pump maintained recirculation at 30 mL/min, an upflow fluid velocity of 8.1 cm/min. The influent line at the base of the bioreactor was split with a T-connector and fed from the medium bottle and the recycle stream. A 20-mL glass vial fitted with a butyl rubber stopper was connected before the medium inlet where 6 mL were maintained so that gas samples representative of the bioreactor constituents could be taken. An analog peristaltic pump (Ismatec, Wertheim, Germany) fed medium from a 5-L glass bottle fitted with a 3-L tedlar bag, to the influent line.

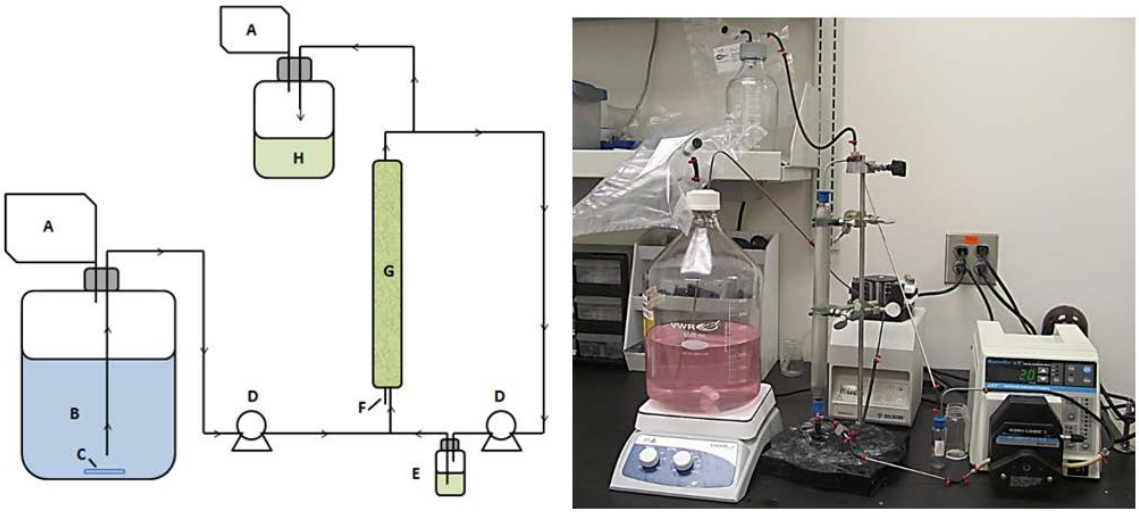

Figure 1 (L) Schematic showing reactor setup A, gas bag; B, medium bottle; C, magnetic stir bar; D, peristaltic pump; E, sampling vial; F, biofloc dispense line; G, UASB; H, effluent bottle.(R) Photograph of reactor setup.

2.2 Inoculum

At startup, the bioreactor was inoculated with 20 mL of ZARA-10 dechlorinating culture, 1 mL of anoxic digester sludge obtained from the Northwest Water Reclamation Plant (NWWRP) in the City of Mesa, AZ and 100 mL of reduced anaerobic mineral medium. ZARA-10 is a mixed dechlorinating culture enriched in *Dehalococcoides*, fermenting and homoacetogenic bacteria and hydrogenotrophic methanogens (Delgado et al. 2013, Delgado et al. 2014). The anoxic digester sludge was previously described by Parameswaran et al. (2009). The reduced anaerobic mineral medium was comprised of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM NaHCO3 and salts and trace minerals as described in Delgado et al. (2013), 500 µg L -1 Vitamin B12, 5 mL L -1 Wolfe’ s vitamins solution (Wolin et al. 1964), 0.4 mM L-cysteine, 0.2 mM

Na2S· 9H2O, and 0.25 µg L -1 resazurin and amended with 15 mM methanol and 5 mM sodium DL-lactate.

2.3 Bioreactor Operation

2.3.1 Bioreactor Startup

Following inoculation with the microbial cultures, the bioreactor ran continuously for 112 days in order to promote biofloc formation and determine optimal operating parameters. Once bioflocs were established, the bioreactor was kept in batch mode with the recycle stream running at 10 mL min-1 for two weeks during which time it was batch-fed 1 mM TCE, 15 mM methanol and 5 mM lactate every 7 days.

2.3.2 Phase I: Synthetic Groundwater Remediation under Optimal Growth Conditions

Synthetic groundwater was made using the aforementioned technique for reduced anaerobic mineral media with 10 mM sodium DL-lactate and 15 mM methanol. The upflow recycle stream was increased to 55 mL min-1. The bioreactor was operated continuously for a total of 24 days in this phase, which corresponds to a total of 85 HRTs. The starting HRT was 10.8-hr. Once pseudo-steady state was achieved in which conversion of ethene stabilized, the HRT was reduced to 7.2-hr and, finally, to a 3.6-hr HRT.

2.3.3 Phase II: Dechlorination of Contaminated Superfund Site Groundwater

Groundwater from the Motorola 52nd Street Superfund Site in Phoenix, AZ was withdrawn from the inlet of RID-92 GAC Wellhead Treatment System. The groundwater

was amended with 0.5 mM TCE, 10 mM HEPES and electron donors lactate and methanol at concentrations of 5 mM and 7.5 mM, respectively. In addition the following nutrients were added to the groundwater: 200 mg/L yeast extract, 50 ug/L vitamin B12, 500 uL/L Wolfe's vitamin solution and 0.2 mM sodium sulfide. The reactor was operated continuously for 43 days starting at an HRT of 12.0 h which was progressively reduced to 9.1 h, 6.1 h and finally 3.2 h. This phase of the experiment ran for a total of 269 HRTs.

Table 2 Chemical analyses of groundwater

Contaminant of Concern	Value[1] (μg/L)	Ion	Value[2]	Unit
Trichloroethene (TCE)	73.6 ± 3.9	Bicarbonate (HCO_3)	180.2	mg/L CaCO3
Perchloroethene (PCE)	13.8 ± 0.7	Sulfate (SO_4)	72.8	mg/L CaCO3
1,1-Dichloroethene (1,1-DCE)	4.6 ± 0.7	Nitrate (NO_3)	8.97	mg/L CaCO3
Chloroform (TCM)	3.1 ± 0.1	Chloride (Cl)	381	mg/L CaCO3
1,1-Dichloroethane (1,1-DCA)	1.8 ± 0.2	Phosphate (PO_4)	<0.080	mg/L CaCO3
1,2-Dichloroethene (cis-1,2-DCE)	7.5 ± 0.5	Iron (Fe)	0.067	mg/L

2.4 Chemical Quantification by Gas Chromatography

TCE, cis-DCE, vinyl chloride, ethene and methane gas concentrations were quantified using a gas chromatograph with a flame ionization detector, GC-FID (GC-2010, Shimadzu). Helium carrier gas eluted the sample in an Rt-QS-BOND capillary column (Restek, Bellefonte, PA) via a temperature gradient. The operating parameters of the GC-FID were as previously described Delgado et al. (2012). Henry's constants had

been previously determined experimentally (Delgado et al. 2013) and were used to determine liquid concentrations for these compounds.

2.5 High-Performance Liquid Chromatography to Track Fermentable Substrates

Liquid samples were taken along the course of the experiment in order to monitor consumption of methanol and lactate and production and of acetate and propionate. 1 mL of culture was sampled from the effluent line of the UASB and filtered through 0.2 µL polyvinylidene fluoride membrane filter (Acrodisc LC 13 mm Syringe Filter with PVDF Membrane, Pall). The concentrations of the aforementioned compounds were measured using a high performance liquid chromatograph, HPLC (LC-20AT, Shimadzu). The HPLC column (Aminex HPX-87H, Bio-Rad) was maintain at 50˚C with a 2.5 mM sulfuric acid eluent at a flow rate of 0.6 mL/min.

2.6 Microbial Ecology Analysis

2.6.1 DNA Extraction

Biomass pellets were made from samples of effluent culture and sludge at each HRT. Effluent pellets were made from 500 µL samples from the effluent line. Pellets from sludge were made by collecting 500 µL cell suspensions from the base of the UASB which were then resuspended in 1 mL of sterile reduced anaerobic mineral medium. The aliquots were then divided equally to make triplicate pellets. DNA was extracted from these biomass pellets as previously described (Ziv-El et al. 2011).

2.6.2 Quantitative real-time PCR (qPCR)

We performed qPCR targeting the 16S rRNA gene of *Dehalococcoides mccartyi* and Archaea, the functional gene of homoacetogens, formyltetrahydrofolate synthase (FTHFS) and the reductive dehalogenase genes of D. mccartyi, i.e. tceA, bvcA and vcrA. The primer, probes, reagent concentrations, and PCR conditions were described previously (Ziv-El et al. 2011). *Dehalococcoides mccartyi* were enumerated by assigning one copy of the 16S rRNA gene to one *Dehalococcoides* cell (Ritalahti et al. 2006).

2.6.3 High-throughput microbial community analysis

The microbial community amplicon sequencing was performed using the Illumina MiSeq platform using Bacterial primers 515F and 806R to amplify the V4 hyper-variable region of the 16S rRNA gene (Caporaso et al 2012). The reads were pair-end and each end of the DNA fragment consisted of 150 bp (2 x 150). Library preparation was performed according to the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). Forward and reverse reads were paired using PANDASeq (Masella et al. 2012). QIIME 1.8.0 pipeline was used to process raw sequences (Caporaso et al., 2010). Sequences were submitted to NCBI Sequence Read Archive and are available under the accession numbers: SAMN03784691, SAMN03784692, SAMN03784693, SAMN03784694, SAMN03784695, SAMN03784696, SAMN03783052, SAMN03783053, SAMN03783054, SAMN03783055, SAMN03783056, SAMN03783005.

Sequences with at least one of the following characteristics were omitted for the downstream analysis: shorter than 250 bps, quality score of 25 or below, 1 or more

primer mismatches, more than 6 homopolymers. From the sequences that passed the quality filtering, OTUs were picked based on 97% sequence similarity, using the uClust algorithm (Edgar 2010). The most abundant sequence of each cluster was picked as the representative sequence. PyNAST was used for alignment of sequences (Caporaso et al. 2010b) to Greengenes database (DeSantis et al., 2006). We used Chimera Slayer (Haas et al., 2011) to identify chimeric sequences for their following removal by filtering. In order to construct BIOM formatted OTU table, we used uClust (Edgar 2010) to assign taxonomy to Greengenes database (DeSantis et al., 2006). We removed OTUs with single sequences (singletons) from the OTU table. To avoid biases that occur when sampling various species in a community, we subsampled (rarefied) the OTU table using the pseudo-random number generator (PRNG) NumPy's, an implementation of the Mersenne PRNG (Matsumoto and Nishimura, 1998).

2.6.4 Scanning Electron Microscopy Imaging

Scanning electron microscope images were taken of bioflocs dispensed at the base of the UASB during Phase I and washed with 3X phosphate-buffered saline solution. Fixation, gradual dehydration and critical point drying in preparation for SEM imaging was performed by David Lowry of the Electron Microscopy Laboratory at ASU (Tempe, AZ) as previously described (Ziv-El et al. 2012). Once mounted on stubs and sputter-coated with gold-palladium, images were captured by a XL-30 Environmental Scanning Electron Microscope with a Schottky Field Emission Source.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Bioreactor Facilitates Fast-Rate Conversion to Ethene

The dechlorination performance of the bioreactor is shown in Figure 2 as aqueous concentration calculated from gas sampling in the headspace of the sampling vial. Percent conversions to ethene were calculated from the measured concentration of chlorinated ethenes and ethene as follows:

$$\%\ Conversion\ to\ ethene = \frac{[ethene]}{[TCE] + [cis - DCE] + [VC] + [ethene]} \times 100$$

For Phase I the influent TCE concentration was maintained at 2 mM and within 4 days of continuous mode operation, the reactor achieved high rates of dechlorination (i.e., >95% conversion to ethene). As seen in Figure 2, the reactor remained in pseudo-steady state (defined here as steady rates of conversion to ethene) for the remaining 20 days of continuous operation. An average conversion of TCE to ethene of 98.0% was maintained as the HRT was sequentially reduced from 10.8-h, to 7.2-h and finally 3.6-h (Table 2).The decrease in HRT to 3.6-h did not result in a detectable decline in dechlorination activity, as seen in Figure 2. At this HRT, we report a remarkably high maximum conversion rate (R_{max}) for TCE of 1.63 $\pm$ 0.012 mmol Cl^- L^{-1} h^{-1} (Table 2). This is thirteen times higher than previously reported values for *Dehalococcoides* cultures grown in batch or continuous reactors (Delgado et al. 2013; Ziv-El et al. 2011;). Previous studies have shown a significant decline in dechlorination with decreasing HRT to ≤1 d in UASB reactors (Zhang, Wang, et al. 2015; Prakash and Gupta 2000; D. T. Sponza 2003; Hwu and Lu 2008).

As the reactor was transitioned from Phase I, synthetic groundwater with 2 mM TCE, to Phase II with actual groundwater and 0.5 mM TCE there was no discernable decline in dechlorination performance. As can be seen in Figure 2 dechlorination performance in this phase was maintained at >99% conversion to ethene as the HRT was decreased proving the system to be robust. At a 3.2 h HRT the reactor achieved an Rmax for TCE of 0.47 $\pm$ 0.006 mmol Cl^{-} L^{-1} h^{-1}. While steady-state CSTR systems are limited by the ability of the rate of biomass proliferation we achieved successful dechlorination in HRTs shorter than the previously reported doubling times of 2.2 days for strain BAV1 and 2-2.5 days for strain GT (Loffler et al. (2013) in a continuous system. We attribute this to the provision of high concentrations of the electron acceptor TCE under optimal growth conditions combined with high biomass retention.

Table 3 Dechlorination performance during both Phases according to maximum conversion rate and average conversion to ethene.

	HRT (h)	Duration (h)	R_{max} (mmol Cl^{-} L^{-1} h^{-1})	Average Conversion to Ethene (%)
PHASE I (2 mM TCE)	10.8	254	0.54 ± 0.01	98
	7.2	174	0.82 ± 0.01	98
	3.6	136	1.63 ± 0.01	98
PHASE II (0.5 mM TCE)	12.0	282	0.13 ± 0.00	100
	9.1	333	0.16 ± 0.00	100
	6.1	219	0.25 ± 0.00	100
	3.2	552	0.47 ± 0.01	100

As the HRT decreased during both Phases we see a decline in the total mass balance. Given the 1:1 molar ratio between TCE and the products of reductive dechlorination the total molar concentration of these compounds should equate to the

concentration of TCE fed. However the high rate of biogas production in the reactor particularly at the lower HRTs resulted in greater stripping of these volatile compounds into the gas phase. Furthermore as the HRT was decreased, methane production increased (Figure 3) which we speculate resulted in the dilution of ethene in the headspace. As such the calculated aqueous concentration of ethene declined with HRT and does not equate to the concentration of TCE fed.

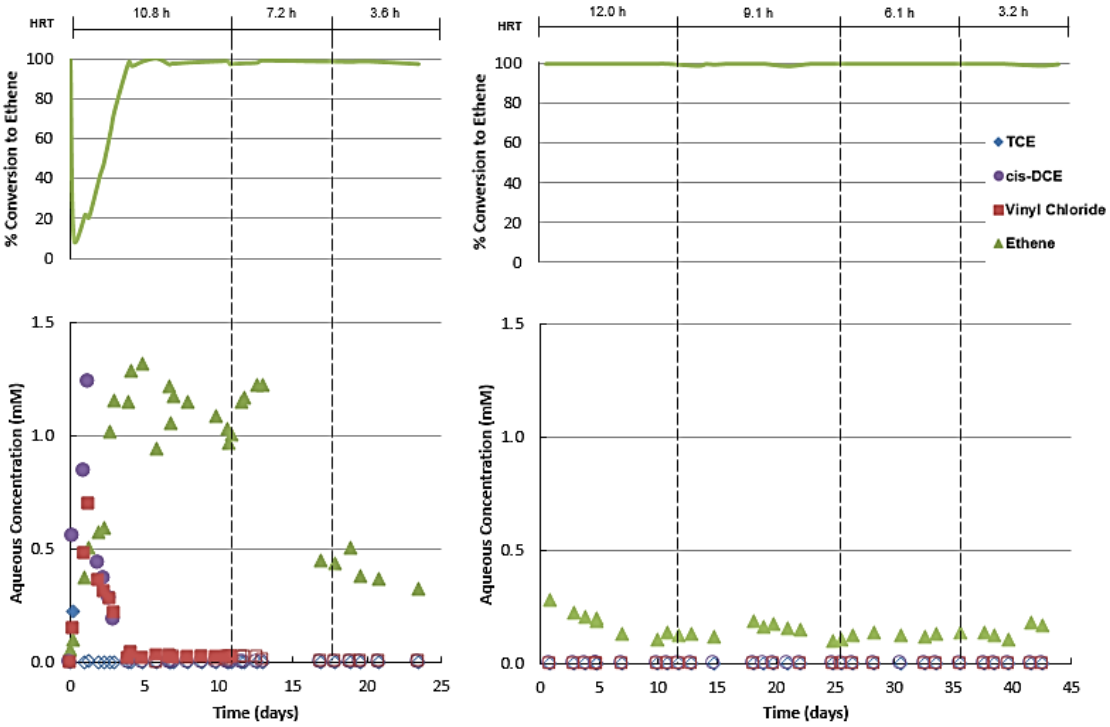

Figure 2 Dechlorination performance for phase I (L) and phase II (R). Open symbols indicate that the compound was below the detection limit i.e. ≤0.018 mM when measured.

Table 4 Summary of previous studies using UASB reactors for the treatment of chlorinated compounds.

Inoculum	Chlorinated contaminant	Electron Donor	HRT	Substrate Removal	Primary product	Reference
Sulfidogenic sludge	0.3 mM TCE	Acetate, propionate, butyrate	12 h (batch)	74%	Ethene	(Guerrero-Barajas, et al. 2014)
Anaerobic granular sludge	0.018 mM PCE	Lactate, sucrose	4 d – 1 d	87% - 51%	Ethene	(Hwu and Lu 2008)
Methanogenic granular sludge	0.24-0.96 mM PCE	Glucose	48-6 h	90%	Not reported	(D. T. Sponza 2001)
Aclimated anaerobic sludge	1.9 mM PCE	Acetate, formate	7.2-1.9 h	99%-93%	Cis-DCE	(Horber, et al. 1998)
Dechlorinating granules, anaeraobic sludge	0.05-0.07mM PCE	Glucose, methanol	4.5 h	80%	Ethene	(Wu, et al. 1993)
Methanogenic granular sludge	0.073 mM PCE, 0.1 mM TCE	Acetate, ethanol	34 h, 65 h (batch)	97%, 95%	Trans-DCE	(Christiansen, et al. 1997)
Anaerobic digester sludge	0.038-0.38 mM PCE	Acetate, methanol, acetone	24-8 h	99%	Vinyl chloride	(Prakash and Gupta 2000)
Methanogenic granular sludge	0.005-1.2 mM PCE	Glucose	48-6 h	90%	Not reported	(D. T. Sponza 2003)
Aclimated anaerobic sludge	0.11-0.56 mM TCE	Glucose, lactate	10 h	86.4-90.0%	Cis-DCE, vinyl chloride	(Zhang, Liu, et al. 2014)
Aclimated anaerobic sludge	0.28 mM TCE	Glucose, lactate	25-5 h	99-85%	Cis-DCE, vinyl chloride	(Zhang, Wang, et al. 2015)
Aclimated anaerobic sludge	0.03-0.18 mM 1,1,2,2-tetrachloroethane	Acetate	36-12 h	99-98%	1,2 dichloroethane	(Basu and Gupta 2010)
Anaerobic sludge	0.34 mM hexachlorocyclohexane	Methanol	48 h	99%	Pentachlorocyclohexene	(Bhatt, et al. 2008)
Anaerobic sludge	0.038-0.38 mM TCE	Acetate, methanol, acetone	8.4 h	86.4-87.4%	Cis-DCE, vinyl chloride	(Ozdemir, et al. 2007)

3.2 Fermentable Substrate Utilization Differs Between Phases

Fermentable substrates lactate and methanol were not within detectable limits in the effluent at any point during continuous operation under Phase I conditions. Acetate accumulation occurred during batch mode as seen in Figure 3 however acetate production declined once the reactor was transitioned to continuous mode. Average pseudo-steady state production of acetate was 8.0 mM during 10.8 h HRT, 7.2 mM during the 7.2 h HRT and 5.9 mM during the 3.6 h HRT. Propionate production averaged 9.9 mM during 10.8 h and 7.2 h HRTs, and 8.9 mM during the 3.6 h HRT. Lactate fermentation produces acetate and propionate however lactate is preferentially fermented to acetate in the presence of H2 scavengers (Seeliger, Janssen, & Schink, 2002). In Phase I there was a greater accumulation of propionate than acetate throughout continuous operation, likely due to TCE suppression of methanogens, and homoacetogens. Pseudo-steady state average methane production increased from 0.76 mM at a 10.8 h HRT, 0.93 mM at a 7.2 h HRT and 1.32 mM at a 3.6 h. The increased methane production is likely explained by higher lactate loadings at lower HRTs which provided increased H_2 and HCO_3^-/CO_2 for the growth of hydrogenotrophic methanogen populations within the reactor.

The HPLC data showed incomplete consumption of the amended electron donors, lactate and methanol for phase II operation. Both residual lactate and methanol were measured in the effluent throughout continuous operation at concentrations of approximately 2 mM methanol and 1.6 mM lactate. At a 12 h HRT, the average acetate concentration was approximately 7.1 mM. Once the HRT was reduced to 9.1 h HRT, the concentration fell to 4.9 mM and was further reduced to 4.1 mM at a 6.1 h HRT. At a 3.2 h HRT however the average acetate concentration was 4.9 mM. This rebound effect was

also seen in propionate concentrations at a 3.2 h HRT. The average propionate concentration during the 12 h HRT was 1.7 mM, reduced to 1.1 mM at a 9.1 h HRT, then 0.8 mM at a 6.1 h HRT. At 3.2 h HRT, the average propionate produced was 1.4 mM. In Phase II there was a greater accumulation of acetate which is expected at lower TCE concentrations.

There was a small observed increase in methane production from approximately 0.6 mM during the 12 h HRT to 0.8 mM during the 3.2 h HRT. The overall low methane production can be attributed to the elimination of additional bicarbonate buffer. Instead the influent was amended with 10 mM HEPES to achieve a pH of 7.5-8. Natural concentrations of bicarbonate in the groundwater were approximately 3 mM. Unlike in Phase I with synthetic feed and 2 mM TCE there is a higher accumulation of acetate than propionate. The reduction in TCE lessened the inhibitory effect on acetogens.

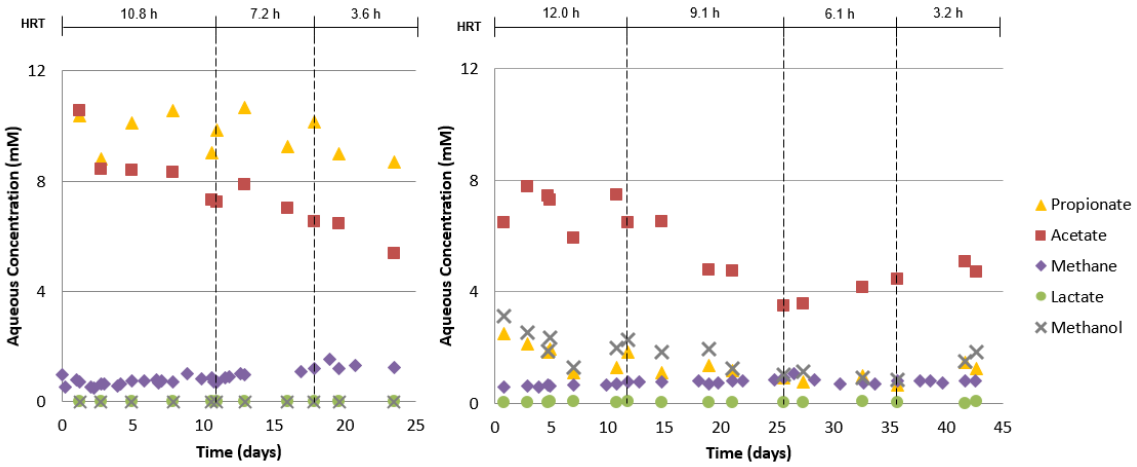

Figure 3 Consumption of electron donors lactate and methanol, and coupled production of propionate, acetate and methane.

Direct comparison of the concentration data in order to determine the significance of electron sinks is not possible as the concentration of electron donors' lactate and methanol was halved in Phase II while the concentration of electron acceptor, TCE was quartered. In order to elucidate better the primary electron sinks the concentration data was converted to an electron distribution for dechlorination, and the formation of acetate, propionate and methane as seen in Figure 4 below.

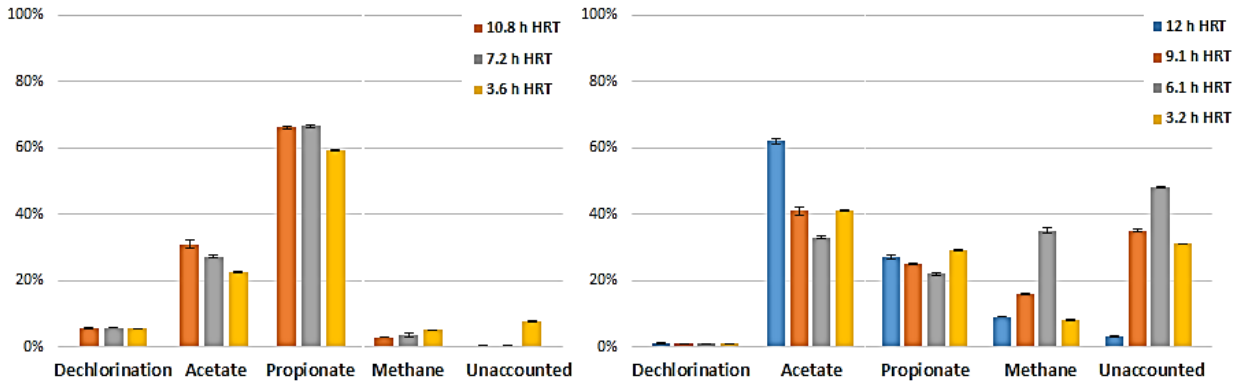

Figure 4 Electron distribution for acetate, propionate and methane formation, and dechlorination at each HRT for (left) Phase I and (right) Phase II of the experiment.

In Phase I it is clear that propionate production is the primary electron sink of the electrons provided by the fermentation of lactate and methanol. However in Phase II, acetate and methane become more prominent electron sinks. This is likely due to the higher concentration of bicarbonate inherent in the groundwater (3.6 mM HCO_3^-) which promotes both hydrogenotrophic methanogenesis and hydrogenotropic acetogenesis. Phase II has a significantly larger portion of unaccounted electrons. The presence of sulfate (0.729 mM SO_4^{2-}) and nitrate (0.179 mM NO_3^-) among other electron acceptors in the groundwater provide a possible explanation for this. The divergence of electrons to sulfate reduction

would corroborate the sequencing data which shows an increase in sulfate reducing bacteria. It is also possible that other fermentation products were present but not measured. Furthermore, dechlorination here accounts strictly for the reductive dechlorination of TCE and does not include that of trace chlorinated contaminants such as TCM.

3.3 Upflow Design Achieves High Biomass Retention

Quantitative real time PCR results revealed an increase in 16S rRNA gene copies of *Dehalococcoides mccartyi* at a 3.6 h HRT in sludge and a decline in FTHFS genes while Archaea remained consistent, as highlighted in Figure 4. In the effluent, there was an observable decline in the concentration of gene copies of *Dehalococcoides* and FTHFS and an increase in Archaea. High concentrations of *Dehalococcoides mccartyi* were maintained in sludge throughout the course of Phase I (Figure 4). At startup sludge contained an average of 2.47×10^{13} cells $L_{culture}^{-1}$, and 1.73×10^{13} cells $L_{culture}^{-1}$ at the end of the 3.6-h HRT run. These are the highest *Dehalococcoides* concentration ever achieved in a bioreactor, up by an order of magnitude from batch or continuous systems (Delgado et al. 2013; Steffan and Vainberg 2012; Vainberg et al. 2009; Ziv-El et al. 2011). The effluent concentrations of *Dehalococcoides mccartyi* declined from 7.93×10^{12} cells $L_{culture}^{-1}$ during a 10.8-h HRT to 1.05×10^{12} cells $L_{culture}^{-1}$ at the final 3.6-h HRT. The increase in *Dehalococcoides* in sludge but decline in effluent concentrations as the reactor transitioned from batch to continuous operation indicates favorable conditions to self-immobilization. The high effluent concentrations indicate that, while *Dehalococcoides mccartyi* are retained in the sludge, a significant amount of cells are also in suspension and eventually exit the reactor. The effluent concentration of *tceA* and

vcrA genes declined with increasing HRT whereas the sludge concentrations remained consistent as with the concentrations of Dehalococcoides. *bvcA* genes was not detectable.

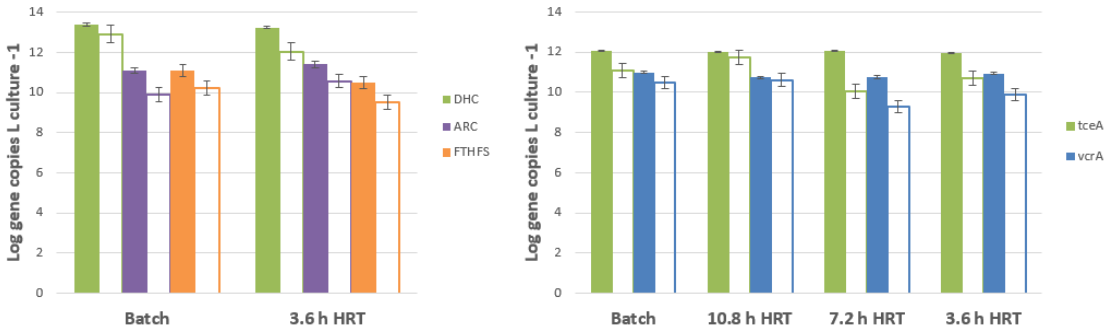

Figure 5 (L) Quantification of Dehalococcoides (DHC), Archaea (ARC) and FTHFS during Phase I, (R) Concentration of reductive dehalogenases genes tceA and vcrA. Filled bars represent sludge samples and unfilled bars represent effluent samples.

The concentration of gene copies of Archaea in the effluent increased by one order of magnitude. However, concentrations in the sludge remained in the same order of magnitude of 10^{11} cells Lculture-1 (Figure 4).The increase in effluent concentration of Archaea correlates with the chemical data that show increased methane production and decreased propionate at low HRTs, indicating an increase in electron donor availability to stimulate hydrogenotrophic methanogen populations. The decline in gene copies of FTHFS in both effluent and sludge also corresponds to the chemical data which showed a decline in acetate concentrations as the HRT was lowered.

In Phase II we witnessed the expected decline in gene copies of Dehalococcoides mccartyi associated with the lower concentration of TCE being fed. During the 3.2 h

HRT sludge maintained a high cell density estimated at 10^{12} cells Lculture-1 of Dehalococcoides mccartyi. The effluent concentration albeit lower, contained approximately 10^{10} cells Lculture-1 which is comparable to the commercial dechlorinating culture KB-1.

Figure 5 shows an aggregation of Dehalococcoides cells in flocs. The disc shape and biconclave indentations on either surface are consistent with known morphology of Strain BAV1 (Loffler et al. 2013). While the biofloc is clearly composed of a mixed microbial community with different cell morphologies, aggregates of disc-shaped Dehalococcoides are observed, showing that these cells are able to aggregate independently forming microcolonies of Dehalococcoides cells.

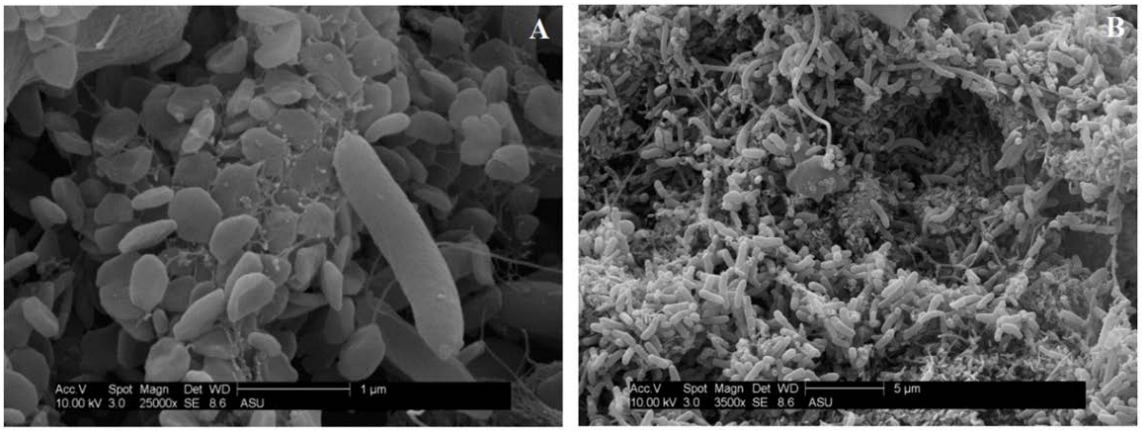

Figure 6 Scanning electron microscope (SEM) images of bioflocs removed from the reactor during a 10.8-h HRT. Images revealed microcolonies of Dehalococcoides (A) within a diverse microbial community (B). Image A is a magnification of the area identified in B.

3.4 High-rate dechlorination maintained under shifts in microbial community structure

High throughput sequencing was performed in order to characterize changes in the microbial community structure associated with the performance of the UASB under different conditions.

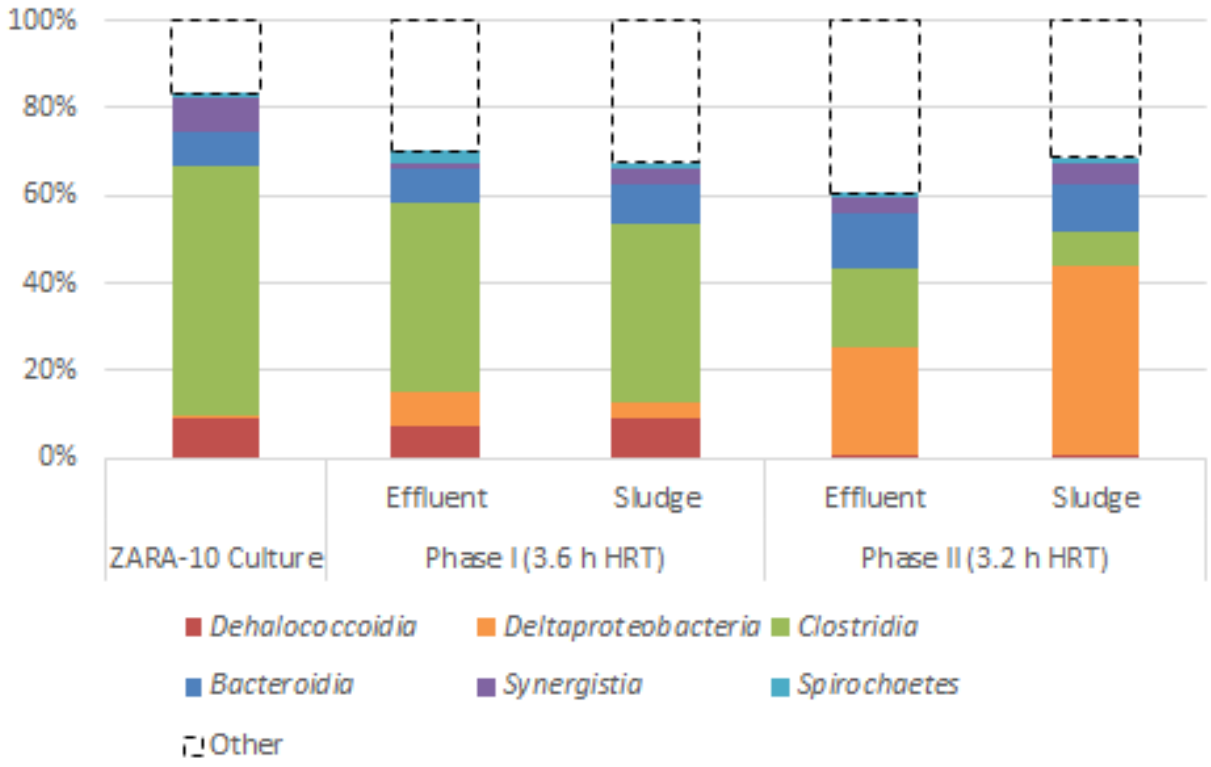

Figure 7 Relative abundance bacterial OTUs at class the level.

The inoculum used in this reactor, ZARA-10 dechlorinating culture, was dominated by sequences belonging to the class of fermenting bacteria *Clostridia* (57.1%) as previously determined (Delgado et al. 2014b). Phylotypes most similar to the organohalide respiring bacteria genera *Dehalococcoides* and *Geobacter* made up 9.6% of the total sequences, with phylotypes most closely related to *Dehalococcoides* representing the dominant organohalide respiring genus and phylotypes most closely

related to *Geobacter* being at a far lower abundance. During Phase I operation, phylotypes most closely related to *Clostridia* continued to dominate the microbial community in both effluent (43.3%) and sludge (41.0%). The abundance of phylotypes closely related to organohalide respiring bacteria, *Dehalococcoides* and *Geobacter* increased to 14.1% in the effluent and 12.2% in sludge compared to the inoculum. Even though we did not see a change in the reductive dechlorination performance of the UASB at 3.6-h HRT (Figure 2), the relative abundance of phylotypes most closely related to *Dehalococcoides* declined in the effluent culture, as seen in Figure 6. At the 3.6 h HRT, *Dehalococcoides* phylotypes represented 9.4% of the total sequences in sludge and 7.4% in effluent culture. There was also an increase in abundance of phylotypes most closely related to *Geobacter* abundance with the opposing trend, having a higher distribution in the effluent culture (6.8%) than sludge (2.8%). The increased abundance of both *Dehalococcoides* and *Geobacter* phylotypes in Phase I strongly indicates that this reactor (with the culturing conditions employed) is an effective culturing tool for dechlorinating cultures.

Under Phase II operating conditions however, the genomic sequences showed a shift towards a dominant *Deltaproteobacterial* class. *Deltaproteobacterial* phylotypes dominated both the effluent (24.8%) and sludge (42.9%) microbial communities, with the family of sulfate reducing bacteria *Desulfovibrionaceae* present as the most dominant phylotype. *Clostridial* phylotypes fell to 18.1% of the total sequences in the effluent culture and 8.2% in sludge. Unlike in Phase I where the synthetic groundwater was designed to target to the growth of *Dehalococcoides*, the groundwater fed in Phase II contained additional electron acceptors including sulfate, nitrate and iron which promote

the growth of other community members. Furthermore, the concentration of fermentable substrates lactate and methanol being fed was reduced which corresponds to the decline in the steady-state acetate and propionate concentration and the abundance of *Clostridia*. *Dehalococcoides* and *Geobacter* accounted for only 1.4% in both the effluent and sludge. This corresponds to the qPCR data which revealed an order of magnitude reduction in the *Dehalococcoides* concentration in both effluent and sludge. Interestingly, although the structure of the community changed, the overall reactor dechlorination performance remained at high rates.

CHAPTER 4

SUMMARY AND CONCLUSIONS

A UASB reactor was employed to facilitate the bioflocculation of a *Dehalococcoides* containing culture and to promote high-dechlorination rates at short HRTs. Previous studies have not been able to achieve complete removal of TCE/PCE nor have they achieved high conversions to ethene. In these previous studies the UASB reactors produced *cis*-DCE or vinyl chloride as the primary product of reductive dechlorination. Our study showed that 100% TCE removal and >98% conversion to ethene can be achieved with a UASB reactor. The improved performance compared to previous studies is the result of three major differences with our system design and operation; (i) inoculation with high-performance dechlorinating culture, (ii) the addition of a recycle stream for improved biomass retention and increased contact time and (iii) minimizing the bicarbonate in the influent.

This study was conducted in two phases, the first of which explored the bioreactor's ability to facilitate complete dechlorination and bioflocculation, and the second of which tested the ability of the bioreactor to remediated contaminated groundwater. Phase I of the study provided conditions to support the growth of *Dehalococcoides*, i.e. high concentration of TCE and excess electron donor/carbon source. Phase II tested the system's ability to maintain high-rates of dechlorination when applied as an ex situ remediation technology.

During Phase I system was able to promote biofloc formation from a heterogeneous dechlorinating culture. Cell retention due to biofloc formation allowed HRTs lower than

known doubling times of *Dehalococcoides* strains under both experimental conditions. Conversion to ethene was maintained between 97.1-99.0% as the HRT was decreased to 3.6-h for a 2 mM TCE feed. The bioreactor produced sludge containing concentrations of *Dehalococcoides* of 10^{13} cells/L and effluent concentrations of 10^{11}-10^{12} cells/L. The system successfully provided a growth environment conducive to bioflocculation and resulted in high-performance, fast-rate dechlorination of TCE to ethene. Similar results were achieved when fed TCE contaminated groundwater from the Motorola Inc. 52nd Street Plant Superfund site during Phase II. Our results show >99% dechlorination of TCE to ethene in contaminated groundwater for HRTs of as low as 3.2 hours.

Although changes in the feed composition between Phases I and II resulted in a change in the microbial community structure and a decline in *Dehalococcoides*, the dechlorination performance of the bioreactor was not affected. Furthermore unlike previous studies, changes in HRT and electron acceptor concentration did not affect the dechlorinating performance of the system. The bioreactor operated successfully as both a high-density culture production tool and as a real-time remediation technology.

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