Trichloroethene and *cis*-1,2-Dichloroethene Concentration-Dependent Toxicity Model Simulates Anaerobic Dechlorination at High Concentrations. II: Continuous Flow and Attached Growth Reactors

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Received 2 March 2010; revision received 20 May 2010; accepted 24 May 2010

Published online 1 June 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.22822

ABSTRACT: A model that was used to describe toxicity from high concentrations of chlorinated aliphatic hydrocarbons (CAHs) on reductively dechlorinating cultures in batch reactors (Sabalowsky and Semprini (in press)) was extended here to simulate observations in continuous flow suspended and attached growth reactors. The reductively dechlorinating anaerobic Evanite subculture (EV-cDCE) was fed trichloroethene (TCE) and excess electron donor to accumulate *cis*-1,2-dichloroethene (cDCE) in a continuous flow stirred tank reactor (CFSTR); and an attached growth recirculating packed column (RPC). A concentration-dependent toxicity model used to simulate the results of batch reactors in part I (Sabalowsky and Semprini (in press) Biotechnol Bioeng) also simulated well the observations for the CFSTR and RPC growth modes. The toxicity model incorporates cDCE and TCE toxicity coefficients that directly increase the cell decay coefficient in proportion with cDCE and TCE concentrations. Simulated estimates of the cDCE and TCE toxicity coefficients indicate reductively dechlorinating cells are most sensitive to high concentrations of cDCE and TCE in batch-fed growth, followed by CFSTR, with attached growth being least sensitive. The greater toxicity of TCE than cDCE, and ratio of the modeled toxicity coefficients, agrees with previously proposed models relating toxicity to partitioning in the cell wall ($K_{M/B}$), proportional to octanol-water partitioning (K_{OW}) coefficients.

Biotechnol. Bioeng. 2010;107: 540–549.

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KEYWORDS: *Dehalococcoides ethenogenes*; reductive dechlorination; toxicity modeling; inhibition; TCE; chemostat

Introduction

In part I, batch-growth experiments with aqueous cDCE concentrations in the range of 1,000–10,000 μ M inhibited reductive dechlorination in mixed cultures both from biogenic cDCE accumulation, and short-term commercial cDCE exposure. Traditional inhibition models, including competitive inhibition and Haldane inhibition, could not simulate long-term exposure data, while the proposed toxicity model could. In this companion paper, we further apply the proposed toxicity model from part I (Sabalowsky and Semprini, in press) to two different growth modes: continuously fed suspended; and continuously fed attached growth.

Inhibition and toxicity from high concentrations of CAHs has been documented in reductive dehalogenation studies (Adamson et al., 2004; Chu, 2004; Duhamel et al., 2002; Yang and McCarty, 2000; Yu and Semprini, 2004). The vast majority of studies in chlorinated aliphatic hydrocarbon (CAH) dechlorination use batch-fed suspended growth systems, due to their simplicity of operation. However, both planktonic and attached cells can both occur in the environment. It is therefore important to understand how batch growth data can be related to results more representative of *in situ* conditions and above ground reactor systems, such as continuously fed suspended- and attached-growth systems. It has commonly been observed that chemostat grown cells exhibit increased resistance to

Correspondence to: Andrew R. Sabalowsky

Contract grant sponsor: U.S. EPA Western Region Hazardous Substance Center

Contract grant number: R-828772

Contract grant sponsor: National Institute of Environmental Health Sciences

Contract grant number: 1P42 ES10338

Additional Supporting Information may be found in the online version of this article.

inhibition or toxicity compared to batch grown cells (Chang and Alvarez-Cohen, 1996; Piringer and Bhattacharya, 1999; Qu and Bhattacharya, 1997), and attached growth cells have greater resistance than suspended cells (Harrison et al., 2007; Stewart and Costerton, 2001; Walters et al., 2003). Comparisons between different growth modes (such as batch-grown, continuously fed, or attached growth) are therefore valuable regarding issues of inhibition or toxicity. Such comparisons can supply important information needed for designing treatment strategies or relating batch-fed microcosms to field observations or predictions. Feasibility demonstrations of biologically enhance tetrachloroethene (PCE) dissolution have been performed in batch- and attached-growth systems, but explaining lost activity from CAH accumulation has yet to be achieved (Adamson et al., 2004; Amos et al., 2007; Cope and Hughes, 2001; Sleep et al., 2006; Yang and McCarty, 2000, 2002).

Only a few studies have been devoted to evaluating biological reductive dechlorination in continuous flow suspended growth systems (Carr et al., 2000; Drzyzga et al., 2001; Yang and McCarty, 1998; Zheng et al., 2001), but we are only aware of one such study that specifically operated at relatively high CAH concentrations (Carr et al., 2000), which resulted in lost dechlorination capabilities as cDCE accumulated above 600 μM . While attached growth studies regarding biological reductive dechlorination are more numerous (e.g., Azizian et al., 2008; Isalou et al., 1998; Kao et al., 2001; Ma et al., 2006; Singhal et al., 2007; Sleep et al., 2006), few studies compare batch suspended growth to attached growth using the same starting culture (Azizian et al., 2008; Schaefer et al., 2009). Several studies have utilized attached growth in pack columns to achieve enhanced dissolution of PCE, resulting in high cDCE concentrations, but comparisons to other growth modes have yet to be made (Cope and Hughes, 2001; Sleep et al., 2006; Yang and McCarty, 2000, 2002).

Reported here are the effects of high CAH concentrations (cDCE $\sim 10,000 \mu\text{M}$ and TCE $> 1000 \mu\text{M}$) on different growth modes, and simulations of the experimental observations. The CAH concentration-dependent toxicity model developed in part I (Sabalowsky and Semprini, in press) for batch-fed growth was applied to two other growth modes: continuously fed suspended growth, and continuously fed attached growth. cDCE accumulation effects on dechlorination activity were studied in a continuous flow stirred tank reactor (CFSTR), and an attached growth recirculating packed column (RPC). The toxicity model presented in part I simulated the transient responses of cDCE and TCE concentrations in both of these systems and the ultimate reactor failure that occurred.

Materials and Methods

Chemicals and Analytical Methods

The chemicals for media and analytic standards utilized in this study are presented elsewhere (Sabalowsky and

Semprini, in press). Gas chromatography for all CAHs and ethene (ETH) were also as previously described (Sabalowsky and Semprini, in press). Additionally, organic acids analyses were analyzed by high pressure liquid chromatography (HPLC) on a Dionex-500 HPLC with a Prevail Organic Acid 5u column (250 mm $\times$ 4.6 mm ID, Alltech, Deerfield, IL) with a 25.0 mM KH_2PO_4 and 17.3 mM H_3PO_4 eluent. An ultraviolet lamp (UV) detector was used at 210 nm for signal quantification. Protein measurements were made with the Pierce Micro BCATM Protein Assay Preparation Reagent Set (Pierce, Rockford, IL) per manufacturer's instructions. Measurements were made with a HP 8453 UV/VIS spectrophotometer on the visible lamp, quantified at 562 nm, with a detection limit of 0.5 mg/L.

Culture

The EV-cDCE subculture described in part I was used for the CFSTR and RPC studies presented here. The culture was maintained under batch growth conditions as previously described prior to adding to the CFSTR and the RPC (Sabalowsky and Semprini, in press).

CFSTR Construction and Operation

A zero-headspace CFSTR was constructed with a 303 mL Kimax bottle fitted with a three-hole stainless steel cap and completely mixed with a Teflon stirbar. The CFSTR reactor was connected to an Orion M361 syringe pump (Thermo Electron Corp., Beverly, MA) with PEEK tubing and valves (Upchurch Scientific, Oak Harbor, WA) for influent and effluent sampling (Fig. 1). Pumping rates were periodically changed in response to reactor performance. Pumping rates and corresponding hydraulic residence times are summarized in Table I. The majority of operation was at an influent flow rate of 18 mL/day, resulting in a hydraulic retention time of 16.8 days.

The CFSTR was inoculated from inside an anaerobic glove box with 5% H_2 , 95% N_2 atmosphere (Coy Laboratory Products, Inc., Grass Lake, MI) with 303 mL of the EV-cDCE culture. The reactor was then capped, exported from the glove box, and connected to influent and effluent waste collection lines as illustrated in Figure 1. More details of reactor construction are supplied by Sabalowsky (2008).

The influent was continuously supplied to the CFSTR using a 100 mL gastight syringe (Hamilton, Leno, NV). Batches of influent solution were made by augmenting basal growth medium (Yu and Semprini, 2004) in a 250 mL media bottle with excess neat TCE to achieve an average aqueous concentration of 7.4 mM ($\pm 0.95 \text{ mM SD}$) and sodium lactate to 25.6 mM ($\pm 4.0 \text{ mM SD}$) as a fermenting substrate. The influent solution was stirred and settled (to remove TCE NAPL from suspension), and one syringe-volume of feedstock was extracted from the N_2 -pressurized media bottle. The influent syringe was refilled, and more TCE-lactate amended medium was prepared as necessary. To

Figure 1. CFSTR schematic diagram. Not to scale.

avoid oxygen exposure, the reactor influent valve was closed and 2 mL of feed were flushed through the line prior to opening the influent valve to the CFSTR. Resazurin was added as an oxygen indicator. It was never oxidized during reactor operation based on the lack of a visible color change. The influent medium was carbonate buffered, and the effluent pH remained between 6.8 and 7.6 throughout the operation.

RPC Reactor Construction and Operation

The RPC reactor (Fig. 2) was constructed using a 30 cm × 2.5 cm (ID) glass Kontes Chromaflex column with PTFE end fittings and 20 µm polyethylene bed support frits

Table I. Reactor operating conditions.

Days of operation	Pumping rate (mL/day)	Residence time (days)
CFSTR ^a		
0–3	0	—
4–5	28	10.8
6–49	18	16.8
50–55	0	—
56	18	16.8
57–84	12	25.3
85–99	0	—
RPC ^b		
0–100	6	11.8
101–155	12	5.9

^aCFSTR influent concentrations (average ± standard deviation): 7.4 (±0.95) mM TCE, 25.6 (±4.0) mM lactate.

^bRPC influent concentrations (average ± standard deviation): 11.3 (±2.5) mM TCE, 22.6 (±6.2) mM lactate.

(Kontes, Vineland, NJ). The column was packed with 2 mm glass beads and connected with 1/8" PEEK tubing (0.062" ID) to a recirculating pump consisting of a Masterflex L/S 7550 computerized driver and Masterflex Micropump (Cole-Parmer Instrument Co., Vernon Hills, IL). The glass beads were etched to enhance roughness and cellular attachment with a chromic and hydrochloric acid procedure as described by Shellenberger and Logan (2002), thoroughly rinsed, autoclave sterilized and oven dried. The column was packed with the glass beads and oriented vertically for upflow operation. T-valve PEEK connections below and above the column allowed influent and effluent liquid sampling. A 25 mL gastight syringe (Hamilton) on an Orion M361 syringe pump was used for feed addition and connected to the influent T-valve connection. A T-connection at the top of the recirculating column system allowed effluent to leave the reactor by pressure into an anaerobically prepared waste collection bottle.

To allow culture assessment without interrupting RPC operation two mini-columns, that could be removed, were connected at the influent and effluent ends of the main packed column (Fig. 2). The inline mini-column cartridges were constructed from 1/4" (ID) glass tubing filled with the 2 mm glass beads, and placed below and above the column, with T-valve connections and 1/16" PEEK tubing bypass loops. Each packed mini-column had a liquid volume of 0.72 mL. The entire RPC assembly liquid volume, including tubing and valves, was determined from a bromide tracer test to be 70.8 mL.

Prior to inoculation, the RPC assembly was bleach sterilized, flushed with sterile anaerobic water, flushed and filled with basal anaerobic medium. To inoculate the RPC, 100 mL of the EV-cDCE subculture was extracted from the batch growth reactor with a 100 mL gastight syringe (Hamilton) within the anaerobic glove box, removed from the glove box, and injected up through the column at a rate of 2 mL/min without recirculation, until 90 mL of culture had been flushed up through the 70.8 mL system. The recirculating and influent pumps were then immediately started. Influent pumping rates, TCE, and lactate concentrations are summarized in Table I. To distribute activity throughout the length of the column, the recirculating pump speed was set to 2 mL/min, resulting in a 35 min circulation time through the system.

Growth media for the RPC were prepared in the same fashion as the CFSTR, by augmenting the basal medium with 60% sodium lactate syrup and excess TCE. As with the CFSTR, the influent syringe was refilled as needed, flushing influent lines each time to maintain anaerobic conditions. Periodic effluent pH monitoring confirmed RPC pH remained between 6.8 and 7.4. For both the RPC and CFSTR, in order to avoid hydrogen limitations on dechlorination, lactate was supplied at approximately 5–6 times the required hydrogen supply to dechlorinate TCE to cDCE, based upon lactate fermentation to acetate and hydrogen, as has been observed in our lab for this culture.

Figure 2. RPC schematic diagram. Not to scale.

CFSTR and RPC Sampling

Liquid samples from the CFSTR and RPC were collected by pumping 1.5 mL from the influent syringe into the system and extracting 1.5 mL of effluent or influent instantaneously from the appropriate sampling port with a disposable 3.0 mL syringe. A headspace sample for GC analysis was created by injecting 1.0 mL of liquid sample into a 2.0 mL crimp-top clear glass vial. The vials were vortexed for 1 min at room temperature (22.5–25.5°C), and 100 μ L of headspace was extracted and injected into the GC-FID injection port for CAH and ETH quantification. Gaseous and aqueous concentrations, and total mass for CAHs, and ETH were calculated based upon sampling temperatures, Henry's Law coefficients (Gossett, 1987; Perry et al., 1997) and the relationships of $H_{cc} = C_g/C_l$, $M_{total} = C_g V_g + C_l V_l$.

Twice during operation, the 0.72 mL mini-columns were taken offline to assess attached growth activity associated with the glass beads. The mini-columns were flushed with

5 mL of fresh basal medium (containing no lactate or TCE), and then TCE- and lactate-augmented medium (1,120 μ M and 25,000 μ M, respectively) was added. The columns were incubated for 18 h at 20°C. The total amount of cDCE formed was determined by extracting the aqueous contents into a 5.0 mL Hamilton Gastight syringe, analyzing TCE and cDCE on a GC-FID fitted with a purge-and-trap apparatus. Details of the purge-and-trap analysis are provided elsewhere (Azizian et al., 2008). Average TCE dechlorination rates were determined by dividing cDCE moles formed by the incubation time and the volume.

Model Development

Michaelis–Menten Kinetics for Reductive Dechlorination

Relevant Michaelis–Menten based kinetics, including inhibition, are presented in part I (Sabalowsky and

Semprini, in press). Here, we expand the previously presented equations to the CFSTR and RPC reactor studies.

CFSTR Modeling

The kinetic and toxicity models for the batch reactors as presented in part I can be incorporated into CFSTR modeling for TCE dechlorination as follows,

$$\frac{dC_{L,TCE}}{dt} = \frac{Q}{V} C_{L,TCEin} - \frac{Q}{V} C_{L,TCE} - \frac{k_{max,TCE} X C_{L,TCE}}{K_{S,TCE} + C_{L,TCE}} \quad (1)$$

where Q is the volumetric flow rate, and V is the reactor volume, and $C_{L,TCEin}$ is the TCE concentration in the influent. All other parameters are as defined and summarized in the Nomenclature of part I. Assumptions are that the dechlorination reaction stops at cDCE, which is a property of the EV-cDCE subculture, and the reactions are not electron donor limited. The CFSTR equation for cDCE (not shown) would include the inflow and outflow terms for cDCE and the cDCE production term, which is the positive form of the TCE transformation rate term shown above.

CFSTR dehalogenating biomass concentration can be modeled by modifying Equation (2) of part I as follows,

$$\frac{dX}{dt} = \frac{Q}{V} X_{in} - \frac{Q}{V} X + Y \left(\frac{k_{max,TCE} X C_{L,TCE}}{K_{S,TCE} + C_{L,TCE}} \right) - k_d^* X \quad (2)$$

where X_{in} would be biomass concentration introduced in the influent, which was zero for these experiments. The k_d^* term represents the concentration based toxicity decay term defined in Equation (8) of part I.

RPC Modeling

The above biomass CFSTR equation was expanded to a simplified RPC model by the incorporation of a fraction of biomass suspended, to account for sloughing of cells, as follows.

$$\frac{dX}{dt} = \frac{Q}{V} X_{in} - \frac{Q}{V} X f_s + Y \left(\frac{k_{max,TCE} X C_{L,TCE}}{K_{S,TCE} + C_{L,TCE}} \right) - k_d^* X \quad (3)$$

where f_s represents the fraction of cells suspended, assumed to be 0.15, meaning 15% of cells in the reactor are subject to loss by advection from the system while the remaining biomass is retained. Measured cell sloughing in a laminar tube reactor has been reported to range from 10% to 25% (Telgmann et al., 2004). A constant rate of cell sloughing, as modeled here, is an approach used by Schaefer et al. (2009). Since this system was operated with high cDCE and TCE concentrations, the biofilm is assumed to be fully penetrated, and therefore that $k_{max,TCE}$ and $K_{S,TCE}$ values are the same as the suspended culture, thus allowing us to model the dechlorination kinetics for the RPC with the same kinetic equations used to describe the CFSTR. A summary of

Table II. Model kinetic parameters for batch, CFSTR, and RPC simulations.^a

Parameter	Batch	CFSTR	RPC
$k_{max,TCE}$ ($\mu\text{mol/mg protein/day}$)	28 ^b	28	28
$K_{S,TCE}$ (μM)	1.8 ^b	1.8	1.8
$K_{t,TCE}$ (μM)	230 ^c	400	770
$K_{t,cDCE}$ (μM)	800 ^b	2,020	2,600
X_0 (mg protein/L)	0.8 ^b	22.5	20

^aAll competitive inhibition and yield coefficients, which are not listed here, are as reported previously for EV culture (Yu and Semprini, 2004).

^bAs supplied in Table I, part I.

^cAs calculated for modeling in Figure 4b, part I. Calculated based upon the $K_{t,cDCE}$ value from part I, and the assumed relationship: $K_{t,TCE}/K_{t,cDCE} = K_{M/B,cDCE}/K_{M/B,TCE}$ per Equations (8) and (10), part I (Sabalowsky and Semprini, in press).

kinetic modeling parameters and values for CFSTR and RPC models utilized in this study are presented in Table II. All models were constructed and solved using the STELLA[®] v.9.0 software (Isee Systems, Inc., Lebanon, NH).

Results and Discussion

CFSTR Observations

The CAH monitoring data, along with model simulations, are presented in Figure 3a. Pumping rates were adjusted throughout the experiment, as presented in Figure 3a and Table I. From days 8 to 31 of operation, with a 16.8-day retention time, the CFSTR maintained nearly complete dechlorination of TCE to cDCE, with no detectable VC or ETH, and only traces of TCE generally below 12 μM . TCE began to increase after day 31, and the reactor was operated in stop-flow mode from days 50 to 56 until TCE was reduced below 110 μM . Pumping was resumed for one day with the 16.8-day retention time, but increasing TCE was observed in the effluent, and the influent rate was then reduced to 12 mL/day, to produce a retention time of 25.3 days. TCE continued to increase in the effluent as pumping was maintained, and pumping was stopped after day 85 to determine if the reactor would recover performance, as it had during stop-flow initiated on day 50. No observable dechlorination was measured from day 85 to 99, and thus the experiment was terminated. Effluent organic acids monitoring revealed excess propionate was always present above 5 mM (not shown) from the fermentation of lactate at an average influent lactate concentration of 25.6 mM.

CFSTR Modeling

The CFSTR model to simulate observed performance included transformation kinetics as described in Equation (1), plus the cDCE and TCE toxicity terms, $K_{t,cDCE}$ and $K_{t,TCE}$, as shown in Equation (8) of part I (Sabalowsky and Semprini, in press). As with other research

Figure 3. CAH monitoring data for the CFSTR with fitted CAH concentration-dependent toxicity model. CAH monitoring data and modeled values (a), and model-predicted biomass (b). TCE data (◆), cDCE data (Δ), modeled TCE (solid line), modeled cDCE (dashed line), and modeled protein concentrations (dash-dot line). Note the different scales for TCE and cDCE concentrations.

investigating CAH dechlorination (Amos et al., 2007; Cupples et al., 2004; Sabalowsky and Semprini, in press), the CFSTR model was calibrated by adjusting the initial biomass (X_0) to fit the observed dechlorination between days 0 and 3 at no-flow conditions (Fig. 3a). The $k_{\max, \text{TCE}}$ from the batch reactor simulation, as well as all other $k_{\max}$ and K_S values listed in part I, were used. Flow changes during operation were included in the model input, and a constant modeled TCE influent concentration of 8,900 μM maintained a mass balance consistent with effluent CAH measurements.

Modeling was initially performed using only cDCE toxicity, and the same $K_{t, \text{cDCE}}$ as used for the batch experiments of part I. Model simulations using these parameters predicted premature CFSTR failure, losing dechlorination activity by day 30, indicating a $K_{t, \text{cDCE}}$ of 800 μM caused too much toxicity to match experimental observations. This result was expected, as continuously fed cells in chemostats, similar to our CFSTR, have exhibited greater tolerance to toxicity than batch-fed cells (Chang and Alvarez-Cohen, 1996; Piringer and Bhattacharya, 1999; Qu and Bhattacharya, 1997). Though the mechanisms have yet

to be determined, acclimation to toxic or inhibitory conditions in continuous cultures or a shift in the microbial community is possible. Acclimation to toxicity in continuous cultures has been previously observed (Gupta et al., 1996; Piringer and Bhattacharya, 1999; Qu and Bhattacharya, 1996). The $K_{t, \text{cDCE}}$ toxicity coefficient was therefore adjusted to reflect the observed TCE accumulation and cDCE decline beginning after day 56. After setting the $K_{t, \text{cDCE}}$ coefficient, the TCE toxicity coefficient ($K_{t, \text{TCE}}$) was adjusted to fit the TCE increase and cDCE decrease observed between days 56 and 90.

Both the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ coefficients are sensitive and were fit to provide good match to the experimental observations. Sensitivity analyses to these coefficients are supplied in the Supplemental data section, including Figures S1a and S1b. As discussed in the Supplemental data section the simulations are very sensitive to changes in the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ coefficients by $\pm 10\%$ of the base case values and illustrate the non-linear nature of the model solutions.

Simulations with the adjusted coefficients better reflected the transient TCE increases observed from days 3 to 8, and days 31 to 56 (Fig. 3a). Modeling the CFSTR data with only cDCE toxicity, or only TCE toxicity could not simulate the transient TCE peaks observed starting on days 3 and 31, and reactor failure commencing at day 56 of operation. Only when both cDCE and TCE toxicity terms were included were reasonable matches to CAH observations obtained (Fig. 3a). Unlike the batch experiments in part I, continuous exposure to high TCE concentrations occurred in the CFSTR, which is why the batch cDCE accumulation experiments (Fig. 2, part I) could be simulated without including TCE toxicity ($K_{t, \text{TCE}}$). Simulated biomass from the toxicity model is shown in Figure 3b. As shown in Equation (1), the TCE dechlorination rate is directly proportional to the amount of biomass present. The decrease in the simulated biomass is fairly continuous throughout the test and is associated the early increase in cDCE and the latter increase in TCE concentration.

The ratio of $K_{t, \text{cDCE}}/K_{t, \text{TCE}}$ in the model simulation that exhibited a good fit to the data was 5.0, similar to the ratio of K_{OW} for TCE/cDCE (3.6), and the $K_{\text{M/B}}$ ratio for TCE/cDCE (3.5), as discussed in part I. This is consistent with previous work regarding the relationship between toxicity and the K_{OW} or $K_{\text{M/B}}$ of a solvent (Chu, 2004; Chu et al., 2006; Isken et al., 1999; Sikkema et al., 1994), as represented by Equation (10) in part I. Addition of Yu and Semprini's (2004) Haldane inhibition terms to the toxicity model could not produce a model fit to the data, nor could modeling with only the Haldane inhibition model previously reported (Yu and Semprini, 2004), which did not predict reactor failure. Adapting the threshold model (Eq. 7, part I) to include the CFSTR advection terms with cDCE threshold inhibition alone, TCE threshold inhibition alone, or both cDCE and TCE threshold inhibition also could not fit CAH observations, either predicting complete reactor failure by day 30, or continued complete dechlorination of TCE to cDCE

without failure (not shown). Various inhibition models were unable to describe CAH data in our CFSTR experiment, but the previously described toxicity model (Sabalowsky and Semprini, in press) fit the data quite well with a small modification to the toxicity parameters. These observations suggest the proposed toxicity model more accurately describes long-term exposure to high CAH concentrations than inhibition models. Direct measurements of the individual toxicity coefficients on short-term experiments in continuous-fed mode are required to directly compare continuously fed cell responses to batch-fed cells and to test the proposed model. Measurements of batch rates with cells harvested temporally from the reactors and application of DNA based molecular methods to quantify the dehalogenating population, as described by Sabalowsky (2008), should be applied in future tests.

RPC Observations

The RPC CAH monitoring data and the simulation using the toxicity model are presented in Figure 4a. TCE dechlorination to cDCE was maintained from days 9 to

78, with no detectable VC or ETH. Only traces of TCE below 41 μM were measured in the effluent, compared to an average influent TCE concentration of 11,300 μM during the 6 mL/day pumping (11.8 days hydraulic residence time). Modeled TCE effluent concentrations were between 5.8 and 75 μM during this time frame. A transient increase in TCE concentration to 1,280 μM observed on day 79 resulted from an overnight pumping problem, which was removed below 18 μM by day 82 under resumed normal operating conditions. On day 101, the influent pump rate was increased to 12 mL/day to decrease the hydraulic residence time from 11.8 to 5.9 days. TCE began to increase in the effluent, remained relatively constant between 894 and 1,350 μM from day 103 to 127, but then sharply increased as cDCE concentrations decreased from days 127 to 155, when the experiment was terminated.

Twice during operation, days 77 and 150, the two mini-columns were removed from the RPC and tested for attached growth TCE transformation activity. Average rates of TCE dechlorination over the 18-h incubations are summarized in Table III along with estimated RPC dechlorination rates and the model's simulated biomass for comparison. Rates in the mini-columns were in the range of the dechlorination rates observed in the RPC on the same days. The rates obtained for each mini-column were within 5–10% of each other, thus TCE transformation rates were essentially the same at the influent and effluent ends of the RPC. These analyses confirmed that TCE dechlorination to cDCE was associated with cells attached to the beads in the mini-columns, and that the 2 mL/min recirculation rate successfully distributed equal activity throughout the length of the RPC. The mini-column rates were approximately 50% higher than the average total RPC reactor dechlorination rates, but are in reasonable agreement considering the potential differences in test conditions. The mini-columns' rates clearly showed the decreased performance with time in the RPC with rates on day 150 decreasing to 49% to 52% of those on day 77, while the RPC data showed a decrease to 59%. The model-simulated total biomass, also shown in Table III, showed a decline to 63% of the biomass present on day 77 by day 150. Thus there is good agreement with the measured decreases in transformation rates and the simulated decreases in biomass.

Effluent samples were periodically collected for organic acid and protein analyses. Effluent organic acids consistently

Figure 4. CAH monitoring data for the RPC with fitted CAH concentration-dependent toxicity model. CAH monitoring data and modeled values (a), and model-predicted biomass (b). TCE data ($\blacklozenge$), cDCE data (Δ), modeled TCE (solid line), modeled cDCE (dashed line), and modeled protein concentrations (dash-dot line). Arrow indicates when pumping increased from 6 to 12 mL/day (day 101). Note the different scales for TCE and cDCE concentrations.

Table III. Mini-column and average RPC dechlorination rates.

Day of operation	Influent column rate ($\mu\text{mol/h/mL}$) ^a	Effluent column rate ($\mu\text{mol/h/mL}$) ^a	Total RPC reactor rate ($\mu\text{mol/h/mL}$) ^b	Model simulated biomass (mg)
77	0.071	0.067	0.042	2.7
150	0.037	0.033	0.025	1.7

^aValue calculated as total moles cDCE formed, divided by total incubation time and reactor volume.

^bValue calculated as cDCE concentration in RPC effluent that day, divided by pumping rate and total RPC reactor volume.

revealed approximately 12 mM propionate and 10 mM acetate were present from lactate fermentation, and that the RPC was not electron donor limited. All effluent protein samples, taken approximately every thirty days, were below the 0.5 mg/L detection limit, indicating that suspended cells were unlikely contributing significant dechlorination activity, and that the modeling assumption of 15% cell sloughing is reasonable. Bacterial growth in the waste collection bottle indicated cells were sloughing and exiting the RPC, despite the inability to detect them through protein analysis of the effluent samples.

RPC Modeling

Because neither competitive inhibition, Haldane inhibition, or threshold inhibition modeling could describe our CFSTR data, we chose to only investigate our toxicity model in analyzing the continuously-fed RPC data. The RPC model was calibrated by establishing the X_0 that fit TCE dechlorination observed between days 5.7 and 9.6, consistent with the approach used in the CFSTR analysis. The same $k_{\max, \text{TCE}}$ from the batch and CFSTR experiments was used for the RPC simulations. After model calibration, the $K_{t, \text{cDCE}}$ coefficient was adjusted until the simulated rate of TCE increase between days 97 and 105 matched the experimental observations. Adjustments of $K_{t, \text{cDCE}}$ alone could not simulate the observed plateau of TCE concentrations observed from days 103 to 127 or the ultimate buildup of TCE and cDCE decreases, beginning on day 128. Adjusting only the $K_{t, \text{cDCE}}$ parameter resulted in either a transient spike of TCE that returned to negligible concentrations by day 110, or predicted premature reactor failure beginning at day 101 (not shown). By including the $K_{t, \text{TCE}}$ term, the observed plateau and then buildup of TCE, accompanied by flushing of cDCE from the RPC could be simulated (Fig. 4a). As with the CFSTR model, the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ coefficients are sensitive and were adjusted to fit the RPC experimental observations. Sensitivity analyses to these coefficients are supplied in the Supplemental data section, including Figures S2a and S2b. The sensitivity analysis, discussed in the Supplemental data section, showed the simulations were very sensitivity to $\pm 10\%$ changes in the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ and illustrate the non-linear nature of the system of equations that are being solved.

The predicted biomass from the toxicity model is shown in Figure 4b. As with the CFSTR model, the RPC modeled biomass decreases at later time are associated with increasing TCE concentrations. Parameters used for the RPC model are summarized in Table II. All but the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ and X_0 , terms were the same as those in the EV-cDCE batch and CFSTR models. Note the ratio of $K_{t, \text{cDCE}}/K_{t, \text{TCE}}$ for the RPC fitted model was 3.7, nearly identical to the ratio of K_{OW} for TCE/cDCE (3.6), and the $K_{\text{M/B}}$ ratio for TCE/cDCE (3.5), and is consistent with the CFSTR simulation results. This further supports previous work relating the degree of solvent toxicity to concentrations in the cell wall (Chu, 2004; Chu et al., 2006; Isken et al., 1999; Sikkema et al., 1994).

Table IV. Impacts of modeled f_s on toxicity coefficients for the RPC.

f_s (unitless)	$K_{t, \text{cDCE}}$ (μM)	$K_{t, \text{TCE}}$ (μM)
0	2,200	605
0.15	2,600	770
0.50	5,000	1,400

Since the modeled f_s (Eq. 3), $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ (Eq. 8, part I) all directly impact cell loss (dX/dt), modifying f_s will impact modeled toxicity, and is therefore an important term to rigorously quantify in more detailed studies. Note, we do not fit f_s to our data, but assumed a typical f_s value (Telgmann et al., 2004) and fit the toxicity terms. Changing f_s from our reported 0.15 to 0 or 0.5 produced different K_t coefficients for equal fits to the data and modeled active biomass (Fig. 4a and b), as summarized in Table IV. With the assumption of no cell sloughing ($f_s = 0$), the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ coefficients had to be lower (more toxic) to achieve sufficient loss of active cells to fit the data. Even in this scenario, the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ coefficients are higher (less toxic) than for the CFSTR or batch-fed cells (Table II), indicating the RPC biomass was more resistant to CAH toxicity than CFSTR biomass. Less inhibition or toxicity in attached growth when compared to chemostats has been observed in previous studies (Anwar et al., 1992; Welin et al., 2003).

Failure of the RPC dechlorination performance happened shortly after increasing influent flows from 6 to 12 mL/day (on day 101). To test if failure was due to increased advection of cells, we performed simulations excluding the $K_{t, \text{cDCE}}$ and $K_{t, \text{TCE}}$ terms while modifying f_s of Equation (3). Simulated RPC failure in the absence of the toxicity terms did not occur until f_s was adjusted above 0.85, and did not produce the transient TCE plateau measured between days 103 and 131. Increasing the influent flow from 6 to 12 mL/day represents only a 0.2% change in the total flow rate (including recirculation). Advective cell loss from the RPC is therefore unlikely a significant factor in lost performance. The proposed model indicates the sudden increase in loading elevated TCE concentrations to enhance toxicity, resulting in the net cell decay rate becoming greater than the net biomass growth rate, which is illustrated by the decline in total active biomass in Figure 4b. This decline is supported by the results of the mini-column batch tests which show approximately a 50% reduction in dechlorination rates from day 77 to 150 (Table III), consistent with the simulated reduction in biomass. Future direct experiments are necessary to test this observation, using approaches such as the mini-column method demonstrated here.

Adding Yu and Semprini's (2004) Haldane inhibition terms, or modeling with Haldane inhibition in the absence of our toxicity terms could not produce a model fit to the data (not shown). Modeling the RPC reactor with only Haldane inhibition ($K_{I, \text{H-TCE}}$) resulted in simulations with either complete dechlorination of TCE to cDCE and no reactor failure (for $K_{I, \text{H-TCE}} \geq 624 \mu\text{M}$), or loss of TCE

dechlorination and biomass (X) by day 30 (for $K_{i,H-TCE} \leq 623 \mu\text{M}$). The toxicity model, with the exclusion of Haldane inhibition however, matches the CAH data very well (Fig. 4a), indicating concentration-based toxicity is a likely reason for lost activity.

Simulations were also run with modified half-velocity, K_S , values to determine whether changing effective K_S for attached growth could predict loss of dechlorination, or impact toxicity coefficients. Increasing reported $K_{S,TCE}$ from values in Table II by a factor of 10 did not produce a simulation resulting in loss of TCE dechlorination if excluding the K_i toxicity terms, and had a small impact on the model outcome while including K_i toxicity terms. This analysis indicates a shift in culture's $K_{S,TCE}$ is not a sensitive factor in the response to the simulated TCE and cDCE concentrations.

Conclusions

Unsuccessful attempts to simulate observations using Haldane or other inhibition for the batch-fed, CFSTR or RPC reactor data indicates that the proposed toxicity model better represents long term effects of high CAH concentration exposure. It is possible that a combination of inhibition and toxicity were experienced by the cultures tested, though the assumption of Haldane inhibition combined with the proposed toxicity model did not result in better model fits to the experimental observations.

The proposed toxicity model produced $K_{t,cDCE}$ values in the different systems, from low to high: batch-fed < CFSTR < RPC (see Table II, and Table I of part I). This suggests batch-fed cells were the most sensitive to toxicity, followed by the CFSTR grown cells, with attached cells in the RPC being the least sensitive. This trend is consistent with other observations of increased resistance often found in chemostat cells compared to batch-fed cells (Chang and Alvarez-Cohen, 1996; Piringer and Bhattacharya, 1999; Qu and Bhattacharya, 1997) and in attached growth cells compared to batch suspended cells (Harrison et al., 2007; Stewart and Costerton, 2001; Walters et al., 2003), or chemostat suspended cells (Anwar et al., 1992; Welin et al., 2003). No attached growth was visually observed in the previous batch experiments (Sabalowsky and Semprini, in press), or in the CFSTR reactor. We therefore believe the different toxicity coefficients reflect differences in the biological and transport processes of suspended versus attached cell systems regarding toxicity or protection from it.

While the long retention times of 16.8 and 25.3 days used through most of our CFSTR operation seem to be of a similar magnitude to the 60-day batch experiments presented in part I (Sabalowsky and Semprini, in press), the CFSTR is distinctly different in the continued slow addition of TCE instantaneously mixed into the reactor versus daily spikes of high ($\sim 800 \mu\text{M}$) TCE concentrations in the batch-fed systems. The reasons for enhanced

resistance in chemostat versus batch-fed cells are still not fully known, though cells in exponential growth, such as can occur in batch-fed systems, appear to be more susceptible to toxic chemical exposure than those in a stationary phase, as occurs in continuously fed systems (Piringer and Bhattacharya, 1999). Toxicity as it relates to exponential growth, stationary cells, acclimated chemostat-grown cells tested in batch-toxicity experiments, and resulting community transcriptional responses would be necessary to illuminate such differences observed in our experiments and subsequent toxicity coefficient differences.

Mechanisms that may improve resistance of attached cells compared to planktonic cells can include cell signaling, diverse phenotypes, diffusion limitations, biosorption, and cell differentiation within biofilms (Harrison et al., 2007). Diffusion limitation is only one of the possible reasons for attached cell resistance, even in a fully penetrated biofilm. For example, a *Pseudomonas* biofilm has exhibited antibiotic resistance despite full antibiotic penetration of the biofilm (Walters et al., 2003). Future experimentation related to attached cell resistance mechanisms described by Harrison et al. (2007) would be required to understand the differences between the RPC and CFSTR responses to high CAH concentrations and related toxicity coefficients modeled here.

The inability of Haldane or threshold inhibition models to describe these CFSTR and RPC data suggests the proposed toxicity/decoupler model best characterizes the observed effects of long-term exposure to high CAH concentrations. The ratios of fitted $K_{t,cDCE}$ and $K_{t,TCE}$ fitted values further supports the concept of toxicity being proportional to cell wall partitioning concentrations (Chu, 2004; Chu et al., 2006; Isken et al., 1999). Future work is required to more directly test the toxicity model proposed in this study, both in batch-fed growth, and in alternative growth modes.

Funding for this research was provided by NSF Integrative Graduate Education and Research Traineeship Program (IGERT), U.S. EPA Western Region Hazardous Substance Center (Grant # R-828772), and National Institute of Environmental Health Sciences (Graduate Training Grant #1P42 ES10338). This article has not been reviewed by the above agencies and no endorsement should be inferred.

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