Sulfide Inhibition Effect on *Dehalococcoides* Strain 195's Ability to Biodegrade Trichloroethene and *Desulfovibrio vulgaris* Hildenborough's Cell Growth

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ABSTRACT

Trichloroethene (TCE) is a prevalent groundwater contaminant across the USA. *Dehalococcoides* is the only known bacterium that can reductively dechlorinate TCE to the benign end product ethene. Sulfate is also a common ubiquitous compound in groundwater. Little research has been conducted on the toxicity of sulfate or sulfide on *Dehalococcoides* and its effect on dechlorination. This study evaluated the inhibitory effects of high sulfate/sulfide concentrations on pure *Dehalococcoides mccartyi* strain 195 (*Dhc*), pure *Desulfovibrio vulgaris* Hildenborough (*DvH*), and a syntrophic co-culture containing *Dhc* and *DvH*. Pure *Dhc* cultures containing 2mM sulfide showed a 40% decrease in the TCE reduction rate and a 38% decrease in cell growth. 5mM sulfide pure *Dhc* cultures showed a 57% decrease in the TCE reduction rate and a 65% decrease in cell growth. DvH was maintained using lactate as the energy and carbon source and sulfate as the electron acceptor. Pure *DvH* cultures containing 5mM sulfide showed no significant differences in cell growth compared to the control; whereas, 10mM and 20mM showed an 80% reduction in cell growth. In the syntrophic co-culture, *DvH* fermented lactate to H_2 and acetate, and *Dhc* used H_2 as the electron donor for dechlorination. The dechlorination rate in cultures containing 5mM sulfate decreased 2 fold and have a 6:1 *DvH* to *Dhc* ratio. These results indicate that sulfide has an inhibitory effect on *Dhc's* dechlorination and cell growth rates. Sulfide inhibition on *Dhc* could negatively impact the efficiency and time frame in which a contaminated site is remediated

KEYWORDS

chlorinated compounds, bioremediation, cell growth, TCE degradation rates, sulfide inhibition.

INTRODUCTION

Available water per capital has plummeted as the global population has grown four fold since 1900 (Terrapin Water Fund). Because of our increasing water demands, it is more important than ever to work towards reliable and sustainable methods of cleaning contaminated fresh water. Over 300,000 groundwater sites in the U.S. are contaminated with chemicals and contaminants used by industrial, agricultural, governmental, and other corporate companies (Kline 2014). In order to relieve the global water crisis, research and focus towards remediation of these contaminants needs to be addressed in order to free up currently unusable water.

Starting in the 1950's, Trichloroethene (TCE) was used as a common metal degreasing agent in the manufacturing, military and food production industry (Bakke et al. 2007). Thirty years later, the Environmental Protection Agency (EPA) discovered TCE was a suspect carcinogen and that its byproducts were known carcinogens (Agency for Toxic Substance and Disease Registry 1997). Unfortunately, by this time, improper management of this hazardous material led to widespread chemical leakages onto the soil and into groundwater (Wisconsin Department of Health Services: Trichloroethylene 2013).

Bioremediation, which is the use of microorganisms remove or neutralize a pollutant has became a popular method used to clean up TCE contaminated sites because of its effectiveness and relatively low cost. There are many bacteria that can degrade TCE; however, the compounds that they degrade it to are just as harmful or more harmful than TCE. To this day, the only known bacterium that can degrade TCE to the benign end product, ethene, is *Dehalococcoides* (*Dhc*) (Panagiotakis et al. 2014). Figure 1 shows the stepwise process of how TCE is degraded to ethene.
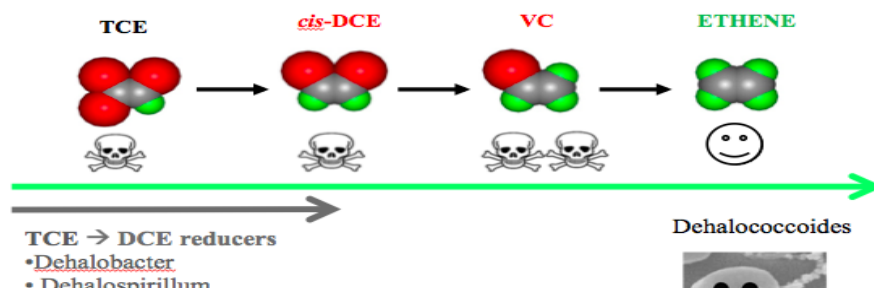
Figure 1. shows the stepwise process through which TCE is degraded to its by-products cis-dichloroethene (cis-DCE), vinyl chloride (VC), and ethene. There are many bacteria such as the ones listed below TCE that can degrade it up until VC, but that is a more toxic version of TCE. Only *Dhc* can degrade TCE to ethene.

Because of its importance to human health through water decontamination, there has been a lot of focus on identifying what other organisms or compounds may hurt or enhance *Dehalococcoides' (Dhc)* ability to biodegrade TCE (Heimann et al. 2005). Research has identified 5 things thus far that it needs to survive which are: acetate as its food source, hydrogen as its electron donor, vitamin B_{12}, TCE as its electron acceptor, and an anaerobic environment. Understanding how *Dhc* interacts with the inorganic and organic components of the subsurface will allow for faster decontamination as well as provide biological insights into this unique microorganism.

Sulfate and sulfide are two of the most commonly found elements that co-exist with TCE in groundwater. They have recently become compounds of concern because research suggests a link between the presence of sulfide and decreased TCE biodegradation rates (Aulenta et al. 2007, He et al. 2005, Panagiotakis et al. 2014). When *Dhc* and sulfide come in contact, *Dhc's* ability to function in the subsurface and utilize TCE decrease (He et al. 2005). This link however, cannot be fully confirmed because researchers have mainly focused on the effects of sulfide on mixed microbial consortiums (MMCs), which are systems with many different types of bacteria (Pantazidou et al. 2011). When researchers use a MMC, they cannot be confident on the mechanisms of inhibition because of the many complex biological pathways. Questions like is sulfide inhibiting *Dhc* or is it the competition for resources have come up frequently? But before we can answer these questions it is important to understand how sulfide is being produced in the subsurface. A group of microorganisms called-- sulfate reducers-- play a large role in this story because

one of the functions they can perform is converting sulfate to sulfide. One of the most common sulfate reducers to co-exist with *Dhc* is a sulfate reducer called *Desulfovibrio vulgaris* Hildenborough *(DvH).* Below shows the sulfate reduction reaction that sulfate reducers carry out in order to obtain energy. The ΔG for this reaction is -151.9 kJ/rxn (Muyzer et al, 2008).

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$$

The two main controversies over the effects of sulfide are: at what concentration does it have a negative effect on *Dhc* and *DvH* and how do these organisms affect each other when there is no sulfate or sulfide present? **My goal** is to resolve the gap in knowledge concerning sulfide inhibition on TCE biodegradation. Knowing what concentrations of sulfide inhibit *Dhc* growth and TCE degradation rates will significantly improve the bioremediation process and tactics used for TCE contaminated plumes.

METHODS

Site description and research design

The cultures, chemicals and analytical skills for this research were obtained through my mentor Xinwei Mao in Dr. Lisa Alvarez-Cohen's laboratory (LAC). My bench scale experiments were also carried out in the LAC lab located in O'Brien Hall.

 All batch reactors were filled with 60mL of sterile liquid medium described by Cole et al 1994. The headspace consisted of a hydrogen and carbon dioxide gas mixture (80:20, vol H_2/vol CO_2) and were sealed with an air tight, blue butyl rubber stopper and crimped with a metal seal. An indicator (resazurin) was also added to ensure that the reactors remained anaerobic throughout the experiment.

Nine reactors were inoculated with a pure *Dhc* culture that was obtained from my mentor, Xinwei Mao. The reactors were then amended with 5mM acetate, 5mM filtered vitamin B12, 7uL of pure TCE and varying concentrations of sulfide. Of the nine reactors, three were allocated to the control (0mM Na_2S), three were fed 2mM Na_2S, and three were

fed 5mM Na_2S (Table 1). Twelve reactors were inoculated with a pure *DvH* culture that was obtained from Weiqin Zhuang (a postdoc in the lab).

Table 1. Experimental Design of Batch Reactors.

Culture Type	Carbon Source	Electron Donor	Electron Acceptor	Headspace	Sulfate/Sulfide Concentration (mM)
Pure strain 195	Acetate	Hydrogen	TCE	H_2/CO_2	Sulfide: 0, 1, 2.5, 5
Pure *DvH*	Lactate	Hydrogen	Sulfate	N_2/CO_2	Sulfide: 0, 5
Co-culture containing strain 195 and *DvH*	Acetate	H_2/Lactate	TCE/Sulfate	N_2/CO_2	Sulfate: 0, 5
Note: All reactors were fed 1mL filtered vitamins					

As indicated in Table 1., the reactors were amended with 1mL filtered vitamin solution, 5mM lactate, 2mM sulfate, and varying concentrations of sulfide (5mM, 10mM, 20mM Na_2S). Of the twelve reactors, three were allocated to the control (0mM Na_2S), three were fed 5mM Na_2S, three were fed 10mM Na_2S, and three were fed 20mM Na_2S (Table 1). The reactors containing a co-culture of *DvH* and *Dhc* were amended with 5mM lactate, 5mM filtered vitamin B12, 7uL of pure TCE and varying concentrations of sulfate (0mM and 5mM SO_4) (Table 1). After cultures were inoculated, they were kept in a dark, 34°C stationary incubator.

Analytical philosophy and data collection details

I measured *Dhc's* ability to reduce TCE using a gas chromatograph (Flame Ionize Detector) (GC-FID). The GC-FID model I used was from Agilent Technologies and it is refurbished every year. I use the "TCE Modified" method and standard curve created by postdoc, Shan Yi. In order to measure the amount TCE reduced, I took 100uL headspace

samples using a glass syringe. TCE is a volatile organic compound and partitions between the headspace and the liquid and is most commonly measured via headspace. I used Henry's constant at room temperature (34°C) to account for the partitioning of TCE between the gas and liquid phase when calculating the total TCE concentration (Gossett 1986).

Cell collection method

In order to quantify the cell concentrations in my reactors, I collected 1.5mL liquid samples from the pure *Dhc*, pure *DvH*, and co-culture (*Dhc* and *DvH*) cultures. I extracted DNA using a DNeasy Blood & Tissue Kit, and then performed quantitative (real-time) polymerase chain reaction using an Applied Biosystems Real-Time PCR System to quantify cell numbers (Ausubel et al. 1998). This allowed me to understand the culture's biomass production rates and resistance to sulfate or sulfide inhibition. I took cell samples based on the culture's doubling rates for the pure and co-cultures. I measured sulfate concentrations using an ion chromatograph to support the biomass data as to whether or not *DvH* is utilizing sulfate or if complete inhibition is occurring.

Data analysis methods

I analyzed the data from the gas chromatograph, ion chromatograph, and qPCR machine via Excel and R Commander. Using these programs, I demonstrated the relevance and significance of the TCE degradation max. rates, biomass growth rates, and sulfate conversion using a student t-test in R. I constructed a scatter plot in Excel from the gas chromatograph/ion chromatograph data and a bar graph from the qPCR data. Error bars and standard deviations were calculated using Excel to account for biological diversity and normality within the triplicates. A paired t-test was applied to the cellular quantification data to determine if the difference in growth between the experimental reactors and the control was significant.

RESULTS

TCE Dechlorination and cell growth of pure *Dehalococcoides* strain 195 cultures

Compared to the control, Pure *Dhc* cultures containing 2mM and 5mM sulfide were significantly inhibited as shown through limited cell growth and reduced TCE degradation rates. The overall dechlorination rates in both experimental cultures decreased when compared to the control—a 40% rate reduction in the 2mM sulfide cultures, and a 57% rate reduction in the 5mM sulfide cultures. In terms of total TCE degraded, the 2mM reactors only degraded 22% of the total TCE and the 5mM reactors only degraded 16% of the total TCE. Also, there was no significant vinyl chloride or ethene production in both sets of reactors amended with 2mM and 5mM sulfide (Figure 2).
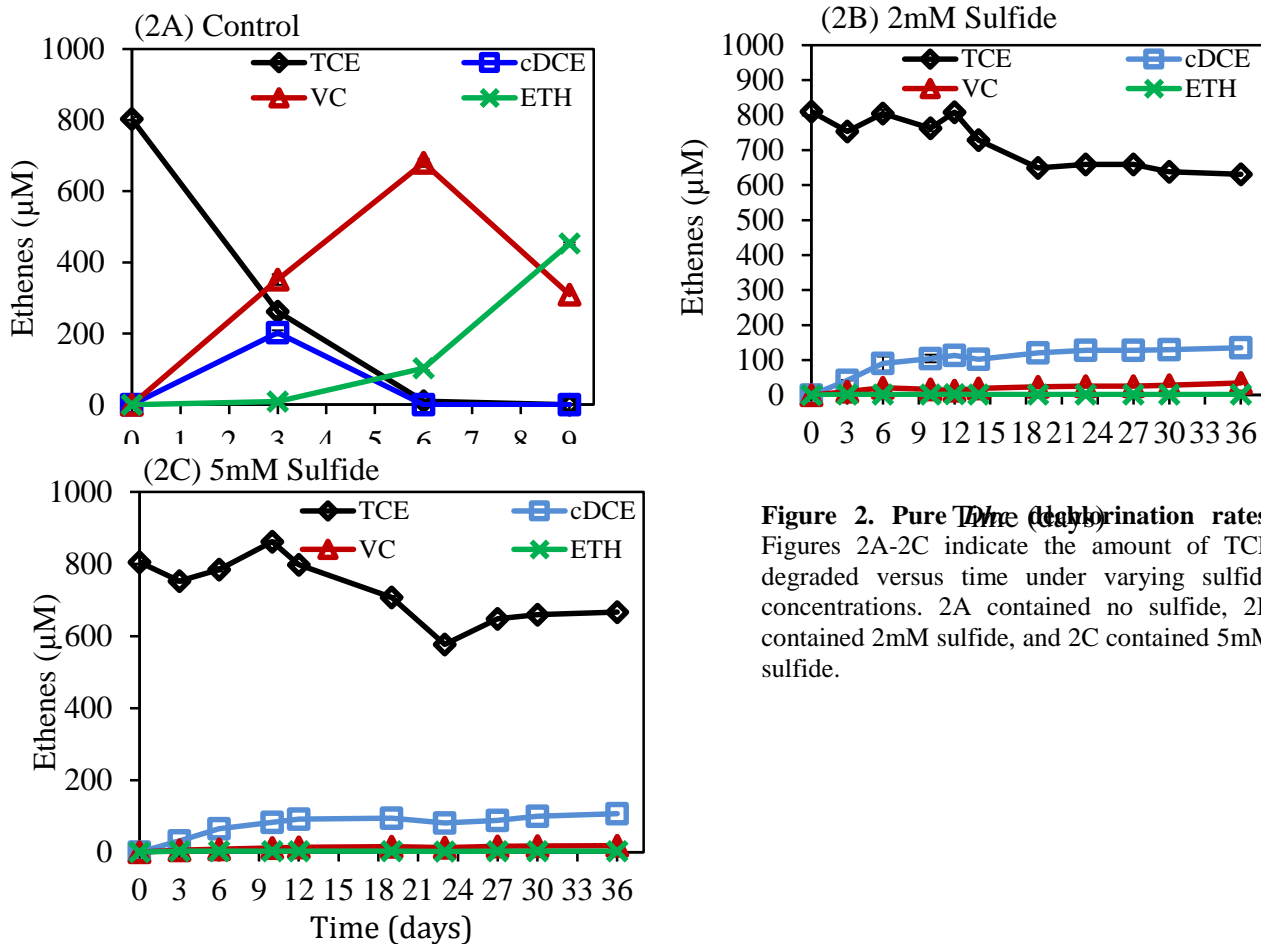

Figure 2. Pure *Dhc* dechlorination rates. Figures 2A-2C indicate the amount of TCE degraded versus time under varying sulfide concentrations. 2A contained no sulfide, 2B contained 2mM sulfide, and 2C contained 5mM sulfide.

Pure *Dhc* cultures containing 2mM and 5mM sulfide showed significantly less cell growth than the control reactors. Compared to the control (9.10E+07 cells/mL), 2mM cultures showed a 38% decrease in cell growth (5.60E+07 cells/mL) at day end[1], and 5mM cultures showed a 65% decrease in cell growth (3.20E+07 cells/mL) at day end (Figure 3).
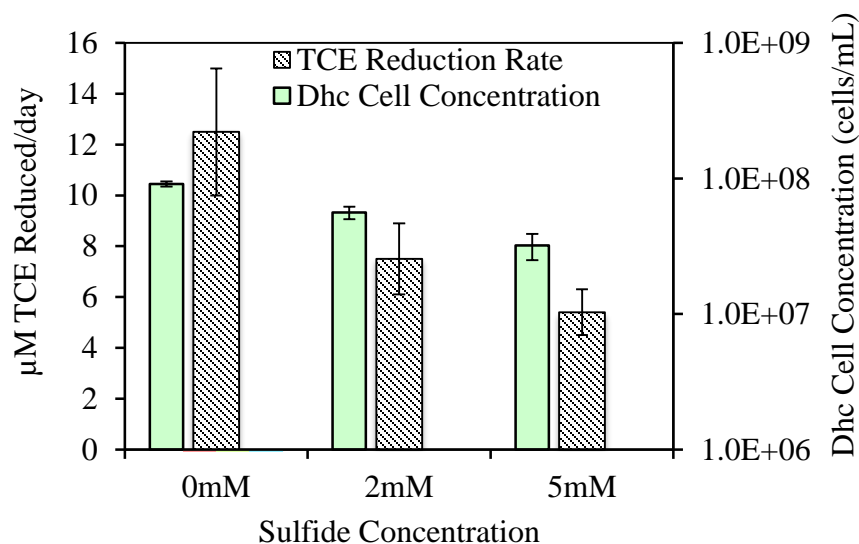

Figure 3. Pure *Dhc* dechlorination rate (µM reduced/day) and cell concentration after TCE degraded. This figure shows the coupling between the TCE reduction rate and cell concentration with increasing concentrations of sulfide.

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[1] Day end refers to the cell collection data point when *Dhc* consumes all TCE. If not all the TCE was consumed then a data point was collected at a representative time that was similar to the control.

Cell growth results of pure *Desulfovibrio vulgaris* Hildenborough

       DvH cultures containing 10mM and 20mM sulfide exhibited diminished cell growth when compared to the control (Figure 4). At day 6, neither culture doubled the time zero concentration of 2.90E+08 cells/mL. At the end of the experiment, the control reactors (1.35E+09 cells/mL) had an order of magnitude greater cell concentration than the 10mM (9.96E+07 cells/mL) and 20mM (1.08E+08 cells/mL) sulfide reactors.

       The cultures exposed to 5mM sulfide were not as inhibited as those exposed to 10mM and 20mM sulfide. The 5mM reactors (1.25E+09 cells/mL) had similar cell concentrations at day to the control (1.35E+09 cells/mL, P-value = 0.1409). Both pure *DvH* cultures containing 10mM and 20mM sulfide had an 80% reduction in cell growth over the 6 day measurement period; whereas, the 5mM sulfide cultures doubled.
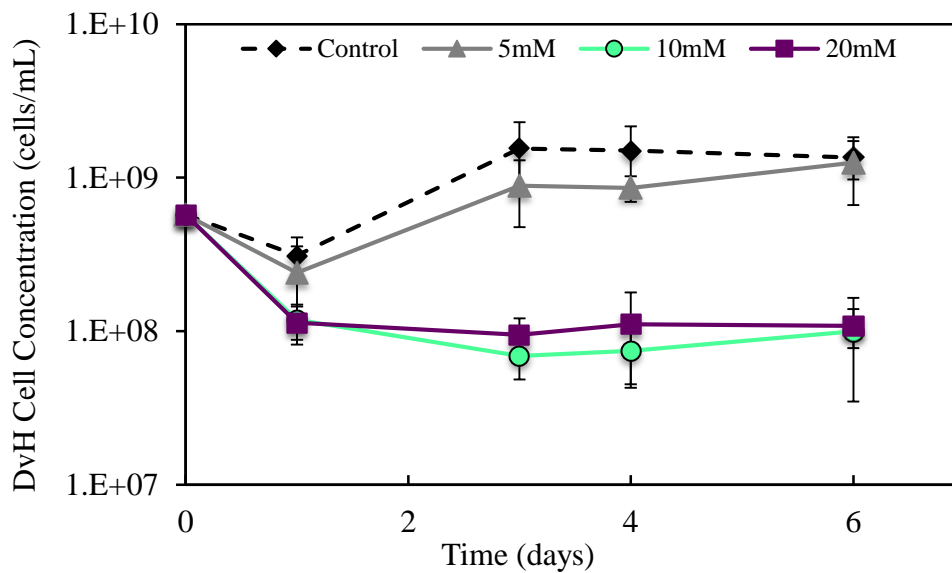

Figure 4. Desulfovibrio *vulgaris* Hildenborough's cell growth.
This figure shows the effects of varying concentrations of sulfide on *DvH*'s cell growth over time.

TCE Dechlorination and Cell Growth Results of a Co-Culture Containing *Dehalococcoides* strain 195 and *Desulfovibrio vulgaris* Hildenborough

The co-cultures containing both *Dhc* and *DvH*, stalled after day 7 in the presence of 5mM sulfate[2]. In the 5mM reactors, TCE was degraded 1.7 times slower than in the control reactors. After the 12-day measurement period, the 5mM sulfate cultures, only converted 69% of the TCE into cDCE and VC, and there was no ethene production.
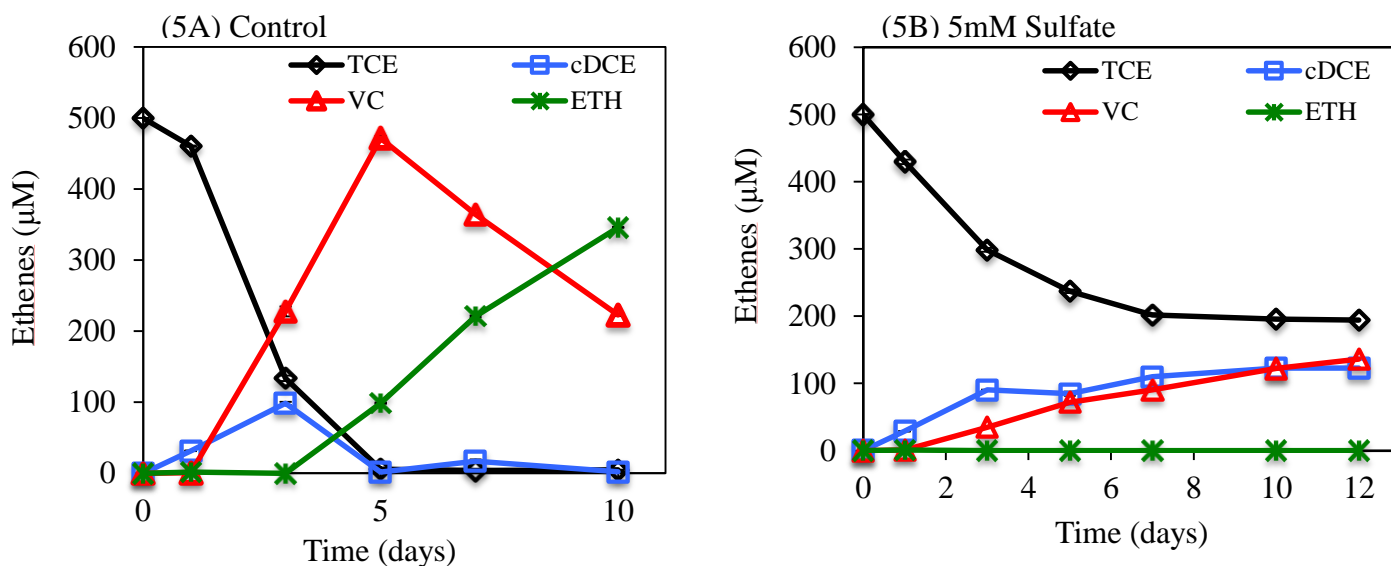

Figure 5. Co-culture containing *Dehalococcoides* and Desulfovibrio *vulgaris* Hildenborough's dechlorination rates. Graph 5A shows the TCE degradation curve of the co-culture without additional sulfate and graph 5B shows the TCE degradation curve of the co-culture with an additional 5mM sulfate.

Due to the carbon source and electron acceptor conditions in the control and 5mM sulfate, there was a distinct difference in the community composition. In the co-cultures that were grown with sulfate (5mM) and lactate (10mM), *DvH* (1.58e+08 cells/mL) grew

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[2] It was assumed that all sulfate was converted to sulfide within the first day due to stoichiometric favorable conditions

in higher concentrations than *Dhc* (2.27e+07 cells/mL), which is a 6:1 ratio. Whereas, in co-cultures that only contained lactate, *Dhc* (9.78e+07) grew at higher concentrations than *DvH* (2.43e+07), which is a 1:4 ratio.
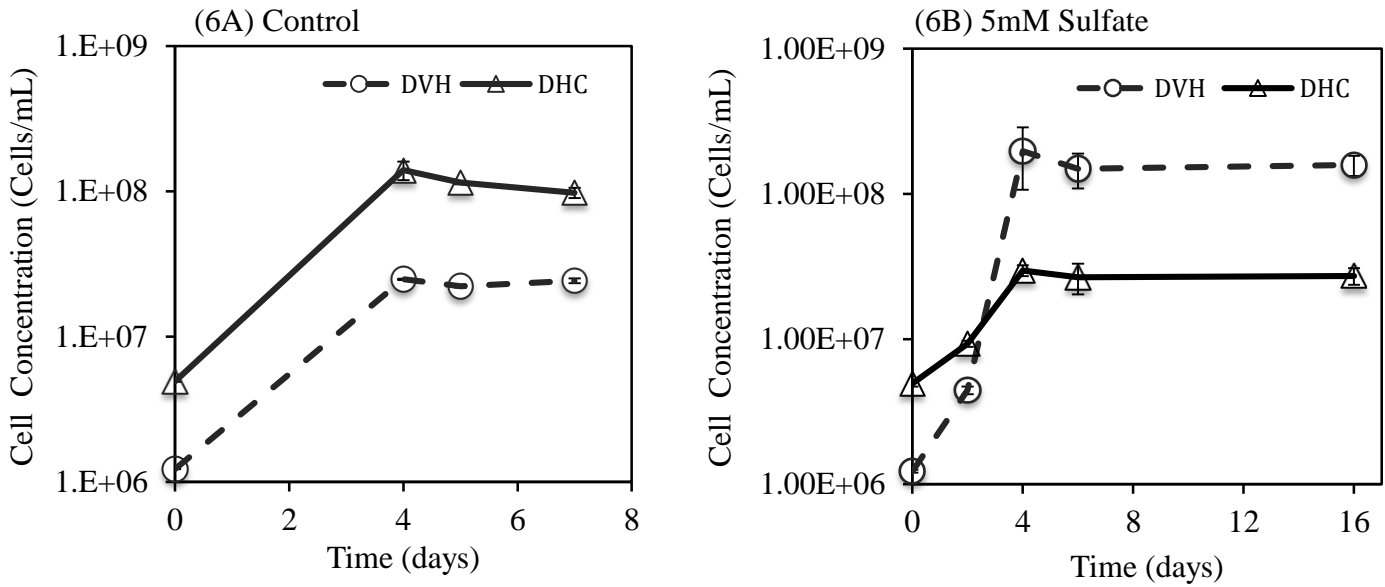

Figure 6. Co-culture containing *Dehalococcoide* and Desulfovibrio *vulgaris* Hildenborough's growth curve. Graph 6A shows the growth curve of the co-culture without additional sulfate and graph 6B shows the growth curve of the co-culture with an additional 5mM sulfate.

DISCUSSION

Pure *Dehalococcoides* strain 195

Biogeochemical inhibitors such as sulfate and sulfide have been a point of interest in the dechlorination field for the past decade. Many sites contaminated with TCE are not fully remediated due to the fact that certain inorganic and organic compounds stall TCE reduction to ethene. There is limited research on the inhibitory effects of sulfate and sulfide on pure and co-cultures containing *Dhc* strain 195 and *DvH*. The only research paper that directly studied the effects of sulfate and sulfide on a pure *Dhc* culture is by He et al. 2005. I used this paper to formulate my experimental design as well as compare my results based on the fact that even though He et al and I did not use the same strain of *Dhc*, the species as a whole is >85% identical at the amino acid level (Morris et al. 2007). This amount of similarity provided the bases for my comparisons of my results. This paper studied the effects of 10mM sulfate and 5mM sulfide on pure culture *Dhc* strain FL2 (*Dhc* FL2), He et al. found that 10mM sulfate showed no inhibitory effects on dechlorination rates or cell growth rates, and that 5mM sulfate resulted in a complete shut down of dechlorination activity. Their findings on sulfate were similar to my preliminary results (not shown), which tested 5mM sulfate on pure *D. mccartyi* strain 195 and showed that there was no inhibitory effect on dechlorination rates. My results also aligned with He et al's regarding the addition of 5mM sulfide to pure *D. mccartyi* strain 195. My study found that 5mM sulfide resulted in a 57% decrease in the TCE reduction rate as well as a 65% decrease in the cell growth rate. These results correspond with He et al's description of *Dhc* FL2's reaction to 5mM sulfide, in which the culture went through a complete shut down of dechlorination activity.

A notable difference between He et al's pure *Dhc* study and mine was that they tested 1mM sulfide on *Dhc* st. FL2 and showed no inhibition; whereas, my study tested 2mM sulfide as the intermediate concentration and showed significant inhibition. When 2mM sulfide was added to my pure *Dhc* st.195 cultures, dechlorination activity as well as cell growth shut down. My study showed a 40% reduction in the TCE dechlorination rate and a 38% reduction in the cell growth rate; whereas, He et al specifically states that, "no

inhibition was observed at 1mM [sulfide] concentration". Our dissimilar results could indicate that there is a sulfide threshold between 1mM and 2mM or that different strains of *Dhc* may have different sulfide resiliencies. My results indicated that both 2mM and 5mM sulfide interfere with *Dhc*'s ability to function and reduce TCE.

Pure *Desulfovibrio vulgaris* Hildenborough

Pure *DvH* reactors that were exposed to 5mM sulfide showed no significant cell growth inhibition *and* had comparable cell growth rates to the control reactors (1.35E+09 cells/mL). This result is consistent with previously reported data that showed *Desulfovibrio* is not affected by 5mM sulfide (Dalsgaard et al. 1993). My research also corresponded with previous studies that acknowledged *Desulfovibrio's* resistance sulfide to a certain degree (Dalsgaard et al. 1993).

When *DvH* was exposed to 10mM and 20mM sulfide concentrations significant inhibition cell growth inhibition was observed. My results showed an 80% reduction in cell growth for the 10mM and 20mM sulfide reactors, which corresponds to previous research that has shown growth inhibition anywhere from 7mM sulfide (Okabe et al. 1994) to 16mM sulfide (Reis et al. 1992). Sulfide inhibition levels for *DvH* could indicate a maximum threshold for a community (Berggren et al. 2013). If *DvH* is inhibited then other microorganisms will not likely thrive as well due to *Desulfovibrio's* higher tolerance to sulfide. *DvH* could indicate the well being of the dechlorinating community for TCE contaminated sites.

Co-Culture containing *Dehalococcoides* strain 195 and *Desulfovibrio vulgaris* Hildenborough

My research on the effects of sulfide on a co-culture containing *Dhc* strain 195 and *DvH* is novel in this particular field. The closest studies I have to compare are ones that test the effects of sulfide on MMCs. These comparisons can be useful; however, it should also be noted that the interactions in a co-culture and a MMC might be very different and

not as comparable. A co-culture study is novel and shall serve as a potential basis for future *Dhc* and *DvH* co-culture studies to be based upon.

My study differed from many of the MMC studies based on the sulfide concentration present in the reactors. I studied the effects of 5mM sulfate, which was converted to sulfide during the exponential phase; whereas, most MMC papers looked at concentrations ranging form 0.6mM-2.5mM. When 5mM sulfate was introduced into the *Dhc* and *DvH* co-culture, reductive dechlorination stalled after 61% of the TCE was degraded; whereas, the control degraded all of the TCE in 5 days. Previous research showed a similar trend where 2.5mM sulfate slowed the dechlorination rate of TCE by a third (Heimann et al. 2005). Once *DvH* converts sulfate to sulfide, the sulfide could be interacting with *Dhc's* cellular function and enzyme activity, thus resulting in a lower dechlorination rate.

In terms of community composition, the presence of sulfate has a large impact on which organism will dominate in the co-culture. When sulfate was present *DvH* was supplied with ample electron donor, electron acceptor, carbon source, and medium to survive. *DvH* did not have to rely on *Dhc* for any assistance. Therefore, *DvH* was able to out compete *Dhc*. However, when sulfate was not added (i.e. the control), *DvH* had to ferment lactate as its carbon source. This resulted in a mutualistic relationship between *DvH* and *Dhc* due to the fact that *DvH* had to rely on *Dhc* to consume hydrogen so that it would continue to be thermodynamically favorable for *DvH* to reduce lactate. There is a limited amount of previous research that has also shown a shift in a community's composition in the presence of sulfate that supports the results from my study as well (Berggren et al. 2013). This result could imply that the available source of electrons may have a large influence on which member of a community will be able to outcompete the other organisms all else equal.

Limitations

One of the main limitations of my study was the access to an ion chromatograph. The ion chromatograph is able to measure the concentration of ionic compounds to very low concentrations (<0.1mM). Because the machine was not working in the lab, I was not able to obtain precise sulfate measurements in the co-culture—before or after *DvH* consumed the amended sulfate. Instead, I had to base my results off of previous data that showed sulfate is consumed within the first few days due to its negative delta G (-262 kJ/rxn) and previous data (not shown) (Heimann et al 2005). Having ion chromatograph data would provide evidence of complete sulfate reduction to sulfide.

Future Direction

Future research could include analyzing *Dhc's* ability to degrade TCE after a third microbe is introduced into the reactor. A possible addition to the co-culture could be the bacterium, Syntrophomonas. *Syntrophomonas* would ferment carbon substrates such as lactate or crotonate and would act as a competitor for resources and electron donor. The addition of another microbe would provide additional pressures on the ecosystem resources. This added pressure would provide a new system to study sulfide inhibition under.

Conclusion/Broader Implications

This research will broaden the scientific community's understanding of the effects of sulfide inhibition on *Dhc* and its behavior under varying sulfate and sulfide concentrations. My research highlighted the discrepancy between the sulfide concentrations that inhibited *Dhc* and the sulfide concentrations that inhibited *DvH*. This research will provide key information concerning the maximum TCE degradation and cell growth rates under environmentally relevant sulfide conditions. Specifically this research was able to better predict the inhibitory ranges of sulfide, which are approximately 5mM HS- and above Also, if there is no sulfate present in the groundwater, *DvH* and *Dhc* can

co-exist and bioremediation using *Dhc* can be implemented. It is important to maintain active *Dhc* populations at TCE contaminated sites in order to optimize the decontamination process. Improving our understanding of the interactions between *Dhc*, *DvH*, and sulfide conditions will allow engineers and scientists to design more effective *in situ* bioremediations systems for TCE contaminated groundwater.

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