REDOX CONDITIONS DETERMINE MICROBIAL COBAMIDE PRODUCTION IN HYPORHEIC SEDIMENT ENRICHMENTS

A Thesis Presented for the

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Degree

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To my graduate advisor, Dr. Frank Löffler, for leading my transition from an animal scientist to microbiologist.

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ABSTRACT

All kingdoms of life rely on cobalamin, a coenzyme produced by a subset of Bacteria and Archaea. Cobalamin belongs to a family of molecules called cobamides. Cobamides all share a tetrapyrrole corrin ring which is joined to one of 17 known naturally occurring lower bases. Until now, little work has explored why one type of cobamide is produced over another. This research explores cobamide production under different redox conditions: glucose fermentation, lactate fermentation, methanogenesis, sulfate reduction, nitrate reduction, and iron reduction. Homogenized sediment from Third Creek (Knoxville, Tennessee) was used for initial microcosms grown under each redox condition and fifth generation or later transfers were used to analyze cobamide production. Type and quantity of cobamide produced under each condition was distinct. Cobamide production was normalized between redox conditions by comparing total cobamide produced to the amount of substrate or electron acceptor consumed. Glucose and lactate fermenters both resulted in 5-hydroxybenzimidazole cobamide and methylbenzimidazole cobamide. Methanogens produced exclusively methylbenzimidazole cobamide. Sulfate reduction and nitrate reduction both produced cobalamin. No detectable cobamide was produced by iron reduction. Maintenance of redox conditions were verified by monitoring the reactants and products for each redox process. This work demonstrates that redox conditions shape the cobamide pool. The results have important implications for microbial ecology including in bioremediation systems where corrinoid-auxotrophic bacteria require certain types of cobamide for metabolic processes that break down pollutants.

PREFACE

This work is a continuation of a project begun by Dr. Burcu Şimşir, a 2015 PhD graduate of the Löffler lab. Work she completed that contributed to this project has been attributed to her where applicable.

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CHAPTER ONE

INTRODUCTION

Microbial Cobamide Production

Cobalamin is an essential enzyme prosthetic group (co-factor) in all kingdoms of life, yet it can only be *de novo* synthesized by some bacteria and archaea. Microbes require cobamides for essential biological functions including methyl group transfer, carbon skeleton rearrangement, and organohalide respiration (Fang, Kang, & Zhang, 2017; Yan et al., 2018). Cobalamin is broadly studied for its impact on animal and human health, but the other sixteen known cobamides remain largely unexplored (Warren, Raux, Schubert, & Escalante-Semerena, 2002; Yan et al., 2018).

Cobamides other than cobalamin are largely understudied since cobalamin is generally considered the only cobamides relevant to human health (Roth, Lawrence, & Bobik, 1996). Much of the detectable corrinoid produced in biological systems, including the human gut, is not cobalamin, and less than 2% of corrinoid found in human feces is cobalamin (Allen & Stabler, 2008). In dairy cattle, the amount of non-cobalamin cobamides present in the digestive tract was found to exceed the amount of cobalamin (Girard, Berthiaume, Stabler, & Allen, 2009).

In recent years, new research has shown that these other cobamides do serve biological functions for microbes and are therefore relevant for human and animal health (Keller et al., 2018; Mok & Taga, 2013; Yan et al., 2018). Many microbes are capable of producing multiple types of cobamides, but it is unknown what causes them to produce one type over another (Crofts, Seth, Hazra, & Taga, 2013). Cobamides are energetically costly so it would be unreasonable for an organism to produce such a molecule if it serves no purpose. Currently there are no known altruistic cobamide producers (Dominique Turkowsky, 2018; Shelton et al., 2018; Warren et al., 2002). Microbes including *Lactobacillus* and *E. coli* have been reported to uptake up to 11 different cobamides to meet metabolic requirements; many microbes have the ability to scavenge complete cobamides or cobamide precursors from the environment and remodel them into

functional types (Yan et al., 2016; Yi et al., 2012). *Dehalococcoides mccartyi* (*Dhc*) is one such corrinoid auxotroph that requires cobamides for its energy metabolism, which is limited to organohalide respiration.

Corrinoid Nomenclature

The term corrinoid refers to any molecule built on a corrin ring ("Nomenclature of Corrinoids," 1975) (Figure 1-1). A complete, functional cobamide consists of four pyrole rings joined together to form a corrin ring with a central coordinated cobalt atom. This tetrapyrrole ring is joined by a nucleotide loop to a lower base and an upper ligand, denoted as an R group (Figure 1-2). A cobamide lacking a lower base is referred to as a cobinamide. Cyanocobalamin**,** synthetic vitamin B_{12}, carries a cyanide as upper ligand and does not occur in nature, but many organisms can replace the cyanide group with a methyl, adenosyl, or hydroxyl group to form biologically functional cobalamin (Froese & Gravel, 2010). Cobamides with the different upper ligands are specifically referred to cyanocobalamin, adenosylcobalamin, or hydroxylcobalamin, respectively. The R group is noted in green as part of the cobalamin structure shown in Figure 1-1.

Seventeen naturally occurring cobamides differing in their lower bases have been discovered to date (Yan et al., 2018; Yi et al., 2012). While the majority of bacteria require cobamides for their metabolism, only about one third possess the genes required for complete de novo cobamide biosynthesis (Shelton et al., 2018). Cobamide lower bases consist of three types: benzimidazolic, phenolic, or purinyl. Benzimidazolic cobamide lower bases include dimethylbenzimidazole, the lower base of cobalamin. Phenolic lower bases are exclusively produced by *Sporomusa ovata* (Mok & Taga, 2013). The phenolic lower bases cannot coordinate to the central cobalt atom; these cobamides are permanently in the “base off” configuration that is not usable for most metabolic processes. The coordination of the lower base to the central

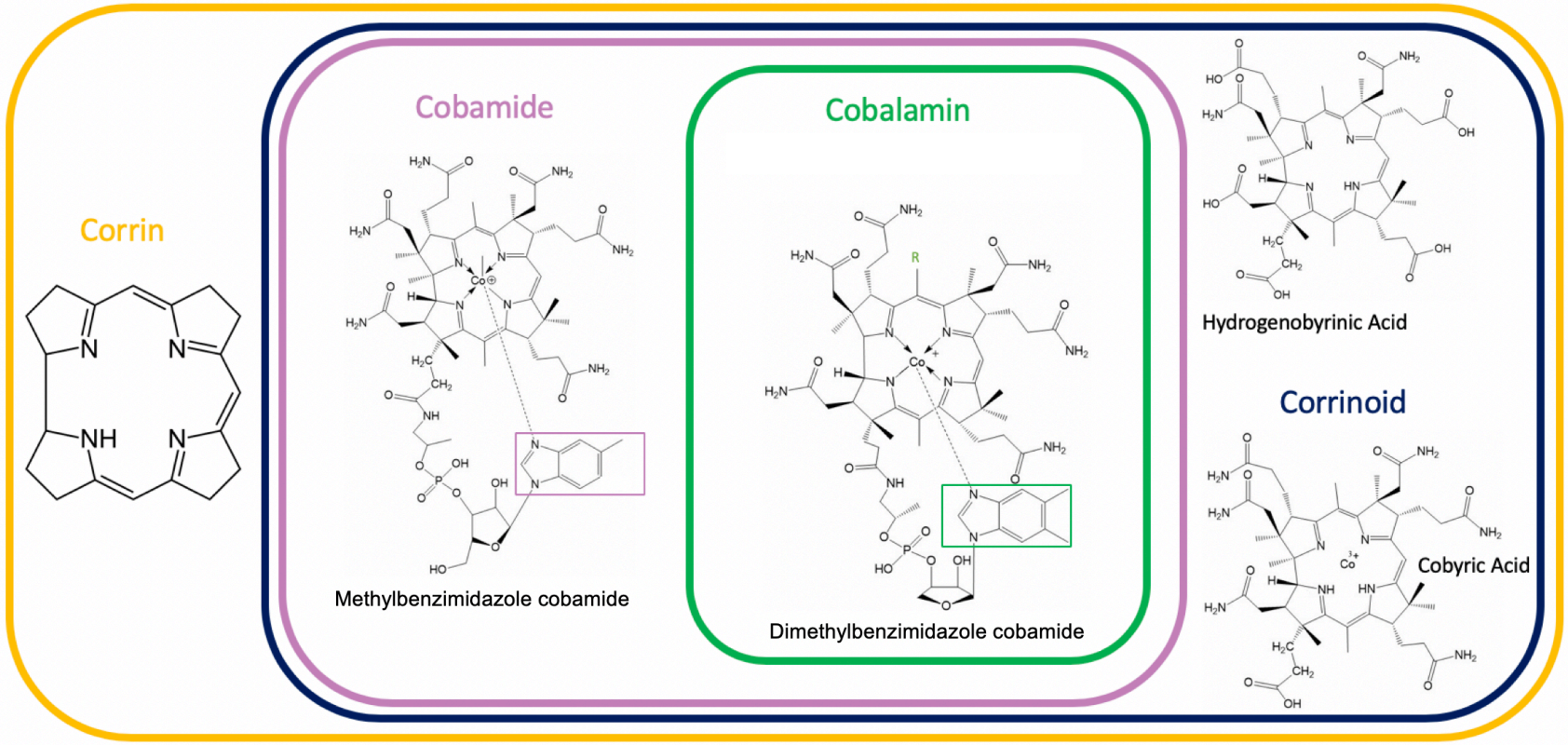

Figure 1-1.Corrinoid nomenclature. Corrinoids include any model built on a corrin ring, while cobamides specifically refer to a corrinoid with a nucleotide loop ending in one of 17 naturally occurring lower bases. Cobalamin is a cobamide with dimethylbenzimidazole as its lower base.

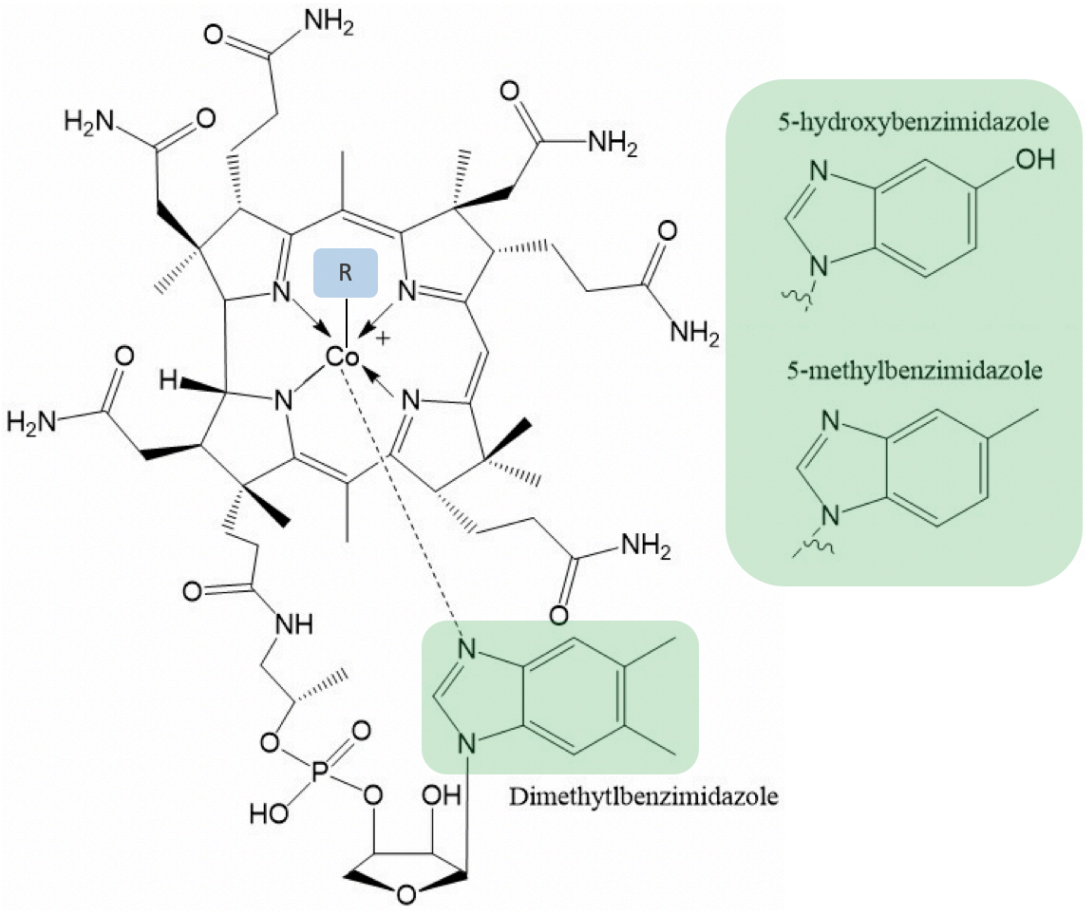

Figure 1-2. Structure of cobalamin with examples of lower bases, highlighted in green. R, in blue, represents where an upper ligand would coordinate with the central cobalt atom.

cobalt atom, the “base on” configuration, provides the functionality of the cobamide in most reactions the cobamide is used for (Roth et al., 1996) Benzimidazolic and purinyl lower bases can form an active “base on” configuration while phenolic cobamides are permanently in a “base off” configuration (Mok & Taga, 2013).

Functions of Cobamides

Cobamides serve an abundance of roles in nature including carbon and nitrogen metabolism, one-carbon metabolism via the Wood-Ljungdahl pathway, and reductive dechlorination (Shelton et al., 2018). The best known role is methyl group transfer in methionine synthesis, a reaction important to human health (Roth et al., 1996). Cobamides’ role in Acetyl CoA synthesis is their best-known use in anaerobes as part of the Wood-Ljungdahl pathway (Zhuang et al., 2014). Cobamides are also critical to methanogens for their function in methyl group transfer in methanogenesis (Roth et al., 1996). Organochlorine reductive dechlorination, utilized for bioremediation of toxic chlorinated solvents, can only be carried out using certain types of cobamides and the type of cobamides affects the rate of dechlorination (Yi et al., 2012). Understanding the conditions that lead to production of cobamides usable by organochlorine-respiring bacteria can improve the efficacy of bioremediation treatment.

Organochlorine Cycling

Organochlorines in pristine environments result from both biotic and abiotic processes. Many microbial taxa produce organochlorines and most of these compounds, including vancomycin and have only been coincidentally discovered in the last forty years (McIntyre, Bull, & Bunch, 1996). This is due to increased interest in finding naturally produced antibiotics (Gribble, 2010). Before discussing microorganisms that respire organochlorines, understanding why organochlorines are produced by other organisms can help to unravel why these organochlorine-respiring microorganisms came to exist.

Ocean-dwelling organisms produce of a plethora of natural organohalogens. While the primary use of these compounds is for defense, some organisms such as sea sponges produce organohalogens for their survival and may live in a mutualistic relationship with bacteria that can respire them (Abrahamsson, Ekdahl, Collen, & Pedersen, 1995; Gribble, 2010; Liu et al., 2017). Other marine animals that produce organohalogens include tunicates, nudibranchs, and soft coral (Gribble, 2010). While the majority of these marine organohalogens are brominated, marine algae *Asparagopsis taxiformis* and *Falkenbergia hillebrandii* show tetrachloroethene (PCE) and trichloroethene (TCE) formation rates that are high enough to be included when formulating global organochlorine production rates (Abrahamsson et al., 1995). Terrestrial organisms also produce organohalogens, more specifically organochlorines, although at a lower level than terrestrial organisms (Leri & Myneni, 2010). Chloromethane is naturally produced by many food crops as a nematicide and this chemical is available commercially for this purpose (Gribble, 2010).

Fungi in soil produce organohalogens and play a role in the incorporation of organochlorines into humus. Basidiomycetes are notable for their ability to synthesize organohalogens, and some produce up to 3% of their biomass dry weight as organically bound halogens (de Jong & Field, 1997). Basidiomycetes are part of the decomposition of forest leaf litter and may make a significant contribution to the natural pool of organochlorine (Gribble, 2010).

Basidiomycetes have also been shown to degrade chloroform and PCE under oxic conditions and could potentially be applied in bioremediation (de Jong & Field, 1997). Partly due to difficulty in identifying organochlorine species in low concentrations in environmental samples, the terrestrial chlorine cycle and role of anaerobic bacteria remains largely understudied. The most notable example of a bacterium producing an organochlorine is *Amycolatopsis orientalis* which produces the chlorinated antibiotic, vancomycin (McIntyre et al., 1996). Organochlorines can also be released from abiotic natural sources such as volcanoes, fires, rocks, and minerals (Gribble, 2010).

Chlorinated ethenes are one of the most widespread global groundwater contaminants. Although these chemicals are naturally present in small concentrations, humans have caused their pervasion in the environment. These highly volatile organic substances are toxic carcinogens that threaten human health and safety (Brandt-Rauf et al., 2012). Produced widely during the 20th century, attention was first brought to chlorinated solvents and groundwater quality when New Orleans residents complained of “chemical” and “oily” flavors in drinking water that was sourced from the Mississippi River; the flavor was attributed to dissolved organic compounds whose exact identity could not be determined at the time because of limited technology (Pankow & Cherry, 1996).

When released into groundwater, chlorinated ethenes and other chlorinated solvents often persist indefinitely until their remediation is directly addressed. This persistence is due to these solvents being more dense than water and having low solubility (Pankow & Cherry, 1996). When the solvents pool at the bottom of an aquifer, these solvents form dense non-aqueous phase liquids, or DNAPLs, which are extremely persistent and difficult to remove.

Trichloroethene (TCE) is an organic solvent broadly introduced into the environment during the 20th century. Used as an industrial solvent and degreaser, it is easily recycled, noncorrosive, and nonflammable. It was even used as a general anesthetic for minor procedures and veterinary medicine (Doherty, 2000). Due to its nonflammable nature, it was the solvent of choice for dry cleaning. During World War II, its use exploded in the United States. Its toxicity became recognized in the 1960s and the 1980s its environmental regulation began. As states begin to outlaw TCE for this use, it has been widely replaced in dry cleaning and degreasing with its cousin perchloroethene (PCE), also referred to as tetrachloroethene. While less toxic than TCE, PCE has been linked to an increase risk of stillborn births (Aschengrau et al., 2018) PCE is still used as a solvent in the dry-cleaning industry.

Also of concern is vinyl chloride (VC). Of the chlorinated ethenes it is the most toxic (Brandt-Rauf et al., 2012). Globally it is produced as a precursor to

polyvinyl chloride, with 90% of vinyl chloride produced globally being for this purpose (Brandt-Rauf et al., 2012). Disposed polyvinyl chloride in landfills is suspected as a source of vinyl chloride in landfill drainage. For many years, vinyl chloride was the largest challenge in implementing bioremediation systems detoxifying chlorinated ethenes as the systems frequently stalled while performing the vinyl chloride to ethene reductive dechlorination step (Figure 1-3).

As discussed previously, microorganisms that can respire chlorinated ethenes and hundreds of other chlorinated compounds have existed in pristine environments long before chlorinated solvents were produced on an industrial scale through the 20th century. The majority of organochlorine-respiring microbes have been isolated from contaminated sites (Bowman, Nobre, da Costa, Rainey, & Moe, 2013; J. He, Ritalahti, Aiello, & Löffler, 2003; Sung et al., 2006). The high concentrations of chlorinated solvents at these sites provide reductively dechlorinating bacteria with a niche allowing for larger populations than in pristine sites without anthropogenic organochlorines.

Dehalococcoides mccartyi

Dehalococcoides mccartyi (*Dhc*) is a remarkable bacterium with a highly streamlined genome. It is disc-shaped and a mere 0.5 µm in diameter (Frank E. Löffler et al., 2013; Maymo-Gatell, Chien, Gossett, & Zinder, 1997). Because of its small genome, it has many specific growth requirements even if grown as a mixed culture. In addition to being a strict anaerobe, *Dhc*'s metabolism relies on reductive dechlorination of organochlorine molecules and the presence of

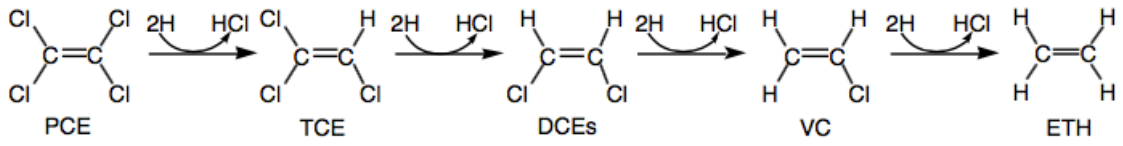

Figure 1-3. Reductive dechlorination pathway of tetrachloroethene to ethene from Máymo-Gatell, Chien, Gossett, & Zinder, 1997.

hydrogen as an electron donor. Without organochlorines present as an electron acceptor, *Dhc* cannot grow as it relies solely on the organochlorine as a terminal electron acceptor for energy conservation. Fortunately, this specificity makes it an excellent candidate for bioremediation.

Dhc is corrinoid-auxotroph meaning that it cannot produce its own cobamide required for reductive dechlorination. Overcoming this obstacle is a challenge in using *Dhc* for bioremediation. There are two possibilities of how *Dhc* obtains the corrinoids needed for reductive dechlorination. The first is that it relies upon other organisms in its environment to produce corrinoids. Cobalamin is the most widely studied cobamide as it is the only one shown so far to have significant biological activity in humans (Yi et al., 2012). The organisms that cannot synthesize their own cobamides must obtain them from their environment. In a lab setting, the easiest way to provide cobamide to *Dhc* is to simply add supplemental vitamin B_{12} to the medium. Additionally, *Dhc* will not utilize just any type of cobamide. This is reflected by an increased reductive dechlorination rate and cell density in the presence of certain cobamides (Yan et al., 2016).

A second pathway by which *Dhc* can obtain its cobamide of choice is through corrinoid scavenging. Interestingly, *Dhc* contains some of the genes for corrinoid synthesis in its small genome which it can use to modify existing cobamide in its environment(Yi et al., 2012). Other bacteria present in dechlorinating microbial communities such as acetogens, methanogens, and sulfate reducing bacteria are cobamide producers, but often they do not produce cobamides that are immediately usable by *Dhc*. *Dhc* can scavenge and remodel these cobamides for use in reductive dehalogenation (Men et al., 2015).

Dhc strain 195 has been shown to only be able to utilize three benzimidazolyl cobamides but can remodel many different cobamides into one of the three usable types when provided with complete cobamides and free lower bases (Yi et al., 2012). It is important to emphasize that the appropriate lower base must be already present in the environment and cannot be synthesized by *Dhc.* The mechanism by which *Dhc* transports lower bases, specifically

dimethylbenzimidazole, the lower base of cobalamin, from its environment into the cell is not well understood. The pathway through which *Dhc* remodels the corrinoid has been proposed (Yi et al., 2012). It is also possible to force bacteria to synthesize specific corrinoids through guided biosynthesis in which they are provided with an excess of the desired lower base (Yan, Im, Yang, & Löffler, 2013). In this case, guided biosynthesis may be used to help *Dhc* synthesize cobalamin. Additionally, *Dhc* has been shown to continue to modify corrinoids even if ample cobalamin, its preferred corrinoid, is present (Yi et al., 2012).

When provided with ample resources in its environment, including cobalamin, *Dhc* can be grown as a pure culture. Co-culture studies have shown that *Dhc* has higher reductive dechlorination rates when grown with other microbes (Men et al., 2014). In mixed culture, *Dhc* primarily grows with acetogens and methanogens that provide it with cobalamin and other nutrients (Jianzhong He, Holmes, Lee, & Alvarez-Cohen, 2007). Of three *Dhc*-containing microbial consortia currently maintained in North America, Donna II, KB-1, and ANAS, completed genomic analysis of each of these consortia found that although they vary phylogenetically, the have similar relative abundances of different metabolic pathways (Hug, Beiko, Rowe, Richardson, & Edwards, 2012). Each consortium contained Firmicutes, Euryarchaeota, and Gamma-Proteobacteria which all appeared to have supporting roles for *Dhc* growth including corrinoid production. This study determined the roles of these supporting microbes which is important for understanding how *Dhc* grows in nature and how its growth can be better supported in bioremediation.

Applications for Bioremediation

Remediation of chlorinated contaminants, especially DNAPLs, is an expensive and labor-intensive process. Many physical remediation processes such as low permeability walls or source-zone containment simply isolate the contaminated area from the main aquifer and do not actually remove the contaminant. The “pump-and-treat” method of removal which involves pulling

water out of a groundwater plume through a capturing well, treating, and returning it, is expensive with mixed results. This method removes vast amounts of groundwater while being unable to totally remove contaminants. For example, a pump that removes 1000 L/min may only extract 2 to 200 drums of DNAPL per year (Pankow & Cherry, 1996). Additionally, the actual total amount of DNAPL in a plume is rarely known.

Biological remediation methods, or bioremediation, show much more promise for actually removal of chlorinated contaminants than physical methods. Bioremediation is also effective for cleaning up residual contaminants after they have been mostly removed by mechanical methods. The most common bioremediation method is biostimulation, adding an electron donor such as vegetable oil or lactate to a contaminated aquifer though a monitoring well. Existing fermentative bacteria break these donors down into volatile fatty acids and hydrogen which are usable by *Dhc* and other organochlorine-respiring bacteria to break down pollutants such as TCE into benign ethene. Supplementation of existing bacteria with *Dhc*, or bioaugmentation, has also been shown to speed up the bioremediation process (Scheutz et al., 2008).

While biostimulation relies on native populations of dechlorinating bacteria, bioaugmentation is a bio remediation strategy in which a culture is injected into a contaminated site along with an electron donor. Bioaugmentation and biostimulation are often combined. *Dhc* occupies a niche unfilled by other bacteria in that it can respire the chlorinated solvents which allows it to grow successfully in contaminated groundwater (Adrian & Löffler, 2016). Today cultures containing *Dhc* are commercially available for remediation of chlorinated solvents. Initially these cultures faced the challenge of being too low in *Dhc* concentration; an exorbitant amount of culture was needed to make an appreciable difference in the *Dhc* population at the remediation site. A concentration of 10^7 cells/L is the target concentration for bioaugmentation. Current commercially available cultures contain 10^{11} cells/L (Adrian & Löffler, 2016)

Although bioremediation of chlorinated ethenes has been implemented with success, many challenges remain for the future. *Dhc* cannot grow in aquifers of a low pH and fractures in bedrock around contaminated aquifers can make it difficult to pinpoint the source of contamination. Heterogeneity of aquifers can complicate bioremediation; however, this is also a problem with physical-chemical remediation methods. These are all issues that will be addressed by engineers and microbiologists in the future in order to create safe and sustainable bioremediation methods to lessen pollution in water sources.

CHAPTER TWO

METHODS

Sample Collection and Microcosm Establishment

Sample collection and establishment of enrichment cultures was completed in prior work (Şimşir, 2016). Briefly, samples were collected from chlorinated solvent-contaminated streambed sediment from Third Creek in Knoxville, TN using direct push tools. Aseptic techniques were used during collection to the fullest extent possible in a field environment. Samples were placed into 1-quart mason jars, covered with creek water, and transported in a cooler with ice. In lab, sediment samples were homogenized in an anoxic glovebox filled with nitrogen and hydrogen gas (97%/3% v/v) then stored at 4° C.

Microcosms were established under each of the conditions studied: Glucose fermenting, lactate fermenting, nitrate reducing, and iron reducing. The exception was the methanogenic condition, which was derived from a glucose fermenting enrichment transfer culture.

Glass serum bottles (160 mL) containing 100 mL anoxic of cobamide-free mineral salts medium prepared as described (F. E. Löffler, Sanford, & Ritalahti, 2005). Medium contained a salt solution with a final concentration of 1.0 g/L sodium chloride, 0.5 g/L magnesium chloride hexahydrate, 0.2 g/L potassium phosphate, .3 g/L each ammonium chloride and potassium chloride, and 0.015 g/L calcium chloride dihydrate. The included tungsten selenium solution was a final concentration of 6 µg/L sodium selenium trioxide pentahydrate, 8 µg/L sodium tungstate dihydrate and .5g/L sodium hydroxide. A trace element solution in hydrochloric acid (.025% solution w/w) was added with final amounts of 15 mg/L ferrous chloride tetrahydrate, .19 mg/L cobalt(II) chloride hexahydrate, .1 mg/L magnesium dichloride tetreahydrate, 70 µg/L zinc diochloride, 6 µg/L boric acid, 36 µg/L sodium molybdate dihydrate, 24 µg/L nickel diochlride hexahydrate, and 2 µg/L copper dichloride dihydrate. L-cysteine hydrate and sodium disulfide nonohydrate were added to a final concentration each of 2 mM. Resazurin, .25 mL/L of a .1% solution, was added as a pH indicator. Medium was buffered with 2.52 g/L sodium bicarbonate.

Redox conditions were established by adding 10 mM amorphous FeOOH, 5 mM nitrate (as $NaNO_3$), or 5 mM sulfate (as $NaSO_4$) as electron acceptors and 5 mM lactate as an electron donor. The glucose fermenting microcosm was amended with 10 mM glucose. 2-bromoethanosulfonate (2 mM) was added to each microcosm to inhibit methanogenesis except in the glucose fermenting microcosm that the methanogenic enrichment was derived from (Webster et al., 2016). Autoclaved controls were included for each condition. Microcosms were transferred repeatedly while maintaining the respective redox conditions in order to obtain sediment-free enrichments. The transfer cultures received 5 mM acetate and 10 mL hydrogen gas as electron donor instead of lactate, except for the lactate fermenting and glucose conditions which continued to receive only lactate or only glucose. All cultures were incubated statically in the dark at 30° C.

Large Scale Cobamide Enrichment Cultures

For corrinoid analysis, all experimental cultures were grown in duplicate with the same defined medium described above for microcosm establishment. All cultures were grown in 2-L glass bottles with 1.6 L of medium and the headspace consisted of a 80% N_2/20% H_2 gas mixture. These large volumes were required to produce sufficient biomass for corrinoid extraction.

The glucose fermenting enrichment cultures were grown in 300-mL bottles with 200 mL of medium. These were the only enrichments grown in smaller bottles. Enrichment cultures were inoculated with 4th or 5th generation transfer cultures from the respective redox condition and were incubated statically in the dark at 30° C. Glucose was reamended (10 mM) when glucose was no longer detectable with a glucose test strip (Precision Laboratories, Cottonwood, AZ). Upon harvest when glucose was depleted a second time, all cultures were checked for production of methane to confirm that BES had successfully inhibited methanogenesis.

Nitrate reducing enrichment cultures were amended with 5 mM sodium nitrate, 10 mM sodium acetate, 2 mM BES, and 120 mL hydrogen gas. Cultures

were monitored every other day for nitrate and nitrite via ion chromatography and checked for nitrous oxide via gas chromatography every other day. MQuant nitrate/nitrite test strips (Sigma-Aldrich, St. Louis, MO) were also used as a quick method to determine if nitrate had been depleted when cultures were sampled daily. Cultures were harvested once nitrate was depleted (<.1mM remaining).

Sulfate reducing cultures were amended with 10 mM sodium acetate, 2 mM BES, 10 mM sodium sulfate, and 120 mL hydrogen gas. Sulfide was measured weekly using a colorimetric assay and quantified using a spectrophotometer as described in analytical procedures. Lactate-fermenting cultures were amended with 10 mM lactate and 2 mM BES. Cultures were sampled daily for lactate measurement and amended with an additional 10 mM lactate when the initial lactate was depleted to <0.5mM.

Methanogenic cultures were amended with 10 mM acetate and monitored twice weekly for methane production via gas chromatography.

Cobamide Extraction and Analysis

Cobamides were extracted as described (Yan et al 2013). Cells from enrichment cultures were collected by centrifugation at 10,000 rpm for 20 minutes with a PTI® F10S-6x500Y rotor (Thermo Fisher Scientific, Waltham, MA) at room temperature. Cell pellets were collected in 300 mL batches of culture fluid in 500 mL plastic bottles and resuspended in a total of 10 mL of deionized water. Potassium cyanide (KCN) was added from a 100 mM stock solution to a concentration of 20 mM and the pH was adjusted to 5-6 using 3% (v/v) acetic acid. The solution was heated in a boiling water bath for 20 minutes and centrifuged in 50 mL conical plastic tubes for 15 minutes at 4° at 10,000 rpm with a Fiberlite® F13 14x50cy rotor (Thermo Fisher Scientific, Waltham, MA). The supernatant, which contained the corrinoids, was collected and the pellet was extracted again. The supernatants from both extractions were combined. A Sep-Pak C18 Cartridge (Waters Corporation, Milford, MA) was primed with 3 mL methanol and flushed with 60 mL deionized water. The combined supernatant

was loaded onto the cartridge dropwise and the column was rinsed with an additional 3 mL water. Corrinoid was eluted from the column using 3 mL methanol. The sample was dried to remove all remaining methanol and suspended in 200 or 300 µL deionized water.

Analytical Geochemical Measurements

Nitrate and nitrite were quantified using ion chromatography. The Dionex ICS-2100 system was equipped with a Dionex IonPac® AS18 4x250 mm analytical column at 30° C. Potassium hydroxide (10 mM) was used as an eluent. For nitrate, the limit of detection was .1 mM and for nitrite limit of detection was .01 mM. Both colorimetric assays used to measure sulfide or ferrous/total iron concentrations used a Thermo Scientific Spectronic 20D+ spectrophotometer to measure light absorbance of resulting precipitates from each method (Cord-Ruwisch, 1985; Riemer, Hoepken, Czerwinska, Robinson, & Dringen, 2004). Iron measurements were reported as a ratio of ferrous to total iron and sulfide was detectable down to .05 mM.

Gases were measured using gas chromatography (GC). Nitrous oxide was measured with an Agilent 7890A gas chromatograph equipped with a HP-Plot Q column (30 m by 0.320mm, 20 µm film thickness), and a micro-electron capture detector. Manual 0.1 mL gas headspace samples were injected using a .1 mL plastic syringe with a 25g needle. Methane was also quantified using 0.1 mL headspace samples on a separate Agilent 7890A gas chromatograph with a DB624 column (30 m x .53 mm, 3 µm film thickness) with a flame ionization detector.

Lactate, acetate, propionate, and glucose were both quantified with an Agilent 1200 series high performance liquid chromatograph (HPLC). Organic acids used an HPX-97H column at 30° C while glucose used a ZORBAX Carbohydrate column (4.6 by 150 mm, 5 µm film thickness) at 80° C. Both methods used deionized distilled water as an eluent and a 20 µl injection volume. Cobamides were identified using an Agilent 1200 series high-performance liquid

chromatography system (HPLC) equipped with an Eclipse XBD-C18 5 μm column and diode array detector set to detect wavelengths of 361 and 355 nm. An auto-injector sampler was used with a 20 μl injection volume. Eluents were 0.1% formic acid in water (eluent A) and 0.1% formic acid in methanol (eluent B) at a flow rate of 1 mL/minute. Initial flow is 82% A/18% B and B is increased linearly to 80%/20% at 34.0 minutes, then increased linearly to 10% A/90% B at 36.0 minutes, held for 3 minutes, and decreased linearly to 82%/18% at 41 minutes.

All IC, GC, and HPLC peak areas were normalized to standard curves for each substance. Each standard curve was created using at least 5 samples of known concentrations ranging from 0.1-10 mM. For propionate, acetate, nitrate, nitrite, ammonium, sulfide, and sulfate, sodium salts of each chemical were added into a 100 mL (total volume) 1M stock solution in deionized distilled water. The stock solutions were serially diluted to 10, 5, 2, 1, .5, .2, and .1 mM solutions. At least 5 of these stocks were used to generate standard curves. The stock solution for glucose was made in an identical manner. Lactate standards were made by weighing 60% sodium lactate syrup (17.96 g) and adding it to deionized distilled water and bringing the solution to 100 mL total. Gas standards for nitrous oxide and methane were prepared in 2L glass bottles containing 1.6L deionized distilled water to reflect enrichment culture volume. Four bottles for each gas were injected with 1, 10, 50, or 100 mL of nitrous oxide or methane gas at room temperature and pressure. Gas volumes were converted to molar amounts using the ideal gas law.

CHAPTER THREE

RESULTS

Enrichment Culture Growth

Reactants and products of each process for each enrichment culture were monitored in order to show that cobamides produced were due to the intended redox condition and to provide a basis for cobamide quantification. All cultures were checked for methane at the end of each experiment before cultures were harvested for corrinoid extraction in order to exclude the possibility that methanogenesis had occurred in the non-methanogenic enrichments.

Glucose-fermenting cultures completely or nearly completely (<.5mM glucose remaining) depleted glucose by 24 hours, were reamended with 10 mM glucose, and harvested for cobamide extraction t 64 hours when glucose was again depleted (Figure A-1). Some propionate (<1 mM) was produced from a total of 20 mM glucose added to the system. Lactate and acetate, direct products of glucose fermentation, were also monitored.

Methanogenic cultures were harvested after 31 days at which time methane production had begun to level off (Figure -2). Methane production steadily increased up until this time.

Lactate fermentation cultures were amended with 10 mM lactate, although measured initial lactate was lower. Measured fermentation products of lactate were acetate and propionate (Figure A-3). Lactate was reamended after 72 hours before lactate was totally depleted (<2mM lactate remaining). After 96 hours, lactate was depleted to under .5 mM and cultures were harvested for cobamide extraction.

Nitrate cultures were amended with 5 mM nitrate (Figure A-4). Even with a low limit of detection, .01 mM, nitrite was scarcely detectable. Detectable nitrous oxide production remained low at <.2 mM. The possibility of dissimilatory nitrate reduction to ammonium (DNRA) in denitrifying cultures could not be completely excluded; a supporting experiment inhibiting N_2O reduction with 6 mL acetylene gas, used to inhibit reduction of N_2O to nitrogen gas, resulted in production of both nitrous oxide and ammonium in two of five replicates (Smith, Firestone, &

Tiedje, 1978). Three replicates showed no increase in ammonium concentration and a stoichiometric amount of nitrous oxide production from denitrification.

Sulfate reducing experiments had a long lag phase of 59 days; no increase in the sulfate reduction product, hydrogen sulfide, was detectable before this point. Cultures were monitored weekly for sulfide and showed only small (<0.05 mM) amounts of sulfide. Cultures were harvested after 86 days after reaching 4.1 mM and 4.6 mM sulfide, respectively, which was near stoichiometric production of sulfide from the 5 mM sulfate originally added and indicated that sulfate was nearly, but not completed depleted with under .5mM sulfate remaining (Figure A-5).

Iron reduction in in iron reducing enrichment cultures was quantified as a ratio of ferrous: total iron (Figure A-6). This ratio increased to 0.6 (ferrous: total iron) at 40 days. Cultures were grown past the 40-day mark in order to increase this ratio, but it continued to fluctuate past this point and never exceeded 0.7.

Cobamide Production

Limits of detection for seven cobamides were obtained from standard curve calculations. Benzimidazole cobamide (Ben-cba) was not detectable in concentrations below 1 mM, 5-hydroxybenzimidazole cobamide (5-OH-Ben-cba) was not detectable below 0.5 mM, and cobalamin (dimethylbenzimidazole cobamide, DMB-cba), methylbenzimidazole cobamide (MeBen-cba), and purinyl cobamide were both detectable down to 0.1 mM. Phenolic cobamide and p-cresol were detectable to 1 mM.

Cobamide production results are summarized in Figure 3-1 and listed individual enrichment cultures in Table 3-1. Product or reactant used to quantify cobamide is listed in Table A-1. Glucose fermentation and lactate fermentation produced both 5-OH-Ben-cba and MeBen-cba. The dominant cobamide produced in enrichments performing glucose fermentation was MeBen-cba, averaging 40.15 nmol MeBen-cba/mmol glucose consumed. Lactate fermentation also produced MeBen-cba in much smaller amounts, averaging

6.44 nm/mmol lactate. Both fermentation conditions also produced 5-OH-Ben-cba with glucose fermentation resulting in an average of 23.26 nmol cobamide/mmol glucose and lactate fermentation averaging 7.31 nmol cobamide/mmol lactate. A small amount of total cobamide was produced by lactate-fermenting cultures, averaging 13.75 nmol total cobamide/mmol lactate. This experiment was repeated multiple times and cobamide yield was consistently low for lactate compared to other redox conditions. Methanogenic enrichment cultures yielded an average of 376.67 nmol 5-OH-Ben-cba mmol methane produced. 5-OH-Ben-cba was the only cobamide produced by methanogenesis.

DMB-cba was produced by both nitrate reducing and sulfate reducing cultures. Sulfate reduction solely produced DMB-cba, averaging 1105.70 nmol cobamide/mmol of sulfate consumed. Nitrate reduction also produced only DMB-cba, averaging 173.0 nm/mmol nitrate consumed. These two conditions were the only ones to produce DMB-cba.

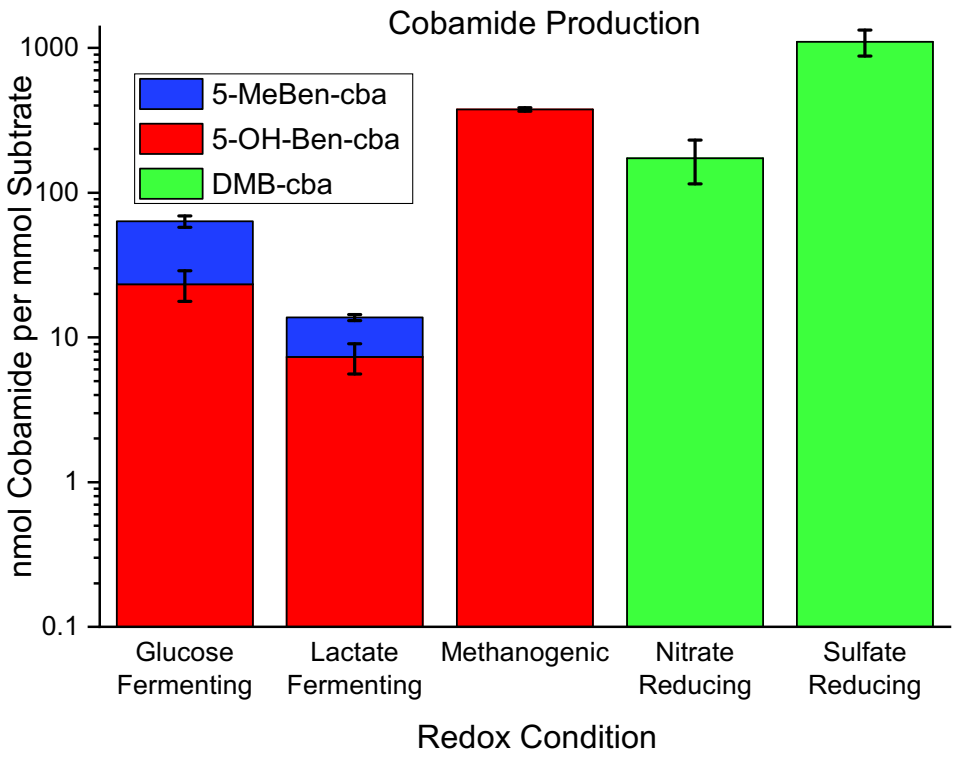

Figure 3-1. Cobamide results for the five cobamide-producing redox conditions. Iron reducing conditions did not produce any detectable cobamide.

Table 3-1 Individual cobamide production reported in nmol per mmol substrate consumed.

Culture	**5-MeBen-cba**	**5-OH-Ben-cba**	**DMB-cba**
Glucose Fermenting A	36.13	27.18	0.00
Glucose Fermenting B	44.18	19.33	0.00
Lactate Fermenting A	5.96	8.54	0.00
Lactate Fermenting B	6.91	6.08	0.00
Methanogenic A	0.00	369.1	0.00
Methanogenic B	0.00	384.2	0.00
Nitrate Reducing A	0.00	0.00	214.0
Nitrate Reducing B	0.00	0.00	132.0
Sulfate Reducing A	0.00	0.00	946.0
Sulfate Reducing B	0.00	0.00	1265.4
Iron Reducing A	0.00	0.00	0.00
Iron Reducing B	0.00	0.00	0.00

CHAPTER FOUR

DISCUSSION

Cobamide was quantified as nanomoles cobamide per millimole fermentable substrate (i.e., glucose) or electron acceptor (nitrate, etc.). This allowed results to be normalized since different conditions consumed different amounts of initial substrate or electron acceptor. Additional reduction products for each redox condition (i.e., propionate from lactate fermentation or nitrite from nitrate reduction) were not included in these calculations; only the reduction of the initial substrate or electron acceptor was considered. In the case of methanogenesis, methane produced was used to quantify cobamide. Initially, cobamide production was going to be normalized using volatile suspended solids, a measure of biomass (American Public Health Association, 1999). This method would account for the differing cell densities between different redox conditions but ultimately was not used.

In a natural environment or bioremediation site, multiple redox conditions would exist simultaneously. Multiple processes, including sulfate reduction, nitrate reduction, and lactate fermentation may all simultaneously occur in the same system. By knowing how much of each electron acceptor is consumed, potential cobamide production is easier to predict than by VSS or a cell counting method (Madrid & Felice, 2005) because cobamide can be assigned to individual substrates, rather than the total biomass from all substrates. Cobamide produced by an individual redox condition can be assigned to a redox condition based on how much electron acceptor is consumed, but it cannot be assigned to a redox condition from a total biomass measurement.

Lactate and glucose fermentation both produced MeBen-cba and 5-OH-Ben-Cba. MeBen-cba is a precursor to the other cobamides observed and was first discovered in methanogenic archaea shortly after (E. Stupperich) (Roth et al., 1996; Schulze, Vogler, & Renz, 1998; Shelton et al., 2018). MeBen-cba may be directly used for cell metabolism or exported from the cell so it can be converted to more broadly used cobalamin (Shelton et al., 2018). In a preliminary experiment glucose fermentation was allowed to proceed into lactate fermentation and shows a similar cobamide profile to glucose and lactate

fermentation. One fermenter, *Clostridium thermoaceticum* was found to produce 5-methoxybenzimidazole-cobamide (5-MeO-Ben-cba) along with a cobinamide, a cobamide lacking the lower base (Koesnandar & Nagai, 1991). 5-MeO-Ben-cba was not found in any cultures for this study.

5-OH-Ben-cba was also found in nitrate reducing and methanogenic enrichments. The respective amount produced under each condition varied greatly. The methanogenic enrichments exclusively produced 5-OH-Ben-cba. Methanogens are known 5-OH-Ben-cba producers so this result is consistent with known literature (Erhard Stupperich, Eisinger, & Schurr, 1990). When methane production levelled off after about 30 days in the experimental cultures, cultures were not visibly dense from cell growth and obtaining any cobamide from extracted cells, even from 1.6 L cultures, was unexpected due to low cell density. Cobamide was quantified according to the molar concentration of methane produced.

The reactants for methanogenesis are difficult to quantify because of the possibility of syntrophic acetate oxidation (Hattori, 2008). Methanogenesis can utilize either acetate or hydrogen and carbon dioxide as reactants. The enrichments for the experiments here used acetate instead of hydrogen and carbon dioxide as the substrate but measuring the change in acetate concentration over the course of the experiment would not accurately reflect all methanogenic activity. Syntrophic acetate oxidation produces hydrogen which can be used by methanogens to produce methane. Hydrogen gas is not measurable because it is consumed immediately upon production. Quantifying cobamides as a function of methane produced still allows a way to normalize cobamide production.

The possibility of glucose and lactate fermenting cultures producing 5-OH-Ben-cba due to methanogens was excluded because of the addition of BES and confirmation via gas chromatography at the end of each experiment that no methane was produced. Therefore, any 5-OH-Ben-cba produced in these conditions can be attributed to glucose or lactate fermentation. Because 5-OH-

benzimidazole, the lower base of 5-OH-Ben-cba, is a precursor to the formation of dimethylbenzimidazole, the lower base of DMB-cba, finding 5-OH-Ben-cba in nitrate reducing cultures is unsurprising. It is possible that 5-OH-benzimidazole was incorporated into complete cobamides.

Sulfate reduction resulted in by far the most cobalamin production of any other substrate, with denitrification (with possible DNRA) being the only process to also produce cobalamin (DMB-cba). Sulfate reduction resulted in the largest amount of total cobamide produced per mmol of substrate. Sulfate reducer *Desulvibrio vulgaris* can produce guaninyl cobamide and hypoxanthyl cobamide *de novo,* but is also capable of synthesizing DMB-cba when supplemented with DMB in its medium (Guimaraes, Weber, Klaiber, Vogler, & Renz, 1994). Several other sulfate reducers, *Desulfobacterium autotrophicum*, *Desulfobulbus propionicus*, and *Archaeoglobus fulgidus*, are all producers of Me-Ben-cba (Krautler, Kohler, & Stupperich, 1988). Because previous work does not include sulfate reducers who can produce DMB-cba, this knowledge makes this new finding more exciting. There is not a documented DMB-cba producing sulfate reducer. In mixed culture, DMB-cba production cannot be assigned to a specific taxon, but that it is produced at all is an important finding.

Iron reduction was not included in the results figure (Figure 3-1) because no cobamide was detected after three experiments with duplicate cultures. Previous work showed a small amount of DMB-cba produced by the same iron reducing culture (Şimşir, 2016). The ferrozine assay gave inconsistent quantitative results for ferric and total iron which made tracking iron reduction difficult. Standards were used with every analysis and gave consistent standard curves; however, total iron measurements varied for the cultures even though total iron measurements should remain consistent for the entirety of the experiments (Figure A-6). Before removing culture fluid for iron measurement, cultures were inverted several times to mix in the layer of iron that settled at the bottom. Visually, cultures were well mixed, and the samples were taken immediately. This step should have resulted in consistent total iron

measurements of 5 mM after the precipitated ferric iron had been dissolved for the ferrozine assay.

The ferrous iron measurements showed no correlation to reduction of iron over the course of the experiments. When ferrous iron was normalized to the total iron readings as a fraction of total iron, a growth curve showing an increase of ferrous iron over the first 40 days of the experiment emerged, shown by a dashed black line in Figure A-5. Assuming total iron remained at 5 mM, this curve indicates that 3.5 mM ferric iron was reduced to ferrous iron after 40 days and that complete iron reduction did not occur. While cultures were mixed immediately before samples were taken, these results show that mixing may have been insufficient to obtain consistent total iron measurements. By normalizing measured ferrous iron to a percentage of total measured iron, ferrous iron can still be reliably quantified by assuming total iron remains at 5 mM in the cultures. Because cultures were not harvested after 40 days when ferrous iron measurements peaked, cell death likely resulted by the time of harvest and explains the irregular ferrous iron measurements taken for the rest of the experiment. If cobamide yield was already low, the cell death would leave few intact cells to extract cobamide from. This explains why no cobamide was detectable for the iron reducing condition. The experiment could not be completed again due to time constraints.

While only three cobamides were detected in enrichment cultures, they were not the only cobamides that were looked for. Standards curves were also generated for p-cresol cobamide, phenolic cobamide, benzimidazole cobamide, and methoxybenzimidazole cobamide. Benzimidazolic cobamides are detectable at a 361 nm wavelength (Yan et al., 2016). In chromatograms from HPLC analysis, some other peaks were detectable outside of the cobamides that standard curves were obtained for; however, it is unlikely that these are complete cobamides because they did not have peak absorbance at 361 nm.

The discovery that nitrate and sulfate reducing conditions result in production of DMB-cba is an important finding for bioremediation of chlorinated

solvents. Of tested cobamides, DMB-cba supports the fastest reductive dechlorination rates in *Dhc* (Yan et al., 2016). Lack of usable type of cobamide is one of several culprits proposed in stalling of dechlorination at bioremediation sites with other possibilities including insufficient electron donor or the presence of nitrous oxide (Fennell, Gossett, & Zinder, 1997; Yin et al., 2019). Further research is needed to determine how available cobamides produced by the community are to other species, specifically *Dhc*. An abundance of DMB-cba does not necessarily mean that the cobamide is available to other cells because the cobamides extracted in this study are measurements of intracellular cobamide.

Outside of the application to bioremediation, this research presents important new findings for microbial ecology. Because enriching the same original sediment under different redox conditions resulted in different cobamide profiles, redox condition is a confirmed factor accounting for why some microbes may produce one type of cobamide over another. Additional factors outside of the community makeup and redox condition could also be at play, but these experiments were completed in a virtually identical manner outside of changing the growth substrate or electron acceptor.

CHAPTER FIVE

CONCLUSIONS & FUTURE DIRECTIONS

The experiments completed in this project represent only the first steps in elucidating the patterns within growth substrates and corrinoid production. Essentially, these experiments form the proof of concept that redox conditions influence the type and quantity of cobamides produced.

Each process can and should be further broken down. For example, determining cobamide production at each step of denitrification from nitrate to nitrite, nitrite to nitrous oxide or nitric oxide, and nitrous oxide to nitrogen gas. This breakdown would determine if certain cobamides are produced during specific metabolic steps. The enrichments for the six redox conditions represented should also be repeated with sediment samples from other geographic locations because the results obtained here are only from one stream system. This work has proven that changing redox condition does change the cobamide profile within the same initial microbial community. It is unknown if the same cobamides would be produced in experiments using samples from different stream systems or types of environment. Literature of known cobamide producers discussed previously indicate that certain taxa within each redox condition (i.e., sulfate reduction, methanogenesis) can result in cobamides that were not observed in these experiments.

Additionally, co-culture experiments with *Dhc* and the cultures used for each redox condition would be beneficial, especially with the sulfate reducing and nitrate reducing conditions which produce DMB-cba. These two redox conditions are the most likely to support *Dhc* because of extensive DMB-cba production.

Chlorinated solvents are only a few of many hazardous chemicals of concern plaguing the United States water supply. This research expands the larger field of bioremediation research as we find ways to prevent stalls of treatment of chlorinated solvents at remediation sites using *Dhc*. This work also expands the body of knowledge of biological cobamide production, an understudied area. This work is the only study of its kind and provides the foundation to further cobamide research both for applications in remediation engineering and fundamental microbiology.

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APPENDIX

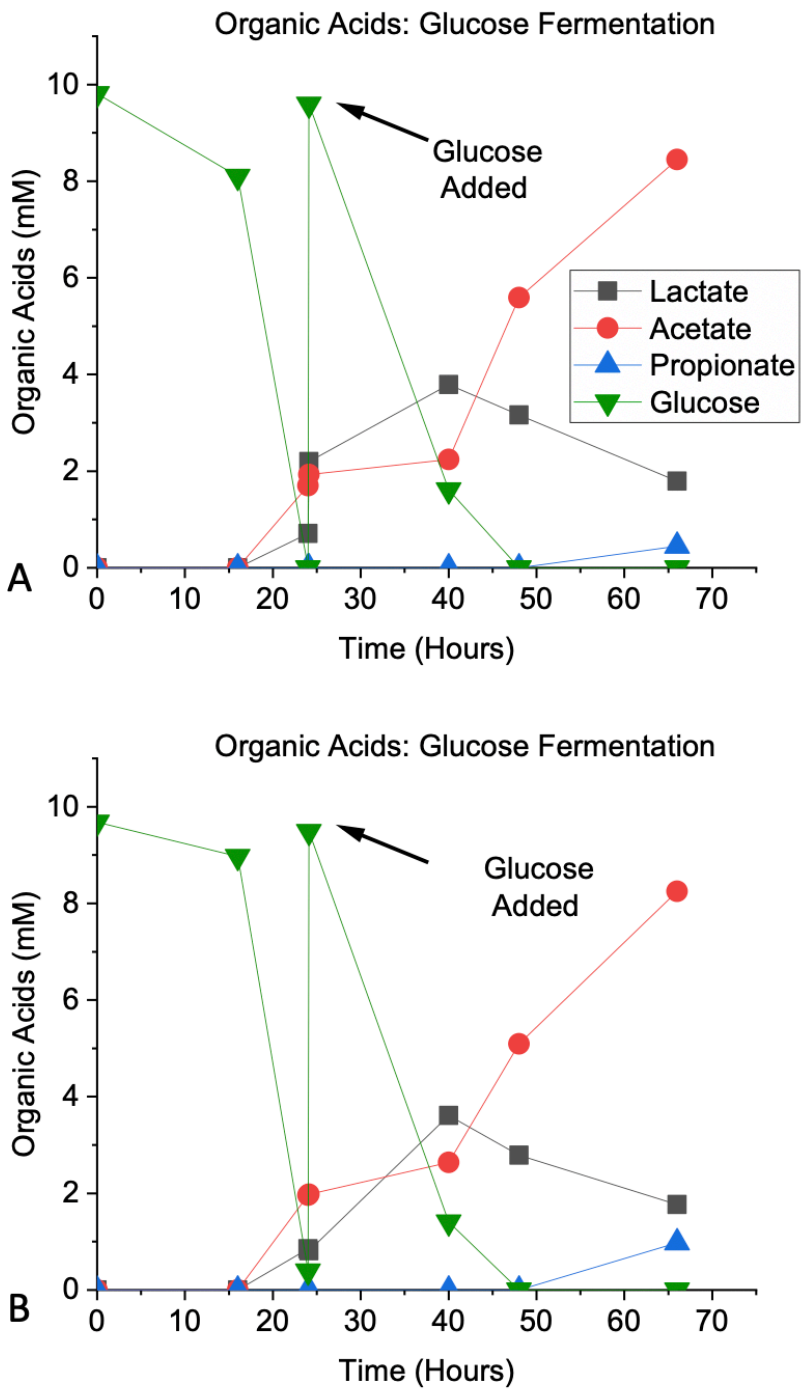


Figure A-1 Glucose fermentation growth curves.

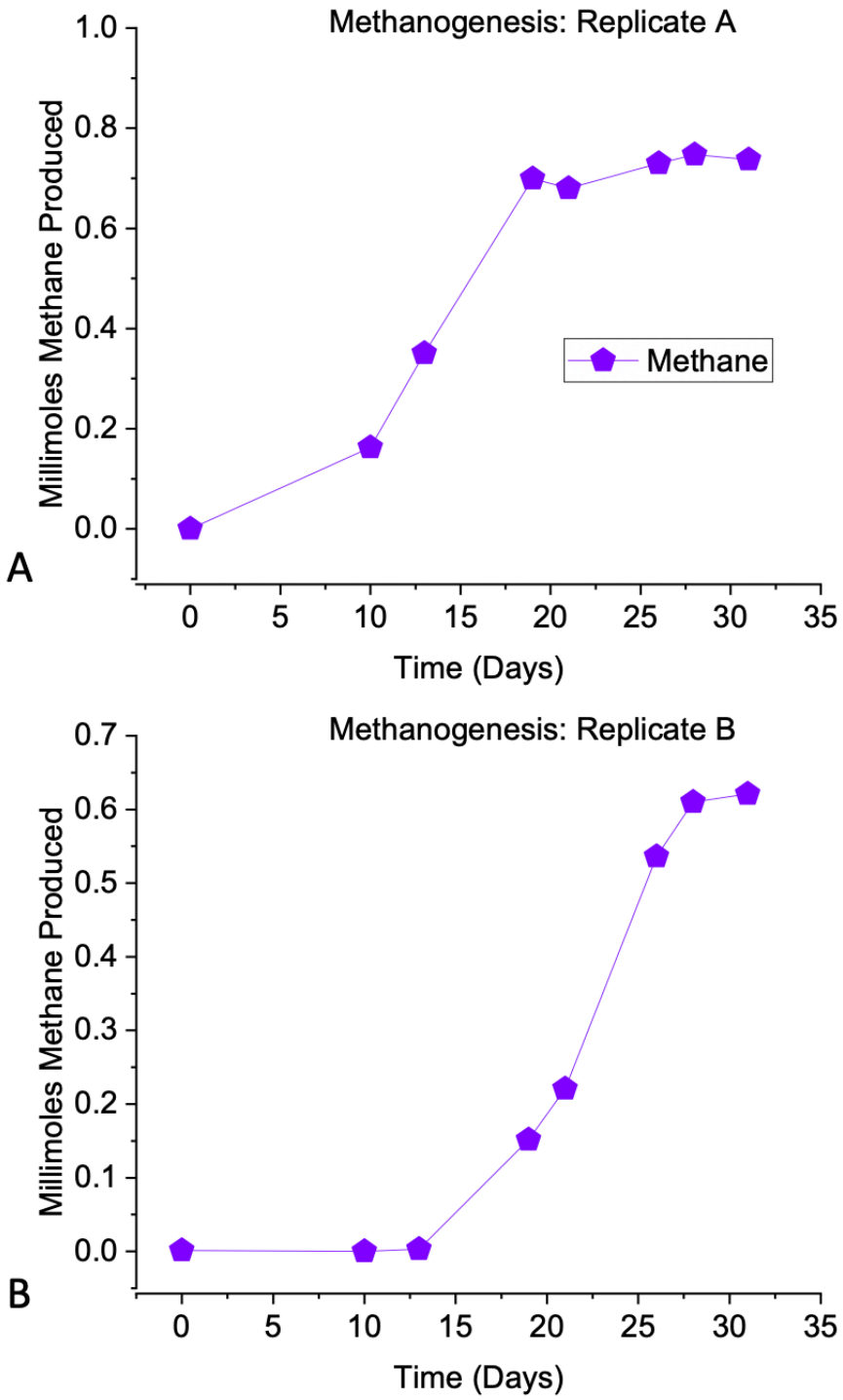


Figure A-2. Methane production in methanogenic cultures.

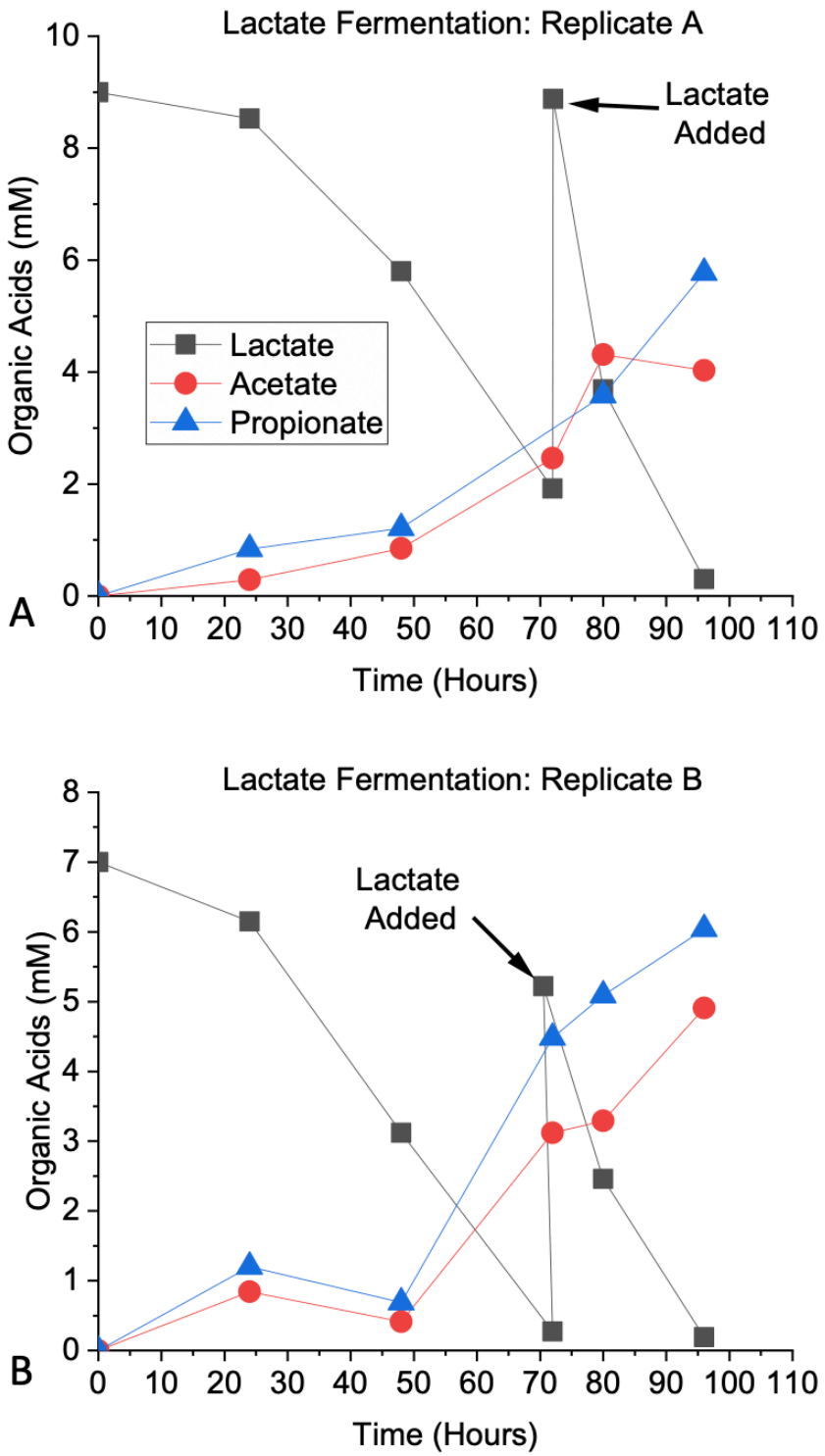


Figure A-3. Lactate fermentation growth curves.

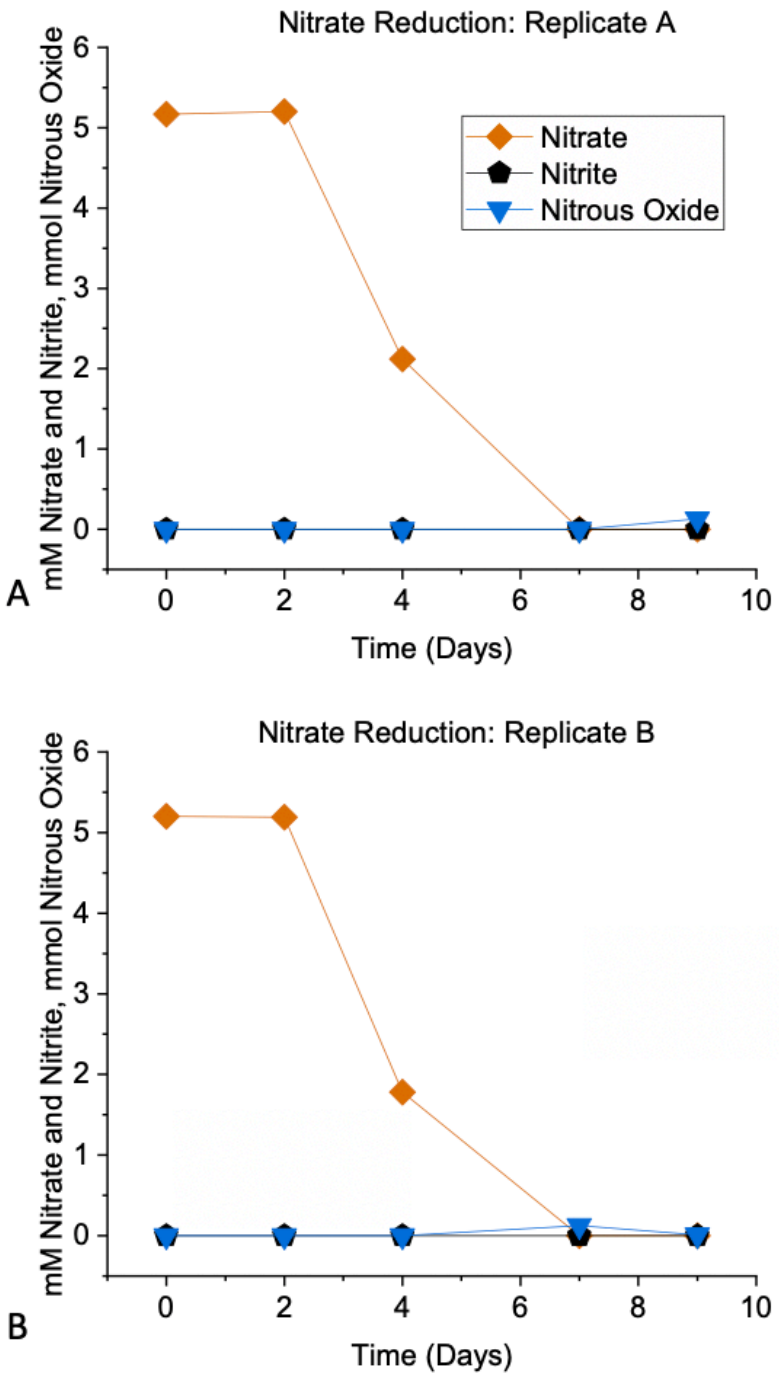
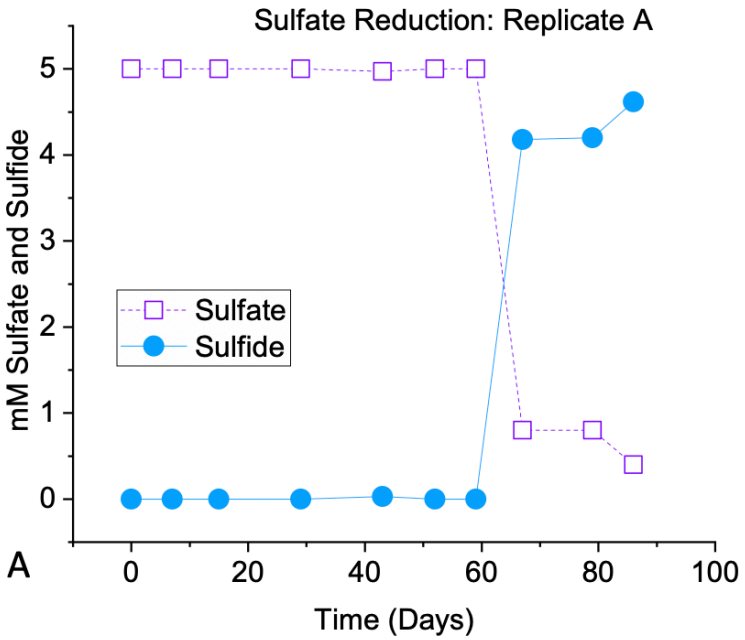


Figure A-4. Nitrate reduction growth curves.



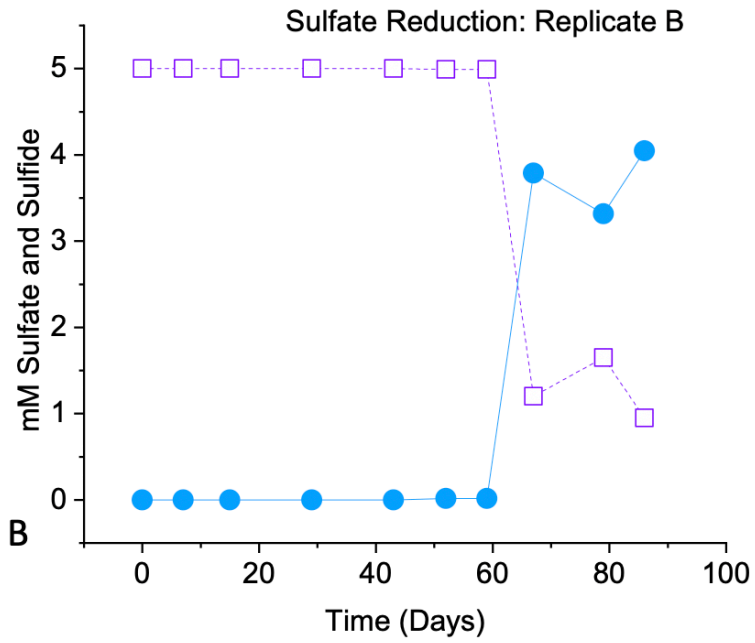


Figure A-5. Sulfate reduction growth curves from each replicate. Sulfide was quantified and sulfate measurements were predicted based on subtraction of the sulfide concentration from total sulfate added to the cultures, 5 mM.

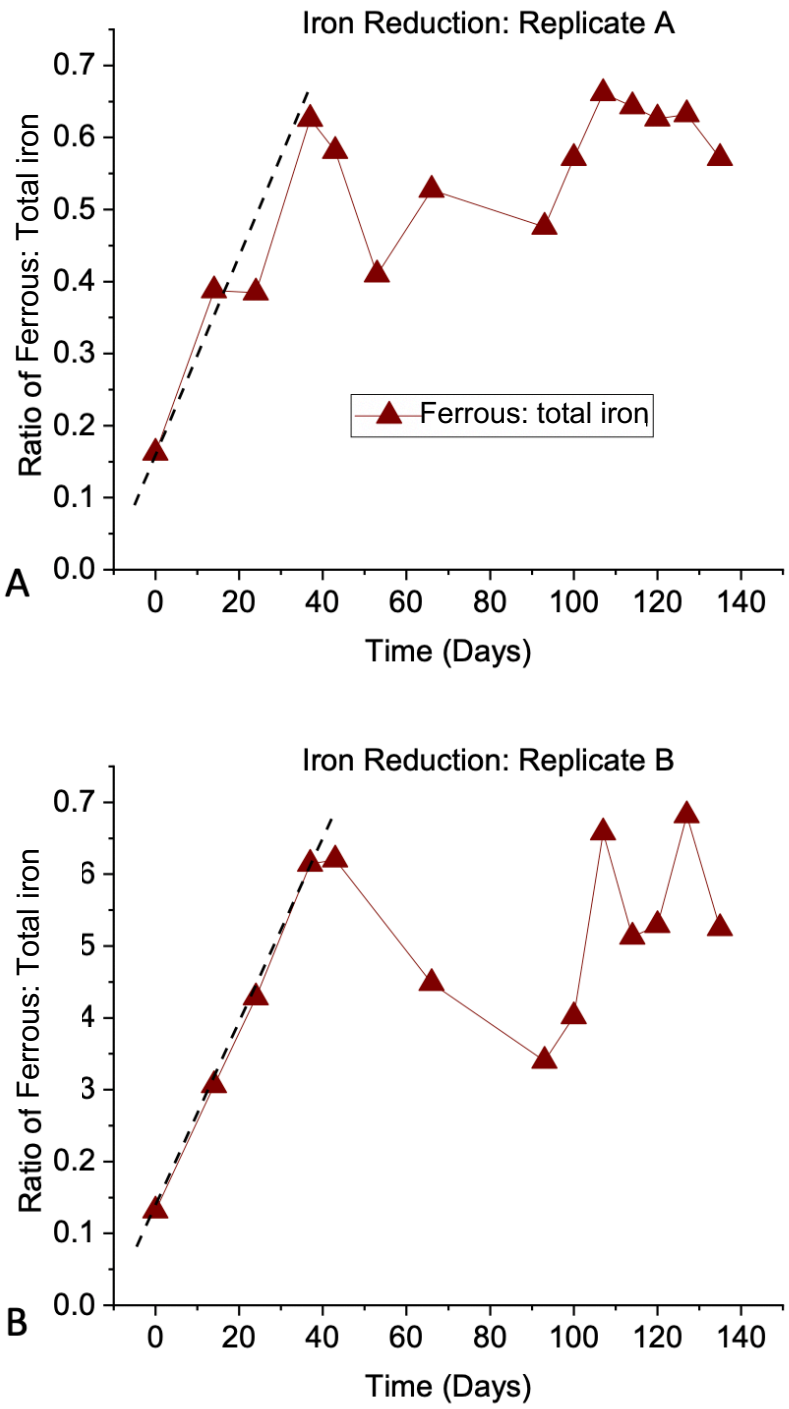


Figure A-6. Iron reduction growth curves represented as a ratio of ferrous (reduced) to total iron. Dashed black line represents actual growth phase.

Table A-1. Substrate consumed under each condition used to calculate cobamide yield. Asterisk refers to the case of methanogenic enrichments, where methane production (in mmol) was used to quantify cobamide production.

Culture	**Substrate Consumed (mM)**	**Substrate Consumed* (mmol)**
Sulfate A	4.62	7.39
Sulfate B	4.05	6.47
Nitrate A	9.85	15.76
Nitrate B	9.47	15.15
Glucose A	19.40	5.82
Glucose B	18.78	5.63
Lactate A	17.84	28.54
Lactate B	18.28	29.25
Methanogen A*	0.56	1.05
Methanogen B*	0.69	1.10

VITA

Laurel A. Seus is a lifelong Tennessee native born in 1993. She attended the University of Tennessee where she received her B.S. in Animal Science in 2015 and returned to the University of Tennessee two years after graduation to pursue her interest in bioremediation. She completed her M.S. in Microbiology at UT in 2019. She has accepted position as a Project Scientist with EHS Support following graduation where she will work as a microbiology and bioremediation consultant.