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Kevin J. McKeage for the degree of Master of Science in Environmental Engineering presented on June 5, 2015.

Title: Low Level Quantification of 1,4-Dioxane and Investigation of 1,4-Dioxane Co-Metabolism by Mycobacterium sp. 1A.

Abstract approved:

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ABSTRACT

1,4-Dioxane (dioxane) is a probable human carcinogen and is often found comingled with chlorinated aliphatic hydrocarbon (CAH) contamination since dioxane is used as a stabilizer in CAH solutions. Dioxane is miscible in water, has a low K_{ow}, low H_{cc}, and is highly recalcitrant in the environment. The presence and potential transformation of dioxane at CAH sites undergoing remediation has been difficult to ascertain due to the difficulty of analysis. Typical analyses performed for CAH's, such as direct liquid injection onto a gas chromatograph equipped with a flame ionization detector, are ineffective at detecting dioxane. Methods have been developed to analyze low levels of dioxane, but they require extraction techniques involving the use of harmful solvents and expensive consumables. A method of analyzing environmentally relevant concentrations of 1,4-dioxane in the sub-µg/L range by heated purge-and-trap coupled with gas chromatography mass spectrometry is presented. This method demonstrates a method detection limit of 0.13 µg/L which is below the EPA risk assessment concentration of 0.35 µg/L. Detection at this level has allowed for the characterization of the 1,4-dioxane degradation capabilities of an Actinomycete culture, *Mycobacterium* sp. 1A (1A). Culture 1A was found to degrade dioxane at rates two to four times faster in the absence of propane than in the

presence of propane. Although propane did inhibit dioxane transformation, dioxane was still observed to be completely transformed in the presence of propane. Culture 1A was able to utilize propane or 2-propanol for growth and concurrently transform 100 μg/L of dioxane in mineral salts growth media (MSM) and in amended and non-amended site groundwater. Although culture 1A grew efficiently on 2-propanol, it exhibited lower rates of dioxane transformation than cells grown on propane.

Low Level Quantification of 1,4-Dioxane and Investigation of 1,4-Dioxane Co-Metabolism by Mycobacterium sp. 1A.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kevin J. McKeage, Author

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CHAPTER 1
INTRODUCTION

1,4-Dioxane (from this point forward, dioxane) has been identified as a possible human carcinogen (Derosa et al., 1996; IARC, 1999). This contaminant poses a risk to groundwater and surface water supplies, as it is fully miscible in water. Dioxane is resistant to conventional remediation techniques due to its low Henry's constant (H_{cc}) and organic partitioning coefficient (K_{oc}). Dioxane's low volatility and hydrophilicity also make this compound difficult to analyze.

Currently there are several methods available for quantifying dioxane. Solid phase extraction coupled with Gas Chromatography Mass Spectrometry (GC-MS) offers the lowest method detection limit (MDL) at 0.02 µg/L, but the method is labor intensive and requires the use of expensive consumables and a large sample volume. Frozen micro extraction coupled with GC-MS has a MDL of 1.6 µg/L, but this method, while not labor intensive, has an MDL above the EPA risk assessment concentration of 0.35 µg/L and requires the use of chlorinated solvents for extraction. A heated purge and trap (P&T) Gas Chromatography with Flame Ionization Detector (GC-FID) method developed by Zenker et al. reported a MDL of 2 µg/L. Draper et al. evaluated the use of P&T GC-MS for use with dioxane but was only able to obtain a quantification limit of 10 µg/L. The difficulties faced with the quantification of dioxane have impeded the evaluation of possible remediation techniques.

Bioremediation, the use of microorganisms to transform contaminants, offers a means to degrading dioxane. If applied as an *in situ* technique, microorganisms capable of metabolizing or co-metabolizing dioxane may provide a means to bioremediate dioxane. While there have been bacteria identified that can metabolize or co-metabolize dioxane, there is only one currently published study addressing field-scale bioremediation of dioxane (Lippincott et al., 2015). In the study, bioaugmentation and biosparging were shown to reduce groundwater dioxane

concentrations to below their detection limit of 2 µg/L, but could not verify remediation to below the EPA risk assessment concentration of 0.35 µg/L.

This document contains an evaluation of a modified heated P&T GC-MS method that exhibits a MDL of 0.135 µg/L, which is below the EPA risk assessment concentration. This method does not require the use of expensive consumables or hazardous chlorinated solvents and is not labor intensive. The utility of this method is explored by evaluating the dioxane degradation capabilities of a recently isolated *Mycobacterium* species under laboratory conditions. Analyses were also performed in groundwater and groundwater amended with solids or mineral media salts.

Research Objectives

- Develop an inexpensive, effective quantification method for sub-µg/L concentrations of dioxane requiring a small volume of sample.
- Select an organism that is capable of degrading dioxane at site relevant concentrations
- Determine the extent to which dioxane degradation is inhibited by growth substrate
- Evaluate the selected organisms potential to grow on propane and degrade 1,4-dioxane under site conditions

CHAPTER 2
LITERATURE REVIEW

2.1 1,4-Dioxane

Dioxane was first characterized by A.V. Lourenço in 1863 (Stumpf, 1956; Flick, 1998). This cyclic ether has a molecular weight of 88.11 g/mol and has the structure shown in Figure 2.1. The symmetry of dioxane's structure makes the compound highly recalcitrant in the environment and relatively immune to ambient bioattenuation processes in most groundwater environments (Zenker et al., 2004; Mohr, 2010). The structure of dioxane should cause this compound to be fairly insoluble (Stoye, 2005), but the dimerization of two dioxane molecules results in the polarization of the homodimer and the compound's apparent near miscibility (Mazurkiewicz and Tomasik, 2006). Dioxane also has low volatility with a dimensionless Henry's Law constant of $2x10^{-4}$ and is hydrophilic in nature with an octanol-water partitioning coefficient of $10^{-0.27}$ (Zenker et al., 2003). The resistance to bioattenuation, near miscibility, low volatility, and hydrophilicity combine to make dioxane difficult to remediate by conventional means, such as with air stripping or carbon adsorption (Zenker et al., 2003, Vainberg et al., 2006).

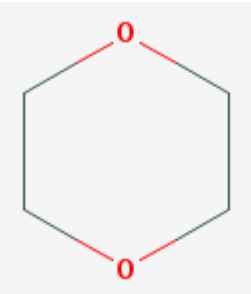

Figure 2.1 The chemical structure of 1,4-dioxane (National Center for Biotechnology Information, 2015).

Dioxane is typically associated with sites contaminated with chlorinated solvents as dioxane was used as a stabilizing agent of chlorinated compounds (Mahendra and Alvarez-Cohen, 2006; Mohr, 2010). It has been used as a wetting

agent in the paper and textile industries and in the manufacture of other organic chemicals (Zenker et al., 2003). Dioxane has also been quantified in paints, lacquers, cosmetics, deodorants, fumigants, and detergents as a solvent (Howard, 1990; Mohr 2010). Unfortunately, the proliferation of dioxane-containing compounds makes the contamination of water resources by dioxane a more widespread issue than other less mobile and less prevalent compounds.

Dioxane has been found in ground, surface, and waste waters, as well as in landfill leachate (Burmaster, 1982; Lesage et al., 1990; Fetter, 1993; Taylor et al., 1997; Johns et al., 1998; Abe, 1999; Jackson and Dwarakanath, 1999; Tanabe et al., 2006; Zenker et al., 2003; Mohr, 2010). Prior to a study by Adamson et al. in 2014, it was believed that dioxane was highly mobile in the aqueous phase (Roy and Griffin, 1985) and thus it would cause vast contamination of groundwater resources (Li et al., 2014). To that end, much of the literature describes dioxane as being found at the leading edge of a groundwater contaminant plume (Patterson et al., 1985; Lesage et al., 1990; Jackson and Dwarakanath, 1999; Mohr, 2010). However, recent findings suggest that plumes of dioxane are comparable in size to that of the chlorinated solvent plumes they are associated with (Adamson et al., 2014).

Dioxane poses health concerns as it has been identified as a probable human carcinogen (Derosa et al., 1996; IARC, 1999). The EPA has labeled dioxane as a drinking water contaminant candidate, which may result in its regulation by the Safe Drinking Water Act (EPA, 2009). There is not an established federal maximum contaminant level, but the EPA recommends that the concentration of dioxane not exceed 0.35 μg/L, the EPA risk assessment concentration, in a drinking water supply (EPA, 2013). This concentration limit represents the 1×10^{-6} cancer risk level as described by the EPA.

Dioxane is commonly present at solvent-contaminated sites, but its fate is often not documented as the compound is particularly difficult to quantify with available analytical techniques (Adamson et al. 2014). Unlike other contaminants, dioxane is found in the environment at dilute concentrations, often in the parts per billion range (Adamson et al. 2014). The study by Adamson et al. (2014) found that

out of 194 dioxane-contaminated sites in California the median maximal concentration was 365 μg/L. The expected concentration of dioxane is significantly lower than we expect for other contaminants, such as tetrachloroethylene (PCE) and trichloroethylene (TCE), that can exhibit high mg/L concentrations when their pure phases are present (Yu and Semprini, 2009).

Since dioxane poses a health risk and is present at low concentrations in the environment, reliable analytics at these low levels are required. In the past, methods used to quantify chlorinated aliphatic hydrocarbon co-contaminants, such as direct headspace injection coupled with gas chromatography, have proven inadequate at quantifying dioxane. Direct aqueous injection (DAI) onto a gas chromatograph equipped with a flame ionization detector has been the standard for gathering dioxane measurements, but this technique is limited to quantification above 100 μg/L (Li et al., 2011). The EPA risk assessment concentration is almost three orders of magnitude below the quantification limit of DAI, which has prompted the creation of new methods that can obtain reliable data at sub-μg/L concentrations (EPA, 2013; EPA, 2014). Solid phase extraction, frozen micro extraction, and heated purge-and-trap methods have been developed to address these quantification limits with varied success. Currently excepted methods for quantifying dioxane are described in the following sections in order of highest to lowest sensitivity.

2.2 Solid Phase Extraction with Gas Chromatography Mass Spectrometry

EPA Method 522 developed by Grimmett and Munch (2009) utilizes solid phase extraction (SPE) to concentrate dioxane for direct liquid injection onto a gas chromatograph mass spectrometer (GC-MS). Solid phase extraction takes advantage of the likelihood of aqueous organic phases to partition onto solid surfaces by various adsorption processes. Extraction of dioxane by solid phase extraction was first developed by Isaacson et al. (2006). The method described by Grimmett and Munch (2009) improved upon Isaacson's method by increasing dioxane detection through a number of adaptations and is currently endorsed by the EPA for the quantification of dioxane. The work performed by Grimmett and Munch (2009) describes large and

small sample volume processes for extracting dioxane and an evaluation of differing commercially available SPE media.

In the large volume experiment, 500 mL of sample were passed through a preconditioned SPE cartridge and the concentrated dioxane eluted from the cartridge with dichloromethane (DCM, Figure 2.2). The DCM was transferred to an autosampler that directly injected the solution onto a GC-MS. The GC-MS was equipped with a CP-Select 624 CB (6% cyanopropyl-phenyl, 94% dimethylsiloxane phase) of 30 m length, 0.25 mm inner diameter and 1.4 μm film thickness. Deuterated tetrahydrofuran (THF-d_8) was used as an internal standard and deuterated dioxane (dioxane-d_8) as a surrogate standard. The GC-MS was operated in selected ion monitoring mode (SIM) at 46, 78, 80 m/z for THF-d_8, 58 and 88 m/z for dioxane, and 62, 64, and 96 m/z for dioxane-d_8. The retention times for THF-d_8, dioxane, and dioxane-d_8 were 6.72, 8.86, and 8.79 minutes, respectively. The small volume method requires approximately one fifth of the sample and reagents used in the large volume method.

Sample Preparation by Solid Phase Extraction (SPE)

- The SPE cartridge was fitted to a vacuum manifold
- The cartridge was conditioned with 3, 6, and 15 mL dichloromethane (DCM), methanol, and water, respectively
- 500 mL of sample were passed through the cartridge at 5-10 mL per minute
- The cartridge was allowed to dry under suction for 10 minutes
- The contents of the cartridge were then eluted with 9 mL of DCM
- This eluent was dried by contact with sodium sulfate
- The eluent underwent internal standard addition and was brought to volume with DCM
- This prepared sample was transferred to an autosampler that directly injected the solution onto a GC-MS

Figure 2.2 Description of the SPE method from Grimmett and Munch, 2009.

The SPE methods have reported MDLs of 0.026 μg/L and 0.020 μg/L for large and small volume methods, respectively, which are far below the 0.35 μg/L EPA recommendation. Grimmett and Munch also reported the single laboratory lowest concentration minimum reporting level, which is similar to a quantitation limit, of 0.047 μg/L and 0.036 μg/L for the large and small volume methods, respectively. EPA Method 522 has been shown to be reliable and exhibit recoveries deviating less

than 10% in all instances. Recoveries refer to the repeatability of samples to produce the same analytical value when run in replicate with 100% recovery indicating identical replicate values. The EPA method for semi-volatile organics, method 8270D, requires that recoveries not deviate greater than or less than 30% from the original sample value (Li et al., 2011; EPA, 2007).

Several benefits have been identified with this method in addition to its low quantification limits. Dioxane recoveries using Method 522 are not affected by the presence of total organic carbon, 1,1,1,-trichloroethane, methanol, or the mineral content of the sample (Grimmett and Munch, 2009). This method also works well in the presence of sodium bisulfate, used as a microbial inhibitor, and sodium sulfite, used as a dechlorinating agent in treating drinking water.

Challenges with Method 522 have also been identified. Vacuum drying may introduce excess error as laboratory air may contain ambient dioxane (Isaacson et al., 2006). This is especially the case if the vacuum drying step occurs in the same fume hood as the preparation of standards and equipment (Isaacson et al., 2006). Reliability and detection are functions of available SPE materials, with increased detection accompanied by an increased price as more effective, proprietary SPE media are made available. The introduction of co-contaminants can reduce recoveries as there may be competition for adsorption sites on the SPE surface (Isaacson et al., 2006).

While Grimmett and Munch accounted for the co-contaminant issue by successfully testing their method on a system with the presence of high concentrations of trichloroethylene (TCE) and total organic carbon (TOC), they did not perform the analysis at solely high TCE or TOC concentrations. TCE has a K_{oc} value of $10^{2.42}$ (Russell et al., 1992) which is greater 200 times greater than the K_{oc} value of dioxane ($10^{-0.27}$) meaning TCE will more readily bind to TOC. Presumably the presence of high levels of TOC and TCE would have a counteracting effect on one another potentially minimizing their interaction with dioxane as the TCE would bind with the TOC thereby reducing the TCE in solution and TOC available to bind with dioxane. This method did show that complex matrices can reduce recoveries, such as when adding copper sulfate, pH 7 Trizma buffer, and ammonium chloride in

conjunction with high TOC recoveries were reduced to 80% (Grimmett and Munch, 2009).

Park et al. in 2005 also mention that matrix interferences and high solids loads on SPE-based methods can result in more labor intensive pretreatment techniques. The time restrictions incurred by pretreatment with SPE methods are apparent as only 24 to 30 extractions of 100 mL samples per day can be performed by a trained laboratory worker (Grimmett and Munch, 2009). In addition, this method requires the use of toxic organic solvents to elute compounds from SPE media and requires the use of expensive consumables. SPE cartridges required for this method are single use and cost greater than $10 per cartridge (VWR, 2015). Finally, large extraction volumes of 100 to 500 mL of sample make it less desirable when working in systems that do not contain large sample volumes.

2.3 Frozen Micro Extraction with Gas Chromatography Mass Spectrometry

Frozen Micro Extraction (FME) of dioxane was first described by Li et al. (2011). This adapted liquid-liquid extraction method capitalizes on the partitioning of dioxane from the aqueous phase into an organic phase during the freezing of the aqueous phase. The organic phase is then directly injected onto a GC-MS.

Aqueous samples were filtered and spiked with internal and surrogate standards. DCM is added to the aqueous sample and placed into a -80^{0}C freezer (Figure 2.3). The DCM extract was then directly injection onto a GC-MS equipped with a HP-5 column of 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness. Analysis was performed in SIM mode at 58 and 88 m/z for dioxane, 64 and 96 m/z for dioxane-d_8, and 115 m/z for 1,4-dichlorobenzene-d4. Retention times for dioxane, dioxane-d_8, and 1,4-dichlorobenzene-d4 were 5.78, 5.69, and 9.78 minutes, respectively.

Quantitation of dioxane was performed from 10 to 1600 μg/L. Dioxane and dioxane-d_8 were eluted separately at concentrations less than 200 μg/L. This resulted in dioxane peaks that were free of chromatographic interferences. Li et al. utilized a seven point calibration combined with a continuing calibration verification (CCV)

standard that was run at the beginning of each analysis. The CCV was found to vary less than 20% from the calibration curve and determined to be a stable instrumental technique. The reported MDL for this method is 1.6 μg/L. Li et al. also reported good recoveries with less than 10% deviation from suspected concentrations.

Sample Preparation by Frozen Micro Extraction (FME)

- 0.3 mL of sample were filtered through a 0.2 μm nylon filter
- 0.2 mL of the sample was spiked with internal and surrogate standard
- 0.2 mL of DCM was added
- The sample was vortexed and placed at an incline into a -80^{0}C freezer for 20 minutes
- The DCM was then extracted via syringe as the water phase had frozen
- Samples were stored at -20^{0}C until run manually via direct liquid injection onto a GC-MS

Figure 2.3 Description of the FME method from Li et al., 2011.

The use of ultra-filtration through 0.2 μm nylon filters has been shown to remove bacteria and not alter the concentration of dioxane (Baker, 2004). The freezing step used in FME is regarded as having a nullifying effect on extracellular enzymes that may remain in solution and serve to alter the concentrations of dioxane through extraneous transformations between experiment and analysis (Li et al., 2011). It has also been suggested that the exclusion of secondary ions from SIM analysis will result in better detection of analytes (Grimmett and Munch, 2009; Li et al., 2011). Recoveries for FME were not altered by 1,1,1-trichloroethane co-contamination or the introduction of large amounts of TOC (Li et al., 2011). The FME method described by Li et al. requires a small sample size of 0.2 mL which is appropriate for bench scale experiments. The method is also relatively cheap and not labor intensive.

The MDL for FME is above 0.35 μg/L, and the instrument was only calibrated to 10 μg/L, not the MDL 1.6 μg/L. This means that the method does not likely have high enough sensitivity to detect sub-μg/L concentrations of dioxane. A challenge faced with FME is that it requires the use of a harmful chlorinated organic solvent, such as DCM. Direct injection of organic solvents onto GC-MS requires the use of a solvent delay. The solvent delay is enacted to protect the instrument from the elution of high concentrations of solvent onto the MS, but incurs a minimum retention time requirement of analytes. This method can only be used to analyze compounds with a

retention time longer than the solvent delay making it less desirable than a method without a solvent delay. Large salt additions have been found to alter dioxane recoveries. The addition of 20% sodium chloride by total sample mass resulted in recoveries deviating above 30% from expected concentrations (data not shown). This indicates that sample matrix may have a significant impact on dioxane recoveries.

2.4 Purge and Trap with Gas Chromatography Flame Ionization Detection

Purge and Trap (P&T) coupled with GC-FID for the detection of dioxane was first described by Zenker et al. (2004). In this method samples are heated and stripped from solution onto a solid phase column, a trap. The system then desorbs the contents of the trap onto the GC-FID with carrier gas. Further description of the method is provided in Figure 2.4.

Sample Preparation by Heated Purge and Trap (P&T)

- 5 mL sample were heated on the sample sparging unit to 80^0C for 15 minutes prior to purging
- The sample was then purged isothermally at 80^0C with high purity helium gas
- The system was then dry purged for 6 minutes to remove excess water vapor from the trap column
- The trap was then desorbed onto the GC-FID with helium carrier gas

Figure 2.4 Description of the P&T GC-FID method from Zenker et al., 2004.

The P&T system used was a Tekmar LSC 3000 installed with a VOCARB 3000 trap and Tekmar 2016 autosampler. The GC-FID was equipped with a 75 m DB-VRX column with 0.45 mm inner diameter, and 2.25 μm film thickness. An internal standard, 1,3-dioxane, was added prior to the purge step. This method yielded a quantitation limit of 2 μg/L. The retention time and reliability of this method were not reported as the scope of the research was not primarily focused on method development.

Ease of use is a readily identifiable benefit of using this method as it does not require the use of harmful chlorinated solvents to extract or elute dioxane from another phase. The trap is usable for multiple samples and needs to be replaced very

infrequently. Continuity between purge extractions is maintained by an autosampler reducing operator time and error.

P&T is often avoided for use with dioxane because it is fully miscible in water and hard to concentrate with extraction efficiencies below 1% (Munch and Eichelberger, 1992). Recoveries for P&T can be improved by the addition of salt to the solution but reproducibility can be affected by high salt concentrations (Epstein et al., 1987). Heating of the sparging unit can improve efficiencies, but may harm the instrumentation used with the introduction of excess water vapor (Zenker et al., 2003). This method does require the use of high purity helium for an extended purging step which can incur greater expense.

2.5 Purge and Trap with Gas Chromatography Mass Spectrometry

P&T GC-MS was first described by Draper et al. (2000). This method operates very similar to the P&T GC-FID method. In this method an aliquot of sample was purged and then desorbed from the trap and eluted onto the GC-MS via helium carrier gas (Figure 2.5).

Sample Preparation by Purge and Trap (P&T)

- 25 mL aliquot of sample was purged for 11 minutes at 30^0C with high purity helium
- The sample was then desorbed from the trap and eluted onto the GC-MS via helium carrier gas

Figure 2.5 Description of the P&T GC-MS method from Draper et al., 2000.

This method utilized a Tekmar LSC 3000 Purge and Trap equipped with a Supelco three-part trap with Tenax GC, coconut charcoal and OV-1 on Chromosorb W. The Varian 3400 GC was installed with a Supelco VOCOL column measuring 75 m long, 0.75 mm inner diameter, and a 1.5 μm film. Dioxane-d_8 was added prior to purging and used as an internal standard. This method was operated in SIM mode and analyzed dioxane at 88, 86, 58, and 43 m/z. Dioxane-d_8 was also observed at 96, 93, 64, and 46 m/z. Retention times for dioxane and dioxane-d_8 were 4.68 and 4.74 minutes, respectively. This method produced a 10 μg/L quantitation limit for dioxane. The instrument calibrated linearly from 10 to 5000 μg/L with a 13% response factor relative standard deviation following isotopic dilution correction. Response factor

refers to the signal to mass ratio of the analyte multiplied by signal to mass ratio of the internal standard. Since the internal standard is a set concentration, the response factor, which under ideal conditions is constant, can be used to solve for the analyte's concentration. Isotopic dilution refers to a correction factor applied to the dioxane concentration that is calculated on a per sample basis by taking the area of the internal standard peak divided by the average of the area of the internal standard peak of the calibration standards. Note that the same isotopic dilution is applied to standard concentration calculation as well.

This method exploits many of the same benefits as the aforementioned P&T GC-FID method. P&T GC-MS does not require the use of chlorinated solvents, the extraction method can be automated, and there is little required in terms of sample preparation. The challenges faced by this method are quite similar to those mentioned for the P&T GC-FID method except that that this method has almost an order of magnitude worse MDL than the reported quantitation limit of P&T GC-FID. Draper et al. also went on to state that P&T GC-MS was only accurate enough for wastewater and leachate containing high concentrations of dioxane, but not for contaminated natural waters with dilute concentrations.

A P&T GC-MS method was later adapted from EPA Method 8260B Revision 2 by Lippincott et al. (2015) with the addition of heating to the P&T system. This resulted in a practical quantitation limit of 2 μg/L. The retention time and reliability of this method were not reported as the scope of the research was not primarily focused on method development.

2.6 Bioremediation

Remediation refers to a number of processes used to remove hazardous compounds from an environment including excavation, transport, soil washing, extraction, pump-and-treat, oxidant addition, and incineration (Doty, 2008). The scope of this document is primarily concerned with the remediation of groundwater, but it is worthwhile to mention that soils, surface water, and other media may be the environment in need of remediation under different circumstances. In the United

States it has been estimated that between 6 and 8 billion dollars are spent each year on remediation efforts and that the world as a whole spends between 25 and 30 billion dollars annually on such efforts (Glass, 1999; Tsao, 2003; Doty, 2008; Kang, 2014). However, conventional remediation efforts are costly (Mahendra and Cohen, 2006; Kang, 2014) and can cause contamination of other resources if performed improperly (Semprini, 1995; Vidali, 2001; Singh et al., 2008). Bioremediation, the use of organisms to degrade hazardous pollutants, provides a less costly and more environmentally friendly method of remediation (Kuiper et al., 2003; Singh et al., 2008; Kang, 2014).

Organisms used for bioremediation can vary from single cell microbes to multicellular plants (Wood, 2008). It is widely accepted that bacteria are capable of degrading a number of hazardous organic compounds (Semprini, 1997; Doty, 2008). Microbial bioremediation focuses on using bacteria to facilitate the degradation of toxic compounds by either intracellular accumulation or enzymatic transformation (Singh et al., 2008). Intracellular accumulation can be used to detoxify an environment but requires the removal and disposal of the bacterial cells in order to effectively remediate the pollutants. Intracellular accumulation by microbes and plants (phytoremediation) is often applied when a contaminant cannot be transformed, such as with metals (Fingerman & Nagabhushanum, 2005; Glick, 2010). Enzymatic transformation can offer a more permanent solution by transforming the pollutant into a more benign form, often resulting in the complete mineralization of organic contaminants (Heitzer and Sayler, 1993; Kuiper et al., 2003). For microbial bioremediation to take place several criteria must be met. The microbes need to be able to survive and remain metabolically active in their environment, the contaminant must be bioavailable, and the necessary enzyme inducers must be present (Doty, 2008).

Degradation of contaminants by microorganisms can happen either metabolically or co-metabolically (Mahendra and Alvarez-Cohen, 2006). Organisms that degrade a contaminant as their sole carbon and energy source perform degradation of the contaminant metabolically (Mahendra and Alvarez-Cohen, 2005).

Organisms that degrade a contaminant metabolically are often desired when large concentrations of contaminant are present at a location as they do not have a limit to how much contaminant they can degrade. Typically metabolically degrading organisms have a lower affinity for the contaminant and exhibit lower growth rates than co-metabolically degrading organisms (Mahendra and Alvarez-Cohen, 2006).

Co-metabolic degradation occurs when there is gratuitous transformation of non-growth substrate by cells (Wackett et al., 1989). This means that the pathway used to degrade a primary growth substrate can also to some extent degrade the contaminant of interest. Semprini (1995) suggested using aerobic co-metabolism as a polishing step in chlorinated solvent remediation as co-metabolic processes can often force contaminant degradation to lower concentrations than those of metabolic processes. This perceivable benefit results from the fact that organisms that must metabolize a contaminant can only utilize it to the point where it becomes too dilute to afford positive growth; whereas, co-metabolic cells utilize a substrate other than the contaminant for metabolic energy (Fournier, 2009; Webster et al. 2013).

Anaerobic or aerobic microbes may be used to carry out bioremediation (Lee et al., 1998; Chomsurin et al., 2008). Anaerobic degradation is a more common approach to bioremediation of chlorinated solvents as the reductive dechlorination pathway has been well studied and effectively applied at multiple locations (Major et al., 2002; Macbeth et al., 2004; Kennedy et al., 2006; Dugat-Bony et al., 2012; Kang, 2014). The anaerobic reductive dechlorination pathway utilizes the chlorinated contaminant as a growth substrate and is considered a metabolic process. Slow growth, pH-sensitivity, and inhibition by co-contamination are some obstacles faced with using anaerobic cultures for bioremediation (Duhamel et al., 2002; Grostern and Edwards, 2006; Kang et al., 2014). Anaerobic cultures are typically more economical to use as they do not require aeration.

Aerobic cultures have been shown to be effective at removing petroleum hydrocarbons and other volatile organic compounds (Adams and Reddy, 2003; Wu et al., 2005; Brar et al., 2006; Kao et al., 2008). Petroleum hydrocarbons can be degraded aerobically or anaerobically, but rates of degradation are higher under

aerobic conditions (Deeb et al., 2003; Moreels et al., 2004; Kao et al., 2008). The higher rate of degradation by aerobic organisms makes them more desirable in some cases, but often the primary issue faced by aerobic organisms is the resupply of oxygen. Many environments are oxygen-limited, such as subsurface aquifers and deep lakes, even shallow ponds with high organic loading. Introducing oxygen to these environments is often costly and may prevent application of aerobic bioremediation.

Bioremediation can be performed as an *ex situ* or *in situ* technique (Dugat-Bony et al., 2012). *Ex situ* remediation implies that the contaminated water is removed from its environment to a different location where it undergoes treatment (de Lorenzo, 2008). An example of an *ex situ* process is a combined pump-and-treat bioremediation technique, where groundwater is removed from the subsurface by pumping and it is treated on the surface by a means of bioremediation, such as in a bioreactor. *In situ* treatment occurs when the treatment happens at the location of the contamination (de Lorenzo, 2008). Biostimulation and bioaugmentation are two examples of *in situ* bioremediation techniques.

Biostimulation takes advantage of native microbial communities able to degrade the contaminant in question. Often natural attenuation rates are slow and can be increased through the addition of substrates, enzymatic inducers, and nutrients called biostimulation (Doty, 2008; Vidali, 2011). Oxygen, gaseous enzymatic inducers, and gaseous substrates can be added through the processes of bioventing or biosparging (Vidali, 2011). Bioventing occurs when gases are pumped into the unsaturated zone above the contaminated groundwater at low pressure (Semprini, 1997); whereas, biosparging employs the introduction of gases directly to the groundwater by injection below the contaminated zone (Kao et al., 2008). Biostimulation has shown to be successful in several sites where reductive dechlorination was applied (Macbeth et al., 2004; Kennedy et al., 2006).

Bioaugmentation occurs through inoculation, or addition, of exogenous microbes capable of degrading the contaminant at the contaminated site. The organisms may then be biostimulated in place as they bioremediate the contaminated

site. There are a number of instances where anaerobic bioaugmentation, primarily for reductive dechlorination, has been successful in the field (Ellis et al., 2000; Major et al., 2002) and in laboratory microcosm experiments (Payne et al., 2011; Justicia-Leon et al., 2014). Co-metabolic bioaugmentation has also been used successfully in several instances of field application (Wilson and Wilson, 1985; Semprini, 1997; Steffan et al., 1999;; Fournier et al. 2009; Lippincott et al., 2015).

Often nonindigenous cultures have difficulties competing with native microbes and thus are incapable of performing adequate degradation (El Fantroussi and Agathos, 2005; Thompson et al., 2005; Vidali, 2011). Failure of bioaugmentation has occurred in many instances (Goldstein et al., 1985; Bouchez et al., 2000; Thompson et al., 2005) but the cause of such failures is often an issue of application oversight. Most bioaugmentation failures transpire because of the wrongful assumption that the native microbes are catabolically unable to degrade the contaminant, while the actual reason is tied to some other physical characteristic such as pH or redox (Thompson et al. 2005). Microbiological methods have only recently been developed to determine why bioaugmentation sometimes fails when all other parameters are accounted for (Dueholm et al., 2015).

2.7 Remediation of Dioxane

Physical properties, such as miscibility, make dioxane difficult to analyze (Li et al., 2011) and remediate (Patterson et al., 1985). Volatilization by air stripping and adsorption by activated carbon do not provide efficient removal of dioxane (Zenker et al., 2003; Vainberg et al., 2006). Aeration for 2.4 hours at an 80:1 air-to-water ratio only resulted in a 3% removal efficiency of dioxane (McGuire et al., 1978). Adsorption by activated carbon resulted in extraction efficiencies of 50 to 67% (McGuire et al., 1978; Johns et al., 1998). Commercialized degradation processes such as photocatalytic, ozone-electrolysis, and UV/hydrogen peroxide do provide a remediation mechanism for dioxane (Hill et al., 1997; Stefan et al., 1998; Kishimoto et al., 2008), but they are often too costly to operate as they require *ex situ* treatment (Mahendra and Cohen, 2006). Transformation by bioremediation provides another

option for the degradation of dioxane (Mahendra et al., 2013), which has shown promise in the laboratory and at one field scale experiment (Lippincott et al., 2015).

Many microbial species of the actinomycete phylum have been identified that can either metabolize (Bernhardt and Diekmann, 1991; Parales et al., 1994; Mahendra and Alvarez-Cohen, 2005) or co-metabolize dioxane aerobically (Zenker et al., 2000; Mahendra and Alvarez-Cohen, 2006; Vainberg et al., 2006, Table 2.1). These microbes transform dioxane through enzymes known as monooxygenases (Mahendra and Alvarez-Cohen, 2006). Monooxygenase enzymes are capable of dividing molecular oxygen with one oxygen going to the oxidation of a substrate and the other to form water. Broad specificity monooxygenases can degrade a number of xenobiotic compounds (Mahendra and Alvarez-Cohen, 2006; Fasan, 2008). Methanotrophs, organisms that oxidize methane, and nitrifying bacteria, organisms that oxidize ammonia, are two examples of organisms that produce broad-substrate specificity monooxygenase enzymes that have been proven useful in co-metabolic bioremediation of hazardous compounds (Dolan and McCarty, 1995; Kim et al., 2002).

The proposed pathway for direct metabolic dioxane degradation by *Pseudonocardia dioxanivorans* is shown in Figure 2.6. The metabolic pathway proposed utilizes dioxane monooxygenase to perform the first oxidation step to 2-hydroxyethoxyacetic acid (HEAA) and then another monooxygenase that allows for HEAA to be degraded to two easily mineralized products (Mahendra et al., 2007). The proposed pathway for co-metabolic dioxane degradation by a tetrahydrofuran-grown microbe, *Pseudonocardia* sp. strain ENV478, is shown in Figure 2.7. The co-metabolic pathway degrades dioxane to HEAA which then undergoes a pH-driven equilibrium reaction to 1,4-dioxane-2-one (PDX, Vainberg et al., 2006). Neither HEAA nor PDX were mineralized or incorporated into biomass in the *Pseudonocardia* sp. strain ENV478 that was found to degrade dioxane (Vainberg et al., 2006).

Organisms that consume dioxane as their sole carbon and energy source are typically unable to degrade the contaminant to implied regulatory standards because

of enzymatic limitations, inability to maintain gene induction, or inability to fully mineralize dioxane metabolites (Vainberg et al., 2006; Lippincott et al., 2015). Microbes grown on tetrahydrofuran, methane, propane, toluene, or ethanol as their primary growth substrate have been shown to co-metabolize dioxane (Burback and Perry, 1993; Kohlweyer et al., 2000; Zenker et al., 2000; Mahendra and Alvarez-Cohen, 2006, Mahendra et al., 2013). Limitations on the rate of co-metabolic degradation of dioxane have been shown to occur in the presence of growth substrate and in the presence of co-contaminants (Mahendra et al., 2013). Most studies have been performed at dioxane concentrations in the parts per million range (Table 2.1) (Mahendra and Alvarez-Cohen, 2006), but as previously mentioned the median concentration at contaminated sites in the U.S. is 365 parts per billion (Adamson et al., 2014). Three studies have explored the degradation of dioxane into the single digit μg/L dioxane range (Lippincott et al., 2015; Li et al., 2010; Zenker et al. 2004), no current studies characterize organisms degrading dioxane to sub part per billion concentrations.

Rhodococcus ruber ENV425, a propane-degrading bacterium, has been used for bioaugmentation and subsequent co-metabolic degradation of dioxane (Lippincott et al., 2015). In the study, dioxane was degraded from as high as 1 mg/L down to below their detection limit of 2 μg/L after 245 days of sparging with propane and air. Nutrients were added with culture in a single slug of water 42 days into biosparging. To the authors knowledge this is the only reported use of bioaugmentation to remediate dioxane contamination. The Lipppincott et al. (2015) bioaugmentation experiment reported 125 times greater *in situ* degradation of propane than a similar biostimulation experiment performed by Kim et al. (2008) to aerobically degrade cis-1,2-dichloroethene and trichloroethene. This bioaugmentation experiment demonstrated successful subsurface bioaugmentation and bioremediation of dioxane via *in situ* propane sparging at a dioxane-contaminated groundwater site. Several studies have also identified intrinsic biodegradation of dioxane in natural, aerobic waters (Li et al., 2010; 2013; Sei et al., 2010; Chiang et al., 2012), but to the authors

knowledge there are no reported remediation attempts with microbes that directly metabolize dioxane.

In this study two cultures were tested as potential bioaugmentation cultures for a field demonstration of co-metabolic dioxane transformation at low concentration levels (< 500 μg/L). Heated P&T GC-MS method was investigated as an analytical tool for the quantification of low concentrations of dioxane. Cultures were evaluated for their ability to transform dioxane when grown on propane or propanol in growth media and site groundwater.

Table 2.1 Various parameters of dioxane degrading organisms. (Adapted from Mahendra and Alvarez-Cohen, 2006).

Reference	Organism	Concentration 1,4-Dioxane (mg/L)	Rate of 1,4-Dioxane (mg dioxane/ mg protein/ hr)	Co-metabolic Transformation Capacity (mg dioxane/ mg protein)	Temperature (^{0}C)
Mahendra and Alvarez-Cohen, 2006	*Pseudonocardia dioxanivorans* CB1190	50	0.19 ± 0.007	N/A (Metabolic)	30
	Pseudonocardia benzenivorans B5	50	0.01 ± 0.003	N/A (Metabolic)	30
	Pseudonocardia K1	25	0.26 ± 0.013	0.22 ± 0.014	30
	Pseudonocardia K1	25	0.16 ± 0.006	0.23 ± 0.015	30
	Methylosinus trichosporium OB3b (soluble methane monooxygenase)	50	0.38 ± 0.02	2.3 ± 0.015	30
	Mycobacterium vaccae JOB5	88	0.40 ± 0.06	1.4 ± 0.007	30
	Rhodococcus RR1	50	0.38 ± 0.03	0.32 ± 0.003	30
	Burkholderia cepacia G4	50	0.10 ± 0.006	0.26 ± 0.008	30
	Ralstonia pickettii PK01	50	0.31 ± 0.007	0.29 ± 0.01	30
	Pseudomonas mendocina KR1	50	0.37 ± 0.04	0.38 ± 0.006	30
	Escherichia coli TG1 (toluene-2 monooxygenase)	50	0.06 ± 0.008	0.29 ± 0.005	30
	Escherichia coli TG1 (toluene-p monooxygenase)	50	0.17 ± 0.01	0.27 ± 0.016	30
	Escherichia coli TG1 (toluene-4 monooxygenase)	50	0.26 ± 0.03	0.30 ± 0.02	30
	Enrichment Culture	200		1.0 ± 0.36	35
Li et al., 2010	*Pseudonocardia dioxanivorans* CB1190	50	0.155 ± 0.038	N/A (Metabolic)	14
	Pseudonocardia dioxanivorans CB1190	50	0.021 ± 0.007	N/A (Metabolic)	4
	Pseudonocardia antarctica DFVS 5a1	50	0.015 ± 0.006		14
	Pseudonocardia antarctica DFVS 5a1	50	0.018 ± 0.004		4
Lippincott et al., 2015	*Rhodococcus ruber* ENV425	1.090 to 0.135	0.864 to 0.504		

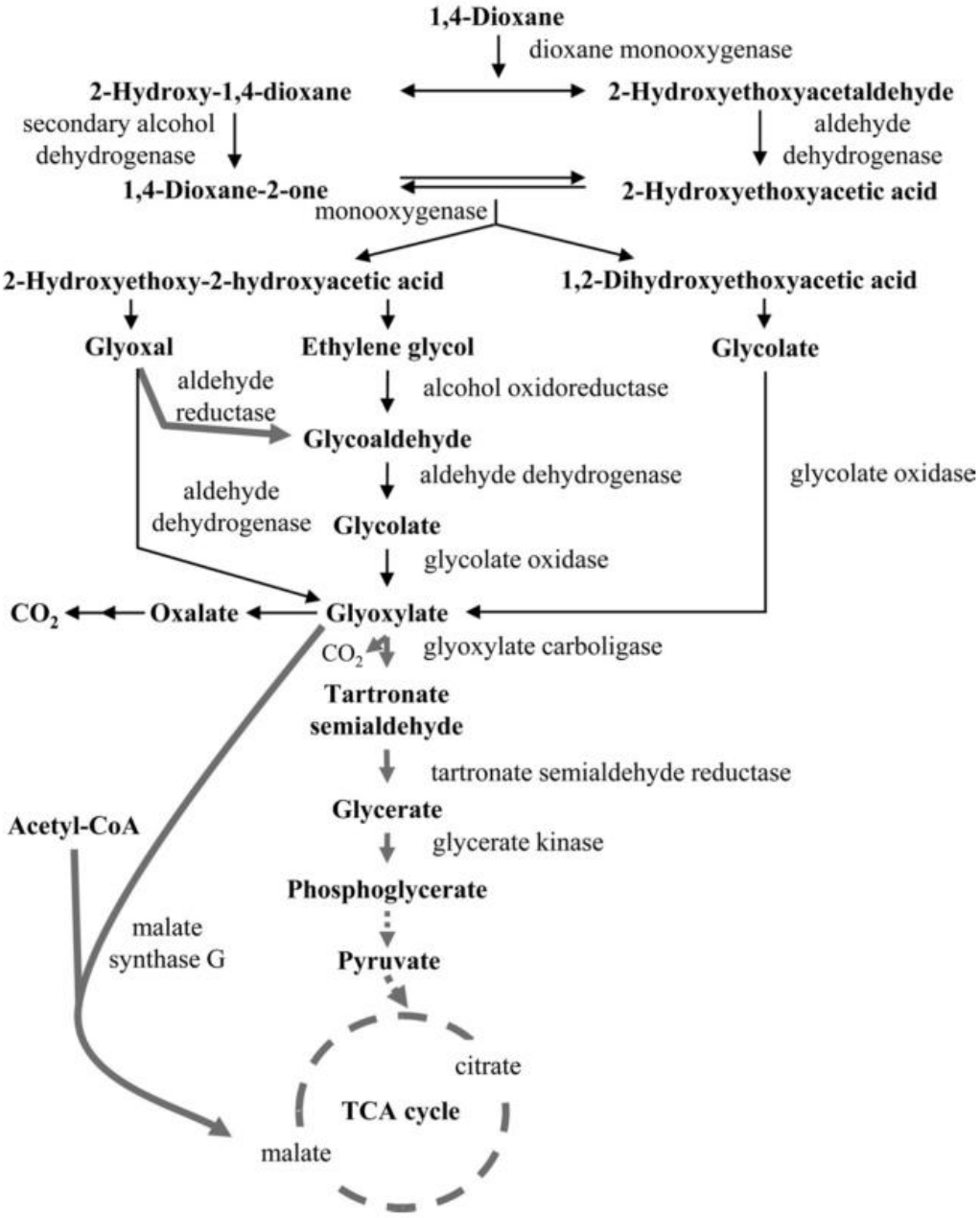

Figure 2.6 Proposed pathways and enzymes involved in dioxane metabolism in *Pseudonocardia dioxanivorans* (Grostern et al. 2012).

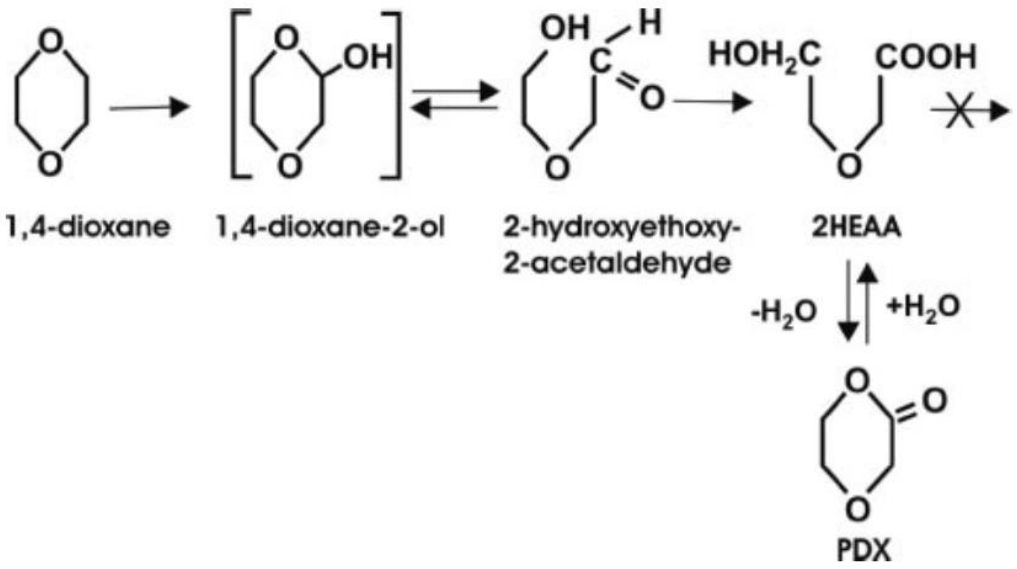

Figure 2.7 Proposed partial pathway for biodegradation of 1,4-dioxane by *Pseudonocardia* sp. strain ENV478 (Vainberg et al. 2006).

CHAPTER 3
MATERIALS AND METHODS

3.1 Reagents

All solid reagents used were American Chemical Society (ACS) grade or better analytical reagents. Propane (99%) and HD5 propane (commercial grade) gases were obtained from commercial suppliers and were fitted with VWR (Radnor, PA) 0.2 μm, cellulose acetate filters for sterility. 1,4-dioxane (99%) and 2-propanol (100.0%) were acquired from J.T. Baker (Center Valley, PA). 1,4-dioxane-d_8 (99% Isotopic) and 1-Propanol (99.5%) were purchased from Alfa Aesar (Ward Hill, MA). Nanopure water refers to laboratory manufactured water that had undergone treatment by a Barnstead (Waltham, MA) nanopure filtration system to a resistance of at least 17.0 MΩ. All glassware, aside from instrumental glassware, glass syringes, and stock or reagent glass containers were autoclaved for at least 50 min prior to use.

3.2 Media Formulation

Precursor media solutions were prepared by adding the salts listed in Table 1 to their appropriate volumes of nanopure water into either 125mL or 500 mL Wheaton (Millville, NJ) glass bottles depending on the amount prepared. Mineral salts growth media (MSM) was prepared by autoclaving nanopure water, buffer precursor, and precursor solutions one through five for 60 min. Once cool, salts were added aseptically to the autoclaved nanopure water. These additions resulted in final media concentrations shown in Table 1. Media was then used immediately or stored at room temperature in a dark cabinet until use.

3.3 Groundwater and Aquifer Solids Preparation

Groundwater was obtained from McClellan Air Force Base, a site contaminated with < 100 μg/L dioxane, < 20μg/L trichloroethene, and < 25μg/L 1,1-dichloroethene,

using non-aseptic techniques. The groundwater was then shipped in 5 gallon plastic drums to the Environmental Engineering laboratory located in Merryfield Hall at Oregon State University. Once received the drums were refrigerated in a 4 ^{0}C cooler until needed for an experiment. Prior to use in an experiment the groundwater was vacuum filtered through Advantec (Dublin, CA) 0.45μm, cellulose acetate filters under aseptic conditions. Aquifer solids were prepared for use in experiments by being autoclaved for one hour on two separate occasions, 48 hours apart.

3.4 Cell Culturing and Cryogenic Preservation

Cultures were acquired on plate count agar media plates and aseptically transferred into 500 mL glass bottles containing 200 mL MSM (Table 3.1) and the remainder of the volume with air containing 30 mL of HD5 propane. Cells were allowed to grow to an optical density (OD) of greater than 0.3 abs. Once grown, cells were preserved until use by transferring 1 mL of cell solution into a 2 mL Corning (Corning, NY) round bottom, cryogenic vial. An additional 1 mL of MSM amended with 20% glycerol was added to the cryogenic vial in order to prevent the formation of ice lattices within the cells during cryopreservation. The vial was then and placed in a -80 ^{0}C freezer. This process was performed multiple times from the same cell stock solution in order to ensure the consistency of culture among experiments.

To grow cells for use in an experiment the vials were removed from the -80 ^{0}C freezer and placed in the 4 ^{0}C cooler to thaw overnight. The next day the vials were taken to the laminar flow hood where they were allowed to thermally equilibrate for at least 30 min. The solution from the cryogenic vials was then aseptically transferred into 500 mL glass bottles containing 200 mL MSM with the remaining volume comprised of air. Propane, HD5, 1-propanol, or 2-propanol were added to the bottle and the cells were allowed to grow to a certain OD defined by experimental requirements. In the event that bottles required additional oxygen it was provided by purging the bottle's headspace in a sterile laminar flow hood with oxygen for 30 seconds followed by letting them rest open for 5 min. After this step additional substrate was added.

Table 3.1 The formulation of mineral salts media used for cell culturing.

Media Precursor	Salt	Stock Solution Final Volume (mL)	Mass Added to Stock Solution (mg)	Final Concentration Stock Solution (mg/L)	Volume Stock Added to Nanopure Water (mL)	Final Concentration SERDPA Media (mg/L)
Buffer Precursor					12.5	
	K2HPO4*3H2O	500	40618	81236		2030.9
	NaH2PO4*H2O	500	14780	29560		739.0
Precursor 1					1	
	MgSO4*7H2O	100	3010	30100		60.2
Precursor 2					1	
	CaCl2	100	555	5550		11.1
Precursor 3					1	
	$(NH3)_{2SO4}$	100	5259.2	52592		105.2
Precursor 4					1	
	NaNO3	100	7650	76500		153.0
Precursor 5					1	
	FeSO4*7H2O	500	1570.75	3141.5		6.283
	MnCl2*4H2O	500	75.2	150.4		0.301
	ZnSO4*7H2O	500	36.65	73.3		0.147
	H3BO3	500	15.45	30.9		0.062
	Na2MoO4*2H2O	500	27.225	54.45		0.109
	NiCl2*6H2O	500	5.95	11.9		0.024
	CuCl2*2H2O	500	4.25	8.5		0.017
	CoCl2*6H2O	500	5.95	11.9		0.024

3.5 Batch Reactors

Batch reactors were created by aseptically adding cell solution and various amounts of media to obtain an experimentally set OD in a 125 or 500 mL glass bottles. The experiments only varied in gas to liquid ratio at a minimum of 2.4:1 up to a maximum of 4.8:1. All experiments were conducted at 30 °C at shaking rates of approximately 200 RPM. Some experiments required the use of groundwater instead of MSM. In cases were groundwater was used, cell solution was centrifuged for 10 min at 9000 RPM and then the liquid decanted and replaced with groundwater. The tubes were vortexed in order to re-suspend the cells, and the cell solution transferred to a batch reactor. Various amounts of propane, HD5, 1-propanol, 2-propanol, and dioxane stock solution were added to these reactors as dictated by experimental parameters. These bottles were then monitored through headspace removal using a Hamilton (Reno, NV) gas tight syringe or liquid removal by plastic syringe. Samples to be analyzed for dioxane were either centrifuged in 1.5 mL VWR micro-centrifuge tubes for 5 min at 12000 RPM prior to frozen micro extraction or filtered through 0.2 μm, cellulose acetate syringe filters into 10 mL BD (Franklin Lakes, NJ) culture tubes for purge and trap analysis.

3.6 Cell Mass and Protein Analysis

Total suspended solids (TSS) measurements were performed by weighing filters used to filter a certain volume of cell solution of known OD. Prior to filtration 0.45 μm, cellulose acetate filters were desiccated in a 105 ^{0}C oven for at least one hour. The filters were then placed within a desiccator, allowed to cool for 5 min, and then were weighed. Once weighed the filters were placed on the vacuum filtration apparatus where various volumes of diluted cell solution were passed through. After filtration the filters were returned to the oven for at least 2 hours. The filters were once again, placed in the desiccator, and then weighed after cooling for 5 min. The initial weight

was subtracted from the final weight to calculate the total cell mass present on the filter and thus in the volume of solution passed through the filter.

Protein analysis was performed on cell solution to quantify the total amount of protein present in cell solutions of differing OD. Bovine serum albumen (BSA) purchased from New England Biolabs (Ipswich, MA) was used as a calibration standard. Samples and standards were lysed and solubilized with 3M sodium hydroxide and heating to 65 ^{0}C for 30 min. The tubes were then centrifuged for 5 min at 12,000 RPM. 100 μL of supernatant was then transferred to a new mircocentrifuge tube, where it was diluted to 400 μL with nanopure water, 100 μL of 3M sodium hydroxide, and 500 μL of biuret reagent. The tubes were then placed in a dark cabinet for 30 min to allow for color development. After the color development stage, samples were analyzed on a Thermo Fisher Scientific Orion AquaMate 8000 spectrophotometer set at 540 nm.

3.7 Cell Density Measurements

Cell concentrations were measured using optical density (OD) measurements which were calibrated to cell dry weights and protein to quickly estimate cell mass and protein concentration within the batch reactor systems. 1 mL samples were taken from the batch reactors by plastic syringe and transferred to 1.5 mL micro-centrifuge tube. The samples were analyzed on an Orion AquaMate 8000 spectrophotometer set at 600 nm. Figures showing the relationships between OD, cell mass, and protein are located in the Appendix. OD and cell mass were correlated using a linear fit and OD and Protein were correlated using a nonlinear fit with the following results:

$$y = 583.1x + 6.0 \; and \; z = 138.4x^2 + 23.4 - 0.84$$

respectively, where y is cell concentration, x is OD, and z is protein concentration.

3.8 Gas Chromatography

Propane analyses were performed by injecting 100 μL of sample headspace onto a Hewlett Packard (HP) (Wilmington, DE) 6890 Gas Chromatograph (GC)

equipped with a flame ionization detector (FID). The GC was installed with an Agilent GS-Q 115-3432 capillary column 30.0 m long and 530 μm inner diameter. The inlet temperature was 250 ^{0}C and the column was held isothermally at 150 ^{0}C for approximately 1.5 min. Flow of helium, hydrogen, air, and nitrogen were 15, 35, 175, and 20 mL/min, respectively. These parameters resulted in the elution of propane at 0.64 min.

Propane calibrations were performed on the GC-FID by adding volumetric amounts of propane to rubber butyl septa sealed 125 mL glass bottles. The GC-FID was calibrated at the beginning of each experiment with experimentally relevant concentrations of propane. Aqueous propane concentrations were calculated assuming gas-water equilibrium using a Henry's constant of 29.38 (mg/L gas)/ (mg/L liquid). The GC-FID was found to be highly stable, reproducible, and calibrated linearly from gaseous concentrations of 0.01 mg/L up to over 100 mg/L.

Propan-1-ol and 2-propanol measurements were obtained using direct aqueous injection onto a 8A Shimadzu (Kyoto, Japan) GC-FID equipped with a Supelco (Bellefonte, PA) 0.2% Carbowax 1500 on 80/100 Carbopack C measuring 6 ft long, 0.125 in inner diameter, and 2.1 mm stainless steel column. The instrument was operated isothermally at 100 °C and had a retention time for 1-propanol and 2-propanol at approximately 1.7 min.

3.9 Frozen Micro Extraction Gas Chromatography Mass Spectrometry

Frozen Micro Extraction was performed via an adaptation to a method first reported by Li et al. (2011). In the adapted method, a 300 μL aliquot of dioxane-containing sample was transferred by glass, gas-tight syringe into a 2 mL glass vial. Then 100 μL of dichloromethane (DCM) was added to the vial. The vial was then closed and vortexed for 30 seconds. Once thoroughly mixed, the vial was placed in a -80 ^{0}C freezer at a 60 to 70 degree angle from the horizontal for at least 2 hours. The sample was then removed from the freezer and approximately 70 to 90 μL of the unfrozen DCM was transferred by plastic pipette into a 200 μL glass, conical vial.

Approximately 0.25 mg of sodium sulfate was then added to the conical vial to remove any excess water. This DCM extract was then analyzed immediately.

Dioxane quantification was performed using a direct liquid injection of 1 μL of DCM extract onto an HP 6890 GC equipped with a HP 5973 Mass Selective Detector. The HP 6890 GC method was adapted from Li et al. 2011 and was installed with a RESTEK (Bellefonte, PA) Rtx-VMS Proprietary Crossbond Phase column measuring 30.0 m long and 250 μm inner diameter with a 1.40 μm thick film. The HP 6890 GC was initially set at 50 ^{0}C for 4.5 min and then ramped at 20 ^{0}C/min to 120 ^{0}C. After reaching 120 ^{0}C the column was then ramped at 50 ^{0}C/min to 250 ^{0}C where upon reaching 250 ^{0}C it was baked for 3 min. The 3 min bake time ensured that all dioxane and DCM was removed from the column and decreased the amount of carryover between samples. The gas flow rate and temperature of the HP 6890 GC were held at 1.3 mL/min and 200 ^{0}C, respectively. The MSD was run in selected ion monitoring mode at 88 m/z for 10 min. A solvent delay was enacted for 5.5 min to protect the MSD from DCM and any other volatile compounds in solution. This resulted in a elution time for 1,4-dioxane at approximately 6.65 min. Li et al. in 2011 had reported a quantification limit of 10 μg/L using this method, however the MDL of this adapted method is discussed later in section 4.1.2.

Dioxane calibrations were applied to the GC-MS by performing frozen micro extractions of standards created by diluting various amounts of dioxane stock solution to experimentally relevant concentrations in class A volumetric flasks with nanopure water. Dioxane stock solution was prepared by weighing 100 mg of dioxane in a septa sealed, glass, gas tight syringe and transferring the syringes contents into a 100 mL volumetric flask. The stock solution and standards were all kept refrigerated in a 4 ^{0}C cooler with minimal light exposure in amber vials. Prior to use, the stock and standard solutions were removed from the refrigerator and placed in a dark cabinet for approximately 20 min to allow thermal equilibration.

3.10 Heated Purge and Trap Gas Chromatography Mass Spectrometry

Dioxane quantification was performed using a Tekmar 3100 Purge and Trap Concentrator (P&T) (Cincinnati, OH) equipped with a Tekmar AQUATek 70 autosampler coupled to a HP 6890 GC equipped with a HP 5973 Mass Selective Detector (MSD). The Tekmar 3100 P&T was fitted with a Supelco VOCARB 3000 trap. The HP6890 GC was fitted and operated as previously mentioned.

The P&T was operated in the same fashion as described in Zenker et al. (2004), except with the following adaptations. The samples were purged for 0.2 min prior to sample heating to transfer the sample to the correct side of the sparger. The setup included a manually set glassware heating which was calibrated by thermocouple to reach 80 ^{0}C with 5mL of aqueous sample present in the sparger. All purging was performed with nitrogen instead of helium gas. The transfer line between the P&T and HP 6890 GC was held constant at 150 ^{0}C.

Each analysis was performed through the introduction of 5 mL of aqueous sample or diluted aqueous sample to the purge and trap sparging apparatus. Observed inconsistencies with results prompted the introduction of an internal standard to all standards and samples. Deuterated 1,4-dioxane (dioxane-d_8) was used as an internal standard for all analyses unless stated otherwise. Dioxane-d_8 shares the same chemical properties as non-labeled dioxane and thus it was eluted from the GC column at the same time as non-labeled 1,4-dioxane, but dioxane-d_8 was detected at a mass over charge ratio of 96 m/z. Therefore the MSD was assigned to monitor both 88 and 96 m/z instead of solely 88 m/z. Dioxane and dioxane-d_8 shared a retention time of 6.7 min under these conditions (Figure 3.1).

Deuterated dioxane stock solution was prepared by weighing 100 mg of dioxane-d_8 in a septa sealed, glass, gas tight syringe and transferring the syringe contents into a 100 mL volumetric flask. The stock solution was kept refrigerated in a 4 ^{0}C cooler with minimal light exposure in amber vials. Prior to use, the stock solution was removed from the refrigerator and placed in a dark cabinet for approximately 20 min to allow thermal equilibration. Internal standard was

consistently spiked into all samples and standards to yield a final concentration of 5 μg/L. This allowed for isotopic dilution of non-labeled dioxane concentrations.

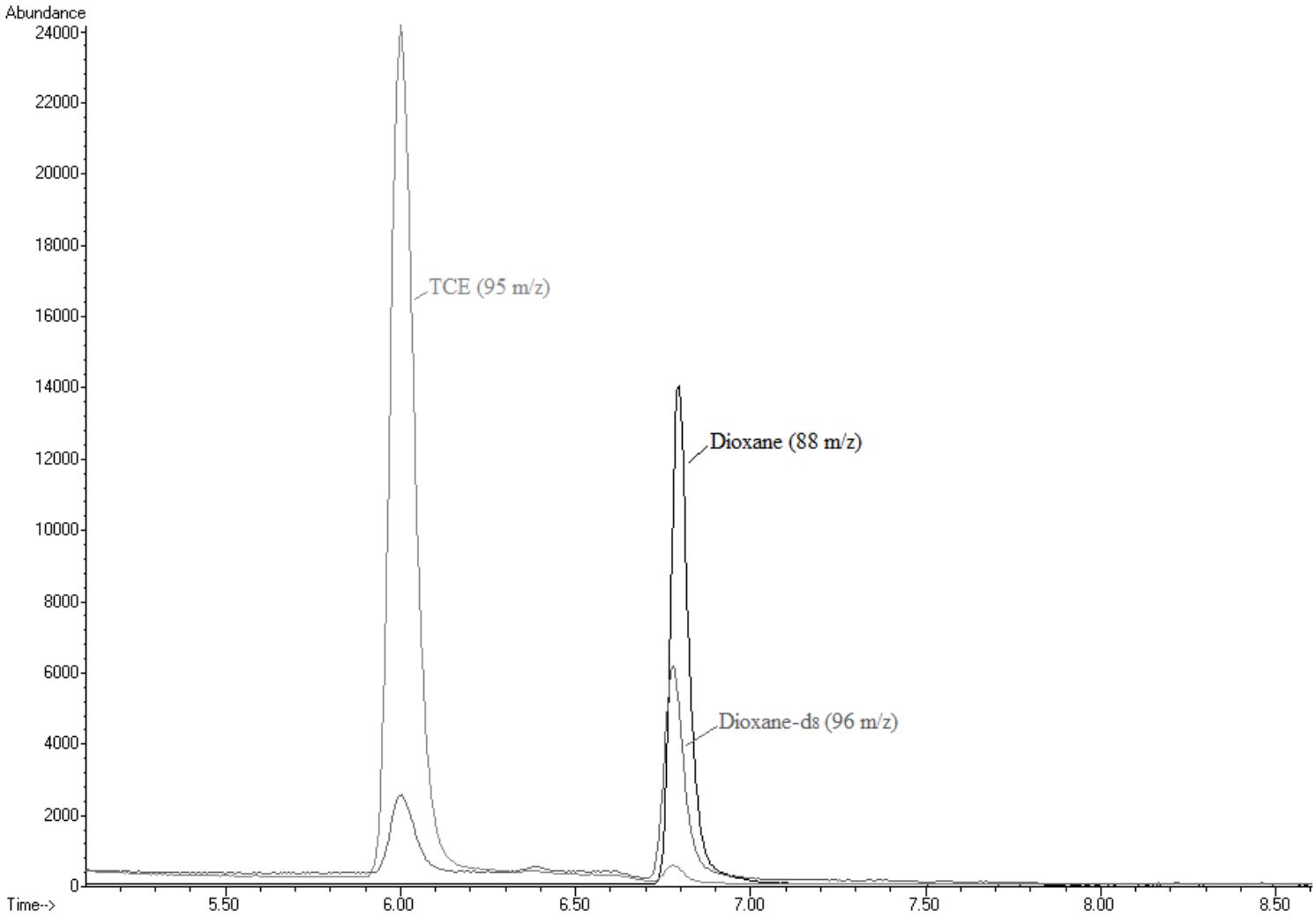

Figure 3.1 A chromatogram showing dioxane (88 m/z), TCE (95 m/z), and dioxane-d_8 (96 m/z).

CHAPTER 4
EVALUATION OF LOW LEVEL ANALYTICS OF 1,4-DIOXANE

Introduction

Development of a method for the quantification of dioxane at sub-µg/L was essential to evaluating possible cultures for use in a bioaugmentation demonstration. In order to determine the best analytical method available for studying dioxane transforming cultures, the FME and Heated P&T methods were evaluated in terms of their analytical performance and method detection limit. This section identifies the limitations of FME, Heated P&T not utilizing isotopic dilution, and Heated P&T with isotopic dilution for the quantification of dioxane.

4.1 Frozen Micro Extraction (FME) without Isotopic Dilution

4.1.1 Analytical Performance

A six point calibration was created using concentrations similar to those reported by Li et al. (2011) to address the range of concentrations found in contaminated groundwater. Standards were prepared at 10, 25, 50, 100, 250, and 500 µg/L dioxane. The calibration curve for the FME method can be found in Figure 4.1. The standards for this method calibrated linearly at concentrations from 25 µg/L up to 500 µg/L, as shown by the > 0.99 correlation coefficient. A four point calibration was performed at the beginning of each daily analysis run with standards at concentrations of 25, 100, 250, and 500 µg/L.

Initial calibration blank verification (ICBV) and initial calibration verification (ICV) quality control standards (QCs) at concentrations of 0 µg/L and 100 µg/L, respectively, were analyzed following each calibration to determine the stability of the instrument following calibration. Continuing calibration verification (CCV) QCs at a concentration of 250 µg/L were analyzed at the end of each analysis period. The calculated concentrations from these QCs would occasionally exceed the EPA

requisite ± 20% threshold indicating some instrumental variability. The largest deviation from the expected concentrations among QCs was recorded in a CCV at 220%. This large of a difference between expected concentration and reported concentration was likely resultant from instrumental carryover between samples and the lack of internal standard to correct method variability.

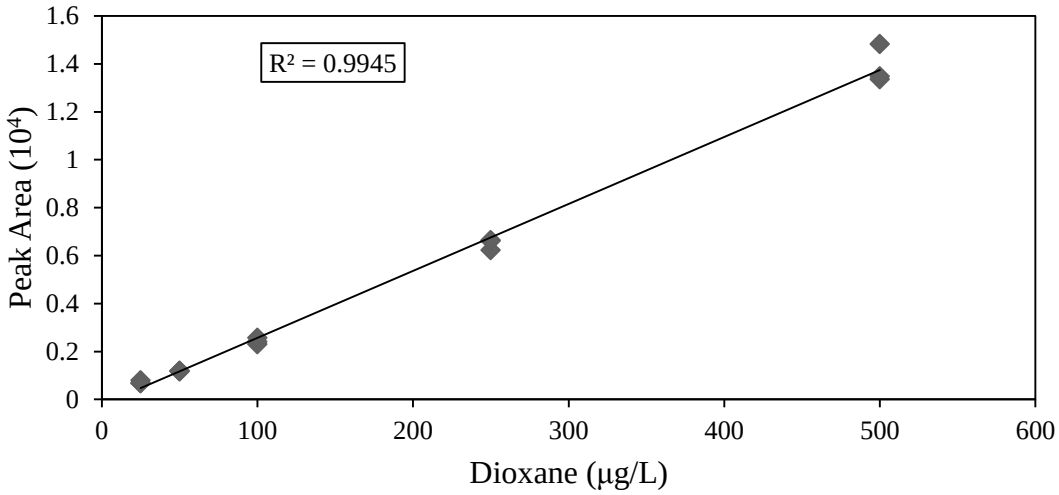

Figure 4.1 Calibration curve of dioxane analyzed by FME without Isotopic Dilution plotted with linear fit and regression coefficient.

4.1.2 Method Detection Limit

Li et al. (2011) reported a MDL of 1.6 µg/L when performing their FME experiments. Four replicates of the lowest concentration standard (25 µg/L) were used to perform an MDL calculation. The MDL was calculated using the follow equation:

$$MDL=\sigma \times T(n-1,1-\alpha=0.99)$$

where σ is the standard deviation of replicate samples from their mean value in µg/L, α is the level of significance, T (n-1,1- α=0.99) is the T value at the 99% confidence level with n-1 degrees of freedom, and n is the number of replicate samples (Kawata et al., 2001; Li et al., 2011).

The FME method described in this work had an MDL of 10.5 µg/L. Deuterated dioxane was not available for use as an internal standard during this method's evaluation and thus the lack of isotopic dilution during analysis likely resulted in a much greater MDL than previously reported. The 10 µg/L calibration

standard was therefore rejected for calibration as it fell below the calculated MDL and during most runs carryover between samples would amount to as large of peak area as the 10 µg/L standard. This MDL is approximately an order of magnitude higher than that reported by Li et al. (2011) using a similar method. Additionally, the difference observed between MDL values may also be a function of the number of replicate analyses performed. Li et al. analyzed seven replicates of their 10 µg/L standard, whereas this evaluation only performed analyses on four replicates of a 25 µg/L standard. The addition of more replicate analyses and incorporation of an internal standard would likely improve the MDL of this method to levels as seen by Li et al. (2011).

4.2 Heated Purge and Trap without Isotopic Dilution

4.2.1 Analytical Performance

A nine point calibration was performed with standards prepared at 0.01, 0.025, 0.05, 0.075, 0.1, 0.2, 0.3 , 0.4, and 0.5 µg/L dioxane (Figure 4.2). While these standards do not cover as large of a range as those selected for the FME method, they attempt to showcase the low level detection capabilities associated with the heated purge and trap method. These standards plotted linearly from 0.025 to 0.5 µg/L with a correlation coefficient of 0.86. Standards below 0.1 µg/L were ignored as the relative standard deviation (RSD) of those standards exceeded the requisite 30% guideline for RSDs in Method 8270D SW-846 provided by the U.S. EPA (2007), and a single outlier was removed from the remaining standards as it was the only point exhibiting a dioxane recovery of greater than 25%. The removal of these standards led to a higher correlation coefficient of 0.95 (Figure 4.3). A six point calibration was performed at the beginning of each daily analysis run with standards at concentrations of 0.075, 0.1, 0.2, 0.3, 0.4, and 0.5 µg/L.

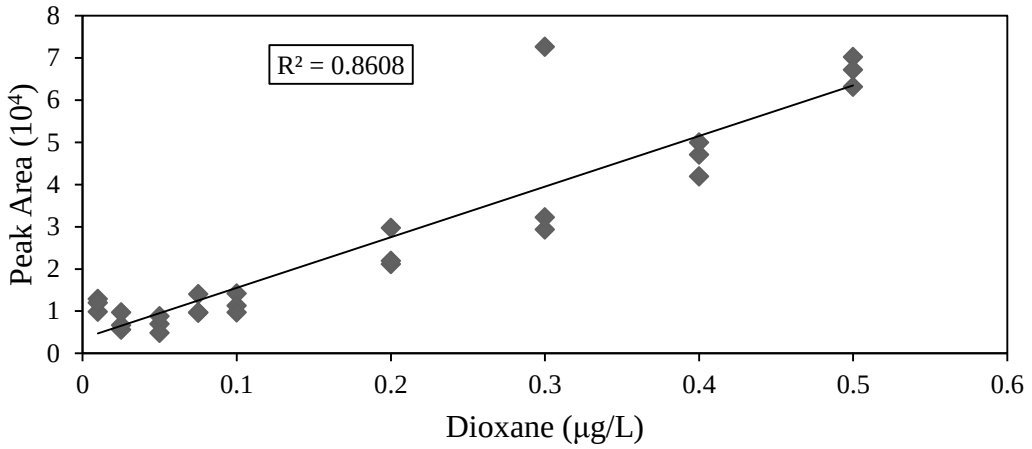

Figure 4.2 Calibration curve of dioxane analyzed by Heated P&T without Isotopic Dilution plotted with linear fit and regression coefficient.

ICBV and ICV QCs were performed at the beginning of each run at concentrations of 0 µg/L and 0.25 µg/L, respectively. CCV QCs were performed intermittently between every 16 samples and at the end of runs at concentrations of 0.1 and 0.5 µg/L. All QCs were highly variable with recoveries of concentrations ranging from 45% up to 150%. This variability between samples resulted from inconsistent sample transfer between the autosampler to the sample sparger. Small modifications to the apparatus and isotopic dilution were selected to rectify the inconsistency in measurements.

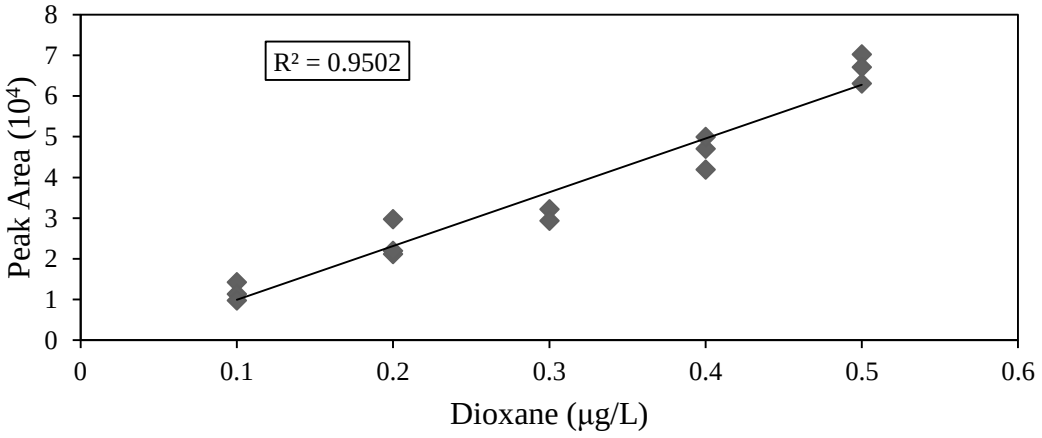

Figure 4.3 Calibration curve of dioxane analyzed by Heated P&T without Isotopic Dilution following the removal of high RSD standards plotted with linear fit and regression coefficient.

4.2.2 Method Detection Limit

Due to the inconsistencies faced with the heated P&T GC-MS method, three replicates of the lowest concentration standard (0.1 μg/L) were used to perform an MDL calculation. Using the aforementioned MDL calculation technique described (Kawata et al., 2001; Li et al., 2011), an MDL of 0.132 μg/L was obtained. Since this MDL was greater than the standard used to calculate the MDL, the MDL calculation technique was performed iteratively until an MDL was obtained at a concentration below that of the standard being used to calculate the MDL. The final MDL for dioxane was calculated as 0.235 μg/L. This MDL falls below the 0.35 μg/L EPA recommended risk concentration, and prompted further investigation of the stability issues faced by this method.

4.3 Heated Purge and Trap with Isotopic Dilution

4.3.1 Analytical Performance

Isotopic dilution was incorporated into the purge and trap method, as isotopic dilution appeared to improve the MDL and stability of the FME method. An eight point calibration was performed with standards prepared at 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, and 10 μg/L dioxane (Figure 4.4). Standards plotted linearly across the entire range of calibration with a correlation coefficient of > 0.999. The highest RSD among the standards was associated with the lowest calibration standard of 0.25 μg/L at 13.4%. The remainder of RSDs fell below 2.5% indicating that the calibration was stable.

ICBV and ICV QCs were performed at the beginning of each run at concentrations of 0 μg/L and 1.0 μg/L, respectively. CCV QCs were performed intermittently between every 15 samples and at the end of runs at a concentration of 2.5 μg/L. All QCs were considered stable with recoveries not deviating larger than 15% from their expected concentrations. The apparent stability of the method resulted in the use of QCs rather than recalibration as an instrumental check prior to each analysis. Li et al. in 2011 prescribed using a similar technique of single calibration as

their CCV QCs did not deviate greater than 20% from expected values and their largest reported RSD was less than 8%. Isotopic dilution with deuterated dioxane is believed to be the main contributor to the increase in stability seen in this method. The improvement by isotopic dilution can be seen by the difference in correlation coefficients between adjusted and non-adjusted standards (Figure 4.4).

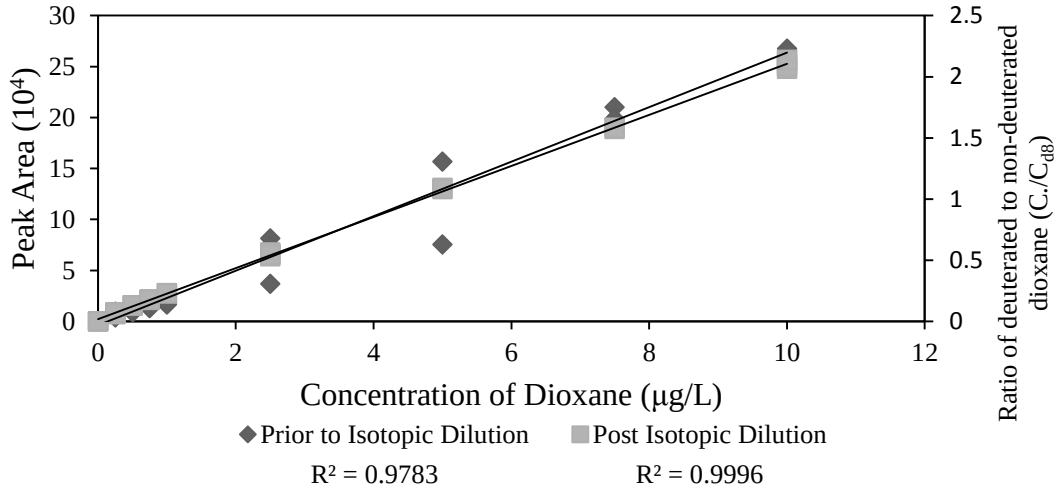

Figure 4.4 The comparison of linear regression coefficients between isotopic dilution adjusted standard values of Heated P&T and non-adjusted standard values.

4.3.2 Method Detection Limit

Four replicates of the lowest concentration standard (0.25 µg/L) were used to perform an MDL calculation. The MDL identified was 0.131 µg/L using the same MDL calculation technique described previously (Kawata et al., 2001; Li et al., 2011). This MDL falls below the 0.35 µg/L EPA recommendation. The MDL and stability of the method imply that heated P&T GC-MS with isotopic dilution could be used to identify hazardous concentrations of dioxane in the subsurface as well as in drinking water supplies, although the MDL would need to be verified using actual groundwater or drinking water.

4.3.3 Impact of Methanol and Propanol as Co-contaminants

Both the SPE method developed by Grimmett and Munch (2009) and the FME method developed by Li et al. (2011) reported that the introduction of high (> 4mg/L) total organic carbon (TOC) did not largely impact dioxane quantification. Unfortunately, high TOC waters were not available for testing their effect on dioxane quantification using heated P&T GC-MS with isotopic dilution. Instead quadruplicate standards containing 100 mg/L of methanol or 2-propanol were analyzed as a proxy for possible microbial growth conditions. Standards containing 100 mg/L of methanol or 2-propanol yielded linear calibrations of $y = 0.21x + 0.01$ and $y = 0.18x + 0.08$, respectively which fall close to the calibration without additions at $y = 0.2x + 0.04$, where y is the ratio of deuterated to non-deuterated dioxane and x is the concentration of non-deuterated dioxane in µg/L (Figure 4.5). Comparison of dioxane recoveries via a student's *t*-test at the 95% confidence interval yielded no statistical difference between standards with methanol or 2-propanol present and those without. This shows that quantities of methanol and 2-propanol below 100 mg/L have no deleterious effect on dioxane recovery. Presumably high loads of TOC would not impact dioxane recoveries as the alcohols used in this experiment were 11 to 25 times more concentrated than the TOC concentrations used in the other two studies. This would need to be verified as the method was only tested with alcohols and not a complex organic matrix that could interact with dioxane via multiple different interactions.

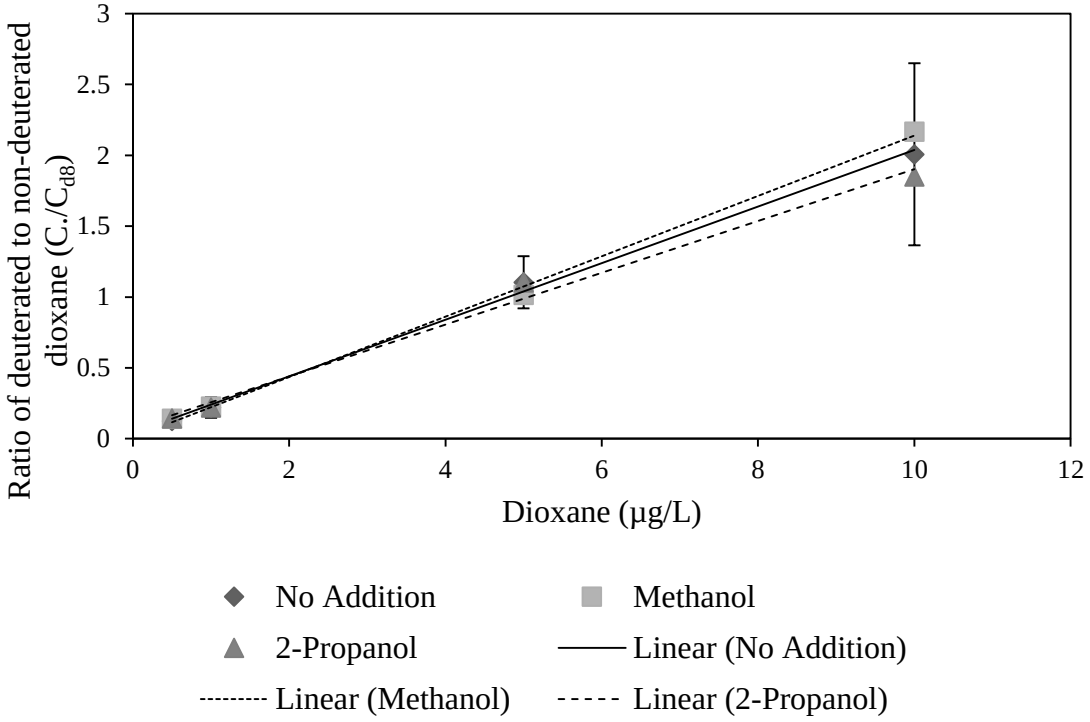

Figure 4.5 Linear calibrations of standards containing no addition, 100mg/L methanol, and 100 mg/L 2-propanol. Note 95% confidence intervals are only presented for the standards without addition.

4.3.4 Impact of Chlorinated Co-contaminants

The SPE method developed by Grimmett and Munch (2009) and FME method developed by Li et al. (2011) reported that the introduction of co-contaminants such as 1,1,1-trichloroethane (TCA) did not largely impact dioxane quantification. Dioxane contamination can be associated with trichloroethene (TCE) contamination (Anderson et al. 2012). Standards containing 1, 2.5, 5, and 10 µg/L TCE were analyzed to evaluate the impact of TCE on dioxane recoveries as well as create a low concentration calibration for TCE. Standards containing 1, 2.5, 5, and 10 µg/L of TCE yielded linear calibrations of $y = 0.17x + 0.01$, $y = 0.16x + 0.06$, $y = 0.18x + 0.01$, and $y = 0.17x + 0.04$, respectively, which fall close to the calibration without additions at $y = 0.2x + 0.04$ (Figure 4.6). Comparison of dioxane recoveries via a student's t-test at the 95% confidence interval yielded no statistical difference between standards with TCE present and those without. This shows that quantities of

TCE below 10 µg/L have no deleterious effect on dioxane recovery. Unfortunately, a linear calibration for TCE could not be established ($R^2 = 0.05$) even though TCE was detected at a retention time of 6.0 minutes at 95 m/z (Figure 3.1). The inability to calibrate TCE may be related to sample treatment, such as the extended purge times and heating, or trap materials. The conditions required for optimal dioxane quantification are likely different from those required for TCE quantification.

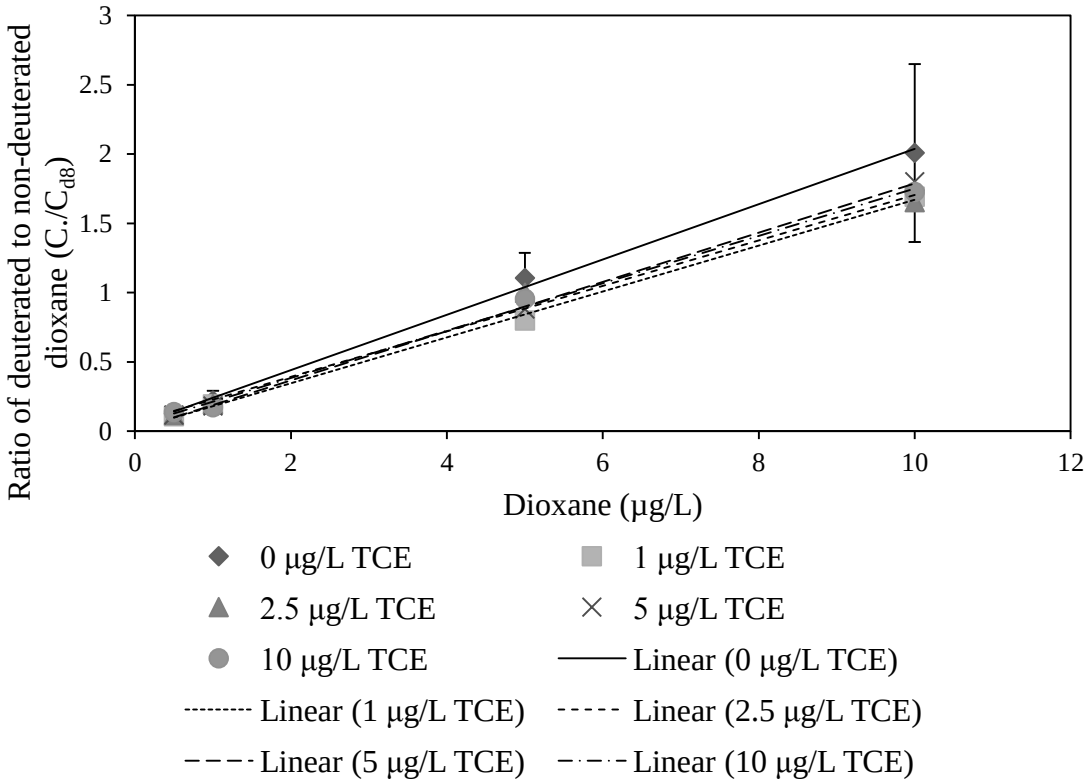

Figure 4.6 Linear calibrations of dioxane standards containing 0, 1, 2.5, 5, and 10 µg/L TCE. Note 95% confidence intervals are only presented for the standards containing 0 µg/L TCE.

4.4 Method Comparisons

The extraction method used for dioxane appears to be the primary difference between MDLs among dioxane quantification methods. Methods used to quantify dioxane report extraction via solid, liquid, or gas phases typically coupled with injection onto a GC-MS operated in SIM mode (Draper et al., 2000; Zenker et al., 2003; Grimmett and Munch, 2009; Li et al., 2011). All methods described herein exclude secondary

ions from SIM detection in order to maximize MDL as suggested in other studies (Grimmett and Munch, 2009; Li et al., 2011). A comparison of parameters shows that Heated P&T with Isotopic Dilution was the only method comparable to the SPE method developed by Grimmett and Munch in 2009, which is currently the standard method utilized by the EPA (Table 4.1). Both FME methods exhibit MDLs higher than the recommended 0.35 μg/L EPA risk assessment concentration. The Heated P&T without isotopic dilution method produced an MDL below the EPA risk assessment concentration, but without the improvement of isotopic dilution the method was compromised by stability issues. The lack of stability was observed when samples' 95% confidence intervals became exceedingly large (> 50% the sample concentration) and quality control samples failed to meet dioxane recovery requirements. The addition of deuterated dioxane allowed for the modification of reported sample constituent concentrations based on the reported concentration of a similar compound present at a known concentration.

The MDL of Heated P&T with isotopic dilution was five times higher than that of the EPA method 522. Further adaptations to the P&T method, such as increasing the sample volume, could result in an improvement in method detection, as more mass should be purged onto the trap and subsequently delivered onto the GC-MS. If extraction efficiencies were to remain the same with a larger sample volume, in theory the achievable MDL would be approximately equal to that of EPA method 522. Five times lower detection could be obtained by purging five times the sample volume used here.

Using the standard 5 mL sample volume employed in this study, the P&T method requires a 16th of the volume per sample and less overall sample preparation when compared to the SPE method. This method is optimal for situations where large sample volumes cannot be acquired such as in bench scale experiments. This analytical tool could be used to identify better remediation practices, as a less costly diagnostic tool for drinking water analysis, and be adapted to facilitate the analysis of other semi-volatile organic compounds of concern.

Table 4.1 Parameters associated with the different dioxane analysis methods evaluated and those presented in the literature.

Parameter	Heated P&T with Isotopic Dilution	Heated P&T without Isotopic Dilution	FME without Isotopic Dilution	FME with Isotopic Dilution	SPE with Isotopic Dilution†
Calibration Range (µg/L)	0.25 to 10	0.01 to 0.5	10 to 500	10 to 1600	0.002 to 1.0
MDL (µg/L)	0.13	0.24	10.5	1.6	0.02
Max RSD (%)	13	59	13	< 8	< 10
R^2	0.9996	0.8608	0.9945	0.9959	Not Reported
Sample Volume (mL)	5	5	0.3	0.2	80
Citation	This Study	This Study	This Study	Li et al. 2011	Grimmett and Munch, 2009

† denotes the currently accepted EPA method for measuring dioxane

CHAPTER 5
1,4-DIOXANE BIODEGRADATION BY MYCOBACTERIUM SP. 1A AND ARTHROBACTER SP. AK19

5.1 HD5 Propane Grown Cells

5.1.1 Culture Selection

Initially two propane-oxidizing cultures were selected for their ability to co-metabolically degrade 1,4-dioxane. *Mycobacterium* sp. 1A (1A) and *Arthrobacter* sp. AK19 (AK19) were obtained from Dr. Hyman at North Carolina State University. Culture 1A was first isolated by the Hyman laboratory group in 2014, has a milky white hue, and grows dispersed in solution. 1A has the ability to grow on propane and co-metabolically degrade tertiary butyl alcohol, a metabolite of methyl tertiary butyl ether (MTBE). AK19 was first isolated in 1972 by McLee et al. from a commercial gas seep growing on n-butane. AK19 is yellow-orange in hue and tends to form aggregates (McLee et al., 1972; Roy and Wayman, 1972). Both cultures are actinomycetes and were selected because they grew on propane and degraded high concentrations of dioxane (> 1 μM). Although it has not been confirmed, 1A and AK19 putatively possess one or more forms of monooxygenase enzyme(s) used for growth on propane and potentially in dioxane transformation as well. The abilities of the cultures to grow on research grade propane, propene, butane, and HD5 commercial grade (HD5) propane that contains a minimum of 90% propane, maximum of 5% propene, and the remaining percent short branch alkanes, were tested (Propane 101, 2011).

Triplicate 715 mL batch reactors containing 33 mg TSS/L of culture 1A or AK19, had approximately 28 mg of HD5 propane added to each reactor. Triplicate 156 mL batch reactors were inoculated with approximately 40 mg TSS/L, of culture 1A or AK19, and had 20 mg of research grade propane, propene, or butane added to

each batch reactor. Following the complete consumption of substrate, the batch reactors were resupplied with air and re-injected with substrate.

In the batch reactors containing HD5 propane, 1A grew to ten times the initial cell mass over the course of ten days; whereas, AK19 grew to only three times the initial cell mass over the same ten days. An average net yield of 0.61 mg of TSS per mg of HD5 propane utilized was calculated for culture 1A. Visual inspection of the reactors showed that 1A remained planktonic in media and AK19 formed aggregates.

Neither research grade propane nor propene supported growth of either culture. Butane, however, did support growth of both cultures. Visual inspection of the butane grown cultures showed that 1A remained planktonic in media and AK19 underwent a change in hue, from orange to white, and did not aggregate to the same extent as cells grown on HD5 propane.

Both 1A and AK19 were capable of utilizing HD5 propane and butane as primary growth substrates. Growth on butane was somewhat expected as gaseous alkane-utilizing organisms, such as *Pseudomonas butanovora*, typically are capable of utilizing C2 through C4 alkanes (Doughty et al., 2006; Kotani et al., 2006). The inability of cells to grow on research grade propane remains a mystery since they surely grew on commercial grade propane. Only one source of research grade propane was tested here, so this result has not been confirmed. Since propene was inaccessible to both 1A and AK19, presumably neither culture contains propene monooxygenase. This result, however, cannot be verified as the presence of significant volumes (5%) of carbon dioxide are requisite for propene monooxygenase to function (Woodland et al., 1995; Chan Kwo Chion et al., 2004).

HD5 propane-grown cultures of 1A and AK19 were tested for the ability to degrade dioxane. Resting cell cultures (350 mg TSS/L of 1A and 70 mg TSS/L of AK19) were incubated with approximately 500 μg/L dioxane and assayed over time for dioxane transformation. Resting cell culture consists of cells which have been grown on growth substrate and then are experimented on in the absence of growth substrate. Dioxane quantification began approximately 1 hour into the experiment.

Culture 1A appeared to exhibit dioxane degradation prior to the acquisition of the first data point (Figure 5.1) with near total dioxane degradation witnessed within 3 hours of the introduction of dioxane. Culture AK19 did not appear to degrade dioxane during the 71 hours of exposure. Dioxane analyses were performed using FME without isotopic dilution, resulting in higher than expected concentrations of dioxane observed in reactors containing cells and large 95% confideince intervals.

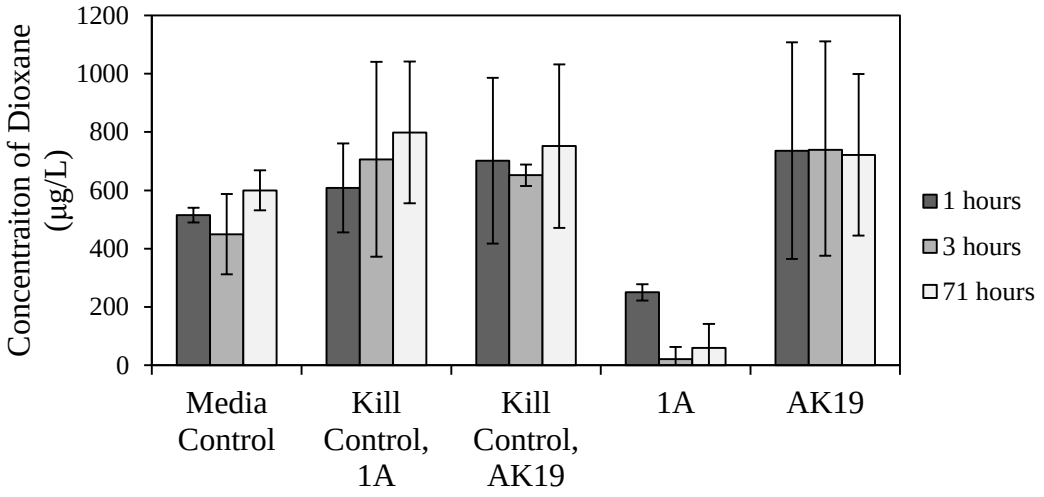

Figure 5.1 Dioxane transformation by resting cells of *Mycobacterium* sp. 1A and *Arthrobacter* sp. AK19. Note that *Arthrobacter* AK19 contained approximately one fifth the initial cell mass of the 1A cell suspensions during this experiment, and dioxane was analyzed using FME without isotopic dilution.

To the author's knowledge there are only several identified cultures shown to degrading dioxane to µg/L concentrations: *Rhodococcus ruber* ENV425 (Lippincott et al., 2015), *Pseudomonas dioxanivorans*, and *Pseudonocardia antarctica* DVS 5a1 (Li et al., 2010). In both studies the cultures presented were bioaugmented into non-sterile microcosms to evaluate their ability to degrade sub-500 µg/L concentrations of dioxane. The microcosms in both studies contained native culture that possessed the ability to naturally attenuate dioxane, creating a mixed culture microcosm, thus confirmation of the bioaugmented cultures' explicit activity was not made, making culture 1A the first isolate to be confirmed to have the ability to degrade dioxane at below 500 µg/L levels.

The difference between expected concentrations of dioxane (e.g., those found in media controls without cells present) and those seen in reactors containing culture is expected to be attributed to the lack of isotopic correction with this analytical method and the sample processing time associated with performing FME. Unfortunately the FME method does not appear to provide fast enough acquisition of samples to be deemed as initial time point data during experiments. Heated P&T GC-MS was selected for future experiments to improve the capture of initial concentrations and increase detection of dioxane. The large error bars associated with the data and the higher than expected concentrations of dioxane in samples with culture present are attributed to the lack of isotopic correction with this analytical method.

AK19 did not appear to degrade dioxane during this experiment, although the cell concentration of AK19 was about one fifth of that used for 1A making it difficult to draw comparable conclusions from the data. The cultures were being evaluated for potential use for subsurface dioxane remediation via bioaugmentation. Most actinomycetes form aggregates during growth under agitation (Lawton et al., 1989) and this shift in morphology is suspected to incur difficulties when attempting to diffuse culture in the subsurface during *in situ* bioremediation (Lippincott et al., 2015). Culture AK19 tended to create aggregates making it a less desirable candidate for use in bioaugmentation. Culture 1A, on the other hand, remained planktonic in solution, and exhibited significantly faster growth kinetics. Therefore, culture 1A was chosen for further investigation for use in *in situ* bioremediation applications.

5.1.2 Growth Substrate Effect on Dioxane Transformation

Batch reactor experiments were conducted to evaluate the ability of culture 1A to degrade dioxane in the presence of its growth substrate, HD5 propane. Triplicate batch reactors containing media with approximately 190 mg TSS/L and 100 μg/L dioxane were injected with 10 mg of HD5 propane and incubated at 30 °C on a shaker table. HD5 propane and/or dioxane were introduced repeatedly to investigate the dynamics of dioxane transformation in the presence and absence of HD5 propane.

Dioxane transformation occurred in the presence and absence of growth substrate, but at a lesser rate when HD5 propane was present (Figure 5.2). Successive spikes of HD5 propane were utilized with corresponding increases in optical density over the course of the experiment. An average net yield of 0.63 mg TSS per mg HD5 propane was obtained for the growth of culture 1A on HD5 propane in the presence of dioxane. The specific rates of dioxane transformation in the presence of HD5 propane increased over the course of the three propane additions. It took less than 21.7, 15.0, and 7.0 hours, respectively, for the three 500 μg/L dioxane additions to be degraded below 10 μg/L. The transformation of successive spikes of dioxane in the absence of HD5 propane took less than 6.0 and 3.3 hours, respectively, to be degraded to below 10 μg/L. Dioxane transformation rates in the absence of HD5 propane were approximately two to four times faster than in the presence of HD5 propane. Although propane inhibited dioxane transformation, concurrent and complete dioxane transformation was observed in the presence of propane, indicating that the effect of propane inhibition was not large. First-order HD5 propane utilization rates remained approximately constant over the course of the experiment at 75 ± 2.0 ng propane per mg protein per hour ($R^2 > 0.863$).

Bioremediation techniques often require that a growth substrate be present in the subsurface for sustainable treatment to occur. In some instances the presence of growth substrate can inhibit an organism's degradation of the target compound. In the case of well-studied chlorinated solvent co-metabolism, various substrates and degradation metabolites were found to cause significant inhibition of contaminant degradation (Kim et al., 2002). The slower rate of dioxane degradation in the presence of HD5 propane likely reflects inhibition of dioxane degradation by propane. However, dioxane was concurrently degraded with the propane, so the inhibition effects were somewhat mild.

It has been shown that when other cultures are exposed to large quantities (> 100 mg/L) of dioxane, the cells experience irreversible toxicity resulting in their inactivation and the loss of propane or dioxane transformation ability (Mahendra and Alvarez-Cohen, 2006). The utilization of HD5 propane and subsequent increase in

optical density following successive spikes of dioxane showed that transformation product toxicity was not as significant at these lower levels of dioxane, an observation that was confirmed in subsequent tests (5.1.3 Dioxane Transformation).

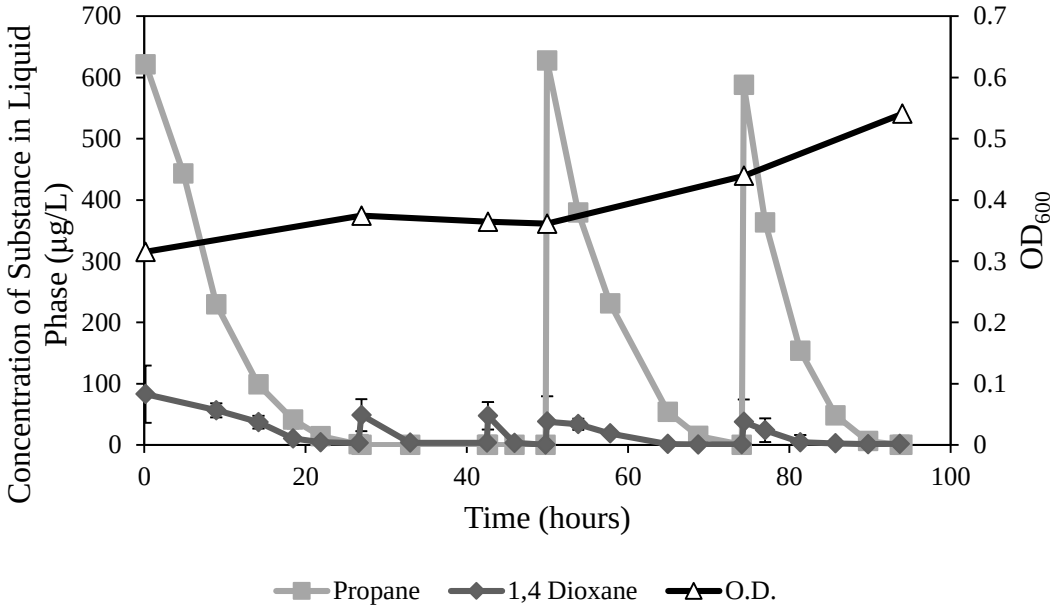

Figure 5.2 Long term exposure of culture 1A to successive injections of HD5 propane and dioxane. Note that 95% confidence intervals are not presented for HD5 propane or optical density, and dioxane samples were analyzed using Heated P&T method without isotopic dilution.

To date, all cultures shown to degrade dioxane to concentrations below 500 µg/L have exhibited first-order rates of dioxane degradation (Li et al., 2010; Lippincott, 2015). The resolution of data points in this experiment did not allow for the determination of a best fit model for dioxane transformation in the absence of HD5 propane and the inhibitory effect of propane blocked the analysis of reaction order in the presence of propane.

The 95% confidence intervals associated with the dioxane data were smaller than with FME analysis, but still in cases close to 50% of the measure quantity. Unfortunately, heated purge and trap without isotopic dilution yields high variability between replicate samples. Additional experimental error was introduced in the initial data points as samples were analyzed following the introduction of cells to the system.

The time required to acquire and process a sample was long enough for significant transformation to occur, effecting the spread of the measured dioxane additions. Fortunately, the 95% confidence intervals get smaller as the data points approach the detection limit. This decrease in the confidence intervals supports the inference that the dioxane was being transformed by the organism. The MDL for dioxane by heated purge and trap without isotopic dilution was calculated to be 0.24 μg/L. Since the samples here were diluted 10 times, culture 1A was observed to degrade dioxane below the detection limit of 2.4 μg/L.

5.1.3 Dioxane Transformation

To investigate the dioxane transformation capabilities of culture 1A, resting cells were incubated with repeated additions of dioxane to determine how much dioxane they could transform and how long the culture was able to express the enzymes needed for dioxane transformation in the absence of growth substrate. Approximately 475 mg TSS/L of culture 1A HD5 propane-grown resting cells were exposed to 500 μg/L dioxane. The culture was able to completely transform the added dioxane in less than 12 hours, when another spike of 500 μg/L dioxane was added. For 7 days, dioxane was added approximately every 12 hours and allowed to be transformed by the resting cell culture.

Cells grown on HD5 propane transformed 47.1 ± 0.6 μg dioxane per mg protein over seven days before degradation rates significantly decreased (Figure 5.3). The nominal mass of dioxane added was greater than the measured mass of dioxane throughout the experiment due to the sampling procedure and the time required to obtain and process samples. Initially, the added 500 μg/L of dioxane was completely transformed within 12 hours. After about 4 days, a detectable slowing of dioxane transformation was observed. Measured concentrations of dioxane after each addition began to exceed the expected concentration of 500 μg/L 4.7 days into the experiment due to residual dioxane remaining from the last addition. Dioxane concentrations in the reactors never exceeded 1 mg/L.

Presumably the cells in this experiment would have continued to degrade dioxane past seven days, but experiment was not continued until a complete loss of transformation ability was found, but was ended after 7 days when the rate fell below 38% of the initial transformation rate. The difference observed between nominal and measured masses of dioxane transformation result from degradation that occurred between the time of dioxane addition and the time the first sample was acquired for measurement. The slow decrease in transformation rate appeared to indicate a gradual loss of reductant supply more than an irreversible loss of activity due to toxicity, but this was not tested with the addition of growth substrate. The renewal of accelerated dioxane degradation rates by culture 1A upon the utilization of additional HD5 propane was witnessed in other unrelated tests (data not shown).

Transformation capacity is a term used to quantify the amount of contaminant a resting organism can co-metabolize before transformation product toxicity or reductant drain results in a loss of transformation capabilities. Loss of transformation ability due to toxicity is generally irreversible where reductant can be regenerated through metabolism of additional growth substrate. To the author's knowledge the only value reported for the transformation capacity of a propane-grown, dioxane-degrading culture is 1.4 ± 0.007 mg dioxane per mg protein (Mahendra and Alvarez-Cohen, 2006). Mahendra and Alvarez-Cohen (2006) tested *Mycobacterium vaccae* JOB5 at an initial dioxane concentration of 88 mg/L for a maximum 2 days of exposure. They concluded that the loss of transformation capability was irreversible and primarily due to (transformation product) toxicity. In the test performed here, culture 1A was exposed to a much lower concentration of less than 1 mg/L over an extended period of 7 days in the absence of growth substrate. While 1.4 mg dioxane per mg protein was transformed in the Mahendra et al. test, only 0.0471 mg of dioxane per mg protein, or about 30 times less dioxane was transformed in this test. Though Mahendra and Alvarez-Cohen (2006) reported that irreversible toxicity from dioxane metabolites on monooxygenase was likely responsible for the loss of the degradation capabilities of culture JOB5, presumably this experiment exhibited a reduction of degradation capability associated with the gradual loss of intracellular

reducing power. The fact that culture 1A was capable of degrading dioxane to below detection limit for up to 4 days with dioxane additions occurring twice each day indicated that culture 1A should be an effective catalyst for the degradation of low concentrations of dioxane with expected treatment to below detection limits.

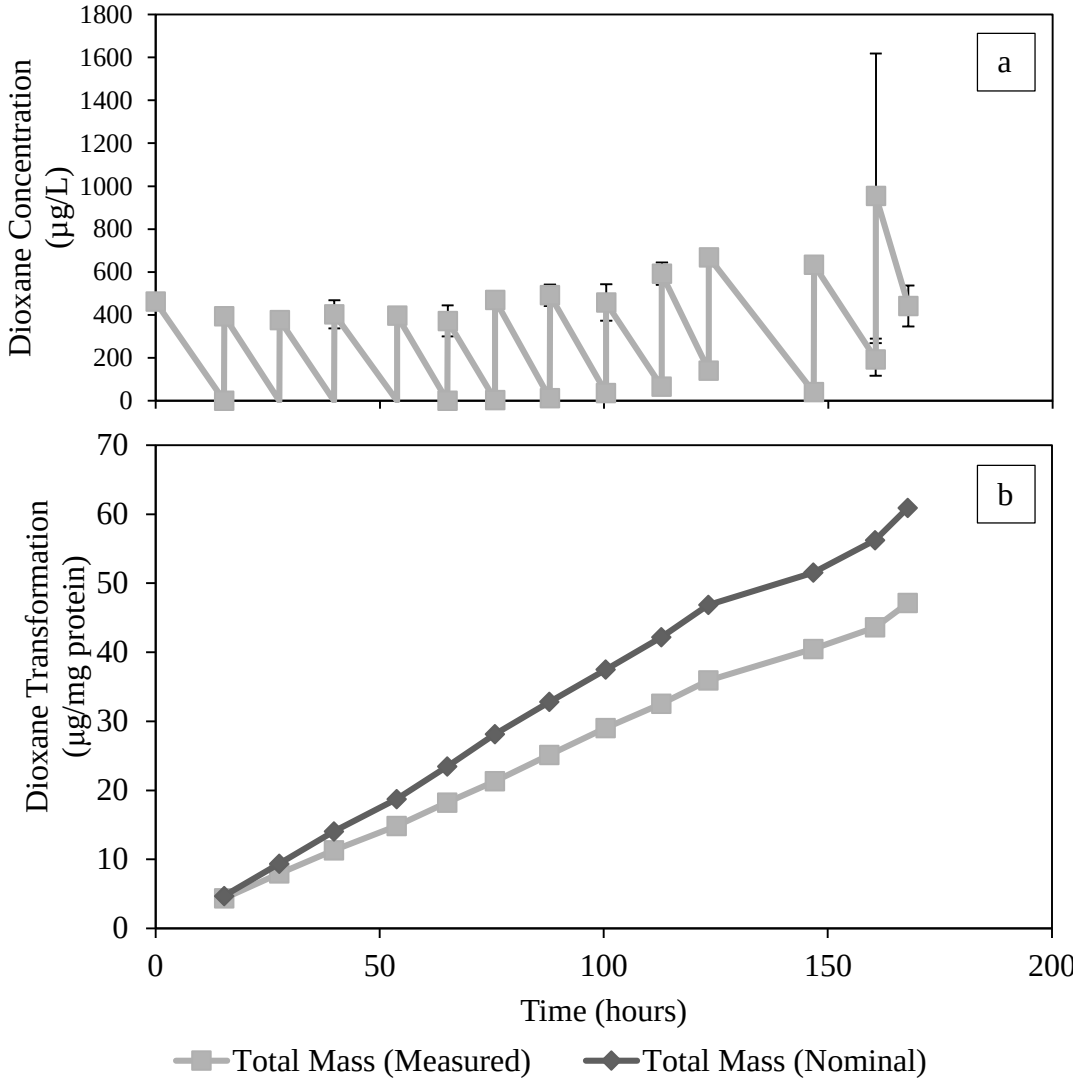

Figure 5.3 Dioxane transformation (a) and the measured and nominal amounts of dioxane transformed (b) by resting cells of HD5 propane-grown culture 1A. Complete degradation of dioxane was observed until 112 hours into the experiment where concentrations of dioxane in the batch reactors began to rise. Dioxane samples were analyzed using Heated P&T with isotopic dilution.

5.1.4 Culture 1A Growth in Site Groundwater

Since culture 1A was being evaluated as a potential candidate for *in situ* bioaugmentation to the subsurface and subsequent dioxane remediation, the culture was tested for the ability to grow on HD5 propane and degrade dioxane in site groundwater or site groundwater amended with either growth media or autoclaved aquifer solids. Typically compounds are amended to site waters as they may not have the necessary nutrients or buffering capacity as is needed to support high concentrations of aerobic microbial populations. Lippincott et al. (2015) reported amending 200 gallons of groundwater with 20 pounds of diammonium phosphate for injection into their bioaugmentation well during their field demonstration of dioxane transformation by *Rhodococcus ruber* ENV425. Minimizing the amount of chemical constituents added to the subsurface during bioaugmentation mitigates extraneous operational costs.

Each batch reactor initially contained 125 mg TSS/L cell solution, 12 mg HD5 propane, and 150 µg/L dioxane. Media and groundwater were formulated and prepared as previously identified (Sections 3.2 and 3.3). Amended groundwater contained an addition of 10% MSM. Additionally, 5 grams of autoclaved aquifer solids were amended to site groundwater and tested to see if non-specific mineral addition effected rates.

Cells utilized one addition of 12 mg of HD5 propane in media, groundwater, and MSM amended groundwater at roughly the same first-order rates of 0.14 ± 0.05, 0.11 ± 0.02, and 0.22 ± 0.05 µg propane per mg protein per hour with$R^2 > 0.90$, respectively (Figure 5.4). A first order rate was not established for groundwater containing solids as optical densities varied with the presence of solids. Complete utilization of HD5 propane and near complete dioxane degradation was witnessed in all of the reactors by 23.5 hours after initial exposure (Figure 5.4). Upon inspection of the dioxane degradation curves (Figure 5.4), dioxane in groundwater or groundwater plus aquifer solids showed delayed degradation of dioxane compared to culture 1A in media or groundwater amended with MSM. However, the high variability in dioxane

measurements at 6 and 15 hours makes it difficult to draw absolute conclusions. In any case, both HD5 propane and dioxane degradation was complete in all tested bottles by 24 hours. Growth was observed in all reactors exhibiting HD5 propane utilization with a corresponding increase in optical density over the 30 hour experiment.

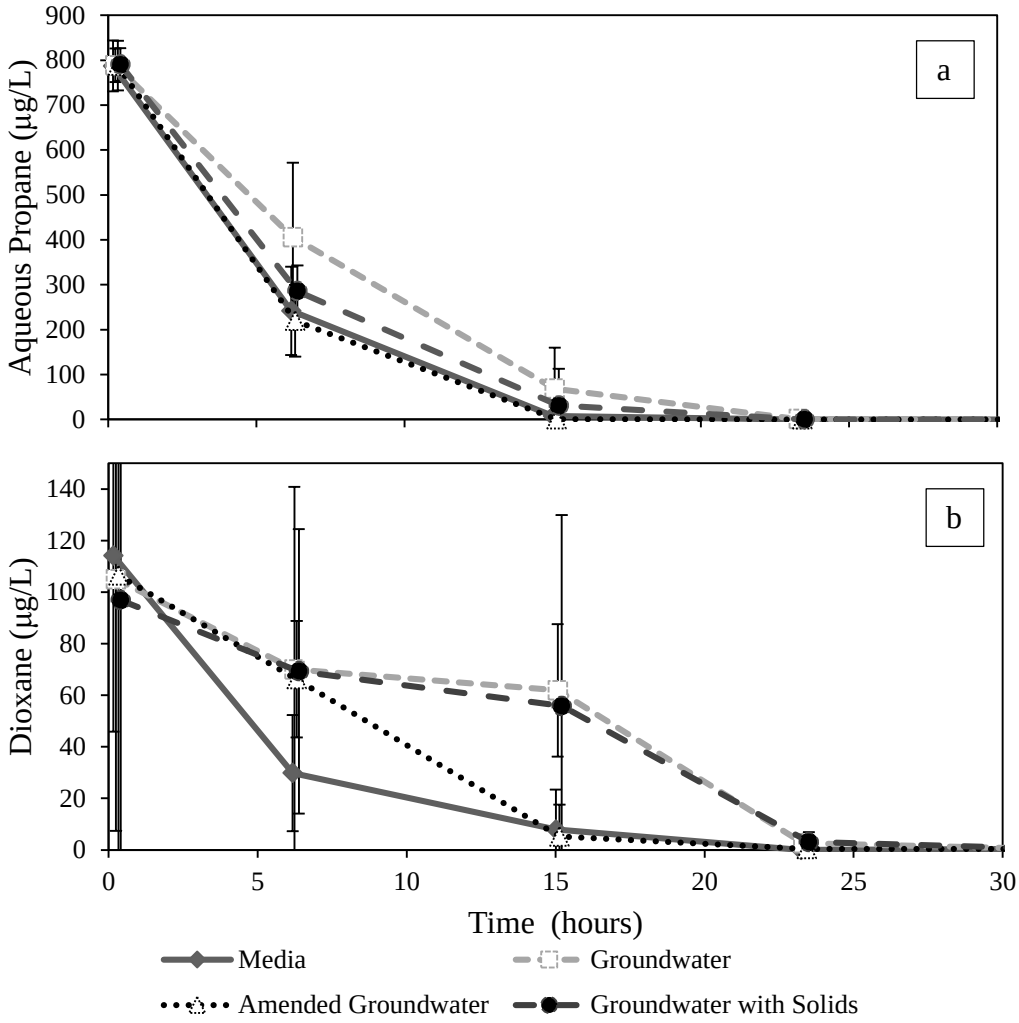

Figure 5.4 HD5 propane utilization (a) and dioxane degradation (b) by HD5 propane-grown culture 1A in the presence of propane and different mediums. Dioxane measurements were quantified using Heated P&T without isotopic dilution.

5.2 Growth of Culture 1A on Propanol

5.2.1 Substrate Utilization

Propane monooxygenase expressing bacteria have been shown to grow on a number of propane metabolites including 1-propanol and 2-propanol (Kulikova and Bezborodov, 2000). Terminal or subterminal oxidation of propane can occur resulting in the formation of 1-propanol or 2-propanol, respectively, which is in turn degraded into other metabolites (Stephens and Dalton, 1986; Ashraf et al., 1994). It has been postulated that terminal, subterminal, or a mix of both oxidations can occur in propane utilizing organisms (Woods and Murrell, 1990; Ashraf et al., 1994), and that subterminal oxidation may be performed through a number of pathways (Kulikova and Bezborodov, 2000).

A batch experiment was performed to determine if culture 1A had the capacity to grow on 1-propanol and 2-propanol, metabolites of propane oxidation. An inoculum of < 1 mg of culture 1A cells was added to batch reactors containing media. Either 1-propanol or 2-propanol were added to each reactor to achieve an initial concentration of 370 mg/L. Culture 1A grew on both 1-propanol and 2-propanol at similar rates, with the optical density increasing from approximately 0.04 to greater than 0.60 over the course of 3 days. The growth rate observed for propanol grown cells was roughly three times faster than that for growth on HD5 propane, that require approximately 10 days to reach similar optical densities.

Since culture 1A was capable of growth on both 1- and 2-propanol, it is possible that culture 1A possess multiple forms of propane monooxygenase, but further study into the genomics of 1A would be needed to verify this possibility. Although the culture was shown to grow on propanol, monooxygenase expression is also critical to effective dioxane transformation and the presence of growth substrate may be needed to induce the necessary (monooxygenase) enzyme expression.

5.2.2 Dioxane Transformation

Culture 1A resting cells grown on 1- or 2-propanol were tested for their ability to degrade dioxane when complemented by small doses of possible enzyme inducing compounds over the course of 7 days. Each batch reactor contained resting cells grown on 1-propanol or 2-propanol. Reactors containing 2-propanol grown cells had roughly half the TSS of reactors containing 1-propanol grown cells. HD5 propane (0.55 mg) or propanol (1.6 mg) were added as potential enzyme-inducing compounds and dioxane was added to a concentration of 250 µg/L (Figure 5.5).

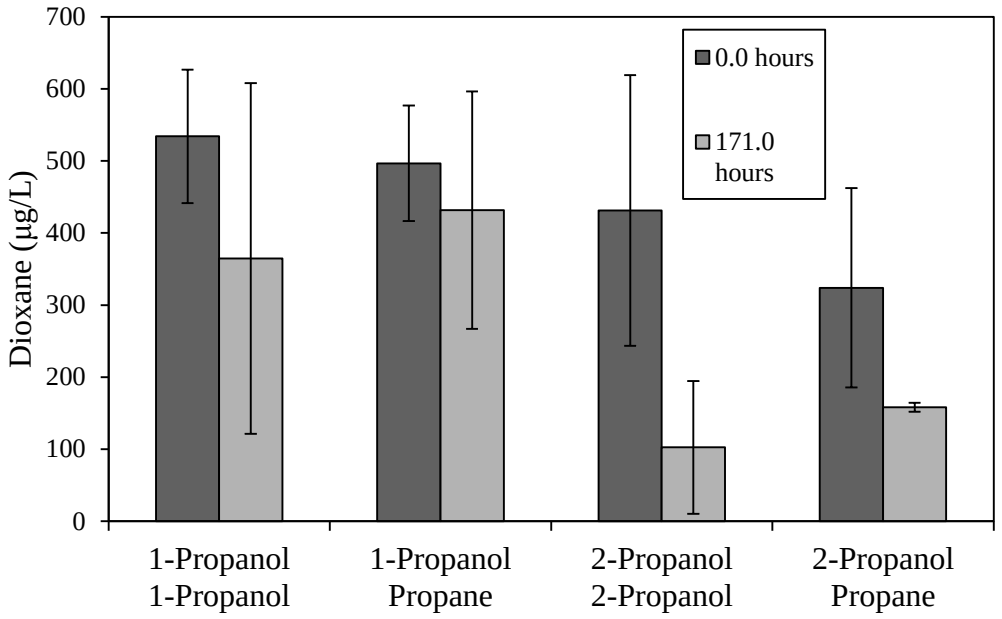

Figure 5.5 Dioxane degradation by cultures grown on 1-propanol or 2-propanol and then exposed to either their growth substrate or HD5 propane. Dioxane measurements were obtained using the FME method without isotopic dilution.

Cells grown on 1-propanol and then incubated with propane or 1-propanol exhibited little or no dioxane transformation over the 7 day test. Cells grown on 2-propanol and then exposed to HD5 propane or 2-propanol as potential enzyme-inducing compounds degraded a significant amount of the dioxane present. Cells incubated with 2-propanol degraded the most dioxane, but again, variability in the dioxane data (FME analysis method) makes absolute conclusions difficult.

It was obvious that cells grown on propanol exhibit a loss of dioxane degradation capability when compared to cells grown on HD5 propane (Figure 5.3). Introduction of small quantities of HD5 propane (50 $\mu g/L_{aq}$) did not appear to re-induce dioxane degradation. It is possible that extended exposure to HD5 propane may increase dioxane transformation capabilities, but this was not explicitly tested. Unfortunately, accurate conclusions about enzymatic induction cannot be draw as the cells were exposed to roughly 25 times the concentration of metabolite alcohol than that of HD5 propane and no apparent increase in dioxane degradation was witnessed in cultures exposed to small amounts of HD5 propane.

It has been documented that monooxygenase-expressing cultures grown on metabolite alcohols sometimes experience lower alkane oxidizing activity and less ability to degrade xenobiotic compounds such as MTBE and 1-chlorobutane (Perry, 1968; Steffan et al., 1997; Smith et al., 2003; Vanderberg, 1994). This was seen in the cells tested herein, likely due to the fact that the utilization of metabolite alcohols does not require the organisms to express the enzymes necessary for oxidizing the parent substrate, propane. Consequently, there can be a loss of dioxane degradation capability if dioxane degradation is associated with monooxygenase enzyme expression.

An experiment was performed to directly compare cells grown for varying times on 2-propanol to HD5 propane-grown cells on a dioxane transformation per protein basis (Figure 5.6). Culture 1A cell suspensions were prepared by either 1 or 3 growth cycles on 2-propanol where a growth cycle is the time taken to growth a diluted cell suspension of below 0.1 OD600 to above 0.6. Cells were harvested, rinsed, and resuspended in the absence of growth substrate and exposed to dioxane over 7 days and assayed for dioxane transformation ability.

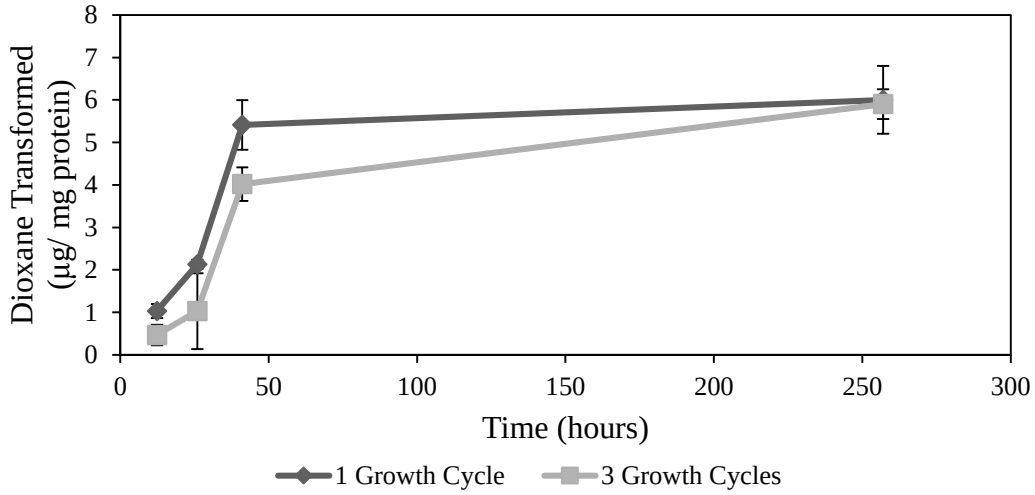

Figure 5.6 Dioxane transformation by culture 1A grown for 1 or 3 growth cycles on 2-propanol. Dioxane was quantified using Heated P&T with isotopic dilution and concentrations of dioxane in the reactor never exceeded 1.5 mg/L.

Cells grown under 1 and 3 growth cycles transformed 6.0 ± 0.8 and 5.9 ± 0.4 µg dioxane per mg protein, respectively (Figure 5.6). Dioxane was not removed to near detection limits nor exceeded 1.5 mg/L at any point during this experiment. Cells grown for a single cycle on 2-propanol have similar dioxane transformation capabilities of cells grown for three growth cycles on 2-propanol over the course of ten days.

In contrast, HD5 propane-grown cells exhibited 8 times the dioxane transformation capability of 2-propanol-grown cells. Although cells were found to grow roughly 3 times faster on propanol than propane, the loss of 8 times the dioxane degradation capacity indicates that growing cells on HD5 propane would be the preferred method for preparing cells for bioaugmentation.

5.2.3 Growth in Groundwater

Culture 1A was tested for the ability to grow on 2-propanol and degrade dioxane in site groundwater or site groundwater amended with growth media or autoclaved aquifer solids. Batch reactors containing 195 mg TSS/L of 2-propanol grown culture 1A cells were injected with 2-propanol (80 mg/L) and dioxane stock solution (150 µg/L). Media and groundwater were formulated and prepared as previously identified

(Sections 3.2 and 3.3). Media-amended groundwater contained an addition of 10% MSM and 5 grams of autoclaved aquifer solids were amended to site groundwater and tested to see if non-specific mineral addition effected rates.

Propanol was consumed at similar rates in all reactors and completely utilized within 29 hours of exposure (Figure 5.7). Dioxane was transformed in all bottles, but cells in growth media degraded dioxane faster and to lower concentrations than the other systems tested (Figure 5.7). By the end of the 42 hour experiment, cells exposed to media, amended groundwater, groundwater, and groundwater with solids had degraded dioxane to 8.9, 18.7, 30.5, and 30.5 µg/L, respectively. Dioxane was transformed at slower rates than those observed for HD5 propane-grown cells.

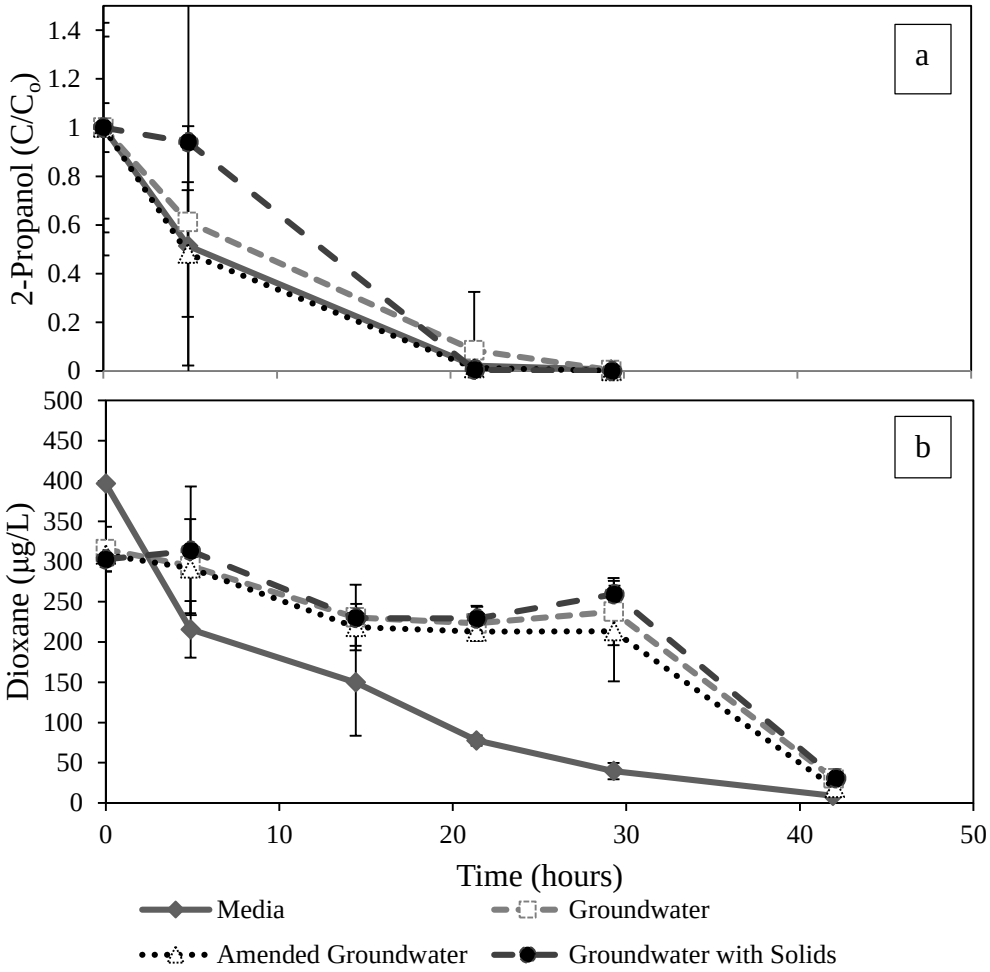

Figure 5.7 2-Propanol utilization (a) and dioxane degradation (b) by 2-propanol grown culture 1A in the presence of 2-propanol and different mediums. Dioxane quantification was performed using Heated P&T with isotopic dilution.

It appeared that dioxane degradation did not occur in any of the groundwater-containing reactors until all of the 2-propanol was utilized. It may be possible that 2-propanol inhibits dioxane transformation, but more study is required to ascertain the effects of 2-propanol on dioxane transformation. Perhaps the groundwater systems were lacking some nutrient or cofactor required for (monooxygenase) enzyme expression and/or dioxane transformation. The best primary substrate and dioxane transformation rates were observed in media systems, indicating the potential lack of an essential nutrient or mineral in the non-media systems. While dioxane concentrations were reduced in all systems, they were not reduced to nearly the same extent here as seen with HD5 propane-grown cells. This may potentially be due to a loss of monooxygenase expression/activity as the cells have been in the absence of their primary growth substrate, propane, for some time.

CHAPTER 6
CONCLUSION

6.1 Heated Purge and Trap with Isotopic Dilution

6.1.1 Conclusions

- The calculated MDL for dioxane analysis using Heated P&T with isotopic dilution was 0.13 μg/L, which is below the EPA risk assessment concentration of 0.35 μg/L.
- Heated P&T with isotopic dilution exhibited a correlation coefficient of greater than 0.999.
- The maximum RSD obtained from the eight point calibration using Heated P&T with isotopic dilution was less than 13%, which is less than the EPA recommended maximum of 30% RSD for semivolatile organic compounds.
- Heated P&T with isotopic dilution does not require the use of hazardous chlorinated solvents nor expensive consumables.
- Heated P&T with isotopic dilution was not impacted by TCE concentrations of less than 10 μg/L nor methanol or 2-propanol concentrations of less than 100 mg/L.

6.1.2 Future Work

Future studies should address the impact of TOC, higher concentrations of TCE, and TCA on dioxane recoveries. Recalculation of the MDL should be performed following the replacement of the mass selective detector filament to see if there is an improvement in detection associated with routine maintenance. This method requires additional trials by exterior laboratories' equipment and personnel to confirm the stability of this method. In time this method will hopefully become a suitable alternative to current methods used to quantify dioxane.

6.2 1,4-Dioxane Biodegradation by Mycobacterium Sp. 1A

6.2.1 Conclusions

- *Mycobacterium* sp. 1A was capable of transforming dioxane and has been confirmed to transform dioxane to below 2.4 μg/L
- Culture 1A was capable of dioxane transformation in the presence and absence of HD5 propane, but higher transformation rates were observed in the absence of HD5 propane.
- Culture 1A was capable of steady transformation of 47 μg of dioxane per mg of protein over the course of 7 days in the absence of growth substrate.
- Cultures grown in site groundwater were capable of growth on HD5 propane and subsequent transformation of dioxane.
- Cells were capable of growth on metabolite alcohols, 1 and 2-propanol, but only 2-propanol grown cells were capable of transforming dioxane.
- Cells grown on 2-propanol exhibited significantly slower rates of dioxane transformation than cells grown on HD5 propane.

6.2.2 Future Work

Enhancement of this study could be performed by identifying the kinetic parameters of cells for propane and dioxane. Since inhibition of dioxane degradation by HD5 propane was observed, identification of the inhibition type and inhibition parameters of HD5 propane on dioxane and vice versa would provide useful insight into the most effective way to apply 1A for *in situ* bioremediation applications. Better resolution of transformation capabilities could help determine how effective 1A works as a biocatalyst at low concentrations of dioxane and help identify what limits the transformation of dioxane. Determining what exposure to HD5 propane is required to reacquire dioxane degradation capabilities of 2-propanol grown cells would assist in culture preparation for *in situ* bioremediation applications.

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APPENDIX

APPENDIX

CELL DENSITY RELATIONSHIPS

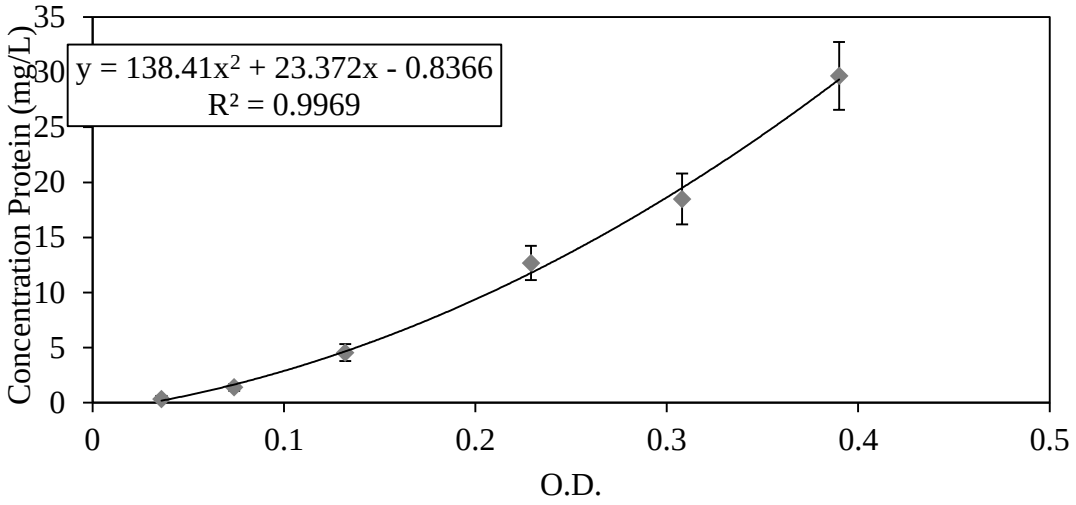

Figure A.1 Concentration of Protein vs. OD.

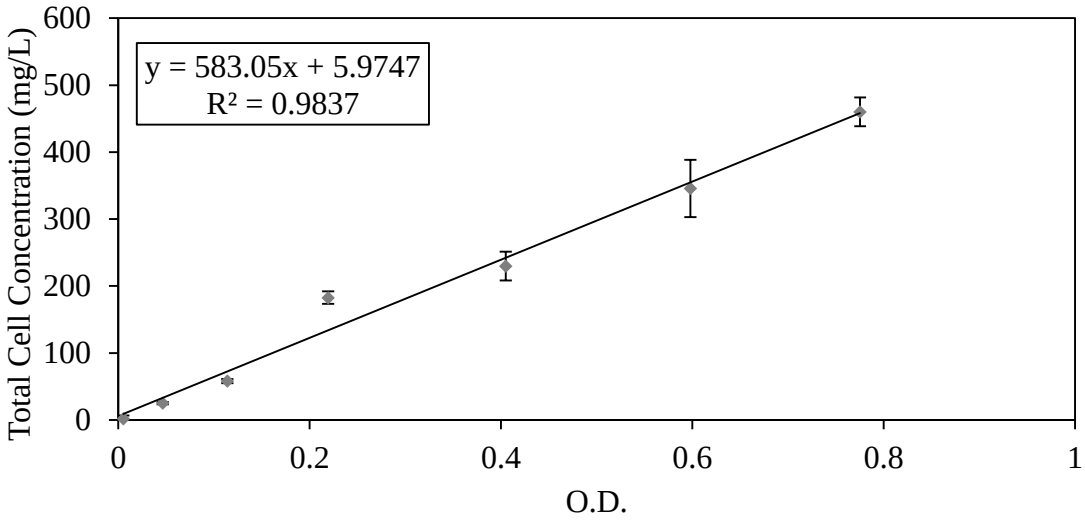

Figure A.2 Total cell concentration vs. OD.

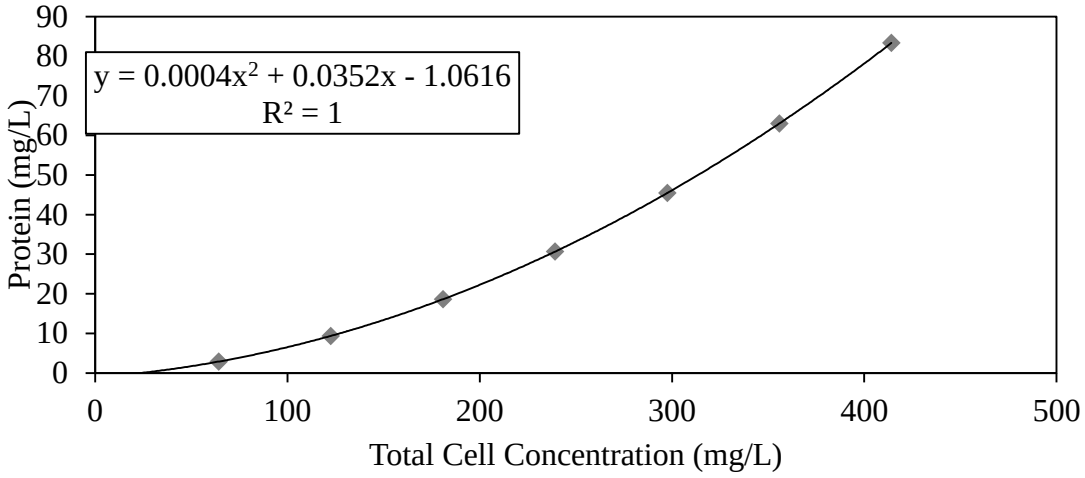

Figure A.3 Protein vs. total cell concentration.