Title
Biodegradation of 1,4-Dioxane in Co-Contaminant Mixtures

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Biodegradation of 1,4-Dioxane in Co-Contaminant Mixtures

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Civil Engineering

by

Shu Zhang

2017
ABSTRACT OF THE DISSERTATION

Biodegradation of 1,4-Dioxane in Co-Contaminant Mixtures

by

Shu Zhang
Doctor of Philosophy in Civil Engineering
University of California, Los Angeles, 2017
Professor Shaily Mahendra, Chair

Bioremediation is a promising technology to degrade or detoxify various organic and inorganic compounds in polluted environments by using microbiological activity, but it is sensitive to biogeochemical conditions as well as co-occurring compounds at impacted sites. This study focused on biodegradation of 1,4-dioxane, which is a carcinogen and an emerging water contaminant. 1,4-Dioxane was utilized as a stabilizer of chlorinated solvents, such as 1,1,1-trichloroethane (TCA); and it has been found widespread in groundwater. Many US states are implementing lower regulatory advisory levels based on the toxicity profile of 1,4-dioxane and the potential public health risks. However, the unique chemical properties of 1,4-dioxane, such as high water solubility, low Henry’s law constant, and importantly, the co-occurrence with chlorinated solvents and other contaminants, increase the challenges to efficiently cleanup 1,4-
dioxane contaminations. The objectives of this research were to measure and model the effects of chlorinated solvents on 1,4-dioxane metabolic biodegradation by laboratory pure cultures, elucidate the mechanisms of the inhibition, and test the effects of mixtures of co-contaminants in samples collected from actual 1,4-dioxane contaminated sites. It was determined that individual solvents inhibited biodegradation of 1,4-dioxane in the following order: 1,1-dichloroethene (1,1-DCE) > cis-1,2-diochloroethene (cDCE) > trichloroethene (TCE) > 1,1,1-trichloroethane (TCA). The results confirmed that 1,1-DCE was the strongest inhibitor of 1,4-dioxane biodegradation, even in chlorinated ethene mixtures. The energy production was delayed, and the genes coding for catalytic enzymes, dioxane monooxygenase (dxmlB) and alcohol dehydrogenase (aldH) were down regulated, in the presence of chlorinated solvents. These results will be useful to scientists in understanding the fundamentals of enzymatic processes that catalyze biological degradation of hazardous compounds, and to environmental engineers by providing quantitative data valuable for the development of in-situ bioremediation approaches for contaminant mixtures.
The dissertation of Shu Zhang is approved.

Michael K. Stenstrom

Jennifer A. Jay

Irwin Mel Suffet

Shaily Mahendra, Committee Chair

University of California, Los Angeles

2017
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<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aldH</em></td>
<td>Gene coding for Alcohol Dehydrogenase (ALDH)</td>
</tr>
<tr>
<td>AMS</td>
<td>Ammonium Mineral Salts</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDL</td>
<td>Below Detection Limit</td>
</tr>
<tr>
<td>cDCE</td>
<td><em>cis</em>-1,2-Dichloroethene</td>
</tr>
<tr>
<td>CB1190</td>
<td><em>Pseudonocardia dioxanivorans</em> CB1190</td>
</tr>
<tr>
<td>CVOCs</td>
<td>Chlorinated Volatile Organic Compounds</td>
</tr>
<tr>
<td>1,1-DCE</td>
<td>1,1-Dichloroethene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>DoD</td>
<td>Department of Defense</td>
</tr>
<tr>
<td>DOE</td>
<td>Department of Energy</td>
</tr>
<tr>
<td><em>dxmB</em></td>
<td>Gene coding for 1,4-Dioxane Monooxygenase (DXMO)</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron Capture Detector</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular Polymeric Substances</td>
</tr>
<tr>
<td>ERD</td>
<td>Enhanced Reductive Dechlorination</td>
</tr>
<tr>
<td>ESTCP</td>
<td>Environmental Security Technology Certification Program</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas Chromatography Flame Ionization Detector</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>ISCO</td>
<td><em>In Situ</em> Chemical Oxidation</td>
</tr>
<tr>
<td>ISCR</td>
<td><em>In Situ</em> Chemical Reduction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>JOB5</td>
<td><em>Mycobacterium austroafricanum</em> JOB5</td>
</tr>
<tr>
<td>MNA</td>
<td>Monitored Natural Attenuation</td>
</tr>
<tr>
<td>NMS</td>
<td>Nitrate Mineral Salts</td>
</tr>
<tr>
<td>NOM</td>
<td>Natural Organic Matter</td>
</tr>
<tr>
<td>ORP</td>
<td>Oxidation Reduction Potential</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMA</td>
<td>Propidium monoazide</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>rpoD</td>
<td>RNA Polymerase subunit D housekeeping sigma factor</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SERDP</td>
<td>Strategic Environmental Research and Development Program</td>
</tr>
<tr>
<td>sMMO</td>
<td>Soluble Methane Monoxygenase</td>
</tr>
<tr>
<td>TCA</td>
<td>1,1,1-Trichloroethane</td>
</tr>
<tr>
<td>TCE</td>
<td>Trichloroethylene</td>
</tr>
<tr>
<td>uspA</td>
<td>Universal Stress Protein encoding gene</td>
</tr>
</tbody>
</table>
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Without my parents’ unconditional love, support, and encouragement, I cannot complete my graduate research and pursue my career goals.
Biographical Sketch

EDUCATION

Peking University

M.S. (2013), Environmental Science

Tianjin University

B.S. (2010), Environmental Science

AWARDS/SCHOLARSHIPS

- Fellow of ASM Science Teaching Fellows Program. 2017
- California Association of Sanitation Agencies (CASA) Education Foundation Scholarship ($5,000). 2016
- Society of Women Engineers at UCLA Scholarship ($500). 2016
- RemTech Student Poster Competition “Honorable Mention”. 2015
- Peking University “WuSi Prize” Award for Academic Excellence ($300). 2012
- Peking University “ChengHuan Prize” Award for Academic Excellence ($300). 2011
- Peking University President’s Award for Academic Excellence. 2011
- Third prize for “Shell Energy and Sustainable Development - Student Research Competition”. 2011
- Tianjin University Dean’s award for academic excellence in school of environment science and technology ($300). 2009 and 2010
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Chapter 1: Background and Objectives
1.1 Introduction

Bioremediation is a promising technology for destroying or rendering harmless various pollutants in contaminated lands and water by using biologically activity. 1,4-Dioxane, an emerging water contaminant, has been found in personal products and aquatic environments over decades. Recent studies revealed that 1,4-dioxane can be biodegraded by many microorganisms, but the presence of co-contaminants has been a common issue challenging the success of 1,4-dioxane bioremediation. My research elucidated the impact of co-occurring pollutants, including chlorinated solvents (TCE, TCA, 1,1-DCE and cDCE) on 1,4-dioxane biodegradation and proposed effective means to overcome this challenge.

1.2. Research Goals and Objectives

The goals of this research were to quantify the substrate interactions of chlorinated solvents on 1,4-dioxane biodegradation, describe the kinetics and mechanisms, and extend the results from laboratory studies to a bioremediation field study.

Objective 1: To determine the impact of chlorinated solvents (trichloroethylene and 1,1,1-trichloroethane) and their biological and abiotic degradation products (1,1-DCE and cis-1,2-DCE) on metabolic biodegradation kinetics of 1,4-dioxane.

Objective 2: To investigate the mechanisms of chlorinated solvents’ inhibition on biodegradation of 1,4-dioxane.

Objective 3: To test the effects of mixtures of co-contaminants in groundwater samples collected from actual 1,4-dioxane contaminated sites.

1.3. Dissertation Overview

This dissertation is organized into 4 Chapters. Chapter 1 briefly introduces the
background, problem statement, goal and objectives of this research. Chapter 2 provides a thorough literature review relevant to this research. Chapter 3 discusses the metabolic biodegradation of 1,4-dioxane in the presence of chlorinated solvent mixtures. Lastly, Chapter 4 summarizes the key findings and discusses future directions.
Chapter 2: Literature Review
2.1 Introduction

1,4-Dioxane is a synthetic chemical used as a solvent and stabilizer of chlorinated solvents, in particular 1,1,1-trichloroethane (TCA) for over a hundred years (Mohr, 2010; USEPA, 2013). Although TCA was eventually phased out of use in the U.S. in the 1990s, 1,4-dioxane has continued to be used as a solvent for paints, oils, polishing compositions, coatings and plasticizers, and can be found as a residue in personal care products and cosmetics (Mohr, 2010; USEPA, 2013). As an organic compound, its miscibility with water renders a high heat capacity, dehydration, and an outstanding polarity in the water-dioxane mixture among other solvent or solvent mixtures (Calvo et al., 1998; Inglese et al., 1983; Mizuno et al., 2003).

1,4-Dioxane is classified as a Group 2B agent by the International Agency for Research on Cancer (IARC), which is possibly carcinogenic to humans due to the inadequate evidence in humans and sufficient research evidence in experimental animals for the carcinogenicity of 1,4-dioxane (IARC, 1999). It was also classified as a likely human carcinogen by the USEPA (USEPA, 2013). Regarding the toxicity profile, EPA’s Integrated Risk Information System (IRIS) determined a chronic oral reference dose (RfD) of 0.03 mg/kg/day based on liver and kidney toxicity in animals and a chronic inhalation reference dose (RfC) of 0.03 mg/m³ based on atrophy and respiratory metaplasia inside the nasal cavity of animals (ATSDR, 2012; USEPA, 2013). Toxicity studies in rats and mice determined 1,4-dioxane increased the occurrence of tumors in these animals (Kano et al., 2009; Kasai et al., 2009). Therefore, EPA’s Health Advisory Level (HAL) of 1,4-dioxane in drinking water was established at 0.35 µg/L (USEPA, 2013). Currently, 1,4-dioxane is not strictly regulated in the US, but several states have established notification levels and guidelines, and the acceptable concentrations of this contaminant are trending lower. Further, its low Henry’s law constant (4.80×10⁻⁶ atm-m³/mol at
25°C), low $K_{ow}$ (Log $K_{ow}$: -0.27) and low $K_{oc}$ (Log $K_{oc}$: 1.23) (USEPA, 2013) limits the success of rapid and high resolution analytical approaches as well as effective remediation technologies. Thus, air stripping, thermal desorption, and soil vapor extraction tend to be inefficient treatment strategies for 1,4-dioxane-contaminated waters.

The long usage history, improper storage and disposal of 1,4-dioxane as a solvent and as a chlorinated solvent stabilizer have resulted in mixed contamination with other common environmental pollutants in groundwater and industrial wastewater. Anderson et al. (2012) reported an important association between 1,4-dioxane and chlorinated solvents where approximately 94% of groundwater monitoring wells with detectable levels of 1,4-dioxane also contained TCA and/or trichloroethylene (TCE). 1,4-Dioxane was initially used as a stabilizer for TCA but not for TCE in the records (Mohr, 2010). However, a recent investigation on the co-occurrence of 1,4-dioxane and TCE determined 65% of 1,4-dioxane detections in monitoring wells were independent of TCA (Anderson et al., 2012). TCE (0.72 µg/L to 650 µg/L) and 1,4-dioxane (1.1 µg/L to 340 µg/L) were also found to be comingled at the United States Air Force Plant No. 44 (AFP 44) aquifers (Chiang et al., 2012). Additionally, poly- and per-fluorinated compounds were also found at 1,4-dioxane contaminated sites (CH2M, 2016).

To better characterize 1,4-dioxane-contaminated sites, a multi-site survey was conducted by Adamson et al. (2014). This study identified the scale of 1,4-dioxane problems at contaminated groundwater sites in California and determined the migration of chlorinated solvents generally occurred beyond 1,4-dioxane plumes (Adamson et al., 2014). Additionally, 1,1-DCE was confirmed as another major co-contaminant associated with 1,4-dioxane contamination (Adamson et al., 2015; Adamson et al., 2014). The co-occurrence of these chlorinated solvents complicates the design of an appropriate remediation system. For instance,
advanced oxidation processes (AOPs) require further optimization when applied to CVOCs and 1,4-dioxane contaminated sites as a result of the different chemical structures and disparate affinities for hydroxyl radicals. Furthermore, recent studies determined the inhibitory effects of chlorinated solvent co-contaminants on the biodegradation kinetics of 1,4-dioxane (Hand et al., 2015; Mahendra et al., 2013; Zhang et al., 2016b). The presence of TCE, cis-1,2-DCE, and 1,1-DCE inhibited 1,4-dioxane metabolism by a dioxane-degrading bacterium producing inhibition constants ($K_I$) of $8.6 \pm 1.74$, and $1.51 \pm 0.26$, mg L$^{-1}$, respectively (Zhang et al., 2016b). $K_I$ was determined using a Michaelis-Menten model and is defined as the inverse measure of the inhibitor affinity for the target enzyme. Thus, lower values of $K_I$ indicated stronger inhibition of solvent.

Heavy metals are also found in 1,4-dioxane contaminated groundwater (Adamson et al., 2015). Thus, understanding the effects of metallic co-contaminants on bacteria and enzymes catalyzing 1,4-dioxane biodegradation is crucial for an improved understanding of field-scale 1,4-dioxane bioremediation. Pornwongthong et al. (2014) evaluated the impact of Cu(II), Cd(II), Ni(II), and Zn(II) on 1,4-dioxane biodegradation in pure cultures and determined dose-dependent inhibitory effects in the order of Cu(II) > Cd(II) ≥ Ni(II) > Zn(II). According to the California GeoTracker database (2015), approximately 30% (number of sites = 67) of 1,4-dioxane sites also have hexavalent chromium contamination issues. The presence of Cr(VI) impeded 1,4-dioxane microbial degradation by affecting monooxygenase transcription and energy production in bacteria (Zhang et al., 2016a). The negative correlation between heavy metals and natural attenuation of 1,4-dioxane determined by Adamson et al. (2015) also suggests that heavy metals inhibit 1,4-dioxane biodegradation by indigenous microorganisms. Therefore, due to the divergent physicochemical properties of each individual chemical in mixed contaminated sites,
different treatment technologies are usually required for environmental clean-up.

2.2 Remediation Strategies

Several chemical, physical, and biological treatment technologies have been developed and applied to remove 1,4-dioxane from impacted environments (DiGuiseppi et al., 2016; DiGuiseppi and Whitesides, 2007). This paper briefly summarizes current AOPs and adsorption treatment processes, but focuses on bioremediation of 1,4-dioxane.

2.2.1 Chemical Treatments

1,4-Dioxane was initially considered as a non-biodegradable environmental contaminant by an early study reporting the persistence of 1,4-dioxane in wastewater treatment plants for over one year (Zenker et al., 2000). Therefore, chemical treatments were primarily considered as effective means to remove 1,4-dioxane. However, the specific absorption wavelength of 1,4-dioxane, ranging from 165 to 191 nm (Pickett et al., 1951), reduces the effectiveness of direct photolysis (Kruithof et al., 2007). Furthermore, in situ 1,4-dioxane remediation using UV is subject to operational challenges (Mohr, 2010). UV/hydrogen peroxide (H2O2) systems have been in development since the 1960s, to improve the treatment effectiveness. These systems utilize a photoinduced oxidation method to significantly destroy the structure of organic chemicals (Omura and Matsuura, 1968; Stefan and Bolton, 1998). The release of hydroxyl radicals from H2O2 is triggered by UV light and drives the rapid decay of 1,4-dioxane following several chemical transformation pathways (Eq. 1-2) to eventually form carbon dioxide (Figure 2.1) (Omura and Matsuura, 1968). The factors influencing the oxidation processes were also investigated in other studies, for example, acidic and alkaline pH (Vescovi et al., 2010) impeded photocatalysis. In contrast, the agitation by ultrasound (Sohmiya et al., 2001) and the presence of titanium dioxide (Maurino et al., 1997) significantly enhance the degradation of 1,4-dioxane.
\[
\begin{align*}
  \text{H}_2\text{O}_2 + hv & \rightarrow 2\text{HO}^\cdot \quad \text{Eq.1} \\
  \text{C}_4\text{H}_8\text{O}_2 + \text{HO}^\cdot & \rightarrow \text{C}_4\text{H}_7\text{O}_2^\cdot + \text{H}_2\text{O} \quad \text{Eq.2}
\end{align*}
\]

AOPs are often selected as the remedial strategy for 1,4-dioxane because of the short timeline and high removal efficiency. For example, a 96% removal of 1,4-dioxane was achieved in a 2-hour reaction using hydrogen peroxide (Kim et al., 2006); over 99% of 1,4-dioxane was removed within 10-hour via a combination of hydrogen peroxide and ferrous ions (Klečka and Gonsior, 1986); and a half-life of 12.2 hours was determined when perozone activated persulfate was applied to degrade mixtures of 1,4-dioxane and chlorinated solvents (Eberle et al., 2016). To further improve treatment timelines, reduce energy consumption and operational costs, multiple oxidation reagents (O\textsubscript{3}/H\textsubscript{2}O\textsubscript{2}, O\textsubscript{3}/UV, O\textsubscript{3}/H\textsubscript{2}O\textsubscript{2}/UV) are generally considered to optimize remediation efficiency (Ikehata et al., 2016). The increasing number of 1,4-dioxane contaminated sites and the urgency of in-situ and ex-situ treatment plans warrants more studies focused on oxidation reagents, such as zero-valent iron (ZVI), hydrogen peroxide, ozone, and persulfate to enhance remediation of 1,4-dioxane and its co-contaminants. These chemicals can directly generate reactive species, such as •OH and SO\textsubscript{4}^\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\·, to decompose organic pollutants (Li and Zhu, 2016; Merayo et al., 2014). Oxidation reactions can also be indirectly activated, for instance, the addition of Fe\textsuperscript{0} and Fe\textsuperscript{2+} significantly accelerated sonication removal of 1,4-dioxane (Son et al., 2006). Further, advanced electrochemical oxidation (AEO) is a technique that generates hydroxyl radicals via water electrolysis and has been reported as an effective treatment strategy for potential in situ and ex situ treatment of 1,4-dioxane (Jasmann et al., 2016).

Importantly, 1,4-dioxane degradation products by AOPs are usually low molecular weight fatty acids (Figure 2.1), such as glycolic, formic, glyoxal, acetic, and oxalic acids, which
can be biotransformed through conventional biological treatments (Adams et al., 1994; Merayo et al., 2014). These findings illustrate the effectiveness and short time-span of chemical oxidation strategies to degrade 1,4-dioxane and suggest a combined AOP and biological treatment approach should be considered as a viable remediation strategy.

![Diagram of 1,4-dioxane degradation pathways](image)

Figure 2.1. Pathways of 1,4-dioxane chemical and biological degradation. **Top:** 1,4-dioxane chemical decomposition by AOPs treatment. Modified from Barndökk *et al.* (2016); Beckett and Hua (2000, 2003). The initiation of 1,4-dioxane transformation needs active free radicals. The products are usually biodegradable small fatty acids. **Bottom:** 1,4-dioxane biodegradation. The intermediates presented in the pathways are major identified products, which can be detected for
the evaluation of 1,4-dioxane degrading microorganisms. Modified from Grostern et al. (2012); Huang et al. (2014); Kim et al. (2009); Mahendra et al. (2007); Sales et al. (2013). Black box outlines highlight the intermediate products can be biodegraded, suggesting the combination of abiotic and probiotic processes. Yellow box outline indicates the unique degradation pathway of *P. dioxanivorans* CB1190. Green box outline underlines that HEAA is the end product of 1,4-dioxane cometabolic degradation.

2.2.2 Physical Treatments

High solubility and low Henry’s law constant of 1,4-dioxane can limit the success of physical remediation outcomes. Thermal destruction technologies and air stripping are generally not considered as efficient means since these are usually applied to treat highly volatile and non-aqueous phase liquids (NAPL) (Triplett Kingston et al., 2010). Yet, 1,4-dioxane is a non-volatile compound, and it’s mobility would considerably require the large-scale construction of these physical treatments. Distillation is applicable to separate chemicals by boiling points (bp) but becomes challenging when separating 1,4-dioxane from water since they share a similar boiling point (1,4-dioxane bp: 101 °C) (Mohr, 2010; Vescovi et al., 2010). These technologies can be considered as a means for 1,4-dioxane remediation, but they demand a considerable amount of energy to achieve the desired levels of 1,4-dioxane removal.

Activated carbon adsorption to remove environmental contaminants has been successfully applied for many decades. Organic compounds such as chlorinated solvents (TCE and tetrachloroethylene) and inorganic metals (copper, zinc, and lead) can be adsorbed by granular activated carbons (GAC) (Crittenden et al., 1988; Goyal et al., 2001; Kilduff et al., 1998; Nakano et al., 2000). Initially, GAC adsorption and air-stripping techniques were not considered as applicable means to remove 1,4-dioxane from water. However, recent studies reported a high removal efficiency of 1,4-dioxane by different sorbents. Ambersorb™ 560 is a polymeric adsorbent synthesized by Dow Chemical Company (Woodard et al., 2014). Its high
surface area, high porosity, hard, spherical shape, and regenerable features produced a high removal efficiency for 1,4-dioxane and other organic compounds (Simon, 2015; Woodard et al., 2014). Yet, this physical treatment is a non-destructive approach and typically results in concentrating 1,4-dioxane on the sorbent which raises concerns for long term stability and potential desorption under dynamic flow conditions.

2.3 Bioremediation

Bioremediation is an attractive technology to destroy or render harmless various pollutants in contaminated lands and water by using biological activity (Bamforth and Singleton, 2005; Vidali, 2001). The broader definition of bioremediation includes the use of plants (phytoremediation), bacteria (bacterial), and fungi (mycoremediation) to remove environmental contaminants during in situ or ex situ treatment. In comparison with traditional abiotic approaches, such as UV oxidation or sorption, bioremediation can be a low-cost, low-technology and highly effective remedy (Vidali, 2001). It was successfully applied in situ for the treatment of various contaminants in surface water, groundwater, industrial wastewater, and drinking water systems (Brierley, 1990; Chapelle, 1999; Wagner-Dobler, 2003).

2.3.1 Phytoremediation

Phytoremediation is an effective technology utilizing plants to degrade, assimilate, metabolize, or detoxify metals, radionuclides and chlorinated solvents (Schnoor et al., 1997; Susarla et al., 2002). Previous research has shown BTEX (benzene, toluene, ethylbenzene and xylene), TCE, 1,1,2,2-tetrachloroethane, and carbon tetrachloride can be removed via phytoremediation (Ma and Burken, 2002; Schnoor et al., 1997). Recent studies also reported that vegetation could remove 1,4-dioxane from contaminated soil and groundwater by root uptake (Ouyang, 2002; Sorensen, 2013). Specifically, Aitchison et al. (2000) first determined the
feasibility of hybrid poplar trees to remove 1,4-dioxane from hydroponic solutions and found that 54.0% ± 19.0% of the 1,4-dioxane can be rapidly removed in 9 days (the initial concentration was 23 mg/L). Another bench-scale study found that combining poplar trees and bioaugmentation with *Amycolata sp.* (now classified as *Pseudonocardia dioxanivorans*) CB1190 removed 100 mg/L 1,4-dioxane until it was below the instrument detection limit in 45 days (Kelley *et al.*, 2001). Dietz and Schnoor (2001) suggested hydrophilic compounds such as methyl-tert-butyl ether and 1,4-dioxane might be oxidized by cytochrome P450s passing along the membrane to the stem and leaves. Therefore, more plant-candidates that are able to remediate organic contaminants, such as *Betula pendula, Vetiveria zizanoides* and *Eucalyptus sideroxylon rosea* (Kuppusamy *et al.*, 2016; Lewis *et al.*, 2015), should be tested for 1,4-dioxane phytoremediation to reveal potentially novel biodegradation enzymes and/or pathways.

### 2.3.2 Microbial Remediation

Microbial activity is a naturally occurring process and is a desirable ‘green’ technology to remove harmful and undesirable environmental contaminants (Singleton, 1994). 1,4-Dioxane was initially considered a non-biodegradable industrial synthetic chemical. However, in the past decade, several studies on 1,4-dioxane biodegradation have been published (Mahendra and Alvarez-Cohen, 2006; Mahendra *et al.*, 2007; Skinner *et al.*, 2009; Sun *et al.*, 2011; Zhang *et al.*, 2016b) suggesting a greater potential for bioremediation in the field (Figure 2.2).
Aerobic conditions are generally favorable to rapidly biodegrade a majority of environmental pollutants (Fritsche and Hofrichter, 2005). To date, little evidence of anaerobic 1,4-dioxane biodegradation has been reported other than a single study using an iron-reducing bacterium to anaerobically grow on 1,4-dioxane (Shen et al., 2008). Thus, this review focuses on aerobic 1,4-dioxane biodegradation processes. A summary of microorganisms that are reported to aerobically biodegrade 1,4-dioxane is presented in Table 2.1. Depending on the role of 1,4-dioxane in cellular growth and energy generation, we characterize these microorganisms into three groups: metabolism, cometabolism, or others. Microbial metabolism is a process in which microorganisms use the organic contaminants as a carbon and energy source to support the growth of microbes (Suthersan, 2017), whereas, microbial cometabolism is defined as a process by which a contaminant is degraded via introducing the other carbon source to induce enzyme or cofactor during microbial metabolism (Luo et al., 2014). In addition, energy is not generated and
carbon is not utilized for growth during cometabolism of the target compound (Alvarez-Cohen and McCarty, 1991; Horvath, 1972) (Figure 2.3).

Figure 2.3. Schematic illustration of microbial metabolism and cometabolism. Mahendra and Alvarez-Cohen (2005); Nakamiya et al. (2005); Patrauchan et al. (2012)

2.3.2.1 1,4-Dioxane Metabolic Biodegradation

Parales et al. (1994) isolated the Actinomycete, CB1190, from industrial activated sludge, and determined that CB1190 was able to aerobically grow on 1,4-dioxane as a sole carbon and energy source. The doubling time of CB1190 was about 30 hours when it was grown in ammonium mineral salts medium at 30 °C amended with 5.5 mM 1,4-dioxane at a rate of 0.33 mg dioxane/hr/mg protein (Parales et al., 1994). Further studies on this organism resulted in its
classification as *Pseudonocardia dioxanivorans* sp. nov. CB1190 (Mahendra and Alvarez-Cohen, 2005), a Gram-positive bacterium with 74% G+C content and a powdery white morphology (Figure 2). This team also reported a cell yield of 0.09 ± 0.002 g protein/(g dioxane), energy production was 19 mM ATP/(mM dioxane) and the key enzyme was a multicomponent monooxygenase (Groster et al., 2012; Mahendra and Alvarez-Cohen, 2005; Mahendra et al., 2007). The findings of the proposed 1,4-dioxane metabolic biodegradation pathway (Figure 4) and the major enzymatic oxidization steps also led to numerous studies on alternative 1,4-dioxane biotransformation pathways. For instance, Kim et al. (2009) isolated *Mycobacterium* sp. PH-06 from contaminated river sediment and determined its ability to metabolize 1,4-dioxane forming 1,4-dioxane-2-ol and ethylene glycol as major metabolites following monooxygenation. The kinetic parameters of monooxygenase activity, such as maximum specific 1,4-dioxane degradation rate ($k_{\text{max}}$) and half saturation concentration ($K_s$), are generally used to evaluate the performance of 1,4-dioxane degrading bacteria (Sei et al., 2013).

A Gram-negative strain *Afipia* sp. D1, isolated from soil samples collected from the drainage area of a chemical factory, had a high $k_{\text{max}}$ of 0.236 mg-1,4-dioxane/mg-protein/hr and a low $K_s$ of 25.8 mg/g (Sei et al., 2010; Sei et al., 2013). Moreover, *Afipia* sp. D1 was able to tolerate high concentrations of the co-contaminant ethylene glycol while grown on 1,4-dioxane (Sei et al., 2013). Huang et al. (2014) isolated another 1,4-dioxane-degrading bacterium, *Acinetobacter baumannii* DD1 (Gram-negative, short rod, and aerobic), from activated sludge in China, and reported a high cell yield of 0.414 mg-protein/mg-1,4-dioxane. However, 2-hydroxyethoxyacetic acid was not detected in pure culture studies degrading 1,4-dioxane which suggests a divergent biodegradation pathway facilitated by *Acinetobacter baumannii* DD1. Recently, *Pseudonocardia carboxydivorans* RM-31 and *Xanthobacter flavus* DT8 were identified to metabolize 1,4-dioxane
with rapid biodegradation rates. A monooxygenase enzyme was not the initiation enzyme in X. flavus DT8 suggesting a novel 1,4-dioxane biodegradation pathway (Chen et al., 2016). The bacterial strain Pseudonocardia benzenivorans B5 was originally identified as a 1,2,3,5-tetrachlorobenzene degrading bacterium (Kämpfer and Kroppenstedt, 2004), and was also able to grow on 1,4-dioxane (Mahendra and Alvarez-Cohen, 2006).

The role of fungi in bioremediation is equally important as that of bacteria for the cleanup of contaminated sites. To date, Cordyceps sinensis is the only fungus capable of metabolizing 1,4-dioxane producing ethylene glycol, glycolic acid, and oxalic acid as intermediate metabolites during 1,4-dioxane biodegradation (Nakamiya et al., 2005). The optimal growth of this fungus was observed under aerobic conditions with an initial 1,4-dioxane concentration of 0.034 M and incubation at 30 °C.

Several microcosm studies have been conducted to understand 1,4-dioxane metabolic biodegradation in environmental samples incubated under controlled conditions. For example, bioaugmentation with CB1190 degraded 1,4-dioxane at rates of 0.16 ± 0.04 and 0.021 ± 0.007 mg-dioxane/day/mg-protein at 14 and 4 °C, respectively (Li et al., 2010). In the presence of a co-occurring chlorinated solvent mixture, bioaugmentation with CB1190 was able to remove approximately 80% 1,4-dioxane (Zhang et al., 2016b). Similarly, Li et al. (2014b) established a bench-scale microcosm study using 14C-labeled 1,4-dioxane to examine the potential for bioremediation in 1,4-dioxane contaminated groundwater samples from California. After 28-week incubation, 75 % of the biological microcosms had significant removal of 1,4-dioxane (Li et al., 2014b), suggesting the existence of indigenous 1,4-dioxane metabolizing microorganisms. Biodegradation of 1,4-dioxane at high concentrations (~80 mg/L) was also demonstrated by the microbial community in activated sludge from an industrial wastewater treatment plant, and
specific microorganisms within this mixed microbial community were able to grow on 1,4-dioxane based on the high positive correlation between 1,4-dioxane removed and increases in CB1190-like bacteria (Gedalanga et al., 2014).

2.3.2.2 1,4-Dioxane Cometabolic Biodegradation

Cometabolism is a biological process to biotransform non-growth supporting substrates via induction of biodegradative enzymatic activities by a primary growth supporting carbon source. Mahendra and Alvarez-Cohen (2006) identified several monooxygenase-expressing strains that can cometabolize 1,4-dioxane, such as Pseudonocardia K1, Burkholderia cepacia G4, Pseudomonas mendocina KR1, and Mycobacterium austroafricanum JOB5. Burkholderia cepacia G4 was originally isolated from TCE contaminated water to cometabolize TCE and aromatic compounds (Kang and Doty, 2014; Nelson et al., 1987), and later it was determined to degrade 1,4-dioxane at a rate of 0.1 ± 0.006 mg/hr/mg-protein by toluene induced monooxygenase (Mahendra and Alvarez-Cohen, 2006). Similarly, 1,4-dioxane biodegradation was observed in Mycobacterium austroafricanum JOB5 cultures growing on several short-chain alkanes, such as propane, n-butane, n-pentane, isobutane, isopentane, and even dextrose (Lan et al., 2013). Pseudonocardia sp. strain ENV478 was identified to biodegrade 1,4-dioxane after growth on the primary substrate THF. The proposed pathway suggested that the major products of 1,4-dioxane cometabolism were 1,4-dioxane-2-one and 2-hydroxyethoxyacetic acid (2HEAA) (Vainberg et al., 2006). Cometabolism of 1,4-dioxane was also identified in the fungus Graphium sp. via propane and THF induced monooxygenase activity (Skinner et al., 2009). Another fungus, Aureobasidium Pullmans, was able to cometabolically degrade 1,4-dioxane after growth on THF (Patt and Abebe, 1995). These microorganisms directly participating in 1,4-dioxane biotransformation can be considered as candidates for biostimulation and
bioaugmentation remedial strategies, and they have been examined in many microcosms set up using contaminated aquifer materials (Zenker et al., 2000), industrial waste (Roy et al., 1994), and wastewater from treatment plants (Sock, 1993).
Table 2.1 List of 1,4-dioxane degrading microorganisms and biodegradation rates. (MO – Monooxygenase; THF – Tetrahydrofuran; TSS – Total suspended solids; N/A – Not available.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Induced enzyme</th>
<th>Biodegradation rate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudonocardia dioxanivorans</em> CB1190</td>
<td>1,4-dioxane MO</td>
<td>0.19 ± 0.007 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2005, 2006)</td>
</tr>
<tr>
<td><em>Actinomycete</em> CB1190</td>
<td>N/A</td>
<td>0.33 mg/min/mg-protein</td>
<td>Parales et al. (1994)</td>
</tr>
<tr>
<td><em>Amycolata sp.</em> CB1190</td>
<td>N/A</td>
<td>0.92 ± 0.29 mg/day/mg-protein</td>
<td>Kelley et al. (2001)</td>
</tr>
<tr>
<td><em>Pseudonocardia benzenivorans</em> B5</td>
<td></td>
<td>0.01± 0.003 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td><em>Mycobacterium sp.</em> PH-06</td>
<td>MO</td>
<td>60 mg/L/day</td>
<td>Kim et al. (2009)</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> DD1</td>
<td>MO</td>
<td>2.38 mg/hr/L</td>
<td>Huang et al. (2014)</td>
</tr>
<tr>
<td><em>Pseudonocardia carboxydivorans</em>. RM-31</td>
<td>N/A</td>
<td>31.6 mg/L/hr</td>
<td>Matsui et al. (2016)</td>
</tr>
<tr>
<td><em>Xanthobacter flavus DT8</em></td>
<td>N/A</td>
<td>Similar as CB1190</td>
<td>Chen et al. (2016)</td>
</tr>
<tr>
<td><em>Afipia sp.</em> D1</td>
<td></td>
<td>0.052 to 0.263 mg/mg-protein/h</td>
<td>Sei et al. (2013)</td>
</tr>
<tr>
<td><em>Cordyceps sinensis</em> (fungus)</td>
<td>MO</td>
<td>0.011 mol/day</td>
<td>Nakamiya et al. (2005)</td>
</tr>
<tr>
<td><strong>Cometabolism</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium austroafricanum</em> JOB5</td>
<td>Propane MO</td>
<td>0.40 ± 0.06 mg/hr/mg-protein</td>
<td>House and Hyman (2010); Lan et al. (2013); Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td><em>Rhodococcus ruber</em> ENV425</td>
<td>Propane MO</td>
<td>10 mg/hr/g TSS</td>
<td>Lippincott et al. (2015); Vainberg et al. (2006)</td>
</tr>
<tr>
<td><em>Pseudonocardia sp.</em> strain ENV478</td>
<td>THF MO</td>
<td>21 mg/hr/g TSS</td>
<td>Masuda et al. (2012); Vainberg et al. (2006)</td>
</tr>
<tr>
<td><em>Rhodococcus RR1</em></td>
<td>N/A</td>
<td>0.38 ± 0.03 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td><em>Rhodococcus jostii</em> RHA1</td>
<td>Propane MO 1-butane MO</td>
<td>N/A</td>
<td>Hand et al. (2015); Li et al. (2013)</td>
</tr>
<tr>
<td><em>Flavobacterium</em></td>
<td>N/A</td>
<td>N/A</td>
<td>Sun et al. (2011)</td>
</tr>
</tbody>
</table>
### Microorganisms with Tetrahydrofuran (THF) and Toluene-2 Methoxy (MO) Activity

<table>
<thead>
<tr>
<th>Organism</th>
<th>Solvent</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudonocardia K1</td>
<td>tetrahydrofuran MO</td>
<td>0.26 ± 0.013 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td>Burkholderia cepacia G4</td>
<td>toluene-2- MO</td>
<td>0.1 ± 0.006 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td>Ralstonia pickettii PKO1</td>
<td>toluene-p- MO</td>
<td>0.31 ± 0.007 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td>Pseudomonas mendocina KR1</td>
<td>toluene-4- MO</td>
<td>0.37 ± 0.04 mg/hr/mg-protein</td>
<td>Mahendra and Alvarez-Cohen (2006)</td>
</tr>
<tr>
<td>Aureobasidium pullmans NRRL 21064</td>
<td>N/A</td>
<td>6-8 mg/L within a day</td>
<td>Patt and Abebe (1995)</td>
</tr>
<tr>
<td>Graphium sp. (ATCC 58400) (fungus)</td>
<td>Propane MO</td>
<td>4 ± 1 nmol/min/mg dry weight</td>
<td>Skinner et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>THF MO</td>
<td>9 ± 5 nmol/min/mg dry weight</td>
<td></td>
</tr>
</tbody>
</table>

### Others

<table>
<thead>
<tr>
<th>Organism</th>
<th>Solvent</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shewanella oneidensis</td>
<td>N/A</td>
<td>27.9 ± 3.37 to 36.2 ± 4.13 µM/hr</td>
<td>Sekar et al. (2016)</td>
</tr>
<tr>
<td>Enriched consortium-FS</td>
<td>MO</td>
<td>0.037 mg/hr/mg-protein</td>
<td>Nam et al. (2016)</td>
</tr>
<tr>
<td>Enriched consortium-AS</td>
<td>MO</td>
<td>0.078 mg/hr/mg-protein</td>
<td>Nam et al. (2016)</td>
</tr>
<tr>
<td>SL-D(propanotroph strain)</td>
<td>N/A</td>
<td>20 mg/L within a day</td>
<td>Innovative Engineering Solutions Inc.IESI (2017)</td>
</tr>
<tr>
<td>CL-OUT® (Pseudomonas putida stain B, Pseudomonas putida strain E and Pseudomonas fluorescens strain G)</td>
<td>N/A</td>
<td>Over 70% removal</td>
<td>Saul (2012)</td>
</tr>
</tbody>
</table>

### 2.3.2.3 Other Biodegradation

Many microorganisms, unable to oxidize 1,4-dioxane via enzymatic reactions, can still play important roles in bioremediation. For example, a recent study constructed a microbially-driven Fenton system to effectively remove 1,4-dioxane and chlorinated solvent mixtures by the Fe(III)-reducing facultative anaerobe, *Shewanella oneidensis* (Sekar and DiChristina, 2014; Sekar et al., 2016). This strategy greatly extends the function of microbes, which will not
necessarily rely on specific enzymes produced during metabolic and cometabolic processes to initialize 1,4-dioxane biotransformation.

Advanced biological treatment technologies are always pursued to match various contaminated sites’ biogeochemical characteristics. The use of microbial consortia, which contains multiple microbial players, expands the range of treatable contaminants. A previous study using mixed communities determined that approximately 93-97% 1,4-dioxane removal could be achieved using a trickling filter with attached microbes by feeding THF (Zenker et al., 2004). A patented method using a CL-OUT® Pseudomonas sp. organisms consortium (Pseudomonas putida strain B, Pseudomonas putida stain E, and Pseudomonas fluorescens strain G) was able to cometabolically biodegrade chlorinated solvents and 1,4-dioxane in groundwater (Saul, 2012). Recently, bacterial consortia isolated from forest soil (FS) and activated sludge (AS) were able to degrade 1,4-dioxane with the rates of 0.037 and 0.078 mg/hr/mg-protein, respectively (Nam et al., 2016). In laboratory microcosms using samples collected from the Vandenberg Air Force Base (VAFB) site, biostimulation with propane and bioaugmentation with Rhodococcus ruber ENV425 was able to remove 1,4-dioxane following first-order decay rates ranging between 0.021 - 0.036 per day (Lippincott et al., 2015). These findings provide evidence of applying natural attenuation, biostimulation, and bioaugmentation as in-situ 1,4-dioxane remediation strategies.

2.4 Monitoring Tools and Field Applications

A comprehensive understanding of 1,4-dioxane biodegradation pathways and specific enzymatic reactions also contribute to the development of novel monitoring tools, which can
better characterize the contaminated site prior to implementing remedies as well as precisely evaluate the performance of the selected active and passive treatments.

Molecular biological tools, including contaminant-specific biomarkers, can provide direct information about the existence of potential contaminant-degrading microorganisms and relevant active processes in the field (Loeffler et al., 2013). Mahendra and Alvarez-Cohen (2006) initially identified the role of monooxygenases in 1,4-dioxane oxidation, providing a reference for targeting genes encoding monooxygenase enzymes as biomarkers for 1,4-dioxane biodegradation. Subsequently, the whole genome sequence of CB1190 was announced by Sales et al. (2011), allowing fundamental examination of 1,4-dioxane metabolism at the genetic level. Grostern et al. (2012) used a microarray to analyze gene expressions along with CB1190 degrading 1,4-dioxane, and identified monooxygenase gene clusters located on the plasmid. Moreover, the up-regulation of THF monooxygenase gene cluster (thmA/dbc), aldehyde dehydrogenases, aldehyde reductase, and alcohol oxidoreductase were observed as associations with the activity of CB1190 growing on 1,4-dioxane (Grostern et al., 2012; Sales et al., 2013). To improve the accuracy of 1,4-dioxane biomarkers, Gedalanga et al. (2014) designed a set of primers targeting multicomponent monooxygenase genes (dxbB and aldH) to predict the 1,4-dioxane biodegradation potential. The presence of nonspecific monooxygenase genes coupled with the up-regulation of aldehyde dehydrogenase confirmed the occurrence of active 1,4-dioxane oxidation (Gedalanga et al., 2014). Li et al. (2013) developed a primer/probe set of thmA/dxmA encoding a group 5 soluble di-iron monooxygenases (SDIMOs) that had a positive relationship with the potential of 1,4-dioxane biodegradation in microcosms studies using contaminated groundwater and soil (Li et al., 2014a; Li et al., 2013). The detection of monooxygenase genes (e.g. the gene abundance) is indeed evidence of the microbial players’
existence, however, it may not serve as a determinative predictor of the enzymatic activities. For example, the up-regulation of monooxygenase can be observed in the presence of enzyme inhibitors such as acetylene and co-contaminants, even when 1,4-dioxane biodegradation remains inhibited (Gedalanga et al., 2014; Zhang et al., 2016b). These molecular biological analyses have been applied to many field samples collected from industrial activated sludge and groundwater samples from Arizona, Alaska, California, and Texas (Chiang et al., 2012; Gedalanga et al., 2014; Li et al., 2014a; Li et al., 2013; Zhang et al., 2016b).

Compound Specific Isotope Analysis (CSIA) is another monitoring tool often used to assess biodegradation processes. Pornwongthong \textit{et al.}, (2011) developed a $^{13}$C-based CSIA method for 1,4-dioxane, and determined a kinetic carbon isotopic fractionation factor ($\varepsilon$) ($\varepsilon = -1.73 \pm 0.14$) during aerobic biodegradation of 1,4-dioxane by \textit{P. dioxanivorans} CB1190. This method was also applied to confirm cometabolic biodegradation of 1,4-dioxane in pure cultures as well as in laboratory microcosms constructed using industrial wastewater or groundwater samples (Pornwongthong et al., 2017b). Optimization of this approach led to the documentation of $^2$H enrichment during biodegradation of 1,4-dioxane (Pornwongthong et al., 2017a) resulting in an improved CSIA method to provide evidence for 1,4-dioxane biodegradation. Indeed, CSIA and 1,4-dioxane specific biomarkers were recently applied directly in groundwater samples collected from a contaminated aquifer, and complemented traditional analyses to provide multiple lines of evidence showing biodegradation of 1,4-dioxane was likely occurring at that site (Gedalanga et al., 2016a).

Aside from a recently published article (Gedalanga et al., 2016b) and our research manuscripts in review (Pornwongthong et al., 2017a; Pornwongthong et al., 2017b), peer-reviewed publications of applications and case studies that have implemented 1,4-dioxane CSIA
are lacking. However, progress has been made using $^{13}$C-labeled 1,4-dioxane stable isotope probing (SIP) technology, such as the detection of $^{13}$C-biomass via microbial incorporation of $^{13}$C-1,4-dioxane suggesting the potential of natural attenuation at AFP 44 (Chiang et al., 2012). This study suggests a promising potential for applying stable isotope-based tools, such as CSIA and SIP, to accurately confirm natural or enhanced bioremediation of 1,4-dioxane.

The stringent regulatory advisory levels of 1,4-dioxane in groundwater and drinking water systems require advanced sensing and analytical methods to reflect changes at trace concentrations. Some efforts were made to improve quantification using spectrophotometric techniques, for example, a heated purge-and-trap pre-concentration and Gas Chromatography/Mass Spectrometry (GC/MS) method was able to quantify 0.15 µg/L 1,4-dioxane (Sun et al., 2016); and addition of a coconut charcoal adsorbent for solid-phase extraction (SPE) and GC/MS can detect 0.016 µg/L 1,4-dioxane in the environmental samples (Stepien and Püttmann, 2013). An on-site real-time 1,4-dioxane analysis using solid phase microextraction followed by thermally desorbing into direct sampling ion trap mass spectrometer (DSITMS) was developed to rapidly quantify 1,4-dioxane in the groundwater and soil to support real-time decision-making in the field (Davis et al., 2016). Furthermore, luminescent assays and novel materials were also developed to simplify sample preparation and detection procedures. Recently, a bimetallic lanthanide metal–organic material and a porous organic polymer, which can specifically bind with 1,4-dioxane molecules and immediately emit detectable colors, has been reported for detecting 1,4-dioxane in aqueous solutions (Ma et al., 2016; Zhou et al., 2015). Full scale or field applications using luminescent assays have not been reported so far.
2.5 Summary

1,4-Dioxane is a contaminant of emerging concern that has been found widespread in groundwater, surface water, and drinking water systems. Many states are implementing lower regulatory advisory levels based on the toxicity profile of 1,4-dioxane and the potential public health risks. However, the unique chemical properties of 1,4-dioxane, such as high water solubility, low Henry’s law constant, and importantly, the co-occurrence with chlorinated solvents and other contaminants, increase the challenges to efficiently cleanup 1,4-dioxane. This chapter summarizes currently available chemical and physical 1,4-dioxane treatment technologies and focuses on recent advances in bioremediation and monitoring tools. It is important to provide useful references to change the industrial and regulatory perception of 1,4-dioxane biodegradability, to understand treatment mechanisms especially in contaminant mixtures, and to direct research for meeting practical needs.
2.6 References


CH2M, 2016. Final supplemental remedial investigation/feasibility study work plan for 1,4-dioxane and perfluorinated compounds at Ashumet Valley, Joint Base Cape Cod, MA, Prepared for AFCEC/JBCC Installation Restoration Program: Otis ANGB, MA. CH2M, Otis ANGB, MA.


Eberle, D., Ball, R., Boving, T.B., 2016. Peroxone activated persulfate treatment of 1,4-dioxane in the presence of chlorinated solvent co-contaminants. Chemosphere 144, 728-735.


Pickett, L.W., Hoeflich, N.J., Liu, T.-C., 1951. The vacuum ultraviolet absorption spectra of cyclic compounds. II. tetrahydrofuran, tetrahydropyran, 1,4-dioxane and furan1. J. Am. Chem. Soc. 73, 4865-4869.


Sei, K., Miyagaki, K., Kakinoki, T., Fukugasako, K., Inoue, D., Ike, M., 2013. Isolation and characterization of bacterial strains that have high ability to degrade 1, 4-dioxane as a sole carbon and energy source. Biodegradation 24, 665-674.


Sock, S.M., 1993. A comprehensive evaluation of biodegradation as a treatment alternative for the removal of 1,4–dioxane. Clemson University, Clemson, SC.


Sorensen, H., 2013. 1,4-Dioxane and the application of phytoremediation at North Carolina hazardous waste groundwater contaminated sites. North Carolina State University, Raleigh, North Carolina.


USEPA, 2013. Integrated Risk Information System (IRIS) on 1,4-Dioxane. National Center for Environmental Assessment, Office of Research and Development, Washington DC.


Zhang, S., Gedalanga, P.B., Guo, S., Mahendra, S., 2016a. Bioprocesses for simultaneously removing hexavalent chromium and 1,4-dioxane. 251st ACS National Meeting Presentation.


Chapter 3: Biodegradation Kinetics of 1,4-Dioxane in Chlorinated Solvent Mixtures
3.1 Introduction

Successful in-situ bioremediation of contaminated aquifers is heavily influenced by site-specific conditions, such as microbial communities, dissolved oxygen, groundwater chemistry, and co-occurring contaminants (Adamson et al., 2015; Adamson et al., 2016; Stroo et al., 2010). These issues are increasingly important for emerging water contaminants, such as 1,4-dioxane, a probable human carcinogen (IARC, 1999). While many laboratory-based studies focus on individual contaminants to determine the feasibility of a biodegradation approach, the complexity of contaminant mixtures and biogeochemical conditions might result in significantly different outcomes (Adamson et al., 2015; Hand et al., 2015; Mahendra et al., 2013; Pornwongthong et al., 2014). Groundwater contamination with 1,4-dioxane is a widespread problem, and affected aquifers are typically impacted with mixtures of multiple chlorinated solvents (Chiang et al., 2012; Mohr, 2010; Zenker et al., 2003). Although 1,4-dioxane was primarily used as a stabilizer of 1,1,1-trichloroethane (TCA), many facilities alternated between trichloroethylene (TCE) and TCA in response to a perceived lower toxicity for TCA or availability and pricing (Mohr, 2010). A recent study determined that co-occurrence of 1,4-dioxane and TCE accounted for 64% of 1,4-dioxane detected in monitoring wells (Anderson et al., 2012), further suggesting that mixed contamination is a potential challenge for 1,4-dioxane remediation. Importantly, the incomplete biological and abiotic degradation of these co-contaminating compounds can result in chlorinated solvent mixtures of TCE, 1,1-dichloroethene (1,1-DCE), cis-1,2-dichloroethene (cDCE), and vinyl chloride at contaminated sites (Brown et al., 2009).

Chlorinated organic solvents are known to inhibit bacterial growth rates (Koenig et al., 2014; Rajagopal, 1996; Segura et al., 2012), reduce membrane permeability (Segura et al., 2012),
and damage nucleic acids (Segura et al., 2012). In studies using pure and mixed methanotrophic cultures, TCE exposure slowed both metabolic and cometabolic biotransformation rates of methane and dichloroethenes, respectively (Alvarez-Cohen and McCarty, 1991; Alvarez-Cohen and Speitel, 2001). Chlorinated solvents have been reported to inactivate specific enzymes (Ely et al., 1995), such as cytochrome P450 related monoxygenases (Oldenhuis et al., 1989). For example, TCE was reported to decrease toluene-2-monoxygenase activities in Burkholderia cepacia G4 (Newman and Wackett, 1997) and inactivated the oxygenase in a methanotrophic culture (Alvarez-Cohen and McCarty, 1991). These findings indicate chlorinated solvents may significantly impact the monoxygenase-driven degradation of 1,4-dioxane by aerobic bacteria. Several studies with Gram-positive bacteria have shown that a large number of stress response proteins are produced under conditions that compromise the cell membrane (Petersohn et al., 2001), such as solvent exposure. The uspA gene encodes a universal stress protein, which is induced during stationary phase growth, starvation, and during exposure to a wide range of stimuli including heat, oxidants, metals, and antibiotics (Kvint et al., 2003).

Pseudonocardia dioxanivorans CB1190 (hereafter CB1190) is a model 1,4-dioxane-degrading bacterium that has previously been shown to grow on a wide range of carbon sources and rapidly degrade 1,4-dioxane at rates ranging from 1.1 to 19.8 mg/min/mg-protein (Mahendra and Alvarez-Cohen, 2005; Parales et al., 1994) (Table 3.1). It was determined, using multiple lines of evidence, that a monooxygenase enzyme (DXMO) was responsible for catalyzing initial biodegradation of 1,4-dioxane, while aldehyde dehydrogenase (ALDH) degraded intermediates in the biodegradation pathway (Mahendra and Alvarez-Cohen, 2006; Mahendra et al., 2007). Whole genome sequence of CB1190 (Sales et al., 2011) revealed genes encoding 1,4-dioxane monooxygenase (dxmADBC) and aldehyde dehydrogenase (aldH), some of which have been
used as biomarkers to predict the biotransformation potential in samples collected from contaminated sites (Gedalanga et al., 2014; Li et al., 2014a; Li et al., 2013; Li et al., 2014b).

Table 3.1. Growth and 1,4-dioxane biodegradation kinetics parameters for *Pseudonocardia dioxanivorans* CB1190.

<table>
<thead>
<tr>
<th>Strains</th>
<th>$q^d$ (mg-dioxane/hr/mg-protein)</th>
<th>$K_s$ (mg/L)</th>
<th>yield (mg-protein/mg-dioxane)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1190$^a$</td>
<td>$1.1 \pm 0.008$ $^a$</td>
<td>$160 \pm 44$</td>
<td>$0.09 \pm 0.002$</td>
<td>Mahendra and Alvarez-Cohen, 2006</td>
</tr>
<tr>
<td>CB1190$^b$</td>
<td>$19.8$ $^b$</td>
<td>-</td>
<td>$0.02$</td>
<td>Parales et al., 1994</td>
</tr>
<tr>
<td>CB1190$^c$</td>
<td>$0.038 \pm 0.012$ $^c$</td>
<td>-</td>
<td>-</td>
<td>Kelley et al., 2001</td>
</tr>
</tbody>
</table>

$^a$Initial 1,4-dioxane = 1000 mg/L  
$^b$Initial 1,4-dioxane = 484 mg/L  
$^c$Initial 1,4-dioxane = 30 mg/L  
$^d$Maximum substrate degradation rate

The objectives of this study were to provide a quantitative analysis of 1,4-dioxane biodegradation kinetics in mixtures with relevant chlorinated solvents (TCE, TCA) and their degradation products (1,1-DCE and cDCE). We also determined the relationship between solvent exposure, cellular energy, and expression of genes coding for stress response and key reactions in the biochemical pathway to better understand mechanisms influencing 1,4-dioxane biodegradation kinetics in mixed contaminant environments.

3.2 Materials and Methods

3.2.1 Chemicals

1,4-Dioxane (99.8%, ACS grade), 1,1-DCE ($\geq$ 99.9%), cDCE (97%), and TCE ($\geq$ 99.5%, ACS grade) were purchased from Sigma-Aldrich. 1,1,1-TCA (> 95%) was obtained from Ultra
Scientific (North Kingstown, RI). Saturated chlorinated solvent stock solutions were prepared as previously described (Alvarez-Cohen and McCarty, 1991).

3.2.2 Bacterial strain and culture conditions

CB1190 was harvested with a 1% (vol/vol) transfer from an actively growing pure culture and incubated at 30°C with 150 rpm agitation to maintain aerobic conditions in the ammonium mineral salts (AMS) medium (Parales et al., 1994) amended with 1,4-dioxane. Cultures (late exponential or early stationary phase) were used to inoculate the experimental bottles when 1,4-dioxane in the stock fell below the detection limit (< 1.0 mg/L).

3.2.3 Experimental approach

3.2.3.1 Biodegradation of 1,4-dioxane in the presence of individual chlorinated solvents

To examine the effects of each chlorinated solvent on 1,4-dioxane biodegradation, each 120 mL serum bottle contained 30 mL active CB1190 culture with 0.5 to 100 mg/L 1,4-dioxane and 1,1-DCE, cDCE, TCE, or TCA at final concentrations of 0.5 mg/L, 5 mg/L, or 50 mg/L. 1,4-Dioxane activity (Equation 3.1; Eq. 3.1) and biodegradation rates (Equation 3.2; Eq. 3.2) were calculated as follows:

Observed activity \( \left( \text{mg} \text{L}^{-1} \text{hr}^{-1} \right) = \frac{C_{\text{Initial}} - C_{\text{Final}}}{\Delta t} \) \hspace{1cm} (Eq.3.1)

Biodegradation Rate \( \left( \text{mg dioxane hr}^{-1} \text{mg}^{-1} \text{protein} \right) = \frac{C_{\text{Initial}} - C_{\text{Final}}}{X_{\text{protein}} \times \Delta t} \) \hspace{1cm} (Eq.3.2)

Where, \( C_{\text{Initial}} \) and \( C_{\text{Final}} \) were the initial and final concentrations of 1,4-dioxane (mg/L), respectively. \( X \) was the biomass measured as total protein (mg/L), and \( \Delta t \) was time (h). In solvent free controls, 1,4-dioxane biodegradation followed first order kinetics in the first 8 hours, thus we use \( \Delta t=8 \) for calculations.
The maximum specific rate of 1,4-dioxane degradation \( (V_{\text{max}}) \), 1,4-dioxane half saturation coefficient \( (K_s) \), and inhibition constants \( (K_I) \) were determined by fitting the equation

\[
v_0 = \frac{V_{\text{max}} \cdot C_{\text{initial}}}{K_s + C_{\text{initial}}}
\]

to match the observed non-volatile 1,4-dioxane concentrations using the Prism version 6.0 (Graphpad Software, Inc., San Diego, CA) as previously described (Mahendra et al., 2013).

3.2.3.2 Microcosm study

A total of 8 L of groundwater was collected from a monitoring well and 2.26 kg soil was collected by direct push sampling near the monitoring well. The well was located in the source zone of a contaminated groundwater plume containing > 700 \( \mu \text{g}/\text{L} \) 1,4-dioxane and chlorinated solvents ranging from 0 - 1000 \( \mu \text{g}/\text{L} \). Microcosms were prepared in sterile 250 mL boston round bottles equipped with mininert valves (Restek Corporation, Bellefonte, PA) and designed according to the following conditions: (1) Untreated Control (Natural attenuation; (+) Solvents; (2) Heat Sterilized Control; (3) Natural attenuation after flushing to remove solvents (Natural attenuation; (-) Solvents); (4) Bioaugmention with CB1190 (Bioaugmented CB1190; (+) Solvents); (5) Bioaugmention with CB1190 without solvents (Bioaugmented CB1190; (-) Solvents). For the (+) and (-) solvents conditions 1 – 3, 50 mL of groundwater were transferred into each bottle. Bioaugmented conditions (4 and 5) received 25 mL of groundwater from each location, 20 mL of ammonium mineral salts (AMS) medium, and 5 mL of CB1190 cultured in AMS medium to a final cell concentration of approximately \( 1.0 \times 10^4 \) cells/mL. Addition of AMS was performed to provide the necessary nutrients for CB1190 as described in a previous microcosm study (Li et al., 2010). All microcosms received 10 g of soil and all (-) solvent conditions were flushed with \( \text{N}_2 \) gas to remove volatile solvents from solution by cycling gas for 15 min followed by 15 min of rest for a total of 5 cycles.
Heat-sterilized controls were prepared by autoclaving microcosm bottles for 30 minutes at 121°C and 15 psi. All microcosms were prepared in triplicates with the exception of a single sterile control for each location. Microcosms were incubated at 30°C with 150 rpm agitation. At each time point, aliquots of 200 µL were collected for 1,4-dioxane quantification and 300 µL samples of the soil and groundwater slurry from each microcosm were collected and stored at -80°C for the extraction of total nucleic acids.

3.2.3.3 Dissolved oxygen (DO), oxidation-reduction potential (ORP), and pH Measurements

DO, ORP, and pH values were measured using an Orion 5-Star Plus multi-parameter meter (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions in samples collected at the beginning and end of microcosm incubations (Table 3.2).

Table 3.2. Geochemical parameters measured in the groundwater used to prepare microcosms. The values changed <5% during microcosm preparation and incubation.

<table>
<thead>
<tr>
<th>Microcosm treatments</th>
<th>DO (mg L⁻¹)</th>
<th>pH</th>
<th>ORP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile control</td>
<td>6.7</td>
<td>8.2</td>
<td>290</td>
</tr>
<tr>
<td>Untreated (+/-) chlorinated solvents</td>
<td>7.3</td>
<td>7.0</td>
<td>410</td>
</tr>
<tr>
<td>CB1190 Bioaugmentation (+) chlorinated solvents</td>
<td>7.2</td>
<td>7.5</td>
<td>360</td>
</tr>
<tr>
<td>CB1190 Bioaugmentation (-) chlorinated solvents</td>
<td>7.1</td>
<td>7.1</td>
<td>390</td>
</tr>
</tbody>
</table>

3.2.3.4 Biodegradation of 1,4-dioxane in solvent mixtures

To rank the relative importance of inhibitory solvents on 1,4-dioxane biodegradation in environmental mixtures, the impact of solvent mixtures in CB1190 culture was evaluated using similar concentrations of chlorinated solvents as determined in the microcosm study described above (Table 3.3). The following chlorinated solvent mixtures were studied for their combined effects on the biodegradation of 1 mg/L 1,4-dioxane. Mixture 1 contained 5000 µg/L 1,1-DCE, 500 µg/L cDCE, 700 µg/L TCE and 120 µg/L TCA, while mixture 2 had 500 µg/L cDCE, 700 µg/L TCE and 120 µg/L TCA, and mixture 3 contained only 700 µg/L TCE and 120 µg/L TCA.
This approach was used to determine chlorinated solvent(s) with the greatest impact on 1,4-dioxane metabolism. The aqueous concentrations of chlorinated volatile organic compounds was calculated based on Henry’s Law constants (Chen et al., 2012). At every time point (0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 hours), 200 µL aqueous samples were collected for 1,4-dioxane analysis by using a 1 mL syringe equipped with a sterile needle (23 gauge, 0.64 mm o.d., 1.5 in. length) and stored at -20°C; 500 µL samples were collected and stored at -80°C for total nucleic acids extraction. Chlorinated solvent concentrations were quantified from 100 µL headspace samples collected from each bottle at 0, 1, 7, and 10 days using a gas chromatograph equipped with a flame ionization detector (GC-FID) or an electron capture detector (GC-ECD).
Table 3.3. Quantification of chlorinated solvents in synthetic mixtures at 0 and 24 hours. Each mixture also contained 1mg/L 1,4-dioxane.

<table>
<thead>
<tr>
<th>Groundwater aqueous concentration* (Total concentration in the experimental bottle)</th>
<th>TCE (mg/L)</th>
<th>TCA (mg/L)</th>
<th>cDCE (mg/L)</th>
<th>1,1-DCE (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abiotic Live (0.72)</td>
<td>0.017 ± 0.011</td>
<td>0.10 ± 0.05</td>
<td>0.14 ± 0.03</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Live (0.45)</td>
<td>0.012 ± 0.010</td>
<td>0.84 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Mixture 1 0 hour</td>
<td>0.91 ± 0.11</td>
<td>0.87 ± 0.07</td>
<td>0.13 ± 0.08</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>24 hour</td>
<td>0.96 ± 0.10</td>
<td>0.86 ± 0.06</td>
<td>0.14 ± 0.02</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Mixture 2 0 hour</td>
<td>0.10 ± 0.05</td>
<td>0.81 ± 0.10</td>
<td>0.12 ± 0.00</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>24 hour</td>
<td>0.92 ± 0.15</td>
<td>0.81 ± 0.10</td>
<td>0.14 ± 0.02</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Mixture 3 0 hour</td>
<td>1.11 ± 0.01</td>
<td>1.10 ± 0.05</td>
<td>0.14 ± 0.01</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>24 hour</td>
<td>1.00 ± 0.13</td>
<td>0.84 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>
*The chlorinated solvent concentrations were the actual measured concentrations in the groundwater. In synthetic mixtures, higher total concentrations of chlorinated solvents were added to ensure final aqueous concentrations similar to those reported in the contaminated groundwater.

Log $K_{ow}$ (TCE) = 2.61; Log $K_{ow}$ (TCA) = 2.49; Log $K_{ow}$ (1,1-DCE) = 2.31; Log $K_{ow}$ (cDCE) = 1.86.

References:
ATSDR, Toxicology profile for 1,1-dichloroethene. 1994.
ATSDR, Toxicology profile for 1,2-dichloroethene. 1996.
ATSDR, Toxicological profile for 1,1,1-trichloroethane. 2006.
ATSDR, Toxicology profile for trichloroethylene. 2015.

### 3.2.4 Analytical Methods

1,4-Dioxane (1-2000 µg/L) was monitored using an Agilent 6890 chromatograph equipped with a 5973 mass spectrometer (GC-MS) and a Supelco SPB-1 Sulfur column (30 m × 0.32 mm id × 4 µm). The collected aqueous sample was prepared for GC-MS analysis using a frozen microextraction procedure as previously described (Li et al., 2011) and 5 µL of processed sample was injected into the GC-MS equipped with a pulsed split-less injection with an inlet temperature of 150°C, 77 kPa and a pulsed pressure of 170 kPa for 2 min, and purge flow of 15.0 mL/min. The oven temperature was initially held constant at 35°C for 5 minutes, then increased to 100°C at a rate of 20°C/min, and further increased to 275 °C at a rate of 50°C/min. The Mass Selective Detector (MSD) was operated to use an electron multiplier (EM) offset of 400 and EM voltage of 2000. The MS quadrupole was programmed for 150°C and source for 230°C.

1,4-Dioxane concentrations in the range of 0.8-100 mg/L were measured, in 2 µL aqueous samples filtered by sterile 0.2-µm syringe filters, using an Agilent 6890 GC, equipped with a Restek® Stabilwax-DB capillary column (30 m ×0.53 mm ×1 µm) and an FID. The injector and detector were set at 220 °C in splitless mode and 250°C, respectively. The oven temperature was maintained at 80 °C for 3 min then increased at 20 °C/min to 140 °C, where it was held constant for an additional 1 min.
TCE, TCA, 1,1-DCE, and cDCE were quantified by GC-FID equipped with a Restek® Stabilwax-DB capillary column (30 m × 0.53 mm × 1 µm) for experiments investigating the influence of individual chlorinated solvents. The injector and detector temperatures were set at 220°C with 7.4 mL/min of flow rate and 250°C, respectively. The oven was initially set at 100 °C for 1 min then increased at 25 °C/min to 150 °C and maintained for 30 seconds.

In experiments with solvent mixtures, a Varian 3500 capillary gas chromatograph equipped with an ECD was used to quantify 100-5000 µg/L TCE, TCA, and 1,1-DCE, with an Agilent DB-5 capillary column (30 m × 0.25 mm × 0.25 µm). Headspace samples of 50 µL were injected into the injector set at 260°C, whereas the detector was set at 310°C. The oven temperature was 35 °C and held for 30 min with 9.6 mL/min of column flow rate. Concentrations of cDCE from 500-5000 µg/L were measured by GC-FID with constant oven temperature at 130 °C for 7 min. The flow rate was 1.7 mL/min and other parameters were the same as those described for individual solvents.

3.2.5 ATP Assay for Energy Generation

ATP production was measured in 100 µL of cell culture using the BacTiler Glo™ assay (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 50 µL of sample was transferred to a 96-well plate and mixed with 50 µL BacTiter Glo™ reagent. Samples were incubated in the dark for 5 minutes at room temperature and luminescence measurements were recorded using the VICTOR 3V plate reader (PerkinElmer, Waltham, MA).

3.2.6 Total nucleic acids extraction and cDNA synthesis

Total nucleic acids were extracted from samples using a modified phenol-chloroform extraction method as described previously (Gedalanga et al., 2014). Briefly, 500 µL cell cultures were centrifuged at 13,000 × g for 3 min and the supernatant was discarded. After adding 250 µL
of lysis buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.1]), 100µl 10% sodium dodecyl sulfate, 1.0 ml pH 8.0 buffer-equilibrated phenol, and 1 g 100 µm-diameter zirconia-silica beads (Biospec Products, Bartlesville, OK), the cells were lysed by heating at 65°C for 2 min, bead beating for 2 min with a Mini-Beadbeater 16 (Biospec Products, Bartlesville, OK), incubating for 8 min at 65°C, and bead beating again for 2 min. The lysate was collected by centrifugation at 13,000 × g for 5 min followed by phenol/chloroform/isoamyl alcohol purification and chloroform/isoamyl alcohol purification. Precipitation of total nucleic acids was performed by addition of 0.1 volume 3 M sodium acetate and 1 volume isopropanol followed by incubation at -20 °C overnight. Nucleic acid pellets were collected by centrifugation at 4°C for 30 min at 20,000 × g. The precipitate was washed with 70% ethanol, and resuspended in 100 µL DNase- and RNase-free water. The concentrations of DNA and RNA were determined by a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE). For gene expression analyses, RNA was isolated from total nucleic acid extracts using a RapidOUT DNase Kit (Thermo Scientific, Waltham, MA). The cDNA was synthesized from purified total RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). All samples were stored at -80°C until further amplification and analyses.

### 3.2.7 Quantitative Polymerase Chain Reaction (qPCR)

To study the influence of chlorinated solvents on gene expression in pure cultures, 1,4-dioxane biomarker targets (dxmB and aldH) were selected because of the significance of monooxygenase and dehydrogenase enzymes in the 1,4-dioxane biodegradation pathways as described previously (Gedalanga et al., 2014). Universal stress gene (uspA) was also selected to investigate the response of CB1190 cells exposed to chlorinated solvents (Nachin et al., 2005). Gene expression was quantified using the 2-ΔΔCT method as described by Livak and Schmittgen.
Gene expression data were first normalized to the housekeeping gene, RNA polymerase sigma subunit D (\textit{rpoD}), followed by normalization to the values obtained at time 0. All reactions were run on a StepOnePlus thermocycler (Life Technologies, Carlsbad, CA) using a total volume of 20 \textmu L containing: 1\times Luminaris Color HiGreen/HiROX qPCR Master Mix (Thermo Scientific, Waltham, MA), 0.3 mM primers, and 2 \textmu L DNA (1-10 ng/\textmu L) template. Primer sequences are listed in Table 3.4 and additional details following MIQE reporting requirements can be found in Table 3.5. The cycling parameters to amplify the gene fragment included sample holds at 50\degree C for 2 min, 95\degree C for 10 min, followed by 40 cycles of 95\degree C for 15 s and 60\degree C for 45 s. All reactions were accompanied by a melt curve analysis to confirm the specificity of qPCR products. Melt curve analyses within 78.1-80.5\degree C (\textit{rpoD}), 81.5-83.6\degree C (\textit{dxmB}, \textit{aldH}, and 16S rRNA), and 86.9-88.7\degree C (\textit{uspA}) were considered specific to target gene.

Table 3.4. Sequences of primers used for amplification of genes by qPCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>CB1190 16S rRNA for</td>
<td>5’-ACGGTCTCGCAGCCCTCTGT-3’</td>
<td>This study</td>
</tr>
<tr>
<td>CB1190 16S rRNA rev</td>
<td>5’-ACCGGGTTATGCGCCGGGACT-3’</td>
<td></td>
</tr>
<tr>
<td>\textit{uspA} for</td>
<td>5’-GACGGCAGGATACCAGCAG-3’</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{uspA} rev</td>
<td>5’-ATGGGAAATGACCACGCTCG-3’</td>
<td></td>
</tr>
<tr>
<td>\textit{rpoD} for</td>
<td>5’-GGGCAGAAGGAATGGTC-3’</td>
<td>Sales \textit{et al.}, 2013</td>
</tr>
<tr>
<td>\textit{rpoD} rev</td>
<td>5’-CAGGGTGCCGTTAGGGAAGAA-3’</td>
<td></td>
</tr>
<tr>
<td>\textit{dxmB} for</td>
<td>5’-CCAAACGGGCCGTCAGTCAT-3’</td>
<td>Gedalanga \textit{et al.}, 2014</td>
</tr>
<tr>
<td>\textit{dxmB} rev</td>
<td>5’-AGAACGTGCGCTCCAAAG-3’</td>
<td></td>
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<tr>
<td>\textit{aldH} for</td>
<td>5’-GCCGACGCCTTTTAGCAGATG-3’</td>
<td>Gedalanga \textit{et al.}, 2014</td>
</tr>
<tr>
<td>\textit{aldH} rev</td>
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<td></td>
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<td>Universal 16S for</td>
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<tr>
<td>Universal 16S rev</td>
<td>5’-ACGGGCCGTTGTAC-3’</td>
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Table 3.5. Minimum information for publication of real-time PCR experiments (MIQE Guidelines).

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Experimental Group</td>
<td>Solvent exposed</td>
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<tr>
<td>Control Group</td>
<td>Solvent-free; Time 0 samples</td>
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<table>
<thead>
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<td>Description</td>
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</tr>
<tr>
<td>Microdissection or Macrodissection</td>
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</tr>
<tr>
<td>Processing procedure</td>
<td>Culture conditions described in methods</td>
</tr>
<tr>
<td>If frozen, how and how quickly</td>
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</tr>
<tr>
<td>Sample storage conditions and duration</td>
<td>-80°C until ready for extraction</td>
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<table>
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<tr>
<th>Nucleic Acid Extraction</th>
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<td>Procedure and/or instrumentation</td>
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<td>Kit and details of any modifications</td>
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<tr>
<td>Details of DNase or RNase treatment</td>
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<tr>
<td>Contamination assessment (DNA or RNA)</td>
<td>Evaluated no enzyme control (NEC)</td>
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<td>Nucleic acid quantification</td>
<td>Performed using spectrophotometer</td>
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<td>Instrument and method</td>
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<td>RNA integrity: method/instrument</td>
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<td>RIN/RQI or Cq of 3' and 5' transcripts</td>
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<td>Inhibition testing</td>
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<table>
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<tr>
<td>Amount of RNA and reaction volume</td>
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<tr>
<td>Primers and concentration</td>
<td>Described/referenced in methods</td>
</tr>
<tr>
<td>Reverse transcriptase and concentration</td>
<td>Described/referenced in methods</td>
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<td>Temperature and time</td>
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<table>
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<tr>
<td>Sequence accession number</td>
<td>Described/referenced in methods</td>
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<tr>
<td>Amplicon length</td>
<td>rpoD = 64 bp; uspA = 199 bp; dxbB = 100 bp; aldH = 120 bp; CB1190 16S = 139 bp</td>
</tr>
<tr>
<td>In silico specificity screen</td>
<td>BLAST confirms specificity</td>
</tr>
<tr>
<td>Location of each primer by exon or intron</td>
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<tr>
<td>What splice variants are targeted</td>
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<th>qPCR oligonucleotides</th>
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<tr>
<td>Primer sequences</td>
<td>Reported in SI and referenced</td>
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<tr>
<td>Location and identity of any modifications</td>
<td>No modifications</td>
</tr>
</tbody>
</table>

| qPCR protocol |  |
Complete reaction conditions Described/referenced in methods
Reaction volume and amount of cDNA/DNA Described/referenced in methods
Primer, probe, Mg2+, dNTP Described/referenced in methods
Polymerase identity and concentration Described/referenced in methods
Buffer/Kit identity and manufacturer Described/referenced in methods
Additives (Sybr, etc.) Described/referenced in methods
Complete thermocycling parameters Described/referenced in methods
Manufacturer of qPCR instrument Described/referenced in methods

qPCR validation
Specificity (gel, sequence, melt, digest) Described/referenced in methods

For SYBR, Cq of NTC rpoD = 33; uspA = N/A; dxmB = N/A; ALDH = N/A; CB1190 16S = N/A;

Calibration curves with slope and y-intercept y = -3.44x + 36
PCR efficiency 95.3%
r2 of calibration curve r2 = 0.99
linear dynamic range 9.0 × 10^1 through 9.0 × 10^6
Cq variation at LOD 0.53
Evidence for LOD p > 0.05

Data analysis
qPCR analysis program StepOne Software ver. 2.3
Method of Cq determination Automatic
Outlier identification and disposition N/A
Results for NTCs Reported above
Justification of number and choice of reference genes Single reference gene (rpoD) based on literature source (Grosten et al. 2012)
Description of normalization method ddCT (Livak and Schmittgen 2001)
Number and stage of technical replicates 3
Repeatability (intraassay variation)
Statistical methods for results significance Student's T-Test; ANOVA
Software Graphpad Prism v6

3.2.8 Quantification of cells with intact membranes

Propidium monoazide (PMA) or ethidium monoazide (EMA) treatment is commonly used to quantify viable cells using qPCR (Gedalanga and Olson, 2009; Kralik et al., 2010; Rudi
et al., 2005). These methods rely on the ability of PMA and EMA to permeate membrane compromised cells and covalently bind to DNA preventing quantification by qPCR. This study used PMA to investigate if chlorinated solvents damaged the cell membrane of CB1190. Pure cultures were exposed to 5 mg/L of individual CVOCs for 24 hours. Solvent-free and heat-killed controls were included to determine whether solvent exposure increased the concentration of membrane-compromised cells. A volume of 0.3 mL was collected for each condition in triplicate and transferred to a clear 96-well plate, where PMA treatment was performed according to the manufacturer’s instructions (Biotium Inc, Hayward, CA). Briefly, PMA was added to samples at final concentration of 50 µM followed by incubation in the dark for 5 min at room temperature. Photoactivation was performed on ice using a 700-W halogen lamp at a distance of 20 cm for 15 min. Samples were also processed without PMA to quantify populations with and without intact cell membranes. CB1190 with intact cell membranes was calculated as a percent using equation 3 (Eq. 3.3):

\[
\text{Percent Intact Cell Membrane} (%) = \left( \frac{X_{\text{PMA} (+)}}{X_{\text{PMA} (-)}} \right) \times 100 \quad (\text{Eq. 3.3})
\]

Where, \(X_{\text{PMA} (+)}\) is the concentration of CB1190 with intact cell membranes (cells/mL), \(X_{\text{PMA} (-)}\) is the concentration of CB1190 with and without intact cell membranes. Cell concentrations were determined using qPCR.

**3.2.9 PMA-qPCR**

DNA was extracted and cells were quantified by qPCR as described in the Methods section with slight modifications. Primers targeting the 16S rRNA of CB1190 (Table 3.4) were used in the qPCR assay. The cycling parameters to amplify the gene fragment included sample holds at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45
s. All reactions were accompanied by a melt curve analysis to confirm the specificity of qPCR products. The standard curve for gene copy number quantification was generated from genomic DNA extracted from pure cultures of CB1190. Additional information to adhere to MIQE guidelines are presented in Table 3.5.

3.3 Results

3.3.1 Kinetics of 1,4-dioxane biodegradation by CB1190 exposed to chlorinated solvents

TCE, TCA, 1,1-DCE, and cDCE were individually examined for their potential inhibitory effects on 1,4-dioxane biodegradation (Figure 3.1). In solvent free controls, CB1190 (32.1 ± 4.2 mg-protein) biodegraded 1 mg/L of 1,4-dioxane to below detection limits in 8 hours at a rate (Mean ± SD) of 100.3 ± 1.0 µg/L/hr, which was as similar rates observed in the presence of 0.5 mg/L TCE and 0.5 mg/L cDCE, 95.4 ± 1.4 µg/L/hr, (p=0.26) and 93.9 ± 8.2 µg/L/hr (p=0.23, respectively. Comparatively, TCA at concentrations of 0.5 mg/L (p=0.37), 5 mg L⁻¹ (p=0.34) and 50 mg/L (p=0.41) had little effect on 1,4-dioxane biodegradation by CB1190 (37.8 mg protein). At 50 mg/L TCE, 1,4-dioxane was inhibited with the biodegradation rate of 35.3 ± 8.5 µg/L/hr. cDCE had a stronger inhibitory effect at 5 mg/L and completely inhibited 1,4-dioxane biodegradation at 50 mg/L. In 1,1-DCE exposed cultures, 1,4-dioxane was degraded in the presence of low 1,1-DCE concentrations at the rate of 79.4 ± 3.7 µg/L/hr in the first 12 hours, but 1,4-dioxane degradation ceased in the following 12 hours. Thus, 1,1-DCE was the strongest inhibitor of 1,4-dioxane biodegradation among tested chlorinated solvents. The inhibition constants (Kᵢ, mg/L) for 1,1-DCE, cDCE, and TCE were determined as 1.51 ± 0.26, 26.63 ± 6.63, and 8.60 ± 1.74 by the Michaelis-Menten model, respectively (Table 3.6).
most appropriate model was selected based on fit (R²; coefficient of determination) and the Akaike information criterion (AIC). AIC uses estimates of goodness-of-fit and model variability to provide a quantitative ranking for different models in their abilities to describe each data set. This approach has been used in several studies to identify best-fit models. (Buckman et al., 2011; Saffron et al., 2006) According to R² values and Akaike’s information criterion, (Kolhatkar and Polli, 2010) the uncompetitive model was the best fit for 1,1-DCE and TCE while the noncompetitive model was the most suitable for cDCE. As observed earlier, the presence of TCA had little impact on 1,4-dioxane biodegradation, so the K₄ could not be determined.
Figure 3.1. Inhibition of 1,4-dioxane biodegradation by chlorinated solvents. *P. dioxanivorans* CB1190 was exposed to final concentrations of 1 mg/L 1,4-dioxane and 0.5, 5, or 50 mg/L A) TCA; B) TCE; C) cDCE; D) 1,1-DCE individually. Error bars represent standard deviation of triplicates.

Table 3.6. Kinetic parameters for 1,4-dioxane degradation in the presence of 1,1-DCE, cDCE, TCE, and TCA. $K_i$ is defined as an inverse measure of the affinity of the inhibitor for the target enzyme. (Mahendra et al., 2013)

<table>
<thead>
<tr>
<th>Chlorinated Solvents</th>
<th>Michaelis-Menten Model</th>
<th>$V_{max}$ (mg-dioxane/hr/mg-protein)</th>
<th>$K_s$ (mg/L)</th>
<th>$K_i$ (mg/L)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,1-DCE**</td>
<td>Competitive</td>
<td>0.079</td>
<td>8.78</td>
<td>0.26±0.10</td>
<td>0.921</td>
</tr>
<tr>
<td></td>
<td>Noncompetitive</td>
<td>0.082</td>
<td>10.28</td>
<td>2.05±0.35</td>
<td>0.977</td>
</tr>
<tr>
<td></td>
<td>Uncompetitive</td>
<td>0.085</td>
<td>12.17</td>
<td>1.51±0.26</td>
<td>0.978</td>
</tr>
<tr>
<td>cDCE**</td>
<td>Competitive</td>
<td>0.069</td>
<td>12.53</td>
<td>4.09±1.47</td>
<td>0.937</td>
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<tr>
<td></td>
<td>Noncompetitive</td>
<td>0.072</td>
<td>14.68</td>
<td>26.63±6.63</td>
<td>0.958</td>
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<tr>
<td></td>
<td>Uncompetitive</td>
<td>0.074</td>
<td>16.63</td>
<td>18.73±5.01</td>
<td>0.957</td>
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<tr>
<td>TCE**</td>
<td>Competitive</td>
<td>0.073</td>
<td>16.61</td>
<td>2.41±1.09</td>
<td>0.906</td>
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<tr>
<td></td>
<td>Noncompetitive</td>
<td>0.074</td>
<td>16.70</td>
<td>12.86±3.28</td>
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<tr>
<td></td>
<td>Uncompetitive</td>
<td>0.078</td>
<td>19.80</td>
<td>8.60±1.74</td>
<td>0.974</td>
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<tr>
<td>TCA**</td>
<td>Competitive</td>
<td>0.081</td>
<td>25.02</td>
<td>6.9×10^{14}*</td>
<td>0.976</td>
</tr>
<tr>
<td></td>
<td>Noncompetitive</td>
<td>0.081</td>
<td>25.02</td>
<td>1.6×10^{21}*</td>
<td>0.976</td>
</tr>
<tr>
<td></td>
<td>Uncompetitive</td>
<td>0.081</td>
<td>25.02</td>
<td>1.3×10^{17}*</td>
<td>0.976</td>
</tr>
</tbody>
</table>

* No inhibition.
** Model fitting figures, presented as below.
a,b,c We selected the model with highest $R^2$ as the best model and compared it with the second highest $R^2$ model by AICc values: 1,1-DCE (-0.927), cDCE (-1.399) and TCE (-9.447). Negative values of AICc indicate our selections are preferred.

Competitive Model:

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{1}{(C_{initial}) V_{max}} \left( K_s + \frac{(I)}{K_i} \right)$$

Eq. 3.4

Noncompetitive Model:

$$\frac{1}{v_0} = \frac{1}{V_{max}} \left( 1 + \frac{(I)}{K_i} \right) + \frac{1}{(C_{initial}) V_{max}} \left( 1 + \frac{(I)}{K_i} \right)$$

Eq. 3.5

Uncompetitive Model:

$$\frac{1}{v_0} = \frac{1}{V_{max}} \left( 1 + \frac{(I)}{K_i} \right) + \frac{1}{V_{max}} \left( 1 + \frac{(I)}{K_i} \right)$$

Eq. 3.6
\( I = \) concentration of chlorinated solvent

**1,1-DCE**

- \( r^2 = 0.9777 \)
- \( \alpha K_i = 1.508 \)
- \( V_{\text{max}} = 0.085 \)

**cDCE**

- \( r^2 = 0.9576 \)
- \( K_i = 26.63 \)
- \( V_{\text{max}} = 0.072 \)

**TCE**

- \( r^2 = 0.9739 \)
- \( \alpha K_i = 8.60 \)
- \( V_{\text{max}} = 0.078 \)
3.3.2 Chlorinated solvents hindered energy production in CB1190

The ability of CB1190 to grow using 1,4-dioxane as the sole energy and carbon source was previously reported (Mahendra and Alvarez-Cohen, 2005). ATP content was measured in actively growing CB1190 cultures exposed to cDCE and 1,1-DCE amended with 100 mg/L 1,4-dioxane and 0.5 mg/L 1,1-DCE or 5 mg/L cDCE. ATP generation correlated with 1,4-dioxane oxidation ($R^2 = 0.91; p < 0.01$) and the total ATP yields were similar in the solvent free control, 1,1-DCE, and cDCE exposed cultures (4.7-5.9 mol-ATP/mol-1,4-dioxane) (Figure 3.2). However, maximum ATP yield was delayed by 4 days in both solvent exposed conditions compared to the unexposed controls (Figure 3.2).
Figure 3.2. Effect of chlorinated solvents on energy production in CB1190. Actively growing *P. dioxanivorans* CB1190 culture (1% transfer of active seed culture) amended with 100 mg/L 1,4-dioxane (lines) was exposed to 0.5 mg/L 1,1-DCE (A) and 5 mg/L cDCE (B). The ATP production (bars) was delayed in the presence of both chlorinated solvents. 1,4-Dioxane (1-100 mg/L) was monitored using an Agilent 6890 GC-FID as previous study described. (Pornwongthong et al., 2014) Error bars represent ranges of duplicates.

### 3.3.3 Solvents inhibit biodegradation of 1,4-dioxane by CB1190 in environmental mixtures

In aerobic microcosms prepared using groundwater and soil from a site contaminated with 1,4-dioxane and chlorinated solvents, no 1,4-dioxane removal occurred in 10 weeks in
untreated bottles (Figure 3.3A). This suggested that the indigenous microorganisms had limited potential to biodegrade 1,4-dioxane. Even when volatile chlorinated solvents were removed by flushing, 1,4-dioxane biodegradation was not observed. However, bioaugmentation with CB1190 successfully removed 1,4-dioxane within 7 days in flushed bottles. The presence of chlorinated solvents slowed 1,4-dioxane biodegradation in CB1190 amended microcosms to a rate of 6.65 µg/L/day over 56 days, which confirmed the inhibitory effects of chlorinated solvents.

A)

![Graph A](image1)

B)
Figure 3.3. 1,4-Dioxane biodegradation in the presence of chlorinated solvent mixtures. A). Microcosm study: Untreated Control (Natural attenuation; (+) Solvents); Heat Sterilized Control; Natural attenuation after flushing to remove solvents (Natural attenuation; (-) Solvents); Bioaugmentation with CB1190 (Bioaugmented CB1190; (+) Solvents; Bioaugmentation with CB1190 without solvents (Bioaugmented CB1190; (-) Solvents). B). Pure culture study: Mixture 1 contained 5000 μg/L 1,1-DCE, 700 μg/L TCE, 450 μg/L cis-1,2-DCE and 120 μg/L TCA; Mixture 2 contained 700 μg/L TCE, 450 μg/L cis-1,2-DCE and 120 μg/L TCA; Mixture 3 contained 450 μg/L cis-1,2-DCE and 120 μg/L TCA. Error bars represent standard deviation of triplicates.

3.3.4 1,1-DCE was the major inhibitor of 1,4-dioxane biodegradation among individual chlorinated solvents and in chlorinated solvent mixtures

Synthetic mixtures of chlorinated solvents were designed to investigate the inhibitory effects caused by mixtures of chlorinated solvents in the environment and to help interpret the results from the microcosms. The concentrations of solvents (TCE, TCA, 1,1-DCE, and cDCE) and 1,4-dioxane were selected to be similar to concentrations found in groundwater at the site used for the microcosm study. 1,4-Dioxane biodegradation was completely inhibited in Mixture 1 containing approximately 5 mg/L 1,1-DCE, but was successful in Mixture 2 and Mixture 3 (Figure 3.3B). These results agreed with our results from individual chlorinated solvents
demonstrating that 5 mg/L 1,1-DCE was the strongest inhibitor of 1,4-dioxane by CB1190 (Figure 3.1).

A:

1,1-DCE

B:

cDCE

cDCE

TCE

TCE

TCA

TCA
Figure 3.4. 1,4-Dioxane degradation and expression of relevant genes in CB1190 exposed to individual chlorinated solvents. A) Kinetics of 1000 µg/L 1,4-dioxane biodegradation in the presence of 5mg/L individual solvent (1,1-DCE, cis-1,2-DCE, TCE and TCA). This figure was extracted from Figure 1 for comparison with gene regulation figure. B) Fold change in transcript copy numbers of dioxane monooxygenase (dxmB) and aldehyde monooxygenase (aldH) at 0, 2, 6 and 12 hours. CB1190 culture with 1000 µg/L 1,4-dioxane was exposed to 5 mg/L of solvents (1,1-DCE, cis-1,2-DCE, TCE, or TCA). The circles represent 1,4-dioxane concentrations.
Figure 3.5. Dose-dependent gene regulation in CB1190 pure culture exposed to chlorinated solvents. CB1190 culture with 1000 µg L\(^{-1}\) 1,4-dioxane was independently exposed to 0.5, 5, or 50 mg/L 1,1-DCE, cis-1,2-DCE, TCE, and TCA. Expression of \(dxmB\) and \(aldH\) was down-regulated (relative to initial levels) with increasing concentrations of 1,1-DCE and cDCE, and to a lesser extent in the presence of TCE and TCA, respectively.
3.3.5 *Dioxane monooxygenase (dxbB)* and *aldehyde dehydrogenase (aldH)* genes were downregulated in the presence of chlorinated solvents in pure cultures.

Figure 3.6. CB1190 population in the presence of 1 mg/L 1,4-dioxane and individual chlorinated solvents. As the cell yield of CB1190 growing on 1,4-dioxane is low, 0.09 mg-protein/mg-1,4-dioxane (Mahendra & Alvarez-Cohen, 2005), the change in gene abundance (16S rRNA) was negligible throughout the duration of the experiments (24 hours). Error bars represent standard deviations of triplicates.
Figure 3.7. CB1190 population in the presence of 1 mg/L 1,4-dioxane and chlorinated solvent mixtures. As the cell yield of CB1190 growing on 1,4-dioxane is low, 0.09 mg-protein/mg-1,4-dioxane (Mahendra & Alvarez-Cohen, 2005), the change in gene abundance (16S rRNA) was negligible throughout the duration of the experiments (24 hours). Error bars represent standard deviations of triplicates.

Gene abundance (Figure 3.7 and 3.7) and expression were quantified in studies of individual chlorinated solvents as well as solvent mixtures. CB1190 exposed to 5 mg/L TCE or cDCE showed up-regulation of dxmB compared to the initial levels in the first 2 hours, during which 1,4-dioxane was being degraded at a high rate (Figure 3.4). When the cells were exposed to 50 mg/L TCE, insignificant 1,4-dioxane degradation occurred in the first 12 hours, but dxmB remained induced by 1,4-dioxane (Figures 3.1 and 3.5), while aldH expression was low due to lack of formation of 1,4-dioxane degradation products containing aldehyde functional groups (Gedalanga et al., 2014). In contrast to TCE and cDCE, lower concentrations of TCA did not inhibit 1,4-dioxane degradation by CB1190, which resulted in dxmB and aldH fluctuating with no significant difference in expression comparing to solvent free control (Figures 3.4 and 3.5). Similar expression levels occurred in the presence of 50 mg/L TCA, at which 1,4-dioxane degradation rate was only marginally lower than that of the solvent free control (Figure 3.5). However, at all concentrations of 1,1-DCE tested, dxmB and aldH were repressed about 6-fold at
6 hours, and no further changes were measured in the following 6 hours (Figure 3.5). In chlorinated solvent Mixture 1, the presence of 5 mg/L 1,1-DCE resulted in decreased expression of both \( dxmB \) and \( aldH \) (Figure 3.5), similar to that observed when 1,1-DCE was added individually (Figure 3.4). This result suggests that 1,1-DCE repressed the transcription of \( dxmB \) and \( aldH \) genes.

![Graph showing dose-dependent uspA gene expression at 6 hours in the presence of 0.5, 5, or 50 mg/L individual chlorinated solvents. All cDNA copy numbers were first normalized to \( rpoD \) housekeeping gene and then to solvent free control. Error bars represent standard deviation of triplicates.](image)

Figure 3.8. Dose-dependent uspA gene expression at 6 hours in the presence of 0.5, 5, or 50 mg/L individual chlorinated solvents. All cDNA copy numbers were first normalized to \( rpoD \) housekeeping gene and then to solvent free control. Error bars represent standard deviation of triplicates.

### 3.3.6 Dose-dependent universal stress protein gene (\( uspA \)) was induced by 1,1-DCE and cDCE

The regulation of \( uspA \) gene was reported to be an important response of bacteria during growth arrest (Kvint et al., 2003), and it was induced by stress to protect the cells from nutrient depletion or accumulation of toxic chemicals (Nystrom and Neidhardt, 1992). The expression of \( uspA \) in CB1190 was examined in the presence of individual chlorinated solvents and mixtures (Figures 3.8 and 3.9). Exposure to TCE or TCA did not contribute to the \( uspA \) induction, which means \( uspA \) was not sensitive to TCE and TCA. Similarly, when low concentrations of cDCE were present in the culture, little influence on \( uspA \) expression was observed. However, \( uspA \)
was significantly over-expressed approximately 5.0-fold and 3.5-fold, when 1,1-DCE or cDCE concentrations were increased up to 50 mg/L, respectively. Interestingly, uspA transcription increased when approximately 50% 1,4-dioxane remained in Mixture 1. In Mixtures 2 and 3, uspA induction in CB1190 was limited without 1,1-DCE, even though 0.5 mg/L cDCE and low concentrations of TCE and TCA were present (Figure 3.9).

![Graph showing gene expression](image)

Figure 3.9. Gene expression in Pseudonocardia dioxanivorans CB1190 exposed to chlorinated solvent mixtures. Expression of dxmB, aldH, and uspA relative to solvent-free controls at 2 hours (approximately 50 % 1,4-dioxane degradation) in 1, 2, and 3 synthetic solvent mixtures. Error bars represent standard deviation of triplicates.

### 3.4 Discussion

The inhibitory effects of individual chlorinated solvents on 1,4-dioxane biodegradation have been previously identified, e.g., the presence of TCE incurred incomplete 1,4-dioxane
cometabolism in JOB5 and *Rhodococcus jostii* RHA1 cultures (Hand et al., 2015), and the presence of 1,1-DCE reduced monooxygenase activity by 80% for *Rhodococcus erythropolis* (Ewers et al., 1990). Comparatively, this study determined that solvents inhibited biodegradation of 1,4-dioxane in the following order: 1,1-DCE > cDCE > TCE > TCA (Figure 3.1). However, Mahendra and coworkers examined the effects of pre-exposing CB1190 to individual chlorinated solvents prior to 1,4-dioxane biodegradation, and reported stronger inhibition for TCA according to $K_I$ values of 1.2 ± 1.0 µM and 3.3 ± 2.9 µM for TCA and 1,1-DCE, respectively (Mahendra et al., 2013). Our study differs from that of Mahendra et al. (2013) by focusing on 1,4-dioxane biodegradation inhibition in the presence of each chlorinated solvent as well as in mixtures and environmental samples (Table 3.6). The present approach adds improved representation of mixed contamination in groundwater, and the results provide a better understanding of chlorinated solvent influences on bioaugmentation strategies in co-contaminated environments. These studies differed significantly in exposure conditions and activity related to low substrate concentrations indicating that cell density, growth phase (Rajagopal, 1996), and the activity of cells (Yeager et al., 2001) vary the tolerance of bacteria against chlorinated solvents. Our study reports an inhibition constant, $K_I$, which was calculated according to initial biodegradation rates. However, this approach proved unreliable in determining overall solvent impacts, such as universal stress, energy depletion, transcriptional regulation and/or product toxicity. For example, $K_I$ values from our study suggested a stronger inhibitory effect of TCE over cDCE on 1,4-dioxane biodegradation (Table 3.6). However, continuous 1,4-dioxane biodegradation still occurred in the presence of TCE, while the degradation ceased after 6 hours in the presence of cDCE (Figure 3.1).
Despite a higher degree of halogenation, TCE and TCA imparted less inhibition towards 1,4-dioxane biodegradation than dichloro-substituted ethenes. Similar results were reported in a mammalian toxicity study (Nakahama et al., 2000) as well as a bacterial cometabolism study (Ely et al., 1995), which were attributed to the chemical structures of the solvents that affect electron density distribution, polarity, and hydrophobicity of the molecules (Ewers et al., 1990). For example, more hydrophilic and polar solvents like 1,1-DCE and cDCE with lower Log K\textsubscript{ow} values (Table 3.2), may abundantly bind to cell membranes (Torres et al., 2011; Zenker et al., 2003). They can change the cell membrane composition resulting in leakage of intracellular metabolites or forming channels for solvents to attack intracellular components (De Smet et al., 1978; Rajagopal, 1996). The unsaturated carbon-carbon bond, isomerization, and number of chlorine atoms further determined the chemical interaction between each chlorinated solvent and macromolecules, such as monooxygenases (Schultz and Yarbrough, 2004). In addition, carbon-carbon single bonds (1.54 Å) have longer interatomic distance than carbon-carbon double bond (1.39 Å), which corresponded to the reduced reactivity of the molecule (Pauling and Brockway, 1937; Pauling et al., 1935). Thus, TCA has fewer cellular interactions than TCE, cDCE, and 1,1-DCE as observed in the present study. Furthermore, three chlorine atoms attached to the carbon-carbon double bond in ethenes balanced resonance better than fewer chlorines attached to the unsaturated double bonds. Even in the abiotic oxidation of chlorinated solvents by potassium permanganate, the ease of attack was determined to be in the following order 1,1-DCE > cDCE > TCE > PCE, suggesting that 1,1-DCE has high reactivity (Yan and Schwartz, 1999).
Figure 3.10. Quantification of chlorinated solvents in CB1190 pure culture study at 0 and 24 hours. Each individual solvent exposed culture also contained 1000 µg/L 1,4-dioxane. Darker colors represent the concentrations at Time 0, and lighter colors represent the concentrations at 24 hours.

Previous studies of 1,4-dioxane biodegradation pathways have revealed that monooxygenases catalyze the initial steps of 1,4-dioxane biodegradation in both metabolic and cometabolic processes (Mahendra and Alvarez-Cohen, 2006; Mahendra et al., 2007). Since these multicomponent enzymes are membrane-bound in many bacteria (Gedalanga et al., 2014; Masuda et al., 2012), their activity is susceptible to changes in structural conformation, covalent binding by inhibitors, and collapse in membrane potential (Ely et al., 1997). A prior study of chlorinated solvent cometabolism by nitrifying bacteria demonstrated that 1,1-DCE, TCE, and 1,2-dichloroethane were able to inactivate enzymes (Ely et al., 1997). It was also reported that 1,1-DCE had stronger inhibition effects than TCE and cDCE on nitrifying bacteria (Ely et al., 1997) as was observed in our study. Further, reactive epoxide formation was suggested as one of the possible causes to pose stronger inhibitory effects (Ewers et al., 1990). However, no significant loss of chlorinated solvents in 24 hours was observed in our study, which indicated inhibition was not associated with product toxicity (Figure 3.10). Additionally, PMA-qPCR determined exposure to cDCE, TCE, or TCA did not impact the abundance of cells with intact
membranes compared to the solvent free controls. Cells exposed to 1,1-DCE had a higher fraction of qPCR amplifiable cell numbers and were significantly different \((p < 0.01)\) than the solvent-free control (Figure 3.11). This finding suggests that 1,1-DCE may bind or even damage nucleic acids preventing PMA treatment. Previous studies determined that TCE and 1,1-DCE were able to inflict DNA damage in mammalian cells (Jeon et al., 2005) and impacted gene transcription (Houde et al., 2015). Those results are consistent with the observations in this study that \(dxmB\) and \(aldH\) were markedly suppressed when CB1190 was exposed to 5 mg/L or 50 mg/L solvents, especially 1,1-DCE. As cell membranes are also associated with energy generation and consumption, this study determined that the ATP production rate was related to 1,4-dioxane degradation rate, and that the total ATP yield was not affected by solvents. Taken together, these findings suggest that cell membrane damage does not explain inhibition at 5 mg/L solvent concentration.

![Bar chart showing non-permeabilized membrane percentages](image)

**Figure 3.11.** Quantification of cells with intact cell membranes. PMA treatment was performed to quantify viable cell numbers in chlorinated solvents exposed cultures. Heat-killed controls were prepared by heating an equal concentration of CB1190 in a water bath at 95°C for 5 minutes. Heat-killed and viable cultures were confirmed by plating on R2A agar.
Exposure to chlorinated solvents influenced the expression of 1,4-dioxane biomarker genes (Figure 3.4 and 3.5). Expression of \textit{dxmB} and \textit{aldH} was down regulated in the presence of 1,1-DCE suggesting potential interactions between 1,1-DCE and the regulatory network of the \textit{dxm} genes. Specifically, 1,1-DCE may bind to the \textit{dxmB} promoter or activator regions in the nucleic acid sequence reducing the affinity of RNA polymerase (Houde et al., 2015). Studies on the toluene-3-monoxygenase of \textit{Burkholderia pickettii} determined the expression of this operon is linked to the expression of the activator TbuT (Byrne and Olsen, 1996). Decreased concentrations of \textit{dxmB} transcripts might result from interactions of 1,1-DCE with CB1190’s two component signaling system (Huang et al., 2015), or repression following passive or active transport of 1,1-DCE through the cell membrane (Bressler and Gray, 2003; Kim et al., 2002). Our gene expression results suggest further research into the regulation of this enzyme will help elucidate key mechanisms of solvent inhibition for bacterial multicomponent monoxygenases.

Chlorinated solvent induced stress on Gram positive bacteria has been linked to general stress response proteins (Torres et al., 2011). We targeted the \textit{uspA} gene encoding a universal stress protein to provide insights into the cellular response to chlorinated solvents. This protein is an important bacterial response against global cellular stress, and induction of \textit{uspA} occurs in response to a wide range of environmental stimuli including a transition into stationary phase growth (Kvint et al., 2003; Liu, 2006). Our study determined that TCE and/or TCA exposure caused little stress on CB1190 cells resulting in down regulation of \textit{uspA} in a dose-dependent manner (Figure 3.6) and biodegradation of 1,4-dioxane was able to proceed (Figure 3.1). In contrast, concentrations of 50 mg L$^{-1}$ cDCE and 1,1-DCE concentrations $> 5$ mg/L induced \textit{uspA}, indicating a cellular stress response related to inhibition of 1,4-dioxane biodegradation (Figures 3.1 and 3.5). In other studies, cell membrane permeabilization (Segura et al., 2012), transport
activity (De Carvalho et al., 2014; Segura et al., 2012), and protein production (Torres et al., 2011) were also influenced by organic solvents. The regulation of uspA is an important new insight into the mechanism of inhibition among various organic solvents, and valuable in evaluating the potential of bioremediation of various contaminants in the field. In pure cultures exposed to synthetic solvent mixtures, the total stress from all solvents resulted in significant down-regulation of dxmB and aldH, and up-regulation of uspA in Mixture 1 (Figure 3.9). Furthermore, our results determined that upregulation of uspA may prove useful in co-contaminated environments for predicting the potential of 1,4-dioxane biodegradation.

In conclusion, individual chlorinated solvents and their mixtures posed time- and dose-dependent inhibition of 1,4-dioxane biodegradation via energy depletion and down-regulation of genes coding for biodegradative enzymes. This study showed that chlorinated solvent inhibition of 1,4-dioxane metabolism was not significantly related to the destruction of the cell membrane or formation of toxic byproducts. Bioaugmentation successfully removed the 1,4-dioxane in the contaminated environmental samples, and low concentrations of TCA, TCE, and cDCE did not have significant effects on metabolic transformation of 1,4-dioxane by CB1190, implying advantages for implementing metabolic biodegradation in certain co-contaminated aquifers. Therefore, aerobic bioremediation should be considered as a viable candidate strategy for 1,4-dioxane-and chlorinated solvent-contaminated groundwater.
3.5 References


Chapter 4: Conclusion and Future Research Direction
4.1 Conclusion and Summary

My study investigated the impacts of individual chlorinated solvents and their mixtures on aerobic 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190. The established association of these co-occurring compounds suggests important considerations for their respective biodegradation processes. In the metabolism study, kinetics and mechanistic studies demonstrated that individual solvents inhibited biodegradation of 1,4-dioxane in the following order: 1,1-dichloroethene (1,1-DCE) > cis-1,2-dichloroethene (cDCE) > trichloroethene (TCE) > 1,1,1-trichloroethane (TCA). The presence of 5 mg L\(^{-1}\) 1,1-DCE completely inhibited 1,4-dioxane biodegradation. Subsequently, I determined that 1,1-DCE was the strongest inhibitor of 1,4-dioxane biodegradation by bacterial pure cultures exposed to chlorinated solvent mixtures as well as in environmental samples collected from a site contaminated with chlorinated solvents and 1,4-dioxane. Inhibition of 1,4-dioxane biodegradation rates by chlorinated solvents was attributed to delayed ATP production and down regulation of both 1,4-dioxane monooxygenase (*dxmB*) and aldehyde dehydrogenase (*aldH*) genes. Moreover, increasing concentrations of 1,1-DCE and cis-1,2-DCE to 50 mg/L respectively increased 5.0-fold and 3.5-fold expression of the *uspA* gene encoding a universal stress.

4.2 Future Research Direction

4.2.1 Laboratory-based Research

*Co-contaminant effects.* Identification of environmental factors that may influence 1,4-dioxane biodegradation plays an important role in the transition from the laboratory to field remediation. The inhibitory effects of chlorinated solvents (TCE, TCA, 1,1-DCE and cDCE) and heavy metals (Cu, Cd, Ni, Zn, Cr) on 1,4-dioxane biodegradation have been identified in
previous work (Mahendra et al., 2013; Pornwongthong et al., 2014; Zhang et al., 2016a; Zhang et al., 2016b), but considering the chemical complexity in groundwater, other 1,4-dioxane associated environmental co-contaminants and their mixtures, including natural organic matters and poly-/per-fluorinated compounds, should be investigated to quantify their interferences on biological treatment of 1,4-dioxane. Further, very few studies have focused on the identification of naturally occurring biological or chemical stimulants to accelerate 1,4-dioxane biodegradation. Microbial growth factors, such as pH, trace metals, nutrients, primary substrates, and enzyme inducers should be recognized to also assist in the selection of key 1,4-dioxane degrading microbes. Evidence of unique 1,4-dioxane biodegradation pathways in isolated microorganisms summarized in Chapter 2 suggests potentially different outcomes to recognize various environmental factors that positively or negatively influence 1,4-dioxane biodegradation.

**Contaminated soil bioremediation.** While aquatic environments remain the focus of 1,4-dioxane bioremediation efforts, soil environments, in particular soil moisture such as the capillary fringe (Kurt and Spain, 2013), might serve as a possible unexplored reservoir of 1,4-dioxane and 1,4-dioxane-degrading microbes. Moreover, the high water solubility of 1,4-dioxane will result in its likely absorption in the vadose zone and moist soil particles in low permeability zones (Adamson et al., 2016). Information on 1,4-dioxane bioremediation in contaminated soil still remains scarce. Column studies are usually constructed to simulate the interface of unsaturated and permeable zones for biodegradation studies (Kurt and Spain, 2013). Hence, laboratory experiments, such as soil column and tank studies, as well as full-scale studies should be considered to bridge the gap of this knowledge on 1,4-dioxane contaminated soil bioremediation.
4.2.2 Treatment Trains

Upon release into the aquifers, 1,4-dioxane contaminated plumes expand rapidly (Adamson et al., 2016). *In situ* biodegradation of 1,4-dioxane is an ideal solution when contamination presents as a large and dilute plume because *ex situ* technologies are typically costly and inefficient under these conditions. However, successful *in-situ* field-scale implementation using bioremediation to remove 1,4-dioxane are still scarce (DiGuiseppi et al., 2016). The most recent *in situ* example using bioaugmentation and propane biosparging at Vandenberg Air Force Base to treat 1,4-dioxane contaminated aquifer was still confronted with an extensive incubation time and limited available nutrients (nitrogen and phosphorus source) (Lippincott et al., 2015). A single technology may not be optimal to overcome these biological treatment difficulties and to remove contaminants, especially under time and spatial constraints. Thus, treatment trains provide many alternatives by using different technologies to complete remediation goals.

Specifically, while many sites have undergone CVOCs remediation for decades, there is an urgent demand that allow the treatments to continuously remove contaminant mixtures containing CVOCs and 1,4-dioxane. For example, Lowry Landfill Superfund Site was contaminated by more than 50 chemicals, including chlorinated solvents, polynuclear aromatic hydrocarbons, metals, and 1,4-dioxane (Colorado Department of Public Health and Environment, 2006). Advanced oxidation procedures coupled with a fixed film moving bed bio-reactor (MBBR) system utilizing Kaldnes® media was applied to remove these co-contaminants to alleviate the inhibitory effects on 1,4-dioxane biodegradation processes at this site (Stanfill et al., 2004). However, problems of lithology and the slow movement of groundwater still challenge the success of 1,4-dioxane bioremediation (Colorado Department of Public Health and
Environment, 2006). Hence, bioremediation combined with AOPs, adsorption, and electrolysis for the rapid and cost-effective in-situ treatment of 1,4-dioxane and co-contaminant mixtures should be further investigated.

### 4.2.3 Microbial Community Characterization

A recent evaluation and large-scale database statistical analysis suggested the occurrence of 1,4-dioxane natural attenuation in groundwater (Adamson et al., 2015). However, most biodegradation studies of 1,4-dioxane mainly focused on cultured microorganisms. Many unknown and unidentified microbes may also play critical roles to decompose 1,4-dioxane and associated co-contaminants. Molecular biology tools, such as biomarkers and enzyme activity probes significantly enhanced the evaluation of the natural attenuation potential (Suthersan et al., 2016). Similarly, microbial community analysis (e.g. high-throughput sequencing and metagenomics), which is well established in medical microbiology and ecology, can be useful in 1,4-dioxane bioremediation to identify novel degrading microbes and biotransformation pathways. Moreover, using microbial community analysis can save efforts of culturing microbes and enhance the site assessment for the potential of 1,4-dioxane biodegradation.

Most remediation strategies present unique advantages and disadvantages to resolve contamination under various conditions. Since 1,4-dioxane biodegradability has been discovered, natural attenuation, or biostimulation and bioaugmentation interventions promise to be a sustainable and cost-effective technology for 1,4-dioxane contamination cleanup. Moreover, the development of advanced monitoring tools, such as biomarkers, CSIA, and analytical techniques, promotes the reliability of applying bioremediation. Knowledge gaps still remain in the areas of 1,4-dioxane biodegradation and continued research is needed on the identification of co-contaminants, biodegradation potential in solid wastes, novel microbial degrading bacterial and
fungal strains, and demonstrated field applications under a wide range of conditions. Research in these areas will promote the success of 1,4-dioxane bioremediation in the future.
4.3 References


Colorado Department of Public Health and Environment, 2006. 1,4-Dioxane and the Lowry landfill superfund site U.S. EPA, Denver, CO.


Stanfill, J., Koon, J., Plaehn, W., Murphy, M., Shangraw, T., Bollmann, D., 2004. 1,4-Dioxane biodegradation pilot study at the Lowry landfill superfund site Water Environment Federation, pp. 851-870.


Zhang, S., Gedalanga, P.B., Guo, S., Mahendra, S., 2016a. Bioprocesses for simultaneously removing hexavalent chromium and 1,4-dioxane. 251st ACS National Meeting Presentation.